การหาลายพิมพ์ดีเอ็นเอของไรโซเบียมถั่วเหลืองที่แยกจากปมรากถั่วเหลืองที่ใส่เชื้อปุ๋ยชีวภาพ NA7 ในตำบลน้ำมวบ จังหวัดน่าน



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาจุลชีววิทยาอุตสาหกรรม ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2552 ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DNA FINGERPRINTING OF SOYBEAN RHIZOBIA ISOLATED FROM NODULES OF SOYBEANS INOCULATED WITH BIOFERTILIZER NA7 IN NAM MOUB SUBDISTRICT,

NAN PROVINCE

Miss Thanpapha Chanthapetch

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Industrial Microbiology Department of Microbiology

Faculty of Science

Chulalongkorn University

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ชันปภา จันทเพ็ชร : การหาลายพิมพ์ดีเอ็นเอของไรโซเบียมถั่วเหลืองที่แยกจากปมรากถั่วเหลืองที่ใส่ เชื้อปุ๋ยชีวภาพ NA7 ในตำบลน้ำมวบ จังหวัดน่าน. (DNA FINGERPRINTING OF SOYBEAN RHIZOBIA ISOLATED FROM NODULES OF SOYBEANS INOCULATED WITH BIOFERTILIZER NA7 IN NAM MOUB SUBDISTRICT, NAN PROVINCE) อ.ที่ปรึกษา วิทยานิพนธ์หลัก : รศ.ดร.กาญจนา ชาญสง่าเวช, 111 หน้า.

ไรโซเบียมถั่วเหลืองเข้าสร้างปมที่รากถั่วเหลืองและเปลี่ยนในโตรเจนจากอากาศให้เป็นแอมโมเนียสำหรับ ถั่วเหลืองใช้ในการเจริญ บรรทัดฐานแรกในการพัฒนาปุ๋ยชีวภาพไรโซเบียมสำหรับถั่วเหลืองประกอบด้วยการ คัดเลือกสายพันธุ์ไรโขเบียมถั่วเหลืองที่สามารถแข่งขันกับไรโซเบียมท้องถิ่น ในการเข้าสร้างปมที่รากถั่วเหลือง ปุ๋ย ชีวภาพไรโซเบียมถั่วเหลืองที่มีจำหน่ายในท้องตลาด ไม่มีวิชีควบคุมคุณภาพที่ระบุว่าสายพันธุ์ไรโซเบียมถั่วเหลืองที่ ใช้ในการผลิตปุ๋ยฯ เป็นไรโซเบียมที่ไม่เกิดการเปลี่ยนแปลงสารพันธุกรรม ในปีเพาะปลูก 2550/2551 มีการทดลอง ผลิตปุ๋ยชีวภาพไรโซเบียมถั่วเหลือง NA7 ในระดับห้องปฏิบัติการ ซึ่งมีลายพิมพ์ดีเอ็นเอประจำสายพันธุ์ และเก็บ รักษาได้ที่อุณหภูมิห้อง เพิ่มจำนวนเขลล์โดยเลี้ยงในอาหารสูตร yeast extract mannitol และคลุกเขลล์กับดินพีต (peat) ในสัดส่วน 2x10° เซลล์ต่อกรัมพีต ใช้ปุ๋ยชีวภาพไรโซเบียมถั่วเหลือง NA7 คลุกกับเมล็ดถั่วเหลืองพันธุ์ เชียงใหม่ 60 (CM60) และปลูกถั่วเหลืองในแปลงทดลองขนาด 15 x 24 ตารางเมตร ที่ ต.น้ำมวบ อ.เวียงสา จ.น่าน วัตถุประสงค์ของงานวิทยานิพนธ์นี้ เพื่อหาประสิทธิภาพในการเข้าสร้างปมของไรโซเบียมถั่วเหลืองสายพันธุ์ NA7 โดย แยกแบคทีเรียจากปมรากถั่วเหลืองหลังการเพาะปลูก 1 เดือน และนำมาหาลายพิมพ์ดีเอ็นเอ โดยวิธี RAPD-PCR โดยใช้ไพร์เมอร์ RPO1 หรือ CRL-7 เพื่อเปรียบเทียบลายพิมพ์ดีเอ็นเอกับลายพิมพ์ดีเอ็นเอของสายพันธุ์ NA7 หากตรวจพบลายพิมพ์ดีเอ็นเอดังกล่าวจากแบคทีเรียที่แยกได้จากปมราก แสดงว่าไรโซเบียมสายพันธุ์ NA7 สามารถแข่งขันกับไรโซเบียมท้องถิ่นในการเข้าสร้างปมภาคสนาม ผลการทดลองได้แยกแบคทีเรียจากปมรากถั่ว เหลือง 198 ไอโซเลต แบ่งเป็นประเภทเพิ่มจำนวนเร็ว 147 ไอโซเลต และประเภทเพิ่มจำนวนช้า 51 ไอโซเลต เนื่องจากสายพันธุ์ NA7 เป็นไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า จึงหาลายพิมพ์ดีเอ็นเอของแบคทีเรีย ประเภทเพิ่มจำนวนข้า 51 ไอโซเลต และในการเปรียบเทียบลายพิมพ์ดีเอ็นเอพบลายพิมพ์ดีเอ็นเอของ NA7 จำนวน 13 ไอโซเลต คิดเป็นสัดส่วนการเข้าสร้างปม 6.6% ผลการหาปริมาณไรโซเบียมถั่วเหลืองในตัวอย่างดินจากแปลง ทดลองที่ต.น้ำมวบ อ.เวียงสา จ.น่าน โดยวิธี Most Probable Number (MPN) พบไรโซเบียมถั่วเหลืองโดยเฉลี่ย 4x10⁴ เขลล์ต่อดินหนึ่งกรัม ผลการจำแนกขนิดไรโซเบียมถั่วเหลือง 7 สายพันธุ์ได้แก่ NA7, NM22-8, NM22-11, NM22-13, NM22-15, NM22-25 และ NM22-30 โดยใช้อนุกรมวิธานแบบพอลิฟาสิก พบสายพันธุ์ NA7 และ NM22-25 เป็นสายพันธุ์เดียวกันคือ Bradyrhizobium elkanii สายพันธุ์ NM22-11, NM22-13 และ NM22-15 เป็น Bradyrhizobium elkanii สายพันธุ์ NM22-8 กับ NM22-30 เป็น Bradyrhizobium japonicum

ภาควิชา	รุลชีววิทยา	ลายมือชื่อนิสิต	Sala
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THANPAPHA CHANTHAPETCH : DNA FINGERPRINTING OF SOYBEAN RHIZOBIA ISOLATED FROM NODULES OF SOYBEANS INOCULATED WITH BIOFERTILIZER NA7 IN NAM MOUB SUBDISTRICT, NAN PROVINCE. THESIS ADVISOR : ASSOC. PROF. KANJANA CHANSA-NGAVEJ, PH.D., 111 pp.

Soybean rhizobia nodulate soybean roots and convert atmospheric nitrogen to ammonia which is utilized by soybeans for growth. The first criterion in the development of soybean rhizobium biofertilizers is selection of soybean rhizobia strains which could compete with indigenous soybean rhizobia in nodulating soybean roots. There is no DNA fingerprints quality control in the production of rhizobium biofertilizers available in the market. Rhizobium biofertilizer NA7 had previously been produced at the lab scale by mixing strain NA7 with peat at the ratio of 2 x 10 8 cells per gram. Soybean seeds cv. CM 60 mixed with the biofertilizer were planted in a 15 x 24 m² experimental plot in 2007/2008 in Nam Moub subdistrict, Nan province. The aim of the thesis is to determine nodulation efficiency of NA7 by isolating bacteria from root nodules of soybean plants after one month cultivation in the experimental plot. DNA fingerprints of the isolates were obtained by RAPD-PCR using either RPO1 or CRL-7 as the primer. Out of the 198 root nodule isolates, 147 were fastgrowers and 51 isolates were slow-growers. Since soybean rhizobium strain NA7 was a slowgrower, DNA fingerprints of the 51 slow-growing isolates were obtained. Comparisons of DNA fingerprints showed strain NA7 in the biofertilizer nodulated 13 out of the 51 isolates which made up 6.6% of nodule occupancy. The average number of soybean rhizobia in soil samples from the experimental plot in Nam Moub subdistrict was determined by the Most Probable Number (MPN) to be 4 x 10⁴ cells per gram soil. Seven soybean rhizobium strains (NA7, NM22-8, NM22-11, NM22-13, NM22-15, NM22-25, and NM22-30) were identified by polyphasic taxonomy. Strain NM22-25 was found to be identical to strain NA7 which was found to be Bradyrhizobium elkanii. Strains NM22-11, NM22-13, and NM22-15 were found to be Bradyrhizobium elkanii while strains NM22-8 and NM22-30 were found to be Bradyrhizobium japonicum.

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CHAPTER I

INTRODUCTION

Soybeans are raw materials which are used in the production of other food products such as soybean oil, soybean milk, soy sauce, soybean paste and tofu. In addition, soybean seeds and soybean meal are used as animal feed. Table 1.1 shows that at present, Thailand imports 85% of local soybean consumption leading to trade deficit and lost opportunities to grow soybeans as rotational crop with economic crops such as rice to improve soil conditions. The reason is because in soybean root nodules there are rhizobia which fix or convert atmospheric nitrogen to ammonia that can be used by soybean. Hence, the use of soybean biofertilizers leads to reduction in usage of chemical fertilizers and water pollution in the form of eutrophication.

Table 1.1 Quantities of soybeans grown locally and soybeans imported from 2005 to2007.

Year	Quantities of soybeans	Quantities of soybeans
Q	grown locally (Tons)	imported (Tons)
2005	230,271	1,607,784
2006	229,059	1,395,370
2007	226,843	1,540,835

Sources :

Customs Office (2008).<u>http://www.feedusers.com/thai/cms/html/Inedible/110.html</u> Office of Agricultural Economics(2008).<u>http://www.oae.go.th/oae_website/oae_imex.php</u>

One reason Thailand relies on soybeans imported at approximately 85% of the soybean consumption is the country's low soybean yields with an average of 250 kg/rai compared with approximately 430 kg/rai in countries which are leading soybean exporters such as the USA as shown in Table 1.2

Table 1.2 Average soybean yields (kg/*ra*i) in Thailand and in countries which are leading soybean exporters.

Ranking	Country	Average soybean yields (kg/rai)			
No.		2004	2005	2006	2007
	Year		10-		
1	USA	454	463	465	370
2 🤜	Brazil	368	357	381	451
3 🥌	Argentina	352	437	429	452
21	Thailand	238	250	250	253

Source : Office of Agricultural Economics (2009).

http://www.oae.go.th/statistic/yearbook50/section2/sec2table25.pdf

An additional factor which could lead to an even lower quantities of locallygrown soybeans is the decease in areas used for soybean cultivation as shown in Table 1.3 as more and more land is used for growing other cash crops which provide growers with higher income such as corn. However, corn cultivation requires large amounts of chemical fertilizer and pesticide usage.

Table 1.3 Sovhean	cultivation areas in	n Thailand and	average sovbean	vields
Table 1.5 Suybean	cultivation areas in	i malianu anu	average suppear	yieius.

Year	Cultivation Area (1000 <i>rai</i>)	Average Soybean Yield (kg/rai)
1998	1,467	234
1999	1,451	227
2000	1,396	232
2001	1,154	236
2002	1,130	238
2003	961	246
2004	945	238
2005	929	250
2006	886	250
2007	831	253

Source : Office of Agricultural Economics (2009).

http://www.oae.go.th/statistic/yearbook50/section2/sec2table26.pdf

One way to increase domestic soybean yields is to popularize the use of soybean rhizobium biofertilizers among soybean growers. Soybean rhizobium biofertilizers consist of 10⁸ rhizobial cells mixed with 1 g peat. Soybean rhizobium biofertilizers available in the market in Thailand need to be kept in cool places or in refrigerator to prevent cell multiplication. If rhizobial cells are more than 10⁸ cells per gram biofertilizer, nodulation efficiency decreases (Loh et al., 2002a, b ; Loh and Stacey, 2003). Rhizobium biofertilizer NA7 had previously been produced at the lab scale by mixing strain NA7 grown in yeast extract mannitol broth with peat at the ratio of 2 x 10⁸ cells per gram peat. Soybean seeds cultivar CM 60 mixed with the biofertilizer had previously been grown in a 15 x 24 m² experimental plot in the cultivation year 2007/2008 in Nam Moub subdistrict, Nan province (Chantapetch and Chansa-ngavej, 2009). The aim of the thesis is to determine the nodulation efficiency of soybean rhizobium strain NA7 used in the lab-scale production of biofertilizer for soybeans as well as to determine the average amount of soybean rhizobia in soil samples from the experimental plot in Nam Moub subdistrict, Nan Province. Strain NA7 and six rhizobial isolates from root nodules grown in the experimental plot will also be identified by polyphasic taxonomy.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

LITERATURE SURVEY

Soybean rhizobia are Gram negative, motile, non-spore forming rods which fix nitrogen in root nodules of soybeans. Cultivation of soybeans in rotation with other economic crops such as rice or corn has led to increased yields of soybeans, rice, and corn, with reduction in nitrogen chemical fertilizers usage. Use of soybean rhizobium biofertilizers has been reported to increase soybean yields. In addition, use of soybean rhizobium biofertilizers alleviates eutrophication which is a form of water pollution due to enrichment of water by agricultural run-off containing nitrate and phosphate from chemical fertilizers.

Bradyrhizobium japonicum is a slow-growing rhizobium which nodulates roots of soybeans. In 1982 Jordan proposed the transfer of slow-growing soybean rhizobia from genus *Rhizobium* to genus *Bradyrhizobium* due to differences in growth rate, number and type of flagella, antibiotic sensitivity and genetic properties. There are two categories of soybean rhizobia : Fast-growing soybean rhizobia and slow-growing soybean rhizobia. At present, five species of soybean rhizobia are recognized as shown in Table 2.1.

Table 2.1 Five recognized species of soybean rhizobia.Fast-growers :

Sinorhizobium fredii (Chen et al., 1988) Sinorhizobium xinjiangense (Peng et al., 2002) Slow-growers :

> Bradyrhizobium elkanii (Kuykendall et al., 1992) Bradyrhizobium japonicum (Jordan, 1982) Bradyrhizobium liaoningense (Xu et al., 1995)

Some differences between fast-and slow-growing soybean rhizobia are shown in Table 2.2.

Properties Soybean rhizobia Fast-growers Slow-growers 1. Doubling time Less than 6 hours More than 6 hours 2. Type of flagella 2-6 peritrichous flagella 1 subpolar flagellum 1 HIU 3. nifHDK nifHDK are in the same operon nifH and nifDK are on separate operons niiD K E niiBhxA niiH 加密核 <u>588</u>

Table 2.2 Some differences between fast- and slow-growing soybean rhizobia (Elkan & Bunn, 1992)

Soybean rhizobium biofertilizers presently available in the market need to be kept in cool places or in refrigerator to prevent soybean rhizobium cell multiplication which may lead to inhibition of nodulation gene expression by quorum-sensing mechanism. Quorum sensing is a cell-density dependent mechanism for communication commonly found in bacteria when cell density is sufficient for the secretion of an autoinducer at levels that trigger changes in gene expression (Sharma et al., 2003). In the years 2001-2003, Loh and co-workers (Loh et al., 2001; 2002a; 2002b; 2003) discovered that when soybean rhizobium *Bradyrhizobium japonicum* grown in minimum medium was approximately 10[°] cells•ml⁻¹ in the stationary phase, the autoinducer Bradyoxetin with the chemical [2-[4-[[4-(3-aminooxetan-2yl)phenyl](imino)methyl]phenyl]oxetan-3-ylamine] formula was secreted in sufficient quantities to induce expression of *nodD*₂. Protein NodD₂ inhibits expression of *nodYABC* which encode enzymes in the production of Nod factor which was involved in root nodule formation. Loh et al. (2002b) constructed 4 mutants : B. japonicum JWS21 (nwsB Sm^rSp^r); B. japonicum JNWS24 (JNWS21 harboring

pBGAlac1 with *nolA-lacZ* translational fusion); *B. japonicum* JNWS31 (JNWS21 harboring pZB32 with *nodY-lacZ* translation fusion); *B. japonicum* JNWS41 (JNWS21 harboring pPRJ1248 with *nodD2-lacZ* translational fusion). The mutants were used to demonstrate that at high cell density (more than 10⁹ cell/ml) the expression of *nodD2-lacZ* increased while that of *nodY –lacZ* decreased and that *nwsB* was essential for the density-dependent full expression of *B. japonicum nodD1*, and *nodYABC*. NwsB was postulated to sense the presence of Bradyoxetin at high cell density which led to the activation of *nodD2* which inhibited the expression of *nodYABC* leading to a decrease in Nod factor synthesis. The results implied that the number of *B. japonicum* cells in a rhizobial biofertilizer should be optimal for optimal expression of nodulation genes *nodYABC* for Nod factor synthesis. Figures 2.1 summarizes nodulation genetics.



Figure 2.1 Summary of slow-growing soybean rhizobium nodulation genetics (modified from Stacey, 1995).

Loh and Stacey (2003) reported nodulation genes in *B. japonicum* included *nodD1, nodD2, nodYABC* and *nwsB.* Soybean roots secrete flavonoids such as genistein which enters the periplasm of *B. japonicum* cells that move towards root hair through chemotaxis along the gradient of genistein (Kosslak et al., 1987). A complex between genistein and NodD1 is formed in the periplasm. This complex acts as a transcriptional activator which binds to the promoter regions of *nodD1* and *nodYABC* which are known as *nodD1* box and *nodYABC* box respectively. Wang and Stacey

(1991) reported the 9 bp repeat sequences of nodD1 box are ATTGCTTTT GCGCGTCTA. Binding of NodD₁-flavonoid complexes to $nodD_1$ box activates the transcription of $nodD_1$. The transcriptional start site of $nodD_1$ lies 44 bp upstream of $nodD_1$ box as shown in Figure 2.2

The promoter of *nodYABC* contains *nodYABC* box which is made up of four 9 bp repeats as follows :

Wang and Stacey (1991) stated that promoters of $nodD_1$ and of nodYABC overlapped with transcriptional start sites of $nodD_1$ and of nodYABC lying in the *nod* box of the opposing transcript as shown in Figure 2.2.



Figure 2.2 Diagramatic representation of a DNA segment of *B. japonicum* nodulation genes showing promoters of $nodD_1$ and nodYABC are overlapped with transcriptional start sites of nodD and of nodYABC lying in the *nod* box of the opposing transcript (modified from Wang and Stacey, 1991).

In addition, $nodD_1$ and nodYABC are activated by the two-component system encoded by nodVW. NodV is a kinase which autophosphorylates and transfers the phosphate group to NodW. Phosphorylated NodW activates transcription of nodD1 and *nodYABC* possibly by influencing DNA bending as in the case of the activation mechanism of NodD1-flavonoid complexes (Loh and Stacey, 2003).

Expression of $nodD_1$ and nodYABC is repressed by NodD₂ which is encoded by $nodD_2$. NolA product from *nolA* regulates the expression of $nodD_2$. Figure 2.3 summarizes the activation and repression of nodulation gene expression.



Figure 2.3 NodD₁-flavonoid complexes bind to $nodD_1$ box and nodY box in the promoters of $nodD_1$ and nodYABC to activate transcription of $nodD_1$ and nodYABC. A protein product NodV autophosphorylates then transfers the phosphate group to NodW. Phosphorylated NodW-P activates the expression of $nodD_1$ and nodYABC. NolA regulates the expression of $nodD_2$ whose protein product, NodD₂, represses the expression of $nodD_1$ and nodYABC (Loh and Stacey, 2003).

Transcription and translation of *nodA*, *nodB*, and *nodC* lead to the synthesis of the first three enzymes in the synthesis of Nod factor which is essential for root hair deformation and nodulation process. NodC, N-acetylglucosaminyl transferase catalyses the joining of N-acetylglucosaminyl units by Beta 1,4 glycosidic linkages. NodB, N-deacetylase, catalyses the removal of an acetyl group of the N-acetylglucoaminyl group at the non-reducing end of the Nod factor. NodA, N-acyltransferase, catalyses the transfer of an acyl group (C18:1) to the N-glycosyl unit at the non-reducing end of the

Nod factor.Nod factor of *Bradyrhizobium japonicum* consists of 5 N-acetylglucosaminyl units with side chains as indicated in Figure 2.4.



Figure 2.4 Synthesis of Nod factor in *Bradyrhizobium japonicum* (Stacey, 1995).

Other genes essential for nitrogen fixation include *nifH*, *nifD*, and *nifK* which encode subunits of the dinitrogenase reductase (NifH, Fe protein), alpha subunits and beta subunits of the dinitrogenase (MoFe protein, NifDK), respectively (Fuhrmann and Hennecke, 1984). Nitrogenase is made up of a dimer of identical NifH subunits and a tetramer of two alpha and two beta subunits of NifD and NifK. Genetic regulation of *nifH* and *nifDK* in *B. japonicum* via NifA is similar to that reported for the free-living nitrogenase and showed that NifA bound at the upstream activator seqence (UAS) of *nifHDK*. The integration host factor (IHF) bound to the region between NifA binding site and the Sigma-54 holoenzyme of RNA Polymerase or the -24/-12-type promoter region of *nifHDK* as shown in Figure 2.5.

NIFA binding site

126

-139

IHF binding site

E σ 54 promoter

.39

Figure 2.5 IHF binding site is between those of NifA binding site and the -24/-12-type promoter of *nifHDK* in *Klebsiella pneumoniae*. IHF bends the DNA to allow close contact between NifA and Sigma-54-RNA Polymerase holoenzyme for activation of transcription of *nifHDK* (Lee et al., 1993; Santero et al., 1989).

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Nitrogenase activity is sensitive to oxygen because the expression of *nifA* is sensitive to oxygen (Fischer et al., 1986; Fischer, 1996).

There has been no report on metal clusters of *B. japonicum* nitrogenase. However, in the free-living nitrogen-fixer, *Azotobacter vinelandii*, each monomer of NifH or Fe protein contains a 4Fe-4S metal cluster (Georgiadis et al., 1992) while the MoFe protein contains an FeMo cluster and a P cluster. An FeMo cluster is made up of 4Fe-3S and 1Mo-3Fe-3S cluster and a P cluster contains two 4Fe-4S clusters as shown in Figure 2.6a, b (Kim and Rees, 1992, Chan et al., 1993)



Figure 2.6 (a) An FeMo cofactor of MoFe protein of the free-living nitrogen-fixer *Azotobacter vinelandii* contains one 4Fe-3S cluster and one 1Mo-3Fe-3S cluster (b) A P cluster contains two 4Fe-4S clusters (Kim and Rees, 1992).

Figure 2.7 shows catalytic activity of nitrogenase where electrons are transported via the electron acceptor Ferredoxin to the Fe Protein and to the nitrogen substrate which binds to the MoFe protein (Voet and Voet, 1995).



Figure 2.7 Catalytic activity of nitrogenase (Voet and Voet, 1995).

The literature survey on the structural and functional activity of nitrogenase indicates that in order to fix nitrogen, at least the following metals should be available in soils for use by soybean rhizobia in fixing nitrogen: Fe, Mo, S.

All soybean rhizobia present in soybean-cultivating areas are potential candidates for the production of soybean rhizobium biofertilizers to increase soybean yields. In December, 2007 soybean rhizobia were isolated from experimental plots in Nam Moub and Klang Wiang subdistricts in Wiang Sa district, Nan province (Figure 2.8). RAPD-PCR fingerprints of the isolated soybean rhizobia using CRL-7 primer were obtained as shown in Figure 2.9a,b (Chansa-ngavej et al., 2009). Figure 2.9a showed that strain NA7 was isolated from Klang Wiang but not from Nam Moub subdistrict. Therefore, any bacteria with NA7 fingerprints isolated from root nodules of soybean

mixed with biolfertilizer NA7 in Nam Moub subdistrict must come from NA7 in the biofertilizer. Figure 2.9b showed that, in 2006, Nam Moub isolates with identical fingerprints could be grouped into 6 groups represented by NA273, NA274, NA82, NA83, NA160, and NA228.



Figure 2.8 Map showing 11 soybean-growing subdistricts in Wiang Sa district, Nan province. Nam Moub subdistrict is located at the border of Thailand and Loas Republic. (http://www.oae.go.th/gis/images/boundary/nan/wiangsa.jp)





(b) Nam Moub

Figure 2.9 RAPD-PCR fingerprints of soybean rhizobia isolated from root nodules of 7 soybean cultivars (ST1, ST2, ST3, SJ4, SJ5, CM2, CM60) grown in soils from (a) Klang Wiang and (b) Nam Moub subdistricts in Wiang Sa district, Nan province (Chansangavej et al., 2009).

Strain NA7 isolated from Klang Wiang subdistrict was selected for field experiments in the cultivation year 2007/2008 (December-March) in Nam Moub subdistrict, Nan province, with a 15 X 24 m² experimental plot. The average yields of soybean seeds cultivar CM60 mixed with rhizobium biofertilizer NA7 were found to be 231.0 kg.*rai*⁻¹ compared with 223.2 kg.*rai*⁻¹ in the control treatment where soybean seeds were not mixed with any rhizobium biofertilizer and 220.2 kg.*rai*⁻¹ where seeds were mixed with market rhizobium biofertilizer before planting. Rhizobium biofertilizer NA7 was found to retain 10⁸ cells·ml⁻¹ 4 weeks after incubation at 40°C and was found to increase soybean cultivar CM60 yield 4% (Chanthapetch and Chansa-ngavej, 2009). One aim of this thesis is to use DNA fingerprints to detect the ability of soybean rhizobium strain NA7 to nodulate field-grown soybean by isolating bacteria from root

nodules of soybean in the experimental plot one month after cultivation. The presence of NA7 DNA fingerprints among those obtained for root nodule isolates indicated strain NA7 could nodulate soybean roots in the experimental plot in Nam Moub subdistrict. In addition, the thesis research aims to find out the Most Probable Number of soybean rhizobia present in soil in the experimental plot in Nam Moub subdistrict. Seven soybean rhizobia isolated from root nodules of soybeans mixed with biofertilizer NA7 as well as soybean rhizobium strain NA7 will be identical by polyphasic taxonomy. The results obtained would contribute to the number of types of indigenous soybean rhizobia in soybean cultivation areas in Thailand.

In countries which are leading soybean exporters there have been large amounts of research on soybean rhizobium strain selection for inoculant production. (Aguilar et al., 2001; Brutti et al., 1998; Chen et al., 2000; de Jensen et al., 2004; Hungria et al., 2001; Thomas-Oates et al., 2003). In addition, there are many patents on soybean rhizobia and soybean inoculant production in these countries. In the US there is an association known as the American Soybean Association (ASA) which requests assistance from the government for soybean growers when the latter extend the market to high risk countries.(<u>http://www.soy growers.com/step</u>). In Thailand there has been relatively few research on soybean rhizobium (Nuntagij et al., 1997; Shutsrirung et al., 2002a,b,c; Teaumroong and Boonkerd, 1998; Thompson et al., 1991; Yokoyama et al.,1996). Most of the research conducted in Thailand concerns with the isolation and identification of soybean rhizobia from various soybean-growing areas (Nuntagij et al., 1997; Thompson et al., 1991; Yokoyama et al., 1996; Ly and Chansa-ngavej, 2006a,b) Teaumroong & Boonkerd (1998) used primer RAPD (Random Amplified Polymorphic DNA, 5'GGAAGTCGCC3') to obtain fingerprints of 18 B. japonicum isolates from root nodules of soybean which the authors did not specify the cultivar. The authors also obtained fingerprints of 4 strains of soybean rhizobia: TAL377, THA7, THA5, and TAL216 and 4 USDA strains (USDA, United States Department of Agriculture) USDA 8-0, USDA 94, USDA 35 and USDA 117.

At present, there is not much information on polyphasic taxonomy of soybean rhizobia in Thailand. There are several methods to identify soybean bradyrhizobia. For example, in 2008, Appunu and co-workers obtained PCR – RFLP of 16S rDNA of 50 isolated soybean rhizobia by cutting PCR-amplified 16S rDNA with 7 restriction enzymes (*Cfol, Ddell, Haelll, Hinfl, Mspl, Ndell* and *Rsal*), cutting PCR products of the

intergenic spacer region between 16S rDNA with and 23S rDNA (IGS) with *Alul*, *Cfol*, and *Hae*III, and cutting PCR-amplified products of *nifH* with *Cfol*, *Hae*III, and *Mspl*. Patterns of RPLFs obtained were used to group the 50 isolates into 8 haploid genotypes or haplotypes. In addition, the IGS – PCR – RFLP patterns were used to group the 50 soybean rhizobium isolates into 6 IGS types (I - VI). PCR – RFLP of *nifH* was used to group the 50 soybean rhizobium isolates to 3 *nif* types (I - III). Construction of three dendrograms, the first one with 938 bp sequences of IGS of representatives from IGS type I – VI isolates as well as those of several type strains *B. yuanmingense* LMG R16434^T (*Lespedeza*), *B. japonicum* LMG 6138^T (*Glycine*) *B. elkanii* LMG 6134^T (*Glycine*) showed representatives of IGS types I – III isolates had close evolutionary relationship with *B. yuanmingese* LMG R 16434^T (*Lespedeza*). The authors could not identify the isolated representative of bradyrhizobia which was grouped in IGS types V and VI were found in the same cluster as *B. liaoningense* LMG 18230^T (*Glycine*) as shown in Figure 2.10.

Construction of the second dendrogram with concatenated sequences of the housekeeping genes, *dnaK*, *glnII*, and *recA* of representative isolates from IGS types I – IV also revealed IGS types I – III isolates had close evolutionary relationship with *B. yuanmingense* CCBAU 10071^T (Figure 2.11). The construction of the third dendrogram with 612 bp sequences of *nifH* of representatives of *nif* types I – III isolates revealed representatives from *nif* types I and II were closely related to *B. yuanmingense* CCBAU 10071^T (*Lespedeza*) type strain with bootstrap value > 70% and representatives from *nif* type III isolates were closely related to *B. liaoningense* LMG 18230^T (*Glycine*) with 100% bootstrap value (Figure 2.12). The authors concluded that 36% of the isolates were *B. yuanmingense* biovar which could nodulate soybeans, 26% of the isolates were *B. liaoningense* and 38% of the isolates were not the two *Bradyrhizobium* strains but another strain with similar symbiotic genotype to those of *B. liaoningense* and *B. japonicum* bv. *glycinearum*. This is the first report of *B. yuanmingense* biovar which could nodulate soybeans (Appunu et al.,2008)



Figure 2.10 Phylogenetic ML tree based on 938-bp alignment of nucleotide sequences of the IGS between the 16S and 23S rRNA genes (Appunu et al., 2008).





Figures 2.11 Phylogenetic ML tree based on 1,493-bp alignment of concatenated nucleotide sequences of *dnaK* (489 bp), *glnll* (519 bp), and *recA* (482 bp) (Appunu et al., 2008).



Figures 2.12 Phylogenetic ML tree based on 612-bp alignment of nucleotide sequences of the *nifH* gene (Appunu et al., 2008).

CHAPTER III

MATERIALS AND METHODS

3.1 Field trial of soybean rhizobium biofertilizer NA7 at Nam Moub subdistrict

A 15 X 24 m² experimental plot as shown in Figure 3.1 with four 7.5 X 6.0 m² subplots as described by Somasegaran and Hoben (1994) had been set up in December 2007 at Nam Moub subdistrict in the northern part of Thailand with latitude 18^{0} 46' 30" N ; longitude 18^{0} 46' 44" E (Chanthapetch and Chansa-ngavej, 2009).





Figure 3.1 Lay-out of a 15 X 24 m² experimental plot (upper diagram) with a 2.0 X 7.5 m² plot (lower diagram) showing 4 rows of soybean plants which were represented by small circles. Soybean seeds in rows 1 and 4 were not mixed with any biofertilizer. Seeds in rows 2 and 3 were mixed with either biofertilizer NA7 or rhizobium biofertilizer available from the market. Early harvest indicated area where plants with root nodules were collected one month after planting for use in the isolation of bacteria to determine if soybean rhizobium strain NA7 used in the production of biofertilizer could successfully nodulate soybean. Dry weight of seeds of plants in the yield harvest area was obtained for the determination of soybean yield. Darkened areas showed plants whose seeds were not obtained for the determination of soybean yield (Somasegaran and Hoben, 1994).

3.2 Isolation of nodules from soybean collected in the early harvest area

Bacteria were isolated from root nodules of soybean collected from the early harvest area (Figure 3.1). Root nodules collected 28 days after planting were surfaced-sterilized with 5% H_2O_2 and rinsed with sterilized deionized water as described by Jordan (1982). Bacteria isolated from root nodules were grown on plates containing yeast extract mannitol (YM) agar medium with 25 µg. ml⁻¹ congo red. Purified isolate was kept in YM agar slant at 4⁰C for short-term storage and in 10% glycerol for long-

term storage. Each isolate was grown in YM broth at 30° C, 200 rpm, for 4 days for RAPD-PCR fingerprinting. Composition of YM medium was as described by Somasegaran and Hoben (1994) as follows: (g/l), mannitol 10.0; K₂HPO₄ 0.5; MgSO₄ •7H₂O 0.2; NaCl 0.1; yeast extract 0.5; deionized water 1 liter with 25 µg.ml-1 congo red.

3.3 Bacterial strains and isolates

Bacterial strains and isolates used in the experiments consisted of those isolates obtained as described in Section 3.2 as well as the following type strains: *Bradyrhizobium elkanii* Type strain NBRC14791

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Bradyrhizobium japonicum Type strain NBRC14783

Bradyrhizobium liaoningense Type strain NBRC 100396.

3.4 Determination of fast-growing isolates and slow-growing isolates

Each isolate was streaked on YMA containing Congo red at the final concentration of 25 μ g. ml⁻¹ and incubated at 25[°] C for 5 days. If visible colonies were observed at least one day after incubation, the isolate was determined to be a fast-grower. On the other hand, if visible colonies were observed at least 5 days after incubation, the isolate was regarded as a slow-grower.

3.5 Isolation of chromosomal DNA

Slant culture was activated by culturing YM agar slants at 30°C for 2 days. One loop of each activated isolate was inoculated into 50 ml YM medium. The culture was harvested after growing at 200 rpm, 30°C, until mid log phase. 100 μ l 2.5 mg.ml⁻¹ lysozyme was added to the cell pellet, mixed thoroughly, and incubated at 37°C for 1 h before 4 cycles of freezing at –20°C for 5 minutes and thawing at 80°C for 5 minutes. 250 μ l of DNAzol[®] (Invitrogen) was added to the solution which was gently mixed by inverting the eppendorf tubes. The mixture was centrifuged at 10,000 rpm, 4°C, for 5 minutes. The supernatant was transferred to a fresh eppendorf tube. DNA was precipitated with 500 μ l ice-cold ethanol at -80° C for 15 min. The mixture was centrifuged at 10,000 rpm, 4°C, for 15 minutes, washed with 70% ice-cold ethanol, air dried and dissolved in high-purity distilled water. Quantity and quality of the isolated DNA were determined by absorbance at 260 nm and OD_{260}/OD_{280} ratios followed by 0.8% agarose gel electrophoresis by standard methods (Sambrook et al., 1989).

3.6 RAPD-PCR fingerprinting (Welsh and McClelland, 1990)

Sequence of RPO1 was as described as Richardson et al. (1995). Sequence of primer CRL-7 was 5'GCCCGCCGCC 3' (Mathis and McMillin, 1996). RPO1 is the 20 mer in the unserved sequence of *nifH* of *Rhizobium trifolii* (Schofield and Watson, 1985). Composition of PCR mixture was as follows: 10x PCR buffer 2.0 µl, 10mM dNTPs 2.0 µl, 100 µM primer CRL-7 0.2 µl,100 µM primer RPO1 0.4 µl, DNA template 60-100 ng) 1.00 µl, *Taq* polymerase (5U. μ I⁻¹) 0.2 µl, distilled water to 20 µl. PCR program: 95 ° C 15 seconds, 55° C 30 seconds, 72° C 90 seconds for 5 cycles, 95° C 15 seconds, 60° C 30 seconds for 25 cycles, followed by 72°C 10 minutes. Isolates with identical RAPD-PCR fingerprints were put into the same groups.

3.7 Authentication test of soybean rhizobia

Five ml of each bacterial strain grown in YMB at 200 rpm, 30[°] C, for 4 days, were added onto germination soybean seeds cultivar ST1, ST2, SJ5, CM2 and CM 60 in Leonard jars. Preparation of Leonard jars was as described by Somasegaran and Hoben (1994). The control jar received no inoculation. Soybeans were fed with Nitrogenfree medium. Composition of Nitrogen-free medium for soybeans was given in Appendix A. Leonard jars were placed in a 28[°]-32[°]C temperature-controlled greenhouse for 28 days before observing root nodulation. If root nodules were detected, the isolate was regarded as a soybean rhizobium.

3.8 Polyphasic taxonomy of soybean rhizobia

3.8.1 Colony morphology

Cells of each strain in slant culture were streaked on petri dish with YMA containing Congo red at the final concentration of 25 μ g. ml⁻¹. Plates were incubated at 25[°] C for 10 days before observing colony morphology.

3.8.2 Bromthymol blue reactions

Cells of each strain in slant culture were streaked on petri dish with YMA containing Bromthymol blue at the final concentration of 25 μ g. ml⁻¹. Plates were incubated at 25[°] C for 5-10 days before observing Bromthymol blue reactions. Resultant yellow colonies indicate cells secreted acidic product(s) which changed the dye from blue to yellow color. Blue colonies indicated cells secreted alkali product(s) which did not change the color of Bromthymol blue (Somasegaran and Hoben, 1994).

3.8.3 Determination of type and number of flagella by negative staining

Cells of each soybean rhizobial strain were streaked on petri dish with YMA containing Congo red at the final concentration of 25 μ g. ml⁻¹, incubated at 25^o C for 5 days. A small drop of distilled water was placed next to a single colony. The plate was tilted to run water through the colony to create a cell suspension. A Pasteur pipet was used to gently place the cell suspension onto an electron microscope copper grid, and left for one minute. The grid was partially dried with the ragged torn edge of a Whiteman no. 1 filter paper. The cells were stained with 1% Phosphotungstic acid for one minute. The grid was immediately, swiftly, and completely dried with the ragged torn edge of a Whiteman no.1 filter paper and left to dry in a desiccator overnight before observing under a transmission electron microscope (JEOL model JEM-2100) at the Scientific and Research Equipment Center, Chulalongkorn University.

3.8.4 Determination of growth at different temperatures

Cells of each soybean rhizobium strain in slant culture were activated by streaking onto plate containing YMA plus Congo red as previously described. Seed culture was prepared by inoculating one loop of activated cells into 50 ml YMB in an Ehrenmeyer flask. The culture was incubated in an incubator shaker at 200 rpm, 30^oC for 4 days. Five ml of seed culture were distributed into 45 ml of YMB in 250 ml Ehrenmeyer flasks. The flasks were placed in temperature-controlled incubator shakers set at 25 °C, 30 °C, 37 °C, and 40 °C. At one day intervals, 0.5 ml samples were taken, serially-diluted and 0.1 ml was plated onto plate containing YMA plus Congo red as previously described and incubated at 25 °C for 5 days before counting colony forming units (CFU) to determine growth as CFU/ml over incubation time.

3.8.5 Determination of the ability to use or not use carbon and nitrogen sources

Biolog test kit was used in the determination of the ability to use or not use 95 carbon and nitrogen sources according to the manufacturer's instruction. Soybean rhizobium cells from each slant culture were streaked onto plates containing YMA plus Congo red as previously described. Plates were incubated for 5 days at 25 °C. Cells were scraped into Biolog's inoculation fluid and the percentage of transmission adjusted to 52% on a spectrophotometer. 150 µl inoculation suspension culture was aseptically added into each well of the Biolog's 96- well plate, incubated at 30° C for 24 hours before obtaining optical density readings at 590 and 750 nanometers. The in-built program of the Biolog processing unit was used to calculate the dual wavelength readings compared with the reading in Well A1 which is a control with neither carbon nor nitrogen sources. Dual wavelength readings of more than twice that of the control well were interpreted as an ability to use the carbon or nitrogen source (+). Two plus symbol (++) and three plus symbol (+++) were used to indicate dual wavelength readings of three and five times of those of the control well respectively.

3.8.6 Isolation, sequencing, and dendrogram construction with sequences of 16S rDNA

16S rDNA of soybean rhizobium strain NA7 as well as six isolated soybean rhizobium strains were obtained by PCR using 27f and 1492r as the primers. Sequences of the primers 27f and 1492r were as described by Dorsch and Stackerbrandt (1992) as follows: 5'GAGTTTGATCCTGGCTCAG3' and 5'ACGGCTACCTTG TTACGACCT3'. PCR mixture consisted of 10x PCR buffer 2 μl, 10mM dNTPs 2 μl, primer 27f (10 pmol•μl⁻¹)

and primer 1492r (10 pmol·µl⁻¹) 0.5 µl, DNA 200 ng, *Taq* polymerase(5 units·µl⁻¹) 0.2 µl, distilled water to 20 µl. PCR program was as follows: 95°C 30 seconds, 95°C 60 seconds, 48°C 60 seconds, 72°C 120 seconds (30 cycles) followed by 48°C 60 seconds, 72°C 300 seconds (1 cycle). PCR mixture was sent to the Genome Institute for DNA sequencing with the following 9 primers : 27f, 1241f, 1492r, 1385r, 1100r, 907r, 787r, 509r, and 343r. Sequences of the 9 primers are as described by Dorsch and Stackerbrandt (1992). Soybean rhizobia were identified by comparisons of 16S rDNA sequences with those deposited with the GenBank data base.

3.9 Determination of soybean rhizobium number by the Most Probable Number Technique (MPN)

3.9.1 Preparation of plastic growth pouches for planting

A paper wick with hole to allow for elongation of soybean root was put in each plastic growth pouch (Figure 3.2). All pouches were sterilized at 121°C for 15 minutes.

3.9.2 Planting seeds in growth pouches

Soybean seeds cultivar CM 60 were surface-sterilized by 95% ethanol and 5% H_2O_2 (Jordan,1982). The seeds were placed on seedling agar (0.75% agar) and incubated at 25°C in darkness for 36 hr. 35-45 well-germinated seeds of similar size and radical length (1-1.5 cm) were selected and placed 1 seed in each pouch. 100 ml of sterilized nitrogen-free medium, pH 6.8, were put in the pouches (Somasegaran and Hoben, 1994). The pouches were arranged in a rack and incubated at 25° C in a temperature-controlled illuminated incubator.

3.9.3 Determination of the MPN

One gram of soil sample from the experimental plot in Nam Moub subdistrict was put in distilled water and five-folded serial dilutions were carried out as indicated in Figure 3.3. One ml of soil suspensions at each dilution level was added into the pouches (four replicates). MPN was calculated after 4 weeks using the MPN Table (Appendix G).



Figure 3.2 (a) Plastic growth pouches in rack (b) 4 weeks old soybean plant (c) soybean plants in plastic pouches in rack.



Figure 3.3 Serial dilutions for MPN (Somasegaran and Hoben, 1994).

จุฬาลงกรณ์มหาวิทยาลัย
CHAPTER IV

RESULTS

4.1 Isolation of fast- and slow-growing bacteria from root nodules

A total of 198 bacteria were isolated from root nodules of soybean cultivar CM 60 mixed with soybean rhizobium biofertilizer NA7 and planted in the experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province. Based on the length of time required for colonies to be visible on YMA (Yeast extract Mannitol Agar with 25 µg•ml⁻¹ Congo red) plates, the bacteria were found to comprise of 147 fast-growing isolates and 51 slow-growing isolates.

4.2 DNA fingerprints of slow-growing bacterial isolates from root nodules

RPO1

2000 1650 1000

Figure 4.1 showed RAPD-PCR DNA fingerprints of soybean rhizobium strain NA7 and 51 slow-growing bacteria isolated from root nodules of soybean cultivar CM 60 mixed with soybean rhizobium biofertilizer NA7 and planted in the experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province. The results showed 13 out of 51 slow-growing isolates or 13 out of the total of 198 isolates had identical fingerprints with those of strain NA7. Assuming that one bacterial isolate was obtained from one nodule, 6.6% of the total number of soybean nodules were found to be occupied by strain NA7.

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Figure 4.1 RAPD-PCR DNA fingerprints of soybean rhizobium strain NA7 and 51 slowgrowing bacteria isolated from root nodules of soybean cultivar CM 60 mixed with soybean biofertilizer NA7 and planted in the experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province. Either primer RPO1 or CRL-7 was used in the DNA fingerprinting. Arrows indicated bacterial isolates with identical DNA fingerprints to those of soybean rhizobium strain NA7. Lane (M) is molecular size standard.

Comparisons of identical fingerprints shown in Figure 4.1 indicated that the 51 slow-growing isolates consisted of 8 groups (including strain NA7) as shown in Table 4.1. and Appendix C. Therefore, out of the 51 slow-growing isolates, 8 groups of slow-growing bacteria were isolated from the root nodules.

In addition, identical DNA fingerprints of isolates in the same groups as NM12-42, NM22-5, and NM22-7 were found to be the same strains as NA160, NA82, and NA273 originally isolated in 2006 (Chansa-ngavej et al., 2009). Four groups of root nodule isolates represented by NA22-2, NM22-3, NM22-18, and NM22-30 were not isolated in 2006. Fingerprints of all the 5 root nodule isolates obtained in this thesis are shown in Appendix C.

Table 4.1 Slow-growing bacteria with identical DNA fingerprints were put into the same groups. A total of 8 slow-growing strains (including strain NA7) were isolated from root nodules of soybean cultivar CM 60 mixed with soybean biofertilizer NA7 before planting.

Groups	Isolates with identical DNA fingerprints	Isolated from Nam Moub in 2006 by Chansa-ngavej et al. (2009)
NA7	NM22-1, NM22-4, NM22-10, NM22-12, NM22-16, NM22-19,	No
	NM22-20, NM22-23, NM22-25, NM22-27, NM22-33, NM22-34,	
	NM22-38	
NM12-42	NM22-15, NM22-21, NM22-24, NM22-29, NM22-31, NM22-35,	Yes (NA160)
	NM22 <mark>-5</mark> 1, N <mark>M3</mark> 2-57	
NM22-2	NM22-44	No
NM22-3	NM22-1 <mark>3, NM22-17, NM22-32, NM22-37, NM22-39, N</mark> M22-41,	No
	NM22-42, N <mark>M</mark> 22- <mark>5</mark> 0, NM32-56	
NM22-5	NM22-9, NM22 <mark>-1</mark> 1, NM22-14, NM22-28, NM22-36, NM22-43	Yes (NA82)
NM22-7	NM22-8, NM22-22, NM22-40, NM22-47	Yes (NA273)
NM22-18	NM22-26, NM32-48	No
NM22-30	NM22-45	No

4.3 Polyphasic taxonomy of 6 bacterial isolates

4.3.1 Authentication tests of 6 bacterial isolates

The following six bacterial isolates : NM22-8, NM22-11, NM22-13, NM22-15, NM22-25, and NM22-30 which were representatives of the members in each group having identical DNA fingerprints were selected for authentication tests to determine if they were soybean rhizobia. Figures 4.2(a)-(e) showed the isolates produced nodules on roots of 5 soybean cultivars (ST1, ST2, SJ5, CM2 and CM60). Therefore, the bacterial isolates were soybean rhizobia.

NM22-8



(a)









NM22-15



(d)



NM22-30



Figures 4.2 (a)-(f) showed the representative isolates from 6 groups produced nodules on roots of 5 soybean cultivars (ST1, ST2, SJ5, CM2 and CM60).

4.3.2 Colony morphology

Figure 4.3 showed colony morphology of soybean rhizobium strains consisting of strain NA7 and the representatives from the 6 groups of bradyrhizobia grown on YMA plus Congo red plates. All colonies did not absorb Congo red. In addition, all colonies produced copious amounts of extracellular polysaccharides which is one characteristics of soybean rhizobia (Jordan, 1982). Two types of colonies were observed : Type 1 (NA7, NM22-11, NM22-13, NM22-15 and NM22-25) with irregular, slimy colonies, and Type 2 (NM22-8 and NM22-30) with round, pearly, and not so slimy colonies.



Figure 4.3 Colony morphology of soybean rhizobium strains including strain NA7 and the 6 selected strains grown on YMA plus Congo red plates.

4.3.3 Bromthymol blue reactions

Figure 4.4 showed Bromthymol blue reactions of soybean rhizobium strains NA7 and the 6 selected strains which were representatives from 6 groups of bradyrhizobia grown on YMA plus Bromthymol blue plates. The results showed strains NM22-8 and NM22-30 strongly secreted acidic products while other strain secreted alkali products. The results showed physiological variability in the selected rhizobium strains. Correlations were observed between the Bromthymol blue alkali reaction and the previously-observed irregular, slimy colonies and between the Bromthymol blue acidic reaction and the previously-observed round, pearly, and not so slimy colonies (Figure 4.3).



Figure 4.4 Bromthymol blue reactions of soybean rhizobium strains including strain NA7 and the 6 selected strains grown on YMA plus Bromthymol blue plates.

4.3.4 Number and type of flagella

Figure 4.5(a)-(e) showed negative staining results for 5 representative strains from the 5 groups of soybean rhizobia. All strains contained one subpolar flagellum as expected (Jordan, 1982).



Figure 4.5 (a)-(e): Negative staining results for 5 selected strains of soybean rhizobia.

4.3.5 Growth at different temperatures

Figure 4.6 showed growth of strain NA7. The results showed strain NA7 did not increase in number when incubated at 37° C and 40° C. Therefore, strain NA7 could be used to produce lab-scale rhizobium biofertilizer based on the ability to maintain cell numbers while the biofertilizer was kept at room temperature.





Figure 4.6 Growth of soybean rhizobia including strain NA7 and representative soybean rhizobium strains from 6 groups at different temperatures.

The results in Figure 4.6 indicated that all the isolated soybean rhizobia decreased in colony number at a faster rate than strain NA7. Therefore, they may not be suitable for use in the production of soybean rhizobium biofertilizers that can be kept at room temperature because cells will decrease in number upon storage at room temperature.

4.3.6 Utilization/Non utilization of carbon and nitrogen sources

Appendix D showed results obtained with the Biolog test kit on the utilization/non-utilization of 95 carbon and nitrogen sources by three reference strains. The consensus results were obtained from 7 determinations. Since the Biolog machine at the Center for Agricultural Biotechnology of Kasetsart University at Kampangsaen campus, Nakorn Pathom province, was used, one reason the Biolog test was conducted 7 times for the reference strains because there had been some delay in the transport of cell cultures from Bangkok to Nakorn Pathom therefore the age of the culture might play a part in the results on the utilization and non-utilization of the carbon and nitrogen sources. However, the Biolog results presented in this thesis were as accurate as possible under the constraint of the time taken to reach the Kamphangsaen campus for the use of the Biolog machine. In addition, in this research, it was planned that the strain identification would be obtained by 16S rDNA sequences followed by confirmation by the Biolog results which formed a part of polyphasic taxonomy employed in this research.

Results of the Biolog test kit by the three reference strains in Appendix D showed that the carbon sources that could be used by all the three reference strains were found to be Tween 40, Tween 80, L-Arabinose, Pyruvic acid methyl ester, Succinic acid mono-methyl-ester, Acetic acid, D-Gluconic acid,
-Hydroxybutyric acid,
-Keto glutaric acid, D,L-Lactic acid, Propionic acid, Sebacic acid, Succinic acid, Bromosuccinic acid and Succinamic acid.

The following carbon and nitrogen sources were not utilized by the three reference strains : -Cyclodextrin, Glycogen, N-Acetyl-D-Galactosamine, N-Acetyl-D-Glucosamine, Adonitol, D-Cellobiose, i-Erythritol, Gentiobiose, m-Inositol, -D-Lactose, Lactulose, Maltose, D-Melibiose, -Methyl-D-Glucoside, D-Psicose, D-Raffinose, L-Rhamnose, D-Sorbitol, Sucrose, D-Trehalose, Tulanose, Xylitol, Cis-Aconitic acid, D-Glucoaminic acid, , D-Glucuronic acid, p-Hydroxy phenylacetic acid, -Keto butyric acid, -Keto valeric acid, Malonic acid, Glucuronamide, L-Alanyl-glycine, L-Asparagine, Glycyl-L aspartic acid, Glycyl-L-glutamic acid, L-Histidine, Hydroxy-L-Proline, L-Ornithine, L-Proline, L-Serine, L-Threonine, D,L-Carnitine, Urocanic acid, Inosine, Uridine, Thymidine, Phenylethyl-amine, Putrescine, 2-Aminoethanol, 2,3-

The following carbon source could be used by both *B. elkanii* and *B. japonicum* : L-Fucose, Citric acid, Formic acid, D-Alanine, L-Pyroglutamic acid.

No carbon nor nitrogen sources could be used by both *B. elkanii* and *B. liaoningense*.

Both *B. japonicum* and *B. liaoningense* were found to use D-Mannose, L-Aspartic acid and L-Leucine.

The following carbon and nitrogen sources could be used by *B. japonicum* only : Dextrin, D-Arabitol, D-Fructose, D-Mannitol, D-hydroxybutyric acid, L-Alanine, L-Phenylalanine, and Glycerol.

The following carbon and nitrogen sources could be used by *B. liaoningense* only: –D-Glucose, D-Galactonic acid lactone, D-Galacturonic acid, Itaconic acid, and r-Amino butyric acid.

Based on the positive Biolog results on the reference strains, the results seemed to suggest a close similarity the between *B. elkanii* and *B. japonicum* because both were found to use 5 common carbon/nitrogen sources which *B. liaoningense* could not use. A close relationship was also observed between *B. japonicum* and *B. liaoningense* because both were found to use 3 common carbon/nitrogen sources which *B. elkanii* and *B. elkanii* could not use. Both *B. elkanii* and *B. liaoningense* did not share the use of common carbon/nitrogen sources.

In conclusion, based on the common positive Biolog results on the carbon and nitrogen sources that could be used by two strains, *B. elkanii* was found to be closely related to *B. japonicum* and *B. japonicum* was found to be closely related to *B. liaoningense*.

Appendix E showed Biolog results of the representative isolates from 6 groups. Table 4.2 summarized the identification of the strain NA7 and 6 representative groups of bradyrhizobium strains using the Biolog test results. Strain NM22-8 was found to use 17, 31, and 24 carbon and nitrogen sources differently from *B. elkanii* NBRC 14791, *B. japonicum* NBRC 14783, and *B. liaoningense* NBRC 100396 respectively. Therefore, NM22-8 was found to be similar to *B. elkanii* NBRC 14791. By the same kind of analysis of results, NM22-11 was found to use 4, 19, and 14 carbon and nitrogen sources differently from *B. elkanii* NBRC 14791, *B. japonicum* NBRC 14783, and *B. liaoningense* NBRC 100396 respectively. Therefore, NM22-11 was found to be closely similar to *B. elkanii* NBRC 14791. NM22-13, NM22-15, NM22-25, NM22-30, and NA7 were also found to be *B. elkanii* (Table 4.2).

Table 4.2 Identification of the strain NA7 and representative strains from 6 groups of bradyrhizobium strains by using Biolog test results on the utilization/non-utilization of 95 carbon and nitrogen sources.

	Number of carb			
Strains	the reference	Identification		
Otrains	B. elkanii	B. japonicum	B. liaoningense	Identification
	NBRC 14791	NBRC 14783	NBRC 100396	
NA7	12 (11.4%)	23 (21.9%)	15 (14.3%)	B. elkanii
NM22-8	17 (16.2%)	31 (29.5%)	24 (22.8%)	B. elkanii
NM22-11	4 (3.8%)	19 (18.1%)	14 (13.3%)	B. elkanii
NM22-13	5 (4.8%)	20 (19.0%)	11 (10.5%)	B. elkanii
NM22-15	8 (7.6%)	17 (16.2%)	14 (13.3%)	B. elkanii
NM22-25	4 (3.8%)	19 (18.1%)	13 (12.4%)	B. elkanii
NM22-30	15 (14.3%)	27 (25.7%)	22 (<mark>20.9</mark> %)	B. elkanii

4.3.7 Identification by 16S rDNA sequences of strain NA7 and representatives of soybean rhizobia from 6 groups

Figure 4.7 showed 16S rDNA nucleotide sequence of soybean rhizobium strain NM22-8. Comparisons of 16S rDNA sequence of strain NM22-8 (Length=1455 bp) with corresponding sequences deposited at GenBank indicated the strain could be Bradyrhizobium japonicum USDA 110 with identities= 1450/1456 (99%), gaps = 5/1456 (0%), or *Bradyrhizobium japonicum* strain USDA 62 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or Bradyrhizobium sp. SEMIA 5083 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or Bradyrhizobium sp. SEMIA 6059 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or *Bradyrhizobium* sp. SEMIA 5021 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or *Bradyrhizobium* sp. SEMIA 5036 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or Bradyrhizobium sp. SEMIA 5043 with identities = 1450/1456 (99%), gaps = 5/1456 (0%) or *Bradyrhizobium* sp. SEMIA 5060 with identities = 1450/1456 (99%), gaps = 5/1456 (0%), or Bradyrhizobium sp. SEMIA 5020 with identities = 1450/1456 (99%), gaps = 5/1456 (0%) or *Bradyrhizobium* sp. SEMIA 510 with identities = 1450/1456 (99%), gaps = 5/1456 (0%). Alignments of 16S rDNA sequences of the above soybean rhizobial strains as shown in Appendix G showed only one or two nucleotides difference among the strains. According to Binde et al. (2009) strains with less than 15 nucleotides difference could be the same strain. If this criterion is accepted, NM22-8 could be identified as Bradyrhizobium japonicum.

1492r M22-0 150 130 120 COOSGAACGT ATTCACCOTO OCOTOCTGAT CCACGATTAC TAOCGATTCC AACTTCATOG OCT 10122-8 CCATOGTOTO ACOGOCOO 220 1385r 10122-8 380 1241f 310 COTAROGOCC ATGREGATTY GROUTCATCC CORCUTTCCT COCOGCUTAT CRCCGOCRGT CTCCTTRERS TECTCARCTA RATEGINGCA ACTAROGREG 10122-0 10122-8 1100r \$10 520 430 540 550 540 570 TTOGA CATOTICAAGE GETGGTAAGE TTCTGCCGET TGCTCGAAT TAAACCACAT GCTG TETETGEGAE COGTECTOGA CATOTEAAGG GETE HH22-8 630 610 620 630 640 650 650 650 FOR THE THE CONCEPTION OF THE THE CONCEPTION OF THE CONC 610 640 650 660 NH22-8 907r 710 907/ 710 720 730 740 750 760 770 780 790 800 MH22-8 ezo 787r eso 840 GTTCTTOCGA ATATCTACGA ATTTCACCTC TACACTOGCA GTTCCACTCA CCTCTCCCGA ACTCAAGATC TTCAGTATCA AAGGCAGTTC TGGAGTTGAG 10122-0 920 940 950 CTCCASGATT TCACCCTCA CTTAAAGACC CGCCTACGCA CCCTTTACGC CCASTGATTC CGAGCAACGC TAGCCCCCTT CGTATTAA MM22-8 1090 519r 1020 1070 1010 1030 1040 1050 1060 1000 1100 CACGAROTTA OCCOORDETT ATTETTOCOS TACCOTCATT ATETTOCCOC ACARARORDE TITACARCE TROSDECTTE ATERCTCRES CORCATORET H122-8 1110 1120 1130 114 GGATCAGGGT TGCCCCCATT GTCCAATATT CCCCACTGCT 1130 1170 1160 1160 1140 1180 TA GEASTITIGES CONTENENA STOCCAATET GECTEATEAT COTOCCAGAC 10122-0 1210 1220 1290 1260 1260 1260 1270 1200 1290 1300 CAGCTACTGA TCGTCGCCTT GGTAGGCCGT TACCCTACCA ACTAGCTAAT CAGACGCGGG CCGATCTTTC GGCGATAAAT CTTTCCCCGT AAGGGCTTAT 10122-0 1310 1920 1930 1340 1350 1960 1970 1900 1990 140 CCGGTATTAD CACABGTITC COTOTOTTOT TOCOBACCAR ARGOTACGTT CCCACGCGTT ACTCACCCCT CTGCCGCTGA CGTATTGCTA CGCCCCGCTCG 10122-8 1450 1410 1420 1430 14 ACTTGCATGT GTTAAGCCTG CCGCCAGCGT TCGCTCTGA 1440 10122-0 27f

Figure 4.7 16S rDNA sequence of soybean rhizobium strain NM22-8. Nucleotide sequences of sequencing primers were shown in boxes.

Figure 4.8 showed nucleotide sequence of 16S rDNA of soybean rhizobium strain 22-11. Comparisons of 16S rDNA sequence of strain NM22-11 (Length= 1456 bp) showed the strain could be *Bradyrhizobium elkanii* strain GZ1 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6099 with identities = 1450/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain S 127 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain S strain SEMIA 6096 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6096 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6414 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6416 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6405 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6416 with identities = 1451/1455 (99%), gaps = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain SEMIA 6416 with identities = 1451/1455 (99%), gaps = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhiz*

SEMIA 5002 with identities = 1451/1455 (99%), gaps = 4/1455 (0%). Alignments of the above-mentioned sequences were shown in Appendix G. The results indicated that strain NM22-11 was *Bradyrhizobium elkanii*.

10122-11 110 1492r 110 1492r 120 130 140 150 160 170 180 190 20 MM22-11 ATOSTOTOA GOGZOOTOTO TACAADOSCC GOGAACGTAT TCACCOTOC GTOCTOATCC ACGATTACTA CCOATTCCAA CTTCATOGOC TCOAGTTCCA 250 260 220 1385r 230 240 210 GAGCCCAATC CGAACTGAGA CGGCTTTTTG AGATTTGCGA AGGGTCGCCC CTTAGCATCC CATTGTCACC GCCATTGTAG C 10122-11 380 1241f 310 320 330 340 350 360 370 10122-11 TAAGGGCCAT GAGGACTTGA CGTCATCCCC ACCTTCCTCG CGGCTTATCA CCGGCAGTCT CCTTAGAGTG CTCAACTAAA TGGTAGCAAC TAA 410 460 420 400 440 450 470 400 490 C OTTOCOGOAC TTAACCCAAC ATCTCACGAA CACCAGETGA CGACAGCCAT GCAGCACCTG TCTCCGGTCC AGCCGAACTG AAGA MM22-11 1100r 810 850 \$70 820 830 540 560 580 \$90 18422-11 TCTCTOGAGT CCGCGACCGG GATGTCAAGG GCTGGTAAGG TTCTGCGGGT TGCGTCGAAT TAAACCACAT GCTCCACCGC TTGTGCGGGGC CC 650 660 620 630 640 670 680 610 690 TTOACT TITAATETTE COACCETACT COCCASCOS AATOCITAAA COSTACCTS COCCACTACT CASTAAACCC ACTAACCECT COCATTCATC MM22-11 907r 710 720 730 740 750 760 770 700 790 800 CCA DOCTATETAA TECTOTITICE TECECACOET ITEGTOCETE ADEGTEADTA TEGODECAGT GAGEGEEETT COCCACTOOT 770 HH22-11 GTTTACGOCG TOGACTACCA 820 787r 810 830 840 850 860 870 880 890 10122-11 GTTCTTGCGA ATATCTACGA ATTTCACCTC TACACTCGCA GTTCCACTCA CCTCTCCCGA ACTCAAGATC TTCAGTATCA AAGGCAGTTC TGGAGTTGAG 940 950 960 970 910 920 930. 990 10122-11 CTCCAGGATT TCACCCCTGA CTTARAGACC COCCTACGCA CCCTTTACGC CCAGTGATTC CGAGCAACGC TAGCCCCCTT CGTA 0001 0501 0101 1040 1050 1060 1070 1000 1090 519r 1100 NER22-11 CACGAAGTTA GCCGGGGCTT ATTCTTGCGG TACCGTCATT ATCTTCCCGC ACAAAAAAGAGC TTTACAACCC TAGGGCCTTC ATCACTCACG CGGCATGGCT 1100 1110 1120 1140 1150 18822-11 00ATCAGGET TOCCCCATT GTCCAATATT CCCCACTOCT OCCTCCCOTA GC 1110 1200 1160 1170 1190 COTA GGAGTITOGG CCGTOTCTCA GTCCCAATGT GGCTGATCAT CCTCTCAGAC 1240 343r 1280 1210 1220 1230 1260 1270 1280 1290 1300 10122-11 CADETACTOR TEGTEGECETT OGTORAGECAT TACCTERECA ACTAGETART CADACGEOGG CEGATETTE GOCGATARAT CTTTECCCCT ARGONCTTAT 1320 1310 1320 1330 1340 1350 1360 1370 1380 140 18822-11 CCOSTATTAS CTOAAGTITC CETCAGTTOT TCCOAACCAR AROSTACGTT CCCACGCGTT ACTCACCCCT CTGCCGCTCA CATATTOCTA TGCCGCGCTG 1430 1450 1440 1410 1420 18422-11 ACTTOCATOT GTTAAGCCTG CCOCCAGCOT TCOCTCTGAG CAO ATCAR ACTORS 27f

Figure 4.8 16S rDNA sequence of soybean rhizobium strain NM22-11. Nucleotide sequences of sequencing primers were shown in boxes.

Figures 4.9, 4.10, 4.11, 4.12, and 4.13 showed 16S rDNA sequences of soybean rhizobia strains NM22-13, NM22-15, NM22-30, NM22-25, and NA7 respectively. Alignments of the sequences as shown in Appendix F indicated that strains NM22-13, NM22-15, NM22-25, and NA7 were the same strain as strain NM22-11 which was *Bradyrhizobium elkanii*. Strain NM22-30 was found to be the same as strain NM22-8 which was found to be *Bradyrhizobium japonicum*.

MH22-13	CTACOGCTAC	CTTGGTTCGA	CTTCACOCCA	40 GTCGCTGACC	CTACCGTGGC	CGGCTGCCCC	CTTTCGGTTA	GCGCACCGTC	TTCAGGTAAA	ACCAACTOCC
10122-13	ATGGTGTGAC	6000000000	TACAAGGCCC	GGGALCGTAT	TCACCGTGGC	GTGCTGATCC	ACGATTACTA	GCGATTCCAA	CTTCATGGGC	TCGAGTTGCA
10122-13	SIGAGCCCRATC	CGAACTGAGA	1385r 230	AGATTTGCGA	LGGGTCGCCC	CTTAGCATCC	CATTGTCACC	GCATTOTAG	CACGTOTOTA	GCCCAGCCCG
10122-13	TALGOGCCAT	GROOPCTTON	0 330 CGTCATCCCC	ACCTTCCTCG	COOCTTATCA	CCGGCAGTCT	CCTTAGAGTG	о 380 стеллетала	1241f 390 TGGTAGCAAC	TAAGGACGCG
10122-13	OGTTOCOCTC	420 00000000000000000000000000000000000	TTAACCCAAC	ATCTCACGAC	ACGAOCTOAC	GACAGCCATO	CAGCACCTGT	ettecootteca	GTCAGAACTO	AAGAACTCCG
MH22-13	1100r BLO	CCGCGACCGG	GATOTCAAGO	GCTOGTAAGG	TTCTOCOCOT	TOCOTCOANT	TAAACCACAT	octocacooc	590 TTGTGCGGGC	CCCCGTCAAT
BH22-13	TCCTTTOAOT	620 1111AATCTT0	COACCOTACT	640 CCCCA00C00	44TOCTTAAA	OCOTTAGCTO	COCCACTAGT	GAGTAAACCC	ACTAACGOCT	00000000000000000000000000000000000000
HH22-13	907r 710	TOGACTACCA	COGTATCTAL	TCCTOTTTOC	TCOCCCOOCT	TTCGAGOCCT	CAGCGTCAGT	ATCGGGCCAG	TGAGCCGCCT	000 11 TCOCCACTOG
MH22-13	TGTTCTTGCG	AATATCTACG	AATTTCACCT	CTACACTOGC	AGTTCCACTC	ACCTCTCCCG	AACTCAAGAT	CTTCAGTATC	AAAGGCAGTT	900
M122-13	GCTCCAGGAT	TTCACCCCTG	ACTTAAAGAC	CCGCCTACGC	ACCETTIACS	CCCAGTGATT	CCGAGCAACG	CTAGCCCCCT	TCOTATTACC	OCGGCTGCTG
10122-13	GCACGAAGTT	0 102	TATTCTTGCG	GTACCGTCAT	0 105 TATCTTCCCG	CACAAAAGAG	CTTTACAACC	0 100 CTAGGGCCTT	0 109 CATCACTCAC	GCGGCATGGC
10122-13	TOGATCAGOC	0 112 TTGCGCCCAT	TGTCCAATAT	TCCCCACTOC	TOCCTOCOT	0 116	e 117 eccetetete	AGTCCCAATG	TGGCTGATCA	TCCTCTCAGA
10122-13	CCAGCTACTG	0 122 ATCGTCGCCT	TGGTGAGCCA	0 124	AACTAGCTAA	0 126 TCAGACGCGG	0 127 GCCGATCTIT	CGGCGATAAA	0 129 TCTTTCCCCG	TAAGGGCTTA
10122-13	TCCOGTATTA	0 192 GCTGAAGTIT	CCCTCAGTTG	TTCCGAACCA	AAAGGTACGT	o 136 TCCCACGCGT	0 137	o 130 TCTGCCGCTG	0 139 ACATATTOCT	0 1400 11 ATGCCCGCTC
10122-13	0ACTTOCATO	TOTTAAOCCT	o 143	TTCOCTCTOA	CA0000ATCA	AACTCAA	0 147	1		

Figure 4.9 16S rDNA sequence of soybean rhizobium strain NM22-13. Nucleotide

sequences of sequencing primers were shown in boxes.

АССССАВ ТСАСТБАССС ТАССБТОВСС С СТТТССАТТАВ СОСАССАТСТ ТСАСБТАААА ССАА 10122-15 110 1492r 130 140 150 160 170 180 190 120 TOUTUTOACO COCOTUTOT A CCCO GGAACGTATT CACCOTOGOG TOCTGATCCA CGATTACTAG CGATTCCAAC TTCATGOGCT CGAGTTGCAG N22-15 220 1385r 210 220 ¹³⁸⁵ 230 240 250 260 270 280 NH22-15 ASCCCARTCC GARCTGAGAC GOCTITITGA GATTGGGAA GGGTGGCGCCC TIASCATCCC ATTGTGACCG CCATTGTAGC ACCTON 230 240 290 TOTAS CCCASCCOST 920 310 260 330 340 350 370 000 1241f 390 AAGGGCCATG AGGACTTGAC GTCATCCCCCA CCTTCCTCGC GGCTTATCAC CGGCAGTCTC CTTAGAGTGC TCAACTAAAT GGTAGCAACT AAGGACG 10122-15 410 420 430 440 450 460 470 480 490 TES TECCOGGACT TAACCCAACA TETERCOACA CONSETURE ACACCENTEE ACCACETOTE TECCOTECAS CEGNACTORA GAAC Qf22-15 1100r 510 610 620 630 640 650 660 670 680 690 70 CAUGTET TAATCTTEGG ACCEDENCE CANGEGORA TOCTTATAGE GTTACCTCC CALCOUTOG CATTCATCCT MI22-15 720 730 740 750 760 770 780 790 CCA00 0TATCTAATC CTOTITIOCTC CCCACOCTIT COTOCCTCAS COTOCATATC GOGCCAGTAA GCCGCCTTCG CCAC 907r 710 10122-15 TTACOGCOTO GA ezo 787r 000 040 850 870 890 810 060 880 10122-15 TCTTGCGAAT ATCTACGAAT TTCACCTCTA CACTCGCAGT TCCACTCACC TCTCCCGAAC TCAAGATCTT CAGTATCAAA GGCAGTTCTG GAGTTGAGCT 910 920 930 940 950 960 970 900 CCRAGATTIC ACCOCTACT TAAAGACCCG CCTACGCACC CTTTACGCCC AGTGATTCCG AGCARCGCTA GCCCCCTTCG T 1000 1010 1020 1030 1040 1050 1060 1070 1060 519r 1090 1100 1010 1020 1030 1040 1050 1060 1070 1060 1070 1060 1100 10122-15 CGAAGTTAGC CGGGGGGTTAT TCTTCCGGGTA CGGTCATTAT CTTCCCGCGCAC AAAAGAGGCTT TACAACCCTA GGGCGTTCAT CACTCACGCG GCATGGCTGG 1100 NH22-15 ATCAGGCTTG CGCCCATTGT CCAATATTCC CCACTGCTGC CTCCCGTAGG AGT 1160 1170 1180 1190 1200 STAGE ASTTTEGECC STETCTCAST CCCAATETES CTEATCATCC TCTCAGACCA 1220 1230 1240 343r 1250 1260 1270 1280 1290 1300 10122-15 остастояте отсосство телесатта сетслесале тлестлател следовосе слеститесь солталиет тессеста соос TATCO 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1922-15 GGTATTACC CALOFTOTIC COALCCARA GGTACGTICC CACCCOTTAC TCACCCOTC GCCCTGAC TATTGCTATG CCCCCTGAC 1410 1420 1430 1440 1450 1422-15 TTOCATOTOT TAROCCTOCC GOCLOCOTTC OCT**CTOROCC ACCOUNTCALA** CTCAL

Figure 4.10 16S rDNA sequence of soybean rhizobium strain NM22-15. Nucleotide sequences of sequencing primers were shown in boxes.

	30		9 3	60	80	60	79	80	90	100
M122-30	TTTACOOCTA	CCTTOTTAC	ACTTCACCOC	AGTCOCTOAC	CCTACCOTOG	CONSCTUCCT	CCATTOCTOS	TTAOCOCACC	OTCTTCAGOT	AAAOCCAACT
10(22-30	CCCATGOTOT	ancooocoo	T OTOTACALOO	0 14	TATTCACCOT	GOCGTOCTOA	TOCACGATTA	CTAOCGATTC	CAACTTCATS	200 COCTCOAOTT
10122-30	GCAGAGCCCA	ATCCGAACT	19 21 5 AGACGGCTTT	0 24 TTGAGATTTG	CGALOGOTOG	CCCCTTAGCA	D 270 TOCCATTGTC	ACCOCCATTO	TAGCACOTOT	OTAGCCCAGC
10122-30	CCGTALGOOC	CATGAGGAC	T TOLOGTCATO	CCCACCTTCC	TCGCGOCTTA	TCACCOGCAG	TCTCCTTAGA	GTOCTCAACT	AAATGGTAGC	AACTAAGGAC
10622-30	00000TT000 1100r	CTCOTTO CO	GACTTARCC	AACATCTCAC	GACACGAGCT	GACGACAGCC	ATOCAOCACC	TOTOTTCCAG	GCTCCGARGA	GAROGTCACA
18622-30	TCTCTOCOAC	соотсство	A CATOTCARO	OCTOOGTANO	ottetococa	TTOCOTCOAL	TTAAACCACA	TOCTCCACCO	CTTOTOCOOS	COCCOTCAA
10622-30	TTUCTTTOAD	THILATOR	T OCOACCOTAC	TCCCCAGOCO	GAATOCTTAA	AGCOTTAGET	GCGCCACTAG	TOROTAAACC	CACTAACOOC	TOOCATTCAT
10122-30	COTTTACOOC	GTOOL CTAC	A ADOUTATETA	ATCETOTITO	стесселеве	TITESTOCET	CAGCOTCAGT	ATCOGGCCAG	TGAGCCGCCT	TEGECACTOS
10122-30	TGTTCTTGCG	AATATCTAC	AATTTCACCT	CTACACTOGC	AGTTCCACTC	ACCTCTCCCG	AACTCAAGAT	CTTCAGTATC	AAAGGCAGTT	900 CTGGAGTTGA
10122-30	GCTCCAGGAT	TTCACCCCT	ACTTAARGAC	CCOCCTACOC	ACCUTTACS	CCCAGTGATT	CCGAGCAACG	CTAOCCCCT	TOOTATTACC	0000CT0CT0
10122-30	OCACGAAGTT	0 10 A00000000	TATTCTTOCO	OTACCOTCAT	TATCTTCCCG	CACAAAAAAA	CTTTACAACC	0 100 CTA000CCTT	CATCACTCAC	0000CAT00C
10(22-30	TOGATCAGOG	TTOCCCCCA	T TOTOCAATAT	TOCCCACTOC	TOCCTOCCOT	a 114	occototete	AGTOCCAATS	0 119 TOOCTOATCA	TCCTCTCAGA
10122-30	CERSCHACTS	ATCOTCOCC	T TOGTAGOCCO	TTACCCTACC	AACTAGCTAA	TCAGACGCOG	GCCGATCTIT	0 129 CGOCGATAAA	0 129 11 TCTTTCCCCG	0 1300 TAAGGGCTTA
HH22-30	TCCOGTATTA	0 11	T CCCTGTGTGT	TTCCGAACCA	AAAGGTACGT	TCCCACGCGT	TACTCACCCG	TCTOCCOCTO	ACGTATTOCT	ACOCCOGCTC
10122-30	GACTTOCATO	TOTTAAOCC	T GCCGCCLOCO	TTCOCTCTO	0 145 000000ATCA	AACTCAAA				

Figure 4.11 16S rDNA sequence of soybean rhizobium strain NM22-30. Nucleotide sequences of sequencing primers were shown in boxes.

20 90 40 50 60 70 80 90 100 AC TICACCEAS TESTRACETASCE ACCEPTESCE GEORGECE TITEGETIAS CEASEGETE TEASGRAAA CEASETECCA NM22-25 1492r 150 160 110 140 170 180 TEGTETEACE GEC CCG GGAACGTATT CACCGTGGCG TGCTGATCCA CGATTACTAG CGATTCCAAC TTCATGGGCT CGAGTTGCAG MM22-25 1241f 230 240 250 210 260 270 280 220 290 AGCCCAATCC GAACTGAGAC GGCTTTTTGA GATTTGCGAA GGGTCGCCCC TTAGCATCCC ATTGTCACCG C TOTOTAG CCCAGCCCGT NM22-25 1385r 370 310 320 330 340 350 360 390 380 ANOGOCCATE AOGACTTERC GTCATCCCCA COTTCCTCCC GCCTATCAC COGCAGTCTC CTTADAGTEC TCAACTALAT GGTAGCAACT AAGGACGGG NM22-25 410 420 430 440 450 460 470 490 50 HTVGCGGGACT TAACCCAACA TCTCACGACA CGAGCTGACG ACGAGCTGAC AGGACTGCTC TCCGGTGCAG CCGAACTGAA GAACTCCGTC 500 MM22-25 1100r 510 530 540 \$70 520 550 560 580 TETEGAGTEC GEGACEGEGA TETEAAGGEE TEGTAAGETT ETEEGEGTTE CETEGAATTA AACCACATEC TECACEGETT ETEEGEGECEE NM22-25 907 510 620 530 640 550 660 570 680 590 700 TTDAGTT TAATCTTOCG ACCETATCC CCAGEGGGAA TOCTTAAAGC GTTAGCTOCG CCATACTGA GTARACCCAG CATACCATCATCGT 660 NM22-25 730 740 750 760 770 780 290 800 720 710 TTACGGCGTG GACT NM22-25 787r 820 870 7877 810 820 830 840 850 850 870 880 900 TETTGCGART ATCTACGART TTCACTCACE TCCACTCACE TCCACGAC TCCAGATCT CACTATCAA GGCAGTTCT GAGTTGAGCT NM22-25 910 920 950 930 940 960 970 COAGGATITE ACCCETEACT TAAAGAECEG CETACGEACE ETTTACGECE AGTEATTECE AGEAACGETA GECECETTE NM22-25 509r 1090 1020 1030 1040 1050 1060 1070 1080 1010 1100 CGANGTTAGE CGGGGGETTAT TETTGEGGTA CEGTEATTAT ETTECEGEAE AAAAGAGETT TACAACCETA GGGEETTEAT EAETEAEGEG GEATGGETGG NM22-25 1140 1150 1160 1170 1180 1170 TIGCTGC CTCCCGTAGG AGTITIGGGCC GTGTCTCAGT CCCAATGTGG CTGATCATCC TCTCAGACCA 1120 1130 1110 ATCAGGETTG CGCCCATTGT CCAATATTCC CCA NM22-25 343r 1240 1230 1300 1210 1220 1250 1260 1270 1280 1290 GCTACTGATE GTEGECETTEG TEAGECATTA CETEACEAAE TAGETAATEA GACGEGEGEE GATETTTEGE CEATAAATET TTEECEGTAA GEGETTATEE NM22-25 1310 1320 1330 1340 1350 1360 1370 1380 1390 140 GGTATTAGCT GAAGTTTCCC CCAGTTGTTC CGAACCAAAA GGTACGTTCC CACGCGTTAC TCACCCGTCT GCCGCTGACA TATTGCTATG CCCGCTCGAC 1400 MM22-25 1430 1440 1420 1450 1410 1410 1420 1700 TTGCATGTGT TAAGCCTGCC GCCAGCGTTC GCT TGAGCG GG2 27f AAAC TCAAA M22-25

Figure 4.12 16S rDNA sequence of soybean rhizobium strain NM22-25. Nucleotide sequences of sequencing primers were shown in boxes.

HA.7	ŤT.	-	20	acceed on	40 COCTORCC CTRO	se corooc caoc	Tocccc CTTT	to COOTTA GOOCA	ecore trea	90 LISTAAA ACCAA	etcec
BA 7	ATISTOTOA	1492r 10 1 10 10 10 10 10 10 10 10 10 10 10 1	120 1 1 1 1	100	240 1 94400TAT TCA	150 1 1 Сотоос отос	169 1 1 16ATCC ACGA	170 1 TTACTA OCOAT	100 1 TCCAA CTTC	199 1 1 1 1 ATOOSC TCOAC	005 TTTSCA
BA7	GAGCOCAAT	10 C CGALCT	220 1385	* 200 	540 ATTTOCA AGG	259 TCGCCC CTTA	CON CATT	ETO	200	299	900 000000
8A7	таарэрсса	10 1 1- 7 GAGGAC	STO TTGA COT	SOO	140 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	363 TTATCA CCGG	969 11 CAGTCT CCTT	920 11	999 CTAAA 1997	398 AGCARC TARGO	400 (AC9
HA7	1100;	100	420 1 1 1000 TTA	ACCTARS AT	440 STCACGAC ACGI	450 100000 0404	400 OCCATS CAGE	470 ACCTGT CTCCO	499 GTCCA OCTO	499 588CTS 88688	590 CTCC0
НА.7	TETETOCAS	10 7 000000	100 001	120 DOLANOS	540 TOGTANOG TTCT	550 OCOCOT TOCO	TEGAAT TAAA	579 CCACAT OCTCC	1000 TTOT	seesse ce	630
88.7		TITAAT	420 0110 COA	450	640	653 1	463 11 TAOCTO 0900	670 L1 ACTAGT GAGTA	693 1 	495 ACOUCT OCCAT	706 TCATC
KA 7	907r -	G TGGA	720	Ta0	740 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	783 CACGCT TTCO	TOCCTC ADCO	179 TCAGTA TCOGG	789 CCAST GAGO	799 COCCTT COCCA	B30 CTG3T
KA.7	GTTCTTOCC	A ATATCT	eco 781	TCACCTC TA	S40	CACTCA COTO	SCO TOCCOA ACTO	BTO	000	CAGTTO TOGAG	900 TTCAC
8A.7	CTCCASGAT	10 1 1 TCACCO	920 TOA CTT.	*20	etacoca coot	HTTACOC SCAS	SAN COAD	920 CARCOC TAGEC	ecctr cert	993	1000
BA7	CACGALOTT	ate 1 0000004	1020 CTI ATT	LOID TA	AD40	teccoc ACAA	LOCO	LOTO	LEED CCTTC ATCA	1090 CTCA00 COOCA	1100 10001
KA7	OGATCAGO	7 790900	LUZO CATT GTO	LANTATT CO	sten cca	1150	1340 1777095 CCGT	LITO	AATGT GGCT	1190 GATCAT COTOT	1200
BA7	CASCTACTO	A TOFFOR	1220 CCTT 00T	LETO	1140 MADE	1210 SCTART CAGA	1200 CGCGGG CCGA	12TO	1200 TAAAT CTTT	1130 CCCCGT AAGGO	1100 CTTAT
88.7	L CCONTATTA	I CTALLO	1920	LANT TOTTOL	EN40	1350 TACOTT CCCA	1940 L COCOTT ACTC	LITO LCCOTT CTOCC	1360 1 ОСТОА САТА	1390 1	14008 1 000100
HA.7	ACTERCATO	T GTTANO	1400 1400 1000	1410 CCAOCOT TO	1440 1 907	1410	1.				

Figure 4.13 16S rDNA sequence of soybean rhizobium strain NA7. Nucleotide sequences of sequencing primers were shown in boxes.

4.4 MPN of soybean rhizobia

Table 4.3 showed the average MPN of soybean rhizobia in the experimental plot to be 4.72×10^4 MPN per gram soil.

Table 4.3 MPN of soybean rhizobia in soil samples from the experimental plot in NamMoub subdistrict, Wiang Sa district, Nan province.

Determination	Soybean rhizobial cells (MPN per g soil)
E a VI	7.06x10 ⁴
2	3.55x10 ⁴
3	3.55x10 ⁴
average	4.72x10 ⁴

CHAPTER V

DISCUSSION

One of the 132 isolated soybean rhizobium strains (NA7) isolated from Klang Wiang subdistrict, Wiangsa district, Nan province was selected for use in the lab-scale production of soybean rhizobium biofertilizer for field testing in a 15 X 24 m² experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province, in the cultivation year 2007/2008. Use of the biofertilizer was found to increase soybean yield 4%(Chanthapetch and Chansa-ngavej, 2009). Detection of bacteria which had identical DNA fingerprints to those of strain NA7 in this thesis (Figure 4.1) was taken as an evidence for the successful nodulation of the strain. However, the nodulation efficiency was found to be only 6.6%. The low percentage increase in yield obtained (4%) indicated that strain selection by high dry weight of plants grown in Leonard jars at the greenhouse scale did not perform well under field conditions. Several researchers have reported poor nodulation ability of strains selected under greenhouse conditions when competed with indigenous soybean rhizobial strains already present in the soils (Botha et al., 2004; Pinochet et al., 1993; Streeter, 1994). In addition, soybean rhizobium biofertilizer produced for use in field trials in this experiment consisted of cells of rhizobial strain NA7 mixed with peat as the carrier. If cell density of strain NA7 in the biofertilizer was not just right, there might be an inhibition in nodule formation through the quorum-sensing mechanism.

Apart from the biotic factor influencing the outcome of the field trial experiment, abiotic factors such as metal contents (Fe, S, Mo) in soils also have an influence in terms of the availability of metals as cofactors of the nitrogenase enzyme. Research for a more effective selection method of soybean rhizobia for field testing might include selection of strains with high efficiency in metal uptake. In 1988 Ohara and co-workers reported on mineral constraints to nitrogen fixation. In 2009, Glass et al. reported on coevolution of metal availability and nitrogen assimilation in cyanobacteria and microalgae. At present, there is no comparable report on the relationship between

metal availability and soybean rhizobia nitrogen fixing potential resulting in higher soybean yields.

In 1987 Lawson and co-workers noted that survival of *Rhizobium leguminosarum* used in biofertilizers depended on their ability to survive in relatively hostile climate. The same could be true for soybean rhizobia used in the production of the inoculants. With global warming, it is imperative to find out the effects of high soil temperatures on the survival of soybean rhizobia used in the commercial production of inoculants.

It is interesting to note that the selected soybean rhizobia identified by polyphasic taxonomy in this study were found to be closely similar to either Bradyrhizobium elkanii or Bradyrhizobium japonicum strains deposited at SEMIA Rhizobium Culture Collection Center in Brazil. Binde et al. (2009) reported that at present, the SEMIA Rhizobium Culture Collection Center in Brazil housed 142 rhizobial strains whith high nodulation and nitrogen fixation efficiency for approximately 47 SEMIA keeps and distributes these rhizobial strains including leguminous plants. soybean rhizobia for commercial production of the inoculants in Brazil. Binde et al. (2009) reported that at SEMIA, rep-PCR DNA fingerprinting using the primer BoxR1 is routinely used for quality control of strains in the culture collection as well as in the quality control of strains being used in the commercial production of inoculants for various leguminous plants. In addition, rep-PCR fingerprinting is proposed for use in the monitoring of the success of inoculant strains in the fields. In this thesis, it is proposed that RAPD-PCR fingerprints with CRL-7 be used in the quality control of strains during production process as well as in the monitoring of the success of soybean root nodulation in the fields. DNA fingerprinting is also a tool in the studies of competitive nodulation among indigeneous soybean rhizobia and the introduced soybean rhizobia used to produce inoculants for soybeans.

Results of identical DNA fingerprints as shown in Appendix C showed 4 new groups (NM22-2, NM22-3, NM22-15, and NM22-30) of slow-growing soybean rhizobia which were not previously isolated by Chansa-ngavej et al (2009). However, results of sequences of 16S rDNA showed no new slow-growing soybean rhizobia were found in the experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province. Although strain NA7 and 6 representative isolates from the 6 groups of isolated

Bradyrhizobia were identified by the Biolog test kit as *B. elkanii* and by 16S rDNA as *B. japonicum* NM22-8, NM22-30) and *B. elkanii* (NM22-11, NM22-13, NM22-15, NM22-25, and NA7) their RAPD-PCR fingerprints were not the same (Appendix C). The results were similar to those reported by Binde et al. (2009) who reported that dendrograms constructed from 16S rDNA sequences did not show genetic diversity of rhizobia when compared with dendrograms constructed from rep PCR fingerprints. The researchers proposed that a new species is obtained when there is a difference of 15 nucleotides or more. If the criterion for a new species proposed by Binde et al. (2009) is used to interpret the results obtained by comparisons of 16S rDNA sequences with those deposited at GenBank, no new species was obtained in this thesis. However, the findings may shed light on the predominance of slow-growing soybean rhizobia in Nam Moub subdistrict. This result is in contrast to those obtained by Dowdle and Bohlool (1985) who reported predominance of fast-growing soybean rhizobia in Hubei province in the People's Republic of China.

Colony morphology of the slow-growing *B. elkanii* (NM22-11, NM22-13, NM22-15, NM22-25, and NA7) was irregular, slimy, while that of *B. japonicum* (NM22-8, NM22-30) were round and pearly (Figure 4.3). It is expected that morphology of root nodules of these two species of slow-glowing soybean rhizobia should be different as well.

Weaver and Federick (1974a,b) added liquid formulation containing up to 10^9 *Rhizobium japonicum* cells to soil samples from 22 sites for growth of soybeans in a greenhouse. The results obtained showed that the liquid soybean did not help increase soybean yields if indigenous soybean rhizobium MPN was more than 10^3 MPN per gram soil. Their field experiments confirmed the findings. Lupwayi et al. (2000) suggested that high quality soybean inoculants should contain 5×10^7 to 1×10^9 soybean rhizobium cells per gram biofertilizer. At this dose, 10^3 , 10^4 , and 10^5 soybean rhizobium cells should adhere to small, medium, and large soybean seeds, respectively. The MPN results of quantities of soybean rhizobia obtained in this research provide a valuable set of data onto which to build new findings on the relationship between the quantity of indigenous soybean rhizobia and the need or dose of soybean inoculation in Thai soils.

CHAPTER VI

CONCLUSION

All soybean rhizobia present in soybean-cultivating areas are potential candidates for the commercial production of soybean rhizobium biofertilizers to increase soybean yields. However, at present, there is not much information on the development of soybean rhizobium inoculants for the biofertilizer industries. The aim of this research is to detect if a soybean rhizobium strain NA7 used in the lab-scale production of soybean inoculant could compete with indigenous soybean rhizobia in a 15 x 24 m² experimental plot in Nam Moub subdistric, Wiang Sa district, Nan province. RAPD-PCR fingerprints using either RPO1 or CRL-7 as the primer were used to detect the presence of bacteria with identical DNA fingerprints to those of NA7. The experimental results showed NA7 could nodulate 6.6% of root nodules of soybean cultivar CM 60 previously mixed with NA7 biofertilizer before planting. Polyphasic taxonomy of 6 selected soybean rhizobia showed they belonged to the slow-growing *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum*. MPN determination of indigenous soybean rhizobia in the experimental plot revealed 4.72 X 10⁴ MPN per gram soil.

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APPENDIX A

BACTERIAL GROWTH MEDIA AND PLANT NUTRIENT SOLUTIONS

Preparation of all bacterial growth media and plant nutrient solutions are as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)							
Mannitol	10.0 g						
K ₂ HPO ₄	0.5 g						
MgSO ₄ .7H ₂ O	0.2 g						
NaCl	0.1 g						
Yeast extract	0.5 g						
Deionize <mark>d</mark> wat <mark>e</mark> r	1.0 g						

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YMA)

YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petri dishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 μ g.ml⁻¹. The medium was autoclaved at 121°C for 15 min.

N-free Nutrient Solutions

Stock Solutions	Chemicals	g/liter
1	CaCl ₂ .2H ₂ O	294.1
2	KH ₂ PO ₄	136.1
3	FeC ₆ H ₅ O ₇ .3H ₂ 0	6.7
	MgSO ₄ .7H ₂ O	123.3
	K ₂ SO ₄	87.0
	MnSO ₄ .H ₂ O	0.338
4	H ₃ BO ₃	0.247
	ZnSO ₄ .7H ₂ O	0.288
	CuSO ₄ .5H ₂ O	0.100
	CoSO ₄ .7H ₂ O	0.056
	Na ₂ MoO ₂ .7H ₂ O	0.048

Warm water was used to prepare stock solutions to get the ferric-citrate into solution. Ten liters of full-strength plant culture solution were prepared as follows:

- To 5 liters of water, add 5 ml of each stock solution and mix,
- Dilute to 10 liters by adding another 5 liters of water,
- Adjust pH to 6.8 with 1 N HCI
- For positive control treatment, 0.05% KNO₃ was added to give final N concentration of 70 ppm.

APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.9 g NaCl, 0.29 g EDTA were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

DNAzol solution (Molecular Research Lab, MRL) was used according to the manufacturer's instruction.
APPENDIX C



APPENDIX D

Utilization/Non-utilization of 95 carbon and nitrogen sources by three reference strains as determined by the Biolog test kit. Consensus results were obtained from 7 determinations.

	Consensus results from 7 determinations								
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	<i>B. liaoningense</i> NBRC 100396						
α-Cyclodextrin		· · ·	-						
Dextrin	3	+	-						
Glycogen	(CA)	-	-						
Tween 40	+++	++	++						
Tween 80	CO +++	++	+++						
N-Acetyl-D-Galactosamine	22	-	-						
N-Acetyl-D-Glucosamine	11110	-	-						
Adonitol	-	-	-						
L-Arabinose	+++	++	+++						
D-Arabitol	-	+							
D-Cellobiose	-		-						
i-Erythritol	-		-						
D-Fructose	-	+	-						
L-Fucose	+	+	-						
D-Galactose	DIONS	01010	5 ++						
Gentiobiose	C) /- 1 d	ΠĒ	- 0						
α-D-Glucose	-	-	+						
m-Inositol	6	<u> </u>	- 9						
α-D-Lactose	19198	1391	2178						
Lactulose	0 01 11	1011	D 101						
Maltose	-	-	-						
D-Mannitol	-	+	-						
D-Mannose	-	+	++						

	Consensus	Consensus results from 7 determinations							
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	<i>B. liaoningense</i> NBRC 100396						
D-Melibiose		-	-						
β-Methyl-D-Glucoside		2 -	-						
D-Psicose		<u>_</u> .	-						
D-Raffinose	9. =		-						
L-Rhamnose	1	-	-						
D-Sorbitol	// 1-1115	-	-						
Sucrose	/	-	-						
D-Trehalose			-						
Turanose		-	-						
Xylitol	Car A		-						
Pyruvic Acid Methyl Ester	+++	++	++						
Succinic Acid Mono-Methyl-Ester	+++	+++	++						
Acetic Acid	+++	++	++						
Cis-Aconitic Acid	Vallano V	-	-						
Citric Acid	+++	+	-						
Formic Acid	+++	++	-						
D-Galactonic Acid Lactone	N. Maran	- (++						
D-Galacturonic Acid	-	- 9	+						
D-Gluconic Acid	+++	++	+++						
D-Glucosaminic <mark>Aci</mark> d	-		-						
D-Glucuronic Acid	-	-	-						
α-Hydroxybutyric Acid		au + 1 a	00						
β -Hydroxybutyric Acid	++	++	+++						
γ-Hydroxybutyric Acid	+++	+	++						
p-Hydroxy Phenylacetic Acid	6 -		- 0.						
Itaconic acid	19192	220	01++ 0						
α-Keto Butyric Acid	8 61 VI	671	0-161						
α-Keto Glutaric Acid	+	+	+++						
α-Keto Valeric Acid	-	-	-						
D,L-Lactic Acid	+++	++	+++						

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	<i>B. liaoningense</i> NBRC 100396				
Malonic Acid		-	-				
Propionic Acid	++	+	++				
Quinic Acid		++	-				
D-Saccharic Acid	+++	++	-				
Sebacic Acid	+++	++	+				
Succinic Acid	+++	++	+++				
Bromosuccinic Acid	+++	++	+++				
Succinamic Acid	++	++	++				
Glucuronamide		-	-				
L-Alaninamide	TOT A	++	-				
D-Alanine	+	++	-				
L-Alanine	A COL	+	-				
L-Alanyl-glycine	Malar /		-				
L-Asparagine	Valeno V	-	-				
L-Aspartic Acid	and the second	++	++				
L-Glutamic Acid	201.27	++	-				
Glycyl-L-Aspartic Acid	A search		-				
Glycyl-L-Glutamic Acid	-	- 9º	-				
L-Histidine	-		_				
Hydroxy-L-Proline	-	- 11	-				
L-Leucine	-	++	++				
L-Ornithine	01000		00				
L-Phenylalanine	FIVER	+	713				
L-Proline							
L-Pyroglutamic Acid	+	++	- 0				
D-Serine	201010	++ 0	202				
L-Serine	6 6 1	1 d / I	CJ -16				
L-Threonine	-	-	-				
D,L-Carnitine	-	-	-				
γ -Amino Butyric Acid	-	-	+				

	Consensus results from 7 determinations							
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	<i>B. liaoningense</i> NBRC 100396					
Urocanic Acid	- / /	-	-					
Inosine		-	-					
Uridine		-	-					
Thymidine		-	-					
Phenyethyl-amine	11	-	-					
Putrescine	// 1-	-	-					
2-Aminoethanol	/ <u>-</u>	-	-					
2,3-Butanediol	Ja-	· ·	-					
Glycerol		+	-					
D,L-α-Glycerol Phosphate	GA		-					
α-D-Glucose-1-Phosphate	1997	-	-					
D-Glucose-6-Phosphate	TIOST	-	-					

APPENDIX E

Determination with the Biolog test kit of the ability to utilize or not utilize 95 carbon and

nitrogen sources by three reference strains.

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. elkanii NBRC 14791							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
α-Cyclodextrin	1	-	-	-	-	-	-	-
Dextrin	//+	11-11	-	-	+	+	+	-
Glycogen	//- \	-	-	-	-	-	-	-
Tween 40	+	+	+	+++	+++	+++	+++	+++
Tween 80	+	+	+	+++	+++	+++	+++	+++
N-Acetyl-D-Galact <mark>osa</mark> mine	-	K- 1	-	-	-	-	-	-
N-Acetyl-D-Glu <mark>c</mark> osamine	10	- A	-	-	-	-	-	-
Adonitol		2-1	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	+++	+++	+++	+++
D-Arabitol	44.19	12-7/	- 1	-	-	-	-	-
D-Cellobiose	2-2	2-3	-	-	-	-	-	-
i-Erythritol		-	-	-	-	-	-	-
D-Fructose	0.00			-	-	-	-	-
L-Fucose	+	+	+	+	+	-	-	+
D-Galactose	~- V	+	-	+	0	-	-	-
Gentiobiose	-	-	-	-	150	-	-	-
α-D-Glucose	-	-	-	-	2	-	-	-
m-Inositol	-	-	-	-		-	-	-
α-D-Lactose	-	-	-	-	<u> </u>	-	-	-
Lactulose	-	-	-	-	-	-	-	-
Maltose	0.1.0		1.	1.0.1	-	-	++	-
D-Mannitol	5 - Y		//	-	-		+++	-
D-Mannose	_	1.0	1		- 11	-	++	-
D-Melibiose	1	-	-	-	-	-	-	-
β -Methyl-D-Glucoside	1.0						-	
D-Psicose	- 1	-1)	- 1	-			1-0	-
D-Raffinose	0.0	N - 17		2	-	_	1.0	
L-Rhamnose	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-

Carbon/Nitrogen sources	B. elkanii NBRC 14791							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
D-Trehalose	_		-		-	-		_
Turanose	-	-	-	-	-	-	-	_
Xylitol	-	-	-	-	-	-	++	-
Pyruvic Acid Methyl Ester	+	+	+	+++	+++	+++	+++	+++
Succinic Acid Mono-Methyl-Ester	+	+	+	× -	+++	+++	+++	+++
Acetic Acid	+	+	+	+++	+++	+++	+++	+++
Cis-Aconitic Acid	- 7	-	-	_	-	-	-	-
Citric Acid	+	+	+	+++	+++	+++	+++	+++
Formic Acid	+	+	+	+++	+++	+++	+++	+++
D-Galactonic Acid Lactone	1 - 1	-	-	-	-	-	-	-
D-Galacturonic <mark>Aci</mark> d	1	-	-	-	-	-	-	-
D-Gluconic Acid	+	+	+	+++	+++	+++	+++	+++
D-Glucosaminic <mark>Acid</mark>	-	+	-	-	-	-	-	-
D-Glucuronic Acid	60	- 4	-	-		-	-	-
α -Hydroxybutyric <mark>Ac</mark> id	+	-	-	-	-	-	++	-
β-Hydroxybutyri <mark>c</mark> Acid	+	+	+	+++	+++	+	+++	++
γ-Hydroxybutyric Ac <mark>id</mark>	+	+	+	+++	+++	+++	+++	+++
p-Hydroxy Phenylacetic Acid	6-6	6-00	-	-	+	-	-	-
Itaconic acid	3. .	5)-	-	-	-	-	-	-
α-Keto Butyric Acid	-	+	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+	-	++	-	+	+
α -Keto Valeric Acid	- X	+	-	-		-	-	-
D,L-Lactic Acid	+	+	+	+++	+++	+++	+++	++
Malonic Acid	-	-	-	- /	1	-	-	-
Propionic Acid	+	+	+	++	++	++	++	+
Quinic Acid	-	-	-	-	0	-	-	++
D-Saccharic Acid	+	+	+	+++	+++	+++	+++	++
Sebacic Acid	+	+	+	+++	+++	+++	+++	++
Succinic Acid	+	+	+	+++	+++	+++	+++	++
Bromosuccinic Acid	+	+	+	+++	+++	+++	+++	++
Succinamic Acid	+	+	+	+++	+++	++	+++	++
Glucuronamide	1.0	100	00	6	0.0	01/	10	01
L-Alaninamide	2-2	+	-		-		1-6	++
D-Alanine	+	+	+	+	+	+	+	++
L-Alanine	-	-	-	-	-	-	-	+
L-Alanyl-glycine	-	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-	

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. elkanii NBRC 14791								
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus	
L-Aspartic Acid	-	-	-	-	-	-	-	++	
L-Glutamic Acid		-	-	-	+	-	-	++	
Glycyl-L-Aspartic Acid	-		-	-	-	-	-	-	
Glycyl-L-Glutamic Acid	-	1/-//	2-	-	-	-	-	-	
L-Histidine	0		-	V -	-	-	-	-	
Hydroxy-L-Proline	-		-	-	-	-	-	-	
L-Leucine	+ -		+	++	-	-	-	++	
L-Ornithine	200	_	-	-		-	-	-	
L-Phenylalanine	(/- \	-	-	-	-	-	-		
L-Proline	1	-	-	-	+	-	-		
L-Pyroglutamic Acid	+	+	+	-	+	-	-	++	
D-Serine	2 50		-	-	-	-	-	++	
L-Serine	-	5-1	-	-	-	-	-	-	
L-Threonine	62	4	-	-	-	-	-		
D,L-Carnitine	14- T	-	-	-	-	-	+++	-	
γ-Amino Butyric Acid	776	-	4-	-	-	-	++		
Urocanic Acid			7 - N	-	-	-	-	-	
Inosine	6	-	-	-	-	-	-	-	
Uridine	2.50	1.5	-	-	-	-	-	-	
Thymidine	-	-	-	-	-	-	-	-	
Phenyethyl-amine	20-3	-	-	-	-	-	-	-	
Putrescine	- V	-	-	-	0		-	-	
2-Aminoethanol	-	-	-	-	133	-	-	-	
2,3-Butanediol	-	-	-	- /	1	-	-	-	
Glycerol	-	-	-	-	-	-	-	+	
D,L- α -Glycerol Phosphate	-	-	-	-	U.	-	-	-	
lpha-D-Glucose-1-Phosphate	-		1	-	-	-	-	-	
D-Glucose-6-Phosphate	010	1.6	201	1.61	0	S	2	-	
ยู่นยางเยทรพยากร									

จุฬาลงกรณ์มหาวิทยาลัย

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. japonicum NBRC 14783							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
α -Cyclodextrin	-	+	1-7	-	-	-	-	-
Dextrin	-	+	1-	-	++	++	++	+
Glycogen	-	+		-	-	-	+	-
Tween 40	+	+	+	+++	++	+	++	++
Tween 80	+	+	+	++	++	++	+++	++
N-Acetyl-D-Galactosamine	-	2-		-	-	-	-	-
N-Acetyl-D-Glucosamine	1-11	+	-	-	-	-	-	-
Adonitol		-	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	++	++	++	++
D-Arabitol	+	+	+	+	+	-	+	+
D-Cellobiose	-	+	2 - 1	-	-	-	-	-
i-Erythritol	1-2	<u>(-</u>)	1	-	-	-	-	-
D-Fructose	+	+	+	-	+	-	++	+
L-Fucose	+	+	7-)/	-	+		+	+
D-Galactose		+	27-X	-	-	-	-	-
Gentiobiose		24	_	-	-	-	-	-
α-D-Glucose	666	+	-	-	· -	-	-	-
m-Inositol	-	-	-	-	-	-	-	-
α-D-Lactose	122	~-~		-	-	-	-	-
Lactulose	-	+	-	-	-	(La)	-	-
Maltose	-	-	-	-	-	3	-	-
D-Mannitol	+	+	+	+	++	+	++	+
D-Mannose	+	+	+	+	++	-	++	+
D-Melibiose	-	-	-	-	-	- ~	-	-
β -Methyl-D-Glucoside	-	+	0	<i>J</i> -	-	-	-	-
D-Psicose	17-6	+	1-5	-	9	Ū.	2.4	-
D-Raffinose		5-7	I - 6	-		-	-	d -
L-Rhamnose	-	-	-	-	-	-	-	-
D-Sorbitol	-	+	-	-		-	-	0
Sucrose	5	-	195	0	0		++	120
D-Trehalose		-	-	-	G	-	-	16- C
Turanose	-	+	<u>_</u>	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	++	+++	++
Succinic Acid Mono-Methyl-Ester	+	+	+	-	+++	++	+++	+++

Carbon/Nitrogen sources on Biolog	B ianonicum NBRC 1/783							
GN2 MicroPlate								
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
Acetic Acid	+	+	+	+++	++	++	++	++
Cis-Aconitic Acid	-		-	-	-	-	-	-
Citric Acid	+	+		+	++	+	++	+
Formic Acid	+	+	+	+++	++	++	++	++
D-Galactonic Acid Lactone	-	+		-	+	-	+	
D-Galacturonic Acid	-	+		-	+	-	++	
D-Gluconic Acid	+	+	+	++	++	++	+++	++
D-Glucosaminic Acid	-	+		-	-	- 11	+	-
D-Glucuronic Acid	- /	+	-	-	+	-	++	
α-Hydroxybutyric Acid		+	+	+	+	-	++	+
β-Hydroxybutyric Acid	+	+	+	+++	+++	++	+++	++
γ-Hydroxybutyric Acid	/-/3	+	+	· - ·	+	-	+++	+
p-Hydroxy Phenylacetic Acid		-	-	-	-	-	+	-
Itaconic acid	1-2	+	-4	-	+	-	++	
α-Keto Butyric Acid	7 -	×	-		-	-	-	-
α-Keto Glutaric Acid	+	+	-	-	+	-	+	+
α-Keto Valeric Acid	-	-	7-6	-	-	-	-	-
D,L-Lactic Acid	+	+	+	+++	+++	++	+++	++
Malonic Acid	S.E.L.	(j-)	-	-	-	-	+	-
Propionic Acid	+	+	+	++	+	-	++	+
Quinic Acid	+	+	+	+++	++	++	++	++
D-Saccharic Acid	+	+	+	+++	++	++	+++	++
Sebacic Acid	+	+	+	++	++	+	+++	++
Succinic Acid	+	+	+	+++	+++	++	+++	++
Bromosuccinic Acid	+	+	+	+++	++	++	++	++
Succinamic Acid	+	+	+	+++	++	++	++	++
Glucuronamide	-	+	- 0		+	-	-	-
L-Alaninamide	+	+	+	++	++	+	++	++
D-Alanine	+	+	+	++	++	+	++	++
L-Alanine	+	+		+	+	-	+	+
L-Alanyl-glycine	-	+	-	-	-	-	++	6.7
L-Asparagine	-	+	100	5	+	00.0	++	00
L-Aspartic Acid	+	+	-//	++	++	-	++	++
L-Glutamic Acid	+	+		+	++	-	+++	++
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	+++	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	++	-
L-Histidine	-	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. japonicum NBRC 14783							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
Hydroxy-L-Proline	-	-	-	-	-	-	-	-
L-Leucine	+	+	+	++	++	+	++	++
L-Ornithine	-	-	1-	-	-	-	-	-
L-Phenylalanine	-	+	-	+	+	-	++	
L-Proline	-	+		-	+	-	+	
L-Pyroglutamic Acid	+	+	-	+	++	-	++	++
D-Serine	-	+	-	+	+	-	++	++
L-Serine		-	-	-	-		-	-
L-Threonine	-/	+		-	+	-	+	
D,L-Carnitine		/ - \	-	-	-	-	++	-
γ-Amino Butyric Acid	/ -	+	-		+	-	++	
Urocanic Acid	1-13	+			-	-	-	-
Inosine		-	5 - \	-	-	-	-	-
Uridine	12	(-)	-4	-	-	-	-	-
Thymidine	-	<u> - 1</u>	-	-	-	-	-	-
Phenyethyl-amine	5- 8		-	-	-	-	-	-
Putrescine	10			· -	-	-	-	-
2-Aminoethanol	-		-	-	-	-	-	-
2,3-Butanediol	1.2	11-11	-	-	-	-	+	-
Glycerol	+	+	+	-	+	-	++	+
D,L- Q -Glycerol Phosphate		(),=1)	-	-	+	-	++	-
α-D-Glucose-1-Phosphate	-	× V	-	-	-	0	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	37	++	-

Carbon/Nitrogen sources	R liconingense NBRC 100306							
on Biolog GN2 MicroPlate			D. 11	aomigen	SE INDIN	5 10039	0	
	1 st	2 nd	3 rd	4 th	5 th	6 th	7^{th}	consensus
lpha-Cyclodextrin	-	1	-	-	-	-	-	-
Dextrin	-	- /	14-1	-	++	+	+	+
Glycogen	-		-	7 -	-	-	-	-
Tween 40	+	+	+	+++	+++	+++	++	++
Tween 80	+	+	+	++	+++	+++	+++	+++
N-Acetyl-D-Galactosamine	~	÷-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	In	-3	-	-	-	-	-	-
Adonitol	11	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	+++	+++	+++	+++
D-Arabitol	-		-	-	-	-	+++	-
D-Cellobiose	1	1	-	-	-	-	-	-
i-Erythritol	1	-	-	-	-	-	-	-
D-Fructose	2	0	4-	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-	-
D-Galactose	+	+	+	+	++	++	++	++
Gentiobiose	1-12	and	4-	-	-	-	-	-
α-D-Glucose	+	+	+	+	++	+	++	+
m-Inositol	6640	321		- 1	-	-	-	-
α-D-Lactose		270		-	-	-	-	-
Lactulose	280	12			-	-	-	-
Maltose	-	-	-	-	-	2	-	-
D-Mannitol	-	-	-	-	- 2	1-	+++	-
D-Mannose	+	+	+	+	++		+++	++
D-Melibiose	-	-	_	-	-	-	-	-
β -Methyl-D-Glucoside	-	-	-	-	1	-	-	-
D-Psicose	-	-	0	-	-	-	-	-
D-Raffinose	161	90	5), - (5-6	12	5	-
L-Rhamnose	+	+	G	7 -		-	-0	-
D-Sorbitol	-	-	-	-	-	-	-	-
Sucrose	- 6	-	-	-6	h - 1	-	-	6
D-Trehalose	2	9-14	2.9.4	7-0	1.67	0	9	2.61
Turanose	0 0	2	7 -	- 6	-	-	-	61-CJ
Xylitol	-	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	-	+++	+++	+++	++
Succinic Acid Mono-Methyl-Ester	+	+	+	-	++	++	++	++
Acetic Acid	+	+	+	-	+++	++	++	++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. liaoningense NBRC 100396							
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus
Cis-Aconitic Acid	-	-	-	-	-	-	-	-
Citric Acid	-	1.5	-	-	-	-	-	-
Formic Acid	+	+	1	-	+++	-	-	-
D-Galactonic Acid Lactone	+	+	+	+	+++	++	++	++
D-Galacturonic Acid	+	+	+	-	+	-	++	+
D-Gluconic Acid	+	+	+	+++	+++	+++	+++	+++
D-Glucosaminic Acid	+	+	-	-	-	-	-	-
D-Glucuronic Acid	1		-	-	_	-	-	-
α -Hydroxybutyric Acid	1/-//	-	-	-	-	+	-	-
β-Hydroxybutyric Acid	+	+	+	+++	+++	+++	+++	+++
γ-Hydroxybutyric Acid	+	+	+	+	+	-	++	++
p-Hydroxy Phenylacetic Acid	+	+	-	-	-	-	-	-
Itaconic acid	+	+	+	+	++	++	++	++
α -Keto Butyric Acid	2.	2-	4-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+	+	+++	+++	+++	+++
α-Keto Valeric Acid	1.676	0,700	0.4	-	-	-	-	-
D,L-Lactic Acid	+	+	+	+++	+++	+++	+++	+++
Malonic Acid	<u> </u>	-	-	-	-	-	-	-
Propionic Acid	+	+	+	++	+	+	++	++
Quinic Acid	-	-	-	-	-	-	-	-
D-Saccharic Acid	+	+	-	-	+	-	+	
Sebacic Acid	+	+	+	+	+	++	+	+
Succinic Acid	+	+	+	+++	+++	+++	+++	+++
Bromosuccinic Acid	+	+	+	+++	+++	+++	+++	+++
Succinamic Acid	+	+	+	+	+++	+++	+++	++
Glucuronamide	+	+	-	-		-	-	-
L-Alaninamide	+	+	0	-	-	-	-	-
D-Alanine	+	+	5	-	2 - 4		-	-
L-Alanine	+	+	G	-	_	-	-6	-
L-Alanyl-glycine	+	+	-	-	+	-	-	-
L-Asparagine	- 6	-	-	- 6	-	-	-	0
L-Aspartic Acid	+	+	+	++	+++	++	++	++
L-Glutamic Acid	+	+	-	- (-	-	-	<u>-</u>
Glycyl-L-Aspartic Acid	-	<u> </u>	3 3			-	+++	-
Glycyl-L-Glutamic Acid	-	-	+	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-				-	-

Carbon/Nitrogen sources	B. liaoningense NBRC 100396								
on Biolog GN2 MicroPlate									
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	consensus	
L-Leucine	+	+	+	++	++	++	++	++	
L-Ornithine	-	1.	-	-	-	-	-	-	
L-Phenylalanine	+	+	-	-	-	-	-	-	
L-Proline	+	+	-	-	-	-	-	-	
L-Pyroglutamic Acid	+	+	-	-	-	-	-	-	
D-Serine	-	-	-	-	-	-	-	-	
L-Serine	2		-	-	-	-	-	-	
L-Threonine	+	+	-	-	_	-	-	-	
D,L-Carnitine	(-	1	-	-	-	-	-	
γ-Amino Butyric Acid	+	+	+	-	-	-	+++	+	
Urocanic Acid	+	+	-	-	-	-	-	-	
Inosine			-	-	-	-	-	-	
Uridine		-	-	-	-	-	-	-	
Thymidine	3- (-	-	-	-	-	-	-	
Phenyethyl-amine	-	-	-	-	-	-	-	-	
Putrescine	1500	3577	5-1	-	-	-	-	-	
2-Aminoethanol	-	1	-	-	-	-	-	-	
2,3-Butanediol		0	-	-	-	-	-	-	
Glycerol		1	-	-	-	-	-	-	
D,L- Q -Glycerol Phosphate	-	-	-	-	-	-	-	-	
Q-D-Glucose-1-Phosphate	9-9	×	-	-	-	-	-	-	
D-Glucose-6-Phosphate	-	-	+	-	-	0	-		

APPENDIX F

Determination with the Biolog test kit of the ability to utilization or not utilization 95 carbon and nitrogen sources by 7 soybean rhizobium strains (NA7, NM22-8, NM22-11, NM22-13, NM22-15, NM22-25 and NM22-30).

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense		NA7		Consensus
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
α -Cyclodextrin	-///		· · · · ·	-	-	-	-
Dextrin		+		-	-	-	-
Glycogen	//-///		A -	-	-	-	-
Tween 40	+++	++	++	++	++	++	++
Tween 80	+++	++	+++	++	++	++	++
N-Acetyl-D-		12/2	4	-	-	-	-
		added to be					
Glucosamine	-		-	-	-	-	-
Adonitol		1200	18 J	-	-	-	-
L-Arabinose	+++	++	+++	+	+	+	+
D-Arabitol	-	+	-	-	18-1	-	-
D-Cellobiose	-	-	-	-		-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose		+	-	-	-	-	-
L-Fucose	+	+		-	-		-
D-Galactose	2 9	n e- 9/	++	-	9	2-1	5 -
Gentiobiose			0-11	-	-	-	0 -
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-		-		-	-	Q.
α -D-Lactose	125	2.9	98-71	-		5-4	126
Lactulose		6	-	0-	-	-	
Maltose		-		-	-	-	-
D-Mannitol	-	+	-	-	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NA7 2 nd	3 rd	Consensus results from 3 determinations
β-Methyl-D-	-		11-	-	-	-	-
D-Raffinose					-	-	
L-Rhamnose	_		_				
						_	
Sucrees		////	-		-	-	-
D Trobologo			-	-	_	-	-
D-Trenaiose			-	-	-	-	-
Turanose		ha	-	-	-	-	-
Xyiitoi			-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	A.++	++	++	++	+	++
Succinic Acid Mono-Methyl- Ester	+++	+++	++	-	++	++	++
Acetic Acid	+++	++	++	++	+	+	+
Cis-Aconitic Acid	1 6	all star)	-	-	-	-
Citric Acid	+++	+	-	-	-	-	_
Formic Acid	+++	++	-	+++	++	++	++
D-Galactonic Acid Lactone	-	-	++	-	9	-	
D-Galacturonic	-	-	+	-		-	-
D-Gluconic Acid	+++	++	+++	+	+	+	+
D-Glucosaminic Acid	12.	0.0100	2 AL		+	-	~
D-Glucuronic Acid	5.9.	12JY	19.11	D	-		3.
α-Hydroxybutyric Acid	000	÷.		8			Sec.
β-Hydroxybutyric Acid	++ 0	6 ++ 6	+++	+++	+++	5	+++
γ-Hydroxybutyric Acid	+++	+	++	-	++	+	+
p-Hydroxy	-	-	-	-	-	-	-

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NA7 2 nd	3 rd	Consensus results from 3 determinations
Phenylacetic Acid			10-				
Itaconic acid	-	- 1/	++	++	++	++	++
α-Keto Butyric Acid			2	-	-	-	-
α-Keto Glutaric Acid	+	7+1.	+++	+	-	-	-
α-Keto Valeric Acid	-//			-	-	-	-
D,L-Lactic Acid	+++	++	+++	+++	++	++	++
Malonic Acid		174-18-18	- I P	++	++	+	++
Propionic Acid	++	+	++	++	++	++	++
Quinic Acid		++		-	-	-	-
D-Saccharic Acid	+++	++	-	++	++	++	++
Sebacic Acid	+++	++	+	-	× .	-	-
Succinic Acid	+++	++	+++	-	-	-	-
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	-	-	-	-
Glucuronamide	- 44	2220.37	2/5-1-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	-	34	-	-
L-Alanine	-	+	-	-	1	-	-
L-Alanyl-glycine	-	-	-	-	- 11	-	-
L-Asparagine	2	-	-	-	<u> </u>	-	-
L-Aspartic Acid	6	++	++	-	-	-	-
L-Glutamic Acid	100	++	5.01	01	9	51	<u>-</u>
Glycyl-L-Aspartic	0.01		l d. /\	C	-	-	d .
Chould Clutomic							
				-	-		
L-Histidine	1715	219	12.1		_		188
Hydroxy-I -Proline		010 01			_		
	_	++	++	++	++	++	++
L-Ornithine	_		-		-	-	
L-Phenylalanine	-	+	-	+	-	-	-

	Consensus	results from 7 det	erminations				
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B.	NA7			Consensus results from 3
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
L-Proline	-		11-	-	-	-	-
L-Pyroglutamic	+	++		-	-	-	-
Acid							
D-Serine	-	++	-	+	+	+	+
L-Serine	-	- i	-	-	-	-	-
L-Threonine	-	1111	-	-	-	-	-
D,L-Carnitine	- /		-	-	-	-	-
γ-Amino Butyric Acid		(//-	+	-	-	-	-
Urocanic Acid	1.	12.23		-	-	-	-
Inosine		/ -	-	-	-	-	-
Uridine		19(3)	A	-	-	-	-
Thymidine			-	-	-	-	-
Phenyethyl-amine		2.02.00		-	· -	-	-
Putrescine	-	- United	// -	-	-	-	-
2-Aminoethanol	-	MY a law	-	-	-	-	-
2,3-Butanediol	- 0	666-0 - P	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L- Q- Glycerol	1	2320-34	Alexandre -				
Phosphate		-	-			-	-
α -D-Glucose-1- Phosphate		-	-	-		-	-
D-Glucose-6- Phosphate		-	-	-	_ -	-	-

Carbon/Nitrogen	Consensus	erminations					
sources on Biolog							_
GN2 MicroPlate	B. elkanii	B. japonicum	B.		NM22-8		Consensus
	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
α -Cyclodextrin	-		11-	-	-	-	-
Dextrin	-	+	1Pa	-	-	-	-
Glycogen	-	Construction of the second		-	-	-	-
Tween 40	+++	++	++	+	+	-	+
Tween 80	+++	++	+++	+	+	-	+
N-Acetyl-D-		1/11 1					
Galactosamine		///		-		-	-
N-Acetyl-D-			1110				
Glucosamine		「人気		-		-	-
Adonitol	-		-	-	-	-	-
L-Arabinose	+++	++	+++	-	-	-	-
D-Arabitol	-	+	-	-	-	-	-
D-Cellobiose	-		-	-	-	-	-
i-Erythritol	-	2.500		-	-	-	-
D-Fructose	-	+	-	+	-	-	-
L-Fucose	+	+		-	-	-	-
D-Galactose			++	-	-	-	-
Gentiobiose	-			-	-	-	-
lpha-D-Glucose	1		+	-	-	-	-
m-Inositol	-	-	-	-	E	-	-
α-D-Lactose	-	-	-	-	31	-	-
Lactulose	-	-	-	-		-	-
Maltose	-	_		-	-	-	-
D-Mannitol	·	+	-	-		-	-
D-Mannose	6	+	++	-	-	-	-
D-Melibiose	2199	n e-i 97	5-91	61	9	21	5 -
eta-Methyl-D-			0.11	Q		_	d
Glucoside							
D-Psicose	-	6	-	-	-	-	0
D-Raffinose	เกร	ลเข	92-71	() - () - (120
L-Rhamnose		b 16 ch	· ·	6	-	-	161 C
D-Sorbitol				-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-

Carbon/Nitrogen	Consensus	results from 7 det	erminations				
sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-8	3 rd	Consensus results from 3 determinations
Xylitol		san11	ll d -	_	_		
Pyruvic Acid Methyl Ester	+++	++	++	+	+	-	+
Succinic Acid Mono-Methyl- Ester	+++	+++	++	+	+	_	+
Acetic Acid	+++	++	++	+	-	-	-
Cis-Aconitic Acid		//-	-	+	+	-	+
Citric Acid	+++	+ =	-	-	+	-	-
Formic Acid	+++	++	- 1	+	+	-	+
D-Galactonic		20	++	-	-	-	-
D-Galacturonic Acid			+	-	-	-	-
D-Gluconic Acid	+++	++	+++	-	-	-	-
D-Glucosaminic Acid				-	-	-	-
D-Glucuronic Acid	- A	ENER SI	11/2-	-	-	-	-
α -Hydroxybutyric Acid		+	-	-	5	-	-
β -Hydroxybutyric Acid	++	++	+++	+	+	-	+
γ-Hydroxybutyric	+++	+	++	-	-	-	-
p-Hydroxy Phenylacetic Acid	ะเวิเ	1819	15.91	21	9	ก	5 -
Itaconic acid			++	-	-		· ·
α-Keto ButyricAcid	000	6					No.
α -Keto Glutaric Acid	1119	644	+++	9	-	2	161 E
α -Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+	+	-	+

Carbon/Nitrogen	Consensus	results from 7 det	erminations				
sources on Biolog GN2 MicroPlate	B. elkanii	B. japonicum	B. liaoningense		NM22-8	3	Consensus results from 3
	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
Malonic Acid	-		11-	+	+	-	+
Propionic Acid	++	+	++	-	-	-	-
Quinic Acid	-	++		+	+	-	+
D-Saccharic Acid	+++	++	-	-	-	-	-
Sebacic Acid	+++	++	+	-	-	-	-
Succinic Acid	+++	++	+++	+	-	-	-
Bromosuccinic Acid	+++	++	+++	-	.	-	-
Succinamic Acid	++	++	++	+	-	-	-
Glucuronamide	1-11	17 203	- 1	-	-	-	-
L-Alaninamide		++	-	-	-	-	-
D-Alanine	+	++		-	-	-	-
L-Alanine		+	-	-	-	-	-
L-Alanyl-glycine	/	0. 5000		-	×.	-	-
L-Asparagine	-	No.	7/-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	1	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	- 13		11.	-	-	-	-
Glycyl-L-Glutamic Acid	_	_	-	-	6	-	-
L-Histidine		_	_	- /	2	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine		++	++	_	<u>.</u>	-	-
L-Ornithine	60	-	0.7	-	-	-	-
L-Phenylalanine	000	n o+1 o/	C-OAL	-	-	0	e .
L-Proline		17.17		7-1	-	-	
L-Pyroglutamic					-		×
Acid	+	++	-	-	-	-	0.7
D-Serine	000	++	0000	20		31/	000
L-Serine		SU L	1/1-	-	-	-	1617
L-Threonine		010 01		<u> </u>	-	-	
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-

Carbon/Nitrogen	Consensus results from 7 determinations						
sources on Biolog GN2 MicroPlate	B. elkanii	B. japonicum	B.		NM22-8	3	Consensus
	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
Urocanic Acid	-		11-	-	-	-	-
Inosine		- //		-	-	-	-
Uridine	-	Down and		-	-	-	-
Thymidine	-		-	-	-	-	-
Phenyethyl-amine	-	1 T	-	-	-	-	-
Putrescine	-	1/11/0	-	-	-	-	-
2-Aminoethanol	- /		-	1	-	-	-
2,3-Butanediol		///-	-	-	-	-	-
Glycerol	-//	+ =	-	-	-	-	-
D,L- A -Glycerol		A STR					
Phosphate						-	-
α-D-Glucose-1-		A 131					
Phosphate		67/20			_	-	-
D-Glucose-6-		2.000	DAN			_	_
Phosphate		audi			_	_	-

Q		anaese anaese anaese anaese			6		
	Consensus	results from 7 de	terminations	1			
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense		NM22-11	1	Consensus
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
lpha-Cyclodextrin		- Y/-	- W	-	-	-	
Dextrin		+	0.11	-		+	· -
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	+++	+++	++	+++
Tween 80	+++	++	+++	+++	+++	++	+++
N-Acetyl-D- Galactosamine			-	-	-	-	
N-Acetyl-D- Glucosamine	-	-	-	-	-	-	-

	Consensus	results from 7 de	terminations				
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense		NM22-17	1	Consensus results from 3
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
Adonitol	-		11-	-	-	-	-
L-Arabinose	+++	++	+++	++	+	-	+
D-Arabitol	-	+		+	-	-	-
D-Cellobiose	-		-	-	-	-	-
i-Erythritol		2.1	-	-	-	-	-
D-Fructose	-	+	-	+	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	- /)	(//-	++	-	-	-	-
Gentiobiose		1.3		-	-	-	-
α-D-Glucose		N 2002	+	-	-	-	-
m-Inositol	//-///		-	-	-	-	-
α -D-Lactose		8 63 A		-	-	-	-
Lactulose		R-AK	-	-	-	-	-
Maltose	D	10000		-	-	-	-
D-Mannitol		+	// -	+	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	- 66	66461419	-	-	-	-	-
β-Methyl-D-							
Glucoside	145			-	-	-	-
D-Psicose	-	-	-	-	£	-	-
D-Raffinose	-	-	-	-	3	-	-
L-Rhamnose	-	-	-	-		-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	- "	-	-
D-Trehalose		-	5	-	-	-	-
Turanose		0101	5	0		5-4	-
Xylitol	0	0.7	0	C)	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	+++	-	++	++
Succinic Acid Mono- Methyl-Ester	+++	+++	++	++	++	++	12+8
Acetic Acid	+++	++	++	++	+	++	++
Cis-Aconitic Acid		-		-	-	-	-
Citric Acid	+++	+	-	+	+	-	+
Formic Acid	+++	++	-	++	+++	+++	+++

	Consensus	results from 7 de	terminations				
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	B. elkanii	B. japonicum	B. liaoningense		NM22-11	1	Consensus results from 3
	NBRC 14791	NBRC 14783	100396	1 st	2 nd	3 rd	determinations
D-Galactonic Acid Lactone	- 2		++	+	-	-	-
D-Galacturonic Acid	-	201-11/	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	+++	++	++	++
D-Glucosaminic Acid	-	2.1	-	++	-	-	-
D-Glucuronic Acid	-	1000	-	-	-	-	-
α -Hydroxybutyric Acid	-	+		-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	++	++	++
γ-Hydroxybutyric	+++	+	++	++	-	-	-
p-Hydroxy Phenylacetic Acid	- b		-	-	-	-	-
Itaconic acid		Diala	++	-	-	-	-
lpha-Keto Butyric Acid		Makawa	- A	-	-	-	-
α -Keto Glutaric Acid	+	+	+++	-	-	-	-
lpha-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	++	++	++	++
Malonic Acid	-	-	-	-	E	-	-
Propionic Acid	++	+	++	++	31	+	+
Quinic Acid	-	++	-	-		-	-
D-Saccharic Acid	+++	++	_	+++	+++	++	+++
Sebacic Acid	+++	++	+	++	+	++	++
Succinic Acid	+++	++	+++	+++	+++	++	+++
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	+++	+	++	++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	++	-	-	01
D-Alanine	+	++	00-01	+	+	+	+ 0
L-Alanine	1 - d	+	7-	d-		-	16 - CI
L-Alanyl-glycine	1.1.1		5.5. 5	-	_	-	
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-

Carbon/Nitrogen	Consensus	results from 7 de	terminations				
sources on Biolog	B. elkanii	B. japonicum	ь. liaoningense		NM22-17	1	Consensus results from 3
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
Glycyl-L-Aspartic Acid	-0		120	-	-	-	-
Glycyl-L-Glutamic Acid				-	-	-	-
L-Histidine		2.11	-	-	-	-	-
Hydroxy-L-Proline		111 3	-	-	_	-	-
L-Leucine		++	++	+	-	-	-
L-Ornithine	-//	(//-	-	-	-	-	-
L-Phenylalanine		+	1	-	-	-	-
L-Proline		12 24-2		-	-	-	-
L-Pyroglutamic Acid	+	++	-	+	-	-	-
D-Serine	-	++		-	+	-	-
L-Serine	-	R TAKA	-	-	-	-	-
L-Threonine	- 4	1575 0577	-	-	-	-	-
D,L-Carnitine		MICH	/ -	-	-	-	-
γ -Amino Butyric Acid		Marana	+	-	-	-	-
Urocanic Acid	1- 68	66461419	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	- 45			-	-	-	-
Thymidine	-	-	-	-	E	-	-
Phenyethyl-amine	-	-	-	-	3	-	-
Putrescine	-	-	-	-		-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-		-	-
Glycerol		+	3	-	-	-	-
D,L- α -Glycerol Phosphate	131	18181	รพ	2	7	2	ă ·
α-D-Glucose-1- Phosphate	-	8	-	-	-	-	i.
D-Glucose-6- Phosphate	กรา	1.1	หา	29	<u>A (</u>	-	121

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-13 2 nd	3 3 rd	Consensus results from 3 determinations
α -Cyclodextrin	-	<u> </u>		_	-	-	-
Dextrin	-	+)	-	-	-	-	-
Glycogen	-	3.1	-	-	-	-	-
Tween 40	+++	++	++	++	++	+++	+++
Tween 80	+++	++	+++	++	++	++	++
N-Acetyl-D- Galactosamine		6-3		-	-	-	-
N-Acetyl-D- Glucosamine			-	-	-	-	-
Adonitol	-			-	-	-	-
L-Arabinose	+++	++	+++	+	-	+	+
D-Arabitol	- 9	+		-	-	-	-
D-Cellobiose	-	12-212	-	-	-	-	-
i-Erythritol	1		-	-	-	-	-
D-Fructose	- 24	+	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose		200 - V 3	++	-	-	-	-
Gentiobiose	-	-	-	-	5	-	-
α-D-Glucose	-	-	+	-)	2	-	-
m-Inositol	-	-	-	- 1	1	-	-
α-D-Lactose	-	-	-		-	-	-
Lactulose	j	-	-	-	-	-	-
Maltose	1.2				-	-	-
D-Mannitol		+	2-W	-	-		- 1
D-Mannose	I G I	+	++	-	-	-	- 0
D-Melibiose	_		-	-	-	-	-
eta-Methyl-D-	-			6			
Glucoside	251		7871				125
D-Psicose	1-0	0 10 0	/ - 1	0-	-	9	
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC	1 st	NM22-13	3rd	Consensus results from 3
		5000	100396	I	2	3	Gelenninations
D-Trehalose	-	-		-	-	-	-
Turanose	-	- //		-	-	-	-
Xylitol	-			-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	++	++	++
Succinic Acid Mono- Methyl-Ester	+++	+++	++	++	++	++	++
Acetic Acid	+++	++	++	+	-	+	+
Cis-Aconitic Acid	1-11		-	-	-	-	-
Citric Acid	+++	+		+	-	-	-
Formic Acid	+++	++	-	+++	+++	+++	+++
D-Galactonic Acid Lactone			++	-	-	-	-
D-Galacturonic Acid	- A	ATT OUT	+	-		-	-
D-Gluconic Acid	+++	++	+++	++	++	++	++
D-Glucosaminic Acid	-	1016 <u>1</u> (016	-	-	-	-	-
D-Glucuronic Acid	- 66	66461419	-	-	-	-	-
α -Hydroxybutyric Acid	- 60		1725	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+++	+++	+++
γ-Hydroxybutyric	+++	+	++	+	+	+	+
p-Hydroxy Phenylacetic Acid	1	-	a.	-	-	-	-
Itaconic acid	0.01	0:01	++	01	9	510	
lpha-Keto Butyric Acid	/	C - 7	d - 1	<u>(</u>	-	-	d -
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α -Keto Valeric Acid	-	6	-	-	-	-	0.7
D,L-Lactic Acid	+++	++	+++	++	++	++	++
Malonic Acid	1 - 1		/-	-	- 1	-	16-71
Propionic Acid	++	+	++	++	+	-	+
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	+++	++	++	++
Sebacic Acid	+++	++	+	+	+	+	+

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-13 2 nd	3 3 rd	Consensus results from 3 determinations
Succinic Acid	+++	++	+++	++	++	++	++
Bromosuccinic Acid	+++	++	+++	+	-	+	+
Succinamic Acid	++	++	++	++	+	++	++
Glucuronamide	-		-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	+	-	-	-
L-Alanine	-//	+	-	-	-	-	-
L-Alanyl-glycine	-//	// -	-	-	-	-	-
L-Asparagine		1.3		-	-	-	-
L-Aspartic Acid		++	++	-	-	-	-
L-Glutamic Acid	/-///	++	-	-	-	-	-
Glycyl-L-Aspartic		A (0)		-	-	-	-
Glycyl-L-Glutamic	//·/>	ATT ON	×-	-	-	_	-
L-Histidine	-	100 <u>6</u> /000	-	-	-	-	-
Hydroxy-L-Proline	- 66	6646 410	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-45			-	-	-	-
L-Phenylalanine	-	+	-	-	0	-	-
L-Proline	-	-	-	-	35	-	-
L-Pyroglutamic Acid	+	++	-	-		-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	- 1	-	-	-
L-Threonine		-	3	-	-	-	-
D,L-Carnitine	00	0100	5.01	01	Ġ	514	-
γ -Amino Butyric Acid	- c /	C 7	+		-	-	d -
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	- E	-	-	-	-	01
Uridine	55	0101	00.01				000
Thymidine	1 - 6		/-	d	- 1	-	6-71
Phenyethyl-amine			5.5. 5.	-		-	
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-

	Consensus	results from 7 de	terminations				
Carbon/Nitrogen sources on Biolog	B. elkanii B. japonicum		B. liaoningense		NM22-13	3	Consensus
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
Glycerol	-	+	10-	-	-	-	-
D,L -α -Glycerol Phosphate	-			-	-	-	-
α-D-Glucose-1- Phosphate	-			-	-	-	-
D-Glucose-6- Phosphate		111		-	-	-	-

	Consensus	results from 7 det	terminations					
Carbon/Nitrogen sources on Biolog	B <mark>.</mark> elkanii	B. japonicum	B. liaoningense		NM22-15	ō	Consensus	
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations	
α-Cyclodextrin	-/313			-	-	-	-	
Dextrin	-	+	-	++	++	+	++	
Glycogen	-	-	-	-	37	- ¹⁰	-	
Tween 40	+++	++	++	++	++	++	++	
Tween 80	+++	++	+++	++	++	++	++	
N-Acetyl-D-	-	-	0.7	-	-	-	-	
N-Acetyl-D- Glucosamine	179	18191	59	21	7	24	ă -	
Adonitol				-	-	-		
L-Arabinose	+++	++	+++	+	+	+	+	
D-Arabitol	000	+	000	+	+	-	+	
D-Cellobiose	(- <u>)</u> (1 /- '	-	-	-	16 2	
i-Erythritol				<u> </u>	-	-		
D-Fructose	-	+	-	-	+	-	-	
L-Fucose	+	+	-	-	-	-	-	
D-Galactose	-	-	++	+	-	-	-	

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-15 2 nd	5 3 rd	Consensus results from 3 determinations
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	Opin Star		-	-	-	-
α-D-Lactose	-			-	-	-	-
Lactulose	-	× - †	-	-	-	-	-
Maltose	-	111 3	-	-	-	-	-
D-Mannitol	-//	+	-	+	+	+	+
D-Mannose	-//	+	++	-	-	-	-
D-Melibiose		4.3	/ /	-	-	-	-
β-Methyl-D- Glucoside				-	-	-	-
D-Psicose		2 63		-	-	-	-
D-Raffinose		in - Kakar	-	-	-	-	-
L-Rhamnose	// -// b	(STG-9)11	-	-	-	-	-
D-Sorbitol		Not olio	-	-	-	-	-
Sucrose		MN6 <u>1</u> (200	-	-	-	-	-
D-Trehalose	- 66	66491012	-	- 1	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-45	999-34		-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	++	++	++
Succinic Acid Mono- Methyl-Ester	+++	+++	++	++	++	++	++
Acetic Acid	+++	++	++	++	++	++	++
Cis-Aconitic Acid	6	-	0	-	-	-	-
Citric Acid	+++	0+00	5-91	01	9	574	× -
Formic Acid	+++	++	0	+	-	+	+
D-Galactonic Acid Lactone	-	6	++	+	-	-	-
D-Galacturonic Acid	550	G 1-0 1	00+07		n c		501
D-Gluconic Acid	+++	++	+++	++	++	++	++
D-Glucosaminic Acid				-	-	-	
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-15	5 3 rd	Consensus results from 3 determinations
β-Hydroxybutyric Acid	++	++	+++	+++	+++	+++	+++
γ-Hydroxybutyric Acid	+++	+	++	++	++	+	++
p-Hydroxy Phenylacetic Acid		En la	ŀ	-	-	-	-
Itaconic acid	-		++	-	-	-	-
lpha-Keto Butyric Acid	-//	(//-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	+	-	-	-
α-Keto Valeric Acid		N 202	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	++	+++	++	++
Malonic Acid		2 631	A - \	-	-	-	-
Propionic Acid	++	+	++	++	+	++	++
Quinic Acid	- 0	++		-	-	-	-
D-Saccharic Acid	+++	++	-	++	++	++	++
Sebacic Acid	+++	++	+	++	++	+	++
Succinic Acid	+++	++	+++	++	++	++	++
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	+++	+++	++	+++
Glucuronamide	-	-	-	-	E	-	-
L-Alaninamide	-	++	-	-	3	-	-
D-Alanine	+	++	-	+	1	-	-
L-Alanine	-	+	_	-	-	-	-
L-Alanyl-glycine	-	-	-	-	- ¹⁰	-	-
L-Asparagine	600	-	0	-	-	-	-
L-Aspartic Acid	109/	++	++	01	9	576	
L-Glutamic Acid		++	d - / \	G	-	-	d -
Glycyl-L-Aspartic Acid	-	6	-	-	-	-	i
Glycyl-L-Glutamic Acid	กรา	ปป	<u>187</u>	29	<u> </u>	-	121
L-Histidine	· · · ·		1 A. A.	-	-	-	
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-	-	-	-	-	-	-

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense		NM22-15	ō	Consensus
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
L-Phenylalanine	-	+	11-	-	-	-	-
L-Proline	-	- 1//		-	-	-	-
L-Pyroglutamic Acid	+	++		-	-	-	-
D-Serine	-	++		-	-	-	-
L-Serine	-	1 · 1	-	-	-	-	-
L-Threonine	-	111 3	-	-	-	-	-
D,L-Carnitine	-	(///-	-	-	-	-	-
γ -Amino Butyric Acid	-//	(// -	+	-	-	-	-
Urocanic Acid		L-Z		-	-	-	-
Inosine		N 201	-	-	-	-	-
Uridine		-	-	-	-	-	-
Thymidine	-	2.69	-	-	-	-	-
Phenyethyl-amine	-	/ A	-	-	-	-	-
Putrescine	- 8	1576-957	-	-	-	-	-
2-Aminoethanol		B IOIO	-	-	-	-	-
2,3-Butanediol	-	Maken M	-	-	-	-	-
Glycerol	1- 16	+	-	-	-	-	-
D,L-α-Glycerol	-						
Phosphate	115			_	-	-	-
α-D-Glucose-1-	_	_	_	_	5	_	_
Phosphate					31		
D-Glucose-6-	-	-	_			-	-
Phosphate							

Consensus results from 7 determinations

01	Consensus						
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense	NM22-25			Consensus
GN2 MicroPlate	N2 MicroPlate NBRC 14791 NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations	
lpha-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-25 2 nd	5 3 rd	Consensus results from 3 determinations
Tween 40	+++	++	++	+++	++	++	++
Tween 80	+++	++	+++	+++	++	++	++
N-Acetyl-D-				-	-	-	-
Galactosamine			-				
N-Acetyl-D-				-	-	-	-
Glucosamine	-	Um a					
Adonitol	- /	(///- \ \		-	-	-	-
L-Arabinose	+++	++	+++	+	+	+	+
D-Arabitol		+	11.1	-	-	-	-
D-Cellobiose		A 2018		-	-	-	-
i-Erythritol	//-///		-	-	-	-	-
D-Fructose		+		-	-	-	-
L-Fucose	+	+		-	-	-	-
D-Galactose	// -// b	576-507	++	+	-	-	-
Gentiobiose		No.	// - \\	-	-	-	-
α-D-Glucose		MNG(GN)	+	-	-	-	-
m-Inositol	- VG	66461419	-	-	-	-	-
α-D-Lactose	-	-		-	-	-	-
Lactulose	-45		15-1-	-	-	-	-
Maltose	-	-	-	-	E	-	-
D-Mannitol	-	+	-	-	31	-	-
D-Mannose	-	+	++	-	1	-	-
D-Melibiose	_	-		-	-	-	-
β-Methyl-D-				-	- ¹	-	-
Glucoside	6	-	U.				
D-Psicose	0.9/	6100	5-91	61	9	574	-
D-Raffinose	- 6 T		d - 1/	Ξ	-	-	d -
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	6-		35	-	-	0
Sucrose	251	5 I 9 I	02-7/	7-0	<u>)</u> -() - (100
D-Trehalose	- 6	0.00	/ -	d-	-	- -	6 C
Turanose		_	8.8.04	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	+++	++	++

	Consensus	Consensus results from 7 determinations					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-28	5 3 rd	Consensus results from 3 determinations
Succinic Acid Mono- Methyl-Ester	+++	+++	++	+++	++	++	++
Acetic Acid	+++	++	++	++	++	+	++
Cis-Aconitic Acid	-	2 - 0	-	-	-	-	-
Citric Acid	+++	+ -	-	-	+	-	-
Formic Acid	+++	++	-	+++	+++	++	+++
D-Galactonic Acid Lactone	-	///·	++	+	-	-	-
D-Galacturonic Acid		1.3	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	+++	++	+	++
D-Glucosaminic Acid	- / /		-	++	-	-	-
D-Glucuronic Acid	-	3.63		-	-	-	-
α-Hydroxybutyric		+		-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+++	++	+++
γ-Hydroxybutyric Acid	+++	+	++	+	++	-	+
p-Hydroxy Phenylacetic Acid	145	ew_~~	alester -	-	0	-	-
Itaconic acid	-	-	++	-	3	-	-
α -Keto Butyric Acid	-	-	-	-	1	-	-
α -Keto Glutaric Acid	+	+	+++	-	-	-	-
α -Keto Valeric Acid	-	-	-	- 4		-	-
D,L-Lactic Acid	+++	++	+++	+++	++	++	++
Malonic Acid	0.01	0101	5-91	01	9	5	
Propionic Acid	++	+	++	++	+	-	+
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	+++	+++	++	+++
Sebacic Acid	+++	++	00+	++	++	+	++ 0
Succinic Acid	+++	++	+++	++	+++	++	++
Bromosuccinic Acid	+++	++	+++	++	+	-	+
Succinamic Acid	++	++	++	+++	++	++	++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-

	Consensus results from 7 determinations						
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense	NM22-25			Consensus results from 3
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
D-Alanine	+	++	11-	+	+	+	+
L-Alanine		+		-	-	-	-
L-Alanyl-glycine	-	Open in		-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic	-/	//-	<u> </u>	-	-	-	-
Glycyl-L-Glutamic	/-//	hā	11-1	-		-	-
L-Histidine		-	-	-	-	-	-
Hydroxy-L-Proline		3		-	-	-	-
L-Leucine		++	++	-	-	-	-
L-Ornithine	// -// b	1000		-	· .	-	-
L-Phenylalanine		+	/ -	-	-	-	-
L-Proline	-	Malana	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-45			-	-	-	-
L-Threonine	-	-	-	-	0	-	-
D,L-Carnitine	-	-	-	-	3	-	-
γ -Amino Butyric Acid	-	-	+	-		-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	- 1	- 1	-	-
Uridine	6	-	3	-	-	-	-
Thymidine	100	0101	5	01	č	5	-
Phenyethyl-amine	- c /	C- 7	d - 1	Ξ	-	-	d -
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	6	-	0	-	-	01
2,3-Butanediol	55	01-01	00	20			000
Glycerol		+	/ -	d	- 1	-	6-71
D,L- Q -Glycerol			1.1.1.1.1				
Phosphate	-	-	-			-	-
α -D-Glucose-1- Phosphate	-	-	-	-	-	-	-

	Consensus						
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense	NM22-25			Consensus
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations
D-Glucose-6- Phosphate	- 0	-	100	-	-	-	-

	Consensus	results from 7 det	erminations				
Carbon/Nitrogen sources on Biolog	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	NM22-30			Consensus
GN2 MicroPlate				1 st	2 nd	3 rd	determinations
α-Cyclodextrin				-	-	-	
Dextrin	//-//A	+		-	-	-	-
Glycogen	- 7		7/7-	-	-	-	-
Tween 40	+++	++	++	++	-	-	-
Tween 80	+++	++	+++	++	+	-	+
N-Acetyl-D- Galactosamine	100	Ment an		-	-	-	-
N-Acetyl-D- Glucosamine	-	-	-	-	9	-	-
Adonitol	-	-	-	++	5-	-	-
L-Arabinose	+++	++	+++	+	-	-	-
D-Arabitol	-	+	-	++		-	-
D-Cellobiose	1 -	-	0.7	-	-	-	-
i-Erythritol	175.0/	0100	2 OAI	65.1	~	5	
D-Fructose	V	+	- M	++	-	-	-
L-Fucose	+	+	0.11	-	-	-	
D-Galactose	-	1	++	+	-	-	
Gentiobiose	00	0.101	000	-	100	110	0.01
α -D-Glucose	1-27	1.1-1.1	+	+	-	-	I A FI
m-Inositol	11.0	010.04		<u>.</u>	_	<u> </u>	INF
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
	Consensus	results from 7 de	terminations				
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Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	1 st	NM22-30) 3 rd	Consensus results from 3 determinations
D-Mannitol	-	+	11-	++	-	-	-
D-Mannose		+	++	++	-	-	-
D-Melibiose	-	Obier and	1/-	-	-	-	-
β-Methyl-D- Glucoside	-		-	-	-	-	-
D-Psicose	-	200		-	-	-	-
D-Raffinose	-/		-	-	-	-	-
L-Rhamnose	- /	(//.	-	-	-	-	-
D-Sorbitol	- /			++	-	-	-
Sucrose		A STR.		-	-	-	-
D-Trehalose	//-///		-	-	-	-	-
Turanose		3. 631		-	-	-	-
Xylitol		83/200		-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	+	+	++
Succinic Acid Mono- Methyl-Ester	+++	+++	++	++	-	-	-
Acetic Acid	+++	++	++	++	+	+	++
Cis-Aconitic Acid	-49		-	++	+	-	+
Citric Acid	+++	+	-	++	+	+	++
Formic Acid	+++	++	-	+++	++	++	++
D-Galactonic Acid Lactone	-	-	++	+		-	-
D-Galacturonic Acid	-	-	+	- 1		-	-
D-Gluconic Acid	+++	++	+++	++	+	-	+
D-Glucosaminic Acid	100	0100	5-01	+	9	5-0	-
D-Glucuronic Acid	- C /	C 1	d - / \	C.	-	-	
α -Hydroxybutyric Acid	-	+	-		-	-	0.7
β-Hydroxybutyric Acid	1+51	1+1	+++	+++	+	+	1215
γ-Hydroxybutyric Acid	+++	+	++	++	-	-	
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	_	-

	Consensus	results from 7 de					
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	<i>B. elkanii</i> NBRC 14791	<i>B. japonicum</i> NBRC 14783	B. liaoningense NBRC 100396	NM22-30) 3 rd	Consensus results from 3 determinations
Itaconic acid	-	- 1/	++	-	-	-	-
α -Keto Butyric Acid	-	- 1//	122	-	-	-	-
α -Keto Glutaric Acid	+	+	+++	-	-	-	-
α -Keto Valeric Acid	-	3 - 0	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+++	+	+	++
Malonic Acid	-	1/11 3	-	+++	+	+	++
Propionic Acid	++	+	++	++	-	-	-
Quinic Acid	- / /	++	-	++	+	-	++
D-Saccharic Acid	+++	++		+	-	-	-
Sebacic Acid	+++	++	+	++	-	-	-
Succinic Acid	+++	++	+++	++	+	+	++
Bromosuccinic Acid	+++	++	+++	++	+	-	+
Succinamic Acid	++	++	++	++	-	-	-
Glucuronamide	- 3	atte (?) //		-	· -	-	-
L-Alaninamide		++	-	-	-	-	-
D-Alanine	+	++	-	+	-	-	-
L-Alanine	- 16	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-45			-	-	-	-
L-Aspartic Acid	-	++	++	+	E	-	-
L-Glutamic Acid	-	++	-	++	3	-	-
Glycyl-L-Aspartic	-	-	-	•	1	-	-
Glycyl-L-Glutamic Acid	-	-	C	-	-	-	-
L-Histidine	0	0101	5-91	-	9	5-6	-
Hydroxy-L-Proline		C- 7	0	-	-	-	-
L-Leucine	-	++	++	+	-	-	-
L-Ornithine	-	6	-	-	-	-	0
L-Phenylalanine	550	+	00-01	+	n (-	50
L-Proline	- 6		/ -	6	7 - 7	-	6-71
L-Pyroglutamic Acid	+	++	5 A. A.	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-

	Consensus	results from 7 de	terminations					
Carbon/Nitrogen sources on Biolog	B. elkanii	B. japonicum	B. liaoningense	NM22-30			Consensus	
GN2 MicroPlate	NBRC 14791	NBRC 14783	NBRC 100396	1 st	2 nd	3 rd	determinations	
D,L-Carnitine	-	-	11-	-	-	-	-	
γ -Amino Butyric Acid	-	- //	+	-	-	-	-	
Urocanic Acid	-			-	-	-	-	
Inosine	-		-	-	-	-	-	
Uridine	-	× - †	-	-	-	-	-	
Thymidine	-	101 3	-	-	-	-	-	
Phenyethyl-amine	-	///- \ \	-	-	-	-	-	
Putrescine	-//	// -	-	-	-	-	-	
2-Aminoethanol			-	-	-	-	-	
2,3-Butanediol		12 <u></u>	-	-	-	-	-	
Glycerol	- / / /	+	-	++	-	-	-	
D,L- Q -Glycerol		2 (0)		-	-	-	-	
α-D-Glucose-1-	/-/P	ATT ON		-	-	-	-	
D-Glucose-6- Phosphate	1			-	_	-	-	

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX G

Alignment of 16S rDNA sequences.

	10	20	30	40	50	60	70	80	90	100
Belkanii62 SEMIA6099 Belkanii51 SEMIA6496 SEMIA6414 SEMIA6416 SEMIA6418 SEMIA6118 SEMIA6118 SEMIA5002 Bpachyrhiz NH22-15_co NH22-13com Clustal Co	ATTAAGGAGG 	TGATCCASCC TGATCCASCC TGATCCASCC TGATCCASCC 	GCAGGTTCCC GCAGGTTCCC GCAGGTTCCC GCAGGTTCCC GCAGGTTCCC GCAGGTTCCC GCAGGTTCCC NCAGGTTCCC NCAGGTTCCC	CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC CTACGGCTAC TACGGCTAC CTACGGCTAC	CTTG TTACG CTTG TTACG	ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC ACTTCACCCC	AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC AGTOGOTOAC	CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG CCTACCOTOG		CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT CCTTTCGGTT
BelkaniiGZ SEMIA6099 BelkaniiS1 SEMIA6496 SEMIA6416 SEMIA6416 SEMIA6405 SEMIA6405 SEMIA6405 Byachyrhiz Byachyrhiz NH22-15_com NH22-13com Clustal Co	AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT AGCGCACCGT	CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA CTTCAGGTAA	2 13: AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC AACCAACTCC	CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA CATGUTUTGA	CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET CEGECCETET	GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC GTACAAGGCC	170 CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA CGGGAACGTA	TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG TTCACCOTGG	194 CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG CGTGCTGATG	CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT CACGATTACT
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SEMIA5060 SEMIA5020 SEMIA510 NM22-8_com NM22-30_co Clustal_Co	AGGGCCTTCA AGGGCCTTCA AGGGCCTTCA AGGGCCTTCA AGGGCCTTCA	TCACTCACGC TCACTCACGC TCACTCACGC TCACTCACGC TCACTCACGC	GGCATGGCTG GGCATGGCTG GGCATGGCTG GGCATGGCTG GGCATGGCTG	GATCAGGGTT GATCAGGGTT GATCAGGGTT GATCAGGGTT GATCAGGGTT	GCCCCCATTG GCCCCCATTG GCCCCCATTG GCCCCCATTG GCCCCCATTG	TCCAATATTC TCCAATATTC TCCAATATTC TCCAATATTC TCCAATATTC	CCCACTGCTG CCCACTGCTG CCCACTGCTG CCCACTGCTG CCCACTGCTG CCCACTGCTG	CCTCCCGTAG CCTCCCGTAG CCTCCCGTAG CCTCCCGTAG CCTCCCGTAG	GAGTTTGGGC GAGTTTGGGC GAGTTTGGGC GAGTTTGGGC GAGTTTGGGC	CGTGTCTCAG CGTGTCTCAG CGTGTCTCAG CGTGTCTCAG CGTGTCTCAG
	121	0 122	0 123	0 124	0 125	0 126	0 127		0 129	0 1300
USDA110 USDA62 SEHLA5083 SEHLA SEHLA5021 SEHLA5026 SEHLA5040 SEHLA5060 SEHLA5020	TCCCAATGTG TCCCAATGTG TCCCAATGTG TCCCAATGTG TCCCAATGTG TCCCAATGTG TCCCAATGTG TCCCAATGTG	GCTGATCATC GCTGATCATC GCTGATCATC GCTGATCATC GCTGATCATC GCTGATCATC GCTGATCATC GCTGATCATC	CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC CTCTCAGACC	AGCTACTGAT AGCTACTGAT AGCTACTGAT AGCTACTGAT AGCTACTGAT AGCTACTGAT AGCTACTGAT AGCTACTGAT	CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG CGTCGCCTTG	GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT	ACCCTACCAA ACCCTACCAA ACCCTACCAA ACCCTACCAA ACCCTACCAA ACCCTACCAA ACCCTACCAA ACCCTACCAA	CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC CTAGCTAATC	AGACGCGGGC AGACGCGGGC AGACGCGGGC AGACGCGGGC AGACGCGGGC AGACGCGGGC AGACGCGGGC AGACGCGGGC	CGATCTTTCG CGATCTTTCG CGATCTTTCG CGATCTTTCG CGATCTTTCG CGATCTTTCG CGATCTTTCG CGATCTTTCG
SENIA510 NH22-8_com	TCCCAATGTG TCCCAATGTG	GCTGATCATC GCTGATCATC	CTCTCAGACC CTCTCAGACC	AGCTACTGAT AGCTACTGAT	CGTCGCCTTG CGTCGCCTTG	GTAGGCCGTT GTAGGCCGTT GTAGGCCGTT	ACCCTACCAA ACCCTACCAA	CTAGCTAATC CTAGCTAATC CTAGCTAATC	AGACGCGGGC AGACGCGGGC	CGATCTTTCG CGATCTTTCG CGATCTTTCG
Clustal Co			*****	*****		******	*****	11111111111	******	11111111111
	101	0 192	0 133	0 134	0 195	0 196	0 197	0 130	0 139	0 1400
USDA110 USDA62 SEHIA5083 SEHIA5021 SEHIA5021 SEHIA5043 SEHIA5043 SEHIA5020 SEHIA5020 SEHIA5020 SEHIA5020 SEHIA5020 Clustal Co	СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС СССАТАЛАТС	TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA TTTCCCCGTA	ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE ACCOUNTATE	COGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC CGGTATTAGC	ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC ACAAGTITEC	CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI CTGIGITIGTI	СССАЛССАЛА СССАЛССАЛА СССАЛССАЛА СССАЛССАЛ	AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC AGGTACGTTC	CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA CCACGCGTTA	CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC CTCACCCOTC
	141	0 142	0 143	0 144	0 145	0 146	0 147	0 140	0 149	0
USDA110 USDA62 SEMIA5083 SEMIA SEMIA5021 SEMIA5036 SEMIA5043	TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC	GTATTGCTAC GTATTGCTAC GTATTGCTAC GTATTGCTAC GTATTGCTAC GTATTGCTAC	GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA	CTTGCATGTG CTTGCATGTG CTTGCATGTG CTTGCATGTG CTTGCATGTG CTTGCATGTG	TTARGECTGE TTARGECTGE TTARGECTGE TTARGECTGE TTARGECTGE TTARGECTGE	CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT	CGCTCTGAGC CGCTCTGAGC CGCTCTGAGC CGCTCTGAGC CGCTCTGAGC CGCTCTGAGC	CAGGATCAAA CAGGATCAAA CAGGATCAAA CAGGATCAAA CAGGATCAAA CAGGATCAAA	CTCT CTCT CTCT CTCT CTCT CTCT CTCT CTC	
SENIA5060 SENIA5020 SENIA510 NH22-0_com NH22-30_co	TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC TGCCGCTGAC	GTATTGCTAC GTATTGCTAC GTATTGCTAC GTATTGCTAC GTATTGCTAC	GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA GCCCGCTCGA	CTTGCATGTG CTTGCATGTG CTTGCATGTG CTTGCATGTG CTTGCATGTG	ТТААССТСС ТТААССТСС ТТААССТСС ТТААССТСС ТТААССТСС ТТААССТСС	CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT CGCCAGCGTT	CGCTCTGAGC CGCTCTGAGC CGCTCTGAGC CGCTCTGA <mark>CA</mark> CGCTCTGAGC	CAGGATCAAA CAGGATCAAA CAGGATCAAA GGGGATCAAA CGGGATCAAA	СТСТ СТСТ СТСТ СТСА СТСАААА	

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APPENDIX H

Table for determination of the most probable number for soybean rhizobia (Brockwell,1963.)

Most probable number of nodule bacteria calculated from the distribution of positive (nodulated) test plants in a plant infection test based on a fivefold dilution series

No. of positive (nodu- lated) test plants (of four) resulting from in- oculation with 1-mi samples		Most probable no. ruspensio	of nodule bacteria per ml of n at dilution level 1	2 fo	io. o lated ur) i oculi	f pos tes-li stion sar	itive t plan ting f with sples	(nod ots () rom 1-m	din-	Most probable no. of nodule bacteria per ml of suspension at dilution level 1					
Fivefo	id di	iluti	n le	rel						ilutie	m le	rel			
1:5	1:25	1:125	1:625	1:3125*	Estimate	Confidence limits (95%)	1.7	1:5	1:25	1:125	1:625	1:3125*	Estimate	Confidence limits (95%)	
0	0	0	0	0	1.1	0.2- 7.9	4	4	4	0	1	0	2.7×100	10.4- 7.0 × 100	
0	0	0	0	0	2.6	0.6-10.1	4	4	4	1	1	0	3.8×100	1.5- 9.8 × 100	
0	0	0	0	0	4.6	1.5-14.1	4	4	4	2	1	0	5.4×100	$2.0-14.4 \times 100$	
0	0	0	0	0	8.0	3.0-21.5	4	4	4	3	1	0	8.2×100	$3.1-22.0 \times 100$	
1	0	0	0	0	1.0	0.1- 7.7	4	4	4	0	2	0	3.5×100	1.4- 9.2 × 100	
1	0	0	0	0	2.3	0.6- 9.6	4	4	4	1	2	0	4.9×100	$1.8-13.0 \times 100$	
1	0	0	0	0	4.0	1.2-12.8	4	4	4	2	2	0	7.1×100	$2.6-19.0 \times 100$	
1	0	0	0	0	6.5	2.3- 18.0	4	4	4	3	2	0	10.9×100	$4.2 - 28.6 \times 100$	
2	0	0	0	0	2.1	0.5- 9.2	4	4	4	0	3	0	4.5×100	$1.7 - 11.9 \times 100$	
2	0	0	0	0	3.5	1.1-11.9	1	11	1.	1.	3	0	6.3×100	$2.3-16.9 \times 100$	
2	0	0	0	0	5.5	1.9-16.0		11	1	2	3	0	9.1 × 100	$3.4-24.2 \times 100$	
2	0	0	0	0	8.7	3.3-23.0	1	1	1	0	3	0	14.1 X 100	5.4- 35.7 × 100	
3	0	0	0	0	3.0	0.9-10.6			4	4	١.	0	14.9 × 100	E E 28 0 V 100	
3	0	0	0	0	4.9	1.6- 14.6	1	12	1	1	1		14.3 X 100	5.5- 36.9 × 100	
3	0	0	0	8	1.2	2.7- 19.6	4	1	17	12	5		20.3 × 100	11.2- \$1.2 × 100	
°		0	0	0	11.3	4.4- 29.2	4	1	17	17	1 A		50.5 × 100	10.0-133.8 × 100	
11		0	0	6	11.4	11.005	4	17	4	12	6	l i l	13.5 × 100	5 2- 35 3 × 100	
5	0	0	0	0	16.9	9.4-29.0	4	4	4	4	ľĭ	l i l	18.8 × 100	7.2-49.0 × 100	
5	0	0	0	0	04.0	0.2- 12.1	4	4	4	4	2	li l	26.9 × 100	10.1-71.8 × 100	
4	ŏ	ŏ	ő	ő	40.4	15 3-106 6	4	4	4	4	3	1 i l	41.0×100	15.3-110.2 × 100	
0	ĩ	ŏ	ŏ	0	10.8	4.2-28.1	4	4	4	4	0	2	17.7×100	6.8-45.9 × 100	
ĩ	î	ŏ	ő	ō	15.1	5.8-39.2	4	4	4	4	1	2	24.5×100	9.2-65.0 × 100	
2	i	Ő	0	0	21.5	8.1-57.4	4	4	4	4	2	2	35.3×100	$13.1 - 95.4 \times 100$	
3	1	0	0	0	32.8	12.2-87.9	4	4	4	4	3	2	54.4×100	20.6-143.8 × 100	
0	2	0	0	0	14.1	5.4-36.6	4	4	4	4	0	3	22.6×100	8.6- 59.7 × 100	
1	2	0	0	0	19.6	7.4-51.9	4	4	4	4	1	3	31.4×100	11.7- 84.7 × 100	
2	2	0	0	0	28.3	10.5-76.1	4	4	4	4	2	3	45.5×100	$17.0-121.4 \times 100$	
3	2	0	0	0	43.6	16.6-114.2	4	4	4	4	3	3	70.6×100	$27.1-184.2 \times 100$	
0	3	0	0	0	18.1	6.9-47.7			Ι.	Ι.	Ι.	1.1			
1	3	0	0	0	25.2	9.4-67.6	4	14	11	14	11		7.1×1000	$2.7-18.6 \times 1000$	
2	3	0	0	0	36.4	13.7-96.8		1.2	1.	1.2	12	2	10.1×1000	3.8- 27.0 × 1000	
3	3	0	0	0	56.5	21.9-146.0		1	4	4	4	4	15.1×1000 25.2×1000	8.6-74.0 × 1000	
4	1	0	0	0	5.7×10	2.2- 14.7 × 10	_	• 1	ive	test	nl	inter	were incculated wi	ith 1 ml samples from	
4	2	0	0	0	8.1 × 10	$3.1 - 21.2 \times 10$	di	luti	on 1	leve	1		nere moconated wi	ten r-an samples from	
1	3	0	0	0	12.1 × 10	$4.5 - 32.4 \times 10$									
11	4	0	0	0	20.2 × 10	7.6-53.3 × 10									
1	0	1	0	0	5.4×10	$2.1-14.0 \times 10$									
1		1	0	0	7.5 X 10	2.9-19.6 × 10									
21	2		0	0	10.8 X 10	4.0-28.7 × 10									
71	0	2	0	0	7 1 × 10	0.1- 43.9 X 10									
11	1	5	0	0	0.8 × 10	2.7-18.3 × 10									
4	2	2	0	ő	14.1 × 10	5.3-38.1 × 10									
4	3	2	0	ő	21.8 × 10	8.3- 57.1 × 10									
4	0	3	0	ő	9.1 × 10	3.4-23.8 × 10									
4	1	3	0	0	12.6×10	4.7- 33.8 × 10									
4	2	3	0	0	18.2×10	6.9-48.4 × 10									
4	3	3	0	0	28.2 × 10	10.9-73.0 × 10									
4	4	1	0	0	2.9×100	1.1- 7.3 × 100									
4	4	2	0	0	4.1 × 100	1.6-10.6 × 100									
4	4	3	0	0	6.0 × 100	$2.3-16.2 \times 100$									
		4	0	0	10.1 × 100	3 8 96 6 ¥ 100									

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APPENDIX I

Number of nodules in the determination of MPN



Dilution level		Repli	cates		Number of pouches	
Dilution level	1	2	3	4	with nodules	MPN of soybean
1	29	16	12	28	4	rhizobium per ml
1:5	20	14	10	12	4	of suspension at
1 : 25	7	9	20	34	4	dilution level 1
1 : 125	15	16	10	19	4	613
1:625	11	17	18	0	3	
1:3125	16	14	9	0	3	7.06x10 ³
	1	71			W.L.T.M.	71107

MPN of soybean rhizobia = 7.06×10^4 MPN/g soil



Dilution loval		Repli	cates		Number of pouches	
Dilution level	1	2	3	4	with nodules	MPN of soybean
1	24	18	19	18	4	rhizobium per ml
1:5	17	1	6	18	4	of suspension at
1:25	11	7	2	17	4	dilution level 1
1 : 125	7	3	12	10	4	กร
1 : 625	23	21	0	0	2	110
1:3125	17	2	0	0	2	3.55 x10 ³

MPN of soybean rhizobia = 3.55×10^4 MPN/g soil



Dilution loval	/	Repli	cates	1232	Number of pouches	
Dilution level	1	2	3	4	with nodules	MPN of soybean
1	37	34	29	3	4	rhizobium per ml
1:5	23	23	3	26	4	of suspension at
1:25	3	12	23	17	4	dilution level 1
1 : 125	16	4	12	5	4	
1:625	16	2	0	0	2	
1 : 3125	13	11	0	0	2	3.55 x10 ³

MPN of soybean rhizobia = 3.55×10^4 MPN/g soil



Author's Biography

Miss Thanpapha Chanthapetch was born on November 4, 1983. She obtained a Bachelor of Science Degree in Microbiology from Prince of Songkla University, Thailand, in 2006.

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