การหาลายพิมพ์ดีเอ็นเอของไรโซเบียมถั่วเหลืองที่แยกจากปมรากถั่วเหลืองที่ใส่เชื้อปุ๋ยชีวภาพ NA7 ในตำบลน้ำมวบ จังหวัดน่าน

นางสาวธันปภา จันทเพ็ชร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

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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Thesis Title	DNA FINGERPRINTING OF SOYBEAN RHIZOBIA ISOLATED FROM NODULES OF SOYBEANS INOCULATED WITH BIOFERTILIZER NA7 IN NAM MOUB SUBDISTRICT, NAN PROVINCE
By	Miss Thanpapha Chanthapetch
Field of Study	Industrial Microbiology
Thesis Advisor	Associate Professor Kanjana Chansa-ngavej, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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

ไรโซเบียมถั่วเหลืองเข้าสร้างปมที่รากถั่วเหลืองและเปลี่ยนไนโตรเจนจากอากาศให้เป็นแอมโมเนียสำหรับถั่วเหลืองใช้ในการเจริญ บรรทัดฐานแรกในการพัฒนาปุ๋ยชีวภาพไรโซเบียมสำหรับถั่วเหลืองประกอบด้วยการคัดเลือกสายพันธุ์ไรโซเบียมถั่วเหลืองที่สามารถแข่งขันกับไรโซเบียมท้องถิ่น ในการเข้าสร้างปมที่รากถั่วเหลือง ปุ๋ยชีวภาพไรโซเบียมถั่วเหลืองที่มีจำหน่ายในท้องตลาด ไม่มีวิธีควบคุมคุณภาพที่ระบุว่าสายพันธุ์ไรโซเบียมถั่วเหลืองที่ใช้ในการผลิตปุ๋ยฯ เป็นไรโซเบียมที่ไม่เกิดการเปลี่ยนแปลงสารพันธุกรรม ในปีเพาะปลูก 2550/2551 มีการทดลองผลิตปุ๋ยชีวภาพไรโซเบียมถั่วเหลือง NA7 ในระดับห้องปฏิบัติการ ซึ่งมีลายพิมพ์ดีเอ็นเอประจำสายพันธุ์ และเก็บรักษาได้ที่อุณหภูมิห้อง เพิ่มจำนวนเซลล์โดยเลี้ยงในอาหารสูตร yeast extract mannitol และคลุกเซลล์กับดินพีต (peat) ในสัดส่วน $2x10^8$ เซลล์ต่อกรัมพีต ใช้ปุ๋ยชีวภาพไรโซเบียมถั่วเหลือง NA7 คลุกกับเมล็ดถั่วเหลืองพันธุ์เชียงใหม่ 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 ผลการทดลองได้แยกแบคทีเรียจากปมรากถั่วเหลือง 198 ไอโซเลต แบ่งเป็นประเภทเพิ่มจำนวนเร็ว 147 ไอโซเลต และประเภทเพิ่มจำนวนช้า 51 ไอโซเลต เนื่องจากสายพันธุ์ NA7 เป็นไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า จึงหาลายพิมพ์ดีเอ็นเอของแบคทีเรียประเภทเพิ่มจำนวนช้า 51 ไอโซเลต และในการเปรียบเทียบลายพิมพ์ดีเอ็นเอพบลายพิมพ์ดีเอ็นเอของ NA7 จำนวน 13 ไอโซเลต คิดเป็นสัดส่วนการเข้าสร้างปม 6.6% ผลการหาปริมาณไรโซเบียมถั่วเหลืองในตัวอย่างดินจากแปลงทดลองที่ต.น้ำมวบ อ.เวียงสา จ.น่าน โดยวิธี Most Probable Number (MPN) พบไรโซเบียมถั่วเหลืองโดยเฉลี่ย $4x10^4$ เซลล์ต่อดินหนึ่งกรัม ผลการจำแนกชนิดไรโซเบียมถั่วเหลือง 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 จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม —

5072647323 : MAJOR INDUSTRAIL MICROBIOLOGY

KEYWORDS : Soybean rhizobium, Soybean rhizobium biofertilizers, Soybean yields, Competition in nodulation

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 2×10^8 cells per gram. Soybean seeds cv. CM 60 mixed with the biofertilizer were planted in a 15×24 m^2 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 fast-growers and 51 isolates were slow-growers. Since soybean rhizobium strain NA7 was a slow-grower, 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 4×10^4 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*.

Department : Microbiology — Student's Signature Thanpapha Chanthapetch

Field of Study : Industrail Microbiology — Advisor's Signature K. Chansangavej

Academic Year : 2009 — Co-Advisor' Signature —

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Contents

Content of Tables

Content of Figures

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 to 2007.

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

Sources :

Customs Office (2008).http://www.feedusers.com/thai/cms/html/Inedible/110.html

Office of Agricultural Economics(2008).http://www.oae.go.th/oae_website/oae_imex.php

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/*rai*) in Thailand and in countries which are leading soybean exporters.

Ranking No.	Country / Year	Average soybean yields (kg/*rai*)			
		2004	2005	2006	2007
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 locally-grown 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 Soybean cultivation areas in Thailand and average soybean yields.

Year	Cultivation Area (1000 *rai*)	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 10^8 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 10^8 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 2×10^8 cells per gram peat. Soybean seeds cultivar CM 60 mixed with the biofertilizer had previously been grown in a 15 x 24 m^2 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.

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 :

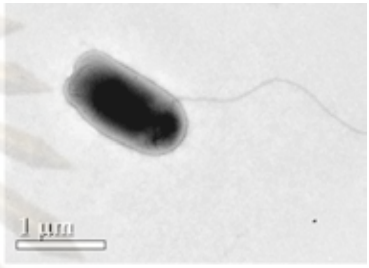
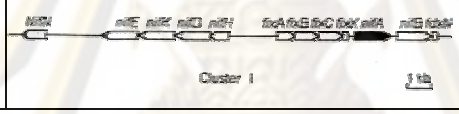
Bradyrhizobium elkanii (Kuykendall et al., 1992)

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.

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

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 
3. *nifHDK*	*nifHDK* are in the same operon 	*nifH* and *nifDK* are on separate operons 

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 10^9 cells·ml^{-1} in the stationary phase, the autoinducer Bradyoxetin with the chemical formula [2-[4-[[4-(3-aminooxetan-2yl)phenyl](imino)methyl]phenyl]oxetan-3-ylamine] was secreted in sufficient quantities to induce expression of $nodD_2$. Protein $NodD_2$ 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 10^9 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.

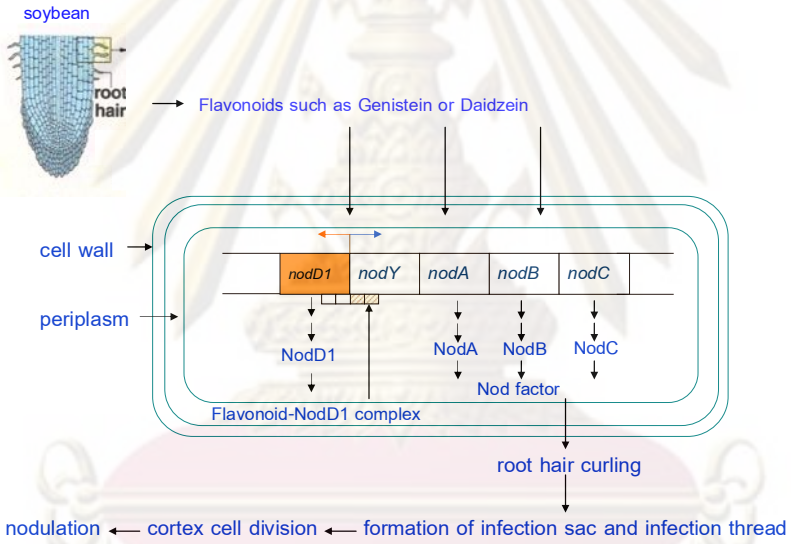


Figure 2.1 Summary of slow-growing soybean rhizobium nodulation genetics (modified from Stacey, 1995).

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 $NodD_1$-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 :

ATCCATCGT	GTGGATGTA	TTCT	ATCGAAACA	ATCGATTTT	ACCAGAT

The consensus sequence of nod boxes are A_{74} T_{90} C_{88} G_{85} A_{93} T_{89} T_{71} G_{74} T_{74} (Wang and Stacey., 1991).

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.

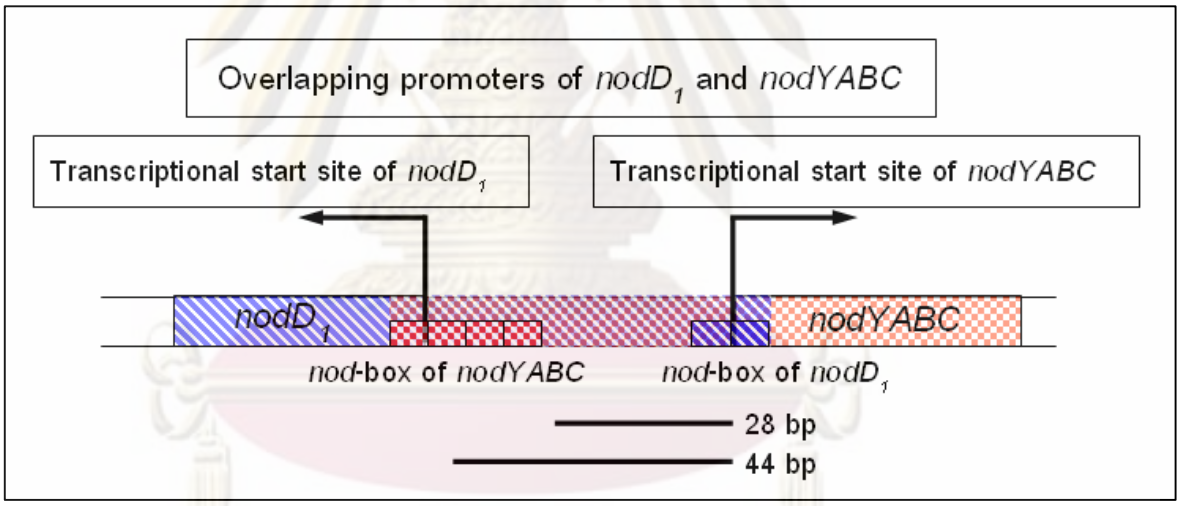


Figure 2.2 Diagramatic representation of a DNA segment of *B. japonicum* nodulation genes showing promoters of $nodD_1$ and *nodYABC* are overlapped with transcriptional start sites of *nodD* and of *nodYABC* lying in the *nod* box of the opposing transcript (modified from Wang and Stacey , 1991).

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 $NodD_2$ 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.

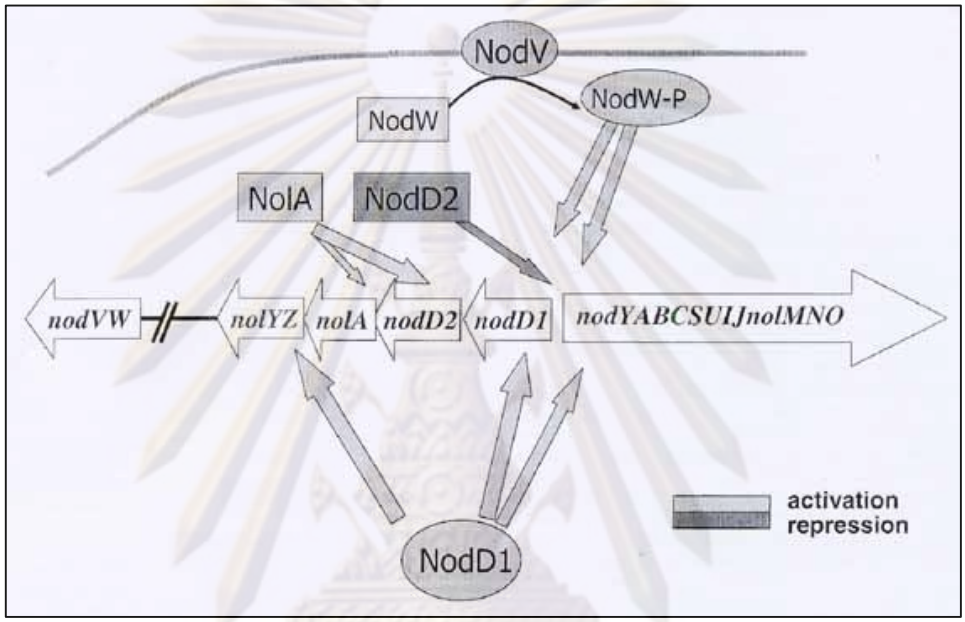


Figure 2.3 $NodD_1$-flavonoid complexes bind to $nodD_1$ box and *nodY* box in the promoters of $nodD_1$ and *nodYABC* to activate transcription of $nodD_1$ and *nodYABC*. A protein product NodV autophosphorylates then transfers the phosphate group to NodW. Phosphorylated NodW-P activates the expression of $nodD_1$ and *nodYABC*. NolA regulates the expression of $nodD_2$ whose protein product, $NodD_2$, 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.

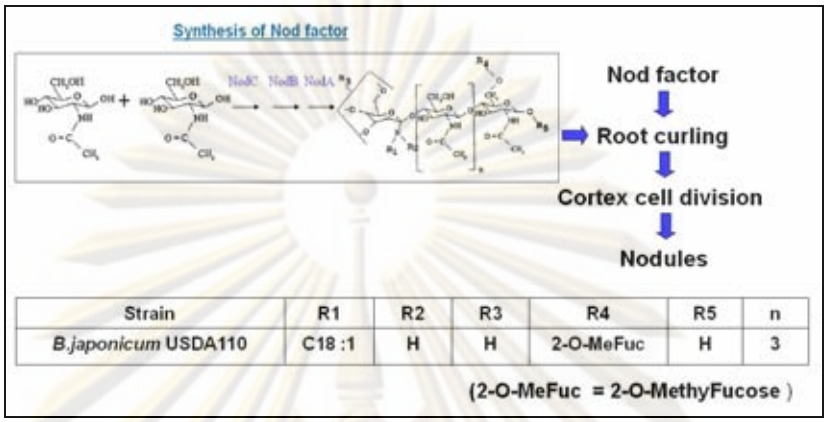


Figure 2.4 Synthesis of Nod factor in *Bradyrhizobium japonicum* (Stacey, 1995).

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 nitrogen-fixer, *Klebsiella pneumoniae*. Lee et al. (1993) purified NifA from *Klebsiella pneumoniae* 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.

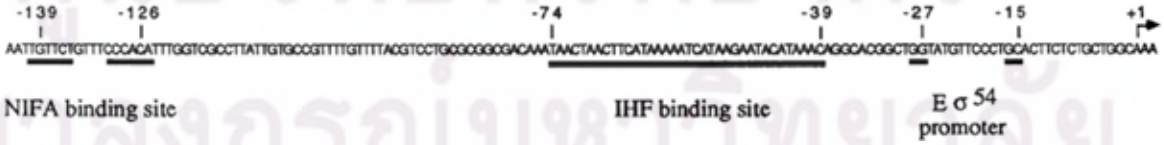
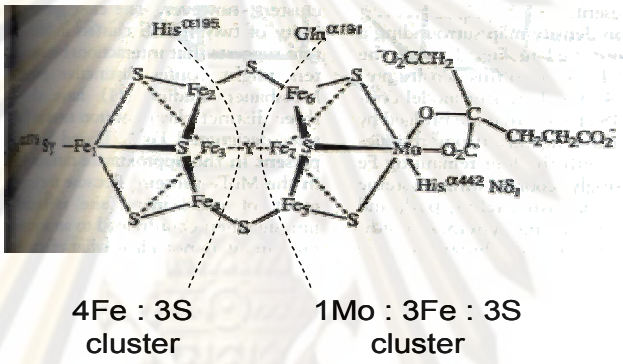


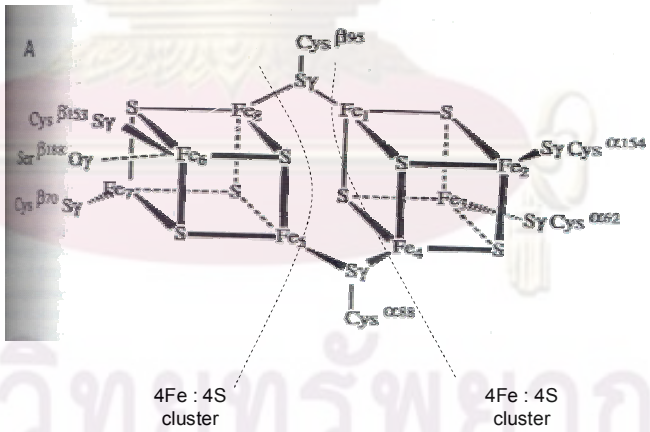
Figure 2.5 IHF binding site is between those of NifA binding site and the -24/-12-type promoter of *nifHDK* in *Klebsiella pneumoniae*. IHF bends the DNA to allow close contact between NifA and Sigma-54-RNA Polymerase holoenzyme for activation of transcription of *nifHDK* (Lee et al., 1993; Santero et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

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)



(a)



(b)

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).

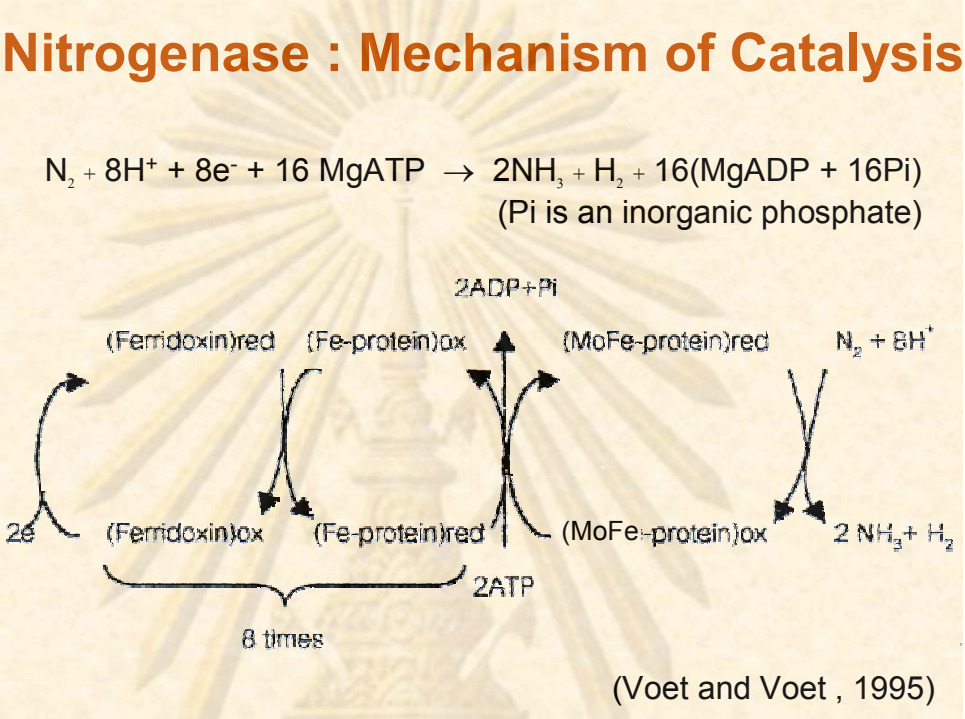


Figure 2.7 Catalytic activity of nitrogenase (Voet and Voet, 1995).

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.

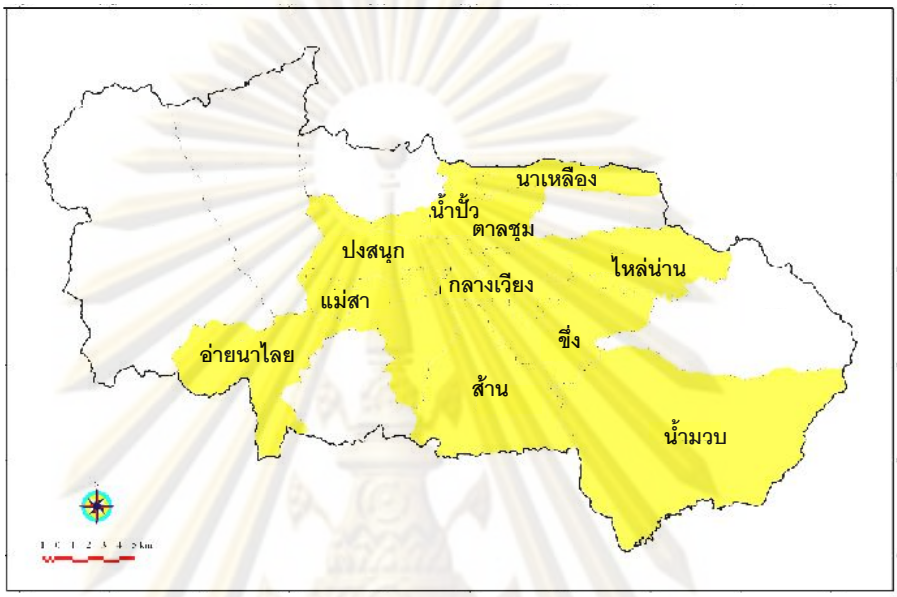
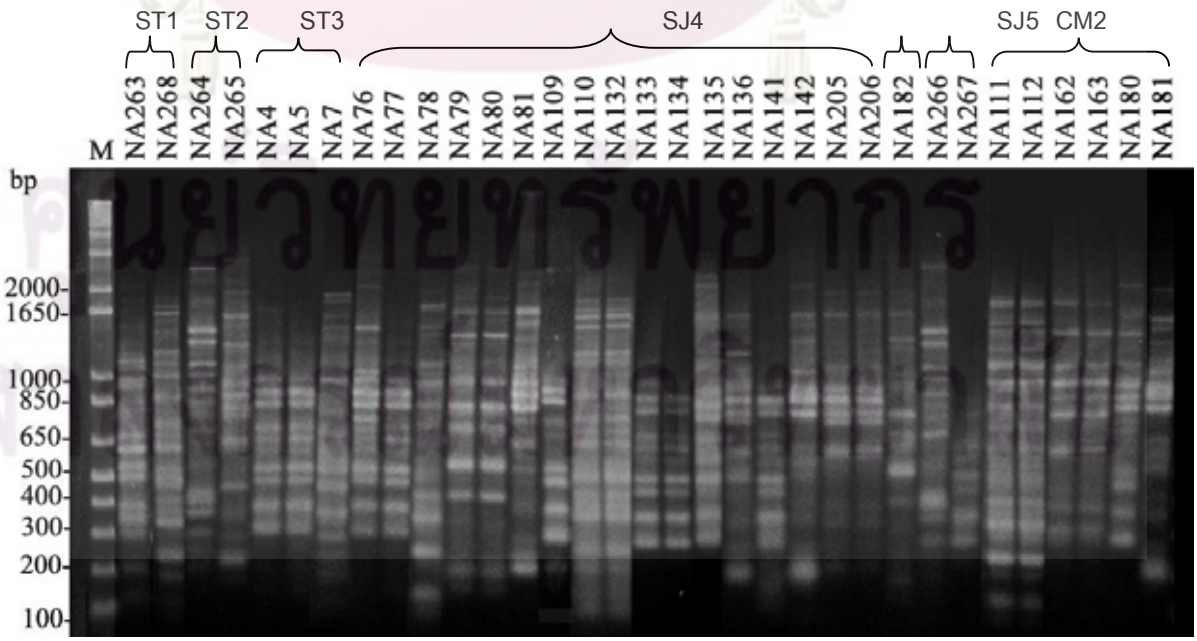
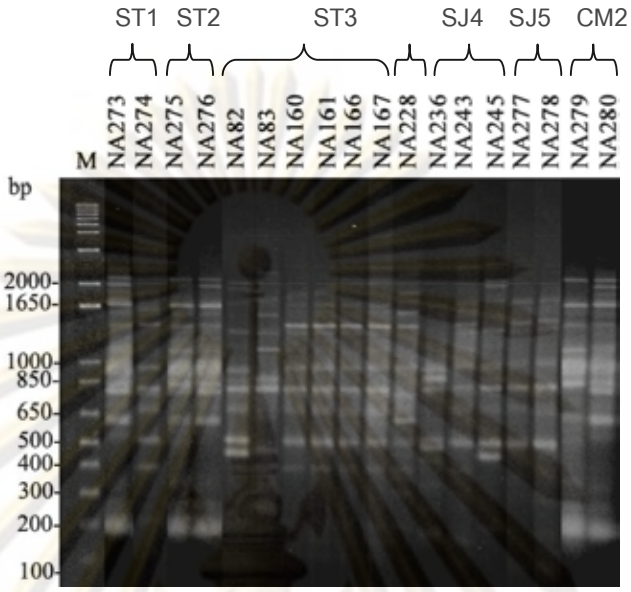


Figure 2.8 Map showing 11 soybean-growing subdistricts in Wiang Sa district, Nan province. Nam Moub subdistrict is located at the border of Thailand and Loas Republic. (http : www.oae.go.th/gis/images/boundary/nan/wiangsa.jp)

(a) Klang Wiang





(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 (Chansa-ngavej 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 m^2 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 10^8 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.(http://www.soy growers.com/step). 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 (*Cfo*I, *Dde*II, *Hae*III, *Hinf*I, *Msp*I, *Nde*II and *Rsa*I), cutting PCR products of the

intergenic spacer region between 16S rDNA with and 23S rDNA (IGS) with *Alu*I, *Cfo*I, and *Hae*III, and cutting PCR-amplified products of *nifH* with *Cfo*I, *Hae*III, and *Msp*I. 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 type IV. However, The representatives of the isolated bradyrhizobia belonging to the 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)

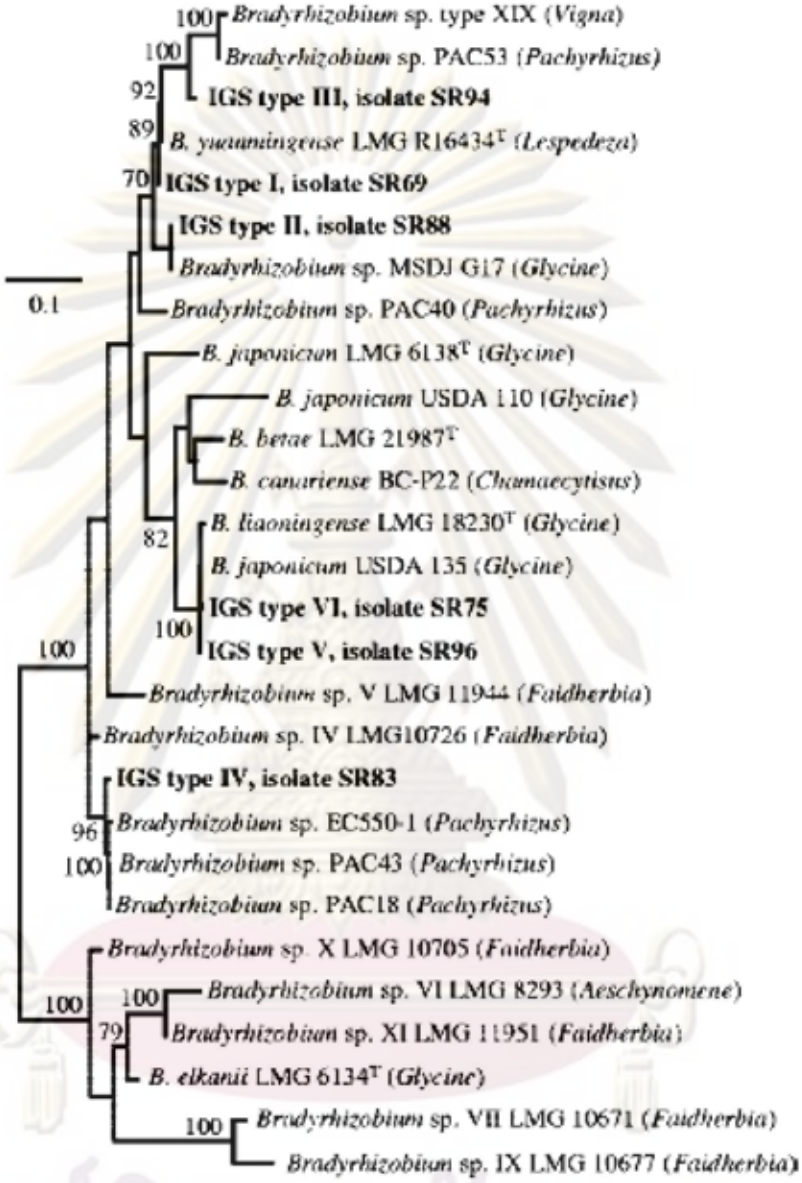
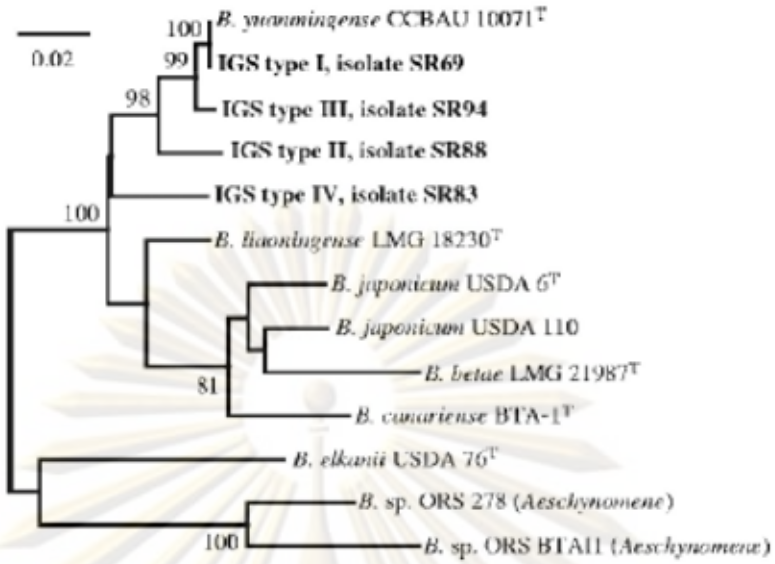
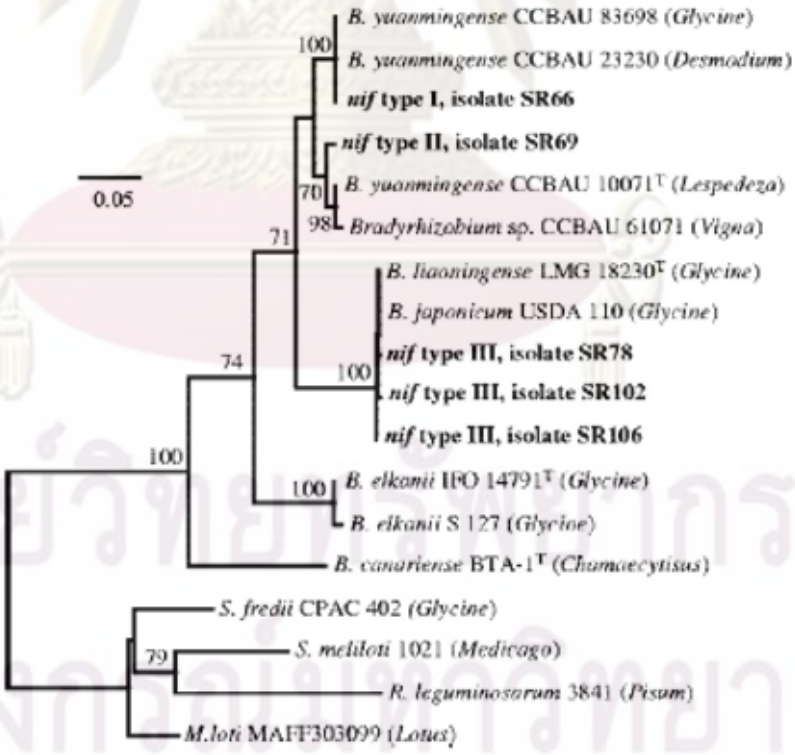


Figure 2.10 Phylogenetic ML tree based on 938-bp alignment of nucleotide sequences of the IGS between the 16S and 23S rRNA genes (Appunu et al., 2008).



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



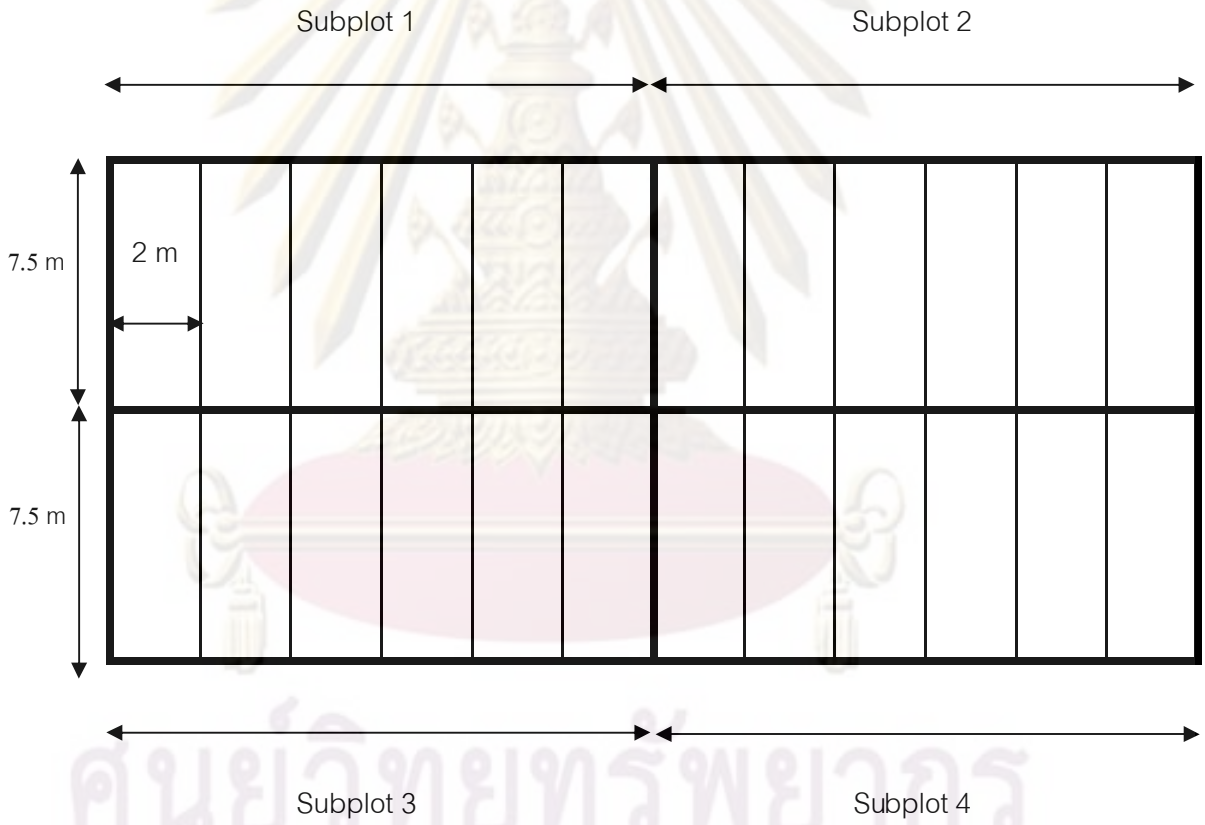
Figures 2.12 Phylogenetic ML tree based on 612-bp alignment of nucleotide sequences of the *nifH* gene (Appunu et al., 2008).

CHAPTER III

MATERIALS AND METHODS

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

A 15 X 24 m^2 experimental plot as shown in Figure 3.1 with four 7.5 X 6.0 m^2 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).



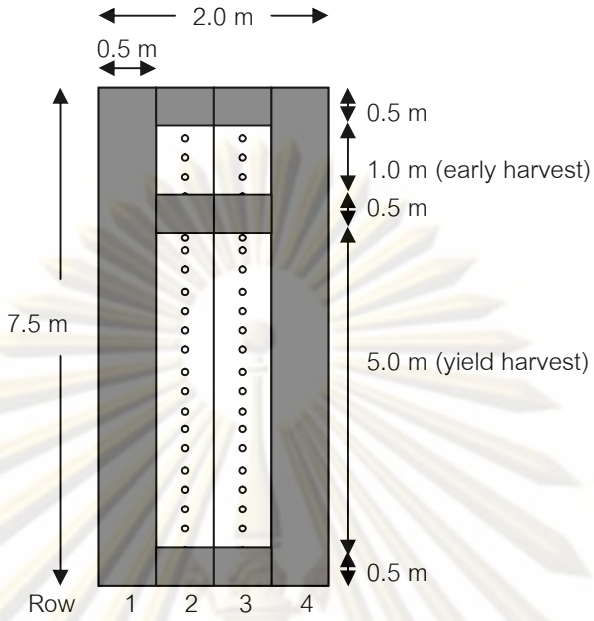


Figure 3.1 Lay-out of a 15 X 24 m^2 experimental plot (upper diagram) with a 2.0 X 7.5 m^2 plot (lower diagram) showing 4 rows of soybean plants which were represented by small circles. Soybean seeds in rows 1 and 4 were not mixed with any biofertilizer. Seeds in rows 2 and 3 were mixed with either biofertilizer 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 4^0C for short-term storage and in 10% glycerol for long-

term storage. Each isolate was grown in YM broth at 30^{0}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; K_2HPO_4 0.5; $MgSO_4$ •7H_2O 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

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 µg. ml^{-1} and incubated at 25^0 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^{o}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^{o}C, until mid log phase. 100 µl 2.5 mg.ml^{-1} lysozyme was added to the cell pellet, mixed thoroughly, and incubated at 37^{o}C for 1 h before 4 cycles of freezing at –20^{o}C for 5 minutes and thawing at 80^{o}C for 5 minutes. 250 µl 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^{o}C, for 5 minutes. The supernatant was transferred to a fresh eppendorf tube. DNA was precipitated with 500 µl ice-cold ethanol at -80^0 C for 15 min. The mixture was centrifuged at 10,000 rpm, 4^{o}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. μl^{-1}) 0.2 µl, distilled water to 20 µl. PCR program: 95 ^{o} C 15 seconds, 55^{0} C 30 seconds, 72^{0} C 90 seconds for 5 cycles, 95^{0} C 15 seconds, 60^{0} C 30 seconds, 72^{0}C 90 seconds for 25 cycles, followed by 72^{0}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^{0} 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 Nitrogen-free medium. Composition of Nitrogen-free medium for soybeans was given in Appendix A. Leonard jars were placed in a 28^{0}-32^{0}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 μg. ml^{-1}. Plates were incubated at 25^0 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 μg. ml^{-1}. Plates were incubated at 25^0 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 μg. ml^{-1}, incubated at 25^0 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^{0}C 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 ^{o}C, 30 ^{o} C, 37 ^{o} C, and 40 ^{o} 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 ^{o}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 ^{o}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^{0}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 25^0 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).

(a) (b) (c)

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

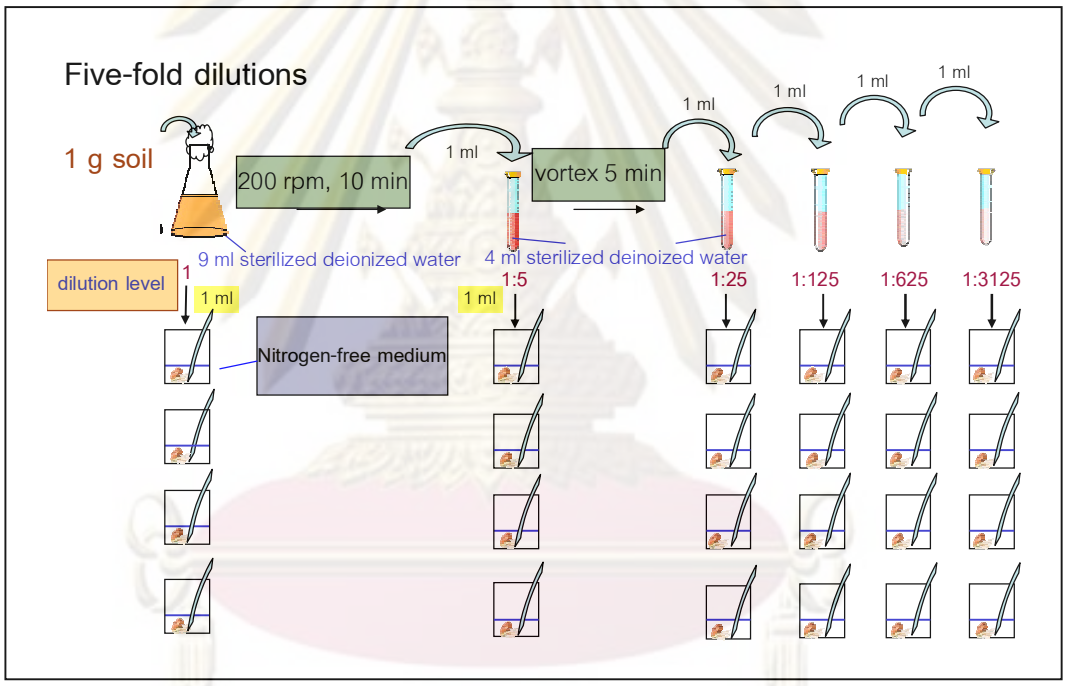


Figure 3.3 Serial dilutions for MPN (Somasegaran and Hoben, 1994).

CHAPTER IV

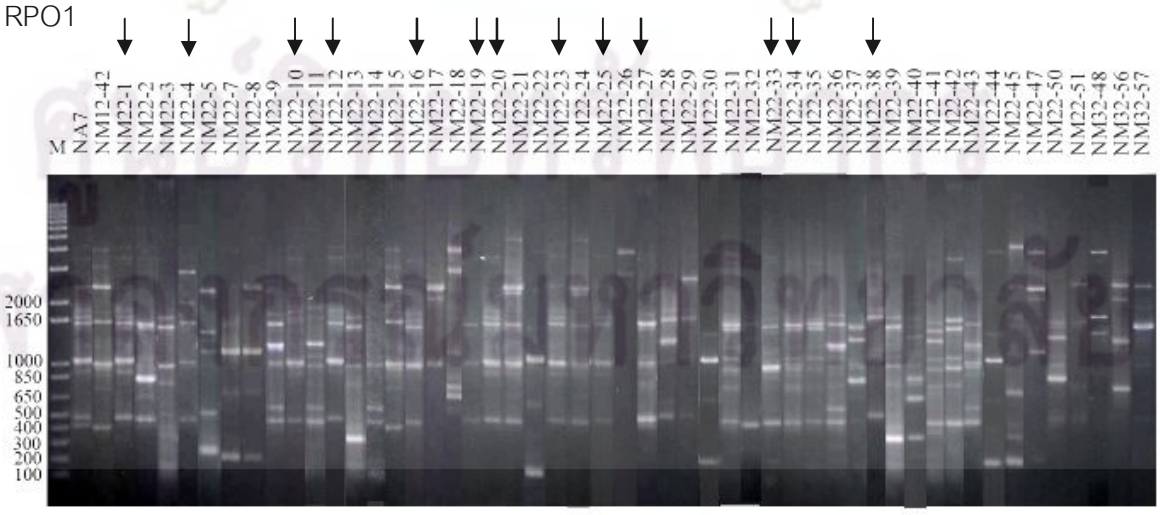
RESULTS

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 $\mu g \cdot ml^{-1}$ 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

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.



CRL-7

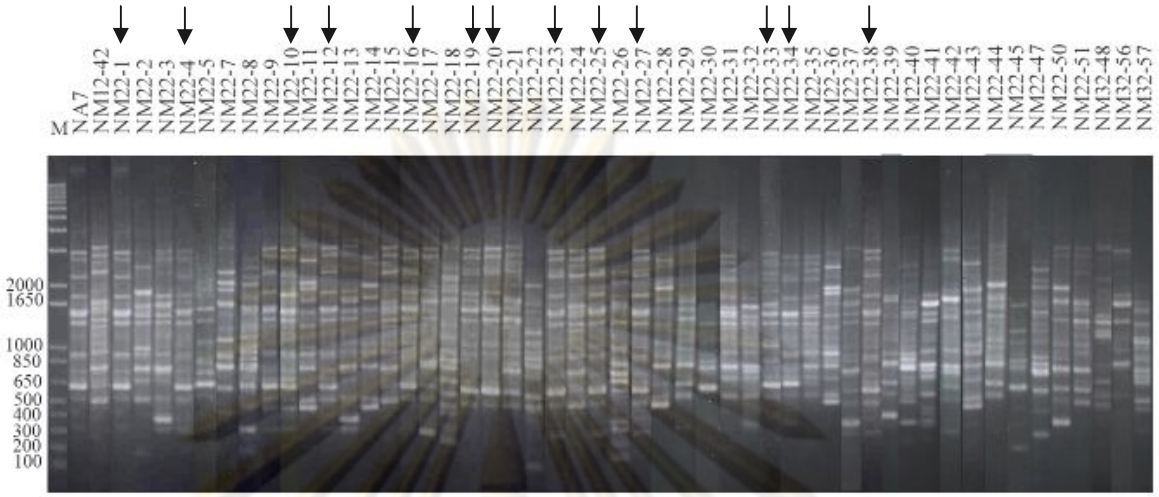


Figure 4.1 RAPD-PCR DNA fingerprints of soybean rhizobium strain NA7 and 51 slow-growing bacteria isolated from root nodules of soybean cultivar CM 60 mixed with soybean biofertilizer NA7 and planted in the experimental plot in Nam Moub subdistrict, Wiang Sa district, Nan province. Either primer RPO1 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, NM22-20, NM22-23, NM22-25, NM22-27, NM22-33, NM22-34, NM22-38	No
NM12-42	NM22-15, NM22-21, NM22-24, NM22-29, NM22-31, NM22-35, NM22-51, NM32-57	Yes (NA160)
NM22-2	NM22-44	No
NM22-3	NM22-13, NM22-17, NM22-32, NM22-37, NM22-39, NM22-41, NM22-42, NM22-50, NM32-56	No
NM22-5	NM22-9, NM22-11, 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

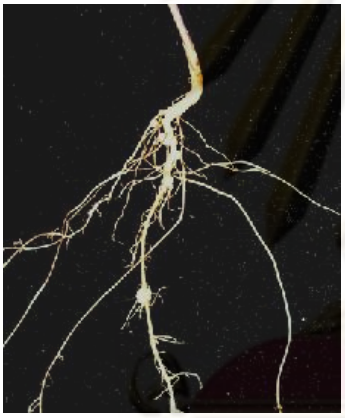
4.3 Polyphasic taxonomy of 6 bacterial isolates

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.

(a) NM22-8

ST1



ST2

SJ5

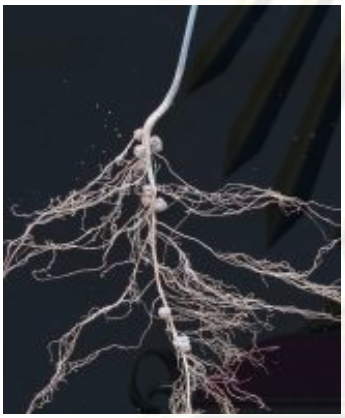
CM2

CM60

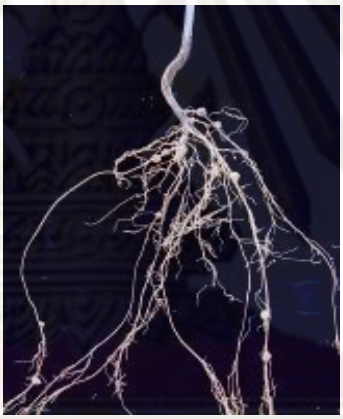
control

(b) NM22-11

ST1



ST2

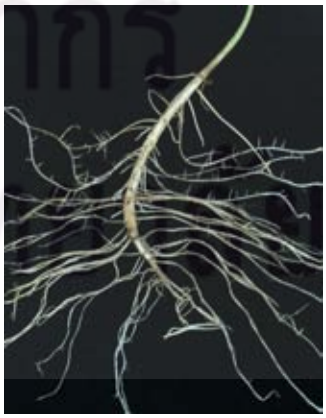


SJ5

CM2

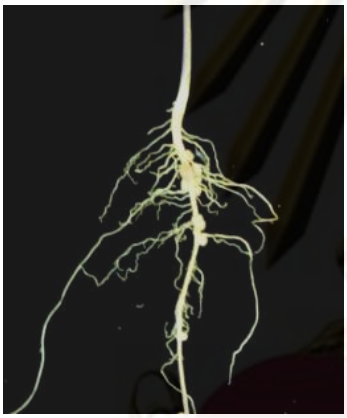
CM60

control

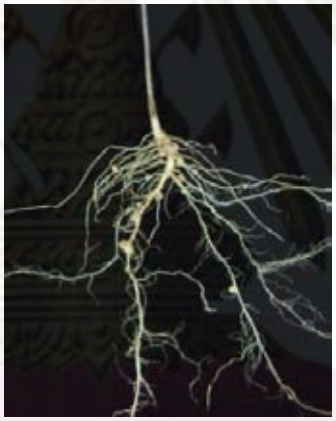


(c) NM22-13

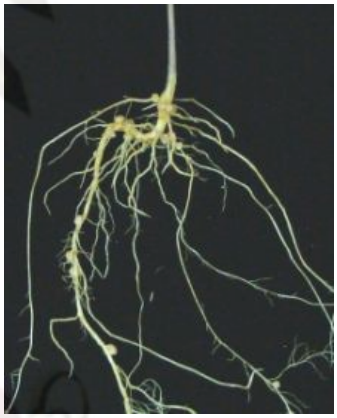
ST1



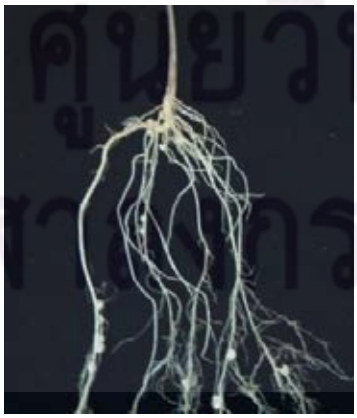
ST2



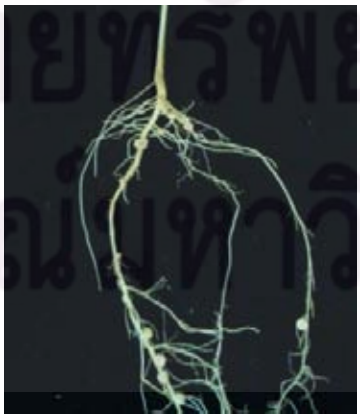
SJ5



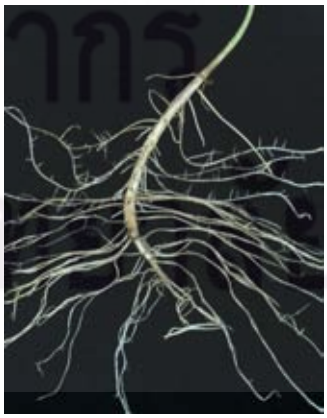
CM2



CM60



control



(d) NM22-15

ST1

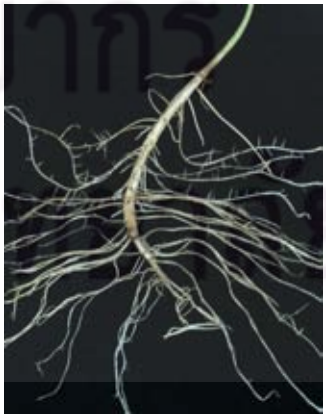
ST2

ST1

CM2

CM60

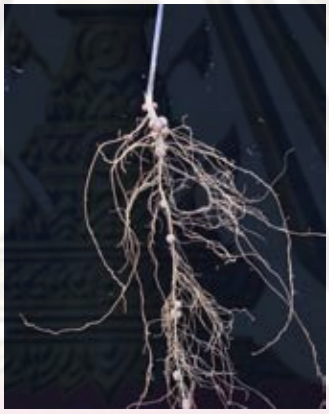
control



(e) NM22-25

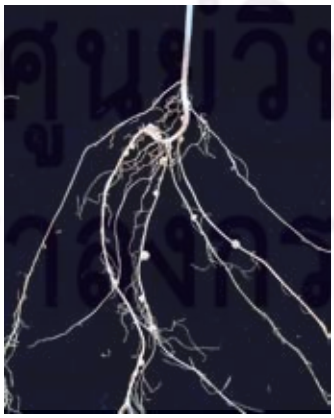
ST1

ST2

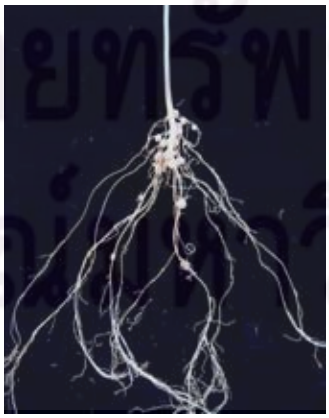


SJ5

CM2



CM60



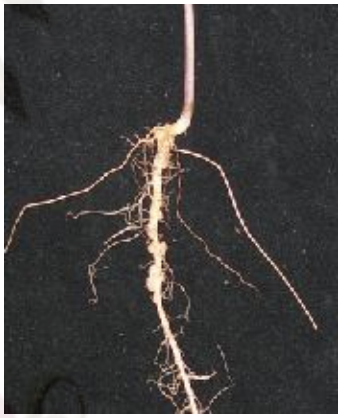
control

(f) NM22-30

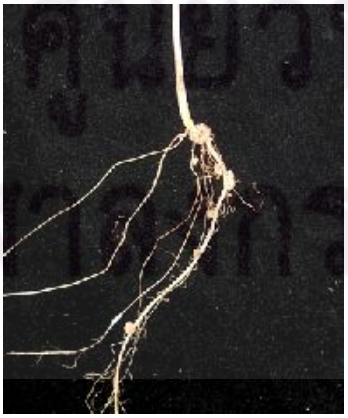
ST1

ST2

ST5

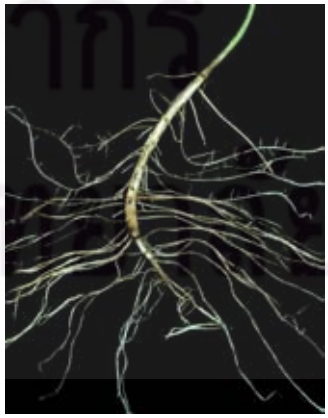


CM2



CM60

control



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.

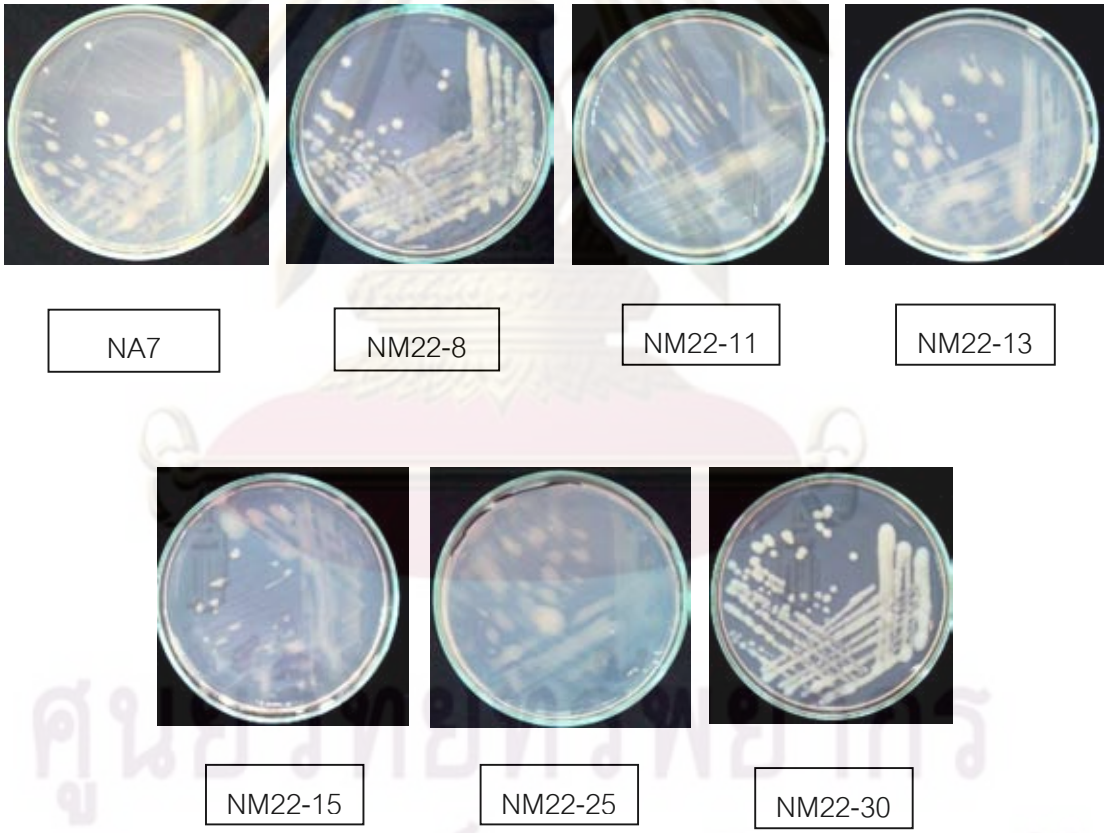


Figure 4.3 Colony morphology of soybean rhizobium strains including strain NA7 and the 6 selected strains grown on YMA plus Congo red plates.

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).

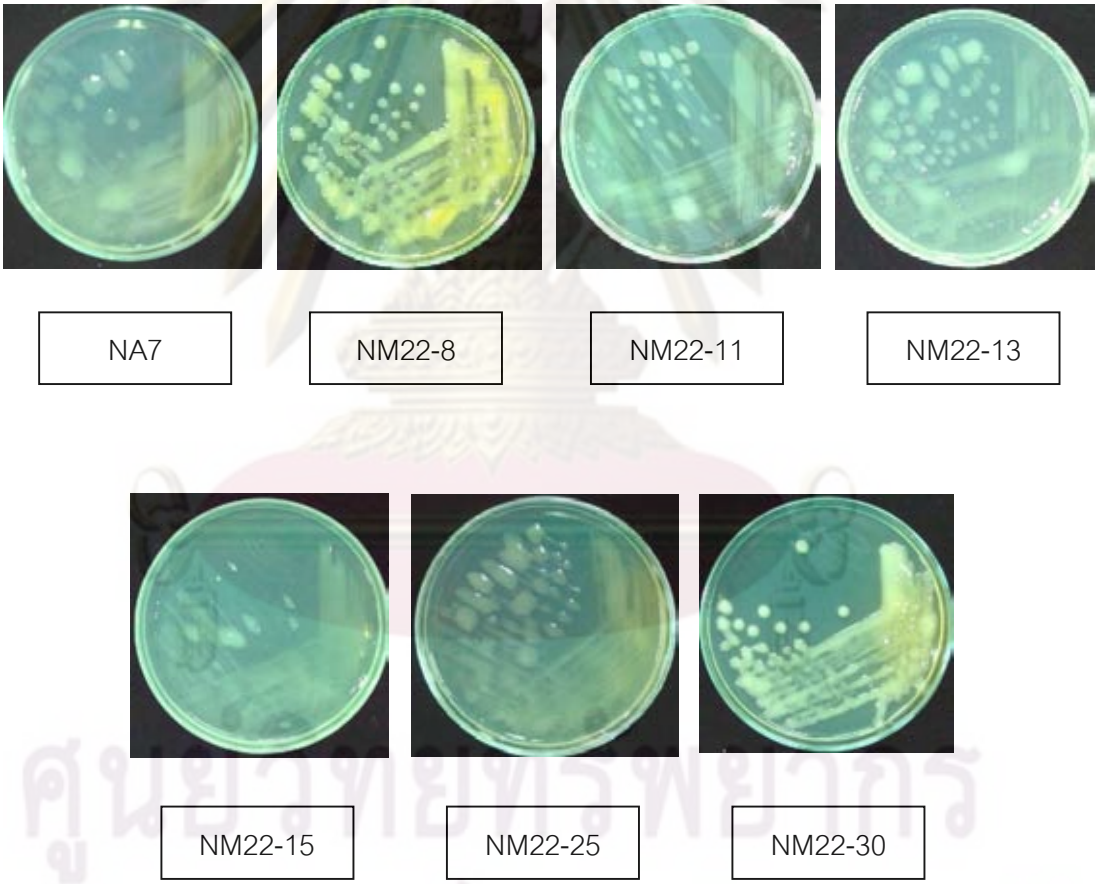
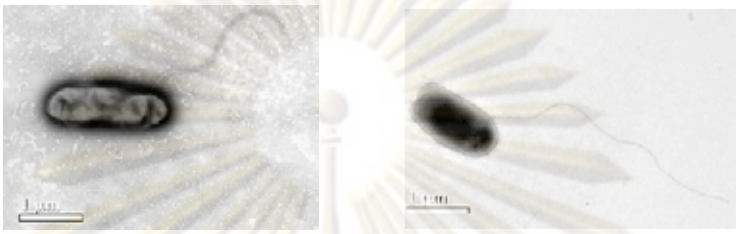


Figure 4.4 Bromthymol blue reactions of soybean rhizobium strains including strain NA7 and the 6 selected strains grown on YMA plus Bromthymol blue plates.

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).

(a) NM22-8 (b) NM22-11



(c) NM22-13 (d) NM22-15 (e) NM22-30

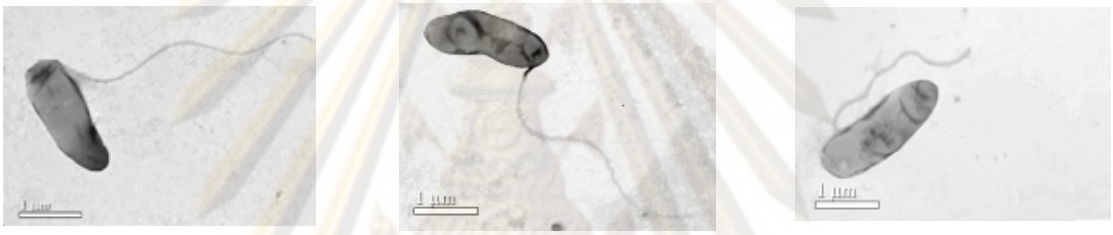
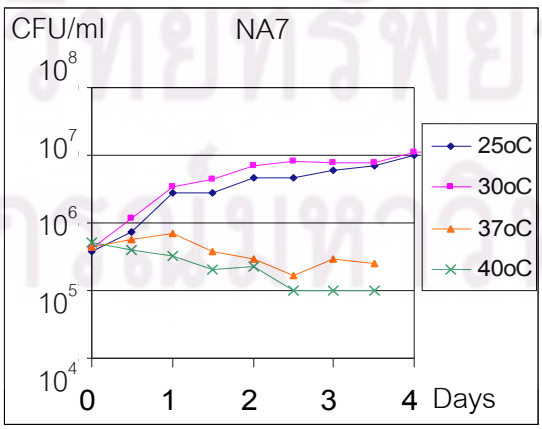


Figure 4.5 (a)-(e): Negative staining results for 5 selected strains of soybean rhizobia.

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 37^0 C and 40^0 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.



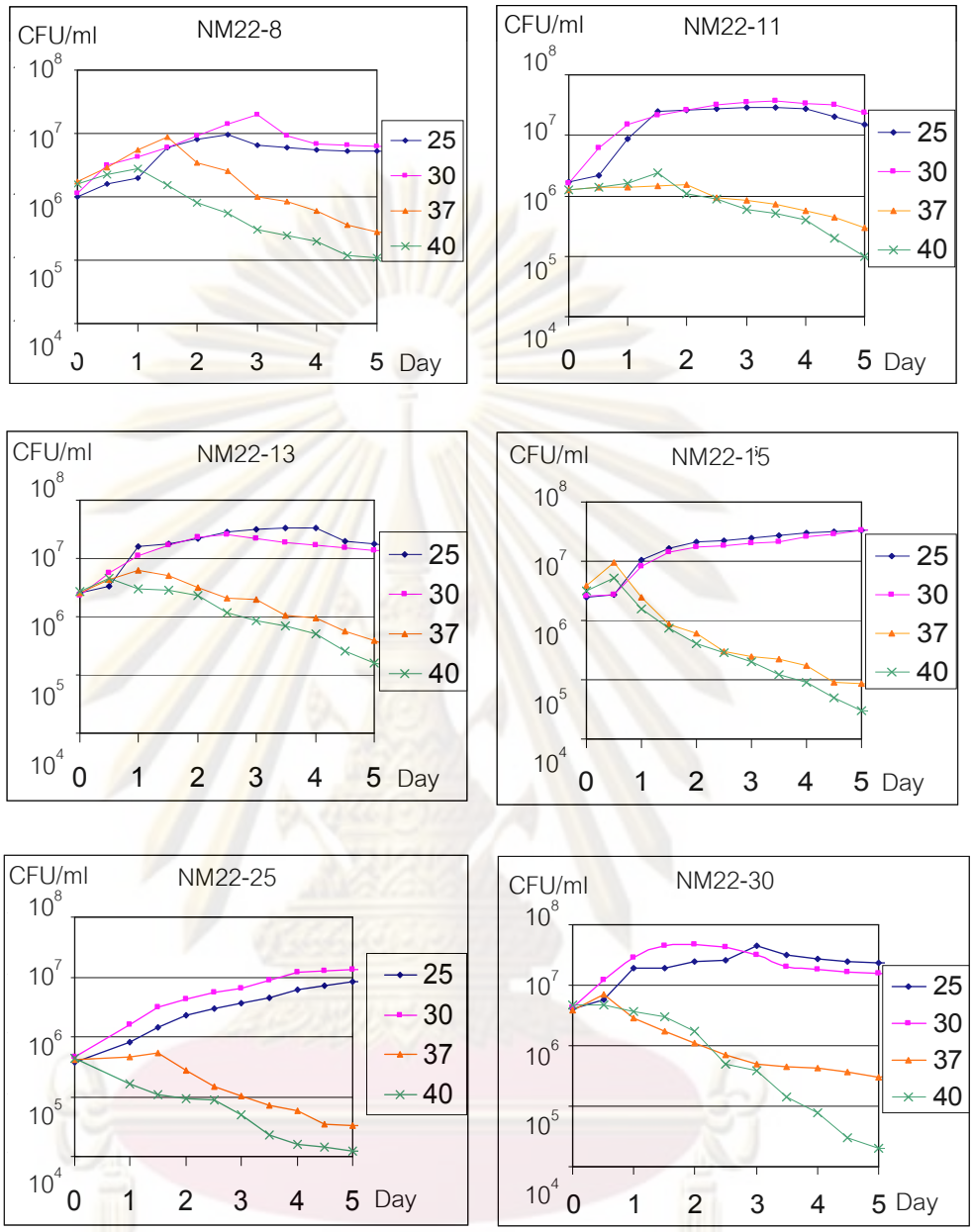


Figure 4.6 Growth of soybean rhizobia including strain NA7 and representative soybean rhizobium strains from 6 groups at different temperatures.

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, □–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-

Butanediol, D,L- □-Glycerol phosphate, □ –D-Glucose-1-phosphate, D-Glucose-6-phosphate.

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, □-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* 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.

Strains	Number of carbon and nitrogen used differently from the reference strains with percentage in brackets			Identification
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* 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 (20.9%)	*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.*

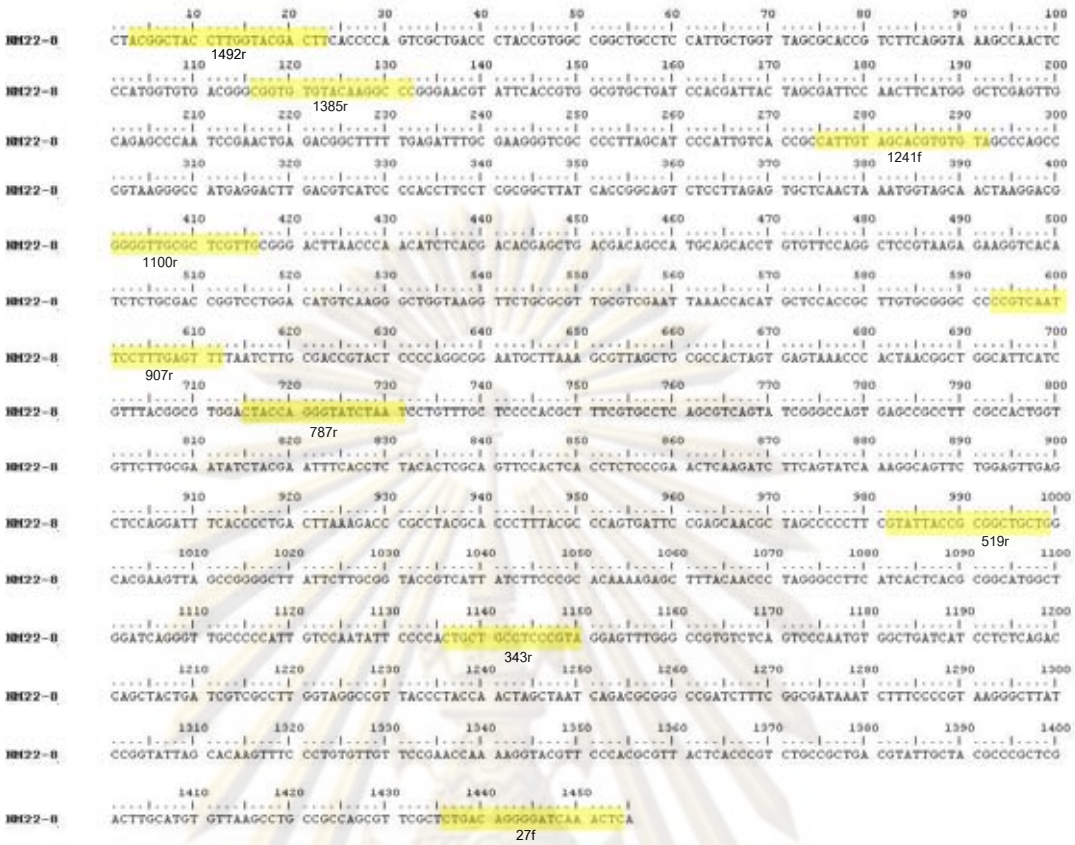


Figure 4.7 16S rDNA sequence of soybean rhizobium strain NM22-8. Nucleotide sequences of sequencing primers were shown in boxes.

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 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* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 4/1455 (0%), or *Bradyrhizobium elkanii* strain

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.*

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.*

Figure 4.9 16S rDNA sequence of soybean rhizobium strain NM22-13. Nucleotide sequences of sequencing primers were shown in boxes.

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

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

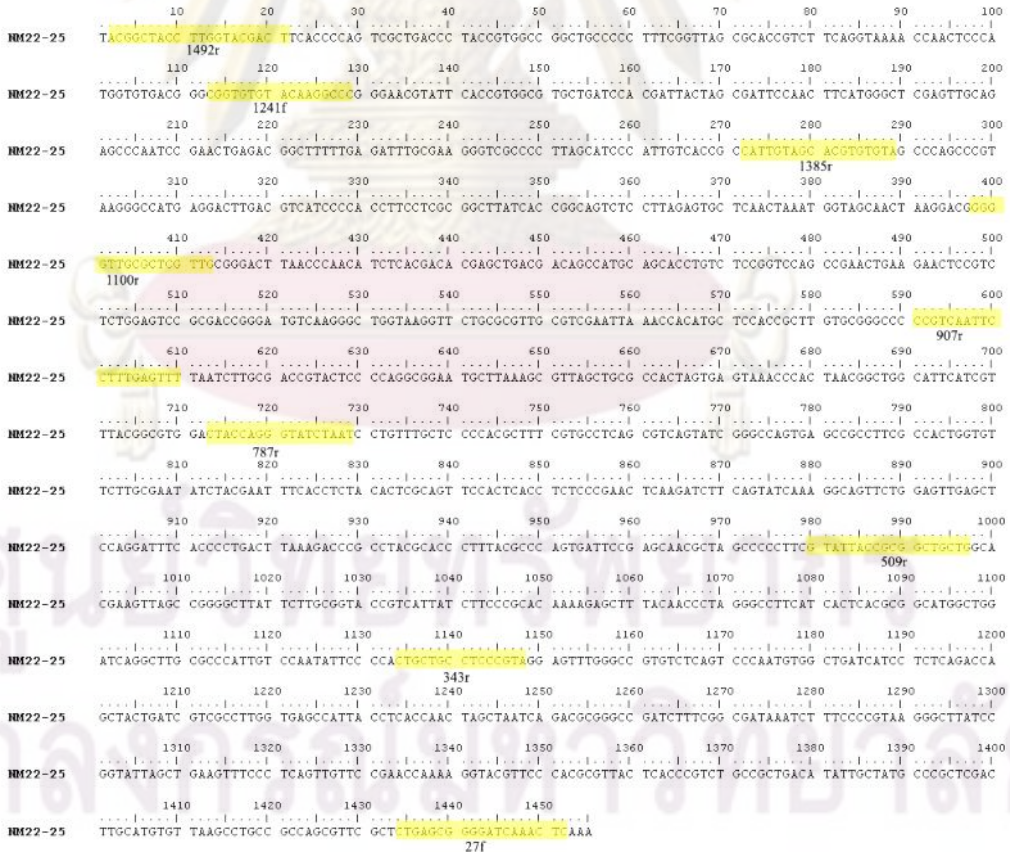


Figure 4.12 16S rDNA sequence of soybean rhizobium strain NM22-25. Nucleotide sequences of sequencing primers were shown in boxes.

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 x 10^4 MPN per gram soil.

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

Determination	Soybean rhizobial cells (MPN per g soil)
1	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 m^2 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 leguminous plants. SEMIA keeps and distributes these rhizobial strains including 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 m^2 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 10^4 MPN per gram soil.

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APPENDICES

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_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	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 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

N-free Nutrient Solutions

Stock Solutions	Chemicals	g/liter
1	$CaCI_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2 MoO_2.7 H_2O$	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% KNO_3 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

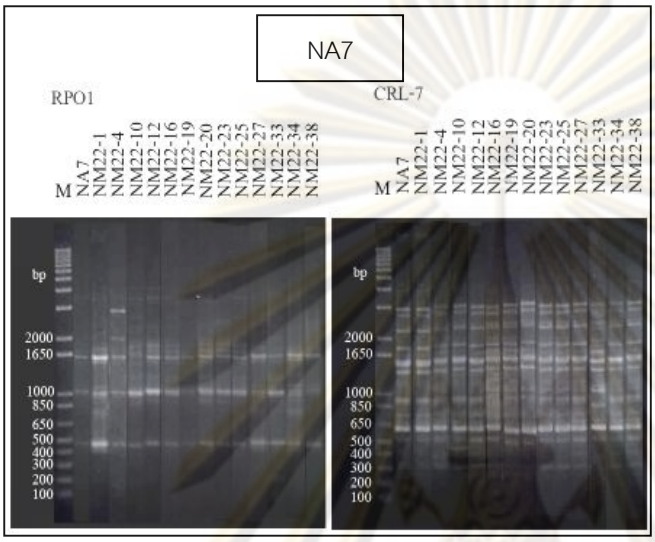
0.9 g NaCl, 0.29 g EDTA were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

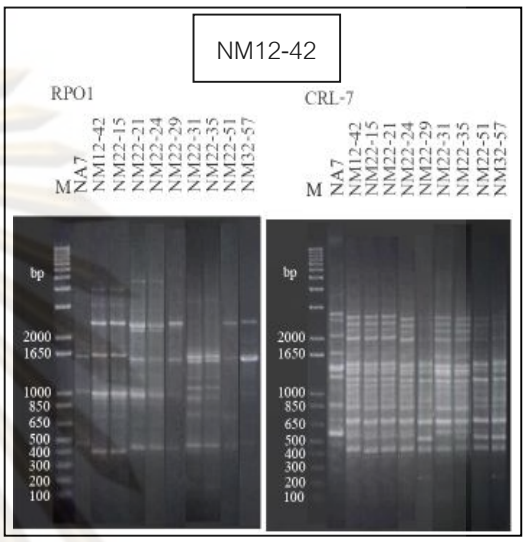
DNAzol

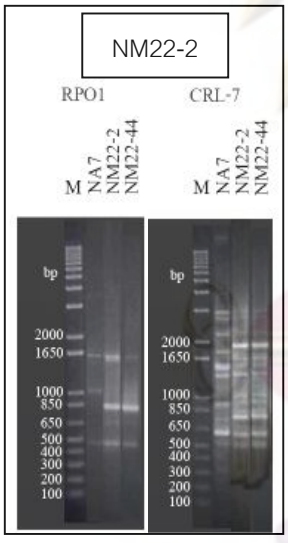
DNAzol solution (Molecular Research Lab, MRL) was used according to the manufacturer's instruction.

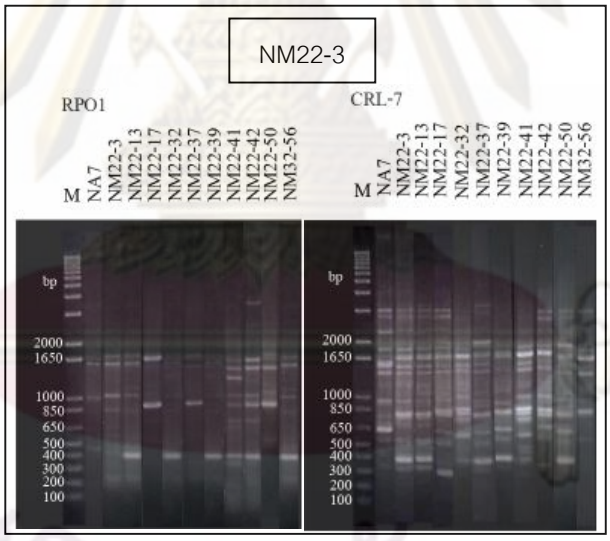
APPENDIX C

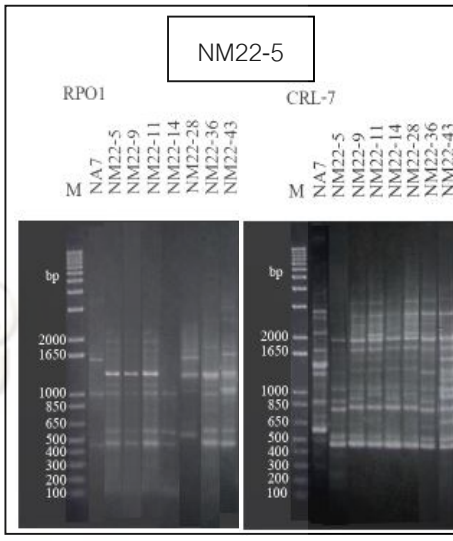
RAPD-PCR FINGERPRINTS OF ISOLATES WITH IDENTICAL FINGERPRINTS

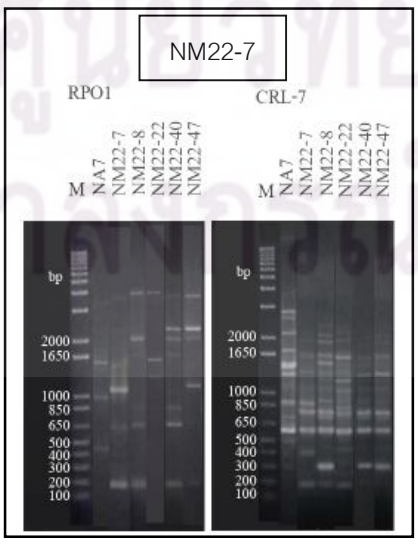


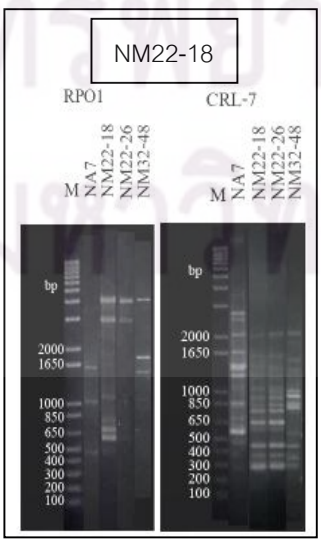


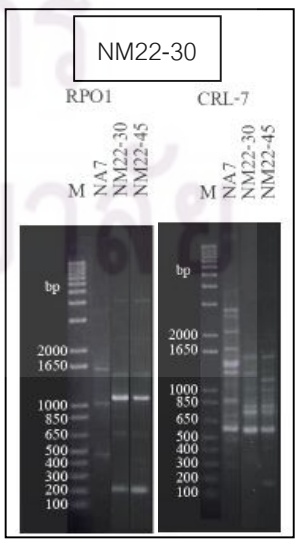












APPENDIX D

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.

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations		
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396
α-Cyclodextrin	-	-	-
Dextrin	-	+	-
Glycogen	-	-	-
Tween 40	+++	++	++
Tween 80	+++	++	+++
N-Acetyl-D-Galactosamine	-	-	-
N-Acetyl-D-Glucosamine	-	-	-
Adonitol	-	-	-
L-Arabinose	+++	++	+++
D-Arabitol	-	+	-
D-Cellobiose	-	-	-
i-Erythritol	-	-	-
D-Fructose	-	+	-
L-Fucose	+	+	-
D-Galactose	-	-	++
Gentiobiose	-	-	-
α-D-Glucose	-	-	+
m-Inositol	-	-	-
α-D-Lactose	-	-	-
Lactulose	-	-	-
Maltose	-	-	-
D-Mannitol	-	+	-
D-Mannose	-	+	++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations		
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396
D-Melibiose	-	-	-
β-Methyl-D-Glucoside	-	-	-
D-Psicose	-	-	-
D-Raffinose	-	-	-
L-Rhamnose	-	-	-
D-Sorbitol	-	-	-
Sucrose	-	-	-
D-Trehalose	-	-	-
Turanose	-	-	-
Xylitol	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++
Acetic Acid	+++	++	++
Cis-Aconitic Acid	-	-	-
Citric Acid	+++	+	-
Formic Acid	+++	++	-
D-Galactonic Acid Lactone	-	-	++
D-Galacturonic Acid	-	-	+
D-Gluconic Acid	+++	++	+++
D-Glucosaminic Acid	-	-	-
D-Glucuronic Acid	-	-	-
α-Hydroxybutyric Acid	-	+	-
β-Hydroxybutyric Acid	++	++	+++
γ-Hydroxybutyric Acid	+++	+	++
p-Hydroxy Phenylacetic Acid	-	-	-
Itaconic acid	-	-	++
α-Keto Butyric Acid	-	-	-
α-Keto Glutaric Acid	+	+	+++
α-Keto Valeric Acid	-	-	-
D,L-Lactic Acid	+++	++	+++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations		
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396
Malonic Acid	-	-	-
Propionic Acid	++	+	++
Quinic Acid	-	++	-
D-Saccharic Acid	+++	++	-
Sebacic Acid	+++	++	+
Succinic Acid	+++	++	+++
Bromosuccinic Acid	+++	++	+++
Succinamic Acid	++	++	++
Glucuronamide	-	-	-
L-Alaninamide	-	++	-
D-Alanine	+	++	-
L-Alanine	-	+	-
L-Alanyl-glycine	-	-	-
L-Asparagine	-	-	-
L-Aspartic Acid	-	++	++
L-Glutamic Acid	-	++	-
Glycyl-L-Aspartic Acid	-	-	-
Glycyl-L-Glutamic Acid	-	-	-
L-Histidine	-	-	-
Hydroxy-L-Proline	-	-	-
L-Leucine	-	++	++
L-Ornithine	-	-	-
L-Phenylalanine	-	+	-
L-Proline	-	-	-
L-Pyroglutamic Acid	+	++	-
D-Serine	-	++	-
L-Serine	-	-	-
L-Threonine	-	-	-
D,L-Carnitine	-	-	-
γ-Amino Butyric Acid	-	-	+

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

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							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
α-Cyclodextrin	-	-	-	-	-	-	-	-
Dextrin	-	-	-	-	+	+	+	-
Glycogen	-	-	-	-	-	-	-	-
Tween 40	+	+	+	+++	+++	+++	+++	+++
Tween 80	+	+	+	+++	+++	+++	+++	+++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	+++	+++	+++	+++
D-Arabitol	-	-	-	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-
L-Fucose	+	+	+	+	+	-	-	+
D-Galactose	-	+	-	+	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-
α-D-Glucose	-	-	-	-	-	-	-	-
m-Inositol	-	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	++	-
D-Mannitol	-	-	-	-	-	-	+++	-
D-Mannose	-	-	-	-	-	-	++	-
D-Melibiose	-	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	*B. elkanii* NBRC 14791							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
D-Trehalose	-	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	++	-
Pyruvic Acid Methyl Ester	+	+	+	+++	+++	+++	+++	+++
Succinic Acid Mono-Methyl-Ester	+	+	+	-	+++	+++	+++	+++
Acetic Acid	+	+	+	+++	+++	+++	+++	+++
Cis-Aconitic Acid	-	-	-	-	-	-	-	-
Citric Acid	+	+	+	+++	+++	+++	+++	+++
Formic Acid	+	+	+	+++	+++	+++	+++	+++
D-Galactonic Acid Lactone	-	-	-	-	-	-	-	-
D-Galacturonic Acid	-	-	-	-	-	-	-	-
D-Gluconic Acid	+	+	+	+++	+++	+++	+++	+++
D-Glucosaminic Acid	-	+	-	-	-	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	+	-	-	-	-	-	++	-
β-Hydroxybutyric Acid	+	+	+	+++	+++	+	+++	++
γ-Hydroxybutyric Acid	+	+	+	+++	+++	+++	+++	+++
p-Hydroxy Phenylacetic Acid	-	-	-	-	+	-	-	-
Itaconic acid	-	-	-	-	-	-	-	-
α-Keto Butyric Acid	-	+	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+	-	++	-	+	+
α-Keto Valeric Acid	-	+	-	-	-	-	-	-
D,L-Lactic Acid	+	+	+	+++	+++	+++	+++	++
Malonic Acid	-	-	-	-	-	-	-	-
Propionic Acid	+	+	+	++	++	++	++	+
Quinic Acid	-	-	-	-	-	-	-	++
D-Saccharic Acid	+	+	+	+++	+++	+++	+++	++
Sebacic Acid	+	+	+	+++	+++	+++	+++	++
Succinic Acid	+	+	+	+++	+++	+++	+++	++
Bromosuccinic Acid	+	+	+	+++	+++	+++	+++	++
Succinamic Acid	+	+	+	+++	+++	++	+++	++
Glucuronamide	-	-	-	-	-	-	-	-
L-Alaninamide	-	+	-	-	-	-	-	++
D-Alanine	+	+	+	+	+	+	+	++
L-Alanine	-	-	-	-	-	-	-	+
L-Alanyl-glycine	-	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-	

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	*B. elkanii* NBRC 14791							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
L-Aspartic Acid	-	-	-	-	-	-	-	++
L-Glutamic Acid	-	-	-	-	+	-	-	++
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-	-
L-Leucine	+	-	+	++	-	-	-	++
L-Ornithine	-	-	-	-	-	-	-	-
L-Phenylalanine	-	-	-	-	-	-	-	
L-Proline	-	-	-	-	+	-	-	
L-Pyroglutamic Acid	+	+	+	-	+	-	-	++
D-Serine	-	-	-	-	-	-	-	++
L-Serine	-	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-	
D,L-Carnitine	-	-	-	-	-	-	+++	-
γ-Amino Butyric Acid	-	-	-	-	-	-	++	
Urocanic Acid	-	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	+
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	*B. japonicum* NBRC 14783							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
α-Cyclodextrin	-	+	-	-	-	-	-	-
Dextrin	-	+	-	-	++	++	++	+
Glycogen	-	+	-	-	-	-	+	-
Tween 40	+	+	+	+++	++	+	++	++
Tween 80	+	+	+	++	++	++	+++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	+	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	++	++	++	++
D-Arabitol	+	+	+	+	+	-	+	+
D-Cellobiose	-	+	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-	-
D-Fructose	+	+	+	-	+	-	++	+
L-Fucose	+	+	-	-	+	-	+	+
D-Galactose	-	+	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-
α-D-Glucose	-	+	-	-	-	-	-	-
m-Inositol	-	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-	-
Lactulose	-	+	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
D-Mannitol	+	+	+	+	++	+	++	+
D-Mannose	+	+	+	+	++	-	++	+
D-Melibiose	-	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	+	-	-	-	-	-	-
D-Psicose	-	+	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-
D-Sorbitol	-	+	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	++	-
D-Trehalose	-	-	-	-	-	-	-	-
Turanose	-	+	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	++	+++	++
Succinic Acid Mono-Methyl-Ester	+	+	+	-	+++	++	+++	+++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	*B. japonicum* NBRC 14783							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
Acetic Acid	+	+	+	+++	++	++	++	++
Cis-Aconitic Acid	-	-	-	-	-	-	-	-
Citric Acid	+	+	-	+	++	+	++	+
Formic Acid	+	+	+	+++	++	++	++	++
D-Galactonic Acid Lactone	-	+	-	-	+	-	+	
D-Galacturonic Acid	-	+	-	-	+	-	++	
D-Gluconic Acid	+	+	+	++	++	++	+++	++
D-Glucosaminic Acid	-	+	-	-	-	-	+	-
D-Glucuronic Acid	-	+	-	-	+	-	++	
α-Hydroxybutyric Acid	-	+	+	+	+	-	++	+
β-Hydroxybutyric Acid	+	+	+	+++	+++	++	+++	++
γ-Hydroxybutyric Acid	-	+	+	-	+	-	+++	+
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	+	-
Itaconic acid	-	+	-	-	+	-	++	
α-Keto Butyric Acid	-	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	-	-	+	-	+	+
α-Keto Valeric Acid	-	-	-	-	-	-	-	-
D,L-Lactic Acid	+	+	+	+++	+++	++	+++	++
Malonic Acid	-	-	-	-	-	-	+	-
Propionic Acid	+	+	+	++	+	-	++	+
Quinic Acid	+	+	+	+++	++	++	++	++
D-Saccharic Acid	+	+	+	+++	++	++	+++	++
Sebacic Acid	+	+	+	++	++	+	+++	++
Succinic Acid	+	+	+	+++	+++	++	+++	++
Bromosuccinic Acid	+	+	+	+++	++	++	++	++
Succinamic Acid	+	+	+	+++	++	++	++	++
Glucuronamide	-	+	-	-	+	-	-	-
L-Alaninamide	+	+	+	++	++	+	++	++
D-Alanine	+	+	+	++	++	+	++	++
L-Alanine	+	+	-	+	+	-	+	+
L-Alanyl-glycine	-	+	-	-	-	-	++	-
L-Asparagine	-	+	-	-	+	-	++	
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							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
Hydroxy-L-Proline	-	-	-	-	-	-	-	-
L-Leucine	+	+	+	++	++	+	++	++
L-Ornithine	-	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	+	+	-	++	
L-Proline	-	+	-	-	+	-	+	
L-Pyroglutamic Acid	+	+	-	+	++	-	++	++
D-Serine	-	+	-	+	+	-	++	++
L-Serine	-	-	-	-	-	-	-	-
L-Threonine	-	+	-	-	+	-	+	
D,L-Carnitine	-	-	-	-	-	-	++	-
γ-Amino Butyric Acid	-	+	-	-	+	-	++	
Urocanic Acid	-	+	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	+	-
Glycerol	+	+	+	-	+	-	++	+
D,L-α-Glycerol Phosphate	-	-	-	-	+	-	++	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	++	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	*B. liaoningense* NBRC 100396							
	1st	2nd	3rd	4th	5th	6th	7th	consensus
α-Cyclodextrin	-	-	-	-	-	-	-	-
Dextrin	-	-	-	-	++	+	+	+
Glycogen	-	-	-	-	-	-	-	-
Tween 40	+	+	+	+++	+++	+++	++	++
Tween 80	+	+	+	++	+++	+++	+++	+++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+++	+++	+++	+++	+++
D-Arabitol	-	-	-	-	-	-	+++	-
D-Cellobiose	-	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-	-
D-Galactose	+	+	+	+	++	++	++	++
Gentiobiose	-	-	-	-	-	-	-	-
α-D-Glucose	+	+	+	+	++	+	++	+
m-Inositol	-	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	+++	-
D-Mannose	+	+	+	+	++	-	+++	++
D-Melibiose	-	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-
L-Rhamnose	+	+	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	-	+++	+++	+++	++
Succinic Acid Mono-Methyl-Ester	+	+	+	-	++	++	++	++
Acetic Acid	+	+	+	-	+++	++	++	++

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

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

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).

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NA7			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	++	++	++	++
Tween 80	+++	++	+++	++	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	+	+	+	+
D-Arabitol	-	+	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	-	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NA7			Consensus results from 3 determinations
				1st	2nd	3rd	
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	++	+	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++	-	++	++	++
Acetic Acid	+++	++	++	++	+	+	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+++	+	-	-	-	-	-
Formic Acid	+++	++	-	+++	++	++	++
D-Galactonic Acid Lactone	-	-	++	-	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	+	+	+	+
D-Glucosaminic Acid	-	-	-	-	+	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+++	-	+++
γ-Hydroxybutyric Acid	+++	+	++	-	++	+	+
p-Hydroxy	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NA7			Consensus results from 3 determinations
				1st	2nd	3rd	
Phenylacetic Acid							
Itaconic acid	-	-	++	++	++	++	++
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	+	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+++	++	++	++
Malonic Acid	-	-	-	++	++	+	++
Propionic Acid	++	+	++	++	++	++	++
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	++	++	++	++
Sebacic Acid	+++	++	+	-	-	-	-
Succinic Acid	+++	++	+++	-	-	-	-
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	-	-	-	-
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	-	-	-	-
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	++	++	++	++
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	+	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NA7			Consensus results from 3 determinations
				1st	2nd	3rd	
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	+	+	+	+
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-8			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	+	+	-	+
Tween 80	+++	++	+++	+	+	-	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	-	-	-	-
D-Arabitol	-	+	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	+	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	-	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-8			Consensus results from 3 determinations
				1st	2nd	3rd	
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	+	+	-	+
Succinic Acid Mono-Methyl-Ester	+++	+++	++	+	+	-	+
Acetic Acid	+++	++	++	+	-	-	-
Cis-Aconitic Acid	-	-	-	+	+	-	+
Citric Acid	+++	+	-	-	+	-	-
Formic Acid	+++	++	-	+	+	-	+
D-Galactonic Acid Lactone	-	-	++	-	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	-	-	-	-
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+	+	-	+
γ-Hydroxybutyric Acid	+++	+	++	-	-	-	-
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+	+	-	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-8			Consensus results from 3 determinations
				1st	2nd	3rd	
Malonic Acid	-	-	-	+	+	-	+
Propionic Acid	++	+	++	-	-	-	-
Quinic Acid	-	++	-	+	+	-	+
D-Saccharic Acid	+++	++	-	-	-	-	-
Sebacic Acid	+++	++	+	-	-	-	-
Succinic Acid	+++	++	+++	+	-	-	-
Bromosuccinic Acid	+++	++	+++	-	-	-	-
Succinamic Acid	++	++	++	+	-	-	-
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	-	-	-	-
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	-	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-8			Consensus results from 3 determinations
				1st	2nd	3rd	
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-11			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	+	-
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	+++	+++	++	+++
Tween 80	+++	++	+++	+++	+++	++	+++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-11			Consensus results from 3 determinations
				1st	2nd	3rd	
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	++	+	-	+
D-Arabitol	-	+	-	+	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	+	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	+	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	+++	-	++	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++	++	++	++	++
Acetic Acid	+++	++	++	++	+	++	++
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+++	+	-	+	+	-	+
Formic Acid	+++	++	-	++	+++	+++	+++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-11			Consensus results from 3 determinations
				1st	2nd	3rd	
D-Galactonic Acid Lactone	-	-	++	+	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	+++	++	++	++
D-Glucosaminic Acid	-	-	-	++	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	++	++	++
γ-Hydroxybutyric Acid	+++	+	++	++	-	-	-
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	++	++	++	++
Malonic Acid	-	-	-	-	-	-	-
Propionic Acid	++	+	++	++	-	+	+
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	+++	+++	++	+++
Sebacic Acid	+++	++	+	++	+	++	++
Succinic Acid	+++	++	+++	+++	+++	++	+++
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	+++	+	++	++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	++	-	-	-
D-Alanine	+	++	-	+	+	+	+
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-11			Consensus results from 3 determinations
				1st	2nd	3rd	
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	+	-	-	-
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	-	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	+	-	-	-
D-Serine	-	++	-	-	+	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-13			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	++	++	+++	+++
Tween 80	+++	++	+++	++	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	+	-	+	+
D-Arabitol	-	+	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	-	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-13			Consensus results from 3 determinations
				1st	2nd	3rd	
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	++	++	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++	++	++	++	++
Acetic Acid	+++	++	++	+	-	+	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+++	+	-	+	-	-	-
Formic Acid	+++	++	-	+++	+++	+++	+++
D-Galactonic Acid Lactone	-	-	++	-	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	++	++	++	++
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+++	+++	+++
γ-Hydroxybutyric Acid	+++	+	++	+	+	+	+
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	++	++	++	++
Malonic Acid	-	-	-	-	-	-	-
Propionic Acid	++	+	++	++	+	-	+
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	+++	++	++	++
Sebacic Acid	+++	++	+	+	+	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-13			Consensus results from 3 determinations
				1st	2nd	3rd	
Succinic Acid	+++	++	+++	++	++	++	++
Bromosuccinic Acid	+++	++	+++	+	-	+	+
Succinamic Acid	++	++	++	++	+	++	++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	+	-	-	-
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	-	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-

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

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-15			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	++	++	+	++
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	++	++	++	++
Tween 80	+++	++	+++	++	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	+	+	+	+
D-Arabitol	-	+	-	+	+	-	+
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	-	+	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	+	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-15			Consensus results from 3 determinations
				1st	2nd	3rd	
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	+	+	+	+
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	++	++	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++	++	++	++	++
Acetic Acid	+++	++	++	++	++	++	++
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+++	+	-	-	-	-	-
Formic Acid	+++	++	-	+	-	+	+
D-Galactonic Acid Lactone	-	-	++	+	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	++	++	++	++
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-15			Consensus results from 3 determinations
				1st	2nd	3rd	
β-Hydroxybutyric Acid	++	++	+++	+++	+++	+++	+++
γ-Hydroxybutyric Acid	+++	+	++	++	++	+	++
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	+	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	++	+++	++	++
Malonic Acid	-	-	-	-	-	-	-
Propionic Acid	++	+	++	++	+	++	++
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	++	++	++	++
Sebacic Acid	+++	++	+	++	++	+	++
Succinic Acid	+++	++	+++	++	++	++	++
Bromosuccinic Acid	+++	++	+++	+	+	+	+
Succinamic Acid	++	++	++	+++	+++	++	+++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	+	-	-	-
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-15			Consensus results from 3 determinations
				1st	2nd	3rd	
L-Phenylalanine	-	+	-	-	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-25			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-25			Consensus results from 3 determinations
				1st	2nd	3rd	
Tween 40	+++	++	++	+++	++	++	++
Tween 80	+++	++	+++	+++	++	++	++
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+++	++	+++	+	+	+	+
D-Arabitol	-	+	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	-	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	+	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
D-Mannitol	-	+	-	-	-	-	-
D-Mannose	-	+	++	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	+++	++	++

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-25			Consensus results from 3 determinations
				1st	2nd	3rd	
Succinic Acid Mono-Methyl-Ester	+++	+++	++	+++	++	++	++
Acetic Acid	+++	++	++	++	++	+	++
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+++	+	-	-	+	-	-
Formic Acid	+++	++	-	+++	+++	++	+++
D-Galactonic Acid Lactone	-	-	++	+	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	+++	++	+	++
D-Glucosaminic Acid	-	-	-	++	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+++	++	+++
γ-Hydroxybutyric Acid	+++	+	++	+	++	-	+
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+++	++	++	++
Malonic Acid	-	-	-	-	-	-	-
Propionic Acid	++	+	++	++	+	-	+
Quinic Acid	-	++	-	-	-	-	-
D-Saccharic Acid	+++	++	-	+++	+++	++	+++
Sebacic Acid	+++	++	+	++	++	+	++
Succinic Acid	+++	++	+++	++	+++	++	++
Bromosuccinic Acid	+++	++	+++	++	+	-	+
Succinamic Acid	++	++	++	+++	++	++	++
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-25			Consensus results from 3 determinations
				1st	2nd	3rd	
D-Alanine	+	++	-	+	+	+	+
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	-	-	-	-
L-Glutamic Acid	-	++	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	-	-	-	-
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	-	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-25			Consensus results from 3 determinations
				1st	2nd	3rd	
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-30			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	-	+	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Tween 40	+++	++	++	++	-	-	-
Tween 80	+++	++	+++	++	+	-	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	++	-	-	-
L-Arabinose	+++	++	+++	+	-	-	-
D-Arabitol	-	+	-	++	-	-	-
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	++	-	-	-
L-Fucose	+	+	-	-	-	-	-
D-Galactose	-	-	++	+	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	+	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-

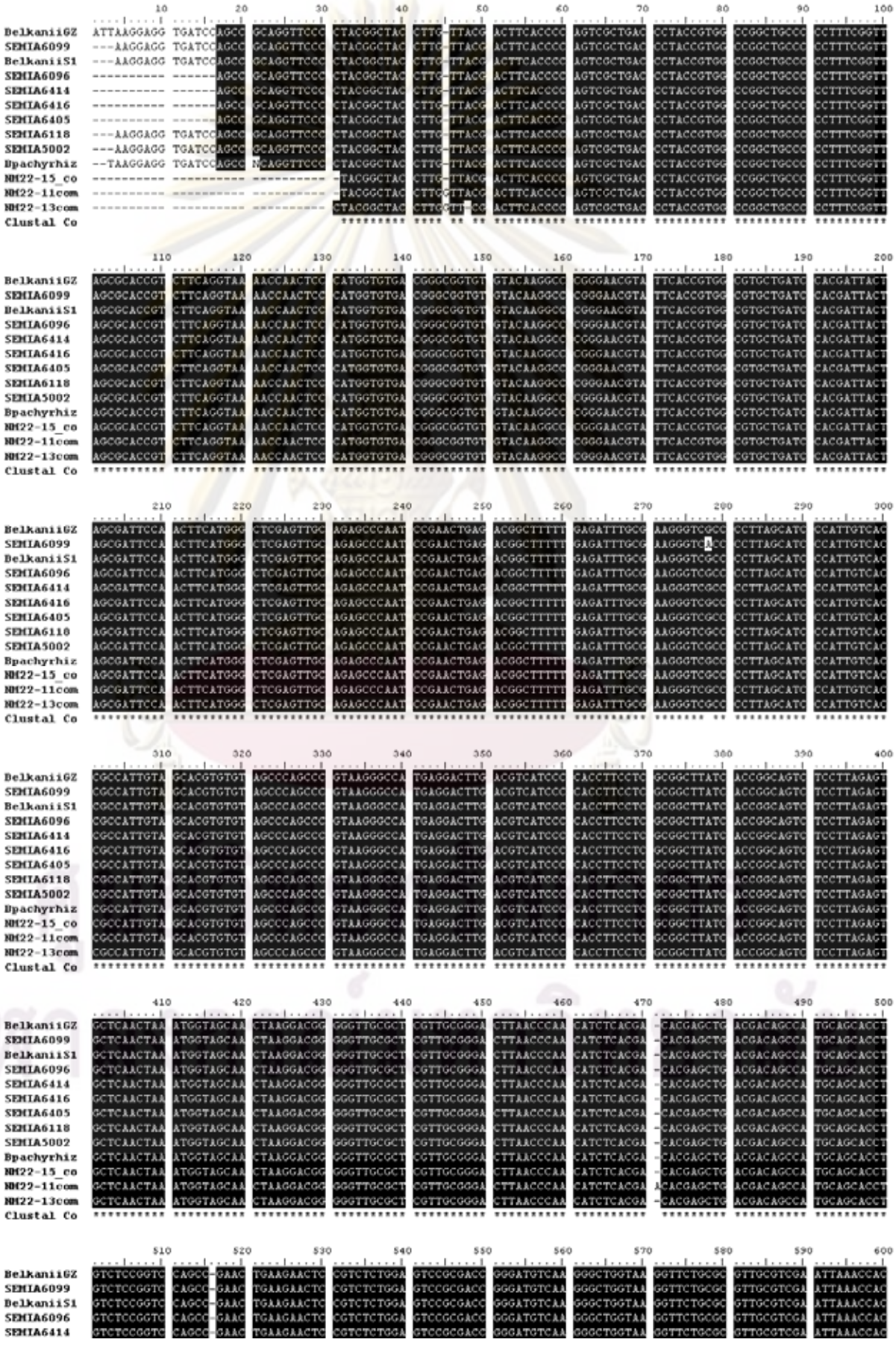
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-30			Consensus results from 3 determinations
				1st	2nd	3rd	
D-Mannitol	-	+	-	++	-	-	-
D-Mannose	-	+	++	++	-	-	-
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	++	-	-	-
Sucrose	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+++	++	++	++	+	+	++
Succinic Acid Mono-Methyl-Ester	+++	+++	++	++	-	-	-
Acetic Acid	+++	++	++	++	+	+	++
Cis-Aconitic Acid	-	-	-	++	+	-	+
Citric Acid	+++	+	-	++	+	+	++
Formic Acid	+++	++	-	+++	++	++	++
D-Galactonic Acid Lactone	-	-	++	+	-	-	-
D-Galacturonic Acid	-	-	+	-	-	-	-
D-Gluconic Acid	+++	++	+++	++	+	-	+
D-Glucosaminic Acid	-	-	-	+	-	-	-
D-Glucuronic Acid	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-	-	-	-
β-Hydroxybutyric Acid	++	++	+++	+++	+	+	+
γ-Hydroxybutyric Acid	+++	+	++	++	-	-	-
p-Hydroxy Phenylacetic Acid	-	-	-	-	-	-	-

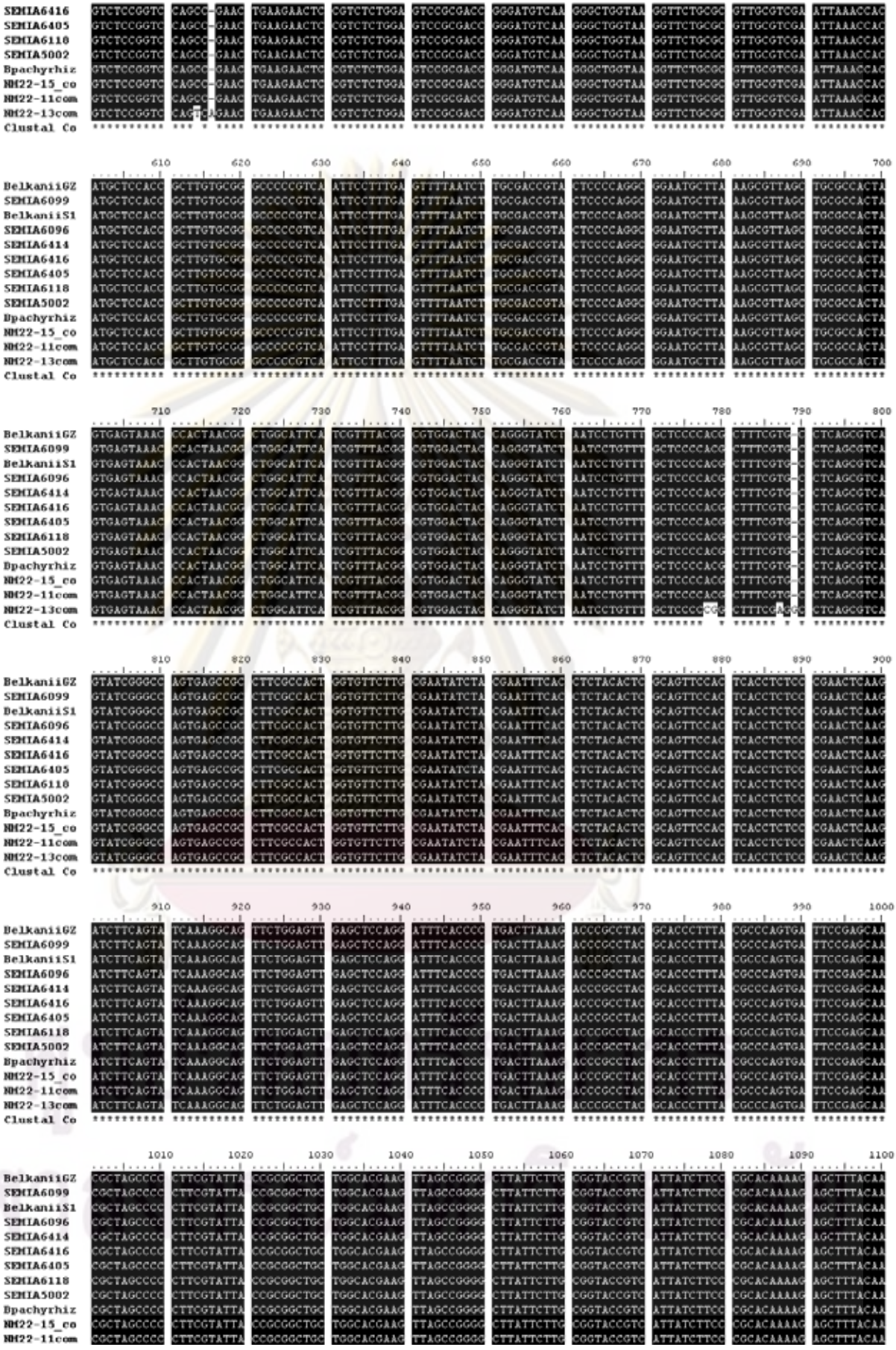
Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-30			Consensus results from 3 determinations
				1st	2nd	3rd	
Itaconic acid	-	-	++	-	-	-	-
α-Keto Butyric Acid	-	-	-	-	-	-	-
α-Keto Glutaric Acid	+	+	+++	-	-	-	-
α-Keto Valeric Acid	-	-	-	-	-	-	-
D,L-Lactic Acid	+++	++	+++	+++	+	+	++
Malonic Acid	-	-	-	+++	+	+	++
Propionic Acid	++	+	++	++	-	-	-
Quinic Acid	-	++	-	++	+	-	++
D-Saccharic Acid	+++	++	-	+	-	-	-
Sebacic Acid	+++	++	+	++	-	-	-
Succinic Acid	+++	++	+++	++	+	+	++
Bromosuccinic Acid	+++	++	+++	++	+	-	+
Succinamic Acid	++	++	++	++	-	-	-
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	-	++	-	-	-	-	-
D-Alanine	+	++	-	+	-	-	-
L-Alanine	-	+	-	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	-	-	-	-	-	-	-
L-Aspartic Acid	-	++	++	+	-	-	-
L-Glutamic Acid	-	++	-	++	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	-	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	-	++	++	+	-	-	-
L-Ornithine	-	-	-	-	-	-	-
L-Phenylalanine	-	+	-	+	-	-	-
L-Proline	-	-	-	-	-	-	-
L-Pyroglutamic Acid	+	++	-	-	-	-	-
D-Serine	-	++	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	NM22-30			Consensus results from 3 determinations
				1st	2nd	3rd	
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	+	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	-	+	-	++	-	-	-
D,L-α-Glycerol Phosphate	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

APPENDIX G

Alignment of 16S rDNA sequences.

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Bpachyrhiz
NM22-15_co
NM22-11com
NM22-13com
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SEMIA6416
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NM22-15_co
NM22-11com
NM22-13com
Clustal Co
910 920 930 940 950 960 970 980 990 1000
BelkaniiGZ
SEMIA6099
BelkaniiS1
SEMIA6096
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SEMIA6416
SEMIA6405
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SEMIA5002
Bpachyrhiz
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NM22-11com
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Clustal Co
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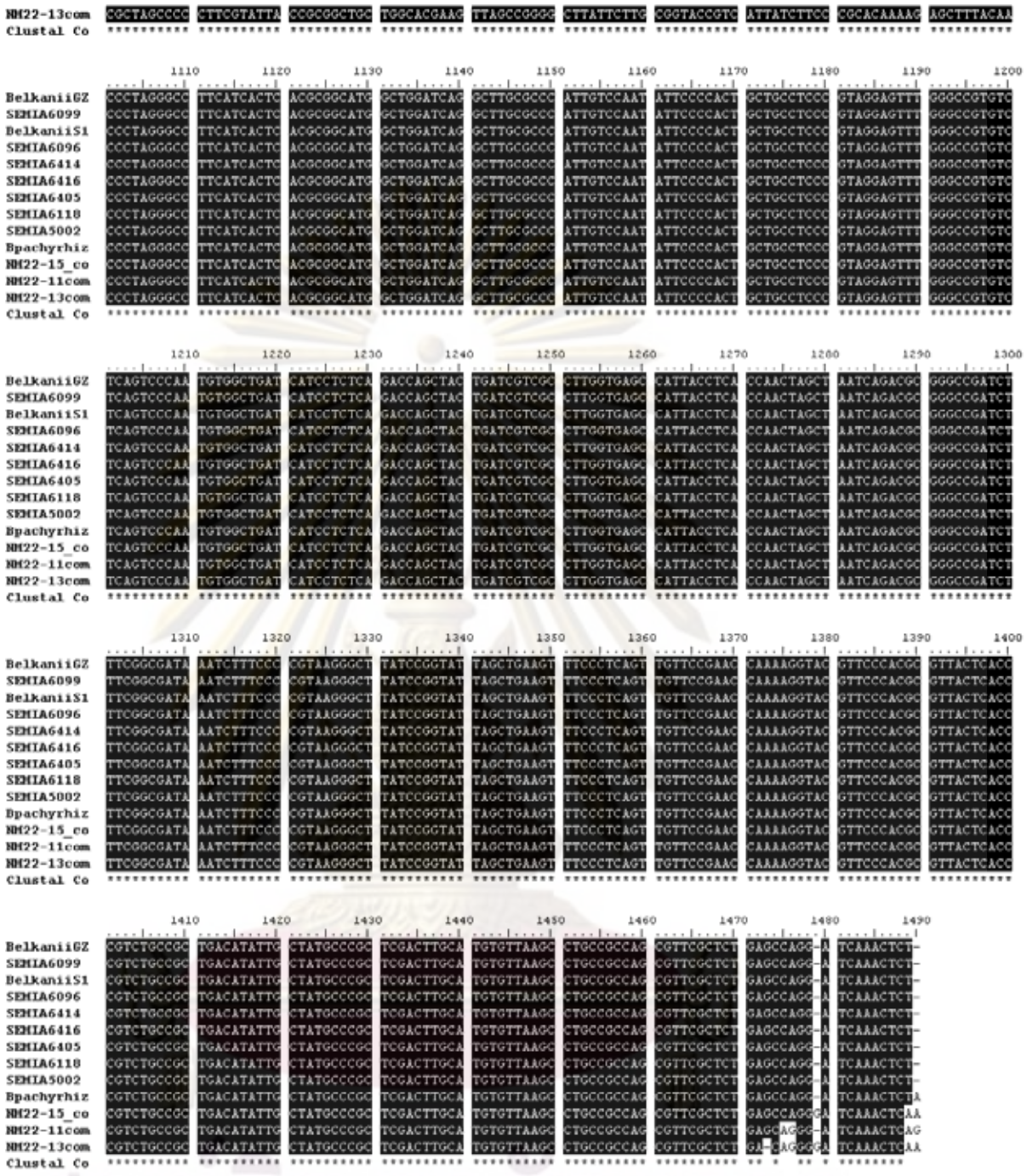

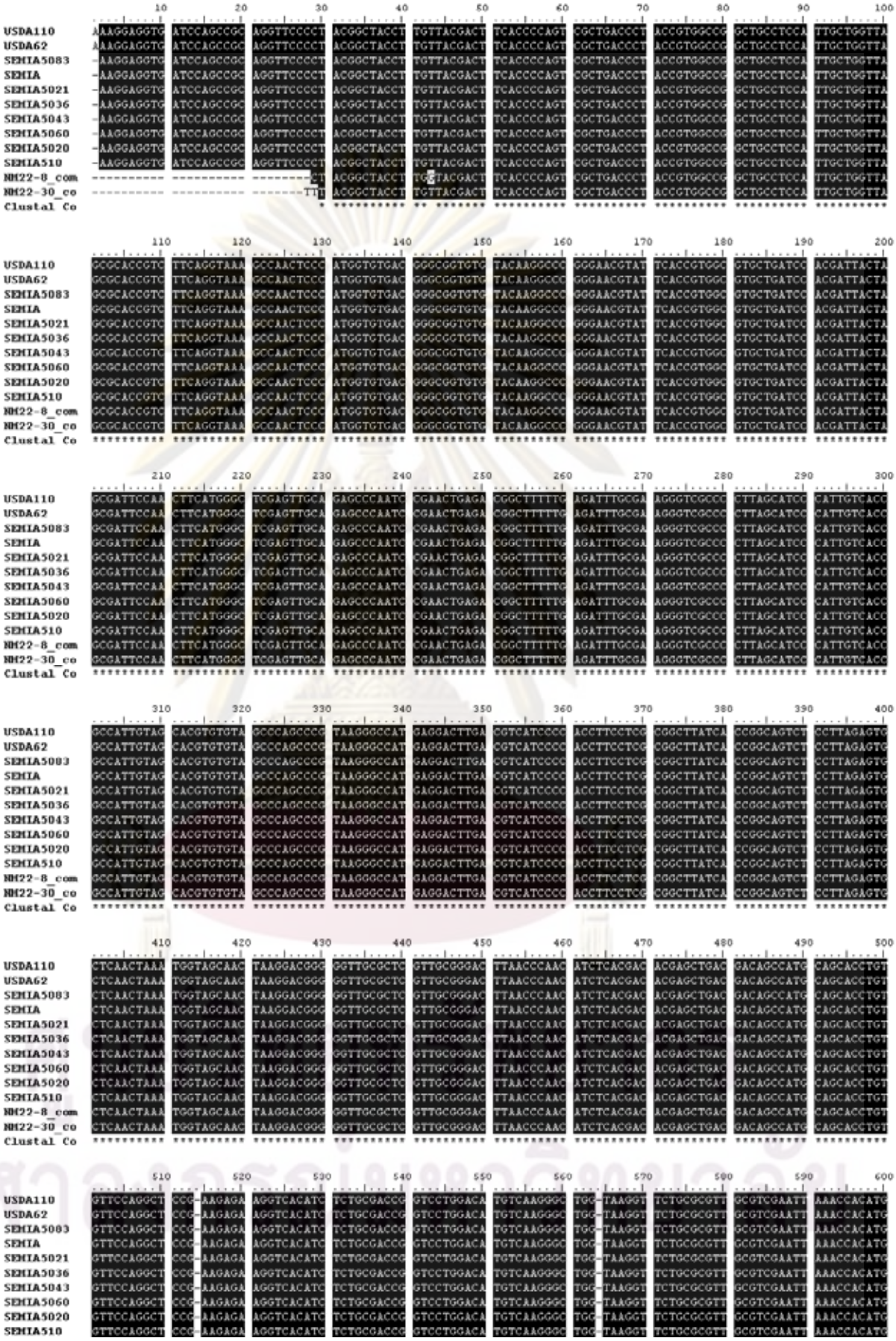
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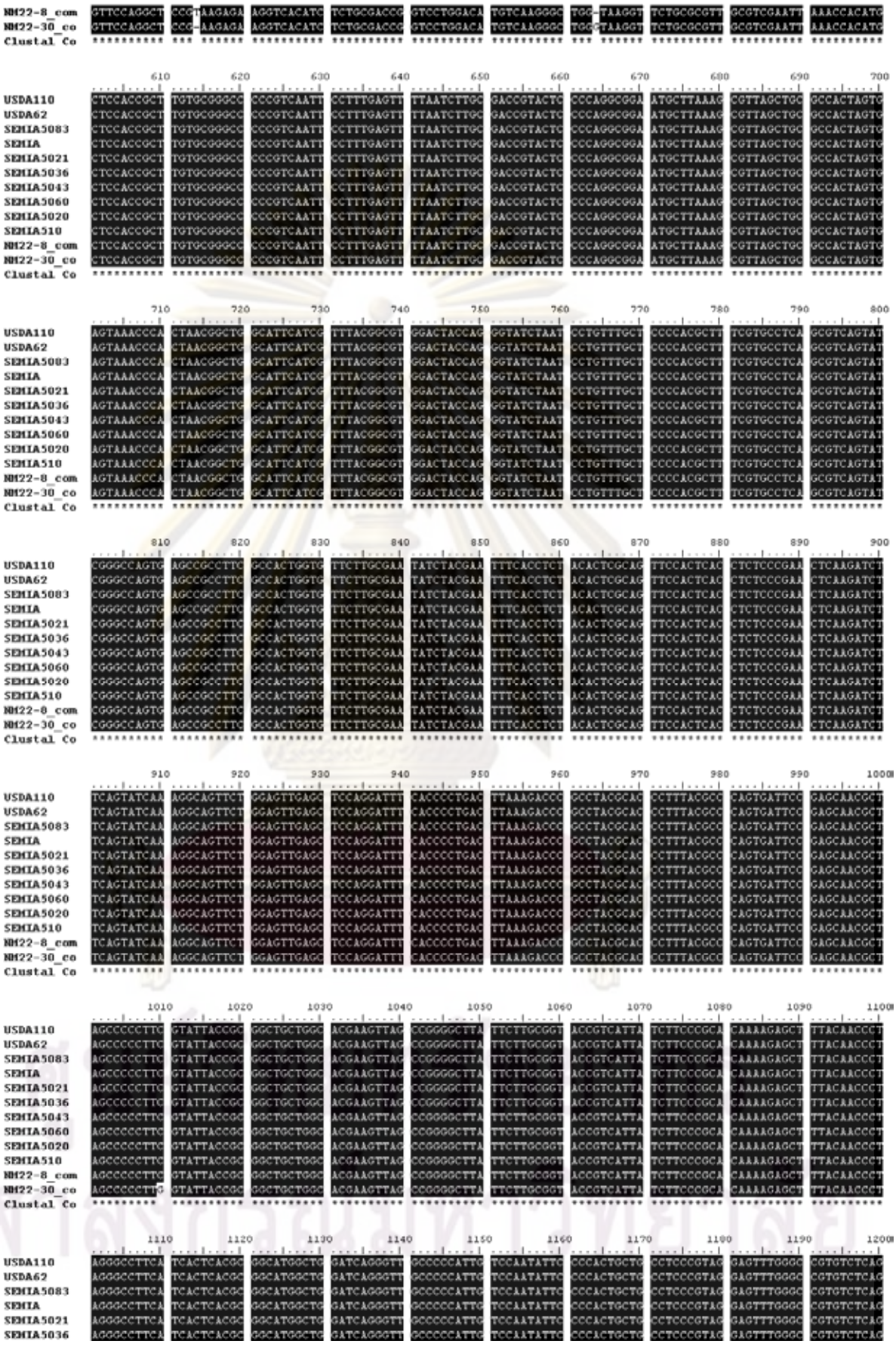
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SEMIA5043
SEMIA5060
SEMIA5020
SEMIA510
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810 820 830 840 850 860 870 880 890 900
910 920 930 940 950 960 970 980 990 1000
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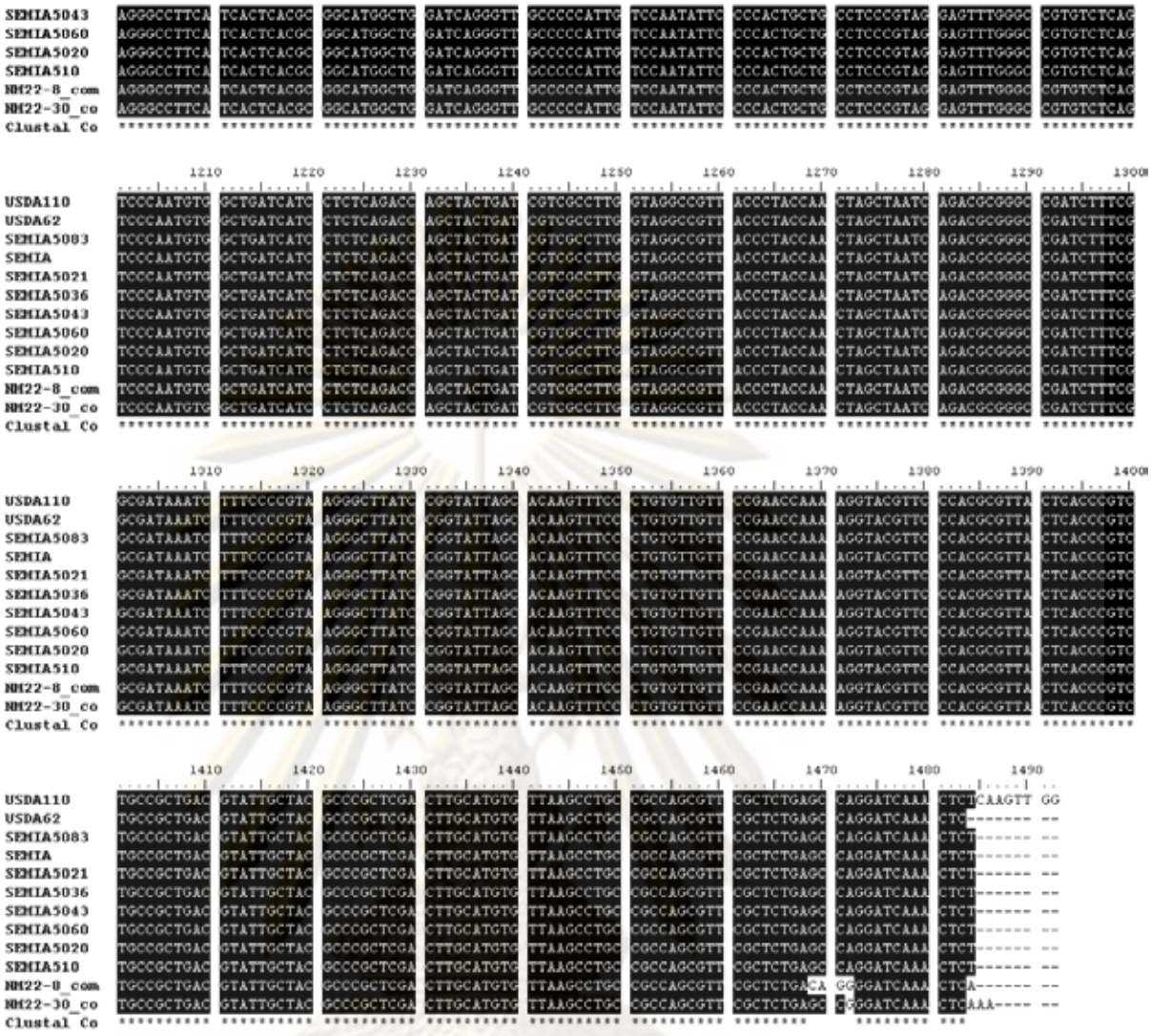
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200



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 (nodulated) test plants (of four) resulting from inoculation with 1-ml samples						Most probable no. of nodule bacteria per ml of suspension at dilution level 1	
Fivefold dilution level							
–	1:5	1:25	1:125	1:625	1:3125*	Estimate	Confidence limits (95%)
1	0	0	0	0	0	1.1	0.2– 7.9
2	0	0	0	0	0	2.6	0.6– 10.1
3	0	0	0	0	0	4.6	1.5– 14.1
4	0	0	0	0	0	8.0	3.0– 21.5
0	1	0	0	0	0	1.0	0.1– 7.7
1	1	0	0	0	0	2.3	0.6– 9.6
2	1	0	0	0	0	4.0	1.2– 12.8
3	1	0	0	0	0	6.5	2.3– 18.0
0	2	0	0	0	0	2.1	0.5– 9.2
1	2	0	0	0	0	3.5	1.1– 11.9
2	2	0	0	0	0	5.5	1.9– 16.0
3	2	0	0	0	0	8.7	3.3– 23.0
0	3	0	0	0	0	3.0	0.9– 10.6
1	3	0	0	0	0	4.9	1.6– 14.6
2	3	0	0	0	0	7.2	2.7– 19.6
3	3	0	0	0	0	11.3	4.4– 29.2
4	1	0	0	0	0	11.4	4.4– 29.5
4	2	0	0	0	0	16.2	6.2– 42.4
4	3	0	0	0	0	24.2	9.0– 64.9
4	4	0	0	0	0	40.4	15.3–106.6
4	0	1	0	0	0	10.8	4.2– 28.1
4	1	1	0	0	0	15.1	5.8– 39.2
4	2	1	0	0	0	21.5	8.1– 57.4
4	3	1	0	0	0	32.8	12.2– 87.9
4	0	2	0	0	0	14.1	5.4– 36.6
4	1	2	0	0	0	19.6	7.4– 51.9
4	2	2	0	0	0	28.3	10.5– 76.1
4	3	2	0	0	0	43.6	16.6–114.2
4	0	3	0	0	0	18.1	6.9– 47.7
4	1	3	0	0	0	25.2	9.4– 67.6
4	2	3	0	0	0	36.4	13.7– 96.8
4	3	3	0	0	0	56.5	21.9–146.0
4	4	1	0	0	0	5.7 × 10	2.2– 14.7 × 10
4	4	2	0	0	0	8.1 × 10	3.1– 21.2 × 10
4	4	3	0	0	0	12.1 × 10	4.5– 32.4 × 10
4	4	4	0	0	0	20.2 × 10	7.6– 53.3 × 10
4	4	0	1	0	0	5.4 × 10	2.1– 14.0 × 10
4	4	1	1	0	0	7.5 × 10	2.9– 19.6 × 10
4	4	2	1	0	0	10.8 × 10	4.0– 28.7 × 10
4	4	3	1	0	0	16.4 × 10	6.1– 43.9 × 10
4	4	0	2	0	0	7.1 × 10	2.7– 18.3 × 10
4	4	1	2	0	0	9.8 × 10	3.7– 26.0 × 10
4	4	2	2	0	0	14.1 × 10	5.3– 38.1 × 10
4	4	3	2	0	0	21.8 × 10	8.3– 57.1 × 10
4	4	0	3	0	0	9.1 × 10	3.4– 23.8 × 10
4	4	1	3	0	0	12.6 × 10	4.7– 33.8 × 10
4	4	2	3	0	0	18.2 × 10	6.9– 48.4 × 10
4	4	3	3	0	0	28.2 × 10	10.9– 73.0 × 10
4	4	4	1	0	0	2.9 × 100	1.1– 7.3 × 100
4	4	4	2	0	0	4.1 × 100	1.6– 10.6 × 100
4	4	4	3	0	0	6.0 × 100	2.3– 16.2 × 100
4	4	4	4	0	0	10.1 × 100	3.8– 26.6 × 100
4	4	4	0	1	0	2.7 × 100	10.4– 7.0 × 100
4	4	4	1	1	0	3.8 × 100	1.5– 9.8 × 100
4	4	4	2	1	0	5.4 × 100	2.0– 14.4 × 100
4	4	4	3	1	0	8.2 × 100	3.1– 22.0 × 100
4	4	4	0	2	0	3.5 × 100	1.4– 9.2 × 100
4	4	4	1	2	0	4.9 × 100	1.8– 13.0 × 100
4	4	4	2	2	0	7.1 × 100	2.6– 19.0 × 100
4	4	4	3	2	0	10.9 × 100	4.2– 28.6 × 100
4	4	4	0	3	0	4.5 × 100	1.7– 11.9 × 100
4	4	4	1	3	0	6.3 × 100	2.3– 16.9 × 100
4	4	4	2	3	0	9.1 × 100	3.4– 24.2 × 100
4	4	4	3	3	0	14.1 × 100	5.4– 36.7 × 100
4	4	4	4	1	0	14.3 × 100	5.5– 36.9 × 100
4	4	4	4	2	0	20.3 × 100	7.8– 53.0 × 100
4	4	4	4	3	0	30.2 × 100	11.2– 81.3 × 100
4	4	4	4	4	0	50.5 × 100	19.0–133.8 × 100
4	4	4	4	0	1	13.5 × 100	5.2– 35.3 × 100
4	4	4	4	1	1	18.8 × 100	7.2– 49.0 × 100
4	4	4	4	2	1	26.9 × 100	10.1– 71.8 × 100
4	4	4	4	3	1	41.0 × 100	15.3–110.2 × 100
4	4	4	4	0	2	17.7 × 100	6.8– 45.9 × 100
4	4	4	4	1	2	24.5 × 100	9.2– 65.0 × 100
4	4	4	4	2	2	35.3 × 100	13.1– 95.4 × 100
4	4	4	4	3	2	54.4 × 100	20.6–143.8 × 100
4	4	4	4	0	3	22.6 × 100	8.6– 59.7 × 100
4	4	4	4	1	3	31.4 × 100	11.7– 84.7 × 100
4	4	4	4	2	3	45.5 × 100	17.0–121.4 × 100
4	4	4	4	3	3	70.6 × 100	27.1–184.2 × 100
4	4	4	4	4	1	7.1 × 1000	2.7– 18.6 × 1000
4	4	4	4	4	2	10.1 × 1000	3.8– 27.0 × 1000
4	4	4	4	4	3	15.1 × 1000	5.4– 42.6 × 1000
4	4	4	4	4	4	25.2 × 1000	8.6– 74.0 × 1000

* Five test plants were inoculated with 1-ml samples from this dilution level.

APPENDIX I

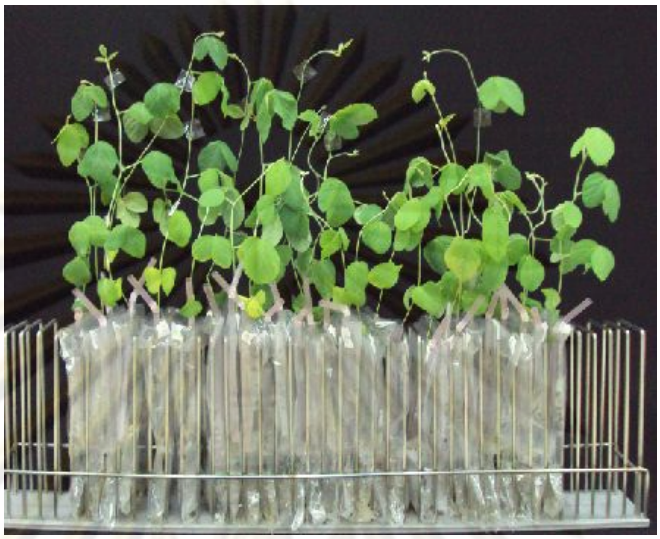
Number of nodules in the determination of MPN

Dilution level	Replicates				Number of pouches with nodules	MPN of soybean rhizobium per ml of suspension at dilution level 1
	1	2	3	4		
1	29	16	12	28	4	
1 : 5	20	14	10	12	4	
1 : 25	7	9	20	34	4	
1 : 125	15	16	10	19	4	
1 : 625	11	17	18	0	3	
1 : 3125	16	14	9	0	3	7.06×10^3

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

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

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



Dilution level	Replicates				Number of pouches with nodules	MPN of soybean rhizobium per ml of suspension at dilution level 1
	1	2	3	4		
1	37	34	29	3	4	
1 : 5	23	23	3	26	4	
1 : 25	3	12	23	17	4	
1 : 125	16	4	12	5	4	
1 : 625	16	2	0	0	2	
1 : 3125	13	11	0	0	2	3.55×10^{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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