การแยก *Bradyrhizobium japonicum* ทนกรดสำหรับตรวจไฮโดรจีเนสแอกทิวิตีและรูปแบบของแถบโปรตีน

นายสุวัฒน์ แสงเกิดทรัพย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOLATION OF ACID-TOLERANT *Bradyrhizobium japonicum* FOR HYDROGENASE ACTIVITY AND PROTEIN PATTERN DETERMINATION

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สุวัฒน์ แสงเกิดทรัพย์ : การแยก *Bradyrhizobium japonicum* ทนกรดสำหรับตรวจไฮโดรจีเนสแอกทิวิตีและรูปแบบของแถบโปรตีน (ISOLATION OF ACID-TOLERANT *Bradyrhizobium japonicum* FOR HYDROGENASE ACTIVITY AND PROTEIN PATTERN DETERMINATION) 104 หน้า. ISBN 974-334-595-7

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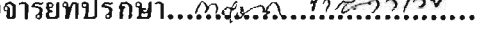
วัตถุประสงค์ของงานวิจัยเพื่อแยก *Bradyrhizobium japonicum* ที่ทนกรด และหาผลของพีเอชเริ่มต้นต่อไฮโดรจีเนสแอกทิวิตีและรูปแบบการเรียงแถบโปรตีนที่แยกจาก *B. japonicum* แยกแบคทีเรียปมรากถั่วเหลืองที่เลี้ยงบนตัวอย่างดิน 13 แห่งที่มีค่าพีเอชในช่วง 4.19-6.89 ได้ 56 ไอโซเลต เป็นแบคทีเรียที่เพิ่มจำนวนรวดเร็ว 6 ไอโซเลต และเพิ่มจำนวนช้า 50 ไอโซเลต ทุกไอโซเลตเป็น *B. japonicum* ที่ทำให้เกิดปมที่รากถั่วเหลือง ผลการทำAnalysis of variance พบว่าความแปรปรวนในค่าน้ำหนักแห้งของลำต้นและน้ำหนักแห้งของปม เกิดจากชนิดของ *B. japonicum* ที่ใช้ มิได้เกิดจากสารอาหารไร้ธาตุไนโตรเจนที่มีค่าพีเอช 4.5 หรือ 6.8 การทดลองใช้ลักษณะต่อไปนี้คัดเลือก *B. japonicum* 4 สายพันธุ์ที่ไม่ซ้ำกัน (#S50, S58, S179, S204) : (1)ประสิทธิภาพในการตรึงไนโตรเจนที่พีเอช 4.5 และ 6.8 (2)ลักษณะโคโลนี (3)ลายพิมพ์ดีเอ็นเอที่ได้จากการใช้วิธี RAPD-PCR โดยมี RPO1 เป็นไพรเมอร์ และ (4)ลักษณะการเจริญของ *B. japonicum* เมื่อเพิ่มจำนวนเซลล์ในอาหารเลี้ยงเชื้อที่มีบัฟเฟอร์ควบคุมพีเอช 9.0 การทดลองพบว่าพีเอชเริ่มต้นของอาหารเลี้ยงเชื้อมีผล 3 แบบ ต่อไฮโดรจีเนสแอกทิวิตี ได้แก่ แบบที่ 1 (#S50) มีฟีโนไทป์ *hup*$^-$ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีค่าพีเอชเริ่มต้น 4.0 แต่มีฟีโนไทป์ *hup*$^+$ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีค่าพีเอชเริ่มต้นเท่ากับ 6.0-9.0 โดยมีไฮโดรจีเนสแอกทิวิตีสูงสุด 30.1 ยูนิตต่อมิลลิกรัมโปรตีน เมื่อค่าพีเอชเริ่มต้นเท่ากับ 8.0 แบบที่ 2 (#S58, S179) มีฟีโนไทป์ *hup*$^-$ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีค่าพีเอช 4.0-8.0 แต่มีฟีโนไทป์ *hup*$^+$ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีค่าพีเอชเริ่มต้นเท่ากับ 9.0 โดยมีไฮโดรจีเนสแอกทิวิตีสูงสุด 29.4 ยูนิตต่อมิลลิกรัมโปรตีน แบบที่ 3 (#S204) มีฟีโนไทป์ *hup*$^-$ เมื่อพีเอชเริ่มต้นของอาหารเลี้ยงเชื้ออยู่ในช่วง 5.0-7.0 แต่มีฟีโนไทป์ *hup*$^+$ เมื่อพีเอชเริ่มต้นของอาหารเลี้ยงเชื้อมีค่า 4.0 โดยมีไฮโดรจีเนสแอกทิวิตีเท่ากับ 43.9 ยูนิตต่อมิลลิกรัมโปรตีน ผลการทดลองแสดงให้เห็นเป็นครั้งแรกว่า เมื่อ*B. japonicum* เจริญแบบอิสระ การแสดงออกของยีนส์ *hup* ขึ้นอยู่กับค่าพีเอชเริ่มต้นของอาหารเลี้ยงเชื้อ ผลการทดลองแยกโปรตีนภายในเซลล์ของไอโซเลต #S50, S58, S179 และ S204 โดยวิธี SDS-PAGE พบว่า ไอโซเลต #S58 มีปริมาณโพลิเปปไทด์ขนาด 28, 33, 37 และ 50 กิโลดาลตันเพิ่มขึ้น เมื่อเลี้ยงในอาหารสูตร YMB ช่วงพีเอช 4.0-8.0 ที่ไม่เติมบัฟเฟอร์ และสร้างโพลิเปปไทด์ ขนาด 29 และ 120 กิโลดาลตันเพิ่มขึ้น เมื่อเลี้ยงในอาหารสูตร YMB ช่วงพีเอช 6.0-8.0 ที่เติมบัฟเฟอร์ ไอโซเลต #S179 สร้างโพลิเปปไทด์ขนาด 25, 28, 33, 37 และ50 กิโลดาลตันเพิ่มขึ้น เมื่อเลี้ยงในอาหารสูตร YMB ที่มีพีเอช 8.0-9.0 ซึ่งเติมบัฟเฟอร์ ไอโซเลต #S204 สร้างโพลิเปปไทด์ ขนาด 120 กิโลดาลตัน เพิ่มขึ้น เมื่อเลี้ยงในอาหารสูตร YMB ที่มีพีเอช 5.0-7.0 ที่เติมบัฟเฟอร์

ภาควิชา จุลชีววิทยา	**ลายมือชื่อนิสิต**..... 
สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม	**ลายมือชื่ออาจารย์ที่ปรึกษา**... 
ปีการศึกษา 2542	**ลายมือชื่ออาจารย์ที่ปรึกษาร่วม**.....

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SUWAT SAENGKERDSUB: ISOLATION OF ACID-TOLERANT *Bradyrhizobium japonicum* FOR HYDROGENASE ACTIVITY AND PROTEIN PATTERN DETERMINATION 104 pp. ISBN 974-334-595-7

THESIS ADVISOR : Associate Professor Kanjana Chansa-ngavej, Ph.D

The aim of this research is to isolate acid-tolerant *Bradyrhizobium japonicum* for use in pinpointing the effects of initial pH on hydrogenase activity and protein patterns. 56 soybean root nodule isolates obtained from 13 soil samples of pH ranging from 4.19-6.89 consisted of 6 fast-growing isolates and 50 slow-growing isolates. Authentication test on soybean plants grown in Leonard jars showed that all isolates were *B. japonicum*. Analysis of variance indicated that the source of variations in the observed plant dry weight and nodule dry weight was the types of isolates and not pH 4.5 or 6.8 of the N-free plant nutrient solutions. The following four criteria were used to select 4 distinct strains of *B. japonicum* (#S50, S58, S179, S204) : (1) nitrogen fixing ability at pH 4.5 and 6.8 (2) colony morphology (3) RAPD-PCR fingerprint patterns obtained when RPO1 was used as the primer (4) extent of *B. japonicum* growth in buffered yeast extract mannitol broth (YMB) at pH 9.0. Initial pH had 3 types of effects on hydrogen uptake hydrogenase activity. Type 1 (#S50) exhibited *hup*$^-$ phenotype when initial pH of the medium was 4.0 but exhibited *hup*$^+$ phenotype when media initial pHs were 6.0-9.0 with maximum specific activity 30.1U. mg^{-1} protein at initial pH 8.0. Type 2 (#S58, S179) exhibited *hup*$^-$ phenotype when media initial pHs were 4.0-8.0 but exhibited *hup*$^+$ phenotype when the initial pH was 9.0 with specific hydrogenase activity of 29.4 U.mg^{-1} protein. Type 3 (#S204) exhibited *hup*$^-$ phenotype when the initial media pH was in the range of 5.0-7.0 but exhibited *hup*$^+$ phenotype when the initial pH was 4.0 with specific hydrogenase activity of 43.9 U. mg^{-1} protein. The results indicated for the first time that expression of *hup* genes in free-living *B. japonicum* depended upon initial pH. SDS-PAGE separation of cellular proteins of isolates #S50, S58, S179, and S204 indicated an increase in quantities of the following polypeptides of isolate S58 (28, 33, 37, 50 kDa) when grown in unbuffered YMB at pH 4.0-8.0 and an increase in quantities of polypeptides 29 and 120 kDa when grown in buffered YMB, pH 6.0-8.0. Isolate #S179 was found to synthesize more of the following polypeptides (25, 28, 33, 37, 50 kDa) when grown in buffered YMB at pH 8.0-9.0. Isolate #S204 contained more 120 kDa polypeptide when grown in buffered YMB at pH 5.0-7.0.

ภาควิชา จุลชีววิทยา	ลายมือชื่อนิสิต.....
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Abbreviations

AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid
BIS-TRIS	Bis(2-hydroxyethyliminotris(hydroxymethyl)methane
bv	biovar
CAPSO	3-(cyclohexyamino)-2-hydroxy-1-propanesulfonic acid
cv	cultivar
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhoff-Parnas pathway
ERIC	Enterobacterial repetitive intergenic consensus
HEPES	N-(2-Hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)
kDa	kilodalton
MES	2-(N-Morpholino)ethanesulfonic acid
MOPS	3-(N-Morpholino)propanesulfonic acid
NEDA	*cis*-endo-bicyclo(2.2.1)hept-5-ene-2,3-dicarboxylicanhydride
OD	Optical density
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PP	pentose phosphate pathway
RAPD-PCR	Ramdom Amplification of Polymorphic DNA-Polymerase Chain Reaction
REP	Repetitive extragenic palindromic
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis

TCA	Tricarboxylic acid cycle
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

Chapter 1

Introduction

Soybeans (*Glycine max*) are considered one of the most important economic crops in Thailand. Thienchai Arayangura (1998) reported that in 1996 demand for soybean seeds in Thailand was more than 800,000 tons, of which more than 400,000 tons were imported. Preliminary data from the Office of Agricultural Economics showed that, in 1998/1999, demand for soybean seeds was up to 926,310 tons, of which 550,000 tons were imported as indicated in Table 1.1.

Table 1.1 Supply and demand for soybean seeds in Thailand during 1995 to 1999

Unit : Tons

Year	Local Production	Import	Demand
1995/1996	385,560	425,652	811,212
1996/1997	359,094	657,360	1,016,454
1997/1998	366,349	574,244	940,593
1998/1999*	376,310	550,000	926,310

Source : Office of Agricultural Economics(2000)

* Preliminary data

The figures in Table 1.1 indicate an increased local demand for imported soybeans in the past five years. Therefore, if quantities of soybean seeds cultivated in Thailand are increased, Thailand will not have to rely on imported

soybeans. Moreover, some of the imported soybean seeds are transgenic which may present future complications regarding the biosafety protocol of labelling transgenic soybean-based value-added products such as soybean oil which will be exported to the European Union (EU) countries(Suthat Sriwatanapongse, 2000). One way to increase soybean seeds grown in Thailand is to increase the acreage of soybean cultivation. The 1999 Annual Report of the Center for Agricultural Information stated that there were 100,000-200,000 rais for soybean cultivation in each of the following provinces : Chiang Mai, Kampaeng Pet, Sukothai, Tak, and there was no soybean cultivation in the following eight border provinces in the north-eastern part of Thailand as shown in Figure1.1 : Amnat Charoen, Buri Ram, Mukdahan, Nakorn Panom, Srisa Ket, Surin, Ubon Ratchathani, and Yasothon. One reason is the acid and infertile soils(Ultisol soils) in the above-mentioned border provinces as indicated in Figure 1.2 (Erb Kaewruenrom, 1998; Brady and Weil, 1996).

Therefore, in order to increase the acreage for soybean cultivation in the north-eastern part of Thailand, both acid tolerant soybean cultivars and acid-tolerant *Bradyrhizobium japonicum* strains have to be obtained by either traditional breeding or mutagenesis or genetically constructed in order to withstand the acidic and often infertile soil conditions. At present, there is no record on acid tolerance property of all soybean cultivars grown in Thailand. Table 1.2 shows soybean cultivars commonly grown in Thailand (Somsak Srisomboon, 1999). However, soybeans are known to be less sensitive to soil acidity compared to other leguminous plants(Department of Land Development,

Figure 1.1 Map of Thailand showing areas of soybean cultivation in 1997/1998 (Source : Annual Report of the Center for Agriculture Information, 1999)

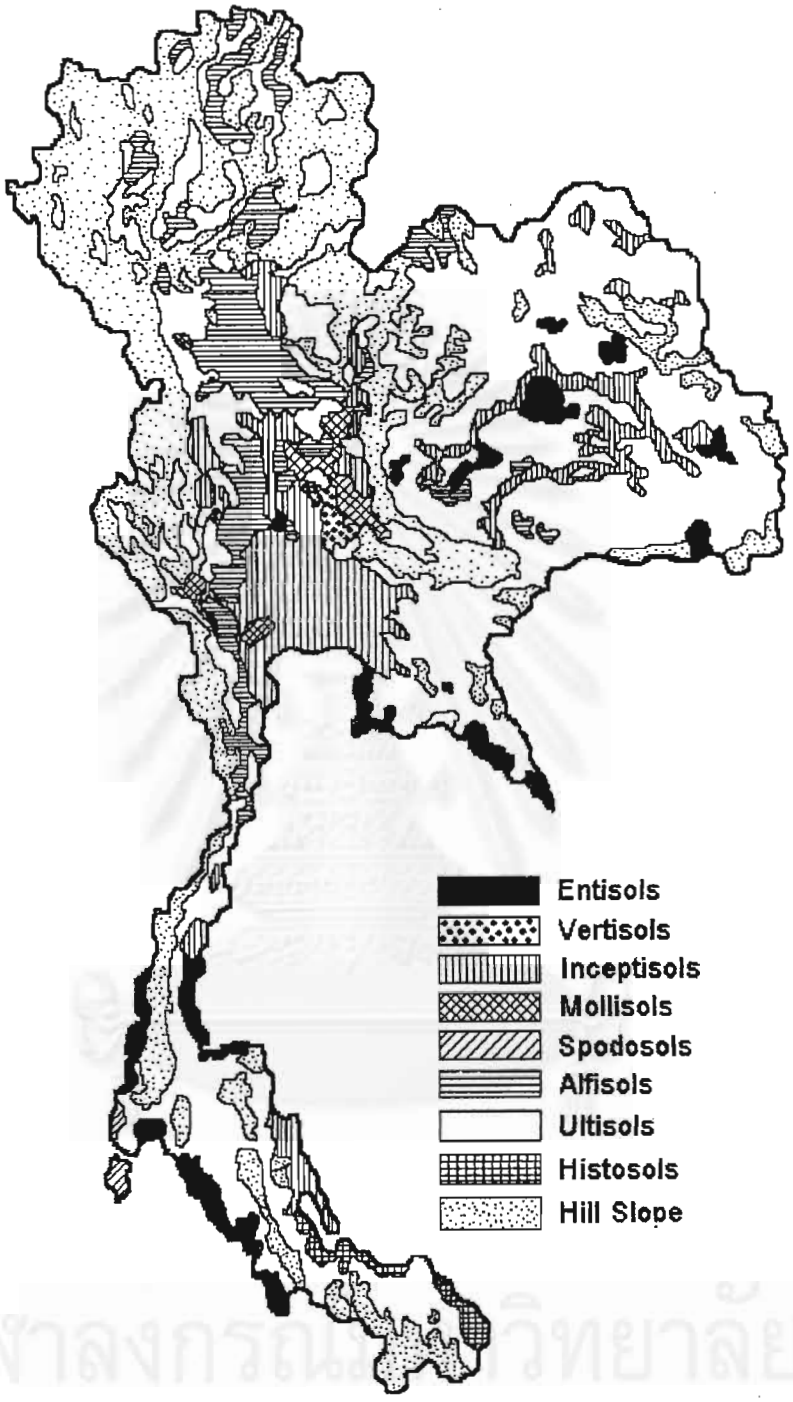


Figure 1.2 Map of Thailand showing distribution of various soils.

(Source : Erb Kaewruenrom, 1998)

1999). In Indonesia, Wahyudi *et al.* (1998) reported an acid-tolerant soybean, *Glycine max* cultivar B-09.

Table 1.2 Soybean cultivars grown in Thailand

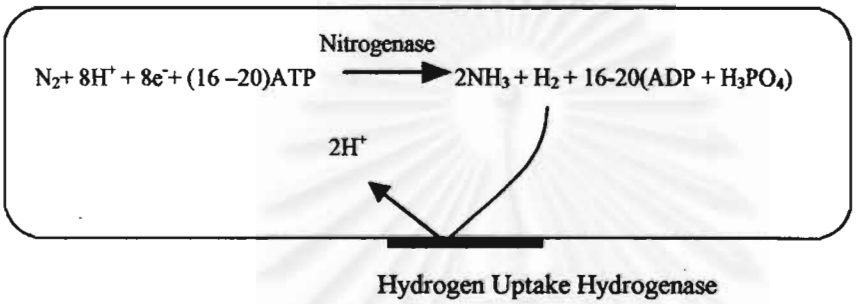
Cultivars	Time to harvest (days)
Nakorn Sawan1, Chiang Mai 2	74-85
SJ4, SJ5, Sukothai1, Sukothai2, Chiang Mai 60	84-112
MK35, Jakrapan1, Ratchamongkol 1	104-120

Source : Somsak Srisomboon, 1999

B. japonicum are symbiotic bacteria in root nodules of soybeans. Normally, *B. japonicum* grow well in medium with neutral pHs (Somasegaran & Hoben, 1994). Low soil pHs have been known to restrict growth of both soybeans and root nodule bacteria (Glenn and Dilworth, 1994). Glenn and Dilworth (1994) proposed possible mechanisms involved in acid tolerance in root nodule bacteria as shown in Figure 1.3 which included a possible involvement of transcriptional activation of low pH-inducible proteins. It is thus expected that protein profiles of representative *B. japonicum* isolates when grown in media with different initial pHs might shed light on a possible mechanism for acidophilicity or acid-tolerance in *B. japonicum*. Moreover, there is an increasing need for a more rapid method to predict nitrogen-fixing potential of *B. japonicum* strains without having to perform acetylene-reduction test on root nodules or

having to inoculate soybeans with *B. japonicum* and determine the plant and the nodule dry weight. Maier *et al* (1978) proposed the hypothesis that *B. japonicum* with the expression of *hup* genes would be more efficient nitrogen fixers. The following diagram summarizes the concept underlying the hypothesis.

B. japonicum cell



The [Ni-Fe] hydrogenases are a well-conserved family of membrane – bound hydrogenases that can catalyse both the uptake and evolution of hydrogen (Black *et al.*, 1994). These heterodimeric proteins consist of a small subunit (~ 30 kDa) and a large subunit (~65 kDa) containing nickel and iron-sulfur clusters. *B. japonicum* [Ni-Fe] hydrogenase consists of two subunits, a 33 kDa subunit and a 65 kDa subunit encoded by the *hupSL* genes. Figure 1.4 indicates some other loci on the hydrogenase gene cluster of *B. japonicum* which are involved in hydrogenase processing (Fu & Maier, 1993), nickel metabolism (Fu *et al* , 1994) and regulation (Kim and Maier, 1990; Kim *et al*,1991).

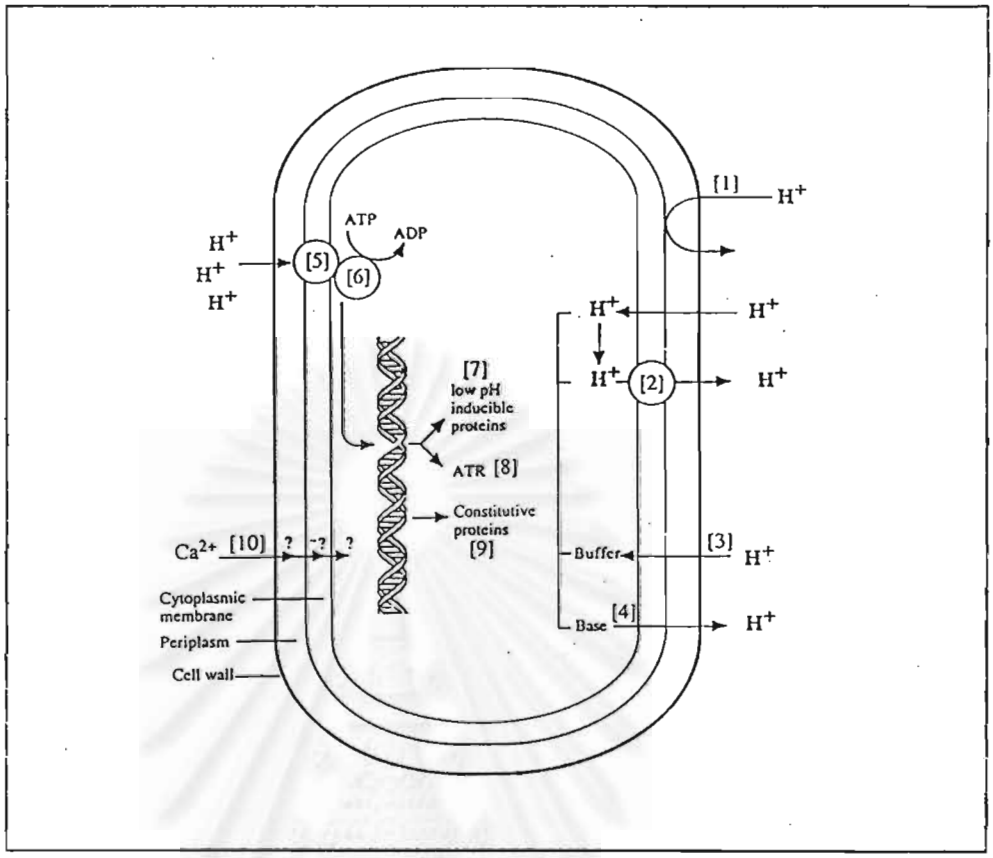


Figure1.3. Possible mechanism(s) involved in acid tolerance in root nodule bacteria. 1: decrease in membrane permeability to H^+; 2: H^+-export mechanism (including ion pumps); 3: cytoplasmic buffering; 4: alkalinization of external medium by base export; 5: postulated environmental pH sensor(s); 6: postulated regulator(s) mediating between sensor(s) and DNA; 7: transcriptional activation of low pH-inducible proteins; 8: transcriptional activation of adaptive acid tolerance response(ATR) genes; 9: potential transcriptional of activation of constitutively expressed *act* genes; 10: the undefined role of Ca^{2+} in acid tolerance.(from Glenn and Dilworth, 1994).

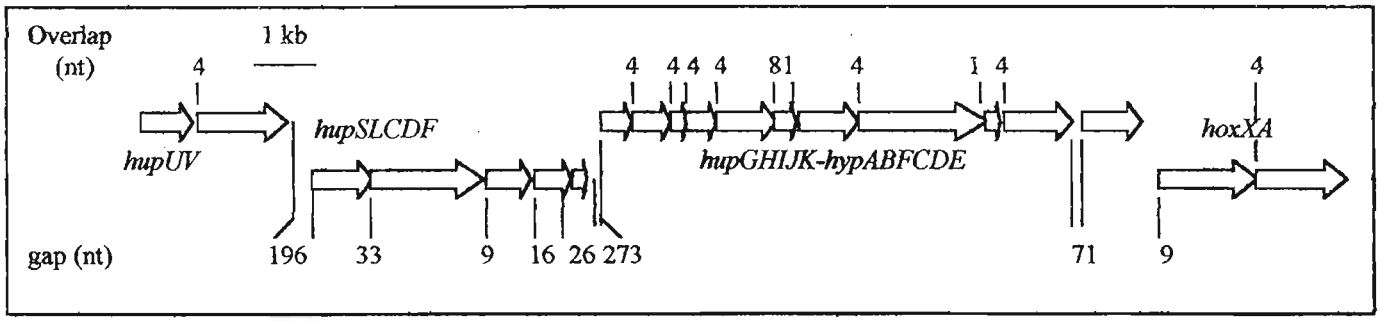


Figure 1.4. Physical map of the *B. japonicum* hydrogenase gene cluster. Each gene is represented by an arrow pointing in the relative direction of transcription, and grouped in one of the four proposed operons, numbers above the figure represent the overlap of the reading frames of adjacent genes. Numbers below the figure indicate gaps between the genes (Olson & Maier, 1997)

Black *et al*(1994) stated that hydrogenases, which can catalyse both the uptake and evolution of hydrogen, play key roles in energy metabolism of many bacteria. Emrich *et al*(1979) provided an experimental evidence to show that in *B. japonicum*, hydrogenase oxidizes the nitrogenase evolved hydrogen, generating H^+ for further uses in redox-reactions. This process, called hydrogen cycling, is believed to recover some of the energy lost during the energy expensive nitrogen fixation reaction.

The aim of this research is to isolate acidophilic or acid-tolerant *B. japonicum* from soils for further characterization of nitrogen fixation potential and for use in the preliminary elucidation of acid tolerance mechanisms. The isolates will be used in the determination of the effects of growth medium initial pH on hydrogen uptake hydrogenase activity and cellular protein patterns as well as the possibility of employing the expression of *hup* genes as one criterion for high nitrogen fixing ability.

Chapter 2

Literature Survey

2.1 *Rhizobium* spp and other rhizobia

According to Elkan and Bunn(1992) there are 4 species of *Rhizobium* namely *R. leguminosarum*, *R. meliloti*, *R. loti* and *R. galegae*. Three former species *R. phaseoli*, *R. trifolii* and *R. leguminosarum* have been combined into *R. leguminosarum*. Recently *R. fredii* which consists of fast-growing rhizobia which nodulate Chinese soybean cultivars have been assigned to a new genus, *Sinorhizobium* with *S. fredii* as the type species (Chen *et al*, 1988).

In 1988 Dreyfus *et al.* described a new genus of nitrogen fixing rhizobia, *Azorhizobium* (type species : *Azorhizobium caulinodans*), which were first isolated from stem nodules of *Sesbania rostrata*. Table 2.1 indicates taxonomic classification of rhizobia.

Table 2.1 Taxonomic classification of rhizobia

Recognized genus	Recognized species
Bradyrhizobium	*B. Japonicum*
	B. elkanii
Rhizobium	*R. leguminosarum*
	R. meliloti
	R. loti
	R. galegae
Azorhizobium	*A. caulinodans*
Sinorhizobium	*S. fredii*

Source : Elkan and Bunn, 1992; Kuykendall *et al.*, 1992

Elkan and Bunn (1992) summarized the differences between fast-growing *Rhizobium* spp and slow-growing *Bradyrhizobium* spp as indicated in Table 2.1.

Table 2.2 The differences between fast- and slow-growing rhizobia

Characteristics	Rhizobial type	
	Fast-growing	Slow-growing
Generation time	< 6 h	> 6 h
Carbohydrate substrate	Uses pentose, hexose, mono-,di-, and tri-saccharides	Uses pentoses and hexoses solely
Metabolic pathways	EMP-low activity ED-main pathway TCA-fully active PP pathway present	EMP-low activity ED-main pathway TCA-fully active Hexose cycle present
Flagellation type	Peritrichous	Subpolar
Symbiotic gene location	Plasmid and chromosome	Chromosome only
Nitrogen-fixation gene location	*nifHDK* on same operon	*nifDK* and *nifH* on separate operons
Intrinsic antibiotic resistance	High	Low

Abbreviations : ED, Entner-Doudoroff pathway, EMP, Embden-Meyerhoff-Parnas pathway; PP, pentose phosphate pathway, TCA, Tricarboxylic acid cycle

Source : Elkan and Bunn, 1992

Figure 2.1 shows the genome map of *B. japonicum* USDA110 (Kündig *et al.* , 1993) indicating that *nifDK* and *nifH* are on separate operons whereas Figure 2.2 shows that *nifHDK* of *R. meliloti* are on the same operon(Fischer, 1994).

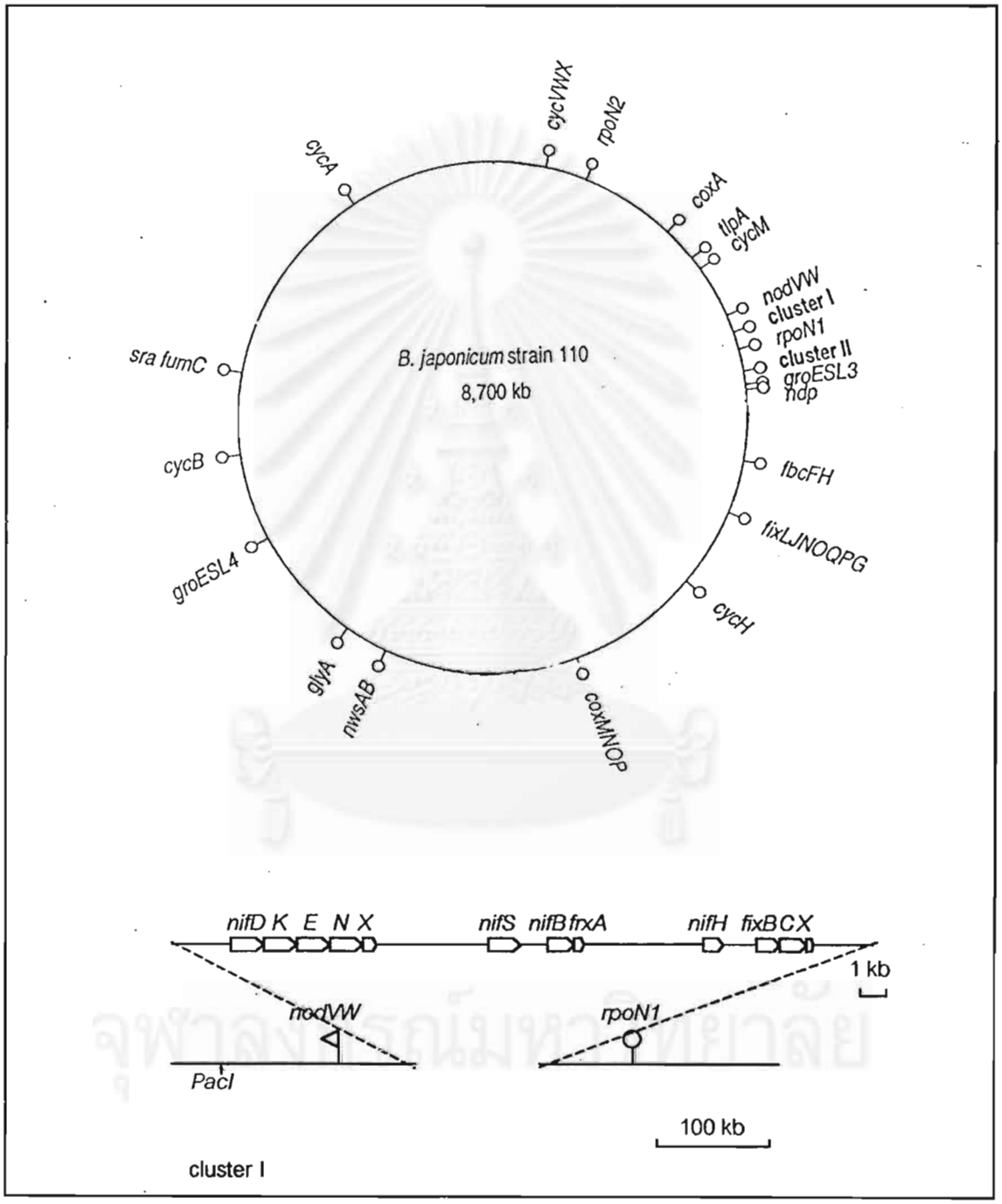


Figure 2.1 Genome map of *B. japonicum* USDA110 chromosome. Positions of gene regions are shown in thc outer periphery. (Kündig *et al*, 1993).

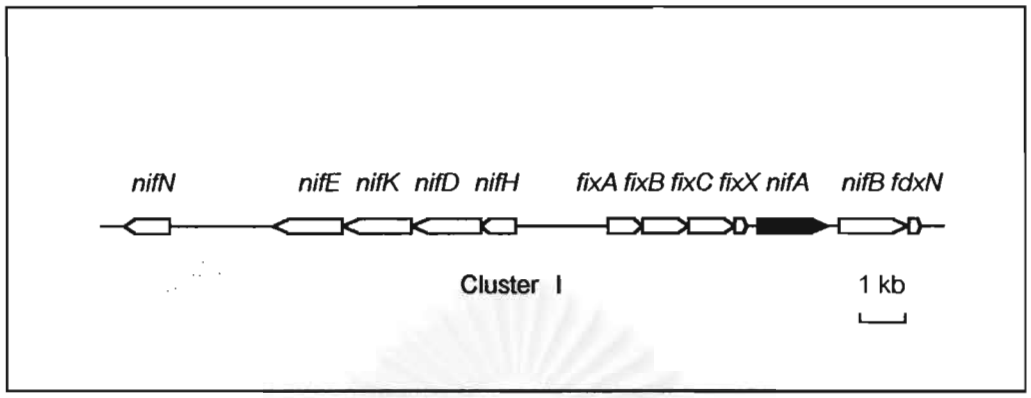


Figure 2.2 Organization of *nif* gene cluster in *R. meliloti* (Fischer, 1994).

2.2 Description of *Bradyrhizobium japonicum*

Jordan (1982) stated that in 1980 members of the International subcommittee on *Agrobacterium* and *Rhizobium* unanimously approved the establishment of a new genus *Bradyrhizobium* for the slow-growing, non-acid producing rhizobia in which the type species is *B. japonicum* Buchanan 1980 with the following description : Gram-negative, aerobic, non-spore-forming, short rods 0.5 to 0.9 μm by 1.2 to 3.0 μm. Motile by one polar or subpolar flagellum. Produce slow growth on yeast extract mannitol broth. Colonies are circular, opaque, rarely translucent, white, and convex, and tend to be granular in texture. Growth on carbohydrate media is accompanied by extracellular slime. *B. japonicum* causes formation of effective root nodules on soybean (*Glycine max*). The guanine-plus-cytosine content of the DNA is 61-65 mole%. Most strains grow on a mineral salts medium containing yeast extract and glucose, galactose, gluconate, glycerol, fructose, arabinose, or mannitol. Maltose is utilized by about 10% of the strains, but lactose, rhamnose, raffinose, trehalose,

sucrose, and dextrin are rarely utilized. Organic acids such as fumarate, malate, succinate, citrate, and pyruvate are utilized, provided the basal medium has sufficient Ca^{2+} and Mg^{2+} to overcome the inhibitory chelating effects of these acids. Cellulose and starch are not utilized. Some strains can utilize ammonium salts or nitrate as a sole source of nitrogen. Certain amino acids (glutamate, histidine, aspartate, and proline) serve as sole nitrogen sources. Casein and agar are not hydrolyzed. Peptone is poorly ulitized. Usually acid tolerant, most strains grow at pH 4.5. Over 30% of the strains will grow at pH 4.0 and a few as low as pH 3.5. Fails to grow above pH 9.0. An alkaline reaction is produced in litmus milk, without the digestion of casein to produce a clear, upper "serum zone". Fails to grow in media containing 2% NaCl and does not produce H_2S. Penicillinase production is common. $NADP^+$-linked 6-phosphogluconate dehydrogenase(EC 1.1.1.43), the key enzyme in the pentose phosphate pathway, is absent or virtually so. Glucose is metabolized largely by the Entner-Doudoroff route. Nitrogenase activity by free-living cells occurs in certain strains but only in media containing selected carbon sources and under reduced oxygen tension. Some strains possess an active uptake hydrogenase which enables them to grow chemolithotrophically in an atmosphere of hydrogen, carbon dioxide, and low levels of oxygen.

In 1984 the genus *Bradyrhizobium* was incorporated into the Family Rhizobiaceae in Bergey's Manual of Systematic Bacteriology. There is only one species in the genus *Bradyrhizobium*, namely, *B. japonicum* which nodulates soybeans. Other bradyrhizobia , for example, the slow-growing bradyrhizobia which nodulate mungbeans have not been classified to the species level. These

bradyrhizobia are identified as *Bradyrhizobium* followed by scientific name(s) of host plant(s). For example *Bradyrhizobium* sp.(*Vigna*); *Bradyrhizobium* sp. (*Lupinus*) (Jordan, 1984; Elkan and Bunn, 1992).

Previous molecular methods which could be employed to find out of the isolates are either genetically related or of the same strain include two-dimensional SDS-PAGE patterns. Roberts *et al.* (1980) employed two-dimensional SDS-PAGE patterns to identify and classify 33 strains of various fast-growing *Rhizobium* species and 24 strains of slow-growing rhizobial strains. They reasoned that the protein pattern on 2D-SDS-PAGE gels reflected the genetic background of the tested strains, similarities in pattern could therefore be used to estimate the relatedness among these strains. The results indicated that patterns on 2D-SDS-PAGE gels could be used to differentiate the fast-growing rhizobial strains into group I with the bulk of visible acidic proteins and slow-growing rhizobial strains into group II which had their most abundant proteins at a much more basic position. Roberts *et al.* (1980) found no obvious similarities in the 2D-SDS-PAGE gel patterns between the group I and group II strains.

2.3 Nitrogenase and hydrogen evolution

It is well known that nitrogenase, which catalyses the reduction of N_2 to NH_3, consists of two proteins :

1. The Fe-protein, an ~ 64 kDa dimer of identical subunits that contains one [4Fe-4S] cluster and two ATP binding sites. *nifH* encodes Fe-protein.

2. The MoFe-protein, an ~ 220 kDa heteromer of subunit structure $\alpha_2\beta_2$ that contains Fe and Mo. *nifDK* encodes Mo-Fe protein. The flow of electrons in the nitrogenase-catalysed reduction of N_2 is as shown in Figure 2.3.

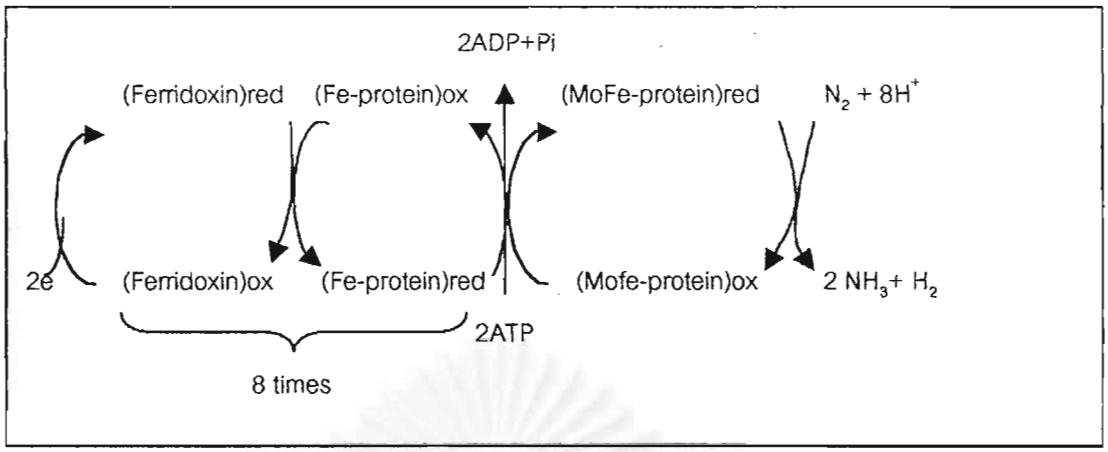


Figure 2.3 The flow of electrons in the nitrogenase-catalysed reduction of N_2 (Voet and Voet, 1995)

According to Chen *et al.* (1993) and Garrett and Grisham (1999) each αβ dimer of MoFe protein of *Azotobacter vinelandii* which serves as the functional unit of nitrogenase contains two types of metal centers, an 8 Fe-7S center known as the P-cluster (Figure 2.4(a)) and the FeMo-cofacter with two MFe_3S_3 (M= Mo or Fe) clusters linked by two binding sulfides and a third ligand, designated Y (Figure 2.4(b)).

P-cluster of the MoFe protein

Figure 2.4 (a) shows that the two Fe_4S_3 clusters of the P-cluster are joined by a disulfide bridge formed between S atoms in each Fe_4S_4 cluster. According to Chen *et al.* (1993) this disulfide bridge is located on the side of the P-cluster pair closed to the binding site for Fe protein.

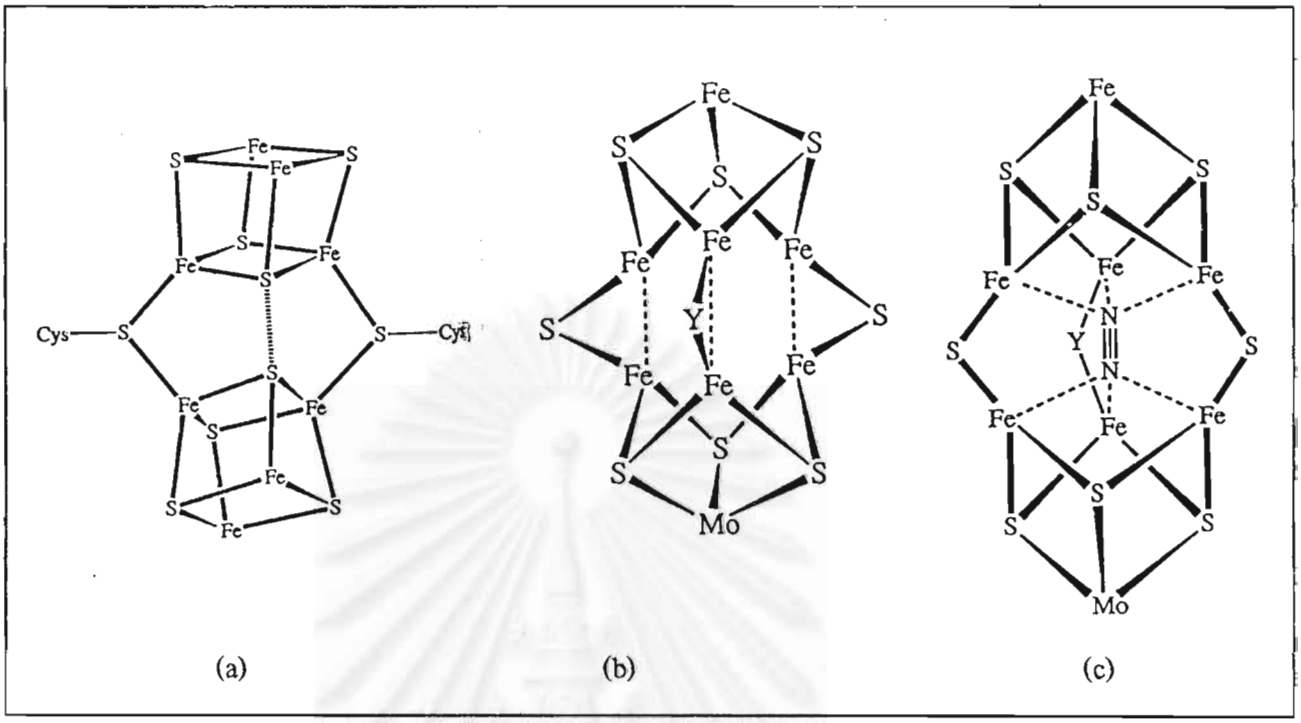


Figure 2.4 Structure of two types of metal clusters found in MoFe protein (a) the P-cluster. Two Fe_4S_3 clusters share a fourth S and bridged by two third ligand from the protein (αCys 85 and βCys 95) (b) The FeMo-cofactor consists of two MFe_3S_3 (M=Mo or Fe) clusters linked by two bridging sulfides and a third ligand, Y. (c) N_2 is suggested to bind in the center of the FeMo-cofactor(Chen *et al,* 1993; Garrett and Grisham, 1999).

The presence of the disulfide bond in the P-cluster suggests that this center can act as a two-electron redox group, involving cleavage and reformation of the disulfide bridge. This disulfide bond may provide a site for H_2 evolution by nitrogenase. Chen *et al.* (1993) suggested it was possible that protonation of the reduced P-cluster might generate H_2 upon disulfide bond formation and that this mechanism could contribute to the hydrogenase activity of nitrogenase.

The disulfide bridge also suggests a possible mechanism linking protein conformational changes to redox reactions at the P-cluster pair (Chen *et al.*,

1993). Since the P-cluster pair, which is at the αβ subunit interface in the MoFe protein, appears to interact with the Fe-protein, ATP hydrolysis by the Fe-protein could affect redox reactions at the P-cluster pair leading to conformational alterations. This postulated linkage between protein conformation and P-cluster pair oxidation state may help explain the requirement of ATP in the form of MgATP in the nitrogenase reaction as shown in Figure 2.5 (Dean *et al.*, 1993). Hydrolysis of one mole of ATP is coupled to the transfer of a pair of electrons.

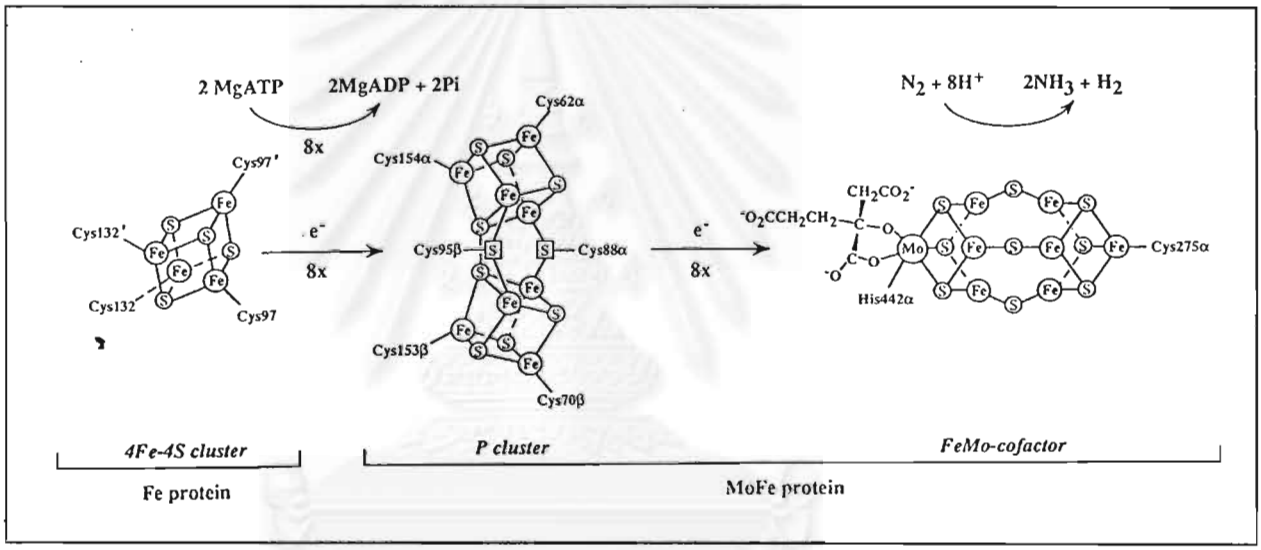


Figure 2.5 Organization and structures of the nitrogenase metalloclusters. Homocitrate is shown to provide two oxo ligands to the Mo atom. Arrows indicate the proposed electron path (Dean *et al.*, 1993)

FeMo-cofactor of the MoFe protein

Chen *et al.* (1993) also reported that the FeMo-cofactor (Figure 2.4(b)) contained three weak Fe-Fe bonds. N_2 could bind in the center of the FeMo-cofactor, thereby replacing the weak Fe-Fe bond as shown in Figure2.4(c)

In summary, the literature surveyed indicated the transfer of eight pairs of electrons in the nitrogenase-catalysed reduction of N_2 with the evolution of H_2 according to the following sequence (Garrett and Grisham, 1999) :

Fe protein → P-cluster → FeMo-cofactor → N_2 → NH_3+H_2

2.4 *B. japonicum* and hydrogen uptake hydrogenase

Friedrich and Schwartz (1993) reported that in 1931 Stephenson and Stickland discovered bacteria which could evolve H_2 and utilize it to reduce artificial and physiological substrates such as Methylene blue and NAD^+ respectively. Since then, [NiFe] containing membrane-bound hydrogen uptake hydrogenases, which catalyse the uptake of H_2 have been purified from a number of chemolithotrophic bacteria including *Rhizobium japonicum* (Arp, 1985). Hydrogen evolution is an inherent characteristic of the nitrogenase reaction and cannot be avoided (Simpson and Burris, 1984). Koch *et al.* (1967) showed that crude extract of *R. japonicum* bacteroids isolated from soybean plants (*Glycine max* cv. Merrit) catalysed the reduction of nitrogen gas under the atmosphere of Argon with the production of hydrogen. Wong *et al.*(1986) stated that most experts believed this loss of hydrogen is a wasteful process that reduces the efficiency of the nitrogen fixation process. As early as 1978 Maier *et al.* hypothesized that *B. japonicum* strains which expressed *hup* genes would be better nitrogen fixers. Professor H. J. Evans at the Nitrogen Fixation Research Unit, Oregon State University, USA, was the first US researcher who conducted research on the relationship between H_2 evolution and nitrogen fixing ability of *B. japonicum* in 1976-1987 (Schubert and Evans, 1976; Evans *et al.*, 1987). Another group of US researchers, actively engaging in research on the

relationship between the expression of *hup* genes and nitrogen fixing potential of *B. japonicum* as well as molecular genetics of *B. japonicum hup* genes, are Professor R. J. Maier and his colleagues who have been conducting research on this topics since 1978 (Maier *et al.*, 1978, Durmowicz and Maier, 1997)

In Japan, Professor K. Minamisawa and his colleagues also conduct experiments on the isolation and DNA fingerprints of hydrogen-uptake positive strains of *B. japonicum* (Minamisawa, 1990; Minamisawa *et al.*, 1999)

Keyser *et al.* (1984) reported the distribution of *R. japonicum* with hydrogenase phenotype in 12 states of the U.S. In Thailand, there have been no extensive reports on the distribution of *B. japonicum* with *hup*$^+$ phenotype. In 1995, Jeeraparn Taweesuksombat found that all the 41 *B. japonicum* isolates obtained from soybean nodules from Sukothai province did not exhibit *hup*$^+$ phenotype. Three isolates out of the 55 isolates (SSN10, SSN46, and SSN52) obtained from soil samples from Nakornnayok province showed *hup*$^+$ phenotype. The nitrogen fixing potential of the three isolates was of medium level. Jeeraparn Tweesuksombat's results did not indicate clear-cut positive correlation between *hup*$^+$ phenotype and nitrogen fixing potential of the isolates tested. In 1999, Supatra Triratrakul found that *B. japonicum* isolate Chaiyapoom 1 which showed *hup*$^-$ phenotype could fix nitrogen better than isolate Chaiyapoom 2 with *hup*$^+$ phenotype. Again, the result did not show that *B. japonicum* with *hup*$^+$ genotype are better nitrogen fixers.

It is notable that before 1981 several researchers provided data to support the hypothesis that *B. japonicum* which expressed *hup* genes were better nitrogen fixers. For example, Carter *et al.* (1978) reported that *B. japonicum*

strains 311b-143, 311b-6, 311b-142 and 311b-110 which took up hydrogen into cells were better nitrogen fixers under field conditions when compared with strains which did not recycle hydrogen. Albrecht *et al.* (1979) reported that *R. japonicum* strains USDA136, USDA110, USDA122, 311b-143, 311b-6 which exhibited hydrogen uptake hydrogenase activity were found to be better nitrogen fixers than those strains which did not exhibit the activity. Hanus *et al.* (1981) found *R. japonicum* inoculants with hydrogen uptake hydrogenase characteristic provided higher yields of soybean seeds with more nitrogen contents. The researchers suggested that *B. japonicum* with hydrogen uptake hydrogenase activity should be used as soybean seed inoculants. However, after 1981 several researchers are of the opinion that increase in soybean yield may depend on several factors other than the use of *B. japonicum* inoculants. For example, Van Soom *et al.* (1993) reported that soybean plant growth also depend on nutrient content in soils especially phosphorus contents. High phosphorus in soil would result in higher extent of nitrogen fixation leading to higher soybean yield. Genetic background of soybeans and their growth conditions such as daylight and light intensity also contribute to high soybean yield.

In summary, the literature surveyed indicated that at present, there is no consensus agreement on whether *B. japonicum* strains with hydrogen uptake hydrogenase activity are better nitrogen fixers or not. In fact, recent research work on hydrogen uptake activity seems to concentrate on genetic characterization of *hup* genes and their regulation. However, the general agreement seems to be that other agronomic factors as well as soybean genetic background may contribute to soybean yield as well. Nonetheless, there is still a

need to identify a rapidly detectable *B. japonicum* property which could be used to predict their nitrogen fixing ability without resorting to conducting the acetylene reduction test or the determination of soybean plant or nodule dry weight, both for soybean-*B. japonicum* symbiosis under neutral to slightly alkali soils and soybean-*B. japonicum* symbiosis under acidic soils.

2.5 Identification of *B. japonicum* by Random Amplification of Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) fingerprints

The traditional microbiological, morphological, and biochemical methods employed in the identification of authenticated *B. japonicum* are often time-consuming and relatively vague(for example + equals 90% or more of the strains positive, - equals 90% or more of the strains negative). Elkan and Bunn(1992) listed biochemical tests on growth of at least 68 carbon sources and 18 nitrogen sources for 17 strains of *Bradyrhizobium*. Therefore, attempts have been made to search for more rapid methods to identify authenticated *B. japonicum* strains. Judd *et al.*(1993) reported that RAPD-PCR fingerprints could differentiate even *B. japonicum* which had previously been found to belong to the same serogroup based on ELISA tests.

Although early species identification of *Bradyhizobium* spp relied mainly on the host plant that was nodulated, the trend in *B. japonicum* species identification at present time involves relatively more modern molecular techniques such as RAPD-PCR fingerprinting and percent homology of 16S rDNA sequences.

RAPD-PCR

deBruijn (1992) used primers REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) to identify and classify *E. coli, Rhizobium meliloti, R. leguminosarum, Azorhizobium caulinodans, Bradyrhizobium japonicum, Agrobacterium tumefaciens, Agrobacterium rhizogens and Pseudomonas spp.* The results suggested that REP- and ERIC-like sequences are highly conserved in rhizobia, agrobacteria and pseudomonads and that the REP and ERIC PCR method can be used to distinguish and classify closely-related *Rhizobium* strains. Judd *et al.*,1993 used REP-PCR and ERIC-PCR to differentiate very closely-related *Bradyrhizobium japonicum* serocluster 123 strains.

Harrison *et al.* (1992) found 25 nucleotide primer of 52 % GC to be effective in amplifying DNA from *Rhizobium leguminosarum* biovar *trifolii.*

Richardson *et al.* (1995) reported that the *nif*-directed RPO1 primer was shown to clearly differentiate a wide range of rhizobial and bradyrhizobial species including *R. meliloti, R. leguminosarum* bv *trifolii, R. leguminosarum* bv. *viciae* and *B. japonicum*(data not shown).

Mathis and McMillin(1996) found that RAPD-PCR with GC rich arbitrary primers, ARP-7: GTACGTGGCG and CRL-7 : GCCCGCCGCC, could detect genetic polymorphisms between each of the *B. japonicum* USDA110 variants. Both primers were high in GC content relative to other primers which failed to separate these closely-related variants. Mathis and McMillin(1996) suggested GC rich bacteria such as *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* required GC rich primers for RAPD-PCR.

From the literature surveyed it is noted that the effectiveness of primers in PCR fingerprinting of *R. leguminosarum* and *B. japonicum* seems to depend upon length and GC content of primers.

2.6 *B. japonicum* and acid tolerance

Acid shock proteins have not been investigated in *B. japonicum* but were investigated by Aarons and Graham(1991) for acid-tolerant *Rhizobium leguminosarum* bv *phaseoli* (UMR 1899). The bacterial strain was found to synthesize higher quantities of four proteins (55, 60, 82 and 145 kDa) and less quantities of other four proteins (43, 48, 50 and 200 kDa). Acid sensitive strain UMR5005(mutant of UMR 1899) was found to produce three proteins (45, 59 and 85 kDa) at pH 4.5 which were not produced by the parent strain at this pH, but fail to synthesize proteins of 36 and 43 kDa which were produced by UMR 1899. Acid sensitive strain UMR1632 increased the production of 65 kDa protein and decreased the synthesis of six proteins (32, 33, 43, 47, 49 and 64 kDa) at pH 4.5. Aarons and Graham(1991)'s studies did not shed any light on the identity of acid-shock proteins in *Rhizobium leguminosarum* bv *phaseoli* indicating that the study of acid shock proteins in rhizobia including *B. japonicum* is still in its infancy stage.

Buffer Solutions

It is well known that rhizobia and bradyrhizobia produce significant amounts of acidic and basic ions in solutions. These ions can affect pH of culture. In study by Glenn and Dilworth (1994) buffers were added into the medium to control the pH. Some of the buffers used to control pH of *Rhizobium* spp. or *Bradyrhizobium* spp. media are shown in Table 2.3.

Table 2.3 : Some of buffers used in *Rhizobium* spp. or *Bradyrhizobium* spp. growth media

Concentration (mM)	pH	Buffers	Rhizobia	References
30	4.0-5.4 5.5-6.5	Malic acid MES	*R. leguminosarum* bv. *trifolii*	Chen, Gartner, and Rolfe(1993)
10	4.5-6.5	NEDA Mellitic acid	*Bradyrhizobium* spp. and *R. tropici*	Graham et al. (1994)
20	5.5-6.0 6.5-7.0	MES PIPES	*Sinorhizobium meliloti*	Del Papa et al. (1999)
20	5.6-6.0 6.5-7.0	MES Bis-Tris	*R. meliloti*	O'Hara et al. (1989)
20	5.6 7.0	MES HEPES	*R. meliloti* WSM419	Clark, Dilworth and Glenn.(1993)
20	5.6 5.5-6.0 6.5-7.0 9.0-10.0	Either acetate MES Bis-tris CAPSO	*R. meliloti*	Goss et al. (1990)
100	5.5-6.0 6.4-6.8 7.3	MES PIPES MOPS	*R. meliloti* 104A14	Perez-Galdona and Kahn.(1994)

Table 2.3(continued)

Concentration (mM)	pH	Buffers	Rhizobia	References
40	< 6.0 6.1 6.8 7.5	Piperazine MES MOPS/PIPES TES	*R. leguminosarum* bv. *viciae*	Kannenberg and Brewin.(1989)
22	7.0	HEPES/MES	*R.. leguminosarum* bv. *phaseoli*	Aarons and Graham.(1991)

Data in Table 2.3 indicate that the following buffers were used to control pH in culture media for rhizobia.

pH 4.0-5.4	Malic acid, NEDA, Mellitic acid
5.5-6.0	MES, Piperazine, Either acetate
6.0-6.5	PIPES
6.5-7.0	HEPES, MOPS, Bis-Tris, TES, MOPS/PIPES
9.0-10.0	CAPSO

Costilow (1981) stated that suitable buffers for use in controlling pH in growth media should not be utilizable as nutrient sources for rhizobia. Therefore, malic acid, mellitic acid and ethylacetate should not be employed in the studies of acid tolerance in rhizobia.

Table 2.4 indicates pH range of some biological buffers. (Dean, 1999)

Table 2.4 : pH range of biological buffers

Buffers	Acronym	pka	pH range
2-(N-Morpholino)ethanesulfonic acid	MES	6.1	5.5-6.7
Bis(2-hydroxyethyliminotris(hydroxymethyl) methane	BIS-TRIS	6.5	6.1-7.5
Piperazine-N,N'-bis(2-ethanesulfonic acid)	PIPES	6.8	6.1-7.5
3-(N-Morpholino)propanesulfonic acid	MOPS	7.2	6.5-7.9
N-(2-Hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)	HEPES	7.5	6.8-8.2
N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	TES	7.5	6.8-8.2
3-[(1,1-Dimethyl-2-hydroxyethyl)-2-hydroxypropanesulfonic acid	AMPSO	9.0	8.3-9.7
3-(Cyclohexyamino)-2-hydroxy-1-propanesulfonic acid	CAPSO	9.6	8.9-10.3

Source : Dean, 1999

In this thesis, criteria for selecting buffers are as stated by Costilow (1981) and Fasman (1989) as follows :

1. pH of culture is in the range of pKa ±1 unit,
2. The buffers do not have inhibitory or toxic effects on *B. japonicum,*
3. *B. japonicum* do not utilize the buffers,
4. The buffers do not bind to di- and trivalent metal ions.

Using the above criteria, the following buffers were chosen for controlling *B. japonicum* media pHs:

pH 4.0-5.0 :cis-endo-bicyclo(2.2.1)hept-5-ene-2,3-dicarboxylicanhydride (NEDA)

6.0 :2-(N-Morpholino)ethanesulfonic acid(MES)

7.0-8.0 :N-(2-Hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)(HEPES)

9.0 :3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO)

General properties of some of the selected buffers are given in Appendix A.

2.7 Analysis of Variance

In order to find out if variations in plant dry weight and nodule dry weight are due to either the plant nutrient solution pH or the bradyrhizobial isolates or both, Analysis of Variance (ANOVA) was carried out. According to Somasegaran and Hoben (1994), the following terms are used in ANOVA :

Total Sum of Squares SS(T)	represents total variations
Treatment Sum of Squares SS(Tr)	represents variations due to bradyrhizobial isolates
pH Sum of Squares SS(pH)	represents variations due to plant nutrient solution pH
Error Sum of Squares SS(E)	represents variations due to experimental errors

Mean Squares (MS) for Treatment, pH, and Error are calculated by dividing the corresponding Sum of Squares with the corresponding degree of freedom.

Calculated F ratio represents $\frac{MS(Tr)}{MS(E)}$ or $\frac{MS(pH)}{MS(E)}$

The calculated F ratios are compared with the F ratios shown in the F distribution table at 95% level of confidence for the same degrees of freedom. A higher calculated F ratio than the tabulated F ratio indicates that the "source" contributes significantly to the variation observed.

Chapter 3

Materials and Methods

3.1 Soil collection sites

One sample collection site was within a factory located on Romklao Road, Meenburi District, Bangkok. Another sample collection site was outside the factory wall. The owner of the factory wished to convert the area within the factory compound into plantation areas. The aim of collecting soil samples here was to see if endogenous *B. japonicum* could be detected in the factory soils. The rest of the sample collection sites were several places in Kampaeng Pet, Petchaboon, Pijit and Nakorn Sawan provinces. Soil samples were placed in a total of 13 sterilized earthernware pots, 30 cm. in diameter.

3.2 Determination of soil pH

Determination of soil samples pHs from each pot were determined followed Peech (1965). Three core soil samples were obtained from each pot. A total of nine pH values for soil samples from each pot was obtained for the top, middle, and bottom parts of each of the three core samples. Ten grams of each soil sample were stirred in 20 ml of 0.01 M $CaCl_2.2H_2O$ in a 50 ml beaker for 30 minutes and left standing for additional 30 minutes. Then, pH was determined with pH meter (HANA)previously calibrated with 4.0, 7.0, and 10.0 buffer standards (Merck). pH value of soil in each pot was reported as the average of 9 replicate pHs.

3.3 Isolation and authentication of *B. japonicum* from soil samples

3.3.1 Seed surface-sterilization and germination

Soybean seeds cultivar SJ5 were surface-sterilized as described by Somasegaran and Hoben (1994). Seeds were placed in a wide-mouthed, sterilized Erlenmeyer flask. The mouth of the flask was covered with half of a sterilized Petri dish. The space the seeds took up was about 25% of the volume of the flask. The Petri dish cover was kept in place throughout the operation. The seeds were rinsed in 95% ethanol for 10 seconds to remove waxy material, after that the ethanol was drained off. Five percent hydrogen peroxide solution was added in sufficient volume to immerse the seeds completely. The content was swirled gently to bring the seeds and 5% hydrogen peroxide into contact. After 3-5 minutes, the liquid was drained off aseptically and the seeds were aseptically rinsed with six changes of sterililzed water. Aseptic procedures were observed throughout the rinsing. After the sixth rinse, the seeds were submerged in water, then left in the refrigerator for 4 hours for seed imbibition. After 4 hours, the seeds were rinsed again for 2 times with sterilized water and plated in 0.75%(w/v) water agar in Petri dishes. Approximately 20-50 seeds were placed per plate and were incubated at 25-30 °C.

3.3.2 Isolation and authentication of the isolates

Three germinating soybean seeds were placed into the soil in each of the 13 pots. Thinning to 1 plant per pot was carried out 7 days after planting. Remaining plants were allowed to grow for 28 days before collection of root

nodules for the isolation of root nodule bacteria using yeast extract mannitol agar (YMA) containing 25 μg ml^{-1} Congo Red. The composition of YMA was as described in Appendix A. Authentication of *B. japonicum* in Leonard jars was carried out as described by Somasegaran & Hoben (1994) employing the more drought-tolerant soybean cultivar SJ4. Stock cultures of the isolates were maintained on YMA slants at 4 °C.

3.4 PCR fingerprinting

3.4.1 Preparation of template DNA

One loop of activated *B. japonicum* was inoculated into 10 ml of YMB, pH 7.0 and incubated at 28 °C for 7 days. Cells were collected by centrifugation in a sterilized Eppendorf microcentrifuge(Model 5402) at 10,000 rpm for 15 minutes. Culture broth was discarded. Cells were treated with 60 μl saline-EDTA solution and 20 μl lysozyme(10 mg/ml) at 37 °C for one hour . The composition of saline-EDTA is described in Appendix B. The mixture was frozen and thawed for 4 times at 80 °C and –20 °C. One milliliter of DNAzol(Gibco) was added to the freeze-thawed mixture. Cell debris was separated by centrifugation at 10,000 rpm for 5 minutes. The supernatant was transferred to a new sterilized Eppendrof tube. Genomic DNA was precipitated by adding 0.5 ml cold absolute ethanol and centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded. Genomic DNA precipitate was washed with 0.6 ml of 70% ethanol. After drying the genomic DNA in oven at 50 °C, DNA was dissolved in 30 μl of high purity distilled water. The isolated DNA was kept at 4 °C until use.

3.4.2 PCR Amplification

RPO1 primer which corresponds to 20 nucleotide-conserved sequences in *nif HDK* promoter of *Rhizobium leguminosarum* bv. *trifolii* was used in PCR fingerprinting as described by Wilson (1995). The primer was synthesized by Gibco BRL Custom Primers, Life Technologies, Inc., USA, with the following sequence :

5' AATTTTCAAGCGTCGTGCCA 3' .

The PCR mixture was composed of :

15 µl 10x PCR buffer
5 µl 50 mM $MgCl_2$
3 µl 40 mM dNTPs, each10 mM
30 µl 10 µM primer RPO1
92 µl ddH_2O
1.5 µl 5U/µl *Taq* polymerase

The mixture was mixed by flicking with fingertip and briefly centrifuged to bring all solution to the bottom of the tube. The mixture was divided into six aliquots, each of 0.2 ml reaction tubes. One µl of genomic DNA of each isolated *B. japonicum* was added into each tubes. Negative control contained no genomic DNA.

Conditions for PCR on a Perkin-Elmer Thermal Cycler (Model 2400) were as follows :

95 °C 15 seconds
50 °C 30 seconds
72 °C 90 seconds
} 5 cycles

95 °C 15 seconds
55 °C 30 seconds } 25 cycles
72 °C 90 seconds

An additional 10-min incubation at 72 °C was carried out after the 25th cycle.

Products from the PCR were separated on 2.0% agarose gels and stained with ethidium bromide (Sambrook, Fritsch and Maniatis, 1989)

3.5 Growth on YMA containing bromthymol blue

Each isolate was streaked on YMA containing bromthymol blue (25 μg ml^{-1}). The plates were incubated at 25 °C for 5 days before examination. Yellow and blue zones surrounding colonies indicate the secretion of acidic and alkali products, respectively.

3.6 Growth characteristics of the isolates

One loop of each stock culture was reactivated by inoculating one loop on fresh YMA slant and incubating at 25 °C for 1 day for fast growers or 7 days for slow growers. One loop of the activated culture was inoculated into 50 ml of yeast extract mannitol broth in a 250 ml Klett flask. The culture was incubated at 28 ± 2 °C on a rotary shaker at 200 rpm for 2 days or 7 days for the fast growers or the slow growers, respectively. Growth was monitored by measuring the optical density at 660 nm at various time intervals. Culture was streaked on YMA containing congo red plates to check for contamination at the end of each experiment. Final pH was also determined for the cell-free culture at the end of

each experiment. Doubling times were calculated by standard methods (Pelczar *et al.*,1993)

3.7 Effects of initial pHs on growth of *B. japonicum*

One loop of activated *B. japonicum* was inoculated into 50 ml of YMB, pH 7.0. The seed culture was grown to mid-log phase before being used as an inoculum at 10% inoculum size for the YMB growth media with initial pHs from 4.00 to 9.00. Growth was monitored by absorbance readings at 660 nm.

3.8 Effects of pH on growth of *B. japonicum*

The experiments were conducted as described in No. **3.7** except growth media were prepared with the addition of appropriate buffers as indicated in Appendix B.

3.9 Effect of initial pHs on Hydrogen Uptake Hydrogenase activity

One loop of activated cells of each isolate was inoculated onto 5 ml of slant culture of hydrogen uptake medium (Maier et al., 1978) in 16 mm x150 mm test tubes and incubated at 30 $^{\circ}$C for 4 days. Cotton wool plugs were changed to sterilized serum stoppers. Composition of the medium was described in Appendix A. Each tube was flushed with nitrogen gas at 10 kg/cm^2 for 5 min, after which a gas mixture of 82 % N_2, 10% CO_2, 5%O_2, and 3%H_2 was flushed into each tube at 10 kg/cm^2 for 5 min. Control tubes contained no *B. japonicum* and was flushed with the gas mixture for 5 min. Four milliliters of gas sample were injected into a Gas chromatography Shimadzu 5890 series II with

Molecular Sieve 5A column, and a thermal conductivity detector. Oven temperature was 40 °C, Helium flow rate was 30 ml/min. All tubes containing *B. japonicum* were left standing to equilibrate for 30 min before 4 ml gas sample from each tube were injected into Gas chromatography. The tubes were incubated at room temperature for 3 days, at the end of which 4 ml of gas sample from each tube were injected into Gas chromatography. Cells were scrapped from agar slants, grounded for 5 min in a pastle and mortar with 50 µlof 50 mM Tris-HCl, pH 7.8. Soluble proteins were obtained after centrifugation at 10,000 rpm, 40 min. Final volumes of the supernate were noted. Protein content in the sample was determinated by Bradford method (Bradford, 1976) employing bovine serum albumin as the standard. The final volume of the supernate was used in the calculation of the total intracellular protein content. Using the criterion as stated by Cunningham et al (1986), *hup*$^+$ phenotype was detected when at least 30 % of hydrogen were lost and *hup*$^-$ phenotype was detected when hydrogen in each test tube was found to increase more than at least 40 %.

One Unit of hydrogen uptake hydrogenase activity was defined as one µmole of H_2 lost after the cut point loss of 30% under the experimental conditions.

3.10 Quantitation of cellular proteins

Aliquots of supernate from No. **3.9** were placed into clean test tubes. The volume in each tube was made up to 500 µl with distilled water. Five milliliters Coomassie brilliant blue solution were added to each tube and vortexed. The mixtures were left standing for 5 min at room temperature. The absorbance was

measured at 595 nm and protein concentration in the sample was determined from the standard curve using BSA as the standard.

3.11 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described by Laemmli (1970). Preparations of solutions for SDS-Polyacrylamide Gel Electrophoresis of proteins are as described in Appendix B.

3.11.1 Preparation of separating gel

The construction was composed of two clean glass plates and two 0.75mm spacers (Bio-Rad, model Protean II 16-cm unit). Glass-plate sandwich was locked to the casting stand.

Composition of 10% separating gel :

9.9 ml Acrylamide and N,N'-methylenebisacrylamide stock solution

5.9 ml Separating gel buffer (5x)

13.9 ml Distilled water

150 µl 10%Ammonium persulfate

The mixture was prepared in a 250-ml sidearm flask and degased under vacuum for 5 min. 30 µl TEMED was addd to the gel solution. The content was immediately transferred to the glass plate sandwich, using a Pasteur pipet. The top of the gel was covered with distilled water to prevent oxygen from inhibiting the polymerization reaction and to render smoothness to the uppermost part of the gel. Gel polymerized after 30 to 60 minutes at room temperature.

3.11.2 Preparation of stacking gel

The water was poured off layer and the gel was rinsed with water.

Composition of 4% stacking gel

1.2 ml Acrylamide and N,N'-methylenebisacrylamide stock solution

1.8 ml Stacking gel buffer (5x)

6.0 ml Distilled water 6.0 ml

30 µl 10%Ammonium persulfate

The mixture was prepared in a 250-ml sidearm flask and degased under vacuum for 5 min. 15 µl TEMED was added to the gel solution. The content was immediately transferred to the glass plate sandwich, using a Pasteur pipet. Teflon comb was inserted into the layer of stacking gel solution. The stacking gel polymerized after 30 to 45 minutes at room temperature.

3.11.3 Preparations of protein samples and loading onto gel

Each of the following four isolates of *B. japonicum* (S50, S58, S179, S204) was cultured in YMB at pH 7.0 until mid-log phase. Five milliliters of each seed culture was transferred to 45-ml of culture with initial pHs ranging from 4.0-9.0 with or without buffers. List of buffers used was given in Appendix B. The cultures were incubated at 28±2°C until mid-log phase was reached. Cells were collected by centrifugation at 10,000 rpm 15 min. Liquid nitrogen was added onto cells in a mortar with 1 g Aluminium oxide before grinding cells with mortar. Proteins were dissolved in 50-µl Tris-Cl buffer pH 7.8 and separated from Aluminium oxide and cell debris by centrifugation at 10,000 rpm, 40 minutes, 4 °C. Soluble protein content was measured by Bradford reagent as previously

described. Aliquots of the 50 μg-protein were diluted 1:1(v/v) with Sodium dodecyl sulfate(SDS) gel-loading buffer (2x) and heated 5 min at 95 °C in sealed microcentrifuge tubes. High range molecular-weight- protein standard(Bio-Rad) was dissolved in Sodium dodecyl sulfate(SDS) gel-loading buffer (2x) and heated for 5 min before loading onto gel.

The comb was removed and distilled water was used rinsed the wells. The lower buffer chamber was filled with 2,250-ml Tris-glycine electrophoresis buffer. The upper buffer chamber was filled with 375-ml Tris-glycine electrophoresis buffer so that the sample wells were filled with buffer. Protein samples were loaded into wells by carefully applying the sample as a thin layer at bottom of wells.

3.11.4 Running the gel and Silver staining

Power supply was connected to cell and run at 15 mA constant current per gel for a 0.75-mm thick slab gel, until the bromphenol blue entered the separating gel. The current was then increased to 20 mA per gel. The polyacrylamide gel was stained by Silver staining kit (Bio-Rad) according to supplier's instructions.

3.12 Statistical Analyses

Analysis of Variance (ANOVA) and linear correlation analysis for soybean plant and nodule dry weight were carried out with SPSS program, version 7.5. All experiments were conducted at least twice.

Chapter 4

Results

4.1 Isolation and characterization of isolates

Table 4.1 indicates that 56 root nodule bacterial isolates were obtained from various acidic soil samples with pH ranging from 4.19-6.89. Mean generation times for the fast- and the slow-growers as calculated from data shown in Figure 4.1 were found to be 3-4 hours and 35-45 hours, respectively. The fast-growers are coded S75, S171, S172, S173, S174 and S175. All isolates (except the fast growers) were found to produce alkali products on YMA containing bromthymol blue. The fast growers were found to produce acidic products. Colony morphology of all slow-growing isolates was found to be of two types as shown in Table 4.2.

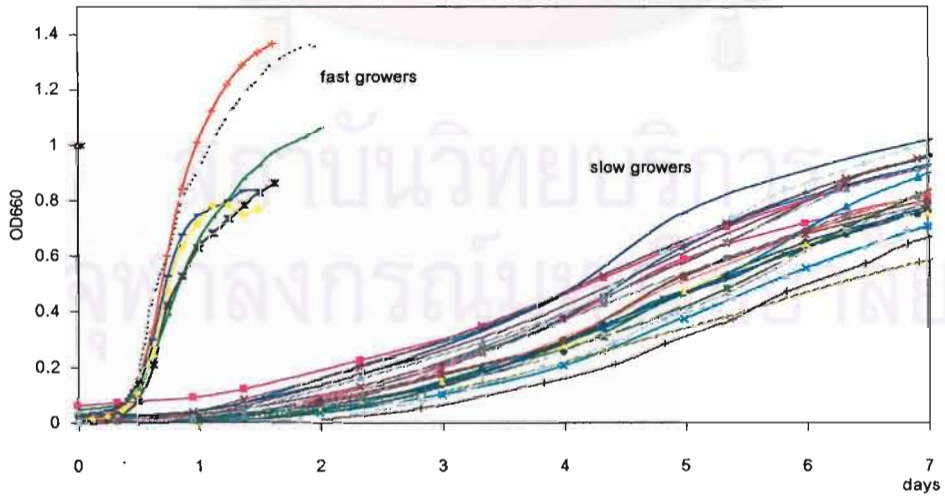


Figure 4.1. Growth curves of 56 root nodule bacterial isolates grown in 50 ml yeast extract mannitol medium at 28°C, 200 rpm

Table 4.1: Isolation of root nodule bacteria from acid soil samples from 5 provinces of Thailand. (Names of provinces are underlined)

Soil collection site	Soil pH in 0.01 M $CaCl_2$*	Isolate code #
<u>Bangkok</u> , Meenburi District		
Inside factory compound	4.28 } (4.78)	S18, S50
	4.57	S139
	5.12	S101, S151
	5.18	S75
Outside factory wall	5.44 } (5.58)	S5, S8
	5.45	S40, S74, S90
	5.66	S57, S58
	5.68	S30, S33, S42
<u>Kampaeng Pet</u>		
Muang district	5.42	S203, S204
Kanuworarakburi district	5.82	S201, S202
<u>Petchaboon</u>		
Tad Mork Falls, Muang district	4.28 } (4.87)	S183, S184, S179,S180,
	4.46	S181,S182, S186
	5.37	
Nuarn Mahatsajan, Kao Kaw district	5.28 } (5.25)	S76, S77, S78, S162,
	5.35	S171, S172, S173, S174, S175, S178, S187
Nuarn Mahatsajan, Muang district	6.82 } (6.86)	S161, S163, S185
	6.89	
<u>Pijit</u>		
Taparn Hin district	4.19	S195
Patale district	4.54	S196, S197, S198, S205, S206
<u>Nakorn Sawan</u>, Banpotpisai district		
Nong Ta Ngu sub-district	4.39	S188, S189, S190,S199
Bung Pla Too sub-district	5.31	S200
Ta seng sub-district	5.73	S191, S192, S193,
Ta seng sub-district	6.16	S194

*Parentheses indicate averages of pHs for soil from each site .

Table 4.1 shows that the six fast-growing isolates # S75, S171-S175 were from soil samples from the compound of a factory in Meenburi district, Bangkok, and from Nuarn Mahatsajan, Kao Kaw district, Petchaboon . The fast growers might be closely related to *Sinorhizobium fredii* which had been reported to nodulate soybean cultivars in People's Republic of China(Elkan and Bunn, 1992).Since this thesis concentrates on the slow-growers, *Bradyrhizobium japonicum*, all the six fast-growing isolates were deposited with Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand, under the culture collection codes TISTR1389-TISTR1394 for future work. So far, lyophilized culture of the six fast-growers have been sent to Dr. Brad L. Reuhs at the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, USA, who also works with *Sinorhizobium fredii* and *Sinorhizobium meliloti* (Reuhs et al., 1998) for ELISA tests using anti-sera against *S. fredii* strains which nodulate *Glycine max* cv. Williams. The ELISA tests were negative, indicating that the isolates were not genetically related to *S. fredii* strains which nodulate *Glycine max* cv. Williams. (Reuhs, pers. comm., Appendix D).

4.2 Authentication of *B. japonicum*

As part of an initial effort to characterize acid tolerance in *B. japonicum* and in soybean cultivars grown in Thailand, soybean plants in Leonard jars were supplied with Nitrogen-free medium of either pH 4.5 or 6.8.

Figures 4.2 (a) and 4.3 (a) showed dry weight of soybean plants cultivar SJ4 grown in Leonard jars with N-free medium pH 6.8 and 4.5, respectively. Figures 4.2(b) and 4.3(b) showed dry weight of soybean cultivar SJ4 root nodules

Plant dry weight(g)

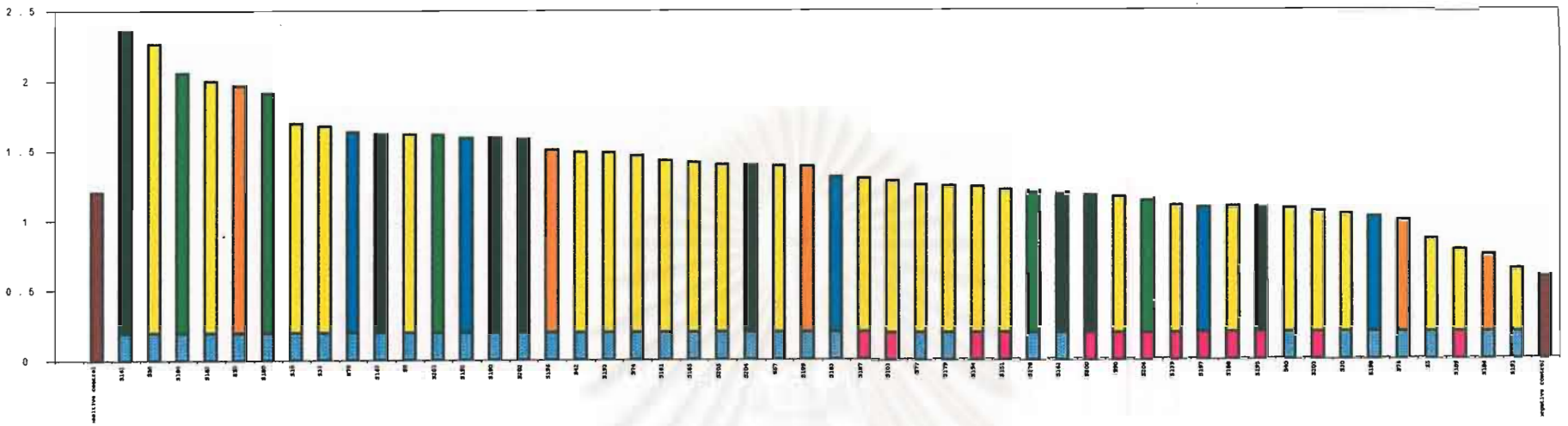


Figure 4.2(a) Dry weight of soybean plants cultivar SJ4 grown in Leonard jars with N-free medium, pH 6.8, for 28 days isolate

Nodule dry weight(g)

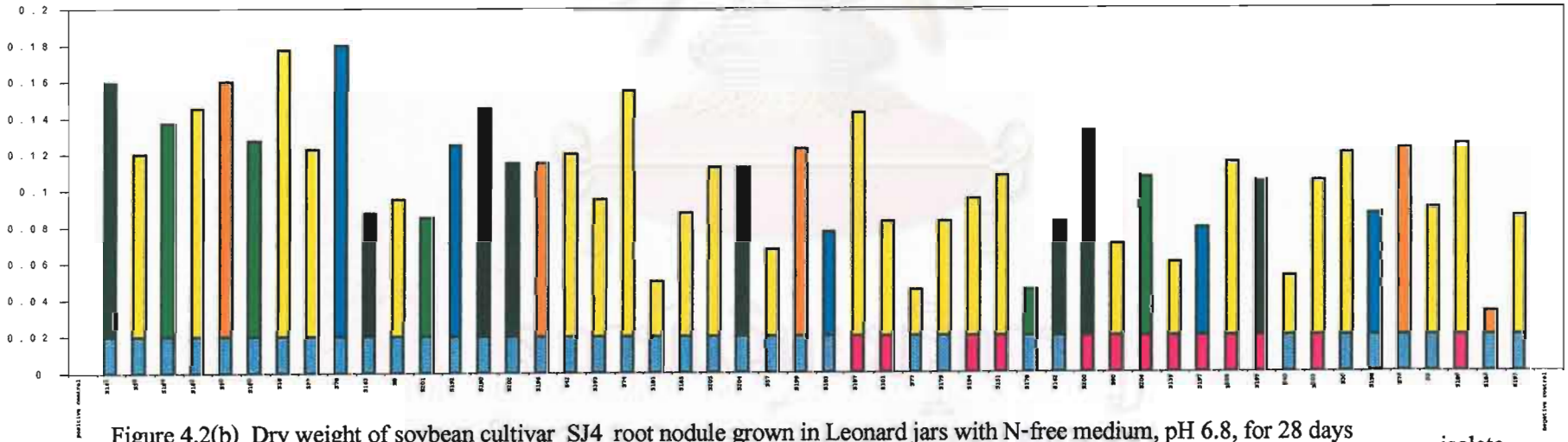


Figure 4.2(b) Dry weight of soybean cultivar SJ4 root nodule grown in Leonard jars with N-free medium, pH 6.8, for 28 days isolate

PCR pattern#1 black #2 green #3 yellow #4orange #5 blue

Colony type I purple typeII pink

Plant dry weight(g)

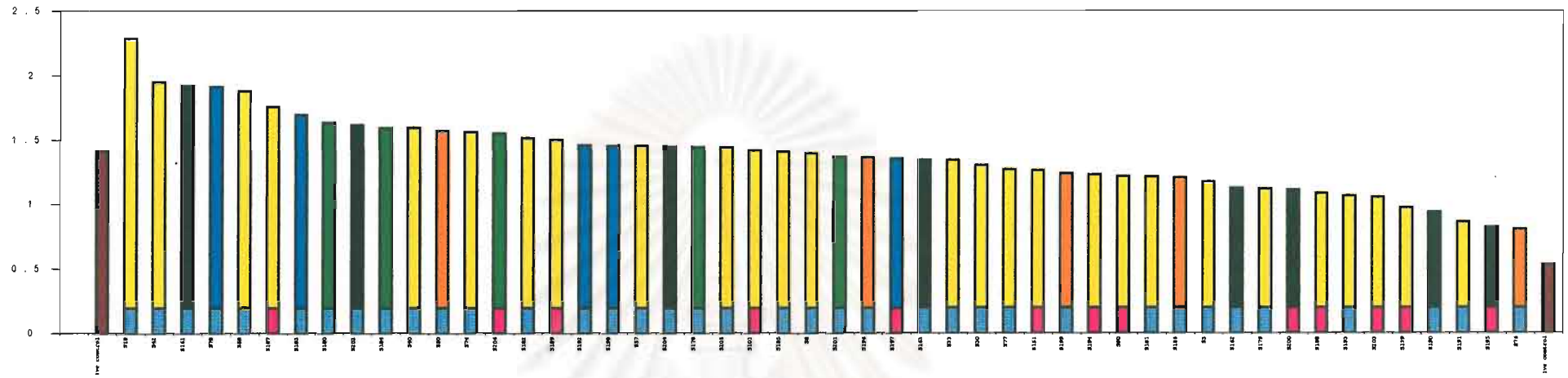


Figure 4.3(a) Dry weight of soybean plants cultivar SJ4 grown in Leonard jars with N-free medium, pH 4.5, for 28 days

isolate

Nodule dry weight(g)

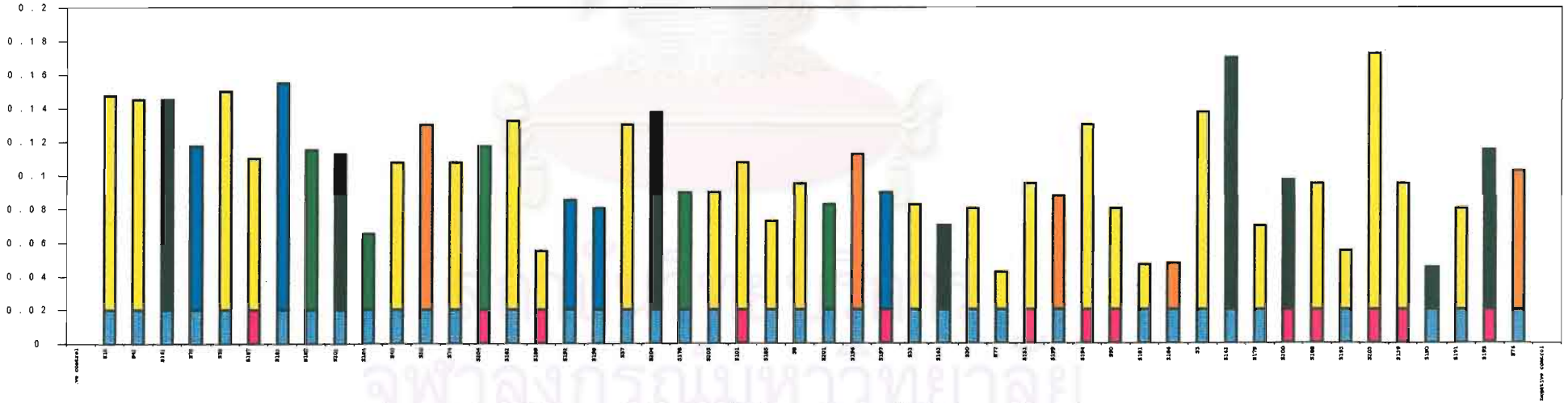


Figure 4.3(b) Dry weight of soybean cultivar SJ4 root nodule grown in Leonard jars with N-free medium, pH 4.5, for 28 days

isolate

PCR pattern#1 black #2 green #3 yellow #4orange #5 blue

Colony type I purple typeII pink

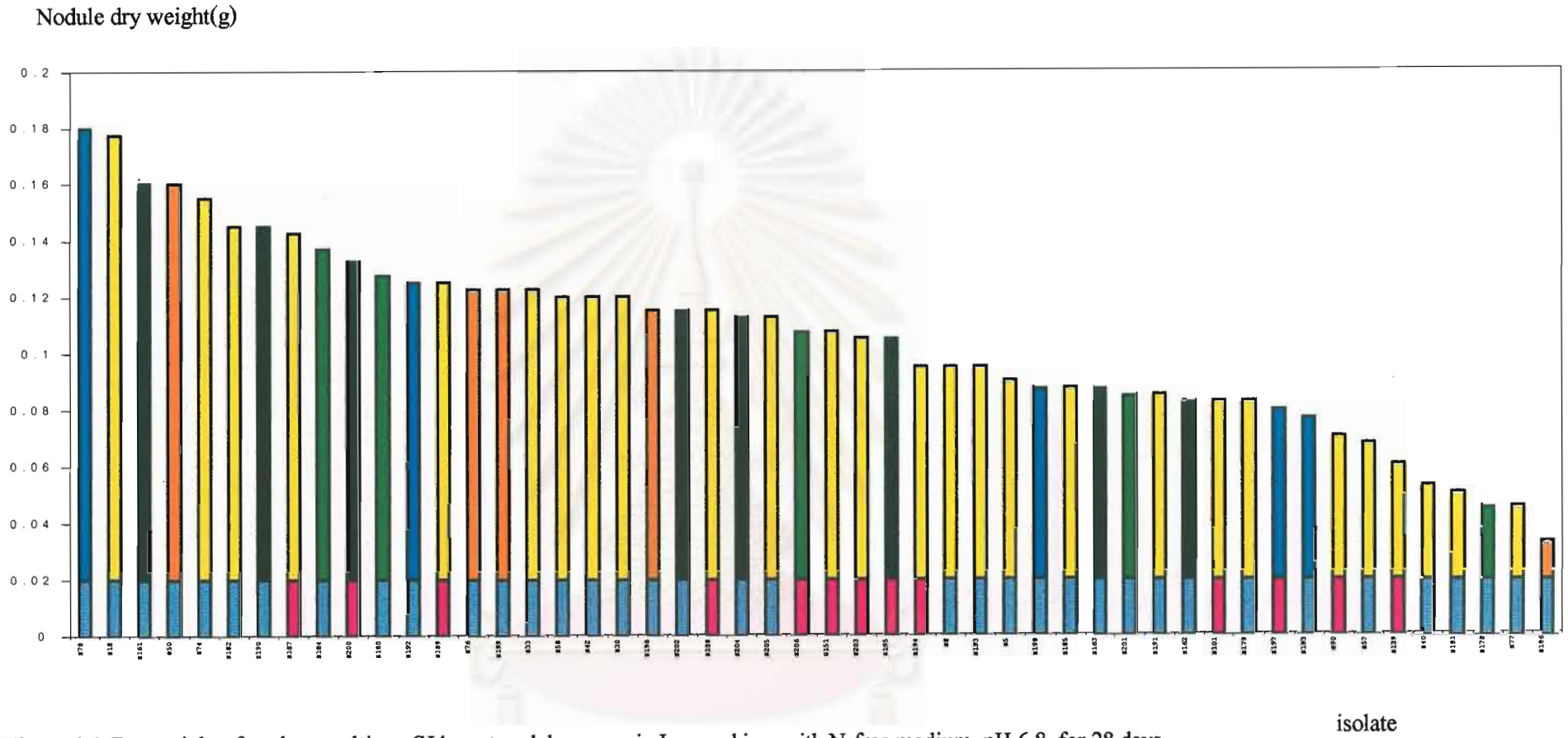


Figure 4.4 Dry weight of soybean cultivar SJ4 root nodule grown in Leonard jars with N-free medium, pH 6.8, for 28 days

PCR pattern#1 black #2 green #3 yellow #4orange #5 blue

Colony type I purple typeII pink

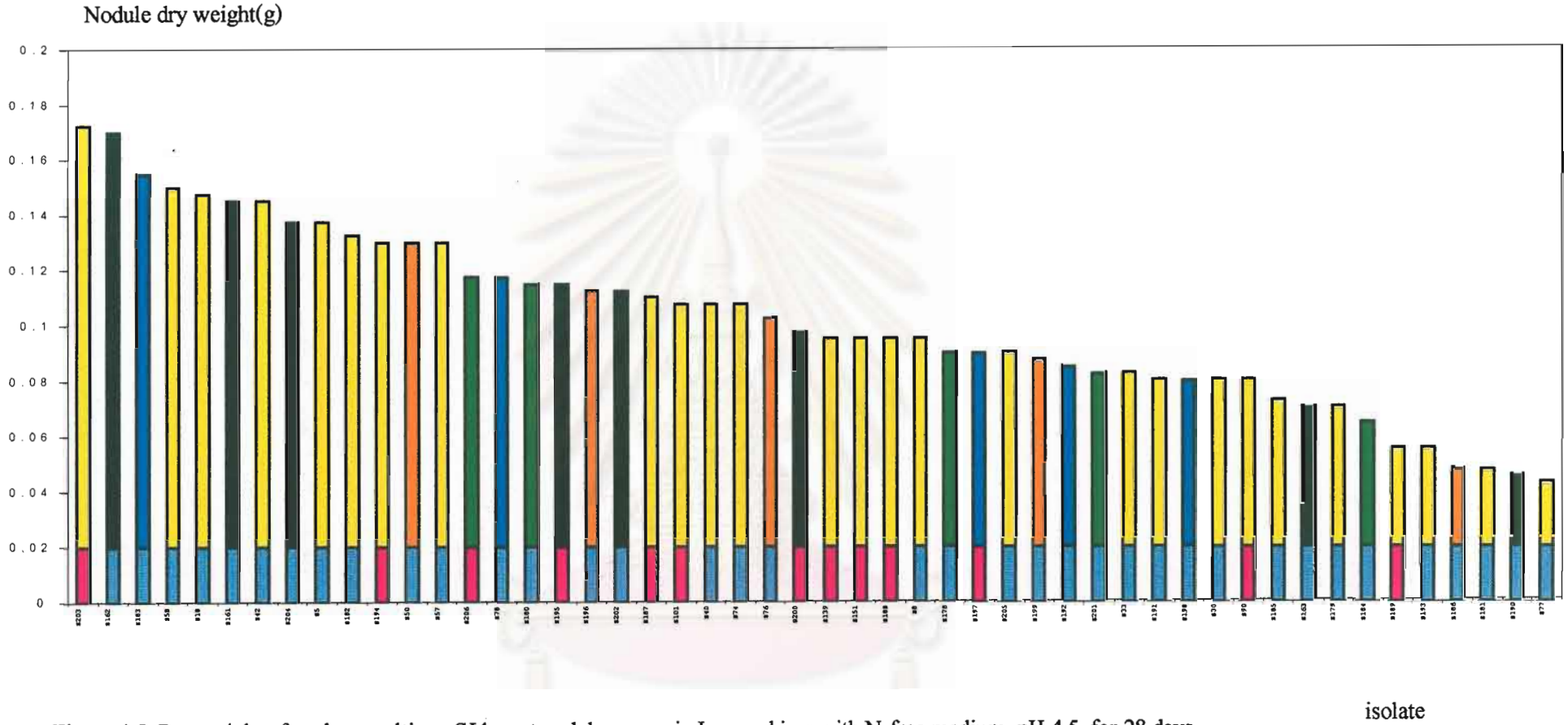


Figure 4.5 Dry weight of soybean cultivar SJ4 root nodule grown in Leonard jars with N-free medium, pH 4.5, for 28 days

PCR pattern#1 black #2 green #3 yellow #4orange #5 blue

Colony type I purple typeII pink

from Leonard jar- grown plants with N-free medium pH 6.8 and 4.5, respectively. Dry weight of root nodules was ranked from high to low values in Figures 4.4 and 4.5.

The results as shown in Figures 4.2(b) and 4.3(b) indicated that high nodule dry weight did not necessarily yield high plant dry weight. One reason was because high nodule dry weight might belong to ineffective nodules. Only effective, pink nodule with leghemoglobin would lead to nitrogen fixation (Hopkins, 1999) Figures 4.2(b), 4.3(b), 4.4 and 4.5 indicated that all the 50 isolated slow-growers were *B. japonicum* which nodulated soybean (*Glycine max* cv. SJ4).

The results shown in Figures 4.2 and 4.3 indicated that the following *B. japonicum* isolates might contribute better growth of soybean cultivar SJ4 when compared with the positive control at different pH of N-free nutrient solutions.

pH 6.8 : isolates # S161, S58, S184, S182, S50, S180

pH 4.5 : isolates # S18, S42, S161, S78, S58

The results indicated 4 situations:

Situation I : Those with good growth and high nodule dry weight when N-free nutrient solutions pH 6.8 and 4.5 were used.

B. japonicum isolates #S58 and #S161 consistently yielded good plant growth even though pH of the N-free nutrient solutions differed. Relatively high nodule dry weight was obtained when isolates # S58 and S161 were used. High nodule dry weight might result in the observed high plant dry weight when isolates #S58 and S161 were used to inoculate soybean plants.

Situation II : Those with good growth and high nodule dry weight when pH 6.8 N-free nutrient solution was used. Relatively poor growth when pH 4.5

N-free nutrient solution was used, probably due to less nodule dry weight. This situation was exemplified by isolate #184.

Situation III : Those with moderately good growth and relatively high nodule dry weight when both pH 6.8 and pH 4.5 N-free nutrient solutions were used. These isolates were also of interest due to their ability to yield moderately good growth and nodule formation under pH 6.8 and 4.5. This situation was observed when isolates #S182, S50, and S180 were used to inoculate soybean plants.

Situation IV : Those with good growth and relatively high nodule dry weight when pH 4.5 N-free nutrient solution was used. Moderately good growth but high nodule dry weight when pH 6.8 N-free nutrient solution was used. This situation was observed for isolates #S18 and S78 and to a lesser extent, isolate # S42.

All the observed results did not seem to clearly indicate if pHs of the N-free nutrient solutions contributed significantly to the variations observed for the plant and the nodule dry weight. Therefore , plant dry weight and nodule dry weight obtained from all the experiments were analysed by Analysis of variance (ANOVA) with either the isolates or the pH of the N-free nutrient solutions as sources of variations observed for the plant and the nodule dry weight. The results indicated in Table 4.2 and 4.3 showed that pH of N-free media did not significantly affect the plant and the nodule dry weight. However, isolates used in soybean inoculation were found to contribute significantly to variations observed in the plant and the nodule dry weight.

Table 4.2 Analysis of variance for plant dry weight

Sources of Variation	Sum of Squares	Degree of Freedom	Mean Squares	F-ratio (Calculated)	F-ratio (Tabulated,95%)
Treatment	10.144	51	0.199	4.047	1.60
pH	0.004	1	0.004	0.077	4.03
Experimental Error	2.507	51	0.049		

Table 4.3 Analysis of variance for nodule dry weight

Sources of Variation	Sum of Squares	Degree of Freedom	Mean Squares	F-ratio (Calculated)	F-ratio (Tabulated,95%)
Treatment	0.07229	49	0.1475	1.917	1.60
pH	0.00035	1	0.00035	0.454	4.03
Experimental Error	0.03771	49	0.00077		

Since pH of the N-free nutrient solution was found to have no significant effects on the plant and the nodule dry weight, all data obtained for dry weight were used in conducting linear correlation analysis between the plant dry weight and the nodule dry weight. Figure 4.6 indicated that the plant dry weight was

slightly correlated with the nodule dry weight with a linear correlation coefficient $r^2 = 0.2122$

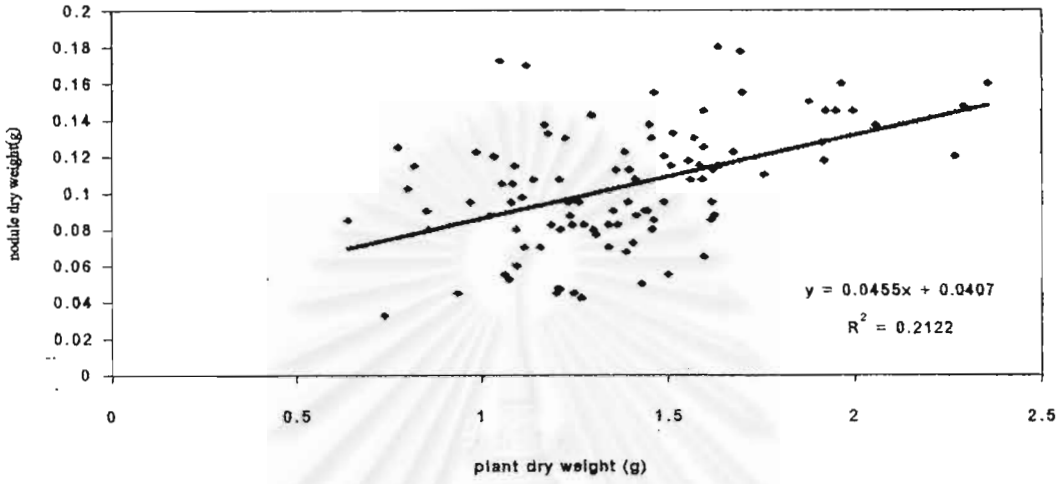


Figure 4.6 Linear correlation analysis between plant dry weight and nodule dry weight

From the results obtained for the plant dry weight and the nodule dry weight (Figures 4.2 –4.5) the acid-tolerant isolates of interest might be # S18 , S42 , S161, S78 and S58 due to their contribution to relatively good soybean growth when compared with plant dry weight for the positive controls. Isolates # S161 and S58 are especially interesting since they were found to yield good growth and nodule formation when pH 4.5 and 6.8 N-free nutrient solutions were used for plant growth. Therefore, these two isolates were initially selected for further experiments on the effects of initial pH on hydrogen uptake hydrogenase activity and protein profiles.

4.3 Characterization of *B. japonicum* isolates

B. japonicum isolates were characterized according to types of colony morphology and RAPD-PCR fingerprints as follows :

4.3.1 Types of colony morphology

Table 4.4 showed groupings of slow-growing *B. japonicum* isolated from acidic soil samples based on two types of colony morphology:

Type I : pinkish-white, opaque, pearl-like with round and smooth margin, dome-shaped, less than 1 mm in diameter. (Figure 4.7)

Type II : light pink, watery-translucent, mucus, flat shape, more than 1 mm in diameter, sometimes with opaque area in the middle. (Figure 4.7)

(A) (B)

Figure 4.7 : Two types of colony morphology of *B. japonicum* isolates.
(A)Type I colony morphology (B) Type II colony morphology. See text for description of colony morphology.

Table 4.4 : Colony morphology of 50 *B. japonicum* isolates from acidic soils

Soil collection site	Isolate number : Type of colony morphology	
	Type1	Type 2
Bangkok ,Meenburi district		
1. Inside factory compound	S18,S50	S101,S139,S151
2. Outside factory wall	S5,S8,S30,S33,S40,S42,S57,S58,S74	S90
Kampaeng Pet		
3. Muang district	S204	S203
4. Kanuworarakburi district	S201,S202	-
Phetchaboon		-
5. Tad Mork Falls, Muang district	S179,S180,S181,S182,S183,S184,S186	
6. Nuarn Mahatsajan, Kao Kaw district	S76,S77,S78,S162,S178	S187
7. Nuarn Mahatsajan, Muang district	S161,S163,S185	-
Pijit	-	S195
8.Taparn Hin district		
9.Patale district	S196,S198,S205	S197,S206
Nakorn Sawan, Banpotpisai district		
10. Nong Ta Ngu sub-district	S190	S188,S189
11. Bung Pla Too sub-district	S199	-
12. Ta seng sub-district	-	S200
13. Ta seng sub-district	S191,S192,S193	S194
Total	37	13

4.3.2 RAPD-PCR fingerprints of *B. japonicum* isolates

Figure 4.8 shows PCR fingerprints of isolates S163, S196, S197, S206, S190, S180, S78, S178, S187, S183, and S184. The results revealed 5 patterns of PCR fingerprints as follows :

Pattern 1 : Two PCR products, 300 and 400 bp (isolates # S163, and S190)

Pattern 2 : Three PCR products, 300, 400 and a faint > 2,000 bp(isolates # S178, S180, S184 and S206)

Pattern 3 : Three PCR products, 300, 400 and 850 bp (isolate # S187)

Pattern 4 : Four PCR products, 300, 400, 850 and a faint >2,000 bp (isolate # S196)

Pattern 5 : Five PCR products 300, 400, 700, 850 and a faint >2,000 bp (isolates # S78, S183 and S197)

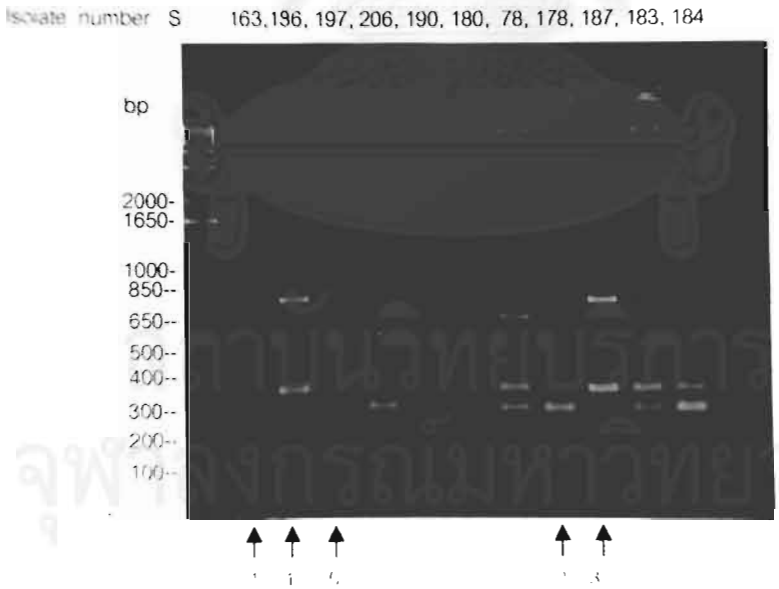


Figure 4.8 : lane 1: molecular weight markers; lanes 2-12 indicate PCR fingerprints of isolates # S163, S196, S 197, S206, S190, S180, S78, S178, S187, S183, and S184 , respectively. Lane 13 is negative control. Numbers 1-5 indicate RAPD-PCR patterns 1-5, respectively.

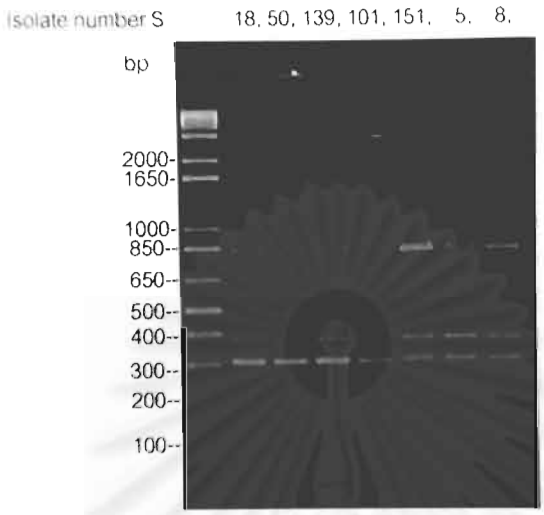


Figure 4.9 : lane 1: molecular weight markers; lanes 2-8 indicate PCR fingerprints of isolates # S18, S50, S 139, S101, S151, S5, S8, respectively.

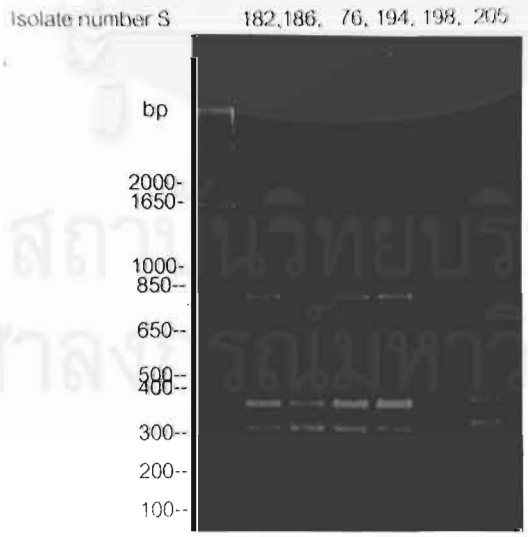


Figure 4.10 : lane 1: molecular weight markers; lanes 2-7 indicate PCR fingerprints of isolates # S182, S186, S 76, S194, S198, S205, respectively.

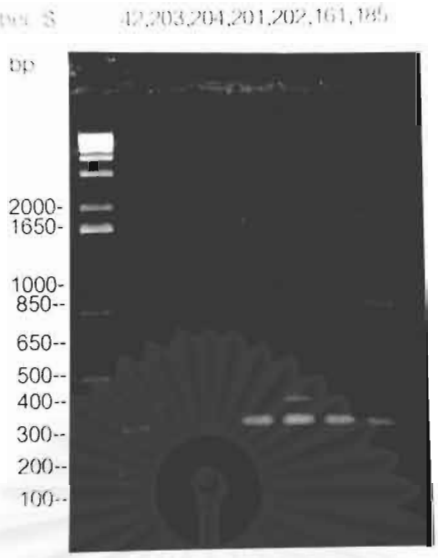


Figure 4.11 : lane 1: molecular weight markers; lanes 2-8 indicate PCR fingerprints of isolates # S42, S203, S204, S201, S202, S161, S185, respectively.

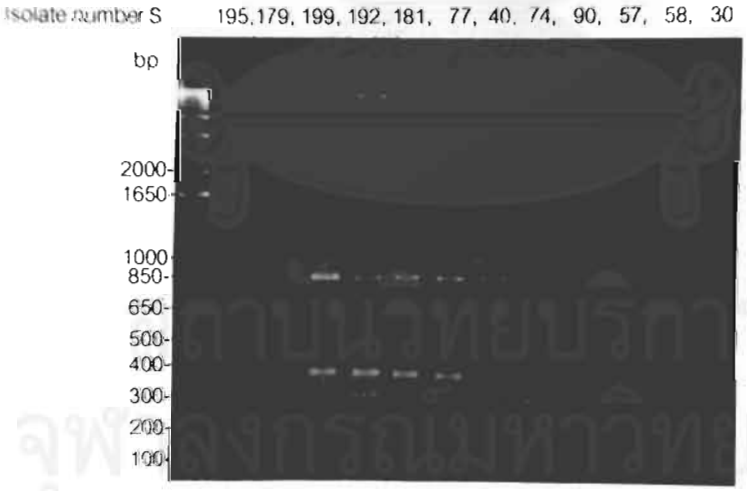


Figure 4.12 : lane 1: molecular weight markers; lanes 2-13 indicate PCR fingerprints of isolates # S195, S179, S199, S192, S181, S77, S40, S74, S90, S57, S58 and S30, respectively.

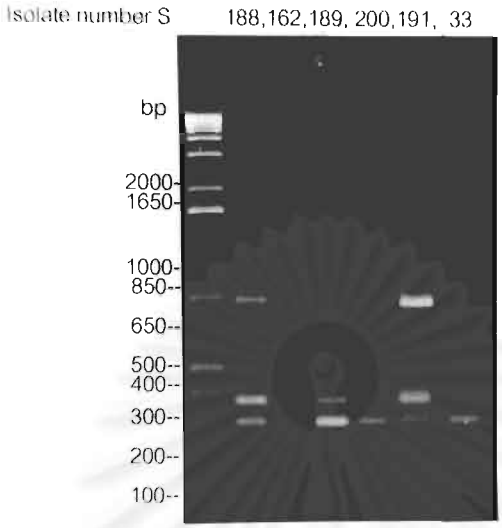


Figure 4.13 : lane 1: molecular weight markers; lanes 2-7 indicate PCR fingerprints of isolates # S188, S162, S189, S200, S191, and S33, respectively.

Patterns of PCR fingerprints of 50 *B. japonicum* isolates as shown in Figures 4.8 –4.13 were summarized in Tables 4.5 and 4.6.

Table 4.7 showed distribution of isolated *B. japonicum* based on colony morphology and PCR fingerprint patterns.

Table 4.5 : Grouping of 50 isolated *B. japonicum* based on different patterns of PCR fingerprints

Pattern	PCR products (bp)	Isolate number	Total
1	300, 400	S161,S162,S163,S190,S195, S200,S202,S204	8
2	300,400,>2,000	S178,S180,S184, S201,S206	5
3	300,400, 850	S5,S8,S18,S30,S33,S40,S42, S57,S58,S74,S77,S90,S101,S139, S151,S179,S181,S182,S185, S187,S188,S189,S191,S193, S194,S203,S205	27
4	300,400,850,>2,000	S50,S76, S186,S196,S199	5
5	300,400,700,850,>2,000	S78,S183,S192,S197,S198	5
		Total	50

Table 4.6 : Distribution of various *B. japonicum* according to patterns of PCR fingerprints

Soil collection site	Number of isolates :PCR fingerprint pattern				
	1	2	3	4	5
Bangkok ,Meenburi District					
1. Inside factory compound	-	-	4	1	-
2. Outside factory wall	-	-	10	-	-
Kampaeng Pet					
3. Muang district	1	-	1	-	-
4. Kanuworarakburi district	1	1	-	-	-
Petchaboon					
5. Tad Mork Falls, Muang district	-	2	3	1	1
6. Nuarn Mahatsajan, Kao Kaw district	1	1	2	1	1
7. Nuarn Mahatsajan, Muang district	2	-	1	-	-
Pijit					
8.Taparn Hin district	1	-	-	-	-
9.Patale district	-	1	1	1	2
Nakorn Sawan, Banpotpisai district	1	-	2	-	-
10. Nong Ta Ngu sub-district					
11. Bung Pla Too sub-district	-	-	-	1	-
12. Ta seng sub-district	1	-	-	-	-
13. Ta seng sub-district	-	-	3	-	1
Total	8	5	27	5	5

Table 4.7 : Colony morphology and PCR fingerprint patterns of 50 *B. japonicum* isolates.

Isolate		Colony Type I					Colony Type II				
		Patterns of PCR fingerprints					Patterns of PCR fingerprints				
		1	2	3	4	5	1	2	3	4	5
Bangkok, Meenburi district											
1. Inside factory compound	S18			✓							
	S50				✓						
	S101								✓		
	S139								✓		
	S151								✓		
2. Outside factory wall	S5			✓							
	S8			✓							
	S30			✓							
	S33			✓							
	S40			✓							
	S42			✓							
	S57			✓							
	S58			✓							
	S74			✓							
	S90								✓		
Kampaeng Pet											
3. Muang district	S203								✓		
	S204	✓									
4. Kanuworarakburi district	S201		✓								
	S202	✓									
Phetchaboon											
5. Tad Mork Falls, Muang district	S179			✓							
	S180		✓								
	S181			✓							
	S182			✓							

	S183					✓				
	S184		✓							
	S186				✓					
6. Nuarn Mahatsajan, Kao Kaw district	S76				✓					
	S77			✓						
	S78					✓				
	S162	✓								
	S178		✓							
	S187							✓		
7. Nuarn Mahatsaja, Muang district	S161			✓						
	S163	✓								
	S185	✓								
Pijit										
8.Taparn Hin district	S195						✓			
9.Patale district	S196				✓					
	S197									✓
	S198					✓				
	S205			✓						
	S206							✓		
Nakorn Sawan, Banpotpisai district								✓		
10. Nong Ta Ngu sub-district	S188									
	S189							✓		
	S190	✓								
11. Bung Pla Too sub-district	S199				✓					
12. Ta seng sub-district	S200						✓			
13. Ta seng sub-district	S191			✓						
	S192					✓				
	S193			✓						
	S194								✓	

Bangkok

Meenburi district (inside factory compound and outside factory wall)

Table 4.7 indicated that of the 15 isolates from the Meenburi soil samples, eleven isolates were found to have Type 1 colony morphology and 4 isolates were found to have Type 2 colony morphology. Of the type 1 colony morphology, ten isolates were found to have pattern 3 PCR fingerprint and 1 isolate with pattern 4 PCR fingerprint. Of the type 2 colony morphology, all the four isolates were of pattern 3 PCR fingerprint. It is likely that isolates # S18, S5, S8, S30, S33, S40, S42, S57, S58, S74 are the same strain and isolates # S101, S139, and S151 constitute another strain while isolates # S50 and S90 are two different strains.

Kampaeng Pet

Muang district and Kanuworarakburi district

Table 4.7 revealed that all the four isolates from Kampaeng Pet soil samples were diverse. However, isolates # S202 and S204 may be the same strain. Isolate #S203 may be the same strain as isolates # S90, S101, S139, and S151 isolated from soil samples from Meenburi district.

Petchaboon

Tad Mork Falls, Muang district

Table 4.7 showed that isolates # S180, and S184 may be the same strain as isolate # S201 isolated from Kampaeng Pet. Isolates # S179, S181, S182 may be the same strain as isolates # S18, S5, S8, S30, S33, S40, S42, S57, S58, and S74 isolated from soils from Meenburi district. Isolate # S186 has the same PCR fingerprint pattern as isolate # S50 (Meenburi district). Isolate # S183 may constitute a different strain.

Nuarn Mahatsajan, Kao Kaw district

Table 4.7 showed that all the 6 isolates from Nuarn Mahatsajan soils, Kao Kaw district, may be of different strains of *B. japonicum*. However, all the 6 isolates may be the same strains as previously described above.

Nuarn Mahatsajan, Muang district

Table 4.7 showed that all the three isolates # S161, S163, S185 have Type I colony morphology and that the isolates may be related to those previous isolates with PCR fingerprint patterns 1 and 3.

Pijit

Taparn Hin district

Table 4.7 indicated that the only isolate # S195 from soil samples from this area was of Type II colony morphology with PCR fingerprint pattern 1 which had not previously been found.

Patale district

Table 4.7 revealed that soil samples from Patale district, Pijit province, also contained different strains of *B. japonicum*, isolates #S196, S197, S198, S205, and S206. Previously, soil samples from Nuarn Mahatsajan, Kao Kaw district, Petchaboon province, were found to contain different varieties of *B. japonicum*.

Nakorn Sawan

Banpotpisai district

Table 4.7 showed that *B. japonicum* isolated from various acidic soil samples from 3 sub-districts of Banpotpisai district, Nakorn Sawan province, exhibited both types of colony morphology with PCR fingerprint patterns 1, 3, 4, 5 for isolates with colony morphology type I and patterns 1, 2, and 3 for isolates

with colony morphology type II. These isolates may be the same strains as the ones isolated from other soil samples as previously described.

For Type I colony morphology :

The results shown in Table 4.7 indicated that soil samples from Nuarn Mahatsajan, Kao Kaw district, Petchaboon province contained all of the variety of *B. japonicum* detected in this study which consisted of isolates with PCR fingerprint patterns 1 to 5.

For Type II colony morphology :

Isolates from Nuarn Mahatsajan, Kao Kaw district and Pijit province with Type II colony morphology were also found to consist of the isolates with PCR fingerprint patterns 1, 2, 3 and 5. No isolates with PCR fingerprint pattern 4 were found.

In summary, the results shown in Table 4.7 indicated a relatively rich variety of *B. japonicum* from Nuarn Mahatsajan , Kao Kaw district, Petchaboon province, and from Taparn Hin district and Potale district, Pijit province.

4.4 Selection of distinct *B. japonicum* strains

As previously stated in Section 4.2, isolates # S58 and # S161 had been selected for further studies. Results of colony morphology and PCR fingerprints as summarized in Table 4.7 indicated that isolates # S161 and the following isolates may be the same strain as isolates # S58 with colony morphology type I and PCR fingerprint pattern 3 : S18, S5, S8, S30, S33, S40, S42, S57, S74, S179, S181, S182, S77, S161, S205, S191, S193

The above finding indicated , unfortunately, that isolates # S18 and S42 which yielded good plant growth and nodule formation under acidic pH 4.5 might be the same strain as # S58 and # S161 as shown above.

Results shown in Table 4.7 indicated that there were no isolates with colony morphology type II and PCR fingerprint pattern 4. Isolate # S197 should be different from the rest of the isolates. However, since isolate # S 197 has type II colony morphology which was associated with relatively less-able nitrogen fixers as indicated by the plant and the nodule dry weight shown in Figures 4.2-4.5. Based on colony morphology and PCR fingerprint patterns, the following isolates were selected for further research on the effects of initial pH on hydrogen uptake hydrogenase activity and protein profiles:

Isolates*	**Colony morphology**	**PCR fingerprint pattern**	**Soil pH**	**Collection site**
S161	I	1	6.82	Nuarn Mahatsajan, Muang district, Petchaboon
S202	I	1	5.82	Kanuworarakburi district, Kampaeng Pet
S204	I	1	5.42	Muang district, Kampaeng Pet
S58	I	3	5.66	Outside a factory, Meenburi District
S179	I	3	4.28	Tad Mork Falls, Muang district, Petchaboon
S50	I	4	4.28	Inside a factory, Meenburi District
S196	I	4	4.54	Patale district, Pijit
S199	I	4	4.39	Nong Ta Ngu sub-district, Nakorn Sawan
S192	I	5	5.73	Ta seng sub-district, Nakorn Sawan

*Isolates with the same colony morphology and PCR fingerprint pattern were selected for further experiments on growth characteristics to find out if they were the same strains.

4.5 Effects of initial pHs on growth

Figures 4.14 and 4.15 showed growth curves of the nine selected *B. japonicum* isolates when grown in unbuffered and buffered Yeast Extract Mannitol broth respectively.

Tables 4.8 and 4.9 showed initial and final pHs of the cell-free unbuffered and buffered culture media at the end of the experiments.

Table 4.8 : Initial and final pHs of unbuffered YMB at the end of the growth curve experiments for nine acid-tolerant *B. japonicum* isolates

		Initial pH					
	Isolates	4.0	5.0	6.0	7.0	8.0	9.0
Final pH	S58	4.8	4.9	6.1	7.0	7.2	7.3
	S192	5.2	5..5	6.2	6.9	7.3	7.3
	S161	4.9	54	6.1	6.8	7.1	7.2
	S179	4.5	5.3	5.9	6.7	7.0	7.2
	S196	4.5	5.2	6.6	6.6	7.1	7.2
	S202	4.3	5.2	5.9	6.8	7.0	7.1
	S199	4.7	5.5	6.1	6.9	7.2	7.3
	S204	5.1	5.6	6.1	6.9	7.3	7.4
	S50	4.2	4.8	6.6	6.7	7.0	7.3

The results shown in Table 4.8 revealed that the nine isolated acid-tolerant *B. japonicum* could maintain acidic pHs in the culture media within 1.0 pH unit when grown in media with acidic initial pHs. However, cells were found to secrete acidic products when grown in media with neutral to alkali pHs.

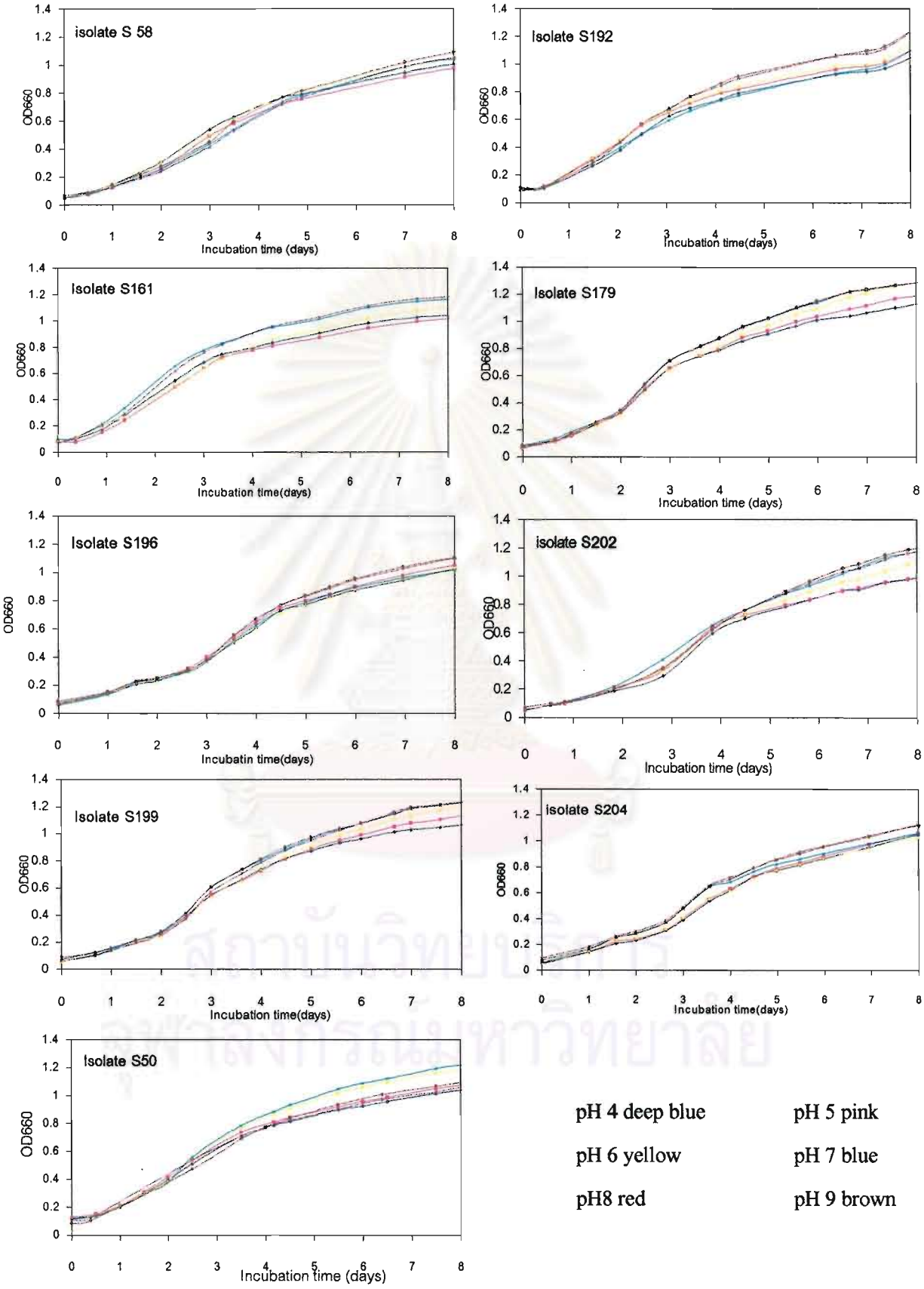


Figure 4.14 : Growth curves of nine isolates cultured in unbuffered yeast extract mannitol broth, pH range 4.0-9.0

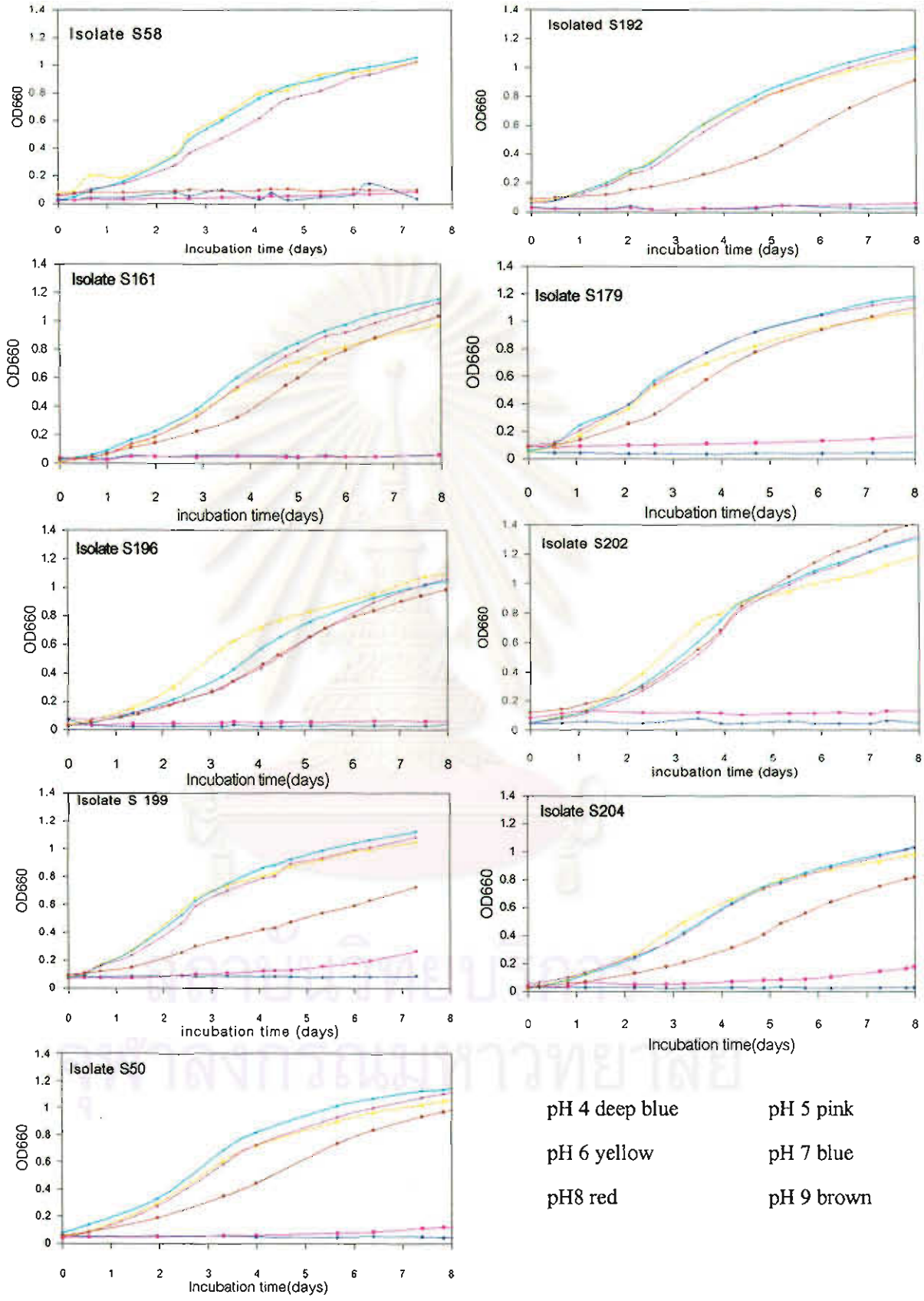


Figure 4.15 : Growth curves of nine isolates cultured in buffered yeast extract mannitol broth pH range 4.0-9.0

Table 4.9 : Initial and final pHs of buffered YMB at the end of the growth curve experiments for nine acid-tolerant *B. japonicum* isolates

	Isolates	Initial pH					
		4.0 NEDA	5.0 NEDA	6.0 MES	7.0 HEPES	8.0 HEPES	9.0 AMPSO
Final pH	S58	4.1	5.4	6.1	7.1	8.0	8.9
	S192	4.1	5.5	6.3	7.3	8.0	8.7
	S161	4.0	5.0	5.9	6.9	7.7	8.3
	S179	4.0	5.4	6.1	7.1	7.8	8.4
	S196	3.9	5.0	5.9	6.9	7.9	8.5
	S202	3.9	5.0	5.8	6.9	7.6	8.2
	S199	4.2	5.8	6.2	7.1	7.9	8.8
	S204	4.0	5.5	6.0	7.0	7.9	8.5
	S50	4.0	5.7	6.1	7.2	8.0	8.6

The results shown in Table 4.9 indicated that the selected buffers could maintain pH of the culture media. All the nine isolates were found to grow in unbuffered yeast extract mannitol broth at pH range 4.0-9.0, as shown in Figure 4.14. However, they were found to exhibit different growth characteristics when grown in buffered media at pH range 4.0-9.0, as shown in Figure 4.15. The finding that all the nine isolates did not grow at pH 4.0 and pH 5.0 when the media were buffered but grew at pH 4.0 and pH 5.0 in unbuffered media

indicated that the buffer NEDA used for controlling of the pH 4.0 or 5.0, might inhibit growth of the *B. japonicum* isolates.

The nine isolates were classified into 3 groups according to the shapes of growth curves as shown in Figure 4.15. The first group consisted of isolate #S58 which did not grow at pH 9.0. The second group consisted of isolates # S50, S192, S199 and S204 which grew moderately at pH 9.0 .The third group consisted of isolates #S161, S179, S196 and S202 which grew well at pH 9.0.

Growth characteristics observed for the nine *B. japonicum* isolates indicated that although the isolates had the same colony morphology and PCR fingerprint patterns, their growth characteristics in buffered media revealed that they belonged to different strains. The results could be summarized as follows :

Colony morphology	PCR fingerprint pattern	Result
I	1	Isolate #S161 may be the same strain as #S202 but differs from #S204
I	3	Isolate #S58 is not the same strain as #S179
I	4	Isolates #S50 and #S199 may be the same strain that is different from isolate#S196
I	5	Isolate #S192

Based on the results obtained the following isolates were selected for further studies : #S50, S58, S179, and S204.

4.6 Effects of initial pHs on hydroegn uptake hydrogenase

4.6.1 Determination of *hup*$^+$ phenotype

A typical GC chromatogram for the determination of H_2 and the standard curve for the determination of μmole of H_2 were shown in Figures 4.16 and 4.17, respectively.

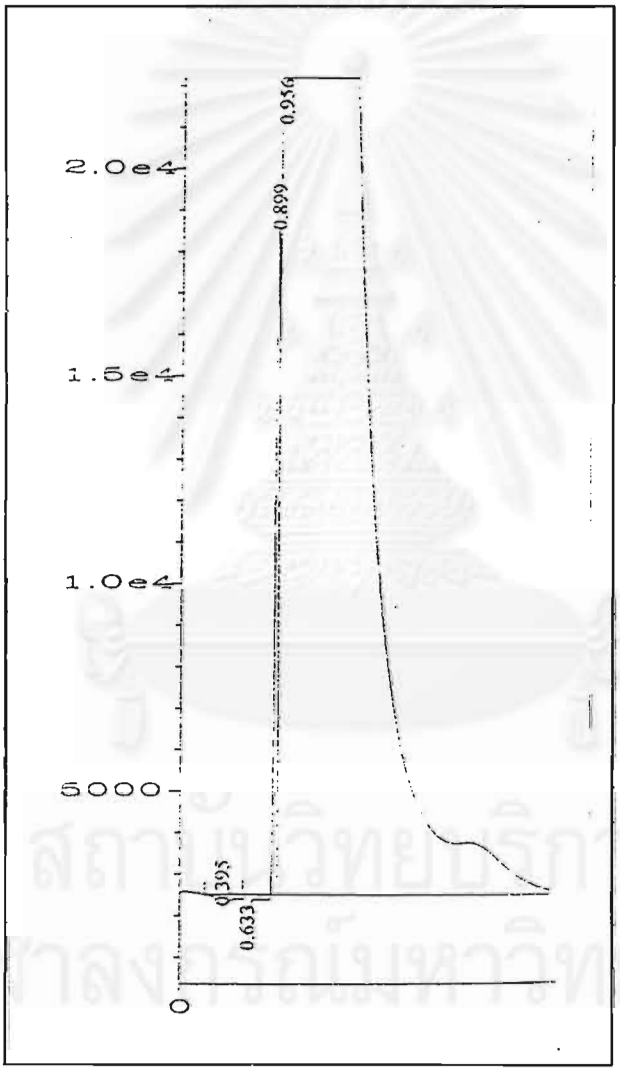


Figure 4.16 Typical GC chromatogram for the determination of H_2. Retention times for CO_2, H_2, O_2, and N_2 were 0.395, 0.633, 0.899, and 0.956 min, respectively

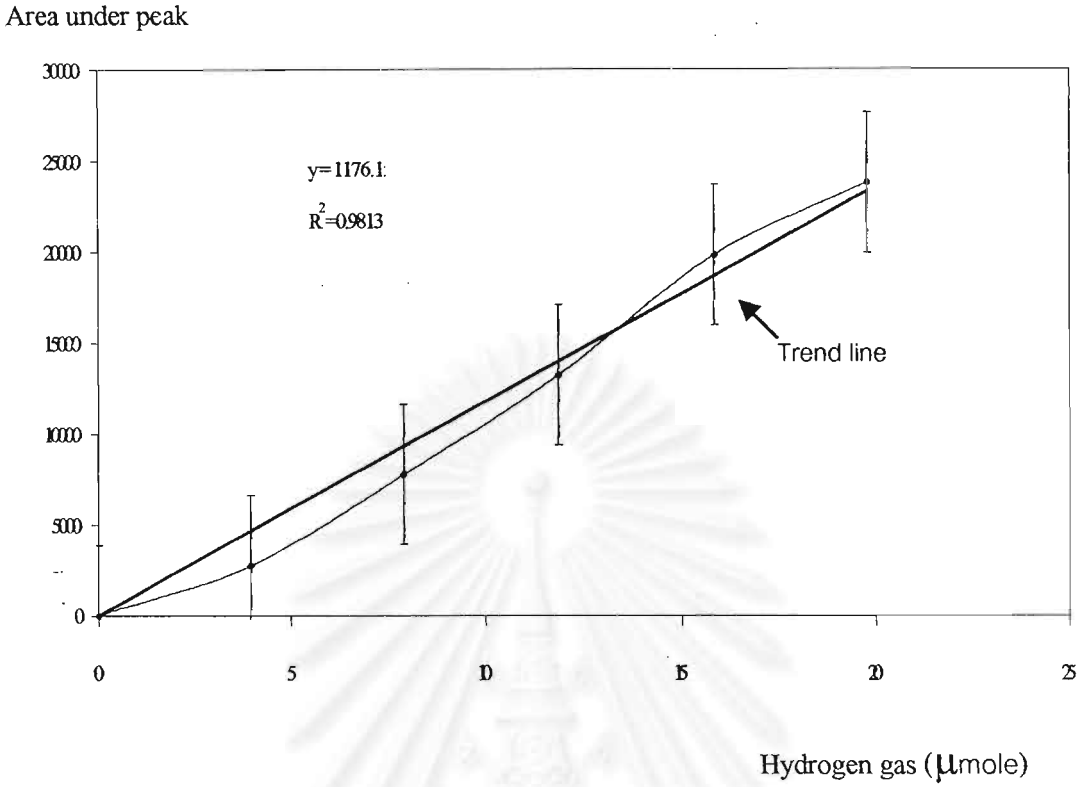


Figure 4.17 Standard curve for the determination of µmole H_2

Table 4.10 indicated an average of 3.3 µmole of H_2 in a 4 ml injection volume were taken from the control tubes which were flushed with a gas mixture of 10 % CO_2, 5 % O_2, 82 %N_2, and 3 % H_2

Table 4.10 µmole of H_2 originally present at the start of the experiment which was taken as at 30 min after flushing control tubes with a gas mixture of 10 % CO_2, 5 % O_2, 82 %N_2, and 3 % H_2

Area under peak		µ mole H_2 in control tube		µ mole of H_2 in 4 ml injection volume
At 30 min	At Day 3	At 30 min	At Day 3	
3826	2903	18.3	13.9	3.24
3885	3342	18.5	16.0	3.29
		Mean = 18.4	Mean = 14.9	Mean = 3.3

Average μ mole H_2 in a control tube at the end of Day 3 = 14.9+3.3 = 18.2 μ mole

Since one gas sample (4 ml) was taken from each tube after 30 minutes, at the end of the 3rd day of experiment, the control tube with culture medium but no *B. japonicum* should contain 18.2 μmoles H_2 which was equal to the sum of μ moles H_2 in the control tube at the end of Day 3 plus μmoles of H_2 in a 4 ml injection volume. Therefore, any loss of hydrogen more than 30% (equivalent to 5.5 μmoles H_2) detected at the end of Day 3 indicated that an isolate exhibited *hup*$^+$ phenotype.

4.6.2 Determination of hydrogen uptake hydrogenase activity

The results on hydrogen uptake hydrogenase activity were shown in Table 4.11.

The results shown in Table 4.11 indicated three types of effects of initial media pH on hydrogen uptake hydrogenase activity of the four selected *B. japonicum* strains :

Type I (#S50) exhibited *hup*$^-$ phenotype when the initial pH of the medium was 4.0 and exhibited *hup*$^+$ phenotype when media initial pHs were 6.0-9.0 with maximum specific activity (30.1 U mg^{-1} protein) when initial pH was 8.0.

Type II (#S50, S179) exhibited *hup*$^-$ phenotype when initial pHs of the media were 4.0-8.0 and exhibited *hup*$^+$ phenotype when the initial pH of the medium was 9.0 with specific hydrogen uptake hydrogenase activity of 29.4 U mg^{-1} protein.

Type III (#S204) exhibited *hup*$^-$ phenotype when the initial media pH was in the range of 5.0-7.0 and exhibited *hup*$^+$ phenotype when the initial pH

Table 4.11 Effects of initial pH of culture media on hydrogen uptake hydrogenase activity of 4 *B. japonicumm* isolates

Isolates	Initial pH	Area under peak		μmole H_2 obtained from standard curve		μmole H_2 at Day 3 +3.3 μmole		*hup* phenotype	Unit of enzyme	Protein (μg)	Specific activity ($U.mg^{-1}$ protein)
		30 min	Day 3	30 min	Day 3		Average				
S58	4.0	5123	4226	24.5	20.2	23.5	23.9	hup^-			
	4.0	6435	4403	30.7	21.0	24.3					
	5.0	6886	3987	32.9	19.0	22.3	22.3	hup^-			
	6.0	4742	3093	22.6	14.8	18.1	18.1	hup^-			
	7.0	3245	4189	15.5	20.0	23.3	20.7	hup^-			
	7.0	6066	3110	28.9	14.8	18.1					
	8.0	5339	3521	25.5	16.8	20.1	20.1	hup^-			
	9.0	3888	426	18.6	2.0	5.3	5.3	hup^+	7.4	251.8	29.4

Isolates	Initial pH	Area under peak		μmole H_2 obtained from standard curve		μmole H_2 at Day 3 +3.3 μmole		*hup* phenotype	Unit of enzyme	Protein (μg)	Specific activity ($U.mg^{-1}$ protein)
		30 min	Day 3	30 min	Day 3		Average				
S50	4.0	4454	3386	21.3	16.2	19.5	15.7	hup^-			
	4.0	4970	1786	23.7	8.5	11.8					
	6.0	3627	779	17.3	3.7	7.0	7.0	hup^+	5.7	592.1	9.6
	7.0	3074	767	14.7	3.7	7.0	7.0	hup^+	5.8	552.6	10.5
	8.0	3416	627	16.3	3.0	6.3	6.3	hup^+	6.5	215.8	30.1
	9.0	4785	1568	22.8	7.5	10.8	10.8	hup^+	2.0	603.1	3.3
S179	6.0	4181	2239	20.0	10.7	14.0	20.2	hup^-			
	6.0	2954	4811	14.1	23.0	26.3		hup^-			
	9.0	3707	658	17.7	3.1	6.4	6.4	hup^+	6.3	517.9	12.2
	9.0	3957	652	18.9	3.1	6.4					
S204	4.0	2296	533	11.0	2.5	5.8	5.8	hup^+	6.9	157.0	43.9
	5.0	3960	2648	18.9	12.6	15.9	15.3	hup^-			
	5.0	3987	2377	19.0	11.3	14.6		hup^-			
	6.0	4217	4373	20.1	20.9	24.2	24.2	hup^-			

was 4.0 with specific hydrogen uptake hydrogenase activity of 49.3 4 U mg^{-1} protein.

4.7 Effects of initial pHs on protein profiles

The results shown in Figures 4.18-4.21 indicated that initial pHs had effects on quantities of cellular proteins of *B. japonicum*, especially isolates #S58 and S179 where some protein bands were present more conspicuously than others. Isolate #S58 increased the synthesis of 28, 33, 37 and 50 kDa polypeptides when grown in unbuffered YMB at pH 4.0-8.0 but increased the synthesis of 120 and 29 kDa polypeptides when grown in buffered YMB pH 6.0-8.0. Isolate #S179 was found to contain more 25, 28, 33, 37, and 50 kDa polypeptides in buffered YMB at pH 8.0-9.0.

Another protein of interest was observed in the cellular proteins of *B. japonicum* isolate #S204 which contained more ~80 kDa polypeptide.

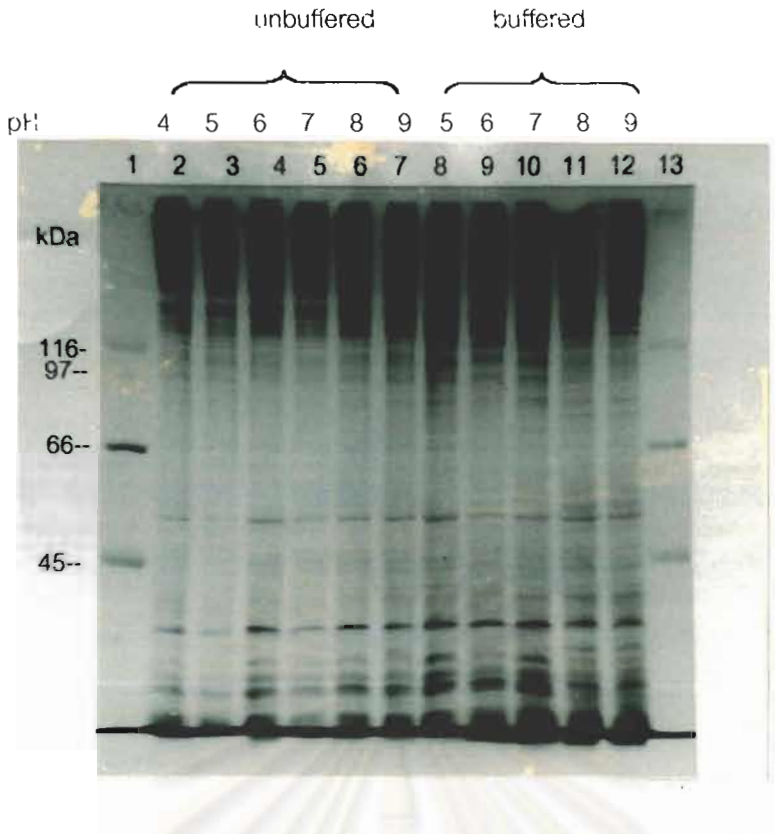


Figure 4.18 SDS-PAGE separation of cellular proteins extracted from mid-log phase *B. japonicum* isolate#S50. : Lanes 1, 13 :molecular weight markers; Lanes 2-7 : cellular proteins of isolate # S50 cultured in unbuffered YMB at pH 4.0-9.0; Lanes 8-10 : cellular proteins of isolate #S50 cultured in buffered YMB at pH 5.0-9.0

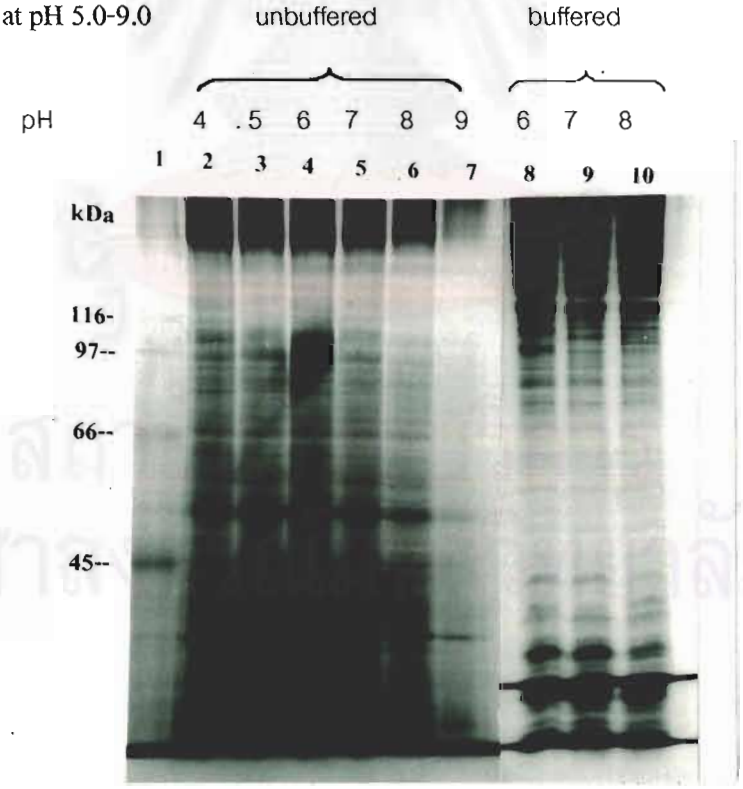


Figure 4.19 : SDS-PAGE separation of cellular proteins extracted from mid-log phase *B. japonicum* isolate#S58. : Lane 1 :molecular weight markers; Lanes 2-7 : cellular proteins of isolate # S58 cultured in unbuffered YMB at pH 4.0-9.0; Lanes 8-10 : cellular proteins of isolate #S58 cultured in buffered YMB at pH 6.0-8.0.

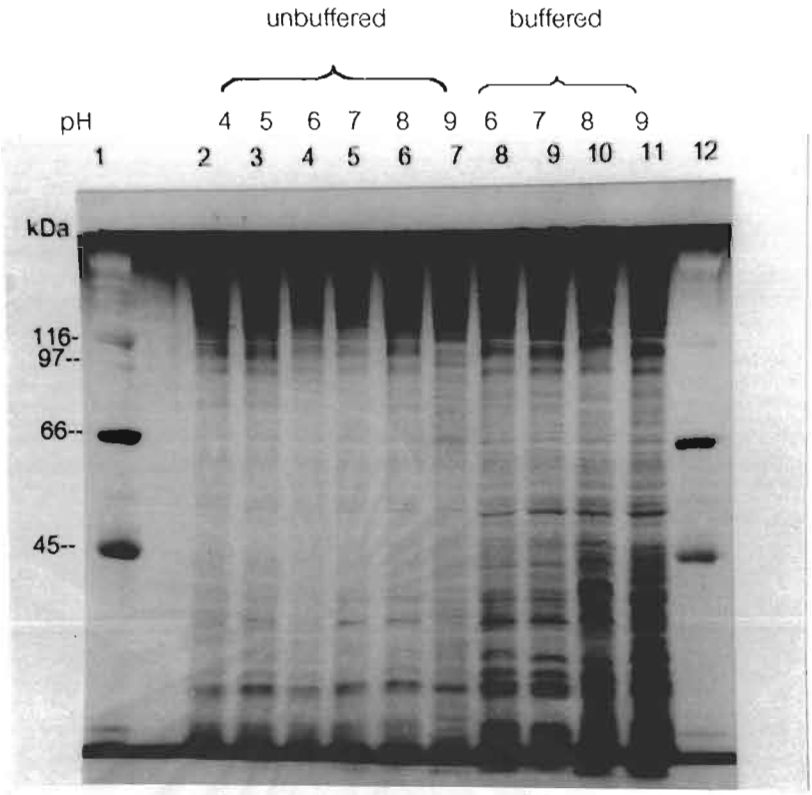


Figure 4.20 : SDS-PAGE separation of cellular proteins extracted from mid-log phase *B. japonicum* isolate#S179. : Lanes 1, 12 :molecular weight markers; Lanes 2-7 : cellular proteins of isolate # S179 cultured in unbuffered YMB at pH 4.0-9.0; Lanes 8-11 : cellular proteins of isolate #S179 cultured in buffered YMB at pH 6.0-9.0.

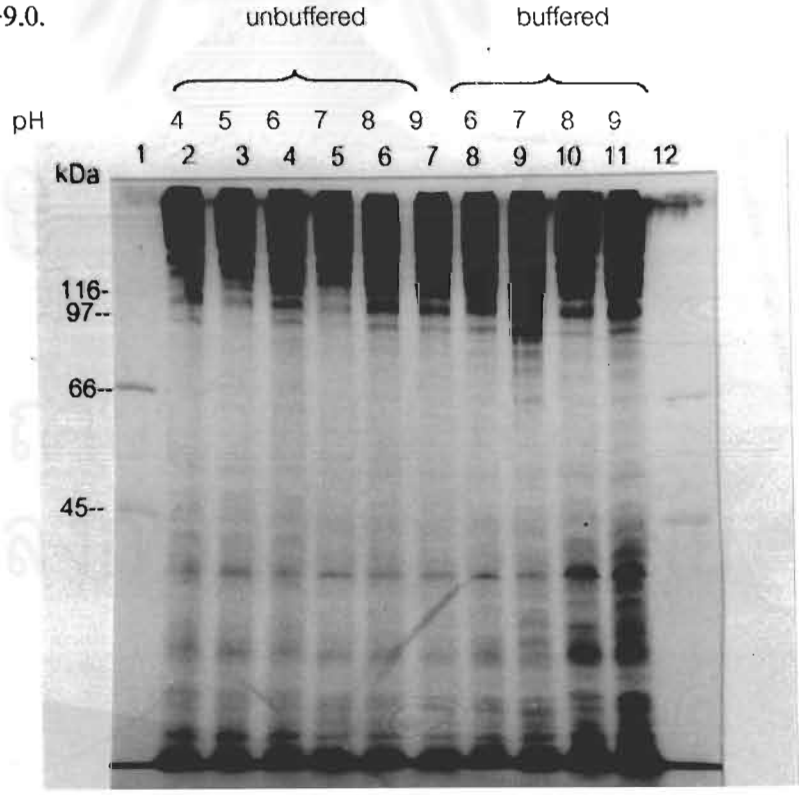


Figure 4.21 : SDS-PAGE separation of cellular proteins extracted from mid-log phase *B. japonicum* isolate#S204. : Lanes 1, 12 :molecular weight markers; Lanes 2-7 : cellular proteins of isolate # S204 cultured in **unbuffered** YMB at pH 4.0-9.0; Lanes 8-11 : cellular proteins of isolate #S204 cultured in buffered YMB at pH 6.0-9.0.

Chapter 5

Discussion

5.1 Identification of Distinct Strains of *B. japonicum*

The results surprisingly revealed that the soil samples from a factory in Meenburi district, Bangkok, contained *B. japonicum*. The results showed that, from the very start of the construction of the factory, soils used in land filling might have been taken from areas once used for soybean cultivation.

At present there has been no report on the absorption of Congo red by *B. japonicum*. However, in 1983, Kneen and Larue separated polysaccharide layers from 8 strains of *Rhizobium leguminosarum* grown in the presence of congo red to show that the 8 strains took up the dye in the capsular polysaccharide layers. The authors cautioned that if the criterion of not absorbing congo red was used to select *Rhizobium leguminosarum*, the collection would include only those which did not absorb the dye.

In this work, colonies of all slow-growing root nodule isolates appeared light pink on congo red yeast extract mannitol agar plates. In fact, during the isolation process, no red colonies appeared on the congo red yeast extract mannitol agar plates. Therefore, the problem of not selecting *B. japonicum* which took up congo red did not occur. However, one drawback of this work was having to pick a large number of pinkish, slimy, slow-growing colonies which might belong to the same *B. japonicum* strains in order to isolate all existing *B. japonicum* in the acidic soil samples (Table 4.1).

Hence, one difficulty encountered in this work was choosing the criteria employed to select distinct strains of *B. japonicum* for further studies on the effects of initial pH on hydrogen uptake hydrogenase activity and protein profiles.

Four criteria were finally employed to select 4 distinct *B. japonicum* strains (#S50, S58, S179 and S204) for further studies. The criteria were

1. High nitrogen fixation ability at pH 4.5 and 6.8
2. Colony morphology type I
3. RAPD-PCR fingerprint patterns when RPO1 was used as the primer
4. Growth characteristics when grown in buffered YMB at pH 9.0

In fact, there are many more criteria for use in the strain identification. Some older criteria had been used to characterize bradyrhizobial isolates in Thailand, for example, serogrouping, intrinsic antibiotic resistance, (Thompson *et al.*, 1991), IAA production, growth characteristics at pH 3.0, growth at different temperatures, enzyme profiles, and RAPD-PCR fingerprints using different primers (Nuntagij *et al.*, 1997). Lately, researchers have tended to employ the more sensitive molecular biology techniques such as RAPD-PCR fingerprints and genetic variety in *nod* gene as identification method of choice for *B. japonicum* isolated in Thailand (Yokoyama et al., 1996; Donnaya Matpatawee *et al.*, 1999). In this work RAPD-PCR fingerprinting should be carried out on the isolates, using other primers such as ERIC. Catalogues of RAPD-PCR fingerprints obtained from using different kinds of primers could pave the way for the use of RAPD-PCR fingerprints in *B. japonicum* strain identification.

5.2 The Effects of Initial pH on Hydrogen Uptake Hydrogenase Activity

In this thesis, research was conducted on the effects of initial pH on hydrogen uptake hydrogenase activity to find out if it is possible to use the expression of *hup* genes (*hup*$^+$ phenotype) as a predictor for better *B. japonicum* nitrogen fixers in acidic soils.

The interesting results obtained from this study indicated that initial unbuffered media pH had 3 types of effects on the expression of *hup* genes in *B. japonicum* isolates#S50, S58, S179, and S204. Some isolate(s) exhibited *hup*$^+$ phenotype when grown at initial pHs of 6.0-9.0(Type I, #S50); other isolates expressed *hup* genes when grown only at initial pH 9.0 (Type II, #S58, #S179); other isolate(s) expressed *hup* genes when grown only at initial pH 4.0 (Type III, #S204). Therefore, soil pH should be taken into account when embarking upon the proving of the hypothesis that *hup*$^+$ phenotype *B. japonicum* are better nitrogen fixers.

The finding that the expression of *hup* genes in *B. japonicum* depends on initial media pH has never been published in literature. This is the first report of the finding.

5.3 Acid Tolerance Mechanisms in *B. japonicum*

The results shown in Table 4.8 indicated that the isolated acid-tolerant *B. japonicum* seemed to adjust to alkali environments by changing pH of the media to near neutrality. Acidic products were exported out of the cells. Holding and Lowe (1971) found that acetic acid, propionic acid, butyric acid, iso-butyric acid and pyruvic acid were acid substances which were produced by *R. trifolii*.

In 1993, Fujihara and Yoneyama found that *Rhizobium fredii* P220 acidified Yeast-sucrose media with initial pH 6.0-9.0 and accumulated high cellular polyamine at pH 4.0. On the other hand, *Bradyrhizobium japonicum* A1017 produced alkali products at pH 5.0-7.0 of this medium. Glenn and Dilworth (1994) reported that alkalinization of external medium by base export was one of several mechanisms in acid tolerance in root nodule bacteria. But in this work, the unbuffered YMB media were not alkalinized to a great extent(Table 4.8). The results indicated that in unbuffered YMB some *B. japonicum* isolates, responded to the acidic initial pH by secreting slight quantities of alkali products and secreting acidic products when the initial pHs were alkali (Table 4.8). However, when YMB was buffered, the isolates were observed to synthesis higher quantities of some polypeptides, notably the 120, 33 and 28 kDa (Figures 4.19-4.21).

Recommendations for Future Research

1. Performing 2D-SDS-PAGE

In order to accurately pinpoint polypeptide(s) responsible for acid tolerance in *B. japonicum*, two-dimensional SDS-PAGE should be employed to separate soluble cellular proteins obtained from *B. japonicum* grown in YMB with initial pHs ranging from 4.0-9.0 with appropriate buffers. (In 1994 Graham *et al.* reported that NEDA had little effect on growth of *B. japonicum* but markedly inhibited growth of *Rhizobium* spp at acidic pH. The results obtained were in contrast to what had been reported by Graham *et al.* (1994). NEDA was found to markedly inhibit growth of the nine *B. japonicum* isolates at pH 4.0 and 5.0). The two-dimensional SDS-PAGE results may be used to group *B. japonicum* strains as had previously been performed by Roberts *et al.*(1980) who

used two-dimensional SDS-PAGE patterns to identify and classify *Rhizobium* strains.

2. The effects of initial pHs on hydrogen uptake hydrogenase activity and (1D-SDS-PAGE) protein profile should be conducted for the rest of the different strains of isolated *B. japonicum* which yielded good plant growth and nodule formation to confirm that the expression of *hup* genes depends on pH.

3. Survey of the distribution of *hup*$^+$ *B. japonicum* in different soybean cultivation areas with different soil pHs in Thailand should be continued and if it is statistically proven that *hup*$^+$ *B. japonicum* strains are better nitrogen fixers, a correlation between RAPD-PCR fingerprints and the *hup*$^+$ phenotype might be conducted for use in the application of PCR fingerprint patterns to predict high nitrogen fixing potential under different soil pHs since PCR fingerprinting is easier to perform and is a more accurate predictor than *hup*$^+$ phenotype.

Chapter 6

Conclusion

1. Fifty-six soybean nodule bacterial isolates were obtained from soil samples from 13 collection sites in Bangkok, Kampaeng Pet, Pijit, and Nakorn Sawan. Soil pH in 0.01 M $CaCl_2$ was found to be in the range of 4.19-6.89. Six isolates were found to be fast-growers and the rest was found to be slow-growers in yeast extract mannitol broth with 3-4 hours and 35-45 hours generation time, respectively. All fast-growing isolates were found to secrete acidic products while all slow-growing isolates were found to secrete alkali products when grown on yeast extract mannitol Agar with bromo thymol blue.

2. The 50 slow-growing isolates were authenticated as being *Bradyrhizobium japonicum.* There were 2 types of colony morphology. Type I colony morphology with pinkish-white, opaque, pearl-like with round, smooth margin, dome-shaped colonies of less than 1 mm diameter. Type II colony morphology with pinklish, translucent, irregular, mucous colonies more than 1 mm in diameter. The 50 slow-growing *B. japonicum* were found to have one of the five patterns of PCR fingerprints when RPO1 primer was used in RAPD-PCR

Pattern 1 : 2 PCR products, 300 and 400 bp (8 isolates)

Pattern 2 : 3 PCR products, 300, 400 and >2,000 bp (5 isolates)

Pattern 3 : 3 PCR products, 300, 400 and 850 bp (27 isolates)

Pattern 4 : 4 PCR products, 300, 400, 700 and >2,000 bp (5 isolates)

Pattern 5 : 5 PCR products, 300, 400, 700, 850 and >2,000 bp

(5 isolates)

Soil samples from Nuern Mahatsajan, Kao Kaw district, Petchaboon province were found to contain the most diverse collection of *B. japonicum* isolates with Type I colony morphology and all the 5 patterns of PCR fingerprints.

3. Analysis of variance indicated that the source of the observed variations in soybean dry weight and root nodule dry weight was the types of isolates used in the authentication test. pH of the N-free plant nutrient solution was not found to be the cause of the observed variations. The linear correlation coefficient for plant dry weight and nodule dry weight was found to be 0.2122.

4. Using the following four criteria, four *B. japonicum* isolates (#S50, S58, S179, and S204) were tentatively identified as being distinct strains :

4.1 Nitrogen fixing ability at pH 4.5 and 6.8

4.2 Colony morphology

4.3 RAPD-PCR fingerprint pattern when RPO1 was used as the primer

4.4 Growth characteristics when grown in buffered yeast extract mannitol broth at pH 9.0

5. The effect of initial pH on hydrogen uptake hydrogenase and protein profiles

5.1 Isolate #S50 was found to exhibit *hup*$^-$ phenotype when grown in media with initial pH 4.0 but exhibit *hup*$^+$ phenotype when the initial pHs were 6.0-9.0 with maximum specific hydrogenase activity of 30.1 unit. mg^{-1} protein when the initial pH was 8.0. This

isolate was found to increase the production of 120 kDa protein when grown in buffered YMB, pH 5.0-7.0.

5.2 Isolates #S58 and #S179 were found to exhibit *hup*$^-$ phenotype when initial pHs of the media were 4.0-8.0 but exhibit *hup*$^+$ phenotype when the initial pH was 9.0 with specific hydrogenase activity of 29.4 unit. mg^{-1} protein.

Isolate #S58 was the only isolate found to increase the synthesis of 28, 33, 37 and 50 kDa polypeptides when grown in unbuffered YMB with initial pH 4.0-8.0. The isolate was found to synthesize more 29 and 120 kDa polypeptides when grown in buffered YMB at initial pH 6.0-8.0.

Isolate #S179 was found t increase the production of 25, 28, 33, 37 and 50 kDa polypeptide when grown in buffered YMB at initial pH 6.0-9.0.

5.3 Isolate #S204 was found to exhibit *hup*$^-$ phenotype when the initial pH of the medium was in the range of 5.0-7.0 but exhibit hup$^+$ phenotype when initial pH was 4.0 with specific hydrogenase activity of 43.9 unit. mg^{-1} protein.

The isolate was found to synthasize more of the 25, 33 and 80 kDa polypeptides when grown in buffered YMB at initial pH of 7.0-9.0.

This is the first report that pH has an effect on the expression of *hup* genes which encode hydrogen uptake hydrogenase in *B. japonicum*.

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Appendix

Appendix A

Bacterial Growth Media and Plant Nutrient Solutions

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

Yeast 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 liter

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

Yeast 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 flask 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 μg ml^{-1}. The medium was autoclaved at 121 °C for 15 min.

YMA with Bromthymol Blue (BTB)

BTB stock solution : 0.5 g BTB dissolved in 100 ml of ethanol. Five milliliers of BTB stock solution were added to 1 liter of YMA to the final concentration of 25 μg ml^{-1}. The medium was autoclaved at 121 °C for 15 min.

Hydrogen Uptake Medium (Maier et al., 1978)

Distilled water	1 L		
$NaH_2PO_4.H_2O$	150 mg	$CaCl_2.2H_2O$	150 mg
$MgSO_4.7H_2O$	250 mg	Iron-EDTA	28 mg
$NaMoO_4.2H_2O$	0.25 mg	$Cu\ SO_4.5\ H_2O$	0.04 mg
$CoCl_2.6\ H_2O$	0.025 mg	KI	0.78 mg
Inositol	100 mg	Thiamine hydrochloride	10 mg
Nicotinic acid	1 mg	Pyridoxal HCl	1.0 mg
Sucrose	0.5 g	L-arabinose	1.0 g
Sodium gluconate	0.5 g	Sodium glutamate	0.5 g
Yeast extract	0.1 g	Bacto agar(Difco)	20 g

The medium was adjusted to pH ranging from 4.0-9.0 with 0.1 N NaOH or HCl. Inositol, Thiamine hydrochloride, Nicotinic acid, Pyridoxal HCl were filter sterilized.

N-free Nutrient Solution

Stock Solutions	Chemicals	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$Mn\ SO_4.H_2O$	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
	$Na_2MoO_2.2H_2O$	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 either 4.5 or 6.8 with 1 N NaOH or HCl.
- 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 determination of soil pH

0.01 M Calcium chloride (Peech, 1965)

1. Stock Calcium chloride solution (3.6M) : 1,059 g of $CaCl_2.2H_2O$ were dissolved in distilled water in a 2-liter volumetric flask. Twenty milliliters of the stock Calcium chloride solution were diluted to 1-liter in a volumetric flask.

2. The Calcium chloride solution was standardized prior to use by titrating 25 ml diluted solution with 0.1 N $AgNO_3$, using 1 ml 5 %$K_2Cr_7O_4$ as indicator. 0.01 M Calcium chloride solution was prepared from the standardized Calcium chloride solution using the formula $N_1V_1 = N_2V_2$where N denotes the normality and V denotes the volume .

2. Solutions for SDS-PAGE

29.2% Acrylamide and 0.8% N,N'-methylenebisacrylamide stock solution (Sambrook, Fritsch, and Maniatis, 1989)

To prepare a stock solution, 29.2 g Acrylamide (Bio-Rad) and 0.8 g N,N'-methylenebisacrylamide (Bio-Rad) were dissolved in distilled water. The volume was brought to 100 ml. The solution was stored in an amber glass bottle. Fresh solutions were prepared every few months.

Note : Unpolymerised Acrylamide is neurotoxic and must be handled with care.

10% Ammonium persulfate (Sambrook, Fritsch, and Maniatis, 1989)

One milliliter of aqueous 10 %(w/v) Ammonium persulfate stock solution was prepared and stored at 4 °C. Ammonium persulfate decomposes slowly, and fresh solutions were prepared weekly.

Separating gel buffer (5x) (Ausubel et al, 1992)

1.875 M Tris HCl, pH 8.8, 0.5% Sodium dodecyl sulfate

22.8 g Trisma base (Sigma) and 0.5 g Sodium dodecyl sulfate (Sigma) were dissolved in approximately 50 ml distilled water. pH of the solution was adjusted to 8.8 with HCl (conc). The resulting solution was made up to a final volume of 100 ml.

Stacking gel buffer (5x) (Ausubel et al, 1992)

0.625 M Tris HCl, pH 6.8, 0.5% Sodium dodecyl sulfate

7.569 g Trisma base (Sigma) and 0.5 g Sodium dodecyl sulfate (Sigma) were dissolved in approximately 50 ml distilled water. pH of the solution was adjusted to 6.8 with HCl(conc). The resulting solution was made up to a final volume of 100 ml.

Sodium dodecyl sulfate gel-loading buffer (2x) (Laemmli, 1970)

The solution was composed of : 200 µl Glycerol

100 µl 2-Mercaptoethanol

200 µl Stacking gel buffer (5x)

500 µl Distilled water

0.04 g Sodium dodecyl sulfate(SDS)

trace amount of Bromophenol blue

50 mM Tris HCl (Sambrook, Fritsch, and Maniatis, 1989)

0.6055 g Trisma base (Sigma) were dissolved in approximately 50 ml distilled water. pH of the solution was adjusted to 7.8 with HCl (conc). The final volume of the solution was 100 ml.

Tris-glycine buffer (Laemmli, 1970)

25 mM Trisma base, 192 mM Glycine and 0.1 % Sodium dodecyl sulfate (SDS)

6.8118 g Trisma base (Sigma), 32.44 g Glycine (Sigma), 2.26 g SDS (Sigma) were added to distilled water. The final volume was made up to 2,250 ml.

Coomassie brilliant blue solution (Bradford, 1976)

One hundred milligrams Coomassie brilliant blue G-250 were dissolved in 50 ml of 95% ethanol. One hundred milliliters of 85% phosphoric acid were added to the solution. The volume was made up to 1 liter with distilled water. The solution was filtered through Whatman no.1 filter paper and stored in an amber glass bottle.

3. 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 up 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 (Gibco BRL) was used according to manufacturer 's instruction.

4. Solutions for RAPD-PCR

Primer RPO1

(Data supplied by Gibco BRL Custom Primers, Life Technologies, Inc., USA)

Molecular weight(μg/μmole)	6415.0
Millimolar Extinction Coefficient (OD/μmole)	216.5
μg per OD	29.6
nmoles per OD	4.6
OD's	8.28
μg's	245.34
nmoles	38.2

Before use : One milliliter of high purity distilled water was added to lyophilized primer to yield 245.34 μg/ml which was equivalent to 245.34/6415 μ mole/ml = 0.04 μmole/ml (40 μM).

10 μM primer solution used in the PCR reaction mixture was prepared from the 40 μM primer solution by using the formula

$$N_1V_1 = N_2V_2$$

where N denotes the normality and V denotes the volume.

10x PCR buffer (Biotools)

50 mM $MgCl_2$ (Biotools)

40 mM dNTPs each 10 mM (Biotools)

10 μM primer RPO1 (Life Technologies)

5 U/μl *Tag* polymerase (Biotools)

5. Buffers

MES; 2-(*N*-morpholino)ethanesulfonic acid(Good and Izawa, 1972)

$pK_a = 6.15$

$\Delta\ pK_a$/degree = -0.011

Very little tendency to bind metal ions.

Comments : MES undergoes some decomposition when autoclaved in the presence of glucose.

HEPES; *N*-2-hydroxyethylpiperazine-*N*'–ethanesulfonic acid (Good and Izawa, 1972)

$pK_a = 7.55$

ΔpK_a/degree = -0.014

Does not bind Mg^{2+}, Ca^{2+}, Mn^{2+}, or Cu^{2+}

Comments : The Folin protein assay cannot be used in the presence of HEPES. The biuret protein assay is unaffected. (HEPES, by the rules of English honetics, should be pronounced in the same ways as " heaps.")

Appendix C

Determination of Hydrogen Concentration

Use of Gas chromatography to determine hydrogen concentration in space above *B. japonicum* grown on 5 ml slants of hydrogen Uptake agar medium in 16 mm x 150 mm test tubes. Air space volume = 27.5-5.0 = 22.5 ml

- Injection volume was 4 ml
- Standard curve was constructed with 10 % hydrogen in Nitrogen as the standard

Volume of hydrogen in 4 ml of 10% hydrogen-nitrogen =(10x4)/100 = 0.4 ml = 400 µl

According to Hanus et al. (1980), at sea level, Hg pressure is 743 mm at 301 °K. Using the gas law relationshin

$$\frac{P_1V_1}{T_1} = \frac{P_2V_2}{T_2}$$

P_1 = 760 mm Hg ; V_1 = unknown ; T_1 = 273 °K

P_2 = 743 mm Hg ; V_2 = 400 µl ; T_2 = 301 °K

Therefore, volume of hydrogen at normal temperature and pressure(NTP)

= 400 x (743/760) x (273/301)

= 354.7 µl

According to molar volume, 1 mole of hydrogen at NTP will occupy 22.4 liters.Therefore, 354.7 µl hydrogen = 354.7/22.4 = 15.8 µmole

$$\text{µmole hydrogen per tube} = \frac{15.8 \times 22.5 \times \text{area under peak}}{4.0 \times \text{area under peak for standard } H_2}$$

Appendix D

Raw Data

Date: Tue, 1 Jun 1999 10:04:51 -0400
From: Brad Reuhs <breuhs@ccrc.uga.edu>
To: Kanjana Chansa-ngavej <Kanjana.C@chula.ac.th>
Subject: Re:

Dr. Chansa-ngavej,

We do not use the McCall here for our studies, that is used by Krishnan and Pueppke. We use soybean cv. Williams, which yields the same phenotype (i.e., only S. fredii USDA191 can infect; all other S. fredii strains are non-infective on Williams). I can send you plenty of seeds for Williams if you would like to use that plant. As for the Peking, we have only a hand full of seeds left. We will be generating more soon, but that will take some time. I can send you ten seeds if you want to use those as the positive control. However, I don't know if that will be enough. You may wish to contact Hari Krishnan at U. of Missouri for more Peking seeds, and McCall. His Email address is KrishnanH@missouri.edu. Please send your mailing address if you want the Williams seeds.

By the way, we have polyclonal antisera that recognize 95% of all S. fredii strains tested, if you would like us to test your strains, just send us the freeze-dried pellet from a 5 ml culture.

Good luck in your work.

Brad Reuhs

Date: Thu, 27 Jan 2000 10:18:04 -0500
From: Brad Reuhs <breuhs@ccrc.uga.edu>
To: Kanjana Chansa-ngavej <Kanjana.C@Chula.ac.th>
Subject: Re: Lyophilised cultures sent for ELISA testing

[Part 1, Text/PLAIN (charset: ISO-8859-1 "Latin 1") 8 lines.]
[Unable to print this part.]

[The following text is in the "iso-8859-1" character set.]
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[Some characters may be displayed incorrectly.]

The results with all antibodies were negative (in both ELISA and immunoblots). If the strains are S. fredii, they do not fall into either of the major LPS serogroups. In addition, the PAGE analysis showed that they were distinct from any S. fredii or S. meliloti that we have worked with. That is all I can tell you. I do recall a paper by Pueppke et al in which they analyzed some fast-growing soybean symbionts from Viet Nam, and those strains were also different than the China isolates.
B. Reuhs

Biography

Mr. Suwat Saengkerdsub was born on January, 2 1975. He obtained a Bachelor of Science Degree in Marine Science from Chulalongkorn University, Bangkok, Thailand, in 1996.

Publication

1. Saengkerdsub, S., Chansa-ngavej, K. 1999. Isolation and authentication of fast- and slow-growing acid-tolerant *Bradyrhizobium japonicum*. Proceedings of the Fifth Asia-Pacific Biochemical Engineering Conference and the 11th Annual Meeting of the Thai Society for Biotechnology. 6 pages. CD-ROM format.

Presentation at Scientific Conferences

1. Saengkerdsub, S., Chansa-ngavej, K. 1999. Effects of media initial pHs on growth and protein profile of *Bradyrhizobium japonicum*. Poster presentation at the 25th Congress on Science and Technology of Thailand. Pitsanulok, Thailand. Abstract book p. 730-731.