Chapter 4

Results

4.1 Selection of *B. japonicum* strains for study

The results as shown in Figure 4.1 a indicated that fifteen of the nineteen isolates exhibited an identical RAPD-PCR fingerprint with a band of approximately 750 bp as the PCR product when RPO1 was used as the primer. Figure 4.1 b indicated that isolates S18, S40, S57, S74, S78 and S205 were the same strain. Isolates S42, S76, S180, S182, S184, S185 and S198 were the same strain. Isolates S162 and S202 were the same strain. Isolates S8, S58, S187 and S192 were different strains. The sizes of the PCR products of each group of strains when CRL-7 was used as the primer are shown in Table 4.1.

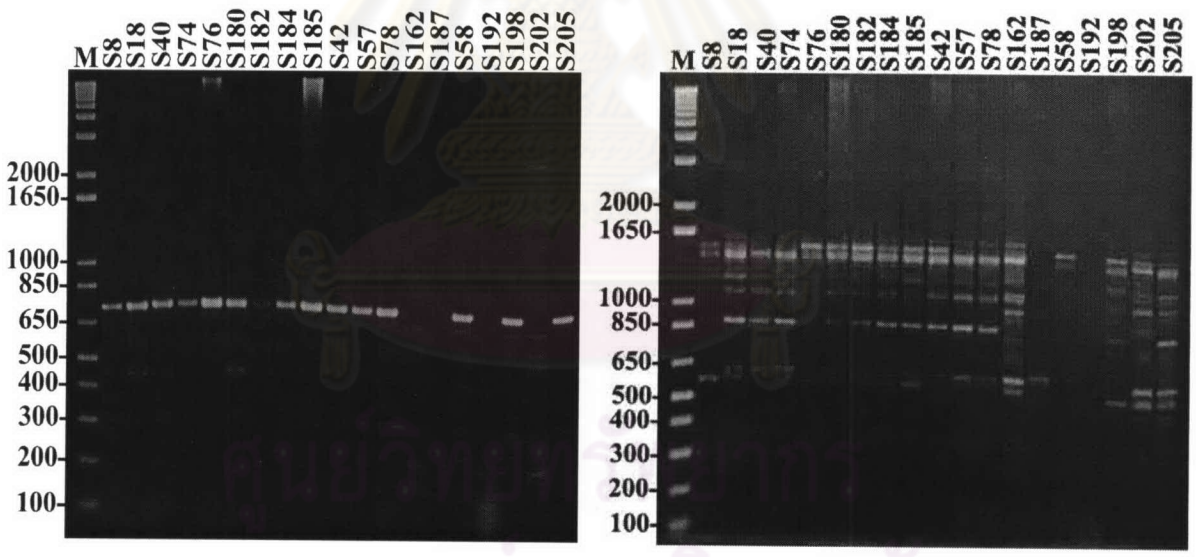

Figure 4.1 RAPD-PCR fingerprints of nineteen isolates of *B. japonicum* when (a) RPO1, and (b) CRL-7 was each used as the primer.

The sizes of the PCR products as shown in Table 4.1 indicated that *B. japonicum* strains S76, S78, and S162 were different strains with 1500 bp, 1400 bp, and 950 bp as

the respective characteristic bands for the groups where *B. japonicum* S76, S78 and S162 belong. Isolates S76, S78 and S162 which were isolated from soil samples from Nern Mahatsajan, Kao Kaw district, Petchaboon province by Suwat Saengkerdsub (1999) were therefore chosen for further studies. Moreover, Patima Permpoonpattana (2002) reported preliminary results of heat shock proteins in these three *B. japonicum* strains. The three strains were therefore selected for use in further experiments.

Table 4.1 Sizes of PCR products of 19 isolates when CRL-7 was used as the primer.

Isolates	Sizes of PCR products (bp)
S18, S40, S57, S74, S78, S205	570, 620, 880, 1100, 1200, 1400, 1500, 1550
S42, S76, S180, S182, S184, S185, S198	570, 620, 880, 1100, 1200, 1400, 1500
S162, S202	570, 620, 950, 1100, 1200, 1400, 1500, 1550
S8	570, 1400, 1500
S58	1400, 1500
S187	620
S192	no PCR products

4.2 Nitrogen-fixing potential of the three *B. japonicum* strains

Figures 4.2-4.5 indicated average Leonard jar grown soybean plant and nodule dry weights when inoculated with either *B. japonicum* S76 or S78 or S162 and watered with nitrogen-free medium of either pH 6.8 or pH 5.0. Figure 4.2 showed that when nitrogen-free medium pH 5.0 was used, average plant dry weights of all the three soybean cultivars were found to be lower than those of the corresponding positive controls indicating that nitrogen fixation by the three strains of *B. japonicum* might not be optimum at pH 5.0. Figures 4.2 and 4.3 showed that the average nodule dry weight of *Glycine max* cv CM 60 inoculated with *B. japonicum* S78 was relatively higher than those obtained when *B. japonicum* S76 and S162 were the inoculants resulting in a relatively

higher plant dry weight. *B. japonicum* S76 and S162 were found to poorly nodulate all the three soybean cultivars at pH 5.0 resulting in the same plant dry weights as those of the corresponding negative controls.

Figure 4.4 indicated that when nitrogen-free medium pH 6.8 was used, inoculation of *Glycine max* cv CM 2 and CM 60 with *B. japonicum* S76 or S78 yielded the same level of average soybean dry weight as that of the positive control for *Glycine max* cv CM 2. Inoculating *Glycine max* cv ST 2 with *B. japonicum* S76 and S78 yielded higher soybean dry weight than that of the positive control while *B. japonicum* S162 was not found to increase soybean cv ST 2 dry weight when compared with the positive control. The results indicated that *B. japonicum* S162 in conjunction with the use of pH 6.8 nitrogen-free medium resulted in the highest average soybean dry weight for *Glycine max* cv CM 60. Since Figure 4.5 indicated that *B. japonicum* S78 and S162 yielded the same level of nodule dry weight when *Glycine max* cv CM 60 was watered with pH 6.8 nitrogen-free medium, the higher plant dry weight when *B. japonicum* S162 was the inoculant for *Glycine max* cv CM 60 might result from higher nitrogen-fixing ability of *B. japonicum* S162 at pH 6.8.

Duncan's Multiple Range test as indicated in Table 4.2 confirmed the findings that when nitrogen-free medium pH 5.0 was used, a combination of *B. japonicum* S78 and *Glycine max* cv. CM 60 yielded the most average plant dry weight (1.44 g.plant^{-1}) which was in the same range as that of the positive control (1.61 g.plant^{-1}). Table 4.4 showed that when nitrogen-free medium pH 6.8 was used, *B. japonicum* S162 was found to yield the most average plant dry weight when *Glycine max* cv. CM 60 was used (1.96 g.plant^{-1}) and the average dry weight obtained was found in the highest statistically significant level. The average plant dry weight obtained was statistically higher than that of the corresponding positive control (1.01 g.plant^{-1}) as shown in Table 4.4. The overall results as shown in Tables 4.2-4.5 indicated that at both pHs *Glycine max* cv. CM 60 yielded the most average plant dry weight and that *B. japonicum* S78 was found to be a good nitrogen-fixer for *Glycine max* cv CM 60 at pH 5.0 while S162 was found to be a good nitrogen-fixer for *Glycine max* cv CM 60 at pH 6.8.

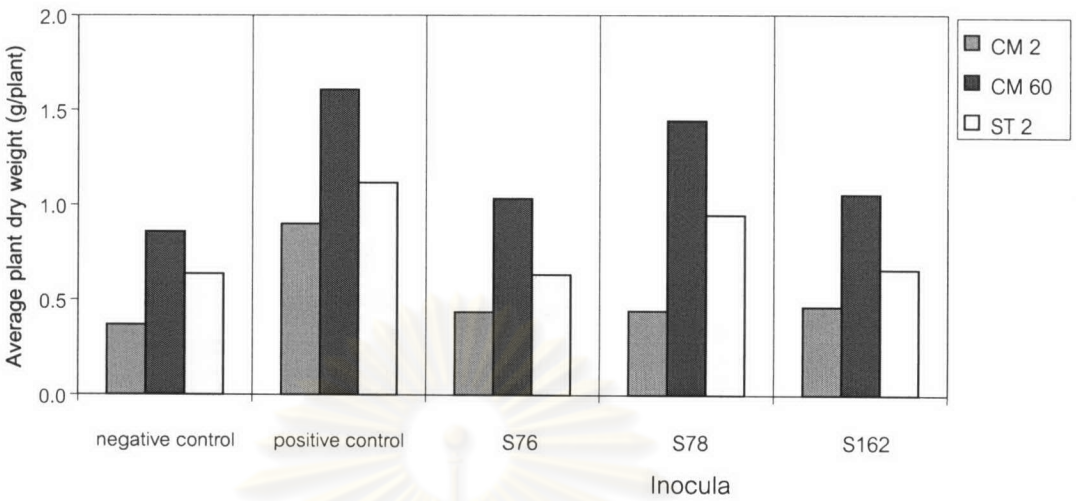

Figure 4.2 Average plant dry weight of soybean *Glycine max* cultivars CM 2, CM 60 and ST 2 after inoculation with either *Bradyrhizobium japonicum* S76 or S78 or S162 in Leonard jars with nitrogen-free medium pH 5.0 for 28 days.

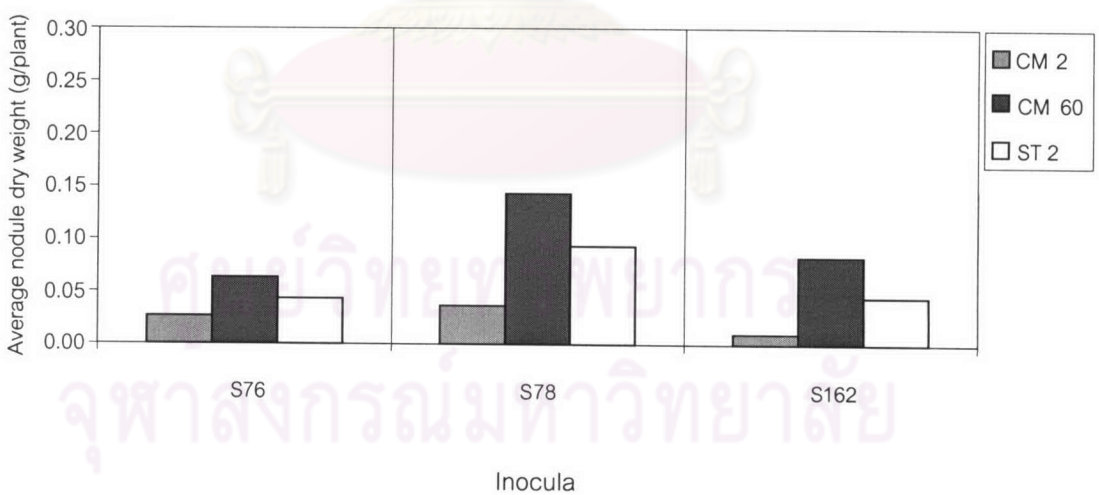

Figure 4.3 Average nodule dry weight of soybean *Glycine max* cultivars CM 2, CM 60 and ST 2 after inoculation with either *Bradyrhizobium japonicum* S76 or S78 or S162 in Leonard jars with nitrogen-free medium pH 5.0 for 28 days.

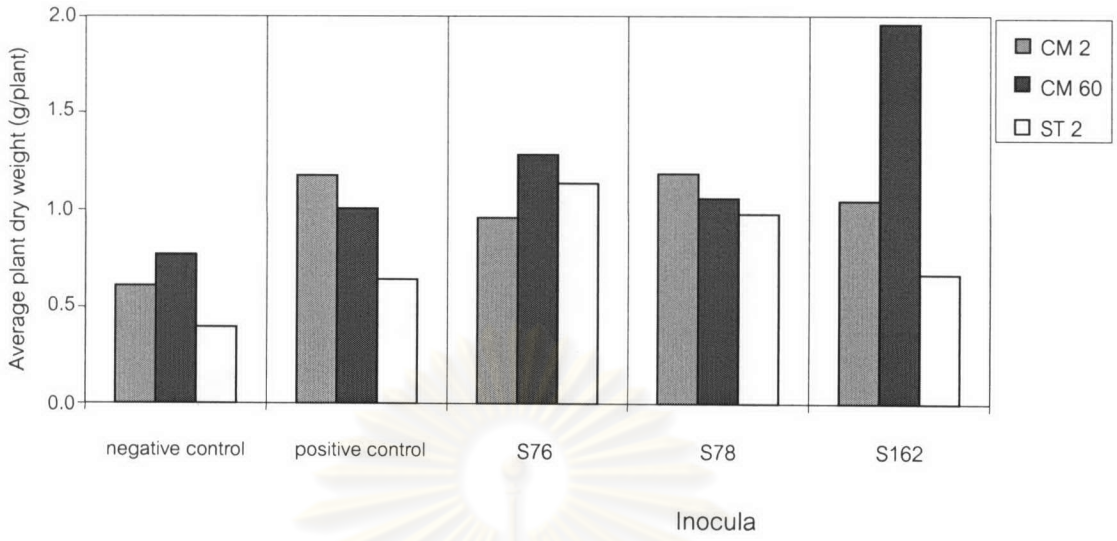

Figure 4.4 Average plant dry weight of soybean *Glycine max* cultivars CM 2, CM 60 and ST 2 after inoculation with either *Bradyrhizobium japonicum* S76 or S78 or S162 in Leonard jars with nitrogen-free medium pH 6.8 for 28 days.

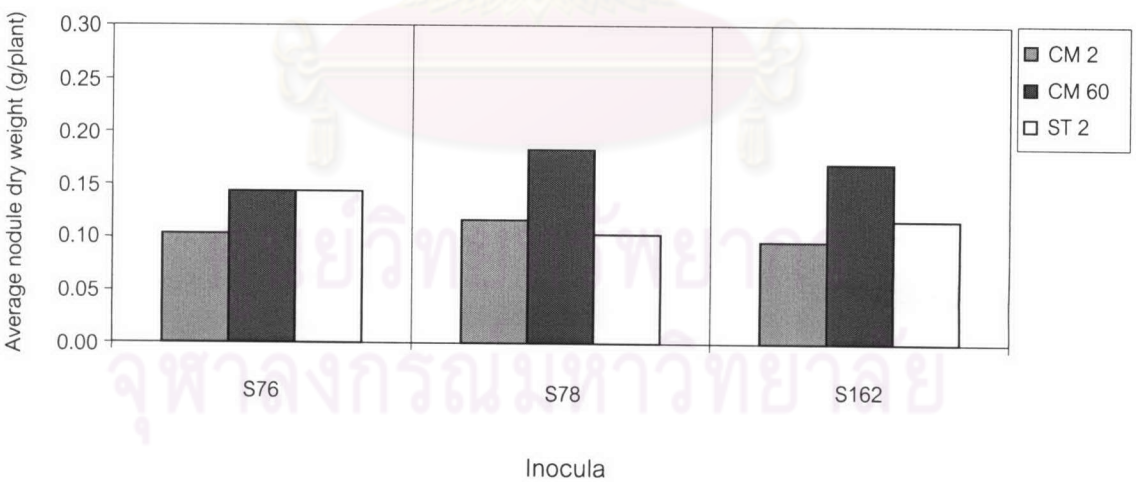

Figure 4.5 Average nodule dry weight of soybean *Glycine max* cultivars CM 2, CM 60 and ST 2 after inoculation with either *Bradyrhizobium japonicum* S76 or S78 or S162 in Leonard jars with nitrogen-free medium pH 6.8 for 28 days.

Table 4.2 Duncan's Multiple Range Test for average plant dry weight when each of *Bradyrhizobium japonicum* strains S76, S78 and S162 was inoculated onto germinating seeds of soybean *Glycine max* cv. CM 2, CM 60 and ST 2 in Leonard jars with nitrogen-free medium pH 5.0 for 28 days. (Level of probability, $\alpha = 0.05$)

Treatment *B. japonicum* strains	Average plant dry weight in grams per plant (*Glycine max* cultivar)		
	CM 2	CM 60	ST 2
S76	0.44^{a}	1.03^{ab}	0.63^{a}
S78	0.44^{a}	1.44^{b}	0.95^{ab}
S162	0.47^{a}	1.06^{ab}	0.66^{a}
Positive control	0.9^{ab}	1.61^{b}	1.12^{ab}
Negative control	0.37^{a}	0.86^{ab}	0.64^{a}

Table 4.3 Duncan's Multiple Range Test for average nodule dry weight when each of *Bradyrhizobium japonicum* strains S76, S78 and S162 was inoculated onto germinating seeds of soybean *Glycine max* cv. CM 2, CM 60 and ST 2 in Leonard jars with nitrogen-free medium pH 5.0 for 28 days. (Level of probability, $\alpha = 0.05$)

Treatment *B. japonicum* strains	Average nodule dry weight in grams per plant (*Glycine max* cultivar)		
	CM 2	CM 60	ST 2
S76	0.03^{ab}	0.06^{ab}	0.04^{ab}
S78	0.04^{ab}	0.14^{c}	0.09^{bc}
S162	0.01^{a}	0.08^{bc}	0.05^{ab}

Table 4.4 Duncan's Multiple Range Test for average plant dry weight when each of *Bradyrhizobium japonicum* strains S76, S78 and S162 was inoculated onto germinating seeds of soybean *Glycine max* cv. CM 2, CM 60 and ST 2 in Leonard jars with nitrogen-free medium pH 6.8 for 28 days. (Level of probability, $\alpha = 0.05$)

Treatment *B. japonicum* strains	Average plant dry weight in grams per plant (*Glycine max* cultivar)		
	CM 2	CM 60	ST 2
S76	0.96^{ab}	1.28^{bc}	1.14^{ab}
S78	1.19^{ab}	1.06^{ab}	0.98^{ab}
S162	1.05^{ab}	1.96^{c}	0.67^{ab}
Positive control	1.18^{ab}	1.01^{ab}	0.64^{ab}
Negative control	0.61^{ab}	0.77^{ab}	0.40^{a}

Table 4.5 Duncan's Multiple Range Test for average noduie dry weight when each of *Bradyrhizobium japonicum* strains S76, S78 and S162 was inoculated onto germinating seeds of soybean *Glycine max* cv. CM 2, CM 60 and ST 2 in Leonard jars with nitrogen-free medium pH 6.8 for 28 days. (Level of probability, $\alpha = 0.05$)

Treatment *B. japonicum* strains	Average nodule dry weight in grams per plant (*Glycine max* cultivar)		
	CM 2	CM 60	ST 2
S76	0.10^{a}	0.14^{a}	0.14^{a}
S78	0.12^{a}	0.18^{a}	0.10^{a}
S162	0.10^{a}	0.17^{a}	0.12^{a}

4.3 Growth of *B. japonicum* S76, S78 and S162 in unbuffered and buffered YMB

The results as shown in Figures 4.6.a-4.6.f and Table 4.6 indicated different growth responses of the three *B. japonicum* strains when grown in unbuffered and buffered YMB pH 4.0-9.0. The three *B. japonicum* strains were found to grow best in unbuffered and buffered YMB, pH 7.0. *B. japonicum* S76 was found to grow poorer in unbuffered YMB pH 5.0, 6.0 and 8.0 when compared to *B. japonicum* S78 and S162. *B. japonicum* S162 was found to grow equally well in unbuffered YMB pH 4.0-9.0. All the three *B. japonicum* strains could not grow in buffered YMB pH 4.0 and pH 5.0. Poor growth was obtained when cells were grown in buffered YMB, pH 9.0.

Table 4.6 Specific growth rates of *B. japonicum* S76, S78, and S162 grown in buffered and unbuffered YMB pH 4.0-9.0. (NG = no growth)

strain	Specific growth rate (h^{-1})						strain	Specific growth rate (h^{-1})					
	unbuffered pH							buffered pH					
	4.0	5.0	6.0	7.0	8.0	9.0		4.0	5.0	6.0	7.0	8.0	9.0
S76	0.017	0.019	0.018	0.038	0.016	0.013	S76	NG	NG	0.016	0.023	0.016	0.005
S78	0.024	0.025	0.024	0.032	0.025	0.012	S78	NG	NG	0.019	0.034	0.015	0.004
S162	0.022	0.024	0.022	0.024	0.023	0.024	S162	NG	NG	0.023	0.033	0.016	0.009

The results as shown in Figures 4.6.a – 4.6.c and Table 4.7 indicated that when there was no buffer in the culture medium, *B. japonicum* S76, S78, and S162 could grow to some extent. One reason is because the cells secreted either acidic or alkali products in response to the medium pH in such a way that the range of the growth medium pH was 4.2-8.0.

Table 4.7 : Final pHs of unbuffered yeast extract medium.

Strain / Initial pHs	4.0	5.0	6.0	7.0	8.0	9.0
S76	4.5	6.1	6.4	7.2	7.7	7.9
S78	4.4	5.4	6.3	7.1	7.7	8.0
S162	4.2	5.9	6.2	7.0	7.6	7.8

4.4 Intracellular protein profiles of mid-log phase *B. japonicum* S76, S78 and S162

The results shown in Figure 4.7 indicated there was no change in intracellular soluble protein profiles when each of the three *B. japonicum* strains was cultured in unbuffered yeast extract mannitol medium. One explanation of the results lies in the findings as shown on Table 4.7 that the initial pHs had been "adjusted" by the cells to more suitable pHs.

Figure 4.8 indicated there was no change in intracellular soluble protein profiles when each of the three *B. japonicum* strains was cultured in buffered yeast extract mannitol medium (YMB). Since no growth was obtained when YMB was buffered at pHs 4.0 and 5.0, cells were grown in YMB, pH 5.5 for the lowest acidic pH.

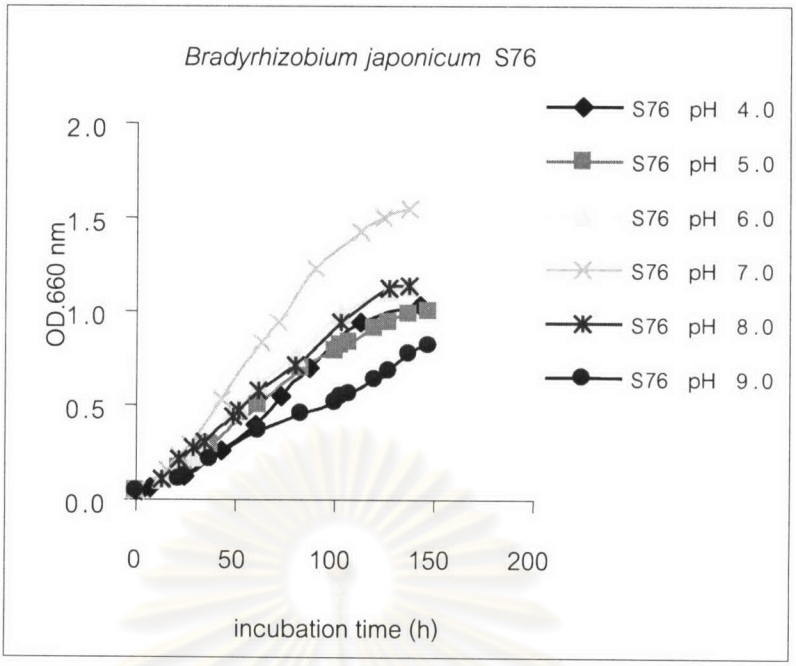

Figure 4.6.a. Growth curves of *B. japonicum* S76 cultured in unbuffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

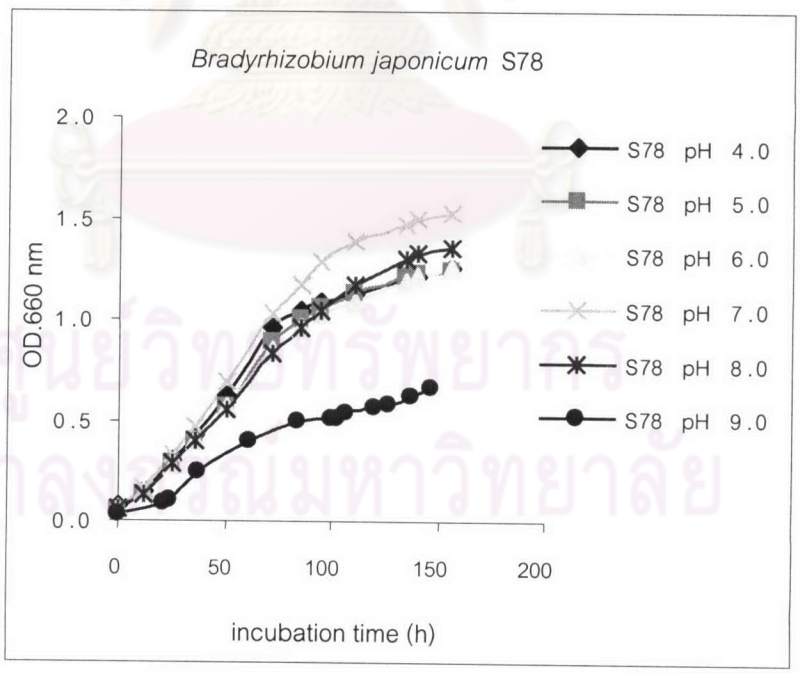

Figure 4.6.b. Growth curves of *B. japonicum* S78 cultured in unbuffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

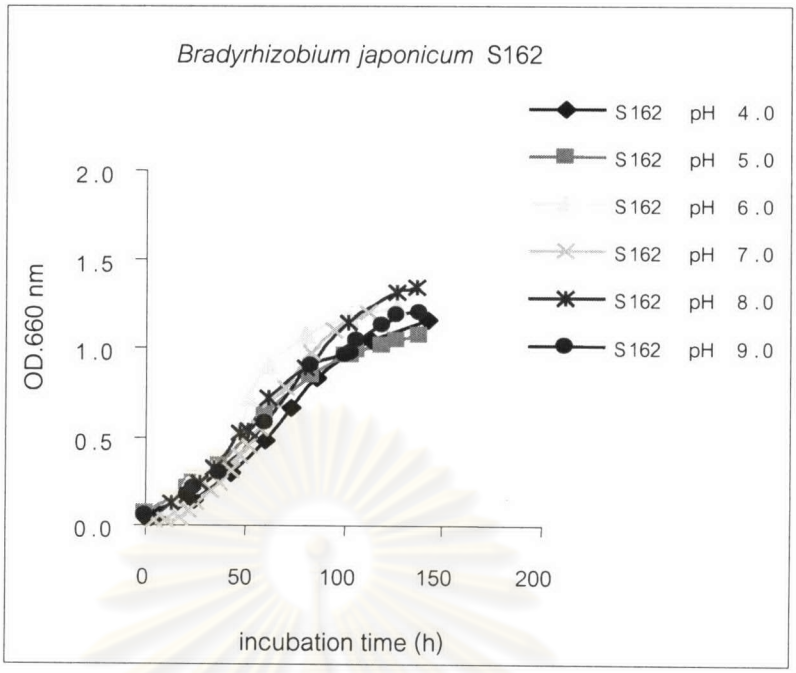

Figure 4.6.c. Growth curves of *B. japonicum* S162 cultured in unbuffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

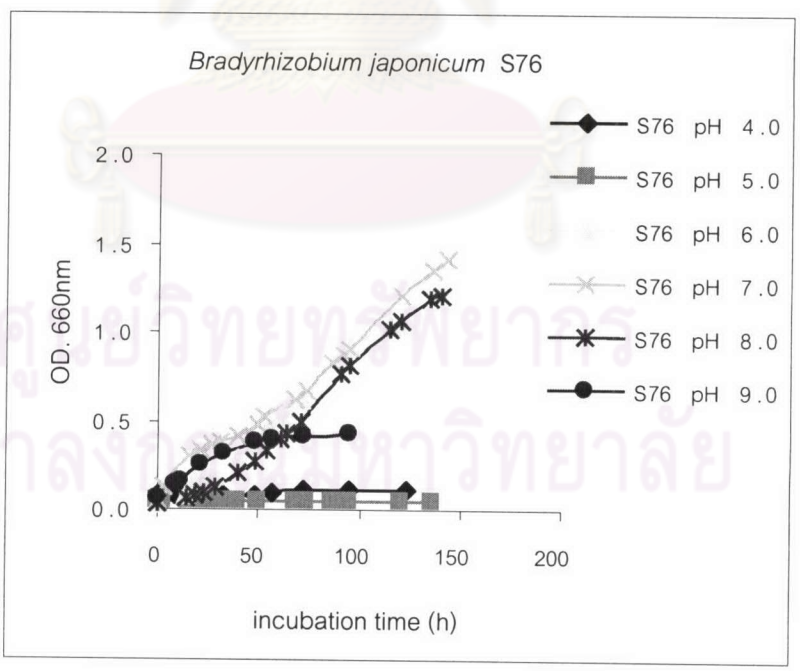

Figure 4.6.d. Growth curves of *B. japonicum* S76 cultured in buffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

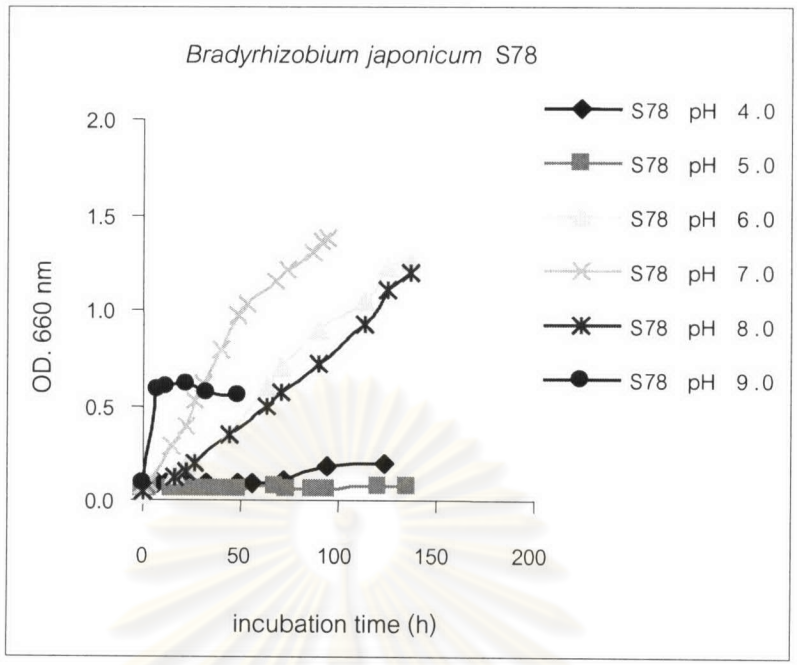

Figure 4.6.e. Growth curves of *B. japonicum* S78 cultured in buffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

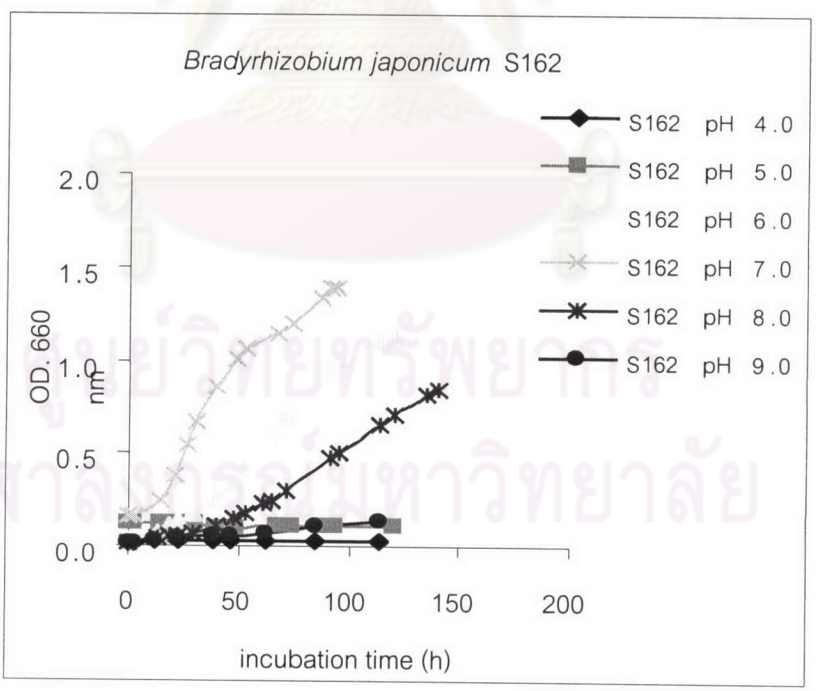

Figure 4.6.f. Growth curves of *B. japonicum* S162 cultured in buffered yeast extract mannitol broth with initial pHs ranging from 4.0 to 9.0 at 200 rpm, 30°C.

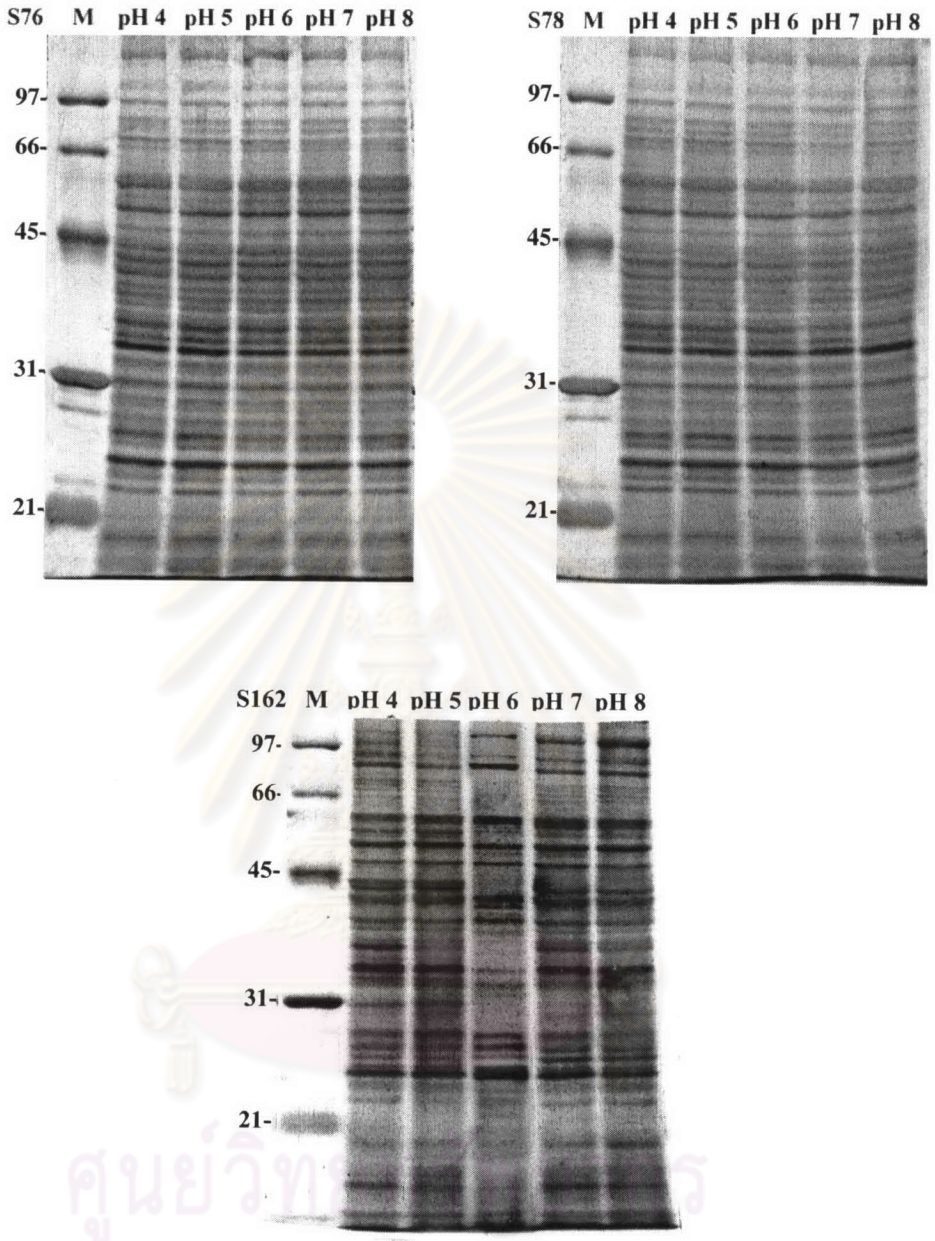

Figure 4.7 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S76, S78 and S162 when cultured in unbuffered yeast extract mannitol medium with initial pHs ranging from 4.0-8.0 at 200 rpm, 30 °C (M = molecular weight markers).

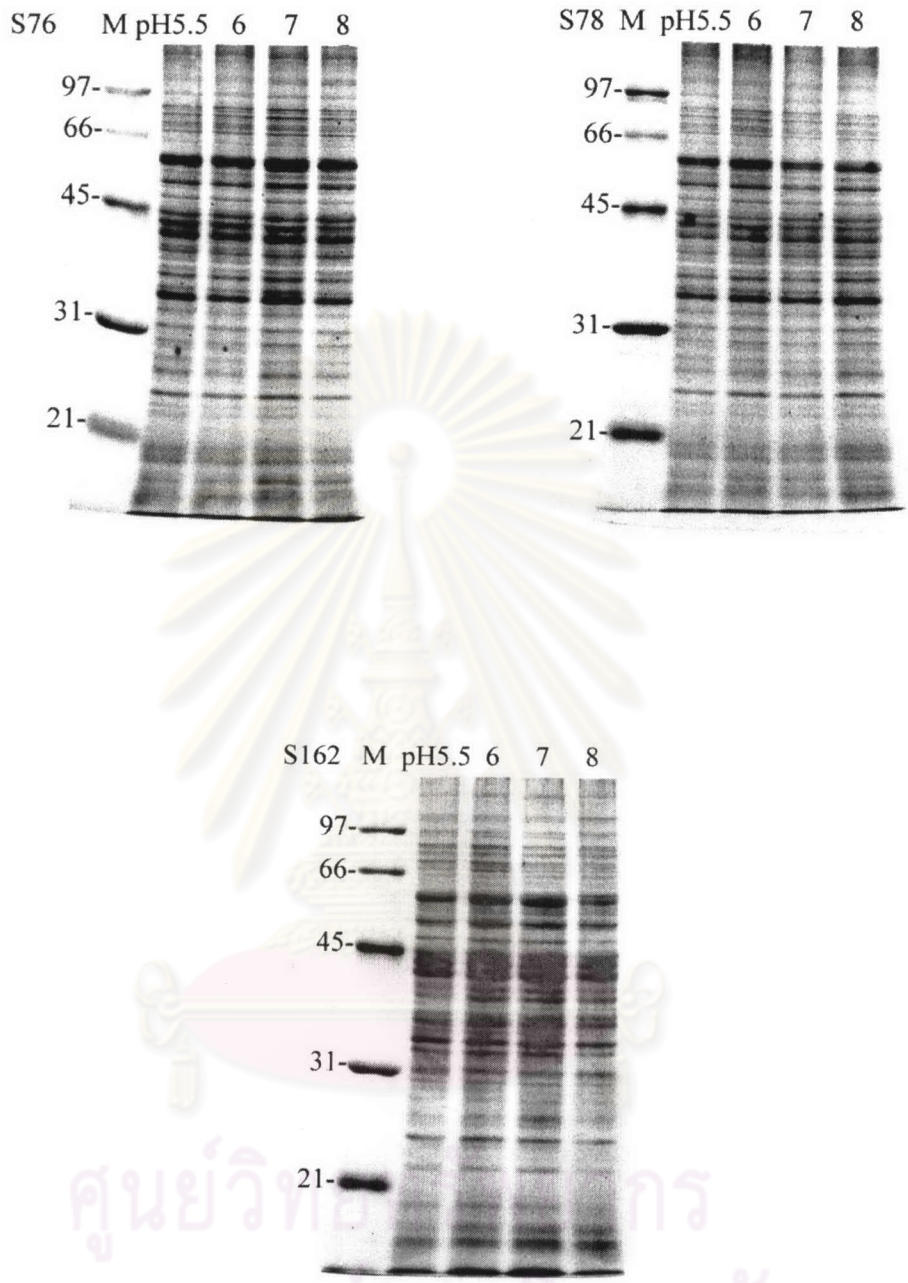

Figure 4.8 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S76, S78 and S162 when cultured in buffered yeast extract mannitol medium with initial pHs ranging from 5.5-8.0 at 200 rpm, 30 °C (M = molecular weight markers).

4.5 Effects of changes of medium pH on intracellular protein profiles

Figures 4.9.a to 4.9.d indicated that when mid-log phase cells of *B. japonicum* S76, S78 and S162 grown in buffered yeast extract mannitol broth (YMB) at pH 5.5 were transferred to buffered YMB at pHs 5.5, 6.0, 6.5 and 7.0, there was the same extent of growth. Moreover, Figures 4.10 - 4.12 indicated that there was no change in the intracellular protein profiles of *B. japonicum* S76, S78 and S162 12h, 18h, 24h, 3 days and 5 days after the pH shifts from 5.5 to 6.0. However, it was noticed that a 53 kDa polypeptide was either absent or reduced in quantity after mid-log phase *B. japonicum* S76 previously grown in buffered YMB pH 5.5 were transferred to buffered YMB at pH 6.0 for 12, 18 and 24 hours (Figure 4.10). Figure 4.11 indicated the absence of the 53 kDa polypeptide even in the seed culture at the time of transfer of *B. japonicum* S76, pH 5.5 to pH 6.0. SDS-PAGE separation of intracellular proteins extracted from *B. japonicum* S76, S78 and S162 during the 24h after the pH shift from 5.5 to 6.0 as indicated in Figure 4.13 offered a possible explanation on the observed disappearance or reduction in quantity of the 53 kDa. Figure 4.13 indicated that there was a 53 kDa polypeptide in *B. japonicum* S76 and S78 during the 24 h after the pH shift from 5.5 to 6.0. The polypeptide might be a labile protein. Moreover, loading of lesser proteins than 50 µg per lane might also lead to the disappearance or reduction in quantity of the 53 kDa polypeptide. The N-terminal amino acid sequence of the 53 kDa polypeptide is being analysed. So far the preliminary results as indicated in Appendix D indicated the first 4 N-terminal amino acids are Asn Ser Val Thr. These results are to be confirmed since the area under peak of each amino acid seemed not to differ significantly (AppendixD). Repeated extractions and SDS-PAGE of intracellular proteins of *B. japonicum* S76, S78 and S162 after medium pH changes from pH 5.5 to pHs 6.5 and 7.0 for 12h, 18h, 24h, 3 days and 5 days revealed no changes in the intracellular protein profiles as shown in Figures 4.14-4.19.

Some occasional disappearance or decrease in quantity of the 53 kDa polypeptide was also observed in SDS-PAGE separations of intracellular proteins extracted from *B. japonicum* S76 after shifting of yeast extract mannitol medium from pH 5.5 to 7.0 for 12 h and 18 h (Figure 4.15), and from pH 5.5 to 6.5 (Figure 4.16) ; in *B. japonicum* S162 after pH shift from 5.5 to 6.5 for 12 h (Figure 4.18) and after pH shift from 5.5 to 7.0 for 12 h, 18 h and 24 h (Figure 4.19).

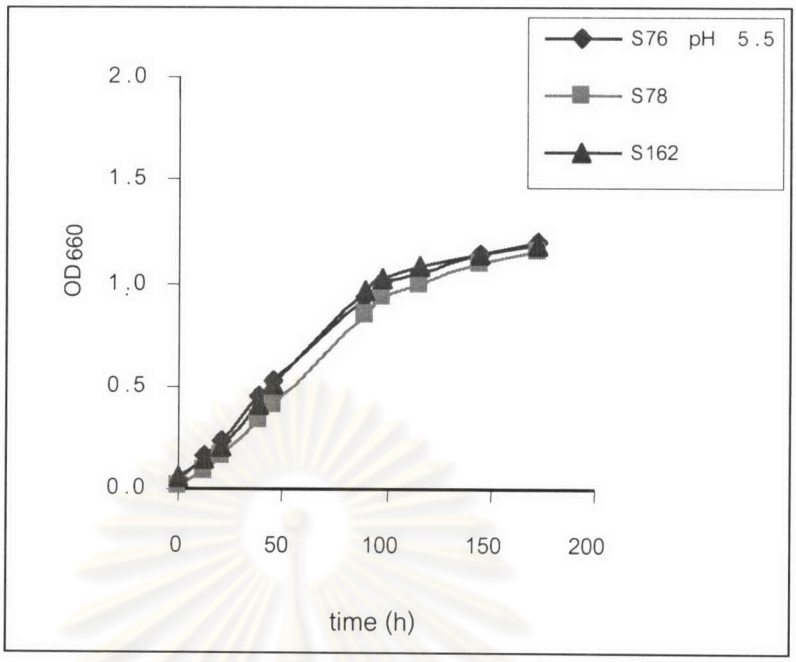

Figure 4.9.a Growth curves of *B. japonicum* S76, S78 and S162 cultured in buffered yeast extract mannitol (YMB), pH 5.5, then shifted to YMB pH 5.5 and cultivated at 200 rpm, 30 °C.

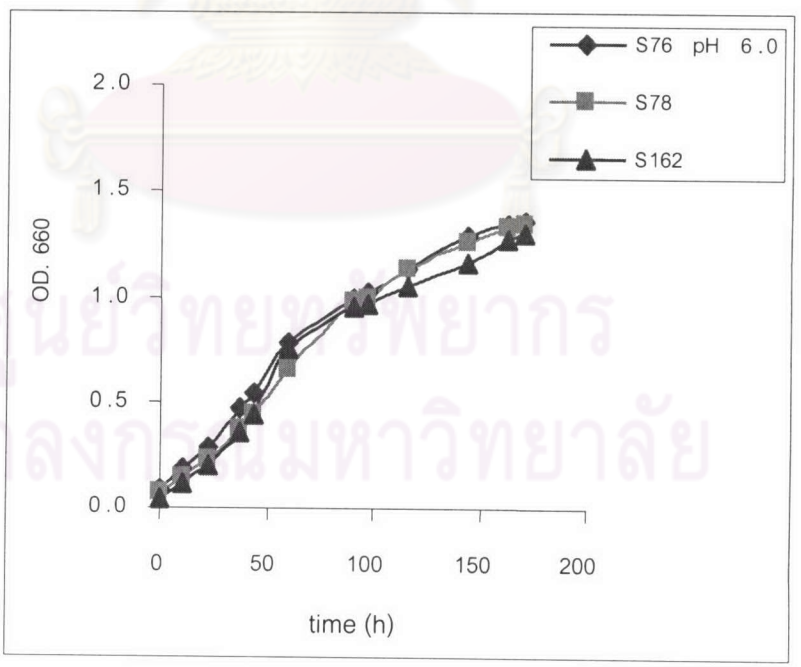

Figure 4.9.b. Growth curves of *B. japonicum* S76, S78 and S162 cultured in buffered yeast extract mannitol (YMB), pH 5.5, then shifted to YMB pH 6.0 and cultivated at 200 rpm, 30 °C.

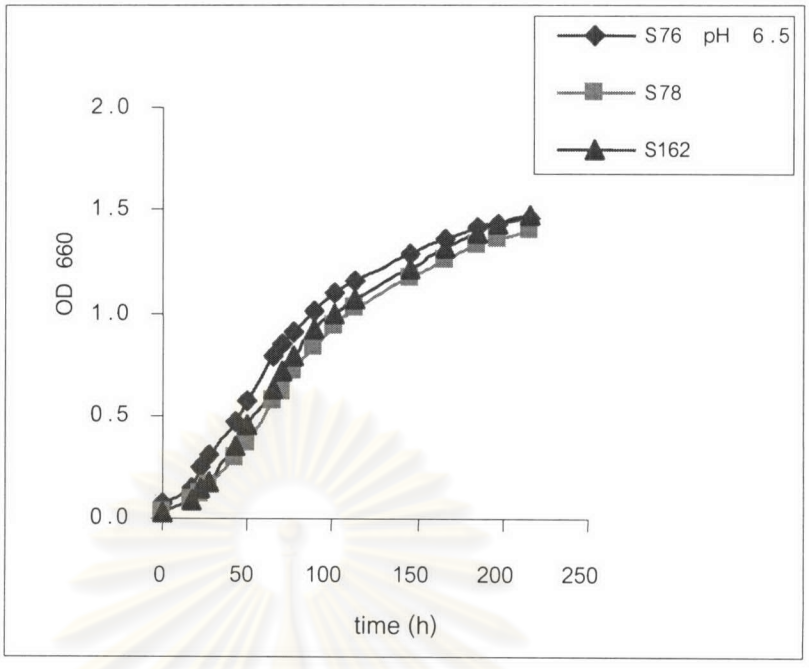

Figure 4.9.c. Growth curves of *B. japonicum* S76, S78 and S162 cultured in buffered yeast extract mannitol (YMB), pH 5.5, then shifted to YMB pH 6.5 and cultivated at 200 rpm, 30 °C.

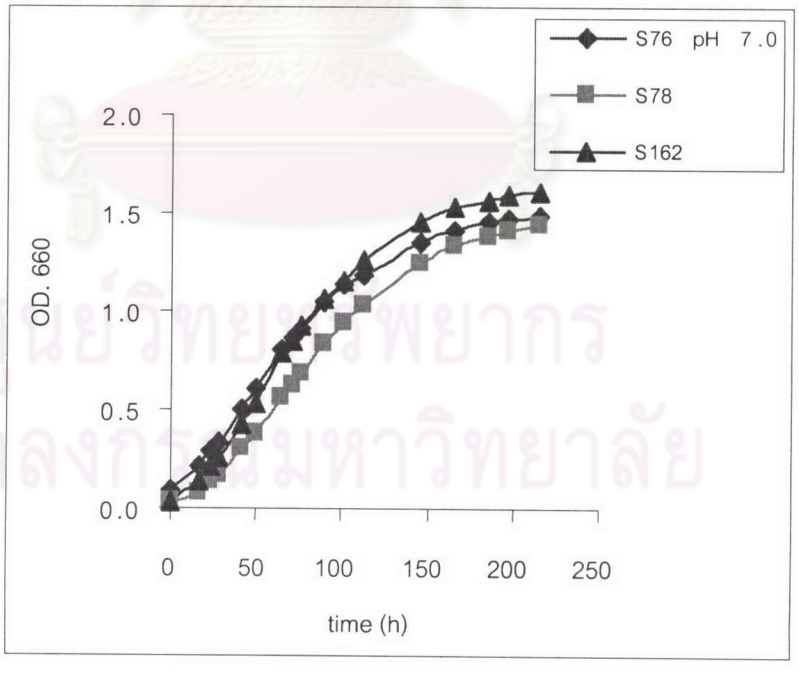

Figure 4.9.d. Growth curves of *B. japonicum* S76, S78 and S162 cultured in buffered yeast extract mannitol (YMB), pH 5.5, then shifted to YMB pH 7.0 and cultivated at 200 rpm, 30 °C.

S76
pH 5.5 → 6.0

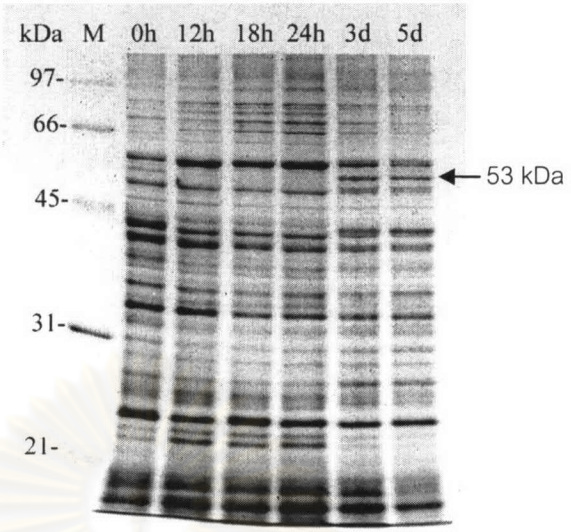

Figure 4.10 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S76 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.0 at 200 rpm, 30 °C.

S78
pH 5.5→ 6.0

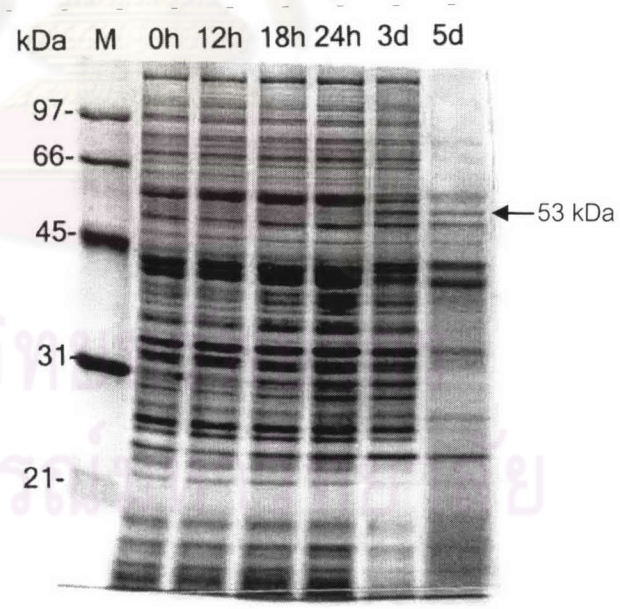

Figure 4.11 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S78 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.0 at 200 rpm, 30 °C.

S162
pH 5.5→ 6.0

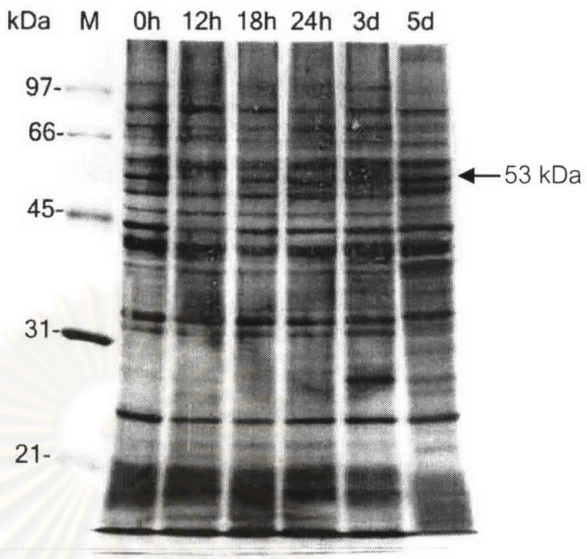

Figure 4.12 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S162 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.0 at 200 rpm, 30 °C.

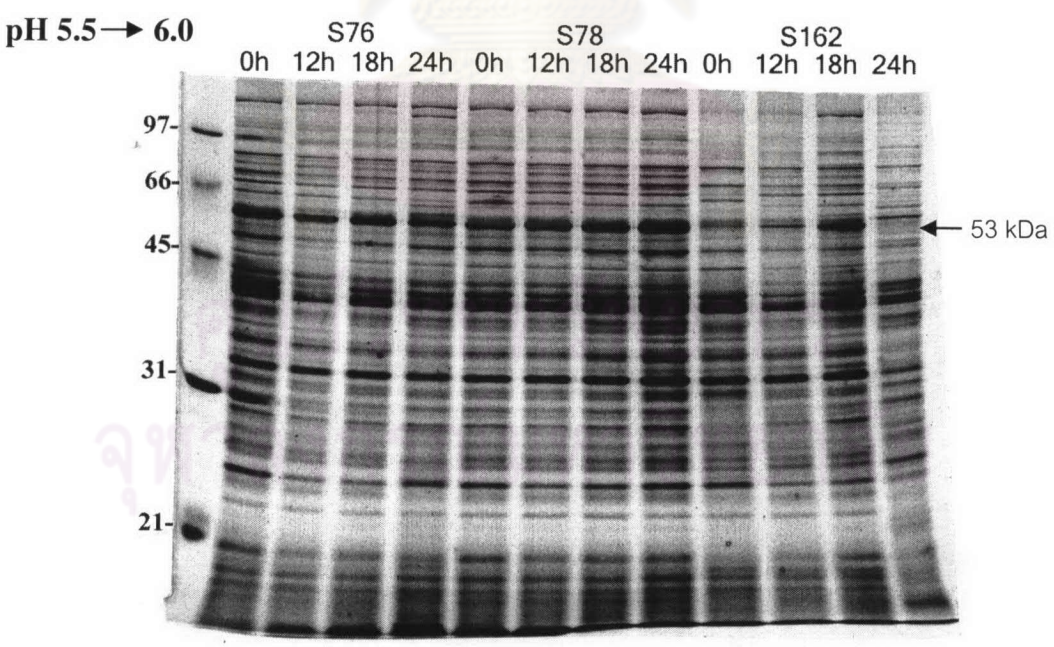

Figure 4.13 SDS-PAGE of intracellular protein profiles of *B. japonicum* strains S76, S78, and S162 cultured in YMB medium, pH 5.5 until mid-log phase, then transferred to YMB medium with pH buffered at 6.0 and cultured for 6 h, 12 h, 18 h and 24 h at 200 rpm, 30°C.

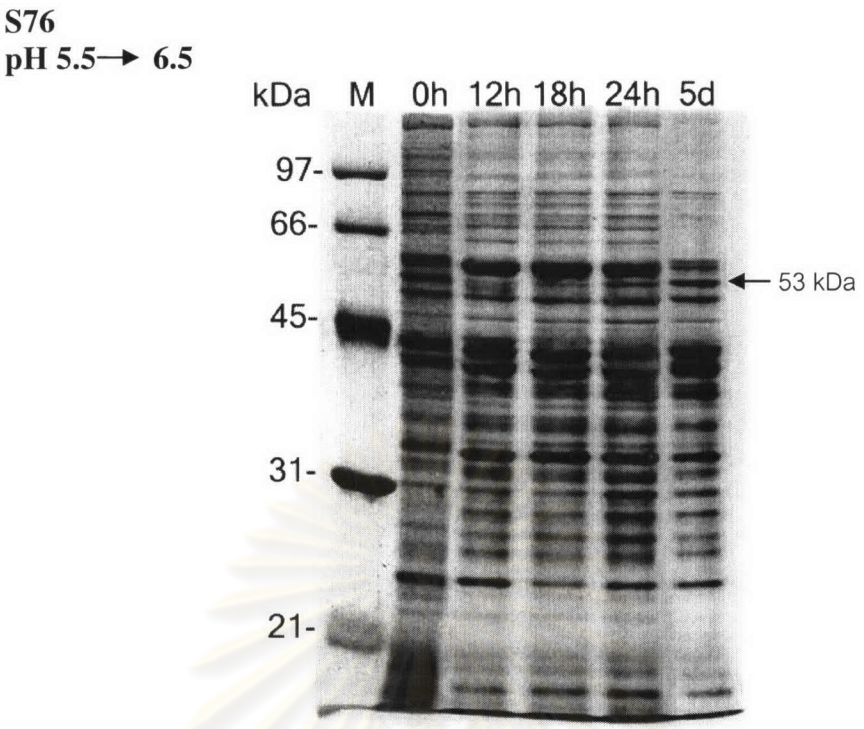

Figure 4.14 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S76 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.5 at 200 rpm, 30 °C.

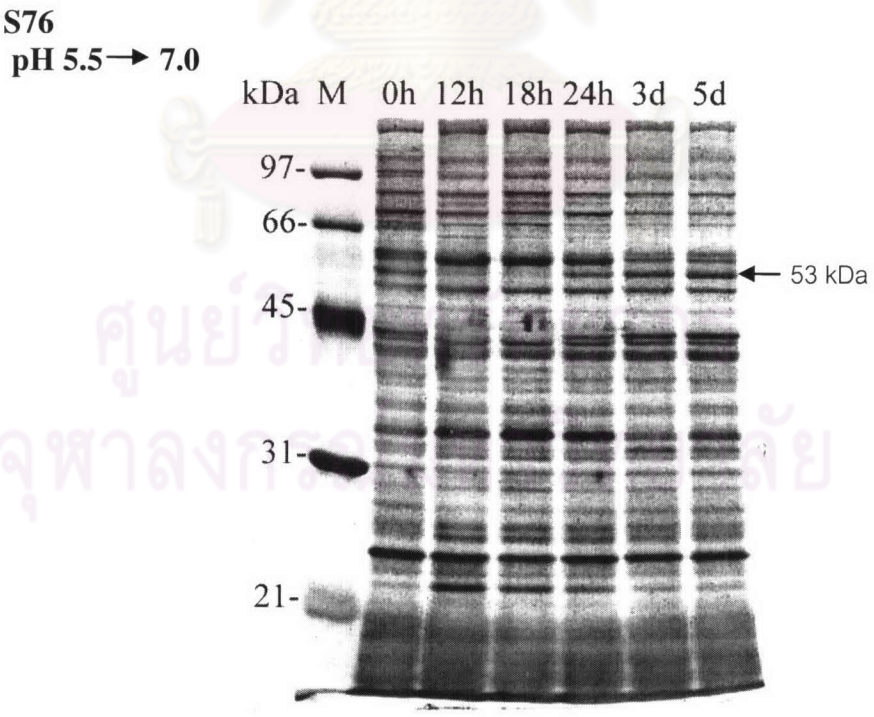

Figure 4.15 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S76 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 7.0 at 200 rpm, 30 °C.

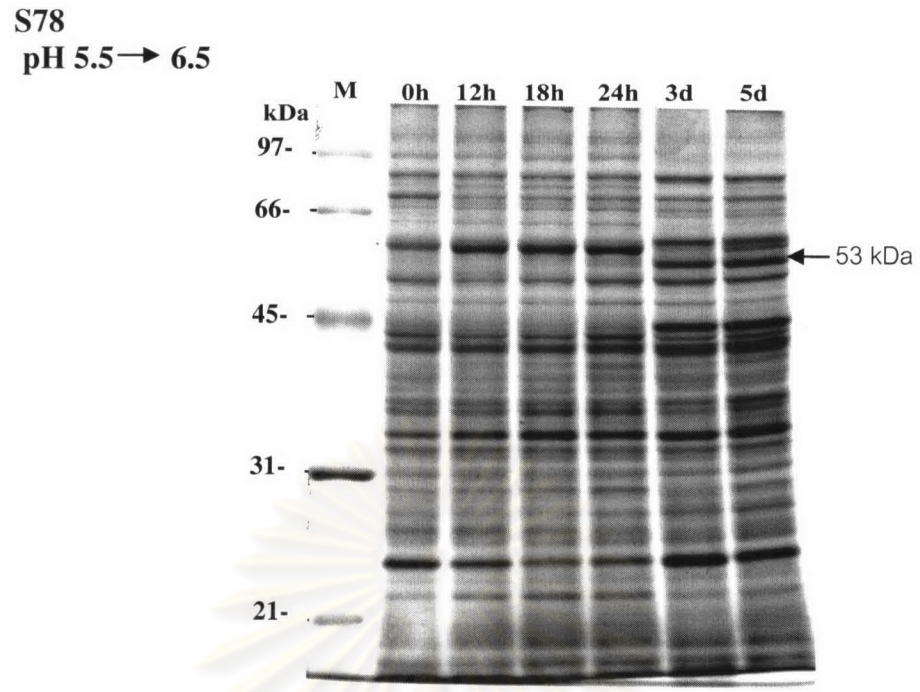

Figure 4.16 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S78 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.5 at 200 rpm, 30 °C.

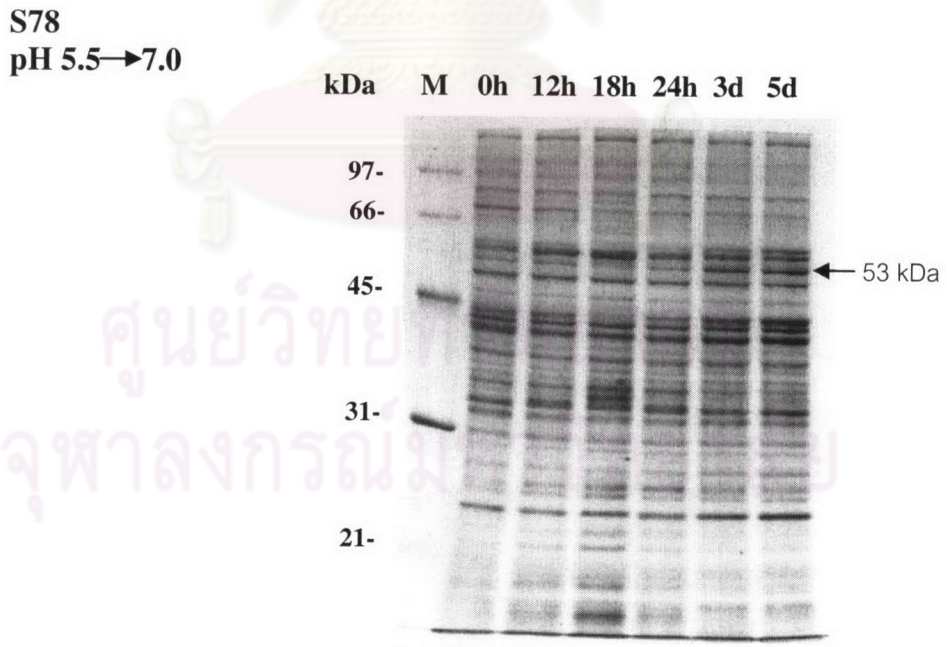

Figure 4.17 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S78 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 7.0 at 200 rpm, 30 °C.

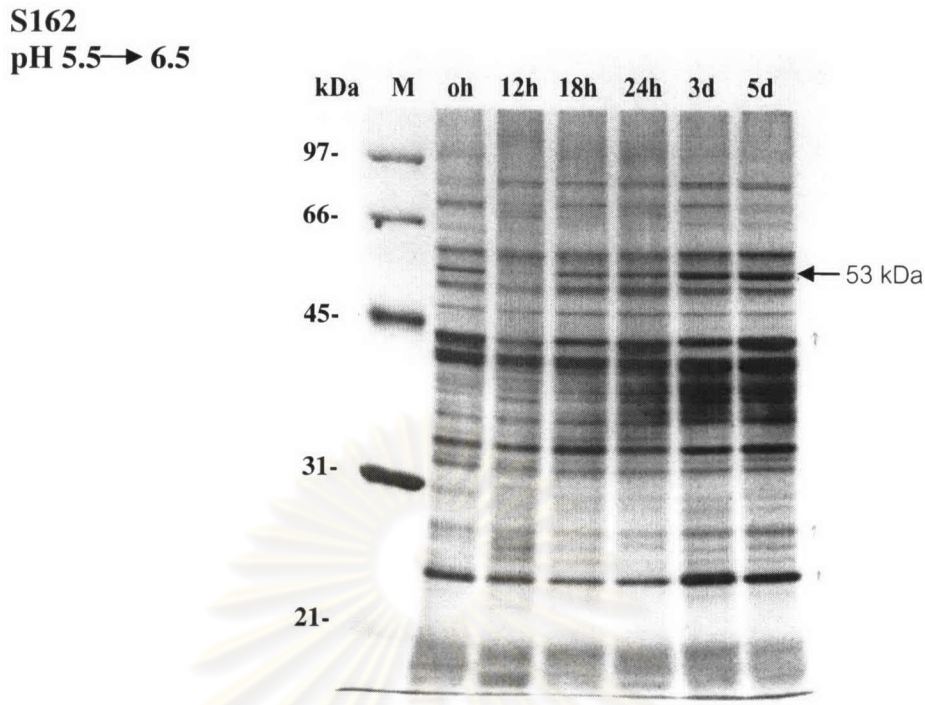

Figure 4.18 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S162 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 6.5 at 200 rpm, 30 °C.

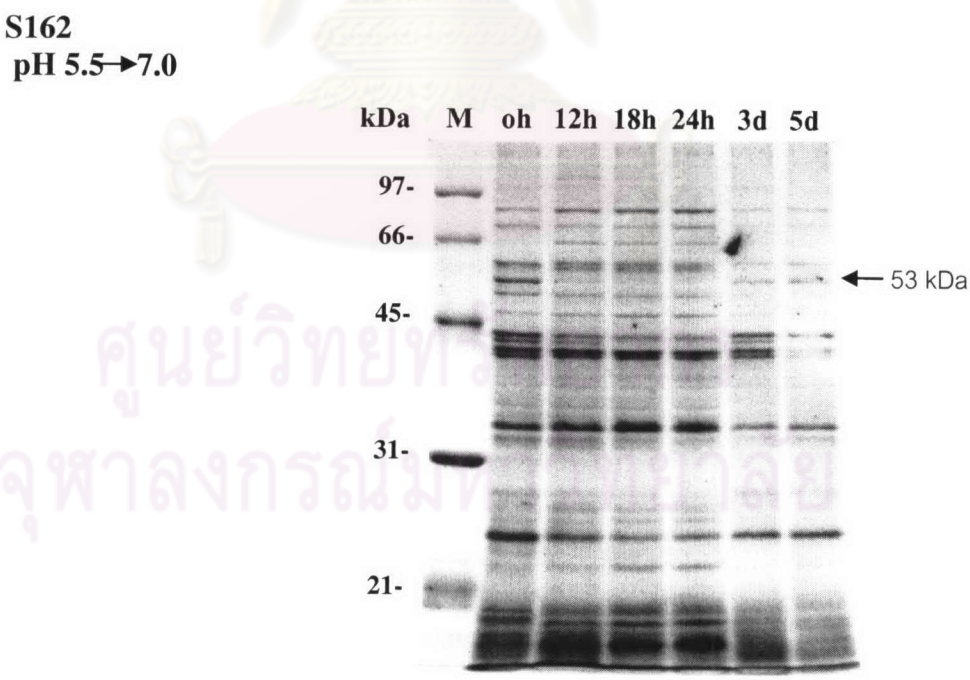

Figure 4.19 SDS-PAGE of intracellular protein profiles of mid-log phase cells *B. japonicum* S162 when cultured in buffered yeast extract mannitol medium pH 5.5 then transferred to pH 7.0 at 200 rpm, 30 °C.