Results

DNA base compositions

Pure DNA samples (A_{260}/A_{280} = 1.8) were isolated from bacterial cultures by the procedures detailed in Materials and Methods. Since these DNA would be used in subsequent reassociation experiments, their purity were checked by observing its denaturation in a temperature controlled spectrophotometer cell. When the DNA melted in 0.1xssc solution, no hyperchromicity was observed at temperatures lower than the DNA denaturation transition, indicating the absence of any RNA or single stranded DNA in the preparation. As a control for this denaturation experiment, calf thymus DNA and E. Coli B DNA were melted in paralleled with the tested bacterial DNA. They melted at Tm = 86.7° and 90.2 °C (Figure 1a, 1b) indicating the G+C content of 42.2 and 50.8 mol %, comparable to published values for calf thymus, 41.9 mol % (21) and E. Coli B DNA, 50.1 mol % (38) respectively.

The Tm values determined in 0.1xssc solution of K. oxytoca 1301, R15 and R17 fell within the range of 91.8 to 92.5°C (Figure 2) and 97.6 to 98.4°C for R25 A. 34H and A. FS (Figure 3) but for Pseudomonas spp., the Tm values ranged from 95.7 to 96.1°C (Figure 4) The G+C content calculated from the Tm's fell within the range of 54.7-56.4 mol %, 68.8-70.8 mol % and 64.2-65.2 mol % respectively (Table 4). Therefore, it seemed to be that the bacterial strains fell into three distinct groups. If significant differences in DNA base composition were to occur between the bacterial groups, this would be the evidence for the existence of more than one genus and the similarity within the

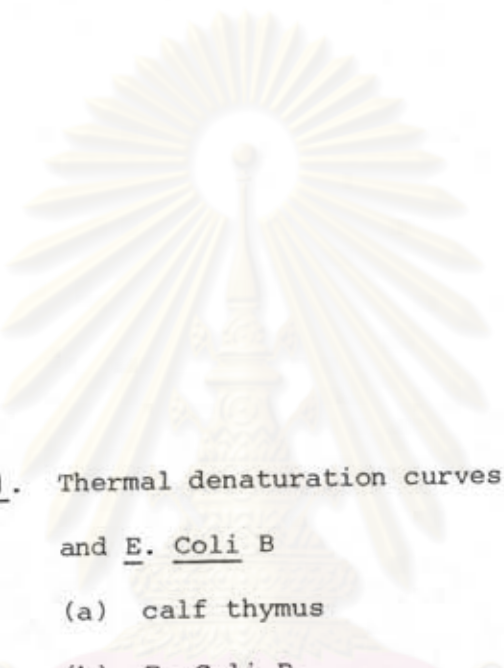
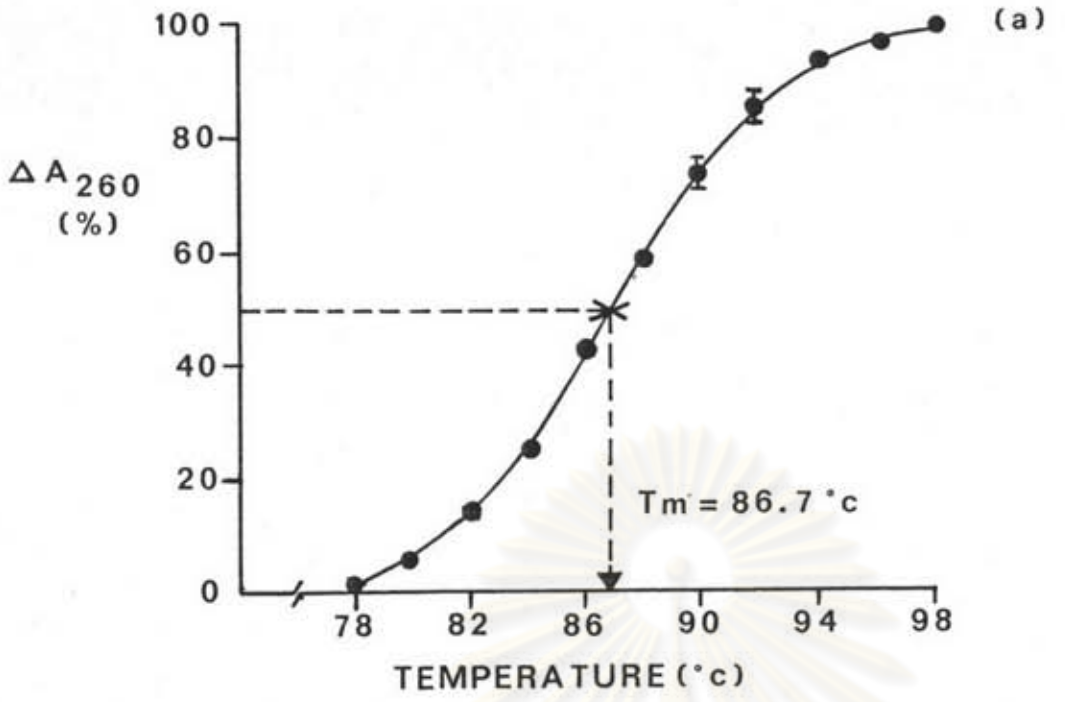


Figure 1. Thermal denaturation curves of DNA form calf thymus and E. Coli B

(a) calf thymus

(b) E. Coli B

Vertical bars represent the standard error of the mean from three replicates.



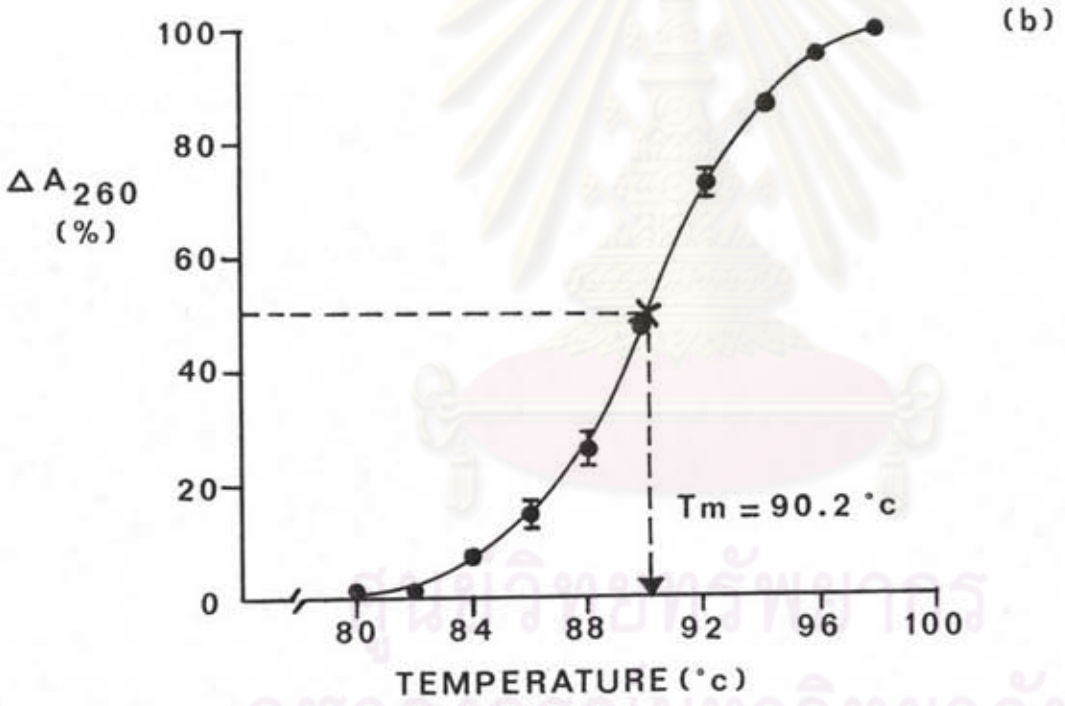


Figure 2. Thermal denaturation curves of DNA from R15, R17 and K. oxytoca 1301

(a) R15

(b) R17

(c) Klebsiella oxytoca 1301

Denaturation curves were determined in 0.1xssc solution (n=7 at each point, except (c) n=5). When the standard error was greater than the diameter of the symbol for the point on the line, an error bar to indicate the standard error is shown.

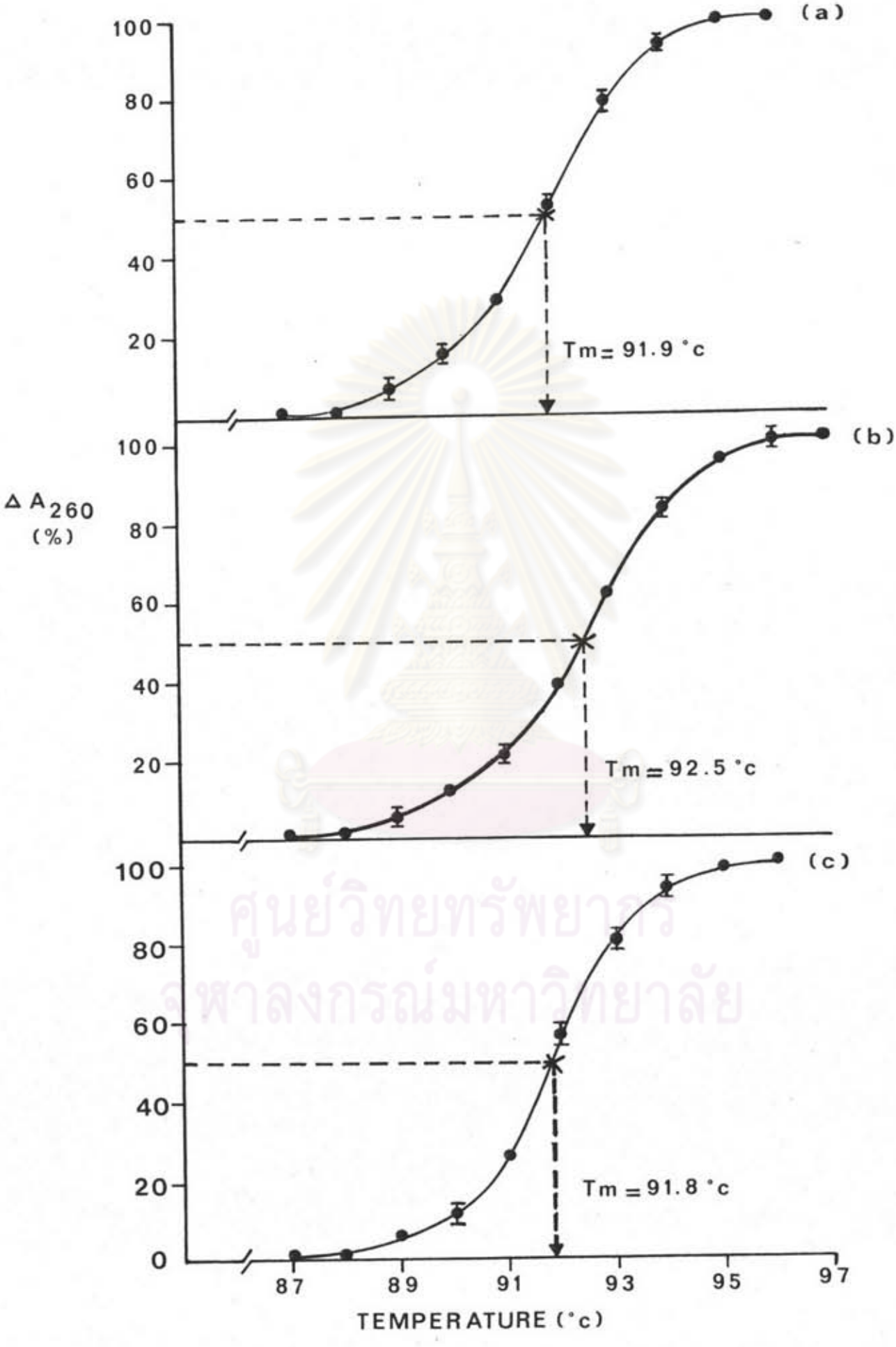


Figure 3. Thermal denaturation curves of DNA from R25 and *Azospirillum spp.*

(a) R25

(b) *A.* 34H

(c) *A.* FS

Denaturation curves were determined in 0.1xssc solution (n=5 at each point, except (a) n=8). When the standard error was greater than the diameter of the symbol for the point on the line, and error to indicate the standard error is shown.

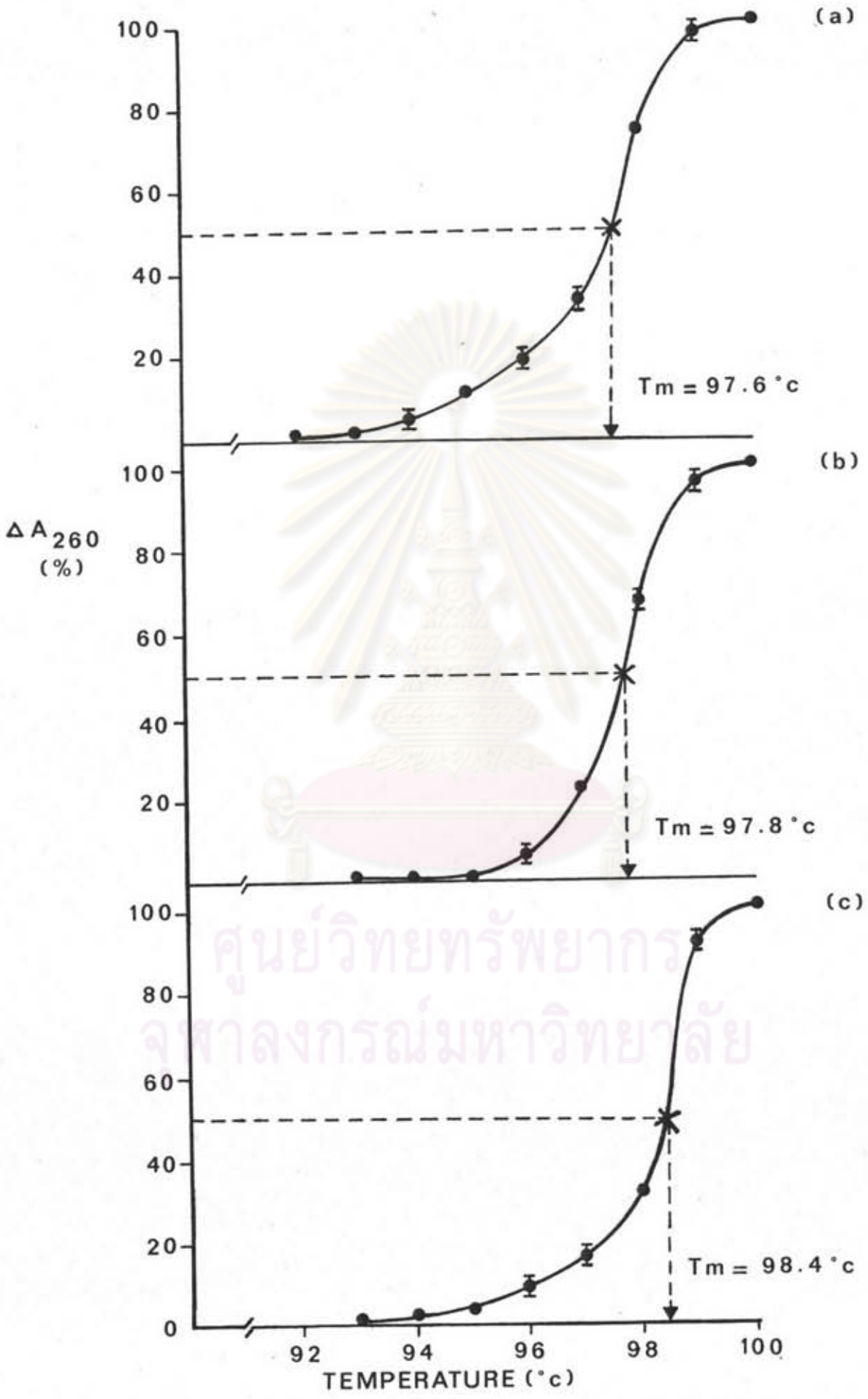


Figure 4. Thermal denaturation curves of DNA from Pseudomonas spp.

(a) P. H8

(b) P. KLH76

Both curves determined in 0.1xssc solution. Each point was carried out on 6 replicates. When the standard error was greater than the diameter of the symbol for the point on the line, an error bar to indicate the standard error is shown.

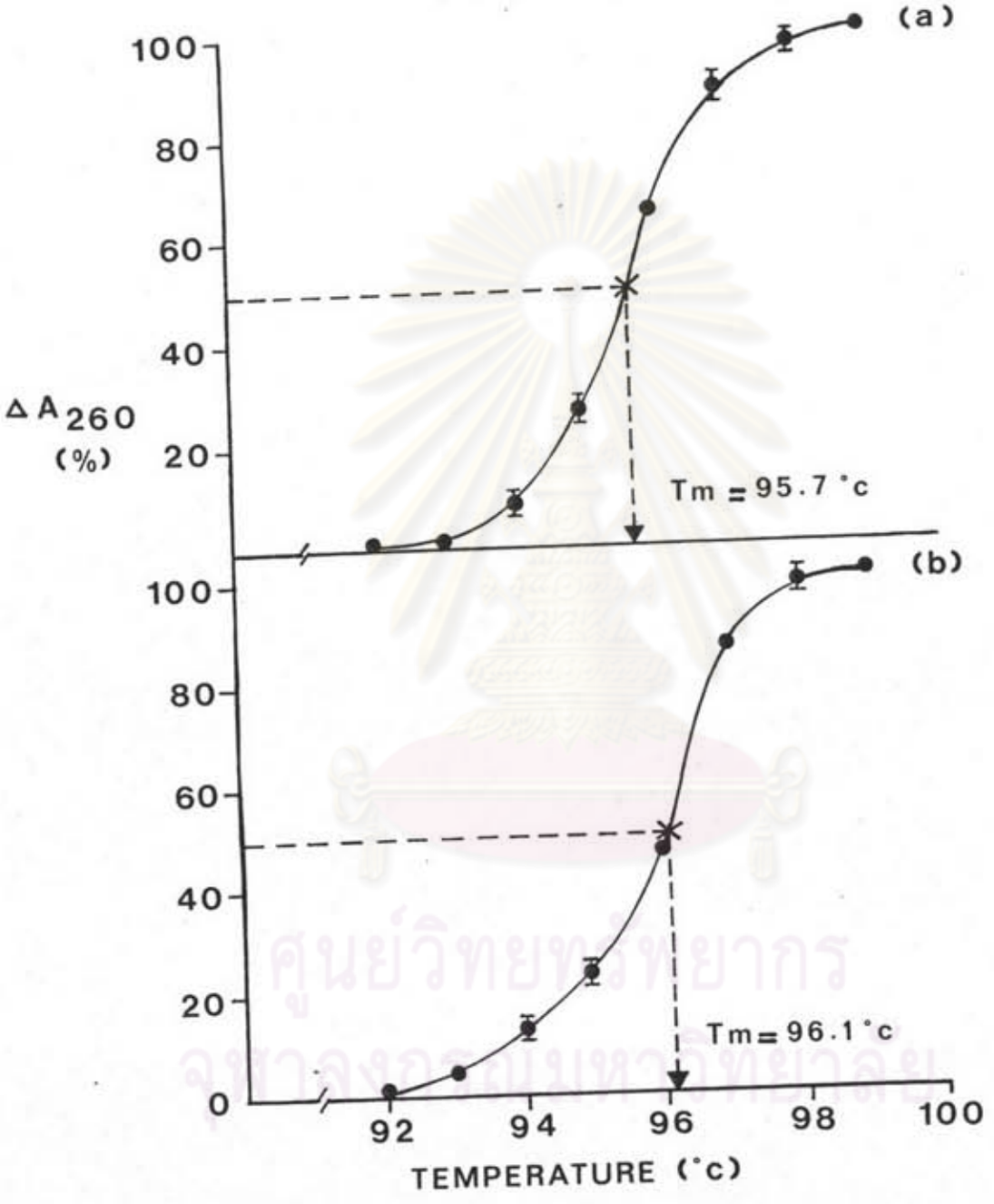


TABLE 4 Melting points (Tm values) and statistical analysis of mole percentage G+C of bacterial DNA using the spectrophotometric technique

Isolate designation	Average Tm, ^{o}C	Percentage G+C	N	S.D	Average (Cells/ml)	Statistical significance (between group)
R15	91.9	54.9	7	0.6	2×10^8	SD+
R17	92.5	56.4	7	0.7	1×10^8	
K. oxytoca 1301	91.8	54.7	5	0.4	3×10^8	
R25	97.6	68.8	8	0.9	8×10^7	SD+
A. 34H	97.8	69.4	5	0.7	1×10^8	
A. FS	98.4	70.8	5	1.1	8×10^7	
P. H8	95.7	64.2	6	1.3	6×10^7	SD+
P. KLH76	96.1	65.2	6	1.3	6×10^7	

SD+ = significant difference at 1 % level

bacterial groups indicated that the strains could possible, but not necessarily, all belong to the same genus. As indicated in Table 4, the mol % G+C values of R15 and R17 was similar to K. oxytoca 1301 but significantly different from standard strains of Azospirillum spp. and Pseudomonas spp. This similarity indicated that they belong to one genus. The G+C content of R25 was very close to the values of both Azospirillum spp. and Pseudomonas spp., but the result from statistical analysis indicated that the value of R25 including A. 34H and A. FS were significantly different from the values of Pseudomonas spp. (Appendix 1). However, to determine the exact genus of R25, its biochemical properties were compared to the properties of standard reference strains of both genus.

DNA sequence homology

In order to compare DNA sequence homology among unknown N_2-fixing bacteria and reference bacteria, denatured DNA were allowed to reassociate in 5M NaCl either between identical strains or hybrids, and the reassociation time (t_r) were measured. After complete reassociation, the remelting temperature (T_r) of these double stranded DNA were determined under high salt condition. The reassociation time (t_r) and the remelting temperature (T_r) of the self-hybridezed DNA was used as control to compare with t_r and T_r of different hybrid DNA. Longer reassociation time (t_r) by two-fold together with reduction in remelting temperature (T_r) by $10^{o}C$ are taken as strong evidence for difference in DNA sequence homology. Comparison of remelting curves among self-hybridized DNA and difference hybrid DNA were shown in Figures 5-9, and the differences in t_r (Δt_r) and T_r (ΔT_r) were shown in Table 5-9. It can be seen that when R15 and R17 were used as the reference strains

Figure 5. The remelting curves of hybrid-DNA with respect to self-hybridized R15

All curves determined in 5 M NaCl. Each point is the average of three replicates, an error bar represents standard error of the mean.

Symbols : (▲) hybrid-DNAs of R15 - P. H8; $T_r = 77.1^{\circ}C$

(O) hybrid-DNAs of R15 - A. 34H; $T_r = 81.1^{\circ}C$

(■) hybrid-DNAs of R15 - K. 1301; $T_r = 86.1^{\circ}C$

(●) hybrid-DNAs of R15 - R15; $T_r = 91.9^{\circ}C$

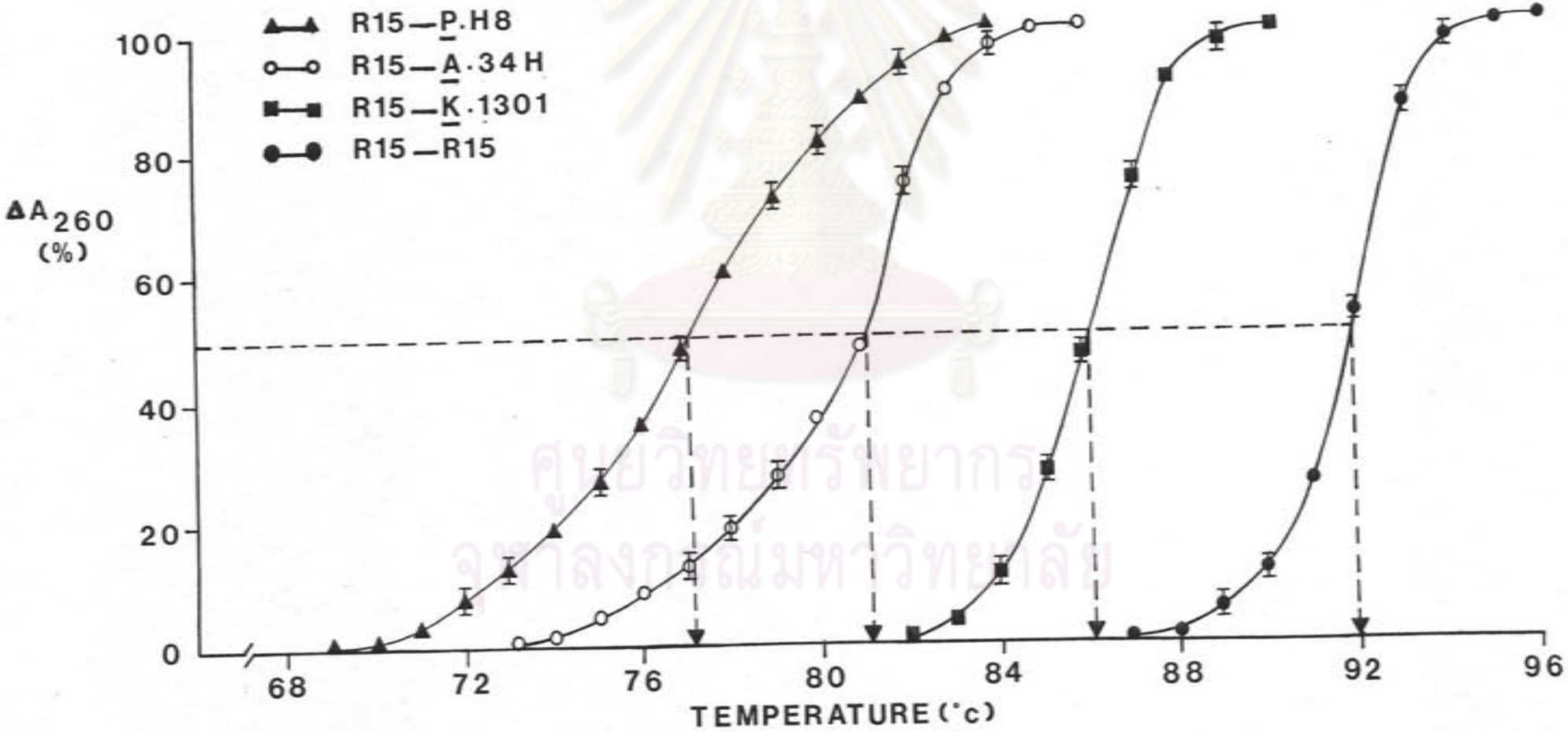


Figure 6. The remelting curves of hybrid-DNA with respect to self-hybridized R17.

All curves determined in 5 M NaCl. Each point is the average of three replicates, an error bar represents standrd erro of the mean.

Symbols : (▲) hybrid-DNA of R17 - P. H8; T_r = 78.6°C

(○) hybrid-DNA of R17 - A. 34H; T_r = 79.8°C

(■) hybrid-DNA of R17 - K. 1301; T_r = 85.4°C

(●) hybrid-DNA of R17 - R17; T_r = 92.5°C

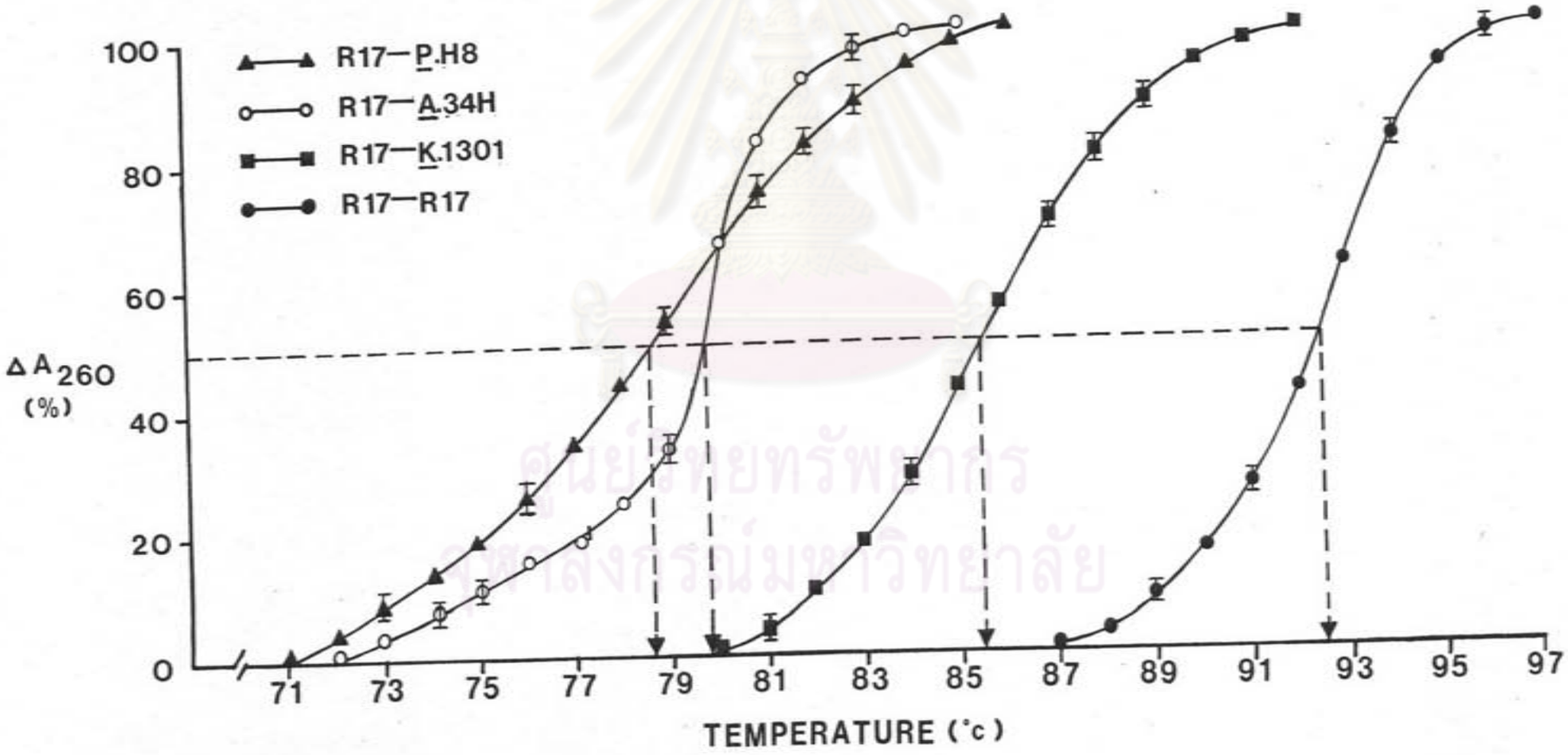


Figure 7. The remelting curves of hybird-DNA with respect to the self-hybridized R25.

All curves determined in 5 M NaCl. Each point is the average of three replicates, an error bar represents standard error of the mean.

Symbols for a; (O) hybrid-DNA of R25- P. H8; T_r = 76.1°C

(▲) hybrid-DNA of R25- P. KLH76; T_r = 79.8°C

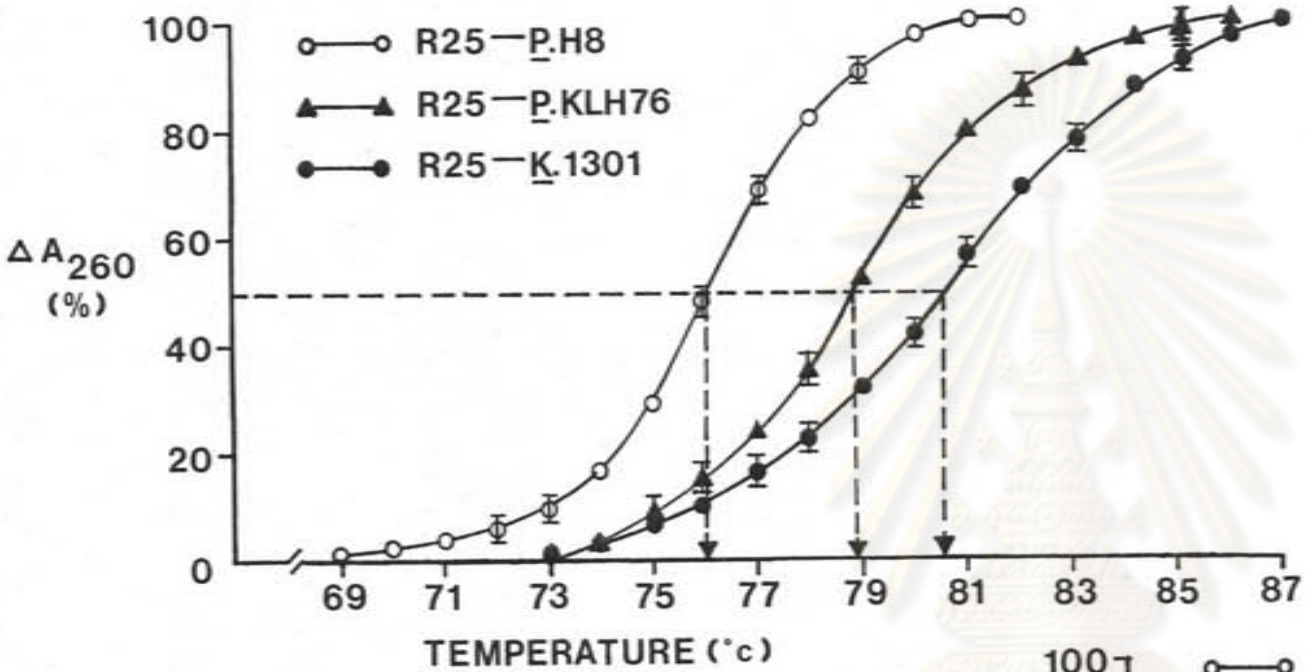
(●) hybrid-DNA of R25- K. 1301; T_r = 80.6°C

b; (O) hybrid-DNA of R25- A. 34H; T_r = 91.7°C

(▲) bybrid-DNA of R25- A. FS; T_r = 92.2°C

(■) bybrid-DNA of A. 34H - A. FS; T_r = 93.4°C

(●) hybrid-DNA of R25- R25; T_r = 97.6°C



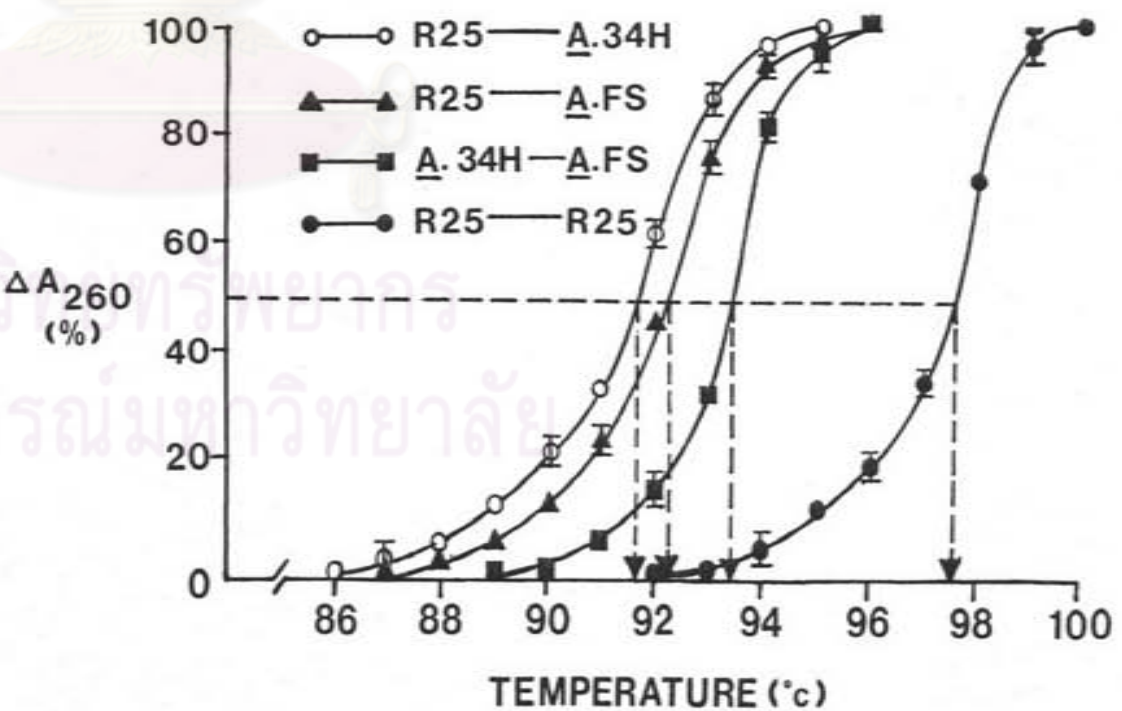


Figure 8. The remelting curves of hybrid-DNA which formed between K. oxytoca 1301 and P. H8 or A. 34H

The curves determined in 5 M NaCl. Each point is the average of three replicates, an error bar represents standard error of the mean.

Symbols: (■) hybird-DNA of K. 1301 - P. H8; T_r = 75.7°C

(●) hybrid-DNA of K. 1301 - A. 34H; T_r = 75.8°C

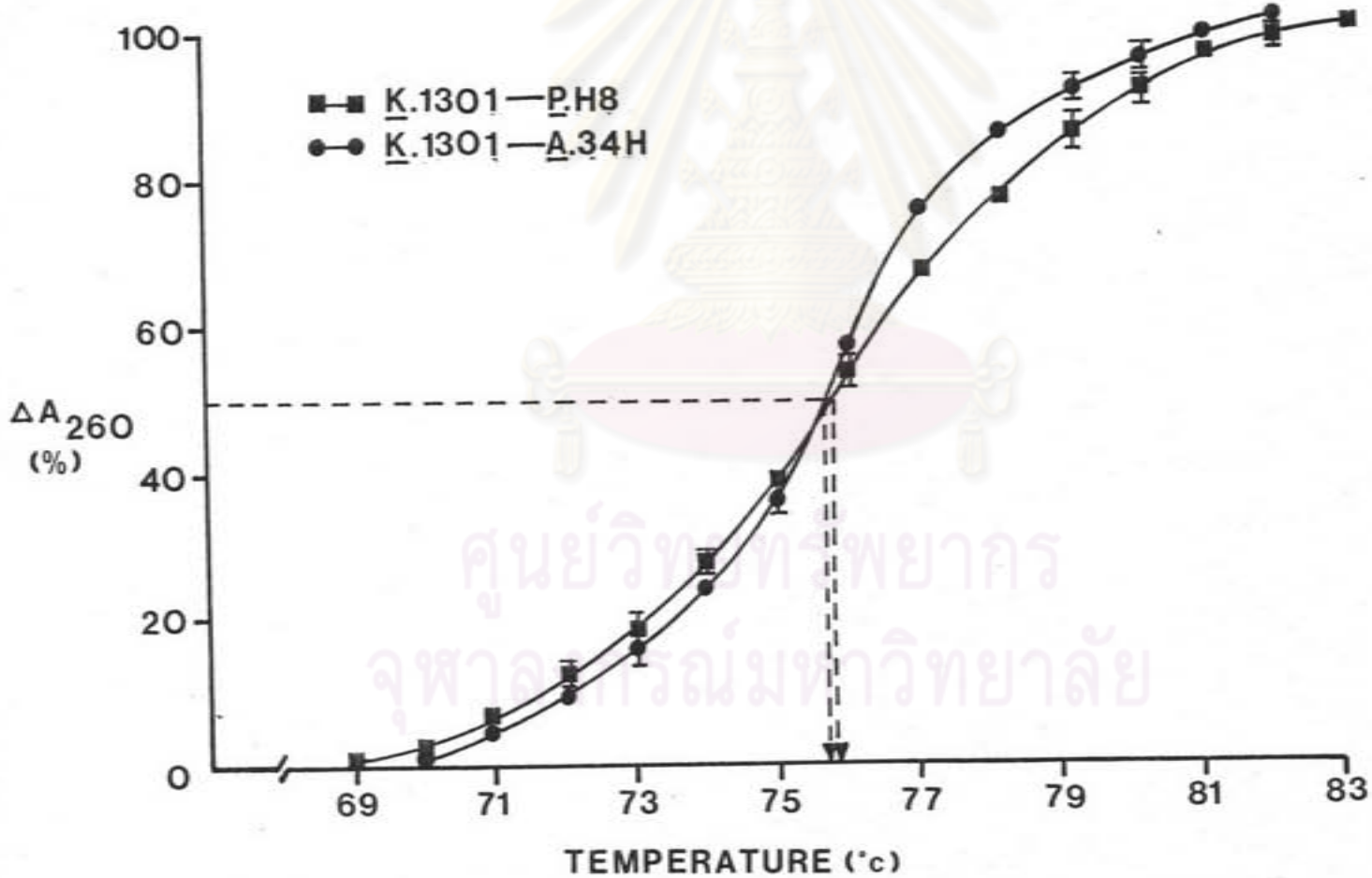


Figure 9. The remelting curves of hybrid-DNA which formed between standard strains of Azospirillum spp. and Pseudomonas spp.

All curves determined in 5 M NaCl, each point is the average of three replicates. An error bar represents standard error of the mean.

Symbols: (○) hybrid-DNA of A. 34H - P. KLH76; T_r = 70.6°C

(▲) hybrid-DNA of A. 34H - P. H8; T_r = 72.8°C

(■) hybrid-DNA of A. FS - P. KLH76; T_r = 74.0°C

(●) hybrid-DNA of A. FS - P. H8; T_r = 74.4°C

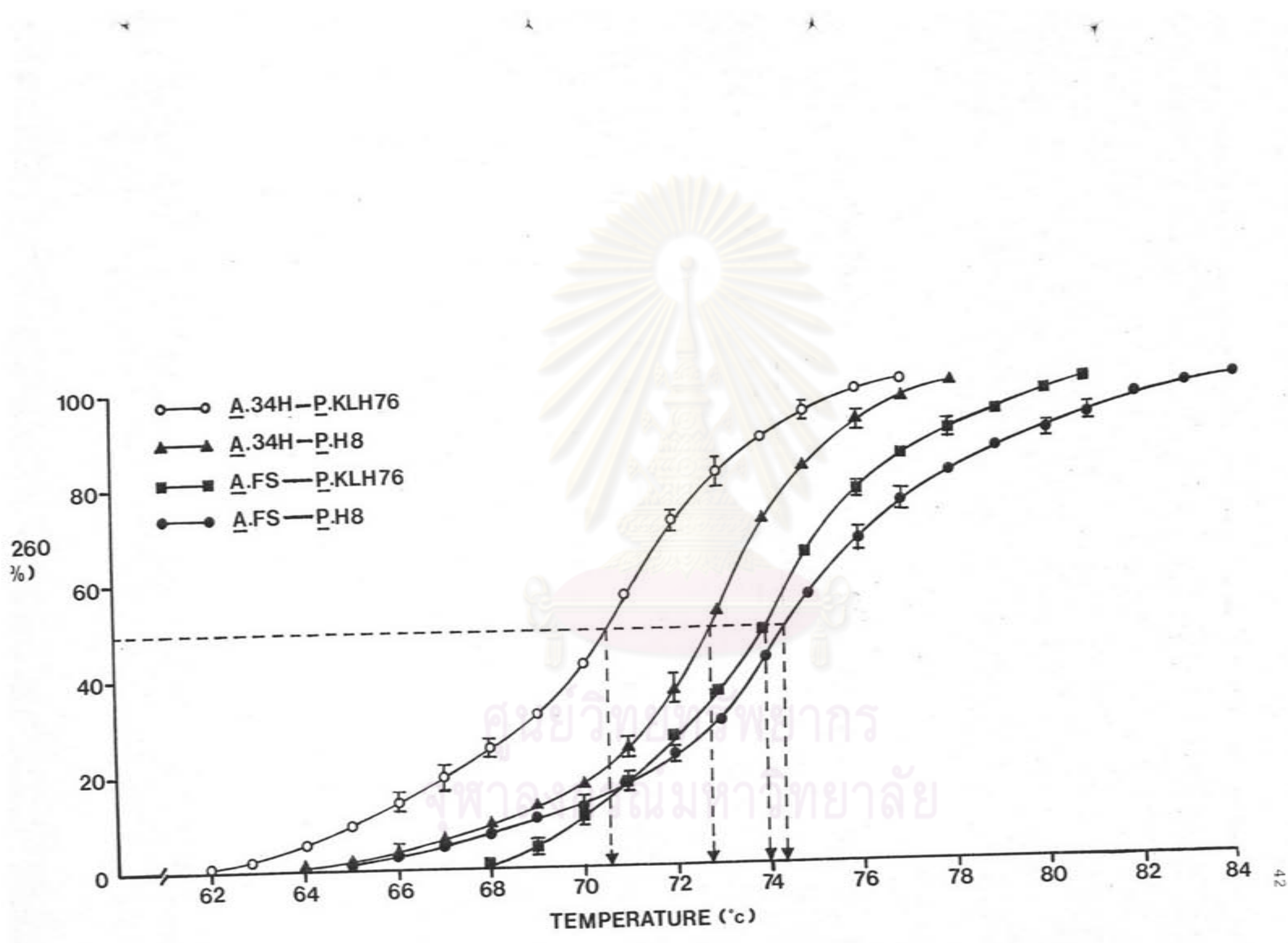


Figure 10. The remelting curves of hybrid-DNA which formed between P. H8 and P. KLH76.

The curves determined in 5 M NaCl, each point is the average of three replicates. An error bar represents standard error of the mean.

Symbols: (▲) hybrid-DNA of P. H8 - P. KLH76; T_r = 89.2°C

(●) hybrid-DNA of P. H8 - P. H8; T_r = 95.7°C

(■) hybrid-DNA of P. KLH76 - P. KLH76; T_r = 96.1°C

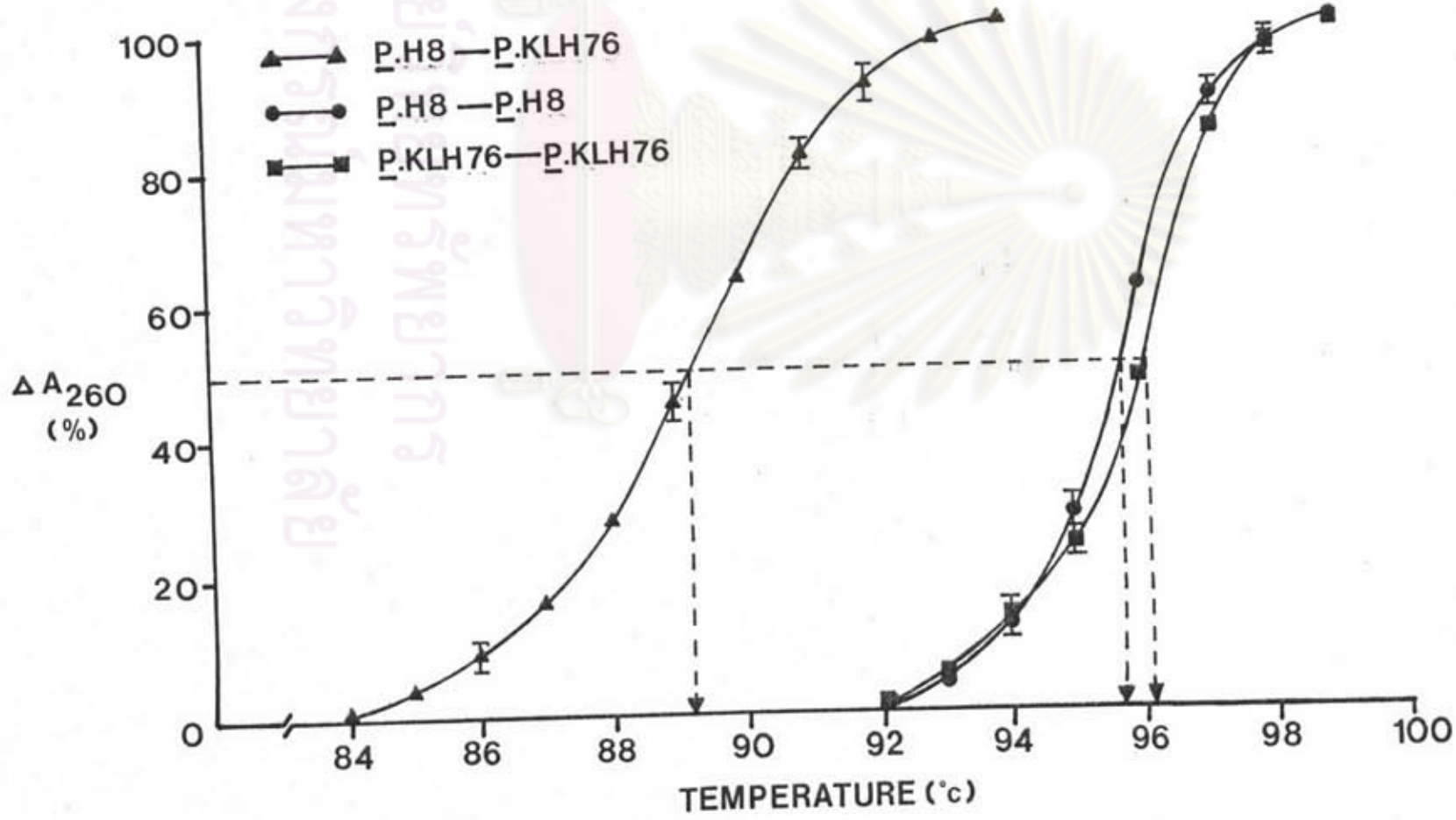

P.H8 —P.KLH76
P.H8 —P.H8
P.KLH76—P.KLH76
100
80
60
40
20
0
ΔA260 (%)
84
86
88
90
92
94
96
98
100
TEMPERATURE (°c)

Figure 11. The remelting curves of hybrid-DNA which formed between unknown bacteria.

All curves determined in 5 M NaCl, each point is the average of three replicates. An error bar represents standard error of the mean.

Symbols: (○) hybrid-DNA of R25- R17; T_r = 75.5°C

(▲) hybrid-DNA of R25-R15; T_r = 77.9°C

(●) hybrid-DNA of R15-R17; T_r = 83.7°C

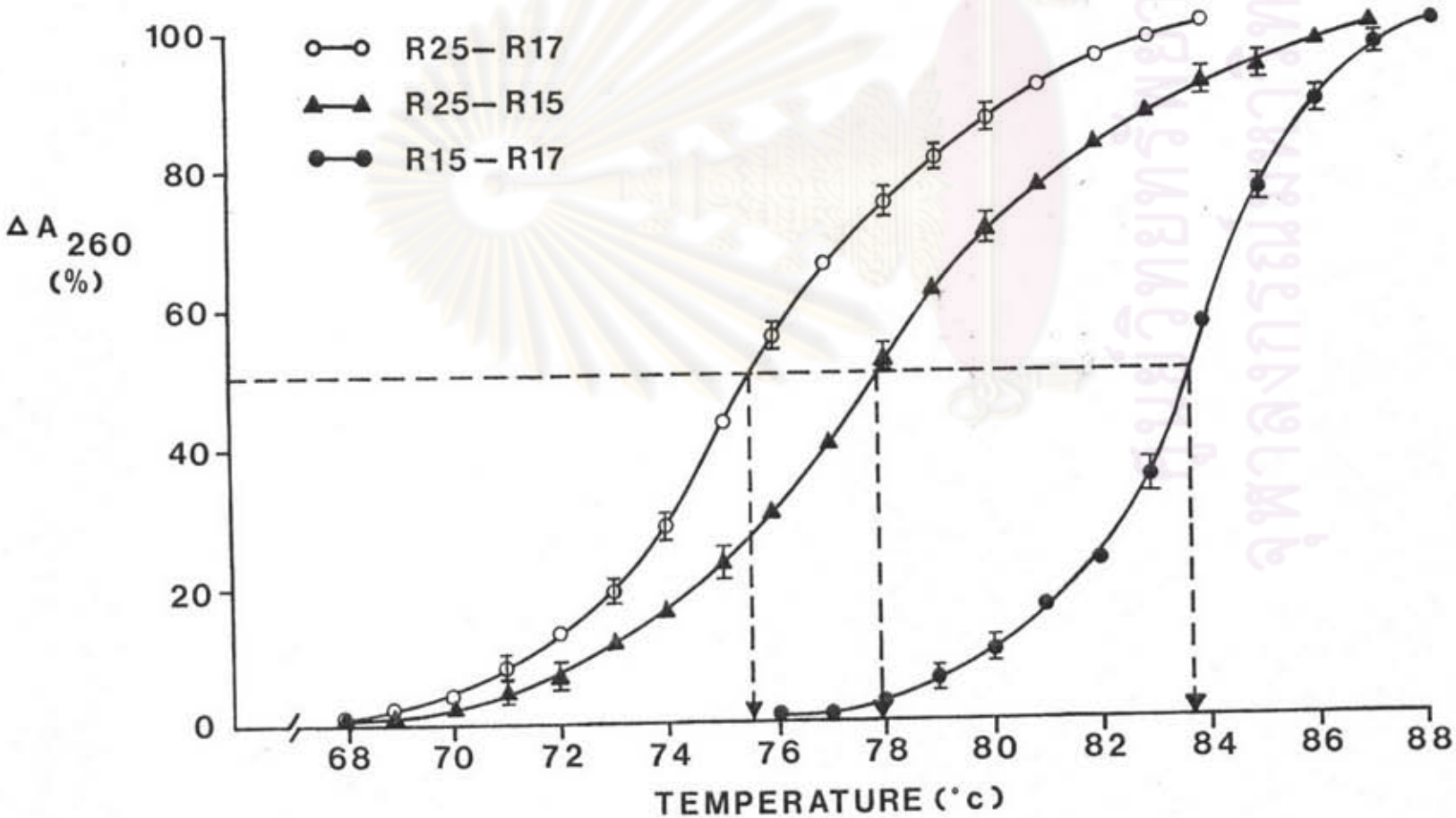

R25–R17
R25–R15
R15–R17
100
80
60
40
20
0
ΔA 260 (%)
68
70
72
74
76
78
80
82
84
86
88
TEMPERATURE (°c)

Figure 12. The remelting curves of hybrid-DNA which formed between R15, R17 and K. oxytoca 1301.

All curves determined in 5 M NaCl, each point is the average of three replicates. An error bar represents standard error of the mean.

Symbols: (O) hybrid-DNA of R15-R17; T_r = 83.7°C

(▲) hybrid-DNA of R17- K. 1301; T_r = 85.4°C

(●) hybrid-DNA of R15- K. 1301; T_r = 86.1°C

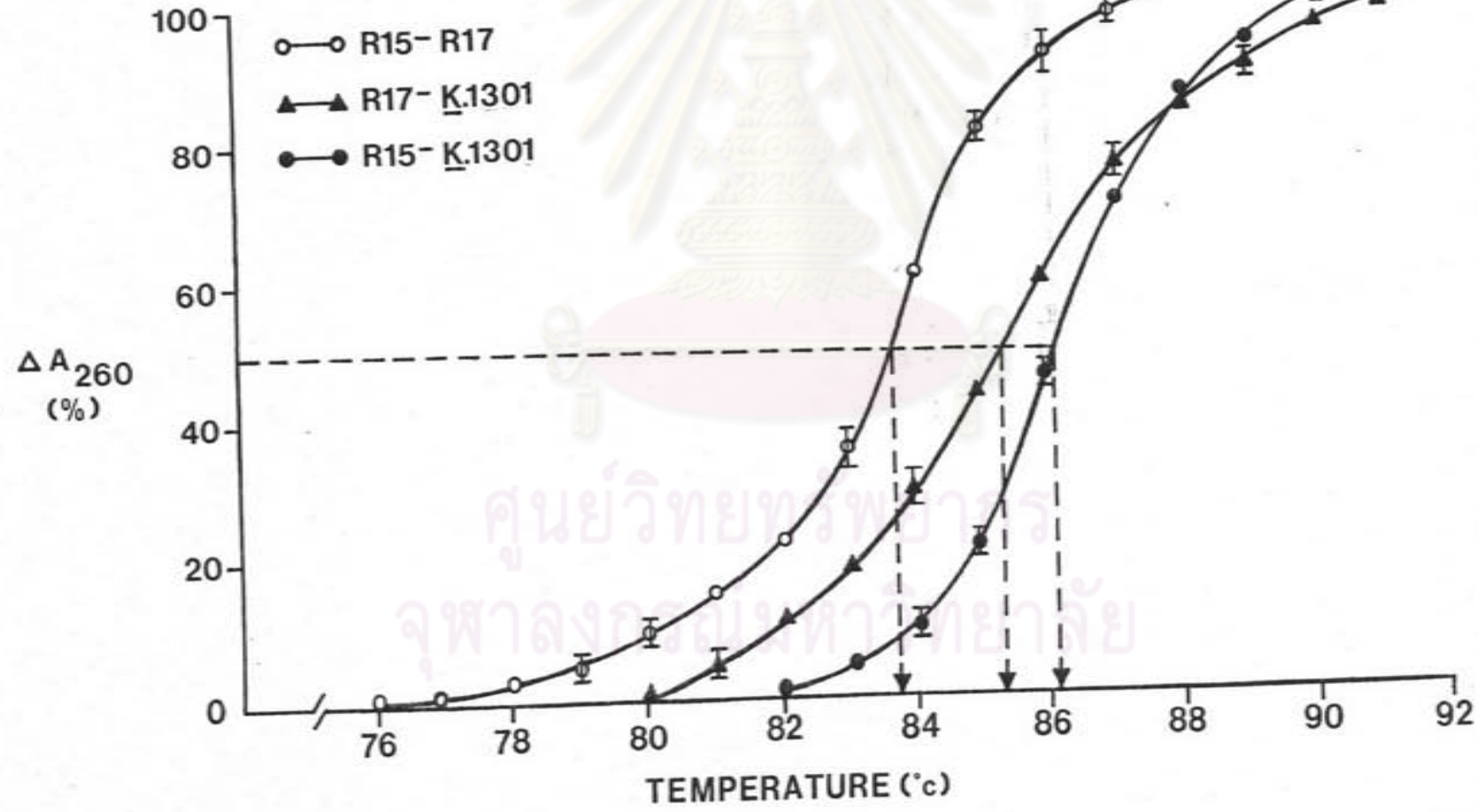


TABLE 5 The reassociation time (t_r) and the remelting temperature (T_r) of the hybrid-DNAs between R15 and standard reference strains (one of each genus)

Source of hybrid-DNAs	t_r (sec) (n=3)	Δt_r (sec) (t_r-27)	T_r (°C) (n=3)	ΔT_r (°C) (T_r-91.9)
R15 - R15	27	-	91.9	-
R15 - K.1301	37	10	86.1	-5.8
R15 - A.34H	63	36	81.1	-10.8
R15 - P.H8	68	41	77.1	-14.8

TABLE 6 The reassociation time (t_r) and the remelting temperature (T_r) of the hybrid-DNAs between R17 and standard reference strains (one of each genus)

Source of hybrid-DNAs	t_r (sec) (n=3)	Δt_r (sec) (t_r-23)	T_r (°C) (n=3)	ΔT_r (°C) (T_r-92.5)
R17 - R17	23	-	92.5	-
R17 - K.1301	36	13	85.4	-7.1
R17 - A.34H	58	35	79.8	-12.7
R17 - P.H8	61	38	78.6	-13.9

TABLE 7. The reassociation time (t_r) and the remelting temperature (T_r) of the hybrid-DNAs between R25 and standard reference strains.

Source of hybrid-DNAs	t_r (sec) (n=3)	Δt_r (sec) (t_r-19)	T_r (^oC) (n=3)	ΔT_r (^oC) (T_r-97.6)
R25 - R25	19	-	97.6	-
R25 - A.FS	27	8	92.2	-5.4
R25 - A.34H	31	12	91.7	-5.9
R25 - K.1301	55	36	80.6	-17.0
R25 - P.KLH76	65	46	78.9	-18.7
R25 - P.H8	72	53	76.1	-21.5

TABLE 8. The reassociation time (t_r) and the remelting temperature (T_r) of the hybrid-DNAs between standard reference strains

Source of Hybrid-DNAs	t_r (sec) (n=3)	Δt_r (sec) $(t_r - \bar{X}_1)$*	T_r (°C) (n=3)	ΔT_r (°C) $(T_r - \bar{X}_2)$**
K.1301 - A.34H	69	47	75.8	-19.0
K.1301 - P.H8	66	45	75.7	-18.3
A.34H - A.FS	27	7	93.4	-4.7
A.34H - P.H8	73	53	72.8	-24.2
A.34H - P.KLH76	75	57	70.6	-26.2
A.FS - P.H8	71	54	74.4	-22.8
A.FS - P.KLH76	69	52	74.0	-23.1
P.H8 - P.KLH76	28	10	89.2	-6.7

* $\bar{X}_1$ = average t_r between two bacteria that formed the hybrid-DNAs

** $\bar{X}_2$ = average T_r between two bacteria that formed the hybrid-DNAs

TABLE 9. The reassociation time (tr) and the remelting temperature (T_r) of the hybrid-DNAs between unknown bacteria

Sorce of hybrid-DNAs	t_r (sec) (n=3)	Δt_r (sec) $(t_r - \bar{X}_1)$*	T_r (°C) (n=3)	ΔT_r (°C) $(T_r - \bar{X}_2)$**
R15 - R17	42	17	83.7	-8.5
R15 - R25	61	38	77.9	-16.9
R17 - R25	67	46	75.5	-19.6

* $\bar{X}_1$ = average t_r between two bacteria that formed the hybrid-DNAs

** $\bar{X}_2$ = average T_r between two bacteria that formed the hybrid-DNAs

(Figure 5, 6 and Table 5, 6), the other strains fell into three distinct groups: that contain strain K. 1301, A. 34H and P. H8 respectively. K. 1301 exhibited high homology with both R15 and R17 because when their DNAs were reassociated, the t_r value increased only about 10-13 sec., and the T_r decreased only 5.8-7.1°C from the t_r and Tm of self-reassociated DNA. A. 34H and P. H8 exhibited low homology with R15 and R17, because they gave the high increasing value of t_r more than 2-fold and greater decreasing value of T_r ($-\Delta Tr > 10^{\circ}C$) which are in the range of 35-41 sec. and 10.8-14.8°C respectively. In the reciprocal situation where self-hybridized DNA from R25 was used as the reference (Figure 7 and Table 9), K. 1301 which had exhibited high homology with R15 and R17 was found to have low homology with R25 DNA. The highest homology was observed between DNA from R25 and those Azospirillum spp. i.e. A. FS and A. 34H, as evident by Δt_r of only 8-12 sec and $-\Delta T_r$ of 5.4-5.9°C

Since the Tm of R25 DNA was found to be close to Tm of both Azospirillum spp. and Pseudomonas spp., so DNA from P. KLH76 and A. FS were used to performed reassociation experiments wiht DNA extracted form R25. The result indicated that DNA from R25 exhibited high homology only with Azospirillum spp. not Pseudomonas spp. because reassociation of DNA from R25 with DNA from P. KLH76 and P. H8 showed Δt_r of 46-53 sec which were greater than 2-fold of R25-R25 (19 sec) and $-\Delta T_r$ of 17-21.5°C as shown in Table 7.

The results of reassociation experiments between DNA from standard strains of various genera were shown in Figure 8-11 and Table 8. When the strains from different genera were reassociated, they gave longer t_r values and respective Δt_r in the range of 45-57 sec and $-\Delta T_r$ values in the range of 18.3-26.2°C, whereas reassociation

within the same genus, the Δt_r values were 7-10 sec and the $-\Delta T_r$ were 4.7-6.7°C respectively.

These results also indicated that, although standard Azospirillum spp. had mol % G+C values similar to those of standard Pseudomonas spp., no DNA sequence homology values higher than K. oxytoca 1301 were observed for these strains.

DNA sequence homology among unknown N_2-fixing bacterial strains R15, R17 and R25 were also determined. As shown in Figure 11 and Table 9, R15 exhibited high homology with R17 but not R25. The results suggested that R15 and R17 could possibly belong to a single species in the same genus but R25 should belong to the distinct and unrelated genus.

Plasmid detection in N_2-fixing bacteria

Screening of plasmids was performed using various strains of N_2-fixers indicated that their plasmids were easily resolved by the protocal described herein. Plasmid of various sized harbored in these bacteria were detected by agarose gel electrophoresis in 7 out of 8 strains tested by comparison to the standard marker DNA (Figure 13). R15 and R17 harbored a plasmid of molecular weight larger than 33 M dal (λ DNA) whereas A.34H, A.FS, P.H8 and P.KLH76 harbored a plasmid of molecular weight very close to the molecular weight of chromosomal DNA. Plasmids of R15, R17, P.H8, P.KLH76 were detected after heating at 65°C for 30 min (Figure 13 a, c) but of R25, A.FS and A.34H were detected after heating for 60 min (Figure 13 b). As seen from the Figure 13 a,b,c the plasmid profile of R15 and R17 were similar to each other but the plasmid profile of R25 was similar to A.FS and A. 34H which were different from P.H8 and P.KLH76. No plasmids was detected in K.1301 and thus it may be considered as plasmidless.

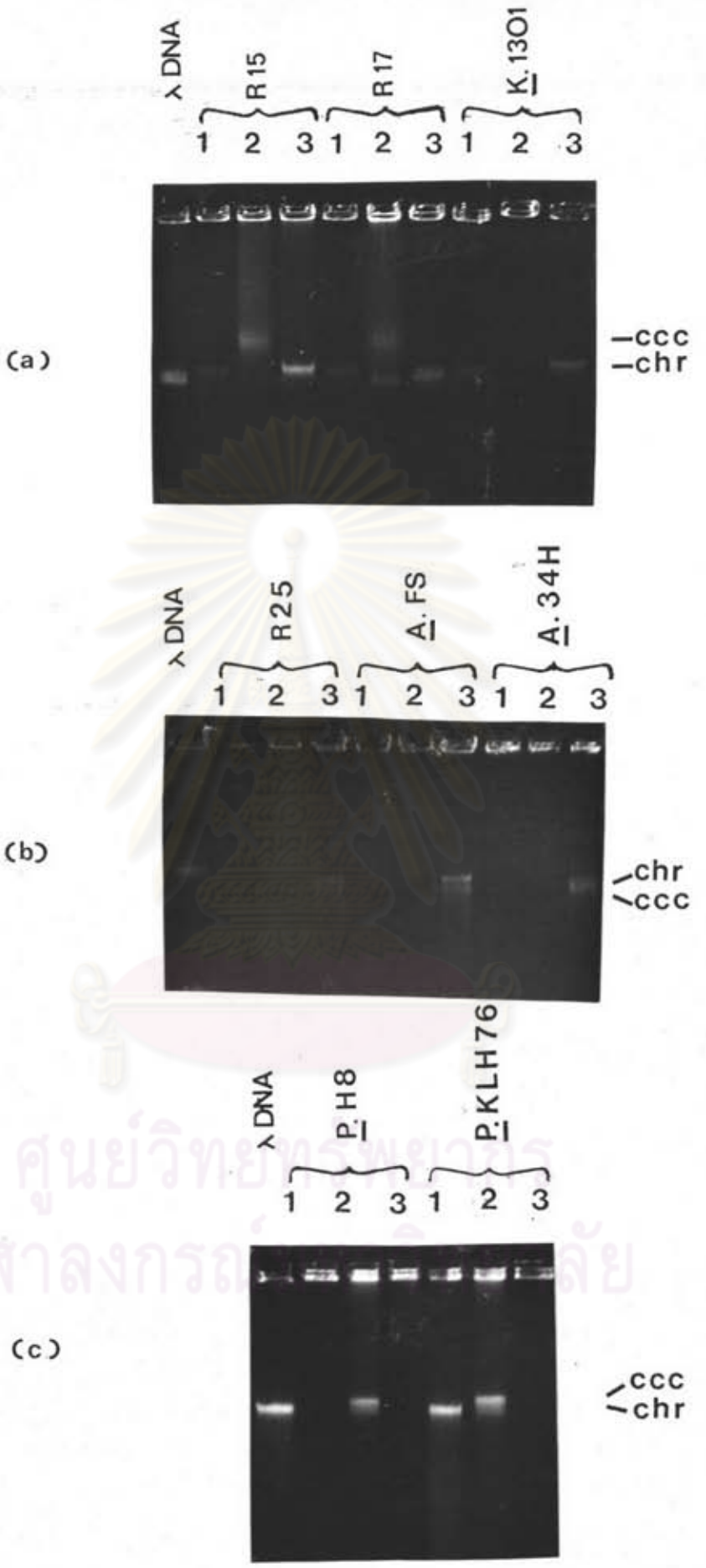
Figure 13. Plasmid profiles of all N_2-fixing bacteria on agarose gel electrophoresis.

(a) R15, R17 and K.oxytoca 1301

(b) R25, A.FS and A.34H

(c) P.H8 and P.KLH76

After cell lysis by SDS treatment, the cell lysate from each bacteria were heated at 65°C for 1) 0 min 2) 30 min and 3) 60 min before phenol-chloroform extraction. The 35 µl-aliquot from each sample preparation was applied to each slot, and λ DNA (33 Mdal) was used as marker DNA: ccc; plasmid DNA, chr; chromosomal DNA.



Physiological characteristics of N_2-fixing bacterial strains

In this experiment, we selected the tests that can distinguish different genera of bacteria from each other. The characters of all strains tested are shown in Table 10. R15, R17 and K.1301 were positive for V.P. reaction, indole production tests and could utilize malonate as a sole carbon source. The interesting results reported hereby were the salt and detergent tolerant properties of R15, R17 and K.1301.

Only R25, A.34H and A.FS exhibited a requirement for biotin. No growth after several successive transfers were observed. In this case, large inoculants (0.1 ml) was used to avoid misjudgement due to starter problems. Instead of successive transfers, the inoculant was also centrifuged and washed twice, then resuspended in biotin-free medium. These strains also failed to grow in such medium.

Also, only these strains formed yellow colonies on rich agar plates and pink colonies on nutrient agar plates after 3 weeks of incubation. They had scanty growth with 3 % NaCl but no growth with 5 % NaCl and they didn't resist to 5 % SDS.

As for P.H8 and P.KLH76, they were negative for V.P. reaction, indole production tests, and they could not utilize malonate as sole carbon source. The two strains were not salt or SDS tolerant. In addition, P.H8 P.KLH76 together with R15, R17 and K.1301 exhibited no requirement of biotin for growth.

Antibiotic resistance

All the strains showed various degrees of resistance to at least one of the 5 antibiotics tested (Table 11). R15, R17 and K.1301 were resistant to ampicillin, kanamycin, streptomycin and sulfanilamide.

TABLE 10. Physiological characteristics of unknown and reference bacterial strains.

Character of test	R15	R17	K.1301	R25	A.34H	A.FS	P.H8	P.KLH76
Yellow colonies on rich agar plate	-	-	-	+	+	+	-	-
Pink colonies on nutrient agar plate	-	-	-	+	+	+	-	-
Biotin requirement	-	-	-	+	+	+	-	-
Growth in presence of; 3 % NaCl	+	+	+	w	w	w	-	-
5 % NaCl	+	+	+	-	-	-	-	-
Voges-Proskauer reaction	+	+	+	-	-	-	-	-
Indole production	+	+	+	-	-	-	-	-
Malonate utilization	+	+	+	-	-	-	-	-
SDS resistance (> 5 %)	+	+	+	-	-	-	-	-

Symbols: +, positive reaction or growth; w, weak and -, negative.

R_{25}, A.34H and A.FS were resistant to ampicillin and sulfanilamide and P.H8 and P.KLH76 to sulfanilamide only. All the strains were sensitive to chloramphenicol.

TABLE 11. Growth response of the unknown and reference strains to antibiotics.

Strain	Amp	Cm	Km	Sm	Su
R15	+(25)	-	+(6.25)	+(6.25)	+(100)
R17	+(25)	-	+(6.25)	+(6.25)	+(100)
K.1301	+(25)	-	+(6.25)	+(6.25)	+(100)
R25	+(50)	-	-	-	+(6.25)
A.34H	+(50)	-	-	-	+(6.25)
A.FS	+(50)	-	-	-	+(6.25)
P.H8	-	-	-	-	+(25)
P.KLH76	-	-	-	-	+(25)

Amp, ampicillin; cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Su, sulfanilamide. +, growth; -, no growth. Numbers in parentheses indicate the maximum concentration in ug/ml at which normal growth was observed.

Growth pattern in nitrogen-free medium

Figure 14 shows aerobic growth as followed by changes in optical density at 420 nm in NF medium with glucose as carbon source, in cultures of K.1301, R15, R17 and R25 at 37°C. It is noticeable that, this aerobic condition supported better growth of R15, R17 and K.1301 whereas only minimal growth of R25 was observed. When 0.05 % agar was added into

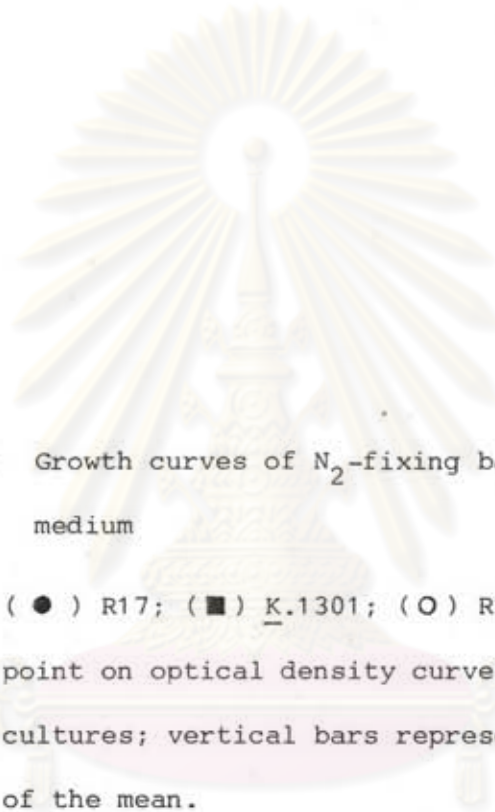


Figure 14. Growth curves of N_2-fixing bacteria in acrobic NF medium

Symbols: (●) R17; (■) K.1301; (O) R15; (□) R25. Each point on optical density curve is average of three cultures; vertical bars represent standard deviation of the mean.

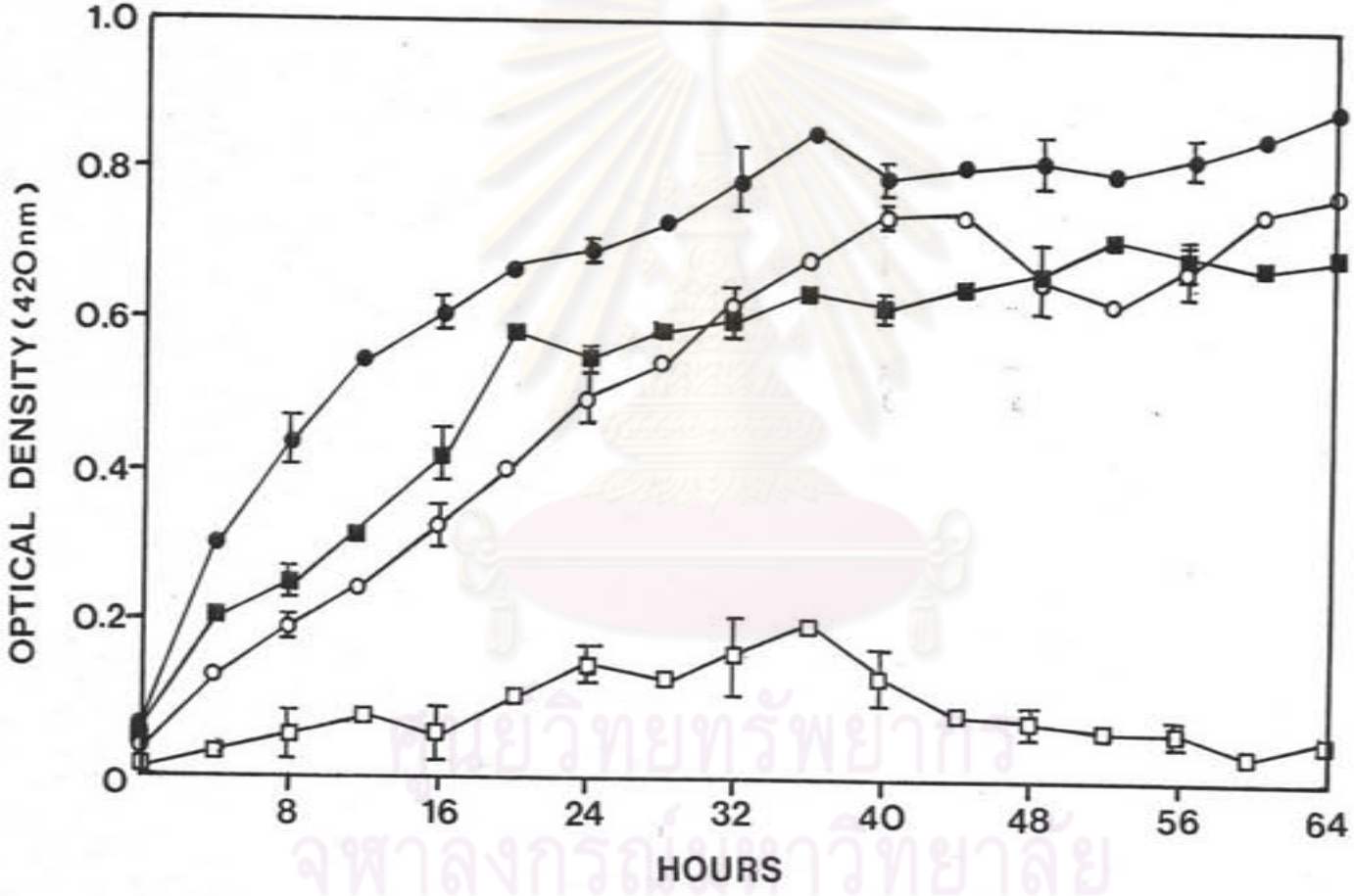


Figure 15. Growth curves of N_2-fixing bacteria in stagnant culture 0.05 % agar

Symbols: (□) R25; (■) K.1301; (●) R17; (○) R15. Each point on optical density curve is average of three cultures; vertical bars represent standard deviation of the mean.

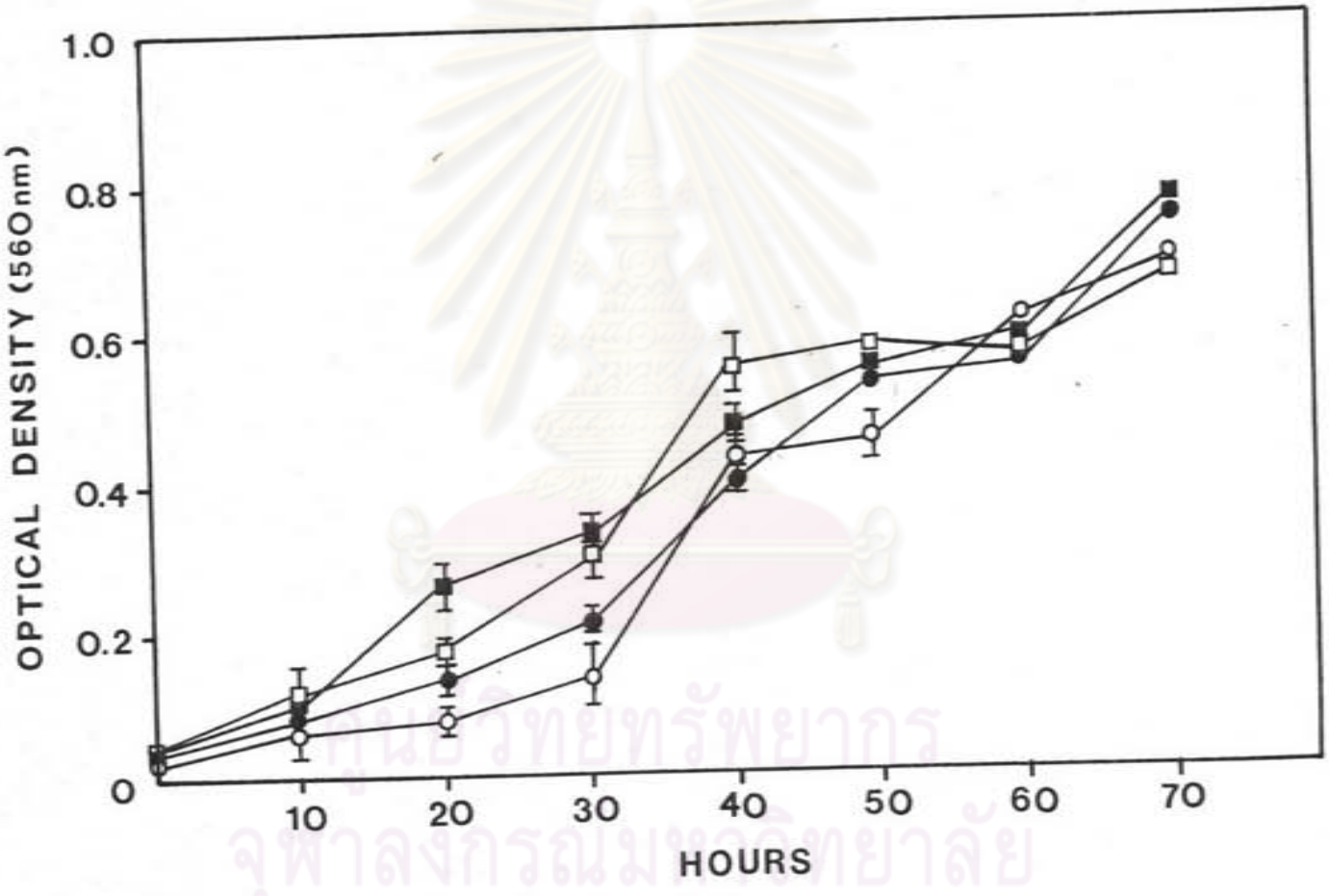


Figure 16. Growth curves of N_2-fixing bacteria in NFb semisolid stagnant culture

Symbols: (□) R25; (▲) A.FS; (△) A.34H; (●) P.H8; (○) P. KLH76. Bacteria were incubated in g-ml tubes with 5 ml of enrichment medium at 30°C. Each measurement was carried out on three replicates. VErtical bars represent standard deviation of the mean.

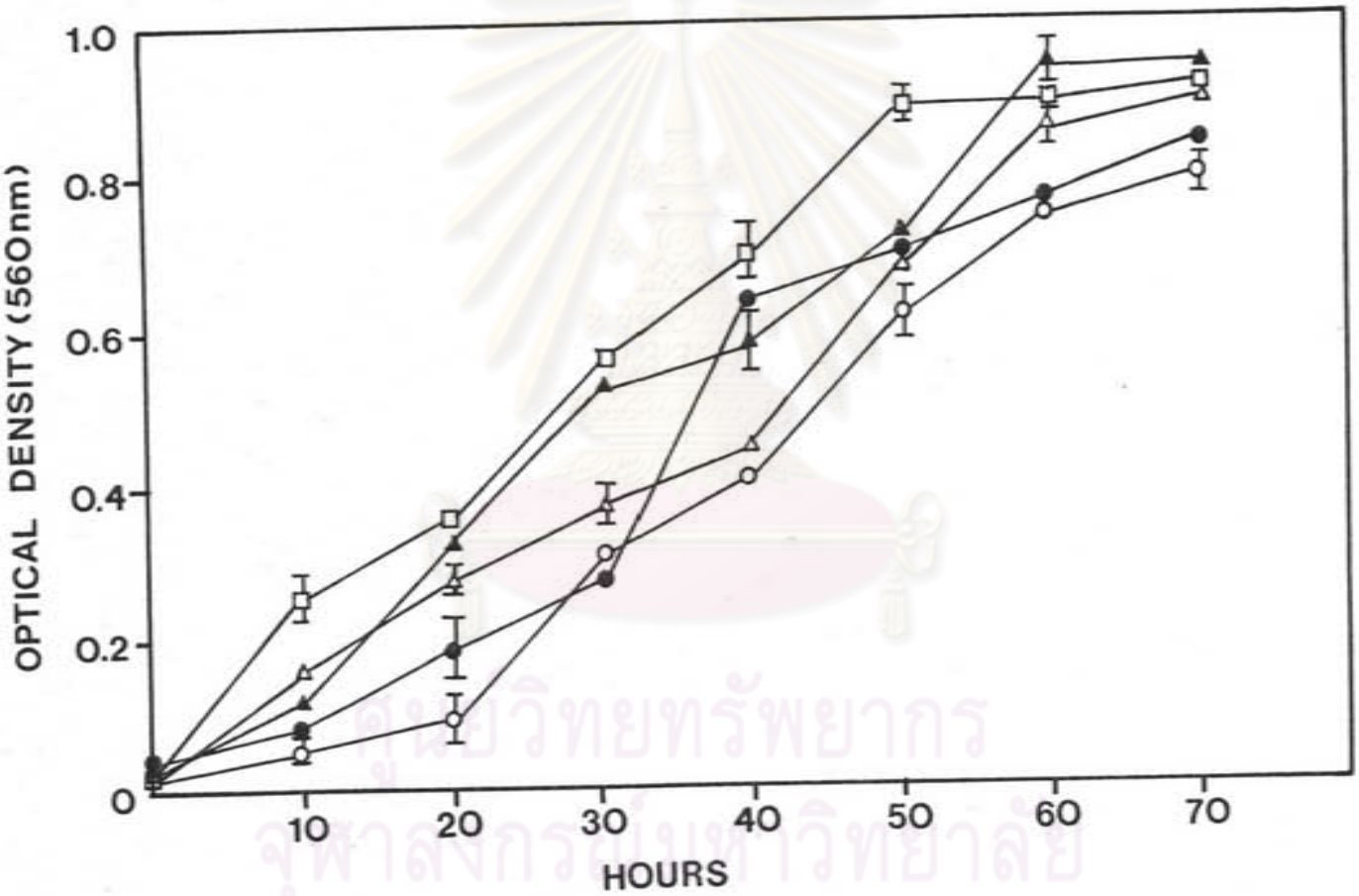
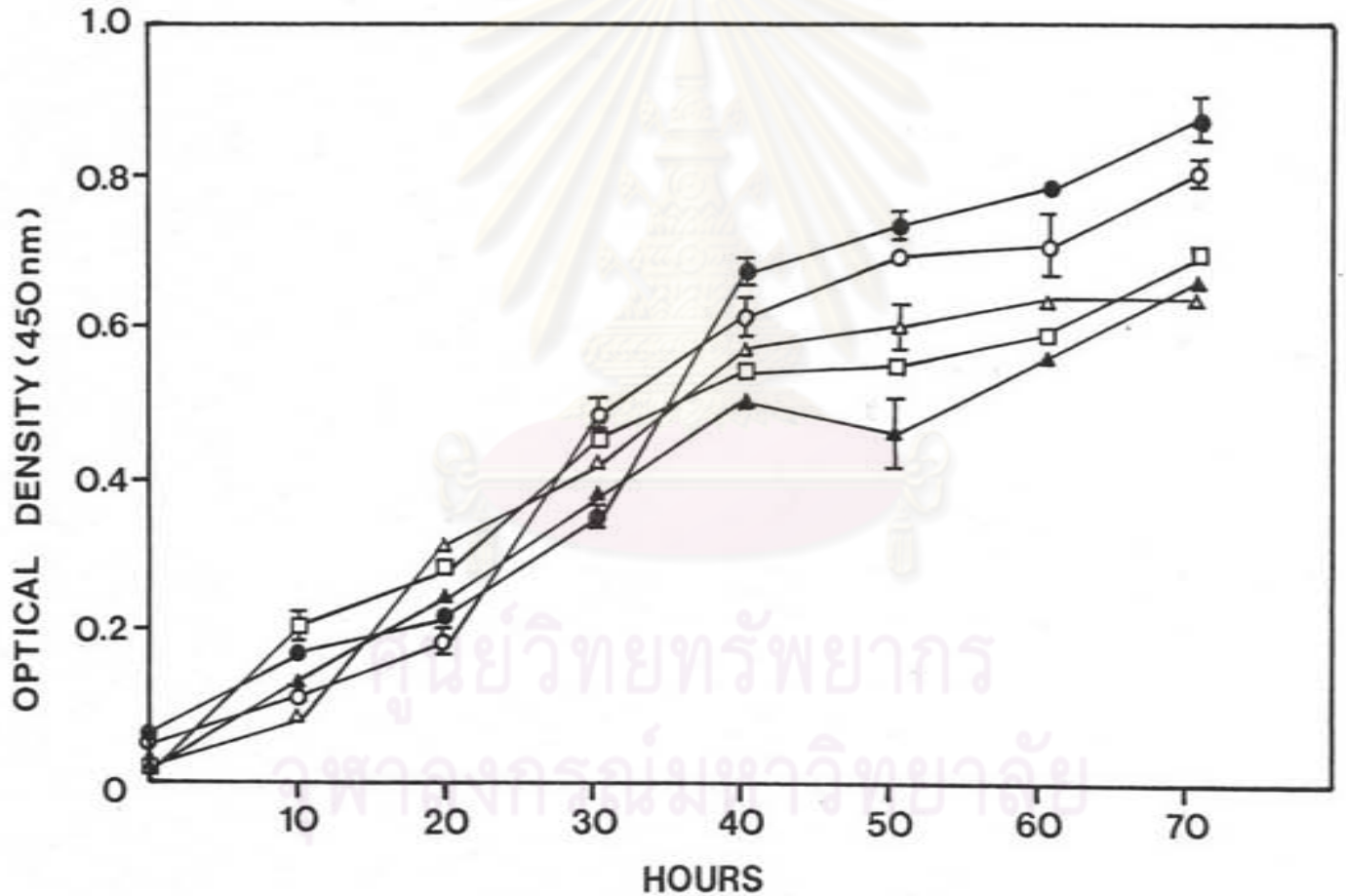


Figure 17. Growth curves of N_2-fixing bacteria in GYE semisolid stagnant culture

Symbols: (●) P.H8; (○) P.KLH76; (△) A.34H; (□) R25; (▲) A.FS. Bacteria were incubated in 9-ml tubes with 5 ml of enrichment medium at 30°C. Each measurement was carried out on three replicates. Vertical bars represent standard deviation of the mean.



the medium and the cultures was incubated by stagnant at 37°C (Figure 15), growth of R25 was enhanced slowly to the same maximal growth of K.1301, R15 and R17 although slower growth rates during the first 30 hours. The presence of 0.05 % agar produces an oxygen gradient in which these nitrogen-fixers grow and typically form pellicle at reduced oxygen tension just below the surface of the medium. An agar concentration of 0.05 % in the medium permitted easy manipulation of bacterial suspensions and direct measurement of their turbidity to estimate growth. This manner is similar to those found with standard strains A.34H and A.FS (unpublished data). Thus R25 was cultured in specific NFb medium with malate as carbon source of Azospirillum spp. as shown in Figure 16. R25 showed similar growth rate and gave the same pattern with the reference Azospirillum FS and 34H. The Pseudomonas spp. had slower growth rate under this condition. Figure 17 is the comparison of growth in GYE semisolid medium with glucose as carbon source. The results indicated that malate supported faster growth of R25 in stagnant cultures than glucose which was similar to A.FS and A.34H. In contrast, P.H8 and P.KLH76 showed similar growth pattern in both malate and glucose (Figure 16,17).

In cases of K.1301, R15 and R17, glucose also supported more rapid growth than did malate (data not shown).

Acetylene reduction activity

In this experiment, colonies from each bacterial cultures were tested for nitrogenase activity by the acetylene reduction assay. Acetylene reduction activity (ARA) was measured for a 24 h period after 2 days growth at 30°C on semi-solid media and 12 h period after 4 h growth at 30°C in liquid media. Population of N_2-fixing bacteria

were determined by dilution plate counts on TSA plates.

All the strains showed acetylene reduction activity under any or both cultured conditions. As shown in Table 12, only K.1301, R15 and had ARA under aerobic condition among which R15 showed the highest specific activity of 234 nmol of C_2H_4. h^{-1}. mg $protein^{-1}$ and the highest population on TSA plate of 2×10^6 cells/ml. K.1301 and R17 were less active, but Azospirillum spp. and Pseudomonas spp. did not grow under this condition.

From Table 13, it is noticeable that when ARA assays were conducted on microaerobic-cultured, the five strains that failed to show ARA under aerobic had nearly 2-fold higher specific activity than did K.1301, R15 and R17. This is becuase of the cells number of the five strains were in all cases higher than K.1301, R15 and R17 As shown in Table 13, the standard error of the mean were greater for a given isolate. This is probable due to difficulty in keeping the subsurface growth band intact during processing for the acetylene reduction assay. Distrubance of the band, exposes the oxygen-sensitive nitrogenase enzyme to suboptimal concentration of oxygen. However, ARA and the viable counts on TSA plates of K.1301, R15 and R17 under both conditions were not significant different. The results suggested that nitrogen should be fixed by these Klebsiella strains whether under aerobic or microaerobic condition in contrast to R25 A.34H, A.FS, P.H8 and P.KLH76 that were able to fix nitrogen only in microaerobic condition at best.

TABLE 12. Specific acetylene reduction activity of unknown bacteria compared to standard reference strains in NF aerobic condition.

Culture	Population of N_2-fixers ($X10^6$ cells/ml)	Mean n mol of $C_2H_4 h^{-1} (tube)^{-1}$	mg of protein/tube	C_2H_4 (n mol h^{-1} mg of protein^{-1})
R15	2	14.02 ($\pm$0.54)*	0.060	234
R17	0.7	7.04 ($\pm$0.70)	0.052	135
K.1301	2	11.58 ($\pm$0.62)	0.057	203
R25	0**	0	0	0
A.34H	0	0	0	0
A.FS	0	0	0	0
P.H8	0	0	0	0
P.KLH76	0	0	0	0

*Mean ARA is based on an average of three replicates, with three tubes in each replicate.

Deviation of the mean is presented as $\pm$ standard error

**The population is not over than 2×10^2 cells/ml

TABLE 13. Specific acetylene reduction activities of unknown bacteria compared to standard reference strains in microaerobic condition.[a]

Culture	Population of N_2-fixers ($X10^6$ cells/ml)	Mean n mol of $C_2H_4 h^{-1} (tube)^{-1}$	mg of protein/tube	C_2H_4 (n mol h^{-1} mg of $protein^{-1}$)
R15	2	12.79 ($\pm$4.54)[b]	0.054	234
R17	2	11.21 ($\pm$3.21)	0.049	229
K.1301	3	15.71 ($\pm$2.75)	0.061	258
R25	26	32.71 ($\pm$6.50)	0.081	404
A.34H	16	25.50 ($\pm$6.75)	0.075	340
A.FS	19	28.92 ($\pm$3.42)	0.072	402
P.H8	7	21.42 ($\pm$6.08)	0.067	320
P.KLH76	7	20.12 ($\pm$3.58)	0.064	314

[a] Incubated 2 days in nitrogen-free culture tubes (0.05 % agar), 30°C.

[b] Average and standard error of 9; 5-mL culture tubes after 24 h. under 10 % acetylene in air.