CHAPTER 3

Results

3.1 Growth characteristics of R15, R17 and R25

3.1.1. In NF medium

Growth curve for each strain was followed by measuring the optical density at 420 nm, and colony-forming unit (cfu) by dilution plating method. Klebsiella strains, R15 and R17, were cultured in NF medium with glucose as carbon source in aerobic condition at 30°C, whereas Azospirillum strain, R25 was grown in NF medium with malate as carbon source at 30°C without shaking. The log of cfu per ml was followed in parallel with the culture turbidity until the culture reached stationary phase. Growth curves were plotted between cell density (OD 420) or log of cfu/ml and time in hour as shown in Fig. 3.1 a-c. OD420 and cfu/ml of the culture of R15 and R17 increased up to about 7h and then OD420 remained constant, whereas, the cfu/ml declined. For the culture of R25 showed, log of cfu/ml increased until about 24h and then began to decrease. Fig. 3.2 a-c showed linear correlation between the

Figure 3.1 Growth curves of Klebsiella R15, R17 and Azospirillum R25

Bacteria (3% inoculum) were cultured in 150 ml NF medium with reciprocal shaking at 30°C, except R25 without shaking and 3 ml aliquot was taken at intervals to measure OD420 and cfu by plate-count method. Three replicative measurements were carried out at each time interval. The symbols (o——o) are growth measured by plate-count method (cfu/ml), and (Δ——Δ) by optical density (OD420).

(a) R15 (b) R17 (c) R25

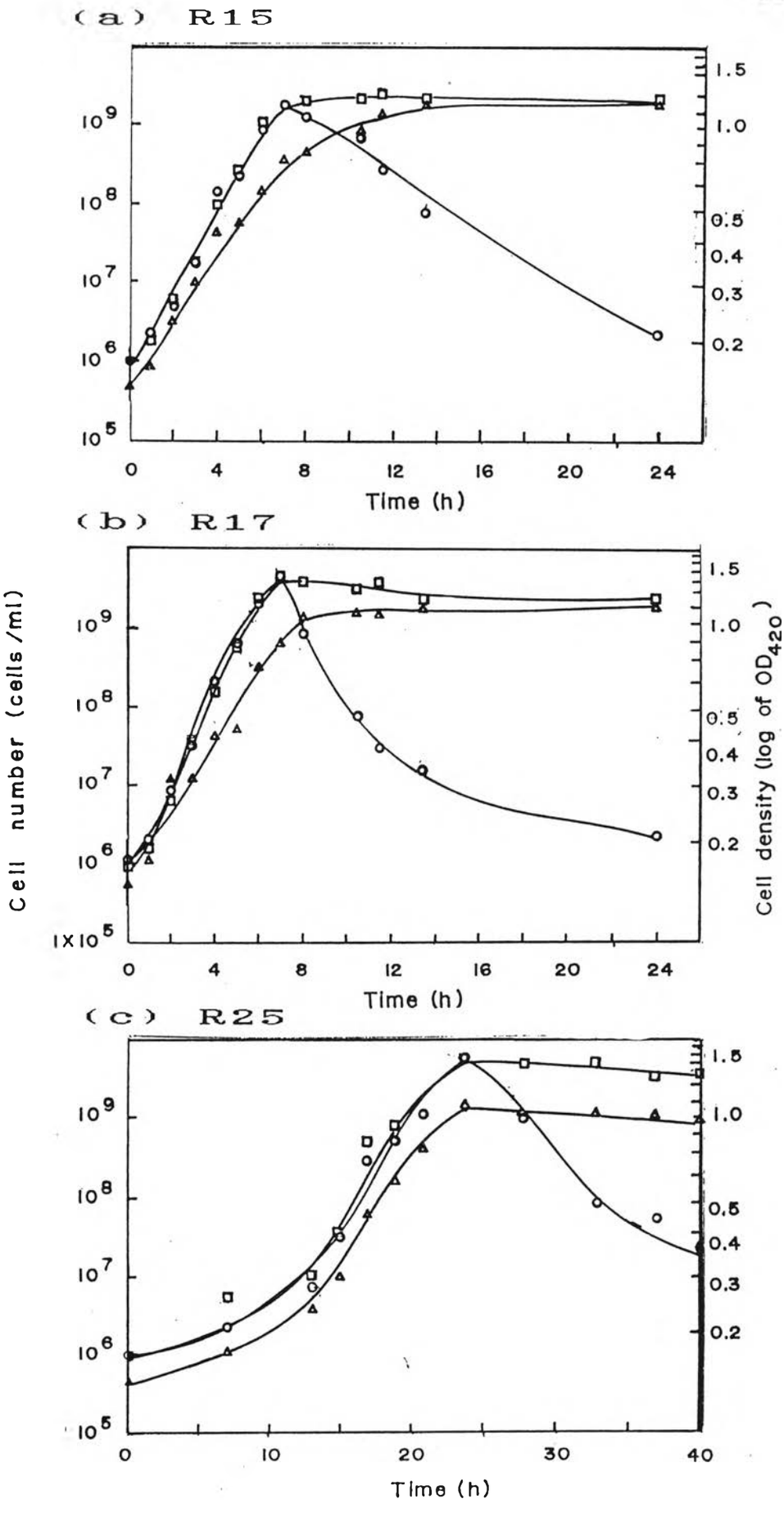
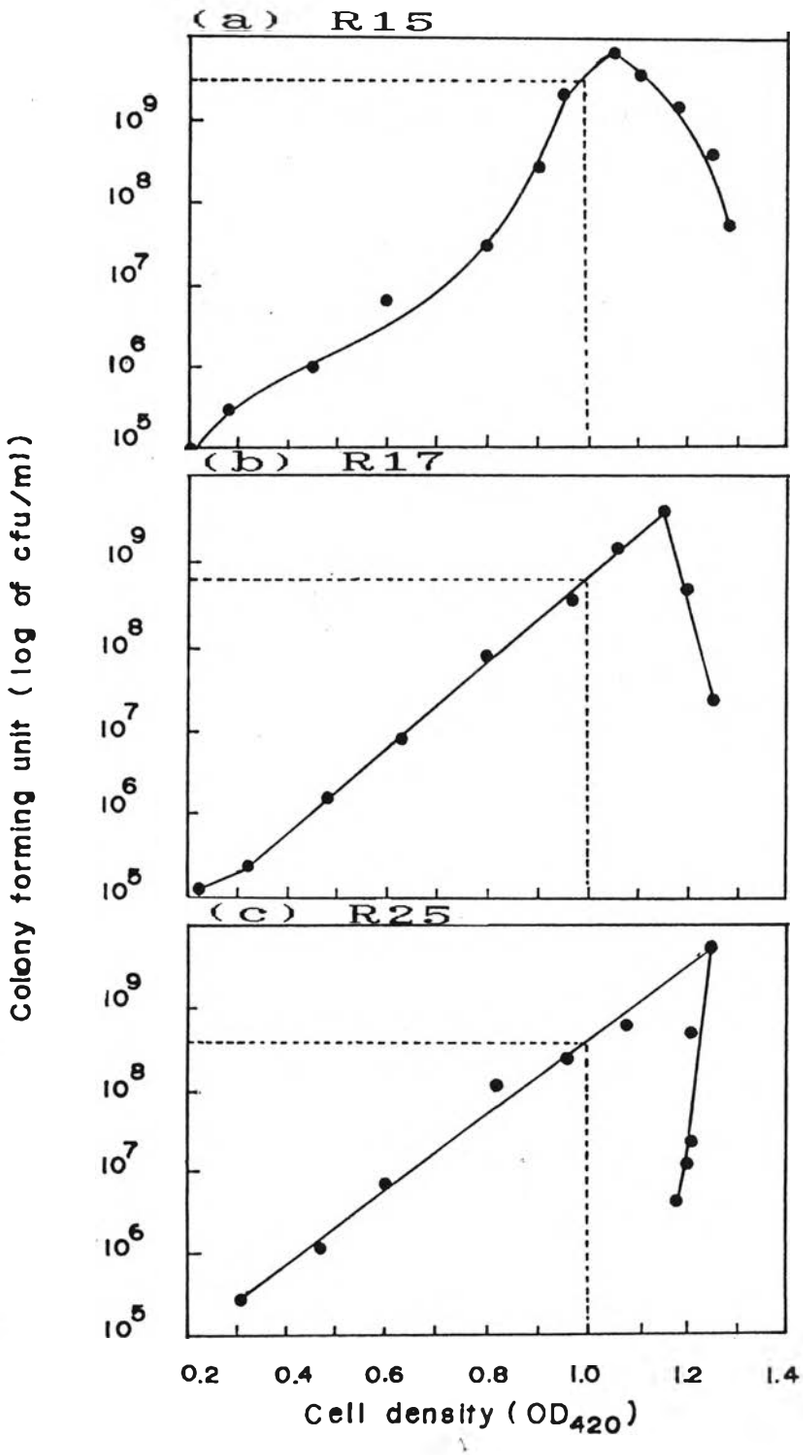

(a) R15
(b) R17
(c) R25
Cell number (cells/ml)
Cell density (log of OD_{420})
Time (h)
10^9
10^8
10^7
10^6
10^5
1X10^5
1.5
1.0
0.5
0.4
0.3
0.2
0
4
8
12
16
20
24
10
20
30
40

Figure 3.2 Correlation between colony forming unit and optical density

The linear correlation between OD420 and colony forming unit (cfu) has been observed in NF medium up to the early stationary phase.

(a) R15 (b) R17 (c) R25


(a) R15
(b) R17
(c) R25
Colony forming unit (log of cfu/ml)
10^9
10^8
10^7
10^6
10^5
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Cell density (OD_{420})

direct cell density (OD 420) and cfu/ml up to OD $\simeq$ 1.0 and cfu in the order of 10^9 cells/ml, which are in the early stationary phase. Beyond this time point, a large number of cells could be inactive and resulting in the drastic decrease in colony forming activity. These curves were also used as standard curves to convert a unit of cell density into cfu/ml. From these standard curves, an OD 420 of 1.0 (1-cm light path) of R15, R17 and R25 corresponded to $1.3x10^9$, $7.0x10^8$, and $4.0x10^8$ cells/ml respectively.

3.1.2 In NF and RM medium supplemented with various concentrations of NaCl

Since saline soil is one of the stress condition for growing rice, the growth pattern of R15, R17 and R25 in nitrogen free medium containing 0.5 - 12.5% NaCl (as a model of non - fertile saline soil) and rich medium containing 2.5 - 15.0% NaCl (as a model of fertile saline soil) were investigated. Table 3.1 shows that in NF medium the best salt tolerant strain is R17 which can grow in the presence of 10% NaCl and showed the maximum cell density of 0.37 with the doubling time of 4.5 h. In the presence of NF medium plus 7.5% NaCl, R15 reached the maximum OD420 of 0.327 with doubling time 6.4 h, and

Table 3.1 Growth character of N_2-fixing bacteria, R15, R17 and R25 in NF and RM medium containing various salt concentrations

Condition of the culture cells	R15		R17		R25	
	Generation time (h)	Max.OD[a]	Generation time (h)	Max.OD	Generation time (h)	Max.OD
NF 0.5% NaCl	0.9	1.1	0.9	1.1	1.1	1.2
2.5%	1.3	0.95	0.9	0.93	2.5	1.1
5.0%	3.0	0.40	1.2	0.88	4.2	0.84
7.5%	6.4	0.327	1.9	0.63	6.8	0.49
10.0%	-	-	4.5	0.371	-	-
12.5%	-	-	-	-	-	-
RM 2.5% NaCl	1.9	1.1	1.2	1.1	1.3	1.2
5.0%	2.6	0.96	1.6	0.98	1.6	0.83
7.5%	3.6	0.82	2.3	0.91	1.9	0.62
10.0%	6.0	0.68	3.1	0.73	2.3	0.439
12.5%	10.8	0.49	7.8	0.54	3.3	0.294
15.0%	-	-	-	-	-	-

[a] Max.OD = Maximun Optical density 420 nm

R25 the maximum OD420 of 0.49 with doubling time 6.8h, respectively (Fig. 3.3 a-c). In rich medium, R15, R17 and R25 could tolerate NaCl upto the concentration of 12.5%, showing the maximum turbidity of 0.49, 0.54 and 0.29 with doubling time of 10.8, 7.8 and 3.3h (Fig 3.4 a-c). The results thus show that R17, the best nitrogen - fixing strain isolated from the saline soil area of Tapra, Khonkan Province, could tolerate high salt concentration significantly better than R15 and R25 in both media.

3.2 Preparation of antiserum against R15, R17, and R25

Antisera against R15, R17, and R25 have been produced in female New Zealand White rabbits. Primary injection was carried out by mixing an equal volume of bacterial suspension (ca $1x10^9$ cells/ml) with Freund's complete adjuvant and injected intramusculary (IM) in the hind legs. Four weeks later, intravenous (IV) booster was weekly injected into the ear vein after a few ml bleeding to check the serum titer. The high agglutination titer of 1:1,600; 1:2,400 and 1:3,200 were detected at the 10^{th}, 10^{th} and 20^{th} weeks for R15, R17 and R25, respectively (Fig. 3.5 a-c). The massive

Figure 3.3 Growth pattern of R15, R17 and R25 in NF-medium containing various concentrations of NaCl

Bacteria were grown in NF medium containing NaCl (0.5 - 12.5%) with reciprocal shaking at 30°C, except R25 without shaking and 3 ml aliquot was taken at intervals to measure OD420. Three replicative measurements were carried out at each time interval.

(○——○) NF containing 0.5% NaCl

(△——△) NF containing 2.5% NaCl

(□——□) NF containing 5.0% NaCl

(●——●) NF containing 7.5% NaCl

(▲——▲) NF containing 10.0% NaCl

(■——■) NF containing 12.5% NaCl

(a) R15 (b) R17 (c) R25

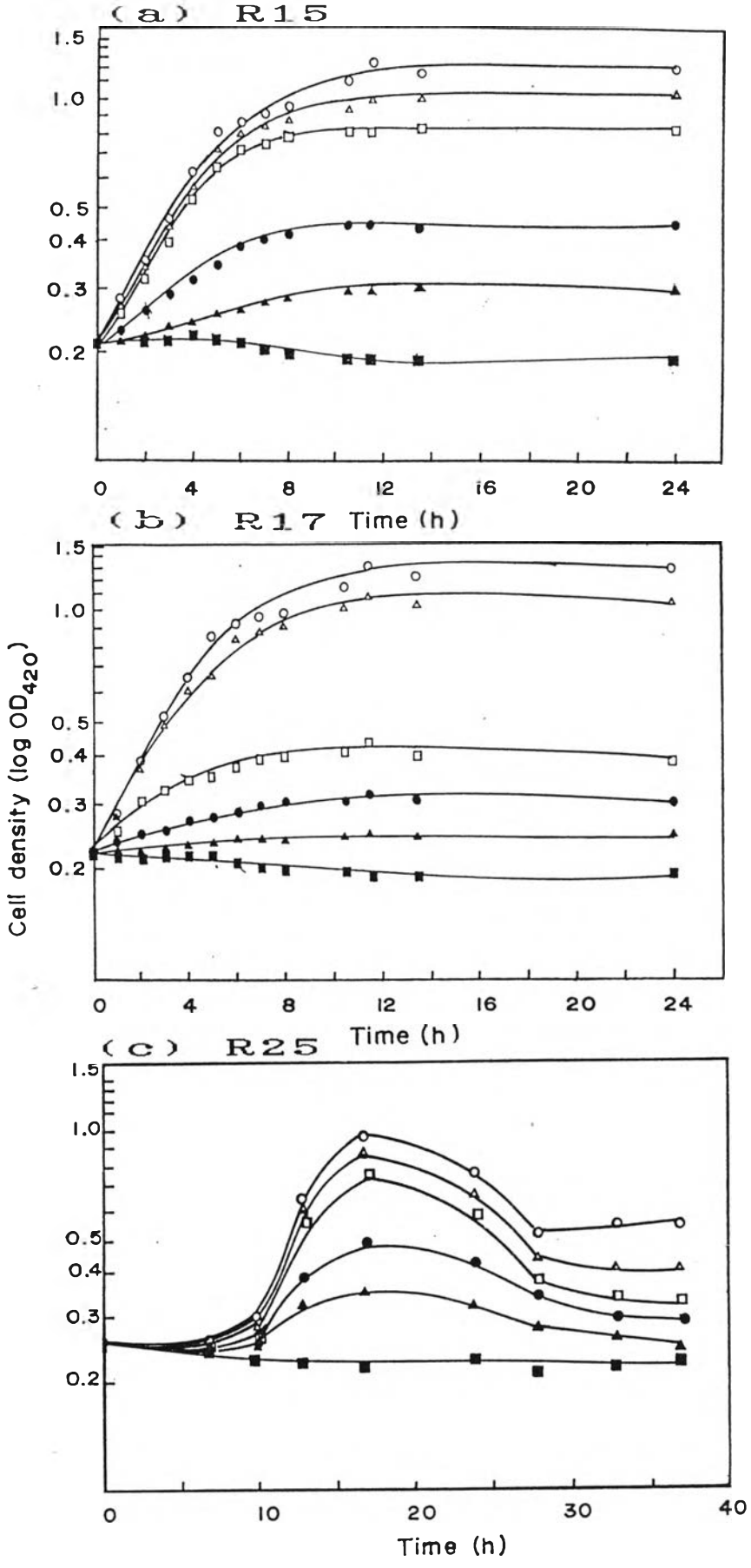

(a) R15
(b) R17
(c) R25
Cell density (log OD_{420})
Time (h)
1.5
1.0
0.5
0.4
0.3
0.2
0
4
8
12
16
20
24
10
30
40

Figure 3.4 Growth pattern of R15, R17 and R25 in RM-medium containing various concentrations of NaCl

Bacteria were grown in RM medium containing NaCl (2.5 - 15.0%) with reciprocal shaking at 30°C, except R25 without shaking and 3 ml aliquot was taken at intervals to measure OD420. Three replicative measurements were carried out at each time interval.

(o——o) RM containing 2.5% NaCl

(△——△) RM containing 5.0% NaCl

(□——□) RM containing 7.5% NaCl

(●——●) RM containing 10.0% NaCl

(▲——▲) RM containing 12.5% NaCl

(■——■) RM containing 15.0% NaCl

(a) R15 (b) R17 (c) R25

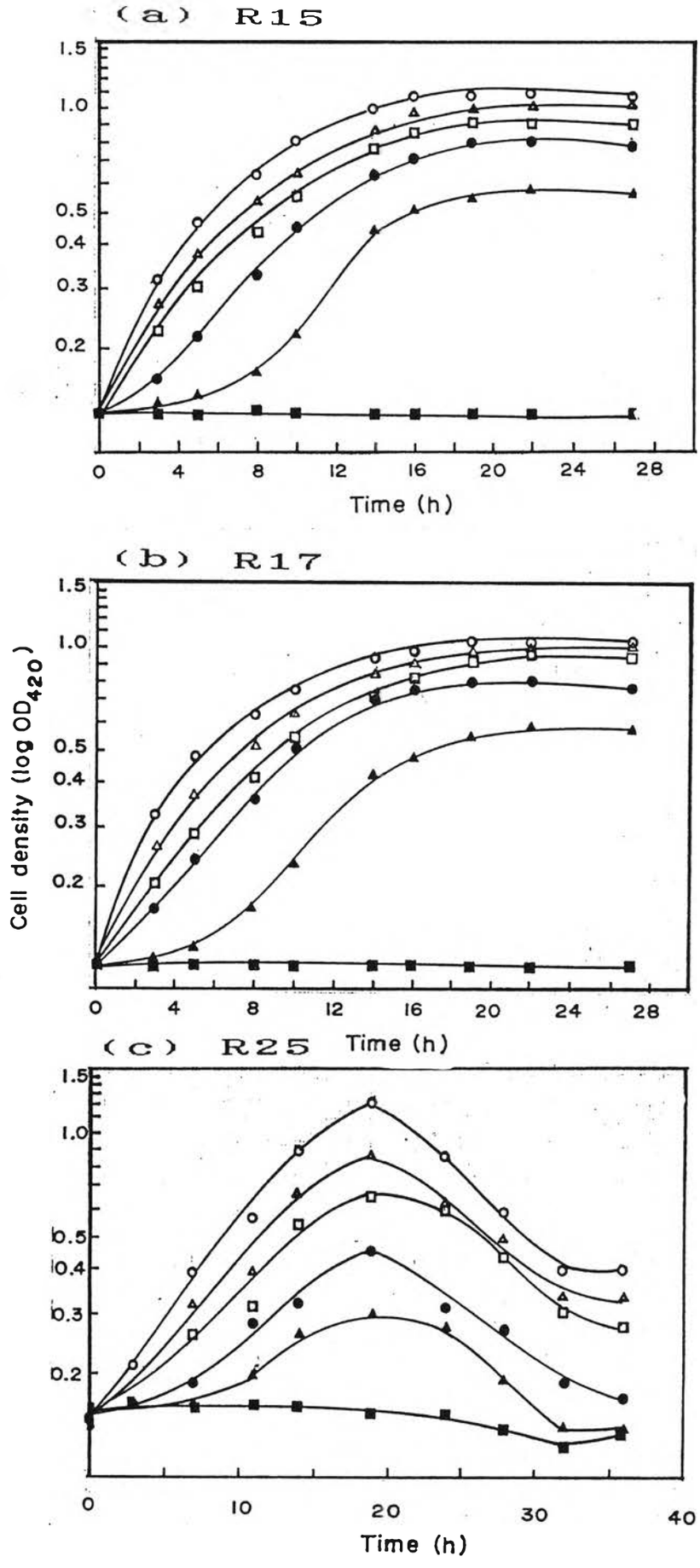
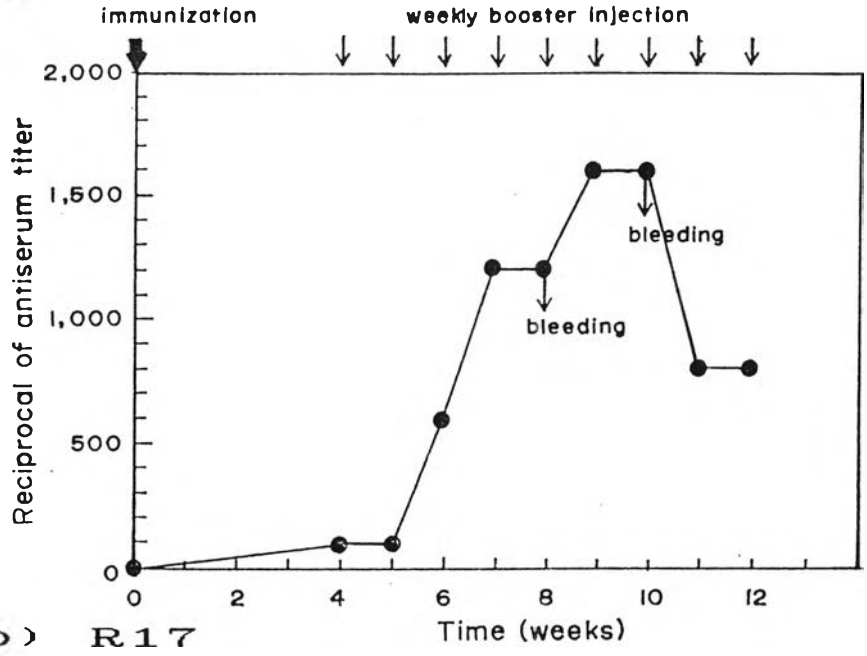

(a) R15
1.5
1.0
0.5
0.4
0.3
0.2
0
4
8
12
16
20
24
28
Time (h)
(b) R17
Cell density (log OD_{420})
(c) R25
Time (h)
0
10
20
30
40
Time (h)

Figure 3.5 Immunization scheme and antisera titer aganist R15, R17 and R25

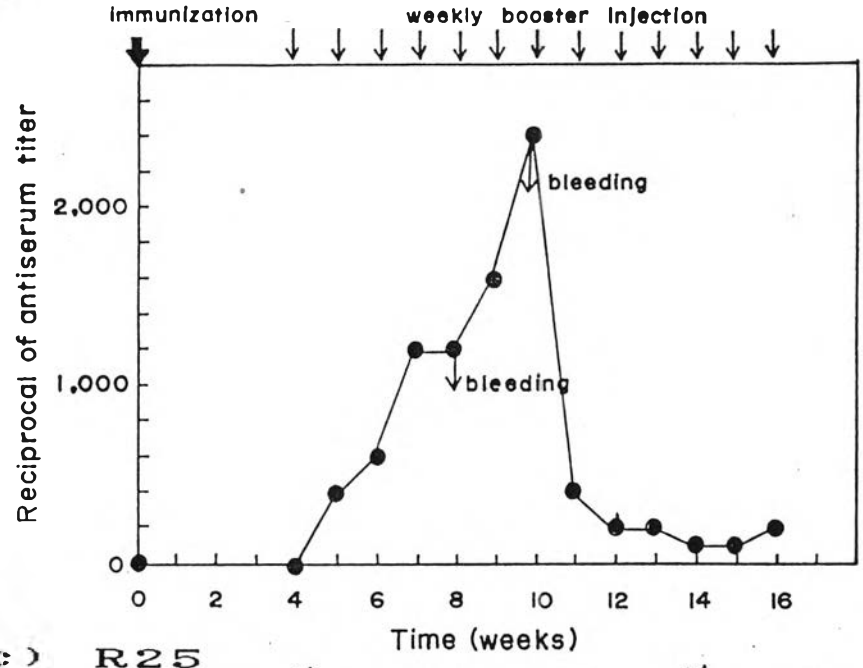
The first injection, containing a mixture of 0.5 ml of heated antigens in normal saline (10^9 cells/ml) and 0.5 ml of complete adjuvant mixture is injected via IM. The weekly injection containing 0.5 ml of cell in normal saline suspension ($1x10^9$ cells/ml) is injected into ear vein after blood sampling. Antibody titer of the serum was determined by agglutination test.

(a) R15 (b) R17 (c) R25

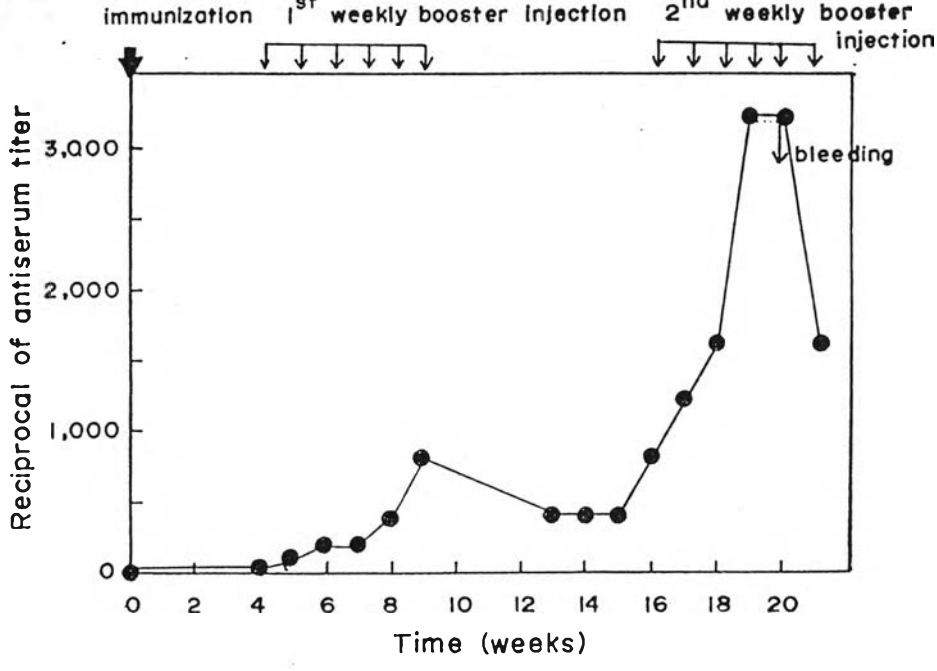
(a) R15



(b) R17



(c) R25



antisera were then collected and used throughout the study. Only one of the two rabbits injected for R15 and one of the four rabbits for R17 and R25 gave satisfactory high titer.

3.3 Indirect immunofluorescence antibody (IND-FA)

3.3.1 Optimization and Specificity

Antisera against R15, R17, and R25 were used as the first antibodies in the indirect fluorescent antibody technique, in which the commercial FITC-conjugated goat antirabbit immunoglobulin was used as the second antibody. Fig. 3.6 shows cell antigens coated on the glass slide, after reacted with the homologous antibody, and FITC-conjugated goat antirabbit immunoglobulin result in bright - green fluorescent antigen-antibody complex when viewed under an epifluorescence microscope.

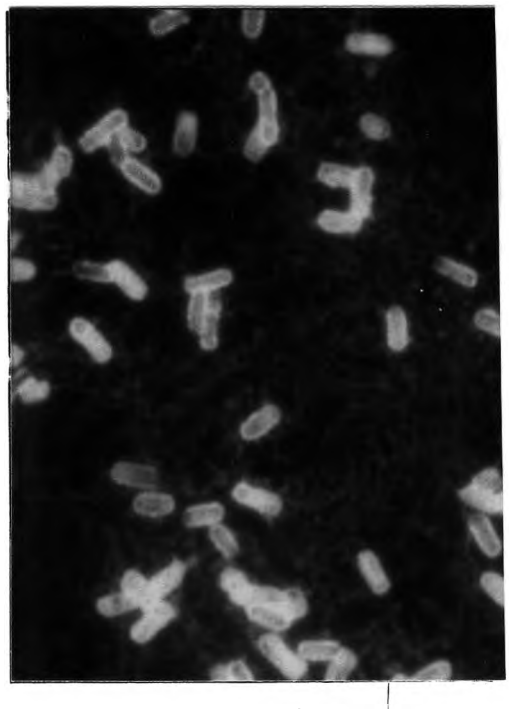
For saving, the minimum concentration of cell antigens and its antiserum which still maintained high fluorescent intensity were determined. Table 3.2 indicates that $1x10^7$ cells/ml of cell antigen and the homologous antisera of 1:100 dilution were the minimum

Figure 3.6 Fluorescent micrographs of R15, R17 and R25 in various concentrations of NaCl

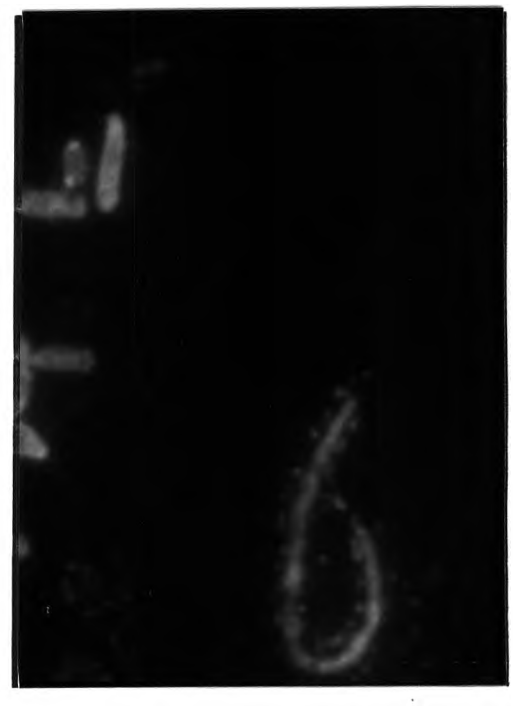
Culture (3% inoculum) were grown in NF and RM media containing various salt concentrations, using the NF medium containing 0.5% NaCl as the control. The procedure of checking the cell surface antigen was described in Method 2.10 and 2.11.

(a) R15 (b) R17 (c) R25

(a) R15

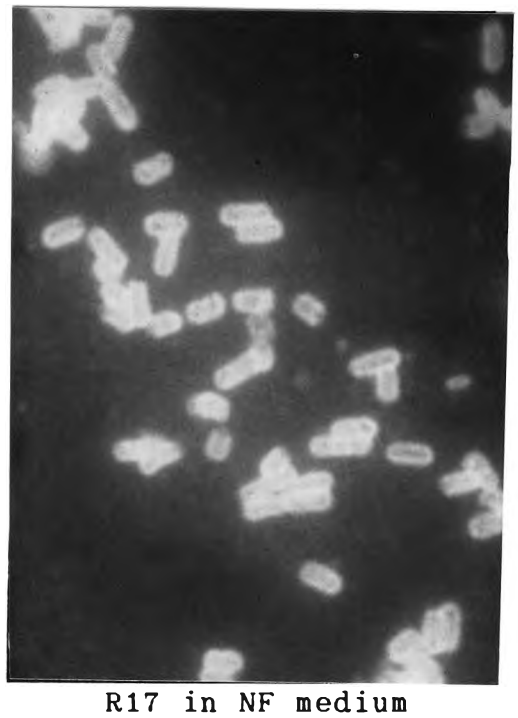


R15 in NF medium
(control)

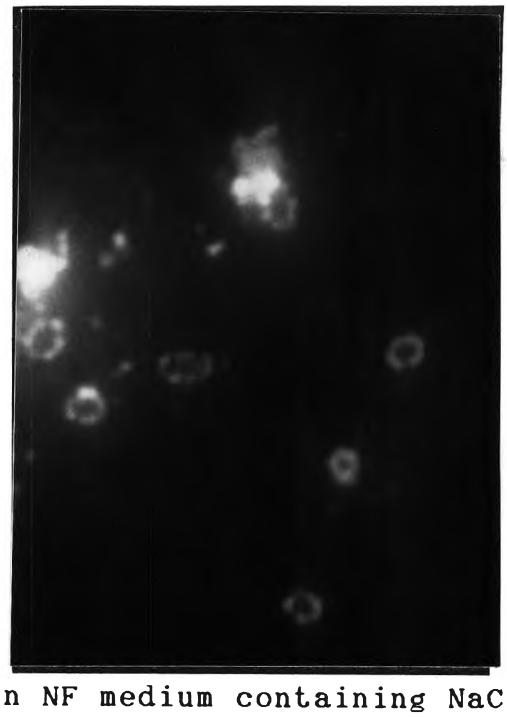


R15 in RM medium containing NaCl 7.

(b) R17

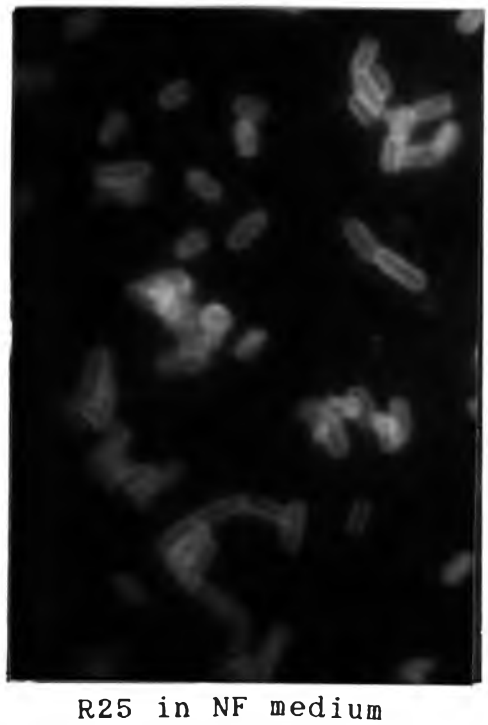


R17 in NF medium
(control)

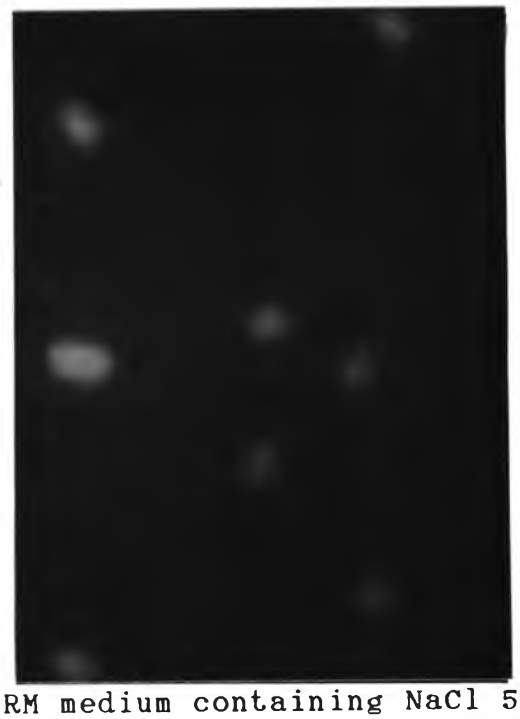


R17 in NF medium containing NaCl 7.5

(c) R25



R25 in NF medium
(control)



R25 in RM medium containing NaCl 5.0%

Table 3.2 Optimization of coating antigens and antisera concentrations for indirect immunofluorescence

Cell concentration	Antisera dilution											
	1:25			1:50			1:100			1:200		
(n=12)	R15	R17	R25	R15	R17	R25	R15	R17	R25	R15	R17	R25
$1x10^7$	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+
$1x10^8$	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
$1x10^9$	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

concentration suitable for the purpose and therefore were used in most of the subsequent experiments.

The specificity of IND-FA was determined by cross - reaction of each antiserum with other 13 non-homologous related strains of bacterial antigens, both heated and unheated. All three strains (R15, R17 and R25) gave maximal 4+ fluorescent intensity with its homologous antiserum, but strains R15 and R17 could not be distinguished by IND-FA method. Both strains are more closely related to K.oxytoca NG13 than other genera tested (Table 3.3). Strain R17 shows sparsely selorogical relationship with Azospirillum spp. (A. R25, A. FS and A. 34H), as shown by FA grade 1+ in unheated cells. Strain R25 strongly cross reacts with unheated A. FS and A. 34H but lessening fluorescent intensity with heated cell antigens. This result suggests that heat treatment of cells antigen should increase the specificity of the antigen - antibody reaction for all strains. Thus, cell antigens were heated before use in future experiments.

3.3.2 Salt effect on immunofluorescent intensity

From the preliminary study on growth

Table 3.3 Cross-reactivity of antisera against R15, R17 and R25 with nonhomologous heated and unheated antigens by IND-FA technique

Each bacterial tested antigen was cultivated from NF medium and adjusted to the final concentration of $2.5x10^8$ cells/ml in normal saline.

Tested antigen	Species, antisera - FA tested					
	K. R15		*K.* R17		*A.* R25	
(n=8)	unheated	heated	unheated	heated	unheated	heated
K. R15	4+	4+	4+	4+	ND	ND
K. R17	4+	4+	4+	4+	ND	ND
A. R25	ND	ND	1+	ND	4+	4+
K. oxytoca NG13	3+	2+	3+	2+	ND	ND
K. pneumoniae M5a1	ND	ND	ND	ND	ND	ND
A. FS	ND	ND	1+	ND	3+	2+
A. 34H	ND	ND	1+	ND	3+	2+
A. Sp7	ND	ND	ND	ND	ND	ND
A. SpRG20	ND	ND	ND	ND	ND	ND
A. SpBr17	ND	ND	ND	ND	ND	ND
P. H8	ND	ND	ND	ND	ND	ND
P. KLH 76	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	ND	ND

(ND) indicates no fluorescent intensity is detected.

characteristics of R15, R17, and R25, smaller coloniesand different cell morphology (Fig. 3.6) were observed in high salt concentration. The effect of salt on the sentivity of IND-FA in strain identification was therefore tested. Antisera (1:100 dilution) was used to react with homologous antigens which had been cultured in either NF or RM medium supplemented with various concentrations of NaCl. Table 3.4 showed that the fluorescent intensity were decreased in higher salt concentration (not less than 2.5% NaCl), but in rich medium, the presence of NaCl showed less effect on fluoresent intensity except for R17, which is the best salt tolerant strain among diazotrophs tested. This result indicates that differences in media and salt concentration both affect the sensitivity of strain identification by immunofluorescence method.

3.4 Determination of associative N_2-fixing bacteria on the rice root by immunofluorescence

3.4.1 One hour adsorption

In this experiment, N_2-fixing bacteria, R15, R17 and R25 were varied in the order of 10^7, 10^8 and 10^9 cells/one ml of PBS and 1 ml of each culture was

Table 3.4 Salt effect on imunofluorescent intensity

Each culture was cultivated from various salt condition and adjusted to final concentration of $1x10^7$ cells/ml in normal saline.

Condition of the culture cells	Nitrogen-fixing Bacterial ($1x10^7$), antisera-FA tested		
	K. R15	K. R17	A.R25
NF (0.5% NaCl)	4+	4+	4+
2.5% NaCl	3+	1+	2+
5.0% NaCl	1+	1+	2+
7.5% NaCl	2+	1+	2+
10.0% NaCl	1+	3+	2+
RM (2.5% NaCl)	4+	2+	3+
5.0% NaCl	2+	1+	3+
7.5% NaCl	3+	2+	4+
10.0% NaCl	4+	3+	4+
12.5% NaCl	4+	3+	3+
15.0% NaCl	-	-	3+

(-) indicates no culture growth is detected

incubated with roots (cut into pieces about 1 cm long) of 3 rice seedlings (2-day-old) for 1 h as described in Methods 2.13. Table 3.5 indicates that when the inoculum size of 10^7 cells/3 plants of R15, R17 and R25 were used, fluorescent antigens could not be detected in any rice varieties tested. At inoculum size of 10^8 cells of these bacteria, positive immunofluorescent reaction can be detected in the root of rice RD7, whereas RD5 shows the positive colonization only when inoculated with R17 and R25. At the inoculum size of $1x10^9$ cells, colonization of fluorescent stained R15, R17 and R25, were detected in 4 varieties of rice; RD5, RD7, RD25 and SPT, whereas RD6, RD23, LPT, and KDML, failed to produce fluorescence labelled bacteria at the dilution of antisera 1:100. This result indicates that RD7 is the best host-plant among 8 cultivars tested for (1h) colonization by the 3 N_2-fixing strains tested, and the inoculum size should not be less than 10^9 cells/ml under this condition.

3.4.2 Long term association

Long term association was conducted in semi-solid medium (deficient in nitrogen) for 15 days, and $5x10^8$ cells/inoculum was selected. After 2, 7 and 12

Table 3.5 Detection of N_2-fixing bacteria, R15, R17 and R25 adhered on the rice roots by IND-FA

Rice root varieties (2-days-old)	Bacterial inoculum (cells/ml)								
	K. R15			*K.* R17			*A.* R25		
	10^7	10^8	10^9	10^7	10^8	10^9	10^7	10^8	10^9
(n=12)	Immunofluorescent reaction of washed roots after 1h								
RD5	-	-	+	-	+	+	-	+	+
RD6	-	-	-	-	-	-	-	-	-
RD7	-	+	+	-	+	+	-	+	+
RD23	-	-	-	-	-	-	-	-	-
RD25	-	-	+	-	-	+	-	-	+
LPT	-	-	-	-	-	-	-	-	-
SPT	-	-	+	-	-	+	-	-	+
KDML	-	-	-	-	-	-	-	-	-

Symbols (+) positive test

(-) negative test

A positive reaction is indicated by brilliant yellow green bacteria on the dark background and negative reaction is indicated by no fluoresent labelled bacteria.

days of inoculation, the roots of 3 plants were cut off and extracted for associative bacteria as described in Methods 2.14 to eliminate the rice debris autofluorescence. Table 3.6 shows the degree of immunofluorescence rate of the extracted cell antigen from the rice root at 2, 7, and 12 days after inoculum. The results show that, 2 days after inoculation, the colonization of fluorescent stained bactria were detected in RD7 and SPT for R15, R17 and R25 whereas RD5 was positive only with R17 and R25. After 7 days, the positive reaction were observed in RD5, RD7 RD25 and SPT for all three bacterial strains. After 12 days of inoculation, the brilliant - yellow green fluorescent intensity were found in RD5, RD7, RD25, LPT and SPT for all strains, whereas RD6 shows positive reaction only with R25. Thus, RD7 and SPT were the most effective rice varieties pertaining associative ability for these bacteria. RD5 and RD25 were the runner-up, whereas KDML was not appropiated for R15, R17 and R25 system.

3.5 Development of IND-ELISA method

The IND-ELISA for the determination of R15, R17, and R25 have been developed by adding the test antiserum

Table 3.6 Detection of N_2-fixing bacteria, R15, R17 and R25 associated with rice roots by IND-FA

Rice varieties inoculated with $5x10^8$ cells/tube (n=9)	Days of inoculation								
	R15			R17			R25		
	2d	7d	12d	2d	7d	12d	2d	7d	12d
	Immunofluorescent reaction of associated bacteria								
RD 5	-	+	+	+	+	+	+	+	+
RD 6	-	-	-	-	-	-	-	-	+
RD 7	+	+	+	+	+	+	+	+	+
RD 23	-	-	-	-	-	-	-	-	-
RD 25	-	+	+	-	+	+	-	-	+
LPT	-	-	+	-	-	+	-	-	+
SPT	+	+	+	+	+	+	+	+	+
KDML	-	-	-	-	-	-	-	-	-

Symbols (+) positive test

(-) negative test

A positive reaction is indicated by brilliant yellow green bacteria on the dark background and negative reaction is indicated by no fluoresent labelled bacteria.

Figure 3.7 Optimum dilution of anti-R15 for IND-ELISA

Each well was coated with 100 µl of antigen in the concentration range of 10^6 - 10^9 cells/ml and incubated overnight at 5°C. The titer of the first antiserum was varied from (○——○) 1:12,800, (△——△) 1:25,600, (□——□) 1:51,200, (●——●) 1:102,400, and (▲——▲) non-immune serum, and incubated with coating antigens at 30°C for 1h. The second antibody was fixed at the dilution of 1:1,500, incubated at 37°C for 3h and the enzymatic reaction was proceeded for 50 min at room temperature. Each point is the mean of 8 replicates.

to a polystyrene microtiter plate which has been previously sensitized by coating with specific antigen. A conjugate of goat antirabbit immunoglobulin chemically linked to the alkaline - phosphatase and the amount of enzyme - labelled antibody was estimated by addition of substrate, p-nitrophenol phosphate. After the yellow coloration of the positive wells had developed, the reaction was stopped and the absorbance at 405 nm was measured.

3.5.1 Optimum dilution of the first antisera

To determine the appropriate dilution (titer) of the first antibody, the concentration of homologous antigens were fixed in the range of $1x10^{9}$ - $1x10^{6}$ cells/ml. 100 ul of antigens were used for coating the well overnight, then 100 ul of the first antibody was added at various dilutions, and followed by 100 ul (1:1,500) of the second antibody and the substrate. The absorbance of coloured products obtained when various dilutions of anti R15, R17, and R25 were compared with that obtained when preimmunized sera of the same rabbit were used (Fig. 3.7, 3.8 and 3.9). The working dilution chosen for each serum was the highest dilution that shows correlation between the concentration of coating

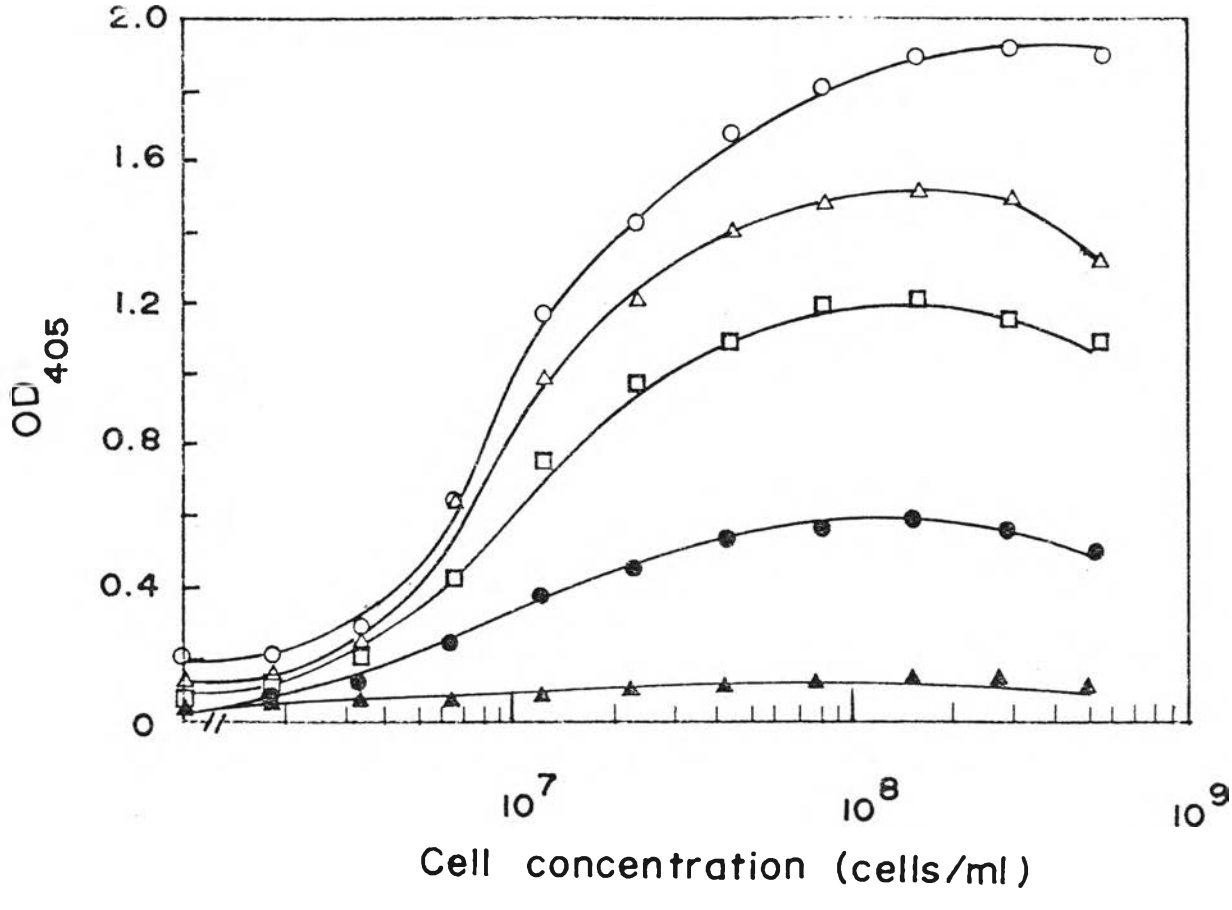

2.0
1.6
1.2
0.8
0.4
0
OD405
10^7
10^8
10^9
Cell concentration (cells/ml)

Figure 3.8 Optimum dilution of anti-R17 for IND-ELISA

Each well was coated with 100 µl of antigen having the concentration range of 10^{6} - 10^{9} cells/ml and incubated overnight at 5°C. The titer of the first antiserum was varied from (o—o) 1:6,400, (Δ—Δ) 1:12,800, (□—□) 1:25,600, (●—●) 1:51,200, and(▲—▲) non-Immune serum, and incubated with coating antigens at 30°C for 1h. The second antibody was fixed at the dilution of 1:1,500, incubated at 37°C for 3h and the enzymatic reaction was proceeded for 50 min at room temperature. Each point is the mean of 8 replicates.

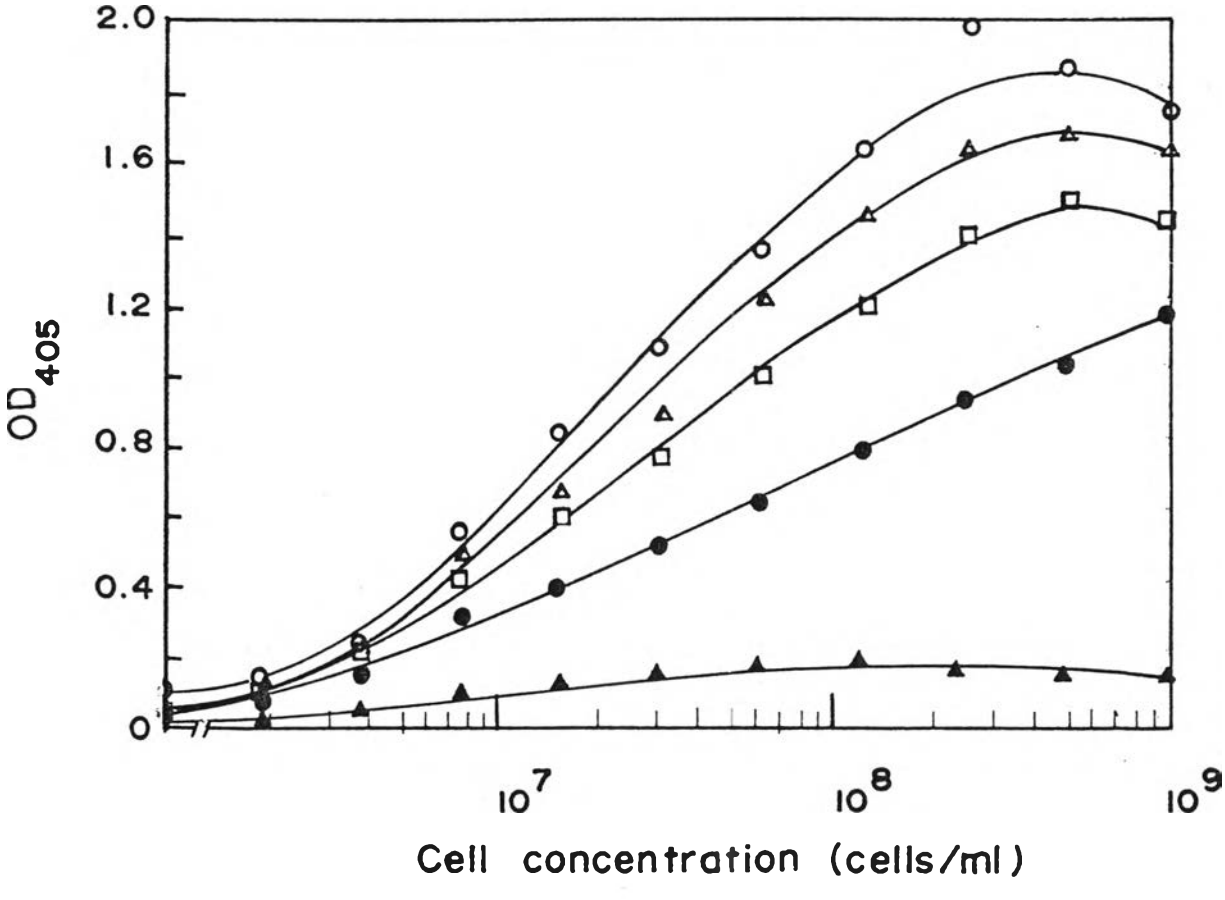
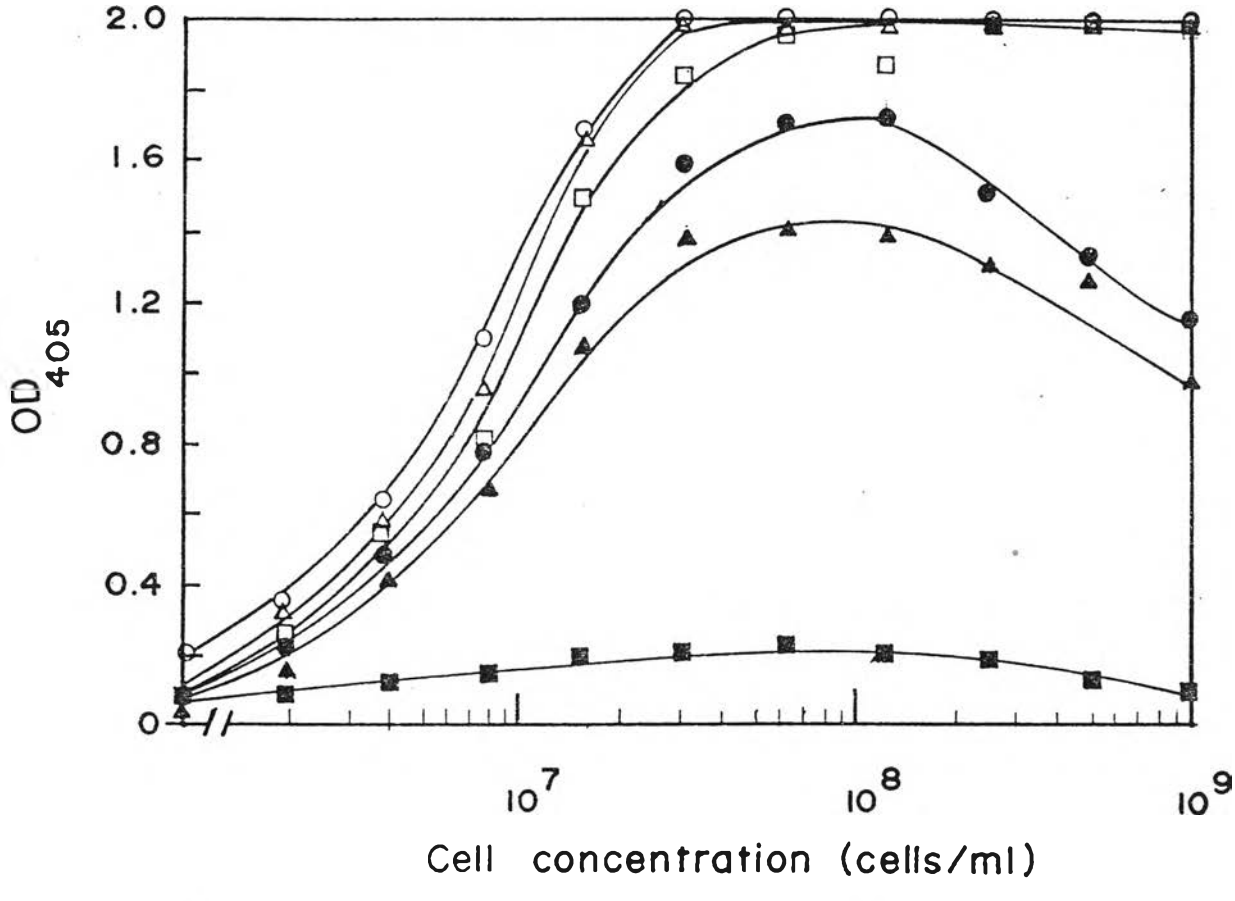

2.0
1.6
1.2
0.8
0.4
0
OD405
10^7
10^8
10^9
Cell concentration (cells/ml)

Figure 3.9 Optimum dilution of anti-R25 for IND-ELISA

Each well was coated with 100 µl of antigen having the concentration range of 10^8 - 10^9 cells/ml and incubated overnight at 5°C. The titer of the first antiserum was varied from (○—○) 1:6,400, (△—△) 1:12,800, (□—□) 1:25,600, (●—●) 1:51,200, (▲—▲) 1:102,400 and (■—■) non-immune serum, and incubated with coating antigens at 30°C for 1h. The second antibody was fixed at the dilution of 1:1,500, incubated at 37°C for 3h and the enzymatic reaction was proceeded for 50 min at room temperature. Each point is the mean of 8 replicates.


2.0
1.6
1.2
0.8
0.4
0
OD 405
10^7
10^8
10^9
Cell concentration (cells/ml)

antigen and OD405, which should be in the range of 1.0 - 1.5. By these criteria, the working titer of anti R15, R17, and R25 are 1:25,600, 1:25,600 and 1:51,200, respectively.

3.5.2 Optimum dilution of the second antibody

From preliminary study, when the concentration of the second antibody (conjugate) was lower than 1:1,000, the OD405 values would approach 2.0 within very short range of increasing antigens (data not shown). The dilution of the conjugate has then been varied between 1:1,500 and 1:2,500. Fig. 3.10 a-c shows that the optimum dilution of the conjugate was 1:1500 for all antisera, since this dilution yields the acceptable absorbance values (OD405 = 1.0 - 1.5).

3.5.3 Optimization of antiserum incubation time

The results in Fig.3.11 a-c indicate that the antiserum incubation time of 0.5 h and overnight (12 h) gave too low and too high absorbance whereas 1.0, 2.0, and 3.0 h show results in acceptable period, regardless of the temperature. Fig.3.12 is the replot of ELISA values (OD405 = 1.0-1.5) at fixed antigens concentrations of 6.75×10^{7} , 3.38×10^{7} and 1.67×10^{7}

Figure 3.10 Optimum dilution of FITC-conjugated goat antirabbit immunoglobulin for IND-ELISA

Each well was coated with 100 µl of antigen, (a) R15, (b) R17 and (c) R25 in the concentration ranged of 10^8 - 10^9 cells/ml and incubated overnight at 5°C. The titer of anti-R15, R17 and R25 were 1:25,600, 1:25,600 and 1:51,200, respectively. Incubated was at 30°C for 1h. The second antibody was varied at (●——●) 1:1,500 and (▲——▲) 1:2,500. Further incubation was at 37°C for 3h and the enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates.

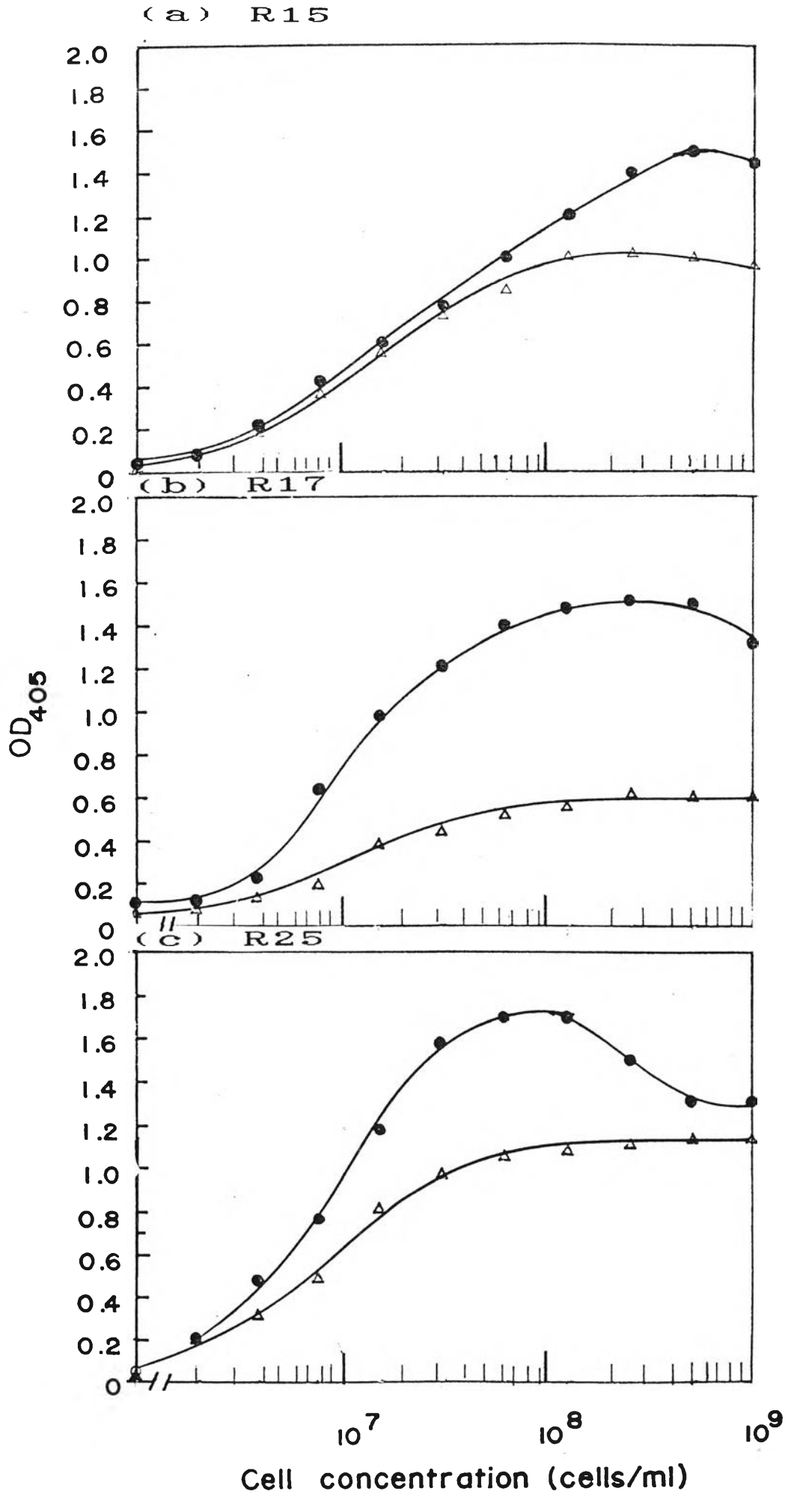

(a) R15
(b) R17
(c) R25
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD405
10^7
10^8
10^9
Cell concentration (cells/ml)

Figure 3.11 Optimum incubation time of the first antibody for IND-ELISA

Each well was coated with 100 µl of antigen, (a) R15, (b) R17 and (c) R25 in the concentration range of 10^8 - 10^9 cells/ml, incubated at 5°C overnight. The titer of anti-R15, R17 and R25 were 1:25,600, 1:25,600 and 51,200 respectively, and the incubation time was varied from 0.5, 1.0, 2.0, 3.0h at 30°C and overnight at 5°C. The titer of second antibody was fixed at 1:1,500. Further incubation was at 37°C for 3h, and the enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates. The symbols of the first antiserum incubation time at 30°C are (o—o) 0.5, (Δ—Δ) 1.0, (□—□) 2.0, (●—●) 3.0h and (▲—▲) overnight at 5°C.

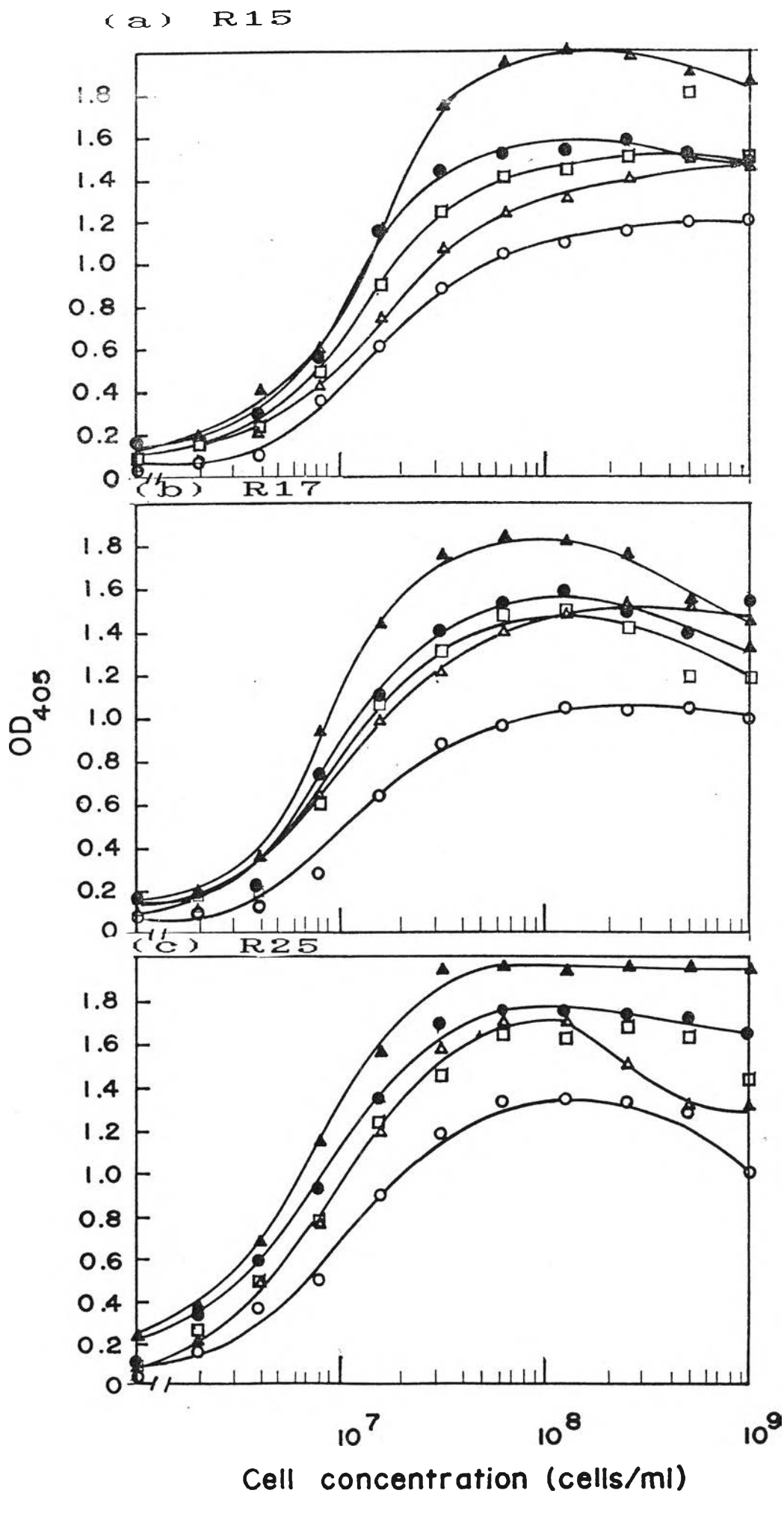
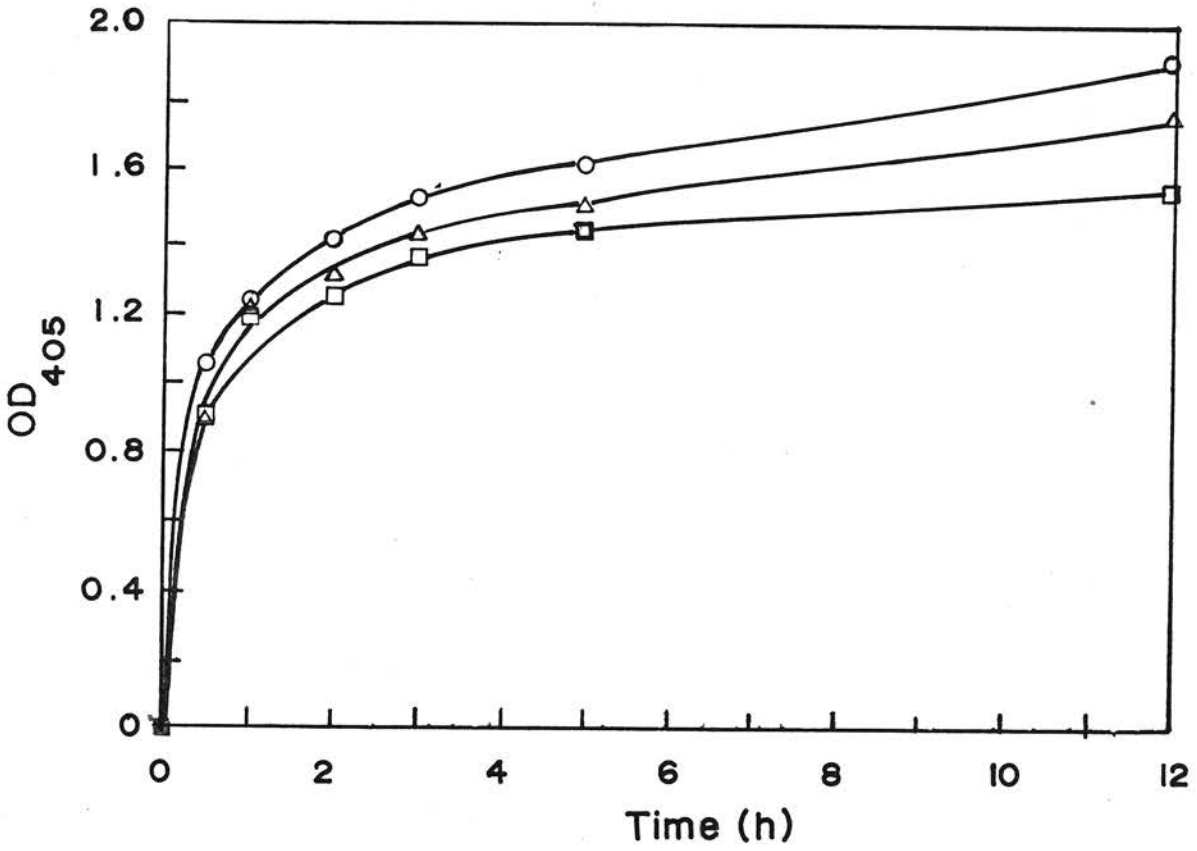

(a) R15
(b) R17
(c) R25
OD405
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10^7
10^8
10^9
Cell concentration (cells/ml)

Figure 3.12 Saturation of the binding of anti-R15, R17 and R25 to the coated antigens

Binding kinetics of anti-R15 (o—o), R17 (Δ—Δ) and R25 (□—□) to their homologous coating antigens' at the selected concentration of 6.75×10^7, 3.38×10^7 and 1.69×10^7 cells/ml respectively, according to Fig. 3.11.


2.0
1.6
1.2
0.8
0.4
0
OD405
0
2
4
6
8
10
12
Time (h)

cells/ml for R15, R17, and R25, respectively versus the incubation time. It was shown that 1 h was the shortest incubation time to attain immunological equilibrium.

3.5.4 Optimization of conjugate incubation time

Conjugate incubation period was varied as performed with the first antibody. Fig. 3.13 a-c shows that 3.0 h was the optimum incubation time to obtain acceptable OD405. The relationship between ELISA values at fixed antigens' concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml for R15, R17, and R25, respectively and incubation time shown in Fig.3.14, confirms that 3.0 h was suitable for this incubation period.

3.5.5 Optimization of substrate incubation time

This experiment was conducted to investigate the effect of substrate incubation time in order to select the most suitable shortest period between 40 and 50 min. By fixing the optimum conditions obtained from the previous experiments, the incubation time of 50 min was selected because the absorbance value fit the acceptable range (Fig. 3.15 a-c).

Figure 3.13 Optimun incubation time of the second antibody for IND-ELISA

Each well was coated with 100 µl of antigen, (a) R15, (b) R17 and (c) R25 in the concentration range of 10^8 - 10^9 cells/ml, and incubated overnight at 5°C. The titer of anti-R15, R17 and R25 were 1:25,600, 1:25,600 and 51,200 respectively, incubated at 30°C for 1h. The second antibody was fixed at 1:1,500, the incubation time was varied to (o—o) 0.5, (Δ—Δ) 1.0, (□—□) 2.0, (●—●) 3.0h at 37°C and (▲—▲) overnight at 5°C and the enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates.

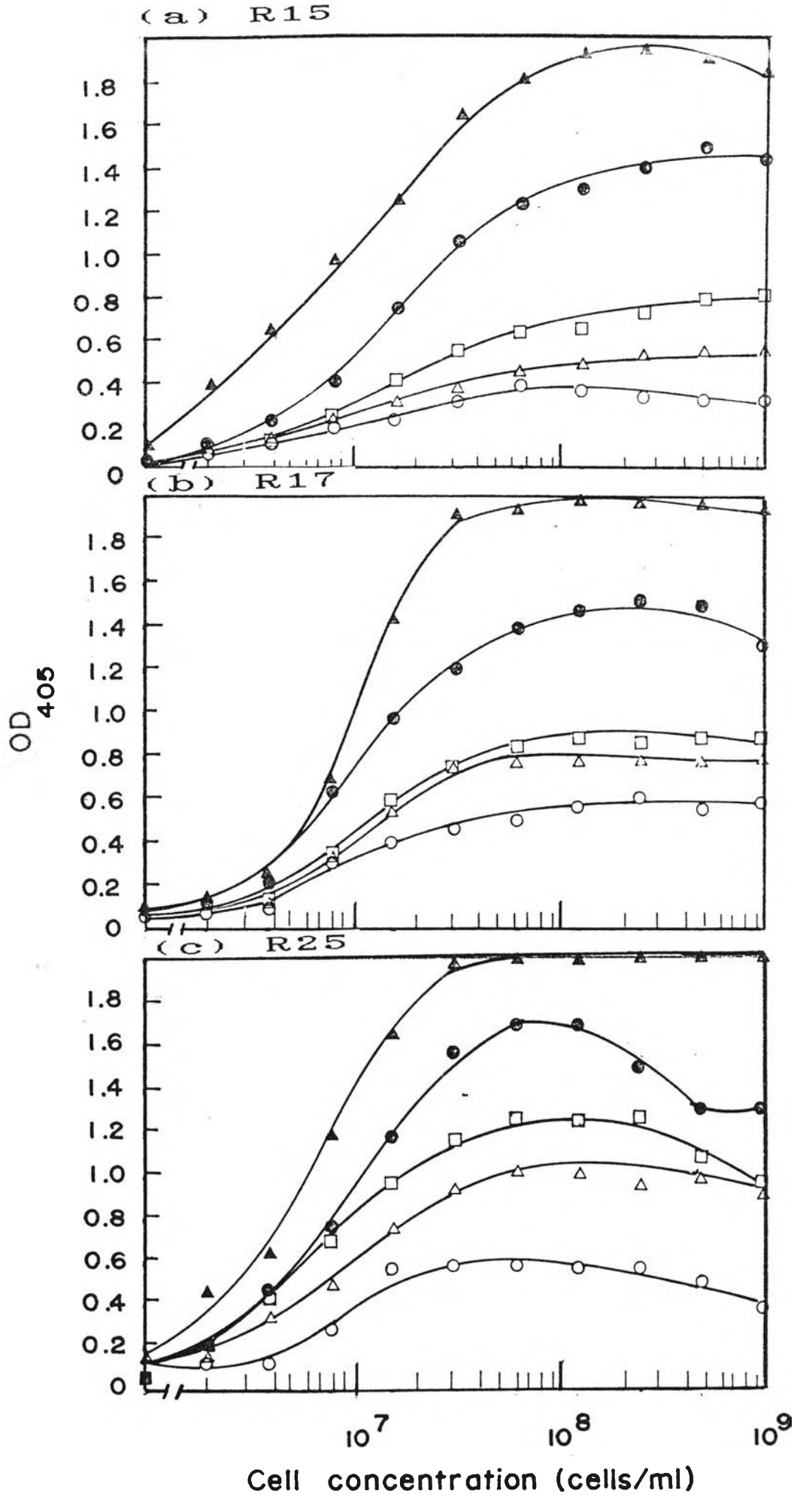

(a) R15
(b) R17
(c) R25
OD 405
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10^7
10^8
10^9
Cell concentration (cells/ml)

Figure 3.14 Saturation of the binding between FITC conjugated goat antirabbit globulin and the first antibodies

Binding kinetics of the conjugate, to the first antibodies: (o—o) anti-R15, (Δ—Δ) anti-R17 and (□—□) anti-R25 at the selected concentrations of coating antigens: 6.75×10^7, 3.38×10^7 and 1.69×10^7 cells/ml, respectively, according to Fig. 3.13.

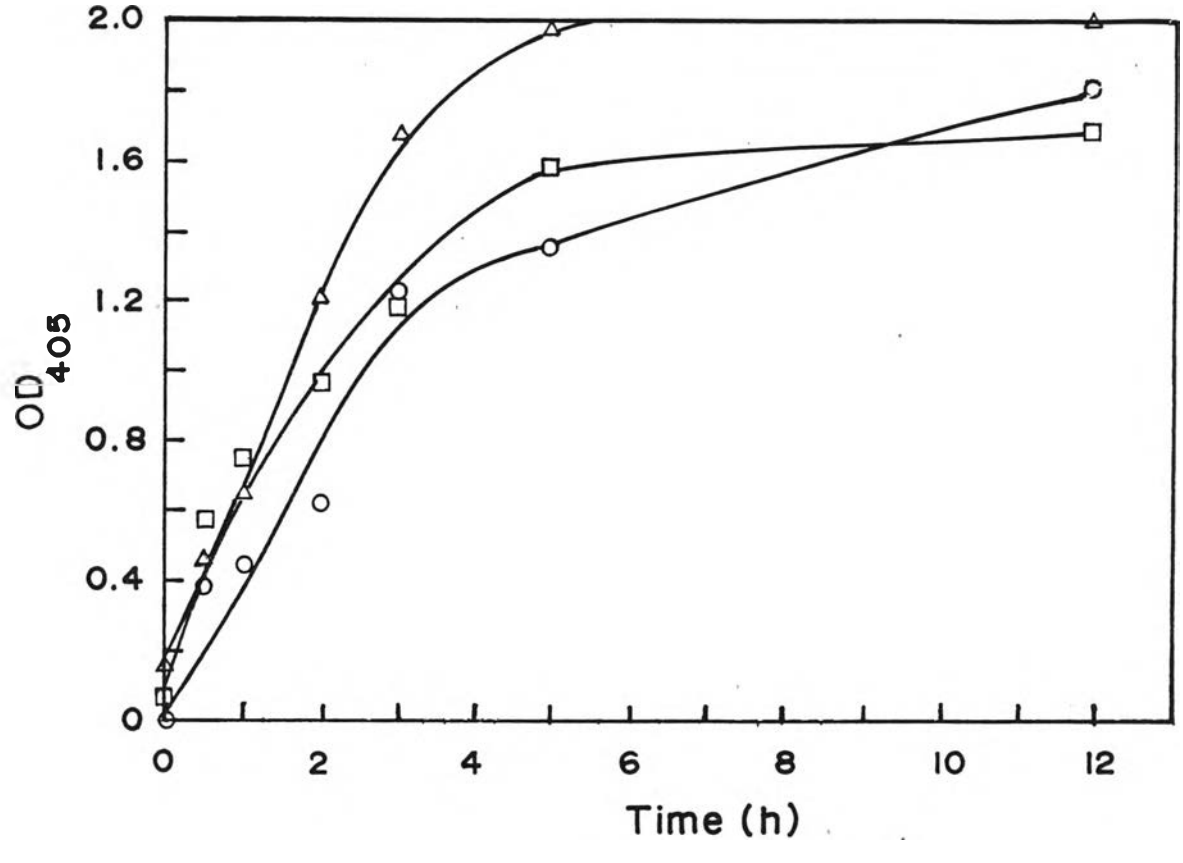
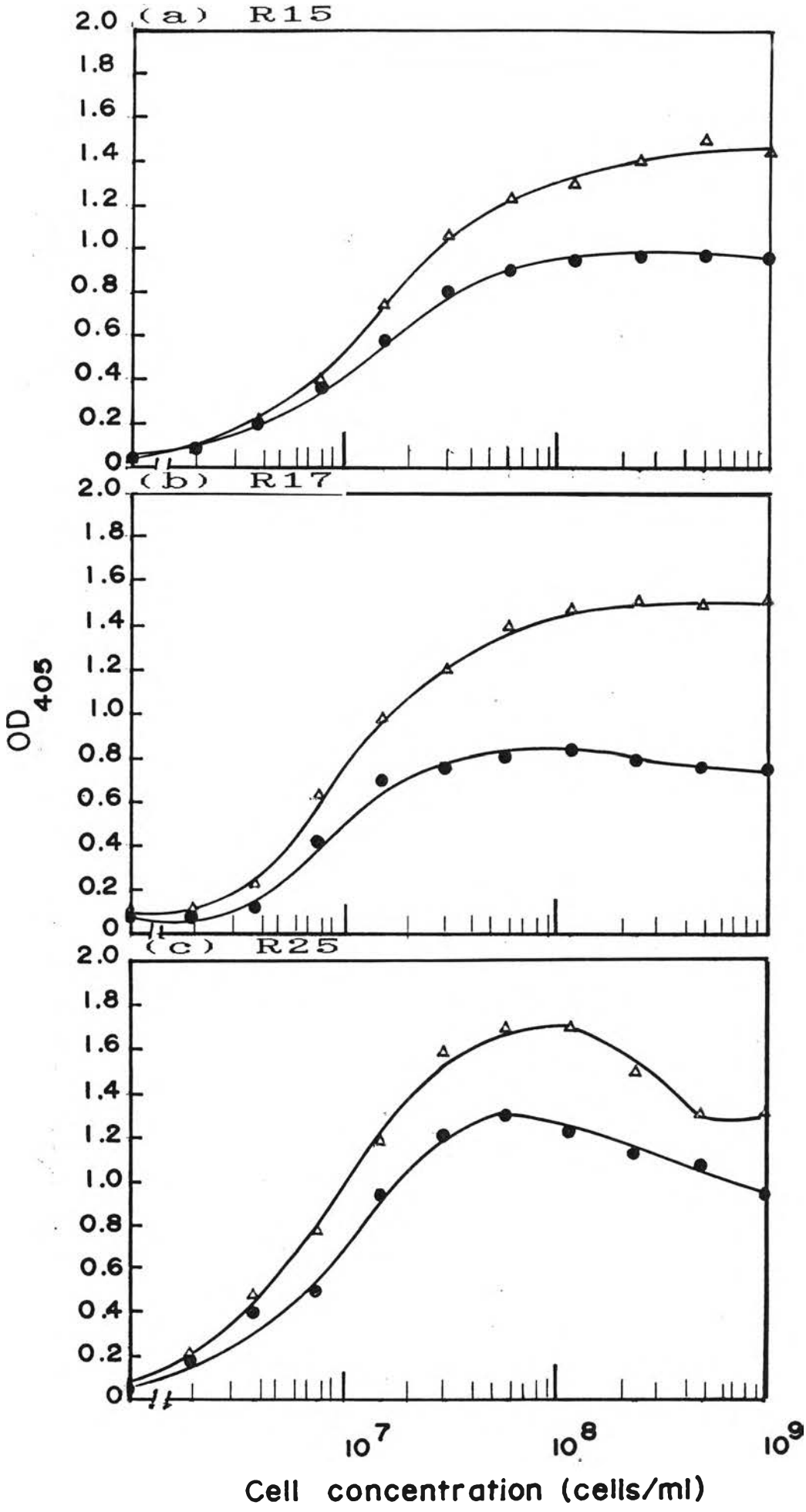

2.0
1.6
1.2
0.8
0.4
0
OD405
0
2
4
6
8
10
12
Time (h)

Figure 3.15 Optimum incubation time with substrate for enzymatic reaction.

The plate was coated with 100 µl of antigen, (a) R15, (b) R17 and (c) R25 in the concentration range of 10^8 - 10^9 cells/ml, incubated overnight at 5°C. The titer of anti-R15, R17 and R25 were 1:25,600, 1:25,600 and 1:51,200 respectively. Incubation was at 30°C for 1h. The titer of second antibody was fixed at 1:1,500. Further incubation was at 37°C for 3h and the enzymatic reaction was varied for (●——●) 40 min and (▲——▲) 50 min at room temperature. Each point is the mean of 8 replicates.


(a) R15
(b) R17
(c) R25
OD405
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10^7
10^8
10^9
Cell concentration (cells/ml)

3.6 Development of competitive indirect ELISA (COM-IND-ELISA)

In order to quantitate the cell antigens (R15, R17, and R25) in natural condition or in mixture containing several unknown bacteria, the ordinary ELISA method would not fit this purpose since other bacteria can interfere at the nonspecific coating step. The COM-IND-ELISA was therefore developed by firstly; coating each well with a definitely known amout of the test antigen, which gives an OD405 in the acceptable range (1.0-1.5). Secondly, a series of soluble test antigens at increasing concentration will be mixed with a constant final concentration of antiserum to compete with the bound antigen coated on the well. The more soluble antigens in the serum mixture, the less OD405 will be observed via bound antigen. Fig. 3.15 a-c show that the suitable concentration of coating antigens are $6.75x10^7$, $3.38x10^7$, and $1.69x10^7$ cells/ml for R15, R17, and R25, respectively. In the next step, a 100ul aliquot from serial dilution of soluble test antigen ($1x10^9$ - $1x10^6$ cell/ml) in its own homologous antiserum is added to each well containing fixed amount of suitable coated antigen. The COM-IND-ELISA provides the serological

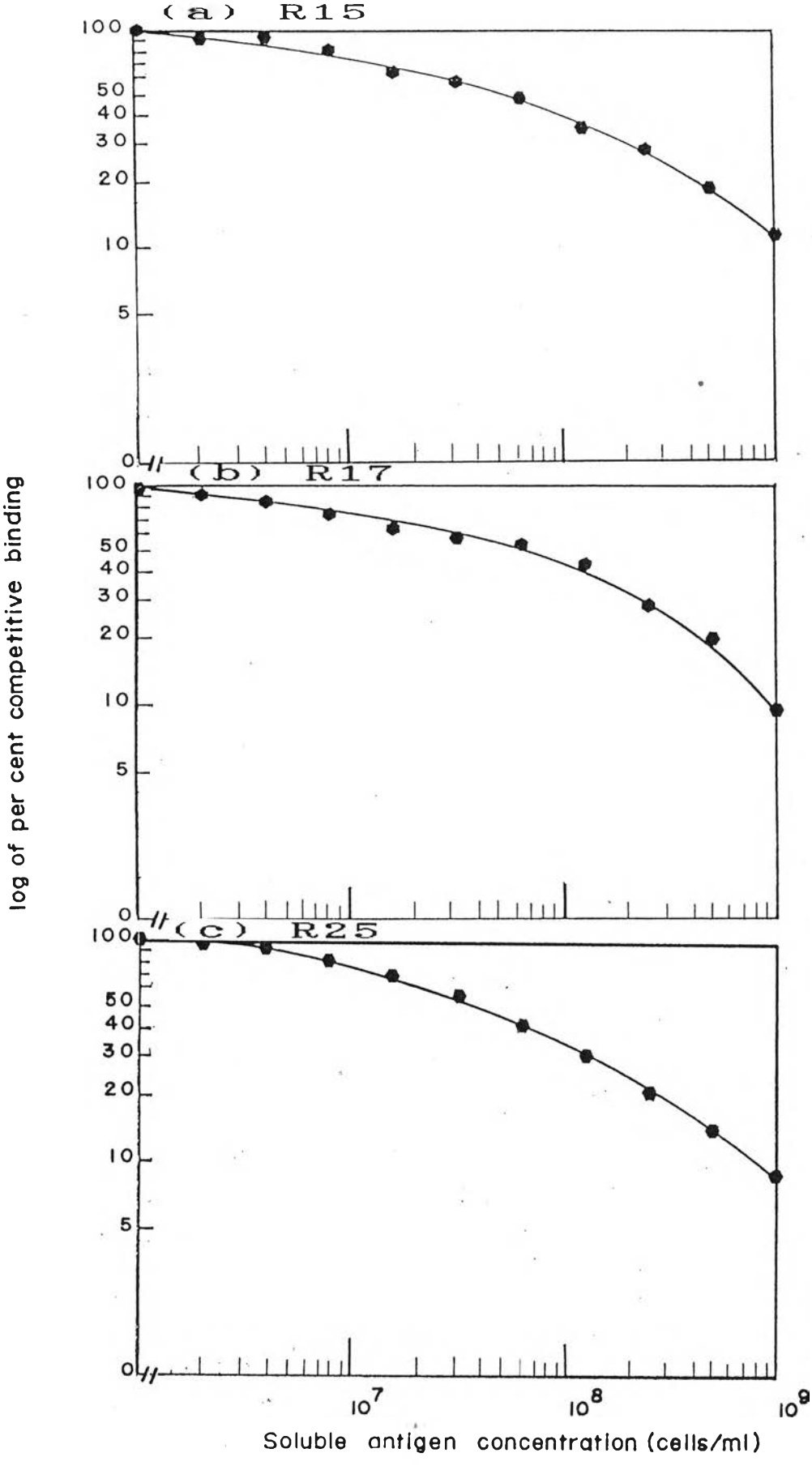
reaction values in terms of per cent competitive binding. The standard curve is a plot of log per cent competitive binding versus the concentration ($1x10^9$ - $1x10^6$ cells/ml) of soluble antigen as shown in Fig. 3.16 a-c. One hundred per cent bound means no addition of soluble antigen in the first antiserum and the zero per cent bound should be for complete binding between soluble antigens in the first antiserum. By this method the correlation between per cent bound and the concentration of soluble antigens lower than $1x10^6$ cells/ml can be detected in all 3 strains, although great variation were obtained in approaching 100% bound. On the other extreme, the concentration of soluble antigens higher than $1x10^9$ cells/ml result in the competitive binding around 4-6 % from bound antigens which does not correlate with the cell number.

3.6.1 Effect of soluble test antigens concentration in COM-IND-ELISA

In order to find the range of soluble antigens concentration that gave reliable competitive per cent binding, or significant difference between ELISA values of control (no competitive soluble antigen) and of a definite antigen concentration of a bacterial strain

Figure 3.16 Standard curves for the determination of R15, R17 and R25 by COM-IND-ELISA

Each 100 µl of antigens' concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml of (a) R15, (b) R17 and (c) R25, respectively was coated to the tested well. Followed by a series dilution of soluble tested antigen mixed with its antiserum to the final concentration of 10^6 - 10^9 cells/ml in appropiate titer of 1:25,600, 1:25,600 and 1:51,200 for R15, R17 and R25 respectively. Incubation was at 30°C for 1h. The titer of the second antibody was fixed at 1:1,500. Further incubation was at 37°C for 3h and the enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates.


(a) R15
(b) R17
(c) R25
100
50
40
30
20
10
5
0
log of per cent competitive binding
10^7
10^8
10^9
Soluble antigen concentration (cells/ml)

(R15, R17 and R25), various antigen concentration ranging from $1.0x10^6$ to $1x10^9$ cells/ml were used to compete with coated or bound antigens. Table 3.7 a-c show that $1.95x10^6$ cells/ml was the minimum soluble antigen concentration which gave significant difference of ELISA values over the control by COM-IND-ELISA. In other words, the sensitivity of this developed method is reliable in the order of $1.95x10^6$ cells/ml for pure culture of antigen and its homologous antiserum.

3.6.2 Specificity of COM-IND-ELISA

When non-homologous soluble antigens (concentration of $2.5x10^8$ cells/ml) were used to compete with the coated bacterial antigen (using the optimum concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml for R15, R17 and R25, respectively), Fig. 3.17, demonstrates that the anti-R15 shows about 96 - 98% cross-reaction with R17 and vice versa. When the antisera of both R15 and R17 were allowed to react with a series of other non-homologous N_2-fixing bacteria and E. Coli, only Klebsiella oxytoga NG13 shows significant cross-reaction (26 - 28%). Antiserum raised against R25 showed no cross-reaction with either R15 or R17, but showed 100% cross-reaction with both reference strains

Table 3.7 Effect of soluble antigen concentration in COM-IND-ELISA

Table 3.7 a R15

Soluble antigen concentration[a]	ELISA values (OD405nm)[b]	Student t-test
control	1.105 ± 0	-
$1.0x10^6$	1.103 ± 0.031	NS[c]
$1.95x10^6$	0.994 ± 0.031	$p < 0.01$
$3.90x10^6$	1.032 ± 0.032	$p < 0.05$
$7.81x10^6$	0.880 ± 0.055	$p < 0.01$
$1.56x10^7$	0.688 ± 0.036	$p < 0.01$
$3.12x10^7$	0.633 ± 0.031	$p < 0.01$
$6.25x10^7$	0.532 ± 0.030	$p < 0.01$
$1.25x10^8$	0.400 ± 0.034	$p < 0.01$
$2.50x10^8$	0.331 ± 0.012	$p < 0.01$
$5.00x10^8$	0.215 ± 0.013	$p < 0.01$
$1.0x10^9$	0.121 ± 0.008	$p < 0.01$

[a] Concentration of soluble antigens in cell/ml

[b] Each value was the mean of eight replicated ± SD.

[c] NS, not significant.

Table 3.7.b R17

Soluble antigen concentration[a]	ELISA values (OD405nm)[b]	Student t-test
control	1.025 ± 0	-
$1.0x10^6$	1.022 ± 0.034	NS[c]
$1.95x10^6$	0.970 ± 0.055	$p < 0.05$
$3.90x10^6$	0.884 ± 0.034	$p < 0.01$
$7.81x10^6$	0.768 ± 0.027	$p < 0.01$
$1.56x10^7$	0.673 ± 0.024	$p < 0.01$
$3.12x10^7$	0.578 ± 0.049	$p < 0.01$
$6.25x10^7$	0.555 ± 0.035	$p < 0.01$
$1.25x10^8$	0.462 ± 0.027	$p < 0.01$
$2.50x10^8$	0.278 ± 0.024	$p < 0.01$
$5.0x10^8$	0.113 ± 0.012	$p < 0.01$
$1.0x10^9$	0.093 ± 0.008	$p < 0.01$

[a] Concentration of soluble antigens in cell/ml

[b] Each value was the mean of eight replicated ± SD.

[c] NS, not significant.

Table 3.7 c. R25

Soluble antigen concentration[a]	ELISA values (OD405nm)[b]	Student t-test
control	1.105 ± 0	-
$1.0x10^6$	1.112 ± 0.044	NS[c]
$1.95x10^6$	1.058 ± 0.026	$p < 0.01$
$3.90x10^6$	1.034 ± 0.167	$p < 0.05$
$7.81x10^6$	0.908 ± 0.037	$p < 0.01$
$1.56x10^7$	0.779 ± 0.059	$p < 0.01$
$3.12x10^7$	0.615 ± 0.059	$p < 0.01$
$6.25x10^7$	0.463 ± 0.041	$p < 0.01$
$1.25x10^8$	0.334 ± 0.026	$p < 0.01$
$2.50x10^8$	0.221 ± 0.013	$p < 0.01$
$5.0x10^8$	0.148 ± 0.011	$p < 0.01$
$1.0x10^9$	0.094 ± 0.008	$p < 0.01$

[a] Concentration of soluble antigens in cell/ml

[b] Each value was the mean of eight replicated ± SD.

[c] NS, not significant.

of Azospirillum lipoferum (FS and 34H). The data suggest a strong serological homology between R15 and R17, and their resemblance to K.oxytoca NG13. Whereas R25 is serologically similar to A.lipoferum.

3.6.3 Precision and accuracy of COM-IND-ELISA

From the intra-assay and inter-assay tests for precision of this developed method, by using per cent coefficience of variation (%CV) as the criteria, Table 3.8 a-c shows that the %CV distribution in the intra-assay was 2.85 - 8.71, 3.34 - 8.71 and 1.61 - 9.63 (range 3 - 10%), whereas in inter-assay wider range of %CV were observed (range 3 - 15%) for R15, R17 and R25, respectively.

The accuracy of COM-IND-ELISA has been evaluated for each antiserum by adding the known amount of homologous soluble antigen to various samples of increasing concentration and assayed for per cent recovery of totally added antigens. From Table 3.9 a-c, it can be seen that per cent recovery of more or less 90 - 120% were obtained for antisera of R15 and R17. The accuracy in the case of R25 was better as indicated by the narrow range of % recovery from 94.5 - 109.5.

Figure 3.17 Cross reaction of related strains with anti-R15, R17 and R25 by COM-IND-ELISA

Each 100 μl of exact concentration of (a) ▨ R15, (b) ▧ R17 and (c) ▨ R25 was coated to the tested well. The first antiserum was mixed with 2.5×10^8 cells/ml of the homologous and nonhomologous antigen and added to the tested well, and incubated at 30°C for 1h. The titer of the second antibody was fixed at 1:1,500. Further incubation was at 37°C for 3h. The enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates.

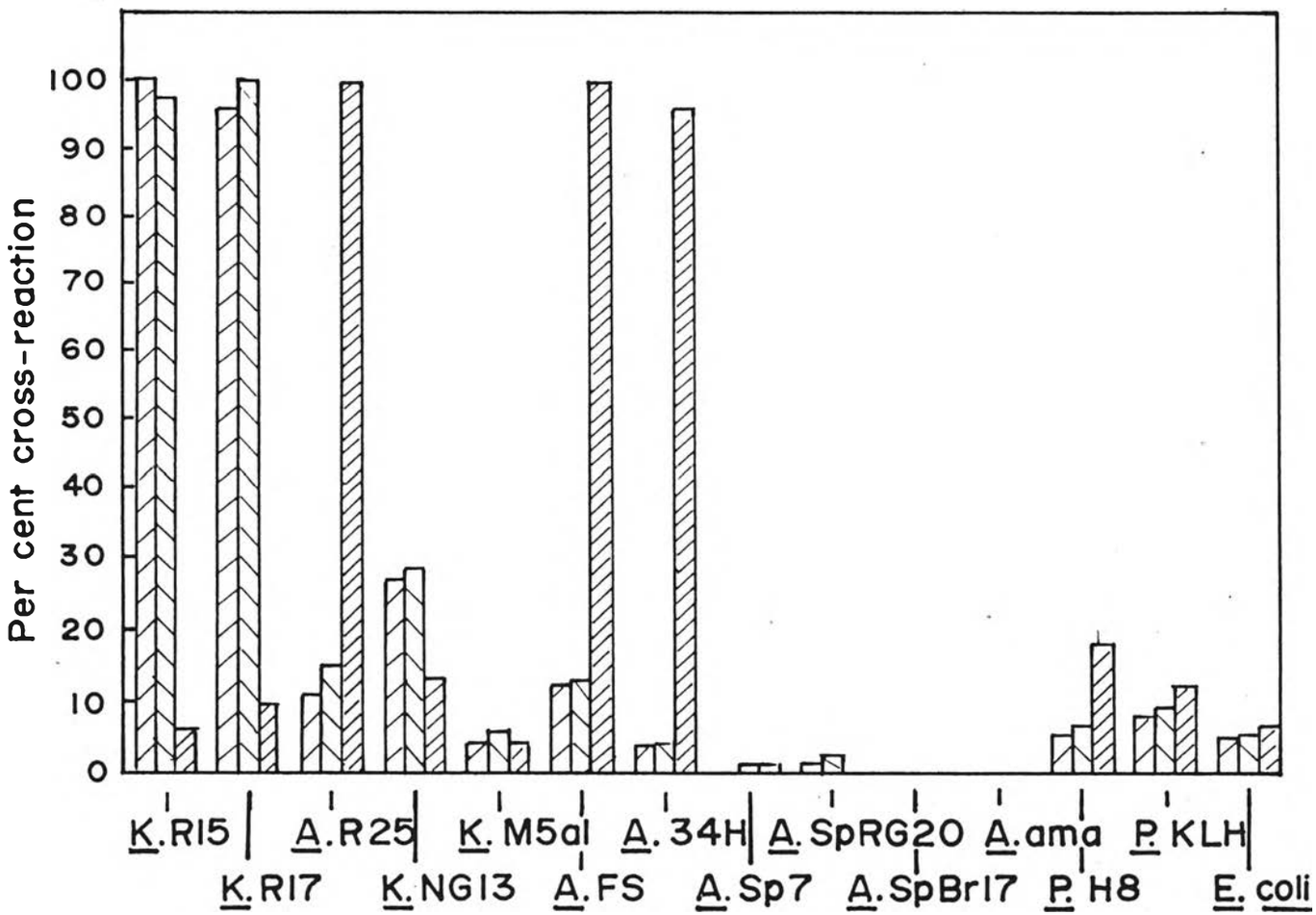

Per cent cross-reaction
100
90
80
70
60
50
40
30
20
10
0
K. R15
K. R17
A. R25
K. NG13
K. M5al
A. FS
A. 34H
A. Sp7
A. SpRG20
A. SpBr17
A. ama
P. H8
P. KLH
E. coli

Table 3.8 The precision of the COM-IND-ELISA method

Each 100ul of antigens concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml of (a) R15, (b) R17 and (c) R25, respectively were coated to the tested well. Followed by a series dilution of soluble antigen which added known amount of cells number, mixed with its antiserum to final concentration of 10^8 - 10^9 cells/ml in appropiate titer of 1:25,600, 1:25,600, and 1:51,200 respectively. Incubation was at 30°C for 1h. The second antibody was fixed at dilution of 1:1,500, incubated at 37°C for 3h. The enzymatic reaction was conducted for 50 min at room temperature.

Table 3.8 a R15

Soluble antigen concentration (cells/ml)	% competitive binding							
	Intra-assay				Inter-assay			
	Mean	SD	N	%CV	Mean	SD	N	%CV
$1.0x10^6$	99.80	2.85	8	2.85	99.66	3.83	8	3.84
$1.95x10^6$	90.00	2.80	8	3.11	90.00	3.66	8	4.07
$3.90x10^6$	93.45	2.90	8	3.10	92.67	3.66	8	3.95
$7.81x10^6$	79.67	4.95	8	6.21	80.66	6.00	8	7.43
$1.56x10^7$	62.33	3.33	8	5.34	62.67	4.33	8	6.91
$3.12x10^7$	57.33	2.80	8	4.88	56.33	3.33	8	5.93
$1.25x10^7$	48.15	2.70	8	5.60	47.33	3.16	8	6.69
$1.25x10^8$	36.16	3.15	8	8.71	34.67	4.00	8	11.54
$2.50x10^8$	29.98	1.12	8	3.73	27.33	1.46	8	5.35
$5.0x10^8$	19.51	1.25	8	6.40	18.33	2.00	8	10.90
$1.0x10^9$	10.96	0.71	8	6.48	11.00	1.33	8	12.10

Table 3.8 b R17

Soluble antigen concentration (cells/ml)	% competitive binding							
	Intra-assay				Inter-assay			
	Mean	SD	N	%CV	Mean	SD	N	%CV
$1.0x10^6$	99.67	3.33	8	3.34	99.67	4.67	8	4.68
$1.95x10^6$	94.67	5.33	8	5.63	93.33	5.33	8	5.71
$3.90x10^6$	86.33	3.33	8	3.86	86.67	4.67	8	5.38
$7.81x10^6$	74.88	2.67	8	3.56	75.67	3.33	8	4.40
$1.56x10^7$	65.67	2.33	8	3.55	64.33	3.33	8	5.18
$3.12x10^7$	56.45	4.78	8	8.46	58.67	6.67	8	11.36
$1.25x10^7$	54.15	3.45	8	6.37	53.66	5.83	8	10.87
$1.25x10^8$	45.14	2.55	8	8.71	34.67	4.00	8	11.54
$2.50x10^8$	27.17	2.30	8	8.46	27.73	3.16	8	11.43
$5.0x10^8$	19.05	1.51	8	6.03	19.67	2.00	8	10.16
$1.0x10^9$	9.13	0.76	8	8.32	9.33	1.33	8	14.29

Table 3.8 c R25

Soluble antigen concentration (cells/ml)	% competitive binding							
	Intra-assay				Inter-assay			
	Mean	SD	N	%CV	Mean	SD	N	%CV
$1.0x10^{6}$	100.00	4.00	8	4.00	100.00	3.99	8	3.99
$1.95x10^{6}$	95.13	2.33	8	2.45	94.67	2.30	8	2.43
$3.90x10^{6}$	93.00	1.50	8	1.61	92.67	2.67	8	2.88
$7.81x10^{6}$	81.67	3.33	8	4.08	81.33	4.00	8	4.92
$1.56x10^{7}$	70.13	5.33	8	7.60	69.33	5.33	8	7.68
$3.12x10^{7}$	55.33	5.33	8	9.63	55.33	6.67	8	12.05
$1.25x10^{7}$	41.67	3.67	8	8.80	42.00	6.00	8	14.28
$1.25x10^{8}$	30.00	2.33	8	7.77	29.99	2.67	8	8.90
$2.50x10^{8}$	19.95	1.20	8	6.01	20.66	1.50	8	7.26
$5.0x10^{8}$	13.33	1.00	8	7.50	13.99	1.00	8	7.15
$1.0x10^{9}$	8.50	0.75	8	8.80	8.60	1.00	8	11.62

Table 3.9 The accuracy of COM-IND-ELISA

Each 100ul of antigens concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml of (a) R15, (b) R17 and (c) R25, respectively were coated to the tested well. Followed by a series dilution of soluble antigen which added known amount of cells number, mixed with its antiserum to final concentration of 10^8 - 10^9 cells/ml in appropiate titer of 1:25,600, 1:25,600, and 1:51,200 respectively. Incubation was at 30°C for 1h. The second antibody was fixed at dilution of 1:1,500, incubated at 37°C for 3h. The enzymatic reaction was conducted for 50 min at room temperature.

Table 3.9 a **R15**

Cells concentration			
Soluble antigen	Known amount of cells added	Observe from COM-IND-ELISA	% Recovery of total cells
$1.00x10^6$	$1.00x10^6$	$2.00x10^6$	100.0
$1.95x10^6$		$3.50x10^6$	118.6
$3.90x10^6$		$4.38x10^6$	89.3
$7.81x10^6$		$8.90x10^6$	101.0
$1.56x10^7$	$1.56x10^7$	$3.31x10^7$	106.0
$3.12x10^7$		$5.19x10^7$	110.8
$6.25x10^7$		$7.38x10^7$	94.4
$1.25x10^8$	$1.25x10^8$	$2.83x10^8$	113.2
$2.50x10^8$		$3.70x10^8$	98.6
$5.00x10^8$		$6.87x10^8$	109.9

Table 3.9 b R17

Cells concentration			
Soluble antigen	Known amount of cells added	Observe from COM-IND-ELISA	% Recovery of total cells
$1.00x10^6$	$1.00x10^6$	$2.00x10^6$	100.0
$1.95x10^6$		$3.04x10^6$	103.0
$3.90x10^6$		$4.89x10^6$	99.8
$7.81x10^6$		$1.02x10^7$	116.3
$1.56x10^7$	$1.56x10^7$	$3.72x10^7$	119.2
$3.12x10^7$		$5.33x10^7$	113.8
$6.25x10^7$		$8.09x10^7$	100.0
$1.25x10^8$	$1.25x10^8$	$2.66x10^8$	106.4
$2.50x10^8$		$3.20x10^8$	85.3
$5.00x10^8$		$6.03x10^8$	96.4

Table 3.9 c R25

Cells concentration			
Soluble antigen	Known amount of cells added	Observe from COM-IND-ELISA	% Recovery of total cells
$1.00x10^6$	$1.00x10^6$	$2.00x10^6$	100.0
$1.95x10^6$		$3.23x10^6$	109.4
$3.90x10^6$		$4.63x10^6$	94.4
$7.81x10^6$		$9.12x10^6$	103.5
$1.56x10^7$	$1.56x10^7$	$3.01x10^7$	96.4
$3.12x10^7$		$4.58x10^7$	97.8
$6.25x10^7$		$7.58x10^7$	97.0
$1.25x10^8$	$1.25x10^8$	$2.48x10^8$	99.2
$2.50x10^8$		$3.88x10^8$	103.4
$5.00x10^8$		$6.25x10^8$	100.0

3.7 Determination of the growth characteristics of N_2-fixing bacteria R15, R17 and R25 by COM-IND-ELISA

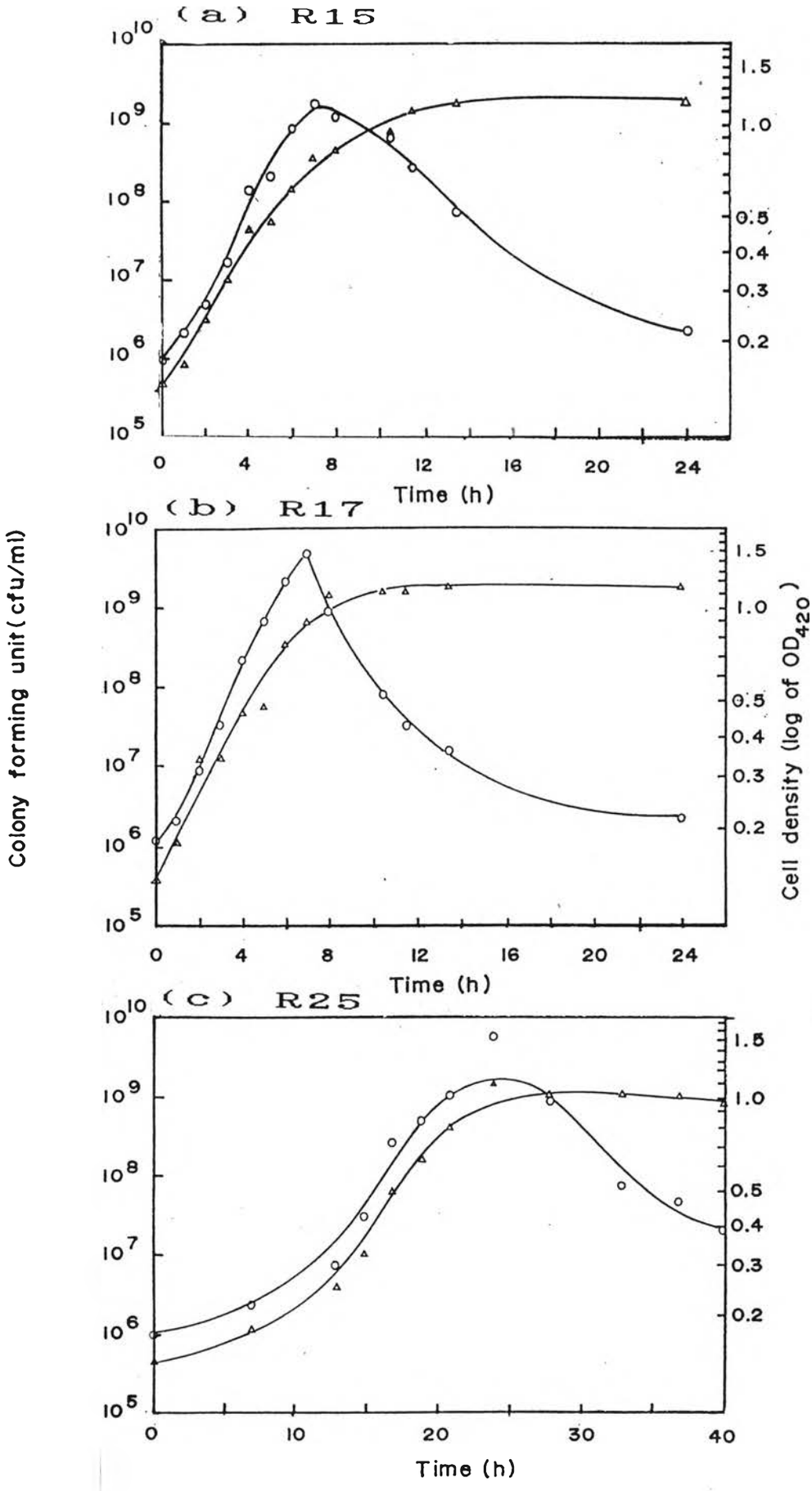
Growth curves of bacteria have been previously measured by cfu/ml and OD420. In this experiment COM-IND-ELISA has been performed in parallel with plate - count method and turbidity measurement. Fig. 3.18 a-c shows good agreement among the 3 methods toward the early stationary phase. After that stage, the direct cells number assayed by COM-IND-ELISA and OD420 remained constant whereas cfu or active cells began to decrease by PMN method. COM-IND-ELISA cannot distinguish active from inactive cells, thus similar to OD420 in counting antigenic particles.

3.8 Quantitation of associative N_2-fixing bacteria by COM-IND-ELISA

In this experiment, a serial dilution of antigens, R15, R17 and R25 ($1x10^9$ - $1x10^6$ cells/ml) were passed through the filtering unit used for the separation of associative bacteria from root debris (as described in Method 2.21) before adding in the antiserum

Figure 3.18 Comparision of growth character by optical density, plate-count method, and COM-IND-ELISA for R15, R17 and R25

Bacteria (3% inoculum) were cultured in 150 ml NF medium with reciprocal shaking at 30°C, except R25 without shaking and 5ml aliquot was withdrawn at intervals to measure (△——△) OD420, (○——○) cfu by plate-count method and (□——□) cell number by COM-IND-ELISA. Three replicative measurements were carried out at each time interval.


(a) R15
(b) R17
(c) R25
Colony forming unit (cfu/ml)
Cell density (log of OD_{420})
Time (h)
Time (h)
Time (h)

and measured for the per cent competitive binding by COM-IND-ELISA. The standard curves obtained for associative system are shown in Fig. 3.19 a-c. Different varieties of rice were grown in modified spermosphere model as described in Methods 2.12. Bacteria, R15, R17, and R25 were inoculated on day 2 after germination at the dose of $5x10^8$ cells in 1 ml normal saline per one tube which contains 3 rice seedlings. Control tubes were added with 1 ml normal saline instead of bacterial suspension. The nitrogen-fixing potential of associative bacteria in each tube were measured by ARA as described in Method 2.18, and reported as net umol ethylene produced after subtracting average values of the control tubes. The colonization potential of each bacterial strain on each variety of rice was the different values of cells number between the inoculated rice seedlings and the uninoculated control as described in Methods 2.20. Fig. 3.20 a-c shows the patterns of increasing colonization potential, nitrogen-fixing potential and the vigor index of rice cv RD7 when inoculated with R15, R17 and R25, respectively. From these results, the maximum colonization potential, N_2-fixing potential and plant vigor index were observed on day 12 after inoculation.

Figure 3.19 Standard curves for determination of associative R15, R17 and R25 by COM-IND-ELISA

Each 100 ul of antigens concentration of $6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml of (a) R15, (b) R17 and (c) R25 respectively was coated to the tested well. Followed by a series dilution of soluble tested antigen (which were passed the filtering unit), mixed with their antisera to the final concentration of 10^8 - 10^9 cells/ml in appropiate titer of 1:25,600, 1:25,600 and 1:51,200 for R15, R17 and R25 respectively. Incubation was at 30°C for 1h. The titer of the second antibody was fixed at 1:1,500. Further incubation was at 37°C for 3h and the enzymatic reaction was conducted for 50 min at room temperature. Each point is the mean of 8 replicates.

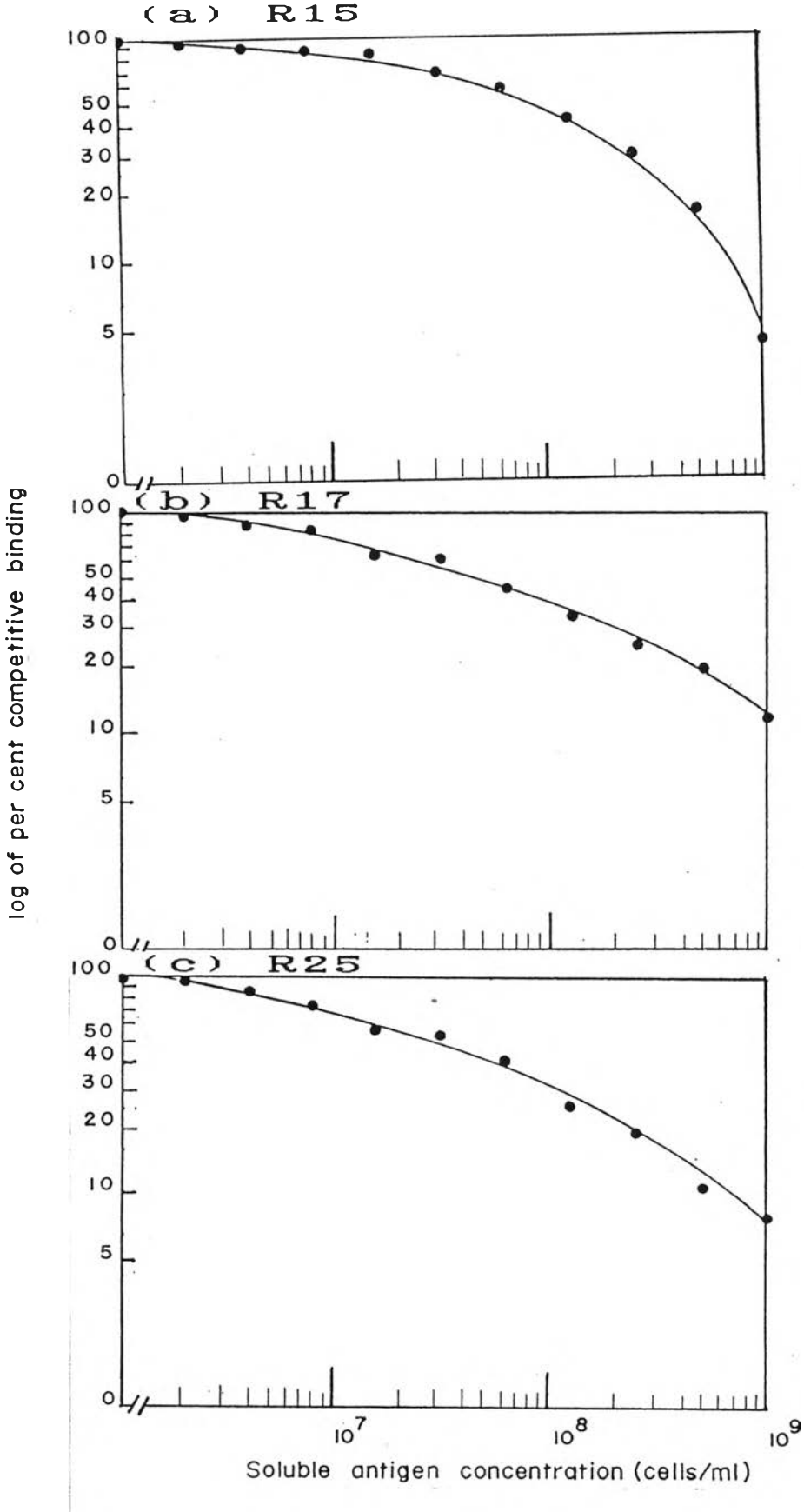
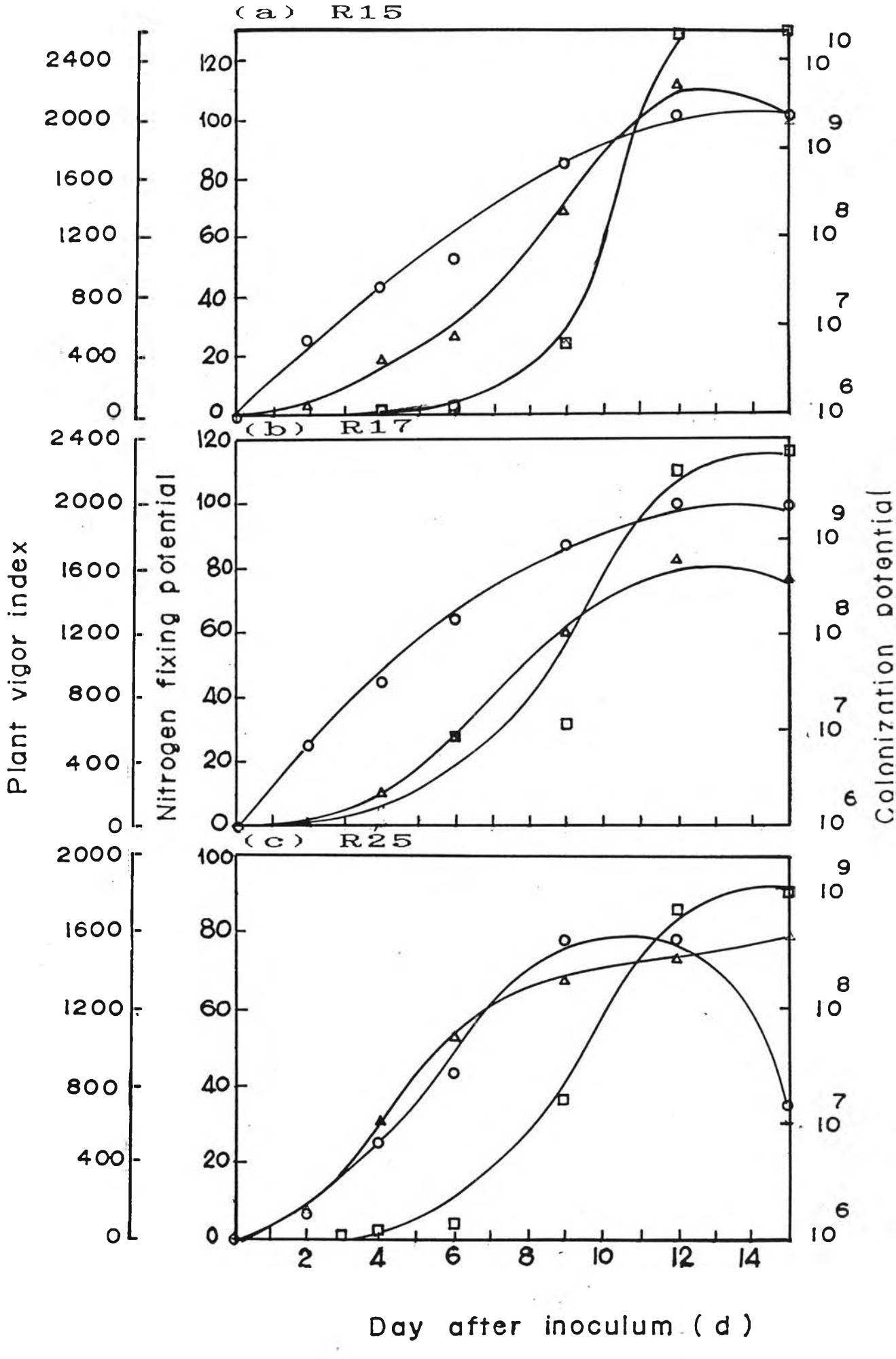

(a) R15
(b) R17
(c) R25
100
50
40
30
20
10
5
0
log of per cent competitive binding
10^7
10^8
10^9
Soluble antigen concentration (cells/ml)

Figure 3.20 Increasing of colonization potential, N_2-fixing potential and plant vigor index for the associative R15, R17 and R25 in rice RD7 variety

The rice were grown in spermosphere model, after inoculation of $5x10^8$ cells/tube. The head space atmosphere was changed to 10% acetylene. For each ARA assay, the plant were measured colonization potential and the plant vigor index. The procedure for measuring these three values are in Method 2.18 -2.21. The symbols (o——o) are colonization potential, (Δ——Δ) nitrogen-fixing potenial and (□——□) Plant vigor index.


(a) R15
(b) R17
(c) R25
Plant vigor index
Nitrogen fixing potential
Colonization potential
Day after inoculum (d)
2400
2000
1600
1200
800
400
0
120
100
80
60
40
20
0
10
9
8
7
6
2
4
6
8
10
12
14

The relationship among these 3 criteria for compatible association between N_2-fixing bacteria and rice were thereforedetermined on day 12 after inoculation as shown in Table 3.10 a-c. RD5, RD7 and RD25 were the rice varieties which behave as good host plants as indicated by the colonization potential of all 3 bacterial strains which were significantly higher than the average values. RD7 and RD5 were the best hosts for colonization of bacteria R15 and R17.

As for the nitrogen-fixing potential of these associative bacteria comparing among 8 varieties of rice used in this experiment, the results show that the best N_2-fixing rice, was RD7, which was a good host for all 3 N_2-fixers tested and also resulted in N_2-fixing potential of 111, 82.84, and 45.08 µmol of C_2H_2/g root dry weight for R15, R17 and R25, respectively. The second best host plant was RD5 in case of bacteria R15 and R17, and SPT in case of R25 inoculation. RD25 was doing well as host plant for colonization, but resulting in lower potential of associative N_2-fixation for all N_2-fixers tested. Under these experimental conditions, RD6, RD23, LPT and KDML did not gain their vigor via associative N_2-fixation. For the inoculated plant, all

Table 3.10 Relationship among colonization potential, N_2-fixing potential and seedling vigor index 12 day after inoculation

The inoculated rice with R15,R17 and R25 were grown under 10% acetylene of head space volume. ARA was measured and reported in term of N_2-fixing potential, the plant was reported in the terms of vigor index, and the cell number were detected by COM-IND-ELISA and reported in the terms of colonization potential.

Table 3.10.a. R15

Rice varieties	Different values from uninoculated control rice		
	Colonization pot. (cells/g root dry wt)	N_2-fixing potential (µmol C_2H_2/g root dry wt)	Vigor index (cm.%)
RD 7	2.23×10^9	111.0	2558
RD 5	2.05×10^9	49.41	1869
RD 25	1.44×10^9	4.96	1555
LPT	3.82×10^8	-	966
KDML	3.11×10^8	-	253
SPT	2.48×10^8	25.2	633
RD 23	1.72×10^8	-	920
RD 6	1.23×10^7	-	918
mean	8.56×10^8	-	1209

Table 3.10.b. R17

Rice varieties	Different values from uninoculated control rice		
	Colonization pot. (cells/g root dry wt)	N_2-fixing potential (µmol C_2H_2/g root dry wt)	Vigor index (cm.%)
RD 5	2.35×10^9	45.12	1749
RD 7	2.10×10^9	82.84	2210
RD 25	1.48×10^9	0.28	1575
LPT	5.98×10^8	-	806
SPT	4.05×10^8	22.48	-136
RD 23	3.45×10^8	-	918
KDML	3.01×10^8	-	793
RD 6	2.50×10^7	-	1401
mean	9.51×10^8	-	1164

Table 3.10.c. R25

Rice varieties	Different values from uninoculated control rice		
	Colonization pot. (cells/g root dry wt)	N_2-fixing potential (µmol C_2H_2/g root dry wt)	Vigor index (cm.%)
RD 5	$4.73x10^8$	12.18	1605
RD 25	$4.30x10^8$	8.56	1550
RD 7	$4.12x10^8$	45.08	1722
LPT	$3.36x10^8$	-	847
RD 6	$2.35x10^8$	-	1912
SPT	$2.03x10^8$	40.24	1636
RD 23	$9.00x10^7$	-	886
KDML	$5.19x10^7$	-	509
mean	$2.80x10^8$	-	1333

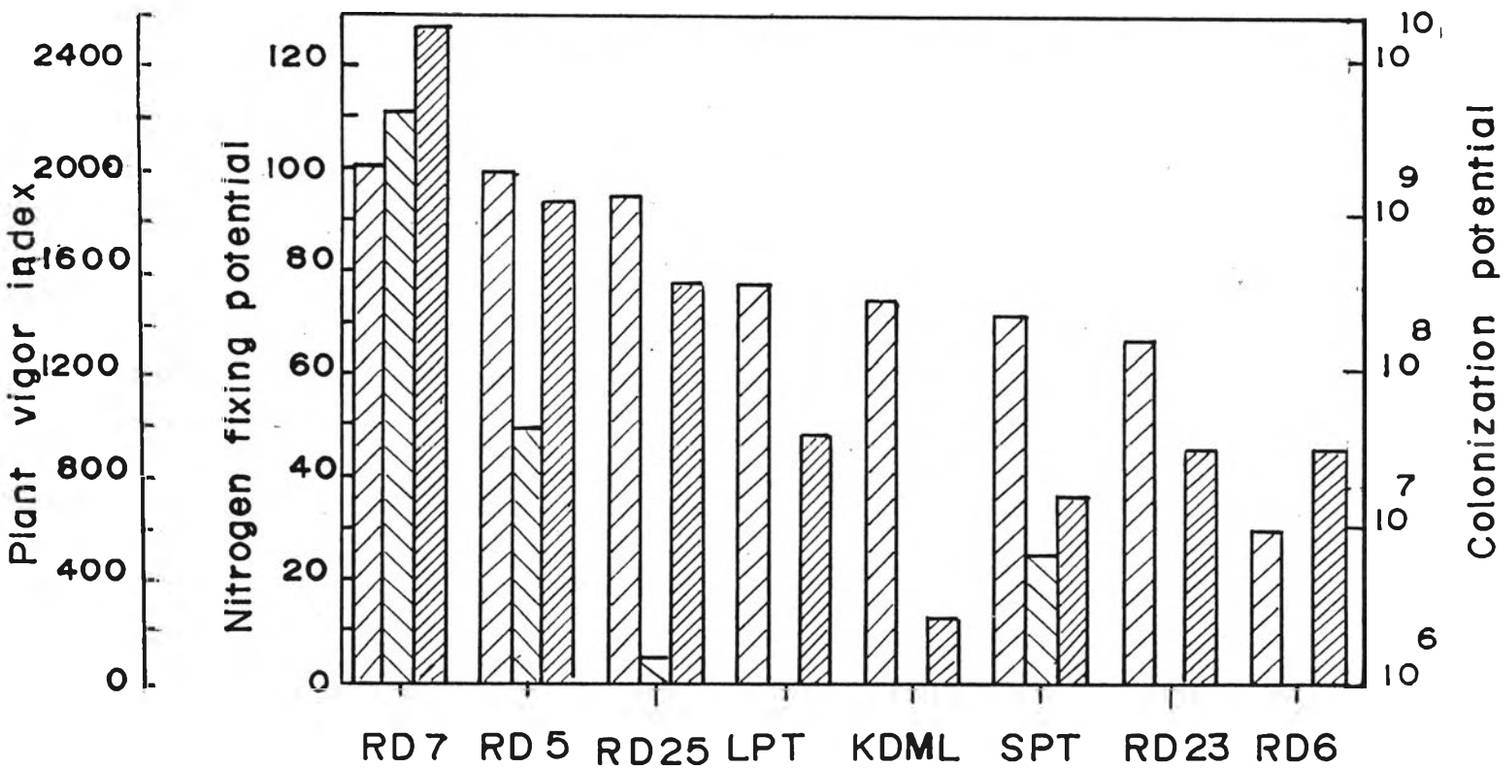
of them showed the net associative vigor index, except when rice CV SPT was inoculated with R17 (-136). The rice varieties which showed a consistently significant increase in vigor index above the means, were RD7, RD5 and RD25 when inoculation with bacteria R15 and R17. RD6 showed the difference of vigor index above the means in R17 and R25, whereas SPT showed only in R25. Among the inoculated rice varieties, RD7 showed the highest net associative vigor index of 2,558 and 2,210 when inoculated with R15 and R17, whereas RD6 showed the highest vigor index of 1,912 when inoculated with R25.

The relationship among colonization potential, nitrogen-fixing potential and plant vigor index can be classified in 2 catagories as summarized in Fig. 3.21 a-c. The former one was the association between rice cv. ; RD7, RD5, RD25, SPT and bacteria R15, R17, R25 in which colonization potential was in the order of 10^{8}-10^{9} cells/g dry weight of root, with a net increase in nitrogen-fixation potential in the range of 0.28-111 µmol C_2H_2/g dry weight of root, together with the positive net increase in plant vigor index ranging from 633-2,558 cm% (except in the association between SPT-R17 that the inoculated plant vigor index was lower than the

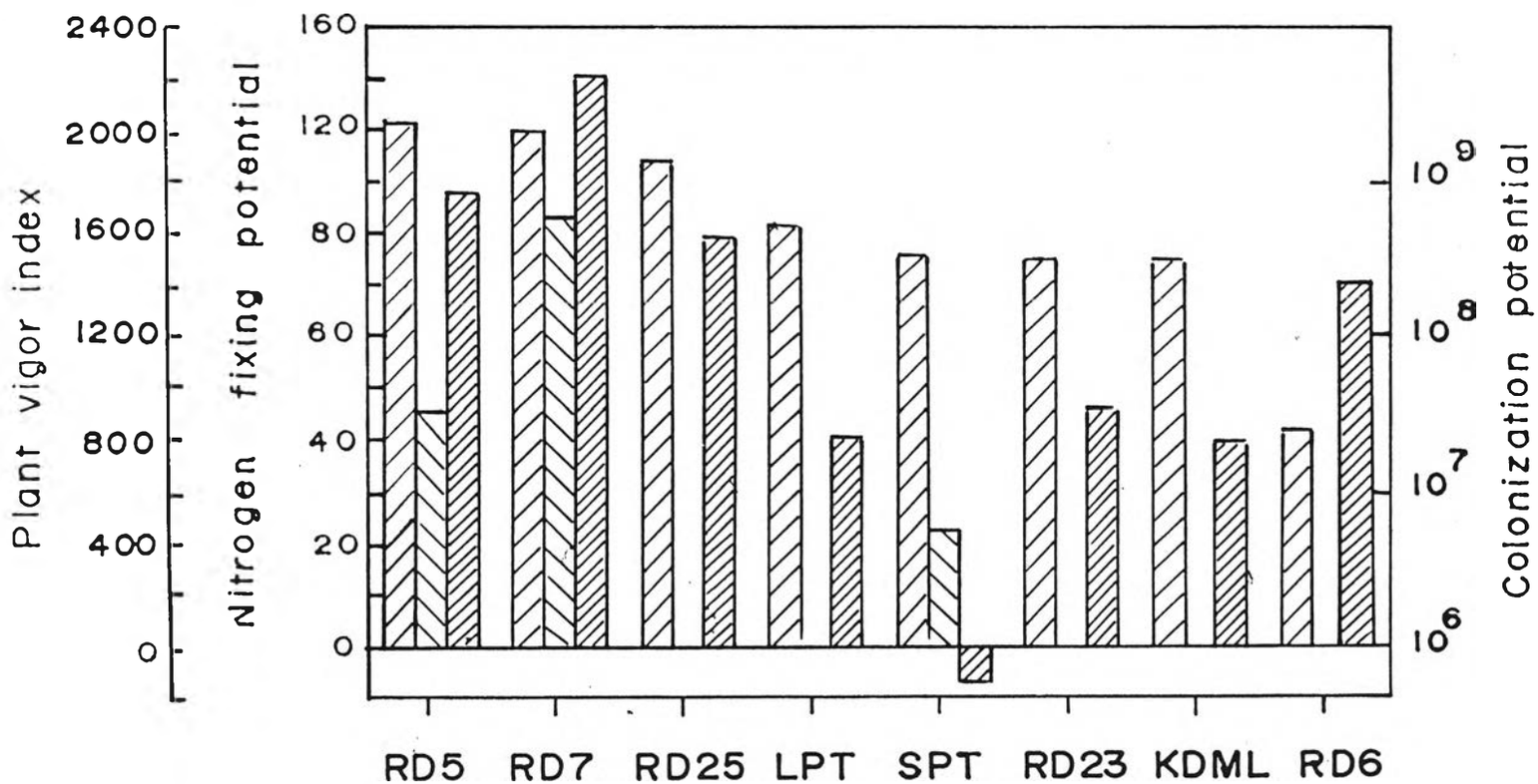
Figure 3.21 Effect of R15, R17 and R25 associated to various varieties of rice (at day 12 after inoculum) on the colonization potential, N_2-fixing potential and plant vigor index.

The rice were grown in spermosphere model, after inoculated with $5x10^8$ cells/tube and the head space atmosphere was changed to 10% acetylene. For each ARA assay, the plants were measured colonization potential and the plant vigor index. Comparision was made among the different values of inoculated and uninoculated cell number/ g.root dry weight, μmol C_2H_2/ g.root dry weight and cm.% versus time at day 12 after inoculum. The procedure of measuring these three values was as described in Method 2.18 -2.21. The symbols are (▨) colonization potential, (▧) nitrogen-fixing pottential and (▨) plant vigor index.

(a) R15


Plant vigor index
2400
2000
1600
1200
800
400
0
Nitrogen fixing potential
120
100
80
60
40
20
0
Colonization potential
10
10
9
10
8
10
7
10
6
10
RD7
RD5
RD25
LPT
KDML
SPT
RD23
RD6

(b) R17


Plant vigor index
2400
2000
1600
1200
800
400
0
Nitrogen fixing potential
160
120
80
60
40
20
0
Colonization potential
10^9
10^8
10^7
10^6
RD5
RD7
RD25
LPT
SPT
RD23
KDML
RD6

(c) R25

Plant vigor index
2000
1600
1200
800
400
0
Nitrogen fixing potential
100
80
60
40
20
0
Colonization potential
10^9
10^8
10^7
10^6
RD 5
RD25
RD7
LPT
RD6
SPT
RD23
KDML

non-inoculated control plants). The latter type was the association between rice cv.;RD6, RD23, LPT, KDML and bacteria R15, R17, R25 in which colonization potential is in the order of 10^7-10^8 cells/g dry weight of root, although without significant increase in nitrogen fixation potential above uninoculated control plants, but resulting in the net positive increase in the plant vigor index ranging from 253-1,912 cm%. In the first type of association, RD7-R15 was the best compatible pair of plant-bacterial interaction as indicated by all 3 criteria. The second good pair was RD7-R17. For the second type of association, RD6-R25 was the best compatible pair of plant-bacterial interaction. Association between RD6 and R17 gave less colonization potential of one order, but resulting in high plant vigor index as comparable to that of RD25-R17.