Appendix - A

Bacterial Identification Tests (Faddin, 1980)

1. Oxidase Test

Bacterial culture of isolate-5 was grown on a nutrient agar medium. A freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride was poured on the plate to cover the surface of bacterial colony, and then decanted. This bacterial strain was shown to oxidase positive with developing a purple color. This test was also done in another way. First, the Whatman filter paper no.1 was soaked in oxidase solution and then a loop of bacterial colony was transferred on that paper. Positive test was noticed with deep purple color.

2. Citrate Utilization Test

This is a test for the ability of an organism to utilize citrate as the sole source of carbon and energy where ammonium salt used as the sole source of nitrogen. Simmons Citrate Agar Medium was used in this experiment.

Sodium chloride, NaCl	5.0 g
Magnesium sulphate ,$MgSO_4$	0.2 g
Ammonium dihydrogen phosphate ,NH_4H_2PO4	1.0 g
Potassium dihydrogen phosphate, KH_2PO_4	1.0 g
Sodium citrate, $Na_3C_6H_5O_7.2H_2O$	5.0 g
Bromothymol blue (1.5 % alcoholic)	0.08 g
Agar	20.0 g

The pH of this medium was adjusted to 6.8. About 5.0 ml of this medium was distributed to each golden cap bottle and sterilized by autoclaving at 121 °C for 15 minutes. After autoclaving the golden cap bottle was placed as slope for making slant. 24 hours old strain culture was inoculated and incubated up to 4-days. Positive test was achieved with blue color and streak of growth.

3. Urea Utilization Test

Bacteria, particularly those growing naturally in on environment exposed urine, may decompose urea by means of the enzyme urease,

$NH_2.CO.NH_2 + H_20 = 2NH_3 + CO_2$

The occurrence of this enzyme can be tested by growing the organisms in the presence of urea, and testing the producing ammonia with phenyl red indicator.

Christensens Urea Agar Medium was used in this test.

Peptone	1.0 g
Sodium chloride, NaCl	5.0 g
Monopotassium phosphate (KH_2PO_4)	2.0 g
Glucose (dextrose)	1.0 g
Urea (20%)	20.0 g
Phenol red	012 g
Agar	15.0 g
Distilled water	1OO0.0 ml

29.0 gm of the dehydrated base was weighed out and dissolved in 100 ml of distilled water. Agar was autoclave at 121 °C for 15 minutes for sterilization ,and then it was cooled down to 50 °C. 100 ml of the filter paper sterilized urea was added asceptically to the 900 ml agar, and added sufficient water to make the final concentration of urea to 10 per cent. 5.0 ml of this media was dispensed into the sterile tubes. 24 hr. old bacterial culture was inoculated to the tubes and incubated at 35 °C. Negative urea test was observed for this bacterial strain because it bacteria was unable to change the color of indicator yellow color (pH 6.8) to pinkish red color (pH 8.4). The result was recorded every 6-hours in the first day and thereafter every day up to 6-days.

4. Sugar Utilization Test

This test was done in two ways to confirm the results,

(i) Gas Production in basal medium: peptone-water base

Peptone	10 g
Sodium chloride, NaCl	5 g
Water	1000 ml

The pH of this medium was adjusted to 7.2. 50 ml of 0.2 per cent of the phenol red solution in 1 N NaOH was added to the medium. 5 ml of this medium was distributed to each test tube with Durhams's tube and autoclaved at 121 °C for 15 minutes. 10 per cent of each sugars (glucose, sucrose, arabinose, rhamnose, inositol and

xylose) solution was prepared separately and sterilized by filtration. 0.25 ml of sugar solution was added to each tube. No gas was found in Durham's tube up to 5-days and also no color changed from purple pink to yellow (acidic condition) was observed.

(ii) Acid production test: Hugh and Leifson`s OF semisolid basal medium

Peptone	2 g
Sodium chloride, NaCl	5 g
Dipotassium phosphate, K_2HPO_4	0.3 g
Bromothymol blue	0.03-0.08 g
Agar	2-3 g
Distilled water	1000 ml

All compounds were weighed out and dissolved in water. Then, it was heated gently into solution. The pH was adjusted to 7.1. The solution was sterilized at 121 °C for 15 minutes, and then cooled down to 40-50 °C. 10 per cent carbohydrates solution (glucose, lactose and maltose) were sterilized by Millipore filter paper (pore 0.45 μ) and added to the basal medium. About 3 ml of this medium was distributed to each test tube. 24 hours old bacterial culture was inoculated to the medium with stabbing and incubated at 35 °C for 4-days. Positive results was observed (Acid production) with changing the green color (pH 7.1) of the indicator bromothymol blue to yellow color (pH 6.0).

5. Motile-Nitrate-Pyocyanin (MNP) Medium

This test is used for non-fermentative, gram-negative bacteria to identify the motility and nitrate reduction, pyocyanin pigment production capacity. The ingredients of this medium is as follows:

Bacto tryptose	20 g
Bacto dextrose	1 g
Disodium phosphate, Na_2HPO_4	2 g
Potassium nitrate, KNO_3	1 g
Sodium chloride, NaCl	5 g
Magnesium chloride, $MgCl_2$	1 .4 g
Potassium sulfate, K_2SO_4	10 g
Glycerol	10 g
Agar	3 g
Water	1000 ml

The pH was adjusted to 7.2 and the ingredient was melted by heating. 3 ml of this medium was distributed to each test tube and sterilized at 121 °C for 15 minutes. The medium was allowed to cool in an upright position. 24 hours old bacterial culture was transferred to each tube. The center of this medium was stabbed with inoculating needle to a depth of a half inch. All tubes were incubated at 35 °C for 48 hr.

(i) Motility test

Positive motility was observed. Because, the selected bacterial strain was migrated from the stab line and diffused into the medium, caused a turbidity. It was exhibited fuzzy streaks of growth.

(ii) Nitrate reduction test

This bacterial strain was able to reduce the nitrate. Gas bubble was found inside the inoculated tube which produced due to the reduction of nitrate to nitrite to N_2 (gas). For confirmation sulphanilic acid and α-naphthylamine was added to the test tube. Positive results with red color was observed.

(iii) Pigment formation

This bacterial strain was formed pyocyanin-fluorescent pigment in MNP medium which was detected under UV at 254 nm.

6. Phenylalanine deaminase Test

DL-Phenylalanine	2.0 g
Yeast-extract	3.0 g
Sodium chloride, NaCl	5.0 g
di-Sodium Phosphate, Na_2HPO_4	1.0 g
Agar	12.0 g
Distilled water	1000 ml

The pH of this medium was adjusted to 7.3 and heated gently into solution. About 4.0 ml of this medium was distributed to each long tube and autoclaved at 121 °C for 15 minutes. The medium was solidified in slanted position and cooled before use. 24 hours old selected bacterial culture was inoculated to each tubes and incubated at 35 °C for 24 hours. After incubation, 4 to 5 drops of 10% $FeCl_3$ solution was added to the tube. No green color reaction was observed within 5 minutes.

7. Gelatin Liquefaction Test

This test is used to determine the ability of an organism for production of proteolytic enzymes (gelatinases) which liquefy gelatin. Nutrient gelatin stab medium was used for this test.

Beef extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
Distilled water	1000.0 ml

First, gelatin was dissolved in water and kept about 30 minutes. Later, it was heated to 50 °C to put gelatin into solution. Beef extract and peptone were added to the solution and again heated at 50 °C to dissolve all constituents and the pH was adjusted to 6.8. About 5.0 ml of this medium was distributed to each screwcap tube and sterilized at 121 °C with pressure 15 lb for 15 minutes. The medium was cooled in an upright position. 24 hours old bacterial culture was inoculated in the tube with stabbing to a depth of half inch and incubated at 35 °C for 24 hours. Positive test with turbidity and liquefaction was observed in this medium.

8. Flagella Staining (Finegold and Baron, 1986):

Solution A,	
Saturated alum aqueous solution	2 ml
5% phenol aqueous solution	5 ml
20% tannic acid	2 ml

Solution B,	
Basic fuchsin	11 gm
95% ethanol	100 ml

9 ml of solution A and 1 ml of solution B were mixed and shaken well. A loop of bacterial colony was transferred to a drop of water on a slide. It was kept a few minutes for movements of bacterial strain. Then, it was dried in air. The dye was poured on the bacterial strain for two minutes, and then washed with water. Flagella was observed under microscope.

Appendix-B

Flow-chart of DNA Isolation:

The following flow-chart was followed for the isolation and preparation of DNA from the selected bacterial strain of isolate-5.

Bacterial culture (A_{600} 1.6)

1.5 ml culture in an eppendarf tube, centrifuged at 13,000 rpm for 2 minutes

Add 300 µl TE buffer (Tris-EDTA) to each tube and shake well

Add 300 µl lysis buffer (20% SDS and 2M NaOH, 1:1)

Incubate at 60 °C for 10 - 15 min

Add 1 mg/ml lysozyme buffer per tube

Incubate in waterbath at 37 °C for 1 hr.

Add 300 µl precipitation buffer (2.55 M KAc, pH 4.8)

↓

Incubate at 4 °C for 1 hour

↓

Centrifuge at 13000 rpm about 10 min.

↓

Transfer supernatent to new tube

↓

Add equal volume of Isopropanol

↓

Keep in overnight at - 20.0 °C or atleast 2 hr. for precipitation

↓

Centrifuge at 13000 rpm for 10 - 15 min

↓

Form very small pellet

↓

Wash with 70 % ethanol (50 µl /tube)

↓

Centrifuge at 12000 rpm for 2 minutes

↓

Dry in air about 30 min or until formation of semisolid

↓

Dissolve the DNA with TE buffer (20 µl/ml)

↓

Keep at - 20.0 °C

↓

Mix with ethidium bromide (about 0.2 µl/ ml)

↓

Run in gel electrophoresis

carbaryl Calibration Report

Printed: 5-MAR-1996 22:43:11

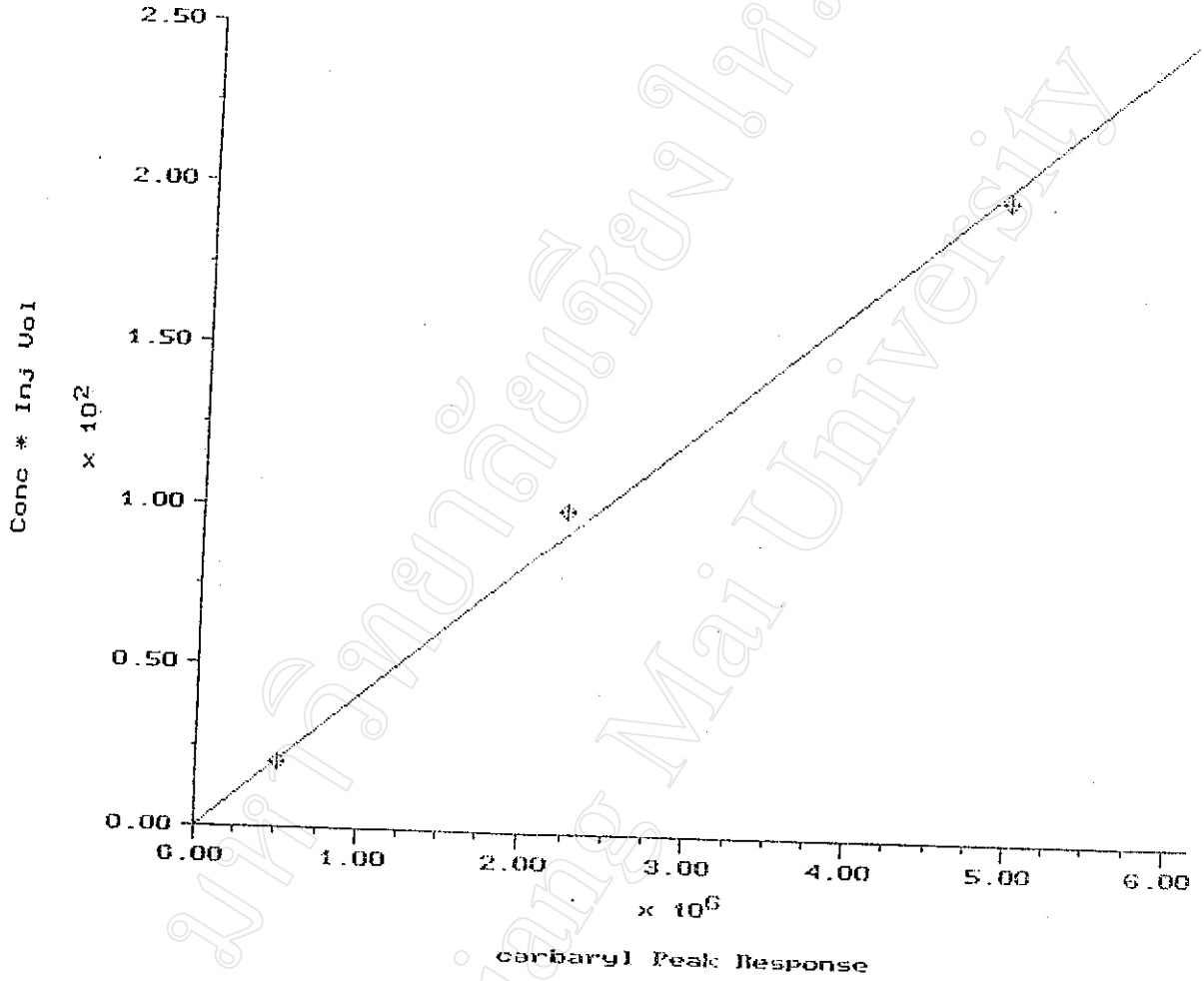
Quant Basis: Area
Curve Type: Linear
Y-axis Label: Concentration

Rejection Tolerance: None
Weighting: None

Internal Standard: None
Forced Through Origin: Yes

Equation: Conc*(Inj Vol) = 4.123532E-05 * R

Sample	File Name	Valid	Concentration	Response	Calc'd Concentration	% Deviation	Response Factor
STD1.0	MARST1	Y	1.000000E+00	5.1380237E+05	1.059340E+00	-5.60E+00	3.892547E-05
STD5.0	MARST5	Y	5.000000E+00	2.2434585E+06	4.625487E+00	8.10E+00	4.457404E-05
STD10	MAR10	Y	1.000000E+01	4.9298740E+06	1.016425E+01	-1.62E+00	4.056899E-05



Appendix - D

Table A. 1. Effect of pH on bacterial degradation of carbaryl

pH	Bacterial growth and carbaryl degradation	Time (hours)						
		0	8	16	24	32	40	48
6.0	A_{600}	0	0	0	0	0	0	0
	Carbaryl, ppm	10	-	9.74	-	9.49	-	9.54
6.5	A_{600}	0	0.093	0.121	0.148	0.160	0.281	0.176
	Carbaryl, ppm	10	7.94	7.66	7.39	7.08	4.4	4.0
6.8	A_{600}	0	0.059	0.065	0.103	0.117	0.073	0.115
	Carbaryl, ppm	10	7.39	7.08	6.75	6.52	6.55	6.0
7.0	A_{600}	0	0.038	0.0	0.094	0.111	0.100	0.077
	Carbaryl, ppm	10	7.82	6.52	6.28	6.07	5.46	5.04
7.2	A_{600}	0	0.059	0.134	0.109	0.101	0.106	0.126
	Carbaryl, ppm	10	7.6	5.50	5.25	4.42	3.87	2.94
7.5	A_{600}	0	0.068	0.004	0.056	0.048	0.09	0.067
	Carbaryl, ppm	10	6.28	3.06	2.86	1.73	1.55	0.80
8.0	A_{600}	0	0.021	0.038	0.064	0.067	0.082	0.081
	Carbaryl, ppm	10	-	0.940	-	0.110	-	0.05
8.5	A_{060}	0	0	0	0	0.042	0.071	0.037
	Carbaryl, ppm	10	-	0.098	-	0.053	-	0
9.0	A_{600}	0	0	0	0	0.037	0.042	0.052
	Carbaryl, ppm	10	-	ND	0	0	0	0

- : Not measured

ND : Non detectable

Table A. 2. Effect of temperature on bacteria in degradation of carbaryl in minimum minerals media

Time (hours)	Temperature							
	30° C		34° C		37 oC		41 ° C	
	A_{600}.	carbaryl, ppm	A_{600}	Carbaryl, ppm	A_{600}	Carbaryl, ppm	A_{600}.	Carbaryl ppm
0	0	10.0	0	10	0	10.0	0	10.0
12	0.068	8.67	0.062	7.11	0.067	7.62	0	7.1
24	0.073	8.34	0.103	6.75	0.08	7.21	0	6.7
36	0.090	8.13	0.112	6.52	0.086	6.80	0	4.27
48	0.079	7.88	0.115	6.0	0.102	6.47	0.001	3.65

Table A. 3. Bacterial degradation of carbaryl in nutrient broth.

Time (hours)	Bacterial growth (A_{600})	Remaining amount of carbaryl (ppm)
0	0	10.00
8	0.521	7.12
16	1.88	5.36
28	1.62	3.15
40	1.44	0.924
48	1.37	0.764

Table A. 4. Carbaryl degradation in minimum minerals media with yeast-extract

Time (hours)	Bacterial growth (A_{600})	Remaining amount of carbaryl (ppm)
0	0	10.00
12	1.36	6.37
33	1.71	6.18
46	1.22	5.86
58	1.18	5.31
70	1.17	4.04
81	1.048	3.12

Table A. 5. Bacterial degradation of carbaryl in MM with vit. B1, B6 and nicotinamide

Incubation Time (hours)	Without vit.		Vit. B1		Vit.B6		Vit. Nicotinamide	
	Growth (A_{600})	Carbaryl, ppm	Growth (A_{600})	Carbaryl, ppm	Growth (A_{600})	Carbaryl, ppm	Growth (A_{600})	Carbaryl, ppm
0	0	10.0	0	10.0	0	10.0	0	10.0
12	0.066	6.95	0.015	6.83	0.041	6.18	0.005	6.864
24	0.068	-	0.017	-	0.049	-	0.007	-
36	0.067	6.49	0.028	6.23	0.068	5.66	0.015	6.228
48	0.077	6.03	0.025	5.76	0.077	5.22	0.030	5.739

- : Not measured

Table A. 6. Degradation of carbaryl in minimum minerals media with carbaryl enriched bacteria

Time (hours)	Growth (A_{600})	Remaining amount of carbaryl (ppm)
0	0	10.0
20	0.428	7.22
44	0.446	7.01
68	0.447	6.97

Table A. 7. Bacterial degradation of carbaryl in MM in presence of carbofuran and carbosulfan.

Incubation time (hours)	Carbofuran		Carbosulfan	
	Bacterial growth (A_{600})	Remaining Carbaryl (ppm)	Bacterial growth (A_{600})	Remaining carbaryl (ppm)
0	0	10.0	0	10.0
12	0.003	8.62	0.014	8.47
24	0.017	8.4	0.025	7.90
36	0	7.9	0.032	7.67
48	0	7.86	0.023	7.43

Table A. 8. Bacterial growth in MM with 1-naphthol and carbofuran

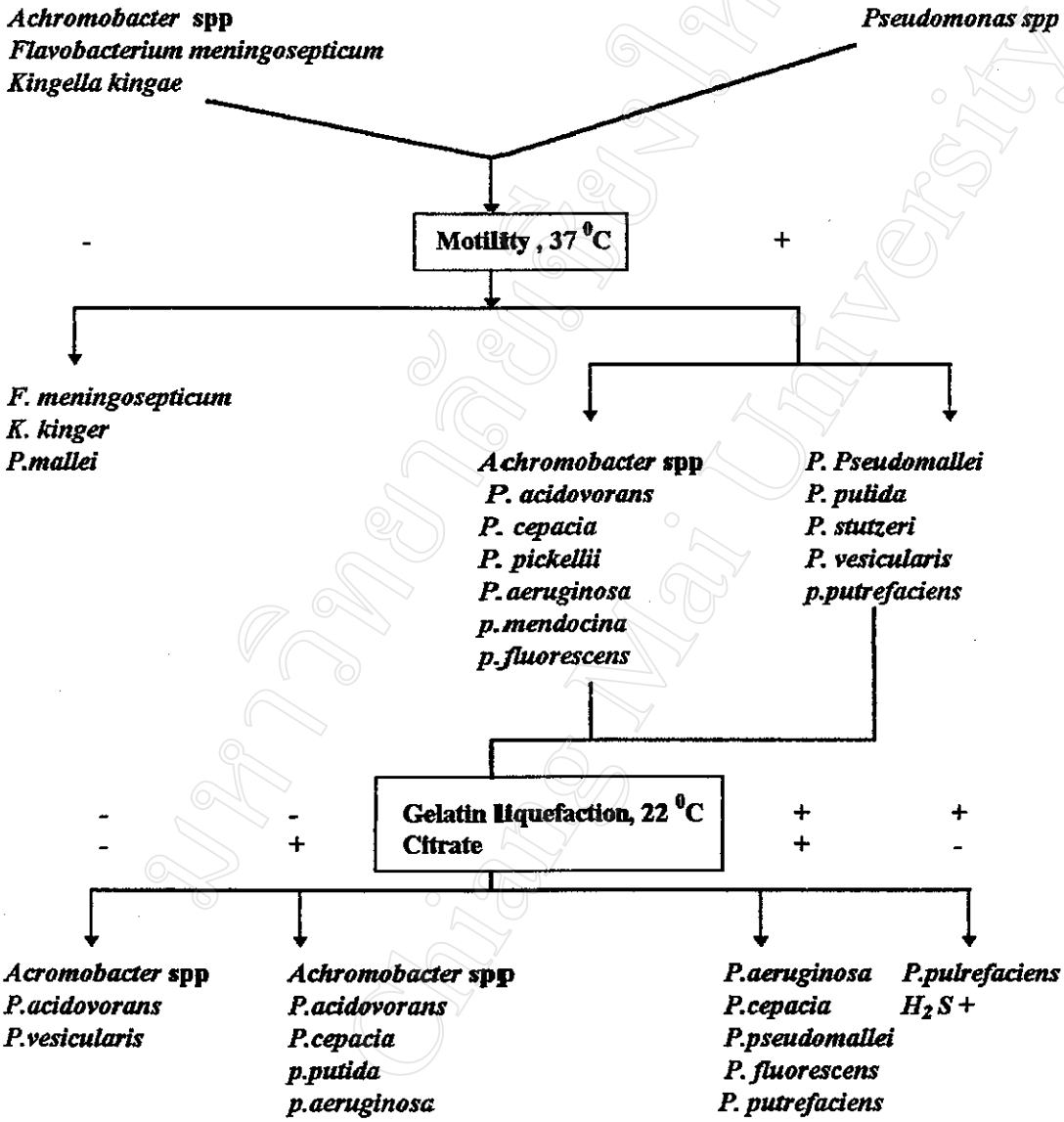
Duration (hours)	1-naphthol		Carbofuran
	Bacterial growth (A_{600})	Remaining 1-naphthol (ppm)	Bacterial growth (A_{600})
0	0	10.0	0
8	0.138	-	0
16	0.121	6.67	0
24	0.110	-	0
32	0.140	6.43	0
40	0.146	-	0
48	0.161	6.24	0

Table A. 9. Effect of UV radiation on the mortality of bacteria

UV-exposing time (sec.)	CFU x 10^6/ml
UV-0	3.79
UV-10	3.20
UV-30	2.87
UV-60	1.96
UV-120	0.083

Appendix - E

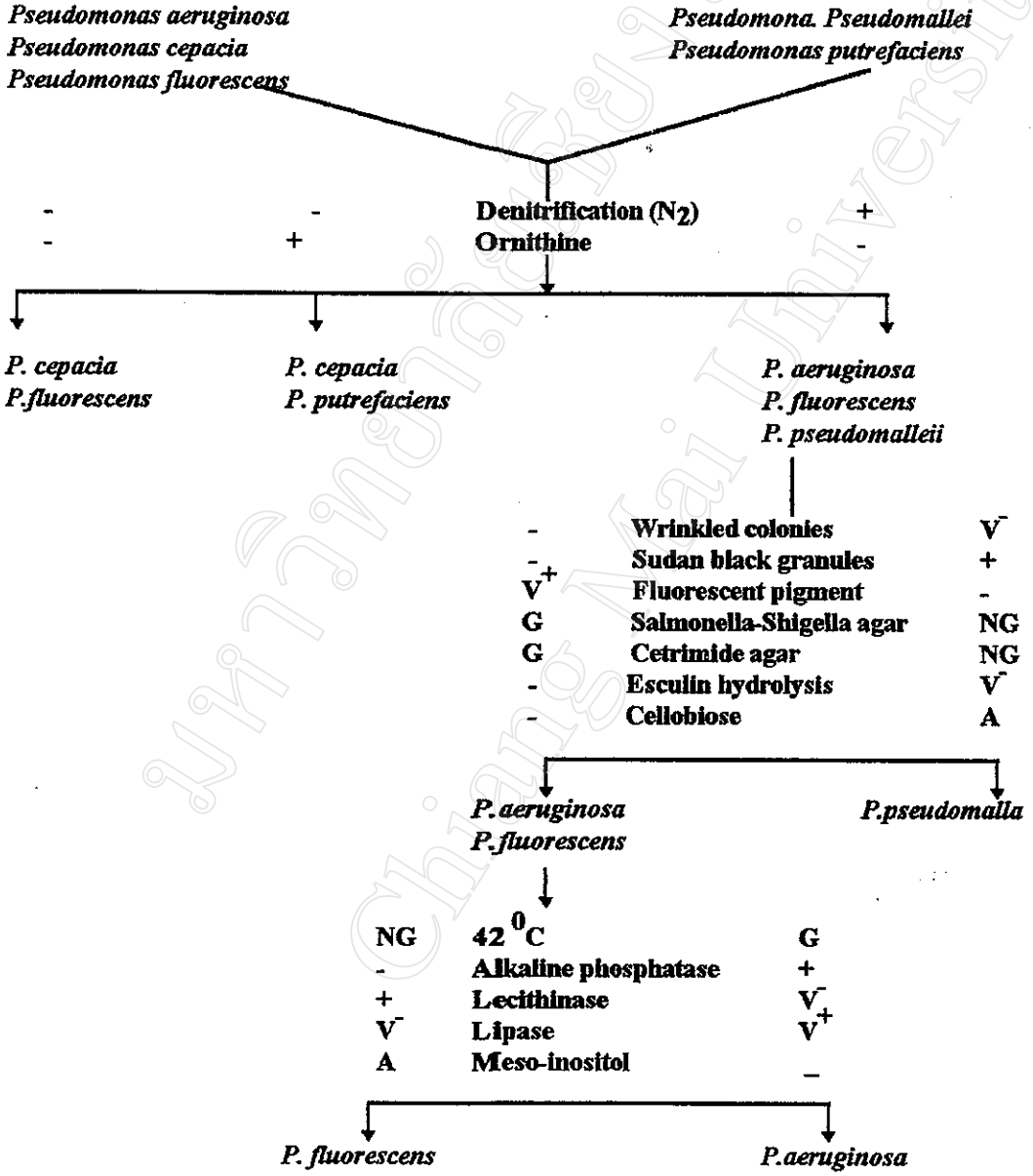
Differentiation of OXIDASE - POSITIVE, OXIDATIVE gram-negative rods (coccobacilli) that grow on ordinary isolation media (Faddin ,1980)



See appendix - F

Appendix - F

Differentiation of OXIDASE - POSITIVE, MOTILE , CITRATE - POSITIVE ,OXIDATIVE gram-negative rods (coccobacilli) that LIQUEFY GELATIN



Note : NG - No growth , v - Variable , G - Growth

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