Chapter 4

Results

I. Isolation of actinomycetes from cave soil

The calculated of total number of actinomycetes isolated from each cave soil and total count under the aerobic condition were shown in Table 5.

Table 5 Microbiological population of cave soil

Cave	Microorganism per Gm		
	Actinomycetes	total count	percent
Mungkorn-tong	1,695	25,958	2.23
Kao-lam	1,423	36,346	3.92
Sarika	4,451	61,575	7.23
Jom-pon	14	8,525	0.16
Kao-bin	269	23,220	1.16
Pothi-sat	7	830	0.84
Net	7,859	206,454	3.81

There was no isolated colony of actinomycetes from any cave soil sample incubated under anaerobic condition.

The strain number of actinomycetes isolated to study from Mungkorn-tong, Kao-lam, Sarika, Jom-pon, Kao-bin and Pothisat were 19,14,49,5,16 and 1 respectively so that the total number of actinomycetes strain were 104.

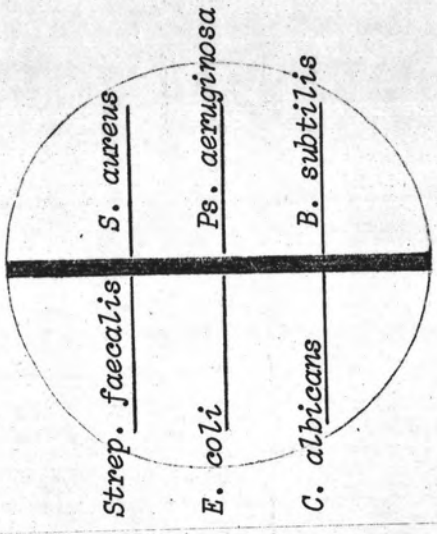
II Determination of antibiotic-producing actinomycetes

According to the screening of cultures for antibiotic production, we have selected 51 active ones out of 104 strains by streak plate method and classified them into 6 group. (Table 6)

Table 6 Classification of 51 active strains of actinomycetes

group	No. of inhibited test organism	No. of actinomycetes	Percent
1	6	6	5.77
2	5	8	7.69
3	4	7	6.73
4	3	7	6.73
5	2	8	7.69
6	1	15	14.42
Net		51	49.04

The strain ST-13-2 isolated from Mungkorn-tong cave inhibited a wide range of test organism(classified in group 1) and much more clear inhibition distances was selected for further study. The photograph of the strain ST-13-2 assay plate against 6 test organisms was shown in Fig 1.



Strain ST-13-2

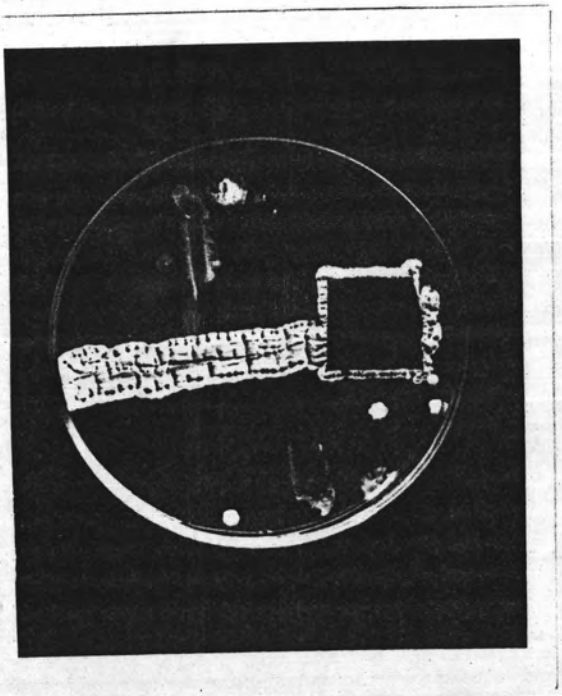


Figure 1 Photograph showing secondary screening of strain ST-13-2 producing antibiotics on assay plate

III Taxonomic studies of strain ST-13-2

A. Morphological characterization

The cultural characteristic of strain ST-13-2 on various media were shown in Table 7.

Table 7 Cultural characteristics of strain ST-13-2

Medium	Growth	Aerial mycelium	Substrate-mycelium	Soluble pigment
Yeast extract-malt extract agar	Abundant	grayish, powdery	Dark brown	Slightly yellow
Oatmeal agar	Abundant	grayish, powdery	Dark brown	Cream
Inorganic salt-starch agar	Abundant	grayish, powdery	Gray	No
Glycerol-asparagine agar	Abundant	grayish, powdery	Brown-gray	No

Strain ST-13-2 formed sporophores monopodially branched, with long, regular, open spirals. The number of spores at the end of mature hyphae was more than 10. (see in Fig 2)

The spore under scanning electron microscopic was cylindrical and 1.09 x 0.5 μm in size as shown in Fig 3. The surface of the spore was smooth.

Morphological characteristics of strain ST-13-2, place it in the genus *Streptomyces*.

Figure 2 Photomicrograph of strain ST-13-2 (on oatmeal agar, x 100)

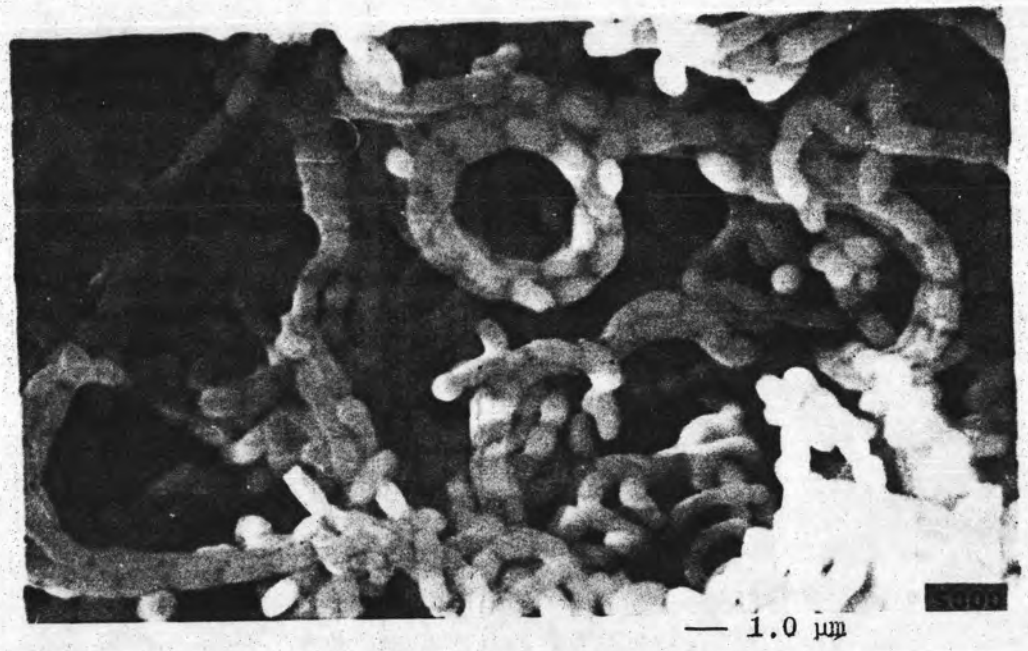


Figure 3 Scanning electronmicrograph of spore surface of strain ST-13-2 (on oatmeal agar, 30°C, 7 days, x 5,000)

B. Physiological characteristics

The melanin production of strain ST-13-2 was negative.

The utilization of carbon sources and biochemical properties were summarized in Table 8 and 9, respectively.

Table 8 Carbon utilization pattern of strain ST-13-2

D-glucose	+	D-mannose	+
L-arabinose	++	Sucrose	±
D-Xylose	+	Raffinose	-
D-fructose	+	D-mannitol	++
Inositol	+	Salicin	+
L-rhamnose	++	Cellulose	++
D-galactose	++	Control	-

Table 9 Biochemical properties of strain ST-13-2

Nitrate reduction	positive
Starch hydrolysis	positive
Gelatin liquefaction	negative
Milk peptonization	positive
Milk coagulation	positive
Melanin formation	negative
Casein decomposition	positive
Tyrosine decomposition	positive
Xanthine decomposition	positive

IV Antibiotic production in liquid culture

A. Development of liquid media

The strain ST-13-2 was fermented in 3 kinds of liquid media. The glucose soybean medium produced the highest activity of antibiotic against all test organisms at approximately day 3 and 5. (see in Table 10)

Table 10 Antibiotic production of strain ST-13-2 in various media

Medium	Fermentation time (days)	Average of inhibition zone in mm against					
		S. aureus	*B. subtilis*	*S. faecalis*	*E. coli*	*Ps. aeruginosa*	*C. albicans*
Glucose peptone medium	3	0	0	0	0	0	0
	5	8.7	9.65	8.6	7.4	9.05	0
	7	8.2	9.85	8.5	0	8.7	0
Glucose soybean medium	3	11.2	12.05	9.2	9.4	8.8	14.45
	5	10.5	12.3	8.7	8.5	8.9	13.9
	7	9.9	11.6	8.7	8.2	8.6	12.5
Maltose soybean medium	3	7.8	7.55	0	0	0	12.3
	5	7.8	8.7	8.2	0	8.0	0
	7	7.4	9.05	8.0	0	7.8	0

B. Determination of optimum pH and temperature

Three sets of glucose soybean medium (pH 4, 5, 6, 7 8 and 9 before sterilization) were fermented with strain ST-13-2 at 23°, 30° and 33°C. The pH changes and antibiotic production during fermentation of temperature 23°, 30° and 33°C were shown in Fig 4, Fig 5 and Fig 6 respectively.

They showed that the pH shifted to approximately pH 7, then slowly increased until the fermentation course were over. Among these temperatures, the temperature 33°C provided the most sharply curve between 2 and 3 days or 3 and 4 days.

The antibiotic productions were dominant when the initial pH before sterilization was in range 6-8 at 23°C, range 7-9 at 30°C, and range 5-7 at 33°C.

Fig 7 showed that the optimum temperature for antibiotic production was 23°C. The highest peak of antibiotic activity of temperature 23°, 30°, and 33°C were 14.0; 10.5 and 7.8 mm respectively. The optimum initial pH before sterilization that gave the maximum of antibiotic at 23°C was 7.

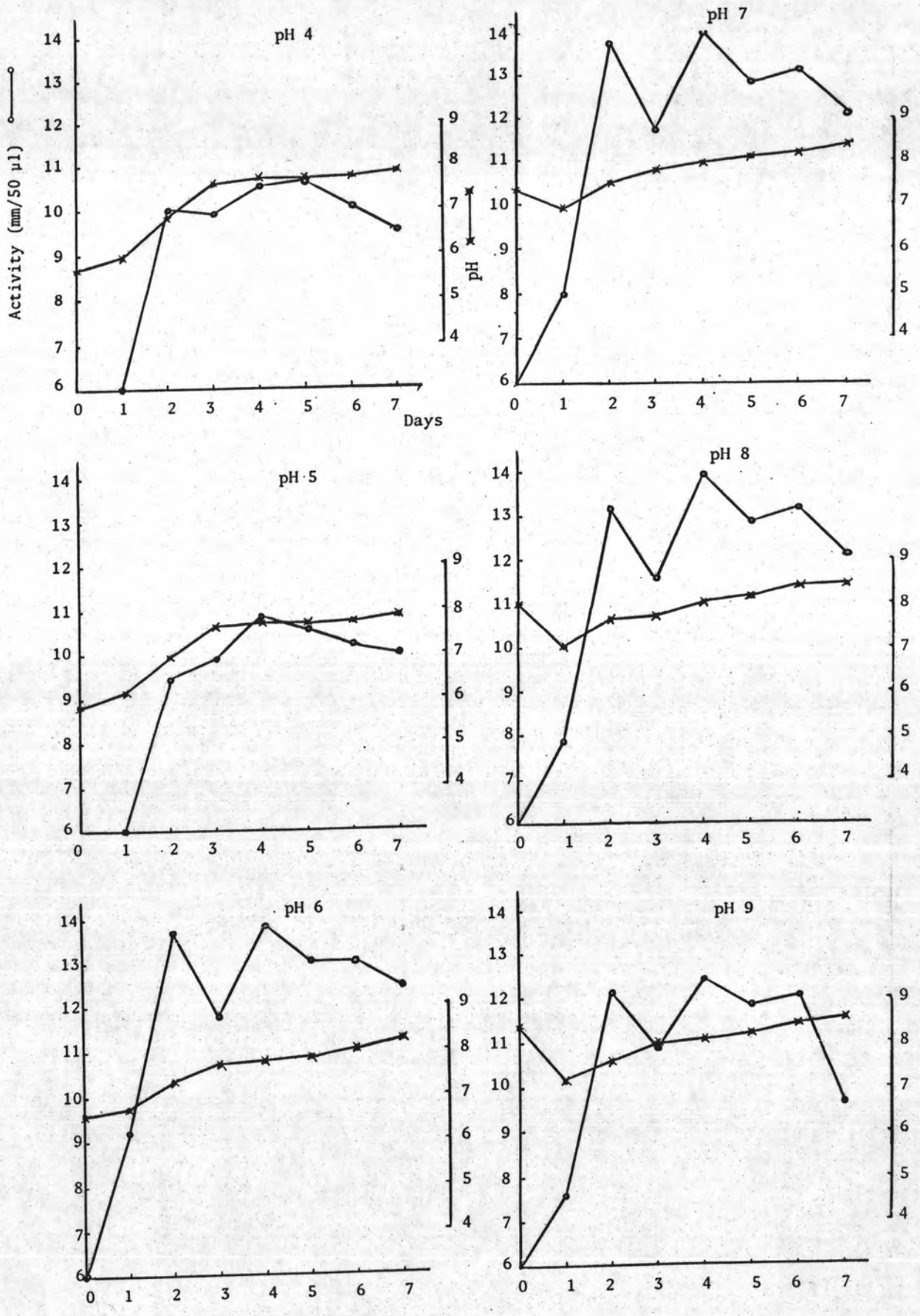


Figure 4 Fermentation of antibiotic from strain ST-13-2 in various pH at 23°C

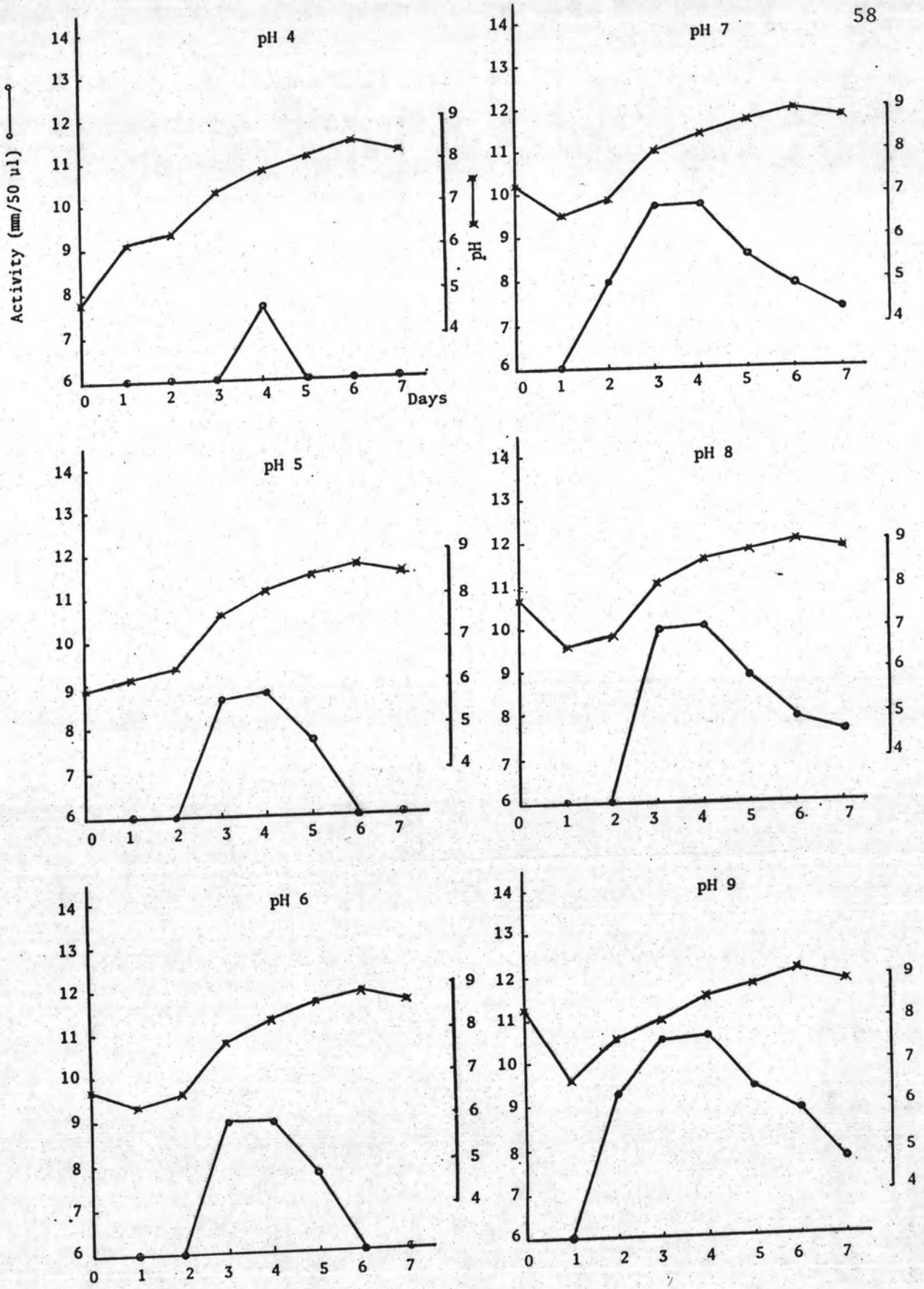


Figure 5 Fermentation of antibiotic from strain ST-13-2 in various pH at 30°C

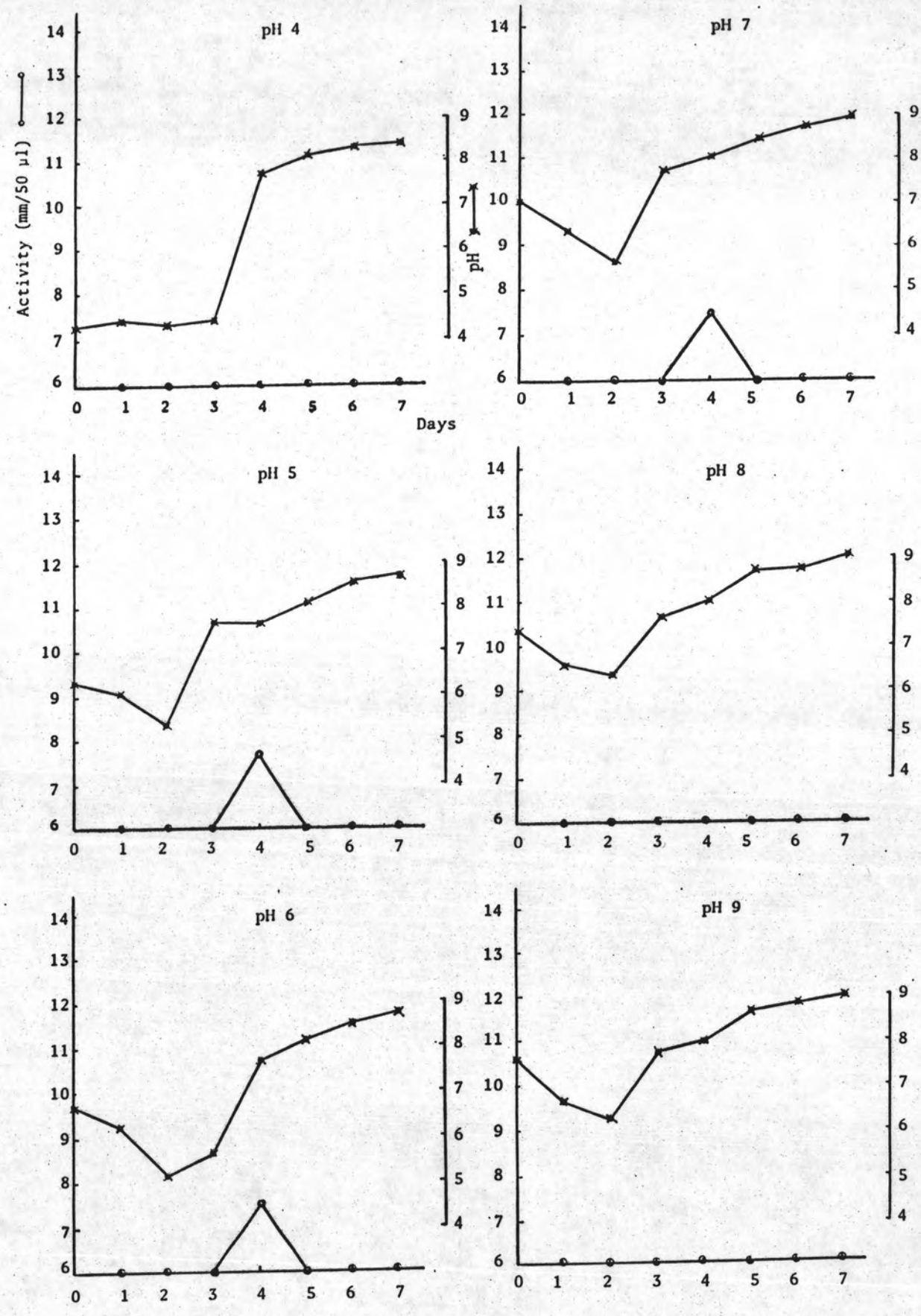


Figure 6 Fermentation of antibiotic from strain ST-13-2 in various pH at 33°C

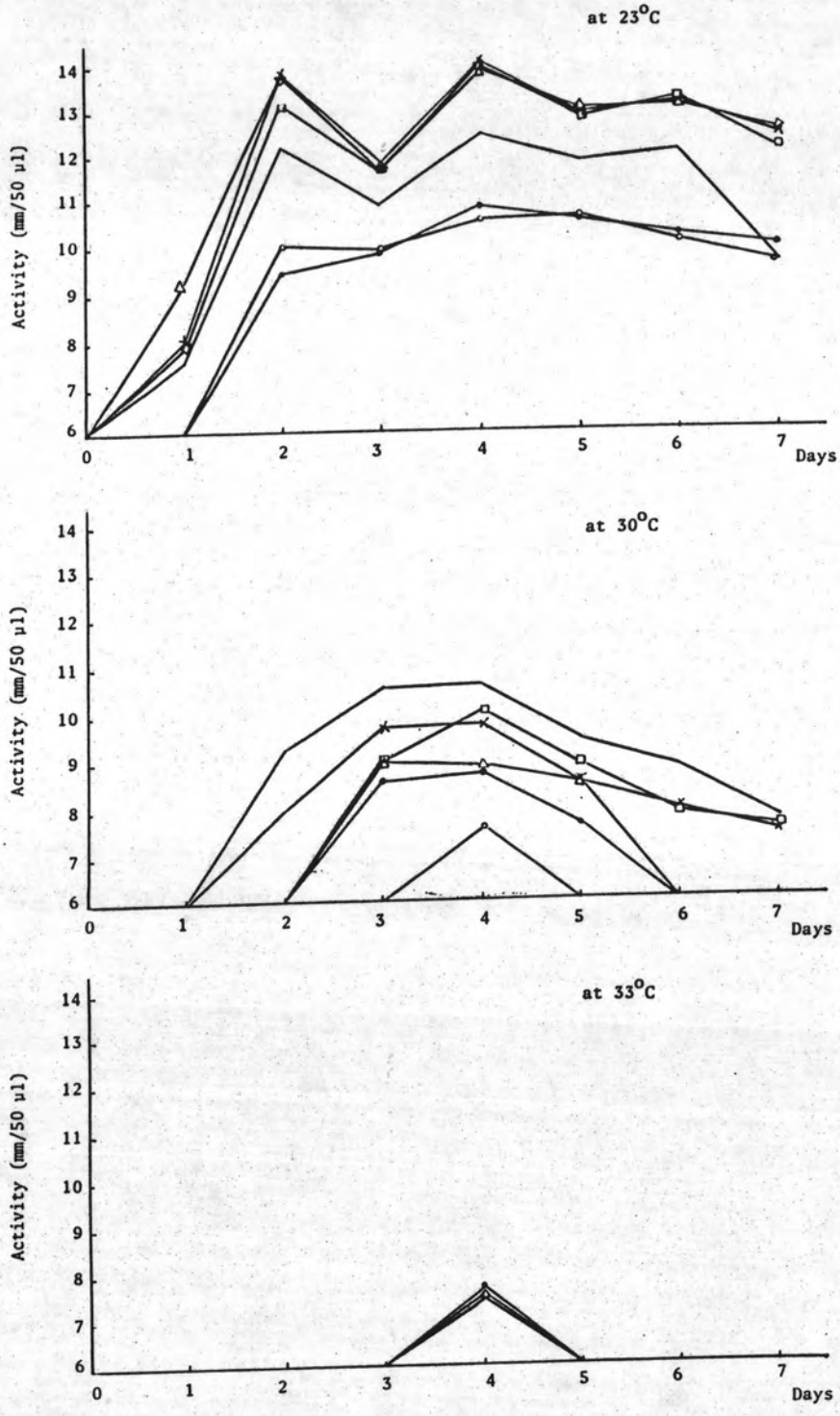


Figure 7 The comparison of antibiotic production pattern of strain ST-13-2 in various pH and temperature

pH 4 ○——○ pH 7 ×——×

pH 5 ●——● pH 8 □——□

pH 6 △——△ pH 9 ——

V Determination of antibiotic substances by TLC

The location of inhibition and colors of the spots were recorded after each treatment. The Rf values were determined from the chromatoplate

$$Rf = \frac{\text{distance of spot moving from start point}}{\text{distance of solvent front from start point}}$$

A. Microbiological detection

The solvent system was varied with the type of antibiotic. For separating water-soluble basic antibiotics, the solvent systems propanol : pyridine : acetic acid : water (15:10:3:10) and chloroform: methanol : 17% ammonia (2:1:1) were used. Peptide antibiotics were separated by use of the solvent system butanol : acetic acid : water (3:1:1). Polyene antibiotics were separated by use of the solvent system ethanol : conc. ammonia : water (8:1:1) and nucleoside antibiotics by use of the solvent system ethyl acetate : methanol (100:15). Antibiotics of macrolide group were separated by use of any of three solvent system ethanol : conc. ammonia : water (8:1:1), butanol : acetic acid : water (3:1:1) and ethanol : water (4:1).

Results of bioautography were present only from the chromatoplates that were developed in solvent system 3 (Propanol : pyridine : acetic acid : water = 15:10:3:10). According to solvent system, it showed that antibiotic substances from strain ST-13-2 were water-soluble basic antibiotics. The crude fermented broth and the extraction with butanol and isopropanol provided inhibition spots more than one. The extraction with acetone and ethanol did not provide any

inhibition spot. The standard antibiotics, penicillin G and kanamycin were also shown in Fig 8.

B. Chemical detection

The application of chromatogenic reagents to the chromatoplate yield the color spot shown in Fig 9. These spots showed no any related location to the inhibition zone of antibiotic substances from strain ST-13-2, but they showed ones in the area of standard antibiotics.

According to primary identification of unknown antibiotic substances from strain ST-13-2, the more standard antibiotic were applied to the chromatoplate. The results of bioautograph were shown in Fig 10. The Rf value of unknown antibiotic substances from crude fermented broth showed one inhibition spot that identical to standard antibiotic, cloxacillin (Rf = 0.26).

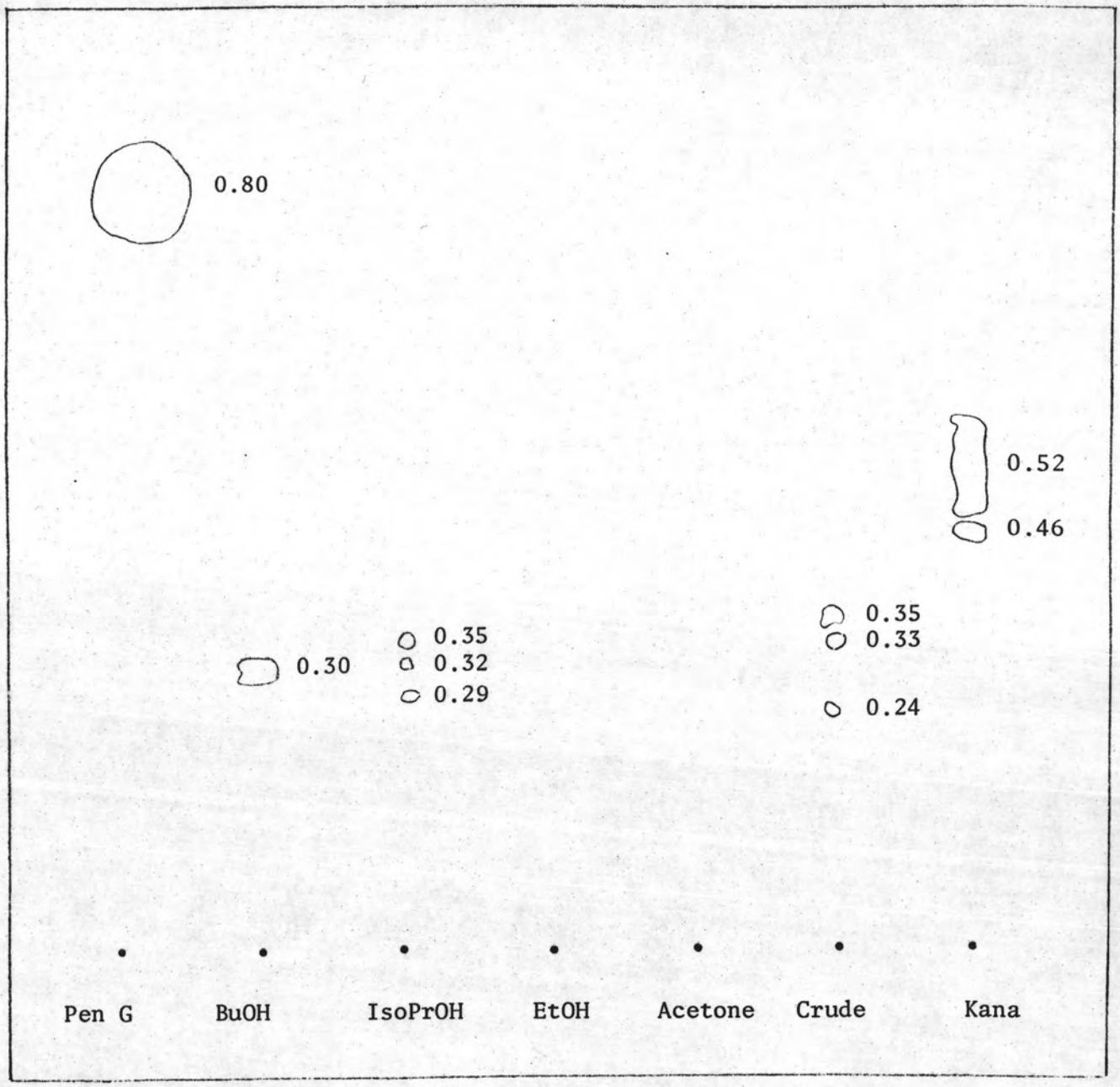


Figure 8 Bioautographic detection of antibiotic on paper, prepared as "reprints" from thin-layer chromatoplate

TLC system :- Propanol : pyridine : acetic acid : water(15:10:3:10)

Organism :- *S. aureus*

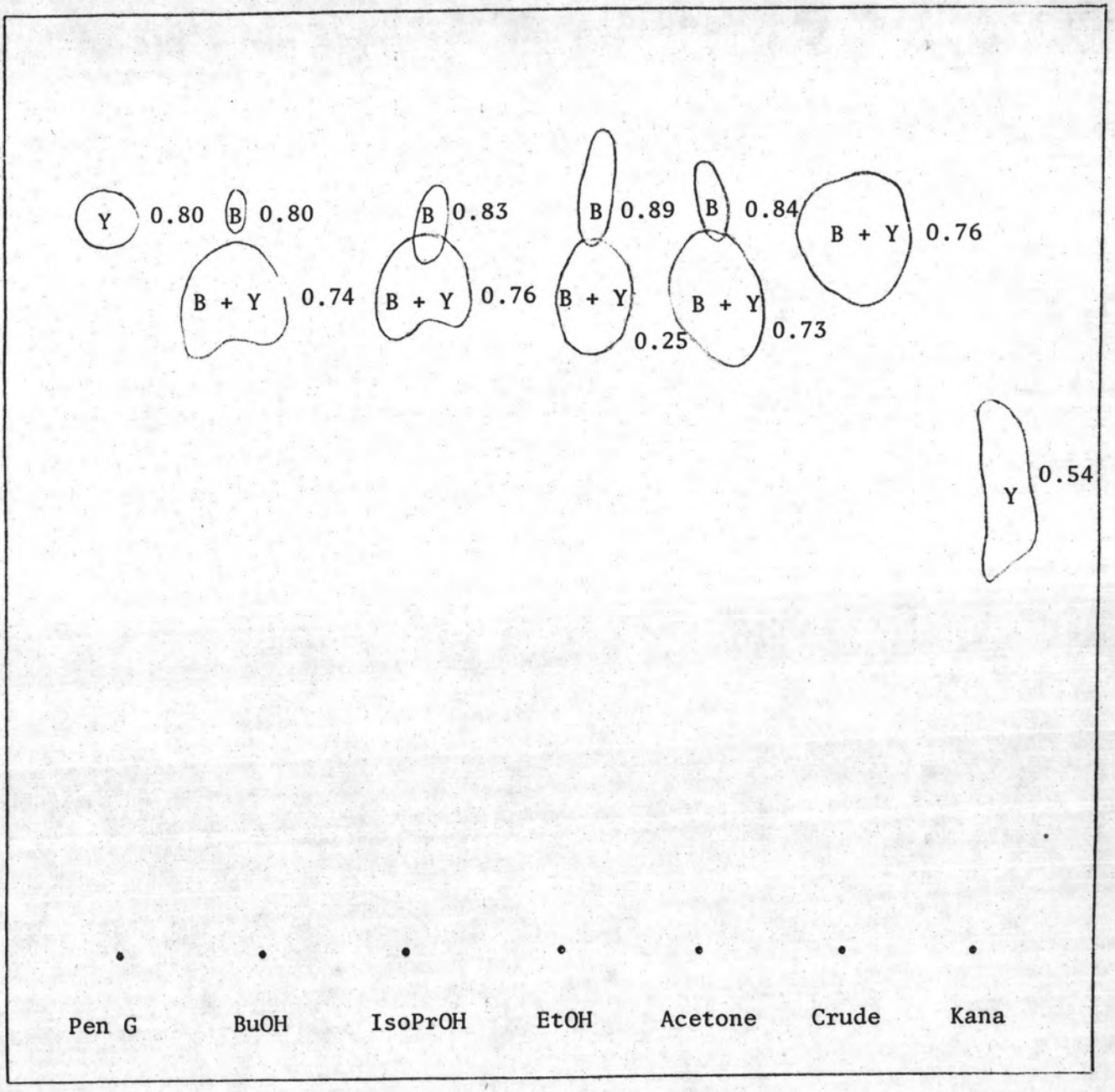


Figure 9 Thin-layer chromatogram of antibiotics sprayed with 10% $KMnO_4$ solution and 0.2% bromphenol blue solution

TLC system :- Propanol : pyridine : acetic acid : water(15:10:3:10)

Color :- B = Brown

Y = Yellow

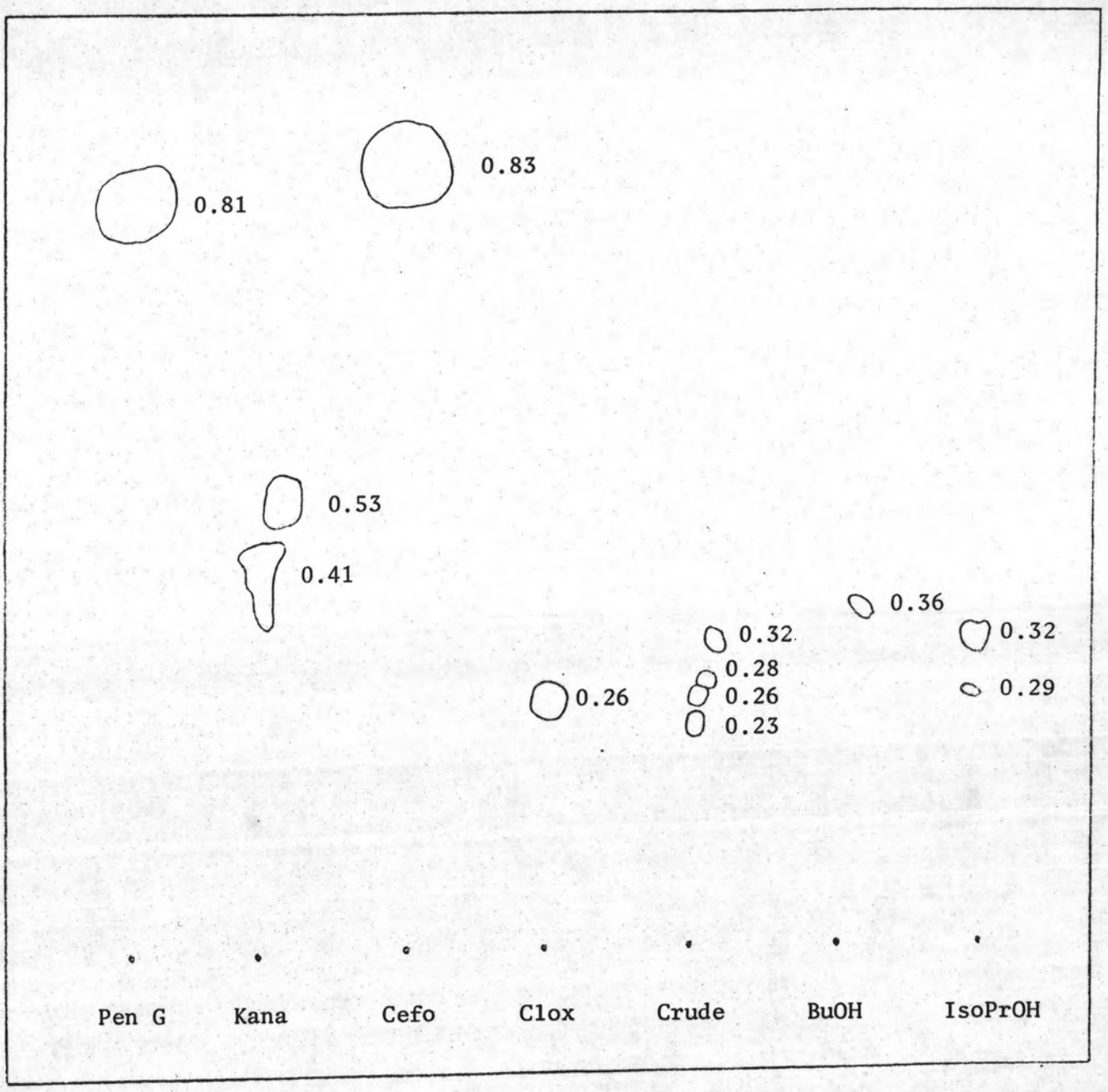


Figure 10 Bioautographic detection for primary identification of unknown Antibiotics on paper, prepared as "reprints" from thin-layer chromatoplate

TLC system :- Propanol : pyridine : acetic acid : water(15:10:3:10)

Organism :- *S. aureus*

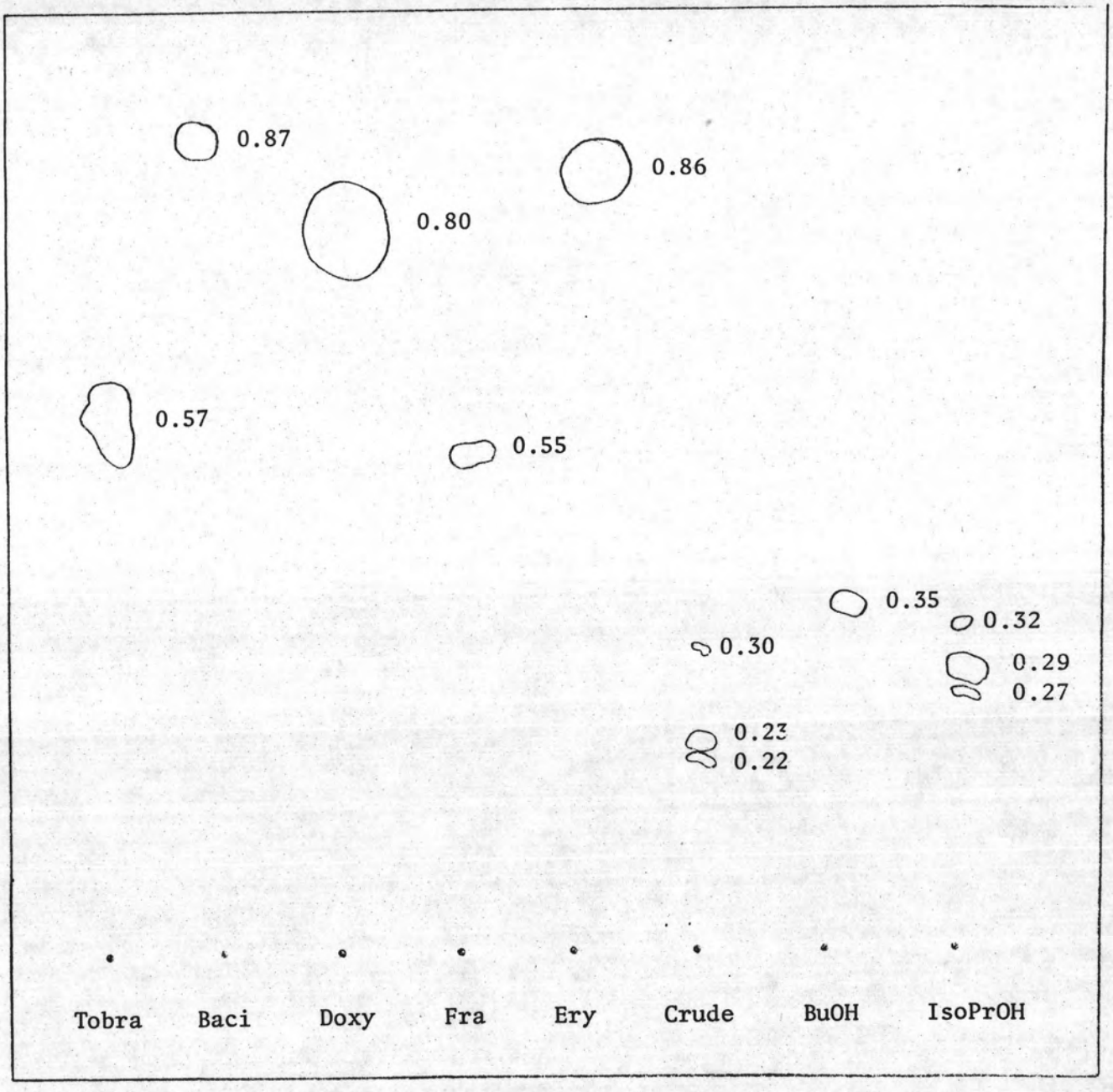


Figure 10 (Continue)

VI Laboratory evaluation of antibiotics from strain ST-13-2

Thirty isolates of each pathogenic organism of various strain derived from the patients were tested for antimicrobial susceptible with 4 dilutions of antibiotic producing strain ST-13-2 broth compared to other 4 antimicrobial agent discs. The results of each test against *S. aureus*, *E.coli*, *Ps. aeruginosa* and *K. pneumoniae* were shown in table 11, 12, 13 and 14 respectively.

Four dilutions of fermented broth from strain ST-13-2 were as follow :

U_1 = Dilute to two-fold (v/v) of the stock broth

U_2 = The stock broth

U_3 = Concentrate to two-fold (v/v) of the stock broth

U_4 = Concentrate to four-fold (v/v) of the stock broth

Table 11 The results of antimicrobial susceptibility test of *S. aureus*

Number	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Erythromycin	Penicillin G	Cloxacillin	Cefotaxime
1	A	13.0	13.9	15.2	16.0	I	R	S	S
2	A	14.6	16.0	17.7	19.2	S	R	S	S
3	B	14.5	14.5	15.8	17.0	S	R	S	S
4	A	13.0	13.5	16.5	17.0	R	R	S	S
5	A	13.0	14.2	14.8	16.3	S	R	S	I
6	A	14.0	15.6	15.6	17.0	S	R	S	I
7	A	12.1	14.0	15.6	17.7	I	R	S	I
8	A	13.0	14.6	15.8	16.3	R	R	S	S
9	A	15.0	15.8	18.5	19.4	I	R	S	I
10	A	14.4	15.7	16.9	18.0	R	R	S	S
11	A	13.8	15.5	15.5	17.7	S	R	S	S
12	A	14.3	15.8	16.0	18.2	S	R	S	I
13	A	14.4	15.4	16.3	17.4	S	R	S	S
14	A	13.6	14.8	15.3	16.4	I	R	S	I
15	A	14.0	15.0	16.0	17.2	R	R	S	S
16	B	13.2	14.1	15.6	16.2	I	R	S	I
17	B	16.4	17.6	18.1	20.0	S	R	S	S
18	B	14.6	16.0	16.2	18.0	I	R	S	I
19	A	14.2	15.0	15.5	17.6	S	R	S	S
20	B	14.0	15.1	15.1	16.1	I	R	S	I
21	B	13.8	15.0	16.1	16.6	S	R	S	S
22	B	15.0	15.2	16.0	17.4	S	R	S	S
23	A	13.7	15.5	17.0	17.1	R	R	S	S
24	A	14.2	14.7	16.4	18.0	R	R	S	S
25	A	13.7	14.5	14.7	16.0	I	R	S	I
26	B	14.2	15.1	16.0	18.0	S	R	S	S
27	A	14.0	15.0	16.2	17.4	I	R	S	I
28	A	14.1	15.7	16.8	18.4	I	R	S	S
29	A	14.0	15.1	15.1	17.0	I	R	S	S
30	A	14.0	14.2	14.2	15.8	R	R	S	S
Total number of susceptible organisms						12	0	30	19
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						40.0	0.0	100.0	63.3

N.B. Hospital A = Siriraj
B = Bangkok Christian

Table 12 The results of antimicrobial susceptibility test of *E.coli*

Number	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Gentamicin	Tobramycin	Ceftriazone	Cefotaxime
1	A	13.7	15.7	17.3	18.0	S	S	S	I
2	A	16.3	17.7	18.0	20.4	S	S	S	S
3	A	15.5	16.8	18.8	21.0	R	R	S	R
4	A	16.0	17.1	17.7	19.0	S	S	S	I
5	A	13.7	15.2	17.0	19.0	S	S	S	I
6	A	16.0	17.4	18.8	21.0	S	S	S	S
7	A	11.6	12.0	13.6	14.2	R	R	S	I
8	A	14.7	16.3	17.3	18.6	S	S	S	I
9	A	12.4	13.7	15.5	16.3	I	I	I	S
10	A	13.6	16.2	17.3	18.3	S	S	S	I
11	A	15.1	15.9	17.6	19.4	S	S	S	S
12	A	14.9	16.6	18.0	20.4	S	S	S	S
13	A	16.2	17.0	17.8	19.2	S	S	S	I
14	A	15.6	17.0	17.7	20.0	S	S	S	I
15	A	16.6	18.0	19.0	21.0	S	S	S	S
16	B	14.6	16.0	18.0	20.0	S	S	S	S
17	B	14.9	16.8	18.0	20.0	S	S	S	I
18	B	16.0	17.3	18.0	18.6	S	S	S	I
19	B	15.0	16.6	18.0	18.6	S	S	S	S
20	B	16.4	17.4	19.0	21.0	S	S	S	S
21	B	15.5	16.3	17.6	19.1	S	S	S	S
22	B	15.0	17.4	18.6	20.3	S	S	S	I
23	B	15.4	16.8	16.0	18.0	S	S	S	I
24	B	15.2	16.5	17.8	20.0	S	S	S	I
25	B	15.0	16.0	17.6	18.5	S	S	S	S
26	B	16.0	16.7	17.8	20.5	S	S	S	I
27	A	16.1	17.1	18.2	20.5	R	R	S	S
28	A	13.5	14.8	15.2	18.0	S	S	S	I
29	A	14.1	15.0	16.1	18.2	R	R	S	I
30	A	15.0	16.9	17.1	19.3	R	R	S	I
Total number of susceptible organisms						24	24	29	12
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						80.0	80.0	96.7	40.0

Table 13 The results of antimicrobial susceptibility test of *Ps. aeruginosa*

Number	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Netilmicin	Amikacin	Moxalactam	Cefotaxime
1	A	0	0	0	0	R	R	R	R
2	A	0	0	0	0	R	R	R	R
3	A	0	0	0	0	R	R	I	R
4	A	0	0	0	9.6	R	R	R	R
5	A	0	0	0	8.3	S	S	R	R
6	A	0	7.9	9.0	9.2	S	S	I	I
7	A	0	0	0	0	S	I	I	I
8	A	7.8	9.0	10.1	12.3	S	S	I	I
9	A	0	0	0	0	R	R	I	R
10	A	0	0	8.5	9.4	S	S	R	R
11	A	0	0	0	0	S	S	I	R
12	A	0	0	0	0	S	R	I	I
13	A	0	7.9	9.0	10.0	R	I	I	I
14	A	0	0	0	0	R	R	I	I
15	A	0	0	0	0	S	S	R	I
16	A	0	0	0	0	S	S	I	I
17	A	0	0	9.1	11.0	S	I	I	I
18	A	0	9.3	10.8	12.0	R	R	R	R
19	A	0	0	0	0	I	R	I	I
20	A	0	0	0	0	S	S	I	I
21	A	16.8	17.4	18.8	20.0	S	S	S	S
22	B	0	0	0	0	S	S	R	I
23	B	0	0	0	0	S	S	I	I
24	B	0	9.0	10.4	11.8	S	I	I	R
25	B	22.2	22.8	23.5	26.0	S	S	S	S
26	B	9.3	9.6	11.2	13.5	S	S	I	I
27	B	0	0	0	8.9	R	R	R	R
28	B	13.8	15.6	17.2	18.9	S	S	S	I
29	B	10.3	11.4	12.8	14.9	S	S	R	R
30	B	0	0	0	0	S	I	R	R
Total number of susceptible organisms						20	15	3	2
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						66.7	50.0	10.0	6.7

table 14 The results of antimicrobial susceptibility test of *K. pneumoniae*

Number	Hospital	Inhibition zone in mm.				Susceptibility			
		U_1	U_2	U_3	U_4	Gentamicin	Tobramycin	Ceftriazone	Cefotaxime
1	B	11.8	12.9	13.7	15.0	R	R	I	R
2	B	13.8	15.6	16.3	18.0	S	S	S	I
3	B	13.4	14.7	15.8	17.1	I	R	I	R
4	B	13.2	15.0	16.6	18.0	R	R	S	I
5	B	11.3	12.6	14.8	16.7	R	I	I	R
6	B	15.9	16.2	17.7	20.3	R	R	I	R
7	B	14.6	15.7	16.7	18.1	I	R	S	I
8	B	11.6	13.2	14.0	16.3	I	R	I	I
9	B	14.9	15.5	15.8	16.9	S	I	I	R
10	B	12.8	13.6	14.1	16.2	I	I	S	I
11	B	12.6	13.7	14.9	16.5	I	I	S	I
12	B	13.9	14.0	15.4	16.8	I	S	S	I
13	B	12.3	13.5	16.0	17.2	I	I	S	I
14	B	13.8	15.3	16.7	18.8	I	S	S	I
15	A	15.0	15.5	16.0	18.5	S	I	S	I
16	A	13.8	15.3	16.1	17.4	R	R	S	I
17	A	14.9	15.1	15.5	16.9	I	S	S	I
18	A	14.9	15.9	18.2	19.3	S	S	S	I
19	A	14.0	14.7	15.6	16.2	S	S	S	I
20	A	12.0	12.8	14.5	15.2	R	I	S	I
21	A	13.7	15.1	16.2	17.7	S	S	S	I
22	A	13.2	14.3	15.5	16.7	R	R	R	I
23	A	14.0	14.7	16.0	17.8	R	R	R	I
24	A	12.1	12.8	14.5	16.9	R	R	R	R
25	A	13.2	14.7	16.2	17.3	R	I	S	R
26	A	17.3	18.1	20.7	22.1	R	R	I	I
27	A	13.4	14.4	16.7	18.9	R	R	R	R
28	A	13.3	15.5	16.0	17.8	S	I	S	I
29	A	14.2	15.0	15.9	18.0	S	S	S	S
30	A	15.6	17.1	18.0	19.5	S	S	S	S
Total number of susceptible organisms						9	9	19	2
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						30.0	30.0	63.3	6.7