STUDY ON ANTIBIOTIC-PRODUCING ACTINOMYCETES FROM CAVE SOIL IN CENTRAL REGION OF THAILAND

การศึกษาเชื้อแอคติโนมัยซีตีสจากดินถ้ำบริเวณภาคกลางของประเทศไทย ที่สามารถสร้างสารปฏิชีวนะ

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ABSTRACT

In preliminary screening for actinomycetes, thirty soil samples collected from six caves in Changwat Kanchanaburi, Ratchaburi and Saraburi were used. The percentage of actinomycetes isolated from caves namely : Mungkorn-tong, Kao-lam, Sarika, Jom-pon, Kao-bin and Pothisat were 2.23, 3.92, 7.23, 0.16, 1.16 and 0.84 respectively. The net per cent of isolated actinomycetes was 3.81. Screening for antibiotic-producing strains was carried out by the streak plate method using Staphylococcus aureus *ATCC 6538-P,* Bacillus subtilis *ATCC 6633,* Streptococcus faecalis *Department of Microbiology, Faculty of Medicine, Chulalongkorn Hospital,* Escherichia coli *ATCC 10534,* Pseudomonas aeruginosa *NCTC 10612 and* Candida albicans *ATCC 10231 as test organisms. Fifty-one out of 104 strains (49.04%) were found active and produced antibiotics. They were classified into 6 groups according to their antibiotic activity. The selected strain ST-13-2 which gave high antibiotic activity in plate assay against all test organisms was selected for further taxonomic study and antibiotic production in liquid media. Taxonomic studies revealed that it is closely related to* Streptomyces parvullus. *Fermentation for antibiotic production in glucose soybean medium, at pH 7.0 (before sterilization), and at temperature 23 ° C was found optimum.*

บทคัดย่อ

การศึกษาหาเชื้อ แอคติโนมัยซีตีส ทำโดยการเก็บตัวอย่างดินถ้ำจำนวน 30 ตัวอย่างจาก ถ้ำ 6 แห่งในจังหวัดกาญจนบุรี ราชบุรี และสระบุรี พบว่าสามารถแยกเชื้อนี้ได้จากถ้ำมังกรทอง 2.23%, ถ้ำเขาแหลม 3.92%, ถ้ำสาริกา 7.23%, ถ้ำจอมพล 0.16%, ถ้ำเขาบิน 1.16% และถ้ำโพธิสัตว์ 0.84% คิดเป็นเชื้อแอคติโนมัยซีตีสแยกได้ทั้งหมด 3.81% เมื่อนำเชื้อที่แยกได้มาทดสอบความสามารถในการสร้างสารปฏิ-ชีวนะบนอาหารแข็งที่มีผลยับยั้งต่อเชื้อ *Staphylococcus aureus* ATCC 6538-P, *Bacillus subtilis* ATCC 6633, *Streptococcus faecalis* Department of Microbiology, Faculty of Medicine, Chulalongkorn Hospital, *Escherichia coli* ATCC 10534, *Pseudomonas aeruginosa* NCTC 10612 และ *Candida albicans* ATCC 10231 พบเชื้อ 51 สายพันธุ์จาก 104 สายพันธุ์ (49.04%) ที่สามารถสร้างสารปฏิชีวนะได้ จำแนกเชื้อตามผลการยับยั้งเชื้อทดสอบออกได้เป็น 6 กลุ่ม คัดเลือกเชื้อ ST-13-2 ที่ให้ผลดีต่อการยับยั้งเชื้อทดสอบทุกชนิดบนอาหารแข็งมาศึกษา เพื่อจำแนกบ่งชี้เชื้อและศึกษาการสร้างสารปฏิชีวนะในอาหารเหลว พบว่าเชื้อที่คัดเลือก ST-13-2 จัดอยู่ในกลุ่มของ *Streptomyces* โดยมีลักษณะใกล้เคียงกับเชื้อ *Streptomyces parvullus* มากที่สุด การเพาะเลี้ยงเชื้อนี้ในอาหาร glucose soybean medium ที่มี pH 7.0 (ก่อนผ่านการฆ่าเชื้อโดยความดัน) ณ อุณหภูมิ 23°ซ. จะให้ผลในการสร้างสารปฏิชีวนะสูงสุด

INTRODUCTION

The field of antibiotics has undergone spectacular developments since the discovery of Penicillin in 1929. Later Waksman and his associates reported the isolation of Actinomycin in 1940, Streptothricin in 1942, Streptomycin in 1943 and Neomycin in 1949. The discovery of Streptomycin, which was found to be particularly useful in treating bacterial infection especially tuberculosis, greatly stimulated the search for useful antibiotics among actinomycetes. The actinomycetes belonging to the genus *Streptomyces* have recently occupied an eminent place because many of them are important producers of antibiotics. This group of microorganisms is the source of many of the currently used antibiotics. Chloramphenicol, the first of the so-called broad-spectrum antibiotics of commercial importance, was first isolated by Ehrlich in 1947. The following year, Dugger described Aureomycin, an antibiotic obtained from cultures of *Streptomyces aureofaciens*. This event opened up the search and the discovery of the important class of antibiotics called the Tetracyclines. Following these discoveries, the pace of new discoveries accelerated.[3] The potentiality of a particular antibiotic for important therapeutic usefulness in the treatment of one or more infectious diseases depends upon its action on the causative agents of the disease and its lack of toxicity of the affected animals. In Thailand, new antibiotics from the actinomycetes were researched from the soils. In 1979, Meevootisom and Nomi[9] isolated antibiotic producing *Streptomyces* from the soil. Kulprecha and co-workers isolated a new species of *Streptomyces*, that was found to produce antifungal antibiotics in the culture filtrate and mycelium, from the soil from a rubber plantation in Thailand.[6]

It was known that the natives treated the infection wound by using cave soil. According to this local practice, cave soil samples were collected from the central region of Thailand for investigation. It is anticipated to find new antibiotics from the actinomycetes that could help solve major problem of chemotherapy especially the prevalence of certain microbes resistant to specific antibiotics.

MATERIALS AND METHODS

Isolation methods [10, 12, 13, 17]

Thirty soil samples were collected from various caves in the central region of Thailand. The soil samples and their sources were shown in Table 1. Fresh soil was taken from the soil surface and down to a depth of 6 cm. The samples were placed in the laboratory at 4 °C in refrigerator.

To isolate antibiotic-producing actinomycetes, 10 g of each soil sample was suspended in sterile distilled water to make 1:10, 1:100, 1:1000 and 1:10000 dilutions. One ml portion of soil dilution was plated with potato dextrose agar (plus nystatin 50 μg/ml) to determine the total count.

The soil suspension of 0.1 ml was applied to the other agar surface by pipette, then spread across the surface with sterile glass rod to isolate selected colonies. Two sets of each soil sample were studied. One was incubated at 28-30°C for 3-5 days under aerobic condition and another under anaerobic condition in gas pak jar.

The isolated colonies of actinomycetes were subcultured and purified by streaking, before making stock cultures on the potato dextrose agar slants and kept in a cold room at 4°C.

Screening of cultures for antibiotic production [2, 16]

The pure cultures were streaked in a narrow band across the centers of the nutrient agar plates, then incubated at 28 -30°C for 5 days or until growth and, possibly, sporulation had occurred. Six test organisms were then streaked from the edges of the plates up to but not touching the growth, they were as follows :

Staphylococcus aureus	ATCC 6538-P
Bacillus subtilis	ATCC 6633
Streptococcus faecalis	Department of Microbiology, Faculty of Medicine, Chulalongkorn Hospital
Escherichia coli	ATCC 10534
Pseudomonas aeruginosa	NCTC 10612
Candida albicans	ATCC 10231

The assay plates were further incubated at 35-37°C to allow growth of the test organisms. The clear inhibition distance over which the growth of each test organism had been inhibited by antibiotic in the vicinity of the actinomycetes was observed. Only those actinomycetes that had produced antibiotics with anticipated microbial inhibition spectra were retained for further testing.

Taxonomic studies of selected strain

Morphological and physiological properties of a selected strain (strain ST-13-2) were determined by media and methods described by Shirling and Gotllieb[14] along with several supplementary tests.

A. Morphological characterization

The culture media were medium 2 (yeast extract-malt extract agar), medium 3 (oatmeal agar), medium 4 (inorganic salts-starch agar) and medium 5 (glycerol-asparagine agar). Each medium was poured into 7 plates.

The suspension of spores or mycelium was inoculated to make the crosshatched culture on the agar surface. The plates were incubated in the dark at 25-28°C. For each culture two plates of each medium were observed after 7 days, 14 days and 21 days. One extra plate was also inoculated to prevent any accidental loss.

Determination of the characteristics of the culture was carried out as follows :

1. Direct light microscopic examination. Observation was done at 100 × - 700 × to establish the presence (or absence) of chains of spores, the number of spores at the end of mature hyphae, the form of the spores chain and spore bearing hyphae.

2. Color determinations. This was done to describe the aerial mass color, the color of substrate mycelium (reverse color), and the soluble colors other than melanoid pigmentation.

3. Scanning electron microscopic examination.[18, 20] The culture was cut to some small cubes (3-5 mm^3) and then primary fixed in a 4% solution of paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at room temperature for 2 h. After that they were washed 3 times with buffer and were treated with secondary fixative, a 1% solution of osmium tetroxide in buffer, and were washed in the same process before dehydrating through a graded ethanol series and finally drying in the critical point dryer. Fix the specimen to a stub and coat with thin film of gold by Sputter coater.

The spore chains were observed in the scanning electron microscope at the magnification of 5,000 × - 10,000 ×.

B. Physiological characteristics

1. Melanin production. A heavy inoculum of spores and aerial mycelium was streaked on the agar slants of medium 1 (tryptone - yeast extract agar), medium 6 (peptone iron agar, Difco, supplement with 0.1% yeast extract), and medium 7 (Shinobu's modification of Masumoto's tyrosine agar). The agar slants were incubated at 25-28°C and melanoid pigments observed after 48 h and 96 h.

In comparing inoculated tubes with uninoculated controls, culture forming a greenish brown to black diffusible pigment or a distinct brown pigment modified by other color was recorded as positive (+).

2. Carbon utilization. Placed 0.05 ml of washed inoculum (culture in broth and washed with normal saline) onto the agar surface. Streak the drop straight across the dish and repeated with a second drop. Inoculated duplicate plates. Carbon sources used in the basal media (medium 8, Pridham and Gottlieb carbon utilization medium) were D-glucose (positive control) L-arabinose, D-xylose, D-fructose, inositol, L-rhamnose, D-galactose, D-mannose, sucrose, raffinose, D-mannitol, salicin and cellulose. The plates were incubated at 25 - 28°C and observed at 10-16 days.

Results were recorded in term of strongly positive utilization (+ +), positive utilization (+), utilization doubtful (±) and utilization negative (−).

C. Biochemical reactions[8]

1. Nitrate reduction

Inoculated two nitrate broth tubes with the culture and incubated at 28 - 30 °C for 4 to 6 days. On the 4th day, transferred 1 ml of the medium into a test tube and add 3 drops of sulfanilic acid reagent, followed by 2 drops of dimethyl-α-napthylamine solution. If nitrites were present, the mixture would become pink. When a positive test for nitrates was obtained, incubated the tubes further, and then performed the qualitative test for ammonia by Nessler's solution. If ammonia was present, a yellow-brown color was developed in the medium.

2. Starch hydrolysis

Streaked the culture in a band across the centers of the starch agar plate and incubated at 28 - 30°C for 5 days. After incubation was complete, flood the surface of the plate with dilute Gram's iodine solution. If starch hydrolysis was present, a dark-blue color did not appear.

3. Gelatin liquefaction

Streaked the culture in a band across the centers of the 0.4% nutrient gelatin agar and incubated at 28-30°C for 5 days. After incubation was complete, flood the surface of the plate with saturated ammonium sulfate solution. Whenever, the gelatin has been hydrolyzed, turbidity did not appear.

4. Litmus milk reduction

Inoculated two tubes of litmus milk with the culture and incubated at 28-30°C, and observed daily through a ten day period for a) reduction of litmus, b) milk coagulation, c) milk peptonization, d) gas production, and e) any changes in pH according to the indicator.

5. Casein decomposition

Made a single streak across the center of the skim-milk plus nutrient agar plate with the culture and incubated at 28-30°C for 5 days. Observed the clear zone if there was any casein decomposition.

6. Tyrosine and xanthine decomposition

Streaked the culture in a band across the centers of the tyrosine agar plate

and xanthine agar plate. The plates were incubated at 28-30°C for 5 days. If the decomposition was present, the clear zone was resulted.

Development of methods for submerged-culture antibiotics production[2, 12]

A. Development of media[15]

1. Liquid media

The selected antibiotic producing actinomycetes strain ST-13-2 which showed broad spectrum and strong inhibition when tested on solid media was tested for its ability to produce antibiotic in 3 kinds of liquid media : glucose peptone medium, glucose soybean medium, and maltose soybean medium. Thirty-five ml of each liquid medium in 250 ml Erlenmeyer flask with 20 glass beads was used in this test.

2. Inoculation for antibiotic production

The inoculum was prepared by adding 3-5 ml of sterile distilled water to the stock slant and using a sterile wire loop to get the spores or mycelial fragments. Each liquid medium was inoculated with 0.5 ml of the inoculum and was incubated on a rotary shaker (180 rpm, 28°C) for 3, 5 and 7 days. By this time, 5 ml of each culture fluid was taken aseptically to assay for antibiotic activity against 6 test organisms by agar diffusion method.

3. Antibiotic assay[5]

3.1 Preparation of inoculum

The fresh cultures of each test organism on antibiotic medium no. 1 slants that had been incubated at 37°C for 16-18 h were used. They were washed out with sterile normal saline and standardized by determining the dilution that would permit 25% light transmission at wave length 525 nm.

3.2 Preparation of assay plate

The flasks with 100 ml medium no. 1 were melted then cooled to 48°C, and inoculated with 0.5 ml of each inoculum suspension. After thorough mixing avoiding air bubbles, the 20 ml of agar was poured into 9 cm sterile petri dishes. A sterile cork borer with 6 mm diameter was used to press upon the hardened agar to make 5 sharp circles to give holes after removing the agar within the circles.

3.3 Assay procedures

The inoculated agar plates were filled in each hole with 50 μl of each fermented liquid medium. The plates were left 1 h at room temperature for diffusion into the agar medium. Then, they were incubated at 37°C for 16-18 h. Compared the diameters of the inhibition zone by measuring with a sliding caliper.

B. Determination of optimum pH and temperature

1. Preparation of liquid media

The selected liquid medium from development of media was dispensed into 3 sets of the 500 ml Erlenmeyer flasks, each set had 6 flasks containing 130 ml of the medium. The pH of the medium in each set was adjusted to pH 4, 5, 6, 7, 8 and 9 with either 1N NaOH or 1N H_2SO_4 before sterilization.

2. Preparation of seed medium

Prepared 3.0 ml of turbid suspension of mycelial growth from strain ST-13-2 in sterile water and inoculated into a 500 ml Erlenmeyer flask containing 130 ml of the selected liquid media (pH 7.0). The flask was incubated on a rotary shaker (300 rpm, 28°C) for 3 days.

3. Fermentation

A 5.0 ml aliquot of the culture from the seed medium was inoculated into each of 500 ml Erlenmeyer flask described in B.1. Fermentation was carried out for each set at 23, 30, and 33°C with agitation (300 rpm).

The pH of the culture fluid was measured and the antibiotic production during fermentation was monitored by agar diffusion method using *S. aureus* ATCC 6539-P as the test organism. An example of a typical time course of the fermentation in each 500 ml Erlenmeyer flask was determined every day until the antibiotic activity was decreased.

RESULTS

Isolation of actinomycetes from cave soil

The calculation of total number of actinomycetes isolated from each cave soil and total count under the aerobic condition were shown in Table 2. There were no isolated colony of actinomycetes from any cave soil sample incubated under anaerobic condition. The numbers of actinomycetes strains 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 strains of actinomycetes were 104.

Determine of antibiotic-producing actinomycetes

According to the screening of cultures for antibiotic production, we have selected 51 active ones out of 104 by streak plate method and classified them into 6 groups (Table 3). 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 distance was selected for further study.

Taxonomic studies of strain ST-13-2

The cultural characteristic of strain ST-13-2 on various media were shown in Table 4. Strain ST-13-2 formed sporophores monopodially branched, with long, regular, open spirals. The number of spore at the end of the mature hyphae was more than 10 (Figure 1). The spore under scanning electron microscopic was cylindrical and 1.09×0.5 μm in size as shown in Figure 2. The surface of the spore was smooth. Morphological characteristics of strain ST-13-2, placed it in the genus *Streptomyces*. The melanin production of strain ST-13-2 was negative. The utilization of carbon sources and biochemical properties were summarized in Table 5 and 6 respectively.

Antibiotic production in liquid media

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 3 and 5 days (Table 7).

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 Figure 3, 4 and 5 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 at 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.

Figure 6 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.

DISCUSSION

To isolate successfully a wide variety of actinomycetes from soil samples it was necessary to eliminate or greatly curtail fungal and bacteria spreaders in the isolation medium without producing an adverse effect on actinomycetes. This could be accomplished in one, or a combination of more than one, of the following ways : 1) control of the medium constituents, 2) addition of inhibitors to the medium, 3) prior treatment of the soil sample.[4]

Fungal contaminants could be virtually eliminated by adding antifungal agents to the isolation medium. In this study, nystatin, added at the level of approximately 50 μg/ml[19] to selective media such as those above, effectively eliminated most undesired contaminants in isolation plates.

The isolation of actinomycetes from cave soil in our primary screening accounted 3.81% of the total microorganisms. Since the soil samples was too shallow, about 6 cm in depth, there was no isolated colony of actinomyces under anaerobic condition. An early representative study was that of Danish soils, the number of actinomycetes varied from none to 13 million / g and the per cent of the total microflora from 0 to 73.[11]

In the antibiotic screening, 51 strains of actinomycetes out of 104 strains (49.04%) were able to elaborate antibiotic substances by the streak plate method as compared to Kuroya and co-workers demonstrated only 360 active ones out of 1,800 strains (20.0%).[7]

The isolated strain ST-13-2 from Mungkorn-tong cave inhibited more clear inhibition distance to all test organisms was selected for further study. The results of morphological characteristics showed that it belongs to genus *Streptomyces*. The cultural and physiological characteristics of strain ST-13-2 were compared with those of the known species of *Streptomyces* described by Waksman[15] and Burgey's Manual of Determinative Bacteriology.[1] The results indicated that strain ST-13-2 was closely related to *S. parvullus*. Difference observed between these two strains were as follows : the spore chain of strain ST-13-2 was long open spiral, spore cylindrical, no soluble pigment on nutrient agar and no gelatin liquefaction while the spore chain of *S. parvullus* was long closed spiral, spore spherical, yellow soluble pigment on nutrient agar and slow gelatin liquefaction. These differences were not sufficient to consider strain ST-13-2 as a new species.

The ability of strain ST-13-2 to produce broad spectrum antibiotics in 3 kinds of liquid medium showed that the glucose soybean medium was superior to glucose peptone medium and maltose soybean medium. The medium supplied nutrients for growth, energy, building of all substance and biosynthesis of fermentation products. Of particular importances were the sources of carbon and nitrogen in the medium, since microbial cells and fermentation products were composed largely of these elements. A poor choice of medium components could cause limited cellular growth and alter the type and ratios of products. Thus the types and amounts of the nutritive components of a medium were critical.[2] According to the composition of 3 kinds of liquid medium, it seemed that glucose and soybean powder were suitable for the sources of carbon and nitrogen in antibiotic production of strain ST-13-2 in this study.

During microbial growth, pH changes can occur for one of several reasons. Obviously, an acidic or alkaline fermentation product can alter the pH picture. Also, an inorganic salt component of the medium can cause pH changes. The media selected are then studied further in several aspects. The initial pH and temperature were varied so as to determine the effect of pH and product yields.[2] The optimum pH and temperature for antibiotic production of strain ST-13-2 fermented in the glucose soybean medium were pH 7 (before sterilization) at 23°C. The incubation peroid of high yield was between 2 and 6 days.

CONCLUSION

The percentage of actinomycetes isolated from Mungkorn-tong, Kao-lam, Sarika, Jom-pon, Kao-bin and Pothisat were 2.23, 3.92, 7.23, 0.16, 1.16 and 0.84 respectively. The net actinomycetes isolated was 3.81%. From these isolated actinomycetes, 51 active ones out of 104 strains (49.04%) were able to produce antibiotics against test organisms, and were then classified into 6 groups.

One of the isolates, strain ST-13-2 was selected for further study. It was identified as a species closely related to *Streptomyces parvullus*. Antibiotic production of strain ST-13-2 was compared in 3 liquid media : glucose peptone medium, glucose soybean medium and maltose soybean medium. The glucose soybean medium was found most suitable. An initial pH of 7.0 (before sterilization) proved to be optimum for antibiotic production and the optimum temperature was 23°C. The high yield was in the incubation peroid between 2 and 6 days.

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Table 1. The cave soil samples and their sources

Sample number	Cave	Changwat
1-5	Mungkorn-tong	Kanchanaburi
6-10	Kao-lam	Kanchanaburi
11-15	Sarika	Ratchaburi
16-20	Jom-pon	Ratchaburi
21-25	Kao-bin	Ratchaburi
26-30	Pothisat	Saraburi

Table 2. Microbiological population of cave soil

Cave	No. of microorganisms/g		
	Actinomycetes	Total count	Per cent
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
Pothisat	7	830	0.84
Total	7,859	206,454	3.81

Table 3. Classification of 51 active strains of actinomycetes

Group	No. of inhibited test organism	No. of actinomycetes strains	Per cent
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
Total		51	49.04

Table 4. 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

Table 5. 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 6. 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

Table 7. 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

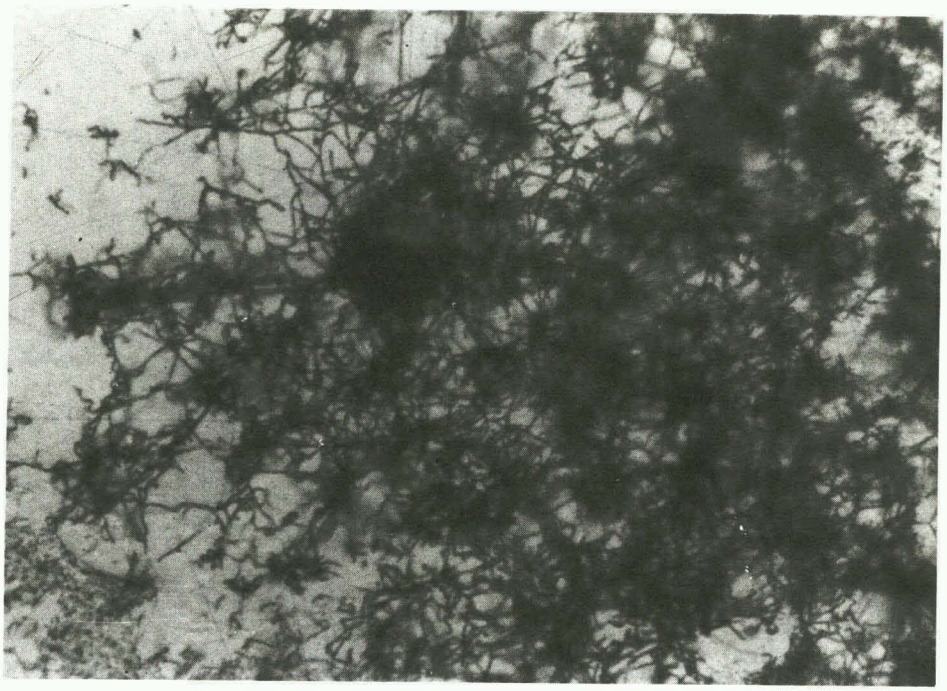

Fig. 1 Photomicrograph of strain ST-13-2 (on oatmeal agar, × 400)

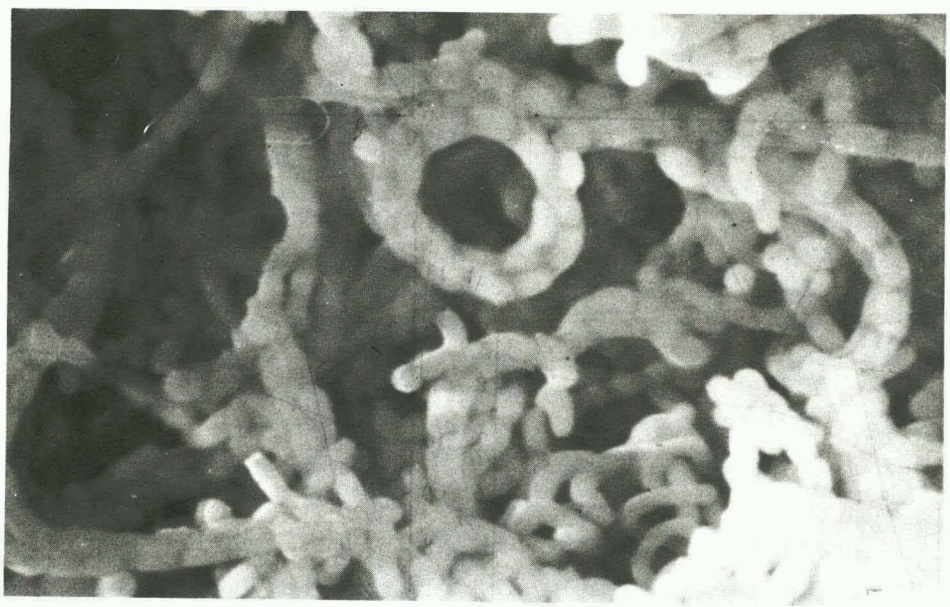

Fig. 2 Scanning electronmicrograph of spore surface of strain ST-13-2 (on oatmeal agar, 30°C, 7 days, × 10,000)

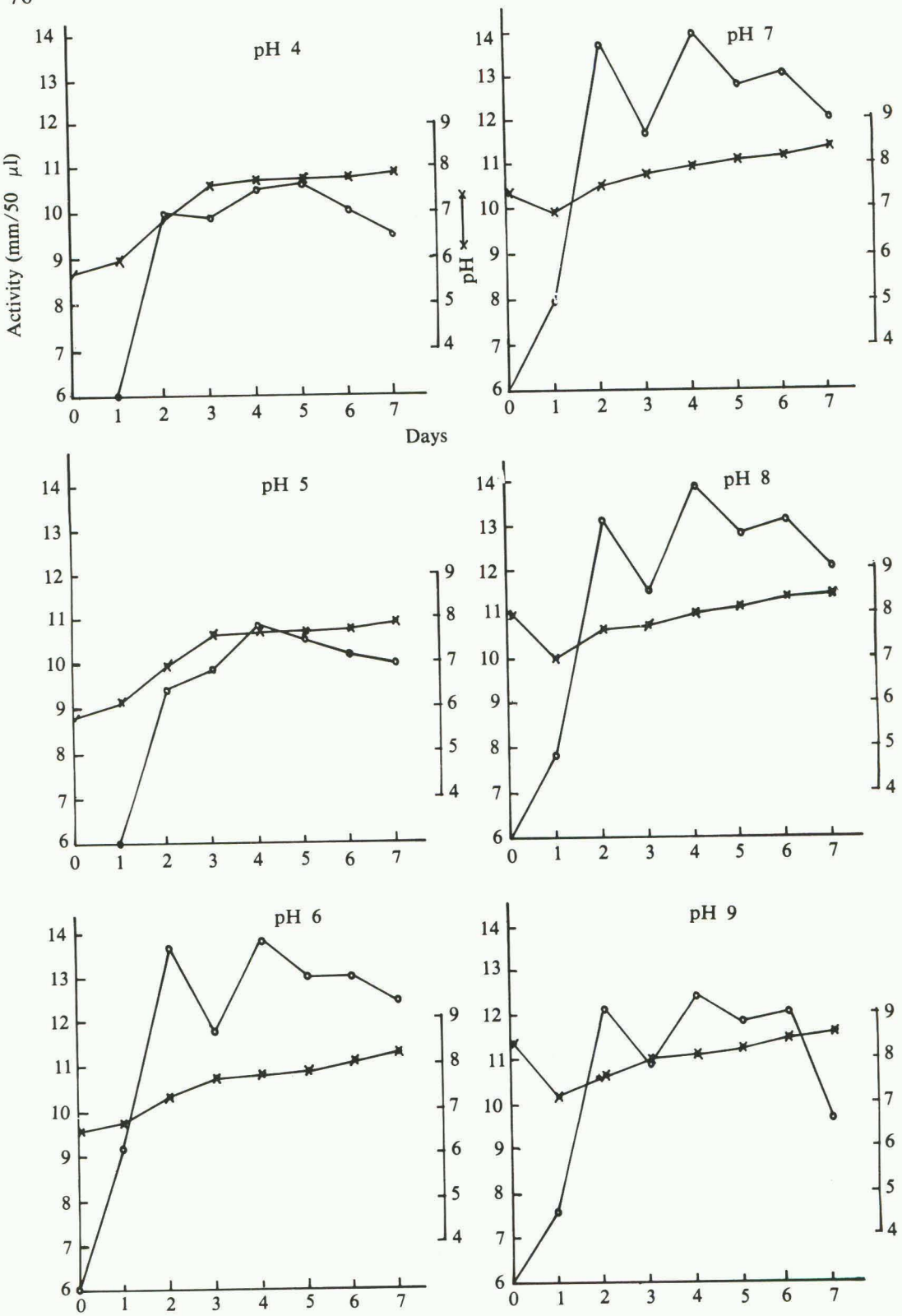

Fig. 3 Fermentation of antibiotic from strain ST-13-2 in various pH at 23°C

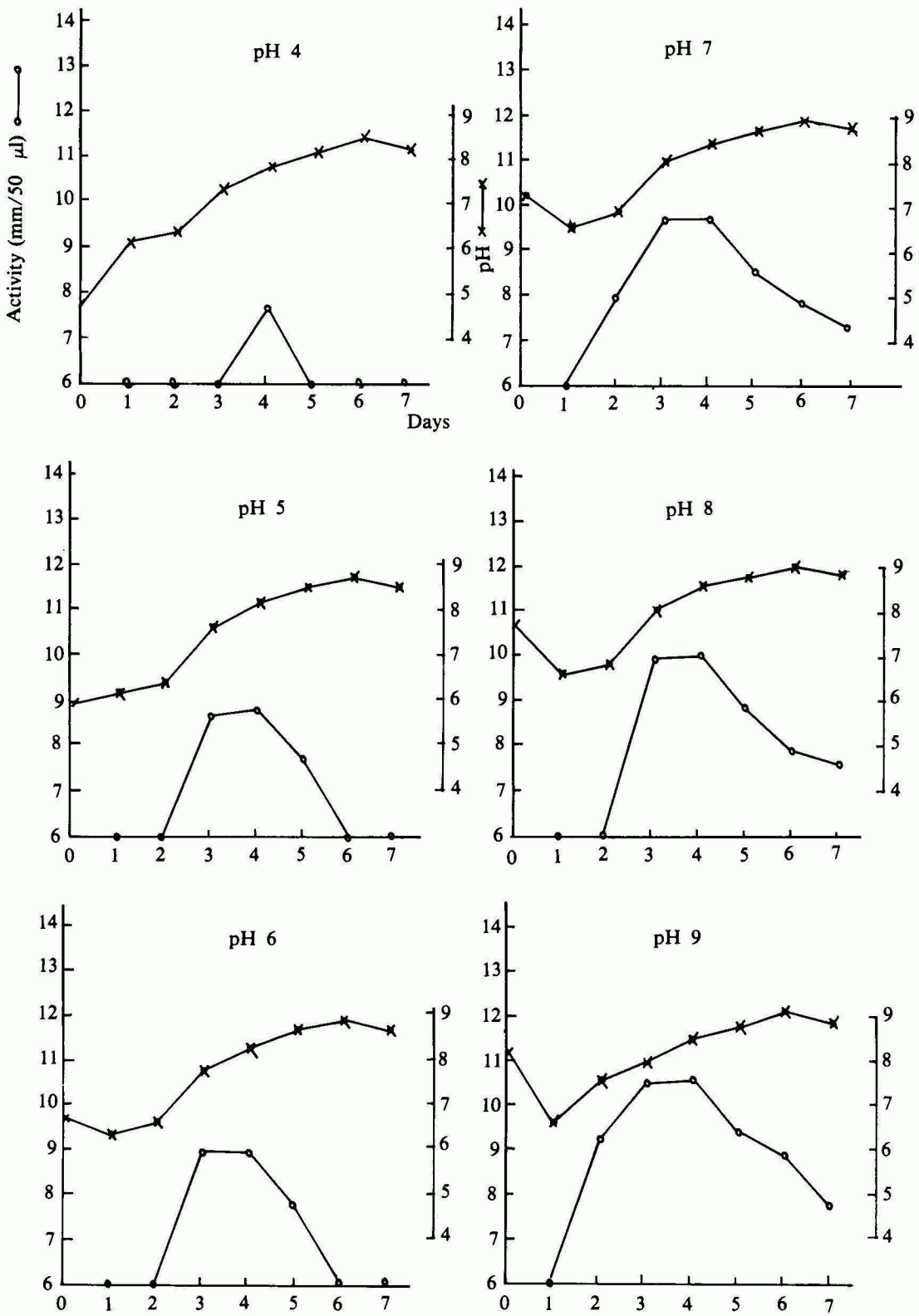

Fig. 4 Fermentation of antibiotic from strain ST-13-2 in various pH at 30°C

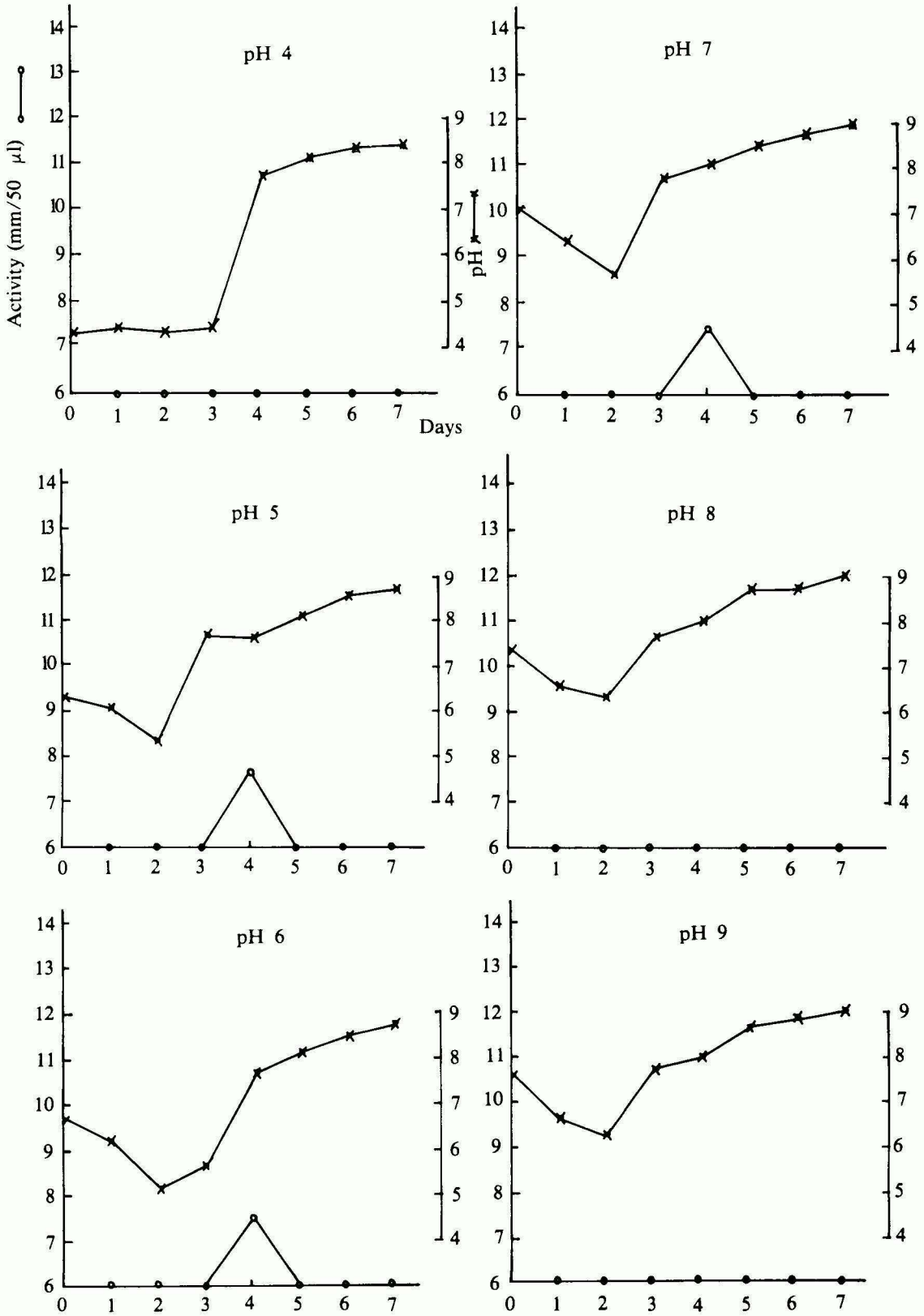

Fig. 5 Fermentation of antibiotic from strain ST-13-2 in various pH at 33°C

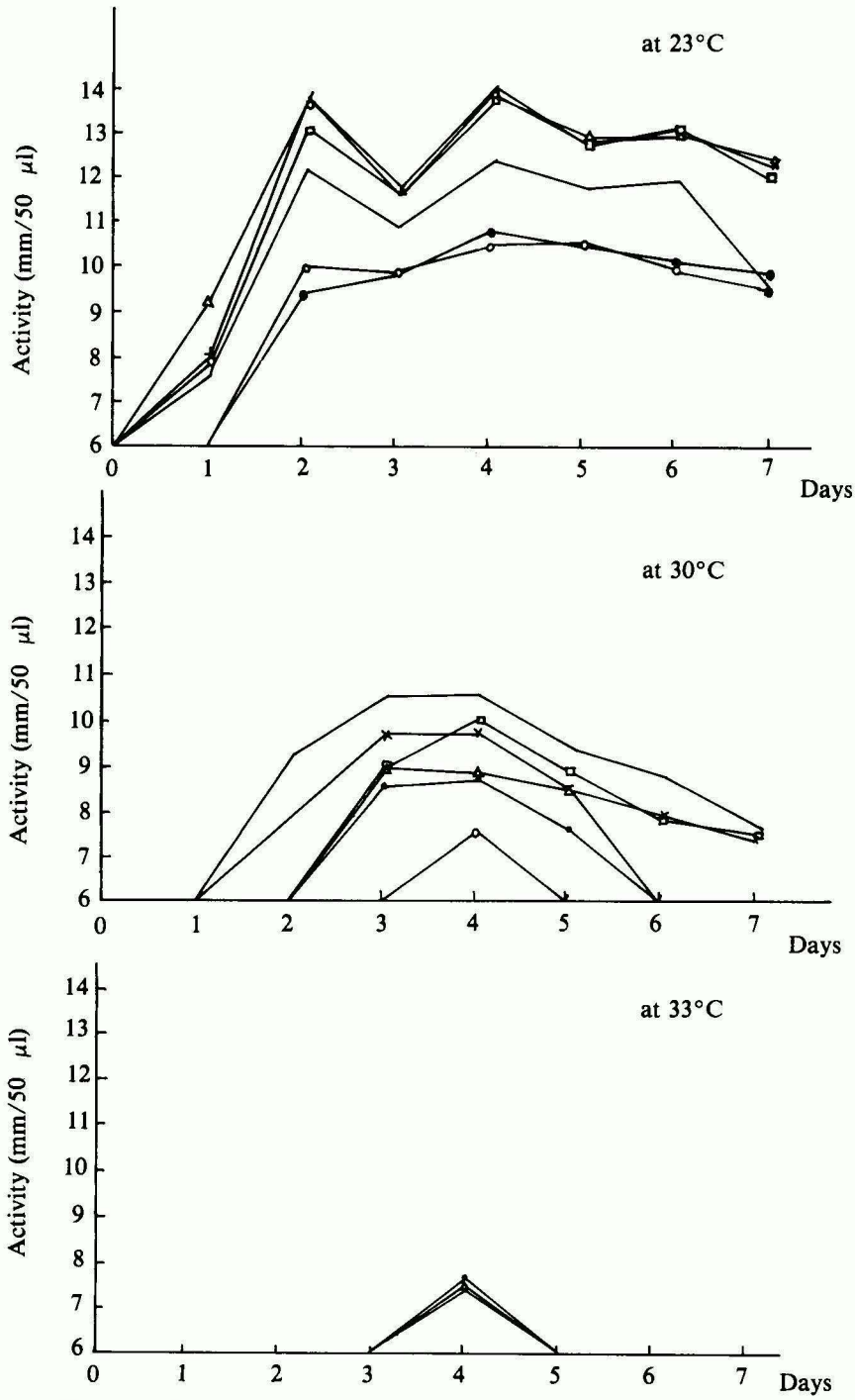

Fig. 6 The comparison of antibiotic production pattern of strain ST-13-2 in various pH and temperature

pH 4 pH 7
pH 5 pH 8
pH 6 pH 9