ACTINOPLANES PHILIPPINENSIS: EFFECT OF CARBON SOURCES ON ZOOSPORE PRODUCTION

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THESIS

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CHAPTER I

INTRODUCTION

Within the past two decades actinomycetes have gained great economic and public health importance as producers of antibiotics, vitamins and enzymes. Selman A. Waksman, the pioneer in this field, started the study of actinomycetes early in this century. His research consisted of the study of cultural characteristics, types of species, their classification, their physical characteristics, their biochemical activities, and their ability to produce antibiotic substances (11, 12).

The actinomycetes are a group of branching unicellular organisms that form a mycelium. They reproduce by fission or by means of special spores called conidiospores (12, p. 3). Actinomycetes have been regarded as a group of organisms occupying a position between the filamentous fungi and bacteria. The properties of actinomycetes, such as the diameter of the filaments and spores, reproduction by fragmentation and oidia, production of aerial mycelium in some cases, and the acid fastness of cells, reveal their close relationship with bacteria. However, the manner of branching in some aerial mycelium, reproduction by means of conidiospores and the growth of colonies on the surface of liquid and solid media, are properties similar to those of fungi

rather than of true bacteria (11, p. 11). Therefore, it has been suggested that actinomycetes form the link between fungi and bacteria, or even that they represent the original prototype from which both of these groups of organisms have evolved (11, p. 1).

In 1949 some cultures of actinomycetes that reproduce by zoospores (2) were discovered accidentally by J. N. Couch, using the techniques commonly employed in the isolation of saprophytic chytrids from soil (1). Such a chytrid trap consists of placing a soil sample in a sterile petriplate to which sterile charcoal water has been added. Charcoal water consists of animal charcoal dissolved in distilled water; after one hour, this solution is filtered and autoclaved before use. Paspalum spp., hemp seed, filter paper and pollen grains are used as a bait in the petriplate.

Couch made his first discovery on February 23, 1948, while examining a piece of filter paper which had been in one of the dishes for several weeks. The organism consisted of branching filaments producing a mycelium. On solid and liquid media it grew as a mass of unicellular mycelium usually designated as a "colony." This is not a colony in a sense comparable to that of bacteria, since it is not an accumulation of many cells, but rather a mass of branching filaments which originate from a spore or from a fragment of mycelium (12, p. 29). The colonies were rough and leathery and different from those of bacteria and fungi. These characteristics thus showed

that this organism clearly belonged to actinomycetales. However, unlike the characteristic feature of this order (i.e., the absence of motile flagellated cells), it formed zoospores. Couch, therefore, included it within actinomycetales and assigned it to a new genus called Actinoplanes (3, 4). In 1955 Couch revised this genus and included it in a new family of Actinomycetales designated Actinoplanaceae (5). By 1963 Couch was able to recognize five distinct generic groups, of which Actinoplanes was the largest. These five genera were Actinoplanes, Ampullaria, Spirillospora, Streptosporangium and Amorphosporangium (6).

Actinoplanes includes species that are aerobic, gram-positive and acid fast. They usually do not form aerial mycelium, and are characterized by bacteria-like, flagellated, swimming spores formed in sporangia. On agar, the colonies are characterized by the production of pink to orange pigments. Actinoplanes philippinensis produces bright orange pigments on agar. The Zoospores possess several polar flagella (Figure 1).

Certain species related to this genus had been seen and described as fungi before the discovery of Actinoplanes (6). But Couch reported that their swimming spores were similar to those of most known fungi, while the zoospores of Actinoplanes were like those found in certain bacteria (3).

The first attempt to study the fine structures of the zoospores of Actinoplanes was made by Hubert Lechevalier and

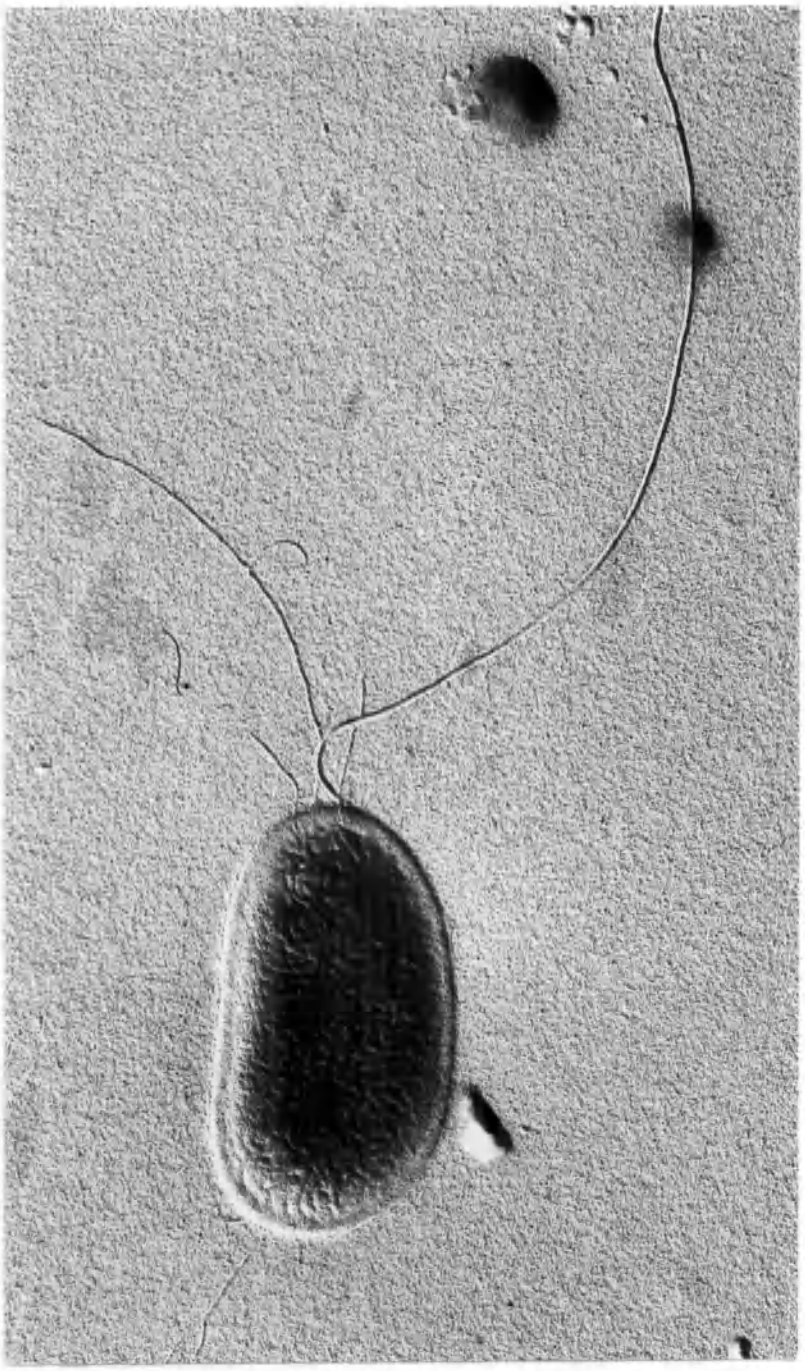

Fig. 1--Actinoplanes philippinensis: Electron micrograph of zoospore showing the polar insertion of the flagella. X 38,500. (Photograph taken by Tom Rogers, graduate student at North Texas State University)

Pauline Holbert in 1965 and 1966 (9, 10). An electron microscope was used to study the formation, structure, and cross section of the sporangia of Actinoplanes sp. strain P 128. Numerous flagella, formed by the helical winding of subfibrils were seen on the poles of sporangiospores.

Further research was conducted by Michael Higgins, who studied the release of sporangiospores by a strain of Actinoplanes sp. He concluded that the motility and swelling of the spore are the main causes of the dehiscence in that particular species (7).

Although sufficient number of species have been discovered to present a modified classification, investigations have been limited to the field of morphological studies, except for the release of sporangiospores and the electron microscopic observation of sporangial structure. Therefore, the purpose of this paper is to study some of the factors affecting the sporulation of one species, Actinoplanes philippinensis.

It is known that the great majority of actinomycetes are able to utilize a variety of organic compounds. Waksman pointed out that like other microorganisms, the growth of actinomycetes is also affected by kind of nutrient, pH, temperature, minerals, etc. Because these are many variables, this study was limited to the effect of six different sugars, viz. Arabinose, Dextrose, Fructose, Galactose, Lactose, and Sucrose and their varying concentrations on the zoospore production of Actinoplanes philippinensis.

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CHAPTER II

MATERIALS AND METHODS

Carbohydrates play a fundamental role in the life of animals and plants. They occupy a unique position among the essential elements required by living organisms. Protoplasm, cell wall, enzymes and nutrient stored within the cells are all carbohydrates. They are an important source of energy as well as a means by which chemical energy is stored.

Actinomycetes are able to utilize a great variety of carbohydrates, the most common example of which is sugar. The particular kind of sugar and its concentration has decisive effect on the growth of microorganisms. The proper nutritional media aids also in the production of spores. Based on this generalization, that the growth and sporulation of microorganisms are greatly influenced by the nature and the concentration of carbohydrates, an attempt has been made to study Actinoplanes philippinensis with respect to this influence.

Test Organism

Actinoplanes philippinensis, discovered by J. N. Couch (1950) was obtained from the American Type Culture Collection (number 12427). The culture is also stored in the culture collection of North Texas State University.

Preparation and Standardization of Inoculum

The inoculum preparation included grinding the colonies of the test organism in a sterile tissue grinder. The colonies were cultured in Czapek-Dox broth medium for this preparation process. A sterile pipette was used to add two c.c. of sterile distilled water to the mortar of the tissue grinder. Using a looped inoculating needle, a few colonies of the test organism were then transferred aseptically into the mortar. The tissue was gently ground by the pestal until a homogeneous suspension was obtained.

Matched calorimeter tubes, filled with five c.c. of distilled water, were then sterilized and cooled to be used in a photometer. A Rouy-photometer was set to read 100 per cent transmittance at a wavelength of 520 millimicrons, using a calorimeter tube containing sterile water as a blank. The ground inoculum was transferred from the tissue grinder to the calorimeter by sterile micropipette. Sufficient inoculum was added to read 90 per cent transmittance.

The content of this calorimeter tube was diluted in a rubber-stoppered serum bottle containing ten c.c. of sterile distilled water. Several petriplates containing Czapek-Dox agar were inoculated with 0.5 ml. of this diluted inoculum. After a forty-eight hour incubation period, the colonies of test organism were counted. The approximate number of colonies on each petriplate was twenty-five per 0.5 ml. of inoculum. This inoculum, standardized at a concentration of approximately fifty colonies per ml., was used throughout the

experiment.

Test Sugars

In order to study the comparative effect of sugars on the sporulation of Actinoplanes philippinensis, different concentrations of sugar were used. The sugars used in this experiment were arabinose, dextrose, fructose, galactose, lactose and sucrose. The five concentrations used for each sugar were 1 gm/l, 2 gm/l, 4 gm/l, 6 gm/l, and 8 gm/l.

Growth Medium

A liquid synthetic medium was selected for the growth of Actinoplanes philippinensis since zoospores are not produced on a solid media. The essential characteristic of any synthetic media is the availability of required chemicals in utilizable form. Table I shows the chemical composition of the medium used in this experiment.

TABLE I

CHEMICAL COMPOSITION OF THE MEDIUM

Chemical	Amount
Sodium Nitrate (crystal)	2 gm.
Potassium Phosphate (dibasic powder)	1 gm.
Magnesium Sulfate (crystal)	0.5 gm.
Ferric Sulfate (powder)	0.5 gm.
Sugar	variable*

*The concentration of the sugar was changed according to the amount required in the experiment.

In preparing this media it was essential to keep the chemical composition of the sugar unchanged. Media are

commonly sterilized by autoclaving. It should be noted, however, that the high temperature of autoclaving may cause alteration of some constituent in the media. Sugars are among the substances most easily altered by autoclaving. The extent of alteration depends upon the specific sugar used, the other constituents of the medium, and the time of autoclaving (1, p. 16). To avoid hydrolysis and caramelization of the sugar by autoclaving, the sugar was dissolved in 100 ml. of distilled sterile water and sterilized by use of a millipore filter (HA 0.45).

The remaining chemicals of the medium were dissolved in 900 ml. distilled water and autoclaved. On cooling, this autoclaved medium was added to the sterile sugar solution. 100 ml. of the above media was measured and placed in sterile cotton-plugged 250 ml. Erlenmeyer flasks. Into each flask 0.5 ml. of standardized inoculum was introduced. All the flasks were placed on a low speed shaker to allow a homogeneous growth of all the organisms.

Counting of the zoospores was begun after forty-eight hours of incubation at room temperature (approximately 25°C.). A Nikon microflex model EAM microscope, under phase contrast and with 40X objective, was used for the counting.

Zoospore Counting Technique

The zoospore of Actinoplanes philippinensis growing on the different sugar media were counted in the Petroff-Hausser and Helber counting chamber. This chamber consists of a special glass microscope slide with a flat rectangular

platform depressed exactly 0.02 mm. below the surface. The platform is marked by a grating of engraved lines giving 400 small squares each 1/20 X 1/20 mm. in separated blocks of sixteen squares.

A looped inoculating needle was used to transfer a drop of medium successively from each flask containing test organism to the counting chamber. The cover slip was then carefully placed without allowing air bubbles under it. The number of zoospores in any sixteen squares was counted. By using the following formula, the number of zoospores per c.c. was calculated.

Zoospores per ml. =

$$\frac{\text{Total zoospore count X 20 mm X 20 mm X 50 mm X 1000}}{\text{Number of squares counted}}$$

After each use, the counting chamber was thoroughly cleaned with disinfectant and alcohol.

Experimental Procedure

A first run of the six sugars for each of five arbitrary concentrations gave good separation between both sugars and concentrations. This stabilized the experimental design, particularly since the concentration range gave intrasugar gradients in zoospore counts. For each sugar and each concentration three aliquots were drawn from the flask for counting. The final number of observations for this run was 150.

The series was repeated two more times, pushing the replicated counts to 450, a number well within the range of

an adequate sample for tests such as Chi^2 and variance analysis should cases of confounded variation arise and variation segments need to be isolated.

As the pattern of variation emerged, the counts on one concentration (4 gm/l) were so different they were obviously of another population. A fourth run on the six sugars for this concentration was added as a confirmatory block of values. Although probably unnecessary in terms of additional information gained it was worth the effort if the need for a better estimate of deviation in the 4 gm/l population was needed.

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CHAPTER III

RESULTS

The average zoospore production of all observations, including three runs and triplicate counts, on the varying concentrations of all six sugars is given in Tables I through VII. The number of zoospores increased in each concentration of each sugar after every interval of counting is given in Tables VIII through XIII. To reiterate, zoospore counts were begun forty-eight hours after incubation and made at twenty-four hour intervals over a period of 144 hours. The zoospores are expressed in millions per milliliter.

The number of zoospore for given concentrations of arabinose is given in Figures 2 and 3. Note that in Figure 2 the maximum zoospore production is obtained in the 4 gm/l concentration. A periodic increase of zoospores is seen in every concentration except for the sudden drop of zoospore count in 2 gm/l after 120 hours incubation. Though the 4 gm/l concentration gave the maximum zoospore production, Figure 3 shows that the increment of zoospores during the third interval in 4 gm/l was nil. An unusual increment in 2 gm/l is also seen after 120 hours incubation which decreases considerably during the next interval.

In Figures 4 and 5 the effect of dextrose concentrations

TABLE II

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF ARABINOSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	9	23	28	26	13
72 hrs.	15	25	38	26	16
96 hrs.	28	26	48	36	32
120 hrs.	42	59	88	60	38
144 hrs.	71	55	157	87	63

*Zoospores expressed as millions/ml.

TABLE III

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF DEXTROSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	39	40	104	65	68
72 hrs.	51	45	190	83	78
96 hrs.	71	55	243	111	119
120 hrs.	79	69	320	123	129
144 hrs.	113	176	380	130	139

*Zoospores expressed as millions/ml.

TABLE IV

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF FRUCTOSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	21	26	70	51	27
72 hrs.	49	39	121	67	61
96 hrs.	91	73	159	90	78
120 hrs.	68	118	206	108	101
144 hrs.	107	158	285	146	137

*Zoospores expressed as millions/ml.

TABLE V

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF GALACTOSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	38	43	43	55	41
72 hrs.	50	81	114	88	78
96 hrs.	78	93	144	103	102
120 hrs.	79	119	176	182	116
144 hrs.	113	140	209	229	149

*Zoospores expressed as millions/ml.

TABLE VI

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF LACTOSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	8	16	24	12	9
72 hrs.	28	24	40	29	21
96 hrs.	40	34	66	64	49
120 hrs.	63	60	93	80	68
144 hrs.	89	83	142	115	99

*Zoospores expressed as millions/ml.

TABLE VII

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON GIVEN CONCENTRATIONS OF SUCROSE

Counting Intervals	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
48 hrs.	20	21	59	50	21
72 hrs.	23	37	104	68	68
96 hrs.	46	71	119	80	106
120 hrs.	55	108	183	110	120
144 hrs.	75	188	252	243	185

*Zoospores expressed as millions/ml.

TABLE VIII

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF ARABINOSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	9	23	28	26	13
48-72	6	2	10	0	3
72-96	13	1	10	10	16
96-120	14	33	40	24	6
120-144	29	-4	69	27	25

*Zoospores expressed as millions/ml.

TABLE IX

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF DEXTROSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	39	40	104	65	68
48-72	12	5	80	18	10
72-96	20	10	53	28	41
96-120	8	14	77	12	10
120-144	34	107	60	7	10

*Zoospores expressed as millions/ml.

TABLE X

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF FRUCTOSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	21	26	70	51	27
48-72	28	13	51	16	34
72-96	42	34	38	23	17
96-120	-23	45	47	18	23
120-144	39	40	79	38	36

*Zoospores expressed as millions/ml.

TABLE XI

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF GALACTOSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	38	43	43	55	41
48-72	12	38	71	33	37
72-96	28	12	30	15	24
96-120	1	26	32	79	14
120-144	34	21	33	41	33

*Zoospores expressed as millions/ml.

TABLE XII

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF LACTOSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	8	16	24	12	9
48-72	20	8	16	17	12
72-96	12	10	26	35	28
96-120	23	26	27	24	19
120-144	26	23	49	35	31

*Zoospores expressed as millions/ml.

TABLE XIII

INCREMENT OF ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS BETWEEN COUNTS ON GIVEN CONCENTRATIONS OF SUCROSE

Increment Between Hours	Sugar Concentration				
	1 gm/l	2 gm/l	4 gm/l	6 gm/l	8 gm/l
0-48	20	21	59	50	21
48-72	3	16	45	18	47
72-96	23	34	15	12	38
96-120	9	37	64	30	14
120-144	20	80	69	133	65

*Zoospores expressed as millions/ml.

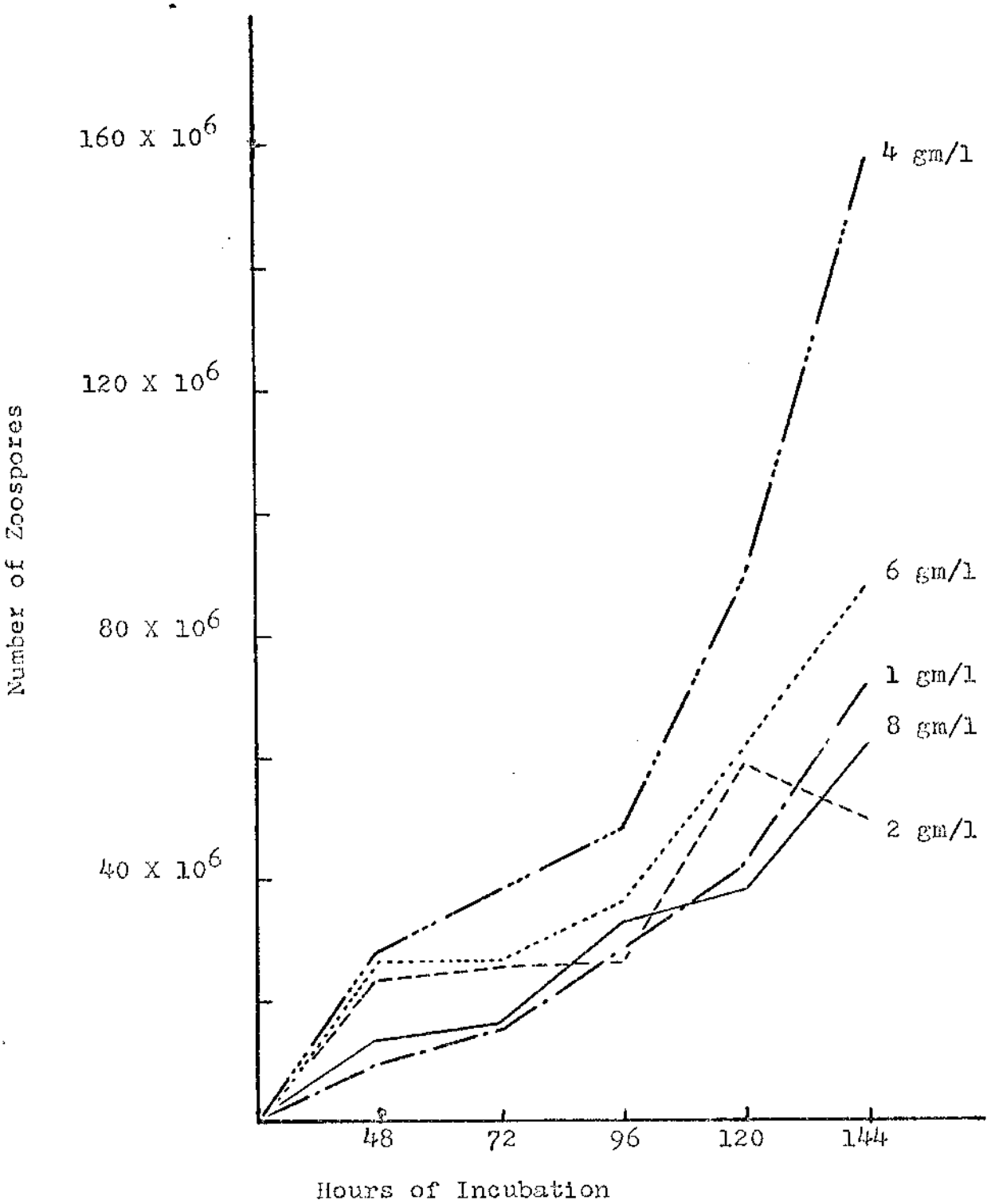

Fig. 2--Total zoospore production of Actinoplanes philippinensis on given concentrations of arabinose: optimum concentration 4 gm/l.

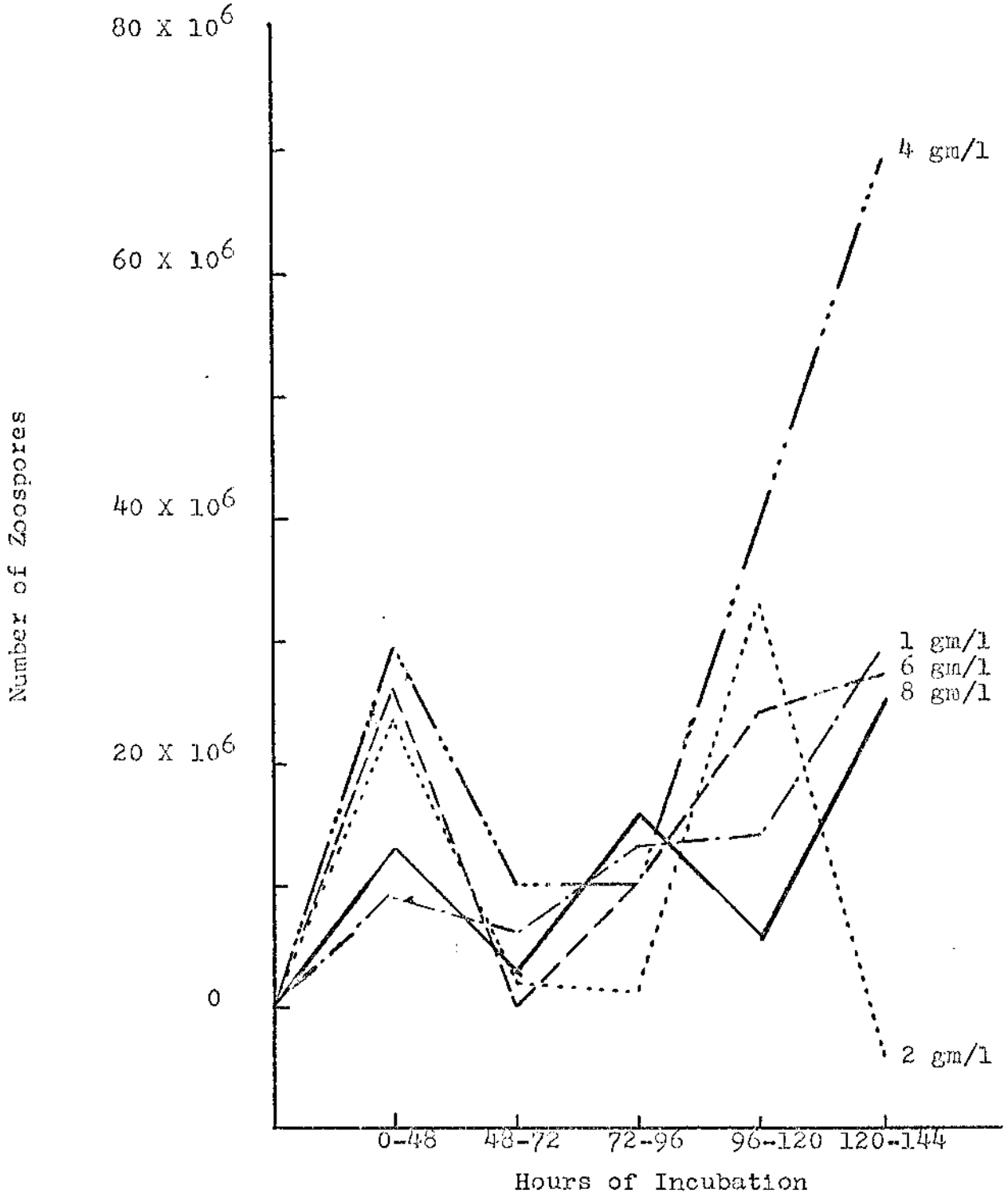

Fig. 3--Increment of zoospore production of Actinoplanes philippinensis on given concentrations of arabinose between different hours of incubation.

on zoospore production is seen. During each count the 4 gm/l concentration gave the best result. Note the approximately equal production of zoospores in concentrations of 6 gm/l and 8 gm/l. A similar relationship is evident between 1 gm/l and 2 gm/l except for the sudden increase in 2 gm/l after 120 hours incubation. Figure 5 shows an alternately increasing and decreasing pattern of zoospore production in 4 gm/l which was found to be the best concentration for the growth.

The zoospore production in fructose (Figure 6) yields only slight variation for concentrations of 1 gm/l, 2 gm/l, 6 gm/l and 8 gm/l. Here again the 4 gm/l concentration of sugar was found to be the most effective in the production of zoospores, with significantly greater yields after each interval. In Figure 7 a sudden drop in the number of zoospores occurs after ninety-six hours incubation in the 1 gm/l concentration of fructose. The greater increment for 4 gm/l takes place after forty-eight hours and 120 hours incubation.

Zoospore amounts for given concentrations of galactose are seen in Figures 8 and 9. After forty-eight hours, 120 hours, and 144 hours of incubation, the number of zoospores in 6 gm/l was higher than the other four concentrations. The 4 gm/l concentration showed higher zoospores production than the other concentrations after seventy-two hours and ninety-six hours of incubation. A periodic increase in the number of zoospores in each concentration of galactose is seen in Figure 8. The number of zoospores increased after each

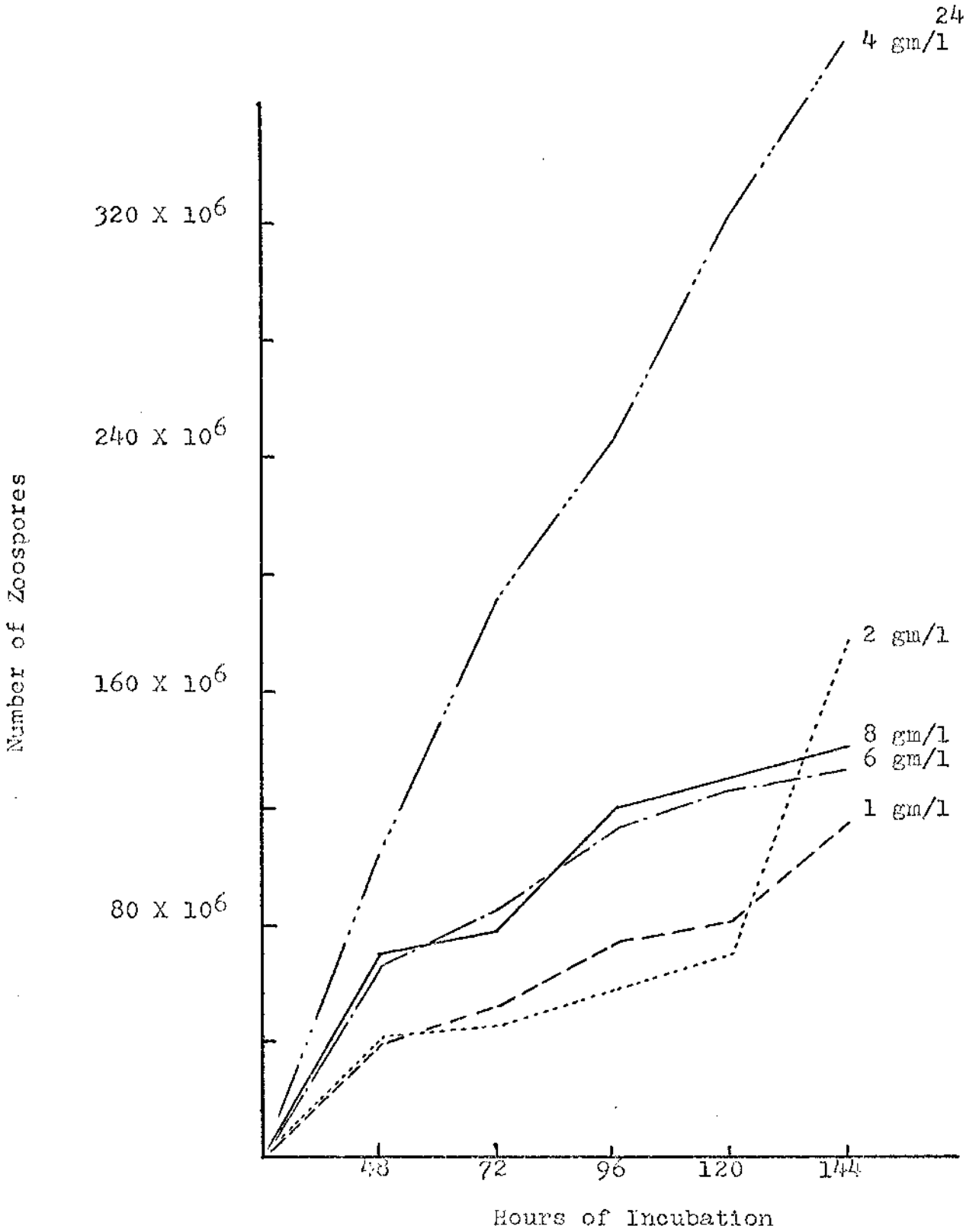

Fig. 4--Total zoospore production of Actinoplanes philipoinensis on given concentrations of dextrose: optimum concentration 4 gm/l.

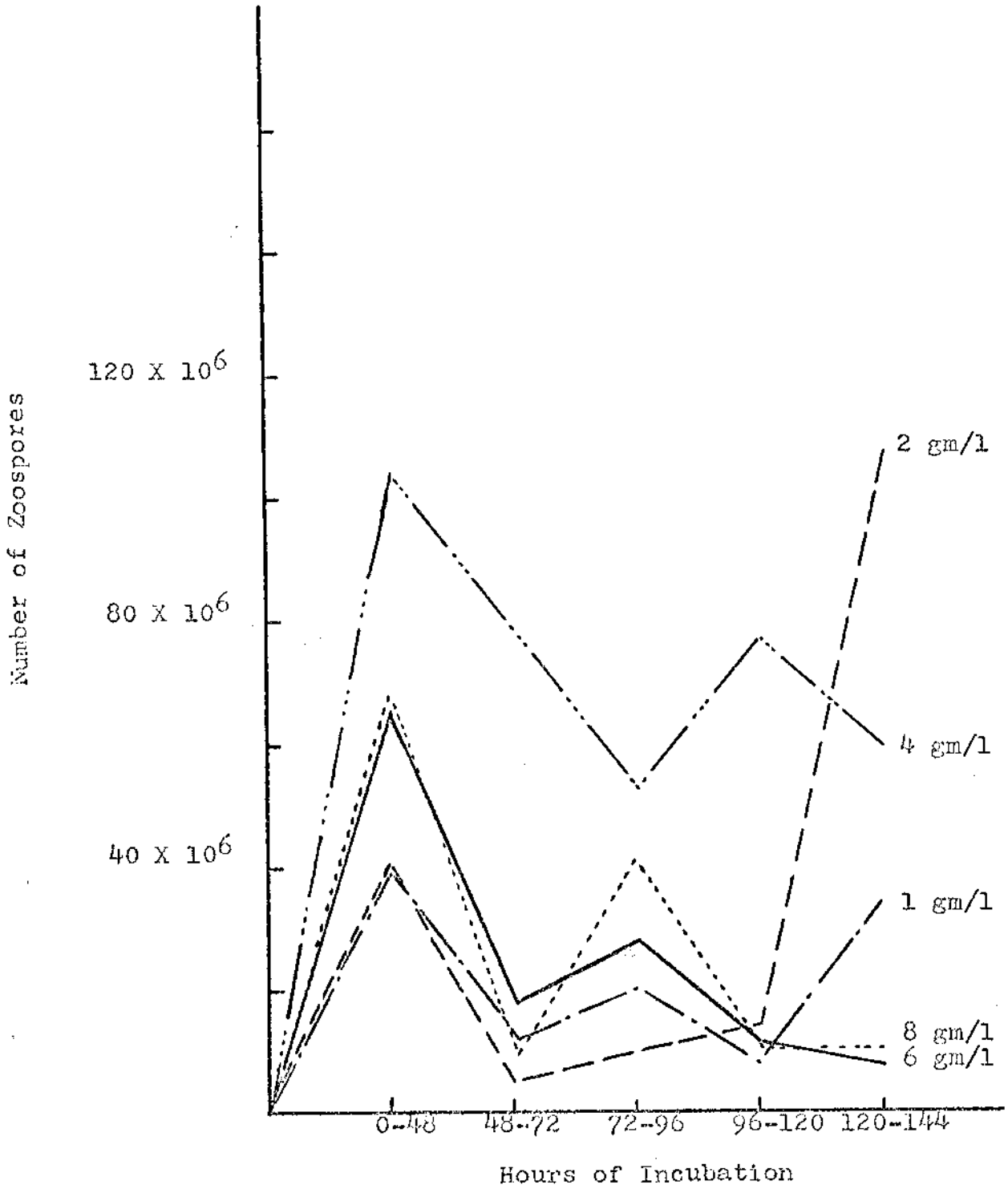

Fig. 5--Increment of zoospore production of Actinoplanes philippinensis on given concentrations of dextrose between different hours of incubation.

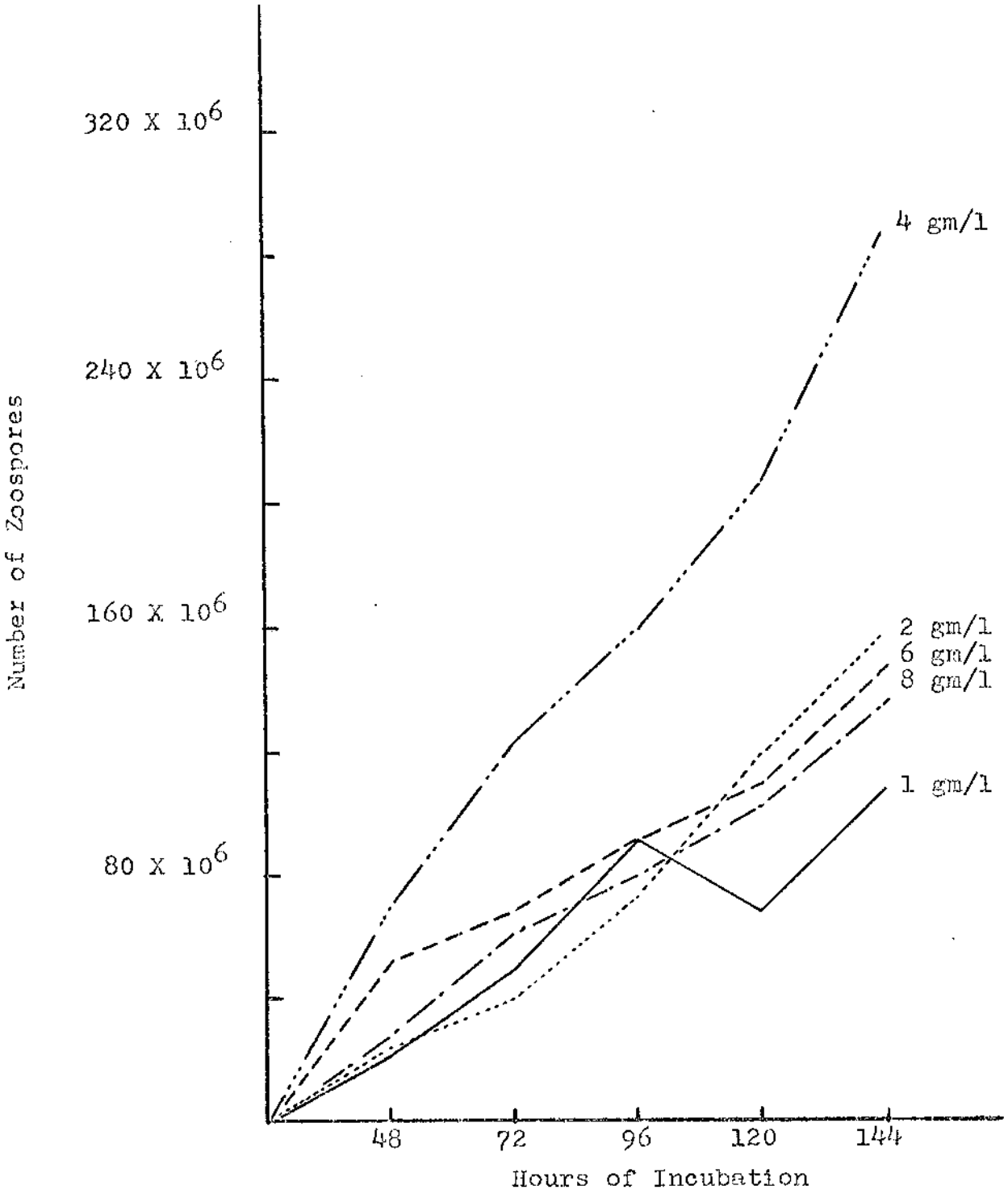

Fig. 6--Total zoospore production of Actinoplanes philippinensis on given concentrations of fructose: optimum concentration 4 gm/l.

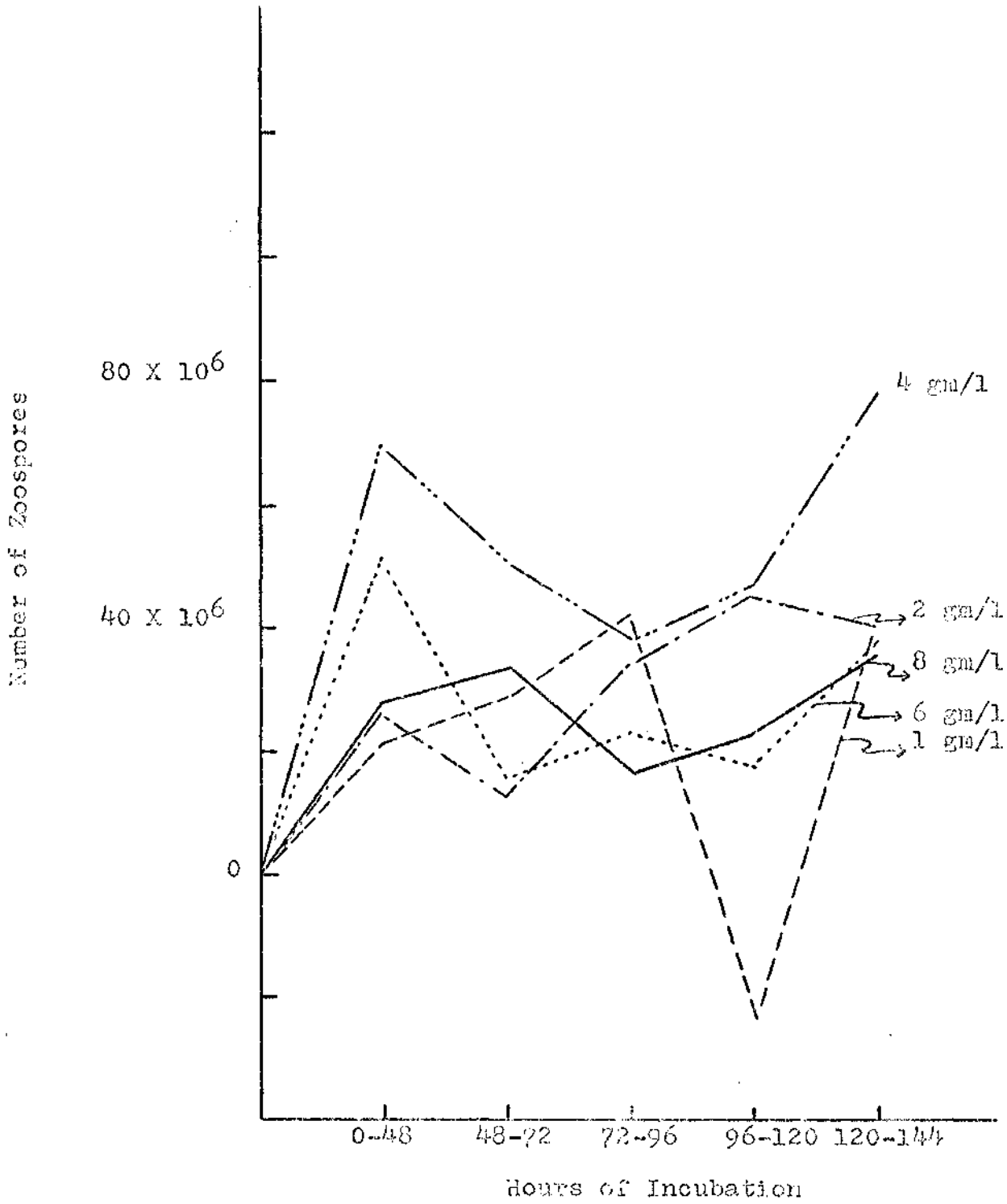

Fig. 7--Increment of zoospore production of Actinoplanes philippinensis on given concentrations of fructose between different hours of incubation.

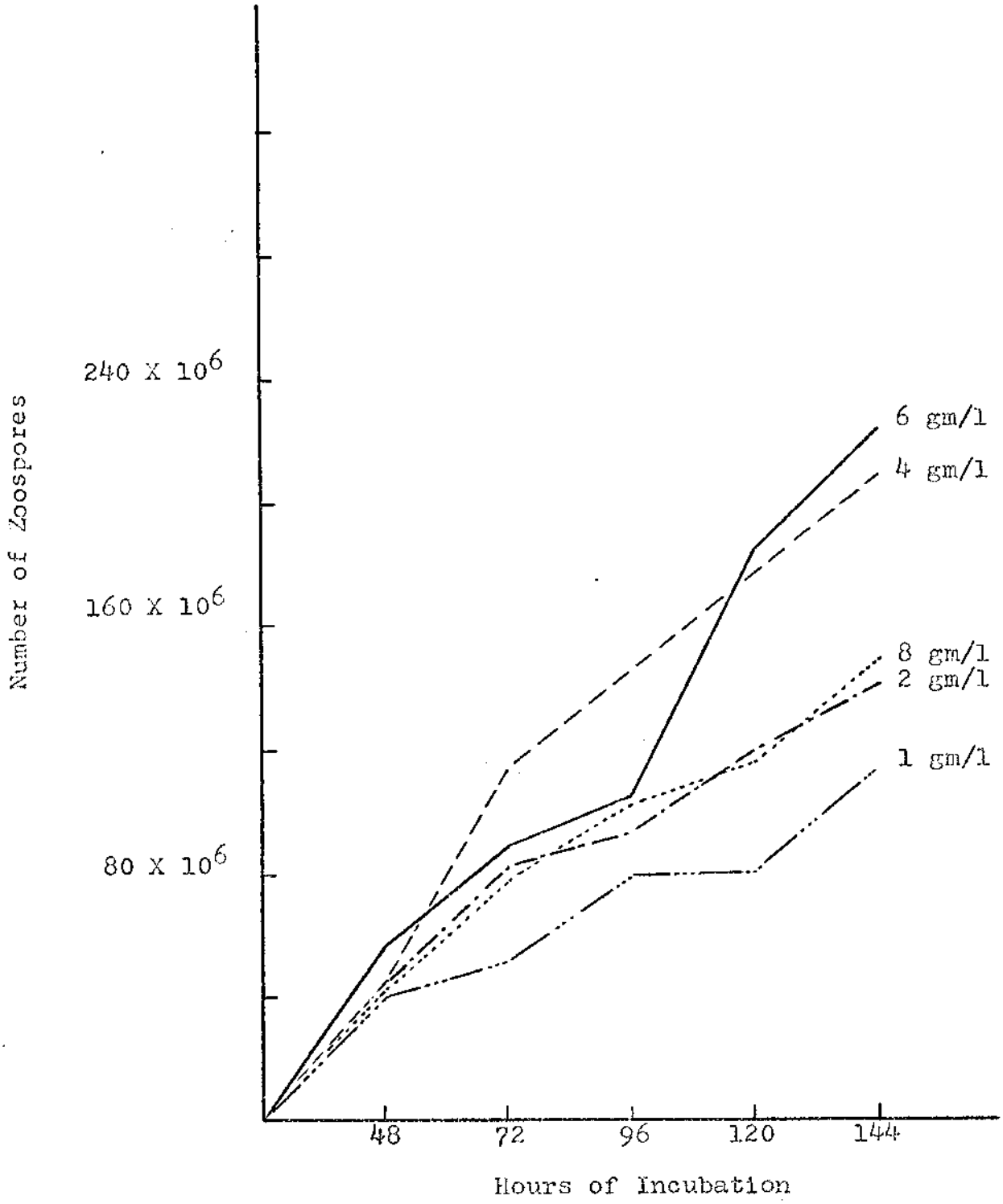

Fig. 8--Total zoospore production of Actinoplanes philippinensis on given concentrations of galactose; optimum concentration 4-6 gm/l.

incubation period (Figure 9) shows that during the first half of the incubation period, a concentration of 4 gm/l proved most effective. The increment in the number of zoospores in 6 gm/l is similar to that of 2 gm/l and 8 gm/l. But the second half of the incubation period showed a remarkable increment of zoospores in the 6 gm/l concentration, while the increment in 4 gm/l was almost negligible.

The average zoospore production in lactose is given in Figures 10 and 11. The difference in zoospore production as seen in the various concentrations was less prominent than in any other sugar. After seventy-two hours incubation, both 4 gm/l and 6 gm/l concentrations showed a greater increase in the number of zoospores produced than the other concentrations. Though 4 gm/l was found to be the best, as is seen in Figure 10, Figure 11 shows that up to 120 hours incubation, zoospore increase after each incubation period in 4 gm/l concentration was less than that of the other concentrations. Note the sudden increment of zoospores in 4 gm/l during the last interval.

The effect of sucrose concentrations on zoospore production is seen in Figures 12 and 13. Here again the best concentration for maximum zoospore production was found to be 4 gm/l. A periodic increase in zoospores is seen in each concentration (Figure 12). Very few zoospores were produced in the 4 gm/l concentration between seventy-two hours and ninety-six hours of incubation; most of the increase was between forty-eight to seventy-two and ninety-six

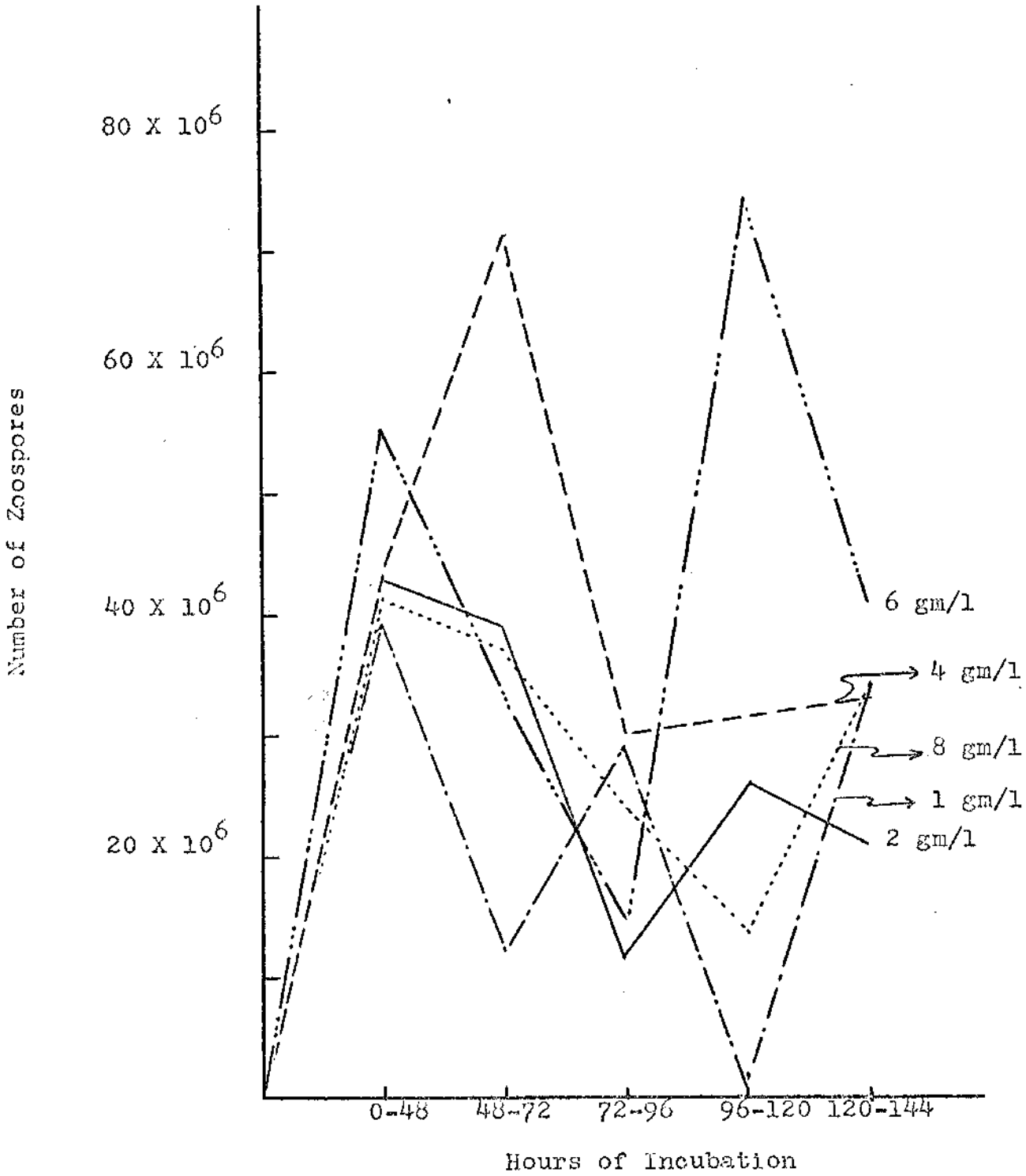

Fig. 9--Increment of zoospore production of Actinoplanes philippinensis on given concentrations of galactose between different hours of incubation.

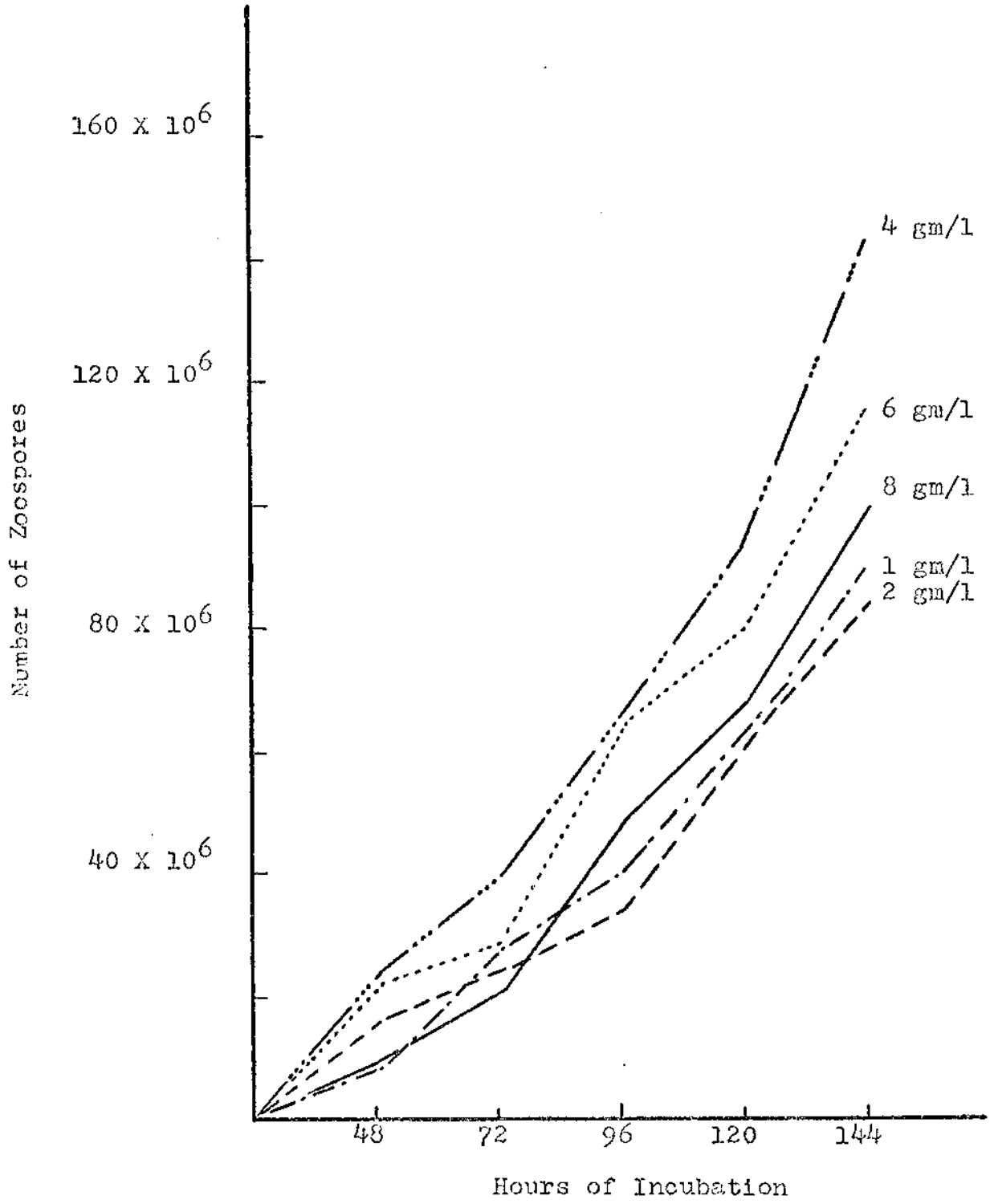

Fig. 10--Total zoospore production of Actinoplanes philippinensis on given concentrations of lactose: optimum concentration 4 gm/l.

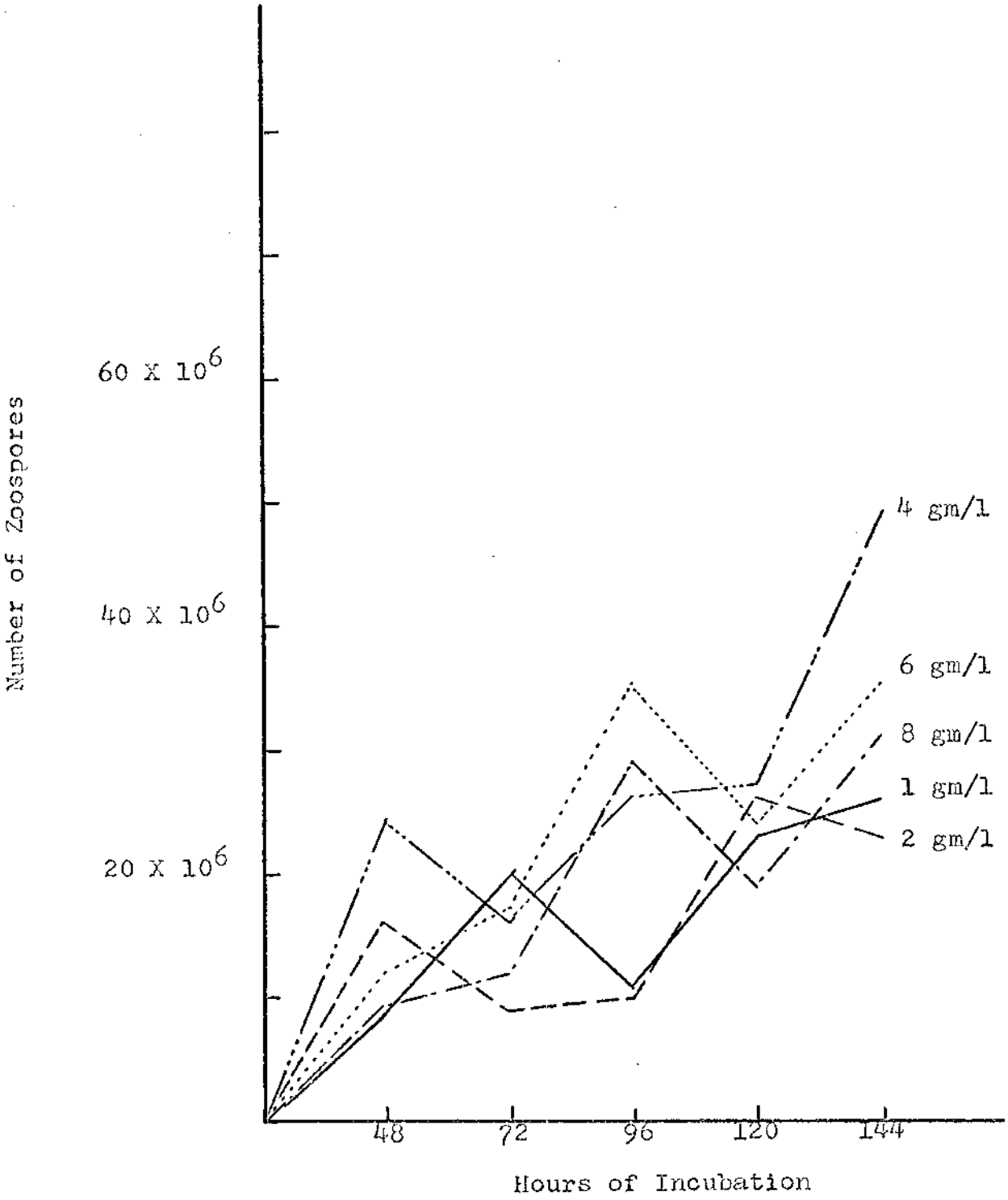

Fig. 11--Increment of Zoospore production of Actinoplanes philippinensis on given concentrations of lactose between different hours of incubation.

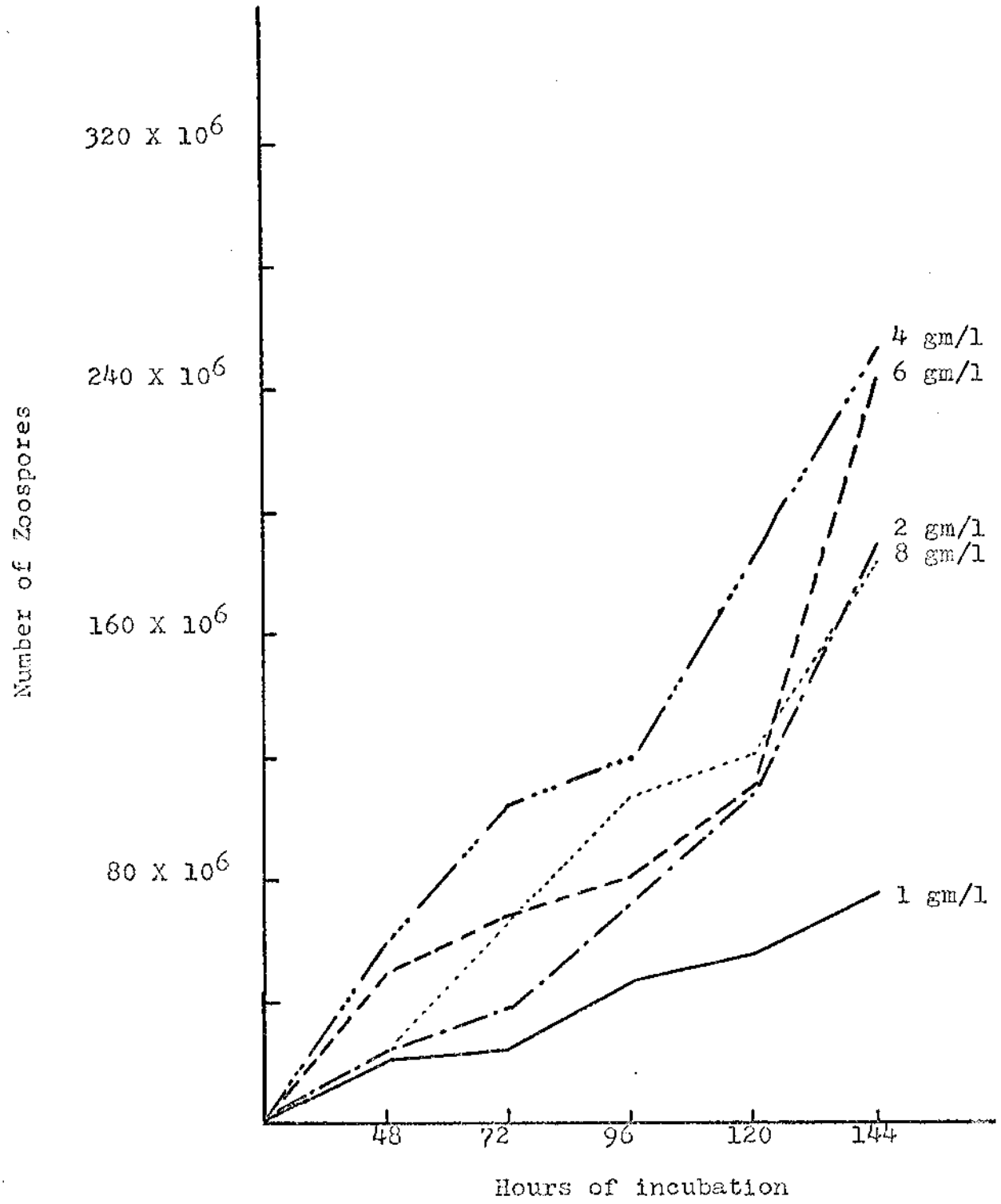

Fig. 12--Total zoospore production of Actinoplanes philippinensis on given concentration of sucrose: optimum concentration 4 gm/l.

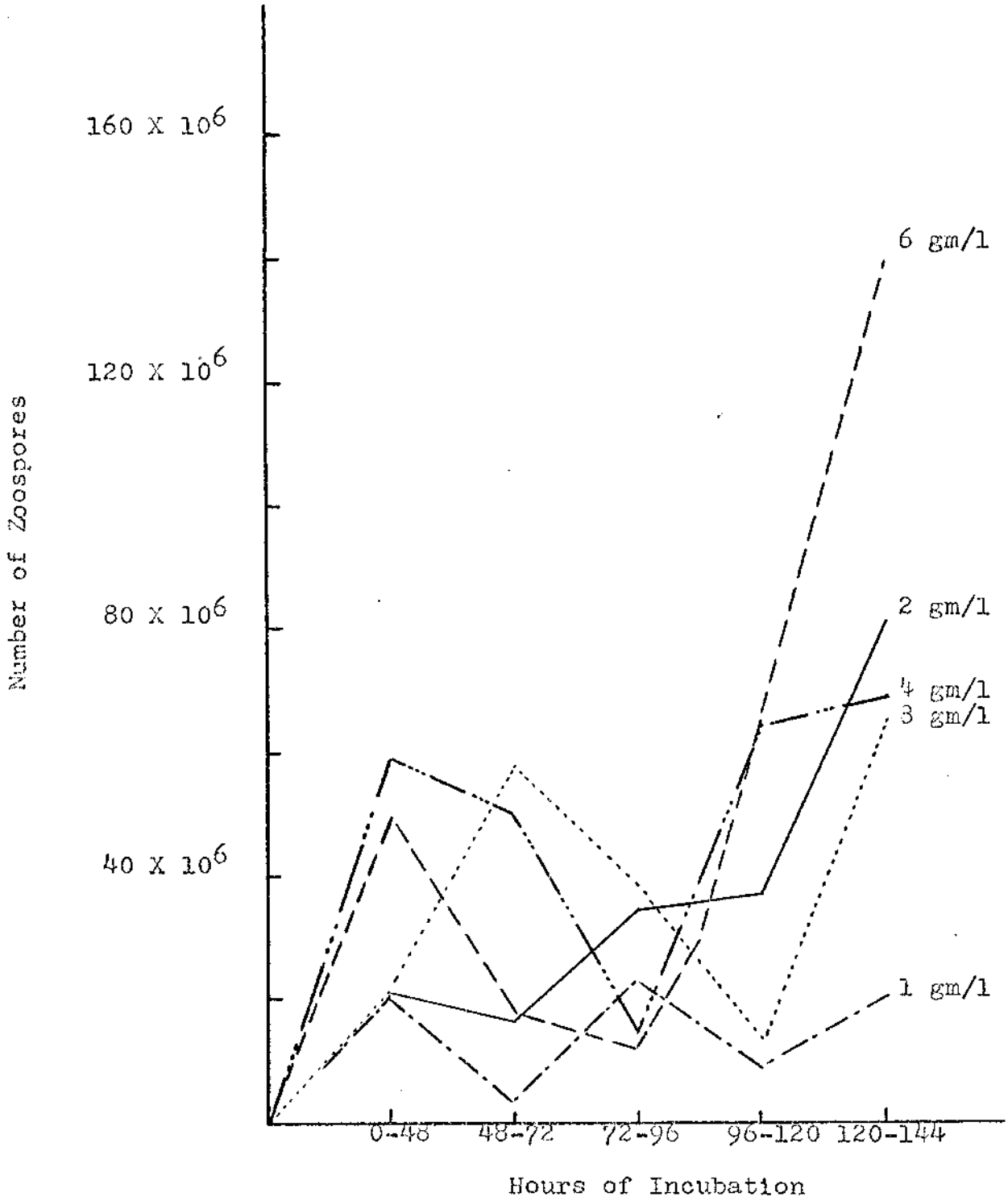

Fig. 13--Increment of zoospore production of Actinoplanes philippinensis on given concentrations of sucrose between different hours of incubation.

to 120 hours incubation. Greater numbers of zoospores were produced in all the other concentrations between 120 hours and 144 hours incubation.

Tables XIV and XV present the comparative data of 4 gm/l concentrations of each sugar. Figure 14 shows that dextrose yields the greatest production of zoospores; while arabinose and lactose gave the smallest production, yielding approximately equal numbers of zoospores for each count. Figure 15 shows that in dextrose media, most of the zoospores were formed in the periods of forty-eight to seventy-two hours and 120 to 144 hours. Dextrose and fructose followed the same incremental pattern. In galactose, very few zoospores were produced during the last incubation period.

TABLE XIV

TOTAL ZOOSPORE PRODUCTION* OF ACTINOPLANES PHILIPPINENSIS ON A GIVEN CONCENTRATION OF SIX SUGARS

Counting Interval	Sugar Concentration 4 gm/l					
	Arabinose	Dextrose	Fructose	Galactose	Lactose	Sucrose
48 hrs.	16	97	73	40	15	53
72 hrs.	38	162	120	88	30	89
96 hrs.	53	220	153	148	55	106
120 hrs.	80	265	165	223	83	138
144 hrs.	108	376	207	243	123	170

*Zoospores expressed as millions/ml.

TABLE XV

INCREMENT OF ZOOSPORE PRODUCTION* BETWEEN COUNTS ON A GIVEN CONCENTRATION OF SIX SUGARS

Counting Interval	Sugar Concentration 4 gm/l					
	Arabinose	Dextrose	Fructose	Galactose	Lactose	Sucrose
48 hrs.	16	97	73	40	15	53
72 hrs.	22	65	47	48	15	36
96 hrs.	15	58	33	60	25	14
120 hrs.	27	45	12	75	28	32
144 hrs.	28	111	42	20	40	32

*Zoospores expressed as millions/ml.

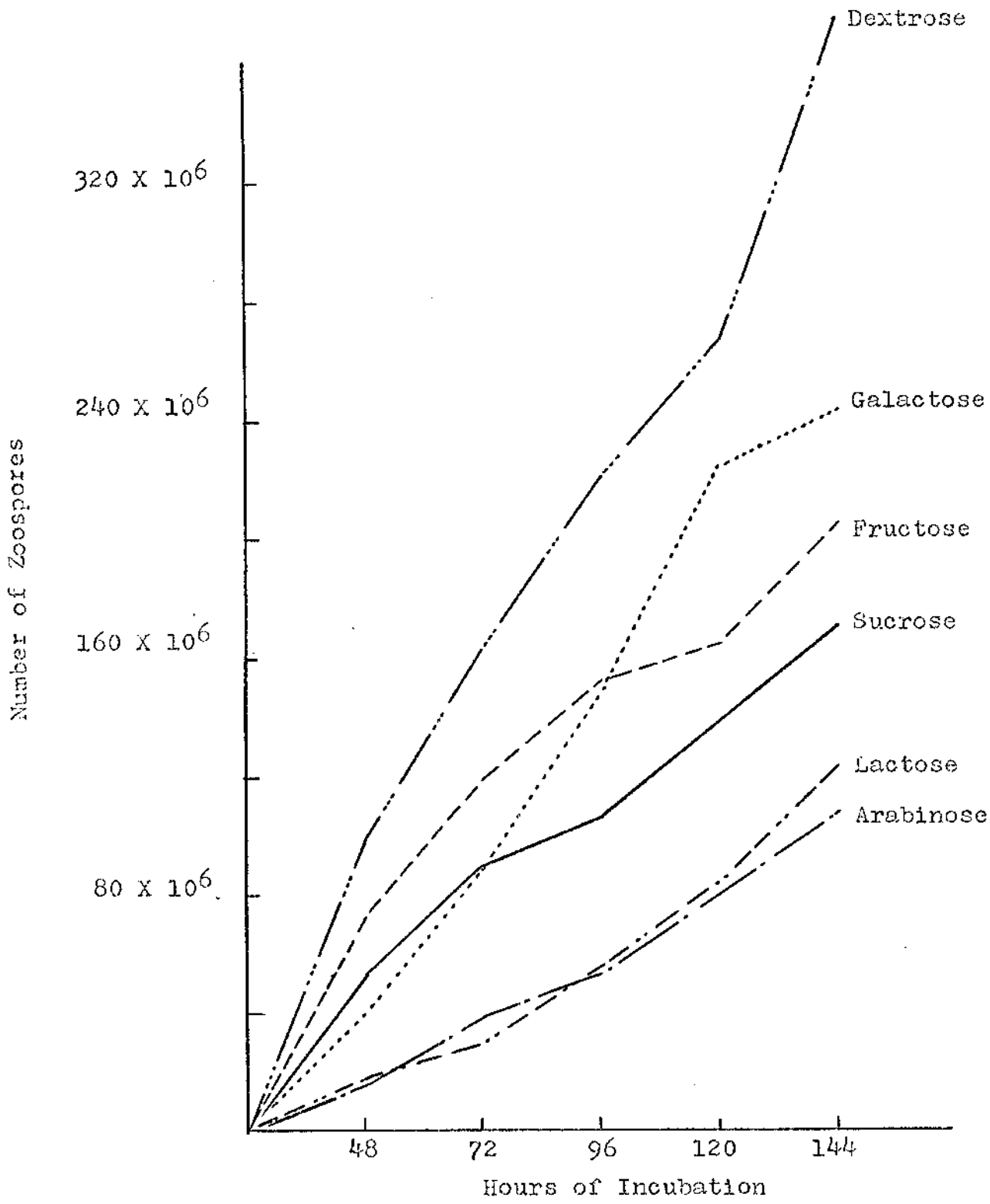

Fig.14--Total zoospore production of Actinoplanes philippinensis on 4 gm/l concentration of six different sugars: optimum sugar dextrose.

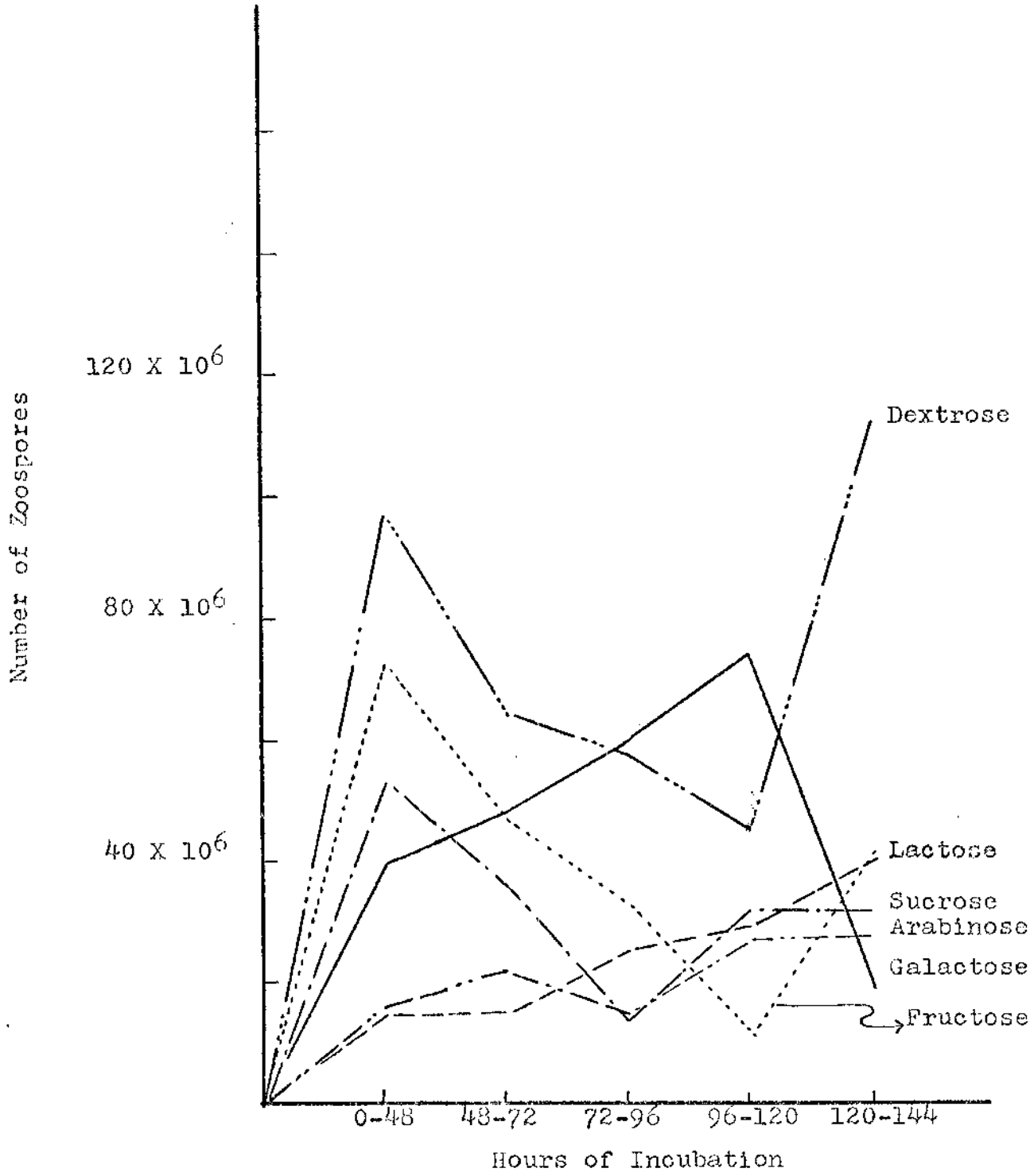

Fig. 15--Increment of Zoospore production of Actinoplanes philippinensis on 4 gm/l concentration of six sugars between different hours of incubation.

CHAPTER IV

DISCUSSION

In this study of the zoosporulation of Actinoplanes philippinensis in a synthetic medium, where the carbon sources and their concentrations were changed, it is evident from the data obtained (Tables II to XV) that some production occurred in all concentrations of each of the six sugars, viz. arabinose, dextrose, galactose, fructose, lactose and sucrose. It is also clear from Figures 2, 4, 6, 8, 10, and 12 that the change in concentration of sugars had a direct effect on zoospore concentration. In each experiment the best concentration of sugar was 4 gm/l except for galactose where the 6 gm/l concentration as well as the 4 gm/l resulted in more vigorous growth.

The number of zoospores produced by the test organism in 1 gm/l and 2 gm/l sugar concentrations was found to be less than the number of zoospores produced in 4 gm/l sugar concentration. The obvious explanation is that the former provided an inadequate supply of carbon. The available carbon compounds are utilized by the cells first for respiration and second for growth. The remaining carbon compounds are used for reproduction. By comparing the number of zoospores, it appears that sugar concentrations lower than 4 gm/l were

probably insufficient to meet little more than the respiratory and growth requirements.

The zoospore production in 6 gm/l and 8 gm/l sugar concentrations was also found to be less than the zoospore production in the 4 gm/l concentration. The excess carbon in the medium seems to suppress the rate of zoosporulation. Similar results were found in experiments conducted with bacteria. Szulmajster and Hanson (7), in studying the physiological control of sporulation of Bacillus subtilis, discovered that in the presence of excess glutamate and glucose (9 gm/l) sporulation was inhibited by about sixty to eighty per cent as compared with a control culture (approximately 1 gm/l).

In Figure 14 it is evident that glucose (dextrose) gave the best zoospore production. Glucose, in a free or combined form, is not only the most common of the sugars but also is probably the most abundant organic compound (4, p. 89). It is readily absorbed by the cells since cell membranes do not present any barrier to the influx of glucose molecules. Once it enters the cell it participates in the glycolysis cycle. Higgins (1, p. 94) studied the release of sporangiospores in a strain of Actinoplanes and concluded that sporulation was greatly enhanced by the addition of glucose.

Pridham and Gottlieb (5) in their study of carbon utilization for the species characterization of actinomycetes

found that all species were able to use d-glucose, d-mannose, starch, dextrin, etc. Certain compounds, such as fructose, sucrose, etc., were utilized by some organisms and not by others. Arabinose was consumed in a very small quantity.

Waksman (8) emphasized the utilization of sugars by Streptomyces griseus. He discovered that pentoses were found to be poor carbon sources; glucose and mannose were best and maltose was the best of the dissacharides.

Fructose was also metabolized quite vigorously and resulted in a significant zoospore production. Like glucose, fructose also plays an important role in the glycolysis cycle. It is also distributed widely in nature and readily used by microorganisms.

The zoosporulation of the test organism in a medium containing galactose (Figure 14) was slightly lower than in glucose. Galactose, like glucose, occurs free in nature and is readily utilized by any organisms. The growth and zoosporulation of the test organism was very poor in the substrate containing arabinose. McClung (2) conducted experiments to study the utilization of carbon compounds by Nocardia species. Glucose and galactose were found also to be excellent for the growth while arabinose showed very slow growth.

Both lactose and arabinose produced a minimum number of zoospores compared to the other sugars used in the experiment. This is probably of little importance since lactose is not readily available in soil because it is almost lacking in the

plant kingdom (4, p. 446). According to McClung (2), species of Nocardia were able to use carbon compounds having an alpha glucoside linkage more readily than those having a beta linkage. Since lactose possesses a beta linkage, it is not utilized readily by Actinoplanes either.

Since the pH of the media was not recorded periodically and since no biochemical tests such as chromatography were carried out, it is impossible to determine the mechanism that triggers zoospore production. Nakata and Halvorson (3), studying the biochemical changes occurring during growth and sporulation of Bacillus cereus, discovered that in glucose-containing media the pH readily decreased during growth; after some time the pH increased to a level higher than the initial pH of the media. They concluded that glucose was dissimilated to some organic acid intermediate during growth that was utilized when sporulation started. In addition, Srinivasan (6) stated that sporulation in Bacillus cereus is triggered by a substance which does not belong to the class of macromolecules, such as proteins or nucleic acids. The activity of this "trigger" is generally manifested only under environmental conditions that are unsuitable for vegetative multiplication. Such a conclusion about Actinoplanes can only be made when the pH is regularly recorded and a qualitative analysis of the media is made from time to time in order to study the production of organic acids.

In the present experiments conducted with Actinoplanes philippinensis, the general graphical pattern of zoospore

production, as seen in Figures 2, 4, 6, 8, 10, and 12, was sigmoid, except for Figures 2 and 4 in which an unusual decrease occurred. This decrease is seen not in all concentrations of sugar but only in one concentration, 2 gm/l in the case of arabinose and 1 gm/l in the case of fructose. There was probably no change in the environment or the graphical drop would have shown up on the other sugars. The only reasonable explanation must be that some error occurred in the counting. The counts were made up of motile zoospores. It is a possibility that during the time of counting some zoospores, though yet alive, were not moving and thus not included in the counts.

The increment graphs in Figures 3, 5, 7, 9, 11, and 13 showed that for different sugars and different concentrations the time of incubation plays an important role. In Figure 5 the increment of zoospores in 2 gm/l concentration of dextrose up to 120 hours of incubation is minimum compared to the zoospore production of the other concentrations. But after 120 hours of incubation the zoospore production in 2 gm/l exceeded all the concentrations. Similarly in Figure 15 the increment of zoospores in dextrose and fructose decreased between seventy-two to 120 hours while the increment of galactose increased considerably. Therefore, it can be concluded that probably a more accurate determination of sugar concentration could be made if the increment patterns were studied for a period of time longer than 144 hours.

Also it has been shown in many instances that a slight change in such environmental factors as temperature, pH, nutrition, etc., can also affect the growth and reproduction of microorganisms. For these reasons the following suggestions might improve the accuracy of counts and provide more insight into the mechanisms of zoosporulation in longer studies. First, extend the incubation period since the zoospore production of Actinoplanes philippinensis lags in some concentrations. Second, record the pH of the media to study the biochemical changes of the media. Third, conduct the experiments under a constant temperature to avoid the effect of temperature changes on the zoosporulation. Fourth, carry out such biochemical analysis as chromatography sequentially to determine the production of intermediate metabolic products. And finally, conduct additional experiments with concentrations varying in tenths of gm/l between four and eight gm/l to determine more precisely the optimum concentration of sugar.

For the length of time this experiment was conducted, the following three conclusions can be made concerning the zoosporulation of Actinoplanes philippinensis: (1) growth and reproduction occurred in all six sugars tested; (2) in each case the 4 gm/l concentration was considered the optimum; and (3) glucose-containing media gave maximum zoospore production.

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CHAPTER V

SUMMARY

The effect of varying concentrations of six sugars (arabinose, dextrose, fructose, galactose, lactose and sucrose) on the zoospore production of Actinoplanes philippinensis was investigated in an effort to discover the most utilizable sugar and its concentration for the maximum zoospore production.

Actinoplanes, a most interesting group of actinomycetes, was discovered by Couch (1950) from a soil sample. It is characterized by the presence of substrate mycelium, aerial mycelium usually lacking. The organisms reproduce by means of spores which are motile and possess several polar flagella.

The method incorporated in this investigation consisted of three major steps: standardization of inoculum, preparation of growth media and the estimation of zoospores produced during a given period of incubation.

The inoculum was standardized at a concentration of approximately fifty colonies per milliliter, using a Rouy-Photometer at a wavelength of 520 millimicrons. The preparation of growth medium included the sterilization of the chemicals other than the carbon sources, and millipore filtration of the sugars. The sugars were sterilized separately in millipore filters to avoid their hydrolysis and caramelization

due to autoclaving. Six sugars in five arbitrary concentrations (1, 2, 4, 6, and 8 grams per liter) were used in the test medium. Equal amounts of medium in flasks were inoculated by standardized inoculum and placed on a low speed shaker to provide a homogeneous growth.

The number of zoospores was counted on a Petroff-Hausser and Helber counting chamber, starting after forty-eight hours and continuing at intervals of twenty-four hours. The experiment was repeated three times, each run of which gave excellent separation between both sugars and their concentrations. The average zoospore production and the increment of zoospores were given in tables as well as plotted graphically.

It was evident from the data obtained that some production of zoospores occurred in all concentrations of each of the six sugars. It was reported previously that a great majority of actinomycetes are able to utilize a variety of complex organic compounds. The amount of sugar in the medium shows its effect on the number of zoospores. The optimum concentration for the maximum zoospore production was 4 gm/l in all of the sugars except for galactose where it was both 4 and 6 gm/l.

Less than the optimum concentration of the sugars was not sufficient to fulfill the carbon requirement of the organism; therefore, zoospore production was not maximum. On the other hand, more than the optimum concentration seemed

to inhibit the sporulation.

The most utilizable sugar was found to be glucose and it resulted in the maximum zoospore production. The availability of glucose and its significant role in the glycolysis cycle explains the reason for its being most effective.

After glucose, the next three most effective sugars, in decreasing order of effectiveness, were galactose, fructose and sucrose. Arabinose and lactose produced minimum zoospores compared to other sugars.

The results and conclusions of this investigation if extended may be made more meaningful if the following factors are considered during the experiment. First, maintain a constant temperature throughout the experiment; secondly, record the pH of the media each time the counts are made; and thirdly, conduct biochemical analysis of the media to study physiological changes and their effect on sporulation.

From the observations made during the investigation it was concluded that Actinoplanes philippinensis can readily utilize all the given sugars and produces zoospores. In each case the optimum concentration was 4 gm/l with the exception of galactose where it was both 4 gm/l and 6 gm/l. Glucose gave maximum zoospore production while arabinose and lactose produced minimum zoospores.

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