A STUDY ON THE BIOLOGICAL ACTIVITY OF TWO THERMOPHILIC ACTINOMYCETES WITH SPECIAL REFERENCE TO PROTEOLYTIC ACTIVITY

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A STUDY ON THE BIOLOGICAL ACTIVITY OF TWO THERMOPHILIC ACTINOMYCETES WITH SPECIAL REFERENCE TO PROTEOLYTIC ACTIVITY

By

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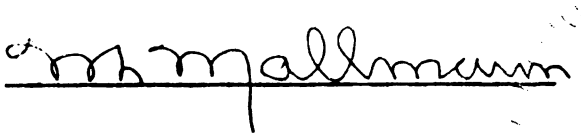
Approved by: 

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AN ABSTRACT

Two thermophilic actinomycetes were isolated at 45 to 50 C from an active vegetative compost. One species produces spores in chain-like fashion at the terminal ends of hyphae and is classified as a thermophilic Streptomyces species. The other species produces single spores at the tips of extremely short sporophores that are side branches of the hyphae. This type of spore formation places this species either in the genus Micromonospora (Bergey) or in the proposed genus Thermoactinomyces (Waksman and Corke). The latter genus is more valid for the thermophilic forms of this type of actinomycete. Both of the organisms are gram positive and non-acid fast.

Temperature studies showed that neither of the species could develope growth at temperatures below 35 C or above 59 C and the optimum temperature is 50 to 52 C. The organisms develope optimum growth between pH 7.0 to 8.5 and very little, if any, growth occurs below pH 6.0 or above pH 9.0.

Growth characteristics were observed on 21 different media. These thermophilic actinomycetes are aerobic since they grow only at the surface of stationary liquid and stab cultures. On media supporting good growth, the Thermoactinomyces sp. produces a very light cream colored vegetative mycelium and a white to light grey aerial mycelium that appears

dry and fragile. The Streptomyces sp. also has a cream colored vegetative mycelium with white aerial mycelium which becomes grey with the production of spores. No pigmentation is produced on any of the media by either of the organisms. Both of the species liquefy gelatin and cause proteolysis of milk after the formation of a curd. Cellulolytic activity is negative.

When inoculated into solutions of maltose, mannitol, dextrin, sorbose, cellobiose, fructose, rhamnose, lactose, sucrose, and glucose, the Streptomyces species ferments all but rhamnose, sorbose, and lactose whereas the Thermoactinomyces species utilizes only glucose, fructose, and sucrose. Of the following organic acids; malic, fumaric, succinic, pyruvic, citric, lactic, oxalic, and tartaric, only pyruvic acid is utilized and then only slowly by Streptomyces sp.

Proteolytic activity was studied using casein, gelatin, and phytone in a basal salts solution. Each organism was inoculated into each type of protein medium contained in a series of flasks and growth was developed in shake culture at 50 C. Samples were removed at various times during a 72 hour incubation period and from each sample, mycelial weights and ammonia were determined. By means of paper chromatography, free amino acids were determined in growth samples of casein and gelatin. Mycelial weights, when plotted against incubation time, produce curves very similar to bacterial growth curves

but the area of the curve that corresponds to decelerated growth in bacteria is actually an autolysis in the actinomycetes. Ammonia was produced from the proteins in amounts considerably in excess of the physiological requirements of the organisms. Graphical interpretation of the data produced sigmoid-type curves which show ammonia production continues even during the period of autolysis. The ammonia is apparently released by deamination during proteolysis.

A total of seventeen amino acids and two amines were identified in the culture filtrates. The number of amino acids occurred in groups of ten or less for each incubation increment of the total 72 hours. Larger numbers of amino acids were found in cultured casein medium than in the cultured gelatin medium. The identified compounds were: cystine, cysteine, lysine, asparagine, histidine, arginine, glutamine, glutamic acid, aspartic acid, serine, hydroxyproline, threonine, alanine, proline, tryptophane, methionine, valine, phenylalanine, and isoleucine. The number of amino acids found, the amount of growth produced, and the amount of ammonia produced from proteins indicate that these thermophilic actinomycetes are strongly proteolytic and possess a very active deaminase system.

REFERENCES

Breed, R.S., Murray, E.G.D., and Hitchens, A. Parker 1948 Bergey's manual of determinative bacteriology. Sixth Edition The William and Wilkins Co., Baltimore, Md.

Waksman, S.A., and Corke, C.T. 1953 Thermoactinomyces Tsiklinsky, a genus of thermophilic actinomycetes. J. Bact. 66, 377.

BIOGRAPHY

Lawrence L. Reed was born November 4, 1921, in Grand Rapids, Michigan. After completion of high school, he was a member of the United States Navy for nearly four years during World War II. He enrolled in college in 1946 and in 1950 received a Bachelor of Arts degree from Wayne University, Detroit, Michigan. In 1951, he was granted a Master of Science degree from Michigan State College and since that time he has been engaged in studies leading to the degree of Doctor of Philosophy. During the period of advanced studies he has been employed as a research assistant.

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The author expresses his sincere appreciation to Professor W.L. Mallmann for counsel and guidance during the years of graduate study which has culminated in the successful completion of this thesis.

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In moderate climates, the majority of forms of life have optimum growth temperatures between 15 and 37 C but there are some forms which are capable of development at temperatures below and above these limits. Those forms which have optimum growth temperatures above 45 C are usually termed thermophilic organisms. Among these latter forms of organisms is a group of microorganisms which have received little consideration. These are the thermophilic actinomycetes. Most of the available information concerning this group was published between 1888 and 1912 and is mainly concerned with the occurrence of these organisms in natural substrates and with their possible action in self-heating plant residues. This study was undertaken with the hope that more knowledge might be acquired in regard to the biological activity of the thermophilic actinomycetes.

The first reported isolation of thermophilic actinomycetes was made by Globig in 1888. He isolated these organisms from soil by developing the culture on potato slices held at 52 to 65 C. He extended his investigations to the examination of animal feces, canal water, tap water, and dust of the floors. Early reports about these organisms described them as thread-like "bacteria". Thus, it is probable that investigators before this time had observed these microorganisms and considered them to be bacteria. Globig's publication created enough interest to promote further investigations.

Rabinowitsch (1895) found thermophilic actinomycetes in the feces of the horse, cow, dog, guinea pig, mouse, and fish. His method of isolation consisted of suspending the fecal material in water for 18 to 24 hours at 62 C and then inoculating agar plates with the suspension. The agar plates, incubated at elevated temperatures, showed colonies in 16 to 24 hours.

Kedzier (1896) carried out a more detailed study of a thermophilic actinomycete which he had isolated from sewage. He described the growth characteristics of this organism on various types of media. Temperature studies indicated that the actinomycete could develope over a temperature range from 35 to 65 C and its optimum growth temperature was 55 C. He also made studies on the resistance of the spores to heat, desiccation, and 5% phenol solution.

Tsiklinsky (1899,1903) isolated thermophilic actinomycetes from composts, soils, and feces. He inoculated potato with the material being examined and incubated it at 53 to 55 C. Isolations were made on agar plates at 55 to 57 C. Two actinomycetes, differing in type of spore formation, were isolated. The one organism produced chains of spores at the ends of hyphae and thus was classified as a true Actinomyces. The morphology of this thermophilic form agreed with descriptions given by other investigators. The other actinomycete produced single round or ovoid spores at the end of short side branches. This organism had an optimum growth temperature at 57 C but could also develope

in the range of 48 to 68 C. It exhibited prominent proteolytic behavior but not amylolytic. The name Thermoactinomycetes vulgaris was given to this organism because it was believed to be widely distributed in nature.

Several strains of a thermophilic actinomycete were isolated by Gilbert (1904) from various soil types. He designated these strains as Actinomyces thermophilus. These strains, when grown on potato, produced a folded white growth which later became grey on the surface. The optimum temperature was 55 C with the growth of most strains ceasing at 45 C but some strains could be adapted to grow at 37 C and even 22 C. The liquefaction of gelatin was slow.

Miehe (1907) isolated thermophilic actinomycetes from self-heated hay and considered these organisms to be characteristic of decomposing plant masses since the spores would survive on hay particles but rapidly lost their viability on other media, especially agar media. One of his cultures produced single spores on side branches and this organism was designated Actinomyces thermophilus (Berestnev). It grew best between 40 and 50 C and not at all at 25 or 60 C. He apparently had another type present also since he reported that one form produced spores in chains.

Representatives of the two spore-bearing types of thermophilic actinomycetes were isolated by Schutze (1908) from decomposing clover hay. One of these was designated

as Actinomyces thermophilus (Berestnev) and the other as Actinomyces monosporus (Lehman and Schutze). The latter organism produced single spores at the end of small side branches. Noack (1912) also isolated a number of thermophilic strains of actinomycetes from moist hay held at 45 to 46 C.

Bernstein and Morton (1934) in studies of pasteurized cheeses repeatedly isolated a thermophilic actinomycete which could withstand temperatures of 140 to 160 F (60 to 71 C). The optimum growth temperature for this organism was found to be 56 C. They suggested that this species be designated as Actinomyces casei since its characteristics were apparently different from species previously described.

Waksman et al. (1939), reporting on studies of soils and composts, found that thermophilic actinomycetes were present in all seasons in all types of soils examined and were especially numerous where soils had received stable manure. Even in frozen soils these organisms were found to average 10,000 to 15,000 per gram. In moist composts held at 50 C for ten days, counts of thermophilic actinomycetes on egg albumen agar were found to be as high as 12,000,000,000 per gram of compost. The greatest number of actinomycetes in horse manure compost occurred at temperatures between 50 and 65 C and also the greatest amount of decomposition was observed in this temperature range.

Katznelson (1940) found that a thermophilic actino-

mycete which he had isolated from horse manure autolyzed on a starch-ammonium sulfate agar after a certain incubation time at 50 C. Investigating this phenomenon, he found that the autolytic process was initiated when the pH of the medium reached 6.0 to 6.5 and the agent of lysis was non-transmissible.

Erikson (1952) isolated an actinomycete from composts of lawn cuttings. He designated this organism as Micromonospora vulgaris (Tsiklinsky). The optimum temperature range for growth was 45 to 60 C. Spores of this culture germinated more readily if they were given a five minute preliminary heat treatment at 75 to 90 C. The spores resisted 100 C for 45 minutes when suspended in a 1% sucrose solution and retained their viability after storage at 2 C for six months. These observations indicate the probable role of spores in surviving unfavorable growth conditions.

The classification of the thermophilic actinomycetes has long been in a state of confusion primarily due to the incomplete descriptions given in the early reports on studies of these microorganisms. From the time of early studies on these organisms, it has been recognized that two distinct types exist. One type produces true aerial mycelium, and spores are formed in either straight or spiral chains with some strains producing whorls of sporophores. Members of this type definitely belong to the genus recognized as Streptomyces. The other type pro-

duces single spores on short sporophores arising from the mycelium. Tsiklinsky (1899) first described a member of this form and proposed the genus Thermoactinomyces for organisms with this characteristic. Orskov (1923)[1] introduced the generic name Micromonospora for single-spore-producing forms and because of a more careful description of this group and insufficient differentiation of Thermoactinomyces, the tendency has been to include the thermophilic forms in this genus (Waksman et al. 1939).

The organism described by Miehe (1907) and designated as A. thermophilus belongs to the Thermoactinomyces-Micromonospora group but the organism described by Krassilnikov (1941)[2] and Mishustin (1950)[3] as A. thermophilus appears to be in the Streptomyces group since they describe a spiral producing form.

Waksman and Corke (1953), after comparative studies of Micromonospora and Thermoactinomyces, proposed that Thermoactinomyces (Tsiklinsky) be established as a genus of thermophilic actinomycetes. The differences between the two genera being mainly that members of Micromonospora do not produce true aerial mycelium and grow readily at 35 to 37 C whereas members of Thermoactinomyces do produce true aerial mycelium and are only thermophilic forms. They isolated a form which produces a rose colored pigment on certain media and suggested the name Thermoactinomyces thalpophilus for it.

1,2,3 as cited in Waksman & Corke (1953), Jour. Bact.66

Schuurmans (1954) cultured a green pigmented thermophilic actinomycete which has an optimum temperature for growth at 55 C and which produces a bactericidal agent. He suggested that this organism be designated as Thermoactinomyces viridis and the antibiotic substance has been named Thermoviridin.

In the study to be presented, two thermophilic actinomycetes have been used, one being a representative of the Streptomyces group and the other from the Thermoactinomyces-Micromonospora group.

MATERIALS AND METHODS

Isolation and Maintenance of Cultures. The organisms used in this study were isolated from a garbage composting project at Michigan State University, East Lansing, Michigan. Isolation was accomplished by plating appropriate dilutions of water suspensions of garbage compost on pryptone-glucose-extract agar (Difco) and incubating the plates at 45 to 50 C for 36 to 48 hours. After sufficient incubation time, typical actinomycete colonies were picked and seeded into nutrient broth tubes. These tubes were vigorously shaken and placed at 45 to 50 C for 12 hours prior to diluting and replating. The procedure for replating was the same as that used for the primary isolation. After development of mycelium on the plates, colonies were picked and inoculated onto slants of tryptone-glucose-extract agar.

Stock cultures of the isolated thermophilic actino-

mycetes were maintained on slants of the isolation medium. This medium was chosen because it readily produced a luxuriant growth. A number of slants were prepared from the isolated actinomycetes and after spore formation occurred, these slants were placed in a refrigerator at a temperature of 2 to 4 C and no transfers were made except to determine viability and comparison of these stored cultures with repeatedly transferred cultures. Other slants were prepared and monthly transfers were made; these cultures were the working stock for the studies described in the following pages.

Taxonomic Studies. Before developing studies upon these cultures, it was necessary to determine the optimum temperature for their growth. This was accomplished by inoculating tryptone-glucose-extract agar slants and tryptone-glucose-extract broth with the actinomycetes and incubating them at the following temperatures: 25, 30, 35, 37, 40, 42, 45, 47, 50, 52, 55, 57, and 60 C. The criterion used for optimum growth temperature was the temperature at which first visible growth occurred. Incubation was carried out in a hot air incubator containing a reservoir of water to give a high humidity and thus prevent desiccation.

The hydrogen ion concentration for optimum growth of these actinomycetes was determined by observing their development in a medium beffered at various levels of pH. Nutrient broth was buffered over a pH range of 5.5 to

8.5 at two-tenths intervals using mixtures of sodium monohydrogen phosphate and potassium dihydrogen phosphate. For pH values above 8.5, the pH of the medium was adjusted with sodium hydroxide solutions. Replicate tubes at the various pH levels were inoculated with the actinomycetes and incubated at 50 C. The criterion used for optimum pH level was the pH at which first visible growth appeared.

The presence or absence of growth and the characteristics of growth on various types of media have been established as part of the system of classification of actinomycetes. Following this system, the following media were used for growth observations: corn meal agar, corn steep agar, Czapek's agar, Emerson's agar, glucose-asparagine agar, glucose-peptone A agar and broth, glucose-peptone B agar and broth, nutrient agar and broth, nutrient glycerol agar, potato slants, Sabouraud's agar, starch agar, tryptone-glucose-extract agar, and yeast-glucoseagar. In the case of solid media, plates were prepared and replicate plates of each medium were streaked with the cultures of the thermophilic actinomycetes. These plates were incubated at 50 C for varying lengths of time depending upon the organisms ability to develope growth on a particular medium. In the case where good growth occurred, the plates were incubated until spore formation developed which usually occurred in 48 to 72 hours. Where little or no growth occurred, the plates

incubated up to 7 days. Broth cultures were also incubated at 50 C and for varying periods of time using the same criteria as those used for solid media. Formulae for the above named media are given in the appendix.

The following biochemical activities were observed: proteolysis, gelatin liquefaction, nitrate reduction, starch hydrolysis, and cellulolytic activity. Litmus milk was used for the observation of proteolysis. Two methods were used to determine liquefaction of gelatin because of the high temperature of incubation. The ordinary tube method was used with 4% gelatin and after varying times of incubation at 50 C the tubes were refrigerated to determine liquefaction. The other method was the plate method of Smith (1946) in which 0.4% gelatin is incorporated in nutrient agar. After sufficient incubation at the desired temperature, liquefaction is determined by flooding the plate with a mercuric chloride-hydrochloric acid solution and observing whether or not clear zones appear around areas of growth. Unchanged gelatin reacts with the reagent to produce opaque areas whereas areas of degradated gelatin remain clear.

Nitrate reduction tests were performed following the usual qualitative method for identification of microorganisms. Nutrient broth containing 0.1% potassium nitrate was inoculated and tested for the presence of nitrite using sulphanilic acid and alpha-naphthylamine reagents. Tests were made at regular intervals during the total

incubation period to insure the detection of the nitrite ion before it may have been further reduced to the ammonium ion.

The ability of these organisms to hydrolize starch was tested by growing the actinomycetes on a starch agar and then flooding the plate with iodine solution at varying times during the growth of the culture. The presence of a clear zone about an area of growth is indicative of starch hydrolysis.

Cellulolytic activity of these organisms was qualitatively examined by inoculating strips of filter paper immersed in Dubos cellulose medium and in a basal salts solution, and also by inoculating a suspension of cellulose in this basal solution. The basal medium has the following composition: Ammonium monohydrogen phosphate, 1.0g; potassium chloride, 0.2g; magnesium sulfate (heptahydrate), 0.2g; and water, 1000ml.

All the above biochemical tests were carried out using replicate tubes or plates. Incubation in all cases was at 50 C for varying periods of time but in no case did the total time exceed 8 days.

For microscopic examination of the cultures two staining procedures were used: (1) the Burke modification of Gram's stain, and (2) the Ziehl-Neelson method of acid fast staining. Growth of mycelium and spores for this examination were obtained by using the agar block method of Riddell (1950).

Utilization of Carbohydrates and Organic Acids. Using the basal medium previously mentioned in cellulolytic activity, 1% solutions were prepared using the following carbohydrates: lactose, sucrose, glucose, maltose, mannitol, dextrin, cellobiose, rhamnose, sorbose, and fructose. Organic acid solutions of 0.5% concentration were also prepared with this basal medium. The acids used were malic, fumaric, citric, succinic, pyruvic, lactic, oxalic, and tartaric. Replicate tubes of each of these resulting solutions were inoculated with the actinomycete cultures and incubated at 50 C for periods up to 96 hours. Ability or inability to grow in these media was observed.

Proteolytic Activity. Preliminary experiments showed that the thermophilic actinomycetes used in this study were capable of utilizing the following proteins for development of growth: keratin, zein, gluten, gelatin, casein, and phytone[1]. Of this group keratin, zein, and gluten are either insoluble or soluble only after treatment with acid or base and because of this undesirable property only gelatin, casein, and phytone were used for further studies. A medium was prepared of each of these using 1.5g of the protein material per liter of the salts of Czapek's solution. Each medium was dispensed into 125ml Erlenmeyer flasks in the volume of 50ml per flask and autoclaved. Twelve flasks of each medium were inoculated with the

1 A peptone preparation of Baltimore Biological Laboratory, Inc.

desired culture, placed on a Burrell wrist-action shaker, and incubated at 50 C. A flask was removed for analysis according to the following schedule of hours of incubation: 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, and 72. Replicate series were run for each organism.

The inoculum of each culture was prepared by inoculating spores of the culture into 50ml of the medium to be used for study. The culture was developed on the Burrell shaker at 50 C for 12 to 18 hours at which time it was blended in a sterile Waring Blendor and the Erlenmeyer flasks were inoculated with 1ml per flask of the resulting mycelial suspension.

The amount of growth occurring during the incubation period was determined by weighing the mycelium present in each sample removed at the specified times. The sample was filtered through tared filter paper which had been dried in a 105 C oven for 12 to 14 hours. After filtering, the mycelium and filter paper were again dried at 105 C for 12 to 14 hours and weighed. The difference in weight between the filter paper alone and the filter paper plus the mycelium gave the dry mycelial weight per sample.

The filtrate from each sample was divided into two equal portions. One portion was immediately frozen to be used for amino acid studies by paper chromatography. The other portion was used for ammonia determinations.

The amount of ammonia in the filtrate for each cul-

ture sample removed at the designated incubation periods was determined by the modified aeration method of Van Slyke and Cullen. A 25ml filtrate sample treated with a saturated solution of potassium carbonate was aerated for 3 hours with air washed by dilute (1:10) sulfuric acid. The freed ammonia was trapped in a 2% boric acid receiver which was then titrated with standard sulfuric acid (0.02 N). Ammonia free water was used for preparing all solutions and for rinsing glassware.

One dimensional paper chromatograms were made on the culture filtrates to determine the presence of free amino acids. Whatman No. 1 filter paper for chromatography ($18\frac{1}{4}$" x $22\frac{1}{2}$") was marked off in twelve divisions so that the various samples from 0 to 72 hours could be spotted on the same sheet of paper. Fifty microliters of each culture filtrate was applied at each spot. Chromatograms were developed by the ascending method at 25-1 C in an insulated Chromatocab[1] using a solvent system of n-butanol-acetic acid-water (4:1:1). After development of the chromatograms, the papers were dried at 100 C in an air circulating oven. Color development of the amino acid spots was obtained by spraying the dried chromatograms with a ninhydrin solution composed of 1g ninhydrin and 500ml of n-butanol. The ninhydrin sprayed papers were dried at 100 C and placed in the dark for 18 to 24 hours to allow full color development. Known amino

1 Product of Research Equipment Corporation, Oakland, California.

acids and amines were chromatographed at the same time and in the same manner as the unknowns.

EXPERIMENTAL RESULTS

Several actinomycete cultures were isolated at 50 C from the composting waste material over a period of two weeks. The cultures could be separated into two groups by gross examination of their growth on tryptone-glucose-extract agar. One group produced a light colored aerial mycelium which gave the appearance of fragility whereas the other group produced a grey aerial mycelium which was compact and appeared more hardy. One specimen from each group was selected for further study. The cultures selected were those which produced the most rapid and abundant growth on the isolation medium.

Taxonomic Studies. Microscopic examination revealed these thermophilic actinomycetes to be of two different genera, Thermoactinomyces (Waksman and Corke, 1953) and Streptomyces (Bergey).

Thermoactinomyces sp. Microscopic examination of this thermophilic actinomycete showed that conidia were produced singly on extremely short conidiophores branching from the hyphae. The conidiophores are so short that in the majority of observations the conidium appears to be resting directly upon the hypha. The spores are round but occasionally a spore appears to be slightly elongate. The diameter of the spore is between 0.8 and 1.0 micron

which is slightly larger than the average hypha diameter of 0.5 to 0.8 micron.

Stained preparations of this organism showed it to be non-acid fast and gram positive in reaction. However, instances were noted when, in stained preparations of older cultures, the mycelium was gram negative but the spores were always gram positive whether in young or old cultures.

The temperature range for growth (Table 1) of this organism is 37 to 59 C with the optimum temperature being 50 to 52 C. No growth is obtained at temperatures below 37 C at which temperature only slight growth occurs after 48 to 72 hours. No growth is observed at 60 C. A vigorous culture produces visible vegetative growth in 7 to 8 hours when incubated at its optimum temperature.

The hydrogen ion concentration range for good growth of this Thermoactinomyces sp. is slightly alkaline and it appears that equal amounts of growth can be obtained over the range of pH 7.0 to 8.5. Mycelial development occurs above and below this range but in diminishing quantities as the medium becomes more alkaline or more acid. In the acid range, little growth appears below pH 5.8, and in the alkaline range growth ceases at pH 9.0.

The cultural and biochemical characteristics of this actinomycete are given below. In general, the

Table 1

Growth Temperatures

Temp. C.	Hours incubated until visible growth.	
	Streptomyces	Thermoactinomyces
25	No growth	No growth
35	24	No growth
37	18	48
40	10	24
42	9	20
45	8	12
47	7	9
50	6	8
52	6	7
55	9	8
57	12	11
59	24	20
60	No growth	No growth

organism is a strict aerobe producing mycelium only at the surface of broth or stab cultures. On media where growth occurs, the vegetative mycelium is a very light cream color and the aerial mycelium varies from white to very light grey and has a characteristic dry appearance. No pigment either of the mycelium or the water-soluble type is produced on any of the media used in this study. The cultural and biochemical characteristics are:

TGE	Large, slightly raised colony of dry white aerial mycelium and light cream colored vegetative mycelium. Center of colony wrinkled.
Nutrient Agar	Growth very similar to that on TGE agar with the exception that there is less sporogenous development.
Emerson's Agar	Flat, rough, dry colony with striations from center to edge. Vegetative mycelium of light cream. Aerial mycelium has the appearance of white and light grey concentric rings.
Nutrient-glycerol Agar	Flat, rough, dry colony of light cream vegetative mycelium and white aerial mycelium. Colony not as large as on the above media.
Yeast-glucose Agar	Colony similar to that on TGE agar only more compact and wrinkled.
Glucose-asparagine Agar	No growth.
Glucose-peptone A Agar	Very small colony formation of light cream vegetative mycelium and no aerial mycelium.
Glucose-peptone B Agar	Very small colonies of vegetative mycelium only.

Czapek's Agar	No growth.
Sabouraud's Agar	No growth.
Corn Meal Agar	No growth.
Corn Steep Agar	No growth.
Starch Agar	Small white colonies with slight zone of hydrolysis.
Nutrient Broth	White, surface pellicle-type growth. Some floccules settle when disturbed.
Glucose-peptone A Broth	Very slight white growth at surface.
Glucose-peptone B Broth	Very slight growth at surface.
Potato Slant	No growth.
Litmus Milk	Coagulation followed by nearly complete digestion in 3 days. Alkaline reaction.
Gelatin	Liquefaction positive but slow.
Nitrate	Reduction to nitrite negative.
Cellulose	No growth.

The ability of this organism to use various sugars and organic acids as energy sources is summarized in Table 2.

Streptomyces sp. Microscopic examination of this thermophilic actinomycete reveals that the spores are borne in the typical manner of members of the genus Streptomyces. The spores are produced in chain-like formation at the terminal ends of hyphal elements. These spores are round and have a diameter of 0.5 to 0.7 micron. The terminal ends of those hyphae which produce spores become slightly larger in diameter than the hyphal element which

Table 2

Growth of *Thermoactinomyces* sp. in various sugar and organic acid liquid media.

Sugars	Growth Character	Amt. Growth	Final pH[1]
Maltose	No growth	-	7.0
Mannitol	No growth	-	7.0
Dextrin	No growth	-	7.0
Sorbose	No growth	-	7.0
Cellobiose	No growth	-	7.0
Fructose	White surface clump	+	6.8
Rhamnose	No growth	-	7.0
Lactose	No growth	-	7.0
Sucrose	White surface clump	+	6.9
Glucose	White surface clump	+	6.4
Acids			
Malic	No growth	-	7.4
Fumaric	No growth	-	7.4
Succinic	No growth	-	7.3
Pyruvic	No growth	-	7.4
Citric	No growth	-	7.5
Lactic	No growth	-	7.3
Oxalic	No growth	-	7.3
Tartaric	No growth	-	7.4

1. Original pH of sugar media was 7.0.
1. Original pH of organic acid media varied from 7.3 to 7.5.

has an average diameter of 0.4 to 0.6 micron. Some of the hyphae appear to form whorls at their terminal ends. This characteristic is also observed in certain species of the mesophilic members of this genus. Staining procedures show this species to be gram positive and non-acid fast.

Growth temperature studies (Table 1) disclosed that this species can develope more growth over a wider temperature range than can the Thermoactinomyces species. No growth is developed at 25 C but slow growth occurs at 35 C. The optimum temperature like that of the Thermoactinomyces species is 50 to 52 C and no growth developed at 60 C.

This Streptomyces grows best in a slightly alkaline medium. Development of growth appears equally well in a range of pH 7.0 to 8.5. On the alkaline end of this range decreasing amounts of growth occur up to pH 9.2 and cease beyond this point. In the range below neutrality, development occurs in decreasing quantities to pH 5.8 below which no growth developes.

Growth characteristics of this organism on various media are as follows:

TGE Agar	Large raised colony with light cream vegetative mycelium. Aerial mycelium is white changing to grey with production of spores. Abundant growth.
Nutrient Agar	Growth good with large raised colony having light cream colored vegetative mycelium and white aerial mycelium turning to grey.

Emerson's Agar	Growth good with slightly raised colony. Colorations similar to above media.
Nutrient-glycerol Agar	Abundant growth with raised colony which is considerably wrinkled. Colorations similar to above media.
Yeast-glucose Agar	Abundant growth with raised, wrinkled colony with extensive aporulation giving a dark grey appearance.
Glucose-asparagine Agar	Poor growth, consisting mostly of vegetative mycelium. Aerial mycelium very sparse with little sporulation.
Glucose-peptone A Agar	Small white colony with no sporulation occurring.
Glucose-peptone B Agar	Small colony formation with sparse aerial mycelium but some sporulation occurring.
Czapek's Agar	Slight vegetative growth only.
Sabouraud's Agar	No growth.
Corn Meal Agar	Slight growth of light cream vegetative mycelium and some white aerial mycelium.
Corn Steep Agar	No growth.
Starch Agar	No growth.
Nutrient Broth	Abundant surface growth with pellicle formation. Abundant sporulation.
Glucose-peptone A Broth	White surface growth but not abundant.
Glucose-peptone B Broth	Slight amount of white surface growth.
Potato Slant	No growth.
Litmus Milk	Soft curd formation with slow digestion. Alkaline reaction.
Gelatin	Positive liquefaction.

Nitrate	Positive nitrite reaction.
Cellulose	No growth.

On media which produce good mycelial development of this organism, the general appearance of the growth consists of a light cream colored vegetative mycelium which gives rise to a white aerial mycelium. The appearance of the colony is dry and wrinkled and becomes decidedly grey with the advent of sporulation.

Development of growth of this organism in various sugar and organic acid solutions is given in table 3.

Mycelial Weights and pH. Growth of the two thermophilic actinomycetes in the protein sclutions of phytone, gelatin, and casein was followed by determining the amount of dry mycelial weight for each sample removed at the various periods of incubation. Results are recorded as milligrams of dry mycelium per 50ml of medium and are given in tables 4 and 5 for both organisms. These mycelial weights have also been plotted against incubation time to produce the curves found in figures 1 and 2.

Changes in pH value during growth of each organism in the protein media were followed by determining the pH of each sample removed at the specified times of incubation. The pH values for respective samples of replicate series of each medium were found to be very similar and the average of these values for each medium have been recorded in tables 6 and 7 for each organism.

Ammonia Determination. The date collected from the

Table 3

Growth of Streptomyces sp. in various sugar and organic acid liquid media.

Sugars	Growth Character	Amt. Growth	Final pH[1]
Maltose	White pellicle	++	6.5
Mannitol	White pellicle	+	6.1
Dextrin	White pellicle	+	6.3
Sorbose	No growth	-	7.0
Cellobiose	White pellicle	++	6.5
Fructose	Grey pellicle	+++	6.2
Rhamnose	No growth	-	7.0
Lactose	No growth	-	7.0
Sucrose	White pellicle	++	6.8
Glucose	Grey pellicle	++++	6.1
Acids			
Malic	No growth	-	7.4
Fumaric	No growth	-	7.4
Succinic	No growth	-	7.3
Pyruvic	Light grey pellicle	--	8.6
Citric	No growth	-	7.5
Lactic	No growth	-	7.3
Oxalic	No growth	-	7.3
Tartaric	No growth	-	7.4

1. Original pH of sugar media was 7.0.
1. Original pH of organic acid media varied from 7.3 to 7.5.

Table 4

Dry mycelial weights of Thermoactinomyces sp. expressed in mg/50 ml of culture medium.

Hours Incubation	Casein				Gelatin				Phytone			
				Ave.				Ave.				Ave.
0	0.4	0.3	0.4	0.4	0.2	0.4	0.3	0.3	0.2	0.4	0.5	0.4
3	0.6	0.5	0.6	0.6	0.4	0.5	0.5	0.5	0.3	0.4	0.6	0.4
6	1.5	1.4	1.7	1.5	0.5	0.6	0.6	0.6	0.5	0.6	0.8	0.6
9	2.2	2.0	2.3	2.2	0.8	0.9	0.8	0.8	10.4	10.7	10.8	10.6
12	7,5	7.4	7.8	7.6	4.5	4.9	4.6	4.7	11.4	11.9	11.9	11.7
15	8.9	8.7	9.0	8.9	6.8	6.9	6.5	6.7	9.5	10.0	10.2	9.9
18	11.5	11.5	11.7	11.6	7.8	8.1	8.0	8.0	5.8	6.1	6.3	6.1
24	10.8	10.5	11.1	10.8	8.5	8.7	8.4	8.5	4.0	4.4	4.3	4.2
30	7.2	7.3	7.6	7.4	7.8	8.0	7.7	7.8	2.9	3.2	3.5	3.2
36	5.7	5.6	5.9	5.7	6.7	6.9	6.5	6.7	2.1	2.4	2.2	2.2
48	3.0	2.8	3.2	3.0	3.3	3.5	3.0	3.3	1.2	1.6	1.5	1.4
72	2.5	2.4	2.7	2.5	1.4	1.8	1.6	1.6	1.1	1.4	1.5	1.3

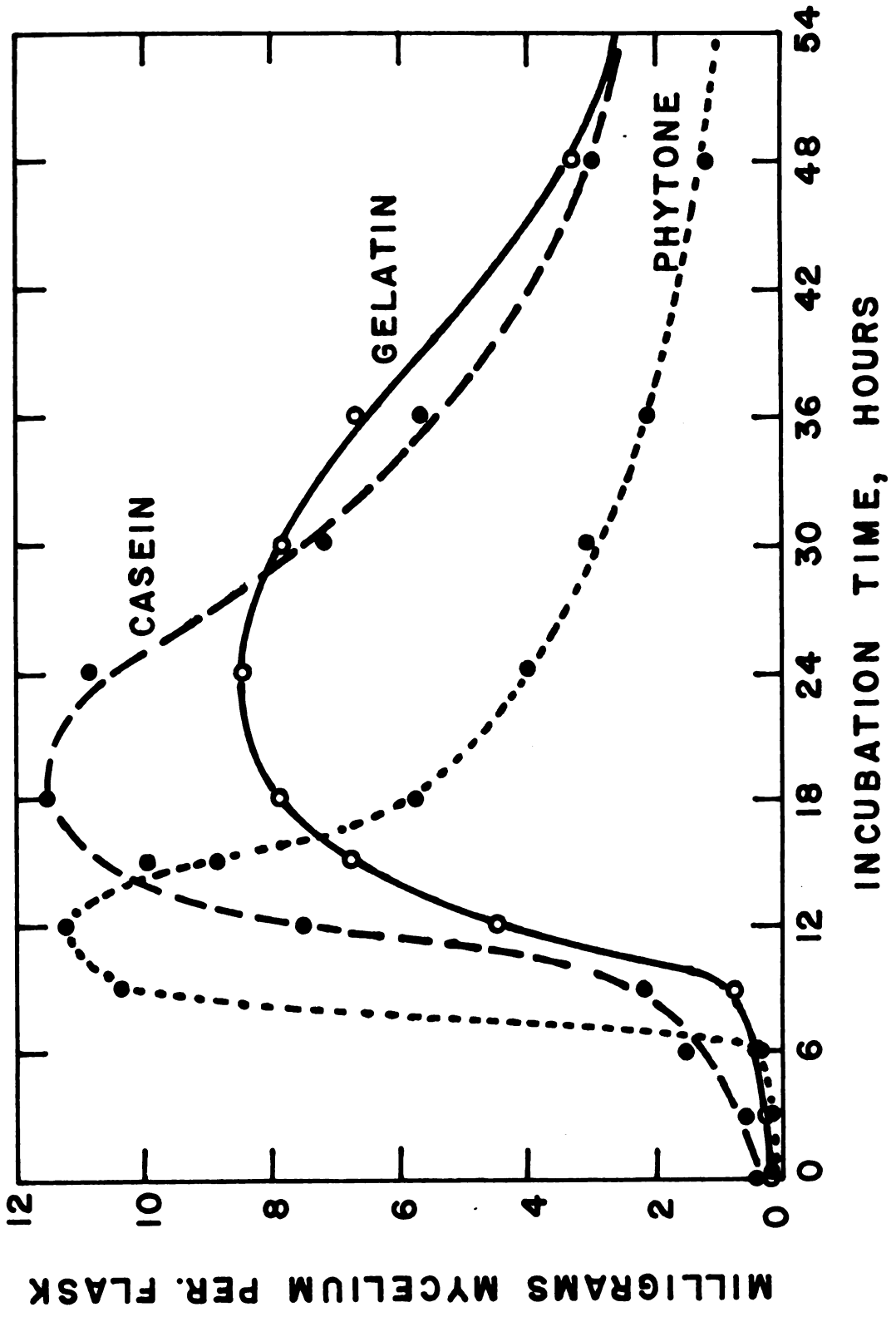

Figure 1. Dry mycelial weights of Thermoactinomyces sp. grown in 50 ml portions of protein media.

Table 5

Dry mycelial weights of *Streptomyces* sp. expressed in mg/50 ml of culture medium.

Hours Incubation	Casein			Ave.	Gelatin			Ave.	Phytone			Ave.
0	0.2	0.2	0.3	0.2	0.5	0.3	0.5	0.4	0.6	0.5	0.3	0.5
3	0.4	0.5	0.4	0.4	1.9	1.4	2.1	1.8	0.9	0.8	0.6	0.8
6	0.6	0.8	0.8	0.7	2.0	1.9	2.4	2.1	14.0	13.6	13.4	13.7
9	7.9	7.7	8.1	7.9	6.0	5.8	6.2	6.0	18.9	18.2	18.1	18.4
12	22.6	21.7	22.2	22.1	9.5	9.2	9.8	9.5	20.2	20.4	19.7	20.1
15	27.6	27.1	28.1	27.6	11.4	11.4	11.9	11.6	17.6	17.6	17.1	17.4
18	26.8	26.6	27.1	26.8	14.7	14.2	15.0	14.6	16.2	16.5	15.9	16.2
24	24.9	24.0	24.5	24.5	12.1	12.5	12.8	12.5	11.0	11.4	10.6	11.0
30	20.2	19.7	20.6	20.2	10.3	10.7	10.5	10.5	9.7	10.1	9.6	9.8
36	14.2	13.8	13.2	13.4	9.5	9.8	9.4	9.6	8.2	8.7	8.0	8.3
48	11.6	11.0	10.8	10.5	9.5	9.3	9.0	9.3	6.5	6.6	6.2	6.4
72	10.7	10.4	10.8	10.4	9.4	9.2	8.9	9.2	5.3	5.5	5.0	5.3

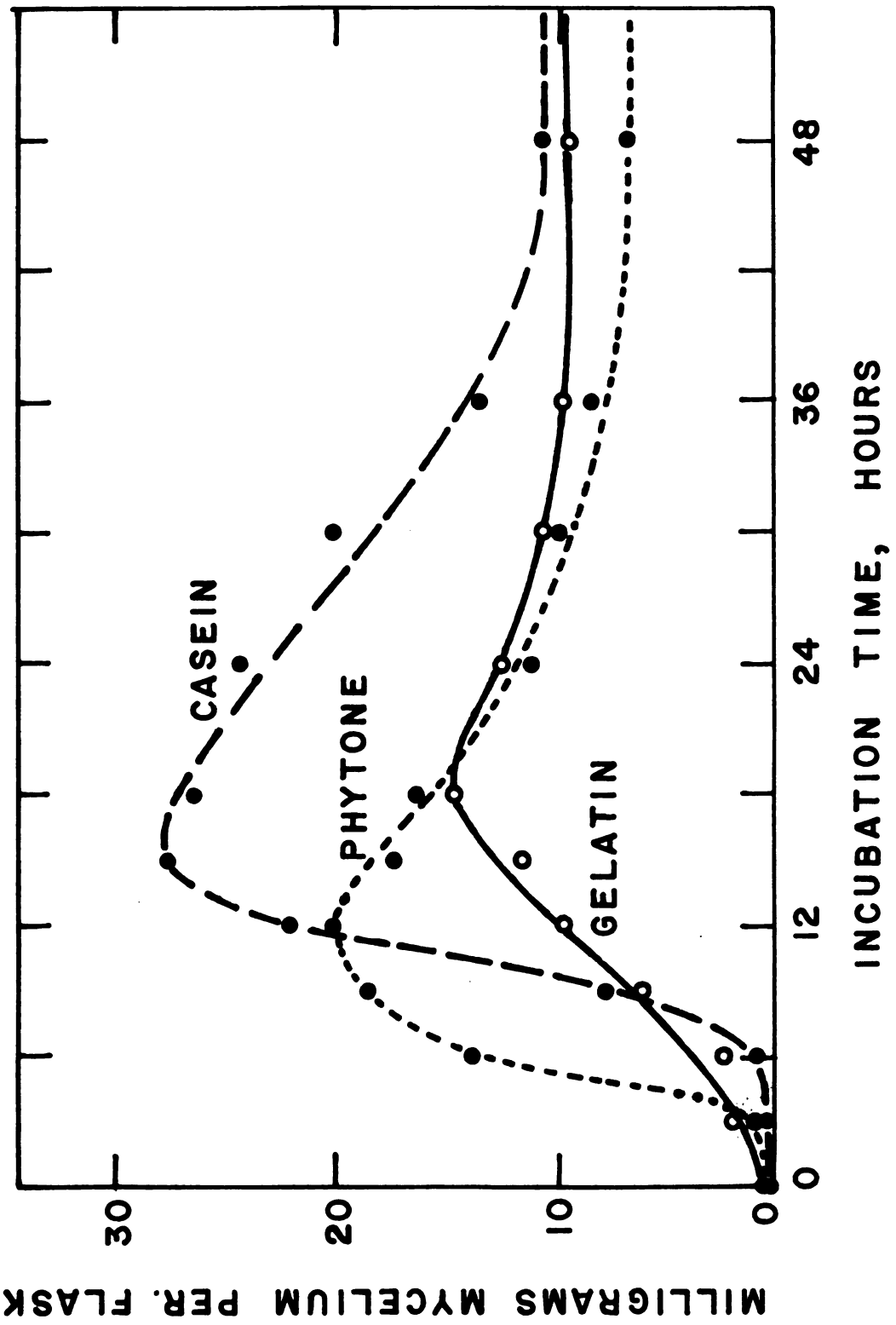

Figure 2. Dry mycelial weights of Streptomyces sp. grown in 50 ml portions of protein media.

Table 6

Average pH values occurring during growth of *Thermoactinomyces* sp. in liquid protein media.

Hours Incubated	Casein	Gelatin	Phytone
0	8.4	7.5	7.7
3	8.3	7.4	7.65
6	8.3	7.35	7.5
9	8.1	7.2	7.3
12	7.8	7.2	7.2
15	7.7	7.2	7.06
18	7.6	7.45	7.4
24	7.8	7.02	7.6
30	7.6	7.2	7.1
36	8.0	7.2	7.0
48	7.3	7.8	6.9
72	7.6	7.1	6.85

Table 7

Average pH values occurring during growth of *Streptomyces* sp. in liquid protein media.

Hours Incubated	Casein	Gelatin	Phytone
0	7.5	7.45	7.6
3	7.7	7.5	7.5
6	7.6	7.55	7.2
9	7.2	7.7	7.3
12	7.2	7.6	7.9
15	7.4	7.48	8.0
18	7.6	7.68	8.0
24	8.4	7.85	8.4
30	8.1	7.2	8.9
36	8.7	7.3	8.7
48	8.8	8.4	8.7
72	8.5	7.55	9.0

titration of ammonia in each culture sample were used to calculate the amount of ammonia present in each milliliter of culture medium after growth of one of the actinomycetes for a designated time. The amount of ammonia per milliliter is so small that calculations were made on the basis of milligrams of ammonia per liter of medium. The results of the replicate determinations are given in table 8 for the Thermoactinomyces sp. and in table 9 for the Streptomyces sp. The average values of the determinations of replicate samples for each incubation period have been plotted as milligrams of ammonia per liter against incubation time so that the amount of ammonia produced from each protein by growth of the organisms can be followed graphically. These graphs are represented in figures 3 and 4.

Paper Chromatography. Chromatograms were made of 18 l-amino acids and 2 amines. The names of these compounds and their corresponding Rf values are given in table 10. Chromatograms of the culture filtrate samples from each protein medium of casein and gelatin were made at the same time that the known compounds were being chromatographed. The Rf values of unknown spots were determined and comparison of these values with the known Rf values identified the unknown spots. The identified amino acids and amines for each sample chromatographed are given in tables 11, 12, 13, and 14.

Table 8

Ammonia* content of Thermoactinomyces sp. culture medium expressed as milligrams NH_3/liter.

Hours Incubation	Casein				Gelatin				Phytone			
				Ave.				Ave.				Ave.
0	3.2	3.5	3.5	3.4	5.4	5.9	5.5	5.6	4.4	4.5	4.5	4.5
6	5.4	5.6	5.8	5.6	8.3	8.7	8.5	8.5	9.6	9.8	10.6	10.0
12	13.7	14.1	14.2	14.0	21.9	22.8	22.5	22.4	32.2	32.8	32.5	32.5
18	33.7	33.9	34.4	34.0	43.8	43.8	44.4	44.0	42.8	43.5	42.7	43.0
24	51.8	51.9	52.4	52.0	63.2	63.4	63.9	63.5	47.2	48.0	47.9	47.7
30	61.8	62.0	62.2	62.0	79.4	79.6	79.5	79.5	49.4	50.4	50.7	50.5
36	69.9	70.6	70.8	70.4	88.0	88.6	88.6	88.4	51.1	51.8	51.6	51.5
48	74.8	74.9	75.3	75.0	100.8	100.5	100.1	100.8	52.8	52.8	53.4	53.0
72	76.9	77.6	77.7	77.4	104.9	105.9	105.7	105.5	53.6	53.7	54.1	53.8

* Ammonia content of uninoculated controls has been subtracted.

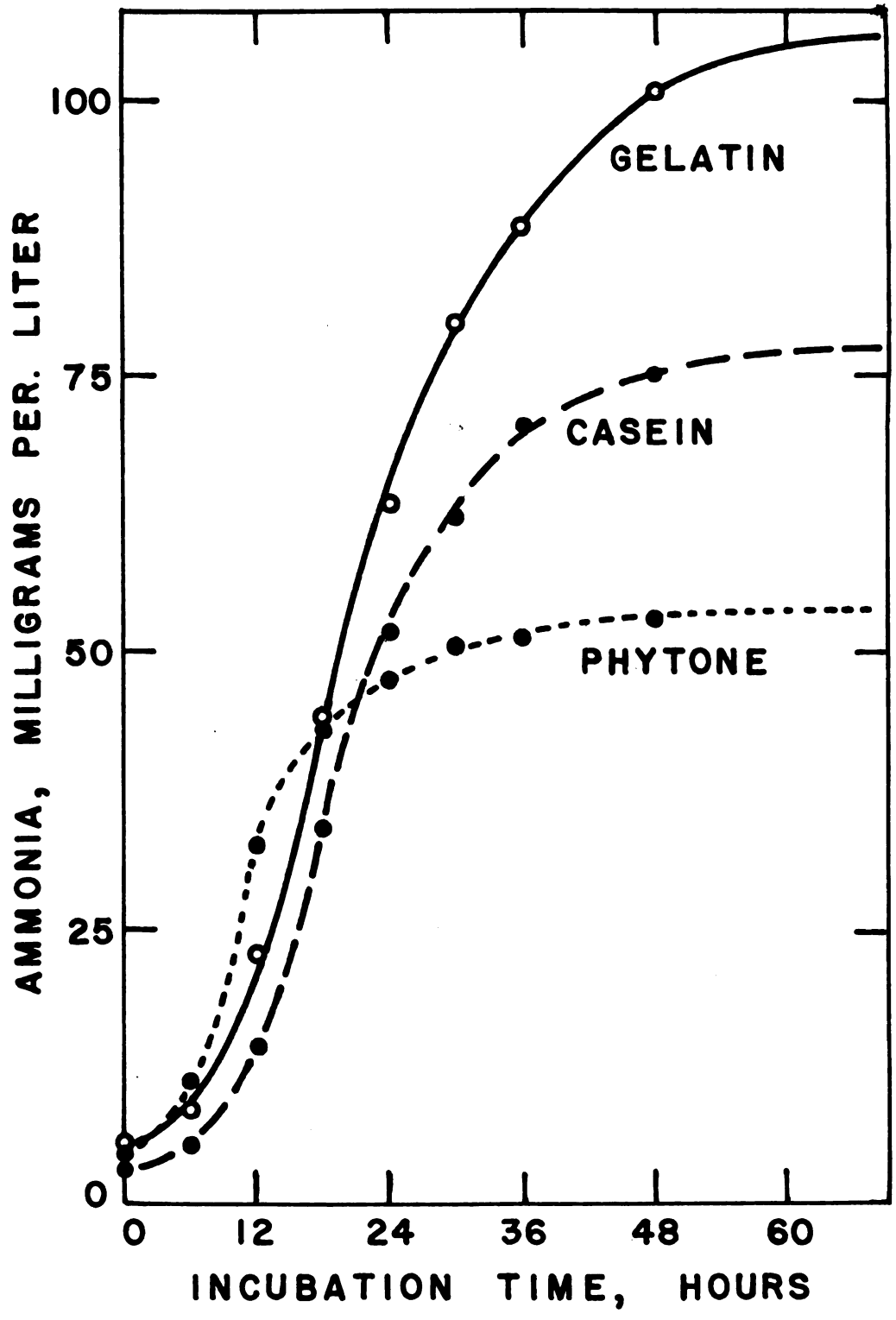

Figure 3. Ammonia produced during growth of Thermoactinomyces sp. in protein media.

Table 9

Ammonia* content of Streptomyces culture medium expressed as milligrams NH_3/liter.

Hours Incubation	Casein			Ave.	Gelatin			Ave.	Phytone			Ave.
0	5.8	6.1	6.2	6.0	5.9	6.1	6.1	6.0	4.7	4.5	4.3	4.5
6	8.7	8.8	9.2	8.9	10.9	11.4	11.3	11.2	16.4	16.4	15.8	16.2
12	39.8	40.4	40.7	40.3	40.2	40.8	40.9	40.3	29.2	29.1	28.7	29.0
18	56.2	56.7	56.6	56.5	58.6	59.2	59.2	59.0	38.1	38.1	37.8	38.0
24	66.9	67.5	67.8	67.4	70.2	70.5	70.9	70.6	43.1	43.3	42.7	43.0
30	76.9	77.4	77.6	77.0	75.7	75.9	76.4	76.0	45.6	45.8	45.1	45.5
36	82.3	82.8	83.0	82.7	80.6	80.9	81.5	81.0	49.7	49.5	48.8	49.4
48	87.8	87.9	88.3	88.0	86.7	87.0	87.3	87.0	52.2	52.1	51.7	52.0
72	92.7	92.9	93.7	93.1	91.0	91.7	91.8	91.5	55.4	54.9	54.7	55.0

* Ammonia content of uninoculated controls has been subtracted.

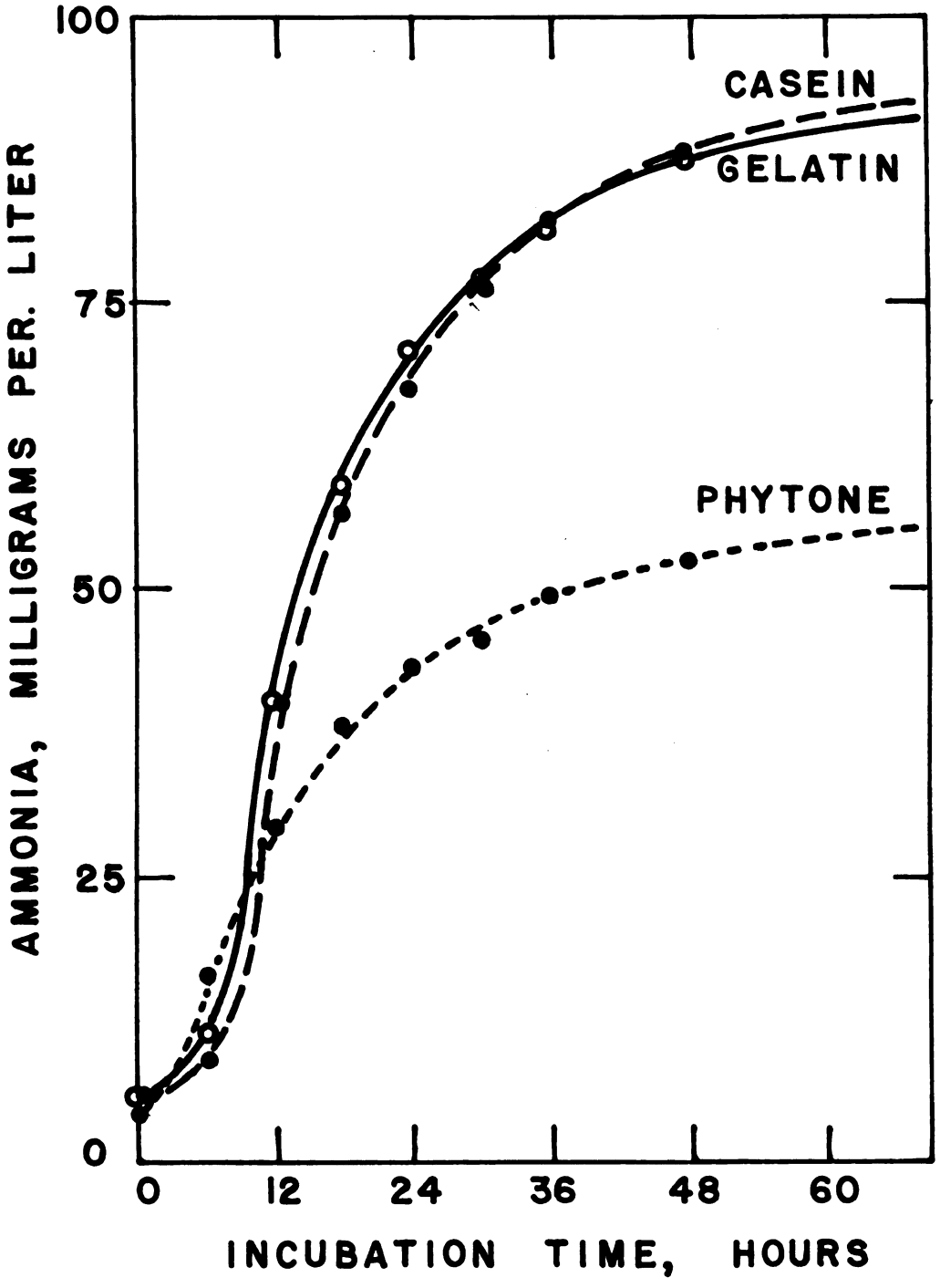

Figure 4. Ammonia produced during growth of Streptomyces sp. in protein media.

Table 10

Rf values for known L-amino acids and amines chromatographed in n-butanol-acetic acid-water solvent (4:1:1) at 25-1 C.

Rf value	Compound	Rf value	Compound
.036	Cystine	.265	Hydroxyproline
.074	Cysteine	.283	Threonine
.09	Lysine	.351	Alanine
.098	Asparagine	.412	Proline
.109	Histidine	.525	Tryptophane
.115	Arginine	.592	Methionine
.126	Glutamine	.627	Valine
.139	Glutamic acid	.665	Phenylalanine
.15	Aspartic acid	.747	Leucine
.185	Serine	.754	Isoleucine

Table 11

Amino acids and amines identified in liquid casein medium after growth of *Thermoactinomyces* sp.

Hours Incubated	Compound	Hours Incubated	Compound
3	Cystine	18	Cysteine
	Histidine		Lysine
	Glutamic acid		Histidine
	Serine		Glutamine
	Alanine		Hydroxyproline
	Proline		Alanine
	Valine		Tryptophane
			Methionine
6	Cystine		Phenylalanine
	Cysteine		Isoleucine
	Histidine		
	Aspartic acid	24	Cysteine
	Serine		Lysine
	Alanine		Glutamine
			Aspartic acid
9	Cystine		Methionine
	Cysteine		Phenylalanine
	Lysine		Isoleucine
	Histidine		
	Glutamine	30	Cysteine
	Aspartic acid		Lysine
	Hydroxyproline		Histidine
	Alanine		Aspartic acid
	Proline		Hydroxyproline
	Valine		Isoleucine
12	Lysine	36	Cysteine
	Histidine		Lysine
	Glutamine		Aspartic acid
	Aspartic acid		Hydroxyproline
	Hydroxyproline		Tryptophane
	Alanine		
	Proline	48	Cysteine
	Valine		Lysine
	Isoleucine		Histidine
			Glutamine
15	Cysteine		Alanine
	Lysine		Tryptophane
	Histidine		
	Glutamic acid	72	Cysteine
	Hydroxyproline		Lysine
	Alanine		Histidine
	Tryptophane		Tryptophane
	Phenylalanine		

Table 12

Amino acids and amines identified in liquid gelatin medium after growth of *Thermoactinomyces* sp.

Hours Incubated	Compound	Hours Incubated	Compound
3	Cysteine	24	Cysteine
	Lysine		Lysine
	Glutamine		Arginine
			Glutamine
6	Cysteine		Threonine
	Lysine		
	Arginine	30	Cysteine
			Lysine
9	Cysteine		Aspartic acid
	Lysine		Serine
	Arginine		Tryptophane
12	Cysteine	36	Cysteine
	Arginine		Lysine
	Serine		Arginine
	Hydroxyproline		Glutamic acid
			Serine
15	Cystine		
	Lysine	48	Cysteine
	Histidine		Lysine
	Glutamine		Arginine
	Hydroxyproline		Serine
	Valine		Hydroxyproline
			Threonine
18	Cystine		
	Cysteine	72	Cysteine
	Asparagine		Lysine
	Glutamine		Serine
	Hydroxyproline		Hydroxyproline
			Threonine

Table 13

Amino acids and amines identified in liquid casein medium after growth of *Streptomyces* sp.

Hours Incubated	Compound	Hours Incubated	Compound
3	Cystine	18	Aspartic acid
	Cysteine		Threonine
	Lysine		Alanine
	Histidine		
	Arginine	24	Lysine
	Glutamic acid		Histidine
	Hydroxyproline		Glutamic acid
	Threonine		Aspartic acid
	Alanine		Threonine
	Proline		Alanine
6	Cystine	30	Cysteine
	Lysine		Lysine
	Asparagine		Histidine
	Arginine		Glutamic acid
	Glutamic acid		Aspartic acid
	Proline		Threonine
	Valine		Alanine
9	Lysine	36	Cysteine
	Asparagine		Asparagine
	Arginine		Glutamic acid
	Glutamic acid		Threonine
	Aspartic acid		Alanine
12	Cystine	48	Cysteine
	Asparagine		Asparagine
	Glutamic acid		Glutamine
	Aspartic acid		Aspartic acid
	Threonine		Threonine
	Alanine		Alanine
15	Cystine	72	Lysine
	Cysteine		Glutamine
	Asparagine		Aspartic acid
	Aspartic acid		Serine
	Threonine		Threonine
	Alanine		Alanine

Table 14

Amino acids and amines identified in liquid gelatin medium after growth of *Streptomyces* sp.

Hours Incubated	Compound	Hours Incubated	Compound
3	Lysine Glutamine	24	Lysine Arginine Hydroxyproline
6	Lysine Glutamine Threonine		Threonine
		30	Lysine Histidine
9	Lysine Glutamine Threonine	36	Lysine Histidine Aspartic acid
12	Lysine Glutamine Threonine	48	Asparagine Aspartic acid
15	Lysine Arginine Threonine	72	Lysine Arginine
18	Lysine Arginine Threonine		

DISCUSSION

The classification of the two thermophilic actinomycetes used in this study has been limited to genue because of the confusion in descriptions given by past investigators and because of the possible variations which have been demonstrated to occur in other cultures of actinomycetes. Although few thermophilic actinomycetes have been recorded in literature, they have been described under seven different genera, two of which are bacteria and one a fungus. The generic names which have been used are: Cladothrix, Streptothrix, Thermomyces, Actinomyces, Streptomyces, Thermoactinomyces, and Micromonospora. The last three generic names are those commonly used of recent times. The general characteristics of the Streptomyces and the Thermoactinomyces-Micromonospora groups have been described earlier in the paper. The generic name Thermoactinomyces has been used to designate one of the organisms in this study because it fulfills the requirements set forth for this proposed genus by Waksman and Corke (1953). Since there has been a lack of interest in this group of microorganisms, no pattern for identification procedures has been established and if further work is to be done, standards must be established to reduce or eliminate the confusion resulting from past descriptions.

The results of the investigations on the isolated Thermoactinomyces species indicate that it most nearly resembles the established species Thermoactinomyces vul-

garis. The characteristics of the species used in this study do not agree with the recognized description in that growth does occur in sucrose medium, there is no growth on potato, and the temperature range and optimum temperature for growth is lower by several degrees. Although these differences make identification doubtful, they are not significantly great to establish a new species especially when the possibility of variation is considered.

The *Streptomyces* species used in this study most nearly resembles *Streptomyces thermophilus* described by Gilbert (1904). It differs from the recognized species in that there is no growth on potato, starch agar, or Czapek's agar and it is unable to develope growth at 28 C. The description given for *Streptomyces thermophilus* is lacking in many respects and thus a true comparison cannot be established.

An examination of table 2 will indicate that the *Thermoactinomyces* species does not produce the necessary enzyme systems for the utilization of sugars. Only the three sugars sucrose, glucose, and fructose were utilized and these were done so with difficulty since the amount of growth after three days of incubation was slight. The inability to use sugars is in agreement with the investigations of Bernstein and Morton (1934). They found that a thermophilic actinomycete isolated from pasteurized cheese was unable to ferment maltose,

mannitol, lactose, sucrose, glucose, dextrin, inulin, xylose, and cellobiose. Schuurmans also found that the Thermoactinomyces he isolated did not utilize carbohydrates readily but grew well on media containing proteins or peptones. Similar results were obtained in this study.

In contrast to the Thermoactinomyces species, the Streptomyces species utilizes all but three of the sugars used for study. The three not fermented are sorbose, rhamnose, and lactose. Sorbose and rhamnose are the only hexoses in the group which are not utilized. With the exception of lactose, it appears that this organism is able to utilize the complex sugars but only up to a certain point of complexity since it was unable to hydrolize starch or cellulose. The Streptomyces species, therefore, has the capacity to produce a number of enzymes effective against several sugars and derivitives of sugars but the Thermoactinomyces species is quite limited in its capacity to produce such enzymes.

In those sugar media which support growth, the reaction of any particular sugar medium becomes more acidic with increased growth. It seems apparent that the metabolism of the sugars by the thermophilic actinomycete produces an acid or acids. It is interesting to note that the carbohydrates that are utilized contain glucose or fructose molecules, or molecules which can be converted to either of these sugars. Numerous microorganisms,

including mesophilic Streptomyces, are known to produce various acids from the metabolism of glucose and fructose.

Thermoactinomyces sp. was not able to grow in any of the organic acid media and Streptomyces sp. was able to grow only in the pyruvic acid medium. These acids are commonly found in vegetative matter and malic, fumaric, succinic, pyruvic, and citric acids are implicated in the tricarboxylic acid cycle of glucose metabolisim. If this cycle is operative in these organisms, it is expected that the intermediates of the cycle would be utilized. However, this was found not to be true with the exception of pyruvic acid in the case of the Streptomyces species. Succinic acid and pyruvic acid might also be expected to be utilized since they can be formed by the deamination of the aspartic acid and alanine respectively and deamination is a primary function when the actinomycetes are grown in protein or amino acid media.

The average mycelial weights which were determined at various periods of incubation when plotted graphically result in curves which bear a close resemblance to growth curves of bacterial cultures. There is a lag phase during the early hours of incubation where the mycelial weight increases but slightly. This occurs even though a vigorous culture is used for the inoculum. This lag phase which lasts for 3 to 9 hours is follow-

ed by a rapid acceleration in growth. With both species, the most accelerated growth is observed when phytone is used as the medium. This would be expected since phytone is a partially hydrolized protein preparation. The slowest acceleration is found with gelatin and growth acceleration on casein is found to be intermediate between phytone and gelatin. The largest amount of mycelium was obtained from the casein medium and this was especially true in the case of the Streptomyces. Casein contains more amino acids than the other two proteins so that it is possible that it furnishes more of the constituents needed for the growth of these organisms. The peak of growth is reached in 12 to 24 hours depending upon the medium and the species of organism.

After the maximum amount of growth occurs, there is a rather rapid decrease is the amount of mycelium obtained. This decrease must be due to an autolysis because without such a phenomenon the weight of mycelium would at least remain at the maximum level. Katznelson (1940) also observed autolysis in a thermophilic actinomycete which he had isolated. The deceleration of growth or decrease in mycelial weight continues until about 36 hours at which time it begins to level off and produces only a very slight decrease between 48 and 72 hours. It is apparent that autolysis of the culture occurs at a more rapid rate than growth of new cells. Subcultures can be made of the organisms during the rapid autolytic

period and also between 48 and 72 hours when the curves indicate that the cultures are essentially stabilized.

During the growth of the actinomycetes on a protein medium there is a considerable amount of ammonia released into the medium. Graphical interpretation of the data collected from ammonia determinations show that a sigmoid type curve is produced. Up to the point of maximum growth, the release of ammonia into the medium closely parallels tha amount of mycelium produced. Beyond this point the amount of ammonia in the medium continues to increase but at a steadily decreasing rate until there is only a slight increase from 48 to 72 hours. The continued increase in ammonia during the time of decreased mycelial weights or autolysis is further evidence that metabolism is occurring during this phase.

The amount of ammonia released into the phytone medium by both species is very similar and it is also much less than that released from the other two proteins. However, since phytone is already partially hydrolized and has a smaller total nitrogen content less amino groups are present for deamination. The ammonia in the casein and gelatin media of the Streptomyces species are essentially the same throughout the entire incubation period. However, nearly twice as much growth, as indicated by mycelial weight, was produced from the casein as from the gelatin. This indicates that more deamination occurred in the casein medium but a great amount of re-

leased ammonia was utilized in the propagation of new cellular material. In the Thermoactinomyces culture about 25 per cent more ammonia was produced from the gelatin than from the casein but from the standpoint of growth about 25 per cent more mycelium was produced from the casein medium. In both cultures more growth was obtained from the phytone medium than from the gelatin but considerably less ammonia was produced from the phytone. It becomes obvious that although there is a positive relationship between growth and ammonia production the amount of ammonia found in a medium does not indicate the amount of mycelium produced.

The pH of the three protein media during growth of the cultures shows some variations within each medium but in general there is a definite trend in pH produced by each organism on all three media. The Thermoactinomyces species has a tendency to lower the pH or increase the hydrogen ion concentration whereas the Streptomyces species tends to increase the pH or decrease the hydrogen ion concentration. The difference in pH trend for each of these organisms suggests that their metabolic products are different and thus their metabolic pathways would also be different.

Although there is a difference in pH trend for each organism, the pH remains in the alkaline range during the total incubation period. Investigations on the desirable pH for these organisms revealed that

they prefer a slightly alkaline medium. It was also observed that these thermophiles prefer a protein or protein-like medium for good growth. When grown in a protein, these cultures produce a quantity of ammonia which is greatly in excess of their requirements and which is released by deamination. Gale (1940) demonstrated that the deaminase system of Escherichia coli functions between pH 6.0 and 8.5 and the optimum pH for the system is 7.5 to 8.0. From the data gathered in this study, it appears evident that the two thermophilic actinomycetes have a strong deaminase system and the desirable conditions of growth and metabolic activity center about this enzyme system. Even when supplied with a fermentable sugar and a usable nitrogen source, the growth is not as abundant as that produced on a desirable protein medium.

A number of free amino acids were identified in the culture filtrates of the two organisms grown in casein and gelatin media. There were also a large number of unidentifiable ninhydrin reactive substances. The majority of these were observed in the early hours of incubation and they were of low Rf values which suggests that they may have been small fragments of protein hydrolysis, e.g. small peptides. These low Rf value spots diminished in number with increasing incubation time which further indicates that they were protein fragments. The amino acids vary somewhat depend-

ing upon the protein source. In general, most of the identified amino acids and amines appeared more than once throughout the entire incubation period.

In the Thermoactinomyces culture of the casein medium, glutamine and the following amino acids were identified: cystine, cysteine, lysine, histidine, glutamic acid, serine, alanine, proline, valine, aspartic acid, phdroxyproline, isoleucine, tryptophane, phenylalanine, and methionine. Essentially the same amino acids with the addition of arginine, threonine, and asparagine were identified in the gelatin medium but in much smaller groups per incubation period. In both of these protein media cystine, cysteine, lysine, hydroxyproline, and histidine appear very frequently if not in every sample throughout the total growth period. Such predominance in both media suggests that these amino acids are metabolized sparingly or not at all by this species of Thermoactinomyces. Some of the other amino acids appear often but not continuously while others appear only once or twice. These amino acids are probably utilized by the growing organism.

The amino acids and amines identified in the casein medium cultured with the Streptomyces species are: cystine, cysteine, lysine, histidine, arginine, glutamic acid, hydroxyproline, threonine, alanine, proline, valine, aspartic acid, glutamine, and asparagine. In the

gelatin medium, lysine, glutamine, threonine, arginine, hydroxyproline, histidine, and aspartic acid appear but mainly only in groups of two or three for each incubation period. Lysine appears very frequently in both of these media which indicates that this amino acid is not utilized to any extent by the Streptomyces species. Glutamic acid also appears frequently in the culture filtrates but it is felt that its appearance is due to the large amount of the amino acid that is contained in casein.

In consideration of both cultures of the actinomycetes, the largest groups of amino acids were produced from the casein medium. At times as many as ten amino acids were identified in a culture filtrate. The abundance of different amino acids found in the culture filtrates and the amount of growth obtained from casein indicates that the proteolytic enzymes of these two organisms are well adapted to the degradation of casein. The gelatin medium contained smaller groups of amino acids and the Streptomyces culture produced the least number of different amino acids from gelatin. The consistent appearance of only a few amino acids in this culture is reflected by the lesser amount of growth of the Streptomyces on gelatin.

The numbers of amino acids identified in these culture media of gelatin and casein cannot be considered as being entirely due to the hydrolytic derivatives of these

two proteins. The culture organisms undergo autolysis and may release amino acids into the culture medium. That such a process occurs is indicated by the presence of asparagine and glutamine early in the incubation period even while the organism is undergoing rapid growth. However, the numbers of amino acids released by autolysis cannot be too pronounced since the number of identified amino acids in the culture filtrates during the period of decreasing mycelial weights does not increase to any extent. This is especially noted in the Streptomyces cultures where the number of amino acids remain the same or decrease. The presence of free amino acids in the culture filtrates during the growth of these organisms indicates that during the hydrolytic process of proteolysis individual amino acids are split-off. It is probable that these amino acids are then deaminated to produce ammonia as a nitrogen source for metabolism and the remaining residue provides the carbon source for metabolic processes.

SUMMARY

From a number of thermophilic actinomycetes isolated from an active vegetative compost, two species, each representing a different group, were selected for further study. Microscopic examination revealed that one species produces spores in chains and thus is a thermophilic member of the genus Streptomyces. The other species produces single spores on extremely

short sporophores branching directly from the hypha. This organism may be included in the genue Micromonospora (Bergey) or the proposed genus Thermoactinomyces (Waksman and Corke). The latter genus is preferred by the author since it has more validity for thermophilic forms. Both of the organisms are gram positive and non-acid fast.

Temperature studies revealed that neither of the organisms could develope growth at temperatures below 35 C or above 59 C and the optimum temperature is 50 to 52 C. Optimum growth of the organisms occurs when the pH of the medium is between 7.0 and 8.5. Very little if any growth is obtained below pH 6.0 or above 9.0.

Observations on growth characteristics of these actinomycetes were made using 21 different media. Both of the species are strict aerobes producing only surface growth in broth and stab cultures. On agar media which produced good growth, the Thermoactinomyces sp. produces a very light cream colored vegetative mycelium and a white to very light grey aerial mycelium which appears dry and fragile. The Streptomyces sp. also has a cream colored vegetative mycelium which produces a white aerial mycelium that becomes grey with the production of spores. Neither of the species produces any pigmentation on the media used. Both of the organisms liquefy gelatin and cause proteolysis of milk after the formation of a curd but they differ in nitrate reaction; the Streptomyces sp. producing a positive nitrite test whereas the Thermoactino-

myces ap. has a negative nitrite reaction. No cellulolytic activity was observed for either species but both developed growth in protein solutions of keratin, gluten, zein, casein, gelatin, and phytone.

Each of the organisms was inoculated into the following carbohydrate solutions of pH 7.0: maltose, mannitol, dextrin, sorbose, cellobiose, fructose, rhamnose, sucrose, and glucose. The Streptomyces species is able to ferment all except sorbose, rhamnose, and lactose. The Thermoactinomyces is able to ferment only fructose, glucose, and sucrose and then only slightly. Malic, fumaric, succinic, pyruvic, lactin, oxalic, and tartaric acids buffered at pH 7.3 to 7.5 were also inoculated but only pyruvic acid is utilized and then only slightly by Streptomyces sp.

The proteolytic activity of these thermophilic actinomycetes was studied using casein, gelatin, and phytone in a basal salts solution. Each organism was inoculated into individual series of flasks with each series containing one of the three protein media. Individual flasks were removed at various periods of incubation up to 72 hours. From each sample flask mycelial weights were determined, amount of ammonia was determined, and paper chromatograms were made to identify free amino acids present in the culture filtrate. Mycelial weights plotted against incubation time gave curves similar to bacterial growth curves but the decelerated growth is

actually due to autolysis of the mycelium. A considerable amount of ammonia in excess of physiological requirements was released into the protein medium. The ammonia is the result of the deamination of amino acids released by the proteolytic processes of the actinomycetes.

Seventeen amino acids and two amines were identified in the culture filtrates. The identified compounds were: cystine, cysteine, lysine, asparagine, histidine, arginine, glutamine, glutamic acid, aspartic acid, serine, hydroxyproline, threonine, alanine, proline, tryptophane, methionine, valine, phenylalanine, and isoleucine. Larger numbers of amino acids were found in cultured casein filtrates than in cultured gelatin filtrates. The number of amino acids found, the amount of growth produced on these proteins, and the amount of ammonia produced indicate that these organisms have a strong deaminase system and their proteolytic activity centers about this system.

APPENDIX

Formulae of Media

Corn Meal Agar

Corn meal, infusion from	50.0g
Glucose	2.0g
Agar	15.0g
Water, distilled	1000.0ml

Corn Steep Medium

Peptone	5.0g
Corn steep	15.0g
Sodium chloride	5.0g
Glucose	10.0g
Water, distilled	1000.0ml

Czapek's Agar

Sodium nitrate	2.0g
Potassium monohydrogen phosphate	1.0g
Magnesium sulfate	0.5g
Potassium chloride	0.5g
Ferrous sulfate	0.01g
Sucrose	30.0g
Agar	15.0g
Water, distilled	1000.0ml

Dubos' Cellulose Medium

Sodium nitrate	0.5g
Dipotassium phosphate	1.0g
Magnesium sulfate	0.5g
Potassium chloride	0.5g
Ferric sulfate	trace
Water, distilled	1000.0ml

Emerson's Agar

Beef extract	4.0g
Peptone	4.0g
Sodium chloride	2.5g
Yeast extract	1.0g
Glucose	10.0g
Agar	20.0g
Water, distilled	1000.0ml

Glucose-Asparagine Agar

Glucose	10.0g
Asparagine	0.5g

Dipotassium phosphate	0.5g
Agar	15.0g
Water, distilled	1000.0ml

Glucose-Peptone A Agar

Peptone	5.0g
Glucose	20.0g
Sodium chloride	5.0g
Agar	15.0g
Water, distilled	1000.0ml

Glucose-Peptone B Agar

Peptone	5.0g
Glucose	10.0g
Potassium dihydrogen phosphate	1.0g
Magnesium sulfate	5.0g
Agar	15.0g
Water, distilled	1000.0ml

Glycerol Agar

Glycerol	10.0g
Sodium asparaginate	1.0g
Dipotassium phosphate	1.0g
Agar	15.0g
Tapwater	1000.0ml

Nutrient Agar

Beef extract	3.0g
Peptone	5.0g
Sodium chloride	5.0g
Agar	15.0g
Water, distilled	1000.0ml

Sabouraud's Dextrose Agar

Peptone	10.0g
Dextrose	40.0g
Agar	15.0g
Water, distilled	1000.0ml

Starch Agar

Starch (Potato or Corn)	10.0g
Dipotassium phosphate	0.3g
Magnesium carbonate	1.0g
Sodium chloride	0.5g
Sodium nitrate	1.0g
Agar	15.0g
Water, distilled	1000.0ml

Neutralize and autoclave for 30 minutes at 110 C.

Yeast Glucose Agar

Yeast extract	10.0g
Glucose	10.0g
Sodium chloride	5.0g
Magnesium sulfate	0.25g
Ferrous sulfate	0.01g
Agar	15.0g
Water, distilled	1000.0ml

REFERENCES

Balston, J.N. and Talbot, B.F. 1952 A guide to filter paper and cellulose powder chromatography. H. Reeve Angel and Company Limited, London.

Bernstein, A. and Morton, H.E. 1934 A new thermophilic actinomyces. J. Bact., 27, 625.

Block, R.J. 1950 Estimation of amino acids and amines on paper chromatograms. Anal. Chem., 22, 1327.

Block, R.J., Durrum, E.L. and Zweig, G. 1955 A manual of paper chromatography and paper electrophoresis. Academic press, Inc., New York.

Breed, R.S., Murray, E.G.D. and Hitchens, A. Parker 1948 Bergey's manual of determinative bacteriology. Sixth Edition. The William and Wilkins Co., Baltimore, Md.

Brimley, R.C. and Barrett, F.C. 1953 Practical chromatography. Reinhold Publishing Co., New York.

Corum, C.J., Stark, W.M., Wild, G.M., and Bird, H.D., Jr. 1954 Biochemical changes in a chemically defined medium by submerged cultures of Streptomyces erythreus. Applied Microbiol., 2, 326-329.

Du Bos, Rene J. 1928 The decomposition of cellulose by aerobic bacteria. J. Bact., 15, 223-234.

Emerson, R.L., Whiffen, A.J., Bohonos, N., and De Boer, C. 1946 Studies on the production of antibiotics by actinomycetes and molds. J. Bact., 52, 357-366.

Erikson, D. 1952 Temperature-growth relationship of a thermophilic actinomycete, Micromonospora vulgaris. Jour. Gen. Microbiol. 6, 286.

Foster, J.W. 1949 Chemical activities of fungi. Academic Press, Inc., New York, N.Y.

Gale, E.F. 1940 Enzymes concerned in the primary utilization of amino acids by bacteria. Bact. Rev. 4, 135.

Gale, E.F. 1948 The chemical activities of bacteria. Academic Press, Inc., New York, N.Y.

Gershenfeld, L. and Bernstein, A. 1951 Detection of a free amino acid in filtrate of E. coli culture medium by paper chromatography. Amer. Jour. Pharm. 123, 237-245.

Gilbert, A. 1904 Uber Actinomyces thermophilus and andere Actinomyceten. Ztschr. Hyg. 47, 383.

Globig, L. 1888 Bakterien-Wachstum bei 50 bis 70. Ztschr. Hyg. 3, 294.

Gottlieb, D., Bhattacharyya, P.K., Anderson, H.W. and Carter, H.E. 1948 Some properties of an antibiotic obtained from species of Streptomyces. J. Bact. 55, 409-417.

Greenberg, D.M. 1951 Amino acids and proteins; theory, methods, applications. Charles C. Thomas, Publ. Springfield, Ill.

Hamilton, P.B. and Ortiz, P.J. 1950 Purification of ninhydrin by crystalization. Anal. Chem. 22, 948-949.

Hawk, P.B., Oser, B.L. and Summerson, W.H. 1947 Practical physiological chemistry. 12th. Ed. The Blakiston Co., Philadelphia, Pa.

Katznelson, L. 1940 Autolysis of a thermophilic Actinomyces. Soil Sci. 49, 83.

Kedzier, D. 1896 Uber eine thermophile Cladothrix. Arch. fur Hyg. 27, 328.

Miehe, H. 1907 Die Selbsterhitzung des Heues. Jena.

Noack, K. 1912 Beitrage zur Biologie der thermophilen Organismen. Jahrb. Wiss. Bot. 57, 593.

Riddell, R.W. 1950 Permanent stained mycological preparations obtained by slide culture. Mycologia. 42, 265.

Robinowitsch, L. 1895 Ueber die thermophilen Bakterien. Ztschr. Hyg. 20, 154.

Schmidt, C.L.A. 1945 The chemistry of the amino acids and proteins. Charles C. Thomas, Publ. Springfield, Ill.

Schutze, H. 1908 Beitrage zur Kenntnis der thermophilen Aktinomyzeten und ihner Sporenbildung. Arch. fur Hyg. 67, 35.

Smith, N.R. 1946 Aerobic mesophilic spore-forming bacteria. U.S. Dept. of Agri. Misc. Publ. No 559.

Society of American Bacteriologists 1944 Manual of methods for pure culture study bacteria. 9th. Ed. Biotech Publications, Geneva, N.Y.

Tsiklinsky, P. 1899 Sur les mucedinees thermophiles. Ann. Inst. Pasteur 13, 500.

Waksman, S.A. 1918 Studies on the proteolytic enzymes of soil fungi and actinomyces. J. Bact. 3, 509-530.

Waksman, S.A. 1920 Studies in the matabolism of actinomyces. III. Nitrogen metabolism. J. Bact. 5, 1-30, 31-49.

Waksman, S.A. 1950 The Actinomycetes; their nature, occurrence, activities, and importance. Chronica Botanica Co., Waltham, Mass.

Waksman, S.A. and Cordon, T.C. 1939 Thermophilic decomposition of plant residues in composts by pure and mixed cultures of microorganisms. Soil Sci. 47, 217-225.

Waksman, S.A., Cordon, T.C. and Hulpoi, N. Influence of temperature upon the microbiological population and decomposition in composts of stable manure. Soil Sci. 47, 83.

Waksman, S.A. and Corke, C.T. 1953 Thermoactinomyces Tsiklinsky, a genus of thermophilic actinomycetes. J. Bact. 66, 377.

Waksman, S.A. and Joffe, J. 1920 Changes in reaction as result of the growth of actinomycetes upon culture media. J. Bact. 5, 31-48.

Waksman, S.A. and Lechevalier, H.A. 1953 Guide to the classification and identification of the actinomycetes and their antibiotics. The Williams and Wilkins Co., Baltimore, Md.

Waksman, S.A. and Lomanitz, S. 1925 Contribution to the chemistry of decomposition of proteins and amino acids by various groups of microorganisms. J. Agr. Res. 30, 263-281.

Waksman, S.A. and Starkey, R.L. 1932 The decomposition of proteins by microorganisms with particular reference to purified vegetable proteins. J. Bact. 23, 405-428.

Waksman, S.A., Umbreit, W.W. and Cordon, T.C. 1939 Thermophilic actinomycetes and fungi in soils and in composts. Soil Sci. 47, 37.