

PRODUCTION AND ISOLATION OF THERMOVIRIDIN,
AN ANTIBIOTIC PRODUCED BY THERMOACTINOMYCES VIRIDIS N. SP.

By

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AN ABSTRACT

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Approved

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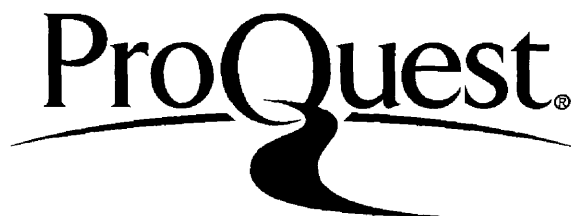
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AN ABSTRACT

A thermophilic actinomycete was isolated from a composted manure pile. Since no previous description of this organism was found, it was considered a new species of the genus Thermoactinomyces (Tsiklinsky). The name Thermoactinomyces viridis was suggested for the organism, and the name thermoviridin for the antibiotic produced by it.

Thermoviridin was produced initially in stationary bottle cultures. Subsequent production was carried out on a Gump rotary shaker for the most part, and also in a 30 liter laboratory fermenter.

The fermentation medium consisted of Bacto tryptone 4%, Bacto beef extract 0.5%, and had a pH of 6.9 - 7.1. The addition of a number of nutrients to this medium did not increase antibiotic potency above the 32 units per ml obtained in the basal medium. Maximum antibiotic production was reached in 27 hours at 45°C.

Recovery of thermoviridin was accomplished by acid precipitation at pH 3. The precipitate was washed with water and then extracted with a quantity of 80% acetone equal to one-tenth the original volume of the antibiotic beer. The acetone extract was evaporated, and the remaining water residue was dried from the frozen state. The solid material obtained by this procedure had 64 units per mg activity against M. pyogenes var. aureus 209P.

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Some purification of the active solid material was accomplished by fractional precipitation from methanol using ethyl ether as the precipitating agent. The countercurrent distribution method of Craig was also an effective method of purification. A preparation having 270 units per mg activity was obtained by this method, using a butanol-water system buffered at pH 6.

Thermoviridin was found to be stable at pH 2 for 21 hours at 37°C, but at pH 10 for the same time period approximately 75% of the antibiotic activity was lost. The stability of thermoviridin to temperatures higher than 37°C was not investigated.

Chemical tests and solubility data have indicated that thermoviridin is an organic acid which contains no sulfur or phenyl groups. The active material appeared to be non-protein and non-carbohydrate in nature. Thermoviridin was dialyzable and could be precipitated with saturated ammonium sulfate.

The antibiotic preparations tested showed maximum absorption in the range of 268-272mμ.

Thermoviridin was primarily active against the gram positive bacteria tested. Preliminary toxicity tests in mice, consisting of a single intraperitoneal injection of 32 mg of material (800 units), did not cause death in any case. An autopsy of the mice showed no abnormalities in the gross appearance of the organs.

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INTRODUCTION

In the course of the continuing search for new and superior antibiotics, the goal has been to isolate cultures which produce antibiotics differing from those already known. For this reason some investigators have attempted to obtain cultures from unusual sources, thereby increasing the chance of isolating organisms which are unique. A second method of obtaining antibiotic-producing organisms which are unique is to investigate some group of organisms which has not as yet been tapped as a source of antibiotic producers.

The thermophilic actinomycetes comprise one such group. A search of the literature indicated that these organisms have not been widely investigated as a source of antibiotic producers. Here then, is an area where any antibiotic-producing organisms isolated might well be different from those previously isolated.

In addition to the apparent neglect of these organisms with respect to antibiotic production, it seemed advantageous to investigate them for two other reasons: 1. It was considered possible that an antibiotic produced during a thermophilic fermentation might be heat stable. 2. Since the respiratory rate of thermophilic organisms is high, the time required for maximum antibiotic production should be shorter than in the case of mesophilic fermentations.

For these reasons a program for the screening of thermophilic actinomycetes was established. After the isolation and screening of 174 thermophilic cultures, one organism was chosen for further investigation. This organism was found to be a new species in the genus Thermoactinomyces (Tsiklinsky). The name Thermoactinomyces viridis was given to the organism, and the antibiotic produced by it was named thermoviridin.

The work done on the selected organism and the antibiotic produced by it is to be presented here. This includes the isolation and classification of the organism, followed by studies on production media and conditions, recovery, purification, chemical properties, toxicity and microbial spectrum of the antibiotic.

HISTORICAL

The first reported investigation of the thermophilic actinomycetes was published in 1888 by Globig (15). He was not the first investigator to observe these organisms, however, for he mentioned the previous observations of Geheimrath Koch, who influenced Globig to turn his efforts to the study of these organisms.

Globig found potato slices to be the most satisfactory medium for the isolation of these organisms. After developing the technique for isolation and cultivation of the thermophilic organisms, he began searching for them in all types of materials. In the course of his investigation he examined the feces of man, dog, guinea pig, rabbit, horse, pigeon and mouse. He also examined the canal water and tap water, as well as bored samples obtained from the earth down to a depth of two meters. He found thermophilic organisms at all levels of depth tested. Soil cores from several foreign countries were also cultured.

Globig's curiosity concerning the possible habitats of these organisms also lead him to examine laboratory benches, floors, dust and the hot-air oven of the Institute of Hygiene in Berlin.

The interest aroused by Globig's report concerning these novel, heat-loving organisms resulted in several subsequent publications dealing largely with the description of organisms

isolated and the sources from which they were obtained. However, there was no clear cut distinction between the bacteria and the actinomycetes. These early reports often refer to the thermophilic actinomycetes as "bacteria" having thread-like forms.

Rabinowitsch (27) examined the excrements of the horse, cow, dog, guinea pig, mouse and fish, and found thermophilic actinomycetes in all cases. Manure suspended in water was incubated 18-24 hours at 62°C. The suspension was then used to inoculate agar plates. Colonies appeared in 16-24 hours.

Kedzior (18) isolated a thermophilic actinomycete from sewage and described its growth on several media. The organism grew at temperatures from 35° to 65°C, with an optimum growth temperature of approximately 55°C. The spores produced by this organism were found to be resistant to heat, 5% phenol, and to desiccation.

Tsiklinsky (34) described three organisms isolated from various composts. The first organism, Thermoactinomyces vulgaris, was isolated from several sources including soil, hay, straw, manure and potato. The hyphae of this organism were 0.5u in diameter, with round or oval spores borne singly on short conidia. The spores were found to survive at 100°C for 20 minutes, and to withstand 5% phenol for 24 hours. The optimal growth temperature for the organism was about 57°C. T. vulgaris grew well on all ordinary liquid and solid media, and was found to be non-pathogenic to mice and guinea pigs.

A second actinomycete culture possessed hyphae of 1.2 - 1.5u in diameter with spores borne in chains. The spores did

not survive for 5 minutes at 100°C. This organism resembled that previously described by Kedzior in spore formation and staining reactions. It differed, however, in that no growth occurred at 35°C, and the culture had a marked odor.

The third organism, Thermomyces lanuginosus, grew well on apple sprinkled with garden soil. Contaminating thermophilic bacteria were eliminated by transferring the organism to white bread, followed by a transfer of spores to an agar plate. This procedure yielded a pure culture. This organism grew well on the usual nutrient media, and had an optimum growth temperature of 54-55°C. On agar the mycelium was downy white, later taking on a dark tint. The spores did not survive for one minute at 100°C.

Sames (29) isolated a "thermotolerant" actinomycete from raw milk. This organism had an optimum growth temperature of approximately 55°C, and grew anaerobically to some extent as well as aerobically. The growth of the organism on various solid and liquid media was described.

Several thermophilic actinomycetes were isolated from various soils by Gilbert (14). Actinomyces thermophilus grew well on potato medium at 55°C, possessed hyphae of 0.5 - 0.6u in diameter and spores of 0.8 - 1u in diameter. The growth of this organism and several other isolates was described.

Schütze (33) described two thermophilic actinomycetes isolated from self-heated hay. One of these organisms was Thermoactinomyces monospora. This organism produced a gray-green aerial mycelium, possessed hyphae of about 1u in diameter, oval spores of 1.5 - 1.8u, and had an optimum temperature

range of 37° - 55°C. The organism being reported in this work resembled T. monospora in all but a few characteristics.

Noack (25) isolated a number of thermophilic actinomycetes and fungi from moist hay held at 45° - 46°C. Miehe (23) also isolated many actinomycetes from hay but only a few of these were thermophilic. He suggested that thermophilic actinomycetes probably arise from self-heating plant masses such as manure.

Lieske (20) discussed the origin of thermophilic actinomycetes and their ability to survive under conditions which are seldom optimum. He suggested that thermophiles might be mutants of mesophilic actinomycetes.

Lieske thought that the origin of these thermophilic forms could be traced back to a sudden change in temperature requirement. He did not believe that thermophilic forms could be developed by gradual temperature rise. This sudden change or mutation involving temperature requirement was also believed to be reversible. Lieske concluded his discussion by stating that no conclusive evidence was available and that a re-examination of the theories expressed up to that time was in order.

With two exceptions, the above reports were published prior to 1910. Methods of isolation, and descriptions of isolates were in many cases incomplete, giving rise to much confusion regarding the true identity of the organisms isolated. The current publications dealing with the thermophilic actinomycetes are relatively few in number.

A thermophilic actinomycete was isolated repeatedly from pasteurized cheese by Bernstein and Morton (2). The organism had an optimum growth temperature of about 56°C. The name Actinomyces casei was suggested for this organism.

Katznelson (17) isolated a thermophilic actinomycete from horse manure compost held at 50°C. When the organism was grown on a starch-ammonium sulfate agar medium, autolysis was noted after a certain period of incubation at 50°C. After a study of this autolytic process, Katznelson concluded that the autolytic mechanism was activated when the pH of the medium reached pH 6.0 - 6.5. No transmissible lytic agent could be demonstrated.

Micromonospora vulgaris (Tsiklinsky) has been isolated by Erikson (12) from composts made from lawn cuttings. The organism was found to have an optimum growth range of 45° - 60°C. Heating a spore suspension of M. vulgaris for 5 minutes at 75° - 90°C induced growth in 18 hours, whereas unheated spores did not germinate during the same interval. Spores suspended in 1% sucrose withstood 100°C for periods up to 45 minutes. Spores borne on the aerial mycelium retained their viability and heat resistant properties after six months storage at 2°C. Erikson suggested that the organism survived periods of unfavorable growth conditions by means of its spores.

Waksman, Umbreit, and Cordon (42) studied the thermophilic actinomycete and fungal population of soils and composts. They noted that thermophilic actinomycetes were present in the soil in all seasons of the year. In winter

actinomycetes composed only a small percent of the thermophilic population, whereas in the spring they made up a large proportion of it. Thermophilic molds were far less abundant than the thermophilic actinomycetes at all seasons.

The survival rate of thermophilic actinomycetes in the soil was determined by treating portions of Sassafras sandy loam with the following: sterilized and unsterilized fresh manure, composted manure, and pure cultures of thermophilic actinomycetes. All samples were held at 28°C for 5 weeks. Plate counts at 3 and 5 weeks showed that the thermophilic population of the soil decreased rapidly at 28°C in all cases except where composts were added. Here the population increased.

In a second experiment the thermophilic population in horse manure was followed. Portions of fresh horse manure were incubated at 50° and 65°C and sampled at intervals. At both temperatures the highest number of thermophilic actinomycetes reached nine billion per gram of moist compost at 65°C, and three billion per gram of moist compost at 50°C. The thermophilic fungi numbered two hundred million per gram of moist compost after 10 days incubation at 50°C. The fungi found were for the most part members of the genus Thermomyces previously described by Tsiklinsky.

In a third experiment sterile manure was inoculated with pure cultures of thermophilic bacteria, actinomycetes, and fungi in an attempt to determine the role of thermophiles in decomposition of manure. After 42 hours incubation at 50°C, results indicated that neither bacteria nor actinomycetes in

pure culture were as effective as the normal mixed population of manure in bringing about decomposition.

The influence of temperature upon the microbial population and upon the rate of decomposition of composting stable manure has been investigated by Waksman, Cordon and Hulpoi (37). Portions of manure were held at 28°, 50°, 65°, and 75°C. Chemical analyses and counts on the number of organisms present were made periodically during 47 days of incubation.

Results indicated that the greatest amount of decomposition occurred in manure held at 50°C. Microbiologically, at 50°C the number of bacteria per gram of moist manure decreased quite rapidly early in the incubation period. This decrease was accompanied by an increase in the actinomycete and fungal population. At 65°C aerobic bacteria and fungi disappeared rapidly, whereas the actinomycete population increased. At 75°C only certain spore-forming bacteria survived.

In 1953 Waksman and Corke (38) described a thermophilic actinomycete, Thermoactinomyces thalpophilus. The organism was isolated from soil and high temperature composts, and had an optimum temperature of approximately 50°C. The growth characteristics of T. thalpophilus on various media were described.

The only previous report of an antibiotic produced by a thermophilic actinomycete was published by Schöne (32). The morphological and biochemical properties of the organism agreed well with Streptomyces thermophilus, first reported by Gilbert (14). The organism had an optimum temperature of about 60°C. Schöne called the antibiotic thermomycin. The bacterial

spectrum of thermomycin was rather narrow. It was active against 19 strains of Corynebacterium diphtheriae and inhibited Listeria monocytogenes only slightly. All other organisms tested were insensitive to thermomycin.

The antibiotic was produced in surface culture on a medium containing 10% blood serum and phosphate buffer. Maximum antibiotic activity was reached in 4 days at 60°C. A serial dilution titer of 256 units per ml was obtained in the culture filtrate, using C. diphtheriae PW8 as the assay organism.

Extraction of the culture filtrate with ether followed by evaporation yielded a yellow powder, which in a concentration of 5% inhibited C. diphtheriae at a dilution of 1:4096. Thermomycin could be completely salted out by one-half saturated ammonium sulfate. The antibiotic was found to be non-dialyzable. Regarding stability, 15 minutes at 75°C reduced the C. diphtheriae titer 25%, whereas 15 minutes at 100°C destroyed activity completely.

Injection of 2 ml of antibiotic beer (256 units per ml) into each of the 20 mice did not cause a toxic reaction. There was also no irritation when crude filtrate was placed on the cornea of a rabbit.

"Schöne noted that S. thermophilus produced rough as well as smooth colonies, which indicated dissociation. Rough colonies were characterized by an irregularly folded surface and edge. Smooth colonies were circular with an even edge, relatively smooth surface, and were smaller than the rough type.

Rough colonies produced an antibiotic titer of 4-8 units per ml in the crude filtrate, whereas smooth colonies produced a titer of 256 units per ml.

MATERIALS AND METHODS

Screening Methods

Thermoactinomyces viridis was isolated from a composted manure pile on property belonging to the Michigan Department of Health. The material was sampled at a depth of 4 to 6 inches from the surface. One gram of sample was diluted 10^{-2} and 10^{-3} , and direct platings of 0.5, 0.2 and 0.1 gram were also made. The dilutions and direct samplings were plated out in duplicate using sodium caseinate agar and a medium which will be referred to as trypticase agar. This medium was very similar to the nutrient agar for penicillin assay specified by the Food and Drug Administration (5). Trypticase was used as the pancreatic digest of casein, and the medium was modified by the addition of sodium chloride. This modified formulation was also used extensively in this work as a liquid medium. The formulae of these agar media are presented in Table I.

It has been found by Murray (24) and Erikson (12) that a water saturated atmosphere is necessary for the cultivation of certain thermophilic organisms on agar media. The isolation plates were, therefore, incubated at 50°C for 3-5 days in an atmosphere saturated with water. Colonies were picked and transferred to sodium caseinate agar slants, which were then incubated in the same manner as the isolation plates. The slant cultures were stored at 25°C. T. viridis was

TABLE I
AGAR MEDIA USED IN SCREENING

Trypticase agar		Sodium caseinate Agar	
Component	Concentration per liter	Component	Concentration per liter
Bacto peptone	6.0 gm	Sodium caseinate	2.0 gm
Trypticase	4.0 gm	Glucose	1.0 gm
Bacto beef extract	3.0 gm	K ₂ HPO ₄	0.2 gm
Glucose	1.0 gm	MgSO ₄ ·7H ₂ O	0.2 gm
Sodium chloride	5.0 gm	Bacto peptone	1.0 gm
Dist. water to make 1000 ml		Glycerine	5.0 gm
pH 7.0		Dist. water to make 1000 ml	
		pH 7.3	

maintained on nutrient agar slants since growth on sodium caseinate agar was poor. The nutrient agar contained Bacto peptone 1%, Bacto beef extract 0.5%, sodium chloride 0.5%, and had a pH of 6.8 - 7.0.

Antibiotic activity against 7 test organisms was determined by an agar cross-streak method similar to that of Waksman's (40). The actinomycete was streaked on nutrient agar and incubated for 3 days at 50°C, at which time it was cross-streaked with 7 test organisms and re-incubated at 37°C. The test organisms were Mycobacterium 607, Salmonella typhimurium, Micrococcus pyogenes var. aureus 209P, Escherichia coli, E. coli 0111, Proteus vulgaris, and Bacillus subtilis 6633.

Cross-streak results were read after 18-24 hours incubation, except for Mycobacterium 607 results which were read after 36-48 hours incubation.

Ultraviolet Irradiation Method

A spore suspension of Thermoactinomyces viridis was subjected to ultraviolet irradiation in an attempt to obtain a mutant which would give increased yields of thermoviridin. The growth from an agar slant culture of T. viridis was suspended in 100 ml of sterile distilled water. The suspension was shaken vigorously for 5 minutes to distribute the spores as evenly as possible. Large clumps and fragments were then allowed to settle, and 21 ml of the suspension was carefully withdrawn and placed in a sterile Petri dish.

The spore suspension in the opened Petri dish was exposed to the ultraviolet rays from a 15 watt General Electric

germicidal lamp placed 6 inches above the spore suspension. The suspension was stirred continuously throughout the exposure period by means of a magnetic stirrer. Samples were withdrawn after 0, 1, 2, 4, 8 and 16 minutes exposure. The samples were plated out in nutrient agar (Bacto peptone 1%, Bacto beef extract 0.5%, sodium chloride 0.5%, pH 6.8 - 7.0).

After incubation at 45°C for 5 days, total counts were made. The exposure time of 2 minutes was chosen as the proper time for subsequent irradiation studies.

After 2 minutes exposure using the above procedure, 4 ml of the irradiated spore suspension was added to 100 ml of sterile distilled water. Forty ml of this dilution was plated out in 1 ml amounts in nutrient agar.

The plates were incubated 4 days at 45°C and colonies were then picked and transferred to nutrient agar slants. After incubation these subcultures were tested for antibiotic activity by the agar cross-streak method.

Assay Methods

In the course of this work antibiotic activity was determined by three assay methods: 1) serial dilution, 2) paper disc, and 3) agar dilution. Unfortunately, no one method was found to be satisfactory for all cases in which an assay of antibiotic activity was desired.

The thermoviridin unit was based on the serial dilution test. The unit was defined as the minimum amount of thermoviridin in 1 ml which inhibited the growth of a 1% inoculum of M. pyogenes var. aureus 209P in trypticase broth for 24 hours at 37°C.

The serial dilution assay method was used for the determination of the bacterial spectrum of thermoviridin, and for following antibiotic activity in studies on media development and purification. The test was performed following a procedure similar to that of Schmidt and Moyer (31). Trypticase broth was inoculated with an 18-24 hour culture of the test organism in the amount of 1% and then dispensed into sterile tubes. In addition to the usual two-fold series, a dilution series which increased by increments of 10 units was frequently used. Controls for the test included sample sterility, broth sterility, and test organism growth.

Samples for assay were sterilized by filtration (Seitz or fritted glass), but in the case of solid preparations, samples were merely dissolved in methanol and assayed without further treatment. Methanol was found to inhibit the growth of M. pyogenes var. aureus 209P, the only organism used under these conditions, up to a dilution of 1:8. Any inhibition found in dilutions greater than 1:8 was attributed to thermoviridin.

As the second method of choice, the paper disc assay was used as a means of following antibiotic activity during studies on media development, recovery and purification. Assay plates contained 21 ml of trypticase agar as base coat and a 4 ml seed coat of the same medium. Seed coats were inoculated with an 18-24 hour culture of M. pyogenes var. aureus 209P in the amount of 1.4%, or with a B. subtilis 6633 spore suspension in the amount of 0.08%. The spore suspension contained 2×10^8 viable spores per ml. Seeded plates were stored at 0°-5°C for no longer than 1 week, and

warmed before use.

Paper discs 13 mm in diameter and capable of absorbing up to 0.1 ml of sample were used. The discs were made from sheets of E and D filter paper #623-.030. Assay plated seeded with B. subtilis 6633 were incubated 18-24 hours at room temperature, and those seeded with M. pyogenes var. aureus 209P were incubated 18-24 hours at 37°C. The zone of inhibition in all cases was considered to be the over-all diameter of the zone in millimeters.

Thirdly, the agar dilution method of assay was selected as the means of testing the sensitivity of several mycobacteria and pathogenic fungi to thermoviridin. The desired dilutions of the sample to be tested were made in the melted agar medium contained in sterile screw-capped tubes. When the slanted tubes had hardened, they were inoculated with the test organism. Tubes inoculated with the mycobacteria were incubated at 37°C and read after 3 and 6 weeks incubation. Tests involving the fungi were incubated at room temperature and read after 3 weeks incubation.

Fermentation Methods

Initial fermentation studies concerning thermoviridin production were carried out in stationary liquid cultures. Rectangular-shaped bottles were placed in a horizontal position and partially filled with 250 ml of culture medium. A layer of glass wool was added to insure adequate aeration and to support the mycelial growth. The bottles were plugged with rather loosely fitting gauze-covered cotton plugs to allow free transfer of gases. The bottle cultures were sterilized by autoclaving one-half hour at 121°C.

Stationary liquid cultures were inoculated by pooling the growth of several slant cultures of Thermoactinomyces viridis in sterile distilled water. Five ml of this suspension was distributed over the surface of the glass wool layer in each bottle. The cultures were then incubated 5 days at 50°C.

Most of the fermentation studies were carried out on a Gump rotary shaker in wide-mouth 500 ml Erlenmeyer flasks. The shaker operated at about 250 rpm, each flask describing a two and one-quarter inch diameter circle. Each flask contained 50 ml of medium and was plugged with gauze-covered cotton held in place over the lip of the flask with a rubber band. The flasks were autoclaved 30 minutes at 121°C except when insoluble particulate matter, such as the various grain products, was present. In these cases the flasks were autoclaved 45 minutes at 121°C.

The inoculum for the fermentations was prepared by transferring the growth from an agar slant culture of T. viridis (not more than 10 days old) to a 1 liter flask containing 100 ml of seed medium. The flask was shaken for about 48 hours at 45°C. A second flask of seed medium was inoculated with 2 ml of vegetative growth from the primary culture, and shaken for about 18 hours at 45°C. This secondary growth was used to inoculate the fermentation flasks, each flask receiving 1 ml of inoculum per 50 ml of medium. The procedure for inoculum preparation was also carried out at 37°C, in which case the primary seed flask was shaken for 72 hours, and the secondary flask for 45 hours. The seed medium contained Bacto tryptone 2%, Bacto beef extract 0.5%, and had a pH of 6.9 - 7.1.

Fermentations carried out at 45°C were shaken 26-28 hours, whereas fermentations at 37°C were shaken 72 hours. Nutrients tested in the development of the fermentation medium were added to a basal medium at levels of 0.5%, 1%, 2%, 4% and sometimes 8%. The basal medium contained Bacto tryptone 4%, Bacto beef extract 0.5%, and had a pH of 6.9 - 7.1. Those materials which appeared to enhance thermoviridin production were then added simultaneously to the basal medium. Each enhancing nutrient was added in its optimum concentration, and also at higher and lower levels. By altering the concentration of all the supplementary constituents, many variations of the same medium were obtained. This alteration of the balance of nutrients was undertaken because the optimum concentration for a given supplement when present alone in the basal medium may not necessarily be its optimum when other supplementing nutrients are present.

Thermoviridin was also produced in a 30 liter laboratory fermenter previously described by Olson, Jennings, Pisano and Junek (26). The fermenter contained 12 liters of fermentation medium and 150 ml (1.25%) of antifoam, which was mineral oil containing 5% octadecanol by weight. The fermenter containing a 12 liter charge was autoclaved 3 hours at 126°C.

The inoculum for the fermenter was 100 ml of a secondary culture obtained by the same procedure as for the preparation of inoculum for shake flask fermentations (see page 18).

During the fermentation, the temperature was 45°C, the air flow 0.6 volume of air per volume of medium per minute, and the impeller speed 530 rpm.

Recovery Methods

Recovery of thermoviridin was first attempted by solvent extraction. Portions of antibiotic beer were adjusted to pH 2, 4, 8 and 10. An equal amount of water immiscible solvent was added to each portion. The solvent-antibiotic beer mixtures were shaken for 1 minute and the layers allowed to separate. This shaking and separating process was repeated 3 times, and after the final separation both layers were assayed by the paper disc method. Solvents were evaporated from the discs at 37°C before being placed on the assay plates. Solvent controls evaporated in like manner were also assayed. Using this procedure the following solvents were tested: benzene, petroleum ether, ethyl ether, chloroform, butanol, amyl acetate, and xylene.

Solvent extraction was found to be an unsuitable method of recovery. The one selected may be called an acid precipitation method. After completion of the fermentation, the crude culture was adjusted to pH 5.5 - 6.5 and filtered using celite 545 as a filter aid. An alternate means used in separating the mycelium from the antibiotic beer was centrifugation.

The culture filtrate was adjusted to pH 3 with phosphoric acid and held at 0° - 5°C for at least 2 hours. Hydrochloric and sulfuric acids were also found to be equally effective as precipitating agents.

The resulting precipitate was recovered and washed with water. Thermoviridin was extracted from the precipitate with an amount of 80% acetone equal to one-tenth the original

volume of the culture filtrate. The acetone extract was then concentrated by evaporation at reduced pressure, and the water solution remaining was dried from the frozen state. The recovery procedure is illustrated graphically in figure 1.

It was found that thermoviridin could be precipitated by saturating the antibiotic beer with ammonium sulfate. This method was not used, however, because of the convenience and simplicity of the acid precipitation method.

Purification Methods

Two methods were found to be of value in the purification of thermoviridin. The first method involved fractional precipitation of the active material from a methanol solution, using ethyl ether as the precipitating agent. Crude dry antibiotic material was extracted with sufficient methanol to give an antibiotic concentration of 300 units per ml. Extraction of the antibiotic activity was effected during a 30 minute period of constant agitation with glass beads on a Gump rotary shaker. A second portion of methanol equal in volume to the first, was added to the insoluble residue, and the extraction process repeated.

The second methanol extract was separated from the insoluble residue and was combined with the first, giving a final antibiotic concentration of 150 units per ml of methanol. A quantity of ethyl ether equal to one-fourth the volume of the combined extracts was added to the methanol solution. The ethyl ether concentration was increased to one-half volume by the addition of a second one-fourth volume. In this manner the ethyl ether content was increased to a total

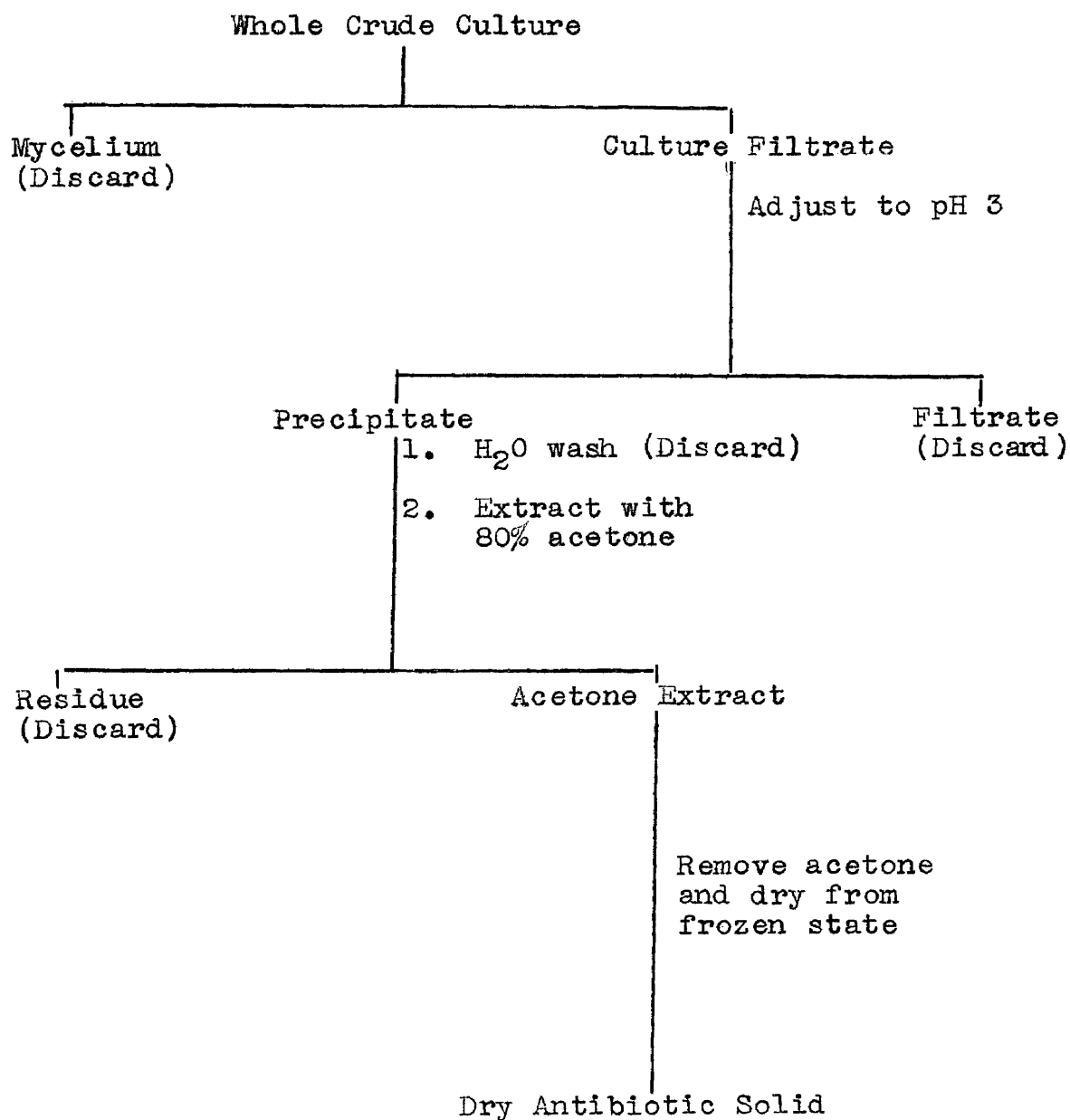


Figure 1. Procedure for the recovery of thermoviridin

concentration of 1, 2, 4, and 7 volumes. The precipitate which formed with the addition of each increment of ethyl ether was recovered and dried under reduced pressure. The dried precipitates were assayed by the serial dilution method.

The countercurrent distribution method of Craig (6) was also of value in the purification of thermoviridin. This procedure was carried out in stoppered test tubes containing equal amounts of n-butanol and 0.04 M phosphate buffer at pH 6, each mutually saturated with the other beforehand.

The pH at which the material to be purified had a distribution coefficient of approximately one was determined by assaying a butanol-water system containing the material after its adjustment to several pH values. Craig (7) found that in a series of any given number of tubes, the purest fraction would appear in the middle of the series when the distribution coefficient of the solute between the solvents approached one.

The antibiotic material to be purified was placed in tube number one containing equal volumes of butanol and buffer. The tube was shaken 25 times in a period of 30 seconds, and then centrifuged for 1 minute at 1,500 rpm to effect complete separation of the two layers. The water layer of tube one was transferred to tube two, which contained an equal volume of butanol. A fresh portion of buffer was then added to tube number one, and the shaking and centrifuging processes repeated. The water layers from tubes one and two were then transferred to the next consecutive tubes in the series (one to two, and two to three), and

buffer again added to tube number one. The above processes were continued until the material being purified was distributed in eight tubes.

Dry weight determinations were made on both the butanol and buffer fractions of all tubes, and on butanol and buffer controls. All fractions were disc assayed and the unitage read from a standard curve. The degree of purification was then calculated from the dry weight data.

Toxicity Study Method

An acute toxicity study of thermoviridin was conducted using mice. Twenty gram White Swiss mice of the Webster strain from the Michigan Department of Health colony were injected intraperitoneally with 800 units of thermoviridin. The solid material used for this study had an antibiotic activity of 25 units per mg.

After injection the mice were weighed periodically and observed for 3 weeks. At that time the mice were sacrificed and an autopsy was performed.

RESULTS

Cultural Characteristics

Thermoactinomyces viridis was found to have an optimum growth temperature of approximately 55°C. The organism grew slowly at 37°C and not at all at 60°C. At the optimum temperature, growth on agar was fully developed in 3 days. At 37°C growth was not maximum even after 14 days incubation. The hyphae were about 0.5u in diameter and bore oval conidia approximately 1.0u by 1.3u. The conidia were borne singly on short sporophores. The microscopic appearance of this organism may be seen in figure 2. The preparation shown in the photomicrograph is a portion of a slide culture of T. viridis prepared after the method of Riddell (28).

T. viridis is not acid fast and is gram negative. This is unusual since most actinomycetes are gram positive (36). However Lieske (20) found that certain thermophilic forms are gram negative. Using Burke's modification of the gram stain, and with M. pyogenes var. aureus 209P mixed in the preparation as a control, T. viridis was gram negative, whereas the control organism gave the typical gram positive reaction. The gram reaction did not depend on the age of the organism since a young vigorously growing shake flask culture and a fully grown shake flask culture both gave the gram negative reaction.

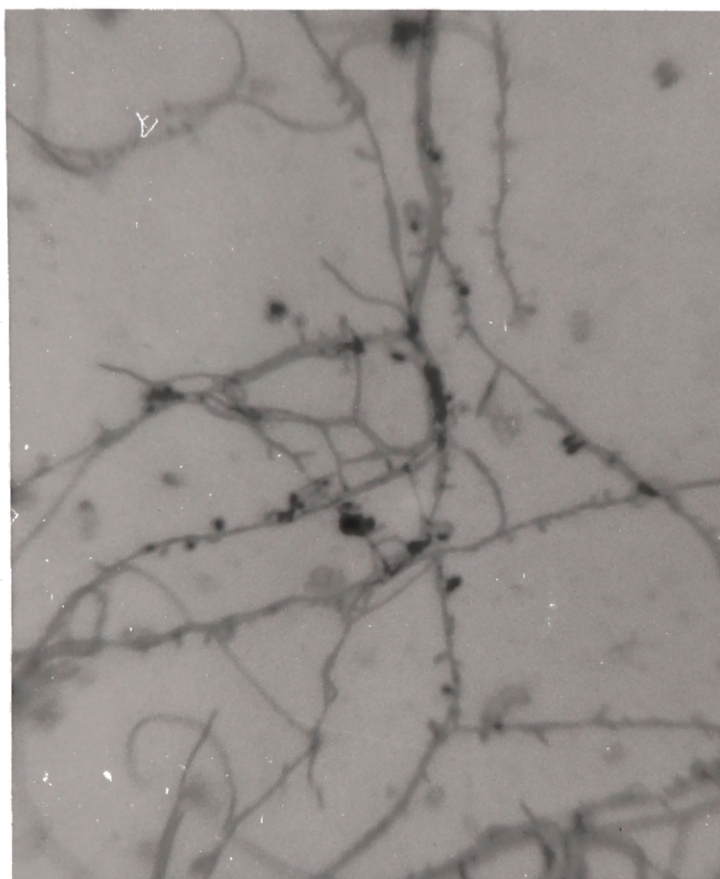


Figure 2. Thermoactinomyces viridis

The growth characteristics and biochemical reactions of T. viridis on various organic media are presented in Table II. Results were read after 7 and 14 days incubation at 45°-50°C. Although growth on most media was fully developed after 7 days incubation, the cultures were incubated for an additional 7 days. A duplicate set of media were incubated at 37°C and the results read after 7, 14 and 21 days incubation. This was done in order to ascertain whether or not the organism had been previously described by its cultural characteristics at 37°C. It was found, however, that results obtained at 37° and 50°C were identical.

The glucose-peptone A and B media, glucose-asparagine agar, and starch agar A, were prepared according to formulae given by Waksman (36). The medium for the testing of cellulose digestion consisted of filter paper strips suspended in a solution of the salts found in Czapek-Dox agar. The nutrient agar contained Bacto peptone 1%, Bacto beef extract 0.5%, sodium chloride 0.5%, and had a pH of 6.8 - 7.0.

The cultural characteristics of T. viridis on nutrient agar were taken as the standard description of the organism. Most of these characteristics may be seen in figures 3-6. Figure 3 shows the degree of growth development after 3 days incubation at 50°C. Figures 4-6 indicate the progressive development of the colonies on consecutive days thereafter.

The color reproduction in these photographs is not entirely accurate, but the series does serve to illustrate in general the various stages of growth on nutrient agar.

TABLE II

CULTURAL CHARACTERISTICS AND BIOCHEMICAL REACTIONS OF
THERMOACTINOMYCES VIRIDIS ON VARIOUS ORGANIC MEDIA

Medium	Description of Growth or Biochemical Reaction after 14 days Incubation at 45°C
Nutrient agar	Mycelial growth wrinkled and close to agar surface; blue-green aerial mycelium; emerald-green water-soluble pigment present
Nutrient broth	Flocculent cream-colored submerged growth; Surface growth blue-green; green pigment present
Glucose-peptone A agar	Colorless colonies 1-2 mm in diameter; no aerial mycelium
Glucose-peptone A broth	Flocculent cream-colored submerged growth
Glucose-peptone B agar	Wrinkled colorless growth; no aerial mycelium
Glucose-peptone B broth	Flocculent cream-colored submerged growth
Czapek-Dox agar	No growth
Sabouraud agar	No growth
Glucose-asparagine agar	No growth
Corn meal agar	Flat colorless colonies 1-2 mm in diameter
Calcium malate agar	Colorless colonies 1-2 mm in diameter; few colonies with blue-green aerial mycelium
Potato plug	No growth
Carrot plug	No growth
Blood plate	Few large folded colonies; many small flat colonies; no aerial mycelium; hemolysis present

TABLE II Continued

Medium	Description of Growth or Biochemical Reaction after 14 days Incubation at 45°C
Skim milk	Coagulation followed by digestion
Nitrate reduction	Not reduced to nitrites
Cellulose digestion	Negative
Gelatin liquefaction	Positive
Starch hydrolysis	Positive

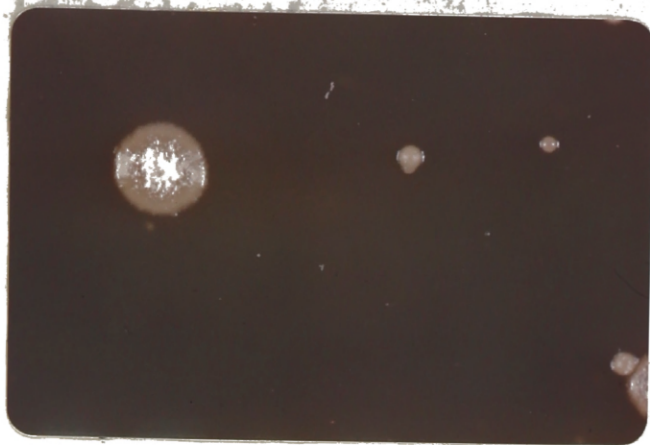


Figure 3. T. viridis after 3 days
incubation at 50°C.

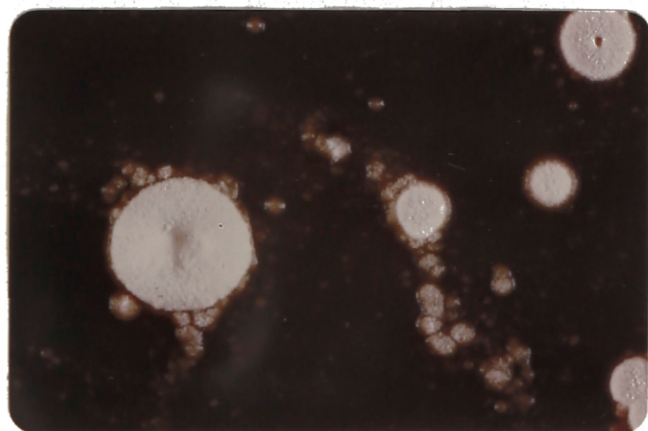


Figure 4. T. viridis after 4 days
incubation at 50°C.

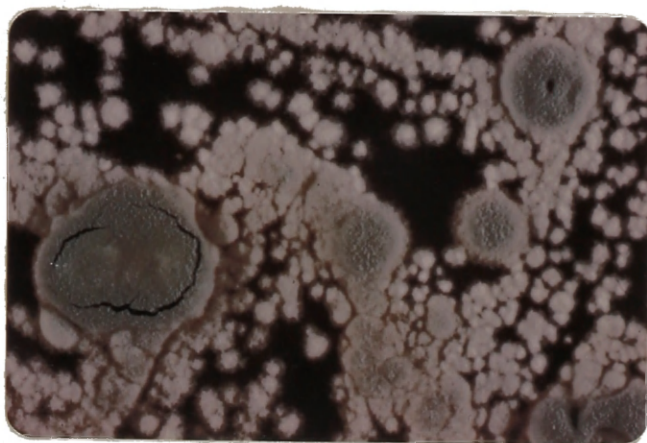


Figure 5. T. viridis after 5 days
incubation at 50°C.

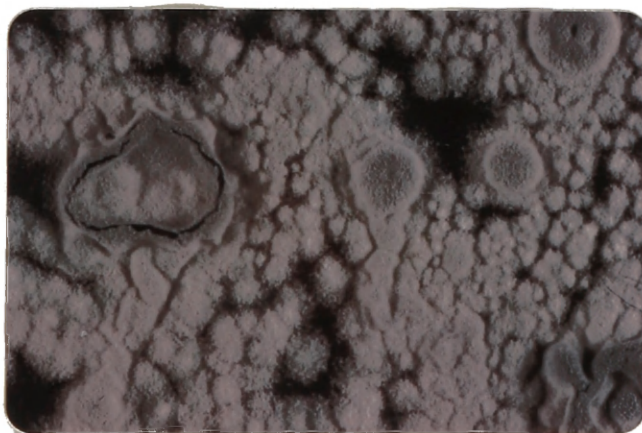


Figure 6. T. viridis after 6 days
incubation at 50°C.

The surface of the fully developed culture of T. viridis was bluish-green, whereas the reverse side of the culture was emerald-green. A water-soluble pigment was present which appeared to be emerald-green in the agar, but more nearly forest-green when extracted into water.

In naming this organism, several factors had to be considered. The organism in many ways resembles Thermoactinomyces monospora, described by Waksman (40). The points of difference, however, are considered significant. T. monospora possesses hyphae with a diameter of about 1 μ , has an optimum growth range of 37° - 55°C, and does not coagulate milk.

The hyphae of the organism reported here were approximately 0.5 μ in diameter, the optimum growth temperature was about 55°C, and milk was coagulated and digested. In addition a green water-soluble pigment was present.

Bergey's Manual of Determinative Bacteriology (3) includes genus Thermoactinomyces in the genus Micromonospora. Waksman (37) has stated more recently that thermophilic organisms which bear spores singly and produce an aerial mycelium should be classified separately under the genus Thermoactinomyces.

For the above reasons, the organism reported here is considered to be a new species for which the name Thermoactinomyces viridis is suggested. The name thermoviridin is suggested for the antibiotic produced by this organism.

Ultraviolet Irradiation

The result of exposing a spore suspension of T. viridis to ultraviolet irradiation for 1-16 minutes is shown in Table III.

TABLE III
THE EFFECT OF ULTRAVIOLET IRRADIATION UPON
THE VIABLE SPORE COUNT OF T. VIRIDIS

Exposure period in minutes	Viable spore count	Percent kill
0	150,000	
1	15,000	90.00
2	6,700	95.50
4	700	99.53
8	170	99.89
16	40	99.97

Upon testing the cultures isolated from a spore suspension which had been irradiated for 2 minutes, it was found that no culture produced significantly more thermoviridin than did the parent strain. However, mutants which differed from the parent strain in pigment production, degree of sporulation, and colonial morphology were isolated.

Fermentation

Production of thermoviridin was first undertaken in stationary liquid cultures. A maximum antibiotic activity of 4 units per ml was obtained after 5 days incubation at 50°C with a medium containing Bacto peptone 2%, Bacto beef extract 0.5%, sodium chloride 0.5%, and having a pH of 6.8 - 7.0.

Fermentation studies using shake flask cultures proved to be most successful. Bacto tryptone was found to be more

suitable than Bacto peptone for antibiotic production, and was therefore substituted for it in the fermentation medium. The medium then contained Bacto tryptone, Bacto beef extract, and sodium chloride. The balance of these constituents was altered to determine the optimum concentration of each nutrient. The formulation which gave maximum thermoviridin production is presented in Table IV.

TABLE IV
TRYPTONE-BEEF EXTRACT FERMENTATION MEDIUM

Constituent	Concentration in grams per liter
Bacto tryptone	40
Bacto beef extract	5
pH 6.9 - 7.1	

Using this medium, an antibiotic level of 32 units per ml was obtained. Buffering the medium did not increase thermoviridin production. An attempt was made to increase the antibiotic potency by supplementing the tryptone-beef extract medium. A list of the nutrients added is given in Table V.

TABLE V
NUTRIENTS TESTED IN THE TRYPTONE-BEEF EXTRACT
MEDIUM FOR STIMULATION OF ANTIBIOTIC PRODUCTION

Glycerol	L-tryptophane
Glucose	DL-tyrosine
Sucrose	Casamino acids

TABLE V Continued

Maltose	Bacto peptone*
Lactose	Bacto tryptose*
Corn starch	Soy peptone*
Corn meal	Soy bean meal
Corn steep liquor	Milk nutrient GG
Whole wheat flour	NZ amine B*
Wheat germ	Brewer's yeast
	Bacto yeast extract

* Nutrients showing some stimulation of antibiotic production

Several nutrients caused a slight increase in antibiotic production, but the increase was not considered significant. The amino acids tryptophane and tyrosine were tested because these are the amino acids in Bacto tryptone which are present in substantial amounts, according to Difco laboratories.

Since in some cases insufficient trace elements may be the limiting factor in antibiotic production, several mineral salts were added to the tryptone-beef extract medium. In the case of thermoviridin production, the addition of various mineral salts had no enhancing effect. The ions, and the concentrations in which they were added, are shown in Table VI.

Failure to improve upon the simple tryptone-beef extract medium to any marked extent indicates the rather specific requirements of T. viridis for antibiotic production. Growth and antibiotic activity have even been obtained using only a 4% tryptone solution as the fermentation medium.

TABLE VI
METALLIC IONS ADDED TO THE
TRYPTONE-BEEF EXTRACT MEDIUM WITHOUT EFFECT

Ion Added	Concentration of Ion in mg/liter	Ion Added	Concentration of Ion in mg/liter
Ca (as CaCl_2)	72 and 144	Zn (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	1 and 10
Mg (as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	19.5 and 39	Fe (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	1 and 10
K (as KCl)	103 and 206	Cu (as CuSO_4)	0.01 and 0.1
Na (as Na_2HPO_4)	25.8 and 51.6	Mn (as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	1 and 10
		Co (as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)	0.1 and 1

Thermoviridin production at 37° and 45°C have been compared using the tryptone-beef extract medium. Antibiotic production was more rapid at 45°C, reaching a maximum in approximately 27 hours. At 37°C, about 48 hours were required for maximum production. The relationship of pH changes and thermoviridin production during fermentation at 37° and 45°C may be seen in figure 7.

Thermoviridin was also produced in a 30 liter laboratory fermenter with the tryptone-beef extract medium. The growth obtained was good, although not so heavy as that in shake flasks. Antibiotic activity did not exceed 4 units per ml. The low antibiotic level obtained may have been caused by sub-optimal growth conditions. Because of the difficulties encountered and the expense of the medium, most of the antibiotic beer used for recovery and purification studies was produced by the shake flask method.

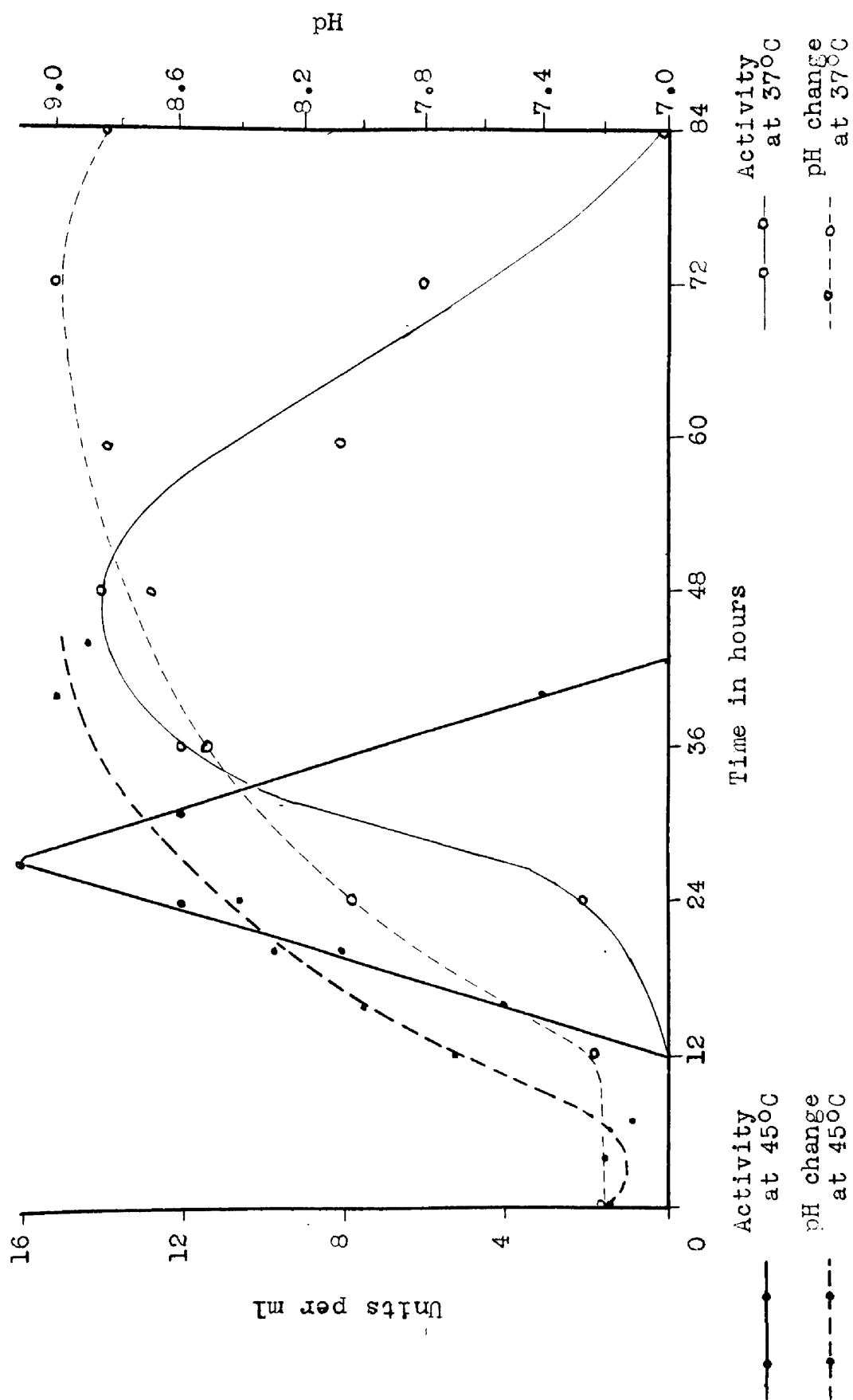


Figure 7. Thermoviridin production and pH changes at 37° and 45°C

Recovery

It was found that the filter aid celite 545 adsorbed as much as 75% of the antibiotic activity when the pH of the crude culture was pH 8.5 - 8.7. By adjusting the pH to a range of pH 5.5 - 6.5 before filtration, the amount of activity adsorbed was greatly reduced, but the problem was not entirely eliminated. Centrifugation was therefore the method of preference whenever the volume of crude culture to be treated was not in excess of 3-4 liters.

Although the solvent extraction method was found to be undesirable for the recovery of thermoviridin, the results obtained from the investigation of this recovery method are given here. Extraction of thermoviridin from a water solution was accomplished with the use of n-butanol. The active material was completely extracted with butanol only when the pH of the system was 5 or lower. Thermoviridin was not extracted from water by any other solvent tested.

Solid active material was also extracted with several solvents. Thermoviridin was found to be soluble in the following solvents, in decreasing order of solubility: ethylene glycol, dihydroxyethyl ether, methanol, pyridine, ethanol, 1,4-dioxane, and isoamyl alcohol. The antibiotic was insoluble in acetone, ethyl acetate, diethylamine, 2-butanone, and cyclohexane.

The selected recovery method has been outlined in figure 1 (see page 22). In order to effect complete precipitation of thermoviridin at pH 3, it was necessary to hold the antibiotic beer at 0°-5°C for not less than 2 hours. Large

volumes of antibiotic beer (8-10 liters) were held overnight at 0°-5°C.

No precipitate was formed when a portion of sterile fermentation medium was adjusted to pH 3, indicating that the precipitate from the antibiotic beer was due to metabolic products of T. viridis.

The washing of the acid precipitate with water did not cause a loss of antibiotic activity, as measured by a paper disc assay of the water wash.

Antibiotic activity was not completely removed from the precipitate by extraction of the washed precipitate with a quantity of 80% acetone equal to one-tenth the original volume of the antibiotic beer. A second extraction with 80% acetone failed to increase the total recovery, therefore the extraction was limited to one-tenth volume. Much of the precipitate was insoluble in acetone and remained after extraction.

Approximately 80% of the activity in the antibiotic beer was recovered in the acetone extract. After evaporation of the acetone, the remaining water fraction was dried from the frozen state yielding a solid possessing up to 64 units per mg activity.

Purification

The solid material obtained from the recovery procedure was purified by fractional precipitation or by countercurrent distribution. The degree of purification obtained by the fractional precipitation method may be seen in Table VII.

TABLE VII
PURIFICATION OF THERMOVIRIDIN
BY FRACTIONAL PRECIPITATION

Fraction	mg in Fraction	Units per mg	Units in Fraction	% Activity Recovered in Fraction
Starting material*	192.0	20	3840	
Residue	89.9	0	0	0
Ppt. from $\frac{1}{2}$ vol. ether	No ppt.			
Ppt. from 1 vol. ether	21.8	30	654	17.0
Ppt. from 2 vol. ether	16.4	80	1312	34.1
Ppt. from 4 vol. ether	18.6	30	558	14.5
Ppt. from 7 vol. ether	13.2	0	0	0
Total recovery	159.9 mg		2524 units	65.6%

* Sufficient methanol added to give an antibiotic concentration of 150 units per ml

The presence of a precipitate with the addition of one-fourth and one-half volumes of ethyl ether depended upon the potency of the solid material being purified. When the starting material was crude, for example 5-10 units per mg, the one-fourth and one-half volume precipitates were heavy. When the starting material was of higher potency, the precipitate in these fractions was light, or more often absent.

In Table VII it will be noted that the two volume fraction showed a four-fold increase in purity over that of the

starting material. It was found that as the potency of the starting material increased, the degree of purification effected decreased. The fact that fewer ether-precipitable impurities were present in starting material of higher potency is probably the reason for the above mentioned occurrence.

In general, as the purity of the fractions increased, the color of the dried precipitates proceeded from tan to dark-brown, but it was found by countercurrent distribution that the brown color was not associated with the antibiotic itself.

Fractional precipitation from methanol was also attempted using acetone, benzene, amyl acetate, cyclohexane, carbon tetrachloride and toluene. None of these solvents was found to be suitable for the precipitation of thermoviridin from methanol.

Countercurrent distribution was the second method used in the purification of thermoviridin. The number of tubes used in this procedure was arbitrarily established, but was based somewhat on the limitations imposed by the time involved in making the transfers from tube to tube with pipettes. Several pieces of apparatus for countercurrent distribution have been described by Craig and Post (8), and with such equipment a large number of transfers may be made automatically, greatly simplifying the performance of the technique.

In transferring the water layers from tube to tube, complete separation and transfer were impossible. Craig (7) has stated that this does not markedly affect the final purification when a large number of transfers are used. Undoubtedly, with only eight tubes, purification was affected by the incom-

plete transfer of the water layers.

Visual observation of the series of tubes after the completion of countercurrent distribution showed the dark-yellow color of the initial water layer to have migrated to tube eight for the most part, whereas the lighter yellow color in the butanol layer of the initial distribution remained largely in tube one. The other tubes in the series were progressively lighter in color proceeding toward the middle tubes, which were colorless.

The degree of purification obtained by the use of countercurrent distribution is presented in Table VIII.

It will be noted that in buffer fractions two and six antibiotic activity is present, but dry weight data indicate that no active material is present in the fractions. These inconsistencies were probably caused by the use of 3 ml portions for dry weight determinations. The amount of solid material in the portions was small, making errors in weighing significant.

Aside from the above-mentioned discrepancies, these data demonstrate that much inactive material is removed by countercurrent distribution, and that considerable purification can be accomplished by this method.

Biological and Chemical Properties

The stability of thermoviridin in the antibiotic beer has been tested up to 21 hours at several temperatures and pH values. The results are presented in Table IX.

Four portions of antibiotic beer were adjusted to pH values of 2, 4, 8 and 10. The four portions were then dispensed

TABLE VIII
PURIFICATION OF THERMOVIRIDIN BY COUNTERCURRENT DISTRIBUTION

Tube Number	mg Active Material in Fraction	Total Units Thermoviridin in Fraction	Approximate Units per mg	% Recovered in Fraction
Butanol Fractions				
1	3.80	0	0	0
2	1.68	84.0	50	5.4
3	1.31	171.0	130	11.0
4	0.57	153.9	270	9.9
5	1.74	0	0	0
6	2.32	0	0	0
7	6.55	0	0	0
8	1.14	0	0	0
Buffer Fractions				
1	3.50	0	0	0
2	0	99.7	--	6.4
3	7.31	121.8	17	7.9
4	7.41	171.0	23	11.0
5	2.32	133.4	58	8.6
6	0	92.8	--	6.0
7	5.83	0	0	0
8	11.22	0	0	0

Total recovery 66.2%

Potency of starting material 40 units per mg

in test tubes in 1 ml amounts and placed at the desired temperatures. At each time interval tubes were removed; those at acid pH were neutralized with solid sodium bicarbonate. The amount of antibiotic activity remaining was then tested by the paper disc assay method.

TABLE IX
STABILITY OF THERMOVIRIDIN TO VARIOUS
pH VALUES AND TEMPERATURES

Time in Hrs.	Percent Antibiotic Activity Remaining											
	9°C				24°C				37°C			
	pH2	pH4	pH8	pH10	pH2	pH4	pH8	pH10	pH2	pH4	pH8	pH10
1	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100	100	100	100	100
8	100	100	100	100	100	100	100	75	100	100	100	50
21	100	100	100	100	100	100	100	50	100	100	100	25

The purpose of the stability studies was to determine the length of time that thermoviridin would withstand acid or alkaline conditions at several temperatures. This information was needed before the investigation of possible recovery methods could be undertaken. A time limit of 21 hours was arbitrarily chosen because in most recovery processes there is no need to hold the antibiotic at acid or alkaline pH values for longer periods.

Thermoviridin has not been found to lose activity in the presence of sheep blood, nor does it hemolyze sheep red blood

cells. This was determined by the paper disc assay method.

The antibiotic appears to be of the bacteriostatic type. Subcultures were made from serial dilution assay tubes which showed inhibition after 24 hours incubation, beginning with the highest dilution showing inhibition and proceeding toward the lowest. In a two-fold series with inhibition at 1:64, subcultures of the 1:64 and 1:32 dilutions grew out, but that of the 1:16 dilution did not. Failure to obtain growth in the subcultures from the 1:16 dilution and below, may have been due to an actual bactericidal effect at the lower dilutions, or to a weakened physiological condition of the cells which might not allow the initiation of growth in the subculture.

Several qualitative biochemical tests have been performed using material having 30 units per mg activity dissolved in sufficient water to give a concentration of 1 mg per ml. The biuret test was negative, indicating the absence of peptide linkages in the preparation. The ninhydrin test was negative, also indicating the absence of proteins and related compounds. No yellow color was noted with the xanthoproteic reaction which would indicate that no phenyl groups were present in the material. The Molisch test was also negative, suggesting the absence of carbohydrates. Sulfur was not present in the active material, as determined by the lead acetate test. The above tests were performed according to the methods described by Hawk, Oser and Summerson (16).

A water solution containing 2 mg per ml of 30 unit per mg material contained 10.3% nitrogen. The micro-Kjeldahl method of Ma and Zuazaga (21) was used for the determination.

The above tests are significant in that all but the test for nitrogen were negative. In a preparation of only partial purity, positive tests do not necessarily give any indication of the chemical nature of the antibiotic itself, since impurities may well be responsible for the positive reaction.

Thermoviridin could be characterized as a chemical compound only if a crystalline product were available, but in the light of the above data and also solubility data, several generalizations concerning its chemical nature can be made.

According to Florey et. al. (13), a compound which has preferential solubility in an organic solvent under acid conditions, is itself acid in nature. This is true of thermoviridin, which in the presence of both water and butanol favors the butanol phase at acid pH and the water phase at alkaline pH.

Thus it appears that thermoviridin is an organic acid which contains no sulfur or phenyl groups, but which may contain nitrogen.

The free acid was found to have only limited solubility in water, whereas the sodium salt was soluble to the extent of at least 300 units per ml. Its solubility was not tested beyond this level.

Thermoviridin was also found to be of such molecular size that it was dialyzable. This was demonstrated in a water system. Dialysis was carried out at room temperature for a 24 hour period. Toluene was added as a preservative.

The ultraviolet absorption spectrum of thermoviridin was determined in a Beckman quartz spectrophotometer. Two preparations from the same experiment but having 30 units per mg and 80 units per mg activity respectively were tested. A water solution of each preparation containing 0.04 mg per ml was used. Therefore, the first solution contained 1.2 units of thermoviridin per ml, and the second, 3.2 units per ml. It was necessary to use a solution of low concentration so that there would be sufficient light transmission for a reading in the lower portion of the ultraviolet spectrum.

The ultraviolet absorption spectrum of a third preparation having 30 units per mg activity was also determined. The water solution tested contained 0.25 mg of material per ml, or 7.5 units per ml. The results of the three determinations are found in figure 8.

The results show that absorption increases with an increase in thermoviridin concentration. The preparations which had 1.2 and 3.2 units activity per ml showed a maximum absorption peak at a wave length of 268mu, whereas the solution contained 7.5 units per ml showed maximum absorption at 272mu. This difference is not considered significant, and may have been due in part to the degree of sensitivity of the spectrophotometer. The absorption shown is attributed to thermoviridin and is considered to be characteristic of it.

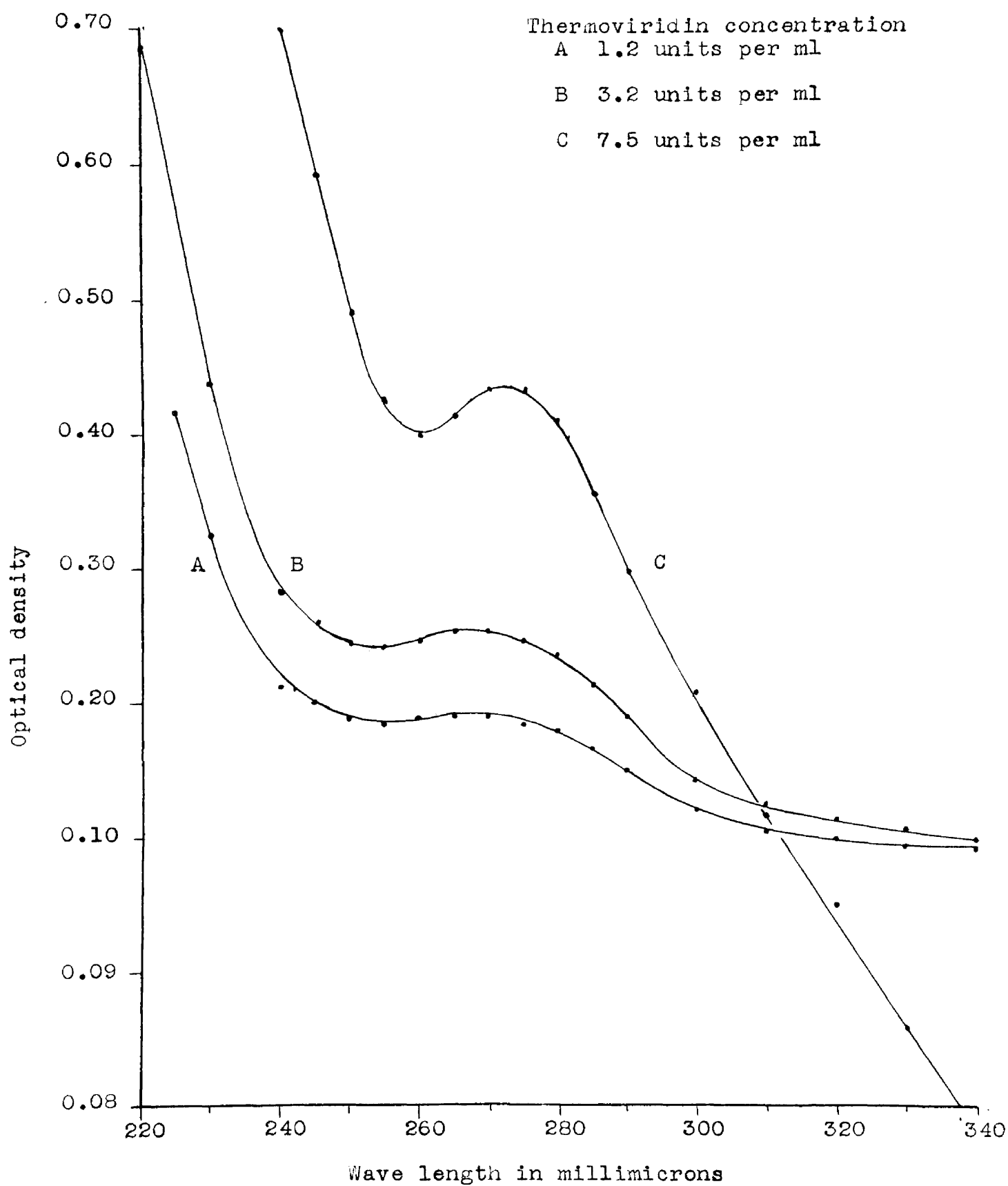


Figure 8. Ultraviolet absorption spectrum of thermoviridin

The microbiological spectrum of thermoviridin is presented in Table X. The sensitivity of M. pyogenes var. aureus 209P was taken as the standard to which the sensitivity of all other organisms was compared. It will be noted that two of the micrococcus strains are penicillin resistant. Strain 193 was found to require 200 times as much penicillin for inhibition as the 209P strain, whereas the 218 strain required 1600 times as much as the 209P strain. Table X shows that a four-fold increase in the concentration of thermoviridin was sufficient for the inhibition of both resistant strains.

The sensitivity of all bacterial cultures except the mycobacteria was tested using the two-fold serial dilution method. The agar dilution method was used in testing the sensitivity of the mycobacteria and fungi.

The streptococci and D. pneumoniae type III were grown in Felton's broth. C. diphtheriae PW8 was grown in veal infusion broth, and the mycobacteria, on glycerol-beef extract agar. The remaining bacteria and fungi were grown on trypticase broth and agar respectively.

Anaerobic conditions were obtained for the growth of Clostridium tetani (Pease) and Vibrion septicum by the use of germinating oats in a desiccator (22). The pressure within the desiccator was reduced by means of a vacuum pump, thereby hastening the attainment of anaerobic conditions.

The values given for all bacteria in Table X except those for the mycobacteria are based on the degree of inhibition after 18-24 hours incubation at 37°C. The values

TABLE X
THE MICROBIOLOGICAL SPECTRUM OF THERMOVIRIDIN

Organism	u/ml required for inhibition
Micrococcus pyogenes var. aureus 209P	1
" " " 218*	4
" " " 193*	4
" " " B314	4
" " " SM	4
Streptococcus viridans 31	16
" " 143	16
Streptococcus hemolyticus SFIV	4
" " K64C	4
" " 316A	16
" " 314B	16
Diplococcus pneumoniae type III	4
Corynebacterium diphtheriae PW8	16
Sarcina lutea PCI-1001W	.25
Bacillus subtilis 231	.5
Clostridium tetani Pease str.	2
Vibrio septicum	.03
Proteus vulgaris 1414	64
Escherichia coli FDA	64
Shigella paradysenteriae 15001	64
Salmonella typhimurium Edward str. 9	64
Mycobacterium tuberculosis var. hominis H37	> 26

TABLE X Continued

Organism	u/ml required for inhibition
<i>Mycobacterium phlei</i> 1201	> 26
<i>Trichophyton mentagrophytes</i>	> 26
<i>Histoplasma capsulatum</i>	> 26
<i>Blastomyces dermatiditis</i>	> 26
<i>Nocardia asteroides</i>	> 26
<i>Aspergillus fumigatus</i>	> 26
<i>Candida albicans</i>	> 26

* penicillin resistant

given for the mycobacteria are based on the degree of complete inhibition after 6 weeks incubation at 37°C, and those for the fungi, on the degree of complete inhibition after 3 weeks incubation at room temperature.

Toxicity

Injection of 32 mg of material containing 25 units per mg activity into each of 3 mice by the intraperitoneal route showed no lasting toxic effect. During the first 24 hours following injection the mice were sluggish and generally inactive, but later appeared healthy and active. The mice were observed for a three-week period following injection and during that time increased their weight from approximately 20 grams to 27 grams. Following the period of observation, the mice were sacrificed and an autopsy was performed. The gross appearance of the internal organs was normal.

DISCUSSION

In considering the production of thermoviridin, it is unusual that the level of antibiotic activity obtained with the tryptone-beef extract medium could not be improved significantly. Throughout the media studies, media which contained various peptone-like products in combination with beef extract were consistently most successful.

In the case of thermoviridin, the factor which limited antibiotic production was not found. The deficiency, if such was the case, may have been in the carbon, nitrogen, vitamin, or mineral source. With the correction of such a deficiency, it would seem feasible that thermoviridin production could be greatly increased.

Waksman (36) has stated that, as a rule, actinomycetes prefer proteins to carbohydrates as a carbon source. Tryptone serves as a source of both carbon and nitrogen for Thermoactinomyces viridis. This is supported by the fact that growth has been obtained using only a 4% tryptone solution as the fermentation medium. The beef extract is believed to serve as a source of supplementary growth factors and minerals.

Of the nutrients added to the tryptone-beef extract fermentation medium, whole wheat and glycerol stimulated the growth of T. viridis to some extent, although there was no significant enhancement of thermoviridin production.

Neither of these materials was therefore included as a permanent constituent of the fermentation medium.

Most antibiotic fermentations require approximately 72 hours to reach maximum production, whereas only 27 hours were required for maximum thermoviridin production at 45°C.

Purification of the dry antibiotic material obtained from the acetone extract was only partially achieved. Countercurrent distribution was found to be the most effective of the two methods used. Had commercial equipment been available so that many more transfers could have been made, a much purer product could likely have been obtained. Battersby and Craig (1) have reported the isolation of crystalline tyrocidine by this method, so that even a crystalline product is possible.

Countercurrent distribution as described by Craig, and fractional precipitation are primarily laboratory tools, and are not practical for large scale purification. Such methods are of great value in purifying an antibiotic and are a great aid in reaching the ultimate goal of purification, a crystalline product.

The stability of thermoviridin at temperatures above 37°C was not tested systematically. However, autoclaving 10 ml of antibiotic beer for 5 minutes at 121°C caused a reduction in activity of approximately 50%. A more thorough investigation of stability at temperatures above 37°C was considered important only if a crystalline product were available, which was not the case.

With the exception of the ultraviolet absorption spectrum, the chemical characteristics of thermoviridin have

been determined for the most part by indirect evidence. The negative qualitative color tests indicate the absence of proteins and carbohydrates in the preparation tested. The solubility characteristics of thermoviridin under acid and alkaline conditions indicate that the antibiotic is an acid. More definite conclusions concerning the chemical nature of this antibiotic cannot be drawn from the data presented.

The microbiological spectrum of thermoviridin is not at all unusual or unique. It is primarily the gram positive bacteria which are inhibited by the antibiotic. The fungi were found to be insensitive to thermoviridin at the levels tested. However, Histoplasma capsulatum and Blastomyces dermatiditis showed some sensitivity to 26 units per ml of thermoviridin when assayed initially, but did not appear sensitive when the assays were repeated. Thus, at higher levels of concentration thermoviridin might be more effective against these organisms. The limited supply of the antibiotic prevented such testing.

Speculation as to the possible chemotherapeutic value of thermoviridin is perhaps in order. Since it does inhibit penicillin resistant micrococci in rather low concentrations, the possible value of thermoviridin would be its use as an adjunct to penicillin. If the two fungi mentioned above were found to be sensitive to thermoviridin at higher concentrations, the future of the antibiotic would then be more promising.

SUMMARY

A thermophilic actinomycete was isolated from a composted manure pile. Since no previous description of this organism was found, it was considered a new species of the genus Thermoactinomyces (Tsiklinsky). The name Thermoactinomyces viridis is suggested for the organism, and the name thermoviridin for the antibiotic produced by it.

Thermoviridin was produced initially in stationary bottle cultures. Subsequent production was carried out on a Gump rotary shaker for the most part, and also in a 30 liter laboratory fermenter.

The fermentation medium consisted of Bacto tryptone 4%, Bacto beef extract 0.5%, and had a pH of 6.9 - 7.1. The addition of a number of nutrients to this medium did not increase antibiotic potency above the 32 units per ml obtained in the basal medium. Maximum antibiotic production was reached in 27 hours at 45°C.

Recovery of thermoviridin was accomplished by acid precipitation at pH 3. The precipitate was washed with water and then extracted with a quantity of 80% acetone equal to one-tenth the original volume of the antibiotic beer. The acetone extract was evaporated, and the remaining water residue was dried from the frozen state. The solid material obtained by this procedure had 64 units per mg activity against M. pyogenes var. aureus 209P.

Some purification of the active solid material was accomplished by fractional precipitation from methanol using ethyl ether as the precipitating agent. The countercurrent distribution method of Craig was also an effective method of purification. A preparation having 270 units per mg activity was obtained by this method, using a butanol-water system buffered at pH 6.

Thermoviridin was found to be stable at pH 2 for 21 hours at 37°C, but at pH 10 for the same time period approximately 75% of the antibiotic activity was lost. The stability of thermoviridin to temperatures higher than 37°C was not investigated.

Chemical tests and solubility data indicate that thermoviridin is an organic acid which contains no sulfur or phenyl groups. The active material appeared to be non-protein and non-carbohydrate in nature. Thermoviridin was dialyzable and could be precipitated with saturated ammonium sulfate.

The antibiotic preparations tested showed maximum absorption in the range of 268-272mμ.

Thermoviridin was primarily active against the gram positive bacteria tested. Preliminary toxicity tests in mice, consisting of a single intraperitoneal injection of 32 mg of material (800 units), did not cause death in any case. An autopsy of the mice showed no abnormalities in the gross appearance of the organs.

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