to 6.5% NaCl and pH 9.6, and fermentation of sorbitol, mannitol, and arabinose. The organism had a lag period of 7 days in pH 9.6 medium as compared with growth in 24 hr for the parent strain. Tolerance to 6.5% NaCl was comparable in both strains. The organisms were serologically identical.

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Studies on the Thermophilic Actinomycetes[1]

I. Methods of Cultivation

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ABSTRACT

TENDLER, M. D. (Yeshiva University, New York, N. Y.), AND P. R. BURKHOLDER. Studies on the thermophilic actinomycetes. I. Methods of cultivation. Appl. Microbiol. **9**:394–399. 1961.—A total of 1,000 isolates of thermophilic actinomycetes representing two genera, *Streptomyces* and *Thermoactinomyces*, were studied. Media for cultivation and for physiological studies were designed. Differences between the two genera are noted and taxonomic criteria for the genus *Thermoactinomyces* are suggested. The importance of the nutritional environment to the thermophilic habit is noted.

The name *Thermoactinomyces* was proposed by Tsiklinsky in 1899 to include monosporous actinomycetes capable of growing at high temperatures. The validity of assigning thermophilic species with the morphology of *Micromonospora* to the genus *Thermoactinomyces* has been discussed by Waksman and Corke (1953). The genus is recognized in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957), with three species listed. A fourth species, *Thermoactinomyces viridis*, has been described by Schuurmans, Olson, and San Clemente (1956). The study here summarized was undertaken as an extension of the previous work of one of the authors (Tendler, 1959) to help in further characterizing isolates of the genus, and to gain additional insight into the physiological aspects of thermophily.

Since this genus is primarily characterized by two distinguishing properties, the thermophilic habit and the micromonospora type arrangement, studies were undertaken to compare members of the genus *Thermoactinomyces* with thermophilic variants of the genus *Streptomyces*. To encourage other students of actinomycete taxonomy and physiology to include the thermophilic forms in their studies, special attention was given to the comparison of media and techniques commonly employed in actinomycete work, with the variations thereof that are necessary for successful handling of thermophilic cultures. The rapid growth rate of the thermophiles and the reduction in con-

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tamination, because of the elevated growth temperature, make these organisms particularly useful for industrial fermentations.

Materials and Methods

One thousand isolates were obtained from soils and composts collected in the United States, Virgin Isles, France, Spain, Italy, Israel, Puerto Rico, Peru, and Chile.

Media were composed and evaluated for use as maintenance media, for the study of specific physiological properties, and for the study of micromorphology. Each isolate was studied to determine its micromorphology, temperature limits for growth, ability to grow on synthetic media containing inorganic nitrogen, spore color, pigment production, nitrate reduction, and H_2S production. The occurrence of five exoenzymes, amylase, caseinase, gelatinase, lipase, and rennin-like activity, was investigated. The media and methods recorded in a previous publication were re-evaluated for this larger number of isolates, in comparison with eight types of media that are commonly employed for mesophilic strains. Additional media were designed and several techniques were modified. Table 1 comprises the media found most useful for the study of thermophilic actinomycetes. Media Ia and VII are recommended for general use in the isolation of cultures. Medium Ia is the preferred maintenance medium. Medium II is most useful for determining pigment production. Media III and IV are helpful in rejuvenating cultures that have lost one or more physiological properties during storage on maintenance media. Medium V should be included in any study of temperature limits for growth. Medium VI is preferred for broth cultures grown with continuous agitation. Medium VII is a useful synthetic medium lacking an organic source of nitrogen. Asparagine or glutamic acid may be added to provide a suitable organic nitrogen source. The addition of 1.0% gelatin hydrolyzate to synthetic medium VII makes it particularly useful for cultivating the blue or blue-green thermophilic actinomycetes that are difficult to grow on other media.

Nitrate reduction tests were done as suggested in the *Manual of Microbiological Methods* of the SAB (1957), using sulfanilic acid and α-naphthol as reagents, and the zinc dust confirmatory test. The rich organic maintenance medium Ia, and the synthetic medium, with addition of 0.1% KNO_3, were used if the isolate could grow on both these media, so as to determine the effect of available organic nitrogen on the sensitivity of the nitrate reduction test. H_2S production was determined by incorporating Fe-citrate and Na-thiosulfate into the medium, and also by hanging a strip of lead acetate paper in each tube, and incubating for 14 days.

Rennin-like activity was determined by adding 0.1 to 1.0 ml of a 24-hr broth culture, grown on a shaker, to a test tube containing 2 ml of double strength (2×) reconstituted skim milk, and water added to give a total volume of 4 ml per tube. The tubes were placed

TABLE 1. *Media useful for cultivating thermophilic actinomycetes**

Maintenance medium Ia		Medium Ib	
Trypticase	0.5%	2.5% raw potato puree added to medium Ia.	
Yeast extract	0.3%		
Sucrose	0.5%		
Dung extract†	0.5 ml/100 ml		
Molasses	0.5 ml/100 ml		
$MgSO_4 \cdot 7H_2O$	0.05%		
$FeSO_4 \cdot 7H_2O$	1.0 mg/100 ml		
Microelement solution‡	0.1 ml/100 ml		
Medium II		**Soil extract medium III**	
dl-Asparagine	0.1%	Soil extract†	5.0 ml/100 ml
Glucose	0.1%	N-Z-Amine B	0.2%
Yeast extract	0.5%	Soluble starch	0.5%
$NgSO_4 \cdot 7H_2O$	0.05%	Glucose	0.1%
$FeSO_4 \cdot 7H_2O$	1.0 mg/100 ml		
Barley medium IV		**Basamin medium V**	
Gerber's barley cereal	1.0%	Basamin (Anheuser-Busch)	0.5%
Tryptone	0.1%	Soluble starch	0.5%
Dung extract†	1.0 ml/100 ml	Tryptose	0.2%
D-Mannitol	0.2%	Glucose	0.2%
Beer medium VI		**Synthetic medium VII**	
N-Z-Amine B	0.5%	$NaNO_3$	0.2%
Yeast extract	0.2%	K_2HPO_4	0.1%
Soytone	0.2%	$MgSO_4 \cdot 7H_2O$	0.05%
Soluble starch	1.0%	KCl	0.05%
D-Mannitol	0.5%	$FeSO_4 \cdot 7H_2O$	1.0 mg/100 ml
$FeSO_4 \cdot 7H_2O$	1.5 mg/100 ml	Microelement solution‡	0.1 ml/100 ml
Microelement solution‡	0.1 ml/100 ml	Sucrose	1.0%
		D-Mannitol	0.5%

* The pH of all media adjusted to 7.0 to 7.2; agar added to 2.0% when desired.

† Dung and soil extracts: 25% dried sheep manure (for dung extract) or 50% air dried garden soil containing 0.1% $CaCO_3$ (for soil extract), suspended in tap water, autoclaved 30 min, filtered, and refrigerated under toluene.

‡ Microelement stock solution (per ml): Fe (as $Fe(NH_4)_2SO_4$), 1.0 mg; Zn (as $ZnSO_4$), 1.0 mg; Mn (as $MnSO_4$), 0.5 mg; Cu (as $CuSO_4$), 0.08 mg; Co (as $CoSO_4$), 0.1 mg; and B (as H_3BO_3), 0.1 mg.

in a 37 C water bath and the coagulation time was noted.

Exoenzyme tests for amylase, caseinase, lipase, and gelatinase, were slightly modified over that previously reported to save time and economize on glassware. For tests of gelatin and starch hydrolysis, 0.4% gelatin and 0.2% soluble starch were added to any medium supporting good growth. One-quarter-inch filter paper discs were dipped into a 24-hr shaker-grown broth culture and placed on the exoenzyme plates. After 22 to 24 hr of incubation, the plates were flooded with one-half strength Lugol's iodine to determine amylase activity. The plates were next left exposed under incandescent illumination until the starch-iodine complex was decolorized, and then flooded with acid mercuric chloride to determine gelatinase production.

Caseinase activity was determined by incorporating 0.4% skim milk powder into a suitable agar medium and noting the zones of clearance. These zones can be accentuated by flooding plates with acid mercuric chloride. Lipase activity was determined, as previously reported, by overlaying the agar plates with 1.0% agar water containing 1% tributyrin or cottonseed oil. Lipase activity was noted by a clearance of the fine oil droplets in the zone surrounding the paper disc impregnated with the culture. Several methods for accentuating the faintly visible zone of lipase activity were tried. Saturated copper sulfate, previously used as a developing reagent, was compared with other methods, e.g., the Nile blue sulfate staining of Hammer and Long (1937), and the use of a saturated alcoholic solution of Sudan-B on the plates. The Sudan-B method was decided upon as significantly superior, both as to clarity of interpretation and reproducibility of results.

Preparation of inoculum. When reproducible inocula are required, as in comparing media or in nutritional and growth rate studies, spore inocula can be easily prepared, if preferred over the vegetative hyphal inoculum previously used for such studies.

Fresh agar slant cultures are grown and washed

TABLE 2. *Distribution of exoenzyme activity in 950 isolates of thermophilic Streptomyces (S) and Thermoactinomyces (T)*

Exoenzyme		No. of cultures	Per cent	Genus	
				T	S
Amylase	Strong	634	67.0	523	111
	Weak	36	4.0	18	18
	Negative	280	29.0	280	0
Caseinase	Strong	937	98.8	809	128
	Weak	10	1.0	9	1
	Negative	3	0.2	3	0
Lipase	Strong	815	86.0	696	119
	Weak	57	6.0	55	2
	Negative	78	8.0	70	8
Rennin enzyme	Positive	412	43.0	408	4
	Negative	538	57.0	413	125

TABLE 3. *Distribution of exoenzyme activity in 267 mesophilic actinomycetes isolated from the same soil samples as the thermophilic forms presented in Table 2*

Exoenzyme		No. of cultures	Per cent
Amylase	Positive	264	98.9
	Negative	3	1.1
Caseinase	Positive	232	87.0
	Negative	35	13.0
Lipase	Positive	237	89.0
	Negative	30	11.0
Gelatinase	Positive	262	98.5
	Negative	5	1.5
Rennin	Positive	11	3.0
	Negative	256	97.0

TABLE 4. *Spore color and micromorphology of 1,000 thermophilic isolates studied in various media*

Spore color	No. of isolates	Type of micromorphology
White	643	*Thermoactinomyces*
Light gray	202	*Thermoactinomyces*
Gray	130	*Streptomyces*
Gray-green	4	*Thermoactinomyces*
Blue	16	*Streptomyces* (9) and *Thermoactinomyces* (7)
Yellow	2	*Thermoactinomyces*
Lavender	1	*Streptomyces*
Nonsporulating	2	Sterile

TABLE 5. *Temperature limits of 500 isolates belonging in the genera Thermoactinomyces (T) and Streptomyces (S)*

No. of isolates	Temperature range, C	No. in each genus	
		T	S
6	67–30	4	2
43	67–45	43	0
10	67–50	10	0
16	65–30	12	4
100	65–45	98	2
3	65–50	3	0
39	60–30	27	12
86	60–45	86	0
1	60–50	1	0
2	60–55	2	0
108	55–30	32	76
66	55–45	63	2
1	55–37	0	1
22	50–30	13	9

down with 5 ml of sterile water with a glass rod or rubber "policeman." A 5- or 10-ml pipette, with the tips enlarged and plugged with glass wool, may be used to draw the spore suspension from the agar slant tube. The spore suspension thus freed of clumps of hypha and bits of agar is transferred to a clean sterile tube. Usually this single procedure is adequate to obtain a homogeneous spore suspension, but if clumps persist, a second filtering, through another glass wool-plugged pipette, suffices. The concentrated spore suspension is washed and then diluted to the desired turbidity reading with sterile saline containing 0.1% agar. The presence of a slight amount of agar prevents the rapid settling out of spores during pipetting of inocula and gives more uniform inocula. Subvisible inocula are sufficient to initiate growth in nutritionally adequate media.

Micromorphology. An agar slab placed on a sterile slide and cross hatched with spores of the organism provided a useful material for microscopic observations. The methods of Drechsler (1919), Nishimura and Tawara (1957), and the cellophane strip methods were tried and found to be less satisfactory. The slide culture method gave results comparable to those obtained by the Petri plate cross-hatching method employed by many workers, and had the additional convenience of fitting the mechanical stage of the microscope. The inoculated slides must be incubated in a moist chamber, such as a Petri plate placed in a canister containing a saturated piece of cellulose sponge. After incubation, the slide can be allowed to dry in the incubator for about 30 min to give a dry surface and thus avoid the fogging of high-power objectives, resulting from the condensation of moisture on the lenses. If the slide culture is to be preserved for subsequent examination, it is useful to place the slides in a staining jar containing a shallow layer of 40% formaldehyde solution. A few drops of formalin added to the Petri plate in which the slide was incubated, will also serve to kill the culture and prevent the disruption of the spore arrangement that occurs if the culture is permitted to grow and become senescent. A cover slip can be placed over the slide culture to permit study with an oil immersion objective. Any nutritional medium that supports good growth and sporulation of the isolate can be used. Superior results are obtained if the nutrients of rich media are diluted to half the usual concentration.

RESULTS

Growth. Various media, including oatmeal-tomato paste, Hickey and Tressner, Emerson, Bennet, Czapek, corn steep, potato dextrose, and tyrosine agars, were evaluated (*see* Pridham et al., 1957) in this study. Only 9 of 805 isolates, showing Micromonospora type spore arrangement, grew on Czapek's medium after being subcultured three times. The use of glucose,

TABLE 6. *Growth responses of 21 isolates at six different temperatures (30, 37, 45, 50, 55, and 60 C) on four media previously found adequate for good growth and sporulation at 50 C*

Isolate no.	Maintenance medium Ia						Potato maintenance medium Ib						Soil extract medium III						Basamin medium V					
	60	55	50	45	37	30	60	55	50	45	37	30	60	55	50	45	37	30	60	55	50	45	37	30
2	—	x	x	x	x	—	—	x	x	x	x	—	—	—	x	x	x	—	—	x	x	x	x	—
25	x	x	x	x	x	—	—	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—
32	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	—	—	x	x	x	x	x	—
33	—	x	x	x	—	—	x	x	x	x	—	—	x	x	x	x	—	—	x	x	x	x	—	—
42	—	x	x	x	x	—	—	—	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	x
49	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	x
56	x	x	x	x	x	x	x	x	x	x	x	—	—	x	x	x	x	x	—	x	x	x	x	x
82	x	x	x	x	x	x	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—
84	x	x	x	x	x	—	—	—	x	x	x	—	—	—	x	x	x	—	x	x	x	x	x	—
111	—	x	x	x	—	—	—	—	x	x	—	—	x	x	x	x	x	—	x	x	x	x	x	—
126	x	x	x	x	x	—	—	—	x	x	x	—	x	x	x	—	—	—	x	x	x	x	x	—
132	x	x	x	x	x	—	—	—	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—
144	x	x	x	x	x	—	—	—	x	x	x	—	x	x	x	x	—	—	x	x	x	x	x	—
175	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	—
182	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	x
409	x	x	x	x	x	—	—	x	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	—
450	x	x	x	x	x	x	—	x	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	x
458	x	x	x	x	x	x	x	x	x	x	x	—	—	x	x	x	x	x	x	x	x	x	x	—
496	x	x	x	x	x	—	—	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	—
501	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	x
505	x	x	x	x	x	—	x	x	x	x	x	—	x	x	x	x	x	x	x	x	x	x	x	—
No. failing to grow	4	0	0	0	2	17	11	6	0	0	2	21	4	2	0	1	4	13	2	0	0	0	1	15

Good growth in repeated tests is indicated by x in the temperature columns.

starch, glycerin, or mannitol, as a substitute for sucrose, does not significantly improve Czapek's medium for maintenance, although it serves as an excellent medium for isolation of cultures from crude materials. Oatmeal-tomato paste agar, highly praised for culturing mesophilic strains, is of little value for thermophilic cultures, supporting growth and sporulation of only 2% of the 805 isolates tested.

Hickey and Tressner, Emerson, Bennet, or corn steep agar, although supporting growth of approximately 32% of the isolates, failed to promote good sporulation in the majority of the strains investigated. Bennet's medium supported growth of 55% of the isolates, but was decidedly inferior to the media routinely used in our laboratories, and listed in Table 1. The addition of microelements to the complex medium Ia, which contained poorly defined crude ingredients, significantly improved sporulation at the upper temperature limits of growth.

H_2S production. Only eight isolates produced H_2S under the conditions provided. All eight were white spored cultures of *Thermoactinomyces*, which can be grouped on the basis of the other physiological characteristics into three species or strains. None of the thermophilic cultures of *Streptomyces* gave a positive H_2S test. There are indications that the sensitivity of the test is inadequate as presently performed.

Nitrate reduction. Only 9 of the 805 *Thermoactinomyces* isolates tested reduced nitrates. These strains were the same as the 9 that could grow on Czapek's medium. In contrast, 113 out of the 121 thermophilic *Streptomyces* isolates that were tested reduced nitrates. Three *Thermoactinomyces* cultures gave a positive nitrate reduction test on synthetic medium VII, but not on rich organic medium Ia.

Exoenzymes. The results obtained for activity of four exoenzymes studied in 950 isolates are summarized in Table 2. Strong amylase reactions were observed in 67% of the thermophiles, with representation in both genera. An appreciable number of isolates belonging in the genus *Thermoactinomyces* failed to digest starch under the conditions of our tests. Almost 99% of the isolates showed strong caseinase activity. The majority of cultures were lipolytic. The rennin enzyme was present in over 40% of isolates belonging in *Thermoactinomyces*, but occurred in very few of the cultures of *Streptomyces*.

A comparison was made of the five exoenzymes in 267 isolates of mesophilic actinomycetes. The data shown in Table 3 indicate that the majority of these cultures have the ability to digest starch, casein, fat, and gelatin. The rennin enzyme is absent in 97% of these isolates.

No gelatinase-negative cultures were isolated. There are significant quantitative variations in gelatinase production, but even this variation lacked reproducibility under the conditions used.

Morphological types. The general micromorphology and spore color of 1,000 thermophilic isolates were studied in various media. A summary of the results is presented in Table 4. The great majority of thermophilic isolates are white or gray. All of our white-spored isolates belong in the genus *Thermoactinomyces*. Gray-spored thermophilic *Streptomyces* cultures are common, and darker in color than the light gray cultures of *Thermoactinomyces*. Gray-green, blue, and yellow strains of *Thermoactinomyces* occur infrequently.

Temperature limits. The temperature limits for growth of 502 isolates belonging in the genera *Thermoactinomyces* and *Streptomyces* were studied in medium Ia. The data of Table 5 show the variations with respect to range of temperature that was tolerated. Organisms that are restricted to fairly narrow ranges of high temperature (up to 65 or 67 C) belong in the genus *Thermoactinomyces*. Only a few *Streptomyces* isolates could grow at temperatures above 60 C, but numerous cultures in both genera grew in the lower ranges, below 55 C down to 30 C. Four isolates of *Thermoactinomyces* and two of *Streptomyces* grew over the wide range from 30 to 67 C.

The effect of the nutritional environment on the temperature limits for growth of the *Thermoactinomyces* isolates can be seen from the results in Table 6. Thirty cultures of *Thermoactinomyces* that differ in one or more physiological characteristics were studied on four media that differ but slightly in their components. All contained natural sources of amino acids and vitamins. All supported excellent growth and sporulation at 50 C. Nine isolates are not reported in Table 6, because five of them proved to be duplicates of isolates 25, 56, 82, 132, and 409, and four isolates did not present discernible differences in growth patterns on the media tested. There are several types of broad and narrow ranges of temperature tolerance. It is significant that addition of potato to the excellent maintenance medium Ia markedly reduced growth of many cultures in the upper range, but had little effect at the lower temperatures.

Discussion

The multiple physiological differences between the isolates of thermophilic *Streptomyces* and *Thermoactinomyces* give support to the validity of the new genus *Thermoactinomyces*. If we accept the premise that evolution in the actinomycetes may have progressed from complex nutritional requirements (poorly developed synthetic ability) to simple nutritional requirements (increased synthetic ability), then it could be thought that the *Streptomyces* have been derived from the *Thermoactinomyces*. When considered as a group, the strains of *Thermoactinomyces* fail to grow on media lacking an organic source (reduced form) of nitrogen, do not reduce nitrates, contain a larger

percentage of amylase-negative and lipase-negative strains than the thermophilic (or mesophilic) *Streptomyces*, show some caseinase-negative strains, evidence little variation in morphology, and possess a larger percentage of strains that grow over a limited temperature range. The rennin-like enzyme is present in more than 46% of the *Thermoactinomyces* strains, but only in 3% of the *Streptomyces* examined. Also the curd formed by the *Streptomyces* is produced in 1 hr or more as compared to a 3- to 5-min curd produced by the *Thermoactinomyces*. Curdling time is inversely related to the quantity of enzyme present.

The dependence of the thermophilic habit on the nutritional environment has been investigated by Baker, Hutner, and Sobotka (1955), and by Long and Williams (1959). The results in Table 5 accentuate the importance of the quantitative composition of the medium. It is not the presence or absence of specific metabolic intermediates, but the interaction of all components in the nutritional environment that appears to be important. Studies presently being conducted to elucidate the role of specific amino acids, vitamins, purines, and pyrimidines in supporting the thermophilic habit, fully confirm the importance of balanced amino acid composition of media permitting growth at both extremes of the temperature range.

The role of this group in the economy of nature is still to be explained. Despite the apparent unavailability in nature of suitable conditions for thermophilic growth, these organisms occur in almost all soils and composts. Attempts to mimic the nutritional environment of the soil by use of sterile soil tubes, soil and manure extracts, and extracts of other soil microorganisms failed to show that under these conditions all the *Thermoactinomyces* can proliferate at mesophilic temperatures, although soil extract medium III does appear to be superior to others for supporting growth at 30 C. It is hoped that other investigators will find these organisms useful in clarifying various aspects of thermophily and general physiology of actinomycetes.

The need for a taxonomic scheme for identifying isolates of the genus *Thermoactinomyces* has led the authors to set up a system of expediency. It seems certain that the three species recognized by Bergey's Manual inadequately represent the genus. Descriptive characters found useful in taxonomic work with our collection of thermophilic actinomycetes are the following:

1) Spore color—white, yellow, gray, green, blue.
2) Ability to grow on synthetic medium VII.
3) Temperature limits on maintenance medium Ia.
4) Soluble pigments on media Ia, II, VI, VII—red to violet, green to black, yellow to brown.
5) Nitrate reduction.
6) H_2S production.
7) Rennin activity.
8) Amylase, caseinase, and lipase exoenzyme spectrum.

Variations in the spore arrangement may also provide a criterion for recognizing certain strains of *Thermoactinomyces*. For example, some strains carry the spores on what seem to be short sterigmata; others bear sessile spores directly on the hyphae. In some isolates, the spores are borne only at the hyphal tips, whereas in others they are found along the entire length of each hypha. We are presently investigating these morphological features to determine how consistent they are with respect to growth at different temperatures of incubation and under various conditions of nutrition. The taxonomic significance of carbohydrate utilization tests also is being evaluated further. Subsequent reports will describe results of taxonomic studies, with proposed new species and strains.

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