STUDIES ON THE PRODUCTION OF ANTIBIOTICS BY ACTINOMYCETES AND MOLDS

R. L. EMERSON, ALMA J. WHIFFEN, NESTOR BOHONOS, AND C. DEBOER

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

Received for publication June 17, 1946

Many important diseases have been brought under control in recent years through rapid advances in the field of chemotherapy. A large number of agents antagonistic to pathogenic bacteria have been obtained from microorganisms. Some of these, penicillin for example, have been found to possess remarkable therapeutic properties. Others, such as streptomycin and streptothricin, (Waksman *et al.*, 1944; Jones *et al.*, 1944) appear promising as agents for combating disease organisms. But despite the fact that progress has been made in the treatment of certain diseases, there are some caused by bacteria, molds, and other agents which have not responded to the chemotherapeutic substances now in use. For this reason a continued search for antibiotic agents of therapeutic value seems justified.

Waksman and coworkers (1941) demonstrated that antagonistic actinomycetes are prevalent and widely distributed in nature; 106 of 244 actinomycetes isolated at random were found to be antagonistic to *Bacillus subtilis*. Seventy-seven of 164 actinomycetes were found to be active against *Staphylococcus aureus*.

Alexopoulos (1941) performed experiments to determine how widespread are *Actinomyces* substances which are toxic to fungi. A total of 80 *Actinomyces* strains were studied; 45 of these inhibited the growth of the test fungus, *Colletotrichum gloeosporoides*, but 35 had no effect. A series of studies on antibiotic production by fungi has been reported by Wilkins and Harris (1942, 1943*a*, 1943*b*, 1944). Of the first 100 fungal species studied, 40 per cent of the *Aspergillus* species and 25 per cent of the *Penicillium* species gave positive results against one of the test organisms, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas pyocyanea* (*P. aeruginosa*). In an examination of another 100 fungal cultures these authors found that penicillia and aspergilli were the most promising. Of 33 penicillia tested, 12 were active against *E. coli*, 31 against *S. aureus*, and 7 against *P. pyocyanea*. Of all the cultures examined by these authors, they found 50 per cent of the aspergilli and 50 per cent of the penicillia to be active; whereas 30 percent of the basidiomycetes and no phycomycetes or ascomycetes showed activity. Surveys of antibiotic production by molds were reported by Atkinson (1943*a*, 1943*b*, 1943*c*) and Atkinson *et al.* (1944). Of one group of 68 penicillia tested, 18 showed some activity against bacteria. In another survey 50 penicillia and 2 aspergilli were studied; 15 of the penicillia and aspergilli were found to inhibit bacterial growth.

In 1944 studies were begun in this laboratory on the isolation and testing of actinomycetes and molds for antibiotic production. The studies reported in this paper deal with screening tests conducted on approximately 1,000 molds and

actinomycetes that were isolated from soils collected at many points throughout this country.

METHODS

The methods employed in this survey were adapted from those of other investigators. For a description of methods used in searching for antibiotic substances, see the recent book by Waksman (1945).

Isolation of cultures. Cultures of molds and actinomycetes were isolated from the soil and obtained in pure culture before tests were made for antibiotic activity. Soil samples were collected in Michigan, Texas, Florida, Oklahoma, California, Arizona, Louisiana, North Carolina, Mississippi, Georgia, Tennessee, New Mexico, and South Carolina. A total of 239 soil samples were studied; 1,007 actinomycetes and 221 molds were isolated. Members of the *Mucorales* and rapidly growing species of the Fungi Imperfecti were ignored. Isolations of molds were confined to aspergilli, penicillia, and a miscellaneous group of Fungi Imperfecti. In selecting colonies of actinomycetes for isolation an effort was made to isolate from each plate only those colonies differing in appearance.

The agar media used for the isolations were as follows: (1) peptone yeast extract glucose agar at pH 7.0, (2) Czapek-Dox agar at pH 6.5 to 7.0, and (3) sodium caseinate glucose salts agar at pH 7.0. Media (1) and (2) were used for molds and (3) was used for actinomycetes.

Screening actinomycetes and molds for antibiotic activity. In screening cultures for antibiotic activity it is desirable to have methods that are rapid and sensitive. After investigations of some of the methods used for screening organisms for antibiotic production, it was decided to use both a primary and a secondary screening. The first screening was used (1) to determine what cultures were active, (2) to get information concerning the extent of the activity, and (3) to get information concerning the groups of organisms against which the cultures were active. The secondary screening was used mainly to select cultures for further studies, and, since various media were used, information was obtained concerning media requirements for antibiotic production.

Primary screening. The streak plate method was used in the primary screening. Single, large colonies of the actinomycetes and molds were grown on agar plates that contained 20 ml of agar. The media employed were as follows:

1. Difco beef extract	4.0	g
Difco peptone	4.0	g
NaCl	2.5	g
Glucose	10.0	g
Solubilized liver	1.0	g
Difco yeast extract	1.0	g
Agar	20.0	g
Dist. H_2O	1,000.0	g
2. Dextrin	10.0	g
Difco tryptone	5.0	g
K_2HPO_4	2.0	g
NaCl	2.0	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Agar	20.0	g
Dist. H_2O	1,000.0	ml
3. Brown sugar	10.0	g
$NaNO_3$	3.0	g
KH_2PO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$FeSO_3 \cdot 7H_2O$	0.01	g
KCl	0.5	g
Difco yeast extract	2.0	g
Agar	20.0	g
Dist. H_2O	1,000.0	g

4. Corn steep	10.0 g
Lactose	20.0 g
$NaNO_3$	3.0 g
KH_2PO_4	0.50 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$ZNSO_4 \cdot 7H_2O$	0.02 g
Agar	20.0 g
Dist. H_2O	1,000.0 ml

5. Difco peptone	1.0 g
Difco yeast extract	1.0 g
Glucose	10.0 g
Agar	20.0 g
Dist. H_2O	1,000.0 ml

All media were adjusted to pH 6.5 to 7.0 before autoclaving. Media 1 and 2 were used for actinomycetes. Media 3 and 4 were used for molds when screening them against bacteria, but media 4 and 5 were used when screening them against pathogenic fungi.

In conducting the screening tests, colonies 20 to 25 mm in diameter were prepared by growing the cultures on these media for 4 to 7 days at 23 C; however, with some molds it was necessary to make tests earlier on account of spreading growth. After sufficient growth of the molds and actinomycetes had occurred, test organisms were streaked from the edge of the test colony to the edge of the agar plate. The plates were incubated and observations made for inhibition of growth. In the case of bacteria, five organisms, representative of various morphological and physiological types, were streaked on each plate from broth cultures. The following bacteria were used: *Bacillus subtilis* (laboratory strain), *Staphylococcus aureus* FDA 209, *Escherichia coli* ATCC 26, *Proteus vulgaris* ATCC 8427, and *Pseudomonas aeruginosa* ATCC 9027. The plates were incubated at 37 C for 24 hours.

The following pathogenic fungi were selected for these studies: *Trichophyton gypseum* ATCC 9533 (*T. mentagrophytes*), *Candida albicans* ATCC 2091, *Sporotrichum schenkii* ATCC 7968, *Blastomyces dermatitidis* ATCC 7967, *Endodermophyton tropicale* ATCC 4568 (*T. concentricum*), and *Cryptococcus hominis* ATCC 1655 (*C. neoformans*). These fungi were selected for study because they represent different morphological types, and they represent groups causing a wide variety of infections, both systemic and cutaneous. Both yeasts and Fungi Imperfecti are included in this group. In making streak tests against these organisms, spore suspensions were prepared from agar slants and streaks were made on the test plates, which were incubated at 30 C for 40 hours, then read to determine whether inhibition of growth had occurred.

Secondary screening. Of the promising cultures from the primary screening 38 molds and 107 actinomycetes were tested against bacteria, whereas 35 molds and 58 actinomycetes were tested against a fungus.

Cultures were grown at 23 C in duplicate 500-ml Erlenmeyer shaker flasks containing 75 ml of medium, and the resulting fluids were tested for antibiotic activity by tube dilution methods. Samples were taken from the culture flasks with sterile 6-mm glass tubes fitted with cotton filters to remove mycelium. In the case of actinomycetes tests were made after 3, 5, and 7 days of growth, and the molds were tested after 4 and 6 days of growth. A variety of culture media

were used in order to increase the possibilities of finding promising antibiotic producers and as an aid in determining whether an antibiotic was a new one. Molds were grown on the following media:

1. Corn steep..... 30.0 g
   Lactose..... 20.0 g
   Tap water..... 1,000.0 ml
   pH—4.2 to 4.5

2. Corn steep..... 30.0 g
   Glucose..... 20.0 g
   Tap water..... 1,000.0 ml
   pH—4.2 to 4.5

3. $NaNO_3$..... 3.0 g
   K_2HPO_4..... 1.0 g
   $MgSO_4 \cdot 7H_2O$..... 0.5 g
   $FeSO_4 \cdot 7H_2O$..... 0.01 g
   Brown sugar..... 20.0 g
   Tap water..... 1,000.0 ml
   pH 6.2 to 6.6

4. Difco peptone..... 10.0 g
   Difco yeast extract..... 1.0 g
   Glucose..... 10.0 g
   NaCl..... 2.0 g
   Tap water..... 1,000.0 ml
   pH 6.5 to 7.0

The following media, all of which were adjusted to pH 6.9 to 7.1, were used for actinomycetes:

1. Difco tryptone..... 5.0 g
   K_2HPO_4..... 2.0 g
   NaCl..... 2.0 g
   $FeSO_4 \cdot 7H_2O$..... 0.01 g
   Glucose..... 10.0 g
   Dist. H_2O..... 1,000.0 ml

2. Difco peptone..... 4.0 g
   Difco beef extract..... 4.0 g
   Wilson liver fraction L.... 1.0 g
   Difco yeast extract..... 1·0 g
   Glucose..... 10.0 g
   Dist. H_2O..... 1,000.0 ml

3. Same as medium 2 except starch replaced glucose.

4. Same as medium 2 except lactose replaced glucose.

5. Corn steep..... 20.0 g
   Glucose..... 10.0 g
   Dist. H_2O..... 1,000.0 ml

6. Smaco casein hydrolyzate..... 3.0 g
   Glucose..... 10.0 g
   Dist. H_2O..... 1,000.0 ml

The culture fluids were tested for antibiotic activity against the bacteria, *Escherichia coli* and *Staphylococcus albus*, and against the fungus, *Cryptococcus hominis*. In conducting the tests against the bacteria, dilutions of the culture fluids were made in the following medium: peptone (7.5 g), yeast extract (2.5 g.), distilled water (1,000.0 ml), pH 7.25. On the first day of testing the following dilutions of the culture fluids were made: 1:10, 1:30, 1:100, 1:300, and 1:1,000. Tubes, containing 10 ml of medium each, were inoculated with 0.1 ml of a 24-hour broth culture of the test organism; they were incubated for 16 to 18 hours at 37 C and read to determine the highest dilution of the culture fluids that prevented growth. If the 1:1,000 dilution of a culture fluid completely inhibited growth of a test organism on the first day of assay, higher dilutions were run on the next day; these dilutions were generally as follows: 1:300, 1:1,000, 1:3,000, 1:10,000, and 1:30,000.

Tests against *C. hominis* were made in a medium of the following composition: $(NH_4)_2SO_4$ (1.0 g), glucose (20.0 g), yeast extract (5.0 g), and distilled water

(1,000.0 ml). The pH was adjusted to 7.5. The same system of dilutions was used for these tests as for those against the bacteria. The inoculum was prepared in flasks shaken for 24 hours at 23 C. Each tube containing 10 ml of medium was inoculated with 0.1 ml of culture. Tubes were incubated for 24 hours at 30 C.

RESULTS AND DISCUSSION

Primary screening. A total of 734 actinomycetes and 210 molds were tested against the five bacteria in the primary screening; 382 actinomycetes and 117 molds showed some inhibitory activity against at least one of the test organisms. A total of 764 actinomycetes and 315 molds were tested against the six pathogenic fungi; 350 actinomycetes and 178 molds inhibited at least one test organism. From these figures it is seen that 53 per cent of the cultures were active against at least one test bacterium, and 49 per cent were active against at least one test fungus.

TABLE 1

Scores made by actinomycetes and fungi in primary screening

BACTERIA AS TEST ORGANISMS			FUNGAL PATHOGENS AS TEST ORGANISMS		
Score	Per cent actinomycetes	Per cent fungi	Score	Per cent actinomycetes	Per cent fungi
0	47.5	46.0	0	53.7	42.5
1 to 3	27.7	15.7	1 to 3	16.1	20.7
4 to 6	13.3	28.6	4 to 6	11.9	13.0
7 to 9	4.9	3.8	7 to 9	10.5	13.3
10 to 12	4.0	5.7	10 to 12	5.0	7.0
13 to 15	2.6	0.0	13 to 15	1.6	2.9
			16 to 18	0.7	0.6

The results of the primary screening are presented in an abbreviated form in table 1. A scoring system was used to compile the data shown in this table. Each mold or actinomycete was scored on the basis of the medium on which it performed best. A culture was given 3 points for each test organism that it completely inhibited, 2 points for strong inhibition, and 1 for slight inhibition. Since there were 5 test bacteria, a mold or actinomycete would make a score of 15 if it completely inhibited the growth of all 5; but a score of 18 was possible against the fungi, as 6 of these were used. In the table are found the percentages of molds and actinomycetes that made particular scores against the test organisms. This table serves to illustrate the order of activity against the two groups of organisms, bacteria and pathogenic fungi.

Figures 1 and 2 show the relative sensitivities of the test organisms to the molds and actinomycetes, and they also give the orders of activity exhibited by the molds and actinomycetes against the bacteria and pathogenic fungi. It is noted that *B. subtilis* was the most sensitive bacterium to the action of both actinomycetes and molds; it was inhibited by more than 50 per cent of the cul-

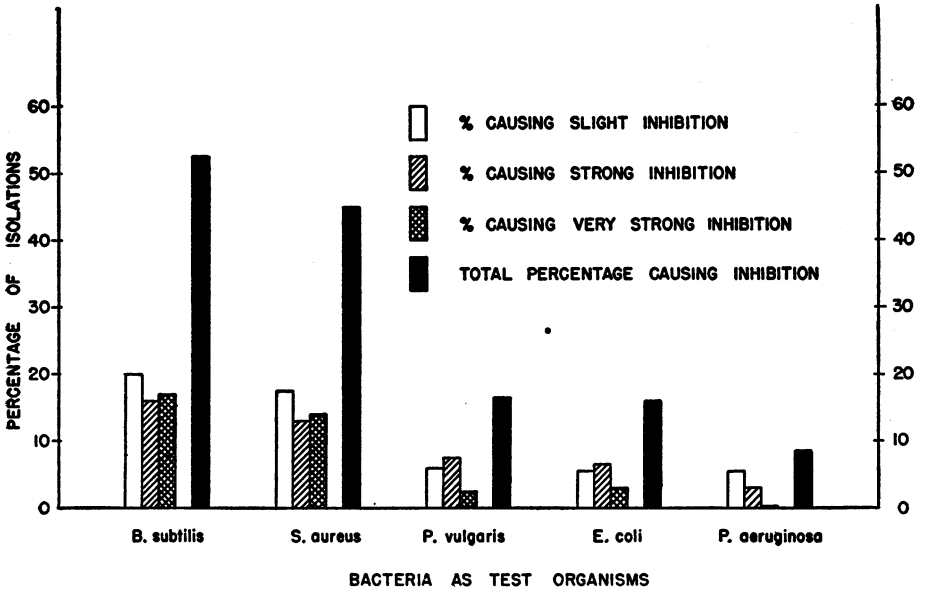

FIG. 1. THE DEGREE OF ANTIBIOTIC ACTIVITY OF ALL FUNGAL AND ACTINOMYCETE ISOLATIONS AGAINST EACH OF FIVE TEST BACTERIA

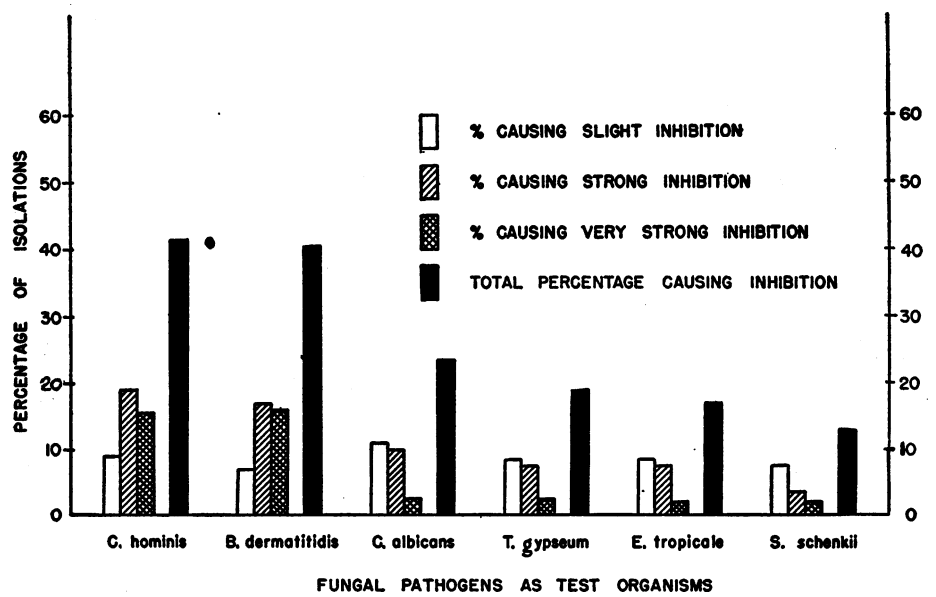

FIG. 2. THE DEGREE OF ANTIBIOTIC ACTIVITY OF ALL FUNGAL AND ACTINOMYCETE ISOLATIONS AGAINST EACH OF SIX TEST FUNGI

tures. *S. aureus* was only slightly less sensitive than *B. subtilis*. *P. aeruginosa* was the most resistant of the test bacteria, being inhibited by 10.9 per cent of the actinomycetes and 5.3 per cent of the molds. Approximately 14 per cent of the

actinomycetes inhibited the growth of *P. vulgaris* and *E. coli*, whereas 18 per cent of the molds inhibited these cultures.

C. hominis, inhibited by 41.0 per cent of the cultures, was the most sensitive organism among the pathogenic fungi; it was more sensitive to actinomycetes than to molds. *B. dermatitidis* was the most sensitive of the pathogenic fungi to molds, but it was more resistant to actinomycetes than was *C. hominis*. *T. gypseum* was the most resistant fungus to the action of the actinomycetes; it was inhibited by 11 per cent of these cultures. *S. schenkii*, inhibited by 10 per cent of the molds, was the most resistant fungus to this group of organisms.

TABLE 2

Distribution of antibiotic activity of actinomycetes and molds according to genus

GENUS	TESTED AGAINST BACTERIA			TESTED AGAINST FUNGAL PATHOGENS			TESTED AGAINST BOTH BACTERIA AND FUNGI: NUMBER ACTIVE AGAINST				
	Number active	Number inactive	Number tested	Number active	Number inactive	Number tested	Bacteria only	Molds only	Both	Neither	Number tested
Streptomyces	399	370	769	353	399	752	149	109	233	237	728
Micromonospora	1	2	3	0	6	6					
Nocardia					8	8					
Total for actinomycetes	400	372	772	353	413	766	149	109	233	237	728
Aspergillus	28	24	52	43	23	66	6	11	22	13	52
Penicillium	89	51	140	124	49	173	21	34	67	15	137
Other genera of hyphomycetes	11	47	58	13	68	81	6	5	3	43	57
Total for fungi	128	122	250	180	140	320	33	50	92	71	246
Total for actinomycetes and fungi	528	494	1,022	533	553	1,086	182	159	325	308	974

Table 2 gives the distribution of antibiotic activity of the actinomycetes and fungi according to genus. Most of the actinomycetes belong to the genus *Streptomyces*. It was found that 52 per cent of the *Streptomyces* strains were active against at least one test bacterium. Three *Micromonospora* strains were tested; one of these showed activity. Forty-seven per cent of the *Streptomyces* strains were active against the fungi. Six *Micromonospora* and 8 *Nocardia* strains were tested against the fungi, but none inhibited their growth. One may also observe in table 2 that 32 per cent of the *Streptomyces* strains that were tested against both bacteria and fungi were active against both groups, but 33 per cent were not active against either group.

Slightly over 50 per cent of the aspergilli inhibited growth of the test bacteria, and 65 per cent were active against the fungi. Sixty-four per cent of the penicillia were active against the bacteria, and 70 per cent were active against the fungi. Forty-two per cent of the aspergilli that were tested against both bacteria and fungi inhibited the growth of both groups, but 25 per cent were not active against either group. Sixty-seven per cent of the penicillia were active against both bacteria and fungi, but 11 per cent were not active against either

group. This activity against both bacteria and fungi may be attributed to the production of a single antibiotic, active against both bacteria and fungi; or it may be due to the production of more than one antibiotic. Also, when evaluating the significance of the percentage of active cultures, it must be borne in mind that one active species may have been isolated many times.

Secondary screening. Since many antibiotic-producing cultures were found by the primary screening, some of the promising cultures from the primary screening were studied by the secondary screening method. A total of 107 actinomycetes and 37 molds were tested against bacteria by the secondary screening method. Fifty-eight actinomycetes and 35 molds were tested against the fungus, *C. hominis*. The results of these studies are presented in table 3; the numbers of cultures that inhibited the test organisms at particular dilution ranges are given. These dilutions represent the highest ones at which complete inhibition of

TABLE 3

Potencies of culture fluids produced by molds and actinomycetes*

CULTURES TESTED	TEST ORGANISM	DILUTIONS				
		No inhibition	1:10 to 1:100	1:100 to 1:1,000	1:1,000 to 1:10,000	>10,000
107 Actinomycetes	*E. coli*	27	20	24	29	7
	S. albus	6	18	24	43	16
37 Molds	*E. coli*	27	10	0	0	0
	S. albus	24	12	1	0	0
58 Actinomycetes	*C. hominis*	1	9	14	24	10
35 Molds	*C. hominis*	2	11	9	8	5

* The figures represent the numbers of cultures that produced culture fluids inhibiting the test organism in the particular dilution ranges shown. These figures represent the maximum potencies of the cultures.

growth occurred on any of the days the culture fluids were tested. Since too much space would have been required to present these data in detail, there is no indication of the relative merits of the different culture media. Suffice it to say that no medium proved generally superior to the others for antibiotic production. However, for any particular actinomycete or mold there were usually great differences in the antibiotic potencies produced in the different culture media.

It is observed that 80 of the 107 actinomycetes produced culture fluids that inhibited the growth of *E. coli*, and 101 produced fluids that inhibited *S. albus*. It is worthy of note that 29 actinomycetes produced culture fluids that prevented the growth of *E. coli* in dilutions of 1:1,000 to 1:10,000, whereas the maximum dilution for complete suppression of growth for culture fluids of 7 actinomycetes was greater than 1:10,000. The results against *S. albus* are even more striking, for 43 of the 107 actinomycetes yielded culture fluids that completely inhibited growth of this organism in dilutions of 1:1,000 to 1:10,000, and 16 cultures produced fluids that prevented growth in dilutions above 1:10,000.

The results obtained with molds were not so encouraging as those obtained with actinomycetes. Only 10 of 37 molds produced culture fluids that inhibited the growth of *E. coli*, whereas 13 produced fluids that inhibited *S. albus*. No mold culture fluid inhibited *E. coli* in a dilution greater than 1:100 and *S. albus* in a dilution greater than 1:1,000. It is entirely possible, however, that further work on the molds will lead to the development of conditions more suitable for antibiotic production.

All except 1 of 58 actinomycetes produced culture fluids that prevented growth of the fungus, *C. hominis*, and all but 2 of 35 molds produced culture filtrates that suppressed growth of this organism. It is noted that culture fluids from 24 of 58 actinomycetes prevented growth in dilutions of 1:1,000 to 1:10,000, and 10 cultures yielded fluids that prevented growth in dilutions above 1:10,000. Eight of 35 molds gave culture fluids that prevented growth in dilutions of 1:1,000 to 1:10,000, and 5 gave fluids that prevented growth in dilutions greater than 1:10,000.

Since many molds and actinomycetes were found to produce antibiotic substances, there are a number of cultures available for further studies. The more promising cultures are being investigated to determine whether the antibiotic substances produced by them are of any therapeutic value. Studies are being conducted on the microbiology, chemistry, and pharmacology of the antibiotic substances.

SUMMARY

Molds and actinomycetes were isolated from the soil and studied for antibiotic activity. Approximately 1,000 cultures were tested.

The cultures were tested against a group of bacteria and a group of pathogenic fungi by the streak plate method. Approximately 50 per cent of the cultures were found to produce inhibitory substances.

A number of cultures that performed well in the primary screening (agar streak) were studied further in shaker flasks. Thirty-seven molds and 107 actinomycetes were grown in this way, and their resulting culture fluids were tested against *Escherichia coli* and *Staphylococcus albus*; 58 actinomycetes and 35 molds were tested against *Cryptococcus hominis*. Ten molds and 80 actinomycetes produced culture fluids that inhibited the growth of *E. coli*, whereas 101 actinomycetes and 13 molds produced culture fluids that inhibited the growth of *S. albus*. All but 1 of 58 actinomycetes produced culture liquors that prevented the growth of *C. hominis*, and all but 2 of 35 molds yielded fluids that prevented growth of this organism.

ACKNOWLEDGMENT

The authors are grateful to Mrs. Millard Bachelder, Miss June Coburn, Mrs. Cornelius Kuivenhoven, and Miss Alyda Vander Salm for their valuable technical help. They also wish to express their appreciation to the salesmen and other employees of The Upjohn Company, who supplied soil samples for these studies.

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