

ACTIVITY OF THE ASPERGILLI ON CELLULOSE, CELLULOSE DERIVATIVES, AND WOOL

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Frequent reference is made in the literature to the activity of various organisms on different industrial materials. Usually the work has been done by individuals more intimately concerned with the nature of the degradation than with the identity of the organisms. Recently, three papers have been published in which the earlier works have been reviewed and new data presented. A wide range of organisms has been studied for ability to attack cellulose by White *et al.* (6), and by Marsh *et al.* (2). The relationship of the black Aspergilli to cellulose degradation has been studied in detail by White *et al.* (8). The identity of all the organisms employed in these investigations was thoroughly established in accordance with the monograph of Thom and Raper (5).

A large collection of microorganisms isolated from deteriorating materials is maintained in this laboratory. One objective of our research on this collection is the determination of the organisms which are active in the degradation of various materials employed by the Quartermaster Corps. A continuous testing program is under way in which all the organisms isolated from deteriorating materials are being evaluated for their activity. The present paper reports the results on cellulose and on autoclaved wool obtained from a study of 422 isolates belonging to the genus *Aspergillus*. Isolates, representative of each group, were examined microscopically. Any organism about which there was any question of proper identification was sent to Dr. K. B. Raper for confirmation.

The complex nature of cellulose degradation is becoming more apparent as experimental data accumulate. In an earlier report (4), it was shown that the ability to hydrolyze the 1,4 β -glucosidic linkages of the cellulose chain does not in itself determine whether an organism can attack cellulose, since many non-cellulolytic micro-

organisms possess the enzyme (Cx) carrying out that reaction. It was postulated that cellulolytic organisms possess an additional enzyme (C₁) capable of converting native cellulose into the form acted on by Cx. This enzyme (C₁) is lacking in non-cellulolytic organisms. The present paper considers both the ability of members of the *Aspergilli* to hydrolyze the 1,4 β linkage, and the ability to degrade cellulose.

EXPERIMENTAL

The method for testing the activity of all isolates was slightly modified from that previously used in this laboratory by White *et al.* (6). Cloth strips ravelled to a width of 1 inch and cut to a length of 3 inches, were placed into 20 \times 150 mm. pyrex test tubes. A nine ml. aliquot of the following nutrient solution was added to each tube: yeast extract 0.01%; MgSO₄·7H₂O, 0.03%; NH₄NO₃, 0.1%; M/100 potassium phosphate buffer pH 5.7; initial pH 6.3 \pm 0.2. After sterilization and cooling, each tube was inoculated with 1 ml. of spore suspension. Incubation was at 30° C. for 2 weeks, at the end of which time the strips were examined for growth and harvested. After the usual conditioning treatment, tensile strength determinations were made by means of a Scott tester. Five replicates of the 3.3 oz. bleached cotton sheeting and four replicates of the wool charmeen were used. For our purpose, we consider as inactive those organisms which effect a loss in tensile strength of less than 15 per cent in two weeks. This is purely arbitrary and organisms which are weakly active are not sharply defined. In addition, more limited tests were made with representative isolates of each group. Twelve oz. grey cotton duck was used in one series of experiments for comparison with results obtained on sheeting. In another experiment, the utilization of wool as a nitrogen source was tested by omitting the NH₄NO₃ of the nutrient solution and adding one per cent sucrose for comparison with the previous results where the wool was the only carbon source. In a third series of experiments, the ability of representative isolates to degrade filter paper was determined by incubation in shake flasks in accordance with the method previously described (3). Loss in weight was used as a criterion of cellulolytic activity.

The practical value of data obtained on wool that has been auto-

claved is questionable, since woolen cloth is never subjected to such treatment in actual service. In field tests, the only fungi found to degrade wool are the dermatophytes and members of the Gymnoascaceae. On the other hand, the results on autoclaved wool may be of some value to those seeking organisms capable of attacking other proteinaceous materials. Eventually, perhaps, the more active of the organisms attacking autoclaved wool may be tested on wool sterilized by some other means. Preliminary experiments from these laboratories (7) indicate that unautoclaved wool is resistant to most of the fungi tested. Under field conditions, most severe degradation of unautoclaved wool seems to be caused by bacteria and actinomycetes rather than by fungi. Such degradation is most rapid when the fabric is in contact with the soil.

The following represents a generalized summary (TABLE 1) of the results. Each *Aspergillus* group is arranged in the order of its activity. *A. terreus*, *A. fumigatus*, and *A. flavipes* were the only groups active on both 3.3 oz. bleached cotton sheeting and wool. *A. clavatus*, *A. flavus-oryzae*, and *A. tamarii* were active on wool but inactive on cotton sheeting and on duck. *A. ochraceus*, *A. nidulans*, and *A. rugulosus* were active on wool and grey duck but not on sheeting. The *A. ustus* group, in general, was inactive on wool and cotton sheeting, but active on grey duck. In the *A. nidulans* group, *A. unguis* was inactive on wool, but both *A. nidulans* and *A. rugulosus* (one isolate) were active. Members of this group were inactive on cellulose. The remaining groups *A. wentii*, *A. versicolor*, *A. glaucus*, and *A. niger* (with the exception of the *A. luchuensis* series on grey cotton duck) proved inactive on both cellulose and on wool.

A close relationship between physiological activity and morphology is shown (TABLE 1). The various isolates of a species are remarkably alike in their physiological properties as determined by these degradation studies. Furthermore, morphologically stable species tend to be more uniform physiologically than species showing greater variation in growth patterns. Thus, the isolates of *A. fumigatus* are similar morphologically and in their abilities to degrade autoclaved wool and cotton sheeting, while the morphologically dissimilar isolates of *A. ustus* tend to show greater variability in their activity on the two substrata used.

TABLE 1
ACTIVITY OF *Aspergillus* ISOLATES ON WOOL AND COTTON

Groups (13) Species (36)	Number isolates	Average % loss in T.S.**				Activity distribution of isolates			
		Wool		Cotton		Wool		Cotton	
		Charmeen	Sheeting 3.3 oz.	Grey duck 12 oz.	Grey duck A* I*	Charmeen A* I*	Sheeting A* I*	Grey duck A* I*	Grey duck (Marsh) A* I*
<i>A. terreus</i> group	36								
<i>A. terreus</i>	35	65	68	77	35-0	35-0	6-0	7-0	
<i>A. niveus</i>	1	38	73	79	1-0	1-0	1-0	1-0	
<i>A. fumigatus</i> group	31								
<i>A. fumigatus</i>	24	53	77	84	24-0	24-0	2-0	9-0	
<i>A. fischeri</i>	7	67	61	55	7-0	5-2	3-0		
<i>A. flavipes</i> group	4								
<i>A. flavipes</i>	4	27	38	71	3-1	4-0	3-0	7-0	
<i>A. clavatus</i> group	4								
<i>A. clavatus</i>	3	68	6	7	3-0	0-3	0-3	8-4	
<i>A. giganteus</i>	1	3	19	58	0-1	1-0	1-0	2-0	
<i>A. flavus-oryzae</i> group	48								
<i>A. oryzae</i>	42	70	8	6	2-0	0-2	0-2	0-2	
<i>A. flavus</i>	4	68	1	5	4-0	0-42	0-7	0-7	
<i>A. parasiticus</i>	2	53	2	8	2-0	0-2	0-2	0-1	
Unclassified	2	57	7	2	2-0	0-2	0-2	0-1	

* A = Active, I = Inactive
 ** Though not justifiable mathematically, an average per cent loss gives a value that can be useful in comparing activities of the different species.

TABLE 1—Continued

Groups (13) Species (36)	Number of isolates	Average % loss in T.S.**				Activity distribution of isolates			
		Wool		Cotton		Wool		Cotton	
		Charmeen	Sheeting 3.3 oz.	Grey duck 12 oz.	Grey duck A* I*	Charmeen A* I*	Sheeting A* I*	Grey duck A* I*	Grey duck (Marsh) A* I*
<i>A. tamarii</i> group	15								
<i>A. tamarii</i>	15	58	5	5	15-0	0-15	0-3	0-7	
<i>A. ochraceus</i> group	5								
<i>A. ochraceus</i>	4	53	0	36	4-0	0-4	3-1	4-0	
<i>A. sclerotium</i>	1	79	0	13	1-0	0-1	0-1	0-1	
<i>A. ustus</i> group	34								
<i>A. ustus</i>	30	12	11	49	7-23	9-21	30-0	3-1	
<i>A. ustus</i> var. <i>laevis</i>	4	0	3	12	0-4	0-4	1-3	1-0	
<i>A. nidulans</i> group	31								
<i>A. unguis</i>	26	5	1	8	1-25	0-26	0-6	0-2	
<i>A. nidulans</i>	4	30	7	25	3-1	1-3	2-2	2-2	
<i>A. rugulosus</i>	1	45	0	50	1-0	0-1	1-0	1-0	
<i>A. wentii</i> group	2								
<i>A. wentii</i>	1	4	8	10	0-1	0-1	0-1	0-5	
<i>A. penemansis</i>	1	0	5	13	0-1	0-1	0-1	0-5	
<i>A. versicolor</i> group	78								
<i>A. sydowii</i>	47	2	2	4	0-47	0-31	0-8	0-6	
<i>A. versicolor</i>	31	2	2	7	0-31	0-31	0-5	0-6	

TABLE 1—Continued

Genus (13) Species (36)	Number of isolates	Average % loss in T.S.**						Activity distribution of isolates				
		Wool		Cotton		Wool		Cotton		Cotton		
		Charmeen	Sheeting 3.3 oz.	Grey duck 12 oz.	Charmeen A* I*	Sheeting A* I*	Grey duck A* I*	Grey duck (Marsh) A* I*				
<i>A. glaucus</i> group	35											
<i>A. repens</i>	14	2	4	6	0-14	0-14	0-3					
<i>A. chenalieri</i>	15	1	2	8	0-15	0-15	0-3					
<i>A. chenalieri</i> var. <i>intermedius</i>	2	0	3	2	0-2	0-1	0-1					
<i>A. chrysogenum</i>	1	0	3	6	0-1	0-1	0-2					
<i>A. restrictus</i>	3	0	3	6	0-3	0-3	0-2					
<i>A. niger</i> group	99											
<i>A. niger</i> series	13	0	0	22	0-13	0-13	1-11					0-3
<i>A. niger</i> var. <i>Tieghem</i>	11	0	3	0	0-11	0-11	1-11					0-3
<i>A. niger</i> mut. <i>cinnamomeus</i>	1	5	0	39	0-1	0-1	1-0					0-2
<i>A. niger</i> mut. <i>schiemannii</i>	1	0	0	0	0-1	0-1	0-1					0-1
<i>A. foetidus</i>	1	0	0	8	0-1	0-1	0-1					0-1
<i>A. phoenicis</i>	1	0	10	0	0-1	0-1	0-1					0-1
<i>A. carbonarius</i> series	1	0	0	1	0-1	0-1	0-1					0-1
<i>A. fonssecaus</i>	1	0	0	1	0-1	0-1	0-1					0-1
<i>A. carbonarius</i>	1	0	0	1	0-1	0-1	0-1					0-1
<i>A. luchuensis</i> series	14	1	2	24	0-14	0-14	8-4					2-3
<i>A. niger</i> group unclassified	66	0	0	1	0-66	0-66	0-25					0-3
Total	422											

Of the 422 isolates examined, only seven appear to differ from others in their respective groups in their behavior on autoclaved wool and on cotton sheeting. Originally, several others also appeared to be exceptions. Closer examination revealed either that these had been improperly identified, or that contaminants were present. In the latter case, after purification of the culture, the organism behaved in a manner characteristic of the species. Because of this uniformity, exceptional behavior on a substratum is suggestive of contamination or mis-identification. The following exceptions have been noted. Two of the seven isolates of *A. fischeri* tested differ from the other twenty-nine members of the *A. fumigatus* group in being unable to attack cotton sheeting. They were, however, active on grey duck, and the inability to attack the sheeting may have been due to a nutritive deficiency. It is interesting to note that both isolates originated in Florida, and that both were somewhat different macroscopically from our other isolates of *A. fischeri*. One isolate of *A. flavipes* differed from the others in its inactivity on wool. All of the other members, however, might be classed as weakly active on this substratum. The results of Marsh (2) relative to the activity of *A. giganteus* were confirmed. This species, of which only one representative was tested, differed from the other members of the *A. clavatus* group in being active on cotton, but not on wool. In the *A. nidulans* group, one of twenty-six isolates of *A. unguis* was able to attack wool, and only one of four isolates of *A. nidulans* was able to degrade cotton sheeting. Three of four isolates of *A. nidulans* attacked wool.

The relationships found above are based on results obtained under a definite set of conditions. The data resulting from other tests are in general agreement with those given. Organisms capable of using autoclaved wool as a carbon source were also able to degrade the same material when it was the only N-source (in the presence of sucrose). In like manner, there is agreement between the results using grey cotton duck and those using 3.3 oz. bleached cotton sheeting, except that (1) the activity is usually greater on the duck; (2) the following organisms are unable to attack 3.3 oz. sheeting but are able to attack duck.

(a) *A. luchuensis* series of the *A. niger* group (four exceptions, unable to attack duck).

TABLE 2
ABILITY OF SPECIES OF *Aspergillus* TO PRODUCE AN ENZYME
CAPABLE OF HYDROLYZING CARBOXYMETHYL CELLULOSE

<i>Aspergillus</i> species		Incubation time (days)	Growth	Cx activity*
Cellulolytic species				
<i>A. fumigatus</i>	QM 45b	9	4+	.14
<i>A. fumigatus</i>	QM 6b	9	2+	.22
<i>A. fischeri</i>	QM 864	9	4+	.13
<i>A. terreus</i>	QM 82j	9	4+	.31
<i>A. terreus</i>	QM 91c	9	4+	.31
<i>A. flavipes</i>	QM 24a	9	4+	.27
Non-cellulolytic species				
<i>A. clavatus</i>	QM 862	9	4+	.11
<i>A. chevalieri</i>	QM 312	20	none	—
<i>A. repens</i>	QM 210	20	none	—
<i>A. nidulans</i>	QM 25b	20	2+	.28
<i>A. unguis</i>	QM 8f	20	4+	.27
<i>A. ustus</i>	QM 29c	9	4+	.56
<i>A. ustus</i>	QM 892	9	4+	.43
<i>A. sydowi</i>	QM 4d	9	4+	.40
<i>A. sydowi</i>	Fla. F 3	9	4+	.38
<i>A. versicolor</i>	QM 17d	9	4+	.31
<i>A. niger</i> v. Tiegh.	QM 458	9	4+	.21
<i>A. carbonarius</i>	QM 331	9	3+	.02
<i>A. tamaris</i>	QM 50b	9	4+	.35
<i>A. tamaris</i>	QM 75b	9	4+	.38
<i>A. flavus</i>	QM 4m	9	4+	.28
<i>A. flavus</i>	QM 63c	9	4+	.20
<i>A. ochraceus</i>	QM 26b	9	4+	.12

* Cx activity: 5 ml. 1% CMC 50T + 1 ml. M/2 citrate pH 5.0 + 3 ml. water + 1 ml. cell-free filtrate. Temperature 50° C; time 1 hour. Results expressed as reducing sugar in terms of glucose in mg./ml. of mixture/hour.

(b) *A. niger* v. Tiegh. One isolate (of 13) represents an exception to the rule that no *black* Aspergilli attack cellulose, the members of the *A. luchuensis* series and *A. niger* mut. *schiemanni* not being black. It is like the latter, however, in being able to attack grey duck but not cotton sheeting. Raper, in verifying the correctness of the identification, states: "this [organism] obviously suffers from some nutrient deficiency as evidenced by its very limited growth on Czapek solution agar. Perhaps this deficiency might in some way be related to its cellulolytic properties."

(c) *A. niger* mut. *schiemanni*.

(d) *A. ustus* was highly variable in its attack on cotton sheeting but all isolates attacked the grey duck.

(e) *A. ochraceus* group (two exceptions, unable to attack duck).

The results of Marsh (2) on duck agree quite well with ours for the same substratum, except perhaps for *A. clavatus*. In this group, Marsh found a preponderance of active strains in contrast to the inactivity recorded for our more limited number of isolates.

Growth of species of Aspergillus on carboxymethyl cellulose. Organisms representing the various groups in the genus *Aspergillus* were tested for their ability to grow on carboxymethyl cellulose (CMC)¹ in shake flasks. At the end of incubation, the cultures were filtered and the filtrates tested for ability to hydrolyze CMC (TABLE 2) by methods previously described (4). The time of incubation was varied in accordance with the rate of growth of the cultures. All of the Aspergilli tested possess the ability to produce the enzyme Cx. The absence of growth by two members of the *A. glaucus* group (QM 312 and QM 210) is not unusual, these being difficult organisms to grow in shake-cultures. The low activity of the *A. carbonarius* filtrate is in opposition to the good growth obtained. The two members of the *A. nidulans* group (QM 25b and QM 8f) showed fair growth but no activity in the filtrates after 9 days incubation. Yet the 20-day filtrates had good activity. This effect of culture age on filtrate activity has been frequently observed.

DISCUSSION

Cellulose which has undergone various chemical and physical steps during purification differs in its susceptibility to degradation by microorganisms. The effects of such treatments may be summarized as follows:

(a) Increase in surface area renders cellulose more easily attacked.

(b) Decrease in crystallinity or decrease in "cross linkages" between cellulose chains. For example, viscose rayon and cuprammonium rayon are more readily attacked than the initial cellulose.

¹ CMC 50T supplied by Hercules Powder Company, Wilmington, Delaware. Degree of substitution 0.52.

(c) Removal of impurities may lead to apparent resistance. Highly bleached and desized cotton cloth may not support growth of an organism due to the absence of growth factors, whereas the same organism may grow well on the untreated fabric. This type of resistance may be overcome by adding the proper vitamins, minerals, etc.

(d) Deposition of chemicals. Incomplete removal of bleaches or other chemicals may inhibit fungus growth.

(e) Chemical modification of the cellulose molecules by substitution tends to increase resistance. As the number of added substituents per anhydroglucose molecule increases, the resistance increases. One substituent on every anhydroglucose unit appears to confer complete resistance.

It is not unusual then, that bleached cotton sheeting differs from the more crude cotton duck in its susceptibility to attack by microorganisms. Where differences occur, the duck is the more rapidly degraded. Though this problem has been considered before (8), it is not yet certain that the answer is simply growth factor deficiency. For instance, treatment of the resistant, bleached sheeting with alkali has been found to permit growth of *A. luchuensis* (QM 873) on fabric which would not otherwise support growth. While these data may indicate that the resistance is due to a toxic chemical present in the fabric, the problem is not so simple since filter paper is also resistant. It seems unlikely that the same chemical impurity would be present there as in the bleached sheeting. As a rule, our results on decomposition of filter paper in shake flasks agree with the data on loss in tensile strength of cotton sheeting.

Since all of the *Aspergilli* seem capable of hydrolyzing the linkages between anhydroglucose units in straight chain molecules derived from cellulose, it appears that the difference between cellulolytic and non-cellulolytic must be in the ability to carry on an earlier step by means of the postulated enzyme (C₁). The ability to produce the enzyme Cx is common to all *Aspergilli*. The non-cellulolytic members of the genus produce as large amounts as do the cellulolytic species. Most of the strains tested produce much more Cx if a substratum is present which contains the 1,4- β -glucosidic linkage in long chains. The enzyme diffuses readily into

the medium, a requirement if such long molecules are to be split up to permit diffusion into the cell.

A close correlation is found in the *Aspergilli* between the morphological entity as exemplified by the species or group, and the physiological activity. Such data are useful in evaluating results given in the literature, even when the organism then used is no longer available. Thus, Basu (1) in a recent paper, reports his strains of *Aspergillus niger* as non-cellulolytic, and *A. ustus*, *A. terreus*, *A. fumigatus*, and *A. sydowi* as cellulolytic. All of these but one are in agreement with the data gathered in this report. It appears unlikely that the organism he calls *A. sydowi* is correctly named since none of 53 isolates of that organism tested by us or by Marsh has any cellulolytic action. Such a conclusion must, however, be accepted with reservations.

For ready reference, the groups may be brought together on the basis of activity as follows:

A. Active on Cellulose

1. Active on wool

a. Active on cotton sheeting and grey duck

A. terreus, *A. niveus*

A. fumigatus, *A. fischeri*

A. flavipes

b. Active on grey duck but not on sheeting

A. ochraceus

A. nidulans, *A. rugulosus*

2. Inactive on wool

a. Active on sheeting and on duck

A. giganteus

b. Active on grey duck but not on sheeting

A. ustus

A. niger mut. *schimmani*

A. luchuensis series (see below)

B. Inactive on Cellulose

1. Active on wool

A. clavatus

A. flavus, *A. oryzae*, *A. parasiticus*

A. tamarii

A. sclerotiorum

2. Inactive on wool

*A. unguis**A. wentii*, *A. panamensis**A. versicolor*, *A. sydowi**A. repens*, *A. chevalieri*, *A. montevidensis*, *A. restrictus**A. carbonarius* series*A. niger* series (except *A. niger* mut. *schiemanni*)*A. luchuensis* series (except for above)

SUMMARY

1. The isolates of any particular species of *Aspergillus* are alike in their ability to attack a particular substratum, such as cellulose or wool.

2. Some species of *Aspergillus* can attack both autoclaved wool and cotton, some one but not the other, and still others can attack neither.

3. Some species of *Aspergillus* are capable of degrading crude cotton duck but not the more pure cellulose of bleached cotton sheeting. These organisms are cellulolytic. Failure to grow on the cotton sheeting must be attributed to other causes.

4. All members of the genus *Aspergillus* appear to be capable of hydrolyzing the 1,4 β -glucosidic linkages found in the cellulose chain.

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