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CELLULOSE DECOMPOSITION BY THE ASPERGILLI WITH SPECIAL REFERENCE TO

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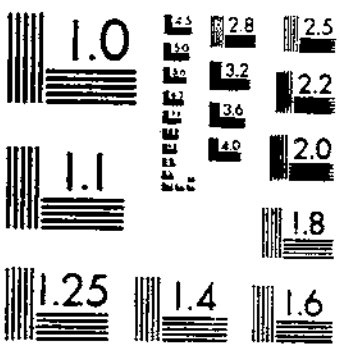

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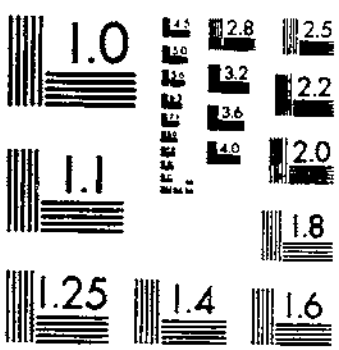

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WITH SPECIAL REFERENCE TO ASPERGILLUS NIGER

By MARION E. SIMPSON and PAUL B. MARSH

Crops Research Division
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CONTENTS

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CELLULOSE DECOMPOSITION BY THE ASPERGILLI

WITH SPECIAL REFERENCE TO ASPERGILLUS NIGER[1]

By MARION E. SIMPSON and PAUL B. MARSH, *Crops Research Division, Agricultural Research Service*

SUMMARY

Most of the 14 major taxonomic groups of the Aspergilli are known to have the ability to decompose cellulose. However, in the *A. niger* group, activity has been reported to be confined to the purple-black forms of the *A. luchuensis* series and has not been found in the truly black types closely related to *A. niger* v. Tiegh. Strength loss of cotton fabric during incubation with the fungus was the criterion of activity.

In experiments reported here, strength loss was caused in a cotton fabric by *A. niger* and other truly black Aspergilli when a small amount of glucose was incorporated into the test medium. The same result occurred with several other soluble carbon sources. Changes in the concentrations of salts in the test medium, in the presence of added glucose, also influenced the degree of decomposition. Inoculation of the test strip by brushing on the spores, rather than by adding them in liquid suspension, resulted in decomposition even in the absence of supplementary glucose. With each of these manipulations of the test conditions, cellulose decomposition was accompanied by a decrease in pH of the test medium as measured at the end of the incubation.

Several members of the *A. luchuensis* series caused strength loss in a cotton fabric without any supplementary carbon compound. With effective isolates, there was an accompanying decrease in the pH of the medium.

In experiments with *A. niger* in the presence of a range of concentrations of supplementary glucose, addition of $CaCO_3$ to the test medium to maintain pH near neutral prevented cellulose degradation even though the fungus grew well on the glucose.

Setting the pH of the test medium initially at a low level resulted in cellulose decomposition by *A. niger* even in the absence of any supplementary carbon compound. This cellu-

[1] This bulletin consists principally of excerpts from theses presented by the senior author to the faculty of the University of Maryland in partial fulfillment of requirements for the M.S. and Ph. D. degrees, June 1959 and June 1962. The work was carried out in the laboratories of the Crops Research Division, U.S. Department of Agriculture, Beltsville, Md.

lose degradation was proved not to be due to a direct effect of pH on the cellulose of the test strip.

The favorable effect of low pH on cellulose decomposition by *A. niger* occurred not only *in vivo* but also with filtrates from the growth medium of the fungus. The action of filtrates was measured in three ways: (1) in an increase in the degree of swelling of enzyme-exposed fiber in alkali, (2) in the production of reducing materials from cotton fiber, and (3) in the production of reducing materials from carboxymethyl cellulose.

In comparative tests, certain other Aspergilli (nonblack) also produced cellulases with optimum activity at rather low pH levels. However, in contrast to the results with *A. niger*, maintaining the pH of the culture medium near neutral with calcium carbonate did not prevent fabric strength loss by *A. terreus* Thom and *A. flavipes* (Bainier and Sartory) Thom and Church nor did setting a low initial pH in the medium cause strength loss by *A. flavus* Lk. ex Fr. Thus, while the pH of the medium was a dominant factor in respect to *in vivo* decomposition of cotton cellulose by the black Aspergilli, it was less so with these other species.

In comparative tests with the well-known cellulose-decomposing fungus *Myrothecium verrucaria* Ditm. ex Fr., glucose favorably affected cellulose decomposition during growth at elevated incubation temperatures without influencing the final pH of the test medium.

The effect of supplementary glucose on cellulose decomposition by *A. niger in vivo* was measurable not only in strength loss but also in the degree of alkali-swelling of the fiber. The alkali-swelling showed a pronounced tendency to parallel the strength loss; this observation supplements previously published evidence indicating that the alkali-swelling response as here employed is principally, if not entirely, a measure of degradation of cellulose rather than of pectin or other materials in the outer wall of the fiber.

When culture filtrates from *A. niger* were tested for cellulase activity in buffered solutions, similar pH-activity curves were obtained over a temperature range from 30° to 75° C. These results suggest that *A. niger* cellulase may be a single enzyme rather than a complex of several enzymes. Absorption of cellulase (S factor) by cotton fiber was greater at low pH levels than at higher ones. The enzyme was at least moderately stable at a pH of 3.0 at room temperature. Increasing the time of the S-activity assay from 1 to 3 hours increased the steepness of pH-activity curves.

Filtrates from the growth of *A. niger* on glucose and on cellobiose as sole carbon sources failed to exhibit major cellulase activity.

BACKGROUND

The investigations reported here originated with the observation that a strain of *Aspergillus niger* could weaken a cotton

fabric if a small amount of glucose were present in the test medium. The particular strain had been used for many years in the specification testing of textiles for mildew resistance and had become widely regarded in both applied and theoretical scientific circles as a "superficial" or "non-cellulose-decomposing organism." This fungus and related forms have now been examined in greater detail for factors that influence their behavior toward cellulose.

In 1948, White and colleagues (*17*)[2] reported on the cellulose-decomposing potentialities of 27 truly black isolates from the *Aspergillus niger* group. In these tests, which employed breaking strength of fungus-incubated cotton fabric as a criterion for cellulose decomposition, all 27 isolates were found to be inactive.

In the same year, White, Siu, and Reese (*18*) concluded from similar tests on 52 members of the *A. niger* group: "None of the truly black Aspergilli were found to be capable of cellulolytic action. Such activity was confined to the ochraceous *A. niger* mutant Schiemanni and to the more or less purple-brown forms which comprise the *A. luchuensis* series. The results seem to warrant the general conclusion that, within the *Aspergillus niger* group, cellulolytic ability is absent in the *A. niger* series and the *A. carbonarius* series but is, in contrast, a fairly constant feature of the *A. luchuensis* series."

In 1949, Marsh and colleagues (*8*) reported results on 13 isolates from the *A. niger* group which were in accord with the above-quoted conclusion in that 2 isolates from the *A. luchuensis* series were found to be active and all of the truly black isolates tested exhibited little, if any, activity.

In 1952, Reese and Levinson (*14*) indicated that *A. niger* TC 215–4247 could decompose wood cellulose but could not decompose cotton and that a single truly black Aspergillus (QM 877) had been found which could weaken cotton cellulose. The former isolate is widely used in specifications for testing mildew resistance of fabrics as a "superficial" organism. It is referred to frequently in this bulletin by the abbreviated designation "TC."

Breaking strength of fungus-incubated cotton fabric has been used in numerous investigations as an index of microbial cellulose decomposition (*16*). However, a more sensitive measure of decomposition is the degree of swelling of the fiber in alkali. An alkali-swelling test has been described (*11*) and information has been presented on its application to cotton fiber previously exposed to micro-organisms or to cellulase-containing filtrates (*7*). In this test, the enzymatic factor whose action is measured has been designated "S factor." Increased swelling in alkali is regarded as an index of cellulose decomposition because filtrates from *Myrothecium verrucaria* were effective in influencing the property only when growth had occurred in

[2] Italic numbers in parentheses refer to Literature Cited, p. 45.

the presence of cellulose (*7*). Other experimental facts supporting this conclusion are reported in this bulletin.

At the outset of our investigations, the only commercially available cellulase preparations were from undisclosed organisms. During the course of the work, we learned that *A. niger* was being used for producing some such preparations. Certain experiments on these cellulases were therefore included in our investigations for comparative purposes.

METHODS

Details on materials and methods are given in the appendix.

Cellulose decomposition was observed (a) with the intact fungus and (b) with filtrates from fungal growth media. In the former type of observation, cotton fabric was incubated in the presence of the fungus and the effect on the fabric was measured in breaking strength and in degree of swelling in alkali. Alkali-swelling was determined by a previously described technique (*7*, *11*). In the latter type of observation, fungal growth-medium filtrates were tested for cellulase action on raw cotton and on carboxymethyl cellulose (CMC). The effects on the fiber were measured in degree of alkali-swelling and in reducing substances produced. Reducing substances were determined colorimetrically with a dinitrosalicylic acid (DNS) reagent. The same reagent was also used to determine production of reducing substances from CMC.

RESULTS

Experiments With the Black Aspergilli

Decomposition of Cotton Fabric by the Growing Fungus

EFFECTS OF SUPPLEMENTARY GLUCOSE.—The investigations described in this bulletin began with a single experiment in which the TC isolate of *Aspergillus niger*, contrary to expectation, caused strength loss in a cotton fabric. As stated earlier, this fungus had been known widely as a non-cellulose-decomposer because of its alleged inability to weaken cotton fabric. This earlier conclusion was based on incubations of fabric with the fungus in the presence of either mineral salts or mineral salts plus a high concentration of glucose. When we incubated fabric with *A. niger* TC for 2 weeks in the presence of mineral salts-agar and an intermediate concentration of glucose (0.5 percent), 10 test strips lost an average of 35 percent of their strength. On the other hand, 10 sterile-incubated control strips had a strength of 108 pounds, as compared with an original strength of 109 pounds.

The purity of the TC culture was verified microscopically. In addition, another culture of the same isolate was obtained from the American Type Culture Collection and similarly

tested. This time there was an even greater strength loss—49 percent.

These experimental results were confirmed and extended. Tests were repeated with a re-isolated culture of *A. niger* TC. "Q" duck and two other unbleached 8-ounce duck samples ("Hooper" duck and "M–2" duck) were tested. As an added modification, the tests were performed with and without added glucose in the mineral salts-agar. The results showed a distinct strength loss in the presence of 0.5 percent of glucose, whereas in the absence of glucose only insignificant losses occurred (table 1). In addition to strength determinations, the sensitive alkali-centrifuge test was employed as a criterion of cellulose decomposition. In two of the three incubated fabrics, the alkali-centrifuge value showed definite increase over the sterile control *even at the zero-glucose level;* all three fabrics showed very large increases at the 0.5-percent glucose level. It had become evident that the TC isolate of *A. niger* could cause cellulosic degradation in a cotton fiber under suitable test conditions.

Following the experiment reported in table 1, the TC isolate and several other black Aspergilli were incubated on Q duck in the presence of a mineral salts-agar medium containing a graded series of glucose concentrations from zero to 3 percent.

The visible growth of these fungi increased progressively from slight at the 0.1-percent glucose level to very heavy at the 3.0-percent glucose level. The results (table 2 and figs. 1 to 4) indicated that (a) of the 12 isolates tested, 8 produced strength losses of 30 percent or more at some glucose concentration, and (b) in general, the greatest strength losses occurred at intermediate glucose concentrations, although one truly black isolate (QM 1005) produced major strength losses with no added glucose.

TABLE 1.—*Effects of supplementary glucose on strength losses and increases in alkali-centrifuge values of unbleached cotton fabrics incubated with A. niger TC*

Fabric [1]	Strength loss in test with		Increase in alkali-centrifuge value in test with	
	No glucose	0.5 glucose	No glucose	0.5 glucose
	Percent	*Percent*	*Points*	*Points*
Q duck	2	44	27	174
Hooper duck	2	46	51	185
M–2 duck	3	47	19	166

[1] Breaking strengths for sterile-incubated controls were 110, 138, and 133 for the three fabrics, respectively, and alkali-centrifuge values were 230, 223, and 220.

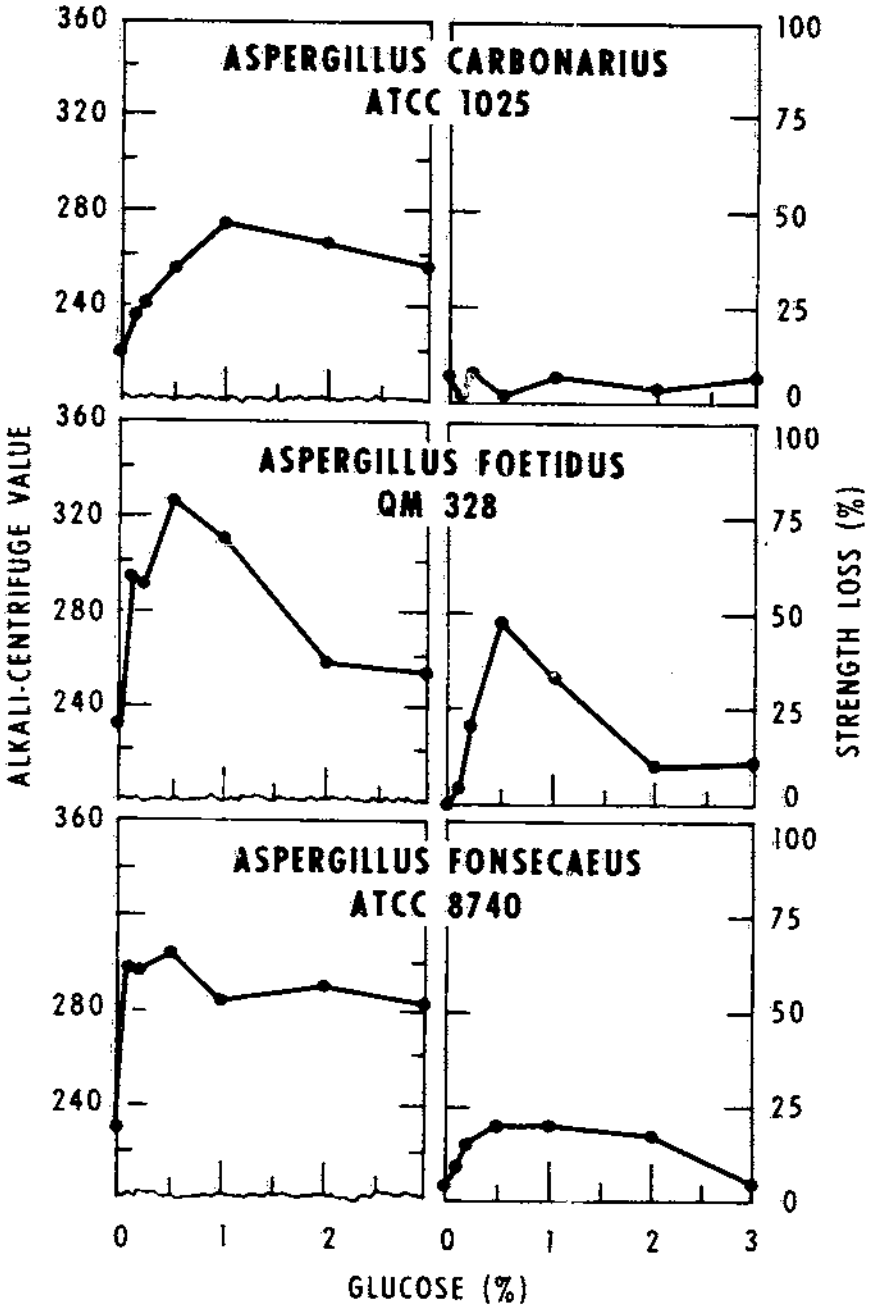

FIGURE 1.—Relation of glucose content of test medium to strength loss and alkali-centrifuge value of cotton fabric incubated with *A. carbonarius* ATCC 1025, *A. foetidus* QM 328, and *A. fonsecaeus* ATCC 8740.

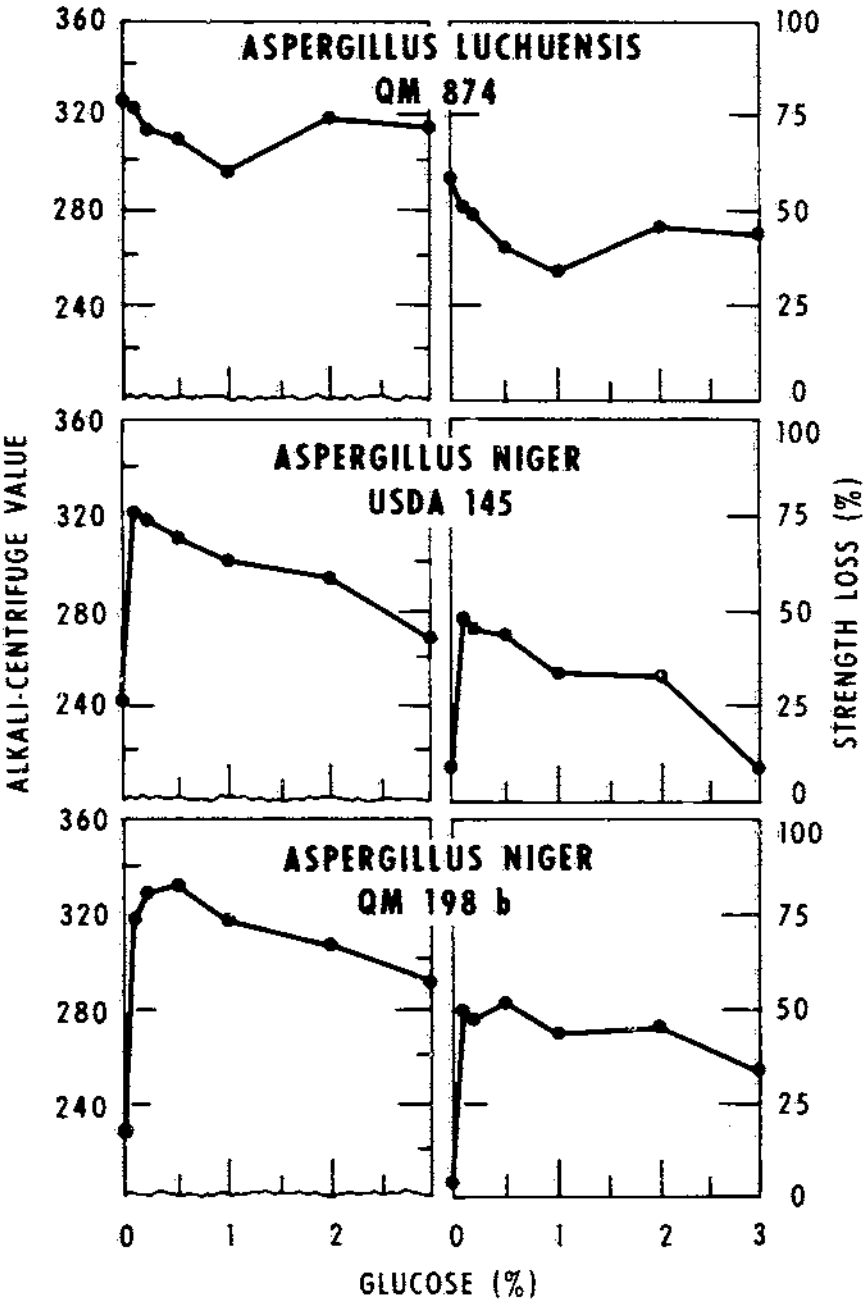

FIGURE 2.—Relation of glucose content of test medium to strength loss and alkali-centrifuge value of cotton fabric incubated with *A. luchuensis* QM 874, *A. niger* USDA 145, and *A. niger* QM 198 b.

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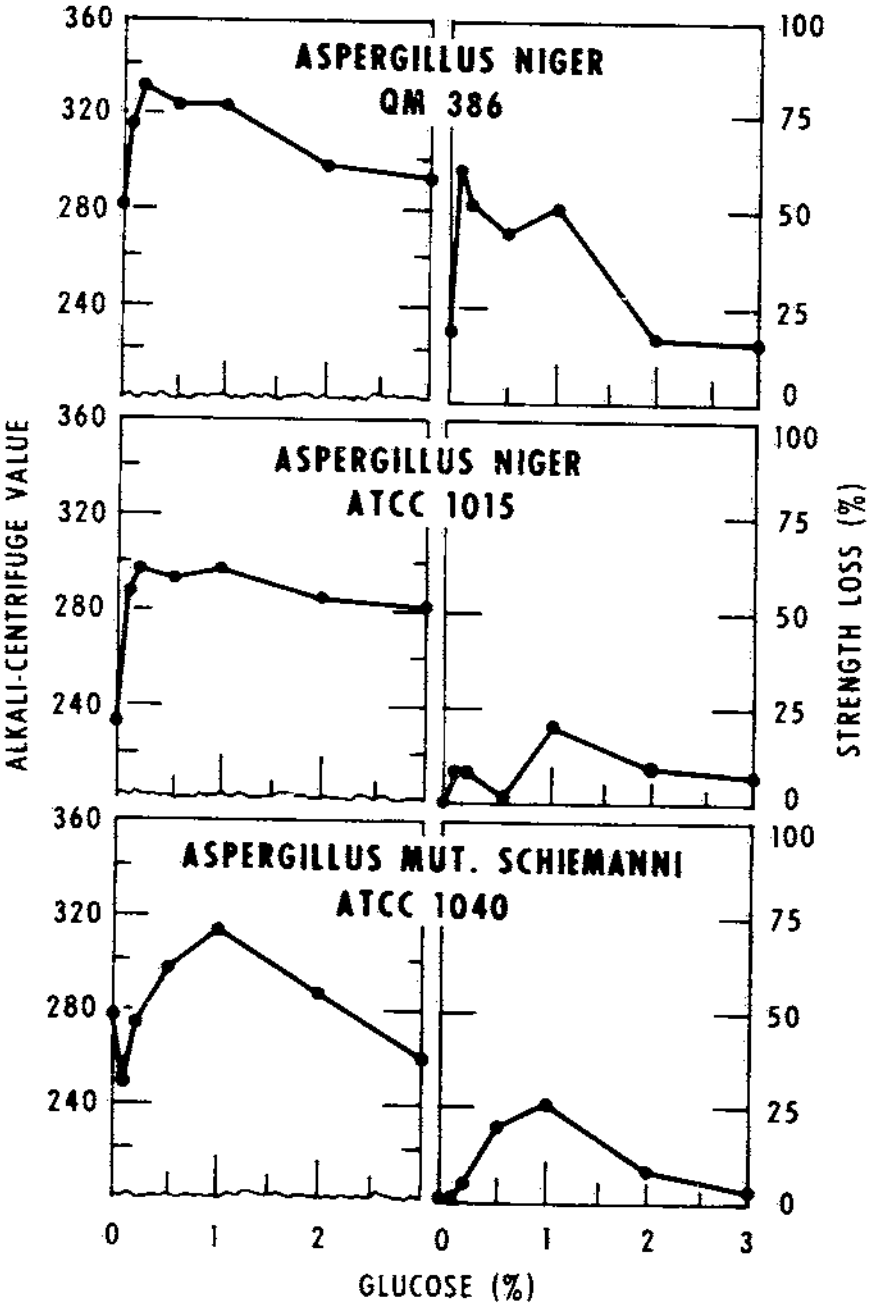

FIGURE 3.—Relation of glucose content of test medium to strength loss and alkali-centrifuge value of cotton fabric incubated with *A. niger* QM 386, *A. niger* ATCC 1015, and *A. niger* mut. *schiemanni* ATCC 1040.

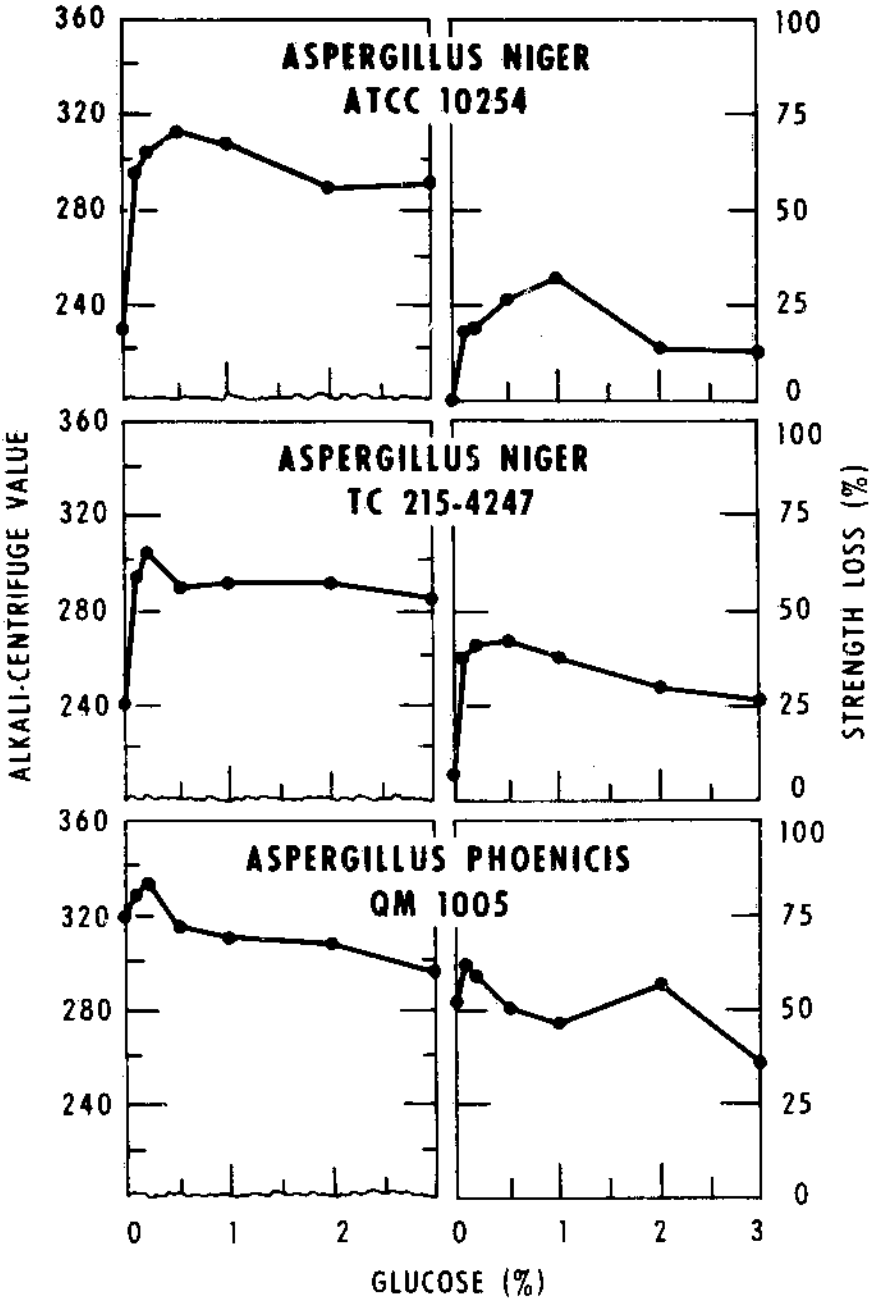

FIGURE 4.—Relation of glucose content of test medium to strength loss and alkali-centrifuge value of cotton fabric incubated with *A. niger* ATCC 10254, *A. niger* TC 215–4247, and *A. phoenicis* QM 1005.

TABLE 2.—*Effects of supplementary glucose on strength loss and alkali-centrifuge value of cotton fabric and final pH of the test medium after 2 weeks' incubation with various black Aspergilli*

[Italics are used to emphasize the relation between strength losses of 20 percent or more and final pH of medium]

Fungus and concentration of glucose (percent)	Strength loss	Alkali-centri-fuge value	Final pH of medium
A. carbonarius ATCC 1025:	*Percent*		
0	7	222	6.3
0.1	2	236	6.1
0.2	8	241	6.0
0.5	2	257	5.7
1.0	7	275	5.3
2.0	4	267	6.1
3.0	7	257	6.6
A. foetidus QM 328:			
0	0	233	6.4
0.1	4	296	6.3
0.2	*20*	292	*6.1*
0.5	*48*	327	*4.2*
1.0	*38*	312	*5.8*
2.0	10	260	7.6
3.0	11	255	7.6
A. fonsecaeus ATCC 8740:			
0	4	232	6.3
0.1	9	299	5.5
0.2	15	298	4.7
0.5	*20*	305	*4.1*
1.0	*20*	285	*4.1*
2.0	17	291	4.7
3.0	5	284	5.5
A. luchuensis QM 874:			
0	*58*	326	*3.7*
0.1	*51*	323	*3.9*
0.2	*49*	315	*3.7*
0.5	*40*	311	*3.8*
1.0	*34*	297	*3.8*
2.0	*46*	319	*4.8*
3.0	*44*	316	*5.6*
A. niger USDA 145:			
0	9	243	6.2
0.1	*48*	322	*3.6*
0.2	*46*	318	*3.5*
0.5	*44*	311	*3.1*
1.0	*34*	302	*3.4*
2.0	*38*	295	*3.7*
3.0	*19*	270	3.0
A. niger QM 198 b:			
0	4	228	6.3
0.1	*50*	319	*4.0*
0.2	*48*	330	*4.0*
0.5	*52*	333	*3.6*
1.0	*44*	318	*3.4*
2.0	*46*	308	*4.0*
3.0	*34*	292	*5.5*

See footnote at end of table.

TABLE 2.—*Effects of supplementary glucose on strength loss and alkali-centrifuge value of cotton fabric and final pH of the test medium after 2 weeks' incubation with various black Aspergilli*—Continued

Fungus and concentration of glucose (percent)	Strength loss	Alkali-centrifuge value	Final pH of medium
	Percent		
A. niger QM 386:			
0	19	282	6.1
0.1	*61*	316	*4.2*
0.2	*52*	332	*4.2*
0.5	*45*	324	*3.7*
1.0	*51*	324	*3.6*
2.0	17	300	6.4
3.0	16	293	6.5
A. niger ATCC 1015:			
0	0	234	6.3
0.1	8	288	6.0
0.2	8	297	5.7
0.5	2	293	5.1
1.0	*20*	297	*4.2*
2.0	9	286	5.4
3.0	7	283	6.2
A. niger mut. *schiemanni* ATCC 1040:			
0	1	278	6.4
0.1	1	250	6.4
0.2	5	275	6.1
0.5	*20*	299	*5.9*
1.0	*26*	314	*5.6*
2.0	8	288	7.0
3.0	3	262	7.2
A. niger ATCC 10254:			
0	0	231	6.4
0.1	18	296	6.2
0.2	19	306	5.6
0.5	*27*	314	*4.4*
1.0	*32*	309	*3.8*
2.0	14	290	6.4
3.0	13	292	6.4
A. niger TC 215–4247:			
0	1	248	6.5
0.1	13	331	6.0
0.2	*50*	340	*5.6*
0.5	*35*	364	*5.0*
1.0	*48*	360	*4.5*
2.0	*21*	349	*6.3*
3.0	16	317	6.6
A. phoenicis QM 1005:			
0	*52*	320	*4.2*
0.1	*62*	329	*4.1*
0.2	*59*	335	*4.0*
0.5	*51*	316	*3.6*
1.0	*47*	311	*3.3*
2.0	*57*	310	*4.2*
3.0	*36*	297	*5.4*

[1] "Sparing action" of the glucose toward cellulose seems evident here.

After the incubated strips shown in figures 1 to 4 were broken, they were tested by the alkali-centrifuge method. For each isolate, there was an interesting parallelism between the breaking strength loss and the alkali-centrifuge value (figs. 1 to 4). Since it is well known that the strength of the fiber is in the cellulose, this parallelism seems to be explainable only on the basis that the alkali-centrifuge test is an index of cellulose decomposition. These data supplement earlier data of a different kind that lead to the same conclusion (*7*). Replicate experiments by the method shown in table 2 and figures 1 to 4 yielded similar but not identical results. The data on the TC isolate in table 2 and figure 4 were obtained in replicate experiments and show this variation. Presumably the amount of carryover of glucose from the inoculum culture, the numbers of spores in the inoculum, or some other incompletely controlled experimental variable must have led to these results.

The pH of the agar test medium at the end of the incubation period in the experiments with supplementary glucose (table 2) bore a clear relation to the degree of cellulose decomposition. Cellulose decomposition was regularly accompanied by a decreased pH in the medium. No interpretive value should be placed on differences in strength loss below the 20-percent level, since such differences might result from surface-frictional rather than cellulose-deterioration phenomena. Because of fiber fragmentation and cellulose solution, alkali-centrifuge values in the highest range may also be questionable. Note

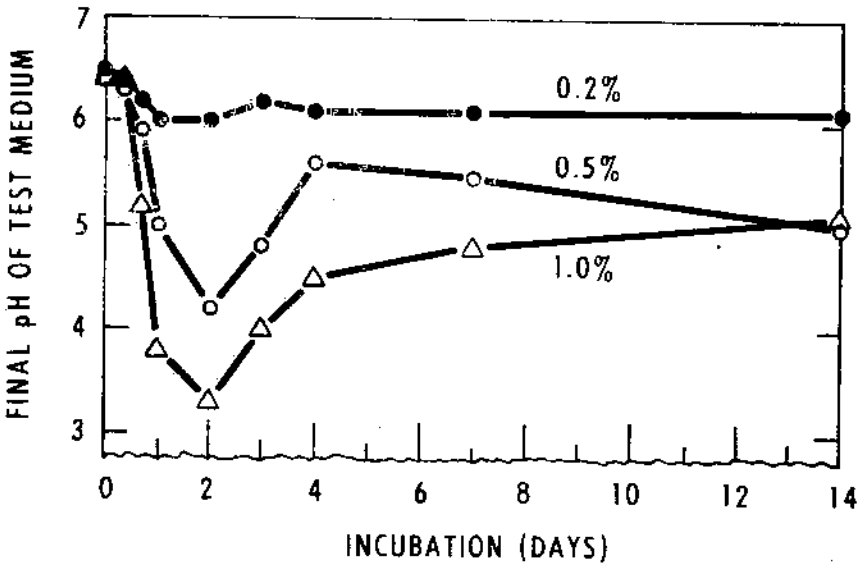

FIGURE 5.—The pH of agar culture medium after different incubation periods with *A. niger* TC. Note decrease in pH after short incubation and increase again as incubation continues.

also that the pH recorded in table 2 is a final figure taken *at the end of the experiment only* and that it could have varied during the course of the incubation. When an experiment conducted according to the method shown in table 2 was terminated after several incubation periods, the data showed this pH shift (fig. 5).

Experiments were performed in which 11 members of the *Aspergillus luchuensis* series, purple-black forms of the *A. niger* group, were tested for ability to decompose cellulose (table 3). The incubations were on mineral salts-agar and on mineral salts-agar plus 0.5 percent of glucose. In accord with earlier data (*8, 18*), eight isolates caused distinct strength losses *at zero percent of glucose;* two others caused losses at 0.5 percent of glucose. Again the final pH in the test medium was lower at the higher levels of fabric strength loss.

EFFECTS OF OTHER SUPPLEMENTARY CARBON SOURCES.—Was the stimulatory effect on cellulose decomposition for glucose specific for this compound or did other fungus-nutritive carbon compounds have a similar effect? In pursuing this matter, supplementary carbon sources were tested singly with *A. niger* TC by the procedures shown in table 2. Progressive increases in growth and sporulation were noted visually over the range of concentration of supplementary compounds. The

TABLE 3.—*Effects of supplementary glucose on strength loss of cotton fabric during incubation for 2 weeks with members of Aspergillus luchuensis series (subgroup of the A. niger group), and pH of the test medium at the end of the incubation period*

Fungus	Mineral salts agar		Mineral salts agar plus 0.5 percent of glucose	
	Strength loss	Final pH of medium	Strength loss	Final pH of medium
A. japonicus:	*Percent*		*Percent*	
QM 155e	11	6.4	52	4.4
QM 332	8	6.4	41	4.3
QM 2018	60	4.2	44	4.1
QM 333	56	4.2	44	4.0
A. violaceo-fuscus:				
QM 6649	63	3.8	55	3.8
A. luchuensis:				
QM 21e	29	6.1	35	4.8
QM 23b	44	4.6	31	4.6
QM 102d	49	4.6	34	4.1
QM 374	58	4.2	36	4.2
QM 873	59	4.3	58	4.7
QM 70c	6	6.5	9	5.9

TABLE 4.—*Effects of several supplementary carbon sources on strength losses and alkali-centrifuge values of cotton fabric, and final pH of the test medium after incubation for 2 weeks with A. niger TC*

[Italics are used to emphasize the relation between (1) strength losses of 20 percent or more, or (2) a single high alkali-centrifuge value of more than 300 and final pH of medium]

Concentration of supplementary carbon source (percent)	Strength loss	Alkali-centri-fuge value	Final pH of medium
Arabinose:	*Percent*		
0	0	244	6.3
0.1	*21*	342	*5.5*
0.2	*37*	342	*4.8*
0.5	*45*	363	*3.8*
1.0	*42*	369	*3.6*
2.0	*23*	355	*5.6*
3.0	5	332	6.3
Cellobiose:			
0	8	232	6.4
0.1	12	298	6.0
0.2	15	305	6.0
0.5	14	324	5.5
1.0	12	*397*	*4.8*
2.0	16	313	6.2
3.0	16	308	6.5
Citric acid (neutralized): [1]			
0	8	226	6.4
0.1	6	230	7.0
0.2	3	236	7.4
0.5	7	235	7.9
1.0	10	256	8.0
2.0	6	246	8.0
3.0	7	240	8.2
d-Galactose:			
0	6	240	6.4
0.1	5	240	6.3
0.2	0	253	5.6
0.5	*24*	326	*3.5*
1.0	12	310	3.0
2.0	13	298	3.8
3.0	10	292	5.7
Glycerine:			
0	0	228	6.5
0.1	12	279	5.6
0.2	*20*	284	*5.3*
0.5	15	279	5.1
1.0	10	289	4.4
2.0	0	257	6.5
3.0	4	269	6.8
Lactose:			
0	0	230	6.5
0.1	4	230	6.4
0.2	2	229	6.2
0.5	6	227	6.1
1.0	5	240	4.3
2.0	0	238	3.6
3.0	3	263	4.8

See footnote at end of table.

TABLE 4.—*Effects of several supplementary carbon sources on strength losses and alkali-centrifuge values of cotton fabric, and final pH of the test medium after incubation for 2 weeks with A. niger TC*—Continued

Concentration of supplementary carbon source (percent)	Strength loss	Alkali-centri-fuge value	Final pH of medium
	Percent		
Maltose:			
0	5	234	6.4
0.1	3	293	6.0
0.2	4	307	6.2
0.5	11	323	5.5
1.0	19	*335*	*4.8*
2.0	6	314	6.4
3.0	7	323	6.2
Mannitol:			
0	13	235	6.6
0.1	10	273	6.2
0.2	8	299	5.8
0.5	6	275	4.4
1.0	13	*319*	*3.8*
2.0	16	307	5.6
3.0	12	284	6.0
d-Sorbitol:			
0	2	252	6.5
0.1	*28*	335	*5.4*
0.2	*34*	331	*5.0*
0.5	*42*	336	*3.9*
1.0	*45*	337	*3.7*
2.0	*34*	338	*5.4*
3.0	*22*	330	*5.9*
l-Sorbose:			
0	0	229	6.3
0.1	16	342	4.7
0.2	*20*	340	*4.3*
0.5	18	330	4.2
1.0	11	323	3.6
2.0	*20*	333	*5.2*
3.0	10	311	5.8
Sucrose:			
0	0	219	6.4
0.1	18	334	6.0
0.2	*28*	335	*5.6*
0.5	*27*	344	*5.3*
1.0	*31*	339	*5.0*
2.0	*24*	339	*6.1*
3.0	13	320	6.3
d-Xylose:			
0	0	227	6.6
0.1	*21*	349	*6.1*
0.2	*21*	362	*5.7*
0.5	*36*	360	*4.4*
1.0	*36*	380	*3.9*
2.0	16	341	5.9
3.0	6	300	6.6

[1] Citric acid neutralized to pH 5.9 with KOH.

spore heads were jet black. Cellulose decomposition was *not* correlated with total visible growth, since degradation generally was not greatest at the highest concentrations of supplementary compounds.

Several carbon sources other than glucose increased strength loss, notably arabinose, d-sorbitol, sucrose, and xylose, but also to a lesser extent galactose and l-sorbose (table 4). Large increases in the alkali-centrifuge value without major strength losses occurred in the presence of glycerine, maltose, cellobiose, and mannitol. These latter results indicated the sensitivity of the alkali-swelling technique as a measure of cellulose decomposition. Strips in the presence of lactose and of neutralized citric acid showed neither strength losses nor definite changes in the alkali-centrifuge value. Again, the higher levels of cellulose decomposition were regularly accompanied by a decrease in the pH of the test medium (table 4).

EFFECTS OF METHOD OF INOCULATION.—When the spores of *A. niger* were *brushed* onto the fabric strips, instead of being pipetted on, a major strength loss occurred even in the absence of any supplementary carbon source (table 5). Although the cause of this experimental finding is not completely understood, it was clearly related to the final pH of the test medium. Note that the 50-percent strength loss for the brush-inoculated sample at zero-glucose was accompanied by a lowered pH in the test medium; i.e., pH 4.8 as compared with pH 6.5 for the corresponding zero-glucose pipette-inoculated sample.

TABLE 5.—*Effect of brush inoculation, in contrast to pipette inoculation, on strength loss and alkali-centrifuge value of cotton fabric, and on final pH of the test medium after incubation with A. niger TC*

Inoculation procedure and concentration of glucose (percent)	Strength loss	Alkali-centrifuge value	Final pH of medium
	Percent		
Brush inoculation:			
0	50	299	4.8
0.1	50	303	4.0
0.2	48	312	4.1
0.5	53	311	3.9
1.0	48	314	3.6
2.0	43	304	5.6
3.0	26	302	6.0
Pipette inoculation:			
0	1	248	6.5
0.1	13	331	6.0
0.2	50	340	5.6
0.5	35	364	5.0
1.0	48	360	4.5
2.0	21	349	6.2
3.0	16	317	6.6

TABLE 6.—*Effects of magnesium sulfate content of the test medium on strength loss and alkali-centrifuge value of cotton fabric, and on pH of the test medium at the end of 2 weeks' incubation with A. niger TC*

$MgSO_4 \cdot 7\ H_2O$/l in medium and concentration of glucose (percent)	Strength loss	Alkali-centri-fuge value	Final pH of medium
1.4 grams per liter:	*Percent*		
0	1	248	6.5
0.1	13	331	6.0
0.2	50	340	5.6
0.5	35	364	5.0
1.0	48	360	4.5
2.0	21	349	6.2
3.0	16	317	6.6
0.7 gram per liter:			
0	0	231	6.3
0.1	41	283	4.6
0.2	55	322	4.2
0.5	57	329	4.0
1.0	48	323	3.9
2.0	31	311	6.1
3.0	38	312	5.9
0.35 gram per liter:			
0	0	249	6.2
0.1	21	331	5.7
0.2	25	299	5.4
0.5	43	315	4.4
1.0	37	305	4.4
2.0	12	285	6.5
3.0	9	286	6.5
0.17 gram per liter:			
0	2	220	6.5
0.1	0	256	6.1
0.2	1	261	5.9
0.5	5	284	5.6
1.0	24	295	5.2
2.0	9	272	6.4
3.0	11	262	6.5
0.09 gram per liter:			
0	0	227	6.4
0.1	0	257	6.4
0.2	4	267	6.2
0.5	4	292	5.9
1.0	16	294	5.5
2.0	2	271	6.6
3.0	2	260	7.1

EFFECTS OF INORGANIC COMPOSITION OF TEST MEDIUM.—Would changes in the inorganic composition of the test medium affect cellulose decomposition? In order to examine this possibility, the salt composition of the medium was varied.

In the experiments shown in table 6, the concentration of the *magnesium sulfate* was varied. The results showed that strength losses increased at one-half the normal concentration of $MgSO_4$ (0.7 gram per liter), but that at lower concentrations

they decreased. As in earlier experiments, the higher strength losses were accompanied by lower final pH values in the test medium (table 6). Note, for example, that at 0.5 percent of glucose, strength losses were 35, 57, 43, 5, and 4 percent and the corresponding pH values were 5.0, 4.0, 4.4, 5.6, and 5.9. Similarly, at the 1-percent glucose level, strength losses were 48, 48,

TABLE 7.—*Effects of nitrogen source and percentage of glucose on strength loss and alkali-centrifuge value of cotton fabric, and on final pH of the test medium after incubation with A. niger TC*

Source of nitrogen and concentration of glucose (percent)[1]	Strength loss	Alkali-centrifuge value	Final pH of medium
NH_4NO_3 (2.0 grams per liter):	*Percent*		
0	1	248	6.5
0.1	13	331	6.0
0.2	50	340	5.6
0.5	35	364	5.0
1.0	48	360	4.5
2.0	21	349	6.2
3.0	16	317	6.6
$(NH_4)_2SO_4$ (3.3 grams per liter):			
0	0	274	6.5
0.1	1	339	6.2
0.2	5	336	6.0
0.5	16	381	5.0
1.0	25	387	3.6
2.0	2	364	2.9
3.0	0	330	2.8
NH_4Cl (2.7 grams per liter):			
0	12	267	6.2
0.1	0	322	6.1
0.2	2	326	5.8
0.5	21	341	4.6
1.0	16	340	3.5
2.0	0	317	3.0
3.0	2	271	2.7
$(NH_4)_2CO_3$ (2.4 grams per liter):			
0	0	234	7.8
0.1	3	254	7.0
0.2	0	277	7.0
0.5	0	286	6.8
1.0	8	290	6.7
2.0	20	322	5.0
3.0	10	266	3.6
$NaNO_3$ (4.2 grams per liter):			
0	0	238	6.7
0.1	0	349	6.4
0.2	13	352	6.0
0.5	21	352	5.8
1.0	26	357	5.6
2.0	30	359	5.5
3.0	30	341	5.3

[1] Media adjusted to contain 0.7 g. of nitrogen per liter.

37, 24, and 16 percent and the corresponding pH values were 4.5, 3.9, 4.4, 5.2, and 5.5. The beneficial effect of lowering the $MgSO_4$ to half strength is reminiscent of the deleterious influence of 0.01 M $MgSO_4$ on the cellulase of *Myrothecium verrucaria* in prior experiments (*7*).

Changes in the *nitrogen source* also affected cellulose decomposition (table 7) and the final pH of the test medium. At concentrations of glucose at which strength losses occurred, the pH values of the agar medium at the end of the incubation period tended to be decreased (table 7). Even with nitrogen sources with which supplementary glucose failed to be accompanied by strength loss, the alkali-centrifuge values were elevated, for example, with $(NH_4)_2CO_3$. These data, together with those of earlier experiments, indicated the sensitivity of the alkali-swelling test to show cellulose decomposition of cotton fiber.

Did a low pH in the test medium favor cellulose decomposition by the black Aspergilli? The data in tables 2 to 7 suggested this possibility. To investigate the question more directly, two types of experiments were performed: (a) calcium carbonate was added to a glucose-containing test medium to maintain a near-neutral pH during incubation, and (b) the pH of the medium was lowered before incubation.

Calcium carbonate added to the glucose-containing test medium *stabilized the pH* and also largely prevented cellulose decomposition (table 8). The growth of *A. niger* TC in the presence of calcium carbonate was not visibly different from that in its absence.

TABLE 8.—*Effect of calcium carbonate added to the basal mineral salts agar incubation medium on strength loss and alkali-centrifuge values of cotton fabric, and on final pH of the test medium after incubation with A. niger TC*

Concentration of glucose (percent)	Strength loss	Alkali-centrifuge value	Final pH of medium
	Percent		
0	0	231	6.6
0.1	3	261	6.4
0.2	4	271	6.3
0.5	5	264	6.4
1.0	2	263	6.5
2.0	3	272	6.7
3.0	4	257	6.9

Lowering the initial pH of the medium clearly favored cellulose decomposition, even in the absence of a supplementary carbon source. The dibasic phosphate was omitted from the usual mineral salts medium and the pH was adjusted to 3.0 with phos-

phoric acid. The mineral salt solution, thus adjusted, was autoclaved with and without glucose present and was added to water-agar in the culture bottles under sterile conditions. The pH of the mixture was about 3.9. Strength losses of 29 and 28 percent accompanied final pH values of 3.8 and 3.4 and alkali-centrifuge values of 389 and 386.

Did cellulose decomposition in a low-pH medium depend on the presence of agar in the test system? A low-pH mineral salts medium was prepared as before but without agar and the incubations were carried out in large test tubes (25×200 mm.). The tubes were incubated in a vertical position and the test strips were placed against their walls with approximately half of each strip immersed in the liquid. The results (table 9) showed that low-pH media stimulated strength losses of fabric even in the absence of agar. The effect was obviously not specific for any particular acidic substance, since lowering the initial pH of the mineral salts solution with HCl and H_2SO_4 (table 10), and with malic and citric acids (table 11) had the same effect. The results for citric acid were contrary to those for the corresponding *neutralized* acid (table 4). Clearly, the effect with citric and malic acids (table 11) was principally one of lowered pH rather than of supplementary carbon available for the growth of the fungus.

TABLE 9.—*Effect of a low initial pH in an agar-free medium on the strength loss of cotton fabric incubated with A. niger TC in the presence of varying concentrations of glucose and sucrose*[1]

Initial pH of medium and concentration of carbon source (percent)	Strength loss with—	
	Glucose	Sucrose
pH 6.4:	*Percent*	*Percent*
0	2	0
0.1	10	25
0.2	22	48
0.5	36	52
1.0	36	56
2.0	37	52
3.0	43	45
pH 3.0:		
0	46	40
0.1	41	38
0.2	41	41
0.5	41	40
1.0	38	37
2.0	24	38
3.0	25	29

[1] Incubations with liquid media in large test tubes. Composition of the media at pH 6.4 is given in detail in the Appendix; at the low pH level, only the monobasic phosphate was used and the pH was adjusted to 3.0 with H_3PO_4.

TABLE 10.—*Effect of a low initial pH, 3.0 (HCl and H_2SO_4), in an agar-free medium on subsequent changes in properties of cotton fabric during incubation with A. niger TC, and final pH of medium*

Kind of acid and concentration of glucose (percent)	Strength loss	Alkali-centri-fuge value	Final pH of medium
Sulfuric acid:	*Percent*		
0	41	376	3.4
0.1	37	383	3.2
0.2	37	375	3.0
0.5	36	374	2.9
1.0	32	368	2.6
2.0	21	368	2.4
3.0	26	368	2.4
Hydrochloric acid:			
0	41	400	3.4
0.1	41	420	3.4
0.2	35	410	3.2
0.5	40	404	2.9
1.0	27	395	2.6
2.0	24	401	2.2
3.0	24	392	2.2

TABLE 11.—*Effect of various concentrations of malic and citric acids on cellulose decomposition of cotton fabric incubated with A. niger TC in large test tubes*

Kind of acid and initial concentration (percent)	Strength loss	Alkali-centri-fuge value	pH of medium	
			Initial	Final
Malic acid:	*Percent*			
0	7	257	6.4	6.0
0.1	40	313	4.0	5.8
0.2	56	310	3.2	4.6
0.5	52	312	2.7	3.5
1.0	50	308	2.7	3.0
2.0	42	303	2.3	2.6
3.0	40	304	2.0	2.4
Citric acid:				
0	1	274	6.4	6.4
0.1	46	377	4.4	5.8
0.2	42	385	3.5	4.5
0.5	54	384	3.1	3.5
1.0	43	383	2.6	3.1
2.0	36	370	2.5	2.7
3.0	40	375	2.4	2.5

Minor elements seemed unlikely as an important factor in cellulose decomposition in the experiments reported here because of the presumably adequate levels of such elements present under the conditions of test. However, the matter was investigated experimentally. Two types of experiments were performed: (a) addition of disodium ethylenediamine tetraacetate (EDTA) to the cultures, and (b) addition of minor elements to the mineral salts medium. If minor elements were present but were unavailable, EDTA should increase their availability. Strength losses of 6 and 4 percent were recorded for incubations in the usual mineral salts-agar procedure in the presence and absence of EDTA (0.25 percent), respectively. When EDTA was incorporated into the spore suspension (0.25 percent), no strength losses occurred in either its presence or its absence during subsequent incubation in the absence of supplementary glucose.

Addition of minor elements to the basal mineral salts-agar medium did not affect cellulose decomposition during incubation with *A. niger* TC. Iron, zinc, copper, and manganese were used together at concentrations of 0.30, 0.30, 0.075, and 0.075 milligrams per liter, respectively, in the basal mineral salts-agar. In the absence of supplementary glucose, strength losses of zero and 5 percent were recorded for incubations with and without the addition of minor elements to the mineral salts-agar medium, respectively. At the 0.5 percent of glucose level, strength losses of 23 and 20 percent were measured in the presence and absence of minor elements. In a second experiment, fabric strips were incubated in large test tubes in mineral salts solution with and without the addition of the minor elements and at initial pH levels of 6.4, 5.4, and 3.5. The results (table 12) indicated that minor elements had no effect on strength loss or on the final pH of the test medium.

Sophorose, a disaccharide impurity in commercial glucose, has been shown by Mandels and colleagues (*3*, *4*) to stimulate

TABLE 12.—*Effect of addition of minor elements to liquid mineral salts media of different initial pH levels on strength loss of cotton fabric incubated in large test tubes with A. niger TC*

pH of medium		Strength loss of fabric with minor elements	
Initial	Final	Present	Absent
		Percent	*Percent*
6.4	6.5	0	0
5.4	5.4	45	43
3.5	3.6	40	41

cellulose decomposition by *Trichoderma viride*. Nothing in our data suggested that sophorose favored cellulose decomposition by *A. niger*, especially since such decomposition could be brought about by simply altering the initial pH of a mineral salts medium. Nevertheless, an experiment was performed with presumably sophorose-free glucose, produced enzymatically from starch (A. E. Staley Co., Decatur, Ill.) and used in a fashion otherwise identical with that shown in table 2. Cellulose decomposition obviously occurred (table 13).

TABLE 13.—*Effect of enzymatically produced glucose on cellulose decomposition of fabric incubated with A. niger in a mineral salts-glucose-agar medium*

Concentration of glucose (percent)	Strength loss	Alkali-centrifuge value	Final pH of medium
	Percent		
0	0	228	6.2
0.1	9	292	6.0
0.2	11	292	6.0
0.5	22	298	5.6
1.0	39	303	4.4
2.0	21	302	5.8
3.0	5	291	6.0

Control experiments were performed to investigate the possibility that the favorable effect of low pH on cellulose decomposition might be caused by (a) strength loss resulting from a direct effect of low pH on the fiber, or (b) some predisposing effect which the lowered pH might exert on the fiber to make it more susceptible to later microbial action. Both alternatives were proved invalid. Also, at the end of several of our experiments, a low pH in the test medium was not accompanied by fabric strength losses. These results proved that the low pH was not a direct cause of the strength loss. Data in point may be seen in the ammonium sulfate, ammonium chloride, and ammonium carbonate experiments shown in table 7. In order to directly investigate question (a) above, fabric strips were incubated under *sterile conditions* for 2 weeks in a medium initially adjusted to pH 3.0 in the test tube culture technique, and strength and alkali-centrifuge values were determined. The results (table 14) showed no significant strength loss nor alkali-centrifuge value increase.

An experiment was performed to determine any possible predisposing effect of autoclaving the fabric at low pH on its subsequent susceptibility to deterioration by *A. niger* (question (b) above). The strips were sterilized either (1) in the presence of the low pH medium or (2) in the absence of the medium, the strips and medium being sterilized separately before in-

TABLE 14.—*Effects of incubation under sterile conditions for 2 weeks at 30° C. in a low-pH medium in test tubes on strength and alkali-centrifuge values of cotton fabric*

Concentration of glucose (percent)	Strength loss	Alkali-centrifuge value		Final pH of medium
		Upper portion	Lower portion	
	Percent			
0	0	203	201	3.3
0.1	3	206	205	3.3
0.2	3	204	207	3.4
0.5	0	209	207	3.4
1.0	3	198	201	3.4
2.0	6	194	205	3.4
3.0	1	197	205	3.5

TABLE 15.—*Effect of sterilizing media and fabric strips together and separately on the properties of fabric strips subsequently incubated with A. niger TC for 2 weeks at 30° C. over a range of concentrations of glucose*

Concentration of glucose (percent)	Strip and media autoclaved together			Strip and media autoclaved separately		
	Strength loss	Alkali-centrifuge value	Final pH of medium	Strength loss	Alkali-centrifuge value	Final pH of medium
	Percent			*Percent*		
0	46	405	3.3	40	372	3.1
0.1	41	416	3.2	44	370	2.9
0.2	41	396	3.2	26	366	2.9
0.5	41	406	3.0	33	361	2.7
1.0	38	401	2.8	29	352	2.7
2.0	24	403	2.5	22	366	2.3
3.0	25	389	2.4	18	350	2.3

oculation. The results (table 15) showed small and probably insignificant differences in cellulosic deterioration between the two sets of strips.

Decomposition of Cellulosic Substrates by Enzyme Preparations

EFFECTS OF PH AND TEMPERATURE OF TEST MEDIUM.—Since a low pH medium clearly favored cellulose degradation by *A. niger*, the effect of pH on the organism's cellulase was investigated. *A. niger* filtrates and commercial preparations from the growth of *A. niger* were tested to determine the optimum pH for cellulase activity. Even though some varia-

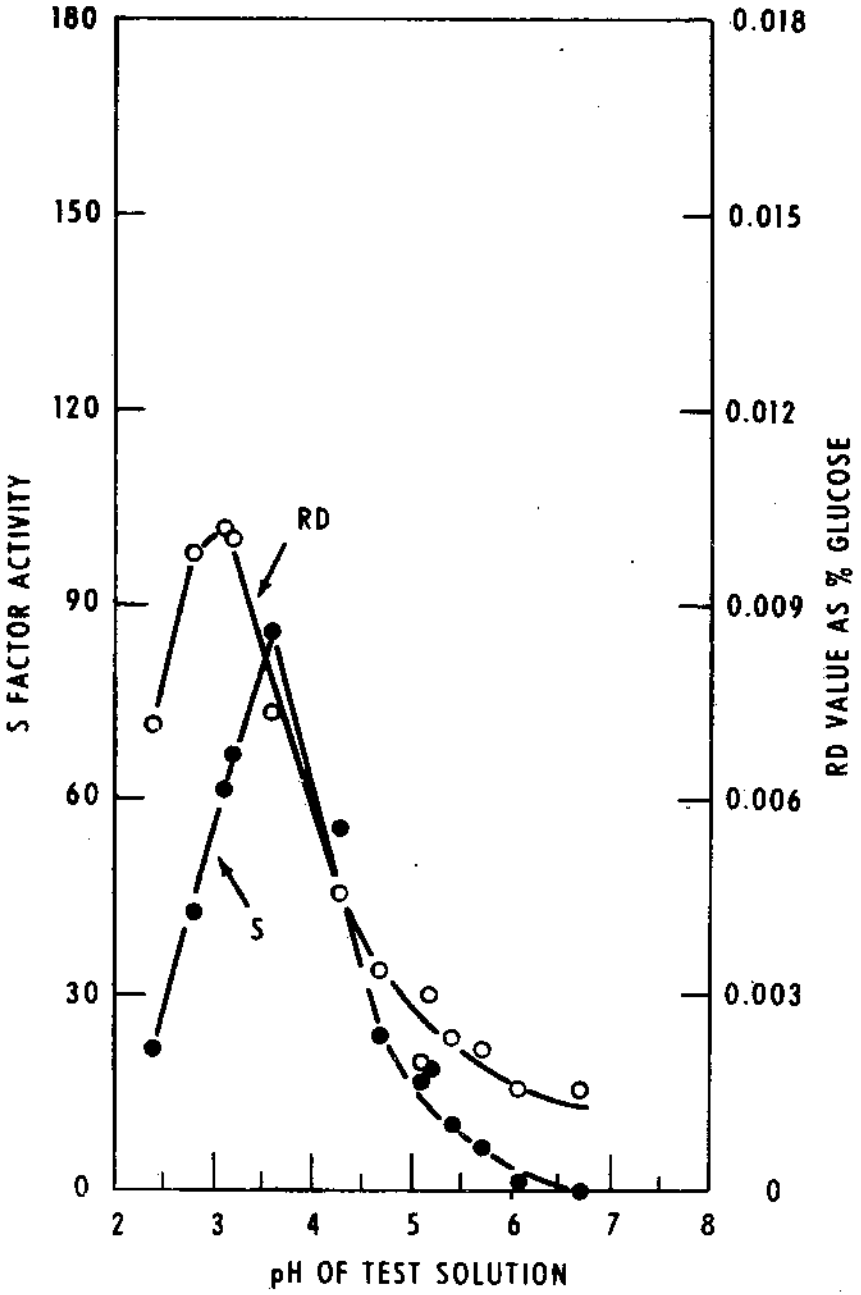

FIGURE 6.—Relation of pH of test medium to decomposition of raw cotton fiber by *A. niger* growth medium filtrates. Results measured as S factor and as amount of reducing substances produced (RD value).

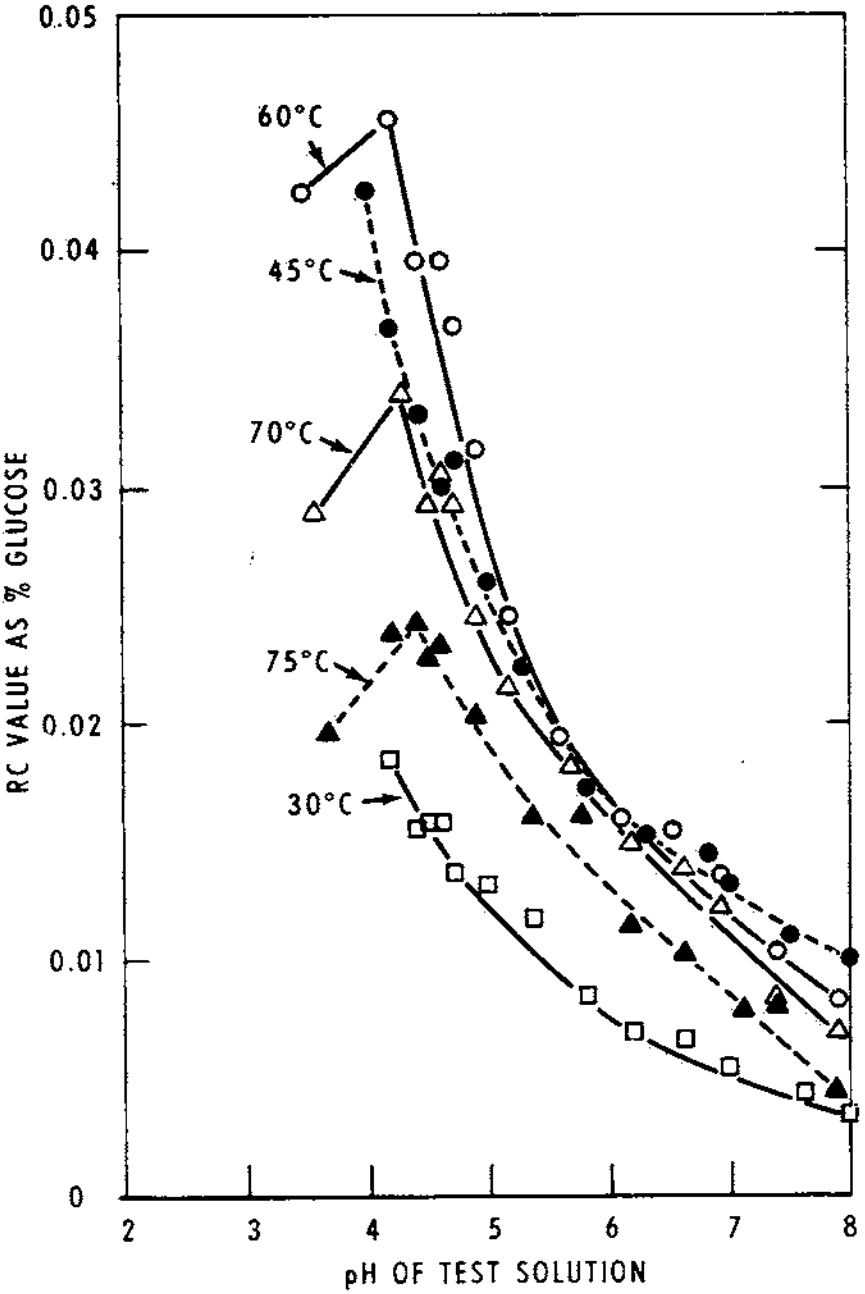

FIGURE 7.—Relation of pH and temperature of test medium to decomposition of carboxymethyl cellulose by *A. niger* growth medium filtrates. Results measured as amount of reducing substances produced (RC value). Final concentration of CMC was 0.5 percent, veronal buffer was 0.014 M, and *A. niger* filtrate was 10 × diluted.

TABLE 16.—*Effect of temperature and pH on cellulase activity of A. niger filtrate as measured by the formation of reducing substances from carboxymethyl cellulose*[1]

pH	RC value × 10⁴			
	30° C.	45° C.	50° C.	60° C.
2.7	66	84	100	88
3.6	80	106	118	112
3.8	72	92	102	102
4.0	62	90	102	94
4.4	52	72	76	68
4.8	26	42	50	48
5.3	18	30	30	32
5.8	4	14	16	18
6.3	4	8	16	10
6.7	0	8	12	6
7.1	0	0	6	4
7.6	0	0	6	0
8.0	0	0	2	4

[1] Final concentration of carboxymethyl cellulose was 0.1 percent, veronal buffer was 0.014 M, and *A. niger* filtrate was 10 × diluted.

tion occurred, tests by three methods on several different preparations all disclosed a rather low pH to be optimum for activity. Such data are shown for a laboratory-produced filtrate in table 16 and figures 6 and 7.

In the RD test, a 250-milligram sample of fiber was exposed to 25 milliliters of *A. niger* filtrate as in the S factor procedure. One milliliter of the reaction mixture was used to determine reducing substances produced during the exposure period. At the peak of enzymic activity (fig. 6) approximately 0.01 percent of reducing substance as glucose was formed per milliliter of reaction mixture. This is equivalent to approximately 2.5 milligrams of total reducing substance formed or about 1 percent of the weight of the fiber.

RC activity (reducing substances from CMC) over a wide range of test temperatures is recorded in table 16 and figure 7. The same optimum pH for activity is demonstrated also at various incubation temperatures for S activity (fig. 8). These data (table 16 and figs. 7 and 8) seem to favor the concept of a single-enzyme type of cellulase rather than the multiple-enzyme interpretation postulated by some workers.

Does the pH-S activity curve change with time of exposure of the fiber to an *A. niger* filtrate? S factor was determined in the usual manner for 1-, 2-, and 3-hour incubation periods. Enzymatic action of the filtrate on the fiber continued for a longer period at the lower pH levels (fig. 9). Thus, essentially all of the activity occurred at the higher pH levels in 1 hour and almost all of it at the intermediate levels in 2 hours, but at the lowest

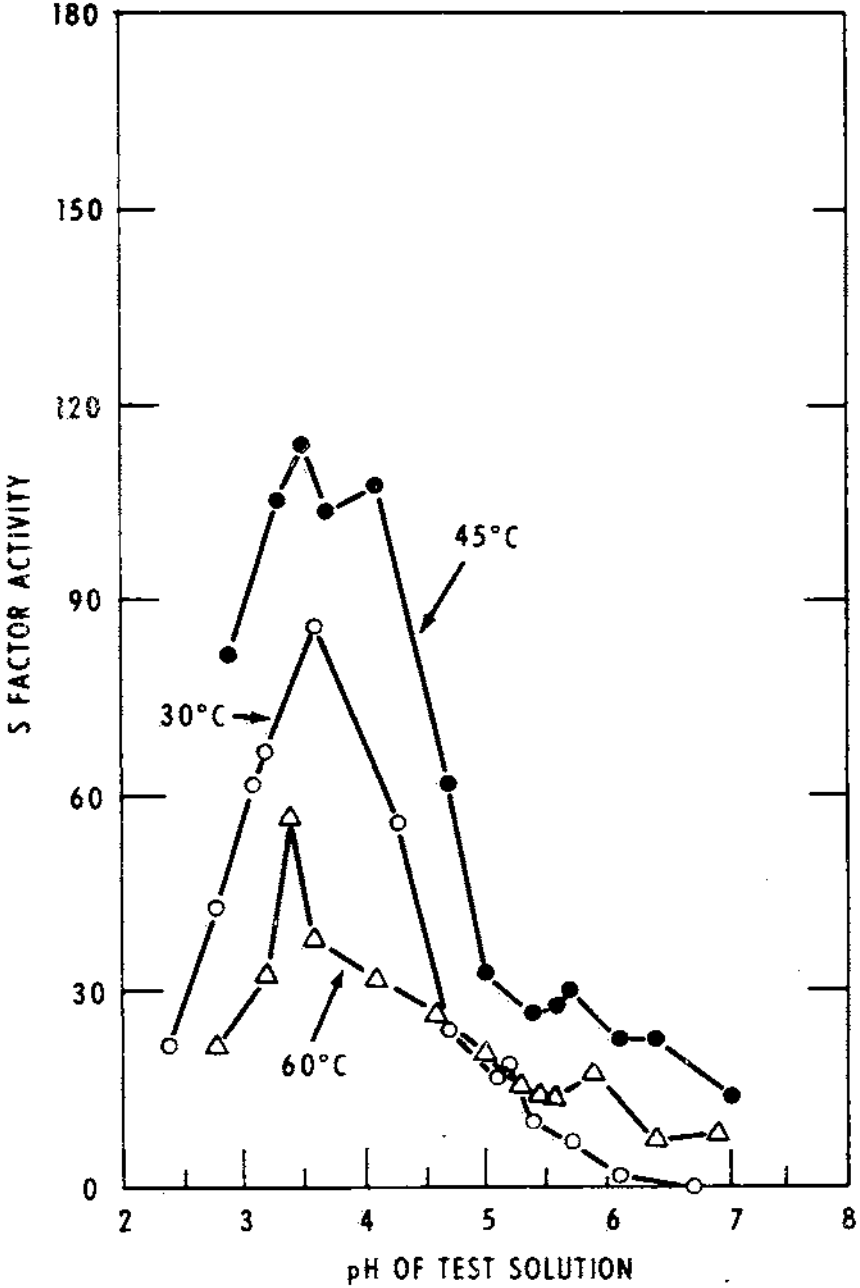

FIGURE 8.—Relation of pH and temperature to S factor activity of *A. niger* growth medium filtrates.

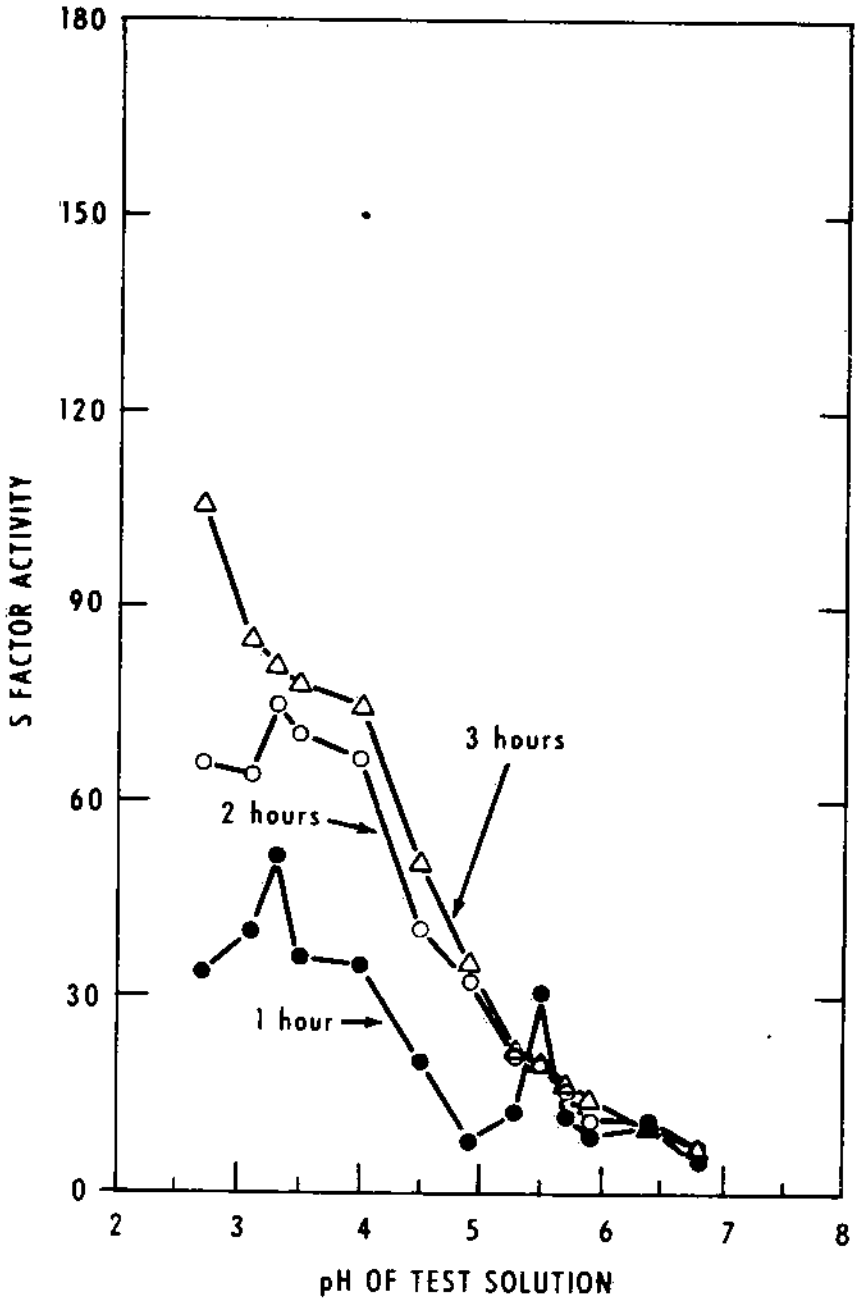

FIGURE 9.—Relation of pH and duration of test to S factor activity of *A. niger* growth medium filtrates.

pH levels activity of cellulase on the fiber continued during the third hour. No certain explanation of this result is available.

The occurrence of high cellulase activity at low pH levels not only is characteristic of filtrates from the growth of *A. niger* TC but also is found with other black Aspergilli (table 17). Data from *Fusarium moniliforme* Sheldon filtrate are included because they present a pH-activity situation obviously different from that encountered with the black Aspergilli.

TABLE 17.—*Effect of pH on cellulase activity (S factor) of filtrates from the growth media of five black Aspergilli and of Fusarium moniliforme*

pH during assay	S factor for filtrates of—					
	A. carbonarius ATCC 1025	*A. phoenicis* QM 1005	*A. niger* QM 386	*A. niger* QM 198 b	*A. luchuensis* QM 874	*F. moniliforme* USDA 1004.1
2.7	.....	.....	127	147	141	.....
3.0	33	143	128	140	142	131
3.3	27	142	128	138	146	147
3.6	22	142	125	138	137	153
3.9	15	137	120	128	135	151
4.2	13	131	110	118	125	139
4.5	10	122	92	92	114	150
4.8	10	109	70	58	106	159
5.1	12	90	36	45	92	162
5.4	10	71	27	27	84	157
5.7	10	62	20	24	60	165
6.0	12	52	17	20	52	151
6.3	7	45	15	18	49	142
6.6	8	44	17	19	46	134
6.9	7	.....	19	.....	.....	147

Three commercial *A. niger* cellulase preparations were tested at pH levels ranging from 2.1 to 7.8. They exhibited their greatest cellulase activity at low pH levels (table 18).

Might some general circumstance in our experimental conditions be conducive to a low pH optimum for activity generally for other hydrolytic enzymes besides cellulase? Apparently not, since tests for invertase activity (fig. 10) in *A. niger* filtrates showed maximum activity at a much higher pH level than had been found for cellulase in the same filtrates.

An experiment involving repeated exposure of cotton fiber to the same sample of filtrate demonstrated that fiber absorbs more *A. niger* S factor at low pH levels than at higher ones. The filtrate was adjusted to pH levels ranging from 2.8 to 6.9 with veronal buffer and 250 milligrams of fiber was exposed in 30 milliliters of the solution for 1 hour. The fiber was then removed, presumably having withdrawn some S factor from the filtrate. A second 1-hour exposure was performed by adding

TABLE 18.—*Effect of pH on cellulase activity of three commercial cellulase preparations* [1]

pH during assay	Cellulase 1000		Cellulase 4000		Cellulase	
	S	RC×10⁴ [2]	S	RC×10⁴ [2]	S	RC×10⁴ [2]
2.1	.....	105	.....	88	.....	200
2.4	.....	130	.....	78	.....	176
2.7	.....	151	42	103	30	207
3.0	.....	155	37	116	31	208
3.3	33	159	34	131	33	210
3.6	37	165	34	138	23	208
3.9	37	177	21	132	16	204
4.2	36	174	17	108	12	184
4.5	28	150	12	89	11	150
4.8	23	128	9	73	10	127
5.1	21	92	5	58	8	102
5.4	15	79	3	46	8	86
5.7	11	70	2	36	8	73
6.0	10	70	1	29	9	66
6.3	10	70	3	21	10	58
6.6	9	70	3	10	9	52
6.9	7	64	1	3	11	49
7.2	6	50	0	8	13	48
7.5	4	0	0	0	12	36
7.8	2	14	0	9	11	37

[1] Activity measured as S factor and as production of reducing substances from CMC.

[2] Final concentrations were: carboxymethyl cellulose, 0.1 percent; veronal buffer, 0.014 M; and cellulase preparations, 1 mg./ml.

TABLE 19.—*Effect of pH on absorption of A. niger S factor by cotton fiber* [1]

pH of filtrate during absorption	Residual S activity, measured at pH 3.0	pH of filtrate during absorption	Residual S activity, measured at pH 3.0
2.8	25	5.3	107
3.1	18	5.5	103
3.3	25	5.6	111
3.5	20	5.9	121
3.9	23	6.4	102
4.4	42	6.9	124
4.9	82		

[1] Fiber placed twice in succession into *A. niger* filtrate at different pH levels, then all filtrate samples adjusted to pH 3.0 and their residual S activity determined.

300 milligrams of fresh fiber to 25 milliliters of the once-used filtrate. Finally, the pH of the filtrate in each beaker was adjusted to pH 3.0, and a 200-milligram sample of fiber was exposed for 2 hours in 20 milliliters of twice-used filtrate. Alkali-centrifuge values were determined on this fiber. The results (table 19) show that residual S activity was greatest when the initial pH levels were highest.

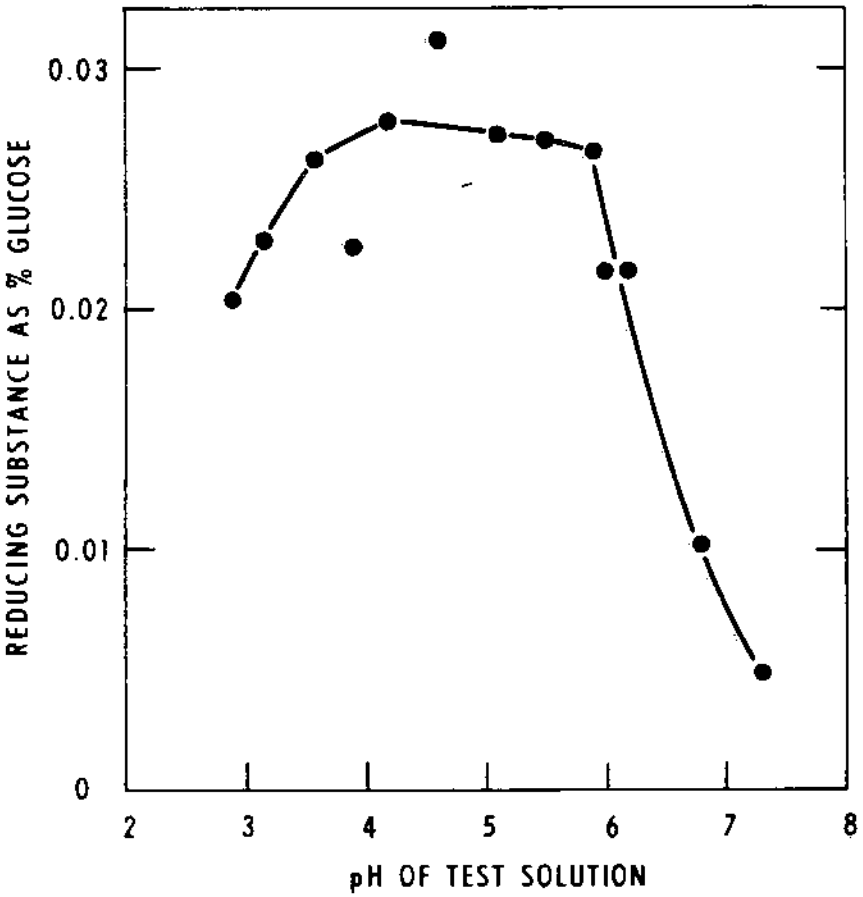

FIGURE 10.—Relation of pH to invertase activity of *A. niger* growth medium filtrates.

EFFECTS OF METHOD OF PRODUCING ENZYME PREPARATION.—Cellulase is known to be adaptively produced by various fungi (*7*, *16*), but no information was found in the literature with respect to the cellulase of *A. niger*. Mandels and Reese (*5*) have considered that with *Trichoderma viride* cellobiose is the true inducer for cellulase but that for unknown reasons it is effective only at low concentrations. An experiment was performed to determine whether growth of *A. niger* on cellobiose

would result in formation of the enzyme. The data (table 20), involving incubations at three initial pH levels, indicated that extremely little S factor was produced under these circumstances even though the concentration of reducing materials in the growth medium fell to very low levels. In a similar experiment with glucose as the carbon source (table 21), *A. niger* TC exhibited essentially similar results. The data in tables 20 and 21 show that *A. niger* grew rapidly at low pH levels. Also, the ability of the organism to grow at low pH is clearly not limited to circumstances involving cellulose.

With the method of producing cellulase with *A. niger* on a cotton mat (described in the appendix), no major change in the shape of the pH-activity curve occurred with time of incubation on the mat. Table 22 shows pH-activity data by three methods for filtrates from cotton mat cultures grown for 3, 6, 10, and 14 days. Maximum activity was attained in 6 and 10 days. The somewhat lower activity at 14 days as compared with that at 10 days may have been a result of a degradative effect of

TABLE 20.—*Production of S factor by A. niger TC during growth on cellobiose*

Original pH of medium	Measurement after growth for—					
	1 day	2 days	3 days	4 days	7 days	11 days
	pH of medium					
3.0	3.0	2.8	2.6	2.4	2.4	2.4
4.6	4.0	2.7	2.5	2.4	2.5	2.6
6.4	6.4	6.2	3.5	3.3	2.6	2.4
	Dry weight of mycelium (mg.)					
3.0	3	73	254	356	342	311
4.6	9	156	354	375	329	303
6.4	3	20	55	72	172	260
	Residual reducing substance as percentage of glucose					
3.0	.83	.68	.42	.12	.02	0
4.6	.74	.52	.13	.01	.01	0
6.4	.80	.78	.72	.77	.34	0
	S factor in medium					
3.0	.....	7	8	15	.....	6
4.6	.....	10	8	12	.....	5
6.4	.....	8	7	9	.....	1

TABLE 21.—*Production of S factor by A. niger TC during growth on glucose*

Original pH of medium	Measurement after incubation for—						
	0 day	1 day	2 days	3 days	4 days	7 days	11 days
	pH of medium						
3.0	3.0	2.9	2.6	2.6	2.5	2.4	2.6
4.5	4.5	3.2	2.7	2.6	2.4	2.4	2.5
6.4	6.4	3.8	3.2	3.0	2.8	2.5	2.3
	Dry weight of mycelium (mg.)						
3.0	0	43	188	172	181	276	292
4.5	0	46	106	151	267	225	233
6.4	0	20	76	100	117	126	195
	Residual reducing substances as percentage of glucose						
3.0	.93	.93	.56	.42	.00	.00	0
4.5	.92	.77	.65	.47	.47	.00	0
6.4	.93	.69	.54	.36	.24	.03	0
	S factor in medium						
3.0		11	8	16	10	11	10
4.5		8	9	11	12	10	7
6.4		11	10	10	14	12	8

low pH on the enzyme. All cultures were started at an initial pH of 3.0 and remained at essentially that level throughout the incubation in which the enzyme was produced. Apparently the cellulase of *A. niger* TC not only is heat stable but also is at least relatively stable during exposure at a pH of 3.0. In a more direct examination of this question, *A. niger* filtrate was adjusted to pH 3.3 and tested for S activity immediately after passing through a Morton filter and after standing under sterile conditions in Morton filter flasks for 1, 2, and 3 days at room temperature. The S activity after 0, 1, 2, and 3 days was 104, 87, 92, and 39, respectively.

Comparative Experiments With Other Fungi

Since a low pH was obviously of dominant importance in both *in vivo* and enzymatic decomposition of cellulose by *A. niger*, might similar relations occur with other fungi? Experiments

TABLE 22.—*Effect of time of incubation of cotton mat with A. niger TC on cellulase activity of filtrates as measured at different pH levels by S factor, RD, and RC methods*[1]

pH during assay	Measured value at incubation time in days											
	S factor				RD value × 10⁴				RC value × 10⁴			
	3	6	10	14	3	6	10	14	3	6	10	14
2.7	57	100	106	99	81	95	106	106	19	72	51	32
3.0	73	100	105	111	98	105	115	110	33	81	60	45
3.3	72	109	107	104	88	94	116	97	46	88	68	54
3.6	77	101	108	98	70	74	108	78	47	83	74	54
3.9	77	97	99	89	58	56	71	65	43	68	80	52
4.2	69	86	91	74	49	48	58	52	34	56	49	41
4.5	57	70	78	53	40	36	44	34	20	44	36	27
4.8	42	34	42	36	30	18	30	20	11	30	30	14
5.1	23	30	31	29	19	12	14	18	5	15	28	9
5.4	13	24	21	23	13	2	12	18	1	12	19	7
5.7	9	21	20	22	12	3	9	18	0	10	14	5
6.0	8	21	13	15	14	1	8	7	0	8	12	2
6.3	7	14	12	11	11	3	8	0	0	5	10	1
6.6	6	12	8	8	8	4	3	0	0	2	6	0
6.9	3	12	.....	.....	4	4	.....	.....	0	4	2	0

[1] Initial pH of cotton mat culture set at pH 3.0.

were performed with various nonblack Aspergilli and with the well-known cellulose decomposer *Myrothecium verrucaria.*

Experiments With Nonblack Aspergilli

A. terreus can weaken cotton fabric during incubation in the presence of mineral salts agar (*8*). When the fungus was grown in this manner, the pH of the test medium decreased as the incubation proceeded (table 23). However, when calcium carbonate was added to the medium and the pH thereby maintained at a near-neutral level, a major strength loss still occurred. Thus, when *A. terreus* 182 was incubated on Q duck in a mineral salts agar system with 2 percent of $CaCO_3$ present, the final pH of the medium was 7.2 and the strips sustained an 88-percent strength loss. The cellulase of the fungus, obtained in the filtrate from a cotton mat culture, exhibited its highest activity at a rather low pH level (figs. 11 and 12). Presumably in this fungus some factor other than the pH of the test medium was a controlling factor in determining the degree of cellulose decomposition. Large differences in the amounts of enzyme produced might possibly counterbalance pH influences on the enzyme activity.

A. flavipes is also known to weaken cotton fabric in the presence of mineral salts agar (*8*). Experiments similar to those described for *A. terreus* were performed with *A. flavipes;* similar results were obtained (table 23 and fig. 13). During the

TABLE 23.—*Change in pH of the test medium and strength loss of Q duck fabric during incubation with A. terreus and A. flavipes in the presence of mineral salts agar*

Days incubated (number)	*A. terreus* 182		*A. terreus* 45		*A. flavipes* 36	
	Final pH of medium	Strength loss	Final pH of medium	Strength loss	Final pH of medium	Strength loss
		Percent		*Percent*		*Percent*
0	6.3	0	6.4	0	6.4	0
1	6.4	11	6.4	0	6.2	9
2	6.4	8	6.2	15	6.3	9
3	6.0	25	5.8	36	6.0	24
4	5.8		4.8	41	5.7	30
7	4.1	32	3.9	68	4.2	54
9	3.9	74	3.7	76	4.0	62
11	3.6	83	3.7	79	3.9	66
14	3.6	86	3.5	84	3.9	71

TABLE 24.—*Strength losses of cotton fabric after 2 weeks incubation with A. flavus isolates on agar media adjusted to different pH levels*

Isolate	Concentration of glucose	pH of medium		Strength loss
		Initial	Final	
	Percent			*Percent*
NRRL 482	0	5.2	6.0	1
Do	.5	5.2	4.9	1
Do	0	3.6	4.2	4
Do	.5	3.6	4.2	1
NRRL 485	0	5.0	5.8	4
Do	.5	5.0	3.8	0
Do	0	3.6	4.0	1
Do	.5	3.6	3.5	0

culture-bottle incubation with $CaCO_3$ present, a strength loss of 83 percent occurred in spite of a final pH of 6.4.

A. flavus isolates have generally shown little tendency to cause strength loss in a cotton fabric (*8*). Although the optimum pH for cellulase activity of this fungus was rather low (fig. 14), adjusting the original pH of the test medium to a low level was ineffective in causing the fungus to decompose a fabric strip (table 24).

Experiments With Myrothecium verrucaria

The addition of any of several soluble carbon sources to the test medium clearly may result in cellulose decomposition by *A. niger* and the effect is at least in part associated with the

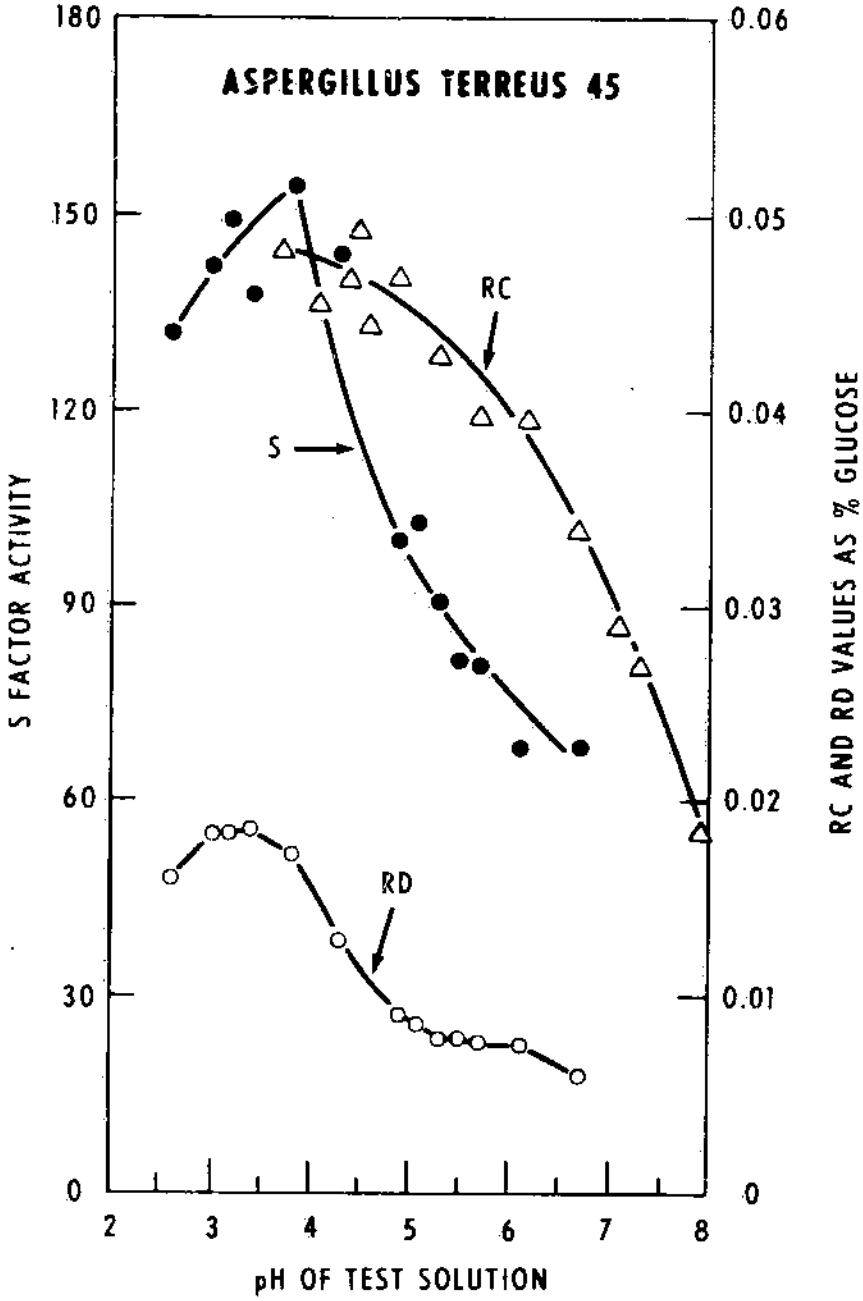

FIGURE 11.—Relation of pH to decomposition of raw cotton fiber (S factor and RD value) and of carboxymethyl cellulose (RC value) by filtrates of *A. terreus* 45.

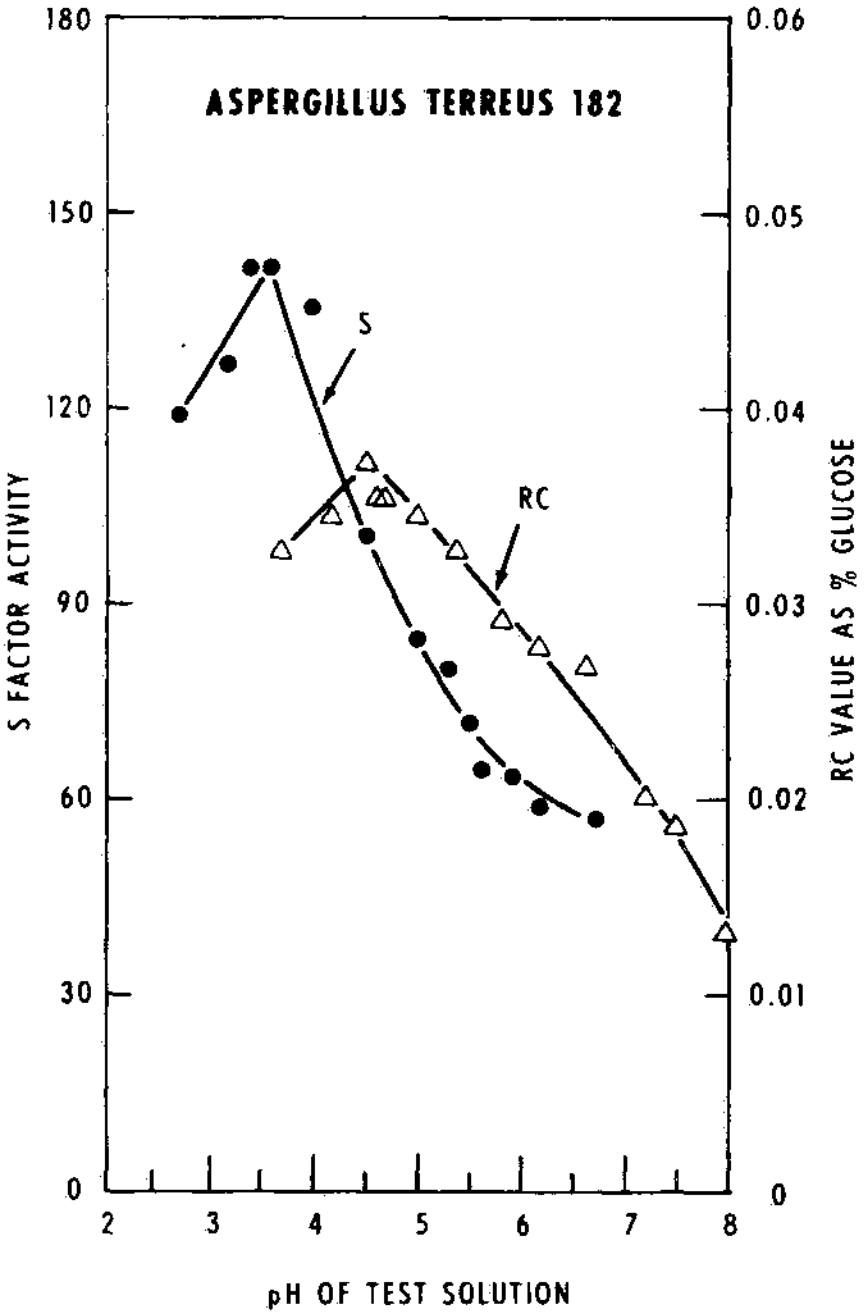

FIGURE 12.—Relation of pH to decomposition of raw cotton fiber (S factor) and of carboxymethyl cellulose (RC value) by filtrates of *A. terreus* 182.

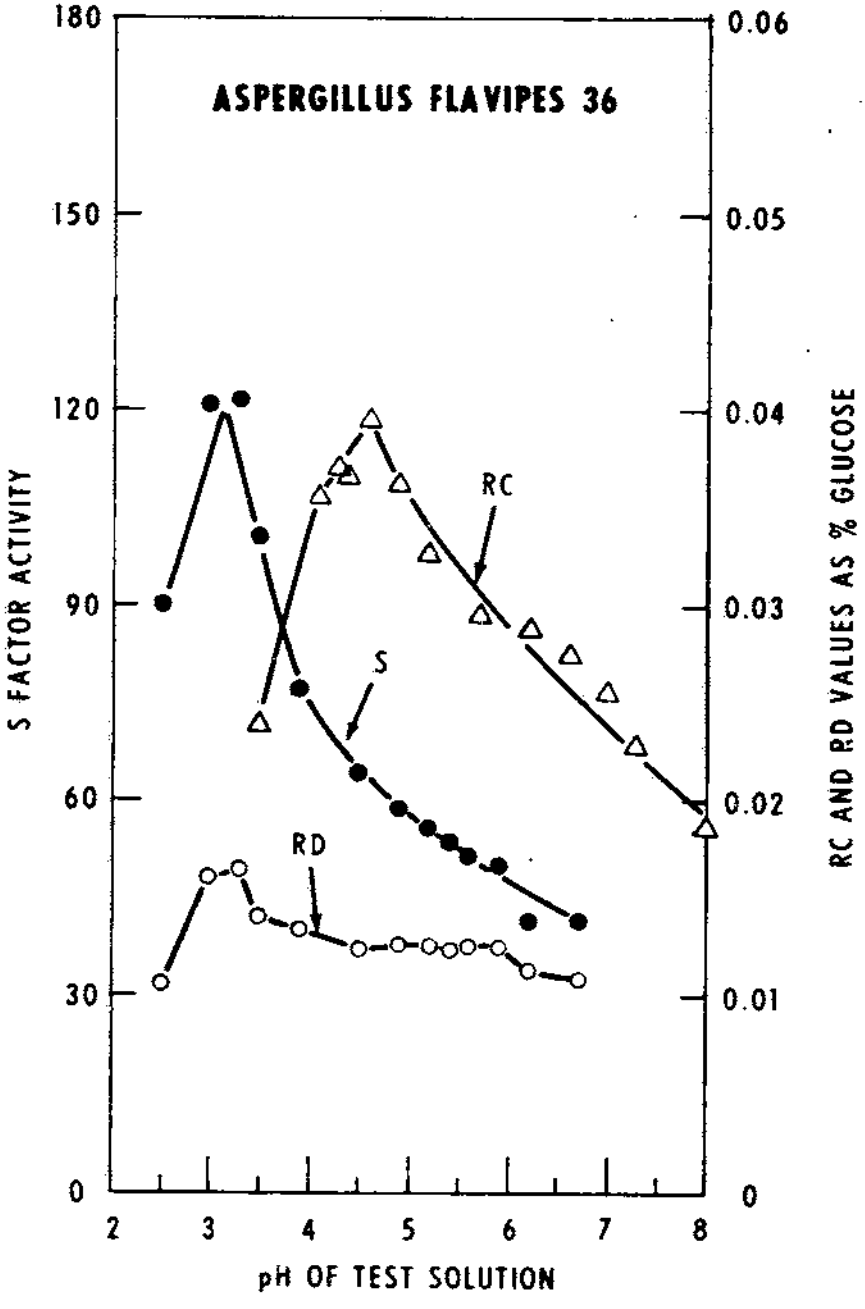

FIGURE 13.—Relation of pH to decomposition of raw cotton fiber (S factor and RD value) and of carboxymethyl cellulose (RC value) by filtrates of *A. flavipes* 36.

710-552 O-64—4

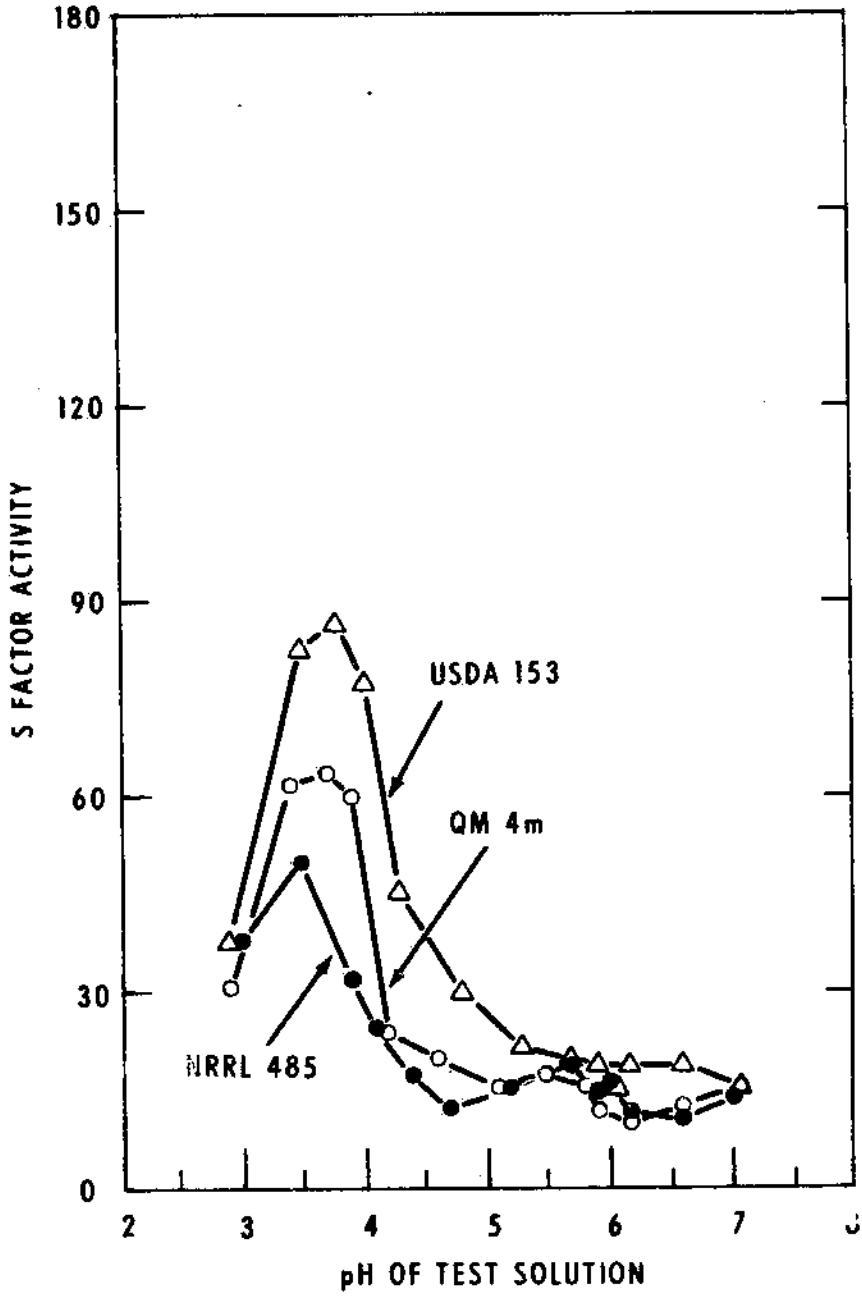

FIGURE 14.—Relation of pH to decomposition of raw cotton fiber by filtrates from three isolates of *A. flavus*.

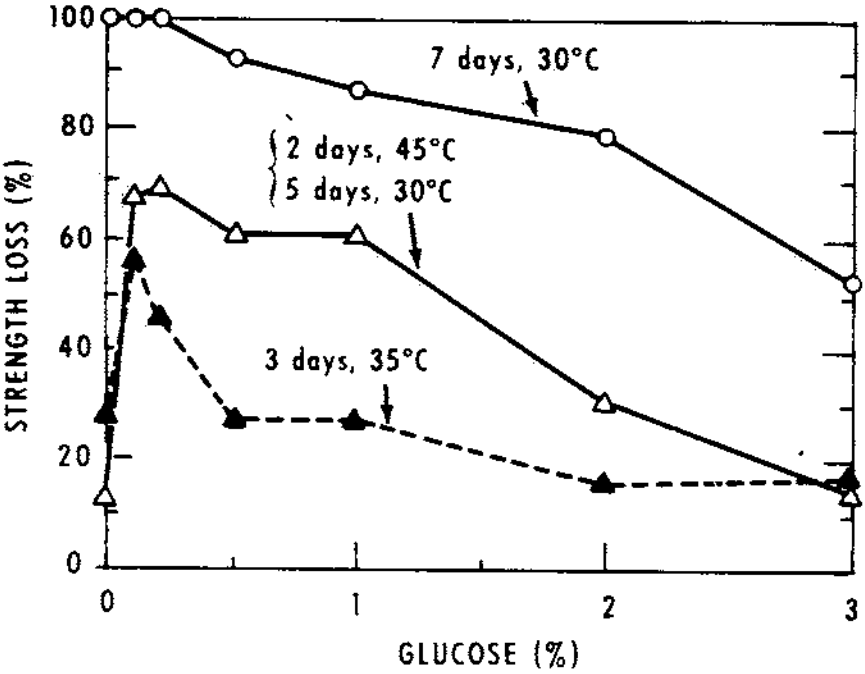

FIGURE 15.—Relation of temperature of incubation and glucose concentration in the medium to strength loss in cotton fabric caused by *Myrothecium verrucaria* USDA 1334.2. Final pH levels in the medium were all in the range 6.0–6.8.

development of a low pH in the test medium. Would such a favoring effect of glucose on cellulose decomposition occur with *Myrothecium verrucaria?* When this organism was grown at elevated incubation temperatures, a favorable effect of a small amount of glucose on cellulose decomposition occurred (fig. 15) even though at higher glucose levels there was a sparing effect toward the cellulose. The results were not associated with pH changes. Apparently the organism grew preferentially on the glucose. By the time the sugar had been exhausted in the cultures with low concentrations of glucose, the fungus had produced an extensive mat of mycelium in contact with the strip, which it then proceeded to decompose. From these data, there seems no reason to conclude that a stimulatory effect of glucose on cellulose decomposition is always with all fungi a result of a pH shift in the medium.

DISCUSSION

Further experiments will be required to answer completely many questions to which partial answers are provided in the present report. Some possible areas for future endeavor are mentioned below.

THEORETICAL CONSIDERATIONS.--In an interesting review on microbial degradation of cellulose, King (*2*) suggests that S factor is probably a nonhydrolytic enzyme, the action of which precedes that of hydrolytic enzymes of the cellulase complex in the decomposition of crystalline cellulose. We do not conclude at present that S factor is or is not hydrolytic, since further and more critical experiments seem necessary to determine the point. However, it seems clear from the measurements of reducing substances liberated during S factor determinations that hydrolytic action did occur coincident with the change in the alkali-swelling response (figs. 6, 11, and 13).

Whether S factor is a "disaggregating" enzyme as suggested by King (*2*) is also not apparent to us. We see no positive proof from any of our experimental results to show that it is. The S-factor determination involves swelling of the fiber in a concentrated sodium hydroxide solution and differs fundamentally from simple absorption of liquid from pure water in that the former procedure places the outer fiber wall under great tension. Under the conditions of our S factor assay, the alkali-centrifuge test is thought to measure enzymatic degradation of the outer wall of the fiber and not to necessarily involve a deep-seated disaggregation of cellulose in the secondary wall.

One possible approach to disaggregation seems to lie in the use of fiber taken from a cotton boll before its initial drying as an enzymatic substrate. Cellulosic degradation of such never-dried fiber by *A. niger* filtrates tends to be distinctly greater than is produced by the same filtrates acting on the usual dried-fiber substrate. Degradation of the never-dried fiber may be measured readily in either weight loss or the production of reducing substances (DNS test).

A relation has been noted between strength loss and increases in alkali-centrifuge value (figs. 1 to 4). Although the strength of the fiber is obviously in its secondary wall, the relation shown in figures 1 to 4 does not indicate that the secondary wall is the site of the enzymatic reaction measured in the alkali-centrifuge test. On the contrary, much evidence suggests otherwise (*11*). The data of figures 1 to 4 are considered more compatible with the concept that a strength loss in the secondary wall and an alkali-centrifuge change in the outer wall are both brought about by a cellulose-decomposing enzyme.

Enzymatic action in the secondary wall was recorded earlier in a fragmentation phenomenon brought about by *Myrothecium verrucaria* filtrates (*6*). With laboratory-produced *A. niger* filtrates, we have not succeeded in obtaining fragmentation. However, an *A. niger* cellulase concentrate from the Miles Laboratories, used at high concentration and a pH of 3.0, did produce in raw cotton fiber a typical transverse fragmentation of the same microscopic appearance as that figured earlier in experiments with *Myrothecium* filtrates (*6*).

The results reported here on the favorable effect of sugars on cellulose decomposition by *A. niger* suggest that the decom-

position of wood cellulose by this fungus in the experiments of Reese and Levinson (*14*) may have been similarly favored by noncellulosic nutrients in the wood.

The sensitivity of the S factor test has been noted at several points in this report. However, the technique here employed is not the most sensitive possible with the test. If the fiber is reduced from 250 milligrams to lower amounts with the amount of filtrate remaining constant, the method becomes even more sensitive. With a well-mixed assay fiber, a 50-milligram sample may be used successfully.

APPLIED CONSIDERATIONS.—In the western part of the U.S. cotton belt, *A. niger* is one of the commonest fungi associated with boll rot. Under laboratory conditions simulating the boll rot situation, *A. flavus* was shown in earlier experiments to be capable of causing considerable fiber strength loss (*9*) even though the same fungus shows little tendency to weaken the unbleached cotton fabric of commerce (*8*). In a simulated-boll rot situation in the laboratory, *A. niger* has also been found to cause definite fiber strength loss. Thus, in an experiment of the type described with *A. flavus* (*9*), Pressley strength losses by the 1-mm.-gap method applied to *A. niger*-incubated fiber were 43, 62, 66, and 84 percent for incubation periods of 2, 3, 4, and 5 days, respectively. The amorphous condition of the cellulose in the never-dried cotton fiber in the boll-rot situation is presumed to cause high susceptibility to microbial fiber deterioration.

We report here that *A. niger* TC 215–4247, which has been called a superficial fungus in many mildew resistance specifications applicable to cotton and other cellulosic materials, actually can cause strength loss in cotton fabric. We do not suggest, however, that this new information carries with it any objection to the continued use of *A. niger* TC in such specifications. On the contrary, much experience in many laboratories has demonstrated the practical usefulness of the fungus in specifications and the interpretability of the data so obtained. No change is proposed in the visual-growth type of indices used. Individuals employing such tests can now be aware, however, that if strength losses or other evidences of cellulose deterioration do occur in cellulosic items in their tests with *A. niger*, these changes are in accord with the behavior of the fungus as here described. The possibility that pH changes in the test medium may influence the efficacy of mildew-preventive agents may require examination.

Work reported from this laboratory several years ago (*7*) made it clear that a cellulase acting on cotton fiber may greatly increase the fiber's degree of swelling in alkali with little or no attendant influence on strength. This situation, combined with current commercial interest in producing cellulase and with the stability of the enzyme, suggests the desirability of experimentation on possible commercial applicability of cellulase-enzyme treatments in cotton textile finishing. In current finishing practice, cotton is frequently subjected to alkali-

swelling. Enzyme treatment of the fiber prior to alkali-swelling might have a desirable effect on the properties of the finished textile. Even if this should not turn out to be the case, enzyme treatments of this kind might be of value in experimental investigation of the fundamental properties of cotton textiles.

CONCLUSIONS

A belief widely held for several years that *A. niger* cannot decompose cellulose was based on insufficient evidence and requires revision. In the presence of suitable amounts of glucose or any of several other soluble carbon sources, the fungus has been found capable of causing strength loss in cotton fabric. The favorable effect of such supplementary materials on cellulose decomposition by the fungus appears to be associated with the production of a low pH in the test medium. In addition to changes in the supplementary carbon source, several other types of manipulations of test conditions also affected strength loss in a cotton fabric during incubation with the fungus. These manipulations involved the method of inoculation, the magnesium sulfate content of the test medium, and the type of nitrogen source employed. Higher strength losses were regularly correlated with a low final pH in the medium. The use of calcium carbonate to prevent pH decrease in the presence of glucose also prevented strength loss, whereas setting the pH initially at a low level resulted in strength loss even in the absence of any supplementary soluble carbon source. The low pH was not in itself a direct cause of strength loss.

Several of the purple-black Aspergilli of the *A. luchuensis* series caused strength loss in a cotton fabric in the absence of any supplementary soluble carbon compound and without the device of setting the initial pH of the test medium at a low level. However, in a series of 11 isolates, there was a marked correlation between the ability of the isolate to cause strength loss and a decrease in pH of the test medium during incubation.

A favorable effect of low pH on cellulose decomposition by *A. niger* occurred not only *in vivo* but also in cellulase enzyme assays with growth medium filtrates. Optimum activity occurred in the pH range 2.7–4.0, with marked decreases in activity at higher pH levels. This result was observed in the alkali-swelling behavior of cotton fiber and in the production of reducing substances from carboxymethyl cellulose (CMC). The shape of the pH-activity curve was rather constant at all temperatures measured from 30° to 75° C. It also exhibited little variation in shape according to the age of the enzyme-producing culture at the time of harvest. The enzyme (S factor) appears to be at least moderately stable at room temperature at a pH of 3.0. Absorption of *A. niger* cellulase (S factor) was greater at low pH levels than at higher ones.

When several black Aspergilli were grown on cotton fabric in the presence of a graded series of concentrations of glucose, the strength loss-percentage of glucose curves varied in shape from one fungus isolate to the next, as did also the alkali-centrifuge value–percentage of glucose curves. However, for each isolate there was a pronounced tendency within suitable ranges of measurement for the alkali-centrifuge values to parallel the strength losses. This result is interpreted as further evidence that the S factor test measures principally, if not exclusively, enzymatic action on cellulose rather than on pectin or other fiber constituents.

Certain other Aspergilli (nonblack) also produced cellulases with optimum activity at a rather low pH. With these fungi, however, cellulose decomposition *in vivo* was not greatly favored by a low pH in the test medium nor was it prevented by a pH maintained near neutral. With these fungi (*A. flavus*, *A. flavipes*, and *A. terreus*), pH of the test medium was obviously a less critical factor in determining cellulose decomposition *in vivo* than it was with *A. niger*.

When *Myrothecium verrucaria* was incubated on cotton fabric at elevated incubation temperatures, a small amount of supplementary glucose in the medium favored strength loss. No correlation was seen between strength loss and the final pH of the test medium.

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APPENDIX

Sources of Fungus Isolates

The fungus isolates came from the U.S. Quartermaster Culture Collection at Natick, Mass.; the American Type Culture Collection in Washington, D.C.; and the U.S. Department of Agriculture at Beltsville, Md. Some isolates were known by one number in the collection from which they were supplied and by another number elsewhere. Table 25 lists the names and isolate numbers. Each isolate is referred to in the text and tables by the number used in the collection from which it was obtained. *A. niger* TC 215–4247 is frequently designated by the abbreviation "TC." When an *A. niger* isolate is mentioned here without isolate symbols, the TC isolate is indicated.

Test Fabric

Except for the experiment recorded in table 1, the fabric used was Q duck, an 8-ounce gray cotton duck employed in a related earlier investigation (*8*). The strips (6 x 1-1/2 inches) were cut from the bolt of fabric and raveled to 1-inch in width before incubation. Approximately 2,000 strips were cut, raveled, and randomized for use in these tests. The fabric has an original breaking strength by the 1-inch raveled strip method of 109 pounds and an alkali-centrifuge value of 224.

Inoculation and Incubation Procedures

As a source of inoculum, each fungus isolate was grown in a 250-ml. Erlenmeyer flask with 40 ml. of a medium containing glucose (0.5%), peptone (0.1%), yeast extract (0.025%), agar (2%), and mineral salts in grams per liter as follows: NH_4NO_3, 2.0 g.; K_2HPO_4, 1.4 g.; KH_2PO_4, 1.8 g.; and $MgSO_4 \cdot 7H_2O$, 1.4 g. At the time the test strips were inoculated, the spores were brought into suspension by adding 50 ml. of sterile distilled water to the inoculum flask and shaking. One ml. of the suspension was then transferred by sterile pipette to the surface of each test strip. The test strips, previously raveled to 1 inch in width, wet in water containing 0.025-percent Aerosol OT (sodium dioctyl sulfosuccinate), and sterilized at 15 pounds' steam pressure for 15 minutes, were placed individually and with sterile precautions on the surface of an agar medium in incubation bottles. The bottles were 500-ml. French square type, each sterilized (15 pounds, 15 minutes) with its contents

TABLE 25.—*Names, isolate numbers, and sources of the fungi used*

Species	Isolate Nos.				Source
	ATCC	QM	USDA	Other	
A. carbonarius	1025	331	248–4030.1	NRRL 369	ATCC
A. flavipes			36		USDA
A. flavus		4m			QM
A. flavus			153		USDA
A. flavus				NRRL 482	NRRL
A. flavus				NRRL 485	NRRL
A. foetidus		328		NRRL 341	QM
A. fonsecaeus	8740	330		NRRL 67	ATCC
A. japonicus		155e			QM
A. japonicus		332			QM
A. japonicus		333			QM
A. japonicus		2018			QM
A. luchuensis		21e			QM
A. luchuensis		23b			QM
A. luchuensis		70c			QM
A. luchuensis		102d			QM
A. luchuensis		873		JQMD 190	QM
A. luchuensis		874		JQMD 745	QM
A. niger			145		USDA
A. niger		198b		PQMD 198b	QM
A. niger	9642	386		SN 26	QM
A. niger	1015		3528.7	TC 167	ATCC
A. niger mut. *schiemanni*	1040	327	222–3534c	NRRL 361	ATCC
A. niger	10254			NRRL 337	ATCC
A. niger	6275	458	TC 215–4247		USDA
A. phoenicis		1005			QM
A. terreus			45		USDA
A. terreus			182		USDA
A. violaceo-fuscus		6649			QM
F. moniliforme			1004.1		USDA
M. verrucaria	9095	460	15[illegible].2	NRRL 2003	USDA

of 35 ml. of mineral salts (as above) agar (2%) fortified with a known concentration (zero to 3%) of a supplementary carbon source.

Five replicate cultures were used for each experimental condition and each breaking strength figure represents an average from breaks on five strips. The incubation temperature, except where otherwise indicated, was 30° C. and the incu-

bation time was 2 weeks. In one experiment (table 5) spores were brushed onto the test fabric rather than being pipetted in liquid suspension. A small, long-handled, sterile brush was used to transfer the spores from the inoculum culture to the test strips.

In several experiments, fabric strips were incubated in 25 x 200 mm. test tubes with 25 ml. of a mineral salts-glucose medium. The strip was in a vertical position against the side of the tube and was about half submerged in the medium. The strips were placed individually in position in each tube prior to sterilization (15 pounds, 15 minutes), having previously been wet in 0.025% Aerosol OT solution. Inoculation was with 1 ml. of spore suspension from a sterile pipette. The mineral salt composition of the medium was as above.

In experiments involving addition of minor elements, copper and iron were added as nitrates, zinc and manganese as chlorides.

Determination of pH

In experiments involving agar media, a 10-g. portion of the medium was removed from the culture bottles at the end of the incubation period and was allowed to stand for several minutes with 10 ml. of boiled-and-cooled distilled water. The pH of the water was then determined with a glass electrode pH meter. The original pH of the standard liquid and agar media was approximately 6.4.

Breaking Strength Test

Following the incubation period, the fabric strips were washed, air-dried in the laboratory, conditioned at 21° C. and 65-percent relative humidity for at least 24 hours, and then broken on a Thwing-Albert pendulum-type strength tester. The control strips, incubated sterile, had a breaking strength of 108 pounds, insignificantly different from the original fabric strength of 109 pounds. No interpretive value is attached to differences in strength loss in the range below 20 percent. Differences below this level may result from changes in surface-frictional properties of the fiber in the fabric.

Alkali-Centrifuge Test

In tests on fabric, ravelings from either side of the break were used for alkali-centrifuge determinations. Only one measurement was made for each group of five strips. The alkali-centrifuge value of the original Q duck was 224. The test is performed as follows:

(1) Weigh a 250-mg. sample of well-mixed cotton fiber from the ravelings of the strips on a chemical analytical balance in a room maintained at 21° C. and 65-percent relative humid-

ity. Place the sample into a 50-ml. beaker with about 25 ml. of 0.05-percent solution of Aerosol OT and allow to stand for 10 minutes.

(2) Remove the fiber from the Aerosol solution with a dissecting needle, blot it free of excess liquid, and place it in a suitable bottle with 50 ml. of a solution containing 180 g. per l. of NaOH, A.C.S. reagent grade. Shake the sample in this solution on a mechanical shaker for 15 minutes at 21° C.

(3) Filter the fiber out of the NaOH solution by the use of a porcelain Gooch crucible, without suction. The crucibles have a capacity of 40 ml. with holes 0.7 mm. in diameter. Except with very badly deteriorated samples, little if any fiber should pass through the crucible in the first filtration; if any small amount does go through, it can be recovered by a second filtration through the fiber mat. Transfer the fiber to an 8-ml. fritted disc filter crucible, medium porosity.

(4) Place three short sections of glass rod or tubing into the bottom of a 40-ml. centrifuge tube, put the fritted-disc crucible containing the swollen fiber on top of the rod or tubing, and cover the centrifuge tube with a rubber cap.

(5) Centrifuge the tube and contents at 1,200 r.p.m. for 10 minutes with a radius of centrifugation of 14.3 cm. (as, for example, on an International centrifuge, size 1). A standard-type, horizontal, 8-place head is used, and the radius of centrifugation is measured to the fritted glass plate in the crucible. A period of 1 minute is allowed to bring the speed to 1,200 r.p.m. and the 10-minute period of centrifugation is measured after this speed is attained. A variac is used in series with the centrifuge for accurate control of the speed which is measured by a standard type of tachometer.

(6) Transfer the centrifuged fiber mass to a weighing bottle, and weigh it on a chemical analytical balance.

(7) Calculate the percentage of increase in weight of the fiber as follows: subtract the original sample weight from the weight after centrifuging to obtain the absolute increase in weight, divide this quantity by the original sample weight, and multiply by 100. The resulting figure is termed the "alkali-centrifuge" value or "AC" value of the fiber.

S Factor Test

The general method for determining S activity was to measure the AC value of a test cotton fiber (as described above) after exposure under certain standardized conditions to a growth-medium filtrate or other solution to be tested. The difference between the AC value of the test sample and the AC value of the unexposed fiber was designated as the S activity. Except as otherwise indicated for particular experiments, a 250-mg. sample of well-mixed cotton fiber was exposed to 25 ml. of filtrate or other solution in a 250-ml. Erlenmeyer flask at 30 °C. for 2 hours. At the beginning of the exposure, the fiber was

tamped with a glass stirring rod until thoroughly wetted by the solution. After the exposure period the fiber was washed in distilled water and in many instances it was dried before the AC value was determined.

Carboxymethyl Cellulose (CMC)

The sample used was a type obtainable from the Hercules Powder Company, Wilmington, Del. It is designated as type "7M" and is indicated to have medium viscosity and a degree of substitution in the range 0.65–0.85.

Preparation of Veronal Buffer

In preparing veronal buffer for use in enzyme assay experiments, 9.714 grams of sodium acetate (with 3 molecules of water of hydration) and 14.714 grams of the sodium salt of veronal (sodium diethylbarbiturate) were dissolved in 500 ml. of distilled water. To each 20-ml. portion of this solution was added 0.1 N HCl with stirring until the desired pH level was attained. The mixture was transferred to a 100-ml. volumetric flask and brought to volume. The concentration of veronal in this buffer was 0.028 M. Since the capacity of the buffer was obviously not adequate at all pH levels, the actual pH values were measured and recorded. No pH changes were observed during the incubation period for enzymatic activity. The data shown in tables 17, 18, and 22 were obtained by graphing the original data and estimating from these curves the cellulase activity at the pH values listed in the tables.

Reducing Substance Tests

The general method employed dinitrosalicylic acid (DNS) and was adapted from the procedure of Marsh and Harrison (*10*). The DNS reagent is prepared as follows: to 300 ml. of 4.5-percent carbonate-free NaOH, add 880 ml. of 1-percent 3,5-dinitrosalicylic acid and 255 g. of Rochelle salt (sodium potassium tartrate). Mix until dissolved and keep tightly stoppered.

The standard curve for determination of reducing substances is performed as follows:

(1) Weigh 100 mg. of glucose into a 100-ml. volumetric flask and add distilled water, containing 0.02-percent Aerosol OT, to the mark. Into a series of matched 15-ml. colorimeter tubes, pipette different amounts of this glucose solution; i.e., 0, 0.1 ml., 0.2 ml., 0.3 ml.—1.0 ml. Add distilled water to bring the volume in each tube to 1.0 ml. This series of tubes then contains from 0 to 1,000 micrograms of glucose.

(2) Heat tubes, in test-tube support, in a boiling water bath for 5 minutes. Add 1 ml. of DNS reagent to each tube, place tubes into boiling water bath for 10 minutes, remove from bath, and cool in running water for at least 5 minutes. Di-

lute the solution in each tube to the 10-ml. mark with water, set the colorimeter at its null point with the "zero glucose" sample, and make readings to provide a standard curve of colorimeter reading versus DNS value. Use a green filter (No. 50), as supplied with the Klett-Summerson colorimeter. For the most accurate determinations, readings should be made at a constant temperature.

The determination of enzymatically produced reducing substances was performed on both CMC and cotton fiber. The former procedure was as follows:

(1) CMC was used at final concentrations of 0.1 percent and 0.5 percent in the presence of 10 x diluted filtrate and half-strength veronal buffer. One-ml. portions of these mixtures were incubated under conditions specified for the individual experiments.

(2) After the incubation period, the test tubes were heated in a boiling water bath for 5 minutes.

(3) One ml. of the reagent was added to each tube and the tubes were heated in a boiling water bath for 10 minutes to obtain maximum color development.

(4) After the boiling period, the tubes were cooled in cold running water for 5 minutes.

(5) The reaction mixture was brought up to 10 ml. in optically matched colorimeter tubes.

(6) The density of color development was determined by the use of a Klett-Summerson colorimeter with a No. 50 filter.

(7) The values obtained for the test samples were compared with a standard glucose curve. In general, a factor of 0.0002 could be used to convert the direct readings to percentage of reducing substance as glucose. The value obtained was called the "RC" value—"R" for "Reducing substance" and "C" for "CMC." A reducing value ("RD") was also obtained for reducing materials produced from raw cotton fiber. This test was carried out on 1 ml. of the liquid remaining after exposure of the fiber in the S factor test.

It is pertinent to the interpretation of the DNS results that glucose and cellobiose yield quantitatively the same degree of reduction in this procedure; the test thus does not distinguish between these two materials.

In the use of the DNS test for reducing substances after action of an *A. niger* filtrate on a cellulosic substrate, it seemed pertinent to know whether such filtrates might contain an oxidase which would act on glucose after its liberation from the cellulose. Experimental evidence indicates that under the conditions here employed, very little or no glucose oxidase is present. Thus, when 300 micrograms of glucose was added to an *A. niger* filtrate buffered at levels between 3.0 and 7.5, the same amount of DNS reduction was measured after a 1-hour incubation period at 30° C. as was measured immediately after adding glucose to the buffered filtrates.

Production of Cellulase-Containing Filtrates

For producing cellulase-containing filtrates, the intended fungus was grown in pure culture in 500-ml. Erlenmeyer flasks containing 1-gram mats of raw cotton fiber saturated with 40 ml. of mineral salts solution. The mineral salts solution was the same standard composition as that used in the culture bottle experiments except that the dibasic phosphate was omitted for use with the Aspergilli. The initial pH of this medium was sometimes lowered to about pH 3.0 with an acid, generally phosphoric. With *Myrothecium verrucaria*, the filtrates were produced by a similar method except that (a) the mineral salts solution contained dibasic phosphate, and (b) the concentration of the salts was one-half the usual concentration. After steam sterilization and cooling, the flasks were inoculated by needle with the fungus and incubated at 30° C., generally for 2 weeks. The culture fluid was drained, pressed out of the fiber mat, and filtered successively with suction through coarse-, medium-, and fine-porosity sintered glass crucibles. The filtrates were stored in a frozen condition until used.

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Sources of Commercial Enzyme Preparations

Cellulase enzyme preparations were obtained as follows: Cellulase 1000, Wallerstein Company; Takamine Cellulase 4000, Miles Chemical Company; and Cellulase, Nutritional Biochemicals Corporation. Mention of these preparations or other commercial products or firms does not imply endorsement by the U.S. Department of Agriculture over other similar preparations.

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