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Factors Influencing the Production of Cellulases by *Sporotrichum thermophile*

A. D. COUTTS[1] AND R. E. SMITH*

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

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Cellulase production and growth of a strain of *Sporotrichum thermophile* were studied by using a mineral salts medium supplemented with yeast extract and insoluble cellulose. The effects of cultural conditions, such as pH, nitrogen source, substrate concentration, and temperature, were examined. Maximum production of C_1 and C_x cellulases occurred at 45 C in 2 to 4 days, in the presence of 1% Solka/Floc as substrate, when $NaNO_3$ or urea were used as sources of nitrogen. Under these conditions, cellulolytic activity of culture filtrates appeared to be similar to that reported for *Trichoderma viride* grown in a favorable environment. However, comparable yields of cellulase were produced by *S. thermophile* in less than one-quarter the time required by mesophilic fungi.

The use of microorganisms or their enzymes for the conversion of cellulose into simple carbohydrates is receiving increased attention. This is the result of growing concern over the accumulation of wastes, and our awareness of the vast quantities of residues, rich in cellulose, which result from agricultural operations and the manufacture of wood products. These potentially valuable sources of food energy are largely unavailable to monogastric animals because of the resistance of cellulose to digestive enzymes. Conversion to cellobiose and glucose by enzymes, however, would provide readily utilizable substrate for the production of microbial protein for use in food supplementation.

The existence of cellulolytic microorganisms is well documented. Foremost amongst these are the filamentous fungi, a number of which are active in the degradation of native cellulose. For example, in 1963 Selby et al. reported that culture filtrates of *Myrothecium verrucaria* could solubilize up to 30% of cotton fibres (17). Mandels and Reese (10) found *Trichoderma viride* to be more active. A mutant of this organism was later obtained which produced yields of cellulase double that of the parent (12). Halliwell (7) showed that culture filtrates of *Trichoderma koningii* could completely degrade cotton fibers in 24 h.

Certain thermophilic fungi are known to be cellulolytic (2-5, 9, 21). The possibility of a high rate of cellulose digestion by these organisms, as a result of their rapid metabolic rates, makes their study particularly attractive. In addition, high incubation temperatures used in their cultivation would greatly limit the number of contaminants able to grow, allowing the use of relatively unsophisticated equipment for large-scale fermentations. Recently, Romanelli et al. reported that *Sporotrichum thermophile* gave a higher rate of cellulose utilization than *Chaetomium thermophile* var. *coprophile* and *Thermoascus aurantiacus* (16). In a previous study of cellulolytic fungi, we obtained similar results in comparisons of *S. thermophile*, *C. thermophile*, and *Humicola insolens* (R. E. Smith and A. D. Coutts, unpublished data). On the basis of these findings, we decided to further investigate the production of cellulases by *S. thermophile*.

MATERIALS AND METHODS

Organism. The strain of *S. thermophile*, UAMH 2015, which we designated as M218, was isolated by G. Semeniuk from alfalfa silage (18), and was kindly donated by J. W. Carmichael of the University of Alberta, Edmonton, Alberta.

Media. Stock cultures were maintained on corn meal agar (CMA) (Difco) slants in 1-ounce screw-capped bottles. To prepare cultures as sources of inoculum, spores and mycelium were transferred to 1-ounce TCYE agar slants (19) and incubated at 45 C. Pettersson medium (14), modified by the substitution of Solka-Floc BW-40 (Brown Co., Boston, Mass.) for the Munktell's cellulose, and the addition of yeast extract (Difco), was used for cellulase production. Solka-Floc BW-40 is a purified wood cellulose product having an average fiber length of 80 μm. Final composition of the medium (per liter) was as follows: Solka-Floc, 10.0 g, $NH_4H_2PO_4$, 2.0 g, KH_2PO_4, 0.6 g, K_2HPO_4, 0.4 g, $MgSo_4 \cdot 7H_2O$, 0.5 g,

[1] Present address: Connaught Medical Research Laboratories LtD., 1755 Steeles Ave., West, Willowdale, Ontario, Canada M2N 5T8.

ferric citrate, 10.0 mg, $ZnSO_4 \cdot 7H_2O$, 4.4 mg, $MnSO_4 \cdot 4H_2O$, 5.0 mg, $CaCl_2$, 55.0 mg, $CoCl_2 \cdot 6H_2O$, 1.0 mg, thiamine hydrochloride, 100 μg, yeast extract, 1.0 g. This medium was designated as MPM. The yeast extract supplied about 28% of the total N. For some experiments, alternate nitrogen sources ($NaNO_3$, NH_4NO_3, and urea) were used in place of the $NH_4H_2PO_4$ in quantities calculated to supply equivalent amounts of nitrogen. The Solka-Floc was dispensed into 500-ml baffled flasks in 0.5-g quantities, and sterilized as 121 C for 30 min. The nutrient medium, previously sterilized as 121 C for 20 min, was added aseptically to provide a 1.0% suspension of Solka-Floc. In some cases, 1.0% D-glucose was used in place of cellulose for growth studies. For pH studies, the medium was prepared in concentrated form without the phosphate, and mixed with sterile citrate-phosphate buffers of required pH to give the correct concentration of nutrients and 0.05 M buffer solutions.

Inoculum. Five-day-old cultures, grown on TCYE agar slants, were used as sources of inoculum. Spores and mycelium were suspended in 10 ml of sterile MPM without cellulose, using a wire loop. The suspensions were transferred to a sterile semimicro Waring blender jar and blended at low speed for 10 s. A 1.0-ml sample of the mixed suspension was added to each replicate flask containing MPM. These were incubated at 45 C on a reciprocal shaker with a 2-cm stroke length and agitated at 80 strokes per min.

Preparation of culture filtrates. Mycelium and residual cellulose were separated from the liquid fraction by filtration through a 9-cm glass fiber filter disk (Reeve-Angel, grade 934AH, Clifton, N.J.), using a Buchner funnel. Filtrates preserved with Thimerosal (Sigma Chemical Co., St. Louis, Mo.), added to give a final concentration of 0.01%, showed no significant loss in cellulase activity when stored at 2 C for 17 days.

Measurement of growth. Growth of *S. thermophile*, with Solka-Floc as carbon source, was measured by monitoring the CO_2 produced during respiration. The medium consisted of MPM buffered at pH 5.0 to prevent bicarbonate formation. A linear relationshp between CO_2 production and mycelium synthesis, expressed as dry weight of mycelial crop, was established previously, using D-glucose in place of Solka-Floc. The CO_2 was continually flushed from replicate flasks with CO_2-free humidified air, prepared by passing sterile air through a column of Lithasorb (Fisher Scientific Co., Toronto, Ontario) and a flask of sterile-distilled water. Effluent CO_2 was trapped in 0.1 N NaOH in Fisher-Milligan gas washing bottles, and measured by determining the conductance of the NaOH solutions and extrapolation from a standard curve.

Cellulase assays. Assays of culture filtrates for C_1 or C_x cellulase activity were based on the production of reducing sugar (RS) from insoluble cellulose or carboxymethyl cellulose. RS was determined by the dinitrosalicylic acid method, using reagents prepared according to the method of Sumner and Howell (20), as modified by Fisher and Kohtés (6). Sucrose was added to the reaction mixture as suggested by Arnold (1) to correct for nonlinearity at low RS values. The enzyme activity in all cases was expressed in terms of RS produced per milliliter of culture filtrate. Blanks were prepared by substituting distilled water for culture filtrates, and all results were corrected for RS present at zero incubation time, by performing a dinitrosalicylic acid assay in the absence of substrate. C_1 assays were based on the method of Mandels and Reese (10), modified by substituting Solka-Floc for cotton sliver, and by conducting reactions at 50 C, after buffering at pH 5.0 with 0.05 M citric acid-phosphate buffer. The estimation of C_x cellulase concentration was carried out using the methods of Reese and Mandels (15), and incubating reaction mixtures at 60 C. Sodium carboxymethyl cellulose (Hercules, Inc., Wilmington, Del., type 41A) was used as substrate. An indication of total cellulolytic activity of filtrates was obtained by the determination of "filter paper activity," using the procedure of Mandels and Weber (11). This was based on RS production from Whatman no. 1 filter paper strips, expressed as mg of RS produced by 1.0 ml of culture filtrate in 1 h at 60 C.

RESULTS AND DISCUSSION

Relationship between growth and cellulase production. *S. thermophile* grew rapidly in shake cultures at 45 C, with maximum C_1 and C_x cellulase activities occurring within 4 days (Fig. 1). C_x appeared in the early stages of growth, before significant C_1 activity was detectable. This finding was not compatible with

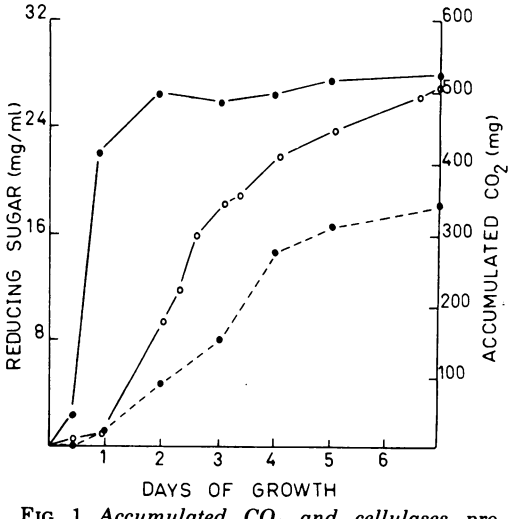

FIG. 1. *Accumulated CO_2 and cellulases produced during growth of Sporotrichum thermophile at 45 C, in MPM buffered at pH 5.0. Enzyme production was measured by standard assay using Solka-Floc for C_1 and sodium carboxymethyl cellulose for C_x cellulase determinations. Activity is expressed as mg of RS produced by 1.0 ml of culture filtrate (see text for details). Symbols: C_1 (●- - -●); C_x (●——●); CO_2 (○——○). Data represent averages of five replicates.*

the widely held supposition that C_1 enzymes initiate the attack on cellulose fibers. Wood and McCrae (23) suggested that if C_x enzymes attack cellulose by randomly breaking internal bonds, they should appear early in the growth of cellulolytic organisms. The C_1 complex, if it splits off cellobiose units, would then have more accessible substrate to attack (i.e., more available chain ends), and would therefore increase in concentration subsequent to the initiation of exponential growth. The data shown in Fig. 1 support this view. A similar sequence of events was reported by Neudoerffer and Smith (13) in studies with *Trichurus cylindricus*, *T. viride* and *M. verrucaria*, when these organisms were grown on supplemented wheat bran. The production of cellulases when CO_2 was monitored (Fig. 1) was superior to that occurring in ordinary shake flasks, presumably becasue of better gas exchange.

Influence of cultural conditions on enzyme production. Figure 2 demonstrates the effect on cellulase production of varying the growth temperature. C_1 and C_x activity appeared most rapidly in cultures incubated at 45 C, whereas lower yields occurred at 35 and 50 C. Maximum production of both enzyme complexes in 4 days appeared to take place at 40 C, but since rate of synthesis was greater at 45 C, this temperature was selected for use in subsequent investigations.

The concentration of Solka-Floc in the MPM affected the production of cellulases as incubation time proceeded (Fig. 3). Assays performed at 48 and 72 h showed that C_1 activity was markedly reduced in culture filtrates if initial

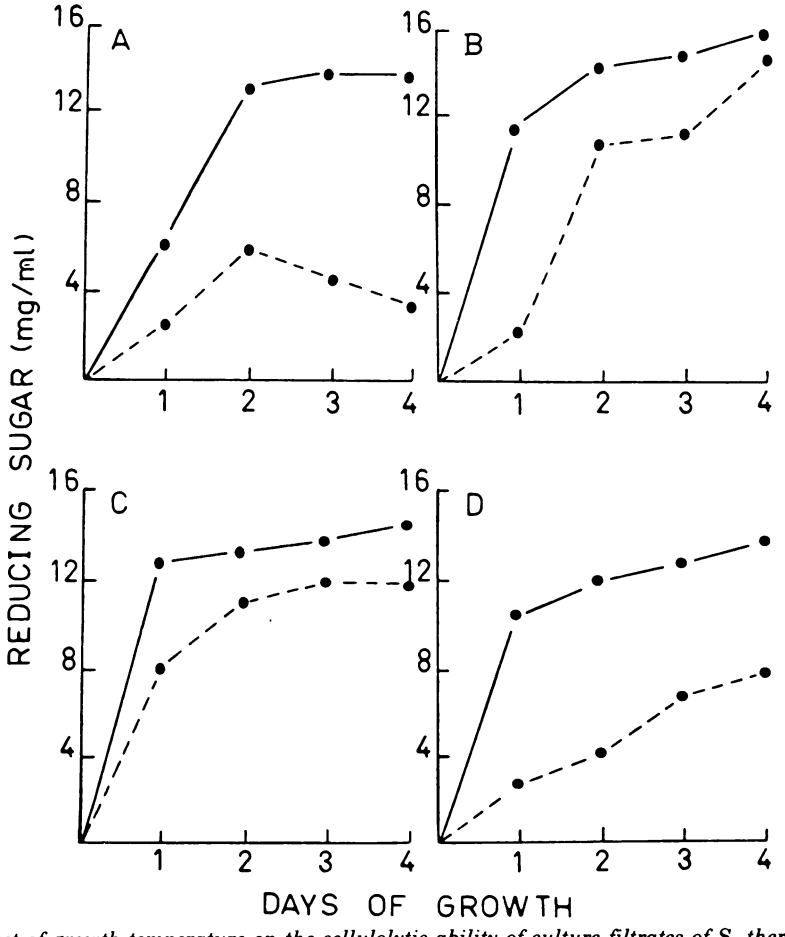

FIG. 2. *Effect of growth temperature on the cellulolytic ability of culture filtrates of S. thermophile. The organism was cultured at 35 (A), 40 (B), 45 (C), and 50 C (D) for 4 days in MPM with $NaNO_3$ as the inorganic N source. Cellulase activity expressed as RS formed by 1.0 ml of culture filtrate during standard assays. Data represent averages of determinations using samples from duplicate flasks. Symbols: C_1 cellulase (●- - -●); C_x (●——●).*

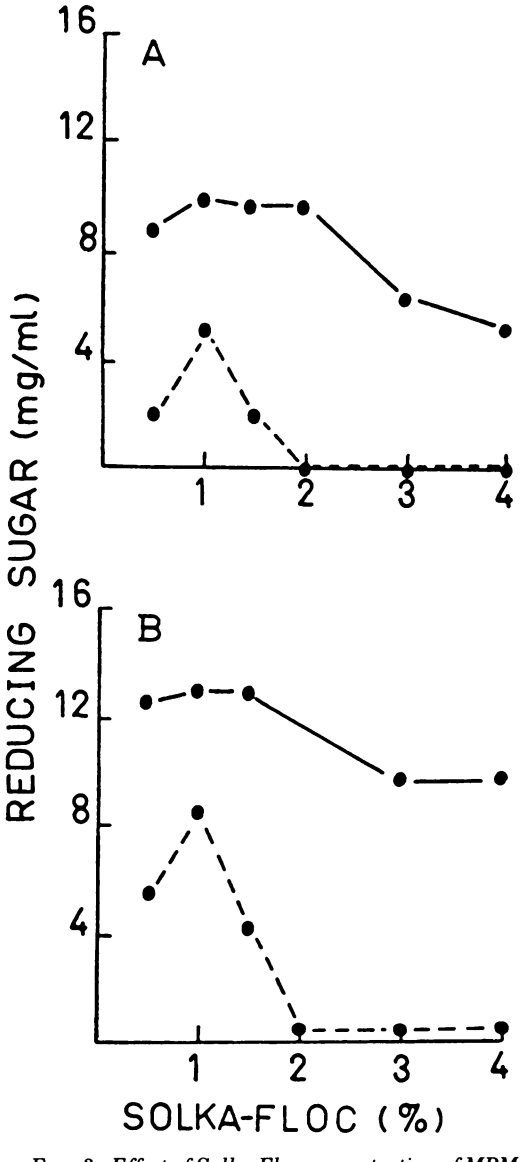

FIG. 3. *Effect of Solka-Floc concentration of MPM on the cellulolytic ability of culture filtrates of S. thermophile. The organism was cultured at 45 C for 48 h (A) and 72 h (B) in MPM with $NaNO_3$ as the inorganic N source. Cellulase activity is expressed as RS formed in standard assays by 1.0 ml of culture filtrate. Data represent averages of results with samples from duplicate flasks. C_1 cellulase: (●- - -●); C_x cellulase (●——●).*

Solka-Floc levels exceeded 1.5%. At levels of 2.0% or higher, C_1 activity was almost undetectable. The effects on C_x production was less dramatic, although enzyme activity declined if Solka-Floc was supplied at rates or more than 2.0%. In the case of both C_1 and C_x, maximum activity appeared in culture filtrates when 1.0% Solka-Floc was used. It was interesting to note that in spite of apparent low yields of C_1 at high cellulose concentrations, growth, as judged by visual inspection, appeared to be abundant. One could postulate that enzyme was produced in excess at low substrate levels, i.e., free enzyme accumulated after all binding sites were saturated. In the case of high substrate concentrations, however, all enzyme was bound to substrate, and no free enzyme remained. If this in fact occurred, binding forces were extremely strong, since little detectable C_1 could be produced by prolonged washing of mycelium and residual substrate, or by disruption in a Waring blender at high speeds. Mandels and Weber (11) reported that the optimum cellulose concentration for *T. viride* cellulase production varied from 0.5 to 1.0% depending on composition of the medium, and that higher concentrations of cellulose were inhibitory. Hulme and Stranks (8) noted that an environment which no longer favored balanced growth of *M. verrucaria* led to increased cellulase production. This phenomenon could be related to a need for deceleration of high growth rates for the induction of cellulases, particularly C_1. This view was supported by the finding that sporulation and cellulase production by *M. verrucaria* seemed to occur at about the same time. We also noted that whenever cellulase activity became apparent in cultures of *S. thermophile*, spores were present in large quantities, but at high substrate concentrations sporulation was minimal. It is well known that declining growth is often accompanied by the induction of sporulation in fungi. When excess substrate is present, one would assume that products of enzyme action are generated at a rate that allows maximum growth of the organism. This response, coupled with the possible accumulation of product, would tend to repress cellulase synthesis. Investigation of this phenomenon is continuing.

It was noted early in the study that the nitrogen source used in preparation of the culture medium appeared to greatly influence cellulase yield. A comparison of $NH_4H_2PO_4$, $NaNO_3$, NH_4NO_3, and urea, used at concentrations to supply equivalent amounts of nitrogen, showed that $NaNO_3$ and urea seemed to be most suitable for C_1 and C_x cellulase production (see Table 1). It was also found that varying the concentration of $NaNO_3$ between 0.05 and 0.4% had little effect on cellulase activity of filtrates of 72-h cultures (Fig. 4).

When *S. thermophile* was grown in MPM

TABLE 1. *Effect of nitrogen source on the cellulolytic activity of culture filtrates of Sporotrichum thermophile*[a]

N source	Mg reducing sugar per ml of culture filtrate[b]	
	C_1 cellulase	C_x cellulase
$NH_4H_2PO_4$	0.3	10.4
$NaNO_3$	9.9	13.6
NH_4NO_3	0.4	7.2
NH_2CONH_2	9.6	14.4

[a] Organism grown in modified Pettersson medium containing equivalent amounts of N supplied as the test compounds, at 45 C for 4 days.

[b] Data represent averages of assays for C_1 and C_x activity of filtrates from two replicate flasks, expressed as RS generated from Solka-Floc.

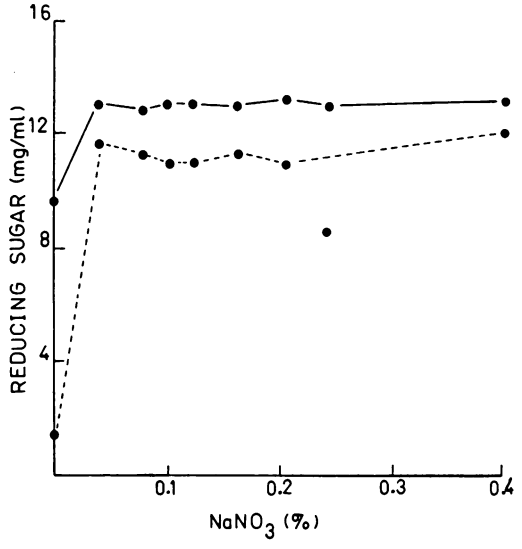

FIG. 4. *Effect of $NaNO_3$ concentration in MPM on the cellulolytic ability of filtrates of S. thermophile cultures. The organism was grown for 72 h at 45 C in media containing different concentrations of $NaNO_3$ as the inorganic source of nitrogen. Cellulase activity was expressed as RS formed in standard assays by 1.0 ml of culture filtrate. Data represent averages of determinations using samples from duplicate flasks. C_1 cellulase (●- - -●); C_x cellulase (●——●).*

with $NH_4H_2PO_4$ as inorganic nitrogen source, the pH invariably dropped to about 3.0 in 24 h. With $NaNO_3$, however, the pH rose to 7.0 or higher in the same time, and remained in the alkaline range throughout the incubation period. Growth of the organism at the higher pH values produced greater yields of both C_1 and C_x. To further investigate this finding, the pH of MPM containing $NH_4H_2PO_4$ was adjusted to 7.0 with 1.0 N NaOH after 24 h of growth of the fungus. Continued incubation for an additional 72 h showed that pH did not decline as usual (Table 2), and enzyme yields approached those obtained with $NaNO_3$. Thus, it appeared that nitrogen per se was not as important as it originally seemed to be for regulating yields of cellulase. Other workers have reported a similar effect with various nitrogenous compounds. Umezurike (22) showed that $NaNO_3$ was superior to ammonium salts for the production of cellulase by *Botryodiplodia theobromae*, and Hulme and Stranks (8) used $NaNO_3$ to prevent the rapid decrease in pH observed when ammonium salts were used for cellulase production by *M. verrucaria*. Alkaline or neutral pH values are not required for this function by all fungi, however, since accounts of highly cellulolytic culture filtrates produced under acidic conditions have been reported (11, 13). It has been suggested that thermophilic fungi are represented by two distinct ecophysiological groups based on pH tolerance: those commonly associated with composts, which function best at alkaline pH values, and those associated with soil but not compost, growing best below pH 6.0 (J. N. Hedger, personal communication). *S. thermophile* belongs to the former group. With respect to cellulose degradation by this organism, however, the apparent beneficial effect of alkaline pH may result from its adverse effect on growth rate. This was tested by growing *S. thermophile* in MPM buffered

TABLE 2. *Effect of pH adjustment of 24-h cultures of Sporotrichum thermophile on final cellulolytic activity of filtrates when $NH_4H_2PO_4$ is used as a source of N*[a]

N source	Culture pH adjusted[b]	Incubation time (h)	RS[c] (mg)		Final pH[d]
			C_1	C_x	
$NaNO_3$	–	72	10.2	13.6	7.2
(control)	–	96	9.9	13.6	7.5
$NH_4H_2PO_4$	–	72	0.0	10.8	3.0
	–	96	0.3	10.4	3.0
$NH_4H_2PO_4$	+	72	11.1	13.6	7.4
	+	96	11.1	13.6	7.4

[a] Organism grown in modified Pettersson medium, containing N in equivalent amounts supplied by $NaNO_3$ or $NH_4H_2PO_4$. Incubation temperature, 45 C.

[b] In one case, pH of replicate cultures was adjusted after 24 h of incubation to 7.0 with 1.0 N NaOH, then incubation was resumed.

[c] Results of standard assays for C_1 and C_x activity of culture filtrates. Data are averages for two replicate flasks, expressed as milligrams of reducing sugar produced from Solka-Floc.

[d] After incubation.

weakly at various pH values (see Materials and Methods), using D-glucose in place of Solka-Floc. Dry weight of the biomass was estimated after 4 days of incubation at 45 C, and pH of culture filtrates was determined. Table 3 shows that best growth occurred at an initial pH of 4.0, resulting in a final pH of about 7.0. At higher initial pH values, final pH was always greater than 7.0 and growth was inferior. Green pigments were produced when the pH became alkaline. Extrapolating to cellulolytic systems, it is probable that a decline in metabolic activity stimulates more cellulase production to maintain adequate growth rates. Paradoxically, *S. thermophile* cellulases function most efficiently at acidic pH values (Fig. 5).

Mandels and Weber (11) grew *T. viride* for 18 days at 29 C in a mineral salts medium containing proteose peptone. They reported that 1.0-ml samples of the culture filtrates produced about 2.0 mg of RS from filter paper in 1 h at 60 C. Somewhat lower yields of enzyme were produced if yeast extract was substituted for the peptone. If Tween 80 was added to the medium, about twice the cellulase activity was obtained. Using identical assay conditions, we found that filtrates of *S. thermophile* cultures, grown for 6 days at 45 C on MPM, produced 2.28 mg of RS in 1 h. We did not examine the effects of peptone or Tween 80 on enzyme production.

On the basis of these data, it can be concluded that one of the major differences between cellulases of *S. thermophile* M218 and those of mesophilic fungi appears to be their rapid rate of production. For example, yields comparable to those produced by *T. viride* in 14 to 18 days (11, 12) were obtained from *S. thermophile* in 3 to 4 days. With respect to optimum conditions for cellulolytic activity, however, the enzymes appear to be quite similar to those of mesophiles. The advantage of rapid production of cellulases is considered by the authors to be important enough to warrant further study of the role of *S. thermophile* in cellulose degradation.

TABLE 3. *Effect of pH on growth of Sporotrichum thermophile in weakly buffered media*[a]

Initial pH	Final pH[b]	Mean dry wt of biomass[c] (mg/g of glucose)
3.0	2.8	28
3.5	5.1	400
4.0	7.1	450
5.0	7.4	370
6.5	8.1	350
7.8	8.1	304

[a] Modified Pettersson medium containing $NaNO_3$ as the inorganic N source, and 1.0% D-glucose in place of Solka-Floc. Incubation time 4 days at 45 C. See text for buffer details

[b] After incubation.

[c] Averages of two replicate flasks.

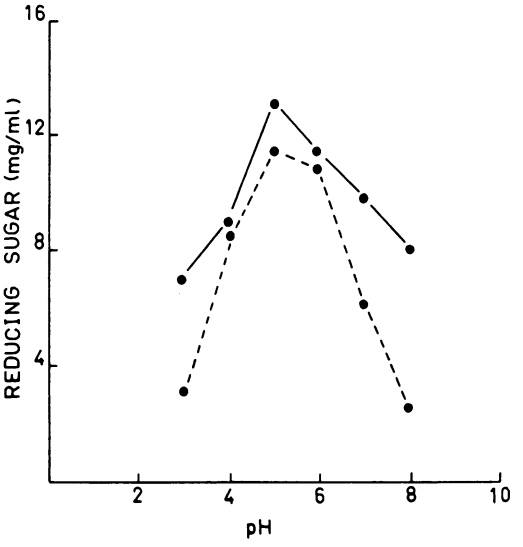

FIG. 5. *Effect of pH of assay systems on C_1 (●- - -●) and C_x (●——●) cellulase activity of culture filtrate. Enzyme activity expressed as mg of RS produced from substrate by 1.0 ml of filtrate under standard conditions. Citric acid-phosphate buffers were used in place of acetate buffer to adjust pH to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 in C_1 assays, and also for C_x assays. Molarities were maintained at 0.05 for C_1 and 0.055 for C_x. Data represent averages of duplicate tests. The culture was grown for 72 h at 45 C, in MPM containing $NaNO_3$ as the inorganic N source.*

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