**Sporotrichum thermophile** Growth, Cellulose Degradation, and Cellulase Activity

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The activity of components of the extracellular cellulase system of the thermophilic fungus *Sporotrichum thermophile* showed appreciable differences between strains; β-glucosidase (EC 3.2.1.21) was the most variable component. Although its endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) activities were markedly lower, *S. thermophile* degraded cellulose faster than *Trichoderma reesei*. The production of β-glucosidase lagged behind that of endoglucanase and exoglucanase. The latter activities were produced during active growth. When growth was inhibited by cycloheximide treatment, the hydrolysis of cellulose was lower than in the control in spite of the presence of both endoglucanase and exoglucanase activities in the culture medium. Degradation of cellulose was a growth-associated process, with cellulase preparations hydrolyzing cellulose only to a limited extent. The growth rate and cell density of *S. thermophile* were similar in media containing cellulose or glucose. A distinctive feature of fungal development in media incorporating cellulose or lactose (inducers of cellulase activity) was the rapid differentiation of reproductive units and autolysis of hyphal cells to liberate propagules which were capable of renewing growth immediately.

Thermophilic fungi are one of the major components of the microflora which develops in self-heating masses of vegetable matter where they contribute to decomposition of plant cell wall polysaccharides (4, 5). Tansey (27) found that the cellulolytic rates of some thermophilic fungi (*Chaetomium thermophile*, *Sporotrichum thermophile*, and *Thermoaecus auranticus*) were two or three times that of *Trichoderma viride*, one of the most cellulolytic mesophilic fungi (15). Romanelli et al. (20) reported that, of the three thermophilic fungi, *S. thermophile* degraded cellulose fastest in liquid shake cultures. Mandels (12) also noted that thermophilic fungi, including *S. thermophile*, degraded cellulose rapidly, but she found that the cellulase activity of their culture filtrates was low. This was repudiated by Tansey (M. R. Tansey, ASM News 45:417, 1979) on the grounds that suboptimal temperatures for growth and enzyme assays were used for the thermophilic fungi. Coutts and Smith (6) reported that *S. thermophile* produced cellulase yields comparable to those produced by *T. viride* and in one-fourth as much time.

In view of the contradictory reports regarding cellulase activity of *S. thermophile*, we have examined several strains of the fungus and made comparisons with *T. reesei*. We have also studied the interrelationship among growth, cellulose utilization, and cellulase production in shake flask cultures and features of growth and development when the fungus was grown under cellulase-inductive conditions.

**MATERIALS AND METHODS**

**Isolation of *S. thermophile***. The procedure used for isolation of the fungus from soil has been described by Maheshwari et al. (11). Strains were maintained on YpsS agar (5) slants at 40°C.

**Cultivation techniques**. Batch cultures of *S. thermophile* in a medium containing cellulose were prepared as follows.

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Spore suspensions were obtained by harvesting growth on YpsS agar medium in bottles (12 by 5 by 3 cm) which had been incubated at 40°C for 10 to 15 days in a humidified incubator. Mycelial inocula were prepared by incubating a spore suspension in a glucose-ammonium dihydrogen phosphate medium (pH 6.0) which contained, per liter: glucose, 10.0 g; NH₄H₂PO₄, 2.0 g; KH₂PO₄, 3.0 g; K₂HPO₄, 2.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.1 g; FeSO₄·7H₂O, 5.0 mg; MnSO₄·4H₂O, 1.6 mg; ZnSO₄·7H₂O, 1.4 mg; CoCl₂·6H₂O, 2.0 mg; yeast extract, 1.0 g; and peptone, 1.0 g. The fungus was grown in 150 ml of medium in a 500-ml Erlenmeyer flask at 50°C for 24 h on a gyratory shaker. Finally, the cellulose cultures were initiated by adding 5 ml of the homogeneous mycelial suspension to the above medium in which cellulolic material (1%) replaced glucose. The cellulolic substrate was blotting paper (manufactured mainly from bamboo pulp). This was pretreated with alkali before use: 20 g was autoclaved with 360 ml of 1% NaOH for 1 h, washed free of alkali, blended in distilled water, and dried at 70°C. In some experiments untreated Whatman no. 1 filter paper, cellulose powder, or absorbent cotton was also tested.

*T. reesei* was grown in the medium of Mandels and Sternberg (14), containing the cellulose described above, at 26 to 28°C on a gyratory shaker (240 rpm). When stated, batch cultures of *T. reesei* and *S. thermophile* were also initiated by using 5 ml of a conidial suspension containing approximately 10⁹ spores ml⁻¹.

**Enzyme assays**. Unless stated otherwise, all assays of *S. thermophile* enzymes were carried out in sodium acetate buffer (pH 5.6) at 50°C for 30 min, which were optimal conditions. In some experiments the cellulolytic activity of culture filtrates was assayed by the release of glucose from filter paper. A reaction mixture (1 ml) containing 50-μg Whatman no. 1 filter paper (1 by 6 cm) was incubated with 0.1 ml of culture filtrate, and the glucose released was quantified by the glucose oxidase-peroxidase method (16).

Filter paper activity was assayed according to Mandels et al. (13), using 0.5 ml of culture filtrate in a 1.0-ml reaction mixture. After 1 h of incubation, reducing sugar was esti-
mated by the dinitrosalicylic acid method (17). The activity, calculated as reducing sugar (milligrams of glucose) × 0.185 was expressed as filter paper units (13). Endoglucanase (EC 3.2.1.4) activity was determined on sodium carboxymethyl cellulose (medium viscosity), using 0.5 ml of 1% (wt/vol) substrate in a 2-ml reaction mixture. The liberation of reducing end groups was measured by the Nelson-Somogyi method (25). Exoglucanase (EC 3.2.1.91) activity was determined on microcrystalline cellulose (Sigma; Chemical Co.) pretreated by boiling for 30 min in 1 M HCl (to remove any frayed ends in particles) and washed free of acid. The reaction mixture (2 ml) contained 1 ml of a 5% suspension of the substrate. The reducing sugar was measured by the Nelson-Somogyi method. β-Glucosidase (EC 3.2.1.21) activity was assayed by using p-nitrophenyl β-D-glucopyranoside. The reaction mixture (1 ml) contained culture filtrate and 0.5 ml of 1 mM substrate solution. The reaction was stopped by adding 2 ml of 5% Na₂CO₃, and the absorbance of the solution was measured at 400 nm. Cellobiase activity was measured by estimating the glucose produced from the hydrolysis of cellobiase.

For T. reesei, cellulase assays were carried out as described above except 0.05 M sodium citrate buffer, pH 4.8 (13), was used.

Reducing sugar. Reducing sugar in culture fluids was determined colorimetrically by the dinitrosalicylic acid method (17), with glucose as standard.

Cellulose estimation. For studying the utilization of cellulose during growth, a 10-ml culture mass (mycelia plus cellulose) was removed from duplicate flasks and filtered through a Whatman glass fiber circle (2.5 cm) by suction. The insoluble material was washed with distilled water and dried at 70°C. Cellulose in the material was estimated by the method of Ullrich (28). The 6-day-old mycelial growth permitted sampling with a broad-tipped pipette.

Growth measurement. Growth of the fungus was followed as the increase in insoluble nitrogen by the Kjeldahl method or as an increase in mycelial dry weight. The latter was determined by subtracting the cellulose content from the weight of the dried insoluble material. Specific growth rate (μ) in media incorporating cellulose material or soluble sugar was determined from the exponential portion of semi-logarithmic plots of growth curves and calculated as follows:

μ = \[(\log x₂ - \log x₁)\]h⁻¹[\(t₂ - t₁\)], where x₂ and x₁ are mycelial protein contents at times t₂ and t₁, respectively.

Samples (5 ml) of culture were removed from duplicate flasks at 3-h intervals, filtered through glass fiber, and washed three times with 10 ml of distilled water. The insoluble material was extracted with 5 ml of 0.5 M NaOH at 90°C for 30 min. The extract was clarified by centrifugation and protein was measured by the Lowry method (10).

Cellulase preparation. Culture suspensions from 6-day-old cultures were filtered through glass wool. Culture filtrate protein was precipitated with 80% saturated ammonium sulfate, desalted by chromatography on a Sephadex G-25 column, and lyophilized before use.

Saccharification and solubilization of cellulose. The lyophilized cellulase powder was dissolved in 50 mM sodium acetate buffer (pH 5.6) and the solution was sterilized by membrane filtration (0.45-μm pore size; Millipore Corp.). Suspensions of cellulosic material (1%) were incubated with the cellulase preparation in sterile Sporotrichum medium at 50°C on a shaker. At intervals, aliquots of the reaction mixture were removed and glucose was estimated (16). The residual cellulose was estimated gravimetrically. Percent saccharification was calculated as follows: [milligrams of reducing sugar × (162/180) × 100]/(milligrams of initial substrate). In this calculation, the factor 162/180 normalizes the conversion for the weight gain caused by addition of water to the glycosyl moiety on hydrolysis.) Percent solubilization was calculated as follows: [(milligrams of total cellulose − milligrams of remaining cellulose) × 100]/milligrams of total cellulose.

Light microscopy. For examination of fungal growth and structural changes in cellulosic material during culture, samples were mounted in lactophenol and examined by phase-contrast and polarized-light microscopy.

Quantitative expression of results. All estimations were made on samples pooled from duplicate or triplicate culture flasks. Experiments were repeated at least once and results were quite reproducible. Data are average or typical values from 2 to 3 experiments.

Biochemicals. Biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Yeast extract and malt extract were from Difco Laboratories, Detroit, Mich. Peptone and cellulose powder were from Hindustan Dehydrated Media, Bombay, India. Blotting paper was purchased from a local paper merchant and the same lot was used throughout.

RESULTS

Morphological characteristics of S. thermophile. Most isolates were buff or cinnamon brown and powdery (e.g., IIS 72 and IIS 101), but a few isolates were white and floccose (e.g., IIS 125 and IIS 220). Despite visible differences, the cultures resembled one another in conidiophore morphology (Fig. 1) and conformed to the description of S. thermophile Apinis given by Semeniuk and Carmichael (23).

Cellulase activity in different strains. Eleven strains were grown in a cellulose-NH₄H₂PO₄ medium to determine their cellulolytic potential and production of extracellular cellulase enzymes and to examine possible differences in their cellulase systems. Microscopic examination of cultures (day 6) showed absence of cellulose particles. The final (day 6) cellulase activity (measured as release of glucose from filter paper) varied from 0 to 0.16 U ml⁻¹.

Four strains, IIS 101, IIS 220, ATCC 42464, and UAMH 2015, were compared for activity of endoglucanase, exoglucanase, and β-glucosidase. Two of these strains, IIS 101 and IIS 220, were selected because their culture filtrates produced, respectively, the lowest and highest amounts of glucose in the above cellulase assay. Strain ATCC 42464 was investigated because it was reported to lack extracellular β-glucosidase activity (3). UAMH 2015 was included because this strain was reported to produce cellulase activity comparable to that of T. viride (6). The activities of endoglucanase and exoglucanase between strains varied 1.4- to 2-fold, respectively, while that of β-glucosidase varied up to 5.3-fold (Table 1). The filter paper activity of the most productive strain, IIS 220, was comparable to that of UAMH 2015 but was <10% of that reported for T. viride 9414 (14).

Additional differences in the cellulase systems of isolates were found when two strains, IIS 101 and IIS 220, were
FIG. 1. *S. thermophile*, phase-contrast micrographs. (A) Strain IIS 72, young conidiophores. (B) Strain IIS 72, mature conidiophores. (C) Strain IIS 220, dendritic branching of a fertile hypha and conidiophores. (D) Strain IIS 220, mature conidiophores. (E) Strain IIS 220, prostrate hyphae bearing lateral conidia. Bars, 50 μm.

TABLE 1. Activities of primary cellulase components in some strains of *S. thermophile*<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular protein (mg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Filter paper activity</th>
<th>Endoglucanase</th>
<th>Exoglucanase</th>
<th>β-Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIS 101</td>
<td>0.07 ± 0.002</td>
<td>0.05 ± 0.01</td>
<td>0.85 ± 0.18</td>
<td>0.09 ± 0.03</td>
<td>0.31 ± 0.09</td>
</tr>
<tr>
<td>IIS 220</td>
<td>0.17 ± 0.005</td>
<td>0.11 ± 0.01</td>
<td>1.20 ± 0.09</td>
<td>0.18 ± 0.01</td>
<td>1.65 ± 0.22</td>
</tr>
<tr>
<td>ATCC 42464</td>
<td>0.13 ± 0.003</td>
<td>0.07 ± 0.006</td>
<td>1.05 ± 0.08</td>
<td>0.10 ± 0.03</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>UAMH 2015</td>
<td>0.41 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>1.00 ± 0.10</td>
<td>0.16 ± 0.02</td>
<td>0.45 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> All strains were grown in cellulose-NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> medium for 6 days.
Table 2. Cellulase activities of *S. thermophile* IIS 110 and IIS 220 in media containing soluble and insoluble inducers

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Strain</th>
<th>Exocellular protein (mg ml⁻¹)</th>
<th>Filter paper activity</th>
<th>Endoglucanase</th>
<th>Exoglucanase</th>
<th>β-Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>IIS 101</td>
<td>0.01 ± 0.005</td>
<td>0.03 ± 0.00</td>
<td>0.03 ± 0.005</td>
<td>0.02 ± 0.005</td>
<td>0.23 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>IIS 220</td>
<td>0.07 ± 0.00</td>
<td>0.08 ± 0.01</td>
<td>0.39 ± 0.005</td>
<td>0.12 ± 0.005</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>Cellulose</td>
<td>IIS 101</td>
<td>0.07 ± 0.02</td>
<td>0.07 ± 0.008</td>
<td>0.89 ± 0.07</td>
<td>0.10 ± 0.01</td>
<td>0.16 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>IIS 220</td>
<td>0.19 ± 0.01</td>
<td>0.12 ± 0.007</td>
<td>1.44 ± 0.09</td>
<td>0.22 ± 0.01</td>
<td>1.29 ± 0.05</td>
</tr>
</tbody>
</table>

* Cultures were grown in media containing 1% lactose or 1% cellulose (blotting paper, Whatman filter paper, cellulose powder, or absorbent cotton). The data for cellulases are average values of enzyme activities on all types of cellulosic substrates tested.

grown in media containing lactose or cellulosic materials as soluble (present observation) and insoluble inducers of cellulase, respectively (Table 2). Strain IIS 220 secreted more protein than IIS 101, 7- and 2.5-fold more in lactose and cellulose media, respectively. The enhanced protein secretion was reflected in the increased absolute levels of all enzymes. Microscopic examination of cultures showed that practically complete conversion of cellulose had occurred by day 2 in cultures of both strains. A qualitative difference in the composition of the cellulase system was also revealed; the specific activities of endoglucanase and exoglucanase in cellulosic media in IIS 101 were 1.7- and 1.2-fold higher, respectively, than in IIS 220. The ratio of endoglucanase to exoglucanase activity was 1.5 in IIS 101 and 3.3 in IIS 220 in lactose-grown cultures and 8.9 in IIS 101 versus 6.5 in IIS 220 in cellulose-grown cultures.

**Cultural conditions for cellulose degradation.** Microscopic examination at 12-h intervals of cultures of some strains tested (IIS 72, IIS 125, IIS 220, and ATCC 42464) showed that degradation of cellulose in shaken flasks also occurred during growth at 30°C, although slowly. Some cellulose particles remained insolubilized in the culture flasks. In contrast, in parallel cultures at 50°C, virtually no trace of cellulose particles remained by 48 h. Sporulation was increased at 30°C. The filter paper activity (units per milliliter) in cultures grown at 30°C for 6 days ranged from 0.08 to 0.10, whereas at 50°C it ranged from 0.10 to 0.13.

Parallel cultures of the above four strains in stationary flasks at 30 and 50°C showed the development of a white mycelial mat on the surface of the liquid. Appreciable amounts of cellulose remained insolubilized until day 12. This indicated that adequate aeration of cultures was essential for the rapid and complete degradation of cellulose.

*S. thermophile versus T. reesei.* Strain IIS 220 was the best cellulase producer among *S. thermophile* strains tested (Table 1). Its cellulolytic activity was compared with that of *T. reesei* (Fig. 2).

Growth curves based on estimates of soluble nitrogen or mycelial dry weight in culture flasks containing cellulosic material were almost identical in *S. thermophile* IIS 220 (data not shown). The agreement between growth parameters in *T. reesei* was also good, except that after maximal growth (day 4) the growth curve based on mycelial dry weight remained stationary with culture age whereas that based on mycelial nitrogen content showed a small decline before becoming stationary. Both fungi degraded the cellulolytic substrate completely, but their cellulolytic behavior differed in a number of ways. *S. thermophile* showed a faster growth rate and a higher culture density than *T. reesei.* Whereas the time of maximal growth and complete cellulose conversion coincided in *S. thermophile*, in *T. reesei* they did not. Estimations revealed that 20 to 40% cellulose remained at the time when the culture density of *T. reesei* attained a maximum value (days 4 to 5). In *S. thermophile*, soon after maximal growth was attained, the culture density declined sharply and was reduced by 50% at day 4. In contrast, the culture density of *T. reesei* showed a slower and smaller reduction with time.

*T. reesei* was superior to *S. thermophile* in terms of extracellular protein, endoglucanase, and exoglucanase activities (Table 3). However, in spite of its lower titer of cellulase in the culture broth, the thermophilic fungus achieved a complete conversion of the cellulosic substrate at a faster rate than *T. reesei.*

**Growth and development in cellulosic media.** Microscopic examination showed that the cellulose substrate (blotting paper) was composed of fibrous and, to a lesser extent, parenchymatous cell types (Fig. 3A). A 24-h culture of *S. thermophile* initiated with mycelial inoculum or spores showed a mass of mycelia (Fig. 3B). The parenchymatous cells had totally disappeared, whereas cellulose fibers had become extensively fragmented. The mycelial growth at this time appeared cream-yellow and the hyphal cells were filled with protoplasm. Samples taken from a 48-h culture (Fig. 3C) contained negligible cellulose particles. Hyphal cells...
were long and vacuolated and the mycelia appeared brittle. Cultures at 72 h of all strains examined invariably showed a visible transformation; the culture had turned greenish-brown and had become thin in consistency. The mycelia had become fragmented (Fig. 3D), and some had spores attached to them. In 96-h cultures, some of the hyphal fragments had renewed growth ("secondary growth phase") by protruding fine, unbranched hyphae of limited length (Figs. 3E and F), presumably because nutrients were exhausted. Asexual reproduction by differentiation of conidia was more abundant in cultures of IIS 101 and ATCC 42464 than in IIS 220. In these cultures, the secondary growth phase was largely composed of germinating conidia.

**Time of cellulase secretion.** An attempt was made to determine the crucial time during which the amount of cellulase enzymes required for cellulose hydrolysis was formed. The approach taken was to inhibit growth at different ages by the addition of cycloheximide, an inhibitor of eucaryotic protein synthesis.

Figure 4A shows the pattern of growth, cellulose utilization, and cellulase (endoglucanase) activity in control flasks of *S. thermophile*. The appearance of cellulase activity in culture broth lagged behind growth apparently because the enzymes were adsorbed on the culture substrate, a well-established phenomenon. In the second set of culture flasks (Fig. 4B) to which cycloheximide was added at 12 h and then again at 12-h intervals, there was neither measurable growth nor cellulose utilization or cellulase production. When cycloheximide additions were begun after 24 h (Fig. 4C), the growth was arrested and the utilization of cellulose proceeded slowly. However, the activities of cellulase enzymes were detected later when approximately 75% cellulose had been solubilized. In the fourth set of culture flasks to which cycloheximide addition was begun at 48 h (Fig. 4D), cell density and cellulose utilization were comparable to those of the control. The exoglucanase (not shown) and endoglucanase activities, however, were 60 and 70% those of the control. From the above observations, the interval between 12 and 24 h appeared to be the crucial time for conversion of the bulk substrate.

**Growth on soluble and insoluble carbon sources.** The specific growth rate and the cell density (mycelial protein) of *S. thermophile* IIS 220 on glucose were marginally higher than on cellulose (blotting paper) (Table 4). Other cellulose substrates such as Whatman filter paper or cellulose powder were also extensively degraded but an appreciable amount of cotton remained in fibrous form. Whether the utilization of a given type of cellulotic material was complete or not, the final cellulase activity of the culture broths was in the range of 0.10 to 0.14 filter paper units ml⁻¹, showing that the cellulase enzyme was induced to similar extents by all types of cellulotic materials. In contrast, culture filtrates of *S. thermophile* grown on glucose, maltose, sucrose, starch, or sodium carboxymethyl cellulose as carbon source did not possess filter paper cellulase or β-glucosidase activities.

Contrary to that in *S. thermophile* ATCC 42464 studied by Canevascini et al. (2), cellobiose did not induce these enzyme in strain IIS 220. Cell-associated cellobiose activity was detected, but the specific activity of the enzyme in mycelia grown with glucose or cellobiose was low (0.13 and 0.8 U mg of protein⁻¹, respectively) compared to that in cellulose- or lactose-grown mycelia (4.7 and 5.0 U mg of protein⁻¹, respectively).

Under noninductive conditions, with either glucose or cellobiose as carbon source, the mycelia remained in a vegetative state and lysis did not occur until day 10. In contrast, under inductive conditions, with either cellulose (blotting paper, Whatman filter paper, and cellulose powder) or lactose as the carbon source, the mycelia formed spores and autolysis of hyphal cells occurred after 48 h, as described before. Mycelial growth on cotton, however, remained in a vegetative state up to day 10, when the experiment was terminated. It was noted that in cellulotic media the level of soluble sugars at all times was very low, indicating that cellulose hydrolysis and uptake of hydrolys products occurred concurrently.

**Cellulolytic activity of culture filtrates.** In experiments to determine cellulolytic activity, the same type (blotting paper) and quantity (1%) of substrate was incubated with the quantity of cellulase estimated to be secreted by *S. thermophile*. For example, on day 6 the total cellulase activity (glucose produced ml⁻¹ min⁻¹) in 100 ml of culture filtrate was nearly 10 U. However, hydrolysis of cellulose by cellulase preparations containing 20 U was only 51%. The results were similar when hydrolysis of cellulose was performed with an amount of cellulase preparation equivalent to the protein secreted in culture broth (18 mg 100 ml⁻¹). The same quantity of cellulase preparation, based on enzyme unit or protein value, hydrolyzed Whatman filter paper to 21 to 25%, although this substrate was also solubilized nearly completely under cultural conditions.

Cellulase may be inactivated under in vitro conditions, whereas it is renewed continuously by the growing fungus in vivo. The effect of a single dose and of multiple doses of the same quantity of cellulase (20 U) was investigated. The initial rate of cellulose hydrolysis was slower when cellulase was added intermittently in portions at 12-h intervals (data not shown). However, the extent of hydrolysis of cellulose after 120 h by both treatments was comparable (70%).

The difference in solubilization of cellulose under in vitro conditions could be due to the accumulation of soluble products, which would be continuously removed by assimilation. However, glucose, the principal product of cellulose hydrolysis by culture filtrates of *S. thermophile* IIS 220 in 24 h (determined by paper chromatography of reaction mixtures), even at 1% caused only 14% inhibition of cellulose solubilization after 96 h, as determined by gravimetric estimation. Therefore, end-product inhibition was discarded as the primary reason for the weak activity of culture filtrates. The results of these experiments, using rather large quanti-
FIG. 3. Growth and development of *S. thermophile* II 220 in medium containing blotting paper in shake cultures examined by phase-contrast microscopy (except panel A). (A) 0 h; initial appearance of cellulosic substrate under polarized light optics. (B) 24 h; primary growth phase consisting of vegetative mycelium. Extensive fragmentation and solubilization of cellulose has occurred. Some insoluble cellulose particles (†) are visible. (C) 48 h; cellulose particles are no longer visible. Hyphae with vacuolated cells. (D) 72 h; fragmentation of mycelia. (E) 96 h; secondary growth phase. Some separated hyphal cells have renewed growth by protruding fine hyphae at one or both ends (†). (F) 96 h; cells showing renewed growth (†) at higher magnification. Bars, 50 μm.
Glucosidase that produce endoglucanase activity (more than in physiological concentrations), showed that culture filtrates, unlike the organism itself, brought about only a modest conversion of cellulose.

**DISCUSSION**

Quantitative and qualitative differences, comparable to those observed in some laboratory-produced mutants of *Trichoderma* sp. (24), were found in *S. thermophile* strains. Extracellular β-glucosidase was the principal variable component of the cellulase system in the latter fungus. It appears that the production and levels of this enzyme are strongly influenced by cultural conditions. For example, Canavescini and Meyer (3) found that *S. thermophile* ATCC 42464 did not produce extracellular β-glucosidase. The level of β-glucosidase in an enzyme preparation strongly affects cellulase assays, in particular, the assay of filter paper activity (1, 9). It was therefore necessary to compare the cellulase productivity of different strains of *S. thermophile* by using a uniform cultural regimen and assay procedure to obtain a true representation of cellulase activity of this fungus.

In spite of its distinctly lower cellulase activity, *S. thermophile* grew more rapidly and degraded cellulose faster than the hyperproducing cellulase mutant *T. reesei* (Table 3). Such behavior has also been reported for the anaerobic bacterium *Clostridium thermocellum* (19). The substrate utilization rate of *S. thermophile* is approximately 2.5 times faster than that of *C. thermocellum* (18). In spite of differences in the cellulase composition among strains, they degraded cellulose at the same rate, as judged by microscopic examination of cultures. These observations raise the question as to whether the levels of cellulase produced are of prime importance in determining the rate or extent of cellulolysis. This point was also brought out by the inability of enzyme preparations from *S. thermophile* or from some other cellulolytic microorganisms (22, 26) to extensively hydrolyze cellulose.

Few attempts have been made to determine the precise relationship among growth, cellulase production, and cellulose utilization. Presumably because of the tedium of measuring growth on an insoluble substrate, investigators have used certain soluble inducers (7, 21) and the experimental results have been interpreted to show that cellulase formation is not directly correlated with mycelial growth, but the enzymes are produced during idiophase. We have found that a close interrelationship exists between cellulase production and cellulose utilization during the trophophase, in both *S. thermophile* and *T. reesei*. Therefore, unlike lignin degradation by the white-rot fungi (8), cellulose degradation is not temporally separated from primary growth. The active growth of the fungus is crucial in cellulolysis. When growth was inhibited (Fig. 4), cellulolysis remained weak, although cellulase enzymes were present in culture broth. Vaheri (29) proposed the participation of an oxidative reaction which is believed to disrupt the hydrogen bonds in crystalline cellulose, rendering it susceptible to attack by endogluccanase. He found that this activity was associated with cell wall in young cells of *T. reesei* in both induced and noninduced conditions. Thus, activities associated with growing cells appear to play a crucial role in the degradation of crystalline cellulose.

Growth of fungi on a polysaccharide is simply viewed as a problem of converting the substrate into soluble sugars for transport within the mycelium for biosynthesis. The observations in this paper illustrate that the physiology of the mycelium is influenced differently by a polymeric substrate and its depolymerized forms. A distinctive feature of development of *S. thermophile* on cellulosic substrates was the precocious differentiation of reproductive units and exten-

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**TABLE 4. Some characteristics of the growth of *S. thermophile* in media containing soluble and insoluble carbon sources**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Specific growth rate (h⁻¹)</th>
<th>Mycelial protein (mg 10 ml⁻¹)</th>
<th>Extracellular (mg ml⁻¹) content of:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
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<td>Glucose</td>
<td>0.10</td>
<td>0.75₁</td>
<td>0.00</td>
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<td>Cellobose</td>
<td>ND⁺</td>
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<td>Lactose</td>
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<td>0.17</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.09</td>
<td>0.72₂</td>
<td>0.03</td>
</tr>
</tbody>
</table>

₁ Carbon sources were present at 1% protein, and reducing sugars in culture filtrates were estimated after 144 h.
₂ Sampled after 24 h.
⁻ ND, Not determined.
₂ Sampled after 28 h.

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FIG. 4. Effect of cycloheximide addition on growth, cellulose utilization, and cellulase production in *S. thermophile* IIS 220. (A) Control. Cycloheximide (20 μg ml⁻¹) added after 12, (B), 24 (C), and 48 (D) h. Cycloheximide (20 μg ml⁻¹) was readded at 12-h intervals following the initial addition (indicated by arrow). Cultures were initiated with a uniform spore inoculum. Estimations were done on pooled samples from duplicate flasks. Symbols: A, growth; O, percent cellulose utilized; △, endoglucanase. The pattern of exoglucanase activity (not shown) was similar to that of endoglucanase activity.

**CELLULOSE DEGRADATION BY *S. THERMOPHILE* 2181**
sive autolysis of hyphal cells to liberate propagules capable of
renewing growth immediately. This type of development
was reflected in the rapid reduction in cell density with time;
the initial dense mycelium which filled the liquid was re-
duced to a thin mass of propagules which, on standing,
settled at the bottom of the culture flask. This observation
shows the strong stimulus that cellulolic substrates can have
on reproduction by fungi. The observation may serve to
explain how vast quantities of vegetable matter are decom-
posed in nature but with only a sparse accumulation of
fungal mycelia.

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