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Studies on Thermophilic Cellulolytic Fungi

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Three thermophilic cellulolytic fungi, *Chaetomium thermophile* var. *coprophile*, *Sporotrichum thermophile*, and *Thermoascus aurantiacus* were studied to determine the conditions for a high rate of cellulose degradation. The range of temperature over which good growth occurred was determined first in a temperature gradient incubator; the optimum temperature was then established in shake flask cultures. *T. aurantiacus* had the highest optimum growth temperature range (46 to 51 C), whereas *S. thermophile* had the broadest range over which good growth occurred (36 to 43 C). Optimum temperatures for the three organisms, *T. aurantiacus*, *S. Thermophile*, and *C. thermophile* were 48, 40, and 40 C, respectively. It was found that the addition of an organic carbon and nitrogen source to a cellulose mineral solution medium markedly increased the rate of cellulose degradation. The surfactant, Tween 80, which has been reported to be of value in the production and recovery of the enzyme, cellulase, was shown to be detrimental to the degradation of cellulose in culture. In the medium used, *S. thermophile* gave the highest rate of substrate utilization; 56% of the cellulose was hydrolyzed in 72 h. The average degree of polymerization of cellulose decreased from 745 to 575.

Cellulose, the major component of all vegetation, is one of the world's most plentiful resources. Unlike other resources, such as oil and minerals, cellulose is constantly replenished by photosynthesis and growth of plants; it accounts for nearly one-half of the 18 to 20 metric tons of organic carbon that is fixed by photosynthesis each year (4). Cellulose is also the major component of municipal solid waste. It constitutes 40 to 60% of the solid waste in American cites and is generated at the rate of 1.5 to 3.5 pounds (ca. 681 to 1589 g) per person per day (1, 3, 7). Additionally, vast quantities of this material are produced from such activities as food processing, lumbering operations, paper making, cereal grain harvesting, and sugar cane processing.

Only a small fraction of cellulose accumulates, since most of it is converted to carbon dioxide by biological oxidation or combustion and returned to the atmosphere. Microbiological degradation is the major cause of cellulose disappearance. Attempts to utilize cellulose as a commercial fermentation substrate, however, have been disappointing. The low rate of hydrolysis by mesophilic microorganisms has been cited as one of the chief obstacles to microbiological conversion (4). Tansey (13, 14) showed that cellulolytic activity of a number of thermophilic species was several times that of the most active cellulolytic mesophiles known. The studies reported here were concerned with optimizing the growth conditions for three highly cellulolytic thermophiles.

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MATERIALS AND METHODS

Organisms. The organisms used were *Chaetomium thermophile* var. *coprophile* QM-9381, *Sporotrichum thermophile* QM-9382, and *Thermoascus aurantiacus* QM-9383. These organisms have been reported (13, 14) to be two to three times as active in hydrolyzing cellulose as one of the most vigorously cellulolytic mesophiles, *Trichoderma viride* QM-6a, which was isolated and studied by Mandels and Weber (10). They were obtained from the Mycology Group of the United States Army Natick Laboratories, Natick, Mass.

Media. The following media were used in this study: yeast glucose broth, which consisted of 0.5% yeast extract, 0.5% peptone, 0.5% glucose; yeast glucose agar, which had the same composition with 1.5% agar; Sabouraud dextrose broth (Difco); Sabouraud dextrose agar (Difco); a cellulose medium prepared by adding 1.5% Solka-Floc BW-200, a purified spruce pulp (Brown Co., Berlin, N.H.), to a mineral solution which contained in grams per liter, KH_2PO_4,

2.0, $(NH_4)_2SO_4$, 1.4, $CaCl_2 \cdot 2H_2O$, 0.3, $MgSO_4 \cdot 7H_2O$, 0.3, and in milligrams per liter $FeSO_4 \cdot 7H_2O$, 5.0, $CoCl_2 \cdot 6H_2O$, 2.0, $MnSO_4 \cdot H_2O$, 1.6, $ZnSO_4 \cdot 7H_2O$, 1.4. Solka-Floc is a readily available cellulose of uniform nature which permitted reproducibility of the medium. Because of its crystalline nature, it is considered less subject to attack than other forms of cellulose and therefore is of special interest. The cellulose medium was supplemented in several experiments with proteose peptone (Difco), 0.075%; urea, 0.03%; and Tween 80 (Difco), 0.05%.

Inoculum. Inoculations were made with conidia of *S. thermophile* grown on yeast glucose agar at 37 C or with ascospores of *T. aurantiacus* produced on Sabouraud dextrose agar at 48 C. *C. thermophile* was grown on Sabouraud dextrose agar at 37 C, and a suspension of hyphal fragments and ascospores was prepared for the inoculum, since ascospores were not produced in abundance on this medium.

Equipment. A model TGI temperature gradient incubator (Scientific Industries, Inc., Mineola, N.Y.) was used to determine the growth temperature range of the fungi. The incubator consists of a block of aluminum which is provided with two rows of 30 holes on 1 3/16-inch (ca. 3.02 cm) centers bored to accommodate tubes 1 1/16 inch (ca. 1.65 cm) outer diameter. A liquid in the block assists in obtaining and maintaining a temperature gradient between the heated and cooled end of the block, which is kept in motion by a gentle rocking action. In addition to the thermocouples at each end and at the midpoint of the block, a movable probe is provided so that the temperature in any tube can be monitored.

The L-shaped culture tubes are 7 to 12 inches (ca. 17.78 to 30.48 cm) long, and are fitted with a side arm 1 inch (2.54 cm) from the open end. Positioning the side arm vertically in a spring steel clip on the gradient block secures the tube and holds it in place during incubation. Since the end of the tube is normally sealed with a polypropylene cap, the side which is stoppered with cotton is also used for inoculation.

An environmental incubator shaker, model G-25 (New Brunswick Scientific Co., New Brunswick, N.J.) was used for shake flask cultures.

Determination of cardinal temperatures. The temperature gradient incubator was used in preliminary studies of growth temperatures to determine the growth temperature range and a range of heavy growth. Slants of selected medium were prepared in the L-shaped tubes, inoculated uniformly and placed promptly in the gradient incubator. The cultures were observed at 24-h intervals; final readings of minimum and maximum growth temperatures and the temperature range of heavy growth were made visually at 72 h.

Determination of optimum temperature for cellulose utilization. The optimum temperature for cellulose utilization was determined in the environmental incubator shaker. For the shake flask cultures, 0.5000-g quantities of Solka-Floc BW-200 were weighed and transferred to 250-ml Erlenmeyer flasks containing 50.0 ml of the mineral solution. Temperatures within the range giving heavy growth in the temperature gradient incubator were selected for study.

Monitoring of cultures. All shake flask cultures were monitored for pH. Residual cellulose, fungal growth, protein production, and change in the degree of polymerization of cellulose were determined at the end of each run.

Determination of residual cellulose and fungal growth. Four cultures which had been prepared in an identical manner were necessary for the determination of residual cellulose and fungal growth. The total mass of two cultures was filtered on tared Whatman no. 1 filter paper, washed with distilled water, and dried to constant weight at 105 C. Subtraction of residual cellulose weight (determined on separate duplicate samples) from the net weight of the total culture mass yielded fungal growth.

For the determination of residual cellulose, the two identical cultures were transferred to 50-ml tubes and centrifuged for five min at 5,000 rpm. The centrifugates (the fungal tissue and residual cellulose combined) were washed twice with distilled water. One of three procedures was then used. (i) The semimicro method of Updegraff (15) using an acetic-nitric acid extraction of the homogenized centrifugate to remove lignin, hemicellulose, and xylans, and subsequent colorimetric determination of cellulose utilizing anthrone reagent. The residual cellulose was completely solubilized in this determination. (ii) Fifty milliliters of 1 N sodium hydroxide was added to the centrifugate which was then heated in a boiling water bath for 5 min to digest the fungus, cooled in an ice bath for 2 min, centrifuged for 5 min at 5,000 rpm, filtered on tared Whatman no. 1 filter paper, washed with distilled water, and dried to constant weight at 105 C. (iii) Fifty milliliters of 1 N sodium hydroxide was added to the centrifugate which was homogenized for 2 min in a Virtis tissue homogenizer. The suspension was then shaken for 2 h at 150 rpm, at 40 C in an environmental incubator shaker to digest the fungus, filtered on tared Whatman no. 1 filter paper, washed with distilled water, and dried to constant weight at 105 C. The three methods of determining residual cellulose were compared; the results are summarized in Table 1. There was good agreement between the semimicro method and the cold alkali treatment. The results of these two methods were consistently lower

TABLE 1. *Residual cellulose in 48- and 72-h shake flask cultures of Sporotrichum thermophile as determined by three methods*[a]

Method	Residual cellulose (mg/ml)	
	48 h	72 h
Semimicro determination[b]	6.40	4.63
Hot alkali treatment	7.16	4.84
Cold alkali treatment	6.48	4.64

[a] Original cellulose concentration: 10 mg/ml.
[b] Reference 15.

than those obtained with the hot alkali treatment. Homogenization was, therefore, a necessary step since it assisted in rupturing the fungal material and allowed a more complete extraction.

Determination of protein. The biuret method of Robinson and Hogden (12) as modified by Herbert et al. (9) was used to determine the protein of the washed, collected fungal tissue. Bovine serum albumin was used as a standard.

Viscosity determination. The filtered cellulose from the residual cellulose determination was air dried and used to obtain intrinsic viscosity using a cupriethylenediamine viscosity test, ASTM designation: D 1795-62 (2). The Mark Houwink equation (5) relates cellulose degree of polymerization (DP) to intrinsic viscosity by $(\eta) = K(DP)^a$, with K = 0.57 and a = 1.0.

RESULTS

The results of the temperature gradient incubator studies are presented in Table 2. *T. aurantiacus* showed the highest optimum growth temperature range, 46 to 51 C. *S. thermophile* grew heavily over the broadest range, 36 to 43 C; whereas *C. thermophile* grew heavily over the narrowest range, 40 to 43 C. These results differ somewhat from those given by Cooney and Emerson (6) in their review of the thermophilic fungi. *T. aurantiacus* is listed as giving best growth at 40 to 45 C, *S. thermophile* at 38 C, and *C. thermophile* var. *coprophile* at 50 to 55 C. The differences may be attributed to strain variation and to the different composition and/or pH of the media.

The results of the determination of the optimum growth temperature for cellulose utilization are presented in Table 3. Since with *C. thermophile* it was possible to determine a narrow range of heavy growth in the incubator, only two temperatures were used. The broader ranges recorded for the other two organisms permitted a comparison of cellulose utilization at three temperatures. *S. thermophile* grown at its optimum, 40 C, degraded the most cellulose (22.9%). *T. aurantiacus* displayed the highest optimum growth temperature (48 C), but degraded significantly less cellulose. *C. thermophile* also degraded cellulose to a lesser extent than *S. thermophile*. Since *S. thermophile* displayed the greatest cellulolytic activity of the three fungi, it was selected for further study.

TABLE 2. *Cardinal temperatures of three thermophilic fungi determined in a temperature gradient incubator*[a]

Organism	Gradient range	Minimal growth temp	Optimal growth range	Maximal growth temp
Chaetomium thermophile var. *coprophile*	20.5–60 C	30 C	40–43 C	54 C
Sporotrichum thermophile	21.5–61 C	23 C	36–43 C	52 C
Thermoascus aurantiacus	28.0–61 C	38 C	46–51 C	55 C

[a] Medium, yeast glucose agar; incubation time, 72 h.

TABLE 3. *Percent utilization of cellulose by three thermophilic fungi in shake flasks*[a] *at selected growth temperatures*

Organism	Optimal growth range[b]	Temp (C)	% Cellulose utilization
Chaetomium thermophile var. *coprophile*	40–43 C	40	13.2
		43	10.0
Sporotrichum thermophile	36–43 C	37	12.6
		40	22.4
		43	15.4
Thermoascus aurantiacus	46–51 C	45	7.1
		48	12.3
		51	8.1

[a] Erlenmeyer flasks (250 ml), 50-ml mineral solution, 1% cellulose; shake rate, 150 rpm, incubation time, 72 h.

[b] From temperature gradient incubator studies.

The results of the shake flask experiments to determine the effect of soluble organic compounds in the medium on the degradation of cellulose by *S. thermophile* and on pH are presented graphically by Fig. 1 and 2, respectively. The effect of proteose peptone, urea, and/or Tween 80 was studied since Mandels (10) had used them in the medium for the cellulolytic mesophile *T. viride*. The cultures were grown at 40 C; shake rate was 150 rpm. From Fig. 1 and 2, it can be seen that: (1) the addition of proteose peptone or urea enhanced the utilization of cellulose, (ii) the addition of both proteose peptone and urea yielded optimum cellulose degradation, (iii) the addition of Tween 80 resulted in reduced cellulose utilization, (iv) the pH of the culture did not drop below 4.0 when both proteose peptone and urea were added, and (v) the addition of proteose peptone reduced the lag period of the culture.

A typical set of growth data for *S. thermophile* with proteose peptone and urea as

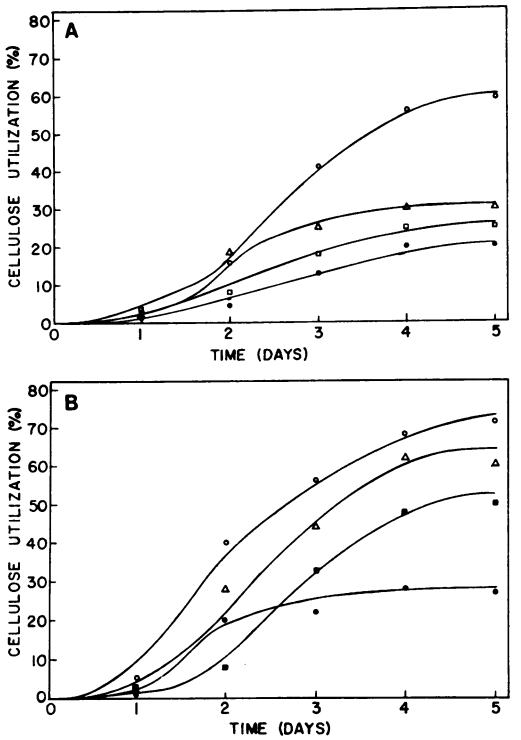

FIG. 1. *(A) The effect of organic additives to mineral solution-cellulose medium on the utilization of cellulose by Sporotrichum thermophile. Symbols:* (□) *Control;* (●) *Tween 80, 0.05%;* (△) *proteose peptone, 0.075%;* (○) *urea, 0.03%. (B) The effect of organic additives to mineral solution-cellulose medium on the utilization of cellulose by Sporotrichum thermophile. Symbols:* (●) *Tween 80, 0.05% and proteose peptone, 0.075%;* (■) *Tween 80, 0.05% and urea, 0.03%;* (△) *Tween 80, 0.05%, proteose peptone, 0.075% and urea, 0.03%;* (○) *proteose peptone, 0.075%, and urea, 0.03%.*

additional carbon, nitrogen, and energy sources, is presented in Fig. 3. Fifty-six percent of the cellulose was utilized in 3 days; 0.37 g of dry cell material was produced per gram of cellulose consumed, 40% of which was protein. It may be seen in Fig. 1b and 3 that most of the hydrolysis of cellulose occurred between 24 and 72 h. The lag period of about 24 h is attributable to the time necessary for the outgrowth of the spore inoculum and production of cellulase.

The results of the determination of untreated and residual cellulose DP are summarized in Table 4. Untreated cellulose had an average DP of 745. The residual samples from cultures showed a DP of approximately 575 when determined on cellulose recovered by the cold alkali method and about 510 when determined on cellulose recovered by the hot alkali method. The hot alkali-treated samples did not dissolve completely in the cupriethylenediamine solution; therefore, the DP is somewhat higher than indicated by this method.

DISCUSSION

The use of the temperature gradient incubator was suggested for initial screening studies as an aid in the selection of the optimal temperatures. The same inoculum could be used and the entire temperature range of interest surveyed for each fungus in a single experiment. Yeast glucose agar was used in the initial studies. Although it was easy to define the minimum and maximum growth temperatures, it was impossible to detect differences visually in the

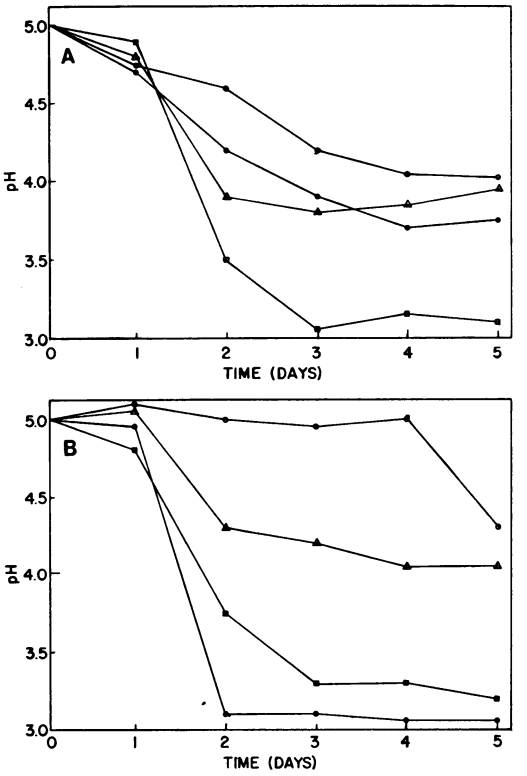

FIG. 2. *(A) The effect of organic additives to mineral solution-cellulose medium on pH of cultures of Sporotrichum thermophile grown at 40 C. Symbols:* (□) *Control;* (●) *Tween 80, 0.05%;* (△) *proteose peptone, 0.075%;* (○) *urea, 0.03%. (B) The effect of organic additives to mineral solution-cellulose medium on pH of cultures of Sporotrichum thermophile grown at 40 C. Symbols:* (●) *Tween 80, 0.05% and proteose peptone, 0.075%;* (■) *Tween 80, 0.05% and urea, 0.03%;* (△) *Tween 80, 0.05%, proteose peptone, 0.075% and urea, 0.03%;* (○) *proteose peptone, 0.075% and urea, 0.03%.*

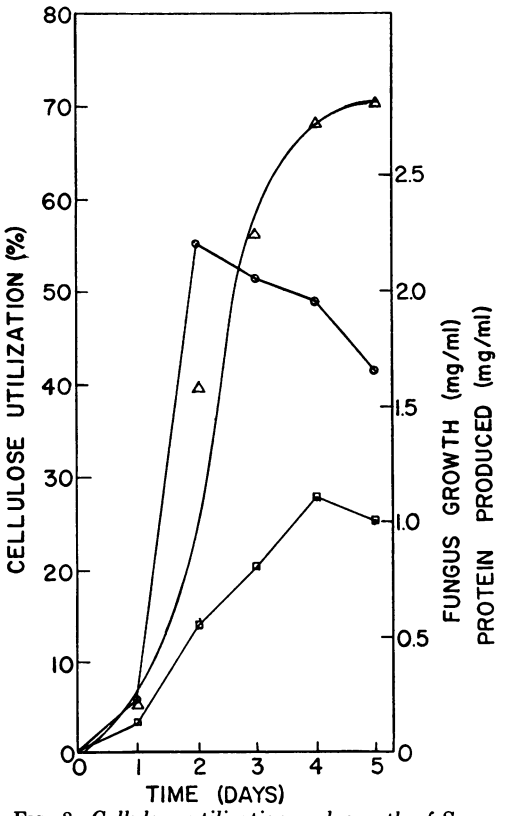

FIG. 3. *Cellulose utilization and growth of Sporotrichum thermophile at 40 C in mineral solution-cellulose medium containing 0.075% proteose peptone and 0.03% urea.* (△) *Cellulose utilization, %;* (○) *fungus growth, mg/ml;* (□) *protein produced, mg/ml.*

amount of growth in a number of tubes displaying heavy growth. Therefore, an optimum range, rather than a single temperature, was recorded. Although the temperature gradient incubator applied in this manner resulted in a considerable savings in time, use of an aqueous medium would have resulted in a greater savings. The weight of fungus growth or the percentage of cellulose utilization in liquid medium would normally be used to determine the optimum growth temperature. However, when yeast glucose broth or cellulose in mineral solution was used in the gradient incubator, the filamentous fungi adhered to and grew on the polypropylene caps outside the gradient block. A modification in design of the gradient incubator is needed for the study of filamentous fungi in liquid media. The determination of a temperature range which gave heavy growth on agar, however, was valuable in establishing an optimum for cellulose utilization in shake flask studies.

Initially it was desired to use a mineral solution with cellulose as the sole carbon source; however, early work in this study and the work of others suggested that the production of the cellulose enzyme system is enhanced by the addition of a small quantity of a soluble organic carbon, nitrogen, and energy source such as peptone (11). Further, it had been suggested that Tween 80 stimulates this production, particularly when peptone is used. The incorporation of the two soluble organic substances, proteose peptone, and urea, yielded optimum cellulose utilization; 56.1% of the cellulose was degraded within 72 h. As stated above, Tween 80 has been shown to be beneficial in the production of the enzyme system. In Reese's study (11), the objective was to increase enzyme production and recovery. In the present study, on cellulose utilization, the addition of the surfactant to the culture medium resulted in less cellulose degradation. It may have acted as a detergent to prevent the adsorption of the extracellular enzyme on cellulose.

The semimicro method was the most rigorous of the three residual cellulose determinations; however, it precludes further analysis of the cellulose (e.g., degree of polymerization) because of complete hydrolysis in the anthrone reagent. Since the cellulose was pure, the lignin extraction of Updegraff (15) was unnecessary. Moreover, a hot or cold dilute alkali treatment is known to solubilize hemicellulose, xylans, and protein (8). Homogenization was a necessary step for complete extraction of the fungal material since the hot alkali extraction of the whole fungal cellulose yielded results consistently higher than those of the semimicro determination. On the other hand, the results of the cold alkali extraction of the homogenized mass

TABLE 4. *Degree of polymerization of unfermented and fermented[a] Solka-Floc BW-200 as determined by ASTM designation: D 1795-62 and the Mark Houwink equation*

Sample	Degree of polymerization		
	Control	48 h	72 h
Unfermented cellulose	745		
Fermented cellulose			
Hot alkali recovery[b]		505	510
Cold alkali recovery		578	571

[a] Recovered from 48- and 72-h cultures of *Sporotrichum thermophile* grown in shake flask culture (150 rpm) at 40 C.

[b] Hot alkali-treated residual cellulose did not dissolve completely; therefore, DP is somewhat higher than indicated.

were in good agreement with those of the semimicro method. The cellulose recovered from the cold alkali treatment dissolved completely in cupriethylenediamine, whereas that from the hot alkali treatment did not. This indicates that extraction of the homogenized mass is not complete in the hot alkali method.

The results of the cupriethylenediamine determination of intrinsic viscosity indicated that the DP of cellulose dropped approximately 23%, from 745 to 575, with little difference in the 48- and 72-h cultures (Table 4). Roughly 56% of the cellulose was utilized in 72 h. These results suggest that once a cellulose chain adsorbs the enzyme, hydrolysis of that particular chain is favored over desorption and random attack on another chain.

Conditions for a high rate of cellulose degradation by the thermophilic fungus, *S. thermophile*, have been established in this study. The lack of information on this and other cellulolytic thermophiles opens a broad area for further investigation. Additional research on methods of enhancing cellulose degradation by these organisms should prove rewarding. Since *S. thermophile* is a rapidly metabolizing aerobe, the quantity of oxygen available to the organisms might be limiting at its relatively high optimum growth temperature. Studies on methods of increasing the supply of oxygen to this organism have been carried out in our laboratories and will be reported elsewhere.

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