Cellulolytic Enzymes of Stereum Gausapatum

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ABSTRACT

The cellulase systems produced by *Stereum gausapatum* had similar pH and temperature stability and activity relationships regardless of whether they were produced on a sawdust substrate or in liquid medium with either cellulose or carboxymethylcellulose as the carbon source. The system has a pH optimum between 4 and 5 and a temperature optimum of 50 C. Dry weight production and cellulase activity varied with both cultural conditions and media substrate. The cellulase system was separated into two components: a cellobiase and a C_x cellulase. Phytopathology 61:134-138.

Additional key words: wood decay, cellulase.

Cellulolytic enzymes play a major role in the decomposition of wood by wood-decaying organisms. Even though these enzymes have been investigated for many years, the relationships between cultural conditions, substrate, growth, and cellulase production are not completely understood. And those enzymes that have been studied usually were collected from cultures grown in liquid media rather than on wood. Because the substrates and cultural conditions in the liquid media are radically different from those in wood, the enzymes produced in liquid culture may not be the same as those produced on wood. In this study we compared the cellulase systems produced by *Stereum gausapatum* (Fr.) Fr. in wood with systems produced in liquid cultures, determined the effects of several cultural conditions on growth and cellulase production in liquid culture, and partially purified the cellulase system produced in liquid culture.

MATERIALS AND METHODS.—To compare the cellulase enzyme systems from *S. gausapatum* grown on different substrates, *S. gausapatum* was grown on two liquid media and sawdust. The liquid media were similar to the one described by Pettersson et al. (9), but were changed by using either 10 g of cellulose (Whatman cellulose powder) or 5 g of carboxymethylcellulose (CMC) and 6 mg of thiamine hydrochloride/liter.

Fifty ml of each liquid medium were placed in separate 250-ml Erlenmeyer flasks and sterilized at 121 C for 20 min. Thiamine was then added to each flask, and each flask was inoculated with 0.2 mg of homogenized mycelium in 0.5 ml of water. The cellulase system was collected by filtration after incubation at room temp for about 3 weeks. The filtrates from several cultures were combined and frozen until needed.

Stereum gausapatum was grown on sawdust by using a modified soil block decay method (1). The wood block was replaced by a plastic screen basket, about 2 cm in diam and 5 cm in height, filled with sawdust from the heartwood of red oak (*Quercus rubra* L.). The basket and sawdust were sterilized with propylene oxide.

After incubating about 30 days at room temp, the mycelium and sawdust were removed from the basket, dried overnight at room temp, and ground in a Wiley mill. The ground material was added at the rate of 1 g per 10 ml to 0.1 M phosphate buffer (pH 6.0) that contained 1 g of insoluble polyvinylpyrrolidone (Polyclar AT, General Aniline and Film Corp.) per 10 ml of buffer. This mixture was soaked and stirred occasionally for 1 hr, then filtered. The filtrate was dialyzed against distilled water at 4 C for 48 hr, then frozen until needed.

Temperature stability of the enzyme extracts was determined by holding fractions of each extract at about 30, 40, 50, 60, and 70 C for 60 min. At the end of the treatment, the cellulase activity of the fractions was measured.

Cellulase activity was measured by adding 0.5 ml of extract to 5 ml of 1.2% CMC (cellulose gum, type 7MF, Hercules Powder Co.) in 0.05 M citrate buffer at pH 4.0. A dinitrosalicylic acid test (4) was made at zero time, and after incubation for 60 min at 30 C. Extract heated at 121 C for 15 min was used as an enzyme blank for each test.

The effect of temp on cellulase activity was determined by measuring enzyme activity as described above, except that incubation temp of about 30, 40, 50, 60, and 70 C were used.

The effect of pH on the stability of the enzyme extracts was determined by concentrating them to one-half of their original volumes in a rotary evaporator, then diluting aliquots of each extract back to their original volume with McIlvaine buffers ranging from pH 2 to 8. After storage at 4 C for 24 hr, the pH was measured, and cellulase activity was measured as before at pH 4.0.

The effect of pH on enzyme activity was determined on a series of solutions containing 1.2% CMC in McIlvaine buffers ranging from pH 2 to 8. After the extracts were added, the pH of the solutions was measured, and cellulase activity was determined.

The relationship between growth and cellulase activity in liquid culture was determined on the CMC medium described earlier. The cultures were grown on a laboratory bench at room temp. After periods of 4, 7, 11, 17, 27, 33, 40, and 47 days, four cultures were harvested by collecting the mycelium on tared filter paper and the filtrate in a side arm flask. The mycelium was washed with 200 ml of distilled water and dried at 100 C for 24 hr, and dry wt was determined. Cellulase activity was measured from the culture filtrate.

To determine the effect of cultural conditions, cul-

tures were grown (i) on a laboratory bench; (ii) on a platform shaker; (iii) in continuous darkness; and (iv) in continuous light from a white fluorescent lamp. The cultures were grown on the CMC medium described earlier.

To determine the effect of carbon source, cultures were grown on the two media described earlier with cellulose or CMC as the carbon source, and on four additional media in which the carbon source was changed to 10 g/liter of either glucose, maltose, sucrose, or cellobiose.

The effect of nitrogen source was determined by growing cultures on the CMC medium described earlier with $NH_4H_2PO_4$ and on three media in which the nitrogen source was changed to either KNO_3 (1.81 g/liter), asparagine (1.12 g/liter), or glutamic acid (2.54 g/liter). The nitrogen concn was the same in all media, and the phosphate concn was held constant by adding phosphoric acid. The pH was adjusted to 5.8 before the media were sterilized.

For the studies of the effects of cultural conditions and carbon and nitrogen sources, eight cultures were prepared for each cultural or media treatment. Four cultures were collected after 9 days and after 20 days, and mycelium dry wt and cellulase activity were determined. Before an assay was run on the filtrates containing reducing sugars, the filtrates were dialyzed against distilled water for 48 hr at 4 C. Dry wt could not be measured in the cultures on cellulose substrate, because the mycelium could not be separated from the cellulose.

The enzyme systems used in the purification experiments were collected from cultures that had grown for about 3 weeks on the CMC medium described earlier. A rotary evaporator, at 35 C, was used to conc 1,450 ml of filtrate to dryness. The residue was then redissolved in 32.5 ml of water. The concd filtrate was next added to a Sephadex G-25 column (2.5 cm × 60 cm) in 10-ml fractions to remove the materials of low mol wt. The column was eluted with 0.01 M phosphate

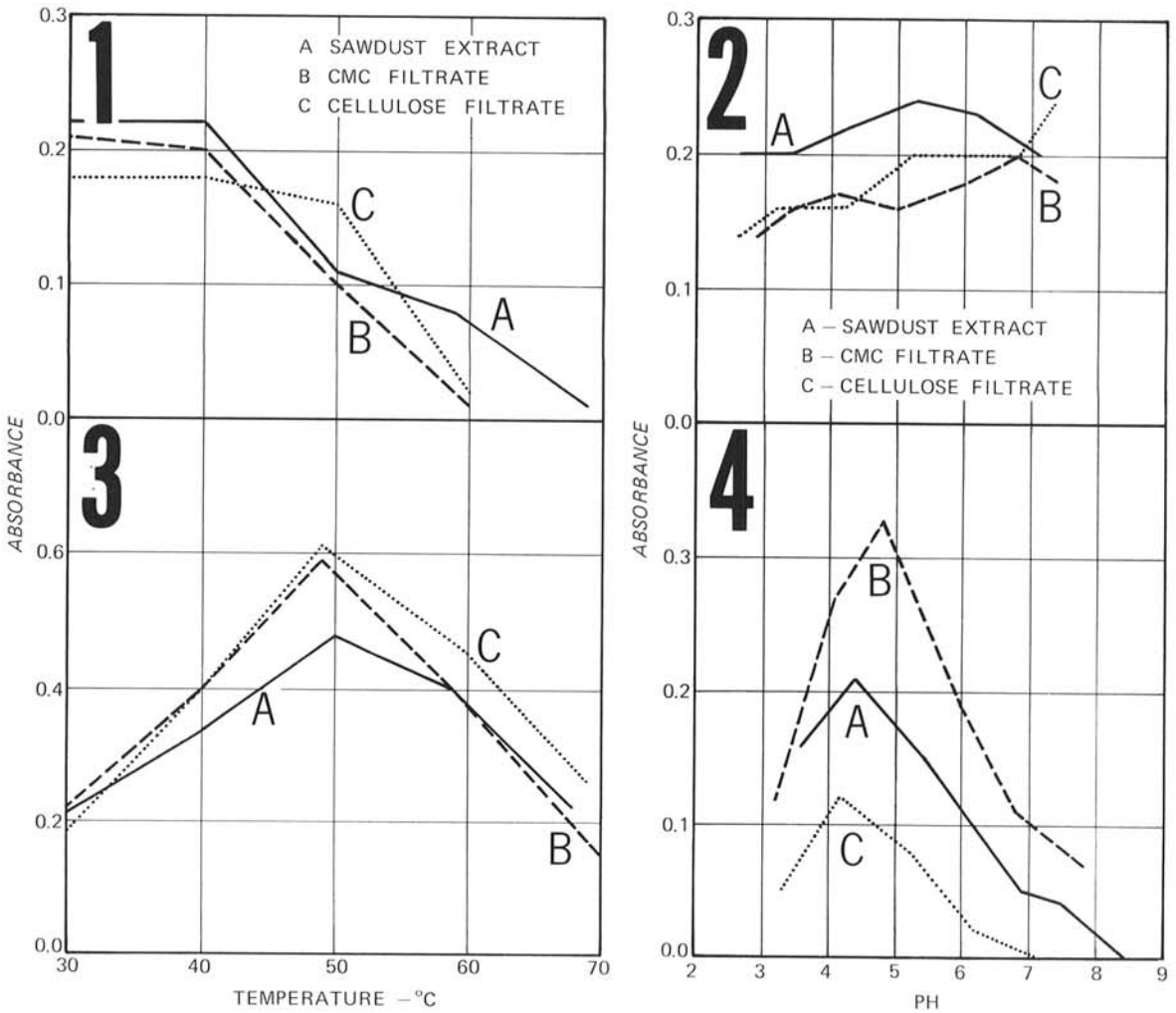

Fig. 1. Effect of temp and pH on stability (1, 2) and activity (3, 4) of *Stereum gausapatum* cellulase produced on different substrates.

buffer at approx 30 ml/hr, and 10-ml samples were collected. The fractions containing activity were combined and were concd to 20 ml on a rotary evaporator. The reconcd material was then placed on a Sephadex G-75 column (2.5 cm × 1,000 cm) in 5-ml fractions. The column was eluted with 0.01 M phosphate buffer at approximately 10 ml/hr, and 5-ml fractions were collected. The Lowry protein assay (7), the cellulase assay, and a cellobiase assay were run on the effluent.

Cellobiase activity was determined by combining 2 ml of 0.001 M *p*-nitrophenyl-β-D glucoside in 0.05 M acetate buffer at pH 5.0 with 0.25 ml of extract. A 0.5-ml sample was immediately withdrawn and combined with 1 ml of 1 M Na_2CO_3. A second 0.5-ml sample was with-

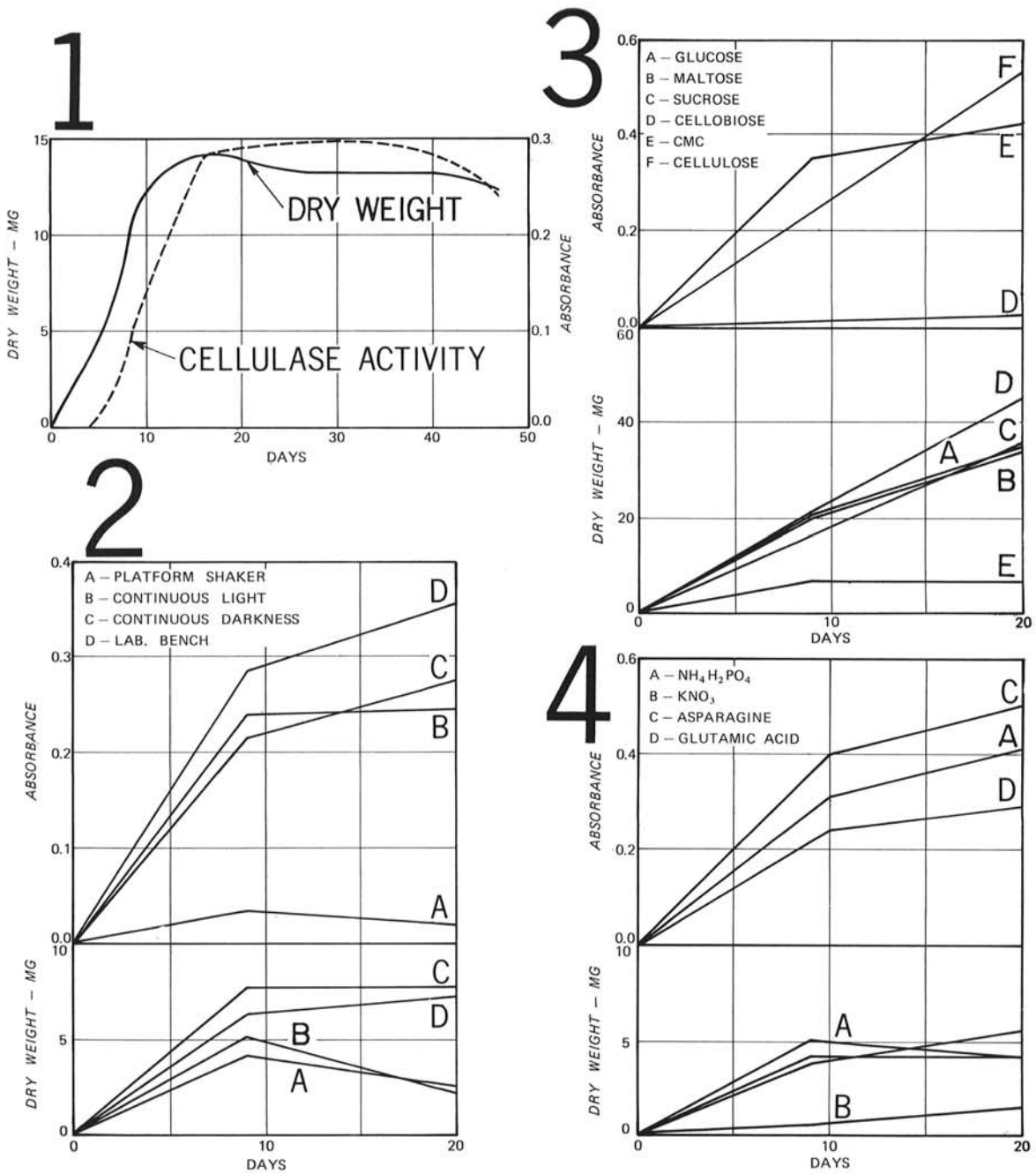

Fig. 2. Relationship between dry wt and cellulase activity in cultures of *Stereum gausapatum* (1), and the effect of cultural conditions (2), carbon source (3), and nitrogen source (4), on dry wt and cellulase activity of cultures of *S. gausapatum*.

drawn after 30 min and added to 1 ml of 1 M Na_2CO_3. The volume of both samples was increased to 4.0 ml with distilled water, and the optical densities at 400 mμ were measured.

RESULTS.—Temperature affected both the stability and activity of the cellulase systems (Fig. 1). Cellulase activity of the heat-treated samples was not affected at 30 and 40 C, but activity decreased with increasing temp at 50 to 70 C. The decrease in activity from the 40-C treatment to the 50-C treatment was greater for the extracts from cultures grown on wood or CMC than from those grown on cellulose. The cellulase activity of all three extracts increased with increasing assay temp from 30 to 50 C, but decreased with increasing temp from 50 to 70 C.

The stability and activity of the cellulase systems were affected by pH. Cellulase activity of the fractions from all three extracts stored at different pH's was fairly stable from pH 2.5 to 7.0. The cellulase activity of all three extracts increased with an increase in assay pH to between pH 4 and 5, but decreased with a further increase in assay pH to 6, 7, and 8.

Cellulase activity and dry wt production followed the same general pattern in liquid culture (Fig. 2). Both increased rapidly to a peak after about 19 days and leveled off. The only difference between the two was that cellulase activity lagged behind dry wt accumulation by several days. After 40 days, both cellulase activity and dry wt decreased.

The production of dry wt and activity of cellulase from cultures on a laboratory bench or in continuous darkness was rapid up to 9 days and continued to increase, but at a slower rate, from 9 to 20 days. Cellulase activity and dry wt of cultures in continuous light or on the shaker increased up to 9 days, but leveled off or decreased from 9 to 20 days. Cellulase activity and dry wt production were greatest in cultures on the laboratory bench or in continuous darkness.

Dry wt was the greatest from *S. gausapatum* grown in a medium containing either a mono- or disaccharide as the carbon source. Conversely, cellulase activity was highest in cultures containing a polysaccharide, CMC or cellulose, as the carbon source. Low cellulase activity was found in the cultures containing cellobiose.

Dry wt production and cellulase activity were highest in cultures containing $NH_4H_2PO_4$, asparagine, or glutamic acid as the nitrogen source. No cellulase activity and little dry wt were found in the cultures containing KNO_3 as the nitrogen source.

The filtrate cellulase system was separated into a cellobiase activity fraction and a cellulase activity fraction (Fig. 3). The cellobiase enzyme came through the column with the sample front and was not held on the column. No relationship was found between protein concn and enzyme activity.

DISCUSSION.—Temp affects the stability and activity of the cellulase system produced by *S. gausapatum*. Between 30 and 40 C, the increase in activity with an increase in temp is due primarily to the influence of temp on the velocity of the enzyme reaction, because the enzyme system is stable over this range of temp.

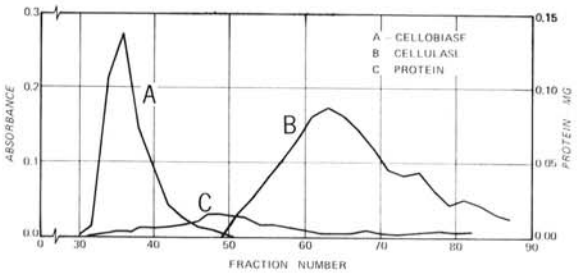

Fig. 3. Enzyme activity and protein concn of fractions collected from a Sephadex G-75, column.

The increase in activity with increasing temp from 40 to 50 C was the result of the influence of temp on the velocity of the enzyme reaction, and also its influence on the stability of the enzyme systems. The activity of the cellulase systems stored at 50 C for 60 min was approximately one-half that stored at 30 or 40 C. Therefore, the increase in activity from 40 to 50 C was probably reduced by degradation of part of the enzyme system during the 60 min of the enzyme assay.

With a further increase in temp from 50 to 70 C, cellulase activity was reduced. However, cellulase activity did not fall to zero, as may have been expected from the relationship between temp and stability of the cellulase systems in which all the cellulase systems were inactivated by the 70 C heat treatment. The reducing materials produced in the 70 C assay were apparently formed shortly after the beginning of the assay before the cellulase systems were inactivated by the high temp, or the CMC substrate used in the assay partially stabilized the cellulase systems so that the inactivation was not as fast as in the stability test.

The pH affected the activity of the cellulase system, but had little effect on the stability of the system within the range of pH tested. Cellulase activity increased with an increase in pH to between 4 and 5, but decreased with a further increase in pH. This change in activity was due to the effect of pH on the velocity of the reaction and not on the stability of the enzyme system, because no large differences were found among the fractions stored for 24 hr at different pH's. This corroborates the results found by Björndal & Eriksson (3) on *S. sanguinolentum* and by Iwasaki et al. (5) on *Trichoderma koningi*.

The pH may affect the velocity of the reaction by changing the attraction between the enzyme and substrate or product (4). For example, when pH changes, the ionization state of all components in the reaction changes. The substrate, enzyme, or some enzyme-substrate complex may have to be in a certain ionic form before the reaction can occur.

The cellulase systems from the three different sources (the two liquid media and sawdust) all exhibited similar pH and temp stability and activity relationships. The temp and pH at which the highest activities were found were very close for all three systems, and all were stable over the pH range tested. The only difference found was that the system in the cellulose filtrate

was more resistant to heat inactivation at 50 C than the systems from the other two sources.

In the experiments on the relationship between growth and cellulase production by *S. gausapatum,* both followed the same general trend over time, but were not directly related. Growth began and terminated 3 to 5 days earlier than cellulase production, but both growth and cellulase production started to decline after 40 days. The initial growth, before cellulase activity was detected, could have been made on materials of low mol wt in the CMC, or on sugars produced by decomposition of CMC by cellulase before cellulase activity could be measured by the assay used. The reduction in growth rate before the reduction in the rate of cellulase production suggests that certain factors necessary for growth are not needed for cellulase production. If both were controlled by the same factors, both should have terminated at the same time.

A direct relationship was observed in the decline of both cellulase activity and dry wt after 40 days. Because decomposition of cellulase in the filtrate occurred after this time, the supply of cellulase must have been continually replenished during the period from 20 to 40 days at a rate equal to the decomposition rate. If the rate of decomposition had exceeded or been less than the production rate, cellulase activity would not have remained steady. As autolysis occurred in the mycelium, the rate of cellulase production probably decreased, thus causing a decline in cellulase activity.

A second explanation of this observation is that cellulase production may have terminated after 20 days, and the enzyme remained stable in the medium. After 40 days, the conditions that caused autolysis of the mycelium may also have caused decomposition of the cellulase enzymes in the medium.

Cultural conditions affected growth and cellulase production of *S. gausapatum.* The organism produced both the best growth and the highest cellulase activity in continuous darkness and on the laboratory bench. Comparable cellulase activity was found in the cultures grown under continous light, but dry wt in these cultures declined after 9 days. Apparently, the organism produces more dry wt if a dark period is included in its growth cycle.

The cultures on the shaker had the lowest cellulase activity. Other authors (2, 8) have also reported that shaking reduces cellulase activity in liquid cultures. The effect of shaking is not completely understood, but Basu & Pal (2) reported that shaking of the cellulase enzyme alone, without the microorganism, reduced enzyme activity.

The composition of the media also affected growth and cellulase production. The best growth was made in media containing either mono- or disaccharides as the carbon source, and ammonium or organic nitrogen as the nitrogen source. Conversely, cellulase production was highest in media containing polysaccharides as the carbon source. So the cellulase system in *S. gausapatum,* as in most organisms, is an adaptive or inducted system. Jennison (6) also reported that a large group of wood-destroying fungi could not utilize nitrate as a nitrogen source.

The purification experiments showed that the cellulase system of *S. gausapatum* contained at least two components: a cellobiase and a C_x cellulase. The mol wt of the two enzymes was not determined, but from their movement on the Sephadex G-75 it can be concluded that the mol wt of the cellobiase fraction is in excess of 70,000 because it came through with the solvent front and was not held on the column.

The trailing effect of the cellulase peak suggests that the cellulase fraction may contain more than one enzyme. An attempt was made to separate the C_x cellulase peak into several fractions and to determine their characteristics, but when the material was concd further, the enzyme activity fell so low that no conclusion could be made.

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