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Influence of Carbohydrate Accessibility on Cellulase Production by *Lenzites trabea* and *Polyporus versicolor*

M. A. Hulme and D. W. Stranks

Department of the Environment, Canadian Forestry Service,
Eastern Forest Products Laboratory, Ottawa, Ontario, Canada K1A OW5.

Abstract

Cellulase and hemicellulase, both free and bound to cellulose, were produced in shake cultures of *L. trabea* and *P. versicolor* grown on ball-milled aspen (*Populus tremuloides*) or filter paper. Most of the enzyme was initially bound to cellulose plus mycelium and could not be readily dislodged, even by ultrasonic disintegration. Proportionally more enzyme could be obtained in the supernatant by reducing the cellulose concentration in the medium. No correlation was observed between the amount of growth and the amount of enzyme production. Rather, enzyme production was shown to be influenced by regulation of the growth rate through the use of cellulosic substrates of varying ease of accessibility and also when cultures were continuously supplied with glucose at a slow rate.

Introduction

Recently, Greaves (1971) showed that the production of cellulase by wood-rotting microorganisms generally increased as the particle size of the cellulosic substrate was decreased, i.e. as the substrate became more accessible. No relationship could be established, however, between cellulase yields and growth measured as production of mycelium. We have been working with similar systems to investigate whether substrate utilization rate rather than just substrate accessibility (total amount of substrate available for metabolism) may largely determine cellulase yields. Our approach has been to establish optimal conditions for cellulase production by the use of cellulosic substrates milled for various periods of time, since Rautela (1967) for example showed that enzymatic solubilization of cellulosic substrates is slower with larger particles. We have also supplied non-cellulosic substrates at levels that will regulate the growth rate of the organism.

Materials and Methods

Whatman No. 1 filter paper and aspen (*Populus tremuloides*) were selected as the cellulosic substrates. Air-dried discs of aspen were chipped, the chips milled (Wiley), and the wood screened to 40–60 mesh particle size prior to ball-milling. Filter paper was also milled (Wiley) and then ball-milled.

The organisms selected were *Lenzites trabea* Pers. ex Fr. A-419, a brown rot, and *Polyporus versicolor* L. ex Fr. S-501, a white rot, obtained from the Eastern Forest Products Laboratory culture collection, Ottawa, Canada.

Ball-milling Procedure

Wood and filter paper were milled with a rotary ball mill in 1·3-gal (5·9-litre) ceramic jars containing 8 lb (3·6 kg) of flint stones (Brownell 1965). The charge was 40 g. Wood that had been milled

for 48 h was about 9% water-soluble. Ball-milled wood was used directly for culture work, but when being used as a substrate for enzyme determinations the soluble material was washed out.

Cultural Procedures

Cultures to determine growth and enzyme production were grown in 250-ml rotary shake flasks containing 80 ml of medium, which consisted of varying concentrations of yeast extract (Difco) plus carbon source. The flasks were inoculated with blended mycelium that had been previously grown and prepared as described by Hulme and Stranks (1971). Shaking was at 130 rev/min and 28°C.

The experiment in which a continuous feed of glucose was supplied (see Fig. 1) was performed with a 4-litre glass fermenter as outlined previously (Hulme and Stranks 1971). The medium was that of Reese and Mandels (1966), which comprised (minus carbohydrate): $(NH_4)_2SO_4$, 1·4 g; KH_2PO_4, 2·0 g; urea, 0·3 g; $MgSO_4.7H_2O$, 0·3 g; $CaCl_2$, 0·3 g; yeast extract, 0·1 g; $FeSO_4.7H_2O$, 5 mg; $MnSO_4.H_2O$, 2 mg; $CoCl_2$, 2 mg; $ZnCl_2$, 1·8 mg; distilled water, 1 litre.

Assay Procedures

(i) *General*

Mycelial dry weight (to determine the amount of growth) and residual glucose (to determine substrate utilization) were assayed as previously described (Hulme and Stranks 1971). Cellulase (EC 3.2.1.4) was determined by viscometry with CM-cellulose as substrate (Hulme and Stranks 1971). This cellulase, often designated C_x, was calculated in international units of activity according to Hulme (1971). Where cellulosic substrates interfered with mycelial dry weight determinations, the mycelial mass was assayed for nitrogen using the microKjeldahl technique (Steyermark 1961) and the mycelial content calculated from a standard obtained for the nitrogen content of mycelium of *L. trabea* grown in glucose medium under shake conditions. The ammonia content of the culture medium was determined by steam distillation of a mixture of culture supernatant (1 ml) and aqueous NaOH (10 ml, 40% w/v) and titration of the liberated ammonia as in the microKjeldahl technique.

(ii) *Reducing sugar assay for cellulase*

Where a very sensitive method for cellulase determination was required, such as in the continuous supply experiment, the viscometric assay with CM-cellulose as substrate was selected. This determined the C_x portion of cellulase activity. To determine all types of cellulase activity, i.e. C_x + hemicellulase + C_1, we used a wood substrate since assays with CM-cellulose as substrate do not necessarily relate to total cellulolytic capacity. Aspen that had been ball-milled for 2 days was attacked by enzyme preparations from cultures grown on wood and cellulose, whereas commercial enzyme preparations termed 'cellulase' were unresponsive. The use of washed aspen made the determination more selective for the cellulase complex that attacks insoluble cellulose and hemicellulose structures. Washed aspen was obtained by first steeping the milled wood in several changes of cold distilled water. This treatment was supplemented by autoclaving a suspension of the wood for 10 min at 15 psi (1055 g/cm²) followed by further washing to remove all soluble wood fractions. The resulting preparation was filtered under vacuum and the wood then freeze-dried and finally stored in a capped brown bottle until required.

For enzyme assays 100–120 mg of washed aspen were weighed into a test tube and covered with 3 ml of 0·2M sodium acetate–acetic acid buffer at pH 5·5. A suitable volume of enzyme solution (culture supernatant or homogenized total culture material) was added and the volume made up to 10 ml. The solutions were incubated for 16 h at 50°C. Controls were prepared in which the equivalent volume of enzyme was autoclaved for 10 min to destroy enzyme activity. After incubation the test material was autoclaved and then clear supernatant from the reaction tubes was assayed for reducing sugar using the method of Somogyi (1952). Linear relationships between enzyme concentration and reducing sugar produced were only observed for low levels of enzyme; therefore test volumes were kept small. A unit of activity was defined as the amount of enzyme that produced 1 mg of reducing sugar under the conditions of assay, and is not related to the international units used for cellulase (EC 3.2.1.4.) determinations (see above).

(iii) *Chromatographic assay of enzymic end-products*

The chromatographic procedure was as reported previously (Hulme and Stranks 1971). To obtain acid-hydrolysed wood products the method of Saeman *et al.* (1954) was followed.

Results

Culture Conditions and Chronology

Cellulase, when first detected, was found in the suspension of mycelium and cellulosic substrate, but in older cultures more activity appeared in the clear supernatant. Table 1 illustrates this sequence for *P. versicolor* grown on ball-milled aspen,

Table 1. Location of cellulase produced by *P. versicolor* during growth on aspen that had been ball-milled for 2 days

Cellulase activity was determined by the reducing sugar assay (see Methods)

Concn of yeast extract (g/l)	Concn of aspen (g/l)	Growth period (h)	Cellulase activity (units/flask): Supernatant	Total	Activity in supernatant (% of total)
1	10	60	32	272	12
1	10	72	48	208	23
1	10	96	40	96	42
2	10	24	24	104	23
2	10	48	88	352	25
2	10	72	120	208	58
2	0·5	48	24	48	50
2	0·5	72	64	88	73
2	0·5	96	120	152	79

and also shows that nitrogen levels are important in determining cellulase yields and locations. Table 2 indicates that 0·2% yeast extract was optimal for cellulase production, probably because unfavourably high pH values developed when more nitrogen was supplied. This was due to accumulation of ammonia from preferential deamination of yeast extract when the carbohydrate supply became limiting. Typically,

Table 2. Effect of concentration of yeast extract on the yield of cellulase from *P. versicolor*

Cultures of *P. versicolor* were grown for 3 days on a 1% suspension of aspen that had been ball-milled for 2 days. Cellulase activity was determined by the reducing sugar assay (see Methods)

Concn of yeast extract (g/l)	Final pH	Cellulase activity (units/flask): Supernatant	Total	Activity in supernatant (% of total)
0·5	4·9	16	88	18
1·0	4·8	32	192	17
2·0	5·3	144	256	56
4·0	7·0	72	192	38
8·0	7·2	32	80	40

cultures at pH 4 had about 5% of the ammonia nitrogen in their yeast extract converted to ammonia whereas at pH 8·0 the proportion was 50%. Other culture parameters such as aeration had comparatively little effect on cellulase production; yields were essentially unchanged, for example, when the culture volume was varied to alter the oxygen transfer rate (McDaniel and Bailey 1969).

Cellulase Production on Cellulosic Substrates

Significant quantities of cellulase were only produced in shake culture when the cellulosic substrate was finely divided, e.g. by ball-milling. Cellulase production was not related to growth as measured by the production of mycelium. Table 3 illustrates

Table 3. Effect on cellulase production of varying the cellulosic substrate

Mycelial dry weight was determined from nitrogen analysis by the micro-Kjeldahl technique (80 μg nitrogen $\equiv$ 1 mg mycelium) and cellulase activity by the reducing sugar assay as described in the Methods

Cellulosic substrate (1% suspension)	Maximum mycelial dry wt (mg/flask)	Maximum total cellulase activity (units/flask)
Lenzites trabea		
20-mesh filter paper	50	8
Blended filter paper[A]	112	8
Filter paper ball-milled for 2 days	132	80
Aspen ball-milled for 2 days	147	176
Aspen ball-milled for 21 days	268	152
Polyporus versicolor		
20-mesh aspen	—	56
Aspen ball-milled for 2 days	—	336

[A] Blended for 1 min as a 1% suspension in a Waring blender.

these points. *P. versicolor* produced six times as much cellulase on ball-milled than on 20-mesh aspen. Similarly, *L. trabea* produced 10 times as much cellulase on ball-milled compared with 20-mesh filter paper. Cellulase yields were not increased by using extensively ball-milled substrates: *L. trabea* produced more cellulase on aspen milled for 2 days that on aspen milled for 21 days, although more growth occurred on the latter substrate. If small amounts of substrate were used most of the cellulase was found in the supernatant liquor—probably because fewer sites were available for adsorption of the enzyme (see also Table 1).

Table 4. Cellulase production by *L. trabea* on 0·1% glucose plus 0·5% yeast extract

Cellulase activity was determined by the reducing sugar assay (see Methods)

Growth period (h)	Final pH	Glucose concentration (mg/flask)	Total cellulase activity (units/flask)
24	4·3	57	0
48	6·5	0	6
72	7·2	—	12
96	7·1	—	0

Cellulase Production on Soluble Carbohydrates

When glucose was used as sole carbohydrate small amounts of cellulase were detected as the glucose concentration became limiting (Table 4). The principle of

using limiting carbohydrate to obtain growth and enzyme production was further explored in cultures grown in the presence of a slow continuous feed of glucose to regulate development. Fig. 1 illustrates production of cellulase (EC 3.2.1.4) by *L. trabea* under these conditions. Results with *P. versicolor* were similar, though the total activity was much smaller and eventually disappeared if the growth period was long enough.

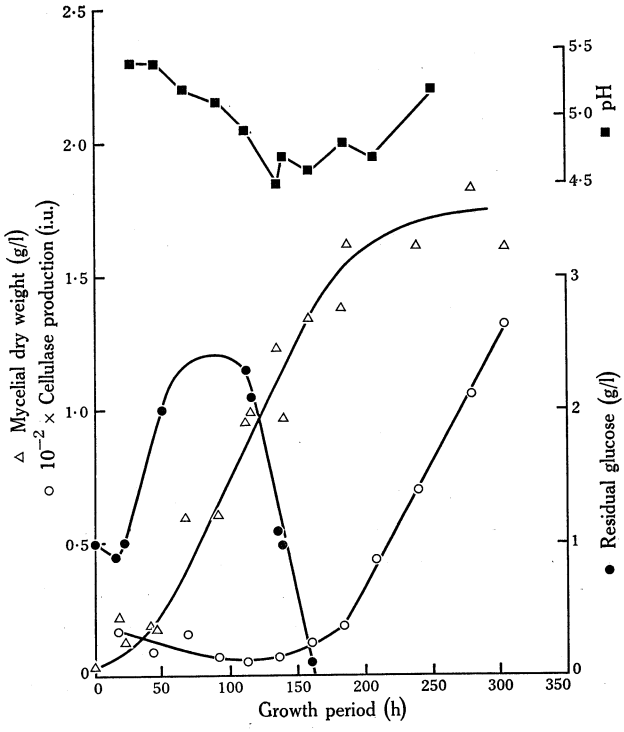

Fig. 1. Production of cellulase (EC 3.2.1.4) from a 4-litre culture of *L. trabea* grown on Reese and Mandels' medium containing 0·1% glucose and continuously supplied after 20 h with a 1% glucose solution at 0·6% per hour. Agitation rate 160 rev/min; aeration rate 0·1 litre/min. Cellulase activity was determined by the viscometric method with CM-cellulose as substrate.

Nature of the Cellulolytic Activity

Both cellulase and hemicellulase were produced under the culture conditions we used. Table 5 shows the chromatographed products obtained from ball-milled aspen incubated with bound cellulase from organisms grown on ball-milled aspen. Cellulase in the supernatant liquor behaved similarly. The products from acid hydrolysis of wood are also shown for comparison. Clearly the enzymes could depolymerize cellulose and hemicellulose to their constituent monomers, and this was achieved via the dimer and trimer (and presumably higher oligosaccharides). Commercial cellulase preparations were also tested under these conditions, but they showed no activity.

It was difficult to release the bound cellulase by any of the standard chemical or mechanical methods. Ultrasonic disruption was the most successful method, but even

here only 12% of the bound cellulase could be recovered in the supernatant after treatment for 15 min at 10°C with a 20-kHz Branson Sonifier operating at full power.

Table 5. Sugars obtained by acid and enzymic hydrolysis of aspen that had been ball-milled for 2 days

Sugars were identified by chromatography (see Methods). +++ represents maximum values

Sugar	R_{xylose}	Acid hydrolysis:[A] I	Acid hydrolysis:[A] II	Hydrolysis by cellulase from: *P. versicolor*	Hydrolysis by cellulase from: *L. trabea*
Xylotriose	0·09	+++		+	++
Cellobiose	0·15		Trace	+	++
Xylobiose	0·31	+++		+++	+++
Glucose	0·50		+++	+++	+++
Mannose	0·65		Trace		Trace
Xylose	1·00	+	+++	++	++

[A] According to Saeman *et al.* (1954). Condition I = 72% H_2SO_4 at 25°C; condition II = 2·5% H_2SO_4 at 120°C.

Discussion

Production of cellulase by the two typical wood-rotting basidiomycetes selected for these tests was clearly affected by the ease with which the substrate could be utilized. The shake culture results showed that relatively inaccessible substrates such as 20-mesh sawdust or filter paper supported little cellulase production, whereas accessible substrates such as glucose supported rapid growth followed by slight cellulase production when the substrate was abruptly exhausted. For maximum cellulase production moderately accessible substrates such as sawdust that had been ball-milled for 2 days were the best of the substrates tried, even though growth was limited. This suggests that a period of relatively slow growth encourages cellulase production, perhaps because the cellulosic substrate acts as a reservoir of carbohydrate which is released to the organism at the correct rate. This idea was reinforced from fermenter culture studies by the finding that slow feeds of glucose (not just cellulose) also favoured cellulase production, an effect that we observed previously in the case of *Myrothecium verrucaria* (Hulme and Stranks 1970). The enzyme was produced during the deceleration phase of growth when the glucose supply became restricted. If, as present results suggest, there is an optimal growth rate (which is not the maximum growth rate) for cellulase production, it is not surprising that no simple correlation was observed in this or previous work (e.g. Greaves 1971; Gupta *et al.* 1972) between mycelial accumulation and cellulase production.

Cellulase yields, of course, represent dynamic equilibria between rates of production and rates of destruction. Furthermore, it appears from our results that the cellulase produced on glucose is relatively unstable (Table 4). This could well be caused by a lack of suitable adsorption sites as has been observed for bound enzymes (Messing 1969), which may confer stability, or by a lack of suitable 'complexing polysaccharides' which for example were shown by Jermyn (1962) to confer stability to cellulase of *Stachybotrys atra*. One significant cause of instability may be concomitant protease production. Such proteases are often responsible for heavy cellulase losses during separation and purification (Selby and Maitland 1967). We have also found

that protease from *P. versicolor* can reduce the activity of supernatant cellulase from *M. verrucaria* (Hulme and Stranks, unpublished data).

The other major culture condition found to affect cellulase yield was concentration of yeast extract. Besides supplying essential nutrients, such complex nitrogenous sources also influence adsorption (Whitaker 1952) and thus the measured enzyme activity. However, an excess of yeast extract results in its eventual deamination and utilization as a carbon source; indeed, the level of ammonia attained has been proposed as a quantitative measure of carbohydrate availability (Sharpe and Woodrow 1972).

With regard to localization of cellulase our results suggest, as expected, that cellulolytic activity first appears in the cellulose and mycelium mixture and later is found in the supernatant. In addition, there is no qualitative difference between 'bound' and 'supernatant' enzyme. In the case of *M. verrucaria*, cellulase was first detected in the bound form (Hulme and Stranks 1971). Later the enzyme was strongly adsorbed onto cellulose so that, for example, one way to obtain more of the total cellulase in the supernatant was to restrict the supply of cellulose and hence the adsorption sites. This strong adsorption onto cellulose probably also explains why so little is released to the supernatant even after ultrasonic disintegration.

Finally, we should point out that ability to produce cellulase in the absence of cellulose in not unique to the two basidiomycetes described here—they were chosen simply as typical rotting fungi. Cellulase production thus appears to be more a condition of a particular metabolic state, as we outlined previously (Hulme and Stranks 1971), than an inductive response to cellulose. In practice, this metabolic state may be too delicate to be obtained artifically without the use of cellulose, either because the correct nutrient balance is elusive in mass culture, or because the unprotected cellulase (*sensu lato*) formed under these conditions is so rapidly degraded that it never accumulates.

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