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Effect of Colloidal Materials on Cellulase Production by *Trichoderma reesei* Rut-C30

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The addition of positively charged colloidal materials to the growth medium markedly increased the concentration of cellulase enzymes produced by *Trichoderma reesei* Rut-C30. Filter paper activities of up to 4 and 13 IU/ml have been achieved by the addition of colloidal materials, using 3% lactose and 3% cellulose, respectively, as a substrate. The particles exert their effect by binding soluble sugars and slowing their uptake by the organism.

Declining reserves of fossil fuels have spurred interest in the development of alternate resources. One such resource possibility is the production of chemical feedstocks through the enzymatic hydrolysis of plant biomass and subsequent fermentation of the monomeric sugars produced. The isolation of high-yielding mutants of *Trichoderma reesei*, a cellulolytic fungus, has made this process more attractive (12, 21); however, enzyme production costs remain a major obstacle blocking commercialization (13).

The production of the cellulase-complex enzymes by *T. reesei* is an inducible phenomenon, triggered by a soluble hydrolysis product of cellulose (6, 7, 15). The exact nature of the inducer is not known. To date, the highest yields of cellulase have been achieved by using cellulose as a carbon source (17). In cellulose-based systems, however, growth and enzyme production are dependent upon hydrolysis of the solid substrate and are thus comparatively slow (1). Lactose, a less potent inducer of cellulase production, offers a number of advantages over cellulose, including increased growth rates and ease of handling. A recent study has indicated that high concentrations of cellulase enzymes could be achieved on lactose media supplemented with cellulose in various forms (19). In the present study, we have examined the effect of addition of colloidal materials on the concentration of cellulase enzymes produced by using both insoluble (cellulose) and soluble (lactose) carbon sources.

MATERIALS AND METHODS

Growth of the organism. *T. reesei* Rut-C30 (12) was obtained from the Agricultural Research Service Patent Collection, Peoria, Ill. The organism was maintained on potato glucose agar slants at 4°C. The basic salts medium used for growth in shake flasks is shown in Table 1. The carbon source used was either lactose or cellulose CF 11 (Whatman Ltd., England). Colloidal materials were added to some media. Sodium citrate (1.5 g/liter) was added as a buffering agent as indicated. The initial pH was adjusted with 5 N NaOH to between 5.5 and 5.7 in all cases. The medium was dispensed into 250-ml Erlenmeyer flasks (working volume, 100 ml) and inoculated with 5 ml of a 3-day-old culture grown in the same medium but without additives such as colloids or metal salts. Cultures were incubated at 27°C with shaking (175 rpm).

Colloidal materials. The following colloidal materials were used in the tests: Alon (Cabot Corp., Boston, Mass.), a positively charged alumina-based colloid; Cab-o-sil M5, MS7, and EH5 (Cabot Corp.), silica-based colloids of three different size ranges; Wesol-P (Wesolite Co., Wilmington, Del.), a positively charged alumina silica sol; and salts of aluminum [$AlK(SO_4)_2$] and iron [$FeCl_3$, $Fe_2(SO_4)_3$], positively charged colloids. Manganese sulfate, a metal salt which does not form a colloid, was used as a control.

Analyses. Samples taken from growth flasks were centrifuged (4,000 × *g*, 20 min) to remove suspended biomass. Cellulolytic activity in the supernatant was monitored by using the filter paper assay described by Mandels et al. (6). Units of filter paper activity (FPA) were converted to international units (IU) by using the multiplication factor described in that study (1 filter paper unit = 0.185 IU). Soluble protein was assayed by the method described by Lowry et al. (5). Glucose was assayed enzymatically by the glucostat method (15). Phosphate was assayed as described in *Standard Methods* (2). Reducing sugar concentration was estimated with dinitrosalicylic acid reagent (10).

Hydrolyses. Cellulase produced in fermentations with lactose as the carbon source was tested for its hydrolytic potential against Solka Floc with enzyme preparations diluted to equal protein concentrations or diluted to equal filter paper activities. Hydrolyses were carried out at 50°C in 125-ml Erlenmeyer screw-capped flasks. Each flask contained 5 to 10 IU of cellulase activity per g of Solka Floc, 0.02% sodium azide (for control of contamination), 0.05 M citrate buffer (pH 4.2), Solka Floc (150 g/liter) and 3 IU of β-glucosidase from *Aspergillus phoenicis* per g of Solka Floc. The method and medium for producing β-glucosidase from *A. phoenicis*, as well as the assay for β-glucosidase activity, have been described previously (12a).

The hydrolytic potential of undiluted cellulase produced in fermentations with cellulose (30 g/liter) as the carbon source was also tested. In these tests, β-glucosidase from two different sources, *A. phoenicis* and almonds (Sigma Chemical Co., St. Louis, Mo.), was used to supplement the cellulase. The enzyme loading ratio in these tests was 40 IU of FPA per g of Solka Floc.

Binding studies. To assess the ability of the colloids to influence the concentration of lactose in solution, binding studies were carried out with lactose, Alon, trace elements, and potassium phosphate in various combinations. In each test, 1% lactose solution was made up, and the test substance was added to it. The tubes were then shaken gently at

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TABLE 1. Medium used for growth of *T. reesei* Rut-C30

Medium component	Concn (g/liter) for the following concn of carbon source:		
	10	20	30
KH_2PO_4	2	4	4
$CaCl_2$	0.3	0.6	0.6
$MgSO_4$	0.3	0.6	0.6
$FeSO_4$	5.0×10^{-3}	1.0×10^{-2}	1.0×10^{-2}
$MnSO_4$	1.6×10^{-3}	3.2×10^{-3}	3.2×10^{-3}
$ZnSO_4$	1.4×10^{-3}	2.8×10^{-3}	2.8×10^{-3}
$CoCl_2$	2.0×10^{-3}	4.0×10^{-3}	4.0×10^{-3}
Urea	0.3	0.6	0.9
$(NH_4)_2SO_4$	1.4	2.8	4.2
Bacto-Peptone	1.0	2.0	3.0
Tween 80	2.0	2.0	2.0

room temperature for 10 min and centrifuged (4,000 × *g*, 20 min), and the lactose concentration in the supernatant was assessed by high performance liquid chromatography (Spectra Physics model SP 8100). The column used, a polypore carbohydrate column (Brownlee Laboratories Inc., Santa Clara, Calif.), was kept at 80°C. Water was used as the mobile phase at a flow rate of 0.3 ml/min.

RESULTS

The final concentration of cellulase produced by *T. reesei* Rut-C30 was much lower when lactose (10 g/liter) was used as a carbon source than when the same concentration of cellulose was used. Typical cellulase activities were 1.5 IU/ml for lactose-grown cultures and 2.5 IU/ml for cellulose-grown cultures (Table 2). The specific activity (IU of FPA per gram of soluble protein) of the cellulase produced was independent of the carbon source used. Extracellular protein concentration reached a maximum more rapidly (2 to 4 days) in cultures grown on lactose than in those grown with cellulose (8 to 10 days) as a carbon source. The use of sodium citrate (1.5 g/liter) as a buffering agent improved the final enzyme concentration by up to 50%.

The addition of positively charged colloidal materials to growth media with 1% lactose as the carbon source resulted in an enzyme production profile similar to cellulose-grown cultures (Fig. 1). Lactose was taken up more slowly in cultures which contained Alon than in those which did not. After 24 h of growth, the lactose concentration in the flasks which contained Alon was more than twice that in the control flasks. Alon (5 g/liter) in the growth medium increased final enzyme concentrations up to twofold for both cellulose- and lactose-grown cultures (Table 2). Higher concentrations of lactose resulted in slightly higher enzyme concentrations.

TABLE 2. Effect of carbon source concentration and colloid addition on enzyme production

Carbon source	Carbon source concn (g/liter)	Alon concn (g/liter)	Maximum FPA (IU/ml)	Maximum protein concn (g/liter)
Lactose	10	0	1.5	1.6
	10	5	3.0	2.7
Cellulose	10	0	2.5	2.6
	10	5	4.5	3.7
	20	0	5.9	5.0
	20	5	7.6	7.0
	30	0	7.1	6.1
	30	5	9.5	9.0

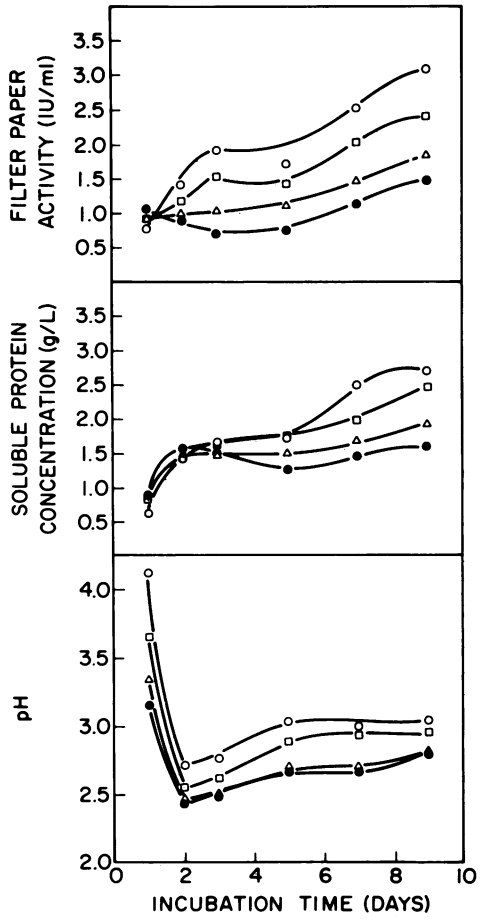

FIG. 1. Effect of Alon addition on pH, extracellular protein concentration, and FPA during growth of *T. reesei* Rut-C30 in shake flasks containing 10 g of lactose per liter. Symbols: ○, 5 g of Alon per liter; □, 3 g of Alon per liter; △, 1 g of Alon per liter; ●, control.

Addition of Alon in concentrations up to 5 g/liter resulted in a linear increase in cellulase production; however, further increases in the concentration of Alon caused a slight decrease in the amount of enzyme produced. The addition of silica-based colloids Cab-o-sil EH5, M5, and MS7 to the growth medium did not increase the cellulase activity or protein concentration of the cultures. The addition of Wesol-P to the growth medium resulted in slightly smaller increases than those produced by Alon.

The addition of iron and aluminum salts resulted in the formation of extensive precipitates. Aluminum and iron salts (Table 3) stimulated enzyme production markedly whereas manganese did not improve the yield of enzyme.

The presence of Alon in the growth medium did not affect the hydrolytic potential of the enzyme produced (Fig. 2). The cellulase from *T. reesei* Rut-C30 was found to be β-glucosidase limited (Fig. 3). When β-glucosidase from almonds was used, conversion of reducing sugars to glucose was observed to increase up to a ratio of 2.5:1 (IU of β-glucosidase/IU of FPA). With this ratio of activities, reducing sugar solutions of up to 120 g/liter have been achieved (Fig. 4). β-Glucosidase from *A. phoenicis* was able to enhance the hydrolysis to nearly the same extent with a

TABLE 3. Effect of the addition of metal salts to citrate-buffered *T. reesei* growth medium

Salt	Salt concn (g/liter)	% Increase in FPA	% Increase in soluble protein concn
$AlK(SO_4)_2$	0.5	11	23
	1.5	13	18
$FeCl_3$	0.5	23	17
	1.5	19	22
$Fe_2(SO_4)_3$	0.5	9	11
	1.5	1	8
$MnSO_4$	0.5	0	0
	1.5	1	3

much smaller quantity of β-glucosidase (0.16 IU of β-glucosidase to 1 IU of FPA).

In growth studies with cellulose as a carbon source, the addition of Alon did not markedly alter pH; however, the production of enzymes was increased (Table 2). Although the relative effect was smaller than when lactose was used as carbon source, enzyme titers at higher cellulose concentrations were much higher than those produced with lactose.

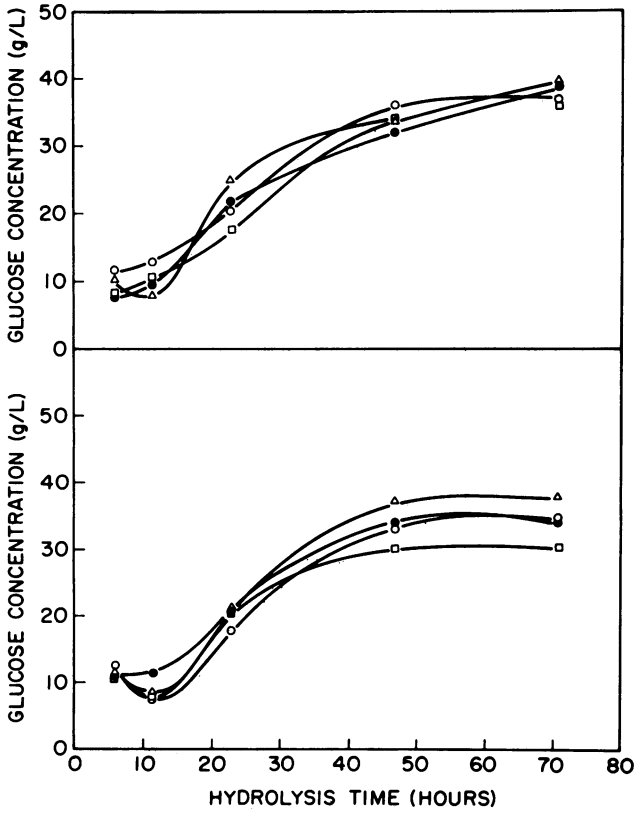

FIG. 2. Hydrolysis of Solka Floc with cellulase from *T. reesei*. Conditions for enzyme induction during the enzyme production step were as follows: 1% lactose (●), 1% lactose plus 0.5% Alon (△), 1% cellulose (□), or 1% cellulose plus 0.5% Alon (○). Top figure shows cellulase preparations diluted to equivalent FPA; in the bottom figure, cellulase preparations were diluted to equal protein concentrations.

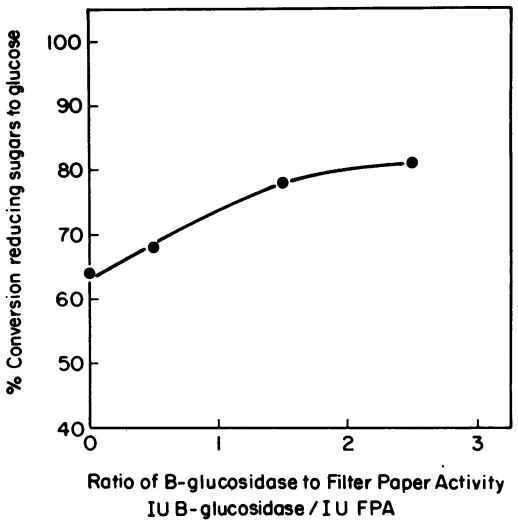

FIG. 3. Effect of increasing β-glucosidase:FPA ratio on hydrolysis of Solka Floc.

Microscopic observation of mycelia grown in the presence of Alon revealed a close association between colloidal particles and the cell wall of the fungus. The presence of the colloid caused the fungus to grow in more dispersed pellet-like growth rather than in the more filamentous form usually observed.

Alon bound ca. 15% of the lactose from a 1% solution (Table 4). The addition of phosphate (KH_2PO_4, 2 g/liter) increased binding to 30% of the total. Approximately one-third of the phosphate in solution was removed with the lactose. The addition of the positively charged metal salts normally supplied as trace elements in the medium appears to have a negative affect on the binding capacity of the colloid. Individually or when added in combination, the salts reduced the binding to 15% in the presence of phosphate and eliminated binding in the absence of phosphate.

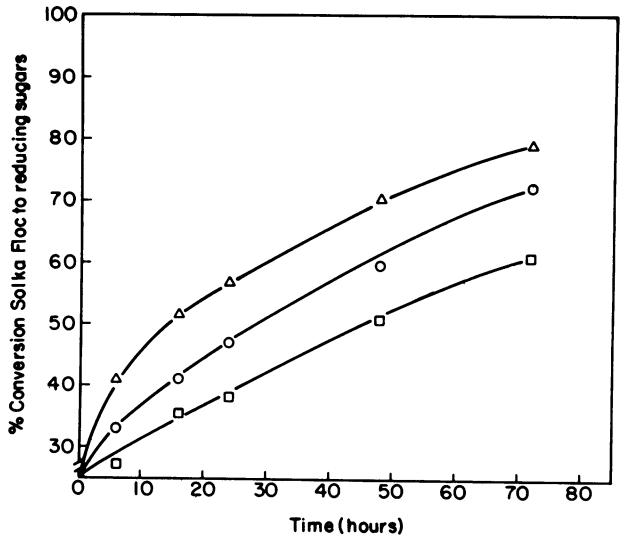

FIG. 4. Effect of addition of β-glucosidase from two sources on hydrolysis of Solka Floc. Symbols: △, 2.5 IU of β-glucosidase (almond) to 1 IU of FPA; ○, 0.16 IU of β-glucosidase (*A. phoenicis*) to 1 IU of FPA; □, control (no added β-glucosidase).

TABLE 4. Effect of metal salts and phosphate on lactose-Alon binding

Colloid	Additive (g/liter)	% Lactose bound
0.5% Alon	None	15
	KH_2PO_4 (2)	33
	KH_2PO_4 (4)	33
	$FeSO_4$ (5×10^{-3})	0
	$MnSO_4$ (1.6×10^{-3})	0
	$ZnSO_4$ (1.4×10^{-3})	0
	$CoCl_2$ (2.0×10^{-3})	0
1.0% Alon	KH_2PO_4 (2)	6.3
	KH_2PO_4 (4)	5.7
0.1% Alon	KH_2PO_4 (2)	40
	KH_2PO_4 (4)	37
0.5% Cab-o-sil M5	None	0
	KH_2PO_4 (2)	0
0.05% $FeCl_3$	KH_2PO_4 ()	15
None	KH_2PO_4 (2)	0

DISCUSSION

The addition of the positively charged colloidal materials, Alon or Wesol-P, to media containing 1% lactose doubled the amount of cellulase produced. Neutral particles (Cab-o-sil M5, MS7, EH5) did not have any effect on the production of the enzyme. Neither was there an improvement when soluble positively charged polymers or soluble metal cations (Mn^{2+}) were added. The effect is dependent upon the presence of small positively charged particles.

The addition of aluminum potassium sulfate and ferric chloride caused an effect similar to that caused by Alon, but Al^{3+} and Fe(III) are well known to form charged colloids in aqueous solutions (3).

The mechanism by which the positively charged colloids exert their effect appears to be related to their ability to bind soluble sugars such as lactose. Metal ions such as iron(III) are known to have a high affinity for ligands which coordinate through oxygen, such as phosphate ions and sugars (3). Alon, in the presence of 0.2% KH_2PO_4, is able to bind approximately one-third of the lactose in a 1% solution. This bound lactose is released slowly, making a constant low concentration of carbon available to the organism. Improvements in enzyme titers achieved by this reduction in effective carbon source concentration are mechanistically similar to those recently achieved by using fed batch fermentations (4). In this type of fermentation, after an initial growth phase, the concentration of sugar in the broth is kept very low. The slow uptake of soluble sugars such as lactose is partially responsible for their ability to induce cellulase production (9).

When the same concentration of Alon (5 g/liter) was added to media containing higher lactose concentrations, smaller improvements in enzyme production were achieved. The influence of Alon was reduced because its binding capacity was low compared with the total amount of lactose present. Attempts to increase the Alon effect at higher lactose concentrations by the addition of higher concentrations of Alon to the growth medium resulted in an increase in viscosity which negated any positive effect which may have existed.

Microbial cells are usually found to be negatively charged in aqueous systems. Thus, there is expected to be an association between the cells and the colloidal particles. Because only the positively charged colloids, Alon and Wesol-P as well as the iron and aluminum hydrates, enhanced enzyme production, it is likely that the positive charge is necessary to bring about the close association between the mycelium and the colloidal particles. It is also possible that the presence of Alon at the cell wall aids in the release of enzyme, thus lowering the internal concentration and favoring an increase in enzyme production.

In fermentations with lactose as a substrate, part of the effect of Alon was a result of the moderating effect which it had on pH. In medium containing no colloids, the addition of sodium citrate is known to increase enzyme production (11, 14). This effect was confirmed in this work. However, Alon improved the enzyme production in citrate-buffered cultures by up to 40%. Also, in fermentations with 1% cellulose as the carbon source, Alon increased the final enzyme concentration by 30%, even though its effect on pH was negligible.

The addition of colloidal materials to cellulose-based fermentations resulted in enzyme titers which are normally only achieved at higher cellulose concentrations. In batch cultures with cellulose (30 g/liter) and Alon (5 g/liter), FPA values of 9 to 10 IU/ml were routinely observed. Maximum values of greater than 13 IU/ml were achieved in several batches. These values compare favorably with the results of other workers with the C30 mutant (4, 18, 20, 21).

It should be noted that the relative increase in enzyme titers observed when colloid was added to cellulose-based media was smaller than that observed for lactose-based media. Because the levels of free sugars normally present in cultures growing on cellulose are very low (<0.2 mg/ml), any effect which the colloids might exert by binding sugars would be markedly reduced. The fact that there is an effect produced in these cultures through the addition of Alon supports the suggestion that there may be a second mechanism by which these particles affect the fermentation.

The cellulase produced by *T. reesei* is deficient in β-glucosidase activity (17). The resultant accumulation of glucose and cellobiose during hydrolysis reduces the rate of sugar production as well as the final concentration of sugar produced (12a). The presence of colloids in the growth medium did not result in the production of an enzyme complex with increased hydrolytic potential (Fig. 2). The addition of β-glucosidase either from almonds or from *A. phoenicis* resulted in marked improvements in the production of reducing sugars (Fig. 3 and 4). The β-glucosidase from *A. phoenicis* showed a much greater degree of synergism with the cellulase produced by *T. reesei* than did the almond β-glucosidase. Sugar syrups ranging in concentration from 100 to 120 g/liter were obtained using β-glucosidase supplementation.

By using one of a variety of inexpensive colloids, it was possible to markedly increase the production of cellulase enzymes in fermentations with either lactose or cellulose as a carbon source. Through the use of the positively charged colloids, available sugar concentrations were kept low, promoting slower growth and increased cellulase yields. The use of these colloids may make conventional batch or continuous processes more economical than the fed batch systems now in use.

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LITERATURE CITED

1. **Allen, A. L., and R. E. Mortensen.** 1981. Production of cellulases from *Trichoderma reesei* in fed-batch fermentation from soluble carbon sources. Biotechnol. Bioeng. **23**:2641–2645.
2. **American Public Health Association.** 1975. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, New York.
3. **Cotton, F. A., and G. Wilkinson.** 1972. Advanced inorganic chemistry, 3rd ed. Interscience Publishers, New York.
4. **Hendy, N., C. Wilke, and H. Blanch.** 1982. Enhanced cellulase production using Solka Floc in a fed batch fermentation. Biotechnol. Lett. **4**:785–788.
5. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
6. **Mandels, M., R. E. Andreotti, and C. Roche.** 1976. Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp. **6**:21–33.
7. **Mandels, M., F. W. Parrish, and E. T. Reese.** 1962. Sophorose as an inducer of cellulase in *Trichoderma viride*. J. Bacteriol. **83**:400–408.
8. **Mandels, M., and E. T. Reese.** 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J. Bacteriol. **73**:269–277.
9. **Mandels, M., and E. T. Reese.** 1960. Induction of cellulase in fungi by cellobiose. J. Bacteriol. **79**:816–826.
10. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugars. Anal. Chem. **31**:426–428.
11. **Montenecourt, B. S., and D. E. Eveleigh.** 1977. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Appl. Environ. Microbiol. **34**:777–782.
12. **Montenecourt, B. S., and D. E. Eveleigh.** 1979. Production and characterization of high yielding cellulase mutants of *Trichoderma reesei*. TAPPI **28**:101–108.

12a. **Murray, W. D., and S. J. B. Duff.** 1984. Studies on the production and use of cellulases for the conversion of cellulose to fermentable sugars and alcohol, p. 179–183. *In* R. Sage and G. B. Maund (ed.), Proceedings of the Sixth International Symposium on Alcohol Fuels Technology, vol. II.

13. **Nystrom, J. M., and A. Allen.** 1976. Pilot scale investigation and economics of cellulase production. Biotechnol. Bioeng. Symp. **6**:55–74.
14. **Panda, T., V. S. Bisaria, and T. K. Ghose.** 1983. Studies on mixed fungal culture for cellulase and hemicellulase production. Part 1: Optimization of medium for the mixed culture of *T. reesei* D1-6 and *Aspergillis wentii* Pt 2804. Biotechnol. Lett. **5**:767–772.
15. **Raabo, E., and T. C. Terkildsen.** 1960. On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. **12**:402–406.
16. **Reese, E. T., and M. Mandels.** 1959. Microbiological process discussion: use of enzymes in isolation and analysis of polysaccharides. Appl. Microbiol. **7**:378–383.
17. **Sternberg, D. E., and S. Dorval.** 1979. Cellulase production and ammonia metabolism in *T. reesei* on high levels of cellulose. Biotechnol. Bioeng. **21**:181–191.
18. **Tagnu, S. K., H. W. Blanch, and C. R. Wilke.** 1981. Enhanced production of cellulase, hemicellulase and β-glucosidase by *T. reesei* (Rut C30). Biotechnol. Bioeng. **23**:1837–1849.
19. **Warzywoda, M., V. Ferre, and J. Pourquie.** 1983. Development of a culture medium for large scale production of cellulolytic enzymes by *Trichoderma reesei*. Biotechnol. Bioeng. **25**:3005–3010.
20. **Warzywoda, M., J. P. Vandecasteele, and J. Pourquie.** 1983. A comparison of genetically improved strains of the cellulolytic fungus *Trichoderma reesei*. Biotechnol. Lett. **5**:243–246.
21. **Watson, T. G., and I. Nelligan.** 1983. Pilot scale production of cellulase by *Trichoderma reesei* (Rut C30). Biotechnol. Lett. **5**:25–28.