Wastepaper Hydrolysate as Soluble Inducing Substrate for Cellulase Production in Continuous Culture of *Trichoderma reesei*

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The enzymatic hydrolysate of wastepaper was evaluated for its cellulase-inducing capability and production characteristics in continuous culture of *Trichoderma reesei* RUT C30. Under the study conditions, i.e., pH 5.0, temperature 25 °C, and typical medium C:N ratio, the apparent cell yield constant was found to be 0.76 (g of dry cell weight/g of reducing sugar), and the maximum specific cell growth rate was 0.26 h^{-1}. The study on the effects of medium C:N ratio confirmed an important role of N sources in the cellulase synthesis. The cellulase production decreased significantly when the feed concentrations of N sources were reduced. An experiment at pH 7.5 with 4-fold N source concentrations also led to poorer cellulase production. When compared with cellulose, the wastepaper hydrolysate was found to have similar cellulase-inducing strength and to induce an apparently complete set of cellulase components. The hydrolysate was also concluded to be a better soluble inducer than sophorose. While comparable at a low dilution rate (0.012 h^{-1}), the specific cellulase productivities of the hydrolysate-supported and the sophorose-induced systems exhibited opposite trends with increasing dilution rates. The specific productivity in sophorose-induced systems decreased with an increase in the dilution rate. On the other hand, with increasing dilution rate the specific productivity in the hydrolysate-supported systems increased from 2.2 FPU/g·h at $D = 0.012$ h^{-1} to 12.2 FPU/g·h at $D = 0.122$ h^{-1} before beginning to decline. The initial increasing trend was attributed to the higher concentrations of inducing oligomer intermediates at larger dilution rates.

Introduction

It has been known for several decades that enzymatic hydrolysis may be used in converting cellulosic materials and wastes to useful products (*1–3*). Its realization, however, has been hindered largely by the high production cost of the cellulase enzymes. For example, the enzyme cost has been estimated to be about 60% of the overall cost for ethanol production from cellulosic materials (*4*).

Most studies for cellulase production were made with batch or fed-batch fermentations of the fungi *Trichoderma* using solid cellulosic materials as the substrate (e.g., *5, 6*). The high solid contents in these systems would burden the agitation and lower the oxygen supply efficiency of the bioreactors. This can prove critical for meeting the cells' respiration need because high cell concentrations are desirable for attaining high production rates.

The cellulase production from cellulosic materials is further affected by the complex metabolic regulation involved. Cellulase synthesis is subject to both induction and glucose repression (*7*). While the intermediates of cellulose hydrolysis provide effective induction (*8*), the concentration of the end product glucose is difficult to control. The glucose concentration is determined by the dynamic balance between the rates of glucose generation (by cellulose hydrolysis) and consumption (by microbial uptake). At low concentrations of cellulase and/or cellulose, the glucose generation may be too slow to meet the need of active cell growth and function. On the other hand, cellulase synthesis can be halted by glucose repression when glucose generation is faster than its consumption. Expensive process control schemes, such as the slow substrate addition enabled by on-line monitoring of glucose concentration or CO_2 evolution (*9*), are required in order to improve the productivity. The solid nature of the cellulosic materials also makes the slow, continuous delivery more difficult to achieve.

These problems can potentially be solved in a continuous culture system using soluble substrates and inducers. The process conditions (e.g., influent substrate concentration and dilution rate) can be optimized so that a high cell concentration can be maintained at a low glucose concentration to maximize the productivity and minimize the glucose repression. Among several saccharides studied, sophorose was the most powerful soluble inducer for *Trichoderma* (*7, 10–12*). Nevertheless, besides being expensive, sophorose was still considered less potent than cellulose for cellulase induction (*13*). The induction by sophorose was also reported to be less complete: some components of the cellulase (*exo-* and some *endo*-glucanases) were absent in sophorose-induced systems (*13*).

The syrup obtained from enzymatic hydrolysis of cellulose has been shown to have cellulase-inducing ability (*9*). However, fundamental knowledge on its inducing and growth-supporting characteristics is lacking. The hydrolysate is readily available in the process of converting cellulosic materials to useful organic products. An integrated design to include the cellulase

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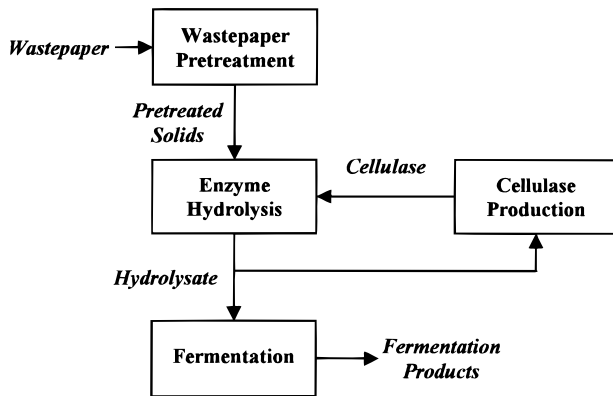
Figure 1. Flowchart showing the hydrolysate-supported cellulase production integrated in an overall process for synthesis of fermentation products from wastepaper.

Table 1. Composition of Media Used in This Study

common components	concn
KH$_2$PO$_4$	2.0 g/L
MgSO$_4$·7H$_2$O	0.3 g/L
CaCl$_2$	0.3 g/L
Tween-80	0.2 mL/L
trace elements	
FeSO$_4$·7H$_2$O	5.0 mg/L
CoCl$_2$	2.0 mg/L
MnSO$_4$·H$_2$O	1.6 mg/L
ZnSO$_4$·7H$_2$O	1.4 mg/L

	concn (g/L)			
nitrogen sources	regular (C:N)	C:0.5N	C:0.25N	C:4N[a]
(NH$_4$)$_2$SO$_4$	0.280	0.140	0.070	1.400
peptone	0.200	0.100	0.050	1.000
urea (NH$_2$CONH$_2$)	0.060	0.030	0.015	0.300

[a] Feed substrate concentration, 2.5 g/L.

production in the overall process (e.g., Figure 1) can offer significant savings in the enzyme costs, particularly those associated with enzyme recovery/purification and denaturation during processing and storage. The use of the hydrolysate as soluble inducing substrate for cellulase production in continuous culture systems deserves more study.

In this work, the ability of wastepaper hydrolysate for cellulase induction has been examined in continuous culture of *Trichoderma reesei* RUT C30. The inducing ability of the hydrolysate has been further compared with those of cellulose, sophorose, and commercial glucose. Other characteristics of cell growth (maximum specific cell growth rate μ_m and cell yield Y) and cellulase production (volumetric and specific productivity) are also reported.

Materials and Methods

Wastepaper Pretreatment and Hydrolysis. Newsprint was pretreated by pulping and high-temperature (160 °C) acid hydrolysis (*4*). A more detailed description is available elsewhere (*14*). The pretreated material was filtered and washed thoroughly with tap water until the pH reached about 5. The analysis of the filtered material indicated a solid content of 21% (w/w), containing 33% lignin. The rest (67%) was assumed to be cellulose because the high-temperature acid hydrolysis was very effective in breaking the glycosidic bonds in hemicellulose and the lignin−hemicellulose bonds, resulting in nearly complete solubilization of the hemicellulose sugars (*4*).

Enzymatic hydrolysis of the pretreated wastepaper was carried out at 50 °C in 500-mL Erlenmeyer flasks. They were shaken at 160 rpm for 3−4 days. The pH was controlled at 4.8 by using the citrate buffer. The flasks contained 10% (w/v) solids and 10 g/L of the enzyme Cellulase TR (Solvay Enzymes, Inc., Elkhart, IN), corresponding to 27.5 FPU (filter paper units)/g of cellulose. These conditions had been established in a previous work done in this laboratory, although the enzyme loading could be much reduced (e.g., to 2 g/L) if a suitable surfactant (e.g., BASF Pluronic F68) was added to enhance the hydrolysis (*14*). The hydrolysis product was filtered (Whatman No. 2) and the filtrate (hydrolysate) used in the later fermentation study.

Microorganism and Media. The fungus used, *T. reesei* RUT C30 (NRRL 11460), was obtained from the United States Department of Agriculture, Agricultural Research Service Patent Culture Collection, Peoria, IL. The culture was maintained with regular subculturing on slants of Potato Dextrose Agar at 4 °C. Cells from the

slants were used to inoculate a 10-mL test tube containing a medium with 10 g/L of glucose. The medium composition was the same as that used by Tangnu et al. (*15*) for 10 g/L of cellulose, with glucose replacing cellulose. After 6 days, these cells were transferred to a stirred flask containing 50 mL of the same medium and incubated for 4 days. The contents of this flask served as the inoculum for the fermentation study.

The composition of the regular feed medium used in the chemostat study was also modified from that used by Tangnu et al. (*15*) for 10 g/L of cellulose. However, in place of the cellulose, either reagent glucose or the enzymatic hydrolysate was used in many cases. Also, the feed reducing sugar concentration was lowered to about 2 g/L, and the concentrations of the nitrogen (N) sources, i.e., peptone, urea, and (NH$_4$)$_2$SO$_4$, were reduced accordingly to keep the C:N ratio constant. The feed N source concentrations were changed in some runs to study the effects of different C:N ratios. The compositions of these media, excluding the source of saccharides, are summarized in Table 1. Antifoam 289 (Sigma), containing silicone and non-silicone (organic) defoamers, was added to all feed flasks in the amount of 1 mL per 10 L. The antifoam was found not to contain any cellulase inducers by comparing the results obtained in the runs with and without the antifoam (results not shown).

The pH in this study was controlled at 5.0 ± 0.2 by automatic addition of 0.4 N HCl or NaOH. It had been found optimal for cellulase production from cellulose by *Trichoderma* (*15*). The reactor temperature was left to fluctuate with the room temperature (25 ± 3 °C).

Chemostat Operation. The continuous culture was made in a 1-L Pyrex Erlenmeyer flask with an overflow glass tube connected to the side. The holding volume was 400 ± 10 mL under the operation conditions. Water-saturated air was continuously pumped into the headspace above the broth. The typical aeration by submerged bubbling was not employed here to avoid the change of gas holdup with different cell and product concentrations in the highly foaming broth. Such a change would otherwise complicate the determination of dilution rate. By using a low reducing sugar concentration in the feed (i.e., 2 g/L), the cell concentrations in the continuous culture were kept low to ensure an adequate oxygen supply to the cells. A dissolved oxygen electrode was used to measure the dissolved oxygen concentrations in a preliminary run. They were found always higher than 50% air saturation.

The chemostat flask contained the regular medium of 2 g/L glucose initially. It was operated at the batch mode
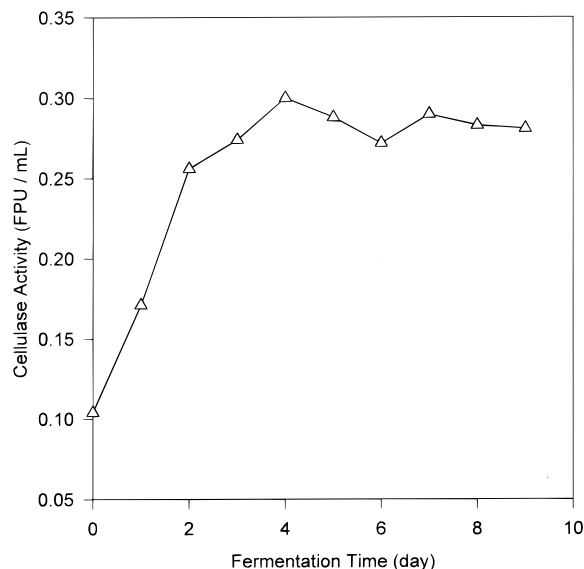
Figure 2. Profile of cellulase activity, after a change of process condition in the continuous culture, followed to indicate the establishment of a new steady state. The specific example is for a run with glucose as the sugar source plus 10 mg/L of sophorose as the inducer.

Figure 3. Steady-state cell (○) and reducing sugar (○) concentrations observed at varying dilution rates in the continuous culture. The wastepaper hydrolysate was used as the soluble inducing substrate, except in the system at the highest dilution rate ($D = 0.26$ h^{-1}), where glucose was the sugar source and sophorose was the inducer. The dashed curve corresponds to the best-fit equation: $X = Y(S_0 - S)$, with $Y = 0.76$.

until the late exponential growth phase. The operation was then switched to continuous culture. The establishment of steady state was determined by following the cellulase activity of the system. An example is shown in Figure 2, where the cellulase activity profile was obtained in a run with glucose as the sugar source and 10 mg/L of sophorose as the inducer. After several successive steady-state data had been collected for a particular study condition, the feed composition and/or the dilution rate was changed to initiate the study for the next condition.

Analysis. Samples taken in the study were analyzed for the cell concentration, reducing sugar concentration, glucose concentration, filter paper activity, CMC-ase (or *endo-β*-1,4-glucanase) activity, and cellobiase (or *β*-glucosidase) activity. The assays for enzyme activities and reducing sugar concentration were made according to the IUPAC procedures (*16*). The glucose concentrations were measured using the enzymatic assay kit from Sigma Diagnostics (Procedure No. 510). Cell concentrations were determined from the optical density values measured at 600 nm on a spectrophotometer. A calibration curve between the optical density and the cell dry weight concentration was first developed for the determination.

Results

Chemostat Culture with Enzymatic Hydrolysate. Continuous culture was first made with the regular medium having the C:N ratio used by Tangnu et al. (*15*). The sugar source in most runs was the filtered enzymatic hydrolysate of wastepaper. For comparison, a few runs were made using glucose, with or without sophorose as the inducer. Regardless the source, the reducing sugar concentration in the feed was kept about 2.0 g/L. It was reported that the carbon source would be limiting in media with such a C:N ratio (e.g., *15, 17*). This was confirmed in batch fermentations made in this laboratory (results not shown).

The results of steady-state cell concentration (*X*) and reducing sugar concentration (*S*) are plotted against the dilution rate (*D*) in Figure 3. The observed profiles are typical for continuous culture (*18*), except that the concentration of the limiting substrate, i.e., reducing
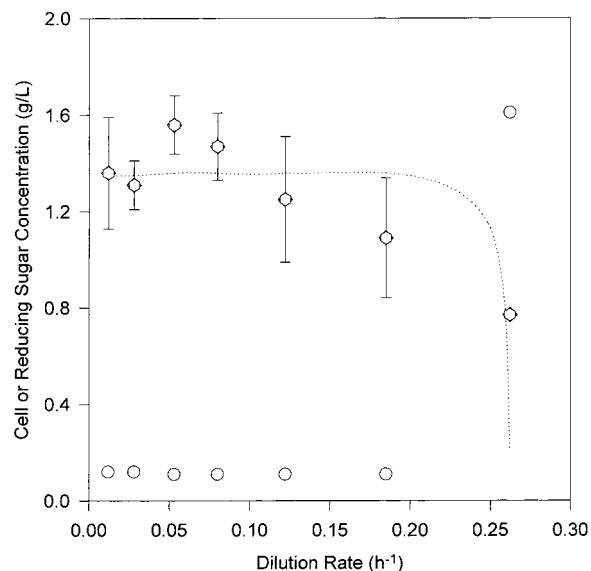
sugar in this case, should have approached zero with diminishing dilution rate. The glucose concentration at $D = 0.012$ h^{-1} was, indeed, undetectable when analyzed by the Sigma glucose assay kit. The small reducing sugar concentrations found at the low dilution rates were, therefore, from some nonmetabolizable interfering materials carried into the bioreactor with the wastepaper hydrolysate.

The apparent cell yield constant $Y_{x/s}^{app}$ ($= X/(S_0 - S)$, where S_0 is the reducing sugar concentration in the feed) was found to be 0.76. This is consistent with the literature values, i.e., 0.64 and 0.80, that have been obtained for *Trichoderma viride* grown at 30 °C with glucose and ammonium as the only C and N sources (*19*). The value obtained in this study was closer to the larger literature value, presumably reflecting the contribution of peptone as an additional C source.

The maximum specific cell growth rate (μ_m) of *T. reesei* RUT C30 under the studied condition was about 0.26 h^{-1} (Figure 3). The value is derived mainly from the results observed in a run with glucose as the sugar source plus 10 mg/L of sophorose as the inducer. It compares well with the value of 0.24 h^{-1} reported in the literature for the fungus grown on glucose (*20*).

The profiles of cellulase concentration (*p*) and volumetric and specific productivities, varying with the dilution rate, are shown in Figure 4 for the hydrolysate-supported continuous culture. The highest cellulase concentration of 0.25 FPU/mL was obtained at the lowest dilution rate of 0.012 h^{-1}. As the dilution rate increased, the cellulase concentration declined. However, the volumetric productivity Q_p ($= Dp$) and specific productivity q_p ($= Q_p/X$) both increased with increasing dilution rate up to $D = 0.122$ h^{-1}. A further increment of *D* then led to the decline of both productivities. While the productivity profiles will be discussed in more detail later, the optimal process condition clearly involves a compromise between the productivity and the effluent cellulase concentration. A certain minimum of the latter must be met for its subsequent usage in enzymatic hydrolysis of
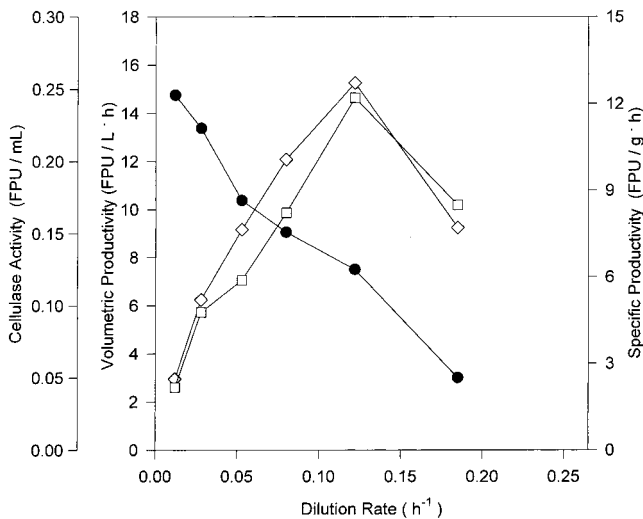
Figure 4. Profiles of cellulase activity (●), volumetric productivity (◇), and specific productivity (□) observed in the wastepaper hydrolysate-supported continuous culture.

Figure 5. Results of cellulase activity, reducing sugar concentrations, and cell concentrations obtained in a run with total elimination of N sources. A slow, continuous feed of wastepaper hydrolysate was maintained until 136 h. The reducing sugar concentration did not decrease afterward, indicating the rapid cell death in the N-free system.

wastepaper. Otherwise, an additional concentration step would be required.

Effects of Feed C:N Ratio. *Total Elimination of Nitrogen Sources.* Cellulase production is generally considered to be non-growth-associated (*21*). To increase the productivity, it had been proposed to raise the cell concentration held in the bioreactor by cell immobilization (*22, 23*). The cells were maintained at stationary phase by omitting one or more nutrients vital for growth but presumably unessential for cellulase synthesis (*23*). Compared to other substrate limitations studied (e.g., $MgSO_4 \cdot 7H_2O$ and all trace elements), the omission of N sources had been found to yield higher enzyme titers. The study was made in a batch mode with *T. reesei* QM 9123, using 1 g/L of glucose as the C source and 10 mg/L of sophorose as the inducer. Only the profiles for cellulase production during a period of 7 days were presented.

In the above study, the exhaustion of C source must have caused cell deterioration and death during the reported period of cellulase production. This was confirmed with the measurement of cell concentration in the experiments repeated in this laboratory using a different strain, i.e., *T. reesei* Rut C30 (results not shown). To differentiate the effects of N source omission from those of C source exhaustion, experiments were designed to include a slow, continuous feed of C source. The goal was to maintain the reducing sugar level in the broth around 0.2–0.3 g/L, so that glucose repression to cellulase synthesis could also be minimized. The level was chosen on the basis of the observation in earlier batch fermentations made in this laboratory.

The cells used in this study were harvested at the late exponential growth phase, centrifuged, washed twice with distilled water, and then transferred to the media without any N sources. Three different systems were examined. Two of them contained initially 5 g/L of cellulose: one as Avicel, and the other as the pretreated wastepaper (prior to the enzyme hydrolysis). A concentrated glucose solution was continuously added to maintain the reducing sugar levels in these systems, by adjusting the addition rate every 8–16 h. The third system examined contained no cellulosic solids. The initial medium had 0.8 g/L of reducing sugars from the hydrolysate. The hydrolysate was also used for the continuous addition.
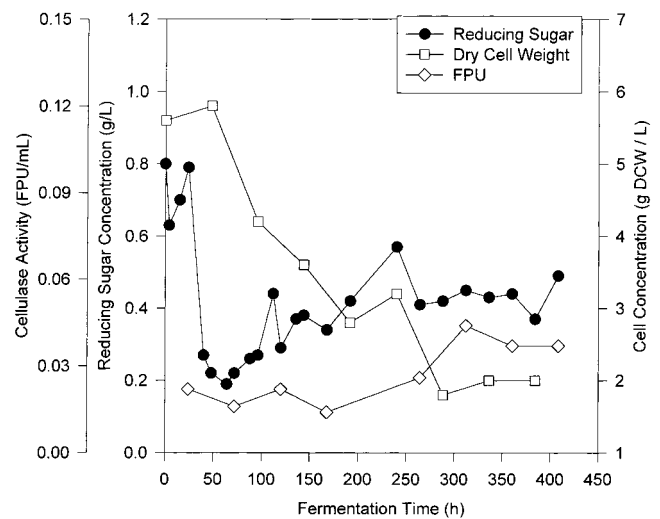
The results obtained in the system with wastepaper hydrolysate are shown in Figure 5. The cell concentration fell steadily from 6 to about 2 g/L, despite the slow addition of C source. The other two systems exhibited similar profiles. The cellulase activity did not increase much, although a higher level (0.1 FPU/mL) was reached in the Avicel-containing system after about 400 h. Clearly, total elimination of N sources would not be feasible for long-term cellulase production with immobilized resting cells.

Different C:N Ratios. Experiments were further made to determine the effects of lower feed N source concentrations on cellulase production in continuous culture. With the ratio used by Tangnu et al. (*15*) being the reference C:N, the feed N source concentrations were decreased proportionately to make the media with C:0.25N and C:0.5N (Table 1). The dilution rate was kept constant at 0.012 h^{-1}. The results obtained are shown in Table 2.

The steady-state cell concentration did not decrease with the *decrease* in the N source concentrations, indicating that the C sources were limiting even in the system of C:0.25N. The cellulase activities, however, declined as the feed C:N ratio was decreased from that used by Tangnu et al. (*15*). The N sources thus played some positive roles in the cellulase synthesis.

A system with higher concentrations of the N sources, i.e., C:4N, was also examined. As shown in Table 2, a significantly higher cell concentration was obtained (beyond the increase expected from the slightly higher feed reducing sugar level, i.e., 2.5 g/L, used in this run). Peptone must have also contributed to the C source for cell growth. Surprisingly, the cellulase production was much poorer than that obtained in the system of regular C:N ratio. The pH in this run was not controlled by acid/base addition. It stabilized around 7.5 at steady state. While the high pH has been shown to have a negative effect on cellulase production (*15*), it is uncertain if the excessively high N source concentrations also caused the channeling of a larger portion of the C source and other resources to cell growth instead of cellulase synthesis.

Comparison of Induced Cellulase Production by Hydrolysate, Sophorose, Commercial Glucose, and Cellulose. Two additional runs of continuous culture were made so that the enzymatic hydrolysate could be

Table 2. Effects of Feed C:N Ratios on Continuous Culture with Wastepaper Hydrolysate as the Inducing Substrate

C:N	dry cell weight (g/L)	red. sugar concn (g/L)	cellulase (FPU/mL)	CMC-ase (units/mL)	β-glucosidase (units/mL)
1:0.25	1.81	0.09	0.133	2.8	0.02
1:0.5	1.74	0.1	0.141	2.9	0.04
1:1	1.36	0.12	0.246	6.8	0.07
1:4[a]	3.45	0.09	0.133	na[b]	na[b]

[a] Results from a run in which pH was not controlled but stabilized at steady state (7.47). Also, feed reducing sugar concentration was 2.5 g/L, indicating that excess peptone might have been used as carbon source. [b] Not analyzed.

compared with sophorose for their inducing capability and characteristics. Both runs were made with $D = 0.012$ h^{-1}. Glucose was the reducing sugar source. One of the runs was supplemented with 10 mg/L of sophorose in the feed; the other was not. The sophorose concentration used (i.e., 10 mg/L) had been reported to yield the maximum induction level attainable with this disaccharide (*22*). The results for the specific productivities of cellulase, CMC-ase, and β-glucosidase obtained in these runs are summarized in Table 3. For comparison, the results from hydrolysate-supported systems at two dilution rates (0.012 and 0.122 h^{-1}) are also included.

The system with only glucose also produced cellulase, although with much lower productivities than the other two systems. The residual inducing capability of commercial glucose was observed by earlier researchers (e.g., *11*). It was attributed to the presence of contaminating sophorose in the commercial glucose (*11*).

At $D = 0.012$ h^{-1}, the specific productivities for FPU, CMC-ase, and cellobiase were all comparable between the hydrolysate-supported and the sophorose-induced systems. However, the comparisons at higher dilution rates were very different. As described earlier (Figure 4), the productivity in the hydrolysate-supported systems increased with increasing dilution rate and reached the maximum at about $D = 0.122$ h^{-1}. The specific productivity of FPU at $D = 0.122$ h^{-1} was nearly 6-fold higher than that at $D = 0.012$ h^{-1} (Table 3). On the other hand, the specific FPU productivity induced by sophorose was reported to decrease when the dilution rate was increased from 0.012 to 0.05 h^{-1} (*17*). The different productivity trends between the two systems are discussed in more detail later. Nonetheless, the enzymatic hydrolysate is shown clearly to be a better soluble inducing substrate than the sophorose-supplemented glucose solution.

A study with *T. reesei* QM 9123 showed that the induction by sophorose (250 mg/L) produced a less complete set of cellulase (*13*). Certain components of cellulase (*exo*- and some *endo*-glucanases) were absent when compared (using electrophoresis) with the enzymes induced by cellulose. The ratio of CMC-ase activity over FPU was 40 for the sophorose-induced system, while that for the cellulose-supported system was 26 (*13*). In the current study, the ratios obtained at $D = 0.012$ h^{-1} for the hydrolysate-supported and sophorose-induced systems were similar: 28 for the former and 27 the latter (Table 3). At higher dilution rates in the hydrolysate-supported systems, the ratios were slightly lower, e.g., 24 for $D = 0.122$ h^{-1}. It should be pointed out that these ratios were comparable to the value of 26 obtained for the cellulose-induced systems in Sternberg and Mandels's study (*13*). The large ratio of 40 obtained with sophorose in their study might have resulted from the low pH value (i.e., 2.8) used. The low pH was found optimal for the

Table 3. Comparison of Cellulase Induction

C source/ inducer	system	specific productivity (units/g·h)		
		FPU	CMC-ase	β-glucosidase
glucose		0.46	8.8	0.08
glucose + sophorose (10 mg/L)	continuous, $D = 0.012$ h^{-1}	2.24	60.0	0.65
hydrolysate		2.17	60.0	0.61
hydrolysate	continuous, $D = 0.122$ h^{-1}	12.20	293.0	1.95
cellulose[a]	fed-batch	1.37−4.20	na[c]	na[c]
cellulose[b]	batch	7.40	na[c]	na[c]

[a] Strain, *T. reesei* Rut C30; volume, 10.5 L; cellulose, αFloc (James River Corp.). The values of specific FPU productivity are for the runs with different fed-batch strategies (*6*). [b] Strain, *T. reesei* QM9414; volume, 300 L; cellulose, spruce sulfite pulp (*24*). [c] Not available.

production of CMC-ase (*13*) but not for FPU (*10*). Of course, the difference in the strains of *T. reesei* employed (i.e., RUT C30 versus QM 9123) might also be a factor. Nonetheless, the results of this study suggest that a complete induction for cellulase synthesis is achievable by wastepaper hydrolysate.

The cellulase-inducing capability of the hydrolysate can be further compared with that of the cellulose reported in the literature. Continuous culture with cellulose was not easy to manage at low dilution rates, especially on a small laboratory scale, because the cellulose solids tended to settle and clog the feed line. The results of some batch and fed-batch fermentations reported in the literature (*6, 24*) have been used to evaluate the specific cellulase productivity in cellulose-supported systems. The values are summarized in Table 3. These values are assessed only for the period of active cellulase production, to minimize the effects of other inhibition, repression, and limitation. The computation was based on the following equation:

$$q_p = \frac{\Delta p}{\Delta t \bar{X}}$$

where Δp is the produced enzyme activity during the period of active cellulase synthesis (Δt). The time-averaged cell concentration ($\bar{X}$) is determined by numerical integration using the trapezoid rule (*25*).

The estimated values of q_p in the cellulose-induced systems (i.e., 1.4−7.4 FPU/g·h) were in the range (i.e., 2.2−12.2 FPU/g·h) obtained in the hydrolysate-supported continuous culture at varying dilution rates. The hydrolysate was therefore shown to have cellulase-inducing capability comparable with that of cellulose. The values obtained in the batch and fed-batch systems were suboptimal because they represented the averaged productivity over the changing metabolic stage. In some cases, they were also affected by glucose repression during the periods of higher glucose levels.

Discussion

Varying with the dilution rate, a unique trend of specific cellulase productivity has been observed in this study for the hydrolysate-supported systems. This is shown in Figure 6, together with the profile reported in the literature for a system using commercial glucose (Cerelose) as the C source (*17*). This source of glucose was known to contain small quantities of sophorose that
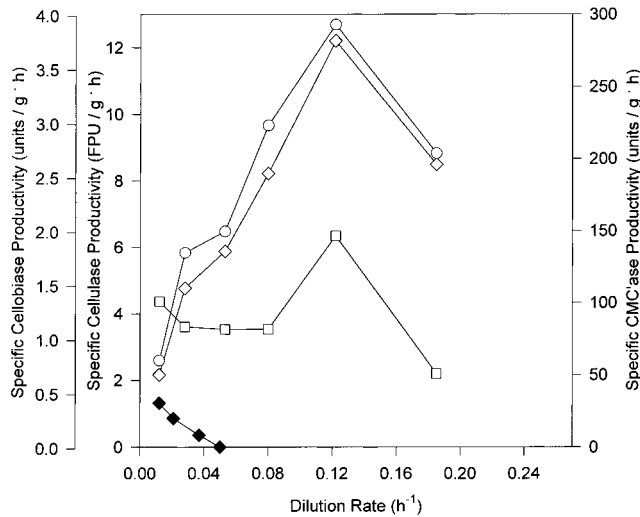
Figure 6. Profiles for specific productivity of cellulase (FPU, ◇), CMC-ase (○), and cellobiase (□) observed in the wastepaper hydrolysate-supported continuous culture as well as that for specific cellulase productivity in sophorose-induced systems (◆) reported in the literature (*17*).

provided the induction for the observed cellulase synthesis (*17*). The trends of FPU productivity observed in these two studies were clearly opposite at low dilution rates, although a much wider range of dilution rate was covered in the current study.

The trend of decreasing specific FPU productivity with increasing dilution rate in the sophorose-induced system is first discussed. Although slowly metabolized if present as the sole sugar source, sophorose is generally considered as a gratuitous inducer for cellulase synthesis by *T. reesei* when glucose is present (*13*). The inducer concentrations in the sophorose-induced systems would be practically constant at various dilution rates: all were equal to the feed sophorose concentration. The variation of specific productivity could not be attributed to the different inducer concentrations. Brown and Zainudeen (*17*) explained the phenomenon by a required maturation of the cells for cellulase synthesis. The lower specific productivity at a higher dilution rate reflected the larger fraction of "immature" cells in the system. Webb et al. (*22*) observed a similar behavior of cellulase productivity in their systems using immobilized cells. They, however, suggested the glucose limitation at the low growth rates being responsible for the increased productivity. Similar phenomena of *apparent* negative dependency on cell growth were observed for production of many other secondary metabolites (e.g., *26, 27*). Some were successfully explained by catabolite repression and/or induction by the accumulated intermediates during slower growth (*26*). However, the responsible mechanism for the decreased cellulase synthesis by *T. reesei* at higher dilution rates is still uncertain.

On the other hand, the initial increasing trend of FPU productivity observed in the hydrolysate-supported systems may be attributed to the higher concentrations of inducers present at larger dilution rates. The intermediate oligomers produced during the process of cellulose hydrolysis are the natural inducers for cellulase (*28*). Sophorose is also found to be present in the cellulose hydrolysate (*8*). It is believed to be formed by the transferase action of β-glucosidase, which is capable of forming β-linked disaccharides (including sophorose) in the presence of glucose and cellobiose (*13*). Other than sophorose, the oligomer inducers are short-living intermediates. Their steady-state concentrations in the biore-

actor are expected to vary with the dilution rate. With increasing dilution rates, the residence times are shorter and the concentrations of hydrolyzing enzymes (cellulase) lower (as shown in Figure 4). Both favor a lower conversion of the oligomers in the bioreactor. Consequently, the inducer concentrations should increase with increasing dilution rates.

At the low dilution rate range (0.012−0.122 h⁻¹), the positive effects of the higher inducer concentrations appeared to overcome the negative dependency described above for the sophorose-induced systems. This led to the increase in cellulase productivity with increasing dilution rates. The eventual decline of the productivity at much higher dilution rates (>0.122 h⁻¹) could result from (*1*) the approach of maximum induction level, so that a further increase in the inducers' concentrations had a diminishing marginal effect, and/or (*2*) the stronger negative dependency, especially glucose repression, at these high dilution rates.

The mechanism proposed above can also be used to explain the trends for CMC-ase and cellobiase observed in this study (Figure 6). The similar increase-then-decrease trend found for the CMC-ase production is anticipated because the induction of CMC-ase also relies strongly on the intermediate oligomer inducers. On the other hand, sophorose is responsible for a main portion of the cellobiase induction (*10*). This would be especially true at small dilution rates when other inducing oligomers were present at much lower concentrations. The relatively constant cellobiase productivity observed initially (D ≤ 0.08 h⁻¹) reflects the balance between the aforementioned negative dependency and the increasing trend caused by the increasing concentrations of other intermediate inducers for cellobiase. The latter appeared stronger at higher dilution rates (0.08 h⁻¹ ≤ D ≤ 0.122 h⁻¹), as suggested by the increased cellobiase productivity (Figure 6). Eventually, the decline trend set in at even higher dilution rates (D ≥ 0.122 h⁻¹), just as that observed for the productivity of PFU and CMC-ase.

The use of enzymatic hydrolysate for cellulase production is economically significant, especially when integrated in the overall process of cellulosic biomass conversion. However, the induction and production characteristics of the hydrolysate-supported systems are complex, as shown in this work. A future study is warranted to provide clearer understanding and to develop a more quantitative kinetic description of the systems.

Conclusions

The enzymatic hydrolysate of wastepaper was used as the soluble inducing substrate for cellulase production in continuous culture of *Trichoderma reesei* RUT C30. The pH was controlled at 5.0 ± 0.2 and temperature at 25 ± 3 °C. With the medium C:N ratio used by Tangnu et al. (*15*), the apparent cell yield constant was found to be 0.76 (g of dry cell weight/g of reducing sugar) and the maximum specific cell growth rate 0.26 h⁻¹.

The study on the feasibility of cellulase production by resting cells indicated that this could not be accomplished by total elimination of N sources from the medium because rapid cell deterioration and death would occur. The study on the effects of medium C:N ratio further confirmed an important role of N sources in the cellulase synthesis: cellulase production was found to decrease significantly when the feed concentrations of N sources were reduced from that used by Tangnu et al. (*15*). An experiment at pH 7.5 with 4-fold N source concentrations also led to poorer cellulase production, although the cell

concentration was much higher due to the contribution of peptone as C source.

The hydrolysate was a much better soluble inducer than sophorose. While they showed comparable specific cellulase productivities at a low dilution rate ($0.012 \ h^{-1}$), their performance with increasing dilution rates exhibited opposite trends. The specific productivity in sophorose-induced systems was reported in the literature to decrease with an increase in the dilution rate. On the other hand, the specific FPU productivity in the hydrolysate-supported systems increased initially with the dilution rate. A 6-fold higher maximum productivity was reached at $D = 0.122 \ h^{-1}$ before it began to decline. A similar trend was observed for the CMC-ase productivity. The cellobiase productivity was, however, relatively constant at low dilution rates, increasing at the middle range, and decreasing eventually as the productivity of FPU and CMC-ase. The phenomena may be explained by the change of inducer concentrations with the dilution rate in the hydrolysate-supported systems.

When compared with cellulose, the wastepaper hydrolysate was found to have similar cellulase-inducing strength and to induce an apparently complete set of cellulase components.

Acknowledgment

References and Notes

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