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Cellulase Production by *Trichoderma reesei* Using Sawdust Hydrolysate

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Abstract

Sawdust hydrolysates were investigated for their ability to support cell growth and cellulase production, and for potential inhibition of *Trichoderma reesei* Rut C30. Simultaneous fermentations were conducted to compare the hydrolysate-based media with the controls having equivalent concentrations of glucose and Avicel cellulose. Six hydrolysates differing in the boiling durations in the hydrolysis procedure were evaluated. The hydrolysates were found to support cell growth and induce active cellulase synthesis. The maximum specific cellulase production rate was 0.046 filter paper units (FPU)/(g of cells · h) in the hydrolysate-based systems, much higher than that (0.017 FPU/[g of cells · h]) in the controls.

Index Entries: Acid hydrolysis; cellulase; induction; inhibition; *Trichoderma reesei.*

Introduction

Plant biomass is the only foreseeable sustainable source of fuels and materials on Earth *(1)*. Lignocellulosic materials are particularly attractive because of their relatively low cost and plentiful supply. More widespread utilization of these renewable resources has been impeded by the absence of low-cost, mild, and environment-friendly technology for their hydrolysis to simple sugars, which can then be converted into useful products. Cellulase is a multicomponent enzyme system that can effectively hydrolyze cellulose to glucose. Effective and economic cellulase production is therefore critically important to the utilization of lignocellulosic materials *(1,2)*. Batch or fed-batch fermentations of the fungi Trichoderma are often used for cellulase production *(3,4)*. Cellulase synthesis in Trichoderma is subject to induction, the natural inducers being the intermediates formed during the cellulose hydrolysis *(5,6)*. Solid

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cellulosic materials were therefore commonly used as both the carbon/energy substrate and the source of inducers in the fermentation *(6–8)*. The use of solid substrates, however, causes certain problems with the fermentation's operation and productivity. In particular, the solids increase the burden on agitation and lower the oxygen supply efficiency of the bioreactors. The compromised oxygen supply, in turn, reduces the employable cell concentration and the attainable productivity. Lower solid concentrations have been shown to yield higher cellulase production *(9)*.

Recently, we have been investigating the feasibility of combining *in situ* foam fractionation with cellulase fermentation *(10)*. The coupled process has the potential to improve cellulase production by minimizing catabolite (glucose) repression and reducing cellulase degradation by proteases. During the study of foaming behaviors of fermentation broths of *Trichoderma reesei* Rut C-30, the foams were also found to remove the solid cellulose (Avicel) present. To minimize the substrate loss, it is desirable to use soluble substrates in the coupled foam-fermentation process. The soluble substitutes must still be able to induce cellulase synthesis. Among several pure saccharides studied, sophorose was the most powerful soluble inducer for *Trichoderma (11–14)*. Nevertheless, sophorose was expensive and still less potent in induction when compared to cellulose *(15)*. Bailey and Taehtiharju *(16)* have recently used the cheaper lactose as the sole inducing C source for cellulase production, using the base addition for pH control as the guiding factor to regulate the substrate feeding. In addition, the enzymatic hydrolysate of pure cellulose or complex cellulosic materials such as wastepaper has been shown to be effective in cellulase induction *(17–19)*.

In the present study, acid hydrolysates prepared from hardwood sawdust *(20)* were investigated for their ability to support cell growth and cellulase production, and for their potential inhibition of microorganisms. The latter was often found with complex hydrolysates and attributed to acid hydrolysis byproducts such as furfural, acetic acid, levulinic acid, and hydroxymethylfurfural (HMF) *(21–23)*. Overliming has been reported to reduce the inhibitory effects *(24)*, in addition to neutralizing the acid used in hydrolysis.

In the present study, simultaneous fermentations were conducted to compare the hydrolysate-based media with their equivalent controls of mixed glucose and Avicel cellulose. The hydrolysates prepared with different procedures could therefore be evaluated for their efficacy in cellulase production.

Materials and Methods

Preparation of Hydrolysate

Mixed hardwood sawdust was obtained from a local sawmill and sieved through a 20-mesh screen. The sawdust had a moisture content of 9.3%, measured by a moisture analyzer (Ohaus MB200). The biomass (220.5 g) was put in a 6-qt bowl of a KitchenAid dough mixer equipped

with a dough hook. Concentrated sulfuric acid (80 wt%) was added slowly to the mixed biomass. A total of 210.0 g of acid was added so that a solid-to-acid ratio of 1.05 was obtained. The acid-biomass "gel" was kneaded for 30 min starting at the addition of acid. A significant reduction in volume resulted from the mixing. The final "gel" was dark and viscous.

At the end of mixing, 609.5 g of distilled water was added to the gel, to dilute the acid to 20%. The dough hook was replaced with a wire whip, and the mixture was stirred for 15 min in order to dissolve all clumps. The unreacted solids were removed by vacuum-filtering the mixture through a plastic sheet (approx 20 mesh). The filtrate was boiled gently, with the addition of water to compensate for vaporization loss, for a total of 120 min. During the boiling, samples of about 100 mL each were removed at 15, 30, 45, 60, 90, and 120 min. The boiled filtrate samples were cooled by immersing in an ice-water bath. The cooled samples had a pH of about 1.8. Calcium hydroxide was then added to adjust the sample pH to 9.0. The "overliming" precipitated out the excess sulfate and was reported to remove/detoxify certain inhibitory compounds generated in the hydrolysis process *(25).* The overlimed samples were centrifuged to remove the solid precipitates. The pH of the supernatants were then adjusted to 7.0, with 1 *N* HCl, and used to prepare the media for the cellulase production study.

Microorganism, Media, and Fermentation

T. reesei Rut C30 (NRRL 11460) was maintained at 4°C on slants of potato dextrose agar (39 g/L, as recommended; Sigma, st. Louis, mo) and subcultured every 3 to 4 wk. The inocula were prepared by transferring three loops of cells from an agar slant to 50 mL of potato dextrose broth (Sigma) in a 250-mL flask. After growing for 4 d in a shaker (Model 4703, Queue Systems, Queue Orbital shaker; Parkersburg, WV) at 200 rpm and 25°C, 1 mL of the culture was inoculated to each system. The composition of the medium was essentially the same as that of Mandels et al. *(26)* except that the 10 g/L of cellulose was replaced with different inducing C substrates. For the hydrolysate-based systems, 10 g/L of combined cellulosic oligomers and glucose was used. For the controls, cellulose powders and glucose were added at the same amounts as the oligomers and glucose, respectively, in the corresponding hydrolysates. Twelve fermentations were conducted: six hydrolysates and six corresponding controls. The concentrations of glucose and cellulose/oligomers in the studied systems are summarized in Table 1; two sets of values were given for each system: measured before and after, respectively, the prepared medium was autoclaved for sterilization (more details on the effects of autoclaving are described later in Results). Different hydrolysates were used in different batches of the hydrolysate-based systems. The difference was in the boiling duration involved in the hydrolysate preparation, as described earlier and given in Table 2 in the corresponding order to the batch number. The cultures were grown for 5 d in shake flasks containing 55 mL of medium each

Table 1
Concentrations of Glucose and Cellulose/Oligomers in Hydrolysate-Based Media and Corresponding Controls[a]

	Hydrolysate-based media					Controls			
	Before autoclaving			After autoclaving		Before autoclaving		After autoclaving	
Batch	Glucose (g/L)	Total sugars (g/L)	Oligomers (g/L)	Reducing sugars (g/L)	Oligomers (g/L)	Glucose (g/L)	Cellulose (g/L)	Reducing sugars (g/L)	Cellulose (g/L)
1	2.1	6.2	7.2	10.4	3.6	2.1	7.2	2.6	6.8
2	3.1	8.3	5.9	13.0	2.8	3.1	5.9	3.5	5.5
3	4.0	9.9	4.7	14.2	2.3	4.1	4.7	4.3	4.5
4	5.3	11.6	3.5	15.0	1.9	5.3	3.5	5.9	3.0
5	7.4	14.1	1.7	16.7	0.7	7.4	1.7	8.5	0.6
6	9.0	16.1	0.5	16.2	1.5	9.0	0.5	9.3	0.3

[a]The remaining concentrations of oligomers and cellulose after autoclaving are estimated using equations similar to Eq. 1.

under the aforementioned conditions for inoculum preparation. Periodically samples were taken and analyzed.

Analytical Methods

Hydrolysate analyses were done after neutralization with calcium carbonate and removal of solids by centrifugation. The supernatants were measured for glucose concentration with a YSI 2700 Select Biochemistry Analyzer. Sugars and degradation products were analyzed using a high-performance liquid chromatography system (Thermal Separation Products Spectra System) with a refractive index detector. The columns used were Bio-Rad Aminex HPX-87P (85°C) and -87H (65°C) for sugar and organic acid analysis, respectively. The mobile phases were water and 0.01 *N* sulfuric acid, respectively, both at 0.6 mL/min.

The fermentation samples were analyzed for reducing sugar concentrations, cell concentrations, and cellulase activities. The methods are described in more detail elsewhere *(10,27).* Briefly, the reducing sugar concentration was measured by the nonspecific dinitrosalicylic acid method. The cell dry weight concentrations could not be measured directly in the controls containing solid cellulose. All samples were therefore analyzed for intracellular protein concentrations, and the corresponding cell dry weight concentrations were estimated using a preestablished correlation: [Cell Concentration] (g/L) = [Intracellular Protein Concentration] (g/L) × 8.0 (±0.5). The total cellulase activity was measured using a standard filter paper assay *(28).*

Results

Hydrolysis

Table 2 summarizes the concentrations of various sugars and degradation products present in the overlimed hydrolysates with different boiling times. A hydrolysate sample was autoclaved at 120°C for 2 h to give the maximum glucose concentration, $[G_{max}]$, attainable. Also included are the oligomer concentrations estimated using the following equation:

$$[\text{Oligomers}] = \left\{[G_{max}] - \left[[G_1] + [G_2] \cdot \left(\frac{360}{342}\right)\right]\right\} \cdot \left(\frac{165}{180}\right) \quad (1)$$

$[G_1]$ and $[G_2]$ are the concentrations of glucose and cellobiose, respectively. The ratio of 360/342 is used to calculate the glucose concentration from cellobiose hydrolysis; 342 is the mol wt of cellobiose, and 360 (=2 × 180) accounts for the 2 glucose mol from hydrolysis of each cellobiose molecule. The glucose concentrations from glucose and cellobiose are thus first subtracted from $[G_{max}]$ to obtain the remainder that should have come from oligomer hydrolysis. The oligomer concentration is then estimated by multiplying the remainder by the ratio of 165/180, assuming that the oligomers

Table 2
Sugars, Degradation Products, and Cellulosic Oligomers in Hydrolysates with Different Durations of Boiling[a]

Hydrolysate boiling duration (min)	Concentration (g/L)										
	Sugars						Degradation products				
	Glucose	Cellobiose	Xylose	Galactose	Arabinose	Mannose	Acetic acid	Levulinic acid	HMF	Furfural	Oligomers
15	2.10	0.83	3.76	<0.10	0.15	0.23	1.61	<0.10	<0.10	<0.10	7.25
30	3.06	1.31	4.44	<0.10	0.38	0.44	1.58	<0.10	<0.10	<0.10	5.91
45	4.05	1.57	4.94	<0.10	0.41	0.54	1.51	<0.10	<0.10	<0.10	4.74
60	5.27	1.70	5.32	<0.10	0.41	0.58	1.48	<0.10	<0.10	<0.10	3.51
90	7.39	1.59	5.59	<0.10	0.42	0.69	1.29	<0.10	<0.10	<0.10	1.66
120	9.04	1.28	5.81	<0.10	0.44	0.79	1.21	<0.10	<0.10	<0.10	0.46
Autoclaved	10.88	<0.10	4.73	<0.10	0.35	0.86	1.64	<0.10	0.09	0.69	—

[a]The autoclaved sample was autoclaved at 120°C for 2 h, to give the maximum glucose obtainable from the hydrolysis. The oligomer concentrations were calculated according to Eq. 1, as described in the text.

have an average degree of polymerization (DP) of 6, i.e., 165 = [180 × 6 – (18 × 5)]/6. The ratio would not change much with different DPs, e.g., 168/180 for DP = 3 and 163.8/180 for DP = 10.

As shown in Table 2, the hydrolysates had similar cellobiose concentrations (0.83–1.70 g/L) but notably different oligomer concentrations, which decreased from 7.25 g/L after 15 min of boiling to 0.46 g/L after 120 min of boiling. None of the hydrolysates contained excessive amounts of potentially inhibitory levulinic acid, HMF, or furfural.

Fermentation

The profiles of cell growth, decrease in reducing-sugar concentration, and cellulase (filter paper units [FPU]) production are shown in Figs. 1 and 2 for the control and hydrolysate systems, respectively.

Cell Growth

Distinctly longer lag phases, ~1 d, were observed for cell growth in all of the hydrolysate systems, presumably because of the presence of hydrolysis byproducts *(21–23,29)*. Nonetheless, the cells proliferated rapidly after the lag phase and reached the stationary phase in 1 d.

As shown in Fig. 1A, glucose supported higher cell growth than cellulose, presumably because of the diffusion limitation of the insoluble cellulose *(30)* and/or the additional resources and energy spent on producing the cellulase required for cellulose hydrolysis (as indicated in Fig. 1C). On the other hand, the maximum cell concentrations were approximately the same in all of the hydrolysate systems (Fig. 2A), indicating the easier assimilation of hydrolysates than the insoluble cellulose. The maximum cell concentrations in the hydrolysate systems were, however, lower than those in the controls (see Discussion).

Reducing-Sugar Consumption

In all systems, the profiles of decrease in reducing-sugar concentration mirrored those of cell growth. Stationary phase was reached at depletion of the sugars, confirming that the systems were C source limited. Reducing-sugar concentrations were higher in the hydrolysate systems than in the controls, because the former contained sugars other than glucose (Table 2) and because large fractions of the hydrolysates' oligomers were hydrolyzed to sugars during the steam sterilization (Table 1). The higher sugar concentrations in the hydrolysates did not yield more cells, although the sugars were all depleted (Fig. 2B).

Cellulase Production

For the controls (Fig. 1C), the cellulase production clearly increased with increasing cellulose contents. The effect was particularly significant when cellulose was 20–60% of the total C substrates (glucose plus cellulose). Below 20%, the cellulose content was too low to provide sustained

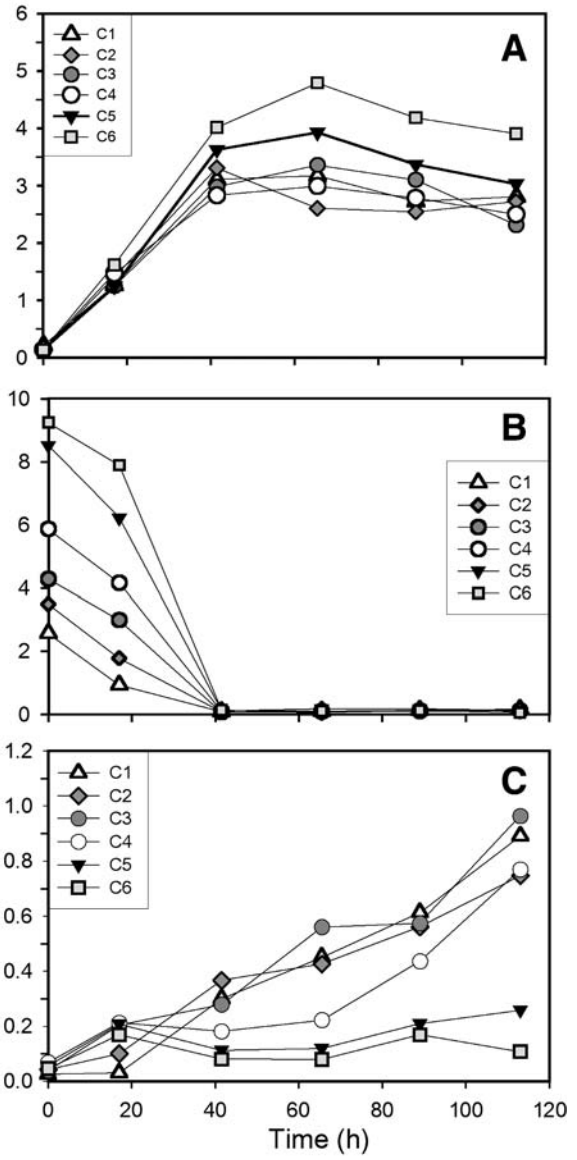

Fig. 1. Profiles of **(A)** cell growth, **(B)** reducing-sugar consumption, and **(C)** cellulase production observed in control systems.

induction, presumably because most of the cellulose was hydrolyzed during the early stage (by the cellulase produced at low, constitutive levels and/or introduced with the inoculum) when the high glucose concentration repressed active cellulase synthesis. At a cellulose content >60%, cellulase production appeared to reach a plateau. Similar but less pronounced effects were also observed in the hydrolysate systems (Fig. 2C). Note that the hydrolysates with lower than 20% of oligomers (H5 and H6) supported active cellulase production whereas the corresponding controls

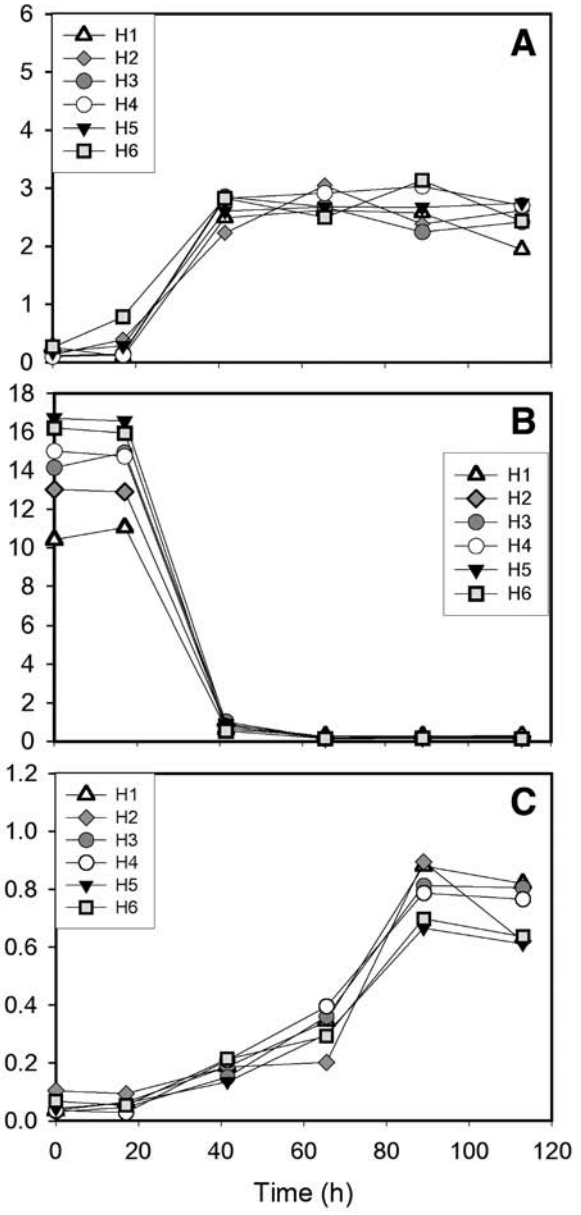

Fig. 2. Profiles of **(A)** cell growth, **(B)** reducing-sugar consumption, and **(C)** cellulase production observed in systems containing sawdust hydrolysates prepared with different lengths of boiling.

(C5 and C6) did not. Xylose is known to induce cellulase synthesis, although not nearly as effectively as cellulose *(31)*. It is likely that the nonglucose sugars present in the hydrolysates facilitated the induction for cellulase synthesis. The cellulase concentrations in the hydrolysate systems leveled off or started to decrease after 90 h, suggesting the depletion of inducing oligomers and sugars. Most important, the effectiveness of the

hydrolysates as soluble cellulase inducers is clearly demonstrated. To be more quantitative, the maximum specific cellulase production rate in each system was calculated for the most productive 1-d period, i.e., 89–113 h for the controls and 65.5–89 h for the hydrolysate-based systems. The rates were then averaged for all productive systems, which for the controls included the four systems with high enough cellulose contents (C1–C4) and for the hydrolysate-based systems included all six systems. The averaged maximum specific rate was 0.046 FPU/(g of cells · h) for the hydrolysate-based systems, much higher than that (0.017 FPU/[g of cells · h]) for the controls.

Discussion

Szengyel et al. *(9)* found that *T. reesei* would consume soluble sugars after all furfural in the hydrolysate of steam-pretreated willow was digested. Palmqvist et al. *(22)* reported that *T. reesei* could digest most of the compounds in the hydrolysate including the putative toxic compounds such as furfural, acetic acid, and HMF. In addition, glucose and xylose were found to be consumed simultaneously. Although the cell growth profiles were not shown in either study, the existence of lag phase could be inferred from the profiles of sugar consumption in their experiments. Our observation of longer lag phases in the hydrolysate-based systems in our work was probably owing to induction of enzymes needed to digest the inhibitory compounds.

As summarized in Table 3, the apparent cell yields from all of the C sources (including sugars and oligomers/cellulose), Y_{Total}, ranged from 0.32 to 0.46 in the controls but only 0.13–0.17 in the hydrolysate-based media. The varying Y_{Total} in the controls was owing to their different fractions of glucose and cellulose. Using the Solver program in Microsoft Excel, the separate cell yields from glucose (Y_{G1}) and cellulose (Y_{Gn}) were estimated from the equation [cell concentration (X)] = $Y_{G1}[G_1] + Y_{Gn}[G_n]$. The best-fit yields were $Y_{G1} = 0.43$ and $Y_{Gn} = 0.25$. Because the experiments were done without pH control, these yields were significantly lower than those reported in the literature under conditions of well-controlled pH. For example, the cell yield was 0.76 from the reducing sugars in an enzymatic hydrolysate of wastepaper at pH 5.0 and 25°C *(19)*. Nonetheless, $Y_{Gn} = 0.25$ was close to the value of 0.29 observed by Schaffner and Toledo *(31)* in similar batch fermentations.

Similarly, the cell yields in the hydrolysate-based systems were estimated as $Y_{G1+G2} = 0.21$, $Y_{Gn} = 0.21$, and Y_{X+A+M} (cell yield from combined xylose, arabinose, and mannose) = 0.08. The outcome of equal Y_{G1+G2} and Y_{Gn} was somewhat artificial because the fitting was done with a reasonable restriction of $Y_{G1+G2} \geq Y_{Gn}$. Nonetheless, the two similar yields confirmed the easy assimilation of oligomers.

Table 3
Cell Yields in Controls and Hydrolysate-Based Systems[a]

	Controls			Hydrolysate-based systems		
Batch	*X (g/L)*	Y_{Total}	*Y* (best fit)	*X* (g/L)	Y_{Total}	*Y* (best fit)
1	3.0	0.32		2.6	0.14	$Y_{G1+G2} = 0.21$
2	2.8	0.32	$Y_{G1} = 0.43$	2.6	0.13	
3	2.9	0.33		2.6	0.14	$Y_{X+A+M} = 0.08$
4	3.2	0.36		2.9	0.16	
5	3.6	0.40	$Y_{Gn} = 0.25$	2.7	0.14	$Y_{Gn} = 0.21$
6	4.3	0.46		2.8	0.17	

[a]*X*, maximum cell concentration; *Y*, apparent cell yield (g of dry cells/g of substrate); Y_{Total}; *Y* based on total C substrates (all sugars, oligomers, and cellulose); *Y* (best fit), best-fit *Y* values calculated using Solver in Microsoft Excel (see text); G_1, glucose; G_2, cellobiose; G_n, oligomers/cellulose; *X+A+M*, xylose, arabinose, and mannose.

Hydrolysates contained nonglucose sugars, particularly xylose in this case. Adenosine triphosphate (ATP) yield from xylose to pyruvate was estimated at 0.67 ATP/xylose for *Escherichia coli (32)*, compared with 2 ATP/glucose via the Embden-Meyerhof-Parnas pathway. Furthermore, yeasts and fungi synthesize xylitol from xylose as a byproduct *(33,34)*. Lower cell yields from hydrolysate-based media, estimated here on the basis of consumed reducing-sugar concentrations, were therefore expected. However, note that Y_{G1+G2} (predominantly from glucose) in the hydrolysate was much lower than Y_{G1} in the controls, and Y_{X+A+M} (primarily from xylose) was much lower than the Y_X (=0.24) observed by Schaffner and Toledo *(31)*. The lower-than-expected yields suggested that certain inhibition was caused by hydrolysates so that either the sugars were less completely catabolized or higher cell death/decay rates were associated with the hydrolysate-based media. The latter would be significant especially because the cell concentrations in our study were determined from intracellular protein concentrations. Damaged or lysed cells would lose their intracellular proteins and, therefore, not be included in the measured values. More studies are needed to identify the responsible mechanism(s) and/or compounds for the negative effects so that the hydrolysate can be more effectively used for cellulase production.

In conclusion, despite the potentially inhibitory effects, the hydrolysates supported cell growth and induced active cellulase synthesis. The maximum specific cellulase production rate in the hydrolysate-based systems was 0.046 FPU/(g of cells · h), much higher than that (0.017 FPU/[g of cells · h]) in the cellulose-based controls. The results of this study encourage the feasibility of using hydrolysates to formulate solid-free media for cellulase production in coupled fermentation and *in situ* foaming, to avoid the loss of solid substrate in the foaming operation.

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