

Whole Broth Cellulase Production for Use in Simultaneous Saccharification and Fermentation

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ABSTRACT

Cellulase, an enzyme that catalyzes the breakdown of cellulose into glucose, is produced inside fungal cells and secreted into the surrounding media. However, the cells retain significant amounts of β -glucosidase, and this extra enzyme could improve the performance of a simultaneous saccharification and fermentation (SSF) process. To test the possible contribution of mycelia-bound enzyme to SSF performance with modern fungal cellulase producers, the performance of SSF using whole culture broth (with cells) was compared to SSF performance using culture filtrate. Cellulase broth was produced using the advanced *Trichoderma reesei* mutant L27. Measurements of cellulase activities showed that the broth had more activity than the filtrate. Subsequent SSF experiments were conducted at 37°C in shake flasks with *Saccharomyces cerevisiae*, and the broth produced 8–25% higher ethanol yields than the cellulase filtrate produced.

Index Entries: Cellulase; cellulose; ethanol; fermentation; *Trichoderma reesei*.

INTRODUCTION

Simultaneous saccharification and fermentation (SSF) is a promising method of economically producing ethanol from lignocellulosics (1). The process uses cellulase enzyme to saccharify cellulose to glucose, which is

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simultaneously converted to ethanol by yeast, all in the same reactor. The immediate removal of glucose prevents inhibition of the cellulase enzyme. Thus, higher ethanol yields are achieved than with separate saccharification and fermentation steps. However, the economic performance of SSF depends on the characteristics and cost of the cellulase (2). For example, differences in SSF performance, resulting from the use of different batches of commercial cellulase, gave 22% differences in the potential price of ethanol. The best performance was produced by the batch with the highest β -glucosidase content, and the performance of every batch was enhanced by the addition of β -glucosidase (3).

Cellulase is produced commercially by the fungus *Trichoderma reesei*. The enzyme is made inside the fungal mycelia and then is secreted into the surrounding media in a soluble form. After the fermentation is complete, the cells are separated from the fermentation broth, and the soluble enzyme is concentrated and sold. Recent work has shown that as much as 50% of the β -glucosidase activity and 7% of the endoglucanase activity remains bound to the cells of *T. reesei* QM 9414 (4). Thus, one possible method of improving SSF performance is to use whole cellulase culture broth (with cells) instead of culture filtrate (without cells), thereby using the enzyme retained in the cells. In the first reported study of SSF, Takagi et al. (5) reported as much as a 25% improvement in ethanol yield when culture broth from the early cellulase-producing strains *T. viride* QM 9123 and *T. viride* QM 9414 were used in place of culture filtrate in SSF experiments with *Saccharomyces cerevisiae* IAM 4178. Furthermore, the addition of whole cell broth will supply cellulose left over from cellulase production to SSF, avoiding a loss of yield while eliminating an additional separation step from the overall process.

Over the past years, many mutants have been developed to improve the release of β -glucosidase into the culture broth, and some questions have existed about the advantages of using whole cell broth in SSF. To test the possible contribution of mycelia-bound enzyme to SSF performance with modern enzyme preparations, we produced cellulase using a mutant of *T. reesei*. Multiple side-by-side experiments were conducted comparing the SSF performance of cellulase filtrate and that of broth using *S. cerevisiae* as the ethanol producing organism.

MATERIALS AND METHODS

Materials

The microorganism used in this study was the yeast strain *S. cerevisiae* D₅A, a SERI strain. Cellulase was produced by *T. reesei* L27, an organism kindly donated by Cetus Corporation in Emeryville, CA. Cellulose for the SSF experiments was Sigmacell 50 obtained from the Sigma Chemical Co. (St. Louis, MO). The substrate for cellulase production was Solka Floc

Table 1
Media for Cellulase Production

		M1	M2	M3
(NH ₄) ₂ SO ₄	g/L	1.4	1.4	1.4
KH ₂ PO ₄	g/L	2.0	2.0	2.0
MgSO ₄ · 7H ₂ O	g/L	0.3	0.3	0.3
CaCl ₂ · 2H ₂ O	g/L	0.4	0.4	0.4
Tween 80	mL/L	0.2	0.2	0.2
Corn steep liquor	g/L	15.0	15.0	15.0
Antifoam	mL/L	-	-	0.7
Glucose	g/L	10.0	-	-
Solka floc	g/L	-	10.0	50.0
Trace Elements ^a				
FeSO ₄ · 7H ₂ O	mg/L	5.0	5.0	5.0
MnSO ₄ · H ₂ O	mg/L	1.6	1.6	1.6
ZnSO ₄ · 7H ₂ O	mg/L	1.4	1.4	1.4
CoCl ₂ · 6H ₂ O	mg/L	2.0	2.0	2.0

^aPrepared as 200X stock solution and used at 5.0 mL/L.

from the James River Corporation (Berlin, NH). All other chemicals and reagents were obtained from the Sigma Chemical Co.

Methods

Cellulase Production

The procedures used for cellulase production and the media composition are essentially from Mohagheghi et al. (6). *T. reesei* was kept as frozen spores in 10% glycerol at -70°C. The culture for the fermenter inoculum was obtained by transferring frozen spores to 100 mL of media M1 and incubating at 28°C in a rotary shaker. The compositions of media M1, M2, and M3 used in this study are defined in Table 1. After 3d, the mycelia was transferred to agar plates consisting of 20% V-8™ vegetable juice. These plates were kept at 30°C for a few days until the culture sporulated, and then the plates were stored at 4°C in a refrigerator.

Cellulase for the initial SSF experiments was produced in a 5-L New Brunswick Scientific Co. (Edison, NJ) Bioflo II fermenter. To begin a fermentation, spores were transferred from a plate to a shake flask containing 200 mL of media M2 that had been adjusted to pH 4.8 by the addition of 5% NaOH. This media was incubated at 28°C for 4 d in a rotary shaker (150 rpm) and then used as the inoculum for the 5-L fermenter. The fermenter media was 3.5 L of M3, also adjusted to pH 4.8. Temperature and pH were maintained at 28°C and 4.8, respectively, for the entire run. The pH was controlled by the addition of either 4N H₃PO₄ or 4N NH₄OH. Dissolved oxygen was maintained above 20% air saturation by the addition

of oxygen. Agitation was set at 275 rpm during the first 3 d of the run, and as the culture thinned over the next few days, the agitation was reduced stepwise to 150 rpm. Foam level was automatically controlled using a capacitance foam probe to activate a peristaltic pump that delivered diluted (1:20) Sigma antifoam B to the fermenter. At the end of the run, the entire fermenter contents was harvested for the cellulase broth.

For the last SSF experiment, only a portion of the fermenter contents was harvested during each of the last few days of production. This allowed us to compare broth performance as a function of harvest day. Since this required a larger volume of cellulase broth, it was produced with a 7-L Chemap fermenter using 6 L of media M3. The inoculum for this fermenter was obtained by transferring spores from a plate to 50 mL of media M1, and after 4 d of incubation, the culture was transferred to 450 mL of M2. The 7-L fermenter was inoculated with the culture after another 3 d of incubation. All procedures and conditions used for the 7-L fermenter were the same as described above for the 5-L fermenter, except diluted (1:10) antifoam was used.

SSF Experiments

Small-scale SSF experiments were conducted according to previously presented methods (7). Media was placed in 250-mL shake flasks constructed to vent carbon dioxide into water traps. All flasks were incubated at 37°C and agitated at 150 rpm in a rotary shaker. The substrate was 7.5% Sigmacell 50, and the media consisted of 1% yeast extract and 2% peptone. A lipid mixture of 5 mg/L ergosterol and 30 mg/L oleic acid was added to improve ethanol tolerance, and penicillin and streptomycin at 10 mg/L were added to minimize contamination. All components were autoclaved together, except antibiotics, which were added after autoclaving. Finally, enzyme broth or filtrate taken directly from the cellulase fermenter and yeast inoculum (10 mL *S. cerevisiae*) were added to each flask. The total volume in each flask was 100 mL. The enzyme broth volume was slightly greater than the filtrate volume such that equal amounts of soluble cellulase were added to each flask. This required knowledge of the volume fraction of cells in the broth, which was determined by filtration of a known broth volume. The soluble cellulase loading was approximately 7 IFPU/g of cellulose for these experiments, and each condition was duplicated 4 x in order to obtain an average ethanol yield. Samples from each flask were taken daily and frozen until they could be analyzed for ethanol.

Concentration Measurements

Glucose was measured by a Yellow Springs Instruments (Yellow Springs, OH) (YSI) Model 27 analyzer. Total sugars (glucose plus cellobiose) were determined by incubating a sample with an equal volume of β -glucosidase solution (2.0 mg/mL) at 37°C for at least 2 h, and then measuring glucose concentration with the YSI analyzer. Ethanol was measured by HPLC using a Bio-Rad (Richmond, CA) HPX-87H column and a refractive

index detector. The column was maintained at 65°C, and the solvent was .012N sulfuric acid at .8 mL/min.

Activity Measurements

Filter-paper activity of the filtrate was determined according to the method recommended by the International Union of Pure and Applied Chemistry (8). The β -glucosidase activity of the filtrate was determined by hydrolysis of *p*-nitrophenol- β -glucopyranoside (PNPG) (9). The concentration of *p*-nitrophenol after hydrolysis was determined by the extinction of 410 nm light after addition of 1.0M NaCO₃. The assay was conducted at 50°C for 30 min, and the unit of activity was defined as the amount of enzyme that cleaves one micromole of substrate/min.

The effective filter-paper activity associated with the broth was determined by conducting a side-by-side measurement of total sugars produced by a broth and filtrate cellulase sample. The ratio of sugars produced by the broth to sugars produced by the filtrate, multiplied by the measured filter-paper activity of the filtrate, is defined as the effective-filter paper activity of the broth. Again, a greater broth volume was used such that the amount of soluble cellulase for the broth assay was the same as that for the filtrate assay. More sugar production by the broth would reflect the extra activity bound to the mycelia. The assay was conducted in 0.05M citrate buffer and 0.025M NaF with 150 mg of Sigmacell 50. The NaF killed the cells, preventing any metabolism of the sugars. Temperature was maintained at 50°C for 1 h, and the reaction was quenched by placing the samples in boiling water for 5 min and then removing them to an ice bath. Effective PNPG activity of the broth was determined using the same method as above with replacement of cellulose by 5 mg of cellobiose, measuring only glucose production, and reduction of the assay time to 30 min. Corrections were made for residual sugars in the enzyme samples and for carry-over of cellulose in the broth samples, which required a measurement of cellulose in the broth. This extra cellulose caused a slight increase in glucose production over controls without the extra cellulose.

Cellulose Measurement in the Presence of Fungi

Cellulose in the broth samples was determined by dissolving the mycelia and weighing the sample to determine the amount of leftover cellulose. The procedure used an acid treatment to dissolve the cell walls and then NaOH to dissolve proteins and other cellular constituents. To begin, a 2-mL broth sample was suspended in 3.7% HCl for 2 h at 80°C. The sample was then repeatedly washed with water and centrifuged until the pH was neutral. The pellet was resuspended in 2.5% NaOH and heated to 65°C for 30 min. Again, the pellet was repeatedly washed and centrifuged until the pH was neutral. Then the acid treatment was repeated to attack any resistant cell wall fractions. After a final wash, the sample was filtered and dried in a 105°C oven for 2 h. Cellulose concentration was calculated from a measurement of dry weight. A correction factor for

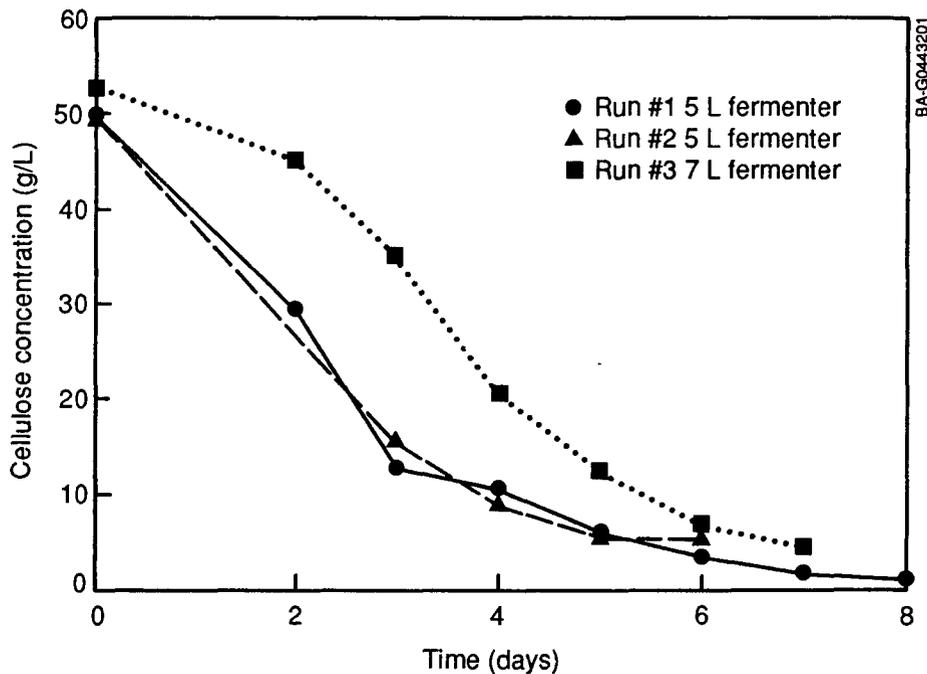


Fig. 1. Cellulose concentration as a function of fermentation time for three cellulase production runs.

cellulose lost during the procedure was determined by using a known weight of Solka Floc as a control.

RESULTS

Cellulase Production

Figure 1 shows the cellulose concentrations in the three cellulase production runs used for the SSF work. The first two runs used the 5-L fermenter and had similar cellulose utilization curves. The third run used the 7-L fermenter, and cellulose utilization lagged behind the first two runs. The activities of the filtrate and the effective activities of the broth for the first run are shown in Fig. 2. Filter-paper activity (as a measure of cellulase activity) for both filtrate and broth peaked on day 7 at 6.2 and 8.1 IU/mL, respectively. Activities on PNPG (as a measure of β -glucosidase activity) were the highest on day 8 at 5.9 IU/mL for the filtrate and 6.3 IU/mL for the broth. This run was harvested on day 8, with corresponding filtrate and broth filter-paper activities of 5.8 and 6.2 IU/mL, respectively, and PNPG filtrate and broth activities of 5.9 and 6.3, respectively.

Since differences between filtrate and broth filter-paper activities were greater during the earlier part of the first cellulase production run, the cellulase fermenter was harvested on day 6 for the second experiment

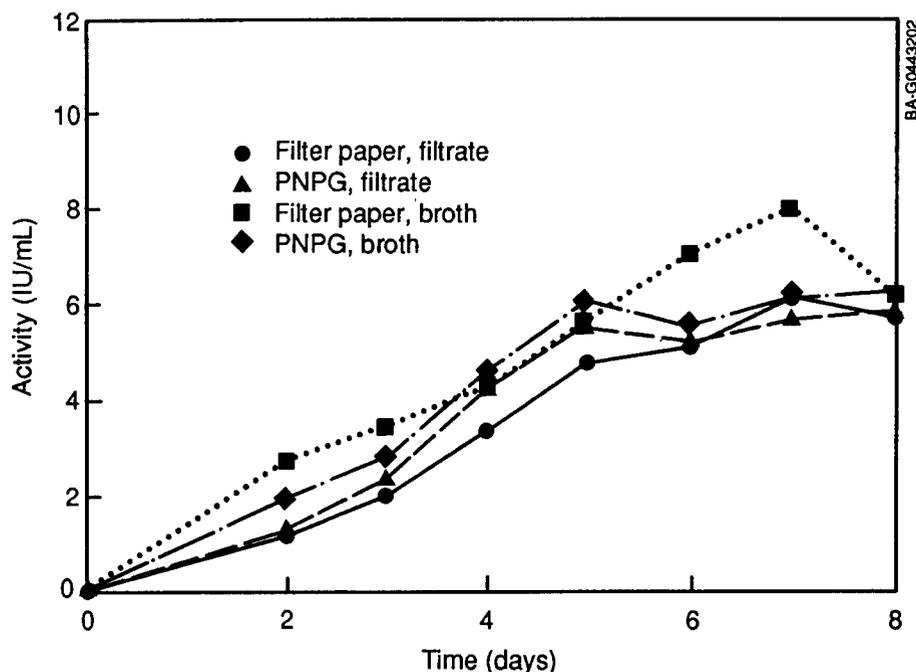


Fig. 2. Filtrate and broth activities as a function of fermentation time for the first cellulase production run.

(Fig. 3). The day 6 filter-paper activities were 5.8 and 8.2 IU/mL for the filtrate and broth, respectively, and PNPg activities were 5.4 and 6.0 IU/mL, respectively.

The third cellulase production run is shown in Fig. 4. Again, because of the slower utilization of cellulose in this run, the activities lagged behind the values obtained for the first two runs. The variability between cellulase production runs for both cellulose utilization and activity values are typical of these experiments. We have observed activity differences of 0–100% (data not shown) for different cellulase batches grown in the same fermenter at the same conditions, which we attribute to variabilities within the microorganisms. Broth was removed from the fermenter beginning on day 5, the first day having sufficient filter-paper activity to run a SSF experiment. Broth was also removed from the fermenter on days 6 and 7. This allowed us to examine the influence of mycelia on SSF performance when broth was harvested on different days. The final day 7 filter-paper activities were 5.8 and 7.9 IU/mL for filtrate and broth, respectively, and PNPg activities were 10.0 and 10.5 IU/mL, respectively.

SSF Experiments

Enzyme from each of the cellulase production runs (1–3) described above was used in SSF experiments 1–3, respectively. The first two experiments had soluble enzyme loadings of 7.7 IFPU/g of cellulose. For the

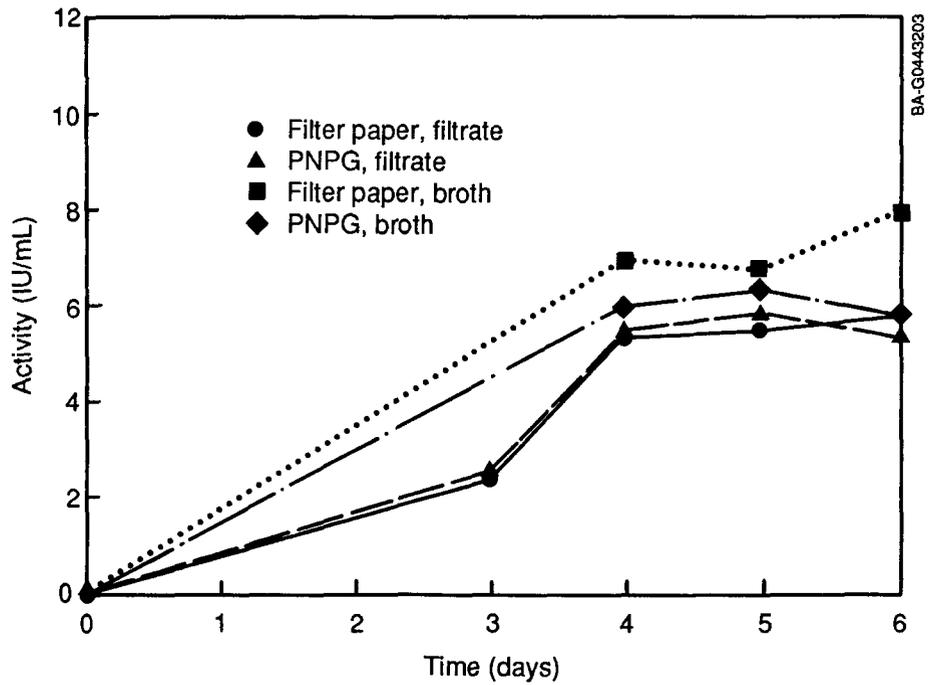


Fig. 3. Filtrate and broth activities as a function of fermentation time for the second cellulase production run.

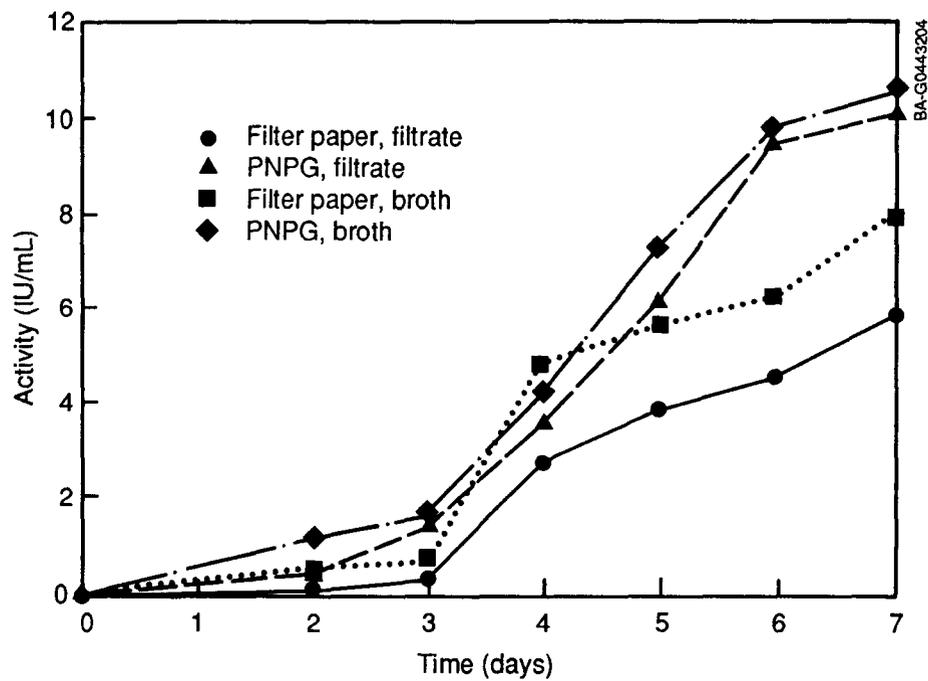


Fig. 4. Filtrate and broth activities as a function of fermentation time for the third cellulase production run.

Table 2
Ethanol Yields^a and Standard Errors for Filtrate and Broth SSF Experiments

SSF exp.	Filtrate	Broth	Process	Percent gain ^b
1	52.4±1.4	56.5±1.5	56.6±1.5	8.0
2	47.7±1.8	59.0±0.4	59.5±0.4	24.7
3/Day 5	50.7±1.9	54.4±1.0	55.7±1.1	9.9
3/Day 6	48.3±2.2	54.1±1.2	54.6±1.2	13.0
3/Day 7	55.8±3.1	61.0±0.3	61.3±0.3	9.9

^ag ethanol/g potential ethanol (*see text for definitions*)

^bProcess over filtrate

third SSF experiment, shake flasks were started with broth from days 5, 6, and 7 of the third cellulase production run and used a constant enzyme loading of 7.0 IFPU/g of cellulose.

The ethanol yields (as percent of theoretical conversion of cellulose to ethanol) from these experiments are shown in Table 2, when the enzyme source is either filtrate or broth. Also shown is the standard error, which is defined as the standard deviation of the measurements divided by the square root of the number of measurements. The first column contains the experiment number and, for the third experiment, the day the broth was harvested. The filtrate yield was calculated as the percentage of the original Sigmacell 50 cellulose converted to ethanol using filtrate as the cellulase source. The broth yield was calculated from the percentage of the original Sigmacell 50, plus Solka Floc carried over from the broth, that was converted to ethanol using broth as the cellulase source. The process yield is calculated as the percentage of the original Sigmacell 50 converted to ethanol using broth as the cellulase source, and thus is higher than the broth yield. The broth yield reflects the advantage gained from using the mycelia, and the process yield reflects the total gain associated with using mycelia and unreacted cellulose from cellulase production. The last column contains the percentage increase in yield when comparing the process yield to the filtrate yield.

DISCUSSION

For all experiments, the broth and process yields are greater than the corresponding filtrate yields, with process yields showing improvements of 8–25% over filtrate yields. There are significant differences in yields between the different cellulase batches, but this is to be expected as there is little reproducibility between batches and daily changes in enzyme composition. This fact, plus experimental error and the variability of the microorganisms, results in the wide range of values that were observed for both yield and percentage gain in yield. However, the broth clearly

does produce higher ethanol yields than the filtrate. Most of this increase is associated with using the mycelia, as there is very little cellulose left in the broth once activities begin to increase. Typically, 70–80% of the cellulose has been consumed before filter-paper activity reaches half of its peak value. This type of result has been observed by other researchers (10,11).

The third SSF experiment did not show any significant difference in broth performance as a function of harvest day (gains ranged from 10–13%). This is not surprising, because the broth activity for the last 3 d of the run (Fig. 4) was a constant amount greater than the filtrate. Thus, the choice of when to harvest the fermenter can be based on either filtrate or broth activity. Furthermore, since all the unreacted cellulose from the cellulase fermenter is effectively utilized in the SSF reactor, the decision as to when to harvest the cellulase fermenter should be based strictly on the criterion of achieving maximum volumetric productivity, and not on the criterion of achieving maximum utilization of the cellulose in the cellulase fermenter.

Finally, it should also be noted that the use of a different organism may result in better performance of the broth if the organism retains more β -glucosidase in the cell. It would be valuable to examine enzyme location in a variety of different cellulase-producing organisms and ultimately to determine their performance with SSF.

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