Production of Cellulase on Mixtures of Xylose and Cellulose in a Fed-Batch Process

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Cellulase production by the RUT-C30 mutant of the fungus *Trichoderma reesei* on mixtures of xylose and cellulose was studied in a fed-batch system. An initial mixture of 30 g/L xylose and 20 g/L cellulose and an intermittent feeding rate of 5 g/L/day xylose and 15 g/L/day cellulose (total of 20 g/L/day) produced the highest titer of enzyme activity of 12.5 IFPU/mL, 57% higher than for a batch system with similar mixed substrate. The total productivity for this feeding strategy was 45.4 IFPU/L/h, a 25% improvement compared to a batch system with similar mixed feed. The study also showed that xylose can be used to replace cellulose in both the initial mixture and the intermittent feed without affecting the maximal enzyme activity and productivity as compared to cellulose alone. However, substituting xylose for more than 25% of the cellulose in the intermittent feed suppressed enzyme production. Although the yield of cellulase in IFPU/g of carbon utilized is less for fed-batch than for a batch process, the fed-batch system achieves higher overall productivities, and any substrate left after enzyme production can be used in subsequent saccharification to sugars for ethanol production.

In the past decade, enzymatic hydrolysis of cellulose has received attention for renewable production of fuels from cellulosics because of the high selectivity of enzymatic hydrolysis compared to acid hydrolysis. In enzymatic hydrolysis, the substrate can in principle be converted completely to sugars, which are clean, and no extra process is required to remove impurities and toxic materials before fermentation. The sugars from hydrolysis can be used in production of liquid fuels or chemicals through fermentation.

In enzymatic hydrolysis, the cost of producing hydrolytic enzymes constitutes a major portion of total production cost, and according to Wright,[1] this cost can constitute approximately 25% of the overall ethanol fuel cost. To improve the economics of the enzymatic hydrolysis process, research has been focused on several areas including improving the strains of microorganisms, selecting an effective mode of operation, and optimizing the operating conditions. Currently, cellulase enzymes are produced by growing different mutants of *T. reesei* such as RUT-C30 and RLP-37 on cellulose.[2-4] However, the growth of the fungus on cellulose is slow. If the growth time could be reduced by growing the fungus on a less expensive and more available soluble carbon source like xylose, then the cost of enzyme production could be lowered substantially.

Several researchers have shown the great potential for improving cellulase production using a fed-batch mode of fermentation.[5-7] These researchers all reported higher maximal enzyme activities and total productivities compared to those achieved in conventional batch systems. In addition to the reports of enhanced production of enzymes, these researchers also reported that in fed-batch cultivation the mycelial mass is lower, which reduces the cost of aeration and agitation.

In an earlier publication,[8] we reported that in batch cultivation, approximately 60% of the cellulose can be replaced with xylose without affecting the enzyme activity and productivity. This result, along with the improved results of other researchers with fed-batch systems for enzyme production, provided the motivation for studying enzyme production on a mixture of xylose and cellulose in a fed-batch process to determine whether better results could be obtained in fed-batch culture with a mixture of xylose and cellulose than on pure cellulose.

MATERIALS AND METHODS

Production Medium and Inoculum

The production media used in these experiements were the same as those described in Mohagheghi et al.,[8] as listed here in Table I. The frozen stock culture was transferred to medium A as preinoculum, and grown culture was then transferred to medium B as inoculum for the fermentor. The growth conditions were an initial pH of 4.8 and a temperature of 28°C.

Fermentation

Fermentations were carried out in a 5-L fermentor (B. Braun, West Germany, Biostat V) with a start-up volume of 2.0 L using medium C of Table I. The fermentation was initiated as a conventional batch culture using a mixture of 30 g/L xylose and 20 g/L cellulose. After 48–

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Table I. Growth media composition.

Component	Medium A	Medium B	Medium C
Glucose (%)	1.0	—	—
Cellulose (%)	—	1.0	5.0
$CaCl_2 \cdot 2H_2O$ (g/L)	0.4	0.4	0.8
$MgSO_4 \cdot 7H_2O$ (g/L)	0.3	0.3	0.6
KH_2PO_4 (g/L)	2.0	2.0	.3.7
$(NH_4)_2SO_4$ (g/L)	1.4	1.4	11.7
Corn steep liquor (%)	1.5	1.5	1.5
Tween 80 (mL/L)	—	0.2	0.2
Trace mineral concentrations (mg/L)			
$FeSO_4 \cdot 7H_2O$		5.0	
$MnSO_4 \cdot H_2O$		1.6	
$ZnCO_4 \cdot 7H_2O$		1.4	
$CoCl_2 \cdot 6H_2O$,		3.7	
prepared as stock solution of 100 × concentration and used 10 mL/L			
Antibiotics			
Penicillin			
Streptomycin			
Prepared 5 mg/mL stock solution and used 2 mL/L			

72 h, when the growth was observed to slow down as determined by a decrease in base addition, specified amounts (10–30 g/L, see Table II) of mixtures of xylose and cellulose or cellulose alone were added to the fermentor. This intermittent addition was repeated daily. The growth temperature was maintained constant at 28°C, and the pH was held at 4.8 by adding NH_4OH (3*M*) and H_3PO_4 (3*M*). Dissolved oxygen was kept above 20% of the saturation value for the medium by varying the agitation rate or supplying pure oxygen instead of air. A dilute solution (1:20) of antifoam B emulsion (Sigma Chemical Co., St. Louis, MO) was used to control foaming. The antibiotic solution mixture given in Table I was added to the fermentor to minimize contamination by bacteria.

ANALYSIS

Filter paper activity (IFPU) was measured by the method recommended by the International Union of Pure and Applied Chemistry (1984).[9] Cellular protein was measured by a modified Lowry method[10] using bovine serum albumin as a standard. The total dry weight, which included residual cellulose and mycelium, was measured by centrifuging 5 mL of culture broth washing the pellets with deionized water and drying it at 90°C overnight. Mycelium dry weight was estimated indirectly using a correlation factor of 0.37 [protein (g/L) per dry cell weight (g/L)] determined in our previous work.[8] Cellulose concentration was calculated from the difference of total dry weight and mycelium dry weight.

Table II. Summary of results on the maximal cellulase activity and correspondent total productivity of RUT-C30 grown on mixtures of xylose and cellulose in a fed-batch system.

Experiment no.	Xylose–cellulose ratio (g/L)		Maximum cellulase activity (IFPU/mL)	Total productivity (IFPU/L/h)	Total cellulose added (g/L)	Total carbon-course added (g/L)	Activity yield (IFPU/g total carbon)	Activity yield (IFPU/g total cellulose used)
	Start-up	Intermittent feed						
1	30:20	0:10	9.5	46.3	100	130	73.1	95.0
2	30:20	0:20	10.8	40.3	220	250	43.2	49.0
3	30:20	0:30	12.0	58.5	320	350	34.3	37.5
4	30:20	5:15	12.5	45.4	170	250	50.0	73.5
5	30:20	10:10	6.5	28.1	100	210	31.0	65.0
6	30:20	15:5	3.1	33.2	70	250	12.4	44.3
7	30:00	0:20	8.8	38.7	200	230	38.3	44.0
8	30:30	0:30	11.6	61.2	330	360	32.2	35.2
9	30:20	2.5:7.5	9.25	41.2	80	130	71.2	115.6
10	30:20	5:5	5.2	31.9	60	130	40.0	86.7
11	30:20	7.5:2.5	2.1	16.7	40	130	16.1	52.5
12	30:20	0:2.5	6.4	43.1	40	70	91.4	160.0
13	30:20	0:5	7.2	40.5	60	90	80.0	120.0
14	30:20	5:10	8.5	38.4	100	170	50.0	85.0
15	0:50	0:20	10.8	40.9	230	230	47.0	47.0

RESULTS

The *T. reesei* mutant RUT-C30[11] was grown via fed-batch cultivation with a mixture of xylose and cellulose as the substrate. The start-up and intermittent addition mixtures are given in Table II. A 10% (v/v), 72-h vegetative inoculum was used to start the initial batch culture. The advantage of fed-batch cultivation is that it maintains high substrate levels with reduced production of cell mass compared to batch cultivation, thus facilitating adequate agitation and aeration. During fed-batch cultivation, the biomass builds up to a certain level and then stays constant; the carbon source added subsequently is used for cell maintenance and enzyme production. On the other hand, in batch enzyme production, the cell mass increases as the initial carbon source concentration increases, and above 5% carbon source, the increased cell mass causes problems with agitation, aeration, and foaming.[8]

Figure 1 shows an example of the growth pattern, substrate consumption, and enzyme activity vs. time for RUT-C30 started on a mixture of 30 g/L xylose and 20 g/L cellulose followed by an intermittent addition of 5 g/L/day xylose and 15 g/L/day cellulose. Summaries of the overall results, as enzyme activity vs. time, are shown in Figures 2 and 3. Table II summarizes total productivity (IFPU/L/h), maximal enzyme activity (IFPU/mL), and enzyme activity yield, defined as IFPU/g carbon source used, for all experiments.

The effect of the presence of cellulose in a start-up mixture was studied by using a mixture of 30 g/L xylose plus 20 g/L cellulose or 30 g/L xylose alone in experiments 2 and 7. Comparing the results of these two experiments showed higher activity, productivity, and enzyme activity yield with cellulose in the start-up mixture. In another case, comparing the results of experiment 2 with 15 in which two different starting mixtures of 30 g/L xylose and 20 g/L cellulose or 50 g/L cellulose alone, both with the same intermittent addition of 20 g/L/day cellulose, were used shows that replacing 60% of cellulose with xylose in starting mixture does not have effect on enzyme production.

Several experiments were performed to study the effect of varying the amount of intermittent feeding of cellulose on

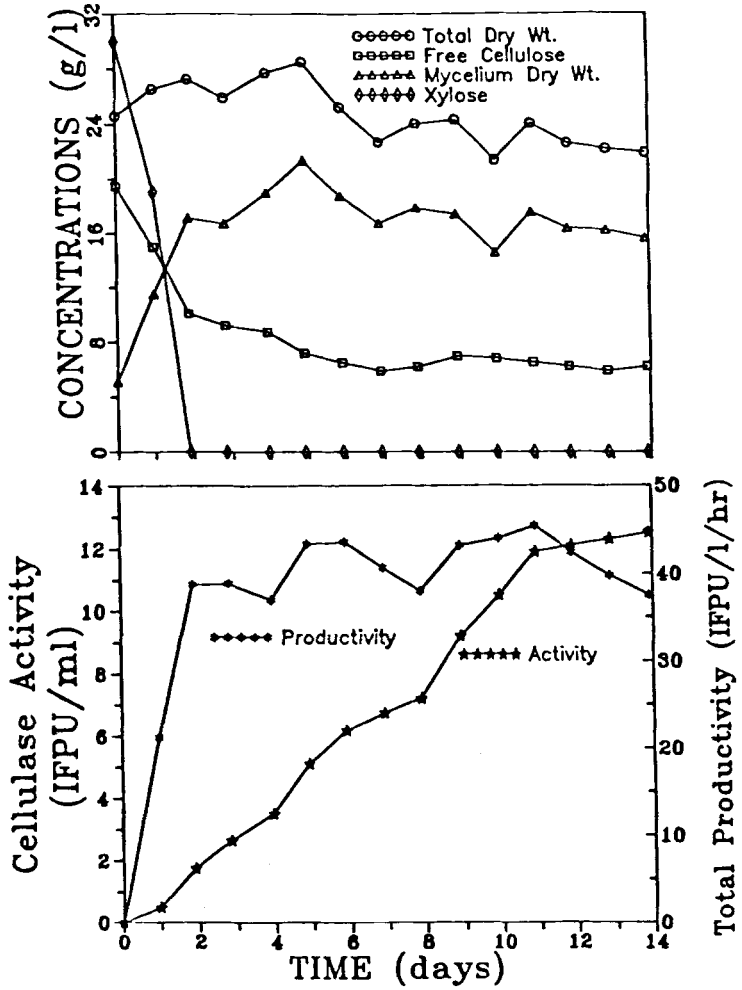

Figure 1. Growth and enzyme production pattern of *T. reesei* RUT-C30 grown on mixtures of xylose and cellulose in fed-batch culture at pH 4.8, T = 28°C. Start-up mixture: 30 g/L xylose, 20 g/L cellulose. Intermittent feed mixture: 5 g/L/day xylose, 15 g/L/day cellulose.

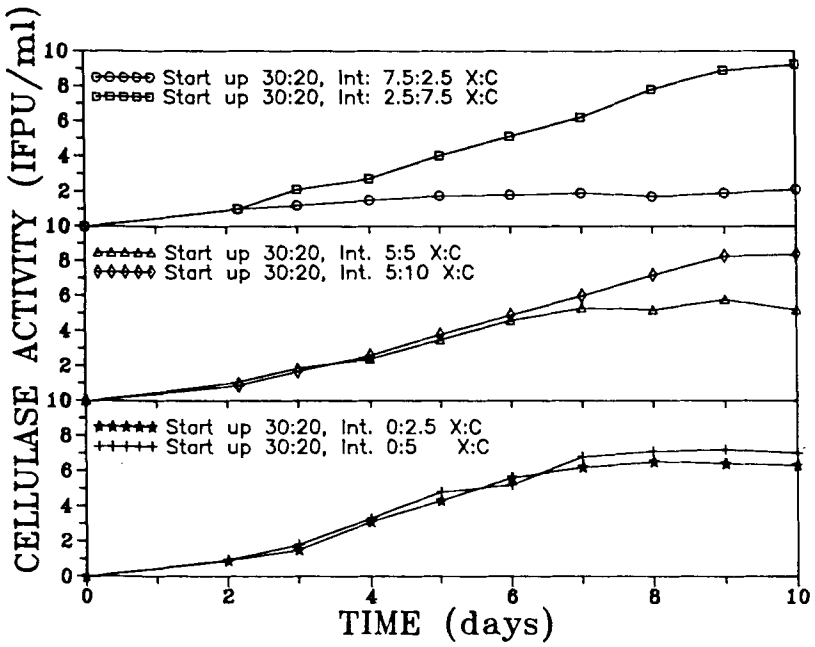

Figure 2. Cellulase production as a function of time by *T. reesei* RUT-C30 grown on xylose–cellulose mixtures in a fed-batch culture at pH 4.8, T = 28°C. X:C represents the xylose to cellulose ratio.

the production of enzyme (experiments 1, 2, 3, 12, and 13). The intermittent feed was varied from 2.5 to 30 g/L/day cellulose, all with the same start-up mixture of 30 g/L/day xylose and 20 g/L cellulose. In these experiments, the maximal enzyme activity and total productivity decreased when the total amount of intermittent feed was decreased while the enzyme activity yield from substrate increased. Thus, by lowering the feed rate, the maximal enzyme activity and total productivity is sacrificed for better yield. This result is compatible with cases such as simultaneous saccharification and fermentation, in which lower enzyme activities of 1–2 IFPU/mL are adequate in the fermentation broth.

In another set of experiments, the effect of adding xylose to the intermittent feed was studied. In this case, we used a total intermittent feed of 10 g/L/day in experiments 1, 9, 10, and 11 and 20 g/L/day in experiments 2, 4, 5, and 6. In both cases, the results showed that replacing up to 25% of cellulose with xylose does not decrease the maximal enzyme activity, productivity, and enzyme activity yield as

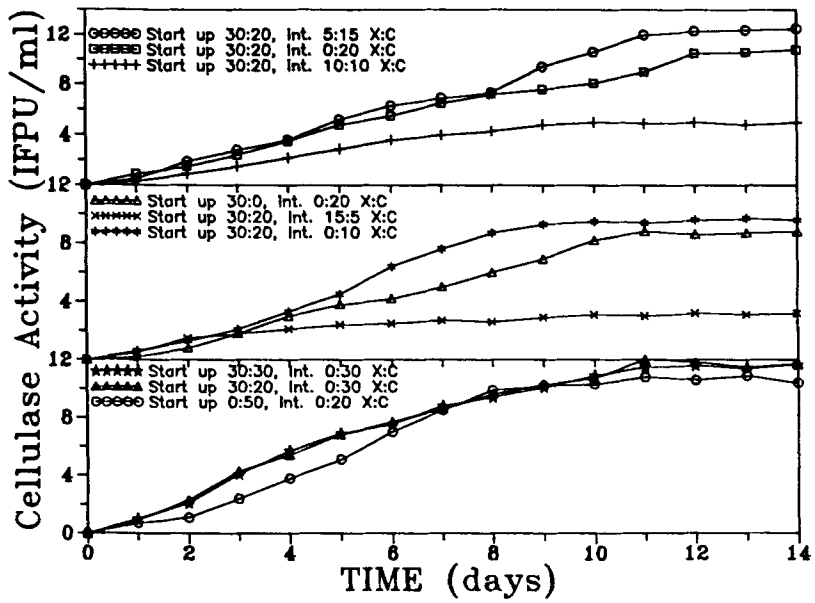

Figure 3. Cellulase production as a function of time by *T. reesei* RUT-C30 grown on xylose–cellulose mixtures or cellulose alone in a fed-batch culture at pH 4.8, T = 28°C.

compared to cellulose alone. Above 25% replacement, xylose suppressed the enzyme production, which results in lower final enzyme activity and yield.

Figure 4 compares batch cultivation using 30 g/L xylose and 20 g/L cellulose as substrate (data from ref. 8) vs. fed-batch cultivation using a 30-g/L-xylose and 20-g/L-cellulose start-up mixture and intermittent addition of 5 and 15 g/L/day xylose and cellulose, respectively. It can be seen from this figure that maximal enzyme activity improved by about 81% and total productivity gained 22% compared to the batch system. The results reported by other researchers show even greater improvement in maximal enzyme activity and total productivity by switching from a batch to a fed-batch system with only cellulose as the substrate (e.g., ref. 5). This difference could be due to differences in enzyme characterization, system operation and procedure, or both.

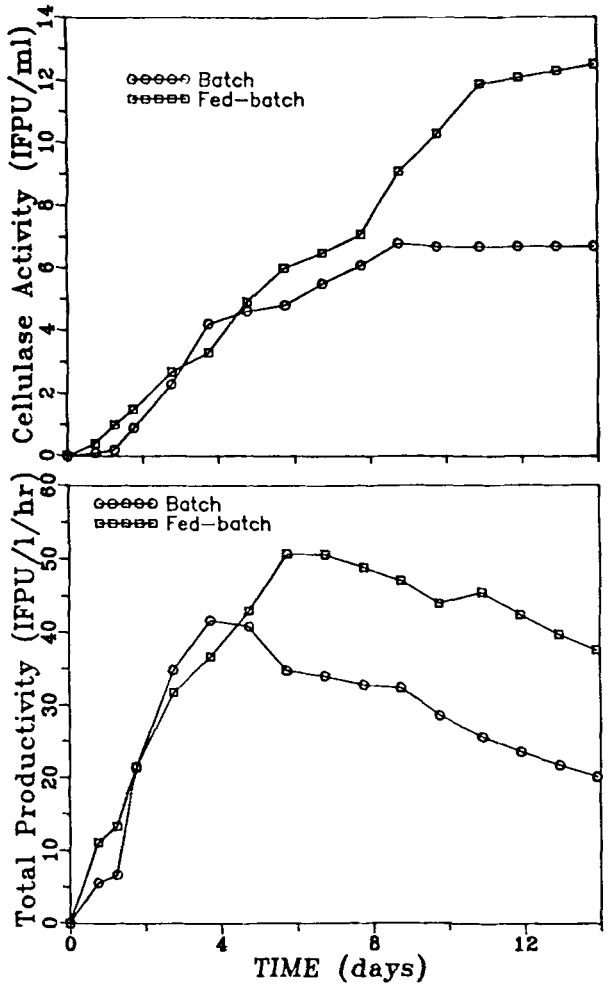

Figure 4. Comparison of cellulase activity and enzyme productivity by *T. reesei* RUT-C30 in batch and fed-batch culture at pH 4.8, T = 28°C.

DISCUSSION

The results of fed-batch cultivation show the potential advantages of enzyme production processes using mixtures of xylose and cellulose as substrate. The results confirmed that almost 60% of cellulose in the initial feed mixture can be replaced with xylose without effecting the enzyme production process. As a result, in most of the experiments the substrate combination of 30 g/L xylose and 20 g/L cellulose were used as start-up mixtures, and only the amount of xylose and cellulose in the intermittent mixture was varied to compare the enzyme production in the fed-batch system using mixtures of xylose and cellulose and cellulose alone as substrate. By increasing the amount of cellulose in intermittent feeding (from 2.5 to 30 g/L/day), the maximal enzyme activity increased but the enzyme activity yield decreased.

This can be supported if the results of experiment 2 are compared with those of experiment 4, each having the same start-up mixture of 30 g/L xylose and 20 g/L cellulose. Experiment 2 was fed 20 g/L/day of pure cellulose and experiment 4 a mixture of 5 g/L/day of xylose and 15 g/L/day of cellulose. The results show (Table II) that substituting xylose for 25% of the cellulose in the intermittent feeding mixture increased the maximal cellulase activity 16%, improved the total productivity 25%, and increased the enzyme activity yield from carbon source 25%. These results show the economic advantage of replacing some portion of cellulose with xylose in the intermittent feeding mixture. The results in Table II also confirm that substituting cellulose with more than 25% xylose in intermittent feed suppresses enzyme production.

In Table III, we compare the maximal enzyme activity, total productivity, and enzyme activity yield of the batch and fed-batch system. The enzyme activity yield is useful for comparing the advantages of batch and fed-batch cultivation. Table III shows that although the fed-batch system improves the maximal enzyme activity and the total productivity, the enzyme activity yield per amount of substrate consumed decreases, indicating that the enzyme activity yield is being sacrificed for better productivity in this system. It should be pointed out, however, that residual substrate from enzyme production could be potentially used in subsequent saccharification and fermentations, and the decreased yield would become immaterial. As we reported previously,[8] replacing about 60% of cellulose with xylose in a batch system does not decrease the maximal enzyme activity and total productivity compared to the results obtained with cellulose alone. For fed-batch systems, it can be concluded that replacing up to 25% of the cellulose with xylose improves the total productivity of enzyme. The amount of cellulose saved by substituting xylose in enzyme production can be used for ethanol production. As a result, the substrate cost can be decreased substantially, making the process more economical.

Table III. Comparison of maximal activity, total productivity, and enzyme activity yield of batch and fed-batch cultivation.

Batch					Fed-batch					
					X–C ratio (g/L)					
X–C	Total carbon-source used (g/L)	Maximum cellulase activity (IFPU/mL)	Total productivity (IFPU/L/h)	Activity yield	Start-up	Intermittent feed	Total carbon-source used (g/L)	Maximum cellulase activity (IFPU/mL)	Total productivity (IFPU/mL)	Activity yield
40:0	40	1.3	6.2	32.5	30:20	0:10	130	9.5	46.3	73.1
30:5	35	1.95	12.1	55.7	30:20	0:20	250	10.8	40.3	43.2
30:10	40	3.5	32.0	87.5	30:20	0:30	350	12.0	58.5	34.3
20:20	40	5.2	39.2	130.0	30:20	5:15	250	12.5	45.4	50.0
25:25	50	5.4	35.0	108.0	30:20	10:10	210	6.5	28.1	31.0
30:20	50	6.9	37.2	138.0	30:20	15:5	250	3.1	33.2	12.4
30:30	60	5.6	37.3	93.3	30:0	0:20	230	8.8	38.7	38.3
50:50	100	7.4	35.2	74.0	30:30	0:30	360	11.6	61.2	32.2
0:40	40	5.3	34.3	132.5	30:20	2.5:7.5	130	9.15	41.2	71.2
0:50	50	7.1	51.0	142.0	30:10	5:5	130	5.2	31.9	40.0
0:100	100	6.7	41.4	67.0	30:20	7.5:2.5	130	2.1	16.7	16.1
					30:20	0:2.5	70	0.4	43.1	90.0
					30:20	0:5	90	7.2	40.5	77.8
					30:20	5:10	170	8.5	38.4	50.0
					0:50	0:20	230	10.8	40.9	47.0

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