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Production of Cellulase on Mixtures of Xylose and Cellulose

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ABSTRACT

Cellulase production by the RUT-C30 mutant of the fungus *Trichoderma reesei* was studied on mixtures of xylose and cellulose. In mixed substrates, the lag phase of the growth cycle was shorter and reached the maximum of total productivity in a shorter time compared to growth on the single substrate, cellulose. A diauxic pattern of utilization of the two carbon sources was observed as well: Xylose was utilized first to support growth, followed by cellulose to induce the cellulase enzyme production and provide an additional carbon source for cellular metabolism. Of the various mixtures of xylose and cellulose used in batch enzyme production, a ratio of 30:30 g/L of xylose to cellulose was optimal. This mixture produced the highest maximal enzyme productivity of 122 IFPU/L h, and its total productivity reached a maximum value of 55 IFPU/L h in less time than others. However, similar total productivities and higher enzyme titers were observed for growth on cellulose alone.

Index Entries: Enzymatic hydrolysis; cellulase; cellulose; *Trichoderma reesei*.

INTRODUCTION

Enzymatic hydrolysis is of interest for fuel production because it avoids many of the problems experienced with dilute acid hydrolysis, a method that has received attention since the early 1900s. Since the sugars produced by hydrolysis are not subjected to severe environments, degradation to byproducts and formation of waste streams such as hydroxymethylfurfural and tars is avoided. Coupled with the high specificity of

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the enzyme systems, the enzymatic hydrolysis allows, at least in principle, complete conversion of the substrate into sugars for fermentation into liquid fuels. The product stream from enzymatic hydrolysis is also clean, and costly processing is not needed to remove impurities and toxins prior to fermentation. Finally, the structure of the lignin portion of the substrate is preserved, and high value applications of the lignin are possible. Thus, enzyme hydrolysis could significantly improve the production cost of liquid fuels from lignocellulosic biomass if kinetic constraints to application of the technology can be overcome.

A key cost element in these investigations is the production of hydrolytic enzymes, and advances are required that will allow production of high cellulase titers at high rates with lignocellulosic feedstock as the carbon source. Detailed studies of current cellulase technology (*1,2*) shows that approximately 50% of the enzyme production cost is for production of glucose from cellulosic materials for feeding the cellulase producing microorganisms.

A major improvement in cellulase technology was the development of the RUT-C30 mutant of *Trichoderma reesei* (*3*), which yields high enzyme activity and productivity. Currently, cellulase enzymes are typically produced by growing up the various mutants of the fungus (*T. reesei*) on cellulose (*4,5*). However, since cellulose is not easily assimilated, the growth rate of the fungus is slow, and batch times on the order of 1–2 w are required to achieve high enzyme titers. On the other hand, liquid carbon souces result in faster growth of the microorganisms, but *T. reesei* does not produce suitable enzyme activity when grown on inexpensive noninducing soluble sugar streams that are readily available in large quantities such as xylose (*6*). By growing the mutant RUT-C30 on lactose, which is a soluble inducer sugar (*7*), higher enzyme activity and productivity can be achieved (*8*), but this substrate is not obtained from lignocellulosics and hence would not be available at the plant in large quantities.

If the fungus could be grown to suitable cell densities on an inexpensive carbon source that is abundant and fed cellulosic substrate to induce cellulase production, perhaps both high growth rates and high enzyme titers could be achieved. The large amounts of xylose produced from biomass during pretreatment could be used to promote rapid cell growth and the resulting dense culture could then be fed cellulose to induce cellulase production. In this fashion, an inexpensive liquid carbon source will support rapid cell growth whereas the addition of cellulose could result in production of high enzymatic activity. Gallo (*9*) reported on enzyme production by mutant MCG77 of *Trichoderma reesei*, using a mixture of xylose, cellulose, and lactose. His result on enzyme production using 1% xylose, 1% cellulose, and 1% lactose showed an increase on filter paper activity from 0.95 to 1.9 IFPU/mL.

To determine the potential of this enzyme production scheme, the RUT-C30 mutant of the *T. reesei* was grown on mixtures of xylose and

cellulose in batch cultures. Even though more advanced mutants of *T. reesei* have been developed, RUT-C30 was selected for this study since considerable information is available on its growth and enzyme production. The objective of the research is to delineate the optimal concentrations and ratios of these substrates for the induction of the cellulase enzyme complex produced in batch cultures containing different initial concentrations of pure xylose and pure cellulose and to compare the results to those possible with cellulose alone.

MATERIALS AND METHODS

Production Medium

Three types of mineral media (media A, B, and C) were used in these experiments, as shown in Table 1. These media were the same as that of Tangnu et al. (*10*) and Wiley (*11*), except that peptone was replaced with corn steep liquor (Sigma Chem. Co., St. Louis, MO) as recommended by Sheir-Neiss and Montenecourt (*12*). Medium A was used for starting fungus from frozen stock culture, medium B was used for preinoculum preparation, and medium C was used for main inoculum and production medium for fermenter. The pH of all the culture media was adjusted to 4.8 before autoclaving. Xylose (Sigma Chem. Co., St. Louis, MO) and solka floc BW200 (James River Corporation, Berlin, NH) were used as carbon source.

Table 1
Growth Media Composition

Component	Medium	A	B	C
Glucose		1.0%	—	—
Cellulose		—	1.0%	5.0%
$CaCl_2 \cdot 2H_2O$		0.4 g/L	0.4g/L	0.8 g/L
$MgSO_4 \cdot 7H_2O$		0.3 g/L	0.3 g/L	0.6 g/L
KH_2PO_4		2.0 g/L	2.0 g/L	3.7 g/L
$NH_4{}_2SO_4$		1.4 g/L	1.4 g/L	11.7 g/L
Corn steep liquor		1.5%	1.5%	1.5%
Tween 80		—	0.2 mL/L	0.2 mL/L
		Trace mineral concentrations		
$FeSO_4 \cdot 7H_2O$			5.0 mg/L	
$MnSO_4 \cdot H_2O$			1.6 mg/L	
$ZnSO_4 \cdot 7H_2O$			1.4 mg/L	
$CoCl_2 \cdot 6H_2O$			3.7 mg/L	
Prepared as stock solution of 100× concentration and used 10mL/L				
Penicillin				
Streptomycin				
Prepared 5 mg/mL stock solution and used 2 mL/L				

Stock Culture

Cultures of *T. reesei* RUT-C30 were streaked on potato dextrose agar (PDA) plates. Single colonies were picked up and streaked again on plates containing 1% cellobiose and 0.125% 2-deoxyglucose in medium A. The function of the 2-deoxyglucose was to suppress revertants. After sporulation, spores were suspended on 10% sterile glycerol (B. S. Montenecourt, personal communication). A 1 mL portion of the suspension was placed in 1.5 mL sterile vials, then frozen at −70°C as a stock culture.

Inoculum

To start up an inoculum one of the vials was thawed, then added to a 100 mL flask containing 25 mL of culture medium A with 1% glucose. The culture was incubated on an orbital shaker at 28°C for 48 h. This grown culture was transferred to a 250 mL flask containing 50 mL of medium B with 1% cellulose and incubated on an orbital shaker at 28°C for 72 h as preinoculum. A 1000 mL flask containing 250 mL of medium C was inoculated from the preinoculum, and incubated again on an orbital shaker for 72 h, to serve as main inoculum for the fermenter. The inoculum was streaked on potato dextrose agar (PDA) plate to check for contamination, before transferring to the fermenter.

Fermentation

Fermentations were carried out in a 5-L fermenter (B. Braun, Biostat V) with an operating volume of 2.5 L, using medium C of Table 1. Temperature was held constant at 28°C, and pH was controlled at 4.8 by addition of NH_4OH and H_3PO_4 both diluted (1:5) v/v with deionized water from concentrated ammonium hydroxide and H_3PO_4 stocks, respectively. Dissolved oxygen was automatically controlled above 20% of the saturation value for the medium by varying the agitation rate or supplying pure oxygen instead of air. The foaming was controlled by addition of (1:20) solution of Antifoam B emulsion (Sigma Chem. Co.) whenever it was needed. To minimize contamination by bacteria, 2 mL of antibiotics (penicillin and streptomycin, 5 mg/mL) were added per liter of fermenter volume after the fermenter and its contents were sterilized.

ANALYSIS

Dry Weights

A 5 mL portion of culture broth was centrifuged, washed with distilled water, then dried in an aluminum dish overnight at 90°C. From the difference of weights, the total dry weight, which included mycellium and residual cellulose, was then determined. Mycellium dry weight was

estimated indirectly from the protein content of the mycellium, using a correlation factor of 0.37 (protein g/L/dry cell weight g/L) which was determined in this work and described in the results section. Free cellulose was determined from the difference of total dry weight and mycellium dry weight.

Filter Paper Activity

Filter paper activity, expressed as international units (IFPU), was measured by the method recommended for the International Union of Pure and Applied Chemistry (*13*). This method measures the release of reducing sugar produced in 60 min from a mixture of 0.5 mL of enzyme dilution and 1 mL of citrate buffer (0.05*M*, pH = 4.8) in the presence of 50 mg of Whatman No. 1 filter paper (1 × 6 cm strip) incubated at 50°C. Activities are expressed as international filter paper units (IFPU/mL = μmol of glucose equivalent/mL/min). The reducing sugar released was measured by the dinitrosalicilic acid (DNS) method (*14*).

Soluble Protein

Cellular protein was measured by modified Lowry method (*15*). Bovine serum albumin was used as a standard.

RESULTS

In this work, the *T. reesei* mutant, Rut-C30, was grown in batch cultures on a mixture of xylose and cellulose, using a 10% v/v, 72 h vegetative inoculum. The age of inoculum, amount of antifoam added to fermenter, and stirring affected enzyme production, as reported by other researchers (*16*). Different ratios of xylose to cellulose, shown in Table 2,

Table 2
Substrates Used in Enzyme Production Experiments

	Concentration	
Experiment #	Xylose, g/L	Cellulose, g/L
1	40	0
2	30	5
3	30	10
4	20	20
5	25	25
6	30	20
7	30	30
8	50	50
9	0	40
10	0	50
11	0	100

were used as the substrate. As a control, several experiments were performed in which only xylose or cellulose were used as substrates. The concentration of xylose and cellulose in the control experiments are also shown in Table 2.

Determination of direct cell dry weight was a major problem because of the presence of the insoluble substrate, cellulose. Other researchers have tried different methods to estimate cell dry weight. Andreotti et al. (*17*) used the correlation between protein content of the cell and total dry weight, by running a calibration experiment in which fungus was grown on a soluble substrate. Their result showed that cellular protein is approximately 40% of total dry weight. Ghose and Sahai (*18*) estimated cell dry weight based on DNA content of the samples. In this work we have employed the same method as used by Andreotti et al. (*17*). Several experiments were performed in which RUT-C30 was grown on xylose only. Samples were taken at different times and analyzed for total dry weight and protein. The result is shown in Fig. 1. From this figure, the correlation between protein content of the cell and its dry weight was determined. The correlation factor varied over the range 0.29–0.42, depending on the stage of the growth. During the log phase it was around 0.37 and changed to 0.42 during the stationary phase of growth. Based on these data, an average correlation factor of 0.37 was used to estimate the cell dry weight and free cellulose in culture.

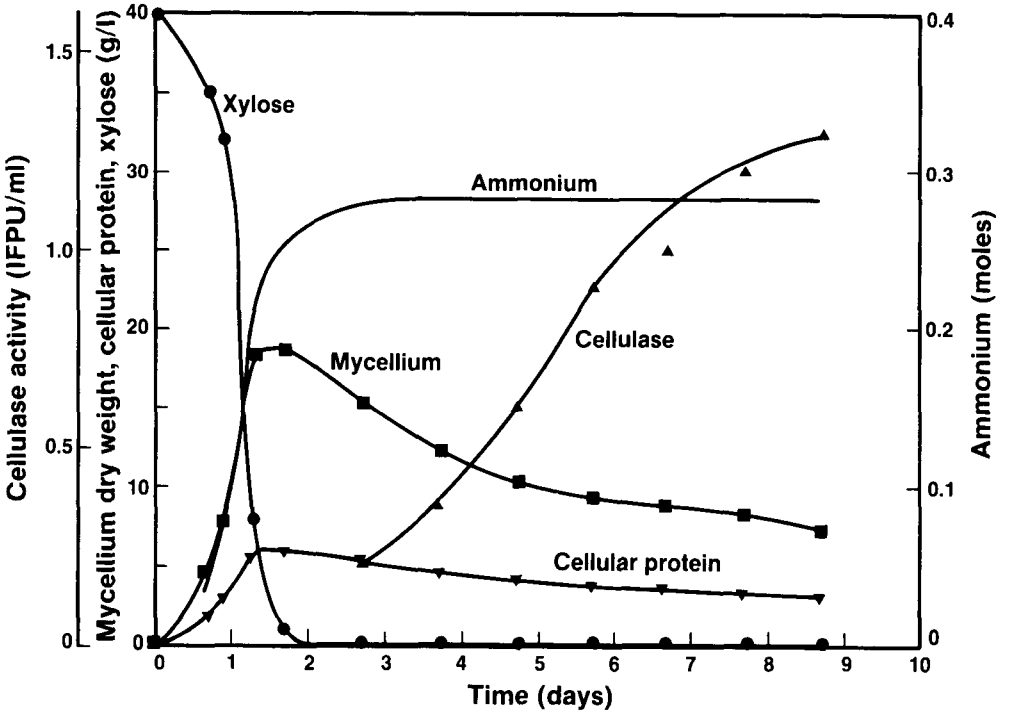

Fig. 1. Growth and enzyme production pattern of *T. reesei* RUT-C30 grown on xylose (40 g/L), at pH = 4.8, T = 28°C.

Figures 1–3 are the examples of growth patterns of Rut-C30 on xylose (40 g/L), cellulose (40 g/L), and a mixture of xylose and cellulose (30:30 g/L), respectively. Figure 4 summarizes the cellulase activity in IFPU/mL as a function of time for all experiments. From this figure, high-titer filter paper activity, which is the maximum of enzyme activity, and the correspondent productivity along with maximal productivity were estimated, as shown in Table 3. Maximal productivity was calculated from the slope of the steepest part of Fig. 4. Total productivity is based on the total time of fermentation including the lag phase, and for this reason its values are lower than maximal productivity.

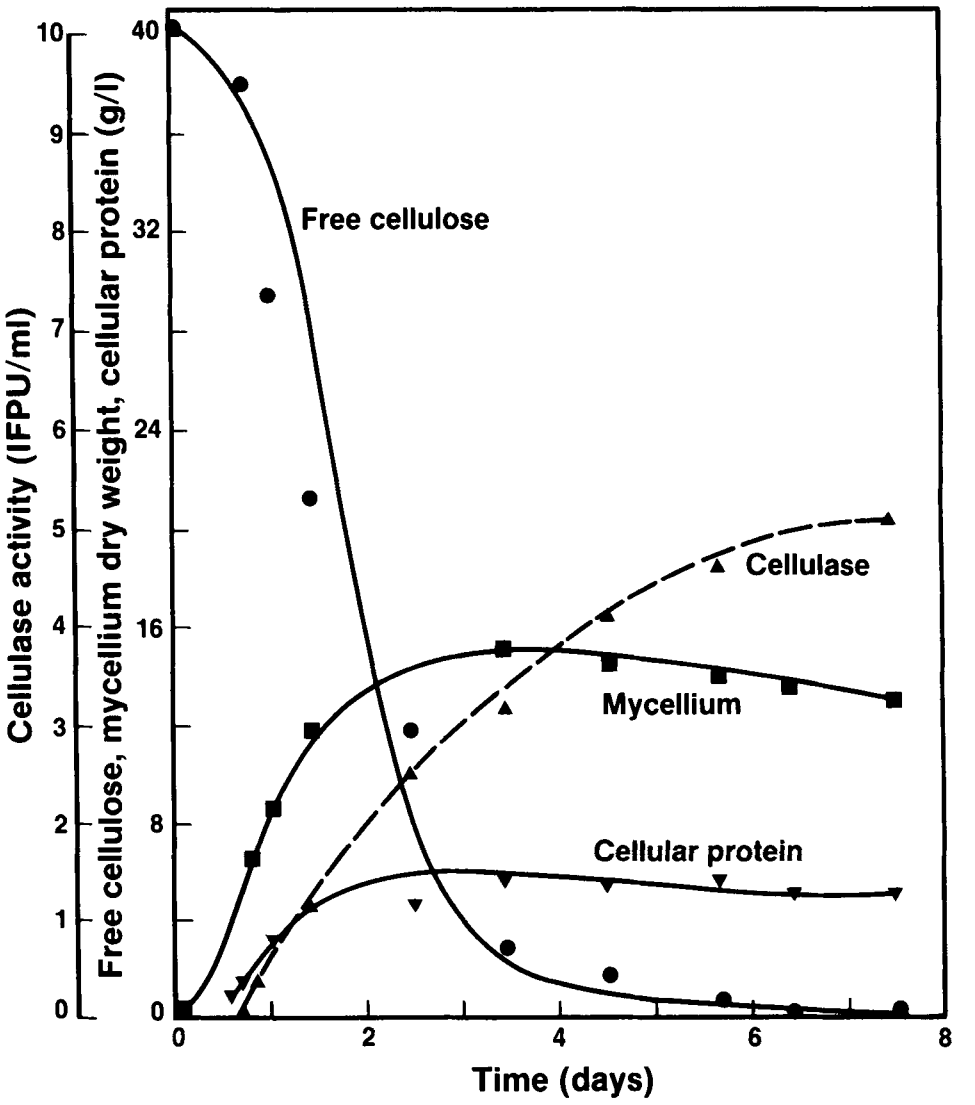

Fig. 2. Growth and enzyme production pattern of *T. reesei* RUT-C30 grown on cellulose (40 g/L), at pH = 4.8, T = 28°C.

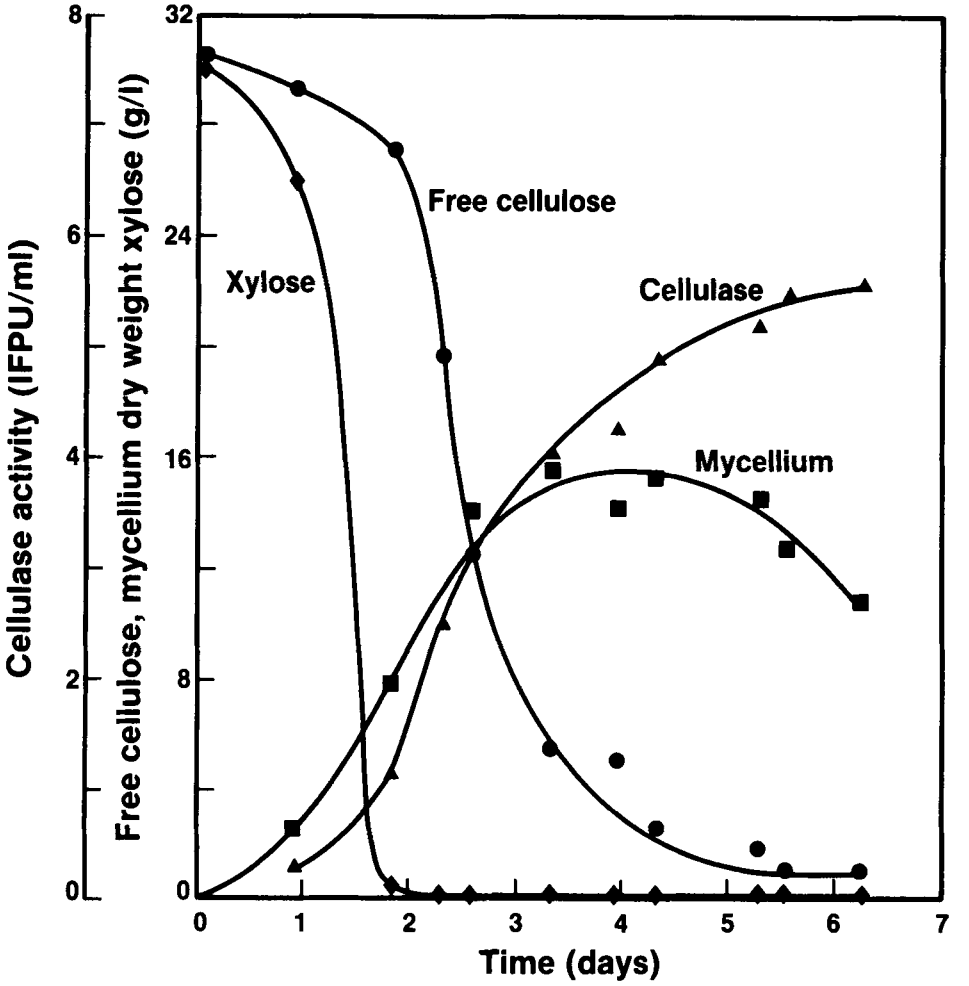

Fig. 3. Growth and enzyme production pattern of *T. reesei* RUT-C30 grown on mixture of xylose:cellulose (30:30 g/L), at pH = 4.8, T = 28°C.

The results obtained in this work show a diauxic patten of utilization of the two carbon sources: Xylose was utilized first to support the growth, followed by cellulose to induce cellulase enzyme production and provide an additional carbon source for cellular metabolism. Figure 3 shows that xylose was depleted at less than 40 h, and this was the case with all other mixed substrate experiments. Enzyme production started in the late log phase and reached its high titer at the late stationary phase of the growth cycle.

Study of the results shown in Table 3 indicates that the highest enzyme activities for all experiments with mixed substrates and a total carbon source of 50 g/L or more were similar (5-7 IFPU/mL). The experiment with a mixture ratio of 30:30 (g/L) xylose to cellulose produced the highest maximal productivity (122.7 IFPU/Lh) for a titer of 5.6 IFPU/mL in 6.25 d. The maximal productivity obtained in experiments with substrate

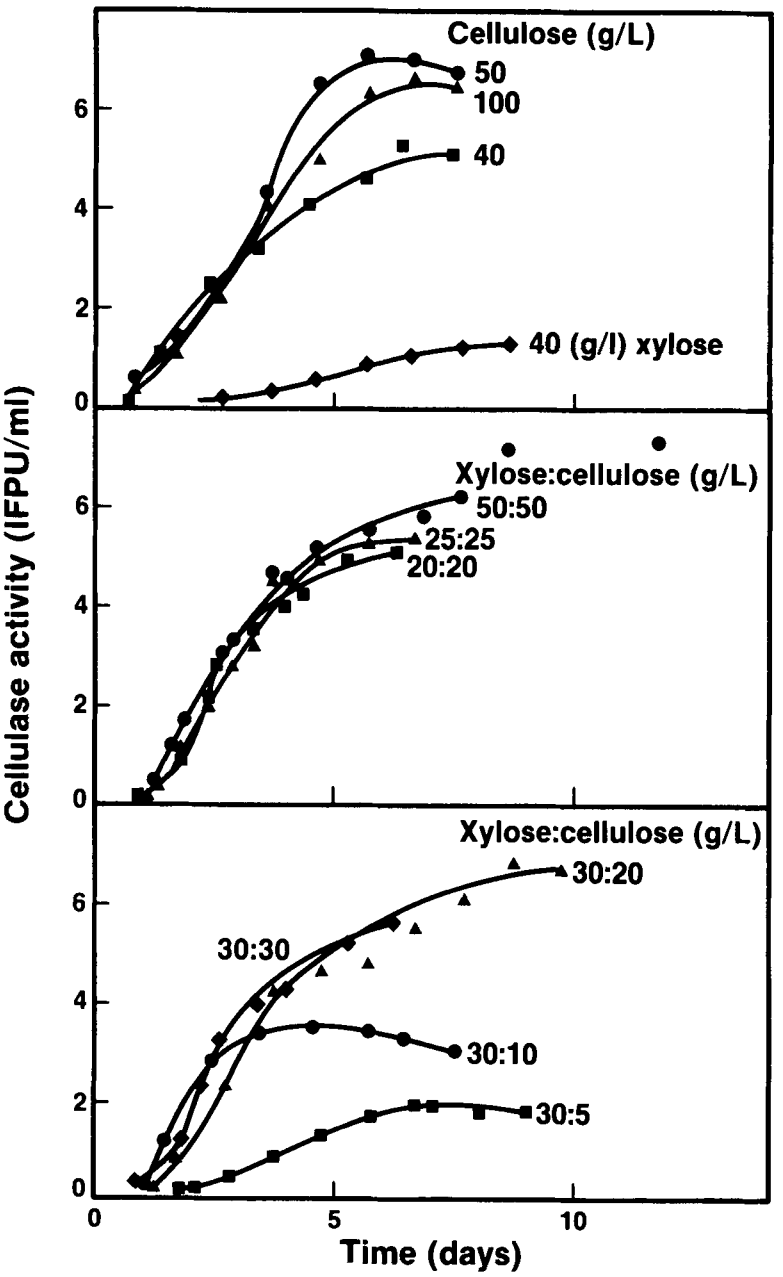

Fig. 4. Cellulase productivity as a function of time by *T. reesei* RUT-C30 grown on different substrate at pH = 4.8, T = 28°C.

ratios of 25:25 and 30:20 are lower than those of experiments with 30:30 and 20:20 g/L of xylose and cellulose, respectively (Table 3). This difference may be explained by uncertainty involved in measuring the filter paper activity by the DNS method and inaccuracies in measurement of the slope, an inaccurate approximation itself.

Table 3
Summary of Results on the High Titer Filter Paper Activity, Correspondent Productivity, and Maximal Productivity of RUT-C30 Grown on Xylose, Cellulose, or Their Mixture

Exp. #	Xylose: Cellulose Ratio, g/L	High titer activity, IFPU/mL	Productivity, IFPU/Lh	
			total	maximal
1	40:0	1.3	6.2	12.5
2	30:5	1.95	12.1	18.5
3	30:10	3.5	32.0	70.8
4	20:20	5.2	39.2	111.0
5	25:25	5.4	35.0	71.0
6	30:20	6.9	37.2	77.1
7	30:30	5.6	37.3	122.7
8	50:50	7.4	35.2	79.2
9	0:40	5.3	34.3	54.2
10	0:50	7.1	51.0	91.7
11	0:100	6.7	41.4	66.7

Figure 5 shows the change of total productivity as a function of time for all experiments. This figure indicates that the total productivity for mixed substrates reaches its maximum in less time than the time required for the single substrate, cellulose, although the final values are similar. The reason for this phenomena is that in mixed substrate experiments, xylose supports the growth and cellulose induces the enzyme production, making the overall process faster. However, for the single substrate, cellulose is required to both support growth and induce enzyme production, and since cellulose is broken down slowly, more time is required for growth. On the other hand, higher activities are obtained for growth on cellulose alone than for the mixed substrate, and since this compensates for the faster growth of the fungi, total productivities for cellulose alone or mixed substrate are similar.

Our results also show that total productivity for mixed substrates reaches its maximum before enzyme activity gets to its high titer value, Fig. 6. In this figure, as an example, the total productivity and enzyme activity are plotted versus time for experiments with 30:30 g/L xylose:cellulose, and 50 g/L cellulose.

Comparing the results of three experiments in which RUT-C30 was grown on xylose (40 g/L), cellulose (40 g/L), and a mixture of the two (20:20 g/L xylose cellulose) (Table 3) shows that by substituting 20 g/L xylose for 20 g/L of cellulose, the maximal productivity has improved by 100%. Also, the other results (Table 3) show that 30:20 and 30:30 g/L mixtures of xylose and cellulose produced the highest enzyme activity for mixed substrate of around 5.5 IFPU/mL in 6.5 days. However, growth of the fungus on cellulose alone attained a higher enzyme titer of 7 IFPU/mL

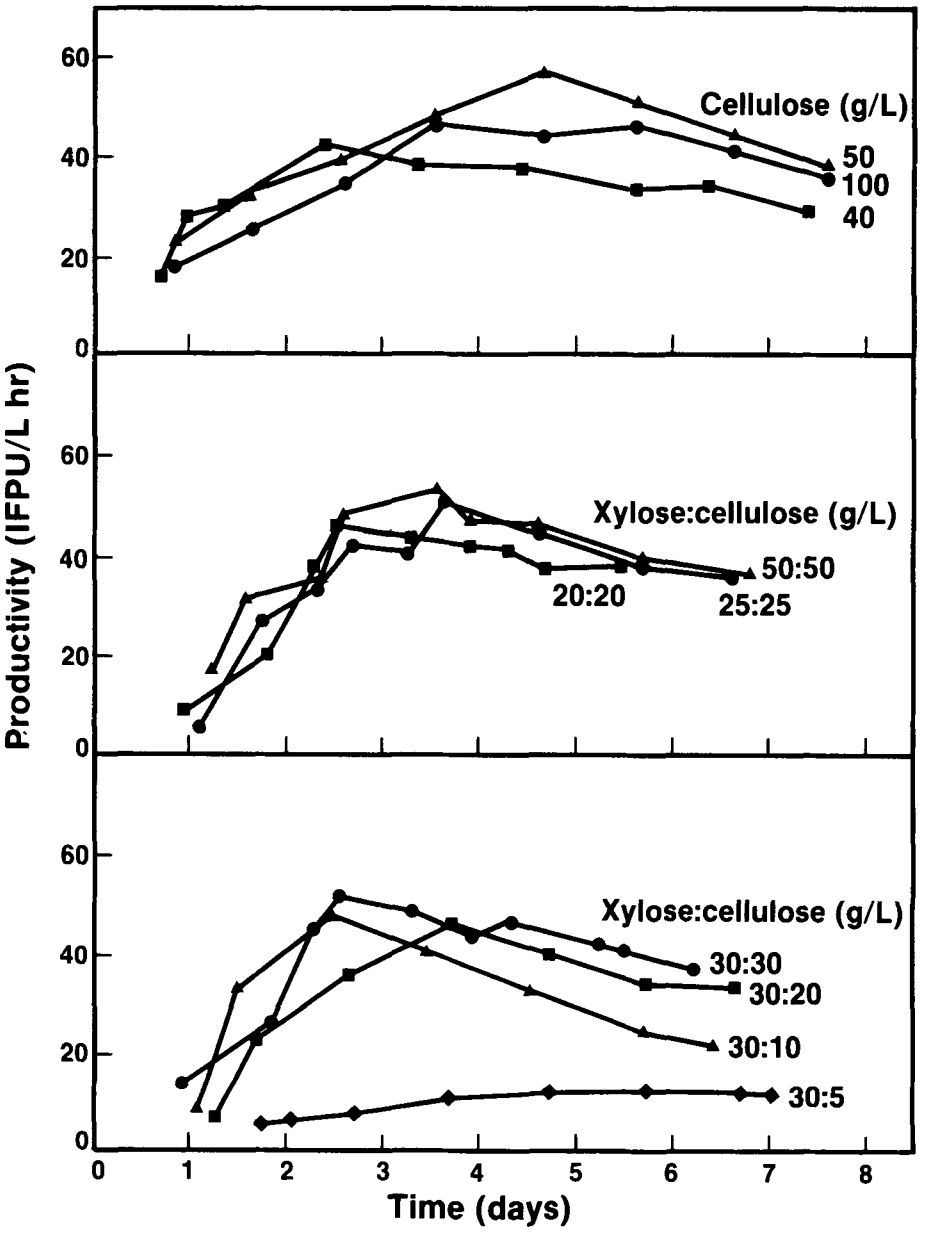

Fig. 5. Productivity of cellulase production as a function of time for *T. reesei* RUT-C30 grown on different substrate, at pH = 4.8, T = 28°C.

in a time period of 6 d. Since the maximal productivity of the 30:30 combination was the highest (122 IFPU/Lh) of all experiments and its total productivity reached maximum value of 55 IFPU/Lh in less time than others (Fig. 5), this ratio seems to be the optimal ratio of xylose to cellulose for enzyme production on mixed substrate in a batch system. The results of this work showed that increasing the total substrate (cellulose or a mixture of xylose and cellulose) concentration above 60 g/L does not improve

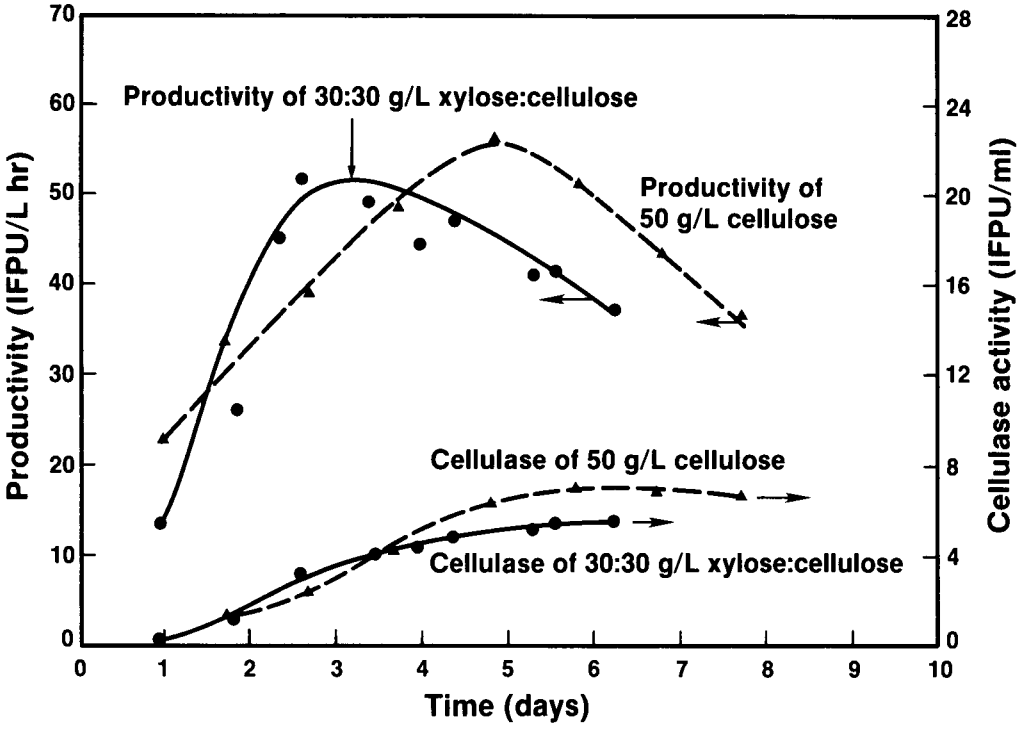

Fig. 6. Productivity and cellulase activity as a function of time for *T. reesei*, RUT-C30 grown on 50 g/L cellulose and 30:30 g/L xylose:cellulose, at pH = 4.8, T = 28°C.

high titer enzyme activity and productivity of enzyme production and is consistent with the results of Sternberg and Dorval (*19*), Ghose and Sahai (*18*), and Hendy et al. (*20*) for cellulose alone.

Using the titration data for ammonium hydroxide consumption versus time, the specific growth rate on cellulose and xylose was calculated. Its value is 0.14 h^{-1} on cellulose, which is in agreement with that of Andreotti et al. (*18*), and 0.22 h^{-1} on xylose.

DISCUSSION

The results show that the RUT-C30 mutant of *T. reesei* can be successfully grown on mixtures of xylose and cellulose. Xylose, which can be readily obtained from pretreatment of real lignocellulosic materials as a coproduct, supports the initial fungal growth, and by supplementing the medium with a smaller amount of cellulose as an inducer, about the same productivity of enzyme can be obtained as when all of the substrate is cellulose.

In order to evaluate the economic advantage of this process, we can compare the result of experiment #4 (20:20 mixture of xylose to cellulose) with that of experiment #9 (40 g/L cellulose). It can be seen that by

substituting 20 g/L of xylose for cellulose (50%), the maximal productivity has improved more than 100%, the total productivity has increased by 14%, and the lag phase of enzyme production has decreased. In another case, compare the result of experiment #9, (40 g/L cellulose) with that of experiment #3 (30:10 mixed substrate). In this case, we have substituted xylose for 75% of the cellulose. Total productivity has remained almost the same, the maximal productivity has improved by about 30%, and the lag phase for enzyme production has decreased substantially.

Our results for production of enzyme with pure cellulose at concentrations of 40, 50, and 100 g/L cellulose showed that the highest maximal productivity of 91.7 IFPU/Lh and a total productivity of 51 IFPU/Lh were achieved in our system at a cellulose concentration of 50 g/L. The results with mixed substrates are comparable to these values, and in some cases, e.g., at 20:20 or 30:30 (g/L), the maximal productivities of mixed substrates are better. Mixed substrates also reach their maximum total productivity in a shorter time, although the enzyme titers are lower than those for pure cellulose. It can be concluded that by replacing part of cellulose with xylose for enzyme production, the maximal productivity of

Table 4
Typical Results Obtained by Researchers on the Production of Cellulolytic Enzymes in Batch Systems Using *T. reesei* RUT-C30

Reference	Substrate and system	Activity, IFPU/mL	Productivity IFPU/Lh	
			Total	Maximum
Montenecourt, B. S., and Eveleigh, D. E. (*21*)	1% Cellulose Shake flask	1	[a]	[a]
Montenecourt, et al. (*22*)	5% Solka floc a	8.	70.	[a]
Andreotti et al. (*8*)	6% Cellulose 10 L Fermenter	7.8	[a]	130
Tangnu et al. (*10*)	5% Cellulose 10 L Fermenter	14.4	[a]	[a]
Hendy, et al. (*20*)	2% Cellulose	4.2	24.2	46
	5% Cellulose	8.0	55.5	140
	7.5% Cellulose	8.4	43.8	83
	10% Cellulose 10 L Fermenter	8.0	26.7	73
Sheir-Neiss, G., and Montenecourt, B. S. (*12*)	5% Solka floc 10 L fermenter	12.2	87.	[a]
This work	5% Solka floc 30:30 g/L	7.1	51.0	91.7
	Xylose:Cellulose 5 L Fermenter	5.6	37.3	122.7

[a]Not available.

batch cultures can be improved, compared to the batch process at similar conditions while the same total productivity can be achieved in less time. The amount of cellulose saved by substituting xylose for it in enzyme production can be used for ethanol production. As a result, the cost of substrate can be decreased substantially, making the process more economical.

Table 4, summarizes the results of most of the research work done on enzyme production to date. As seen from this table, our high titer activity and total productivity for growth on cellulose alone is slightly less than that of others at similar cellulose concentrations. The differences could be due to variations in filter paper activity measurements, mutated strains, or culturing conditions. Finally, our approach has the advantage over that of Gallo (*8*), who used a similar system for enzyme production, since we are not using lactose, which is a substrate with limited availability. The optimal concentrations of xylose and cellulose in more productive fed batch cultures will be investigated in future experiments.

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