0273-2289/99/77–79/0293/$14.25

Enzyme Production, Growth, and Adaptation of *T. reesei* Strains QM9414, L-27, RL-P37, and Rut C-30 to Conditioned Yellow Poplar Sawdust Hydrolysate

Scientific Note

Tammy Kay Hayward,* Jenny Hamilton, David Templeton, Ed Jennings, Mark Ruth, Arun Tholudur, James D. McMillan, Mel Tucker, and Ali Mohagheghi

National Renewable Energy Laboratory, 1617 Cole Blvd., Golden, CO 80401, E-mail: tammy_hayward@nrel.gov

Abstract

National Renewable Energy Laboratory (NREL) has developed a conditioning process that decreases acetic acid levels in pretreated yellow poplar hydrolysate. *Trichoderma reesei* is sensitive to acetic acid and this conditioning method has enabled applied cellulase production with hardwoods. *T. reesei* strains QM9414, L-27, RL-P37, and Rut C-30 were screened for growth on conditioned hydrolysate liquor. Tolerance to hydrolysate was found to be strain-dependent. Strain QM9414 was adapted to grow in 80% (v/v) conditioned hydrolysate (40 g/L of soluble sugars and 1.6 g/L acetic acid from pretreated poplar). However, enzyme production was highest at 20% (v/v) hydrolysate using strain L-27. Cellulase titers of 2–3 International Filter Paper Units (IFPU)/mL were achieved using pretreated yellow poplar liquors and solids as the sole carbon sources.

Index Entries: Cellulase; pretreated poplar hydrolysate; *Trichoderma reesei*; furfural; acetic acid.

Introduction

Economical cellulase enzyme production is key to developing enzyme-based processes for converting lignocellulosic biomass to fuel ethanol. Using pretreated lignocellulosic substrate to produce enzyme has several

*Author to whom all correspondence and reprint requests should be addressed.

advantages. First, the pretreated substrate contains cellulose, xylose, and glucose that can be used as carbon sources for enzyme production. Second, it has been shown that enzyme complex proteins produced by growing *Trichoderma reesei* on pretreated wood are more effective at hydrolyzing pretreated wood than enzyme produced on pure cellulose *(1)*. The fungus literally tailors the enzyme complex for that substrate. Finally, using pretreated yellow poplar for both the cellulase unit operation and the ethanol-production unit operation provides a streamlined process and reduces costs.

The goal of this study was to determine which strain of *T. reesei* (Rut C-30, L-27, RLP-37, or QM9414) should be used for cellulase production from pretreated yellow poplar sawdust. Evaluation of the strains included their ability to grow on the soluble sugars and acetic acid produced during pretreatment as well as their ability to make enzyme on the cellulosic fraction of the pretreated biomass slurry. Adaptation of the strains was also investigated as a means of improving performance.

Proposed Process Description

Yellow poplar sawdust is being tested as a model feedstock for enzyme production and ethanol fermentation. The sawdust undergoes co-current dilute acid pretreatment in a Sund's pilot scale reactor, followed by a solid–liquid separation. Most of the pretreated sawdust slurry is fed to the simultaneous saccharification and cofermentation (SSCF) tanks to produce ethanol. After separation, the liquor undergoes a proprietary conditioning process. Then a small portion of the xylose-rich conditioned liquor is used to feed the seed tanks containing a traditional submerged culture *T. reesei*. The cellulose-rich solids containing pretreated sawdust and minimal amounts of entrained conditioned liquor are then used as the sole carbon source for the cellulase production tanks. Only a small amount of the pretreatment stream is used for cellulase production.

Research to test the feasibility of growing *T. reesei* on xylose-rich conditioned hydrolysate liquor and making cellulase enzyme with cellulose-rich pretreated solids containing small amounts of entrained hydrolysate liquor was conducted. Previous research with hardwood feedstocks has been limited by the sensitivity of *T. reesei* to acetic acid *(2)*. The pretreated yellow poplar hydrolysate liquor contained approximately 12 g/L acetic acid (Fig. 1). The hydrolysate underwent a continuous proprietary conditioning process that reduced the acetic-acid concentration to 2 g/L, increasing the likelihood of *T. reesei* growth on the hydrolysate liquor. Figure 2 shows the effect on some of the major components in the liquor. After conditioning, the hydrolysate contained some acetic acid, furfural, 5-hydroxymethylfurfural, glaucine, syringaldehyde, vanillin, caproic acid, and other complex hardwood degradation products *(3)*. The conditioned hydrolysate was dark brown in color. The effect of these complex chemical compounds on the fungal organism was unknown.

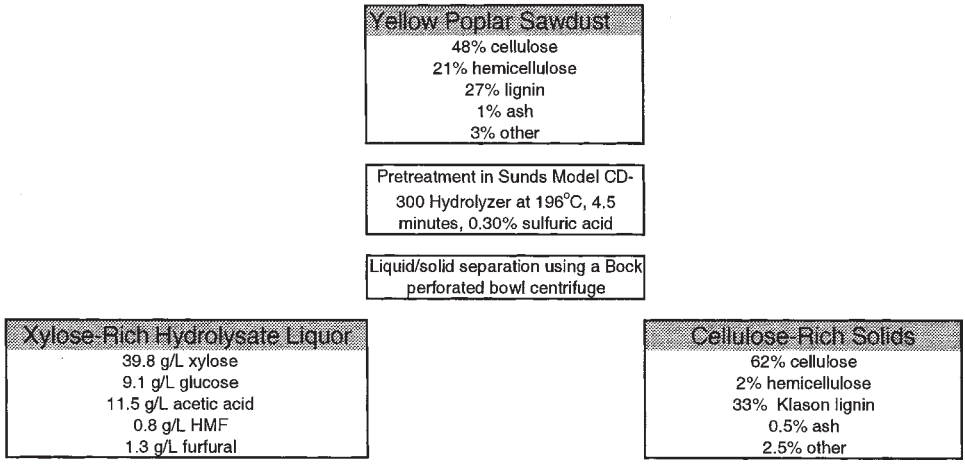

Fig. 1. Composition of yellow poplar sawdust before and after pretreatment.

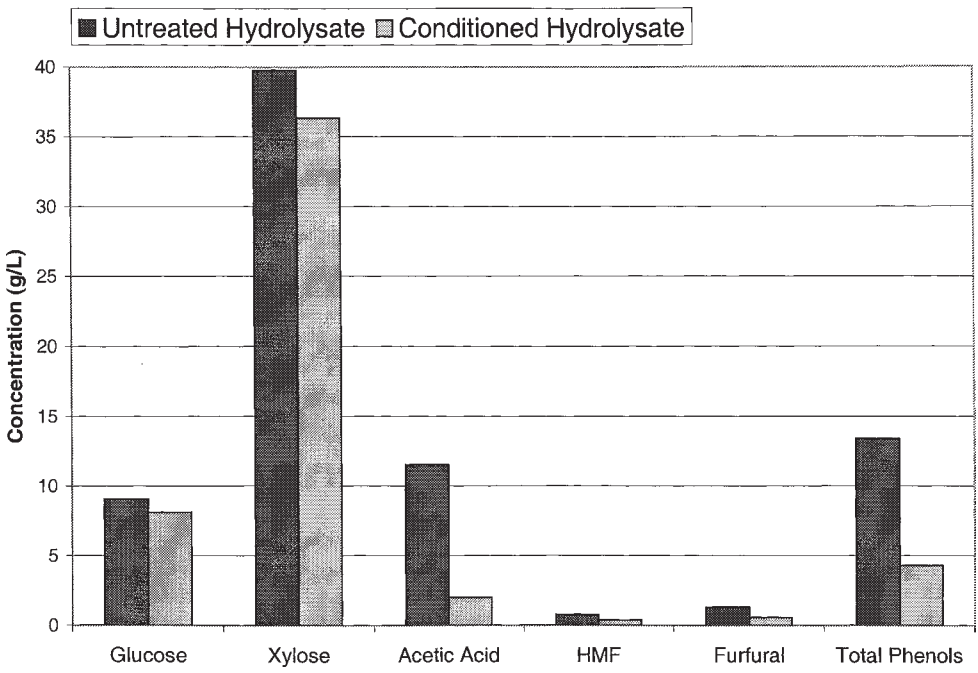

Fig. 2. Composition of yellow poplar sawdust hydrolysate liquor before and after conditioning.

Experimental Approach

Growth on conditioned hydrolysate is critical to use hydrolysate as carbon source for fungal inoculum production. Screening studies were performed to determine the *T. reesei* strain (Rut C-30, L-27, RL-P37, or

QM9414) and the hydrolysate concentration (% v/v) that was effective for growth. Hydrolysate liquor was mixed with growth media on a volumetric basis to determine a feasible hydrolysate concentration (% v/v) for seed production. Controls with sugar, acetic acid, and furfural were used to determine the tolerance of each strain and to provide information for further development of pretreatment and conditioning processes. Also, experience with hydrolysate liquors indicated that some microorganisms could be adapted to tolerate higher concentrations of hydrolysate liquor. Thus, a limited adaptation study was also conducted with several strains of *T. reesei*.

The first step in the bench-scale work was to test enzyme production in the presence of the conditioned hydrolysate liquor and Solka-floc. This was important for two reasons. First, the glucose in the hydrolysate represses enzyme activity (feedback inhibition). Second, toxins in the conditioned hydrolysate may reduce enzyme production by inhibiting or interfering with the fungal organism. By testing the hydrolysate with pure cellulose, the effect of liquid hydrolysate on cellulase production was assessed independently of the solid pretreated cellulosic substrate.

The second step in the bench-scale work was to use washed yellow poplar solids as a carbon source for cellulase production. The pretreated yellow poplar is 62% (w/w) cellulose and 33% (w/w) lignin on a dry weight basis (Fig. 1). Klason lignin adsorbs cellulase *(4)*. Washed pretreated solids were used to test the effect of lignin on cellulase production. Historically, fed-batch operation has been used to increase enzyme titer and productivity *(5)*, so runs using pretreated lignocellulosic substrate were conducted in batch as well as fed-batch mode. The effect of the pretreated wood solids was assessed independently of the hydrolysate liquor. Lastly, the washed pretreated lignocellulosic substrate and conditioned hydrolysate liquor were used together to simulate the use of a solids process stream containing modest amounts of hydrolysate.

Methods

Biomass Pretreatment and Conditioning

Hardwood yellow poplar *(Liriodendron tulipifera)* sawdust was pretreated in the continuous pilot plant pretreatment reactor Sunds model CD-300 (Sunds Defibrator Inc., Norcross, GA). The material used for enzyme production studies was produced in October 1996. Pretreatment conditions were 196°C, 4.5-min residence time with 0.3% (w/w) sulfuric-acid concentration. The pretreated biomass contained approximately 22% total solids. The pretreated slurry was separated into liquid and wet solids fractions using a Bock perforated bowl centrifuge (Bock Engineered Products Inc., Toledo, OH). The separated liquid hydrolysate contained almost 4% (w/v) xylose. Dewatered solids cake was 44% (w/w) total solids and contained 62% cellulose on dry mass basis (Fig. 1).

Pretreated solids were washed to remove inhibitory unconditioned hydrolysate liquors. The primary washing step involved repeatedly slurry-

ing up approx 20 kg wet solids (at about 44% [w/w] solids) in 40 kg water, agitating this slurry for 30 min, and separating the solids from the water using the aforementioned Bock perforated bowl centrifuge. The recovered solids cake was then removed. This procedure was repeated to achieve a total of three primary washes. The secondary washing protocol consisted of mixing approx 500 g of the primary washed wood with 4 L of deionized water and vacuum filtering it through muslin cloth. This procedure was repeated to achieve a total of three secondary washes. The final highly washed material obtained after vacuum filtrations was gravimetrically assayed and determined to contain approx 30% (w/w) solids.

The hydrolysate liquors directly recovered from dewatering the pretreated slurry were subjected to a proprietary pilot scale conditioning process to remove 80% of the acetic acid. The hydrolysate was then acidified to pH 2.0 with sulfuric acid and stored at 4°C prior to overliming. Hydrolysate overliming was typically performed just before experiments were run using standard conditions, as described previously *(3,6)*. The composition of the hydrolysate after conditioning was 36.7 g/L xylose, 8.9 g/L glucose, and 2.0 g/L acetic acid (Fig. 2).

Microorganisms

T. reesei strains Rut C-30, RL-P37, QM9414, and L-27 were grown on potato dextrose agar plates until sporulation occurred. Conidia were resuspended in 15% w/v glycerol and nutrient media, then dispensed into cryovials and stored at –70°C. These four strains are among the most widely published *T. reesei* strains. As delineated by Kadam *(7)*, all the strains derive from a common parent strain, QM6A. Strains Rut C-30 and RL-P37 are hyperproducing mutants developed by Rutgers (Newark, NJ). Strain QM9414 is a product of similar efforts performed at the Natick Laboratory (US Army Natick Research Development and Engineering Center, Natick, MA). Strain L-27, developed by Cetus (San Francisco, CA), is derived from strain QM9414 and is reported to be derepressed in its sensitivity to glucose catabolite repression.

Seed culture was grown in one stage for growth experiments and two stages for enzyme production. The first stage involves inoculating a single cryovial of frozen stock culture into a 250-mL shake flask (50 mL working volume) containing 2% (w/v) glucose, then incubating this flask for 36 h on a temperature-controlled orbital shaker operating at 200 rpm and 28°C. The second stage was used to condition the organism to enzyme production and scale up to the required volume. The stage one glucose-grown mycelia was transferred to a 1-L second stage flask containing 300 mL of medium with 20% (v/v) conditioned hydrolysate (i.e., approx. 1% soluble sugars) and 1% w/v Solka-floc and incubated for another 3 d.

Nutrient Media

The standard nutrient medium used for seed growth and enzyme production is a modified Mandels medium. It contained 1% (v/v) filter-

Table 1
Composition of Basal Salts and Trace Minerals in the Media

Basal salts	Trace minerals
2.0 g/L potassium phosphate	5.0 mg/L ferric sulfate heptahydrate
1.4 g/L ammonium sulfate	1.6 mg/L manganese sulfate monohydrate
0.4 g/L calcium chloride dihydrate	1.4 mg/L zinc sulfate monohydrate
0.3 g/L magnesium sulfate	3.7 mg/L cobalt chloride monohydrate

sterilized corn steep liquor (CSL) (Grain Products Corp., Muscatine, IA) and 2.5 g/L sodium citrate, basal salts, trace minerals, and 0.5 mL/L Tween 80 (enzyme production only). Table 1 shows the composition of the basal salts and trace minerals solutions. The pH of the medium was adjusted to 4.8 before use. This medium is similar to the modified Mandels medium already published by NREL *(8)*.

Shake-Flask Protocol

Shake-flask experiments for growth and adaptation were routinely carried out at an initial working volume of 20 mL in 125-mL baffled shake flasks. Enzyme production experiments used 250-mL baffled shake flasks with sponge caps (Bellco Glass Inc., Vineland, NJ) containing 50 mL working volumes. Enzyme production at the shake-flask scale was performed using 1% (w/v) Solka-Floc 10 NF (FS&D Corporation, St. Louis, MO) plus 20% (v/v) conditioned hydrolysate or the equivalent amount of pure xylose (8 g/L) and glucose (2 g/L) for the pure sugars controls. Prepared flasks were inoculated at 5% (v/v), then placed in an orbital shaker and incubated at 200 rpm and 28°C. Samples from each flask were collected periodically for compositional analysis. No pH control was used in shake-flask experiments, and pH varied from 3.5–5.3.

Bench-Scale Protocol

Strain L-27 was used for all scale-up experiments. Batch and fed-batch fermentor experiments were carried out at an initial working volume of 2.5 L in 5 L Bioflo 3000 fermentors (NBS Scientific, New Brunswick, NJ). Fermentors were prepared by filling with cellulose (i.e., Solka-floc or washed wood), water, sodium citrate, mineral salts, and corn oil, and autoclaved at 121°C for 45 min. Filter-sterilized conditioned hydrolysate liquor was then added to reach the effective hydrolysate level of 20% (v/v) of full strength. Then filter-sterilized clarified CSL, trace minerals, and Tween-80 were added.

As in shake-flask experiments, fermentors were inoculated at 5% (v/v). Fermentors were controlled at 28°C and pH 4.8 using 2 *N* phosphoric acid and 4 *N* sodium hydroxide. The agitation rate varied from 150–350 rpm, depending on the apparent viscosity of the culture. The dissolved oxygen concentration was controlled at 20% of air saturation using air and oxygen-

gas blending. Aeration rate varied, but was typically in the range of 1–3 standard L/min or 0.4–1.2 volumes of gas/vol of culture/min (vvm). Feeding was initiated in fed-batch experiments after 2–4 d, depending on the experiment. Thereafter, autoclaved washed pretreated lignocellulosic solids were fed daily at a rate of 0.5% (w/v) cellulose/d based on an assumed constant 2.5-L culture working volume in the fermentor. The pretreated substrate was fed using cut-off autoclaved preweighed 30-cc syringes, and introduced by flaming and opening a port on the headplate, quickly injecting it, then flaming and closing the port. Concentrated ammonium sulfate solution was added to achieve 100 ppm of free ammonia with each feeding. The nitrogen solution was added through a septum in the headplate at the same time the wood was fed.

Sample Analysis

Samples were taken daily for off-line analysis of pH, ammonium concentration, soluble sugars, total insoluble solids, and cellulase enzyme activity. Microscopic observations and plating were performed to check culture morphology and purity. Samples were typically spun down for 5 min at 4800 rpm using a Beckman GS-15R centrifuge. The solid pellets, consisting of residual cellulosic solids (Solka-floc or washed pretreated wood) and cell mass, were stored at 4°C before gravimetric analysis. Time-course samples were routinely analyzed off-line using an Orion pH meter and pH probe to measure or confirm culture pH. Selected samples were analyzed for residual ammonium using an ammonia probe. Both probes were calibrated using standard reference solutions. Early time-point samples were routinely analyzed by using an HP1090 high-performance liquid chromatograph (HPLC) running on Chemstation Software. The HPLC was equipped with a Bio-Rad Aminex HPX-87H column that used a 0.01 *N* sulfuric acid mobile phase at a flow rate of 0.6 mL/min and an oven temperature of 65°C to measure the concentration of glucose, xylose, and acetic acid. Samples were also assayed for cellulase enzyme using the method recommended by the International Union of Pure and Applied Chemistry *(9)*. Enzyme yield was calculated based on International Filter Paper Units (IFPU) divided by the total amount of sugars added to the culture. Productivity was calculated by dividing the titer in IFPU/L by the total hours in the run at each time point.

Results

Growth of T. reesei *Strains on Conditioned Hydrolysate*

An experiment containing 28 125-mL shake flasks showed that growth on hydrolysate was strain dependent. Control flasks contained 2% (w/v) glucose in nutrient media and no inhibitors. Conditioned hydrolysate was tested at 20% (v/v), 40% (v/v), and 50% (v/v). The 20% (v/v) hydrolysate flasks were supplemented with glucose to contain 2% (w/v) soluble sugars. The 40% (v/v) hydrolysate flasks contained 16 g/L xylose and 4 g/L glu-

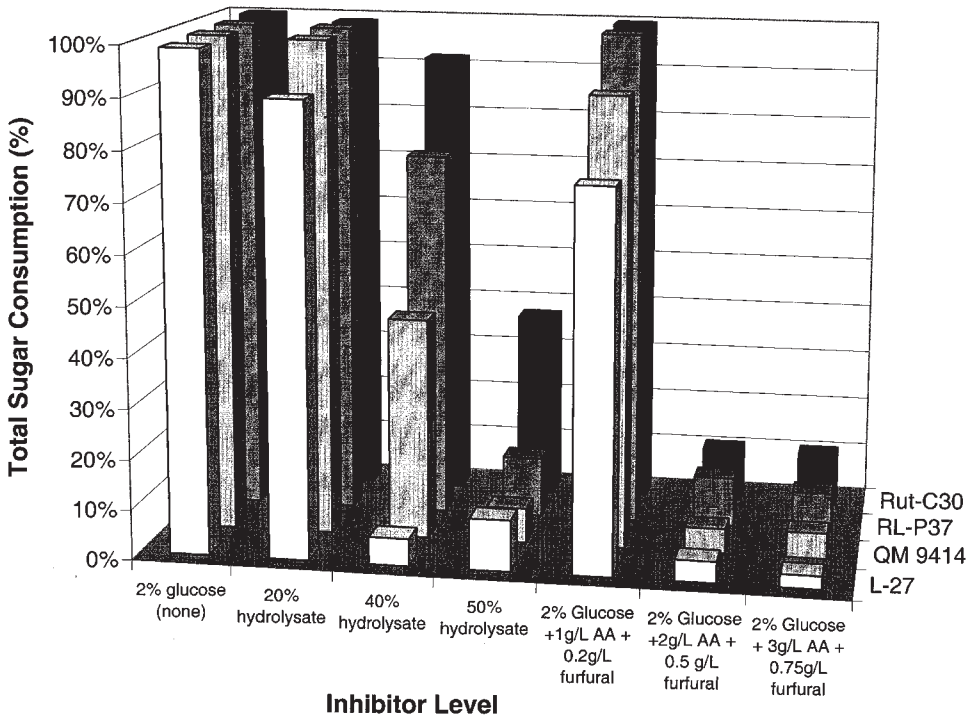

Fig. 3. Sugar consumption in the presence of acetic acid and furfural or conditioned hydrolysate.

cose or 2% (w/v) soluble sugars from pretreated biomass and were not supplemented. Accordingly, flasks above 40% (v/v) hydrolyzate were not supplemented.

Acetic acid and furfural inhibit the growth of strain Rut C-30 *(1)*. Shake flasks containing 2% glucose in nutrient media were spiked with three levels of acetic acid and furfural. The first level contained 1 g/L acetic acid and 0.2 g/L furfural, the second level was 2 g/L acetic acid and 0.5 g/L furfural, and the final level was 3 g/L acetic acid with 0.75 g/L furfural. These levels correspond to potential levels in pretreated biomass hydrolysates.

Figure 3 shows the 48-h sugar consumption as a percentage of initial sugar for *T. reesei* strains Rut C-30, RL-P37, QM9414, and L-27. All four strains consumed 100% of the initial glucose in the pure sugar controls, suggesting that the inocula were in good health, and in the absence of inhibition, 48 h was sufficient for the complete consumption of 2% (w/v) soluble sugars. There was a substantial difference in sugar utilization between the four strains at 40% (v/v) hydrolysate. Rut C-30 consumed the most sugar, followed by RL-P37, QM9414, and lastly L-27. This same trend was evident in the flasks containing acetic acid and furfural. For all the strains, sugar utilization was limited at acetic acid and furfural levels above 1 g/L and 0.2 g/L respectively, and above 40% v/v hydrolysate. Figure 4 shows cell mass concentrations at 48 h in the shake flasks. These results

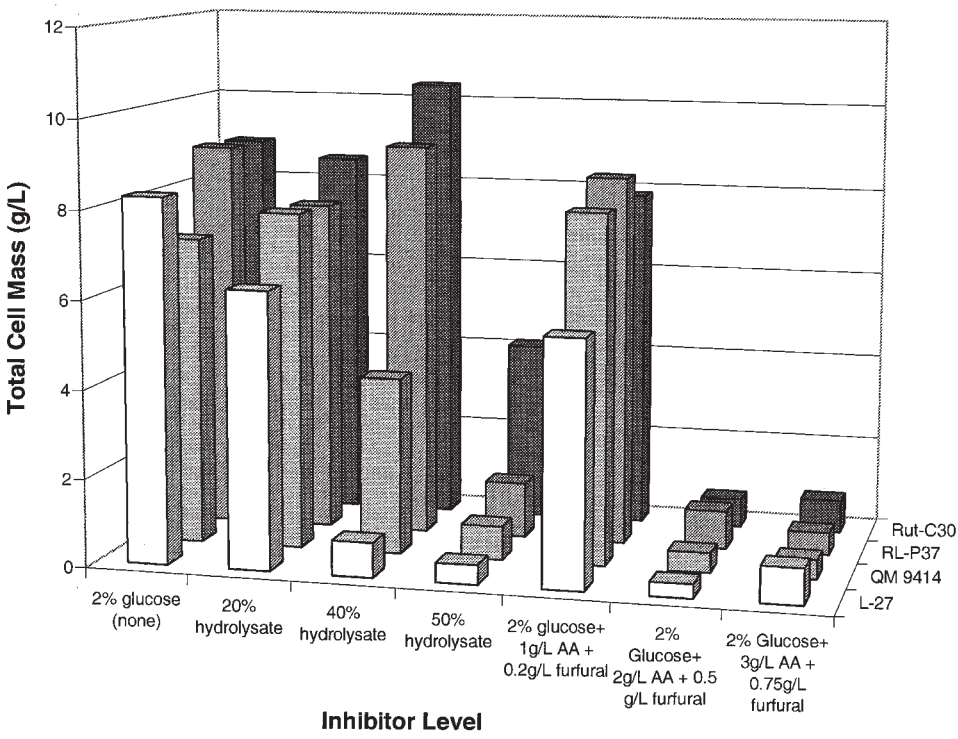

Fig. 4. Cell mass production in the presence of acetic and furfural or conditioned hydrolysate.

confirm that the strains did not grow well in 50% (v/v) hydrolysate or on 2 g/L acetic acid and 0.5 g/L furfural.

HPLC analysis of the 48-h sample points of the flasks with significant sugar utilization and growth showed a disappearance of acetic acid and furfural peaks (not shown). This supports work by Szengyel et al. *(2)*, who noted that Rut C-30 consumes low levels of furfural and acetic acid while growing. In the flasks where no growth occurred, acetic acid and furfural concentrations remained the same, suggesting that the disappearance of these compounds is owing to biological processes. The ability to consume furfural and acetic acid does not appear to be directly strain-dependent, but simply dependent on the growth of the organism.

Adaptation of T. reesei *Strains to Conditioned Hydrolysate*

Experience with pretreated lignocellulosics has shown that many microorganisms can be adapted to grow in the presence of toxins from pretreated biomass. Given the low tolerance for hydrolysate, and the desire to be able to grow *T. reesei* on the biomass sugars, a limited adaptation experiment was conducted. Because the process of sequential transfers is labor-intensive, this work focused on only three of the four strains: QM9414, RL-P37, and L-27. QM9414 was chosen because it performed well in the

Table 2
Summary of Growth and Enzyme Production
in the Presence of Conditioned Hydrolysate by Selected *T. reesei* Strains
Before and After Strain Adaptation[a]

Strain	Conditioned hydrolysate concentration (% v/v)							
Growth of *T. reesei* strains before adaptation	0	10	15	20	40	50	60	80
L-27	Y	Y	Y	Y(lag)	Y(lag)	N	N	N
RL-P37	Y	Y	Y	Y(lag)	Y(lag)	N	N	N
QM9414	Y	Y	Y	Y	Y	Y	N	N
Enzyme production by *T. reesei* strains before adaptation[b]	0	10	15	20	40	50	60	80
L-27	Y	Y	Y	Y	M	ND	ND	ND
RL-P37	Y	Y	Y	Y	M	ND	ND	ND
QM9414	M	M	M	M	M	ND	ND	ND
Growth of *T. reesei* strains after limited adaptation	0	20	40	60	80			
L-27	Y	Y	Y	Y(lag)	N			
RL-P37	Y	Y	Y	Y(lag)	N			
QM9414	Y	Y	Y	Y	Y			
Enzyme production by *T. reesei* strains after limited adaptation[c]	0	20	40	60	80			
L-27	Y	Y	M	N	N			
RL-P37	Y	Y	M	N	N			
QM9414	M	M	M	N	N			

[a]Abbreviations: Y, yes; Y(lag), growth with a larger than normal lag phase; N, no growth; ND, not detected; M, marginal.
[b]Y, at least 1.5 IFPU/mL; M, 0.5 IFPU/mL.
[c]Y, at least 1 IFPU/mL; M, 0.5 IFPU/mL.

screening experiments and was reported by Gracheck et al. and Aiello et al. to be relatively robust *(10,11)*. Strains RL-P37 and L-27 were selected for their documented ability to make high titers of cellulase *(12,13)*.

Using 125-mL shake flasks, strains were grown on 2% (w/v) pure xylose, then sequentially transferred to increasing levels of hydrolysate. Table 2 shows that all three strains were able to grow at higher levels of hydrolysate using this sequential adaptation approach. L-27 and RLP-37 did not grow in hydrolysate-based media above 40% (v/v) before adaptation, but did grow with a lag in 60% (v/v) hydrolysate-based media after adaptation. Even more progress was made with strain QM9414. Although the nonadapted strain did not grow in hydrolysate-based media above 50% (v/v) before adaptation, it grew in 80% (v/v) hydrolysate-based media after adaptation. Adapted strains were then transferred to flasks to determine whether enzyme would be produced at the high levels of hydrolysate. Unfortunately, enzyme production was not detected (i.e., the enzyme titer was at or below 0.5 FPU/mL) at the 60 or 80% hydrolysate levels. Despite

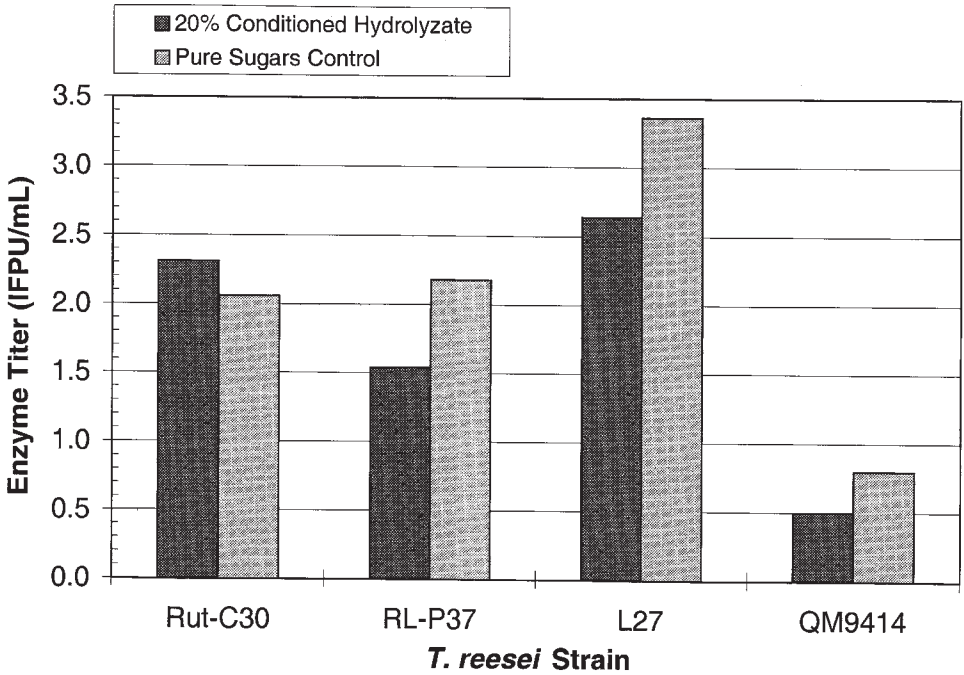

Fig. 5. Comparison of shake-flask enzyme titers at 168 h by *T. reesei* strains using 1% (w/v) cellulose and pure sugars or conditioned hydrolysate.

improvements in growth tolerance to hydrolysate liquor, adaptation did not yield strains with increased enzyme production in the presence of hydrolysate liquor.

Strains Comparison of Enzyme Production Using 20% (v/v) Hydrolysate

Because all four strains grew without adaptation in the presence of 20% (v/v) hydrolysate, this level was chosen to screen for enzyme production. Glucose at the levels present in this dilution may repress enzyme production, so controls containing no hydrolysate, but the amounts of glucose and xylose equivalent to the 20% (v/v) level (i.e., 8 g/L xylose and 2 g/L glucose) were included. All eight 250-mL shake flasks contained 1% (w/v) Solka-floc. Enzyme titers at 168 h for each of the four strains are shown in Fig. 5. Strain QM9414 had the lowest titers both with and without hydrolysate. L-27 produced the highest titers and was chosen for use in the bench-scale experiments.

Bench-Scale Cellulase Production by L-27 Using 20% (v/v) Hydrolysate

The first phase of the scale-up research was to confirm the ability to make cellulase in the presence of 20% (v/v) conditioned hydrolysate and

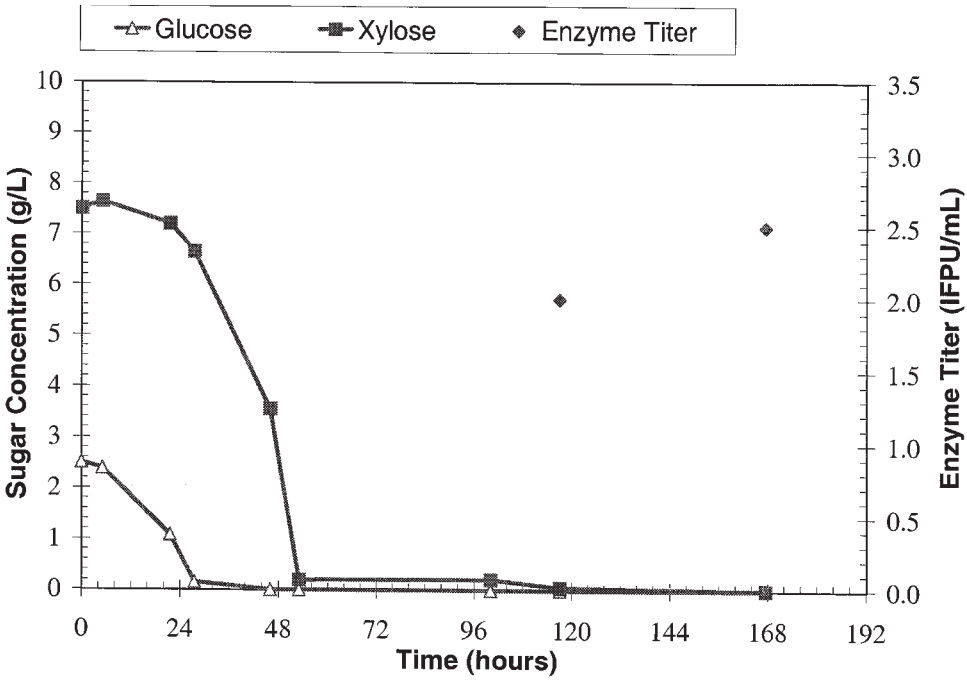

Fig. 6. Batch cellulase enzyme production by *T. reesei* strain L-27 using 1% cellulose and 20% (v/v) conditioned hydrolysate.

1% w/v Solka-floc. Figure 6 shows the glucose and xylose consumption profiles and enzyme titers at 120 and 168 h in a 5-L vessel. The organism utilizes hydrolysate glucose first, then xylose, then cellulose, and later in the process, produces measurable enzyme titers. The culture turned bright yellow after 36 h. At 120 h, there was an enzyme titer of 2 IFPU/mL and at 168 h, the enzyme titer was 2.5 IFPU/mL. However, the culture was thin and sporulated, so the experiment was terminated. These experiments confirmed that L-27 could produce enzyme on cellulose in the presence of moderate amounts of conditioned hardwood hydrolysate liquor.

Fed-Batch Cellulase Production with Washed Pretreated Solids

The second phase of the scale-up research examined the effect of using washed pretreated solids as the sole carbon source for enzyme production. Unlike Solka-floc, the pretreated yellow poplar solids produced considerably more foam in the aerobic cultures. Over time, the thick foam would carry the pretreated biomass out of the solution and deposit it onto the glass walls of the vessel. Figure 7 shows data for a 5-L vessel charged with 7.5% pretreated wood (5% [w/v] cellulose). The insoluble solids (pretreated wood solids and cell mass) of the sampled slurry portion of the vessel dropped quickly during the first 24 h because of caking on the vessel walls (Fig. 7). Fed-batch feeding was very limited, with only one addition

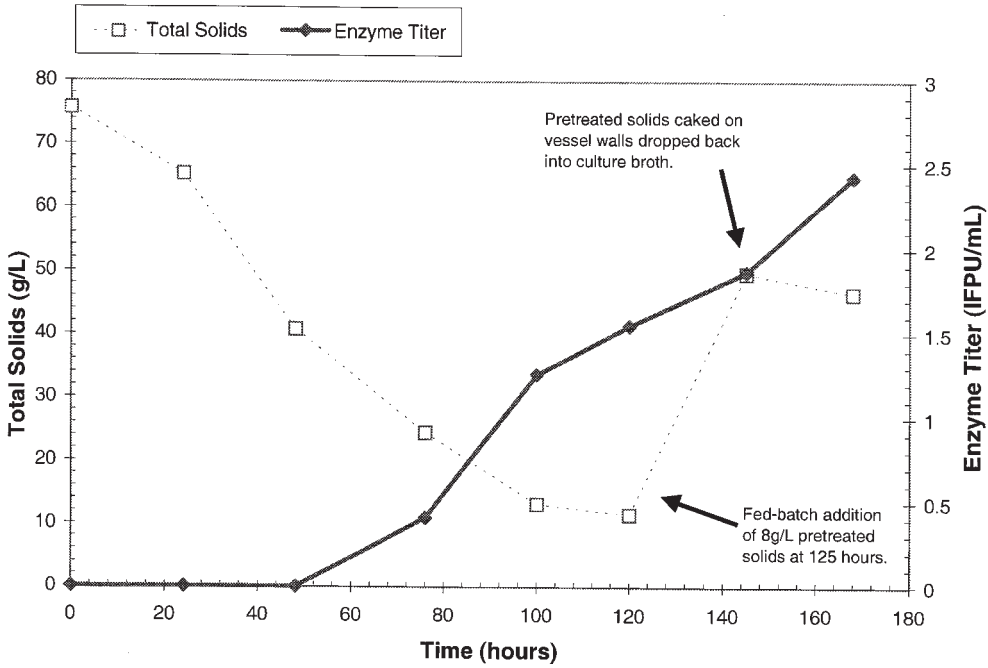

Fig. 7. Fed-batch cellulase enzyme production by *T. reesei* strain L-27 using only washed pretreated solids.

at 125 h, of 0.5% (w/v) cellulose as wood. Foaming continued to be a problem throughout the run. The total solids in the sampled culture dramatically increased at 145 h when the wood on the sides of the vessel dropped back into the solution after shaking the vessel. Despite these operational difficulties, the culture produced 2.5 IFPU/mL of cellulase.

Fed-Batch Cellulase Production Using Both Hydrolysate and Washed Pretreated Solids

The final phase was to combine both the liquid hydrolysate and the washed pretreated solids to simulate the use of process solids containing entrained hydrolysate. Part of the planned solution to the problems encountered in the previous run was to use a lower amount of washed wood along with more frequent fed-batch additions. This would limit the effective amount of solids in the vessel at any given time. A method for feeding washed autoclaved solids to the vessels was developed (*see* Methods section). Two levels of initial solids were tested: 3% (w/v) solids (2% cellulose) and 1.5% (w/v) solids (1% cellulose). Daily fed-batching of solids both vessels started at 120 h at 0.5% equivalent cellulose (0.75% [w/v] pretreated wood solids) and continued to 192 h. Figure 8 shows the cumulative levels of equivalent cellulose added to each vessel. Hydrolysate glucose and xylose consumption was slower in the presence of the pretreated wood than Solka-floc. However, the rate of glucose and xylose consump-

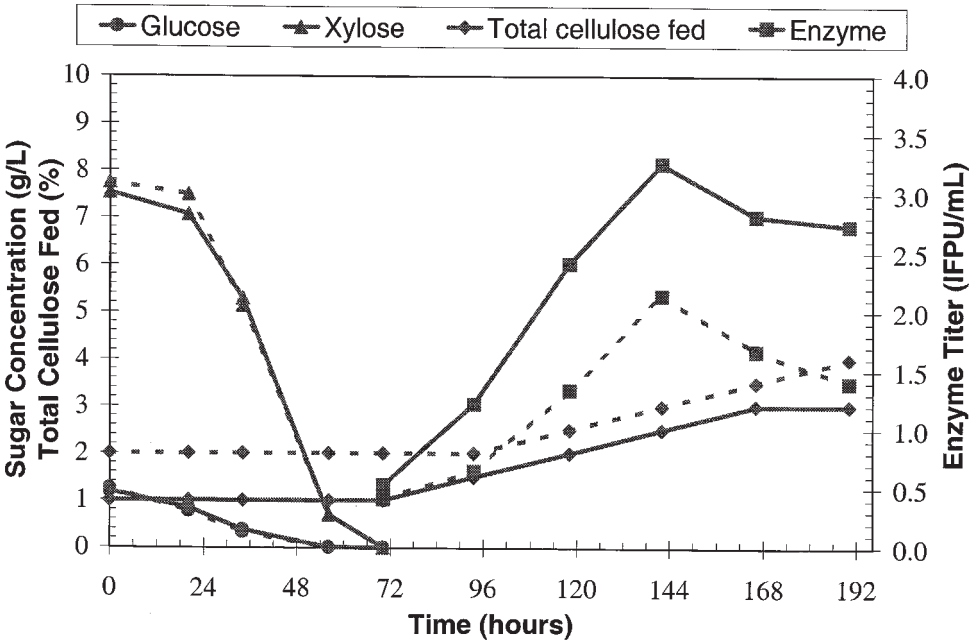

Fig. 8. Sugar consumption and enzyme titer profiles during fed-batch cellulase enzyme production using *T. reesei* strain L-27 on both 20% (v/v) conditioned hydrolysate and 1% (solid line) or 2% (dashed line) cellulose as washed pretreated solids.

tion was identical for both the 1.5% and 3% (w/v) wood conditions (Fig. 8). Moreover, doubling the initial cellulose (as washed pretreated wood) did not increase enzyme titer. A significant amount of foaming and caking on the sides of the vessels occurred in both vessels, with somewhat greater foaming in the vessel containing 3% (w/v) pretreated wood solids. The enzyme titer (3.3 IFPU/mL) was highest in the vessel containing 1.5% (w/v) initial pretreated wood solids.

Discussion

Four strains of *T. reesei*, QM9414, L-27, RL-P37, and Rut C-30 grew on 20% (v/v) conditioned hydrolysate liquor-based media without adaptation. Tolerance to higher levels of conditioned hydrolysate was strain-dependent. Strains QM9414 and Rut C-30 were the most tolerant to the presence of hydrolysate. Strain QM9414 was adapted by sequential transfers to grow in 80% (v/v) hydrolysate-based media. Thus, *T. ressei* cell mass can be produced using hydrolysate liquor as the sole carbon source.

Strain QM9414 has been grown on pretreated biomass substrates by several researchers *(10,11)*. In spite of its tolerance and adaptability to hydrolysate-based media, QM9414 produced the least amount of enzyme in the presence of 20% (v/v) hydrolysate and 1% Solka-floc. In contrast, strains L-27, RL-P37, and Rut C-30 produced higher cellulase titers. L-27

Table 3
Comparison of Titer, Yield, and Productivity by Strain L-27 with Pretreated Yellow Poplar Hydrolysate Liquors and Solids

		6-Day data			
Yellow poplar process stream	Substrate concentrations	Titer (IFPU/mL)	Total sugars fed in monomeric equiv. (g/L)	Yield (IFPU/g of sugars fed)	Productivity (IFPU/L *h)
Liquor	1% (w/v) Solka-floc with 20% (v/v) hydrolysate	2.2	21.1	104	15
Solids	7.5% (w/v) washed wood	2.5	64.4	39	17
Liquor plus solids	3% (w/v) washed wood with 20% hydrolysate	2.1	43.3	48	15
	1.5% (w/v) washed wood with 20% hydrolysate	3.3	37.8	87	23

has consistently produced high enzyme titers using cellulosic substrates *(13)*, and produced the highest enzyme titer in 20% (v/v) hydrolysate.

In bench-scale cellulase production experiments, strain L-27 produced enzyme in all three scenarios:

1. Hydrolysate liquor and Solka-floc;
2. Washed pretreated solids; and
3. Washed pretreated solids combined with hydrolysate.

Despite concerns about the effect of sugars and toxins in the hydrolysate liquor, the enzyme titers were similar for all three cases (Table 3). Higher substrate concentrations had a negative effect on overall cellulase yield (IFPU/g substrate fed to the vessel). The condition with the lowest amount of substrate, 1% Solka-floc with 20% hydrolysate, and had the highest yield. Increasing the amount of pretreated wood solids fed to the reactor did not increase enzyme titers. This is could be owing to the caking of the wood solids on the walls of the vessel, resulting in no net increase in substrate available for the submerged fungal culture. Six-d productivity values ranged from 15–23 IFPU/L-h. The highest enzyme titer achieved was 3.3 FPU/mL using the hydrolysate liquor combined with the washed pretreated solids. Thus, cellulase enzyme was produced using pretreated solids and hydrolysate liquors.

Future experiments will focus on increasing enzyme titer and productivity using pretreated yellow poplar sawdust. L-27 has produced more than 5 IFPU/mL on Solka-floc *(13)*. Mechanical improvements may reduce the build-up of pretreated solids on the vessel walls. This should increase the effective amount of substrate available to the submerged culture and thereby increase titers and cellulase yields based on the substrate fed to the system. Fed-batch protocols still need to be improved. In these experiments, fed-batch additions were performed only once a day. The ability to mechanically deliver small amounts of pretreated solids every hour is expected to further improve cellulase productivity.

Acknowledgments

J. Farmer, B. Lyons, and D. Schell are gratefully acknowledged for assisting with the pretreatment of the yellow poplar sawdust in NREL's pilot plant. This work was funded by the Biochemical Conversion Element of the Office of Fuels Development of the U. S. Department of Energy.

References

1. Vinzant, T. B., Adney, W. S., Decker, S. R., Baker, J. O., Himmel, and M. E. (1998), Poster, 20th Symposium on Biotechnology for Fuels and Chemicals, Humana Press, Totowa, NJ.
2. Szengyel, Z., Zacchi, G., and Reczey, K. (1997), *Appl. Biochem. Biotech.* **63/65,** 351–362.
3. Ranatunga, T., Jervis, J., Helm, R., McMillan, J. D., and Hatzis, C. (1997), *Appl. Biochem. Biotech.* **67,** 185–198.

4. Tatsumoto, K., Baker, J. O., Tucker, M. P., OH, K. K., Mohaghegi, A., Grohmann K., and Himmel, M. E. (1988), *Appl. Biochem. Biotech.* **18,** 159–174.
5. Hendy, N. A. Wilke, C. R., and Blanch, H. W. (1984), *Enzyme Microb. Technol.* **6,** 73–77.
6. McMillan J. D. (1994), in *Enzymatic Conversion of Biomass for Fuels Production*, American Chemical Society, Washington, DC, pp. 411–437.
7. Kadam, K. L. (1996), in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, pp. 213–252.
8. Mohagheghi, A., Grohmann, K., and Wyman, C. E. (1988), *Appl. Biochem. Biotechnol.* **17,** 263–277.
9. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
10. Gracheck, S. J., Rivers, D. B., Woodford, L. C., Giddings, K. E., and Emert, G. H. (1981). *Biotech. Bioengineer. Symp.*, No. 11., Wiley, New York, pp. 47–65.
11. Aiello, C., Ferrer, A., and Ledesma, A. (1996), *Bioresource Technol.* **57,** 13–18.
12. Sheir-Neiss, G. and Montenecourt, B. S. (1984), *Appl. Microbiol. Biotechnol.* **20,** 46–53.
13. Schell, D. J., Hinman, N. D., Wyman, C. E., and Werdene, P. J. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 287–296.