

Final Technical Report

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Development of Optimal Process Conditions for Fungal Cellulase Production

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Preface

This is the final report on a study of the process and economics of cellulase production. The overall goal is to reduce the cost of cellulase production to the point where ethanol production from biomass is economically feasible. Due to our lack of data on the entire ethanol process, this report only deals with the cellulase producing unit. It begins with an economic analysis of the process under several different operating scenarios. The data for the study was taken from both the best results in literature and personal correspondence with NREL through Steve Thomas. Following this is a brief discussion concerning which operating variables should be improved. Also included is a summary of lab results on fermentations performed since the last report submitted in March 1996. Finally there is a section on what directions the study should take in the future.

Appendices include full data on two economic scenarios and a comprehensive bibliography of all the literature used in this study over the past nine months.

Special thanks should be made to Dan Stevens and Marc Becker for their significant contributions to this study.

Process Simulation and Economic Evaluation

The goal of this simulation was to model a plant that produces cellulase by *Trichoderma reesei* which delivers sufficient enzyme for the production of 60×10^6 gallons of ethanol per year. BioPro Designer Version 2.1 (Academic Version) by INTELLIGEN Inc. was used to design the process and evaluate the price of the produced enzyme.

Scenarios

Three different scenarios were looked at to obtain a value for the required amount of cellulase; an SHF process published in a paper by Ballerini (1994), an SSF process using the data from Ballerini, and an SSF process based on a combination of data obtained from literature and from NREL. For the SHF Ballerini scenario, data based entirely on a large-scale wood to ethanol pilot plant in Soustons, France was considered.⁸ This SHF process was not included in process design and economic simulation. For the SSF Ballerini scenario, the data for the cellulase production step was taken from the French pilot plant; however, the ethanol production step used modified data from a SSF process evaluation.^{35,122} For the NREL scenario, averaged data from the literature^{24,83} (which was in agreement with values used at NREL provided by Steve Thomas) was used for the cellulase production step. The ethanol production step used the same process evaluation data^{35,122} as the SSF Ballerini scenario.

The two different operating scenarios based on a SSF process were examined in the BioPro economic analysis. Four different cases were simulated for each scenario. In the main case, lactose was used as substrate and the selling price for cellulase was set to result in a payback time of 5 years for the plant. The lactose was then substituted by cheaper cheese-whey permeate. Finally, the effect of a variation of the selling price for cellulase using lactose as carbon source was investigated. Following this is a brief discussion on which operating variables may be improved. Appendix A includes full data on the main lactose case study for both economic scenarios.

Estimation of the Required Amount of Cellulase

Ballerini Scenario, SHF

Table 1. Enzyme production data from Ballerini⁸ (1994)

Fermentation time	151 hours
Air flow	0.5 VVM
Final volume	30.4 m ³
Lactose consumption	2473 kg
Total lactose concentration	81.349 kg/m ³
Mycelium dry weight	10.3 kg/m ³
Soluble proteins	34.4 kg/m ³
Protein yield on lactose	42.2%
Enzyme activity	37.9 FPU/ml
Enzyme productivity	251 FPU/ml/h

The large scale enzyme production within the French plant using lactose as carbon source yielded an enzyme activity of 37.9 FPU/ml and a productivity of 251 FPU/l/h (Table 1). These results are among the best that have been reported so far.

Assuming all the soluble protein is cellulase one can make the correlation that 1.102×10^6 FPU equals 1 kg of cellulase.

$$\frac{37.9 \text{ FPU / ml}}{34.4 \times 10^{-6} \text{ kg cellulase / ml}} = 1.102 \times 10^6 \text{ FPU / kg cellulase}$$

In the enzymatic hydrolysis step, an enzyme load of 16 FPU/g cellulose resulted in a nearly complete (>95%) hydrolysis of the polysaccharides. Taking into account the enzyme activity calculated above, the amount of cellulose that can be hydrolyzed per kilogram of enzyme is:

$$\frac{1.102 \times 10^6 \text{ FPU / kg cellulase}}{16,000 \text{ FPU / kg cellulose}} = 68.9 \text{ kg cellulose / kg cellulase}$$

The yield of fermentable sugars, mainly glucose, on cellulose is reported to be 53.9% (w/w). In the ethanol fermentation step the yield of ethanol on glucose is reported to be 45.1% (w/w). Thus using the density of ethanol one can calculate the volume of ethanol that can be produced with one kg of cellulase.

$$\left(\frac{68.9 \text{ kg cellulose}}{1 \text{ kg cellulase}}\right) * \left(\frac{0.539 \text{ kg glucose}}{1 \text{ kg cellulose}}\right) * \left(\frac{0.451 \text{ kg ethanol}}{1 \text{ kg glucose}}\right) * \left(\frac{1 \text{ l ethanol}}{0.7873 \text{ kg ethanol}}\right)$$

$$= 21.3 \frac{\text{l ethanol}}{\text{kg cellulase}} = 5.6 \frac{\text{gal ethanol}}{\text{kg cellulase}}$$

Using this scenario and a target amount of 60×10^6 gallons of ethanol per year, a production of 1.07×10^7 kg of cellulase per year is necessary. The value of 5.6 gal EtOH/kg cellulase corresponds to a value of 2.0×10^5 FPU/gal EtOH, which is high compared to a reference number of 3.1×10^4 - 6.2×10^4 FPU/gal EtOH needed for an ethanol production plant (Jim Doncheck, Bio-Technical Resources L.P.).

Ballerini Scenario, SSF

According to Wright,¹²² the enzyme load required in a SSF process to hydrolyze cellulose to an extent of 88% within 7 days is 7 IU per gram of the fed, pretreated cellulose. Hinman³⁵ reported a more realistic optimal use of 13 IU per gram of cellulose, which is also in accordance with design data used at NREL. Roughly estimating that IU and FPU are interchangeable and taking the correlation that 1.102×10^6 FPU is equal to 1 kg cellulase,⁸ one can calculate the amount of cellulase hydrolyzable by 1 kg of cellulase.

$$\frac{1.102 \times 10^6 \text{ FPU / kg cellulase}}{13,000 \text{ FPU / kg cellulase}} = 84.8 \text{ kg cellulase / kg cellulase}$$

Wright also states that 88% of the cellulose feed is hydrolyzed to fermentable sugars, of which 90% are fermented to ethanol in the SSF process. Again, one can calculate the volume of ethanol that can be produced with one kg of cellulase.

$$\left(\frac{84.8 \text{ kg cellulase}}{1 \text{ kg cellulase}}\right) * \left(\frac{0.88 \text{ kg glucose}}{1 \text{ kg cellulase}}\right) * \left(\frac{0.9 \text{ kg EtOH}}{1 \text{ kg glucose}}\right) * \left(\frac{1 \text{ l EtOH}}{0.7873 \text{ kg EtOH}}\right)$$

$$= 85.3 \frac{\text{l EtOH}}{\text{kg cellulase}} = 22.5 \frac{\text{gal EtOH}}{\text{kg cellulase}}$$

In this case a production of 2.66×10^6 kg of cellulase per year is necessary for the targeted amount of 60×10^5 gallons of ethanol per year. The result of 22.5 gal EtOH/kg cellulase corresponds to a required enzyme load of 4.9×10^4 FPU/gal EtOH.

NREL Scenario, SSF

In this scenario, the assumption was made that the cellulase fermentation step yields 600,000 FPU/kg protein. This value is reproducibly achievable with most of the hyperproducing strains according to the literature.^{24,83} Here the amount of cellulose hydrolyzable by 1 kg of cellulase is:

$$\frac{600,000 \text{ FPU / kg cellulase}}{13,000 \text{ FPU / kg cellulase}} = 46.2 \text{ kg cellulose / kg cellulase}$$

According to Steve Thomas the design data considered at NREL is 50 kg cellulose/kg cellulase, which matches with this estimate. The volume of ethanol that can be produced with one kg of cellulase is:

$$\begin{aligned} & \left(\frac{46.2 \text{ kg cellulose}}{1 \text{ kg cellulase}} \right) * \left(\frac{0.88 \text{ kg glucose}}{1 \text{ kg cellulase}} \right) * \left(\frac{0.9 \text{ kg EtOH}}{1 \text{ kg glucose}} \right) * \left(\frac{1 \text{ l EtOH}}{0.7873 \text{ kg EtOH}} \right) \\ & = 46.4 \frac{\text{l EtOH}}{\text{kg cellulase}} = 12.3 \frac{\text{gal EtOH}}{\text{kg cellulase}} \end{aligned}$$

In this case, a production of 4.89×10^6 kg of cellulase per year is necessary for the targeted amount of 60×10^5 gallons of ethanol per year. The enzyme load per gallon of ethanol is 4.9×10^4 FPU/gal EtOH

Table 2. Comparison of cellulase requirements for the production of 60×10^5 gallons ethanol/year by enzymatic hydrolysis

	Cellulase Stream Flowrate	Specific Activity
Ballerini Scenario, SHF	1.07×10^7 kg/yr	2.0×10^5 FPU/gal EtOH
Ballerini Scenario, SSF	2.66×10^6 kg/yr	4.9×10^4 FPU/gal EtOH
NREL Scenario, SSF	4.89×10^6 kg/yr	4.9×10^4 FPU/gal EtOH

Comparing the values of the different scenarios in Table 2, it becomes evident that the required amount of cellulase decreases significantly in a SSF process because of the much better overall yield of ethanol on carbon source. This is the reason why only the two SSF scenarios were taken for process design and economic evaluation.

The higher stream flowrate requirement of the NREL scenario results from the lower enzyme load of 0.6×10^6 FPU/kg cellulase of the stream compared to 1.102×10^6 FPU/kg cellulase in the Ballerini scenario.

Assumptions and Input for the BioPro Model

Large-Scale Process

Two fermenters were included for inoculum growth, providing 5% of the final working volume of the next larger fermentation step (BioPro flowsheet in Appendix A, Figure A.1). The fermentation was scheduled to a batch time of 151 hours plus an additional downtime of 15 hours and an annual operating time of 7,920 hours.

It was assumed that no further down stream processing is necessary for a SSF process, so the whole product stream can be used for the following process step.

Component Prices

The purchase prices for the carbon sources were set to 1.2125 \$/kg for lactose (Chemical Marketing Reporter April 15, 1996) and 0.05 \$/kg for cheese whey permeate.²⁶ No further pretreatment costs for the whey permeate were taken into account. The lactose fraction in whey permeate is roughly 5% (w/v).³⁶

The ammonia stream was entered for the simplified stoichiometric equation in the fermentors, but would not be required in a real process. Accordingly the purchase price for ammonia was set to 0 \$/kg and does not effect the economic simulations.

The media used in the French pilot plant for the CL-847 fermentations was described by Pourquoi⁸⁵ (Table 3). A media optimization led to the replacement of yeast extract and Tween 80 by the cheaper cornsteep liquor.

Table 3. CL-847 fermentation medium used for BioPro simulations

Component	Concentration (kg/m ³)	Purchase Price (\$/kg)
Ammonium Sulfate	2.8	0.08
Calcium Chloride	1.2	0.21
Cobaltous Chloride	0.004	21.78
Cornsteep Liquor	1.0	1.00
Ferrous Sulfate	0.01	0.01
Magnesium Sulfate	0.6	0.35
Manganeous Sulfate	0.0032	0.48
Phosphoric Acid	2.9	0.71
Potassium Hydroxide	1.66	3.28
Zinc Sulfate	0.0028	0.80

Prices are from the Chemical Marketing Reporter from April 15, 1996. To make the results of both scenarios comparable, it was presumed that the same medium could be used in the NREL scenario.

It was assumed that water for the fermentation broth would be available at low cost and would not effect the economic evaluation. The necessary oxygen was supplied by an air stream of 0.5 VVM.

Stoichiometric Equations for the Fermentors

A general equation for an aerobic process was used.

carbon source + oxygen + ammonia →

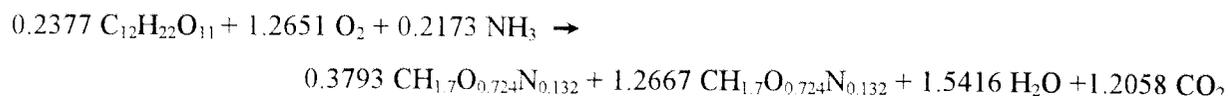
mycelium + cellulase + water + carbon dioxide

Lactose was provided as the carbon source. An empirical formula for *T. reesei* mycelial cells was used as described by Harima.³³ In addition it was assumed that cellulase has the same empirical formula as mycelial biomass.

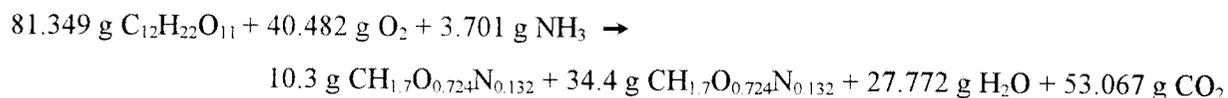
Ballerini Scenario

Enzyme production data from the French pilot plant⁸ was used entirely for the stoichiometric equation in this scenario (Table 1).

Using molar weights, one can set up the stoichiometric equation on a mole basis for 1 liter.



The BioPro stoichiometric equations in the fermentors are based on mass terms accepting three decimal places. Using molar weights one can convert the molar stoichiometric coefficients to mass stoichiometric coefficients (for 1 liter).

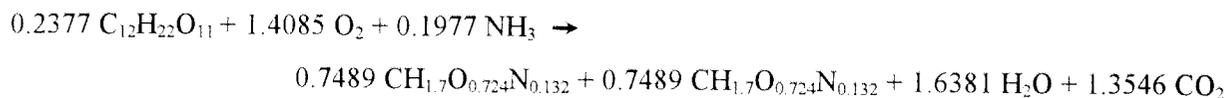


The feed requirements (lactose, ammonia) for each fermentor were calculated applying these concentrations. Oxygen was found to be sufficiently supplied by an air stream of 0.5 VVM.

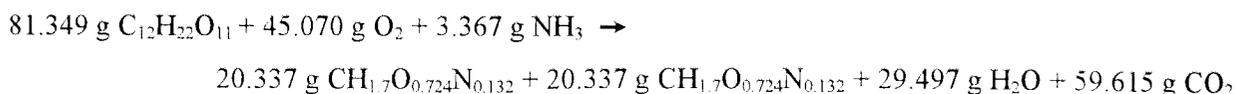
NREL Scenario

The total lactose concentration was set to 8.13% (w/v), making the conversion of carbon source to cellulase as well as the economic data comparable to the Ballerini scenario. Averaged data from Esterbauer²⁴ state that various *T. reesei* strains produce roughly 0.25 g mycelial biomass/g carbon source and 0.25 g cellulase protein/g carbon source. These values are also used for calculations at NREL.

This leads to the following stoichiometric equation on a mole basis for 1 liter.



Converting the molar stoichiometric coefficients to mass stoichiometric coefficients yields the following equation for 1 liter.



In this scenario, the assumption was made that 600 FPU/g protein are obtained. If 20.337 g cellulase protein/l are produced, one can calculate the enzyme activity to 12.2 FPU/ml. This value is reproducibly achievable with most of the improved strains according to the literature.^{24,83}

Results and Discussion

In the Ballerini scenario, an enzyme production of 2.84×10^6 kg per year was reached designing a plant with 12 fermentors of 200 m³ total volume. In the NREL scenario, an enzyme production of 4.90×10^6 kg per year was reached designing a plant with 35 large fermentors of 200 m³ total volume. Additional main equipment specifications for the two scenarios leading to the targeted enzyme production are compared in Table 4. Detailed design data and process flow sheet are shown in Appendix A.

The total equipment cost in the NREL scenario is about threefold the total equipment cost in the Ballerini scenario. This emphasizes the importance of further optimization of the selected strain with respect to higher enzyme titers and productivity.

Table 4. Comparison of the equipment specification for the two scenarios

	Ballerini Scenario	NREL Scenario
Compressors	2	4
Heat Sterilizers	3	3
Fermentors (3 volumes)	12 for each volume	35 for each volume
	(maximum volume of 200 m ³)	
Air Filters	6	8
Pumps	7	16
Total Equipment Cost	22,860,000 \$	65,175,000 \$

Table 5. Comparison of the profitability analysis for the main lactose case

	Ballerini Scenario	NREL Scenario
Substrate	lactose	lactose
Production Unit Cost (\$/kg)	18.71	31.08
Cellulase Selling Price (\$/kg)	29.47	48.90
Specific Activity (FPU/kg)	1.102x10 ⁶	0.6x10 ⁶
Cellulase Selling Price (\$/10 ⁶ FPU)	26.74	81.50
Payback Time (years)	5	5

Neither the equipment specifications, nor the material balances are affected by the variations of substrate purchase price and cellulase selling price that were investigated in the different case studies (Appendix A, Tables A.1, A.2, A.6, A.7).

Main Lactose Case

In this case, lactose was utilized as substrate and the payback time for the plant was adjusted to 5 years by varying the selling price of cellulase. A partial summary of the BioPro economic evaluation output for the two scenarios is compared in Table 5. A detailed description of the BioPro output for the main lactose case of both scenarios is listed in Appendix A.

The economic evaluation gave a production unit cost and a cellulase selling price of 18.71 \$/kg and 29.47 \$/kg in the Ballerini scenario and of 31.08 \$/kg and 48.90 \$/kg in the NREL scenario respectively.

If the annual capital and operating cost per equipment are compared, (Appendix A, Tables A.4 and A.9), only minor differences are found between the two scenarios. About 28% of this cost is attributed to the compressors and 70% to the fermentors.

Whey Case

In this case, the effect of a decrease in substrate raw material price was simulated. The lactose was substituted by cheaper whey permeate, decreasing the price per kg of carbon source by over 95%. Again, the payback time for the plant was adjusted to 5 years by varying the cellulase selling price (Table 6).

Table 6. Comparison of the profitability analysis for the whey case

	Ballerini Scenario	NREL Scenario
Substrate	whey permeate	whey permeate
Production Unit Cost (\$/kg)	15.90	26.34
Cellulase Selling Price (\$/kg)	26.65	44.15
Specific Activity (FPU/kg)	1.102x10 ⁶	0.6x10 ⁶
Cellulase Selling Price (\$/10 ⁶ FPU)	23.82	73.58
Payback Time (years)	5	5

The economic evaluation gave a production unit cost of 15.90 \$/kg in the Ballerini scenario and 26.34 \$/kg in the NREL scenario. The selling price for cellulase was 26.65 \$/kg in the Ballerini scenario and 44.15 \$/kg in the NREL scenario.

As expected, the whey case is less expensive than the lactose case. The production unit cost and the cellulase selling price were reduced by 2.81 \$/kg and 2.82 \$/kg in the Ballerini scenario and by 4.74 \$/kg and 4.75 \$/kg in the NREL scenario respectively. This significant reduction in the selling price indicates that effort should be taken to find less expensive substitutes for substrate and medium components.

Novo Case

Novo Industry sells their enzyme preparation for 12 \$/l which has a guaranteed activity of 80,000 FPU/l. These numbers correspond to a selling price of 150 \$/10⁶ FPU. In this case the effect on payback time if the cellulase product stream could be sold at Novo prices was considered (Table 7). Downstream processing cost for purifying the enzyme were not subtracted from the Novo selling price. The payback time of 0.63 years in the Ballerini scenario compared to 2.21 years in the NREL scenario again shows the good performance of the CL-847 strain.

Production Unit Cost Case

What happens if the cellulase stream is used for a SSF step in a biomass to ethanol plant and thus the intention is not to sell the cellulase?

Table 7. Comparison of the payback time at Novo selling prices

	Ballerini Scenario	NREL Scenario
Novo Selling Price (\$/10 ⁶ FPU)	150	150
Specific Activity (FPU/kg)	1.102x10 ⁶	0.6x10 ⁶
Novo Selling Price (\$/kg)	165.30	90.00
Payback Time (years)	0.63	2.21

Table 8. Cost of cellulase per gallon of ethanol

	Ballerini Scenario	NREL Scenario
Lactose Case	0.83 \$/gal EtOH	2.53 \$/gal EtOH
Whey Case	0.71 \$/gal EtOH	2.14 \$/gal EtOH

If the selling price is set equal to the production unit cost for the different scenarios, the gross profit for the plant is zero. In the BioPro simulation program the production unit cost includes the annual return payments for the initial capital investment. The payback time is 11.1 years in these simulation scenarios. Therefore the production unit cost would be the price used for the enzyme production cost in a biomass to ethanol plant.

Table 8 shows the breakdown of the enzyme production cost per gallon of ethanol for lactose and whey case. For example in the lactose case these results can be calculated.

$$\text{Ballerini Scenario: } \frac{18.71 \text{ \$ / kg cellulase}}{22.5 \text{ gal EtOH / kg cellulase}} = 0.83 \text{ \$ / gal EtOH}$$

$$\text{NREL Scenario: } \frac{31.08 \text{ \$ / kg cellulase}}{12.3 \text{ gal EtOH / kg cellulase}} = 2.53 \text{ \$ / gal EtOH}$$

The absolute values are higher than values found in the literature.⁸⁴ It needs to be investigated if the enzyme load per gallon of ethanol could be reduced using recycling or immobilization techniques.

Summary

The previous cases considered show there is potential to decrease the price of cellulase production in a large-scale plant.

As it was shown, one goal is to improve enzyme titer and volumetric productivity of the fungus. This leads to a decrease in total required fermentation volume, thus lowering not only equipment cost but also annual capital and operating cost (especially utility cost).

A second goal is to further substitute expensive substrates and media components. Particularly the research on alternative carbon sources like cheese whey permeate, poplar wood, hydrolyzed whole wheat flour, crops, or a combination of these should be intensified. If similar results could be accomplished with less expensive substrates as with lactose in the pilot plant in Souston, France, the production of cellulase would become more economically feasible.

Lab-Scale Fermentations

Materials and Methods

The variable tested since the March report was the substrate, one of which was an acid pretreated poplar provided by NREL. The product received was 25% dry matter and the average chip size was 4 mm. The composition of the dry matter was inferred based on data from poplar pretreated in an identical fashion, and is outlined in Table 9. A second substrate tested was the acid hydrolysate of wheat flour (AHW). This was prepared by a 2 hour hydrolysis of 25% solids (wheat flour) at 121 °C in 0.3 M HCl. To neutralize the slurry, CaOH was added until the pH reached 5.0. Table 10 gives the composition of an identically prepared substrate.

Two poplar fermentations and one AHW fermentation have been completed since March. The first, designated Pop1, used 1% cellulose as substrate and had a liquid volume of 25 l in a 30 l vessel. The second, designated Pop 2, used 5% cellulose as substrate and had a liquid volume of 3 l in a 5 l vessel. Both poplar fermentations used the media outlined in Table 11. The cause for change in vessel size was a lack of available funds to continue using the larger pilot plant facilities. The AHW fermentation was carried out at 3 l in a 5 l vessel. The media consisted of 1.2 l of AHW slurry and 0.2 g/l of PPG Macol DF 6000k antifoam. No salts or trace metals were added because of the small protein content of the wheat flour. This corresponds to a glucose concentration of 5.7% and an oligosaccharide content of 0.8%.

Table 9. Composition of the pretreated poplar feed.*

Component	% on dry basis
Cellulose	68.0
Xylan	0.4
Mannan	0.3
Klason Lignin	30.0
Acid Soluble Lignin	1.3

* Based on personal correspondence with Rafael Nieves of NREL.

Table 10. Composition of the acid hydrolyzed wheat.*

Component	g/l
Glucose	166.82
Xylose	13.40
Galactose	1.85
Arabinose and Mannose	8.35
Oligosaccharides	23.94
Total Sugars	214.36
CaOH	6.8

* As reported by Wayman and Chen (1992) (Ref. 120).

Table 11. Media composition used in poplar fermentations. *

Components	Concentration (g/l)
(NH ₄) ₂ SO ₄	11.6
KH ₂ PO ₄	3.8
MgSO ₄ 7H ₂ O	0.6
CaCl ₂ 2H ₂ O	0.8
Urea	0.6
Peptone	2.9
Tween-80	0.2
	Concentration (mg/l)
FeSO ₄ 7H ₂ O	5.0
MnSO ₄ H ₂ O	1.6
ZnSO ₄ 7H ₂ O	1.4
CoCl ₂	2.0

Tangnu et al., *Biotechnology and Bioengineering*, Vol 23, pp 1837-1849, (1981)

Results and Discussion

The results of Pop1 and Pop2 are shown in Figures 1 and 2, respectively. Pop1 attained a maximum activity of 0.51 FPU/ml and Pop2 attained 0.23 FPU/ml. This is unexpected as Pop2 had 5 times the

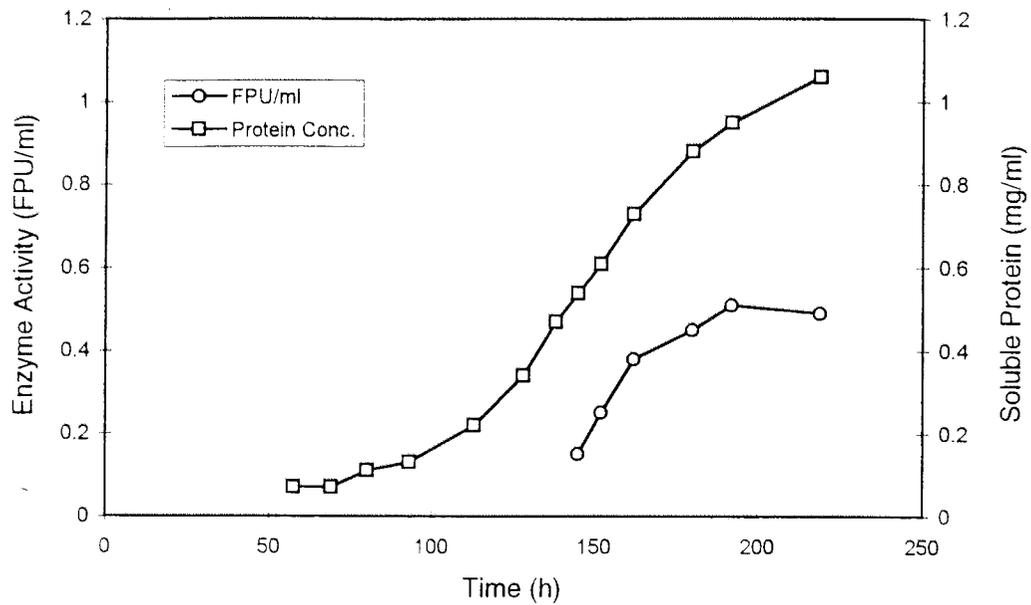


Figure 1. Enzyme activity and soluble protein concentration on a 1% cellulose fermentation using a 67% cellulose poplar feedstock in a 30 l fermentor

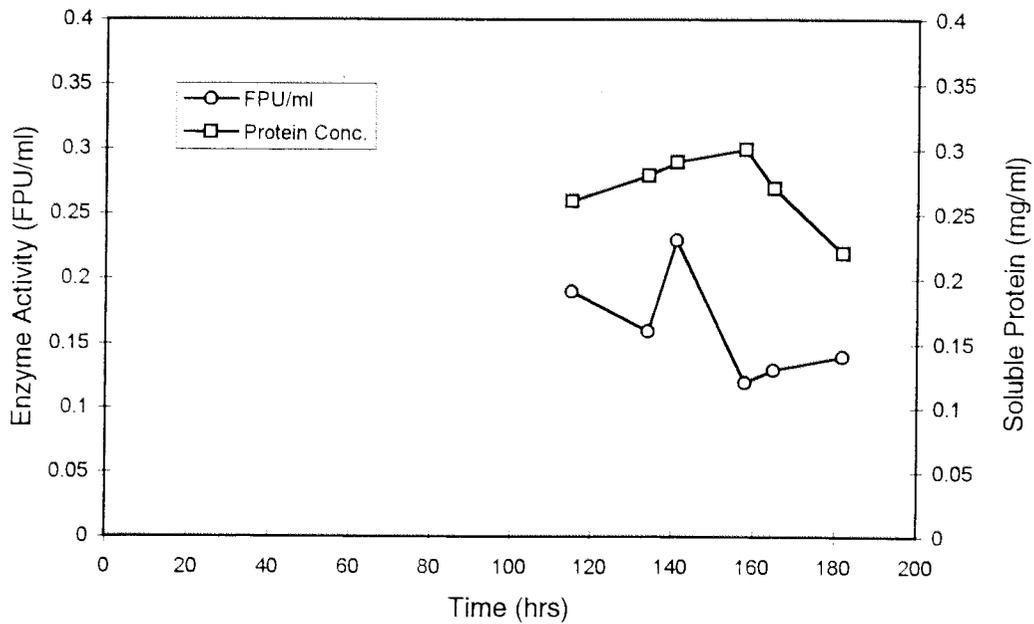


Figure 2. Enzyme activity and soluble protein concentration on a 5% cellulose fermentation using a 67% cellulose poplar feedstock in a 5 l fermentor

cellulose concentration as Pop1. The titer of these fermentations is so low that the productivity of either never exceeds 3 FPU/l/h.

No assay to accurately determine cell density was employed; however, it was qualitatively apparent that the cell density of either fermentation never attained the relatively high level present in the Solka-Floc fermentation. Linear growth was ruled out as the product curves of Figure 1 and 2 do not appear linear. This assumes that linear growth would produce linear production of enzyme. Based on these two fermentations, it is not recommended that poplar be used as the exclusive carbon source.

Although neither Pop 1 nor Pop 2 became contaminated, a third poplar fermentation was started that became contaminated in the first 24 hours. This is another testimony to the robustness of this organism. Once it has grown even a small amount past the inoculum phase, contamination has never been observed. Even with the small amount of growth in Pop1 and Pop2, no contaminant was found.

It is uncertain what caused the poor performance of Pop2. Inhibition from antifoam has been ruled out because the initial antifoam concentration (0.05%) was not inhibitory in other fermentations. It is possible that there was a problem with maintaining DO. The liquid level of the vessel was too low to completely submerge a DO probe into the broth, meaning no DO control could be employed. Also, the sparger became partially plugged with poplar debris, causing a decrease in available air flow.

The AHW fermentation attained a maximum activity of 1.15 FPU/ml at 144 hours. Although this is low, the purpose of the fermentation was to demonstrate the potential of mixed substrate fermentations using inexpensive natural carbon sources. The AHW is primarily glucose with a small amount of dimers which are commonly thought to induce cellulase production. The glucose fraction is used to attain a high cell density. The cell density of this fermentation was 26.7 g/l at 66 hours, which is twice as much as the 13.5 g/l attained in the 4% Solka-Floc fermentation described in the March report. Another benefit of the AHW is that it can be used without supplemental salts or trace metals, reducing the cost of the media.

Future Directions

Based on the poor growth of *T. reesei* on the poplar, it is a poor choice for a substrate when used by itself. In keeping with the goal of producing cellulase from a cellulosic source, some study should be made on

mixed substrate fermentations. A soluble carbon source for growth would be optimal as it would decrease the time of the growth phase and increase productivity. Once cell density is high enough, the poplar may then be able to induce appreciable enzyme production.

The use of AHW should be investigated further. The 5.7% glucose concentration used was excessively high. The AHW should be kept low so that the glucose concentration will only support a short growth phase, perhaps 2% or 3%. Along with the AHW, poplar should be added to the media to see if it would work as a good inducer after the growth phase.

Effort should be made to attain *T. reesei* CL-847. The current literature suggests that this strain produces the highest level of enzyme activity.^{8,85,115,116,117} Steady research with CL-847 at the Institut Francais du Petrole has produced increasingly better fermentations on lactose (Table 12). The 1994 data is taken from a fermentor that is currently in use in an ethanol producing plant in Soustons, France. There is no literature we have found that indicates this level of success with Rut-C30. The strain can be obtained by contacting:

Monsieur Ballerini
 Institut Francais du Petrole
 1 avenue de Bois-Preau
 92500 Reuil Malmaison (France)
 Tel. (33) 16 1-47 52 60 00 -- Fax. (33) 16 1-47 52 70 00

Table 11. The trend in the development of *Trichoderma reesei* CL-847 for the production of cellulase.

Date	Substrate	Volume (L)	Activity (FPU/ml)	Soluble Protein (mg/ml)	Reference
1983	5% Whatman CC41 cellulose	2	13.7	17.9	13
1988	Lactose	2	17.5	22.0	10
1988	Lactose	2	23.2	35.8	10
1988	Lactose	1,700	-	32.0	10
1994	Lactose	30,400	37.9	34.4	1

Appendix A) BioPro Economic Analysis Output

The tables in this section contain the numbers from the output of the BioPro simulation programs for the main lactose case. Selected data from the stream report, economic evaluation report and itemized cost report will be listed for the Ballerini scenario and the NREL scenario.

Ballerini Scenario

Table A.1. Overall material balance (kg/yr)

Component	In	Out	(Out-In)
Air	434,331,946	430,988,816	-3,343,130
Ammonium Sulfate	231,233	231,233	0
Ammonia	305,640	0	-305,640
Biomass	0	850,606	850,606
Calcium Chloride	99,100	99,100	0
Carbon Dioxide	0	4,382,439	4,382,493
Cellulase	0	2,840,860	2,840,860
Cobaltous Chloride	330	330	0
Corn Liquor	82,583	82,583	0
Ferrous Sulfate	826	826	0
Lactose	6,718,055	0	-6,718,055
Magnesium Sulfate	49,550	49,550	0
Manganese Sulfate	264	264	0
Phosphoric Acid	239,491	239,491	0
Potassium Hydroxide	137,088	137,088	0
Water	82,272,620	84,565,541	2,292,921
Zinc Sulfate	231	231	0
Total	524,468,958	524,468,958	0

Table A.2. Major equipment specification and FOB cost (1995 prices)

Quantity/Stand-by		Description	Unit Cost (\$)	Cost (\$)
2/0	G-101	Compressor Pressure change = 5.0 bar Power = 2342.0 kW	1,685,000	3,370,000
1/0	M-103	Flow Splitter	0	0
1/0	M-104	Flow Splitter	0	0
1/0	ST-101	Heat Sterilizer Diameter = 0.00 m Length = 16.71 m	76,000	76,000
12/0	R-101	Fermentor Volume = 0.5 m ³ Power = 1.11 kW	59,000	708,000
1/0	AF-101	Air Filter Throughput = 0.0 m ³ /s	4,000	4,000
1/0	AF-102	Air Filter Throughput = 0.0 m ³ /s	12,000	12,000
1/0	ST-102	Heat Sterilizer Diameter = 0.01 m Length = 112.61 m	130,000	130,000
12/0	R-102	Fermentor Volume = 10.0 m ³ Power = 22.50 kW	186,000	2,232,000
1/0	AF-103	Air Filter Throughput = 0.1 m ³ /s	4,000	4,000
1/0	AF-104	Air Filter Throughput = 0.2 m ³ /s	12,000	12,000
1/0	ST-103	Heat Sterilizer Diameter = 0.03 m Length = 62.80 m	329,000	329,000
12/0	R-103	Fermentor Volume = 200.0 m ³ Power = 450.00 kW	926,000	11,112,000
1/0	AF-105	Air Filter	14,000	14,000

		Throughput = 1.3 m ³ /s		
1/0	AF-106	Air Filter	75,000	75,000
		Throughput = 3.6 m ³ /s		
1/0	P-101	Pump	6,000	6,000
		Pressure change = 1.0 bar		
		Power = 0.0 kW		
1/0	P-102	Pump	14,000	14,000
		Pressure change = 2.0 bar		
		Power = 1.4 kW		
5/0	P-103	Pump	38,000	190,000
		Pressure change = 2.0 bar		
		Power = 14.3 kW		
1/0	M-101	2 - Stream Mixer	0	0
1/0	M-102	2 - Stream Mixer	0	0
		Cost of Unlisted Equipment		4,572,000
		20.0 % of Total		
Total Equipment Purchase Cost				22,860,000

Table A.3. Profitability analysis (unless mentioned values are in \$, 1995 prices)

A. Direct Fixed Capital	155,102,000
B. Working Capital	784,000
C. Startup Cost	7,755,100
D. Total Investment (A+B+C)	163,641,000
E. Revenue Stream Flowrate (kg/yr)	2,840,860
Cellulase in S-123	
F. Production (Unit) Cost (\$/kg)	18.71
Cellulase in S-123	
G. Selling Price (\$/kg)	29.47
Cellulase in S-123	
H. Revenue (\$/yr)	82,046,000
Cellulase in S-123	
I. Annual Operating Costs	52,080,000
J. Gross Profit (H-I)	29,966,000
K. Taxes (40%)	11,986,000
L. Net Profit (J-K+Depreciation)	32,715,000
Gross Margin	0.37
Return on Investment	20 %
Payback Time (years)	5.00

Table A.4. Annual capital and operating cost per equipment (\$/kg of product, 1995 prices)

Unit	Annlzd. Cap. Cost	Labor	Utilities	Maint.	Subtotal	%
Compressor G-101	0.18	0.00	1.21	0.18	1.57	28.04
Fermentor R-101	0.05	0.06	0.00	0.04	0.15	2.68
Fermentor R-102	0.16	0.06	0.09	0.12	0.43	7.68
Fermentor R-103	0.80	0.06	1.84	0.60	3.30	58.93
Others	0.05	0.02	0.03	0.05	0.15	2.68
Total	1.24	0.20	3.17	0.99	5.60	100.00

The raw material costs of 3.02 \$/kg of product were excluded from Table A.4. Adding this cost component leads to a total operating cost of 8.62 \$/kg of product.

Table A.5. Summary of operating cost per equipment (1995 prices)

Product Amount (kg/year)	2,840,860	
Plant Batch Time (hours)	470	
Number of Batches per Year	46	
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Operating Cost		
\$/year	24,486,078	
\$/batch	532,306	
\$/kg of product	8.62	
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Cost Item	\$/year	%
Capital	3,446,140	14.07
Raw Materials	8,912,078	36.40
Consumables	0	0.00
Labor	547,495	2.24
Utilities	8,836,599	36.09
Maintenance	2,743,766	11.21
Waste Treatment/Disposal	0	0.00
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Total	24,486,078	100.00
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NREL Scenario

Table A.6. Overall material balance (kg/yr)

Component	In	Out	(Out-In)
Air	1,266,801,510	1,255,945,613	-10,855,897
Ammonium Sulfate	674,429	674,429	0
Ammonia	811,001	0	-811,001
Biomass	0	4,898,522	4,898,522
Calcium Chloride	289,041	289,041	0
Carbon Dioxide	0	14,359,314	14,359,314
Cellulase	0	4,898,522	4,898,522
Cobaltous Chloride	963	963	0
Corn Liquor	240,867	240,867	0
Ferrous Sulfate	2,409	2,409	0
Lactose	19,594,328	0	-19,594,328
Magnesium Sulfate	144,520	144,520	0
Manganese Sulfate	771	771	0
Phosphoric Acid	698,516	698,516	0
Potassium Hydroxide	399,840	399,840	0
Water	239,961,808	247,066,675	7,104,867
Zinc Sulfate	674	674	0
Total	1,529,620,678	1,529,620,678	0

Table A.7. Major equipment specification and FOB cost (1995 prices)

Quantity/Stand-by		Description	Unit Cost (\$)	Cost (\$)
4/0	G-101	Compressor Pressure change = 5.0 bar Power = 3415.5 kW	2,393,000	9,572,000
1/0	M-104	Flow Splitter	0	0
1/0	M-103	Flow Splitter	0	0
1/0	ST-101	Heat Sterilizer Diameter = 0.00 m Length = 48.02 m	76,000	76,000
35/0	R-101	Fermentor Volume = 0.5 m ³ Power = 1.10 kW	59,000	2,065,000
1/0	AF-101	Air Filter Throughput = 0.0 m ³ /s	4,000	4,000
1/0	AF-102	Air Filter Throughput = 0.0 m ³ /s	12,000	12,000
1/0	ST-102	Heat Sterilizer Diameter = 0.01 m Length = 82.18 m	181,000	181,000
35/0	R-102	Fermentor Volume = 10.0 m ³ Power = 22.50 kW	186,000	6,510,000
1/0	AF-103	Air Filter Throughput = 0.2 m ³ /s	4,000	4,000
1/0	AF-104	Air Filter Throughput = 0.5 m ³ /s	20,000	20,000
1/0	ST-103	Heat Sterilizer Diameter = 0.04 m Length = 102.87 m	459,000	459,000
35/0	R-103	Fermentor Volume = 200.0 m ³ Power = 450.00 kW	926,000	32,410,000
1/0	AF-105	Air Filter	34,000	34,000

		Throughput = 3.8 m ³ /s		
3/0	AF-106	Air Filter	73,000	219,000
		Throughput = 3.5 m ³ /s		
1/0	P-101	Pump	6,000	6,000
		Pressure change = 1.0 bar		
		Power = 0.1 kW		
1/0	P-102	Pump	22,000	22,000
		Pressure change = 2.0 bar		
		Power = 4.2 kW		
14/0	P-103	Pump	39,000	546,000
		Pressure change = 2.0 bar		
		Power = 14.3 kW		
1/0	M-101	2 - Stream Mixer	0	0
1/0	M-102	2 - Stream Mixer	0	0
		Cost of Unlisted Equipment		13,035,000
		20.0 % of Total		
Total Equipment Purchase Cost				65,175,000

Table A.8. Profitability analysis (unless mentioned values are in \$, 1995 prices)

A. Direct Fixed Capital	443,047,000
B. Working Capital	2,255,000
C. Startup Cost	22,152,350
D. Total Investment (A+B+C)	467,454,000
E. Revenue Stream Flowrate (kg/yr)	4,898,522
Cellulase in S-123	
F. Production (Unit) Cost (\$/kg)	31.08
Cellulase in S-123	
G. Selling Price (\$/kg)	48.90
Cellulase in S-123	
H. Revenue (\$/yr)	234,747,000
Cellulase in S-123	
I. Annual Operating Costs	149,225,000
J. Gross Profit (H-I)	85,522,000
K. Taxes (40%)	34,209,000
L. Net Profit (J-K+Depreciation)	93,402,000
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Gross Margin	0.36
Return on Investment	20 %
Payback Time (years)	5.00

Table A.9. Annual capital and operating cost per equipment (\$/kg of product, 1995 prices)

Unit	Annld. Cap. Cost	Labor	Utilities	Maint.	Subtotal	%
Compressor G-101	0.30	0.01	2.05	0.30	2.66	28.33
Fermentor R-101	0.09	0.10	0.01	0.06	0.26	2.77
Fermentor R-102	0.27	0.10	0.16	0.20	0.73	7.77
Fermentor R-103	1.35	0.10	3.12	1.01	5.58	59.42
Others	0.05	0.01	0.04	0.06	0.16	1.70
Total	2.06	0.32	5.38	1.63	9.39	100.00

The raw material costs of 5.10 \$/kg of product were excluded from Table A.9. Adding this cost component leads to a total operating cost of 14.49 \$/kg of product.

Table A.10. Summary of operating cost per equipment (1995 prices)

Product Amount (kg/year)	4,898,522	
Plant Batch Time (hours)	470	
Number of Batches per Year	46	
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Operating Cost		
\$/year	71,003,227	
\$/batch	1,543,548	
\$/kg of product	14.49	
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Cost Item	\$/year	%
Capital	9,867,534	13.90
Raw Materials	25,993,560	36.61
Consumables	0	0.00
Labor	1,513,724	2.13
Utilities	25,809,464	36.35
Maintenance	7,818,945	11.01
Waste Treatment/Disposal	0	0.00
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Total	71,003,227	100.00
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Figure A.1. BioPro flowsheet

