

# Lawrence Berkeley National Laboratory

## Lawrence Berkeley National Laboratory

**Title**

PROCESS DEVELOPMENT STUDIES ON THE BIOCONVERSION OF CELLULOSE AND PRODUCTION OF ETHANOL

**Permalink**

<https://escholarship.org/uc/item/05k7h2wx>

**Author**

Wilke, C.R.

**Publication Date**

1978-12-01

RECEIVED  
LAWRENCE  
BERKELEY LABORATORY

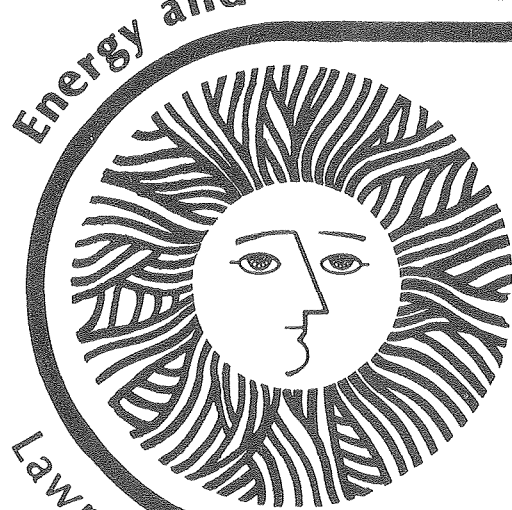
MAR 13 1979

LIBRARY AND  
DOCUMENTS SECTION

TWO-WEEK LOAN COPY

*This is a Library Circulating Copy  
which may be borrowed for two weeks.  
For a personal retention copy, call  
Tech. Info. Division, Ext. 6782*

Energy and Environment Division



Process Development Studies on  
the Bioconversion of Cellulose  
and Production of Ethanol

*Charles R. Wilke and  
Harvey W. Blanch*

December 1978

Lawrence Berkeley Laboratory University of California/Berkeley

Prepared for the U.S. Department of Energy under Contract No. W-7405-ENG-48

— LEGAL NOTICE —

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

PROCESS DEVELOPMENT STUDIES ON THE BIOCONVERSION OF CELLULOSE  
AND PRODUCTION OF ETHANOL

under auspices of

DEPARTMENT OF ENERGY--SOLAR ENERGY DIVISION  
Fuels from Biomass program

Lawrence Berkeley Laboratory

Charles R. Wilke, Principal Investigator  
Harvey W. Blanch, Co-Principal Investigator

Report of Work Progress

December 29, 1978

TABLE OF CONTENTS

I. RAW MATERIALS AND PROCESS EVALUATION	
A. Analysis of Bagasse. Ray P. Freitas .....	3
B. Enzymatic Hydrolysis of Original Materials.....	3
Aldo F. Sciamanna	
(a) Dilute Acid Pretreatment Followed by Enzymatic Hydrolysis.....	3
Aldo F. Sciamanna	
C. Sulfuric Acid Hydrolysis and Mechanical Shear.....	3
Ray P. Freitas and Aldo F. Sciamanna	
(a) Solubility of Glucose in Various Solvents.....	9
Aldo F. Sciamanna	
II. ENZYME FERMENTATION STUDIES	
A. Pilot Plant Process Development and Design Studies.....	11
(a) Continuous Cellulase Production.....	11
Kishen Tangnu	
B. Evaluation of New Enzyme Sources.....	11
Ren-Der Yang	
C. Mixed Enzyme Studies.....	22
Mohammad Riaz	
III. ETHANOL FERMENTATION STUDIES	
A. Fermentation of Enzymatic Hydrolyzates to Ethanol.....	32
Ren-Der Yang	
B. Media Requirements for Ethanol Fermentation.....	37
Harry Wong	
IV. HYDROLYSIS REACTOR DEVELOPMENT	
A. Hydrolysis Kinetic Studies.....	38
Richard L. Lindsey and Ken Alder	
V. UTILIZATION OF HEMICELLULOSE SUGARS	
A. Xylanase Production.....	40
Kishen Tangnu and Bill Long	
B. Xylose Fermentation.....	48
Steven L. Rosenberg and Tonya Hill	
References.....	51
Published Reports.....	52

## I. RAW MATERIALS AND PROCESS EVALUATION

### A. Analysis of Bagasse

Approximately 2 pounds of bagasse stalks and stems were received through the courtesy of the Ander Cane Co., of Naples, Florida. The material was air dried, Wiley milled through a 2 mm screen, and the assay was determined according to (1). The results are shown in Table 1.

### B. Enzymatic Hydrolysis of Original Materials

The initial studies on the above bagasse have been completed. Samples of the 2 mm Wiley milled bagasse were enzymatically hydrolyzed in 5 w% suspensions with Trichoderma viride cellulase enzyme, filter paper activity of 4.3, as performed previously for agricultural residues (LBL-6859, Jan. 1977). The results are shown in Table 2. As can be seen, the bagasse is not readily hydrolyzed, as shown by the quite low carbohydrate conversion of less than 6%.

#### (a) Dilute Acid Pretreatment and Pentosan Extraction

Pretreatment of the bagasse in a 6.4 w% suspension with 0.9 w% (0.09M) sulfuric acid at 100°C for 5 1/2 hours improved the yield of sugar produced by about a factor of 5.6, that is, an over all carbohydrate conversion (including the sugar in the acid liquor) of 35%. The results are shown in Tables 3 and 4, respectively.

### C. Sulfuric Acid Hydrolysis with Mechanical Shear

A very interesting process was reported in Russia a number of years ago (2). In this process, corn stalks were subjected to mechanical shear in the presence of 85 w% sulfuric acid, to effect depolymerization of the cellulose. Basically, a slow moving ball mill was used to accomplish a roll-shear effect, rather than the usual faster falling ball type, to disperse the acid throughout the substrate. As will be shown, this is a very effective way of reducing the

Table 1

BAGASSE<sup>(a)</sup>

ASSAY<sup>(b)</sup>

<u>(%) Carbohydrate</u>	<u>(%) Sugar Equivalent</u>
34.3 Glucan	38.1 Glucose
1.0 Galactan	1.1 Galactose
20.5 Xylan	23.3 Xylose
2.2 Arabinan	2.5 Arabinose
58.0 Carbohydrate	65.0 Sugar Equivalent
18.4 Lignin	
2.8 Ash	
1.7 Azeotropic Benzene-Alcohol Extractives	
1.0 Other Acid Insolubles (Organic)	
3.0 Protein	

(a) Courtesy of Ander Cane Company, 1310 Cabio Crt., Naples, Fla. 33942

(b) 2 mm Wiley milled, 0.25-0.35 mm fraction, 100% dry.

Table 2

40 hour Enzyme Hydrolysis of Original Bagasse

Basis: 100 lbs. of 2 mm Wiley milled material

Glucose	= 3.04 lbs.
Xylose	= 0.44
Arabinose	= 0.06
Other	= 0.19

---

$\Sigma$  = 3.72 lbs., which is equal to 5.7%  
carbohydrate conversion, and a residue of 87.44 lbs.

---



Table 3

Acid Extraction of Bagasse

Basis: 100 lbs of original material

Sugars in acid liquor

Glucose	= 0.22
Xylose	= 1.21
other	= 0.25

---

$\Sigma$  = 1.95 lbs. of sugar and a treated  
residue of 67.5 lbs. for enzymatic hydrolysis.

---

Table 4

40 Hour Enzyme Hydrolysis of Acid Treated Bagasse

Basis: 100 lbs of original material

Glucose	= 17.8
Xylose	= 2.7
Arabinose	= 0.15

---

$\Sigma$  = 20.7 lbs. of sugar and a residue of  
46.2 lbs. The carbohydrate conversion is 31.8%, and  
with the sugars in the acid liquor it is 34.8%.

---

sulfuric acid used compared to the work done here on Populus tristis #1 and reported in the previous progress report (3).

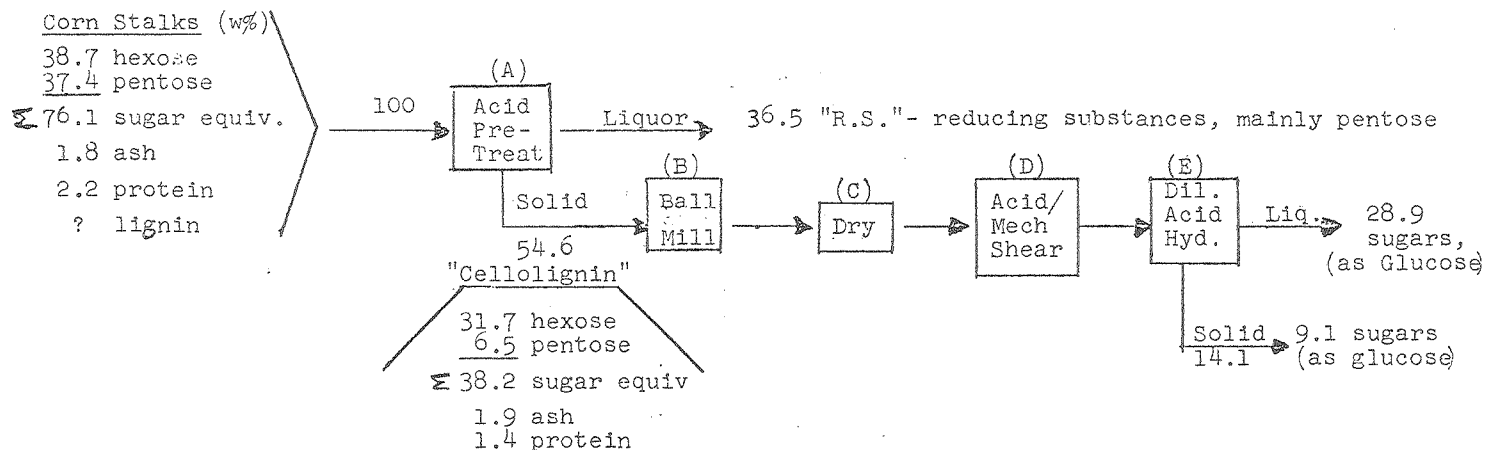
Since the translated version of the Russian report (2) may not be readily available a summary of this work is presented as a flow diagram shown in Fig. 1. As can be seen, the key feature of the process is step "D", whereby shearing was used to disperse a minimal amount of 85 w% sulfuric acid. The rest of the process appears to be common.

A series of experiments were performed on 1 gram samples of the wood Populus tristis #1. Since a comparison was desired with the work reported previously (3), the acid pretreatment step "A", of the Fig. 1 was deleted. The 2 mm Wiley milled populus was ball milled in a small laboratory procelain 1 liter jar with alumina balls and sieved to -200 mesh ( $<75\mu\text{M}$  particles). The sulfuric acid was made to 85.1 w% by diluting reagent grade to 15.42 M and a density of 1.7767 g/ml at 20°C. The experiments were then started at step "D" of Fig. 1 and carried out essentially as reported (2). The shear was accomplished by the use of two rows of four each of cylindrical alumina rods of about 3/4 inch in diameter and long, laid end to end in a pint jar with a teflon lid liner. By rolling the jar at about 60 RPM, the substrate was effectively sheared and dispersed the various amounts, from 0.2 to 1.2 grams, of sulfuric acid per gram of wood (in the report (2), this is the "0.2 to 1.2 module"). The shear time was essentially duplicated as reported, that is, the mixture rolled at room temperature for various times of 0 to 2 hours, followed by 0 to 1 hour at 70°C, and then rolled 5 to 15 minutes thereafter. Secondary hydrolysis, step "E" was done as specified.

The results of these acid hydrolyses are shown in Table 5. As can be seen, there is a considerable decrease in the amount of sulfuric acid used per pound of glucose produced compared to the results reported in (3). In the experiment

Figure 1

RUSSIAN- ACID/SHEAR PROCESS (2)



- (A) 9.09 w% (1:10) suspension of 5-10 mm corn stalks in 2.5w% (0.24M)  $H_2SO_4$ , 100°C. for 2 hours.
- (B) 1. Ball mill for 2 hours @ 64-70 RPM  
2. Substrate moisture cannot exceed 6%, otherwise material sticks to surfaces and does not grind down to size.  
3. 17.24 liter ball jar with ~ 350 (25 mm) steel balls.  
4. Substrate milled to particle size such that 67-70% was -230 mesh ( $< 64 \mu M$ )
- (C) Dry to 98% dry so that in step (D) substrate does not ball up or cake into chunks.
- (D) 1. Add 0.2 module of 85w% (15.42 M)  $H_2SO_4$  (0.2 wgt part acid per part substrate).  
2. Ball mill at 64-70 RPM for 2 hours at 40°C. (usual equilibrium temp.)  
3. " " " " " " 1 hour at 70°C. (with external heating)
- (E) Dilute mixture with water to 5w% (0.48 M) acid and boil the 27w% suspension at 100°C. for 5 hours, or 121°C. for 25 minutes.

series #3, only 2.33 lbs. of sulfuric acid is used per pound of glucose produced at 69% carbohydrate conversion. In Table 7, reference (3), 6.31 lbs of sulfuric acid was used per pound of glucose produced, at a carbohydrate conversion of 93%.

In experimental series #1, the low yield was due to an insufficient amount of sulfuric acid because in series #2, where the mixture was also subjected to shearing, the yield was still quite low with a consequent relatively high acid requirement for glucose and or sugar production.

From the results shown in Table 5, it would appear that 0.60 to 0.62 lbs. of 100% sulfuric acid per pound of wood to be the optimum for a minimum consumption of 2.4 lbs of sulfuric acid per pound of glucose, or 1.6 lbs of acid per pound of total sugars produced. In comparing this with the corn stover where only 0.2 "module" was used (2), it is very reasonable since we have found populus considerably more resistant to hydrolyses than corn stover. Also, it was found, in series #5, that rolling 1/2 hour at room temperature, 1/2 hour at 70°C followed by a 5 minute roll was more than sufficient to accomplish the desired carbohydrate conversion.

The use of mechanical shear for sulfuric acid dispersion would appear to be a distinct advantage, and it should also be noted that carbohydrate conversion of 70 to 80% are sufficient in acid hydrolyses for minimal acid used per unit of sugar produced.

(a) Solubility of Glucose in Various Solvents

In the course of studies on the acid hydrolysis of cellulose materials, it was found necessary to determine the solubility of glucose in various solvents at 25°C.

- 1) In water:  $C_{\max} = 563 \text{ mg G/ml}$

Table 5

Summary of the "Russian" Shear/Acid Process (2)  
Balled milled Populus and sieved to -200 mesh(<75 $\mu$ M)

Exp Series #	15.42M H <sub>2</sub> SO <sub>4</sub> (ML)	100% H <sub>2</sub> SO <sub>4</sub> (GM)	(a) Populus (GM)	(b) Shear Time (HRS)	Sugar Found (GM)	Glucose Found (GM)	H <sub>2</sub> SO <sub>4</sub> Used per GM of		Sugar Conv. (%)	Glucose Conv. (%)
							SUGAR	Glucose		
1	0.132	0.200	1	0/0 Σ3.25	0.181	0.0359	1.11	5.57	29	9
2	0.132	0.200	1	2/1 Σ3.25	0.217	0.0455	0.92	4.40	34	11
3	0.4	0.605	1	2/1 Σ3.25	0.437	0.260	1.38	2.33	69	65
4	0.5	0.752	1	2/.5 Σ2.583	0.484	0.311	1.55	2.42	77	78 (c)
5	0.5	0.752	1	.5/.5 Σ1.083	0.482	0.304	1.56	2.47	77	76 (d)
6	0.5	0.752	1	0/0 Σ3.25	0.350	0.203	2.15	3.70	56	51 (e)
7	0.78	1.182	1	2/1 Σ3.25	0.513	0.358	2.30	3.31	84	89

(a) 10.0 gm. samples of Populus tristis with 63.0% sugar (equivalent) and/or 40% Glucose (equivalent)

(b) acid/shear time is shown as hours rolled at room temperature, then the incubation hours at 70°C, and then tumbled for 5 or 15 minutes. "Σ" is the total contact time of the concentrated acid on the substrate before dilution to 0.48 M sulfuric acid and then autoclaved for 35 minutes at 121°C.

(c) The washed and 100% dry residue was 3.41 gms. (34.1%)

(d) 35.2% final residue.

(e) 55.6% final residue

2) In 72 w% (24.1N)  $H_2SO_4$ :  $C_{max} = 66.7$  mg G/ml

In this mix there occurs approximately a 11% decomposition in 2 hours and nearly 50% in 14 hours.

3) In anhydrous methyl alcohol:  $C_{max} = 17.4$  mg. G/ml.

4) In a mixture of 3.5 grams (4.4 ml) of methyl alcohol per 1.3 ml of 72 w%(24.1N)  $H_2SO_4$ :  $C_{max} = 22.1$  mg. G/ml.

This mixture of these two solvents is a close approximation of the ratios as published for the Purdue projects in the April, 1978 Biomass Newsletter, page 7.

## II. ENZYME FERMENTATION STUDIES

### A. Pilot Plant Process Development and Design Studies

#### (a) Continuous Cellulase Production

Efforts have been directed toward optimization of pH and temperature in a two stage continuous culture system for cellulase production. Current studies are continuing on Trichoderma viride strain QM9414, with peptone and substrate concentration being varied. It was found that a reduction of the Tween 80 concentration from 0.2% to 0.1% in the inlet feed to the first-stage increased the filter paper activity by about twofold. A lower pH (pH 4) appears to increase FPA activity in the cellulase induction stage. A comparison of current studies at pH 4 and earlier results at higher pH's is presented in Table 6.

The high yielding constitutive strain T. viride C-30, developed by the Rutgers' group, is currently being investigated in a similar two-stage system. Results will be summarized in a later report.

### B. Evaluation of New Enzyme Sources

The objective of this program is to develop a test system for a comparison of effectiveness among various mutants and new cellulase producing strains

Table 6  
Comparison Studies on Cellulase Production

FERMENTATION SYSTEM	PRESENT PROCESS <sup>(b)</sup>			PAST PROCESS (d)		
	FPA	Stage 1 pH	Stage 2 pH	FPA	Stage 1 pH	Stage 2 pH
Single Stage	4.0	4.0		2.7	4.8	
Single Stage with recycle	-	-	-	3.7	4.8	
Two Stage	5.5	4.0	4.0	2.5	4.8	4.8
Two Stage with recycle	-	-	-	4.25	4.8	4.8

(a) temperature 28°C in both stages

(b) inlet substrate concentration 25.0 gm/l

(c) dilution rate -- stage 1 0.02 hr<sup>-1</sup>  
stage 2 0.02 hr<sup>-1</sup>

(d) inlet substrate concentration 15.0 gm/l, temperature 30°C.

obtained from other contractors. The organization of the effort is shown in Fig. 2.

1) The effects of temperature, pH, dissolved oxygen, carbon and nitrogen sources and concentrations, C/N ratio and additives is studied using both batch and continuous fermentation systems.

2) Enzyme activities are measured as filter paper,  $C_x$ ,  $C_1$  and  $\beta$ -glucosidase activity. Culture filtrates from certain mutants are fractionated to determine the enzyme composition. Long-term hydrolysis using actual cellulosic substrates are conducted, and the resulting hydrolyzate tested for its fermentability for ethanol production by yeast.

3) Preliminary economic assessments of the relative effectiveness of alternate mutants is made.

The fungus Trichoderma reesei (viride) has been shown to be the most powerful cellulase producer in most studies so far. Since the isolation of the wild-type strain (QM6a, considerable improvements have been obtained through mutation (Fig. 3). Strain QM9414 developed at the Natick Laboratory in 1974 produced up to several times the activity of the original wild-type strain. Most recently, two mutants, Rut-NG-14 and Rut-C-30, were developed at Rutgers University. Both strains are hypercellulase producers. Rut-C-30 is also catabolite repression resistant.

In the present study, evaluations were made of strain NG-14 and C-30 comparing their effectiveness with strain QM9414 upon which most of our previous process development studies were based.

All fermentation runs were conducted in 5-liter New Brunswick fermentors. Medium I (Table 7) was used for Run 1 to Run 4, and medium II was used for Run 5. Ball-milled Solka Floc (BW200) at 5.5% concentration (4.8% for Run 1) was used



Figure 2

EVALUATION OF NEW ENZYME SOURCES

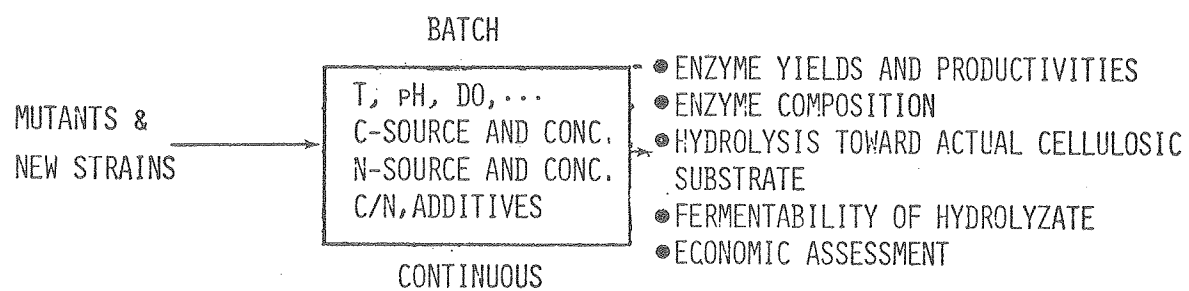
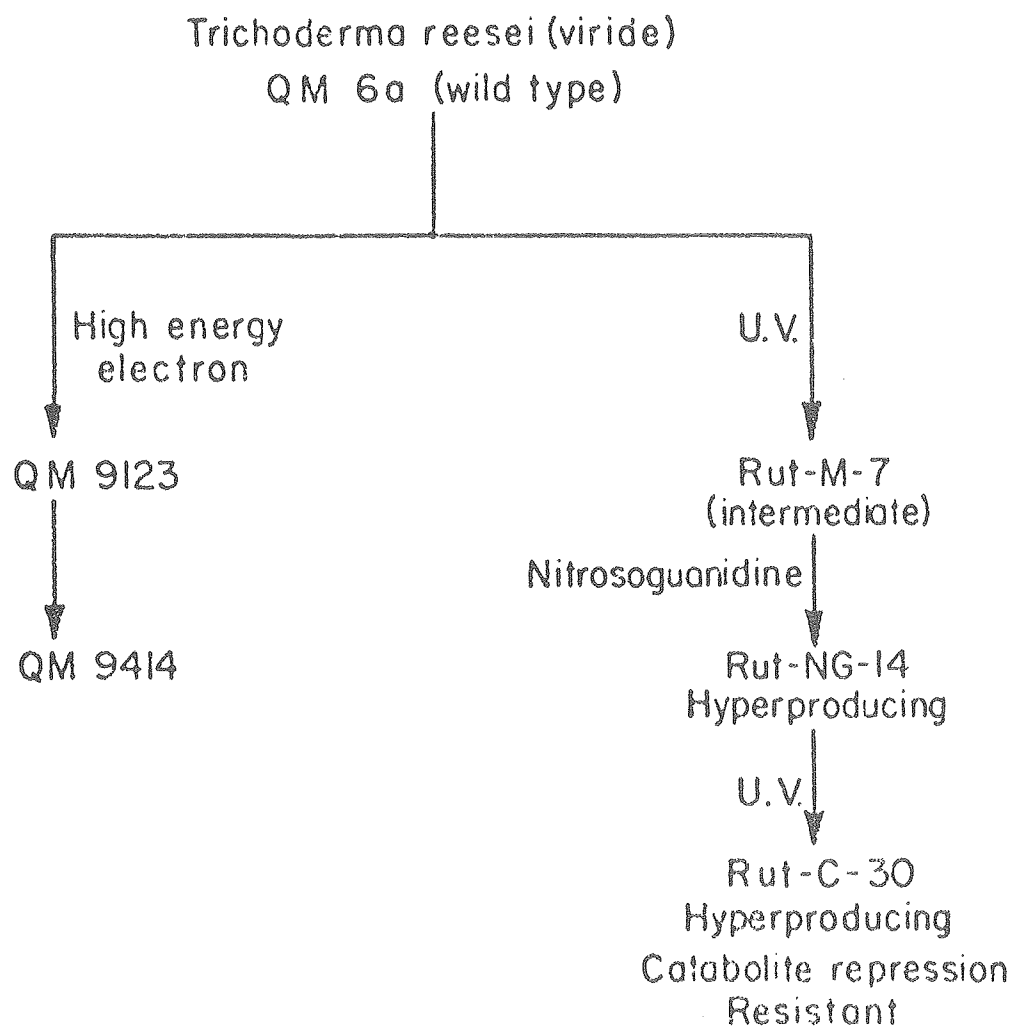


Figure 3

# GENEOLOGY OF HIGH YIELDING CELLULASE MUTANTS



XBL 7810-5980

as a carbon source. Three-day old, 20% mycelium inoculum was used for all fermentations. pH was controlled with addition of millipore sterilized 4N  $\text{NH}_4\text{OH}$  which was also used as an additional nitrogen source. Except for Run #2, all fermentations were conducted with temperature and pH programming in which pH = 4.5 and  $T = 31^\circ\text{C}$  were maintained for the first 48 hours and pH > 3.3 and  $T = 28^\circ\text{C}$  were maintained throughout the rest of the fermentations. In run #2, pH > 3.3 and  $T = 28^\circ\text{C}$  were maintained throughout the fermentation. Measurement of FPA,  $\beta$ -glucosidase,  $C_1$  and  $C_x$  were based on standard assay procedures (4).

Figure 4 shows a typical batch fermentation for Rut-C-30 with temperature and pH programming. The maximum yield of about 8 FP units/ml was obtained in 8 days.

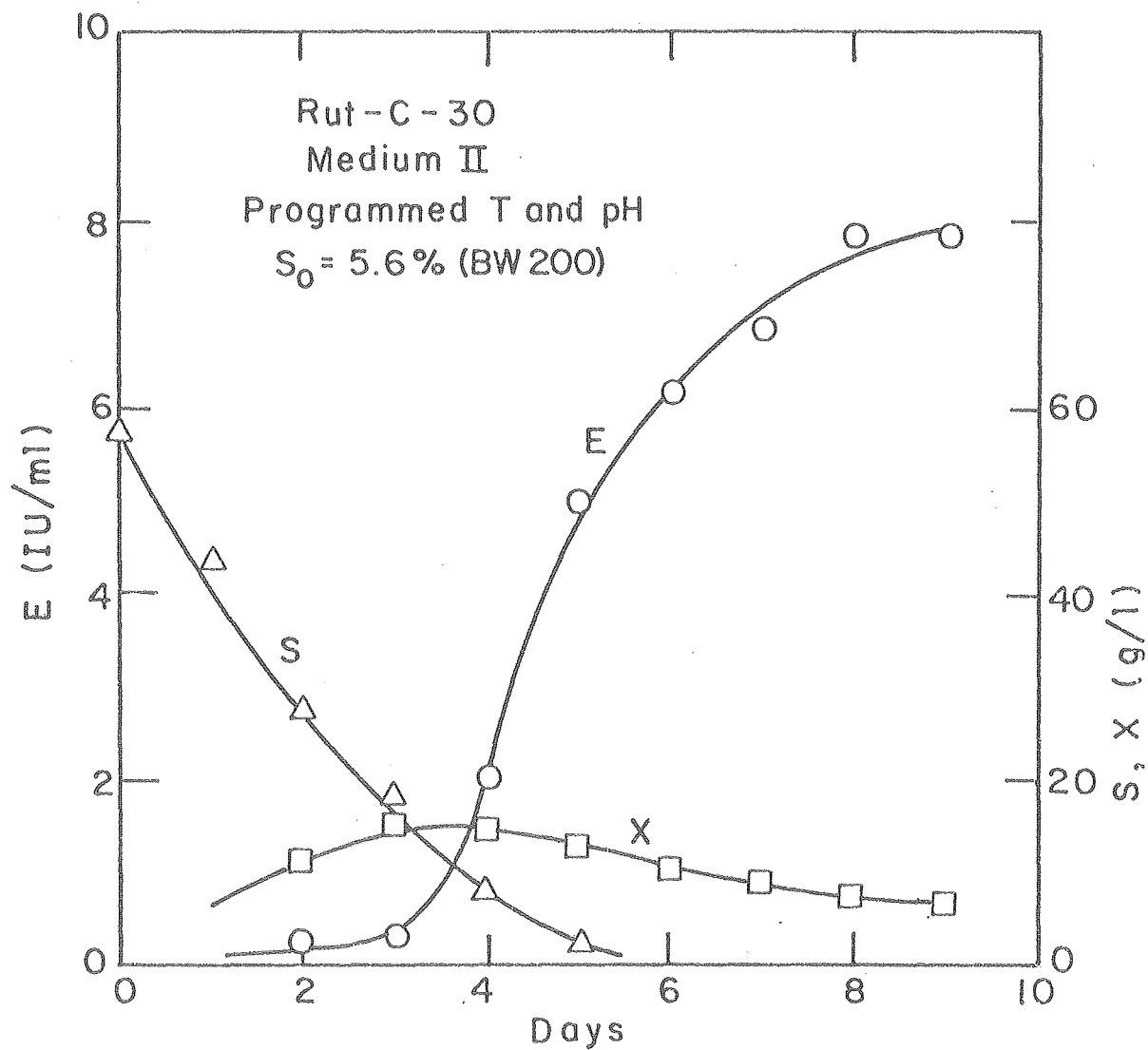
Figure 5 compares the results of two fermentation runs with strain Rut-C-30, one with temperature and pH programming and the other without. The former gives approximately 4% higher Filter Paper Activity, with considerable higher  $\beta$ -glucosidase,  $C_x$  and  $C_1$  activities.

Figure 6 compares the results obtained from the three strains under investigation. About 8 Filter Paper units/ml (soluble protein = 18g/l) and 2 FP units/ml (soluble protein = 5g/l) were obtained in 8-9 days from strains Rut-C-30 and Rut-NG-14, respectively. The results for Rut-C-30 are comparable to those obtained by the Natick Laboratory (5) (17-12 FP units/ml in 4-7 days). However, Rut-NG-14 gives a considerably lower yield than that obtained by Natick (up to 7 FP units/ml). Less than 0.5 FP units/ml were obtained for QM9414 in the present study. This yield is abnormally low compared to at least 1 FP unit/ml obtainable with the use of 1% non-ball-milled Solka Floc (Pharm Grade, 40C)

Experiments repeated for QM9414 using a different medium (Medium II in Table 7) also did not produce a satisfactory enzyme yield (Fig. 7). It is not

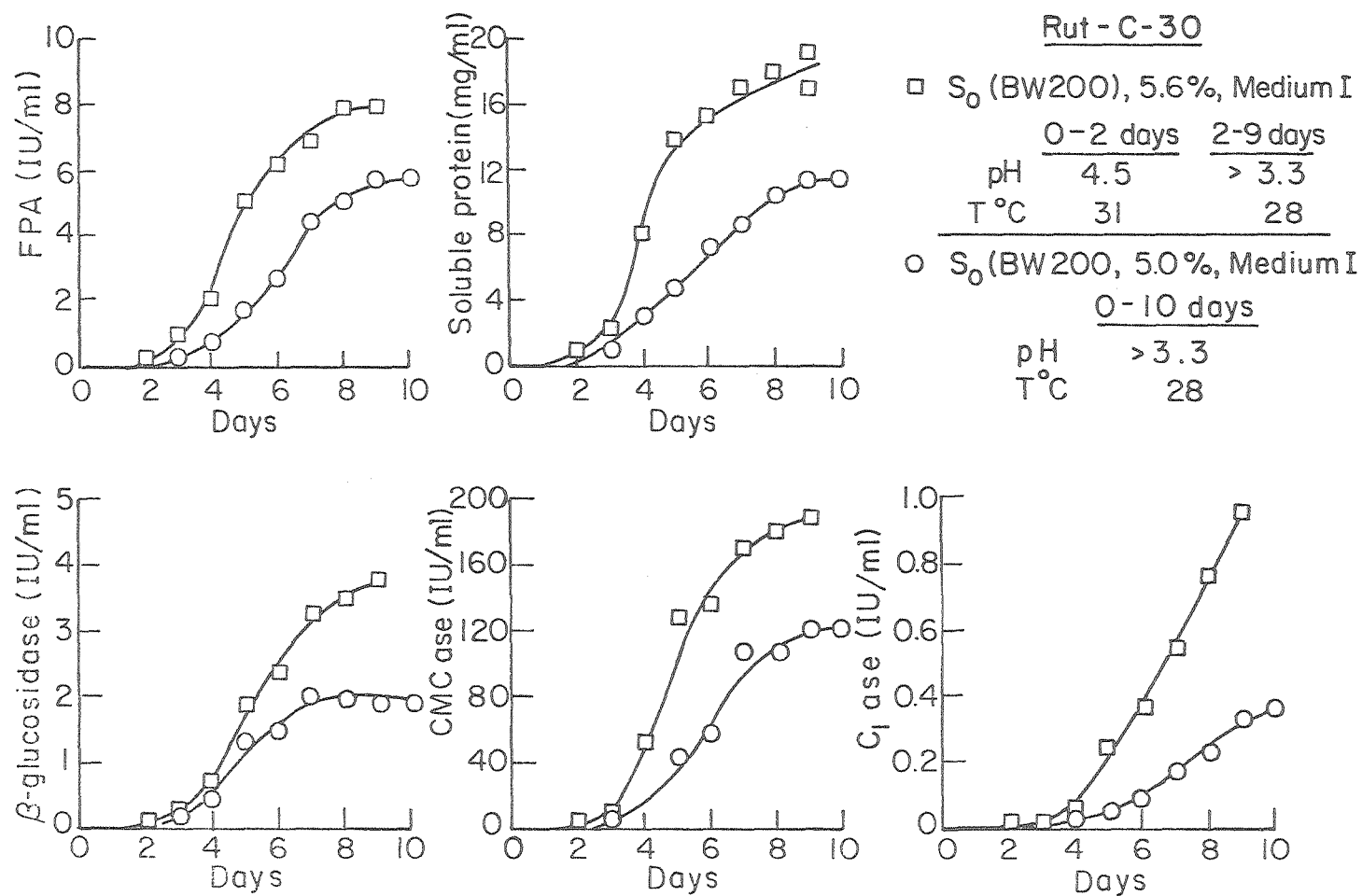
Table 7  
Composition of Mediums Used in the Various Experiments

<u>Standard Medium (MS)</u>		Medium I (MI)	Medium II (MII)
	<u>g/l</u>	<u>g/l</u>	<u>g/l</u>
$(\text{NH}_4)_2\text{SO}_4$	1.4	1.4	14
$\text{KH}_2\text{PO}_4$	2.0	2.0	4.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3	0.3	0.7
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.4	0.4	1.0
Urea	0.3	-	0.7
Protease peptone	0.5	1.0	3.4
Trace elements	1 ml	1 ml	1 ml
Solka Floc	10 (BM40)	45-60 (BM200)	60 (BM200)
Tween 80	-	0.2	0.2
pH adjustment	2N NaOH	4N $\text{NH}_4\text{OH}$	2N NaOH
C/N	8.2	8-9	8.0



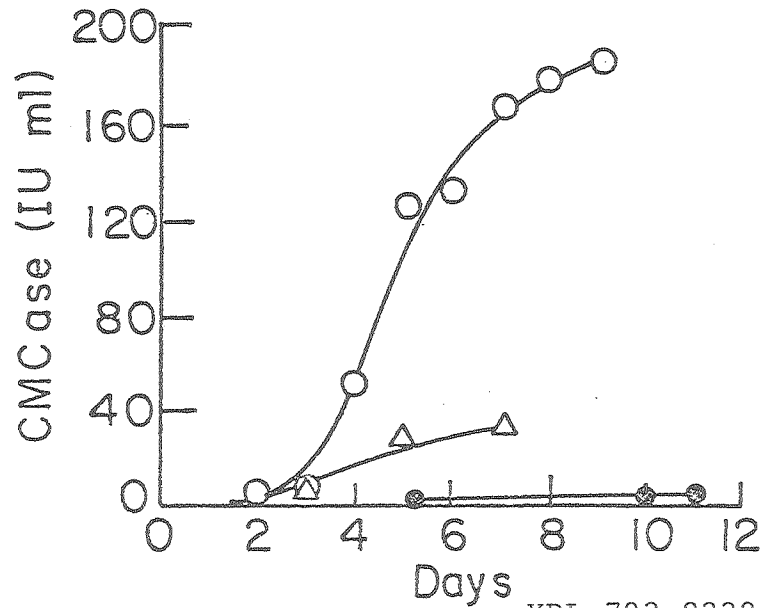
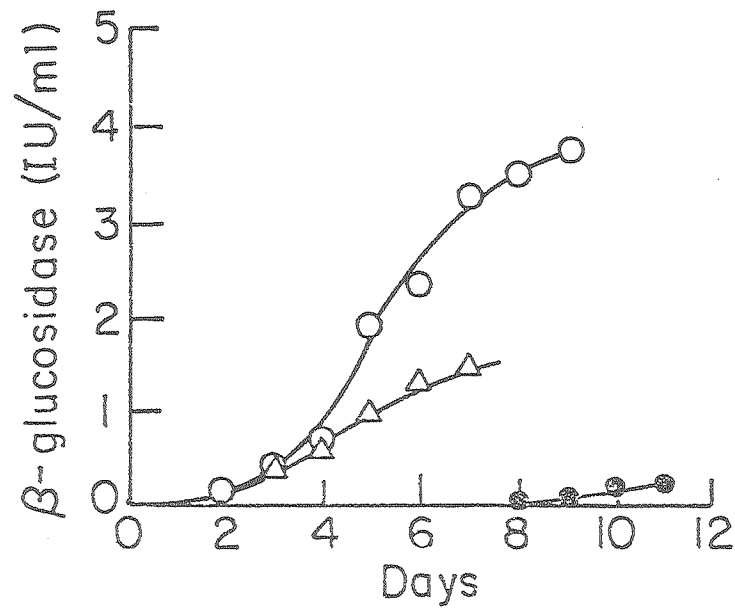
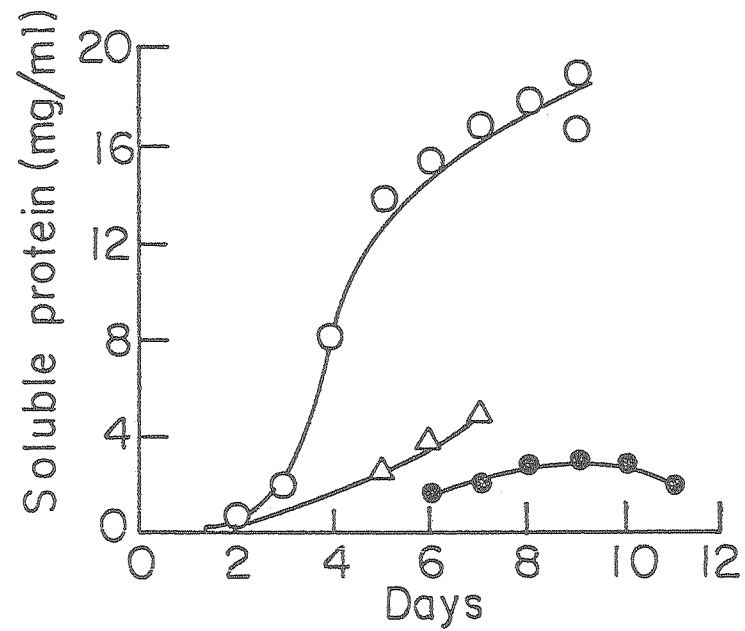
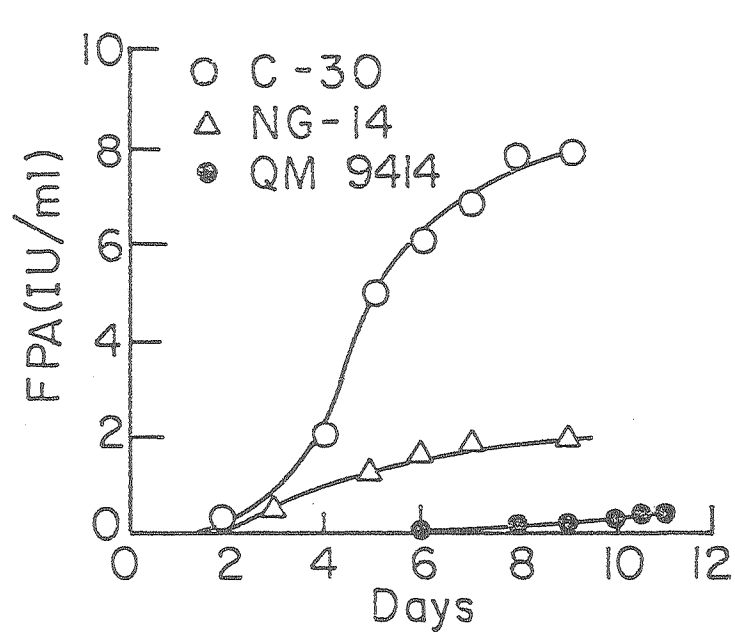
XBL-792-8230

Figure 4. Batch Fermentation for Rutgers C-30 with temperature and pH Programing.



XBL-792-8229

Figure 5. Typical Batch Fermentation for Rutgers C-30 Strain with Temperature and pH Programming (□) and without programming (○).



XBL-792-8228

Figure 6. The Typical Fermentation of the Three Strains with 5.6% Solka Floc (BW200) and Medium I.

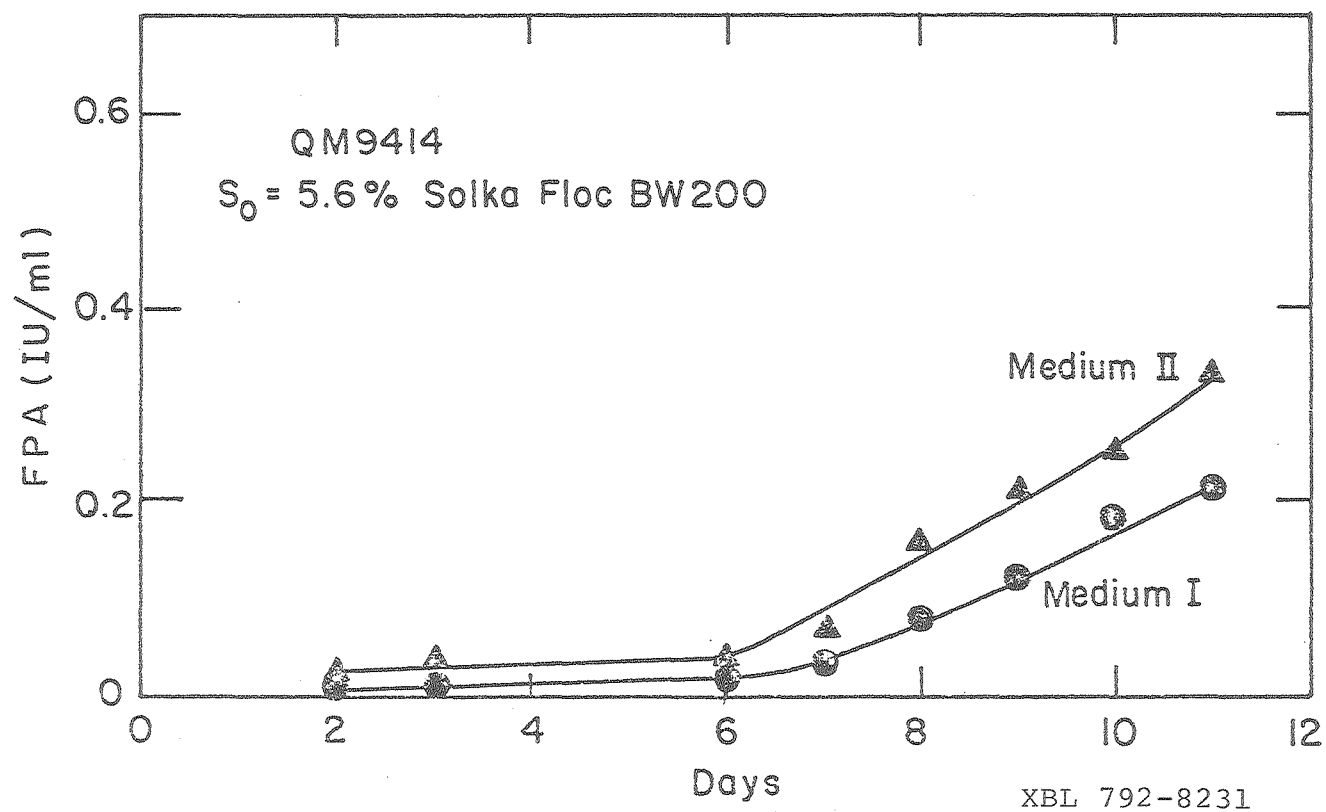


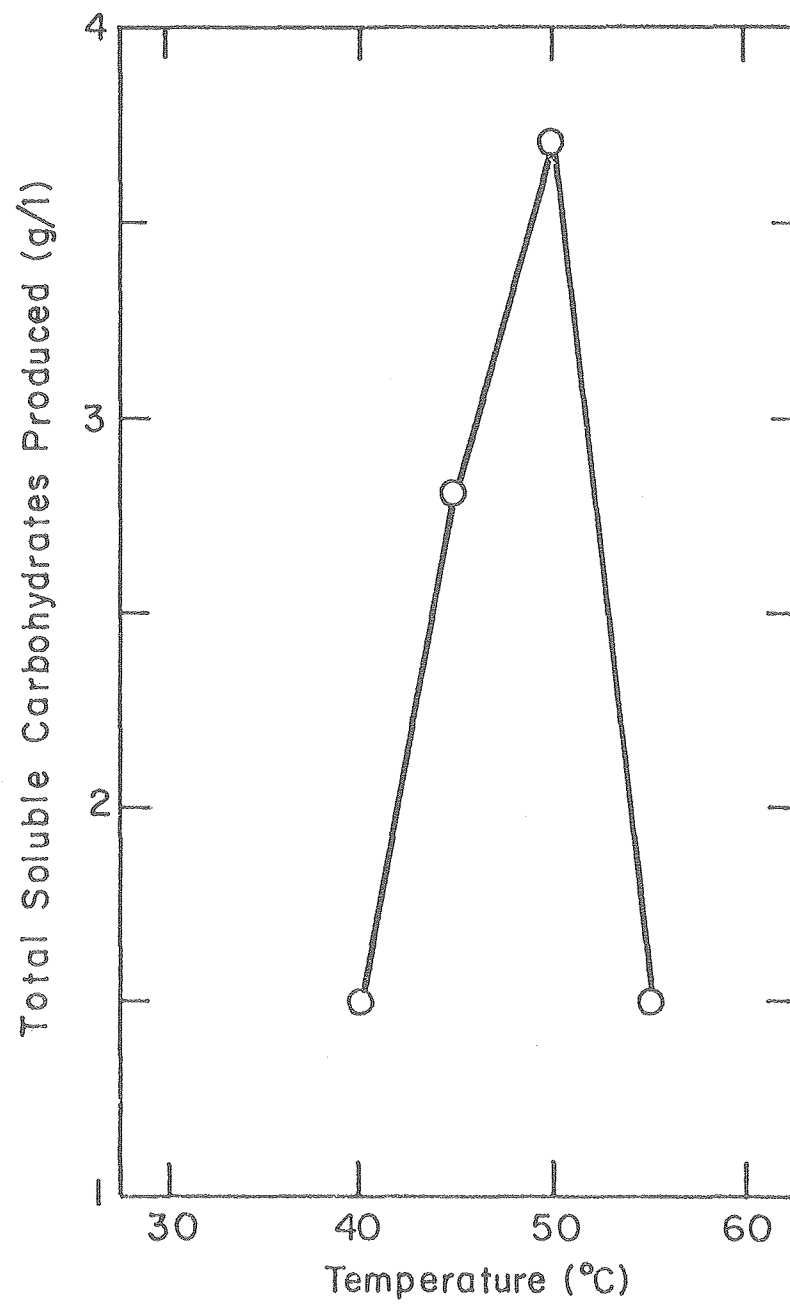
Figure 7. Comparison of Fermentations of QM9414 with Medium I and II.



not yet clear whether the low enzyme yields obtained from Rut-NG-14 and QM9414 can be attributed to persistent foaming problems and/or the use of rather accessible ball-milled cellulose. Non-ball-milled cellulose at different concentrations will be investigated in fermentors with mechanical foam breakers.

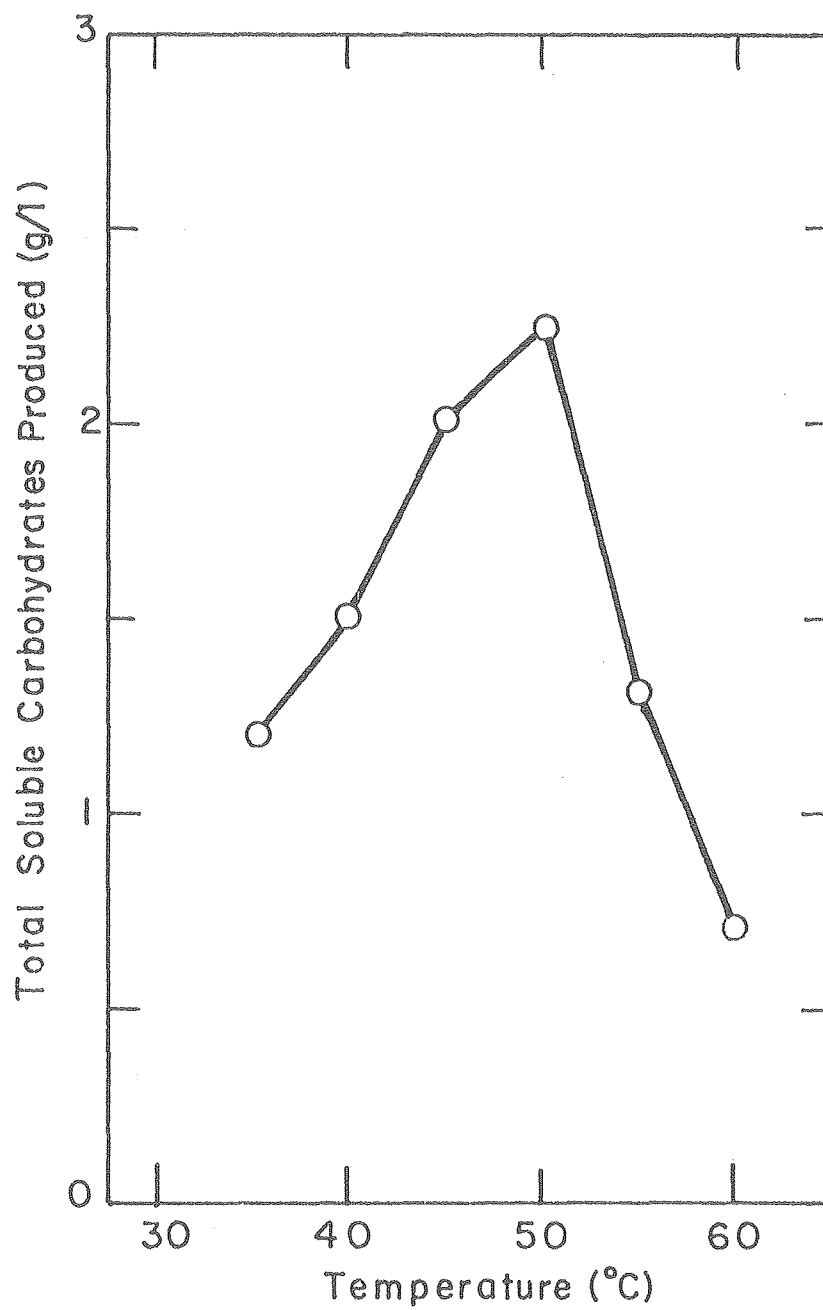
### C. Studies on Development of Mixed Enzyme System

The complete enzyme system may be comprised of two cellulases, component  $C_2$  or endocellulase and component  $C_1$  or cellobiohydrolase and three  $\beta 1 \rightarrow 4$  dextrinases exo- and endo-CMcellulase and cellobiase or  $\beta$ -glucosidase. It may well be that some cellulolytic micro-organisms have evolved pathways where all the five components are not necessarily required. Relative concentration of these five components may vary in preparations from different sources. The most effective system would be comprised of all five components in their optimal concentrations. The overall objectives of the studies initiated at this laboratory is the development of a mixed enzyme system that will be a more effective agent for converting cellulosic wastes to glucose than cellulase obtained from a single source such as Trichoderma viride. Cell-free preparations from two Trichoderma species, namely viride and koningii were subjected to comparison for their relative  $C_x$ , endocellulase (component  $C_2$ ) and cellobiohydrolase ( $C_1$ ) activities. Temperature optima for both cellulase preparations was the same, i.e., 50°C for cellobiohydrolase activity (Fig. 8 and Fig. 9) and 40°C for endocellulase activity (Figs. 10 and 11). Both preparations exhibited wider temperature optima from 45°C to 60°C for  $C_x$  activity (Fig. 12). Preparation from T. koningii cellulase was stable up to 70°C for  $C_x$  activity. Cell-free preparations from both species were adjusted to approximately the same FPA. Using the same assay procedures and conditions,  $C_x$ , cellobiohydrolase and endocellulase activities were determined. Results are summarized in Table 8. Preparations from T. viride seems to possess a relatively stronger



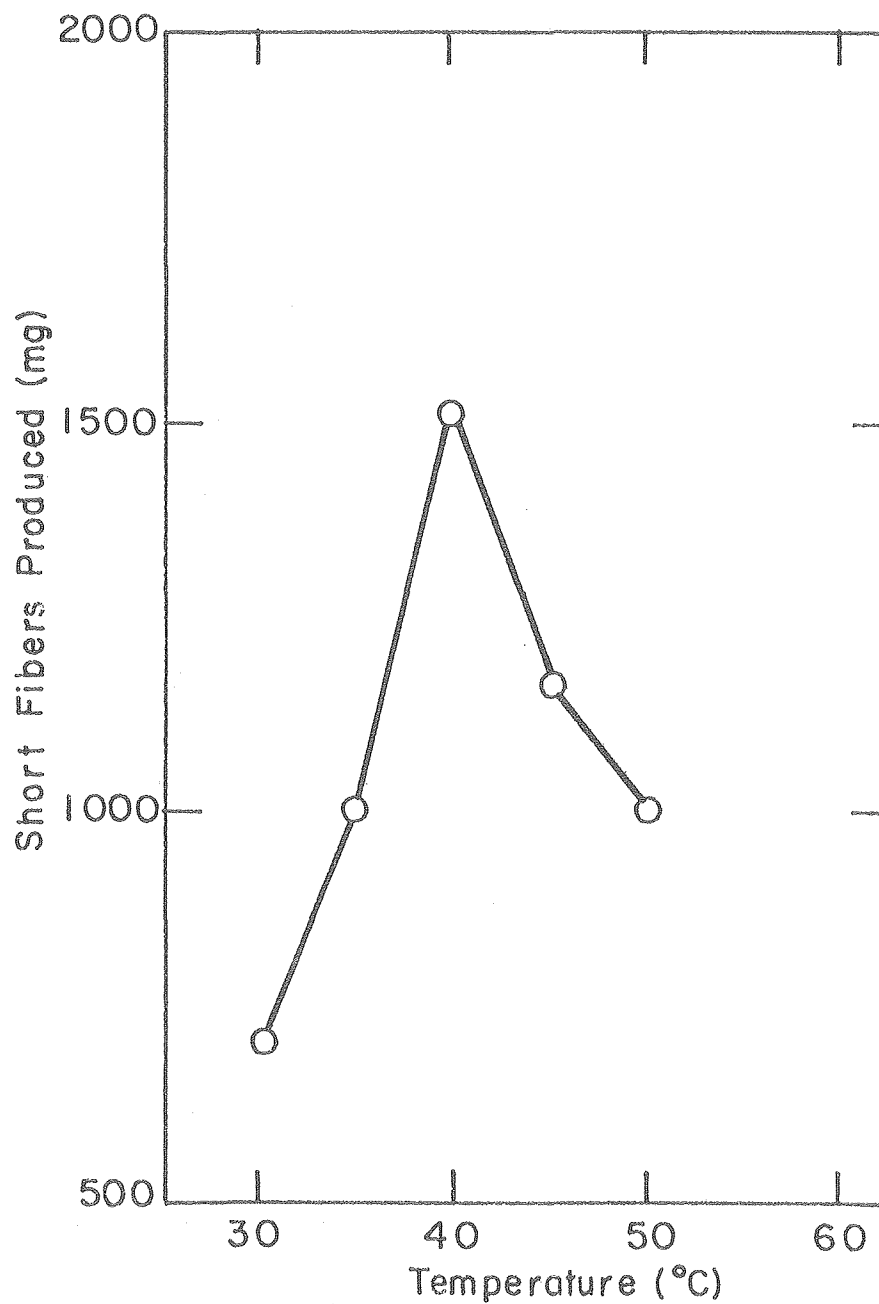
XBL- 792-8232

Figure 8. Temperature Optimum for Cellobiohydrolase ( $C_1$ ) Activity in T. viride cellulase.



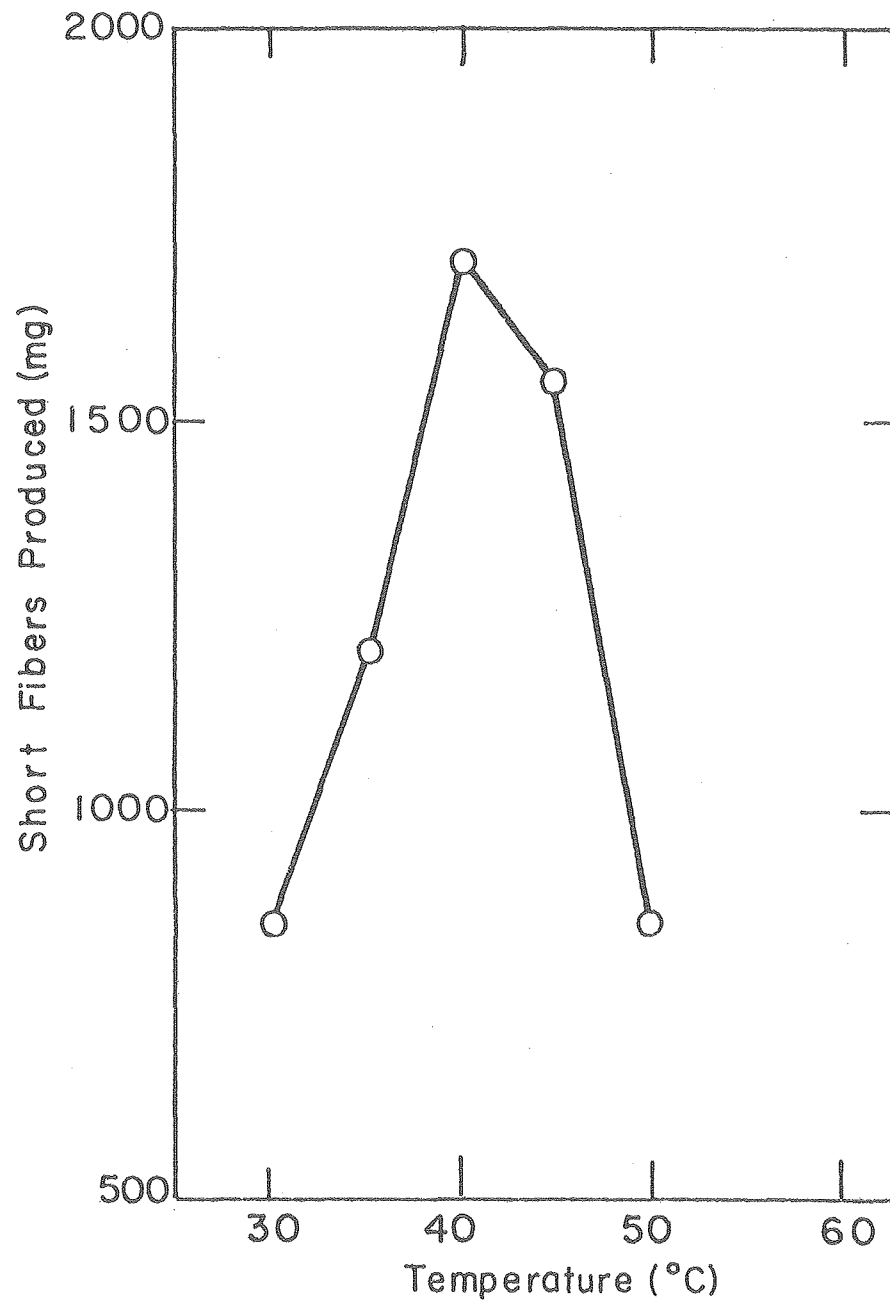
XBL 792-8235

Figure 9. Temperature Optimum for Cellobiohydrolase (C<sub>1</sub>) Activity in *T. koningii* cellulase.

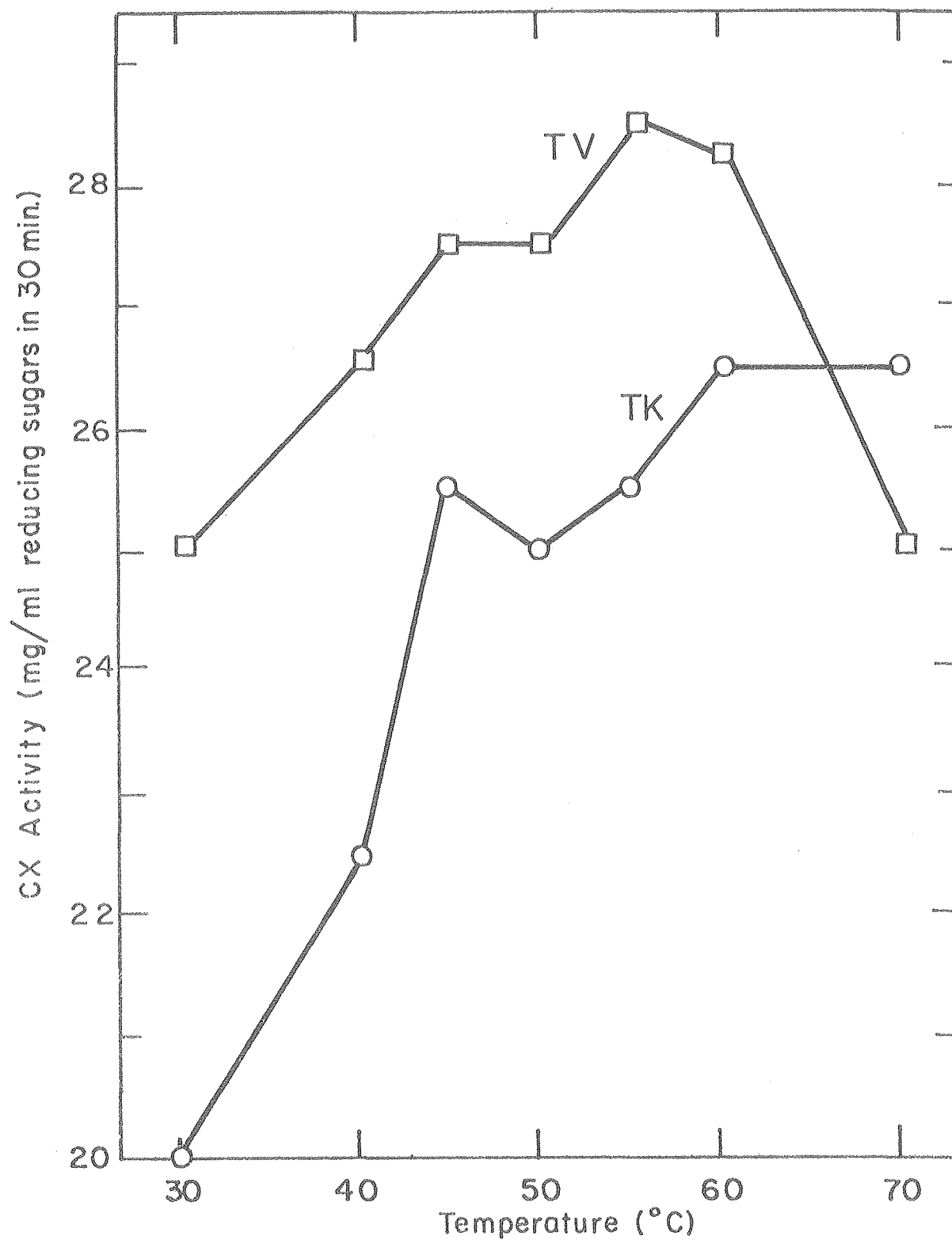


XBL 792-8234

Figure 10. Temperature Optimum for Endocellulase ( $C_2$ ) Activity in T. viride Cellulase.



XBL 792-8233  
Figure 11. Temperature Optimum for Endocellulase (C<sub>2</sub>)  
Activity in T. koningii Cellulase.



XBL 792-8236

Figure 12. Temperature Optimum for  $\beta$ 1-4 Glucanase (C<sub>x</sub>) Activity in *T. viride* and *T. koningii* Cellulase.

Table 8

T. viride and T. koningii Enzyme Components

FILTER PAPER ACTIVITY (FP)		$\beta$ 1 $\rightarrow$ 4 DEXTRINASE (CM-CELLULASE) (C <sub>x</sub> ) ACTIVITY	ENDOCELLULASE (COMPONENT C <sub>2</sub> ) (SF)ACTIVITY	CELLOBIOHYDROLASE (C <sub>1</sub> ) ACTIVITY
<u>T. viride</u>	2.00	1.3	1300  65% Segmen- tation	4.2
<u>T. koningii</u>	2.00	1.6	1900  95 $\rightarrow$ 100% Segmentation	3.4

cellobiohydrolase component and exhibited 19 percent more activity compared with preparations from T. koningii. T. koningii cellulase is relatively rich in the endocellulase component and showed 32 percent more activity compared with T. viride cellulase. The rate of hydrolysis of Solka Floc with T. viride and T. koningii preparations (of approximately 1.5 FPA) alone and in combination were studied, and results are shown in Fig. 13. The amount of each preparation used for hydrolysis is stated on the graphs. The rate of hydrolysis of Solka Floc during the first 50 hours was faster with T. viride cellulase than with T. koningii preparation, which proved to be more effective during the later stage of hydrolysis. T. koningii cellulase possesses relatively strong endocellulase (component C<sub>2</sub>). This may explain its relatively increased effectiveness towards the remaining crystalline and resistant part of Solka Floc structure. This effect becomes more obvious on extended period of hydrolysis (Fig. 14). The mixed enzyme preparation has a higher rate of hydrolysis, and this becomes more obvious after 50 hours. Both preparations possess relatively weak  $\beta$ -glucosidase. Since the rate of conversion is linear during the early stage of hydrolysis, it may be possible that greater amount of cellobiose and cellodextrins are produced and that these have inhibitory action on the cellulolytic components (cellobiohydrolase and endocellulase). As the reaction proceeds, the inhibition effect is less and the effectiveness of the mixed enzyme preparation is dominant over the individual preparations. This effect was studied in the next experiment where mixed preparation of T. viride and T. koningii were supplemented with a  $\beta$ -glucosidase component from A. niger and the rate of hydrolysis of Solka Floc was studied. Reaction products were analyzed for the production of cellobiose and glucose. At present the results are being evaluated.



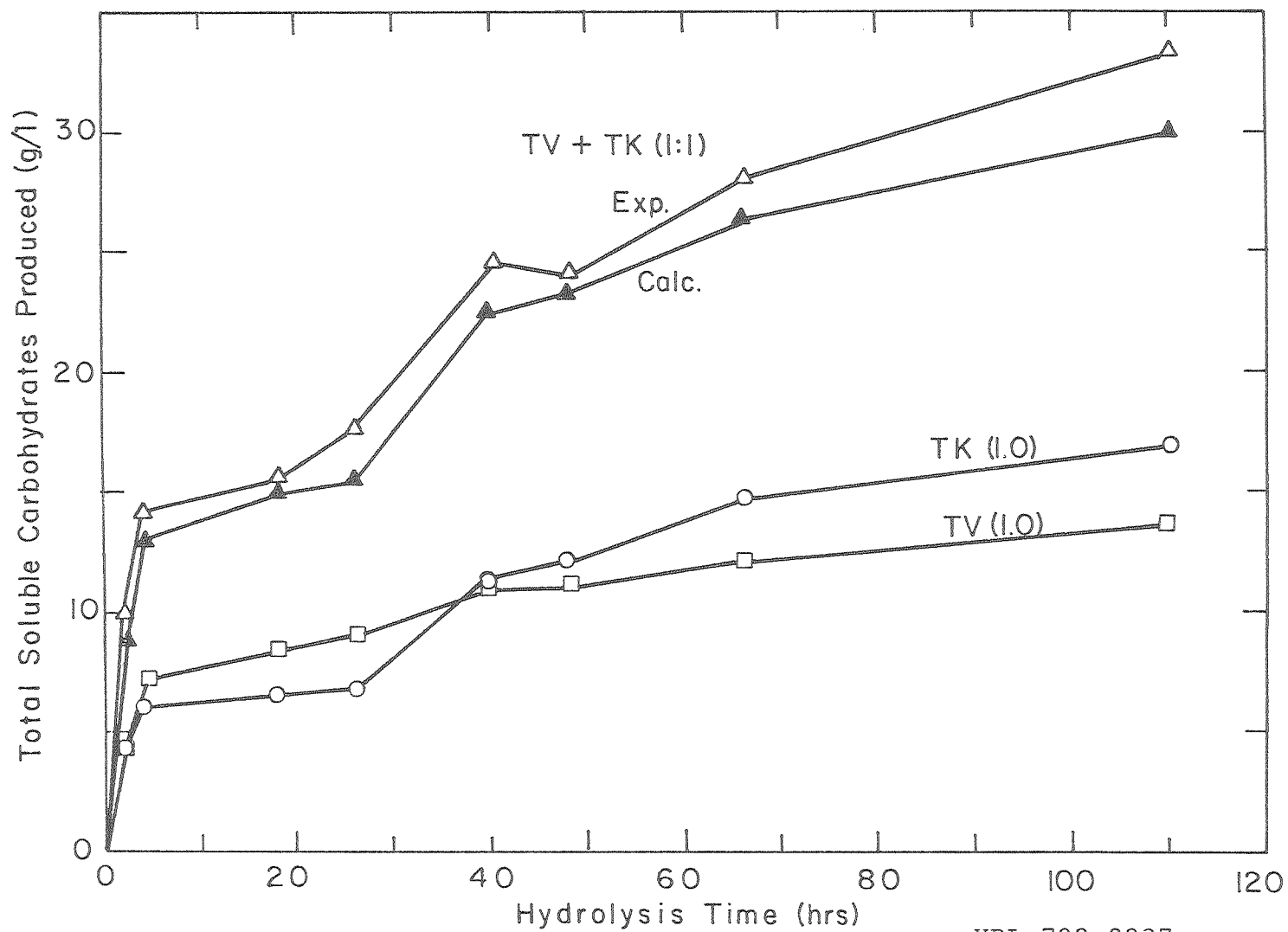


Figure 13. Comparison of Preparations of *T. viride* and *T. koningii* (FPA  $\approx$  1.5 Respectively) Alone and in Combination on the Hydrolysis of 5 wt% Suspension of Solka Floc.

XBL-792-8237

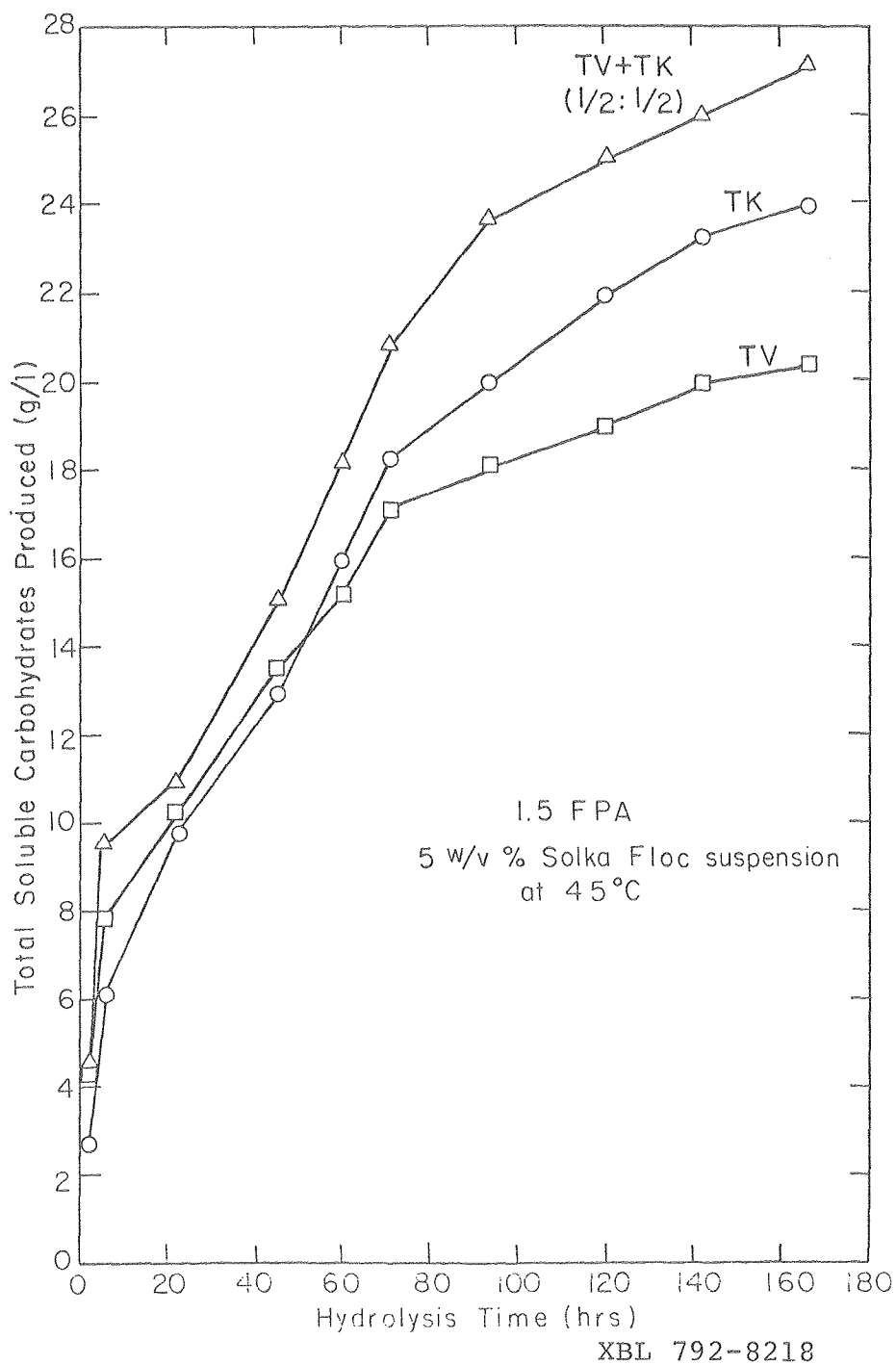


Figure 14. Comparison of Preparations of *T. viride* and *T. koningii* (FPA ~ 1.5, Respectively) Alone and in Combination on the Extended Hydrolysis of 5 wt% Suspensions of Solka Floc.

### III. ETHANOL FERMENTATION STUDIES

#### A. Fermentation of Enzymatic Hydrolyzates to Produce Ethanol

Experiments were conducted to test the fermentability of sugar present in hydrolyzates produced using several cellulase enzyme systems.

All the experiments were run under sterile conditions in a reactor of 600 ml volume. The reactor was saturated with air before inoculation, but was run under anaerobic conditions. The reactor was gassed with nitrogen during sampling. All enzyme preparations (culture supernates) were boiled prior to use, and the coagulated protein was removed by filtration. Figure 15 shows the normal growth and ethanol production in a mineral medium containing 10% glucose.

In a series of experiments on the fermentability of 10% glucose in a culture supernate derived from T. viride, QM 9414, sodium hydroxide was used to control the pH during enzyme production. The resulting preparation was concentrated sevenfold and either used directly or subjected to charcoal filtration. The concentrated preparation inhibits glucose fermentation by the yeast. This inhibition can be largely removed by filtration through activated charcoal. The results of these experiments are shown in Fig. 16.

When the Rutgers mutant C-30 was used as the enzyme source, no inhibition of fermentation was observed with the unconcentrated boiled supernate, shown in Figure 17, or the sevenfold concentrated boiled supernate shown in Figure 18. The supernates were prepared from cultures where ammonium hydroxide, rather than sodium hydroxide was used to control the pH. When the sevenfold concentrate preparation from QM 9414 was used, which had been produced from a culture where ammonium hydroxide was used for pH control, there was a long lag followed by apparently normal yeast growth and ethanol production as shown in Figure 18.

Removal of the protein by boiling or other means seems to be necessary to allow fermentation to proceed in the presence of any supernate. Once this has

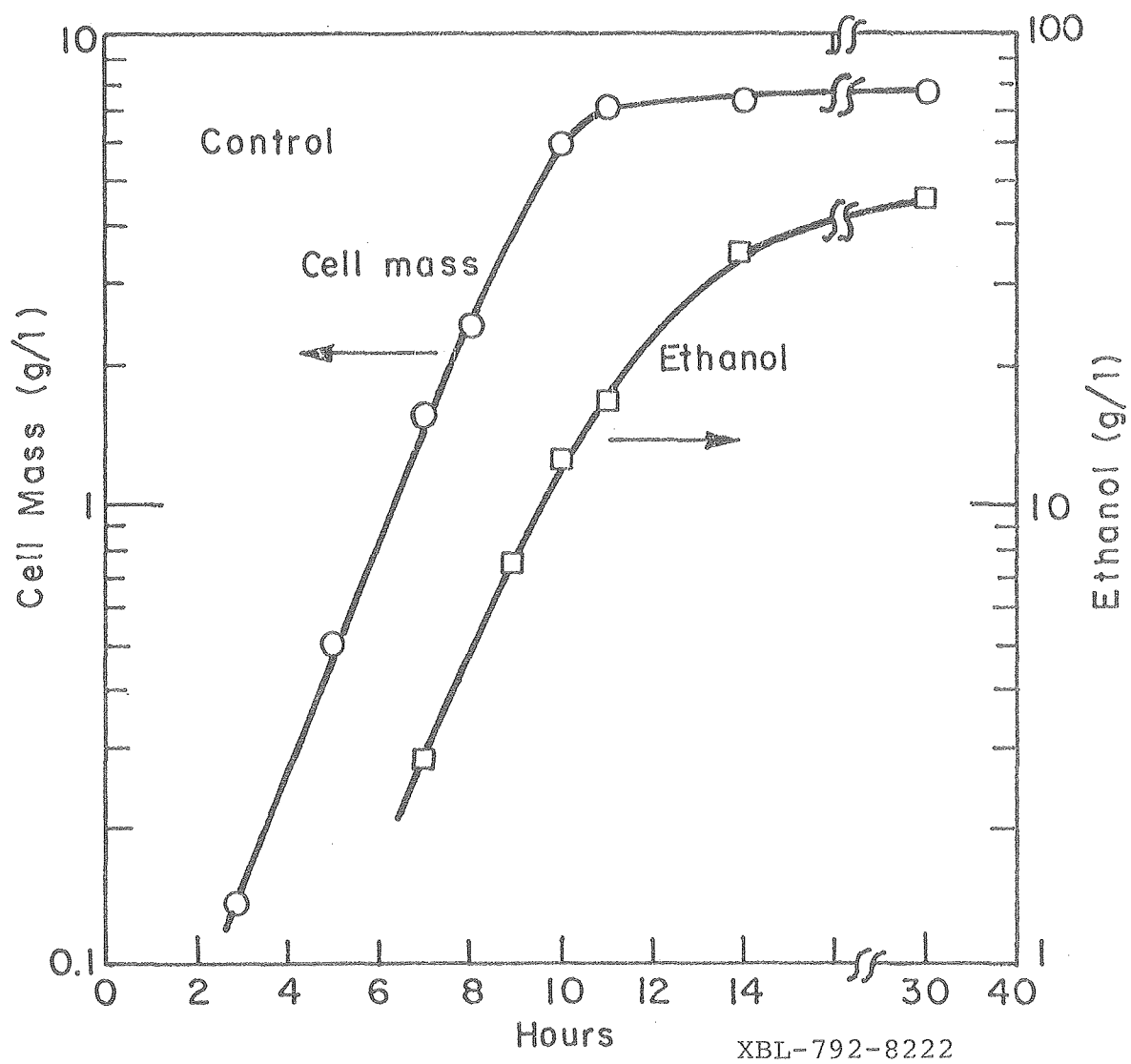
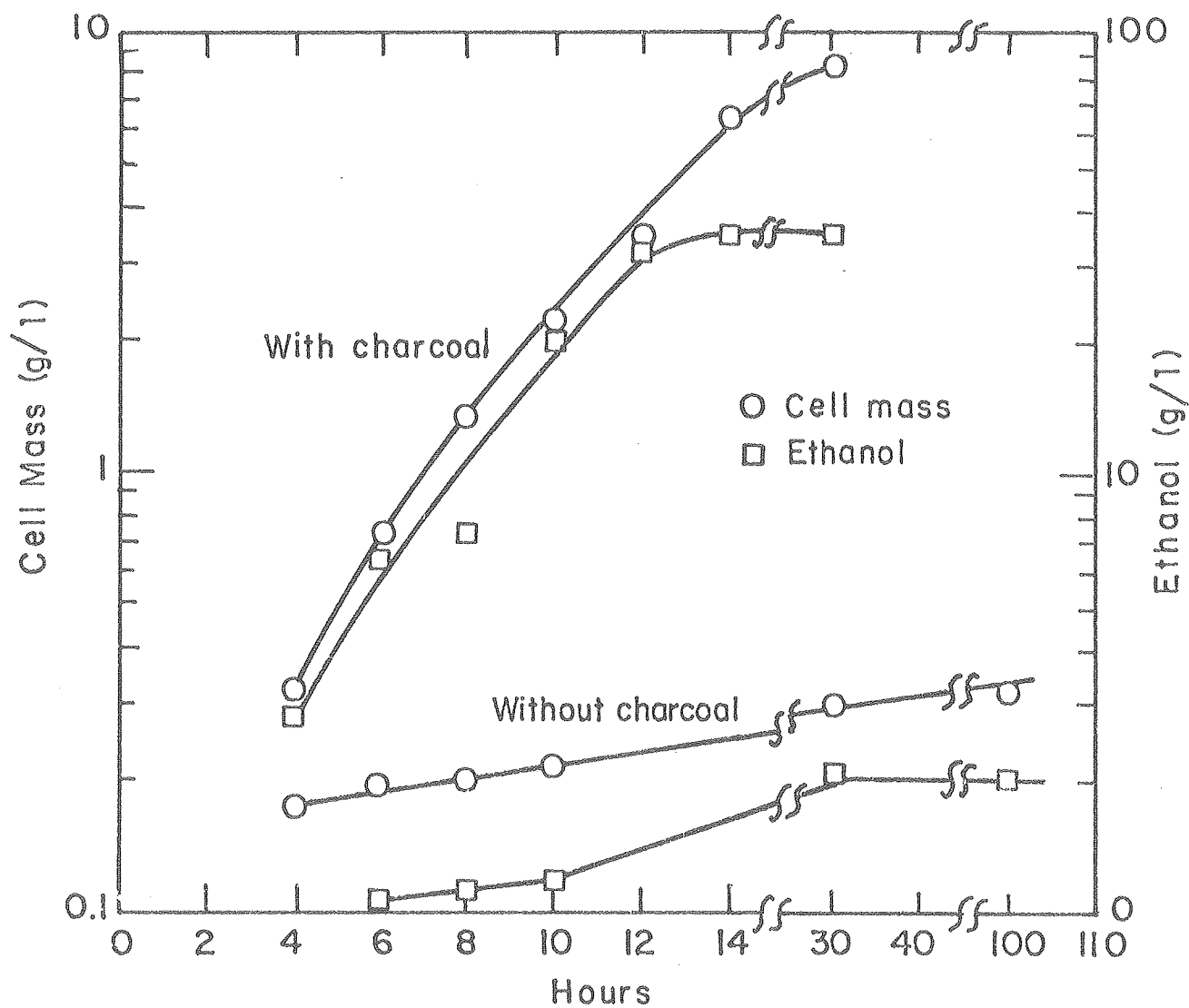


Figure 15. Production of Ethanol and Cell Mass in a Mineral Medium Containing 10% Glucose.



XBL 792-8219

Figure 16. Production of Ethanol and Cell Mass with the Indicated Extracts from *T. viride* (QM 9414). The Enzymes Were Sodium Hydroxide Controlled During Production and then Concentrated Seven Times.

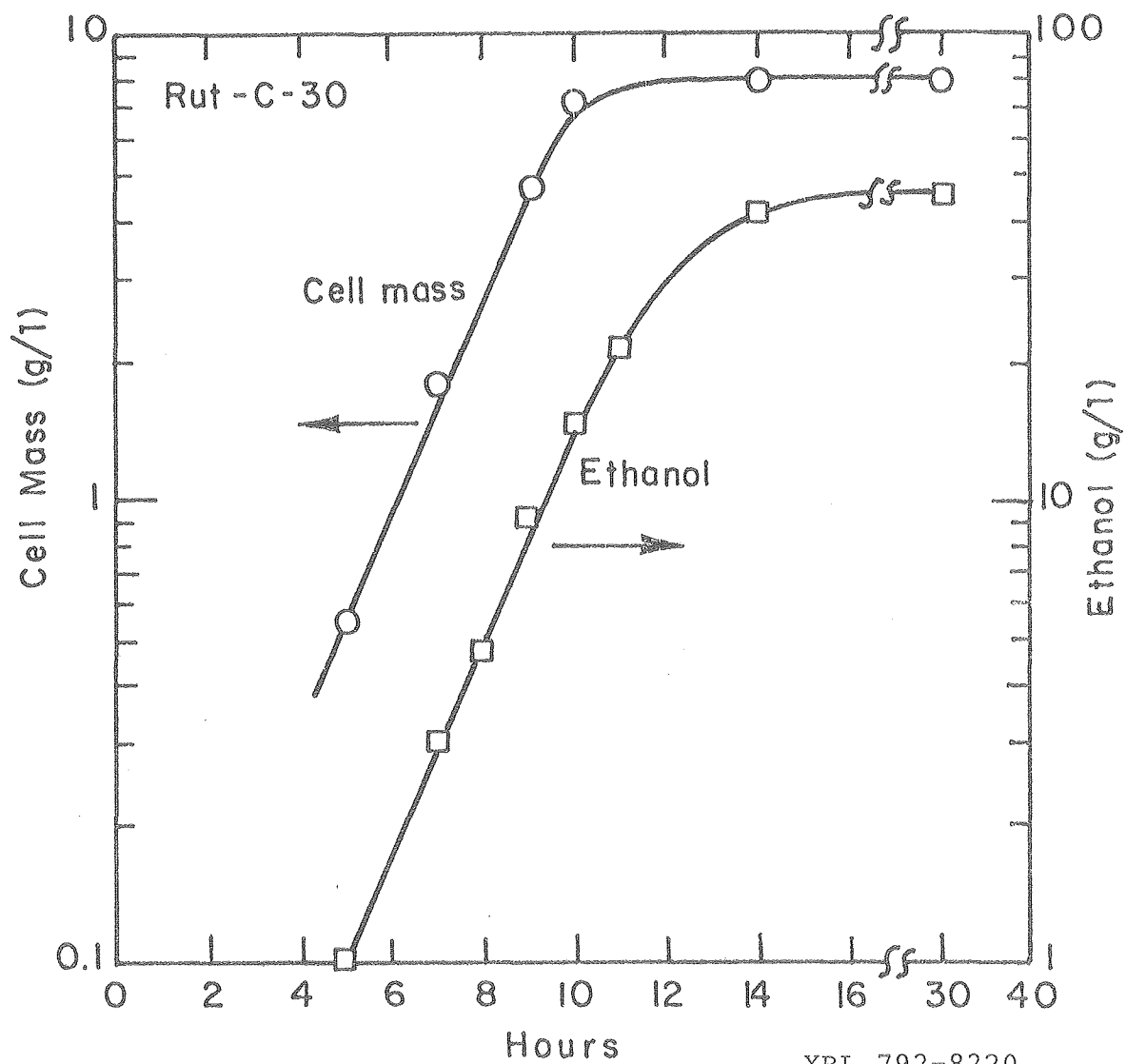


Figure 17. Production of Ethanol and Cell Mass in an Extract Derived from the Rutgers C-30 Mutant.

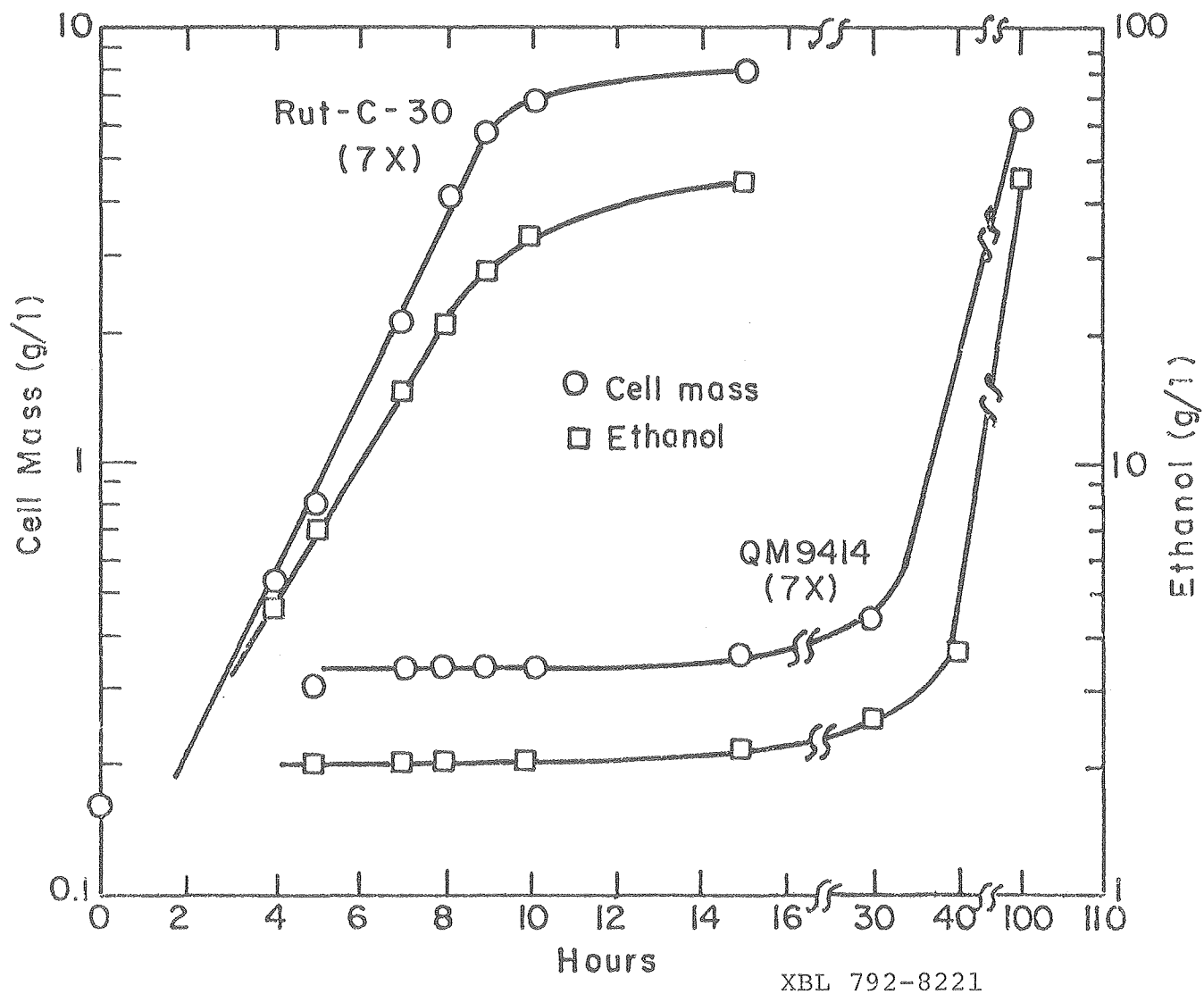


Figure 18. Production of Ethanol and Cell Mass From Seven Times Concentrated Extracts Derived from *T. viride* QM 9414 and Rutgers C-30. Both Enzyme Solutions Were Obtained by Ammonium Hydroxide Controlled Production.

been done, preparations from C-30 appear to need no further treatment, while those from QM 9414 still contain an inhibitor which must be removed. It is interesting to note that QM 9414 produces a yellow pigment which is removed by activated charcoal, while C-30 produces no visually detectable pigment.

The culture supernates are presently being concentrated sevenfold in order to mimic the actual process which would require concentration of the hydrolyzate to give a sugar concentration of approximately 10%. Future experiments will involve the use of actual hydrolyzates produced from natural cellulosic residues.

B. Preliminary Conclusions from Batch Culture Studies of the Media Requirements for Ethanol Fermentation.

Saccharomyces cerevisiae ATCC 4126 was able to grow and produce ethanol in a completely synthetic media without growth factors to produce a cell yield comparable to media with synthetic and complex growth factors. However, the growth and ethanol production rates were much slower without growth factors.

The most important growth factor is biotin. The growth rate in a biotin deficient media with six other vitamins present was roughly the same as in the media without any growth factors. Media deficient in the other six vitamins in various combinations produced considerably faster growth rates than biotin deficient media. The fastest growth rate occurred with the complete set of seven vitamins. However, the cell and ethanol yields were independent of the growth factors.

Growth rates comparable to those obtained by Cysewski (6) with his yeast extract supplemented media was obtained with a synthetic media supplemented with vitamins and amino acids. An even higher growth rate was achieved with this synthetic media supplemented with one-eighth the level of yeast extract used by Cysewski. The yeast extract above a minimum level did not affect the



growth or ethanol production rates but only the cell yield; the ethanol yield was also unaffected.

Adding a small quantity of amino acids to a synthetic media with vitamins raised the growth and ethanol production rates appreciably. Further addition of amino acids by a factor of five resulted in no change.

Increasing the level of vitamins in an amino acid free media did increase the growth rate and yield significantly for an initial glucose concentration of 100 g/l; ethanol yield was unaffected. However, there are declines in growth and ethanol production rates relative to an initial glucose concentration of 50 g/l.

#### IV. HYDROLYSIS REACTOR DEVELOPMENT

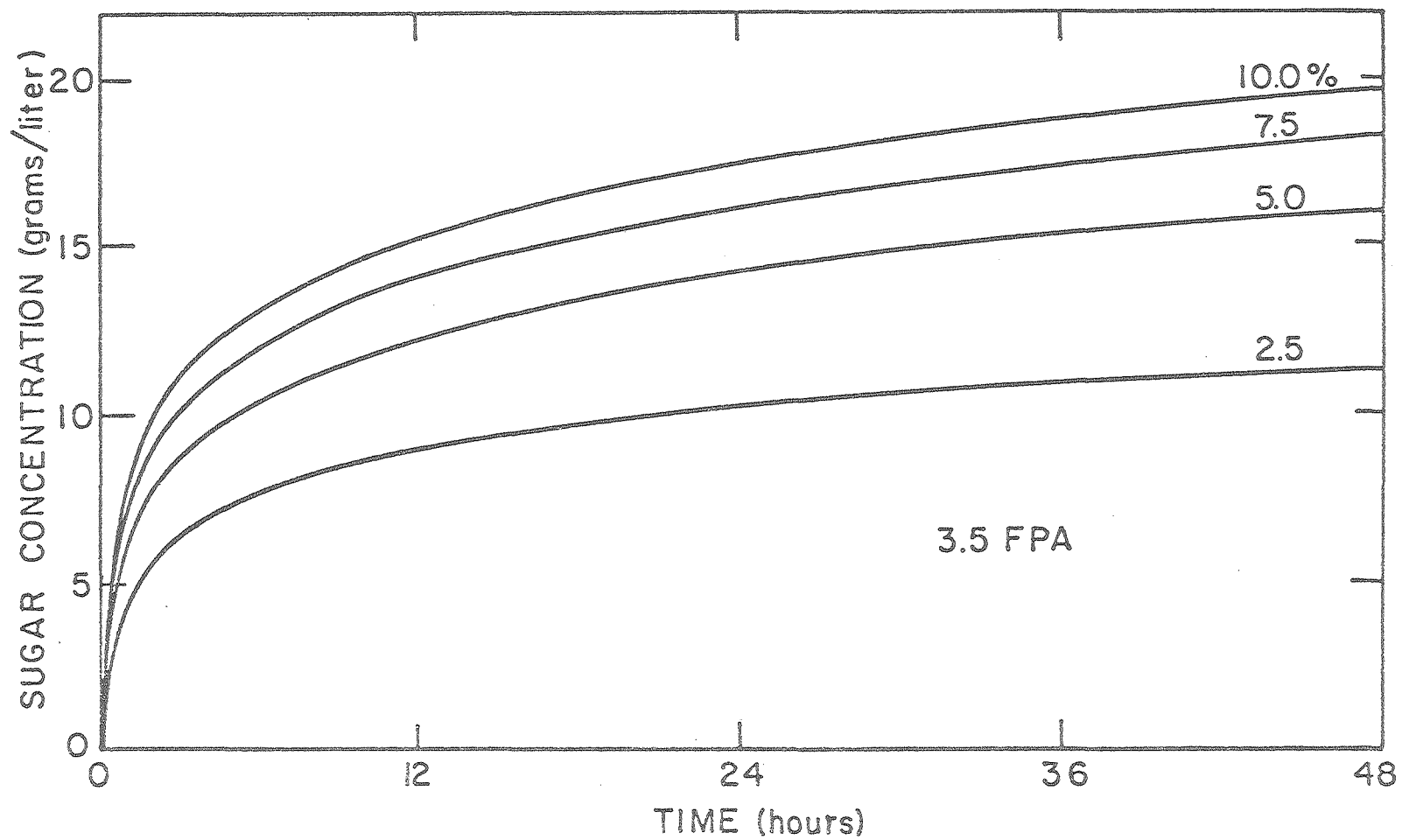
##### A. Hydrolysis Kinetic Studies

In the report PROCESS DESIGN AND OPTIMIZATION OF CELLULOSE HYDROLYSIS (LBL-7864), a kinetic model of the following form was proposed:

$$\frac{dP}{dt} = \frac{U_m e^{-K_1 P} (S_0 - P)}{\frac{K_m}{\alpha_0} \left( 1 + \frac{P}{K_2} + e^{-K_1 P} (S_0 - P) \right)}$$

Based on this model and subsequent initial rate studies it was possible to construct a computer program to predice the hydrolysis curves for various initial substrate concentrations and enzyme activities. A typical example of such predictions is shown in Fig. 19. The distribution of the hydrolysis curves for the various substrate concentrations is standard with each Filter Paper Activity.

To verify the existence of such a family of curves for each Filter Paper Activity, a series of experiments with initial substrate concentrations of 2.5,



XBL 788-5576

Figure 19. Computer Predictions for Batch Hydrolysis (Various Substrate Concentrations).

5.0, 7.5 and 10.0 w% were performed. To provide enzyme solutions of various activities, an enzyme of high activity was produced and diluted to each appropriate activity. Activities of 2.4, 3.1, 3.9 and 4.7 FPA were used to correlate with the range of those considered in the computer simulation (the dilution curve is shown in Fig. 20).

Results of these experiments are shown in Figs. 21 through 24. The experimental curves show a strong resemblance to those of the computer model; and it should be noted that the curves would not be expected to match the model because two different enzyme solutions were used (and these would have different kinetic constants). For this set of experiments, time did not allow for a study of initial rates for the particular enzyme solution used, but the resemblance between the curves (experimental and predicted) is too strong to be overlooked. In Fig. 23 there is a crossover of the 10 and 7.5 w% curves; this can probably be explained by mixing effects. The computer model assumes perfect mixing in all cases, where in actual experimentation mixing can vary greatly when substrate concentrations are high (poor mixing of the suspension would tend to give results lower than predicted).

Based on these results, the computer model can give a reasonable prediction of hydrolysis curves when held within limits. However, this by no means indicates that the model is descriptive of actual events occurring; it is only a means to provide an approximate fit to experimental hydrolysis curves. This allows a computer optimization of a process design (as in LBL-7864) to provide a deeper insight into strengths and weaknesses of the process.

## V. UTILIZATION OF HEMICELLULOSE SUGARS

### A. Xylanase Production and Saccharification

The organism used in this study was Streptomyces xylophagus, nov sp. The

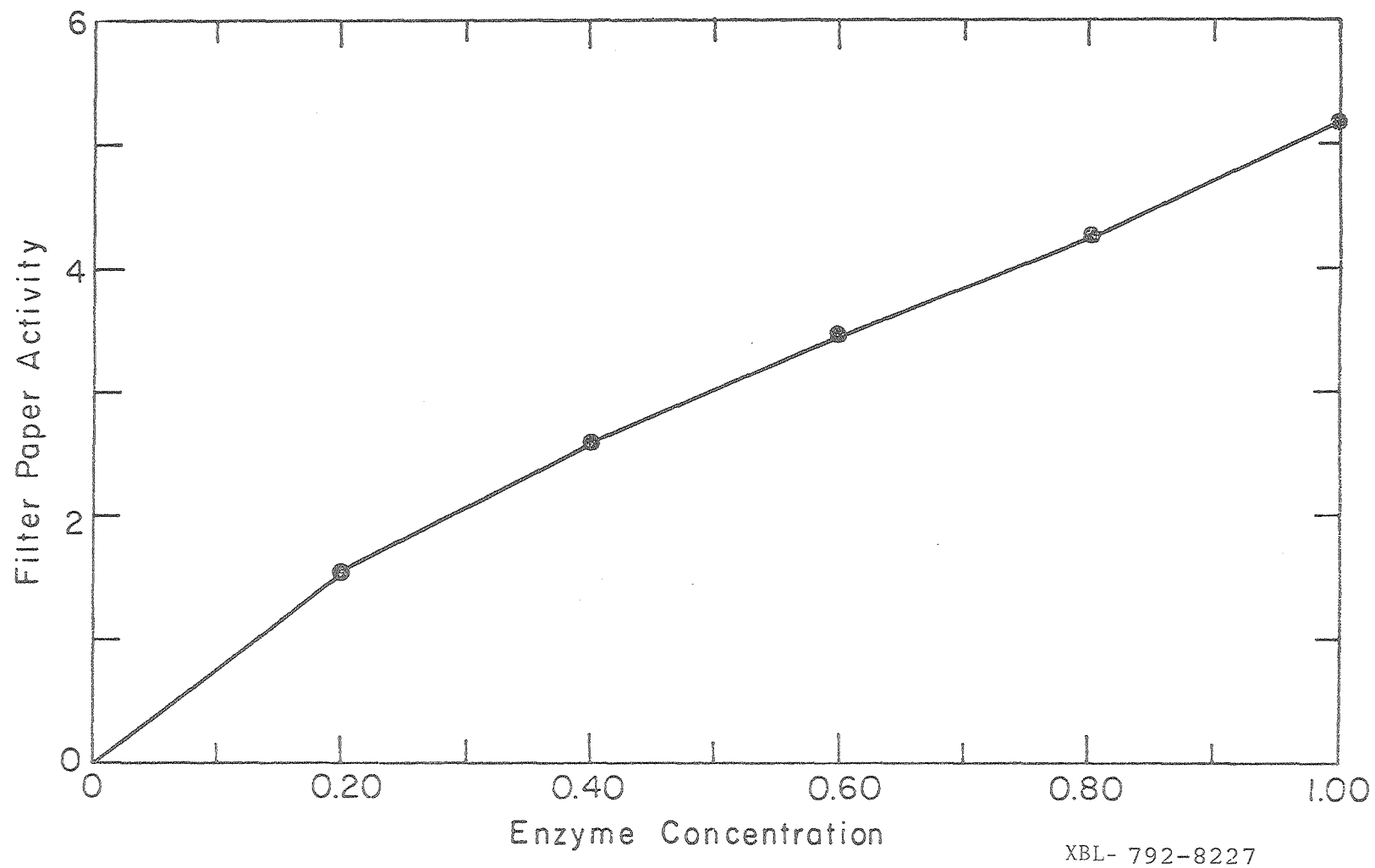
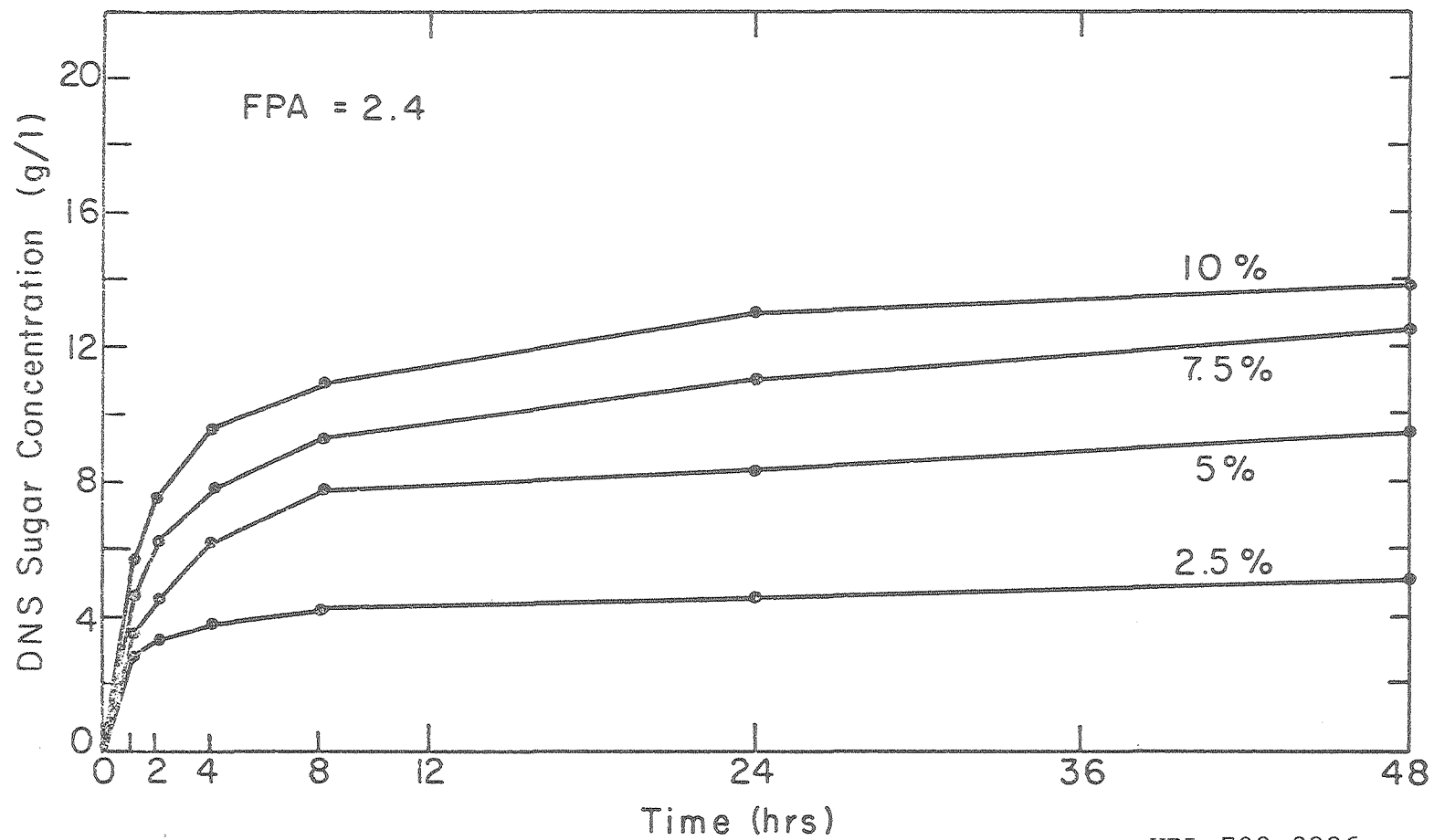


Figure 20. Typical Enzyme Dilution Curve of T. viride Cellulase Solution.



XBL-792-8226

Figure 21. Hydrolysis of Various Amounts of 2 MM Wiley Milled Corn Stover by *T. viride* Cellulase with FPA = 2.4.

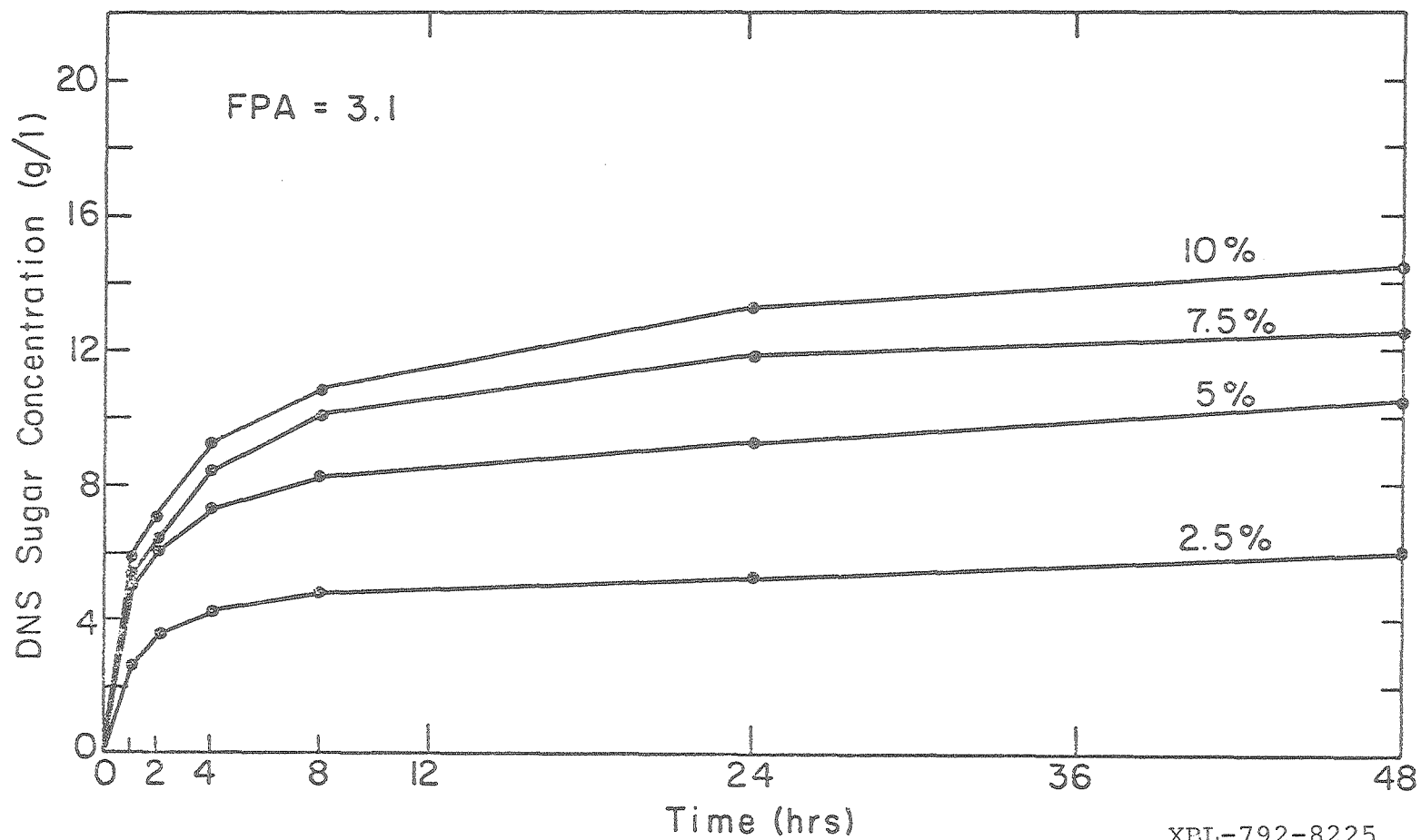


Figure 22. Hydrolysis of Various Amounts of 2MM Wiley Milled Corn Stover by *T. viride* Cellulase with FPA = 3.1.

XBL-792-8225

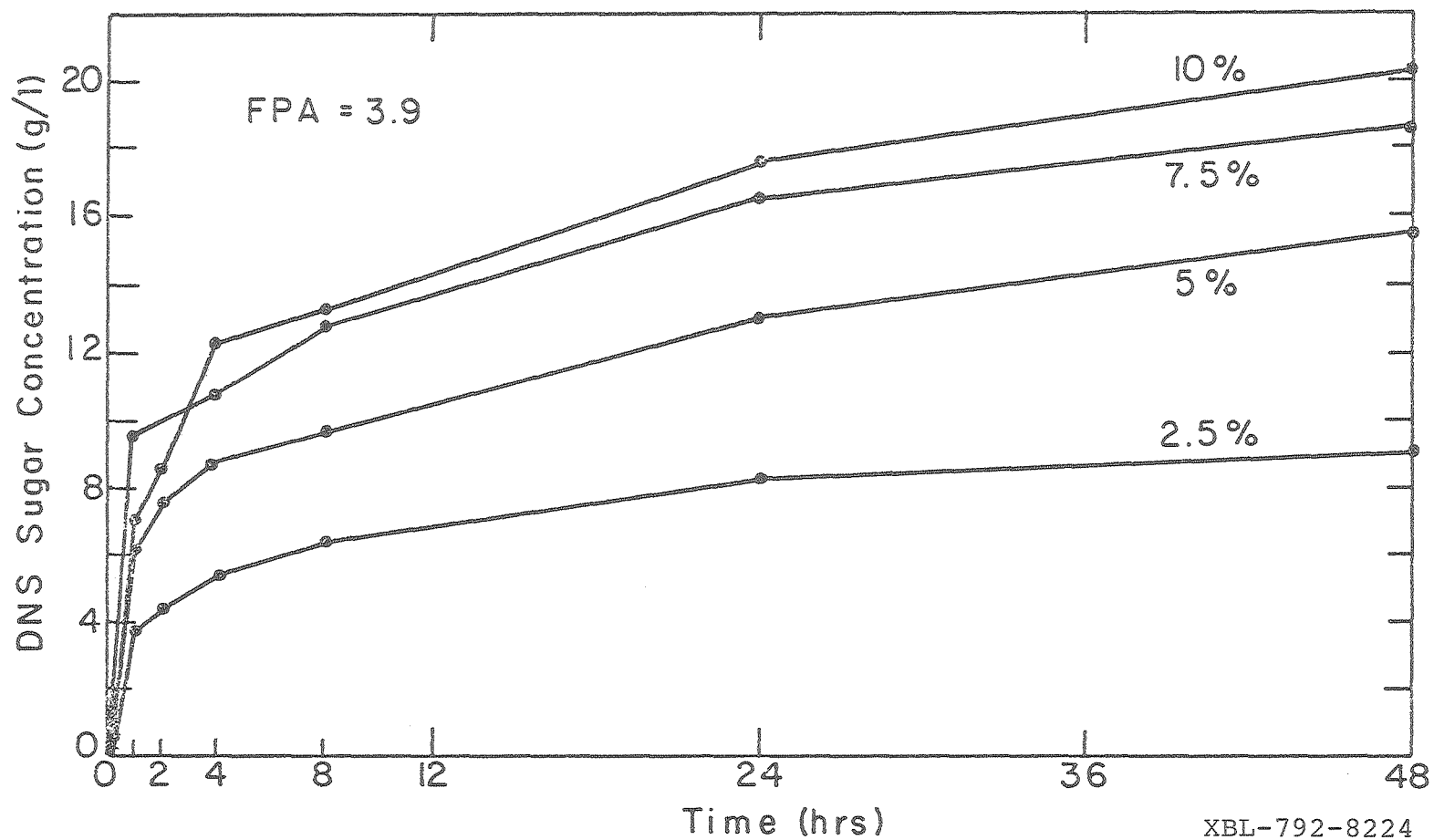


Figure 23. Hydrolysis of Various Amounts of 2MM Wiley Milled Corn Stover by T. viride Cellulase with FPA 3.9.

XBL-792-8224

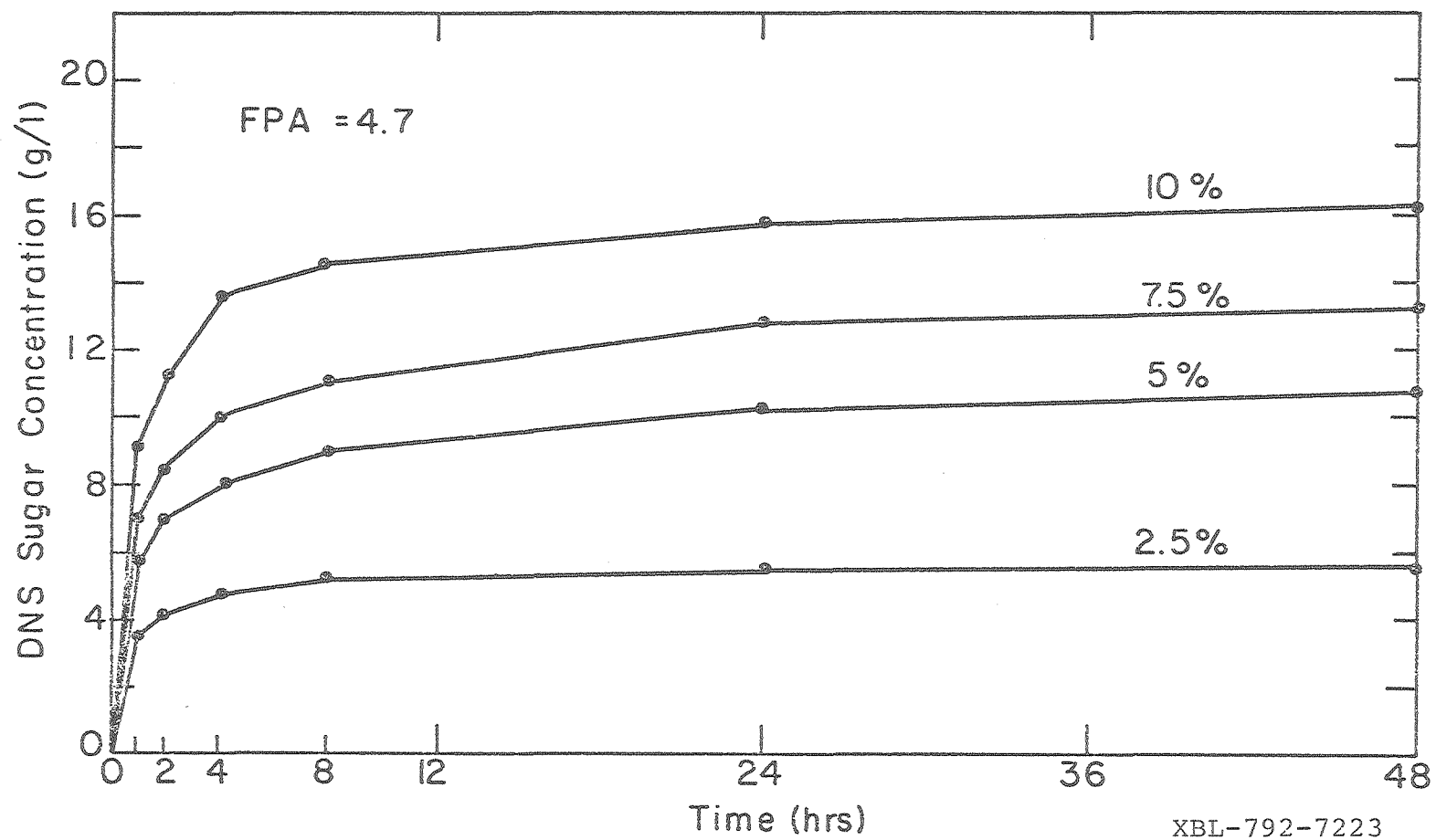


Figure 24. Hydrolysis of Various Amounts of 2 MM Wiley Milled Corn Stover by *T. viride* Cellulase with FPA 4.7.



standard salts medium was the one developed by Kawaminami and Iizuka (7). The fermentations were carried out in 14 liter fermentors equipped with pH control units. The dissolved oxygen was automatically controlled at a level greater than 40% of the saturation value for the media. This was accomplished by varying the agitation rates in response to changes in the dissolved oxygen. In most cases, "one sided control" was used ( $D.O. \leq$  the low set point). Temperature and pH were kept at 30°C and 8.4 respectively. The pH was controlled during the fermentations by the addition of 2N sodium hydroxide. The results of batch and continuous fermentations are given in Table 9.

The xylan used in the saccharifications was from purified larchwood, obtained from Sigma Chemical Co., St. Louis, Mo. The hydrolyses were carried out by suspending 2.5 to 20 w% xylan in buffered (phosphate buffer, pH 5.2) culture filtrates in 20 ml test tubes containing 4 ml of the reaction mixture and incubating them at 55°C with constant shaking at 150 RPM. Samples were taken at intervals and 2N NaOH was added to stop the reaction. Total sugars were measured by the DNS method after removal of the solids by centrifugation. The results are given in Table 10.

#### B. Fermentation of Xylose

Work in this period involved selection of a simple mineral medium, design and construction of a controlled-pH, anaerobic cultureing system and growth of Bacillus macerans at a number of pH's in order to find the optimum for growth and to note any effects of pH on the distribution of fermentation products. Some of the results of these first experiments are shown in Table 11. The optimum pH for growth is approximately 6.0. Ethanol production seems to be greater at lower pH's. The inverse relationship appeared to hold true for

Table 9

Xylanase Production

TYPE OF SUBSTRATE	MODE OF OPERATION	RESULTS
Xylan (Larchwood) 1%	Batch	Xylanase Activity = 9.82 Dry wt. = 5.62 g/l } After 72 hours
Xylan (Wood Gum) 1%	Batch	Xylanase Activity = 8.11 mg/ml Soluble Protein = 9.92 mg/ml Dry wt. = 6.02 g/l } After 72 hours
Wheat-bran (Washed) 7%	Batch	Xylanase Activity = 9.44 mg/ml After 72 hours
Xylan (Wood Gum) 1%	Continuous	A steady state value of 6.75 mg/ml in terms of enzyme activity after 3.5 residence time ( $D = 0.02 \text{ hr}^{-1}$ ) was obtained.

Table 10  
Enzymatic Hydrolysis of Xylan

SUBSTRATE CONC (%)	% XYLANASE		
	100	50	25
2.5	10.8 <sup>+</sup> (1) <sup>++</sup>	10.4 (1)	9.6 (1)
5.0	20.0 (3)	19.2 (3)	16.9 (3)
10.0	29.3 (1)	27.6 (1)	26.5 (1)
	39.1 (7)	36.9 (7)	34.2 (7)
20.0	30.1 (1)	18.0 (1)	12.6 (1)
	45.6 (7)	44.3 (7)	33.9 (7)

+ Reducing sugar formed

++ The reaction time, (hours)

Table 11.

Fermentation Parameters Associated with Growth of B. macerans at Various pH's

---

% Conversion (g product/g xylose Fermented) <sup>a</sup>			
pH	Ethanol	Acetone	$\mu_{\max}$ Hr <sup>-1</sup>
7.5	15.0	0.5%	0.077
7.0	19.8	1.0	0.116
6.5	19.4	1.0	0.146
6.0	19.6	1.0	0.163
5.5	21.9	1.0	0.103
5.0	No growth after five days.		

---

a) 1% Initial xylose concentration, all sugar was fermented.

---

acetic acid production (data not shown). Acetone concentrations were low in all fermentation broths. In the literature conversion to acetone of 6-10% are commonly reported, and our low figures may be due to the fact that no effort was made to recover material lost from the fermentor by CO<sub>2</sub> or nitrogen stripping. Ethanol values are also probably low for the same reason.

The  $\mu_{\max}$  for this organism is nine times that demonstrated by Fusarium oxysporum growing by fermentation of xylose. There is still room for improving the growth rate of B. macerans since the pH and temperature optima found are only approximate, and concentrations of medium components have not been optimized.

Under some conditions the organism can be induced to form pellets. This is a useful property since continuous operation of the fermentation is contemplated, and the pelleted cell mass can be easily recycled to build up the cell concentrations in the fermentor.

At present, fermentations are being conducted at increasing initial xylose concentrations to find the maximum level tolerated. We are planning experiments to examine the effects of ethanol, acetone and acetic acid concentrations on the rate of fermentation and the concentrations of products produced.

Future work will be directed toward identifying all the fermentation products, maximizing the growth (and fermentation) rate and increasing the production of neutral products at the expense of acetic and other acids.

## References

1. R.P. Freitas, W. Long, A.F. Sciamanna and C.R. Wilke, Procedures for Analysis of Solids and Liquors from Cellulosic Sources, LBL-5967, December 1977.
2. E.E. Shnaiier, M.E., Shpultova, S.V. Chepigo, A Combined Method of Hydrolysis of Corn Stalks with Concentrated Sulfuric Acid, Gidroliznaya i Lesokhimicheskaya Promyashlenost, 15(7), 1-4 (1960).
3. C.R. Wilke, Process Development Studies on the Bioconversion of Cellulose and Production of Ethanol, LBL-7880, September 1978.
4. Private Communication, Bill Long, University of California, Lawrence Berkeley, Laboratory, 1978.
5. Private Communication, Douglas Everleigh, Biochemistry, Rutgers University, New Brunswick, N.J., 1978.
6. G.R. Cysewski, C.R. Wilke, Fermentation Kinetics and Process Economics for Production of Ethanol, LBL-4480, March 1976.
7. T. Kawaminami, H. Iizuka, Agr. Biol. Chem., 33, 1787 (1969).

#### Published Reports

1. Utilization of Immobilized  $\beta$ -Glucosidase in the Enzymatic Hydrolysis of Cellulose (with Steven Howard Issacs) M.S. Thesis, LBL-7857 (1978).
2. Process Design and Optimization of Cellulose Hydrolysis (with Richard R. Lindsey) M.S. Thesis, LBL-7864, August 1978.
3. Process Development Studies for the Production of  $\beta$ -Glucosidase from Aspergillus phoenicis (with Mary Jane Howell) M.S. Thesis, LBL-7867, September 1978.
4. Effect of Nitrogen Oxide Pretreatments on Enzymatic Hydrolysis of Cellulose (with Ronald K. Borrevik and David L. Brink) M.S. Thesis, LBL-7879, September 1978.
5. Studies of Desorption of Cellulase from Cellulose with Various Reagents (with Mohammad Riaz) LBL-7889, October 1978.

#### Reports in Preparation or in Press

1. Composition of Utilization of Cellulose from Agricultural Residue for Chemicals, LBL-5966 (in press).
2. Comparison of Batch and Continuous Cellulose Hydrolysis with and without Ethanol Fermentation (with Takeshi Harima). Report in preparation.
3. Enhancement of Enzymatic Hydrolysis of Cellulose by Coupling with Ethanol Fermentation (with Takeshi Harima). Report in preparation.
4. Studies of the Adsorption of Cellulase Enzyme Components on Cellulose (with M. Riaz, M. Castanon, U. Von Stockar) LBL-7890 (in press).

APPENDIX

CELLULOSE BIOCONVERSION AND PILOT PLANT STUDIES  
LAWRENCE BERKELEY LABORATORY

Project Staff

Faculty Investigators

Charles R. Wilke, Professor of Chemical Engineering, Principal Investigator

Harvey W. Blanch, Associate Professor of Chemical Engineering,  
Co-Principal Investigator

Postdoctoral Investigators

Mohammad Riaz, Microbial Biochemistry, University of Wales, U.K., Ph.D. 1969.

Steven Rosenberg, Bacteriology, University of California, Berkeley, Ph.D. 1970.

S. Kishen Tangnu, Chemical Engineering, Indian Inst. of Tech., Delhi, India  
Ph.D. 1976.

Ren Der Yang, Chemical Engineering and Biochemical Engineering,  
University of Pennsylvania, Ph.D. 1974.

Graduate Students

Douglas Hemphill, Chemistry, New Mexico State Univ., B.S. 1977, M.S. Candidate

Richard Lindsey, Chemical Engineering, Illinois Inst. of Tech., B.S., 1976  
M.S. 1978.

Brian Moriella, Chemical Engineering, Massachusetts Inst. of Tech., B.S. 1978  
M.S. Candidate.

Chris Rhodes, Chemical Engineering, Purdue Univ., B.S. 1978, M.S. Candidate.

Dale Wiley, Chemical Engineering, University of California, Santa Barbara,  
Ph.D. 1977, M.S. Candidate.

Harry Wong, Chemical Engineering, Massachusetts Inst. of Technology, M.S. 1973,  
Ph.D. Candidate.

Support Staff

Aldo Sciamanna, Chemist, M.S. in Chemistry, Univ of San Francisco, 1952

Ray Freitas, Biochemist, B.A. in Biochemistry, 1974, B.S. Chemistry 1975,  
University of California, Berkeley

Bill Long, Technician

Tonya Hill B.A. Biochemistry, University of California, Berkeley,  
1978.





This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Department of Energy to the exclusion of others that may be suitable.

TECHNICAL INFORMATION DEPARTMENT  
LAWRENCE BERKELEY LABORATORY  
UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA 94720