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UNIVERSITY OF CALIFORNIA

## ENERGY & ENVIRONMENT DIVISION

Submitted to Biotechnology and Bioengineering

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ENHANCED PRODUCTION OF CELLULASE, HEMICELLULASE  
AND  $\beta$ -GLUCOSIDASE BY TRICHODERMA REESEI (RUT-C-30)

by

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Submitted to Biotechnology and Bioengineering

## SUMMARY

The production of cellulases and hemicellulases was studied with Trichoderma reesei Rut-C-30. This organism produced, together with high cellulase activities, considerable amounts of xylanases and  $\beta$ -glucosidase.

Three cellulose concentrations (1, 2.5 and 5.0%) were examined to determine the maximum levels of cellulase activity obtainable in submerged culture. Temperature and pH profiling to increase viable cell mass to maximum levels and thereby enhancing fermentor productivity at the higher substrate levels is discussed.

The effect of temperature, pH, Tween-80 concentration, carbon source and substrate concentration on the rates of mycelial growth and extra-cellular enzyme production are described.

## INTRODUCTION

The overall conversion of biomass to ethanol through the enzymatic conversion of cellulose to glucose (1,2,3) and the subsequent fermentation of the glucose syrups to ethanol has been hampered by two economic bottlenecks--the high costs associated with delignification and with enzyme production (4,5). A doubling of cellulase productivity is possible by increasing the cellulose concentration (6,7) in the medium from 2.5 to 5.0%, increasing the nitrogen concentration and controlling pH during growth (8). Because of the resulting increase of enzyme strength, culture filtrates from 2.5% cellulose cultures can reduce the hydrolysis time for practical saccharification to one half that required by

culture filtrates from 1.0% cellulose cultures.

The cellulose complex in T. reesei consists mainly of three enzymes: cellobiohydrolase, endo- $\beta$ -glucanase and  $\beta$ -glucosidase. The synthesis of each of these enzymes is subject to specific end product inhibition. The Rut-C-30 strain, developed by Montanencourt and Eveleigh (9) not only hyperproduces the cellulase, as did its parent Rut-NG-14, but is also resistant to catabolite repression.

This paper reports on batch culture studies of T. reesei Rut-C-30, with the objective of delineating optimal conditions for cellulase production.

#### MATERIALS AND METHODS

##### Inoculum

Viable cultures of Trichoderma reesei Rut-C-30 were maintained on PDA slants. The inoculum build up scheme was altered slightly from that reported previously as this scheme resulted in a faster rate of enzyme production and a decrease in foam formed during the fermentation.

A 5 day old PDA slant is incubated in 200 ml mineral salts medium [containing 1% glucose, Tween-80 (0.01%) and antifoam (0.1%)] in 500 ml Erlenmeyer flasks for 96 hours on an orbital shaker at 28°C. The culture was then transferred and incubated in 200 ml mineral salts medium [containing 1% solka floc, Tween-80 (0.01%) and antifoam (0.1%)] in 500 ml Erlenmeyer flasks for 5-6 days on an orbital shaker at 28°C. This then served as inoculum for the fermentor.

### Production Medium

A modified media was employed, based on that reported by Mandels and Weber (10). Modifications are listed in Table 1.

### Fermentation

Fermentations were carried out in a 14-liter fermentor (New Brunswick Magnaferm Model MA 114) with an operating volume of 10 liters. Temperature, pH, agitation, dissolved oxygen and foam were all controlled. The dissolved oxygen was automatically controlled at a level greater than 20% of the saturation value for the medium, by varying the agitation rates in response to changes in the dissolved oxygen tension. In most cases one-sided control was used.

### Extracellular Components

Activities are expressed in international units (I.U.), as micromoles of glucose produced per minute.

The filter paper activity, as described by Mandels, et al., (11) was measured by the release of reducing sugar produced in 60 minutes from a mixture of 1 ml diluted enzyme, 1 ml acetate buffer and 50 mg Whatman No. 1 filter paper incubated at 50°C.

Carboxymethyl cellulase (CMC'ase) was determined by the increase in reducing sugar in 30 minutes from a mixture of 0.1 ml diluted enzyme and 1 ml solution of 2.0% CMC (in acetate buffer), incubated at 50°C.

C<sub>1</sub>-activity was measured by the release of reducing sugar produced in 24 hours from a mixture of 1 ml diluted enzyme; 1 ml acetate buffer and 50 mg Red Cross absorbent cotton, incubated at 50°C.

β-glucosidase activity was measured by the release of glucose

Table 1  
Cellulase Production Medium<sup>10</sup>

Components (g/l)	Cellulose Concentration		
	10 g/l	25 g/l	50 g/l
$(\text{NH}_4)_2\text{SO}_4$	1.4	3.9	11.6
$\text{KH}_2\text{PO}_4$	2.0	2.0	3.8
$\text{MgSO}_4$	0.15	0.15	0.3
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.4	0.4	0.8
$\text{NH}_2\text{CO NH}_2^*$	0.3	0.3	0.6
Peptone	1.0	1.6	2.9
Tween-80 (ml)	0.2	0.2	0.2
Trace elements (mg/l)			
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5.0	5.0	5.0
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.6	1.6	1.6
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.4	1.4	1.4
$\text{COCl}_2$	2.0	2.0	2.0

\*Urea was deleted from the media unless otherwise mentioned.



in 15 minutes as determined by the glucose oxidase-peroxidase assay, from a mixture of 0.1 ml diluted enzyme and 1 ml of 1.25% cellobiose solution (in acetate buffer), incubated at 50°C.

#### Soluble Protein

Extracellular protein was estimated by the Lowry (12) method (without precipitation) using bovine serum albumin as the standard.

#### Dry weights

About 40 ml aliquot of the culture was filtered by suction through a tared 5 micron Nucleopore filter, washed with distilled water and dried at least overnight at 70°C. Dry weight, which included mycelium and residual cellulose, was then determined.

### RESULTS AND DISCUSSION

#### Effect of Tween 80 Concentration

Three concentrations (0.01, 0.02 and 0.1%) were studied for their effect on filter paper activity. There was a slight difference in filter paper activity with 0.01% or 0.02% Tween 80 concentrations, but when the concentration of Tween 80 was increased to 0.1%, a substantial decrease (40%) in filter paper activity results. A similar effect was observed on other extra cellulase enzyme components. The mechanism of enhancement by Tween at low concentrations is not understood but may be related to the increased permeability of the cell membrane, allowing for more rapid secretion of the enzymes which in turn leads to greater enzyme synthesis. It may also allow the excess glucose to flow out of the cell into medium (thus relieving internal inhibition) due to

alteration of the transport phenomenon across the cell membrane. Tween 80 addition (0.1%) during saccharification increased the hydrolysis rate as well as glucose accumulation by about 30%. This increase may be due to increase in the specific activity of  $\beta$ -glucosidase whereby cellobiose in the hydrolyzate is kept at a very low level.

#### Effect of pH and Temperature

It has been observed by previous workers (8) that 31°C and a pH of 4.5 for the first 48 hours and then 28°C and maintaining pH above 3.3 for the rest of the fermentation time period was optimum for cellulase production in T. reesei.

Various temperature and pH profiles were investigated with the Rut-C-30 strain to optimize growth and enzyme production. When the temperature was maintained at 31°C for two days and lowered to either 28°C or 25°C for the rest of the fermentation, little difference was noted. The pH was 4.0 for the first two days and then maintained at greater than 3.3. By increasing the pH to 5.0, higher filter paper activities were observed with the same temperature profile as above. In addition, the higher pH also increased  $\beta$ -glucosidase and  $C_1$  activities. These results are shown in Figures 1 and 2.

It was observed that  $\beta$ -glucosidase activity was strongly dependent on the temperature profile used during the growth and enzyme production phases. Various profiles were investigated; (i) maintaining the temperature constant at 25°C, (ii) maintaining temperature constant at 28°C, and (iii) temperature at 28°C for two days and then at 25°C and (iv) maintaining temperature at 31°C for nine hours and then at 25°C for the duration of the fermentation.

Of these combinations, maintaining the temperature at 25°C for the entire fermentation appeared optimal. Significant increase in  $\beta$ -glucosidase as well as  $C_x$  activity were observed.  $C_x$  increased from 40 to 82 units/ml. The results of the various temperature profiles are shown in Figures 3 and 4.

#### Effect of Urea Addition

The cellulase production by Trichoderma viride QM-9414 is reduced unless urea, in addition to ammonium sulfate and proteose peptone, is added. In both the experiments the temperature was maintained at 25°C, while pH was controlled not to fall below 4.0. The longer lag phase and low filter paper activity (Fig. 5) demonstrates the effect of urea addition. For the initial five days of fermentation,  $C_1$  and  $C_x$  were higher with urea (Fig. 6), but at the end of the fermentation  $\beta$ -glucosidase,  $C_1$  and  $C_x$  activities were nearly the same. If one is interested in producing an enzyme mixture with higher  $C_1$  and  $C_x$  activities in shorter fermentation time period (say 4 days), then addition of urea is advantageous.

#### Effect of Higher pH Levels

In all the experiments (Fig. 7) the temperature was kept at 25°C, while pH was controlled at 4, 5 and 6, respectively. Cellulase production was higher between the 2nd and 3rd day at pH 6 and was nearly constant between the 5th and 7th day at pH 5 and 6. The amount of  $\beta$ -glucosidase obtained within three days of fermentation at pH 6 was equivalent to that obtained after 8 days at pH 4 or 5. Higher levels of  $C_1$ ,  $C_x$  and soluble protein were obtained at pH 4.0. Decrease in extra cellulase activities as well as

soluble protein at the end of the fermentation at pH 6.0 may be due to the denaturation of the enzyme because of the prolonged incubation at higher pH levels.

#### Fermentation with 2.5 and 5.0 wt% Cellulose Concentrations

In both the experiments the temperature was controlled at 25°C. pH was controlled at 5.0 and  $\geq 5.0$  for 2.5 and 5.0 wt% substrate concentrations, respectively. After the third day of fermentation (Fig. 8) the rate of cellulase production for 2.5% substrate concentration was negligible as compared to 5.0%. The lower rate of cellulase production was a result of pH being controlled at 5.0. This may have controlled the release of enzyme into the fermentation broth during the process. A similar type of phenomenon (Fig. 9) was observed for  $\beta$ -glucosidase,  $C_1$ ,  $C_x$  and soluble protein.

#### Carbon Source

From a practical standpoint a cheap cellulosic substrate must be sought for cellulase production. Corn stover, wheat straw, avicel, lactose and solka floc were employed (Table II) at different concentrations. Acid and alkali treated corn stover gave much better activity than acid treated corn stover. Removal of lignin by alkali treatment may be responsible for higher activity. The reference carbon sources, solka floc, avicel or lactose, are in fact too expensive for industrial production of cellulase.

#### Xylanase Production

The results obtained suggested that cellulase in the Rut-C-30 is an inducible enzyme, the formation of which is not controlled by so-called catabolite repression. This organism produced, together with higher cellulase activities, considerable amounts of

Table II

Influence of Substrate on Cellulase Production in Submerged Culture of Rut-C-30.

Substrate (g/l)	Filter Paper Activity (u/ml)
1. Solka Floc (10)	3.1
2. Avicel (10)	2.4
3. Lactose (10)	2.9
4. Corn Stover	
a) acid-treated (10)	0.28
b) acid treated (20)	0.12
c) acid-base treated (20)	2.0
5. Rice straw	
a) acid-treated (10)	0.1
b) acid-treated (20)	0.43

xylanase. Tables III and IV give the comparison of different strains and demonstrates the superiority of Rut-C0-30 in terms of higher xylanase activity. Thus, an enzyme mixture of Rut-C-30 can conduct a simultaneous enzymatic hydrolysis of the cellulose and hemicellulose of an agricultural residue.

Table IV provides a comparison of Rut-C-30 with T. viride QM-9414. Comparing runs 2 and 3, the filter paper activities are nearly the same, but the  $\beta$ -glucosidase activity in run 2 is higher by about 9 fold.

If we compare runs #1 and 3, there is an increase of about 2.8 , 25 and 2.5 times in filter paper activity,  $\beta$ -glucosidase activity and soluble protein, respectively.

#### CONCLUSION

The biosynthesis of cellulase in a medium containing cellulose as the carbon source is greatly influenced by the overall profile of the batch fermentation. Complex interaction exists between the medium composition, the starting pH, the inoculum size and condition, and aeration capacity. The formation of foam is one of the recurring problems in carrying out fermentations. Considerable loss of volume can also occur with a resultant change in the agitation pattern of the fermentation. The modified inoculum buildup scheme resulted in the faster rate of enzyme production and decrease of foam during the fermentation. The various disadvantages of uncontrolled foam formation, which is a main consideration in the scale-up of a fermentation, were resolved.

The initial lag is common to all fermentations and can be controlled by the inoculum age and size. The initial rapid growth

Table III

## Comparison of Xylanase Producing Strains

Run #	Substrate Concentration (g/l)	Strain	Xylanase Activity	Remarks
1	10 (Solka Floc)	Rut-C-30	114 I.U./ml	pH > 5.0 Temp = 25°C Proteose peptone = 1.0 g/l Produced 90 I.U./ml xylanase activity when glucose instead of cellulose was used as a substrate.
2	10 (Larchwood Xylan)	<u>Streptomyces</u> <u>xylophagus</u>	8.8 IU/ml	pH = 7.4 Temp. = 30°C Bacto-peptone = 3 g/l
3	10 (Larchwood xylan)	<u>Chaetomium</u> <u>trilaterale</u>	6.6 I.U./ml	pH = 7.0 Temp = 28°C Yeast Extract = 1 g/l.

Table IV  
Comparison of Rut-C-30 and QM-9414

Run #	S <sub>0</sub> (gh)	Strain	FPA U/ml	B-Glucosidase U/ml	Soluble Protein mg/ml	Remarks
1	50	Rut-C-30	14.4	26	20	pH=5.0, T-80 level= 0.02%, 25°C
2	25	Rut-C-30	5.2	10	8.2	pH = 5.0, T-80 level = 0.2%, 25°C
3	50	QM-9414	5.1	1.0	12.7	(0-1 Day) pH Allowed to fall to 4  (1-2 Day) pH Allowed to fall to 2.9  (2 Day) raised to 3.3 and controlled not to go below pH 3.3.
4	25	QM-9414	4.3	1.2	5.9	same as above



rate is due to proteins in the peptone which are more available than cellulose. The ratio of carbon to nitrogen addition, and the form in which nitrogen is supplied is of special significance. Ammonium sulphate and proteose peptone are the best nitrogen sources, for they increase the enzyme activity and cell mass, respectively, and reduce the generation time. Addition of urea to the media resulted in the decrease of extracellular enzyme activities except for  $C_1$  activity.

For maximum production of cellulase and  $\beta$ -glucosidase, pH 5 and temperature of 25°C were optimum. Higher temperature (31°C) for initial fermentation time period (18 hours) could substantially increase the  $\beta$ -glucosidase activity in the system. pH 6 can produce as much of  $\beta$ -glucosidase in 3 days as one can obtain in 8 days at pH of 4 or 5. With the help of environmental control it is possible to produce enzyme mixture with different ratios of FPA,  $C_1$ ,  $C_x$ ,  $\beta$ -glucosidase and xylanase.

Economic factors in the assessment of various cellulosic substances as chemical and energy resources are many and complex. No substrate nor conversion process can be singled out as significantly advantageous.

#### Acknowledgement

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Figure Captions

Figure 1 Effect of initial higher temperature on growth and cellulase production.

-O-O-	pH controlled at 4 up to 48 hours decreased to 3.3 after 48 hours and was controlled at $\geq 3.3$	{ 31°C 0-2Days (D) 28°C 2-8 D
-□-□-	Same as above	{ 31°C 0-2 D 25°C 2-8 D
-①-①-	Controlled at $\geq 4.0$	{ 31°C 0-2 D
-②-②-	Controlled at $\geq 5.0$	{ 25°C 2-8 D

Figure 2 Effect of initial higher temperature on extracellular enzymes and soluble protein.

-O-O- ; -O-①- As in Figure 1.  
-□-□- ; -□-②-

Figure 3 Effect of higher incubation temperature on growth and cellulase production in cellulose cultures.

-O-O-	} Controlled at $\geq 5.0$	25°C 0-8 D
-□-□-		{ 31°C 0-9 hours (H) 25°C Rest of the fermentation time (RT)
-O-①-		28°C 0-8 D
-③-③-		{ 28°C 0-2 D 2 °C 2-8 D

Figure 4 Effect of higher incubation temperature on extracellular enzymes and soluble protein.

-O-O-; -①-①-; -□-□- ; -③-③- As in Figure 3



XBL 805-5203

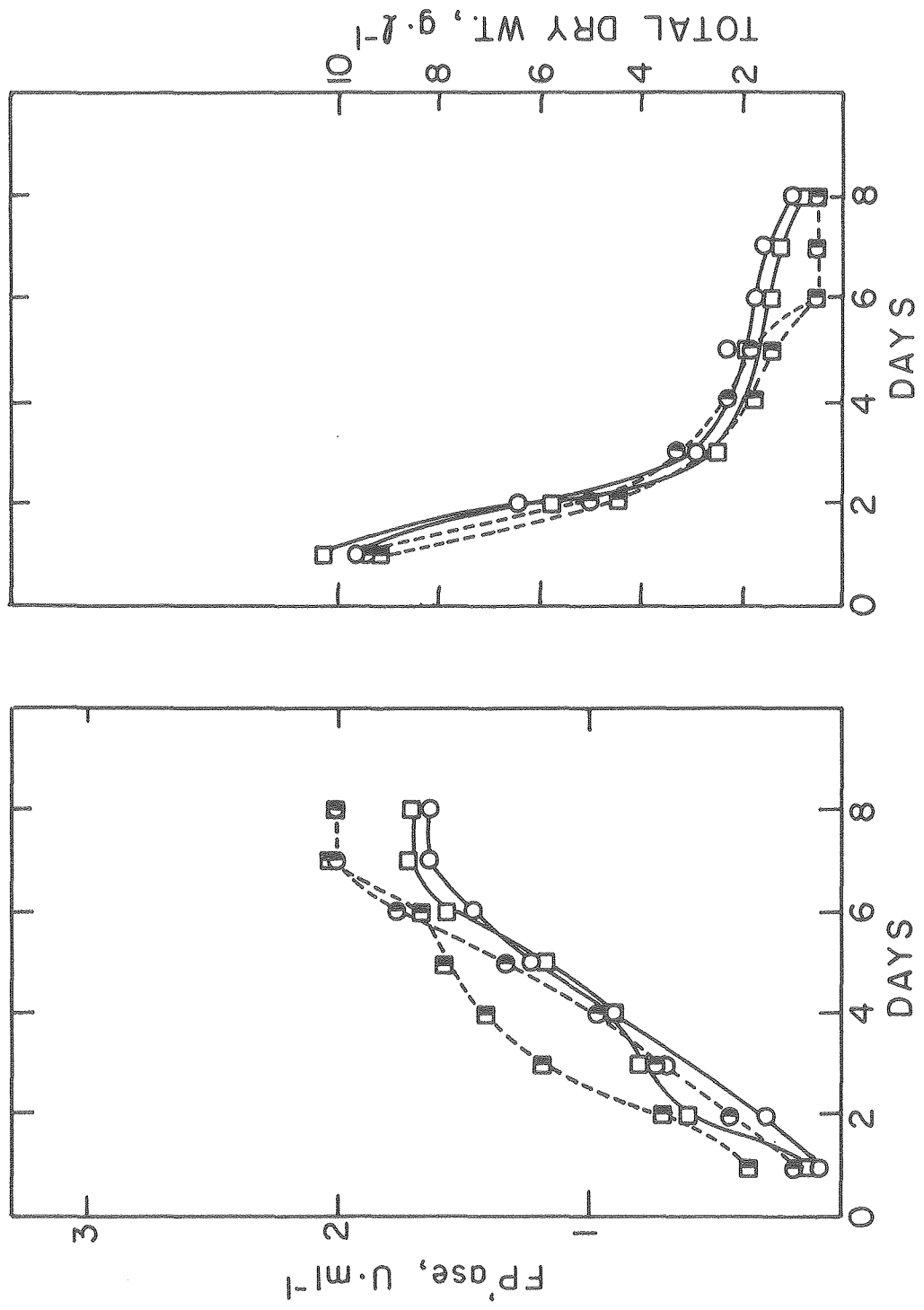


Figure 1.

XBL805-5204

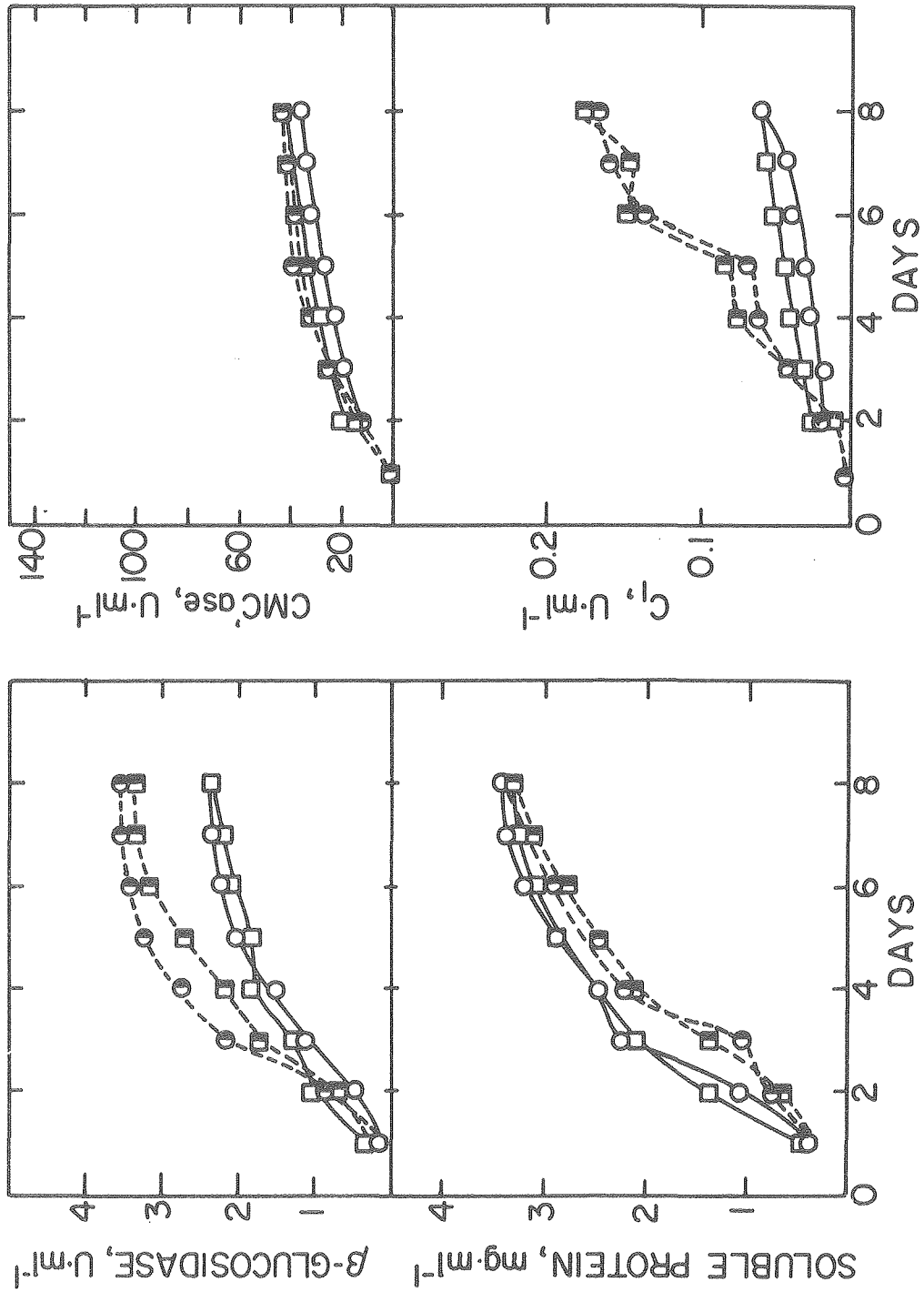


Figure 2.

XBL 805-5205

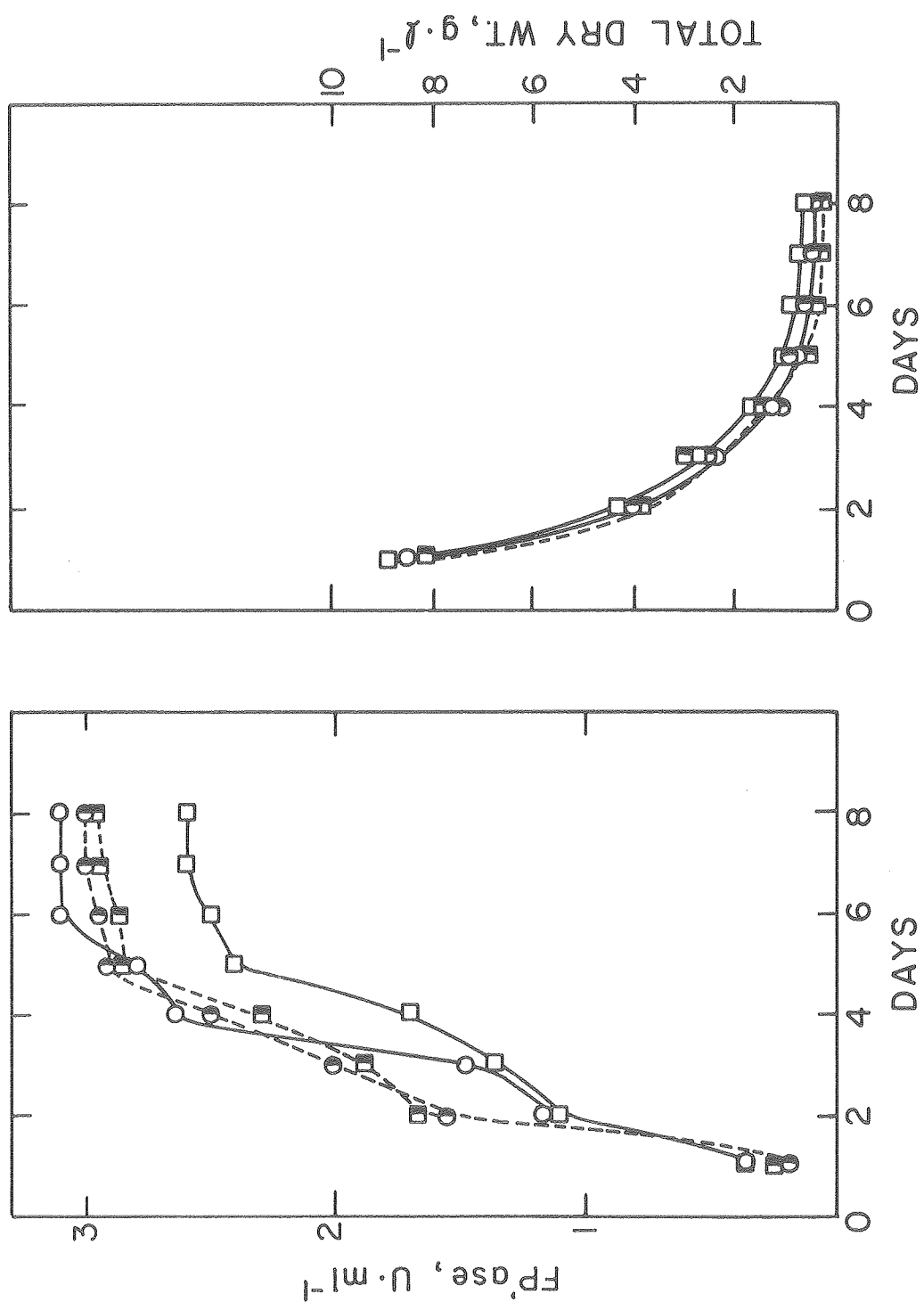


Figure 3.



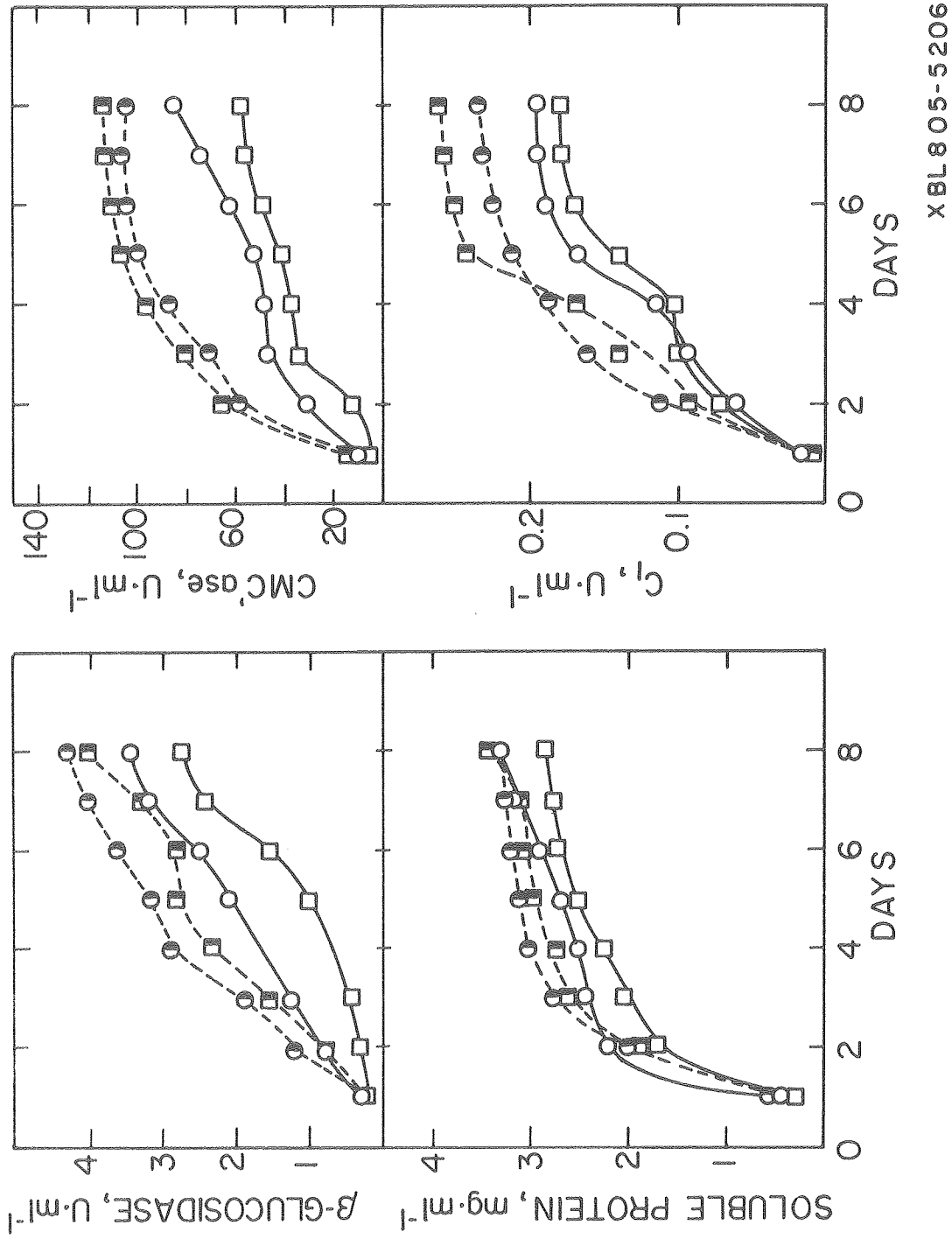


Figure 4

XBL 805-5207

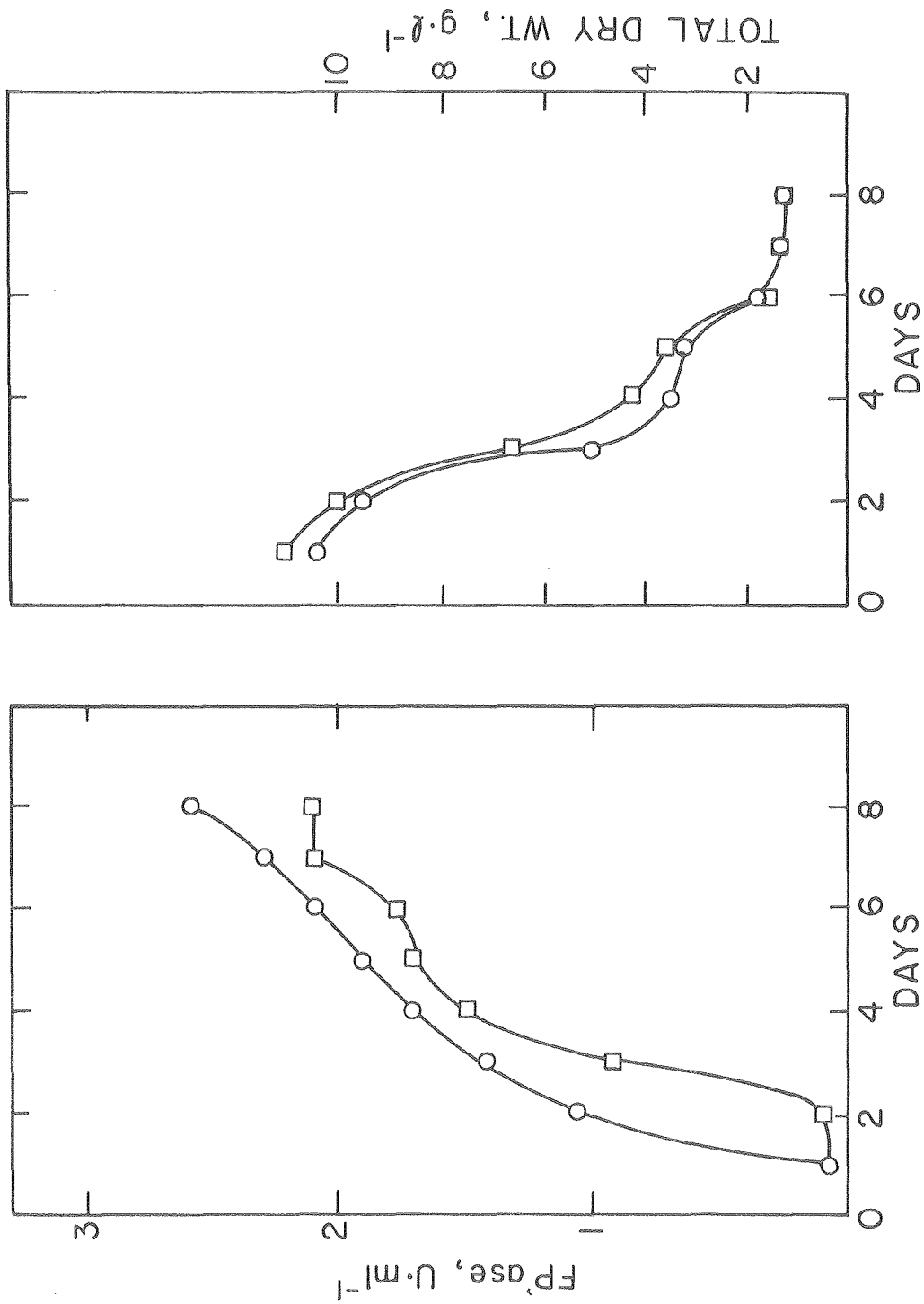


Figure 5

XBL 805-5208

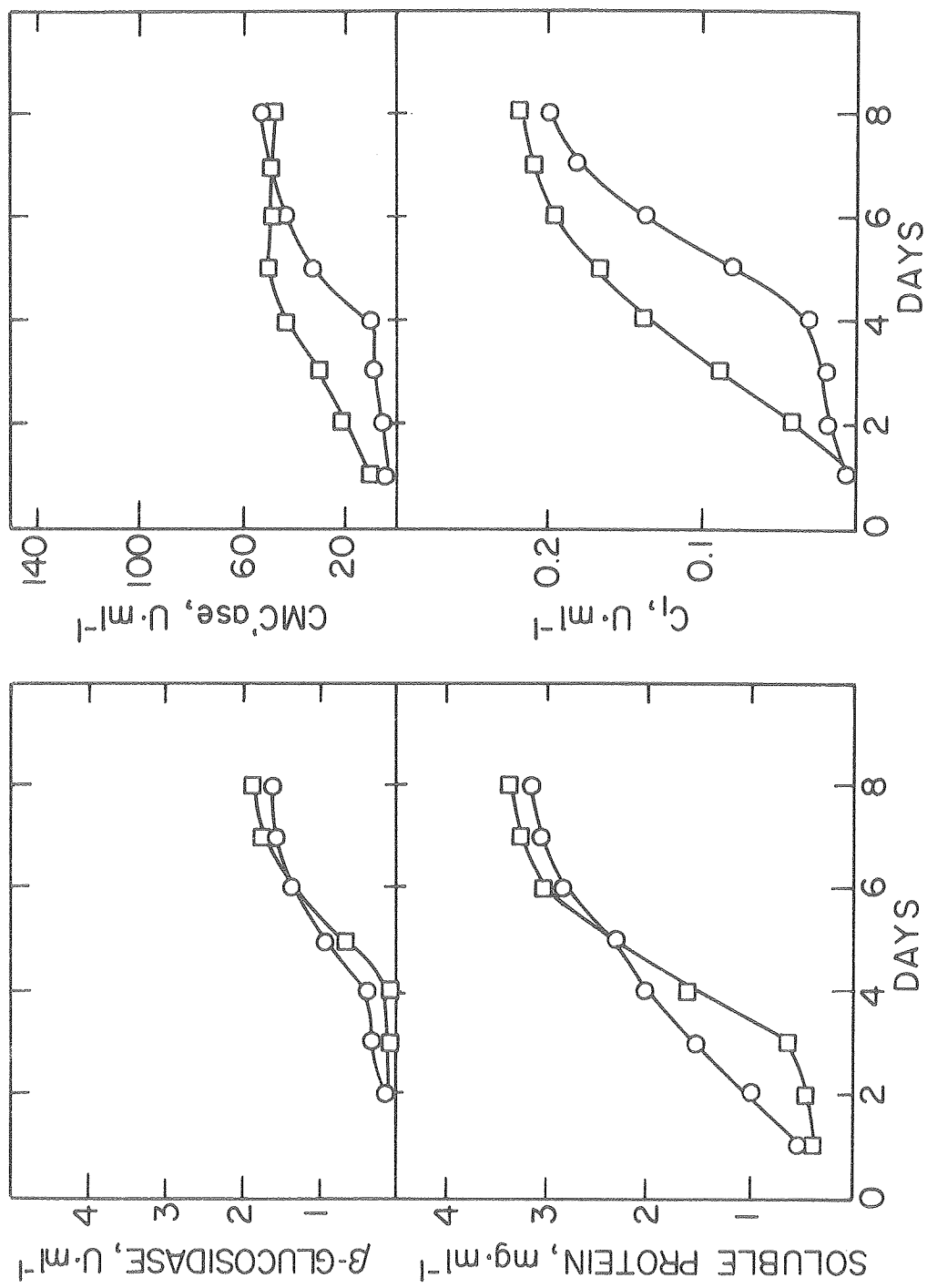
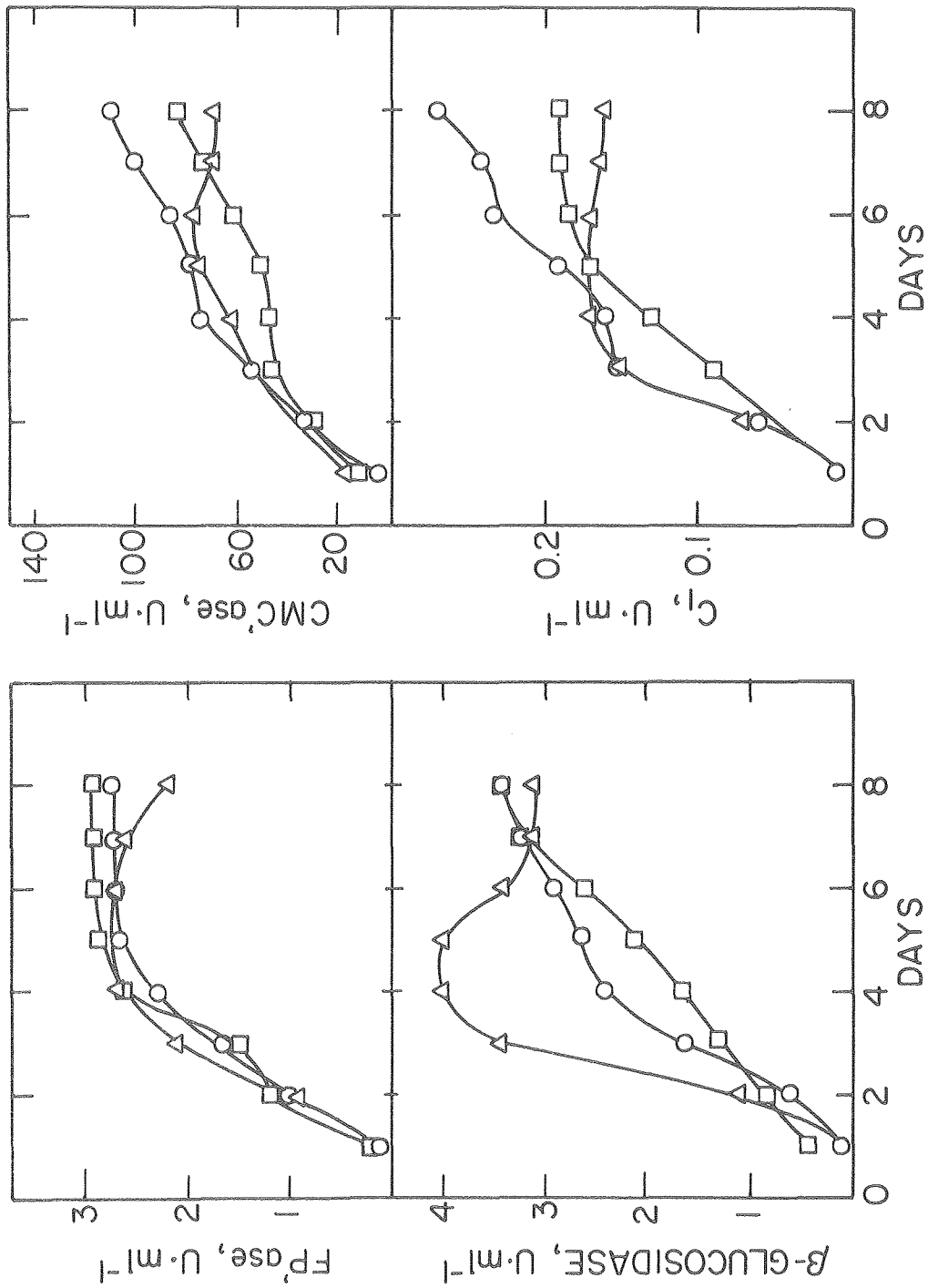
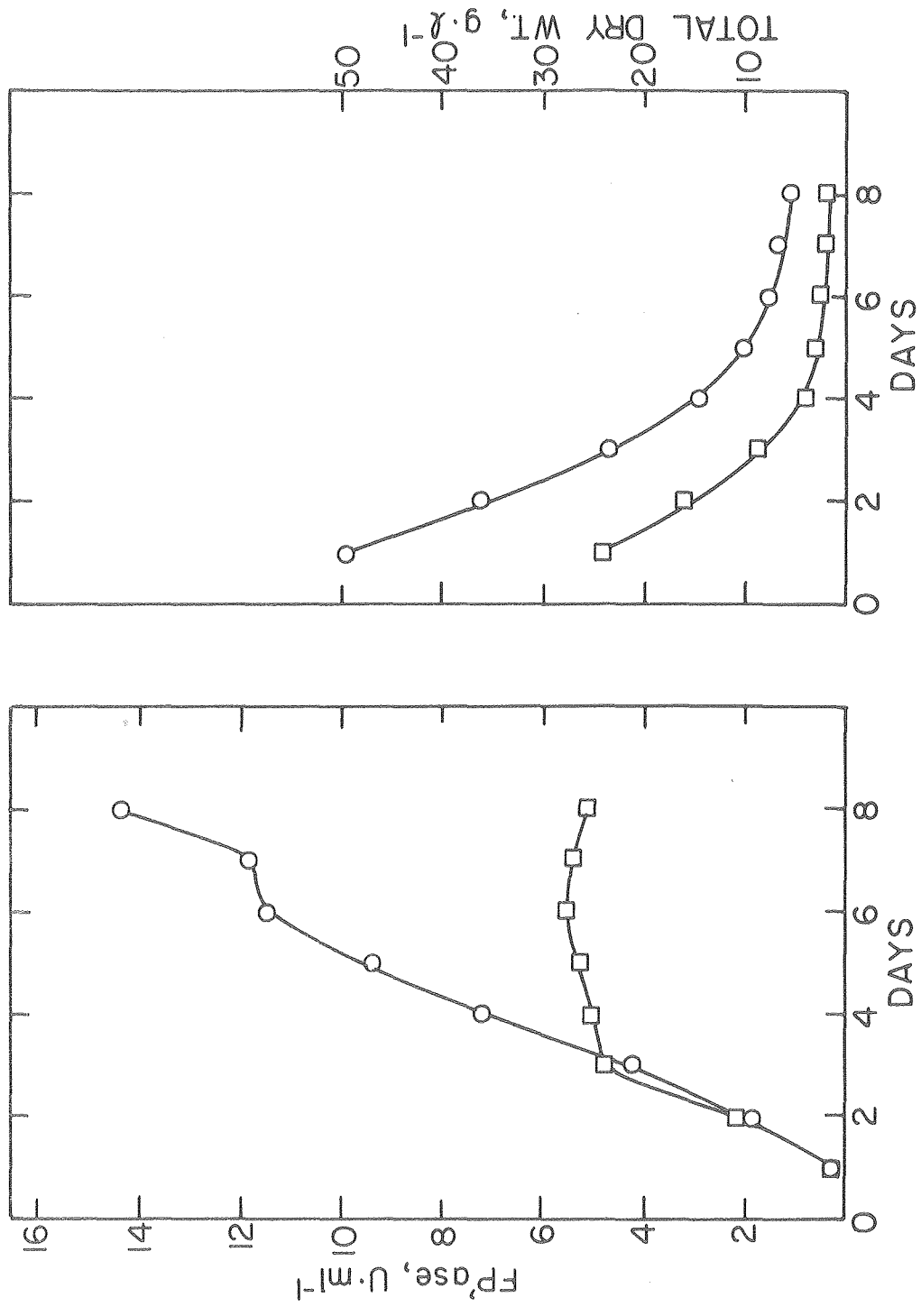


Figure 6



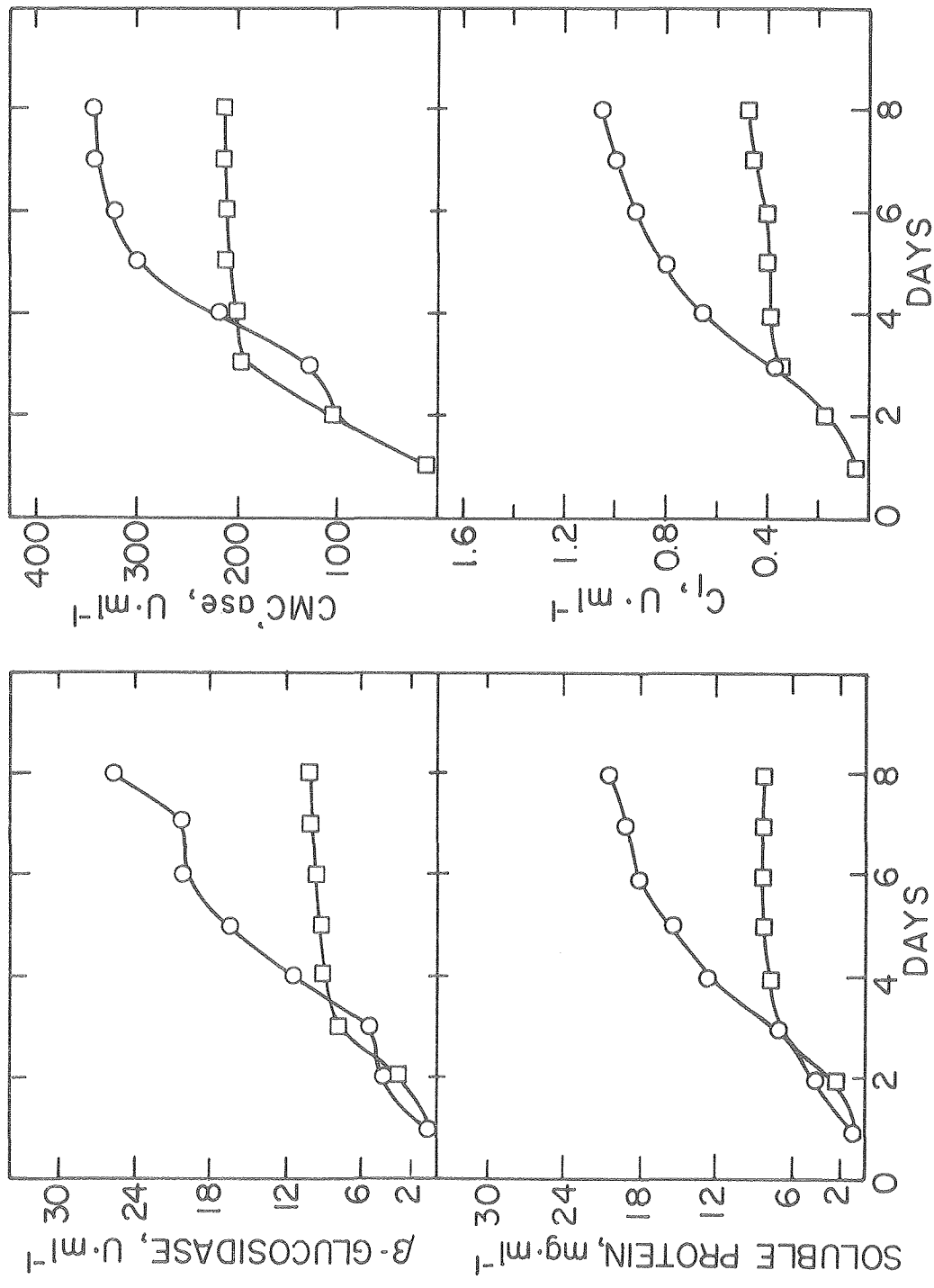
XBL 805-5209

Figure 7.



XBL 805-5210

Figure 8.



XBL805-5211

Figure 9.

