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CONTINUOUS CELLULASE PRODUCTION

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CONTINUOUS CELLULASE PRODUCTION*

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SUMMARY

Trichoderma viride QM 9414 growth characteristics on soluble sugars was investigated in single stage C.S.T.R. operation and growth parameters μ_{max}, K_s, Qo_2 identified. Two stage continuous operation with the second stage primarily utilized for enzyme induction yielded results in general agreement to theoretical predictions. Compared to reported values for single stage operation significant increase in enzyme productivity was obtained.

*Work performed under the auspices of the U. S. Atomic Energy Commission.

INTRODUCTION

Cellulase obtained from fungus Trichoderma viride is generally regarded[1,2,3] to be the most suitable for enzymatic hydrolysis of waste cellulosic materials. Several reports[4,5,6] have been published in recent years investigating the hydrolysis step in great detail but little quantitative work has appeared for the production of the enzyme in a continuous submerged culture fermentation. Mandels and Weber's[1] semicontinuous operation with insoluble substrate utilizing Trichoderma viride QM 6a is probably the only report in this area.

The present investigation looks into the growth of the fungus and production of the cellulase enzyme in greater detail. A two stage C.S.T.R. operation with the first stage utilizing soluble sugars for growth only and the second stage utilizing pure spruce wood cellulose for enzyme induction was investigated. The pertinent factors identified should prove useful to the general overall technological problem of economically converting waste cellulosic materials to reducing sugar solutions.

MATERIAL AND METHODS

Organism

All experiments were conducted with Trichoderma viride QM 9414 obtained from Army Natick Laboratories at Massachusetts. This is a mutant strain obtained by irradiating condia of Trichoderma viride QM 6a with a linear accelerator.[7] The nutrients used for growth were the same as reported by Mendels and Weber.[1]

Assay Procedures

Reducing sugar was assayed by Dinitrosalicyclic acid method proposed by Sumner and Somers[8] and by the modified Somogyi method.[9] Cellulase was chemically

estimated utilizing suggested methods of Viles and Silverman[10] as modified by Updegraff.[11] Protein was assayed by the modified Biuret method as suggested by Koch and Putnam.[12] Enzyme activities were measured according to Mandels and Weber.[1]

Qo_2 Measurement

During exponential growth on soluble sugars specific respiration rate was estimated by dynamic measurement of dissolved oxygen concentration with a fast response dissolved oxygen probe according to the method suggested by Bandyopadhyay and Humphrey.[13]

C.S.T.R. Operation

New Brunswick Scientific Company 14 liter culture vessels were utilized for C.S.T.R. operations. Operating volume was kept between 5 and 10 liters. Five gallon glass carboys were utilized as feed tanks, the liquid being delivered to the fermentor vessel by a variable speed Masterflex pump (Cole Parmer, Chicago). Constant volume of the fermentor liquid was maintained by a variable length outlet pipe attached to the house vacuum (17" Hg) through the product receptacle. Temperature was maintained at 30°C by circulating water through the hollow baffles of the fermentor. Aeration at all times was kept at 0.5 V.V.M. Oxygen concentration in the liquid was monitored by a New Brunswick membrane bound dissolved oxygen probe.

For the two-stage operation pure spruce wood cellulase (Solka Floc, Brown Company, Providence, R. I.) was added continuously to the second stage by a low rate volumetric disc feeder (B.I.F. Omega model 22-01, General Signal Corporation, Providence, R. I.). pH of the second stage was maintained at 4.80 by Leeds Northrup pH electrodes attached to a Beckman model 900 pH

controller-analyzer (On-Off type) coupled to acid/base peristaltic pumps. The schematic arrangement for the two stage operation is shown in Fig. (1).

RESULTS AND DISCUSSIONS

Three sets of experiments were carried out to determine the specific oxygen demand, Qo_2, during exponential batch growth of the fungus on 1% glucose at 30°C. The rate of depletion of oxygen with time after air turn off at three different biomass concentrations is shown in Fig. (2). The rate of oxygen consumption for these three cases is shown in Table I. Based on the experimental values of Qo_2 and the cell yield constant, $Y_{x/s} = 0.43$, it can be shown by calculation that for C.S.T.R. runs on 1% glucose the maximum oxygen utilization is only 1.7% of that entering. Hence cell growth was not limited by aeration.

C.S.T.R. runs for growth of the fungus were made with 0.5% and 1% glucose as carbon source. Steady state was achieved within two fermentor residence times. pH of the effluent fluid was between 3.20 and 3.32. Single stage C.S.T.R. results are shown in Fig. (3). A maximum cell productivity of $0.47 \frac{mg}{ml \times hr}$ and $0.92 \frac{mg}{ml \times hr}$ were obtained at a dilution rate of $0.21\ hr^{-1}$ with inlet sugar concentrations of 0.5% and 1.0%, respectively.

Variations of biomass (X) and substrate concentration (S) with time were measured in the unsteady state. The specific growth rate μ was computed from the equation:

$$X_1 = X_1^1 e^{(\mu - D_1)t}, \text{ provided at } t = 0,\ D_1 \neq \mu$$

with boundary conditions at $t = 0,\ X = X_1^1$

$$t = t,\ X = X_1$$

where: μ is the specific growth rate

t is the elapsed time

X is the fungal biomass

S is the substrate concentration

D_1 is the dilution rate

Figure (4) presents the relationship between specific growth rate and substrate (glucose) concentration directly as a Lineweaver-Burke plot. The corresponding Monod equation constants are $\mu_{max} = 0.294\ hr^{-1}$ and $K_s = 0.083$ mg/ml.

For two stage operation the first stage was operated as described above for maximum biomass productivity. The second stage was operated with a liquid volume of 8 liters at pH 4.80. Enzyme activity with varying dilution rates for the two inlet biomass concentrations (corresponding to 0.5% and 1% glucose concentrations as the growth substrate in the first stage) is shown in Figure (5). Cellulose concentration was maintained at 0.5% and 1%, respectively, for these two cases. Increase in inlet biomass concentration from 2.20 mg/ml to 4.40 mg/ml resulted in approximately doubling the enzyme activity. Increasing dilution rates beyond $0.02\ hr^{-1}$ resulted in washing out of the enzyme activity. Dilution rates below $0.02\ hr^{-1}$, however, resulted in a slow progressive decrease in enzyme activity. To determine the reason for these observations samples from the second stage were assayed for insoluble protein content and one such set of readings for 2.2 mg/ml inlet biomass concentration to the second stage is included in Fig. (5). Insoluble protein corresponding to the 2.2 mg/ml biomass out of the first stage was 0.72 mg/ml (31.3% of fungal dry weight). In the second stage at dilution rates below $0.02\ hr^{-1}$ the insoluble protein decreased gradually from 0.72 mg/ml at $D_2 = 0.02\ hr^{-1}$ to 0.45 mg/ml at $D_2 = 0.0125\ hr^{-1}$.

Fungal biomass grown in presence of glucose shows a sizable lag for cellulase induction in presence of pure spruce wood cellulose (Solka Floc). Batch experiments of Rosenbluth and Wilke[14] placed this lag around 30 hours for T. viride QM 6a. Herbert's analysis[15] may be slightly modified for the present two-stage operation where the second stage is primarily utilized for enzyme induction. From the residence time distribution in the induction stage it is assumed that only that fraction of biomass with average residence time greater than the induction lag succeeds in excreting cellulase.

Let $Z \rightarrow$ concentration of the inducer

$X_1 \rightarrow$ biomass concentration

$P \rightarrow$ enzyme activity

$t \rightarrow$ elapsed time

$z \rightarrow$ fraction of biomass residing in the induction stage for a residence time greater than the induction lag

The kinetics of enzyme induction in the second stage is approximated by a Michaelis-Menten type relation:

$$\frac{dP}{dt} = Kx_1 z \frac{Z}{K_z + Z} \qquad (1)$$

where

K_z is the Michaelis Constant

K is a metabolic coefficient with units of $\frac{\text{enzyme activity}}{\text{mg biomass} \times \text{hr}}$

for

$K_z << Z$, Eq. (1) reduces to

$$\frac{dP}{dt} = Kx_1 z \tag{2}$$

if

D_2 is the dilution rate in the second stage

$$\frac{dP}{dt} = -D_2 P + Kx_1 z \tag{3}$$

At steady state Eq. (3) yields

$$P = \frac{Kx_1 z}{D_2} \tag{4}$$

For cell growth in the first stage we have

$$X_1 \simeq Y S_o \text{ (for total exhaustion of growth substrate)} \tag{5}$$

where

$S_o \rightarrow$ inlet growth substrate concentration to the first stage

$Y \rightarrow$ yield constant $\frac{\Delta X}{\Delta S}$ for growth

Hence from Eqs. (4) and (5) we obtain

$$P = \frac{K Y S_o z}{D_2} \tag{6}$$

The present set of data for the second stage is explained for incoming biomass of 2.2 mg/ml in terms of Eq. (6). Insoluble protein from the effluents of the second stage is a measure of viability of the fungal biomass and hence from the experimental results the effective viable biomass in the second stage (fungal protein = 31.3% of fungal biomass, dry basis) is calculated. This is

equivalent to the term $Y S_o$ in Eq. (6). The induction stage was subsequently divided into two equal parts and the enzyme activity comparison for these two cases is shown in Table II. Residence time distribution of equal volume multistage stirred tanks in series has been presented by McMullin and Weber.[16] Equation (6) can be utilized to obtain

$$\frac{(D_2P)_{\text{two stage induction}}}{(D_2P)_{\text{single stage induction}}} = \frac{z_{\text{two stage}}}{z_{\text{single stage}}} \qquad (7)$$

The left-hand side of the Eq. (7) is evaluated from the experimental data presented in Table II and is equal to 1.19. For overall residence time of 50 hours ($D_2 = 0.02\ hr^{-1}$) and an induction lag of 30 hours[14] the right-hand side of Eq. (7) is evaluated according to the analysis of McMullin and Weber[16] and it is equal to 1.21. Hence, the experimental observations give a fairly close corroboration of the theoretical analysis.

The enzyme productivity of the present mode of operation (one growth stage followed by two induction stages) utilizing 1% glucose for growth and 1% pure cellulose for induction achieved a maximum value of $32.5 \times 10^{-3} \frac{\text{Filter Paper Units}}{\text{ml} \times \text{hr}}$. The corresponding single stage semicontinuous operation results with T. viride QM 6a calculated from the published data of Mandels and Weber[1] was $5.39 \times 10^{-3} \frac{\text{Filter Paper Units}}{\text{ml} \times \text{hr}}$.

NOMENCLATURE

D_1 dilution rate in the first stage, hr^{-1}

D_2 dilution rate in the second stage, hr^{-1}

K metabolic coefficient, $\frac{\text{enzyme activity}}{\text{mg biomass} \times \text{hr}}$

K_s saturation constant, mg/ml

K_z Michaelis constant, mg/ml

P enzyme activity, filter paper units/ml

Qo_2 specific oxygen demand, $\frac{\text{millimoles oxygen}}{\text{gm dry wt} \times \text{hr}}$

S_1 substrate concentration in the growth stage, mg/ml

S_o inlet substrate concentration to the growth stage, mg/ml

t elapsed time, hr

X_1 biomass concentration in growth stage at time t, mg/ml

X_1^1 biomass concentration in growth stage at time t^1, mg/ml

Y yield constant for growth $\frac{\Delta X}{\Delta S}$

z fraction of biomass residing in the induction stages for a time greater than the induction lag

Z inducer concentration, $\frac{\text{mg}}{\text{ml}}$

Greek letters

μ specific growth rate, hr^{-1}

μ_{max} Max^m specific growth rate, hr^{-1}

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Table I. Specific oxygen demand, Qo_2, for exponentially growing T. viride.

Biomass Concentration (gm/l)	Rate of O_2 Consumption $\frac{\text{millimoles}}{\text{liter} \times \text{min}}$	Qo_2 $\frac{\text{millimoles } O_2}{\text{gm dry wt} \times \text{hr}}$
0.99	1.70×10^{-2}	1.03
2.05	3.42×10^{-2}	1.0
2.81	5.15×10^{-2}	1.1

Table II. Comparison of enzyme productivity between single stage and two stage induction.

Biomass (mg/ml)	Enzyme Activity (Filter paper units/ml) with overall $D_2 = 0.02\ hr^{-1}$	
	Single Stage	Two Stage
2.20	0.70	0.83

FIGURE CAPTIONS

Fig. 1. Schematic arrangement of the 2-stage C.S.T.R. operation. Controls and accessory equipments not shown for simplicity.

Fig. 2. Dissolved oxygen decrease with time after air turn off for exponentially growing T. viride QM 9414 on 1% glucose at 30°C.

Fig. 3. Single stage continuous culture operation. D_1 → dilution rate, S_1 → outlet sugar concentration, X_1 → outlet fungal biomass concentration.

Fig. 4. Unsteady state observations, S → substrate concentration, μ → specific growth rate, μ_{max} → max^m specific growth rate, K_s → saturation constant.

Fig. 5. C.S.T.R. operation of the induction stage. D_2 → overall dilution rate of the induction stage.

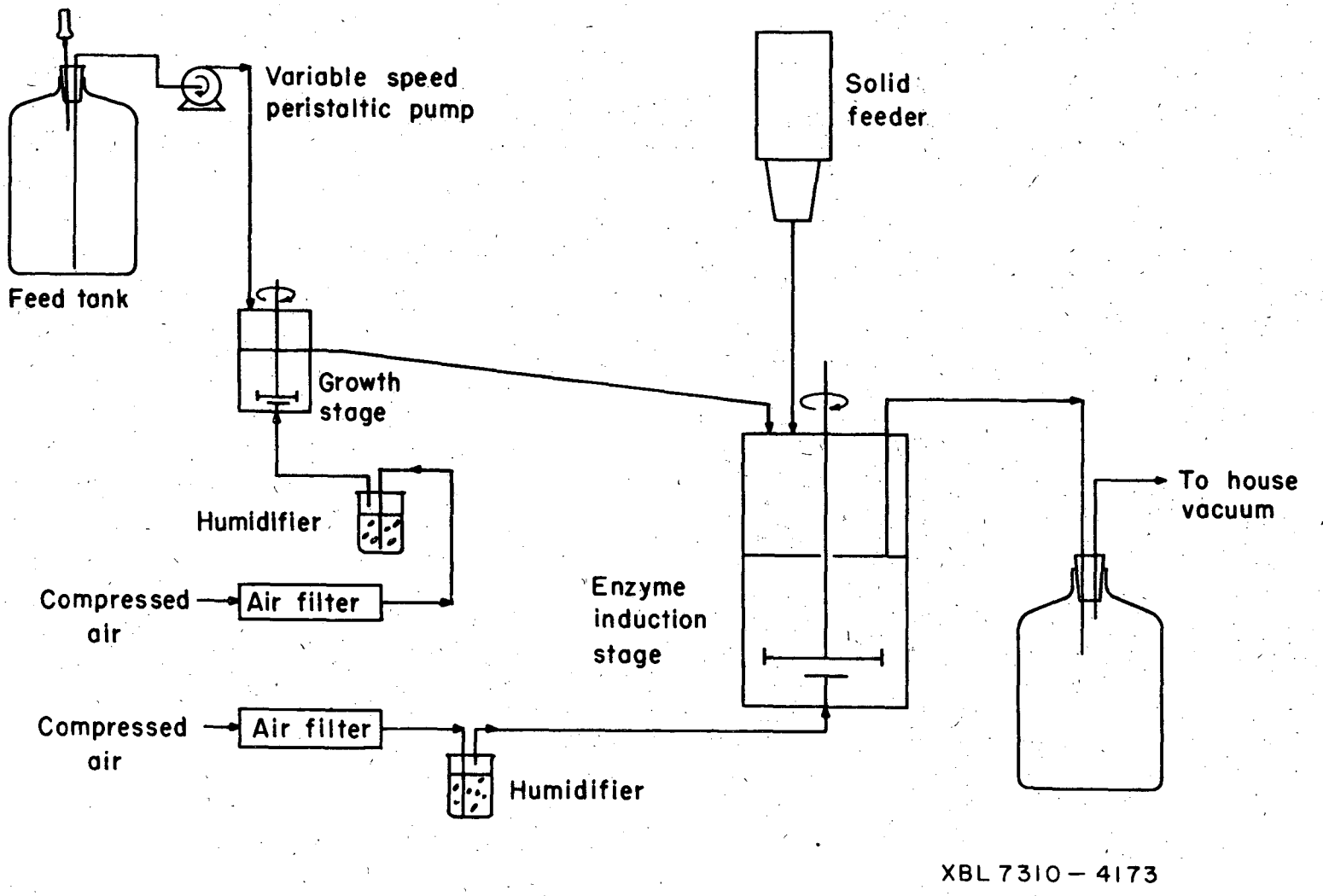

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Fig. 1.

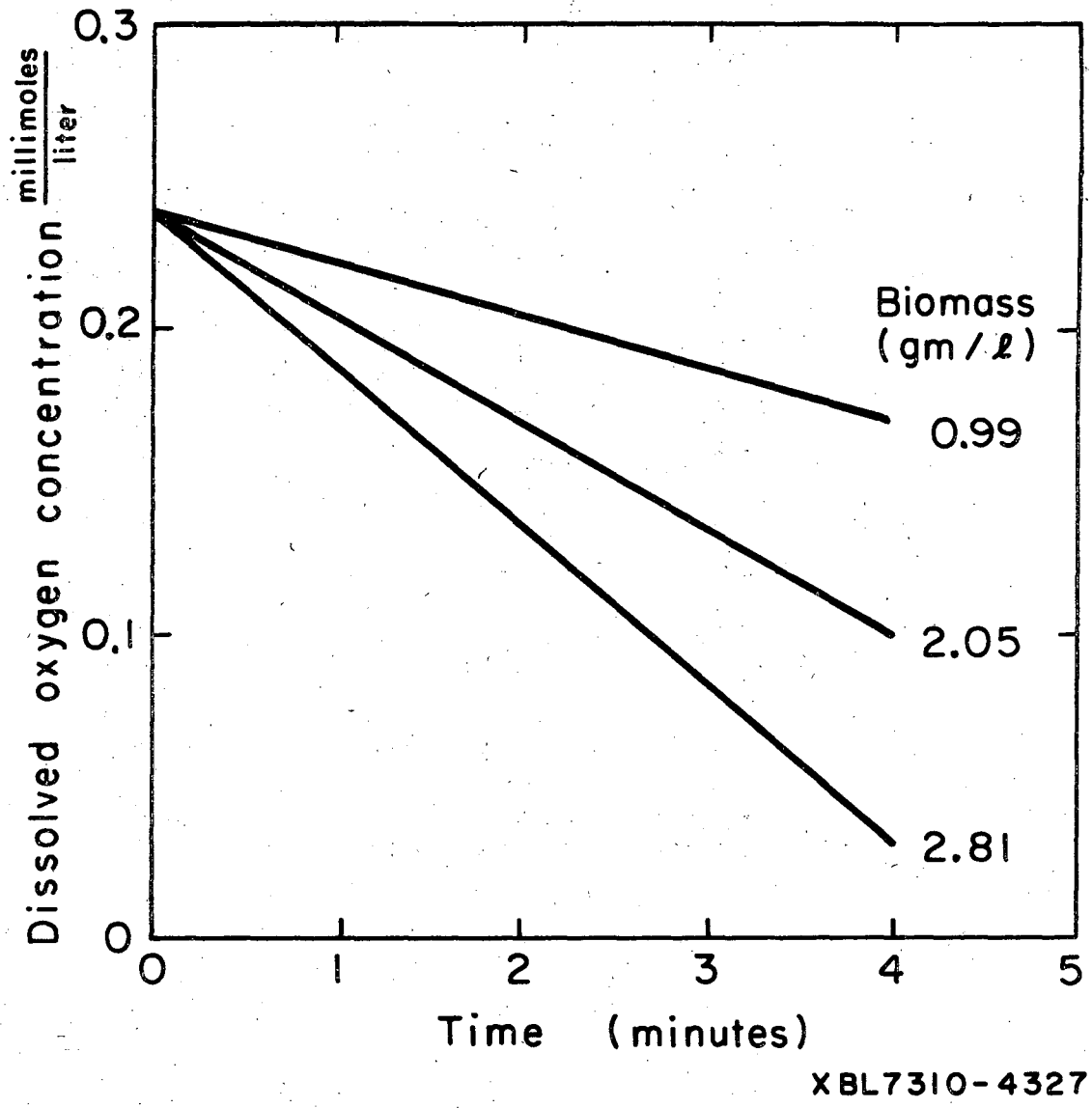
0.3
0.2
0.1
0
Dissolved oxygen concentration millimoles / liter
Biomass (gm / ℓ)
0.99
2.05
2.81
0
1
2
3
4
5
Time (minutes)
XBL7310-4327

Fig. 2

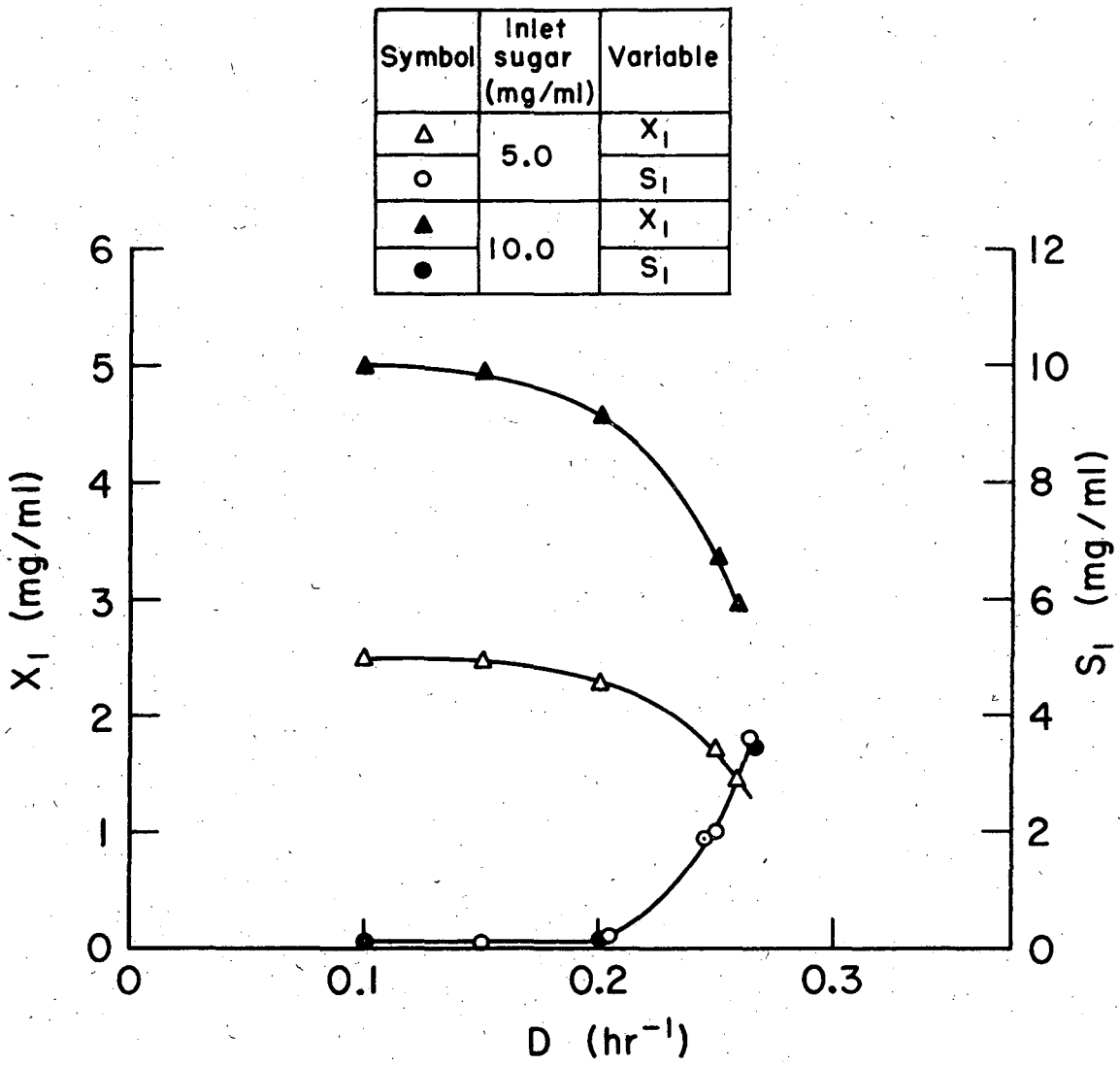
Symbol
Inlet sugar (mg/ml)
Variable
5.0
X_1
S_1
10.0
X_1
S_1
X_1 (mg/ml)
S_1 (mg/ml)
D (hr^{-1})
0
0.1
0.2
0.3
1
2
3
4
5
6
8
10
12
XBL7310-4169

Fig. 3

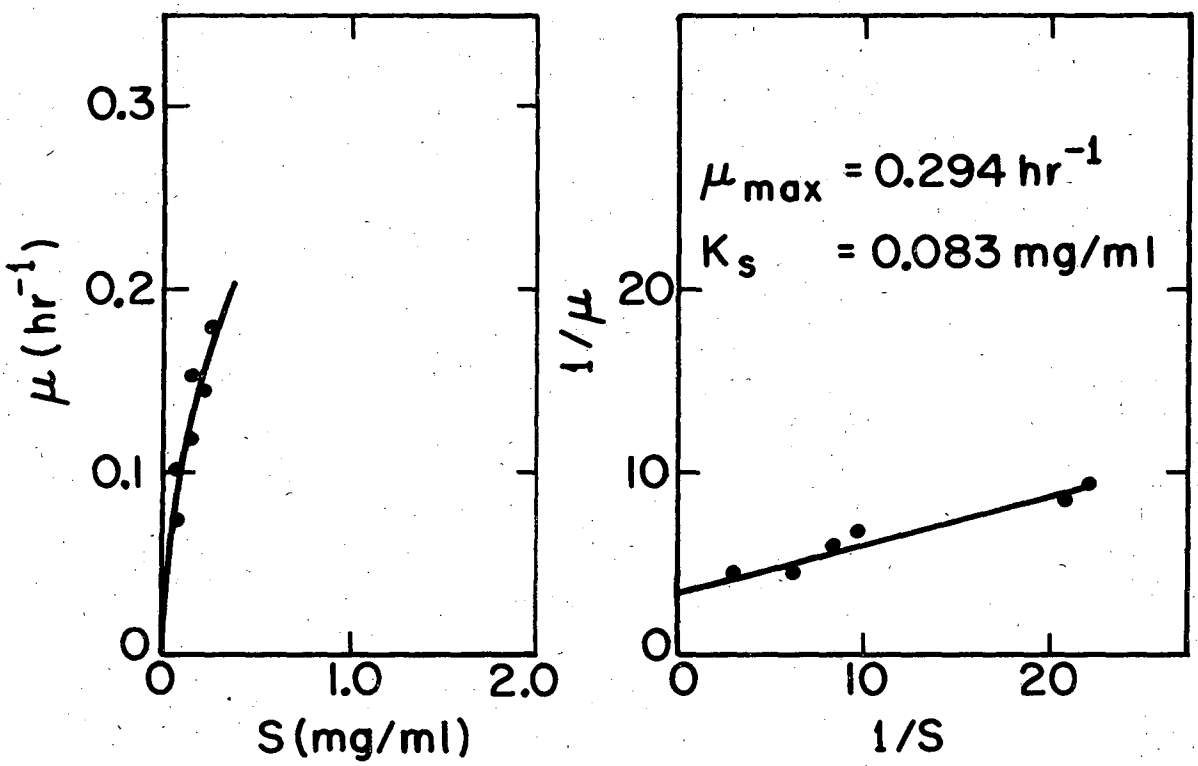
0.3
0.2
0.1
0
μ (hr⁻¹)
0
1.0
2.0
S (mg/ml)
μmax = 0.294 hr⁻¹
Ks = 0.083 mg/ml
1/μ
20
10
0
0
10
20
1/S
XBL7310-4172

Fig. 4

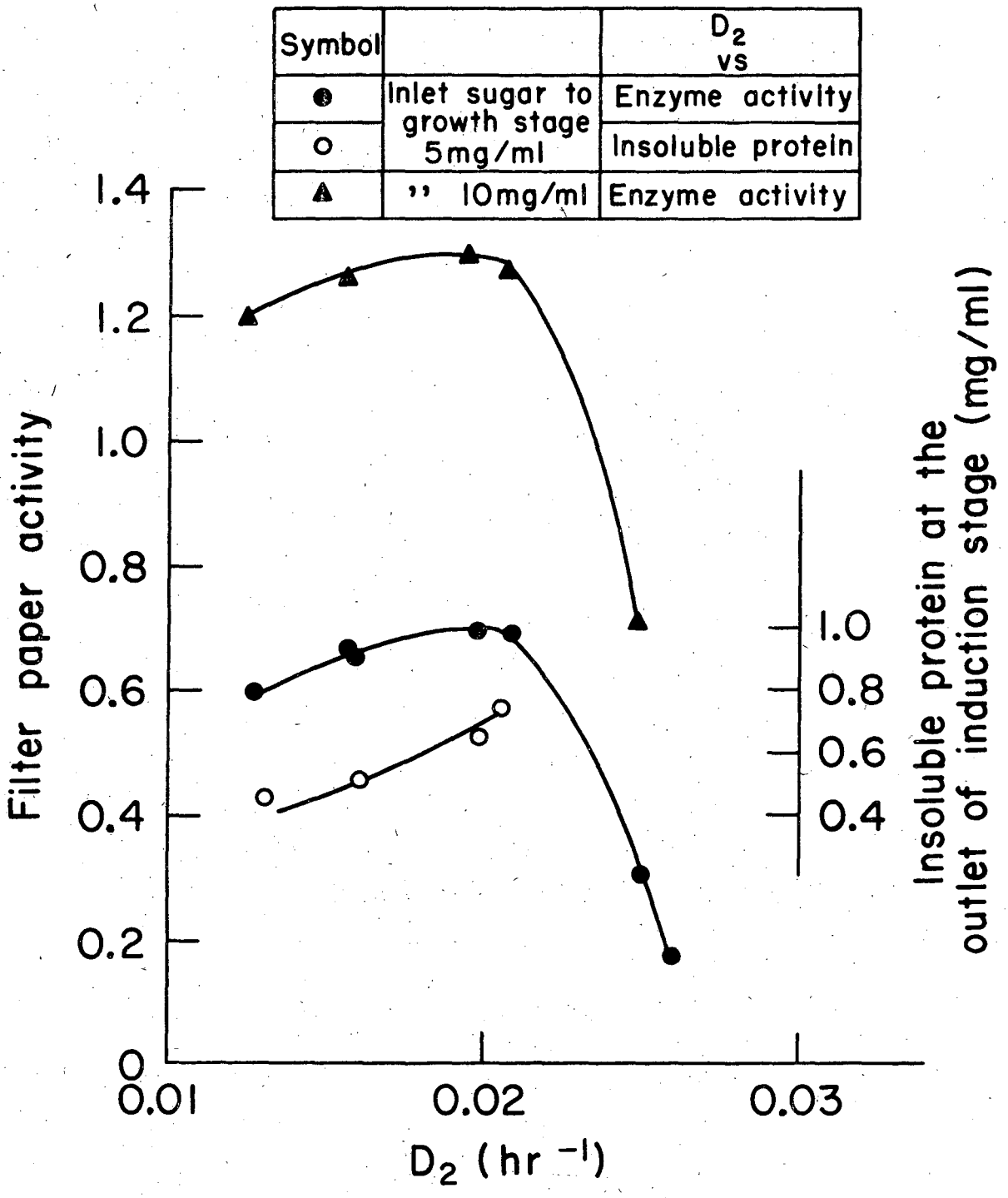
Symbol
D2 vs
Inlet sugar to growth stage 5mg/ml
Enzyme activity
Insoluble protein
" 10mg/ml
Enzyme activity
Filter paper activity
Insoluble protein at the outlet of induction stage (mg/ml)
D2 (hr -1)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.01
0.02
0.03
XBL 7310-4168

Fig. 5

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