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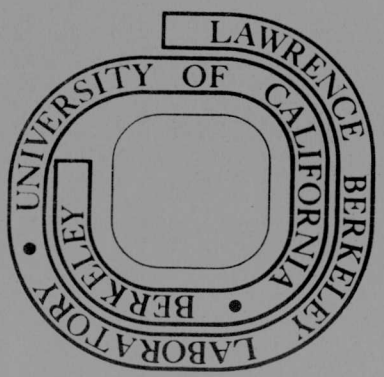

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ETHANOL EFFECTS ON THE KINETICS OF A CONTINUOUS FERMENTATION WITH SACCHAROMYCES CEREVISIAE

by

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Abstract

Alcoholic fermentation by Saccharomyces cerevisiae ATCC 4126 was studied. The aim of this work was to find the effect of the product concentration (ethanol) on the specific rates of cell growth and alcohol production. The experiments were conducted in a continuous culture chemostat where glucose was a limiting substrate, and the data were always obtained at steady state. Some preliminary batch experiments were also done. A Monod type kinetic model was applicable.

A noncompetitive type of product inhibition was found. The data showed that at an ethanol concentration above 93 g/ℓ the cells neither grow nor produce ethanol.

Empirical equations are proposed to represent the inhibitory effect studied. Ethanol concentration, P, vs specific growth and ethanol production rates μ and ν may be correlated in the following form: $\mu_{max} = \mu_o - \frac{aP}{b - P}$ and $\nu_{max} = \nu_o - \frac{a'P}{b' - P}$, where a, a', b

and b' are empirical constants.

A comparison with previous research work is also presented. It is shown that the differences found are not only due to the various microorganisms used but also to the conditions of the experiments and the manner of analyzing the data obtained.

I. Introduction

Studies of growth and alcohol production limitation by alcohol have been previously made by several researchers (1,2, 3,4) for other types of yeasts. The inhibition was found to be of a noncompetitive type (2).

Holzberg et al. (1) used enriched grape juice as the carbohydrate source, and the yeast employed was Saccharomyces cerevisiae var. ellipsodeus. They found that an equation of a linear type could represent their data:

$$\mu_{max} = \mu_o - qP \qquad (1)$$

where q was a constant and μ_o the value for μ_{max} at P equal to zero.

Aiba et al. (2,3,4) used a strain of a respiration deficient mutant of baker's yeast. The equation obtained to describe the effect of ethanol added into a growing culture were the following:

$$\mu_{max} = \mu_o e^{-K_1 P} \qquad (2)$$

$$\nu_{max} = \nu_o e^{-K_2 P} \qquad (3)$$

where K_1 and K_2 were empirical constants and μ_o and ν_o are the values when P = 0. The effects of P on the values of μ and ν were confirmed by the Lineweaver-Burk plot to involve non-competitive inhibition.

Since the foregoing results cannot be extrapolated from one microorganism to another, the goal of this work was to obtain the kinetic pattern of product inhibition for the strain Saccharomyces cerevisiae ATCC No. 4126.

II. Experimental Procedures

The experimental line followed was, first to determine the maximum ethanol concentration the cells could tolerate and still be viable and capable of producing alcohol. Once this upper limit was set, experiments at various alcohol concentrations below that maximum were done to determine the behavior of the strain in both batch and continuous culture.

The experimental conditions were a constant temperature of 30°C, a fairly constant pH of 4.0 (it varied from only 3.90 to 4.15 during all the fermentations) and a limiting amount of substrate (1% glucose).

The medium used had the following composition (for one liter of medium):

Tap water	1 liter
Glucose	10 g
Yeast extract	1.5 g
NH_4Cl	2.5 g
$Na_2HPO_4 \cdot 7H_2O$	5.5 g
KH_2PO_4	3.0 g
$MgSO_4$	0.25g
$CaCl_2$	0.01g
Citric acid*	5.0 g
Sodium citrate*	2.5 g

*These substances were added as a buffer to maintain the desired pH, varying the amount of sodium citrate to obtain a pH of 4.0

For the inoculum the same medium was used except that the glucose concentration was increased to 5% and yeast extract increased to 7.5 g/l. The inoculum was prepared by transferring the microorganisms from agar slants into Erlenmeyer flasks (300 ml) containing 100 ml of this rich medium. The flasks were closed with cotton stoppers and stirred within a water bath to maintain the temperature at 30°C. After 24 hours the inoculum was taken with a sterile syringe and added to the fermentor.

The experiments, as noted below, were divided among batch experiments to establish the upper limit of alcohol concentration, and continuous experiments to find the kinetic model.

1. Batch Experiments in Minifermentors.

These experiments were performed in one liter fermentor vessels equipped as in Figure 1, using a working volume of 500 ml. The fermentors were sterilized and inoculated with 10 ml of inoculum. Hot water maintained constantly at 30°C was pumped through a pipe inside the fermentor with a Haake E12 pump/heater. Magnetic stirrers were used for mixing, and the sampling was made every hour through a sampling valve near the bottom of the fermentor.

These experiments were carried out in two phases, (1) adding the ethanol to the sterile medium before inoculation, that is, having an initial concentration of ethanol different from zero; and (2) injecting the ethanol when the cells were in the logarithmic phase of growth. In this manner it was possible to establish categorically the highest concentration of alcohol at which the cells could still grow and produce ethanol.

The data obtained from these experiments gave the guideline for the setting of the continuous runs that finally determined the kinetic behavior of the yeast.

2. Continuous Experiments

These experiments were carried out also in minifermentors (Fermentation Design Inc.) having a working volume of 500 ml. See Figure 2.

The medium reservoir had an initial volume of 15 liters of sterile medium, and ethanol was added if required. A Sigmamotor pump (peristaltic) from Zero-max was used to pump the medium to the fermentor. This type of pump was very useful because the maximum flow rate was 6.5 ml/min. using rubber listing of 3/8" O.D., and the feed rate remained constant during the experiment. Another advantage was that the whole apparatus could be sterilized together including the piping.

The medium reservoir, the fermentor, and the piping were sterilized in an autoclave at 120°C for 25 minutes. After cooling, pure ethanol was added to obtain the desired initial concentration, P_o , in the fresh medium.

The fermentor was inoculated as for the batch experiments and when the cells were in the logarithmic phase of growth a constant feed rate of medium was started. Sampling was made every two or three hours to confirm whether or not steady state had been achieved. If the cell concentration did not change after two or three samplings steady state was assumed. Then, a 10 ml sample was taken, centrifuged, and the supernatant was used to determine the glucose concentration, S, and the ethanol

concentration, P.

The above procedure was carried out over a range of increasing dilution rates. When the maximum dilution rate was employed, that is, when the culture had the minimum attainable concentration of cells, feed rates were decreased to corroborate if the data obtained were reproducible.

The above procedure was repeated at various initial concentrations of alcohol in the medium.

The cells were examined under the microscope at daily intervals to check for contaminations. Also viability of the cells was determined routinely using the staining technique with methylene blue (5).

The analytical methods for obtaining cell mass, X, and glucose and ethanol concentrations, S and P, were the following.

Cell mass was determined by optical density in a Fischer Electrophotometer using a wavelength of 650 mμ, and converting it to dry cell weight per liter of medium by means of a linear calibration that was done previously. This method was chosen because of the frequent measurements needed.

Glucose was determined by two methods, depending on the concentration. If high concentrations of glucose were to be measured the DNS method was employed (6), and for concentrations of glucose less than 0.2 g/l the Somogyi-Nelson method (7) was used. A Beckman spectrophotometer with a wavelength of 600 Å was used (with a calibration curve to correlate the readings with the concentrations of sugar in g/l).

Ethanol concentration was measured by gas chromatography because the amount of sample necessary for this analysis was very

small. (40 µl) and the results were accurate. Also, with this method it was possible to obtain the results almost immediately. Standard ethanol solutions were injected before and after each run to obtain a calibration curve that correlated areas vs concentrations. An Aerograph 1520 chromatograph was employed for these measurements. (The column was of a Chromosorb W-Acid Wash type, 60-80 mesh, six feet long, and 1/4" diameter). The column temperature was 100°C, and the detector and injector temperatures 150°C. Hydrogen flow was 40 ml/min, helium flow 60 ml/min and air 200 ml/min.

It is relevant to point out that the experiments carried out in this work were not performed at strictly anaerobic conditions. The fresh medium was not deaerated, and therefore, was saturated with atmospheric oxygen. As ethanol was added to this medium, measurements of the saturation concentration of oxygen were done at zero and maximum ethanol concentrations. The values were for P_o = O 7.12 ppm, and for P_o = 80 g/l 7.6 ppm.

As the concentration of oxygen in the continuous runs was essentially constant, it is assumed that the various experiments were conducted under roughly comparable oxygen conditions. It was not possible to measure the very low oxygen level which existed in the fermentor, although this would have been desirable.

III. Kinetic Data.

Figure 3 shows in a semologarithmic scale, growth of the cells, ethanol production and glucose consumption vs time for the first type of batch experiments (those with ethanol added

in the medium at the beginning of the fermentation), each with a different initial concentration of ethanol. The data obtained are summarized in Table 1. Values of μ_{max} were obtained from the slopes of the plots of ℓn X vs t and values of ν_{max} were calculated from the table taking average values for X using $\nu = \frac{1dP}{Xdt}$ for each set of points. The highest value of ν was considered ν_{max} for that ethanol concentration. As a result of these runs P_{max} can be assumed to be higher than 80 g/l but smaller than 100 g/l, because no growth was observed in the run that had P_o = 100 g/l.

On the basis of the results of the first batch experiments in the minifermentors, alcohol was added to the reactors, not at the beginning but after 5.5 hours of fermentation (when the cells were in the logarithmic phase of growth) to see if increasing the concentration of alcohol had really an inhibitory effect in the growth of the cells, and thus, in the production of ethanol. The data obtained are shown in Table II and Figure 4. It is clearly observed that growth and ethanol production are stopped with an addition of ethanol to give 100 g/l (curves C and C') while at 80 g/l (curves B and B') some growth (from 0.489 to 0.702 g/l) and increase in ethanol concentration (3.5 to 3.9 g/l) are noticed. Curves A and A' show the increase of X and ΔP with time when no alcohol is added to the culture. It is also interesting to notice how glucose consumption is affected by the same effect (curves A", B", and C").

The analysis of the results obtained in this batch experimentation showed that the maximum initial concentration P_o at which it was possible to obtain alcohol, in small amounts, and to maintain

the cells viable was below 100 g/l.

Therefore, with this limit in mind, the continuous runs were made to give the data on which to build a model that represented the kinetics of this culture.

The initial concentration of ethanol, P_o, ranged from 0 to 80 g/l. The specific rate of ethanol production in this case was calculated at $\frac{D}{X}(P - P_o)$ and μ was equal to each dilution rate, D. The experimental data are summarized in Table III. Figures 5 and 6 represent the behavior of cell mass, X, and the ethanol production, ΔP, with respect to an increase in ethanol in the incoming medium. In order to analyze this behavior, Lineweaver-Burk type of plots were drawn (Figures 7 and 8). It can be seen that the inhibition produced by the ethanol was of a noncompetitive type, giving a K_s of 0.238 (for μ) and a K_s of 0.329 g/l (for ν). The intercepts of these lines with the ordinate axis were taken as the maximum values of μ and ν for each concentration of alcohol. These lines were drawn corresponding to each initial concentration of ethanol. Of course, they should have been drawn at a constant P, but the variation among the different points at the same P_o was so small that it did not make a considerable difference.

For correlating μ and ν vs P an average value $\bar{P}$ was used. Figure 9 shows this correlation.

At this point, attempts to fit and equation to these values of μ_{max} and ν_{max} were made. The easiest one, with only two constants, was a parabolic type

$$\mu_{max} = \mu_o\left(1 + \frac{\bar{P}}{P_{max}}\right)^{1/2} \qquad (4)$$

and

$$\nu_{max} = \nu_o \left(1 + \frac{\bar{P}}{P_{max}}\right)^{1/2} \qquad (5)$$

where μ_o and ν_o are the values for $\bar{P}$ equal to zero. These equations did not quite fit the data (the standard deviations found were 0.0135 for μ and 0.0868 for ν). The values of the constants are the following:

$$\mu_o = 0.448 \qquad \nu_o = 1.913$$

$$P_{max} = 93.60 \qquad P_{max} = 99.02$$

Observing Figure 5.5 of Aiba et al. (8), it was noticed that the shape of the curve for X resembled the data, and a similar equation was generated. The standard deviations for this case were 0.00298 and 0.0172, respectively. The equations are the following:

$$\mu_{max} = \mu_o - \frac{a\bar{P}}{b - \bar{P}} \qquad (6)$$

$$\nu_{max} = \quad o - \frac{a'\bar{P}}{b' - \bar{P}} \qquad (7)$$

The values of the constants found by a least square fit were

$$\mu_o = 0.428 \qquad \nu_o = 1.802$$

$$a = 0.182 \qquad a' = 0.506$$

$$b = 133.78 \qquad b' = 119.25$$

μ_o and ν_o would correspond to the values of μ_{max} and ν_{max} when $\bar{P}$ is zero.

If μ_{max} and ν_{max} are equal to zero, the value of P_{max} can be evaluated from these equations. For this case P_{max} was 93.84 and 93.1 g/l, respectively.

These equations would be expected to give smaller standard deviations than equations 4 and 5 because they contain an additional constant.

Table I. Data from the batch miniferms experiments (1)

	P_o = 0			P_o = 50			P_o = 60			P_o = 80			P_o = 100		
t	X	S	ΔP	X	S	ΔP	X	S	ΔP	X	S	ΔP	X	S	ΔP
1	0.16	9.83	0.83	0.158	-	0.60	0.126	-	-	-	-	-	0.07	9.8	-
2	0.28	8.00	0,91	0.205	8.60	0.75	0.189	8.90	0.50	0.158	8.70	0.45	*		
3	0.43	5.50	1.25	0.260	-	-	0.248	-	-	-	-	-			
4	0.66	4.27	2.10	0.406	-	-	0.375	-	1.10	0.323	6.20	0.80			
5	-	-	-	0.568	5.30	1.75	0.536	4.98	1.60	-	-	-			
6	1.48	0.80	4.00	0.844	4.56	2.75	0.773	4.90	-	0.560	4.10	1.50			
7	-	-	-	1.144	3.54	3.85	1.065	3.80	-	-	-	-			
8	1.660	-	4.75	-	-	-	-	-	-	1.020	-	3.00			
9	-	-	-	1.577	2.64	4.60	1.427	2.58	3.75	1.220	1.23	3.50			
10	1.700	-	-	1.640	0.25	-	-	-	-	-	-	-			
11	-	-	-	1.664	0.03	-	-	-	-	-	-	-			
12	-	-	-	1.632	0.02	4.60	1.459	0	3.85	-	-	-			
13	1.71	-	4.75	1.632	0	4.60	1.546	-	3.95	1.420	0.02	3.55			
μ	0.4487			0.3687			0.3654			0.2880					
ν	1.56			1.294			1.098			0.949			0		

*Not noticeable growth in the following sampling.

Table II. Data from the batch miniferms (2)

	(1)			(2)			(3)		
t	X	S	ΔP	X	S	ΔP	X	S	ΔP
1	0.174	7.7	1.04	0.118	-	0.65	0.174	6.61	1.04
2	0.181	5.4	1.04	0.126	6.50	0.72	0.174	4.81	1.09
3	0.276	3.8	1.43	0.217	4.72	0.96	0.260	2.86	1.43
4	0.363	-	1.87	0.260	4.48	1.56	0.379	2.51	1.87
5	0.647	2.1	3.74	0.489	2.93	2.86	0.686	1.75	3.74
5.5*	-	-	-	0.536	2.85	3.50	0.765	1.40	4.25
6.5	1.262	0.95	4.75	0.591	2.65	3.60	0.781	1.40	4.20
7.5	1.538	-	4.75	0.631	-	3.70	0.749	-	-
8.5	1.632	0.375	4.60	0.647	2.50	3.70	0.797	1.40	4.10
9.5	1.656	-	-	0.702	2.40	3.90	0.808	1.35	4.30

*At 5.5 hours of fermentation ethanol was added:

(1) 0 g/l
(2) 80 g/l (57 ml to 500 ml of medium)
(3) 100 g/l (73 ml to 500 ml of medium)

Table III. Data from the continuous runs.

$D = \mu$	$\frac{1}{\mu}$	S_o	S	$\frac{1}{S}$	P_o	ΔP	X	ν	$\frac{1}{\nu}$	P	$\bar{P}$
0.072	13.88	9.40	0.048	20.80	0	4.75	1.50	0.230	4.40	4.75	4.37
0.192	5.21		0.230	4.30		4.60	1.40	0.630	1.60	4.60	
0.264	3.79		0.375	2.67		4.75	1.27	0.990	1.01	4.75	
0.270	3.70		0.400	2.50		4.75	1.28	1.000	0.99	4.75	
0.420	2.38		4.500	0.22		3.00	0.69	1.830	0.55	3.00	
0.060	16.67	9.05	0.045	22.20	26.22	4.56	1.38	0.190	5.04	30.78	29.19
0.132	7.58		0.122	8.20		4.56	1.25	0.480	2.08	30.78	
0.252	3.97		0.477	2.10		3.79	0.92	1.040	0.96	30.01	
0.312	3.21		5.340	0.20		1.83	0.46	1.240	0.81	28.05	
0.376	2.66		6.760	0.15		0.13	0.18	0.270	-	26.35	
0.073	13.66	8.35	0.087	11.49	58.54	4.45	1.18	0.276	3.62	62.99	61.29
0.138	7.25		0.270	3.70		3.58	1.03	0.479	2.08	62.12	
0.202	4.96		0.470	2.13		2.30	0.68	0.654	1.53	60.74	
0.258	3.88		5.100	-		0.75	0.28	0.691	1.45	59.29	
0.038	26.04	9.10	0.083	12.05	78.40	4.00	1.10	0.139	7.16	82.40	81.3
0.069	14.37		0.230	4.35		3.85	0.78	0.344	2.91	82.25	
0.074	13.44		0.270	3.70		3.85	0.90	0.318	3.14	82.25	
0.096	10.42		0.480	2.08		2.50	0.54	0.646	2.25	80.90	
0.101	9.92		0.48	2.08		2.50	0.39	0.444	1.55	80.90	
0.138	7.25		6.00	0.17		0.70	0.13	0.743	1.35	79.10	

IV. Conclusions.

A three constant equation was obtained to correlate the growth and ethanol production of Saccharomyces cerevisiae ATCC 4126 with respect to the ethanol concentration.

This equation shows that there is a limiting ethanol concentration beyond which the cells cannot grow or produce ethanol.

An analysis of the results of other researchers (1,2,3,4) was also done. (See Figure 10). It seems, however, that the difference in the results is due not only to the microorganisms used but also to the conditions of the experiments and the manner of studying the data obtained.

The highest P_o that Aiba et al. (2,3,4,) used in their experiments was around 50 g/l, and for the case of μ, if all the points are taken into account, it can be found that the curve drawn may be extended to obtain a P_{max} of 76.4 g/l (with a standard deviation of 0.024). This, however, does not occur with ν; on the contrary, it shows that the cells are capable of continuing the production of ethanol indefinitely. (See Figure 11).

As for Holzberg et al.(1), for concentrations of ethanol higher than 26 g/l, a definite linear relationship is found between μ and P. Here is also found a limit concentration of ethanol (in this case around 70 g/l). Unfortunately, it was not possible to obtain a similar analysis for ν from their data, and thus, there is not a strict criterion to judge the difference between the behavior of ATCC 4126 and the baker's mutant yeast used by Aiba et al.

There exists the possibility of finding adaptation of the cells to the ethanol medium (9) and, if glucose is not limited,

to find the cells producing alcohol even after they cease to grow. But this phenomenon was not studied in this work because these continuous experiments did not deal with sufficiently long times to offer the possibility for the cells of adapting to the medium; and, in the case of the longest residence time (27 hr), there was not sufficient substrate for them to produce more ethanol.

This could be a feasible reason why Aiba's experiments show different results, that is, as glucose was still present in the fermentor the culture could have adapted to that ethanol concentration and utilized the glucose to produce more alcohol giving a smooth decreasing rate of ethanol production instead of an abrupt stop at a certain maximum P.

As explained previously some oxygen was present. Previous workers (10, 11) found that oxygen was an essential growth factor necessary for the synthesis of unsaturated fatty acids and steroids. No attempt was made to quantify this effect, and further research should be done in this field to optimize ethanol production.

An investigation of cell adaptation to high concentrations of ethanol would be an interesting approach for a new set of experiments, since in commercial alcohol production high concentrations of alcohol are desired for economy in the distilling towers.

Acknowledgment

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Nomenclature

a, a'	empirical constants, hr^{-1}
b, b'	empirical constants, g/l
D	dilution rate, hr^{-1}
K_1, K2	Aiba's constants
K_s, K'_s	saturation constants, g/l
P	product concentration, g/l
P_{max}	maximum value of P at which cells are still viable, g/l
P_o	ethanol concentration in fresh medium, g/l
$\overline{P}$	average value of P in the continuous runs, g/l
q	Holzberg's constant
S	substrate concentration, g/l
S_o	substrate concentration in fresh medium, g/l
t	time, hr
X	dry cell mass concentration, g/l

Greek symbols

μ	specific growth rate, hr^{-1}
μ_{max}	maximum specific growth rate, hr^{-1}
μ_o	maximum specific growth rate at P-O, hr^{-1}
ν	specific ethanol production rate, hr^{-1}
ν_{max}	maximum specific ethanol production rate, hr^{-1}
ν_o	maximum specific ethanol production rate at P=O, hr^{-1}

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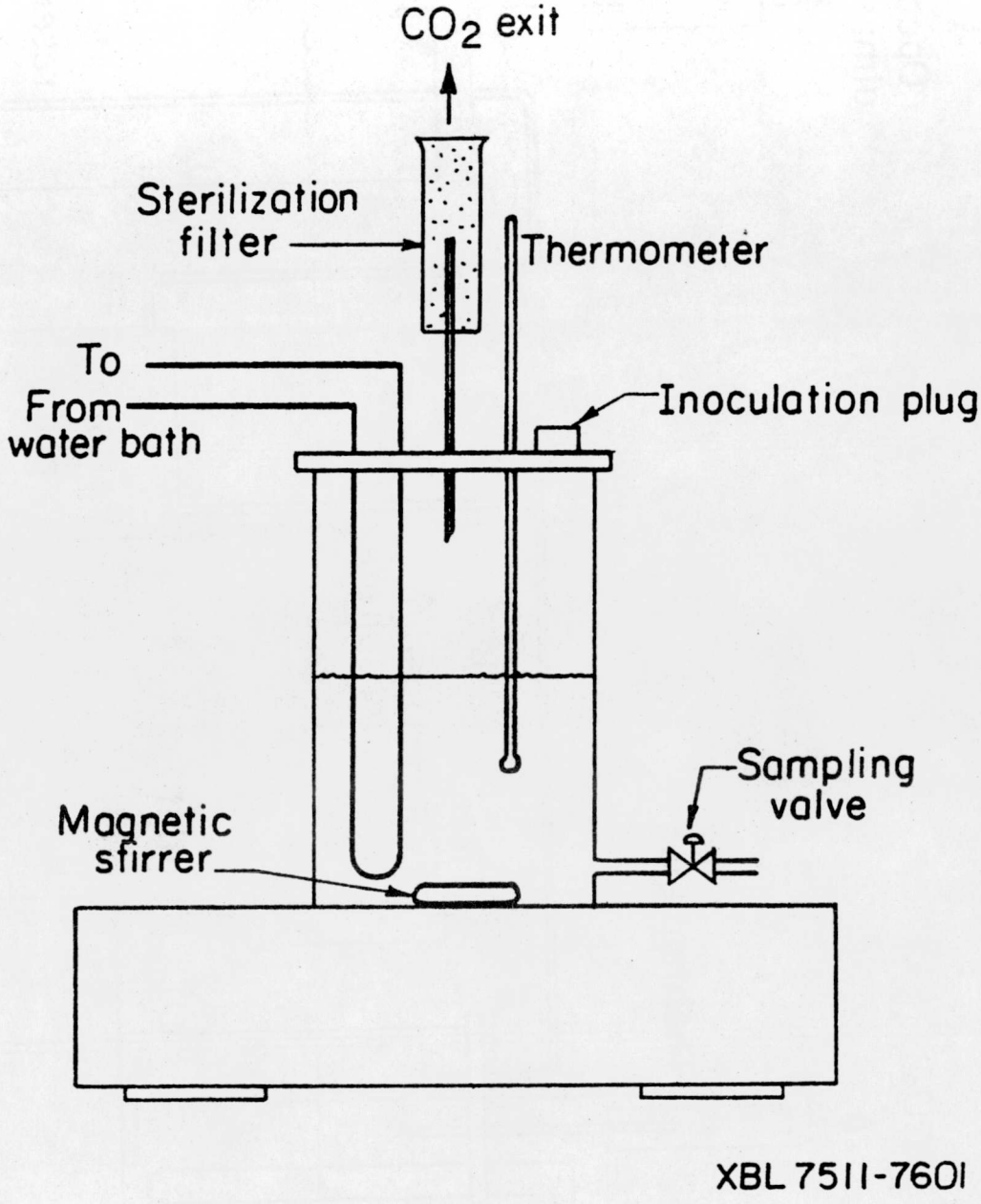

FIGURE 1. MINIFERMENTOR FOR BATCH EXPERIMENTS.

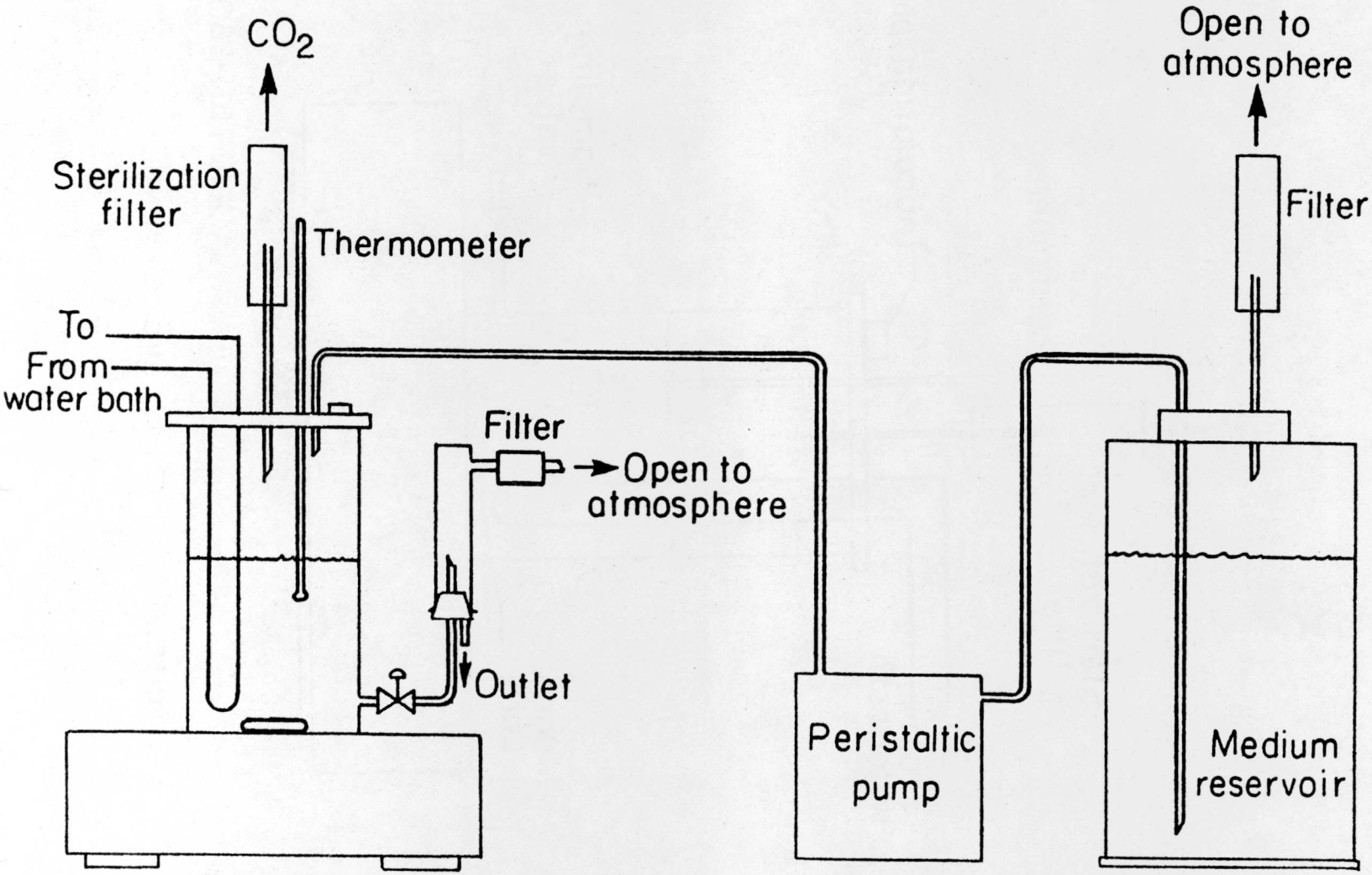

FIGURE 2. MINIFERMENTOR FOR CONTINUOUS RUNS.

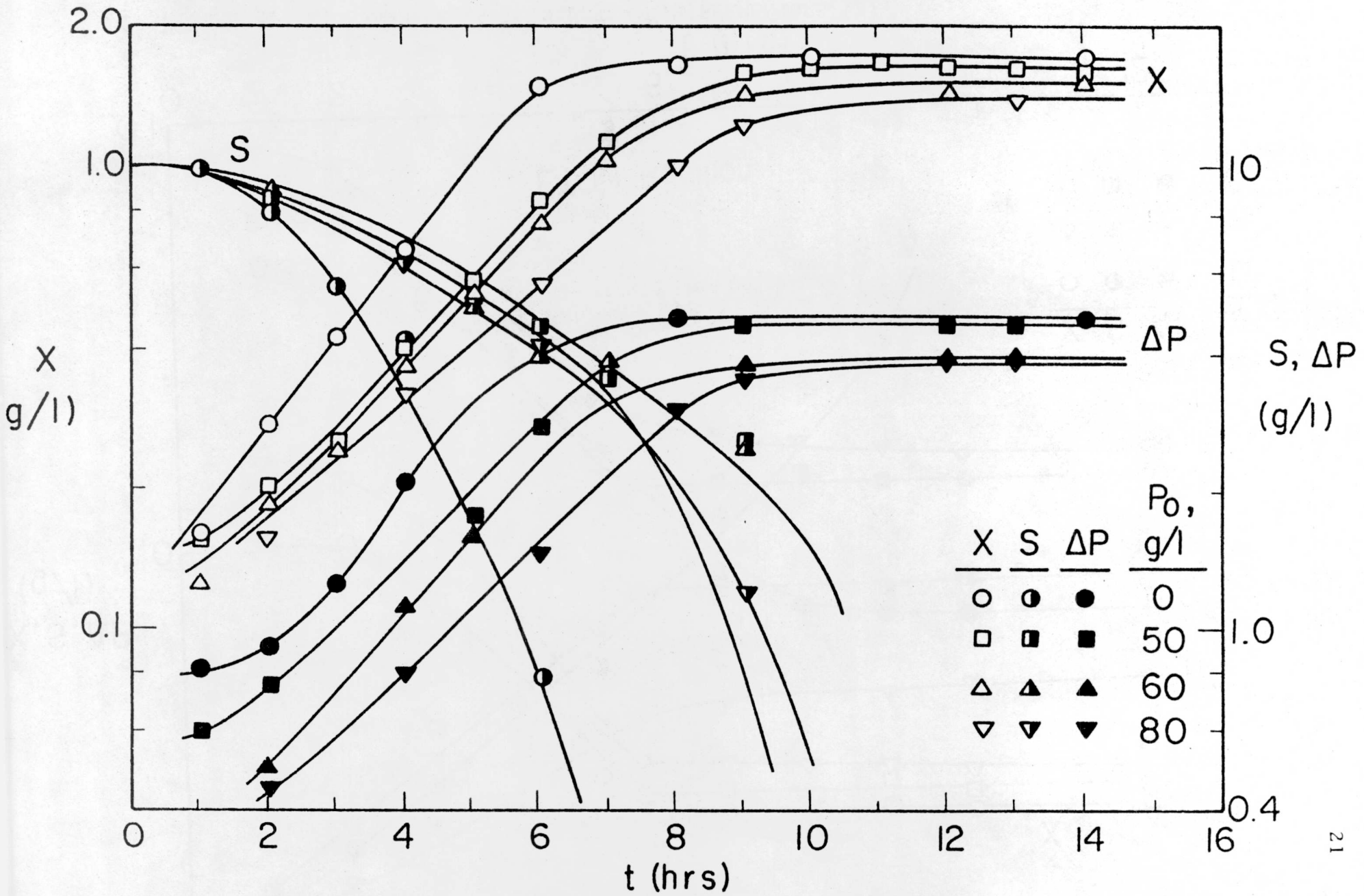

FIGURE 3. X,S AND ΔP VS T IN BATCH MINIFERMENTORS (1).

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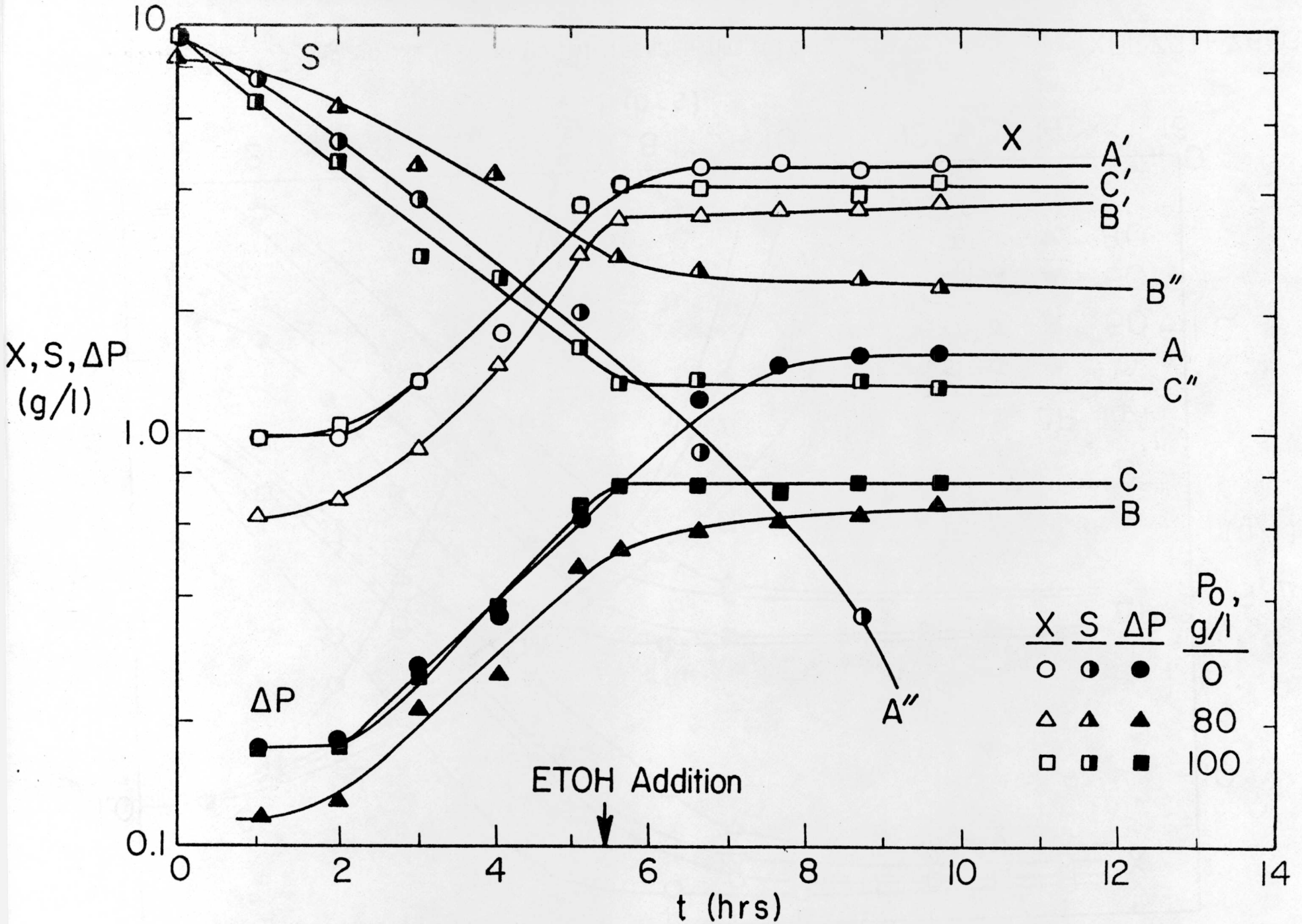

FIGURE 4. X, S AND ΔP VS T IN BATCH MINIFERMENTORS (2).

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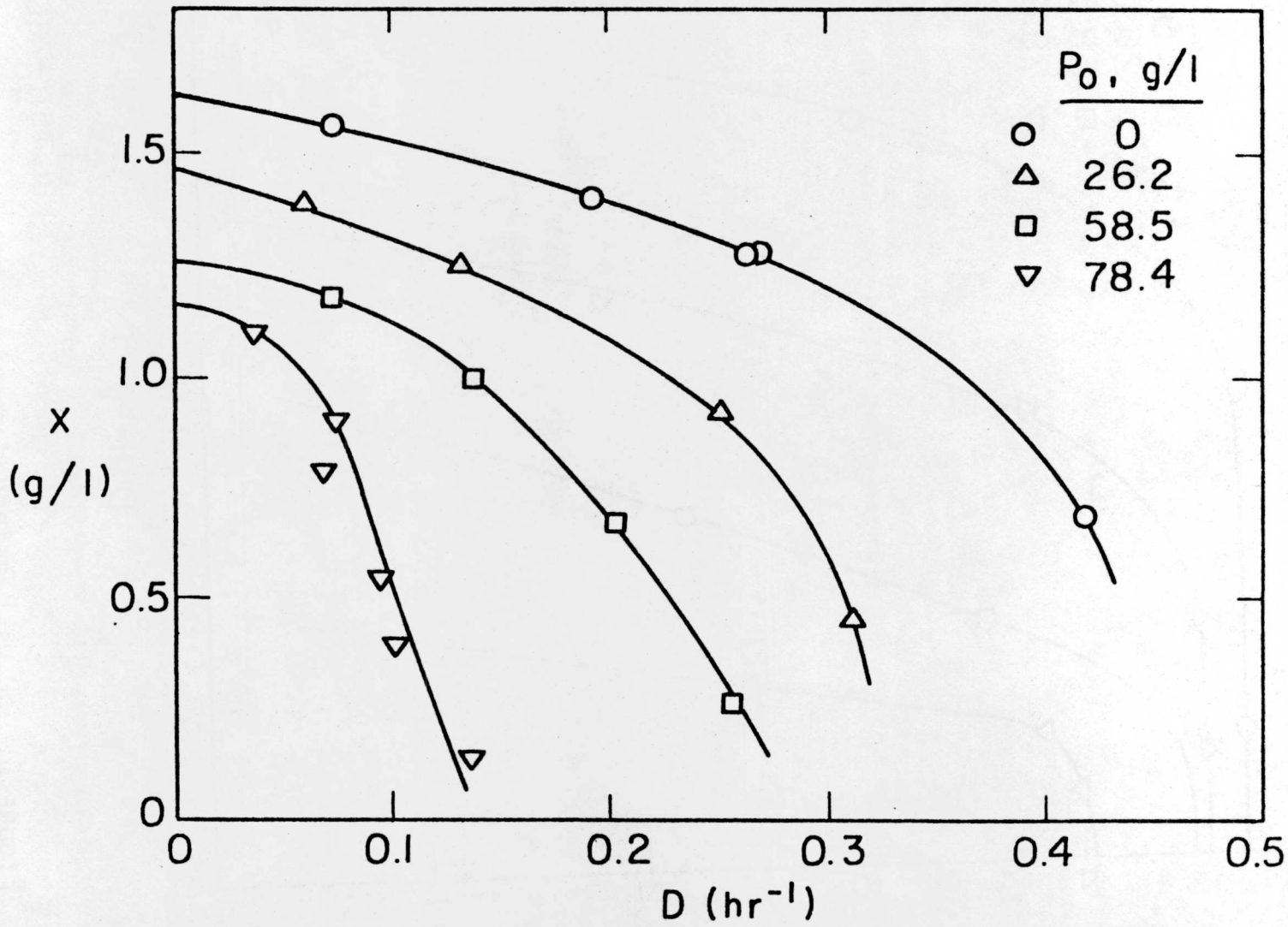

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FIGURE 5. X VS D RESPONSE TO INCREASING P IN THE MEDIUM.

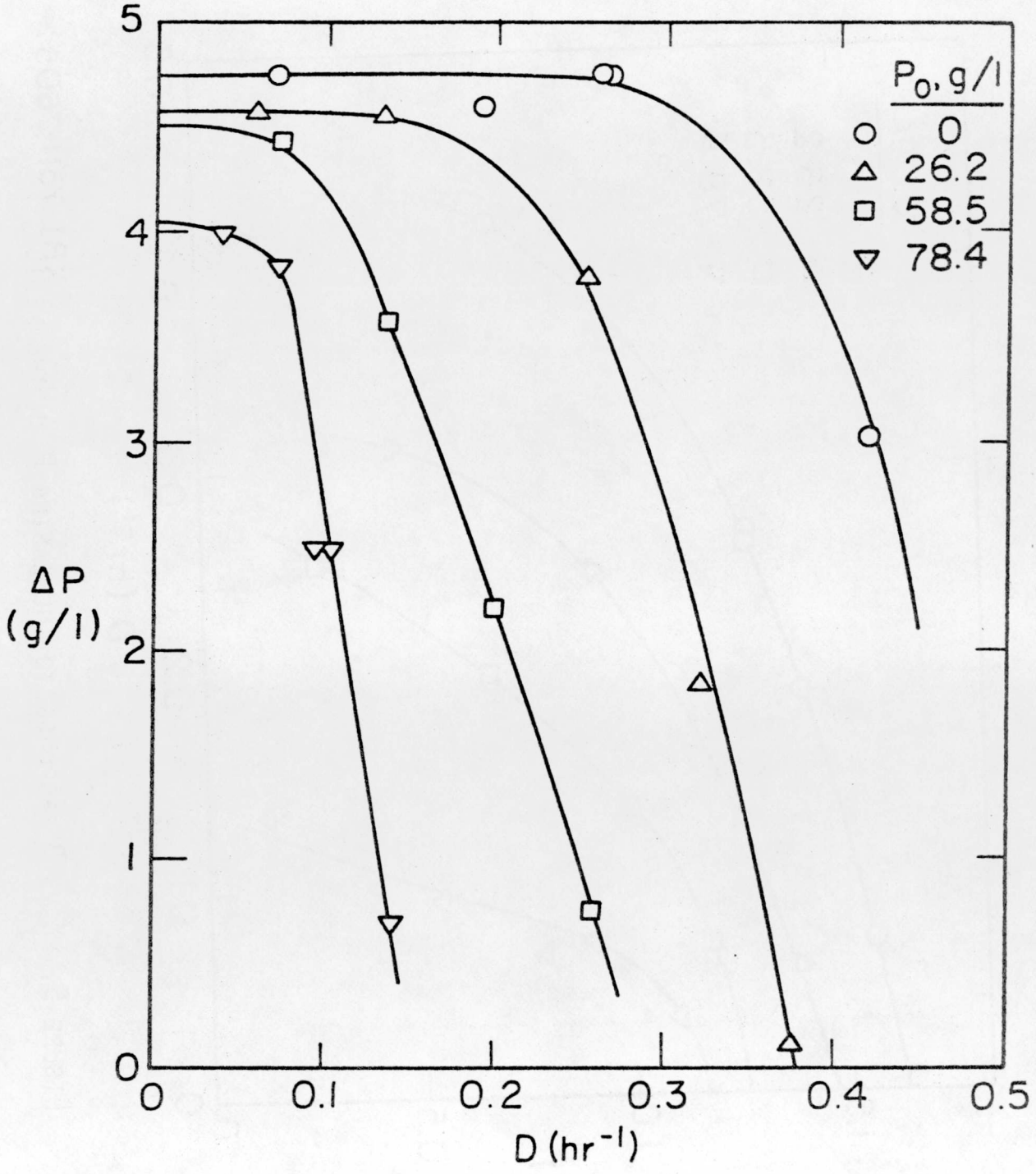

FIGURE 6. ΔP VS D RESPONSE TO INCREAING P IN THE MEDIUM.

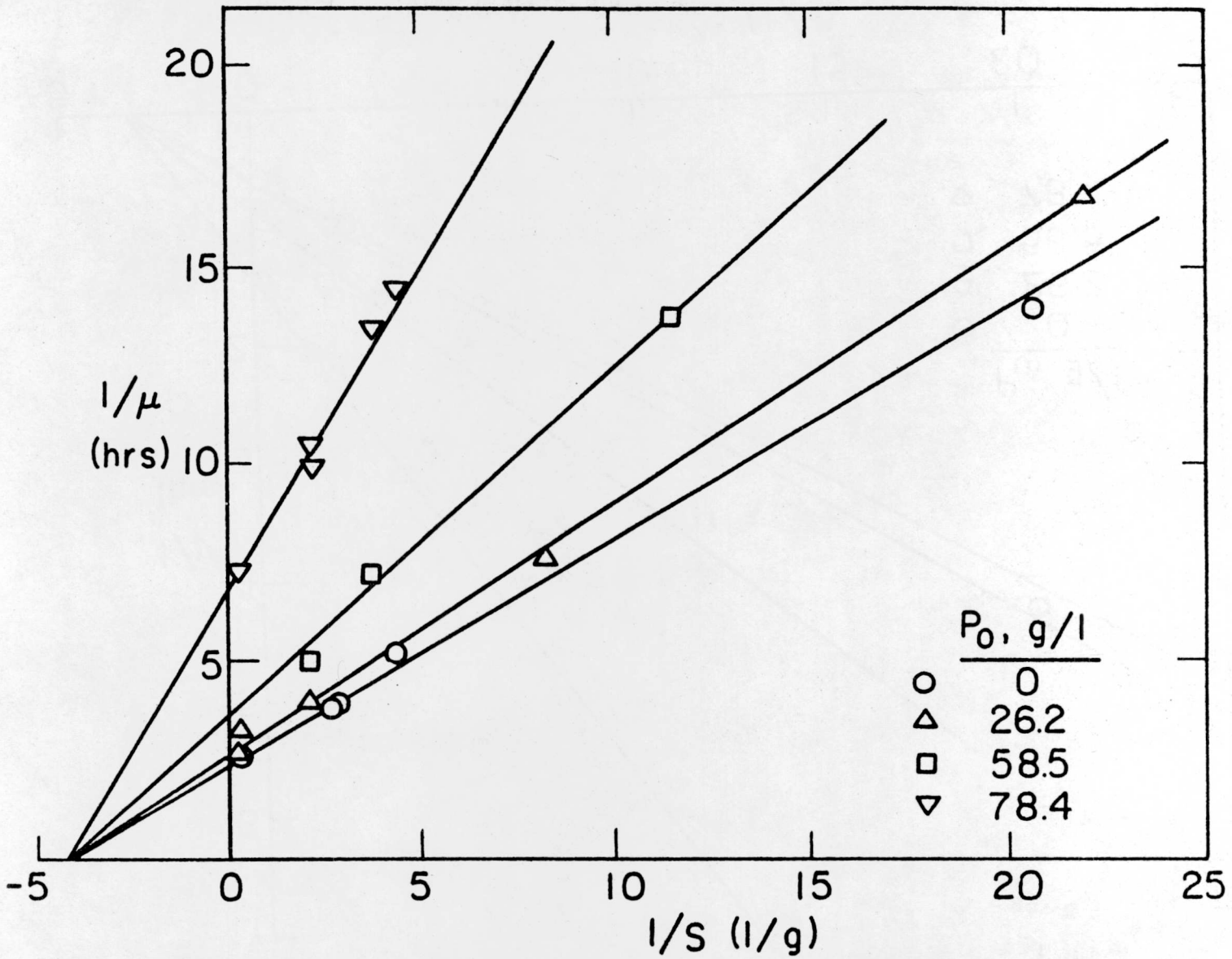

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FIGURE 7. LINEWEAVER-BURK PLOT FOR μ.

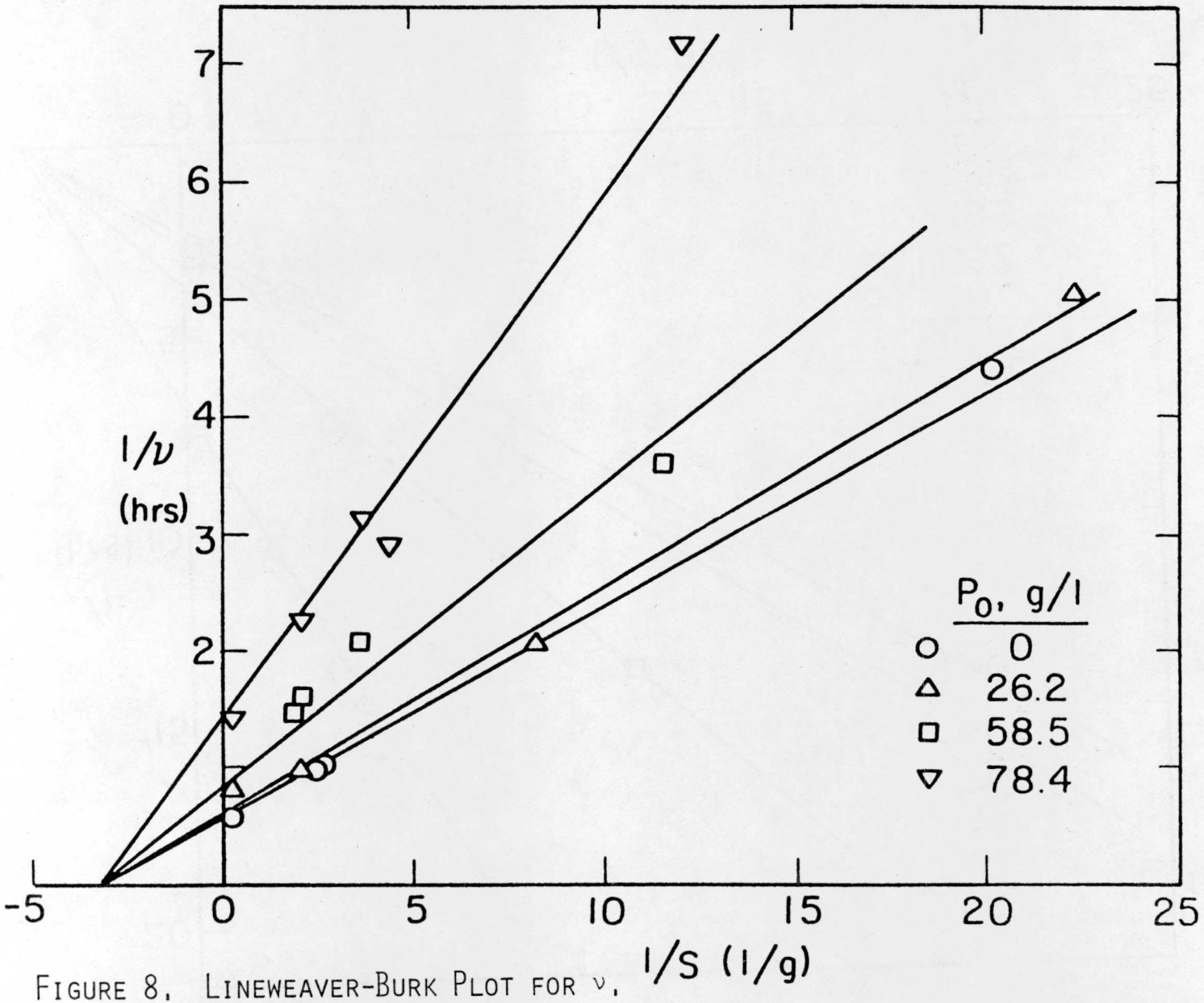

FIGURE 8. LINEWEAVER-BURK PLOT FOR ν.

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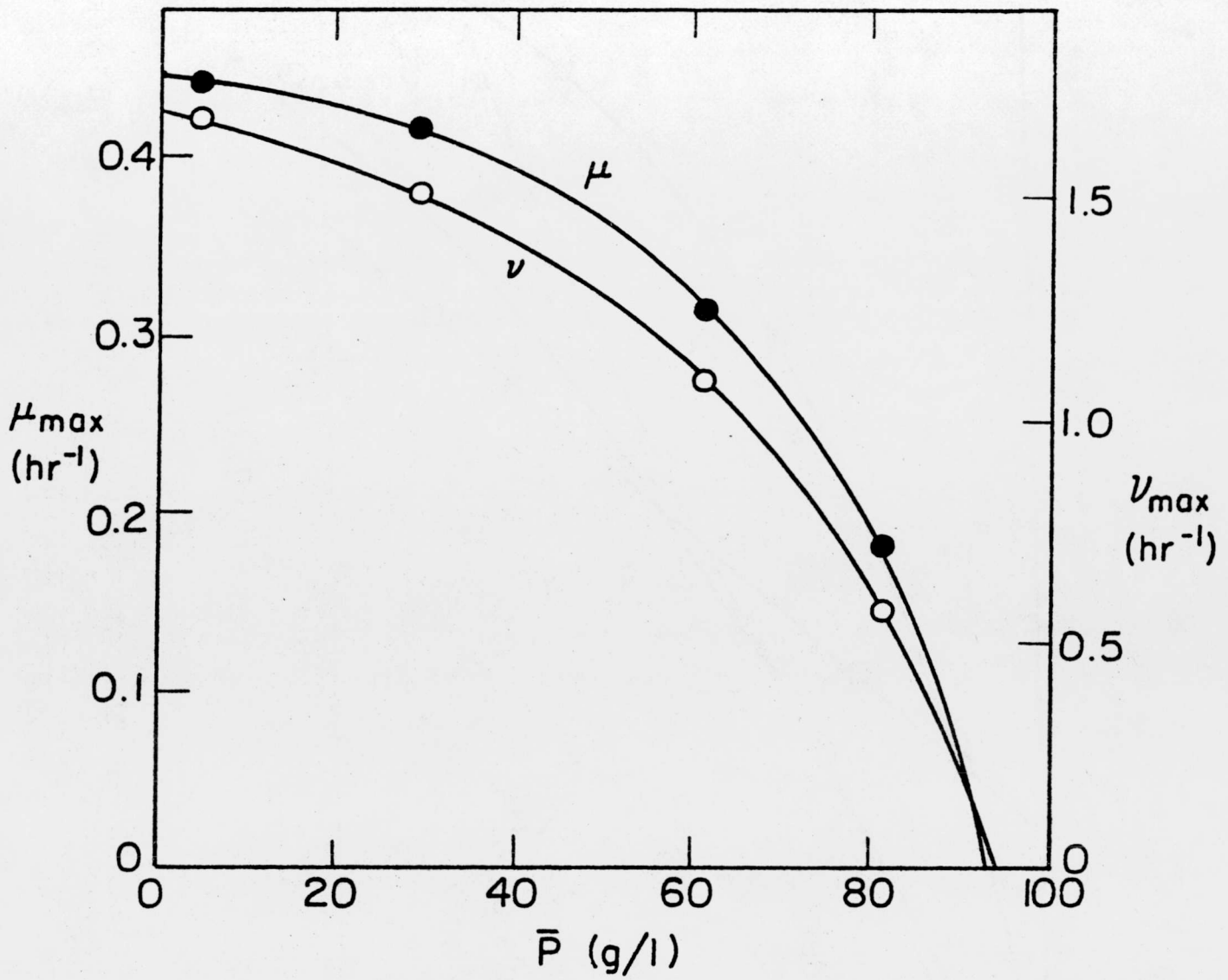

FIGURE 9. CORRELATION BETWEEN μ_{MAX} AND ν_{MAX} $\bar{P}$. (FROM EQS. 6 AND 7) XBL7511-7609

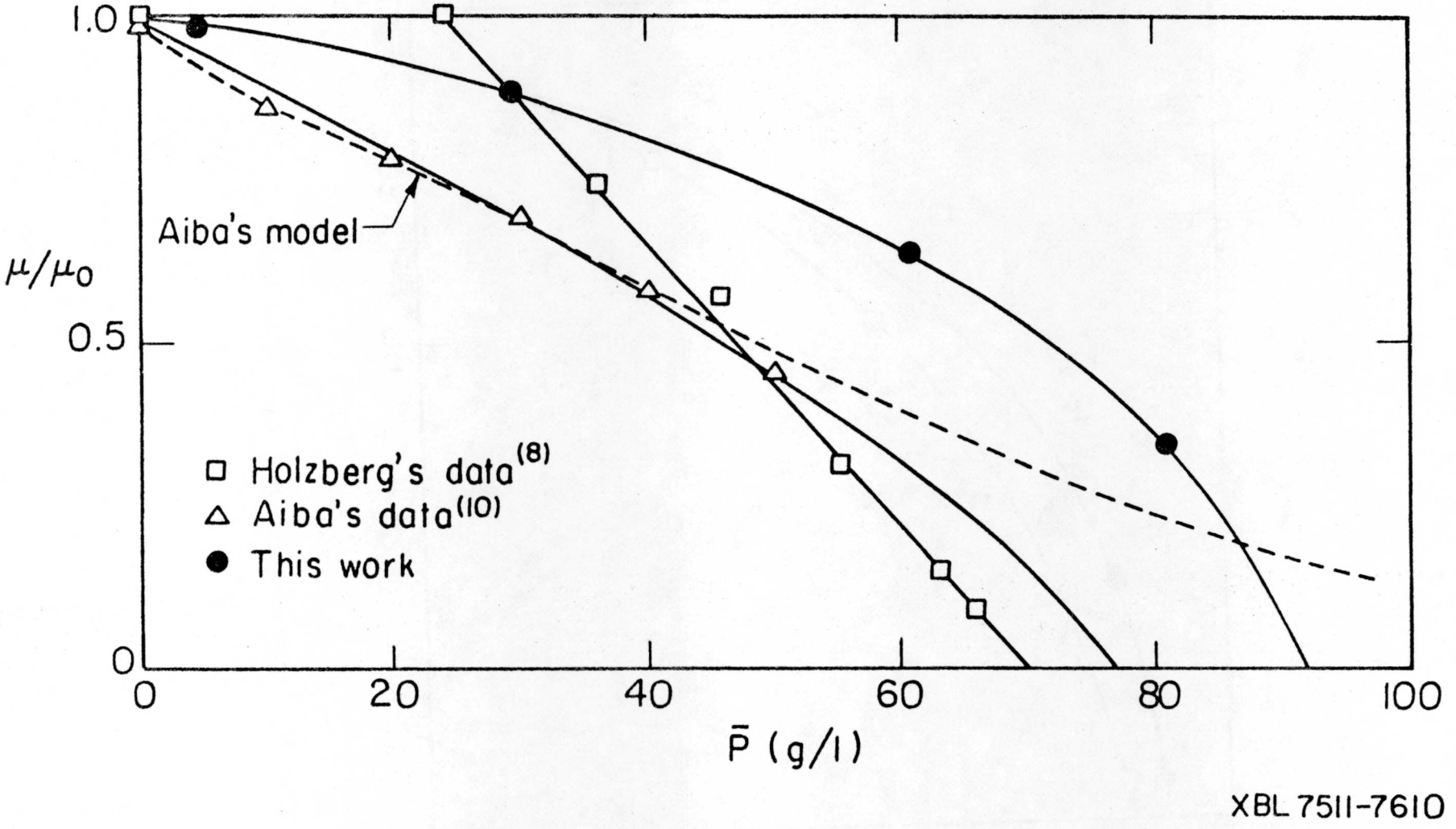

FIGURE 10. COMPARISON BETWEEN DATA FROM OTHER RESEARCHERS AND THIS WORK.

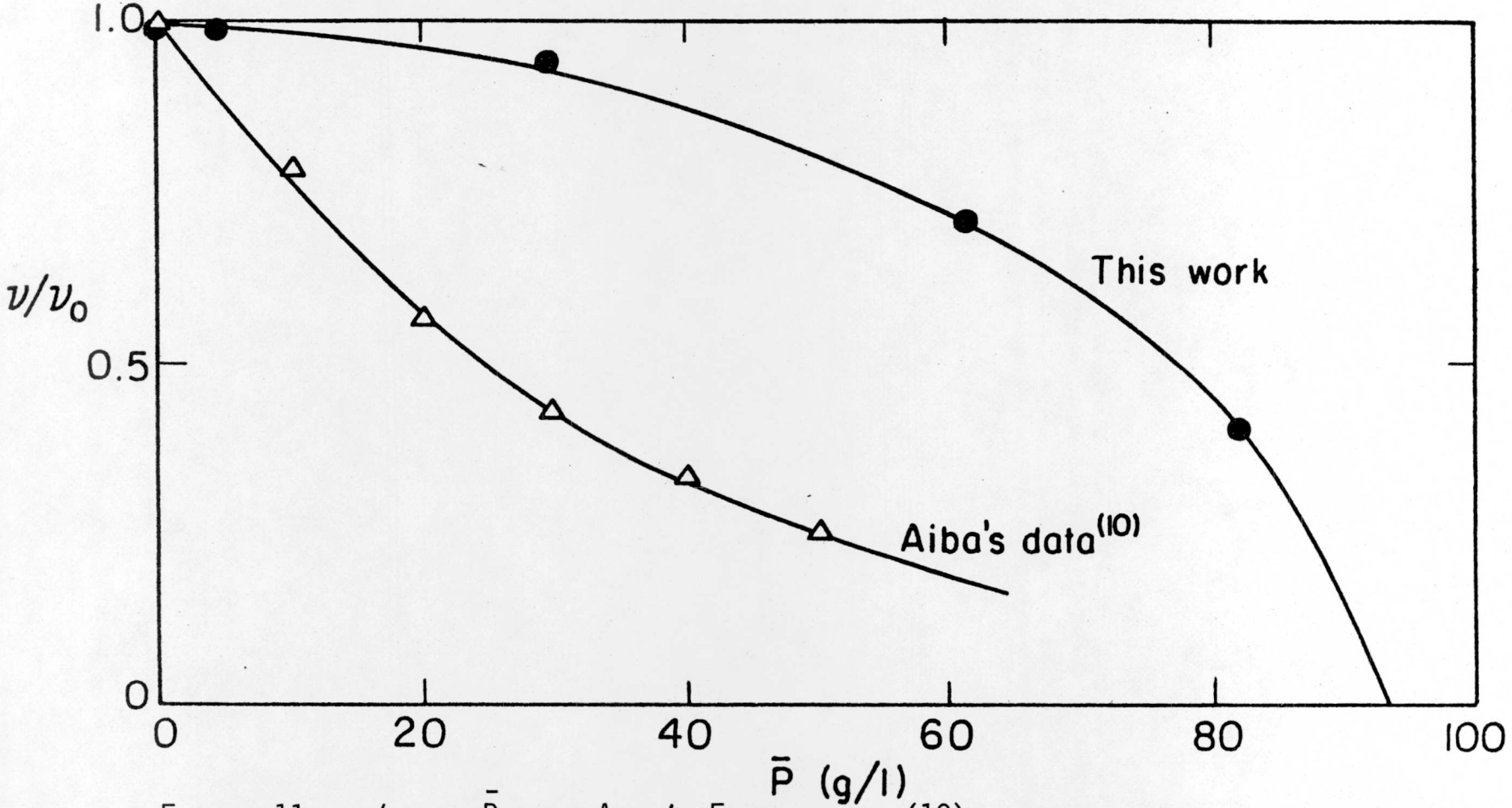

FIGURE 11. v/v_0 VS $\bar{P}$ FROM AIBA'S EXPERIMENTS (10) AND THIS WORK.

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