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A STUDY ON THE GROWTH AND ASPARAGINASE PRODUCTION OF *VIBRIO SUCCINOGENES* IN A CHEMOSTAT

by
Preston David Krautheim

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Masters of Science in Chemical Engineering
1979

APPROVAL SHEET

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ABSTRACT

Title of Thesis: A Study on the Growth and Asparaginase Production of Vibrio succinogenes in a Chemostat

Preston David Krautheim, Master of Science in Chemical Engineering, 1979

Thesis directed by: Dr. T. Greenstein, Associate Professor of Chemical Engineering

A continuous fermentation assembly was constructed and studies on the growth of the organism, Vibrio succinogenes, and its production of asparaginase was conducted. Results were compared with that already performed on a batch system and showed that a continuous fermentation produced 31.83 I.U. enzyme/mM ammonium formate compared to 17.4 I.U. enzyme/mM ammonium formate for batch.

Some of the runs performed indicated that the organism was washing out or giving very low specific growth rates. It was decided that this was not due to operational parameters of the chemostat, but possible chemical contamination in the medium. With all things considered, a continuous fermentation on producing the enzyme, asparaginase, was

almost doubled when compared with that of batch

making this form of fermentation a viable alternative.

FOREWORD

The research, which has been performed and reported in this thesis, deals with studies in Biochemical Engineering. With the nation's food and energy shortages an ever increasing problem, the growing need for extensive research work in fermentation technology and techniques will be needed in order for these problems to be solved.

Biochemical Engineering at New Jersey Institute of Technology is still a new field and lacks vital equipment which is essential if extensive studies are to be conducted. Because of this, the need for expertise outside the college, mostly in the area of microbiology, had to be sought. One such person was Dr. David Kafkewitz of the Department of Zoology and Physiology, at Rutgers University, Newark, N.J. I wish to express my deepest appreciation to Dr. Kafkewitz for all his help, guidance and support in the conduction and completion of this thesis. Dr. Kafkewitz not only supplied some of the chemicals and equipment, but maintained the organism, Vibrio succinogenes, for continual work and performed all of the enzyme assay

tests reported in this thesis.

I also wish to thank my brother, Kenneth Krautheim, for drawing all the graphs and plots contained within this report. His expertise with engineering documentation and the time he spent on the graphs is greatly appreciated since it left me with time to write and arrange the remainder of the thesis.

Last but not least, I would like to thank Dr. Teddy Greenstein for his support, guidance and patience throughout my graduate studies. His devotion to my need for a new project at a critical time, when a previous one had failed, helped greatly in completing my requirements for the Masters. The experience and training gained under the guidance of Dr. Greenstein will never be forgotten and aided in my development as an engineer.

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INTRODUCTION

Vibrio succinogenes is an unusual anaerobe which was orginally isolated from the bovine rumen (5). It has been shown to produce large amounts of the enzyme L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1). Since this enzyme has shown antileukemic activity, studies on its production have been performed (3).

Experimental studies to maximize the production of Vibrio succinogenes (V. succinogenes) has been undertaken by Dr. David Kafkewitz and associates at Rutgers University, Newark N.J. Their studies were done on a batch fermentation system and provided the ground work for the continuous studies to be discussed in this thesis. The ground work of main concern, in these studies, was the composition of the medium to be used. Also, during the batch fermentation experiments, questions were raised as to how the production of the enzyme, asparaginase would be affected by a continuous fermentation and if an upper limit existed.

A continuous fermentor was therefore set up at New Jersey Institute of Technology (see Figure 1). Fermentations with two types of mediums were done. One was a "Sodium Medium", composed of the materials listed in Tables I and

IV. The other was an "Ammonium Medium" which was composed of the materials listed in Tables II, III, V and VI.

Each continuous fermentation took approximately 100 hours to complete. Samples were taken periodically and processed for enzyme assay studies, performed at Rutgers University by Dr. D. Kafkewitz. For complete details of the methodology for preparing the fermentation, running it, taking and processing samples and performing the enzyme assays, refer to Appendix A.

THEORY: CONTINUOUS FERMENTATION

A continuous culture is a flow system in which individual cells are suspended in a (nearly) constant volume, at or near a steady state of growth established by the continual removal of part of the culture (6).

In practice, the study of cells were mainly performed in batch cultures, in which a particular medium was inoculated, with the organism and the system allowed to grow. In batch systems, certain properties of the cell such as size, composition and other functional characteristics vary greatly during the growth of the organism. This makes interpretations of the results difficult. The introduction of continuous cultures; therefore, had advantages over their predecessors, the batch cultures. For one, the rates of growth and division are more easily controlled and maintained for long periods. Secondly, the cell concentration can be set and maintained independently of growth rate. Third, the cells can be grown for longer periods in a constant chemical environment. Also, very low levels of critical nutrients, growth factors, mutagens, or toxix agents can be maintained during the growth of

the culture. Finally, the cell sizes and biochemical composition are more easily selected and maintained with a given strain since these cell characteristics depend upon the rate of growth (6).

Controlling a continuous culture (chemostat culture) is normally done by limiting a particular factor of nutrient required for growth, such as the carbon source, amino acid or an inorgainc ion. In a chemostat, all of the limiting nutrient is utilized by the culture with the result that the cell concentration reaches a constant value which is essentially proportional to the concentration of the limiting nutrient (6).

A continuous fermentor was first initiated in the early 1920's when fodder yeast was produced by a continuous process. Since that time, continuous cultures of micro-organisms has been carried out by numerous workers. During the latter half of this century, growing interest has been focused on the continuous cultivation of microbes (1).

Today, it is difficult to determine the extent of the application of continuous cultures since industrial information is not available and mostly proprietary. However, what information is available does indicate how wide spread the use of continuous cultures has on society.

Continuous fermentations are used in the production of beer, sake, alcohol and acetic and lactic acids, of vitamins (B_{12}) and drugs (alkaloids) and of antibiotics (penicillin and chlortetracycline). The continuous culture process is also used for steroid conversion and the production of vaccines (6).

There are four basic components which mostly all chemostats consist of. They are as follows (6):

(1) A culture vessel or fermentor in which the cells are grown isolated from contamination by other organisms.

(2) A nutrient supply vessel which delivers sterile medium of a desired composition via a metering pump.

(3) A system for agitation of the culture, either by the sparging of sterile air containing oxygen for aerobic fermentations or inert gas (such as nitrogen) for anaerobic fermentations. Agitation is also achieved by an impeller system connected by a direct shaft arrangement or magnetically coupled. Agitation can also be achieved by a combination of both systems.

(4) A system for the removal of the broth which exits the fermentor at the same rate at which fresh medium enters. This system also allows for the escape of effluent gases from the system.

One of the most important parameters for the 'chemostat' is the dilution rate, defined as the fractional rate of replacement of nutrient medium. The dilution rate, w,

can therefore be expressed as:

$$w = W/V_o \qquad (1)$$

where W, is the rate of flow of medium (cc/hr) and V_o is the working volume of the culture broth (cc) (6).

The dilution rate determines the rate of cell division in the fermentor for continuous cultures and the residence time the cells remain in the culture vessel. Since the dilution rate can also be thought of as the wash out rate, a relationship can be derived so as to determine the rate of decrease of the cells, which is given as:

$$\frac{dN}{dt} = -wN \qquad (2)$$

Solving for N, the number of cells:

$$N = N_o EXP(-wt) \qquad (3)$$

where N_o is the initial number of cells in the volume V_o before the medium is added at flow rate W. To work with N, the number of cells or particles, is somewhat difficult; therefore, one can relate this to the cell concentration, C, by:

$$C = N/V_o \qquad (4)$$

or

$$N = CV_o \tag{5}$$

Substitution of equation (5) into (2) gives:

$$\frac{d(CV_o)}{dt} = -wV_oC \tag{6}$$

Solving,

$$\frac{d(CV_o)}{CV_o} = -wdt \tag{7}$$

Since the volume V_o is independent of time, equation (7) can be rewritten as:

$$\frac{dC}{C} = -wdt \tag{8}$$

Solving for C at any time t yields:

$$C = C_oEXP(-wt) \tag{9}$$

where C_o is the initial cell concentration before any flow is initiated. Equation (9) can be rewritten as follows, by taking the natural logarithm of both sides.

$$LnC = LnC_o - wt \tag{10}$$

A plot; therefore, on semilog paper of C versus time, t, can therefore be made for all runs of interest to determine the growth characteristics for each medium and determine whether any washing out of the organism exists. The plot would consist of a theoretical wash out plot versus the actual growth curve. The theoretical plot is made by taking the initial cell concentration, C_o, known at the beginning of the run (first sample taken after the pump is turned on) and the slope of the line which is w, the dilution rate. Then the actual curve is made by plotting the cell concentration at various times throughout the run.

Another paprameter of interest is known as the specific growth rate, μ, defined as (1):

$$\mu = \frac{1}{C} \frac{dC}{dt} \qquad (11)$$

If μ is constant, equation (11) represents the exponential growth, in a batch culture, where the growth is proportional to the mass of the cells present. The specific growth rate can be related to the mass doubling time, which is defined as that time which must transpire for the cell mass to increase two-fold, by the following

equation (1):

$$t_d = 0.693/\mu \quad \frac{Ln2}{\mu} \qquad (12)$$

where t_d is the doubling time. Therefore, knowing the t_d for the batch culture, with no limiting nutrient, one can find the μ_{max} which would be the maximum specific growth rate; hence, dilution rate is limiting for the continuous fermentation. In other words, to have a valid chemostat run, one must operate it below μ_{max}, determined from the batch system.

MATERIALS LIST

Basically there were two types of runs performed. One, called a sodium run, consisted of sodium formate as the limiting nutrient. The other called an ammonium run, consisted of ammonium formate as the limiting nutrient. For the concentrations, weights and composition of each medium for each run, refer to Appendix A for listings.

The complete listing of all chemicals used in the mediums is as follows:

(A) Ammonium Chloride, certified ACS, Mol. Wt. 53.49 gm/gm mole, purchased from Fisher Scientific Company, Lot No. 751617, Cat. No. A-661.

(B) Ammonium Formate, certified, Mol. Wt. 63.06 gm/gm mole, purchased from Fisher Scientific Company, Lot No. 780108, Cat. No. A-666.

(C) Fumaric Acid (purified), Mol. Wt. 116.07 gm/gm mole, purchased from Fisher Scientific Company, Lot No. 770671, Cat. No. A-120.

(D) L(+)-Cysteine Hydrochloride, Reagent Grade Monohydrate, Mol. Wt. 175.64 gm/gm mole, purchased from Fisher Scientific Company, Lot No. 774077, Cat. No. C-562.

(E) Sodium Chloride, Mol. Wt. 58.50 gm/gm mole, normal table salt of suitable grade.

(F) Sodium Formate, Mol. Wt. 68.01 gm/gm mole, suitable grade.

(G) Sodium Hydroxide, Mol. Wt. 40.00 gm/gm mole, pellets, reagent grade

(H) Yeast Extract, "Difco" certified, purchased from Difco Laboratories, Control No. 639994, Cat. No. 0127-01.

EQUIPMENT LIST

The continuous fermentation runs involved the use of a variety of equipment. The following is a list of this equipment.

(A) Autoclave-	New Brunswick Scientific Autoclave, Model No. 57114.
(B) Centrifuge-	Angle Centrifuge Ivca Sorvall, Inc., 7500 rpm max. with variable controller.
(C) Collector-	1000 cc flask with #11 stopper (2 holes).
(D) Fermentor-	500 cc flask modified (see fig. 2) with #7 stopper with inlet ports.
(E) Flow Meter-	#1-Matheson Flow Meter. model # 620 bbw)603. #2-unknown make-standard type as above.
(F) Medium Vessel-	6000 cc flask with #10 stopper (3 holes).
(G) Peristaltic Pump-	Manostat Cassette pump, Serial No. 120 with cassette on slow pump rate connection.
(H) PH Meter-	Corning Model 610A expand, portable pH meter with Fisher Electrode Standard Combination pH 0-14, -5 to 110°C.

(I) Spectrophotometer-	Bausch & Lomb Spectronic 20 spectrophotometer, wavelength 550 nm.
(J) Temperature Controller-	Yellow Springs Instrument Co., Inc., Model 63RC, Thermistemp temperature controller with standard thermocouple (shielded).
(K) Tubing-	*tygon tubing S-50-HL 3/16 in. I.D. used for peristalic pump. *silicon rubber 3/16 in. I.D. used for peristalic pump. *latex tubing 3/16 in. I.D. used for all lines connecting equipment.

MAINTENANCE OF ORGANISM

Vibrio succinogenes has been maintained at the Rutgers University laboratory for a number of years. The organism is listed by the American Type Culture Collection as ATCC 29543 and by the Anaerobe Laboratory of the Virginia Polytechnic Institute and State University, Blacksburg, as VPI no. 10659. The organism is transferred from culture tube to culture periodically to maintain its viability. For further details on this procedure and the composition of the growth medium, refer to Appendix A.

PREPARATION OF THE MEDIUM

All continuous fermentation runs were performed in a 5.5 liter medium volume. The following procedure was used:

All necessary chemicals were weighed and the amounts recorded (for the amounts used for each run, see Tables I-VI). Their mixing were done in three phases. First, 1.0 liter of distilled water was measured out and poured into a 3.0 liter beaker. To this, the yeast extract was added in order to facilitate mixing. Secondly, 2.5 liters of distilled water was added to another beaker. Then the Sodium or Ammonium Formate, L(+)-Cysteine Hydrochloride and Sodium Chloride or Ammonium Chloride (when used) was added and mixed thoroughly. To this mixture, the yeast extract mixture was added and mixed. Then the Fumaric Acid and Sodium Hydroxide were added to the now 3.5 liter mixture and the pH measured. Adjustment was made by adding 1 normal Sodium Hydroxide solution until a pH of 7.5 was obtained. The third phase was to adjust the volume of the mixture to 5.5 liters by adding distilled water. The finished mixture was poured into a 6 liter

flask, sealed with brown paper and then sterilized. For a more detailed description of the procedure, refer to Appendix A.

APPARATUS STERILIZATION AND ASSEMBLY

As with all biological work, sterilization of the equipment being used is essential if aseptic conditions are to be maintained.

The continuous fermentation assembly, shown in Figure 1, was sterilized in two phases. The first phase, included sterilization of the fermentor, collecting vessel, and associated piping and tubing. The second phase, involved sterilization of the fermentation medium in its appropriate vessel. Sterilization of the temperature probe was also required. It involved immersion of the probe in 70% alcohol for one hour then rinsing with sterile distilled water before insertion into the fermentor. For further details see Appendix A.

PROCEDURE USED FOR A TYPICAL FERMENTATION RUN

After sterilization and assembly of the apparatus, the following procedure was used for a typical run. First, the medium, from the supply vessel, was pumped into the fermentor (approx. 235 cc). Next, with the temperature probe already in the fermentor, the heating lamp was turned on and the controller set so as to maintain a temperature of 37°C in the fermentor. The nitrogen supply, to the fermentor, was turned on and sterile nitrogen sparged into the medium. When all systems were set, the organism, Vibrio succinogenes, was inoculated into the medium. The system was then left for a period of 16-20 hours to allow the organism to grow under batch conditions.

After the appropriate time had elapsed, the peristaltic pump was turned on and set at the desired flow rate (30-50 cc/hr). This then began the process of collecting samples. It involved collecting a suitable volume of broth (75-125 cc), recording its volume and the time taken. The pH, of the broth, and its optical density (absorbance), was then taken. The broth was centrifuged, the effluent discarded, resuspension of the cells with 0.85% Sodium Chloride

solution and centrifuged again discarding effluent. The sample was then labled and stored in the freezer until needed for analysis. Samples of broth were taken periodically throughout the entire length of the fermentation (approx. 75-100 hours). At the end of this time, the system was shut down, the apparatus cleaned and prepared for another run, if planned. For further details of this procedure and of the sampling procedure, refer to Appendix A.

ROUTINE ASSAY FOR ENZYME (L-ASPARAGINASE)

Once the samples were processed, as described previously, the amount of enzyme produced for that sample had to be determined. An assay of the sample was therefore performed so that the specific activity (International Units (I.U.) of enzyme per milligram protein) could be determined. The procedure used was as follows. (see Appendix A for further details of the procedure).

The cell pellets, obtained from each sample, were suspended in 0.1 M H_3BO_3 buffer (adjusted to pH 8.5 by NaOH) and sonicated to obtain a cell-free extract. Cell debris was removed by centrifugation for 30 min. at 24,000 x g at 4°C. The extracts were assayed immediately for L-asparaginase activity. The assays were conducted at 37°C. Liberated ammonia, by the hydrolysis of L-asparaginase, was determined with Nessler reagent. Protein determination was by the Biuret method using bovine serum albumin (fraction V) as the standard. Specific Activities were reported as international units of enzyme per milligram protein. An international unit is defined as that amount

of enzyme which catalyzes the formation of 1 micromole of ammonia per minute under the conditions of the assay. The assay was sensitive to 0.01 I.U. of enzyme activity.

DISCUSSION OF PLOTS

Calibration Curves

The two calibration plots made, for the pump, showed a linear relationship between the pump setting and the flow. The maximum flow obtainable using either of the two types of tubing was approximately 140-150 cc/hr. The tygon tubing was found to be adequate in the beginning of the experiment, but as time progressed it was found that tygon didn't hold up to either the sterilization procedure or the pump itself. Even by replacing the tubing for each run, problems still existed. It was decided; therefore, to switch to silicon rubber tubing. Although only two runs using this tubing were performed, previous test with it, prior to experimentation, proved it to have better withstanding properties than tygon. Silicon rubber can be repeatedly sterilized and used in the pump without it breaking down. It is therefore recommended to use silicon rubber tubing in all subsequent runs using the equipment as it stands now.

The other calibration plot made was that of cell

concentration versus optical density readings. Since optical density is known to be directly related to the cell concentration, the best straight line, for the points, by Least Squares Analysis, was drawn. Upon plotting, it was found that an optical density reading of 1.0 corresponds to a cell concentration of 0.69 mg dry weight cells/cc broth. This was for the Spectronic 20 spectrophotometer, wavelength 550 nm, used throughout all the runs (A-F). The other calibration curve represents that relationship between optical density and cell concentration for the Gilford Model 240 spectrophotometer, wavelength 550 nm. It shows that an optical density of 1.0 corresponds to a cell concentration of 0.4 mg dry weight cells/cc broth. Since the data for this plot was only one point, from Reference 3, it was assumed that for an optical density of 0.0, zero cell concentration was present. A straight line was then drawn, connecting these two points and the plot obtained. This plot was used to convert the data from the batch culture run to comparable units used in the continuous runs. All these plots are in Appendix D.

Optical Density versus Fermentation Time

Presented in Appendix F arethe plots of optical density versus fermentation time. These plots are a graphical representation showing the effect time has on the growth of the organism (Vibrio succinogenes) at the particular dilution rates noted. By definition, a chemostat is operated at a dilution rate which is below μ_{max}, determined from the batch culture. It was determined from the batch system (3) that the doubling time (generation time) was about 75 minutes. Therefore, solving for μ_{max}, from equation (12), one finds that the maximum dilution rate (μ_{max}) for the chemostat is 0.55 hr^{-1}.

If one looks at the plot for run A, the following can be noted. First of all, it was operated at two different dilution rates below μ_{max}. This run informs one that operating the chemostat at a dilution rate of 0.2 hr^{-1} will result in steady state (no change in optical density) being reached sooner. This was therefore the basis for subsequent runs having for their dilution rates a value of approximately 0.2 hr^{-1}.

The plot of run B shows that the optical density remained at a fairly constant value throughout the run.

From the results of run A, one dilution rate was chosen at a value of approximately 0.2 hr^{-1} . Unfortunately, the samples obtained for this run were ruined so no enzyme assays were obtainable. A similar run, using the same medium was performed later on, labeled as run C.

The plot on run C shows that the optical density remained at a constant value throughout the run. This run was compared with the batch system performed (3). It can be noted that the optical density in the batch run reached a value of about 1.0; whereby, the continuous run was below this value. Since there was an excess of ammonium formate in the batch run (100 mM) and the continuous run had an ammonium formate concentration of 75 mM, it shows that ammonium formate was the limiting nutrient.

The plot of run D showed somewhat opposite results than that of run A. Using the dilution rate, which run A showed to give steady state readings, a dilution rate of 0.2 hr^{-1} showed an indication of a washing out of the organism. Decreasing this rate to 0.085 hr^{-1} had the organism regrowing. This is somewhat unexplainable, but could be due to a chemical effect of either the organism or the medium. Due to this erractic behavior of the

sodium medium, it was decided to discontinue these runs and concentrate on the ammonium medium. These growth plots are shown in runs E and F.

The plots of runs E and F, however, again show results which are unexplainable at this time. The optical density in both cases was decreasing with time. These plots are interesting in the fact that their mediums were similar to run C and their dilution rates were similar. A question as to why the organisms optical density decreased is still unanswerable.

Wash Out Plots

A wash out plot of each run was made and compared with its theoretical wash out plot (Appendix G). With these plots, an indication as to whether the organism was washing out or not could be determined. For the plot of run A, one can see that initially the organism seemed to be washing out, but quickly recovered and steadily increased.

The wash out plots for run B and C shows what a good chemostat run should look like. In determining the specific growth rate, one knows that the slope of the actual plot minus the slope of the theoretical wash out plot (dilution rate) will give the specific growth rate (1). Runs B and C had a specific growth rate equal to approximately their dilution rates, which is the desired growth rate.

The plot of run D is explainable only in the fact that a definite decrease occurred with a dilution rate of 0.2 hr^{-1}. With a lower dilution rate, the growth rate increased. Explanations for this are still unknown.

The wash out plot of run E shows two effects. The

one plot, which had a dilution rate of 0.204 hr^{-1}, showed a definite washing out of the organism since the actual plot paralleled the theoretical one. For the other plot, dilution rate 0.196 hr^{-1}, the specific growth rate was 0.053 hr^{-1} initially then changed to 0.154 hr^{-1} for the duration of the run.

The plot of run F had only one dilution rate equal to 0.214 hr^{-1}. Its specific growth rate was 0.19 hr^{-1} then changed to 0.154 hr^{-1}.

Both of these plots (E and F) did show that some growth of the organism was occurring, but at a very low growth rate related to its dilution rate, the desired growth rate.

Comparison Plot

In order to determine whether more enzyme was produced through a batch fermentation or a continuous fermentation, a comparison plot was made which plotted international units of enzyme per cc broth versus fermentation time. One can see from the plot that, over the same time span, the continuous run produced more enzyme than the batch. This result is only an estimate of what is actually happening. Since the batch run had a higher concentration of ammonium formate than the continuous run, some sort of normalization of the two runs had to be done. First of all, the time factor had to be eliminated as well as differences in concentration of the ammonium formate. Table XXIV shows this comparison.

As shown, the continuous run produced almost double the amount produced by the batch fermentation.

OVERALL DISCUSSION

Studies on the batch fermentation of Vibrio succinogenes and its production of asparaginase were performed by Dr. D. Kafkewitz and associates at Rutgers University, Newark, N.J. Their studies showed that the maximum production of asparaginase, by using excess ammonium formate in their medium, yielded 17.40 I.U. enzyme per millimole ammonium formate. In order to determine the yield of asparaginase, by a chemostat, a continuous fermentation assembly was constructed at New Jersey Institute of Technology, Newark, N.J. The medium used in the fermentation was similar to that used in the batch fermentation. Results showed that a production of enzyme of 31.83 I.U. enzyme per millimole ammonium formate was obtained. This is about double the production rate of batch indicating that if one is interested in producing the most amount of enzyme one should use a continuous fermentation.

This, however, is only a preliminary study on this fermentation. No economic study for either case was determined. This would include such items as cost of

the equipment and the turn around time for batch operations versus continuous. If one were to do this, it would be based on one year of operation of the two plants. The difference between the two yields does open up this other avenue of producing the enzyme (continuous fermentation) and makes it one to be considered strongly.

The negative side of this argument, is that experienced when runs E and F were performed. Only slight differences in their mediums were made, but the fermentation runs just did not produce the organism as it did in run B and C. No logical explanation on their results can be drawn and only sheer speculation on what caused such results can be made. These include a chemical imbalance in the mediums unrelated to the proper pH, which was tested in each case and found to be within limits (7.3-7.5). This imbalance could be due to unseen contamination which only inhibited the growth of the organism, but didn't curtail it completely. Investigations into this possibility would probably be a waste of needed experimentation time and only the following is recommended: purchase new and fresh chemicals, namely the ammonium formate and fumaric acid.

The sodium formate medium, runs A and D, gave results worth reporting, but only proved, as in Reference (3), that sodium formate does not give as good a production of the enzyme as ammonium formate.

CONCLUSION

Based on the present data correlated, it has been decided that a continuous fermentation of the organism, Vibrio succinogenes, produced a greater yield of the enzyme, asparaginase, than did its counterpart, the batch fermentation. This was based on the data obtained from run C which was a duplicate of run B, whose samples were ruined. Further studies on the economics of the two processes must be performed and compared if one is to make a viable study of the processes for later production of the enzyme.

It has been decided, that the peculiar readings of runs E and F were due mainly to a possible chemical contamination within the medium which could have inhibited the growth of Vibrio succinogenes and not the operational parameters of the chemostat itself. The chemical contamination is only speculation and further investigation on this will just prove to be superfluous and not worth the time and expense. Further studies done on this organism would require the purchasing of new chemicals for the medium; namely, ammonium formate and fumaric acid.

The higher production rate, of the enzyme, in the continuous fermentation compared to the batch fermentation, opens up some interesting avenues to be followed for actual production of the enzyme, asparaginase. The yield of the continuous fermentation, being almost double that of batch fermentation, makes this process a viable alternative.

RECOMMENDATIONS FOR IMPROVEMENTS OF EQUIPMENT

Throughout the course of the fermentation studies, various malfunctions and problems with the existing equipment occurred. Examining the apparatus, one can find and list various improvements which can be incorporated into the assembly. No cost for these improvements will be given since it will depend on when they are made and from which dealer one will purchase the necessary equipment. A list of improvements is given below.

1) An improved peristaltic pump in which the flow rates could be controlled and maintained throughout the run.

2) An improved temperature controller and method for heating the fermentation broth to the desired temperature. The new temperature controller should use an electric relay switch instead of the mechanical one presently used. This mechanical relay has the disadvantage of malfunctioning in high humidity conditions.

3) For the present system, the use of silicon rubber tubing for the pump is recommended since the tygon tubing broke down during sterilization and continuous pumping. Even periodic replacement could not always be counted on to insure trouble free operation.

4) The installation of a temperature controller on the autoclave. The present system has none and requires constant monitoring of the

temperature of the autoclave and constant adjustment in its controls. A temperature controller would allow the experimenter to perform other duties while the apparatus is being sterilized.

FUTURE WORK RECOMMENDED

The following recommendations are a list of suggestions for future work on a continuous fermentation using the organism, Vibrio succinogenes, and its production of the enzyme asparaginase. Future work is listed as follows:

1) An economic study on the continuous fermentation compared to batch operations. This would involve computer simulations of the two processes based on operating conditions used in this work.

2) With the purchase of a new peristaltic pump, the variation of the dilution rate and its relationship on the production of asparaginase investigated.

APPENDIX A

OPERATING MANUAL

OPERATING MANUAL

Maintenance of Organism

Vibrio succinogenes is a rumen anaerobe that obtains its energy by coupling the oxidation of either hydrogen or formate to the reduction of either fumarate or nitrate (4). The organism was isolated by Wolin et. al. (3) and has been maintained at Rutgers University by Dr. D. Kafkewitz for a number of years. Vibrio succinogenes was obtained from M.J. Wolin at the University of Illinois and has been kept viable as described by Wolins' paper (1961). This culture, unlike others obtained, which are normally in a freeze -dried state and must be rejuvenated, was received in a suitable culture medium developed by Wolin. The composition of this medium follows.

When performing biological work, involving micro-organisms, these organisms must be grown in a medium which meets certain requirements in order to insure proper growth. It must maintain the organism over a period of time before it must be transferred to another vessel or culture tube, containing fresh medium. This transfer was done by dipping a sterile wire loop into the tube

containing the organism, in its medium, awaiting transfer. Sterilization of the wire loop was done either by sticking it in a flame until red hot, then cooled by touching it on the inside of the culture tube, or by inserting it in an electric heating element and performing the same steps as before.

The medium consisted of $(NH_4)_2SO_4$, 0.1%; K_2HPO_4, 0.5%; fumaric acid, 0.3%; sodium formate, 0.3%; yeast extract (Difco), 0.1%; $MgCl_2 \cdot 6H_2O$, 0.02%; and $FeSO_4$, 0.001%. The pH was set at 7.0 to 7.2. Sterile, autoclaved sodium thioglycolate (Difco) was added aseptically before inoculation to a final concentration of 0.05% and provided sufficient anaerobiosis for routine transfers. This medium was placed in culture tubes and kept frozen until ready for use.

The organism, Vibrio succinogenes, once inoculated into each tube, was incubated for 24 hours at 37°C then stored in a refrigerator at 4°C until ready for use. One thing to note, however, is that freezing of the organism will result in its death. The organism was transferred to fresh medium every 7-10 days so as to maintain its viability for future experimentation.

Weights of Materials for Runs

The tables, on the following pages, are a listing of the chemical composition for the mediums used in each run (A to F).

TABLE I

Medium For Run A: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Sodium Formate	28.111	0.0752 M
Fumaric Acid	70.245	0.11 M
Yeast Extract	44.005	8.gm/liter
L(+)-Cysteine Hydrochloride	2.757	0.5 gm/liter
Sodium Chloride	8.033	0.025 M
Sodium Hydroxide	44.08	2 equivalents NaOH/ mole Fumaric Acid

TABLE II

Medium For Run B: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Ammonium Formate	26.027	0.075 M
Fumaric Acid	70.253	0.11 M
Yeast Extract	44.01	8. gm/liter
L(+)-Cysteine Hydrochloride	2.765	0.5 gm/liter
Ammonium Chloride	7.359	0.025 M
Sodium Hydroxide	44.098	2 equivalents NaOH/ mole Fumaric Acid

TABLE III

Medium For Run C: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Ammonium Formate	26.06	0.0751 M
Fumaric Acid	70.288	0.11 M
Yeast Extract	44.03	8. gm/liter
L(+)-Cysteine Hydrochloride	2.771	0.5 gm/liter
Ammonium Chloride	7.359	0.025 M
Sodium Hydroxide	44.03	2 equivalents NaOH/ mole Fumaric Acid

TABLE IV

Medium For Run D: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Sodium Formate	28.131	0.0752 M
Fumaric Acid	70.246	0.11 M
Yeast Extract	44.167	8.03 gm/liter
L(+)-Cysteine Hydrochloride	2.762	0.5 gm/liter
Sodium Chloride	8.037	0.025 M
Sodium Hydroxide	44.071	2 equivalents NaOH/ mole Fumaric Acid

TABLE V

Medium For Run E: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Ammonium Formate	34.693	0.10 M
Fumaric Acid	76.624	0.12 M
Yeast Extract	44.011	8. gm/liter
L(+)-Cysteine Hydrochloride	2.759	0.5 gm/liter
Sodium Hydroxide	48.036	2 equivalents NaOH/ mole Fumaric Acid

TABLE VI

Medium For Run F: (Basis: 5.5 Liters medium, pH 7.5)

Chemical	Amount (grams)	Composition
Ammonium Formate	34.68	0.10 M
Fumaric Acid	38.283	0.06 M
Yeast Extract	44.008	8. gm/liter
L(+)-Cysteine Hydrochloride	2.757	0.5 gm/liter
Sodium Hydroxide	26.111	2 equivalents NaOH/ mole Fumaric Acid

Preparation of Medium

Throughout all the continuous fermentation runs, a 5.5 liters medium volume was used. In order to save time for the runs, preparations to make the medium was done prior to the fermentation. This involved weighing the necessary chemicals and storing them in separate containers. The procedure used was as follows.

(1) The necessary chemicals, used for the run, were weighed out. (For the exact amounts used in each run see Tables I-VI)

(2) Each chemical was stored in an individual container and labeled.

(3) One (1) liter of distilled water was measured and poured into a 3 liter beaker.

(4) To this beaker, the yeast extract was added. This was done in order to aid in the dissolving of the yeast extract. It was mixed thoroughly.

(5) Distilled water, 2.5 liters, was added to another beaker which had a volume of at least 4 liters.

(6) To this, the sodium formate or ammonium formate was added and mixed.

(7) Next, the L(+)-cysteine hydrochloride and sodium chloride or ammonium chloride, depending upon the run was added to the mixture, in step (5) and mixed thoroughly.

(8) The yeast extract mixture was added to the mixture above. It was mixed until a uniform

color resulted. (Note: the resulting color was a golden yellow)

The next step, of the procedure, was to add the sodium fumarate (fumaric acid) to the mixture. Sodium fumarate comes as the free acid, fumaric acid and will not dissolve in the distilled water. Therefore, in order to facilitate this, it was neutralized with sodium hydroxide. The fumaric acid is a dicarboxylic acid and requires two equivalents of sodium hydroxide per mole of fumarate. Depending upon the amount of fumaric acid used in each run determine the amount of sodium hydroxide used. The next step in the procedure was as follows.

(9) The mixture, in step (8), which now had a volume of 3.5 liters, had the fumaric acid and sodium hydroxide added and mixed until it was completely dissolved.

(10) The pH of the mixture was taken. On most runs the pH was found to be 4.5-5.5.

(11) The pH of the mixture was adjusted by adding sodium hydroxide solution (1 normal) until a pH of 7.5 was reached. It normally required approximately 200-300 cc of sodium hydroxide to meet the desired pH. Care was taken when adding the NaOH when reaching pH's above 7.0 since a possibility of an overshoot could have resulted.

(12) After the desired pH had been achieved, the volume of the mixture was adjusted to 5.5 liters. This was done by remeasuring the existing volume while pouring it into the 6 liter flask, which

served as the medium storage vessel. Adjustment was made by adding distilled water. The amount added depended upon the amount of NaOH added to the 3.5 liter mixture.

(13) When the final volume had been adjusted to 5.5 liters, the medium was mixed thoroughly. This was aided by pouring some of the mixture out into a beaker, mixing the remaining medium in the flask, then pouring all of it back into the 6 liter flask.

(14) Prior to sterilization of the medium, the flask was sealed with brown paper. Care was taken so as to tape the brown paper to the opening of the flask securely since during sterilization pressure built up inside the flask and without a tight seal, the paper could have popped off.

This, therefore, concludes the procedure for preparing the fermentation medium. The next step involved will be to sterilize it. This procedure is covered in the next section.

Apparatus Sterilization and Assembly

When one performs any type of biological work which requires asceptic conditions, the sterilization of all glassware, chemicals and associated equipment, which will come into contact with the microorganism, must be performed. This procedure must be capable of either destroying or reducing the population of any organism which could prove harmful to the microorganism under study. One such procedure, used in this experiment, was to sterilize the equipment and chemicals with steam under pressure. The use of an NBS (New Brunswick Scientific) Autoclave, Model No. 57114, was used. The unit consisted of a control switch which was used to set the desired temperature so that one had a check on the temperature and pressure of interest. For complete details on its operation, refer to the operations manual on the unit.

The conditions used for sterilization were 121°C, 15 psig, which are standard conditions recorded in all standard Biochemical Engineering texts. The sterilization of the continuous fermentation assembly, shown in Figure 1, was done in two (2) phases. On the day the fermentation run was to be started, a procedure was developed which made effective use of the time which was inherent of the run.

The procedure was as follows.

(1) The apparatus was assembled in three (3) sections (see Figure 2). Section one was the fermentor with the exit line. The second section was the piping and air filters leading from the medium storage vessel to the fermentor. The third section was the collecting vessel which collected the samples during the run.

(2) Each of these sections was wrapped in brown paper and sealed by taping the ends. This step was done carefully so that it resulted in a tightly wrapped package which wouldn't allow any contamination from reaching the apparatus during transfer from autoclave to the assembly sight.

(3) Next, in a flask, approximately 150-200 cc of distilled water was added and the top of the flask wrapped with brown paper. This water, which was sterilized, was used to complete the sterilization procedure used on the temperature probe. Its procedure for sterilization is covered later on in this section.

(4) All the glassware and tubing, described above, was placed in the autoclave. The proper level of distilled water covering the heating coils at the bottom of the autoclave was checked beforehand. This level had to be at least 3 inches above the coils.

(5) The top of the autoclave was closed and the wing nuts tightened. They were tightened in pairs, each pair opposite one another so that a uniform seal was insured. The relief valve, for controlling the pressure, was opened fully. The unit was plugged in and the control switch turned to the high position. The temperature was allowed to reach 100°C. At this time, the relief valve was closed. The temperature then rose to 121°C, 15 psig which was the desired level for proper sterilization. This entire process required approximately 45 minutes.

(6) During the initial warm up time, in the autoclave, the preparation of the medium was made. Refer to the section Preparation of Medium for details.

(7) When the desired temperature had been reached, the control switch was switched to medium. This step required the experimentor to remain within the area of the autoclave to insure that the temperature did not drop below 121°C. If it began falling the control switch was switched back to its high position until the temperature was again reached.

(8) The equipment was left within the autoclave to sterilize at this temperature for 20 minutes.

(9) At the end of this time, the switch was turned off and the temperature allowed to drop 3-5 degrees.

(10) When this was achieved, the pressure in the autoclave was vented by opening the relief valve located on the top of the unit. This was done slowly, otherwise the flask of water in the autoclave would have boiled. Also, it was checked that steam was exiting the valve opening at a constant rate. If pressure was not released and the autoclave was allowed to cool, the unit may have imploded. This was prevented by not loading the autoclave up to the top thus blocking the valve opening.

(11) When the temperature had fallen to 90°C and the pressure read 0. psig, the autoclave was opened and the contents removed. They were placed on a suitable table or box to insure noncontamination.

(12) The already prepared medium was then placed into the autoclave and the top closed. All necessary checks were done as before. The temperature control switch was set at the high position.

(13) When it reached 100°C, the relief valve was

closed. The temperature, inside the autoclave, was then allowed to reach 121°C, 15 psig at which time the control was set at the medium position. This required approximately 1 hour due to the volume of the medium. The temperature was maintained and controlled as before. The medium was sterilized for approximately 45 minutes.

During the time the medium was in the autoclave, the fermentor and associated piping were assembled. The temperature probe also underwent sterilization. The procedure used will now be given beginning with the sterilization of the temperature probe.

(14) In a graduated cylinder 100 cc of a 70% (by volume) ethyl alcohol solution was added.

(15) The alcohol was acidified by adding 1 drop of sulfuric acid (H_2SO_4) (concentrated).

(16) The temperature probe was immersed in this mixture. Care was taken not to immerse the probe beyond the wire located at the top of the probe.

(17) The probe was allowed to soak for 1 hour.

Assembly of the apparatus was done in three (3) phases. Care was taken to insure that sterile conditions were maintained at all times. Before beginning, a bunsen burner was lit and set to show a blue flame.

(18) The fermentor, with the exit line attached, was connected to the assembly via a clamp. (see Figure 1)

(19) The paper from the exit line stopper was unwrapped. It was left semi-covered while the paper from the collecting vessel was unwrapped totally.

(20) The opening of the collecting vessel was flamed. Then the paper was removed from the exit line stopper and the opening flamed.

(21) The exit line was connected to the collecting vessel by inserting the rubber stopper in its top. The vessel was clamped to a stand and placed in an ice bucket.

(22) The paper,from the inlet tubing to the fermentor, was partially removed. It was in such a state that one could remove the inlet tubing simply by grabbing the rubber stopper and lifting it out of itsprotective wrapping.

(23) The paper from the top of the fermentor was unwrapped. It was flamed with a bunsen burner then the inlet lines were quickly inserted. The rim of the fermentor was reflamed. Care was taken so as not to burn the tubing.

(24) The air filters were clamped to their respective clamps and connected to the nitrogen supply. This was done carefully, but didn't require flaming since any contaminates were trapped by the filter.

(25) The silicon tubing was placed over the peristaltic pump, but was not clamped in place.

(26) The inlet lines, to the medium, were gently clamped and left until the medium was ready to be connected to the assembly.

(27) When the 1 hour time span had elapsed for the temperature probe, it was gently lifted out of the graduated cylinder and rinsed with the sterile distilled water.

(28) The stopper, covering the temperature well, was

removed on the fermentor and the probe inserted into it. Care was taken not to let the probe touch anything that was not sterilized since contamination would have resulted.

When this procedure was completed, a check on the progress of sterilizing the medium was done. When the allotted time had passed for complete sterilization, 45 minutes at 121°C, 15 psig, the switch on the autoclave was turned to the off position. The same procedure used to vent off the pressure in the autoclave for the first sterilization phase was used here. When the autoclave had been vented and cooled, the top was opened and the medium removed. It was transported over to the assembly and connected to the other equipment as follows.

(29) The medium vessel was placed on a suitable support. This support was steady enough to hold the vessel for the entire run.

(30) The paper wrapping was removed from the top of the medium vessel. It was not taken off completely at this time.

(31) The inlet lines of the medium were unwrapped and while the paper from the medium vessel was removed, they were inserted into the vessel.

(32) The top lip of the vessel was flamed with a bunsen burner taking care not to burn the tubing.

(33) When the rubber stopper was securely in place, nitrogen was sparged into the medium in order to expel excess air.

(34) Nitrogen was sparged slowly into the medium at a rate of approximately one bubble every 2 seconds.

(35) When the medium had cooled slightly, the peristaltic pump was connected up by clamping down the line which had previously been lying on the pumping apparatus.

(36) Nitrogen was sparged into the fermentor at a very low flow rate (approximately 0.5 on scale).

This completes the sterilization and assembly of the continuous fermentation apparatus. The section to follow will describe the steps which were used for a typical run.

Procedure Used for a Typical Fermentation Run

After the steps, given in the previous section, were performed, one was almost ready to begin the fermentation run. Before the necessary steps were taken, the following had to be done. A 0.85% sodium chloride solution was made which was used to wash the cells taken from the broth medium. This was done by adding 17 grams of sodium chloride to 2 liters of distilled water and placing the mixture in the refrigerator. Once done, the following final steps were taken.

(1) The peristaltic pump was started and set at the highest setting (approximately 140 cc/hr).

(2) The medium was pumped into the fermentor. Some time was required until the first liquid began to come into the fermentor. The nitrogen supply was turned on slowly and aided in delivering the medium into the fermentor.

(3) When a sufficient amount of medium had been added to the fermentor (235 cc), the pump was turned off. It required approximately 2.5 hours before the fermentor had reached its working volume.

(4) When the required volume had been obtained, the heating lamp was plugged in and with the lamp under the fermentor's bottom and the controller set at the desired temperature (37°C), previously calibrated, began heating the medium to temperature.

(5) The system was checked to be sure that no leaks

were present. The ice bucket, which contained the collecting vessel, was filled with ice.

(6) When the medium had reached the desired temperature, inoculation of the organism (Vibrio succinogenes) was done. One knew when the temperature of the medium had reached the desired level when the heating lamp turned on and off periodically.

(7) The culture tube, containing the organism, was removed from the refrigerator and the nitrogen supply to the fermentor turned on at a setting of 8.0 on scale.

(8) The rubber diaphram, of the culture tube, was sterilized by moistening a cotton ball with 70% alcohol.

(9) A 3 cc syringe (Plastic Pack Disposable) was inserted into the culture tube and 0.25 cc of the inoculant withdrawn.

(10) The inoculant port, on the fermentor, was sterilized in the same manner as used on the culture tube.

(11) Via the syringe, the organism was inoculated into the fermentor by inserting it in the inoculant port.

(12) The date and time was recorded.

(13) A final check on the system was performed to be sure all systems checked. This included whether the temperature controller was working, that sufficient nitrogen was being supplied to the fermentor and that no leakage was occurring.

(14) The system was allowed to remain in this state for 16-20 hours to allow the organism to grow.

The organism was now grown under batch conditions.

The next phase of the operation was to turn on the pump and begin sampling. The steps used were as follows.

(15) After the appropriate time had elapsed, a check on the fermentor was done to see whether any growth had occurred. This was determined by noting an apparent change in the turbitity of the medium.

(16) If growth had occurred, the pump was turned on and set at the desired flow rate (most runs in this report used flow rates of 30-50 cc/hr). Time and date were recorded.

(17) The ice bucket was refilled with ice, dumping the water that had collected over the time period passed.

(18) The ice was placed around the collecting vessel so as to kill the organism upon collecting thus stopping further growth.

(19) Collecting samples of the broth began. A volume of at least 75-125 cc of the broth was required before any processing of the samples were performed.

Samples of the fermentation broth were now taken. The steps that follow, describes how the samples were processed so that enzyme assays of them could be taken. They are as follows.

(20) When a suitable volume of broth had been collected, the collecting vessel was disconnected and its contents poured into a 500 cc graduated cylinder.

(21) The time, date and volume collected were recorded.

(22) The cylinder was placed in ice to keep the contents cold.

(23) In a 50 cc beaker, approximately 40-45 cc of the broth was poured. This was used to record the pH of the broth.

(24) The pH of the broth was recorded using a standard pH meter. A Corning Model 610A with a Fisher Model combination electrode was used to record the pH. Calibrations were done for every sample using a 7.0 pH buffer solution.

(25) Next, the optical density of the broth was taken by using a Spectronic 20 spectrophotometer set at a wavelength of 550 nm. Zero absorbance was set by using distilled water as the blank. The readings were recorded.

(26) After the pH and the optical density of the broth were taken, the broth was centrifuged down.

(27) Exactly 30 cc of the broth was measured and poured into a centrifuge tube. It was then inserted into the centrifuge, making sure to balance it with another tube filled with distilled water and placed directly opposite the broth tube.

(28) The cells, contained within the broth, were centrifuged down by setting the controls on the centrifuge to their maximum setting and spun for 5-10 minutes.

(29) When completed, the centrifuge was turned off. When stopped, which was aided by placing ones hands (potected by gloves) on the sides of the centrifuge, a portion of the effluent (approx. 5 cc) was collected and the rest discarded.

(30) Another 30 cc of the broth was remeasured and poured in the same centrifuge tube. The cells

were spun down as before and all the effluent discarded. This step was repeated until all of the collected broth had been centrifuged. The total volume used was recorded.

(31) The cells, in the centrifuge tube, were re-suspended using a 0.85% NaCl solution previously prepared. The volume of salt solution used was 30 cc.

(32) The cells were centrifuged down as before and the effluent discarded.

(33) The samples were labeled with the date, time and sample number. The top, of the tube, was stoppered with a cotton plug and stored in the freezer along with the effluent of the broth (which had also been labeled).

(34) More ice was placed around the collecting vessel to make sure it was kept cold.

(35) The above procedure was repeated for subsequent samples when sufficient volume had been collected.

Samples of the broth were taken periodically throughout the entire run. Each run took approximately 75-100 hours to complete. At the end of this time, the system was shutdown by the following procedure.

(36) When the final sample had been taken and processed, the fermentor was shutdown.

(37) The pump and nitrogen supply was turned off.

(38) The heating lamp was unplugged.

(39) The temperature probe was taken out and placed back into the graduated cylinder containing the 70% alcohol. The probe was rinsed with distilled water before immersion in order to clean off the

broth.

(40) The apparatus was disassembled and all glassware cleaned in preparation for the next run, if one were planned.

(41) The silicon rubber tubing or the tygon tubing, used in the pump, was replaced.

This completes the procedure used in a typical continuous fermentation run. The samples, which were stored in the freezer, were assayed for enzyme (asparaginase). This was performed at Rutgers University by Dr. Kafkewitz. The procedure he used is described in the next section.

Routine Assay for Enzyme (L-Asparaginase)

As was described in the previous section, the samples collected were stored in the freezer. The samples were kept in this condition so as to perserve them until the assay test could be conducted. This test was performed at Rutgers University, Newark, N.J. by Dr. D. Kafkewitz. The procedure, for the assay, has been documented in the literature (2). The procedure used, in the article and hence by Dr. Kafkewitz, will now be described.

(1) A 0.1 M H_3BO_3 buffer was prepared and adjusted to a pH of 8.5 by adding NaOH.

(2) The centrifuge tubes were taken from the freezer and the cells, contained within them, were resuspended in the buffer.

(3) The cells were disrupted by sonicating the cell pellet suspension.

(4) The mixture was centrifuged so as to remove the cell debris. This was done for 30 minutes at 24,000 x g and 4°C.

(5) The supernatant was poured off and stored at 4°C.

(6) The sediment was resuspended in the buffer and centrifuged once again at the same conditions described in step (4).

(7) The supernatant was collected and combined with the previous one.

L-Asparaginase activity was measured by determining the amount of ammonia produced upon the hydrolysis of L-asparagine. The reaction mixture consisted of 5 to 200 µl of enzyme solution diluted to 2 cc with a 0.1 M borate buffer, pH 8.5.

(8) The reaction was initiated by adding 2 cc of the 0.08 M L-asparagine solution to the extract from centrifugation.

(9) The reaction mixture was incubated at 37°C.

If all the samples collected during the fermentation run were to be assayed all together, then steps (8) and (9) were repeated for each sample separately.

(10) Approximately 0.3 cc of sample, that was incubated, was removed at 1 minute and various intervals thereafter up to 30 minutes of incubation and placed in a suitable test tube. Approximately 5 samples were taken.

(11) The reaction was terminated in each tube by adding 0.3 cc of a 5% trichloroacetic acid and maintaining the samples in ice for at least 10 minutes.

(12) Any precipitate was removed by centrifuging the tubes for 10 minutes at 21,000 x g.

(13) A 0.2 cc aliquot, of the supernatant, was taken, placed in a test tube and diluted to 3.5 cc with distilled water.

(14) To this, 0.5 cc of Nessler's reagent was added. It sat at room temperature for 10-15 minutes.

(15) The absorbance of each tube was measured. This was done on a spectrophotometer set at a wavelength of 420 nm.

(16) A least squares analysis was done on the absorbance measurements versus time from when the enzyme solution was added to when the final sample was removed. The slope of this line was recorded.

Enzyme and substrate blanks were included in all assays along with a standard curve prepared with ammonium sulfate. Protein concentration was determined routinely by the Biuret Method using crystalline bovine serum albumin as the standard. The Biuret Method is a standard technique covered in all microbiology laboratory manuals.

The enzyme activity was expressed as international units (I.U.) enzyme per milligrams of protein. For all runs and those done by D. Kafkewitz (3), it was assumed that the cells contained 50% protein.

Procedure used for Calibration Curve
(Cell Concentration versus Optical Density)

When a typical run was performed, the absorbance (optical density) of the broth with respect to time was taken. A relation between optical density and cell concentration had to be determined so that the results would take on a meaniful relationship. Although a calibration curve of this type is a standard technique covered in most microbiology text, some modification of the technique was incorporated in order to faciliate the equipment on hand. The procedure used was as follows.

The first step, in the procedure, was to construct a small batch fermentor. The total volume of the fermentor was one (1) liter and had a working volume of 0.7 liters (700 cc). The set up is shown in Figure 3. The next step was to weigh out the necessary chemicals which would be used as the medium. Since the volume of the medium would only be 700 cc versus 5500 cc, used in each run, adjustments on the amounts used had to be made. Also, from previous work done on the organism, unpublished work by D. Kafkewitz, it had been found that the volume of cells produced did not change sufficiently by the use of either of the mediums used in this report. Therefore, the following

medium was decided upon to be used in constructing the calibration curve. It is shown below.

Chemical	Amount (grams)	Composition
Ammonium Formate	3.3166	0.0751 M
Fumaric Acid	8.9460	0.1101 M
Yeast Extract	5.6057	8.01 gm/liter
L(+)-Cysteine Hydrochloride	0.3545	0.51 gm/liter
Ammonium Chloride	0.9380	0.0251 M
Sodium Hydroxide	6.0395	2 equivalents NaOH/ mole Fumaric Acid

Preparation of the medium was done as is described in the section labeled Preparation of Medium. The rest of the procedure varied slighly and will be described.

(1) Once the medium had been prepared, it was poured into the 1 liter fermentor. The nitrogen inlet lines and inoculation port assembly were inserted.

(2) The air filter was connected and the hosing secured with copper wire.

(3) The top of the fermentor was wrapped with brown paper. It was wrapped securely so that the top did not pop off during sterilization.

(4) A 250 cc flask was filled with distilled water and the top wrapped securely. This was used to rinse the temperature probe. The sterilization

of this probe was the same as was done previously. Refer to the section Apparatus Sterilization and Assembly, steps (14)-(17) for details.

(5) The fermentor, containing the medium, and the distilled water was placed in the autoclave. The necessary checks on the autoclave, as was done previously, was made.

(6) The top of the autoclave was closed, the unit plugged in and the control switch set to the high position. The relief valve, on the top cover of the autoclave, was opened fully.

(7) The temperature was brought up to 100°C. It remained there for a few minutes to ensure that all the air had been released via the relief valve.

(8) The valve was closed and the temperature brought up to 121°C, 15 psig.

(9) When the temperature had been reached, it was controlled by switching the control switch to the medium position.

(10) The temperature was maintained and the contents sterilized for 40 minutes at 121°C, 15 psig.

(11) When completed, the valve was opened and the pressure released slowly. It was cooled to 90°C then the autoclave was opened and the contents removed (fermentor assembly and distilled water).

(12) The paper from the fermentor was removed and the fermentor clamped to the assembly alone with the connection of the air filter to the nitrogen supply. (see Figure 3)

(13) Nitrogen was sparged into the fermentor at a sufficient rate to ensure complete saturation.

(14) The temperature probe was inserted into the fermentor. The probe was rinsed with the sterile

distilled water before inserting it into the fermentor.

(15) The heating lamp was plugged in and the medium brought to a temperature of 37°C. Note, some time was required to do this since the contents had to cool down. This was speeded up by rubbing ice on the outside of the fermentor. When this was done, the lamp was moved out of the way so that no ice could fall on it thus causing a short.

(16) When the desired temperature had been obtained, noted by the heating lamp turning on and off at regular intervals, the inoculation of the organism was ready to commence.

(17) The same procedure, as was described previously (see steps (8)-(12) in the section Procedure used for a Typical Fermentation Run), was used to inoculate 0.75 cc of the organism, Vibrio succinogenes, into the fermentor via the inoculation port.

(18) When all systems checked, no leakage, nitrogen supply adequate, the system was left to grow for approximately 20 hours.

At the end of this time, a check on whether any growth had occurred was made by noting a change in the tubitity of the broth. Then the heating lamp was turned off, the fermentor unclamped and immersed in ice to stop the growth. Then the following steps were performed.

(19) A sample of the broth was taken and its optical density measured on the Spectronic 20 set at 550 nm wavelength.

(20) This sample, which had a volume of 60-100 cc,

was poured into a centrifuge tube and the cells spun down. The total volume used was recorded.

(21) The effluent was discarded and the sample labeled. It was stored in ice.

(22) Next, another sample from the broth was taken and diluted with a 0.85% sodium chloride solution. The dilution was such that when the optical density was taken, a range of readings were obtained. An example was 50 cc of culture and 10 cc salt solution gave an optical density of 0.58 compared to an optical density of 0.7 obtained for the broth medium alone.

(23) The cells were spun down and the effluent discarded once again. The volume used was recorded and the sample labeled.

(24) Steps (22)-(23) were repeated for a total of 6-7 samples.

(25) When completed, the culture tubes, containing the cells, were placed in an oven set at 50°C. This was a preliminary drying so that the cell masses may be removed from the centrifuge tubes.

(26) The tubes remained in the oven for approximately 24 hours.

(27) At the end of this time, the tubes were removed and the cells collected and placed in weighing dishes for further drying. Each dish was labeled with the sample number.

(28) The drying dishes, containing the cells, were placed in an oven set at 95-100°C. The temperature did not exceed 110°C; otherwise, the cells would have burned.

(29) They were dryed for 3-5 hours.

(30) The cells were removed from the oven and weighed.

(31) The weight of cells, for each sample, were obtained. This was done by weighing the containers, containing the cells, first, then washing them completely off the plates and reweighing. Cell weight was obtained by subtraction. When the cells were removed from the oven, their weights were taken as soon as possible. If one were unable, then the cells were stored in a desiccator since dry cells absorb water rapidly.

With the weight of the cells known and the volume of broth centrifuged to collect that amount of cells, the relationship between optical density and cell concentration for the spectrophotometer being used was obtained. The data used, to plot the calibration curve in this experiment, was as follows.

Sample Number	Optical Density	Cell Concentration (mg dry weight/cc broth)
60	0.70	0.407
61	0.58	0.383
62	0.48	0.295
63	0.38	0.256
64	0.25	0.09
65	0.13	0.049

A plot was made of optical density versus cell concentration. Since there exist a direct relationship

between optical density and cell concentration, a straight line was drawn. A least squares analysis on the points was made. Refer to Appendix B to see the results of the analysis.

Procedure used for Calibrating Peristaltic Pump

Throughout all the fermentation runs, the use of a peristaltic pump was used to achieve flow of medium to the fermentor. Before one could use this pump, it had to be calibrated so that the relationship between pump settings and actual flow (cc/hr) could be noted. This calibration was done in the following manner.

First of all, a vessel containing distilled water was placed on the assembly bench and tubing leading from it to the pump was placed. The water pumped was collected in a graduated cylinder so that the volume could be recorded.

Next, the pump was turned on and set at various pump settings. Then by noting the time elapsed and the volume collected over that time, the flow rate (cc/hr) versus pump setting could be constructed.

There were two such plots made. One for 3/16" I.D. tygon tubing (S-50-HL), and the other for 3/16" I.D. silicon rubber tubing. During the course of the runs, it was found that tygon tubing broke down during sterilization thus causing leakage problems and collaspsing of the tubing in the pump. It was therefore decided to go to silicon

rubber tubing which gave no such problems and performed adequately. The data for the two calibration curves are given.

For 3/16" I.D. tygon tubing (S-50-HL)
Used for Runs A-D

Pump Setting	Volume Collected (cc)	Time (minutes)	Flow Rate (cc/hr)
4.0	13.	20.	39.0
6.0	26.	20.	78.0
8.0	39.	20.	117.0
10.0	49.	20.	147.0

For 3/16" I.D. silicon rubber tubing
Used for Runs E-F

Pump Setting	Volume Collected (cc)	Time (minutes)	Flow Rate (cc/hr)
4.0	14.	20.	42.0
4.5	25.	30.	50.0
5.0	20.	20.	60.0
5.5	23.	20.	69.0
7.0	33.	20.	99.0
9.0	34.	15.	136.0
10.0	37.	15.	148.0

The plot was therefore made of pump setting versus flow rate and the best straight line was drawn thus establishing the calibration curve.

Procedure used for Calibrating Temperature Controller

In all fermentation runs performed, the temperature of the medium had to be maintained at 37°C. This was done by a thermocouple connected to a temperature controller. Before this piece of equipment could be used it had to be calibrated. The procedure used was one of a One Point calibration procedure. It involved the following.

A flask (approximate volume 500 cc) was filled with 240 cc distilled water and clamped in place. The heating lamp was then placed beneath it and the temperature probe immersed in the water. A thermometer was then placed in the water and the lamp/controller plugged in an electric outlet. The controls, on the controller, were then set at various settings until the right ones were found which maintained the water at 37°C. The settings were then noted and left untouched throughout the run. Periodically, this procedure was repeated in order to check the settings and make any adjustments necessary.

APPENDIX B

FERMENTATION ASSEMBLIES

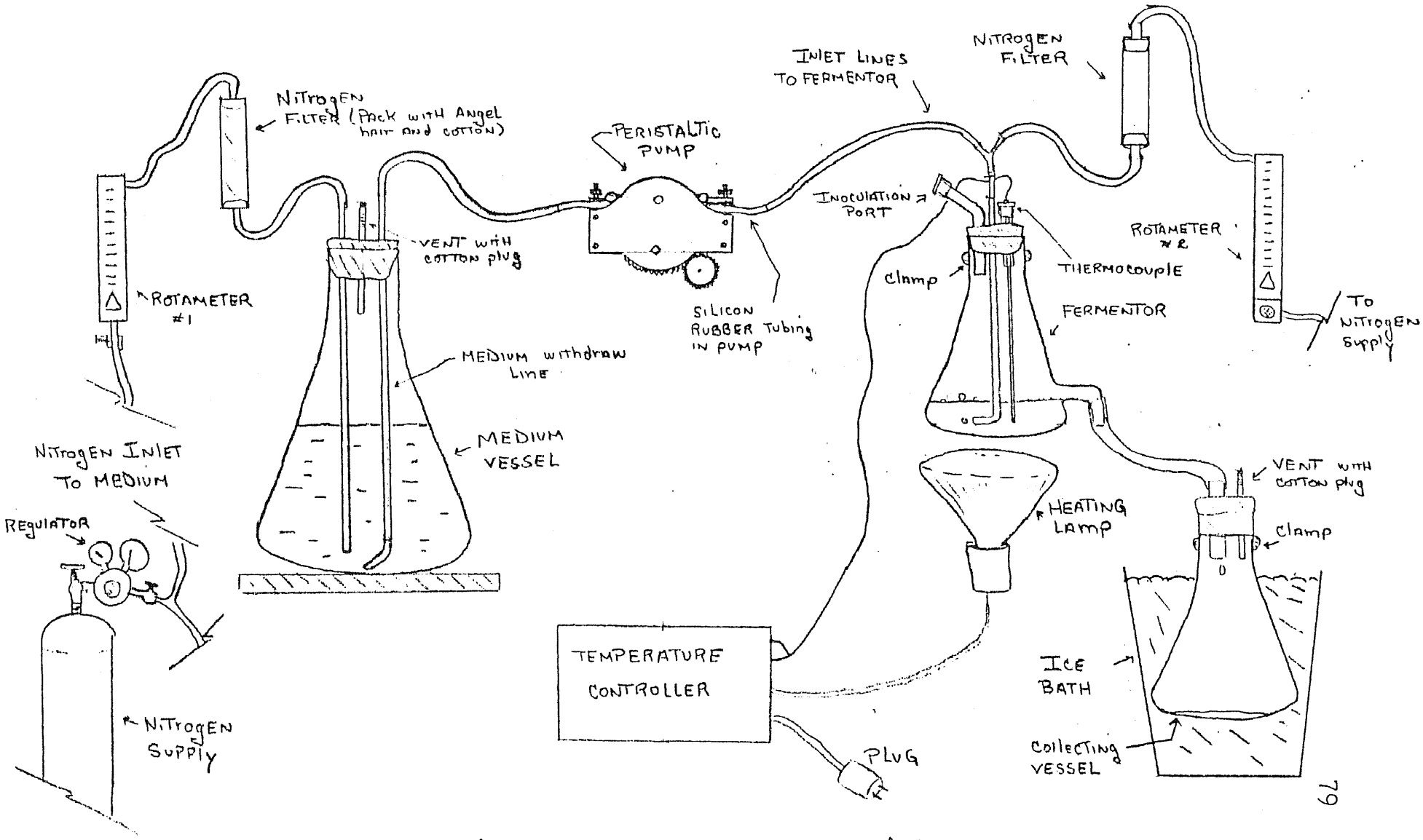


FIGURE 1 : CONTINUOUS FERMENTATION APPARATUS.

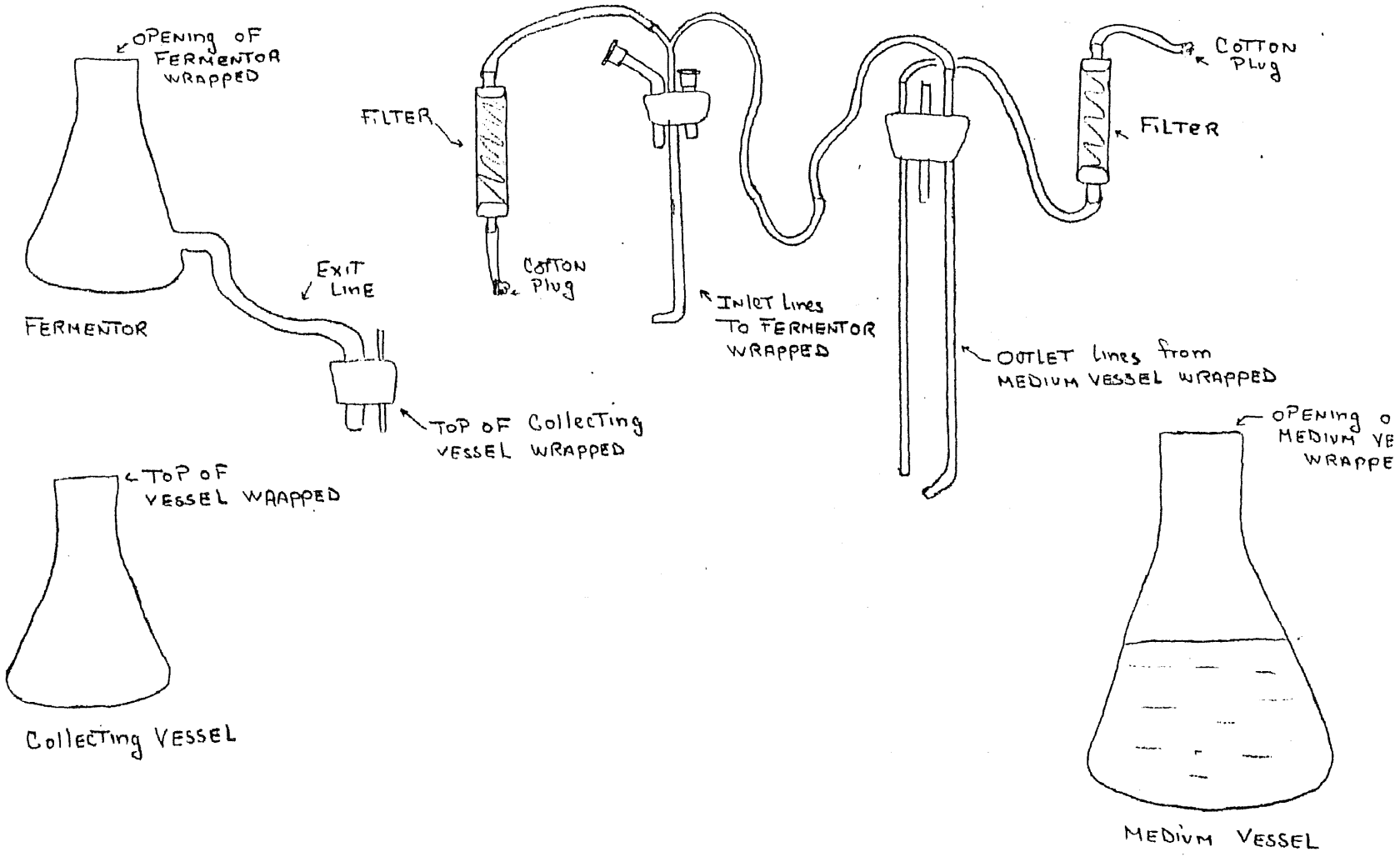


FIGURE 2 BREAKDOWN OF APPARATUS FOR STERILIZATION

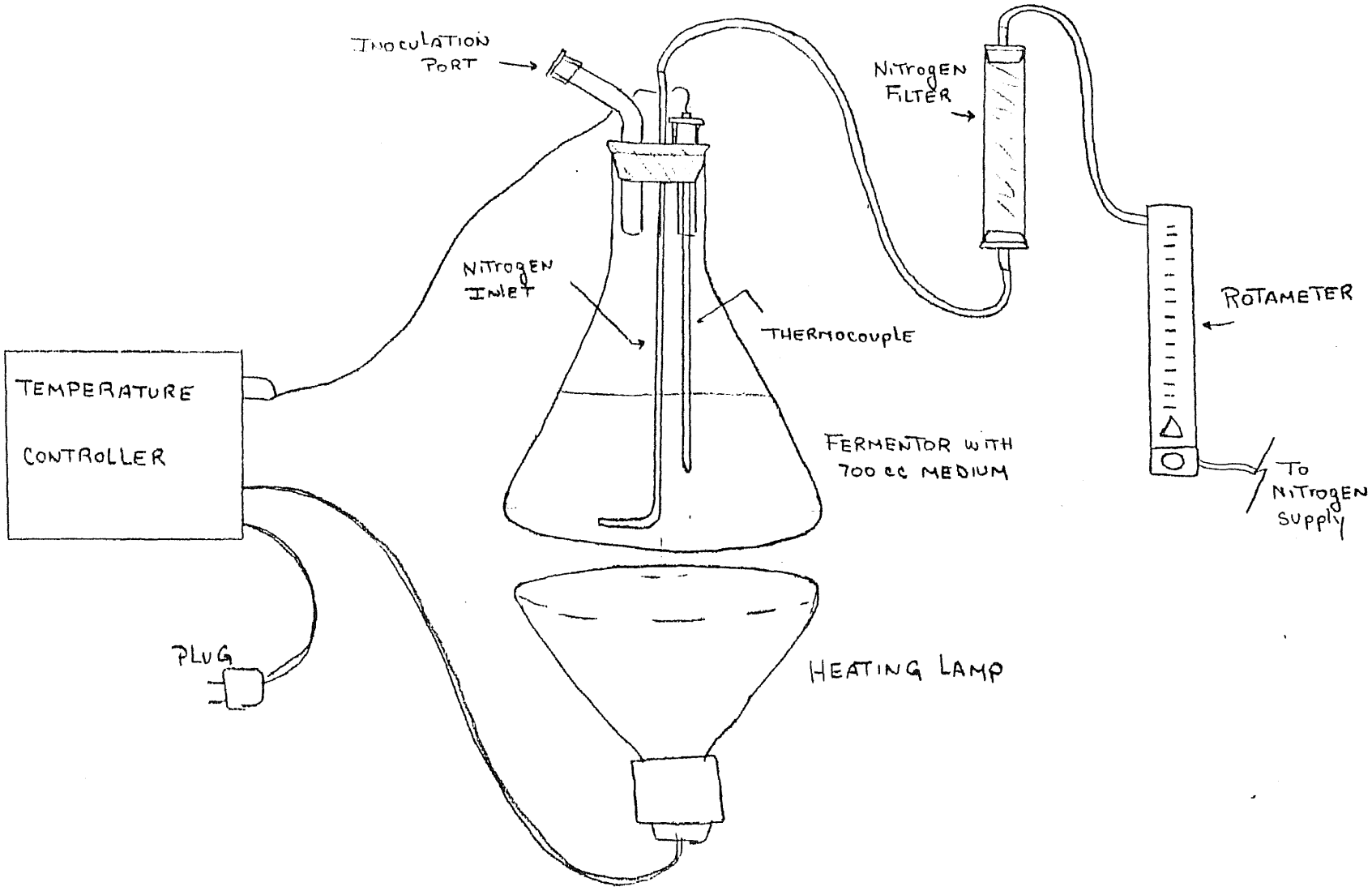


FIGURE 3: BATCH FERMENTATION APPARATUS FOR CALIBRATION CURVE

APPENDIX C

SAMPLE CALCULATIONS

SAMPLE CALCULATIONS

Dilution Rate

$$w = W/V_o \qquad (1)$$

* working volume $V_o = 235.$ cc (constant)

For Run A: sample #1 $W = 32.4$ cc/hr

$w = (32.4 \text{ cc/hr})/(235. \text{ cc}) = 0.138 \text{ hr}^{-1}$

Theroetical Wash Out Curve

$$\text{Ln}C = \text{Ln}C_o - wt \qquad (10)$$

For Run F:

$C_o = 0.295$ mg dry weight/cc broth

$w = 0.214 \text{ hr}^{-1}$

intercept of theoretical plot = 0.295 mg dry weight/cc broth
slope of plot = -0.214 hr^{-1}

Specific Growth Rate

For Run F:

slope of actual curve, $\mu_a = 0.404 \text{ hr}^{-1}$

slope of theoretical curve, $\mu_t = 0.214 \text{ hr}^{-1}$

specific growth rate, $\mu = \mu_a - \mu_t = 0.404 - 0.214$

$= 0.19 \text{ hr}^{-1}$

International Units of Enzyme per cc Broth

For Run C: sample #28

C = 0.355 mg dry weight/cc broth

S.A.=9.1 I.U. enzyme/mg protein

*assume 50% protein per cell

$$\frac{\text{I.U. enzyme}}{\text{cc broth}} = \left(0.355 \frac{\text{mg dry weight}}{\text{cc broth}}\right) * \left(0.5 \frac{\text{mg protein}}{\text{mg cell}}\right) * \left(9.1 \frac{\text{I.U. enzyme}}{\text{mg protein}}\right)$$

$$= 1.62 \text{ I.U. enzyme/cc broth}$$

S.A. specific activity
I.U. international units

International Units of Enzyme per mM Ammonium Formate

For Run C: Continuous Fermentation

I.U. enzyme (cumulative) = 6576.15 I.U. enzyme (Table XXX)

Total volume used = 2.755 liters medium (Table XII)

Concentration ammonium formate =
75 mM ammonium formate/liter medium

I.U. enzyme/mM ammonium formate =

(6576.15 I.U. enzyme)*(1/2.755 liter medium)*
(liter medium/75 mM ammonium formate)

= 31.83 I.U. enzyme/mM ammonium formate

For Run C': Batch Fermentation

I.U. enzyme/cc broth = 1.74 I.U. enzyme/cc broth (Table XXIX)

Concentration ammonium formate =
100 mM ammonium formate/liter medium

I.U. enzyme/mM ammonium formate =

$$\left(1.74 \frac{\text{I.U. enzyme}}{\text{cc broth}}\right) * \left(\frac{1000 \text{ cc broth}}{\text{liter broth}}\right) * \left(\frac{\text{liter broth}}{100 \text{ mM ammonium formate}}\right)$$

= 17.40 I.U. enzyme/mM ammonium formate

APPENDIX D

CALIBRATION PLOTS

FIGURE 4

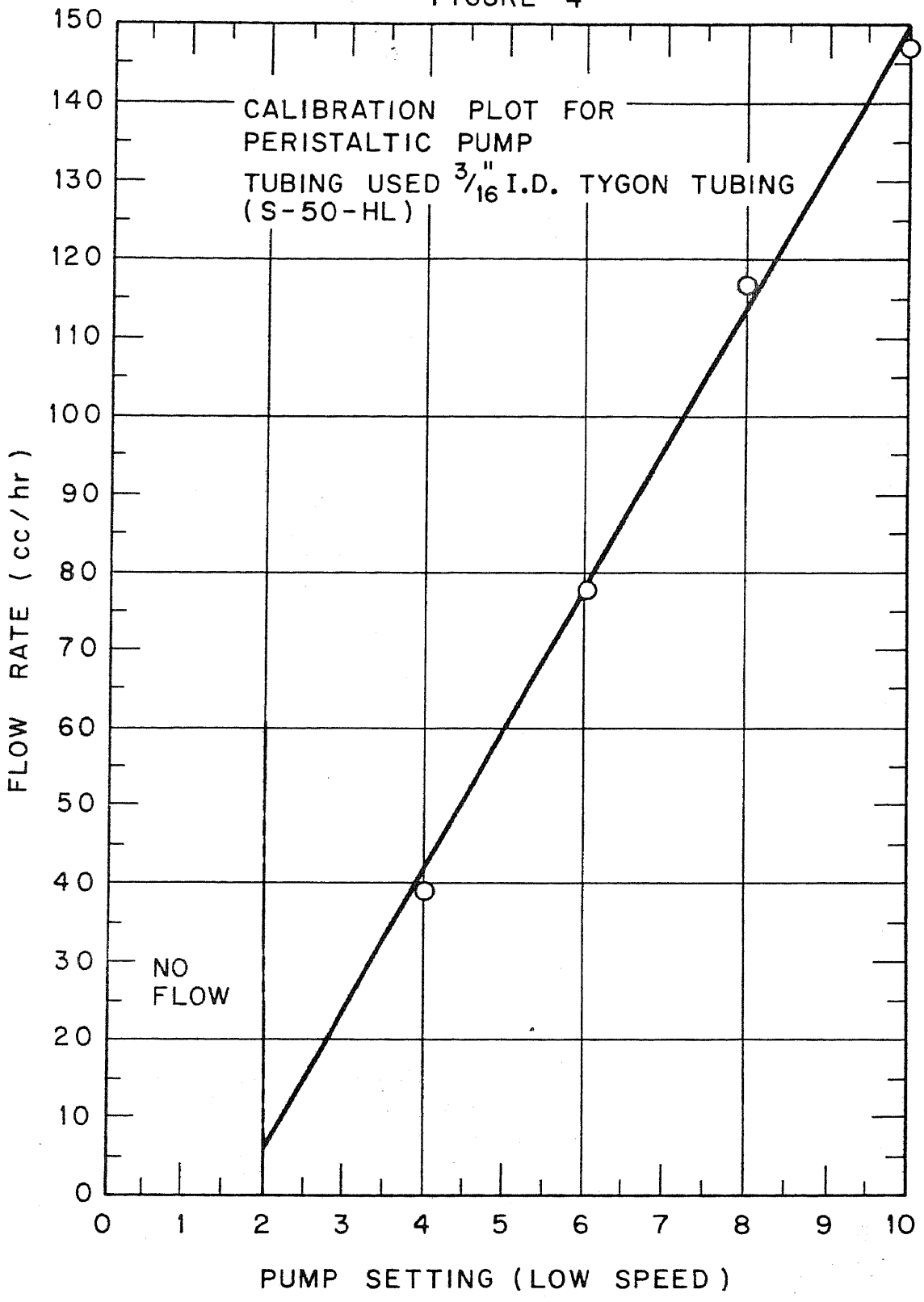

CALIBRATION PLOT FOR
PERISTALTIC PUMP
TUBING USED 3/16" I.D. TYGON TUBING
(S-50-HL)
NO
FLOW
FLOW RATE (cc/hr)
PUMP SETTING (LOW SPEED)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
0
1
2
3
4
5
6
7
8
9
10

FIGURE 5

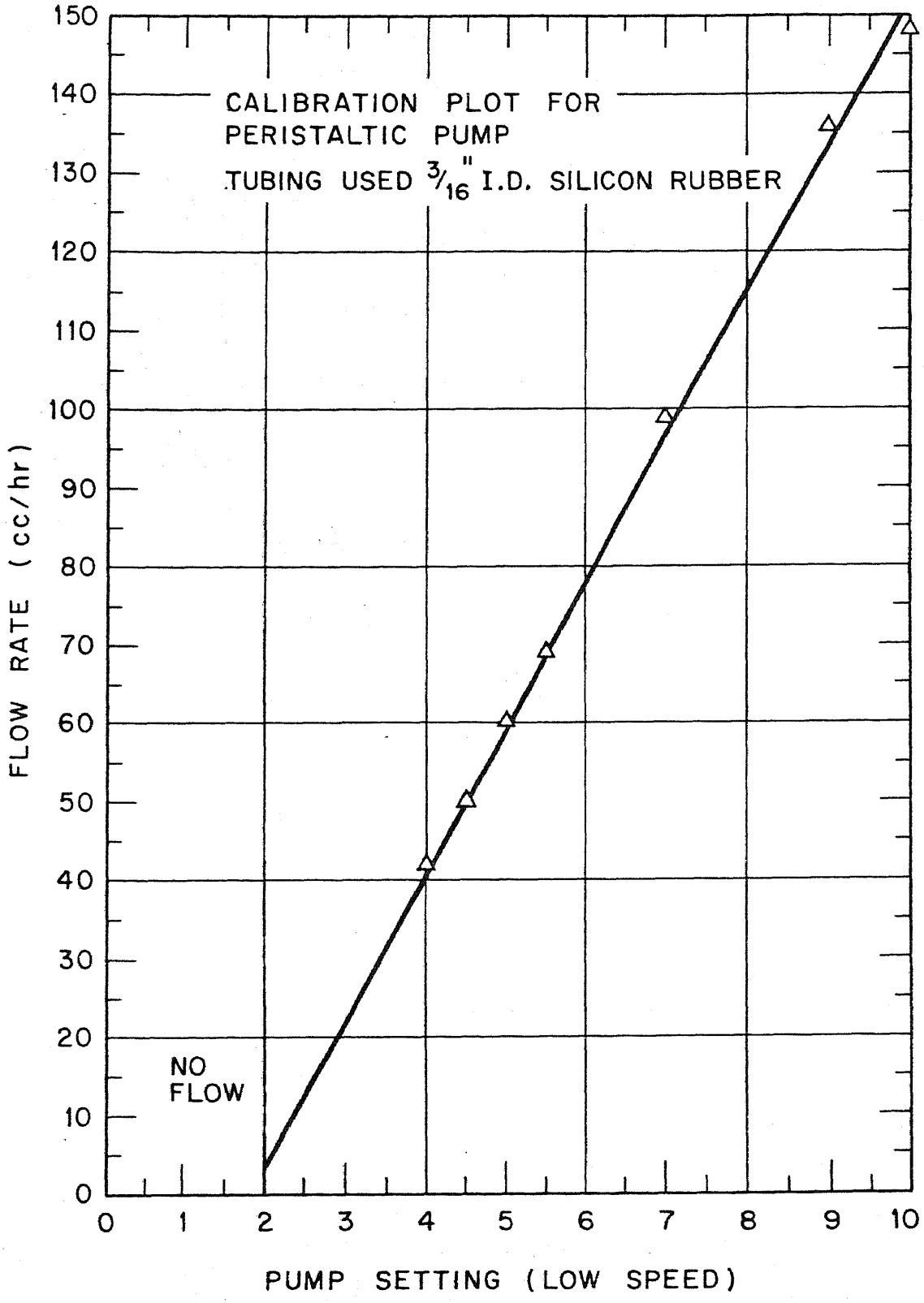

CALIBRATION PLOT FOR
PERISTALTIC PUMP
TUBING USED 3/16" I.D. SILICON RUBBER
NO
FLOW
FLOW RATE (cc/hr)
PUMP SETTING (LOW SPEED)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
0
1
2
3
4
5
6
7
8
9
10

FIGURE 6

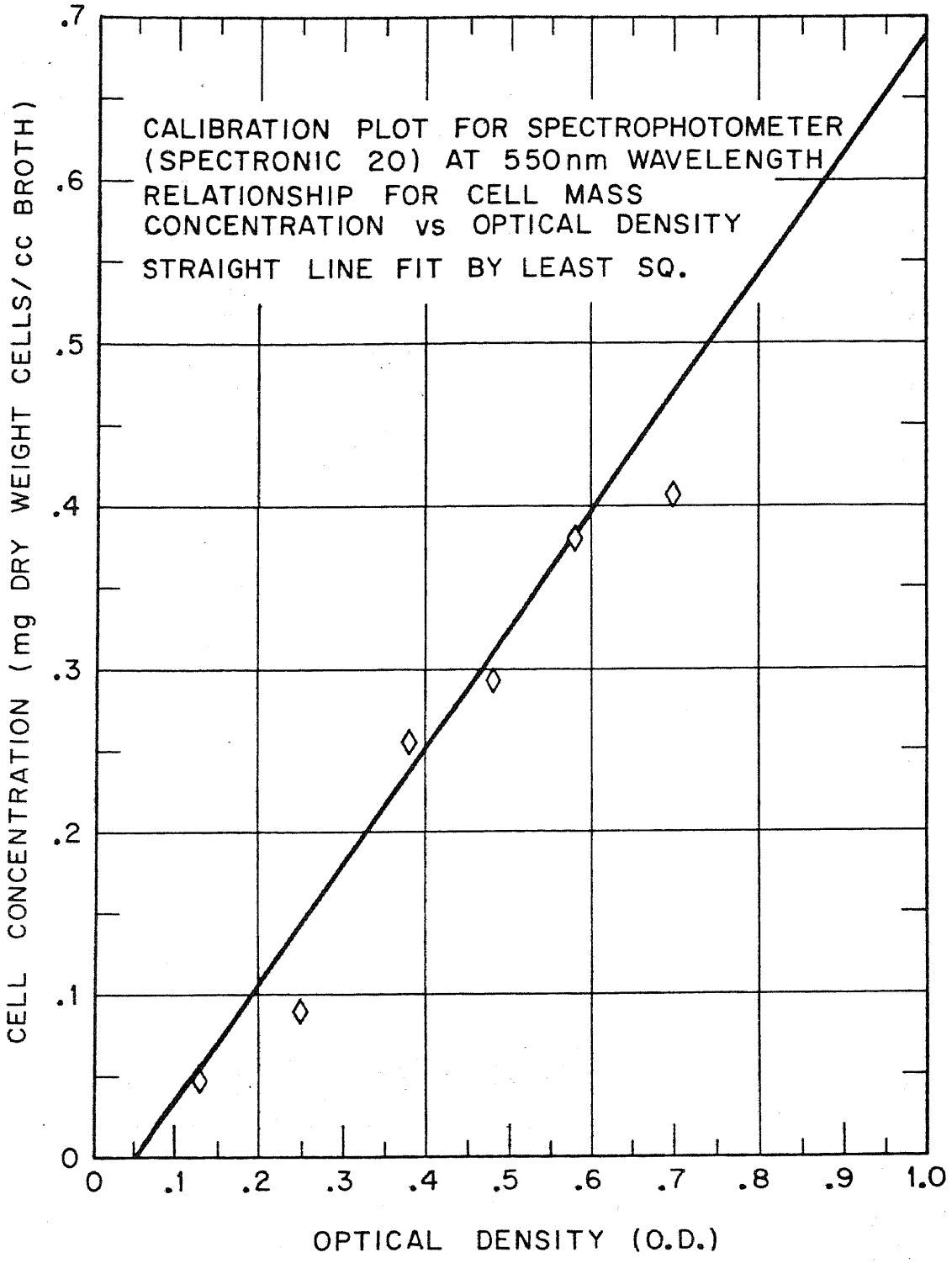

CALIBRATION PLOT FOR SPECTROPHOTOMETER
(SPECTRONIC 20) AT 550nm WAVELENGTH
RELATIONSHIP FOR CELL MASS
CONCENTRATION vs OPTICAL DENSITY
STRAIGHT LINE FIT BY LEAST SQ.
CELL CONCENTRATION (mg DRY WEIGHT CELLS/cc BROTH)
0
.1
.2
.3
.4
.5
.6
.7
OPTICAL DENSITY (O.D.)
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0

FIGURE 7

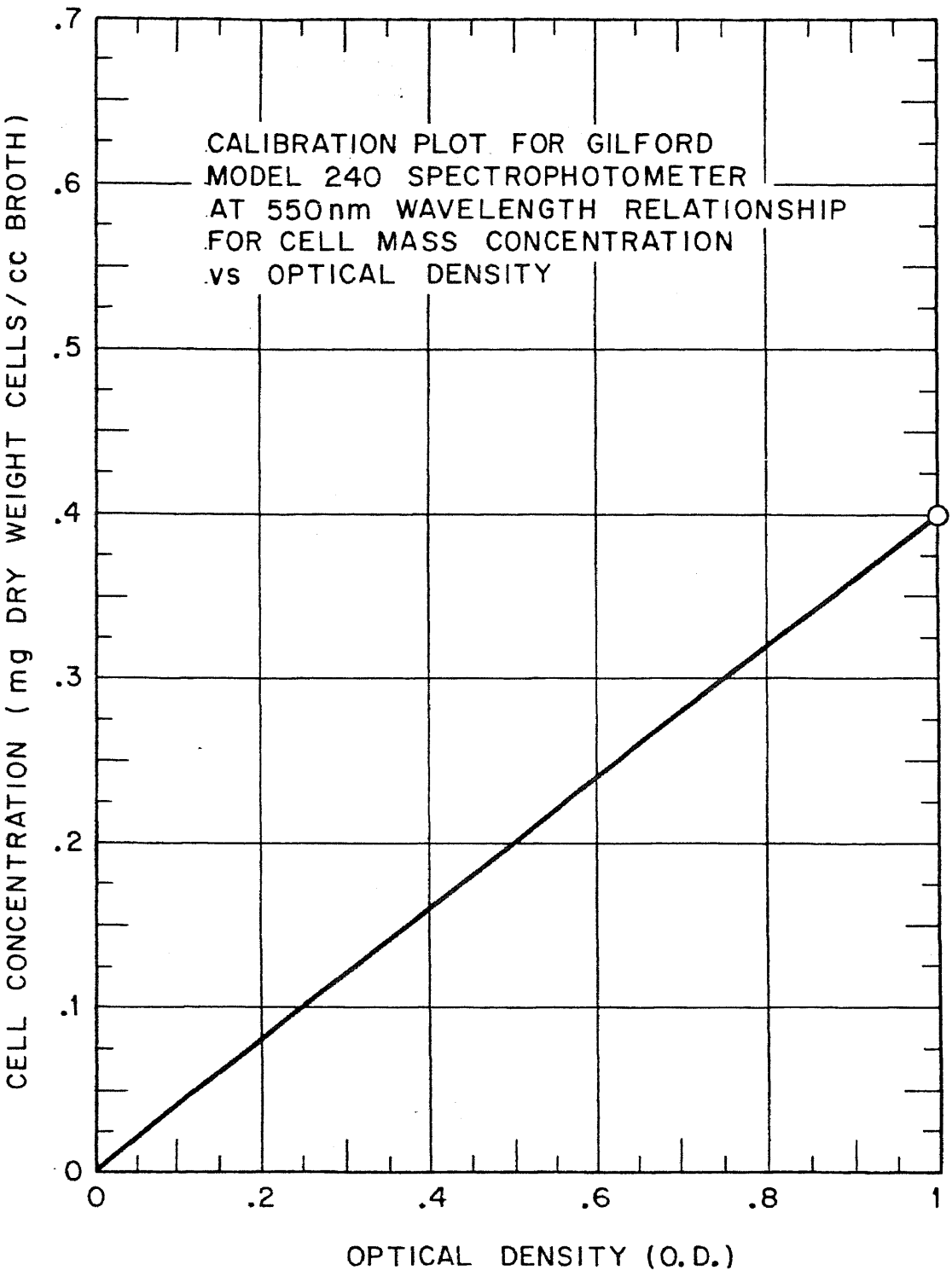
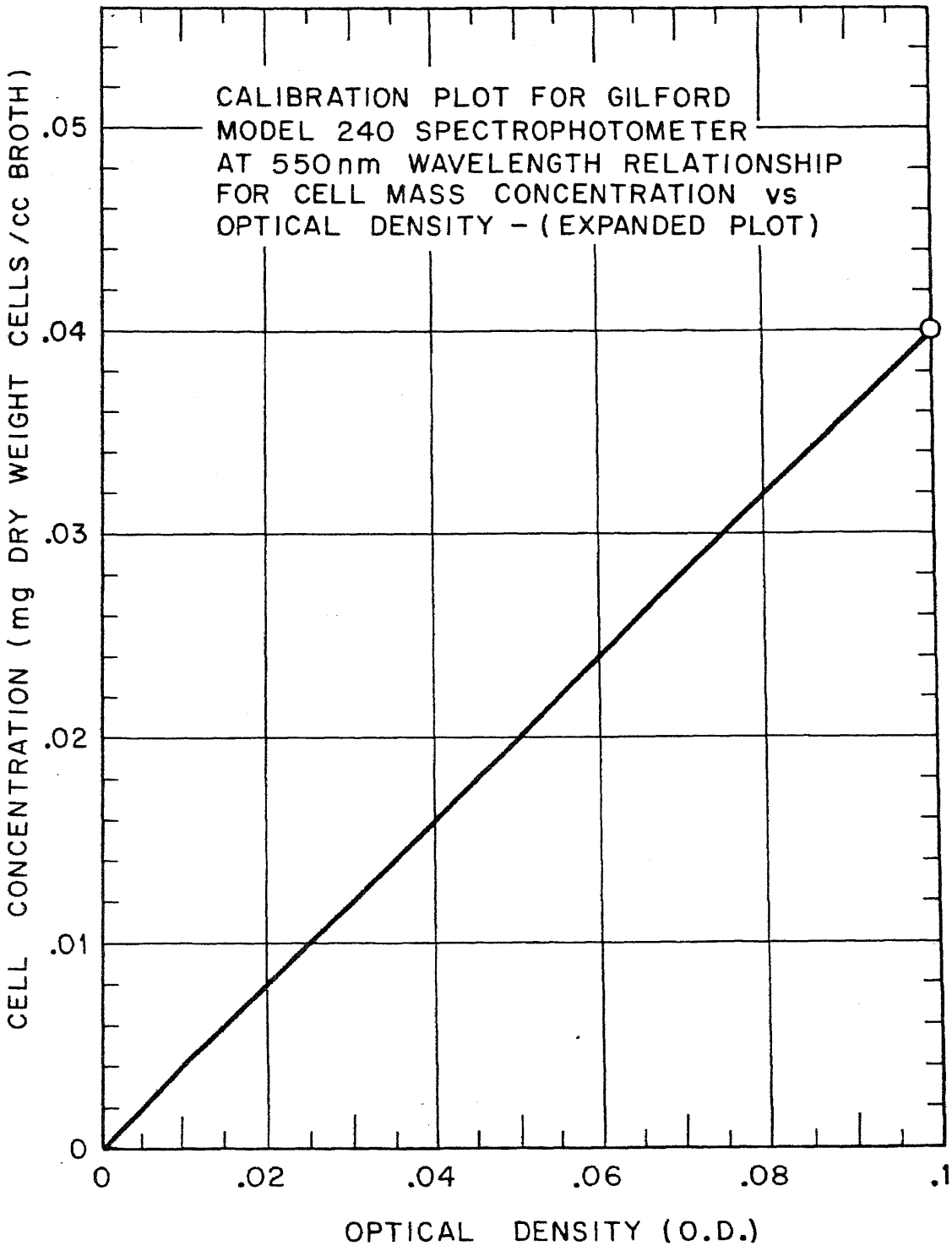

CALIBRATION PLOT FOR GILFORD
MODEL 240 SPECTROPHOTOMETER
AT 550nm WAVELENGTH RELATIONSHIP
FOR CELL MASS CONCENTRATION
vs OPTICAL DENSITY
CELL CONCENTRATION (mg DRY WEIGHT CELLS / cc BROTH)
.7
.6
.5
.4
.3
.2
.1
0
0
.2
.4
.6
.8
1
OPTICAL DENSITY (O.D.)

FIGURE 8


CALIBRATION PLOT FOR GILFORD
MODEL 240 SPECTROPHOTOMETER
AT 550nm WAVELENGTH RELATIONSHIP
FOR CELL MASS CONCENTRATION vs
OPTICAL DENSITY - (EXPANDED PLOT)
CELL CONCENTRATION (mg DRY WEIGHT CELLS/cc BROTH)
.05
.04
.03
.02
.01
0
0
.02
.04
.06
.08
.1
OPTICAL DENSITY (O.D.)

APPENDIX E

DATA

TABLE VII

Hydraulics of Run A: (sodium formate limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
1	73.	73.	32.4	.138
2	140.	213.	33.3	.142
3	600.	813.	33.3	.142
4	73.	886.	31.7	.135
5	NS	886.	NR	NR
6	625.	1511.	29.3	.125
7	75.	1586.	34.7	.148
8	135.	1721.	33.4	.142
9	850.	2571.	47.3	.201
10	95.	2666.	50.0	.213
11	120.	2786.	51.3	.218
12	75.	2861.	52.5	.223

NR = no reading of item, Flow Rate, w, etc., taken for that particular sample number.

TABLE VIII

Experimental Data of Run A:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
1	23.4	9.0	.52	.34
2	27.6	8.8	.34	.205
3	45.6	8.7	.135	.055
4	47.9	8.5	.15	.065
5	50.3	8.6	.64	.425
6	69.2	8.8	.56	.365
7	71.4	8.8	.72	.485
8	75.4	8.7	.64	.425
9	93.4	8.7	.79	.535
10	95.3	8.7	.78	.525
11	97.6	8.7	.78	.525
12	99.1	8.7	.78	.525

TABLE IX

Processed Data for Run A:

Sample Number	Cell Conc. (mg protein / cc broth)	S.A. (I.U. enzyme / mg protein)	(I.U. enzyme / cc broth)	Sum (I.U. enzyme / cc broth)
1	.34	1.5	.255	.255
2	.103	UO	NR	.255*
3	.028	UO	NR	.255*
4	.033	UO	NR	.255*
5	.213	UO	NR	.255*
6	.183	UO	NR	.255*
7	.243	3.9	.948	1.203
8	.213	6.9	1.47	2.673
9	.268	9.1	2.44	5.113
10	.263	9.8	2.58	7.693
11	.263	8.7	2.29	9.983
12	.263	7.8	2.05	12.033

* Since no specific activity of enzyme was determined for the samples, cumulative totals (Sum) assumed unchanged from sample 1.

TABLE X

Hydraulics of Run B: (ammonium formate limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
17	125.	125.	49.6	.211
18	150.	275.	47.3	.201
19	150.	425.	45.2	.192
20	700.	1125.	45.1	.192
21	160.	1285.	47.1	.200
22	130.	1415.	46.4	.197
23	800.	2215.	44.6	.190
24	145.	2360.	46.0	.196
25	125.	2485.	45.8	.195
26	800.	3285.	45.0	.192
27	140.	3425.	46.4	.198

TABLE XI

Experimental Data of Run B:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
17	18.4	8.5	.62	.245
18	21.6	8.5	.60	.24
19	24.9	8.5	.57	.225
20	40.4	8.5	.58	.23
21	43.8	8.5	.58	.23
22	46.6	8.6	.56	.224
23	64.6	8.6	.56	.224
24	67.7	8.6	.56	.224
25	70.4	8.6	.55	.22
26	88.2	8.6	.54	.215
27	91.2	8.6	.56	.224

TABLE XII

Hydraulics of Run C: (ammonium formate limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
28	140.	140.	52.5	.223
29	140.	280.	52.2	.222
30	135.	415.	50.9	.217
31	800.	1215.	49.1	.209
32	125.	1340.	50.0	.213
33	125.	1465.	50.6	.215
34	125.	1590.	50.0	.213
35	785.	2375.	47.6	.203
36	120.	2495.	48.0	.204
37	125.	2620.	50.0	.213
38	135.	2755.	50.9	.217

TABLE XIII

Experimental Data of Run C:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
28	18.3	7.9	.54	.355
29	21.0	7.9	.68	.455
30	23.7	7.9	.65	.435
31	40.0	8.0	.53	.345
32	42.4	7.9	.48	.31
33	44.9	7.9	.48	.31
34	47.4	7.9	.48	.31
35	63.9	7.9	.50	.325
36	66.4	7.9	.49	.315
37	68.9	8.0	.48	.31
38	71.6	7.9	.46	.295

TABLE XIV

Processed Data for Run C:

Sample Number	Cell Conc. $\left(\frac{\text{mg protein}}{\text{cc broth}}\right)$	S.A. $\left(\frac{\text{I.U. enzyme}}{\text{mg protein}}\right)$	$\left(\frac{\text{I.U. enzyme}}{\text{cc broth}}\right)$	Sum $\left(\frac{\text{I.U. enzyme}}{\text{cc broth}}\right)$
28	.178	9.1	1.62	1.62
29	.228	12.4	2.83	4.45
30	.218	15.4	3.36	7.81
31	.173	14.2	2.46	10.27
32	.155	20.8	3.22	13.49
33	.155	16.1	2.50	15.99
34	.155	18.9	2.93	18.92
35	.163	12.4	2.02	20.94
36	.158	13.2	2.09	23.03
37	.155	15.1	2.34	25.37
38	.148	16.1	2.38	27.75

TABLE XV

Hydraulics of Run D: (sodium formate limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
43	115.	115.	42.6	.181
44	120.	235.	47.6	.203
45	115.	350.	51.1	.217
46	375.	725.	21.4	.091
47	60.	785.	19.8	.084
48	65.	850.	21.9	.093
49	370.	1220.	20.6	.088
50	55.	1275.	18.3	.078
51	55.	1330.	18.0	.077
52	355.	1685.	21.0	.089
53	60.	1745.	19.7	.084

TABLE XVI

Experimental Data of Run D:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
43	19.7	7.8	.41	.255
44	22.2	7.8	.35	.215
45	24.5	7.7	.22	.120
46	42.0	7.8	.24	.135
47	45.0	7.5	.30	.175
48	48.0	7.6	.28	.16
49	66.0	7.5	.26	.145
50	69.0	7.5	.34	.205
51	72.0	7.5	.38	.235
52	89.0	7.5	.40	.25
53	92.0	7.5	.44	.28

TABLE XVII

Processed Data for Run D:

Sample Number	Cell Conc. (mg protein / cc broth)	S.A. (I.U. enzyme / mg protein)	(I.U. enzyme / cc broth)	Sum (I.U. enzyme / cc broth)
43	.128	3.8	.486	.486
44	.108	3.9	.421	.908
45	.06	3.9*	.234	1.142
46	.068	3.9	.265	1.407
47	.088	3.6	.317	1.724
48	.08	2.7	.216	1.94
49	.073	2.8	.204	2.144
50	.103	3.5	.361	2.505
51	.118	4.2	.496	3.001
52	.125	6.2	.775	3.775
53	.14	7.3	1.022	4.797

* No specific activity determined for sample 45. S.A. assumed to be average of sample 44 & 46.

TABLE XVIII

Hydraulics of Run E: (ammonium formate limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
67	84.	84.	47.2	.201
68	100.	184.	48.8	.208
69	105.	289.	48.0	.204
70	500.	789.	28.5	.121
71*	55.	55.	39.9	.170
72	55.	110.	47.8	.203
73	50.	160.	49.0	.209
74	190.	350.	47.2	.201
75	80.	430.	47.1	.200

* reinoculation of organism, Vibrio succinogenes, into fermentor

TABLE XIX

Experimental Data of Run E:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
67	19.7	9.2	.58	.38
68	21.7	9.1	.41	.255
69	24.0	8.8	.27	.155
70	41.5	8.7	.14	.065
71*	25.3	9.0	.29	.17
72	26.4	9.0	.26	.145
73	27.5	8.9	.23	.125
74	31.5	8.8	.21	.11
75	33.2	8.5	.19	.095

* reinoculation of organism, Vibrio succinogenes, into fermentor

TABLE XX

Hydraulics of Run F: (fumaric acid limiting nutrient)

Sample Number	Vol. Coll. (cc)	Sum Vol. (cc)	Flow Rate (cc/hr)	w (hr^{-1})
78	125.	125.	52.7	.224
79	75.	200.	50.0	.213
80	NS	200.	NR	NR
81	105.	305.	50.0	.213
82	NS	305.	NR	NR
83	95.	400.	48.7	.207
84	NS	400.	NR	NR

TABLE XXI

Experimental Data of Run F:

Sample Number	Time Ino. (hr)	pH	O.D.	C (mg dry weight/cc broth)
78	25.2	9.1	.46	.295
79	26.7	8.9	.32	.19
80	27.8	NR	.27	.15
81	28.8	8.7	.23	.12
82	29.8	NR	.19	.095
83	30.8	8.6	.17	.08
84	47.3	8.4	.10	.03

TABLE XXII

Wash Out Curve Data for Run A:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
1	2.25	.34
2	6.45	.205
3	24.45	.055
4	26.75	.065
5	29.17	.425
6	48.09	.365
7	50.25	.485
8	54.29	.425
9	72.25	.535
10	74.15	.525
11	76.49	.525
12	77.92	.525

$w^{*} = .138\ hr^{-1}$

* dilution rate determined by geometric average

TABLE XXIII

Wash Out Curve Data for Run B:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
17	2.51	.245
18	5.68	.24
19	9.00	.225
20	24.53	.23
21	27.93	.23
22	30.73	.224
23	48.68	.224
24	51.83	.224
25	54.56	.22
26	72.33	.215
27	75.35	.224

$w^{*} = .197\ hr^{-1}$

* dilution rate determined by geometric average

TABLE XXIV

Wash Out Curve Data for Run C:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
28	2.67	.355
29	5.35	.455
30	8.00	.435
31	24.28	.345
32	26.78	.31
33	29.25	.31
34	31.75	.31
35	48.25	.325
36	50.75	.315
37	53.25	.31
38	55.9	.295

$w^{*} = .213\ hr^{-1}$

* dilution rate determined by geometric average

TABLE XXV

Wash Out Curve Data for Run D:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
43	2.7	.255
44	5.2	.215
45	7.5	.120
46**	7.66	.135
47	10.66	.175
48	13.66	.16
49	31.66	.145
50	34.66	.205
51	37.66	.235
52	54.66	.25
53	57.66	.28

w_1* = .2 hr^{-1}, for samples 43-45

w_2* = .085 hr^{-1}, for samples 46-53

* dilution rate determined by geometric average

** start of new dilution rate, 17.34 hr elapsed from sample 45

TABLE XXVI

Wash Out Curve Data for Run E:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
67	1.81	.38
68	3.86	.255
69	6.05	.155
70	23.61	.065
71*	1.38	.17
72	2.53	.145
73	3.55	.125
74	7.58	.11
75	9.28	.095

$w_1^{**} = .204\ hr^{-1}$, for samples 67-69

$w_2^{**} = .196\ hr^{-1}$, for samples 71-75

* reinoculation of organism, Vibrio succinogenes, into fermentor

** dilution rate determined by geometric average

TABLE XXVII

Wash Out Curve Data for Run F:

Sample Number	Time from Pump Activation (hr)	Cell Conc. (mg dry weight/cc broth)
78	3.02	.295
79	4.52	.19
80	5.6	.15
81	6.62	.12
82	7.57	.095
83	8.57	.08
84	25.05	.03

$w^{*} = .214\ hr^{-1}$

* dilution rate determined by geometric average

TABLE XXVIII

Experimental Data for Run C': (Batch Fermentation, ref. (3))

Sample Number	Time Ino. (hr)	O.D.	C (mg dry weight/cc broth)
A1	19.	.065	.026
B2	20.	.094	.037
C3	21.	.20	.08
D4	22.	.41	.156
E5	23.	.65	.26
F6	24.	.88	.35
G7	25.	1.0	.40
H8	26.	1.1	.44
I9	27.	1.1	.44
J10	39.	1.05	.42
K11	42.	1.0	.40
L12	46.	.99	.395

TABLE XXIX

Processed Data for Run C':

Sample Number	Cell Conc. (mg protein / cc broth)	S.A. (I.U. enzyme / mg protein)	(I.U. enzyme / cc broth)	Sum (I.U. enzyme / cc broth)
A1	.013	NR	NR	NR
B2	.0184	2.7	.05	.05
C3	.04	2.5	.10	.15
D4	.078	4.4	.343	.493
E5	.13	5.0	.65	1.14
F6	.175	7.0	1.23	2.37
G7	.20	6.5	1.3	3.67
H8	.22	7.8	1.72	5.39
I9	.22	9.8	2.16	7.54
J10	.21	10.5	2.21	9.75
K11	.20	9.5	1.90	11.65
L12	.198	8.8	1.74*	13.39

* Total I.U. enzyme (asparaginase) produced in the batch run used for comparison with the continuous run (C)

TABLE XXX

Final Units of Enzyme Produced for Run C:

Sample Number	Vol. Coll. (cc)	(I.U. enzyme / cc broth)	(I.U. enzyme)	Sum (I.U. enzyme)
28	140.	1.62	226.8	226.8
29	140.	2.83	396.2	623.0
30	135.	3.36	453.6	1076.6
31	800.	2.46	1968.0	3044.6
32	125.	3.22	402.5	3447.1
33	125.	2.50	312.5	3759.6
34	125.	2.93	366.3	4125.9
35	785.	2.02	1585.7	5711.6
36	120.	2.09	250.8	5962.4
37	125.	2.34	292.5	6254.9
38	135.	2.38	321.3	6576.2*

* Total I.U. enzyme (asparaginase) produced in the continuous run used for comparison with the batch system (C')

APPENDIX F

OPTICAL DENSITY VERSUS FERMENTATION TIME

FIGURE 9

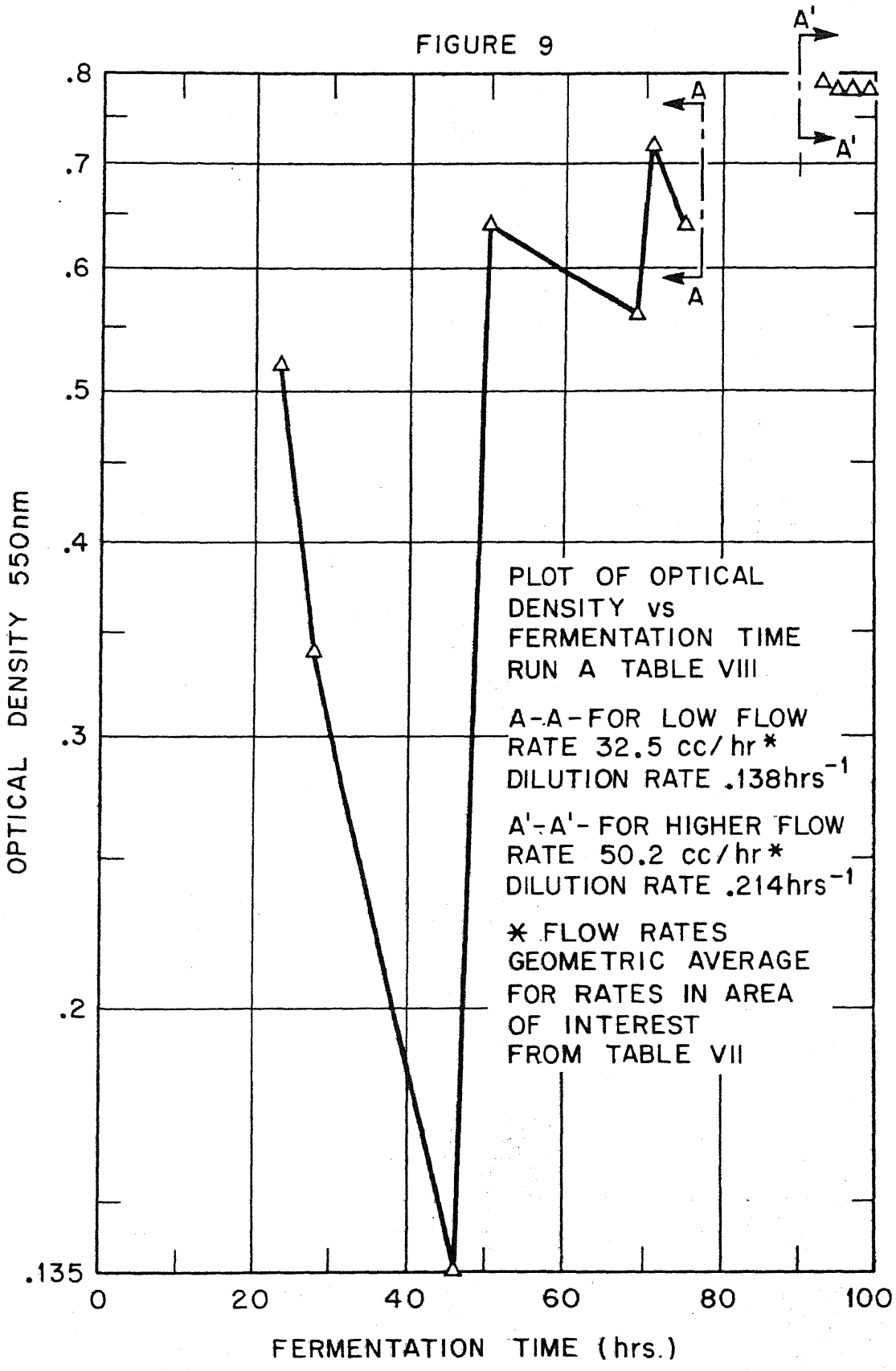

A'
A
A
A'
.8
.7
.6
.5
.4
.3
.2
.135
OPTICAL DENSITY 550nm
0
20
40
60
80
100
FERMENTATION TIME (hrs.)
PLOT OF OPTICAL DENSITY vs FERMENTATION TIME RUN A TABLE VIII
A-A- FOR LOW FLOW RATE 32.5 cc/hr*
DILUTION RATE .138hrs^{-1}
A'-A'- FOR HIGHER FLOW RATE 50.2 cc/hr*
DILUTION RATE .214hrs^{-1}
* FLOW RATES GEOMETRIC AVERAGE FOR RATES IN AREA OF INTEREST FROM TABLE VII

FIGURE 10

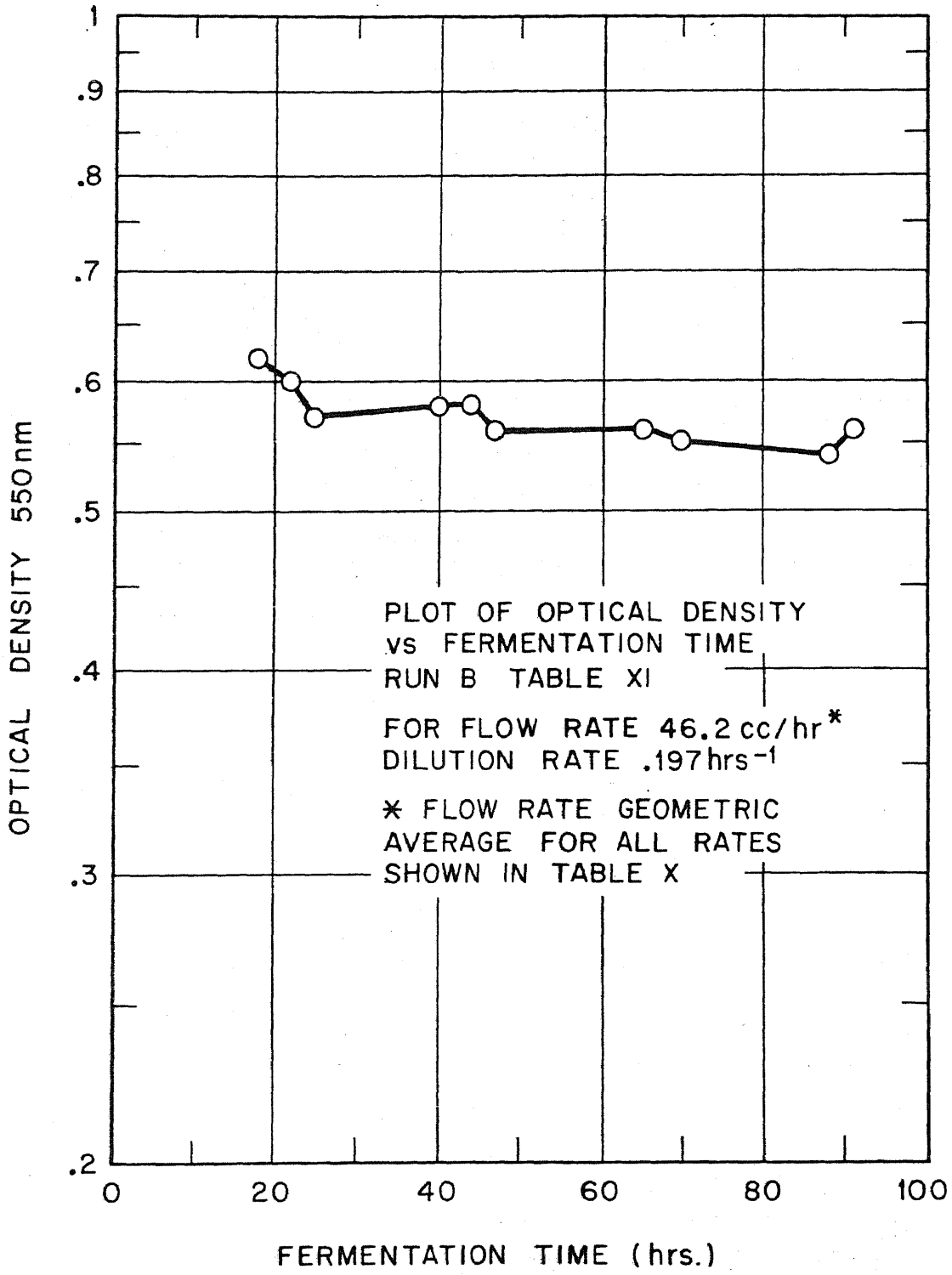

OPTICAL DENSITY 550nm
1
.9
.8
.7
.6
.5
.4
.3
.2
0
20
40
60
80
100
FERMENTATION TIME (hrs.)
PLOT OF OPTICAL DENSITY
vs FERMENTATION TIME
RUN B TABLE XI
FOR FLOW RATE 46.2 cc/hr*
DILUTION RATE .197 hrs⁻¹
* FLOW RATE GEOMETRIC
AVERAGE FOR ALL RATES
SHOWN IN TABLE X

FIGURE 11

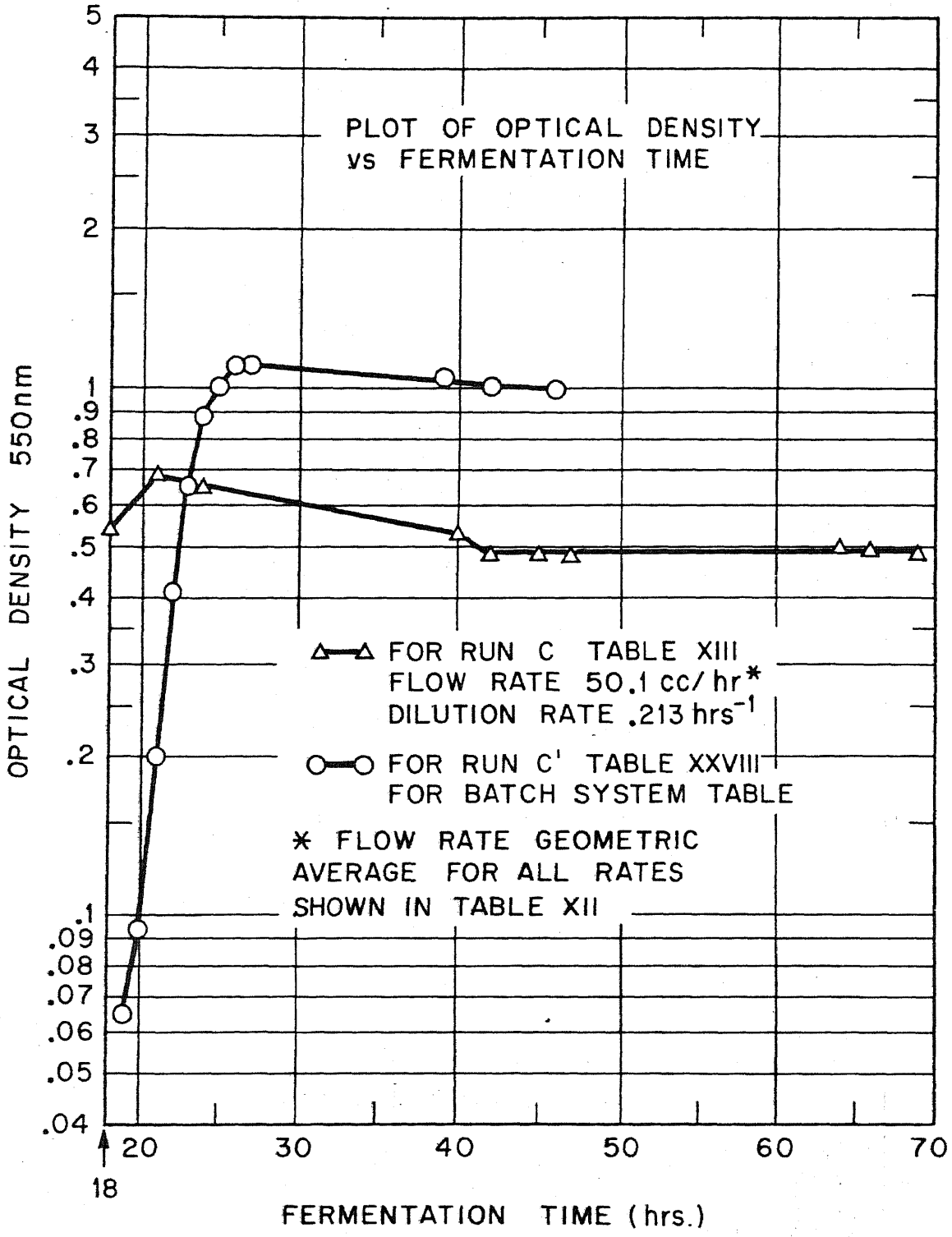

PLOT OF OPTICAL DENSITY
vs FERMENTATION TIME
OPTICAL DENSITY 550nm
5
4
3
2
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
20
30
40
50
60
70
18
FERMENTATION TIME (hrs.)
FOR RUN C TABLE XIII
FLOW RATE 50.1 cc/hr*
DILUTION RATE .213 hrs^{-1}
FOR RUN C' TABLE XXVIII
FOR BATCH SYSTEM TABLE
* FLOW RATE GEOMETRIC
AVERAGE FOR ALL RATES
SHOWN IN TABLE XII

FIGURE 12

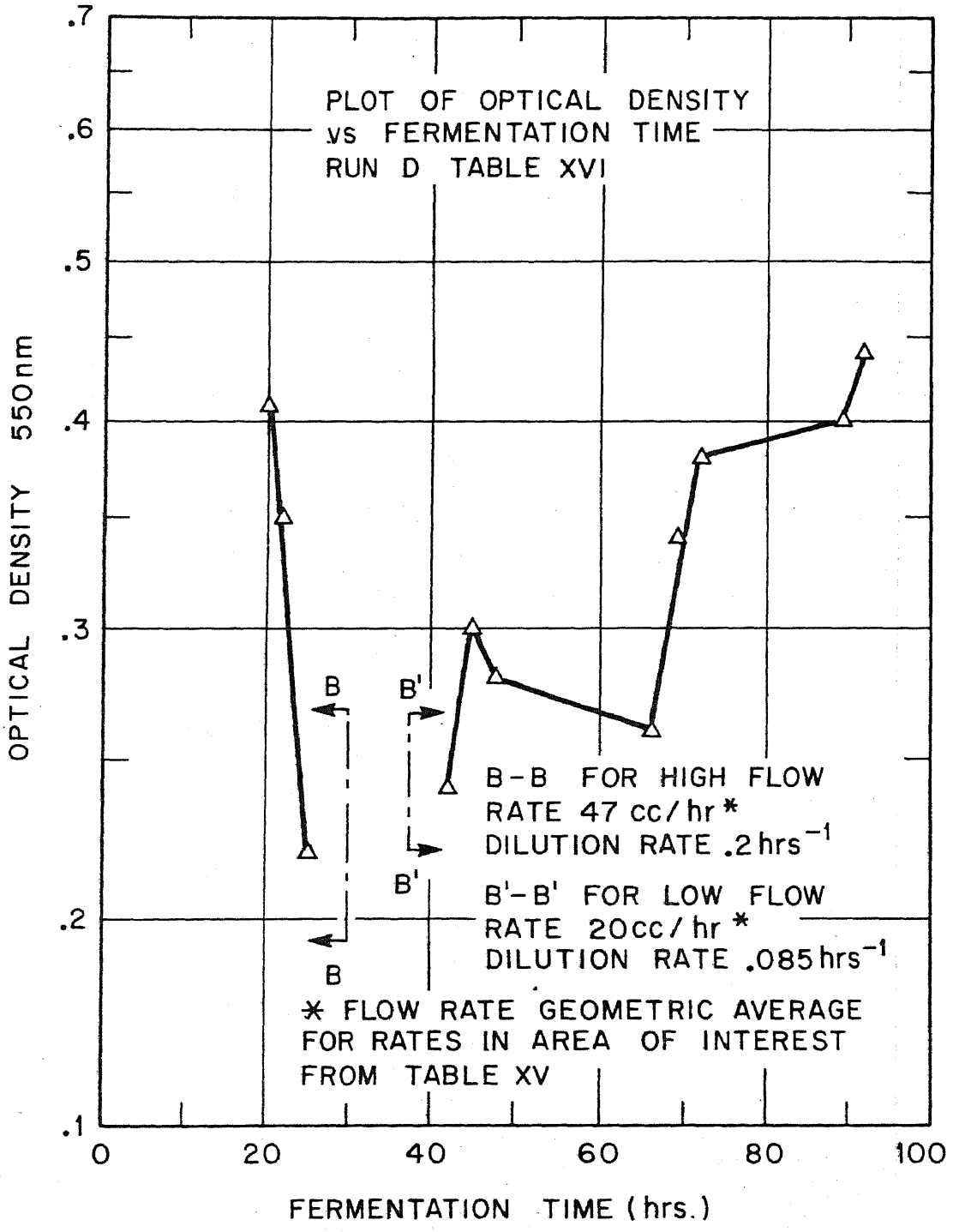

PLOT OF OPTICAL DENSITY
vs FERMENTATION TIME
RUN D TABLE XVI
OPTICAL DENSITY 550nm
.7
.6
.5
.4
.3
.2
.1
0
20
40
60
80
100
FERMENTATION TIME (hrs.)
B
B'
B'
B
B-B FOR HIGH FLOW
RATE 47 cc/hr*
DILUTION RATE .2 hrs^{-1}
B'-B' FOR LOW FLOW
RATE 20cc/hr *
DILUTION RATE .085 hrs^{-1}
* FLOW RATE GEOMETRIC AVERAGE
FOR RATES IN AREA OF INTEREST
FROM TABLE XV

FIGURE 13

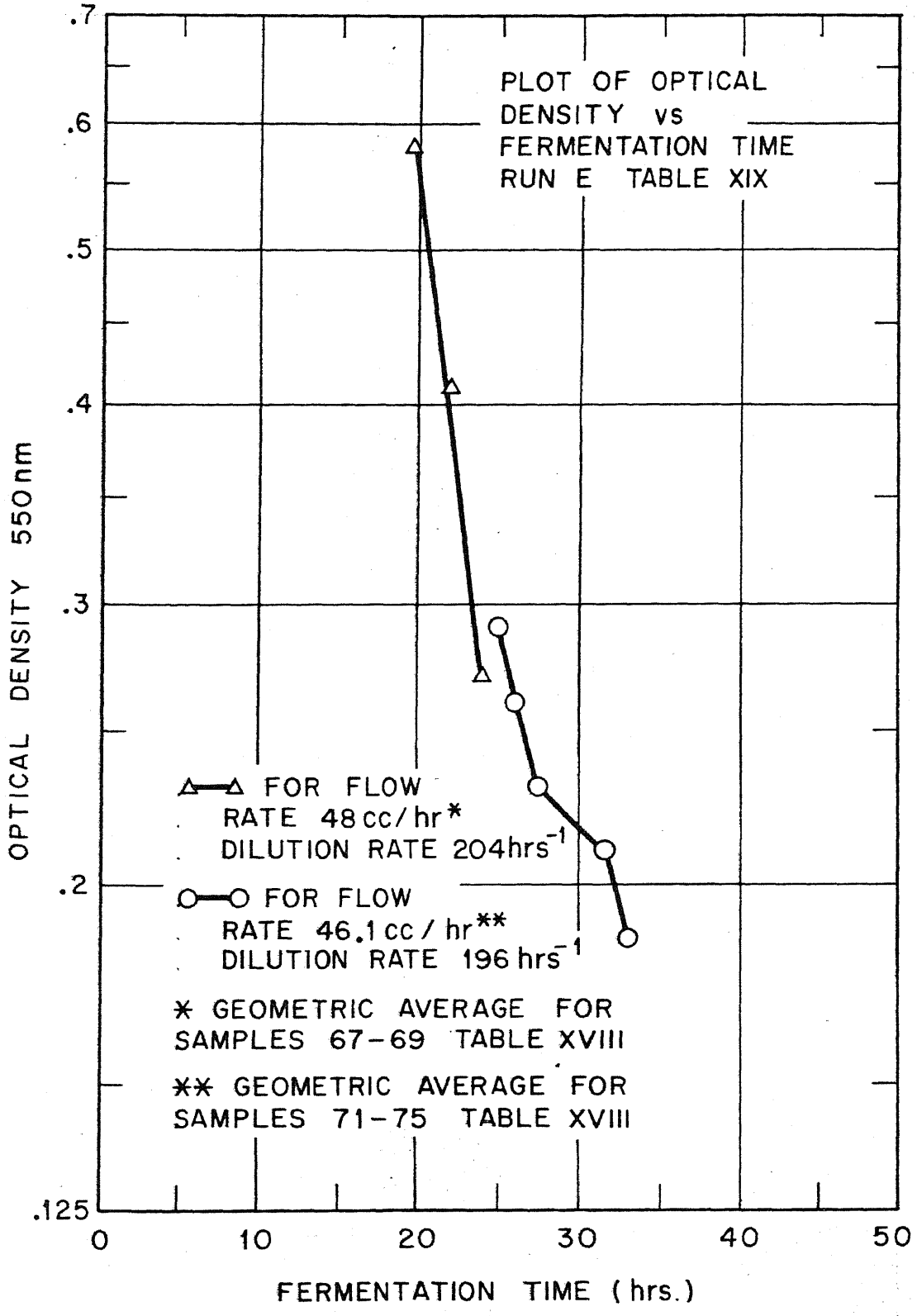
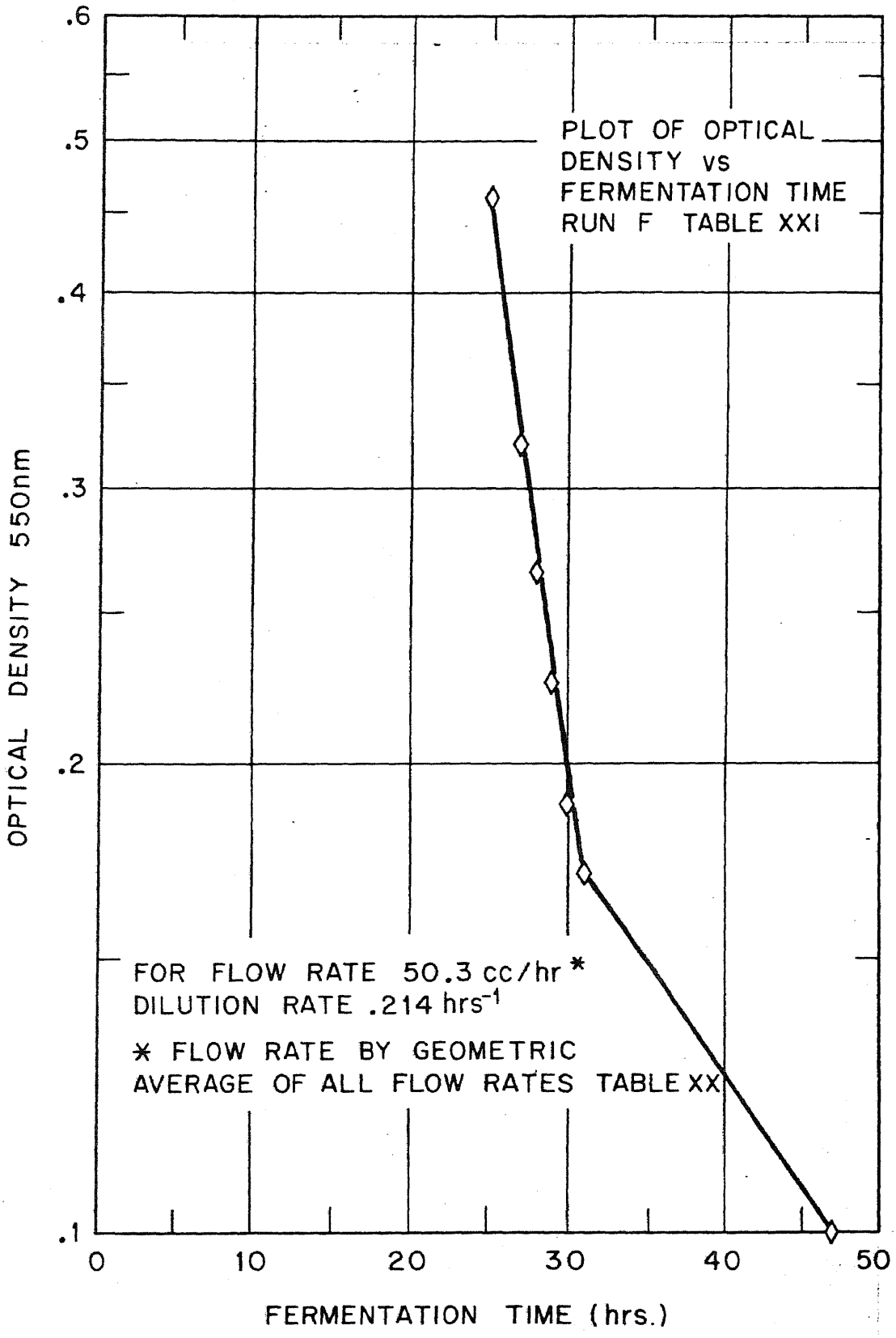

PLOT OF OPTICAL DENSITY vs FERMENTATION TIME RUN E TABLE XIX
OPTICAL DENSITY 550nm
.7
.6
.5
.4
.3
.2
.125
0
10
20
30
40
50
FERMENTATION TIME (hrs.)
FOR FLOW RATE 48 cc/hr*
DILUTION RATE 204 hrs⁻¹
FOR FLOW RATE 46.1 cc/hr**
DILUTION RATE 196 hrs⁻¹
* GEOMETRIC AVERAGE FOR SAMPLES 67-69 TABLE XVIII
** GEOMETRIC AVERAGE FOR SAMPLES 71-75 TABLE XVIII

FIGURE 14


PLOT OF OPTICAL DENSITY vs FERMENTATION TIME RUN F TABLE XXI
FOR FLOW RATE 50.3 cc/hr *
DILUTION RATE .214 hrs⁻¹
* FLOW RATE BY GEOMETRIC AVERAGE OF ALL FLOW RATES TABLE XX
OPTICAL DENSITY 550nm
.6
.5
.4
.3
.2
.1
0
10
20
30
40
50
FERMENTATION TIME (hrs.)

APPENDIX G

WASH OUT PLOTS

FIGURE 15

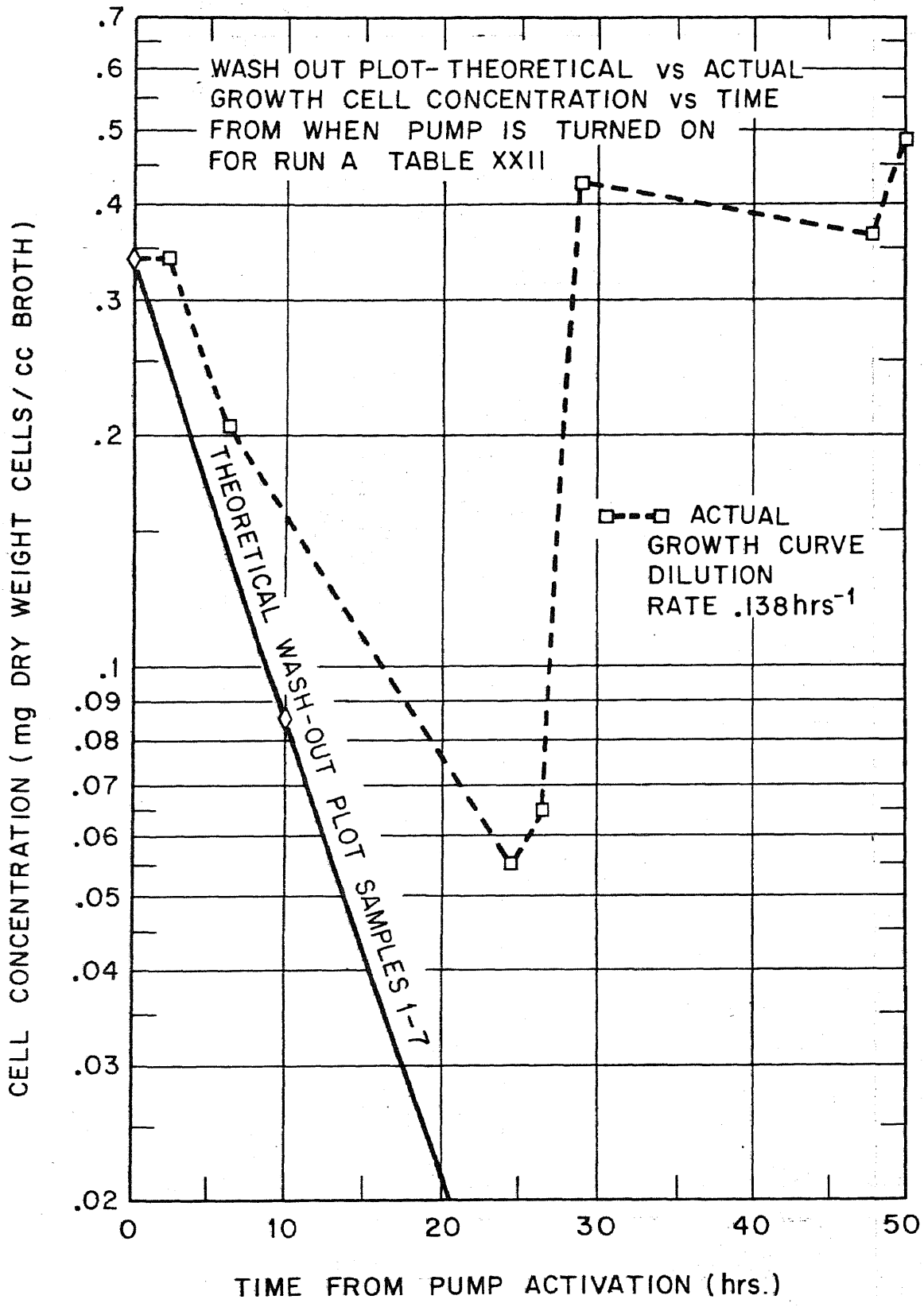

WASH OUT PLOT- THEORETICAL vs ACTUAL
GROWTH CELL CONCENTRATION vs TIME
FROM WHEN PUMP IS TURNED ON
FOR RUN A TABLE XXII
ACTUAL
GROWTH CURVE
DILUTION
RATE .138hrs⁻¹
THEORETICAL WASH-OUT PLOT SAMPLES 1-7
CELL CONCENTRATION (mg DRY WEIGHT CELLS / cc BROTH)
.7
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
0
10
20
30
40
50
TIME FROM PUMP ACTIVATION (hrs.)

FIGURE 16

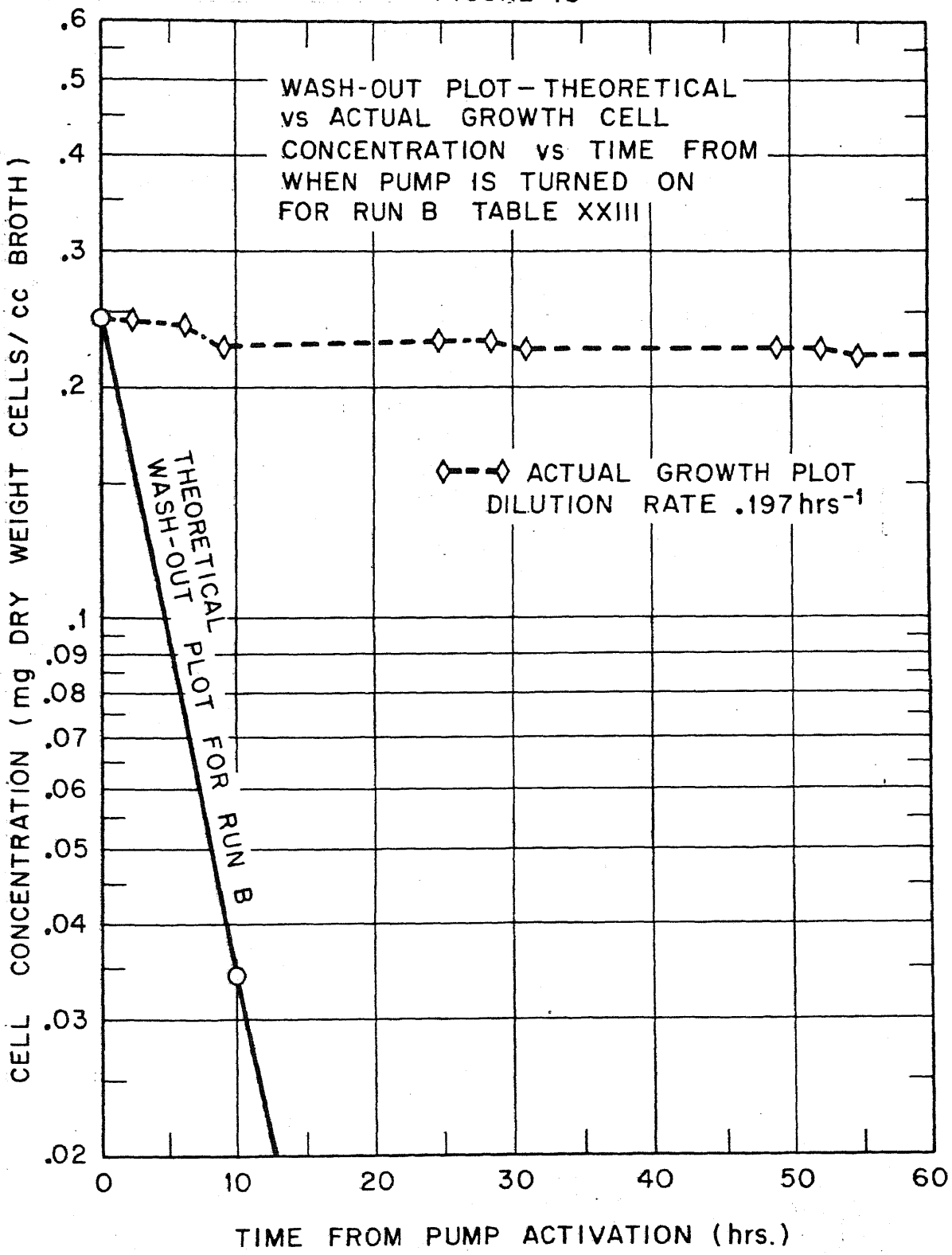

WASH-OUT PLOT – THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN B TABLE XXIII
ACTUAL GROWTH PLOT DILUTION RATE .197 hrs⁻¹
THEORETICAL WASH-OUT PLOT FOR RUN B
CELL CONCENTRATION (mg DRY WEIGHT CELLS/cc BROTH)
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
0
10
20
30
40
50
60
TIME FROM PUMP ACTIVATION (hrs.)

FIGURE 17

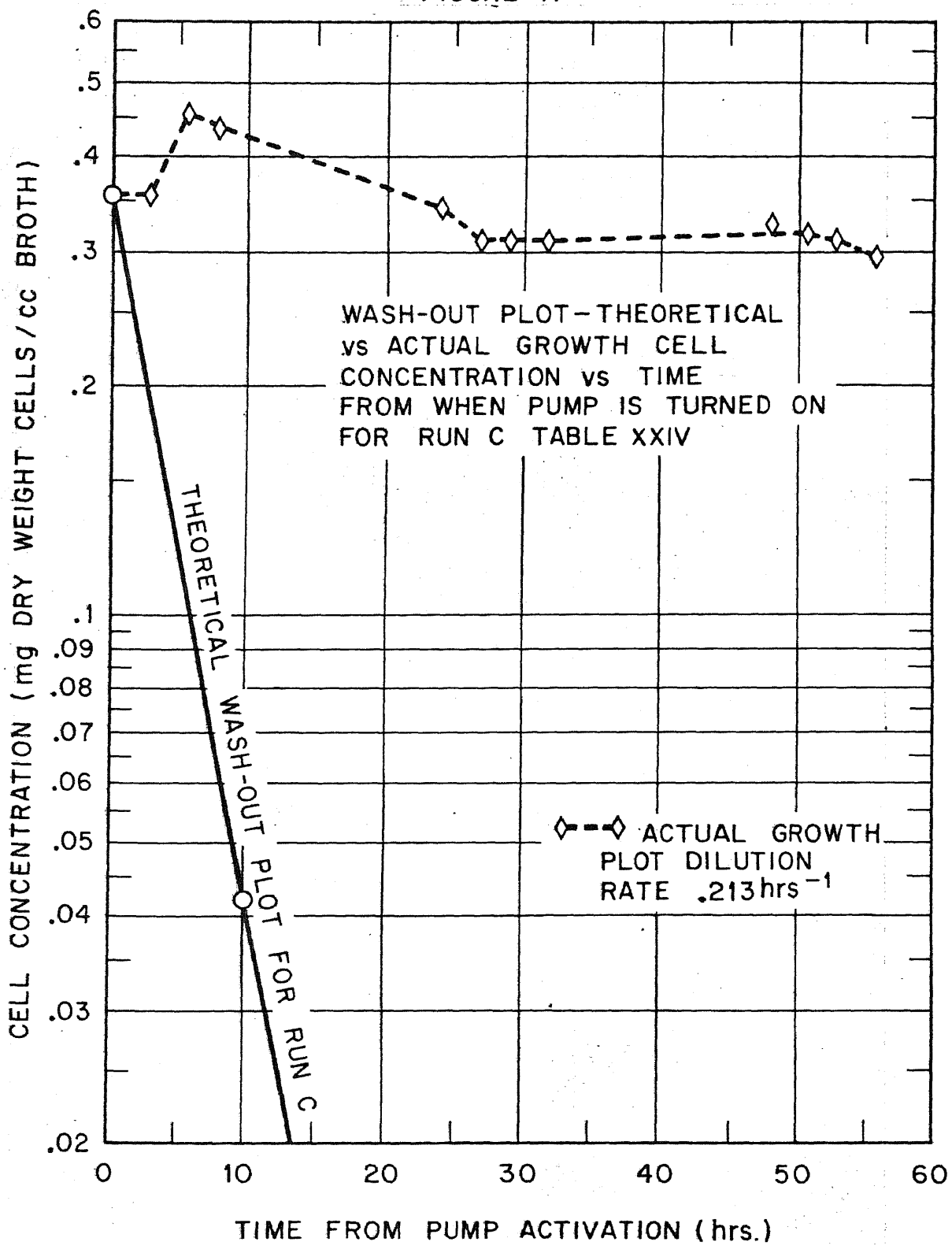

.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
0
10
20
30
40
50
60
CELL CONCENTRATION (mg DRY WEIGHT CELLS / cc BROTH)
TIME FROM PUMP ACTIVATION (hrs.)
WASH-OUT PLOT-THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN C TABLE XXIV
THEORETICAL WASH-OUT PLOT FOR RUN C
ACTUAL GROWTH PLOT DILUTION RATE .213 hrs⁻¹

FIGURE -18

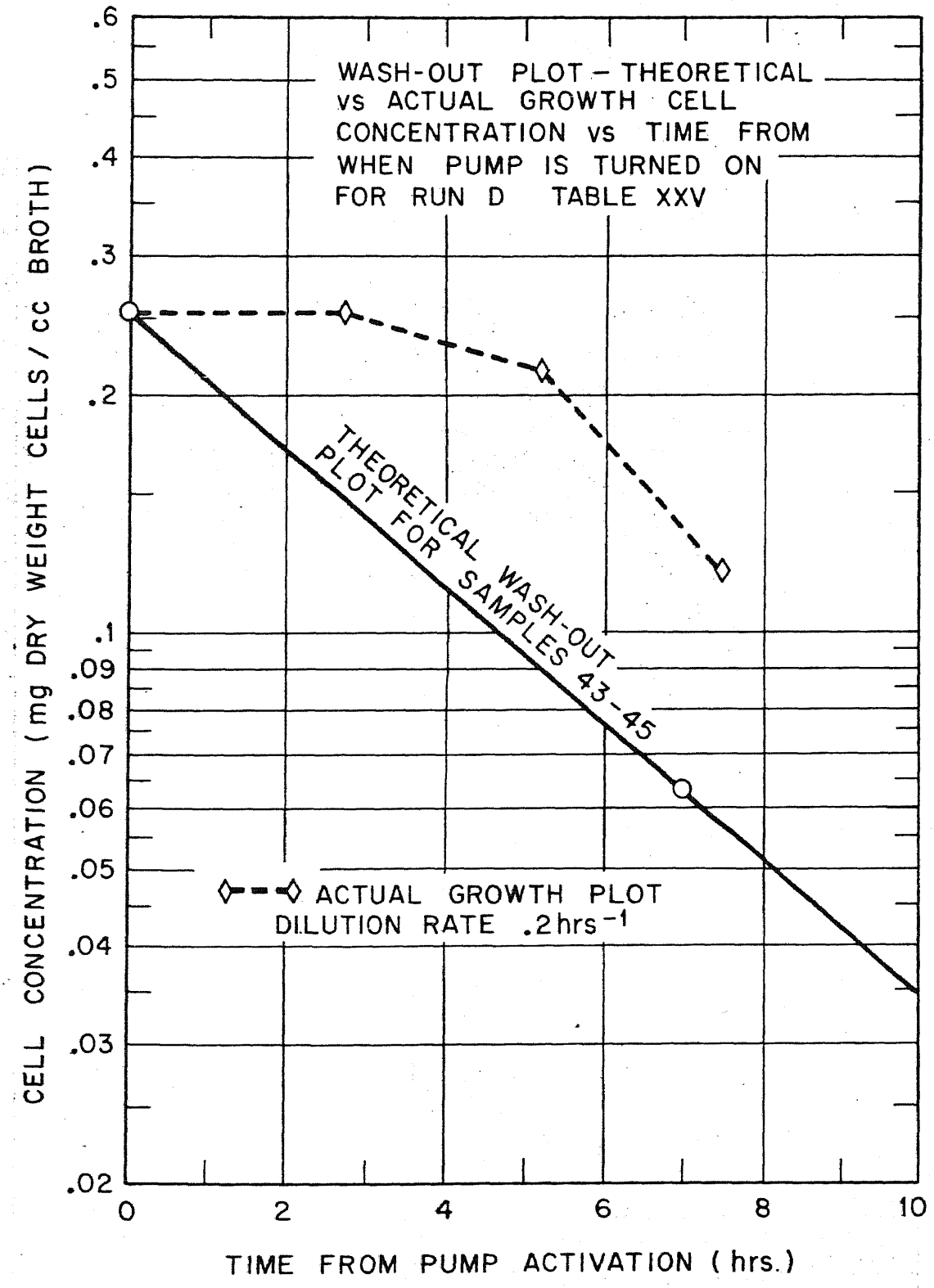

WASH-OUT PLOT – THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN D TABLE XXV
THEORETICAL WASH-OUT PLOT FOR SAMPLES 43-45
ACTUAL GROWTH PLOT DILUTION RATE .2 hrs⁻¹
CELL CONCENTRATION (mg DRY WEIGHT CELLS / cc BROTH)
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
0
2
4
6
8
10
TIME FROM PUMP ACTIVATION (hrs.)

FIGURE 19

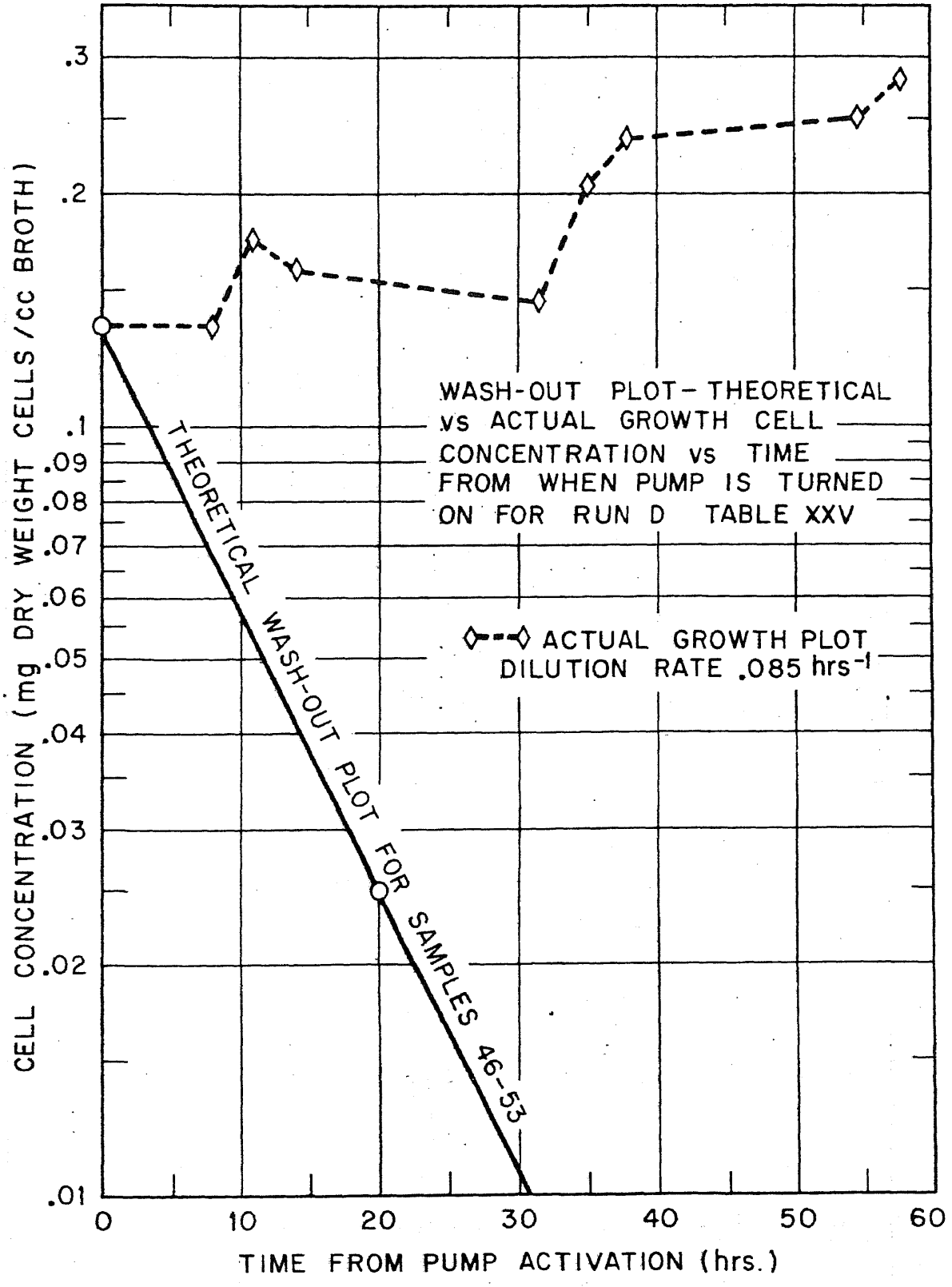

.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
CELL CONCENTRATION (mg DRY WEIGHT CELLS/cc BROTH)
0
10
20
30
40
50
60
TIME FROM PUMP ACTIVATION (hrs.)
WASH-OUT PLOT- THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN D TABLE XXV
ACTUAL GROWTH PLOT DILUTION RATE .085 hrs-1
THEORETICAL WASH-OUT PLOT FOR SAMPLES 46-53

FIGURE 20

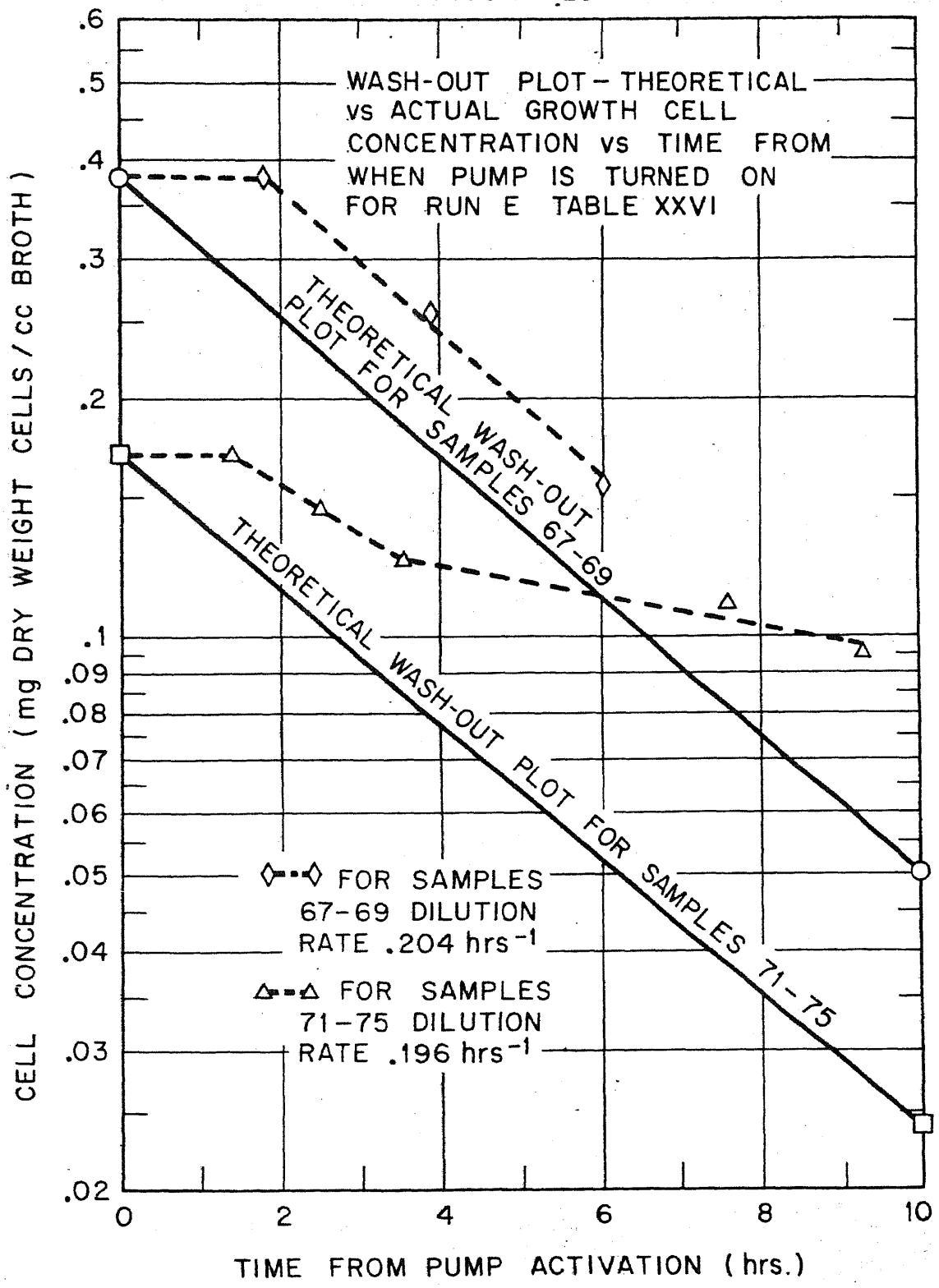
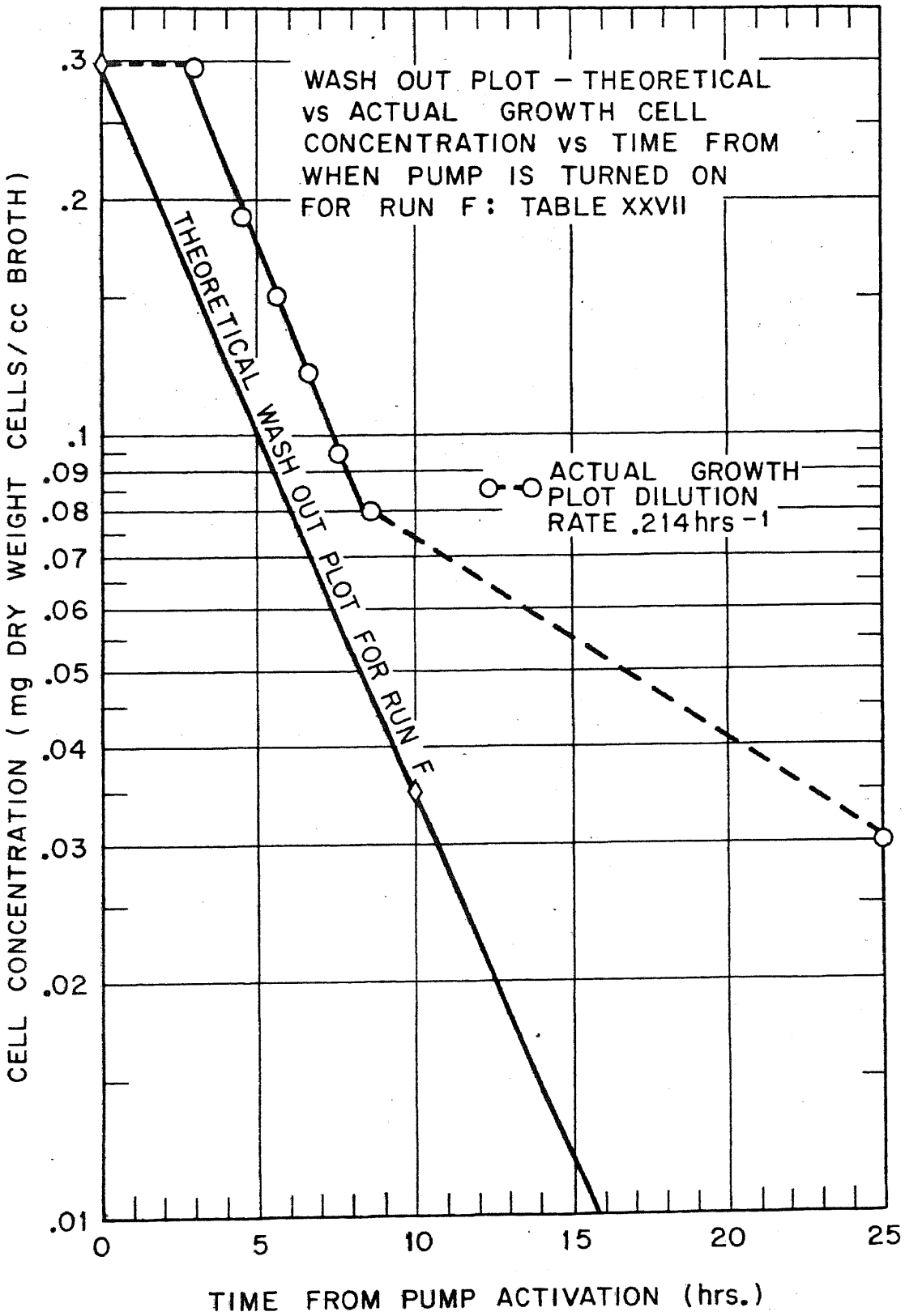

WASH-OUT PLOT – THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN E TABLE XXVI
THEORETICAL WASH-OUT PLOT FOR SAMPLES 67-69
THEORETICAL WASH-OUT PLOT FOR SAMPLES 71-75
◇--◇ FOR SAMPLES 67-69 DILUTION RATE .204 hrs⁻¹
△--△ FOR SAMPLES 71-75 DILUTION RATE .196 hrs⁻¹
CELL CONCENTRATION (mg DRY WEIGHT CELLS / cc BROTH)
.6
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
0
2
4
6
8
10
TIME FROM PUMP ACTIVATION (hrs.)

FIGURE 21


WASH OUT PLOT – THEORETICAL vs ACTUAL GROWTH CELL CONCENTRATION vs TIME FROM WHEN PUMP IS TURNED ON FOR RUN F: TABLE XXVII
THEORETICAL WASH OUT PLOT FOR RUN F
ACTUAL GROWTH PLOT DILUTION RATE .214 hrs -1
CELL CONCENTRATION (mg DRY WEIGHT CELLS/cc BROTH)
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
5
10
15
20
25
TIME FROM PUMP ACTIVATION (hrs.)

APPENDIX H

COMPARISON PLOT AND TABLE

FIGURE 22

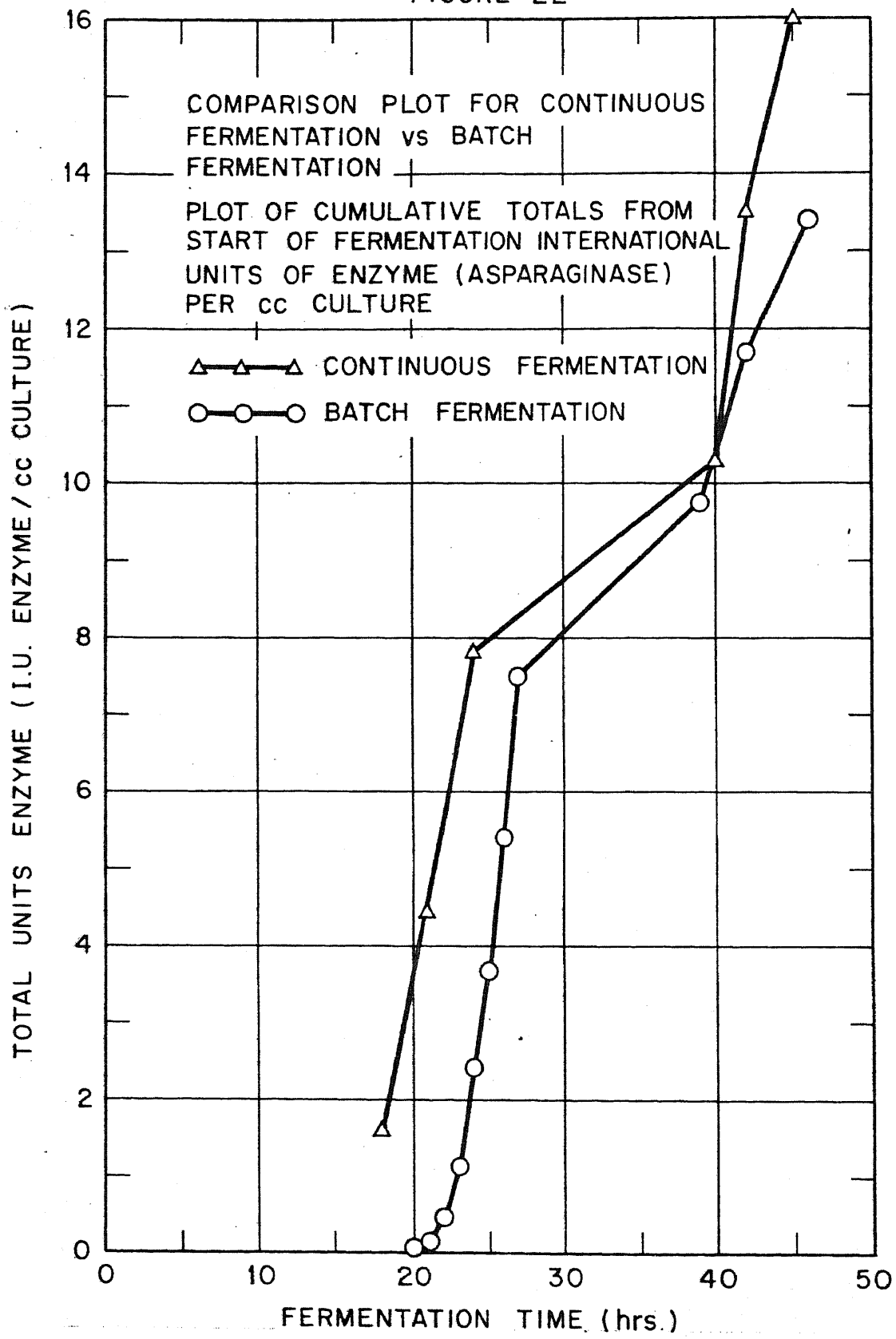

COMPARISON PLOT FOR CONTINUOUS FERMENTATION vs BATCH FERMENTATION
PLOT OF CUMULATIVE TOTALS FROM START OF FERMENTATION INTERNATIONAL UNITS OF ENZYME (ASPARAGINASE) PER cc CULTURE
CONTINUOUS FERMENTATION
BATCH FERMENTATION
TOTAL UNITS ENZYME (I.U. ENZYME / cc CULTURE)
FERMENTATION TIME (hrs.)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50

TABLE XXXI

Comparison between Batch Fermentation and Continuous Fermentation

System Used	$\left(\frac{\text{I.U. enzyme}}{\text{Liter Broth}}\right)$	$\left(\frac{\text{I.U. enzyme}}{\text{mM ammonium formate}}\right)$
Batch	1740.0	17.40
Continuous	2386.99	31.83

APPENDIX I

NOMENCLATURE

NOMENCLATURE

C	= cell concentration, mg dry weight cells/cc broth
cc	= cubic centimetrs, volume measured, 1cc 1 milliliter
I.U.	= international units, amount of enzyme which catalyes the formation of 1 micromole of ammonia per min.
N	= number of cells
NR	= no reading of item, in question, obtained
NS	= no sample of broth taken at time in question
O.D.	= optical density of broth, related to cell concentration via calibration curve
pH	= measurement of hydrogen ion concentration in each sample during the fermentation run
S.A.	= specific activity of each sample, I.U. enzyme per milligram cell protein
Sum Vol.	= summation of the volume collected from the beginning of the fermentation run, cc
Sum	= summation of the amount of enzyme, asparaginase, produced from the beginning of the run, I.U. enzyme/cc broth
t	= fermentation time, hr
t_d	= doubling time; time required for cell population to double, hr
Time Ino.	= time from when organism, Vibrio succinogenes, was first inoculated into the broth to the sampling time, hr
UO	= unobtainable reading due to lack of adequate cell volume after centrifuging

V_o = working volume of broth in fermentor, cc

W = flow rate of medium into fermentor, cc/hr

μ = specific growth rate, hr^{-1}

μ_a = actual specific growth rate of run, hr^{-1}

μ_{max} = maximum specific growth rate determined by batch system, hr^{-1}

μ_t = theoretical specific growth rate determined from actual dilution rate and initial cell concentration for run, hr^{-1}

w = dilution rate, hr^{-1}

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8) Wolin, M.J., E.A. Wolin and N.J. Jacobs, "Cytochrome-Producing Anaerobic Vibrio, Vibrio succinogenes, Sp. N.," Journal of Bacteriology, vol. 81, no. 6, June, 1961, pp. 911-917.