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ABSTRACT

Title of Thesis: The Catabolism of Arginine by *Lactobacillus brevis* in a Chemostat.

Jeanette Carque, Master of Science in Chemical Engineering, 1985

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

A total of thirty chemostat runs were evaluated for steady state biomass concentration of *L. brevis* and arginine deiminase activity at dilution rates ranging from 0.022 to 0.107 h^{-1} for a substrate feed consisting of 1.0% glucose and 0.5% arginine (w/v). Biomass concentration and enzyme activity responses were also evaluated for variations in glucose concentration from 0.05% to 1.5% and variations in arginine concentrations from 0.25% to 1.0% at a constant dilution rate (0.06 h^{-1}).

The system exhibited multiple steady states for the range of dilution rates from 0.022 to 0.107 h^{-1}. A steady state biomass concentration of 0.131 mg cells/ml and an enzyme activity measuring 0.065 units/mg p were obtained at a low dilution rate (0.036 h^{-1}). At a higher dilution rate (0.097 h^{-1}) the biomass was 0.049 mg cells/ml and the enzyme activity was 0.022 units/mg p. Wash-out of biomass occurred above a dilution rate of 0.107 h^{-1}.

Optimum enzyme activity in the chemostat occurred when the glucose concentration was reduced to 0.05% with an arginine concentration of 0.5%. Batch results demonstrated an even higher activity for a stationary phase culture with the same initial substrate concentrations. The resulting optimum activities for chemostat and batch cultures were 0.083 and 0.114 units/mg p, respectively.

The possibilities of glucose-mediated catabolite repression or other mechanisms regulating arginine catabolism are discussed for both batch and continuous conditions.

THE CATABOLISM OF ARGININE BY LACTOBACILLUS BREVIS IN A CHEMOSTAT

by

JEANETTE CARQUE

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
Master of Science in Chemical Engineering
1985

APPROVAL SHEET

Title of Thesis: The Catabolism of Arginine by Lactobacillus brevis in a Chemostat

Name of Candidate: Jeanette Carque
Master of Science in Chemical Engineering, 1985

Thesis and Abstract Approved:

_______________ May 10, 1985
Dr. T. Greenstein Date
Professor
Department of Chemical Engineering

_______________ May 10, 1985
Date

_______________ 5/10/85
Date

VITA

Name: Jeanette Carque.

Permanent Address:

Degree and date to be conferred: Master of Science in Chemical Engineering, May, 1985

Date of birth:

Place of birth:

Secondary education: West Morris Regional H.S., 1972.

Collegiate institutions attended:

Institution	Dates	Degree	Date of Degree
Allegheny College	9/72-3/76	BS	1976
N.J. Institute of Technology	9/80-5/83	BS ChE	1983
N.J. Institute of Technology	9/83-5/85	MS ChE	1985

Major: Chemical Engineering

Positions held:

Laboratory Technician, 1974-1976, Allegheny College, Meadville, Pa. 16335.

Field Representative, 1976-1982, N.J. State Dept. of Health, Div. of Epidemiology, East Orange, N.J. 07018.

PREFACE

Continuous-flow culture systems have found widespread use as a research tool to examine the interaction between microbial cells and their environment. Their potential use within the fermentation industry has not yet been fully exploited. With the current developments in biotechnology and genetic engineering, the prospective use of genetically engineered microorganisms has created momentum within the fermentation industry. The design of new processes for large-scale production of microbial cells and their products are expected to include an increase in continuous culture applications.

This upsurge in continuous fermentation technology is made evident by a review of the periodical literature. The number of publications pertaining to continuous-flow culture systems has significantly increased within the past decade. Research dealing with the preparation of microbial fermentation products (e.g., ethanol, lactic acid, lipids, antibiotics, etc.) is most prominent.
In conjunction with product development, many articles describe apparatus modifications and design improvements, such as cell recycle and multistage systems. Mathematical

modelling of reaction and growth kinetics and elements of process control are also considered.

From a research standpoint, the content of this manuscript is restricted to those references which describe the fundamental theory of continuous fermentation and pertinent prior work related specifically to the experimental system involved. For additional references on continuous fermentation, the reader is directed to two journals available at NJIT, Biotechnology and Bioengineering and Applied Chemistry and Biotechnology. The Derwent Biotechnology Abstracts is an excellent comprehensive source of recent publications (1982$^+$). These abstracts are available at the Library of Science and Medicine, Rutgers University, New Brunswick.

To any experimenter planning research in the area of continuous fermentation, it should be emphasized that the chemostat apparatus is the integral factor in determining the success of the experimentation. Inadequacies in hand-constructed, laboratory-scale equipment frequently lead to contamination and unreliable experimental results. A sufficient amount of time must be devoted to perfecting the fermentation apparatus.

Acknowledgement is due to the Duponts de Nemoirs Corporation for partial financial support during this project. I would like to express personal appreciation to Dr. David Kafkewitz, Chairman of the Department of Zoology, Rutgers University, Newark, for his guidance and supervision during the term of my laboratory research. Without his encouragement and support this project might not have been completed. I would also like to thank Dr. Teddy Greenstein for his unrelenting patience during times of equipment failure, contaminated cultures and other operational difficulties. Special thanks to Nancy Myers for typing this manuscript. Finally, I would like to thank all of my family and friends for their emotional support, especially my parents and Ron Ashley.

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I. INTRODUCTION

Current applications of industrial fermentation involve any system in which microorganisms are cultivated for the manufacture of commercial products. Such products include vaccines, antibiotics, hormones, vitamins, enzymes, alcoholic beverages, and single-cell protein (7).

Fermentation can proceed utilizing either a batch or continuous-flow system. With batch industrial processes well established since the early twentieth century, the development of large-scale continuous culture applications has been slow. Since Monod (33) first outlined the theory of continuous fermentation in 1950, the method has been widely used as a laboratory research tool. Applications in this area have been effective in generating an increased understanding of the characteristics of microbial growth and physiology.

This manuscript describes the investigation of a continuous fermentation system utilizing the microorganism <u>Lactobacillus brevis</u> for the production of the enzyme L-arginine deiminase (EC 3.5.3.6). The potential therapeutic applications associated with arginine

deiminase (21) prompted evaluation of this particular enzyme. Although arginine deiminase has been established in lactobacilli, arginine catabolism is not well characterized within the species *L. brevis*.

A description of Monod's theory of continuous culture fermentation is given in Section II of this manuscript. The theory of the chemostat is preceded by a general description of bacterial growth and followed by specific advantages and applications of continuous culture in both research and industry.

Section III gives an overview of the particular system being investigated. A general introduction to the therapeutic uses of enzymes is followed by a closer description of the enzyme arginine deiminase, arginine catabolism and the cultivation of lactic acid bacteria. The intent of this section is to review any pertinent prior work dealing with the various aspects of this experimental system.

Detailed descriptions of the fermentation apparatus and the experimental procedure are given in Sections IV and V respectively. The remaining sections present an analysis and discussion of the experimental results, conclusions and recommendations derived from this research experience.

II. PRINCIPLES OF CONTINUOUS CULTURE FERMENTATION

A. Bacterial Growth

Analysis of the growth of microorganisms can be performed in both open and closed systems. A batch-grown culture is representative of a closed system, since no nutrients are allowed to enter and no metabolites are allowed to leave the system during fermentation. The extent of growth is limited by the batch environment of a closed system. An open system, such as with continuous flow cultures, allows input of nutrients and outflow of biomass and metabolites (20).

Formulation of the theory of continuous culture fermentation begins with an understanding of the growth characteristics of a microorganism in a batch system. The batch growth cycle of microorganisms can be divided into a series of phases based on changes in the rate of growth. According to Pirt (37) there are six phases:

I. Lag
II. Accelerating Growth
III. Exponential Growth
IV. Decelerating Growth
V. Stationary
VI. Decline

FIGURE 1 schematically illustrates these phases in reference to changes in biomass as fermentation time proceeds.

Although this generalized scheme accurately represents the growth pattern of a batch culture, the length and characteristics of each phase will vary among organisms. In addition, changes in environmental conditions will influence the growth characteristics for the same species. Temperature, pH, nutrient supply, and the concentration of dissolved oxygen can each contribute to the viability of the organism (4).

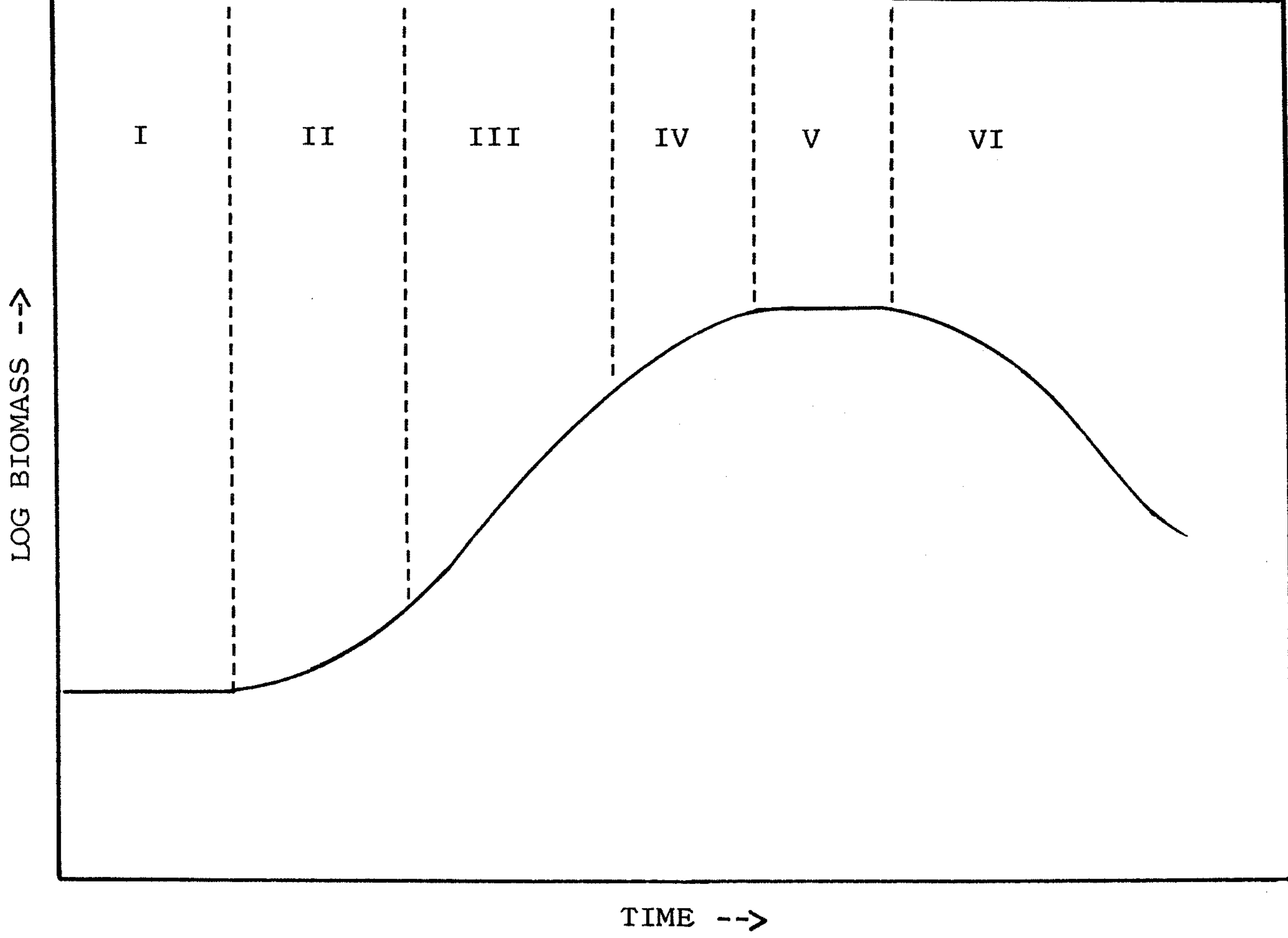
The initial lag period represented by PHASE I of FIGURE 1 is attributed to the time needed for the organism to adapt to a new environment. Changes in nutrient composition or the age and size of the inoculum will affect the length of this lag period (4).

After the organism has adjusted to the new environment, it will begin to multiply (PHASE II). The increase in biomass is directly proportional to the amount of biomass present at any given time (37). This pattern of growth can be represented mathematically by the following equations:

$$\frac{dX}{dt} = uX \tag{1}$$

FIGURE 1

<u>THE BATCH GROWTH CURVE</u>. The six phases of microbial growth are: I, lag; II, accelerating; III, exponential; IV, decelerating; V, stationary; and VI, decline.



$$\mu = \frac{1}{X} \frac{dX}{dt} \qquad (2)$$

The differential dx/dt in equation (1) represents the rate of change of biomass X per unit time t, or the population growth rate. Equation (2) defines the parameter μ as the population growth rate per unit amount of biomass, or the specific growth rate. The units of μ are reciprocal time (1/t).

During the exponential growth phase (PHASE III) the specific growth rate is constant and at its maximum value. This constant parameter is referred to as μ_{max}. Simple integration of the rate equation will result in an equation for biomass as a function of fermentation time. That is,

$$X = X_o e^{\mu_{max}(t - t_{lag})} \qquad (3)$$

where X_o is the amount of biomass present at the time of onset of the exponential growth phase, t_{lag}.

The doubling time, t_d is defined as the time required for the biomass to increase from X_o to $2X_o$. Substituting into equation (3) and rearranging,

$$t_d = \frac{\ln 2}{\mu_{max}} \qquad (4)$$

This parameter, along with the specific growth rate constant, μ_{max} are interchangeably used to characterize the exponential growth phase of the system being described (20).

Monod (32) more specifically describes microbial growth with a functional relationship between the specific growth rate and the substrate concentration by the following classical equation:

$$\mu = \frac{\mu_{max} S}{S + K_s} \tag{5}$$

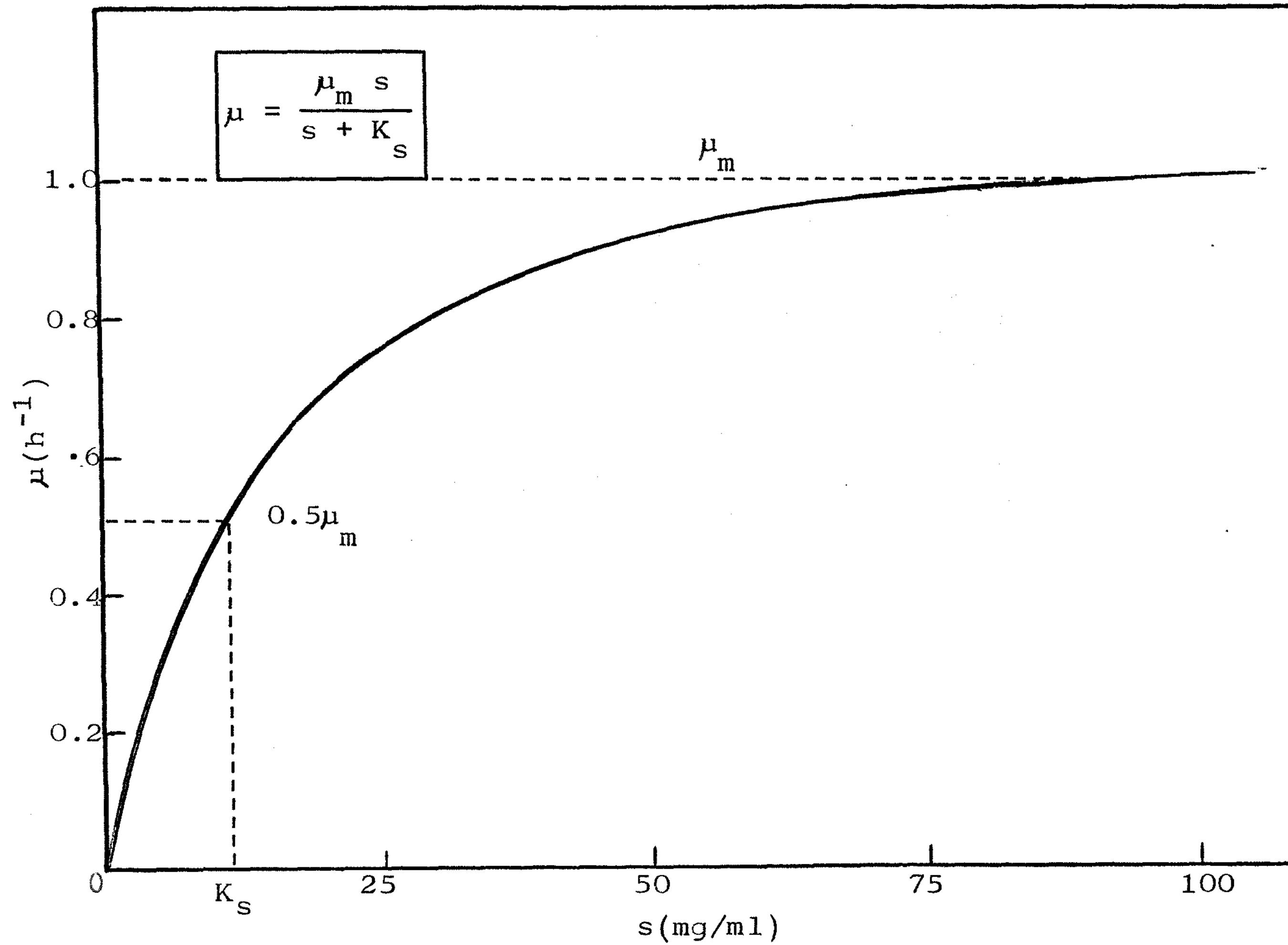
where S is the substrate concentration and K_s is defined as the saturation constant. This equation is illustrated in FIGURE 2. The units of K_s must be equivalent to the units of substrate concentration. The physical significance of the saturation constant is that the substrate concentration, S is equal to the saturation constant, K_s when the specific growth rate μ is at half of its maximum value.

Monod's equation is similar in form to the Michaelis-Menten equation for enzyme catalyzed reactions with a single substrate:

$$v = \frac{v_{max} S}{S + K_m} \tag{6}$$

FIGURE 2

<u>MONOD'S EQUATION FOR BACTERIAL GROWTH</u>. Specific growth rate, μ versus substrate concentration, s; μ_m=1.0 h^{-1}, K_s=10 mg/ml.



where v is the reaction velocity, v_{max} is the maximum velocity and K_m is the Michaelis constant.

Comparison of these two equations leads to the hypothesis that bacterial growth is limited by the rate of active transport of glucose. Active transport rates have been observed to vary with external nutrient concentrations in the same way as enzyme reaction rates. Therefore, the specific growth rate of a culture will vary with nutrient concentration in a similar way (4). For this proposed system, the saturation constant would represent the Michaelis constant for the permease carrier molecule. The specific growth rate constant would then be an incorporation of v_{max} for permease and other various physical factors involved in the cell's nutrient utilization (4).

Since K_s is usually much smaller than S, μ is equivalent to μ_{max} at least at the onset of the exponential growth phase. As the substrate concentration approaches the saturation constant, the specific growth rate will decrease.

The duration of the exponential growth phase can therefore be considered a function of the growth-limiting nutrient. In many systems, however, stationary phase

(PHASE V in FIGURE 1) is reached prior to nutrient starvation. An accumulation of metabolic products will often inhibit growth. There is also a maximum packing density of biomass which helps to regulate perpetual reproduction of microorganisms (37).

During stationary phase some cells are dividing while others die. Utilization of internal nutrients released from lysed dead cells help maintain the population size. The cell population eventually enters the declining phase (PHASE VI). Decay of the culture is usually exponential (4).

Several additional parameters are important in describing bacterial growth. The growth yield, Y is defined as the negative of the change in biomass divided by the corresponding change in substrate concentration:

$$Y = \frac{-dX}{dS} = \frac{\text{mass of cells formed}}{\text{mass of substrate consumed}} \qquad (7)$$

Equation (7) is useful in the determination of the quantitative nutritional requirement of an organism (37). The growth yield should have a constant value for constant experimental conditions.

The metabolic quotient, q represents the rate of consumption of a substrate in a culture at a specific

point in time. Equation (8) defines q in terms of the growth yield, Y and the specific growth rate, μ.

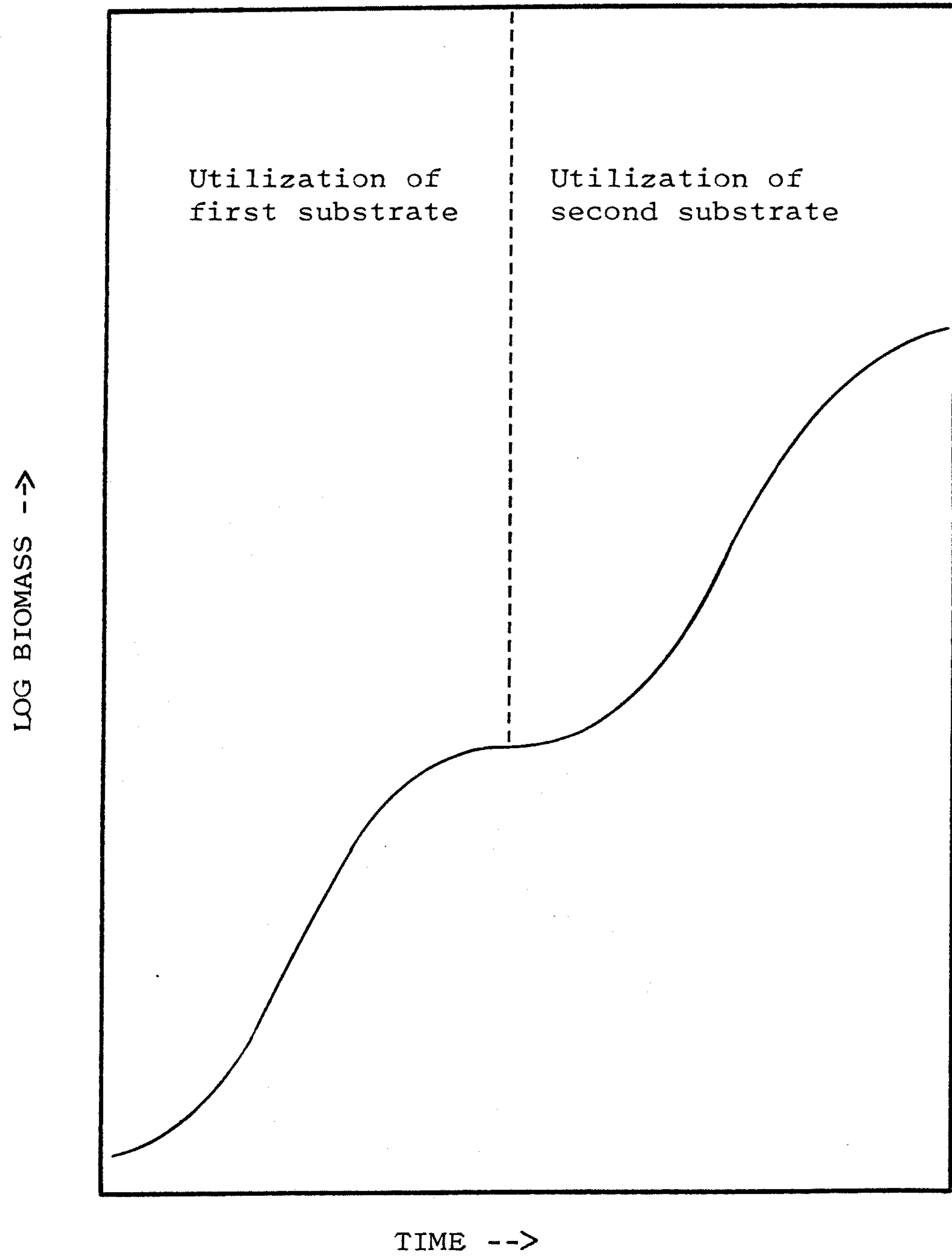
$$q = \frac{\mu}{Y} \tag{8}$$

This relationship enables the estimate for substrate requirements at different growth rates (37). It should be noted that all parameters are defined for simple, homogeneous batch cultures.

There are several variations in the generalized growth pattern illustrated in FIGURE 1 (37). In some systems there is a decrease in cell density before entering stationary phase. Another variation demonstrates a higher increase in the cell population at the onset of growth as compared with the normal slope of the exponential growth curve. FIGURE 3 illustrates a third variation in the generalized growth pattern. This pattern, known as diauxic growth, represents sequential utilization of substrates. After the organism completes utilization of the primary substrate, a lag period occurs to accommodate the metabolic adaptation to the change in utilizable substrates. The culture then initiates a second phase of exponential growth. Diauxie usually occurs as a result of catabolite repression with glucose as the primary substrate (36).

FIGURE 3

DIAUXIC GROWTH



B. Theory of the Chemostat

A continuous flow system is essentially one in which reactants flow at steady state into a reactor and products emerge at that same rate (20). The reactor must be maintained free of contamination and provide rapid mixing of inflowing nutrients (44). FIGURE 4 gives a schematic representation of a continuous flow, stirred tank reactor for cell cultivation. This reactor type is commonly referred to as a chemostat.

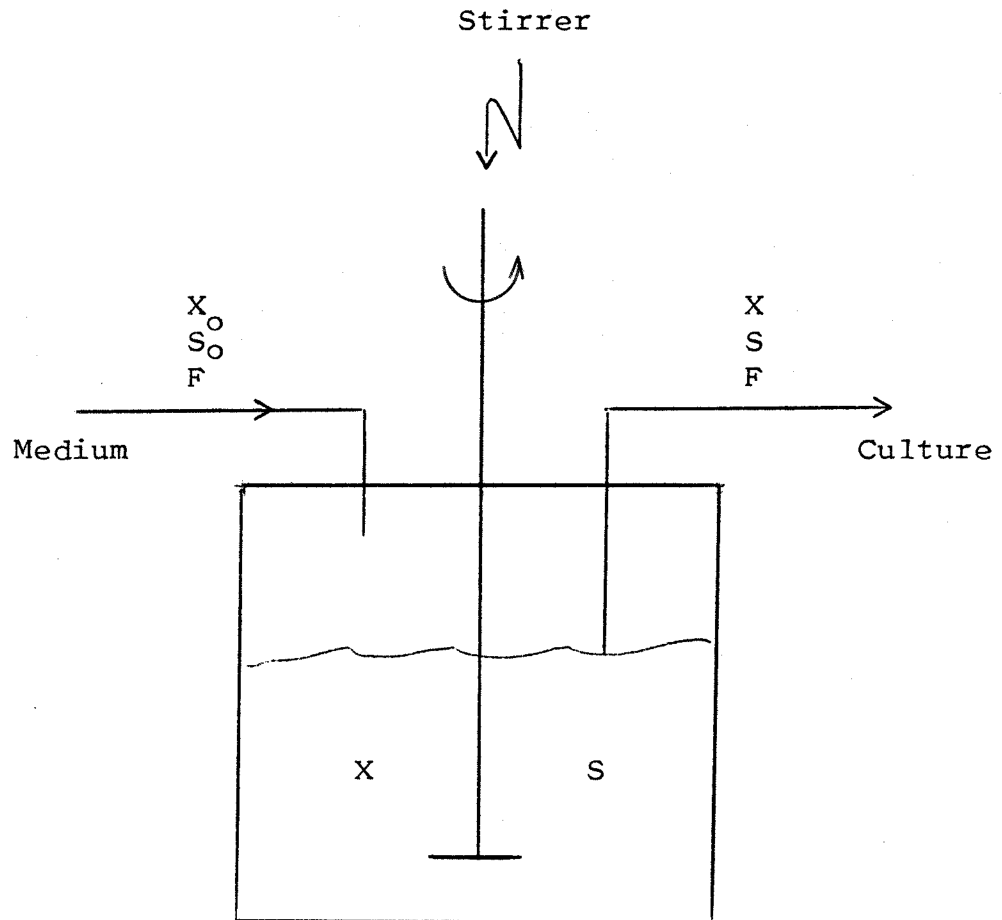
The current theory of continuous culture fermentation is founded on the research of Monod (33) and Novick and Szilard (35). These classic studies on continuous microbial cultivation proposed that the specific growth rate of a culture could be fixed at any positive rate less than μ_{max}. This concept opposed the traditional opinion that the maximum specific growth rate occurring during the exponential growth phase of a batch-grown culture was the only stable growth rate (37).

An important parameter which is characteristic of continuous flow systems is the dilution rate, D. D is defined as the steady state volumetric flow rate, F divided by the fixed reactor volume, V in a continuous flow reactor. Hence,

$$D = \frac{F}{V} \qquad (9)$$

FIGURE 4

THE CHEMOSTAT



The units of D are reciprocal time (1/t).

The dilution rate is more useful than the flow rate in characterizing the growth rate of an organism in a chemostat (44). A more specific, physical description of the dilution rate is the number of culture volumes of medium passing through the vessel per unit time (4). The reciprocal of the dilution rate (1/D) is defined as the mean residence time of the reactor or the culture volume turnover time, $\bar{t}$.

A material balance for a continuous flow system in steady state operation can be expressed as:

$$\text{INPUT} + \text{GROWTH} = \text{OUTPUT} + \text{ACCUMULATION} \tag{10}$$

This expression can be represented mathematically in terms of the physical parameters of the system (4):

$$F(X_o - X) + Vr_x = 0 \tag{11}$$

where X is the exit concentration of biomass, X_o is the feed stream biomass concentration and r_x is the rate of biomass formation (dX/dt). F and V have been previously defined as the volumetric flow rate and reactor volume, respectively. For ideal operation, the exit concentration of the biomass and the concentration of biomass within the reactor are equivalent.

Using the definitions for the specific growth rate, μ and the dilution rate, D given in equations

(2) and (9), equation (11) can be rewritten as,

$$DX_o = (D - \mu)X \tag{12}$$

When dealing with sterile nutrient feed, X_o=0. Therefore for steady state operation with a nonzero cell population within the reactor, the dilution rate and the specific growth rate must be equivalent.

$$D = \mu \tag{13}$$

It follows that the maximum operating dilution rate, D_{max} is equal to the maximum specific growth rate, μ_{max}.

Monod's equation for bacterial growth, equation (5), is used to define the specific growth rate as a function of the substrate concentration. Substituting this expression for μ into equation (12), it becomes:

$$\left[\frac{\mu_{max} S}{S + K_s} - D\right] X + DX_o = 0 \tag{14}$$

A material balance on the substrate can similarly be derived to yield the equation,

$$D(S_o - S) - \frac{\mu_{max} SX}{Y(S + K_s)} = 0 \tag{15}$$

Equations (14) and (15) represent the general model proposed by Monod to describe bacterial growth

in a chemostat. For sterile nutrient feed, the following equations are obtained to define the biomass and substrate concentrations within a chemostat (4):

$$X = Y\left[S_o - \frac{DK_s}{\mu_{max} - D}\right] \tag{16}$$

$$S = \frac{DK_s}{\mu_{max} - D} \tag{17}$$

This pattern of biomass concentration and substrate concentration as a function of the dilution rate is illustrated in FIGURE 5.

The output of biomass, DX, as a function of dilution rate is also described in FIGURE 5. The equation for this curve is obtained by multiplying equation (16) for the biomass concentration by the dilution rate, D (4). The dilution rate producing maximum biomass output is obtained by setting the first derivative of this equation equal to zero and solving for D:

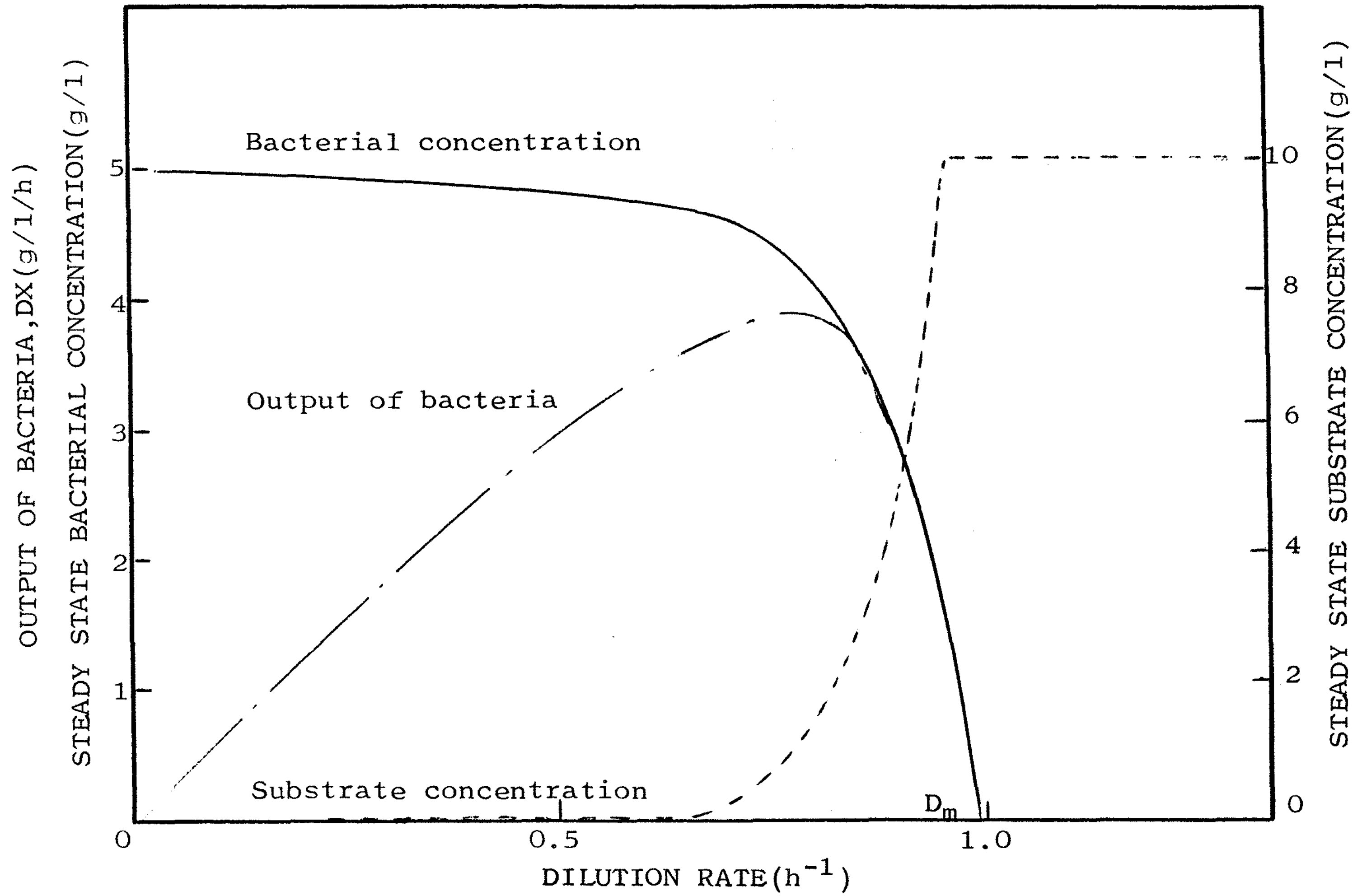
$$\frac{d(DX)}{dD} = 0 \tag{18}$$

$$D_{\text{output max.}} = \mu_{max}\left[1 - \left(\frac{K_s}{K_s + S}\right)^{0.5}\right] \tag{19}$$

FIGURE 5

STEADY STATE RELATIONSHIPS IN A CONTINUOUS CULTURE

$u_m = 1\ h^{-1}$, $K_s = 0.2$ g/l, Y=0.5



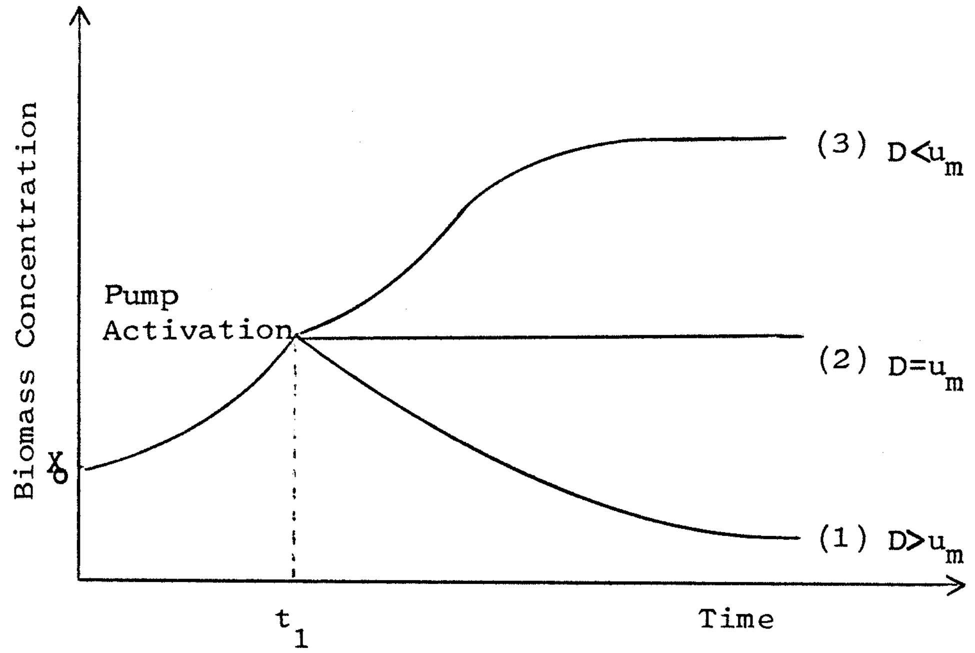
Several generalizations can be made from evaluation of equations (16), (17) and (19) and FIGURE 5. S approaches zero as D approaches zero, indicating that the entire concentration of limiting substrate is utilized. As D is increased, S increases at a linear rate while X decreases correspondingly. This situation suggests that the decrease in biomass is a function of medium flow rate. Finally, X becomes zero and S becomes S_o as D approaches and exceeds the value of D_{max}. This condition is known as washout of the continuous flow system.

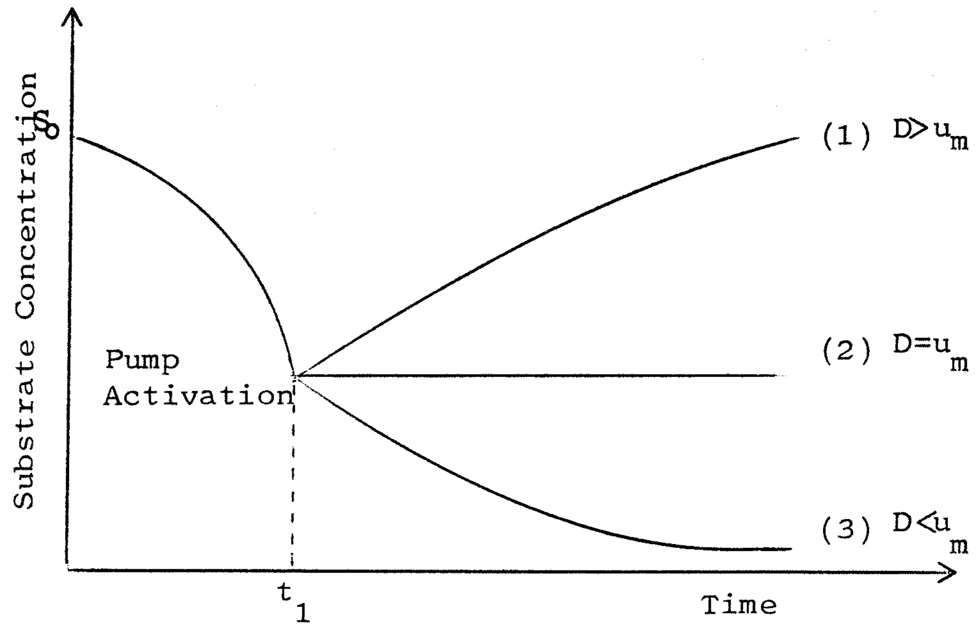
A schematic representation of biomass and substrate concentration as a function of fermentation time can be used to help illustrate the conditions encountered during chemostat operation at different dilution rates (FIGURE 6). A batch culture has been allowed to grow within the chemostat culture vessel. Continuous flow is initiated at time t_1. Curve 1 describes the complete washout of cells when the operating dilution rate is greater than D_{max}. The substrate concentration correspondingly approaches S_o.

A steady state response is observed for a dilution rate equivalent to D_{max} (Curve 2). It should be noted

FIGURE 6

POSSIBLE VARIATIONS IN BIOMASS AND SUBSTRATE CONCENTRATION WITH TIME IN A CHEMOSTAT





that this system is extremely sensitive to minor fluctuations in dilution rate as D approaches D_{max}. Therefore, although the dilution rate at which maximum biomass output occurs is usually quite close to D_{max}, it is not uncommon to operate a given system at a lower, more stable flow rate (12).

Curve 3 depicts the steady state response observed when the operating dilution rate is less than D_{max}. The biomass increases at a maximum rate of growth (μ_{max}) until restricted by the concentration of limiting substrate. The specific growth rate decreases until the rate of growth is equivalent to the operating dilution rate. The steady state conditions obtained for dilution rates less than D_{max} are stable and self-regulating. Minor fluctuations will result in compensatory change in growth rate by the culture in order to restore steady state conditions (12).

It can thus be seen that stable chemostat operation is performed at a flow rate corresponding to a dilution rate less than D_{max}. The steady state concentration of biomass obtained under these conditions is restricted by the concentration of limiting nutrient in the feed medium. If that limiting substrate concentration is

varied, a corresponding change should occur in the steady state biomass concentration. This pattern is illustrated in FIGURE 7. Three different substrate concentrations are depicted for an arbitrarily selected system. As the substrate concentration is increased, the steady state biomass concentration at the same dilution rate increases proportionally. Operation at dilution rates approaching D_{max} becomes even more unstable as the biomass concentration is increased.

C. Advantages and Applications of the Chemostat

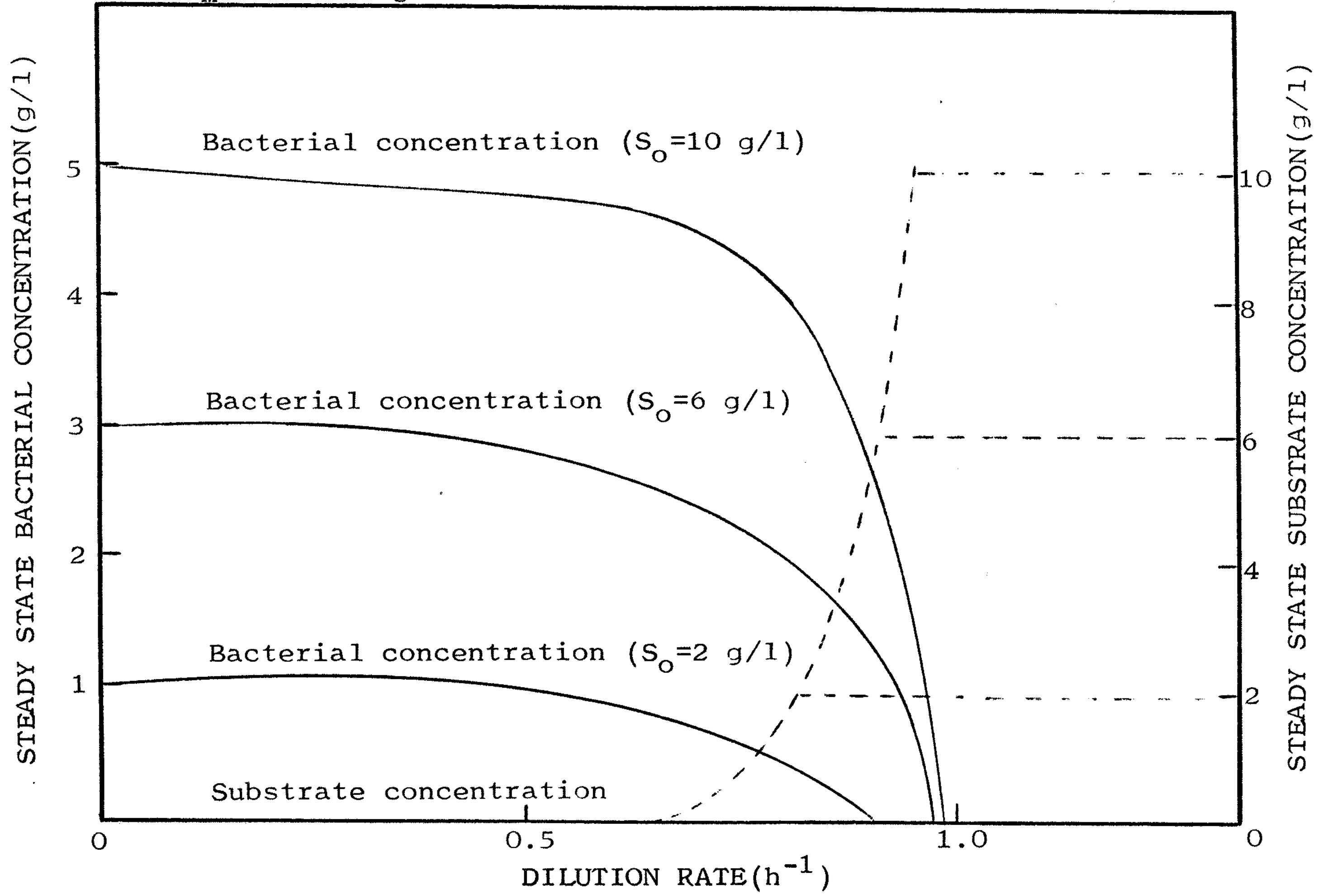
Batch culture methods have attained widespread use for investigation of microorganisms. A batch system can be operated simply and aseptically, providing a useful tool for close examination into the characteristics of pure cultures. Studies with batch-grown cells have provided an abundance of experimental data pertaining to all aspects of Microbiology.

It has been pointed out, however, that the behavior of microorganisms in a batch system is considerably different from microbial existence in natural environments (20). It is unlikely that a culture would be able to naturally propagate at its maximum

FIGURE 7

CHEMOSTAT RESPONSE TO DIFFERENT SUBSTRATE CONCENTRATIONS

$u_m = 1\ h^{-1}$, $K_s=0.2$ g/l, $Y=0.5$



specific growth rate. This would require that essential nutrients be present in excessive amounts. In addition, a naturally occurring culture would not be protected by a closed, homogeneous environment. Mixed microbial populations would predominate. It is therefore an advantage to utilize non-traditional experimental approaches to investigate microbial growth.

The primary use of the chemostat as a research tool is evaluation of microbial growth under substrate limiting conditions (44). The ability of microorganisms to change themselves phenotypically with changing environmental conditions limits the evaluation of many aspects of microbial physiology. A controlled, open environment is more responsive than a batch environment to the characterization of many structural and functional adaptations which are elicited by that specific physical environment.

Research applications are limitless. Malek (29) supports the use of the chemostat to help elucidate the physiologic state of the cell, including content and structure of DNA and RNA, and both quantitative and qualitative description of the composition of ribosomes. Tempest describes investigations into cation metabolism,

cell wall synthesis and enzyme synthesis using a chemostat (44). In addition, the chemostat may find applications in the cultivation of cells of higher orders (29).

Two main disadvantages have been cited with respect to continuous microbial fermentation. Earlier studies using the chemostat were limited as much by the difficulty of maintaining sterility during operation as by the lack of acceptance of Monod's theoretical basis. Maintaining a pure culture is a limitation of the apparatus as opposed to the process and can therefore be controlled.

A second disadvantage is the occurrence of microbial mutation during steady state, continuous operation (20). Microorganisms are fiercely selective, with those organisms best adapted to the environmental conditions surviving. Low concentrations of a growth limiting nutrient will eventually create selective pressures causing mutation. Duration of continuous operation is ultimately restricted by this characteristic of microbial populations. Mutation in a chemostat has been well investigated (27).

The key to incorporation of the chemostat into industry as well as research is the importance of

controlled environmental conditions of operation. The self-regulatory nature of chemostat operation further increases the potential for industrial use. Equally important to industrial applications is the productivity of continuous culture fermentation as opposed to batch fermentation processes. An equation has been derived to theoretically predict the maximum biomass output of a chemostat with respect to the biomass output of a batch culture (37), such that

$$\frac{\text{rate of chemostat output}}{\text{rate of batch output}} = \ln \frac{X_m}{X_o} + \mu_{max} t_1 \qquad (20)$$

where X_o is the initial biomass concentration (inoculum of the batch culture), X_m is the output biomass concentration and t_1 is the initial lag period associated with the batch growth cycle.

The ratio $X_m{:}X_o$ is usually 10 or greater, making the maximum output rate of the chemostat at least 2.3 times greater than the batch rate for zero lag. Obviously this is a minimum value since there is usually some lag time, t_1 associated with a batch system.

The relationship in equation (20) describes higher productivity of biomass which, in many cases, corresponds

to higher yield of products. There are, of course, processes for which this is not the case. In addition, industrial potential is ultimately influenced by economic advantages and disadvantages of a system.

Continuous culture in industry can basically be applied to four main types of microbiological processes (14):

I. Production of biomass or 'single cell protein'

II. Production of primary metabolites

III. Chemical Transformations

IV. Production of secondary metabolites

Ethanol, lactic acid, acetone and butanol are examples of primary metabolites whereas antibiotics and vitamins are secondary metabolites. Chemical transformation of steroids is a common batch process which could be performed under continuous operation.

There is evidence that continuous operation would be economically superior to batch processes for the first three process types listed above (14). However, the only important example of continuous fermentation in industry is continuous brewing. In order for continuous fermentation to become an established industrial process, the continuous process must be (a) economically

superior to the batch process, (b) required to satisfy an increasing product demand, and (c) designed for trouble-free operation.

A number of variations and elaborations on the simple chemostat structure have been developed in attempts to improve continuous culture operations (6 , 14). Some of these modifications are described in TABLE 1.

TABLE 1

CLASSIFICATION OF CONTINUOUS OPERATION REACTOR TYPES

REACTOR TYPE	DESCRIPTION	ADVANTAGES AND APPLICATIONS
Single Stage	. single culture vessel with continuous steady state input and outflow	. simplicity . optimum for cell growth or growth related product
Semicontinuous	. intermittent input and outflow for single vessel	. compromises features of batch and continuous operation
Turbidostat	. steady state flow is determined by photocell regulation of turbidity	. allows stable operation at dilution rates near D_{max}
Biomass Feedback	. internal or external recycling of cells . effluent recirculation until complete conversion occurs	. most effective use of medium in biosynthesis of product rather than cells . growth limiting substrate is dilute or solubility is low . decreases product inhibition
Multiple Stage	. several culture vessels in series	. more efficient use of substrate with higher product yield . combination of component functions is possible . use of all substrates in media with multiple substrates

III. AN OVERVIEW OF THE EXPERIMENTAL SYSTEM

A. Therapeutic Uses of Enzymes

Enzymes are extremely powerful, highly specific catalysts which accelerate chemical reactions in biological systems. Their value as therapeutic agents arises from their specificity and high, near-optimal activity at physiological pH and temperature. It is important that the affinity of the therapeutic enzyme for its substrate is high, therefore having a reasonably low value for the Michaelis constant, K_m. The enzyme must also be stable in blood and other body fluids (21, 22).

Ongoing research into the therapeutic applications of enzymes continues to disclose numerous possibilities. Initial applications were limited to crude preparations with proteolytic and hydrolytic activity which are administered topically or orally. Current research is developing applications in the treatment of genetic defects, clotting disorders, inflammation, digestive problems, drug toxicities, kidney failure and cancer (21).

Parenteral treatment of these disorders with purified, soluble enzyme extracts is limited by a number of factors (22). Upon administration to the bloodstream,

the systemic distribution of the enzyme is random and the protein is rapidly removed or degraded by the body's natural defenses. The enzyme may be subject to adverse immunologic response. It follows that current objectives in the area of enzyme therapy are to (a) eliminate antigenicity to the drug, (b) inhibit removal and degradation of the enzyme within the circulatory system, and (c) modify the drug in such a way as to direct it to the specific target tissue (21).

The degree of response elicited by the enzyme from the body's immune system is a key factor in determining the source of the therapeutic enzyme. The effectiveness of an enzyme can be complicated by circulating antibodies already present in the bloodstream. The recognition associated with a native enzyme results in rapid inactivation. Although a foreign protein also elicits an immune response and in fact can cause severe allergic reaction in the host, therapeutic effectiveness is generally increased by selecting a species that has limited association with man as the enzyme source (47). Microorganisms are an important source of enzymes for a wide variety of applications, including therapeutic applications. An important reason for their popularity

is the ability to manipulate enzyme levels within a microbial culture through environmental and genetic variations (7).

Modifications to enzyme structure have been used to improve the therapeutic properties. Soluble and insoluble conjugates can be used to effectively increase the molecular size, thus interfering with the antigen-antibody recognition process and inhibit clearance from the bloodstream. An alternate method used to decrease antigenicity is encapsulation of enzymes within biodegradable or inert material. The microcapsule creates a barrier between the blood and the circulating enzyme. Modifications of this nature may eventually lead to successful concentration of the enzyme in the specific target area (18).

Enzyme therapy of neoplasia (cancer) involves tumor cell deprivation of specific amino acids through localized degradation of those amino acids by enzymes (21). All nonessential amino acids are potential targets for degradation, since normal cells have the capacity to produce these substances.

This clinical approach to cancer therapy originated with the observation that certain tumor cells do not have the enzyme needed to synthesize asparagine,

asparagine synthetase (22). These diseased cells obtained the asparagine required for growth from the circulating bloodstream. Elimination of asparagine from the bloodstream would result in starvation of the tumor cells. Asparaginase and glutaminase are currently used in the treatment of human acute lymphocytic leukemia (22).

In addition to asparagine and glutamine, nonessential amino acids with possible therapeutic applications include arginine and citrulline (21). Since most tumor cells can utilize citrulline in place of arginine, successful therapy may eventually involve combined depletion of both these amino acids. However, at present citrulline degrading enzymes evaluated have not been found suitable for therapeutic use (21). Arginine can be degraded by arginine deiminase, arginase and arginine decarboxylase, and research has demonstrated an absolute requirement for arginine in a small number of rodent tumors (21). It has been demonstrated that arginine deiminase is effective in inhibiting the growth of L1210 and L5178y mouse leukemic lymphoblasts in vitro (15,24).

B. Arginine Deiminase

Microorganisms catabolize arginine by one of four major pathways (1):

1) The Arginase-Urea Amidolyase Pathway
2) The Arginine Transamidinase Pathway
3) The Arginine Dihydrolase Pathway
4) The Arginine Decarboxylase Pathway

The arginine dihydrolase pathway has proven to be a major source of energy for a number of anaerobic organisms. The pathway has been documented in *S. faecalis* (1), *P. putida* (40), *M. hominis* (39), *M. arthritidis* (13), *S. lactis* (9) and *L. buchneri* (30). The presence of arginine deiminase also supports the existence of the pathway in species of Clostridium, Treponema, Halobacterium, Chlorella, Bacillus, Aphanocapsa, Plectonema and Tetrahymena (1).

Arginine deiminase is the first enzyme of the pathway and it catalyzes the conversion of arginine to citrulline and ammonia. The second enzyme, ornithine transcarbamylase converts citrulline to carbamylphosphate and ornithine. Carbamate kinase catalyzes the final breakdown of carbamylphosphate to carbon dioxide and

ammonia with the formation of one mole of ATP. The pathway is illustrated in FIGURE 8.

Arginine deiminase can be purified using affinity chromatography. Crystalline arginine deiminase is a homogenous preparation. Its dimer form consists of two apparently identical subunits (42). Some general characteristics of this enzyme are summarized in APPENDIX C.

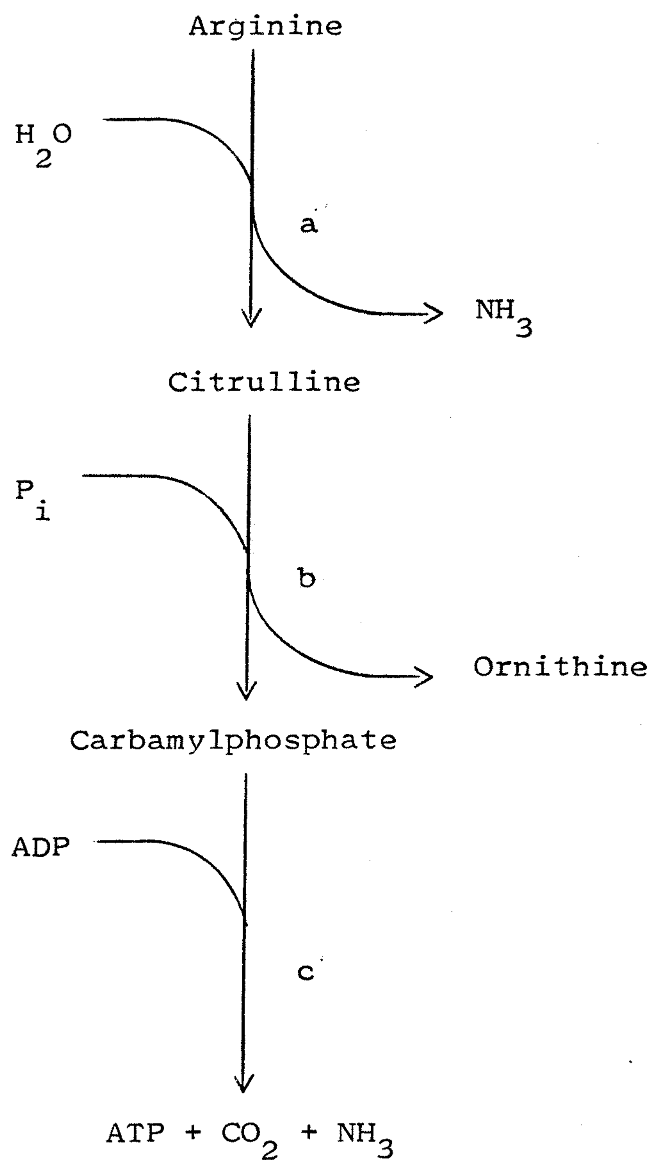
The action of arginine deiminase involves the hydrolysis of a C-N bond in the guanidino group of arginine. A proposed mechanism for the catalytic reaction converting arginine to citrulline and ammonia consists of three steps (42):

1) Formation of the Michaelis complex
2) Formation of an amidino-enzyme intermediate accompanied by the liberation of ammonia
3) Hydrolysis of the amidino-enzyme complex, the rate determining step

As stated previously, arginine deiminase has been purified from several microbial sources. Values for the Michaelis constant from some of these sources are summarized in TABLE 2. The potential for therapeutic use of this enzyme is supported by these very low reported values for the substrate K_m. In addition,

FIGURE 8

THE ARGININE DIHYDROLASE PATHWAY FOR ARGININE CATABOLISM



Enzymes: a, arginine deiminase; b ornithine transcarbamylase; c, carbamate kinase

TABLE 2

CHARACTERISTICS OF L-ARGININE DEIMINASE ISOLATED FROM SEVERAL MICROBIAL SOURCES

ENZYME SOURCE	ACTIVITY units/mg p	OPTIMUM pH	SUBSTRATE K_m, mM	MOLECULAR WEIGHT
Streptococcus faecalis	41.0	6.8	0.15	
Mycoplasma hominis	53.0	6.5-6.7	0.1-0.4	78,300
Tetrahymena pyriformis	47.5	9.3	2.84	
Pseudomonas putida	58.8	6.0	0.20	120,000

the enzymes isolated from *S. faecalis* and *M. hominis* have optimum pH values near physiological pH.

Enzyme activities are also reported in TABLE 2. Arginine deiminase activity is usually determined using assays for citrulline production based on the method of Archibald (3). One unit of activity represents the formation of one micromole of citrulline per minute at the specified temperature (15). The specific activity would then be equivalent to units of activity per milligram of protein.

C. The Cultivation of Lactic Acid Bacteria

Fermentation has been traditionally defined in terms of anaerobic metabolism of microorganisms. The reaction mechanism for extraction of energy in the absence of oxygen utilizes organic compounds as both electron donors and acceptors in ATP yielding oxidative pathways. Carbohydrates are the most important class of nutrients for fermentation (4).

Fermentation by lactic acid bacteria has been well investigated. The role of these organisms in the production of dairy products such as cheese, yogurt, and fermented milk products has significant economic

importance (8a). Lactic acid also has industrial potential as an intermediate in the plastics industry (14). In many cases the effect of these organisms is detrimental, as in the souring of milk by streptococci. In addition, lactic acid bacteria are frequently responsible for excess acidity in beer and wine.

In general, species of the genus Lactobacillus can be classified into three groups according to their glucose fermentation products (8a):

1) Obligate heterofermenters
2) Obligate homofermenters
3) Facultative homofermenters

Obligate heterofermenters produce a variety of products from glucose, including lactic acid, acetic acid, ethanol and carbon dioxide. The absence of fructose 1,6-diphosphate aldolase activity eliminates Embden-Meyerhof glycolysis as the primary fermentative pathway for these organisms. The presence of glucose 6-phosphate dehydrogenase and 6-gluconate dehydrogenase suggest the use of the hexose monophosphate shunt.

Embden-Meyerhof glycolysis is characteristic of obligate homofermenters, with lactic acid as the resulting fermentation product. The presence of the

enzymes from both pathways enables facultative homofermenters to produce either lactic acid as the sole fermentation product or a variety of organic products.

Lactobacilli are usually microaerophilic, but grow best in the absence of oxygen. The nutritional requirements of most species are extremely complex. The species used for this investigation, Lactobacillus brevis has an absolute requirement for a large number of vitamins and amino acids, including arginine (28). L. brevis is an obligate heterofermenter with lactic acid comprising about fifty percent of the fermentation products. Copious carbon dioxide production and excessive amounts of volatile acids are characteristic of this species (48). A list of general characteristics for this group of microorganisms is given in APPENDIX C.

Lactic acid bacteria are particularly suited for continuous culture fermentation with respect to their commercial applications. These organisms and their fermentation products usually involve simple biochemical conversions (14). In addition, normally acidic operating conditions often restrict the problems associated with contamination.

A procedure for continuous lactic acid fermentation was reported as early as 1931 (5). Both laboratory and

pilot plant scale operations using *Streptococcus lactis* to ferment whey were successfully implemented. That particular process was not put into large-scale use. The continuous culture fermentation of whey by lactic acid bacteria continues to be investigated as a commercial method for the production of lactic acid (25).

The use of lactic acid bacteria for the production of dairy products with continuous fermentation systems has also been investigated (5). Continuous cultivation of *Lactobacillus* and *Streptococcus* species in one and two-stage flow systems for the manufacture of active biomass for dairy starters has been reported (23). Results of experimentation with both pure and mixed cultures demonstrated physiological stability and high cellular activity. The authors did emphasize the importance of properly defining the complex nutritional parameters for cultivation of lactic acid bacteria.

Academic research describing lactic acid fermentation has provided evidence for some interesting physiological variations during continuous culture. A number of publications discuss a shift from normally homofermentative to heterofermentative catabolism of glucose for different environmental conditions.

In one study using Lactobacillus bulgaricus, a shift from acidic to basic pH of the medium triggers the shift to heterofermentative metabolism. A decrease in lactic dehydrogenase (LDH) enzyme synthesis also accompanied the shift to alkaline conditions. In addition, the occurrence of the phosphoro-clastic split in pyruvate conversion was apparently associated with alkaline conditions (38). These experimental results suggest that LDH concentration controls pyruvate metabolism.

Pyruvate metabolism has also been found to be dependent on the dilution rate in a glucose limited chemostat. A shift from generally heterofermentative metabolism to homofermentative metabolism occurs with a shift from lower to higher dilution rates. In L. casei, LDH activity is controlled by the concentration of fructose-1,6-diphosphate. Therefore the pathway of pyruvate conversion is regulated by the concentration of fructose-1,6-diphosphate, which is limited by the concentration of glucose (10).

Thomas, et. al., have performed similar research with the organism Streptococcus lactis (45). The shift from homofermentative to heterofermentative metabolism corresponded to a shift from high to low dilution rates.

Specific monitoring of the intracellular fructose-1,6-diphosphate concentrations supported the hypothesis that this compound controls pyruvate conversion.

D. The Regulation of Arginine Catabolism

The regulation of arginine deiminase synthesis and activity has been investigated for a number of micro-organisms in batch-grown cultures. In most cases the arginine dihydrolase pathway is used as an alternate energy yielding pathway supplemental to a carbohydrate energy source.

For aerobic organisms, arginine deiminase is sometimes inducible by anaerobic conditions. In *Mycoplasma hominis*, the actual enzyme levels are shown to double and the specific activity increases as much as ten-fold for an increase in arginine concentration of 4 mM to 34 mM (13). These results are not observed when the organism is grown aerobically. In aerobically grown cultures, the arginine deiminase levels are observed to increase only towards the end of exponential phase (13). Thus, arginine is used as an alternate energy source. It is suggested that an increasing concentration of ADP might be involved in the regulation of the arginine dihydrolase pathway (13).

Research done with *Pseudomonas* species also describes the effect of oxygenation on the arginine dihydrolase

pathway (31). It has been proposed that the transition from aerobiosis to anaerobiosis leads to a drastic change in the nucleotides triphosphate pools. Thus, ADP and cyclic AMP stimulate enzyme synthesis. The presence of arginine is also required for induction in some species.

The occurrence of catabolite repression of arginine deiminase activity with glucose as the primary substrate has been documented by several authors (31,46). Catabolite repression is a reduction in the rate of synthesis of certain enzymes, particularly degrative enzymes, in the presence of glucose. Another regulatory mechanism in which glucose exerts control on enzyme activity rather than on formation is referred to as catabolite inhibition (36). In most cases evaluated, arginine deiminase activity is subject to the control by elevated glucose concentrations.

Arginine metabolism in species of *Streptococcus* has been investigated in both batch and continuous fermentations. The arginine dihydrolase pathway of *S. faecalis* was determined to be both induced by arginine and repressed by glucose, fumarate and oxygen (41). These researchers also specify the drop in ATP and increase in cyclic AMP concentrations as a regulatory factor.

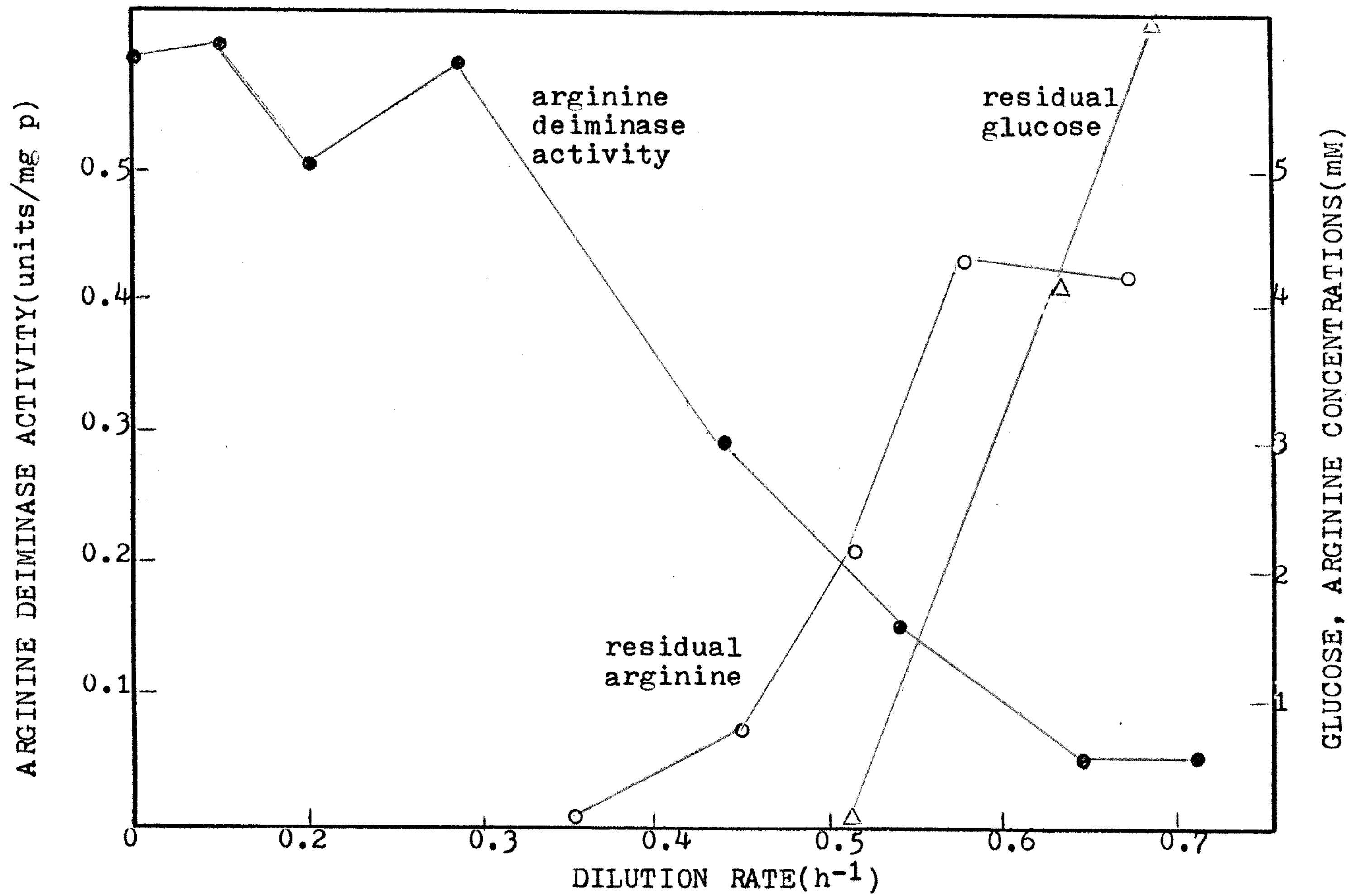
In a chemostat culture of *S. faecalis*, arginine utilization was observed only in the presence of small amounts of glucose (34). Under glucose-limiting conditions, the

biomass output was directly dependent on the arginine concentration.

Crow and Thomas (9) verified these findings with an evaluation of the characteristics of S. lactis growth in a chemostat. Their results are illustrated in FIGURE 9. Glucose was in excess at high dilution rates, with a corresponding low level of arginine deiminase activity. At low dilution rates, where glucose was limiting, arginine deiminase activity increased ten-fold. The characteristics of this system suggest that arginine is metabolized sequentially to glucose. Batch data reports concurrent metabolism in a galactose-arginine system.

FIGURE 9

ARGININE METABOLISM BY S. LACTIS IN A CHEMOSTAT



IV. DESCRIPTION OF THE FERMENTATION APPARATUS

A. Batch Fermenter

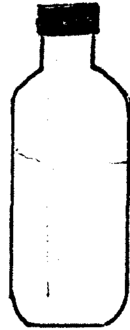
A fermenter is a reactor which provides the conditions suitable for growth of a microorganism. In its simplest form, a batch fermenter consists of a flask or container (the culture vessel) filled to a specified volume with growth medium (the culture volume) and maintained at a specified temperature. The culture volume and temperature should remain constant throughout the fermentation. Three types of batch fermenters were required during this experimental procedure. (FIGURE 10)

Batch Fermenter Type 1 is a 150 ml borosilicate glass culture bottle with a polyethylene screw cap. The culture volume is 100 ml. This type of fermenter was used in the preparation of the stock culture of bacteria. It was selected for its low surface-to-volume ratio, which is preferable for the growth of anaerobic organisms.

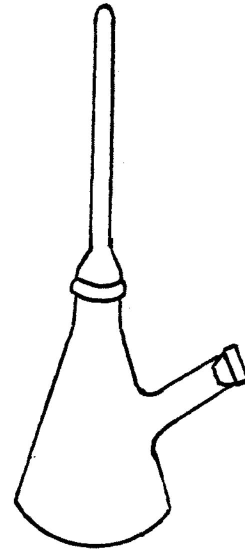
Batch Fermenter Type 2 is a 250 ml Erlenmeyer flask with a nephelo tube attachment and a sidearm. The nephelo tube is attached to the flask by a ground joint and the sidearm is sealed with a 1/2 in. red

FIGURE 10

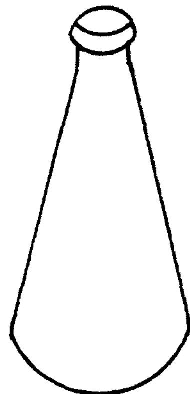
EXPERIMENTAL BATCH FERMENTERS



TYPE 1



TYPE 2



TYPE 3

rubber stopper. This type of fermenter was used in the growth analysis of the experimental microorganism. The nephelo tube attachment allows direct measurement of the optical density without sampling the culture volume.

Batch Fermenter Type 3 is a 500 ml narrow-mouthed Erlenmeyer flask (Pyrex brand) with a culture volume of 250 ml. The flask is sealed with a sponge rubber stopper and covered with aluminum foil. This type of fermenter was used when a larger culture volume was required, as in the determination of biomass concentration as a function of the optical density of the culture.

A constant temperature is maintained for all three types of fermenters by placing the culture vessels into a Precision Scientific cabinet-type incubator.

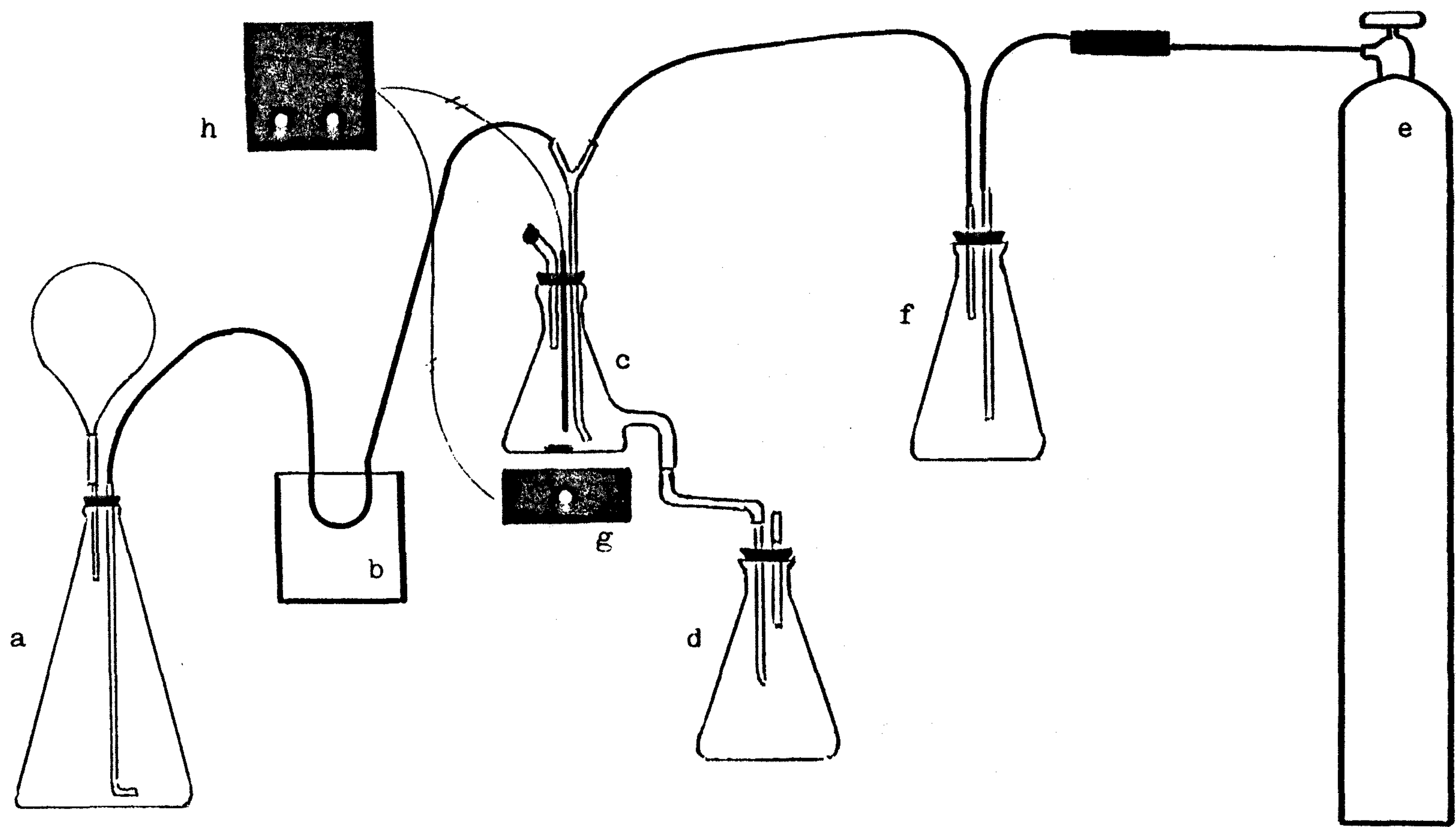
B. Continuous Culture Fermenters

A continuous culture fermenter, or chemostat, is designed by modifying the culture vessel to permit the addition of nutrients without changing the culture volume. Two chemostat set-ups were used during this experimental procedure.

Chemostat Type 1 is illustrated in FIGURE 11. The medium reservoir (a) is a six liter Erlenmeyer flask

FIGURE 11

CHEMOSTAT TYPE 1



with a two-hole rubber stopper (size 10). A 1/4 in. diameter glass pasteur pipet filled with cotton is inserted in one of the holes to filter air entering the flask. If assembled while the growth medium is still hot, a 10 in. diameter balloon filled with nitrogen gas is attached to the end of the filter to help create an oxygen-free atmosphere within the medium reservoir.

The nutrient feed is supplied through a 1/4 in. diameter glass tube extending into the medium reservoir through the second hole in the stopper. Pharmacia Peristaltic Pump Model P-1 (b) is used to transport the feed through latex or silicone rubber tubing to the culture vessel (c).

The culture vessel is a 500 ml Erlenmeyer flask with an inverted L-shaped sidearm extending from the bottom of the flask at approximately the 200 ml level. There is a magnetic stirring bar within the culture vessel. A 1/2 in. diameter glass tube, a 1/4 in. diameter glass tube and the temperature probe pass through a three-hole rubber stopper (size 10) which seals the culture vessel. The larger diameter glass tube is used as an inoculation port and is sealed with a 1/2 in. rubber stopper. The smaller glass tube is

the nutrient feed line.

The sidearm of the culture vessel is connected to a 1/4 in. diameter glass tube with latex tubing. This glass tube enters a one liter Erlenmeyer flask through a two-hole rubber stopper (size 7). This flask is the effluent reservoir (d). A filter consisting of a pasteur pipet filled with cotton is inserted in the second hole.

Before entering the culture vessel, the nutrient feed line is joined with a nitrogen gas feed line using a Y-shaped glass tube connector. The gas feed originates at a cylinder of nitrogen (e), passing through a filter (3/4 in. diameter, 6 in. length) and a saturation flask (f) before joining with the nutrient feed. The saturation flask is a one liter wide-mouthed Erlenmeyer flask filled with approximately 500 ml of distilled water and sealed with a two-hole rubber stopper (size 11). A 1/2 in. diameter glass tube passing through the rubber stopper and extending into the distilled water provides entry of the nitrogen gas into the flask. The 1/4 in. diameter glass tube passing through the other hole allows saturated gas to continue to the culture vessel.

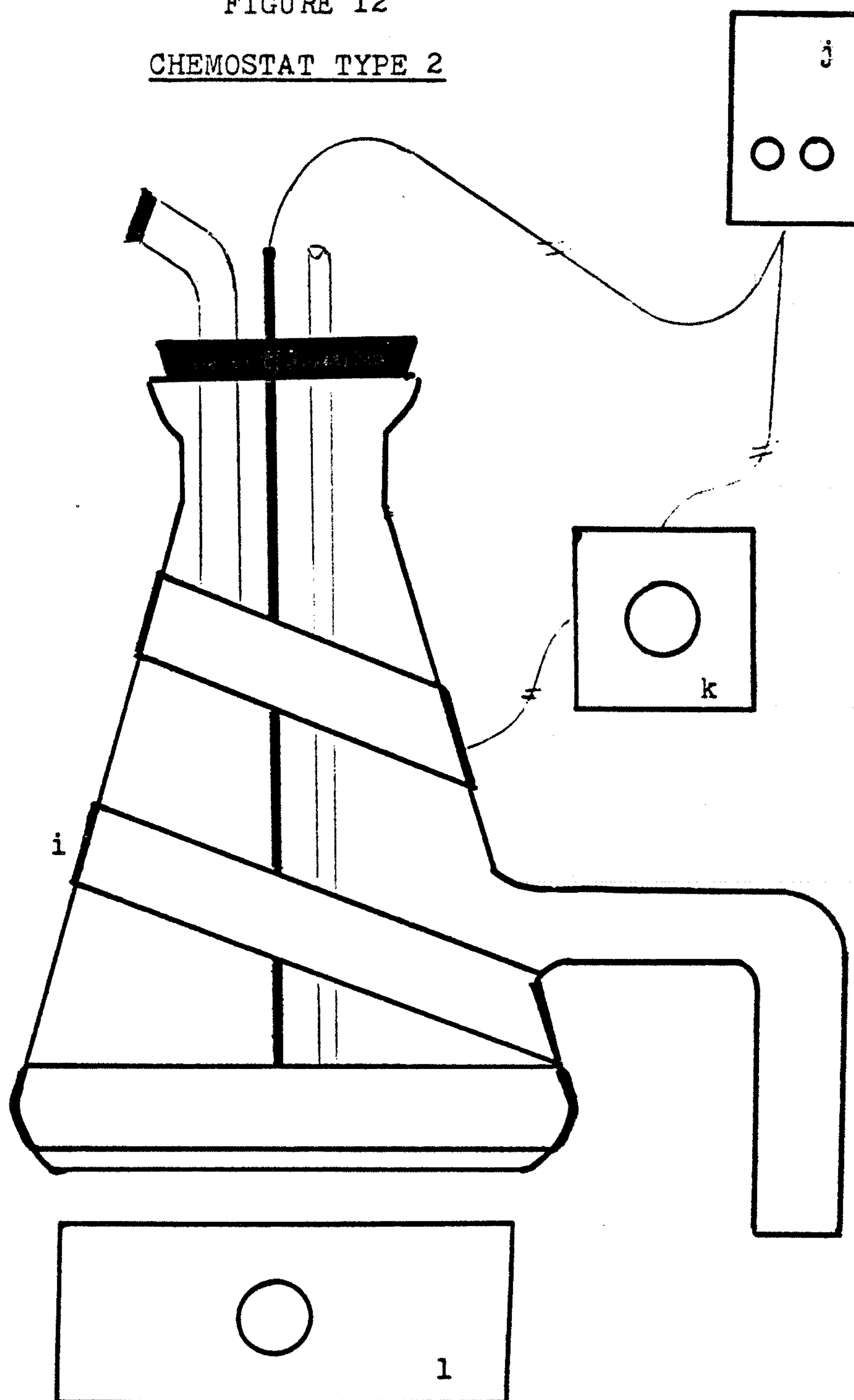
The heat source is a Corning Model PC-351 Hotplate/

Stirrer (g) which is connected to the Cole Parmer Versa-Therm Model 2158 Temperature Controller (h). The magnetic stirrer provides mixing of the culture when the heat source is activated.

Chemostat Type 2 is identical to Chemostat Type 1 with the exception of the metering pump and the heating and stirring mechanism. Pharmacia Peristaltic Pump Model P-3 is used to transport the nutrient feed to the culture vessel. Mixing of the culture is supplied continuously by a Fisher Model 120 MR Magnetic Stirrer (l). The heat source is a 1/2 in. x 2 ft. Thermolyne Briskheat Electric Heating Tape (i) wrapped around the culture vessel. The temperature is regulated by a YSI Thermistemp Model 63 RC Temperature Controller (j) through an Electrothermal Power Regulator Model MC 228 (k) (see FIGURE 12).

FIGURE 12

CHEMOSTAT TYPE 2



V. EXPERIMENTAL PROCEDURE

A. Chemostat Assembly and Operation

The chemostat is assembled as illustrated in FIGURE 11 to verify that all equipment is operating properly. The rubber stopper in the culture vessel is removed and a thermometer is inserted. The temperature probe is removed from the stopper and reinserted into the vessel with the thermometer. The temperature controller is then calibrated against the thermometer for the exact temperature of operation by setting the coarse and fine adjustments. The normal operating temperature is 30° C. A reasonably close first approximation of the dial settings can be determined by referring to the temperature controller calibration plots given in APPENDIX E. The temperature probe is removed and inserted in a 100 ml graduated cylinder filled with a formaldehyde disinfecting reagent and covered with aluminum foil. The probe is chemically sterilized by soaking it for 24 hours in this reagent. The operating volume within the culture vessel is then measured and recorded.

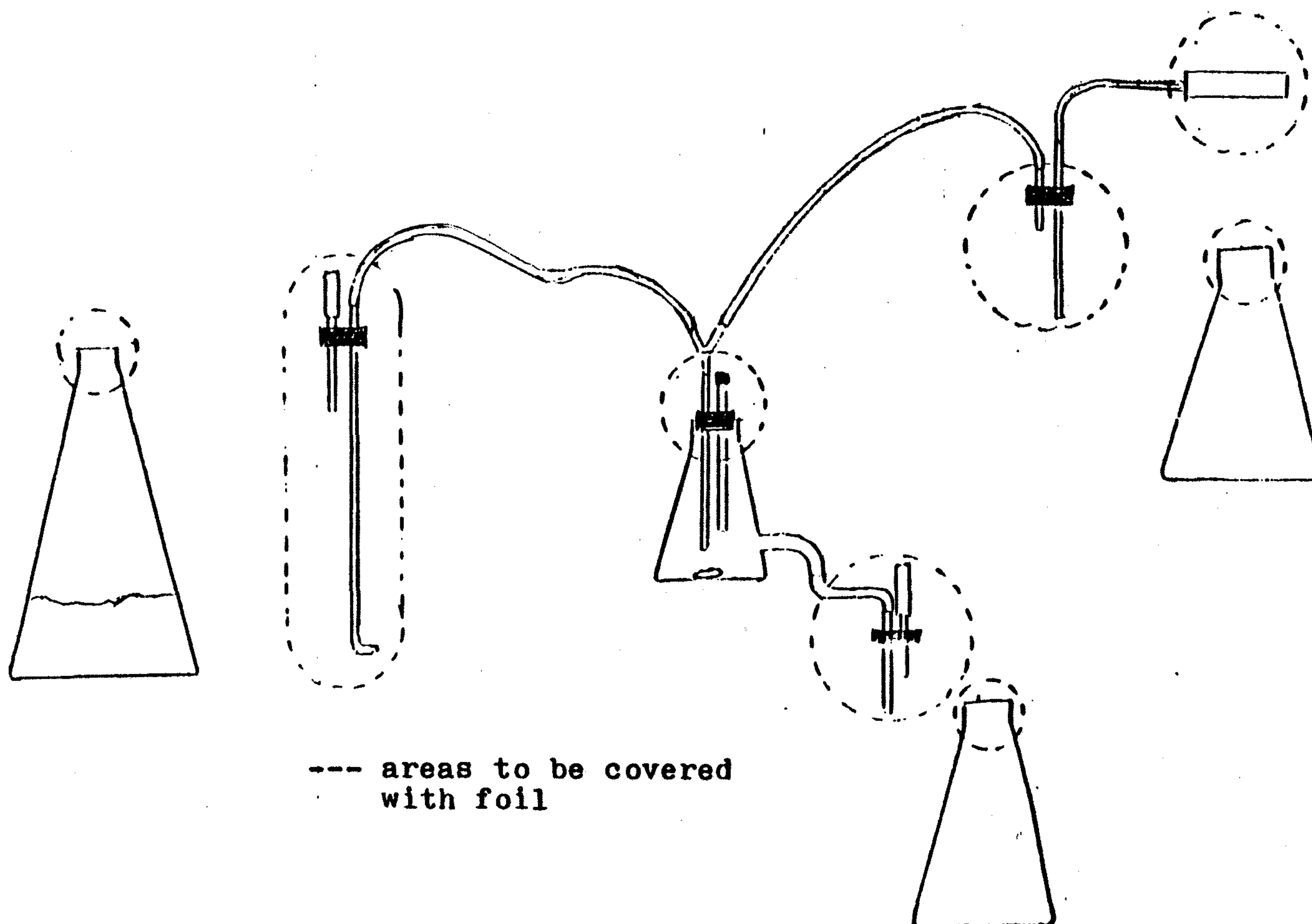
Before sterilization of the remainder of the apparatus, the tubing throughout the chemostat is cleaned

by filling the medium reservoir with a detergent solution and pumping approximately 100 ml of that solution through the apparatus. An equal amount of distilled water is then pumped through to rinse the detergent from the tubing. The apparatus is then disassembled. The medium reservoir, culture vessel, effluent reservoir and saturation flask can be cleaned separately with a detergent solution and rinsed with distilled water.

The parts of the chemostat requiring steam sterilization are illustrated in FIGURE 13. The medium reservoir is filled with one to four liters of growth medium, depending upon the desired period of operation. An estimated one liter is required for each run. The saturation flask is filled with approximately 500 ml of distilled water. The mouths of the flasks and any other openings which might expose the interior of the chemostat to contamination are carefully sealed with aluminum foil, as indicated in the illustration. Sterilization procedures are given in detail in APPENDIX B. In addition, approximately 150 ml of distilled water in a 250 ml Erlenmeyer flask, a 100 ml graduated cylinder and two latex gloves are appropriately sealed with foil and sterilized with the chemostat.

FIGURE 13

CHEMOSTAT PARTS REQUIRING STEAM STERILIZATION



The apparatus is assembled immediately after sterilization while liquids are still hot. Aluminum foil is removed and connections are made as quickly as possible. Approximately 100 ml of sterile distilled water is poured into the sterile graduated cylinder. The temperature probe is quickly removed from the disinfectant and dipped into the hot distilled water for rinsing. It is then quickly inserted through the stopper in the culture vessel. The possibility of contamination can be minimized by using the sterile gloves and flaming exposed areas while making connections.

Once assembled, the pump is turned on until the culture vessel is filled with growth medium (i.e., filled to the overflow tube). The pump is then switched off. After approximately 24 hours, the culture vessel and medium reservoir are examined for contamination. This would be indicated by turbidity in the liquid. If contamination is evident in the culture vessel only, the medium reservoir can be sealed with a sterile rubber stopper and retained for future use. The remainder of the apparatus must be cleaned and resterilized.

If no contamination is noted after 24 hours, the nitrogen gas is turned on and allowed to bubble through

the growth medium in the culture vessel. The pump is turned on to allow a 5 ml sample of pure medium to be taken. A 10 ml sterile nephelo culture tube is used and the sample is taken from the effluent portal. The pump is switched off and the chemostat is ready for inoculation. A time record is initiated at this point (see APPENDIX J).

A test tube containing the pure culture inoculum of *Lactobacillus brevis* is taken from refrigeration and the cells are mixed well using a vortex. Three milliliters of inoculum is drawn from the test tube using a sterile five milliliter syringe under a biohazard safety hood. Sterile latex gloves are used during this procedure. The area around the inoculation port of the culture vessel is flamed and the inoculum is injected quickly into the port. A purity analysis (as described in APPENDIX B) is done on the culture from which the inoculum was taken. The temperature controller is turned on and the cells in the culture vessel are allowed to incubate for approximately two days or until significantly turbid. Turbidity samples can be removed from the effluent portal at periodic intervals to establish when the culture has reached stationary phase.

When a mature culture is present, the pump is started and the time is recorded. The desired flow rate is predetermined, as described in APPENDIX D. The pumps were calibrated by measuring the volume collected in a specific amount of time for various tubing diameters. Pump calibration plots are given in APPENDIX E.

The volume in the culture vessel (approximately 150 ml) is allowed to turnover at least four times for each run. At the completion of a run, the pump is stopped and the cells in the culture vessel are allowed to incubate for approximately 24 hours. The chemostat is then ready to begin the next run.

B. Sample Collection

Chemostat samples are collected from the effluent portal of the culture vessel in a 250 ml Erlenmeyer flask. The sample flask is kept on ice continuously to inhibit further growth of the organism. Sampling times (start and finish) and the volume of the sample collected are recorded. The average sample volume should be approximately 120 ml, which can be divided into four parts: 100 ml for assay analysis, 5 ml for turbidity analysis, 5 ml for purity analysis and 10 ml for pH analysis.

Turbidity analysis involves measurement of the optical density at a wavelength of 550 nm in a Spectronic 20 against a pure medium blank. If it is necessary to dilute the sample to read the optical density (when the absorbance is greater than 0.5), the dilution ratio must be recorded. Pure, sterile medium is used as the diluent. A reference plot (FIGURE E-5) can be used to determine the biomass concentration of *L. brevis* from the optical density of each sample.

The pH meter is standardized at pH 7.0 before measurement of the pH of the sample. The pH and optical density measurements are performed at 22° C and are recorded on a Sample Data Sheet (see APPENDIX J) along with the fermentation time.

The purity of the culture is analyzed as outlined in APPENDIX B. Analysis of the assay sample for arginine deiminase enzyme activity is described in sections C, D and E of this experimental procedure.

The first series of samples collected from the chemostat are used to evaluate turbidity, pH and enzyme activity for various dilution rates. The selection of dilution rates to be examined is based upon the growth analysis described in APPENDIX D. Dilution rates

ranging from 0.02 to 0.12 are evaluated. During this part of the experimentation, sampling is done at a minimum sampling interval equivalent to the time required for one culture vessel volume turnover. Samples are taken for a total time interval equivalent to the time required for approximately six culture vessel volume turnovers. Plots are made of enzyme activity and biomass concentration as a function of fermentation time.

After selection of an operating dilution rate, cell growth and enzyme activity are evaluated against variations in nutrient concentrations. The first parameter evaluated is the limiting nutrient, dextrose. Flow rate analysis was done using a nutrient medium containing 1% dextrose. This series of experiments involve varying the dextrose concentration from 0.05 to 1.5%. Samples are collected after a time interval equivalent to at least the time required for four culture vessel volume turnovers. At this point the system has achieved steady state conditions.

Samples are taken for variation in arginine concentration in a similar manner. The arginine concentration is varied from 0 to 1.0% with the

dextrose concentration held constant at 0.5%. All nutrient concentrations have the units of milligrams per 100 ml volume. Plots are made of enzyme activity and biomass concentration as a function of dextrose and arginine concentrations.

Enzyme activity is also evaluated for batch-grown cells in exponential and stationary phases. The dextrose concentration is varied from 0.05 to 1.0% for arginine concentrations of either 0% or 0.5%. Where appropriate, enzyme activities of chemostat-grown cultures are compared with the batch results. In addition, the enzyme activity of several samples, both batch and continuous, is determined at a temperature of 37° C as well as at the normal operating temperature (30° C). The original samples are well mixed and divided for this purpose.

C. Preparation of the Enzyme Suspension

Approximately 25 ml of the 100 ml assay sample is poured into a 50 ml capacity round-bottom polyethylene centrifuge tube. To expedite the procedure, several tubes can be used and the cells combined at a later time. The tubes are arranged in the Sorvall Refrigerated

Centrifuge Model RC 2-B such that the weight is evenly distributed. The sample is centrifuged at a speed of 10,000 RPM for ten minutes. A pellet of cells will adhere to the bottom of the tube.

The liquid above the pellet (supernatant) is poured off and any original sample remaining is added to the tube. The pellet is dispersed (the cells are resuspended in solution) using a vortex, and the centrifugation process is repeated. The supernatant is again poured off. The cells are washed by adding several milliliters of 0.1 M sodium phosphate buffer (given in APPENDIX A) and then pouring it off. If the sample was distributed in several tubes, it is convenient to combine the cells at this point in the procedure. Several milliliters of the buffer are added to each tube. The cumulative volume should be about 10 ml. The cells are then resuspended and combined in one tube, being certain to collect all the cells. Rinse the empty tubes with additional buffer solution to be certain all of the cells are retrieved.

The suspension is centrifuged again at 10,000 RPM for ten minutes, then the washing process is repeated.

The final pellet of cells should be kept on ice until used in the assay. The pellet can be frozen for up to four weeks (39) if not being used immediately.

In preparation for the assay, the pellet of cells is thawed and resuspended in exactly 10 ml of 0.1 M sodium phosphate buffer solution. The cells are then disrupted by high frequency vibration using an Ultrasonics Sonifier Model W-185. During the sonication process, the polyethylene centrifuge tube containing the cell suspension is kept on ice continuously. A polyethylene or other non-glass beaker must be used to hold the ice, since glass will be shattered by the sonifier.

The probe of the sonifier is lowered into the tube. The probe should not be in contact with any surface. Earphones are used by the operator for protection. The intensity is set at the highest power and the cells are disrupted for thirty seconds. The unit is switched off and a thirty second cooling interval is allowed. The ice around the tube will melt, therefore the water must be poured off and new ice added periodically.

Sonication and cooling is repeated for a total of eight paired intervals. A series of experiments

involving different intervals of sonication were performed to determine the sonication pattern required for this experimental procedure. These experiments are described in detail in APPENDIX F.

The sonicated cells are centrifuged in the refrigerated unit at a speed of 14,500 RPM for 20 minutes. The supernatant is retrieved for assay use and maintained on ice until that time.

D. Assay for Citrulline Production

A colorimetric assay for the determination of citrulline in the reaction mixture of L-arginine and the enzyme extract is used to measure arginine deiminase activity. The procedure is based on the method of Archibald (3). Archibald's exact method results in a peach-colored product of the reaction between citrulline and diacetylmonoxime in acid solution. A more intense red color can be obtained with the addition of phenylhydrazine hydrochloride to the reaction mixture (11). The final assay method used in this experimental procedure is described below. Compositions of assay reagents are given in APPENDIX A.

Six milliliters of the enzyme suspension is put

in a separate tube and brought to a temperature of 30° C in a heated water bath. A 2 ml amount is put in a nephelo culture tube and kept on ice for use in determining the protein content of the sample. Two milliliters of 24 mM L-arginine in buffer solution is also heated to 30° C in the same water bath.

A series of approximately 20 sample tubes is prepared and labeled for the timed sample each will represent. The suggested time schedule for sampling is given in APPENDIX J. One milliliter of acid reagent is added to each tube to stop the reaction when the timed sample is added to the tube. One tube is labeled as a blank.

Two milliliters of the L-arginine solution is added to the 6 ml enzyme suspension and mixed well. This is the reaction mixture. Timed samples are collected from the reaction mixture using a 0.1 ml micro-pipet. Each sample is immediately injected into the corresponding sample tube. Time is equal to zero at the instant the supernatant is mixed with the L-arginine solution. Samples are taken for three hours. Plain buffer solution is added to the test tube labeled as a blank in place of a timed sample.

After the samples have been taken, 0.15 ml phenylhydrazine hydrochloride, 1.6 ml distilled water and 0.15 ml diacetylmonoxime are added to each tube, respectively. The final volume in each test tube should be 3 ml. The tubes are then mixed well using a vortex. A marble or loose-fitting cap is used to cover each tube, allowing air to escape from the tube when heated. The tubes are then placed in a covered boiling water bath for ten minutes.

The sample tubes must be protected from light from the point at which the diacetylmonoxime is added. The samples are allowed to cool overnight after removal from the boiling water bath. After the cooling period, each sample tube is vortexed and the optical density is read against the assay blank. The Gilford Stasar III Spectrophotometer is used at a wavelength of 495 nm. If it is necessary to read the optical densities sooner, the cooling interval can be shortened by placing the samples in a cold water bath for at least one hour. These samples are allowed to reach room temperature before analysis. Proper precaution must be taken to protect the samples from light until the point at which the optical density is read.

E. Protein Content Determination

The Biuret Method (16) is used during this experimental procedure for the determination of the protein content within the enzyme preparation. The sensitivity of this method is 0.1 to 10 mg protein. The determination is based on a colorimetric reaction which occurs between the peptide bonds of the enzyme-protein and copper salts in alkaline solution. The resulting reaction complex is purple.

In this method, two milliliters of 6% sodium hydroxide solution is added to two milliliters of the enzyme suspension. Biuret reagent is added using a 0.2 ml micro-pipet and the complete mixture is vortexed. A standard tube is also prepared by adding 2 ml of 6% sodium hydroxide and 0.2 ml of Biuret reagent to 2 ml of 0.1 M sodium phosphate buffer and mixing well. Reagent compositions are given in APPENDIX A.

After thirty minutes at room temperature, the optical densities are read using a Bausch and Lomb Spectronic 20 at a wavelength of 540 nm against a distilled water blank.

VI. DISCUSSION OF RESULTS

A. General Considerations

A total of thirty chemostat runs were evaluated for biomass concentration of *L. brevis* and L-arginine deiminase activity. Runs 21, 22 and 26 through 30 were performed at an average dilution rate of 0.060 h^{-1} with variations in dextrose (0.5 to 1.5%, w/v) and/or arginine (0.25 to 1.0%, w/v) substrate concentrations. The remaining runs were operated at various dilution rates ranging from 0.022 to 0.147 h^{-1} for a substrate feed consisting of 1.0% dextrose and 0.5% L-arginine hydrochloride.

Seven batch-grown samples were also evaluated for enzyme activity at various substrate concentrations. In addition, the effect of temperature on arginine deiminase activity was considered for four samples (30-C, 30-D, 30-E and batch sample B-2).

The selection of the range of operating dilution rates was based on the results of the batch growth analysis performed on *L. brevis* and described in APPENDIX D. The specific growth rate determined by early experimentation had an approximate value of 0.050 h^{-1}. This value was based on growth curves generated with optical density

data measured against a distilled water blank. This was later determined to be an inaccurate method of measurement. Subsequent growth curves revealed a specific growth rate of approximately 0.10 h^{-1} for this organism.

Since the dilution rate is specified by the specific growth rate, earlier experimentation was performed at very low dilution rates. A switch was then made to a higher range based on new data available. Finally, intermediate dilution rates were evaluated to complete this segment of experimentation.

The chemostat system was assumed to have reached steady state conditions after four culture vessel turnovers (26). This is equivalent to 182 h for the lowest and 27 h for the highest dilution rate investigated. Other researchers measured the time relative to six doubling times (9) and ten mean generation times (10). With respect to *L. brevis*, this is equivalent to 42 h and 69 h, respectively. All samples representing steady state conditions were at least consistent with the lesser of these two values.

At the onset of these experiments (Runs 1 to 5), samples were limited to evaluation of steady state conditions. It was found that more frequent sampling during the transient

start-up period was necessary to better characterize the system. The optical density of each sample was immediately recorded on a semi-log plot as a function of fermentation time. This enabled observation of the changes occurring within the chemostat as time progressed. An example of this plot for Run 19 is given in FIGURE 14.

A theoretical wash-out curve was generated from the dilution rate and is included for comparison in FIGURE 14. This line represents the rate at which nongrowing cells would be removed from the system. Theoretically, the specific growth rate can be estimated from the difference between the slopes of the experimental plot and the theoretical wash-out plot.

The optical density was later converted to biomass concentration using the appropriate reference plot in APPENDIX E (FIGURE E-5). The corresponding plot of biomass concentration as a function of fermentation time for Run 19 is illustrated in FIGURE 15. A similar plot for each run subsequent to Run 5 is included in APPENDIX H.

Operation of the chemostat system described in Section IV presented more problems than anticipated with respect to contamination. The modified catalase test described in APPENDIX B is very reliable if performed on

FIGURE 14

SEMI-LOG PLOT OF OPTICAL DENSITY AS A FUNCTION OF FERMENTATION TIME. Run 19.

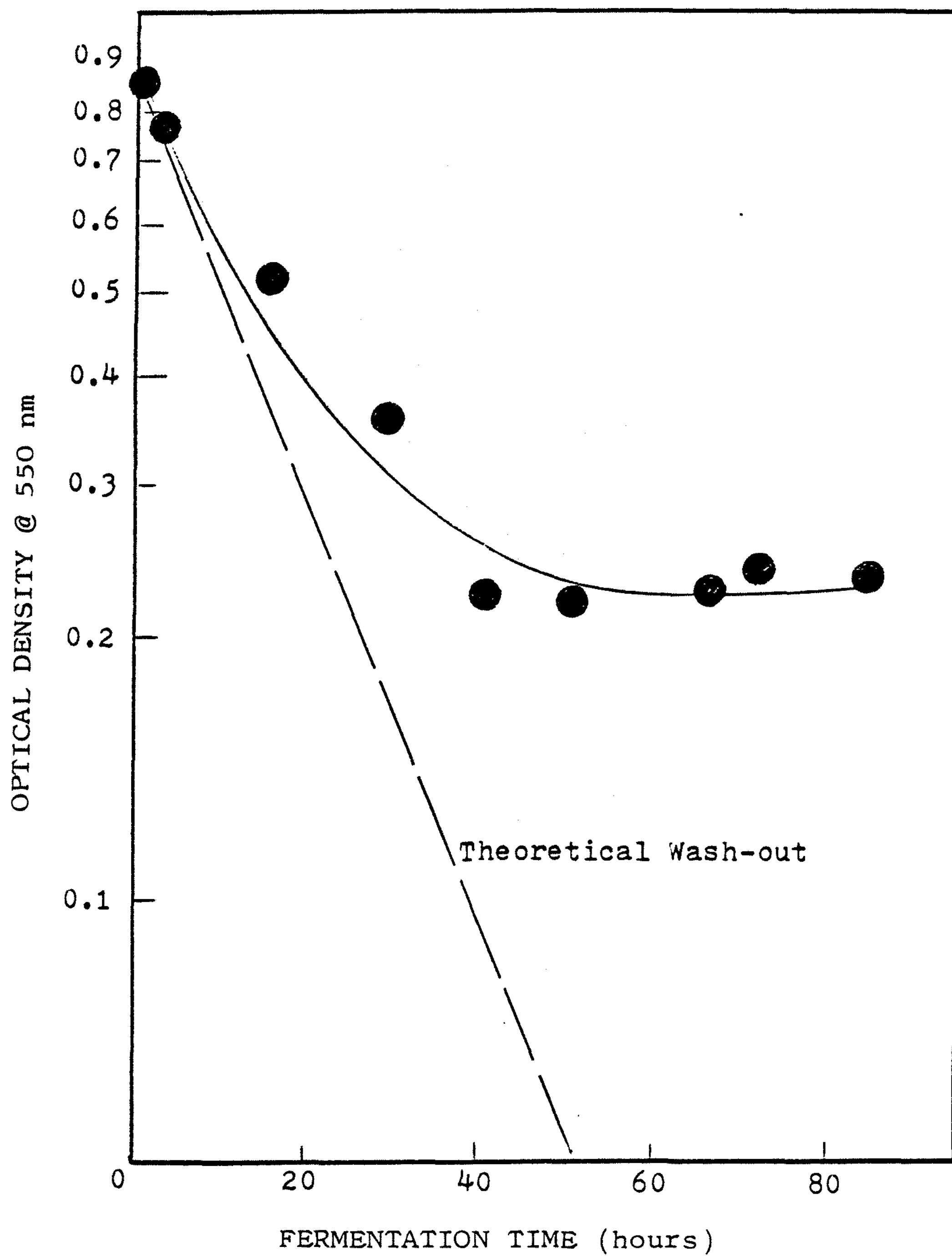
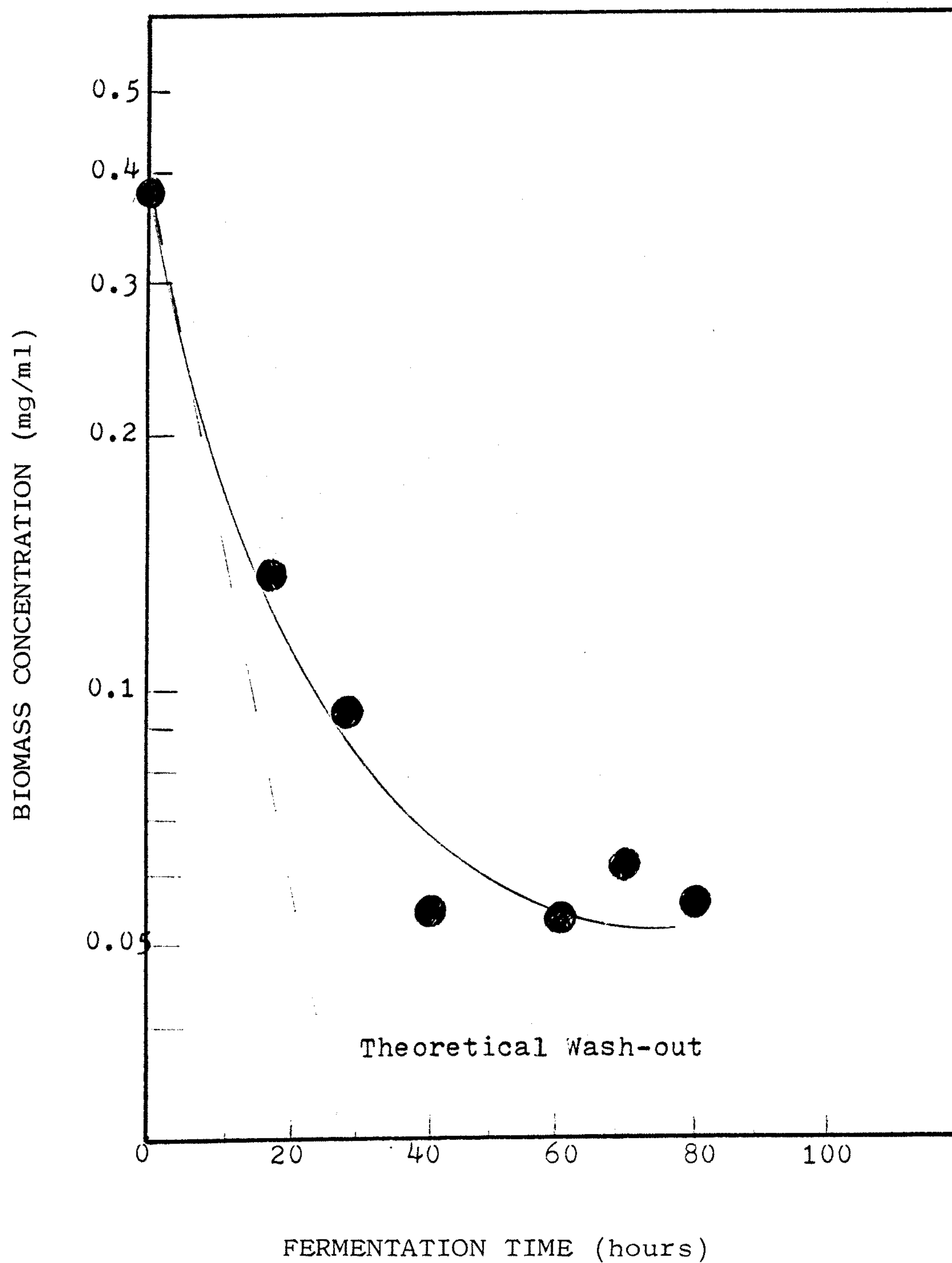


FIGURE 15

SEMI-LOG PLOT OF THE BIOMASS CONCENTRATION AS A FUNCTION OF FERMENTATION TIME. Run 19.



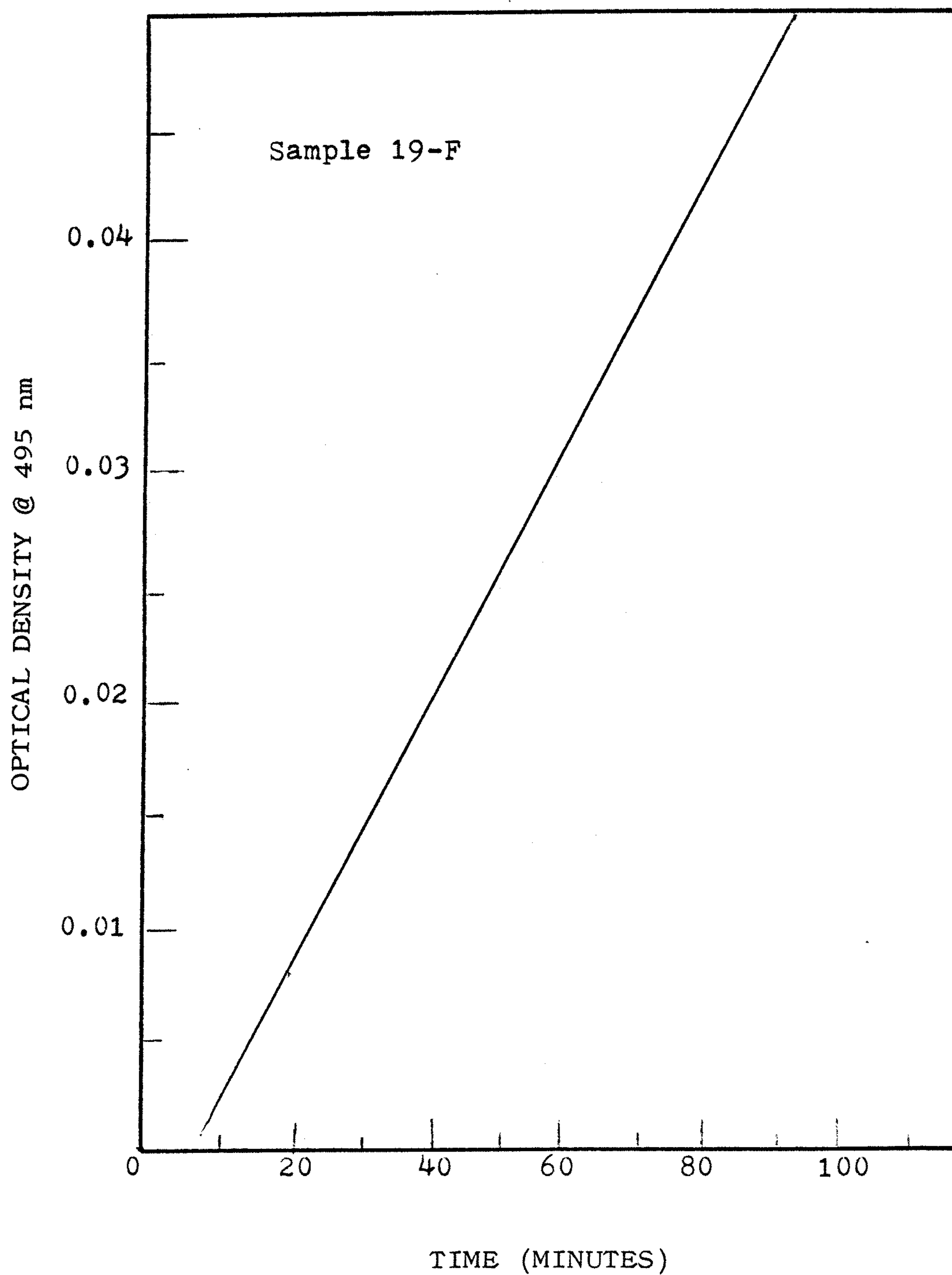
a concentrated cell sample. For approximately 20 runs, the test was performed with the culture diluted by the medium broth. Depending upon its concentration within the culture, the presence of a contaminant was sometimes not clearly evident. Fortunately, a frozen sample of cells from each run after Run 6 was retained and available for re-evaluation.

Analysis of the samples for enzyme activity was fairly straightforward once the exact procedure had been established. Some samples were lost by oversonicating the cells. It was then necessary to revise the sonication procedure as described in APPENDIX F. Modification of Archibald's original assay method (3) by the addition of phenylhydrazine hydrochloride (11) created a stable and reproducible color for spectrophotometric evaluation.

Before reading the optical densities, the samples were examined and any tubes containing excessive or insufficient liquid were not considered reliable. FIGURE 16 illustrates the plot of optical density as a function of time for the citrulline assay performed on Sample F of Run 19. A similar plot for each sample evaluated for enzyme activity is given in APPENDIX H. Linear regression analysis resulted in excellent correlation coefficients

FIGURE 16

ASSAY FOR CITRULLINE PRODUCTION:
OPTICAL DENSITY VERSUS REACTION TIME



for most samples. These values are summarized in TABLE H-1 of APPENDIX H.

By the final stages of this research it was concluded that assay samples should be collected for approximately three hours to obtain a reasonable estimate of enzyme activity. Unfortunately many samples were lost because timed samples from the assay reaction mixture were only collected for one hour. In general, linearity was observed for two hours or to an optical density of 0.100. This optical density corresponds to a citrulline concentration of less than 1 mM which would indicate that the arginine concentration remaining was at least 5 mM. This concentration is sufficient to maintain a linear rate of citrulline production (30), hence other factors must be influencing the system as time progresses.

All the experimental data has been tabulated and is located in APPENDIX G. In general, pH values did not vary considerably in the well-buffered medium. These values ranged from approximately 6.5 to 7.0. Except where pertinent to the discussion of results, this data is restricted to the appendix. Results of linear regression analysis and a summary of the parameters used for the determination of enzyme activity are given in APPENDIX H along with the graphical results.

B. Variations in Dilution Rate

Of the 23 runs performed at different dilution rates, Runs 7, 9, 14 and 18 were determined to be contaminated. The contamination of Runs 14 and 18 was detected immediately using the catalase test and microscopic identification. In addition, a pH value of 5.8 for both runs was significantly less than the average pH value of 6.7. Enzyme activity was not evaluated in either case.

Contamination in Runs 7 and 9 was not initially observed. Later re-evaluation of all stored samples indicated a substantial degree of contamination. Upon examination of the plots of biomass concentration versus fermentation time for these two runs (FIGURE H-1), it can be seen that the biomass was not at a steady state, but climbing. It might be suggested that contamination occurred at some point during chemostat operation, causing the inflection upward evident in both graphs. This can be compared to the circumstances of Run 14 which was apparently contaminated from the onset. In addition, enzyme activities determined for Runs 7 and 9 were much lower (0.006 and 0.005 units/mg p, respectively) than most other runs.

The remaining runs are tabulated according to their biomass response after four culture vessel turnovers. TABLE 3 includes those runs which exhibited complete wash-out of cells. In each case, the dilution rate was obviously greater than the specific growth rate of L. brevis. In general, arginine deiminase activity decreased as the cells were washed out.

Run 16, operating at a dilution rate of 0.100 h^{-1}, appeared to start leveling off but then the biomass concentration suddenly dropped to zero. If the specific growth rate of L. brevis is 0.10 h^{-1}, then this system was operating in a highly unstable region. If all conditions were ideal, the biomass might have leveled off to a steady state value in this case.

TABLE 4 summarizes the results from those runs exhibiting a low steady state biomass concentration. It appears as though this steady state plateau occurs at approximately o.o54 mg cells/ml, however Runs 12 and 17 level off at lower values. Run 12 is operating at a dilution rate greater than 0.10 h^{-1}. Since wash-out for a 0.10 h^{-1} dilution rate has been previously noted (Run 16), this run is operating in a region of instability.

TABLE 3

EFFECTS OF VARIATIONS IN CHEMOSTAT DILUTION RATES: COMPLETE WASH-OUT OF CELLS

RUN	DILUTION RATE, h^{-1}	SPECIFIC ACTIVITY[a] units/mg p
10	0.140	0.023
11	0.147	0.035
15	0.107	0.014
16	0.100	0.004

[a] last measurement before wash-out

TABLE 4

EFFECTS OF VARIATIONS IN CHEMOSTAT DILUTION RATES: LOW STEADY STATE BIOMASS CONCENTRATIONS

RUN	DILUTION RATE, h^{-1}	BIOMASS CONCENTRATION[a] mg cells/ml	SPECIFIC ACTIVITY[a] units/mg p
12	0.107	0.014	-
13	0.097	0.049	0.022
17	0.054	0.020	0.003
19	0.056	0.058	0.031
20	0.067	0.058	0.025
24	0.054	0.049	0.009

[a] measured for steady state conditions after four culture vessel turnovers

Some carmelization occurred during sterilization of the media for Runs 16, 17 and 18. Carmelization is caused by a breakdown of glucose to potentially toxic substances in the presence of salts. Run 18 was lost to contamination and Run 19 was apparently unaffected. It is a possibility that growth was inhibited by toxins in the carmelized medium, or that the available substrate concentration was reduced. However, this reasoning is not supported experimentally since Run 19 was unaffected.

Runs 13, 19 and 20 demonstrated reasonably close values for enzyme activity, averaging 0.026 units/mg p. Runs 17 and 24 exhibited lower values of 0.009 and 0.003 units/mg p, respectively. There is no evidence available to explain this discrepancy.

Run 13 represents an upper limit for operational dilution rates. Although stable conditions are present for this run, continuous operation at a dilution rate so close to the specific growth rate would eventually lead to fluctuations in steady state conditions.

TABLE 5 summarizes the results from those runs exhibiting a higher steady state biomass concentration. Runs 1, 2, 3, 8, 23 and 25 exhibited an average biomass concentration of 0.125 mg cells/ml. A corresponding

TABLE 5

EFFECTS OF VARIATIONS IN CHEMOSTAT DILUTION RATES: HIGH STEADY STATE BIOMASS CONCENTRATIONS

RUN	DILUTION RATE, h^{-1}	BIOMASS CONCENTRATION[a] mg cells/ml	SPECIFIC ACTIVITY[a] units/mg p
1	0.040	0.115	0.053
2	0.022	0.120	0.062
3	0.031	0.107	-
4	0.042	0.656	0.008
5	0.060	0.372	0.004
6	0.042	0.296	0.006
8	0.036	0.159	-
23	0.036	0.131	0.065
25	0.033	0.118	0.059

[a] measured for steady state conditions after four culture vessels turnovers

average value for arginine deiminase activity of 0,06 units/mg was obtained. Activities from Runs 3 and 8 were not determined due to oversonication and insufficient sampling time.

Runs 4 to 6 exhibited a higher average, steady state biomass concentration of 0.441 mg cells/ ml. In addition, the corresponding average for enzyme activity was comparatively low (0.006 units/mg p). Unfortunately, samples from these three runs were not available for re-evaluation. In consideration of the excessive biomass concentration and low enzyme activity, it is very likely that these runs were contaminated. There is, however, no legitimate support for disregarding this data.

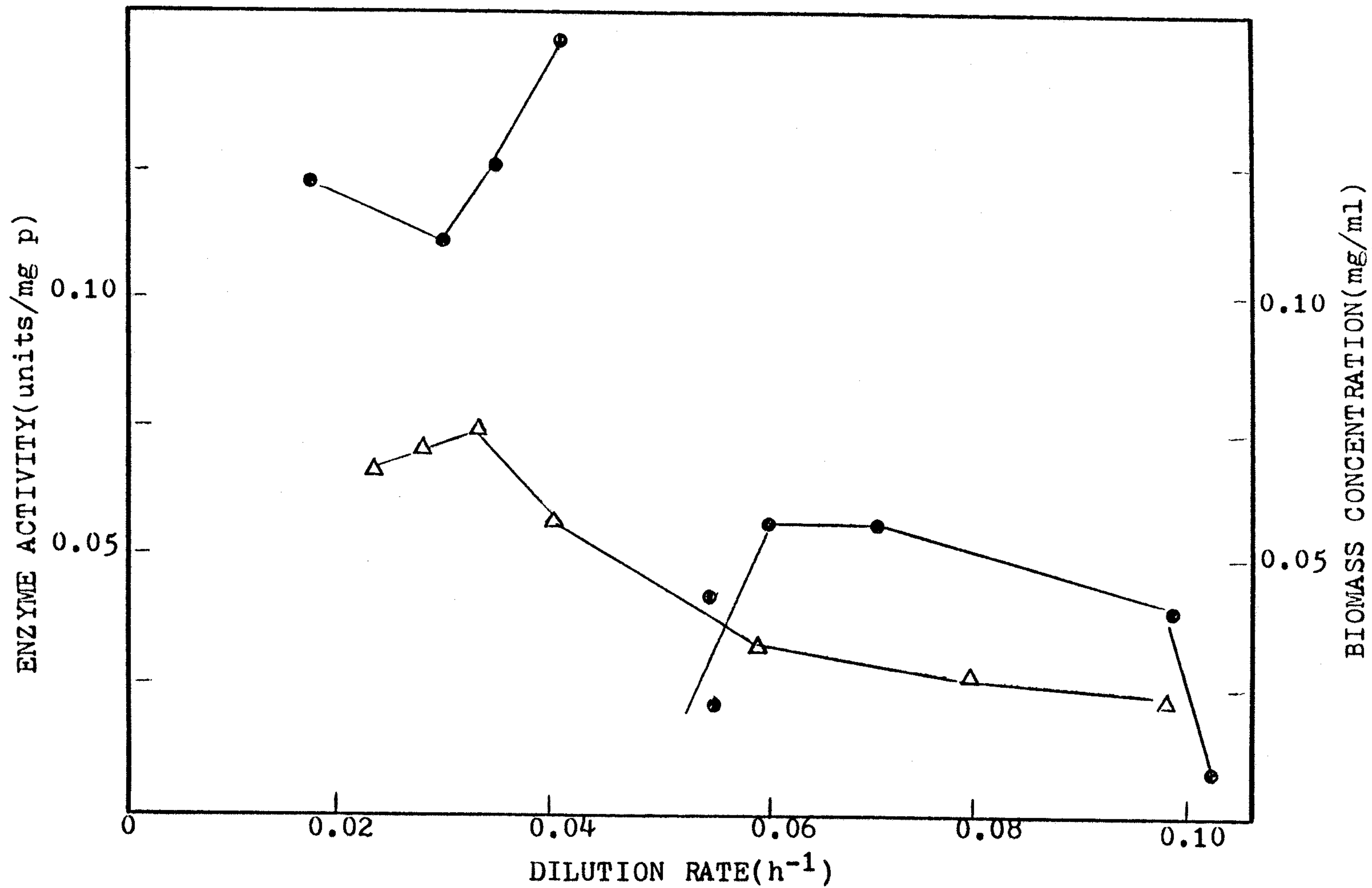
Arginine deiminase activity and _L. brevis_ biomass concentration for steady state conditions are illustrated in FIGURE 17. The biomass concentration and enzyme activity level off at a higher steady state plateau for low dilution rates. Where glucose concentrations are limiting, the arginine catabolic pathway is induced. At higher dilution rates, excesses glucose represses enzyme activity. This data is consistent with the research involving _S. lactis_ described in Section III-D. In addition, _Lactobacillus_ and _Streptococcus_ species are considered to be quite similar in their biochemical characteristics (2).

The exact regulatory mechanism cannot be determined from this experimental data. The prior work described in

FIGURE 17

STEADY STATE ENZYME ACTIVITY AND BIOMASS RESPONSE TO VARIATIONS IN CHEMOSTAT DILUTION RATES. Experimenta Results.

Δ arginine deiminase activity ● biomass concentration



Section III-D suggests the involvement of high concentrations of ADP present after complete utilization of a glucose substrate. Diauxic growth of L. brevis in a glucose-arginine batch culture was described in APPENDIX D. Multiple steady states are found to occur in systems exhibiting diauxic growth in a batch culture (37).

C. Variations in Substrate Concentration

Having established the fact that limiting the glucose concentration can induce arginine catabolism, the chemostat was operated at a constant dilution rate (0.06 h^{-1}) and the substrate concentrations were varied. Runs 19, 21 and 26 to 30 were operated with arginine concentrations ranging from 0.25 to 1% and dextrose concentrations ranging from 0.05 to 1.5%, w/v (see TABLE 6).

An increase in glucose concentration from 1.0 to 1.5% (Run 21) resulted in an increase in biomass concentration (0.071 mg/ml). As expected, there was no effect on the enzyme activity in the presence of an even higher concentration of glucose.

Operation at this dilution rate and a substrate composition of 1.0% glucose and 0.5% arginine resulted in a steady state biomass concentration of 0.058 mg/ml and an enzyme activity of 0.031 units/mg p (Run 19). The enzyme activity doubled for a decrease in glucose concentration

TABLE 6

EFFECTS OF VARIATIONS IN CHEMOSTAT SUBSTRATE CONCENTRATIONS

RUN	DILUTION RATE, h^{-1}	ARGININE CONCENTRATION g/100 ml	GLUCOSE CONCENTRATION g/100 ml	BIOMASS CONCENTRATION[a] mg cells/ml	SPECIFIC ACTIVITY[a] units/mg p
21	0.060	0.5	1.5	0.071	0.031
19	0.056	0.5	1.0	0.058	0.031
26	0.072	0.75	0.05	0.112	0.080
27	0.053	0.5	0.05	0.115	0.083
28	0.059	0.5	0.5	0.099	0.063
29	0.067	0.25	0.5	0.080	0.062
30	0.058	1.0	0.5	0.109	0.027

[a] measured for steady state conditions after four culture vessel turnovers

to 0.5%. Approximately the same activity value was obtained for an arginine concentration of 0.5% (Run 28) and 0.25% (Run 29). However, the biomass concentration decreased from 0.099 to 0.080 mg/ml with the decrease in utilizable substrate.

Increasing the arginine concentration to 1.0% resulted in a decrease in activity. The highest arginine deiminase activity and biomass concentrations were obtained with a glucose concentration of 0.05% (Runs 26 and 27). Increasing the concentration of arginine from 0.5% (Run 27) to 0.75% (Run 26) did not effect the steady state conditions. An average steady atate biomass concentration of 0.114 mg/ml and a specific activity of 0.08 units/mg p were the optimum values obtained for chemostat operation.

D. Comparison to Batch Results and Temperature Effects

TABLE 7 summarizes the specific activities of arginine deiminase in exponential and stationary phase batch cultures for different glucose concentrations. Batch Runs B-5 to B-7 contained no arginine added to the MIB broth. The activity is observed to increase slightly for the transition from exponential to stationary phase, but the activity for all three samples (averaging 0.016 units/mg) was low.

The highest activity obtained for either batch-grown or continuous cultures was 0.114 units/mg for Batch Run B-4. The glucose and arginine concentrations were 0.05% and 0.5%,

TABLE 7

ARGININE DEIMINASE ACTIVITY IN BATCH-GROWN CULTURES

RUN	GLUCOSE CONCENTRATION[a] g/100 ml	PHASE	SPECIFIC ACTIVITY units/mg p
B-1	0.5	stationary	0.092
B-2	0.5	stationary	0.047
B-3	0.05	exponential	0.018
B-4	0.05	stationary	0.114
B-5	1.0	exponential	0.013
B-6	1.0	exponential	0.017
B-7	1.0	stationary	0.018

[a] arginine concentration was 0.5% for B-1 to B-4 and 0% for B-5 to B-7

respectively. The culture was well into stationary phase. A much lower specific activity was obtained for an exponential culture with the same substrate composition (B-3). Stationary cultures of 0.5% arginine and 0.5% glucose had an average specific activity of 0.070 units/mg p.

The low activity for the exponential culture in Batch Run B-3 is consistent with the values obtained from the samples with no arginine concentration (B-5 to B-7). These results, in turn are relatively consistent with the results obtained from chemostat samples under conditions of excess glucose and high dilution rates.

The five-fold increase in activity observed for the transition from exponential to stationary cultures (B-2 and B-3) would indicate that arginine deiminase is induced by exhaustion of the glucose substrate in the presence of arginine.

Comparing the resulting activities for chemostat and batch samples with the same initial substrate concentrations, the batch results in stationary phase cultures are slightly higher (see TABLE 8). In addition, increasing the reaction temperature to 37°C resulted in a 2.5-fold increase in enzyme activity for three of the four samples tested (see TABLE 9).

The activity results obtained in this experiment are based on a crude protein extract and can therefore not be compared with those values listed in TABLE 2.

TABLE 8

A COMPARISON OF BATCH AND CONTINUOUS RESULTS

GLUCOSE CONCENTRATION[a] g/100 ml	BATCH SPECIFIC ACTIVITY[b] units/mg p	CONTINUOUS SPECIFIC ACTIVITY[c] units/mg p
0.05	0.114	0.083
0.5	0.070	0.063

[a] arginine concentration was o.5%

[b] stationary phase results

[c] steady state conditions

TABLE 9

THE EFFECT OF TEMPERATURE ON ARGININE DEIMINASE ACTIVITY

SAMPLE	SPECIFIC ACTIVITY @ 30°C, units/mg p	SPECIFIC ACTIVITY @ 37°C, units/mg p
30-C	0.026	0.040
30-D	0.016	0.036
30-E	0.027	0.070
B-2	0.047	0.117

VII. CONCLUSIONS

1. A chemostat culture of *L. brevis* grown on a nutrient feed of 1.0% glucose and 0.5% arginine exhibited multiple steady state conditions for biomass concentration and arginine deiminase activity. The steady state biomass concentration increased from 0.049 mg/ml at a high dilution rate (0.097 h^{-1}) to 0.131 mg/ml at a low dilution rate (0.036 h^{-1}). The arginine deiminase activity also increased with decreasing dilution rate, demonstrating a maximum of 0.065 units/mg p at a dilution rate of 0.036 h^{-1}.
2. Increased steady state values for enzyme activity can also be obtained at high dilution rates by decreasing the concentration of glucose in the substrate feed. The optimum activity for a substrate feed of 0.05% glucose and 0.5% arginine was 0.083 units/mg p as compared to 0.026 units/mg p for the same dilution rate (0.060 h^{-1}) and higher glucose concentration (1.0%).
3. Stationary phase batch cultures exhibited the highest activity (0.114 units/mg p) when compared to the chemostat results for the same conditions (0.083 units/mg p for a substrate feed of 0.05% glucose and 0.5% arginine).

VIII. RECOMMENDATIONS FOR FUTURE WORK

The following recommendations include suggestions for research related to this investigation of arginine catabolism by *L. brevis* in a chemostat. These suggestions are followed by proposed chemostat modifications to help eliminate operational difficulties.

1. Measurement of dextrose and arginine concentrations in the effluent stream would provide data to validate the experimental findings for this research project.
2. Measurement of biomass output and enzyme activity for variations in pH and temperature for a fixed dilution rate would merit investigation for several reasons. Optimum enzyme activity does not always coincide with optimum biomass output with respect to operating temperature or pH. In addition, pH has demonstrated a definite influence on various metabolic processes of lactic acid bacteria.
3. Before undertaking any continuous culture experimentation, it should be verified that sterility can be maintained within the chemostat. Several improvements to the equipment described in this manuscript are possible:

- Use of glass tubing, thereby minimizing the use of rubber tubing, will limit the need for replacement of worn rubber. Cracks within worn rubber are a potential source of contamination.
- Sterilization of the chemostat as a single unit would eliminate contamination during apparatus assembly.
- Use of millipore filters within the gas filter will minimize a surprisingly active source of contamination. An alternative or addition would be the use of presterilized compressed nitrogen.
- By increasing the culture vessel volume, the flow rate would necessarily be increased to maintain the same dilution rate. Samples could then be collected over a shorter period of time.

IX. NOMENCLATURE

A	-	enzyme activity or rate of citrulline production, units of activity (1 unit = 1 μmole citrulline produced per minute)
D	-	dilution rate, h^{-1}
F	-	volumetric flow rate, ml/h
f	-	fraction of protein sample in assay reaction mixture
K_s	-	saturation constant, μmoles/l
K_m	-	Michaelis constant, consistent units of concentration
OD_s	-	optical density of sample @ 540 nm
OD_x	-	optical density of sample @ 550 nm
OD_B	-	optical density of biuret standard @ 540 nm
P	-	protein concentration, mg dry weight / ml
p	-	protein mass, mg dry weight
S	-	substrate concentration, mg/ml or M
S_o	-	initial substrate concentration, consistent units with S
s	-	slope of plot of assay results, OD/min
s_A	-	slope of assay standardization plot, μmoles/OD
s_B	-	slope of biuret standardization plot, mg protein/OD
s_C	-	slope of biomass standardization plot, mg cells/ml/OD
S.A.	-	specific activity, units/mg protein
t	-	time or fermentation time, h
$\bar{t}$	-	mean residence time, h

t_d - culture doubling time, h

μ - specific growth rate, h^{-1}

μ_{max} - maximum specific growth rate, h^{-1}

V - culture vessel volume, ml

v - enzyme reaction rate, umoles/min

v_{max} - maximum enzyme reaction rate, μmoles/min

X - biomass concentration, mg cells/ml

X_o - initial biomass concentration, mg/ml

Y - yield constant

APPENDIX A

EQUIPMENT AND CHEMICAL LISTS, EQUIPMENT SPECIFICATIONS AND CHEMICAL REAGENT COMPOSITIONS

MAJOR EQUIPMENT LIST

TYPE	MODEL	MANUFACTURER
Cell Disrupter	Sonifier-W185	Heat Systems-Ultrasonics, Inc. Plainview, N.Y.
Hotplate/Stirrer	PC-351	Corning Glass Works
Flexible Electric Heating Tape	BriskHeat	Thermolyne Corporation (Sybron Corporation, Iowa)
Electrothermal Power Regulator	MC-228	Electrothermal Engineering Limited, England
Magnetic Stirrer	14-511-1V2	Fisher Scientific Co. Springfield, N.J.
Peristaltic Pump	P-1	Pharmacia Fine Chemicals
Peristaltic Pump	P-3	Pharmacia Fine Chemicals
Nitrogen Regulator Valve	8-580	Matheson Corporation East Rutherford, N.J.
Temperature Bath	F-4391	Haake Instruments, Inc. Saddle Brook, N.J.

MAJOR EQUIPMENT LIST (cont'd)

TYPE	MODEL	MANUFACTURER
Automatic Refrigerated Centrifuge	Sorvall Superspeed RC2-B	Sorvall Instruments Wilmington, De.
Spectrophotometer	Stasar III 1367X5B	Gilford Instrument Laboratories, Inc. Oberlin, Ohio
Biohazard Safety Hood	740	Contamination Control Co. Lansdale, Penn.
Temperature Controller	Thermistemp 63-RC	Yellow Springs Instrument Co. Yellow Springs, Ohio
Temperature Controller	Versatherm 2158	Cole Parmer Instrument Co. Chicago, Ill.
Incubator	Catalog #31485	Precision Scientific Co.
Autoclave	Eagle Series Vacamatic 2023	Amsco Co.
Autoclave	UE-650	Barnstead Co. div. of Sybon Corp., Boston, Mass.
Mixer	Vortex Genie	Scientific Industries, Inc.

MAJOR EQUIPMENT LIST (cont'd)

TYPE	MODEL	MANUFACTURER
Mixer	Maxi-Mix, M-16715	Thermolyne Corporation
Peristaltic Pump	Variable speed, multichannel, cassette pump	Manostat Corporation
Spectrophotometer	Spectronic 20	Bausch & Lomb
pH Meter	Accumet	Fisher Scientific Co.
Temperature probe	K-2174-10	Yellow Springs Instrument Co. Yellow Springs, Ohio

MISCELLANEOUS EQUIPMENT

TYPE	DESCRIPTION
rubber tubing	silicone and amber latex, bore range - 1/8 to 1/4 in.
plastic tubing	Tygon, bore range - 1/32 to 1/4 in.
glass tubing	borosilicate, O.D. range - 3 to 6 mm.
tubing clamps	stainless steel, for use with above tubing
tri-grip clamp	vinyl dipped jaws to grasp wide-mouth 500 ml flask, smaller size for tubing suspension
support stand	rectangular, steel, rod size - 1/2 x 24 in.
Erlenmeyer flasks	1 liter (2), 6 liter, 4 liter, standard; 500 ml wide-mouth with outlet near base; 250 ml wide-mouth; 250 ml, standard with nephelo tube attachment and sidearm
rubber stoppers	sizes - 6 (2), 7, and 10 (2), black
rubber stopper	vaccine type, red
centrifuge tubes	round-bottom, polypropylene, 50 ml capacity
culture tubes	screw-cap, borosilicate glass, approx. 25 ml capacity
culture tubes	disposable, 10 ml capacity nephelo

MISCELLANEOUS EQUIPMENT (cont'd)

TYPE	DESCRIPTION
culture dishes	disposable, sterile petri
graduated cylinders	50 ml and 100 ml sizes with unit graduations
pipetters	manually operated, plunge type; 1 ml, 0.2 ml sizes
pipet tips	standard, plastic; 250 µl, 1000 µl sizes
pipets	small, disposable pasteur
aluminum foil	heavy duty

LIST OF CHEMICALS

CHEMICAL	MANUFACTURER
diacetyl monoxime (2,3-butanedione-monoxime)	Sigma Chemical Co. St. Louis, Mo.
sulfuric acid (conc.)	Fisher Scientific Co. Springfield, N.J.
phosphoric acid, 85%	Fisher Scientific Co.
phenylhydrazine hydro-chloride, crystalline	Matheson Co., Inc. East Rutherford, N.J.
sodium hydroxide	Fisher Scientific Co.
sodium phosphate, monobasic	Fisher Scientific Co.
sodium phosphate, dibasic	Fisher Scientific Co.
L-citrulline	Sigma Chemical Co.
L-arginine hydrochloride	Sigma Chemical Co. Fisher Scientific Co.
lysozyme	Sigma Chemical Co.
formaldehyde germicide	Becton Dickenson & Co. Rutherford, N.J.
Micro-inoculum Broth	Difco, Inc. Detroit, MI.
Bacto-Tryptone	Difco, Inc.
Nutrient Agar	Difco, Inc.
Bacto-Agar	Difco, Inc.

LIST OF CHEMICALS (cont'd)

CHEMICAL	MANUFACTURER
Tween 80 (polyoxyethylene sorbitan monooleate)	Sigma Chemical Co.
cupric sulfate	Fisher Scientific Co.
Yeast Extract	Difco, Inc.
potassium phosphate, monobasic	Fisher Scientific Co.
sodium tartrate, crystalline	JT Baker Chemical Co. Phillipsburg, N.J.
sodium carbonate, crystalline	Fisher Scientific Co.
hydrogen peroxide, 3%	A & P, Inc. Montvale, N.J.
Phenol Reagent Solution, 2 N (Folin-Ciocalteau)	Fisher Scientific Co.
Bacto-Dextrose	Difco, Inc.
nitrogen gas, prepurified	Matheson Co., Inc.
sodium citrate	Fisher Scientific Co.
copper sulfate pentahydrate	Fisher Scientific Co.

FERMENTATION EQUIPMENT SPECIFICATIONS

Yellow Springs International
Thermistemp Model 63RC Temperature Controller

Temperature Control Range: -100° to +500° F; 0.1° F sensitivity

Settability and Accuracy: Dependent on the accuracy of the reference standard used to set the controls; the control point can be reset within ±0.1° F

Control Point Stability: Better than ±0.07° F with line voltage varying ±20 VAC

Operating Temperature: Ambient temperature can range from 0° to 120° F

Relay: Single pole, double throw plug-in relay with contacts rated at 10 amps at 115 VAC and 5 amps at 230 VAC, noninductive load; the relay is not part of a power source

Power Requirements: 115 VAC, 50/60 Hz, 5 watts operation

Size: 9" x 4 1/2" x 2 3/4", 5 lbs

Cole Parmer
Versa-Therm Model 2158 Temperature Controller

Temperature Control Range: -100° to +500° F; less than 0.05° F sensitivity

Settability: Better than ± 0.05° F using appropriate probe

Control Point Stability: Less than 0.05° F change in set temperature per degree ambient temperature change; less than 0.1° F change in set temperature thru the voltage input range of 105 to 125 VAC

Relay: DPDT relay with silver cadmium contacts UL rated at 10 amps at 125 VAC, non-inductive load

Size: 7" x 7" x 2 1/2"

EQUIPMENT SPECIFICATIONS (cont'd)

Pharmacia
Peristaltic Pump Model P-1

Flow Rate Range: 0.6-500 ml/h depending on tubing diameter and range selector (x1 or x10)

Standard Tubing: i.d. 1.0 mm, 2.1 mm and 3.1 mm silicone rubber

Voltage: 100 or 120 V $\pm$ 10%; 220 or 240 V $\pm$ 10%

Frequency: 50-60 Hz

Maximum Power: 20 W

Temperature Range: 0-40° C

Size: 145 x 112 x 115 mm, 1.7 kg

Pharmacia
Peristaltic Pump Model P-3

Flow Rate Range: 6-500 ml/h depending on tubing diameter

Standard Tubing: i.d. 1.0 mm, 2.1 mm and 3.1 mm silicone rubber

Voltage: 115 VAC

Frequency: 50-60 Hz

Maximum Current: 120 mA

Size: 290 x 112 x 115 mm, 2.7 kg

EQUIPMENT SPECIFICATIONS (cont'd)

Thermolyne
Briskheat Electric Heating Tape

Voltage: 120 VAC

Maximum Power: 104 W

Maximum Temperature: 482° C

Size: 1/2" x 2'

Corning Model PC-351 Hotplate/Stirrer

Maximum Temperature: 510° C

Heating Surface: 35 sq. in.

Stirring Speed Range: 250-1000 RPM

Voltage: 120 VAC

Frequency: 50-60 Hz

Maximum Power: 615 W

Size: 7" x 7" x 5"

Fisher Model 120 MR Magnetic Stirrer

Maximum Stirring Speed: 1650 RPM

Plate Surface Area: 17.7 sq. in.

Size: 4 3/4" x 2 3/4"

EQUIPMENT SPECIFICATIONS (cont'd)

Electrothermal Power Regulator Model MC 228

Supply Voltage: 100-125 VAC

Maximum Current: 10 A - intermittent, 5A - continuous

Output: Cyclic switching with continuously variable on/off ratio; arbitrary scale

Switching: single pole

Indicators: Amber - power output on

Input Power Connection: 2 m flying lead with moulded 3-pin plug

Output Power Connection: 1 m flying lead

CHEMICAL REAGENT COMPOSITIONS

phosphate buffer - 9.52 g sodium monophosphate and 8.45 g sodium phosphate, dibasic per 1 liter for a o.1 M buffer solution (17)

L-arginine HCl in buffer - 0.5045 g L-arginine HCl per 100 ml phosphate buffer for a 24 mM solution

Reagents for Assay Use (3):

acid reagent - 1:3 volumetric ratio of concentrated sulfuric (H_2SO_4) to phosphoric (H_3PO_4) acid; 500 ml stock solution

diacetylmonoxime - 3 g per 100 ml distilled water; wrap in foil for stability; shelf-life of 2-4 weeks

phenylhydrazine hydrochloride - 14.5 mg per 100 ml distilled water; make fresh daily (11)

Reagents for Protein Mass Determination (16):

biuret reagent - 173 g sodium citrate and 100 g sodium carbonate per 500 ml distilled water, warm gently to dissolve; 17.3 g copper sulfate pentahydrate per 100 ml distilled water; combine solutions and bring to 1 liter final volume - one year shelf-life

sodium hydroxide solution - 6 g NaOH per 100 ml distilled water for a 6% solution

APPENDIX B

ASEPTIC TECHNIQUE AND CULTURE PURITY

ASEPTIC TECHNIQUE

The techniques described in this section have been developed through trial-and-error to suit the needs of this experimental procedure. The original basis for the methods of sterilization and pure culture technique can be found in most college-level Microbiology laboratory manuals (4).

Steam Sterilization

Effective studies on pure cultures require that all equipment be free of contamination. Steam-under-pressure sterilization using an autoclave is the primary method used throughout this procedure.

1. Flasks containing liquids should not be more than half-full, or loss of liquid may occur. Sponge stoppers are inserted in the mouth of the flask with aluminum foil placed over it.
2. Any potential opening to the atmosphere on any item to be autoclaved (glassware, tubing, etc.) must be covered with foil before sterilization. However, no opening should be tightly sealed--screw caps should be loose and rubber stoppers should be avoided.

3. The water level in the bottom of the autoclave must be checked to ensure a sufficient amount of steam (Barnstead Model UE-650).
4. Items are placed in the autoclave and the unit is tightly sealed.
5. The sterilization time is selected and both the temperature and pressure are allowed to rise to 121° C and 15 psig, respectively. The sterilization time is usually 15 minutes for media and 25 minutes for dry goods.
6. After completion of sterilization, the temperature control is then switched off automatically and the steam is slowly evacuated from the autoclave. The sterile items can be removed when the pressure in the chamber returns to 0 psig.

Chemical Sterilization

Chemical sterilization is required for certain pieces of equipment which can not be steam sterilized, such as the temperature probe of the temperature controller. This procedure involves simply soaking the piece of equipment in a formaldehyde disinfecting

reagent for 24 hours. Rinsing with sterile distilled water is required before use.

Pure Culture Technique

In addition to making certain that equipment is sterile, it is necessary to employ an aseptic technique when handling the pure culture.

1. Preparation of the medium - Broth medium can be sterilized in a batch amount and then carefully poured into smaller, sterile jars or tubes. Alternately, the broth can be distributed into smaller containers before autoclaving (being careful not to overfill). Agar media must be sterilized in a batch amount and then distributed to sterile petri plates or other appropriate containers. The agar will solidify when cooled.
2. Transfer of the organism - Aseptic conditions are essential when transferring a pure culture from one container to another. The transfer should be made as quickly as possible.
   a) A loop or a sterile, disposable needle and syringe is used for the transfer.

The inoculating loop is flamed before retrieving the inoculum.

b) The area around each container should also be flamed before inserting and after removing the inoculating loop from that container (i.e., the open mouth of a test tube can easily be held over the flame of a bunsen burner).

c) Sufficient inoculum will free itself from the loop upon insertion in broth medium.

d) For agar medium, the inoculum must be applied to the solid surface. The cells are gently spread across the surface such that isolated colonies will be obtained after incubation. This will require flaming the inoculating loop at different points within the streaking process to minimize the number of cells being carried.

e) Use of a biohazard safety hood, a sterile inoculating room (with UV light), a face mask, sterile gloves or any other logical

precaution is recommended when
transferring both the organism and
sterile medium.

MAINTENANCE OF A PURE CULTURE OF L. BREVIS

A pure culture of *L. brevis* is needed for inoculation of the culture vessel of the chemostat. The original, freeze-dried culture was obtained from the U. S. Department of Agriculture. Pure stock cultures are maintained under refrigeration (4° C) in low surface-to-volume ratio, screw-capped 150 ml jars with approximately 100 ml of growth medium. Every two months the stock cultures are transferred to fresh medium. Simultaneously, the pure cultures needed for inoculum are prepared. The procedure is as follows:

1. Petri plates of medium are streaked with inoculum from the stock cultures.
2. The plates are incubated at 30° C in an anaerobe jar (oxygen is burned off by lighting a candle in the jar and then sealing it) until isolated colonies are identifiable (3-4 days).
3. A second series of plates are prepared using these colonies as inoculum.
4. If there is any evidence of contamination, a third series of plates should be prepared

using colonies from the second series as inoculum.

5. The second-to-last series of plates can be used to test for the desired organism (*L. brevis*). That is, if four series of plates are prepared, the third series can be used for the following tests:

   a) Plates of nutrient agar (NA) are streaked, incubated and observed for growth (NA can not sustain the growth of *L. brevis*)

   b) Microscopic identification based on the characteristics of *L. brevis*

   c) Gram stain

   d) Catalase test

   These tests are described in detail later in this section.

6. Three 150 ml screw-capped jars with 100 ml of medium are inoculated with colonies obtained from the last series of plates. After 3-5 days of incubation they are stored under refrigeration (4° C). These are the fresh stock cultures.

7. Twelve 20 ml screw-capped test tubes with 10 ml of sterile PGM are also inoculated from isolated colonies of the last series of plates. After approximately 3 days of incubation, the test tubes are refrigerated until needed as inoculum. Each test tube is used only once as inoculum for the chemostat to reduce chances of contamination.

PURITY ANALYSIS OF L. BREVIS

Growth on Nutrient Agar

Ingredients of 23 g Difco Nutrient Agar:

3 g	Bacto-Beef Extract
5 g	Bacto-Peptone
15 g	Bacto-Agar

1. Add 23 g Nutrient Agar to 1000 ml of distilled water.
2. Sterilize the medium and prepare petri plates for inoculation.
3. Inoculate a petri plate with the culture to be tested for purity; incubate the plate at 30° C.
4. After 2-3 days of incubation, examine the plate for growth: *L. brevis* growth on nutrient agar is extremely poor (48), so any substantially occurring growth is a contaminant.

Microscopic Identification

Microscopic examination of a sample of the culture to be tested for purity is a qualitative test.

L. brevis are short and straight rods with rounded ends (0.7-1.0 by 2.0-4.0 um) which occur singly or in short chains (8). The presence of any other type of microorganism would indicate contamination.

Gram Stain (39a)

1. Place a small drop of the sample on a clean microscope slide. If testing a colony from an agar plate, place a drop of water on the slide and mix the cells with the water. Spread to form a thin film.
2. Air dry or hold the slide over a flame to dry the cells. When dry, pass the slide three times through a flame to fix cells. Heat is necessary, but excess exposure should be avoided.
3. Add several drops of crystal violet dye and stain for 30 seconds.
4. Rinse with distilled water. Purple color remains.
5. Cover the film with gram's iodine mordant for 30 seconds.
6. Rinse with distilled water. Purple color remains.

7. Decolorize with 95% alcohol until solution drippings from the slide no longer have color--10-20 seconds.
8. Rinse with distilled water.
9. Counterstain with safranin for 20-30 seconds.
10. Rinse with distrilled water and blot dry.
11. Examine under the oil-immersion objective of the microscope.
12. Results - If the organism is gram positive, the purple stain should remain on the firm throughout the test. If the organism is gram negative, the film will become completely colorless when rinsed with 95% alcohol (step 7) and will stain pink with safranin (step 9). *L. brevis* is a gram positive organism, turning gram negative with increasing age (8). Therefore, although there will be some evidence of pink stain, purple color should predominate.

Catalase Test (39a)

1. Streak an agar plate with the sample to be tested.

2. Incubate at 30° C for approximately 3 days.
3. Add a drop of 3% hydrogen peroxide (H_2O_2) to an isolated colony on the plate.
4. Wait at least 10 seconds and examine the area for gas bubbles.
5. Results - Catalase catalyzes the formation of water and oxygen gas from hydrogen peroxide. This enzyme is not present in *L. brevis* (8), therefore no bubbling should be observed. Gas formation will be observed in the case of most common contaminants.

Modified Catalase Test

1. Use a sterile loop to transfer a drop of broth culture to a microscope slide.
2. Add one drop of 3% H_2O_2 and quickly place a cover slip on the mixture.
3. Examine the slide for bubbles. A pure catalase + contaminant will demonstrate sufficient gas to produce bubbles under the cover slip.
4. If results are questionable, prepare a concentrated pellet of bacterial cells by

centrifuging a broth culture and pouring off the liquid supernatant.

5. Use a sterile loop to transfer a drop of concentrated cells to a microscope slide.

6. Repeat steps 2 and 3.

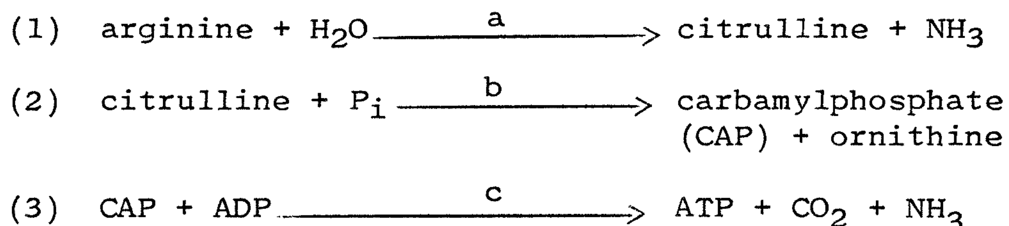
APPENDIX C

ENZYME PROPERTIES AND ORGANISM CHARACTERISTICS

PROPERTIES OF THE ENZYME ARGININE DEIMINASE

General characteristics of the enzyme arginine deiminase (EC 3.5.3.6) as described by Abdelal (1) include:

THE ARGININE DEIMINASE PATHWAY: Arginine deiminase is the first enzyme in a pathway present in some microorganisms which catabolizes arginine to generate ATP. The pathway is represented by the following series of reactions:



Enzymes: a - arginine deiminase
b - ornithine transcarbamylase
c - carbamate kinase

DISTRIBUTION: The arginine deminase pathway has been reported to occur in the following microorganisms:

Streptococci, Mycoplasma arthritidis, Pseudomonas putida, Pseudomonas aeruginosa, Clostridium perferingens, Clostridium botulinum, Clostridium sporogenes, Treponema denticola, Lactobacillus mesentroides, Lactobacillus fermenti, Lactobacillus leichmanii, Halobacterium salinarium, Chlorella vulgaris, Bacillus licheniformis, Aphanocapsa Tetrahymena pyriformis

SPECIFICITY: Highly specific for L-arginine

INHIBITORS: Creatinine, guanidinoacetate, L-homoarginine canavanine, long-chain diguanidines; carbon catabolite repression, ammonia repression

INDUCERS: Histidine; anaerobosis; Hyperbolic arginine saturation curves in some species

PSEUDONYMS: Arginine dihydrolase, L-arginine iminohydrolase

The following characteristics are specific to the enzyme isolated from *M. Hominis* (39):

STABILITY: The enzyme is stable @ 4 °C for four weeks without loss of activity.

pH OPTIMUM: 6.5 - 6.7 in 0.1M potassium phosphate buffer

KINETIC PARAMETERS: K_m for L-arginine is 1 to 4×10^{-4} M

METAL REQUIREMENTS: None after dialysis against 0.01M EDTA between 5.0 and 8.5 pH

SEDIMENTATION CONSTANT: 5.65×10^{-13} @ 25 °C in 0.05M potassium phosphate buffer, pH 7.0

FREE DIFFUSION CONSTANT: 6.75×10^{-7}

ESTIMATED MOLECULAR WEIGHT: 78,300, based on the above free diffusion coefficient and an assumed partial specific volume of 0.73

STRUCTURAL CHARACTERISTICS: Homogeneous preparations, dimer form

CHARACTERISTICS OF THE GENUS LACTOBACILLUS

The following characteristics of the genus *Lactobacillus* and the species *L. brevis* are taken from Bergey's Manual of Determinative Bacteriology (8):

The Genus Lactobacillus

Long and slender to short coccobacilli *rods*; Chain formation in later logarithmic phase of growth, motility by peritrichous flagella when present; non-sporing

Gram-positive turning gram-negative with increasing age

Fermentative metabolism; aero-tolerant with anaerobic growth on isolation

Nitrate reduction unusual

Gelatin not liquified

Casein not digested

Indole and H_2S not produced

Catalase and Cytochrome negative

Benzidine reaction negative

Yellow or orange to rust or brick red pigment production, when present

Surface growth on solid media enhanced by anaerobiosis and 5-10% CO_2

Optimum temperature 30-40°C

Optimum pH 5.5 - 5.8 or less

DNA G+C content ranges from 34.7±1.4 to 53.4±0.5 moles % (buoyant density)

Found in dairy products and effluents, grain and meat products, water, sewage, beer, wine, fruits and fruit juices, pickled vegetables, sourdough and mash; also parasitic in the mouth, intestinal tract and vagina of many homothermic animals including man

Pathogenicity highly unusual

The Species L. Brevis

0.7-1.0 by 2.0-4.0 µm short and straight rods with rounded ends occurring singly or in short chains

Gram or methylene-blue stains may reveal bipolar or other granulations

Generally non-pigmented, nearly translucent, rough or intermediate, flat colonies; some strains pigmented orange to red

Heterofermentative:

- Acid and gas from glucose in media
- Only aerobic catabolism of separately sterilized glucose
- Acid and gas from fructose anaerobically
- Growth at expense of 4% gluconate with copious CO_2 production
- D-Ribose fermented to lactic and acetic acids without gas
- Pyruvate dismutation anaerobically yields lactate, acetate and CO_2
- Aerobically, lactate slowly yields acetate and CO_2

Enzyme activity:

- Glucose metabolized by the hexose monophosphate shunt

Glucose 6-phosphate dehydrogenase activity, but low 6-phosphogluconate activity
No fructose 1.6-diphosphate aldolase activity

Mannitol produced from fructose

-Methyl-D-glucoside fermented by 80% of strains; little or no acidity in milk

DL-Lactic acid produced (50% of total glucose carbon)

Acetate, ethanol and CO_2 are produced

Catalase negative, rare strains decompose peroxide by a pseudocatalase

Ammonia produced from arginine

Cell walls contain glycerol teichoic acid; peptidoglycan is an L-lysine-D-aspartate type

Serological group E

Nutritional requirements: calcium pantothenate, niacin, thiamine, folic acid

Riboflavin, pyridoxal, and vitamin B_{12} not required for growth

Growth at 15°C, no growth at 45°C; optimum growth at 30°C

G+C content of the DNA is 42.7 ± 1.5 to 45.4 ± 1.0 moles % (buoyant density). Variable DNA composition suggests that the species is internally heterogenous.

Isolated from: milk, kefir, cheese, sauerkraut, spoiled tomato products, sourdough, certain soils, ensilage, cow manure, feces, and the mouth and intestinal tract of humans and rats

APPENDIX D

GROWTH ANALYSIS OF *L. BREVIS*

GROWTH ANALYSIS OF LACTOBACILLUS BREVIS

Introduction

An evaluation of the batch growth characteristics of the species *L. brevis* is required for the selection of the dilution rate to be used for chemostat operation. The dilution rate should be less than or equal to the maximum specific growth rate, μ_{max} of the batch culture grown in the same nutrient medium.

A general description of the genus *Lactobacillus* is given in Section III-C and APPENDIX C. The species *L. brevis* is described in detail in APPENDIX C. The purpose of this investigation is two-fold:

(1) To determine a suitable medium for the cultivation of *L. brevis*, and

(2) To estimate the maximum specific growth rate, μ_{max} of a batch culture grown in that medium.

In consideration of the complex nutritional requirements of this group of organisms, a complex component such as yeast extract or some other general source of nutrients must be used in the base medium. Ledesma (28) reports a synthetic medium for the purpose of Lactobacillus cultivation. This synthetic medium is compared to a commonly used complex medium known as $LAPTg_{10}$. Difco also

prepares a medium for Lactobacillus cultivation called Micro-Inoculum Broth (MIB).

The nature of this experimentation does not require the use of a completely defined synthetic medium. Due to the availability of MIB, this medium was selected over $LAPTg_{10}$ as the base medium. Compositions of both $LAPTg_{10}$ and MIB are given in TABLE D-1.

Method

The first series of data were taken from cultures grown in Batch Fermenter Type 1 (FIGURE 10) with MIB at two different pH values. The pH of MIB is normally 6.7. A pH of 5.8 was obtained by addition of HCl. The medium was sterilized and proper precautions taken to inhibit contamination throughout this entire series of growth analyses. The fermenter was inoculated with approximately 3 ml of culture in a 5 ml syringe. Samples (5 ml) were removed periodically with a sterile pipet and the optical density was read at 550 nm against a distilled water blank.

Series II consisted of Spectronic 20 culture tubes filled with 5 ml of medium, sterilized and inoculated with a loop of _L. brevis_ cells. The optical density was then read directly from the tubes without changes in volume

TABLE D-1

COMPOSITION OF LACTOBACILLUS MEDIA

	Micro-Inoculum Broth	$LAPTg_{10}$
YEAST EXTRACT[a]	20	10
GLUCOSE	10	10
TRYPTONE	-	10
PEPTONE	5	-
TWEEN 80	0.1	10
MONOPOTASSIUM PHOSPHATE	2	-
pH	6.7	6.5

[a] all concentrations are as g/l

or exposure to contamination. MIB was evaluated alone and in combination with 0.6% Bacto-Tryptone and 0.5% L-arginine hydrochloride, w/v.

Nephelo-culture flasks (Batch Fermenter Type 2) were available for subsequent batches. These flasks were filled with 120 ml of the specified medium, sterilized and inoculated with 3 ml of culture in a 5 ml syringe. The sidearms were covered with plastic culture tube caps. Series III evaluated MIB with tryptone and arginine in the same concentrations as in Series II. An analysis of growth in $LAPTg_{10}$ was also performed in this series. From this point on, the optical densities of the samples were read against a medium blank rather than a distilled water blank.

Series IV was performed using the chemostat culture vessel as a batch fermenter. The equipment was sterilized and assembled as described in Section V-A. A 3 ml inoculum was used. Samples (5 ml) were withdrawn from the overflow tube during exponential growth. The medium consisted of MIB, 0.6% tryptone and 0.5% arginine.

Batch Fermenter Type 2 was used for Series V and VI, however the sidearms were now tightly sealed with a rubber stopper. Series V used a variation of MIB including 0.5%

dextrose rather than 0.1% of that carbohydrate. The medium also included 0.5% arginine and 0.6% tryptone. The optical density was measured against a distilled water blank as well as a medium blank for comparison.

Series VI used the same medium as Series V, with evaluation of a dextrose concentration of 0.05% also included. This series was performed using a larger concentration of biomass in the inoculum to minimize the initial lag period. This inoculum was prepared by centrifuging 25 ml of a batch-grown culture and resuspending it in sterile medium.

Discussion of Results

Reading the optical density of a culture against a distilled water blank did not accurately represent the growth characteristics of the organism. As can be seen from the results of Series I and II, the estimated maximum specific growth rates (μ_{max}) were very low (see TABLE D-2). The data from Series V was used to determine whether the results obtained from Series I and II could be correlated with the accurate results obtained using a pure medium blank.

General equations relating the measured absorbance to the percent transmittance were used to convert the data based on a distilled water blank to a linear form.

TABLE D-2

MAXIMUM SPECIFIC GROWTH RATES OF L. BREVIS FOR DIFFERENT GROWTH MEDIA

BATCH	MEDIUM COMPOSITION[a]	$\mu_{max}(h^{-1})$
I-1	MIB, 5.8pH	0.047
I-2	MIB, 6.7pH	0.050
II-1	MIB	0.013
II-2	MIB, .6% tryptone	0.016
III-1	MIB	0.089
III-2	MIB, .6% tryptone	0.088
III-3	MIB, .5% arginine	0.089
III-4	$LAPTg_{10}$	0.109
IV-1	MIB, .6%tryptone, .5% arginine	0.110
V-1	MIB(.5% glucose), .6% tryptone, .5% arginine	0.144
VI-1	same as V-1, larger initial inoculum	0.142
VI-2	MIB(.05% glucose), .6% tryptone, .5% arginine	0.163

[a] % concentration in g/100 ml (w/v)

The values were then adjusted with respect to the percent transmittance of the pure medium. The data was next converted back to absorbance and compared to the directly measured experimental data. The maximum specific growth rate was estimated at 0.13 h^{-1}. The corresponding value determined from data measured against a pure medium blank was 0.144 h^{-1}. Thus, a fairly reasonable correlation can be made between the two sets of data.

Actual conversion of the original data was not necessary since the results were evaluated relative to each batch within a series. In Series I, both 6.7 pH and 5.8 pH resulted in a reasonably close slope averaging 0.048 h^{-1}, with the pH value of 6.7 being slightly higher. Therefore, adjustment of the pH of MIB was not incorporated into media preparation.

Series II demonstrated that addition of o.6% Bacto-tryptone gave a slightly larger slope (0.016 h^{-1}) than pure MIB (0.013 h^{-1}). This data was the basis for subsequent assition of tryptone to the base medium composition. It was later demonstrated in Series III that the addition of either tryptone or arginine to MIB had no effect on μ_{max} based on early exponential data.

Series II was performed in an extremely limited environment of only 5 ml volume. The slopes obtained should at least compare to the slopes obtained in Series I,

since both series involved data measured against a distilled water blank. The lower values for Series II could indicate that the culture was already entering stationary phase when the optical density became measurable. This method was estimated to be too restricted to reflect an accurate value for μ_{max}.

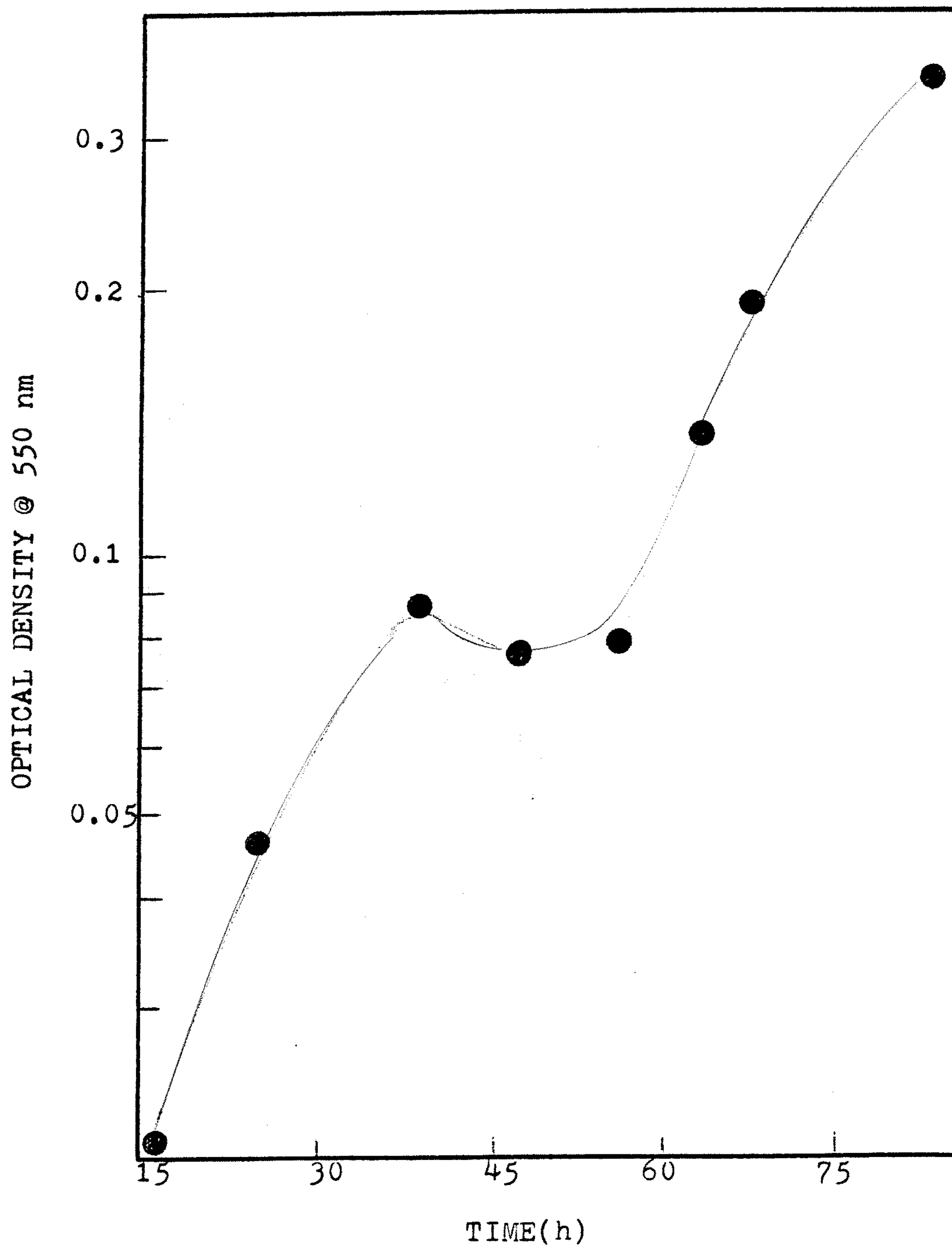
This series did demonstrate some very interesting growth characteristics in the batches containing 0.5% arginine with 0.6% tryptone. FIGURE D-1 clearly demonstrates the occurrence of diauxic growth of L. brevis in this system. This phenomenon is supportive of the steady state characteristics observed in the chemostat experimentation. Sequential utilization of substrates can cause the occurrence of multiple steady states in a chemostat.

The results of Series III with data measured against a pure medium blank gives a more accurate representation of the maximum specific growth rate of L. brevis. A rate of 0.089 h^{-1} was obtained for media consisting of MIB, MIB-tryptone and MIB-tryptone-arginine compositions. Attempts to reproduce the diauxic growth curve in large volume batches were unsuccessful. $LAPTg_{10}$ resulted in a higher rate of 0.109 h^{-1}. This difference was not sufficient to elicit a change in the growth medium used.

Although this data indicates the ineffectiveness of additional tryptone, the component was retained to ensure constant operating conditions. The original selection

FIGURE D-1

DEMONSTRATION OF DIAUXIC GROWTH OF L. BREVIS IN A MEDIUM CONTAINING GLUCOSE AND ARGININE



was made based on the composition of $LAPTg_{10}$. It was later revealed that these organisms do not readily digest proteins (48).

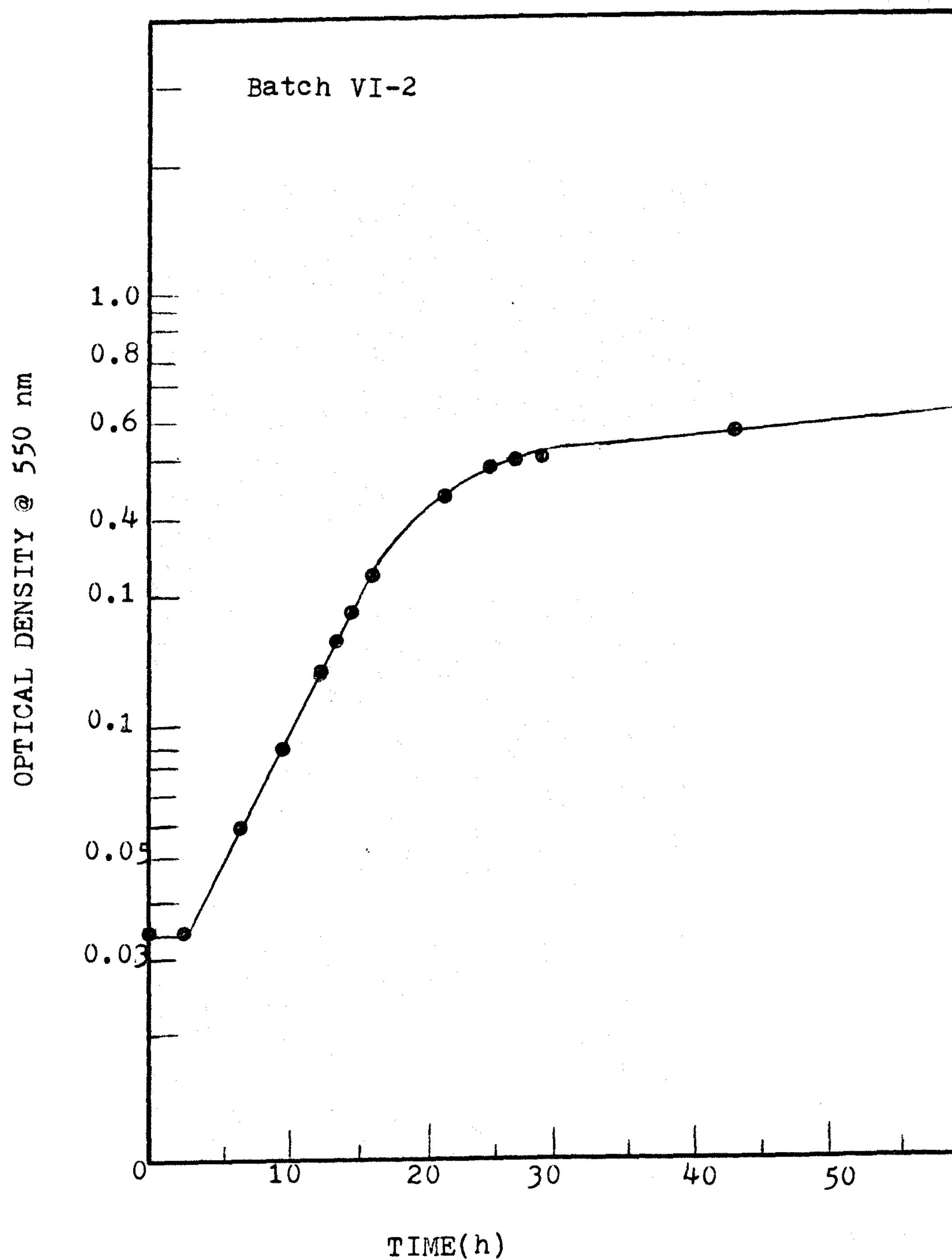
Series IV resulted in a slightly higher growth rate for the MIB-tryptone-arginine medium composition (0.110 h^{-1}). This could be attributed to the maintenance of anaerobic conditions with nitrogen gas.

Series V and VI result in much higher specific growth rates than previously obtained. These values seem to correspond to a decrease in glucose concentration, with the highest rate equivalent to the lowest sugar concentration. A concurrent physical effect may be resulting from the tightly-sealed nature of the fermentation vessel, with *L. brevis* producing copious CO_2. It would be interesting to observe the effects of CO_2 gas within a batch culture comparable to Series IV.

FIGURE D-2 represents the general form of the growth curve obtained in most of these batch growth series.

FIGURE D-2

SEMI-LOG PLOT OF OPTICAL DENSITY VERSUS TIME FOR A BATCH-GROWN CULTURE OF L. BREVIS



Conclusions

1. Pure medium must be used to blank the timed samples when generating data for the determination of μ_{max}.

2. An average μ_{max} of 0.10 was obtained for a medium composition of MIB with 0.5% arginine and 0.6% tryptone. The medium would be used for chemostat operation at different dilution rates.

3. L. brevis exhibits diauxic growth in a restricted glucose-arginine environment.

4. A higher value of μ_{max} is obtained for decreases in the glucose concentration of the medium.

APPENDIX E

CALIBRATION AND STANDARDIZATION METHODS AND RESULTS

CALIBRATION METHODS

Peristaltic Pump

Both peristaltic pumps were calibrated by measuring an amount of water collected in a graduated cylinder over a specific period of time for each pump setting. A larger number of data points were evaluated in the general operating region. The resulting calibration plots of volumetric flow rate as a function of pump setting were used to estimate the operating flow rate for each run. The exact dilution rates were determined from the volumetric flow rate measured during operation.

Temperature Controller

The temperature probe and a thermometer were placed into a flask containing ice and water. The fine control setting for the temperature controller was set at zero. A heat source regulated by the controller was applied to the flask and the solution was uniformly mixed. The temperature at which the controller switched off the heat source was recorded for each coarse controller setting in the region of operation. The calibration curves generated were used to estimate the operating temperature. The controllers were recalibrated at an exact temperature of 30°C using the coarse and fine controller settings for each chemostat run.

STANDARDIZATION METHODS

Biomass Concentration

A standardization plot of biomass concentration as a function of optical density was obtained using eleven batch-grown cultures of *L. brevis*. The organism was grown in 300 ml of growth medium (Batch Fermenter Type 3, FIGURE 10 to various optical densities. The optical density was read at 550 nm and ranged from 0.03 to 1.0. Each batch culture was divided into three 100 ml samples. Each sample was then centrifuged and the cells were washed according to the procedure outlined in Section V-C. The samples were allowed to dry within the centrifuge tubes for 24 hours. The cells were then placed in pre-tared weighing dishes and dried for three days in a 100°C dry-heat oven . The biomass was measured using a sensitive analytic balance. The three values obtained for each culture were averaged to give a final estimate of biomass concentration for each optical density reading.

Citrulline Assay

A solution of 1 mM citrulline was prepared by dissolving 17.5 mg of citrulline in 100 ml of sodium phosphate buffer This solution of citrulline was used in different proportions

with water to prepare a series of ten 1.7 ml samples. The final citrulline content in the samples ranged from 0.03 to 0.80 micromoles. Acid reagent (1 ml), phenylhydrazine hydrochloride (0.15 ml) and diacetylmonoxime (0.15 ml) were added to each sample tube in the specified amounts. The samples were heated and the optical densities read according to the procedure outlined in Section V-D. The slope of this standardization plot relating citrulline concentration to optical density was used in the calculations for enzyme activity.

Protein Content Determination

A solution containing 50 mg of lysozyme in 50 ml of distilled water was prepared. This solution was diluted by different amounts of water to prepare nine 2 ml samples ranging in protein concentration from 0.03 to 1.0 mg/ml. Biuret reagent (0.2 ml) and 6% sodium hydroxide (2 ml) were added to each sample. The samples were analyzed according to the procedure outlined in Section V-E. The slope of the standardization plot relating protein content and optical density was used in the calculations for specific activity.

TABLE E-1

CALIBRATION DATA FOR THE THERMISTEMP
TEMPERATURE CONTROLLER MODEL 63 RC
Fine control set at 0

CONTROLLER SETTING	TEMPERATURE (°C)	
	CONTROLLER #1	CONTROLLER #2
1.0	0.0	0.0
1.5	11.5	12.0
2.0	22.5	24.0
2.5	30.5	32.0
3.0	36.5	37.0
3.5	43.0	43.5
4.0	49.0	49.0
4.5	54.5	54.0
5.0	61.0	60.5

TABLE E-2

CALIBRATION DATA FOR THE VERSATHERM TEMPERATURE CONTROLLER MODEL 2158

Fine control set at 0

CONTROLLER SETTING	TEMPERATURE (^{o}C)
40	65.5
50	51.7
60	37.8
65	32.2
70	26.7
75	21.1
80	17.2

TABLE E-3

CALIBRATION DATA FOR THE PHARMACIA PERISTALTIC PUMP MODEL P-1

PUMP SETTING	VOLUMETRIC FLOW RATE (ml/h) TUBING ID, 1 mm	TUBING ID, 1 mm*	TUBING ID, 1.6 mm	TUBING ID, 2.1 mm
1.0	0.9	9.1	4.0	4.0
2.0	1.9	15.7	6.0	6.7
3.0	2.2	22.1	7.2	9.3
3.5	-	-	8.8	-
4.0	2.9	27.8	9.6	12.5
4.5	-	-	11.1	-
5.0	3.6	35.9	13.6	15.7
6.0	4.3	44.4	15.5	19.4
7.0	4.9	52.3	-	22.6
8.0	5.7	57.7	20.0	25.2
9.0	6.3	65.8	21.8	27.3
10.0	7.0	71.4	24.2	30.5

* x10 magnitude for peristaltic pump setting

TABLE E-4

CALIBRATION DATA FOR PHARMACIA PERISTALTIC PUMP MODEL P-3

PUMP SETTING	VOLUMETRIC FLOW RATE (ml/h) TUBING ID, 1 mm	TUBING ID, 1.6 mm
1.0	3.0	9.3
1.6	-	9.6
1.8	-	12.0
2.0	6.0	15.6
2.2	-	16.8
2.5	-	24.0
3.0	12.0	32.4
4.0	18.6	48.6
5.0	25.5	63.0
6.0	31.5	-
7.0	38.0	96.7
8.0	44.0	-
9.0	51.0	128.2
10.0	54.0	136.7

TABLE E-5

STANDARDIZATION DATA FOR THE DETERMINATION OF BIOMASS CONCENTRATION AS A FUNCTION OF OPTICAL DENSITY

SAMPLE	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
1	0.03	0.005
2	0.08	0.012
3	0.10	0.028
4	0.16	0.039
5	0.21	0.045
6	0.33	0.092
7	0.45	0.112
8	0.56	0.150
9	0.75	0.275
10	0.90	0.475
11	1.00	0.650

TABLE E-6

STANDARDIZATION DATA FOR THE CITRULLINE ASSAY

SAMPLE	CITRULLINE CONTENT (μM)	OPTICAL DENSITY @ 495 nm
1	0.03	0.025
2	0.05	0.093
3	0.10	0.266
4	0.20	0.574
5	0.30	0.960
6	0.40	1.210
7	0.50	1.641
8	0.60	1.960
9	0.70	2.333
10	0.80	2.670

TABLE E-7

STANDARDIZATION DATA FOR THE BIURET METHOD OF PROTEIN CONTENT DETERMINATION

SAMPLE	PROTEIN CONTENT (mg/ml)	OPTICAL DENSITY @ 540 nm
1	1.00	0.205
2	0.50	0.135
3	0.67	0.150
4	0.33	0.110
5	0.25	0.100
6	0.10	0.078
7	0.05	0.073
8	0.03	0.070
9	0.0	0.068

TABLE E-8

SUMMARY OF LINEAR REGRESSION RESULTS FOR PUMP CALIBRATION DATA

PUMP	SETTING RANGE	TUBING DIAMETER (mm)	REGRESSION COEFFICIENT	SLOPE	INTERCEPT
P-1	1-10	1.0	0.999	0.68	0.20
P-1[a]	1-10	1.0	0.999	7.10	1.15
P-1	1-10	1.6	0.996	2.33	1.03
P-1	1-10	2.1	0.999	3.01	0.78
P-3	2-9	1.0	0.998	6.42	-6.98
P-3	1.6-9	1.6	0.999	16.14	-16.81

[a]Pump setting at x10 magnitude

TABLE E-9

SUMMARY OF LINEAR REGRESSION RESULTS FOR STANDARDIZATION DATA (TABLES E-5 to E-7)

DEPENDENT VARIABLE	REGRESSION COEFFICIENT	SLOPE	INTERCEPT
Biomass concentration (mg/ml)	0.995	0.273 (mg/ml/OD)	-0.005 (mg/ml)
Citrulline concentration (micromoles)	0.999	0.291 (umoles/OD)	0.03 (umoles)
Protein concentration (mg/ml)	0.998	7.36 (mg/ml/OD)	-0.48 (mg/ml)

FIGURE E-1

CALIBRATION PLOT FOR THE THERMISTEMP TEMPERATURE CONTROLLER MODEL 63 RC

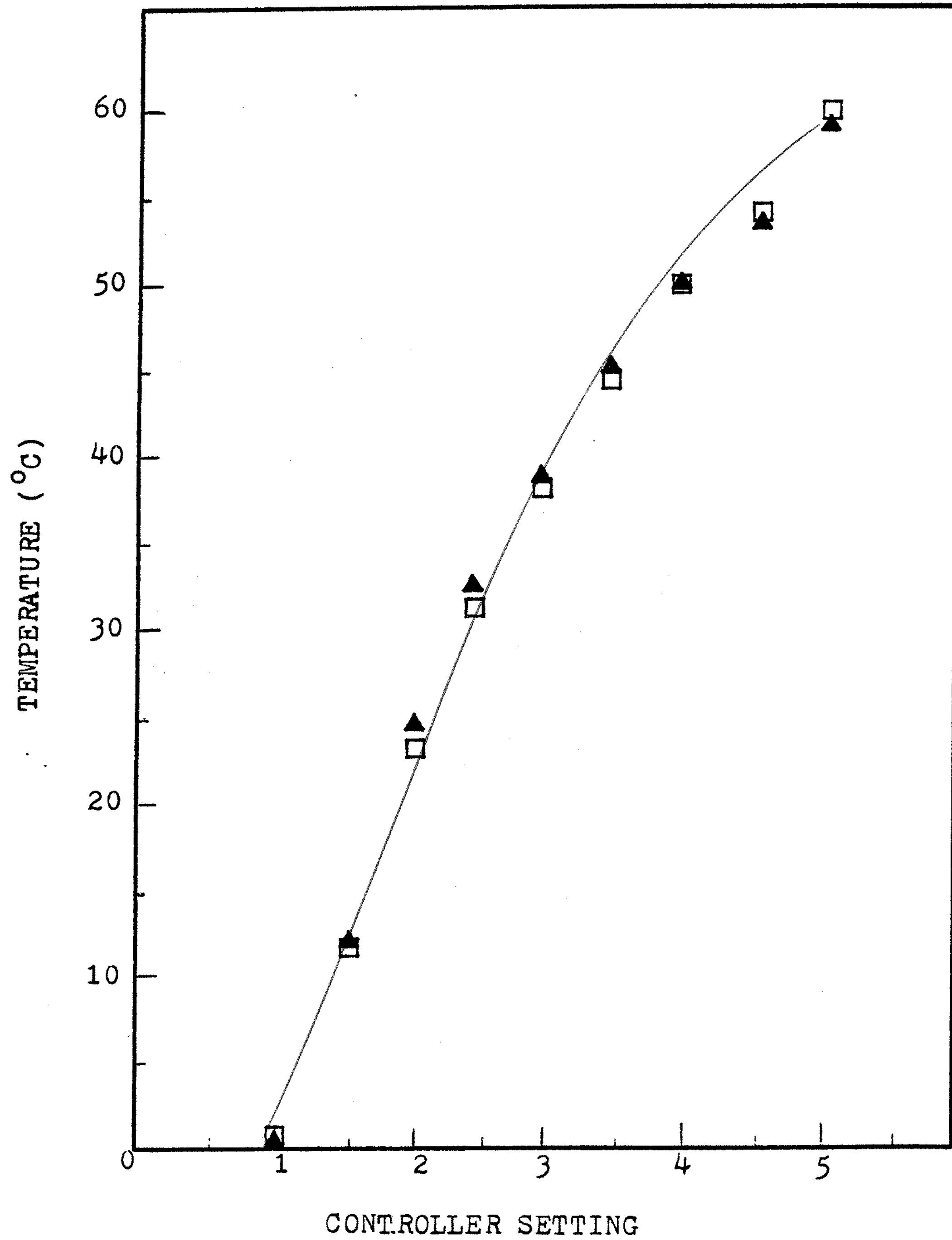


FIGURE E-2

CALIBRATION PLOT FOR THE VERSATHERM TEMPERATURE CONTROLLER MODEL 2158

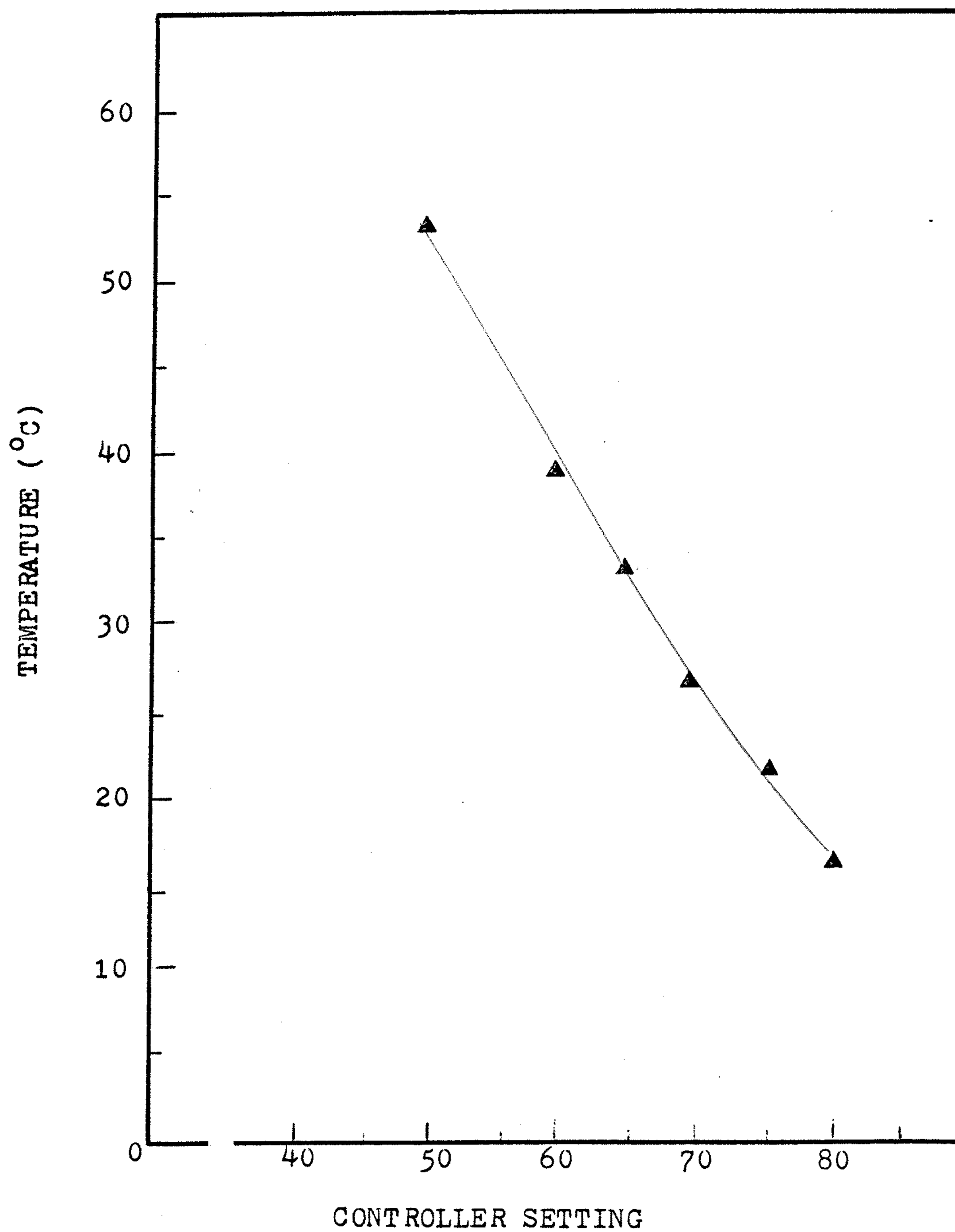


FIGURE E-3

CALIBRATION PLOTS FOR THE PHARMACIA PERISTALTIC PUMP MODEL P-1

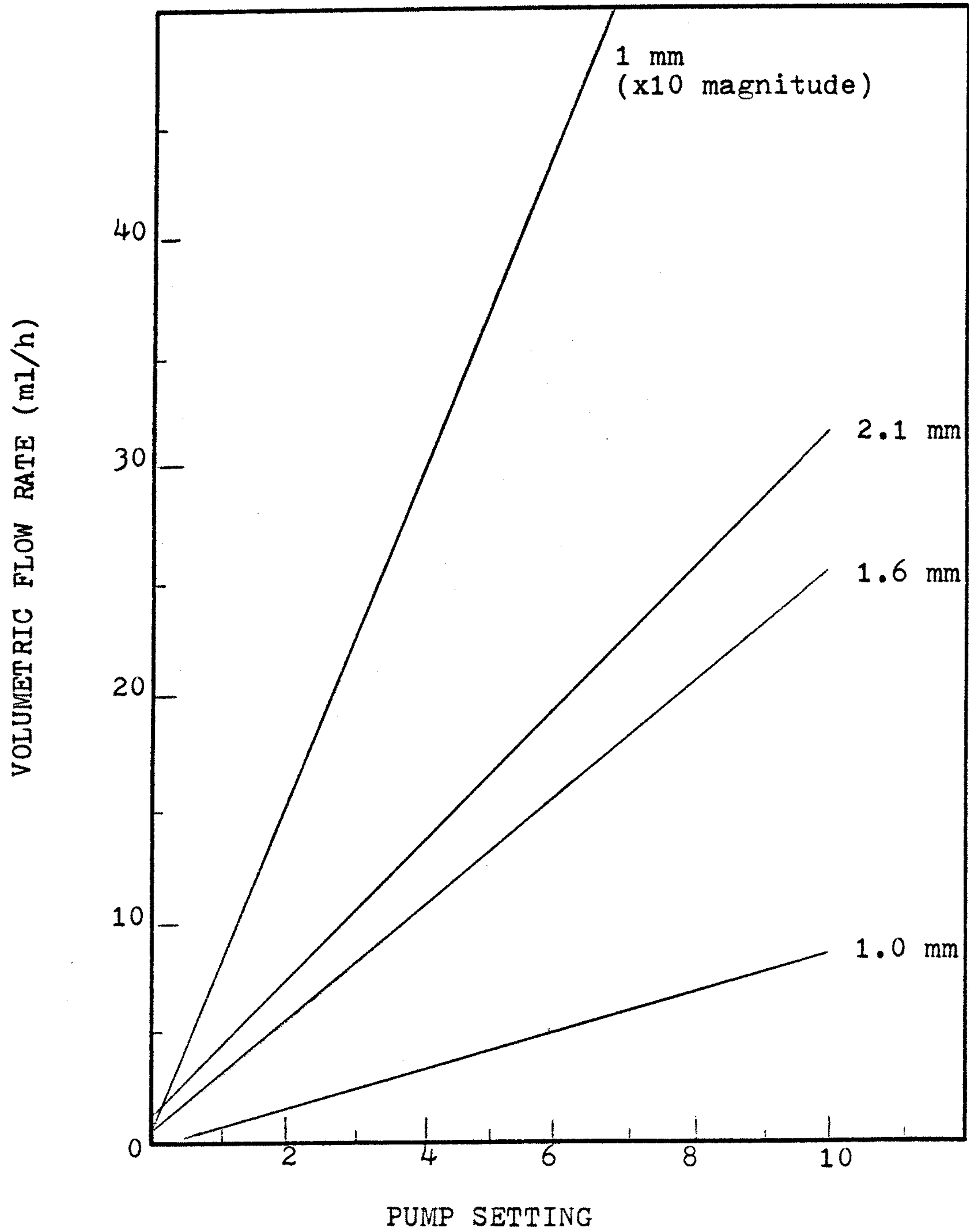


FIGURE E-4

CALIBRATION PLOTS FOR PHARMACIA PERISTALTIC PUMP MODEL P-3

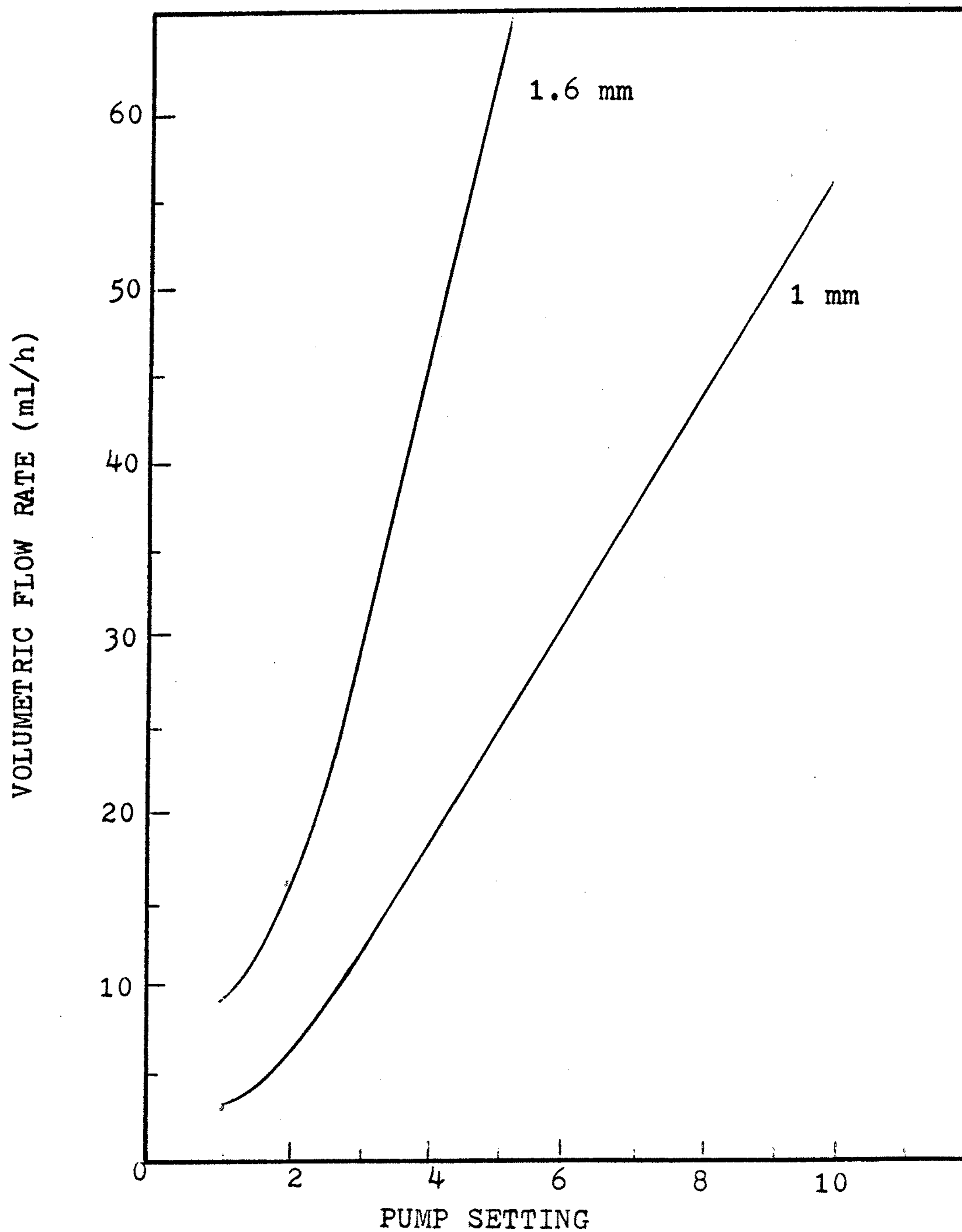


FIGURE E-5

STANDARDIZATION PLOT FOR THE DETERMINATION OF BIOMASS CONCENTRATION AS A FUNCTION OF OPTICAL DENSITY

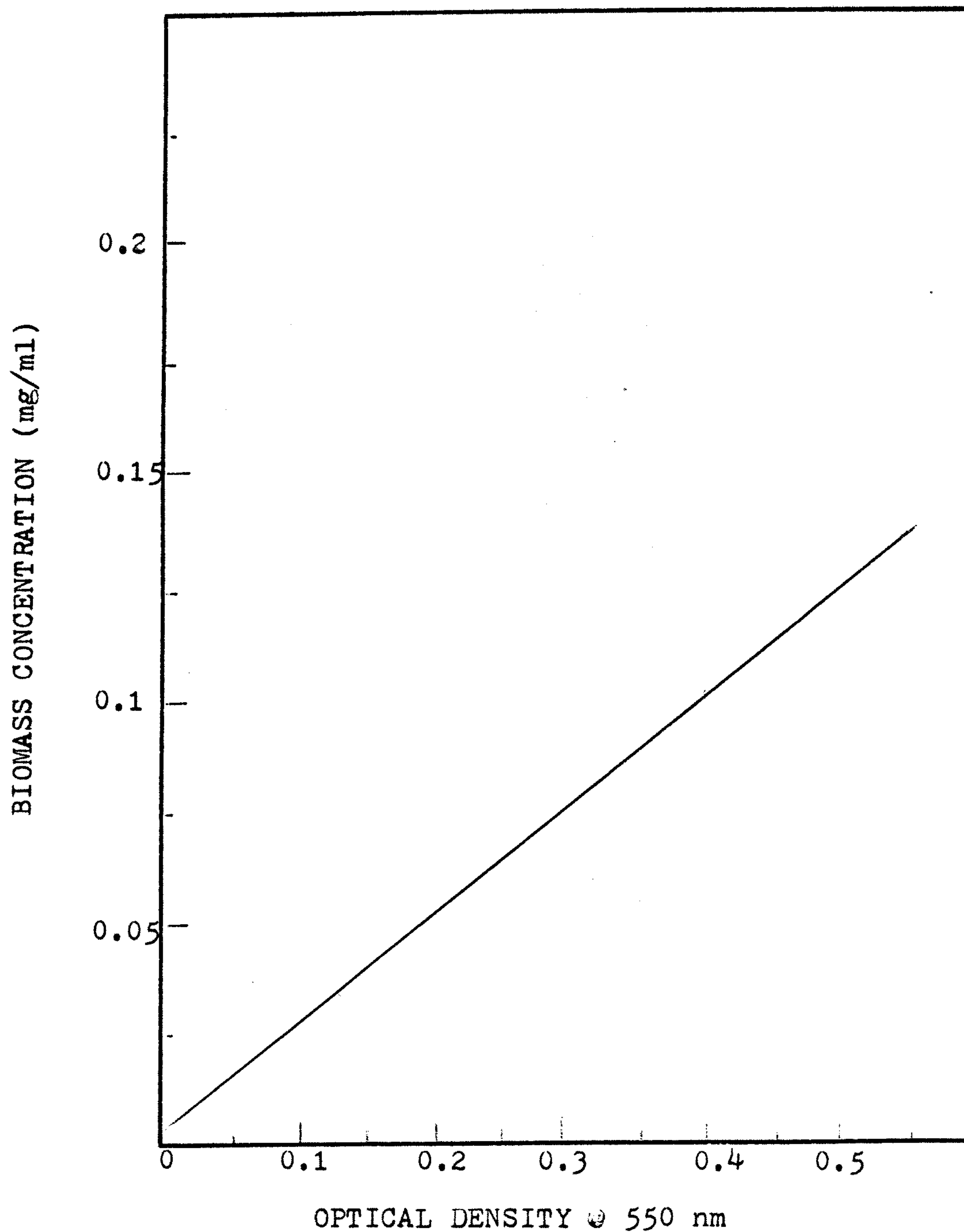


FIGURE E-6

STANDARDIZATION PLOT FOR THE CITRULLINE ASSAY

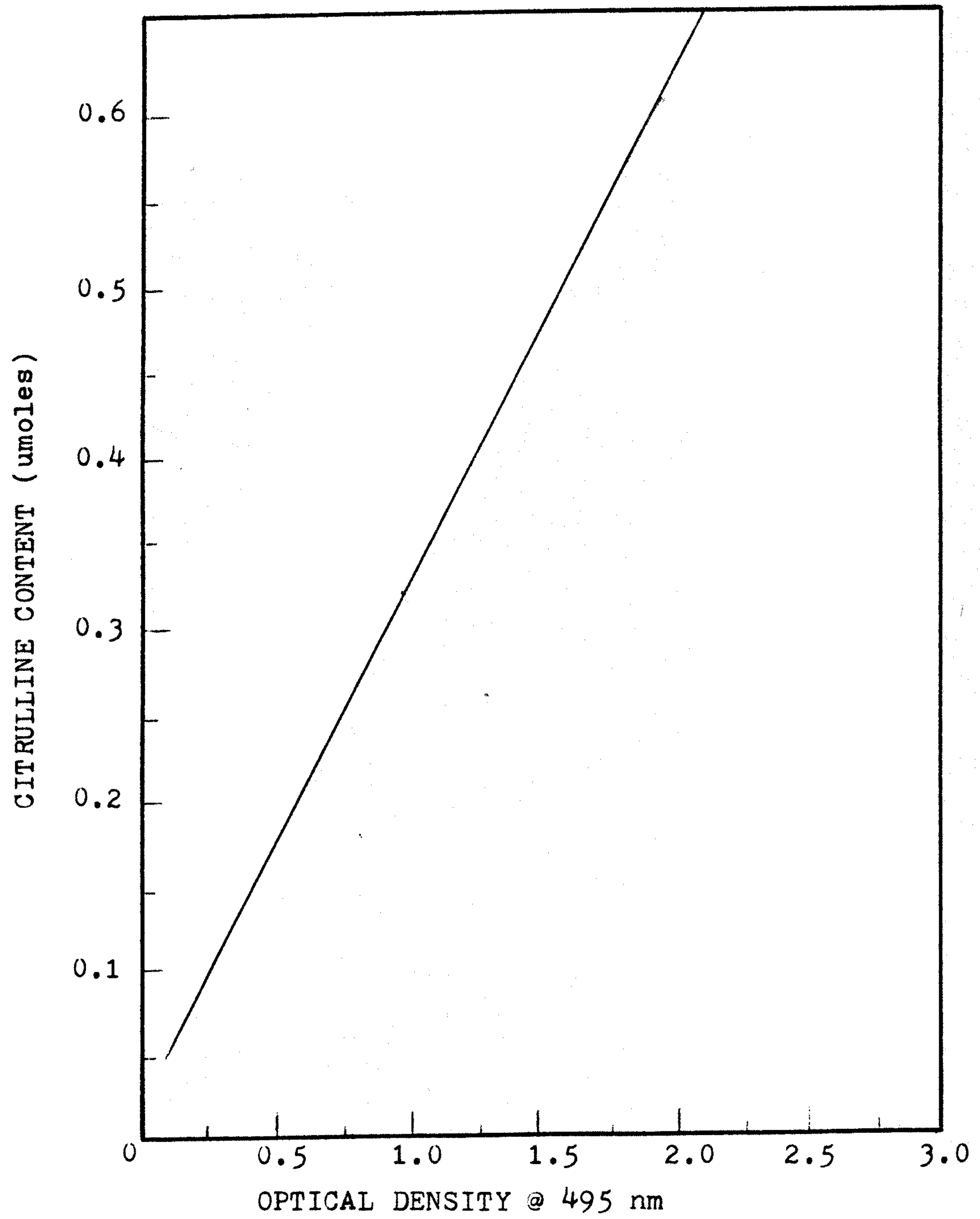
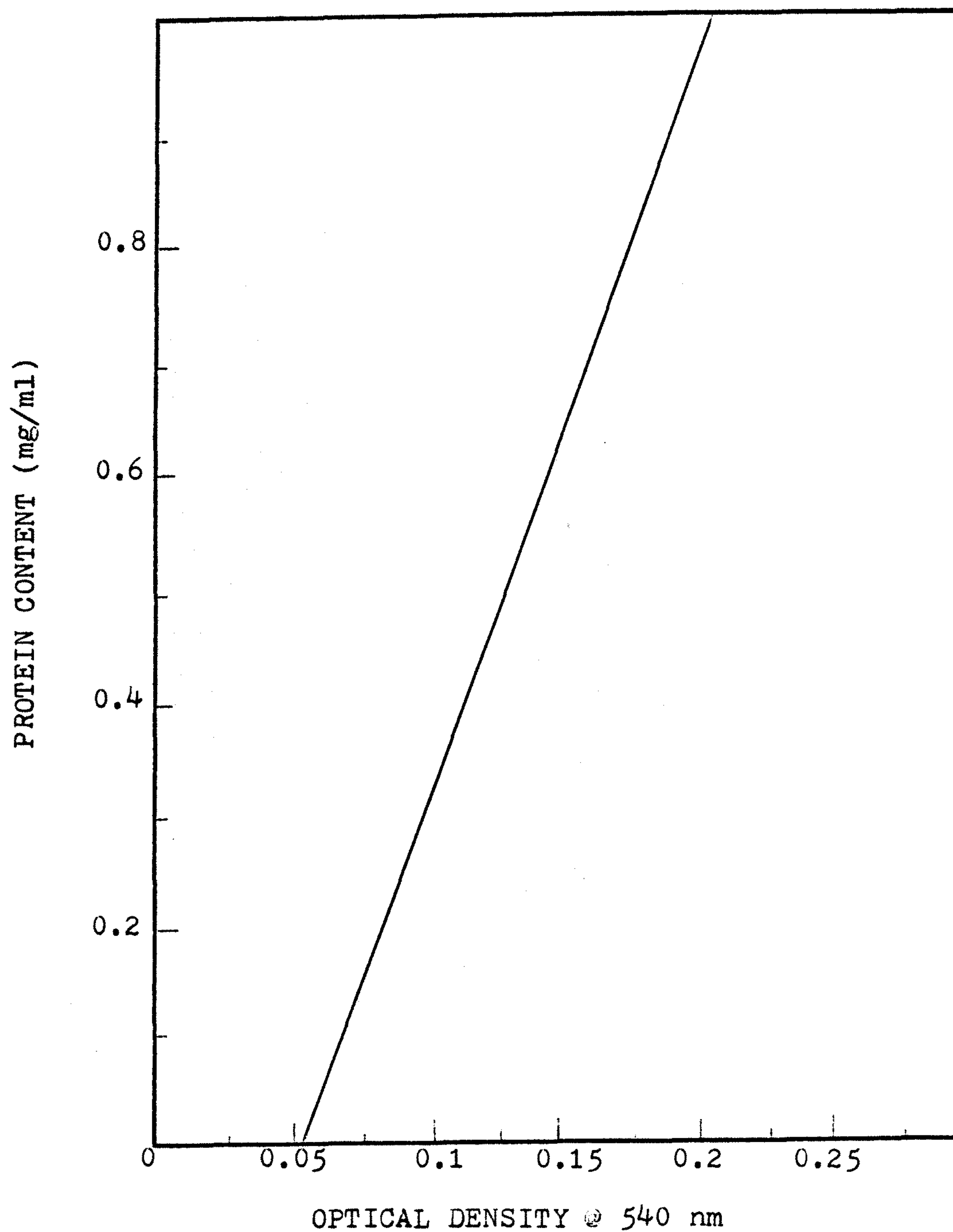


FIGURE E-7

STANDARDIZATION PLOT FOR THE BIURET METHOD OF PROTEIN CONTENT DETERMINATION



APPENDIX F

SONICATION STUDIES ON *L. BREVIS*

SONICATION STUDIES ON L. BREVIS

Introduction

Arginine deiminase as produced by the species *Lactobacillus brevis* is an intracellular enzyme. For the purpose of evaluating the activity of this enzyme, it is necessary to extract a suitable amount of enzyme from the *L. brevis* cells.

Ultrasonic disruption of the cell wall, or sonication, is a simple and commonly used physical method of cell disintegration. When liquids are exposed to high frequencies (ultrasonics), gaseous cavitation occurs. As gas bubbles collapse, forming cavities, shock waves are created which physically disrupt the walls of the cells. The effectiveness of the sonication method depends upon the temperature, pH and ionic strength of the solution. Sonication of cells is usually performed at 4° C (on ice) and the cells are suspended in a suitably buffered solution (43).

The procedure initially followed in this experiment was based upon the work done by Manca de Nadre, et al, with the organism *Lactobacillus buchneri* (30). In order to extract a sample of arginine deiminase from

L. buchneri, cell samples suspended in sodium phosphate buffer (6.5 pH) were exposed to eight 3 minute periods of sonication with 2 minute cooling intervals. The frequency was specified as maximum intensity.

Since lactobacilli are Gram positive organisms which possess thick, dense peptidoglycan cell walls (48), it is not unreasonable to expect intense, high frequency exposure to be required for cell disruption. In addition, *L. brevis* is considered to be quite similar to *L. buchneri* in most physical characteristics (8). Therefore, the procedure for sonication outlined above was initially followed.

After several runs of experimentation, it was observed that enzyme activity was absent in some of the samples. These results were inconsistent with previous results and difficult to interpret. When no results were obtained from cell samples collected from Chemostat Run #3, the assay was repeated on an unused portion of the original sample. Instead of sonifying the cells, several drops of toluene were added to the sample and incubated at room temperature (22° C) for one hour. Toluene is often used to chemically disrupt the cells, although its action on Gram positive

organisms is marginal. The second assay indicated some conversion of arginine to citrulline, but to a limited extent.

It was hypothesized that the first assay sample was oversonicated, resulting in enzyme-protein denaturation. The activity observed in the second assay sample was limited by the small amount of enzyme extracted from the toluenized cell. It was therefore determined necessary to further investigate the method of cell disruption to be used before continuing the primary experimentation.

Method

A 500 ml batch culture of *L. brevis* was allowed to incubate 113 hours to a final optical density of 0.95 at 550 nm.+ The pH after incubation was 6.85 at a temperature of 22.0^{o} C. After uniform mixing of the culture, five 50 ml samples were taken and cell suspensions were prepared as described in Section V-D of the Experimental Procedure. These cell extracts were then exposed to varying intervals of sonication (see TABLE F-1). A Heat Systems-Ultrasonics, Inc. Sonifier Model W185 was used at a maximum intensity setting (40W). The enzyme extract was then obtained

by centrifuging the sonicated cells in a refrigerated centrifuge for 20 minutes at a speed of 14,500 RPM. The supernatant was retrieved as the enzyme extract. This extract was assayed for arginine deiminase activity as described in Sections V-E and V-F of the Experimental Procedure.

+ The incubation time and optical density are arbitrary parameters.

TABLE F-1

METHODS OF CELL DISRUPTION USED IN SONICATION STUDIES ON L. BREVIS

SAMPLE	DESCRIPTION
A	addition of 2-3 drops of toluene with one hour incubation (22° C)
B	eight 30 second periods of sonication with 30 second cooling intervals
C	addition of 2-3 drops of toluene with one hour incubation (22° C); followed by eight 30 second periods of sonication with 30 second cooling intervals
D	addition of a granule of lysozyme+ with one hour incubation (22° C); followed by four 3 minute periods of sonication with 3 minute cooling intervals
E	eight 3 minute periods of sonication with 3 minute cooling intervals

+ the amount of lysozyme is minute and does not contribute to the total protein content determination.

Discussion of Results

No arginine deiminase activity was obtained from Sample A. In addition, the amount of protein released from the cells was insufficient to measure by the Biuret method. It is obvious from these results that addition of toluene alone is inadequate as a method of cell disruption for L. brevis under the conditions specified.

Since all samples were the same cell density from the same original culture, the enzyme activities obtained from each sample should theoretically be identical. All samples demonstrated reasonably close activities approximately 0.125 units/ mg protein.

It had been expected that the sample exposed to the longest period of sonication (Sample E) would demonstrate a reduction in enzyme activity due to protein denaturation. Based on the data available for evaluation, Sample E resulted in a comparable value for enzyme activity. Analysis is limited, however, to the first two data points, after which linearity is absent. The maximum plateau achieved by Sample E in the assay plot of Optical Density versus Time (FIGURE F-1) indicates that the reaction mixture was arginine--limited at a concentration that is inconsistent with

the results from Samples B, C and D. This result is unexplainable.

In comparing Samples B, C and D, the only significant difference is that Sample D had a much larger amount of protein released. Hence, it can be assumed that treatment with lysozyme before sonication enhances the ultimate release of protein from the cells.

All results are summarized in TABLE F-2.

Conclusions

It has been demonstrated that cell suspensions of *L. brevis* can be adequately disrupted at a reduced period of sonication than originally applied. In addition, treatment with lysozyme before sonication apparently enhances protein release. The lysozyme, however, is not essential to the release of a sufficient amount of enzyme for assay analysis. Future enzyme extracts will be prepared simply by disrupting the cells at maximum intensity for eight 30 second periods of sonication with 30 second cooling intervals.

TABLE F-2

SUMMARY OF SONICATION STUDIES RESULTS

SAMPLE	PROTEIN CONTENT (mg)	ENZYME ACTIVITY (units/mg protein)
A	----	----
B	0.02525	0.1150
C	0.01832	0.1316
D	0.06437	0.1300
E	0.02922	0.1233

TABLE F-3

LINEAR REGRESSION ANALYSIS ON ASSAY RESULTS FOR SONICATION STUDIES ON L. BREVIS: OPTICAL DENSITY VERSUS TIME

SAMPLE	RANGE (MIN)	REGRESSION COEFFICIENT	SLOPE	INTERCEPT
B	5-30	.994	0.0100	-0.0248
C	5-20	.999	0.0083	-0.0220
D	5-30	.990	0.0288	-0.1048
E	5-10	---	0.0124	-0.0500

FIGURE F-1

PLOT OF OPTICAL DENSITY AS A FUNCTION OF REACTION TIME FOR THE CITRULLINE ASSAY
Sonication studies.

FIGURE F-1.1

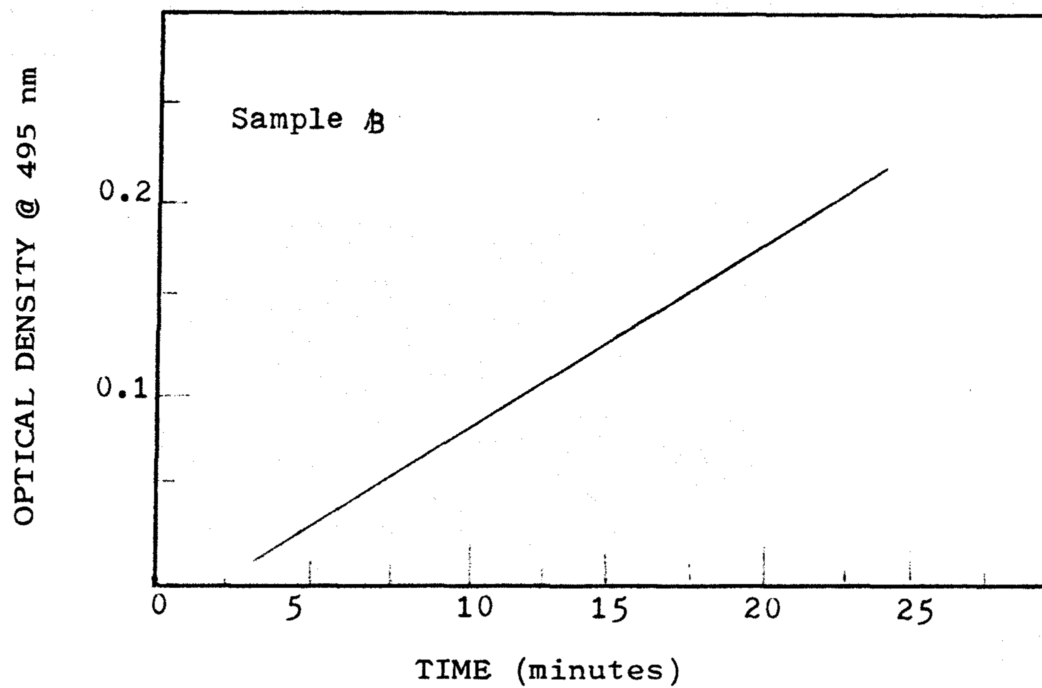


FIGURE F-1.2

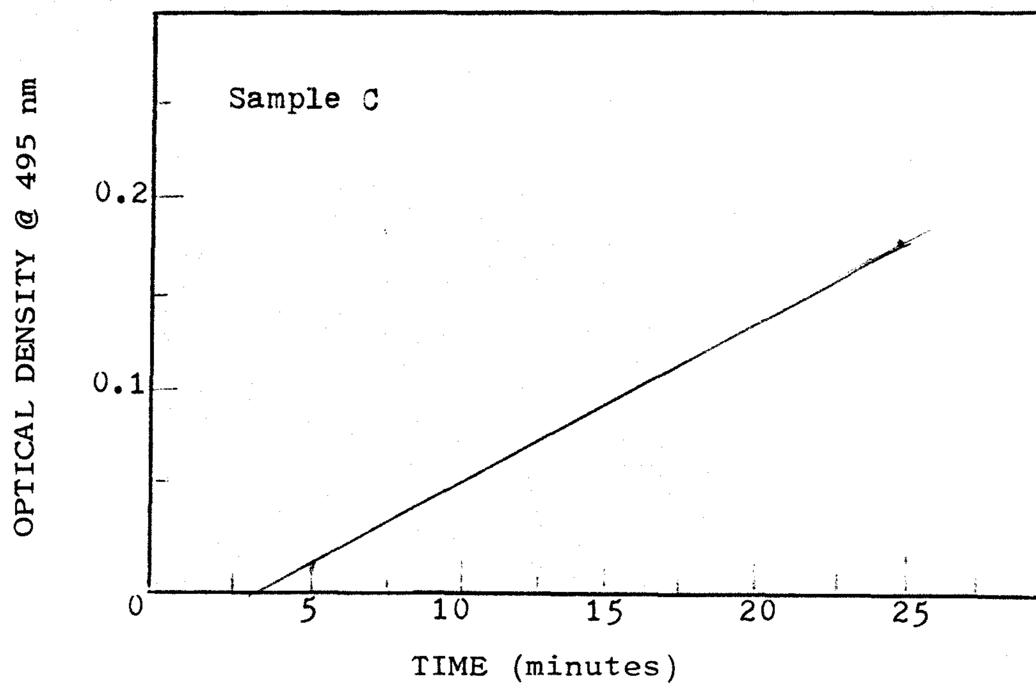


FIGURE F-1.3

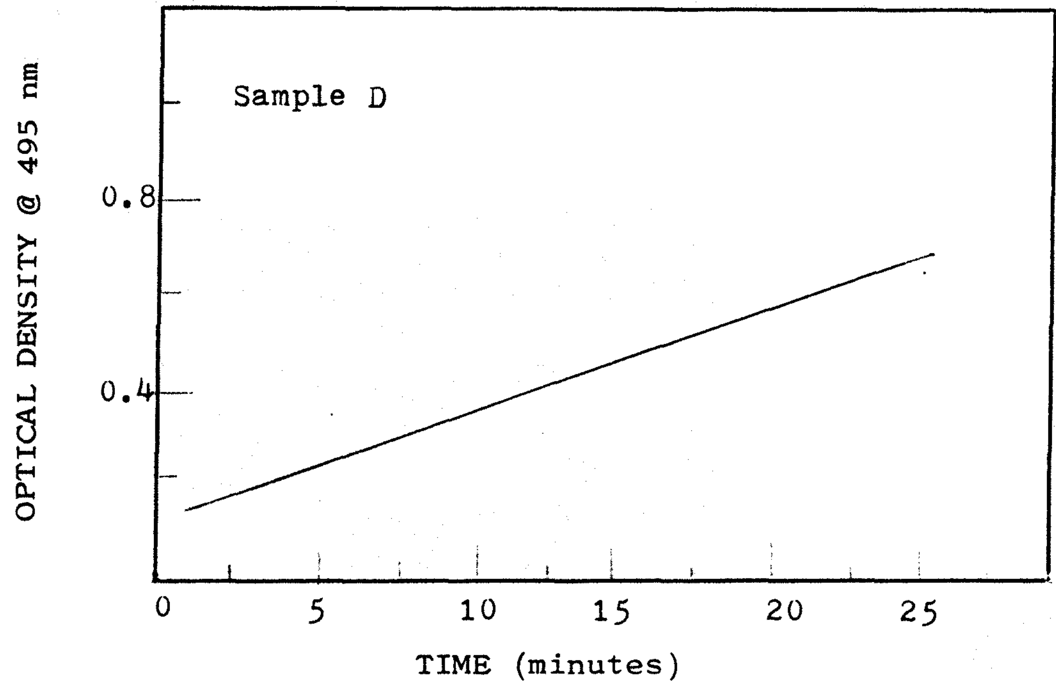
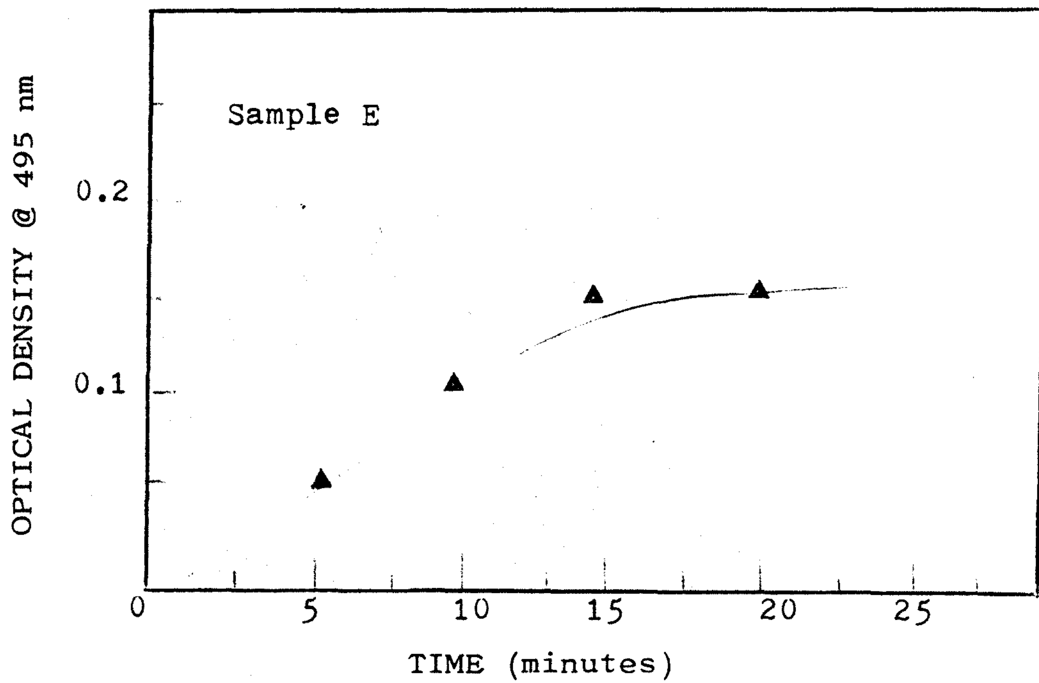


FIGURE F-1.4



APPENDIX G

EXPERIMENTAL DATA

TABLE G-1.1

EXPERIMENTAL DATA FOR CHEMOSTAT OPERATION

Runs 1 to 11

RUN	PUMP MODEL	PUMP SETTING	TUBING DIAMETER (ID, mm)	CULTURE VESSEL VOLUME (ml)	ESTIMATED VOLUMETRIC FLOW RATE (ml/h)
1	P-1	2	2.1	170	6.8
2	P-1	1	2.1	170	3.8
3	P-1	7	1.0	170	5.0
4	P-1	5	1.0	170	3.6
5	P-3	2.2	1.0	140	7.2
6	P-3	2.0	1.0	140	5.9
7	P-1	6	1.0	170	4.3
8	P-1	9	1.0	170	6.3
9	P-3	2.5	1.0	140	9.1
10	P-3	4.0	1.0	140	18.8
11	P-1	3.5(x10)	1.0	170	26.0

TABLE G-1.2

EXPERIMENTAL DATA FOR CHEMOSTAT OPERATION
Runs 12 to 22

RUN	PUMP MODEL	PUMP SETTING	TUBING DIAMETER (ID, mm)	CULTURE VESSEL VOLUME (ml)	ESTIMATED VOLUMETRIC FLOW RATE (ml/h)
12	P-1	2 (x10)	1.0	150	15.3
13	P-3	3.2	1.0	140	13.6
14	P-1	3.5	2.1	150	11.3
15	P-1	5	1.6	140	13.5
16	P-3	2.0	1.6	170	15.6
17	P-1	4	1.6	170	13.2
18	P-1	4	2.1	140	12.8
19	P-3	1.7	1.6	150	10.6
20	P-3	1.7	1.6	180	10.6
21	P-3	1.6	1.6	170	14.0
22	P-3	1.8	1.6	170	11.9

TABLE G-1.3

EXPERIMENTAL DATA FOR CHEMOSTAT OPERATION

Runs 23 to 30

RUN	PUMP MODEL	PUMP SETTING	TUBING DIAMETER (ID, mm)	CULTURE VESSEL VOLUME (ml)	ESTIMATED VOLUMETRIC FLOW RATE (ml/h)
23	P-1	2.5	1.6	140	5.1
24	P-3	1.0	1.6	150	8.0
25	P-3	1.0	1.6	170	9.5/5.5/7.6
26	P-3	1.0	1.6	140	8.0
27	P-3	1.0	1.6	150	8.0
28	P-3	1.0	1.6	150	8.0
29	P-3	1.0	1.6	150	8.0
30	P-3	1.0	1.6	170	8.0

TABLE G-2.1

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 1 to 7

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
1-A	7.8	53	6.8
2-A	22.5	85	3.8
2-B	14.5	55	3.8
3-A	10.0	53	5.3
4-A	17.0	122	7.2
4-B	-	125	-
5-A	9.5	80	8.4
6-A	14.8	82	5.6
6-B	16.5	101	6.1
6-C	16.5	96	5.8
6-D	-	132	-
7-A	16.5	65	3.9
7-B	18.5	77	4.2
7-C	20.8	92	4.4
7-D	24.0	117	4.9
7-E	22.0	85	3.9

TABLE G-2.2

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 8 to 11

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
8-A	23.0	133	5.8
8-B	23.2	166	7.1
8-C	9.5	50	5.3
8-D	12.5	70	5.6
8-E	19.0	135	7.1
8-F	-	173	-
9-A	12.0	202	16.8
9-B	19.0	171	9.0
9-C	20.5	200	9.8
9-D	-	165	-
10-A	7.8	143	18.5
10-B	14.5	322	22.2
10-C	4.5	73	16.2
10-D	6.5	140	21.5
11-A	5.8	150	26.1
11-B	14.5	365	25.2
11-C	4.5	100	22.2
11-D	4.0	106	26.5
11-E	-	170	-

TABLE G-2.3

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 12 tO 14

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
12-A	7.0	131	18.7
12-B	15.0	255	17.0
12-C	8.0	100	12.5
12-D	16.5	228	13.8
12-E	4.5	82	18.2
13-A	7.3	90	12.4
13-B	15.0	200	13.3
13-C	16.5	188	11.4
13-D	8.5	190	22.4
13-E	16.5	110	6.7
13-F	4.5	70	15.6
14-A	5.0	60	12.0
14-B	16.5	155	9.4
14-C	23.0	210	9.1
14-D	20.0	177	8.9

TABLE G-2.4

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 15 & 16

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
15-A	3.0	37	12.3
15-B	1.8	26	14.9
15-C	2.5	39	15.6
15-D	12.5	185	14.8
15-E	2.0	39	19.5
15-F	5.0	61	12.2
15-G	3.0	46	15.3
15-H	16.0	234	14.6
16-A	3.0	49	16.3
16-B	1.8	34	19.4
16-C	2.5	50	20.0
16-D	12.5	148	11.8
16-E	-	5	-
16-F	5.0	80	16.0
16-G	3.0	49	16.3
16-H	16.0	294	18.4

TABLE G-2.5

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 17 & 18

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
17-A	2.8	40	14.6
17-B	18.0	173	9.6
17-C	3.0	12	4.0
17-D	25.5	231	9.1
17-E	19.3	165	8.6
17-F	1.3	-	-
18-A	4.0	33	8.3
18-B	17.3	184	10.7
18-C	-	5	-
18-D	2.0	32	16.0
18-E	18.3	179	9.8
18-F	-	5	-
18-G	24.0	255	10.6

TABLE G-2.6

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 19 to 21

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
19-A	3.0	28	9.3
19-B	19.0	168	8.8
19-C	6.0	34	5.7
19-D	17.3	144	8.4
19-E	2.5	21	8.4
19-F	18.3	152	8.3
19-G	1.0	10	10.0
19-H	24.0	199	8.3
20-A	3.0	36	12.0
20-B	19.0	242	12.7
20-C	6.0	55	9.2
20-D	17.3	205	11.9
20-E	2.5	39	15.6
20-F	18.3	231	12.7
20-G	1.0	10	10.0
20-H	24.0	291	12.1
21-A	19.0	200	10.5
21-B	19.0	166	8.7
21-C	27.0	300	11.1

TABLE G-2.7

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 22 & 23

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
22-A	20.0	216	10.8
22-B	1.0	10	10.0
22-C	21.5	195	7.1
22-D	12.0	158	13.2
23-A	21.5	110	5.1
23-B	1.0	-	-
23-C	19.0	100	5.3
23-D	2.5	13	5.2
23-E	25.8	129	5.0
23-F	2.0	10	5.0
23-G	18.0	90	5.0
23-H	22.0	110	5.0
23-I	23.5	108	4.6

TABLE G-2.8

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION
Runs 24 & 25

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
24-A	1.5	12	8.0
24-B	21.5	175	8.1
24-C	1.0	-	-
24-D	19.0	158	8.3
24-E	2.5	20	8.0
24-F	25.8	203	7.9
24-G	-	3	-
24-H	18.0	140	7.8
25-A	1.5	14	9.3
25-B	21.5	214	10.0
25-C	1.0	-	-
25-D	19.0	103	5.4
25-E	6.5	36	5.5
25-F	25.8	146	5.7
25-G	19.0	142	7.5
25-H	1.0	-	-
25-I	22.0	174	7.9

TABLE G-2.9

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 26 & 27

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
26-A	8.0	84	10.5
26-B	14.5	150	10.3
26-C	3.5	34	9.7
26-D	20.5	203	9.9
26-E	2.0	-	-
26-F	11.5	114	9.9
26-G	12.5	120	10.4
26-H	6.3	62	9.9
27-A	8.0	65	8.1
27-B	14.5	118	8.1
27-C	3.5	28	8.0
27-D	20.5	173	8.4
27-E	2.0	-	-
27-F	11.8	93	7.9
27-G	12.5	85	6.8
27-H	19.5	169	8.7

TABLE G-2.10

EXPERIMENTAL DATA FOR CHEMOSTAT SAMPLE COLLECTION

Runs 28 to 30

SAMPLE	SAMPLING TIME(h)	VOLUME COLLECTED(ml)	VOLUMETRIC FLOW RATE(ml/h)
28-A	8.0	85	10.6
28-B	14.5	147	10.1
28-C	3.5	34	9.7
28-D	20.5	203	9.9
28-E	2.0	-	-
28-F	11.5	121	10.5
28-G	12.5	125	10.0
28-H	19.5	180	9.2
29-A	3.0	31	10.3
29-B	19.8	202	10.2
29-C	1.8	18	10.3
29-D	24.5	245	10.0
29-E	1.0	10	10.0
29-F	21.0	205	9.8
30-A	17.0	190	11.2
30-B	28.0	270	9.6
30-C	21.0	205	9.8
30-D	24.0	210	8.8

TABLE G-3.1

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS (mg/ml)
1-A	123.6	4.9	6.5	0.44	0.115
2-A	194.5	4.3	6.4	0.45	0.118
2-B	213.0	4.8	6.5	0.46	0.120
3-A	140.0	4.4	6.5	0.41	0.107
4-A	177.5	7.5	7.2	0.48(1:3)	0.503
4-B	185.0	7.8	7.3	0.50(1:4)	0.656
5-A	100.3	5.1	6.9	0.36(1:3)	0.372
6-O	0.0	-	7.2	0.52(1:4)	0.683
6-A	16.1	0.7	7.2	0.50(1:4)	0.656
6-B	39.3	1.6	7.1	0.39(1:1)	0.200

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.2

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION(h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS(mg/ml)
6-C	62.0	2.6	7.1	0.48(1:1)	0.252
6-D	120.8	5.0	6.9	0.38(1:2)	0.300
7-O	0.0	-	6.7	0.55	0.145
7-A	31.0	0.8	6.7	0.21	0.052
7-B	80.5	2.0	6.9	0.30	0.077
7-C	102.1	2.6	6.8	0.48	0.126
7-D	127.3	3.2	7.1	0.45	0.118
7-E	150.5	3.8	6.9	0.31(1:1)	0.159

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.3

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS (mg/ml)
8-O	0.0	-	6.8	0.42(1:2)	0.328
8-A	37.3	1.4	6.8	0.38	0.099
8-B	61.1	2.2	7.0	0.32(1:2)	0.247
8-C	79.0	2.9	7.2	0.52	0.137
8-D	90.0	3.3	6.9	0.34(1:1)	0.175
8-E	110.5	4.0	7.2	0.41(1:1)	0.213
8-F	120.0	4.4	7.1	0.31(1:1)	0.159
9-O	0.0	-	6.9	0.50(1:4)	0.656
9-A	16.8	1.1	7.0	0.46(1:2)	0.361
9-B	37.5	2.5	6.6	0.31	0.079

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample:medium diuent)

TABLE G-3.4

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS (mg/ml)
9-C	59.8	4.0	6.6	0.38	0.099
9-D	70.0	4.7	6.6	0.45	0.118
10-O	0.0	-	6.7	0.44(1:1)	0.230
10-A	3.9	0.6	6.7	0.41	0.107
10-B	15.0	2.1	6.7	0.15	0.036
10-C	24.5	3.4	6.6	0.03	0.003
10-D	30.0	4.2	6.7	0.01	0.002
11-O	0.0	-	7.2	0.37(1:1)	0.192
11-A	2.9	0.4	7.2	0.39	0.101

[a]Samples 'O' are for initial conditions

[b]Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.5

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY[b] @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
11-B	13.0	1.9	6.7	0.11	0.024
11-C	22.5	3.3	6.7	0.01	0.002
12-O	0.0	-	6.2	0.48(1:1)	0.252
12-A	3.5	0.4	7.1	0.52	0.137
12-B	16.0	1.7	6.8	0.23	0.058
12-C	27.5	2.9	6.6	0.12	0.028
12-D	41.3	4.4	6.7	0.07	0.014
12-E	52.3	5.6	6.7	0.07	0.014

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample : medium diuent)

TABLE G-3.6

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE	TIME FROM PUMP ACTIVATION(h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS(mg/ml)
13-O	0.0	-	6.9	0.52	0.137
13-A	3.6	0.4	7.0	0.31	0.079
13-B	16.0	1.6	6.7	0.11	0.025
13-C	40.3	3.9	6.6	0.18	0.044
13-D	52.8	5.1	6.8	0.23	0.057
13-E	67.8	6.6	6.6	0.20	0.050
13-F	78.8	7.7	6.6	0.26	0.066
14-O	0.0	-	6.0	0.42(1:1)	0.219
14-A	3.5	0.2	5.9	0.40	0.104
14-B	15.3	1.0	5.7	0.55	0.145

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.7

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
14-C	25.0	1.6	5.7	0.38	0.099
14-D	37.0	2.4	5.6	0.40	0.104
14-E	49.0	3.2	5.7	0.43	0.112
14-F	63.0	4.1	5.8	0.40	0.104
15-O	0.0	-	6.8	0.35	0.090
15-A	2.2	0.2	6.8	0.39	0.101
15-B	5.5	0.6	6.8	0.22	0.055
15-C	7.8	0.8	6.8	0.18	0.044
15-D	16.3	1.7	6.7	0.15	0.036
15-E	23.5	2.5	6.6	0.11	0.025

[a]Samples 'O' are for initial conditions

TABLE G-3.8

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION(h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS(mg/ml)
15-F	27.0	2.9	6.6	0.05	0.009
15-G	31.0	3.3	6.6	0.05	0.009
15-H	40.5	4.3	6.6	0.03	0.003
16-O	0.0	-	6.7	0.30	0.077
16-A	2.5	0.3	6.7	0.27	0.069
16-B	5.5	0.6	6.7	0.28	0.071
16-C	7.8	0.8	6.7	0.26	0.066
16-D	16.3	1.6	6.7	0.20	0.049
16-E	24.5	2.4	6.7	0.19	0.047
16-F	27.0	2.7	6.7	0.16	0.039

[a] Samples 'O' are for initial conditions

TABLE G-3.9

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY[b] @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
16-G	31.0	3.1	6.7	0.15	0.036
16-H	40.5	4.0	6.6	0.06	0.011
17-O	0.0	-	7.1	0.42(1:1)	0.219
17-A	1.9	0.1	7.1	0.37(1:1)	0.192
17-B	14.5	0.8	7.4	0.23	0.090
17-C	25.0	1.4	7.4	0.23	0.058
17-D	52.0	2.8	7.4	0.08	0.017
17-E	62.6	3.4	7.5	0.09	0.020
17-F	72.9	3.9	7.5	0.10	0.022

[a]Samples 'O' are for initial conditions

[b]Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.10

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS (mg/ml)
18-O	0.0	-	6.6	0.47	0.123
18-A	2.0	0.2	6.6	0.40	0.104
18-B	12.6	1.0	6.6	0.35	0.090
18-C	21.5	1.7	6.0	0.22	0.055
18-D	22.8	1.8	5.7	0.20	0.049
18-E	34.1	2.7	5.7	0.16	0.039
18-F	43.8	3.5	5.8	0.08	0.017
18-G	56.3	4.5	5.7	0.06	0.011
19-O	0.0	-	7.2	0.48(1:2)	0.377
19-A	1.8	0.1	7.2	0.53(1:1)	0.279

[a] Samples 'O' are for initial conditions

[b] Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.11

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION(h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS(mg/ml)
19-B	15.0	0.8	7.3	0.52	0.137
19-C	27.5	1.5	7.2	0.36	0.093
19-D	39.1	2.2	7.2	0.22	0.055
19-E	49.0	2.7	7.0	0.22	0.055
19-F	60.6	3.4	7.0	0.23	0.058
19-G	70.3	3.9	6.9	0.24	0.060
19-H	82.7	4.6	6.9	0.23	0.058
20-O	0.0	-	7.5	0.48(1:1)	0.252
20-A	1.8	0.1	7.5	0.53(1:1)	0.279
20-B	15.0	1.0	7.2	0.52	0.137

[a]Samples 'O' are for initial conditions

[b]Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.12

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION(h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS(mg/ml)
20-C	27.5	1.8	7.2	0.43	0.112
20-D	39.1	2.6	6.8	0.40	0.104
20-E	49.0	3.3	6.8	0.30	0.077
20-F	60.6	4.1	6.7	0.23	0.058
20-G	70.3	4.7	6.7	0.24	0.060
20-H	82.8	5.5	6.6	0.23	0.058
21-O	0.0	-	6.7	0.50	0.131
21-A	12.0	0.7	6.8	0.33	0.085
21-B	33.5	2.0	7.0	0.25	0.063
21-C	57.0	3.4	6.8	0.28	0.071

[a]Samples 'O' are for initial conditions

TABLE G-3.13

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm[b]	CONCENTRATION OF BIOMASS (mg/ml)
22-O	0.0	-	7.2	0.43(1:3)	0.449
22-A	10.0	0.6	7.2	0.54	0.142
22-B	21.0	1.3	7.0	0.52	0.137
22-C	33.3	2.0	6.9	0.45	0.118
22-D	123.0	7.4	6.8	0.40	0.104
23-O	0.0	-	7.4	0.37(1:1)	0.192
23-A	11.8	0.4	7.4	0.54	0.142
23-B	23.0	0.8	7.4	0.49	0.129
23-C	33.0	1.2	7.4	0.41	0.107
23-D	44.5	1.6	7.3	0.38	0.099

[a]Samples 'O' are for initial conditions

[b]Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.14

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
23-E	58.6	2.1	7.2	0.29	0.074
23-F	72.5	2.6	7.0	0.29	0.074
23-G	82.5	3.0	6.9	0.38	0.099
23-H	102.5	3.7	6.9	0.50	0.131
23-I	153.0	5.5	6.9	0.52	0.137
24-O	0.0	-	7.0	0.56	0.148
24-A	0.8	0.04	6.9	0.55	0.145
24-B	13.3	0.7	7.1	0.43	0.112
24-C	24.5	1.3	7.1	0.33	0.085
24-D	34.5	1.8	7.1	0.26	0.066

[a]Samples 'O' are for initial conditions

TABLE G-3.15

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
24-E	46.0	2.5	7.0	0.19	0.047
24-F	60.1	3.2	6.9	0.19	0.047
24-G	73.0	3.9	6.9	0.20	0.049
24-H	82.0	4.4	6.8	0.20	0.049
25-O	0.0	-	6.7	0.38	0.099
25-A	0.8	-	6.7	0.38	0.099
25-B	13.3	-	6.6	0.31	0.079
25-C	24.5	-	6.6	0.28	0.070
25-D	34.5	-	6.6	0.24	0.060
25-E	46.0	-	6.6	0.35	0.090

[a]Samples 'O' are for initial conditions

TABLE G-3.16

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
25-F	60.1	—	6.6	0.36	0.093
25-G	82.5	—	6.6	0.45	0.118
25-H	92.5	—	6.6	0.43	0.112
25-I	104.0	—	6.6	0.38	0.099
26-O	0.0	—	7.3	0.44	0.115
26-A	5.0	0.4	7.2	0.35	0.090
26-B	16.3	1.2	7.2	0.42	0.109
26-C	25.3	1.8	7.2	0.50	0.131
26-D	37.3	2.7	7.4	0.50	0.131
26-E	48.5	3.5	7.3	0.48	0.126

[a]Samples 'O' are for initial conditions

TABLE G-3.17

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
26-F	55.3	4.0	7.3	0.47	0.123
26-G	67.3	4.9	7.3	0.42	0.109
26-H	76.6	5.5	7.3	0.43	0.112
27-O	0.0	-	7.1	0.48	0.126
27-A	5.0	0.3	7.0	0.44	0.115
27-B	16.3	0.9	6.9	0.38	0.099
27-C	25.3	1.4	6.9	0.40	0.104
27-D	37.3	2.0	6.9	0.44	0.115
27-E	48.5	2.6	6.9	0.43	0.114
27-F	55.3	3.0	6.9	0.45	0.118

[a] Samples 'O' are for initial conditions

TABLE G-3.18

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY[b] @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
27-G	67.3	3.6	7.0	0.46	0.120
27-H	83.2	4.4	6.9	0.44	0.115
28-O	0.0	-	6.7	0.58	0.153
28-A	5.0	0.3	6.7	0.58	0.153
28-B	16.3	1.0	6.7	0.35(1:1)	0.181
28-C	25.3	1.5	6.7	0.42(1:1)	0.219
28-D	37.3	2.2	6.8	0.35(1:1)	0.181
28-E	48.5	3.3	6.8	0.52	0.137
28-F	55.3	3.7	6.7	0.40	0.104
28-G	67.3	4.0	6.5	0.38	0.099

[a]Samples 'O' are for initial conditions

[b]Dilution ratio in parenthesis, (sample : medium diluent)

TABLE G-3.19

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
28-H	83.2	4.9	6.5	0.38	0.099
29-O	0.0	-	7.3	0.42	0.109
29-A	2.3	0.2	7.2	0.35	0.090
29-B	13.6	0.9	7.1	0.33	0.085
29-C	24.4	1.6	7.1	0.35	0.090
29-D	37.5	2.5	7.1	0.32	0.082
29-E	50.3	3.4	7.1	0.33	0.085
29-F	61.3	4.1	7.1	0.31	0.079

[a] Samples 'O' are for initial conditions

TABLE G-3.20

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE CHEMOSTAT SAMPLES

SAMPLE[a]	TIME FROM PUMP ACTIVATION (h)	NUMBER OF TURNOVERS, N_T	pH	OPTICAL DENSITY @ 550 nm	CONCENTRATION OF BIOMASS (mg/ml)
30-O	0.0	-	6.5	0.51	0.134
30-A	8.5	0.5	6.6	0.40	0.104
30-B	31.0	1.8	6.6	0.32	0.082
30-C	55.5	3.2	6.5	0.19	0.047
30-D	78.0	4.5	6.3	0.25	0.063
30-E	90.0	5.2	6.3	0.26	0.066

[a]Samples 'O' are for initial conditions

TABLE G-4

EXPERIMENTAL DATA FOR pH AND BIOMASS CONCENTRATION ANALYSIS OF THE BATCH SAMPLES

SAMPLE	pH	OPTICAL DENSITY[a] @ 550 nm	BIOMASS CONCENTRATION mg cells/ml
B-1	6.8	0.40(1:3)	0.416
B-2	6.3	0.42(1:3)	0.437
B-3	6.2	0.29	0.074
B-4	7.0	0.27(1:3)	0.274
B-5	6.4	0.15	0.359
B-6	6.3	0.58	0.153
B-7	6.4	0.38(1:3)	0.394

[a] dilution ratio in parenthesis, (sample:medium)

TABLE G-5.1

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 1, 2 & 4.

REACTION	SAMPLE OPTICAL DENSITY @ 495 nm				
TIME (min)	1-A	2-A	2-B	4-A	4-B
15	0.006	0.020	-	0.007	0.013
25	-	0.028	-	-	-
30	-	-	-	0.018	0.020
32	0.013	0.034	0.020	-	-
40	-	0.044	-	0.026	0.026
45	0.030	-	0.026	-	-
50	-	0.053	-	0.035	0.030
60	0.034	0.058	0.040	0.039	0.035
75	0.048	-	0.055	-	-
90	0.055	-	0.067	-	-
105	0.065	-	0.079	-	-
120	0.073	-	0.083	-	-

TABLE G-5.2

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 5 & 6.

REACTION TIME (min)	SAMPLE OPTICAL DENSITY @ 495 nm				
	5-A	6-A	6-B	6-C	6-D
15	0.007	0.003	0.009	0.006	0.005
30	0.012	0.012	0.014	0.008	0.008
40	0.014	0.017	0.016	0.010	0.013
50	0.016	0.022	0.017	0.011	0.014
60	0.017	0.029	0.023	0.015	0.017
100	0.025	-	0.032	0.022	0.026
110	0.029	-	0.035	0.024	0.028
120	0.031	-	0.038	0.029	0.031

TABLE G-5.3

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 7 & 9.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	7-E	9-A	9-C	9-D
15	-	0.009	0.014	0.011
30	-	0.017	0.021	0.016
40	-	0.023	0.027	0.021
50	-	0.025	0.035	0.030
60	-	0.031	0.040	0.032
90	0.017	-	-	0.047
110	-	-	-	0.056
120	0.023	-	-	0.075
150	0.029	-	-	-

TABLE G-5.4

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 10 & 11.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm				
	10-A	10-B	10-C	11-A	11-B
1	0.014	0.006	-	0.005	-
3	0.027	0.011	-	0.009	-
5	0.055	0.018	0.007	0.012	0.005
7	0.077	0.027	-	0.017	-
10	0.108	0.037	0.009	0.022	0.009
15	0.176	0.059	-	0.037	-
20	0.241	0.075	0.016	0.052	0.016
25	0.321	0.096	-	0.068	-
30	0.408	0.123	0.019	0.082	0.021
40	0.523	-	0.031	-	-
50	-	-	0.038	-	0.041
60	-	-	0.041	-	0.049

TABLE G-5.5

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION, Runs 13 & 15.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm		
	13-D	13-E	15-H
5	0.002	-	0.007
10	0.005	-	0.012
15	0.009	0.002	-
20	0.010	-	-
25	0.012	-	0.017
30	0.017	0.008	0.019
35	-	-	0.020
40	0.021	-	0.023
50	0.028	0.017	0.025
60	0.035	0.021	0.030
70	-	-	0.034
80	-	0.025	0.041
90	-	-	0.047
100	-	-	0.052

TABLE G-5.6

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION, Runs 16 & 17.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	16-D	16-H	17-B	17-E
15	0.006	-	-	-
35	0.012	0.008	0.008	-
50	0.015	-	0.011	0.005
60	-	-	0.015	-
70	0.022	-	0.019	-
80	-	0.017	0.021	0.008
90	0.026	0.020	0.023	-
100	0.029	0.023	0.025	0.009
120	0.032	0.026	-	0.010
140	-		-	0.012
160	-		-	0.015

TABLE G-5.7

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 19.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	19-B	19-D	19-F	19-H
5	0.005	0.007	-	-
7	0.007	0.009	-	0.003
10	0.011	0.013	-	-
15	0.019	0.017	0.008	0.007
20	0.020	0.023	-	0.009
25	0.023	0.029	-	0.011
30	0.027	0.034	0.014	-
35	0.029	0.039	0.017	0.013
40	0.037	0.047	0.020	0.018
50	0.044	0.059	0.024	0.019
60	-	-	0.031	0.024
70	-	-	0.036	-
80	-	-	-	-
90	-	-	0.051	-

TABLE G-5.8

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 20 & 21.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm		
	20-F	20-H	21-C
3	-	0.012	-
5	-	0.019	-
7	0.005	0.021	-
10	-	0.024	0.009
11	0.011	-	-
15	0.017	0.035	-
20	0.026	0.049	0.014
25	0.038	0.057	0.018
30	0.043	0.073	0.022
35	0.046	-	0.029
40	0.064	-	0.032
50	0.082	-	0.042
60	0.103	-	0.052

TABLE G-5.9

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 23.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm 23-E	23-G	23-H	23-I
5	0.003	0.003	0.009	0.006
7	-	-	0.013	-
9	0.006	0.005	0.017	0.009
11	-	-	0.025	-
15	0.008	0.010	0.029	0.012
20	0.016	0.014	0.043	0.017
25	0.018	0.017	0.058	0.020
30	0.022	0.020	0.063	0.022
35	0.024	0.025	-	0.024
40	0.031	0.028	-	0.029
50	0.039	0.034	-	-
60	0.042	0.041	-	-
70	-	0.049	-	-
80	-	0.056	-	-
90	-	0.064	-	-

TABLE G-5.10

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 24.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm		
	24-D	24-F	24-H
5	-	-	0.005
7	-	0.008	-
10	-	-	0.011
11	-	0.012	-
15	0.012	0.016	0.015
20	0.014	0.020	0.019
25	-	0.025	0.023
30	0.019	-	0.025
35	0.023	0.030	0.029
40	0.026	-	0.033
50	0.035	-	0.039
60	0.044	-	-
70	0.053	-	-

TABLE G-5.11

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 25.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm				
	25-B	25-D	25-F	25-G	25-I
3	-	-	0.009	0.010	0.012
5	-	0.006	0.012	0.014	0.015
7	-	-	0.013	0.016	0.020
10	0.005	-	0.015	-	0.024
11	-	-	-	0.030	-
15	-	0.010	0.019	0.040	0.034
20	0.012	-	0.024	0.065	0.055
25	0.016	0.012	0.029	0.075	0.061
30	0.022	0.014	0.035	0.097	0.069
35	-	0.016	0.040	-	0.089
40	0.025	0.018	0.044	-	-
50	0.031	0.023	0.052	-	-
60	0.041	0.025	0.059	-	-
70	0.045	0.029		-	-
80	-	0.034		-	-
90	-	0.036		-	-
100	-	0.039		-	-
120	-	0.045		-	-

TABLE G-5.12

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 26.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm		
	26-F	26-G	26-H
3	0.007	-	-
5	-	0.008	0.015
7	-	-	0.019
9	0.030	0.012	0.022
11	0.037	0.015	0.033
15	0.050	0.017	0.043
20	0.073	0.023	0.053
25	0.097	0.029	0.064
30	0.118	0.033	0.073
35	-	0.040	0.091
40	-	0.044	-
50	-	0.051	-
60	-	0.054	-

TABLE G-5.13

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION Run 27 & 28-A.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	27-F	27-G	27-H	28-A
3	0.010	0.008	0.013	-
5	0.018	0.012	0.018	0.008
7	0.024	0.018	0.024	-
9	0.029	0.024	0.033	0.012
11	0.035	0.031	0.046	0.014
13	-	-	-	0.016
15	0.043	0.046	0.060	0.018
20	0.061	0.068	-	0.024
25	0.073	-	-	0.027
30	-	-	-	0.036
35	-	-	-	0.039

TABLE G-5.14

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Run 28, Samples B,D,F,G,H.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm				
	28-B	28-D	28-F	28-G	28-H
3	-	-	0.009	-	-
5	0.009	0.014	0.012	0.015	-
7	0.010	0.018	0.015	-	-
9	0.012	0.022	0.023	0.019	0.011
11	-	0.028	0.030	-	-
13	0.019	0.032	0.039	-	-
15	-	0.037	-	0.027	0.020
20	0.029	0.050	0.060	0.032	0.032
25	0.036	0.057	0.074	0.043	0.039
30	0.044	0.078	-	0.047	0.045
35	0.045	-	-	0.050	-
40	-	-	-	0.062	-
50	-	-	-	-	-

TABLE G-5.15

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Runs 29 & 30.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm				
	29-D	29-F	30-C	30-D	30-E
3	0.004	-	-	-	-
5	0.010	0.008	0.002	0.004	-
7	0.015	-	-	-	-
9	0.020	0.011	-	-	-
11	0.026	-	-	-	-
13	0.029	0.016	-	-	-
15	0.034	0.019	-	0.009	0.005
20	0.052	0.028	0.007	0.010	-
25	0.064	0.035	-	0.012	0.009
30	0.076	0.043	0.011	0.016	0.010
35	-	0.048	-	0.017	0.013
40	-	-	0.017	0.023	0.018
50	-	-	0.018	0.026	0.023
60	-	-	0.026	0.028	0.028
70	-	-	0.033	-	0.030
80	-	-	0.039	-	0.038
90	-	-	0.041	-	0.042

TABLE G-5.16

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Batch Runs B-1 to B-4.

REACTION TIME (min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	B-1	B-2	B-3	B-4
3	0.014	-	-	0.020
5	0.027	-	-	0.031
7	0.045	0.005	-	0.047
9	0.065	-	0.006	0.069
11	0.080	0.011	-	0.084
13	0.095	-	-	-
15	-	0.015	0.010	-
20	-	0.024	0.013	-
25	-	0.030	-	-
30	-	0.038	-	-
35	-	0.047	-	-
40	-	0.051	0.028	-
50	-	0.062	0.038	-
60	-	0.083	0.041	-

TABLE G-5.17

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION. Batch Runs B-5 to B-7

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm		
	B-5	B-6	B-7
3	-	0.003	-
5	-	0.007	0.004
7	-	-	-
9	-	-	-
11	-	0.015	0.011
15	-	0.020	0.014
20	-	0.023	0.020
25	-	0.027	0.026
30	-	0.033	0.032
40	0.015	0.040	-
50	0.020	-	-
60	0.027	-	-
70	0.031	-	-
80	0.036	-	-
90	0.043	-	-
100	0.045	-	-
110	0.050	-	-
120	0.056	-	-

TABLE G-5.18

EXPERIMENTAL DATA FOR THE DETERMINATION OF CITRULLINE PRODUCTION, Reaction Mixture at 37°C.

REACTION TIME(min)	SAMPLE OPTICAL DENSITY @ 495 nm			
	30-C	30-D	30-E	B-2
3	-	-	-	0.007
5	-	-	0.010	0.014
7	-	-	-	0.020
9	-	-	0.012	0.028
11	-	-	-	0.038
13	-	-	-	0.040
15	0.005	0.007	0.022	0.048
20	-	0.010	0.031	0.069
25	-	0.017	0.035	-
30	0.011	0.021	0.040	-
40	0.017	0.029	0.053	-
50	0.019	0.035	0.063	-
60	0.023	0.040	0.088	-
70	0.028	0.047	-	-
80	0.030	0.054	-	-
90	0.033	0.068	-	-
100	0.037	0.073	-	-
110	0.043	0.082	-	-

APPENDIX H

EXPERIMENTAL RESULTS

TABLE H-1.1

SUMMARY OF LINEAR REGRESSION RESULTS FOR THE CITRULLINE ASSAY

SAMPLE	REGRESSION COEFFICIENT	SLOPE ($x10^{-4}$ OD/min)	INTERCEPT (OD)
1-A	0.992	6.57	-0.004
2-A	0.994	8.97	0.006
2-B	0.992	7.81	-0.006
4-A	0.995	7.40	-0.004
4-B	0.998	4.95	0.006
5-A	0.995	2.16	0.005
6-C	0.991	2.11	0.002
6-D	0.997	2.42	0.002
7-E	-	-	-
9-C	0.995	5.96	0.002
9-D	0.998	4.94	0.003
10-B	0.998	39.80	-0.001
10-C	0.989	6.62	0.003
11-A	0.996	27.00	-0.001
11-B	0.996	8.00	0.0001
13-D	0.992	4.48	0.005
13-E	0.996	5.84	-0.001
14-F	-	-	-
15-H	0.991	3.29	-0.001

TABLE H-1.2

SUMMARY OF LINEAR REGRESSION RESULTS FOR THE CITRULLINE ASSAY

SAMPLE	REGRESSION COEFFICIENT	SLOPE ($x10^{-4}$ OD/min)	INTERCEPT (OD)
16-D	0.995	2.54	0.003
16-H	0.998	2.09	0.001
17-B	0.991	2.75	-0.002
17-E	0.984	0.84	0.001
19-F	0.994	5.71	-0.003
19-H	0.990	3.81	0.001
20-F	0.999	18.53	-0.010
20-H	0.994	21.61	0.005
21-C	0.994	8.92	-0.003
23-H	0.994	22.63	-0.002
23-I	0.997	6.55	0.003
24-F	0.994	7.90	0.004
24-H	0.995	7.34	0.003
25-F	0.998	9.03	0.007
25-G	0.994	32.67	-0.004
26-G	0.997	9.92	0.003
26-H	0.995	24.52	0.003
27-G	0.994	35.57	-0.006
27-H	0.992	41.07	-0.002

TABLE H-1.3

SUMMARY OF LINEAR REGRESSION RESULTS FOR THE CITRULLINE ASSAY

SAMPLE	REGRESSION COEFFICIENT	SLOPE ($\times 10^{-4}$ OD/min)	INTERCEPT (OD)
28-G	0.993	13.06	0.008
28-H	0.994	9.57	-0.011
29-D	0.998	27.00	-0.004
29-F	0.996	14.24	-0.001
30-C	0.991	4.90	-0.003
30-D	0.990	4.68	0.002
30-E	0.995	5.08	-0.004
30-C$^{\mp}$	0.996	3.79	0.0001
30-D^{+}	0.994	7.65	-0.004
30-E$^{\mp}$	0.991	13.43	0.001
B-1	0.998	83.43	-0.012
B-2^{+}	0.997	35.89	-0.004
B-2	0.997	14.26	-0.005
B-3	0.996	7.25	-0.001
B-4	0.994	83.00	-0.008
B-5	0.996	5.04	-0.004
B-6	0.994	10.30	0.003
B-7	0.997	11.78	-0.003

$^{+}$ 37°C reaction temperature

TABLE H-2.1

SUMMARY OF RESULTS FOR THE DETERMINATION OF ARGININE DEIMINASE ACTIVITY

SAMPLE	PROTEIN CONCENTRATION mg/ml	PROTEIN MASS $x10^{-2}$mg	ACTIVITY $x10^{-4}$units	SPECIFIC ACTIVITY units/mg p
1-A	0.149	0.490	1.91	0.039
2-A	0.149	0.490	2.61	0.053
2-B	0.111	0.368	2.27	0.062
4-A	0.386	2.895	2.15	0.007
4-B	0.237	1.782	1.44	0.008
5-A	0.208	1.559	0.63	0.004
6-C	0.215	1.615	0.61	0.004
6-D	0.149	1.114	0.70	0.006
7-E	0.200	1.336	0.58	0.004
9-C	0.312	2.079	1.73	0.008
9-D	0.401	2.673	1.44	0.005
10-B	0.483	3.620	11.57	0.032
10-C	0.111	0.835	1.92	0.023

TABLE H-2.2

SUMMARY OF RESULTS FOR THE DETERMINATION OF ARGININE DEIMINASE ACTIVITY

SAMPLE	PROTEIN CONCENTRATION mg/ml	PROTEIN MASS $x10^{-2}$mg	ACTIVITY $x10^{-4}$units	SPECIFIC ACTIVITY units/mg p
11-A	0.156	1.169	7.85	0.067
11-B	0.089	0.668	2.33	0.035
13-D	0.178	1.336	1.30	0.010
13-E	0.104	0.780	1.70	0.022
14-F	-	-	-	-
15-H	0.089	0.668	0.96	0.014
16-D	0.148	1.114	0.74	0.007
16-H	0.193	1.448	0.61	0.004
17-B	0.112	0.835	0.80	0.010
17-E	0.119	0.891	0.24	0.003
18-G	-	-	-	-
19-F	0.207	1.555	1.66	0.011
19-H	0.048	0.362	1.11	0.031

TABLE H-2.3

SUMMARY OF RESULTS FOR THE DETERMINATION OF ARGININE DEIMINASE ACTIVITY

SAMPLE	PROTEIN CONCENTRATION mg/ml	PROTEIN MASS $\times 10^{-2}$ mg	ACTIVITY $\times 10^{-4}$ units	SPECIFIC ACTIVITY units/mg p
20-F	0.119	0.891	5.39	0.060
20-H	0.333	2.500	6.28	0.025
21-C	0.111	0.835	2.59	0.031
23-H	0.134	1.000	6.58	0.066
23-I	0.042	0.309	1.90	0.064
24-F	0.097	0.724	2.30	0.032
24-H	0.333	2.501	2.13	0.009
25-F	0.064	0.480	2.63	0.054
25-G	0.215	1.615	9.50	0.059
26-G	0.047	0.356	2.88	0.032
26-H	0.119	0.889	7.13	0.080
27-G	0.170	1.278	10.34	0.081
27-H	0.191	1.433	11.94	0.083

TABLE H-2.4

SUMMARY OF RESULTS FOR THE DETERMINATION OF ARGININE DEIMINASE ACTIVITY

SAMPLE	PROTEIN CONCENTRATION mg/ml	PROTEIN MASS $\times 10^{-2}$ mg	ACTIVITY $\times 10^{-4}$ units	SPECIFIC ACTIVITY units/mg p
28-G	0.085	0.639	3.80	0.059
28-H	0.059	0.444	2.78	0.063
29-D	0.215	1.611	7.85	0.049
29-F	0.088	0.667	4.14	0.062
30-C	0.074	0.556	1.43	0.026
30-D	0.111	0.833	1.36	0.016
30-E	0.074	0.556	1.47	0.027
30-C+	0.037	0.278	1.10	0.040
30-D+	0.081	0.611	2.22	0.036
30-E+	0.074	0.556	3.90	0.070
B-1	0.348	2.611	24.25	0.093
B-2+	0.119	0.889	10.43	0.117

+ 37°C reaction temperature

TABLE H-2.5

SUMMARY OF RESULTS FOR THE DETERMINATION OF ARGININE DEIMINASE ACTIVITY

SAMPLE	PROTEIN CONCENTRATION mg/ml	PROTEIN MASS $x10^{-2}$mg	ACTIVITY $x10^{-4}$units	SPECIFIC ACTIVITY units/mg p
B-2	0.119	0.889	4.15	0.047
B-3	0.156	1.167	2.11	0.018
B-4	0.281	2.111	24.13	0.114
B-5	0.148	1.111	1.46	0.013
B-6	0.237	1.778	2.99	0.017
B-7	0.252	1.889	3.42	0.018

FIGURE H-1

<u>SEMI-LOG PLOT OF BIOMASS CONCENTRATION AS A FUNCTION OF FERMENTATION TIME</u>
Runs with variations in dilution rates:
Run 6 to 20, 23, 24 and 25.

* dashed line represents the theoretical wash-out plot for that dilution rate.

FIGURE H-1.1

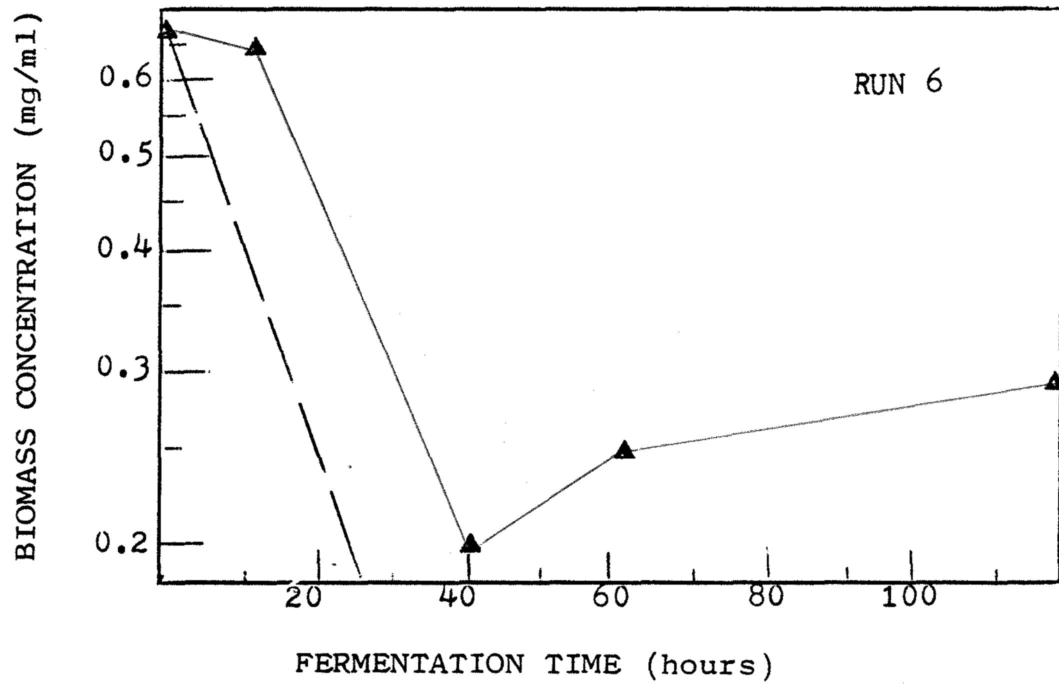


FIGURE H-1.2

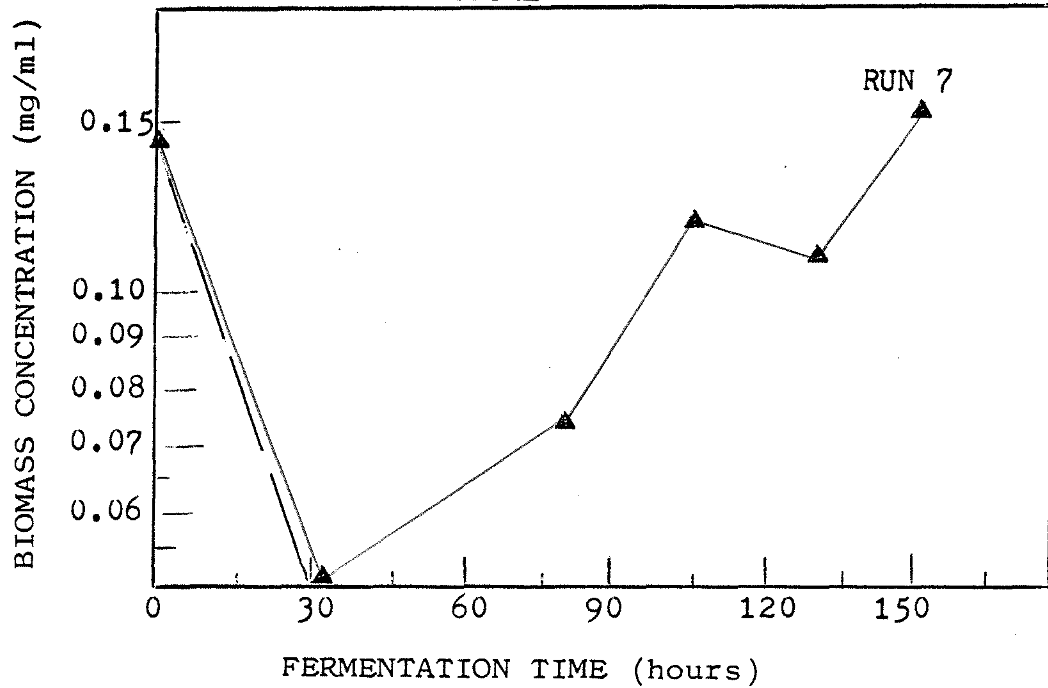


FIGURE H-1.3

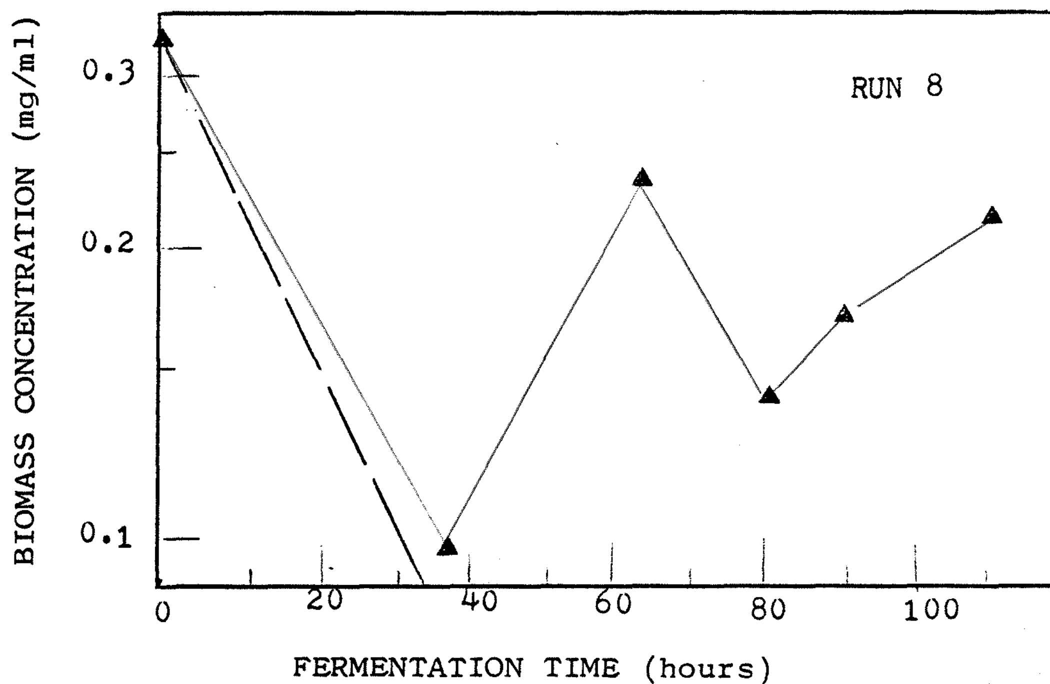


FIGURE H-1.4

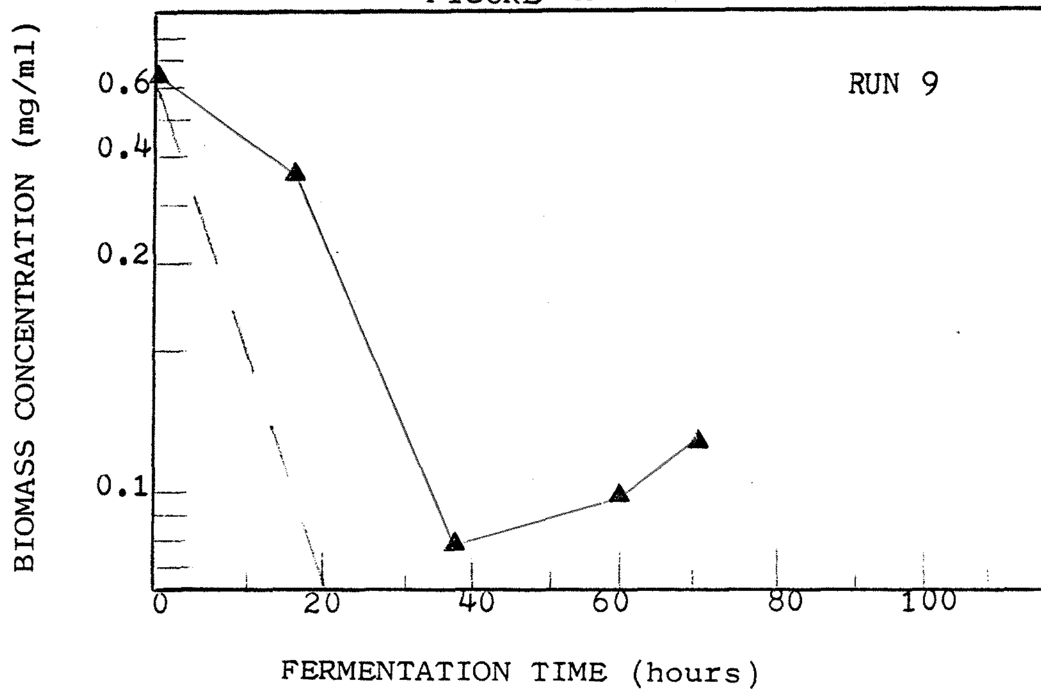


FIGURE H-1.5

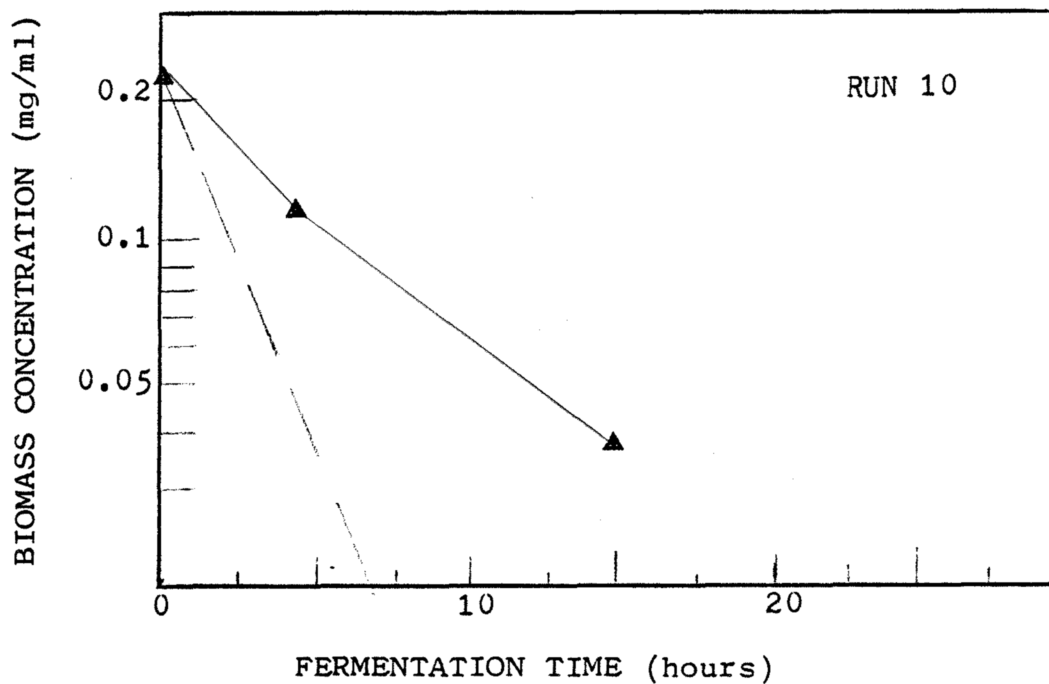


FIGURE H-1.6

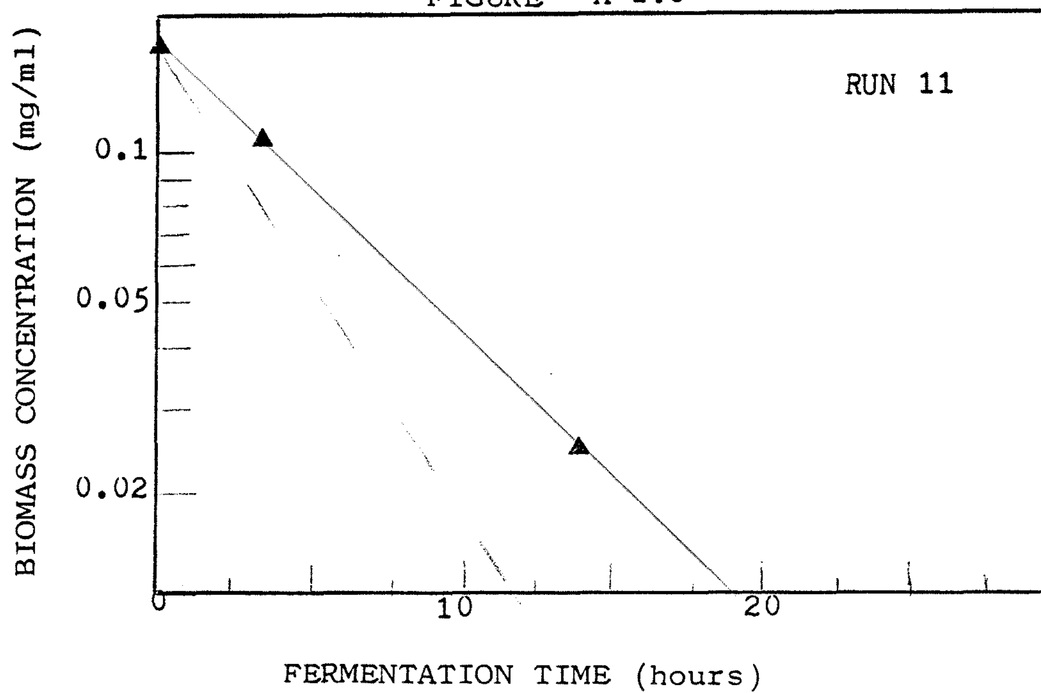


FIGURE H-1.7

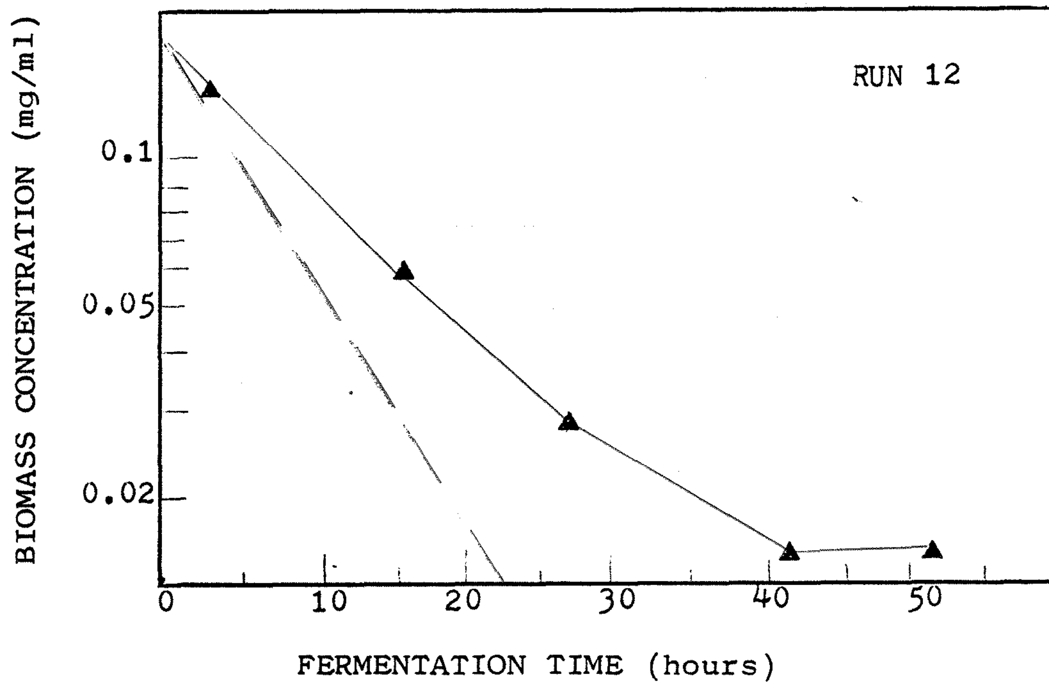


FIGURE H-1.8

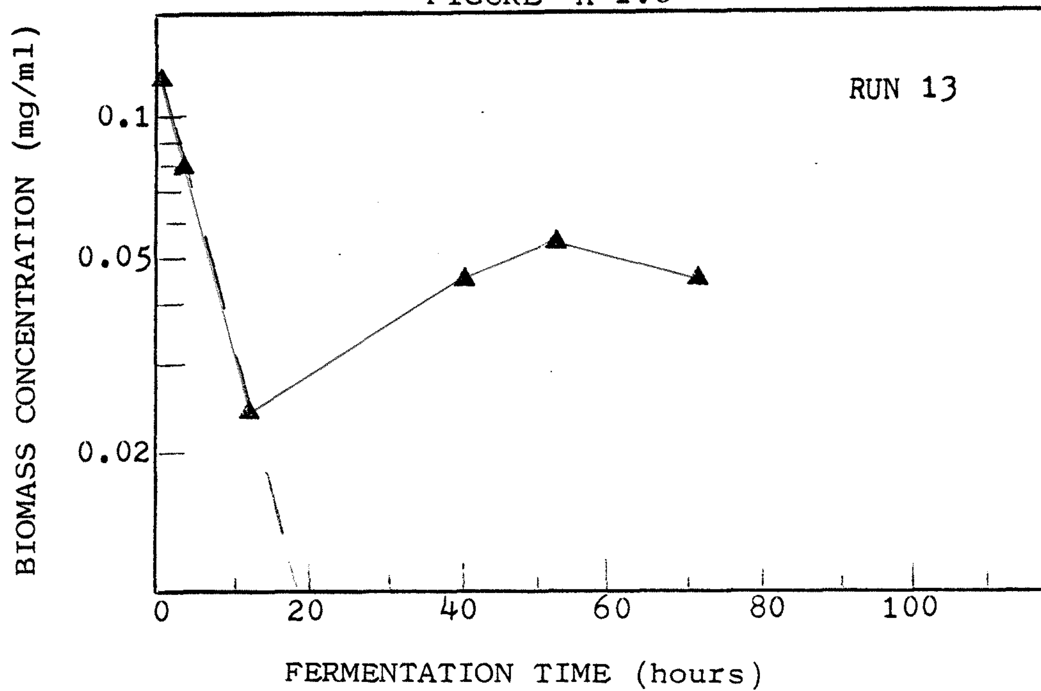


FIGURE H-1.9

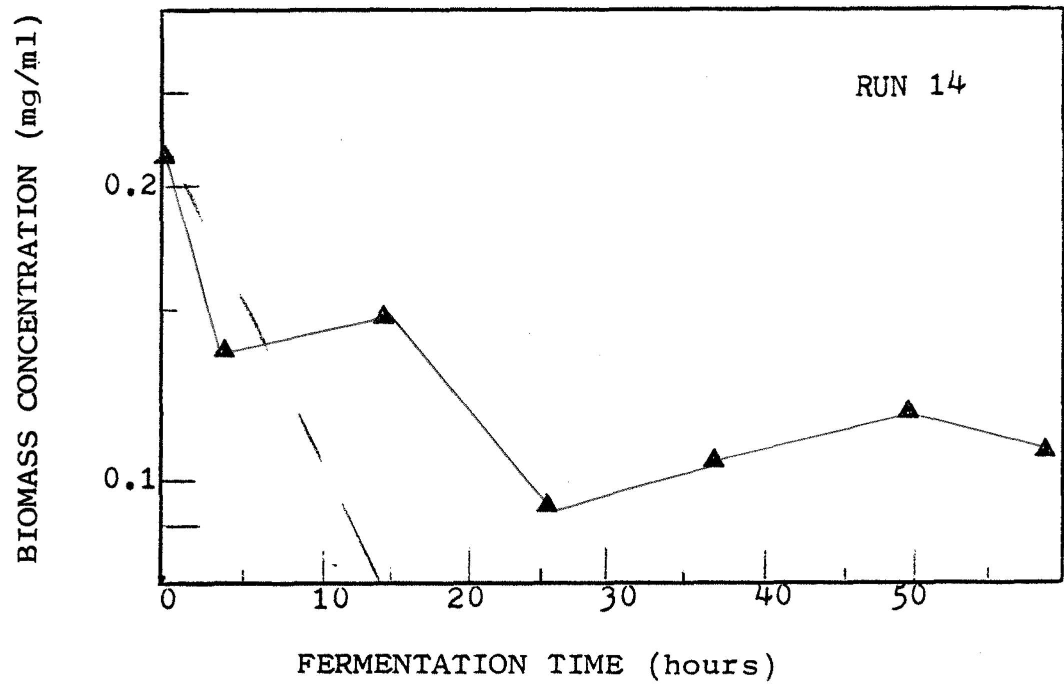


FIGURE H-1.10

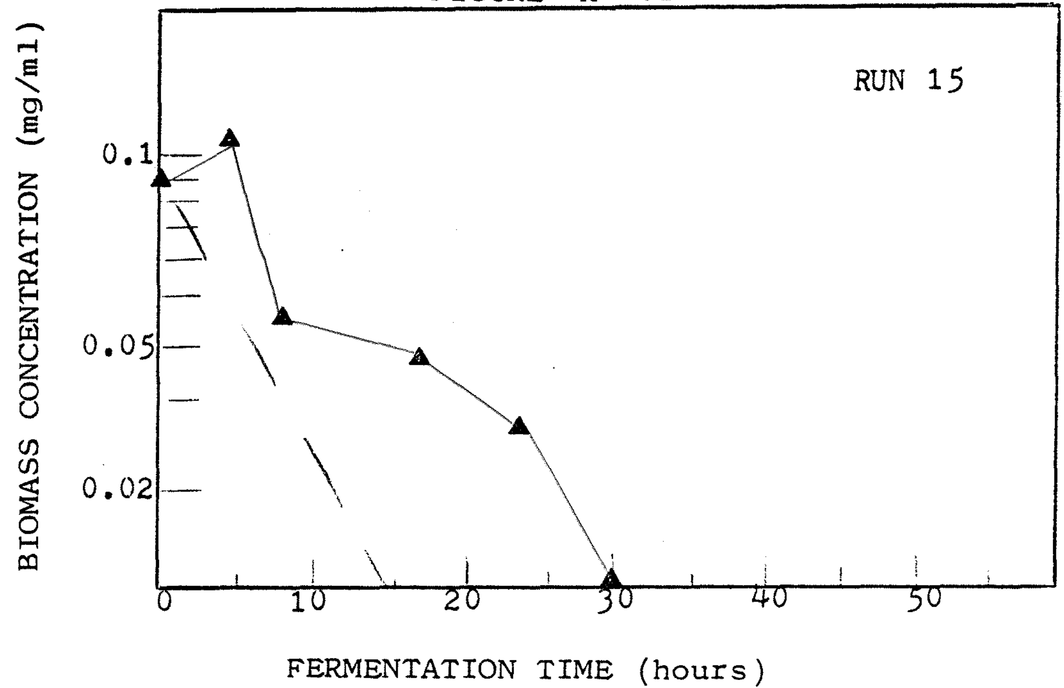


FIGURE H-1.11

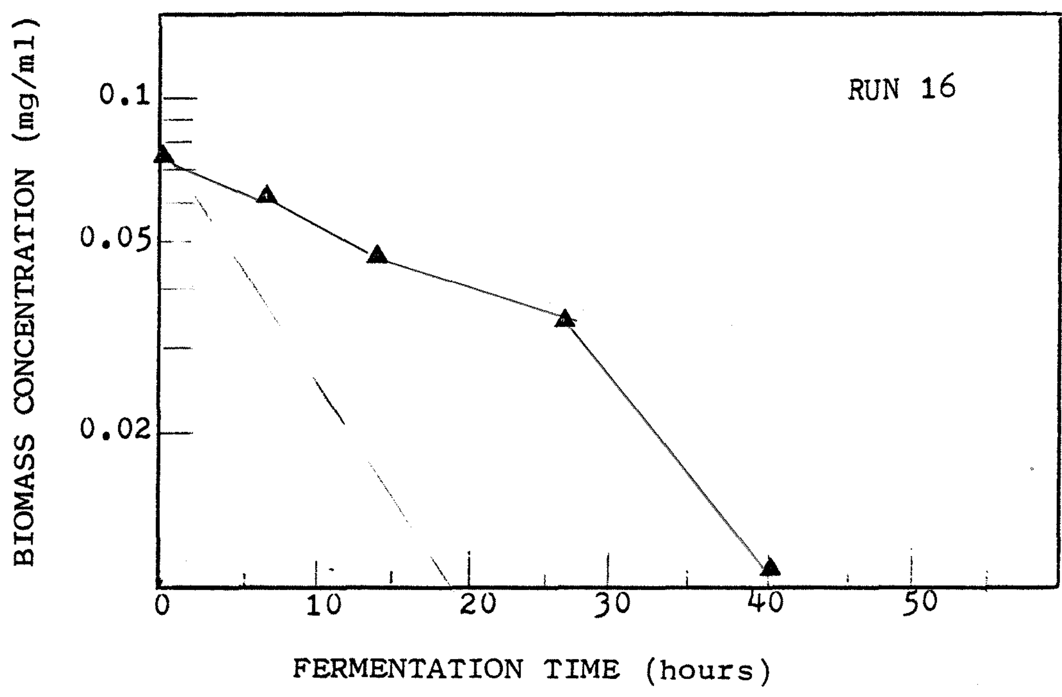


FIGURE H-1.12

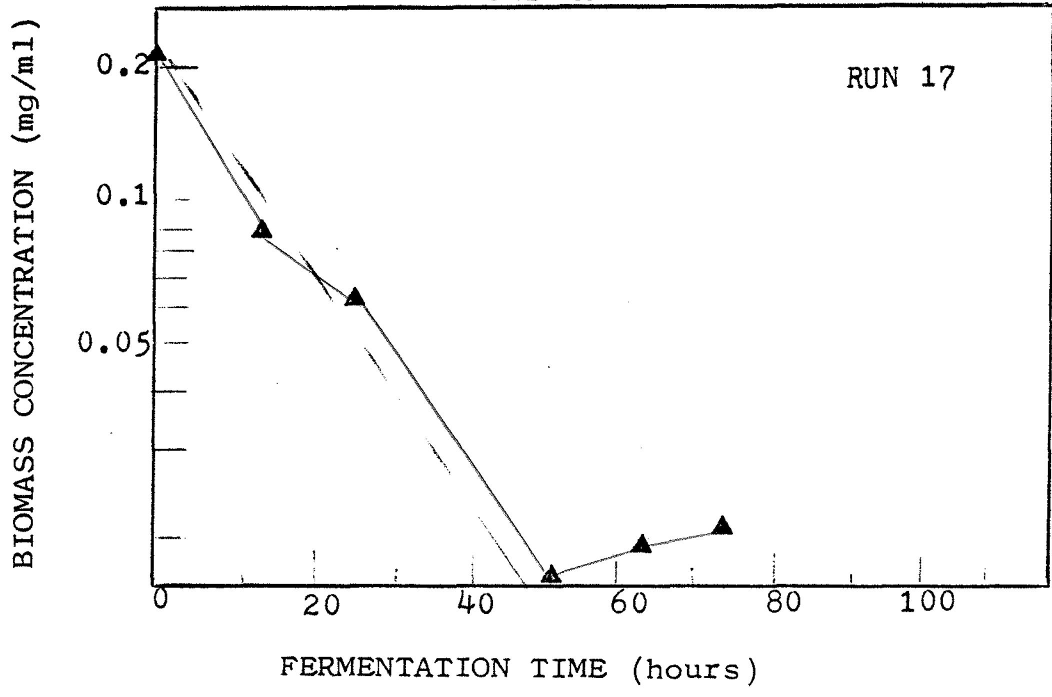


FIGURE H-1.13

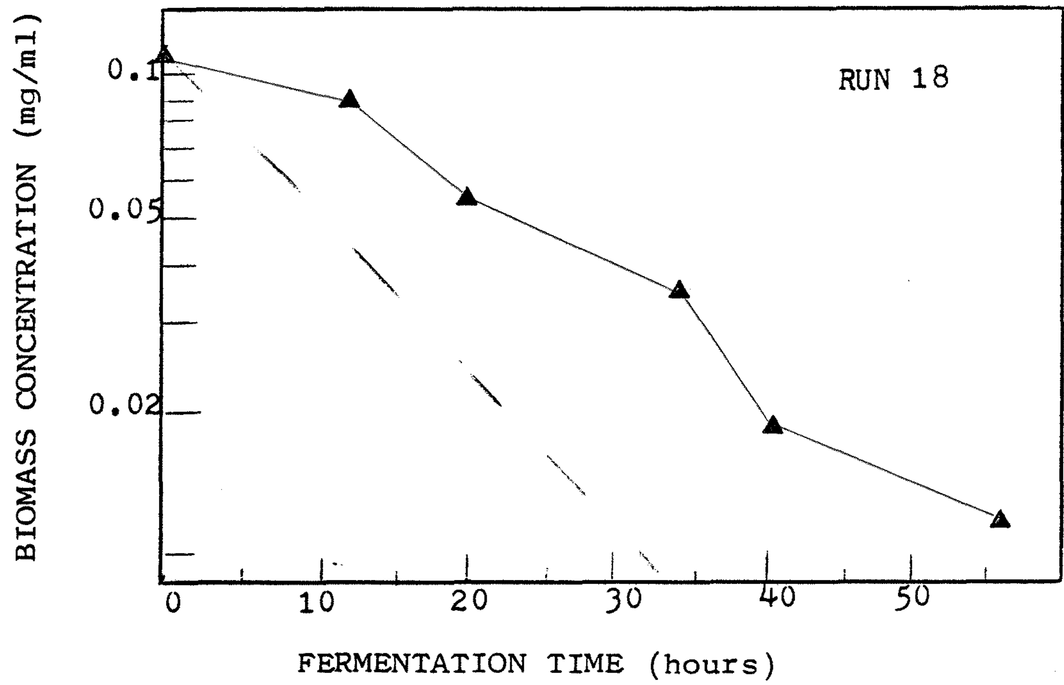


FIGURE H-1.14

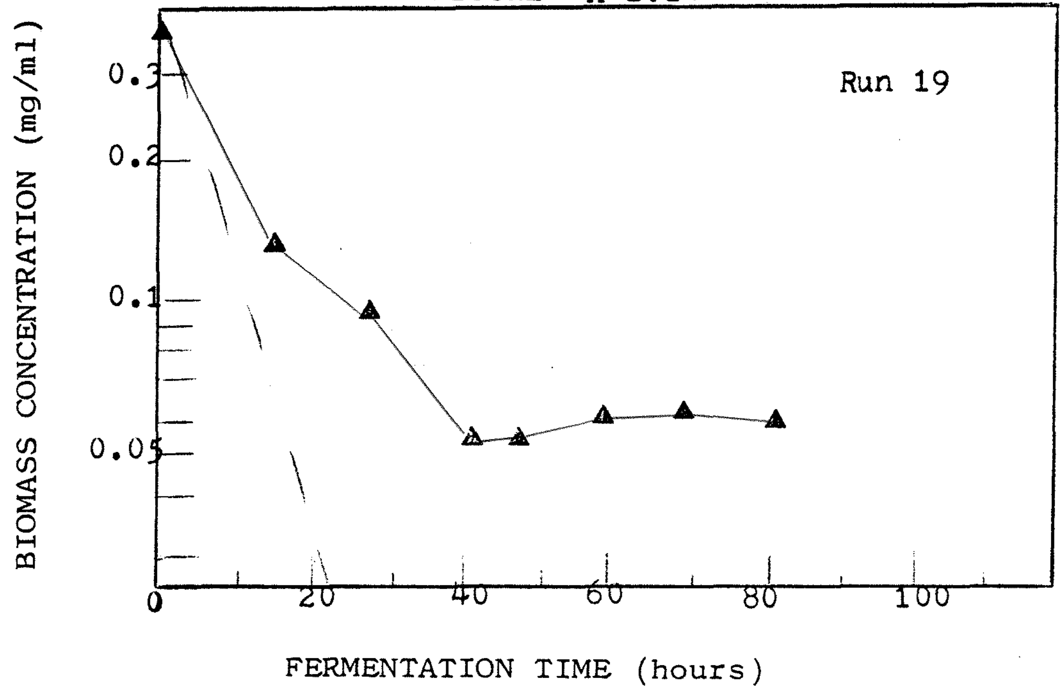


FIGURE H-1.15

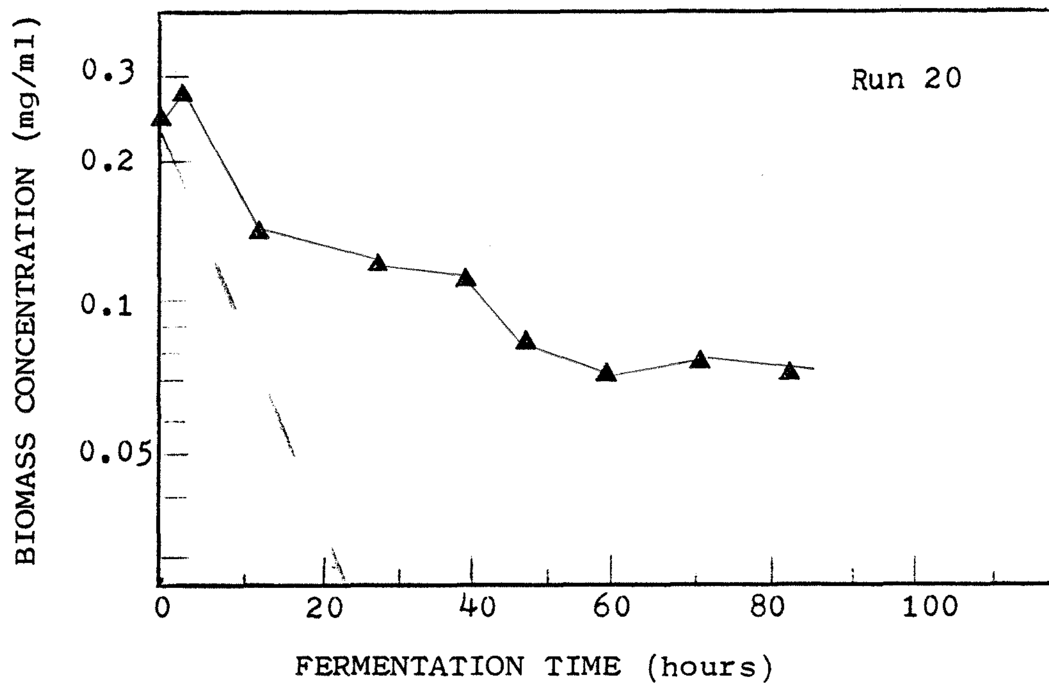


FIGURE H-1.16

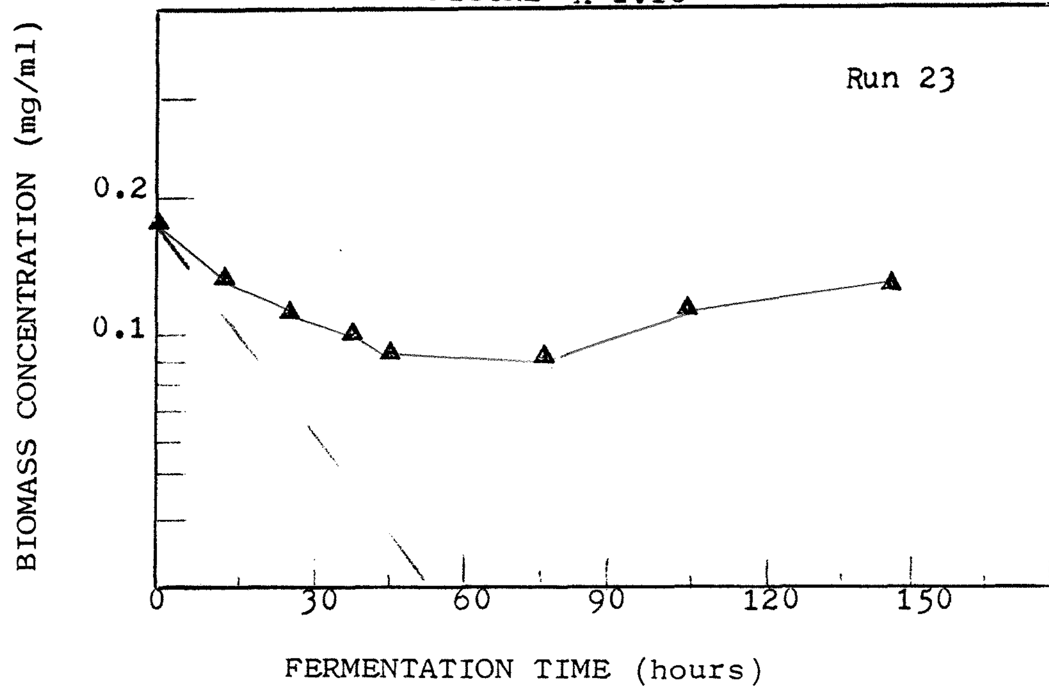


FIGURE H-1.17

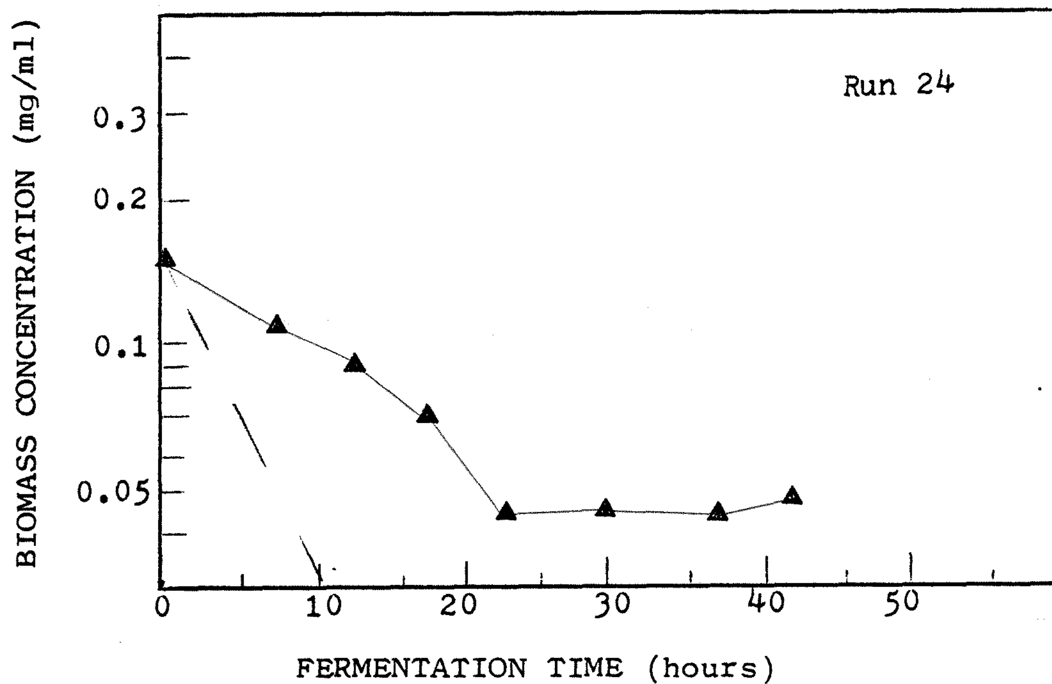


FIGURE H-1.18

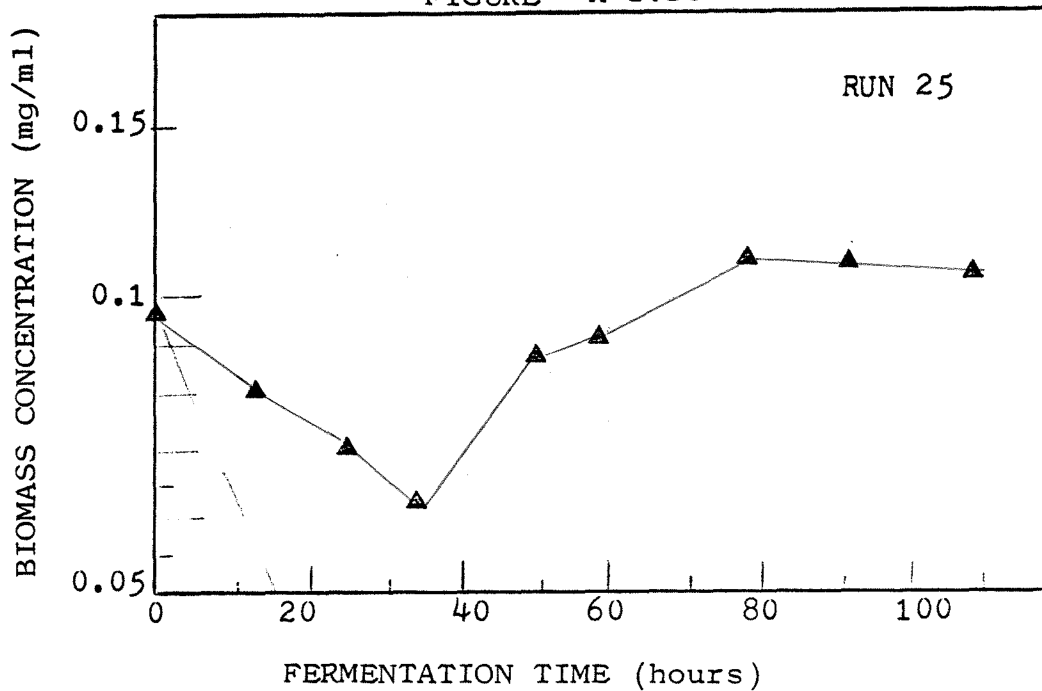


FIGURE H-2

PLOT OF OPTICAL DENSITY AS A FUNCTION OF REACTION TIME FOR THE CITRULLINE ASSAY.
Chemostat Runs 1 to 30, Batch Runs B-1 to B-7.

FIGURE H-2.1

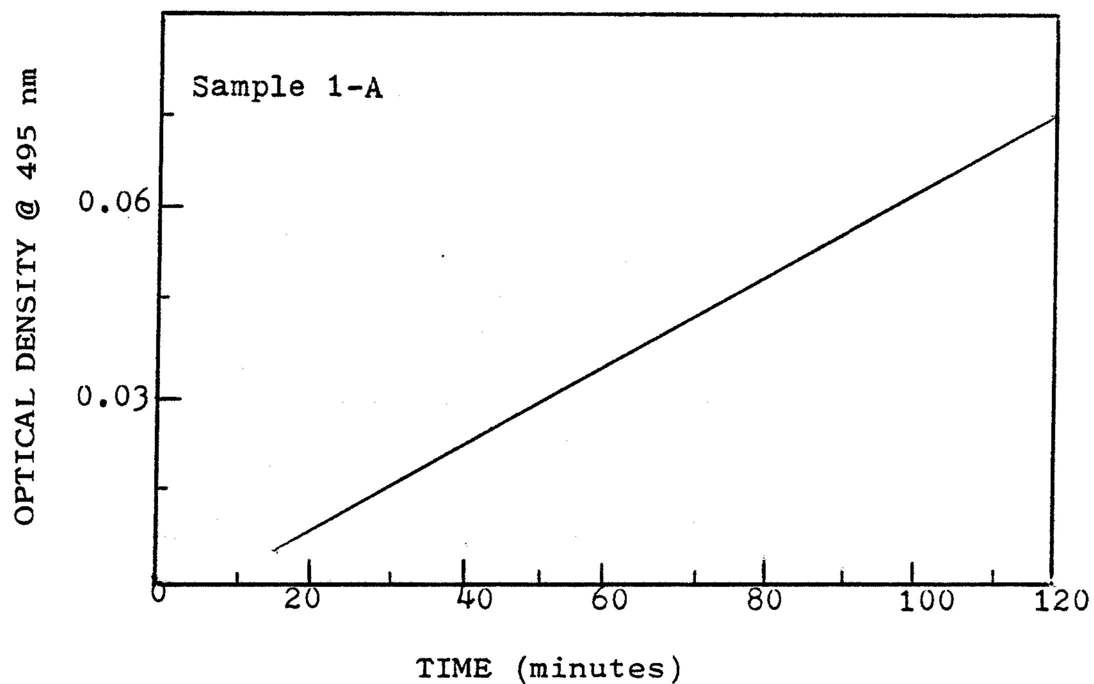


FIGURE H-2.2

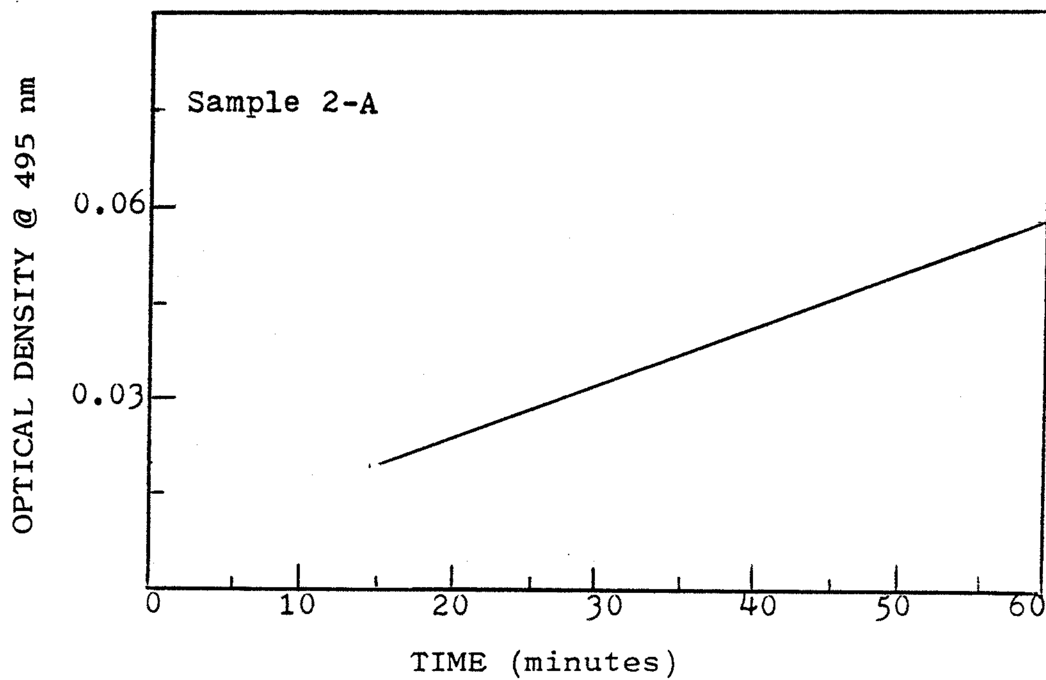


FIGURE H-2.3

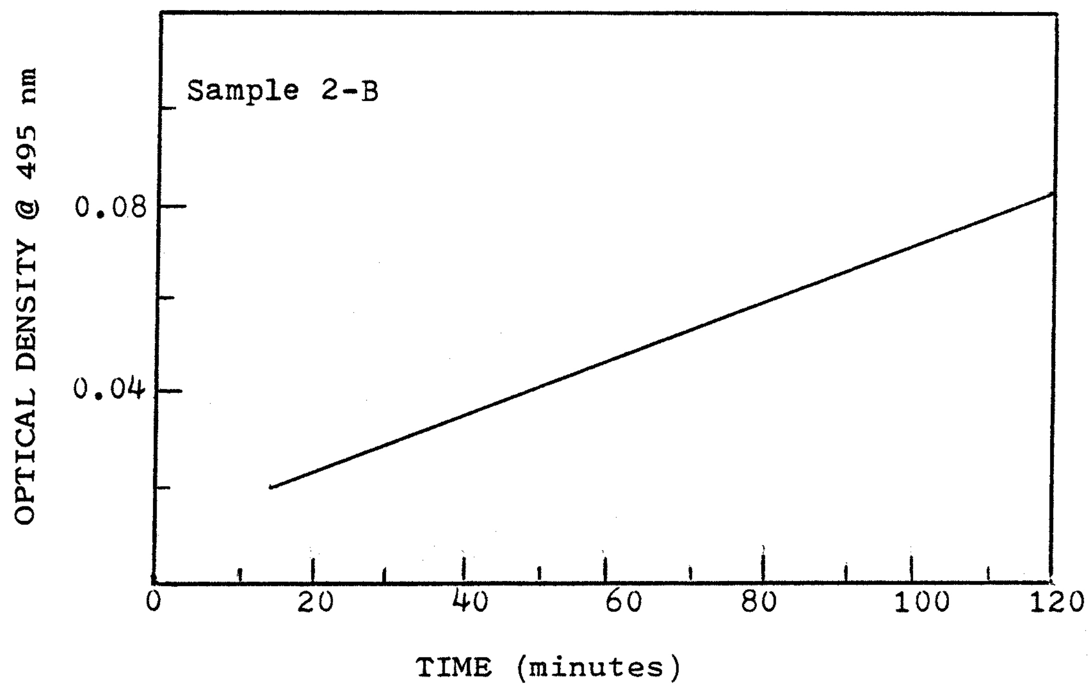


FIGURE H-2.4

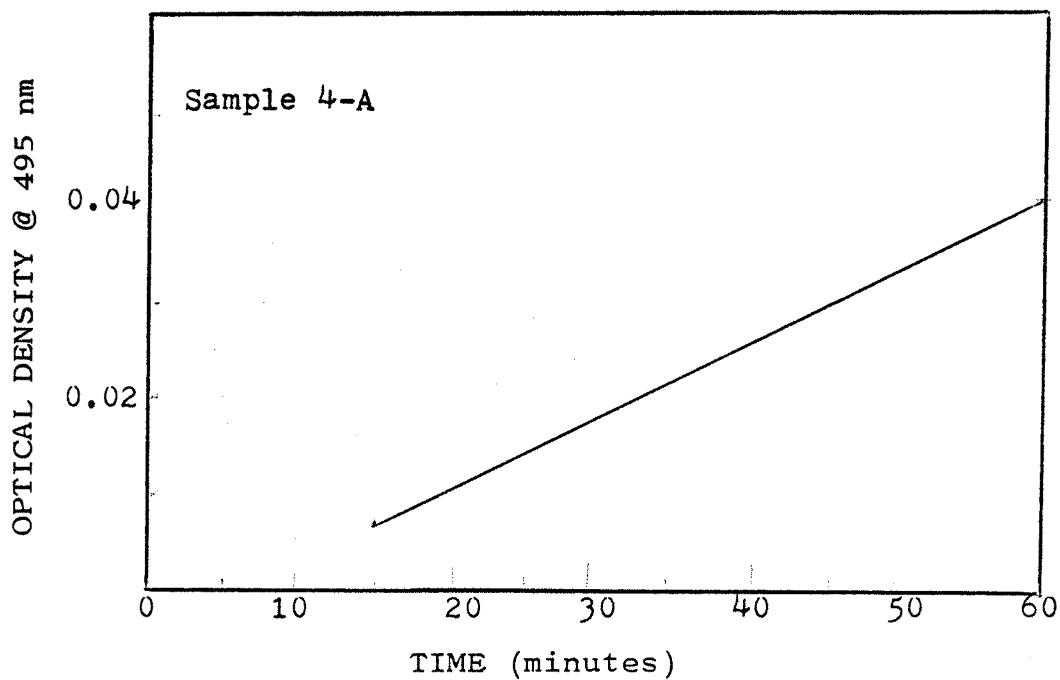


FIGURE H-2.5

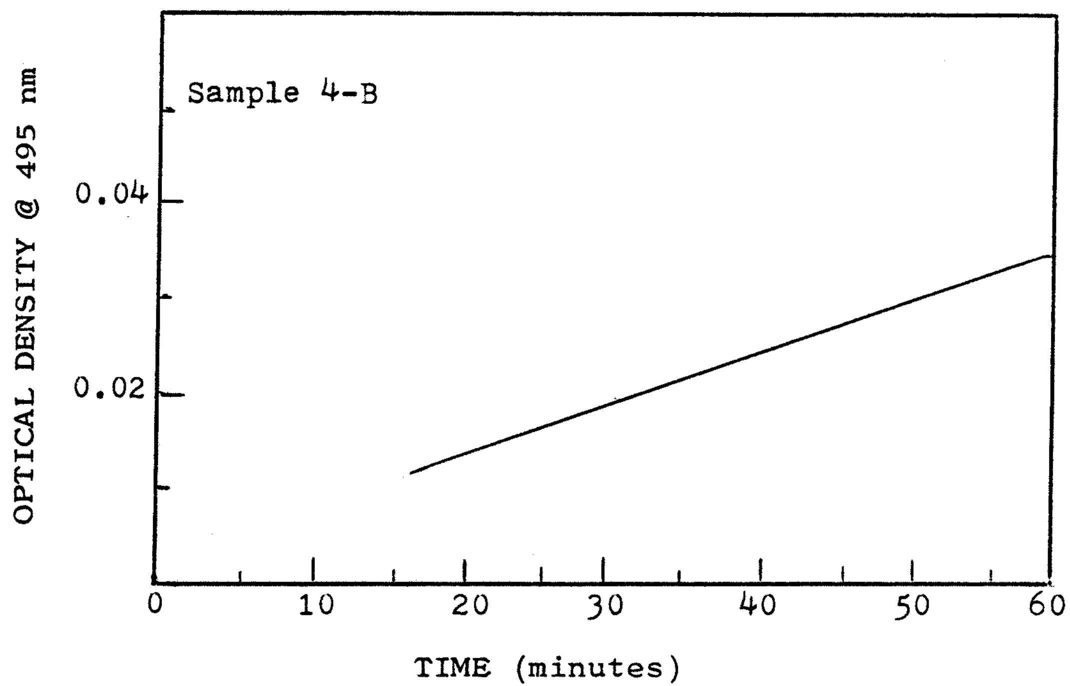


FIGURE H-2.6

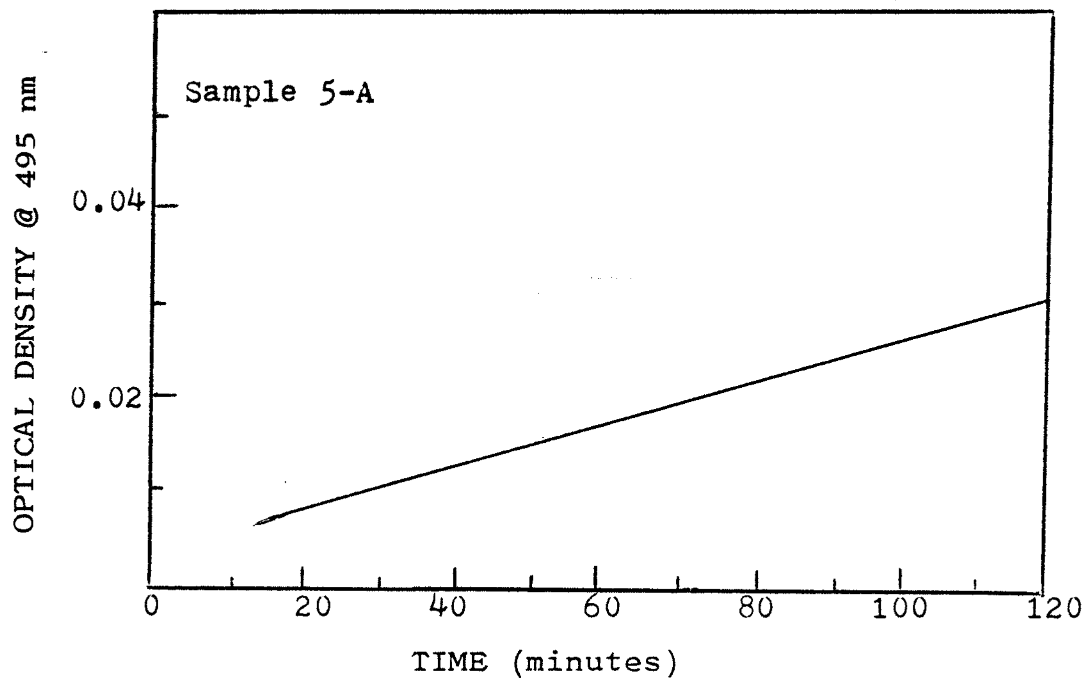


FIGURE H-2.7

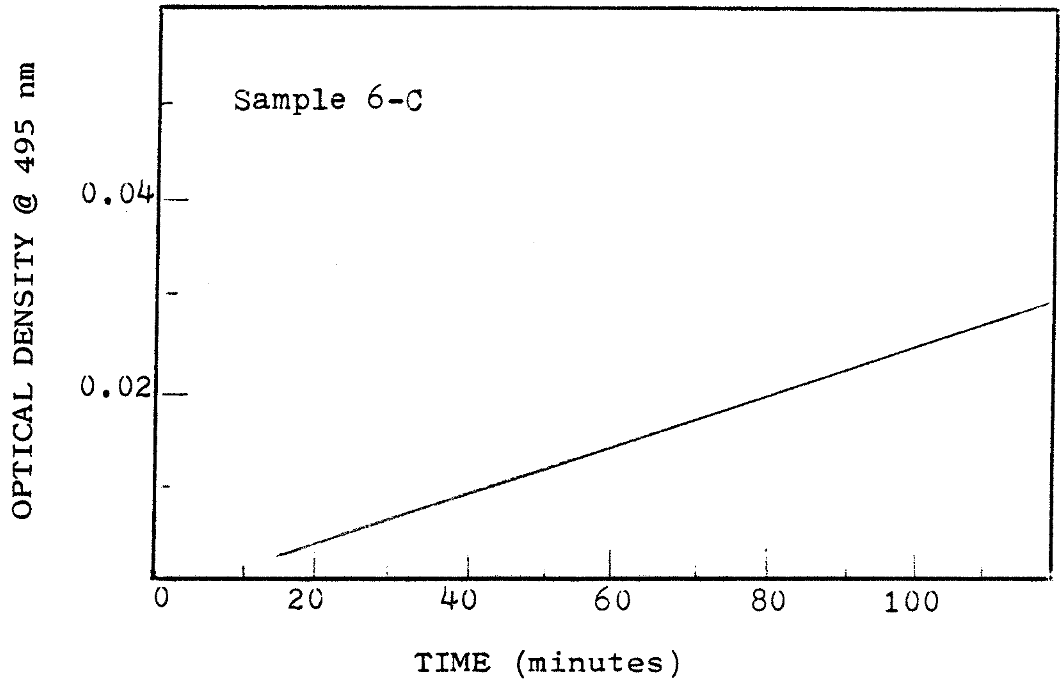


FIGURE H-2.8

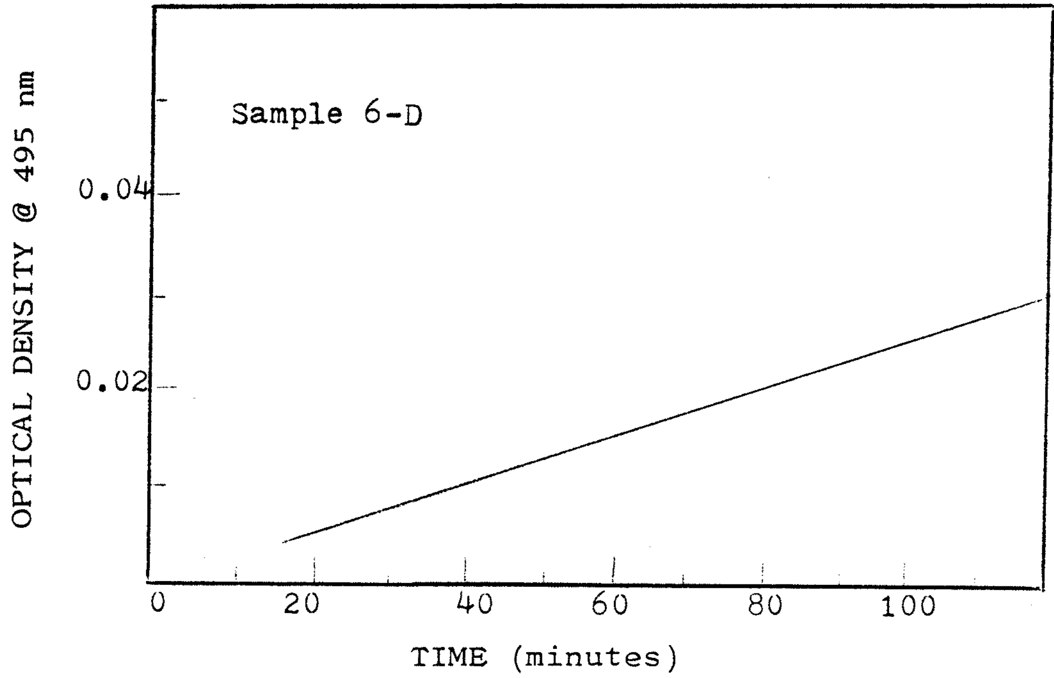


FIGURE H-2.9

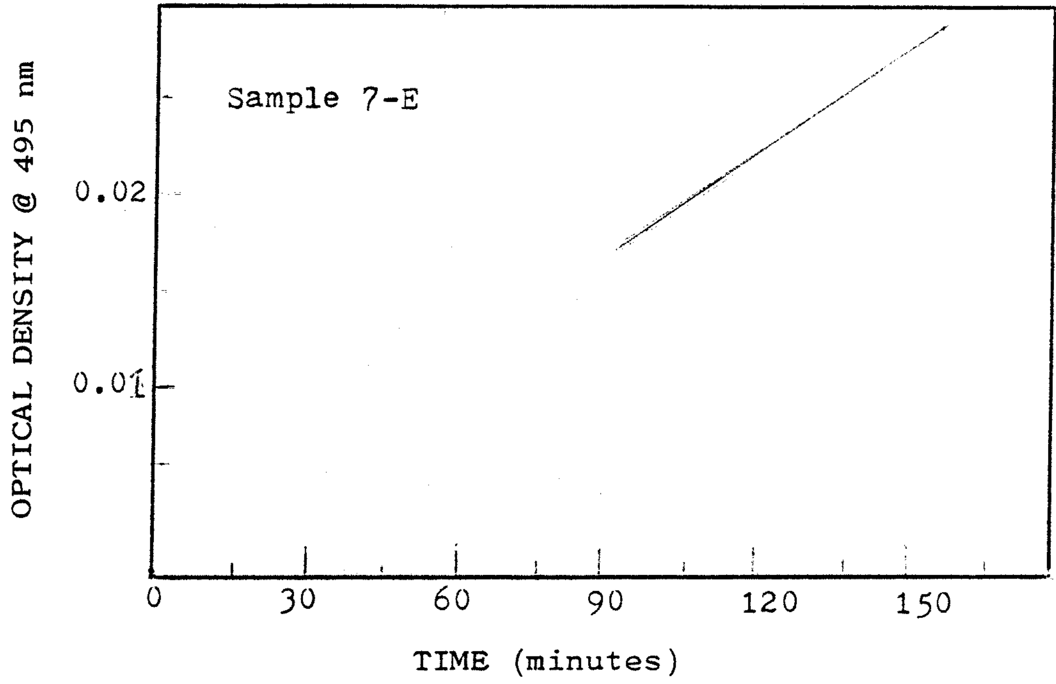


FIGURE H-2.10

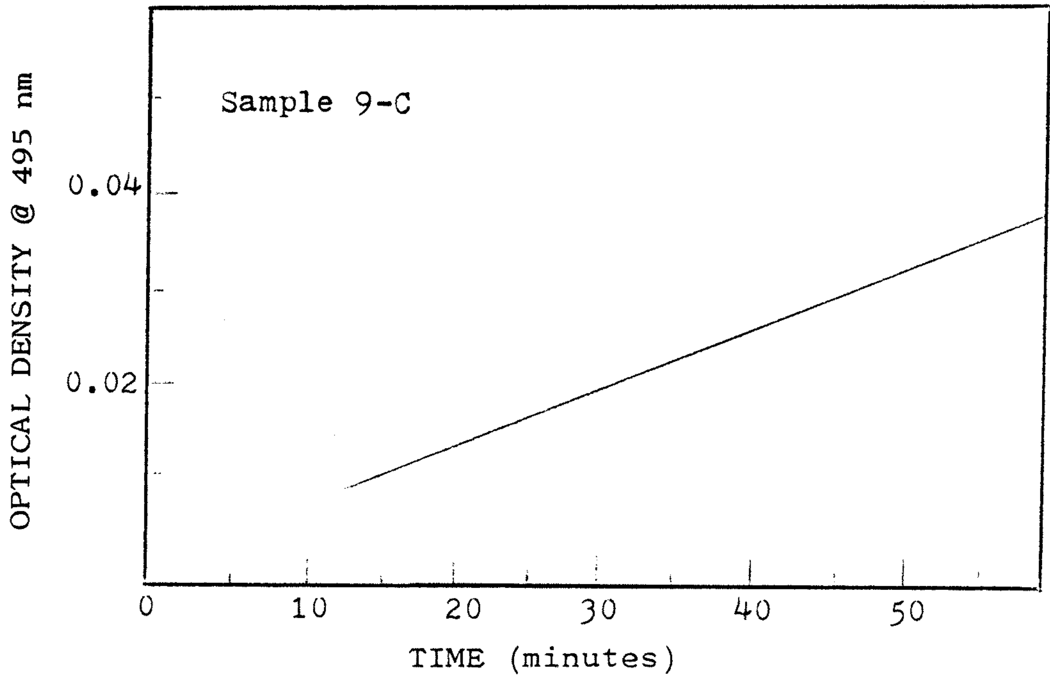


FIGURE H-2.11

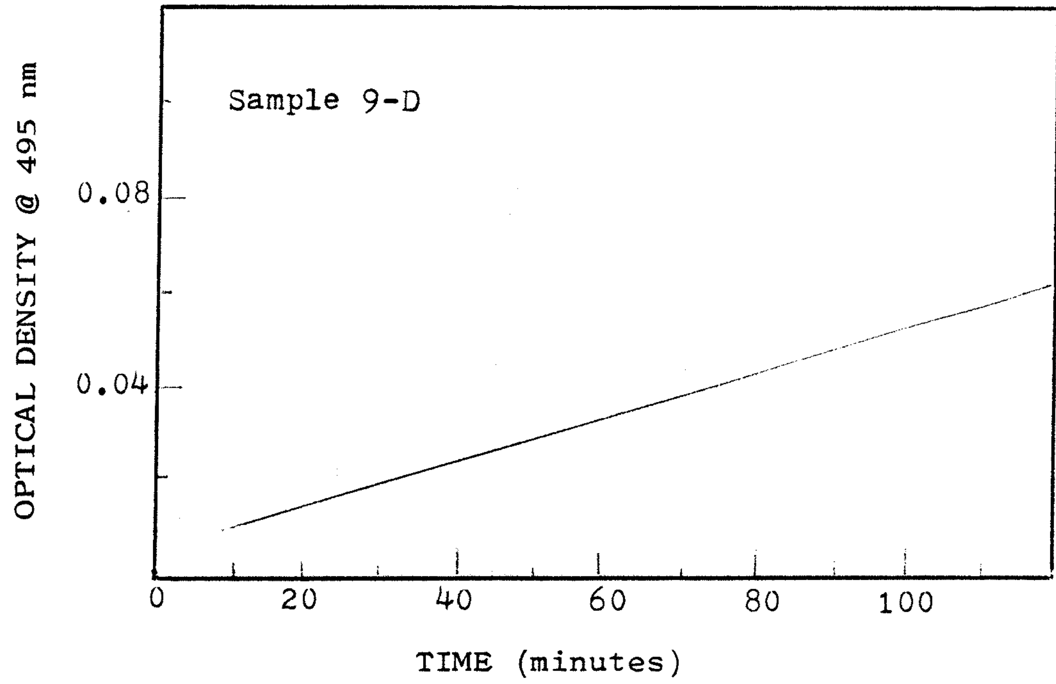


FIGURE H-2.12

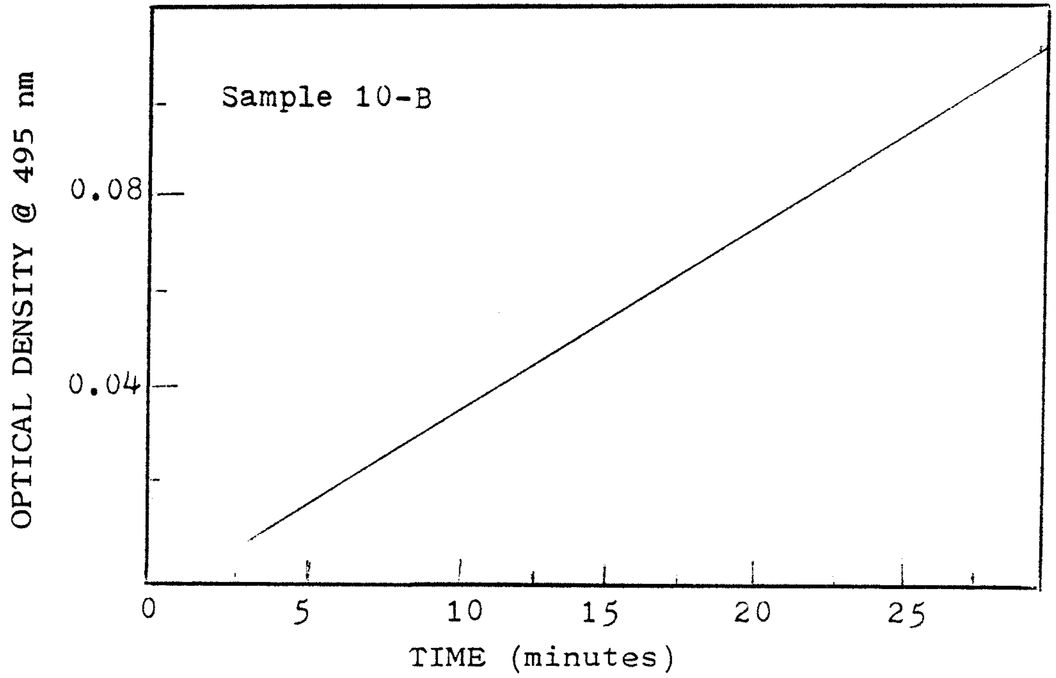


FIGURE H-2.13

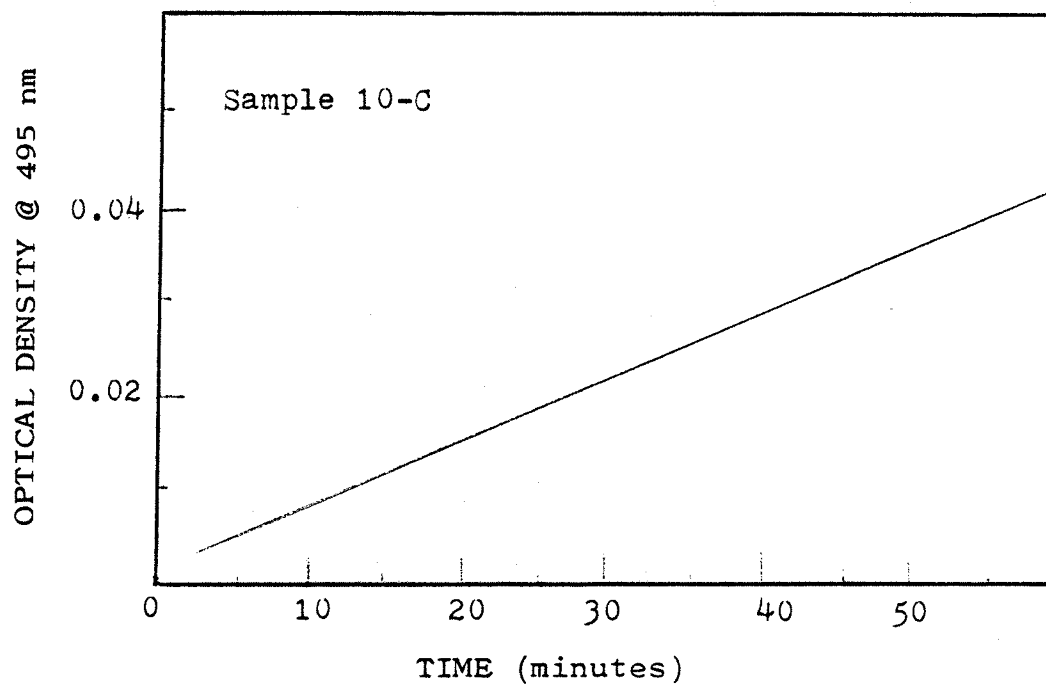


FIGURE H-2.14

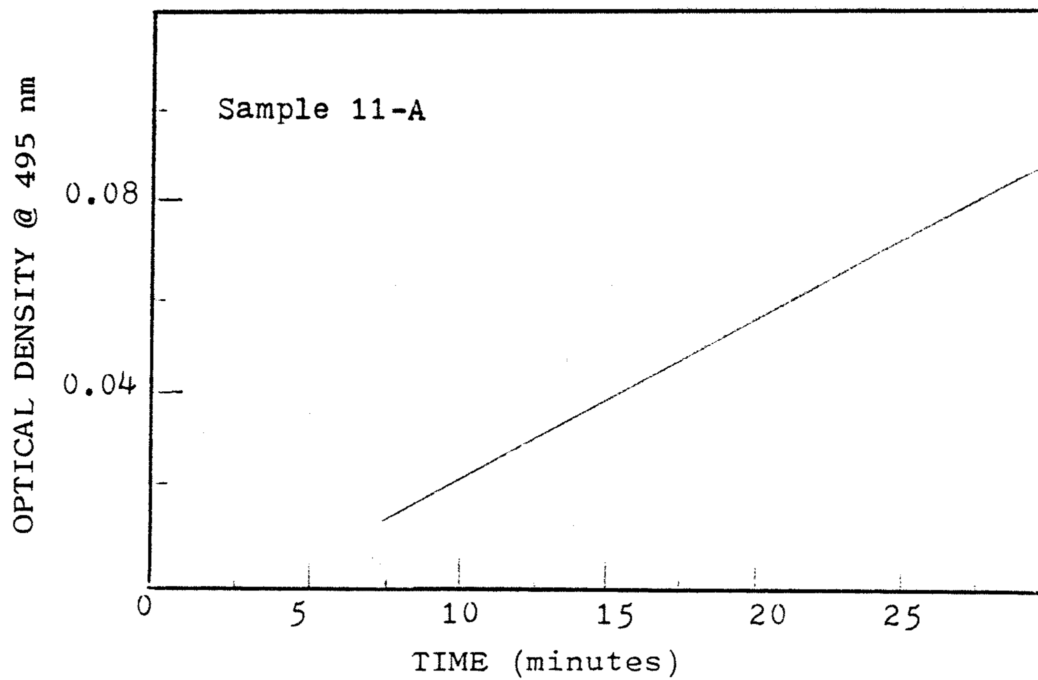


FIGURE H-2.15

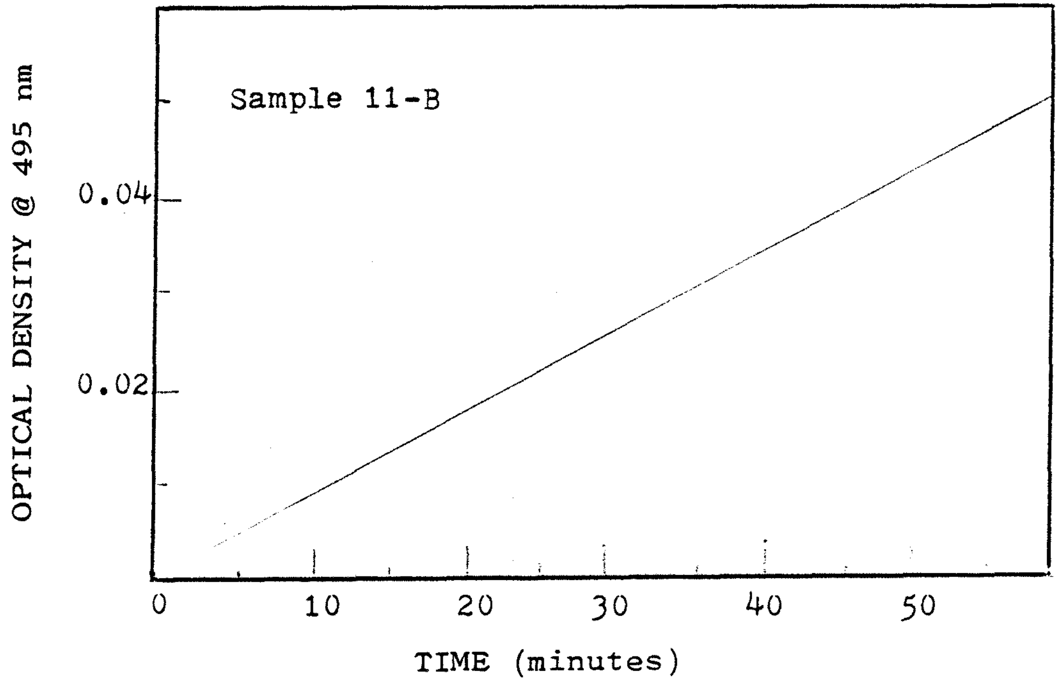


FIGURE H-2.16

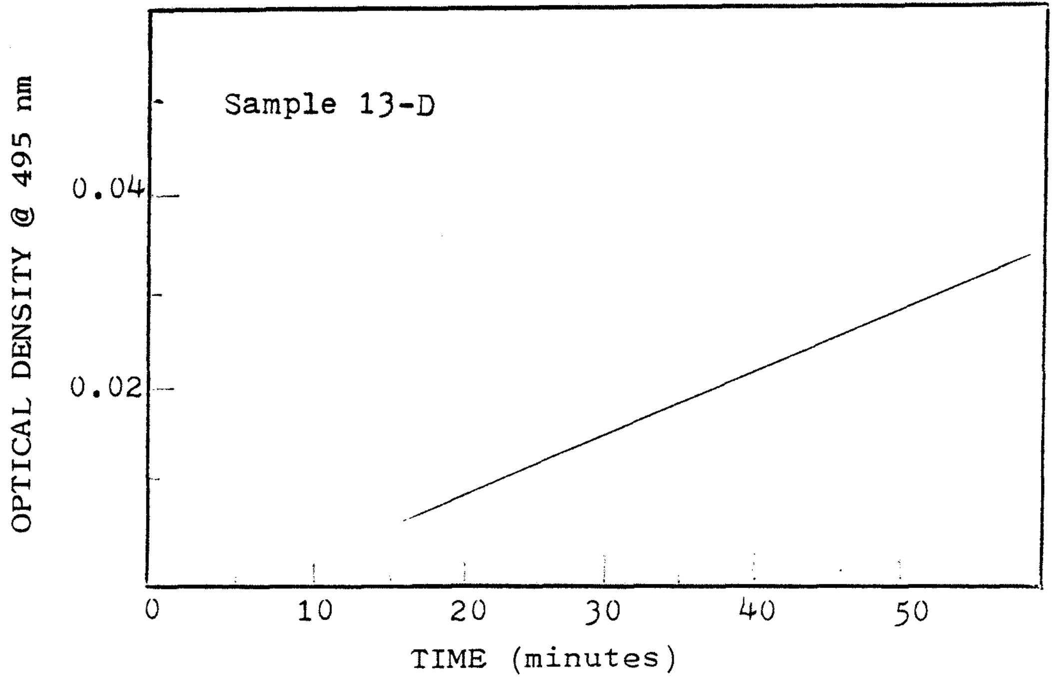


FIGURE H-2.17

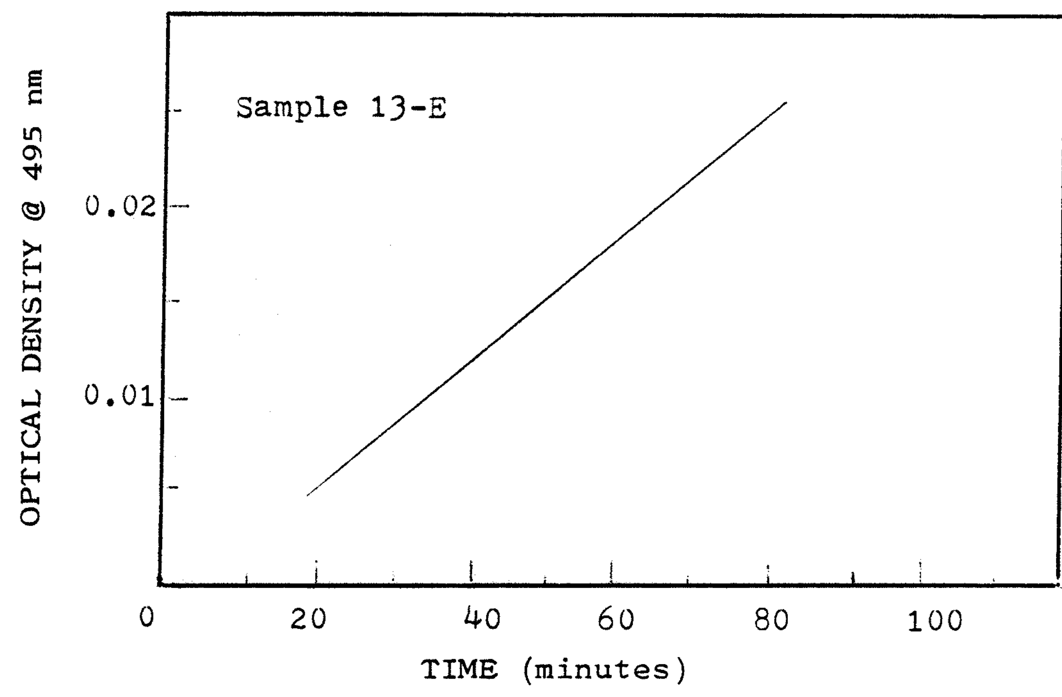


FIGURE H-2.18

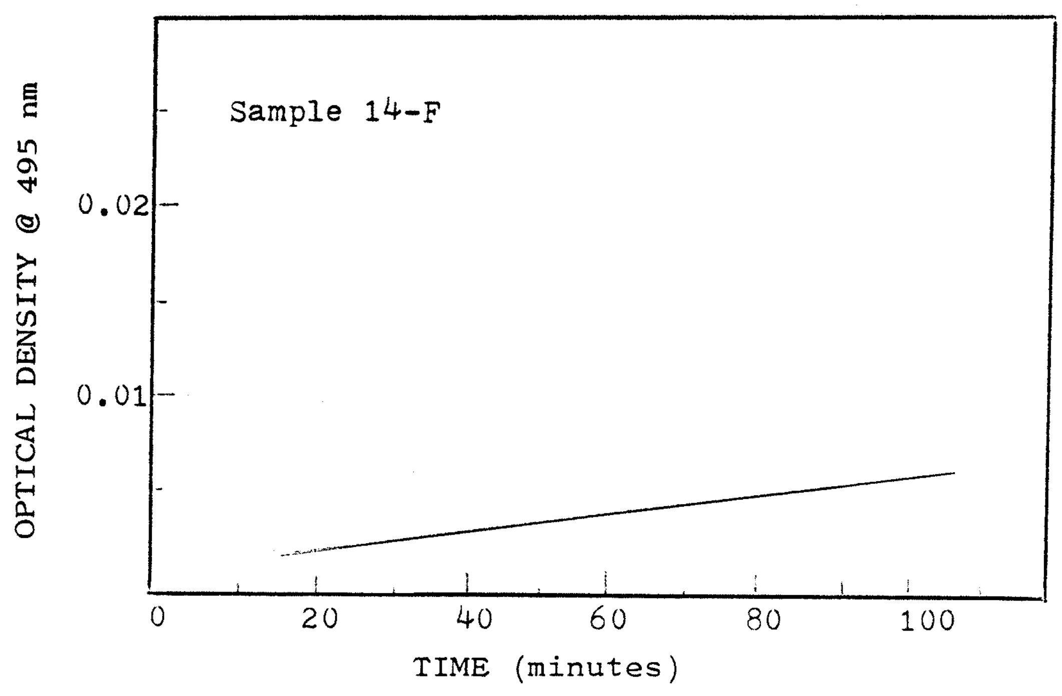


FIGURE H-2.19

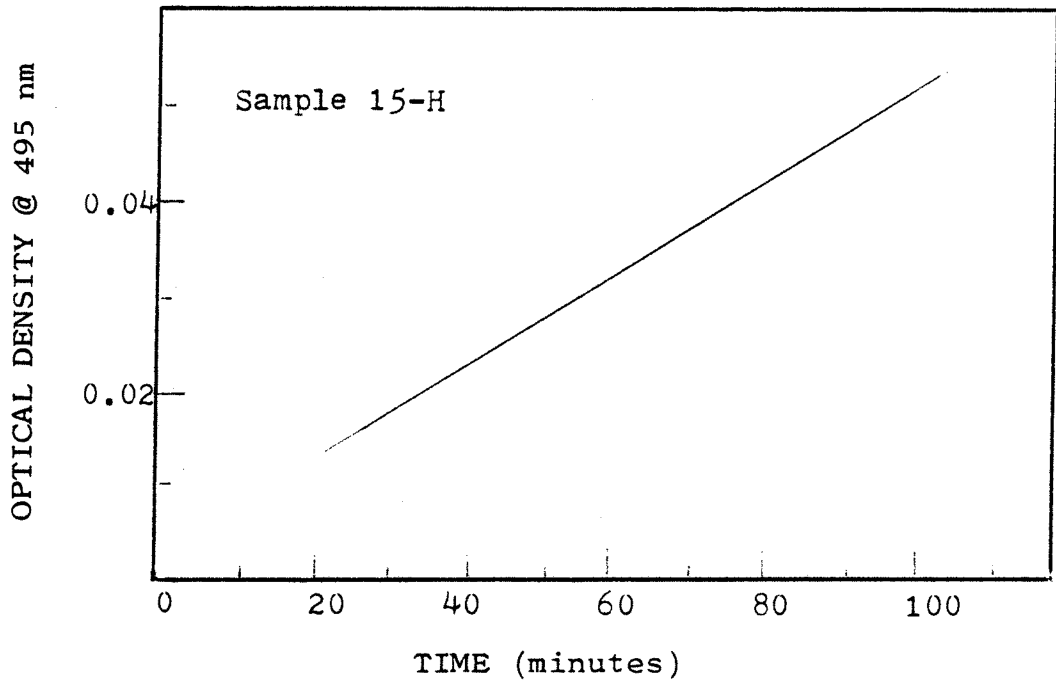


FIGURE H-2.20

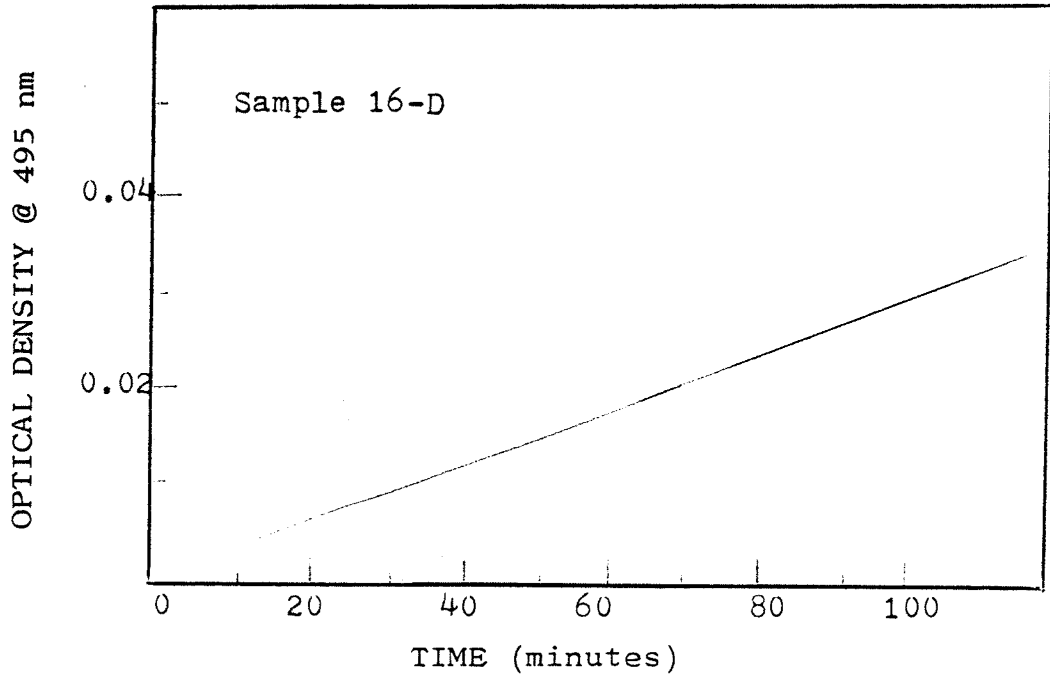


FIGURE H-2.21

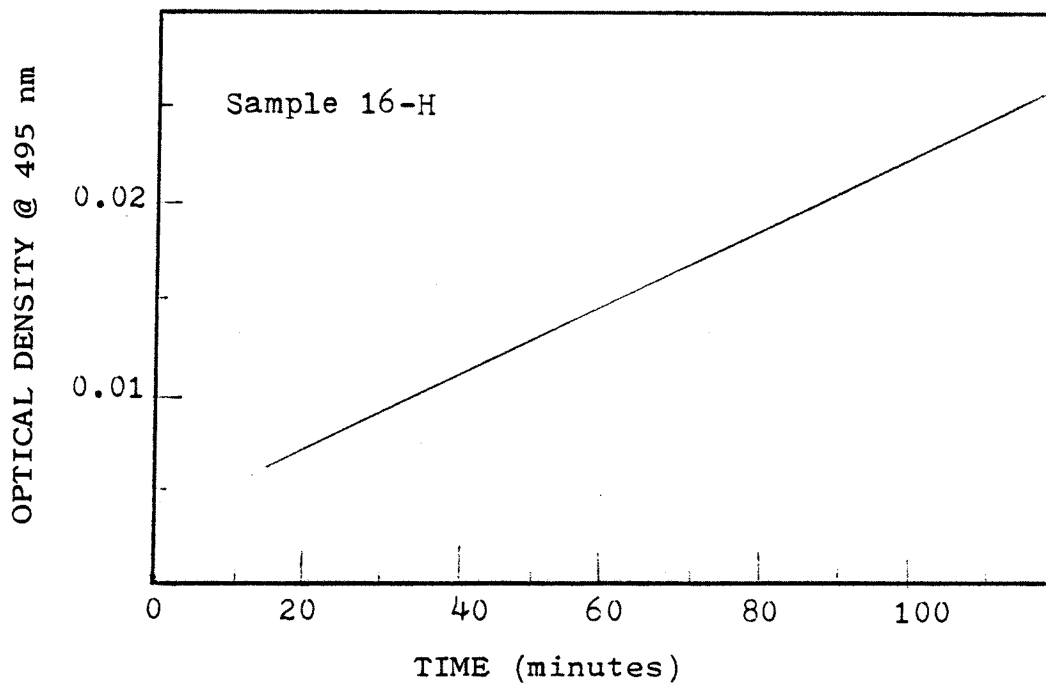


FIGURE H-2.22

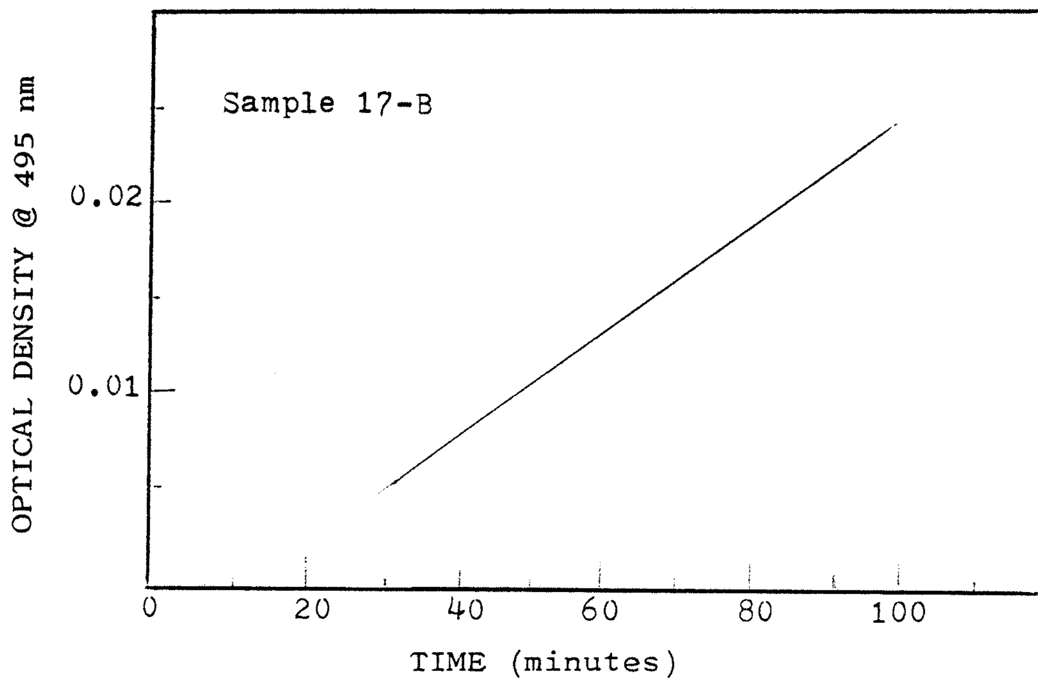


FIGURE H-2.23

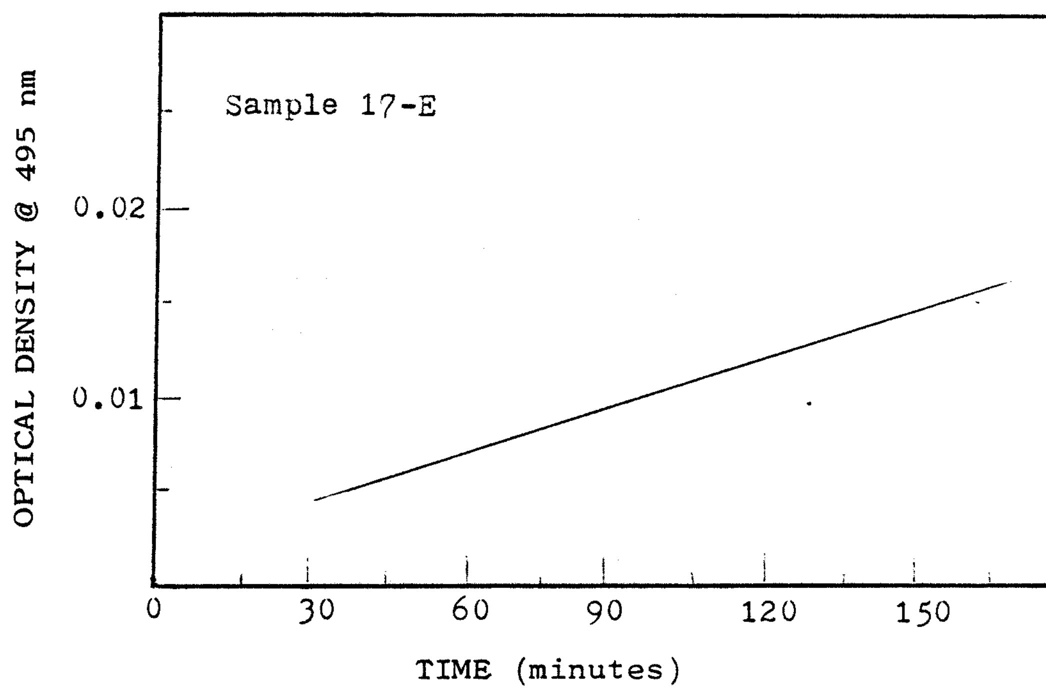


FIGURE H-2.24

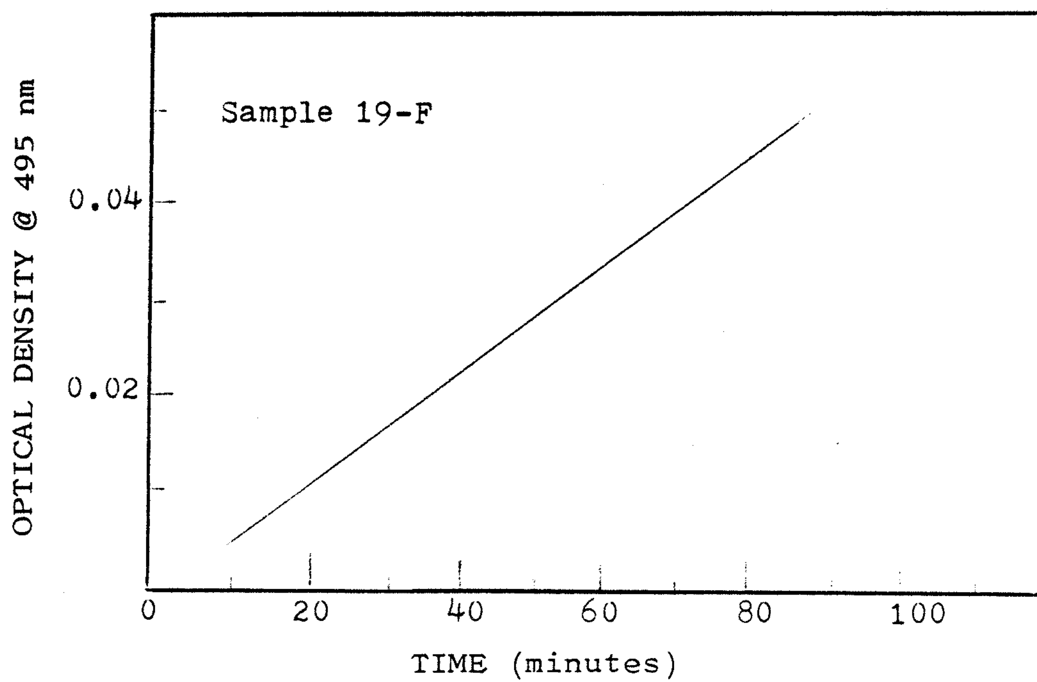


FIGURE H-2.25

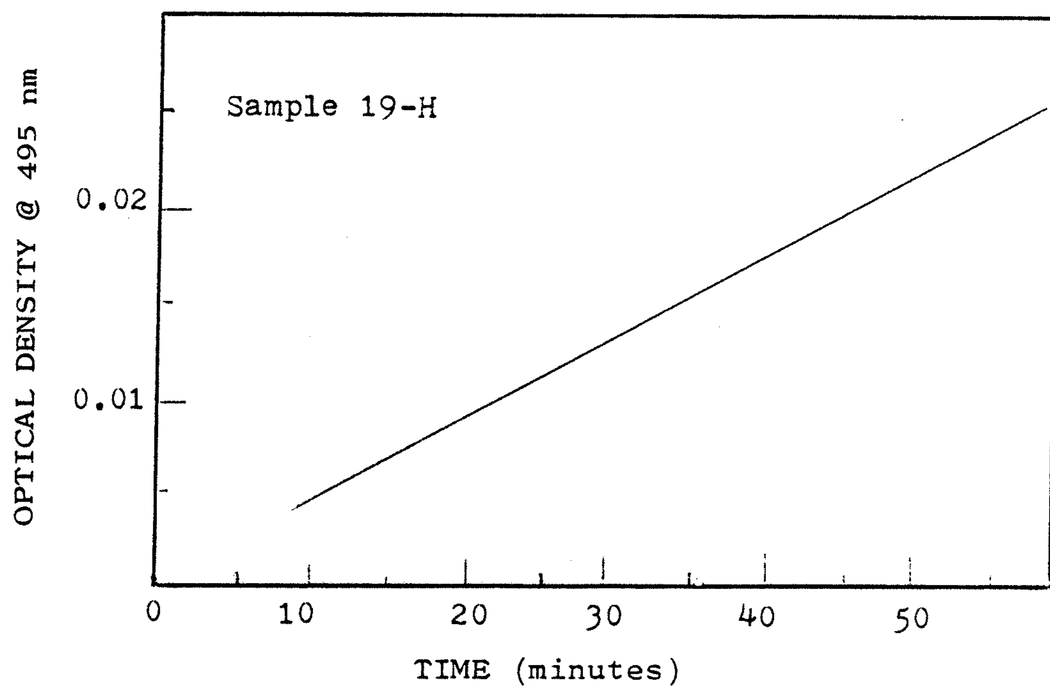


FIGURE H-2.26

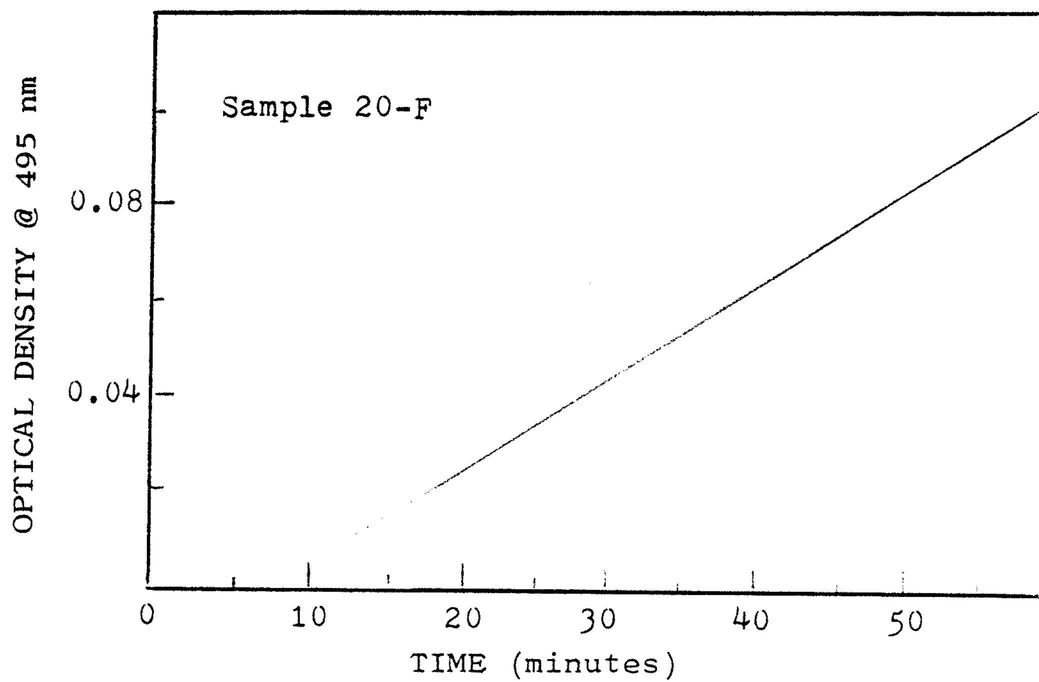


FIGURE H-2.27

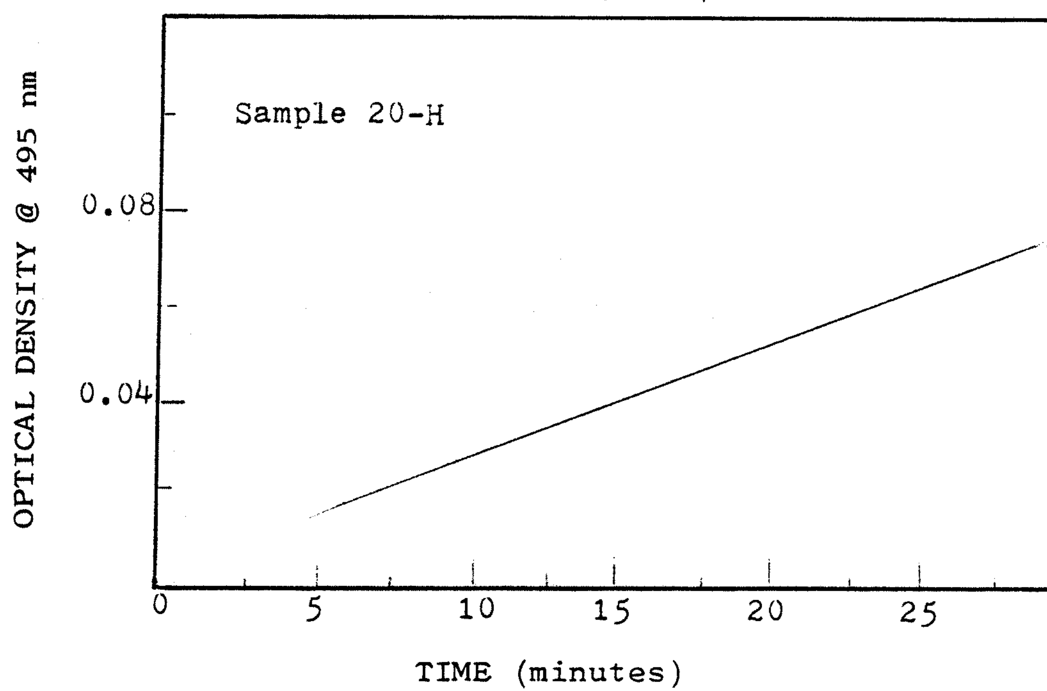


FIGURE H-2.28

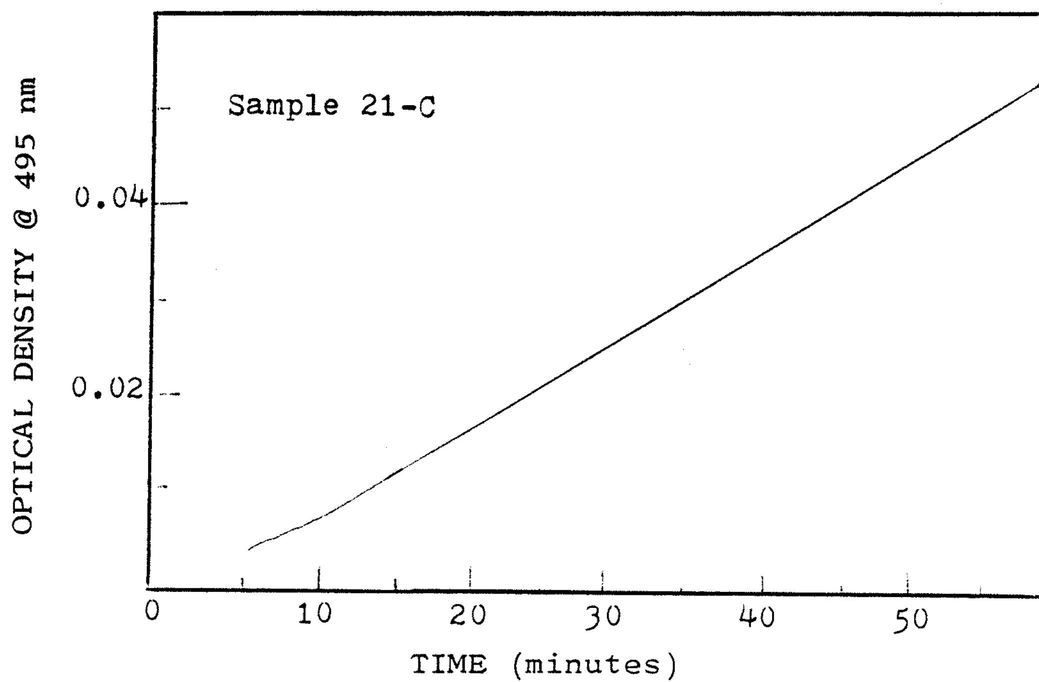


FIGURE H-2.29

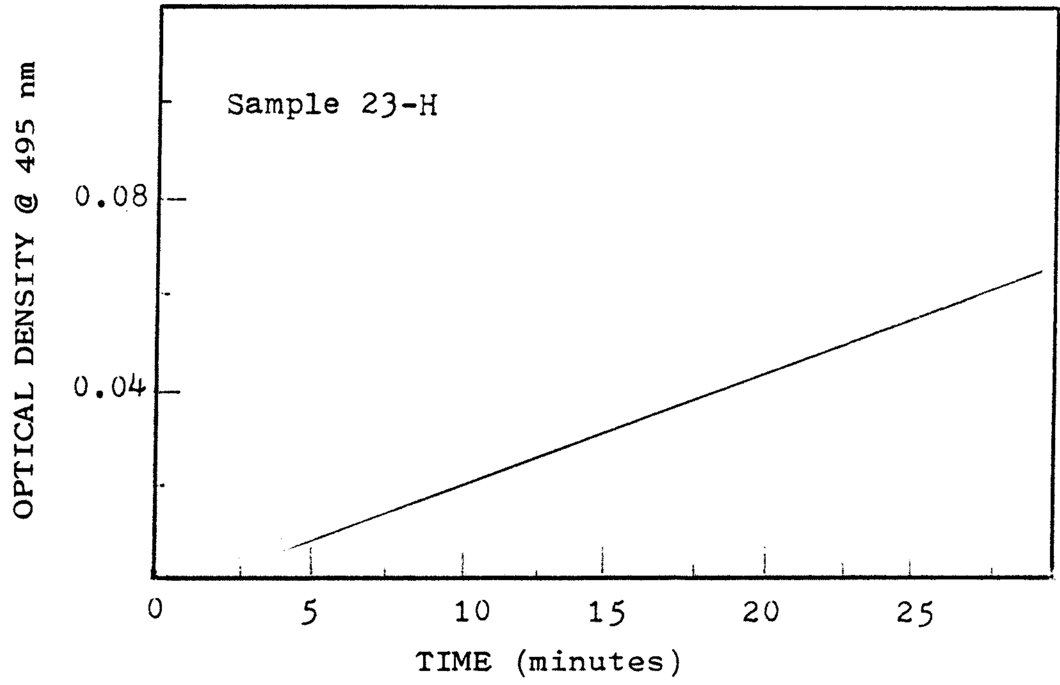
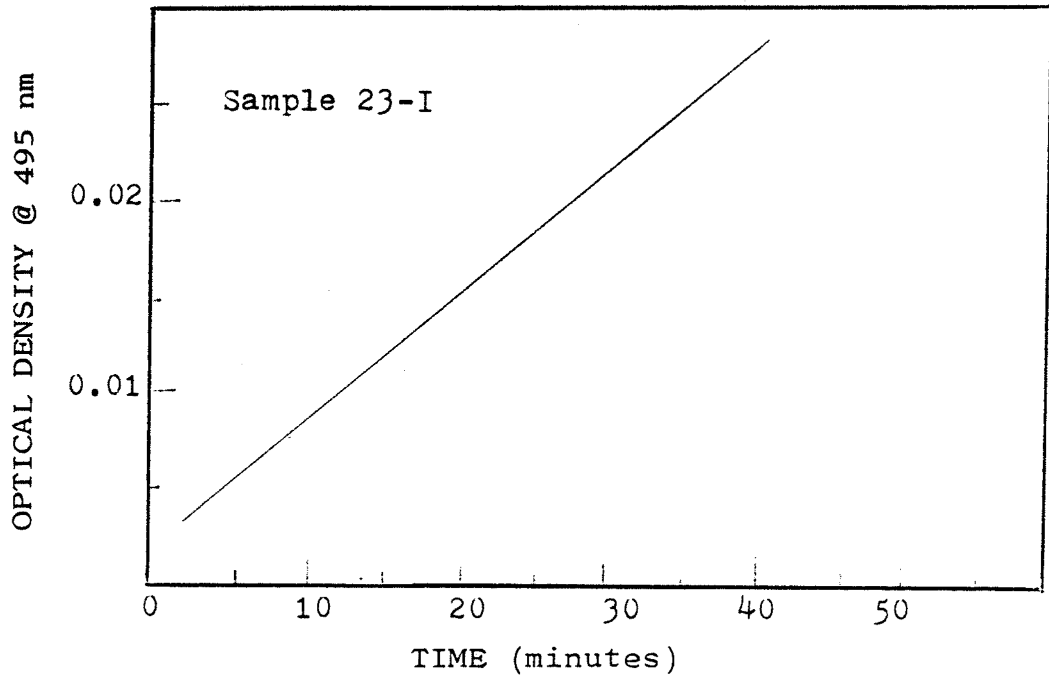


FIGURE H-2.30



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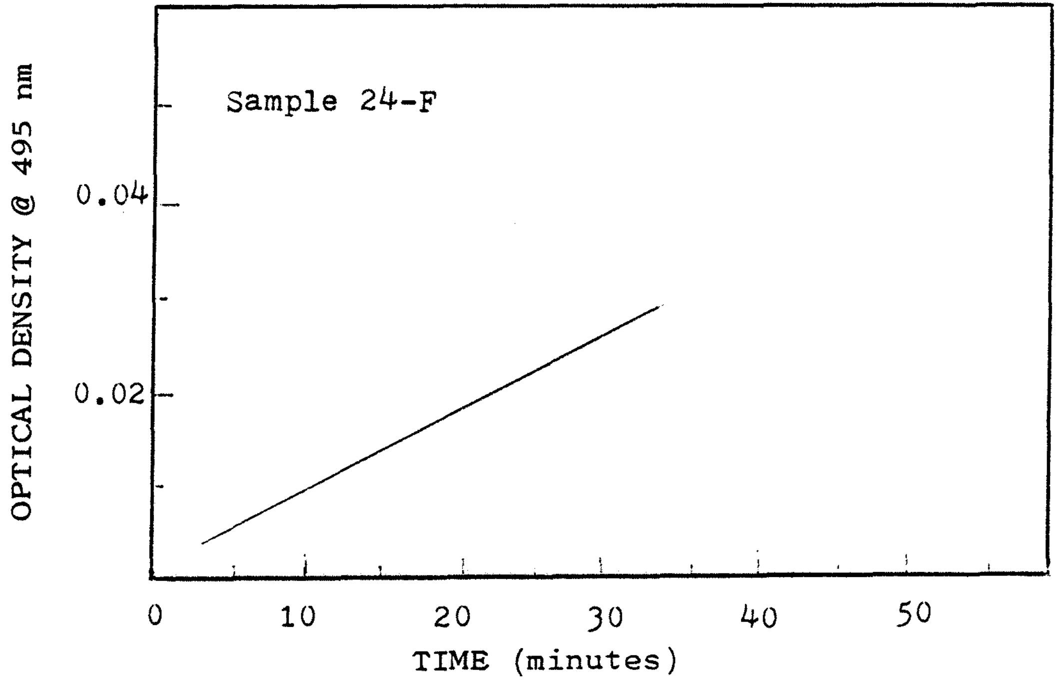

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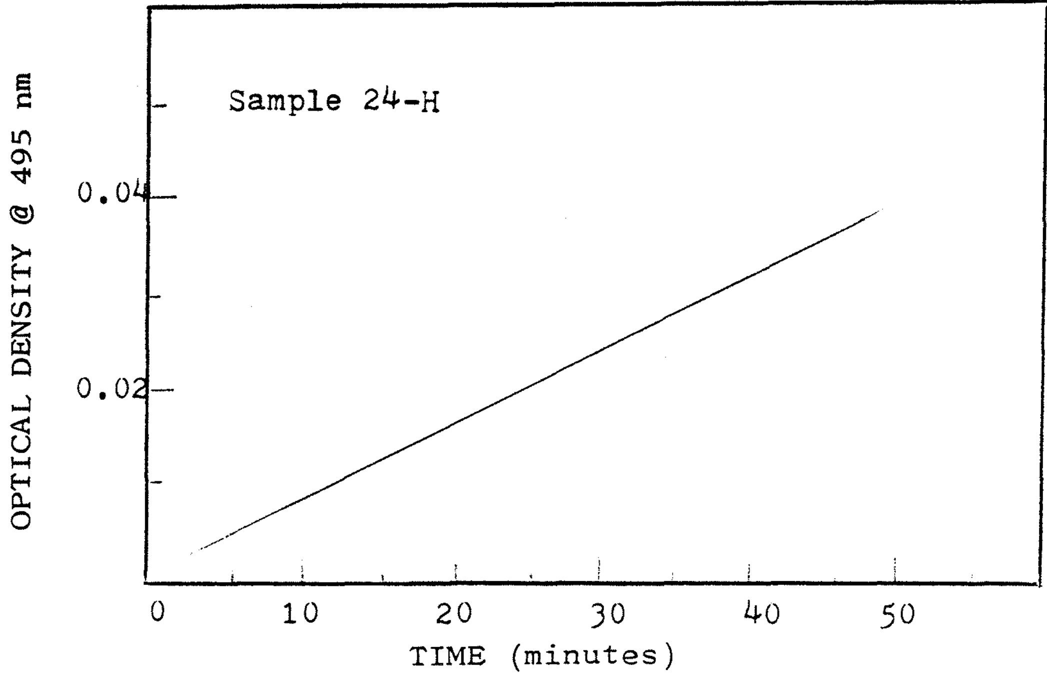

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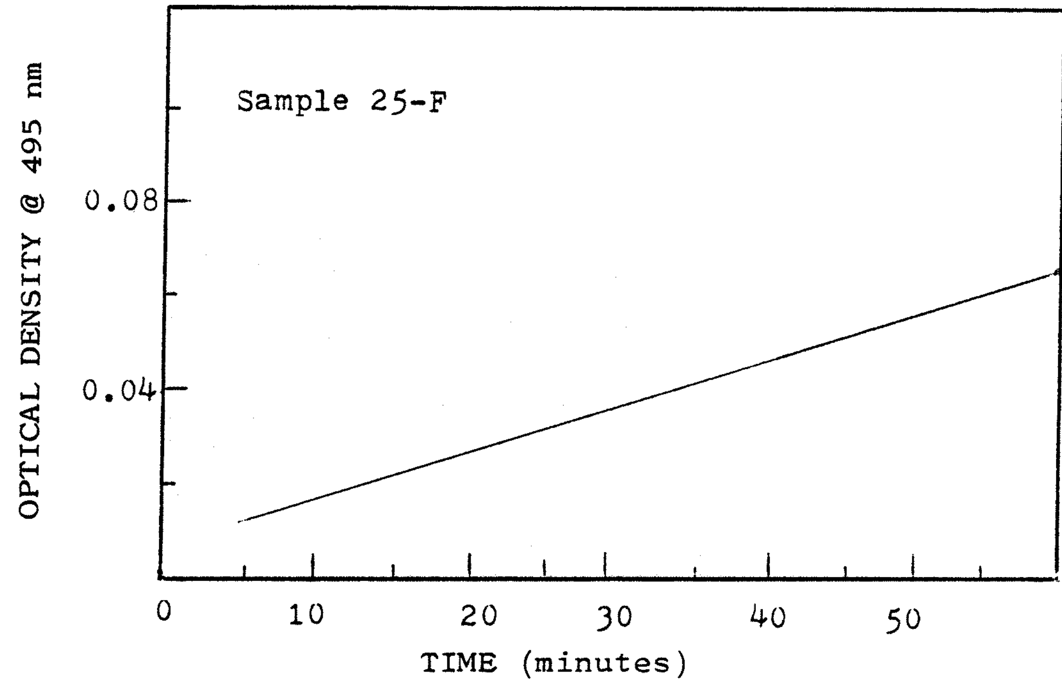

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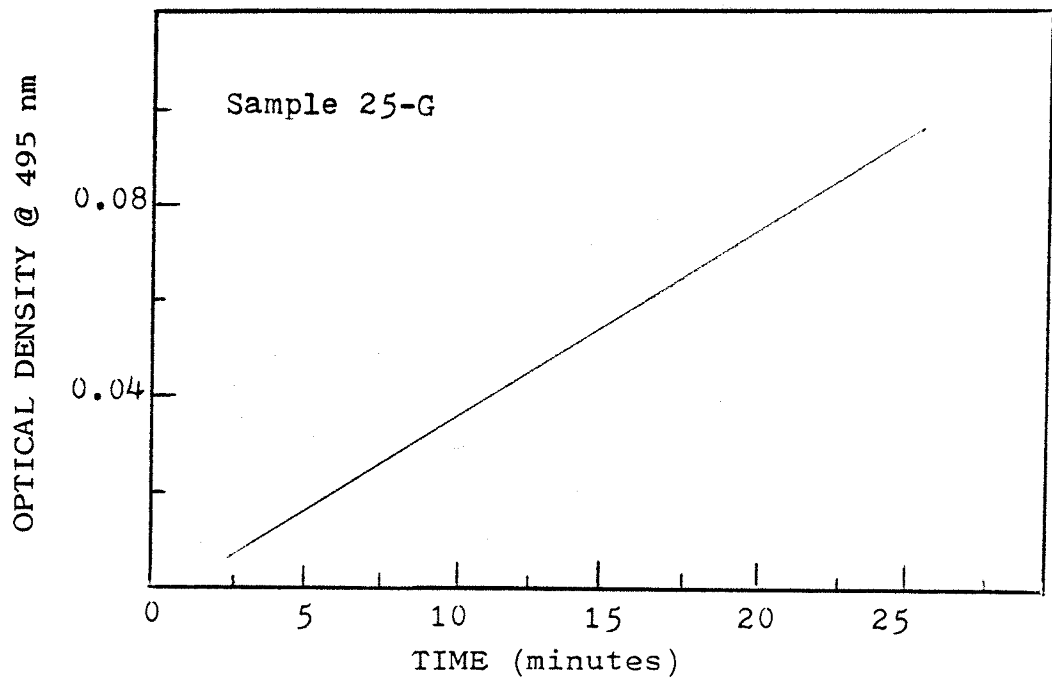

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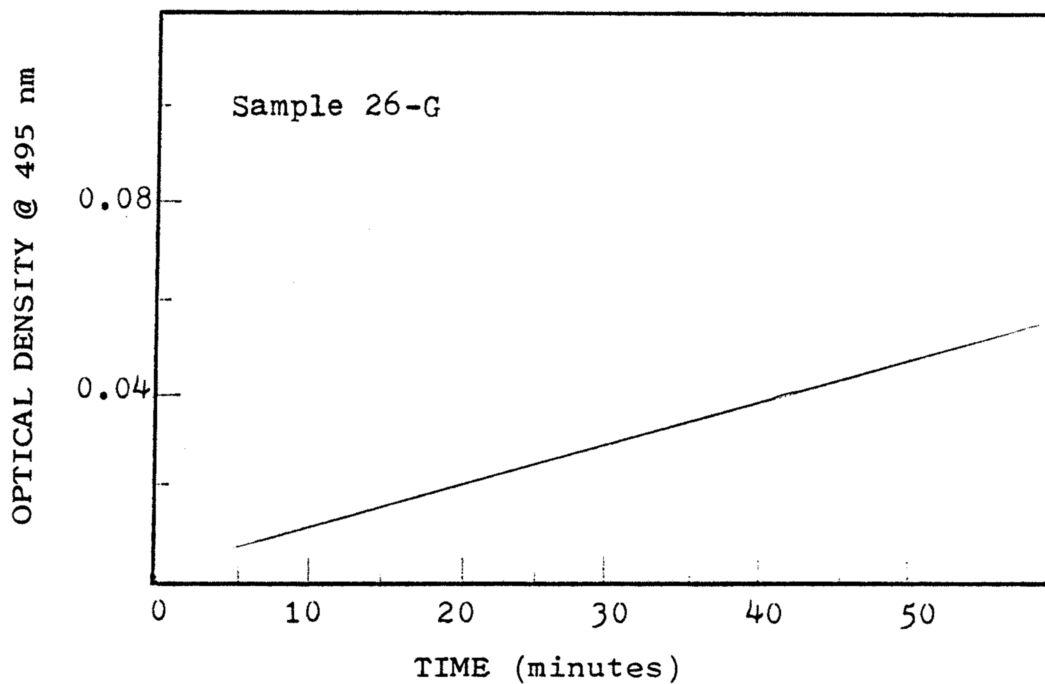

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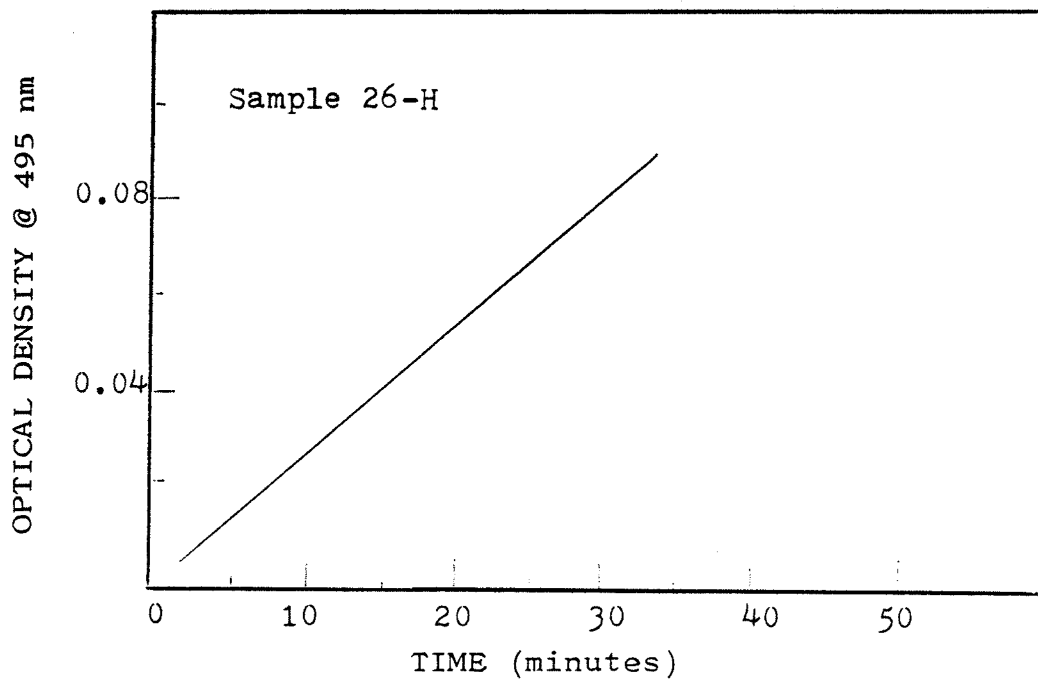

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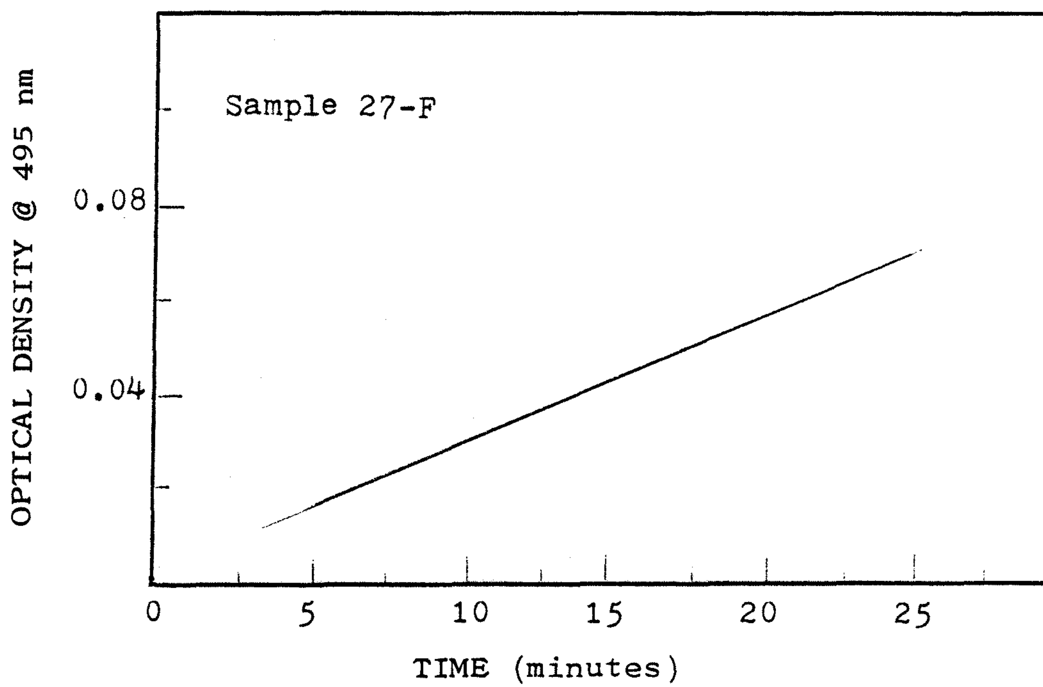

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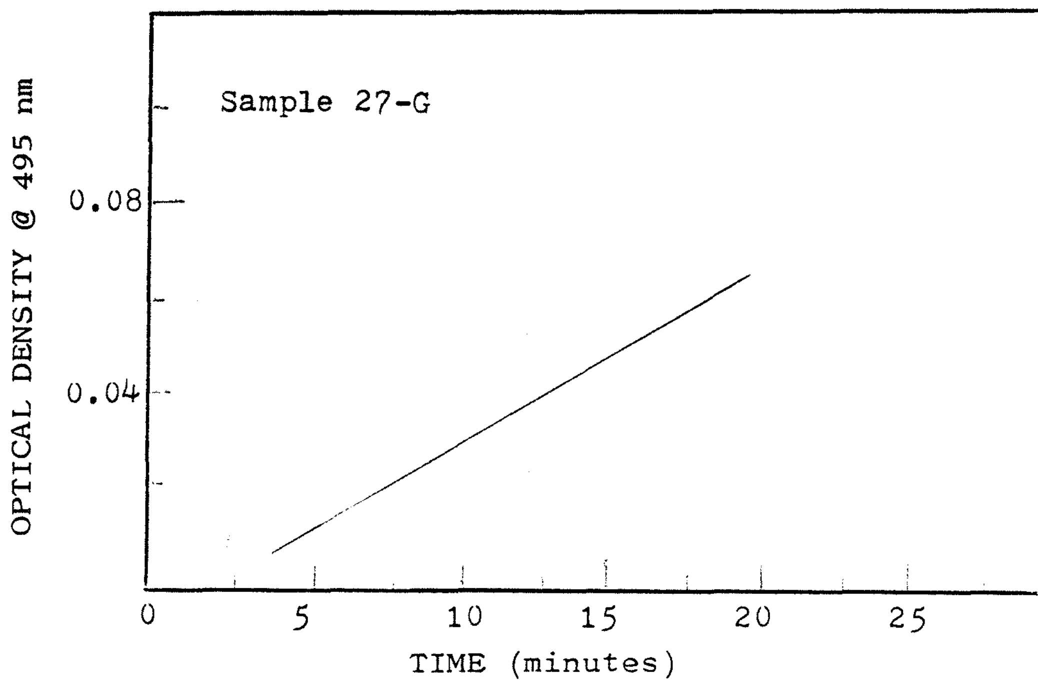

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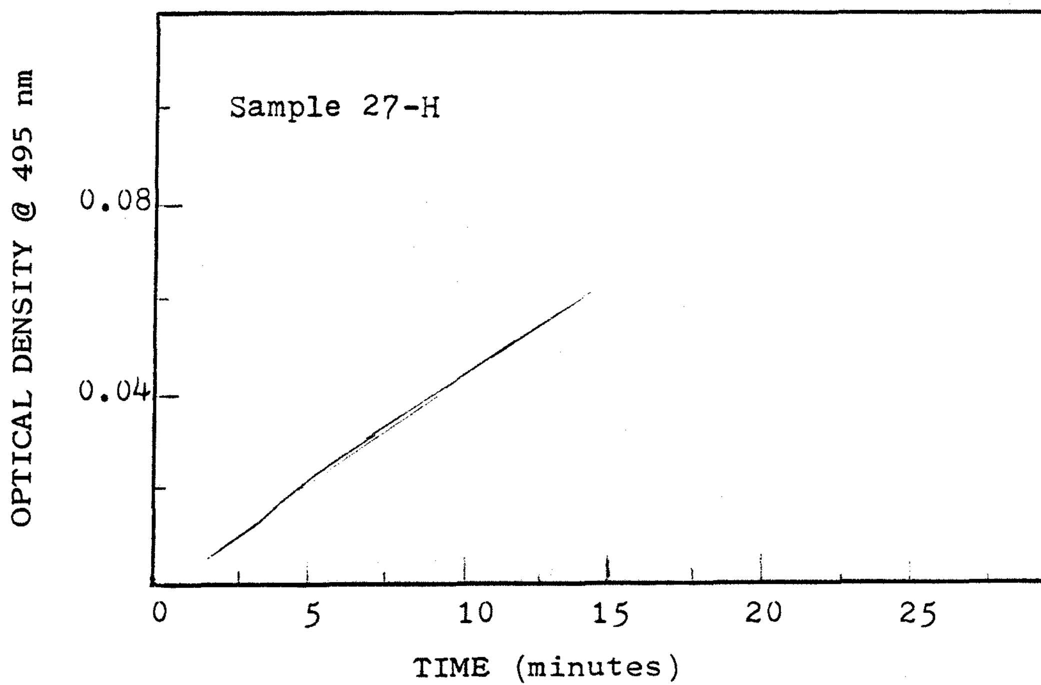

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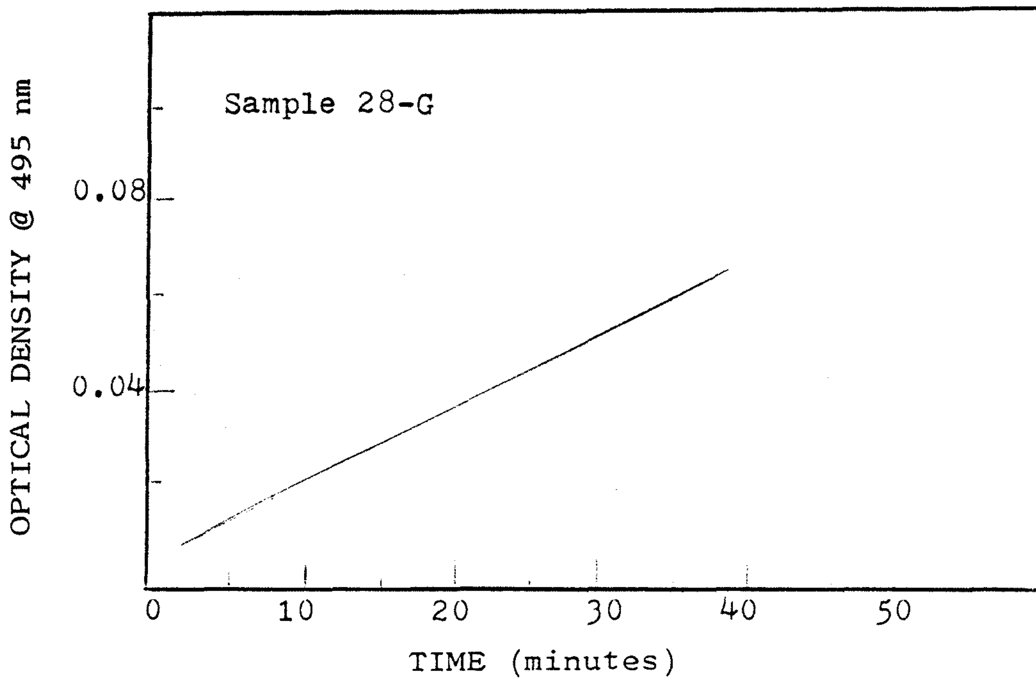

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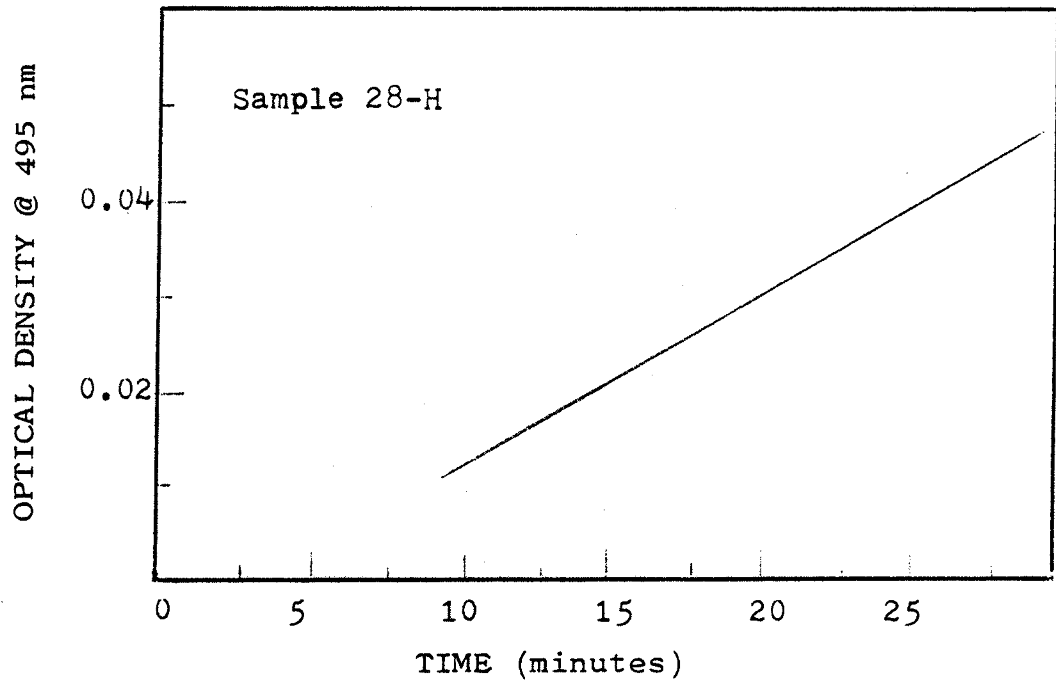

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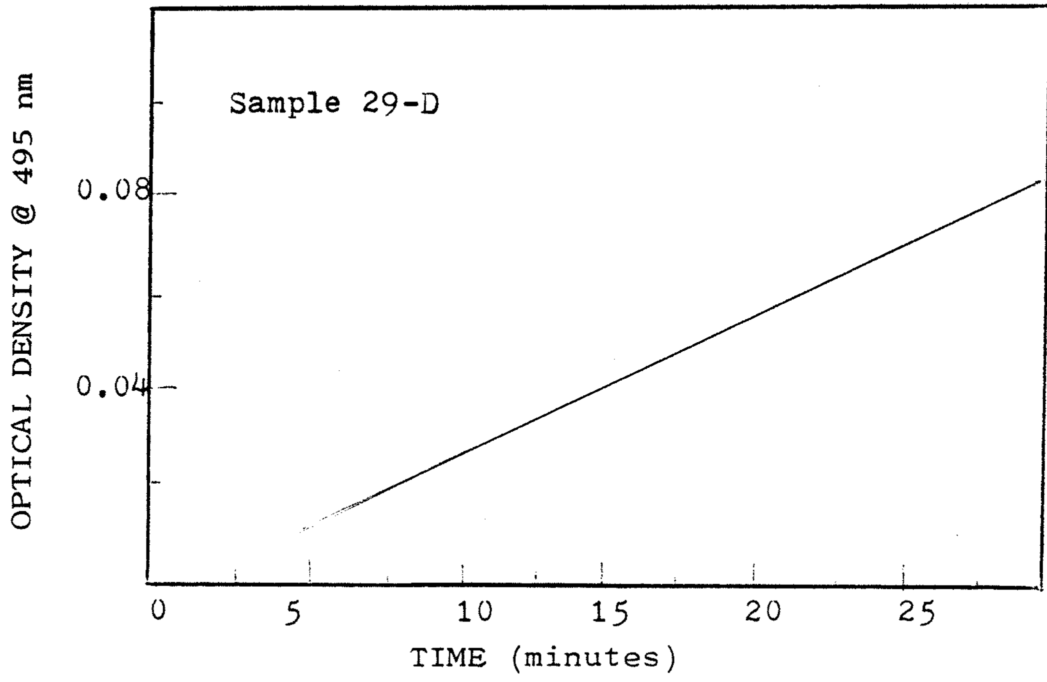

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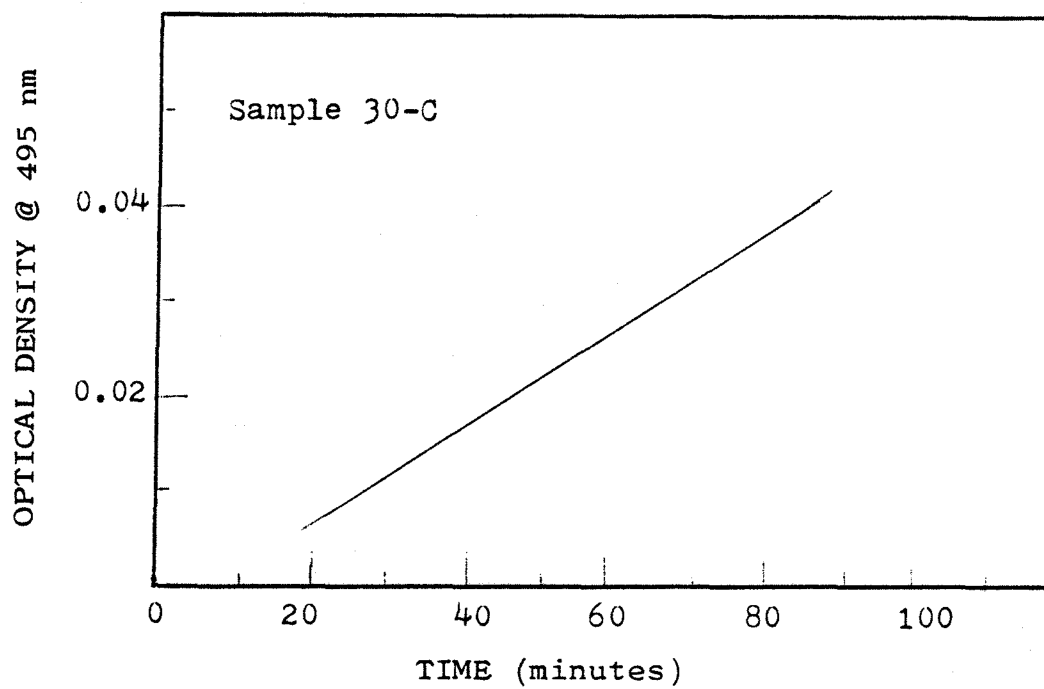

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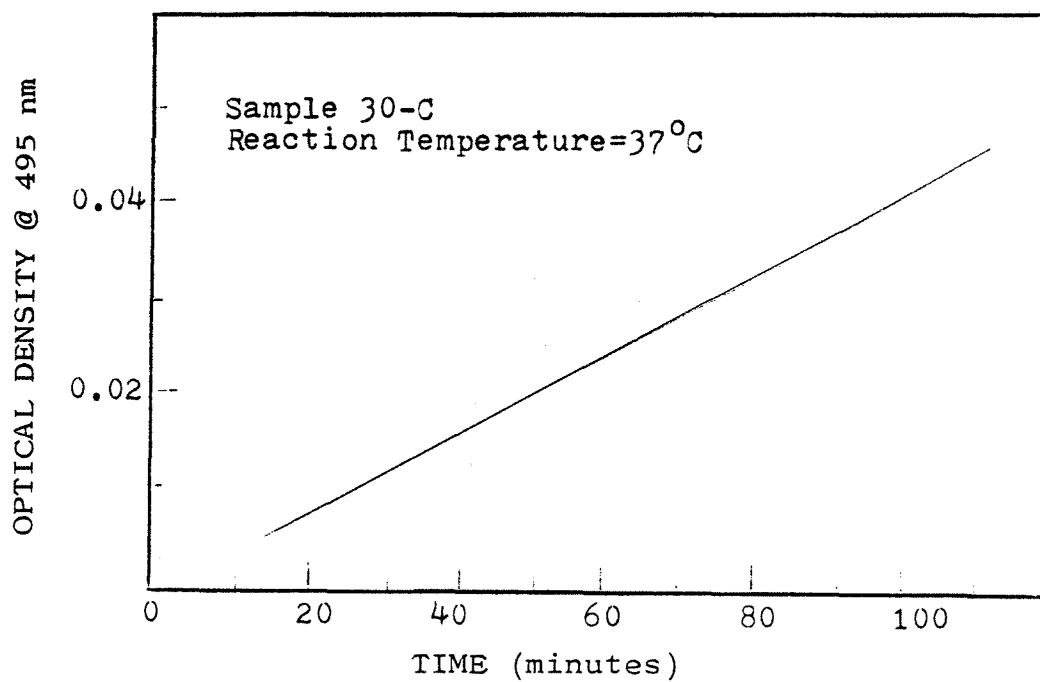

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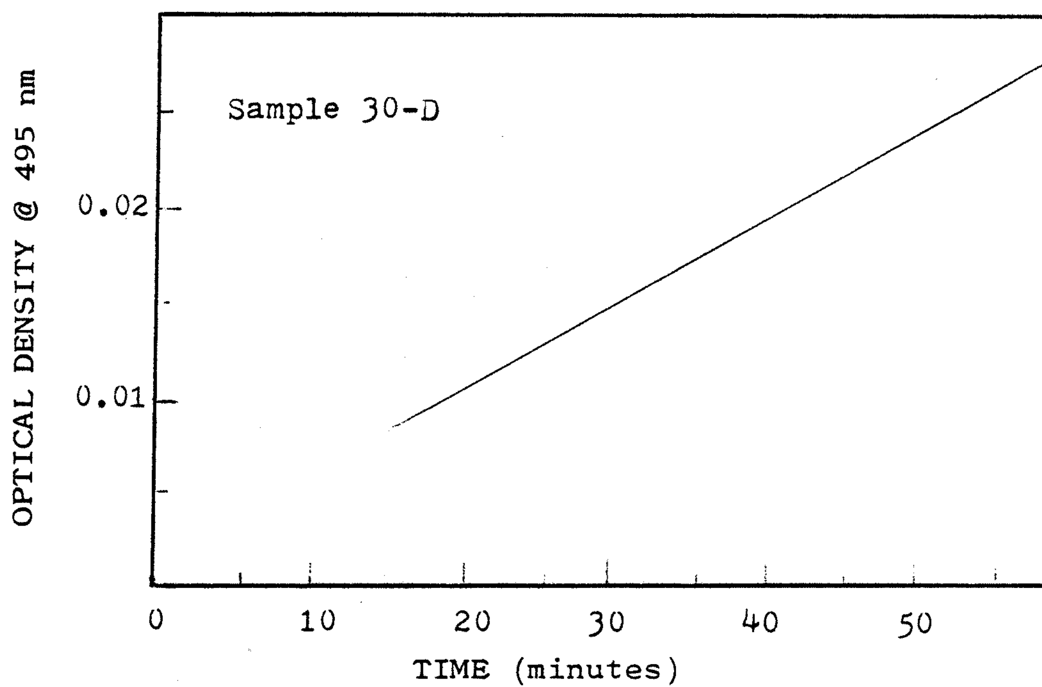

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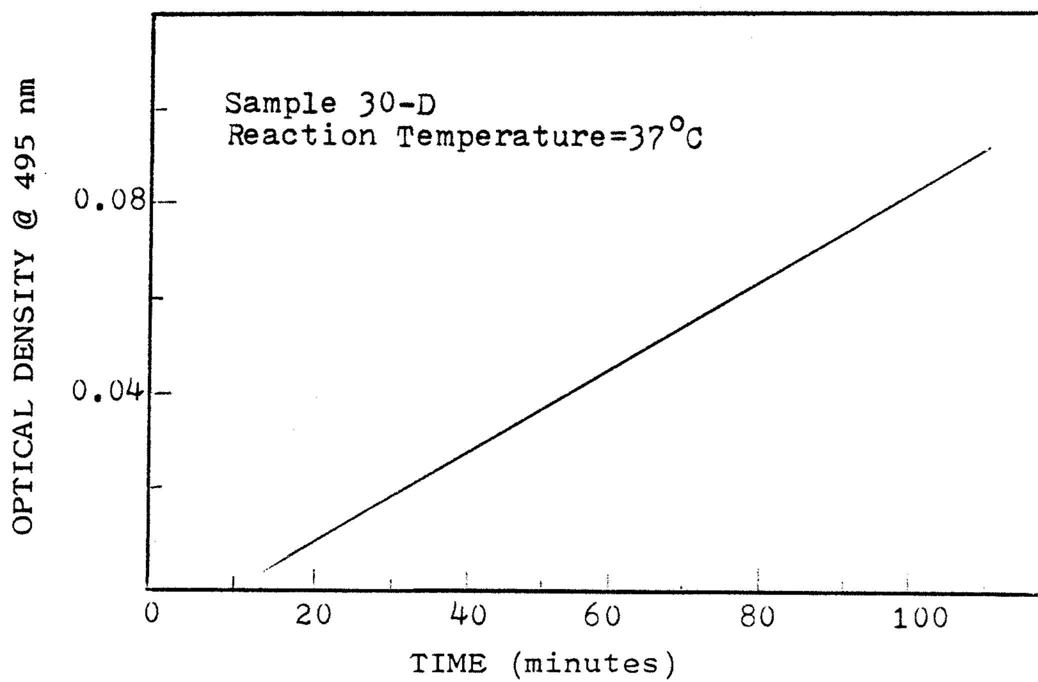

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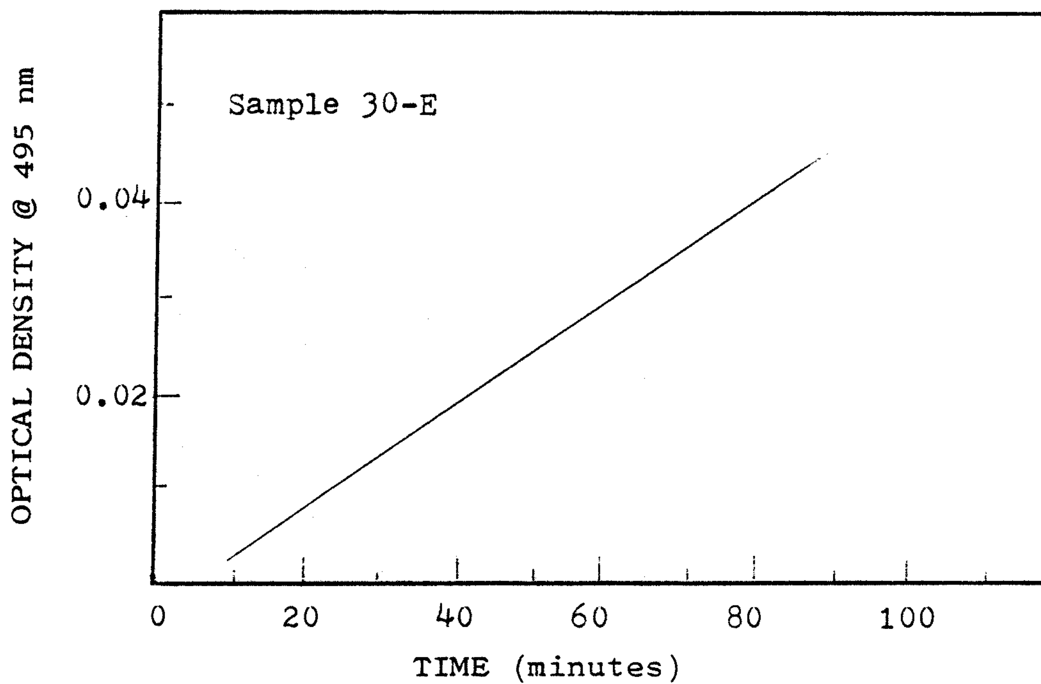


FIGURE H-2.48

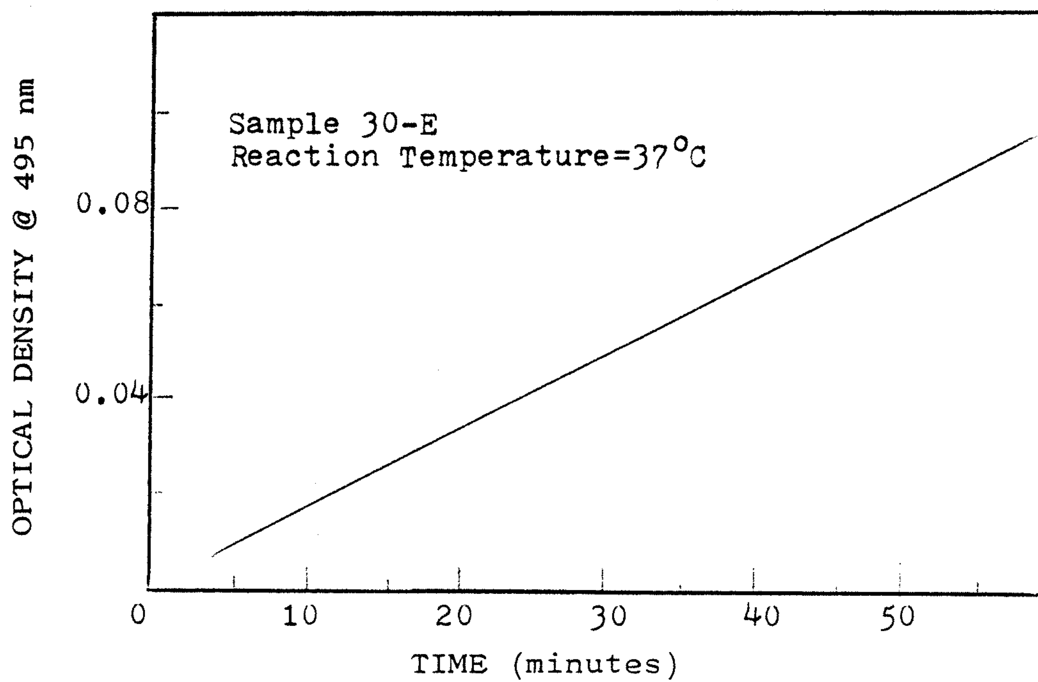


FIGURE H-2.51

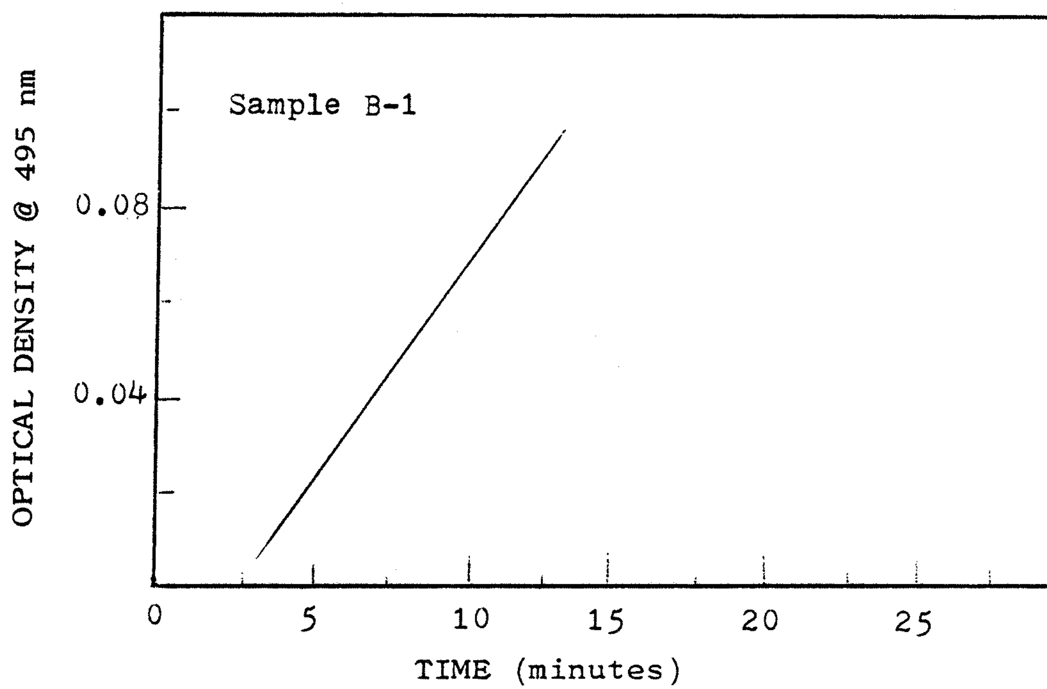


FIGURE H-2.52

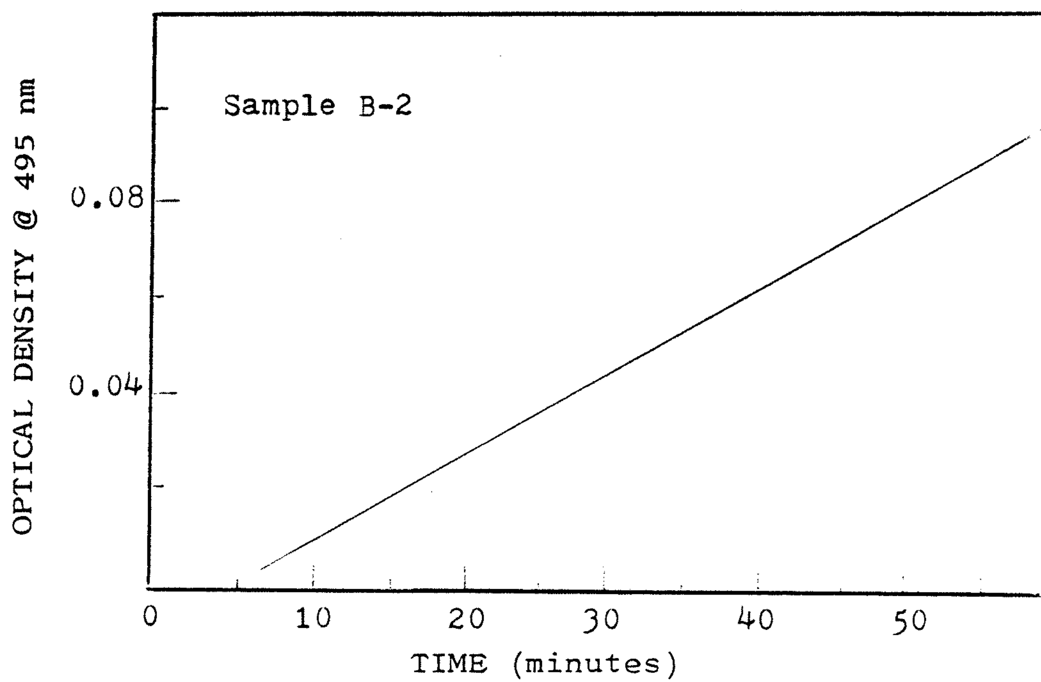


FIGURE H-2.49

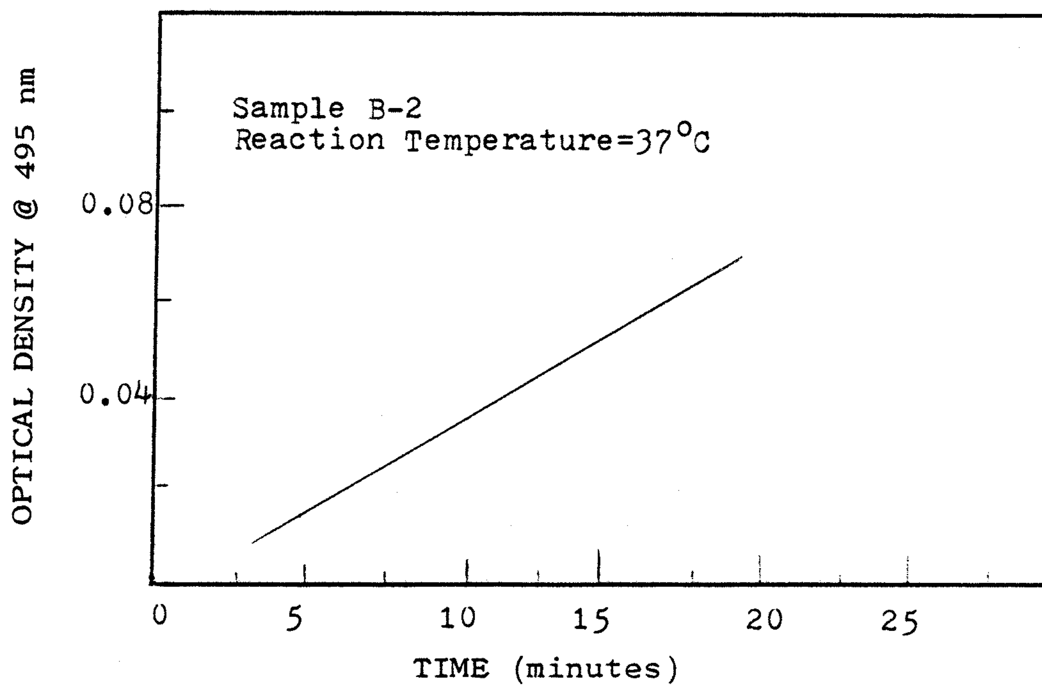


FIGURE H-2.50

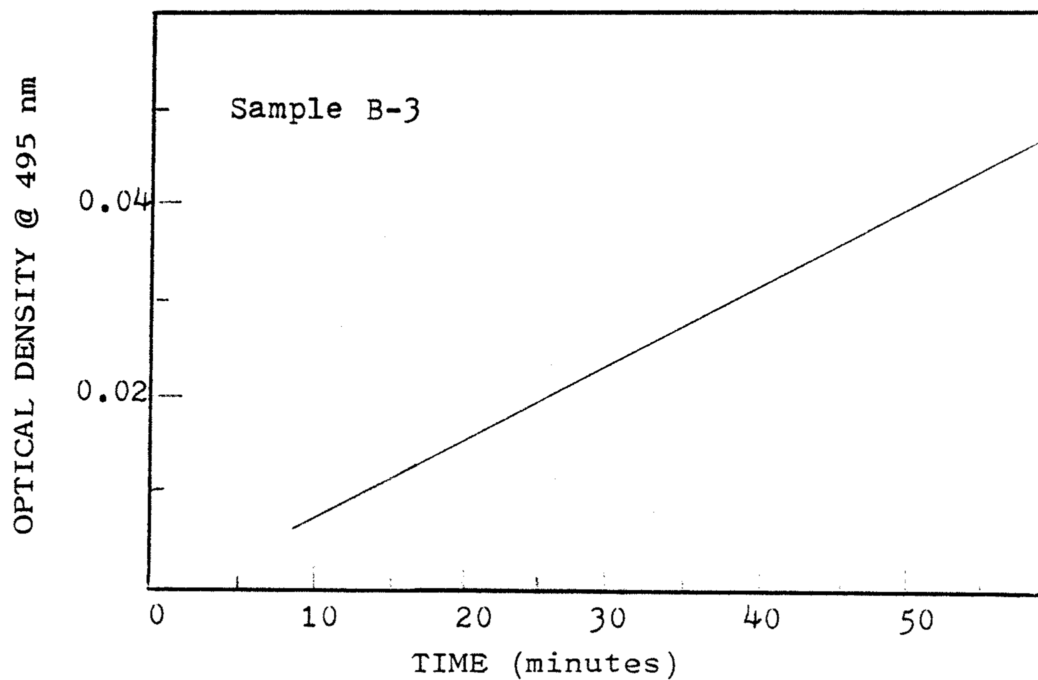


FIGURE H-2.53

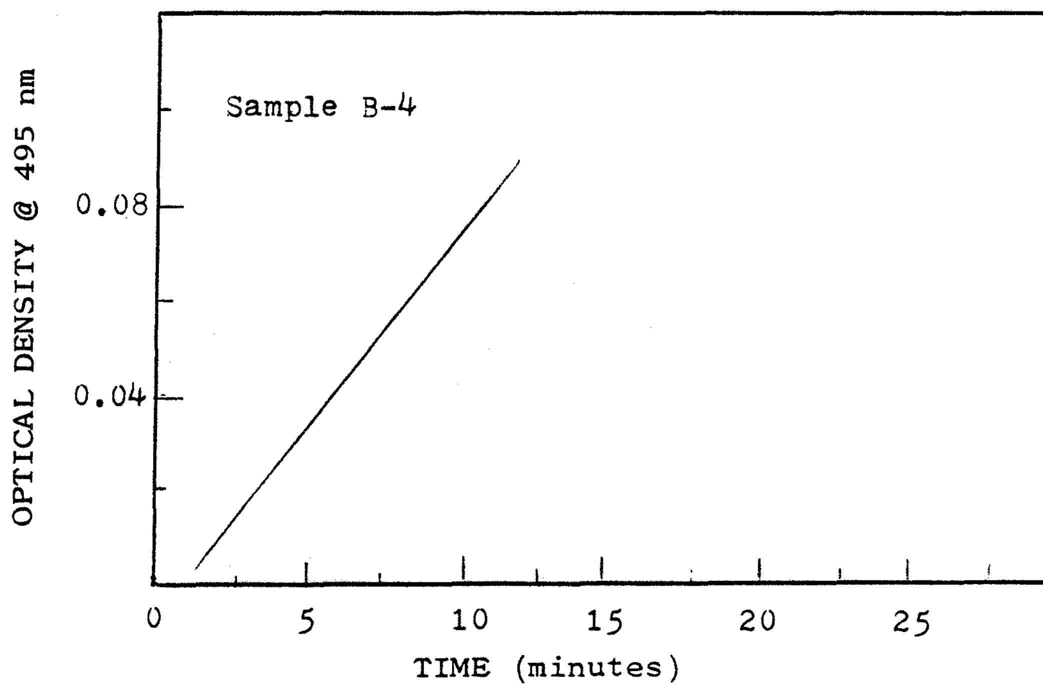


FIGURE H-2.54

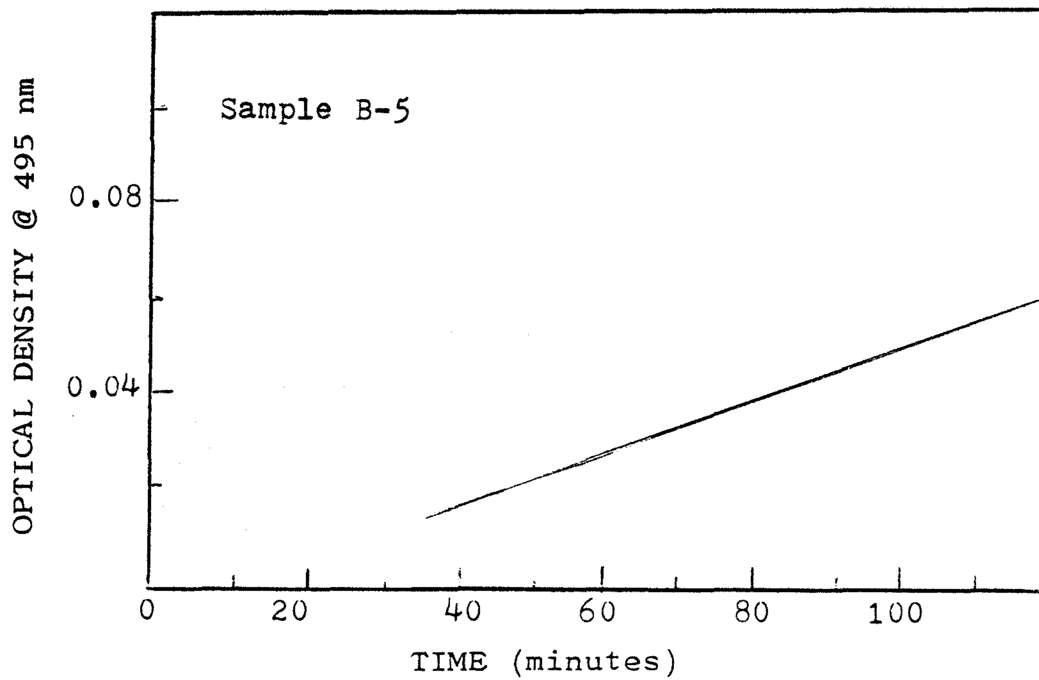


FIGURE H-2.55

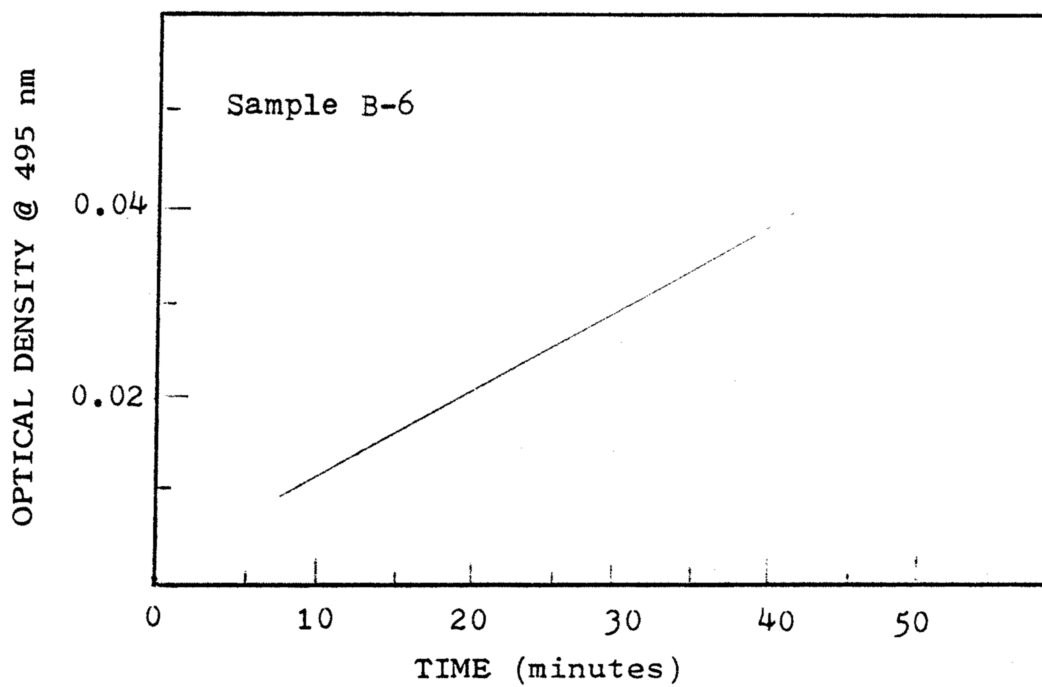
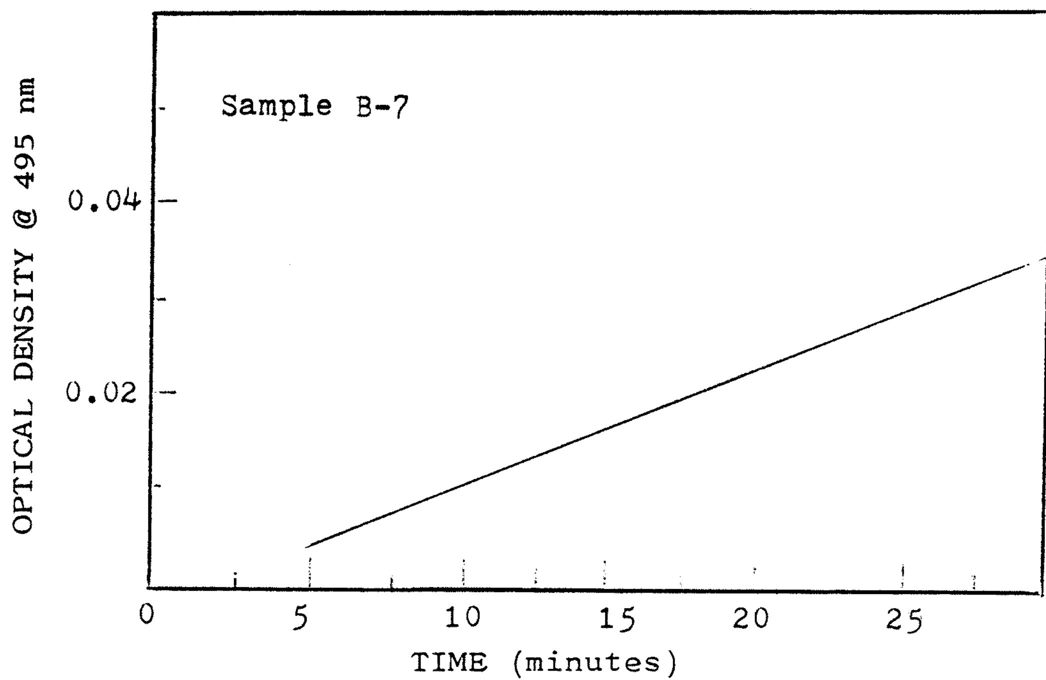


FIGURE H-2.56



APPENDIX I

SAMPLE CALCULATIONS

SAMPLE CALCULATIONS

Dilution Rate

The dilution rate, D is defined as the volumetric flow rate, F divided by the culture vessel volume, V:

$$D = F/V$$

For Run 27,

$F = 8.0$ ml/h (based on an average of all the measured flow rates for this run)

$V = 150$ ml

$$D = F/V = 8.0/150$$

$$D = 0.053\ h^{-1} \quad \text{(Run 27)}$$

Mean Residence Time

The mean residence time is equivalent to the time required for complete displacement of the volume within the culture vessel. This can be represented by the reciprocal of the dilution rate:

$$\bar{t} = 1/D$$

For Run 27,

$$\bar{t} = 1/D = 1/0.053$$

$$\bar{t} = 18.9\ h \quad \text{(Run 27)}$$

Number of Turnovers

The number of turnovers, N_T is used to characterize the conditions of a flow system irrespective of the specific flow rare or culture vessel volume. The operations of two completely different systems can thus be compared. N_T can be considered a dimensionless time parameter and is defined as the fermentation time, t divided by the mean residence time, $\bar{t}$:

$$N_T = t/\bar{t}$$

For Sample H of Run 27,

$$t = 83.2 \text{ h}$$

$$N_T = t/\bar{t} = 83.2/18.9$$

$\underline{N_T = 4.4}$ (Sample H, Run 27)

Biomass Concentration Determination

The biomass concentration, X in a chemostat or batch culture sample of *L. brevis* is obtained by multiplying the optical density of the sample, OD_x by the slope of the biomass standardization plot (FIGURE E-5), s_c:

$$X = s_c(OD_x)$$

For Sample H of Run 27,

$$OD_x = 0.44$$

$$s_c = 0.273 \text{ mg cells/ml/OD}$$

$$X = s_c(OD_x) = 0.273(0.44)$$

$$\underline{X = 0.12 \text{ mg cells/ml}}$$

(Sample H, Run 27)

Protein Concentration Determination

The concentration of the protein in the reaction sample, P is given by the difference between the optical densities of the sample (OD_s) and the Biuret standard (OD_B) multiplied by the slope of the Biuret standardization plot (FIGURE E-7), s_B:

$$P = s_B(OD_s - OD_B)$$

For Sample H of Run 27,

$$s_B = 7.36 \text{ mg protein/ml/OD}$$

$$OD_s = 0.078$$

$$OD_B = 0.052$$

$$P = s_B(OD_s - OD_B) = 7.36(0.078 - 0.052)$$

$$\underline{P = 0.191 \text{ mg protein/ml}}$$

(Sample H, Run 27)

Protein Mass Determination

The protein mass content in each assay sample is determined by multiplying the protein concentration of the reaction sample, P by the assay sample aliquot (o.1 ml) and the fraction of reaction sample in the total reaction mixture, f:

$$p = 0.1(f)(P)$$

For Sample H of Run 27,

$$f = 0.75$$

$$p = 0.1(0.75)(0.191)$$

$$\underline{p = 0.0143 \text{ mg protein}}$$

(Sample H, Run 27)

Arginine Deiminase Activity

Arginine deiminase activity, A corresponds to the rate of citrulline production in the reaction mixture. It is obtained by multiplying the initial rates slope of the assay plot of optical density versus time for the sample (s) by the slope of the assay standardization plot (FIGURE E-6) s_A:

$$A = s_s(s_A)$$

For Sample H of Run 27,

s_A = 0.291 umoles of citrulline/OD

s_s = 0.00411 OD/min

$$A = s_s(s_A) = 0.00411(0.291)$$

A = 0.00120 µmoles/min
A = 0.00120 units of activity

(Sample H, Run 27)

Specific Activity

The specific activity (S.A.) of an enzyme can be determined by dividing the activity, A by the protein mass content of the reaction sample, p:

$$S.A. = A/p = 0.00120/0.0143$$

S.A. = 0.084 units/mg protein

(Sample H, Run 27)

APPENDIX J

OPERATING MANUAL

OPERATING MANUAL

This operating manual summarizes the procedures used for operation of the chemostat and sample analysis. It can be used for quick reference during experimentation. Sample worksheets for recording pertinent data are also included.

Chemostat Assembly

1. The chemostat should be assembled before sterilization to verify that all equipment is operating properly (see Fig. J-1).

2. The temperature controller should be calibrated for a temperature of 30° C in the culture vessel each time the chemostat is assembled.

3. The operating volume within the vessel must be measured and recorded.

4. The chemostat is then disassembled and cleaned thoroughly.

5. The parts of the chemostat which need to be sterilized are autoclaved as indicated in Fig. J-2.

6. The temperature probe must be chemically sterilized by soaking it overnight in an antibacterial formaldehyde disinfectant.

7. 4-5 l. of medium are prepared in the 6 l. medium reservoir and autoclaved immediately.

8. The chemostat is reassembled. As the foil is removed from sterile segments, connections should be made as quickly as possible. Specific precaution should be made to avoid contamination when inserting the temperature probe into the culture vessel.

FIGURE J-1

THE CHEMOSTAT ASSEMBLY

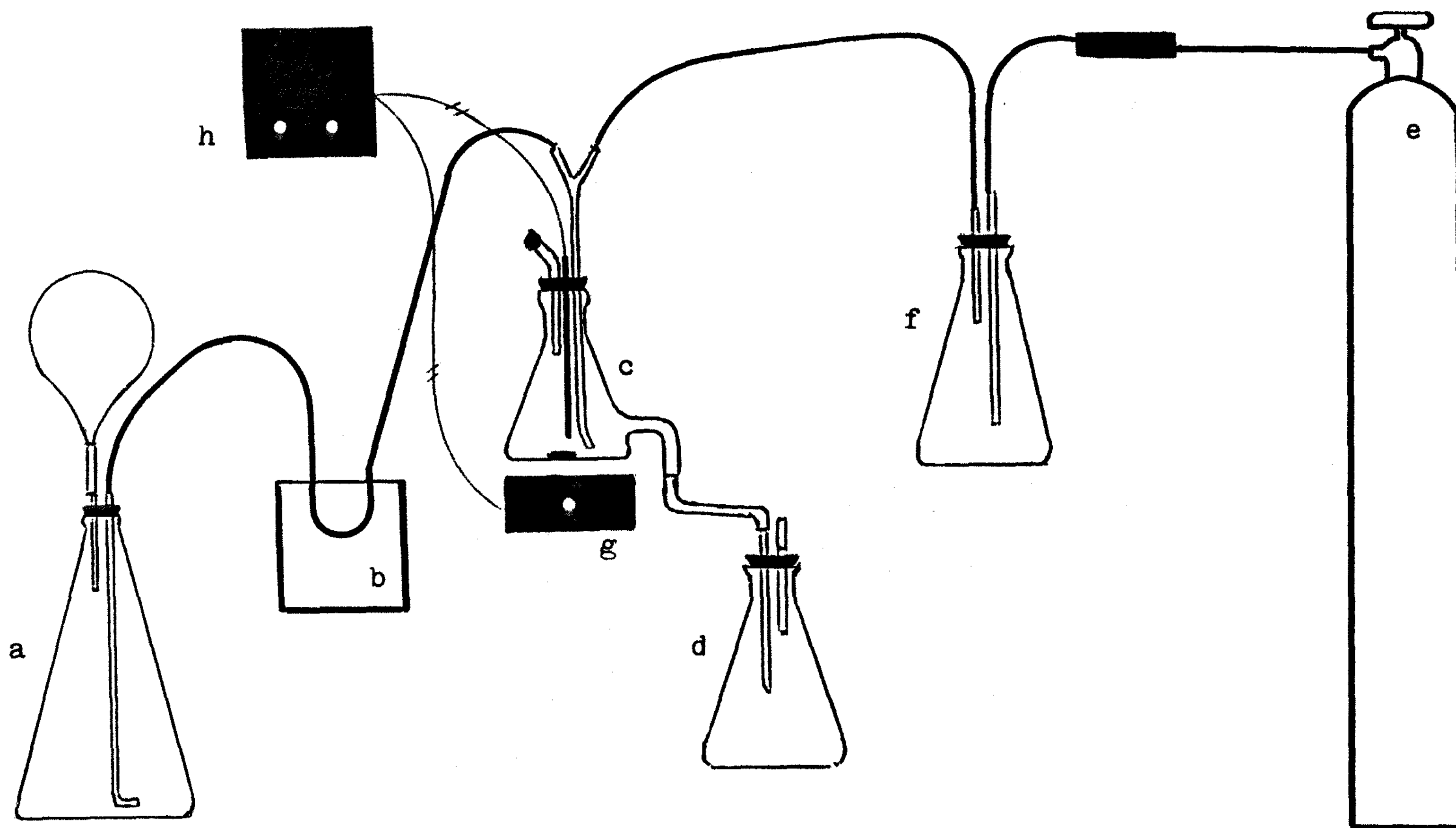
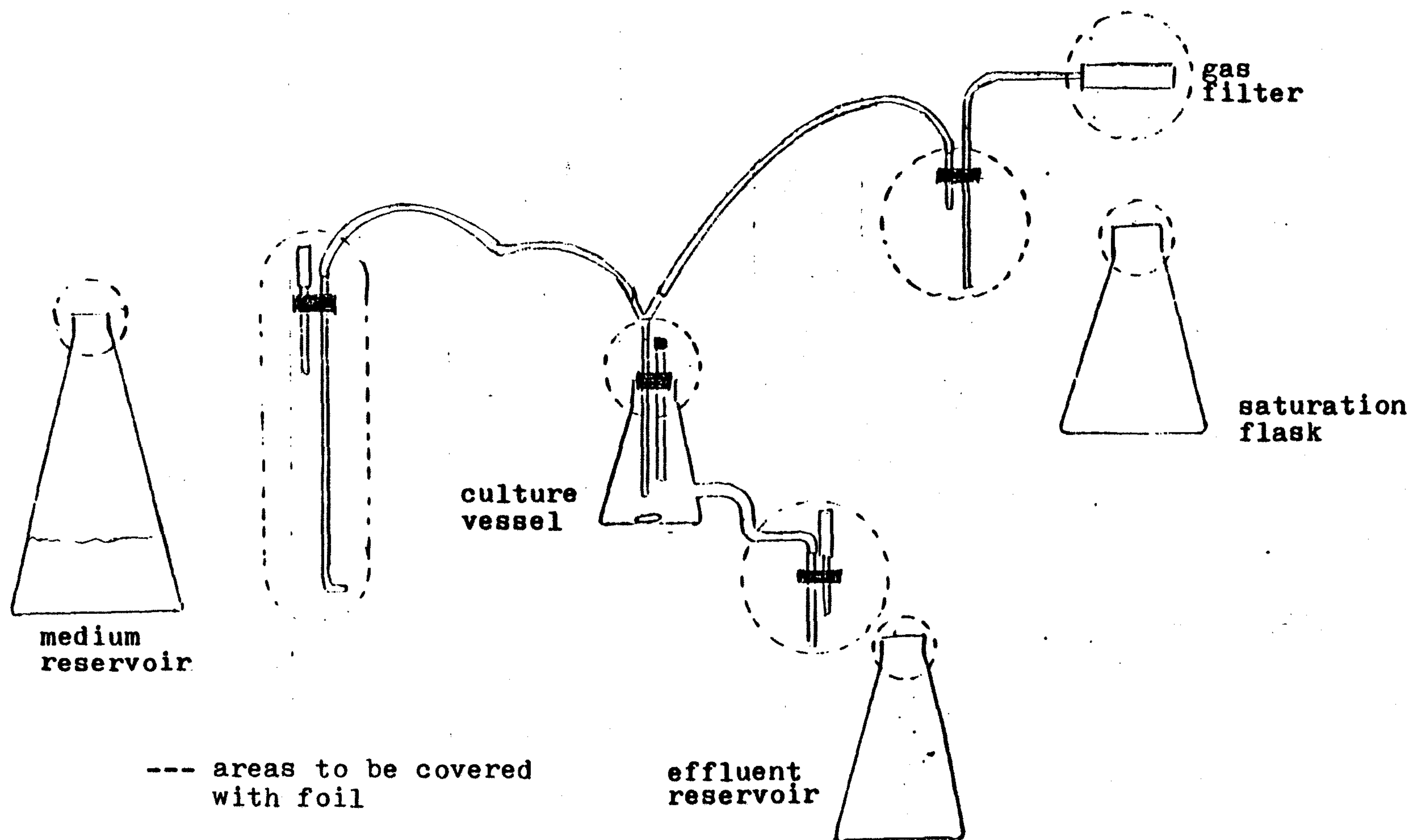


FIGURE J-2 CHEMOSTAT PARTS REQUIRING STEAM STERILIZATION



Chemostat Operation

1. Once assembled, the pump is turned on until the culture vessel is filled with medium (i.e., filled to the overflow tube). The pump is then switched off.

2. After approximately 24 hours, the culture vessel and medium reservoir are examined for contamination (turbidity in the liquid). If contamination is evident in the culture vessel only, the medium reservoir can be sealed with a sterile rubber stopper and retained for future use. The remainder of the apparatus must be cleaned and resterilized.

3. If no contamination is noted after 24 hours, the nitrogen gas is turned on and allowed to bubble through the growth medium. The pump is turned on to allow a 5 ml sample of pure medium to be taken. A 10 ml sterile culture tube is used and the sample is taken from the effluent portal.

4. The pump is switched off and the chemostat is ready for inoculation. A time record and check-off sheet is initiated at this point in the procedure (attached).

5. A test tube containing the pure culture inoculum is taken from refrigeration and the cells are mixed well--using a vortex, if available. 3 ml of inoculum is drawn from the test tube using a sterile 5 ml syringe.

6. The area around the inoculation port of the culture vessel is flamed and the inoculum is injected quickly into the port.

7. The temperature controller is turned on and the cells in the culture vessel are allowed to incubate for 2-3 days or until significantly turbid.

8. When turbid, the pump is started and the time is recorded. Incubation time can be then estimated.

9. The volume in the culture vessel (approx. 150 ml) is allowed to turnover at least four times for each run. Sampling is done at predetermined intervals.

10. A 120 ml sample is collected from the effluent portal in a 250 ml flask. The sample flask is kept on ice continuously to inhibit further growth of the organism. Sampling times (start and finish) are recorded.

11. The sample is divided into four parts: 100 ml for assay analysis, 10 ml for pH analysis, 5 ml for turbidity analysis, and 5 ml for purity analysis.

12. The pump is stopped and the cells in the culture vessel are allowed to incubate again until significantly turbid.

13. Steps 8-12 are repeated for each 1000 ml of growth medium available, using a different flow rate for each run.

<u>Analysis of Samples</u> (not used in the assay)

1. The 5 ml sample for purity analysis is tested in the same way as described in APPENDIX B. That is, the following tests are performed:

   a) Growth on nutrient agar
   b) Microscopic identification
   c) Gram stain
   d) Catalase test

2. The optical densities of the turbidity analysis samples are measured against a pure medium blank and recorded.

Preparation of the Cell Suspension

1. It is necessary to prepare a .1 M sodium phosphate buffer solution with a pH of 6.5.

2. Approximately 25 ml of the 100 ml assay sample is poured into a 50 ml capacity round-bottom polyethylene centrifuge tube. To expedite the following process, several centrifuge tubes can be used and the cells combined at a later time.

3. The tubes are arranged in the refrigerated centrifuge such that the weight is evenly distributed.

4. The sample is centrifuged at a speed of 10,000 RPM for 10 minutes. A pellet of cells will adhere to the bottom of the tube.

5. The liquid above the pellet (supernatant) is poured off and any original sample remaining is added to the tube. The pellet is dispersed (the cells are resuspended in solution) using a vortex, and the centrifugation process is repeated.

6. The supernatant is again poured off. The cells are washed by adding several milliliters of the buffer solution and then pouring it off.

7. If the sample was distributed in several tubes, it is convenient to combine the cells at this point. Several ml of the buffer solution are added to each tube. The cumulative volume should be about 10 ml. The cells are then resuspended and combined in one tube, being certain to collect all cells. Rinse the empty tubes with additional buffer solution if necessary.

8. The suspension is centrifuged again at 10,000 RPM for 10 minutes, then the washing process is repeated.

9. The pellet of cells should be kept on ice until used in the assay. The cells can be frozen if not being used immediately.

10. Before sonication, the pellet of cells is thawed and resuspended in 10 ml of buffer.

Cell Disruption

Immediately before the assay, the cells are disrupted by high frequency vibrations using an ultrasonic dismembrator (sonifier) by the föllowing procedure.

1. The polyethylene centrifuge tube containing the cell suspension is kept on ice at all times. A polyethylene (or other non-glass) beaker must be used to hold the ice, since glass will be shattered by the sonifier.

2. The probe of the sonifier is lowered into the tube. The probe should not be in contact with any surface. Ear phones are used by the operator for protection.

3. The intensity is set at the highest power, and the cells are disrupted for 30 seconds. The unit is switched off and a 30 second cooling interval is allowed.

4. The ice around the tube will melt, therefore the water must be poured off and new ice added periodically.

5. Sonication and cooling is repeated for a total of 8 30-second periods of sonication with 30-second cooling intervals.

6. The sonicated cells are centrifuged in a refrigerated unit at a speed of 14,500 RPM for 20 minutes.

7. The supernatant is retrieved for assay use and maintained on ice until that time.

Assay Procedure

1. The following reagents are prepared:

   -L-arginine HCL in buffer solution
   -Sulfuric/Phosphoric acid reagent
   -Diacetylmonoxime
   -Phenylhydrazine HCl

2. 6 ml of the supernatant is put in a separate tube and brought to a temperature of 30° C in a heated water bath. A 2 ml amount is put in a test tube and kept on ice for use in determining the protein content. The remaining supernatant is frozen.

3. Two milliliters of the L-arginine solution is also heated to 30° C in the same water bath.

4. A series of 21 sample tubes is prepared and labeled for the timed sample each will represent. One milliliter of the acid reagent is added to each tube to stop the reaction at each specified time. One test tube is labeled as a standard. (Time schedule is attached)

5. Six milliliters of the supernatant is added to the 2 ml L-arginine solution. This is the reaction mixture.

6. Timed samples are collected from the reaction mixture using a 0.1 ml micro-pipet. Each sample is immediately injected into the corresponding sample tube. Time is equal to zero at the instant the supernatant is mixed with the L-arginine solution. Samples are taken for 3 hours.

7. 0.1 ml of plain buffer solution should be added to the test tube labeled as a standard.

8. After the samples have been taken, 0.15 ml diacetylmonoxime, 0.15 ml phenylhydrazine HCl and 1.6 ml distilled water are added to each tube. The final volume in each test tube should be 3 ml.

9. The tubes are well mixed using a vortex. A marble or cap is used to cover each tube, allowing air to escape from the tube when heated. The tubes are placed in a boiling water bath for 10 minutes. From this point on, the sample tubes must be protected from light at all times.

10. The sample tubes are allowed to cool overnight (protected from light) after removing them from the boiling water bath. If it is necessary to read the optical density sooner, the tubes can be cooled in a cold water bath for at least 1 hour.

11. After the cooling period, each sample tube is vortexed and the optical density is read against a distilled water blank. The Gilford spectrophotometer is used at a setting of 495 nm. The samples are still light sensitive at this point, so proper precaution should be taken.

Protein Content Determination

1. It is necessary to prepare the following reagents:

   -Biuret reagent
   -6% sodium hydroxide

2. 2 ml of the 6% NaOH solution is added to 2 ml of the supernatant.

3. 0.2 ml of the Biuret reagent is added and the mixture is vortexed.

4. A standard tube is prepared by adding 2 ml of 6% NaOH and 0.2 ml of the Biuret reagent to 2 ml of 0.1 M sodium phosphate buffer (6.5 pH) and mixing well.

5. After 15 minutes at room temperature, the optical densities are read at a wavelength of 540 nm against a distilled water blank.

TIME SCHEDULE FOR ASSAY SAMPLING

1*	50
3	60
5	70
7	80
10	90
15	100
20	120
25	140
30	160
35	180
40	

* time intervals measured in minutes

CHEMOSTAT TIME RECORD

RUN____________

PUMP__________________ CULTURE VESSEL VOLUME (ml)______

PUMP SETTING _________ TURNOVER TIME (h)________________

TUBING ID (mm)________ DILUTION RATE (h^{-1})_____________

FLOW RATE (ml/h)______

MEDIUM COMPOSITION____

- -

TIME RECORD

INOCULATION___________ START PUMP_______________________

SAMPLE	START	END	TIME (h)	VOLUME (ml)
A				
B				
C				
D				
E				
F				
G				
H				

ASSAY DATA SHEET

SAMPLE ______

REACTION TIME(min)	OPTICAL DENSITY @ 495 nm			AVERAGE
	READING 1	READING 2	READING 3	

PROTEIN CONTENT

OPTICAL DENSITY @ 540 nm: STANDARD______ SAMPLE_______

SAMPLE DATA SHEET

RUN ___

SAMPLE	ELAPSED TIME(h)	NUMBER OF TURNOVERS	pH	OPTICAL DENSITY @ 550 nm

Dilution ratio given in parenthesis (S:M)

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