Title
PRODUCTION OP L-ASPARAGENASE BY COULTURES OP ERWINIA AROIDEAE

Authors
Murphy, Thomas F.
Wilke, Charles R.

Publication Date
1972-10-01

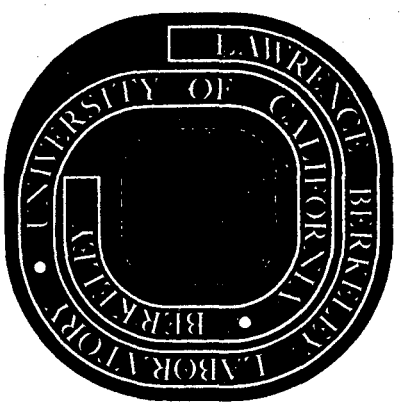
PRODUCTION OF L-ASPARAGINASE BY CULTURES OF ERWINIA AROIDEAE

Thomas F. Murphy* and Charles R. Wilke

October 1972

Prepared for the U.S. Atomic Energy Commission under Contract W-7405-ENG-48

*Filed as a M.S. Thesis



DISCLAIMER

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PRODUCTION OF L-ASPARAGINASE BY CULTURES OF ERWINIA AROIDEAE

Thomas F. Murphy and Charles R. Wilke

Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

ABSTRACT

Since 1967, L-asparaginase from E. coli has been used successfully in the treatment of certain human leukemias. Although more than half the patients treated in early stages undergo one remission, treatments for subsequent relapses often fail because patients develop immunity to the E. coli enzyme. Erwinia aroideae and other microorganisms produce L-asparaginases which are immunologically distinct from E. coli L-asparaginases and could be used successfully with patients immune to E. coli L-asparaginase. The production of L-asparaginase by cultures of Erwinia aroideae was studied to provide basic background information for large scale fermentation of the organism for use as an alternate source of L-asparaginase.

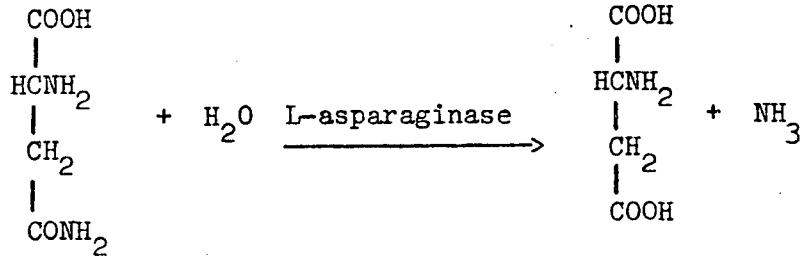
Five liter stirred jar batch and continuous cultures were used to analyze the effects of medium composition, temperature, pH, dissolved oxygen concentration, and growth rate on production of L-asparaginase by submerged fermentations of Erwinia aroideae. Experimental work was directed toward maximizing specific L-asparaginase activity. Maximum specific activity leads to minimum recovery and purification costs. These costs are probably primary in determining the cost of L-asparaginase produced for medical use.

Survey of complex and chemically defined media showed that L-asparaginase is inducible in Erwinia aroideae and that high specific activities are obtained when L-asparagine is the sole carbon and nitrogen source. In this case, production of L-asparaginase is growth associated and specific activity, growth rate, and cell mass yield are all maximized by the same set of fermentation conditions. The optimum temperature is 29°C and the optimum pH is 7.0. Operation with pH control is essential for optimum L-asparaginase production. Submerged cultures must be vigorously aerated for best L-asparaginase production, although dissolved oxygen tensions above atmospheric are not beneficial. Growth of Erwinia aroideae in L-asparagine limited continuous culture is characterized by specific L-asparaginase activity independent of growth rate and cell mass yield which decreases with increasing growth rate.

Two areas of further investigation are indicated by this study. First, two stage cultures, batch or continuous, may be advantageous in increasing the efficiency of L-asparagine use for L-asparaginase induction. Second, other more readily available substances may also be effective L-asparaginase inducers. In the event production of L-asparaginase from Erwinia aroideae becomes a reality, these areas should be investigated.

INTRODUCTION

L-asparaginase is an enzyme which catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia:



L-asparaginase activity is measured in International Units (IU), defined as the amount of enzyme able to produce one micromole of ammonia per minute at 37°C when the enzyme is saturated. L-asparaginase is present in a variety of plant tissues, animal tissues, and microorganisms.

Current interest in the enzyme is largely due to its use in the treatment of certain human leukemias. Unlike other anti-cancer drugs, L-asparaginase is toxic only to neoplastic cells. Early workers hoped that the enzyme would later prove useful against a wide variety of cancers, and thus make it an ideal cancer treatment. L-asparaginase is now known to be effective against only three cancers: acute lymphoblastic leukemia, leukemic lymphosarcoma, and lymphosarcoma. More than half the patients treated in early stages achieve a "complete" remission (median duration, sixty days). Far fewer achieve a permanent remission. Patients typically suffer side effects such as nausea and anorexia when treated and about twenty percent show an allergy to the drug. Early patients also suffered fevers when treated with L-asparaginase, although the availability of high

purity preparations has now minimized this problem. One of the most serious limitations of L-asparaginase chemotherapy is that patients often develop an immunity to the drug and will not respond to treatment with it for a relapse following an earlier remission. Despite its limitations, L-asparaginase, used alone or in combination with other drugs, is considered the most effective treatment for several forms of leukemia at this time.

The first L-asparaginase available for clinical trials was taken from guinea pig serum. A typical treatment leading to a complete remission involves intravenous injection of about one million IU over a period of several weeks. Production of this much L-asparaginase would require approximately 20,000 guinea pigs. Growth of L-asparaginase producing microorganisms in submerged culture presents a much more feasible alternative source of the drug. Escherichia coli and other microorganisms produce cancer inhibitory L-asparaginases and are now the source of the enzyme for clinical use.

Most L-asparaginase fermentation research has been with E. coli and it is the source of the currently available L-asparaginase preparations. Chemically and immunologically distinct L-asparaginases with antitumor activity have been found in other microorganisms, such as Erwinia aroideae. The availability of such different L-asparaginases in amounts sufficient for clinical use would be of considerable practical advantage in treating patients who develop immunity to E. coli L-asparaginase. For this reason, a systematic analysis of L-asparaginase production in Erwinia aroideae fermentations was made. This analysis includes batch

and continuous cultures. Medium composition, temperature, pH, dissolved oxygen concentration, and age of culture were studied for their effects on specific L-asparaginase activity (IU per mg. cellular protein), growth rate, and yield of cell mass in batch cultures. In continuous culture, the effect of specific growth rate (i.e. dillution rate) on specific L-asparaginase activity and concentrations of cell mass, substrate, and L-asparaginase was studied. The importance given to specific activity (IU/mg. protein) as opposed to L-asparaginase concentration (IU/ml.) comes from the premise that the cost of producing an enzyme of acceptable purity for medical use is determined largely by purification and not synthesis operations.

This research does not constitute a true optimization of L-asparaginase activity in Erwinia aroideae fermentations. Each variable of interest was studied independently of all others, which were controlled at constant values. Rigorously, the effect of any one parameter on L-asparaginase activity is a function not only of its own value, but of all the other parameters as well. A true optimization of four variables would require at least 1000 experiments. Nevertheless, the experimental results of this study are significant in showing the sensitivity of the fermentation to each variable independent of the others, showing the influence of the different variables relative to each other, and comparing batch and continuous fermentation.

BACKGROUND

L-asparaginase as an Antitumor Drug

Like a number of other events in scientific history, the discovery of the antitumor property of L-asparaginase began fortuitously. In 1953, Kidd (37) reported the results of experiments in which mice bearing transplanted lymphomas were injected with a mixture of serum from rabbits immunized against lymphoma and guinea pig serum. Kidd was hopeful that antiboidies in the rabbit serum would have an immunological reaction with the rat tumor and that compliment in the guinea pig serum would enhance the immune response. Instead, he observed that tumors in mice injected with the sera mixture and in control mice injected only with guinea pig serum both regressed. Although Kidd concluded that guinea pig serum was active against tumors, the active component and its mechanism of action were unknown.

Broome (5), working in the same laboratory as Kidd, conclusively demonstrated in 1961 that L-asparaginase was the active antitumor component of guinea pig serum. This conclusion was further supported by Clementi's (15) observation in 1922 of high L-asparaginase levels in the blood of guinea pigs and by several demonstrations of an L-asparagine requirement for *in vitro* growth of several tumors (43,25).

In 1966, Dolowy *et al.* (20) reported the use of a partially purified L-asparaginase preparation from guinea pig serum in the treatment of an eight year old boy with acute lymphoblastic leukemia. Although this patient died of complications in ten days, a reduction in blast cells was noted. Hill *et al.* (32), using *E. coli* L-asparaginase, reported the

first complete remission of acute lymphoblastic leukemia in 1967. Oettgen et al. (44) also reported some success with E. coli L-asparaginase treatments in 1967.

The mechanism of L-asparaginase toxicity to neoplastic cells is not known at this time. Many kinds of tumor cells do not contain L-asparagine synthetase and require an external supply of L-asparagine synthesized by normal cells for growth. When L-asparaginase is injected into the bloodstream, it depletes the available L-asparagine and thus stops proliferation of the dependent tumor cells. While not all tumors requiring an exogenous L-asparagine source are treatable with L-asparaginase, those tumors which are treatable with the enzyme are all dependent on an external supply of L-asparagine. Death of dependent tumor cells when treated with L-asparaginase may be associated with increased ribonuclease activity observed in regressing tumors. It has been suggested that L-asparaginase may remove an inhibitor of ribonuclease which, by increasing ribonuclease activity, could result in unbalanced protein synthesis and death of the tumor (42). It is significant that L-asparaginases from different sources are not all toxic to susceptible neoplasms. The failure of some L-asparaginases to stop tumor growth is often associated with rapid clearance of these enzymes from the patients' blood.

Acute lymphoblastic leukemia is the most sensitive human cancer to L-asparaginase chemotherapy. Typically, doses of 200 to 1000 IU per kg. body weight are administered by daily intravenous injection over a period of two to four weeks. Reported complete remission rates vary widely, averaging a little more than fifty percent. The complete remission rate

is apparently independent of the dose size within the above limits, although the duration of the remission may increase for large doses of 1000 IU/kg. or more. Other cancers have been successfully treated with L-asparaginase. However, the response rates have been less than that for acute lymphoblastic leukemia.

Cytotoxic agents such as vincristine, cytosine arabinoside, actinomycin D, daunorubicin, adriamycin, and various cytosine antagonists have had a synergistic effect when applied with L-asparaginase in treatment of mouse tumors and may prove useful against human cancers (18). The success of L-asparaginase chemotherapy has stimulated interest in the use of inhibitors of L-asparagine synthesis in combination with L-asparaginase (10). Similarly, amino acid analogues of L-asparagine may be useful theraputic agents (14).

L-asparaginase has a reported half life of eight to thirty hours in plasma, independent of the dosage or disease (10). The enzyme's high clearance rate from the blood along with patients' acquired immunity to it are major weaknesses of L-asparaginase as an antileukemia drug. The use of extra-corporeal devices and insolubilized L-asparaginase inside the body have been suggested as solutions to these problems. Hasselberger et al. (27) found that E. coli L-asparaginase could be bonded covalently to several water insoluble matrices, increasing its in vitro stability. They suggested the use of insolubilized L-asparaginase in an extracorporeal L-asparagine filter, thus reducing problems of in vivo instability and immunity to the drug. Weetal (59) covalently bonded E. coli L-asparaginase to a Dacron vascular prosthesis and observed that the immobilized enzyme

retained 44.5% of its initial activity after seven days' implantation in a dog. Chang (12) microencapsulated E. coli L-asparaginase and injected it into mice bearing transplanted lymphosarcoma. The time preceding lymphosarcoma appearance was much increased when compared to that of mice injected with L-asparaginase solution. Apple (2) dialyzed blood of leukemia patients against L-asparaginase in a small cellulose capillary dialyzer and demonstrated an eighty percent removal of L-asparagine from the blood.

Production of L-asparaginase by Microorganisms

Despite the promising early observations of tumor inhibition by guinea pig serum L-asparaginase, sufficient amounts for treatment of a large number of patients were not initially available. In 1964, Mashburn and Wriston (41) reported that an L-asparaginase extracted and purified from Escherichi coli B had activity against tumors comparable to guinea pig serum L-asparaginase. E. coli is an attractive source of large amounts of L-asparaginase because it can be grown quickly in large scale submerged fermentations using inexpensive media. A number of other microorganisms produce L-asparaginases, although not all of these enzymes are tumor inhibitory. Pseudomonas species produce an L-asparaginase-L-glutaminase complex with only weak antitumor activity (21). Cancer inhibitory L-asparaginases have also been discovered in Aspergillus terreus (19) and an avirulent strain of Mycobacterium tuberculosis (49). Presently, the most attractive organisms for large scale L-asparaginase production are E. coli, Serratia marcescens, Erwinia carotovora, and Erwinia aroideae.

These are similar enteric bacteria which produce high yields of tumor inhibitory L-asparaginases when grown on inexpensive media in submerged culture. All of the L-asparaginase manufactured commercially at this time is from E. coli. Interest in the development of large scale L-asparaginase production using other organisms has decreased in recent years as the limited applicability of L-asparaginase chemotherapy became apparent. Nevertheless, there is a need for immunologically different L-asparaginases from other sources for treatment of leukemia patients with an acquired immunity to E. coli L-asparaginase.

The properties of E. coli L-asparaginase have been widely studied and, as a result, the enzyme is now fairly well characterized. Campbell et al., (8) found two distinct L-asparaginases in E. coli, which they designated EC-1 and EC-2. Only the EC-2 L-asparaginase was found to be tumor inhibitory. The two enzymes have different pH optima which can be used to determine the proportion of each enzyme in E. coli extracts. Whelen and Wriston (61) reported a molecular weight of 139,000 for EC-2 L-asparaginase. This value has been corroborated by other investigators. Some early confusion existed concerning the subunit nature of the EC-2 enzyme, although it is now generally accepted that there are four subunits of about 35,000 molecular weight with one active site each. The amino acid composition of the enzyme has been determined, indicating a large number of L-asparagine residues (61). The specific activity of pure EC-2 L-asparaginase is about 300 IU/mg. (33), although higher values have been reported. Reported isoelectric points vary, although the value

of 4.85 is typical (61). The pH optimum is broad, with maximum activity occuring between pH 6 and pH 8 (8). EC-2 L-asparaginase has a Michaelis constant of 1.25×10^{-5} M (6). E. coli L-asparaginase usually exhibits a small amount of L-glutaminase activity (8). D-asparagine and L-asparagine analogues such as the β-hydrazide and β-hydroxamate of L-asparagine are inhibitors of E. coli L-asparaginase (17). Ammonia has also been reported to inhibit at basic pH's (9).

Since the discovery of a tumor active L-asparaginase from E. coli, there has been considerable effort in developing fermentations of this organism with high yields of L-asparaginase. Schwartz et al. (53) grew E. coli K12 in a stirred jar fermentor and observed that L-asparaginase activity of well aerated cultures increased by more than one order of magnitude when dense cultures were held without aeration for twenty minutes and then harvested. Roberts et al. (50) screened a number of bacteria, molds, and yeasts for L-asparaginase activity in corn steep liquor shake flask cultures at 37°C. E. coli HAP was found to have the highest activity in corn steep medium. Other media were tried with this organism, but none was superior to corn steep liquor. These investigators observed a strong maximum in specific activity (IU/g. dry weight) during the late exponential growth phase. They also found that specific L-asparaginase activity decreased in proportion to the initial glucose content of the medium, and that aerobic growth resulted in twice as much activity as anaerobic growth on corn steep medium. Cedar and Schwartz (11) grew E. coli K12 in a tryptone-yeast extract-glucose medium in shake flasks. They found a quick

100 to 1000 fold rise in specific activity forty minutes after shaking was stopped. Specific activities of anaerobic cultures were about the same as the two cycle shake flask cultures. They found that additions of L-asparagine or other amino acids to the medium did not induce L-asparaginase and that L-asparagine could not be used aerobically or anaerobically as the sole carbon source. Robison and Berk (52) screened several E. coli strains for L-asparaginase activity in casein hydrolysate-soy peptone-glucose shake flask cultures and found E. coli B to have the highest specific activity. They found that a low sugar or glycerol concentration in the medium was necessary for highest specific activity and that supplements of L-asparagine, L-aspartic acid, and L-glutamic acid were not beneficial. Essentially growth associated L-asparaginase production was observed. The temperature for growth yielding maximum specific activity was found to be 25°C. Bilimoria (3), working with E. coli UM, found that stationary flasks with sparged air had dense growth but low specific activity. Cultures with rotary shaking had both dense growth and high specific activity. A casein hydrolysate-yeast extract medium was superior to a peptone-beef extract-L-asparagine medium in this study. Boeck et al. (4) grew a phage resistant mutant of E. coli B in stirred jars using a casein hydrolysate-soy medium. A two cycle system of aerated growth followed by static holding without aeration was found to increase L-asparaginase activity. An addition of glucose at the beginning of the static cycle and control at pH 7.5 lead to highest activity. These workers found L-asparaginase activity in well aerated cultures to be very high and non-growth associated, with maximum activity occuring during the mid-exponential phase of growth. The two cycle

technique resulted in no improvement in activity when tried with cultures of several other gram negative L-asparaginase producing microorganisms. Erwinia aroideae was not among those tested. Hernadi et al. (31) screened 136 E. coli strains for L-asparaginase activity in 2% corn steep liquor shake flasks at 37°C. They followed production of the two L-asparaginases by the highest producing E. coli strain and observed that the EC-1 enzyme has maximum activity during exponential growth, while the EC-2 enzyme has maximum activity during stationary phase.

Another organism whose fermentations have been studied for possible large scale development is Serratia marcescens. Heinemann and Howard (28) screened several S. marcescens strains in 4% autolyzed yeast extract shake flask cultures for L-asparaginase activity. With their most productive strain, they found 4% autolyzed yeast extract medium to be superior to several other complex media and that the presence of sugars in the medium inhibits L-asparaginase synthesis. These investigators observed that synthesis of L-asparaginase in shake flask cultures took place only after the dissolved oxygen concentration reached zero. Heinemann et al. (29) later reported more conclusive evidence that S. marcescens synthesizes L-asparaginase only in oxygen limited cultures. In pilot fermentor experiments, fermentations with a low oxygen transfer rate which were oxygen limited had about twice the L-asparaginase activity of well aerated, non-oxygen limited fermentations. Khan et al. (36) found that S. marcescens L-asparaginase is inducible by the presence of L-asparagine or L-glutamic acid in the medium. Addition of 0.3% L-asparagine to a 1% peptone-0.5% beef extract-0.5% yeast extract shake flask medium increased specific

L-asparaginase activity about fourfold. These workers also reported that addition of sugars to the medium caused small increases in activity.

Erwinia aroideae, like S. marcescens, produces a tumor inhibitory L-asparaginase which is immunologically different from E. coli L-asparaginase EC-2 (47). These enzymes may be useful in treating cases where antigenic reactions to the EC-2 enzyme are expected. Although Erwinia aroideae L-asparaginase has not been as widely studied as E. coli L-asparaginase, a number of its properties have been reported. Peterson and Ciegler (47) first reported high yields of an L-asparaginase from Erwinia aroideae, which was serologically distinct from both E. coli and S. marcescens L-asparaginases and active against tumors in mice. Unlike E. coli, Erwinia aroideae produces only one L-asparaginase of about 122,500 moleculr weight (55). The specific activity of the pure enzyme is between 550 and 600 IU/mg. (55). Erwinia aroideae L-asparaginase is a basic protein with an isoelectric point of 8.2 (55). The pH optimum is between pH 7 and pH 8, and is less broad than that of the EC-2 enzyme (55). The enzyme has a Michaelis constant of 3×10^{-3} M, which is much larger than that of EC-2 L-asparaginase (47). British researchers have also experimented with L-asparaginase from Erwinia carotovora and suggest that this microorganism may be another attractive alternate source of the enzyme (57).

Relatively little has been published concerning submerged fermentation of Erwinia aroideae for production of L-asparaginase. Peterson and Ciegler (47) grew Erwinia aroideae in shake flasks on a 0.5% tryptone-0.5% yeast extract-0.1% glucose phosphate buffered medium at 28°C. They observed that during the first three hours of growth, the pH dropped from

pH 7.5 to pH 6.8. No L-asparaginase activity was detected during this interval. Later, the pH rose to a final value of pH 8.4 and essentially growth associated L-asparaginase production took place. This pH-time profile suggests that glucose probably was metabolized first, resulting in acid production, followed by metabolism of free amino acids in the medium and consequent base (NH_3) formation. If this is the case, then synthesis of L-asparaginase by Erwinia aroideae is associated only with metabolism of the free amino acids. These investigators also studied the effect of aeration on L-asparaginase activity of stirred jar fermentations of Erwinia aroideae. Mild aeration (low air flow, low agitation rate, fermentor without baffles) promoted highest specific activity, while vigorous aeration (high air flow, high agitation rate, fermentor with baffles) resulted in lowest specific activity. This behavior is similar to the dissolved oxygen dependence of S. marcescens. Peterson and Ciegler (48) later reported further analysis of medium and aeration influences on L-asparaginase production by Erwinia aroideae. Each component of the medium described above was added in two different concentrations to the original medium in shake flasks in an effort to determine which was responsible for L-asparaginase synthesis. Only yeast extract promoted L-asparaginase synthesis in proportion to the original substrate concentration. The other components appeared to have little influence. Substitutes for yeast extract were tested with the original medium, although none proved superior. The effect of aeration on shake flask cultures growing at several yeast extract concentrations was studied. Highest activity (IU/ml.) occured when the yeast extract concentration was 5% and aeration conditions were most vigorous. The

results of this aeration study are difficult to interpret because L-asparaginase activities were reported on a volumetric (per ml.) basis rather than a specific (per g. dry weight or per mg. protein) basis. The published data actually suggest that yeast extract as a substrate in low concentrations may result in constant specific activity and decrease specific activity at higher concentrations. Liu and Zajic (38) analyzed the separate roles of carbon sources and nitrogen sources in L-asparaginase production by Erwinia aroideae. The carbon source which gave highest specific activity in shake flasks also containing tryptone and yeast extract was lactose. The nitrogen source which gave highest specific activity in shake flasks also containing lactose was 0.5% tryptone-0.5% yeast extract. Actually, the terms "carbon source" and "nitrogen source" used in this research are misleading in that Erwinia aroideae is able to use the free amino acids in the various nitrogen sources as both carbon and nitrogen source. Therefore, the medium promoting highest L-asparaginase specific activity need not be a mixture of a carbohydrate and an organic nitrogen source.

A summary of L-asparaginase specific activities reported for E. coli, S. marcescens, and Erwinia aroideae by various investigators is given in Table 1.

Table 1. Specific L-asparaginase Activities of Several Microbial Fermentations

Organism	Maximum L-asparaginase Activity (IU/g. dry wt.)	Investigators
E. coli K12	48*	Schwartz et al. (53)
E. coli HAP	960	Roberts et al. (50)
E. coli K12	130	Cedar and Schwartz (11)
E. coli B	533	Robison and Berk (52)
E. coli B	848	Bilimoria (3)
E. coli B†	6600	Boeck et al. (4)
E. coli	402	Hernadi et al. (31)
S. marcescens	400**	Heinemann and Howard (28)
S. marcescens	57***	Khan (36)
Erwinia aroideae	1250	Peterson and Ceigler (47)
Erwinia aroideae	1080	Liu and Zajic (38)

*Reported as 0.096 IU/mg. protein. Estimated by assuming 50% of dry weight is protein.

**Reported as 100 IU/g. wet cells. Estimated by assuming 75% of wet weight is water.

***Reported as 0.113 IU/mg. protein. Estimated by assuming 50% of dry weight is protein.

†Phage resistant mutant.

THEORY

Batch Culture of Microorganisms

Growth of microorganisms on a single limiting substrate in batch culture characteristically is in five different phases: (1) lag phase, (2) exponential phase, (3) period of decreasing growth rate, (4) stationary phase, and (5) death phase. A typical batch growth curve is shown in Fig. 1. A lag period of no growth may follow inoculation of a fresh medium with cells unadapted to the new medium. During the lag period, cells and synthesize additional enzymes needed to begin growing in the new medium. A lag period is usually not observed when cells for inoculation are grown in the same medium found in the new culture. Exponential growth of microorganisms follows first order kinetics:

$$\frac{dN}{dt} = \mu N \quad . \qquad (1)^*$$

For well mixed, constant volume systems this reduces to

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

When one or more substrates in the medium near depletion, the growth rate decreases from its maximum value (exponential phase) to zero (stationary phase). The dependence of growth rate on substrate concentration is often modeled by the Monod equation:

$$\mu = \frac{\mu \text{ max } S}{Ks + S} \quad , \qquad (3)$$

where Ks is usually quite small. When oxygen is the substrate which limits growth, a different terminolgy is often used.

*Refer to Nomenclature of p. 83.

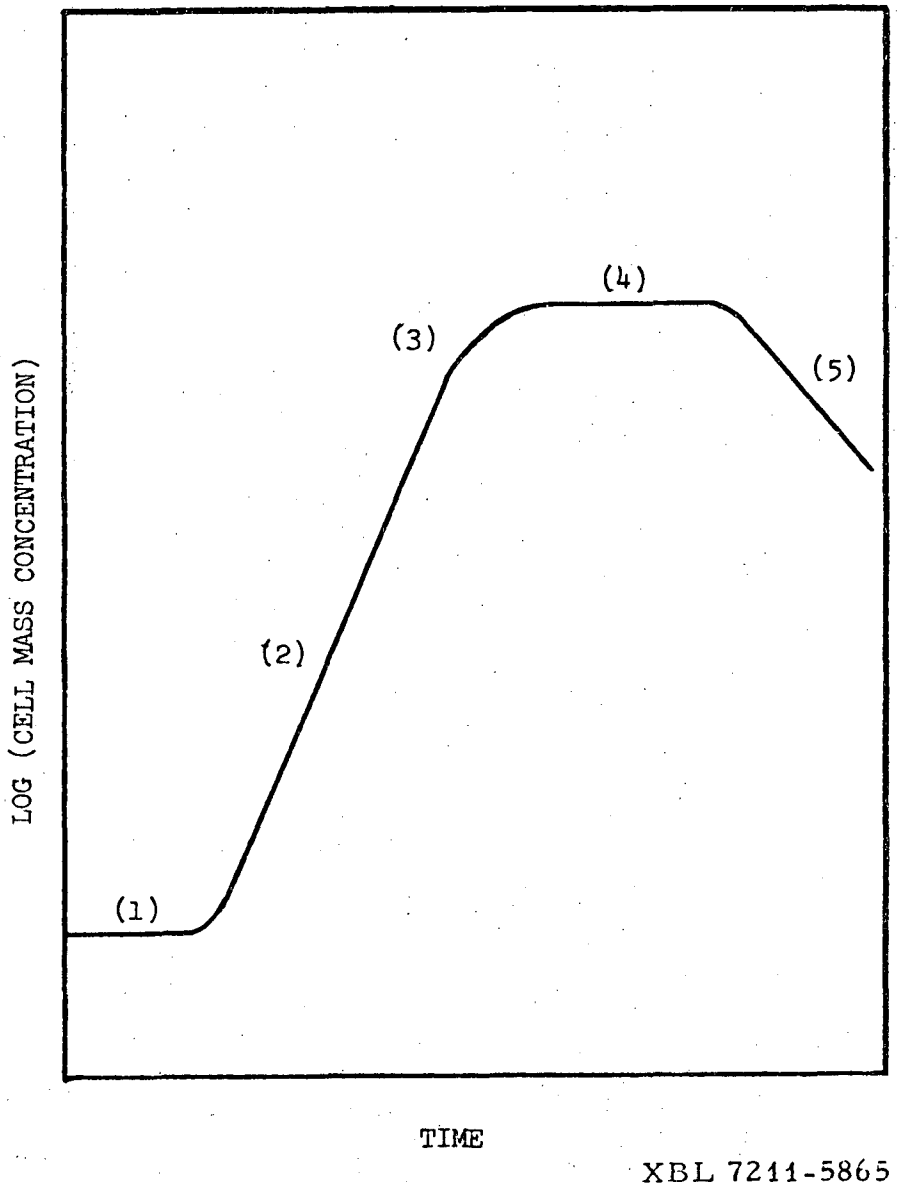


Fig. 1. Typical batch growth curve showing (1) lag phase, (2) exponential phase, (3) period of decreasing growth rate, (4) stationary phase and (5) death phase.

The respiration rate is defined (S = dissolved oxygen concentration) as

$$Q_{O_2} = \frac{1}{X}\frac{dS}{dt} \quad . \tag{4}$$

The dissolved oxygen concentration at which the respiration rate first becomes less than its maximum value is called the critical oxygen concentration.

In order to use the Monod equation to describe uptake of substrate or production of cells, it is necessary to define a yield factor for substrate conversion to cell mass:

$$Y_{x/s} = \frac{dX/dt}{-dS/dt} \quad . \tag{5}$$

Often it is assumed that $Y_{x/s}$ is a constant, independent of S for a given culture system and thus

$$Y_{x/s} = \frac{X - Xo}{So - S} \quad . \tag{6}$$

The validity of this assumption can be tested, as will be described later. A yield factor for product (extracellular or intracellular) formation can be defined in terms of cell mass concentration:

$$Y_{p/x} = \frac{dP/dt}{dX/dt} \tag{7}$$

If the product is growth associated, $Y_{p/x}$ is a constant independent of X and

$$Y_{p/x} = \frac{P - Po}{X - Xo} \quad . \tag{8}$$

The yield of product can also be written in terms of substrate concentration:

$$Y_{p/s} = \frac{dP/dt}{-dS/dt} = Y_{p/x}Y_{x/s} \quad . \tag{9}$$

When $Y_{x/s}$ and $Y_{p/s}$ are both constant, $Y_{p/s}$ is also a constant:

$$Y_{p/s} = \frac{P - Po}{So - S} \quad . \tag{10}$$

When S is large compared to Ks and $Y_{x/s}$ is constant, the integrated forms of Eq. (2) reduce to

$$X = Xo\, e^{\mu max\, t} \quad , \tag{11}$$

$$S = So - \frac{Xo}{Y_{x/s}} \left(e^{\mu max\, t} - 1\right) \quad , \tag{12}$$

$$P = Po\, e^{\mu max\, t} \quad . \tag{13}$$

General implicit relationships between X, S, P, and t can be found by integration of Eq. (2) with appropriate substitutions, however no explicit equations can be written.

Continuous Culture of Microorganisms

Batch submerged fermentation can be modified to incorporate continuous flow of fresh nutrients into the fermentor and continuous flow of cell suspension out of the fermentor. Several assumptions are made to simplify mathematical modeling of continuous culture: (1) the tank is well mixed so that the condition of the exit stream is identical to the condition of the fluid in the tank, (2) only one substrate limits the growth rate, (3) the Monod equation describes the relationship between limiting substrate concentration and growth rate, and (4) $Y_{x/s}$ is a constant, independent of the growth rate.

A cell mass balance can be written around the continuous fermentor (also called a chemostat):

$$\begin{matrix}\text{cell mass} \\ \text{flow in}\end{matrix} + \begin{matrix}\text{cell mass} \\ \text{generation}\end{matrix} = \begin{matrix}\text{cell mass} \\ \text{flow out}\end{matrix} + \begin{matrix}\text{cell mass} \\ \text{accumulation}\end{matrix} \qquad (14)$$

At steady state, there is no accumulation of cell mass and

$$FXo + V\frac{dX}{dt} = FX \quad . \qquad (15)$$

$\frac{dX}{dt}$ is given by Eq. (2). If no cells enter the fermentor, Xo = 0 and

$$\mu = \frac{F}{V} = D \quad . \qquad (16)$$

If Xo $\neq$ 0 (e.g. in systems with recycle or more than one stage),

$$\mu = \left(\frac{X - Xo}{X}\right) D \quad . \qquad (17)$$

Similarly, a substrate mass balance can be written around the chemostat:

$$\begin{matrix}\text{substrate} \\ \text{flow in}\end{matrix} = \begin{matrix}\text{substrate} \\ \text{flow out}\end{matrix} + \begin{matrix}\text{substrate} \\ \text{depletion}\end{matrix} + \begin{matrix}\text{substrate} \\ \text{accumulation}\end{matrix} \qquad (18)$$

At steady state, there is no accumulation of substrate and

$$DSo = DS + \left(\frac{dS}{dt}\right)_{\text{growth}} \qquad (19)$$

Using $\left(\frac{dX}{dt}\right)_{\text{growth}} = \mu X$ from Eq. (2) and Eqs. (3), (6), (9), and (16), Eq. (19) can be factored to give

$$S = \frac{DK_s}{\mu max - D} \quad , \qquad (20)$$

$$X = Y_{x/s}\left(So - \frac{DK_s}{\mu max - D}\right) \quad , \qquad (21)$$

$$P = Y_{p/s}\left(So - \frac{DK_s}{\mu max - D}\right) \quad . \qquad (22)$$

The volumetric rate of production of cell mass and product are defined as DX and DP respectively:

$$DX = Y_{x/s}D\left(So - \frac{DK_s}{\mu max - D}\right) , \tag{23}$$

$$DP = Y_{p/s}D\left(So - \frac{DK_s}{\mu max - D}\right) . \tag{24}$$

Typical plots of Eqs. (20), (21), (22), (23), and (24) are shown in Fig. 2. Setting the first derivative of either Eq. (23) or (24) equal to zero and solving for D gives the dilution rate at which productivity is maximum:

$$D = \mu max\left(1 - \sqrt{\frac{Ks}{Ks + So}}\right) . \tag{25}$$

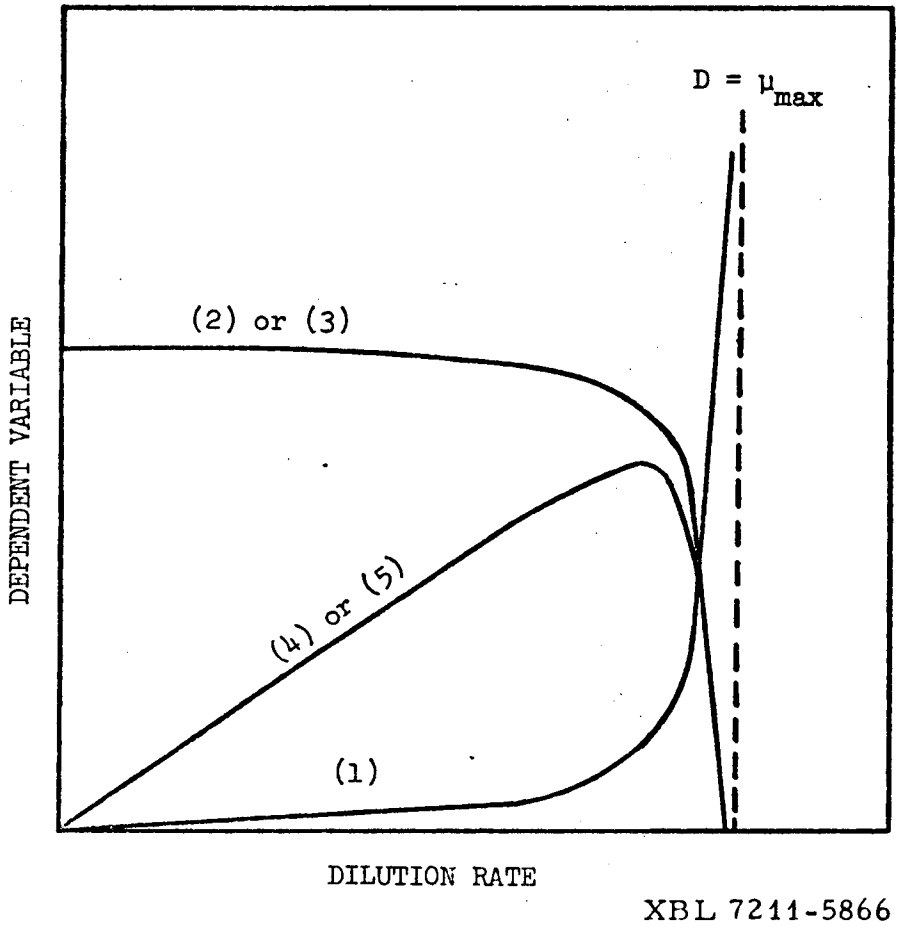


Fig. 2. Typical steady state dependence of (1) substrate concentration, (2) cell mass concentration, (4) cell mass productivity, and (5) growth associated product productivity on dilution rate based on Monod equation.

EXPERIMENTAL

Analytical Procedures

a. Cell Mass Concentration Determination

Cell mass concentrations of microbial suspensions are sometimes measured by first oven drying or freeze drying suspensions of known volume and then weighing the dried material. However, cell mass concentrations found in this manner can often be correlated to light absorbance measured with a spectrophotometer, thus permitting rapid indirect cell mass concentration determination by absorbance measurement of cell suspensions. Samples aseptically withdrawn from a growth vessel were either diluted with distilled water and placed in glass cuvettes or, when the samples were sufficiently dilute, placed directly in cuvettes. The cuvettes were inserted into a Fisher Electrophotometer II and their absorbances read at 650 mμ against a distilled water blank. Dilute cultures growing in sidearm flasks were analyzed simply by inserting the suspension filled arm of the flask into the spectrophotometer. Absorbances of uninoculated media were measured at the appropriate dilutions and then subtracted as blank readings from cell suspension absorbances. The net absorbance values were then converted to cell mass concentrations by use of a standard curve.

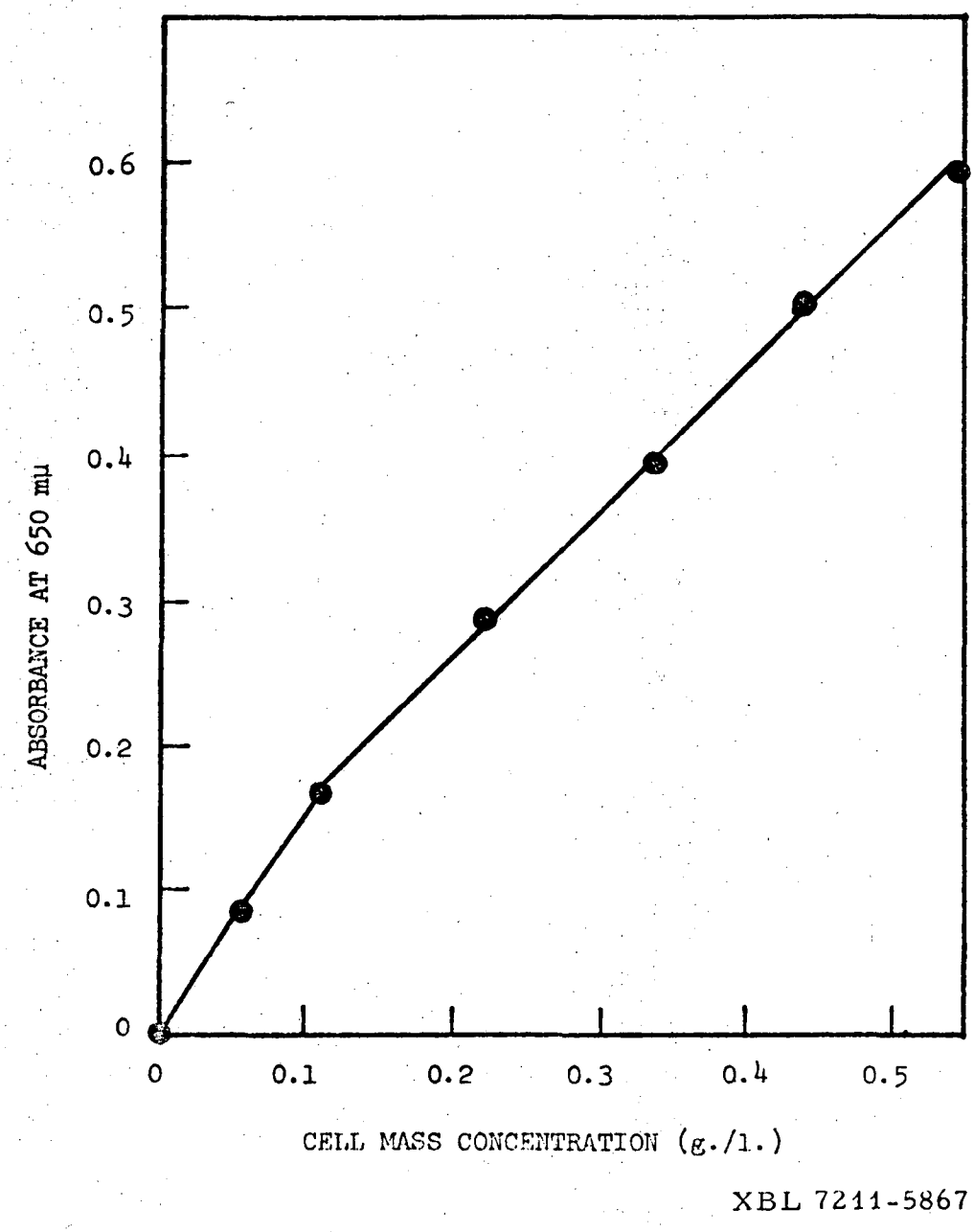
A standard curve of absorbance versus cell mass concentration was prepared for Erwinia aroideae. A culture was grown to high concentration in a complex medium and then divided into two parts. One part, of measured volume, was centrifuged in several tubes with the supernatant solutions discarded. The cell pellets were washed with distilled water, recentrifuged, and the supernatants again discarded. The tubes, previously

tared, were dried in a 90°C oven for 24 hours, placed in a dessicator for 22 hours, and weighed. In this manner, the cell mass concentration of the original suspension was measured. The second part of the original culture was diluted with fresh medium to several different concentrations. The absorbances of these suspensions were read and corrected for the background absorbance of fresh medium. Net absorbance was then plotted against cell mass concentration, calculated from the dilution factors and the concentration of the original suspension. The standard curve generated by this procedure is shown in Fig. 3.

b. L-asparaginase Assay

L-asparaginase activity was measured by a method adapted from that of Mashburn and Wriston (40). Cell suspension samples were aseptically removed from the growth vessel and centrifuged in a Sorvall Superspeed RC-2 automatic refrigerated centrifuge at 10,000 RPM for fifteen minutes. The supernatant fermentation liquor was decanted and saved. The cell pellet was resuspended in 0.1 M sodium phophate buffer at pH 8.0. Both the liquor and resuspended cells were assayed for L-asparaginase. Erwinia aroideae samples quickly lose L-asparaginase activity when frozen and stored for later assay. For this reason, assays were always performed on fresh samples.

Resuspended cells were assayed intact for L-asparaginase activity. Intact cells and cell free extracts prepared by sonic disruption were found to have the same L-asparaginase activity under the conditions of the assay. Whole cell assays have also been reported by other workers (28). 0.1 ml. of resuspended cells and 0.1 ml. of supernatant liquor were added to separate tubes containing 2.5 ml. of 0.2 M tris (hydroxymethyl) aminomethane buffer



XBL 7211-5867

Fig. 3. Standard curve for Erwinia aroideae cell mass determination.

at pH 8.0 and 2.5 ml. of 0.133 M L-asparagine in a 37°C bath. The enzymatic reaction between L-asparaginase and L-asparagine was allowed to proceed for thirty minutes. At the end of this interval, protein was precipitated by the addition of 0.25 ml. of 1.5 M trichloroacetic acid and the reaction was stopped. Blank samples were prepared in an analogous manner to enzymatic reaction samples except that 0.25 ml. of 1.5 M trichloroacetic acid were added before the 0.1 ml. supernatant liquor or resuspended cell addition and the thirty minute incubation of 37°C was not carried out.

Ammonia formed by L-asparaginase action on L-asparagine or present in the sample from other sources was detected by a sensitive color reaction with Nessler's reagent. 1.0 ml. of solution from enzymatic reaction tubes or blank tubes was diluted with 3.0 ml. of distilled water. 1.5 ml. of Nessler's reagent was added and the absorbance at 420 mμ was read after exactly one minute with a Beckman DU-2 spectrophotometer using a glass cuvette. By the use of a standard curve for Nesslerization, prepared with ammonium sulfate, rates of ammonia production, and hence L-asparaginase activity, were computed. The Nesslerization standard curve is shown in Fig. 4.

c. Protein Assay

Bacterial protein concentrations were assayed by the method of Lowry et al. (39). Samples for protein analysis were aseptically removed from the growth vessel, transferred to small bottles, chilled in an ice bath to stop growth, frozen, and stored for later analysis. Frozen samples were thawed and diluted in distilled water, if necessary, before

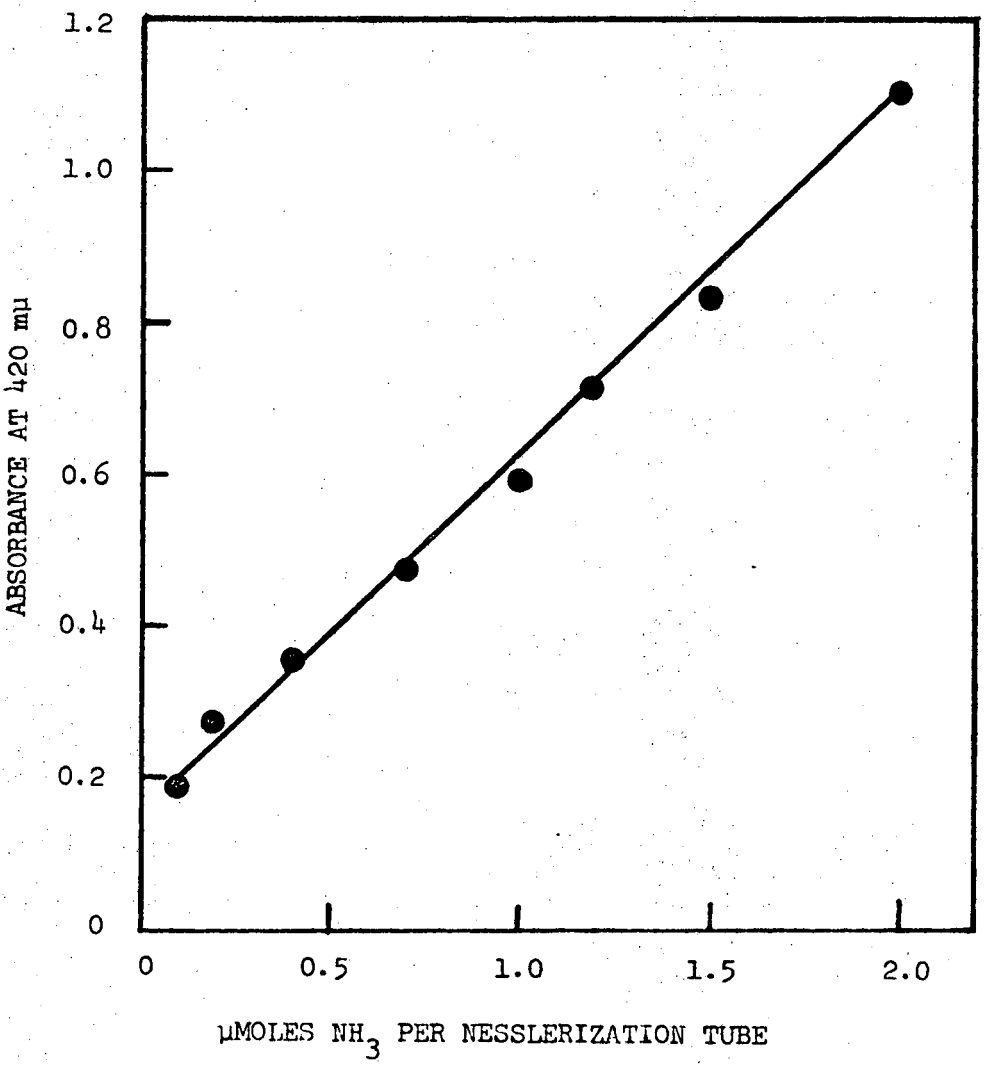


Fig. 4. Standard curve for ammonia concentration measurement by Nesslerization in L-asparaginase assay.

analysis. 2% sodium carbonate-0.1 N sodium hydroxide, 1% cupric sulfate, and 2% sodium tartrate solutions were mixed in the ratio 100:1:1 immediately before use. 0.8 ml. of cell sample was added to 4.0 ml. of the reagent solution and the mixture was placed in a boiling water bath for fifteen minutes. 0.4 ml. of Folin-Ciocalteau reagent was then added with simultaneous mixing. Samples were held at room temperature for sixty minutes, at which time they were transferred to glass cuvettes and their absorbances read at 520 mμ against distilled water in a Beckman DU-2 spectrophotometer. A standard curve was prepared by analyzing bovine serum albumin solutions of known concentration by this method and plotting absorbance versus protein concentration. Absorbance readings of experimental samples were converted to protein concentration by use of the standard curve shown in Fig. 5. An assumption in using this standard is that bovine serum albumin has the same proportion of tyrosine as Erwinia aroideae. Tyrosine is the amino acid detected by the Lowry assay.

d. L-asparagine Assay

L-asparagine concentrations were measured colorimetrically by the method described by Snell and Snell (54). Samples were taken and stored in the same manner as protein samples. L-asparagine samples were centrifuged to remove cells and then diluted in distilled water so that the concentration of L-asparagine was less than 0.01 M. Equal parts of 16.4% hydroxylamine hydrochloride and 14.0% sodium hydroxide were mixed. 2.0 ml. of this mixture were added to 1.0 ml. of L-asparagine solution and placed in a 60°C bath. After three hours at 60°C, the tubes were cooled to room temperature. 1.0 ml. of 4 M hydrochloric acid and 1.0 ml. of 20.0%

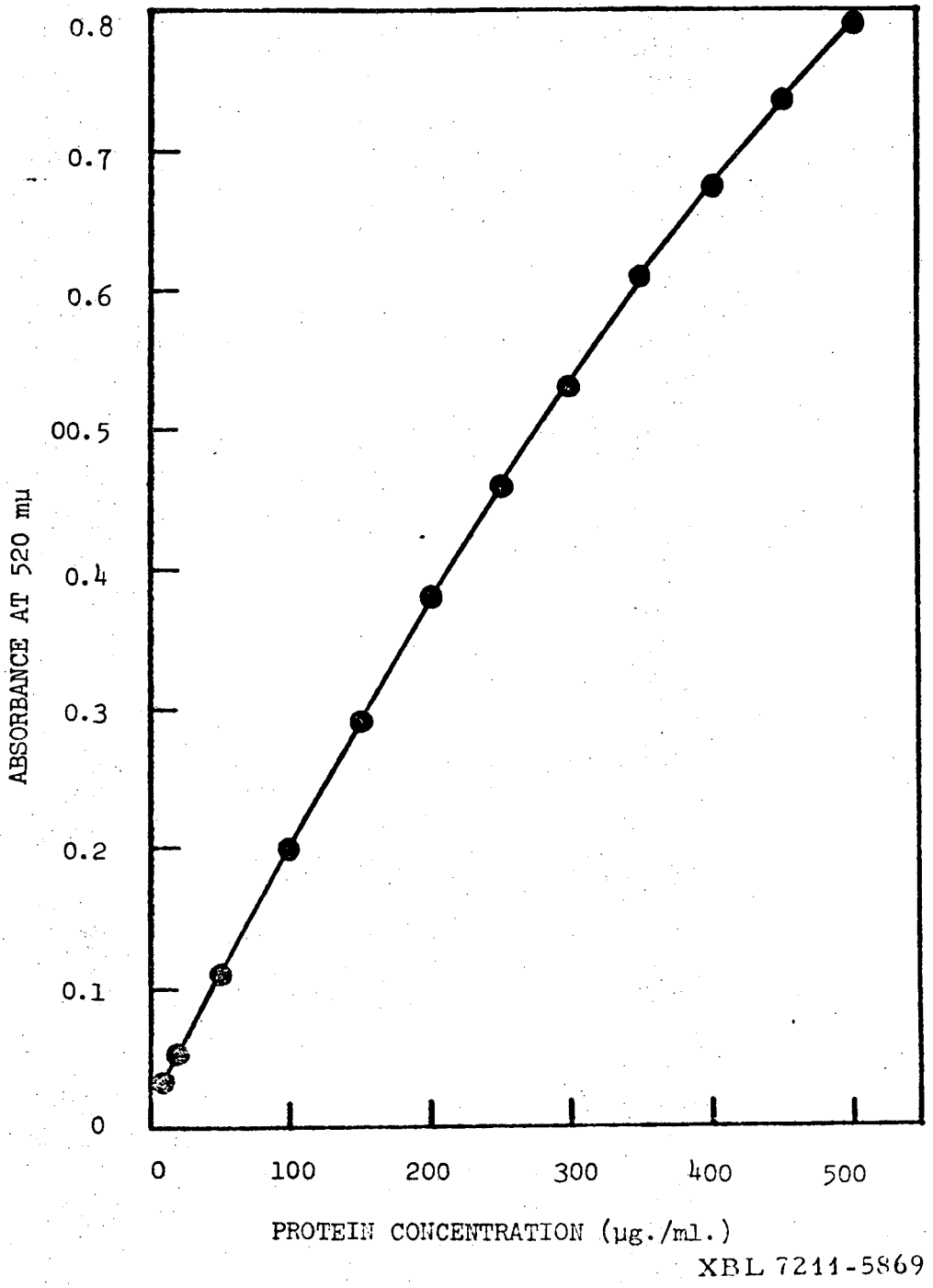


Fig. 5. Standard curve for protein assay with bovine serum albumin standard.

ferric chloride hexahydrate-0.075 M hydrochloric acid were added to the cooled tubes and mixed. Solutions were transferred to glass cuvettes and the absorbances read at 540 mμ against distilled water in a Beckman DU-2 spectrophotometer. Standards of known L-asparagine concentration were analyzed with the experimental samples.

Experimental Apparatus

a. Batch Fermentation

Batch culture experiments were done with a Fermentation Design Model MA501 five liter stirred jar fermentor system. The fermentor contained 3.6 liters of medium initially for batch experiments. The fermentor and accessories are shown schematically in Fig. 6.

Agitation was supplied by two six-bladed turbines on a vertical shaft attached to a magnetic rotor which rested on the bottom of the jar and was coupled with an external variable speed magnetic drive. The fermentor was fitted with four baffles attached to the head plate. Air was forced first through a rotometer, then into a glass wool packed filter for particle entrapment to achieve sterilization, and finally through a sintered glass sparger in a column of sterile water for humidification before entering the fermentor through a single hole sparger under the lower turbine. Exhaust from the fermentor also passed through a glass wool filter. Temperature was controlled by alternating flow of hot and cold water through a coil inside the fermentor. A thermistor was inserted into a well in the head plate to sense the liquid temperature and transmit a signal to an on-off temperature controller. Temperature was measured

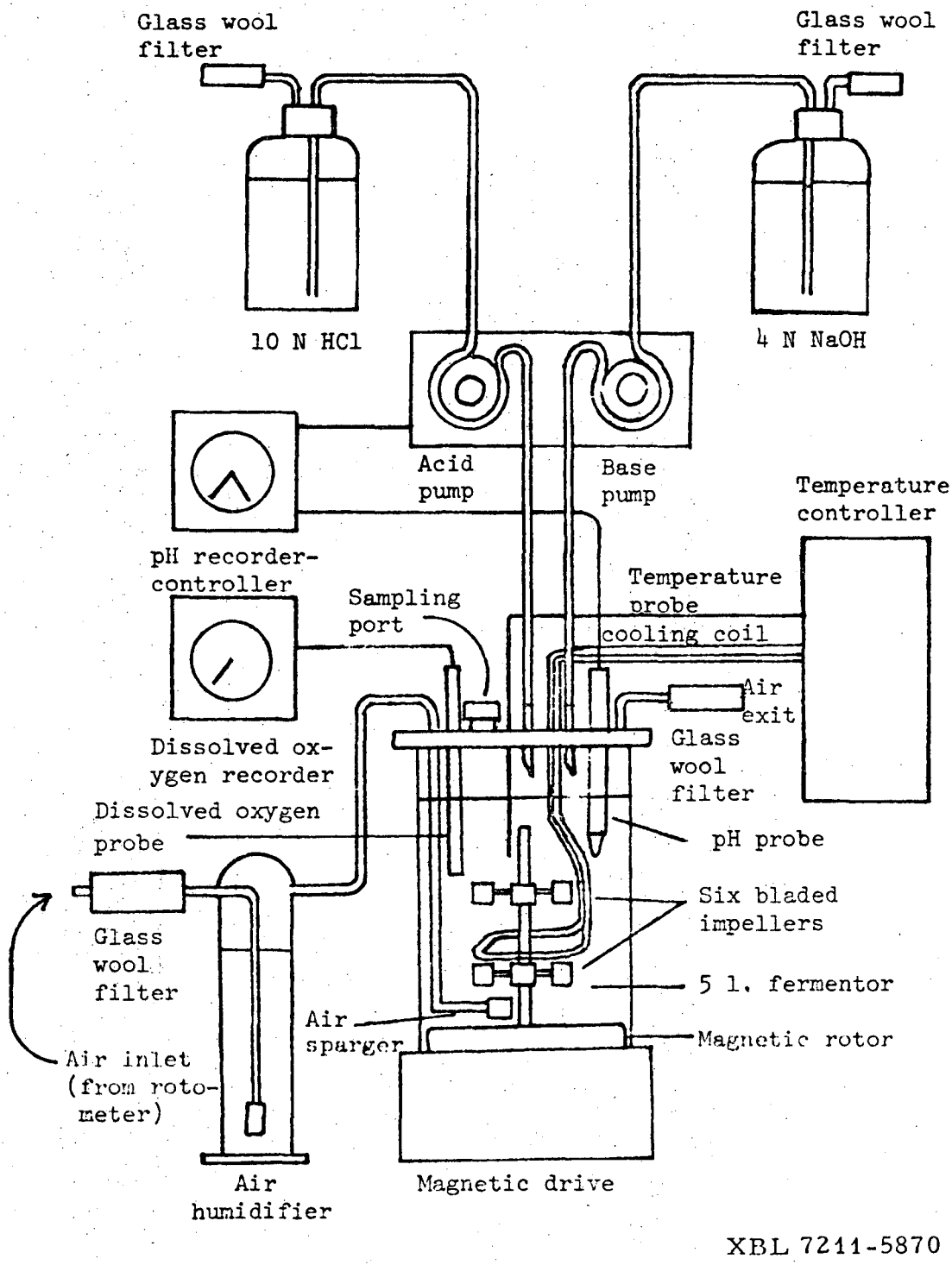


Fig. 6. Batch fermentation system.

with a thermometer placed in another well in the head plate. The magnetic drive, air supply, and temperature control system were all contained in one module.

pH was measured with an Ingold 761-351 B combination pH electrode. The signal from this electrode went to a Fermentation Design PH-RT recorder-controller module. The on-off controller actuated Sigmamotor AL-4 peristaltic pumps which added either 10 N hydrochloric acid or 4 N sodium hydroxide to the fermentor to maintain constant pH.

Dissolved oxygen concentration was measured with a Fermentation Design E100-9 teflon membrane type electrode. The current from this probe was passed through an appropriate resistance and the voltage drop recorded with a Leeds and Northrup Speedomax Type G recorder. In this system, voltage was proportional to dissolved oxygen concentration in the fermentor.

The fermentor was steam sterilized in a vertical autoclave, with a holding time of at least thirty minutes at 121°C. Both the pH and dissolved oxygen probes were steam sterilizable, but were short circuited during sterilization to avoid rapid decomposition of electrolyte solutions. The inlet air filter and humidifying column were autoclaved separately and connected to the fermentor aseptically after sterilization. The acid and base reservoirs for pH control were autoclaved individually to avoid vapor phase chemical reactions during sterilization.

b. Continuous Fermentation

Continuous culture studies were done in a New Brunswick MMF-05 five liter stirred jar fermentor. The continuous culture system, shown

schematically in Fig. 7, was constructed of apparatus from a variety of sources, in contrast to the modular Fermentation Design batch culture apparatus described above.

The fermentor was fitted with four baffles and two six-bladed turbines. The agitator shaft was coupled through an aseptic seal and two universal joints to an overhead 1/4 HP. General Electric Statotrol variable speed motor.

Air was supplied to the fermentor in the same manner as it was supplied to the batch fermentor, passing through a rotometer, glass wool filter, humidifier, and single hole sparger in the fermentor. Exhaust from the fermentor passed through a glass wool filter.

Temperature was controlled by circulation of water by an Eastern Industries Model B-1 centrifugal pump from a heated bath through the hollow baffles in the fermentor which also served as a temperature control coil. A thermistor in a well in the head plate transmitted a signal to a Yellow Springs Instruments Model 72 proportional controller which regulated power input to the heater immersed in the temperature control bath. Temperature was measured with a thermometer in another well in the head plate.

pH was measured with Leeds and Northrup reference and measuring electrodes. The signals from these electrodes went to a Beckman Model 900 on-off pH controller-analyzer coupled to a New Brunswick PA-6 peristaltic pump which added 10 N hydrochloric acid to the fermentor to maintain constant pH.

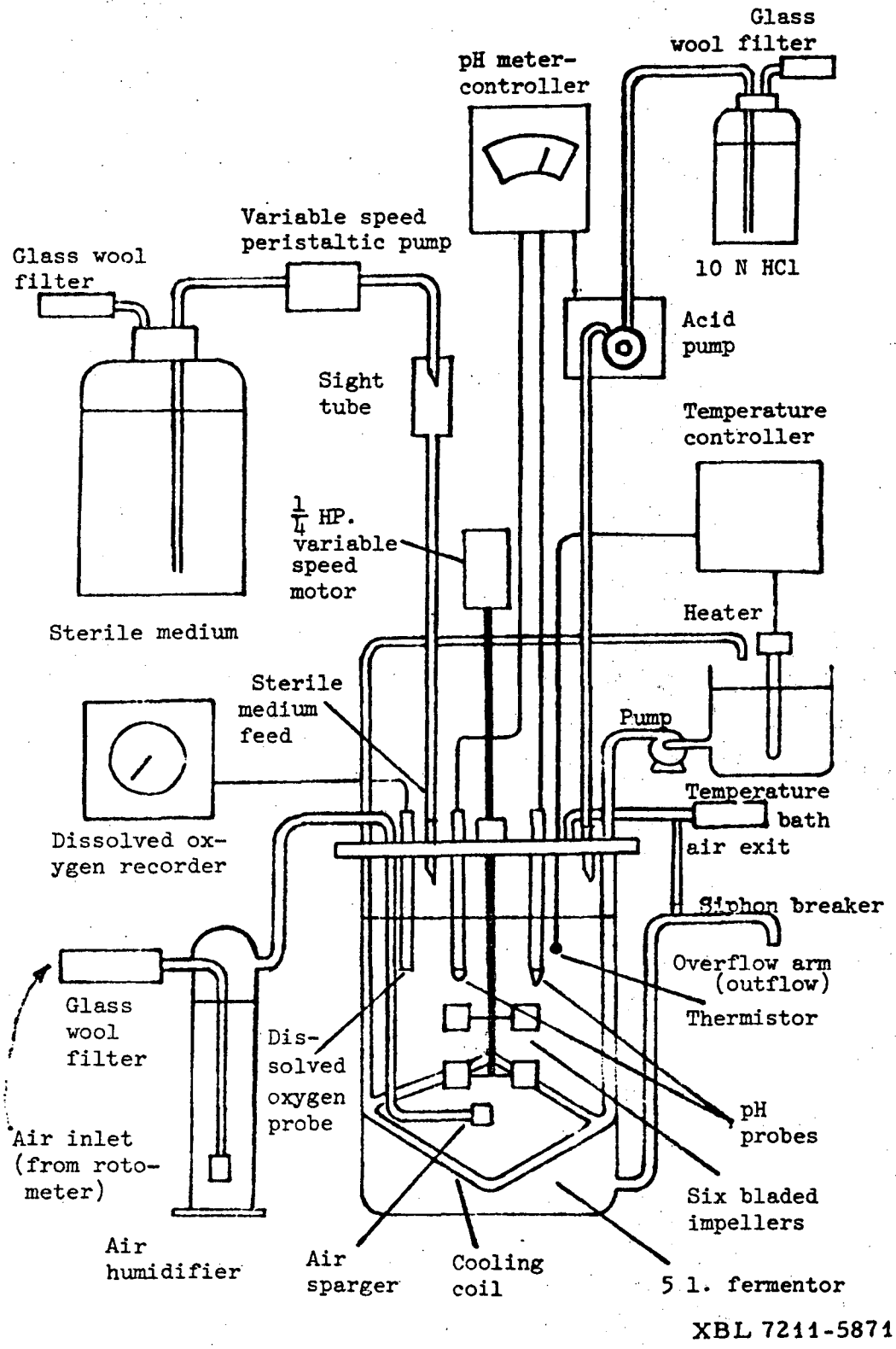


Fig. 7. Continuous fermentation system.

Dissolved oxygen concentration was measured with a teflon membrane type electrode. The current from the electrode was converted to a voltage drop by an appropriate resistance, and the voltage was recorded with a Leeds and Northrup Speedomax Type G recorder. Recorded voltage was proportional to dissolved oxygen concentration in the fermentor.

Sterile medium in a five gallon glass carboy was pumped into the fermentor by a Sigmamotor Model T8 peristaltic pump coupled to a Westinghouse Type FH 1/8 HP. motor by a Zeromax Model E variable speed drive. Before entering the fermentor, medium passed through a glass sight tube, at which point the flow separated into distinct pulses. The discontinuity in flow at the sight tube inhibited growth of microorganisms back up into the sterile medium reservoir. Microbial culture left the fermentor through an overflow arm whose height was adjusted to maintain a constant culture volume inside the fermentor. The overflow arm and the air exhaust were connected by a siphon breaker line to equalize the pressures in both lines.

For continuous operation, the fermentor filled with medium, medium feed line, inlet air filter, and humidifying column were steam sterilized in a vertical autoclave with a holding time of at least thirty minutes. The pH electrodes and oxygen electrode (shorted) were steam sterilizable. Additional medium was steam sterilized in five gallon carboys, holding at least two hours in a vertical autoclave. Carboys of fresh sterile medium were connected aseptically to the medium feed line as required to maintain continuous operation. Acid for pH control was sterilized separately.

Experimental Procedures

a. Culture Maintenance

A lyophilized culture of Erwinia aroideae NRRL B-138 was obtained from the American Type Culture Collection (ATCC 25206). All reported Erwinia aroideae L-asparaginase studies have been with this strain. The lyophilized culture was revived by aseptically transferring the bacterial pellet from a glass ampule to a small flask of sterile nutrient broth. The nutrient broth culture was grown to high cell density and then streaked on a nutrient agar slope. The culture was maintained by aseptic transfer to fresh nutrient agar slopes at approximately three week intervals. Freshly streaked slopes were allowed to grow for one day and were then stored in a refrigerator for latter use.

b. Media for L-asparaginase Production

All submerged cultures of Erwinia aroideae were grown in the same mineral base solution, irrespective of the carbon source. The formulation of this solution is shown in Table 2. When a source of combined organic nitrogen (e.g. amino acids) was not present naturally with the carbon source of a medium, inorganic nitrogen was added in the form of 3.0 g./l. of ammonium chloride. A mineral base solution of greater complexity was compared with the simple solution of Table 2, but resulted in no improvement in growth rate or yield of Erwinia aroideae in shake flasks with glucose as carbon source.

A variety of carbon sources were screened in shake flask cultures for their influence on the production of L-asparaginase by Erwinia aroideae. To avoid caramelization, two carbohydrate substrates of interest, glucose

Table 2. Mineral Base Solution for Submerged Growth of Erwinia aroideae.

Constituent	Concentration (g./l.)
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.02
$MnCl_2 \cdot 4H_2O$	0.002
$NaMoO_4 \cdot 2H_2O$	0.001
Ferric-EDTA solution	1.ml./l.
Antifoam Emulsion (General Electric AF-72)	0.25 ml./l.

Ferric-EDTA solution was prepared by first dissolving 17 g. of (ethylenedinitrilo) tetraacetic acid (EDTA) and 3.23 g. of KOH in 186 ml. of distilled water. Then a solution of 13.7 g. of $FeSO_4 \cdot 7H_2O$ in 364 ml. of distilled water was mixed with the first solution. The mixture was aerated overnight to oxidize the iron and was then filtered and stored in a dark bottle.

and pectin, were autoclaved in the absence of mineral salts, which were added aseptically from a sterile concentrated solution after cooling. Corn steep water was prepared by the method of Roberts et al. (50). Other media were generally prepared in single batches, except when small amounts of glucose were required. Then glucose was added aseptically from a separately sterilized 10% glucose solution. All media were adjusted to pH 7.0 by addition of concentrated hydrochloric acid or sodium hydroxide before sterilization.

Cultures were grown in 100 ml. of medium in 300 ml. sidearm flasks. Flasks were placed in a New Brunswick Aquatherm Water Bath Shaker Model G86 at 30°C and 225 RPM. All cultures were inoculated with 1 ml. of a dense liquid culture grown on the medium of interest. Lag periods preceding the exponential growth phase were thus avoided by inoculation with a nutritionally adapted culture. Inoculum cultures were started by direct aseptic transfer of cells from a nutrient agar slope of Erwinia aroideae.

Absorbance readings were made on the cell suspensions throughout the growth of the cultures. Cell mass concentrations were found from this data and the growth curves were plotted. L-asparaginase acitivity was assayed at two different periods in the growth of each culture: during exponential growth and at the onset of the stationary phase.

c. Temperature Effects on L-asparaginase Production

The influence of temperature on growth and production of L-asparaginase by Erwinia aroideae in batch culture was studied with the five liter batch fermentation system. The medium giving highest specific activity, determined by the screening of media in previous experiments, was used in

the temperature study. Stirred jar fermentations were run at five different controlled temperatures: 21.0°C, 25.0°C, 29.2°C, 32.9°C, and 37.0°C. Fermentor temperature was controlled within 0.3°C of the set point temperature by the on-off temperature control system. Other conditions influencing the fermentation were kept uniform for all temperatures. pH was controlled at pH 7.0. Aeration was vigorous (360 l./hr. air flow rate and 700 RPM agitation rate) so that dissolved oxygen concentration was always near saturation level.

The five liter fermentor was inoculated with 100 ml. of a dense, nutritionally adapted culture of Erwinia aroideae, grown at a temperature close to that of the fermentor. Samples were removed from the fermentor with sterile pipettes for absorbance measurements throughout the growth of a culture. Growth curves were plotted from this data. L-asparaginase activity of the cultures was assayed only once during a run, at a time when cultures were in the exponential growth phase. Small samples of the cell suspension were frozen and later assayed for protein. Specific activities of cultures growing at various temperatures were compared on this basis. Later experiments indicated that specific L-asparaginase activity is at an essentially constant maximum value for rapidly dividing cells of Erwinia aroideae under conditions of constant temperature and pH and vigorous aeration. Hence, measurement of enzyme activity at more than one time during a run would have been superfluous.

d. pH Effects on L-asparaginase Production

The influence of medium pH on growth and production of L-asparaginase was studied in five liter batch fermentor experiments. The medium

and temperature giving highest specific activity, based on previous experiments, were used in these experiments. Three runs were made at constant, controlled pH's of 6.0, 7.0, and 8.0. The pH of the buffered medium was controlled within 0.05 pH units of the set point by the on-off pH control system. The procedure for inoculation and sampling of the culture, aeration and agitation conditions, and the basis for measurement of L-asparaginase activity were the same as those used in the temperature study.

Although industrial fermentations are typically run with some form of temperature control, they often are run without external pH control, relying only on the buffering capacity of the medium. An additional five liter batch run was made without external pH control to characterize this mode of operation and to compare it with batch fermentation with pH control. Unlike the pH controlled fermentations where the important environmental parameters were all invarient with time and specific L-asparaginase activity was constant during growth of the culture, growth without pH control introduces a time variant pH which presumably makes specific L-asparaginase activity also a function of time. During the batch run without pH control, cell suspension samples were assayed for L-asparaginase activity at several times during the growth of the culture. Corresponding samples for protein assay were frozen and assayed later.

e. Dissolved Oxygen Concentration Effects on L-asparaginase Production

The influence of dissolved oxygen concentration in the fermentation medium on growth and production of L-asparaginase by Erwinia aroideae was

studied with both flask and five liter fermentor cultures. The medium, temperature, and pH of these fermentations were those which resulted in highest specific activity in the experiments described above.

Two experiments were done to characterize the role of dissolved oxygen concentration in the growth of Erwinia aroideae. Air and nitrogen blended in several ratios were supplied at different times to a single batch culture. Growth was allowed to proceed for several hours at each measured dissolved oxygen concentration in equilibrium with an air-nitrogen gas mixture until uniform exponential growth was observed (determined by absorbance data). The change in exponential growth rate with dissolved oxygen concentration was used to estimate the critical oxygen concentration. During exponential growth of Erwinia aroideae with the dissolved oxygen tension in equilibrium with pure air, aeration was stopped for several minutes. The depletion of dissolved oxygen during this time was found to be linear with time and the slope of the oxygen uptake curve was used to find Q_{O_2}.

Four experiments were done to determine the influence of dissolved oxygen concentration on the production of L-asparaginase by Erwinia aroideae. A flask culture sparged with pure nitrogen was checked for growth and L-asparaginase production under anaerobic conditions. A five liter fermentor culture was grown under conditions of vigorous aeration (700 RPM agitation and 360 l./hr. aeration) and constant, controlled temperature and pH. The culture was inoculated and sampled in the same manner as previous stirred jar fermentations. Samples of the culture were assayed

for L-asparaginase activity at several times during its growth. Corresponding samples for protein analysis were frozen for later assay. Another experiment, identical to the experiment described above except that agitation was at only 212 RPM, was done to characterize growth and L-asparaginase production by Erwinia aroideae in oxygen limited batch culture. A final stirred jar culture was grown with aeration by an approximately 1:1 mixture of oxygen and air and agitation at 700 RPM. L-asparaginase activity and protein concentration were measured at one time during exponential growth of this culture.

f. Production of L-asparaginase by Continuous Culture

Erwinia aroideae was grown in continuous culture using the medium, temperature, pH, and dissolved oxygen concentration which gave highest specific activity in batch experiments. The five liter continuous culture apparatus was used for this work. In this experiment, the effects of steady state growth rate (i.e. dilution rate) on L-asparaginase production were analyzed.

To begin the continuous culture experiment, the chemostat was inoculated with 100 ml. of nutritionally adapted shake flask culture of Erwinia aroideae. The fermentor was operated batchwise until the culture reached stationary phase. At this time, inflow of sterile medium and outflow of culture were initiated and the continuous fermentation allowed to proceed at a constant flow rate until variations in the cell mass concentration were no longer observed (based on absorbance measurements). A sample of the steady state culture was then aseptically pipetted from the fermentor jar for analysis. After steady state was attained, a step

change in medium flow rate was made and the approach of the culture to a new steady state observed. A sample was taken and the procedure repeated until a total of nine steady states had been analyzed. At the last flow rate tested, the culture "washed out" and the experiment ended. Flow rates were calculated by marking fluid levels in the sterile medium reservoir at different times and later measuring the corresponding volumes. As flow rate was increased, aeration and agitation were also increased to maintain dissolved oxygen concentration above the growth limiting level. Changes in aeration and agitation produced different gas hold-up and steady state fluid volume in the fermentor at each flow rate. The steady state level of ungassed liquid in the fermentor was marked and the corresponding volume measured at each condition. Cell suspension samples were analyzed for protein and substrate (L-asparagine) concentrations and L-asparaginase activity. Samples were frozen for protein and substrate (L-asparagine) assays, which were performed later. The L-asparaginase assays, however, were done on freshly sampled culture.

g. Production of L-asparaginase by Two Stage Batch Culture

Previous experiments showed that L-asparaginase is inducible in Erwinia aroideae by the presence of L-asparagine in the medium. Erwinia aroideae was grown in two stages in a five liter batch fermentor. In the first stage, cells were grown to a high density on a non-inducing substrate. In the second stage, L-asparagine was added to the dense culture to cause induction of L-asparaginase.

1% glucose was used as a readily available, non-inducing substrate for the first stage. The culture was started with a glucose-adapted shake

flask culture. When glucose was exhausted and the culture reached stationary phase, the second stage was begun by addition of L-asparagine crystals to the culture to make a 1% solution. During both stages, the fermentor was controlled at the conditions of temperature, pH, and aeration which gave highest specific L-asparaginase activity in previous experiments. Growth of the culture in both stages was followed by absorbance measurements on cell suspension samples. L-asparaginase activity was assayed for with fresh samples. Samples were frozen for later protein assay.

RESULTS

Media for L-asparaginase Production

The results of shake flask screening of different media for the production of L-asparaginase by Erwinia aroideae are shown in Table 3. Specific L-asparaginase activity is lowest in cells grown on carbohydrate substrates (glucose and pectin) and much higher in cells grown on complex substrates such as peptone, tryptone, beef extract, and corn steep water. The 0.5% tryptone, 0.5% yeast extract, 0.1% glucose-mineral medium listed in Table 2 is the medium reported by Peterson and Ciegler (47) in their early studies. Erwinia aroideae was also grown with 0.1% L-asparagine·H_2O as sole carbon and nitrogen source. The high specific L-asparaginase activity of this culture indicates that L-asparaginase is inducible in Erwinia aroideae. Another shake flask experiment showed that supplementation of the original medium of Peterson and Ciegler with 0.2% L-asparagine·H_2O significantly increases specific L-asparaginase activity, forming further evidence of L-asparaginase inducibility.

A shake flask experiment was done to find if the presence of an L-asparaginase inhibitor, such as D-asparagine, in the medium would enhance the induction of L-asparaginase by Erwinia aroideae. It was hypothesized that an inhibitor might stimulate overproduction of the enzyme by reducing its apparant activity in the cell and create a need for larger amounts of enzyme. However, when a mixture of 0.2% L-asparagine·H_2O and 0.75% D-asparagine·H_2O was the sole carbon and nitrogen source, L-asparaginase activity was actually diminished and with 1.0% D-asparagine as sole carbon and nitrogen source, no growth was observed.

Table 3. Production of L-asparaginase by Erwinia aroideae in Several Media

Medium Composition	Exponential Growth Rate ($hr.^{-1}$)	Cell Mass Yield (g. dry wt./l.)	Specific L-asparaginase Activity (IU/g.dw)
1.0% glucose-mineral	0.640	4.19	79
1.0% pectin-mineral	0.547	4.21	101
1.0% peptone-mineral	0.966	0.57	341
0.5% tryptone, 0.5% yeast extract, 0.1% glucose-mineral	1.012	2.19	428
1.0% tryptone-mineral	0.966	0.81	503
5.0% corn steep water-mineral	0.742	3.77	506
0.5% tryptone, 0.5% yeast extract, 0.1% glucose, 0.2% L-asparagine·H_2O-mineral	0.966	2.95	565
1.0% beef extract-mineral	---	0.35	610
1.0% L-asparagine·H_2O-mineral	0.611	1.69	780
0.25% L-asparagine·H_2O, 0.75% D-asparagine·H_2O-mineral	0.325	0.56	464
1.0% D-asparagine·H_2O-mineral	0	0	---

100 ml. shake flask cultures grown at 30°C and initial pH of 7 in 300 ml. side arm flasks with 225 RPM shaking.

Additional experiments with 1.5% and 2.0% L-asparagine·H_2O (not shown in Table 3) revealed that Erwinia aroideae cultures grown in media containing 2.0% L-asparagine·H_2O have less than maximum activity. Most later experiments analyzing other fermentation parameters were done with 1.5% L-asparagine·H_2O-mineral medium, which gave the highest specific L-asparaginase activity of the media examined in this first study.

Temperature Effects on L-asparaginase Production

The effects of temperature on growth and production of L-asparaginase by Erwinia aroideae in 1.5% L-asparagine·H_2O mineral medium are shown in Fig. 8. Growth rate, yield of cell mass, and specific L-asparaginase activity all are highest at 29°C. Although cell mass yield is essentially independent of temperature between 21°C and 29°C, growth rate and specific L-asparaginase activity exhibit stronger and somewhat parallel dependences on temperature. Subsequent experiments analyzing other parameters were done at 29°C.

pH Effects on L-asparaginase Production

The effects of pH controlled at three different levels on growth and production of L-asparaginase by Erwinia aroidea are summarized in Table 4. The results show that growth rate, yield, and specific L-asparaginase activity are near optimum when pH is controlled at pH 7 or pH 8, but when pH is controlled at pH 6, yield and specific activity decrease quickly.

Batch growth of Erwinia aroideae without external pH control is characterized by the curves of Fig. 9. Ammonia liberated during the metabolism of L-asparagine caused an almost linear rise from pH 7.0 to

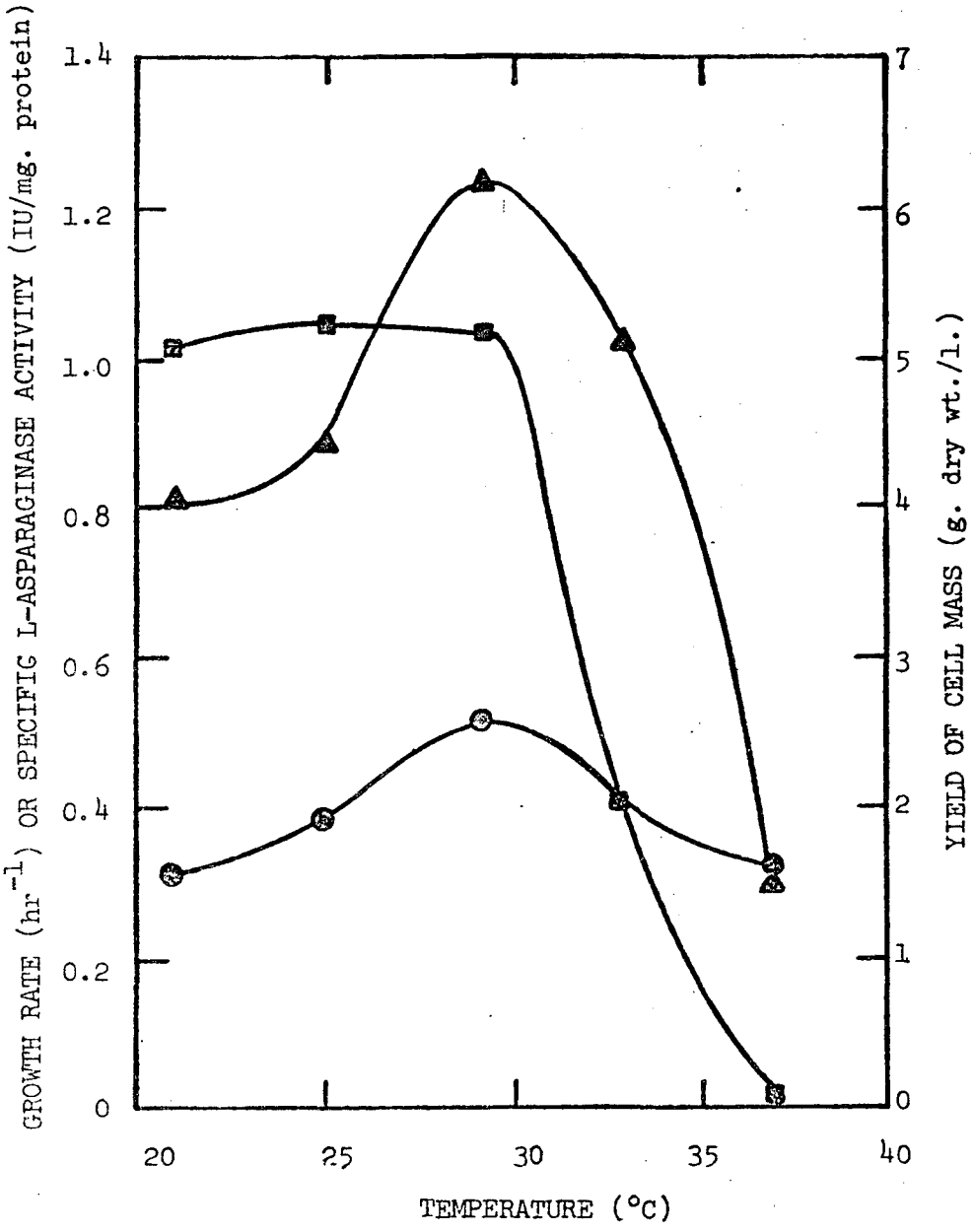
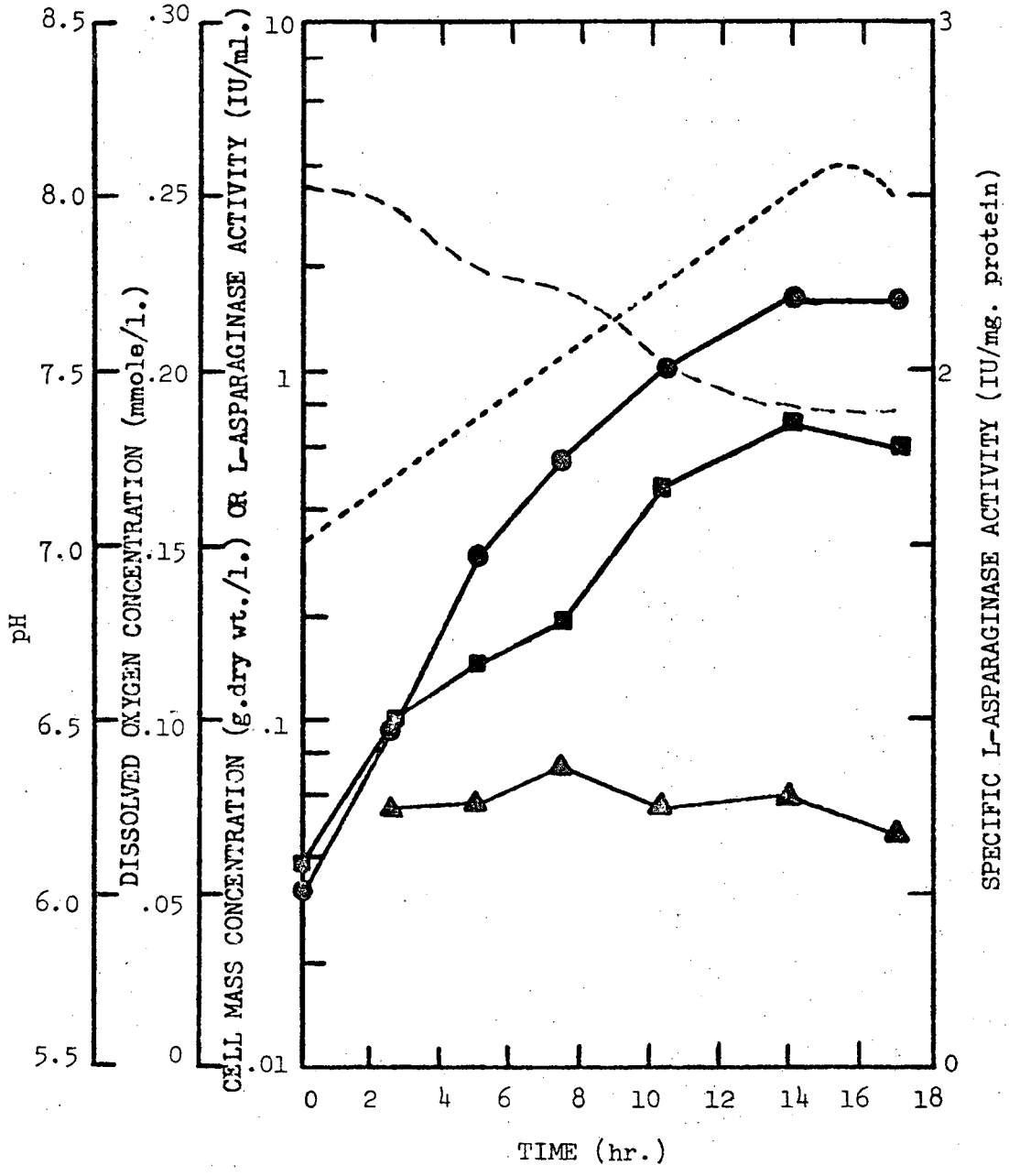


Fig. 8. Effect of temperature on exponential growth rate, specific L-asparaginase activity, and cell mass yield of Erwinia aroideae submerged fermentation.



XBL 7211-5873

Fig. 9. Batch growth of Erwinia aroideae without pH control. Cell mass concentration (●), L-asparaginase activity (■), specific activity (▲), dissolved oxygen (dashed line), and pH (dotted line) versus time. Medium = 1.5% L-asparagine·H_2O-mineral, temperature = 29°C, aeration rate = 360 l./hr., agitation = 700 RPM, inoculum = 100 ml. dense, adapted culture, 5 l. fermentor.

Table 4. Production of L-asparaginase by Erwinia aroideae in pH Controlled Fermentations.

Medium pH	Exponential Growth Rate ($hr.^{-1}$)	Cell Mass Yield (g. dry wt./l.)	Specific L-asparaginase Activity (IU/mg. protein)
6.0	0.420	2.51	0.588
7.0	0.514	5.17	1.233
8.0	0.420	4.55	0.942

Cultures were grown in 1.5% L-asparagine·H_2O-mineral medium in 5 l. fermentor with pH and temperature control. Temperature = 29°C, aeration = 360 l./hr., RPM = 700, inoculum = 100 ml. dense, adapted culture.

pH 8.1 during growth of the culture. In late stages, growth of this culture was nonexponential and specific L-asparaginase activity was at all times less than that of the culture controlled at pH 7. Later experiments were all done with control at pH 7.

Dissolved Oxygen Concentration Effects on L-asparaginase Production

Figure 10 shows the dependence of the exponential growth rate of Erwinia aroideae on dissolved oxygen concentration in 1.5% L-asparagine·H_2O-mineral medium at 29°C and pH 7. At dissolved oxygen concentrations between about 3 μmoles/l. and zero, the exponential growth rate decreased from its maximum value to zero. The critical oxygen concentration for growth of Erwinia aroideae at the conditions specified above is estimated to be 3 μmoles/l., although critical oxygen concentration is actually defined as the concentration at which the respiration rate (rather than growth rate) first deviates from its maximum value.

Figure 11 represents the uptake of dissolved oxygen by an exponential phase culture of Erwinia aroideae growing in 1.5% L-asparagine·H_2O-mineral medium at 29°C and pH 7 without air input. The slope of the linear part of this curve divided by the cell mass concentration equals the repsiration rate under the conditions of the experiment. The respiration rate found by this method is 9.18 mmoles/g. dry wt.-hr.

Growth of Erwinia aroideae and production of L-asparaginase in well aerated batch culture are characterized by the curves shown in Fig. 12. Cell mass concentration and L-asparaginase activity increased essentially in a parallel, exponential manner, while specific L-asparaginase activity remained almost constant during the fermentation. Although dissolved

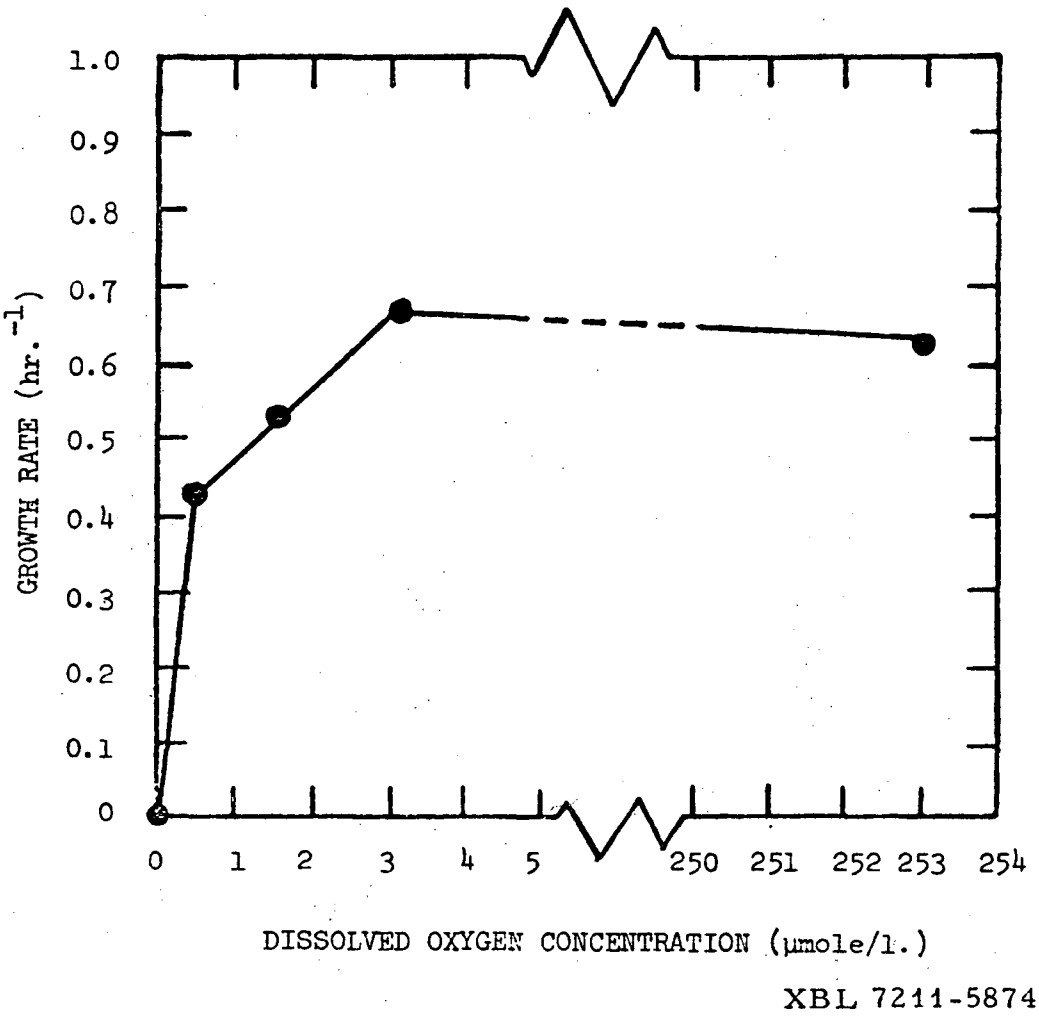


Fig. 10. Effect of dissolved oxygen concentration on exponential growth rate of Erwinia aroideae in submerged culture. Cultures were grown on 1.5% L-asparagine·H_2O-mineral medium, pH = 7.0, temperature = 30°C.

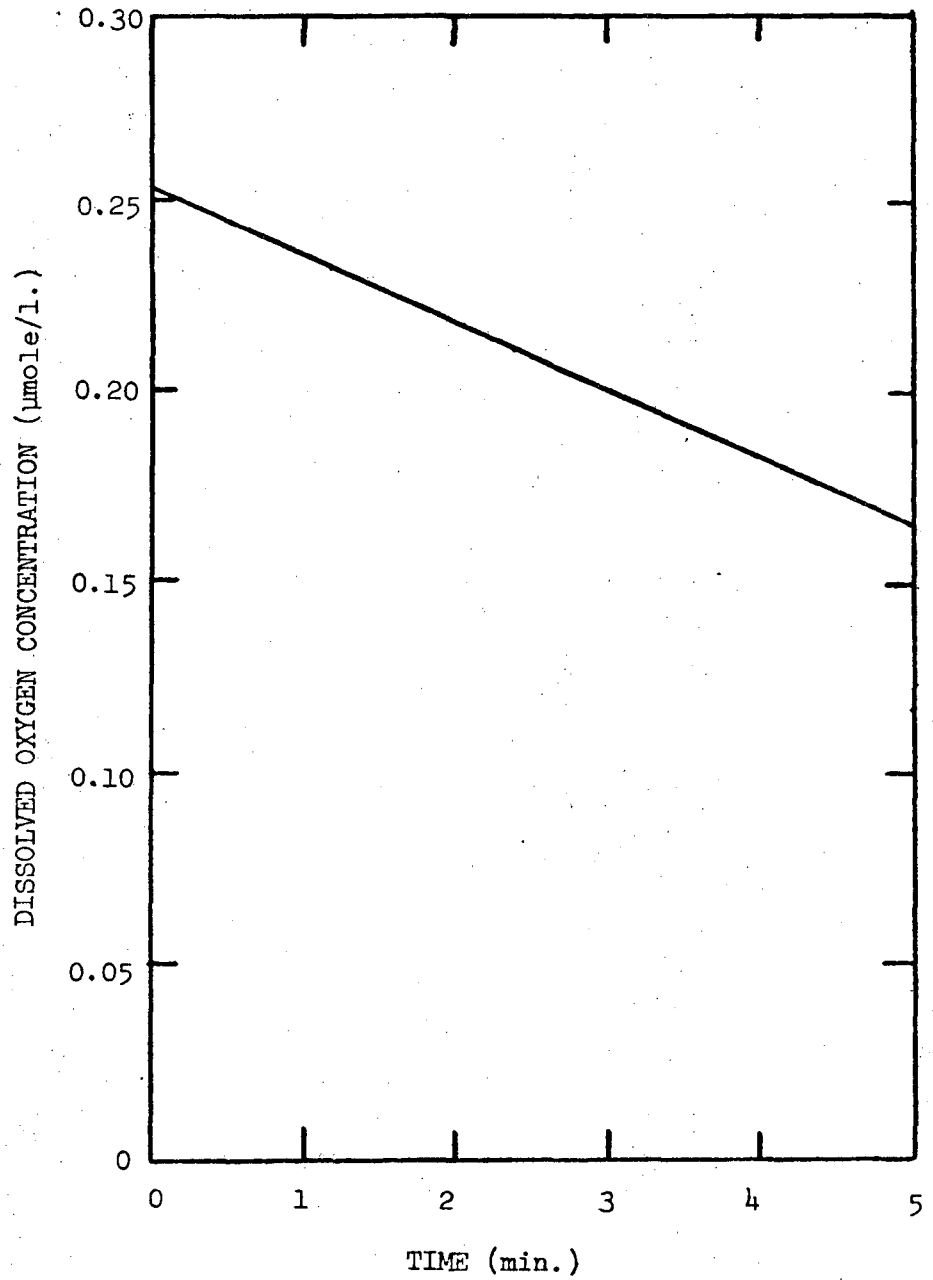
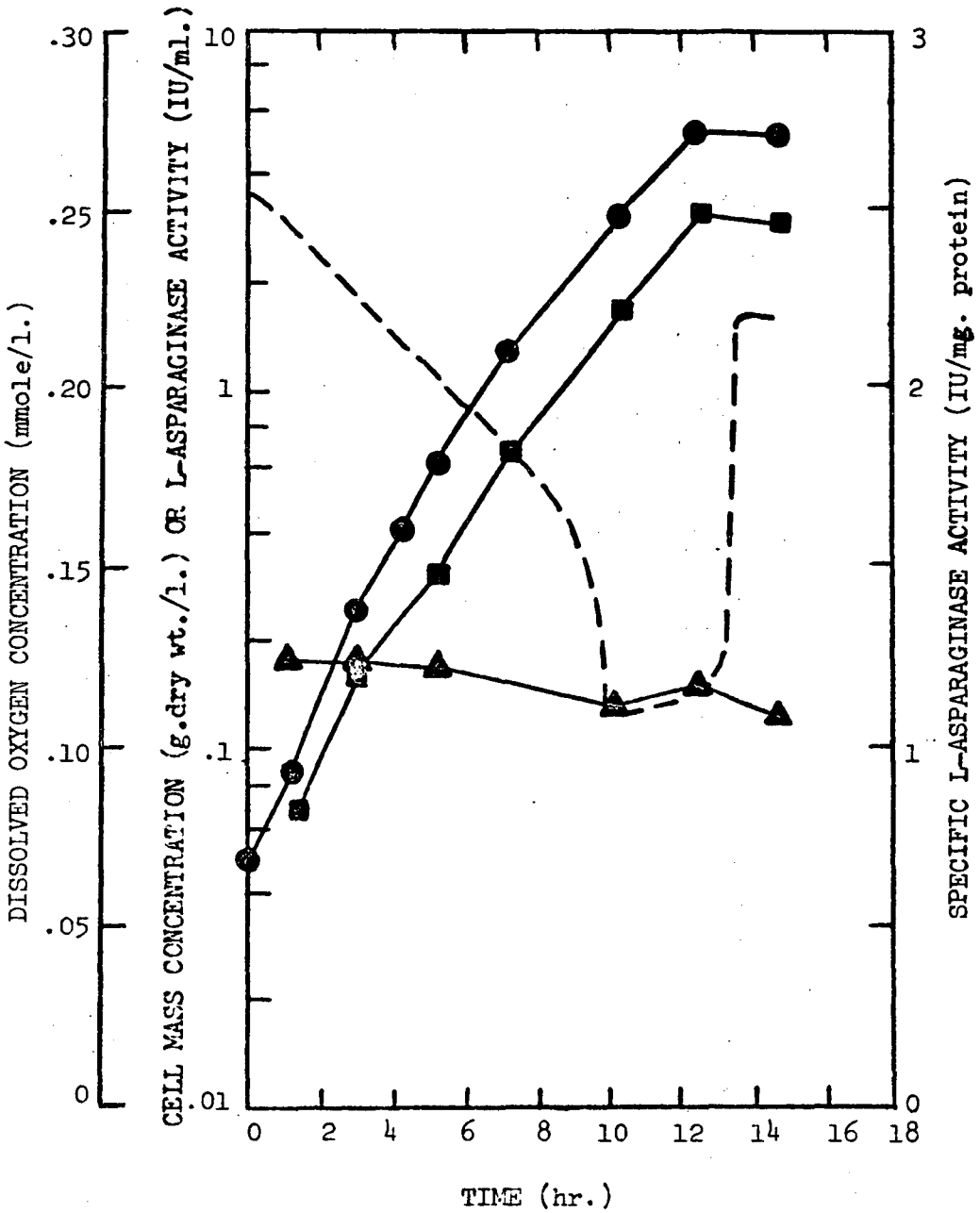


Fig. 11. Oxygen uptake by Erwinia aroideae in submerged culture with air input stopped. Initial medium composition: 1.5% L-asparagine-mineral, cell mass concentration = 0.114 g.dry wt./l., pH = 7.0, temperature = 28.6°C.



XBL 7211-5876

Fig. 12. Growth of Erwinia aroideae in well aerated batch culture. Cell mass concentration (●), L-asparaginase activity (■), specific activity (▲), and dissolved oxygen concentration (dashed line) versus time. Medium = 1.5% L-asparagine·H_2O-mineral, temperature = 29°C, pH = 7.0, aeration rate = 360 l./hr., agitation = 700 RPM, inoculum = 100 ml. dense, adapted culture, 5 l. fermentor.

oxygen concentration dropped to about forty percent of the saturation value near the end of the fermentation, it was always well above the critical concentration.

Growth of Erwinia aroideae and production of L-asparaginase in a poorly aerated batch culture are characterized by the curves shown in Fig. 13. Dissolved oxygen quickly became depleted during this fermentation and both cell mass concentration and L-asparaginase activity increased nonexponentially. After oxygen became depleted, specific L-asparaginase activity decreased to between sixty and eighty percent of its value in the well aerated fermentation.

Table 5 summarizes the relationship of growth and production of L-asparaginase by Erwinia aroideae to aeration and dissolved oxygen concentration. Included in this table are characteristics of a fermentation with dissolved oxygen tension above atmospheric and an anaerobic culture. Erwinia aroideae is unable to ferment L-asparagine in the absence of oxygen.

Production of L-asparaginase by Continuous Culture

The steady state values of cell mass concentration, L-asparaginase activity, and L-asparagine (substrate) concentration are shown for several dilution rates in Fig. 14. Washout, indicated by the rapid decrease in cell mass concentration and L-asparaginase activity and rapid increase in substrate concentration, apparently occurs when the dilution rate approaches the value of μ_{max} measured in batch operation (see Table 4). Although substrate concentration follows a characteristic Monod form, cell mass concentration and L-asparaginase activity, which both decrease steadily with increasing dilution rate, do not (see Fig. 2).

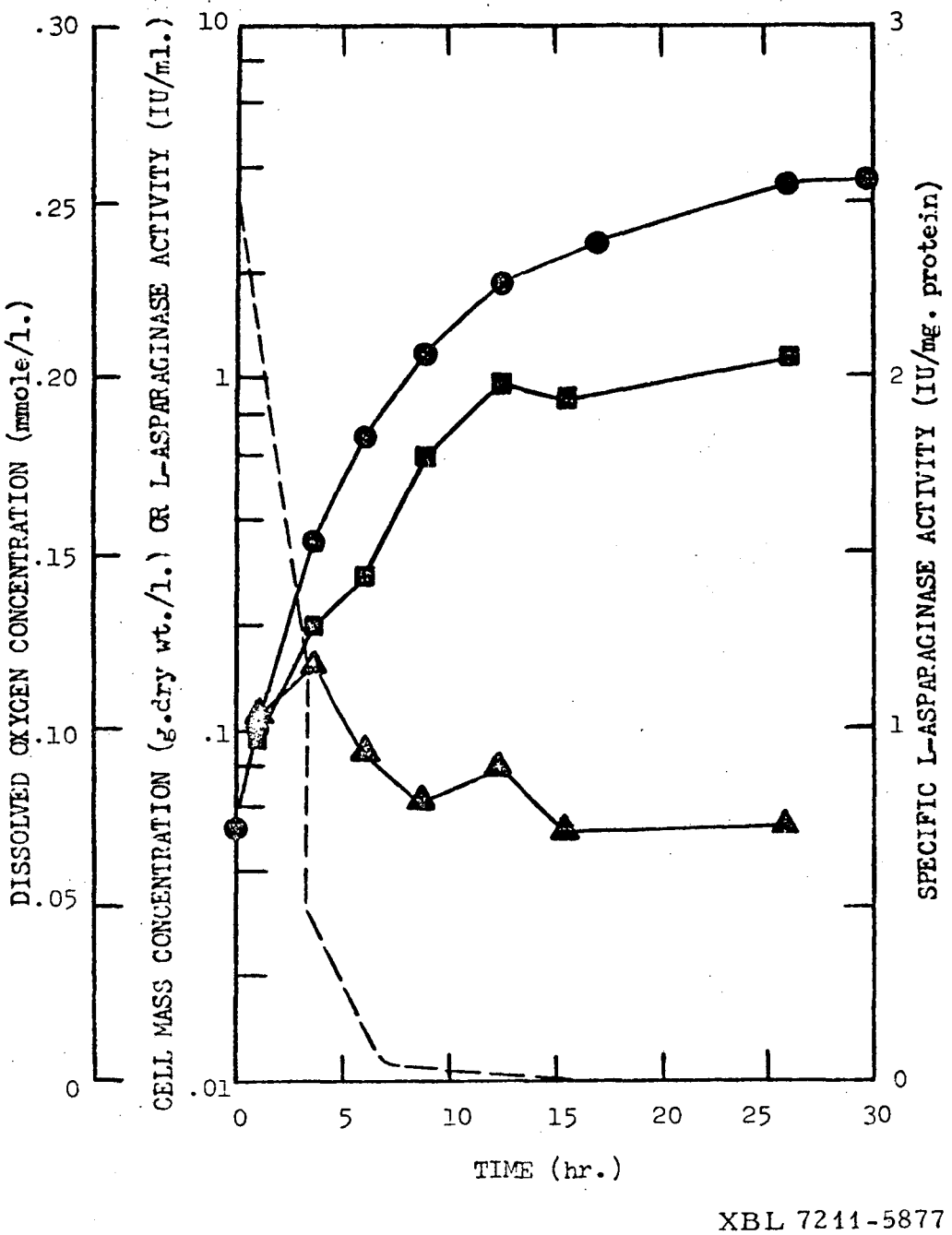
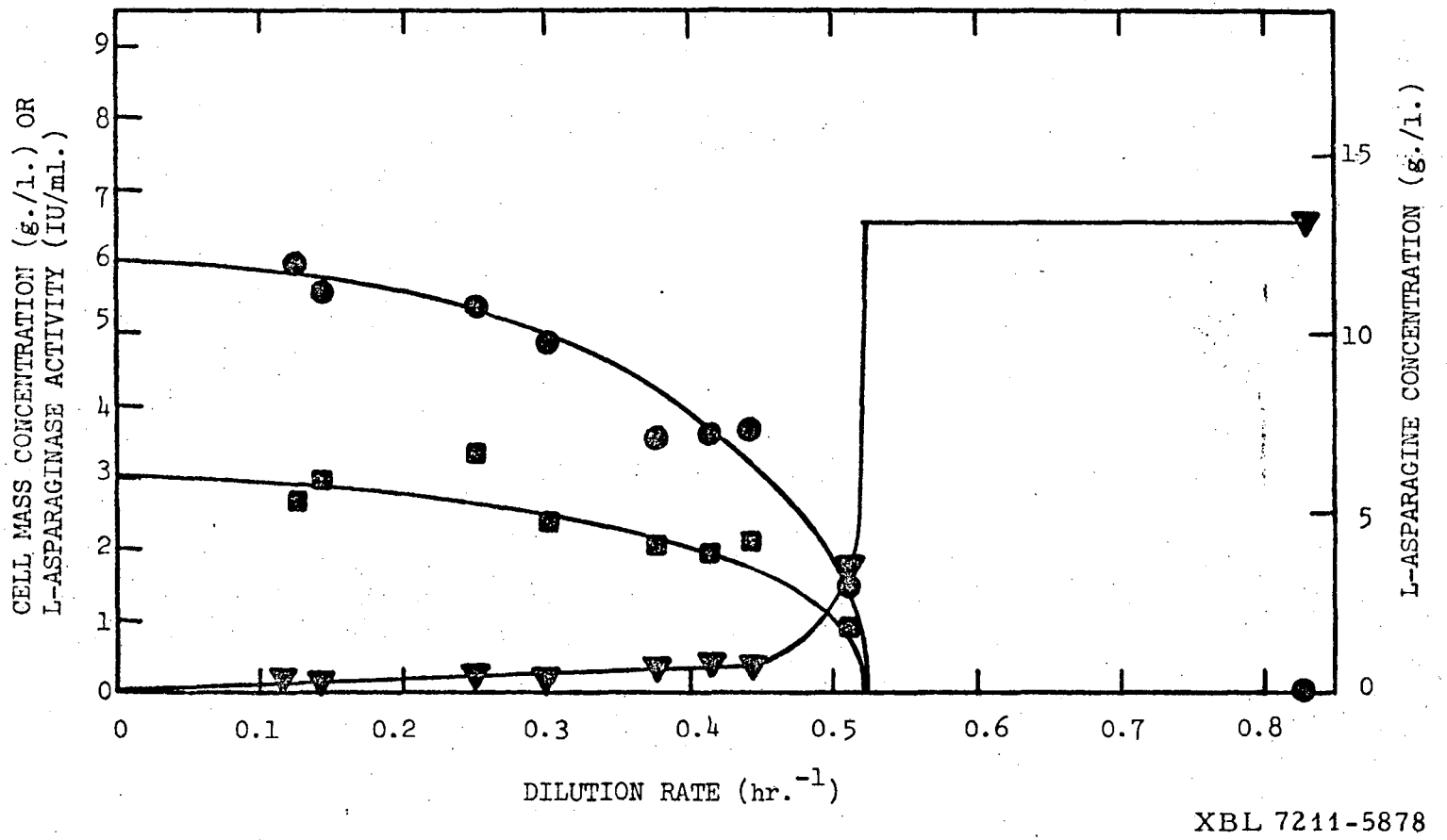


Fig. 13. Growth of Erwinia aroideae in poorly aerated batch culture. Cell mass concentration (●), L-asparaginase activity (■), specific activity (▲), and dissolved oxygen concentration (dashed line) versus time. Medium = 1.5% L-asparagine·H_2O-mineral, temperature = 29°C, pH = 7.0, aeration rate = 360 l./hr., agitation = 212 RPM, inoculum = 100 ml. dense, adapted culture, 5 l. fermentor.



XBL 7211-5878

Fig. 14. Cell mass concentration (●), L-asparagine (limiting substrate) concentration (▲) and L-asparaginase activity (■) variation with dilution rate in steady state continuous culture of Erwinia aroideae. 1.5% L-asparagine·H_2O-mineral medium feed, temperature = 29.0°C, pH = 7.0, vigorous aeration and agitation.

Table 5. Production of L-asparaginase by Erwinia aroideae under Several Conditions of Aeration and Dissolved Oxygen Concentration

Dissolved Oxygen Concentration (mmole/l.)	Exponential Growth Rate ($hr.^{-1}$)	Cell Mass Yield (g. dry wt./l.)	Specific L-asparaginase Activity (IU/mg. protein)
0 (completely anerobic culture)	0	0	---
0 (oxygen transfer limited culture)	nonexponential growth	3.66	0.736
0.253 - 0.112 (well aerated culture)	0.514	5.17	1.233
0.545 (oxygen supplemented culture)	0.344	4.39	0.765

All cultures were grown in L-asparagine-mineral medium at 29 - 30°C and pH 7. Aeration and agitation conditions and growth vessels varied and are described in the Appendix.

The dependence of steady state productivities of cell mass and L-asparaginase on dilution rate are shown in Fig. 15. These curves also differ from the characteristic Monod form at high dilution rates.

Steady state specific L-asparaginase activity is shown as a function of dilution rate in Fig. 16. Within the limits of accuracy of the analyses, specific L-asparaginase activity is independent of the growth rate on L-asparagine.

The yield factor for conversion of substrate to cell mass, $Y_{x/s}$, decreases significantly as dilution rate increases, as can be seen in Fig. 17. The variation of yield factor with dilution rate is a deviation from the constant yield factor case of Eq. (21) and Fig. 2 and accounts for the apparent inconsistency between the cell mass concentration and substrate concentration curves of Fig. 14.

The specific rate of L-asparaginase production, $\frac{1}{X}\frac{dP}{dt}$, is shown as a function of specific growth rate for batch and continuous cultures in Fig. 18. In continuous culture, $\frac{1}{X}\frac{dP}{dt}$ is equal to $Y_{p/x}D$, based on Eqs. (2), (7), and (16). In batch culture, $\frac{1}{X}\frac{dP}{dt}$ can be found by measuring slopes off a P versus t curve and dividing by cell mass concentration. Similarly, μ for batch culture can be found by dividing slopes off an X versus t curve by X. The data of Fig. 12 was analyzed in this manner for comparison of batch and continuous production of L-asparaginase. Figure 18 shows that batch and continuous production rates are essentially the same and that the yield of L-asparaginase from cell mass, $Y_{p/x}$, is a constant (565 IU/g. dry wt.) independent of specific growth rate.

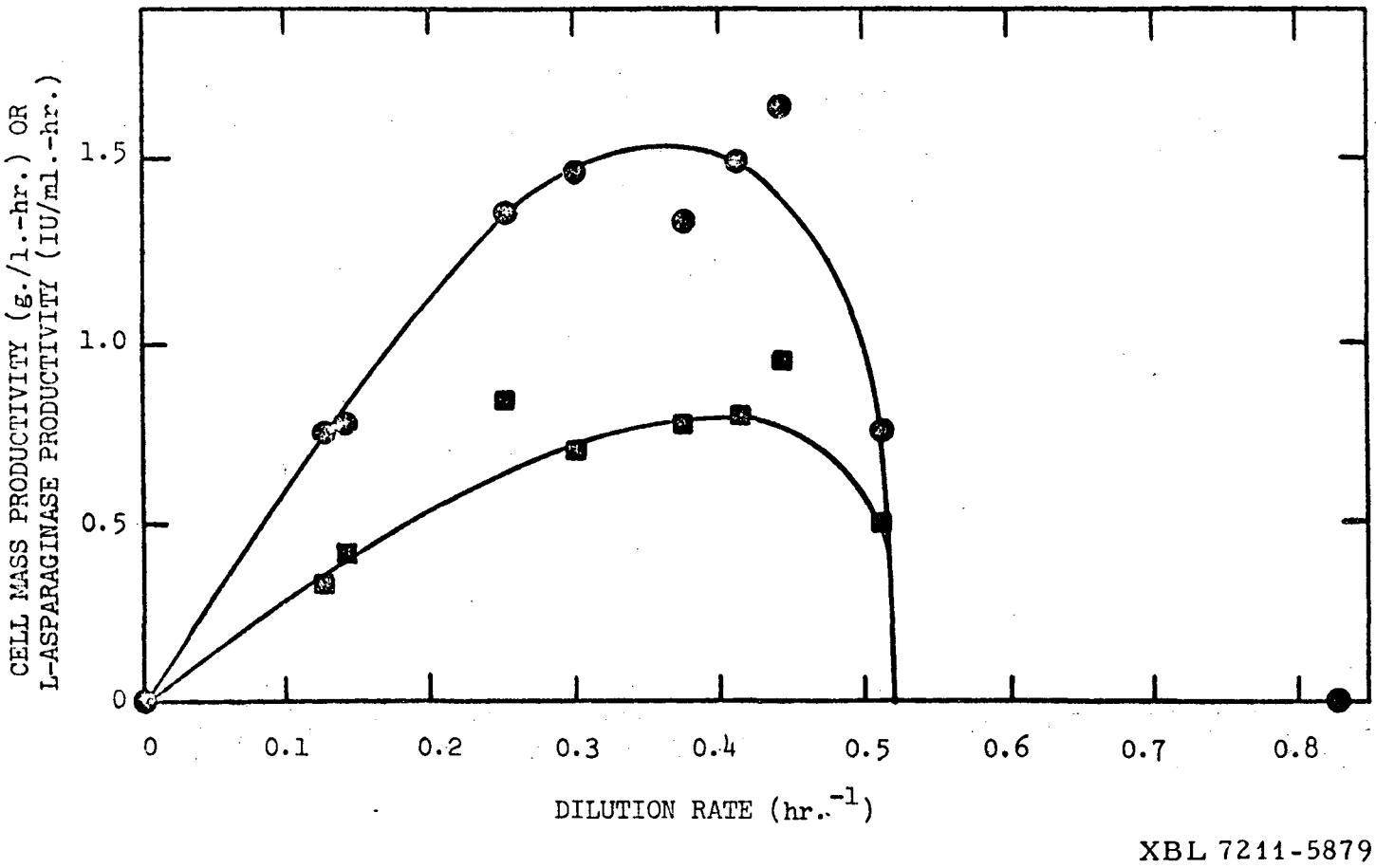
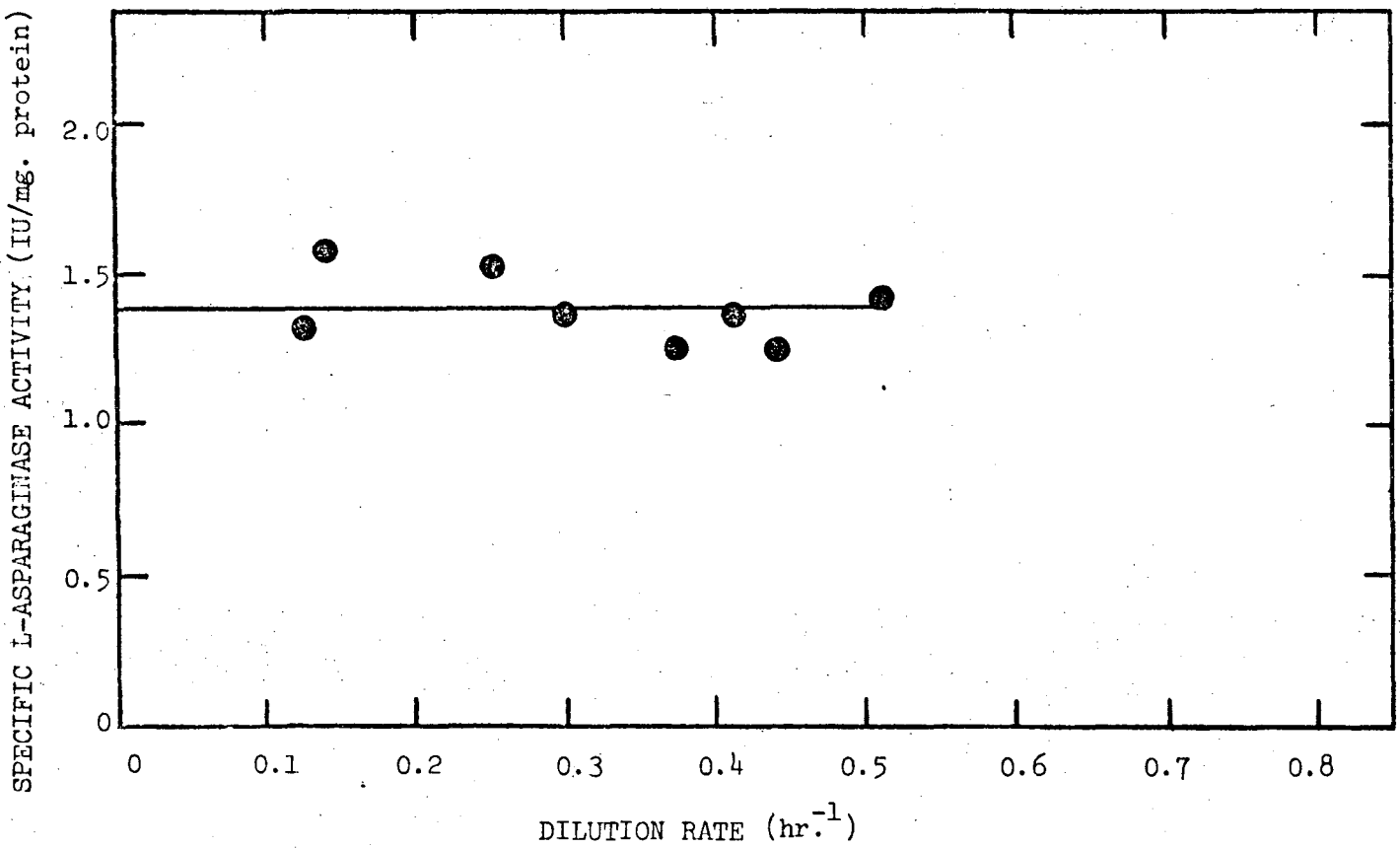


Fig. 15. Cell mass productivity (●) and L-asparaginase productivity (■) variation with dilution rate in steady state continuous culture of Erwinia aroideae. 1.5% L-asparagine·H_2O-mineral medium feed, temperature = 29.0°C, pH = 7.0, vigorous aeration and agitation



XBL 7211-5880

Fig. 16. Specific L-asparaginase activity dependence on dilution rate in steady state continuous culture of Erwinia aroideae. 1.5% L-asparagine.H_2O-mineral medium feed, temperature = 29.0°C, pH = 7.0, vigorous aeration and agitation.

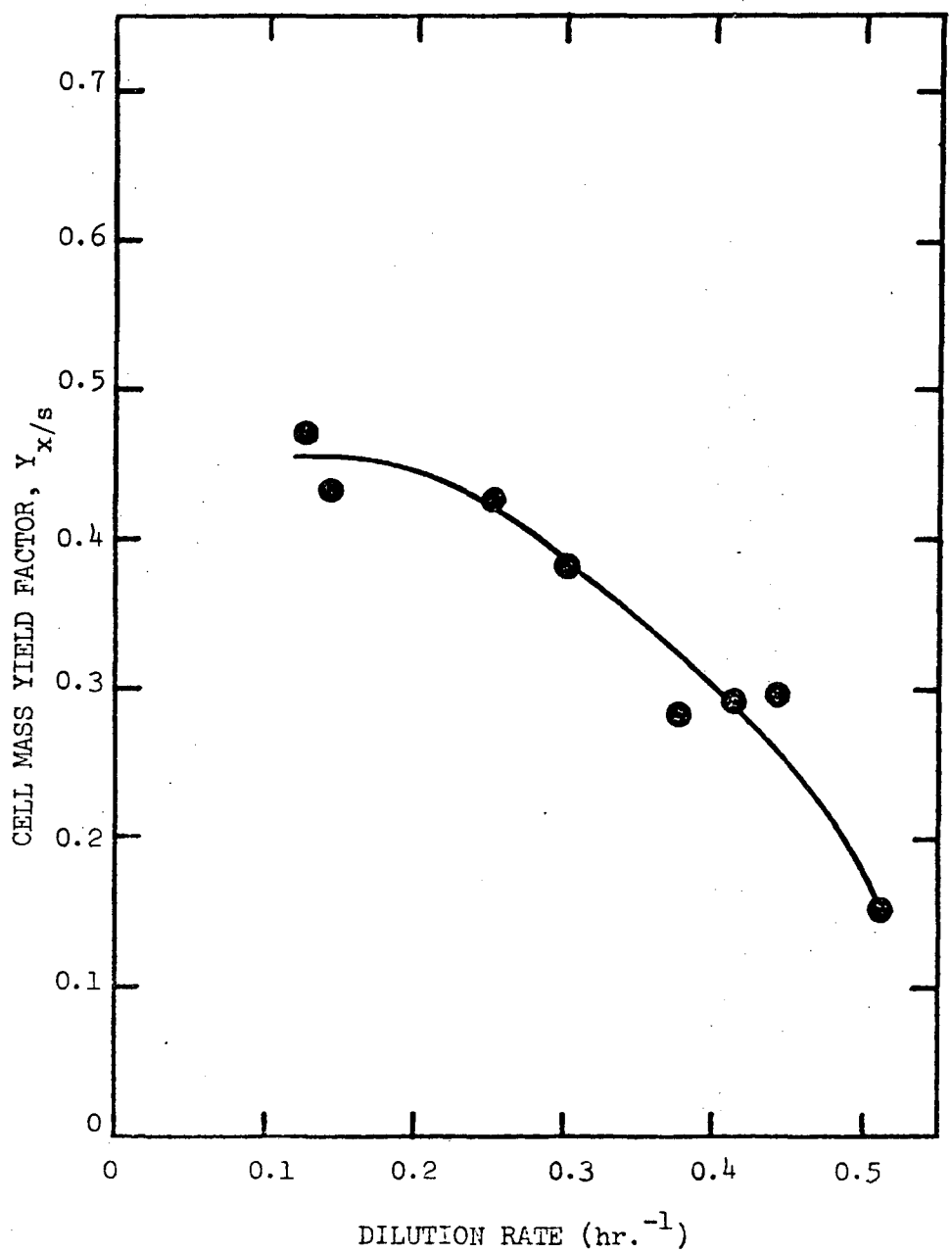


Fig. 17. Dependence of cell mass yield factor on dilution rate in steady state continuous culture of Erwinia aroideae. 1.5% L-asparagine·H_2O-mineral medium feed, temperature = 29.0°C, pH = 7.0, vigorous aeration and agitation.

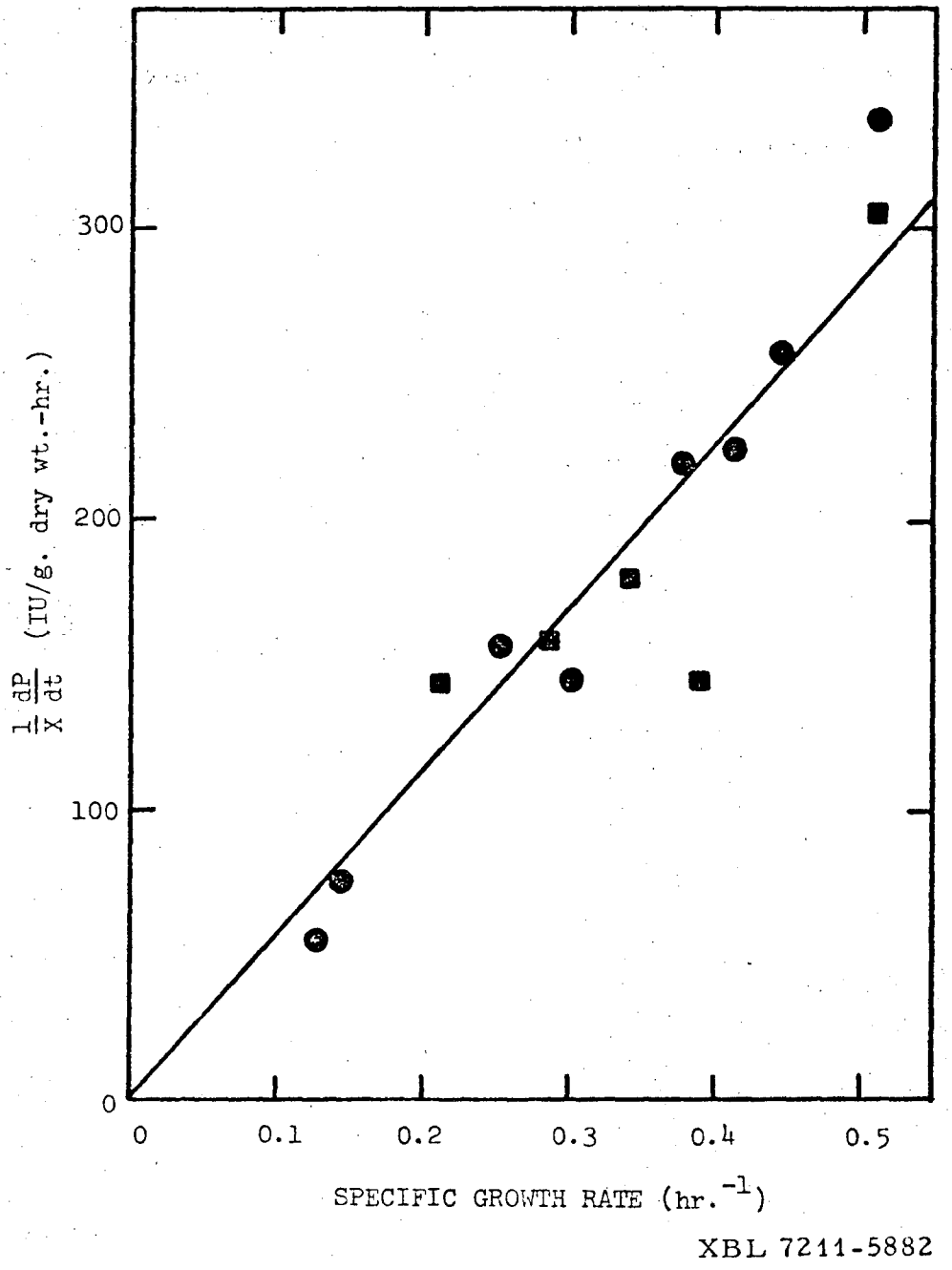
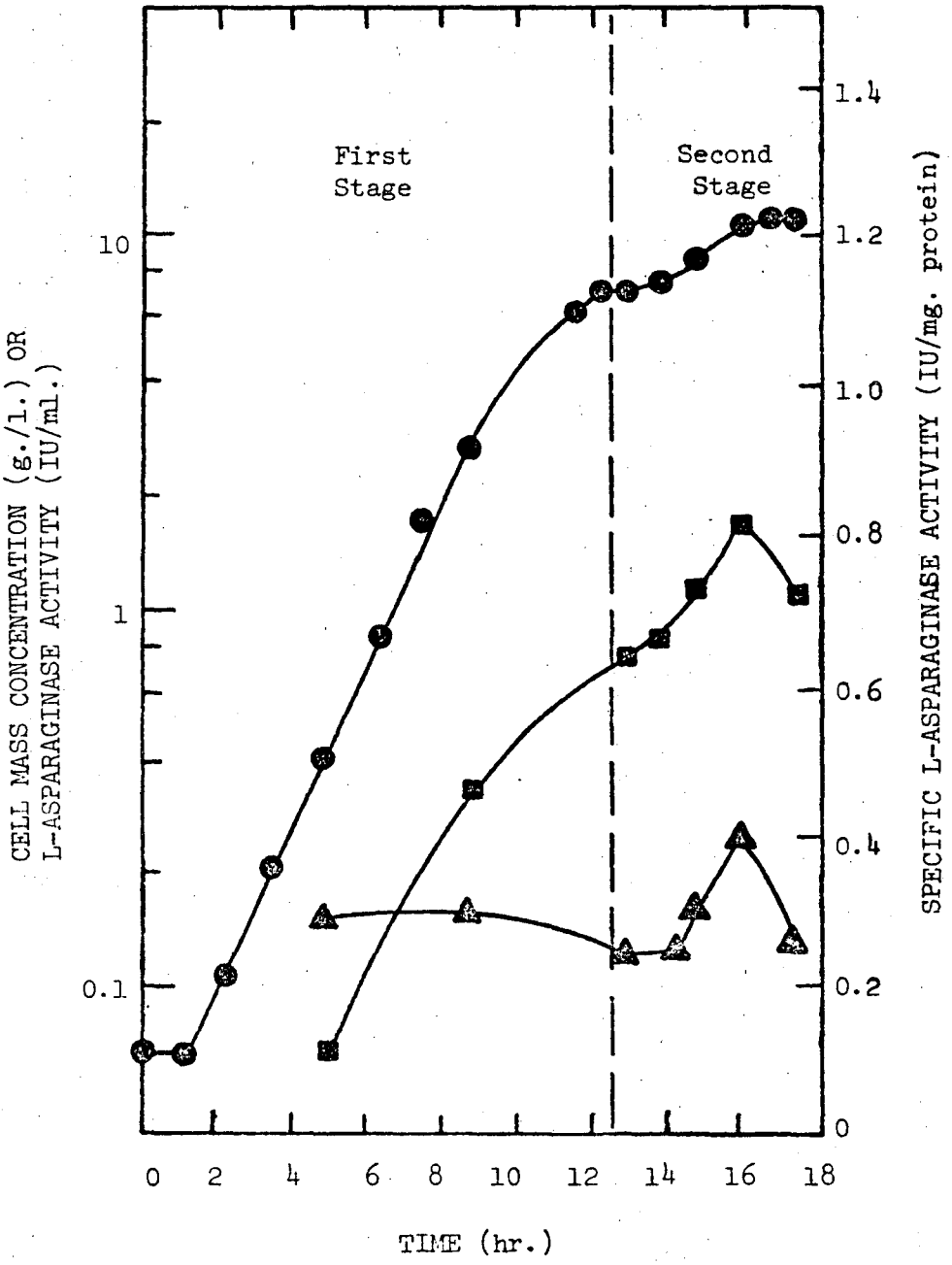


Fig. 18. Dependence of $\frac{1}{X}\frac{dP}{dt}$ on specific growth rate in batch (■) and continuous (●) culture of Erwinia aroideae. Medium = 1.5% L-asparagine·H_2O-mineral medium, temperature = 29.0°C, pH = 7.0, vigorous aeration and agitation.

Production of L-asparaginase by Two Stage Batch Culture

Growth and production of L-asparaginase by Erwinia aroideae in a two stage batch culture are characterized by the curves in Fig. 19. Growth on 1% glucose in the first stage was exponential. However, production of L-asparaginase in glucose limited medium was not exponential and was not growth associated. Specific L-asparaginase activity decreased slightly with the age of the culture. The addition of L-asparagine beginning the second stage was followed by immediate, but slow, growth. The rate of L-asparaginase production and specific activity increased during the second stage, indicating L-asparaginase induction. However, as L-asparagine depleted, L-asparaginase activity dropped markedly.



XBL 7211-5883

Fig. 19. Growth of Erwinia aroideae in two stage batch culture. First stage: growth on 10 g./l. glucose. Second stage: addition to 10 g./l. L-asparagine and subsequent growth. Cell mass concentration (●), L-asparaginase activity (■), and specific activity (▲) versus time. Temperature = 29°C, pH = 7.0, aeration rate = 360 l./hr., agitation = 700 RPM, inoculum = 200 ml. dense, glucose-adapted culture, 5 l. fermentor.

DISCUSSION

Interpretation and Implications of Experimental Results

The screening of several media for L-asparaginase production, summarized in Table 3, shows that specific L-asparaginase activity is lowest in Erwinia aroideae cells grown on carbohydrate substrates, higher in cells grown on complex media, and highest in cells grown on L-asparagine as sole carbon and nitrogen source. These observations indicate that L-asparaginase is inducible in Erwinia aroideae by the presence of L-asparagine (and perhaps other similar molecules) in the growth medium. Complex media such as peptones, tryptones, yeast extract, beef extract, and corn steep water all contain a variety of amino acids, including potential L-asparaginase inducers such as L-asparagine, L-aspartic acid, L-glutamine, and L-glutamic acid. The presence of these molecules in low concentrations apparently leads to varying degrees of partial L-asparaginase induction when Erwinia aroideae is grown in complex media. When the inducer content of a complex medium was increased by supplementation with L-asparagine, specific activity increased but remained below the completely induced level of cells grown on pure L-asparagine. Erwinia aroideae grown in the absence of any inducer molecules exhibits a small, constitutive L-asparaginase activity.

Investigators working with E. coli generally observed that complex media give highest specific L-asparaginase activities. However, supplementation of complex media with possible inducers or growth on inducing substrates fails to increase activity. The apparent conclusion is that certain precursor molecules in a complex medium are beneficial to

L-asparaginase synthesis, but the enzyme is not inducible in E. coli. Whether the presence of precursor molecules in complex media are benefical to L-asparaginase synthesis by Erwinia aroideae is uncertain. It is clear, however, that the enzyme is inducible in Erwinia aroideae. L-asparaginase is also inducible in Serratia marcescens by L-asparagine. Erwinia aroideae is taxonomically closer to Serratia marcescens than to E. coli, which may be associated with both microorganisms having inducible L-asparaginases.

The cell mass yields and growth rates reported in Table 3 follow typical patterns. Highest growth rates result with complex media, whereas defined media give lower growth rates. Cells growing in a simple, defined medium must synthesize a variety of precursor molecules for themselves which may be naturally available to cells growing in a complex medium. Growth rate in a simple medium may, therefore, be limited by slow anabolic reactions while growth rate in a complex medium may be limited by faster catabolic reactions. Cell mass yield, however, is highest in defined media and lower in complex media. Although biosynthesis is more efficient in complex media due to the presence of biosynthetic precursors, much of the material in a complex medium is unsuitable for energy metabolism and the net effect is reduced cell mass yield.

The attempt to stimulate overproduction of L-asparaginase by growing Erwinia aroideae on an inducer-inhibitor mixture of L-asparagine and D-asparagine resulted in lower growth rate and specific activity than growth on L-asparagine alone. Toxicity of D-amino acids to Erwinia species has been reported elsewhere (24). D-asparagine probably reduces

the growth rate of Erwinia aroideae on L-asparagine, to some extent, by reducing the rate of L-asparagine hydrolysis in the presence of the inhibitor. D-asparagine may also compete with L-asparagine for attachment to the repressor of L-asparaginase synthesis. However, D-asparagine may not be effective in releasing the repressor from the operon and L-asparaginase synthesis in the presence of mixtures of D and L-asparagine may be partially repressed. The reduced L-asparaginase activity of the culture may also have been due to failure of the microorganism to effectively distinguish between L-asparagine and D-asparagine during synthesis of L-asparagine polypeptides. E. coli L-asparaginase, for example, is known to have a large number of L-asparagine residues.

Figure 12 shows the growth associated L-asparaginase production by Erwinia aroideae when L-asparagine is the only carbon and nitrogen source. A theoretical maximum growth rate, if L-asparagine conversion to L-aspartic acid is the growth rate limiting reaction, was calculated from the L-asparaginase activity and cell mass yield of this culture. This theoretical maximum growth rate is several times larger than the observed maximum growth rate, indicating that the hydrolysis of L-asparagine is not growth rate limiting.**

** $\frac{1}{X}\frac{dS}{dt} = 565 \frac{\mu\text{mole L-asparagine}}{\text{g. dry wt. cells min.}} \; 60 \frac{\text{min.}}{\text{hr.}} \; 1.32\times10^{-4} \frac{\text{g.}}{\mu\text{mole}} \text{L-asparagine}$

$\frac{1}{X}\frac{dS}{dt} = 4.47 \frac{\text{g. dry wt. cells}}{\text{g. L-asparagine hr.}}$

$Y_{x/s} = 0.392 \frac{\text{g. dry wt. cells}}{\text{g. L-asparagine}}$

Although L-asparagine as sole carbon and nitrogen source gives highest specific L-asparaginase activity in Erwinia aroideae, its use as a substrate in larger scale fermentations may be of limited practicality due to the unavailability of bulk quanities of the amino acid. The two stage batch culture described by Fig. 19 was an attempt to produce a completely induced culture of Erwinia aroideae with less L-asparagine consumption. The procedure was not effective because the rate of L-asparaginase induction was too slow in glucose adapted cells. L-asparagine added to the culture to cause induction was depleted by cellular metabolism before induction was complete. Only about a sixteen percent increase in specific activity from the constitutive level toward the completely induced level was achieved when glucose and L-asparagine were used in equal concentrations. The sudden drop in specific activity after second stage growth stopped is peculiar and is not consistent with the observed stability of L-asparaginase in stationary phase Erwinia aroideae grown only on L-asparagine.

Other schemes for producing fully induced Erwinia aroideae cultures with less L-asparagine consumption might be more effective than the method discussed above. For example, second stage induction using

**(Footnote cont.)

$$(\mu_{max})\ \text{L-asparagine limited growth} = \frac{1}{X}\frac{dX}{dt} = Y_{x/s}\frac{1}{X}\frac{dS}{dt}$$

$$(\mu_{max})\ \text{L-asparagine limited growth} = 1.75\ \text{hr.}^{-1}$$

$$(\mu_{max})\ \text{experimentally observed} = 0.51\ \text{hr.}^{-1}$$

a nonmetabolizable inducer (if available) could be allowed to go to completion without premature consumption of the inducer. Similarly, a growth inhibitory substance could be added with L-asparagine in the second stage. In addition, complex media could be used in the first stage to reduce the degree of induction necessary in the second stage.

The temperature study, summarized in Fig. 8, shows that, fortuitously, all three parameters characterizing L-asparaginase production by Erwinia aroideae are optimum at 29°C. No compromise of specific activity, enzyme yield, or rate considerations with fermentation temperature is necessary for optimal operation.

Up through 33°C, growth rate and specific L-asparaginase activity have an essentially parallel dependence on temperature. Continuous culture experiments showed, however, that specific activity does not change in response to changes in growth rate (see Fig. 16). The parallelism is probably only coincidental. The increase in specific activity with temperature below 29°C may be the result of decreasing affinity of the repressor of L-asparaginase synthesis for the operon. At high temperatures, the rapid drop in specific activity may be due to an uncoupling of energy metabolism and biosynthesis of L-asparaginase or to thermal denaturation of the enzyme. At high temperatures, the dependence of growth rate on temperature is somewhat unusual. Usually a more rapid drop in growth rate is observed after temperature exceeds the optimum value. Cell mass yield is essentially constant at low temperatures but drops rapidly above 29°C. This phenomenon is typical and is due to a breakdown in the coupling of energy yielding metabolism and biosynthesis at high temperatures. Extensive thermal denaturation of cellular proteins may also be in effect at 37°C.

All three parameters characterizing the production of L-asparaginase by Erwinia aroideae are optimum when the pH is controlled at pH 7. Like the temperature dependence discussed above, this situation also obviates the need to compromise parameters having different pH optima to achieve an optimal fermentation. Variation of the measured parameters was small between pH 7 and pH 8. However, cell mass yield and specific activity were significantly decreased by operation at pH 6. L-asparaginase from Erwinia aroideae exhibits maximum activity between pH 7 and pH 8, but has about thirty percent lower activity at pH 6 (47). This L-asparaginase may be in the class of enzymes which are only effectively induced when cellular pH is near the pH optimum of the enzyme (22). The sharp decrease in yield at pH 6 could be due to increased cellular protein denaturation and the resulting increase in biosynthetic energy requirement followed by an overall reduced cell mass yield. However, such a strong effect at a pH close to neutral seems improbable. The observed phenomena are not explainable by variations in the ionic nature of L-asparagine and its permeability to cells at different pH's. Between pH 6 and pH 8, L-asparagine is almost entirely in the Zwitterion form which can permeate the cells.

Figures 9 and 12 show that growth of Erwinia aroideae without pH control is clearly inferior to growth with pH control for production of L-asparaginase. Growth is nonexponential and cell mass yield and specific activity are significantly less than maximum. These effects are not due only to the increase from pH 7.0 and pH 8.1 during the fermentation because the effects are more severe than they are when a culture is maintained at pH 8.0 from the beginning. During growth controlled at a constant pH, ammonia produced by metabolism of L-asparagine was neutralized

by automatic acid addition and accumulated in the medium. However, without pH control, ammonia can only be neutralized by phosphate buffer in the culture medium. After the buffering capacity was saturated by ammonia, further ammonia produced by metabolism of L-asparagine was no longer neutralized but was stripped from the culture by the air flow through the fermentor. In this situation, much of the nitrogen needed for growth was lost as ammonia in the exit air stream (a strong ammonia odor was noticed). Growth may have been limited by nitrogen supply, rather than carbon supply, explaining the low yield. Nitrogen limitation may also have adversely affected L-asparaginase synthesis.

The critical oxygen concentration for growth of Erwinia aroideae on L-asparagine at pH 7 and 30°C is estimated to be 3 μmoles/l based on the data in Fig. 10. This is consistent with reported critical oxygen concentrations for bacteria and yeast which are in the range 0.08 to 16 μmole/l (26). Low critical oxygen concentration has the processing advantage of somewhat reducing the minimum aeration and power requirements needed to maintain a nonlimiting dissolved oxygen concentration in dense cultures.

The respiration rate measured for Erwinia aroideae growing exponentially on L-asparagine is high (9.18 mmole/g. dry wt.-hr.) but consistent with respiration rates reported for other fast growing microorganisms (7). This high respiration rate necessitates vigorous aeration of fermentations to avoid oxygen limitation and diminished specific L-asparaginase activity. It is important to notice that the oxygen uptake curve of Fig. 11 does not include an initial lag period which

could indicate an oxygen probe response too slow to measure the oxygen uptake rate without error.

The four fermentations run at different aeration conditions show that vigorous agitation and aeration with air is most effective for production of L-asparaginase by Erwinia aroideae. Growth rate, cell mass yield, and specific activity were all highest when dissolved oxygen tension was close to atmospheric. Poor aeration, resulting in oxygen limited arithmetic growth, leads to lower L-asparaginase activity and cell mass yield. These effects are not due to a partial switch from aerobic to anaerobic metabolism of L-asparagine because Erwinia aroideae is unable to ferment L-asparagine anaerobically. When growth is limited by dissolved oxygen supply, it may be that biosynthesis is more heavily directed toward synthesis of respiratory machinery, resulting in less efficient growth and reducing the cellular content of enzymes such as L-asparaginase. Growth and production of L-asparaginase by Erwinia aroideae in submerged culture with dissolved oxygen tension above atmospheric is also inferior to growth at lower, nonlimiting concentrations of oxygen. In this situation, the reduced L-asparaginase activity may be due to the enzyme's instability in strong oxidizing environments. Reduced growth rate and cell mass yield may also be due to the oxygen lability of certain cellular components.

Comparison of the experimental continuous culture curves in Figs. 14 and 15 with the curves in Fig. 2 shows that only substrate concentration, of the parameters plotted, qualitatively follows a Monod form. The steady, substantial decrease in cell mass concentration with increasing dilution

rate indicates that the yield of Erwinia aroideae cell mass from L-asparagine substrate decreases as growth rate increases, as can be seen in Fig. 17. The dependence of yield factor on dilution rate explains the atypical forms of the other curves in Figs. 14 and 15. The observed results are not due to oxygen starvation, which can produce similar results. At each steady state, dissolved oxygen concentration was well above the critical level. The steady state yield is higher than batch culture yield at low dilution rates and less than batch culture yield at high dilution rates. Since growth rate decreases during the course of batch growth, the final batch yield is actually an average value, resulting from integration of a growth rate dependent yield factor.

Growth of Erwinia aroideae in steady state continuous culture has no other unusual features. Washout occurs when dilution rate equals μ_{max}, indicating adequate mixing of nutrients and cells and the absence of wall growth. There is no large requirement for maintenance energy, which would be indicated by a drop in cell mass concentration as dilution rate approaches zero. More data at low dilution rates would be necessary however, to characterize accurately a maintenance energy requirement.

The RNA content of microorganisms growing in continuous culture is usually several times higher at high dilution rates than it is at low dilution rates (30). This fact may help explain the decrease in yield of Erwinia aroideae with increasing dilution rate in L-asparagine limited continuous culture. L-aspartic acid is a principal precursor in the biosynthesis of the purine and pyrimidine molecules in RNA. When growing on L-asparagine, Erwinia aroideae is able to produce L-aspartic acid for

nucleotide synthesis in one step by hydrolysis of L-asparagine. Loss of large amounts of L-asparagine to RNA synthesis may significantly decrease the portion of L-asparagine which can be oxidized to produce energy (i.e. ATP) to drive biosynthetic reactions, thereby reducing cell mass yield. The drain on L-asparagine and resulting loss of yield increases as dilution rate increases and RNA synthesis presumably increases. Microorganisms growing on substrates less closely related to RNA precursors would not ordinarily experience such large decreases in energy production and yield as RNA synthesis increases at high dilution rates. Usually the substrate is able to pass partially through the TCA cycle, producing some energy, before being shunted off for nucleotide synthesis.

Specific activity, measured on a protein basis, is independent of growth rate, as indicated by Fig. 18. Figure 18 compares the specific rate of L-asparaginase production in batch and continuous culture. The linearity of this plot shows that specific activity, measured on a cell mass basis, is also independent of growth rate. Although the specific L-asparaginase production rates of batch and continuous cultures are comparable, the usual productivity advantage of continuous culture over batch culture does not exist because of the low yield of cell mass at high dilution rates.

Economic Considerations

The experimental work presented above was directed toward maximizing specific L-asparaginase activity rather than L-asparaginase productivity of Erwinia aroideae fermentations. The premise behind this objective is that purification costs are primary in determining the cost of pure

L-asparaginase for medical use and that fermentation costs are of minor importance. A rough estimation of manufacturing costs in L-asparaginase production was made to give some perspective of the actual cost breakdown.

In 1972, approximately 360 million IU of L-asparaginase were distributed for worldwide medical use. A process for producing twenty percent of this volume (72 million IU per year) was proposed and the manufacturing costs were estimated. The design was based on the fermentation data for Erwinia aroideae presented above and the recovery and purification data for E. coli presented by Ho et al. (34). A process flow diagram and details of the cost estimates are in Appendix II. The recovery and purification scheme presented is a representative design, however no experimental data are available to verify its applicability to Erwinia aroideae L-asparaginase.

Production of L-asparaginase involves six basic operations: fermentation, culture harvest, cell disruption, ammonium sulfate fractionation, ethanol fractionation, and lyophilization. All steps after fermentation must be done at 5°C, necessitating construction and operation of a refrigerated cold room. The breakdown of yearly manufacturing costs (equipment, labor, materials, and utilities) for each of the six basic operations (plus refrigerated cold room) is shown in Table 6. In general, utilities and equipment are minor costs compared to labor and materials costs. Fermentation costs and combined ammonium sulfate and ethanol fractionation costs are about equal and together account for about three-fourths of the manufacturing cost. Cell disruption is also important in determining the manufacturing cost, while cold room, culture harvest, and lyophilization costs are minor.

Table 6. Estimated Annual Manufacturing Costs for L-asparaginase Production from Erwinia aroideae.

	Equipment (depreciated at 10% per year)	Labor	Materials	Utilities	Total
Fermentation	$ 977	$18,000	$41,167	$ 107	$ 60,251
Refrigerated Cold Room	$ 916	$ 0	$ 0	$1,400	$ 2,316
Culture Harvest	$1,983	$ 7,200	$ 0	$ 40	$ 9,223
Cell Disruption (Enzyme Release)	$4,176	$ 7,200	$21,892	$ 8	$ 33,276
Ammonium Sulfate Fractionation	$ 361	$ 9,000	$31,200	$ 8	$ 40,569
Ethanol Fractionation	$ 322	$19,800	$ 108	$ 0	$ 20,230
Lyophilization	$ 135	$ 3,600	$ 0	$ 0	$ 3,735
TOTAL	$8,870	$64,800	$94,367	$1,563	$169,600

Manufacturing Cost: $169,600 per year.

Production Basis: 72 Million IU per year.

Unit Cost: $0.00235 per IU.

Economic analysis indicates that fermentation cost is a significant portion of the total manufacturing cost of L-asparaginase. Modification of the fermentation process could perhaps reduce the total manufacturing cost. If L-asparagine were replaced by a less expensive substrate, such as a casein hydrolysate, without substantial change in productivity of L-asparaginase, the cost for fermentation materials could be reduced from $41,167.00 to about $13,000.00, while other fermentation costs would remain about the same. However, specific L-asparaginase activity of Erwinia aroideae is significantly reduced when L-asparagine is replaced by complex media (see Table 3). This alteration would have several effects. First, more fractionation steps would be required to produce an L-asparaginase of acceptable purity from crude protein with a lower initial L-asparaginase content. The principal change resulting would be an increase in labor cost. Second, the fraction of L-asparaginase recovered could drop substantially with the addition of extra purification steps. This, in turn, would result in an increase in the scale of all operations to produce the same amount of L-asparaginase. Equipment and, more importantly, materials costs would also be uniformly increased. In conclusion, savings in media expense could be counterbalanced by an increase in purification steps and reduction in the overall efficiency of purification.

The estimated total manufacturing cost of L-asparaginase is $0.00235 per IU. L-asparaginase for non-medical use is priced at about $0.15 per IU (Worthington Biochemical Corporation). No information is available concerning the cost of L-asparaginase for clinical use. If the latter cost is comparable, then the manufacturing cost is only about 1.6% of the final

cost and the remainder would be attributable to various fixed costs or high profits. If this cost distribution is correct, optimization of the production process would be of little value. Commercial manufacturers may be writing off several years' expenses in developing the drug. The selling price may decrease substantially after these expenses have been accounted for and processing costs will increase in importance.

CONCLUSIONS

Careful control of conditions during growth on L-asparagine significantly increases specific L-asparaginase activity of Erwinia aroideae. However, the improvements due to process optimization are less substantial than those typically resulting from genetic strain improvement procedures. Penicillin production is one case in point. The significance of the processing variables and schemes analyzed in this work is summarized below.

Erwinia aroideae possesses an inducible L-asparaginase. L-asparagine used as the sole carbon and nitrogen source for fermentation produces higher specific activity than any of the other complex or defined media tested. The presence of an L-asparaginase inhibitor (D-asparagine) in the culture medium does not increase specific L-asparaginase activity and actually restricts growth and synthesis of L-asparaginase. Because L-asparagine is not available in bulk quantities, alternatives to its use should be considered. Other potential inducers such as glutamic acid and glutamine are produced in bulk and might serve as equally effective substrates, although no experimental evidence of this exists. Supplementation of a complex medium with L-asparagine is effective in increasing the specific L-asparaginase activity. Two stage batch culture involving glucose and L-asparagine was not effective. However, other combinations could prove effective.

Temperature has significant effects on production of L-asparaginase by Erwinia aroideae. The temperature resulting in maximum specific L-asparaginase activity, growth rate, and cell mass yield with L-asparagine mineral medium is 29°C. Temperatures above the optimum of 29°C are especially deleterious to specific activity and cell mass yield.

pH control is also an important factor in optimizing L-asparaginase production by Erwinia aroideae. Growth rate, specific activity, and especially cell mass yield are adversely affected by fermentation without pH control. Control at pH 7 is superior in all respects to control at pH 6 or pH 8. Automatic addition of acid was used to control pH, although increased buffering might also be satisfactory.

Vigorous aeration is essential for growth and production of L-asparaginase by Erwinia aroideae in L-asparagine-mineral medium. The organism is unable to ferment L-asparagine anaerobically and grows poorly when dissolved oxygen concentration drops below the critical level of 3 μmoles/l. Dissolved oxygen concentrations above atmospheric appear to be harmful.

Growth of Erwinia aroideae in L-asparagine limited continuous culture is characterized by cell mass yield which decreases as dilution rate increases. Continuous culture is ordinarily more productive than batch culture. However, in this case, the advantage of continuous culture is offset by cell mass yields lower than batch culture. Production of L-asparaginase is growth associated in continuous culture. There is no optimum dilution rate, therefore, associated with maximum specific L-asparaginase activity.

An important assumption of this work is that it is more important to maximize specific L-asparaginase activity than productivity of L-asparaginase because most of the cost of manufacturing L-asparaginase is in purification rather than in fermentation. A crude economic analysis was done which is supportive of this assumption. Much more information concerning purification of Erwinia aroideae L-asparaginase is needed before any assumption about fermentation objectives can be made with certainty, however.

ACKNOWLEDGMENTS

Several people are to be thanked for their generous assistance in this work. Professor Charles R. Wilke is especially thanked for his advice and direction. Dr. William Wernau's discussion of this research, especially concerning dissolved oxygen effects, is appreciated. The assistance of Mr. Paul Carroad in the experimental aspects is also acknowledged. The contributions of Mr. Lynn Williams in assembling the background for this research, assisting in experimental work, and discussing the results were invaluable.

This work was performed under the auspices of the United States Atomic Energy Commission and was supported by a National Science Foundation Traineeship.

NOMENCLATURE

Symbol	Definition	Units
D	dilution rate	$hr.^{-1}$
F	volume flow rate	l./hr.
Ks	Monod constant	g./l.
N	dry cell mass	g./l.
Po	initial or inlet product concentration	IU/l. (L-asparaginase)
P	product concentration	IU/l. (L-asparaginase)
Q_{O_2}	respiration rate	$\frac{\text{mmole}}{\text{g.-hr.}}$
So	initial or inlet substrate concentration	g./l.
S	substrate concentration	g./l.
t	time	hr.
V	fluid volume	l.
Xo	initial or inlet cell mass concentration	g./l.
X	cell mass concentration	g./l.
$Y_{x/s}$	yield of cell mass from substrate	--
$Y_{p/x}$	yield of product from cell mass	--
$Y_{p/s}$	yield of product from substrate	--
μmax	maximum specific growth rate	$hr.^{-1}$
μ	specific growth rate	$hr.^{-1}$

Experiment 1.1 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 0.5% tryptone, 0.5% yeast extract, 0.1% glucose-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0.1	-	0.025	-	-	-	-	-	-
1.0	-	0.047	-	-	-	-	-	-
2.2	-	0.139	-	-	-	-	-	-
3.1	-	0.362	-	-	-	-	-	-
4.2	-	0.595	-	-	-	-	-	-
5.8	-	1.08	-	-	-	-	-	-
7.1	-	1.47	-	-	-	-	-	-
8.7	-	1.99	-	-	-	-	-	-
9.6	-	2.05	-	-	-	-	0.877	-
11.7	-	2.22	-	-	-	-	-	-
15.9	-	2.60	-	-	-	-	0.900	-
27.8	-	2.22	-	-	-	-	-	-

Experiment 1.2 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% glucose-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0.1	-	0.0025	-	-	-	-	-	-
1.2	-	0.0025	-	-	-	-	-	-
2.2	-	0.0044	-	-	-	-	-	-
3.4	-	0.010	-	-	-	-	-	-
4.6	-	0.021	-	-	-	-	-	-
6.0	-	0.050	-	-	-	-	-	-
7.6	-	0.175	-	-	-	-	-	-
9.0	-	0.462	-	-	-	-	-	-
11.8	-	1.89	-	-	-	-	-	-
15.1	-	3.22	-	-	-	-	0.254	-
23.4	-	4.21	-	-	-	-	0.265	-
27.4	-	3.96	-	-	-	-	-	-

Experiment 1.3 - Media Analysis

Apparatus: 300 ml. side arm flask

Temperature: 30°C

Fluid Volume: 100 ml.

pH: initially 7.0, not controlled

Medium: 1.0% L-asparagine·H_2O-mineral

Aeration: none

Inoculum: 1 ml. dense, adapted culture

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0.1	-	0.014	-	-	-	-	-	-
1.0	-	0.017	-	-	-	-	-	-
2.2	-	0.034	-	-	-	-	-	-
3.1	-	0.056	-	-	-	-	-	-
4.2	-	0.102	-	-	-	-	-	-
5.8	-	0.315	-	-	-	-	-	-
7.1	-	0.550	-	-	-	-	-	-
8.7	-	0.750	-	-	-	-	-	-
10.1	-	1.54	-	-	-	-	1.372	-
11.7	-	1.50	-	-	-	-	-	-
16.1	-	1.41	-	-	-	-	1.260	-
27.8	-	1.41	-	-	-	-	-	-

Experiment 1.4 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% pectin-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0.1	-	0.021	-	-	-	-	-	-
1.2	-	0.022	-	-	-	-	-	-
2.2	-	0.023	-	-	-	-	-	-
3.4	-	0.025	-	-	-	-	-	-
4.6	-	0.030	-	-	-	-	-	-
6.1	-	0.039	-	-	-	-	-	-
7.6	-	0.059	-	-	-	-	-	-
9.0	-	0.128	-	-	-	-	-	-
11.8	-	0.582	-	-	-	-	-	-
15.1	-	1.87	-	-	-	-	-	-
16.5	-	2.29	-	-	-	-	0.231	-
23.1	-	3.33	-	-	-	-	0.386	-
27.5	-	4.23	-	-	-	-	-	-

Experiment 1.5 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 0.5% tryptone, 0.5% yeast extract, 0.1% glucose, 0.2% L-asparagine· H_2O-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.040	-	-	-	-	-	-
0.9	-	0.106	-	-	-	-	-	-
2.6	-	0.510	-	-	-	-	-	-
4.1	-	1.19	-	-	-	-	-	-
5.6	-	1.78	-	-	-	-	1.062	-
7.1	-	2.17	-	-	-	-	-	-
8.7	-	2.78	-	-	-	-	-	-
12.4	-	2.99	-	-	-	-	1.465	-

Experiment 1.6 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% tryptone-mineral

Inoculum: 1 ml. dense adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.023	-	-	-	-	-	-
0.9	-	0.042	-	-	-	-	-	-
2.6	-	0.217	-	-	-	-	-	-
4.1	-	0.519	-	-	-	-	-	-
5.7	-	0.676	-	-	-	-	-	-
6.8	-	0.740	-	-	-	-	0.373	-
8.8	-	0.750	-	-	-	-	-	-
12.3	-	0.830	-	-	-	-	0.518	-

Experiment 1.7 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% peptone-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.020	-	-	-	-	-	-
0.8	-	0.027	-	-	-	-	-	-
2.6	-	0.135	-	-	-	-	-	-
4.0	-	0.353	-	-	-	-	-	-
5.3	-	0.475	-	-	-	-	-	-
6.8	-	0.528	-	-	-	-	0.180	-
8.5	-	0.554	-	-	-	-	-	-
12.1	-	0.585	-	-	-	-	0.306	-

Experiment 1.8 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% beef extract-mineral

Inoculum: 1 ml. dense adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	-	-	-	-	-	-	-
0.7	-	-	-	-	-	-	-	-
2.3	-	0.006	-	-	-	-	-	-
5.5	-	0.139	-	-	-	-	-	-
6.9	-	0.209	-	-	-	-	-	-
8.5	-	0.228	-	-	-	-	0.139	-
12.2	-	0.354	-	-	-	-	0.160	-

Experiment 1.9 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 5.0% corn steep water-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0.2	-	0.063	-	-	-	-	-	-
2.9	-	0.398	-	-	-	-	-	-
4.5	-	0.760	-	-	-	-	-	-
5.6	-	0.890	-	-	-	-	0.450	-
7.1	-	1.14	-	-	-	-	-	-
8.0	-	1.38	-	-	-	-	-	-
11.4	-	2.73	-	-	-	-	0.792	-
13.9	-	2.51	-	-	-	-	-	-
15.0	-	3.35	-	-	-	-	-	-
27.4	-	3.83	-	-	-	-	-	-

Experiment 1.10 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 0.25% L-asparagine·H_2O,
0.75% D-asparagine·H_2O-mineral

Inoculum: 1 ml. dense, adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.018	-	-	-	-	-	-
0.6	-	0.025	-	-	-	-	-	-
1.4	-	0.028	-	-	-	-	-	-
1.9	-	0.030	-	-	-	-	-	-
2.4	-	0.034	-	-	-	-	-	-
2.8	-	0.038	-	-	-	-	-	-
3.7	-	0.049	-	-	-	-	-	-
6.5	-	0.135	-	-	-	-	-	-
8.5	-	0.291	-	-	-	-	0.135	-
10.4	-	0.561	-	-	-	-	-	-

Experiment 1.11 - Media Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 100 ml.

Medium: 1.0% D-asparagine·H_2O-mineral

Inoculum: 1 ml. dense, L-asparaginase adapted culture

Temperature: 30°C

pH: initially 7.0, not controlled

Aeration: none

Agitation: 225 RPM shaking

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.001	-	-	-	-	-	-
1.0	-	0.002	-	-	-	-	-	-
3.0	-	0.003	-	-	-	-	-	-
4.1	-	0.004	-	-	-	-	-	-
5.0	-	0.002	-	-	-	-	-	-
5.8	-	0.001	-	-	-	-	-	-
7.2	-	0.001	-	-	-	-	-	-
8.1	-	0.002	-	-	-	-	-	-

Experiment 2.1 - Temperature Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 21.0°C average

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.002	-	0.291	21.2	7.05	-	-
1.8	-	0.002	-	0.291	21.0	7.00	-	-
4.1	-	0.002	-	0.291	21.1	7.00	-	-
6.2	-	0.006	-	0.285	21.0	6.98	-	-
8.2	-	0.021	-	0.283	21.0	7.00	-	-
10.8	-	0.038	-	0.283	21.0	7.00	-	-
12.6	-	0.078	-	0.269	21.0	7.02	-	-
13.9	-	0.117	-	0.252	21.0	7.02	-	-
23.9	-	1.30	-	0.070	21.0	7.07	0.687	0.850
28.2	-	3.02	-	0.155	21.0	7.04	-	-
31.2	-	5.07	-	0.201	21.1	7.06	-	-
37.9	-	4.69	-	0.238	21.0	6.99	-	-

Experiment 2.2 - Temperature Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 25.0°C average

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.017	-	0.268	24.8	6.96	-	-
1.6	-	0.020	-	0.271	24.7	6.92	-	-
3.1	-	0.039	-	0.270	25.0	6.96	-	-
4.8	-	0.072	-	0.266	25.2	6.97	-	-
6.2	-	0.124	-	0.261	25.2	6.98	-	-
7.8	-	0.198	-	0.260	25.2	6.98	-	-
9.4	-	0.292	-	0.257	24.9	7.00	-	-
12.1	-	0.458	-	0.248	25.2	6.98	0.153	172
14.0	-	0.678	-	0.241	25.0	7.00	-	-
24.2	-	2.10	-	0.202	25.0	7.00	-	-
27.3	-	2.62	-	0.192	25.1	7.00	-	-
30.0	-	3.03	-	0.186	25.2	7.01	-	-
31.8	-	3.66	-	0.187	25.0	7.01	-	-
48.6	-	5.25	-	0.218	25.2	6.96	-	-

Experiment 2.3 - Temperature Analysis

Apparatus: 5 liter batch fermenter

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 32.9°C average

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.032	-	0.233	33.0	6.97	-	-
1.4	-	0.060	-	0.240	33.3	7.02	-	-
3.2	-	0.121	-	0.238	33.2	6.98	-	-
4.8	-	0.225	-	0.233	32.9	7.05	-	-
6.6	-	0.416	-	0.216	32.6	7.07	-	-
8.0	-	0.565	-	0.213	32.8	7.04	0.216	212
9.7	-	0.710	-	0.205	32.9	7.04	-	-
14.6	-	1.31	-	0.192	32.8	7.03	-	-
24.8	-	2.02	-	0.194	33.0	7.01	-	-
29.8	-	2.08	-	0.193	32.7	7.02	-	-
32.0	-	2.08	-	0.193	32.7	7.02	-	-

Experiment 2.4 - Temperature Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 37.0°C average

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.037	-	0.223	36.7	7.07	-	-
2.2	-	0.080	-	0.225	37.1	7.04	-	-
4.6	-	0.109	-	0.223	37.1	7.04	-	-
6.8	-	0.116	-	0.218	37.2	7.02	0.0276	92
8.9	-	0.116	-	0.229	37.0	7.04	-	-

Experiment 3.1 - pH Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.1°C average

pH: 6.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.024	-	0.252	29.0	6.02	-	-
1.4	-	0.038	-	0.253	29.2	6.00	-	-
3.0	-	0.084	-	0.247	29.2	6.06	-	-
4.7	-	0.188	-	0.236	29.3	6.07	-	-
6.1	-	0.305	-	0.228	29.3	6.06	-	-
8.0	-	0.430	-	0.217	28.8	6.05	0.137	233
9.6	-	0.595	-	0.209	29.0	6.05	-	-
27.0	-	2.53	-	0.118	28.7	6.09	-	-

Experiment 3.2 - pH Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.0°C average

pH: 8.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.035	-	0.247	29.0	7.84	-	-
1.2	-	0.070	-	0.253	29.1	7.98	-	-
2.6	-	0.112	-	0.251	28.9	8.01	-	-
3.7	-	0.180	-	0.253	29.2	8.00	-	-
5.2	-	0.317	-	0.223	29.1	8.00	-	-
6.8	-	0.610	-	0.182	29.0	8.05	0.246	261
7.9	-	1.01	-	0.173	29.0	8.04	-	-
10.2	-	1.96	-	0.077	29.0	8.05	-	-
11.4	-	2.53	-	0.154	29.1	8.08	-	-
24.2	-	4.59	-	0.227	29.0	8.00	-	-

Experiment 3.3 - pH Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.1°C average

pH: not controlled

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.032	-	0.253	29.2	7.00	0.0382	18
2.6	-	0.094	-	0.245	28.9	7.12	0.096	129
5.0	-	0.296	-	0.229	29.1	7.33	0.140	184
7.4	-	0.552	-	0.223	29.1	7.54	0.192	225
10.3	-	1.01	-	0.202	29.0	7.72	0.470	640
14.0	-	1.64	-	0.190	29.1	8.02	0.706	925
15.8	-	1.64	-	0.188	29.1	8.08	-	-
17.0	-	1.64	-	0.190	29.0	8.04	0.598	910

Experiment 4.1 - Dissolved Oxygen Analysis

Apparatus: 1 liter batch fermentor

Fluid Volume: 0.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 5 ml. dense, adapted culture

Temperature: 29.3°C

pH: not controlled, initially 7.0

Aeration: three different mixtures of O_2 and N_2 were used at different times

Agitation: magnetic stirrer at high RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (f./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.0075	-	0.00313	29.2	-	-	-
0.55	-	0.0075	-	0.00313	29.7	-	-	-
1.07	-	0.0087	-	0.00313	28.5	-	-	-
1.67	-	0.0128	-	0.00313	28.5	-	-	-
2.23	-	0.0191	-	0.00313	28.5	-	-	-
2.83	-	0.0284	-	0.00313	30.5	-	-	-
3.48	-	0.0278	-	0.00151	-	-	-	-
4.02	-	0.0319	-	0.00151	29.2	-	-	-
4.53	-	0.0406	-	0.00151	29.1	-	-	-
4.98	-	0.0516	-	0.00151	29.1	-	-	-
5.48	-	0.0680	-	0.00151	29.2	-	-	-
5.97	-	0.0820	-	0.00043	29.3	-	-	-
6.50	-	0.0980	-	0.00043	29.8	-	-	-
7.07	-	0.133	-	0.00043	29.8	-	-	-
7.48	-	0.160	-	0.00043	29.9	-	-	-

Experiment 4.2 - Dissolved Oxygen Analysis

Apparatus: 300 ml. side arm flask

Fluid Volume: 150 ml.

Medium: 1.0% L-asparagine·H_2O-mineral

Inoculum: 2 ml. dense, adapted aerobic culture

Temperature: 30°C

pH: initially 7.0, not controlled

Ae ation: slow flow of pure N_2

Agitation: none

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.016	-	-	-	-	-	-
0.9	-	0.016	-	-	-	-	-	-
2.3	-	0.016	-	-	-	-	-	-
4.3	-	0.019	-	-	-	-	-	-
5.7	-	0.020	-	-	-	-	-	-
25.8		0.020	-	-	-	-	-	-

Experiment 4.3 - Dissolved Oxygen Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.2°C

pH: 7.0 average

Aeration: 360 liters/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.050	-	0.253	29.3	7.04	-	-
1.1	-	0.087	-	0.251	29.0	7.01	0.068	54
3.0	-	0.247	-	0.238	29.1	7.02	0.164	132
4.2	-	0.409	-	0.225	29.2	7.01	-	-
5.2	-	0.630	-	0.215	29.2	7.01	0.306	248
7.3	-	1.325	-	0.196	29.1	7.06	-	-
10.2	-	3.12	-	0.112	29.2	7.04	1.671	1490
12.6	-	5.22	-	0.123	29.5	7.00	3.11	2630
14.7	-	5.10	-	0.219	29.0	7.00	2.87	2630

Experiment 4.4 - Dissolved Oxygen Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.2°C

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 212 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.052	-	0.253	29.4	7.00	-	-
1.0	-	0.101	-	0.207	29.2	7.04	0.097	95
3.5	-	0.338	-	0.093	29.4	7.15	0.189	159
6.0	-	0.675	-	0.004	29.0	6.97	0.266	290
8.7	-	1.18	-	0.001	29.0	7.12	0.602	755
12.4	-	1.83	-	0	29.2	7.08	0.949	1080
15.7	-	2.30	-	0	29.3	7.05	0.849	1215
17.0	-	2.46	-	0	29.0	7.08	-	-
26.1	-	2.56	-	0	29.0	7.05	1.111	1510
29.8		3.71	-	0	29.1	7.00	-	-

Experiment 4.5 - Dissolved Oxygen Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.0°C average

pH: 7.0 average

Aeration: 105 liter/hr. of 75% air - 25% nitrogen mixture

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Tempera-ture (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.022	-	0.605	29.1	6.98	-	-
1.3	-	0.030	-	0.613	29.2	7.16	-	-
2.7	-	0.053	-	0.588	29.1	7.05	-	-
4.2	-	0.115	-	0.588	29.1	7.08	-	-
5.8	-	0.224	-	0.572	28.9	7.09	-	-
7.1	-	0.319	-	0.581	28.9	7.01	0.133	174
8.8	-	0.479	-	0.547	28.9	6.98	-	-
10.2	-	0.720	-	0.563	28.8	6.98	-	-
11.8	-	1.10	-	0.557	28.8	6.96	-	-
13.2	-	1.29	-	0.580	29.2	6.96	-	-
23.3	-	3.54	-	0.332	29.1	6.95	-	-
25.9	-	4.17	-	0.547	29.0	6.98	-	-
29.0	-	4.63	-	0.607	28.8	6.99	-	-

Experiment 5.1 - Continuous Culture Analysis

Apparatus: 5 liter continuous fermentor

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.0°C average

pH: 7.0 average

Flow Rate (ml./min.)	6.8	12.5	15.3	20.2	42.1
Fluid Volume (l.)	2.88	2.49	2.44	2.73	2.88
Agitation (RPM)	950	1100	1200	1250	1300
Aeration (l./hr.)	300	360	360	360	360
Cell Mass Concentr. (g./l.)	5.58	4.48	3.54	3.74	0
Substrate Concentr. (g./l.)	0.34	0.38	0.73	0.76	13.20
L-asparaginase Activity (IU./ml.)	2.98	2.35	2.06	2.15	0
Protein Concentr. (µg./ml.)	1880	1800	1790	1920	0
Temperature (°C)	29.1	29.0	29.0	29.1	29.0
pH	7.00	7.00	6.95	7.00	7.00
Oxygen Concentr. (mmole/l.)	0.107	0.166	0.180	0.133	0.253

Experiment 5.2 - Continuous Culture Analysis

Apparatus: 5 liter continuous fermentor

Medium: 1.5% L-asparagine·H_2O-mineral

Inoculum: 100 ml. dense, adapted culture

Temperature: 29.0°C average

pH: 7.0 average

Flow Rate (ml./min.)	5.5	10.7	19.2	23.0
Fluid Volume (l.)	2.65	2.55	2.78	2.71
Agitation (RPM)	1200	1200	1200	1200
Aeration (l./hr.)	360	360	360	360
Cell Mass Concentr. (g./l.)	6.00	5.40	3.60	1.48
Substrate Concentr. (g./l.)	0.44	0.51	0.87	3.44
L-asparaginase Activity (IU/ml.)	2.67	3.37	1.93	0.98
Protein Concentr. (µg./ml.)	2030	2190	1410	685
Temperature (°C)	29.1	29.0	29.0	29.1
pH	7.00	6.90	7.00	7.00
Oxygen Concentr. (mmole/l.)	0.175	0.105	0.066	0.141

Experiment 6.1 - Two Stage Batch Culture Analysis

Apparatus: 5 liter batch fermentor

Fluid Volume: 3.7 liters after inoculation

Medium: 1.0% glucose-mineral (first stage)
1.0% L-asparagine-mineral (second stage)

Inoculum: 200 ml. dense, glucose-adapted culture

Temperature: 29.0°C average

pH: 7.0 average

Aeration: 360 liter/hr.

Agitation: 700 RPM

Time (hr.)	Flow Rate (ml./min.)	Cell Mass Concentr. (g./l.)	Substrate Concentr. (g./l.)	Oxygen Concentr. (mmole/l.)	Temperature (°C)	pH	L-asparaginase Activity (IU/ml.)	Protein Concentr. (µg./ml.)
0	-	0.067	-	0.253	28.8	6.96	-	-
1.2	-	0.066	-	0.249	29.1	7.04	-	-
2.2	-	0.106	-	0.248	28.9	7.00	-	-
3.5	-	0.203	-	0.240	28.9	6.99	-	-
4.8	-	0.403	-	0.229	29.0	7.02	0.066	0.236
6.2	-	0.850	-	0.205	29.0	7.00	-	-
7.4	-	1.75	-	0.184	29.0	6.96	-	-
8.7	-	2.69	-	0.155	29.5	7.00	0.328	1.08
11.5	-	6.18	-	0.100	29.0	6.99	-	-
12.2	-	6.88	-	0.184	28.9	7.06	-	-
12.7	* * * * * * * * * * * * * ADD 10 g./l. L-ASPARAGINE * * * * * * * * * * * * * * * * *							
12.8	-	6.88	-	0.133	29.2	7.00	0.749	3.20
13.8	-	7.34	-	0.108	29.4	7.04	0.834	3.36
14.7	-	8.38	-	0.081	29.0	7.03	1.107	3.69
15.9	-	10.14	-	0.046	28.7	7.00	1.697	4.26
16.7	-	10.74	-	0.062	28.7	7.03	-	-
17.2	-	10.74	-	0.147	28.5	7.00	1.096	4.34

APPENDIX II

APPENDIX II

Economic Analysis

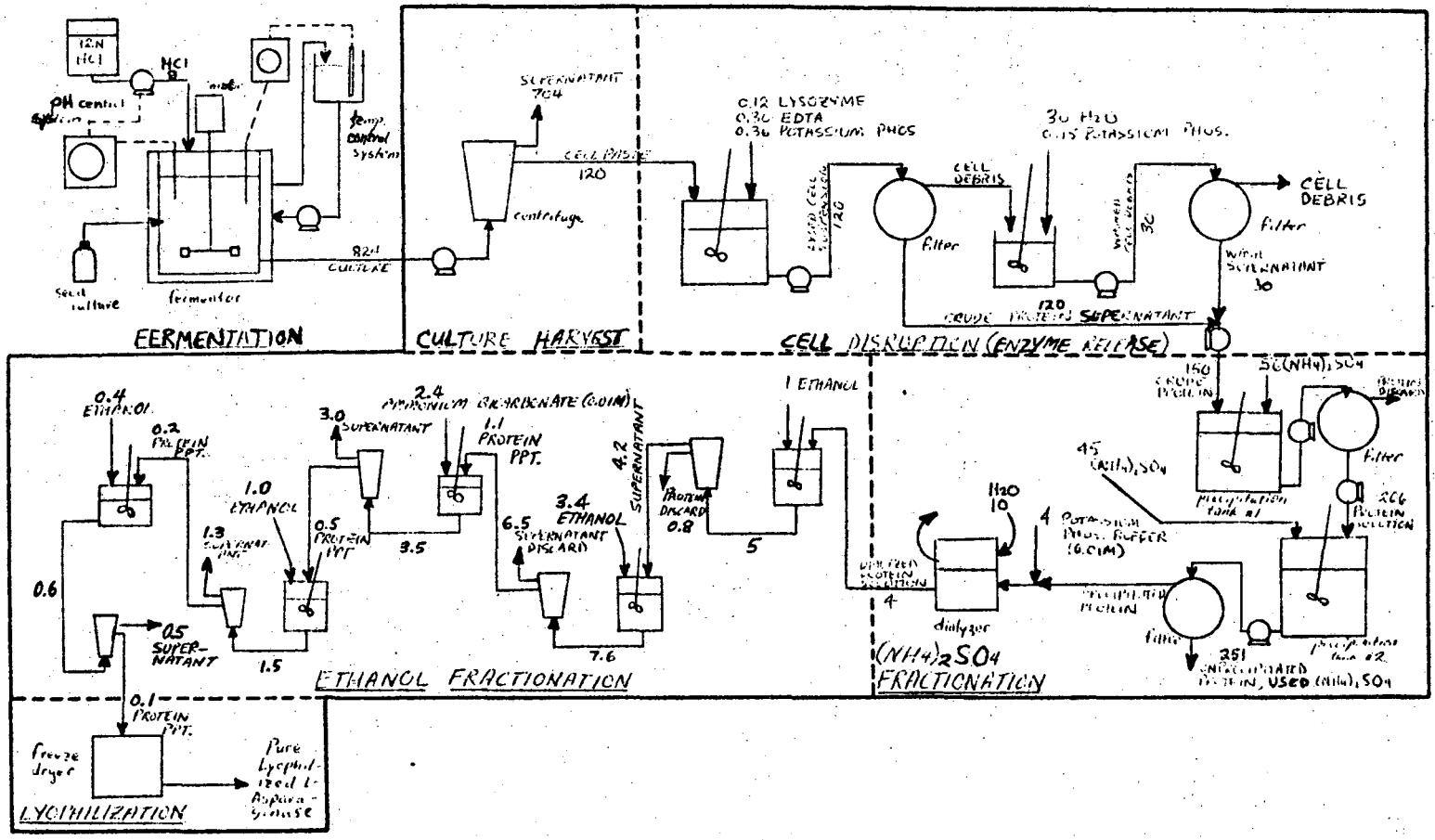
A typical process for production of L-asparaginase is shown schematically in Fig. 1A. The production basis was 72 million IU per year and 7200 operating hours per year. It was assumed that batch fermentation would take 16 hours, including down time, and that all other operations would take a total of 64 hours. It was also assumed that 25% of the L-asparaginase produced by fermentation could be recovered as final product.

Production of L-asparaginase takes place in six basic operations: fermentation, culture harvest, cell disruption, ammonium sulfate fractionation, ethanol fractionation, and lyophilization. All steps after fermentation must be done at 5°C. The recovery and purification operations were adapted from the procedures described by Ho et al. (34) for purification of E. coli L-asparaginase.

I. Fermentation

A. Process Equipment

(1) Fermentation was scaled up from the batch culture data presented previously. With a 3% inoculum, approximately 10 hours are required for a culture to reach stationary phase. Assuming a total of six hours are required to harvest the culture, prepare fresh medium, and sterilize, the complete fermentation cycle is 16 hours. If the maximum batch L-asparaginase yield is 3.1 IU/ml., then each batch must contain 206 l. of culture to produce 72 million IU per year of pure L-asparaginase.



XBL 7211-5864

Fig. 1A. Proposed process for production of L-asparaginase from Erwinia aroideae. Dark border surrounds operations inside 5°C cold room. Flow rates are approximate and are in kg. per 64 hour operation.

A 300 l. fermentor is sufficient, therefore. Sterilization of media can be done batchwise in the fermentor. A turbine mixer with sufficient power to provide oxygen transfer comparable to bench scale equipment is necessary. A 1 HP. motor is easily able to provide equal power input per unit volume of fluid. Given equal power input per unit volume, a necessary condition for insuring equal mass transfer rate in scale up is that superficial air velocities be equal. An air flow of 3.9 SCFH is required. The cost of a 300 l. jacketed, stainless steel fermentor is about $4,000.00. More sophisticated scale up procedures are available for estimating power and aeration requirements. However, the estimated requirements are quite modest and further refinement of the design is unwarranted for the present purpose.

(2) Air must be sterilized by filtration before entering the fermentor. Based on the example of Aiba (1. p. 235), a glass wool packed cylindrical filter 20 cm. in diameter and 100 cm. long is sufficient. The cost of fabricating such a filter is estimated to be $100.00.

(3) Temperature is controlled by circulation of water through a jacket surrounding the fermentor. With a heat transfer coefficient of 1 BTU/hr.ft.^{2}^{0}F, the estimated heat loss from the fermentor jacket to the air is 200 BTU/hr. A 1 kw. heater ($100.00) immersed in a 10 l. water recirculation tank ($50.00) is more than sufficient to make up the heat loss. A 50 ml./min. centrifugal pump ($250.00) is used to recirculate water. The cost of a proportional controller-recorder is about $500.00, including thermistor. The total cost of temperature control, therefore, is $900.00.

(4) pH is controlled by addition of 10 N HCl to the fermentor. Approximately 2 liters are required per batch for neutralization of ammonia. A small (10 ml./min.) positive displacement pump ($130.00) is needed for acid addition along with a reservoir ($10.00). The cost of an on-off controller-recorder is about $450.00 and pH electrodes are about $200.00. The total cost of pH control, therefore, is $790.00.

(5) Other expenses related to fermentation equipment were estimated as follows. Seed culture apparatus need only be a 10 l. jar with air sparger and magnetic stirrer, costing about $50.00. Pipe and pipe fittings were assumed to be 66% of the fermentor cost ($2,640.00). Installation was assumed to be 30% of the fermentor cost ($1,200.00). Electrical wiring was assumed to be 5% of the cost of the two control units ($90.00).

B. Labor

The cost of labor was assumed to be $8.00 per hour, including overhead expenses associated with labor. During a batch fermentation, it is assumed that an operator is required for four of the six hours down time between batches to clean the fermentor, prepare fresh medium, sterilize the fermentor with medium, cool the medium, and inoculate. A total of one additional hour per batch is needed for periodic observation of the operation. Therefore, a total of five man-hours is required per batch, or 2,250 man-hours per year, costing $18,000.00.

C. Materials

The cost of materials was found by direct scale-up of the media formulation listed in Table 2, including 15 g./l. of L-asparagine·H_2O.

The approximate costs of major media constituents are listed below. Materials costs were generally taken from Chemical Marketing Reporter (13).

(1) 1350 kg. of L-asparagine·H_2O are required per year. At $30.00 per kg. (Sigma Chemical Co.), the cost of L-asparagine is $40,500.00 per year.

(2) 180 kg. of potassium phosphate are required per year. At $0.32 per kg., the cost is $57.00 per year.

(3) 1000 kg. of hydrochloric acid are required per year for control of pH. At $0.49 per kg., the cost is $490.00 per year.

(4) About 40 kg. of miscellaneous salts are required per year to complete the formulation of the medium. The estimated cost is $120.00 per year.

D. Utilities

(1) About 1000 kw-hr. of electricity are required annually for operation of the fermentor drive and temperature control heater. At $0.02 per kw-hr., the cost is $20.00 per year.

(2) About 90,000 l. of city water are required annually for making medium. At $0.10 per 1000 l., the estimated cost is $9.00 per year.

(3) A heat balance was made to find the steam requirement for media sterilization. Sterilization is accomplished by injection of 100 psig steam into the medium, raising it to 15 psig (saturated liquid), holding for about 20 minutes, followed by release of pressure and cooling. The estimated annual steam consumption is 33,700 kg. At $2.20 per 1000 kg., the cost is $74.00 per year.

(4) About 28,000 SCF per year of dry, filtered air are required by fermentation. At $0.12 per 1000 SCF, the cost is $4.00 per year.

II. Refrigerated Cold Room

A. Process Equipment

(1) All purification operations must be carried out at 5°C. A 40' × 40' × 10' refrigerated cold room is large enough to contain all purification equipment. The estimated cost of insulating the walls of the room with one foot thick concrete blocks and installing an automatic door is $4,160.00.

(2) The heat leak into the cold room from the surroundings was estimated by assuming a thermal conductivity of 0.4 BTU/hr.ft.°F. Assuming that a cold room would be kept refrigerated 365 days per year, the annual refrigeration load is 6.73×10^8 BTU or 6.4 tons. The estimated cost of a 6.4 ton refrigeration unit is $5,000.00.

B. Labor

No labor is associated with cold room operation.

C. Materials

No materials are associated with cold room operation.

D. Utilities

Refrigeration is the primary utility cost in the production of L-asparaginase. 673 million BTU must be removed per year. At $0.60 per ton-day, the cost is $1,400.00 per year.

III. Culture Harvest

A. Process Equipment

(1) A tubular bowl, high speed centrifuge is needed for harvesting and concentrating the culture. Perry (45) suggests that a 3 HP., 4"

diameter bowl tubular high speed centrifuge may have a minimum capacity of 30 gal./hr. for difficult separations. This implies about a two hour harvesting time. The cost of a stainless steel unit is about $10,000.00. The centrifuge is fed from the fermentor by a 200 l./hr. slurry pump ($170.00).

(2) Pipe and pipe fittings were estimated to be 66% of the equipment cost ($6,610.00) and installation was assumed to be 30% of the equipment cost ($3,050.00).

B. Labor

Four fermentation batches must be harvested during each 64 hour cycle. A total of eight man-hours per cycle are estimated for this operation. At $8.00 per hour, the cost is $7,200.00 per year.

C. Materials

No materials are used in culture harvest.

D. Utilities

About 200 kw-hr. of electricity are required annually for operation of the high speed centrifuge. At $0.02 per kw-hr., the cost is $40.00.

IV. Cell Disruption (Enzyme Release)

A. Process Equipment

(1) Because four fermentation batches can be run during the course of one purification batch, provision must be made for storing harvested culture. Assuming that culture is concentrated into 15% of its original volume after centrifugation, a maximum of 120 l. of cell cream must be stored. A 200 l. stainless steel tank and mixer costs $450.00.

(2) Cell disruption is accomplished by addition of 1 g./l. of lysozyme, 3 g./l. of EDTA, and 3 g./l. of potassium phosphate to the stored cell cream. About 16 hours are required for disruption.

(3) Cell debris must be separated from crude enzyme solution after cell disruption. A vacuum drum filter can be used for this purpose. Since solids concentration and filtration rates are unknown, estimation of the filter size is difficult. Assuming a filtration rate of 3×10^{-3} ml./cm.^{2}sec. for very fine particles, a 24 ft.^{2} filter area is indicated for a 30 minute filtration. The estimated cost of the filter is $20,000.00. A 400 l./hr. pump ($250.00) transfers crude enzyme solution to the filter.

(4) Filtered solids from cell disruption must be washed with phosphate buffer (about 150 g. of potassium phosphate in 30 l. of water) in a 40 l. stainless steel tank with mixer ($170.00) in order to recover all of the original L-asparaginase. The washing should require approximately 30 minutes. Refiltration of the solids should also require 30 minutes. A 200 l./hr. slurry pump ($170.00) transfers washed solids to the vacuum drum filter (one filter is used for both operations). A 400 l./hr. slurry pump ($250.00) transfers released enzyme solution and wash supernatant solution to a tank for ammonium sulfate precipitation.

(5) Pipe and pipe fittings were estimated to be 66% of the equipment cost ($14,070.00) and installation was assumed to be 30% of equipment cost ($6,400.00).

B. Labor

A total of eight man-hours per cycle are estimated for cell disruption. At $8.00 per hour, the cost is $7,200.00 per year.

C. Materials

(1) A total of 57.4 kg. of potassium phosphate are required annually for cell disruption. At $0.32 per kg., the cost is $18.00 per year.

(3) A total of 13.5 kg. of lysozyme are required annually for cell disruption. At $1.60 per g. (Sigma Chemical Co.) the cost is $21,600.00 per year.

D. Utilities

About 400 kw-hr. of electricity are required annually for operation of the vacuum drum filter for cell debris filtration. At $0.02 per kw-hr., the cost is $8.00 per year. Other utility costs are insignificant.

V. Ammonium Sulfate Fractionation

A. Process Equipment

(1) Crude enzyme filtrate and wash solutions are combined in a 300 l. stainless steel tank with mixer ($570.00) for ammonium sulfate precipitation. About 56 kg. of ammonium sulfate are added for a first precipitation of protein. The precipitate is filtered with the vacuum drum filter described above and the filtrate is returned to a second 300 l. stainless steel tank with mixer ($570.00) for addition of another 45 kg. of ammonium sulfate. The protein precipitated by this step is eluted with 4 l. of phosphate buffer. Two 400 l./hr. slurry pumps ($500.00) are required for this operation. Precipitations and filtrations require a total of about three hours.

(2) 4 l. of partially purified L-asparaginase must be dialyzed to remove ammonium sulfate before further purification. A bench scale

dialyzer ($200.00) can remove more than 99% of the salt in 12 hours. About 10 l. of water are required.

(3) Pipe and pipe fittings were estimated to be 66% of the equipment cost ($1,220.00) and installation was estimated to be 30% of the equipment cost ($550.00).

B. Labor

Ten man-hours per cycle are estimated for ammonium sulfate precipitation. At $8.00 per hour, the cost is $9,000.00 per year.

C. Materials

113,600 kg. of ammonium sulfate are used annually for protein precipitation. At $0.275 per kg., the cost is $31,200 per year.

D. Utilities

About 400 kw-hr. per year of electricity are required for operation of the vacuum drum filter during ammonium sulfate precipitations. At $0.02 per kw-hr., the cost is $8.00 per year.

VI. Ethanol Fractionation

A. Process Equipment

Several successive fractionations with ethanol are required for final purification of L-asparaginase. The volumes handled are quite small as indicated in Fig. 1A and the operations are bench scale. Three stainless steel tanks with mixers ($220.00) are required since a tank can be used for more than one fractionation. A small (15 l./hr.) centrifuge ($3,000.00) is needed for separating protein fractions between steps. The final step in the purification is the slow recrystallization of pure

L-asparaginase, requiring about 16 hours. The total time for ethanol fractionations is about 22 hours. Approximately 6 l. of absolute ethanol are needed per cycle.

B. Labor

Twenty-two man-hours per cycle are estimated for ethanol fractionation. At $8.00 per hour, the cost is $19,800.00 per year.

C. Materials

The only important material cost for ethanol fractionation is for 675 l. per year of absolute ethanol. At $0.16 per l., the cost is $108.00 per year.

D. Utilities

Utility costs for ethanol fractionation are insignificant.

VII. Lyophilization

A. Process Equipment

In the final processing step, a small amount of wet L-asparaginase crystals must be lyophilized before standardization and packaging. During lyophilization, both water and ethanol are removed. The cost of a bench scale freeze dryer, suitable for this operation, is about $1,350.00. Approximately an eight hour residence time is required.

B. Labor

Four man-hours per cycle are estimated for lyophilization. At $8.00 per hour, the cost is $3,600.00 per year.

C. Materials

No materials are consumed during lyophilization.

D. Utilities

Utility costs for lyophilization are insignificant.

Manufacturing costs for L-asparaginase production are tabulated and totalized in Table 1A. It is important to realize that equipment cost is only a minor portion of the total cost. Even if relatively large errors exist in the rough equipment cost estimates, the significance in the overall cost estimate is small. The estimations of labor and materials costs are of primary importance in determining production cost. In estimating labor costs, it was assumed that labor could be used on a part time basis (i.e. only when actually needed). Therefore, labor costs were itemized. If labor must be used on a full time basis, the labor costs would be approximately twice as high. The relationship of the seven cost categories to each other would remain similar, however.

Table 1A. Summary of Annual Estimated Costs for Production of L-asparaginase from Erwinia aroideae.

I.	Fermentation	
	A. Process Equipment (depreciated @ 10% per yr.)	
	(1) Fermentor	$400
	(2) Air filter	10
	(3) Temperature control system	90
	(4) pH control system	79
	(5) Seed culture apparatus	5
	(6) Pipe and pipe fittings	264
	(7) Installation	120
	(8) Electrical wiring	9
	Total	$977
	B. Labor	$18,000
	C. Materials	
	(1) L-asparagine	$40,500
	(2) Potassium phosphate	57
	(3) Hydrochloric acid	490
	(4) Miscellaneous salts	120
	Total	$41,167
	D. Utilities	
	(1) Electricity	20
	(2) Water	9
	(3) Steam	74
	(4) Air	4
	Total	$107
	Fermentation Total	$60,251

(continued)

Table 1A (cont.)

II.	Refrigerated Cold Room	
	A. Equipment (depreciated @ 10% per yr.)	
	(1) Insulated room	$416
	(2) Refrigeration unit	500
	Total	$916
	B. Labor	$0
	C. Materials	$0
	D. Utilities	
	(1) Refrigeration	$1,400
	Refrigerated Cold Room Total	$2,316
III.	Culture Harvest	
	A. Process Equipment (depreciated @ 10% per yr.)	
	(1) Centrifuge	$1,000
	(2) Slurry pump	17
	(3) Pipe and pipe fittings	661
	(4) Installation	305
	Total	$1,983
IV.	Cell Disruption (Enzyme Release)	
	A. Process Equipment (depreciated @ 10% per. yr.)	
	(1) Mixing tank (200 l.)	$ 45
	(2) Mixing tank (40 l.)	17
	(3) Vacuum drum filter	2,000
	(4) Two pumps (400 l./hr.)	50

(continued)

Table 1A (cont.)

(5)	Pump (200 l./hr.)	17
(6)	Pipe and pipe fittings	1,407
(7)	Installation	640
	Total	$4,176
B.	Labor	$7,200
C.	Materials	
(1)	Potassium phosphate	$ 18
(2)	EDTA	274
(3)	Lysozyme	21,600
	Total	$21,892
D.	Utilities	
(1)	Electricity	$8
Cell Disruption Total		$33,276
V.	Ammonium Sulfate Fractionation	
A.	Process Equipment (depreciated @ 10% per yr.)	
(1)	Two mixing tanks (300 l.)	$114
(2)	Two pumps (400 l./hr.)	50
(3)	Dialyzer	20
(4)	Pipe and pipe fittings	122
(5)	Installation	55
	Total	$361
B.	Labor	$9,000
C.	Materials	
(1)	Ammonium sulfate	$31,200

(continued)

Table 1A (cont.)

D. Utilities	
(1) Electricity	$8
Ammonium Sulfate Fractionation Total	$40,208
VI. Ethanol Fractionation	
A. Process Equipment (depreciated @ 10% per yr.)	
(1) Three mixing tanks (10 l.)	$ 22
(2) Centrifuge	300
Total	$322
B. Labor	$19,800
C. Materials	
(1) Absolute ethanol	$108
D. Utilities	$0
Ethanol Fractionation Total	$20,230
VII. Lyophilization	
A. Process Equipment (depreciated @ 10% per yr.)	
(1) Freeze dryer	$135
B. Labor	$3,600
C. Materials	$0
D. Utilities	$0
Lyophilization Total	$3,735

REFERENCES

1. S. Aiba, A. E. Humphrey, and N. F. Millis, Biochemical Engineering (Academic Press, New York, 1965).

2. M. Apple, Hemodialysis Against Enzymes as a Method of "Gene Replacement" in Cases of Inherited Metabolic Diseases, Proceedings of the Western Pharmacology Society 14, 125-130 (1971).

3. M. H. Bilimoria, Conditions for the Production of L-asparaginase 2 by Coliform Bacteria, Appl. Microbiol. 18, 1025-1030 (1969).

4. L. D. Boeck, R. W. Squires, M. W. Wilson, and P. P. K. Ho, Effect of Glucose and Low Oxygen Tension on L-asparaginase Production by a Strain of Escherichia coli B, Appl. Microbiol. 20, 964-969 (1970).

5. J. D. Broome, Evidence that the L-asparaginase Activity of Guinea Pig Serum is Responsible for its Antilymphoma Effects, Nature 191, 1114-1115 (1961).

6. J. D. Broome, Factors Which May Influence the Effectiveness of L-Asparaginases as Tumor Inhibitors, Brit. J. Cancer 22, 595-602 (1968).

7. D. E. Brown, Aeration in the Submerged Culture of Microorganisms, Methods in Microbiology, 125-174 (Academic Press, New York, 1970).

8. H. A. Campbell, L. T. Mashburn, E. A. Boyse, and L. J. Old, Two L-Asparaginases from Escherichia coli B. Their Separation, Purification, and Antitumor Activity, Biochem. 6, 721-730 (1967).

9. H. A. Campbell and L. T. Mashburn, L-Asparaginase EC-2 from Escherichia coli. Some substrate Specificity Characteristics, Biochem. 8, 3768-3775 (1969).

10. R. L. Capizzi, J. R. Bertino, and R. E. Handschumacher, L-Asparaginase, Ann. Rev. Med. 21, 433-444 (1970).

11. H. Cedar and J. H. Schwartz, Production of L-Asparaginase II by Escherichia coli, J. Bacteriol. 96, 2043-2048 (1968).

12. T. M. S. Chang, The in vivo Effects of Semipermeable Microcapsules Containing L-Asparaginase on 6C3HED Lymphosarcoma, Nature 229, 117-118 (1971).

13. Chemical Marketing Reporter 202, 13, 27-37 (1972).

14. T. C. Chou, D. A. Cooney, W. A. Creasey, and R. E. Handschumacher, Evaluation of Asparagine Biosynthesis in Neoplastic Cells and Effects of Asparagine Analogues, Pharmacologist 10, 170 (1968).

15. A. Clementi, Desamidination Enzymatique de L'Asparagine, Archives Internationales de Physiologie 19, 369-398 (1922).

16. E. E. Conn and P. K. Stumpf, Outlines of Biochemistry, Second Edition (John Wiley and Sons, Inc., New York, 1967).

17. D. A. Cooney and R. E. Handschumacher, L-Asparaginase and L-Asparagine Metabolism, Ann. Rev. Pharmacol., 421-440 (1970).

18. D. Crowther, L-Asparaginase and Human Malignant Disease, Nature 229, 168-171 (1971).

19. L. C. DeAngeli, F. Pocchiari, S. Russi, A. Tonolo, V. E. Zurita, E. Ciaranfi, and A. Perin, Effect of L-Asparaginase from Aspergillus terreus on Ascites Sarcoma in the Rat, Nature 225, 549-550 (1970).

20. W. C. Dolowy, D. Henson, J. Cornet, and H. Selin, Toxic and Antineoplastic Effects of L-Asparaginase, Cancer 19, 1813-1819 (1966).

21. F. El Asmar and D. M. Greenberg, Mechanisms of Tumor Growth and Inhibition by Glutaminase from a Pseudomonad, Proceedings of the American Association for Cancer Research 6, 17 (1965).

22. E. F. Gale, Factors Influencing the Enzymic Activities of Bacteria, Bacteriol. Rev. 7, 139-173 (1943).

23. S. Goldsweig, (National Cancer Institute, Bethesda, Maryland), private communication, September, 1972.

24. E. A. Grula, Cell Division in a Species of Erwinia II. Inhibition of Division by D-Amino Acids, J. Bacteriol. 80, 375-384 (1960).

25. E. E. Haley, G. A. Fischer, and A. D. Welch, The Requirement for L-Asparagine of Mouse Leukemia Cells L5178Y in Culture, Cancer Res. 21, 532-536 (1961).

26. D. E. F. Harrison, D. G. MacLennan, and S. J. Pirt, Responses of Bacteria to Dissolved Oxygen Tension, Fermentation Advances, D. Perlman, ed. (Academic Press, New York, 1969).

27. F. X. Hasselberger, H. D. Brown, S. K. Chattopadhyay, A. N. Mather, R. O. Stasin, A. B. Patel, and S. N. Pennington, The Preparation of Insoluble Matrix-supported Derivatives of Asparaginase for Use in Cancer Therapy, Cancer Res. 30, 2736-2738 (1970).

28. B. Heinemann and A. J. Howard, Production of Tumor-inhibitory L-Asparaginase by Submerged Growth of Serratia marcescens, Appl. Microbiol. 18, 550-554 (1969).

29. B. Heinemann, A. J. Howard, and H. J. Palocz, Influence of Dissolved Oxygen Levels on Production of L-Asparaginase and Prodigiosin, Appl. Microbiol. 19, 800-804 (1970).

30. D. Herbert, The Chemical Composition of Microorganisms as a Function of their Environment, Eleventh Symposium of the Society of General Microbiology 11, 401 (1961).

31. F. Hernadi, K. Polya, and A. Daroczy, Production of L-Asparaginase Isoenzymes by Strains E. coli, Arch. Mikrobiol. 77, 151-154 (1971).

32. J. M. Hill, J. Roberts, E. Loeb, A. Khan, A. MacLellan, and R. W. Hill, L-Asparaginase Therapy for Leukemia and Other Malignant Neoplasms, J. Am. Med. Assoc. 202, 882-888 (1967).

33. P. P. K. Ho, B. H. Frank, and P. J. Burck, Crystalline L-Asparagine from Escherichia coli B, Science 165, 510-511 (1969).

34. P. P. K. Ho, E. B. Milikin, J. L. Bobbitt, E. L. Grinnan, P. J. Burck, B. H. Frank, L. D. Boeck, and R. W. Squires, Crystalline L-Asparaginase from Escherichia coli B, I. Purification and Chemical Characterization, J. Biol. Chem. 245, 3708-3715 (1970).

35. G. E. Jones, L-Asparagine Degradation in Saccharomycees cerevisiae: Genetic Control and Biochemical Properties of Yeast L-Asparaginase, University of California, Lawrence Radiation Laboratory, Report UCRL-19480 (1970).

36. A. A. Khan, S. P. Pal, S. R. V. Raghavan, and P. K. Bhattacharyya, Studies on Serratia marcescens L-Asparaginase, Biochem. Biophys. Res. Commun. 41, 525-533 (1970).

37. J. G. Kidd, Regression of Transplanted Lymphomas Induced in vivo by Means of Normal Guinea Pig Serum, J. Exptl. Med. 98, 565-581 (1953).

38. F. S. Liu and J. E. Zajic, L-Asparaginase Synthesis by Erwinia aroideae, Appl. Microbiol. 23, 667-668 (1972).

39. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem. 193, 265-275 (1951).

40. L. T. Mashburn and J. C. Wriston, Tumor Inhibitory Effect of L-Asparaginase, Biochem. Biophys. Res. Commun. 12, 50-55 (1963).

41. L. T. Mashburn and J. C. Wriston, Tumor Inhibitory Effect of L-Asparaginase from Escherichia coli, Arch. Biochem. Biophys. 105, 450-452 (1964).

42. L. T. Mashburn and J. C. Wriston, Change in Ribonuclease Concentrations in L-Asparaginase-treated Lymphosarcomata, Nature 211, 1403-1404 (1966).

43. R. A. Neuman and T. A. McCoy, Requirement of Walker Carcinosarcoma 256 in vitro for Asparagine and Glutamine, Science 124, 124-125 (1956).

44. H. F. Oettgen, L. J. Old, E. A. Boyse, H. A. Campbell, F. S. Philips, B. D. Clarkson, L. Tallal, R. D. Leeper, M. K. Schwartz, and J. H. Kim, Inhibition of Leukemias in Man by L-Asparaginase, Cancer Res. 27, 2619-2631 (1967).

45. R. H. Perry (editor), Chemical Engineers' Handbook, Fourth Edition (McGraw-Hill Book Company, New York, 1963).

46. M. S. Peters and K. D. Timmerhaus, Plant Design and Economics for Chemical Engineers, Second Edition (McGraw-Hill Book Company, New York, 1968).

47. R. E. Peterson and A. Ciegler, L-Asparaginase Production by Erwinia aroideae, Appl. Microbiol. 18, 64-67 (1969).

48. R. E. Peterson and A. Ciegler, Factors Influencing L-Asparaginase Production by Erwinia aroideae, Appl. Microbiol. 23, 671-673 (1972).

49. V. V. S. Reddy, H. N. Jayaram, M. Sirsi, and T. Ramakrishnan, Inhibitory Activity of L-Asparaginase from Mycobacterium tuberculosis on Yoshida Ascites Sarcoma in Rats, Arch. Biochem Biophys. 132,

50. J. Roberts, G. Burson, and J. M. Hill, New Procedures for Purification of L-asparaginase with High Yield from Escherichia coli, J. Bacteriol. 95, 2117-2123 (1968).

51. C. W. Robinson and C. R. Wilke, Mass Transfer Coefficients and Interfacial Area for Gas Absorption by Agitated Aqueous Electrolyte Solutions, University of California, Lawrence Radiation Laboratory, Report UCRL-20472 (1971).

52. R. S. Robison and B. Berk, L-Asparaginase Synthesis by Escherichia coli B, Biotechnology and Bioengineering 11, 1211-1225 (1969).

53. J. H. Schwartz, J. Y. Reeves, and J. D. Broome, Two L-Asparaginases from E. coli and their Action Against Tumors, Proceedings of the National Academy of Sciences 56, 1516-1519 (1966).

54. F. D. Snell and C. T. Snell, Colorimetric Methods of Analysis, Vol. 4 (D. van Nostrand Co., Inc., New York, 1954).

55. J. Staerk, O. Zwisler, and H. Ronneberger, Comparison of L-Asparaginase from Erwinia aroideae and from Escherichia coli: Biochemical and Biological Properties, Experientia 27, 250-252 (1971).

56. R. Y. Stanier, M. Douderoff, and E. A. Adelberg, The Microbial World, Third Edition (Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1970).

57. H. E. Wade, R. Elsworth, D. Herbert, J. Keppie, and K. Sargeant, A New L-Asparaginase with Antitumor Activity, Lancet 2, 776-777 (1968).

58. H. E. Wade and D. A. Rutter, Asparaginase-Treatment for Leukemia, Science Journal 6, 62-67 (1970).

59. H. H. Weetal, Insolubilized L-Asparaginase Implant: A Preliminary Report, Journal of Biomedical Materials Research 4, 597-599 (1970).

60. S. Weinberger, L-Asparaginase as an Antitumor Agent, Enzyme 12, 143-160 (1971).

61. H. A. Whelen and J. C. Wriston, Purification and Properties of Asparaginase from Escherichia coli B, Biochem. 8, 2386-2393 (1969).

62. T. B. Young, A dynamic Analysis of Microbial Growth in a Chemostat Continuous Culture, Ph.D. Thesis, Department of Chemical Engineering, Clemson University, Clemson, S. C., 1971.

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720