FOAMING AND THE PRODUCTION OF THE ANTIBIOTIC BACILLOMYCIN L

by

Stephen G. Kuzak, B.Eng.

Department of Chemical Engineering
McGill University

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McGill University
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ABSTRACT

An examination was made of the effect of a variety of process parameters on the foam capacity of a fermentation broth. The foaming was found to be directly related to the concentration of bacillomycin L, a surface active antibiotic produced by a strain of Bacillus subtilis. Changing the pH was found to have no direct effect on foam capacity. Lowering the incubation temperature reduced foaming but only by decreasing the production of bacillomycin L. Likewise, reductions in the concentration of glutamic acid, the nitrogen source in the medium, resulted in corresponding reductions in foam capacity, bacillomycin concentration and biomass concentration. When glutamic acid was replaced with sodium nitrate, the effects were similar. However, when glutamic acid was replaced with ammonium chloride, cell growth was poor and bacillomycin production was very low. When the pH was controlled by increasing the amount of phosphate in the medium, growth returned to normal while bacillomycin concentration and foam capacity remained relatively low. When the phosphate was replaced with Tris buffer, both bacillomycin production and foam capacity increased to normal levels, indicating that the production of bacillomycin is inhibited by the presence of inorganic phosphates. Finally, when the overall electrolyte concentration of the Tris-buffered medium was increased by 3-4 times, bacillomycin production was high but much of the foaming was suppressed.

RÉSUMÉ

L'effet de différents paramètres sur la formation de mousse dans un milieu de fermentation a été étudié. On a constaté que la formation de mousse est directement liée à la concentration de bacillomycine L, un antibiotique tensio-actif produit à partir d'une sous-espèce du Bacillus subtilis. Changer le pH n'a pas d'effet direct sur la formation de mousse. Abaisser la temperature d'incubation réduit la formation de mousse, mais seulement en réduisant la production de bacillomycine L. De même, abaisser la concentration en acide glutamique, la source d'azote dans le milieu, fait diminuer la formation de mousse, les concentrations de bacillomycine et de biomasse. Remplacer l'acide glutamique par du nitrate de sodium produit des effets semblables. Cependant, lorsque l'acide glutamique est remplacé par du chlorure d'ammonium, la croissance des cellules faiblit, ainsi que la production de bacillomycine. En controlant le pH par augmentation du taux de phosphate dans le milieu, on obtient une croissance des cellules normales pour des concentrations en bacillomycine et une formation de mousse qui demeurent relativement faibles. Quand le phosphate est remplacé par un tampon organique, la production de bacillomycine et la formation de mousse retournent à des niveaux normaux, indiquant par là que la production de bacillomycine est rendue impossible par la présence de phosphates inorganiques. Finallement, quand la concentration en electrolyte du milieu organique tampon est multipliée par trois ou quatre, on obtien une production de bacillomycine important tandis que la formation de mousse se trouve grandement réduite.

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1.0 INTRODUCTION

Foaming is a widespread problem in many chemical process industries. It is especially prevalent in biochemical reactors because fermentation broths routinely contain surface active substances either produced by the microorganisms or resulting from cell lysis. This study of the foaming phenomenon, as it appeared in a continuously phased system of Bacillus subtilis grown on a glucose-based medium, is an attempt to determine the effect of a variety of process variables on foaming. The use of antifoam agents or a hydrocarbon substrate to suppress the foaming was ruled out for reasons discussed in Chapter 5.0. Similarly, the foaminess of the broth was not exploited as a means of concentrating and separating the product (as in Gehle and Schugerl, 1984) because of limitations arising from the continuous phasing method itself.

1.1 Continuous Phasing Method

Batch fermentation is still the most widely used mode of production in the biochemical industry. While batch culturing does have some obvious advantages, it poses a fundamental process control problem. Since the biochemical engineer must deal with large populations of cells, any physical measurement that he makes will be a statistical quantity, an average value. The batch is made up of a great number of cells at many different stages of the growth and reproductive cycle. Therefore, these averaged quantities are often insufficient to characterize the system thoroughly enough to allow for a proper control scheme

(Dawson, 1972).

One answer to this problem is to synchronize the metabolism of the cells within a given population. If the cells can be made to reproduce at the same time, then the population may be treated as a homogenous entity (Zeuthen, 1958). Induction synchrony can be achieved through the use of a chemostat by introducing the growth-limiting nutrient into the reactor with a frequency equal to the doubling time of the cells (Goodwin, 1969). Another method of inducing synchrony is to dilute the culture periodically with an equal volume of fresh broth with a frequency equal to that of the cell doubling time. One half of the broth is then removed to maintain constant volume in the reactor. This is known as continuous phasing (Dawson, 1965, 1971). The advantage of this method over the pulsed chemostat is that each cycle produces two identical portions of synchronized cells; one is retained in the reactor and one is harvested (Sheppard, 1989).

One difficulty with the method of continuous phasing is that the cycle time is not always constant over successive generations (Sheppard, 1989). If the actual doubling time of the organism becomes longer with time, then the cells will be cycled before division has occurred, resulting in cell washout. If the doubling time becomes shorter over successive generations, this will result in periods of starvation. Successive periods of starvation may lead to a loss of cell viability. This problem was overcome after it was noted that there is a close correlation between the concentration of dissolved oxygen in the broth and

the concentration of growth-limiting nutrient (Sheppard, 1989). The level of dissolved oxygen increases sharply after the limiting nutrient is exhausted, due to a decrease in cellular respiration. By adding a dissolved oxygen probe to the system, a feedback control loop may be established. This eliminates the cycle time as an independent variable and allows the nutrient environment to be adjusted automatically.

It is quite easy to understand why the level of dissolved oxygen in the medium should increase upon exhaustion of the limiting nutrient. However, it is less obvious that this period should correspond to the cell doubling time. The work of Burns (1964) is helpful in explaining this phenomenon. A limiting nutrient is limiting because it is an absolute requirement for a biochemical pathway or process in the cell. This process must be a step that is necessary either for cell division or for a particular stage in cell growth. Once the concentration of the limiting nutrient reaches zero, cells that have reached this step will be arrested at this point of development. These cells reach a sort of metabolic stasis, neither growing nor dividing. Organisms that are at earlier stages of the life cycle will continue to grow until they arrive at the step requiring the rate limiting nutrient. In this way, they "catch up" with the other metabolically blocked cells. When the limiting nutrient is pulsed to these cells, they will begin growing and dividing again. After several generations, the entire population should be dividing with the same frequency. The experiments carried out by Sheppard (1989), using the cyclone fermentor seen in Figure

1.1, provide strong evidence that the doubling time corresponds with the period of limiting nutrient addition in a continuously phased system with feedback control. This relationship between limiting nutrient exhaustion and cell doubling time was confirmed by the work of Brown and Cooper (1991).

When the continuous phasing method was used to grow bacillomycin-producing Bacillus subtilis in the cyclone fermentor, the resulting foaming problem was very severe. Leaks occurred at some of the seals and at the air filters, leading to the contamination of the reactor. The foam also made it impossible to take uniformly sized samples from the fermentor by preventing proper drainage into the collection vessel. This was a serious problem because, as it has been shown, it is crucial that exactly half of the biomass be removed after each cycle. It should also be obvious that it would not be possible to recover the product by continuous foam flotation in this system.

1.2 Bacillomycin L

The organism under examination in these experiments is a bacterium called Bacillus subtilis ATCC no.10774. This is an organism which is found in the soil and produces the antibiotic bacillomycin L. This compound is a broad spectrum antifungal agent belonging to the iturin family, which is a group of peptido-lipidic compounds (Besson et al, 1978). Bacillomycin L (Figure 1.2) is a cyclic lipopeptide containing seven α-amino acids and one β-amino acid (Chevanet et al, 1986).

The synthesis of bacillomycin L begins only after the growth of the bacteria has virtually ceased. It belongs to a

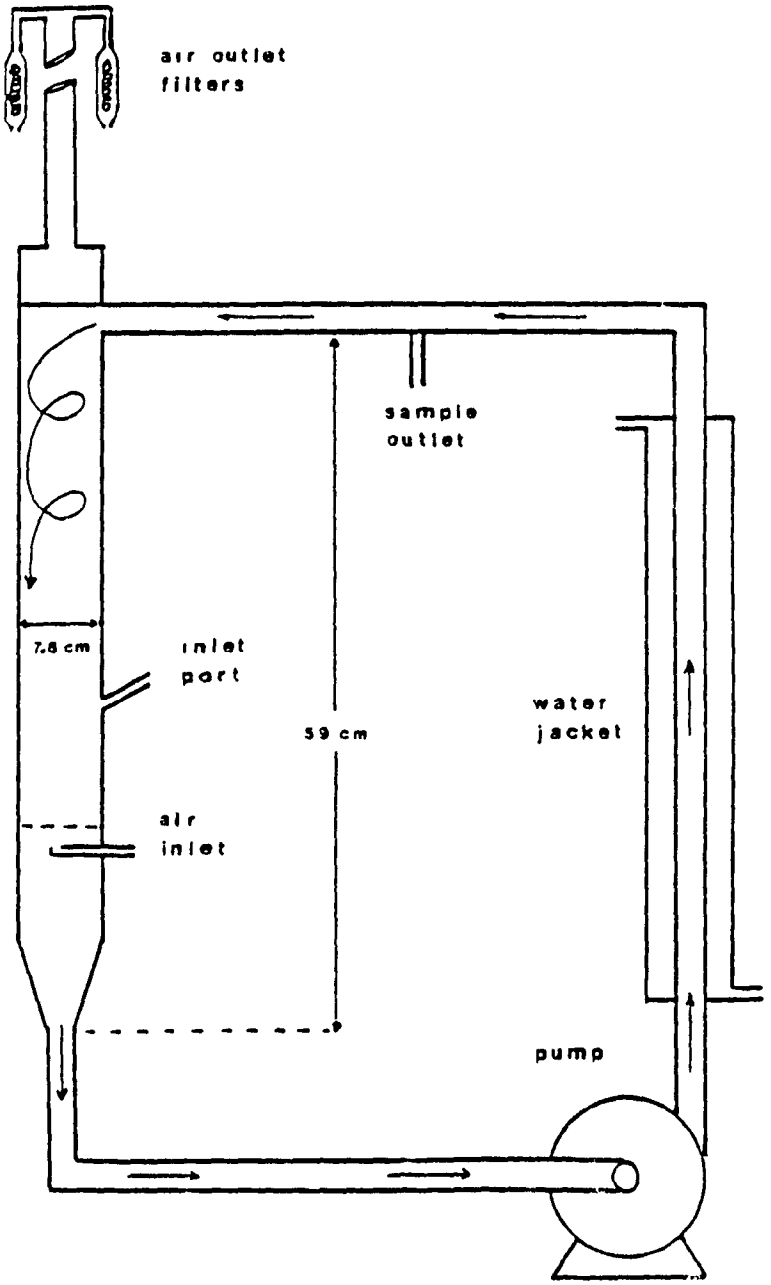

Figure 1.1: The cyclone fermentor (Sheppard, 1989)

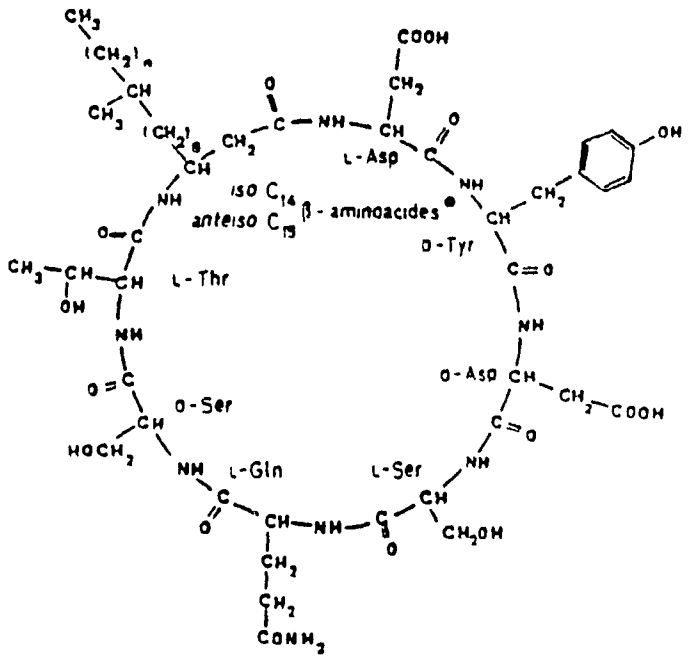

Figure 1.2: Structure of bacillomycin L (Besson and Michel, 1977)

class of microbial products called secondary metabolites. These products are so named because their synthesis is not associated with growth. Such compounds are produced when the environment becomes less viable in an acute way. For example, Chevanet et al (1986) have found that bacillomycin L production begins only once all of the glucose in the medium has been exhausted. Like bacillomycin, many of these secondary metabolites display antimicrobial activity, thereby giving a competitive advantage to the organisms that produce them.

1.2.1 Antibiotics

An antibiotic is a chemical substance produced by one microorganism that is able to kill or inhibit the growth of other microorganisms. Thousands of antibiotics have been discovered but only a relative few are of practical value in medicine (Aharonowitz and Cohen, 1981).

Antibiotic-producing microorganisms are especially common in the soil. Three groups of microorganisms are responsible for most of the antibiotics used in medicine: (1) fungi, especially those of the genus Penicillium, which produce antibiotics such as penicillin and griseofulvin; (2) bacteria of the genus Bacillus, which produce antibiotics such as bacitracin and polymyxin; and (3) actinomycetes of the genus Streptomyces, which produce antibiotics such as streptomycin, chloramphenicol, tetracycline, and erythromycin (Brock, 1979). It is among members of the genus Streptomyces, a genus of organisms widespread in soil, that most of the antibiotics have been discovered (Brock, 1979).

Antibiotics are a diverse group of compounds, but they

can be grouped in families with similar chemical structures. The antibiotics of one group usually have similar types of activity and find similar uses in practice. For instance, there is a large group of penicllins, most of which have similar activity, although certain penicillins are more active against Gram-negative bacteria and others are more active against Gram-positive bacteria (Brock, 1979).

The antibiotics were the first industrially produced microbial metabolites which were not major metabolic end products. The yields, calculated in terms of conversion of the major carbon source into antibiotic, are low and are greatly influenced by the other cultural conditions. These facts have encouraged intense research directed toward improving yields. For this purpose, genetic selection has proved remarkably successful. The wild type strain of Penicillium chrysogenum first used for penicillin production yielded approximately 0.1 gram of penicillin per liter (Aharonowitz and Cohen, 1981). From this strain a mutant was selected which produced 8 grams per liter under the same growth conditions, a 60-fold improvement in yield (Aharonowitz and Cohen, 1981). Subsequent strain selection following chemical mutagenesis has led to the development of new strains with even greater capacity for antibiotic production. By such sequential genetic selection, improvements of antibiotic yield as great as 1000-fold have often been obtained (Aharonowitz and Cohen, 1981). Most genetic improvement has been empirical; large numbers of mutagenized clones are evaluated for their abilities to produce larger quantities of the antibiotic.

However, with increased knowledge of the pathways of biosynthesis of antibiotics, more rational approaches are being exploited. It is now possible to select strains in which control of the synthesis of known precursors of an antibiotic has been altered by mutation. Such strains produce larger amounts of the precursor, and sometimes also larger amounts of the antibiotic end product (Hopwood, 1981).

1.2.2 Biosurfactants

While the antibiotic properties of bacillomycin L have been known since the experiments of Landy et al (1948), its surfactant properties have not been well documented. This study has shown that bacillomycin L can reduce the surface tension of a fermentation broth to as low as 26 mN/m, which would allow it to be classified as a biosurfactant.

A biosurfactant is a microbially produced compound that has both a hydrophilic portion and a hydrophobic portion. The hydrophobic part is usually a type of lipid, while the hydrophilic part may be a carboxylate or hydroxyl function or a complex mixture of phosphate, carbohydrate, amino acids, etc. (Cooper, 1986). These molecules tend to either associate with each other to form micelles, or to collect at surfaces (Parkinson, 1985). When the molecules aggregate in this manner, one result is the reduction of the surface tension of the water from 72 mN/m to around 30 mN/m. The degree to which surface tension is reduced is called the effectiveness of a surfactant, while the degree of surface tension reduction as a function of surfactant concentration is known as the efficiency (Parkinson,

1985). Increasing the surfactant concentration above a characteristic value, known as the critical micelle concentration (CMC), results in no further lowering of the surface tension. At concentrations higher than the CMC, additional surfactant molecules aggregate into micelles in the bulk phase and do not contribute significantly to the reduction of surface tension (Parkinson, 1985). In the region where surface tension changes rapidly with concentration (i.e. below the CMC) the slope of the curve defines the Gibbs surface excess, which is a measure of the excess of surfactant at the interface compared to its concentration in the bulk phase; this parameter is important in assessing foam and emulsion stabilization (Parkinson, 1985).

The strains of Bacillus subtilis that produce bacillomycin and surfactin are exceptional in that they produce very effective surfactants while being grown on carbohydrates, as opposed to most microorganisms which synthesize biosurfactants while metabolizing hydrocarbons (Cooper, 1984). However, the mode of production differs between these two strains, as bacillomycin is produced after the stationary growth phase has been reached, while surfactin synthesis begins in early exponential growth phase (Cooper, 1984). This pattern varies widely among different biosurfactants; some even act as growth stimulators while still others inhibit cell growth (Parkinson, 1985).

Biosurfactants may find applications in any area where synthetic surfactants are now used. They have the advantage of being non-polluting because of their biodegradability. They can

compete with the synthetics on the basis of overall performance but more work must be done to improve yields and lower product separation costs if they are really going to challenge in the marketplace (Cooper, 1986).

1.3 Foam

A foam is basically a two phase system in which a distinct gas bubble phase is surrounded by a continuous liquid lamellar phase. This type of dispersion usually involves a large gas-liquid interface. Since interfaces between immiscible phases are under tension, the expansion of these interfaces requires energy, which is to say work is performed in forming them and energy is released on their relaxation (German et al, 1985). From a thermodynamic standpoint, foams are unstable dispersions by their very nature and should eventually break into individual component phases in the direction of decreasing total surface free energy (Heller and Kuntamukkula, 1987). Surfactants, being amphiphilic, orient at an interface and lower the energy or tension of that interface (German et al, 1985). These compounds enhance both foam capacity and foam stability by hindering bubble coalescence. As the surfactant molecules accumulate at the gas-liquid interface, their hydrophilic groups become oriented into the liquid film surrounding the gas bubble, thus creating repulsive forces when two bubbles come close to each other (Keitel and Onken, 1982). The concentration of the hydrophilic molecules at the surface increases with surfactant concentration (up to a maximum) and results in a lower surface tension (Bukur and Patel, 1989). Therefore, the ability of a surfactant to

rapidly reach an interface, effectively lower the interfacial tension and stabilize new surface determines its ability to form foams (German et al, 1985).

As Walstra (1988) demonstrates, the relationship between surface tension and surfactant concentration is thermodynamically controlled and is given by the Gibbs equation:

$$d\gamma = -RT\Gamma\, d(\ln a)$$

where γ is surface tension, Γ is Gibbs surface excess, a is activity, T is temperature, and R is the universal gas constant. However, in an energetic system such as a bubble column, there are a number of dynamic effects which operate in the direction of stabilization. As air is bubbled through the broth, fresh interface is created quickly and existing interfaces are extended. When this happens, there is a tendency for the surfactant adsorption process to lag behind the increase in surface area. Since the adsorption normally lowers the surface tension of the interface, the result is to raise the effective surface tension for these transient events. The opposite effect, of course, occurs if the surface area rapidly decreases. The change

$$\Delta\gamma = \gamma_{Static} - \gamma_{Dynamic}$$

is referred to as the Marangoni effect and it confers an elasticity on the surface, since changes in area in either direction are opposed (Hunter, 1987). This effect is most pronounced in solutions of strongly surface active materials, especially high molecular weight non-ionic detergents and proteins (Hunter, 1987).

A second contribution to surface elasticity is the Gibbs effect which refers to the fact that if an interface is rapidly expanding in area, the absolute amount of adsorbate may become limiting so that the interfacial tension begins to rise (Hunter, 1987). The Gibbs surface elasticity for a thin liquid film of area A is given by:

$$E = A\ (d\gamma/dA)$$

This is referred to as a "quasi-equilibrium" effect because the thinning lamella surfaces can establish local adsorption equilibrium with the liquid but cease to have effective contact with the bulk solution (Hunter, 1987).

In any dynamic system, both the Marangoni and Gibbs effects tend to maintain a uniform thickness of the film because:

> (1) at any point where the external forces are tending to drastically thin down the lamella, the local surface tension rises and this opposes thinning;
>
> (2) the gradient of surface tension pulls not only the surface layer but also drags with it (by viscous forces) some of the underlying liquid. The result is a tendency to heal any potential points of rupture by surface transport;
>
> (3) thickened areas are subject to a fall in surface tension and so tend to lose liquid to the adjoining thinner areas. (Hunter, 1987)

In general, the Gibbs effect would be expected to be more significant for foams and creams where most of the lamellae have only a tenuous connection with the bulk solution. The Marangoni effect is more important for emulsions in which the stabilizer is a high molecular weight polymer or protein (Hunter, 1987).

The rupture of a foam film or lamella is preceded by its thinning, due to drainage of the liquid core under the influence

of gravity and capillary suction (Djabbarah and Wasan, 1985). This drainage leads to bubble coalescence. There is significant spontaneous interdiffusion of gas from small bubbles into adjacent larger bubbles through the interfacial film, due to the higher capillary pressure associated with smaller bubbles (Heller and Kuntamukkula, 1987). This process results in shrinkage of the small bubbles and expansion of the neighbouring larger bubbles, causing the foam films to become thinner and eventually rupture (Heller and Kuntamukkula, 1987). Interfacial viscosity and elasticity of lamellae may promote foam stability by retarding liquid drainage and by resisting deformations induced by ambient fluctuations in temperature or pressure.

2.0 OBJECTIVES

Research is currently being conducted in the Department of Chemical Engineering at McGill University in the area of fermentation engineering using the continuous phasing method. This study is in response to the foaming problems that have been encountered in trying to use this method to achieve high product yields when cultivating organisms that cannot be grown on hydrocarbons. The objectives of this work are as follows:

(1) To establish a reliable method for quantifying the foaming capacity of a liquid;

(2) To determine the effect of a variety of process variables on the foam capacity of the fermentation broth;

(3) To determine if these parameters can be controlled in such a way as to suppress foaming while allowing for acceptable levels of cell growth and antibiotic production.

3.0 MATERIALS AND METHODS

3.1 Microorganism

The organism used in these experiments was the bacterium Bacillus subtilis ATCC no. 10774. It was obtained from the American Type Culture Collection and produces the antibiotic/ biosurfactant bacillomycin L.

3.2 Media Composition

The basic medium used in these experiments was taken from Chevanet et al (1986) with a few modifications. It is given below:

Table 3.1: Standard Medium

Ingredient	Concentration (g/L)	Molarity
$Na_2 \cdot EDTA \cdot 2H_2O$	0.37	1.0×10^{-3}
KH_2PO_4	1.0	7.3×10^{-3}
KCl	0.5	6.7×10^{-3}
$MgSO_4 \cdot 7H_2O$	0.5	2.0×10^{-3}
$MnSO_4 \cdot H_2O$	4.5×10^{-4}	2.6×10^{-6}
$FeSO_4 \cdot 7H_2O$	8.3×10^{-4}	3.0×10^{-6}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-4}	1.0×10^{-5}
L-glutamic acid	5.0	3.4×10^{-2}
glucose	10.0	5.6×10^{-2}

The pH is adjusted to 7.7 with NaOH before autoclaving.

When the nitrogen source was changed, the glutamic acid was replaced with equimolar amounts of either sodium nitrate (2.89 g/L) or ammonium chloride (1.82 g/L). In order to control the pH of the ammonium-based medium, the total phosphate concentration was increased by adding 9.7 g/L of K_2HPO_4. The medium is described below:

Table 3.2: Ammonium-Based High Phosphate Medium

Ingredient	Concentration (g/L)	Molarity
$Na_2 \cdot EDTA \cdot 2H_2O$	0.37	1.0×10^{-3}
K_2HPO_4	9.7	5.6×10^{-2}
KH_2PO_4	1.0	7.3×10^{-3}
KCl	0.5	6.7×10^{-3}
$MgSO_4 \cdot 7H_2O$	0.5	2.0×10^{-3}
$MnSO_4 \cdot H_2O$	4.5×10^{-4}	2.6×10^{-6}
$FeSO_4 \cdot 7H_2O$	8.3×10^{-4}	3.0×10^{-6}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-3}	1.0×10^{-5}
NH_4Cl	1.82	3.4×10^{-2}
glucose	10.0	5.6×10^{-2}

The repression of bacillomycin production by phosphate ions was studied by using an ammonium-based medium and replacing the phosphates with an equivalent amount of Tris buffer. The resulting medium is shown below:

Table 3.3: Ammonium-Based Tris Buffered Medium

Ingredient	Concentration (g/L)	Molarity
$Na_2 \cdot EDTA \cdot 2H_2O$	0.37	1.0×10^{-3}
Tris	7.66	6.3×10^{-2}
KCl	0.5	6.7×10^{-3}
$MgSO_4 \cdot 7H_2O$	0.5	2.0×10^{-3}
$MnSO_4 \cdot H_2O$	4.5×10^{-4}	2.6×10^{-6}
$FeSO_4 \cdot 7H_2O$	8.3×10^{-4}	3.0×10^{-6}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-3}	1.0×10^{-5}
NH_4Cl	1.82	3.4×10^{-2}
glucose	10.0	5.6×10^{-2}

In order to study the effect of electrolyte concentration on foam capacity, the MM Medium of Nakano et al (1988) was modified slightly and used to cultivate the bacteria. The new medium is shown below:

Table 3.4: Modified MM Medium

Ingredient	Concentration (g/L)	Molarity
$Na_2 \cdot EDTA \cdot 2H_2O$	0.37	1.0×10^{-3}
K_2HPO_4	14.0	8.0×10^{-2}
KH_2PO_4	6.0	4.0×10^{-2}
$MgSO_4 \cdot 7H_2O$	0.5	2.0×10^{-3}
$MnSO_4 \cdot H_2O$	4.5×10^{-4}	2.6×10^{-6}
$FeSO_4 \cdot 7H_2O$	8.3×10^{-4}	3.0×10^{-6}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-3}	1.0×10^{-5}
sodium citrate	1.0	4.0×10^{-3}
L-glutamic acid	5.0	3.4×10^{-2}
glucose	10.0	5.6×10^{-2}

The phosphate in the Modified MM Medium was replaced with Tris buffer in order to reduce the phosphate repression of bacillomycin production. Sodium chloride and potassium chloride were added to make the solution even more hypertonic. The result is given below:

Table 3.5: Modified MM Medium + Tris Buffer

Ingredient	Concentration (g/L)	Molarity
$Na_2 \cdot EDTA \cdot 2H_2O$	0.37	1.0×10^{-3}
Tris	14.5	1.2×10^{-1}
$MgSO_4 \cdot 7H_2O$	0.5	2.0×10^{-3}
$MnSO_4 \cdot H_2O$	4.5×10^{-4}	2.6×10^{-6}
$FeSO_4 \cdot 7H_2O$	8.3×10^{-4}	3.0×10^{-6}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-3}	1.0×10^{-5}
NaCl	3.6	6.0×10^{-2}
KCl	4.6	6.0×10^{-2}
sodium citrate	1.0	4.0×10^{-3}
L-glutamic acid	5.0	3.4×10^{-2}
glucose	10.0	5.6×10^{-2}

3.3 Experimental

3.3.1 Cultivation

The bacteria were inoculated from nutrient agar plates directly into a 500 ml Erlenmeyer flask containing 100 ml of the

appropriate growth medium. This flask was then placed into a controlled-environment shaker set at 37°C and 270 rpm. After 24 hours, 1.0 ml of the resulting broth was then transferred into 100 ml fresh medium and this was incubated again for 24 hours. A 1.0 ml aliquot of this broth was then taken again and used to inoculate another 100 ml of fresh medium. After twelve hours of growth, this broth was used to provide a set of 1% inocula for an experimental run typically involving 20-22 100 ml samples. These flasks would be inoculated at the same time and left to grow in the shaker. Samples would then be taken periodically to monitor the progress of the fermentation.

3.3.2 Tests

Once a flask was removed from the shaker, several tests would be performed. First the pH would be measured with a Perkin-Elmer pH meter. Then the surface tension of the broth would be obtained using a Fisher Autotensiomat. This device measures the tension on a platinum ring as it passes through the liquid-air interface.

The foam capacity of the broth was measured using the device seen in Figure 3.1. A 1.0 ml sample of broth is first introduced onto the diffuser using a micropipette. The air flow rate is kept constant at 0.15 L/min. The foam capacity is defined as the height in centimeters of the resulting column of foam. The measurement is always taken upon the appearance of the first bubble large enough to span the width of the column.

In order to measure the biomass concentration, 20 ml samples of the broth must first be centrifuged at 10,000 rpm for

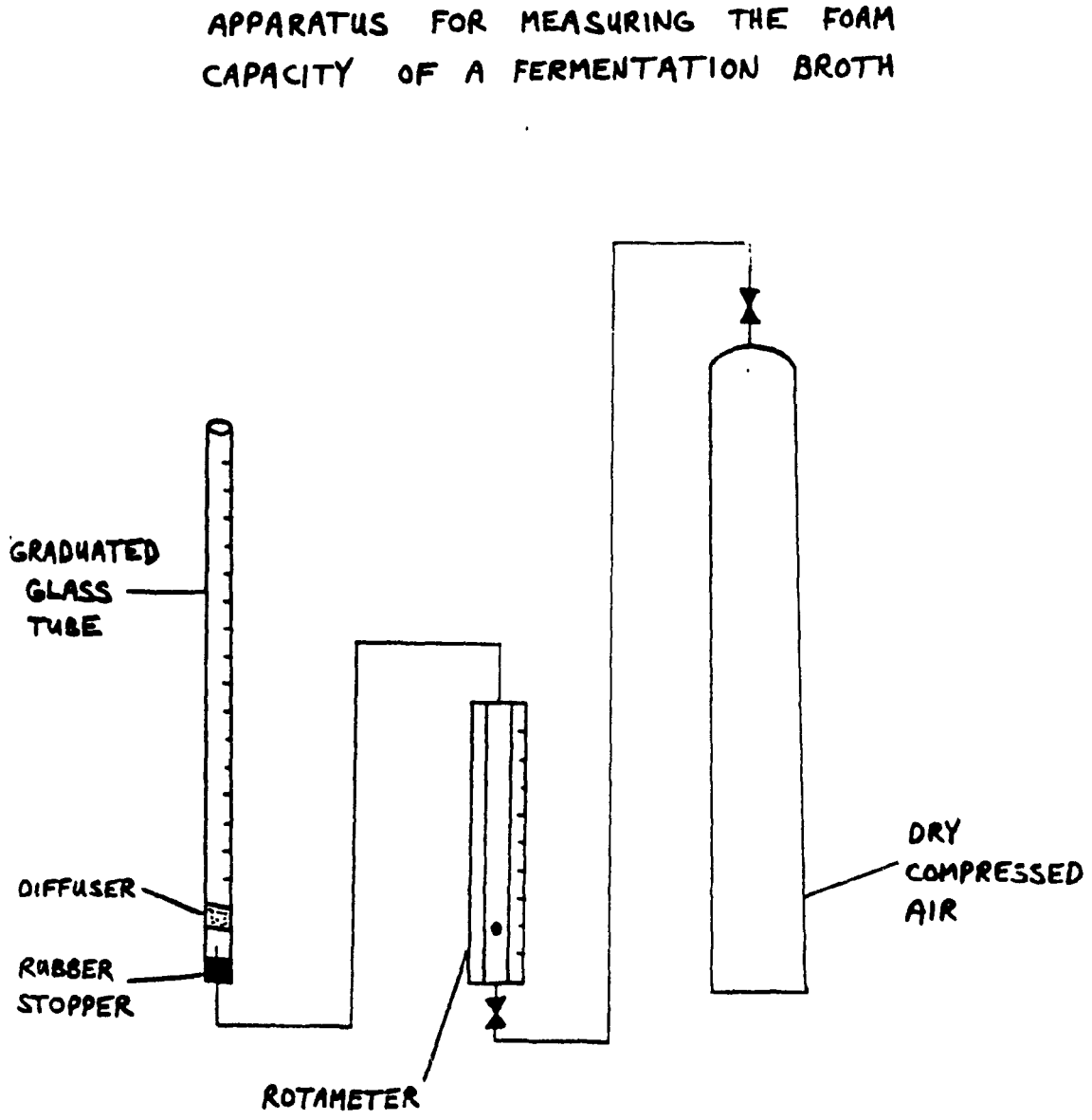

Figure 3.1: Device for measuring foam capacity

ten minutes. The supernatant is then decanted and retained, while the pellet is removed from the tube, washed with water and placed in a weighed aluminum dish. The dish is left overnight in an oven set between 90 and 100°C. The dish is then weighed and the biomass concentration is the total dry wt/vol measurement.

The supernatant that is removed from the centrifuged broth contains the bacillomycin L. It is first acidified to pH 2.5 by the addition of concentrated hydrochloric acid, which precipitates the bacillomycin. This sample is then centrifuged again at 10,000 rpm for ten minutes. The supernatant is then decanted and discarded. The pellet itself is then washed with 10 ml of diethyl ether and dissolved in 5 ml of absolute ethanol. The optical density of this solution can then be measured at 277.0 nm using a Varian UV-Spectrophotometer. This reading gives an accurate indication of the amount of bacillomycin in solution.

3.3.3 Thin Layer Chromatography

Thin layer chromatography was the method used to confirm the presence of bacillomycin L in the fermentation broth. First of all, the bacillomycin must be extracted into ethanol by the procedure described in Section 3.2. However, in this case 100 ml samples of broth are used instead of 20 ml samples. After the bacillomycin has been dissolved, the solution is made more concentrated by evaporating about 80% of the ethanol. The concentrated solution is then spotted onto a silica gel TLC plate using a micropipette tip. The spot on the plate should be made as concentrated as possible. However, in order that the spot does not grow too big, care must be taken to allow it to dry

thoroughly between applications. Once the desired concentration has been achieved, the position of the starting spot is marked and the plate is placed into the TLC chamber containing the chromatographic solvent mixture. The plate is removed once the solvent front has moved about 4/5 of the way up the plate. After it has dried off, the plate is sprayed with concentrated sulfuric acid and allowed to dry again. The plate is then placed in an oven set around 90°C for 10-12 hours. The distance from the starting point to the final position of the solvent front is taken as unity. The displacement of the spot made by each constituent, expressed as a fraction of the distance moved by the solvent front, is known as the Rf value of that constituent. This Rf value is characteristic of a given substance in a given solvent mixture.

4.0 RESULTS

4.1 Fermentation Profile of Standard Case

In order to obtain a reference for this set of experiments, Bacillus subtilis was grown in the Standard Medium described in Section 3.2, at a temperature of 37°C. The fermentation profile is shown in Figure 4.1 and is quite typical of what was seen in most of these experiments.

First of all, the biomass curve is exactly what would be expected for a batch fermentation. The growth of biomass exhibits a short lag phase, exponential growth over the space of a few hours, and finally reaches a maximum of 1.88 g/L as it enters the stationary phase after about twenty hours. The concentration of biomass then decreases slowly after the limiting nutrient is exhausted.

The pH profile is also typical of a batch run with this organism. The pH declines from an initial value of 7.7 to a level between 6.0 and 6.5. After the midway point of the exponential growth phase has been reached, the pH begins to increase again up to around its original value.

Bacillomycin concentration was monitored by measuring the optical density at 277.0 nm. of an ethanol extract prepared by the method described in Section 3.3. The presence of bacillomycin L was confirmed by the use of thin layer chromatography on silica gel. The results are recorded in Table 4.1. A calibration curve was also generated so that actual weight per volume values of bacillomycin concentration could be obtained from the OD 277 readings. This calibration curve is

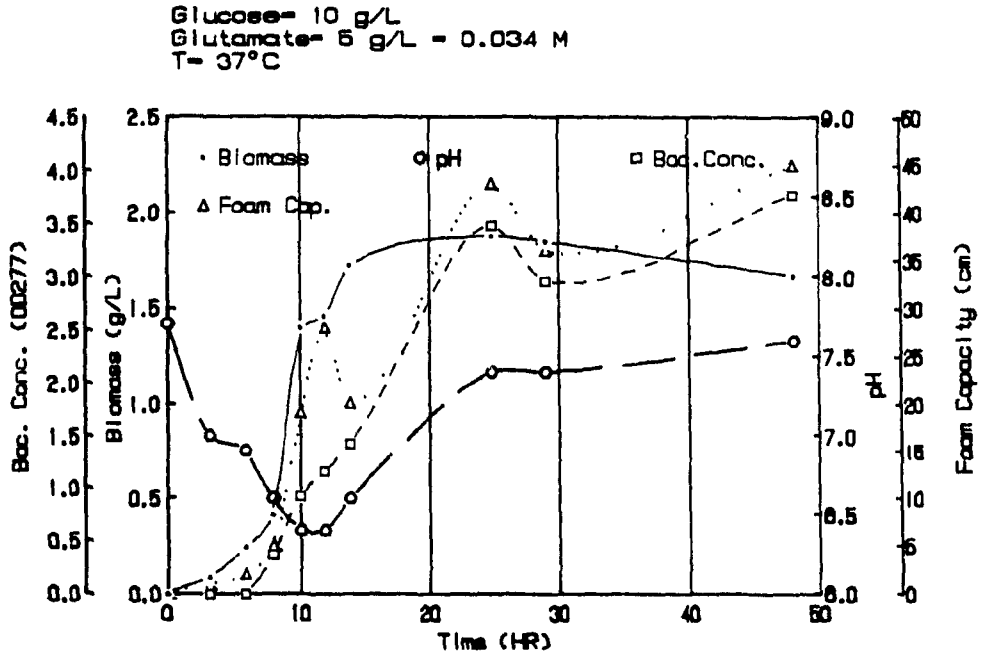

Figure 4.1: Fermentation profile of glutamate-based medium (0.034 M) at T= 37°C

Table 4.1: Thin Layer Chromatography of Fermentation Broth

Substance	Rf	
	Solvent A	Solvent B
Fermentation Broth	strong bar at 0.16 light bar at 0.41 very light bar at 0.85	light bar at 0.21 strong bar at 0.37 very light bar at 0.49 light bar at 0.89
Bacillomycin L	0.16	0.38

Solvent A : Chloroform-methanol-water (65:25:4)

Solvent B : Butanol-acetone-water (4:6:1)

The values for bacillomycin L are from Besson and Michel (1987).

shown in Figure 4.2.

The concentration profile of bacillomycin indicates that it is a secondary metabolite. There is no significant production until the end of exponential growth, after which the production increases sharply and remains high. Bacillomycin is an effective surfactant, so this increase in concentration is accompanied by a corresponding reduction in the surface tension of the broth. The surface tension decreases asymptotically to 26 mN/m after 24 hours (not shown in figure 4.1). However, the most important aspect of Figure 4.1, for this work, is the close relationship shown between the bacillomycin concentration and the foam capacity of the broth. The tight linkage between these two parameters was observed in almost every experiment. In fact, the relationship in most cases is quite linear, with coefficients of correlation between 0.97 and 1.00 for plots of bacillomycin concentration vs. foam capacity (Figure 4.3).

An attempt was made to further examine the relationship between bacillomycin concentration and foam capacity. An experimental run was conducted using Standard Medium at 37°C. Samples were taken over a period of 72 hours and measurements were made for biomass, bacillomycin concentration (OD 277), and foam capacity. Then the bacillomycin was isolated and its concentration measured on a weight per volume basis. This bacillomycin was then redissolved in water and the foam capacity was remeasured. The data for this experiment are displayed in Table 4.2. These results indicate that a loss of 33% to 54% of foam capacity occurs after extraction, isolation, and

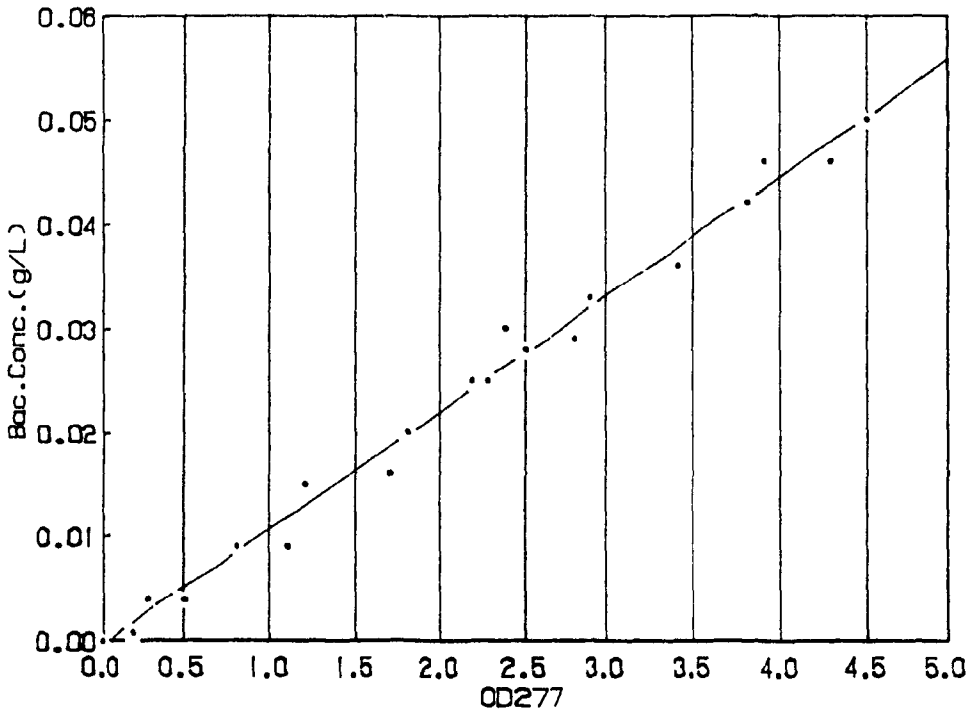

Figure 4.2: Bacillomycin L concentration as a function of the optical density at 277.0 nm of an ethanol extract

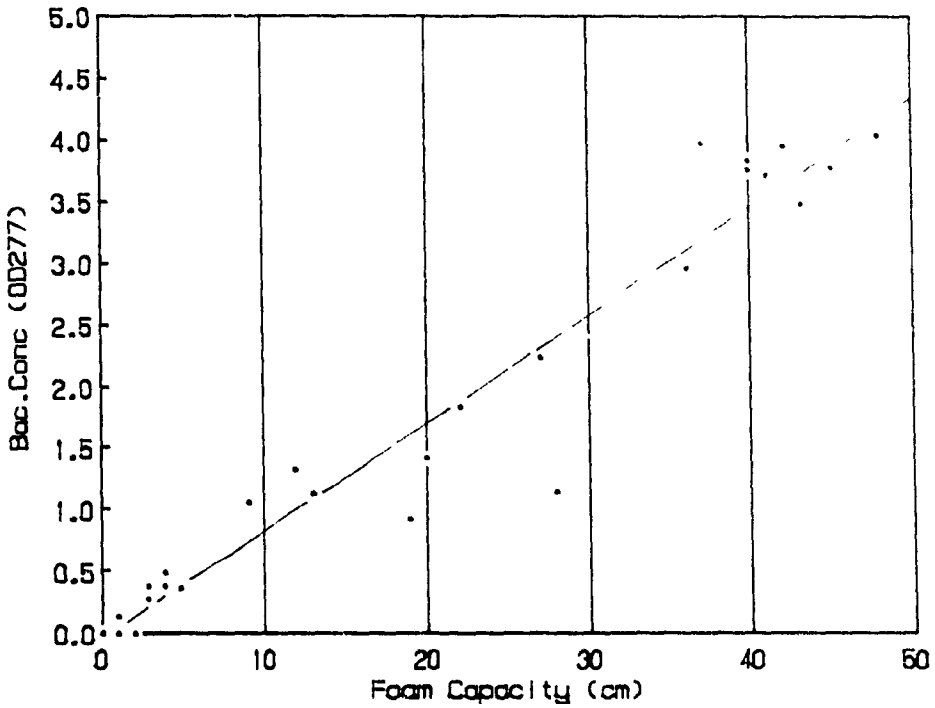

Figure 4.3: Effect of bacillomycin L concentration on the foam capacity of the broth

Table 4.2: Bacillomycin Concentration and Foam Capacity

Time (HR)	Biomass (g/L)	Bac.Conc. (OD277)	Foam Cap. of Broth	Bac.Conc. (g/L)	Foam Cap. after Redissolution (cm)
4	0.11	0	0.5	-	-
8	0.57	0.39	4	-	1
12	1.65	0.45	3	-	1
24	2.07	3.90	43	0.045	24
32	1.94	3.62	41	0.039	19
36	1.92	4.28	42	0.053	28
48	1.81	4.13	43	0.051	26
72	1.69	3.88	36	0.040	17

redissolution of bacillomycin.

Another experiment was carried out in which the role of bacillomycin concentration was investigated. Samples of fermentation broth were centrifuged to produce cell-free broth. Measurements were then made for surface tension, bacillomycin concentration, and foam capacity. The broth was then subjected to a chloroform wash and the surface tension and foam capacity were measured again. The results are shown in Table 4.3.

4.2 Effect of pH

Figure 4.1 would indicate that there might be some connection between the pH of the broth and its foam capacity. In order to investigate this, an experiment was carried out using Standard Medium and an incubation temperature of 37°C. Samples of broth were removed after 14 and 24 hours of fermentation, and their foam capacities measured. Two aliquots of each sample were then taken and the pH of one pair of aliquots was raised to 8.2 by the addition of sodium hydroxide, while the pH of the other pair was lowered to 5.6 by the addition of hydrochloric acid. The foam capacity of each sample was measured after each pH adjustment. The results are shown in Table 4.4. There is no significant change in the foam capacity of the broth when the pH of the system is either raised or lowered.

4.3 Effect of Incubation Temperature

The relationship between incubation temperature and foam capacity was examined by carrying out runs in Standard Medium at temperatures of 37°C (Figure 4.1), 30°C (Figure 4.4), and 23°C

Table 4.3: Role of Bacillomycin in Foam Production

Surface Tension (mN/m)		Bacillomycin Concentration (OD277)		Foam Capacity (cm)	
before chloroform wash	after chloroform wash	before chloroform wash	after chloroform wash	before chloroform wash	after chloroform wash
27	30	2.67	2.49	24	21
26	28	3.72	3.39	39	34

Table 4.4: Effect of pH on Foam Capacity

Incubation Time(hr)	Initial pH	Initial Foam Cap. (cm)	Foam Cap. after pH=5.6 (cm)	Foam Cap. after pH=8.2 (cm)
14	6.4	17	15	17
24	7.0	37	35	35

NOTE: Values for bacillomycin concentration and foam capacity are averages of the measurements made after the end of the exponential growth phase.

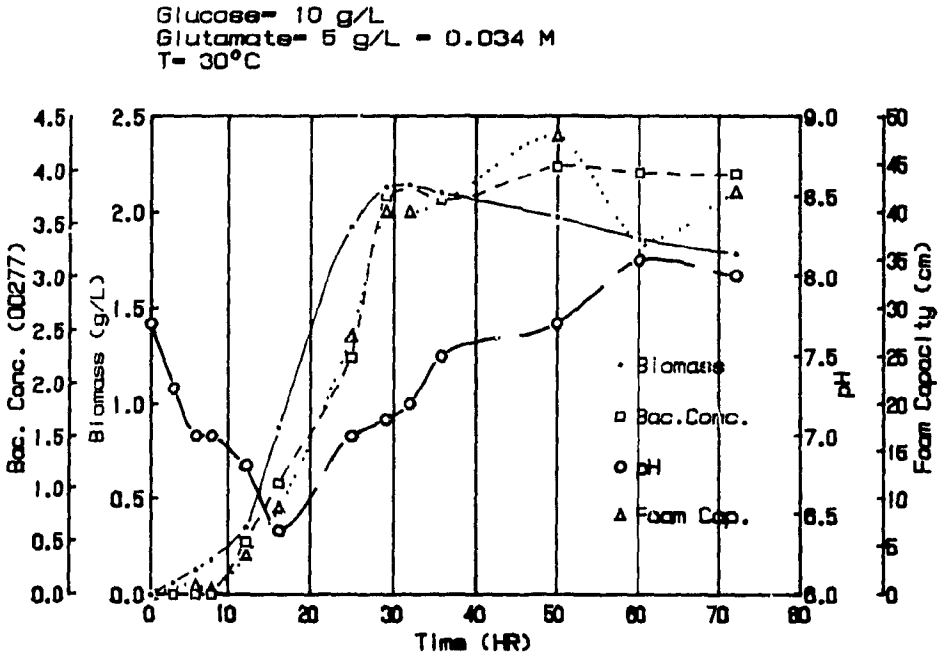

Figure 4.4: Fermentation profile of glutamate-based medium (0.034 M) at T= 30°C

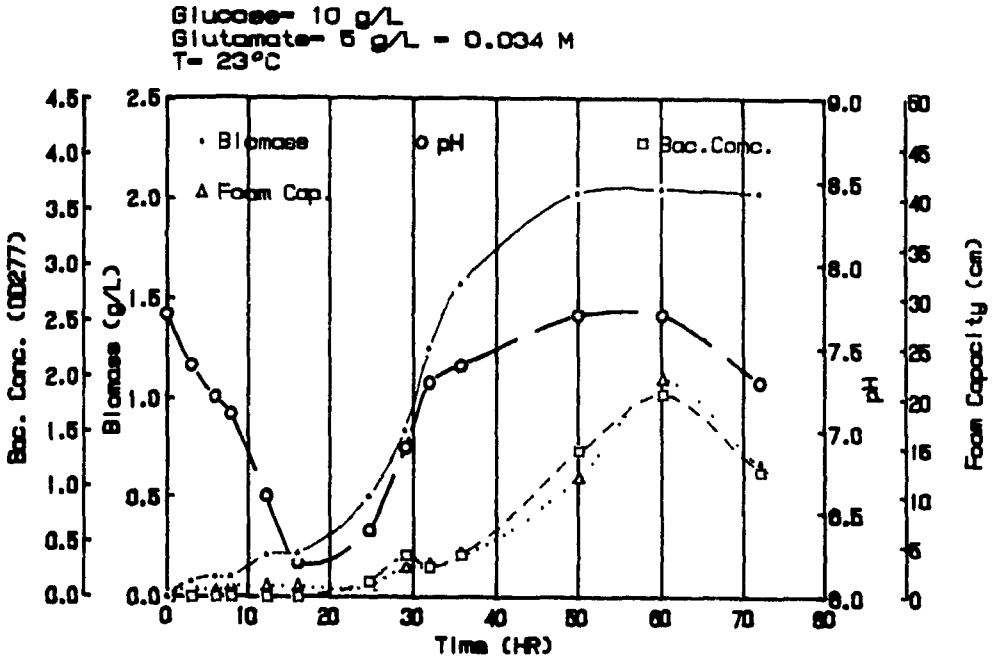

Figure 4.5: Fermentation profile of glutamate-based medium (0.034 M) at T= 23°C

(Figure 4.5).

Temperature has little effect on the maximum level of biomass. However, it does play a role in determining how long it takes to achieve that level of growth. At 37°C, it takes about twenty hours to reach the end of the exponential phase. At 30°C, thirty hours are required. When the temperature is 23°C, about fifty hours are needed to reach the maximum biomass level.

The temperature has little effect on the pH profile of the broth. The graphs of the two lower temperature systems have pH plots similar to the characteristic profile in Figure 4.1.

All three experiments exhibit linkage between bacillomycin concentration and foam capacity. However, the levels of both were about 60% lower at 23°C. The results of these runs are summarized in Table 4.5.

4.4 Effect of Glutamate Concentration

The effect of the nitrogen source concentration on foam capacity was investigated by carrying out three experiments with glutamate levels of 5.0 g/L (Figure 4.1), 2.5 g/L (Figure 4.6), and 1.0 g/L (Figure 4.7).

A two-fold decrease in glutamate concentration leads to a decrease in total biomass of only about 15%. However, a five-fold decrease reduces the maximum biomass level by 75% and results in a slower growth phase.

Halving the level of glutamate in the medium has little effect on the behaviour of the pH. However, reducing the concentration to 1.0 g/L results in a highly erratic pH profile.

A two-fold decrease in glutamate concentration reduces

Table 4.5: Effect of Incubation Temperature on Foam Capacity

Incubation Temperature (°C)	Maximum Biomass (g/L)	Bacillomycin Concentration (OD277)	Foam Capacity (cm)
37	1.88	3.40	41
30	2.05	3.87	41
23	2.14	1.43	16

NOTE: Values for bacillomycin concentration and foam capacity are averages of the measurements made after the end of the exponential growth phase.

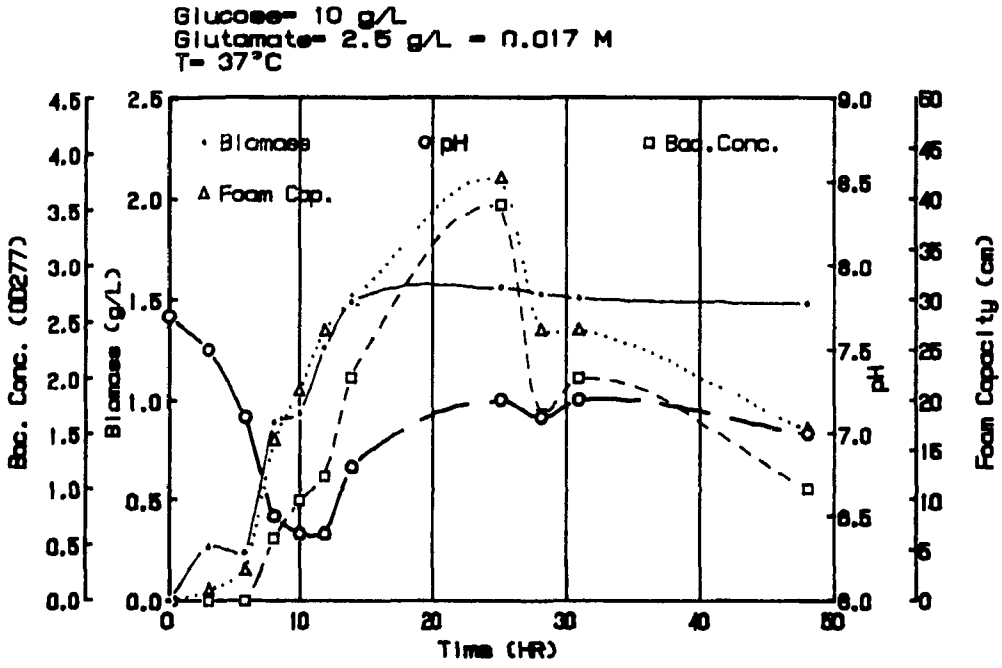

Figure 4.6: Fermentation profile of glutamate-based medium (0.017 M) at T= 37°C

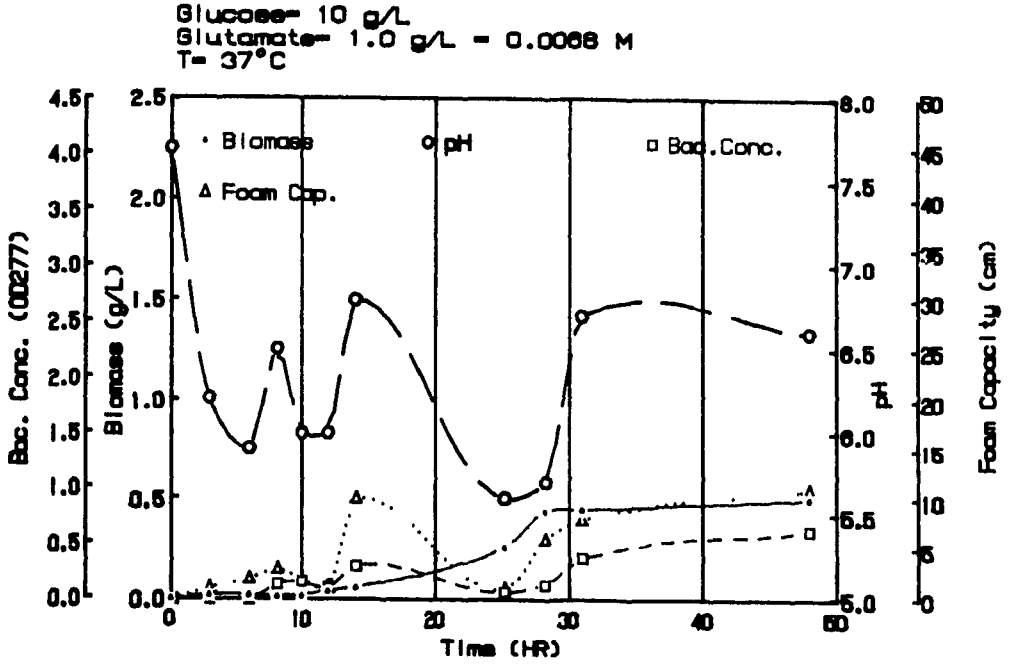

Figure 4.7: Fermentation profile of glutamate-based medium (0.0068 M) at T= 37°C

bacillomycin concentration by 40%, whereas a five-fold reduction in glutamate results in almost a 90% decrease in production. It should also be noted that, once again, these experiments demonstrate the strong relationship between bacillomycin concentration and foam capacity, even at low concentrations. A summary of these experiments is found in Table 4.6.

4.5 Effect of Other Nitrogen Sources

Experiments were carried out to determine if other nitrogen sources would have any effect on the foam capacity. Using the Standard Medium from Figure 4.1, glutamate was replaced by equimolar quantities of sodium nitrate (Figure 4.8) and ammonium chloride (Figure 4.9). The results of these runs are shown in Table 4.7.

The first thing to notice is that there is still strong linkage between bacillomycin concentration and foam capacity; changing the nitrogen source had no effect here. However, there is a big difference between the performance of the nitrate-containing medium and that of the one containing ammonium. Although the total biomass of the nitrate-based system is only about half that of the glutamate medium, the pH profiles are the same and the amount of antibiotic produced is comparable. On the other hand, growth in the ammonium-based system is very poor. The maximum biomass is about 70% less than in the case with glutamate. Furthermore, there is no production of bacillomycin and consequently little foaming. These results demanded further study.

Table 4.6: Effect of Glutamate Concentration on Foam Capacity

Glutamate Concentration (Molarity)	Maximum Biomass (g/L)	Bacillomycin Concentration (OD 277)	Foam Capacity (cm)
0.034	1.88	3.40	41
0.017	1.56	2.05	28
0.0068	0.50	0.38	8

NOTE: Values for bacillomycin concentration and foam capacity are averages of the measurements made after the end of the exponential growth phase.

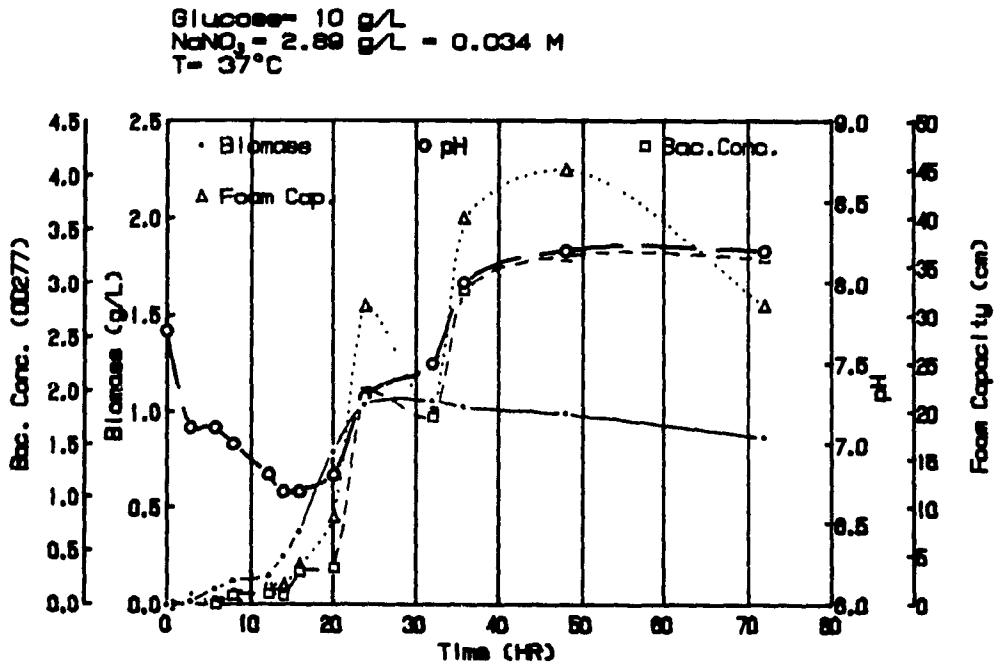

Figure 4.8: Fermentation profile of nitrate-based medium (0.034 M) at T= 37°C

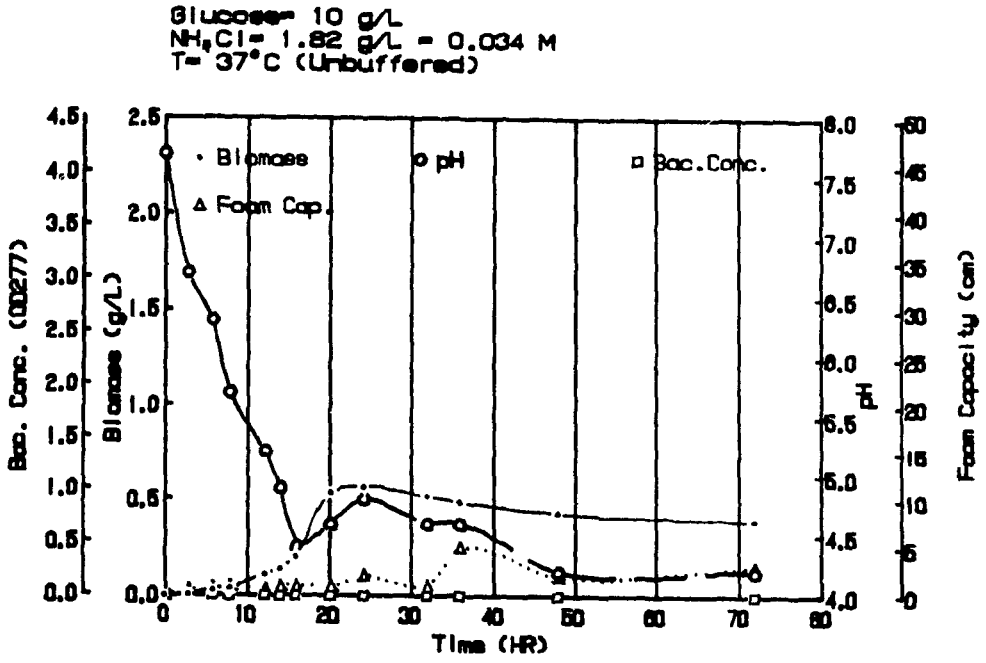

Figure 4.9: Fermentation profile of ammonium-based medium (0.034 M) at T= 37°C

Table 4.7: Effect of Various Nitrogen Sources on Foam Capacity

Nitrogen Source [0.034M]	Maximum Biomass (g/L)	Bacillomycin Concentration (OD 277)	Foam Capacity (cm)
glutamic acid	1.88	3.40	41
sodium nitrate	1.06	3.14	39
ammonium chloride	0.57	0	3

NOTE: Values for bacillomycin concentration and foam capacity are averages of the measurements made after the end of the exponential growth phase.

4.5.1 Effect of Nitrate Concentration

The performance of nitrate-based media was examined and compared to that of the glutamate-based media. Experiments were carried out using the same media used in Figures 4.1, 4.6 and 4.7, but replacing the glutamate with equimolar amounts of sodium nitrate (Figures 4.8, 4.10, and 4.11). A summary of the results can be found in Table 4.8.

In general, the nitrate-based systems behave in much the same manner as the glutamate-based systems. The total biomass tends to be lower for the media containing nitrate but the pH profiles are the same and the amount of bacillomycin produced is similar. Although the product concentration and foam capacity are still strongly coupled, these results are interesting if only for the fact that sodium nitrate performs satisfactorily while being a less expensive nitrogen source than glutamic acid.

4.5.2 Effect of Ammonium Chloride

The most striking feature of the ammonium-based experiment is the pH profile (Figure 4.9). This pH profile is radically different from the ones seen for glutamate or nitrate based media. Instead of dropping from 7.7 to around 6.0 and then rising again to around 8.0, the pH drops rapidly to about 4.5 and then sinks slowly to just above 4.0. It was thought that this low pH level was responsible for the poor growth and the lack of antibiotic production. To examine this phenomenon, a simple experiment was performed, the results of which are found in Table 4.9. After four days of fermentation, the pH of the ammonium based medium was steady at 4.2 and no bacillomycin was present.

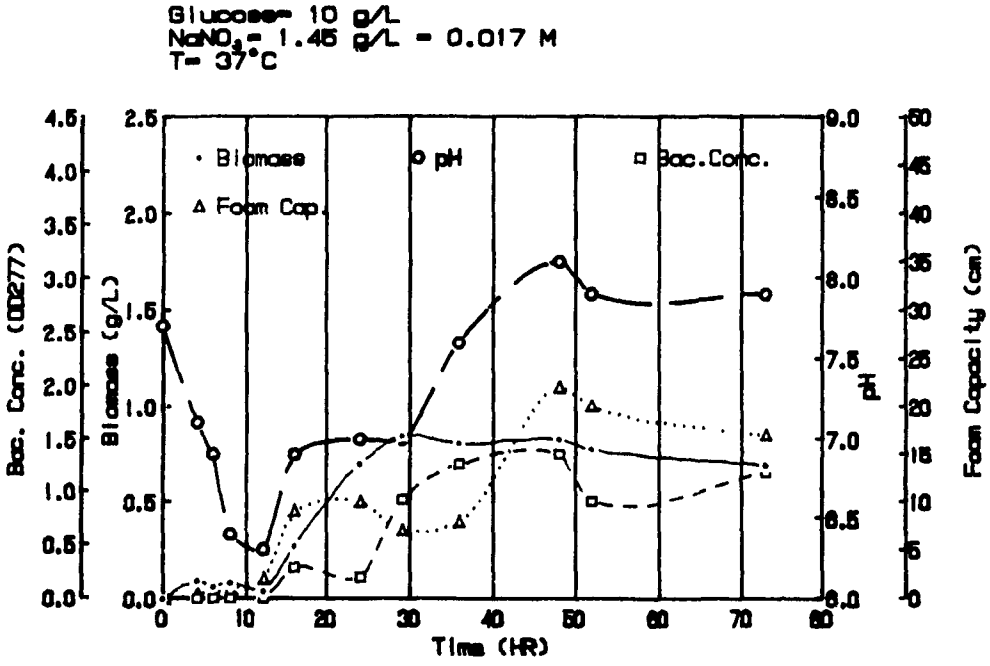

Figure 4.10: Fermentation profile of nitrate-based medium (0.017 M) at T= 37°C

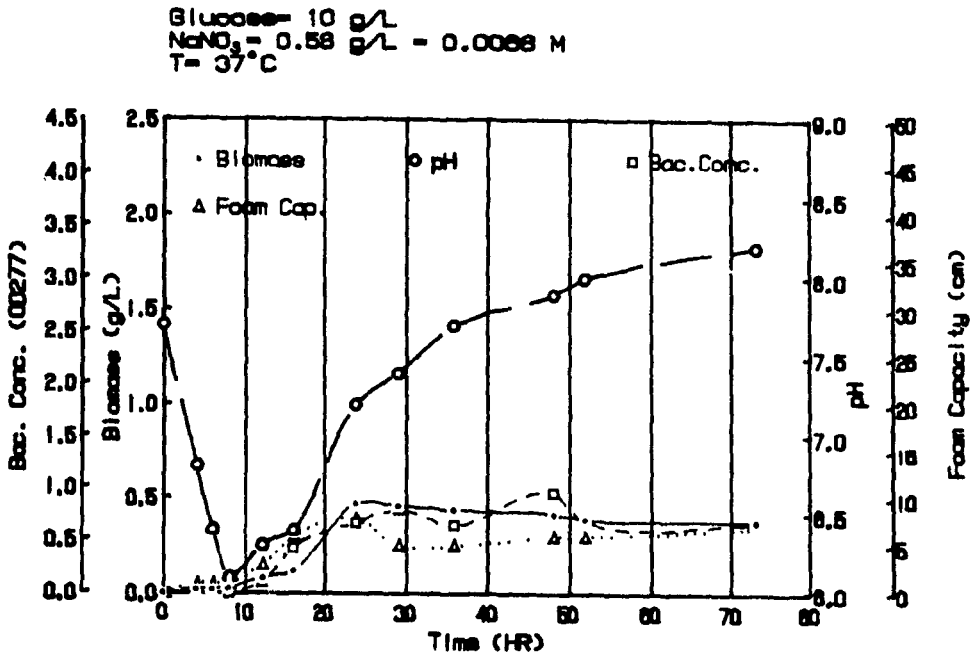

Figure 4.11: Fermentation profile of nitrate-based medium (0.0068 M) at T= 37°C

Table 4.8: Effect of Sodium Nitrate Concentration on Foam Capacity

Nitrate Concentration (Molarity)	Maximum Biomass (g/L)	Bacillomycin Concentration (OD 277)	Foam Capacity (cm)
0.034	1.06	3.14	39
0.017	0.85	1.97	12
0.0068	0.47	0.75	6

Glutamate Concentration (Molarity)	Maximum Biomass (g/L)	Bacillomycin Concentration (OD 277)	Foam Capacity (cm)
0.034	1.88	3.40	41
0.017	1.56	2.05	28
0.0068	0.50	0.38	8

NOTE: Values for bacillomycin concentration and foam capacity are averages of the measurements made after the end of the exponential growth phase.

Table 4.9: Effect of pH on Bacillomycin Production in an Ammonium Based Medium

	pH	Bac.Conc.	Foam Cap.
After 4 days of fermentation	4.2	-	3
After addition of Na_2CO_3	6.7	-	3
After 2 hrs. of further fermentation	6.0	0.14	4
After 3.5 hrs. of further fermentation	5.6	0.83	16

The pH was raised to 6.7 by the addition of 1M sodium carbonate. After two hours of further fermentation, the pH dropped to 6.0 and trace amounts of antibiotic were found. After three and a half hours of fermentation, the pH dropped to 5.6 but there was a significant amount of bacillomycin present. These results indicate a linkage between pH and antibiotic production, and this led to further investigation.

An experiment was carried out wherein an ammonium based system was used but the buffering capacity of the medium was enhanced by increasing the phosphate concentration by about nine times. The results of this run are shown in Figure 4.12. Although the pH profile is somewhat uncharacteristic, the additional buffer in the system prevents the pH from dropping below 6.0. The biomass increases rapidly to a maximum of 2.15 g/L, while the bacillomycin concentration is significant and accompanied by a proportional amount of foaming. This confirmed the fact that both growth and antibiotic production are suppressed by low pH.

4.5.3 Effect of Phosphate

In order to determine the role played by phosphate in these fermentations, an experiment was performed in which the same ammonium-based medium seen in Figure 4.12 was used, but the phosphate was replaced by an equivalent amount of Tris buffer. The results shown in Figure 4.13 indicate that the biomass level is still high at 1.92 g/L, and the pH follows the expected profile. Due to the low level of phosphate in this case, the bacillomycin concentration is comparable to that seen in the

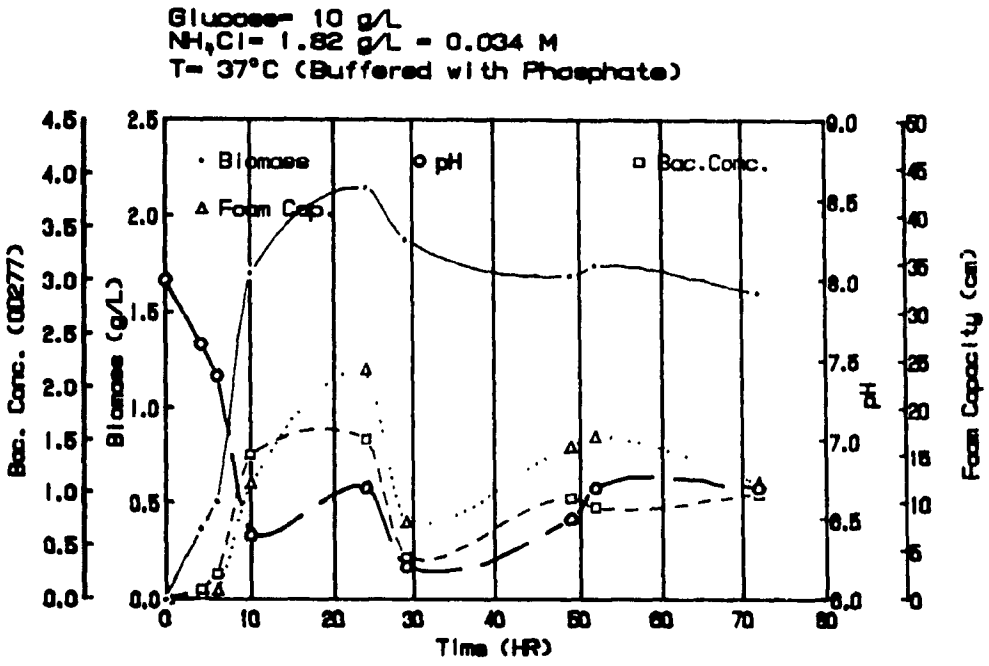

Figure 4.12: Fermentation profile of ammonium-based (0.034M) phosphate-buffered medium at T= 37°C

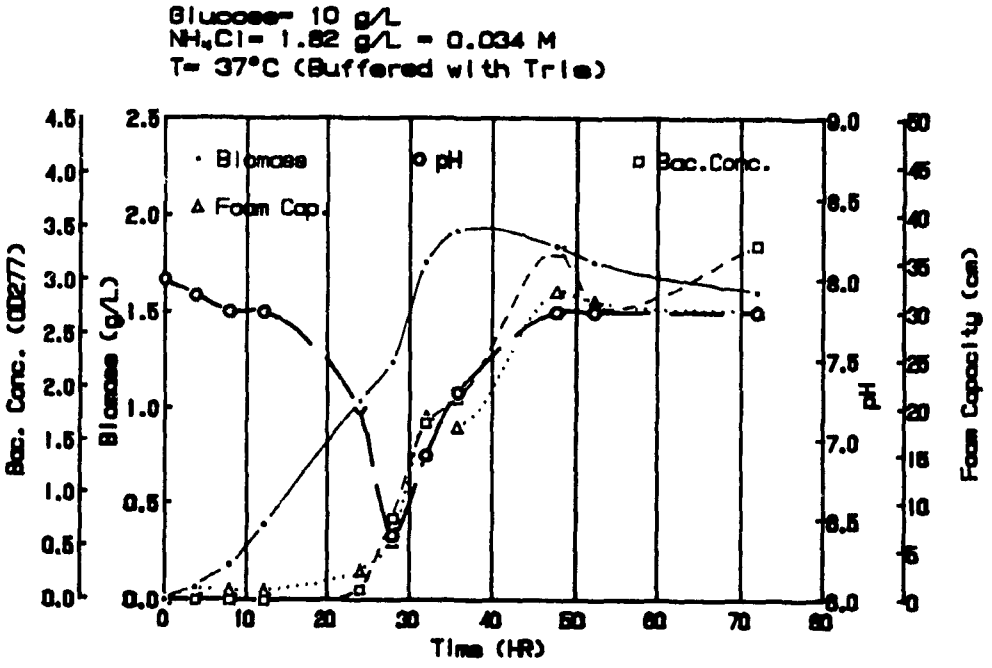

Figure 4.13: Fermentation profile of ammonium-based (0.034 M) Tris-buffered medium at T= 37°C

glutamate and nitrate-based systems.

4.6 Effect of Overall Electrolyte Concentration

The effect of electrolyte concentration was examined by conducting an experiment using the Modified MM Medium described in Section 3.2. The Modified MM Medium is three to four times more hypertonic than the Standard Medium. The results shown in Figure 4.14 demonstrate that the cell growth is mediocre, as the total biomass level gets no higher than 0.76 g/L. No bacillomycin is produced and therefore, there is little foaming.

An experiment was performed in which Modified MM Medium was used but the phosphate was replaced by Tris buffer. The results of this run are shown in Figure 4.15. The level of growth is quite good, with a maximum at 1.32 g/L, and the bacillomycin concentration is higher than in any other experiment. In this case, much less foam is produced than is seen for similar concentrations of bacillomycin at lower electrolyte concentrations.

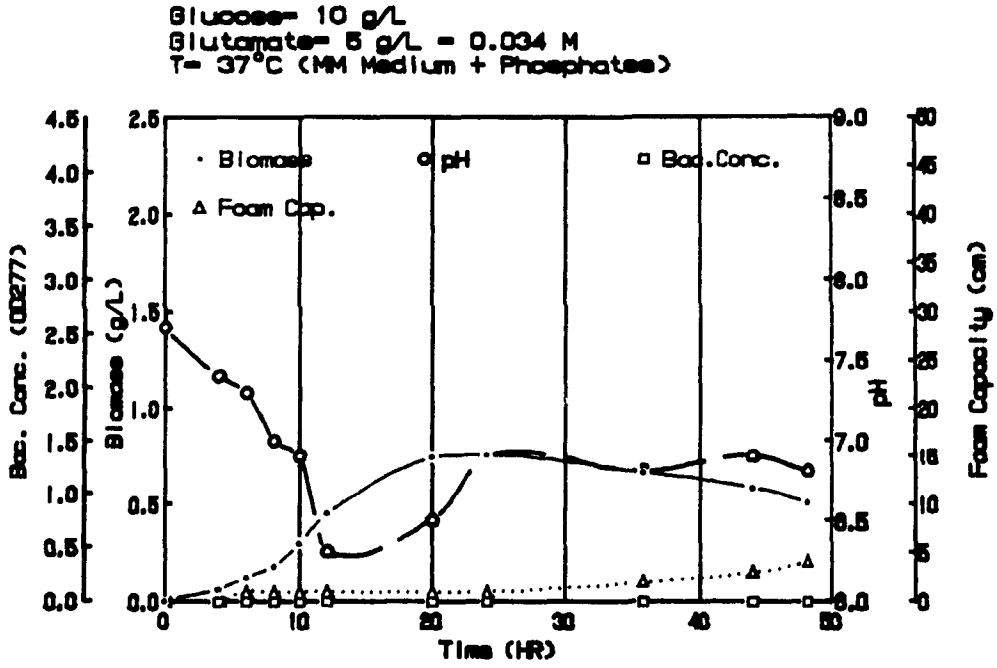

Figure 4.14: Fermentation profile of phosphate-buffered Modified MM Medium at T= 37°C

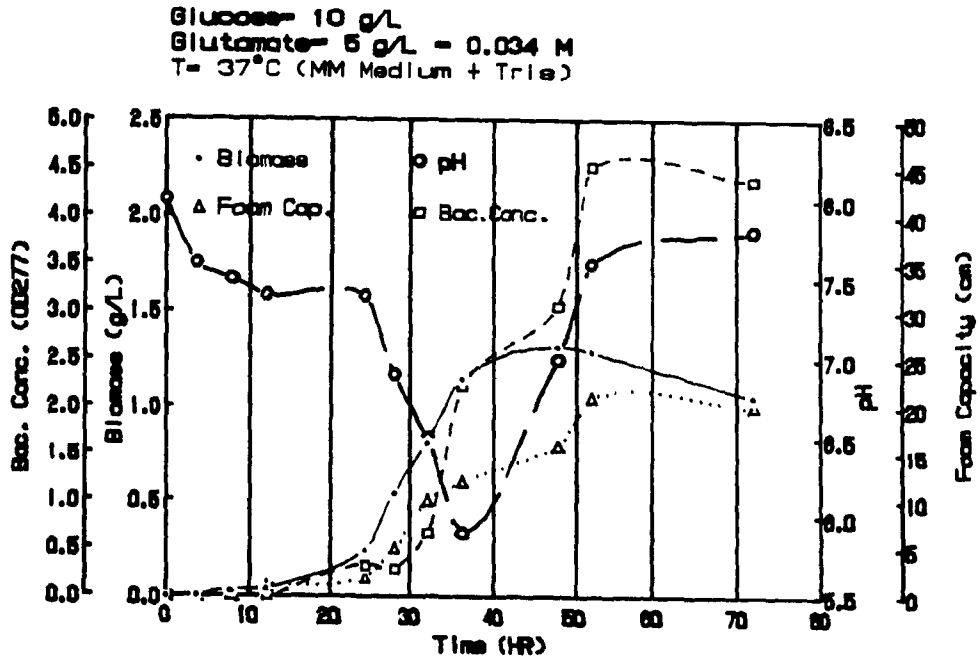

Figure 4.15: Fermentation profile of Tris-buffered Modified MM Medium at T= 37°C

5.0 DISCUSSION

Foaming occurs in many fermentation reactors. It is generally not desirable because it increases capital costs by requiring more reactor space or the addition of mechanical foam breakers. Foaming may also increase the operating cost of a system if antifoaming additives are used.

This work grew out of a project in which the continuous phasing method was used to cultivate Bacillus subtilis in a cyclone reactor. The bacillomycin L produced by the bacteria caused a dramatic drop in the surface tension of the fermentation broth. This led to severe foaming problems that made it impossible to take the uniform samples that are required for the continuous phasing method to work. The foaming also resulted in the contamination of the reactor on numerous occasions. The use of antifoam agents was ruled out in this case because the product, bacillomycin, is itself surface active and the addition of another surface active compound would have created a serious downstream separation problem. Likewise, changing the carbon source to a hydrocarbon and thereby suppressing foaming by the addition of another liquid phase was not a viable option because this strain of Bacillus subtilis does not grow well on hydrocarbons. A study was therefore carried out with the intention of determining what factors affect the foam capacity of the fermentation broth. It was hoped that these factors could be identified and controlled in such a way that foam capacity would be suppressed while still allowing for good bacterial growth and antibiotic production.

5.1 Fermentation Profile of Standard Case

The run shown in Figure 4.1 serves as a reference for this set of experiments. It was carried out following the methods of Chevanet et al (1986).

The biomass curve is typical of a batch fermentation. There is a short lag phase, followed by a period of exponential growth, and finally maximum growth is achieved. This plateau represents the stationary phase and it occurs once the limiting nutrient is exhausted (Brock, 1979). Eventually the total biomass begins to decrease as cells start to lyse (Brock, 1979).

The pH profile for this run follows a pattern seen throughout these experiments. The pH decreases from its original value of 7.7 to around 6.0. About halfway through the exponential growth phase, the pH rises to around 8.0. These results corroborate the findings of Chevanet et al (1986).

The concentration of bacillomycin was obtained by measuring the optical density of an ethanol extract at 277.0 nm. The ultraviolet absorption spectrum of bacillomycin L shows a strong peak at 277.0 nm. (Besson and Michel, 1987). This is due to the phenyl group on the tyrosine present in its peptide ring (Figure 3.2). However, many proteins and other substances with phenyl rings also absorb strongly in this region. It is not clear that this should be an accurate means of determining bacillomycin concentration. However, this method was justified by two types of experiments. First of all, the majority of extracellular material produced by these bacteria is bacillomycin L. This was confirmed qualitatively by the thin layer

chromatographs obtained by following the method of Besson and Michel (1987). These results are shown in Table 4.1. Secondly, very little of the bacillomycin remains in the cells; almost all of it ends up in the broth (Chevanet et al, 1986). Furthermore, Figure 4.2 represents a calibration curve generated by a set of experiments that demonstrates a strong linear relationship between bacillomycin concentration and OD277. These results, taken together, provided confirmation of the legitimacy of using optical density readings to represent the concentration of bacillomycin in the broth.

From Figure 4.1, it is clear that there is no significant production of bacillomycin until the end of the exponential growth phase, as is typical of a secondary metabolite. The production of bacillomycin reduces the surface tension of the broth to 26 mN/m after 24 hours. It is this surface activity of the product that is essential to the formation and stabilization of the foam (Walstra, 1988).

5.1.1 Surfactant Concentration, Surface Viscosity, and Foaming

Figure 4.1 indicates that there is a strong connection between bacillomycin concentration and the foam capacity of the broth. This linkage was exhibited in almost every experiment. The relationship was found to be quite linear, as seen in Figure 4.3.

It was not expected that bacillomycin concentration and foam capacity would be so closely related. The level of bacillomycin contributes to the foam capacity by reducing the surface tension but the surface tension reaches a minimum quite

quickly while foam capacity and bacillomycin concentration continue to increase. It is believed that the increase in bacillomycin concentration causes an increase in the surface viscosity of the foam. This in turn enhances foam stability, which has a strong effect on the foam capacity. The surface viscosity may play a larger role than the viscosity of the bulk liquid. This is due to the fact that a disproportionate amount of surfactant ends up in the foam because it is adsorbed at the large surface area, resulting in a relatively large amount of surfactant concentrated in an area of relatively small liquid volume (Callaghan, 1988).

The connection between surface viscosity and foam capacity and stability has been reported widely (Bikerman, 1953; Joly, 1972; Tabor, 1972; Dickinson, 1988; Nutt and Burley, 1988). Djabbarah and Wasan (1985) have observed a strong relationship between the concentration of lauryl alcohol, surface viscosity and foam stability, in a lauryl alcohol-sodium lauryl sulfate-water system. In general, high surface viscosity increases foam stability by slowing gravitational drainage of liquid through the lamellae and by retarding the process of bubble coalescence (Heller and Kuntamukkula, 1987). When two bubbles approach each other, liquid must be pushed aside. Viscosity slows down this process. Therefore, if viscosity is great enough, coalescence does not occur or occurs very slowly. However, if the viscosity is too large, what is obtained is a gas-in-liquid emulsion rather than a foam. Therefore, foam is produced when the surface viscosity of thick liquid layers is small, thereby enabling the

bubble to quickly cover the major part of the distance to the neighbouring bubble. However, if the liquid layers are thin, as is the case for this fermentation broth, the foam is stabilized by a high surface viscosity because it makes it difficult for a bubble to traverse the width of the lamella and merge with another bubble (Bikerman, 1953).

5.1.2 Bacillomycin and Foaming

The results shown in Table 4.2 demonstrate further the connection between bacillomycin concentration and foam capacity. The foam capacity of the solutions made from the redissolved bacillomycin tend to be 1/3 to 1/2 less than the foam capacity of the corresponding whole broth sample. This could be due to three possible reasons: loss of surfactant during the isolation process, inactivation of the surfactant during extraction, or loss of some other substances that were acting in a synergistic relationship with bacillomycin.

Some product is always lost in an extraction and this case can be no exception. In general, high levels of purity are associated with low yields. It is also quite likely that some bacillomycin remained in the system in an inactive state. The surfactant is precipitated by the addition of concentrated hydrochloric acid and it is probable that high local acid concentrations might have caused some denaturation. Many different combinations of surfactants have been seen to exhibit synergistic effects. Synergism in foam capacity has been shown to be related to synergism in surface tension reduction effectiveness (Rosen and Zhu, 1988). While a synergistic co-

surfactant may be present in the broth, this explanation of the foam capacity reduction is less likely than the previous two. The results shown in Table 4.3 demonstrate that the surface tension and foam capacity of the fermentation broth are not greatly affected by a chloroform wash. The chloroform-soluble substances extracted by such a procedure would include most lipids and many other nonpolar prcducts (Solomons, 1988). Unlike many surfactants, bacillomycin is insoluble in chloroform (Besson et al, 1977). This would indicate that bacillomycin is largely, if not solely, responsible for the reduction in surface tension and the production of foam. While it is not possible to completely discount the presence of synergistic substances, all chloroform-soluble co-surfactants can be ruled out.

5.2 Effect of pH

The fermentation profile shown in Figure 4.1 suggests that there might be some connection between the pH of the broth and its foam capacity. It appears that the foam capacity does not really begin to increase until the pH has started to rise after reaching a minimum. Table 4.4 contains the results of an experiment conducted to determine the effect of pH on foam capacity. Within the range of pH examined, there is no significant change in the foam capacity of the broth. It may therefore be concluded that foam capacity is not a simple function of pH.

5.3 Effect of Incubation Temperature

The effect of incubation temperature on foam capacity was examined in experiments represented in Figures 4.1, 4.4 and 4.5.

The data is summarized in Table 4.5.

Temperature has little effect on the maximum level of biomass or the pH profile. However, as the incubation temperature is decreased, the amount of time required to achieve maximum growth increases. This is due to the fact that for most enzymes in microbial systems, the rate of reaction increases with temperature until a temperature is reached where the enzyme is no longer stable. At low and moderate temperatures, where the enzyme is stable, the increase in activity with temperature is a function of the activation energy for the reaction. For many enzymes, the rate increases by a factor of about two for a 10°C rise in temperature (Zubay, 1988). These enzymes catalyse all reactions in the cell, so it is not surprising that growth and reproduction are slowed at lower temperatures.

All three experiments demonstrate linkage between bacillomycin concentration and foam capacity. However, the levels of both were about 60% lower at 23°C. Bikerman (1953) reports that increased temperature usually decreases foam capacity for a variety of reasons, most notably the reduction of interfacial viscosity. However, any change in foam capacity for these experiments is simply attributable to the change in bacillomycin concentration caused by altering the incubation temperature. In fact, the foaming tests themselves were all carried out at the same temperature.

5.4 Effect of Glutamate Concentration

Chevanet et al (1986) have shown that glutamate has an effect on growth, antibiotic production, and sporulation.

Experiments were carried out to determine how glutamate concentration affects foam capacity. The results are shown in Figures 4.1, 4.6 and 4.7, with a summary in Table 4.6.

A two-fold decrease in glutamate concentration has little effect on total biomass, whereas a five-fold decrease reduces the final biomass level drastically and results in slower growth. These results are in keeping with those of Chevanet et al (1986).

Halving the level of glutamate in the medium has no effect on the behaviour of the pH. However, reducing the concentration to 1.0 g/L results in a highly erratic pH profile that is probably responsible for the low level of antibiotic production.

A two-fold decrease in glutamate concentration reduces bacillomycin concentration by 40%, while a five-fold reduction in glutamate results in almost a 90% decrease in production. Again, a strong relationship between bacillomycin concentration and foam capacity was observed. While the production of bacillomycin is affected by the level of glutamate in the medium, foam capacity cannot be controlled by changing the glutamate concentration if high yields are desired.

5.5 Effect of Other Nitrogen Sources

Experiments were conducted to determine if foam capacity was affected by replacing glutamate with sodium nitrate (Figure 4.8) or ammonium chloride (Figure 4.9). The results are shown in Table 4.7.

Changing the nitrogen source did not affect the strong relationship between bacillomycin concentration and foam

capacity. However, the media containing nitrate and ammonium behave very differently. The overall performance of the nitrate-containing medium is very much like that of the one containing glutamate, with the exception of a reduced level of biomass. The ammonium-based medium, though, exhibits very poor growth, with no production of bacillomycin and consequently little foaming. Although this result is reported by Chevanet et al (1986), it is surprising because the vast majority of bacteria are able to use ammonium as a nitrogen source (Brock, 1979). This is due to the fact that glutamate dehydrogenase is ubiquitous among eukaryotes (Zubay, 1988). As shown in Figure 5.1, it is this enzyme (in the presence of NADPH) which allows ammonium to be incorporated into α-ketoglutarate to form glutamate (Zubay, 1988). Directly or indirectly, the amino groups are all derived from ammonia by way of the amino groups of L-glutamate (Zubay, 1988). On the other hand, nitrate must be reduced to ammonium before it can be utilized. Some bacteria cannot metabolize nitrates because they lack nitrate reductase, the enzyme that reduces the oxidation state of nitrogen in the nitrate form (Brock, 1979). Therefore, the behaviour of the ammonium-based medium would normally be expected to be at least as good as that of one based on nitrate. This warranted further investigation.

5.5.1 Effect of Nitrate Concentration

Experiments were carried out to compare the performance of nitrate-based media with media containing comparable amounts of glutamate. The relevant Figures are 4.1, 4.6, 4.7 and 4.8, 4.10, 4.11. The results are summarized in Table 4.8.

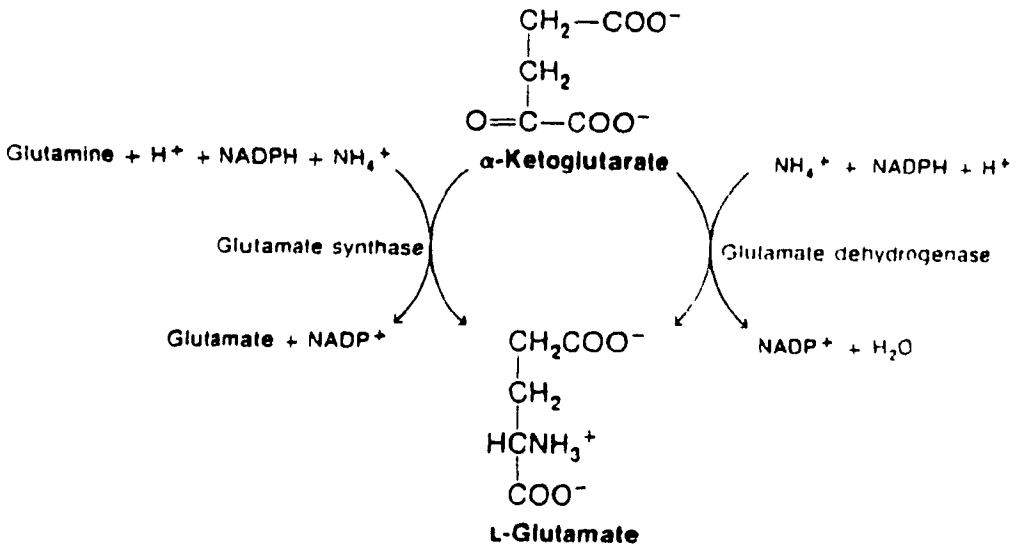

Figure 5.1: Conversion of ammonium into the α-amino group of glutamate (Zubay, 1988)

Nitrate-based media behave in much the same way as media containing glutamate. Total biomass tends to be lower for nitrate-based systems but similar profiles are observed for pH and bacillomycin concentration. Once again, foam capacity is strongly coupled to bacillomycin concentration, so using nitrate as a nitrogen source is not the solution to this problem. However, the similarity in the performance of the nitrate and glutamate would tend to make sodium nitrate more attractive for a commercial application because it is about 50% cheaper than glutamic acid.

5.5.2 Effect of Ammonium Chloride

The poor growth and lack of antibiotic production in the ammonium-based medium seemed to be connected with the pH profile (Figure 4.9). Instead of dropping down to 6.0 and then rising up again, the pH fell sharply to around 4.0 and stayed there. When a sample of ammonium-based broth that had been fermenting for several days was subjected to an increase in pH, bacillomycin production could be detected over the next few hours (Table 4.9). These results demonstrate that bacillomycin production is suppressed by the low pH developed in the ammonium-based medium.

Another experiment was carried out using the ammonium-based medium but with an eight to nine-fold increase in phosphate concentration. The results are shown in Figure 4.12. The additional buffer in the system prevents a drastic fall in pH. The microbial growth is very good, suggesting that the low pH of the unbuffered system suppresses cell growth as well as antibiotic production. The bacillomycin concentration seen in

Figure 4.12 is significant and accompanied by a proportional amount of foaming. However, the amount of bacillomycin present is at only about 1/3 the level seen in a glutamate or nitrate-based medium. This became the subject of the next stage of this research.

5.5.3 Effect of Phosphate

The production of some antibiotics is suppressed by the presence of inorganic phosphates in the fermentation broth (Gonzalez et al, 1990). Control usually occurs at the transcriptional level, although direct inhibition of antibiotic synthesizing enzymes has been observed in some cases (Liras et al, 1990). An experiment was undertaken in which the same medium seen in Figure 4.12 was used, but the phosphate was replaced by an equivalent quantity of Tris buffer. The results shown in Figure 4.13 indicate good growth and a normal pH profile. In the absence of phosphates, the bacillomycin concentration is comparable to that seen in the glutamate and nitrate-based systems. It may be concluded therefore that the production of bacillomycin is inhibited by phosphate. It may also be concluded that ammonium chloride is an acceptable nitrogen source if the pH is controlled by a buffer other than inorganic phosphate. This would make ammonium chloride an even more attractive candidate than sodium nitrate if commercial production were to be considered. Ammonium chloride is eight times cheaper than glutamic acid and four times cheaper than sodium nitrate.

5.6 Effect of Overall Electrolyte Concentration

Increasing the overall electrolyte concentration of the nutrient medium was considered as a means of controlling the foaming problem. Added solutes can reduce foaming in a number of different ways: 1) by reacting chemically with the surfactant; 2) by decreasing the dispersity, and thus the surface activity of a surfactant; 3) by altering the solubility of the surfactant; 4) by simply displacing the surfactant at the interface; 5) by changing the surface rheology of a system (Bikerman, 1953).

An experiment was run using the Modified MM Medium found in Section 3.2. The Modified MM Medium is about three to four times more hypertonic than the Standard Medium used in most of these experiments and it was hoped that this would depress the foam capacity while still allowing for adequate cell growth and antibiotic production. The results seen in Figure 4.14 were disappointing. Cell growth is not very good, as the total biomass level gets no higher than 0.76 g/L. The pH profile is close to being normal but there is still no bacillomycin produced and consequently very little foaming. However, it seemed reasonable to assume that the antibiotic production was being suppressed by the exceptionally high level of phosphates in the Modified MM Medium. A new experiment was therefore carried out wherein the phosphate in the Modified MM Medium was replaced by an equivalent amount of Tris buffer, while some potassium chloride and sodium chloride were added to make the new medium isotonic to that seen in Figure 4.14. The results of this experiment are shown in Figure 4.15. The growth of the biomass

is acceptable, with a maximum at 1.32 g/L. The pH profile follows the characteristic pattern, while the level of bacillomycin production is higher than ever. The most important thing to note, however, is that for the first time the amount of foam produced for a given concentration of bacillomycin is actually lowered. It may be concluded, therefore, that the foam capacity of the fermentation broth can be decreased by increasing the overall electrolyte concentration of the medium. Furthermore, it is possible to have high levels of bacillomycin production with a significantly reduced foam capacity.

6.0 CONCLUSIONS

The first objective of this study led to a very practical result. The bubble column method outlined in Section 3.3 was found to be an accurate and repeatable means of characterizing the foam capacity of a liquid. It was this method that made it possible to study in a quantitative way the effect of various process parameters on foaming.

Figure 4.2 indicates that the concentration of bacillomycin L in the fermentation broth was found to be directly related to the optical density of an ethanol extract produced following the procedure outlined in Section 3.3. The putative reasons for this phenomenon are discussed in Section 5.1. This relationship provided a straightforward method of monitoring the bacillomycin concentration in the fermentation broth.

One common feature seen throughout these experiments is the close relationship between the concentration of bacillomycin and the foam capacity of the broth. Figure 4.3 indicates that this could properly be described as a direct relationship. It is believed that an increase in bacillomycin concentration increases the interfacial viscosity of the foam. This increases the foam capacity and foam stability of the broth by retarding liquid drainage through the foam lamellae and preventing quick bubble coalescence. Needless to say, bacillomycin concentration also increases foam capacity by reducing tension at the interfaces, up to the critical micelle concentration.

Many of the parameters that were examined had no direct effect on foam capacity. It was found that there is no simple

relationship between pH and foam capacity, within the range of pH normally seen in this fermentation. Incubation temperature had no real effect on foaming, other than that produced by changes in the bacillomycin concentration. At the relatively low temperature of 23°C, bacillomycin production was lowered and the foam capacity was reduced for this reason alone. The level of glutamic acid in the medium had predictable effects on the amount of biomass and bacillomycin produced. Biomass levels and bacillomycin concentrations tended to be higher with higher levels of glutamate, and foam capacity varied in accordance with the concentration of bacillomycin.

When sodium nitrate was used as a nitrogen source, the results were quite similar to those recorded for glutamate-based media. However, when glutamate was replaced with ammonium chloride, cell growth was inhibited and antibiotic production completely suppressed. The cause of this poor performance was the sharp drop in pH that often happens in ammonium-based media. When trials were carried out using the same medium but with the phosphate concentration increased by an order of magnitude, biomass levels returned to normal and bacillomycin concentration increased to about 1/3 its normal level. The reduction of bacillomycin production, and the consequent reduction in foam capacity, was due to the increased level of phosphate in the medium. When the phosphate was replaced by an equivalent amount of Tris buffer, both biomass and bacillomycin levels went back to normal. However, the foam capacity remained linked to the bacillomycin concentration.

A final experiment was carried out wherein the overall salt concentration of a glutamate-based Tris-buffered medium was increased by about three to four times. This resulted in adequate growth of biomass, and excellent antibiotic production. More importantly, foam capacity was seen to be decoupled from bacillomycin concentration for the first time. At high concentrations of bacillomycin, foam capacity was repressed to relatively low levels. The more likely explanations of this phenomenon would be that the added salt displaces the surfactant at the interface and changes both the dispersity and the rheology of the system.

For future fermentations, it is recommended that a simple test be carried out to see if the synthesis of the desired product is affected by the presence of inorganic phosphates. It is also suggested that increasing the electrolyte concentration in the medium would seem to be a good means of controlling foaming as long as levels affecting the viability of the organisms are not reached.

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