

SPORULATION AND TOXIN FORMATION IN BACILLUS SPHAERICUS 1593

by

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(ABSTRACT)

The effects of temperature, dissolved oxygen, and pH on the growth, sporulation, and mosquito larval toxin formation by B.sphaericus 1593, were investigated in shaken flasks and a fermentor. The bacteria grew well at temperatures of 25 to 40°C, however, toxin-formation and sporulation were poor at temperatures above 30°C. Cell lysis became evident after about 30 hours into the fermentation. particularly in case of high temperatures. Mature spores, as well as most of the toxin, were formed by 24 to 30 hours of growth.

Controlling the pH at 7.0, gives about ten-fold more toxicity as compared to experiments with no pH control. Simultaneous assay of ammonia concentration indicated a close parallel between the ammonia and pH profiles.

Assays of carbohydrate, protein, and phosphorous indicated that none of these nutrients were limiting at any time, thus none of them could have been the limiting nutrient that triggers sporulation.

It was found that dissolved oxygen concentrations dropped nearly to zero in case of fermentation with sparged air, but were significant in the case of fermentation with sparged oxygen even at their minimum. The toxicity was approximately equal in both the cases, however sporulation was poor in the case of experiments sparged with pure oxygen. Measurements of oxygen uptake rates confirmed that the cells respire more when the cells are in the vegetative phase of growth.

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The opportunity to thank some of the most influential persons in one's career comes but occasionally in a lifetime. It should not be frittered away. Yet a list of impersonal names followed by desultory prose would surely seem inadequate. Words can be only approximations of feelings, hence, knowing that I will be genuine, let me be concise.

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Chapter I

INTRODUCTION

In most of the developed world today, cases of disease transmitted by insects are rare. However, in the developing countries of Africa and Asia, such cases are often measured by the hundreds of thousands. In these parts of the world there is an urgent need for insecticides that can help prevent the spread of these diseases.

Chemical insecticides have been in use for over forty years. They have been considerably successful in eliminating insect-vectors, ie those insects which carry disease spreading microorganisms. However, in recent years, deleterious effects of these chemicals have come to light. One such chemical is Dichlor Diphenyl Trichlorethane, DDT. This causes death in insects by acting upon their central nervous system. However, this chemical can also act on mammalian systems and is suspected to be a carcinogen. Furthermore, it persists in the environment, in that DDT is very slowly biodegradable. This dangerous chemical has also been traced to cattle, and human consumption of affected beef and milk products can be harmful. As a result, DDT has been banned from use in many countries. There are several other similar examples.

Microbial insecticides offer a viable alternative to the use of many of these chemicals. Microorganisms might also have harmful effects, however, with careful screening it is possible to discard those with unwarranted pathogenic effects. Microbes can become a natural part of the existing ecological balances, thereby perturbing them only to a small extent. Selected insect vectors, which are a part of the ecosystem could be eliminated by microorganisms pathogenic to them. Furthermore, when insects often develop immunity to certain chemical insecticides after prolonged application, microorganisms would circumvent this. Those microbes which do not have exotic growth requirements can grow and reproduce themselves within the given environmental conditions, thus requiring less frequent applications. Several microorganisms, e.g. Bacillus thuringiensis, have been commercially exploited in this regard.

The mosquito is responsible for the spread of many diseases. Anopheles, Culex and Aedes species are vectors for malaria and other diseases widespread in tropical locations. Reducing the mosquito population can contribute significantly to controlling the spread of such diseases. The reproduction cycle of the mosquito consists of four major stages: the fertilised egg, the larva, the pupa and the adult mosquito. The larva proceeds through four ages (instars). It

should be mentioned here that when in the larval stage the mosquito is the most vulnerable, and thus most susceptible to insecticides.

Various types of bacteria, notably Bacillus popillae, Bacillus thuringiensis, and Bacillus sphaericus have shown insecticidal properties (1,2,3,4). Bacillus sphaericus has received considerable attention recently, and is the focus of this research. Spores and sporulating cells of many strains (eg. 1593, SSII-1, 1404, 1691) are pathogenic to mosquito larvae. The actual larvicidal activity varies widely with the type of strain, the mosquito species, the growth conditions of the bacteria and the field conditions during application such as moisture and temperature. For example, strain 1593 appears to be more pathogenic than strain SSII-1, and *Aedes* species seem to be more resistant than *Culex* or *Anopheles* species. However, the overall response has been very encouraging. Concentrations of as low as 1000 cells/ml in pond conditions, or 6700 cells/ml in field conditions resulted in a 90 to 100 % mortality in two days (5). Also, Bacillus sphaericus grows into larval tissue and reproduces itself after the larva has died (6). This causes daughter cells and spores to be released in the immediate vicinity of the carcass. Thus, the soil can not only continue to remain larvicidal, but the activity of the soil might even increase.

The toxic factor of these pathogenic strains has been shown to be quite stable under normal terrestrial conditions. Soil samples exposed to cycles of alternate drying and flooding retained most of their activity for up to nine months after application (7). The activity was also largely retained in the aqueous environment of tree-holes that froze during winter and later thawed (8). Under these conditions, the microbe probably survives in the more resistant endospore form, rather than as vegetative cells. Nevertheless, the capacity to retain activity suggests that the bacteria is good for practical use.

It has also been demonstrated as safe to humans according to the World Health Organisation regulations. The pathogenic strains have been tested against various non-target organisms, including mammals, and shown to have no adverse effects (4,5,9,10). Bacillus sphaericus was once implicated in a case of meningitis and one case of food poisoning (8,11), however, further extensive tests with rabbits clearly demonstrated that there is no mammalian toxicity. As of now, large scale field trials remain the only WHO requirement to be considered before B.sphaericus might be considered for marketing (7).

B. sphaericus grows on a variety of inexpensive media, and has no costly or exotic requirements (12). The growth rate is quite rapid, enabling the production of many batches in a short fermentation. Thus, industrial production should be economical and feasible. The above considerations indicate Bacillus sphaericus is indeed a viable microbial insecticide.

There still remain several areas of concern. As of today, standardization of the insecticide is a formidable problem. The chemical nature of the toxin is as yet unknown, and thus the exact concentrations cannot be specified. The activity is specified as LC_{50} , ie the cell concentration lethal to 50 % of the test organisms. This is a vague criterion, subject to fluctuations, and at best only an approximation of the toxicity. The activity is also affected by many factors. For example, one study showed Anopheles albuminus to be 1000 times more tolerant to a toxic strain than Culex quinquefasciatus under field conditions (13), while another study showed it to be more susceptible (12). Under field conditions, the naturally available food particles compete with the bacterium for ingestion by the larvae (13). In other words, the larvae could take in food particles along with the bacterial cells. It may be necessary to bait the product to make it compete more favourably. That is, combine

the insecticide with some other food. In addition, it may be advantageous to complement B.sphaericus preparations with other species so as to prepare a broad spectrum insecticide. For example, B.thuringiensis serovar israelensis is more toxic against Aedes species, while B.sphaericus is more toxic against Culex and Anopheles species. A combination of the two would be effective against most mosquitoes. Apart from standardisation and formulation, much other work is required in developing an optimal fermentation process for large-scale industrial production.

The goal of this research was to study the factors affecting bacterial growth and toxicity, such as media composition, temperature, pH and dissolved oxygen concentration. With this information, we should be able to propose a combination of operating parameters which can be used to maximize the production and activity of the product.

Chapter II

LITERATURE REVIEW

Since utilisation of B.sphaericus as a microbial insecticide is a relatively new concept, there is a scarcity of literature reporting on the properties of this bacteria. A brief outline of the existing reports is given below.

2.1 BIOLOGICAL CONSIDERATIONS

Bacillus sphaericus is a rod-shaped, motile, aerobic, gram-variable bacteria. The vegetative phase cells stain gram positive, and the stationary phase cells stain gram negative (14). The bacteria are sensitive to penicillin (which inhibits cell-wall peptidoglycan biosynthesis) and tetracycline (blocks protein synthesis). However, B.sphaericus is somewhat more resistant to chloramphenicol and streptomycin, which inhibit protein biosynthesis at the ribosomal level (8,15). In case of substrate limitation, or any other trigger, the bacteria change from the vegetative to stationary phase and forms a nearly spherical endospore. The spore is contained in a swollen sporangium at one terminal end of the cell. This organism grows rapidly on many different media, both complex and synthetic. The complex media, in addition to being inexpensive, also supply most of the nutritional

requirements. Bacillus sphaericus has another peculiar characteristic in that it cannot utilise carbohydrates, including the simplest of sugars viz. glucose (16). It appears to subsist on proteinaceous substrates and the synthetic media require amino-acid supplements, such as leucine, valine, lysine, methionine, isoleucine and glutamic-acid. Vitamin supplements such as thiamin and biotin are also required (17). These nutritional properties and behaviour towards antibiotics has been utilised in selective retrieval of these bacteria from soil habitat (7). The pathogenic strains are facultative parasites and saprophytes, ie they can grow on living or dead tissue.

Recently, Krych et al. (18) attempted a classification of 62 strains of B. sphaericus based on DNA homology studies. In this method, the DNA coding sequence of a given strain is compared to a previously designated one. Different strains may then be designated a species name and/or classified into groups, depending on the extent and characteristic of the genetic similarity (homology). It is generally accepted that if the two strains have over 70 % homology then they belong to the same species. Using these homology techniques, Krych et al. recognised five genetically distinct groups of B. sphaericus. Of these group II is further subdivided into

IIA and IIB. The two subgroups have considerable homology with each other, the only distinguishing characteristic is phenotypic, being that all known mosquito pathogens fall into the subgroup IIA. Furthermore, while the pathogens bear less than 20 % homology to the type strain of B.sphaericus (ATCC 14577, group I), they have over 79 % homology to the reference strain of this subgroup: WHO-1593. If the above mentioned criterion for assigning a species name were rigorously enforced, the pathogens would have to be called by some name other than than Bacillus sphaericus. However, usual phenotypic tests indicate that the pathogenic strains are B.sphaericus, and thus the name has been continued.

Other methods of strain classification include phagetyping and serotyping. In the former technique, the cells are exposed to a particular virus. This virus is a lytic bacteriophage, ie it can infect a bacterial cell and eventually cause death by lysis. Thus, upon exposure to this phage, the bacterial cell- count decreases. Several lytic bacteriophage that are specific to particular strains of B.sphaericus, have been isolated from soil (19). The strains which are susceptible to a particular phage are classified as that phagetype. Similarly, the serotype is defined by using flagella H-antigens. It has been mentioned before that B.sphaericus is a motile microorganism with flagella. The

flagella are composed of proteins (the H-antigens), and antibodies prepared against the bacteria, react with the bacteria specifically. There exist chemicals called H-antigens which react with this flagella in a typical antigen-antibody reaction. As with the phagetypes, the antigens are also specific to the strain, and can be used to differentiate the serotype (20). Pathogenic B.sphaericus strains have been classified into phagetypes and serotypes. It is interesting to note that all those pathogenic strains belonging to the same serotype also belong to the same phagetype, and have similar levels of toxicity as measured by the LC_{50} (the concentration lethal to 50 % of the test species) (21). For example, strains 1593, 1691, and 1881 are all of the phagetype 3, serotype H5 and have a final broth LC_{50} of the order of 10^{-4} $\mu\text{g/ml}$. On the other hand, strains Kellen K and Q are of the phagetype 1, serotype H1a and have an LC_{50} of the order of 10^{-1} $\mu\text{g/ml}$.

2.2 PATHOGENESIS

The gut (intestine) of the mosquito larva contains a variety of microbial flora. Some of these organisms may be symbiotic in nature (that is, help the larva in digestion), while others may be parasitic (living off the larva). There exist some early publications regarding this flora

(22,23,24,25). Different investigators found that over 80 % of the flora consisted of gram-negative rods. Compared to Culex or the Anopheles mosquitoes, Aedes had less microbial flora in the midgut. It is interesting to note that later studies have revealed that Aedes larvae are more resistant to pathogenic B.sphaericus strains (12). This might be due to their capacity to keep the microbes out of their gut.

Davidson and coworkers have studied the ultra-structural events in the larval midgut, leading to death (6). These studies provide important clues to the nature of pathogenesis of B.sphaericus (sequence of events leading to death). Larval death could result when the bacteria penetrate the larval systems and interfere with their function (an infection). On the other hand, the process could be a toxin-mediated one, where the bacteria release a component toxic to the larva. It is apparant that if the bacteria do not need to penetrate the larval tissue in order to cause death, then the process is not an infection, but is rather mediated by the toxin. This information is important, even from an industrial standpoint, for it would establish whether high specific toxicity was the criterion for process optimisation. Davidson et.al. (26) found that the cell-count in the larval midgut decreased sharply after ingestion and remained at a low level for as long as the larva was alive. The count

began to increase only after death. Furthermore, the cells contained in the live larva were confined to the peritrophic membrane, ie the midgut-lining. They began growing into the tissue only after death. Singer (27) and Myers (28) have also independently confirmed that bacterial replication is not essential to pathogenesis. B.sphaericus cells were rendered non-viable by treatment with chloroform and these proved to be as pathogenic as viable cells. Use of antibiotics such as bacitracin (which prevent bacterial replication) gave the same levels of mortality as the control (without bacitracin). It is possible to conclude from the above information that this pathogenesis is not a true infection, and that death occurs due to a toxic component of the cell.

2.3 LOCATION OF THE TOXIN

In a comparative study, Myers and Yousten have shown that spores of 1593 strain are definitely more toxic than the vegetative cells (29), whereas sporulation does not seem to affect the toxicity of the SSII-1 strain. Vegetative cells of the 1593 strain have a high LC_{50} (to the order of 10^8 cells/ml), which drops sharply as the cells begin to sporulate (to the order of 10^2 to 10^3 cells/ml). Mn^{++} , Ca^{++} , and Mg^{++} ions are known to aid sporulation. Exclusion of these ions from the medium caused the pathogenicity of the 1593

culture to drop. In another study, using different genetic techniques, oligosporogenic mutants of strain 1593 were developed. These mutants sporulate poorly and the broth developed therefrom had 100-1000 times less insecticidal activity. These effects were not observed for the SSII-1 cultures. However, it seems unlikely that the toxin of strain 1593 is fundamentally different from that of strain SSII-1. Strain 1593 probably produces a greater quantity of the toxin, or modifies the chemical nature so as to give a more potent toxin.

It would also be useful to know the location of the toxin within the spore. Myers and Yousten isolated the cell-wall, cell-membrane and the cytoplasmic contents of the sporulating cell, through an involved fractionation scheme (21,28,30). Using previously established bioassay techniques (31), different fractions were assayed for toxicity, and it was revealed that most of the activity was confined to the cell-wall. Other components also have some toxicity, but lower by several orders of magnitude. While conducting these experiments it was confirmed that there was no contamination of one fraction by another. Furthermore, fully mature spores were about ten times more toxic as the cell-wall fraction of sporulating cells. It is possible that the spore-coat develops greater toxicity as sporulation pro-

gresses. Davidson has observed that the outer wall of the bacterial cell disappears quickly in the midgut (26). There is good evidence that it has been digested. She also demonstrated that all the toxic activity is associated with the cell fraction. The findings suggest a possible localisation of the toxin in the cell-wall. Enzymes like glycosidases, peptidases and amidases are known to solubilise bacterial cell-walls, and could play a key role in releasing the toxin (32). The fact that gut-enzymes of mosquito larvae can solubilise high levels of the toxin lends to this hypothesis (33).

It is known that B.thuringiensis has a parasporal inclusion body that is responsible for most of its toxic activity (3). This body, a crystal of the toxin, is contained outside the spore-coat, and outside the outer exosporium layer. B.sphaericus SSII-1 strain does not have such an inclusion. However, recent reports suggest that aged sporulated cells of B.sphaericus 1593 do indeed contain such a body. These bodies show considerable similarity to the cell-wall toxin, including similar chemical characteristics. These are suspected to play a role in pathogenesis, but the exact nature of this role remains to be determined (34).

In a recent investigation, Davidson detected significant levels of cytoplasmic toxin as well (36). The chemical responses and gross symptoms of the larval intoxication by this toxin are very similar to those of the cell-wall toxin. There appears to be a relation between these two forms. The toxin is probably synthesised at the cytoplasmic level and then rapidly incorporated into the cell-wall.

Sporulating cells of strain 1593, treated with trypsin and protease, did not release any reasonable quantity of the toxin. This indicates that the toxin is not just loosely associated with the cell-wall. Pathogenic, as well as non- as non-pathogenic strains of B.sphaericus, do have an outermost proteinaceous layer (30,35). This layer is smooth for pathogenic strains and rough for non-pathogenic strains (36). It was suspected that this difference may account for the toxicity. However, Yousten dissolved this layer in 8M urea and assayed it for toxicity. His results were negative, indicating that this is probably not the site of the toxin. The toxin is probably closely trapped within a matrix of cell-wall subunits. It could also be a spore-structural protein, probably located in the spore-coat.

2.4 CHEMICAL PROPERTIES OF THE TOXIN

It was mentioned before that Myers and Yousten fractionated the cell-wall component in order to assay it for toxicity (21,30). This preparation was found to be stable to freezing, lyophilisation, sonication, refrigeration (4°C in sterile water for two weeks) and heat (80°C for 12 minutes). In other words, the above techniques did not reduce the toxicity of the cell-walls.

Toxin of strain 1593 was more stable than that of strain SSII-1, though 1% L-cysteine does stabilise the SSII-1 toxin considerably (37). It should be mentioned here that the toxin is destroyed by boiling for 10 minutes and by ultraviolet radiation at 24 ergs/sq.m-sec, for 30 seconds (8).

Experiments have been conducted to find whether the toxin was a lipid, protein or a carbohydrate. The latter can be denatured by periodate oxidation. However, the toxicity was not decreased by such a treatment, indicating that the toxin is probably not a carbohydrate. Furthermore, the toxin was not solubilised in a chloroform-methanol-water solution and Triton X-100, which are lipid solvents. Thus, it is not a lipid either. However, protein denaturing techniques do cause a sharp drop in the toxicity. For example, the toxin was destroyed by alkali (0.1 N NaOH at 22°C for 30 mins). It

is also known that many microbial pathogens have protein components in their spore-coats and have proteinaceous toxins (38). The consensus seems to be that the toxin of Bacillus sphaericus is a protein.

An attempt was made to identify solvents for the toxin (33). The toxin does not dissolve in 8M urea after 30 minutes of contact. However, it was soluble in mercaptoethanol and 3M guanidine hydrochloride, when suspended therein for two hours. A preparation of the gut enzymes of mosquito larvae, suspended in 0.04M sodium carbonate, also dissolved the toxin. Assay of the solubilised toxin indicated a higher LC_{50} (lower toxicity). This would suggest that the cell-wall fraction had lost its toxicity, with only a small amount surviving in the supernatant. It is more likely, however, that the toxin is more effective when not solubilised, and that soluble toxin is not ingested as efficiently.

2.5 SPORE CHARACTERISTICS AND SPORULATION

Bacillus sphaericus endospores are quite similar to those of other spore-formers. One peculiar characteristic seems to be that the cell-wall preparations of sporulating cells and spores lack in dipicolinic acid. This component, together with calcium, is important in giving structural integrity to

the spore. It also appears to be devoid of teichoic acid, which regulates the autolytic machinery of this bacillus. In short, there are less controls on the autolytic system. As if to compensate, the autolysins of this system are also weak. Thus, although mature spores are formed by 8 hours, the mother cell lyses and releases the spores only after 16 hours. The general details of the bacterial endospore have been studied by Tipper and Gauthier (39).

Holt et al. have researched the sporulation cycle of strain 9602, which is a nonpathogenic strain (14). The cycle is fairly typical. Bacillus sphaericus 1593 has a similar cycle. A spore septum is formed initially. This is a sort of internal cross-wall in the cytoplasm that separates the premeval spore, the forespore, from the cell. This septum seems to be similar to the vegetative cell septum that occurs during cell reproduction.

The forespore is enclosed by two membranes, and is contained within the mother cell. A dark staining material soon appears between these membranes, which is suspected to be the peptidoglycan which will form the spore cortex. This then leads to the formation of other spore-coat components. Spores mature by 8 hours after the cessation of exponential growth.

B.sphaericus shows a commitment effect to sporulation two hours after initiation of the cycle. Around this time, the sporangium begins swelling and sporulation is then carried on to completion, regardless of external factors. Antibiotics such as vancomycin, which inhibit sporulation, and addition of fresh nutrients which have a similar effect, are effective only if added before the cells are committed to sporulation. Biochemical events during sporulation have been summarised by Hanson and coworkers(40).

There are differences between spore and vegetative-cell peptidoglycan, viz. the former contain diaminopimelic acid, whereas the latter do not. The enzymes responsible for the synthesis of the spore wall differ from those for the vegetative cell wall (41,42).

Chapter III

EXPERIMENTAL PROCEDURE

The study was conducted using two cultivation techniques: shaken flasks and stirred tank fermentations. Temperature studies were conducted using the shaken flasks, and dissolved oxygen and pH studies were conducted using the fermentor. Nutrient studies were conducted using both the techniques.

3.1 SHAKE FLASK EXPERIMENTS

3.1.1 Microorganism

Bacillus sphaericus 1593, was obtained from Samuel Singer, Western Illinois University. It was maintained on Nutrient Broth- Yeast Extract agar slants, at 4°C in a refrigerator. The strain was transferred once every week.

3.1.2 Media

Bacillus sphaericus 1593 was grown in 8 gm/l of nutrient broth (Difco), supplemented with 0.05% w/v of yeast extract to supply the vitamins. It is known that inorganic salt ions aid sporulation. Therefore, the media was also supplemented with 0.5% v/v of a stock solution consisting of 0.14M calci-

um chloride, 0.2M magnesium chloride and 0.01M of manganese chloride. The media components were dissolved in distilled water prior to steam sterilisation. This was called NYSM (Nutrient broth-Yeast extract-Salts-Medium).

3.1.3 Inoculum

The inoculum was prepared by stirring a one μ l loopful of Bacillus sphaericus from NY agar slants (Nutrient broth, Yeast extract), into sterile test tubes containing 4.5 ml of NY media (same composition as described in the media section, except the salts are excluded). The tubes were allowed to stand at room temperature for 20 h, prior to inoculation. Sterile side-arm shake flasks (500 ml each), containing 50 ml of the NYSM medium, were inoculated at 2% v/v concentration from the tubes.

3.1.4 Experimental Technique

The shake flasks were agitated in Gyrotory water-bath shakers, model G-76 New Brunswick Scientific Company. The shakers were allowed to equilibrate at their pre-set temperatures for several hours prior to commencement of the experiment. The shaker agitation rate was 160 rpm.

3.1.5 Measurements

Absorbance was measured periodically by tilting a portion of the growing broth into the side-arm of the shake flask. This was then examined using a Klett Colorimeter, Model 800-3, Klett Manufacturing Company, set to 660 nm. Klett readings, K , were converted to Absorbance, A , by the relation $A = 2K/1000$. Measurements were taken hourly up to the end of the exponential growth phase.

Spore and viable-cell-counts were done by the pour-plate technique. Different dilutions of the cell-broth were mixed with molten NY agar (23 gm/l Gibco nutrient agar, 0.05% yeast extract), and plated. The agar was allowed to set and the plates were incubated at 30°C for 48 h. The number of cells were determined by using a colony-counter. For the spore-count, whole broth was heat shocked at 80°C for 12 minutes in a mineral oil bath, before making dilutions. Serial dilutions of the heat-shocked broth were then plated to find the spore-count.

Dry cell weights were estimated using standard techniques. The broth was first centrifuged and the cells resuspended in an equal volume of distilled water in order to eliminate dissolved solids in the broth. One ml of this cell suspension was placed in a previously heated and desiccated

weighing boat, and the boat was heated at 110°C to dry the cells. The difference between the initial and the final weights gave the dry cell weight. All weights were taken on an analytical balance.

3.1.6 Chemical Assays

Protein assay was performed by the technique described by Lowry et al. (43). Reagent C was prepared by mixing 50 ml of 5% sodium carbonate and one ml of 0.5% solution of copper sulphate hydrated in 1.0% sodium citrate. To assay the protein, 5 ml of Reagent C was added to one ml of a 1:50 dilution of the sample. After incubating at room temperature for 10 min, 0.5 ml of 1N Folin's Reagent was added. The sample was further incubated at room temperature for 30 minutes. Absorbance was determined at 500 nm in a spectronic 20 photometer (Bausch and Lomb). Standard curves were prepared by using concentrations from 0 to 500 µg/ml of bovine serum albumin. Distilled water subjected to the above procedure was used as a blank.

Carbohydrate was assayed by the phenol-sulphuric acid method of Hanson and Phillips (44). One ml of 5% phenol was added to one ml of a 1:50 dilution of the sample. This was followed with 5 ml of concentrated sulphuric acid, and the

tube was allowed to stand hot. It was then incubated for 20 minutes in a 25-30°C water bath. Absorbance was determined at 490 nm, using distilled water treated as above as blank. Standard curves were prepared with glucose, using concentrations varying from 0 to 100 µg/ml.

Phosphorous was assayed using the technique of Herbert and coworkers (45). A 1:50 dilution of the sample was prepared. To 5 ml of the sample, 0.4 ml of perchloric acid, 0.3 ml of a 0.02% ascorbic acid, and 0.5 ml of 5% ammonium molybdate in the given sequence, mixing well between each addition. Blue color develops within 10 minutes. This was measured at 730 nm using a Hitachi Spectrophotometer. Deionised water treated as above was used as a blank. Standards were prepared with dibasic potassium phosphate. In the above assays, the 1:50 dilution was prepared to ensure that the reading fell within the linear region of the standard curves. Supernatant for the assays was prepared by centrifuging the whole broth at 18000 rpm for 7 minutes, and then removing the cell pellet.

3.1.7 Bioassay

The toxicity was measured in terms of LC_{50} . The test species were second instar larvae of Culex quinquefasciatus

mosquitoes. The bioassay technique has been previously described in the literature(31). One change was that the results were observed after 72 h rather than 96 h.

The broth was first centrifuged at 8000 rpm, for 5 minutes in a Beckman Centrifuge Model J-21C. The supernatant was decanted, and the cells resuspended in deionised water, to an equal volume to the broth. A number of cups were prepared containing 18 ml of distilled water. To these cups, 10 second instar larvae were added. In this process, around 1 ml of water in which the larvae were previously suspended, was also transferred. Three such cups were assigned to each dilution of the cell-suspension. One ml of each dilution of the suspension was pipetted into three cups. The total volume of liquid in each cup was then 20 mls. All the cups were then incubated at 30°C for 72 h. At the end of this period, the number of dead larvae in each cup was estimated. For each dilution, the average number of three cups was used for each dilution. The dilution corresponding to 15 dead larvae (50% of the total of 30 larvae) was used to define the LC_{50} . This was read off the graph of dilution versus number of dead larvae as plotted on a log-probit paper. The dilution was then combined with the dry cell weight measurements to give the LC_{50} in terms of $\mu\text{g/ml}$ of cell suspension.

3.2 FERMENTATION EXPERIMENTS

3.2.1 Fermentor Assembly

A two liter fermentor (Multigen-2, New Brunswick Scientific Company, New Brunswick, N.J.) was used for all the experiments. The temperature was controlled by a built in system within +/- 1°C. The broth was aerated by sparging air or pure oxygen through the fermentor. The gases were supplied from a high pressure tank and passed through a sterile glass wool filter before sparging. A rotameter was included in the lines to measure the flow rate. The off gases were also exhausted through a sterile glass-wool filter.

The dissolved oxygen concentration was measured using a galvanometric probe, connected to a dissolved oxygen controller (New Brunswick Scientific Company, N.J.). This displayed the reading on a meter, and was also connected to a chart recorder (Scientific Products, McGraw Park, Illinois), in order to keep a continuous record. The chart recorder also permitted the estimation of oxygen uptake rates. To calibrate the probe, the media was sparged with air or oxygen, depending on the requirements of the experiment, for 30 minutes. The scale was set to 100 % after the media had been saturated. Then nitrogen was bubbled through the media for 30 minutes to purge the oxygen from solution, and the scale

was set to 0 %. This was checked with the electrical zero setting built into the unit.

The pH was measured using a standard type probe (In-gold, Model 465), connected to a pH controller-meter (New Brunswick Scientific Company, N.J.) or digital meter (Radiometer Copenhagen, PHM 63 Digital), depending upon the experiment. The probe was calibrated using a 6.9 pH buffer.

3.2.2 Media

Media composition was the same as that used for the shake-flask experiments. One liter of this media was placed in the fermentor and autoclaved for 45 minutes. After cooling, the dissolved oxygen and pH probes were introduced in their respective ports, after first wetting them with ethanol to ensure sterility.

3.2.3 Inoculum

A loopful of B.sphaericus 1593 (approximately one μ l) was transferred from NY agar slants to a sterile test-tube containing 4.5 ml of NY media, and was incubated at 30°C for 8 h. Two ml of this were then transferred to 250 ml shake-flasks, containing 48 ml of NY media. These were shaken in a 30°C incubator-shaker for 8 hours. 20 ml of this broth was introduced to the fermentor using a sterile syringe.

3.2.4 Accessory arrangements

Foam was controlled using a silicone antifoam (A.H. Thomas Company, Philadelphia, Pennsylvania), that was introduced from sterile syringes. The pH was controlled by periodic additions (once every twenty minutes) of 10 % sterile sulphuric acid.

Temperature was measured using a standard mercury thermometer immersed in a thermal well filled with water.

3.2.5 Fermentor Operation

The fermentor was operated at 30°C and an agitation rate of 300 rpm. The gas flow rate (air or oxygen) was 1 liter/minute at atmospheric pressure. The chart recorder was operated at a speed of 10 cm/hr.

3.2.6 Measurements and Assays

Except for those mentioned below, all the assays and measurements were carried out using the same techniques as the shake-flask experiments. The absorbance was measured using a Varian Techtron Spectrophotometer Model 635 at 660 nm. Uninoculated broth was used as a blank. The samples were diluted to a level that the optical density fell within the 0 to 0.5 range.

Ammonia concentration was measured using an ammonia probe (Orion, Model 95-10), which was connected to a meter that read the voltage generated (Orion Ionalyser, Model 901). Samples were diluted to 1:100 to ensure that the readings fell within the calibration range. One-half ml of 10 M NaOH was added to 50 ml of this dilution and the sample stirred while the probe remained immersed. The reading was taken when it had stabilised. Calibration was done with ammonium chloride in concentrations of 10^{-3} , 10^{-4} , and 10^{-5} M, subject to the same treatment as before.

Oxygen uptake rates were measured by two different techniques. In the first technique, the oxygen or air supply to the medium was shut off. This caused the dissolved oxygen concentration to decrease, since the cells were still respiring. This instantaneous rate of decrease (the oxygen uptake rate), was recorded on a chart recorder, as the slope after the oxygen/air supply is shut off. The slope (% satn./time), was divided by the optical density at that time, to give a measure of the specific oxygen uptake rate. In the second method, the broth was centrifuged and the cell-pellet washed and resuspended in 0.1 M MOPS buffer containing a different nutrient, either glycerol or glutamate. The oxygen uptake rate of the cells on this nutrient was measured in a respirometer as $\mu\text{l O}_2/\text{h}/\text{mg}$ dry cell weight.

Chapter IV

RESULTS AND DISCUSSION

4.1 SHAKE FLASK STUDIES

4.1.1 Temperature Effects

The preliminary studies are concerned with the effect of temperature on various parameters such as the growth rate, spore-count, toxicity (measured as LC_{50}), and supernatant nutrient concentrations, such as carbohydrate, protein and phosphorous. Figures (1), (2) and (3) show the graphs of absorbance versus time, for various fermentation temperatures.

Bacillus sphaericus ATCC 1593 reproduces by binary fission, as many other bacteria do. Here, each cell divides into two daughter cells, and each of these daughter cells subdivide into two more cells. Thus, the overall bacterial cell count follows the equation,

$$dX/dt = \mu.X \quad \text{or} \quad \ln(x) = \mu.t$$

where μ = Specific growth rate.

Thus, when the substrate concentrations are non-limiting, the bacteria exhibits an exponential growth pattern. This can be observed from figures (1), (2) and (3). The shape of

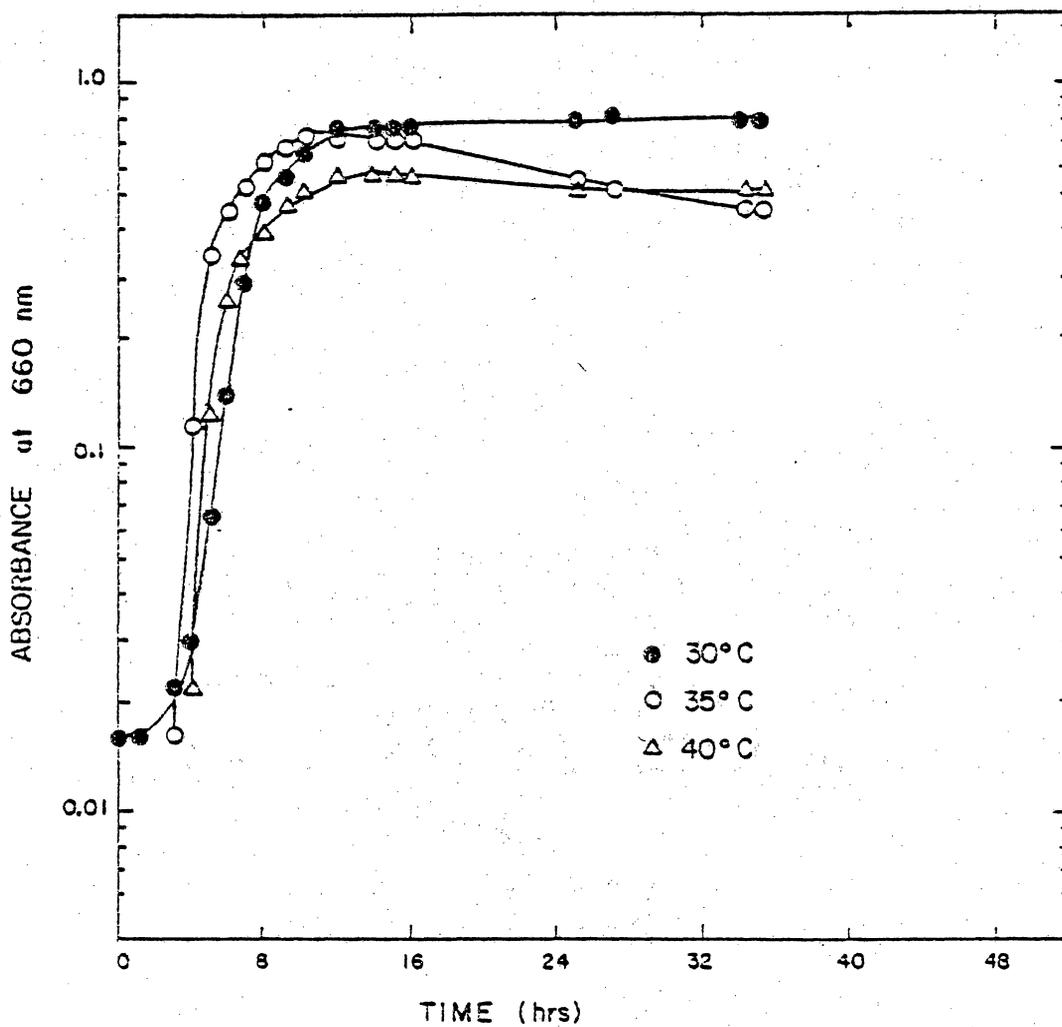


Figure 1: Time temperature dependence of absorbance

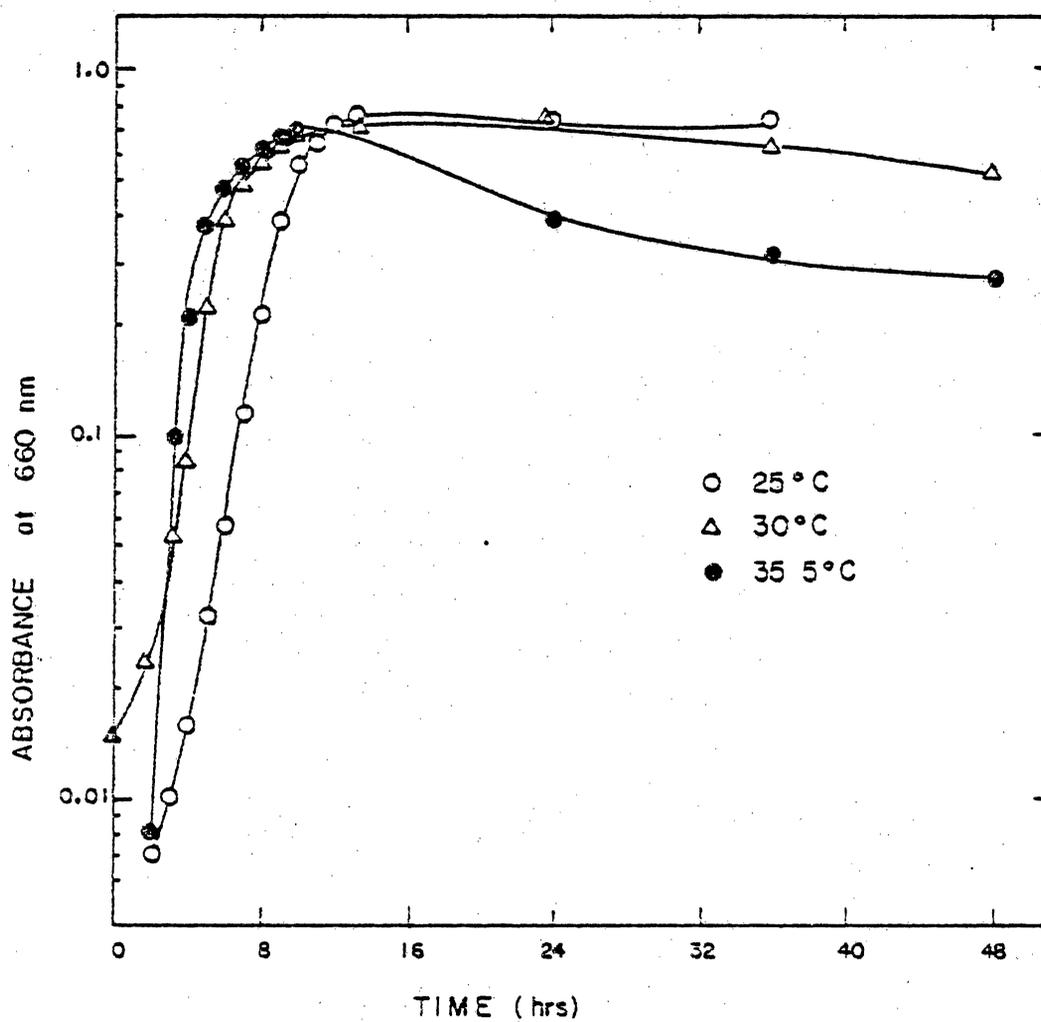


Figure 2: Time temperature dependence of absorbance

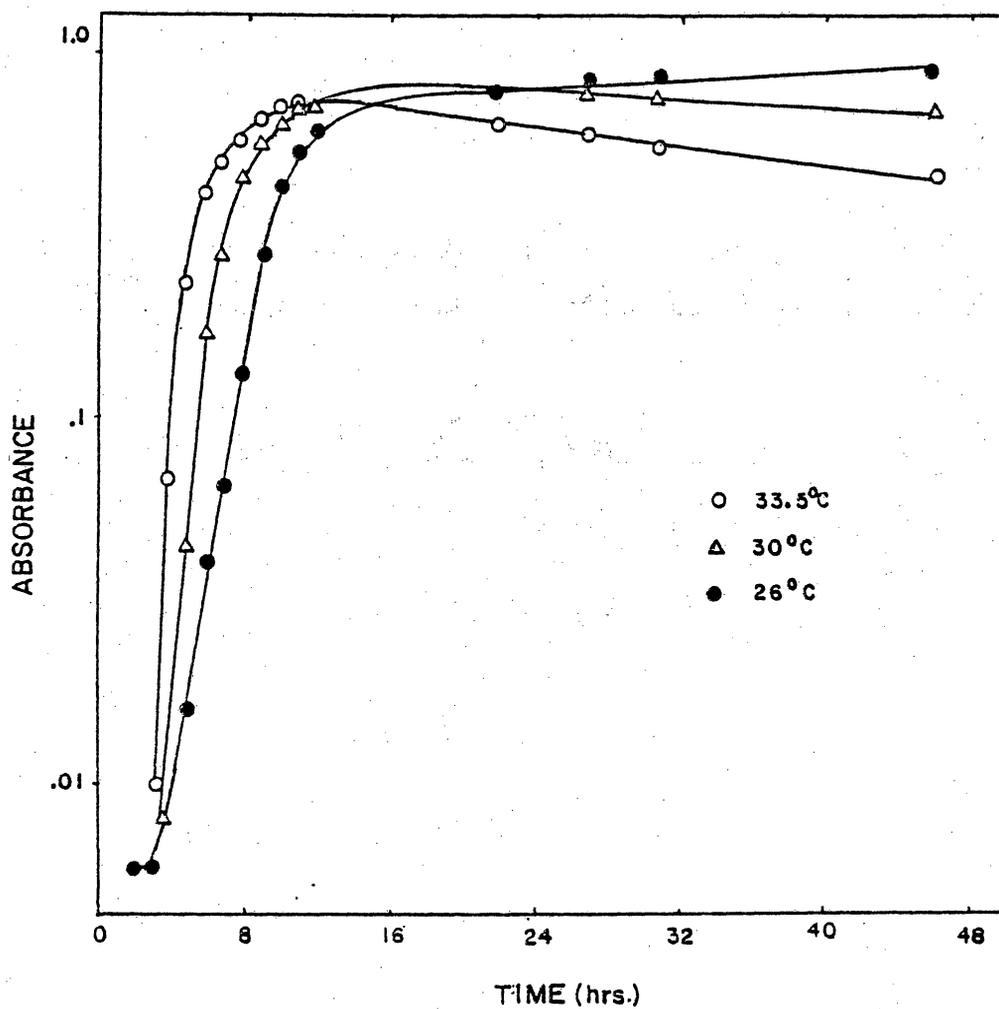


Figure : Time temperature dependence of absorbance

the curves is typical of Monod kinetics. However, it is unwarranted to fit the graphs using the Monod or any other growth model, as the substrate being utilised is a complex one (largely undefined composition), and thus one or more components may be limiting.

At higher temperatures, (ie at 30°C or higher), the optical absorbance curve appears to go through a maxima, decreasing with time in the stationary phase. This could be an indication of cell lysis, for the optical absorbance is directly proportional to the number of cells. However, it should be mentioned that other parameters, such as the cells becoming more refractile as they sporulate, also affects the optical density of the broth. Figure (3) illustrates the effect of higher lysis at higher temperatures. Figure (1) appears to suggest more lysis at 35°C as compared to 40°C. This could be ascribed to a variety of factors such as those mentioned above, as well as to experimental aberration. There exist other indications pointing to cell lysis, such as a minima in the supernatant nutrient concentrations curve, and the fact that some cell debris is visible when the broth is examined under a phase contrast microscope. There also seems to be an inverse relationship between cell lysis and sporulation. These aspects will be examined in more detail below.

The slopes of the growth curves (Figures (1), (2), and (3)) in their exponential phase should be mentioned. It appears that the slopes are smaller at lower temperatures and larger at higher temperatures. Since these results are plotted on the log-axis of a semi-log plot, the slope is a direct measure of the specific growth rate, μ , and one may conclude that the growth rate is higher at higher temperatures. However, this effect is not very significant and the growth rate (absolute value) is very rapid at all temperatures tested.

It is interesting to note that the lag phase is more prolonged at lower temperatures than higher temperatures. For example, in one experiment, the lag phase was nearly 4.5 hours at 26°C, but was only 1.5 hours at 33.5°C (this data is not indicated in the thesis). The enzyme systems of metabolism are activated more rapidly at higher temperatures, as a result of which the growth commences earlier.

Maximum absorbance, reached approximately at the onset of stationary phase, is an indication of the total cell mass produced. By comparing these maximum absorbances, it can be noticed that slightly more total cell mass is produced at lower temperatures. It is possible that at high temperatures, maintenance requirements are higher, and thus less

substrate is apportioned towards growth. However, it is more likely that the cells lyse in greater numbers at higher temperatures, even during exponential growth. This is born out by the fact that autolytic enzymes are more active at higher temperatures.

Obviously, a desirable temperature would yield the greatest cell mass in the shortest time, ie a higher temperature. However, this will increase the rate of lysis and since these are conflicting requirements, the optimum temperature has to be balanced between the two factors. In addition, engineering parameters for this fermentation (eg. cooling the fermentor) also need to be considered before making the final choice of the optimum temperature.

Figures (4) and (5) show toxicity (measured as LC_{50}) and spore count (as heat stable spores) versus fermentation time at two temperatures. Table (1) shows the data from another experiment, for three temperatures. Note that decreased LC_{50} corresponds to increased toxicity. The graphs of LC_{50} and spore count approximately mirror each other, confirming that the cell mass becomes more toxic as the spore count increases. However, this mirror effect is not exact and close observation of the data reveals that the spore count lags toxicity. In other words, toxicity seems to develop before the

spore count has significantly increased. Since the spore count is measured as heat stable spores, it is apparent that the formation of the toxin is associated with an earlier stage of the sporulation cycle. Samples of the growing bacteria were examined under a phase contrast microscope, and it appears that the toxin begins forming approximately at the point when the cells begin to swell as a prelude to sporulation. Other reports have confirmed that different spore-wall components are synthesised at different stages of the sporulation cycle (41,42).

Examining the data given in table (1) reveals that the toxicity of the broth appears to reach a maxima for 25°C and 30°C temperatures, after which it decreases (LC_{50} increases). This suggests that there may be some degradation of the toxin, if the fermentation is continued for too long. Since there are fluctuations in the bioassay, it is difficult to estimate an optimum harvest time based on the criterion of the most toxic product. However, for a temperature of 30°C, 24 hours appears to be a suitable period. Beyond this, the larvicidal activity begins to decrease.

The most pertinent observation is that the product is less toxic when produced at higher fermentation temperatures. The LC_{50} value, as well as the total cell mass are

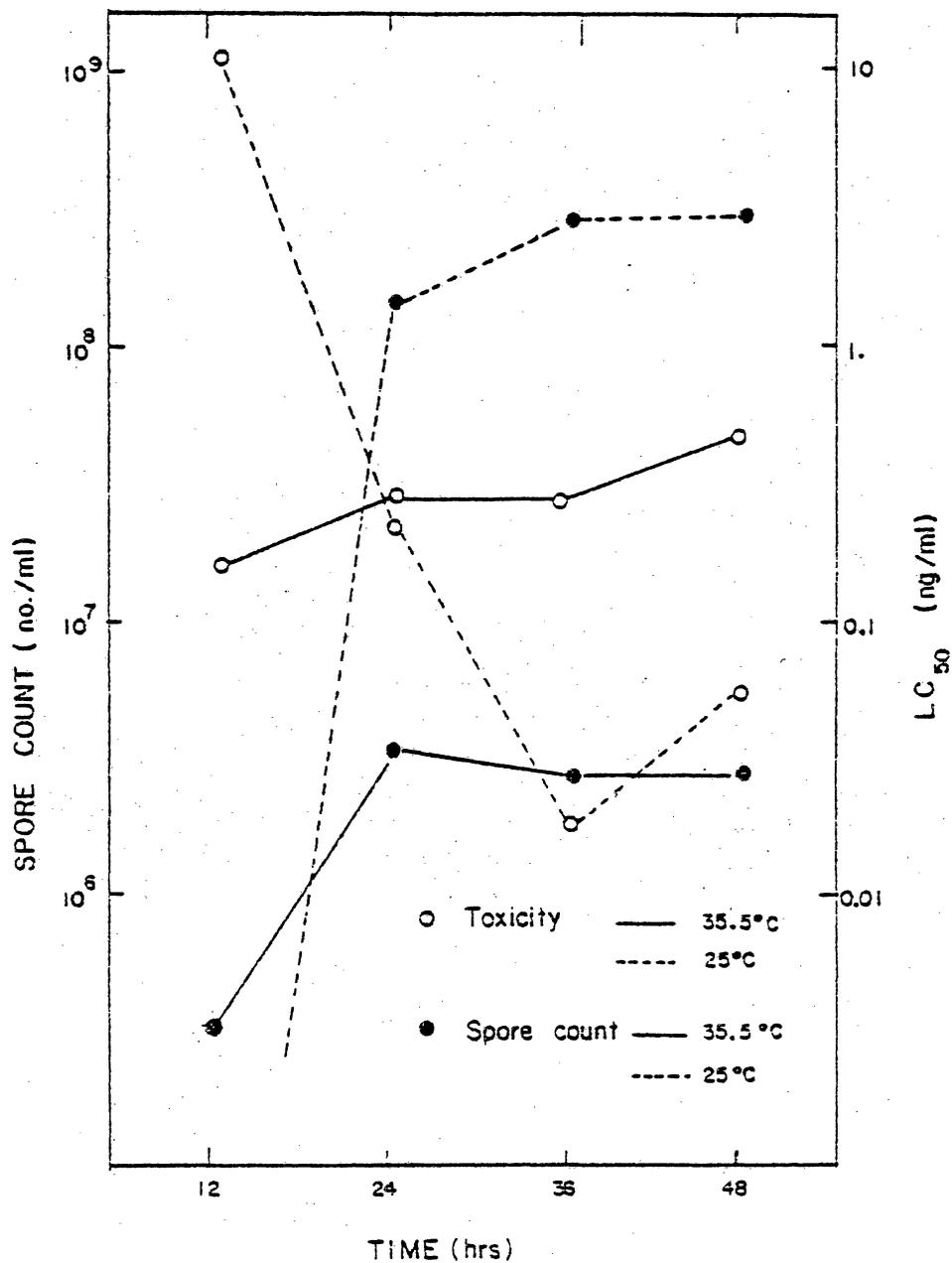


Figure 4: Time temperature dependence of toxicity and spore-count

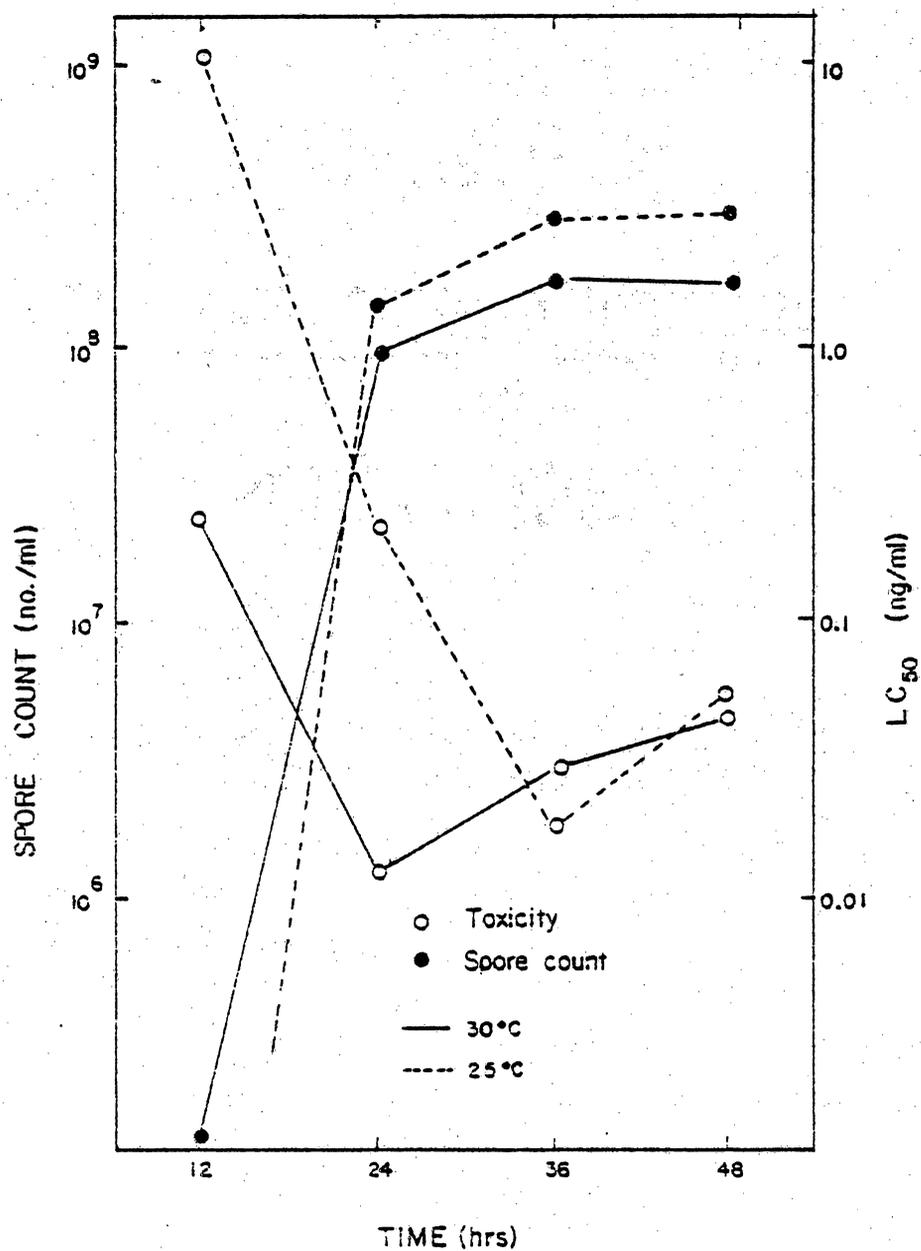


Figure 5: Time temperature dependence of toxicity and spore-count

lower under these conditions. Experiments were also conducted at 40°C, and the results showed that the LC₅₀ was always greater than 200 ng/ml, which is a negligible toxicity when compared to the values for the lower temperature cultures. While the products formed at 26°C and 30°C have approximately equal toxicity, all higher temperatures yielded a distinctly lower toxicity.

It can also be noted that while the total spore count is lower at higher temperatures, the process of sporulation begins earlier. This is probably due to the fact that at higher temperatures growth rate is more rapid, leading to an earlier exhaustion of the nutrient, and an earlier onset of sporulation cycle.

From the standpoint of obtaining a high cell mass with high toxicity in a minimum time, a temperature range of 25-30°C is suitable. However, since heat is given off during the fermentation, it would be desirable to choose 30°C so as to minimise the cooling requirements.

It is recognised that once the cell population has entered stationary phase; the cells may either lyse or sporulate. Cell-lysis appears to be more pronounced at higher

TABLE 1

Variation of Spore-Count and Toxicity with
Time for Three Different Temperatures

SPORE COUNT (cells/ml)

Time(hrs)	25°C	30°C	35°C
12	2.95×10^3	1.36×10^5	3.25×10^5
24	1.41×10^8	9.75×10^7	3.31×10^6
36	2.95×10^8	1.72×10^8	2.70×10^6
48	3.05×10^8	1.63×10^8	2.80×10^6

TOXICITY (LC₅₀, µg/ml)

Time(hrs)	25°C	30°C	35°C
12	8.80×10^{-2}	1.30×10^{-3}	1.50×10^{-3}
24	3.00×10^{-3}	1.70×10^{-3}	1.60×10^{-3}
36	2.67×10^{-4}	5.80×10^{-4}	2.50×10^{-3}
48	8.14×10^{-4}	5.54×10^{-4}	4.40×10^{-3}

temperatures, while the cells sporulate in greater numbers at lower temperatures. This would suggest that the temperature optima of the autolytic enzyme system appears to be higher than that of the enzymes required for the transformation of the cells into spores.

4.1.2 Limiting Nutrient Studies

The NYSM media was used for these studies. This is a complex media composed of nutrient broth, yeast extract and mineral salts. Due to its complex nature, it has been difficult to pinpoint one or more precise limiting substrate(s), that could be used to fit the fermentation data to a mathematical model. However, it is reasonable to assume that the cells enter stationary phase upon the exhaustion of some component of the media. Working on this premise, the supernatant of the broth was assayed for protein, carbohydrate and phosphorous. The results of these assays are given in figures (6), (7), and (8), for three growth temperatures. The three nutrients for a single fermentation at 30°C are also plotted on figure (9), where the time axis extends only upto 30 hours (instead of 48 hours of the previous figures) in order to better compare the shapes.

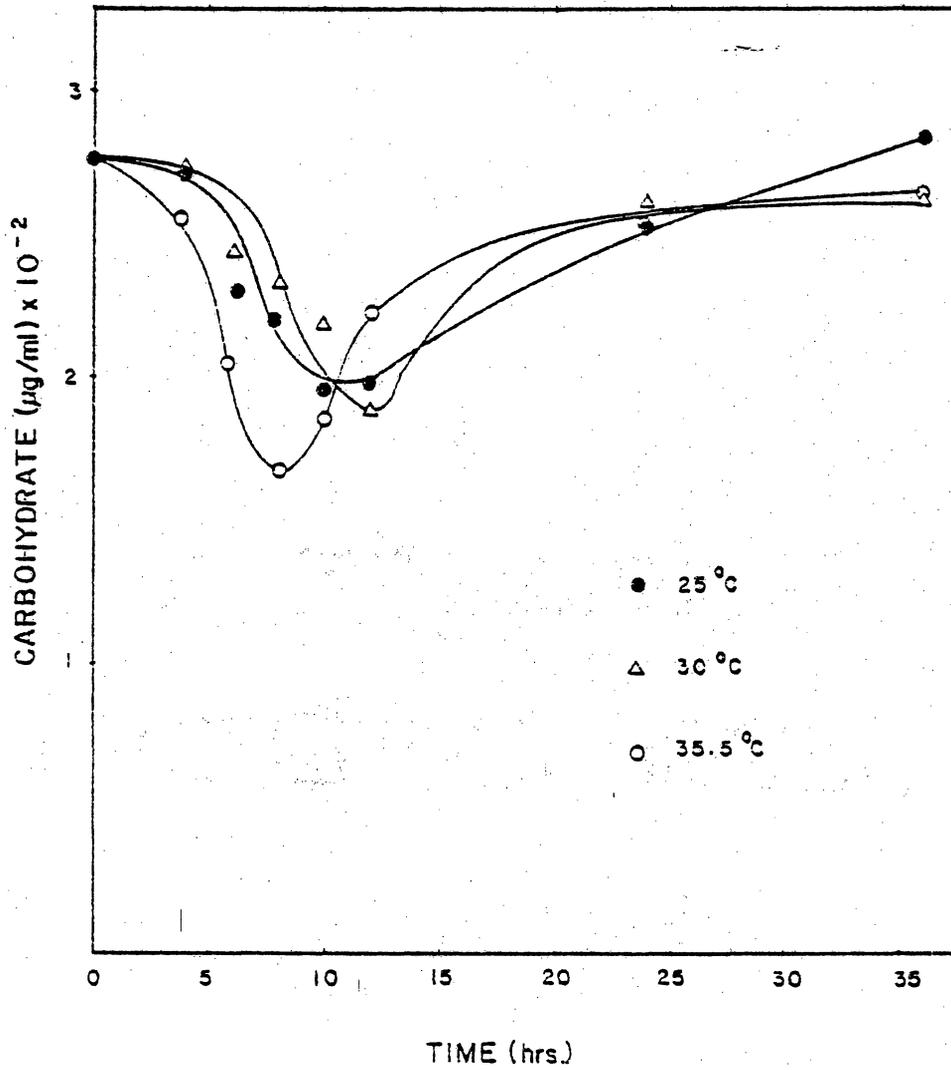


Figure 6: Time temperature dependence of supernatant carbohydrate

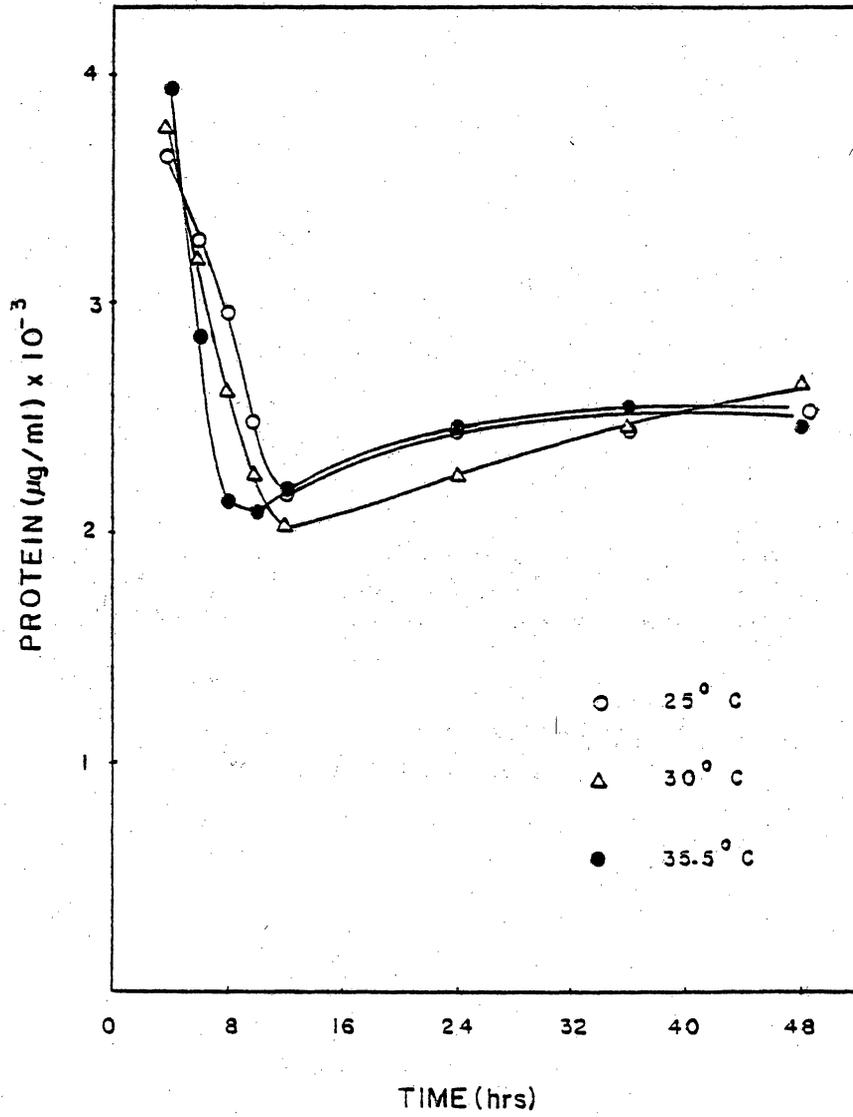


Figure 7: Time temperature dependence of supernatant protein

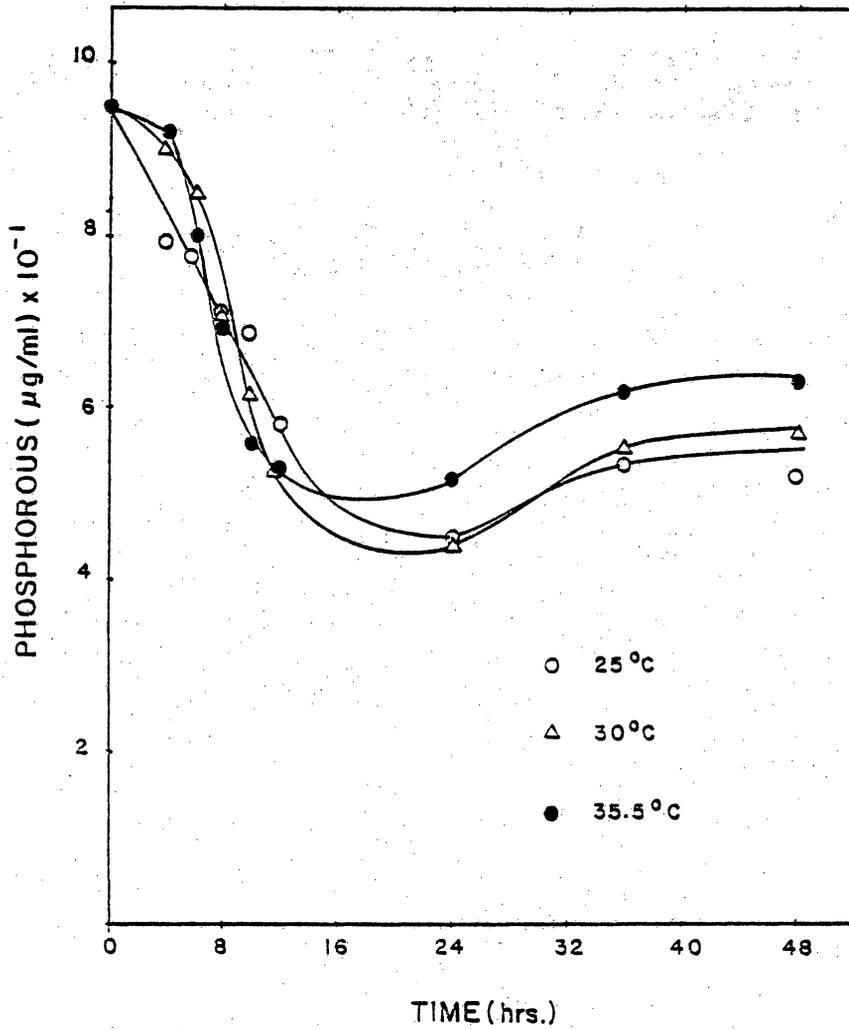


Figure 8: Time temperature dependence of supernatant phosphorus

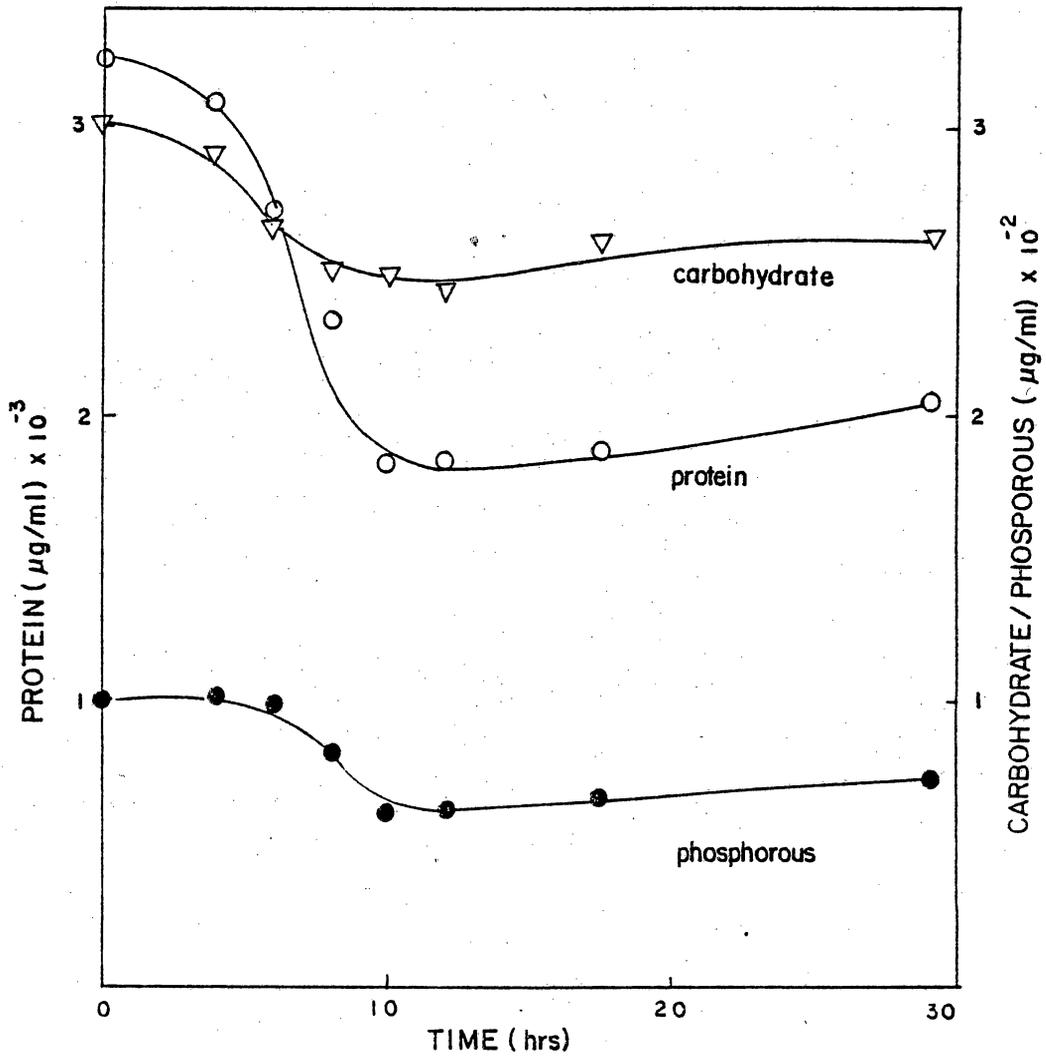


Figure 9: Time temperature dependence of supernatant nutrients. Temperature = 30°C

Each of the nutrient concentrations pass through a minima. The substrate is obviously being consumed in the growth phase, accounting for the decreasing nutrient concentrations. Later, during the stationary phase, cells lyse and release their protoplasmic contents into the medium, and these contain assayable protein, carbohydrate and phosphorous. At the same time, there is little or no utilisation of substrate, causing the net nutrient concentration in the supernatant to rise. As may be expected, the minima coincides with the end of the growth phase in most cases. Furthermore, if the nutrient concentration profiles from the same fermentation are compared (figure (9)), it can be noticed that they all go through a minima at about the same time, and have similar shapes. This would suggest that these nutrients are required and assimilated by the cell in a constant proportion.

It appears that the slope of the nutrient profiles in the region where nutrients are being consumed is steeper at higher temperatures, suggesting that at higher temperatures the nutrients are being consumed more rapidly. This is compatible with the earlier observation of a higher specific growth rate at higher temperatures. Furthermore the higher

temperature curves seem to reach their minima earlier, a logical deduction from the abovementioned idea, since faster growth would imply faster utilisation and exhaustion of the limiting substrate.

It should be noted that while the carbohydrate concentration does decrease initially, it returns almost to its original level towards the end of the fermentation. Thus, while there is a temporary decrease in the concentration of carbohydrate in the supernatant, there is no net decrease. Other nutrients do not exhibit this behaviour. Apparently, protein and phosphorous have been at least partially assimilated, but carbohydrate has been released back into the medium. The fact that the concentration of carbohydrate does decrease initially would suggest that there is some utilisation of this nutrient. However, this is a contradiction to previous reports that Bacillus sphaericus does not utilise any carbohydrate, including the simplest of sugars such as glucose (16). A possible explanation for this phenomenon is that the cells take up carbohydrate, perhaps because it occurs in a form associated with other nutrients (viz. proteins), but do not utilise it in metabolism. For example, carbohydrate may be adsorbed onto the surface of vegetative cells. As the cells sporulate, the surface properties of the cells, such as surface tension also change, and the carboh-

hydrate may then be desorbed from the surface and released into the medium. The cells may also be taking up carbohydrate, and converting it to another chemical form, such as a polymer, and excreting it as such.

The most striking observation about this data is that at the onset of stationary phase (the minima of nutrient concentrations) approximately 50 % of the protein, 75 % of the carbohydrate and 60 % of the phosphorous is left in the broth. Considerable amount of nutrients being still available, it could be expected that the cells would continue growing in the exponential phase. Yet the cells enter stationary phase, eventually lysing or sporulating, a phenomenon that occurs only when some nutrient is exhausted or a toxic metabolite accumulates. The answer to this disparity lies in the assays conducted and a lack of knowledge of the precise limiting nutrient. For example, a particular amino acid or peptide could be exhausted and thus be limiting. However, the Lowry method would assay the total protein and not the concentration of that particular amino acid or peptide. It is highly probable that some amino acid is the precise limiting substrate. It should be mentioned that no evidence exists to support the idea of the accumulation of any growth suppressing metabolites.

4.2 STIRRED TANK FERMENTOR STUDIES

4.2.1 pH and Ammonia profiles

The supernatant ammonia concentration was measured for a fermentation at 30°C. This data has been plotted versus time, with the corresponding pH and optical absorbance values on figure (10). The two profiles are similar, in the sense that the values of ammonia concentration and pH show similar trends of increase. It can be concluded that the ammonia concentration rises, most probably from deamination of proteins during metabolism, and the abovementioned correlation suggests that this is responsible for the increase in pH. However, at a pH of 8.0, ammonia tends to vaporise from the broth, particularly since the broth is being sparged with a gas. As a consequence, the concentration of ammonia in the broth does not increase as rapidly after this value of pH, but the pH itself continues to increase. This is probably due to increasing utilisation of acids in the broth during the stationary phase, a fairly common phenomenon.

The pH results for three different temperatures are plotted on figure(11). The graphs indicate that pH increases more rapidly at higher temperatures than lower temperatures. The 25°C pH curve also shows a sort of lag phase. Faster utilisation of substrate (deamination of proteins), as a

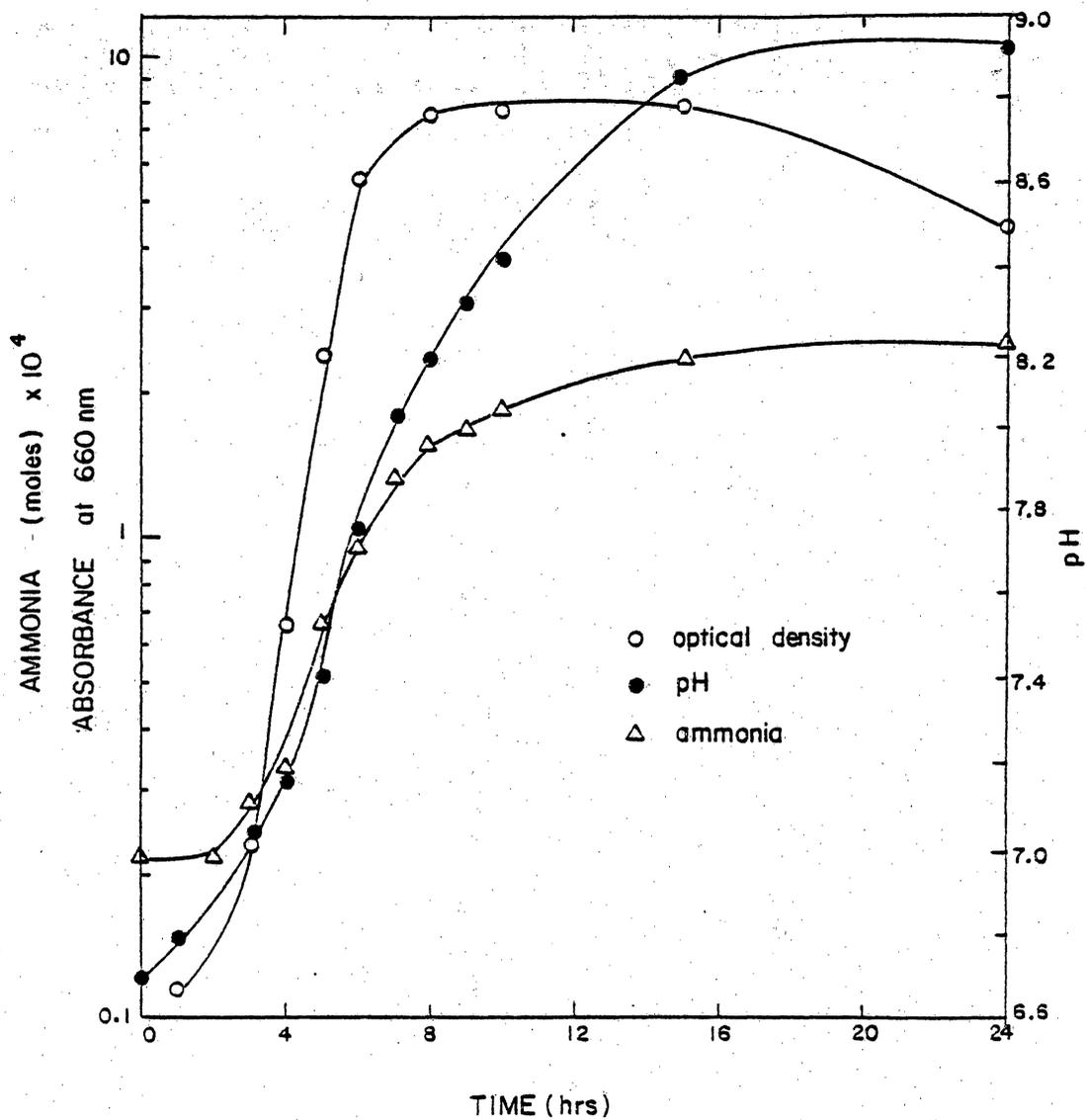


Figure 10: Comparison of growth, pH and ammonia curves. Fermentation with air. Temperature = 30°C.

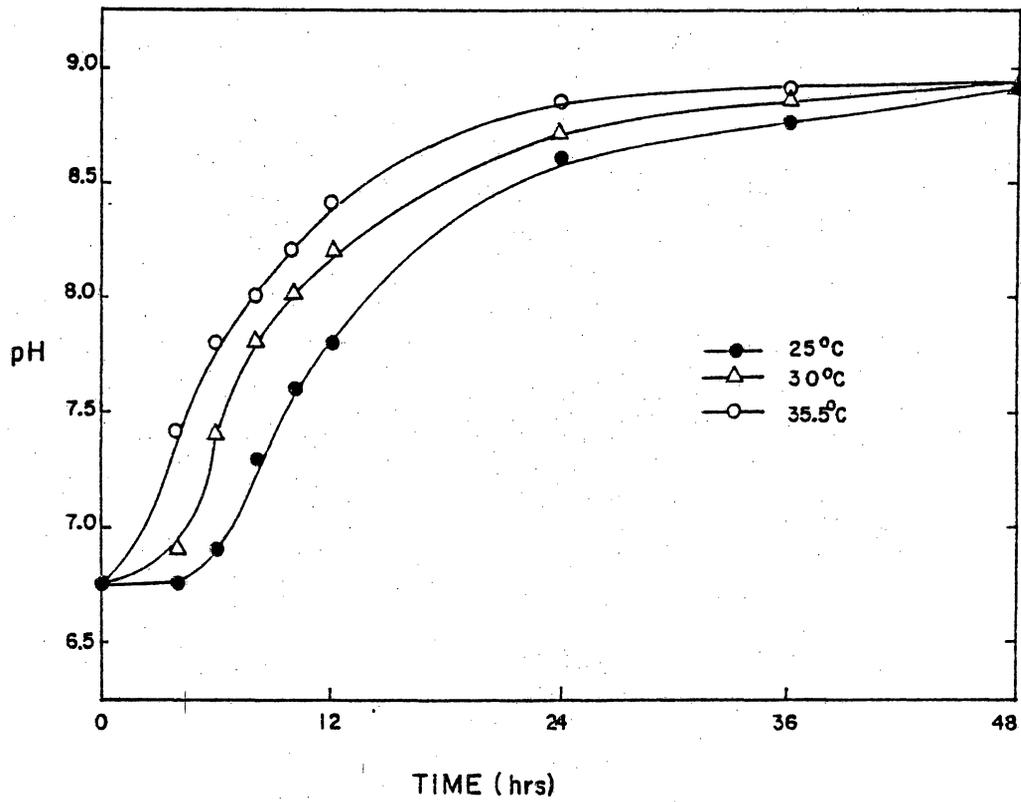


Figure 11: Time temperature dependence of pH

consequence of rapid growth at higher temperatures, would indeed lead to sharper increases in the pH. After the fermentation has progressed to a considerable extent, the pH for the three temperatures tends to converge to a single point. This probably means that the substrate is utilised equally after the cessation of metabolic activity. Yet the maximum absorbance and the final cell mass are different. This could be ascribed to lysis instead of substrate utilisation.

4.2.2 Effect of Dissolved Oxygen Concentration

Studies for the effect of dissolved oxygen were combined with some other parameters such as the pH. Figures (12) and (13) show the results for fermentations sparged with air, and figures (14) and (15) illustrate the results using pure oxygen. In each of these cases, pH was not controlled. The abscissa is the percent of oxygen saturation with that gas used for the experiment.

The dissolved oxygen curves may be divided into three sections, increasing, constant and decreasing concentrations.

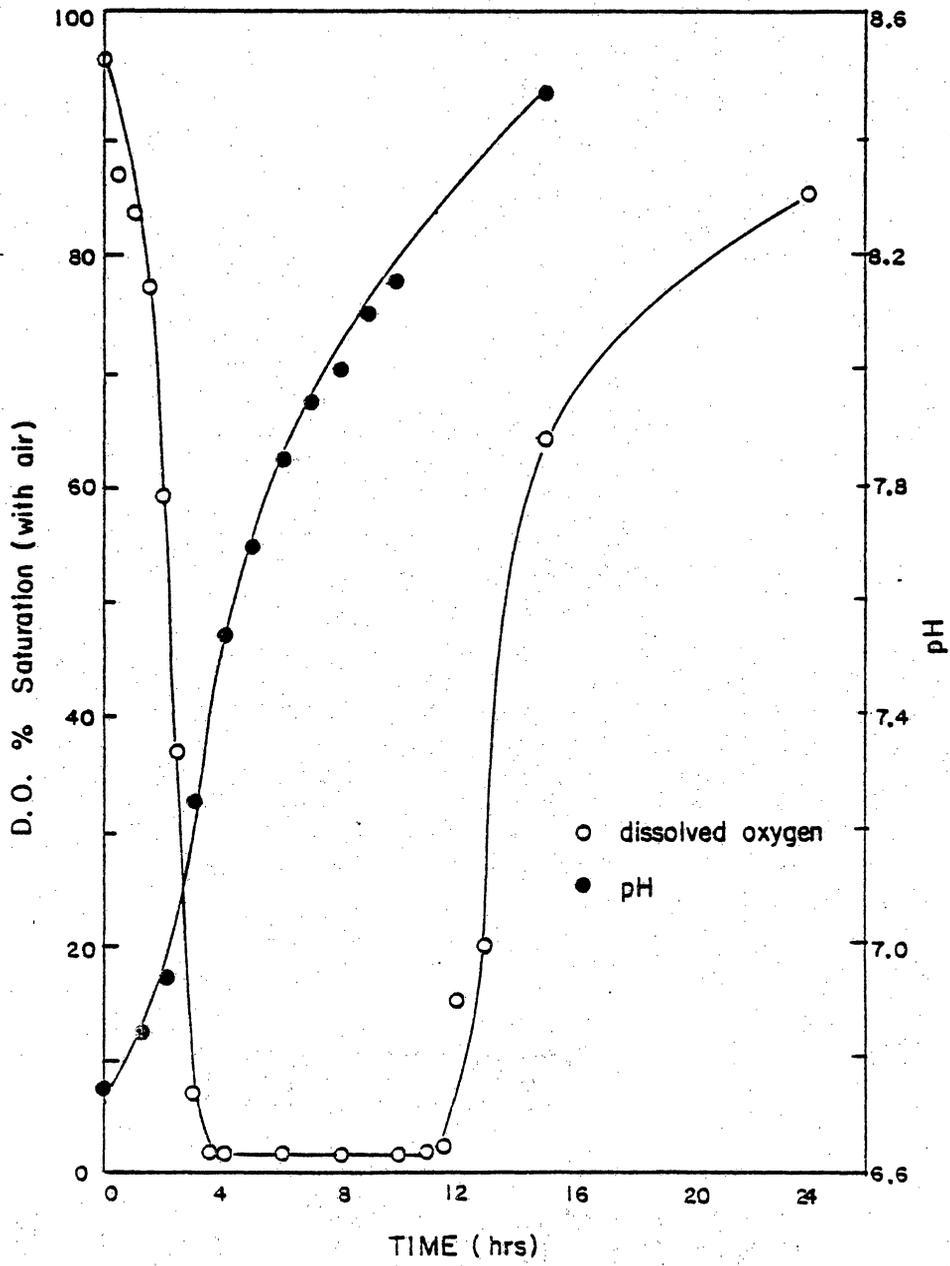


Figure 12: Time dependence of pH and dissolved oxygen. Fermentation with air. Temperature = 30°C.

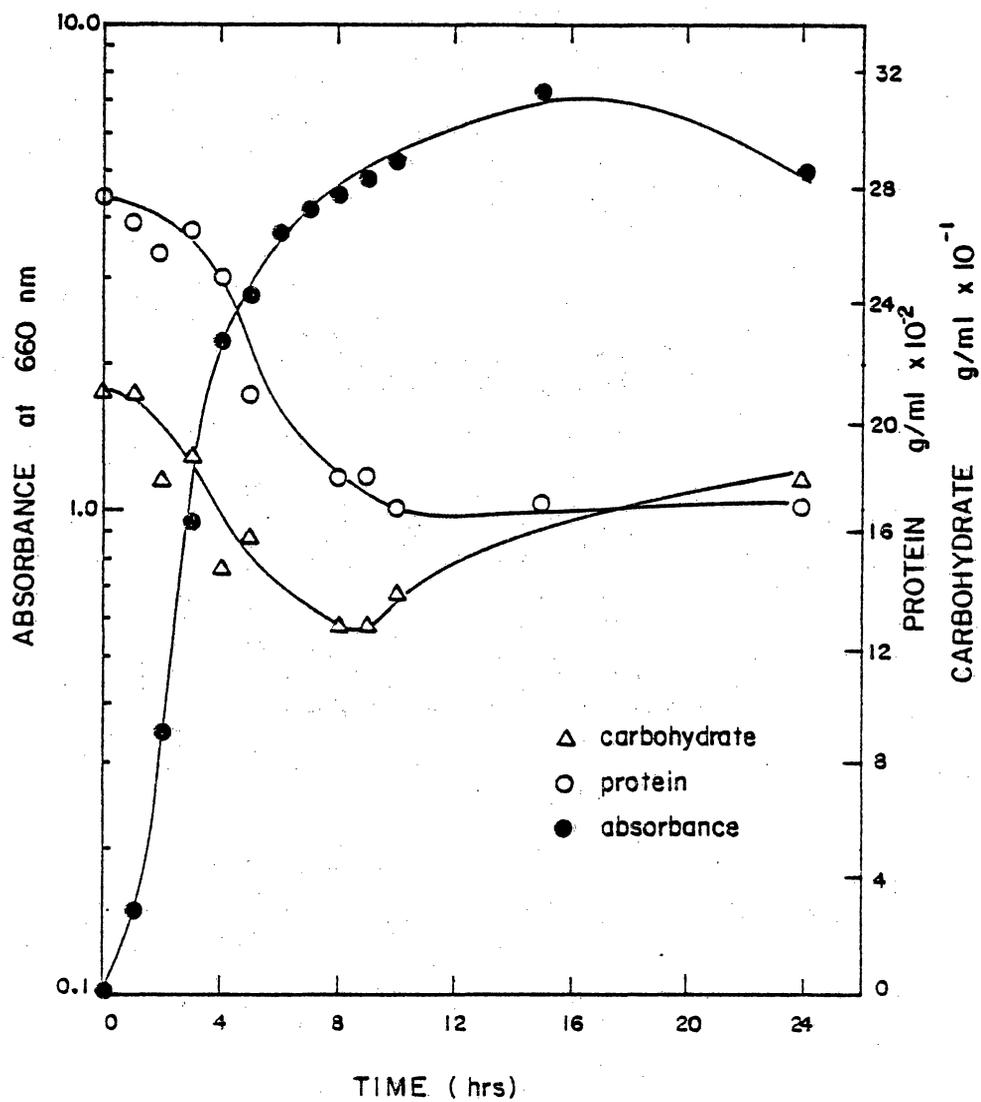


Figure 13: Time dependence of supernatant nutrients.
 Fermentation with air. Temperature = 30°C.

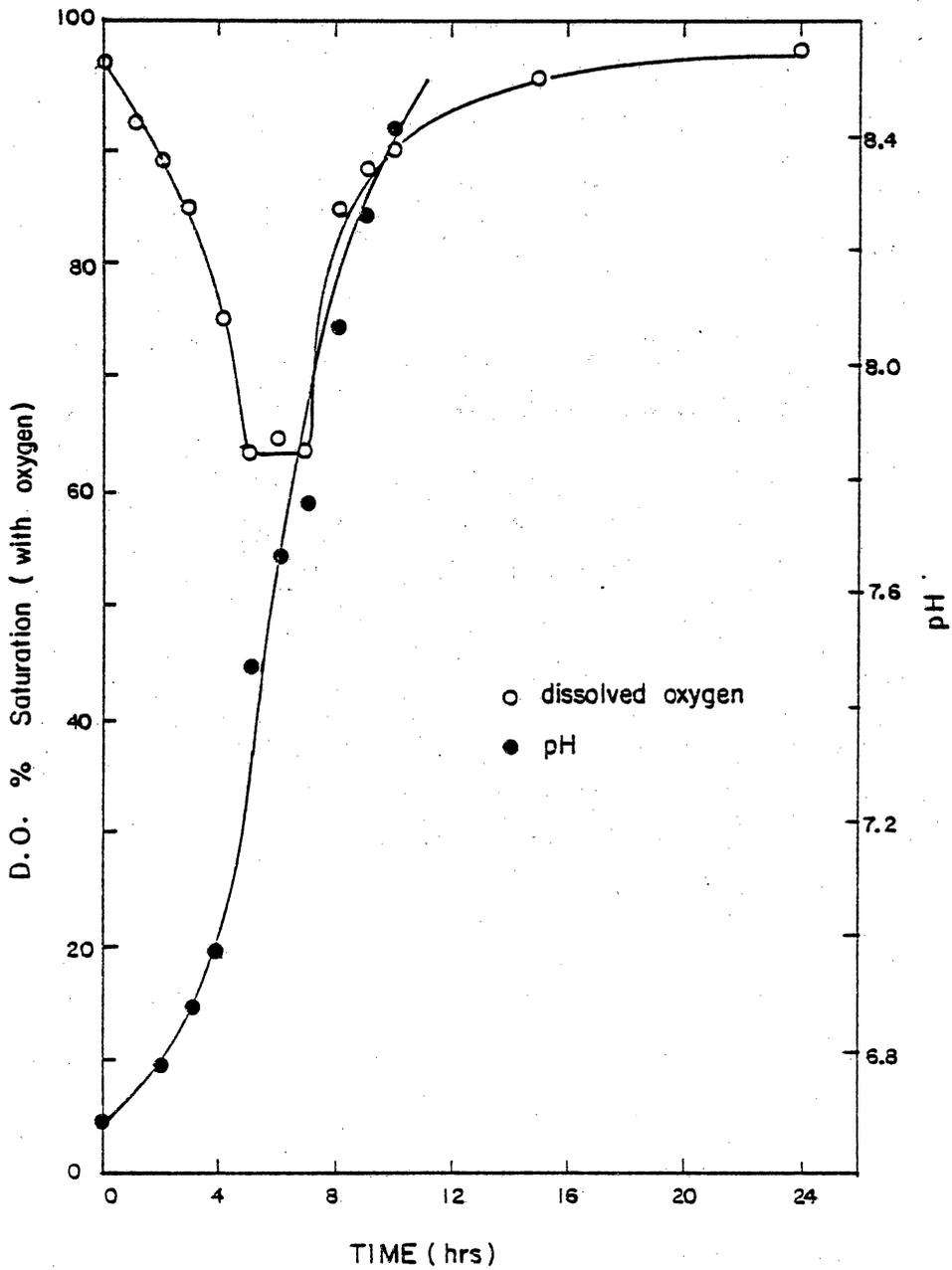


Figure 14: Time dependence of pH and dissolved oxygen concentration. Fermentation with oxygen. Temperature = 30°C.

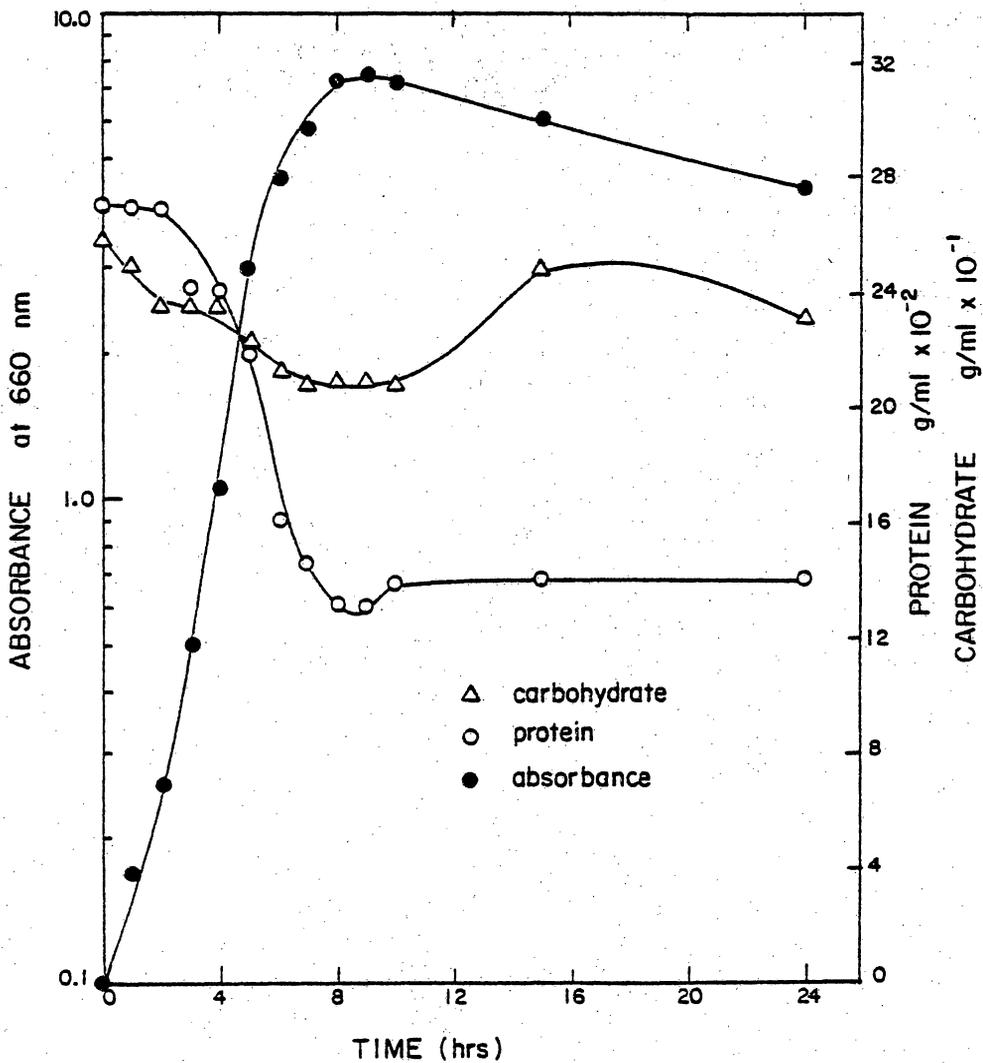


Figure 15: Time dependence of supernatant nutrients. Fermentation with pure oxygen. Temperature = 30°C.

It is apparant that the dissolved oxygen concentration in the medium represents a balance between the rate of dissolution of oxygen from the gas bubble (air or pure oxygen) to the medium (referred to as the 'supply rate') and the uptake of the oxygen so dissolved from the medium by the microorganisms (referred to as the 'uptake rate'). Furthermore, the supply rate is proportional to the difference in concentrations of oxygen in the gas phase (bubble) and the liquid phase (medium). Thus, if the dissolved oxygen concentration is higher or the oxygen concentration in the gas phase is lower (air sparging) ,the supply rates would be lower.

In the first section of the graphs, the slope of the dissolved oxygen curve is negative, implying that the uptake rate exceeds the supply rate. This is plausible, since the cells are young and physiologically very active (vegetative phase). It will also be shown later that the specific oxygen uptake rates are also high in this region. Furthermore, the cell mass is increasing exponentially, and the overall uptake rates also increase. The slope of the dissolved oxygen curve in this region is increasingly negative.

In the second section, slope of the curve is zero, suggesting that the supply rate is just balanced by the uptake rate here. In case of air sparged fermentations the dissolved oxygen concentration (balance point), is nearly zero; however, this concentration is nearly 60% of saturation in the case of pure oxygen sparged fermentations. Examining the growth curve reveals that the cell mass is still increasing just before the second stage commences, and the cells are still physiologically active. Thus, the actual uptake rates would still be high in this section. Particularly in the case of air-sparged fermentations, the cell mass is deficient in its oxygen requirements. However, in oxygen sparged experiments, oxygen appears to be available at all times. It will be discussed later that this difference in the two experiments is probably responsible for the higher spore-counts of air fermentations.

In both the fermentations, dissolved oxygen concentration begins to increase almost coincidentally with the cessation of growth. This occurs at an optical density value of 6.5. The oxygen supply rate exceeds the uptake rate at this point. After the fermentation has progressed into the stationary phase, metabolic activity drops to a very small value as the cells lyse or sporulate. As a consequence, the uptake rates also decrease and the dissolved oxygen concen-

tration begins to increase, reaching a saturation level, approximately the same level as it was at originally. In this third section, the slope is initially a large positive value, eventually decreasing to zero.. The reason for this is that as the dissolved oxygen concentration begins to increase, the supply rates decrease. This fact has been mentioned earlier in this section.

The toxicity and spore-count results for oxygen and air fermentations are shown on tables (2) and (3). The spore count for fermentation with air is almost two orders of magnitude higher than for fermentation with oxygen. It was discussed before that oxygen deficiency led to reduced growth rates in the case of air sparged fermentations as compared to those oxygen sparged. It is possible that oxygen deficiency has triggered a metabolic shift. For example, the cells may rely on stored nutrients like poly-hydroxy-butyrate granules to meet the energy requirements of metabolism, instead of the regular respiratory pathways, which are now oxygen limited. Such shifts often lead to a pseudostationary phase. This could have caused an earlier and longer sporulation phase. However, there is little difference in the toxicity of the two final products. It was shown that toxicity develops early on during sporulation, while the

TABLE 2

Spore-Count and Toxicity Data. Fermentation
with sparged air. No pH control.

Time(hrs)	LC ₅₀ ($\mu\text{g/ml}$)	Spore-Count(cells/ml)
24	2.61×10^{-4}	1.5×10^8
30	2.58×10^{-4}	1.2×10^8

TABLE 3

Spore-Count and Toxicity Data. Fermentation
with sparged oxygen. No pH control

Time(hrs)	LC ₅₀ ($\mu\text{g/ml}$)	Spore-Count(cells/ml)
24	4.98×10^{-4}	3.8×10^6
30	3.84×10^{-4}	7.3×10^6

heat-stable factor develops later on. This suggests that sporulation in oxygen sparged experiments is incomplete, for the toxin has formed without the heat stable factor, most of the sporulating cells have already synthesised the toxin. Examination of the broth under a microscope also revealed many swollen cells, but few completed spores.

In general, most sporulation processes are triggered by nutrient limitation. This is undoubtedly true in the case of B.sphaericus as well. However, as mentioned above, dissolved oxygen concentration does seem to have some indirect effect.

4.2.3 Oxygen Uptake Rates

Studies were also conducted measuring the oxygen uptake rate. The results are given in table (4). It is apparent from the results that the oxygen uptake rates at 2 h, 15min and 5 h are nearly the same, within limits of experimental error. However, the values at 10 h are distinctly lower. This is because younger cells are still in the exponential phase of growth and in a vegetative state, whereas the older 10 h cells have passed into the stationary phase. The energy requirements, and thus respiration, and oxygen uptake rates would be lower during in the stationary phase.

TABLE 4

Oxygen Uptake Rates. Fermentation with
pure oxygen. No pH control.

Time(hrs)	2 h 15 min	5 h	10 h
Graph [*]	43.65	6.20	1.46
Glutamate ^{**}	72.22	113.47	19.86
Glycerol ^{**}	30.76	33.27	7.45

Legend:

'*' : Units: % saturation / time / unit optical
absorbance (660 nm)

'**': Conducted in 0.1 M MOPS buffer + indicated
substrate + washed and resuspended cells.
Units: $\mu\text{l O}_2$ / h / mg dry cell weight.

4.2.4 Limiting Nutrient Studies

The nutrient concentration profiles exhibit patterns very similar to those discussed earlier for shake flask experiments. Much of the carbohydrate is recovered in the supernatant, again implying the small or negligible role of carbohydrate in metabolism. Protein, on the other hand, is utilised. Considerable amounts of nutrients are still left in the broth at the onset of sporulation. It appears again that more protein is utilised in the oxygen sparged fermentations, as compared to other nutrients. The maximum optical absorbance for the oxygen experiments is also higher, implying greater total cell mass. As compared to the oxygen experiments, the cells entered the stationary phase earlier in the air experiments, perhaps due to oxygen limitation.

4.2.5 pH control

Figures (16), (17), (18), and (19) illustrate the air and oxygen studies with the pH controlled at 7.0. The same analysis as for no pH control can be applied here.

The toxicity and spore-count for the experiments with pH control are shown on tables (5) and (6). Controlling the pH does not affect the spore count to any great extent. Yet the toxicity, particularly at 24 hours, is higher with pH con-

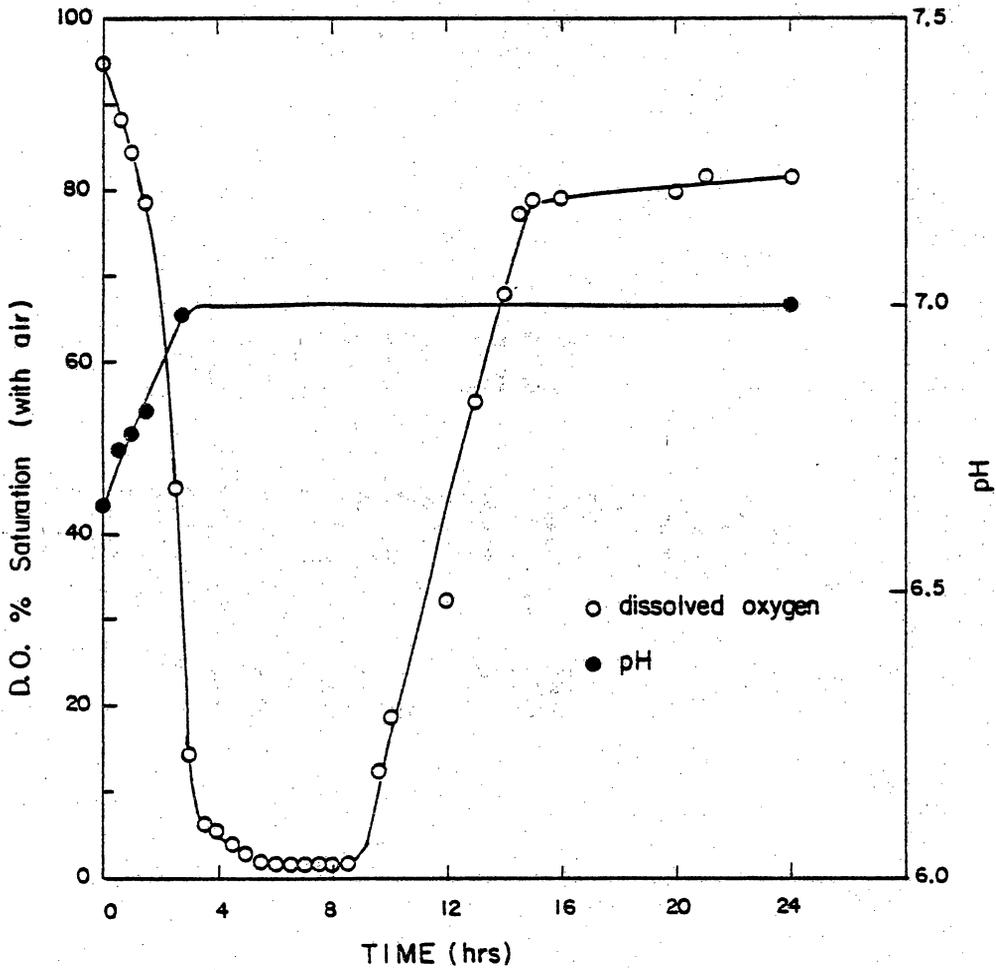


Figure 16: Time dependence of pH and dissolved oxygen concentration. Fermentation with sparged air. pH controlled at 7.0, Temperature = 30°C.

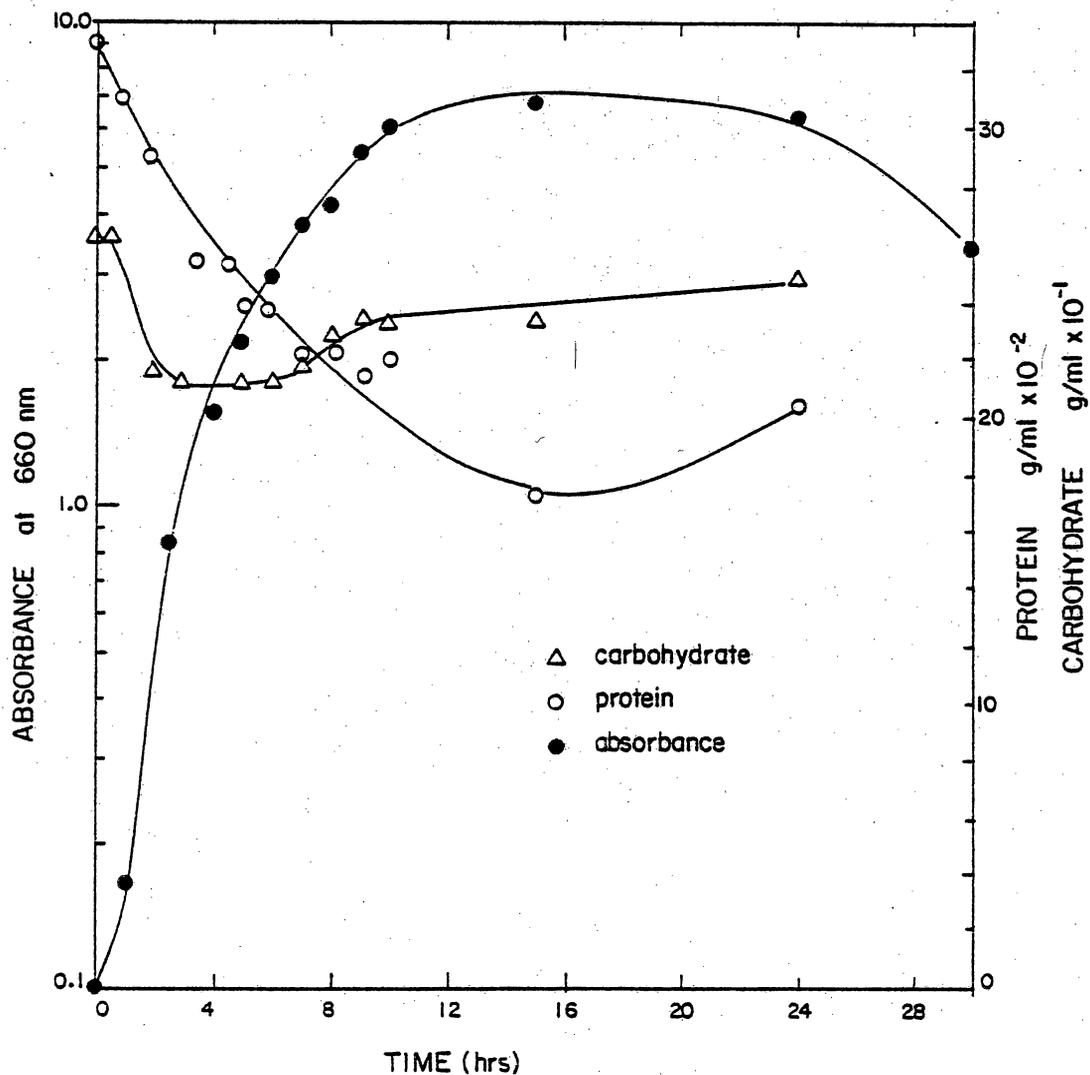


Figure 17: Time dependence of supernatant nutrients. Fermentation with air. pH controlled at 7.0. Temp. = 30°C.

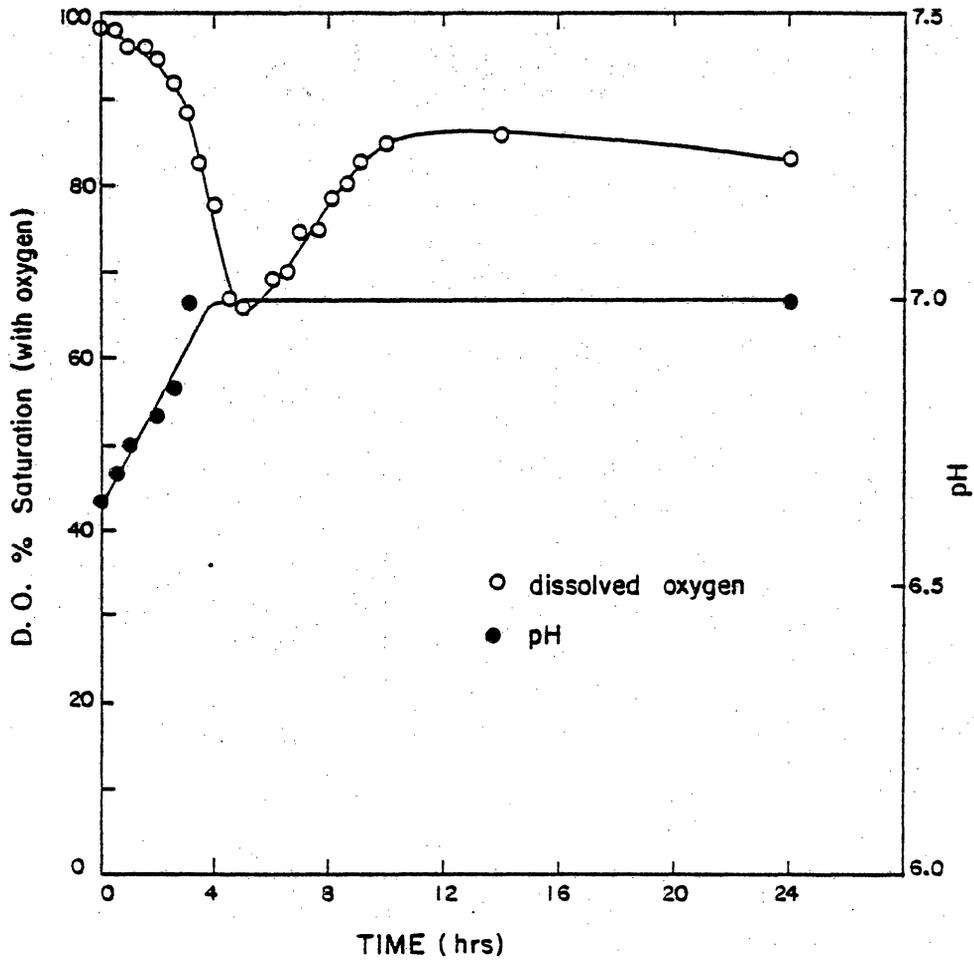


Figure 18: Time dependence of pH and dissolved oxygen concentration. Fermentation with pure oxygen. pH controlled at 7.0. Temperature = 30°C.

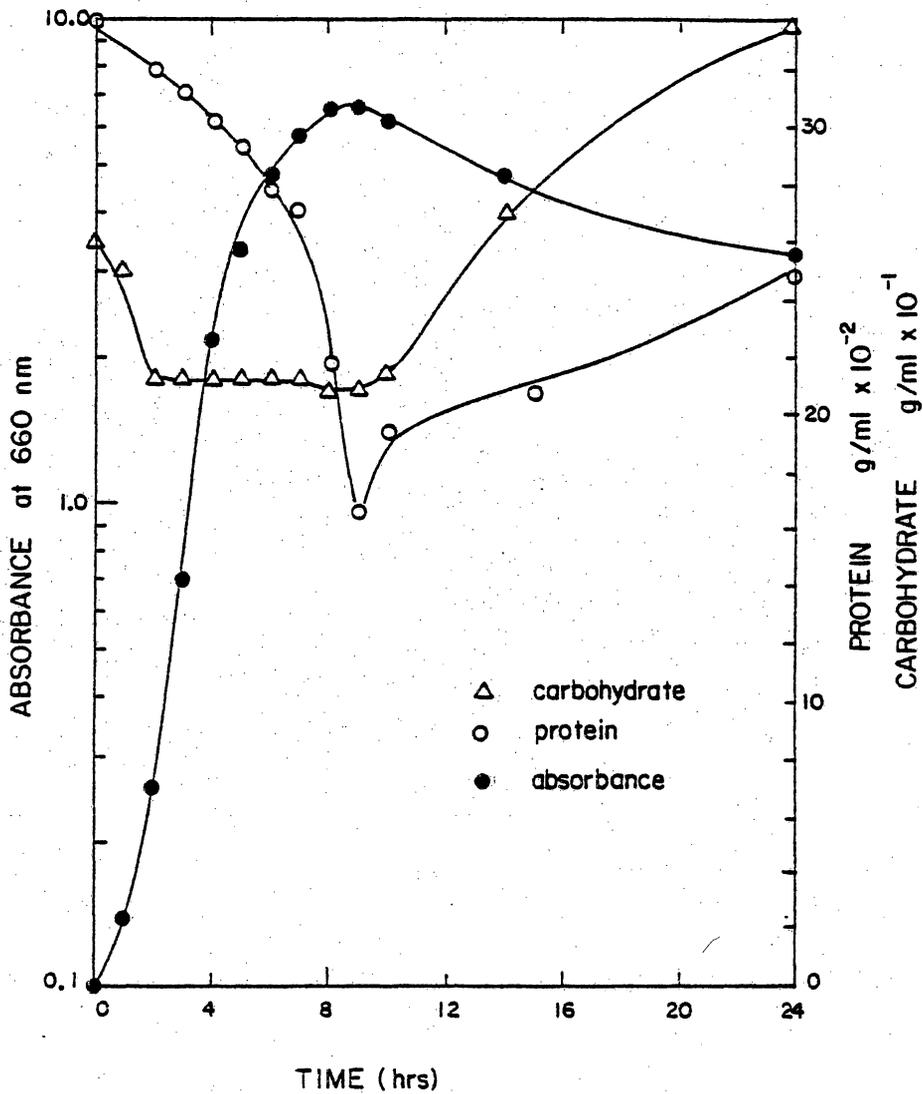


Figure 19: Time dependence of supernatant nutrients. Fermentation with pure oxygen. pH controlled at 7.0. Temperature = 30°C.

trol. This suggests that low pH aids the formation of the toxin. As of today, the chemical nature of the toxin is unknown, as are the biosynthetic pathways leading to its formation in the cell. Therefore, it is difficult to analyse the effects of pH any more precisely. However, it is known that the toxin is solubilised by alkaline conditions (33). Maintaining a low pH could be having some sort of a reverse effect in assimilating the toxin into the spore-coat. The recent discovery of a cytoplasmic toxin, which is hypothesised to be assimilated into the spore coat during sporulation, lends to this idea (35).

It would be reasonable to expect that higher dissolved oxygen concentrations would lead to greater cell-mass. Furthermore, it was also shown that air sparged fermentors gave a more toxic product. Thus, maintaining a high dissolved oxygen concentration in the growth phase, followed by a low concentration in the stationary phase would be expected to give a higher cell-mass with more toxicity. Based on this reasoning, the last experiment consisted of controlling the pH at 7.0, and changing over from oxygen to air sparging after eight hours of fermentation, approximately at a time when the cells enter stationary phase.

TABLE 5

Spore-Count and Toxicity Data. Fermentation
with sparged air. pH controlled at 7.0.

Time(hrs)	LC ₅₀ ($\mu\text{g/ml}$)	Spore-Count(cells/ml)
24	3.30×10^{-5}	7.40×10^8
30	3.06×10^{-5}	4.70×10^8

TABLE 6

Spore-Count and Toxicity Data. Fermentation
with sparged oxygen. pH controlled at 7.0.

Time(hrs)	LC ₅₀ ($\mu\text{g/ml}$)	Spore-Count(cells/ml)
24	2.99×10^{-5}	3.40×10^6
30	2.33×10^{-4}	3.60×10^6

The data for this run is shown in table (7). The toxicity levels and the spore-count appear to be the similar to the air sparged fermentations with pH control (table (5)). Using the dissolved oxygen strategy does not seem to have much of an effect. However, other strategies for different growth conditions need to be examined, and these may have a significant impact on the product.

TABLE 7

Spore-Count and Toxicity Data. Fermentation commenced with sparged oxygen, changed over to air at 8 hours. pH controlled at 7.0.

Time(hrs)	LC ₅₀ ($\mu\text{g}/\text{ml}$)	Spore-Count(cells/ml)
24	3.89×10^{-5}	1.40×10^8
30	2.82×10^{-5}	9.30×10^7

Chapter V

CONCLUSIONS AND RECOMMENDATIONS

Effects of temperature, pH and dissolved oxygen concentration on growth, sporulation and toxin formation in Bacillus sphaericus were investigated.

It was found that growth rate is faster at higher temperatures, but total cell mass produced is approximately equal to that at lower temperatures. Lag phase appears to be longer at lower temperatures. Sporulation begins earlier at high temperatures, but the total spore-count and specific toxicity are higher at lower temperatures.

The pH of the growing broth increases as the fermentation progresses. There is some similarity between the pH and the ammonia profiles. It was also found that controlling the pH at a value of about 7.0, gives about ten-fold more toxicity, as compared to the broths where the pH was not controlled.

Oxygen sparged fermentations seem to increase the growth rate and the total cell mass produced. The spore-count of air-sparged fermentation broths is 1-2 orders of magnitude higher than oxygen sparged broths, but the specific toxicity is nearly the same in both the cases. Switching from oxygen to air sparging at the onset of stationary phase, gave a

product similar to air sparged fermentations (in terms of toxicity and spore-count).

Protein appears to be the primary nutrient utilised. Carbohydrate, taken up by the cells, but is released later on. Concentrations of carbohydrate, protein and phosphorous all go through a minima, but none of the nutrients are ever completely exhausted.

A number of recommendations can be made. For example, it would be worthwhile to conduct a complete amino acid analysis of the fermentation broth at different points in time. This would give an idea of which, if any, amino acid is limiting. Conducting the experiments with different concentrations of the nutrient- broth -yeast extract- salts medium, or with different types of nutrients, may give better toxicity. This has to be further investigated.

Controlling the pH at 7.0 led to increased formation of the toxin. It would be interesting to observe the effects of controlling the pH at an even lower value.

From an industrial standpoint, much work remains to be done. Scale-up studies, testing the stability of the toxin, formulation and so forth are the areas to be considered.

This research was conducted using primarily batch type fermentors. Scale-up studies still remain an open question. Furthermore, It may be fruitful to study different reactor configurations for this system. It is difficult to visualise a single continuous stirred tank type fermentor for the formation of the final product takes place in two distinct stages, the vegetative phase and the stationary phase, with the cell mass itself being the final product. However, a two-stage reactor system, with growth taking place in the first stage and sporulation in the second where the dilution rates would be lower is worth consideration.

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