# A STATISTICAL APPROACH TO MEDIUM OPTIMIZATION FOR GROWTH AND TOXIN PRODUCTION BY THE BACTERIUM *BACILLUS THURINGIENSIS*

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## SUMMARY

A study has been made to determine the optimum medium composition, on a shake-flask scale, for maximum growth, sporulation and crystal protein toxin production by *Bacillus thuringiensis*. This medium-screening exercise was performed using statistical experimental design and mathematical modelling techniques. These techniques have been widely used in the chemical industry for process optimisation, but have only rarely been applied to microbiological studies. Thus, it was hoped to determine whether the techniques could be successfully applied to this situation.

Five nutrients were used for the medium-screening exercise, *viz* glucose, magnesium sulphate, ammonium chloride, yeast extract and potassium dihydrogen phosphate. The data obtained, and analysis of the data, provided strong evidence that these nutrients did not provide the complete growth requirements of the organism. For this reason, the optimum medium composition was not satisfactorily determined.

The modelling techniques used proved satisfactory, particularly in recognising interactions between nutrients, and pointing the experimenter in the correct direction, but because of the incomplete growth medium used their full potential for optimisation was not realised.

However, the work does suggest that the modelling techniques will have a role to play in microbiological exercises of this type,

provided that there is sufficient knowledge of the growth requirements of the organism under study.

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# INTRODUCTION

Bacillus thuringiensis is a Gram positive spore-forming bacterium. Knowledge of its pathogenicity against the larval stages of certain lepidopterous insects has been known for over 70 years. Ishiwata (1902) was the first to isolate it from dying silkworm larvae. Later Berliner (1915) isolated it from sick larvae of Anagasta kuhniella. He noted the existence of a parasporal body or "Rostkorper" in sporulated cells. These observations were confirmed by Mattes (1927), but it was not until 1953 that Hannay (1953) characterized the parasporal body as a crystal. The crystal has since been known to play a key role in the pathogenicity towards the most susceptible lepidopterous larvae. In recent years a considerable degree of interest has been aroused on the use of biological methods for insect control as against the use of chemical insecticides. Among many biological products considered as insect control agents, the crystal produced in the bacterium B. thuringiensis is one of the most hopeful. Industrial organisations in several countries are presently engaged in fundamental research and commercial scale production of insecticidal preparations based on this bacterium (Falcon, 1971; Pendleton, 1969).

Apart from the crystal, which might be considered as an enterotoxin, a number of exotoxins are also known or postulated to be produced by B. thuringiensis. Heimpel (1967a) suggested the following nomenclature: δ-endotoxin, or the proteinaceous crystal; α-exotoxin, a lecithinase C or phospholipase C; β-exotoxin, a thermostable exotoxin or "fly factor"; γ-exotoxin

(an enzyme that clears egg yolk agar, not yet identified). The toxicity for insects of the β-exotoxin and the δ-endotoxin has been substantiated, but the efficiency of the so-called Υ-exotoxin has not been proved. Quite recently Krieg (1971) suggested that the α-exotoxin is not identical with lecithinase C. He concluded that the α-exotoxin is a thermosensitive exotoxin of proteinaceous character which is produced during growth phase by strains of B. thuringiensis and of B. cereus. Krieg (1970) also isolated a relative heat stable bacteriocin produced by B. thuringiensis which he called Thuricin. He suggested that this is a polypeptide and can cause inhibition of growth of Gram positive bacteria and antagonism between several strains of B. thuringiensis.

In this review will be considered the taxonomy of crystal-forming B. thuringiensis, its growth, morphology and physiology and the δ-endotoxin, or proteinaceous crystal. This toxin is of theoretical interest since it is a crystalline protein, and it is also of great economical interest.

## 1. Taxonomy

The group of microorganisms referred to in Bergey's manual (Buchanan and Gibbons, 1974) as B. thuringiensis is "distinguished from B. cereus by pathogenicity for larvae of Lepidoptera and by the production of a crystalline protein body, rarely two or three, in the cell during the phase of spore formation. This body stains like other cell material; it is formed outside the exosporium and it separates readily from the liberated spore. In the larval gut toxin is released from the crystal by enzymatic action. The

capacity to form crystals may be lost by laboratory cultures."

Extensive taxonomic studies have been conducted on the numerous isolates of B. thuringiensis. Norris (1964) suggested the use of vegetative cell esterase activity pattern by electrophoretic analysis as a taxonomic tool. Heimpel (1967b) proposed to use the characteristics of production of -exotoxin and toxicity of B. thuringiensis varieties for silk worm. Rogoff and Yousten (1969), however, questioned the use of such indirect evidence as taxonomic criteria. The problem of taxonomy seems to have been conclusively resolved by the very thorough studies of serological and biochemical characteristics of a large number of B. thuringiensis strains by de Barjac and Bonnefor (1962, 1967, 1973). These authors devised a classification system dependent on the presence of a specific flagellar H antigen, and 12 serotypes are now recognized.

## 2. Growth, Morphology and Physiology

B. thuringiensis is rod-shaped, about 1u wide x 3-6u long, and at the latter stages of growth it produces a spore and a parasporal body within the cell. Breakdown of the cell wall by autolysis then ensues with release of spores and parasporal bodies into the culture medium. Until very recently most investigations concerning the organism have been centered on selection of strains to control economically important insects, efficient mass production techniques, product formulations, field trials and further development of crystal-containing preparations as a "broad spectrum" bacterial insecticide. The uses of the bacterium as such have considerably outpaced our understanding of

the cause of crystal formation, and its chemical formation vis-a-vis sporulation. In particular there is a lack of knowledge on the nutrition and physiology of the organism relating to growth, sporulation and crystal formation.

Most of the early work on growth conditions was carried out using semidefined media containing yeast-extract. The general pattern of metabolism in a glucose-yeast-extract-salt medium was observed by Yousten et al (1969). During the exponential phase of growth, glucose was utilized, acetic and pyruvic acids accumulated in the unbuffered culture broth, and the pH decreased to 4.8-5.0. Following a period of 2 or 3h at low pH, the pH rose and reached neutrality after 2 or 3h due to the metabolism of acetic acid. Agents which interfered with acetate metabolism, e.g. fluoroacetate and α-picolinic acid inhibited both sporulation and crystal synthesis.

Nickerson, St. Julian and Bulla (1974), during studies on carbohydrate metabolism, reported that glucose metabolism was predominantly via the Embden-Meyerhoff-Parnas pathway. Only slight pentose-phosphate pathway activity was noticed, probably to support biosynthetic needs. Scherrer, Luthy and Trumpi (1973) studied the production of the protein crystal as a function of glucose concentration and noted that increase in glucose concentration in the growth medium led to a larger crystal inclusion with a higher content of protein and insecticidal activity. However, the rates of change of protein and toxin concentrations were not proportional. Maximum yield of protein and δ-endotoxin was obtained in a semi-synthetic medium with glucose concentration of 6-8g/l.

Singer, Goodman and Rogoff (1966) reported that *B. thuringiensis* grew only slowly and sporulated poorly on completely defined media containing glucose and salts, and growth could be improved by addition of certain amino acids or casein hydrolysate. However, Connor and Hansen (1967) and Singer and Rogoff (1968) noticed extreme sensitivity of many strains when inhibitory concentration of certain amino acids e.g., valine, leucine, isoleucine, were added to a minimal medium. These observations seemed to be at variance with normal growth control mechanisms and have not been more fully explained until very recently by Nickerson and Bulla (1974). These authors described a basal medium which contained only glucose and salts. They noted that the basal medium did not support growth of any of the strains studied, but when supplemented with 0.2% citrate, aspartate or glutamate, abundant growth and sporulation were observed. These authors suggested that the reported growth in a glucose-salts medium by Singer *et al* (1966) and Singer and Rogoff (1968) was misleading because the "salts" used included certain amounts of citrate and acetate. Since then, Nickerson and Bulla (1974, 1975) have tested and eliminated many hypotheses regarding the reason why the basal medium was deficient, such as vitamin deficiency, trace metal deficiency... They showed that the absolute requirement for citrate and related compounds was not caused by defective citrate or glucose transport or metabolism. They also reported that the TCA cycle was not necessarily required for sporulation, and glutamate appeared to regulate the TCA cycle as well as to influence heat resistance and production of dipliconic acid (Nickerson, de Pinto and Bulla, 1974). Nickerson and Bulla (1975) further expanded their findings by noting that the

auxotrophic requirement for glutamate, aspartate or citrate could be by-passed if the basal medium was instead supplemented with cystine, thiosulphate or ethylene diaminetetra-acetic acid. They demonstrated that there is a direct correlation between compounds that permit growth in defined media and that stimulate fatty acid synthesis in the presence of chloramphenicol.

## 3. The protein crystal

### 3.1. Preparation and purification

Four basic properties of the spores and the crystals can be used to separate them. These are relative density, surface properties, solubility and germination of the spore. Each property can be exploited alone or in combination with others to obtain highly purified preparations of spores and crystals.

One of the most successful methods has been a two phase liquid-liquid extraction. This involves the preparation of an emulsion of an aqueous suspension of spores and crystals in suitable organic solvents. The crystals, being hydrophilic, remain in the aqueous phase while the spores, being hydrophobic, are extracted into the organic phase.

Angus (1959) devised the first of these techniques using trifluorotrichlorethane while other authors have used more common solvents. Lecadet (1965) used tetrabromoethane and Pendleton and Morrison (1966) used carbon tetrachloride. Murray and Spencer (1966) obtained a very satisfactory yield by emulsifying with chloroform followed by passage through a membrane filter. Many of the published methods are tailored to suit particular strains of *B. thuringiensis*, since a method successful with one strain

will not necessarily be the best for another. Recently, Cooksey (1971) mentioned that Norris used a combination of surface charge and density in the continuous electrophoresis apparatus described by Hannig (1958). Lately, Fast (1972) developed a more rapid method using density gradient centrifugation technique. Here a 25-45% w/w CsCl gradient was used, and the crystal band was harvested with a syringe.

3.2. Structure

The mature crystal often exceeds 1μ in length and 0.5μ in width and may account for 30% of total dry weight of the sporulated cell. Thus it is easily visible under the light microscope, either within the cell or released into the culture medium after autolysis of cells. Also, it stains well with strong stains, e.g. carbol fuchsin, naphthalene black, crystal violet, giemsa... Norris (1971) suggested a satisfactory method to stain a heat fixed smear with strong carbol fuchsin and observe it with phase contrast optics. The mature crystals differ somewhat in shape in the various strains of B. thuringiensis. In many strains it is a regular bi-pyramid, but in others, it is much more irregular. Hannay and Fitz-James (1955) were the first to use electron microscopy to show that the parasporal body was a regular bi-pyramidal crystal. For electron microscopy, the techniques used for fixation, embedding and sectioning are critical to avoid artifacts, and interpretation of the electron micrographs is not simple, especially for the study of the fine ultrastructure present in the crystal of B. thuringiensis. Hannay and Fitz-James (1955) observed prominent surface striations on the crystal, spaced at approximately 29nm apart. Thus indicated the presence

of repeating subunits in the crystal. Labaw (1964), in a more detailed study using the carbon replica technique, concluded that the basic protein subunit was a sphere approximately 8.7nm in diameter. Holmes and Monro (1965), however, used X-ray diffraction and demonstrated that the subunit was rod or dumb-bell shape. Their observations were later confirmed by Norris (1969) who, using micrographs of thin sections through crystals, suggested that the subunit was a rod shape body, approximately 4.7nm by 11.8nm. More recent studies involving the use of negative staining techniques (Somerville, James, Ruffell and Norris, 1971) and freeze-etching technique (Norris, 1971; Short, Walker and Thomson, 1974) have revealed that the crystal structure involves a repeating sequence of 3 rows separated by equal spaces, followed by one row separated on either side by a slightly greater space. The particles evident in the three equally-spaced rows are arranged hexagonally in a row-to-row spacing of approximately 7.5nm.

### 3.3. Chemical composition and stability

Chemical composition of the crystal was first studied by Hannay and Fitz-James (1955) and Angus (1956). Using quantitative techniques on paper chromatography, Angus found that a pure suspension of the crystal contained 16.5-17.8% N and traces of phosphorus, and included all the amino acids normally found in proteins. Later, Lecadet (1965) used a more sensitive automatic amino acid analyser and obtained similar results. The total sum of amino acids amounted to 95 to 98% of the weight of the analysed product. Lecadet also noted that the dicarboxylic amino acids accounted for 25% and the basic amino acids 13% of all the amino acids present. The general characteristics of the amino acid

composition did not differ greatly from one strain to another, and no unusual amino acids were detected. It must be assumed that the amino acids are in their natural L-form since their configuration has not been described.

Faust and Esters (1966) reported finding an average of 0.36% silicon by weight in the crystals of 3 strains of B. thuringiensis studied, and postulated that silicon acted as a skeletal lattice on which the protein was deposited. Recently, Faust, Hallam and Travers (1973) have performed spectrographic elemental analysis of the crystal and have reported the presence of significant concentrations of C, Ca, Fe, H, Mg, O, S and Si, lesser concentrations of Al, Cr, Ni, Ti and Zn, and trace amounts of B, Co, Cu, Mn and P. No explanation has been offered for the importance of these elements in the structure of the crystal.

Intact crystals are stable over a large pH range and insoluble in water or organic solvents, but can be dissolved above pH 11.5 (Angus, 1956). The toxicity and the ability to react as an antigen is destroyed by exposure to above pH 12 or below pH 3.3 (Angus, 1956; Cooksey, 1968). The crystal can also be dissolved using disulphide bond reducing agents (Young and Fitz-James, 1959; Lecadet, 1965), and through the actions of several proteolytic enzymes obtained from the gut juices of larvae susceptible to the crystal toxin. Chloroform, methanol and acetone destroyed the toxicity of dissolved crystal protein but not intact crystals (Angus, 1956; Watanabe, Tsutsui and Iwahana, 1967). The protein is denatured by heat, although it is more stable than dissolved toxin, e.g. dissolved protein was

totally inactivated by treatment at 65°C for 1h, but crystals were not inactivated even at treatment at 80°C for 20 min (Cooksey, 1971). Spores of B. thuringiensis are inactivated by ultraviolet light, but crystals retain their toxicity after long exposures (Cantwell and Franklin, 1966; Cantwell, 1967). Burges, Hillyer and Chanter (1975) have reported similar results, and have found no reduction of insecticidal activity of the crystal as a result of exposure to severe gamma or ultraviolet irradiation.

### 3.4. Biochemistry

As mentioned above, there is nothing remarkable about the amino acid composition of the protein crystal, and no unusual amino acids have been detected. To study the toxic properties, it has been necessary to know what are the components, and the type of chemical bonds involved in this dense and strongly organized structure. As it is impossible to study an insoluble protein, it is essential to dissolve the crystal first. This may be followed by some chemical modification of the protein when it is necessary before separation of the components of the crystal protein by such techniques as electrophoresis, ultracentrifugation, chromatography and immunodiffusion.

The various ways by which crystal dissolution has been obtained seem to point to the presence of several types of covalent and non covalent linkages in the crystal structure. Angus (1956) investigated the dissolution of the protein crystal by alkaline buffers. Young and Fitz-James (1959) suggested the crystal becomes insoluble durings its synthesis in vivo due to

the formation of disulphide bonds. This hypothesis has been confirmed by Lecadet (1966, 1967a) who described the action of reducing agents on the crystal by measuring the rate of decrease in turbidity of a suspension of crystals, and by following the swelling and the gradual disappearance of the crystal structure. The author demonstrated that dissolution in thioglycolate is possible at whatever the pH, as low as pH 7.5, but that it is all the more rapid as the pH increases. Lecadet (1967a) also used urea, a protein denaturant known to be effective in "unwinding" the secondary structure of a protein by breaking H-bonds. She reported that urea caused important modification of structure, loss of refractility, swelling of the crystal, but no liberation of the protein constituent chains occurred. The process is almost entirely reversible, for when urea is eliminated through dialysis or dilution, the original structure reappeared. Moreover the action of urea quickens dissolution by reducing agents which are then activated at very low concentrations. These evidences strongly suggested that H-bonds play an important role in the crystal structure. Crystals are also soluble without reducing agents or urea when placed in sodium hydroxide solution at above pH 12 (Angus, 1956). This indicates that ionic bonds between side chain carboxyl amino groups are of significance.

Thus a number of factors and types of linkages appear to be involved in this highly organized structure, but it has not yet been possible to determine the respective roles of the various factors and linkages.

One of the difficulties of working with a solution of the crystal protein appears to be maintaining it in solution. The protein is known to undergo reversible subunit aggregation (Somervill, Delafield and Rittenberg, 1968). This means that the protein chains liberated by H-bond and disulphide bond breakage, reaggregate to form larger entities which finally precipitate. Lecadet (1966) has partially overcome this problem by alkylation of the free sulphide linkage with p-chloromercuribenzoate.

The types of linkages that bind the constituent protein chains of the crystal raise several interesting questions such as the nature of the basic units, the number of polypeptides, and whether or not separate toxic moieties exist in the protein molecule. Evidence is overwhelming in favour of the thesis that the crystal contains several different polypeptide chains linked by disulphide bridges and H-bonds. Holmes and Monro (1965) reported that ultracentrifugation analysis of the protein solution in alkali reveals the presence of two major components. These are in equal concentration and have sedimentation constants of 4.1 S and 0.4-0.8 S respectively. By treatment with thioglycolate at pH 9.5 to release constituent protein chains, followed by alkylation treatment, Lecadet (1966) found at least 3 components by ultracentrifugation with sedimentation constants at 20°C of 9.6, 4.8 and 0.83 S. The last component is formed in proportions varying from 30-80% of the total, according to the

conditions of the reduction and the strain studied. This result has been explained by the reversible association-dissociation phenomenon in the protein solution.

Using gel filtration techniques in the study of B. thuringiensis var. sotto, Watanabe et al (1967) found that after crystal dissolution at pH 11.5 and chromatography at pH 7.8 in 0.1 M phosphate buffer, the solution separated into 2 toxic compounds. These were, however, minor rather than major components of the original solution. Cooksey (1968) found similar results with the Mattes isolate, except that the toxic components were major ones and immunologically distinct.

It was shown by Cooksey (1968) that chymotrypsin, trypsin and subtilisin as well as Pieris brassicae enzymes were able to hydrolyse dissolved and reprecipitated crystal protein in carbonate buffer, pH 9.5, to yield toxic fragments of similar size distribution, as judged by Sephadex G-200 chromatography. Lecadet and Dedonder (1967) studied the proteinaceous fraction excluded by G-75 of molecular weight about 50,000. This fraction was not a single entity as it gave rise to 6 amino-terminal amino acids and 6 bands in starch gel electrophoresis. The authors concluded that there are 6 proteinaceous components of the lysate. Glatron, Lecadet and Dedonder (1969) claimed to have obtained total solubilization of the crystal by treatment with mercaptoethanol in the presence of guanidine hydrochloride at neutral pH. Ultracentrifugation analysis gave a single component of molecular weight of about 80,000, the N terminal amino acid being phenylalamine and the C terminal amino acid arginine. This evidence suggested that the crystal might result

from the association of identical subunits.

3.5. Immunology

The study of the antigenic properties of the crystal toxin proves of basic interest in the characterization of the constituent protein chains. Comparisons of immunochemical properties of the crystals from different strains should help in their identification and eventually allow the establishment of the relationship between structure and toxicity. Review of many papers concerning investigations conducted along this line is complicated by the different techniques in use for the study of precipitin reactions. Some workers used whole crystals as antigen for the antisera preparations (e.g. Pendleton and Morrison, 1966b), whereas others used dissolved crystal preparations (e.g. Cooksey, 1968). These authors used rabbits to prepare antisera, whereas Krywienczyk and Angus (1960) used guinea pigs. The latter investigators showed that the crystals from 3 serotypes have several antigenic determinants. de Barjac and Lecadet (1961) observed that the crystals of the 3 different strains studied have a common antigen-antibody system, while a second precipitation system is common to only 2 of the 3 strains. In their studies on the Berliner, entomocidus and sotto strains, Krywienczyk and Angus (1965) found that the crystals of these strains have a common antigen. They also reported (Krywienczyk and Angus, 1966) that the crystal from the entomocidus strain has another antigen which does not exist in the sotto strain crystal. These experiments thus have demonstrated similarities as well as differences in the structure of crystals from various strains.

Pendleton and Morrison (1966a) examined the antigenic properties of crystals from a large number of B.thuringiensis strains using anti-crystal sera only. They have found 28 different serotypes containing 9 different antigens (called a, b, c, d, e, f, g, h and i) distributed according to different patterns. They suggested that these antigenic properties could be used as a complementary taxonomy criterion for the classification by flagellar antigen (Bonnefoi and de Barjac, 1963) and vegetative cell esterases (Norris, 1964). Pendleton and Morrison (1966b) found only one major precipitin line per strain of dissolved crystal challenged with homologous antiserum. But de Barjac and Lecadet (1961) reported that when alkali-dissolved crystal protein, as opposed to intact crystals, was used to immunize rabbits, the antisera reacted with soluble protein to give an increased number of precipitin lines. Cooksey (1971), however, reported that he had detected 2 major antigens with antisera produced with both dissolved protein and intact crystal of the Mattes isolate of B. thuringiensis. Cooksey (1968) found 2 major antigens in B. thuringiensis Mattes isolate when dissolved protein was challenged with homologous antiserum. There was another faint line in the Ouchterlony double diffusion test, but this was not always seen. On occasions, the 2 major lines were seen to be double ones spaced very close together, making a total of 5 lines. This totally agreed with the results published by Krywienczyk and Angus (1967). Pendleton and Morrison (1967) scanned a larger number of B. thuringiensis isolates for intact crystal serotype and compared the same isolates on the basis of serology of digested crystal. They found that many of the antigenic patterns were changed.

These few examples demonstrate the complexity of the problems and difficulties of an immunochemical study comparing crystals of different strains.

In the study of serology of the protein crystal, it is of particular importance to be sure that the antigenic property of the crystal is related to its toxic action. One of the practical interests is in the replacement of slow toxicology assay by a more rapid immunological assay. Krywienczyk and Angus (1960) were among the first to establish a relationship between toxicity and antigen reaction. They showed that the 3 varieties of B. thuringiensis var. Berliner, sotto, and entomocidus contained a common antigen. When this antigen was removed by precipitation with an antiserum, the supernatant protein solution was atoxic. They noted however that the strains were serologically quite different Later Cooksey (1968) showed that there was a quantitative relationship between the concentration of a particular antigen in a dissolved protein preparation from the Mattes isolate and the toxicity of the same preparation.

## 3.6. Number of polypeptides and separate toxic moieties in the protein molecule

From electron microscopical studies it is well established that the protein crystal is composed of individual subunits. Earlier evidence from biochemical and immunological studies is overwhelmingly in favour of the thesis that the crystal contains several different peptide chains linked by disulphide bridges, H-bonds and other minor linkages. But the questions of their number, and how many are toxic are still not fully answered. The answers are clouded by the use of different strains, solubilizing

agents, separation methods and especially by the tendency of the polypeptides to reaggregate.

Lecadet (1966) found at least 3 fractions in an alkylated protein solution separated by ultracentrifugation. Anderson and Rogoff (1966) reported 5 different zones from partially solubilized crystal protein by paper electrophoresis. By reduction of crystals and electrophoresis in the presence of sodium dodecyl sulphate which would have prevented association, Cooksey (1971) found 6 distinct bands. The same author also used a different alkylation method developed by Anfinsen and Haber (1961), and again disc electrophoresis demonstrated 6 bands. He suggested this as evidence of at least 6 chains. How the chains are organized into the subunits which make up the crystal is still not fully known.

Earlier studies have also detected a number of different antigens in crystals from each of several strains of B. thuringiensis implying a multiplicity of protein substituents. Pendleton and Morrison (1967) found a maximum of 5 antigens in the crystal of a particular isolate of B. thuringiensis. This could be evidence in favour of multiple antigenic sites on one protein or single sites on several proteins. But the multiplicity of antigens could merely reflect the protein association phenomenon. Antibodies may be elicited to proteins in different states of association and this may give a false picture of the number of distinctly different proteins present. However, other workers have given further evidence to support the multiplicity hypothesis Lecadet (1967) and Norris (1971) both observed that the crystal antigens are synthesized at different stages in the

sporulation of the cell. Rogoff (1966) stated the separate genetic control of synthesis of 2 antigens in mutant strains. Rogoff, Ingoffo, Singer, Gand and Prieto (1970) reported the existence of a heterogeneous population of small peptides with molecular weight of 1000-1500 Daltons. Herbert, Gould and Chain (1971) reported the separation of 2 polypeptide chains having molecular weight of 55,000 (A) and 120,000 (B). The ratio of A and B in the crystal appears to be 2:1 on a molar basis but B does not appear to be a dimer of the smaller A. The full toxicity of the unfractionated protein is retained by fraction A whereas fraction B is non toxic. Glatron et al (1969, 1972) discovered only a single subunit of 80,000 Dalton, using treatment with mercapto-ethanol in the presence of guanidine-hydrochloride at neutral pH. Dulmage (1975) has viewed his own personal opinion that the pathogenic actions of different δ-endotoxins may derive from a single basic unit.

## 3.7. Biosynthesis

The ability to produce a crystalline protein is the main characteristic that distinguishes strains of B. thuringiensis from the closely related but non entomopathogenic B. cereus. Synthesis of the toxin as a crystal raises some very interesting problems for the following reasons. Firstly, the production of the crystal is concomitant with the formation of the endospore. Secondly, the crystal represents an important fraction of the proteins formed during sporulation. Lastly, the biosynthesis of this highly organised structure raises the more general problem of formation of insoluble protein structure in a cell.

Several workers have studied the formation of the crystal and its relationship to the spore. Young and Fitz-James (1959b) made detailed studies of the cytological changes using phase contrast microscopy. They showed that the inclusion appeared at very early stages of development of the forespore. Fitz-James (1962) concluded from an electron microscopical study that the inclusion was associated with the forespore membrane and was completed in the sporangial cytoplasm.

Young and Fitz-James (1959a) tried to extract the crystal protein at different pH values throughout the sporulation. They reported that during early development the crystal protein remained in a form which can be extracted at a lower pH value without the aid of a disulphide reducing agent. During the later stages of sporulation, however, a maturation process conferred on the crystal insolubility and refractility. Monro (1961), by means of immunological techniques, showed that the crystal antigens are not present in the vegetative cells; Delafield, Somerville and Rittenberg (1968) have reported similar observations. The antigens are believed to appear during the sporulation process sometime before the development of heat resistance (Monro, 1961; Norris, 1969).

Lecadet (1967b) reported that the several crystal antigens are not synthesized concurrently. One antigen was synthesized in the first step of sporulation after septum formation but before the crystal was visible Aronson and Fitz-James (1968) found that spore coat precursor protein was synthesized during forespore development, i.e. at about the same time as the "early" crystal antigen reported by Lecadet (1967b).

More recently, Somerville (1971) used immunological assays and pulse-chase and label-chase experiments with $[^{14}C]$ leucine to demonstrate that the crystal protein is synthesized at the time of appearance of crystal antigens. From immunological comparisons. Monro (1961) reported that the crystal and the spore did not have common components. But later studies disputed his statement. Delafield et al (1968) showed that immunologically the crystal and endospore contain one or more common proteins Somerville et al (1968) in studying the biochemical homology between crystal and spore protein of B. thuringiensis concluded that a significant portion of the spore protein is identical with the crystal protein. This immunological and biochemical similarity is confirmed by more recent studies (Somerville et al, 1970; Lecadet, Chevrier and Dedonder, 1972).

An interesting question raised by these studies concerns the significance and the use of the crystal to the bacterium. Heimpel and Angus (1960) suggested that the crystal is a waste depository for unwanted metabolites. Monro (1961) provided evidence from radioactive labelling studies that the bulk, if not all, of the crystal protein is formed de novo from amino acids during the sporulation process. Yousten and Rogoff (1969) have reported similar results. Somerville et al (1968) supported the suggestion that the crystal is a result of over production of spore coat protein.

Very recent structural studies seem to give a more detailed picture of the relationship between crystal formation and sporulation. Somerville (1971) showed that during its formation, the crystal is closely associated with the developing exosporium.

It was suggested that the exosporium forms a template for crystal assembly and the crystal may be synthesized on this membrane. Recently, Short et al (1974) examined the ultrastructure of B. thuringiensis spores, using the freeze-etching technique. They provided evidence supporting the relationship between the deeper layers of the spore coat and the crystal protein, and proposed that it is possible that spore coat proteins produced during sporulation escape through the pits in the exosporium to be reassembled on a template on the outer surface.

Thus crystal or toxin formation seemed to be closely bound to spore production, and it appeared as if it was a secondary phenomenon of sporulation. Yet in many cases the two processes can be dissociated, since several mutants have been reported which have lost the ability to form the crystal (Fitz-James and Young, 1959; Toumanoff, Lapied and Malmanche, 1955; Smirnoff, 1965). Nishiitsutsuji-urvo, Wakisaka and Eda (1975) isolated mutants of B. thuringiensis that are completely lacking in ability to form spores. The crystals of the mutants have the same regular shape and the same insecticidal activity as those of original strains. There are even strains which can form 2 or more crystals in each sporangium (Norris, 1971).

Smirnoff (1963) also reported the desynchronisation of crystal formation and sporulation as a result of growth at a suboptimal temperature of 14°C to 14.7°C where metabolism of the bacterial cell was disturbed Arescaldino (1969) has reported that treatment with erythromycin caused the same desynchronisation where normal sporulation was disturbed while maintaining crystal formation.

The finding by Luthy, Hayashi and Angus (1970) that the endotoxin is produced not only during the sporulation process but it is also synthesized in small quantities during the vegetative growth is very interesting and deserves further investigation.

## 3.8. Toxicity and mode of action

When a susceptible larva ingests food containing or coated with the spore and parasporal crystal of B. thuringiensis the toxic manifestations vary from one species to another; yet a typical and general sequence of events occurs (Pendleton, 1969). The crystals are lysed enzymatically in the highly alkaline environment of the insect gut and a protein toxin is released which apparently damages the epithelial cells lining the gut wall. Feeding and digestive functions of the insect cease due to breakdown of the selective permeability barrier between gut contents and haemolymph. The pH of the normally alkaline gut contents falls to about pH 8 and the pH of the haemolymph rises. Finally the insect dies in less than an hour or after a few days, depending on dosage and species of insect.

The number of species of insects susceptible to B. thuringiensis is very large. Heimpel (1967a) quoted that the number is about 140, and approximately 100 are among the lepidoptera. The range of activity of B. thuringiensis varies from one strain to another and depends essentially on the ability of the strain to produce the different toxins mentioned earlier. Apparently, the crystal endotoxin is active only on lepidoptera larvae (Lecadet, 1970).

The mode of action of the crystalline endotoxin has been the subject of numerous investigations for the past 25 years. Results obtained are difficult to interpret because of the diversity of the susceptible species and the observed variations in the efficacy of the crystals from different strains of B. thuringiensis. Angus (1956a) considered the crystal as a protoxin, and that it must be dissolved and/or dissociated before it becomes effective as a toxin. This author (Angus, 1954, 1956b) demonstrated that the crystal alone, or their alkaline solutions, are toxic for Bourbix moris larvae, and that the spores alone are not toxic. But Heimpel and Angus (1959) later classified one group of susceptible species (type Anagasta kuhniella) that are not affected by either the crystal or the spore alone. The spore must be associated with the endotoxin to bring about the toxemia which leads slowly to death without the symptoms of paralysis. Later Somerville, Tanada and Omi (1970) observed that in addition to the B. thuringiensis endotoxin, the whole spore may play an important role in killing the insect larvae. Recently, Somervill and Pockett (1975) isolated a toxin from spore extracts which is active against lepidopterous larvae. The toxic material is located in the outer layers of the spore and can be released by incubation with proteolytic enzymes and gut juice from susceptible insect larvae, and it can be inactivated by crystal-specific antiserum.

Fast and Angus (1965) showed that the toxin altered the selective permeability of the silk worm gut to glucose and to carbonate ions. Louloudes and Heimpel (1969) confirmed these results and extended them to show that acetate and leucine also

appear in the haemolymph more slowly in insects intoxicated by δ-endotoxin. They also agreed that the toxin affects the cell membrane to inhibit the active transport mechanism of the cell. In their study Fast and Angus (1965) also observed what appeared to be a stimulation of glucose transport in the early stages of intoxication. Fast and Ponaghue (1971) further noticed that the uptake is inhibited only 11 min after administration of crystal. Recently Faust, Travers and Hallem (1974) tried to explain the stimulation of glucose uptake at the molecular level: the initial stimulation of glucose uptake could result from the initial effect of the δ-endotoxin on the respiratory system, that is, glucose uptake increases as metabolic respiration increases but without the benefit of respiratory chain-linked ATP production. When endogenous ATP becomes limited, glucose uptake ceases and the observed changes between 15 and 25 min would follow, i.e. ion transport upset, "ballooning" or extrusion of the columnar cells, bursting from uncontrolled osmotic effects disintegration of the mid-gut wall and release of the cell contents.

Ramakrishnan (1968) reported that B. moris larvae, that had ingested a lethal dose of δ-endotoxin, had 5 times as much $K^+$ ions in the haemolymph compared with healthy larvae approximately 90 min after ingestion. Pendleton (1970) showed a similar increase of $K^+$ levels in haemolymph of Philasomia ricini larvae.

Cooksey, Donninger, Norris and Shankland (1969) showed that δ-endotoxin digested by P. brassicae gut juice or by gut enzymes blocked nerve conduction whereas undissolved crystals did not.

All these lines of evidence seem to suggest that the function of the toxin may be to disrupt $K^+$ ion regulation. However,

Fast and Morrison (1972), in their study on the effect of δ-endotoxin on ion regulation by mid-gut tissue, showed that the changes in ion levels in the haemolymph appear to be secondary effects resulting from breakdown of gut metabolism that occurs between 10 and 15 min after the toxin is fed, i.e. the disruption of ion regulation is not the primary toxic mechanism.

Luthy (1973) suggested another possible explanation for the mode of action of the endotoxin. This author studied histological changes induced by gut juice with and without endotoxin when applied to both inside and outside of freshly dissected gut tissues. Microscopical observation showed the first signs of damage after 15 min and there was no difference between the effect of toxic and normal gut juice. Luthy proposed that the endotoxin initiates self-digestion and induces the breakdown of the epithelium of the gut by reacting with the enzyme system or with the membrane of the gut epithelium.

4. Large-Scale Production of B. thuringiensis

Since the first commercial preparation of B. thuringiensis was made available in the late 1950's, the production and usage of the organism in commercial pest control has increased dramatically (Hall, 1963). In recent years commercial preparations have been produced by at least 12 manufacturers in 5 countries (Falcon, 1971). In the U.S.A., hundreds of tons are manufactured each year for registered use on a number of important vegetable and fruit crops, in addition to forestry, for the control of more than 20 insect species. The increased usage is mainly due to the need to replace certain dangerous chemical insecticides, but also

reflect the improvements made in selection of more potent strains, production methods and product formulation. These latter efforts have resulted in better and more reliable commercial products. With the current interest in environmental pollution and the need to develop integrated control and pest management programmes, the use of B. thuringiensis should accelerate in the future (Falcon, 1971).

Since all commercial B. thuringiensis formulations are sold primarily for control of lepidopterous pests, industry is primarily interested in the crystalline toxin. However the spore does play a secondary role in the insecticidal activity and the heat stable -exotoxin has practical potential. All these 3 components are commercially produced in both semi solid and in submerged fermentations.

4.1. Semi-solid fermentation

A typical semi-solid fermentation process consists of the following major steps: the fresh inoculum from a slant is transferred through several passages in shake flasks, each involving a larger volume. The flasks are incubated for 4-6h at 30°C, and each stage is transferred during its maximum rate of growth. The seed for the semi-solid fermentation is then produced by inoculating the organism into a seed fermentor. The fermentor seed is then used to inoculate a semi-solid medium where production of the spores and toxins occurs.

Mechalas (1963) described a large scale production process of toxin on a bran-based semi-solid medium composed of 545g wheat bran, 380g expanded perlite, 62g soybean meal, 36g dextrose,

3.6g lime, 0.9g NaCl, 0.29g $CaCl_2$ and 160 ml $H_2O$. After partial sterilization with flowing steam for 1h, the mixture weighed about 1Kg. Approximately 400ml of fermentor seed was used to inoculate each Kg of the semi-solid medium, and after mixing this gave a moisture content of 60% by weight at pH 6.9. The actual fermentation was carried out in bins containing about 250Kg of inoculated semi-solid medium, for about 36h. The bins had perforated bottoms through which air at 30-34°C and 95-100% RH was passed at about 0.4-0.6 volume/volume inoculated medium/min for the first 3h and 1.0-1.2 volume/volume/min thereafter. At the end of the fermentation, the pH rose to 7.5 and moisture content dropped to about 53%. At this time dry air at 50-55°C was passed through the bran for another 36h. The bran medium was then harvested and ground through an 80-mesh screen. The final product had a moisture content of 4% and a pH of 7.0.

The main advantage of the semi-solid fermentation is that it is rather simple and cheap but there are many problems concerning contamination and control. The bran itself is pasteurized but not sterilized during the steaming process. The air normally used is clean but not sterilized. However, the lower layer of bran acts as an air filter and prevents contaminants from spreading throughout the mixture. When the air passes through the bran it tends to develop channels, this results in poor aeration and temperature control in parts of the bran mass. This can be overcome by periodical mixing of the bran during the fermentation, but mixing may spread contaminants from the bottom layer throughout the bran mass. All the problems can be overcome by sterile-filtering the air and replacing the bin containers with

tumbling perforated drums (Takamine, 1914). Such procedures however are costly and decrease the economic advantages of the semi-solid process. Dulmage and Rodes (1971) reported that in 1964 Dulmage studied the production of insecticidal activity by a single strain of B. thuringiensis in 6 semi-solid fermentation runs using identical media for all runs and the same assay insect. Dulmage showed that because of poor process control the fermentation conditions varied and this resulted in variations in potency of the final product. He also showed that the potency produced did not correlate with growth and sporulation of the organism.

Majumder (1968) developed a simple laboratory-scale method of tray culture using nutrient agar solid medium. Later, Nagamma, Ragunathan and Majumder (1972) attempted to decrease the quantity of costly agar used in the medium by replacing agar with ground nut cakes and tamarind kernel powder, so that a cheap semi-solid medium can be used for commercial scale production.

## 4.2. Submerged fermentation

Standard techniques of industrial scale submerged fermentations are readily applicable to the production of B. thuringiensis. Ignoffo (1967) has described a typical procedure used by the International Mineral and Chemical Corporation to produce their flowable formulation of B. thuringiensis spores and toxins. The fermentation proceeds as follows: Inoculum for a production fermentor is prepared by passing the organism sequentially through two shake-flask media and then to a seed fermentor. Each shake flask seed is incubated for 24 h.

The seed fermentor is allowed to incubate until vigorous vegetative growth is obtained, at which stage it is used to inoculate the production tank. Adequate aeration throughout the fermentation is critical. When the sporulation is complete the culture medium is passed through a fine screen to remove any coarse particle, centrifuged, diluted with various stabilizing agents and packed in a flowable formulation.

Most commercial media used in submerged culture are based on cheap substrates such as molasses, corn steep liquor, fish meal, soybean meal, etc... Pendleton (1969) mentioned a successful medium which contained 2% fish meal, 1% starch, 0.1% $CaCO_3$, 0.001% niacin and a salt mixture. In a patented process Drake and Smythe (1963) described a typical medium containing 6.8% corn starch, 0.64% sucrose, 1.94% casein, 4.7% corn steep liquor, 0.6% yeast extract and 0.6% phosphate buffer. The final liquor before harvest contained $14 \times 10^9$ viable spores/ml. These authors did not believe that it was critical to exhaust the carbohydrate and N sources near the onset of sporulation. Megna (1963), however, believed that the composition of the medium could vary but was limited to 3-4% solids and was so designed that the fermentable carbohydrate and N available for growth were exhausted at approximately the same time as the commencement of sporulation A typical medium in Megna's patent contained 1.86% beet molasses, 1.4% cotton seed flour, 1.7% corn steep liquor and 0.1% $CaCO_3$. The final fermentation beer contained $2\text{-}5 \times 10^9$ viable spores/ml. Bennefor (1960) patented the production of spores and toxins in an aerated liquor containing 0.6-1.0% amino acids, 1-2% sugars and traces of Ca, zinc, manganese and magnesium.

Shell International Research (1969) used a medium containing 2.3% casein, 1% molasses and 2.3% Scotaferm (a by-product of the fermentation of alcoholic beverage).

Different workers have reported variations in production efficiency from culture to culture, and with each culture, from medium to medium. Dulmage (1970) studied crystal production by different strains of B. thuringiensis in submerged culture. He observed that the production of the insecticidal activity was dependent both on the strain and on the medium. No one strain and no one medium was necessarily superior.

4.3. Harvest, recovery and formulation

The semi-solid process yields a wettable powder or dust formulation containing spores, crystals and exotoxins simply by drying and grinding the final bran cake. The product, being dry, is inherently stable, and has been a satisfactory dust base. However, the bran is difficult to wet, and tends to swell when wetted, and it is difficult to use the product as a wettable powder.

In the submerged fermentation, product recovery is usually by centrifugation. The concentrated paste fluid obtained after centrifugation can be filtered to retain spores and crystals, and the moist filter cake can then be dried in a vacuum oven followed by grinding or milling to produce a fine powder. Alternatively suspensions of the spore-crystal complex are used to produce a flowable liquid formulation and this can be used as a spray.

The aim of formulating a microbial insecticide is to enhance

the possibility of infection and avoid practices that might inhibit or harm the pathogen. With B. thuringiensis one must also avoid using any compounds capable of denaturing the protein comprising the toxic crystal. Angus and Luthy (1971) have discussed in some detail the formulation of microbial insecticides. In the case of a dust formulation of B. thuringiensis cultured on a semi-solid medium, an inert filler such as bentonite is used (Pendleton, 1969). The filler may account for about 90% of the weight of the product.

Angus and Luthy (1971) have described the use of an oil suspension where the dry microbial insecticide is firstly wetted with an emulsifier-water mixture before final dispersion in the oil carrier to give a uniform oil suspension. However, some manufacturers omit the drying of the creamy paste of crystal-spore mixture obtained after centrifugation, and suspend the paste in a concentrated emulsion that can later be diluted in water (Smirnoff, 1967, Jacques, Stultz and Huston, 1968).

Originally, flowable formulation did not contain the exotoxins. However, that they can be recovered from the culture medium along with the spores and crystals has been described by Dulmage, Correa and Martinez (1970). They developed a method using coprecipitation with lactose as a means of recovering the spore-crystal complex together with the heat-stable -exotoxin. Dulmage (1970a) also claimed that such a product (the HD-1 formulation) had much improved potency over 2 commercially available products.

Pendleton (1969) mentioned spray drying the whole liquor

culture to produce a preparation containing the -exotoxin and possibly other soluble toxins, as well as the spores and crystals.

The composition of a particular emulsion will depend on a number of factors including the emulsifier used to emulsify the oil and water phase, the salt additive used to achieve isotonicity and possible wetting agents and/or stickers. Spreaders or wetting agents ensure wetting of the surface to be sprayed by forming a stable liquid-solid interface (Frear, 1968). Many materials have been used including dried milk, powdered casein, dry blood gelatin and detergents (Angus and Luthy, 1971). Addition of adhesives or stickers ensures the formation of a resistant film on foliage (Frear, 1968). This is especially important for B. thuringiensis preparation since it is a "stomach poison" and must be ingested with the foliage.

The spores of B. thuringiensis on treated crops are highly susceptible to damage by exposure to sunlight (Pendleton, 1969) and chemical protectants are useful for their protection. Angus and Luthy (1971) mentioned several commercial formulations of the organism which have been treated to increase resistance to damage by sunlight. Greaves, Rowe, Redfern and Ayres (1968) described a protection system whereby spores and crystals are "encapsulated"; the product proved to be less sensitive to sunlight while toxicity was unaffected.

From time to time, it has been suggested that chemical pesticides and fungicides compatible with the spores and crystals can be combined for use in agriculture and forestry. Such additives, however, should not interfere with the activity of the

microbial insecticide, but knowledge of inhibitory or synergistic effects of such mixture is limited

The commercial B. thuringiensis preparations contain living spores of the organism so that after application in the field the bacteria could continue to propagate and provide a lasting insecticidal effect. However there is the potential danger that with repeated sprinkling for agricultural purposes the organism might accumulate and/or spread in the field to cause a disturbance of the ecological balance. Another danger is that the living organism may be carried by wind and cause damage in other agricultural areas. In order to avoid these risks, recent interest has centred on developing preparations free of viable spores yet preserving the crystal toxin. Two general methods for this have been investigated Firstly, extraction and purification of the crystalline toxin (Pendleton and Morrison, 1966; Gingrich, 1968; Somerville, 1973) Secondly, inactivation or killing of the spore without reducing crystal toxicity A French patent (International Mineral and Chemical Corp , 1966) described the use of electromagnetic radiation of 300-400 nm wavelength to inactivate the spores in the presence of a protective agent (propyl gallate) to prevent loss of crystal toxicity by oxidation. Utsumi (1971) has described a sterilization method. Recently Nishiitsutsuji-uwo et al (1975) have isolated 5 asporogenous mutant strains of B. thuringiensis, the crystals of which have the same insecticidal activity as that of original strains. They suggested that these mutants may serve as a biochemical starting material for a microbial insecticide based on δ-endotoxin and having no viable spores.

In the control of damage by spruce budworm, Smirnoff (1971, 1973, 1974) has shown that addition of the enzyme chitinase to a B. thuringiensis preparation increased the activity of the microbial insecticide, with earlier larval mortality and higher protection to foliage. Penetration of the spores in the haemolymph was accelerated by chitinase hydrolysing the chitinous layer of the insect gut. The author suggested that this formulation would be valuable in the control of spruce budworm.

4.4. Standardization

In the final formulation stage a microbial insecticide based on B. thuringiensis is a very complex material indeed. It must be assayed to determine its effectiveness and then standardized before it is marketed. The requirements for assay and standardization for the B. thuringiensis based microbial insecticide are as strict as for chemical insecticides and the problems involved are much more complex.

Producers of B. thuringiensis use viable count and/or counts of particles in stained film as a presumptive assay (Dulmage and Rhodes, 1971). However, these counts are only an apparent measure of activity (Burges, Fisher and Briggs, 1964). Burges and Thompson (1971) have shown the errors and variation involved and the low precision in counting the particles and viable spores of B. thuringiensis. Heimpel (1964) has reviewed other possible measurable entities related directly or indirectly to the spore and/or actual activity of the toxin, e.g. a measure of diplicolinic acid, which appears and rapidly increases in concentration during sporulation. A quantitative serological

test can also be used as an indicator that may be correlated with actual activity of the toxin preparation. So far, however, no good correlations have been found. A biological assay is at present the only method that measures the real activity of a preparation of B. thuringiensis.

Dulmage, Boening, Rehnborg and Hensen (1971) stated that spore count is not a reliable index of potency. Standardization of preparations containing the δ-endotoxin cannot be accomplished by spore count alone and a bioassay is needed.

In 1966, it was recommended that E-61, a formulation of B. thuringiensis from the Institut Pasteur, Paris, France, be adopted as an international standard and be assigned a potency of 1000 International Units (I.U.)/mg. It was also recommended that the antilepidopterous activity of all formulations of B. thuringiensis be standardized by bioassay, comparing the $LD_{50}$ values of these materials with E-61, and their potencies be expressed in I.U./mg (Burges, 1967).

Dulmage et al (1971) proposed a standardized bioassay for formulation of B. thuringiensis based on this I.U. unit against the cabbage looper, Trichoplusia ni.

However it is important to realize that bioassay is not the final and only answer to standardization, since different δ-endotoxin produced by different strains of B. thuringiensis differ in the spectra of their insecticidal activity. At present about 8 species of insects with widely differing responses to the insecticide have been recommended for assay work (Pendleton, 1969).

* * * * * * * * * *

The work described in this thesis was undertaken with a view to determine the optimum medium composition for maximum production of the crystal protein toxin by B. thuringiensis. The optimum conditions for growth and sporulation were also to be determined so that a comparison of the 3 processes could be made to determine whether there is any correlation among them.

Careful considerations in experimental design led to the opinion that unless a rational design was employed, it might be very difficult or even impossible to derive valid conclusions from the resulting experimental data. The conventional one-factor-at-a-time experimental technique was considered inefficient, uneconomical, and incapable of detecting "joint effects" or interactions among 2 or more nutrients. In solving the medium-screening problem, which is essentially an optimisation process, it was decided that Response Surface Methodology (RSM) should be used. This is an experimental strategy initially developed and described by Box and Wilson (1951). Hill and Hunter (1966) have given a brief review of some of the important features of the method. RSM is essentially a set of procedures involving experimental strategy, mathematical methods and statistical inference which, when combined, enable the experimenter to make an empirical exploration of the system under study where some feature of the system, the response, is influenced by a large number of inputs or independent variables. The experimenter is often interested in finding a suitable approximating function for the purpose of predicting future response. This is the mathematical model building phase which involves 3 major steps of performing statistically designed experiments, estimating the

coefficients in the response surface equation, usually approximated by a low order polynomial, and checking the adequacy of the equation or mathematical model. The next phase is the optimization phase where the response surface in the region of interest is studied so that values of the independent variables for an optimum response can be determined.

RSM has been employed with considerable success in a wide variety of situations, especially in the fields of chemistry and chemical engineering (Hill and Hunter, 1966). Practical applications of the method in other areas, e.g. agriculture, food and textile industries, are also cited by Hill and Hunter (1966). However, applications in biology are much fewer, especially in the field of microbiological studies. Thus a second purpose of the work in this thesis was to determine whether RSM can be used for microbial medium screening studies. If so, it could represent a great saving in materials and labour for such studies.

# EXPERIMENTAL PROCEDURES

## 1. Materials and methods

### 1.1. Equipment

Standard microbiological equipment was used throughout this work. No special apparatus was required.

### 1.2. Organism

All experiments were conducted using a strain of *Bacillus thuringiensis* var. Bakthane. This strain was obtained from Dr I.R. Pendleton, Microbiology Department, University of Glasgow, U.K.

The bacterium was maintained on nutrient agar slopes and stored at 5°C. Subculturing was done every three months.

### 1.3. Chemicals

All chemicals used were of analytical grade and were obtained from the following sources:

a) British Drug House Chemical N.Z. Limited, Palmerston North:
Ammonium chloride, Basic fuchsin, Folin and Ciocalteu's phenol reagent, glucose, magnesium sulphate, potassium dihydrogen orthophosphate, sodium carbonate, sodium tartrate.

b) Oxoid Limited, London, U.K.
Casein hydrolysate, nutrient agar, peptone water, yeast extract.

### 1.4. Semi-synthetic media

All media used contained the five basic nutrients: glucose,

magnesium sulphate, ammonium chloride, yeast extract and potassium dihydrogen orthophosphate. The actual concentration of each nutrient in a particular medium depended on the levels chosen for each particular series of experiments.

All media were adjusted to pH 7.5 with 1N NaOH before sterilization.

1.5. Sterilization of media

Media were sterilized at 15 psig for 15 min in an autoclave.

1.6. Inocula preparation and culture conditions

The inoculum for each series of experiment was prepared as follows: one loopful of the growth from a 24 h nutrient agar slope, held at 30°C, was used to inoculate a seed flask of medium (50 ml of medium/150 ml flask) having the same composition as that of the centre points for that particular series of experiments. The flask culture was incubated on a rotary agitator at 200 rpm and 30°C. This inoculum culture was ready for use after a 9 h period of growth when the culture was still in the exponential growth phase.

The nutritional studies were carried out in 250 ml Erlenmeyer flasks containing 100 ml batches of media. Flasks were inoculated with 1 ml of the inoculum culture and shaken for 72 h on a New Brunswick model G10 gyrotory shaker at setting 9. The shaker was installed in an incubation room with a constant temperature of 30 ± 1°C.

1.7. Staining technique and microscopic observation

A heat fixed smear was prepared from the culture and stained

with carbol fuchsin for 1 min, followed by rinsing with water and drying. Observation was performed using an Olympus Photomax light microscope with an oil immersion objective.

## 1.8. Assay methods

### 1.8.1. Total viable count and spore count

The pour plate method on nutrient agar was used after preparing serial dilutions in sterile peptone water (0.1% w/v). Duplicate plates of 3 dilutions were used for each determination.

For the spore count, dilutions were exposed to a temperature of 65°C for 15 min before plating out.

### 1.8.2. Growth rate

This was estimated by measuring the absorbance changes of the culture medium at 625 nm using a Hitachi (model 101) spectrophotometer. Readings were taken at hourly intervals during the exponential growth phase. All culture suspensions were diluted by a factor of 3 to avoid absorbances greater than 1.0 where Beer's Law is not obeyed. The growth rate was then estimated by calculating the slope of the absorbance curve during the exponential growth phase, using the linear regression method, and was expressed as change in absorbance units/h.

### 1.8.3. Protein determination

After 72 h, the culture had lysed, as judged by microscopic examination. The spores, crystals and cell debris were harvested by centrifugation (Sorvall SS-3 superspeed centrifuge) at 6000 x g for 15 min in 50 ml tubes containing 40 ml batches of culture. The

pellet collected was resuspended and washed twice in distilled water. The final pellet was resuspended in known volume of 30 to 40 ml 0.04 N NaOH and held for 30 min with regular agitation to dissolve the crystals in the spore-crystal mixture. The protein solution was then separated from the spores by a final centrifugation step and the volume adjusted to give a protein concentration of up to 500 µg/ml.

The concentration of the protein solution was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). This involved the addition of 5 ml of 0.1 N NaOH, containing 2% (w/v) sodium carbonate, to 1 ml of sample followed by 0.5 ml of Folin-Ciocalteau phenol reagent (previously diluted 1:1 with distilled water). After 30 min the absorbance value at 750 nm was measured using an Hitachi (model 101) spectrophotometer. The concentrations were now determined from a calibration curve prepared with casein.

1.8.4. Determination of toxicity

Larvae of cabbage white butterfly (Pieris rapae) were used as the target insects. These were collected from a market garden near Palmerston North. No control of age or state of health of the larvae was possible. However attempts were made to use active larvae of similar size. The protein solutions, after adjustment to pH 7 using 0.1 N HCl and dilution with distilled water to known concentration, were then tested on batches of 10 caterpillars kept in large glass petri dishes.

Cabbage leaves, cut to standard-sized pieces (approx. 2 cm x 2 cm), were treated with the toxin by immersing the leaves in the

protein solution for 20 min with regular agitation. The treated leaves were then offered to the larvae for feeding. A control group of caterpillars where the cabbage leaves had been dosed with distilled water was also established.

The mortality was evaluated on the 24th hour of the experiment, and results were expressed as the ratio of percentage dead in treatment over percentage dead in control.

2. Data processing

Multiple regression analysis was performed using a Burroughs 6700 computer of Massey University Computer Unit, using the B6700 BASIS subprogramme PROCEDURE MULTR.

Predictions from the constructed models for maximum responses were obtained from computations using a direct-search programme written in FORTRAN. Computations were also performed using the Burroughs 6700.

3. Experimental design

Typical kinetic patterns of fermentation are known to be non-linear, the most common ones being the exponential and the asymptotic curves. Most microbial growth and metabolism responses belong to these categories (Aiba, Humphrey and Millis, 1965). For this reason, linear regression with a first order model, which is useful only where there is little curvature in the response function, was considered inadequate for accurate description of the growth and protein toxin production of *B. thuringiensis* as functions of various nutrients. Thus it was

decided to investigate the use of a 2nd order approximating function for this experimental work.

Initial screening experiments to determine the important process variables were considered unnecessary, since the five nutrient variables chosen, namely glucose, magnesium sulphate, ammonium chloride, yeast extract and potassium dihydrogen orthophosphate are well established as essential and basic nutrients for bacterial growth and metabolism.

Experimental design for fitting a 2nd order response surface must involve at least 3 levels of each variable, so that all the coefficients in the mathematical model can be established. A $3^K$ factorial experiment was considered unsuitable in this case because a relatively large number of variables were under study ($K = 5$), and the number of experimental observations required would be excessive. Thus a central composite design (ccd), a more useful and versatile class of experimental design, was chosen for this work. The ccd is a special class of composite design devised by Box and Wilson (1951) as a workable alternative to the $3^K$ factorial system. It is essentially a $2^K$ factorial or fractional factorial design augmented by additional centre points and axial (or star) points to allow estimation of the coefficients of a 2nd order surface. It requires fewer experimental observations, is more flexible, and the independent variables are investigated at 5 levels rather than 3. It is often useful for building empirical models where, as in this case, not enough fundamental or theoretical knowledge of the underlying mechanisms are available to build a mechanistic model for the system. The experimental design can be made orthogonal and rotatable so that determination of the model

coefficients can be simplified and made independently. Also, the variance (i.e., accuracy) of the estimated response is a function only of the distance from the centre of the design and not of the direction. Another advantage of the ccd is that it allows effective study of multiple responses so that the various responses can be compared and evaluated for correlation.

3.1. Design matrix and the mathematical model

The concentrations of the 5 following nutrients were studied as the independent variables: glucose ($X_1$), magnesium sulphate ($X_2$), ammonium chloride ($X_3$), yeast extract ($X_4$) and potassium dihydrogen orthophosphate ($X_5$). The method for construction of the design matrix is given by Myers (1971). The actual concentrations of the X's were coded to the 5 levels -2, -1, 0, +1 and +2 in the usual fashion for a factorial design so that estimation of the coefficients in the model was simplified. Subsequent decoding of the variables in the model could be achieved using a set of coding equations.

The design matrix, in terms of the coded variables is given in Table 1.

The approximating second order mathematical model is given by an equation of the form:

$$Y = b_o + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_5 X_5 + b_{12} X_1 X_2 + b_{13} X_1 X_3$$
$$+ b_{14} X_1 X_4 + b_{15} X_1 X_5 + b_{23} X_2 X_3 + b_{24} X_2 X_4 + b_{25} X_2 X_5$$
$$+ b_{34} X_3 X_4 + b_{35} X_3 X_5 + b_{45} X_4 X_5 + b_{11} X_1 X_1 + b_{22} X_2 X_2$$
$$+ b_{33} X_3 X_3 + b_{44} X_4 X_4 + b_{55} X_5 X_5$$

Table 1:

Factor levels of experimental trials

| Trial no. | Coded design levels | | | | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ |
| 1 | -1 | -1 | -1 | -1 | +1 |
| 2 | +1 | -1 | -1 | -1 | -1 |
| 3 | -1 | +1 | -1 | -1 | -1 |
| 4 | +1 | +1 | -1 | -1 | +1 |
| 5 | -1 | -1 | +1 | -1 | -1 |
| 6 | +1 | -1 | +1 | -1 | +1 |
| 7 | -1 | +1 | +1 | -1 | +1 |
| 8 | +1 | +1 | +1 | -1 | -1 |
| 9 | -1 | -1 | -1 | +1 | -1 |
| 10 | +1 | -1 | -1 | +1 | +1 |
| 11 | -1 | +1 | -1 | +1 | +1 |
| 12 | +1 | +1 | -1 | +1 | -1 |
| 13 | -1 | -1 | +1 | +1 | +1 |
| 14 | +1 | -1 | +1 | +1 | -1 |
| 15 | -1 | +1 | +1 | +1 | -1 |
| 16 | +1 | +1 | +1 | +1 | +1 |
| 17 | -2 | 0 | 0 | 0 | 0 |
| 18 | +2 | 0 | 0 | 0 | 0 |
| 19 | 0 | -2 | 0 | 0 | 0 |
| 20 | 0 | +2 | 0 | 0 | 0 |
| 21 | 0 | 0 | -2 | 0 | 0 |
| 22 | 0 | 0 | +2 | 0 | 0 |
| 23 | 0 | 0 | 0 | -2 | 0 |
| 24 | 0 | 0 | 0 | +2 | 0 |
| 25 | 0 | 0 | 0 | 0 | -2 |
| 26 | 0 | 0 | 0 | 0 | +2 |
| 27 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | 0 |

where:

$Y$ = response or dependent variable

$X_i$'s = independent variables

$b_o$ = constant

$b_i$'s = first order coefficients (main effects)

$b_{ij}$'s $(i \neq j)$ = mixed quadratic coefficients (interaction terms).

$b_{ii}$'s = pure quadratic coefficients.

Three-factor or higher order interaction terms were assumed to be negligible. This assumption is valid and applicable in most practical cases.

The first 16 points in the design matrix are the usual half replicate of a $2^5$ factorial design for fitting a first order model. The two levels of each variable are coded to the usual -1, +1. These points are called cube points and they give estimates of the 2-factor interaction terms $b_{ij}$ $(i \neq j)$. The following 10 points (from the 17th to the 26th inclusive) are the axial points (or star points), two on each axis at a distance $\alpha = 2$ from the design origin, i.e. each variable is actually studied at 5 levels from -2 to +2. The remaining 6 points are the centre or origin of the design. The response at a centre point estimates $b_o$; the difference between the average of the responses at the 2 axial points on the ith axis and the average at the centre points gives an estimate of the pure quadratic coefficient $b_{ii}$.

# RESULTS AND DISCUSSION

## SECTION 1 PRELIMINARY INVESTIGATIONS

The purpose of these preliminary investigations was to obtain necessary information concerning the growth and toxin production of B. thuringiensis var. Bakthane in order that subsequent experiments could be planned with regard to culture conditions.

### 1.1. Staining

Firstly, it was considered necessary to be able to recognize and differentiate between spores and crystals so that the timing of cell growth, sporulation, crystal formation and cell lysis could be followed by microscopic examination.

A number of stains were tried, e.g., naphthalene black, amido black, crystal violet and malachite green, but carbol fuchsin was found to be the most satisfactory. The stained smear showed that the crystals stained deep red while the oval spores remained unstained except for their coloured margins (Fig. 1).

### 1.2. Growth studies

The aim of the initial experiments was to investigate certain shake flask culture conditions, namely aeration rate and incubation time, so that these could be standardized throughout the subsequent study.

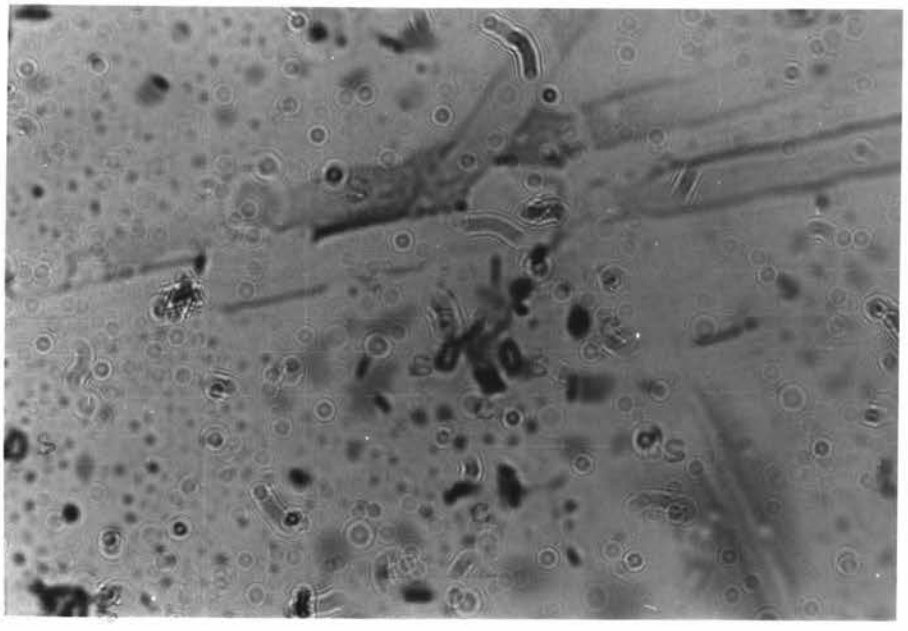



Fig. 1. Photomicrograph of Bacillus thuringiensis var. Bakthane (x2200). S = spore; C = protein crystal.

From the literature, it is well established that the optimum growth temperature for B. thuringiensis is around 30°C, while the optimum pH is in the neutral range of pH 6.9-7.5. In this study, semi-defined media were used rather than industrial media as this minimized the number of uncontrolled nutrient variables. Since the semi-defined media were not well buffered, and the pH of the media tends to decrease during the initial growth period, a high starting value of pH 7.5 was arbitrarily chosen. Temperature was maintained at 30°C.

Aeration is known to be one of the major factors influencing growth and crystal protein production by B. thuringiensis (Ribbons, 1966; Scherrer et al, 1973), but unfortunately it is difficult to monitor and control accurately on a shake flask scale. In a shake flask culture, the rate of absorption of oxygen into the medium is limited mainly by the following factors: diffusion of air through the cotton wool plug of the flask, solubility of oxygen in the liquid (which in turn depends on medium concentration, temperature and air partial pressure in the atmosphere) and finally the depth (and therefore the volume of fluid) in the shake flask. Under the culture conditions used, only the control of the liquid depth was possible, and this only to a certain extent, by controlling the speed of agitation. The swirling motion agitates the liquid and the centrifugal force on the medium displaces the fluid into a relatively thinner layer aerated by diffusion through the liquid surface. Thus in this study, it was necessary to ensure that sufficient agitation was maintained to facilitate the diffusion of air into the medium.

To determine the necessary agitation required, agitation speeds at settings ranging from 6 to 9 on the New Brunswick model G10 gyrotory shaker were investigated. The effect of agitation on the total viable counts for a number of media of widely varying composition was observed and the results are summarized in Table 2.

At setting 6, the counts were much lower than those obtained at settings 8 and 9, while there was no great difference between the latter 2 settings. Thus it was decided that for all further experiments an agitation speed of setting 9 would be used.

The size of the oval-shaped spores ranged from 0.5-0.7 μ wide and 1.5-2 μ long, while the crystals were of various shapes and sizes. The effect of the rate of aeration on crystal size was observed and this agreed with that reported by Scherrer et al (1973): under high aeration rate smaller crystals were obtained whereas a reduced oxygen supply led to relatively large crystals.

A typical growth curve (Fig. 2) shows that the exponential growth phase begins 4-5 h after inoculation. This lasts for a further 4 to 5 h and is followed by a long retardation phase. The stationary phase is not reached until around 65 to 72 h. Sporulation and the concomitant crystal formation were observed to commence at the beginning of the retardation phase. Autolysis of cells, together with the release of spores and crystals, occurs at around 21 h and is largely complete at 72 h. Since the medium used for these particular data was considered to be "rich", thus it was decided that all future experiments would be terminated at 72 h.

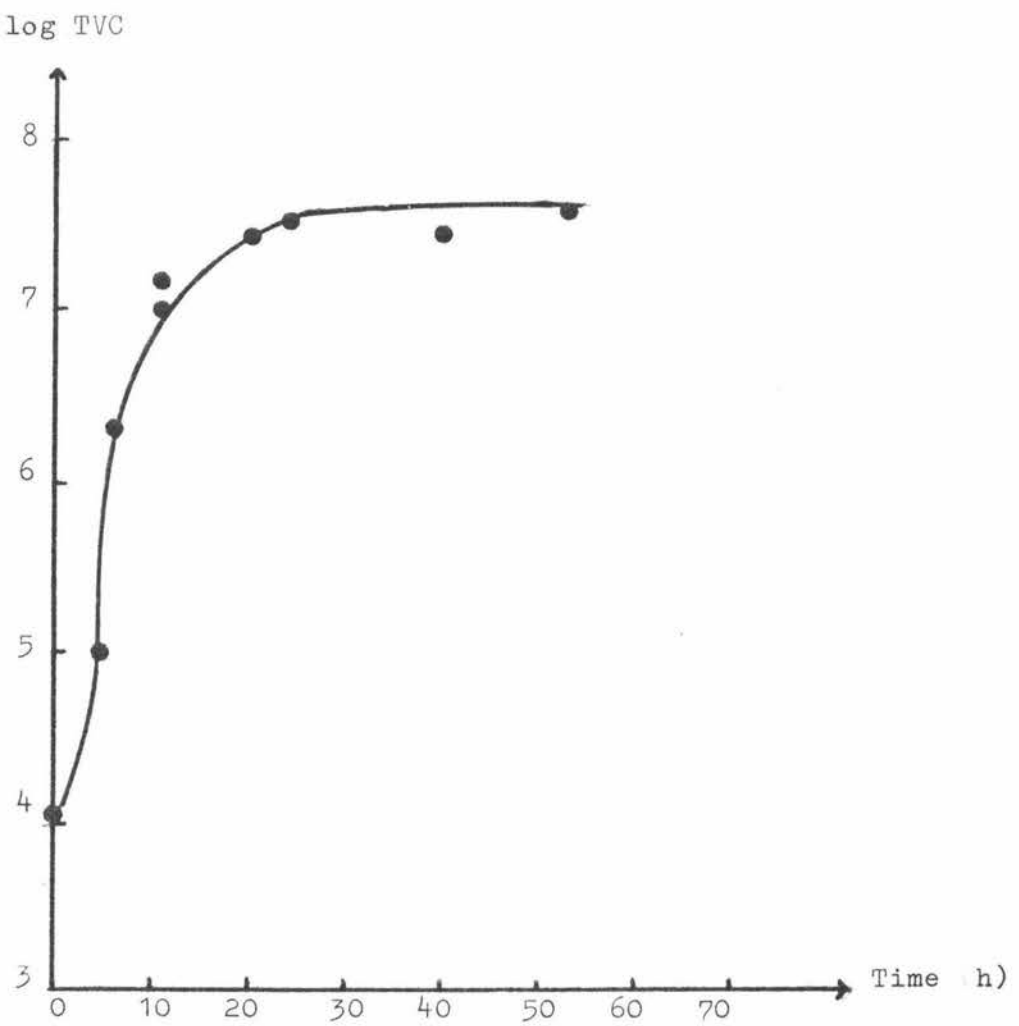



Fig. 2. Typical growth curve of B. thuringiensis var. bakthane in a medium containing glucose (3%), $NH_4Cl$ (0.5%), yeast extract (0.01%), $MgSO_4.7H_2O$ (0.005%) and $KH_2PO_4$ (0.1%), starting medium pH 7.5, incubation temperature at 30°C, agitation speed at setting 9.

TABLE 2: THE EFFECT OF AGITATION SPEED ON TOTAL VIABLE COUNTS

| Medium number | Total viable counts $x10^6$/ml | | |
|---|---|---|---|
| | agitation speed setting | | |
| | 6 | 8 | 9 |
| 1 | 7 | 15 | 14 |
| 6 | 22 | 120 | 119 |
| 7 | 3 | 95 | 105 |
| 8 | 19 | 35 | 34 |
| 9 | 20 | 277 | 261 |
| 17 | 198 | 350 | 270 |
| 18 | 13 | 50 | 71 |
| 23 | 0.008 | 35 | 34 |
| 24 | 171 | 270 | 294 |

Refer to Tables 1 and 3 for medium composition. The medium number corresponds to the trial number shown in Table 1. Table 3 defines the actual nutrient levels used.

1.3. Determination of toxicity

One of the overall purposes of this study was to determine optimum nutrient conditions for production of the crystal toxin by B. thuringiensis var. Bakthane. Hence a reliable method of measuring the toxicity was required. The most logical method to use would be an insect bioassay, since it is a direct and conclusive measure of the toxin activity. However it was considered that the methods of biological assay are too laborious, and the results subject too much error to be accurate and reproducible. Quantitative serological testing is time consuming and was considered impractical. Thus an attempt was made to show a direct relationship between the biological activity and a chemical assay of the protein of the crystal toxin. It was hoped that the time-consuming biological assay could be avoided and replaced by the simpler and more reproducible chemical assay. This was considered essential because of the large number of samples involved in each series of experiments.

The relationship between mortality rate (toxicity) and protein concentration is shown in Fig. 3. Under the experimental conditions used, there appears to be a threshold value for protein concentration. At concentrations up to 1.5 mg/ml the relative mortality remained at the low value of 1.7. From concentrations 1.5 to 2.5 mg/ml the mortality-dose curve rose significantly as concentration increased. Although the result shown here is by no means adequate for establishing a quantitative correlation between toxicity and crystal protein concentration, it provides a clear indication of the direct relationship between the

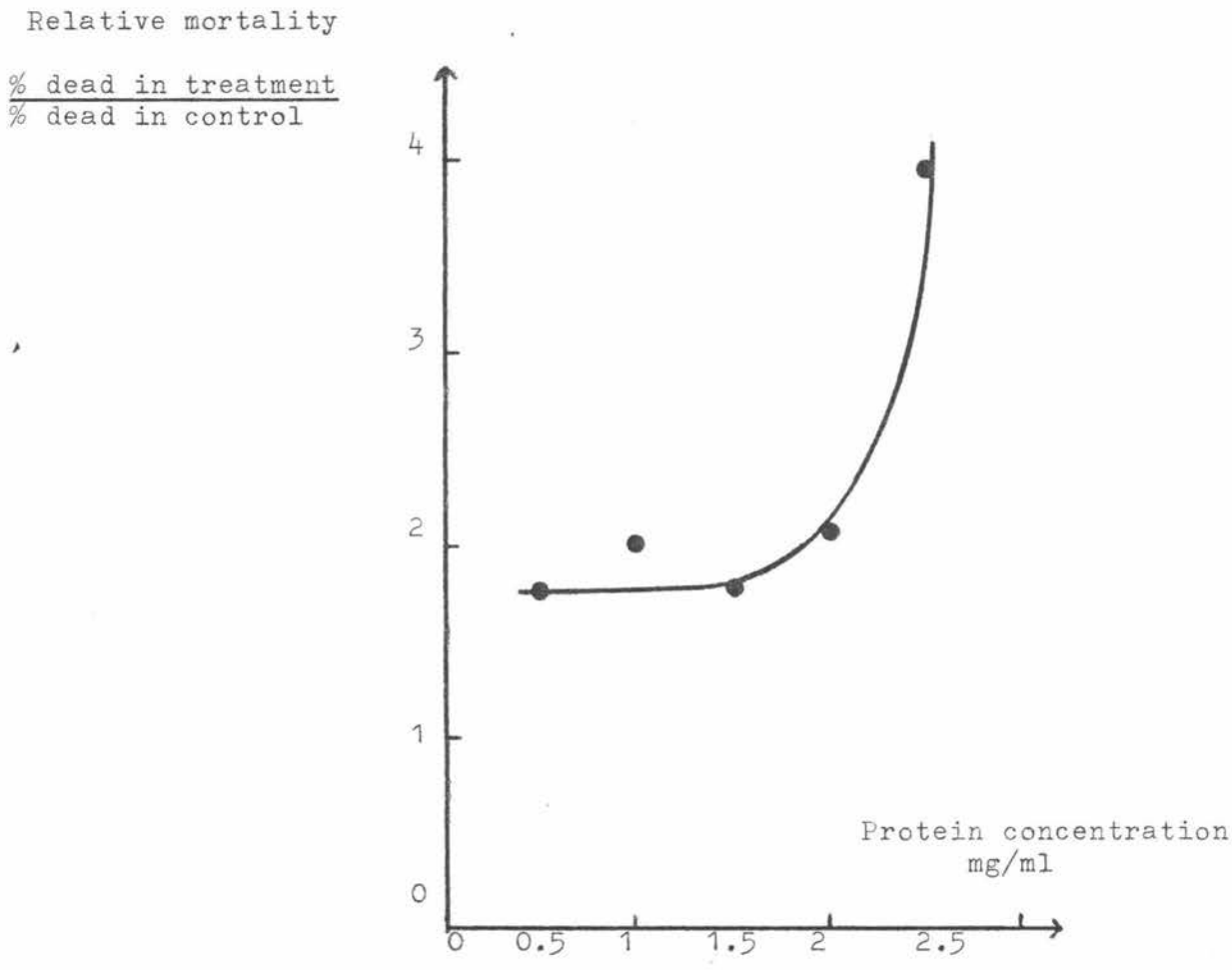



Fig. 3. Relative mortality vs Protein concentration. P. rapae larvae were used as target insect, experiment conducted as outline in "material and methods".

two. This agrees with results observed by Scherrer *et al* (1973).

Thus it was decided that in all further work the estimation of protein would be used as an indirect measure of crystal protein toxicity.

## SECTION 2 EXPERIMENT 1

Ideally, in a study of this type, the range of the nutrient variables should be carefully chosen so that the experiment can be conducted in the "region of optimum", thus allowing elaborate analysis to be performed on the data, and meaningful interpretation of the response system to be obtained. In this particular study, however, previous knowledge of the system and the vicinity of the optimum was rather limited. Thus the design region for this first experiment was chosen rather arbitrarily, in the hope that even if an optimum were not detected enough information would be obtained to successfully design a second, more meaningful, experiment. At the very least, this first experiment would provide experience with the techniques involved.

The levels of the nutrients were chosen so that they covered a reasonably wide range; the coded and measured levels for these independent variables are given in Table 3.

Note that equal spacing of the actual measured factor levels was chosen, e.g. one unit for the coded variable $X_1$ in the response function was assigned as the equivalent of 0.5% $(\frac{w}{v})$ in glucose concentration.

The experimental conditions were chosen as discussed in Section 1, namely an incubation temperature of 30°C, initial medium pH 7.5, agitation speed at setting 9 and incubation time of 72 h. The 32 experimental trials were performed in 4 separate "blocks" or groups, each block consisting of 8 randomly chosen trials.

TABLE 3: DEFINITION AND LEVELS OF INDEPENDENT VARIABLES

| Independent variable % w/v | Symbol | Factor levels | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | 1 | 2 |
| Glucose (G) | $X_1$ | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 |
| $MgSO_4.7H_2O$ (Mg) | $X_2$ | 0.0 | 0.005 | 0.01 | 0.015 | 0.02 |
| $NH_4Cl$ (N) | $X_3$ | 0.4 | 0.45 | 0.5 | 0.55 | 0.6 |
| Yeast extract (YE) | $X_4$ | 0.0 | 0.005 | 0.01 | 0.015 | 0.02 |
| $KH_2PO_4$ (P) | $X_5$ | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 |

Coding equations for converting coded levels into actual measured concentrations are given by the following:

$$X_1 = \frac{G - 3.5}{0.5}$$

$$X_2 = \frac{Mg - 0.01}{0.005}$$

$$X_3 = \frac{N - 0.5}{0.05}$$

$$X_4 = \frac{YE - 0.01}{0.005}$$

$$X_5 = \frac{P - 0.2}{0.1}$$

where G, Mg, N, YE and P are concentrations in % $(\frac{w}{v})$ of glucose, $MgSO_4.7H_2O$, $NH_4Cl$, yeast extract and $KH_2PO_4$ respectively.

The 3 responses studied were total viable count (TVC), spore count (SPC) and crystal protein concentration (PC). Raw experimental data are shown in Table 4. For the purpose of multiple regression analysis, figures for TVC and SPC were transformed into the more commonly used log values, since the count number/ml varied over too wide a range. The values for PC were kept unchanged and expressed in ug/ml, since they did not show such wide variation.

Results of multiple regression analysis and statistical analysis are given in Tables 5 and 6. In Table 5, the coefficient of determination shows the fraction of observed variation in response Y that can be explained by the model. Its complement is

TABLE 4: RAW (UNPROCESSED) EXPERIMENTAL RESULTS

| Trial number | TVC $x10^6$/ml | SPC $x10^6$/ml | PC ug/ml |
|---|---|---|---|
| 1 | 14.1 | 10.7 | 215 |
| 2 | 9.1 | 9.1 | 162 |
| 3 | 14.0 | 10.5 | 240 |
| 4 | 8.7 | 10.3 | 170 |
| 5 | 6.7 | 2.6 | 340 |
| 6 | 119.0 | 5.5 | 140 |
| 7 | 105.0 | 7.7 | 175 |
| 8 | 34.0 | 2.7 | 130 |
| 9 | 261.0 | 0.42 | 230 |
| 10 | 34.0 | 4.8 | 280 |
| 11 | 24.0 | 1.31 | 260 |
| 12 | 26.5 | 6.7 | 315 |
| 13 | 67.0 | 2.3 | 265 |
| 14 | 16.5 | 16.5 | 300 |
| 15 | 46.5 | 4.12 | 375 |
| 16 | 58.5 | 3.35 | 240 |
| 17 | 270.5 | 89.5 | 375 |
| 18 | 71.0 | 8.3 | 335 |
| 19 | 87.5 | 8.15 | 325 |
| 20 | 119.0 | 11.05 | 370 |
| 21 | 0.14 | 0.005 | 78 |
| 22 | 187.5 | 8.55 | 210 |
| 23 | 0.34 | 0.102 | 33 |
| 24 | 290.0 | 3.35 | 510 |
| 25 | 11.2 | 2.6 | 45 |
| 26 | 66.5 | 4.65 | 320 |
| 27 | 72.0 | 3.4 | 460 |
| 28 | 24.1 | 4.8 | 380 |
| 29 | 104.0 | 4.3 | 390 |
| 30 | 21.5 | 5.3 | 450 |
| 31 | 47.0 | 5.7 | 470 |
| 32 | 54.0 | 2.4 | 370 |

TABLE 5: REGRESSION COEFFICIENTS OF THE VARIABLES IN THE MODEL

| VARIABLE | Coefficient Response | | |
|---|---|---|---|
| | $Y_1$ | $Y_2$ | $Y_3$ |
| CONSTANT | 7.6493 | 6.5375 | 416.4205 |
| $X_1$ | -0.0783 | 0.0125 | -18.4583 |
| $X_2$ | 0.1175 | 0.0208 | 8.4583 |
| $X_3$ | 0.0175 | 0.0292 | 2.6250 |
| $X_4$ | 0.3408** | 0.0208 | 68.6250*** |
| $X_5$ | 0.3325** | 0.1792 | 14.8750 |
| $X_1X_2$ | 0.0700 | -0.0688 | 12.0625 |
| $X_1X_3$ | -0.0238 | -0.1188 | - 1.6875 |
| $X_1X_4$ | -0.0913 | 0.1688 | 23.3125 |
| $X_1X_5$ | 0.1088 | -0.0563 | -20.4375 |
| $X_2X_3$ | -0.0350 | -0.0563 | - 5.1875 |
| $X_2X_4$ | -0.1375 | -0.0938 | - 0.1875 |
| $X_2X_5$ | -0.1950 | -0.0438 | -18.9375 |
| $X_3X_4$ | -0.0713 | 0.0063 | 16.0625 |
| $X_3X_5$ | 0.1538 | -0.0688 | -13.9375 |
| $X_4X_5$ | -0.1763 | 0.1938 | 6.0625 |
| $X_1X_1$ | 0.1319 | 0.2375* | -12.6705 |
| $X_2X_2$ | -0.0456 | 0.0125 | -55.7955*** |
| $X_3X_3$ | 0.0982 | 0.1250 | -14.5455 |
| $X_4X_4$ | -0.1543 | -0.1750 | -33.5455* |
| $X_5X_5$ | -0.2268 | -0.1625 | -65.4205*** |
| Coefficient of determination | 0.7066 | 0.6120 | 0.8048 |

Responses studied: $Y_1$ = log TVC
$Y_2$ = log SPC
$Y_3$ = PC

* coefficient significant at 90% level
** " " " 95% "
*** " " " 99% "

TABLE 6: ANALYSIS OF DATA VARIANCE

Table 6.1. For $Y_1$ = log TVC

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 11.4871 | 0.5744 | 1.32 |
| Error | 11 | 4.7700 | 0.4336 | |
| Total | 31 | 16.2571 | 0.5244 | |

Confidence level of F (20, 11) = 67.76%

Table 6.2. For $Y_2$ = log SPC

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 6.6539 | 0.3327 | 0.87 |
| Error | 11 | 4.2183 | 0.3835 | |
| Total | 31 | 10.8722 | 0.3507 | |

Confidence level of F (20, 11) = 37.57%

Table 6.3. For $Y_3$ = PC

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 378401.4356 | 18920.0718 | 2.27 |
| Error | 11 | 91760.4394 | 8341.8581 | |
| Total | 31 | 470161.8750 | 15166.5 | |

Confidence level of F (20, 11) = 91.80%

the unexplained variation fraction due to pure error and lack-of-fit. Both $Y_1$ = log TVC and $Y_2$ = log SPC have very low coefficients of 0.7066 and 0.6120 respectively; $Y_3$ = PC has the highest value of 0.8048, but this is still low compared with the more acceptable practical level of 0.90 or higher.

Analysis of variance for the equations relating the 3 responses to the 5 nutrients are summarized in Tables 6.1, 6.2 and 6.3. The F ratios between the mean square due to regression and mean square due to error are 1.32, 0.87 and 2.27 for $Y_1$, $Y_2$ and $Y_3$ respectively. Only the F value for $Y_3$ is significant at the 10% level (confidence level = 91.8%). This means that for $Y_1$ and $Y_2$ the regression was not significant and did not fit the observed data.

For $Y_3$, since the regression was significant only at a rather low level of confidence of 91.8%, a lack-of-fit test was carried out. This was performed by making a comparison, in terms of an f-ratio, between the lack-of-fit mean square, and the pure experimental error mean square. In this instance, the $f_{6.5}$ value is 6.81. Since this is greater than $f_{6.5,\ 0.05} = 4.95$, there is highly significant lack-of-fit. Thus it was concluded that the model for $Y_3$ was not an adequate representation of the observed data, and the same conclusion applied to $Y_1$ and $Y_2$. Further statistical analysis of the results was considered to be not worthwhile, but closer examination of the data was considered justified in an attempt to understand the situation.

From Table 4, the average TVC was in the order of $10^7$/ml,

with the highest value at $2.9 \times 10^8$/ml. The average for SPC was in the order of $10^6$/ml, with the highest value at $8.95 \times 10^7$/ml. The results show that both growth and sporulation were poor. Production of the crystal protein ranged from 33-510 ug/ml.

Residuals for the 3 responses are shown in Tables 7.1, 7.2 and 7.3. The plots of isolated responses against each individual independent variable (with the other 4 variables at the 0 level) are shown in Figs. 4, 5, and 6. Responses at $X_i = 0$ is the average of the 6 centre points. Responses at $X_i = \pm 2$ are the actual observed single axial points. Responses at $X_i = \pm 1$ are not the actual observed experiment points, but are average of the 8 points in the half replicate of the $2^5$ factorial, with $X_i$ levels at +1 or -1. These average points are shown just for the sake of comparison.

Examination of the tables of residuals and Figs. 4, 5 and 6 reveals that most of the largest residuals occur at observations with independent variables at the -2 and +2 levels. This lack-of-fit indicates that the concentration ranges of nutrient variables covered were too wide.

The general pattern of the response curves appears to be asymptotic (Figs. 4, 5 and 6), and the observed data were mainly on the plateau region 'cd' of the curve as illustrated in Fig. 7.

For the response $Y_1$ (log TVC), over the range of glucose used, it is possible that the high glucose concentration inhibited growth. This can be shown by the negative coefficient for $X_1$, and by Fig. 4.1. One possible explanation is that acetate and

TABLE 7.1: TABLE OF RESIDUALS FOR $Y_1$ = log TVC

| Trial No. | Actual | Predicted | Residual |
|---|---|---|---|
| 1 | 7.1400 | 6.7652 | 0.3748 |
| 2 | 6.9500 | 6.4311 | 0.5189 |
| 3 | 7.1400 | 6.7027 | 0.4373 |
| 4 | 6.9400 | 6.5136 | 0.4264 |
| 5 | 6.8200 | 6.8177 | 0.0023 |
| 6 | 8.0700 | 8.0786 | -0.0086 |
| 7 | 8.0200 | 8.1102 | -0.0902 |
| 8 | 7.5300 | 7.4761 | 0.0539 |
| 9 | 8.4100 | 8.3494 | 0.0606 |
| 10 | 7.5300 | 7.4802 | 0.0498 |
| 11 | 7.3800 | 7.4119 | -0.0319 |
| 12 | 7.4200 | 7.3077 | 0.1123 |
| 13 | 7.5600 | 8.0269 | -0.4669 |
| 14 | 7.2100 | 7.5327 | -0.3227 |
| 15 | 7.6600 | 8.0644 | -0.4044 |
| 16 | 7.7600 | 8.1752 | -0.4152 |
| 17 | 8.4300 | 8.3337 | 0.0963 |
| 18 | 7.8500 | 8.0204 | -0.1704 |
| 19 | 7.9400 | 8.0070 | -0.0670 |
| 20 | 8.0700 | 8.0770 | -0.0070 |
| 21 | 5.1400 | 6.0770 | -0.9370 |
| 22 | 8.2700 | 7.4070 | 0.8630 |
| 23 | 5.5300 | 6.3504 | -0.8204 |
| 24 | 8.4600 | 7.7137 | 0.7463 |
| 25 | 7.0400 | 7.2320 | -0.1920 |
| 26 | 7.8200 | 7.7020 | 0.1180 |
| 27 | 7.8500 | 7.6493 | 0.2007 |
| 28 | 7.3800 | 7.6493 | -0.2693 |
| 29 | 8.0100 | 7.6493 | 0.3607 |
| 30 | 7.3300 | 7.6493 | -0.3193 |
| 31 | 7.6700 | 7.6493 | 0.0207 |
| 32 | 7.7300 | 7.6493 | 0.0807 |

TABLE 7.2: TABLE OF RESIDUALS FOR $Y_2$ = log SPC

| Trial No. | Actual | Predicted | Residual |
|---|---|---|---|
| 1 | 7.0300 | 6.7417 | 0.2583 |
| 2 | 6.9600 | 6.3500 | 0.5500 |
| 3 | 7.0200 | 6.7083 | 0.2917 |
| 4 | 7.0100 | 6.4750 | 0.5250 |
| 5 | 6.4100 | 6.4833 | -0.0833 |
| 6 | 6.7400 | 6.5500 | 0.1500 |
| 7 | 6.8000 | 6.9083 | -0.1083 |
| 8 | 6.4300 | 6.2167 | 0.1833 |
| 9 | 5.6200 | 5.6667 | -0.0667 |
| 10 | 6.6800 | 6.4333 | 0.1667 |
| 11 | 6.1200 | 6.1917 | -0.0917 |
| 12 | 6.8300 | 6.6000 | 0.2300 |
| 13 | 6.3600 | 6.7667 | -0.4067 |
| 14 | 7.2200 | 7.3750 | -0.1550 |
| 15 | 6.6100 | 7.0333 | -0.4233 |
| 16 | 6.5300 | 6.7000 | -0.1700 |
| 17 | 7.9500 | 7.4625 | 0.4875 |
| 18 | 6.9200 | 7.5125 | -0.5925 |
| 19 | 6.9100 | 6.9792 | -0.0692 |
| 20 | 7.0400 | 7.0958 | -0.0558 |
| 21 | 4.7200 | 5.5292 | -0.8092 |
| 22 | 6.9300 | 6.2458 | 0.6742 |
| 23 | 5.0000 | 5.7958 | -0.7958 |
| 24 | 6.5300 | 5.8792 | 0.6508 |
| 25 | 6.4100 | 6.5458 | -0.1358 |
| 26 | 6.6700 | 6.6292 | 0.0408 |
| 27 | 6.5300 | 6.5375 | -0.0075 |
| 28 | 6.6800 | 6.5375 | 0.1425 |
| 29 | 6.6300 | 6.5375 | 0.0925 |
| 30 | 6.7200 | 6.5375 | 0.1825 |
| 31 | 6.7600 | 6.5375 | 0.2225 |
| 32 | 6.3800 | 6.5375 | -0.1575 |

TABLE 7.3: TABLE OF RESIDUALS FOR $Y_3$ = PC

| Trial No. | Actual | Predicted | Residual |
|---|---|---|---|
| 1 | 215.0000 | 196.8598 | 18.1402 |
| 2 | 162.0000 | 92.0265 | 69.9735 |
| 3 | 240.0000 | 170.1932 | 69.8068 |
| 4 | 170.0000 | 168.9432 | 1.0568 |
| 5 | 340.0000 | 279.6932 | 60.3068 |
| 6 | 140.0000 | 148.4432 | -8.4432 |
| 7 | 175.0000 | 183.6098 | -8.6098 |
| 8 | 130.0000 | 86.7765 | 43.2235 |
| 9 | 230.0000 | 202.1932 | 27.8068 |
| 10 | 280.0000 | 320.9432 | -40.9432 |
| 11 | 260.0000 | 301.1098 | -41.1098 |
| 12 | 315.0000 | 304.2765 | 10.7235 |
| 13 | 265.0000 | 315.6098 | -50.6098 |
| 14 | 300.0000 | 298.7765 | 1.2235 |
| 15 | 375.0000 | 373.9432 | 1.0568 |
| 16 | 240.0000 | 307.6932 | -67.6932 |
| 17 | 375.0000 | 402.6553 | -27.6553 |
| 18 | 335.0000 | 328.8220 | 6.1780 |
| 19 | 325.0000 | 352.9886 | -27.9886 |
| 20 | 370.0000 | 363.4886 | 6.5114 |
| 21 | 78.0000 | 124.9886 | -46.9886 |
| 22 | 210.0000 | 184.4886 | 25.5114 |
| 23 | 33.0000 | 144.9886 | -111.9886 |
| 24 | 510.0000 | 419.4886 | 90.5114 |
| 25 | 45.0000 | 176.3220 | -131.3220 |
| 26 | 320.0000 | 210.1553 | 109.8447 |
| 27 | 460.0000 | 416.4205 | 43.5795 |
| 28 | 380.0000 | 416.4205 | -36.4205 |
| 29 | 390.0000 | 416.4205 | -26.4205 |
| 30 | 450.0000 | 416.4205 | 33.5795 |
| 31 | 470.0000 | 416.4205 | 53.5795 |
| 32 | 370.0000 | 416.4205 | -46.4205 |

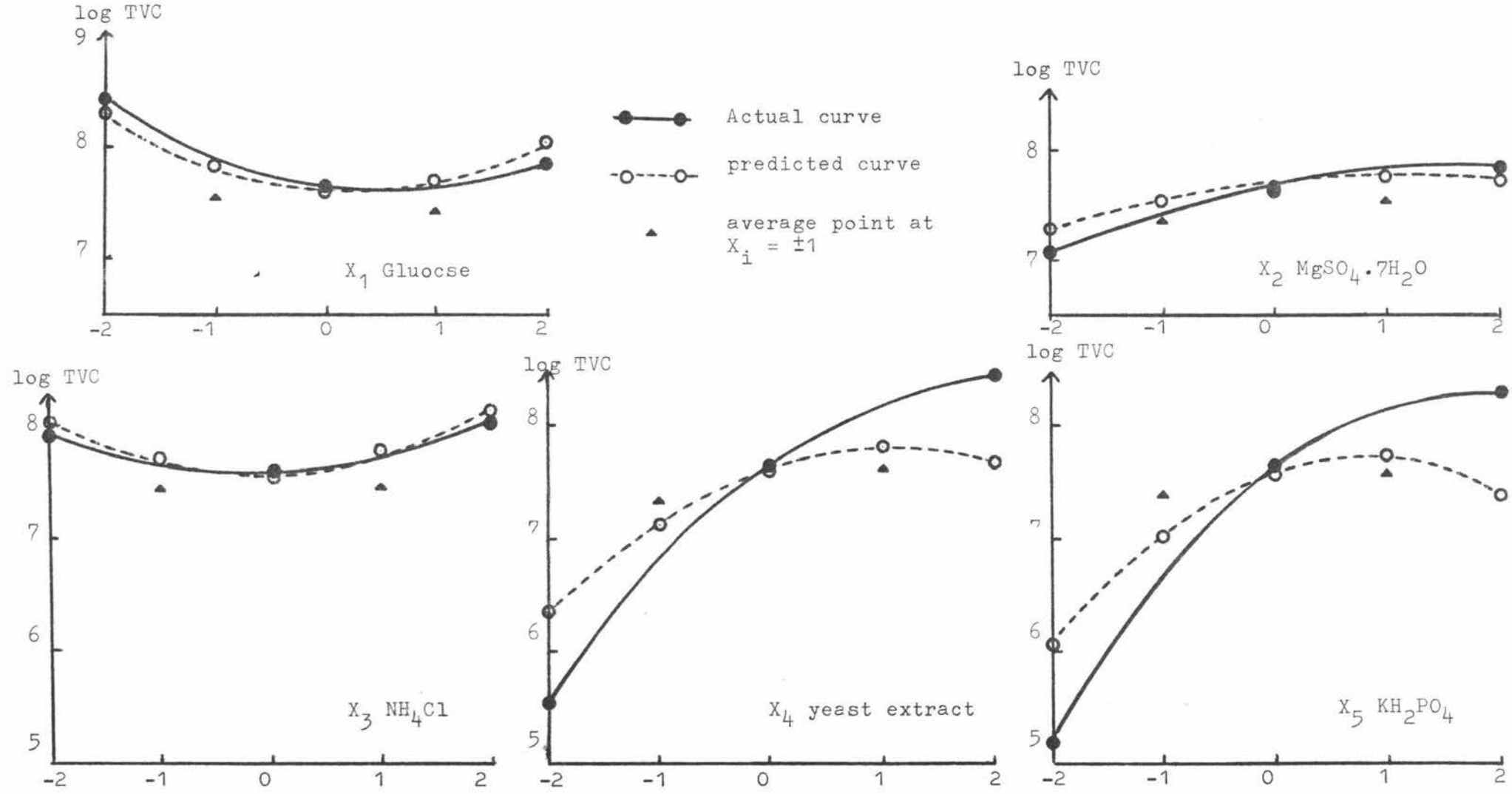



Fig. 4. log TVC as a response of each individual nutrient coded level. Other nutrients were kept constant at coded zero levels.

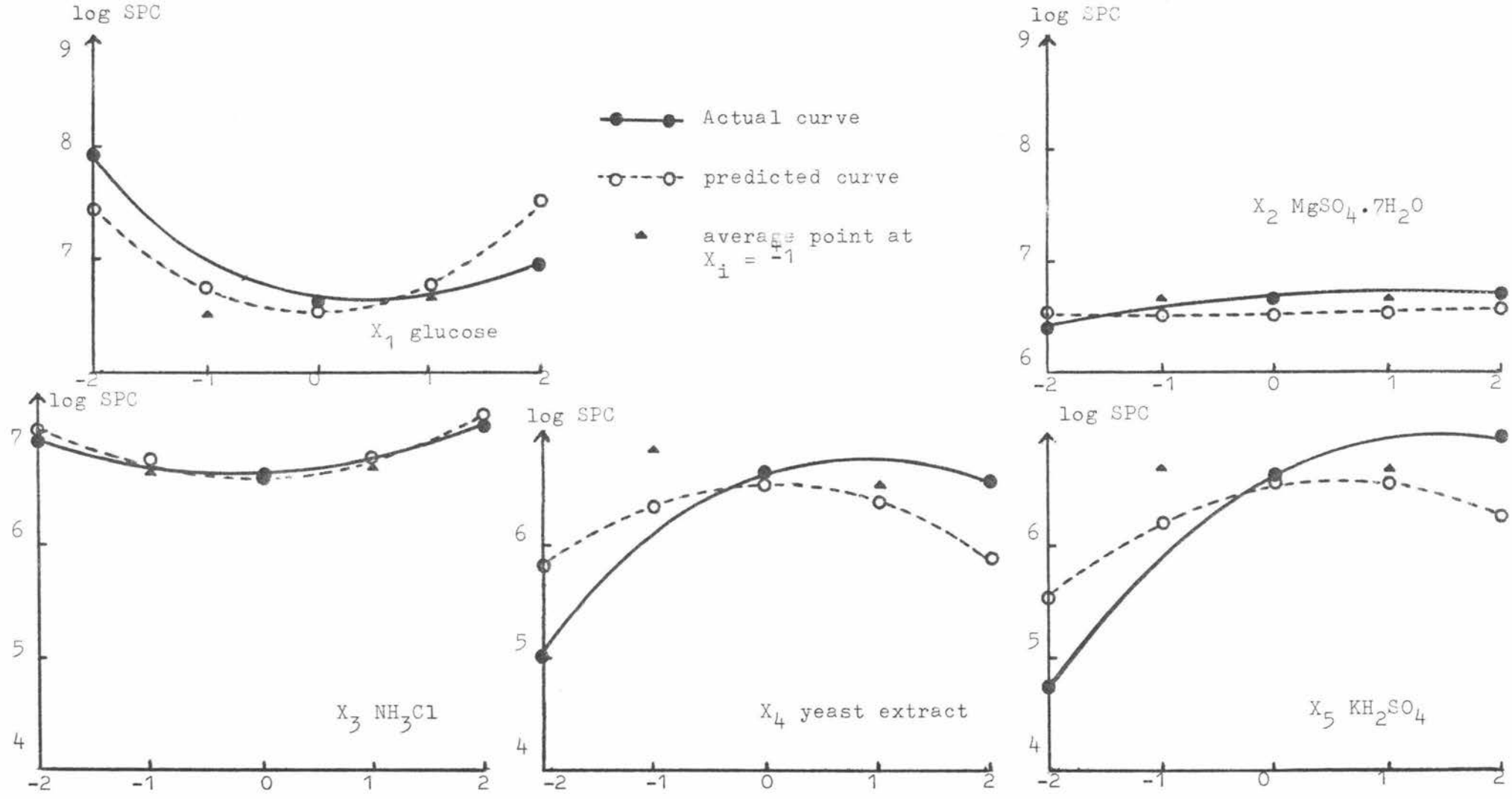



Fig. 5. log SPC as a response of each individual nutrient coded level. Other nutrients were kept constant at coded zero levels.

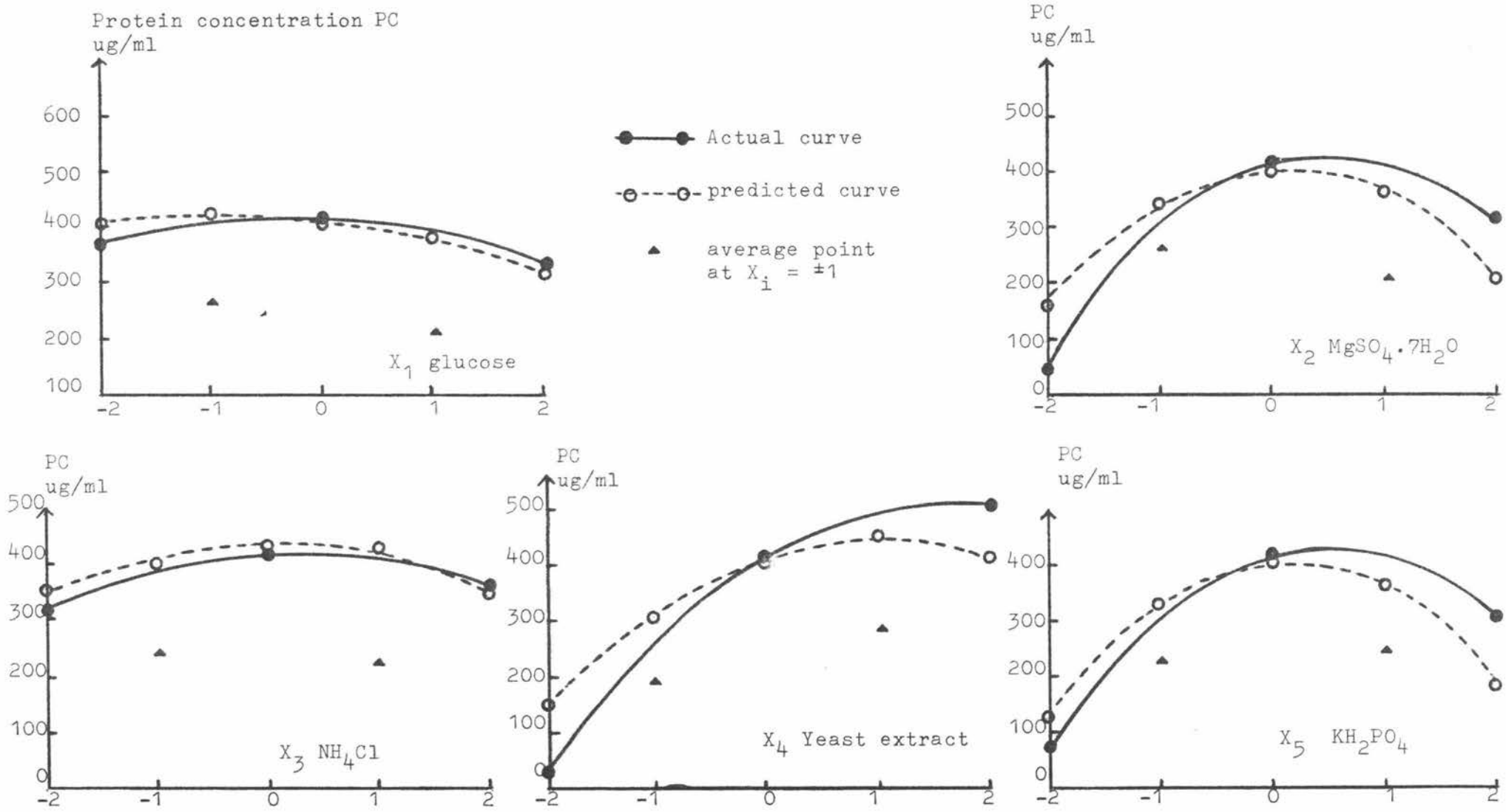



Fig. 6. Protein concentration as a response of each individual nutrient coded level.

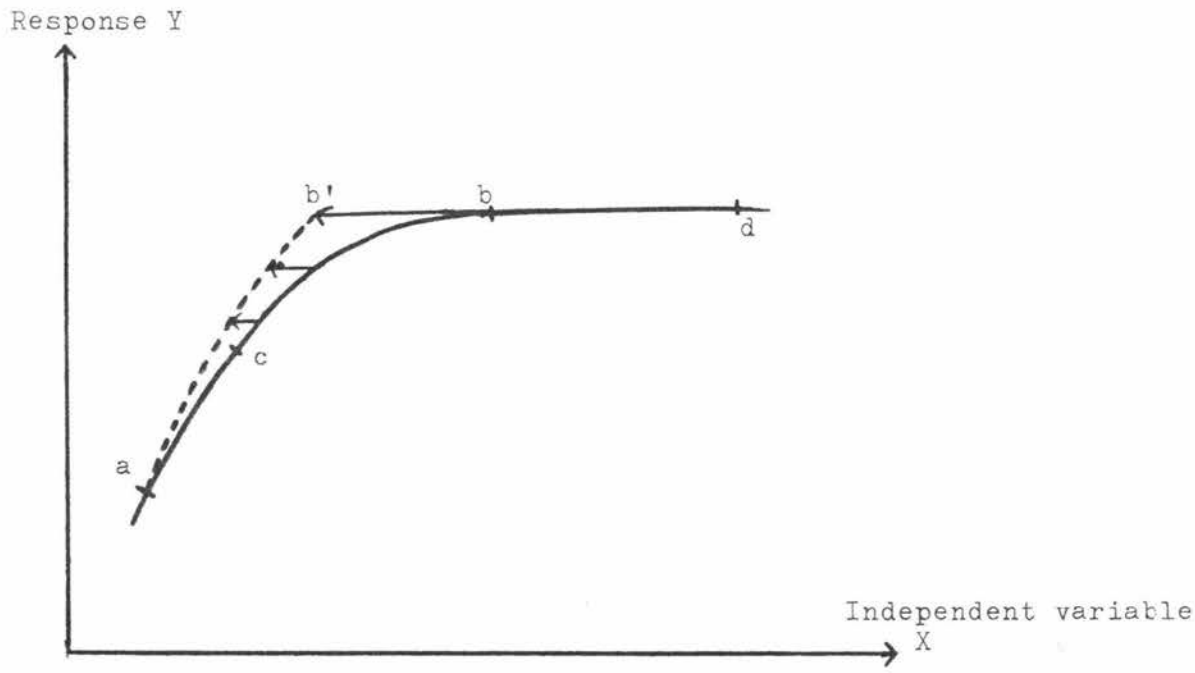



Fig. 7. General asymptotic pattern for the responses studied.

pyruvate accumulated from initial utilization of glucose, and were not metabolized in the later stages because of the high glucose concentration present. Thus the resulting low pH caused inhibition. The curve of $Y_1$ vs $NH_4Cl$ (Fig. 4.3)appears to follow the pattern of that for glucose (Fig. 4.1). Examination of the regression coefficients in Table 5 shows that none of the coefficients differ significantly from zero except for $X_4$ and $X_5$, which are significant at the 95% level. Evidently the levels of the two corresponding nutrients used, namely yeast extract and $KH_2PO_4$, were rather low for growth, since the curves of $Y_1$ vs $X_4$ and $Y_1$ vs $X_5$ (Figs. 4.4 and 4.5) have not reached a maximum. The level of $MgSO_4$ seemed to be sufficient under the conditions used, since a change in its concentration above the 0 level did not cause any further increase in $Y_1$.

The effects of all the nutrients on $Y_2$ (log SPC) are similar to that for $Y_1$. None of the regression coefficients are significant except for the single case of $X_1X_1$. Fig. 5.1 shows that the inhibition of higher glucose concentration to sporulation seemed to be more profound than that to growth. The levels of yeast extract and $KH_2PO_4$ used seemed to have reached the optimum, since higher concentrations did not increase the spore count any further.

For the crystal protein production, $Y_3$ (PC) has reached a maximum value at the levels of glucose and $NH_4Cl$ used. Yeast extract has a very significant coefficient at the 99% level, i.e. the protein concentration is greatly affected by the yeast extract concentration. From Fig. 6.4, it appears that further

increase in yeast extract concentration might result in further increase in the crystal protein production. The curves shown in Figs. 6.2 and 6.5 indicate that around the 0 levels for $MgSO_4$ and $KH_2PO_4$, protein production reached a maximum, since a further increase in substrate resulted in a fall in the response. The two highly significant pure quadratic terms $X_2^2$ and $X_5^2$ describe the importance of this curvature in the response to $X_2$ and $X_5$.

For all 3 responses, it is interesting to note that none of the coefficients for the 2-factor interaction terms $X_iX_j (i<j)$ are significant. This is possibly an indication that, in general, the levels of most of the nutrients chosen were too high, and the effect of substrate limitations was minimized.

Correlation coefficients were calculated to test whether there is any linear relationship between any two of the 3 responses. The scattergrams are shown in Figs. 8, 9 and 10 together with the corresponding correlation coefficients r's. All the r's were very low and the scattergram confirmed that there was no obvious linear relationship among the responses.

To summarize, the 2nd order response model assumed for the chosen region of the nutrient variables did not describe well the observed data for the 3 responses studied. There are two possible explanations for this lack-of-fit. Firstly, the responses appeared to be asymptotic while the 2nd order model can only best describe responses which are linear or parabolic in nature. Secondly, the nutrient concentration levels were not

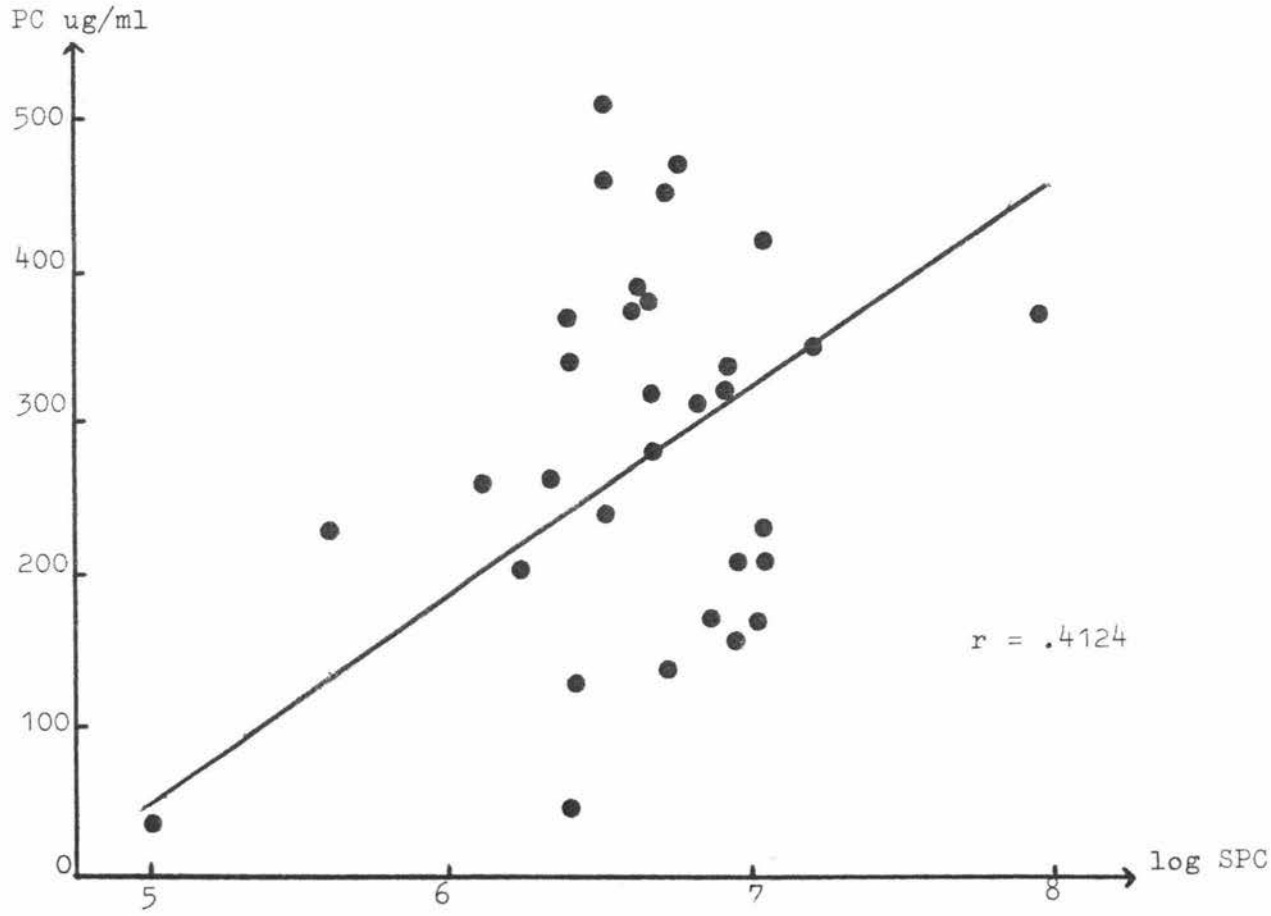



Fig. 8. Protein concentration (PC) vs log SPC

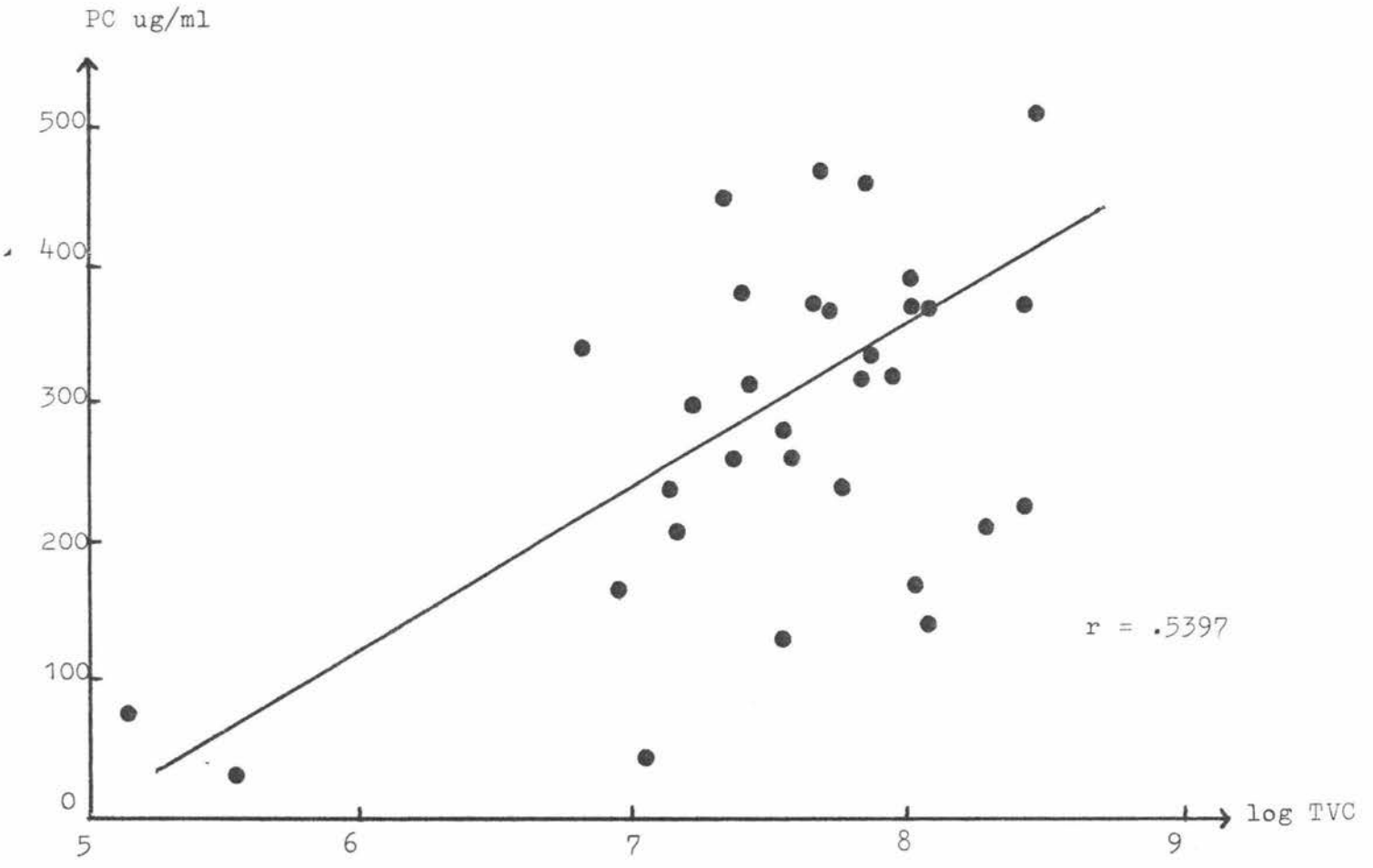



Fig. 9. PC vs log TVC

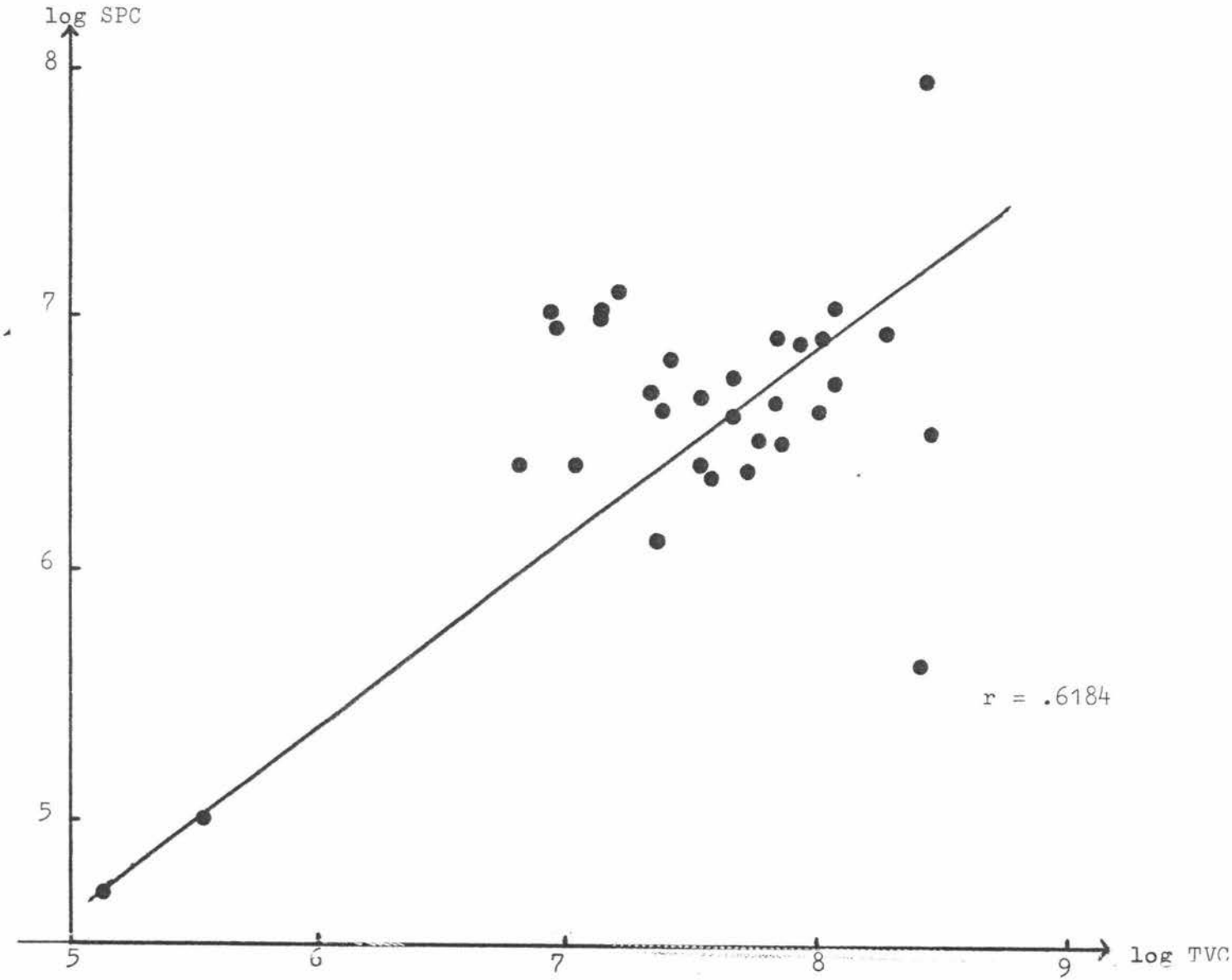



Fig. 10. log SPC vs log TVC

well chosen. In general, the levels for glucose and $NH_4Cl$ were too high; the level of yeast extract was definitely low for growth and crystal protein production; $KH_2PO_4$ level was also low for growth, while the level of $MgSO_4$ seemed to be in the correct range for protein production but had minimal effect on growth and sporulation.

Although the 2nd order model did not describe well the observed responses, it was still considered the model of choice, since to fit the asymptotic regression requires very complex methods and techniques, especially when a rather large number of independent variables are studied. Thus it could still be possible to use the simpler 2nd order model to approximate the response functions, provided the levels and ranges of the nutrient variables could be readjusted such that the narrower region as shown in Fig. 7 could be covered where the response could be approximated as parabolic. Transformation operations could also be carried out on the responses and/or independent variables, since this could be helpful in minimizing the lack of fit in the model.

## SECTION 3 EXPERIMENT 2

From the results and conclusions derived from the first experiment, the levels and ranges of the nutrients for this second experiment were readjusted as shown in Table 8. The concentration of glucose was lowered considerably to the range of 0.5-2.5% ($\frac{w}{v}$), compared with the previous range of 2.5-4.5%. The concentration for $NH_4Cl$ was also lowered to the new range 0.1-0.4% (cf. 0.4-0.6%). The concentration of yeast extract was increased significantly to a high level and range of 0.005-0.1%. The maximum level for $KH_2PO_4$ was increased only slightly to 0.5%, while the range for $MgSO_4.7H_2O$ remained unchanged.

At this point it was decided that an additional response, the growth rate, would be studied in this second experiment. The growth rate was chosen as a more reliable and reproducible measure of growth by the bacterium, as compared with the total viable count where large errors are involved in the pour plate count method.

Results from Experiment 1 also suggested that a transformation of the dependent and independent variables might be helpful in improving the empirical model. Transformation of the responses or dependent variables, if required, can always be performed after collecting the experimental raw data; but transformation of the independent variables needs to be performed before running the experiment, i.e. transformation is part of the experimental design. Referring to Fig. 7, the "ab" region of the

TABLE 8: DEFINITION AND LEVELS OF INDEPENDENT VARIABLES FOR EXPERIMENT 2

| Independent variable % w/v | Symbol | Factor levels | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | +1 | +2 |
| Glucose (G) | $X_1$ | 0.5 | 0.75 | 1.12 | 1.67 | 2.5 |
| $MgSO_4.7H_2O$ (Mg) | $X_2$ | 0.00125 | 0.0025 | 0.005 | 0.01 | 0.02 |
| $NH_4Cl$ (N) | $X_3$ | 0.1 | 0.14 | 0.2 | 0.28 | 0.4 |
| Yeast extract (YE) | $X_4$ | 0.005 | 0.011 | 0.022 | 0.047 | 0.1 |
| $KH_2PO_4$ (P) | $X_5$ | 0.1 | 0.15 | 0.22 | 0.335 | 0.5 |

response curve was to be modified to obtain a closer approximation to a linear or parabolic curve. It was decided that one way of doing this was to use equal spacing of the log values of the nutrient concentrations, instead of equal spacing of the actual values themselves. For example, from Table 8 we have

$\log G$ (at $X_1$=+2) - $\log G$ (at $X_1$=+1) = $\log G$ (at $X_1$=+1) - $\log G$(at $X_1$=0)

Thus in the plot of response Y vs the coded variable $X_i$ (Fig. 7), the upper part of"ab' would be "condensed" on the $X_i$ dimension and be "shifted" to the left in the"ab"direction.

Coding equations for the five nutrients are given by the following:

$$X_1 = \frac{\log G - .048}{0.175}$$

$$X_2 = \frac{\log Mg + 2.301}{0.301}$$

$$X_3 = \frac{\log N + 0.698}{0.151}$$

$$X_4 = \frac{\log YE + 1.65}{0.325}$$

$$X_5 = \frac{\log P + 0.650}{0.175}$$

The raw experimental data are shown in Table 9. As in the previous experiment, total viable count and spore counts were converted to log values, while transformation of the protein concentration (PC) and growth rate (GR) was considered unnecessary. Results of multiple regression analysis and analyses of variances are given in Tables 10 and 11.

In Table 10, the coefficient of determination for log TVC

TABLE 9: RAW EXPERIMENTAL RESULTS FOR EXPERIMENT 2

| Trial no. | TVC $x10^6$/ml | SPC $x10^6$/ml | PC µg/ml | GR Abs unit/h |
|---|---|---|---|---|
| 1 | 145 | 19 | 74 | 0.031 |
| 2 | 35 | 41 | 221 | 0,049 |
| 3 | 177 | 12.7 | 123 | 0,046 |
| 4 | 203 | 23 | 459 | 0.035 |
| 5 | 88 | 11 | 244 | 0.054 |
| 6 | 225 | 24 | 126 | 0.028 |
| 7 | 255 | 43 | 530 | 0.051 |
| 8 | 68 | 22 | 264 | 0.053 |
| 9 | 37 | 42 | 630 | 0.187 |
| 10 | 42 | 22 | 272 | 0.103 |
| 11 | 55 | 21 | 209 | 0.136 |
| 12 | 87 | 8.9 | 300 | 0.115 |
| 13 | 119 | 22 | 273 | 0.171 |
| 14 | 37 | 13 | 481 | 0.160 |
| 15 | 5.4 | 86 | 376 | 0.186 |
| 16 | 76 | 60 | 974 | 0.162 |
| 17 | 76 | 35 | 272 | 0.112 |
| 18 | 21 | 16.4 | 625 | 0.069 |
| 19 | 75 | 13.3 | 155 | 0.082 |
| 20 | 67 | 26 | 317 | 0.076 |
| 21 | 62 | 27 | 268 | 0.017 |
| 22 | 35 | 26 | 539 | 0.103 |
| 23 | 24 | 20 | 395 | 0.029 |
| 24 | 252 | 17 | 710 | 0.311 |
| 25 | 184 | 14.5 | 443 | 0.093 |
| 26 | 84 | 24 | 238 | 0.081 |
| 27 | 78 | 26 | 502 | 0.075 |
| 28 | 84 | 28 | 546 | 0.086 |
| 29 | 45 | 34 | 545 | 0.105 |
| 30 | 82 | 25 | 527 | 0.080 |
| 31 | 37 | 29 | 532 | 0.105 |
| 32 | 43 | 42 | 527 | 0.102 |

TABLE 10: REGRESSION COEFFICIENTS OF THE VARIABLES IN EXPERIMENT 2

| VARIABLE | Coefficient Response | | | |
|---|---|---|---|---|
| | $Y_1$=logTVC | $Y_2$=logSPC | $Y_3$=PC | $Y_4$=GR |
| CONSTANT | 7.7351 | 7.4608 | 532.6591 | 0.0923 |
| $X_1$ | -0.0394 | -0.0441* | 56.0000*** | -0.0101** |
| $X_2$ | -0.0064 | 0.0530** | 51.5833*** | -0.0005 |
| $X_3$ | -0.0349 | 0.0391 | 63.4167*** | 0.0140*** |
| $X_4$ | -0.0678 | 0.0213 | 87.6667*** | 0.0599*** |
| $X_5$ | 0.1871** | 0.0482* | -5.5000 | -0.0065* |
| $X_1X_2$ | 0.0934 | -0.0454 | 55.0000*** | 0.0031 |
| $X_1X_3$ | 0.0487 | -0.0172 | 12.8750 | 0.0024 |
| $X_1X_4$ | 0.1012 | -0.1049*** | 27.5000 | -0.0077* |
| $X_1X_5$ | -0.0382 | 0.0606* | 53.2500*** | 0.0022 |
| $X_2X_3$ | -0.1517 | 0.1832*** | 70.3750*** | 0.0048 |
| $X_2X_4$ | -0.0834 | 0.0271 | -31.7500* | -0.0028 |
| $X_2X_5$ | -0.0003 | 0.0508 | 121.2500*** | 0.0063 |
| $X_3X_4$ | -0.0553 | 0.0557* | 25.3750 | 0.0071 |
| $X_3X_5$ | 0.1315 | 0.0427 | 49.8750*** | 0.0032 |
| $X_4X_5$ | -0.0092 | -0.0244 | -24.7500 | -0.0012 |
| $X_1X_1$ | -0.0116 | -0.0063 | -23.1591* | -0.0005 |
| $X_2X_2$ | 0.0507 | -0.0338 | -76.2841*** | -0.0034 |
| $X_3X_3$ | 0.0051 | 0.0046 | -34.4091** | -0.0082** |
| $X_4X_4$ | 0.0607 | -0.0348 | 2.8409 | 0.0193*** |
| $X_5X_5$ | -0.0133 | -0.0335 | -50.1591*** | -0.0014 |
| coefficient of determination | 0.5930 | 0.8968 | 0.9651 | 0.9730 |

* significant at 90% level

** " " 95% "

*** " " 99% "

TABLE 11: ANALYSIS OF DATA VARIANCE IN EXPERIMENT 2

Table 11.1: For $Y_1$ = log TVC

| Source | Degree of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 2.3834 | 0.1192 | 0.80 |
| Error | 11 | 1.6356 | 0.1487 | |
| Total | 31 | 4.0190 | | |

Confidence level of F (20, 11) = 32.06%

Table 11.2: For $Y_2$ = log SPC

| Source | Degree of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 1.2599 | 0.0630 | 4.78 |
| Error | 11 | 0.1450 | 0.0132 | |
| Total | 31 | 1.4049 | | |

Confidence level of F (20, 11) = 99.47%

Table 11.3: For $Y_3$ = PC

| Source | Degree of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 1176231.6278 | 58811.5814 | 15.19 |
| Error | 11 | 42591.8409 | 3871.9855 | |
| Total | 31 | 1218823.4688 | | |

Confidence level of F (20, 11) = 100.00%

Table 11.4: For $Y_4$ = GR

| Source | Degree of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 0.1121 | 0.0056 | 19.79 |
| Error | 11 | 0.0031 | 0.0003 | |
| Total | 31 | 0.1151 | | |

Confidence level of F (20, 11) = 100.00%

has a very low value of 0.5930, i.e. only just over half of the observed variation in TVC could be explained by the regression. This poor fitting by the model is further confirmed by the results of analysis of variance (Table 11.1). The F-ratio for log TVC is low (0.80) and has a very low confidence level of 32.06%. Thus there is a very significant lack-of-fit in the model, and the results for TVC were not analysed further.

The coefficient of determination for log SPC is only 0.8968, this also is not very satisfactory. Coefficients for protein concentration (PC) and growth rate (GR) are 0.9651 and 0.9730 respectively. Analysis of the variances (Tables 11.2, 11.3, 11.4) show that the F-ratio's are highly significant (confidence level of F at 99.47%, 100%, 100% levels for log SPC, PC and GR respectively). Lack-of-fit tests were carried out, and the corresponding f-ratio's determined to be 2.58, 26.79 and 2.00 (cf. $f_{6,5,0.05} = 4.95$). Thus it was concluded that the second order regression model now explains reasonably well the 3 responses log SPC, PC and GR, although there is a significant lack-of-fit in the case of PC. These results show a great improvement over those obtained from the first experiment, and this is confirmed by examination of the isolated plots of Y vs $X_i$ (Figs. 11, 12 and 13).

The regression coefficients shown in Table 10 give estimates of the effects of the individual nutrients, the interactions between them and the quadratic effects which describe the curvature in the responses. Relative importance of the different effects was studied by examining the magnitude of these regression

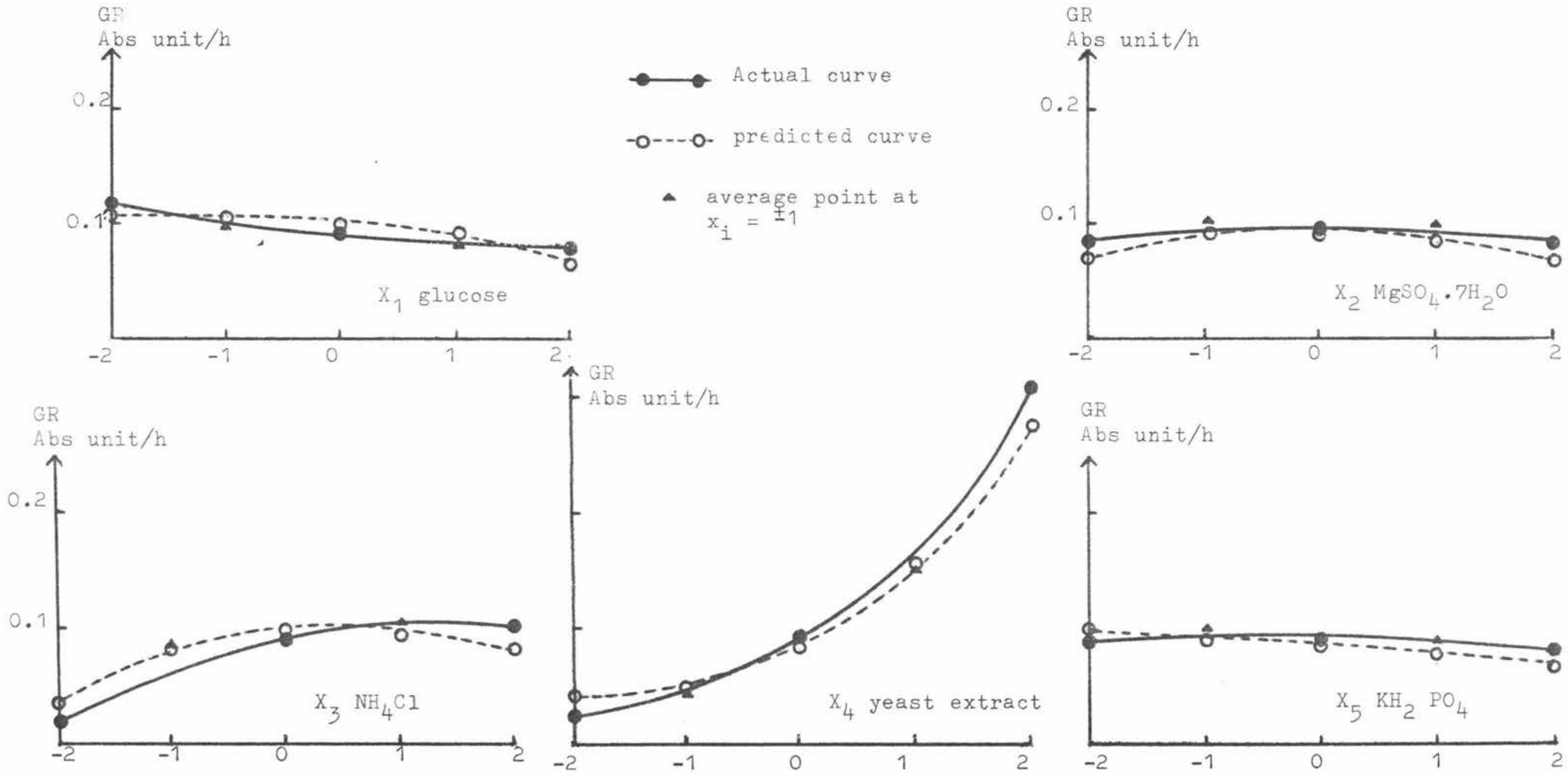



Fig. 11. Growth rate GR as a response of each individual nutrient coded level.

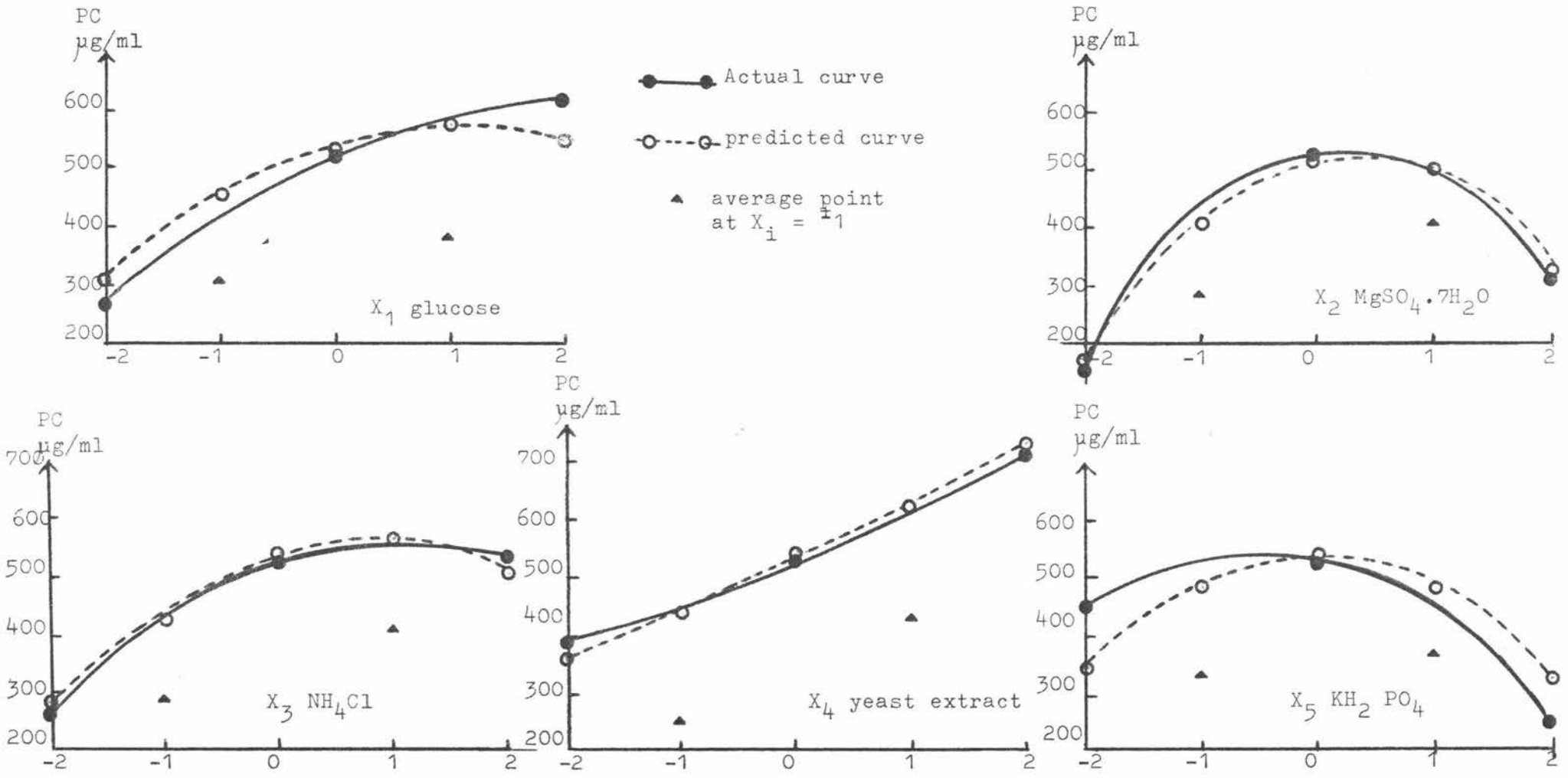



Fig. 12. Protein concentration PC as a response of each individual nutrient coded level.

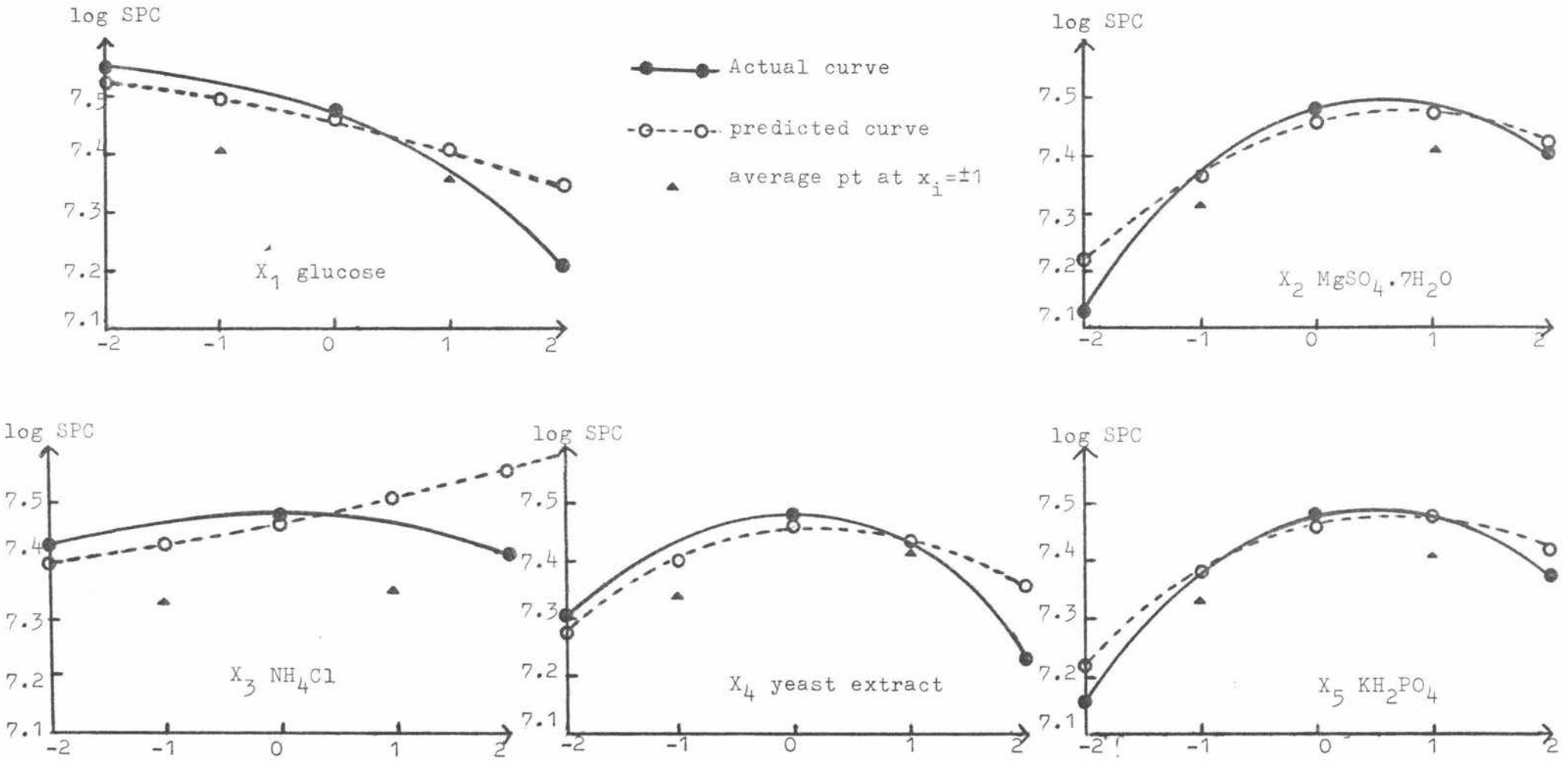



Fig. 13. log SPC as a response of each individual nutrient coded level.

coefficients together with the results of the significance tests. The +ve or -ve sign of the coefficient indicates a stimulatory or inhibitory effect over the experimental range studied.

Comparing the results of the significance tests indicates that the 3 responses studied, namely log SPC, PC and GR, all have quite different nutritional requirements. As in Experiment 1, calculated correlation coefficients and scattergrams did not indicate any linear relationship between any of the responses mentioned above.

For GR, $NH_4Cl$ and yeast extract are the most important nutrients, but there was no significant interaction between the two. Glucose and $KH_2PO_4$ also have significant effects and there appears to be some interaction between glucose and yeast extract (the coefficient for $X_1X_4$ is significant at 100% level). Possibly, metabolism of glucose required one or more of the nutrilites present in yeast extract. $MgSO_4.7H_2O$ seems to have a minimal effect on GR over the concentration range used.

Sporulation is quite different from growth. $NH_4Cl$ and yeast extract have no major effects over the experimental range studied but $MgSO_4 7H_2O$, $KH_2PO_4$ and glucose are more important. Interaction between glucose-yeast extract, glucose-$KH_2PO_4$ and $MgSO_4.7H_2O$-$NH_4Cl$ seem to be even more important than the main effects. This suggests that sporulation is dependent not only on the levels of the individual nutrients, but on interactions between them. For example, the optimum level of $MgSO_4.7H_2O$ for sporulation varies dependently on the level of N.

Results for crystal protein synthesis indicate that there are strong interactions between the nutrients. All the main effects studied were significant, except for $KH_2PO_4$, and most of them seem to interact with each other. $KH_2PO_4$ although not significant in itself, does influence the optimum levels for glucose, $MgSO_4.7H_2O$ and $NH_4Cl$. Thus the optimum level of any particular nutrient is very dependent on the levels of the other nutrients.

Substituting the appropriate coefficients for each response into the general second order equation, a regression equation representing the response was obtained. For example, the response function for growth rate (GR) is given by:

$$\begin{aligned} Y4 = {} & 0.0923 - 0.101\,X_1 - 0.0005X_2 + 0.0140X_3 + 0.0599X_4 \\ & - 0.0065X_5 + 0.0031X_1X_2 + 0.0024X_1X_3 - 0.0077X_1X_4 \\ & + 0.0022X_1X_5 + 0.0048X_2X_3 - 0.0028X_2X_4 + 0.0063X_2X_5 \\ & + 0.0071X_3X_4 + 0.0032X_3X_5 - 0.0012X_4X_5 - 0.0005X_1^2 \\ & - 0.0034X_2^2 - 0.0082X_3^2 + 0.0193X_4^2 - 0.0014X_5^2 \end{aligned}$$

By differentiating the derived equations and equating the differentials to zero, the coordinates (or composition) of the stationary points were predicted. These are shown in Table 12 together with the estimated responses. However, these stationary points are not necessarily those that maximize the responses, since a stationary point can be either a maximum, a minimum or a saddle point. To learn more about the nature of the stationary point requires plotting of contours of constant response, followed by a reduction of the response surface function

to a canonical form which is more easily and clearly analysed and interpreted.

Before starting the canonical analysis, a direct search method was used to ascertain the highest value for each response estimated by the regression equation around the experimental region with each nutrient concentration ranging from the -2 to +2 level. Results of the direct search are shown in Table 13, which gives the highest responses estimated within the region covered, together with the nutrient composition.

Comparison of the values in Tables 12 and 13 shows that the stationary points in Table 12 are not the maximum points. The stationary point for GR could be a minimum since the predicted $Y_0$ = 0.029 Abs units/h corresponds with the lowest value observed in the experiment (see Table 9, trial no. 6). The nature of the stationary points for log SPC and PC is not clear, but obviously they are neither maximum nor minimum points in the experimental region studied. At this stage it was decided that it was not essential to perform the canonical analysis, since in this experimental work only the maximum points were of interest.

The results and analysis obtained so far indicated that it was not possible to search for the maximum responses using the present nutrient concentration levels, i.e. the maxima appear to be outside the ranges used. Thus subsequent studies were centered on determining how adequate the regression models describe and predict responses within the limited experimental region, with independent variables covering the -2 to +2 range.

TABLE 12: COORDINATES (IN CODED VALUES) OF THE STATIONARY POINTS FOR GR, PC AND log SPC AS PREDICTED BY THE REGRESSION MODELS

| Response | $X_{1,0}$ (Glucose) | $X_{2,0}$ ($MgSO_4$) | $X_{3,0}$ ($NH_4Cl$) | $X_{4,0}$ (yeast extract) | $X_{5,0}$ ($KH_2PO_4$) | Predicted $Y_0$ |
|---|---|---|---|---|---|---|
| Growth rate GR | -2.44 | +2.23 | +1.24 | -1.04 | +2.75 | 0.029 Abs unit/h |
| Protein concentration PC | -1.53 | +0.11 | -0.88 | -4.12 | -0.16 | 0.271 µg/ml |
| log SPC | -0.07 | -0.22 | -0.44 | -0.104 | +0.238 | 7.4527 $ml^{-1}$ |

TABLE 13: OPTIMUM NUTRIENT COMPOSITIONS IN % w/v FOR PREDICTED HIGHEST RESPONSES WITHIN THE EXPERIMENTAL REGION. CORRESPONDING CODED FACTOR LEVELS SHOWN IN PARENTHESES

| Response | $X_1$ Glucose | $X_2$ $MgSO_4$ | $X_3$ $NH_4Cl$ | $X_4$ yeast extract | $X_5$ $KH_2PO_4$ | Predicted highest response |
|---|---|---|---|---|---|---|
| Growth rate GR | 0.5(-2) | 0.0012(-2) | 0.23(0.4) | 0.1(+2) | 0.1(-2) | 0.397 Abs unit/h |
| Protein concentration PC | 2.5(+2) | 0.02(+2) | 0.4(+2) | 0.1(+2) | 0.5(+2) | 1750 µg/ml |
| log SPC | 0.5(-2) | 0.02(+2) | 0.4(+2) | 0.1(+2) | 0.34(+1) | 9.2607 $ml^{-1}$ |

From Table 13, the predicted highest responses are much higher than any of the experimental points observed in Table 9. Thus further isolated trials were carried out to check the validity of these predicted points. The results are shown in Table 14. In general, there are considerable discrepancies between the predicted highest values and the actual observed ones. For example, for GR the predicted value is 0.397 Abs unit/h, but the actual value obtained experimentally is 0.335 Abs unit/h. However, this still represents an increased value compared with the highest experimental observation of 0.311 Abs unit/h in Table 9. The discrepancy is even higher for PC and log SPC.

Further experiments were also performed to examine the effect of glucose on growth rate, protein production and sporulation, where the actual and predicted response curves would be compared. The results are shown in Figs. 14, 15 and 16.

As expected, the protein production increased with increase in glucose concentration, but the actual increase was not as great as that predicted (Fig. 15). Under the experimental conditions used, the protein production reached a highest value of around 1360 μg/ml and appeared to reach a plateau at a glucose concentration of 2.5% ($\frac{W}{V}$). This asymptotic response could not be detected by the predicted curve where the PC value kept increasing to the level of 1750 μg/ml (Fig. 15).

From Fig. 14, it is very obvious that under the experimental conditions and glucose levels used, glucose inhibited growth (as measured by the growth rate). However, discrepancy

TABLE 14: COMPARISONS OF PREDICTED AND ACTUAL HIGHEST RESPONSES WITHIN THE EXPERIMENTAL REGION

| Response | Predicted value | Actual value | Highest experimental points in Table 9 |
|---|---|---|---|
| growth rate GR Abs unit/h | 0.397 | 0.336 | 0.311 |
| protein concentration PC µg/ml | 1750 | 1360 | 974 |
| log SPC | 9.26 | 7.90 | 7.90 |

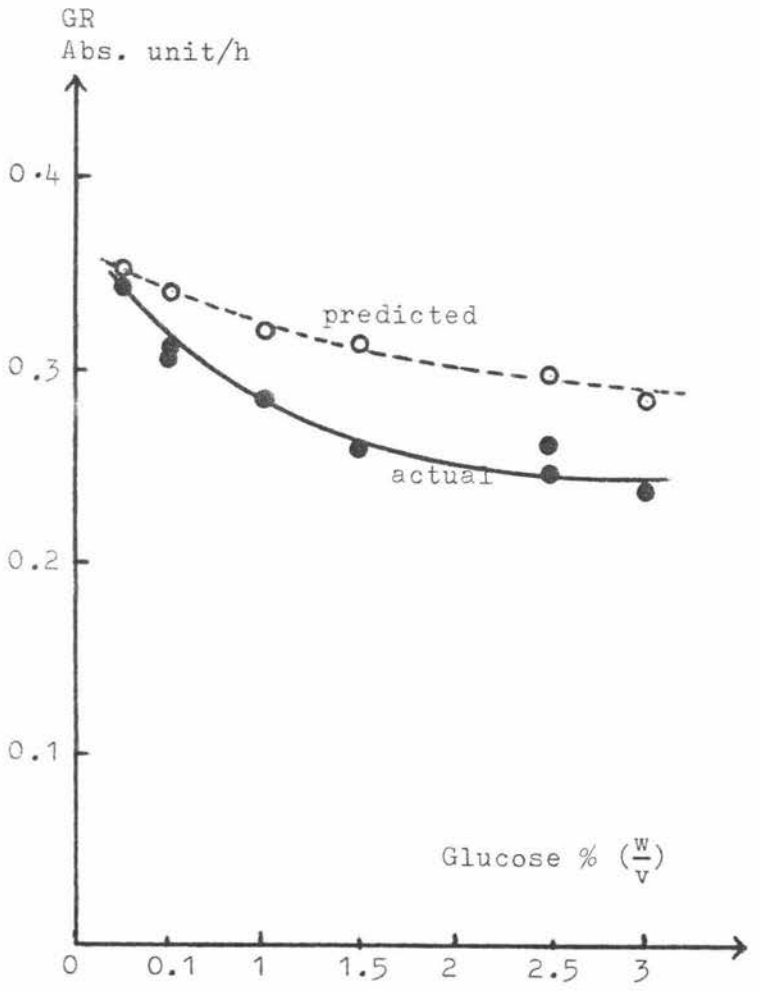



Fig. 14. The effect of glucose concentration on growth rate GR. Basal medium composition % ($\frac{W}{V}$), $MgSO_4.7H_2O$ = .00125%, $NH_4Cl$ = 0.23%, yeast extract = 0.1%, $KH_2PO_4$ = 0.1%.

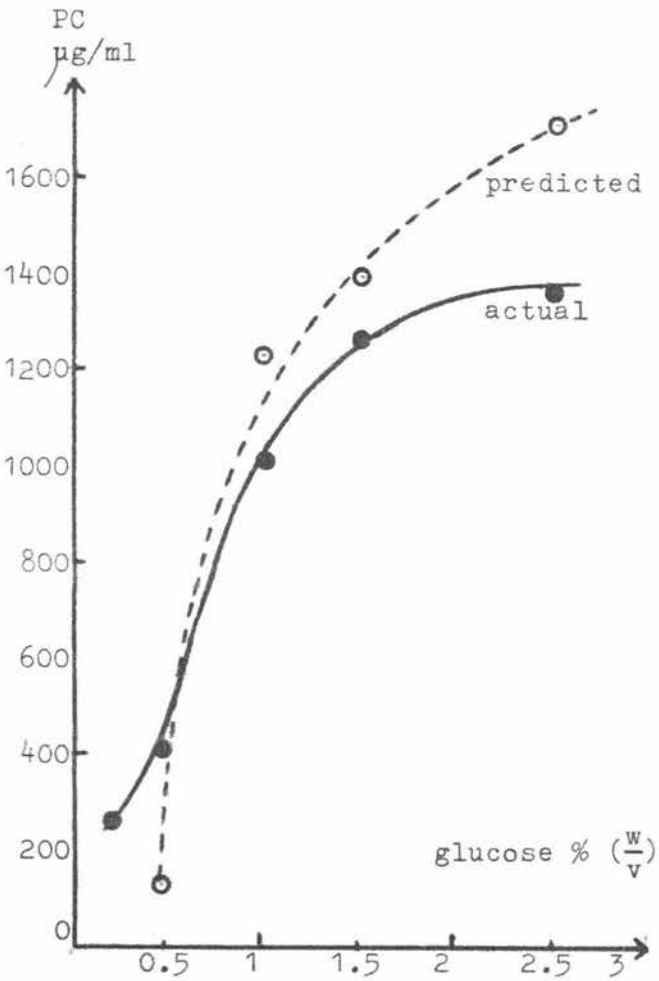



Fig. 15. The effect of glucose on protein production.
Basal medium composition % ($\frac{W}{v}$), $MgSO_4.7H_2O$ = 0.02%,
$NH_4Cl$ = 0.4%, yeast extract = 0.1%, $KH_2PO_4$ = 0.5%.

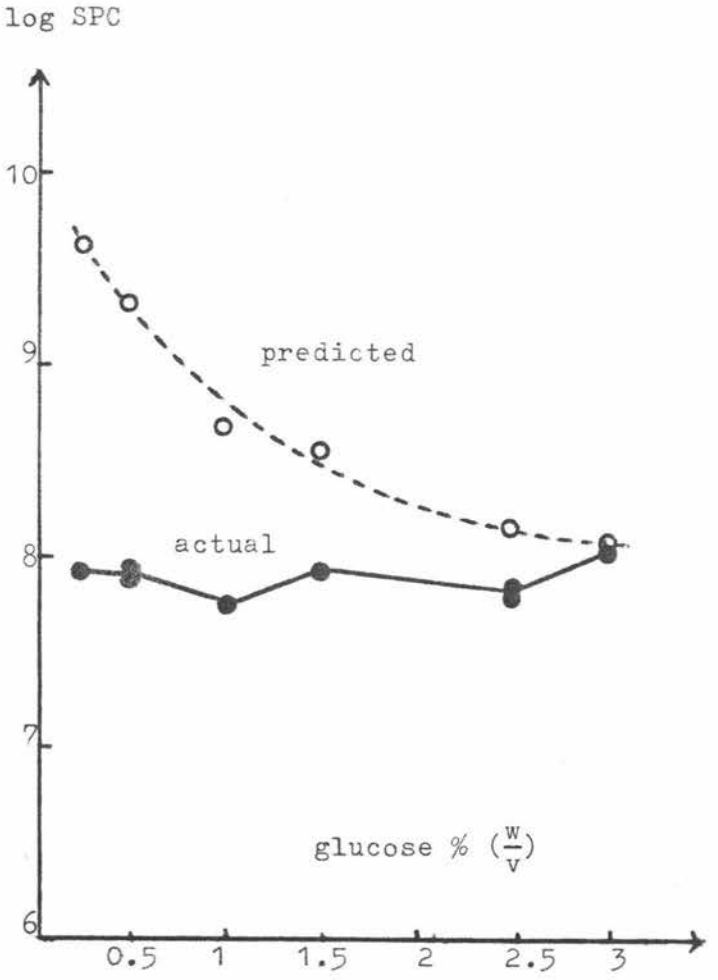



Fig. 16. The effect of glucose on log sporulation. Basal medium composition: $MgSO_4.7H_2O$ = 0.02%, $NH_4Cl$ = 0.4%, yeast extract = 0.1%, $KH_2PO_4$ = 0.5%.

between the actual and predicted curves still exists with a difference of about 0.05 Abs unit/h at 1% glucose concentration or higher. But the shapes of the 2 curves run parallel to each other and follow the same trend.

From Fig. 16, prediction for the effect of glucose on sporulation is very poor. In reality, glucose has no effect on the SPC under the experimental conditions used, but the model predicted that the SPC starts at a very high log value of 9.6 and decreases gradually to the actual level of 8.0 at 3% glucose.

To summarize, there are considerable discrepancies between the predictions by the model and the actual observed values. The main reason for this is probably the fact that the predictions shown in Table 13 all have at least one nutrient concentration at the extreme end of the experimental region, i.e. with $X_i = -2$ or +2. From a design and statistical point of view, the further the prediction is away from the central experimental region, the worse the prediction accuracy is. This is because the model extrapolates from the data.

For growth rate and protein production, of all the nutrients, yeast extract had the largest and most significant positive regression coefficient. Furthermore, the predictions in Table 13 reveal that yeast extract is a limiting nutrient under the experimental conditions used. Thus, it was thought possible that one or more of the components of yeast extract were playing a key role. Of the large number of components present in this material, it was considered that $Ca^{2+}$ ions, because of their role in sporulation, or organic nitrogen, since the main source of

nitrogen in the medium was inorganic, could be limiting nutrients. Thus, experiments were performed to check these hypotheses The results, shown in Figs. 17 and 18, indicate that neither $Ca^{2+}$ nor organic nitrogen are limiting. $Ca^{2+}$ appears to enhance growth rate and protein production to only a very small extent (Fig. 17.1 and 17.2). However, at a $Ca^{2+}$ concentration greater than 0.005%, inhibition is evident. Casein hydrolysate does not affect the growth rate (Fig. 18.1), but quite unexpectedly, as a source of amino acids or organic N, it does not enhance the protein production but actually inhibits it (Fig. 18.2).

Thus it appears that under the experimental conditions used, one or more of the added nutrients are limiting, and it is possible that some unidentified component of yeast extract may also be involved. This latter aspect is puzzling since it was considered that sufficient yeast extract was present in the medium to supply any vitamin or trace element required.

At this point it was decided that subsequent studies would be limited to the response PC only, since this is the response that is most directly related to the production of the crystal protein toxin. The predicted optimum nutrient composition for PC shown in Table 13, with all the nutrients at the +2 level, suggested that in the planning for a further experiment all the nutrient concentrations should be raised to a higher level. However, this appears to be contradictory to the conclusion derived from Experiment 1 that at high nutrient concentrations inhibitions occur. Comparison of the concentration predicted in Table 13 with those at the +2 level in Experiment 1 (Table 3)

Fig. 17.1. Effect of $Ca^{2+}$ on growth rate GR

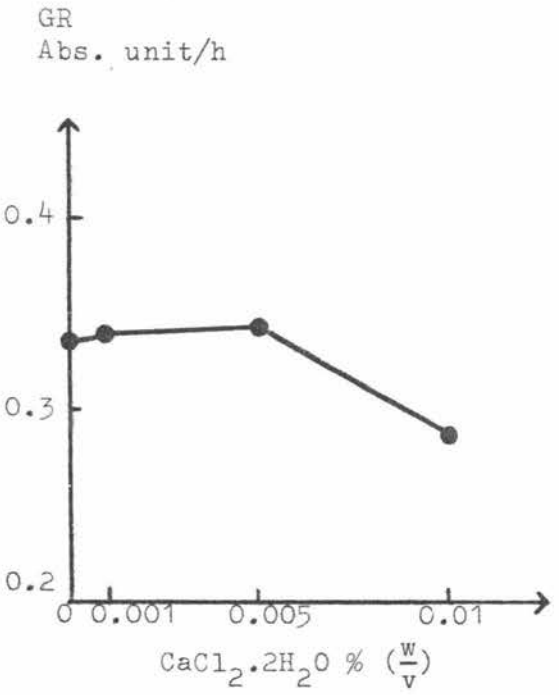
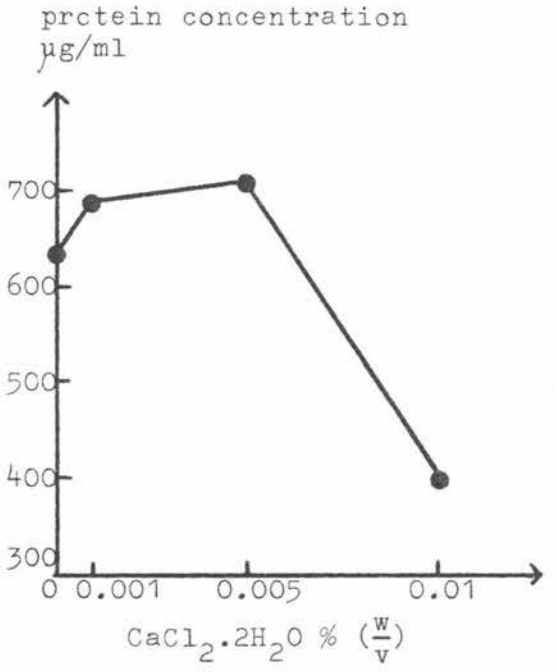



Fig. 17.2. Effect of $Ca^{2+}$ on protein production




Basal medium: glucose = 0.5% $(\frac{w}{v})$,
$MgSO_4.7H_2O$ = 0.00125%, $NH_4Cl$ = 0.23%,
yeast extract = 0.1%, $KH_2PO_4$ = 0.1%.

Fig. 18.1. Effect of casein hydrolysate on growth rate GR

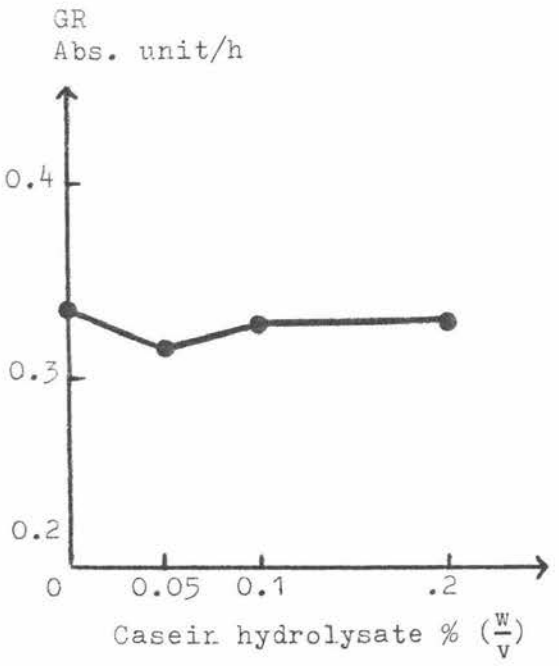
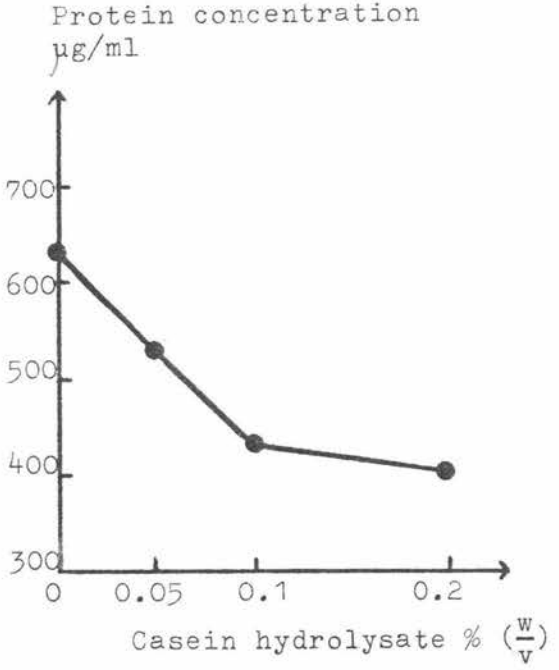



Fig. 18.2. Effect of casein hydrolysate on prctein production




Basal medium: glucose = 0.5% ($\frac{W}{v}$),
$MgSO_4.7H_2O$ = 0.00125%, $NH_4Cl$ = 0.23%,
yeast extract = 0.1%, $KH_2PO_4$ = 0.1%.

reveals that the concentrations of four of the nutrients are quite similar, but for yeast extract the concentration was five times higher in Table 13. Thus the contradictory conclusions can possibly be explained by the great difference in yeast extract and the other nutrients could have caused the relief of inhibition in Experiment 2, especially if some component(s) of yeast extract was the limiting factor in Experiment 1. If this is true it emphasizes how dependent the nutrients are upon each other, and how a change in concentration of one of them can markedly affect the optimum levels of the others.

Thus, for the next experiment it was decided to raise the nutrient levels from those used in Experiment 2, but not by too great an amount since if inhibitory levels were reached the second order model would not be able to describe the response.

## SECTION 4 EXPERIMENT 3

In this experiment, all the +2 levels used in Experiment 2 were chosen as the 0 levels for Experiment 3. The difference in log values for each increase in one coded unit was half of that used in Experiment 2, i.e. concentrations used at +1 levels in Experiment 2 became those for -2 levels in Experiment 3. The complete actual measured levels and ranges for the nutrients are shown in Table 15. The experimental results for PC and the usual analyses are shown in Tables 16, 17 and 18.

The values in Table 16 show that 75% of the actual protein concentrations observed in Experiment 3 are above the 1000 µg/ml mark, with the highest value at 1920 µg/ml. This latter value corresponds with trial no. 24, at the highest (+2) level of yeast extract used. The increased yield in crystal protein production over that obtained in Experiment 2 is obvious and highly significant.

Examination of the significant levels of the regression coefficients for PC shown in Table 17, and comparison of these with those shown in Table 10, for Experiment 2, show that the main effects of the individual nutrients and the pattern of their interactions have changed dramatically. In Experiment 3, with slightly higher nutrient levels, only the effects of yeast extract and $MgSO_4$ were significant. This contrasts with results in Experiment 2 (Table 10) where all the main effects were highly significant, except for $KH_2PO_4$. The number of significant

TABLE 15: DEFINITION AND LEVELS OF INDEPENDENT VARIABLES FOR EXPERIMENT 3

| Independent variables (% w/v) | Symbol | Factor levels | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | +1 | +2 |
| glucose | $X_1$ | 1.67 | 2.06 | 2.5 | 3.03 | 3.68 |
| $MgSO_4.7H_2O$ | $X_2$ | 0.01 | 0.014 | 0.02 | 0.028 | 0.04 |
| $NH_4Cl$ | $X_3$ | 0.28 | 0.33 | 0.4 | 0.48 | 0.57 |
| Yeast extract | $X_4$ | 0.047 | 0.07 | 0.1 | 0.15 | 0.21 |
| $KH_2PO_4$ | $X_5$ | 0.335 | 0.41 | 0.5 | 0.61 | 0.74 |

TABLE 16: RAW EXPERIMENTAL RESULTS AND TABLE OF RESIDUALS FOR EXPERIMENT 3

| Trial no. | Protein concentration (PC) µg/ml | Predicted PC | Residual |
|---|---|---|---|
| 1 | 1098 | 1011.9886 | 86.0114 |
| 2 | 1056 | 971.4886 | 84.5114 |
| 3 | 945 | 933.4053 | 11.5947 |
| 4 | 1080 | 1098.9053 | -18.9053 |
| 5 | 1215 | 1103.2386 | 111.7614 |
| 6 | 936 | 854.7386 | 81.2614 |
| 7 | 900 | 891.6553 | 8.3447 |
| 8 | 1176 | 1169.1553 | 6.8447 |
| 9 | 1470 | 1529.0720 | -59.0720 |
| 10 | 750 | 839,5720 | -89.5720 |
| 11 | 1620 | 1782.4886 | -162.4886 |
| 12 | 669 | 832.9886 | -163.9886 |
| 13 | 1005 | 1067.3220 | -62.3220 |
| 14 | 954 | 1017.8220 | -63.8220 |
| 15 | 972 | 1108.7386 | -136.7386 |
| 16 | 1800 | 1967.2386 | -167.2386 |
| 17 | 1140 | 1105.2727 | 34.7273 |
| 18 | 1035 | 936.2727 | 98.7273 |
| 19 | 893 | 1004.1061 | -111.1061 |
| 20 | 1596 | 1351.4394 | 244.5606 |
| 21 | 1005 | 915.7727 | 89.2273 |
| 22 | 1005 | 960.7727 | 44.2273 |
| 23 | 754 | 1006.4394 | -252.4494 |
| 24 | 1920 | 1534.1061 | 385.8939 |
| 25 | 1410 | 1372.2727 | 37.7273 |
| 26 | 1680 | 1584.2727 | 95.7273 |
| 27 | 1050 | 1340.4091 | -290.4091 |
| 28 | 1129 | 1340.4091 | -211.4091 |
| 29 | 1470 | 1340.4091 | 129.5909 |
| 30 | 1440 | 1340.4091 | 99.5909 |
| 31 | 1380 | 1340.4091 | 39.5909 |
| 32 | 1440 | 1340.4091 | 99.5909 |

TABLE 17: REGRESSION COEFFICIENTS FOR THE RESPONSE PC IN EXPERIMENT 3

| Variable | Coefficient | |
|---|---|---|
| constant | 1340.4091 | |
| $X_1$ | -42.2500 | |
| $X_2$ | 86.8333 | * |
| $X_3$ | 11.2500 | |
| $X_4$ | 131.9167 | ** |
| $X_5$ | 53.0000 | |
| $X_1X_2$ | 86.2500 | |
| $X_1X_3$ | 147.0000 | ** |
| $X_1X_4$ | -61.5000 | |
| $X_1X_5$ | 43.1250 | |
| $X_2X_3$ | 49.8750 | |
| $X_2X_4$ | 67.8750 | |
| $X_2X_5$ | 159.0000 | ** |
| $X_3X_4$ | 10.8750 | |
| $X_3X_5$ | -5.2500 | |
| $X_4X_5$ | 93.0000 | * |
| $X_1X_1$ | -79.9091 | * |
| $X_2X_2$ | -40.6591 | |
| $X_3X_3$ | -100.5341 | ** |
| $X_4X_4$ | -17.5341 | |
| $X_5X_5$ | 34.4659 | |

TABLE 18: ANALYSIS OF VARIANCE FOR PC IN EXPERIMENT 3

| Source | Degrees of Freedom | Sum of Squares | Mean Square | F |
|---|---|---|---|---|
| Regression | 20 | 2470813.9612 | 123540.6981 | 2.13 |
| Error | 11 | 637154.5076 | 57923.1371 | |
| Total | 31 | 3107968.4688 | | |

Confidence level of F (20, 11) = 90.13%

2-factor interaction terms was also reduced.

The analysis of variance (Table 18) gave a much lower F-ratio of 2.13 (cf. 15.19 in Experiment 2) and a lower confidence level of 90.13% (cf. 100%). Although further analysis of the error source did not reveal any significant lack of fit, the low F-ratio and the large residuals observed in Table 16 do indicate that the accuracy of approximation by the chosen model has decreased when compared with Experiment 2.

A search for the highest response in the experimental region, with independent variables ranging from the -2 to +2 levels, predicted a highest PC value of 3366 µg/ml, with all the nutrients at the new +2 levels. These results, similar in nature to those derived from Experiment 2, suggested a further increase in the nutrient levels to obtain higher yields of protein. Comparison of the concentration for the +2 levels in Experiments 1 and 3 again reveals that the only significant difference is in the yeast extract levels used (0.21% cf. 0.02%, a 10-fold difference). Thus the results again suggest that unidentified component(s) in yeast extract might be playing a key role for the functioning of the crystal protein biosynthesis system.

At this stage of the experimental work, information became available which could possibly explain some of the more puzzling aspects of the above results. Thus, Nickerson and Bulla (1974, 1975) reported their findings that poor growth and sporulation by B. thuringiensis on semi-defined media were not due to a vitamin or trace metal deficiency, but rather to an absolute requirement for citrate or related compounds that stimulate fatty acid

synthesis and promote growth. Since the only source of these compounds in the media used for the present experimental work would be the yeast extract, and the concentrations would probably be very low, it is very possible that they could be the limiting nutrients for the three responses SPC, PC and GR. Thus, extremely high levels of yeast extract may be needed to maximize the responses, unless of course, citrate were independently added to the medium.

To summarize, in this third experiment, although a further increase in the protein concentration was obtained, the result is still far away from the goal of obtaining an "optimum" nutrient composition for a maximum yield. Also, it appears that further repetition of the same experimental procedures would not be helpful in reaching this goal. In the light of the more recent information concerning the growth requirements of B. thuringiensis, it appears that a re-definition of the medium constituents is required, possibly incorporating citrate at defined levels.

# FINAL DISCUSSION

The purpose of this study was to determine an optimum medium composition for maximum production of the crystal protein toxin by *B. thuringiensis*, on a shake flask scale, using a central composite experimental design and empirical mathematical modelling techniques. The optimum conditions for growth and sporulation were also to be determined. Unfortunately, however, these goals have not been successfully achieved.

The reasons for this lack of success can be grouped into two categories. Firstly, those that are contributed by the complex microbiological systems under study and, secondly, those caused by the inadequacy of the model and statistical techniques used.

From the results obtained during the later stages of this work it was realized that the physiological responses of *B. thuringiensis* under study, namely growth, sporulation and production of the crystal protein toxin, are more complex than was initially thought. It was realized at the very beginning of the work that fundamental and theoretical knowledge about the underlying mechanisms is very limited, hence an empirical modelling approach was chosen. Because of the practical constraint imposed by experimental conditions and procedures, and the experimental design and statistical analysis chosen, the number of nutrient variables that could be studied was limited to five. Thus a sound knowledge of the nutrient requirements is very essential in the choice of the most important nutrient variables to be studied.

Based on the literature available at the beginning of the work, it was thought that a simple glucose-salt basal medium supplemented with sufficient amount of yeast extract as a source of vitamins, trace minerals and other growth factors, would meet all the growth requirements of the organism. However, in light of the findings of Nickerson and Bulla (1974, 1975), it is clear that the rather poor growth and sporulation observed in this work were due mainly to an inadequate supply of citrate or related compounds which were probably present at very low levels in the yeast extract. Thus, unknowingly, the medium used was probably extremely limiting in the nutrients, and this would explain the inability to achieve an optimum medium composition to achieve maximum response. Thus, it seems clear that before an optimization programme is commenced, all the growth requirement of the organism must be known.

The medium used in this work, because of the presence of yeast extract, was only partially defined. If a fully defined medium had been used by the addition of known and excess amount of vitamins and trace minerals, even without the addition of citrate it should have been possible to arrive at an optimum composition for that particular defined medium. The corresponding "limited" maximum response obtained would be the valid "maximum" for that particular defined, although not perfect, medium.

Results from the 3 experiments carried out indicate that the 3 responses growth, sporulation and crystal protein formation have quite different nutritional requirements. No correlation was observed among them under the experimental conditions used. However,

this does not necessarily mean that a correlation would not exist if all the responses were maximized. Literature available to date shows clear physiological, biochemical, immunological and morphological relationship between sporulation and crystal protein formation. Thus the nutritional requirements for these two processes might be similar.

Turning to the modelling techniques, the central composite design and the second order mathematical model chosen for this work are most suitable for linear or parabolic responses. Unfortunately all the responses under study appeared to be exponential or asymptotic in nature. To fit this type of response requires regression methods and statistical techniques too complex to handle, especially when a rather large number of independent variables are studied.

The problem of fitting the second order model was solved to a large extent by a logarithmic transformation on the coded independent variables. However, this transformation seems to be effective only within a certain rather narrow experimental range. The second order model described reasonably well the observed data in the immediate experimental region, but predictions obtained from the model by extrapolation were not accurate and discrepancy occurred between the predicted and actual values.

Although the modelling technique used failed to predict optimum medium compositions for the three responses studied, this was probably a result of the medium deficiencies rather than the technique itself. However, the technique has been useful in that

it has successfully shown the extremely important interaction between nutrients. When the concentration of one nutrient is changed the technique successfully detects the changes in optimum levels of other nutrients. A conventional one-factor-at-a-time experimental approach would not be able to detect the significance and importance of these interactions.

The experimental technique also succeeds in describing the response functions within the experimental regions and helps in predicting the changes in the responses outside and adjacent to the experimental zones. Although there are discrepancies in predictions due to extrapolation, at least the model pointed in the right direction.

It is believed that the application of the experimental design and modelling technique to the determination of optimum nutrient composition for maximum response would be more successful in future study if a well defined medium were used following initial screening experiments to determine the growth requirements. It is obvious that citrate or a related compound should be included as one of the chosen nutrient variables for such a study.

To summarize, the results from this experimental work have provided very encouraging indications that the central composite design and second order and mathematical modelling techniques have great potential applications in the field of microbiological screening studies. However, this potential has not been realized in this particular study due to a lack of knowledge of the growth requirements of the organisms. In general, it is believed that,

in the near future, the whole concept and approach of the Response Surface Methodology technique will have a place beyond the traditional chemical and engineering fields, and will be used in fields such as microbiology, especially for medium screening and improvement of fermentation processes and yields.

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