STUDIES ON IMPROVED ALPHA-AMYLASE PRODUCTION USING U.V. IRRADIATED STRAIN OF BACILLUS LICHENFORMIS B5. 1.

A DISSERTATION

BY

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DECLARATION.

I hereby declare that the contents of this thesis is purely a record of my research findings. No part of this work has been submitted or accepted in any previous application for a degree or qualification elsewhere. All quotations and literature cited from other sources are fully acknowledged.



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APRIL 1991.

CERTIFICATION.

This thesis by IFEDAPO ABDULMAJEED RAJI meets the regulations governing the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for acceptance in partial fulfilment of the requirements for the degree.

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DEDICATION.

This work is dedicated to

KASSNAM.

Long live KASSNAM.

and

To my wife and children when they come.

ABSTRACT

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The increament of a-amylase production by locally isolated strain of Bacillus, licheniformis. B5-1 using ultraviolet radiation was carried out. It was observed that after the first application of ultraviolet radiation about 270% increament in a-amylase production was the highest level recorded while the second round of application produced a mutant Bacillus lichenlformis B5-1 strain E22 with about 600% increament in a-amylase production. The auxanographic method was used to confirm the successful mutation of this strain. This method also revealed that the mutant strain unlike the wild type strain had lost the ability to utilise niacin and thiamine which are both vitamins of the B group. On the basis of this finding and in line with the standard procedure, this mutant strain was designated Bacillus licheniformis B5-1 nia-thia-.

This strain was cultured for the production of a-amylase enzyme. The enzyme extracted was purified and characterised. The Km was 7.87% starch and the V_{max} was 2.22mg glucose/min. An optimum temperature of 60°C was recorded for the enzyme activity and a pH of 6.5 - 7.0 in 0.02M phosphate buffer was recorded.

The effect of different metal ions on the activity of the enzyme was studied, with calcium ion enhancing the a-amylase activity best while mercuric ion inhibited a-amylase activity. The rate constant (CKo) of the enzyme was found to be 0.22/week (at room

temperature), 0.0534/week (in the refrigerator) and 0.0144/week (in the freezer). The half life was detemined as 3.15 weeks (room temperature), 12.98 weeks (refrigerator) and 48.13 weeks (freezer).

The effect of different chemicals and vitamins on growth and *cc*-amylase production by the mutant strain Bacillus. licheniformis B5-1 nia-˙ thia- when incorporated into the culture medium at the beginning of the fermentation was studied. It was observed that only folic acid enhanced both growth and a-amylase production while glycine and triton X-100 reduced bacterial growth and *a*-amylase produced. Riboflavin and calcium panthothenate though increased bacterial growth, could not increase the level of *a*-amylase production.

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CHAPTER 1

INTRODUCTION

The industrial cultivation of microorganisms for some of their products is widely practised throughout the world. The success of this cultivation is due to the fast growth rate, versatile metabolism and the ease with which they can be cultivated. Among the products of microorganisms that have found wide application in the industries are enzymes and antibiotics. Some industries, on the other hand, propagate relevant microorganisms and harvest the biomass as the primary product which may be subsequently used as food or feed (for example single-cell protein from yeast), or as inocula in the food and beverage industries such as bakeries, breweries, cheese and yoghurt making industries. For the production of these substances, microorganisms such as bacteria, yeasts and moulds have been successfully involved.

Members of the genus Bacillus have been reported to rank high among organisms of industrial importance. The genus Bacillus has found great use in the fermentation and spoilage of some food and food products, in the production of microbial insecticides (Lacey and Mulla, 1977) and the production of enzymes (Priest, 1977). The enzymes they produce include amylases and proteases. There are many sources of commercial enzymes, but enzymes produced by microorganisms seem to predominate (Underkofler et al, 1958).

Members of the genus Bacillus which belong to the family Bacilliaceae are Gram positive, motile and spore-forming bacteria (Buchanan and Gibbons, 1974). They have the ability to form heat resistant endospores and are very ubiquitous in nature. They can grow over a wide range of temperature, between 5°C - 45°C (minimum) and 25°C - 75°C (maximum); most members are mesophiles growing between 30°C and 40°C (Nester et al, 1978). A few species such as Bacillus stearothermophilus are thermophiles which can grow at 65°C and above, while a few psychrophilic species can grow at 0°C (Marshall and Ohye, 1966). They also grow over a wide range of pH values ranging from 2.0 - 8.0.

The use of mutagenic factors in the improvement of enzyme production properties of microorganisms is well knowmn (Meyrath et al, 1971). The process of mutation involves the nucleus of the organism which might lead to increased productivity of its product (Calam, 1970). The mutagens commonly used are ultraviolet, X-rays, gamma rays, fast neutrons and chemicals. Successful mutation will result in improved performace together with the production of fewer unwanted by-products. Thus, whenever a fermentation process is being used for production purposes the search for improved strains should be regarded as an important aspect of the devcelopment programme.

Amylase is one of the most industrially useful enzymes produced by microorganisms especially members of the genus Bacillus (Priest, 1977). The industrial uses of amylase are

generally on the increase. It enjoys wide application in many industries especially the textile, brewing, pharmaceutical, paper, baking, laundry and confectionary industries (Underkofler et al, 1958). Since there is no industry in Nigeria that require amylase for their processes will probably be importing the amylase they use, thereby consuming the scarce foreign exchange. The local production of amylase in this country will certainly help to conserve this foreign exchange. Also since new techniques keep developing in which the need for amylase is becoming increasingly important, for example, the production of beer from cassava (Rajagopal, 1977), a process in which starch saccharification is obligatory, the need for local production of amylase is very necessary.

This work is undertaken with the following objectives in view.

(a) To improve α-amylase production by Bacillus licheniformis B5.1 by subjecting it to U.V. irradiation and isolating high amylase producing mutants.

(b) To extract, purify and characterize the α-amylase enzyme produced by the improved strain of Bacillus licheniformis B5.1.

(c) To determine the effect of some environmental conditions on the α-amylase production by the improved strain of Bacillus licheniformis B5.1.

CHAPTER 2

LITERATURE REVIEW

2.1 GENUS BACILLUS

The genus Bacillus is classified in the family Bacilliaceae. Members of this genus are Gram positive, rod-shaped organisms. They form endospores which differ from vegetative cells in being more refractive and less susceptible to staining and in having greater resistance to heat and other destructive agents. Their spores contain a central cell (the core) which is enclosed by a cortex of peptidoglycan and an outer spore coat. Sporulation in this genus is not repressed by exposure to air. Majority of the members of this genus are motile by lateral flagella (Buchanan and Gibbons, 1974).

2.1.1 Distribution

Members of the genus Bacillus can be isolated from a wide range of environments which include soil, water, air and foods. Some species could be pathogenic, while some because of their saprophitic nature could be found contaminating some materials. Larkin and Stokes (1966) have reported the isolation of some psychrophillic species from Antartic ice, snow, mud and plant debris. The presence of Bacillus species in lakes has been reported by previous workers. Koleshko et al (1982) reported

that in USSR a bacteriological study was carried out on a lake in which the number of Bacillus species isolated was highest among all the bacteria isolated with a count of ten Bacillus species out of a total of thirty-two bacteria species belonging to nine genera. Also in Japan, Bacillus species was most predominant in a lake among the Gram positive bacteria contributing about fifty-five percent (Konda and Tezuka, 1979). The isolation of members of the genus Bacillus from soil has been well established. Siopengco and Aspiras (1981) have reported the predominance of some Bacillus species over the fungal population in a commercial manure inoculant preparation which allows the use of the manure as feed. Wakisaka and Koizumi (1982) have gone further to group Bacillus species isolated from the soil into two (major and minor soil populations). Bacillus subtilis, Bacillus megaterium, Bacillus cereus and Bacillus sphaericus have been considered as the major soil Bacillus population. Members of the genus Bacillus are also found in air, grain storing and processing plants, tobacco plants and in poultry houses, which shows their presence in the general atmosphere (Dutkiewicz, 1980). A few species of Bacillus have been isolated from the larva of insects, such as the honey bee and Japanese beetle (Skerman, 1967).

The presence of different species of Bacillus in various foods, such as meat and meat products, milk and milk products, fish and fish products, beverage drinks and spices have been

reported by previous workers (Frazier and Westhoff, 1978). Fields (1970) reported the ability of the thermophilic Bacillus species, such as Bacillus stearothermophilus to cause flat sour spoilage in under-processed canned foods. In different meat products, Bacillus megaterium, Bacillus subtilis, Bacillus circulans, Bacillus pumilus and Bacillus coagulans have been isolated occuring predominantly together with some Clostridium species (Konuma et al, 1980). Sulzbacher and Mclean (1951), and Kraft (1971) reported the presence of Bacillus species in different animal and poultry products, respectively. They could also cause surface slime in inproperly handled meat (Jensen, 1954), and the thermophilic species could cause spoilage of inproperly cooked meat (Rieman, 1963). The isolation of Bacillus cereus and Bacillus megaterium from some foreign foods such as pozol and tesquino, which are fermented beverages in Mexico has been reported (Ulloa, 1981). They have also been incriminated together with some other water-borne organisms in the contamination of thawing frozen blood products (Dietz and Burnie, 1982). Alawiye (1984) reported a high level of Bacillus cereus in dried local spices. There has been the isolation of different species of Bacillus from various milk and milk products. Wood and Hodge (1985), reported the isolation of different Bacillus species from taette, which is a viscous coagulated, acidic milk product of Northern European countries. The presence of Bacillus cereus, Bacillus subtilis and some atypical strains Bacillus

have been isolated in ultrahigh temperature processed milk (Westhoff and Dougherty, 1981).

2.1.2 Identification

The identification of members of the genus Bacillus has been approached from different points. Smith et al (1946) have classified members of this genus into species on the basis of their morphology. Gordon et al (1973) have divided the genus into three on the basis of their spore shape, swelling of the sporangium by the sport and the diameter of the rod.

The first group (group I) consists of species with ellipsoidal or cylindrical spores which are centrally or terminally located within the cells that have bigger diameter than the spore. This group consists of species such as Bacillus cereus, Bacillus megaterium, Bacillus licheniformis, Bacillus subtilis and Bacillus pumilus. The second group (group II) is made up of species with their sporangia swollen as a result of oval spores which are centrally or terminally located within cells smaller than the spore diameter. Most members of this group are catalase positive. The group consists of Bacillus polymyxa, Bacillus macerans, Bacillus circulans, Bacillus stearothermophilus, Bacillus alvei, Bacillus lateresporus, Bacillus brevis, Bacillus larvae, Bacillus popillae and Bacillus lentimorbus. Group III is made up of species with swollen sporangia which bear spherical spores that are terminal or subterminal.

Members of this group include Bacillus spaericus and Bacillus pasterurii. However, Gordon et al (1973) reported that there are some species which could not be given a clear-cut grouping; an example is the case of Bacillus coagulans in which some strains have sporangia of the type in group I and others with the type in group II.

Buchanan and Gibbons (1974) have classified the genus by dividing it into two groups. Group I, is made up of twenty-two species which are differentiated on the basis of shape and position of the spores, distention of the sporangium and growth on glucose medium, while group II which contains twenty-six species has so far received less widespread recognition and their differentiation is based on similar characteristics as those for group I. Among organisms grouped together in this classification some differences have been established in their characteristic reactions. For instance, Bacillus licheniformis, which is in group I, has the ability to form acetoin while Bacillus megaterium which is in the same group does not form acetoin (Wolf and Barker, 1968).

Recently, members of the genus Bacillus have been classified by the method of numerical taxonomy (Logan and Berkeley, 1981). Bonde (1975) and Priest et al (1981) have corroborated the use of numerical taxonomy for the classification of the genus Bacillus when they classified some strains of Bacillus isolated from natural habitats using this method.

2.1.3 Physiology

A feature of the genus Bacillus in contrast to other bacterial genera, is the great diversity in the properties of the organisms it embrances. Nutritional requirements for vegetative growth of members of the genus vary from simple inorganic carbon and nitrogen at one extreme, to distinctly complex needs at the other (Gordon et al, 1973). An example is in the case of one species, Bacillus fastidiousus, which grows with uric acid or allantion as the sole source of both carbon and nitrogen, while strains of Bacillus polymyxa and Bacillus macerans that assimilate molecular nitrogen have been reported (Buchanan and Gibbons, 1974). The maximum temperature for vegetative growth ranges from about 25°C to above 75°C, and the minimum from about -5°C to about 45°C. Most members of this genus are mesophilic, with their growth temperature between 30°C to 40°C (Nester et al, 1978). A few species have however been reported to grow at extemes of temperature (Marshall and Ohye, 1966). The pH value for growth varies from 2.0 to 8.0 and salt tolerance ranges from less than 2% to 25% sodium chloride.

Spore formation has proved to be a generally reliable diagnostic character of the genus Bacillus. It is controlled by a number of genes which occur in different parts of the chromosome, and under laboratory conditions mutants may be obtained in which sporulation is diminished or does not occur. Nevertheless, asporogenous mutants of these organisms appear to have little capacity to survive in nature (Prasad et al., 1972).

Sporulation has been reported to occur when either carbon or nitrogen or both are lacking in the growth medium (Holmes and Levinson, 1967), or when cells are transferred from a rich medium to a poor medium (Freese, 1977). Reports of Bacillus megaterium sporulating when transferred at any time of growth to a minimal sporulation medium containing acetate as a sole carbon source have been given by Greene and Slepecky (1972).

Schaeffer (1969) described sporulation in six stages. Stage I is the beginning of the sporulation process in which the chromatin is visible as a single axial rod. This is followed by stage II which involves the formation of a spore septum which divides the nuclear material in the cell into two, as a result of the invagination of the cytoplasmic membrane. The carving out of an entirely intraacytoplasmic forespore from the mother cell marks the end of stage III. Stages IV and V are characterised by the formation of new envelopes around the forespores and in stage VI, which is the final stage, the thermoresistance of the enclosed spore is being built up.

2.2 INDUSTRIAL IMPORTANCE OF THE GENUS BACILLUS

Members of the genus Bacillus have been reported to rank high among organisms of industrial importance. It has found great use in the production of antibiotics (Katz and Demain, 1977), microbial insecticides (Lacey and Mulla, 1977), fermented food (Frazier and Westhoff, 1978) and enzymes (Priest, 1977).

2.2.1 Antibiotic Production by the genus Bacillus

Hopwood and Merrick (1977) defined antibiotics as secondary metabolites which are inhibitory to organisms outside the immediate relatives of the producing microorganisms. They are produced by certain fungi, eubacteria, and actinomycetes. Berdy (1974) credits the eubacteria with about 360 antibiotics. However, members of the genus Bacillus produced nearly half the total, and Pseudomonads nearly a quarter. The genus Bacillus is by far the most significant commercially, nearly all the interesting eubacteria antibiotics being cyclic or linear peptides are produced by members of this genus (Sadoff, 1972). Of the total antibiotics produced by this genus, sixty-six different peptide antibiotics are elaborated by strains of Bacillus subtilis and twenty-three are products of Bacillus brevis. Apart from the peptide antibiotics synthesized by this genus, they also produce antibiotics belonging to other chemical classes. For example, Bacillus circulans produce butirosin which is an aminoglycoside and Bacillus licheniformis var mesentericus synthesises proticin, a phosphorous-containing triene (Anderson et al, 1972). Some of the antibiotics produced by members of this genus have been successful clinically. Polymyxins and the closely related colistin, bacitracin, the tyrothricin complex and gramicidin S have been used to some extent for antibacterial therapy. Most of these peptide antibiotics are active against Gram positive bacteria. However, some such as polymyxin, colis-

tin and circulin exhibit activity almost exclusively on Gram negative bacteria, whereas bacillomycin, mycobacillin and fungistatin are effective agents against moulds and yeasts (Katz and Demain, 1977).

Peptide antibiotic synthesis by bacilli is initiated after the organism has passed the rapid growth phase. This was demonstrated using antibiotics such as polymyxin, bacitracin and gramicidin S (Bernlohr and Novelli, 1963; Paulus and Gray, 1964; Tomino et al, 1967). Demain (1972) has reported that antibiotics are sometimes produced by this genus during growth, and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase. The possibility that peptide antibiotic synthesis is controlled by carbon and nitrogen catabolite repression or growth rate control has been reported by Matteo et al (1976). Haavik (1974), reported that bacitracin production was inhibited when glucose was added to the medium, an effect which suggested that bacitracin production is controlled by catabolite repression by glucose. The lowering of the pH in a growth medium has also been reported to have no effect on the production of some antibiotics, such as bacitracin synthesis by Bacillus licheniformis.

2.2.2 The genus Bacillus in the Production of Bioinsecticides

Some species of Bacillus such as Bacillus lentimorbus, Bacillus popillae and Bacillus larvae have been reported by

Skerman (1967) to be pathogenic to the larvae of some insects. Among the different Bacillus species, Bacillus thuringiensis which has a wide host range covering various orders of insects has been developed commercially as a bioinsecticide. Lysenko and Kucera (1971), reported that the insecticidal property of this Bacillus thuringiensis will not be unconnected with the diamond shaped crystal body called parasporal body which it synthesises during sporulation. The production of primary powder of Bacillus thuringiensis subsp. israelensis as an insecticide has been tested on different media containing various local legumes. The medium E containing bambara beans was found to give the optimal yield of all the media tested. The results obtained with medium E compared favourably with the standard medium (IPS 78) (Obeta and Okafor, 1984). Bacillus thuringiensis bioinsecticide has been reported to be effective against the larvae of black fly (Lacey and Mulla, 1977). It has also been successfully used in the control of European cornborer (Raun, 1963) and also as a cornmeal bait to kill the tobacco budworm (Allen, 1961). This bioinsecticide has also been used in the control of vegetable invasion by different pests (Nester et al, 1978). There had been reported cases in which Bacillus thuringiensis serotype H-14 had been used effectively for the control of larvae of medically important dipterans, such as mosquitoes (Goldberg and Margalit, 1977).

Some workers had reported the use of other Bacillus species other than Bacillus thuringiensis as successful bioinsecticide. Kellen et al (1965) reported the use of Bacillus sphaericus as an insecticide against mosquitoes because of the pathogenic effect of the organism on the larvae of various species of mosquitoes. Bacillus sphaericus is best cultivated on medium with high content of "proflo". This organism is highly effective against the larvae of Culex quinquefasciatus, fairly active against Anopheles gambiae and least active against Aedes aegypti.

2.2.3 Food Fermentation and Spoilage by the Genus Bacillus

The fermentation of different food and food products in which members of the genus Bacillus have been implicated has been reported. Different Bacillus species have been isolated in the fermentation of some foreign foods such as "shi", which is a Chinese food product obtained from fermented soya beans. Bacillus subtilis is also involved in the production of "natto", which is a popular food product in Japan, some parts of China and Thailand (Yokotsuka, 1985). Bacillus cereus and Bacillus megaterium have been isolated in 'pozol' and 'tesquino' which are fermented beverages in Mexico (Ulloa, 1981). Bacillus species have been implicated in the production of 'taette', which is a viscous coagulated, acidic milk product of Northern European countries (Wood and Hodge, 1985), and also in the fermentation of 'kishk' which is a common food in Egypt and most of the Arab

World (Morcos et al, 1973). Different Bacillus species have been implicated in the local fermentation of vegetable proteins. Bacillus subtilis and Bacillus licheniformis have been isolated in the local fermentation of locust bean seed to give 'iru' (Odunfa, 1981). These organisms cause proteolysis in the fermentation processing. Bacillus species are isolated in the fermentation of castor oil, sesame seed, melon seed and fluted pumpkin bean seed to give 'ogiri-agbor', 'ogiri-saro', 'ogiri' and 'ogiri-nwan', respectively (Odunfa, 1985). Bacillus species and such other bacteria as Lactobacillus, Klebsiella, Leuconostoc and Corynebacterium have been isolated from 'fufu' a fermented cassava product (Odunfa, 1985). Bacillus subtilis has been reported to be important in the fermentation of oil bean seed to give 'Ugba' (Obeta, 1983).

Bacillus species cause the spoilage of many types of food products. Their ability to survive high temperatures have made them a unique nuisance in most food processing industries. The thermophilic species of Bacillus such as the Bacillus stearothermophilus causes flat sour spoilage in underprocessed canned foods (Fields, 1970). The spores of these thermophiles can survive heating in underprocessed canned foods thereby causing souring of the canned foods if the condition in the can favours their germination into vegetative forms. This type of spoilage cannot be detected by the examination of the unopened can, except by cultural methods because the ends of the can retain their

concavity. This type of spoilage occurs mainly in low acid foods such as peas (Jay, 1978). Bacillus cereus which is a widely distributed organism has been reported to cause spoilage of different foods such as rice, spices, meat, egg and dairy products (Johnson, 1984).

Bacillus cereus has been reported by Ahmed et al (1983) to be present in contaminated milk and milk products such as raw milk, pasteurized milk, cheese and ice-cream. The growth of this organism significantly spoils the quality of dairy products causing sweet curdling and 'bitter' cream (Davies and Wilkinson, 1973). Bacillus cereus was incriminated in the spoilage of washed potatoes, vegetables. minced meat, liver, sausage, puddings and soups in a study carried out in Netherlands by Mossel et al (1967).

Bacillus polymyxa causes spoilage of milk by producing gas and acid in the milk and the acid production is undesirable, except in fermented milk products. The contamination of milk by some proteolytic species of Bacillus such as Bacillus subtilis and Bacillus cereus results in the production of bitter flavour in the milk (Frazier and Westhoff, 1978). The spoilage of sugar and sugar products such as raw juice in which the sugar concentration is not very high can be caused by osmophillic Bacillus species. Bacillus subtilis and Bacillus licheniformis cause ropiness of bread, especially home-baked bread during hot weather. The spores of this species can withstand the temperature

of the bread during baking which does not exceed 100°C and can germinate and grow in the loaf if conditions are favourable. The ropy condition apparently is the result of capsulation of the bacillus together with hydrolysis of the flour protein by proteinases of the organism and of starch by amylase to give sugars that encourage rope formation. The area of ropinesss is usually yellow to brown in colour and is soft and sticky to the touch (Russ et al, 1961). Generally, canned cured meat and fish exhibit a major type of spoilage by Bacillus species that results in their softening and souring. Canned cured meats given a heat process insufficient for sterilization such as ham or luncheon meats may be subject to the production of carbon dioxide, nitrogen oxide or nitrogen gas by Bacillus licheniformis, Bacillus coagulans, Bacillus cereus or Bacillus subtilis (Ayres and Adams, 1953).

2.2.4 The Genus Bacillus in Enzyme Production

Enzymes are defined as proteins with catalytic properties due to their power of specific activation (Whittaker, 1972). Microbial enzymes have become important in such diverse fields as medicine, food and beverage industries and timber preservation. The advantages of microbial enzymes over chemical catalysts include high specificity of action on substrates, their rapid rate of action in low concentrations and the ease with which they can be inactivated (Pariza and Foster, 1983).

Members of the genus Bacillus have played a great role in the development of microbial enzymes. Reasons for the predominance of this group of bacteria in this area of study include the fact that, they comprise a group of chemoorganotrophs that can be easily maintained and cultivated and yet are markedly heterogenous in character; also they are well represented by psychrophiles, mesophiles and thermmophiles in addition to alkalophilic, neutrophilic and acidophilic species (Priest, 1977).

Enzymes produced by the genus Bacillus can be classified as either extracellular (exoenzyme) or membrane-bound. The extracellular enzymes are those that are completely dissociated from the cell and found free in the surrounding medium while the membrane-bound enzymes are attached to cell membrane (Ramaley, 1979). However, the division between these two classes of enzymes is narrow. Some enzymes have been reported as membrane-bound in young cells and released as exoenzymes as the culture enters its stationary phase (Cercignani et al, 1974) or solubilized by relatively mild procedures including washing the cells with water or concentrated salts (Wood and Tristram, 1970). There is enough evidence now to suggest that α-amylase also exists as a membrane-bound and as an extracellular enzyme. Some organisms in which membrane bound α-amylases have been described include Bacillus subtilis marburg; Bacillus natto:, Bacillus amyloliquefaciens and Bacillus subtilis var amylosaccharitious.

In Bacillus subtilis the membrane-bound and extracellular α-amylases are derived from the same structural gene (Nagata et al, 1974).

The genus Bacillus synthesizes a large variety of exoenzymes with the amylases and proteases probably being the most popular (Priest, 1977). α-Amylases characteristically hydrolyse the α-1,4-glucosidic bonds in starch and related polysaccharides. The two pricipal types of α-amylase produced by the bacilli are the liquefying and the saccharifying enzymes (Matsuzaki et al, 1974b). They can be distinguished primarily by their action on starch, the increase in reducing power produced by the saccharifying enzyme being about twice that of the liquefying enzyme. Traditionally, α-amylase and the cyclodextrin glucosyl transferase of Bacillus macerans were thought to be the only starch degrading enzymes produced by the bacilli but β-amylases (Shinke et al, 1974) and α-1,6-hydrolysing starch debranching enzymes (Nakamura et al, 1975) were later detected in various species. Other extracellular carbohydrases from the bacilli include cellulases, hemicellulases and a variety of β-glucanases. The extracellular proteases produced by the genus Bacillus with very few exceptions are either serine or metal enzymes (Priest, 1977). The serine proteases are characterised by the presence of a serine residue at their active sites. The metal proteases are a group of metallo endopeptidases that show maximal activity at or near neutrality. They are sensitive to metal-chelating agents and do not possess esterase activity (Matsubara and Feder, 1971).

Penicillinase (β-lactamase) is the enzyme that hydrolyses the amide bond in the β-lactam ring of 6-amino-penicillanic acid or 7-aminocephalosporanic acid and their N-acyl derivatives (Citri, 1971). This enzyme has been reported to be produced by many Bacillus species, but the penicillinase from Bacillus cereus and Bacillus licheniformis have been studied in most detail (Fogarty et al, 1974). Bacillus cereus produces two types of extracellular penicillinases (β-lactamase I and β-lactamase II), which are coded for by different genes with no immunological affinities detected between them (Kuwabara and Lloyd, 1971; Thatcher, 1975). Bacillus licheniformis on the other hand synthesizes a single extracellular penicillinase that can also be detected as a membrane-bond hydrophobic enzyme. The Bacillus licheniformis extracellular penicillinase is closely related to the β-lactamase I of Bacillus cereus with their molecules cross-reacting serologically, and a comparison of their structures has revealed significant homology (Ambler and Meadway, 1969). Members of the genus Bacillus also produce nucleases. The most extensively studied of these nucleases produced is the extracellular RNase from Bacillus amyloliquefaciens. This enzyme is insensitive to EDTA and hydrolyzes ribonucleic acid (RNA). The hydrolysis is via the cyclic terminal phosphate intermediates (Rushizky et al, 1963). Pullulanase which hydrolyzes 1,6-α-glucosidic linkages in glycogen and amylopectin is also produced by some bacilli. A strain of Bacillus isolated from the soil

produced an extracellular alkaline pullulanase with an optimum pH between 8.5 and 9.0. This value is higher than that recorded for other debranching enzymes (Nakamura et al, 1975).

Other enzymes produced by members of the genus Bacillus include chitinase, dextranase, lipase, phospholipase and maltase.

2.3 DESCRIPTION OF BACILLUS LICHENIFORMIS

Bacillus licheniformis is a Gram positive, rod shaped bacteria generally measuring approximately 0.6μ to 0.8μ by 1.5μ to 3.0μ. They are motile usually with peritrichous flagella. Their spores are ellipsoidal or cylindrical, central or paracentral and swelling the sporangia only slightly if at all (Buchanan and Gibbons, 1974). They are mesophilic with minimum growth temperature of about 20°C and maximum between 50 to 55°C.

Bacillus licheniformis together with Bacillus subtilis and Bacillus pumilus have been classified together to form the subtilis-group or subtilis spectrum. This is because they all have a good number of properties in common and there are few characteristics by which they can be separated (Gordon et al, 1973). Smith et al, (1946) reported the possibility of dividing this group by using two criteria, anaerobic production of acid and gas from glucose and gas production from nitrate by anaerobic cultures. Their macroscopic appearance on agar was not a reliable means of differentiation as they all gave similar appearances. The separation of Bacillus licheniformis from

Bacillus subtilis on the basis of anaerobic growth, gas production from nitrate in anaerobic culture, growth at 56°C and the presence of arginase has been tried by Hanakova-Bauerova et al, (1965). Serological tests have also provided a reliable separation between Bacillus subitilis and Bacillus licheniformis because their spore antigens have been distinguished in agglutination and precipitation tests (Buchanan and Gibbons, 1974). Bacillus pumilus which is the third and last member of this subtilis group is closely related to Bacillus subtilis (Gordon et al, 1973). However, one of the differences established between Bacillus pumilis and Bacillus subtilis is that the former requires biotin for growth whereas the latter grows at 37°C on ammonium basal medium without added growth factors (Knight and Proom, 1950).

2.4 SOME INDUSTRIALLY IMPORTANT ENZYMES PRODUCED BY MEMBERS OF THE GENUS BACILLUS

2.4.1 Proteases

Microbial proteases have been divided into four groups on the basis of their mechanism of action (Morihara, 1974). They are, serine proteases; metal (or metal-chelator sensitive) proteases, thiol protease and acid protease. With very few exceptions, the extracellular proteases of the genus Bacillus are either serine or metal enzyme (Priest, 1977).

The serine proteases have molecular weights in the range of 25,000 to 30,000 daltons and are characterised by the presence of a serine residue at their active site. Serine proteases are stabilized at high temperatures by calcium ion. These enzymes are inhibited by diisopropyl-fluorophosphate but, possessing no metal ion requirement, they are resistant to ethylene diamine tetraacetic acid (EDTA) and their production is associated with sporulation (Dancer and Mandelstam, 1975). The alternative name, alkaline protease reflects their high pH optimum of 9.0-11.0 (Matsubara and Feder, 1971). Serine proteases can be divided into two groups on the basis of their general importance. Group A is typified by the enzymes from Bacillus licheniformis (subtilisin carlsberg) and Bacillus pumilus (Aunstrup et al, 1972) and group B serine proteases are related to subtilisin Novo, which is identical to subtilisin BPN the enzyme from Bacillus amyloliquefaciens (Smith et al, 1968). Serine proteases from Bacillus subtilis Marburg and Bacillus subtilis var amylosacahariticus also belong to the group B serine proteases. Generally, the group A enzymes are less stable below pH 9.5 and have a higher esterase activity than the group B enzymes (Keay, 1972).

Metal proteases are a group of metallo endopeptidases that show maximal activity at or near neutrality. They are sensitive to metal chelating agent and do not possess esterase activity

(Mutsubara and Feder, 1971). Zinc and calcium ions are essential for the enzyme activity and stability, respectively. Their optimum pH is around pH 7.0, so they are also called neutral proteases. Metallo-proteases are found alone or together with other proteases in many microorganisms. They are produced by several Bacillus species, for example, Bacillus subtilis, Bacillus cereus, Bacillus megaterium and Bacillus sterothermophilus (Aunstrup, 1980). Metal proteases generally are the least stable of all the Bacillus proteases (Ohta, 1967).

Glucose and maltose have been reported to be good carbon sources for protease production and rice bran and defatted rice bran have also been reported to be good sources of nitrogen for the biosynthesis of protease by Bacillus species (El-Saied and Ghali, 1976).

The industrial production of protease is usually by Bacillus licheniformis (Keay and Willi, 1970; Li and Yousten, 1976). Most proteases are specific in relation to the peptide likage they can split. The use of the protease enzyme for either limited proteolysis or complete hydrolysis of the protein substrate to animo acids can be achieved with proper selection of enzymes and appropriate conditions of time, temperature and pH (Smith, 1951). Metalloproteases have been used for the hydrolysis of barley protein in the brewing process where an inhibitor in the barley inhibits serine proteases, but not the metalloproteases. Metalloproteases are generally more specific than serine

proteases and this has led to the belief that they could be used as microbial rennet (Aunstrup, 1980).

Proteases have wide applications in the clinics and the pharmaceutical industries where they are used for the debridement of wounds by injection to relieve inflammation. They are also used in the laundary industries, in the leather manufacturing industries, where they are used for baiting and unhairing hides. Their use in the photographic industries for the recovery of silver from photographic films has also been reported (Underkofler et al, 1958).

2.4.2 Amylases

Amylases are starch degrading enzymes. They are distributed widely in the microbial, plant and animal kingdoms. They are divided into three types namely: α-amylase, β-amylase and amylogucosidase (glucoamylase) (Thoma et al, 1971). The α-amylase (α-1,4-glucan 4-glucanohydrolase EC 3.2.1.1) is an endosplitting enzyme which hydrolyzes the α-1,4-glycosidic bonds and bypasses α-1,6-linkages in amylopectin and glycogen, thereby liberating products of varying levels of glucose units which are linked with the α-configuration (Robyt and French, 1963). The action of the α-amylase causes a rapid decreases in the iodine staining power together with rapid liquefaction and marked reduction in the viscousity of starch, hence the name liquefying amylase (Fogarty and Kelly, 1980). The β-amylase (α-1,4 glucan

maltohydrolase, EC 3.2.1.2) is an exosplitting enzyme which characteristically hydrolyses the penultimate bonds at the non-reducing ends of glucose chains in glycogen or amylopectin to yield maltose as the main product together with a residual component known as β-limit dextrin. The inability of the β-amylase to bypass the α-1,6 linkages results in the formation of the β-limit dextrin (Fogarty and Griffin, 1973). The amyloglucosidase (α-1,4-glucan glucohydrolase EC 3.2.1.3) is also an exosplitting enzyme and hydrolyzes amylose, amylopectin and dextrins to give glucose as the sole end product (Whitaker, 1972).

The production of amylase is generally widespread among living organisms. Other than microorganisms and plants, the production of amylase has been reported in the organs of some animals. McGeachin and Potter (1967) reported the synthesis of amylase by the pancrease and salivary glands of animals. The production of amylase by the liver has also been reported (McGeachin and Potter, 1960; Rutter et al, 1961).

Other tissues and organs reported to produce amylase in different animals include the intestinal tract, lungs, duodenum and kidney (McGeachin et al, 1958).

Silano et al (1975) and Powers and Gulberston (1982) have reported the production of amylase by some insects such as: Tenebrio molitor (yellow meal worm), Tribolium castaneum (red flour beetle) and Tribolium confusum (confused flour beetle).

Some marine organisms such as the squid, the cuttle fish and the octopus and some animals such as turkey, quail and monkey have also been reported to produce amylase. In this review emphasis will be on the amylase produced by members of the genus Bacillus

The three types of amylase can be distinguished from each other on the basis of these criteria (Whitaker, 1972) as summarized in the table below.

	Relative Rates		
Criterion	α-amylase	ß-amylase	glucoamylase
Reducing group formation	Fixed and equal	Fixed and equal	Fixed and equal
Loss in Viscousity	fast	slow	slow
Loss in iodine colour	fast	slow	slow
maltose production	slow	fast	none

2.4.2.1 α-Amylase

α-Amylase (α-1,4-glucan 4 glucanohydrolase EC 3.2.1.1) occurs widely in microorganisms. It hydrolyzes α-1,4-glucosidic bonds in amylase, amylopectin and glycogen in an endo fashion but the α-1,6-glucosidic linkages in the branched polymers are not hydrolysed (Fogarty and Kelly, 1980). The source of the enzyme is an indication of its properties and mechanisms of action. The endo-acting characteristic of the enzyme is responsible for the

rapid decrease in the iodine staining power together with a rapid decrease in the viscosity of starch solutions. The hydrolysis of amylose by α-amylase affects its complete conversion into maltose and maltotriose initially which is then followed by the hydrolysis of maltotriose which is a poor substrate (Walker and Whelan, 1960a).

The hydrolysis of amylopectin by α-amylase also yields glucose and matlose in addition to a series of branched α-limit dextrins containing four or more glucose residues containing all of the α-1,6-glucosidic linkages of the original structure. The second stage of α-amylase degradation in amylopectin or glycogen, involves slow hydrolysis of maltotriose as well as slow hydrolysis of specific bonds near the branch points of the α-limit dextrins. Different α-amylases produce different α-limit dextrins (Fogarty and Kelly, 1980).

Roberts and Whelan (1960) have reported the production of multiple-branched dextrins in addition to singly branched α-limit dextrins by some α-amylases. In these reactions, isomaltose is not formed because the α-1,6 linkages are resistant to α-amylases and they thereby confer some stability on certain α-1,4 linkages near the branch points.

Pazur and Okada (1966) reported that members of the genus Bacillus produce both the saccharifying and liquefying α-amylases which are distinguished on the basis of their mechanisms of starch degradation by the fact that the saccharifying α-amylase

produces an increase in reducing power about twice that of the liquefying enzyme. Bacillus subtilis marbug, Bacillus natto and Bacillus subtilis var amylosaccharitícus are examples of species of Bacillus that produce saccharifying α-amylase (Matsuzaki et al, 1974b) and Bacillus amyloliquefaciens produces large quantities of liquefying α-amylase (Welker and Campbell, 1967).

The molecular weight of α-amylase is within the range of 30,000-60,000 daltons. In the case of Bacillus subtilis, α-amylase, monomer-dimer transformation was reported with the dimer having a molecular weight of 100,000 daltons (Menzi et al, 1957). From the reports of Mitchell et al (1973) and Robyt and Ackerman (1973), the α-amylase of Bacillus subtilis has a basic subunit of 24,000 dalton molecular weight. A lower molecular weight of 22,500 has been reported for the α-amylase of Bacillus licheniformis (Saito, 1973). α-Amylase is usually stable in the pH range of 5.5 to 8.0 and to extremes of pH value when supplied with a full complement of calcium ions. Their optimum pH values generally occur between pH 4.8 and 6.5 (Manning and Campbell, 1961), but with significant differences in the shapes of the pH - activity curves for the different enzymes (Fogarty and Kelly, 1980). Bacillus acidocaldarius produces a thermoacidophilic α-amylase which has an optimum pH value at 3.5 and an optimum temperature of 75°C (Buonocore et al, 1976), whereas the enzyme

from Bacillus licheniformis has a broad range of activity between pH 5.0 and 9.0 and a temperature optimum of 76°C (Saito, 1973). In contrast, the amylase produced by alkaline Bacillus species is not thermostable, although it has an alkaline pH optimum (Boyer and Ingle, 1972), whereas the enzyme produced by Bacillus stearothermophilus is thermostable and has little activity under alkaline conditions.

The activity of most highly purified α-amylases is lost quickly above 50°C but the presence of calcium ions may slow down this inactivation (Fogarty and Kelly, 1980). All α-amylases are calcium metallo-enzymes having a minimum of one atom of this metal per molecule of enzyme, and the strength of binding of metal to protein depends on the origin of the enzyme (Fischer and Stein, 1960). The metal is required for catalytic activity.

In the presence of calcium ions α-amylases are quite resistant to extremes of pH value, temperature, treatment with urea or exposure to some proteases. However, calcium-free amylases are highly susceptible to denaturation by similar treatments (Stein and Fischer, 1958). Once calcium-containing α-amylases have been denatured, they are susceptible to protease degradation (Fogarty and Kelly, 1980).

The fungal α-amylase also requires calcium ion for activity as is the case with bacterial α-amylase. The α-amylase of Aspergillus niger contains one gram-atom of calcium, while the α-amylase from Aspergillus oryzae binds ten gram-atoms of calcium

per mole of enzyme (Arai et al, 1969). Other divalent metals such as strontium, barium and magnesium, could replace calcium without appreciable loss in activity but it appears that these metals can replace calcium only when the original conformation of the protein is retained and the active site has not been altered (Whitaker, 1972). Stein and Fischer (1958) have reported that heavy metals such as silver, lead, copper and mercury inhibit the activity of α-amylases.

Until recently, the organism used in the commercial production of bacterial α-amylase was described as Bacillus subtilis. The highly amylolytic strains of Bacillus subtilis that produce liquefying α-amylase have been re-classified as Bacillus amyloliquefaciens by Welker and Campbell (1967) on the basis of physiological and biochemical properties. It is this Bacillus amyloliquefaciens that is now mostly widely used in the commercial production of α-amylase (Fogarty and Kelly, 1980). Saito and Yamamoto (1975) recently reported that Bacillus licheniformis produces an α-amylase with very good thermostability properties which allow its use at temperature in excess of 100°C.

2.4.2.2 β-Amylase

β-amylase (EC 3.2.1.2, α-1,4-glucan maltohydrolase) occurs widely in higher plants. Its occurrence as an extracellular enzyme in microorganism was recently established

(Fogarty and Griffin, 1973). β-Amylase degrades amylose, amylopectin and glycogen in an exo-fashion from the non-reducing chain ends by hydrolysing alternate α (1,4)-glucosidic linkages. The enzyme is incapable of bypassing α-1,6-glucosidic linkages in branched polymers and degradation is therefore incomplete (Fogarty and Kelly, 1980). Unlike α-amylase, the β-amylase can not hydrolyse maltoriose but can hydrolyse maltotetraose (Robyt and Ackerman, 1971). Among members of the genus Bacillus, Bacillus polymyxa has been reported to produce appreciable amounts of β-amylase (Robyt and French, 1964).

Hydrolysis of amylopectin by this enzyme produced β-maltose and a limit dextrin similar to that produced by sweet-potato β-amylase. Many of its properties are similar to typical plant β-amylase (Fogarty and Griffin, 1975).

The optimum pH value is 6.8 which is higher than that recorded for plant β-amylase. It is quite stable in the pH range of 6.4-7.2, but less stable in more acidic pH conditions. The optimum temperature for its activity was reported to be 37°C and it decreased rapidly when exposed to temperatures above 37°C (Fogarty and Kelly, 1980). The plant β-amylases are thiol enzymes with no obvious requirement for metal ions (Rowe and Weill, 1962). As reported for microbial β-amylases, plant β-amylases are also inactivated by thiol reagents such as p-chloromercuribenzoate and by oxidation.

Oxidation occurs in dilute solution and is overcome by the presence of serum albumin and reduced glutathione (Walker and Whelan, 1960b). Other members of the genus Bacillus that have been reported to produce β-amylase include Bacillus megaterium and Bacillus cereus var Mycoides (Higashihara and Okada, 1974).

2.4.2.3 Amyloglucosidase

Amyloglucosidase (EC 3.2.1.3, glucoamylase, γ-amylase) is an exo-acting enzyme that yields β-D-glucose by hydrolysing α-1,4 linkages consecutively from the non-reducing ends of amylose, amylopectin and glycogen. It also hydrolyzes α-1,6 and α-1,3 glycosidic linkages (Pazur and Klepp, 1962). Unlike α-amylases and β-amylases which are products of both bacteria and fungi, amyloglucosidase (glucoamylase) occurs almost exclusively in fungi.

The rate of substrate hydrolysis is affected by molecular size and structure, and by the next bond in sequence (Abdullahi et al, 1963). Thus, the rate of hydrolysis of α-1,4 bonds increases with the molecular weight of the substrate. Similarly, in hydrolysis of α-1,6 linkages the size of the substrate and the position of the linkage play a significant role in their susceptibility to hydrolysis (Krzechowsk and Urbanek, 1975).

Amylogucosidase has optimum temperature for activity between 40°C and 60°C and optimum pH between 4.5 and 5.0, but generally

stable on the acid side of pH 7.0. Preparations of amyloglucosidase have the unique property of polymerizing glucose. The reverse reaction is accompanied by elimination of water and formation of maltose, isomaltose and other oligosaccharides (Hehre et al, 1969). The enzyme transglucosidase often occurs as an impurity in the crude preparations of amyloglucosidase (Pazur and Ando, 1961). Among the many genera of fungi that produce amyloglucosidase, Aspergillus species are prominant (Pazur and Ando, 1960). Other important genera include Penicillium and Rhizopus. The production of amylase by microorganism other than bacteria and mould, have been reported. Upton and Fogarty (1977) reported the production of amylase by an actinomycete Thermomonospora viridis. Lammel et al (1980) also reported the production of amylase by a yeast Saccharomyco-psis fibuligera.

Burbidge and Collier (1968) have reported that bacteria amylase has the advantage of being more thermostable than fungal amylase. Bacteria amylase preparations remain operative at temperatures exceeding 80°C while fungal amylase loses its activity at temperatures higher than 60°C. At these elevated temperatures the bacteria amylase gives rapid liquefaction of starch while fungal amylase gives good saccharification at low temperatures (Underkofler et al, 1958).

2.5 INDUSTRIAL APPLICATIONS OF AMYLASES

The use of amylases in industries such as the textile, pharmaceutical, food and beverages is wide. In the brewing industries, bacterial amylase because of its stability at high temperatures is used in supplementing low diastatic malt and the initial liquefaction of adjuncts such as rice or corn grits (Ononogbu, 1981). Fungal amylases are used as a saccharifying agent in grain alcohol fermentation mashes (Rose, 1961). It has also found use in the conversion of partially hydrolysed starch to sweet syrups. The proper control of the type and proportion of enzymes used usually produces syrups of almost any desired proportions of glucose, maltose and dextrins (Underkofler et al, 1958). In the textile industries, bacterial amylase is used in the continous process for desizing of textile fabrics and in preparing modified starch sizing for textiles.

In the paper industries, microbial amylase could be used for starch coatings and removal of wall papers. It could also be used for the preparation of cold water starches in laundry industries (Underkofler et al, 1958). Fungal amylases are used for starch removal for flavouring extracts, for fruit extracts, juices and in preparing clear, starch-free pectin (Pazur and Ando, 1961).

Other uses of microbial amylases are in treating of vegetables for canning, processing cereals, preparing breakfast foods, in the confectionary and candy industries (Godfrey, 1985).

Since most of these industries mentioned here also operate in Nigeria, the need for amylase in these industries can not be overemphasized. In the laundry industries particularly, since most of the laundry services are carried out in cold water, the use of amylase in this industry will be very important.

2.6 IMPORTANT FACTORS TO BE CONSIDERED IN THE PRODUCTION OF AMYLASE FOR COMMERCIAL USES

In developing the enzyme of any organism for commercial applications, a number of factors will have to be considered.

Medium Composition

The production of amylase by a given organism is affected by the composition of the medium. A careful balance of carbohydrate and nitrogen constituents together with semi-micro and macro components is important (Burbidge and Collier, 1968). Coleman and Grant, (1966) reported that amylase is an inducible enzyme in which case the medium for its cultivation should contain an inducer. Starch is widely used as a carbon source and inducer in the production of α-amylase (Upton and Fogarty, 1977). The use of wheat bran as a carbon source for a number of strains of Bacillus subtilis and baked bean waste for high level production of α-amylase and amyloglucosidase by Aspergillus foetidus was reported by Hang and Woodams, (1977). In relation to induction, yield of amylase is considerably higher when the medium is based

on naturally occurring materials such as wheat, maize, barley, malt, rather than on artificial media largely composed of simple molecules (Nyiri, 1971). In this respect cassava will be a suitable inducer in Nigeria because it is a readily available naturally occurring material. A variety of nitrogen sources have been used for α-amylase production. Examples of these include ammonium citrate, mycological peptone, cornsteep liquor, calcium nitrate and ammonium sulphate (Upton and Fogarty, 1977).

Apart from the carbon and nitrogen which are basic requirement for growth and α-amylase production by microorganism, some other compounds and elements have been reported to play prominant roles in the synthesis of α-amylase by various microorganisms. Markkanen and Bailey (1975) reported that the formation of α-amylase by Bacillus subtilis was stimulated by carbon dioxide (CO_2). Fogarty and Kelly (1980) also reported that calcium ions and in some cases manganous ions are significant for α-amylase synthesis and production and are usually incorporated in their production medium. The introduction of glucose into the production medium on the other hand have been reported to repress amylase synthesis by several species of Bacillus including Bacillus subtilis and Bacillus licheniformis (Heineken and O'Conor, 1972; Meers, 1972).

Other parameters such as pH and aeration have also been shown to be important in the production of α-amylase by various microorganisms. In their study of the effect of pH value and

aeration on amylase production by Bacillus subtilis. Mazza and Ertola (1970) observed that if the pH value is controlled during fermentation, aeration of the culture can be discontinued at the same time as cessation of growth, and α-amylase yields will be similar to those of an aerated culture. Markkanen and Bailey (1975) also showed that α-amylase production by Bacillus subtilis was notably increased by decreasing aeration at the end of the exponential phase of growth.

Temperature variation is also an equally important parameter for α-amylase production. Markkanen and Bailey (1974) reported that temperature used for α-amylase production by Bacillus subtilis is between 37°C and 40°C. Also is Bacillus licheniformis, temperature within the range of 37°C was used for α-amylase production (Outtrup and Aunstrup, 1975).

Strain Selection

The selection of train of organism for enzyme production depends on what the enzyme is going to be used for. As regards amylase production, the strain and type of organism used affect the type of amylase produced. Amylase used in food related industries are produced by microbial cultures that have been tested and established as non-pathogenic, non-toxigenic and non-antibiotic producing (Pariza and Foster, 1983). Also in cases where fungal amylases are needed, mycotoxin-producing fungi are not used for enzyme production. The purpose for which the enzyme

is needed also affects the type of organism for its production. For example, amylase to be used in the brewing industries are produced from bacterial sources because bacterial amylase is more thermostable than fungal amylase (Burbidge and Collier, 1968). Once a given type of microorganism is chosen, the species and strain would then be determined by a comparison of the relative potential of many isolates under a given set of experimental conditions.

Strain Improvement

The overall goal in selecting and improving strains of microorganisms is to optimise the behaviour of the strain in the particular process under consideration. Essentially, this amounts to the strain providing optimal efficiency in both quantitative and qualitative forms of product formation and thereby maximal cost effectiveness in commercial terms (Johnston, 1985). Apart from increased product yield which might be the primary objective, other characteristics should also be given adequate consideration. For example, some undersirable bye-products such as pigments which may be formed along with the products may need to be eliminated, and some chacteristics related to fermentation technology, such as ease of separation of the product from the organism may prove important (Calam, 1970).

The improvement of the strain could be achieved through mutagenesis. Different mutagenic agents could be used for the

improvement of amylase production by different microorganisms (Yoneda and Maruo, 1975). The mutagenic agents frequently used include ultra-violet rays, X-rays, gamma rays, and chemicals such as ethyleneimine, ethylmetanesulphonate, N-methyl-N-nitro-N-nitroso-guanidine (NTG) and β-propiolactone (Gratzner and Sheeham, 1969).

2.7 GENETIC IMPROVEMENT OF INDUSTRIAL MICROORGANISMS

One of the most important, if not the most important method of improving the efficiency of fermentation processes is the production of improved strains by mutation and selection (Backus and Stauffer, 1955). Strain improvement and selection depend on alternative process of diversification, selection and rediversification, so that better and better strains are successfully picked out and further improved (Hopwood, 1970).

As earlier reported, mutation is a process by which microorganisms could be improved for the production of industrial products. This mutation could be induced or spontaneous. The spontaneous mutation which is known to occur randomly occur relatively rarely even among bacteria. The application of induced kind of mutation to microorganisms is favoured by the fact that these organisms have short generation time and give rise to enormous population which can be easily obtained and manipulated under controlled conditions while a few mutants arising in the population can be detected by selective methods

(Calam, 1970). Even, where programmes of mutation or hybridization have been followed, the screening of large numbers of isolates in order to detect a promising variant has often been on a random trial-and-error basis (Johnston, 1985).

2.7.1 Hybridization

A hybridization method which has aroused a great deal of attention is the parasexual process, which allows crossing to take place between asexual organisms such as the fungi imperfect and actinomycetes. In addition to this, other processes exist, such as those involving the intervention of phages. Sexual crossing is also used especially in the case of yeasts (Calam, 1970).

The parasexual process was originally described in fungi, by Pontecorvo and Sermonti (1954). The method of hybridization has been successfully used in the improvement of penicillin production from a penicillin-producing organism (Macdonald et al, 1963). Alikhanian (1962) has desribed a process of hybridization in actinomycetes in which transformation and induction processes were been used. The use of hybridization technique as a means of strain improvement in yeasts has been of interest for many years (Rose and Harrison, 1969).

With bacteria, hybridization, or transfer of properties is possible through three routes: conjugation by a sexual process, transduction via a phage and transformtion by means of DNA which

carries genetic materials from one cell to another. Alikhanian and Iljina (1957) have reported the mutagenic effect of an actinophage on Actinomyces olivaceus, with about 99% of the isolates representing new forms. The relation between viruses and bacteria and the participation of viruses in the transfer of genetic material is attracting much interst at the present time. Apart from transduction and transformation, parts of viruses can become permanantly incorporated into the chromosomal system of bacteria, bringing with them new metabolic activities (Anderson and Cowles, 1958). Lysogeny is a particularly well known example of this, where the "transfer factors" carry determinants for drug resistance, especially to antibiotics. The good understanding of all these mechanisms presents the possibility that they could be used for strain improvement.

2.7.2 Mutagenesis

This is a process by which a change in the genetic make up of microorganisms can be achieved. These changes can be achieved through induced mutation. Mutation usually results from structural alterations in genes but sometimes from rearrangement at the chromosome level. The induction of mutations by physical and chemical agents has been extensively used in strain improvement programmes featuring both bacteria and fungi (Hopwood, 1970). Whereas mutation alters gene structure, 'recombination' combines genes from two or more parental strains

in one individual (recombinant) organism. In microorganisms, recombination can take place by a wide variety of mechanisms resulting in complete chromosome reassortment at one extreme to substitution of a single gene or part of a gene at the other (Spencer and Spencer, 1983).

It is an empirical fact that the use of different mutagens usually results in isolating different 'spectra' of mutants. Probably most of the differences are due to factors affecting the likelihood of recovery in viable colonies of mutations in particular genes, rather than to real differences in their frequency of occurrence (Calam, 1970). Current notions on gene structure and the mechanism of mutagenesis by different agents led to the prediction that any mutagen should be capable of inducing mutations at some sites in any gene, although particular sites (depending on the base pair in the DNA) will be mutable by some, but not by all mutagens. Furthermore only certain mutagens lead to particular classes of mutational events, such as the deletion or addition of single bases (frame shift mutation) (Hopwood, 1970).

Iyer and Szybalski (1958) reported that, to find a mutagen effective in a little known organism, atest for mutagenesis may be useful. This test uses reverse mutation as a criterion of mutagenesis, and the likelihood is that a mutagen effective in this test will also be suitable for the induction of the forward mutations that are usually required in isolating desired mutants.

2.7.3 Methods of Mutation and Choice of Mutagen

The mutation process involves two steps, the treatment of the organism with the mutagen and then the isolation of the mutants prior to testing and selection. Mutagens commonly used have been classified on the basis of their mode of generations.

2.7.4 Radiations

Ionising radiations which include X-rays, gamma-rays, fast neutrons and ultraviolet light are generally used for the induction of mutation in different organisms. The use of X-rays on Drosophila as source of mutation was first reported by Muller (1928). Later, Promptov (1932) reported the use of ultraviolet light to induce mutation in Drosophila. Ultraviolet light is an extremely convenient mutagen provided that the cells to be treated are appreciably transparent to it (Hopwood, 1970). Mutagenic wavelengths lie between about 200-300nm, the peak of absorption by nucleic acids. It is thus an extremely fortunate coincidence that a convenient source of ultraviolet emits a very high proportion of its energy at 254nm close to the most effective wavelength (Meynell and Meynell, 1965).

The photolytic action of ultraviolret light attacks the cytosine nucleotides much more than the thymine nucleotides (Shugar, 1960). The actual effect of ultraviolet light on the DNA has been explained to be really a dimerisation of the thymine nucleotides which thereby results in the formation of thymine

dimers, whic eventually leads to the weakening of the bonds between ther and a distortion of the double helix. This distortion i erferes with replication because it gives rise to dimerisation of thymines on opposite strands which eventually causes cross nkage of the strands and thereby prevents their separation (K lner, 1953; Beukers and Berends, 1960). The other ionizing irra iations are used occasionally for the routine induction of mutations for microorganisms only when other mutagens are neffective. This is because of the comparatively high likeliho l that chromosomal breakage will occur, which might lead to struc ural changes such as translocation and inversions which will co plicate subsequent genetic analysis of the mutants (Kafer, 1965 . The successful improvement of α-amylase production by Bacillus subtilis using ultraviolet light as the mutagen has be n reported by Bailey and Markkanen, (1975).

2.7.5 Chemica Mutagens

I numerable compounds are mutagenic, but few are convenient fo the routine isolation of mutants. Chemical mutagens can g nerally be grouped into three classes on the basis of their mode f action (Calam, 1970).

The firs class of chemical mutagens are those that cause chemical chang s in one or more of the nucleic acid bases, while they remain in situ in the molecule. They can, therefore, operate on non-replica ng nucleic acids. The second class are those that

mimic a natu al base closely enough to be mistaken for it and inserted int a newly synthesized strand during nucleic acid replication (he base analogues). Finally, there are those that cause loss o addition of one or two bases in DNA during its replication o repair (the frame shift mutagens).

Some of the chemical mutagens used include the alkylating agents such s ethylmethane sulphonate, ethylethane sulphonate and diethyls lphonate. These mutagens act specifically on guanine at t e N-7 position. It labilizes the deoxyriboside linkage so th t 7-alkyl guanine is released from the DNA even at 37°C and pH 7) (Brookes and Lawley, 1960). Of the large class of alkylating gents, these three compounds seem to have achieved the greatest se as routine mutagens. There are other chemical mutagens such as the dyes of the acridine series. Orgel and Brenner (196 , who studied systematically, the relationship between mole lar structure and mutagenicity reported that certain acrid es although strongly mutagenic for bacteriophages T2 and T4, ar very weak or non-mutagenic in other systems, for example, bacte ia.

Another hemical mutagen is nitrous acid which act on the nucleic acid b se of resting DNA in such a way that the specific pairing with ther bases is changed (Kaudewitz, 1959). For example, aden e is deaminated to form hypoxanthine which then pairs with cy sine instead of thymine. It can also deaminate cytosine to fo m uracil (Hayes, 1964). Lastly, another group

includes the base analogues such as the 5-bromouracil and 2-aminopurine in which case the base analogues replace the normal bases of DNA during replication (Orgel, 1965). Freese (1959) reported that the ease with which these base analogues cause mutation could be due to the destabilization of their hydrogen atoms by the bromine atom which has a higher electronegativity than the substituted methyl group.

CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganism

The parent strain and reference organism for all mutant strains was Bacillus licheniformsB5.1 described previously as a good producer of amylase (Raji, 1987). Strains were cultivated at 35°C and maintained on Nutrient Agar slants stored in the refrigerator at 4°C.

3.2 Media

3.2.1 Mineral-Salt-Glucose (MSG) medium (Raji, 1987) containing $(NH_4)_2HPO_4$, 0.5%; sodium citrate, 0.2%; $MgSO_4.7H_2O$ 0.05%; $CaCl_2$, 0.08%; $FeSO_4.7H_2O$, 0.001%; glucose, 1.0% and peptone 1.0%; was used for developing the incoulum for mutagenic treatment and for inoculation of shake flask.

3.2.2 Mineral-Salt-Cassava (MSC) medium (Raji, 1987) containing sodium citrate, 0.2%; $(NH_4)_2HPO_4$, 0.5%; $MgSO_4.7H_2O$, 0.05%, $CaCl_2$ 0.08%; $FeSO_4.7H_2O$, 0.001%; "dawadawa", 1.0% and cassava starch, 4.0% was the culture medium for shake flask.

Mineral-Salt-Glucose (MSG) medium (50ml) was dispensed into 250ml Erlenmeyer flasks while 150ml of Mineral-Salt-Cassave (MSC)

medium was dis ensed into 500ml Erlenmeyer flasks. The pH of the MSC medium was adjusted to 6.5 before flasks were antoclaved at 121°C for 15 m utes.

3.3 Mutagenic gent

The ult violet irradiation was used and this was applied continously to selected strains. Standard conditions for mutagenic treat ent as reported by Bailey and Markkenen, (1975) were adopted.

3.4 Standard co lition for ultraviolet treatment

A 12-hour ld culture of cells was centrifuged aseptically for 10 minutes, ashed in a 0.14M saline and recentrifuged. The cells whi were obtained after recentrifugation were suspended in 15 saline in a sterile petri dish which was placed 6cm under a V lamp and stirred magnetically. Initial concentration of cells of the order of 10^7 cfu/ml was used in order to provi a treated suspension suitable for plating. Samples were tak n at different time intervals and cultivated on Nutrient Agar a 35°C using sterile bent glass rod. To avoid photoreactivatio and dark repair, post irradiation manipulations were carried out n buffer with 0.2% caffeine and the environment was illuminated ith yellow light using sodium vapour lamp as source (Asato, 19 2).

3.5 Auxanogr phic method for screening of the irradiated strains for conf med mutation

Minima medium (K_2HPO_4, 28g; KH_2PO_4, 12g; $(NH_4)_2SO_4$, 4g; sodium citrat . 2g; glucose, 0.2g; agar, 15g; and distilled water 1000ml) which allows only for the growth of the wild type strain and not for the mutant strain, and complete medium (sodium chloride, 5g; Lab-Lemco powder, 1g; yeast extract, 2g; peptone, 5g; agar, 15g and distilled water, 100ml) which allows for the growth of bot the wild-type and mutant strains were prepared and 20ml of each dispensed into universal bottles and sterilized in the autoclave at 121°C for 15 minutes.

The grow requirement of the mutant strain that was unable to grow on th minimal medium was determined. This was achieved by inoculatin minimal medium agar plates on which various growth substances h been placed in marked positions with heavy inoculum of t irradiated strain.

For this particular experiment, the first step involved the addition of three different growth substances (vitamin-free casein hydro ysate, yeast nucleic acid and yeast extract) simultaneousl on the same Petri dish and growth around any of the substance recorded. This was carried out in duplicate, so as to indicat consistency, as distinct from accidentl response. The results btained from this step will indicate the pattern the second s p will take viz:

If the organism grows arround vitamin-free casein hydrolysate it indicates a need for amino-acids for growth, if the organism grows around yeast nucleic acid, it indicates a need for purine and pyrimidine while if the organism grows around yeast extract, it indicates need for vitamins of the B group.

3.6 Cultural method for enzyme production

After mutagenic treatment surviving cells were allowed to subdivide before plating to give any further changes a chance to express themselves and thus avoid the production of unstable forms. Successful mutant cells were then collected and cultivated on agar slants for 48 hours at 35°C (Hopwood 1970). After inoculation of duplicate slants, cells were transferred to mineral-salt-glucose (MSG) medium which was used for the development of the inoculum.

In this process, a loopful of the isolate was transferred from Nutrient Agar slant to 50ml of the sterile mineral-salt-glucose medium in the Erlenmeyer flask. The culture was then incubated in a orbital shaker (Lab-line Instrument Inc. U.S.A.) at 200r.p.m for 24 hours at 30°C. Subsequently 5.0ml amounts were transferred into duplicate 500ml flasks containing the mineral-salt-cassava (MSC) medium which served as the fermentation medium for amylase production.

The inoculated medium was incubated in the orbital shaker at 30°C for 4 days hours at 275 r.p.m. With every series of cultivations, two cultivations of Bacillus licheniformis B5.1 were present as standards.

3.7 Recovery of crude amylase

At the end of the growth period in the mineral-salt-cassava (MSC) medium, the contents of the flasks were pooled and centrifuged at 5,000r.p.m for 15 minutes. The supernatant, now the crude enzyme, was then saved for amylase assay and the sedimented bacterial cells and other unwanted components of the flask were discarded.

3.8 Determination of α-amylase activity

α-Amylase activity was assayed according to the method of Fischer and Stein (1961) with some modifications. A 1% solution of soluble starch (Difco) was prepared in 0.02M phosphate buffer (pH 6.8). To 10ml of the buffered starch solution were added 0.5ml of 0.5M $CaCl_2$ and 2.0ml of distilled water. One milliliter of the buffered starch solution was added to 1ml of an enzyme solution diluted in 0.02M phosphate buffer (pH 6.8) in a test tube. After exactly 5 minutes of incubation at room temperature, the reaction was stopped by adding 2.0ml of dinitrosalicylic acid reagent and the test tube placed in boiling water for 5 minutes after which it was cooled under running tap water at ambient

temperature and brought to a final volume of 24ml with distilled water. The tubes were thoroughly mixed and the resulting orange coloured solution was decanted into cuvettes. The absorbance was read at 540nm in a spectrophotometer (WPA S101) Linton Cambridge, U.K). The amount of reducing sugars released by α-amylase action on starch was derived from a standard curve previously calibrated with glucose as standard.

An amylase activity unit was defined as that amount of enzyme that produced 1mg of glucose under the above conditions.

3.9 Preparation of glucose standard curve

The method of Miller (1959) was adopted with some modifications: Stock solution of glucose was prepared by dissolving 360mg of glucose in 100ml of distilled water to give a final concentration of 3.6mg/ml. From this stock solution, varying glucose concentrations ranging from 300µg/ml to 3,000µg/ml were prepared, To 2ml of each resulting glucose concentration, 2ml of dinitrosalicylic acid reagent was added in 14mm test tubes. The blank was prepared by adding 2ml of dinitrosalicylic acid reagent (dinitrosalicylic acid, 1%; phenol, 0.2%; sodium sulphite, 0.05%; and sodium hydroxide, 1%) to 2ml of distilled water in a 14mm test tube. The mixtures were heated in a boiling water bath for 5 minutes after which it was cooled under running tap water at ambient temperature and brought to a final volume of 24ml with distilled water. The absorbance of the coloured reaction products was measured using a spectrophotometer

(WPA S10] Linton Cambridge) set at 540nm. The absorbance of the contents in each tube was plotted against the corresponding glucose concentration, to give a standard glucose curve.

3.10 Protein determination

The protein content of the enzyme preparation was determined by the Folin-Lowry's method (Lowry et al 1951) using bovine serum albumin as standard.

3.10.1 Reagents

A. 50% Folin - Coicalteau reagent (1:1% H_2O).

B. 2% sodium potassium tartrate

C. 1% $CuSO_4.5H_2O$

D. 2% Na_2CO_3 in 0.1N NaOH.

3.10.2 Procedure

To 50ml of Na_2CO_3 in 0.1N NaOH was added 0.5ml of 2% sodium potassium tartrate and 0.5ml of 1% $CuSO_4.5H_2O$.

Five milliliters of the resulting "alkaline solution" was added to each tube containing the protein sample to be measured, mixed thoroughly and allowed to stand at room temperature for 10 minutes after which 0.5ml of the diluted Folin-Ciocalteau reagent (freshly prepared) was added to each tube and mixed well immediately. The mixture was allowed to stand for 30 minutes for colour development. The absorbance of the contents in the tubes

3.13.1 Acetone Precipitation

The method of Fischer and Stein (1961) was adopted with some modifications: Two tire beakers (pyrex) with glass stirrer for gentle stirring was used. To minimise the effects of heat of dillution of acetone, the supernatant from the centrifuged growth broth containing the crude enzyme was placed in an ice bath. The acetone was also pre-cooled to about -10°C.

To 500ml of the crude enzyme 400ml (80%) of cold acetone was added gently. The temperature of the solution during precipitation was made not to exceed 5°C. Stirring was continued for 30 minutes after all the acetone had been added, and the suspension was then centrifuged at 5000rpm for 15 minutes. The sticky pellet (residue) was then discarded and the collected supernatant solution was treated as above with 500ml acetone. This was left to stand for 2 hours after which the precipitate obtained had settled nicely. The supernatant was siphoned off with remainder of the suspension centrifuged at 5000 rpm for 15 minutes. The supernantant solution was discarded and the slightly coloured pellet dissolved in cold 0.02M phosphate buffer (pH 6.8).

3.13.2 Gel-filtration

The method described by Stock and Rice (1974) was adopted with some modification: The enzyme solution from acetone fractionation was passed through Sephadex G-200, which was allowed to swell for five days in the refrigerator by suspending

15g in 500ml 0.02M phosphate buffer pH 6.8. The gel in the column (3 x 26cm) was equilibrated with 0.02M phosphate buffer (pH 6.8). Fifty fractions, each of 5ml were collected at a flow rate of 0.63ml/min. The enzyme was eluted between fractions 17-23. The active fractions (numbers 17-23) were pooled for further purification using ion-exchange chromatography. The α-amylase activity and protein concentration of each fraction were determined as earlier described.

3.13.3 Ion-exchange chromatography

The method described by Saito (1973) was adopted with some modifications using DEAE-Sephadex G150-200. It was swollen for five days by suspending 20g in 500ml of 0.02M phosphate buffer (pH 6.8) and kept in the refrigerator. The concentrated enzyme solution (10ml) obtained from gel-filtration was passed through the DEAE-sephadex column (3 x 26cm) which had been equilibrated with 0.02M phosphate buffer (pH 6.8). A linear gradient elution was carried out in 0.02M phosphate buffer (pH 6.8) between 500ml each of 0.8M NaCl and 0M NaCl.

Fifty fractions, each of 5ml were collected. The enzyme was eluted between fractions 19-23 corresponding to 0.14 and 0.22M NaCl. Active fractions (numbers 19-23) were pooled as purified enzyme.

The α-amylase activity and protein concentration in each fraction were determined as earlier described.

3.14 Properties of the enzyme

3.14.1 Effect of starch concentration on α-amylase activity

Soluble starch solutions ranging from 1% to 10% were prepared and 1.0ml of each concentration was reacted with 1.0mlof the purified enzyme dilution in a test tube. The reaction was allowed to stand for five minutes at room temperature after which it was stopped by adding 2ml of dinitrosalicylic acid reagent to each tube and placed in boiling water bath for five minutes after which the absorbance was read at 540nm and the enzyme activity (V) determined. This enzyme activity (V) was plotted against substrate concentration (S). K_m and V_{max} were determined using the Lineweaver-Burk plot.

3.14.2 The effect of temperature on the activity of α-amylase

This study was conducted at the following temperatures: 25°C; 30°C; 35°C; 40°C; 45°C; 50°C; 55°C; 60°C; 65°C; 70°C; 75°C; 80°C and 85°C. The assay method was the same as described above and in 0.02M phosphate buffer (pH 6.8) as the assay medium. Enzyme activity (V) was plotted against the temperature.

Activation energy was determined from Arrhenius plot of log V_o against 1/T in °K. The slope of which gave the activation energy (Ea) from the equation:

$$\text{Slope} = \frac{Ea}{2.303R}$$

3.14.3 pH - Activity profile of α-amylase

Buffers of different pH values ranging from 3.5-9.0 were prepared. Substrate consisting of 1% soluble starch solution was prepared with each buffer.

Buffers used were, 0.02M acetate buffer pH 3.5-5.5; 0.02M phosphate buffer pH 6.0 - 7.5 and 0.02M tris buffer pH 8.0 - 9.0.

3.14.4 Determination of molecular weight of the α-amylase

The molecular weight estimation of the enzyme was done by passing the enzyme through a sephadex G-200 column (3 x 26cm) according to the method of Andrews (1965). The column was eluted using 0.02M phosphate buffer pH 6.8. The fractions collected (5ml each) were analysed for enzyme activity and protein concentration.

Several marker proteins such as myoglobin, haemoglobin, egg albumen and myosin were run through the same column and their different elution volumes (V_e were determined).

Blue dextran was used to determine the void volume (V_o). The V_e/V_o values of the marker proteins were plotted against their log molecular weights. The purified α-amylase was passed through this column and the V_e/V_o determined, and the result was used to extrapolate its molecular weight from a plot of V_e/V_o against log molecular weights as shown in Fig 7.

3.14.5 Effect of metal ions on the activity of α-amylase enzyme

The chlorides of calcium, zinc, copper, lead and mercury were prepared to give a final concentration of 0.02M. One millilitre of the buffered starch solution containing 0.02M of each metal ion was transferred into each test tube and 1.0ml of the purified enzyme dilution added. Each reaction tube was allowed to stand at 27°C for 5 minutes after which the reaction was stopped with 2.0ml of dinitrosalicylic acid reagent. The tubes were heated in a boiling water bath for 5 minutes, after which they were cooled under running tap water at ambient temperature and brought to a volume of 24ml with distilled water.

The absorbance was read at 540nm in the spectrophotometer (WPA S101 Linton Cambridge) and the amylase activity determined as earlier described.

3.14.6 Stability of the α-amylase in storage

The purified enzyme sample was stored under different conditions such as the freezer (-5°C), refrigerator (4°C) and at room temperature (27°C). The activity of the enzyme as described earlier was determined every week for eight weeks.

3.15 Effect of some chemical substances on α-amylase production in an incubation medium

The induction studies were carried out with the following chemicals and vitamins as inducers at the following concentrations.

Chemical substances	Concentration (%)
Triton X-100	1,2,3,4,5
Glycine	0.2,0.4,0.6,0.8,1,2,3,4,5,6.
Folic acid	0.02,0.04,0.06,0.08,0.10.
Riboflavin	0.02,0.04,0.06,0.08,0.1.0.4.
Calcium panthothenate	0.2,0.4,0.6,0.8,1.0.

5ml of each concentration of each inducer was added into the liquid growth medium (2 flasks each) in an inoculating hood under aseptic conditions after the bacteria have been inoculated. The organism was grown in an orbital shaker as described in section 3.6. Control flasks containing only the bacteria without the chemicals were subjected to the same growth conditions as those containing inducers.

After 96 hours of growth, the α-amylase activity and protein concentration were determined as described in sections 3.8 and 3.10.

CHAPTER 4

RESULTS

4.1 U.V. Irradiation and mutant generation

Table 1 shows the effect of the ultraviolet (U.V) radiation applied, along with the survival rates and average values of the activity of α-amylase produced after the treatment. It was observed that the α-amylase activity was highest in cases where the survival rates were less than 1.0%. However there were cases where the survival rates were less than 1.0% and yet α-amylase activity was low. The further exposure of the improved strains from the first irradiation to a second dose of ultraviolet irradiation increased the α-amylase production of some of these strains tremendously (Table 2).

Having shown in Table 2, that Bacillus licheniformis B5.1 strain E22 gave the highest yield of α-amylase after successful mutation by ultravoilet radiation, the strain was screened for mutation using the auxanographic method. The result of this screening showed lack of growth of this strain on minimal medium while growth was recorded on the complete medium. When the minimal medium was spotted with different growth substances (vitamin-free casein hydrolysate; yeast nucleic acid and yeast extract), growth was only observed around the yeast extract.

Table 1 Effect of treatment of Bacillus licheniformis B5.1 with ultraviolet radiation on survival and α-amylase production by the organism

Strains	Treatment time (min)	Survival %	α-Amylase activity (units/ml)
Control	-	100	0.70
A1	25	1.0	0.50
A2	25	1.0	0.72
A3	25	1.0	0.61
B1	45	1.0	0.38
B2	45	1.0	0.36
B3	45	1.0	0.40
C1	60	1.0	0.47
C2	60	1.0	0.49
C3	60	1.0	0.49
D1	60	1.0	0.90
E1	60	0.1	0.43
E2	60	0.1	2.63
E3	60	0.1	2.62
E4	60	1.0	1.90

Data - means of duplicate determinations

For strains of Bacillus licheniformis B5.1 radiated for 5-20 minutes, the survival rates recorded were more than 1.0% and α-amylase production level of the surviving colonies did not increase.

Strains E2 and E3 were isolated and further exposed to a second round of ultraviolet radiation as shown in Table 2.

Table 2

Effect of further U.V treatment of Bacillus licheniformis B5.1 strains E2 and E3 on their α-amylase production

Strains	Treatment time (min)	*α-Amylase activity	% increase
Control	-	0.70	-
E21	20	2.60	270%
E22	20	4.82	560%
E31	40	3.90	460%
E32	40	2.88	300%

Data - means of duplicate determinations

* = α-Amylase activity (units/ml)

This indicated the need for vitamins of the B group by the mutant for growth and not amico acids or purines and pyrimidines. The incorporation of vitamins of the B group (riboflavin, thiamine, folic acid, calcium panthothenate, pyridoxin, niacin) into the growth medium and emitting one or more components in successive tests indicated the ability of the organism to utilize riboflovin, folic acid, calcium panthothenate and pyridoxin for growth, while it was unable to utilize thiamine and niacin (Table 3).

As a result of this, Bacillus licheniformis B5.1 strain E22 was used for further studies.

4.2 Enzyme purification

The purification profile of the α-amylase produced by the mutant strain of Bacillus licheniformis B5.1 is shown in Table 4. The purification procedures used included acetone precipitation, gel-filtration, and ion-chchange chromatography (using DEAE Sephadex). The specific activity obtained after ion-exchange chromatography is 42.5 with a percentage yield of 8.5% and purification factor of about 40. This values show a good production of the α-amylase by the organism into the culture medium. Figs 1 and 2, represent the elution profiles for both the gel-filtration and ion-cxhange chromatography respectively. The α—amylase best eluents in terms of activity were between fractions 17-23 using gel-filtration and between fractions 19-23 using ion-exchange chromatography.

Table 3: Growth factors requirements of mutant strain E22 of *Bacillus licheniformis* B5.1

	Mutant Strain E22	Wild type
Growth on minimal medium	-	+
Growth around casein-hydrolysate	-	+
Growth around yeast nucleic acid	-	+
Growth around yeast extract	+	+
Growth with some vitamins of the B group		
Riboflavin	+	+
Pyridoxin	+	+
Folic acid	+	+
Panthothenic acid	+	+
Thiamin	-	+
Niacin	-	+

+ = Growth was observed

- No growth was observed

Table 4

Purification table of α-amylase from Bacillus licheniformis E5.1 strain E22.

Enzyme	Volume (ml)	Protein Conc (mg/ml)	Total Protein (mg)	Enzyme activity (unit/mg)	Total enzyme activity (units)	Specific activity (units /protein)	% Purify-ield	Purification factor
Crude extract	500	2.8	1,400	3.0	1,500	1.07	100	1.00
Aceton Precipitation	100	1.2	120	5.0	500	4.16	33.33	3.88
Gel-Filtration	45	0.27	12.15	7.8	321	28.88	22.73	26.99
Ion-exchange chromatography	15	0.20	3.0	8.5	127.5	42.5	8.5	39.71

Data - means of duplicate determinations.

FIG. 1:

GEL-FILTERATION PROFILE of E22 α-amylase in relation to protein content

Symbols: -•- Amylase activity (units/ml)

-x- Protein concentration (mg/ml)

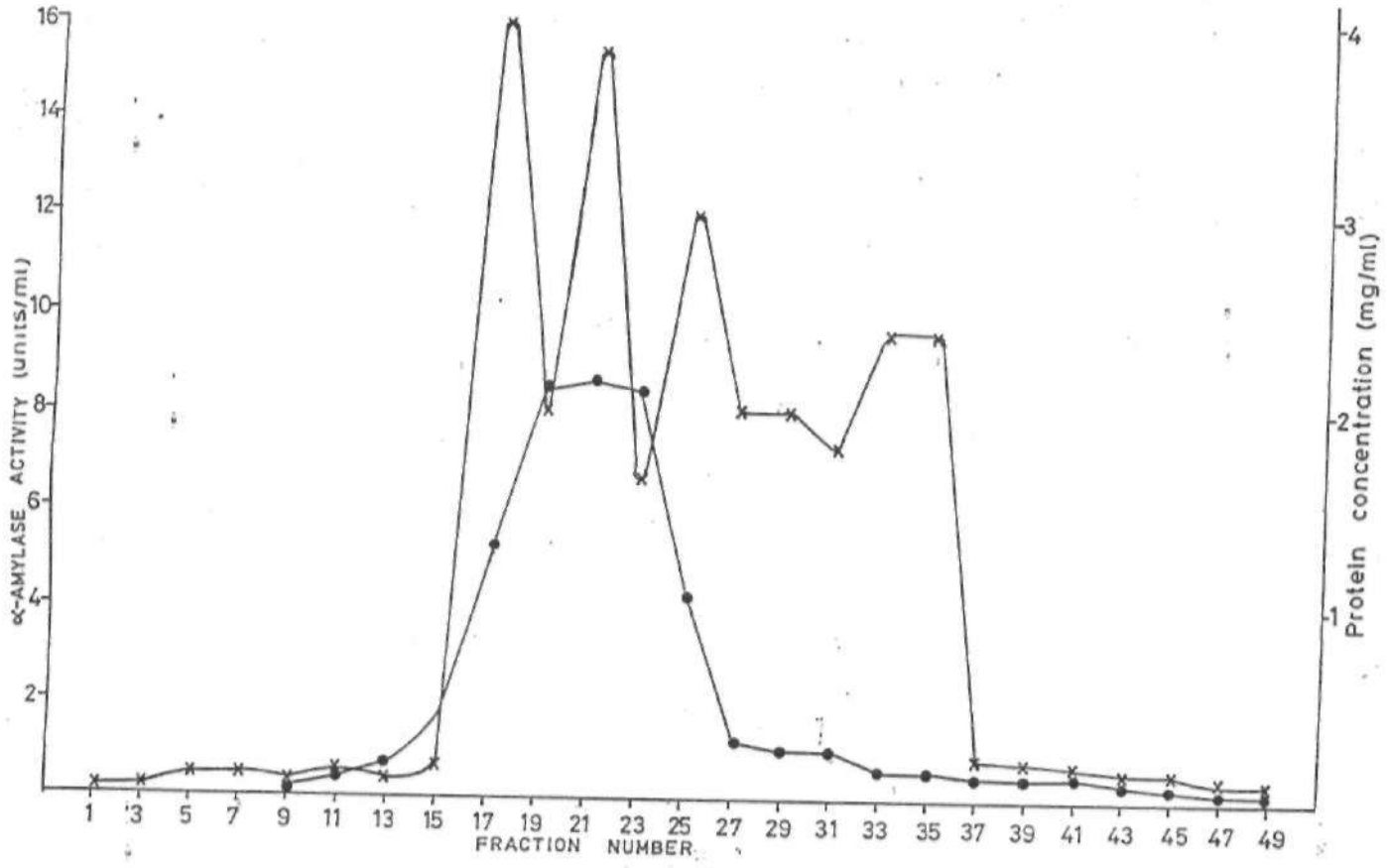

α-AMYLASE ACTIVITY (UNITS/ml)
Protein concentration (mg/ml)
FRACTION NUMBER
16
14
12
10
8
6
4
2
4
3
2
1
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49

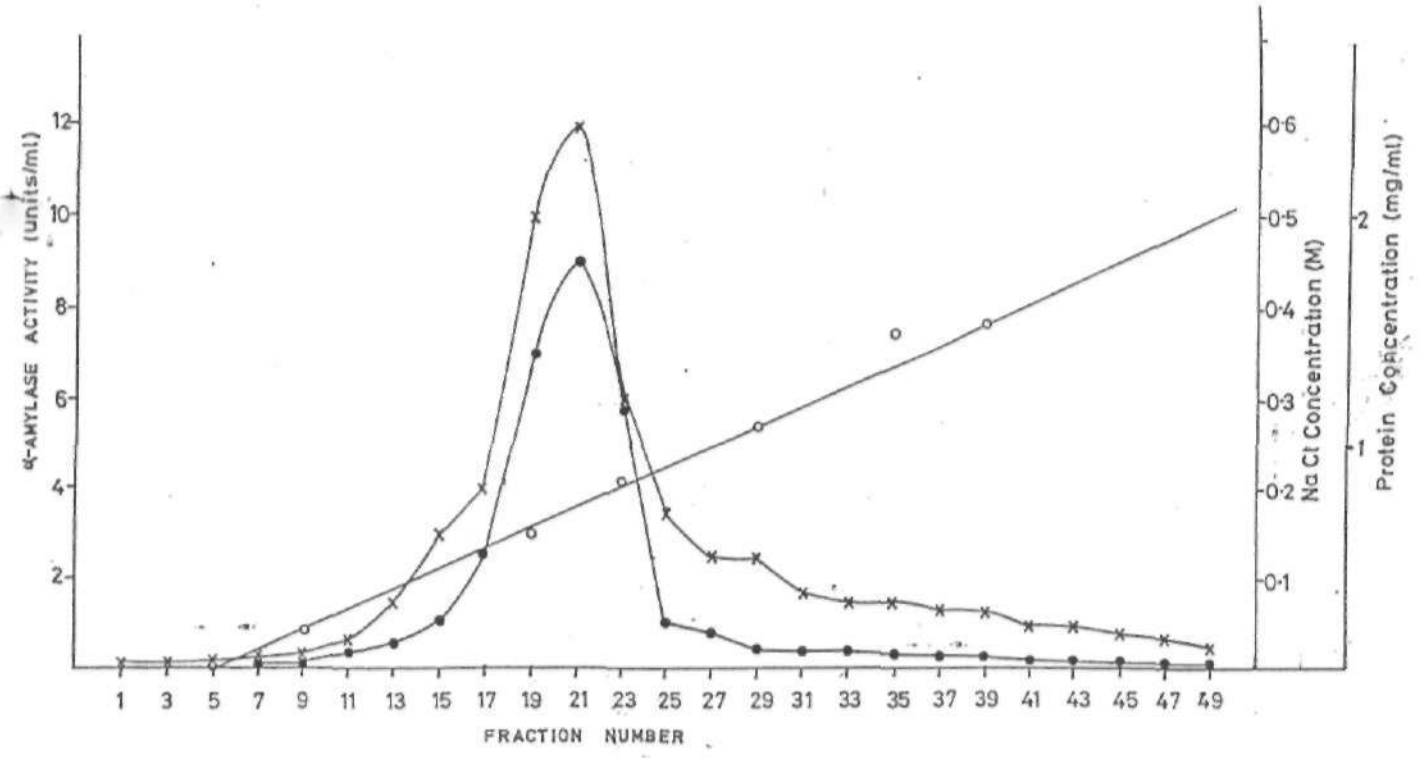
FIG. 2:

ION-EXCHANGE CHROMATOGRAPHY PROFILE of E 22 α-amylase in relation to protein and NaCl concentration

Symbols: -•- Amylase activity (units/ml).

-x- Protein concentration (mg/ml).

-•- NaCl concentration (M).


α-AMYLASE ACTIVITY (units/ml)
Na Cl Concentration (M)
Protein Concentration (mg/ml)
FRACTION NUMBER
2
4
6
8
10
12
0·1
0·2
0·3
0·4
0·5
0·6
1
2
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49

4.3 Enzyme characterization

Results obtained from the effect of different starch concentrations (w/v) (%) on the α-amylase activity were used to prepare the Line weaver-Burke plot (Fig 3) from which the k_m V_{max} values were found to be 7.87% starch and 2.22mg glucose/min respectively. The activation energy (Ea) of the enzyme was determined to be 20.01J/mol using the Arrhenius plot (Fig 4).

4.3.1 Effect of temperature on α-amylase activity

The effect of temperature on the α-amylase activity (Fig 5) showed that the enzyme exhibited maximum activity at 60°C. More than 50% of the α-amylase activity was retained between 40°C and 70°C. This shows that the enzyme is thermostable.

4.3.2 Effect of pH on α-amylase activity

The result in Fig 6 shows the effect of pH on the activity of the enzume. The enzyme was most active between pH 6.5 and 7.0 which there is a decrease in activity. The enzyme retained about 50% of its activity between pH 4.0 and 7.0.

4.3.3 Molecular weight determination of the α-amylase

In determining the molecular weight of the enzyme, a standard curve was first generated from plot of V_e/V_o against log molecular weight of marker proteins. The V_eV_o of the α-amylase was then determined and its molecular weight value extrapolated

FIG 3:

LINEWEAVER-BURKE PLOT OF E22 α-AMYLASE ACTIVITY.

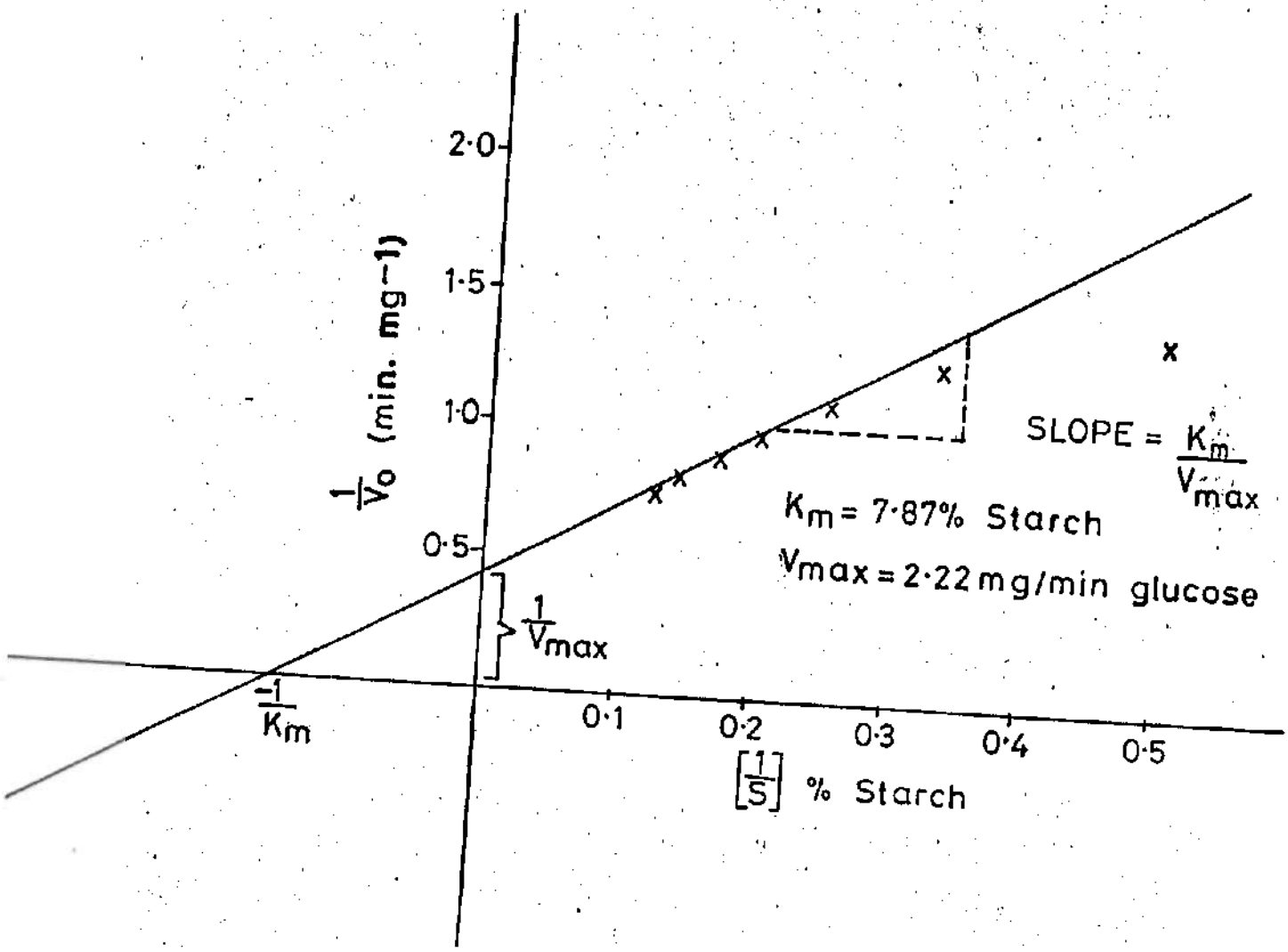

2·0
1·5
1·0
0·5
$\frac{1}{V_o}$ (min. mg⁻¹)
SLOPE = $\frac{K_m}{V_{max}}$
K_m = 7·87% Starch
V_{max} = 2·22 mg/min glucose
$\frac{1}{V_{max}}$
$\frac{-1}{K_m}$
0·1
0·2
0·3
0·4
0·5
$\left[\frac{1}{S}\right]$ % Starch

FIG. 4:

ARRHENIUS PLOT FOR E 22 α-AMYLASE ACTIVITY.

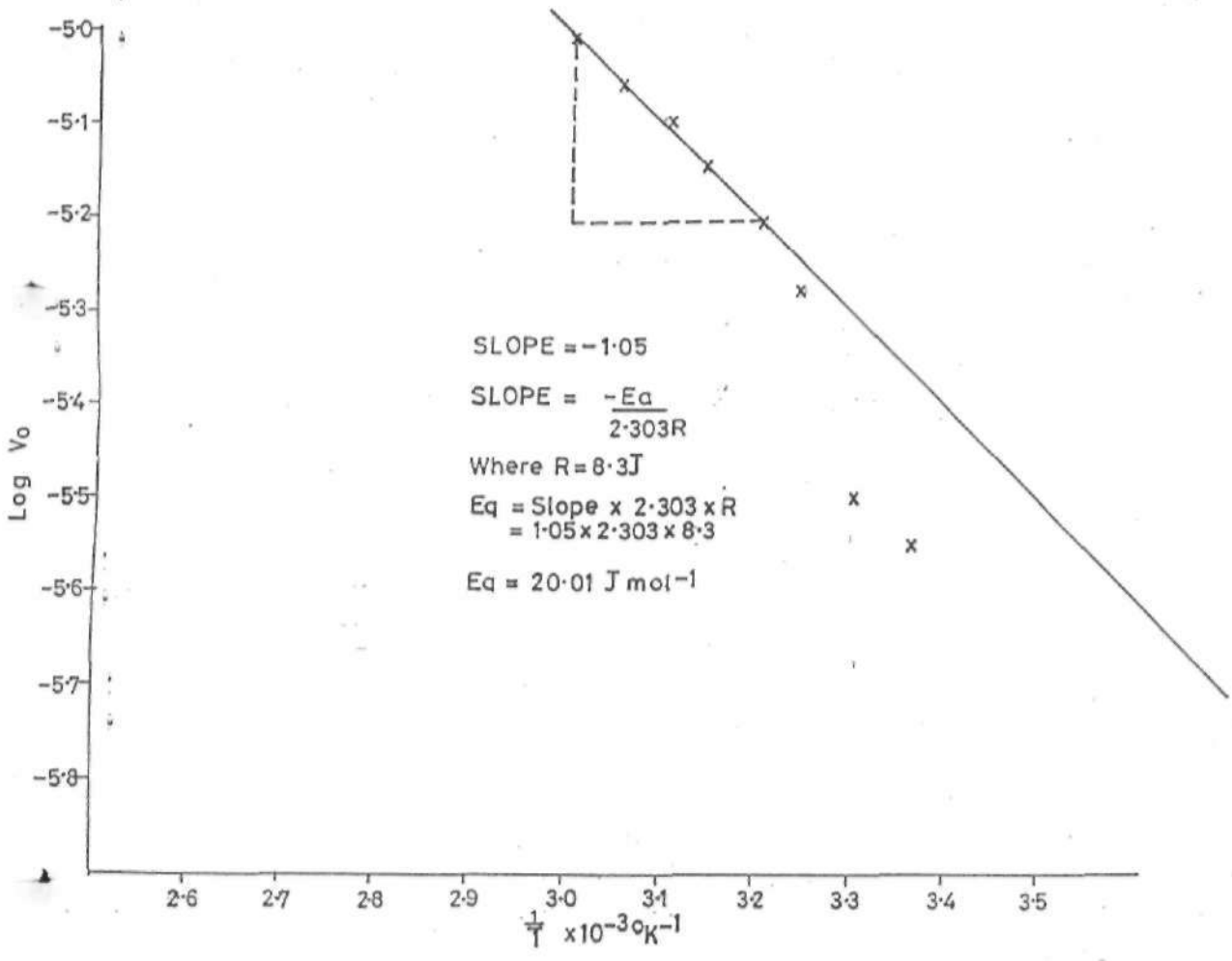

-5·0
-5·1
-5·2
-5·3
-5·4
-5·5
-5·6
-5·7
-5·8
Log Vo
2·6
2·7
2·8
2·9
3·0
3·1
3·2
3·3
3·4
3·5
1/T x10⁻³ °K⁻¹
SLOPE = -1·05
SLOPE = -Ea / 2·303R
Where R = 8·3J
Eq = Slope x 2·303 x R
= 1·05 x 2·303 x 8·3
Eq = 20·01 J mol⁻¹

FIG. 5:

TEMPERATURE-ACTIVITY PROFILE FOR BACILLUS LICHENIFORMIS B5.1 E22 α-AMYLASE

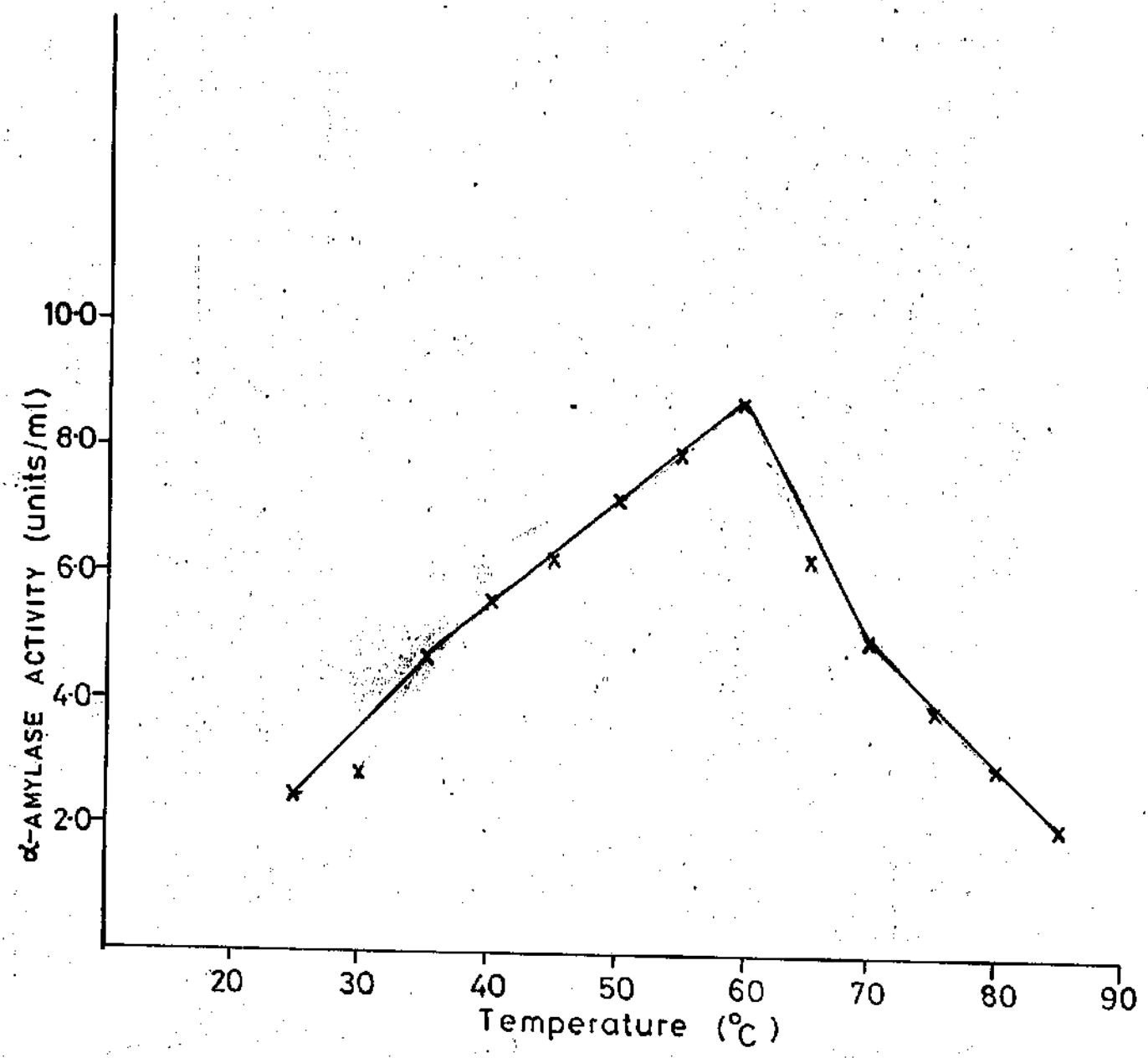
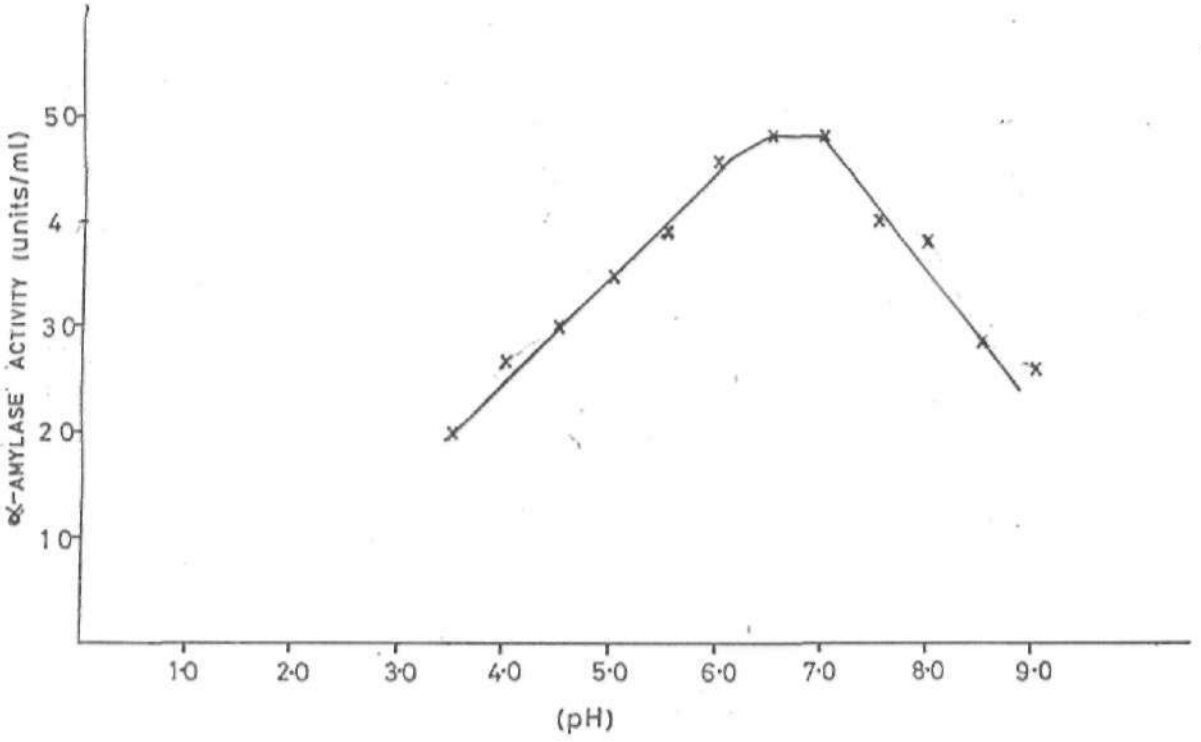

α-AMYLASE ACTIVITY (units/ml)
10·0
8·0
6·0
4·0
2·0
20
30
40
50
60
70
80
90
Temperature (°C)

FIG 6:

EFFECT OF pH ON ACTIVITY OF α-AMYLASE OF BACILLUS LICHENIFORMIS B5.1 E 22


α-AMYLASE ACTIVITY (units/ml)
50
40
30
20
10
1·0
2·0
3·0
4·0
5·0
6·0
7·0
8·0
9·0
(pH)

from the standard curve (Fig 7). The molecular weight for this enzyme was determined as 33,113 daltons.

4.3.4 Effect of metal ions on α-amylase activity

The effect of six different metal ions at 0.02M was determined on the α-amylase activity. Of all the metal ions tested, calcium ion gave the highest α-amylase activity while mercurric ion gave the lowest activity (Table 5).

4.3.5 Stability of the α-amylase in storage

The stability of the enzyme under different storage conditions was studied (Table 6). This also helped to determine the half life of the enzyme at the different storage conditions (Figs 8, 9, 10). The enzyme was most stable in the freezer in which it retained 90% of its activity after eight weeks of storage. It was also very stable in the refrigerator, and at room temperature it retained about 20% of its original activity after eight weeks of storage.

4.4 Effect of some chemical substances on α-amylase production by the mutant strains of Bacillus licheniformis B5.1 B22

Results in Figs 11-15 show the patterns of effect on the α-amylase synthesis by different chemicals and vitamins. Glycine did not show any support, instead the level of α-amylase synthe-

FIG. 7:

LOGARITHM OF MOLECULAR WEIGHT OF E22 α-AMYLASE AS A FUNCTION OF THE RATIOS OF THE ELUTION VOLUME TO THE VOID VOLUME (V_e/V_o).

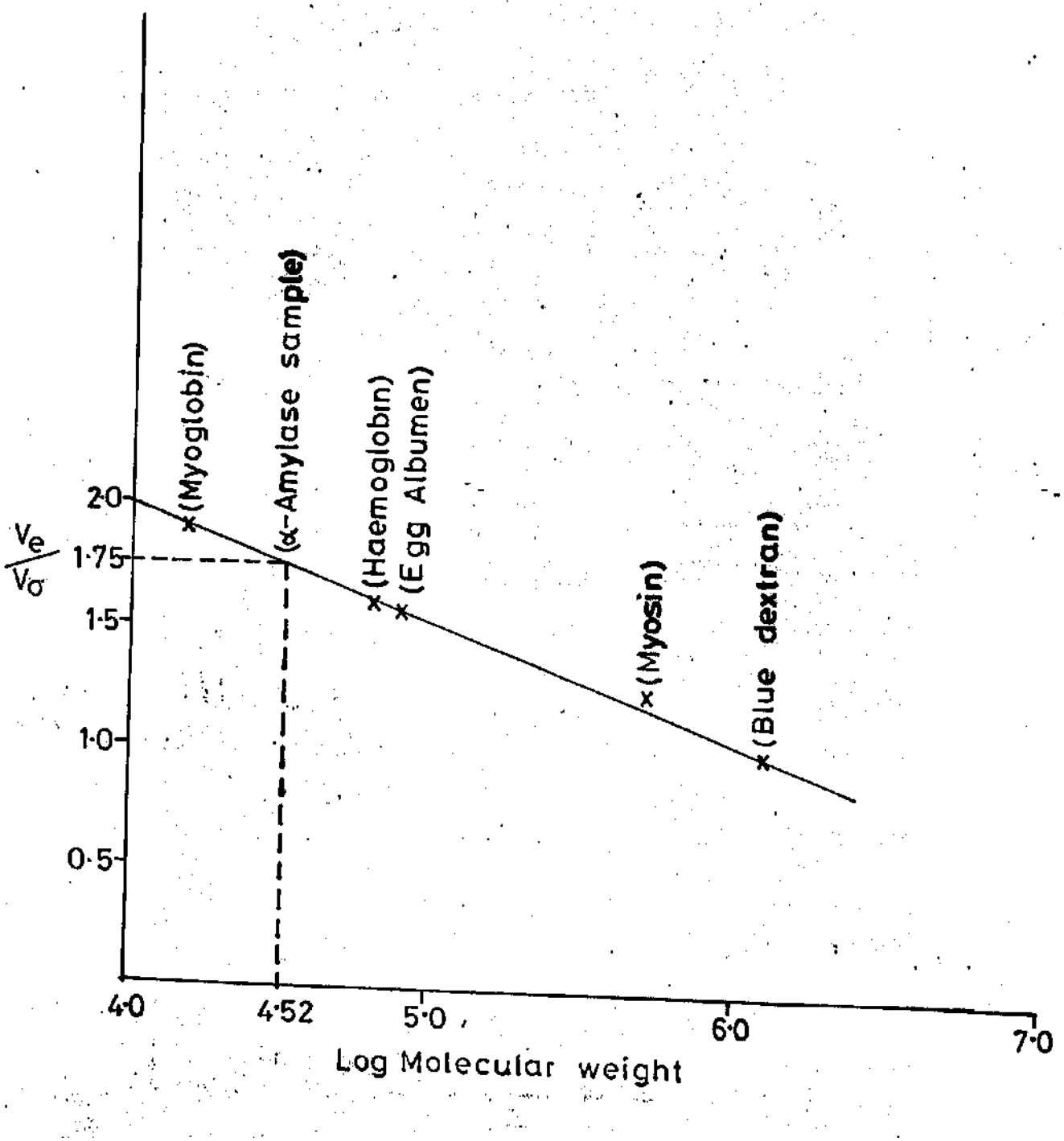

(Myoglobin)
(α-Amylase sample)
(Haemoglobin)
(Egg Albumen)
(Myosin)
(Blue dextran)
Ve/Vo
2·0
1·75
1·5
1·0
0·5
4·0
4·52
5·0
6·0
7·0
Log Molecular weight

Table 5

Effect of different metal ions at 0.02M on the activity of E22 α-amylase

*Metallic ions	α-Amylase activity (units/ml)
Calcium	8.0
Iron	5.0
Zinc	4.9
Lead	4.1
Copper	2.1
Mercury	1.9

*Metal ions were added in their chloride salts

Data - means of duplicate determinations

Table 6

Effect of storage on enzyme stability

Storage period (weeks)	Residual α-amylase activity (%) Room temperature 27°C	Refrigerator (4°C)	Freezer (-5°)
1	96.0	100.0	100.0
2	64.8	90.0	100.0
3	51.6	87.4	96.6
4	40.0	80.8	94.6
5	33.2	76.8	93.8
6	28.4	72.8	91.8
7	22.6	69.4	91.8
8	22.0	69.4	90.0

Data - means of duplicate determinations

FIG. 8:

RATE CONSTANT AND HALF-LIFE DETERMINATION OF E22 α-AMYLASE STORED AT ROOM TEMPERATURE.

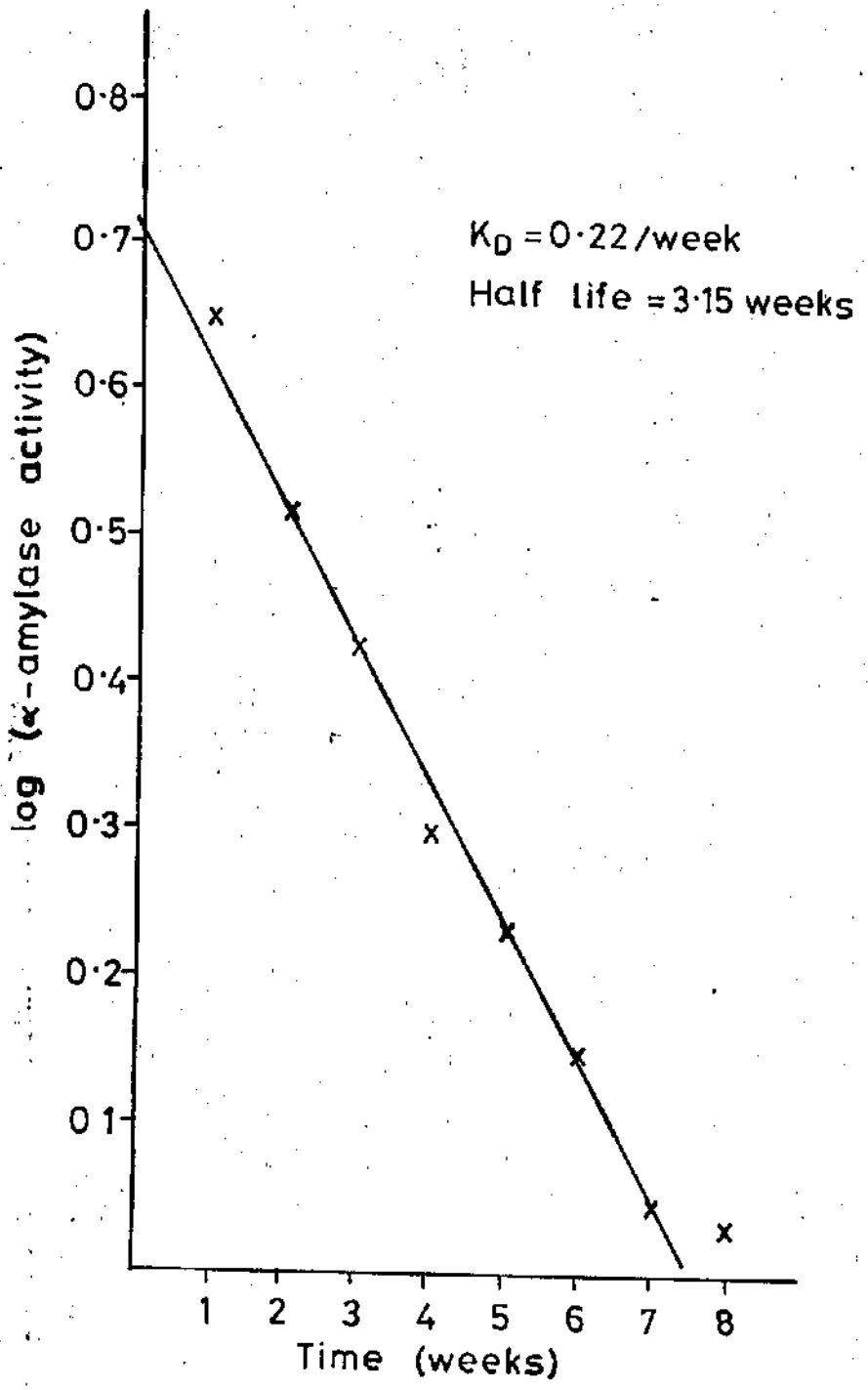

K_D = 0·22/week
Half life = 3·15 weeks
log (α-amylase activity)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0 1
1 2 3 4 5 6 7 8
Time (weeks)

FIG. 9:

RATE CONSTANT AND HALF-LIFE DETERMINATION OF E22 α-AMYLASE STORED IN THE REFRIGERATOR.

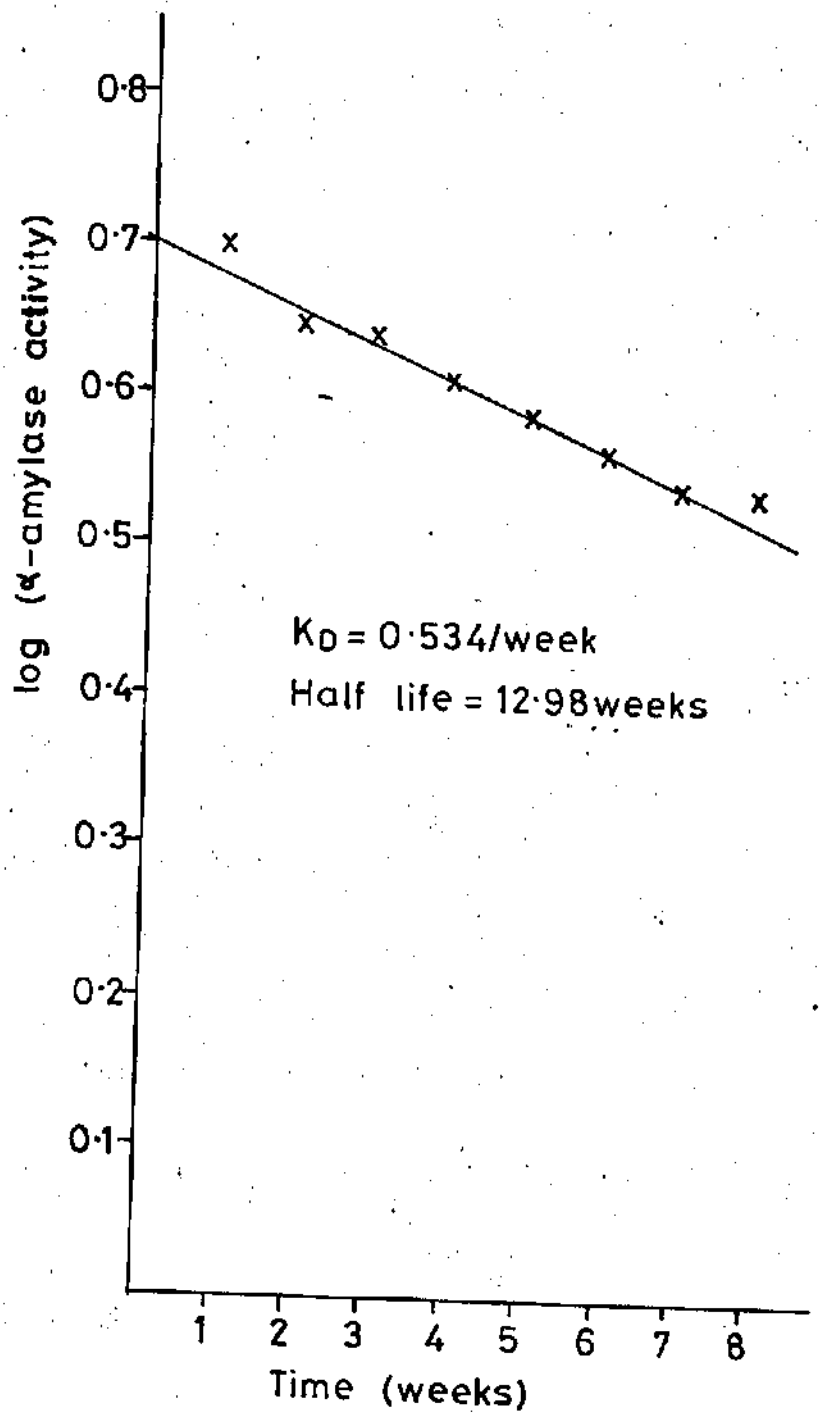
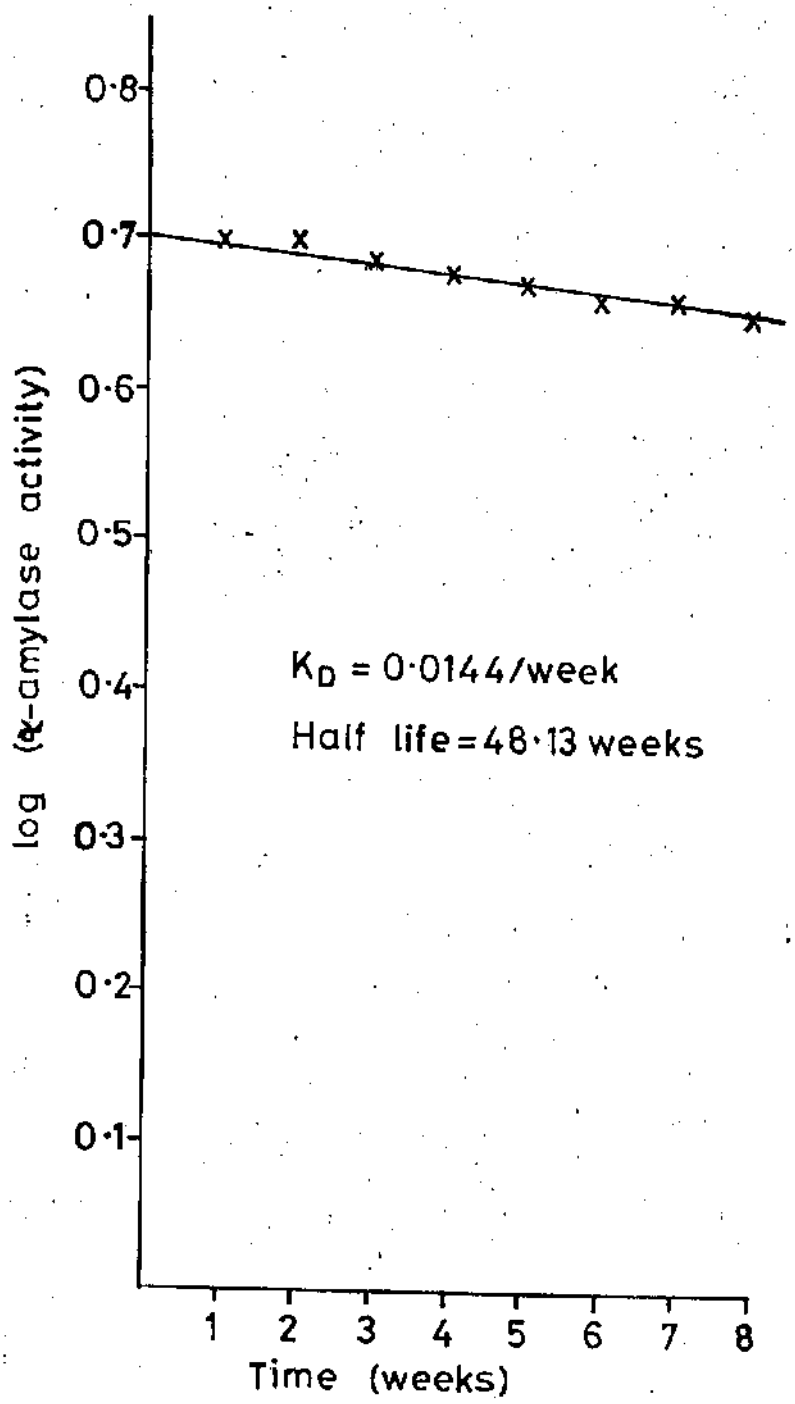

log (α-amylase activity)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
K_D = 0·534/week
Half life = 12·98weeks
1
2
3
4
5
6
7
8
Time (weeks)

FIG. 10:

RATE CONSTANT AND HALF-LIFE DETERMINATION OF E 22 α-AMYLASE STORED IN THE FREEZER.


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
log (α-amylase activity)
K_D = 0·0144/week
Half life = 48·13 weeks
1
2
3
4
5
6
7
8
Time (weeks)

sis declined as the concentration of glycine increased (Fig 11). The bacterial growth followed a similar pattern in which there was no noticeable increase in growth, except at 0.8% glycine and after which a considerable decline in bacterial growth was recorded. Triton X-100 did not show any increase in α-amylase synthesis. At triton X-100 concentrations of 1.0% to 5.0%, the α-amylase level was much less compared to the level in the growth medium without trixon X-100 (control). The bacterial count, as is the case with α-amylase production, dropped considerably as the triton X-100 concentration increased (Fig 12).

The effect of folic acid was different from that of glycine and triton X-100. There was a steady increase in α-amylase production with folic acid with the highest level being recorded at 0.06% folic acid. The bacterial count, as is the case with α-amylase production, increased as the folic acid concentration increased with the highest level also recorded at 0.06% folic acid (Fig 13).

Riboflavin and calcium panthothenate, at the concentrations tested did not show any appreciable increase in α-amylase production. The bacterial count increased as the concentration riboflavin increased (Fig 14), while with calcium panthothenate the bacterial count increased as the panthothenate concentration increased, although the bacterial counts at lower concentrations were less than that obtained with the control (Fig 15).

Fig 11

Effect of glycine on growth of, and α-amylase production by, Bacillus licheniformis B5.1 E22

Symbols:

-x- Amylase activity (units/ml)

-●- Protein concentration (mg/ml)

-○- Specific activity (units/mg protein)

-Δ- $\log_{10}$ Bacterial count

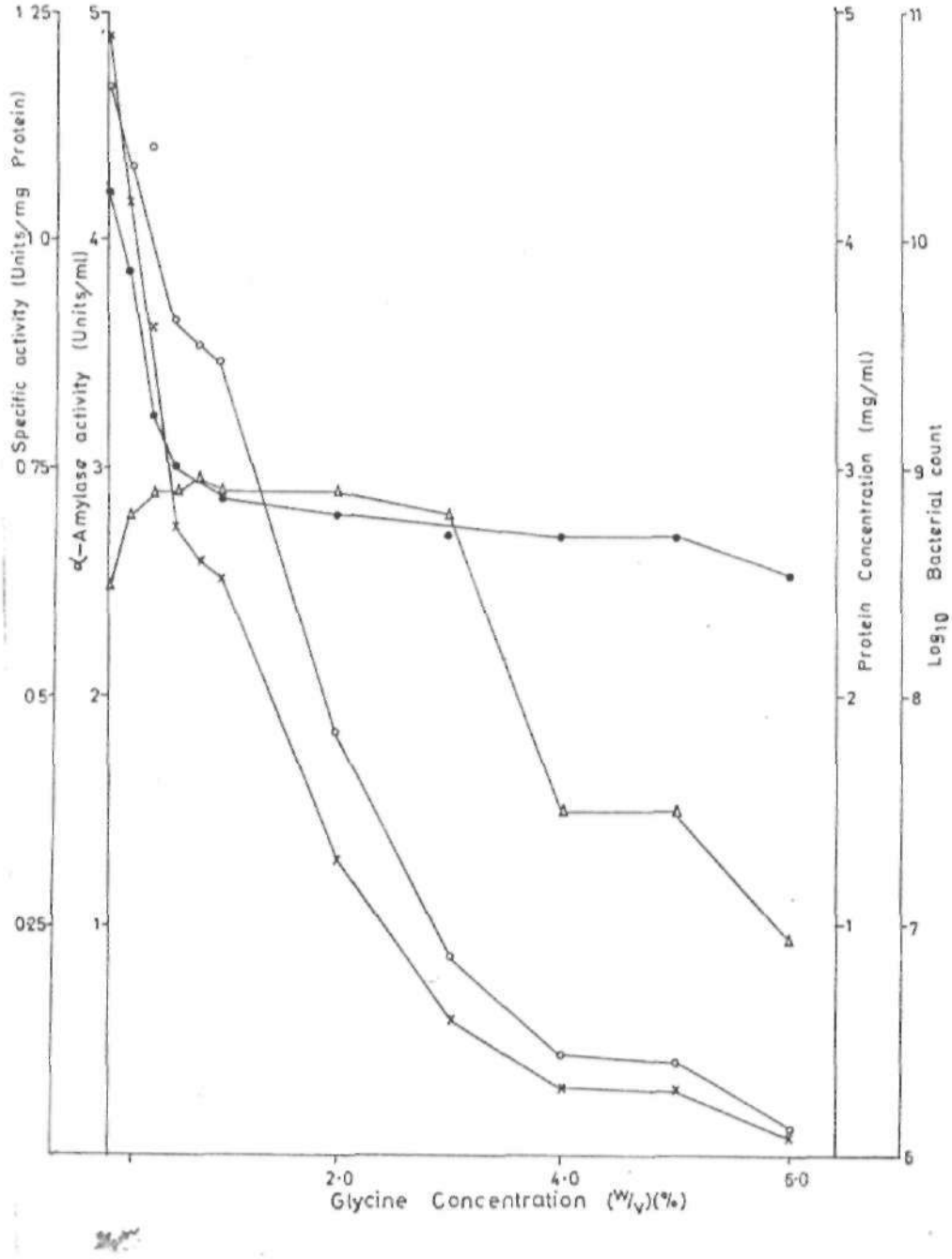

Specific activity (Units/mg Protein)
α-Amylase activity (Units/ml)
Protein Concentration (mg/ml)
Log10 Bacterial count
Glycine Concentration (W/V)(%)
1·25
1·0
0·75
0·5
0·25
5
4
3
2
1
11
10
9
8
7
6
2·0
4·0
5·0

Fig 12

Effect of triton X-100 on growth of, and α-amylase production by, Bacillus licheniformis B5.1 E22

Symbols:

-x- Amylase activity (units/ml)

-●- Protein concentration (mg/ml)

-o- Specific activity (units/mg protein)

-Δ- $\log_{10}$ Bacterial count

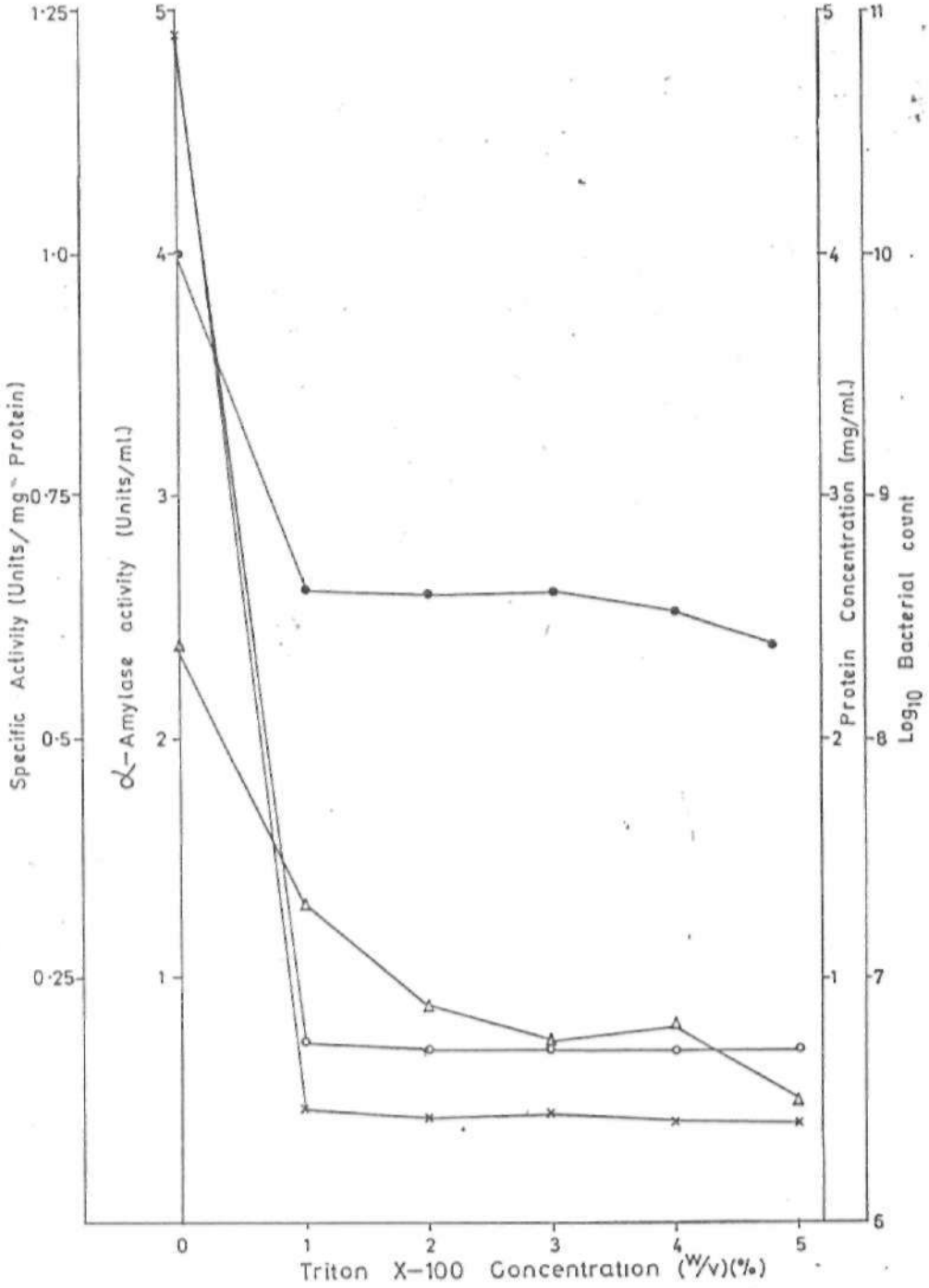

Specific Activity (Units/mg Protein)
α-Amylase activity (Units/ml.)
Protein Concentration (mg/ml.)
Log10 Bacterial count
Triton X-100 Concentration (w/v)(%)
1·25
1·0
0·75
0·5
0·25
0
1
2
3
4
5
6
7
8
9
10
11

Fig 13

Effect of folic acid on growth of, and α-amylase production by, Bacillus licheniformis B5.1 E22

Symbols:

-x- Amylase activity (units/ml)

-●- Protein concentration (mg/ml)

-□- Specific activity (units/mg protein)

-Δ- $\log_{10}$ Bacterial count

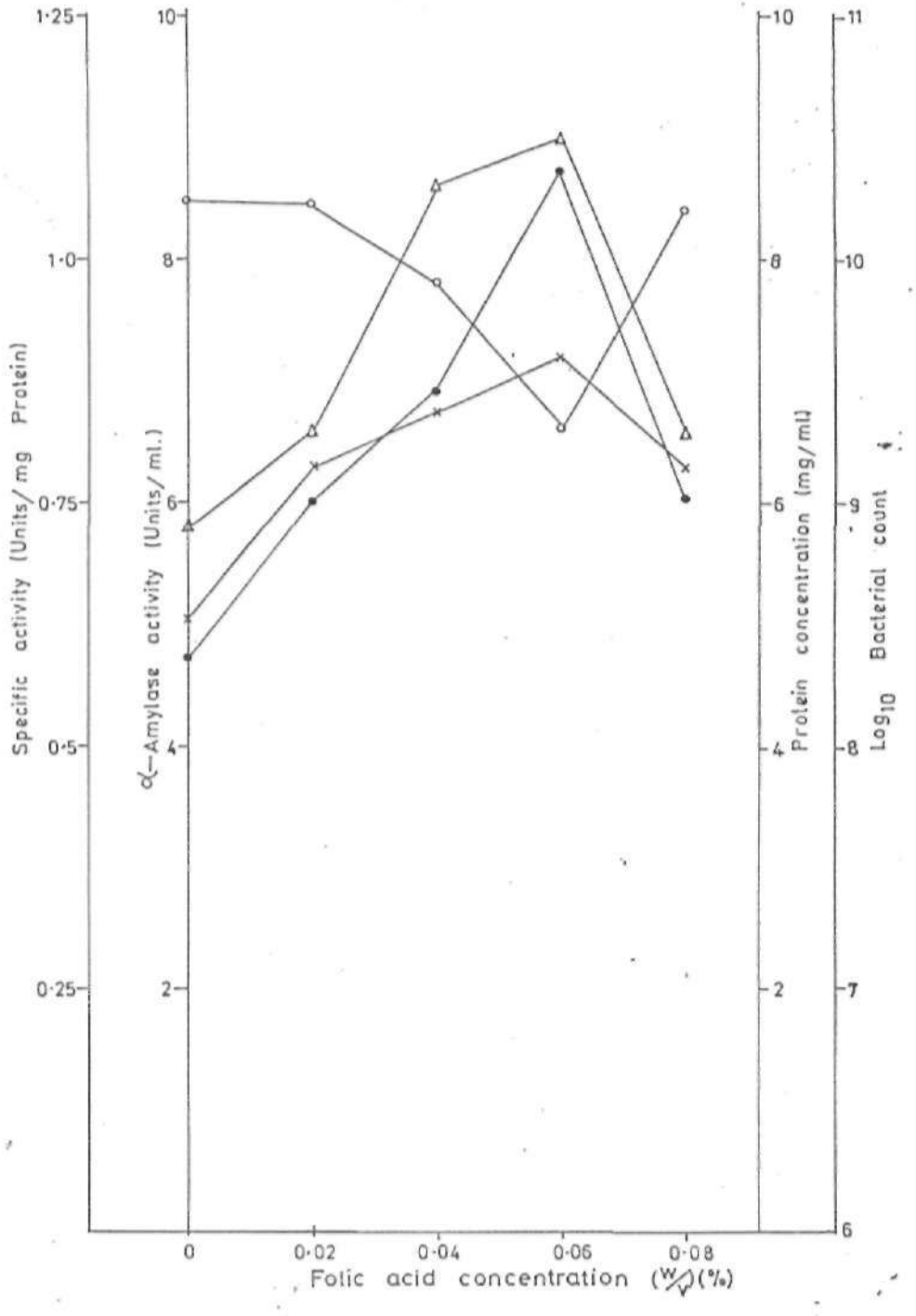

Specific activity (Units/mg Protein)
α-Amylase activity (Units/ml.)
Protein concentration (mg/ml)
Log10 Bacterial count
Folic acid concentration (W/V)(%)
1·25
1·0
0·75
0·5
0·25
10
8
6
4
2
0
0·02
0·04
0·06
0·08
11
9
7

Fig 14

Effect of riboflavin on growth of, and α-amylase production by, Bacillus licheniformis B5.1 B22

Symbols:

-x- Amylase activity (units/ml)

-●- Protein concentration (mg/ml)

-o- Specific activity (units/mg protein)

-Δ- $\log_{10}$ Bacterial count

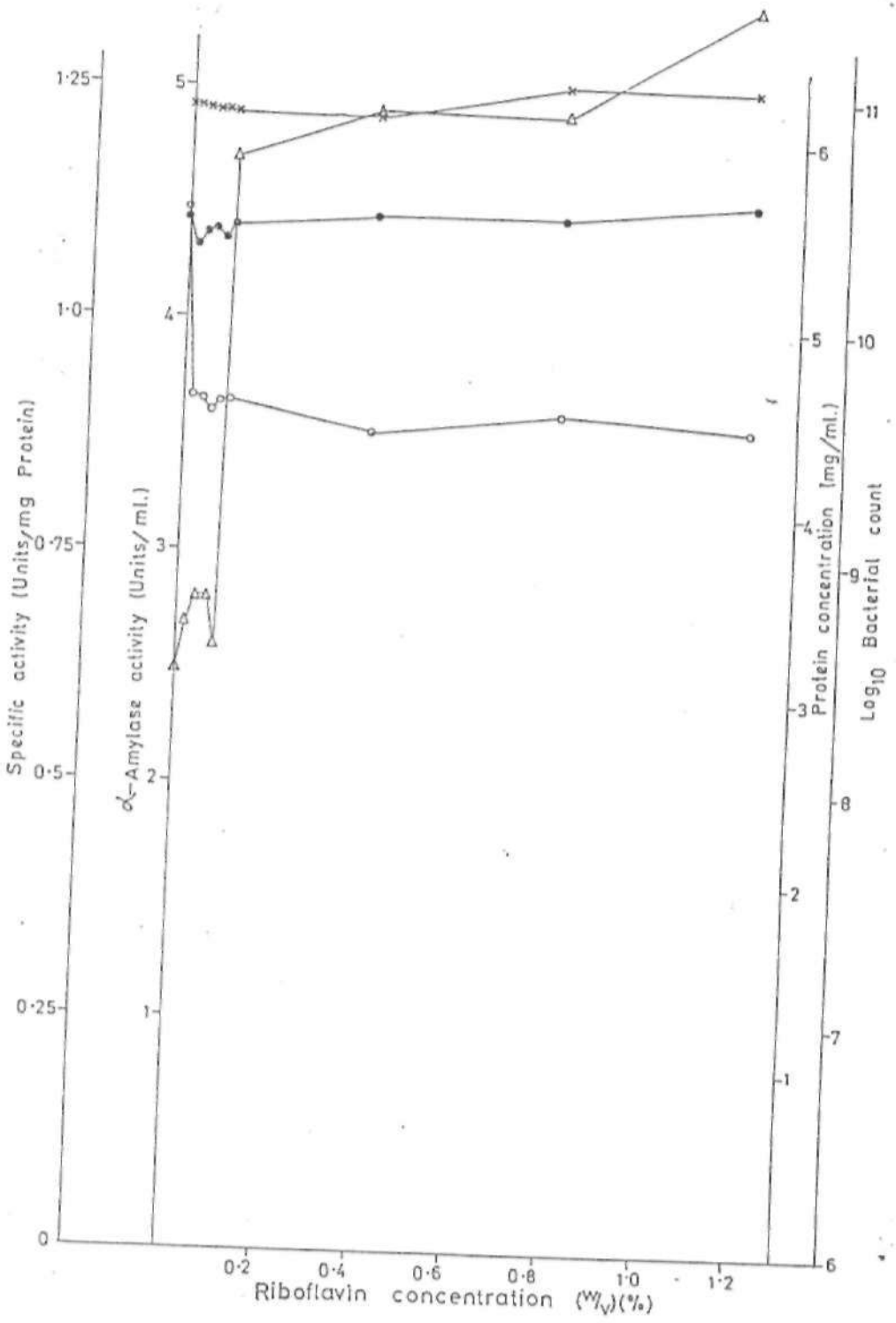

Specific activity (Units/mg Protein)
α-Amylase activity (Units/ml.)
Protein concentration (mg/ml.)
Log10 Bacterial count
Riboflavin concentration (w/v)(%)
1·25
1·0
0·75
0·5
0·25
0
5
4
3
2
1
6
5
4
3
2
1
11
10
9
8
7
6
0·2
0·4
0·6
0·8
1·0
1·2

Fig 15

Effect of calcium panthothenate on growth of, and α-amylase production by, Bacillus licheniformis B5.1 E22

Symbols: -x- Amylase activity (units/ml)

-●- Protein concentration (mg/ml)

-o- Specific activity (units/mg protein)

-Δ- $\log_{10}$ Bacterial count

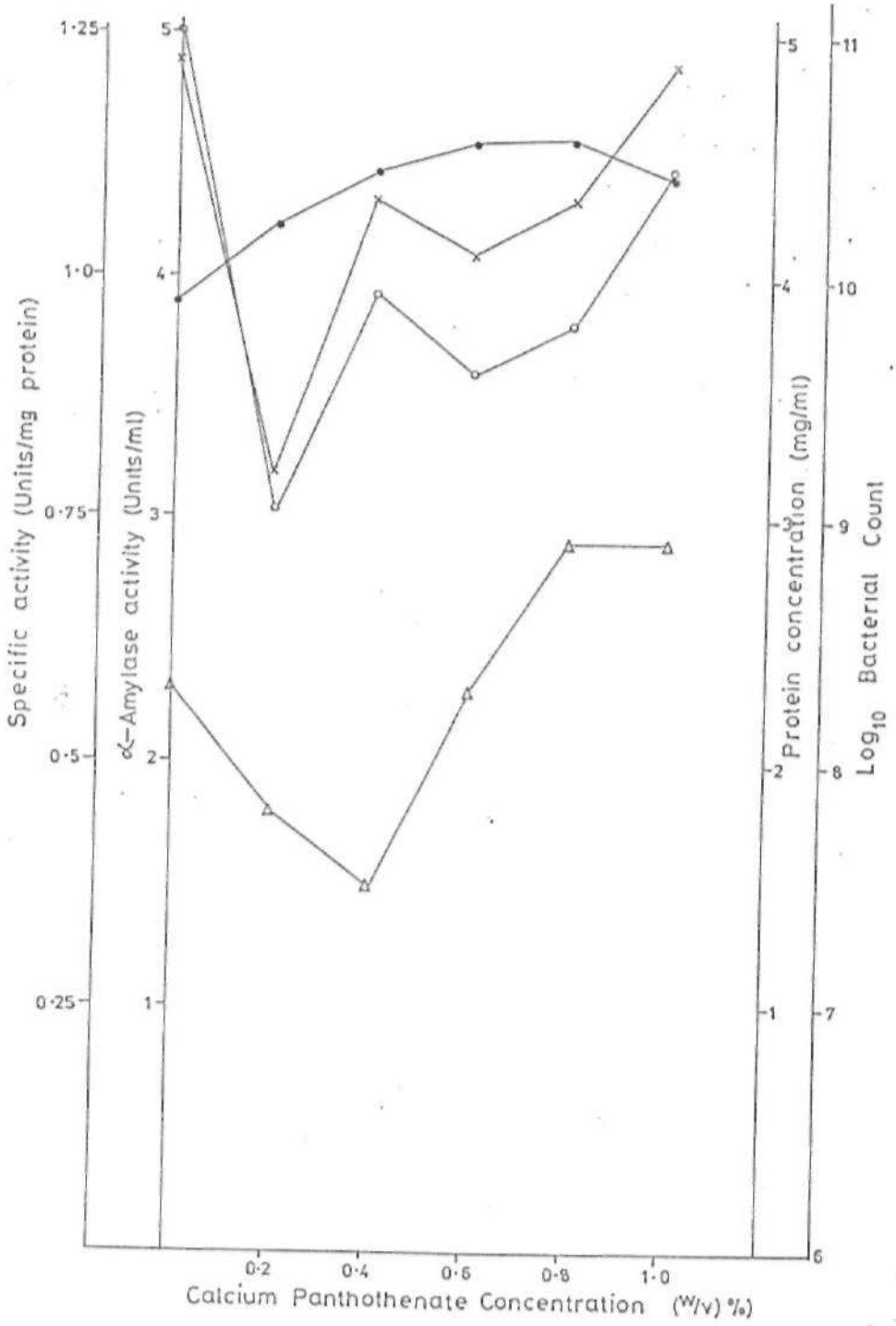
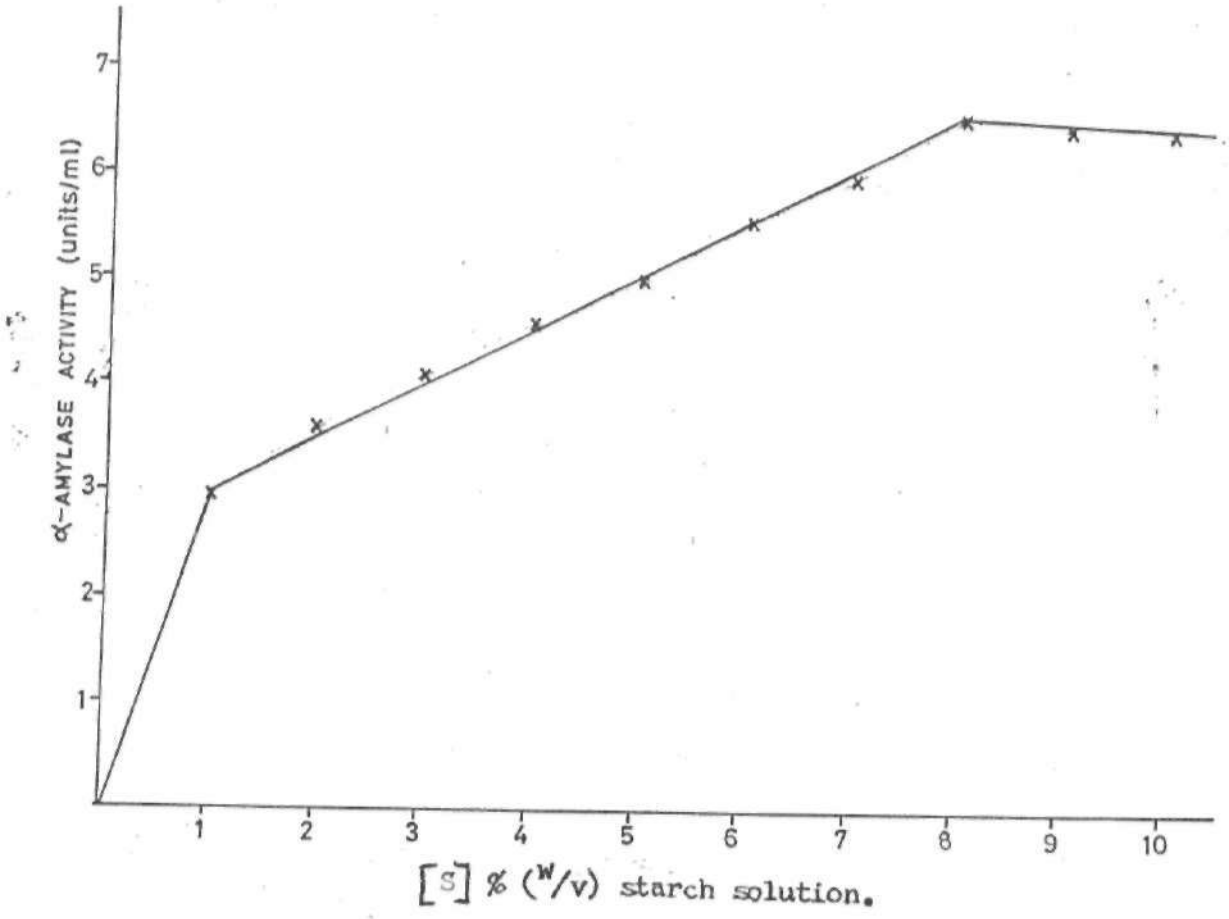

Specific activity (Units/mg protein)
1·25
1·0
0·75
0·5
0·25
α-Amylase activity (Units/ml)
5
4
3
2
1
Protein concentration (mg/ml)
5
4
3
2
1
Log10 Bacterial Count
11
10
9
8
7
6
0·2
0·4
0·6
0·8
1·0
Calcium Panthothenate Concentration (W/v) %

Fig 16

EFFECT OF STARCH CONCENTRATION ON E22 α-AMYLASE ACTIVITY


α-AMYLASE ACTIVITY (units/ml)
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
[S] % (W/v) starch solution.

CHAPTER 5

DISCUSSION

The Bacillus licheniformis B5.1 used in this study produced α-amylase in a growth medium containing cassava starch and 'dawadawa' (fermented locust bean seed) as the sole source of carbon and nitrogen, respectively. This agrees with the work of Raji (1987), who reported cassava and 'dawadawa' as the optimum carbon and nitrogen sources among those tested respectively for α-amylase production by this organism.

Table 1 shows the effect of ultraviolet radiation on survival rate of, and α-amylase production by, Bacillus licheniformis B5.1. As indicated in the table, the duration of exposure of the organism to ultraviolet radiation is not a direct function of the rate at which α-amylase production by the organism increased. Instead, percentage of survival after irradiation appeared to be related to increase in α-amylase production. Strains of Bacillus licheniformis B5.1 that were irradiated for between 25-60 minutes and gave survival rates of 1.0% did not show any increase in their α-amylase production, while strains irradiated for 60 minutes which gave survival rates of about 0.1% had about 270% increase in α-amylase production. This agrees with Calam (1970), who indicated that for mutagenic agents to be very successful they should be able to record a kill of at least 99.9%. Also from the work of Bailey and Markkanen (1975) α-amylase activity

was highest among strains that had been exposed to mutagenic agents to give a final survival rate of between 0.1-0.0001%. The strains with the highest α-amylase yield in Table 1 (strains E2 and E3) were subjected to another round of ultraviolet irradiation lasting from 20-40 minutes (Table 2). Two strains, E22 and E31 which were exposed for 20 and 40 minutes, respectively, gave an increased α-amylase yield of approximately 560% and 460%, respectively. Strains E21 and E32 did not improve their α-amylase production after a second application of ultraviolet radiation. Alikhanian (1962) has observed that the application of a mutagenic agent to a wild type strain gives a good initial improvement in enzyme production that could be followed by a plateau effect in which further applications of the mutagenic agent, regardless of dosage, cease to have any positive effect.

Bacillus licheniformis B5.1 strain E22 which had the highest increase in α-amylase production of approximately 600% was selected for further study of enzyme production. This strain was screened for successful mutation using the auxanographic method. In this method, as shown in Table 3, the mutant strain could not grow on a minimal medium, while the wild type strain did. This is explained by the fact that a strain which is successfuly mutagenized must have had its gene structure damaged in which case the ability to synthesize its own basic growth requirements will be lacking and can only grow on a medium which has complete growth requirements. Therefore, when grown on a minimal medium

which lacks some essential growth requirements, growth of the organism will not occur. In an experiment in which the organism was grown on a minimal medium with three gowth factors (casein-hydrolysate, yeast nucleic acid and yeast extract) added, showed growth only around the yeast extract and none around the other two compounds. This growth around the yeast extract indicated that this organism needs vitamins of the B group for growth. Their incorporation into the minimal medium (Table 3) supported the growth of the organism in the presence of riboflavin, pyridoxin, calcium pathothenate, and folic acid, while no growth was recorded in the presence of niacin and thiamine. For the wild type strain, growth was recorded in the presence of all the vitains of the B group tested. This shows that the genes responsible for the utilisation of niacin and thiamine which are functional in the wild type strain have been damaged in this mutant strain. The production of this mutant strain of Bacillus licheniformis B5.1 using ultraviolet radiation has not only produced a strain with increased α-amylase production but it has also produced a strain that is unable to utilise niacin and thiamine. Mutation using ultraviolet radiation has been reported to be achieved through dimerization of the thymine nucleotides, resulting in formation of thymine dimers (Beukers and Berends, 1960). This irradiation leads to a weakening of the bonds between them and a distortion which later gives rise to dimerization of thymines on opposite strands, causing crosslinka-

kages of the strands and preventing their separation, thus interfering with replication (Kelner, 1953). The inability of this mutant strain to utilise some of the vitamins which the wild type strain can utilise as evident from the auxanographic studies and some other visible changes such as increased α-amylase production and size, is used in this study as a confirmation of mutation of the wild type strain of Bacillus licheniformis B5.1. The mutant strain is, therefore, given a new configuration on the basis of the standard procedure as Bacillus licheniformis B5.1 nia⁻thia⁻.

Table 4 summarizes the steps invovled in the purification of α-amylase from Bacillus licheniformis B5.1 strain E22. The crude enzyme extract was purified using acetone precipitation, gel-filtration with sephadex G-200 and ion-exchange chromatography. The final purification step (ion-exchange) gave a preparation that is 40-fold pure and a high specific activity of 42.5 unit/mg protein, with a percentage yield of α-amylase activity of 8.5%. There was a considerable increase in the specific activity after ion-exchange chromatography as compared to what it was after gel-filtration. This goes to explain that the enzyme preparation obtained after ion-exchange chromatography was much purer than that obtained after gel-filtration of this enzyme. Although the purification factor obtained in this work is lower than what was reported by Matsuzaki et al (1974a) for α-amylase produced by a transformable strain of Bacillus subtilis 6160, it is, however, better than the purification factor reported by Okon and Uwaifo

(1984) in which 5-fold purification was reported for β-amylase. Gel-filtration chromatograpgy using sephadex G-200 which was used to purify the enzyme extract further after acetone fractionation showed (from the elution profile) more than one protein peak (Fig 1). Fractions 17-23 which contain higher α-amylase activity were pooled and subsequently, further purified by ion-exchange chromatography on DEAE-sephadex G150-200. Figure 2, is the elution profile obtained after this chromatography. It can be seen from this profile that most of the α-amylase activity was recovered in fractions 19-23. The fact that only a single peak of activity was observed rules out the possibility of the existence of isoenzymes of this α-amylase preparation.

The method of ion-exchange chromatography has been employed successfully for the purification of proteins. As is the case with gel-filtration, it is a gentle operation, thereby preventing the destruction of labile proteins and having a high resulotion and reproducibility. Ion-exchange chromatography has the advantage of being a test of purity since all proteins of equal charges will show a single peak of elution (Okon and Uwaifo, 1984).

The K_m and V_{max} of this α-amylase enzyme were determined by the Lineweaver-Burk plot (Fig 3) using the data in Table 5. The fact that the plot shows saturation features indicate that Bacillus licheniformis B5.1 α-amylase obeys the Michaelis-Menten kinetics. This enzyme achieved maximum enzyme activity at a starch

concentration of 8%(w/v)(Fig 16). A K_m value of 7.87% starch and a V_{max} of 2.22mg glucose/min were obtained for this enzyme. The K_m value of this α-amylase is high compared to the value obtained for a commercially available purified α-maylase from Bacillus amyloliquefaciois (Matsuzaki et al, 1974a). The V_{max} of this α-amylase compares favourably with that obtained from a commercially purified α-amylase which is 1.03mg glucose/min. The K_m value for this α-amylase from Bacillus licheniformis B5.1 strain E22 suggests that the affinity of this enzyme for the substrate is comparable to that of some other α-amylases (Matsuzaki et al, 1974a). The lower K_m recorded for the commercially purified enzyme could be due to the fact that the commercial enzyme has undergone a lot of purification steps which are much more superior than the steps used in this work. The V_{max} of this α-amylase has been shown to compare favourably with other α-amylases from other sources (Okon and Uwaifo, 1984). This high V_{max}, suggestive of high activity, is a function of several factors such as duration of the assay, the mechanism of the reaction, the conformation of the protein, pH, temperature, ionic strength, enzyme and cofactor concentrations, and buffer (Ikediobi and Ukoha, 1985).

Enzymes being proteins are polyelectrolytes. They possess amino acids at their active sites and the ionization states of these amino acids are pH dependent. Enzymes, therefore, have a pH

optimum at which their activity is maximum. This α-amylase displayed a relatively broad pH-activity curve (Fig 6), in which more than 80% of the activity was retained between pH 5.5 and 7.5 with the optimum at pH 6.5-7.0. This agrees with Saito (1973) who reported that the activity of an α-amylase isolated from Bacillus licheniformis 584 is within the range of 5.0-8.0. Boyer and Ingle (1972) have also reported that most bacterial amylases have their pH optima in the neutral range, but a few have theirs in the alkaline range. Contrary to the report of Manning and Campbell (1961), that optimum pH values for α-amylases generally occur between pH 4.8 and 6.5, activity for this enzyme was low at pH less than 5.0 as compared to what it wws at pH 6.0-7.0. However, amylases, when supplied with full complements of calcium ions are usually stable at extremes of pH values (Fogarty and Kelly, 1980).

The effect of temperature on the α-amylase activity is shown in Fig 5. The temperature-activity profile was similar to that of Bacillus stearothermophilus α-amylase (Takagi et al, 1971). The optimum temperature for this enzyme activity is 60°C, though more than 50% of the enzyme activity was found between 40°C and 70°C. The gradual increase in activity from 25°C to 60°C is in agreement with the theory of general rate increase in a chemical reaction by temperature increase. The Q10 value for this enzyme (which is the factor by which the velocity is increased on raising the temperature by 10°C) is between one and two, which

means that the α-amylase activity almost always doubles for every 10°C increase in temperature.

The Arrhenius plot (Fig 4) gave one straight line with only one single activation energy (Ea) between 25°C and 60°C. The activation energy (Ea) was determined from the slope to be 20.01J/mol. This Ea is lower than that reported for amylase from Pseudomonas stutzeri (Robyt and Ackerman, 1971), and also for sorghum β-amylase (Okon and Uwaifo, 1984). The fact that the activation energy of this enzyme is lower than that for some amylases from other sources indicates that reactions catalysed by this enzyme are faster since the energy barrier to be overcome before the reaction occurs is lower. The higher the Ea value, the slower the reaction, and vice versa, other conditions being constant. Robyt and Ackerman (1971) have reported instances where the Arrhenius plot of some amylase enzymes gave two distinct lines, in which case two energies of activation could be calculated. Bacillus subtilis var amyloliquefaciens amylase has a break at 12°C, Pseudomonas saccharophila amylase has a break at 15°C and barley malt β-amylase and wheat β-amylase have breaks at 20°C. The significance of the breaks in the Arrhenius plots to give two activation energies, they further said, has not been determined.

For the determination of its molecular weight, the purified enzyme was run through a gel-filtration column. Since gel-

filtration operates on the basis of molecular size, the proteins with higher molecular weights move faster than the smaller proteins, thereby making the heavier proteins have a lower V_e/V_o ratio. This α-amylase was eluted as a single band at a V_e/V_o of 1.75, which corresponds to a molecular weight of 33,113 dalton (Fig 7). This molecular weight is high compared to 22,500 which was reported by Saito (1973) for α-amylase from Bacillus licheniformis 584, but it is within the range recorded by Fogarty and Kelly (1980) who reported that α-amylases from microbial origin have their molecular weights between 30,000 and 60,000 daltons.

The effect of metal ions on the activity of α-amylase indicated that calcium ion at 0.02M enhanced the activity of the enzyme most among the metal ions studied (Table 5). The enhancement of α-amylase activity by calcium ion has been reported by previous workers. Whitaker (1972) reported that the addition of calcium ion makes the enzyme very active and more stable. The role of the calcium ion in α-amylase appears to be to maintain the secondary and tertiary structures of the molecule. In the prsence of calcium ions α-amylases are quite resistant to extremes of pH values, temperature, treatment with urea or exposure to some proteases. However, calcium-free amylases are highly susceptible to denaturation by similar treatments (Stein and Fischer, 1958).

Other divalent metals, such as strontium, barium, and magnesium could replace calcium without appreceable loss in activity, but it appears that these metals can replace calcium only when the original conformation of the protein is retained and the active site has not been altered (Whitaker, 1972). The reduction of α-amylase activity by zinc, lead, copper and mercury in this study agrees with Fogarty and Kelly (1980) who reported the inhibition of α-amylase activity by heavy metals such as silver, lead, copper and mercury. Welker and Campbell (1963) further went ahead to report that calcium ion should be incorporated in the reaction medium to enhance amylase activity when assaying for the enzyme.

The stability of the α-amylase when stored under different conditions (Table 6) shows that the enzyme is most stable under frozen condition. Logarithm transformation of the stability data revealed a first order kinetics with the rate constants (K_D) of 0.22/week, 0.0534/week and 0.0144/week for storage at room temperature, refrigerator and freezer, respectively. The half life of the enzyme was also determined for each of the different storage conditions. It was found to be 3.15 weeks, 12.98 weeks and 48.125 weeks for storage at room temperature, refrigerator and freezer, respectively (Figs 8, 9, 10). This showed that the best storage condition for this enzyme is in the freezer. The stability of this enzyme over a longer period when stored in the freezer is typical of many enzymes, since enzymes are proteins

which can easily be denatured at relatively high temperatures. After eight weeks of storage, the enzyme still retained about ninety percent of its activity in the freezer, about seventy percent when stored in the refrigerator and about twenty percent when stored at room temperature.

Judging from the rate of loss of enzyme activity at room temperature, it is obvious that α-amylase is reasonably stable at this temperature. This also means that the initial enzyme assay and purification can be carried out at room temperature, if the means to do so in a cold room is not available. This is confirmed by the fact that after one week of storage at room temperature over 90% of the enzyme activity was still retained. This is important under conditions of unstable power supply.

The results obtained from the studies of the effect of some chemical substances and α-amylase production (Fogs 11-15) showed that only folic acid enhanced appreciable increase in the level of enzyme production.

Glycine did not induce α-amylase production by this organism. This is contrary to what was reported by Zhang et al (1983) in which glycine was found to increase α-amylase production by Bacillus amyliciquefaciens. The fact that α-amylase activity in the growth medium reduced with increase in glycine concentration indicated that, glycine rather than inrease α-amylase production, inhibits it. The glycine had a similar effect on the growth of this organism. This then goes to explain that the reduced α-

amylase production might be due to the inhibition of bacterial growth by this compound.

Triton X-100, which is a surfactant, inhibited amylase production by this organism tremendously. This is contrary to what was reported by Ikediobi and Ogundu (1985) in which triton X-100 was reported to have increased linamarase production. As is the case with glycine, the reduced bacterial count in cultures grown with triton X-100 could be responsible for the reduced α-amylase production.

Riboflavin and calcium panthothenate, which are both vitamins had no effect on the level of α-amylase production. They both supported an increase in bacterial growth. This goes to explain that these vitamins though could not increase α-amylase production in this mutant strain, could nevertheless serve to enhance good growth of this organism. These vitamins, from the auxanographic studies, have been shown to favour the growth of this mutant strain (Table 3).

Folic acid which is also a vitamin was able to increase α-amylase production to about one and half fold, together with an increased bacterial growth of equal magnitude. The ability of this mutant strain to utilise folic acid has also been indicated in auxanographic studies (Table 3).

These results go to explain that some compounds could be used as appropriate enhancers of growth and α-amylase production by this mutant strain of Bacillus licheniformis B5.1, while some

only improve the bacterial growth but do not play any role in enhancing enzyme production.

The fact that some of these chemicals did not increase α-amylase production by this organism does not make them a non-ehnancer of enzyme synthesis, because previous workers such as Reese and Maguire (1969) and Ikediobi and Ogundu (1985) have reported cases in which surfactants generally and triton X-100 in particular had served as very good enhancers of enzymes synthesis by some organisms.

These studies have shown that the production of α-anylase by a locally isolated strain of Bacillus licheniformis B5.1 can be increased appreciably using mutagens such as the ultraviolet irradiation. This finding is of great inportance, because as earlier reported amylase enzyme whcih is of immense importance in may industries can now be produced at an appreciable level using locally sourced materials. The α-amylase produced was also characterized and its activity was found to compare favourably with other α-amylases reported. This goes to explain the fact that this α-amylase with necessary purification and preservation can be used in place of the imported enzyme.

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APPENDIX A: Typical characteristics used for identifying various species of Bacillus (Adopted from Gordon et al (1973).

Characteristic Feature or Reaction	1	2	3	4	5	6	7	8	9	10
Morphological type	rods	rods	rods	rods	rods	rods	rods	rods	rods	rods
Gram reaction	+	+	+	+	+	+	+	+	+	+
Spore shape	oval	oval	oval	oval	oval	oval	oval	oval	oval	oval
Spore location	C	C	CST	C	C	CST	CST	C	T	T
Catalase test	+	+	+	+	+	+	+	+	±	+
Voges-Prausker test	+	+	+	−	+	+	−	−	−	−
Anaerobic growth	+	−	+	−	+	+	+	−	−	+
Growth at 50°C	+	+	+	+	+	−	+	±	+	+
Growth in 7% NaCl	+	+	−	+	±	−	+	+	−	−
Gelatin liquefaction	+	+	+	+	+	+	+	+	±	+
Growth at pH 5.7	+	+	+	+	+	+	±	+	−	−
Starch hydrolysis	+	+	+	+	+	+	+	−	±	−
Growth at 65°C	−	−	−	−	−	−	−	−	+	−
Citrate utilization	+	+	−	+	+	−	±	+	−	−
pH in V.P. Broth < 6.0	+	+	±	+	+	+	+	+	+	+
Casein decomposition	+	+	+	+	+	+	±	+	+	+
Egg Yolk reaction	−	−	−	−	+	−	−	−	−	−
Acid from glucose	+	+	+	+	+	+	+	+	+	+
Acid from arabinose	+	+	±	±	−	+g	+	+	−	−
Acid from xylose	+	+	+	+	−	+g	+	+	−	−
Acid from mannitol	+	+	+	+	−	+g	+	+	−	+

1. B. licheniformis
2. B. subtilis
3. B. coagulans
4. B. megaterium
5. B. cereus
6. B. polymyxa
7. B. circulans
8. B. pumilus
9. B. stearothermophilus
10. B. laterosporus

C = Central CST = Central, subterminal or terminal

T = Terminal +g = Positive with gas production.

Appendix B: Some extracellular enzymes of the genus Bacillus (Priest, 1977).

Enzymes	Species	Comments
α-amylase	B. amyloliquefaciens B. coagulans B. licheniformis B. macerans B. stearothermophilis B. subtilis B. subtilis var amylosaccharitieus	Endohydrolysis of the α-1,4-glucosidic linkages in polysaccharides. The different species produce enzymes with different properties
β-Amylase	B. cereus B. megaterium B. polymyxa	Exohydrolysis of the α-1,4 glucosidic linkages in polysaccharides yielding β-maltose
Cellulase	B. brevis B. firmus B. polymyxa B. pumilus B. subtilis	Hydrolysis of carboxymethyl cellulose to cellobiose

Enzymes	Species	Comments
Dextranase	B. megaterium B. subtilis	cell-bound enzymes catalizing the exohydrolysis of dextran to glucose
Galactanse	B. amyloliquefaciens B. subtilis var amylosaccharitícus	Hydrolysis of the β-1,4-galactosidic linkages in soyabean arabinogalactan.
Maltase	B. subtilis	Hydrolysis of the α-1,4 linkages of maltose and maltotriose
Pullulanase	Alkalophilic Bacillus species	Endohydrolysis of the α-1,6-linkages of pullulan.
PROTEASES		
Metal protease	B. amyloliquefaciens B. cereus B. licheniformis B. megaterium B. polymyxa B. subtilis B. thuringiensis	Enzymes require Ca^{2+} for stability and Zn^{2+} for activity, pH optimum at or near neutrality

Enzymes	Species	Comments
Serine protease	B. amyloliquefaciens B. licheniformis B. subtilis B. pumilus	The subtilisins, alkaline pH optima, serine residues at or near the active site
PENICILLINASES		
β-lactamase	B. anthracis B. cereus B. megaterium B. licheniformis B. subtilis	Hydrolysis of the amidebond in the β-lactam ring of penicillins and cephalosporins
Pencillin amidase	B. megaterium	Hydrolysis of the peptide linkage of penicillin
NUCLEASES AND PHOSPHATASES		
Alkaline phosphatase	B. cereus B. amyloliquefaciens B. subtilis	Often cell bound. The enzyme is extracellular in this species.
Deoxyribo-nuclease and ribonuclease	B. amyloliquefaciens B. cereus B. pumilus B. subtilis	A large number of DNAses, RNAses and phosphodiesterases with individual properties have been purified.

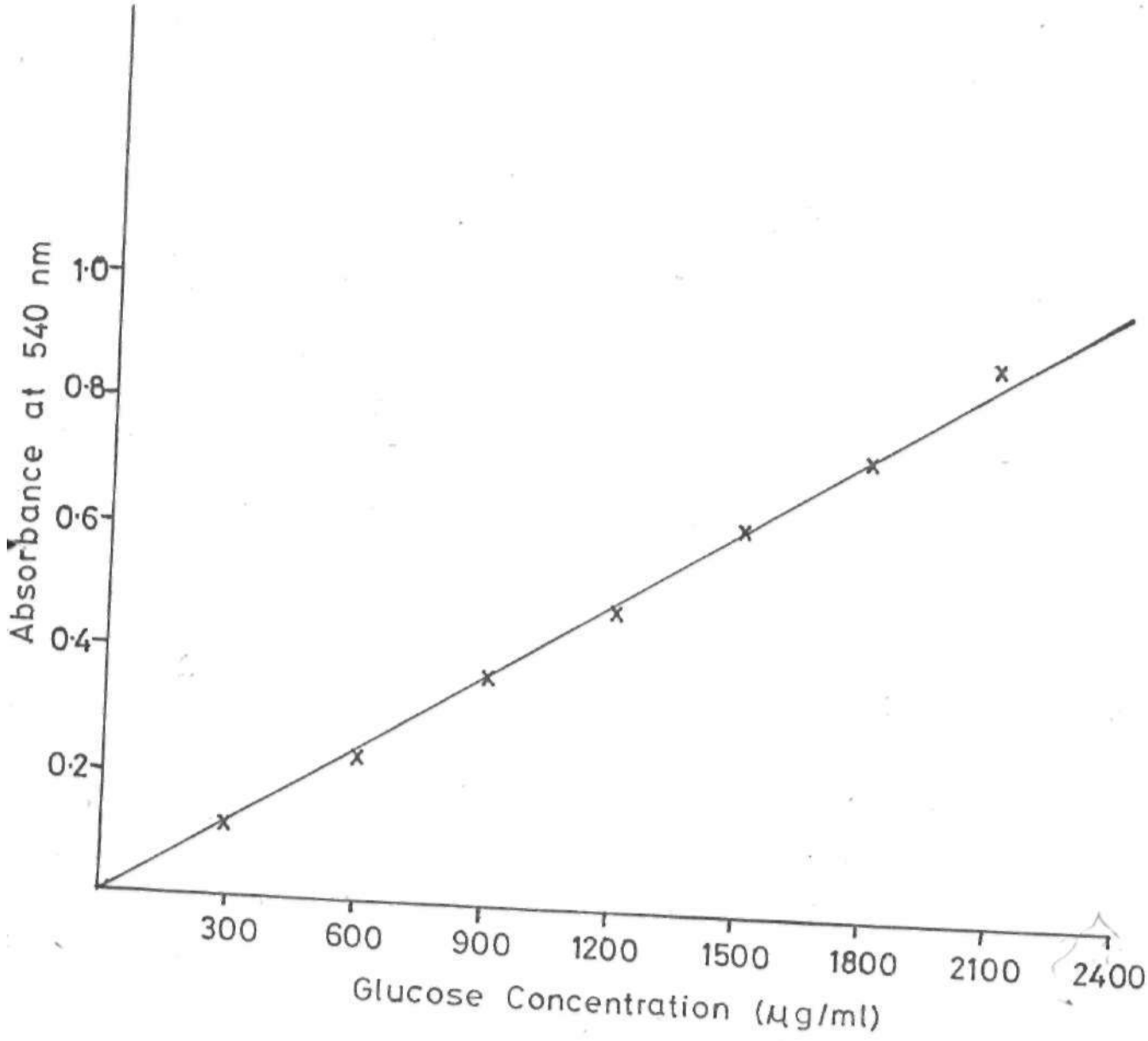
Enzymes	Species	Comments
LIPASE	B. licheniformis	Hydrolysis is triacyl glycerol to diacylglycerol and a fatty acid anion.
PHOSPHOLIPASE C.	B. anthracis	Responsible for the 'egg yolk' reaction
	B. cereus	
	B. thuringiensis	

Appendix C: Some antibiotics elaborated by species of the genus *Bacillus* (Katz & Demain, 1977)

Species	Antibiotics
Bacillus brevis	Gramicidin S
	Tyrocidine
	Linear gramicidin
	Brevin
	Edeine
	Eseine
	Bresseine
	Brevistin
Bacillus subtilis	Mycobacillin
	Subtilin
	Bacilysin
	Bacillomycin
	Fungistatin
	Bulbiformin
	Bacillin
	Subsporin
	Bacillocin
	Mycosubtilin
	Fungocin
	Iturin
	Neocidin
	Eumycin

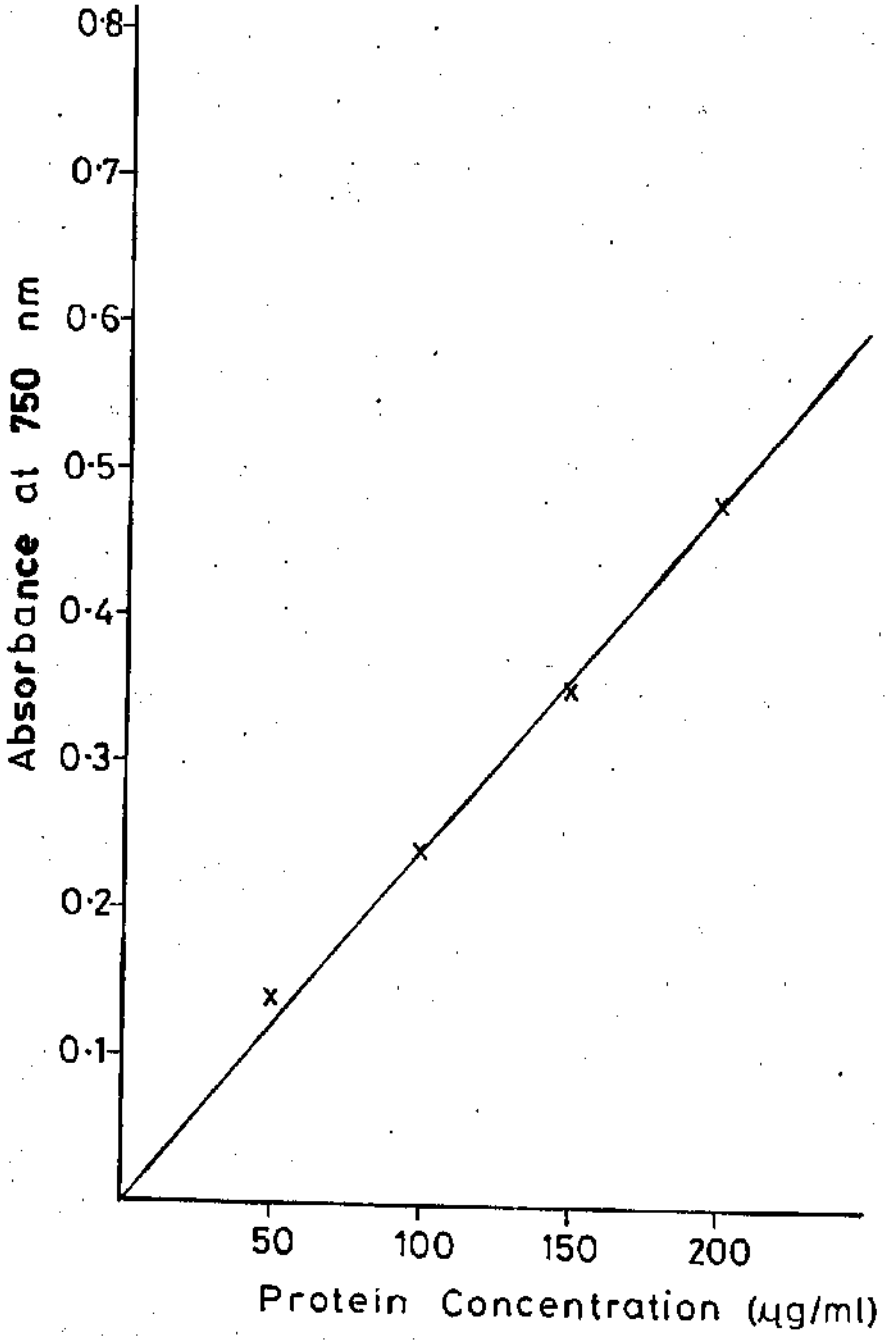
Species	Antibiotics
Bacillus pumilus	Micrococcin P
	Pumilin
	Tetain
Bacillus mesentericus	Esperin
Bacillus thiaminolyticus	Octopytin (Thianosine)
	Baciphelacin
Bacillus licheniformis	Bacitracin
	Licheniformin
	Proticin
Bacillus polymyxa	Polymyxin
	Colistin
	Gatavalin
	Jolipeptin
Bacillus circulans	Butirocin
	Circulin
	Polypeptin
	EM-49
	Xylostatin

Species	Antibiotics
Bacillus laterosporus	Laterosporamine
	Laterosporin
Bacillus cereus	Biocerin
	Cerexin
	Thiocilin


Absorbance at 540 nm
1·0
0·8
0·6
0·4
0·2
300
600
900
1200
1500
1800
2100
2400
Glucose Concentration (µg/ml)

APPENDIX E

STANDARD CURVE FOR THE ESTIMATION OF PROTEIN CONTENT


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Absorbance at 750 nm
50
100
150
200
Protein Concentration (µg/ml)