EVALUATION OF CORNBRAN ('DUSA'), YAM AND BANANA PEELS AS POSSIBLE SUBSTRATES FOR AMYLASE PRODUCTION BY LOCAL STRAINS OF *ASPERGILLUS NIGER* CA-3 AND OR-5

BY

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DECLARATION

I hereby declare that the contents of this thesis is an original work carried out by myself and no part of it has been presented elsewhere for a degree or diploma. The work of other investigators is fully acknowledged and referred to in this presentation.

SALEH ALHAJI ADO

OCTOBER, 1993

CERTIFICATION

This thesis entitled "Evaluation of Cornbran ('Dusa'), Yam and Banana peels as possible substrates for amylase production by local strains of Aspergillus niger CA-3 and OR-5, meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Chairman, Supervisory Committee	Date
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Above all, I wish to express my immeasurable gratitude to Almighty Allah who allowed me to witness this part of my life successfully.

S.A. Ado

OCTOBER, 1993

DEDICATION

To

My Wife and Children when they come.

ABSTRACT

A total of forty five strains of Aspergillus niger were isolated from various sources and screened for amylase producing ability using Nutrient agar containing soluble starch as the substrate. A. niger CA-3 and OR-5 were selected as the highest amylase producers. The two selected strains were studied further for amylase production in submerged cultures using corn bran ('Dusa'), yam and banana peels as the substrates with soluble starch as the control. When grown on soluble starch, the highest amylase activity for A. niger CA-3 was 24 units/ml while it was 27, 297 and 150 units/ml when grown on corn bran ('Dusa'), yam and banana peels respectively. Similarly, the highest amylase activities in the culture filterates of A. niger OR-5 grown on soluble starch, corn bran ('Dusa'), yam and banana peels were 36, 66, 222 and 144 units/ml respectively. The optimum concentration of yam peel powder for amylase production by A. niger CA-3 was 3.5% (w/v), while the optimum pH and temperature were 7.0 and 30°C respectively. It was concluded that yam peel was the best, and banana peel and corn bran ('Dusa') are also better substrates for amylase production by both strains than soluble starch.

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CHAPTER 1

INTRODUCTION

A wide variety of microorganisms such as bacteria, yeast and moulds are involved in the production of many commercially desirable products such as enzymes, alcohol, organic acids, antibiotics and biomass. There are many sources of commercial enzymes but those produced by microorganisms seem to predominate (Under Kofler *et al.*, 1958). Among the moulds, members of the genus *Aspergillus* are good producers of hydrolytic enzymes such as amylases, carbohydrases; cellulases, pectinases, proteinases, lipase and others which are used in a wide fields of industry (Raper and Fennell, 1965; Beckhorn *et al.*, 1965; Gandhi *et al.*, 1974).

Microbial amylases are used in many industries such as textiles, brewing, paper, baking confectionary and pharmaceuticals. Many of these industries exist in Nigeria, but the enzymes they use are imported. It is likely that industrial use of amylases may increase in the near future. This is evident in the increase in number of industries using these enzymes. In order to conserve foreign exchange, there is need to explore the possibility of producing industrial amylases locally by exploiting commercially feasible sources. Since microorganisms of the genus *Aspergillus* are versatile producers of amylases and are easy to maintain on culture, they are suitable source for investigation.

Since amylase is an inducible enzyme (coleman and Grant, 1966; Meers, 1972), the medium should contain an inducer. In

most cases commercial soluble starch is used, although sugars such as Maltose, lactose and galactose may be used. These substrates are expensive for large scale cultivation of amylase producing organisms, hence the need for alternative cheaper carbon substrates for amylase production. Yield of amylase has been found to be considerably high when the medium is based on naturally occuring materials such as wheat, maize, barley and malt, rather than artificial media largely composed of single molecules (Nyiri, 1971). Corn bran ('Dusa'), Yam and Banana peels may be suitable substrates in Nigeria, because they are readily availble starchy waste materials derived from corn, yam and banana. Hence they are suitable source for investigation.

This work is therefore undertaken with the following objectives in view:

(1) To isolate local strains of Aspergillus niger from various sources

(2) To screen the isolates for amylase producing ability

(3) To select strains with high level of enzyme production

(4) To study the selected strains for amylase production using corn bran ('Dusa'), yam and banana peels as substrates

(5) To determine the effect of various environmental factors on amylase production using the most inducing local substrate.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 GENUS ASPERGILLUS

The genus Aspergillus is a group of free living saprophytic Moulds. Members of the genus are filamentous consisting of the following structures: foot cell, conidiophore stalk, vessicle, sterigmata and the conidia or spores. The colour, shape, size and arrangement of the conidial heads are characteristic of the species and to a lesser extent of the group to which they belong for example, the head of Aspergillus fumigatus and A. nidulans are small green, and compactly columnar; heads of A. niger, A. ochraceus, and A. wentii are large, black and radiate, ochraceus and yellow brown respectively. Heads in other species notably A. flavus and A. candidus usually show characteristic variability in size and shape within the same colony and are yellow- green and white respectively (Raper and Fennel, 1965).

2.1.1 Distribution

The Aspergilli are widely distributed in nature and are undoubtedly one of the most ubiquitous of all groups of fungi. The world wide distribution of the genus varies from 0.1 to 22% of the total air spora samples (Mullins, 1974), being the most common organism in some countries to 15th in order of abundance in others. They are found almost everywhere because of their ability to grow on different substrates under a wide

range of environmental conditions. The black aspergilli are probably more common than any other group within the genus. They occur in and upon the greatest variety of substrata including grains, forage products, soiled fruits, vegetables, exposed cotton textiles, fabrics, leather, dairy products, other protein rich substrata, and decaying vegetation in the field. They are abundant in all soils examined and appear to be particularly abundant in soils from tropical and sub-tropical areas (Raper and Fennel, 1965).

2.1.2 Identification

Identification of Aspergillus is mainly based on their morphological, developmental and cultural characterization. Raper and Fennell (1965) recognised 132 species and 18 varieties in the genus. These they classified in 18 groups of one to several species having certain basic characteristics in common. The characters utilized for delineation of the groups include:

Head colour - black, white, yellow, brown and various shades of green.

Head shape - globose, radiate, loosely or compactly columnar.

Sterigmata - in a single or double series developing directly from the vesicle or from melulae

Vesicle shape - clavate, globose, subglobose, subclavate, turbinate, hemispherical, or terminally flattened and with or without a constriction in the conidiophore immediately below.

Conidiophores - smooth or variously roughened, coloured or uncoloured.

These major separating characters are usually clearly evident and with this information alone, a group identification of an unknown can be made with nearly complete confidence. There are exceptions and an occasional species shows characters that appear to aligh it with more than one group. In these instances, species are assigned where preponderance of their character indicate.

2.2 INDUSTRIAL IMPORTANCE OF THE GENUS ASPERGILLUS

Members of the genus Aspergillus rank high among microorganisms of industrial importance. They are of great importance in the production of enzymes, organic acids, food fermentation and biodeterioration.

2.2.1 Enzyme Production by the Genus Aspergillus

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

Members of the genus Aspergillus contribute greatly to the development of microbial enzymes. Some of the important enzymes produced by the Aspergilli include Amylases, proteases and pectinases (barbesgaard, 1977). Others are Glucose oxidase, catalase, β-glucanase, Tannase, Naringinase, Lipase, α-galactosidase, β-galactosidase, Pentosanase (Xylanase) and cellulase.

2.2.1.1 Amylases:

Amylases are starch-degrading enzymes. They are widely distributed in the microbial, plant and animal kingdoms. They are divided into three types namely: α-amylase, B-amylase and amyloglucosidase (glucoamylase) (Thoma et al, 1971). The α-amylase (α-1,4-glucan 4-gluconohydrolase EC 3.2.1.1) is an endosplitting enzyme which hydrolyses the α-1,4-glucosidic bonds and by passes 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 liquefaction and marked reduction in the viscosity of starch, hence the name liquefying amylase (Fogarty and Kelly, 1980). The β-amylase (α-1,4 glucan

maltohydralase 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 residual component known as β-limit dextrin. The ability of the β-amylase to by pass the α-1,6 linkages results in the formation of β-limit dextrin (Fogarty and Griffin, 1973). The amyloglucosidase (*a* -1,4-glucanglucohydrolase EC 3.2.1.3) *is also an exosplitting enzyme and hydrolyses amylose,* amylopectin and dextrins to give glucose as the sole end product (Whitaker, 1972; Barbesgaard, 1977).

The production of amylase is generally widespread among living organisms. Among the microorganisms, moulds and bacteria are the main producers of the enzyme. The moulds include Aspergillus, Penicillium and Rhizopus (Pazur and Ando, 1960). However Aspergillus species mainly A. niger and A oryzae are the most commonly used for the industrial production of fungal amylases. Many genera and groups of bacteria can produce amylases. Examples are Bacillus, Clostridium, Corynebacterium. However, the genus Bacillus has become eminent in industrial production of the enzyme. Amylase has also been produced by microorganisms other than moulds and bacteria. Upton and fogarty (1977), reported the production of amylase by an actionomycete, Thermomonospora viridis and Lammel et al (1980) also reported the production of amylase by

a yeast Saccharomyces fibuligera.

Other than microorganisms and plants, the production of amylase has been reported in the organs of some animals. Mc Geachin and Potter (1967) reported the synthesis of amylase by the pancreatic 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 Gulberstor (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), and some marine organisms such as squid, cuttle fish and octopus. In this review emphasis will be on amylase produced by members of the genus Aspergillus who produces mainly α-amylase and amyloglucosidase (glucoamylase).

2.2.1.1.1 X-amylase

α-amylase (α-1,4-glucan-4-glucanohydrolase EC 3.2.1.1) occurs widely in microorganisms. It hydrolyses α-1,4-glucossidic bonds in amylose, amylopectin and glycogen in an endo fashion but the α-1,6-glucosidic linkages in the

branched polymers are not hydrolysed and the reaction products are mainly maltose and maltotriose (forgarty and Kelly, 1980). The source of the enzyme is an indication of its properties and mechanisms of action. The endosplitting 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, 1960).

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

Production of multiple-branched dextrins in addition to singly branched α-limit dextrins by some α-amylases have

been reported by Roberts and Whelan (1960). In these reactions, isomaltose is not formed because the α-1,6 linkages are resistant to α-amylases and they therefore counter some stability on certain α-1,4 linkages near branch point.

Some members of the genus Aspergillus such as A. niger, A. oryzae, A. batatae, A. foetidus and A. terricola are known to produce α-amylase (Fogarty and Kelly, 1980). Aspergillus niger produces acid-stable and acid-unstable α-amylases (Arai et al, 1969). Strains of A. oryzae are most widely used for commercial production of the enzyme. The enzyme production is carried out originally by cultivating the fungus on moist bran, but it is also possible to produce the enzyme in submerged culture. When the fungus is grown on moist bran it will form several enzymes such as protease and lactase in good yield together with the α-amylase, where as only α-amylase is produced in submerged culture (Barbesgaard, 1977).

The molecular weight of α-amylase is within the range 30,000 - 60,000 daltons. The acid-unstable and acid stable α-amylase of Aspergillus niger have molecular weights of 61,000 and 58,000 respectively (Arai et al, 1969). In case of bacterial α-amylase, mono-dimer transformation was reported

in Bacillus substilis α-amylase with the dimer having a molecular weight of 100,000 daltons (Menzi et al, 1957). From the reports of Mitchel et al (1973) and Robyt and Ackerman (1973), the α-amylase of Bacillus subtilis has a basic submit of 24,000 dalton molecular weight. A lower molecular weight of 22,500 has been reported for 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. The optimum pH values generally occur between pH 4.8 and 6.5 (Manning and Campbell, 1961), but with significant differences in the shapes of pH activity curves for the different enzymes (Fogarty and Kelly, 1980).

The activity of most highly purified α-amylase is lost quickly above 50°C but presence of calcium ions may slow down this inactivation. All α-amylases are calcium metallo enzymes having a minimum of one atom of this metal per molecule of the 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-amylase is quite resistant to extreme 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-amylase has been denatured, they are susceptible to protease degradation (Fogarty and Kelly, 1980).

Stein and Fischer (1958) have reported that heavy metals such as silver, lead, copper and mercury inhibits the activity of x-amylase. The α-amylase of Aspergillus niger contain one gram atom of calcium while the α-amylase of A. 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).

2.2.1.1.2 Amyloglucosidase

Amyloglucosidase (EC 3.2.1.3, glucoamylase, α-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 hydrolyses α-1,6 and α-1,3 glycosidic linkages (Pazur and Klepp, 1962). The enzyme is primarily used to hydrolyse starch completely to glucose. Unlike α-amylase and β-amylase which

are products of both bacteria and fungi, amyloglucosidase occurs almost exclusively in fungi. Among many genera of fungi that produce the enzyme Aspergillus species especially members of A niger group: A. niger, A. awamori, A. phoenicis, and A. foetidus are the prominent (Pazur and Ando, 1960; Barbesgaard, 1977). Other important genera that produce the enzyme include Penicillium and Rhizopus.

The rate of substrate hydrolysis by amyloglucosidase 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 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 (Krzechchwsk and Urbanek, 1975).

Amyloglucosidase 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 accompained 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 preparation of amyloglucosidase (Pazur and Ando, 1961).

In certain instances, two or more forms of

amyloglucosidase have been detected. Strong debranching activity and ability to degrade raw starch are properties of amyloglucosidase I from Aspergillus awamori var. kawachi, whereas weak debranching activity and weak ability to hydrolyse raw starch are properties of amyloglucosidase II from the same organism (Forgarty and Kelly, 1980). In submerged culture, A awamori var kawachi produces three types of amyloglucosidase - I, I' and II. Type I hydrolyses raw starch and limit dextrins almost completely. Type I' and II does not degrade raw starch, and hydrolyses limit dextrin 50% and less than 10% respectively.

Structural characterization of amyloglucosidase I and II from Aspergillus niger has been carried out. Amyloglucosidase I and II have molecular weights of 74,900 and 54,300, and 13% and 18% carbohydrate respectively (Fogarty and Kelly, 1980). The enzymes have different amino acid composition.

2.2.1.1.3 Industrial uses of amylases

The use of amylases in industries such as food and beverages, textile, and pharmaceuticals is wide. Fungal amylases are used as saccharifying agent in grain alcohol fermentation mashes (Rose, 1961). It has also been used in the conversion of partially hydrolysed starch to sweet syrups. The proper control of type and proportion of the enzyme used usually produces syrup of almost any desired proportions of glucose, maltose and dextrins (Under Kofler et al, 1958). Fungal amylases are also used for starch removal for

flavouring extracts, for fruit juice extracts and in preparing clear, starch-free pectin (Pazur and Ando, 1961).

In paper industries, microbial amylases 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 (Under Kofler et al, 1958).

In textile industries, microbial amylases are used in continous process for desizing of textile fabrics and in preparing modified starch sizing for textiles.

Microbial amylases are used generally in other industries as in treating vegetable for canning, processing cereals, preparing breakfast foods, in the confectionary and candy industries (Godfrey, 1985). It is used in mild concentrations as a digestion aid in the pharmaceutical industries, and in the fortification of flour in the baking industries.

2.2.1.1.4 Important factors to be considered in the production of Amylases for commercial uses

A number of factors are considered in developing the enzyme of any organism for commercial applications.

2.2.1.1.4.1 Choice of Organism and Strain

The strain and type of organism used for amylase production affects the type of amylase produced. The use for which the enzyme is intended also determines what type of organism is to be used. For example, amylases used in food and

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). Once a given type of organism is chosen, the species and strain would then be determined by a comparison of the relative potentials of many isolates under a given set of experimental conditions.

2.2.1.1.4.2 Medium Composition

Amylase production by a given organism is affected by the composition of the medium. A balance of carbohydrate and nitrogen constituents, together with semi-micro and micro components is important (Burbidge and Collier, 1968). Since the enzyme is inducible (Coleman and Grant, 1966), the medium should contain an inducer. Starch is widely used as a carbon source and inducer in the production of amylase (Upton and Fogarty, 1977). The use of wheat bran as a carbon source by 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). High level production of a raw starch saccharifying amylase by Bacillus alvei grown on sorghum, rice, corn, yam, cassava and potato starch was reported by Achi and Njoku-obi (1992). Sani et al (1992) also obtained high yield of amylase by A. niger and A. flavus using cassava peel as the sole carbon source. Ogundama and Odoh (1984) prepared fungal

culture media with infusions of wastes derived from agricultural products. The wastes include banana, plantain, pineapple, pawpaw, cassava and yam tuber peels. Other enzymes have also been produced using wastes from agricultural products. For example high level of cellulases (exoglucanase and endoglucanase) were produced by co-culturing of Aspergillus niger and Trichoderma reesei on cellulosic wastes. The wastes include among others bagasse, corn cobs and sawdust (Madamwar and Patel, 1992). In relation to induction, yield of amylase is considerably higher when the medium is based on naturally occuring materials such as wheat, maize, barley, malt rather than an artificial media largely composed of single molecules (Nyiri, 1971).

A variety of nitrogen sources has been used for amylase production. Examples of these include ammonium citrate, mycological peptone, corn steep liquor, calcium nitrate and ammonium sulphate (Upton and Fogarty, 1977).

After the strain of organism has been chosen and cultured in an appropriate medium, some parameters such as pH, temperature, aeration requirements would be properly determined for the system. The need for aeration would be minimal if a solid state fermentation would be used.

2.2.1.1.4.3 Strain improvement

Even after suitable medium composition has been developed for a fermentation system, yield improvement is still possible by improving the strain chosen. This is achieved by mutagenesis. *Different* mutagenic agents could be used for the improvement of amylase production by different micro organisms (Yoneda and Maruo, 1975). The mutagenic agents frequently used include ultraviolet irradiation, X-rays, gamma rays and chemical mutagens such as ethyl methane sulphonate, N-methyl-N-nitro-N-nitroso-guanidine (NTG) and B-propiolactone (Gratzner and Sheeham, 1969).

2.2.1.2 Other Enzymes from the Genus Aspergillus

2.2.1.2.1 Proteases

The Commission on Enzyme Nomenclature (1972) recognizes three groups of proteolytic enzymes namely - EXO-peptidases (EC.3.4.11-15) which participate in, but can not themselves complete, the digestion of proteins; Dipeptide hydrolase (EC. 3.4.13-14) and peptide endohydrolase (EC 3.4.21-24), conveniently called proteases.

The further classification of proteases is principally in terms of catalytic mechanism usually determined by reactivity towards inhibitors that react with particular residues in the active site *region. using these criteria endo-proteases* fall into four broad classes: alkaline proteases, (EC.3.4.21) with a reactive serine residue; thiolproteases (EC 3.4.22); acid

proteases (EC. 3.4.23) and neutral proteases (EC 3.4.24) generally dependent on a metal ion. Proteases of many Aspergilli fit into this classification (Nakagawa, 1970; Matsubara and Feder, 1971). Not all species however, are known to produce enzymes of each class. Thus for example the Aspergillus niger group, including A. awamori, A. niger and A. saitoi (Synonymous with A. Phoenicis), are chiefly known as active producers of extracellular acid protease only (Aunstrup, 1980). However A. niger alone has also been reported to produce what seems to be an alkaline protease (Bossman, 1973). A oryzae produces acid, neutral and alkaline proteases (Davidson et al, 1975; Aunstrup, 1980).

Proteases have wide applications. They are used in clinics and pharmaceutical industries for the debridment of wounds by injection to relieve inflammation. They are also used in the brewing industry to prevent chill haze (a precipitate of protein and tannin formed by cold storage of beer), in the baking industry to adjust the properties of flour gluten, in the leather manufacturing industries, where they are used for bating and unhairing of hides, in the meat industry for meat tenderization to improve its quality and in the photographic industries for the recovery of silver from photographic films (Under Kofler et al, 1958; Aunstrup, 1980).

2.2.1.2.2 Pectinases

Pectin is universally distributed throughout the plant kingdom and likewise pectinase is formed by a large number of microorganisms. Pectic substances are built of galacturonic acid units linked together in alpha - 1,4 bonds. The carboxyl groups may be free as in pectic acid or esterified by methoxy groups. Thus pectins refers to substances with a varying degree of methoxylation. commercial pectinases are all derived from Aspergillus species mainly A niger (Frank and Walker, 1980). Other pectolytic Aspergillus species include A. awamori, A. foetidus and A. japomicus. all commercial pectinases are mixtures of several pectic enzymes. The classification scheme includes two types: pectic methyl esterase which demethylates pectin to pectic acid, and polygalacturonase which degrade pectic acid further by hydrolysing the 1,4-glucosidic links thus finally splitting the molecule into units of galacturonic acid (Frank and Waler, 1980). Most commercial preparations contain these two enzymes. Some species of the genus Aspergillus have the ability to produce the two enzyme types. For example A usamii was reported to produce both pectin esterase and polygalacturonase in culture (Gaetano and Arturo, 1975).

Pectinases are used mainly in the wine and fruit juice industries for two main purposes.

(i) They are used as filtering enzymes in the pressing process. The high content of pectic substances in grapes

and fruits retain water and thus decreases the yield of juice. After degradation of the pectins by use of pectinases a much higher yield is obtained (Babesgaard, 1977; Frank and Walker, 1980). Furthermore, a better extraction of flavour and colour substances is obtained.

(ii) They are also used as clarification enzymes (Shingetaka and Tamatsu, 1971). Clarification seems to be a result of degrading pectin, since the colloidally suspended pectin may hold other substances in suspension. Freshly-pressed juices are rather viscous and have a persistent turbidity. Addition of pectinase results in a rapid drop in viscosity and break of turbidity. cloudy particles agglomerate, forming flocs which settle out. A clear juice is then obtained either by filteration or by centrifugation.

2.2.2 Organic Acid Production by the Genus Aspergillus

Members of the genus Aspergillus produce a rich variety of organic acids such as citric acid and gluconic acid by A. niger (Lockwood, 1975); itaconic acid by A. terreus and A itaconicus (Smith et al 1974). Other organic acids produced by Aspergillus species include succinic acid, glutaric acid, fumaric acid, glycolic acid, malic acid, itatartaric acid and glyoxylic acid.

The production of citric acid by fermentation using Aspergillus niger has been a commercial process since 1923

(Berry et al, 1977). Strains of A. niger vary considerably in their capacity to produce citric acid and excrete it into the growth medium at high concentration. The efficiency of the acid production by A. niger has been shown to be a function of two groups of factors, the characteristics of producing organism and the cultivation conditions (Lockwood, 1975). Other Aspergillus species known to produce critic acid include A. awamori, A. wentii, A. Saitoi, A. usamii, A. phoenicus, A fumaricus, A. flavus and A. lanosus. The production process of critic acid is divided into three phases viz: the preparation of the substrates and the inoculum, the fermentation stage, and product recovery. Molasses have been most widely used as the carbon substrate, while substrates such as starch hydrolysates have also been used. When crude substrates are used it is necessary to reduce the level of iron and other trace metals which interfere with critic acid production (Miall, 1978). This is normally achieved either by precipitation with ferricyanide or by the use of cation exchange resin. Successful fermentation has also been obtained using date syrup which had been deionised using cation exchange column (Berry et al, 1977).

Itaconic acid production by Aspergillus terreus is an important industrial process providing from cheap raw materials, a high yield of valuable product for commercial use. The acid was first isolated as a fungal metabolite from a culture of A. itaconicus while later it was shown to be

formed by certain strains of A terreus (Jakubowska, 1977; Miall, 1978). The acid is produced by surface and submerged fermentation methods. A itaconicus was recommended for fermentation of beet and cane molasses, black strap molasses and refined weed hydrolysates (Kobayashi, 1967). Another acid, itatartaric acid was shown to accumulate in fermentation liquors of itaconic acid by A terreus and A.itaconicus. This acid was first discovered by Stodola et al, (1945) with A. terrus resulting from genetic changes induced by mutation.

2.2.3 Food Fermentation by the Genus Aspergillus

The use of microorganisms to process foods goes back to ancient times. Fermented foods are essential parts of diets in all regions of the world. Food fermentation processes have several advantages, such as improving keeping quality and flavour (Hesseltine, 1983), and addition of desirable nutrients not present in the original product (Steinkraus, 1983a). Fermentation does not greatly alter the amino acids in cereals and soybeans, but often it makes proteins more available (Au and Fields, 1981). In many fermentions using soybeans, undesirable beany flavours are destroyed (Van Veen and Steinkraus, 1970), trypsin inhibitor is inactivated and flatutence factors are eliminated (David and Verma, 1981). In many instances, the use of micro organisms to produce tasty, nutritious food requires less energy than conventional processing (Steinkraus 1983b).

Although fungi belonging to several genera are employed in various fermentations, the genus Aspergillus is pre-eminent both in number of products made through its activities, and in the commercial importance of certain of these products. Notably soy sauce, and 'miso', the genetic term for a range soy based pastes (Wood, 1977). Other fermentation products from the genus include tamari, tampeh (Hesseltine, 1965), Sake' (Murakami, 1972), Cheese, Kaffir bear and a host of others.

In soy sauce production, Aspergillus oryzae or A. Soyae is used in the first stage of the fermentation while lactic acid bacteria and yeast are involved in the second stage (Wood, 1977). A mixture of cooked beans and wheat flour is inoculated with spore suspension of the mould and placed in a humid room at 30°C for 72 hours (Young and Wood, 1976). The product formed (Koji) is mixed with 18% brine, and this mixture (Moromi) undergoes prolonged fermentation in which first lactic acid bacteria and then yeast are involved. The finished product is pressed, clarified and pasteurized, bottled or marketed either alone or in a mixture with caramel, molasses and other additives depending on the preferences of the market being served. Enzymes like amylases and cellulases produced by the mould during the koji production stage remain active in the moromi, slowly hydrolysing the protein and polysaccharide present in the mash, and so enhancing the quality of the finished product.

Tamari is another type of sauce that is more common in China, in which a greater amount of soybeans is used than wheat (Hesseltine, 1965), often with the addition of other materials such as rice. The flavour of the tamari sauce differs from that of soy souce. This difference in flavour is due to the use of Aspergillus tamarii, this mould being the dominant micro organism in this process. The fermentaion period is shorter than that of soy sauce fermentation.

Miso is another fermentation product from the genus Aspergillus. Basic raw materials are soybeans, grain and salts, and it is marketed as a thick paste, and is used as a source of flavour and salt in cooking and food preparations. Shibaski and Hesseltine (1962) have described the making of this product and its characteristics. Three basic types of miso can be recognised (Hesseltine, 1965; Shibasaki and Hesseltine, 1962). There is kome miso, made from beans and rice; mugi miso, made from beans and barley; and mame miso (sold as *hatcho miso in the United Kingdom*) made from soybeans alone. For the production of kome and mugi misos, Aspergillus oryzae strains are used for the fermentation.

Tempeh is another fermented food common in Indonesia and other countries in South east Asia. It has also been produced in Ethiopia using some legumes such as horse-bean, pea and chickpea (Ashenafi and Busse, 1991). The fermentation of tempeh generate meat like flavours and improve nutritional value. Indonesian tempeh offers the world a process of making

low-cost protein-rich meat analogues of excellent nutritional quality (Steinkraus, 1983b). Tempeh has been suggested as a possible source of low-cost protein for child feeding programs in developing countries (Autret and Van veen, 1955). Although the most important and popular type of tempeh is made from soybeans, it may also be made from a variety of other substrates (Djien and Hesseltine, 1979; Ashenafi and Busse, 1991). Most authorities agree that mould involved in the fermentation belong to the genus Rhizopus, but Hesseltine (1965) reported that Aspergillus oryzae is also involved in the fermentation.

Sake' is a traditional alcoholic beverage made from rice, known as 'rice wine' because its alcoholic strength approximates that of wine (Wood, 1977). Its production depends upon the hydrolysis of rice by a koji produced by growing Aspergillus oryzae on a portion of the rice analogous with production of grain whisky where malted barley is used to hydrolyse much larger quantities of unmalted grains, or indeed with the sort of modern beer production where adjuncts such as maize are hydrolysed by malted barley assisted by mould enzymes Arima (1957) has given a detailed account of sake' production.

Some other minor fermentations in which Aspergillus species are involved include:

Kaffir beer: This is common in South Africa. The grain used (Sorghum or maize) are saccharified through the action of

Aspergillus flavus and Mucor rouxii. After the starch has been hydrolysed, the mixture undergoes a mixed lactic acid bacteria plus yeast fermentation. This beverage is an important source of dietary niacin and riboflavin (Hesseltin, 1965).

Chee-Fan: A type of soy-curd cheese in which Aspergillus glaucus may participate, as well as the more usual Mucor and Actino mucor. A. oryzae is also utilized in cheese fermentation (Nakanishi, 1973).

Fermented Minchin: This is a fermentation of wheat gluten, practised in Northern China. At least seven fungal genera including Aspergillus spp are thought to participate.

Hamanatto:- A product of soybean fermentation using A oryzae (Wood and Young, 1975). It is very common in Japan.

Katsoubushi: This is an extraordinary fermentation of fish by Aspergillus ochraceus and possibily A glaucus (Wood, 1977).

2.2.4 Biodeterioration by the Genus Aspergillus

Biodeterioration is a deterioration caused by biological agents or biodeteriogens. fungi are frequently implicated in biodeterioration problem probably due to their common saprophytic role and ability to utilize a wide range of substrates in a variety of ecological niches, and due to the nature of their growth which enables them to exploit a substrate (Eggins and Allsopp, 1975).

Members of the genus Aspergillus are often identified as biodeteriogens probably due to a combination of reasons: The

large number of species comprising the genus represents a broad spectrum of various ecological types capable of utilizing a wide variety of substrates (Raper and Fennell, 1965). Many of the Aspergilli are amongst the most commonly occuring fungal species - various species are frequently isolated from soil vegetation, air and certain substrates undergoing deterioration (Hudson, 1969). The isolation of the Aspergilli from soil, vegetation and the air, has been related to their growth on various materials including cotton farics paints, plastics, leather and glass (Upsher, 1972).

Aspergillus species are involved in the spoilage of a wide variety of stored products such as cereal grains - barley, corn, oats, rice, sorghum and wheat (Christensen and Kaufman, 1974); Cocoabeans (Hensen et al, 1973); Coffee beans (Vincents Nissen, 1967); Dried fruits (Pitt, 1975); Molasses (Eskin et al, 1974); Olives (Eggins and Coursey, 1968); Palm fruit (Raymond, 1966); Raw sugar, Butter, Cheese and meat (Wu et al, 1974). In many of these cases, other fungal species may be involved in causing spoilage apart from other spoilage microorganisms as well as insects and rodents. However in many instances the Aspergillus species are among the major spoilage organisms and in others they are the predominant cause of spoilage.

The species of Aspergillus found actively causing biodeterioration in any particular stored product depends on the nature of the substrate of that product, but even on the

same substrate the Aspergillus species vary depending on physical factors especially moisture and temperature. A number of Aspergillus species germinate and grow at low water activities - the limit of which vary with temperature (Pitt, 1975). This property is a major factor in the predominance of Aspergillus species as storage fungi on dry stored products including members of the A. restrictus, A. glaucus, A. candidus and A. flavus group (Christensen and Kaufman, 1974).

The infestation of cereals and seed by Aspergillus species causes discolouration, loss of germinability, heating, mustiness, caking and decay. Loss of germinability reduces the worth of the produce for use as viable seeds; heating may result in combustion; while the other consequences of spoilage reduce the quality of the produce in terms of food value. Apart from the damage to the stored produce, contaminated and infested stored produce can present health hazards. Some Aspergillus species are known to be pathogenic and thus their presence and growth on stored produce increases risk to the people handling the produce of becoming infected (Dennis, 1973). More in the context of true biodeterioration is the production of mycotoxins on stored produce which renders the food unfit for consumption by humans and animals (Davies et al, 1975).

Raw cotton besides consisting of cellulose provides other nutrients for microbial growth. Aspergillus niger, A. nidulans, A. fumigatus, A.versicolar and A. glaucus have been

found to cause damage to raw cotton fibres (Turner, 1967), which may occur before harvesting. This is not strictly biodeterioration, but such damages may cause uneven dyeing during processing of the fibric (Siu, 1951). Cotton textiles depending on type of finish, and temperature and humidity conditions are subjected to spoilage by a range of microorganisms. Some cause spoilage by merely growing on the textiles utilizing water soluble nutrients of the cotton and dressings such as starch; while other organisms attack the cellulose causing strength and weight losses of the fibric (Turner, 1967). Spoilage apart from visible microbia growth may produce musty odours and stains. A. terreus has been cited as one of the organisms known to cause diamond-spot stains and A roseus produces a pigment causing red stains - though stains are not always due to microbial pigments, but may be caused by interaction between microbial metabolites and finishing materials particularly dyes (Siu, 1951).

Aspergillus species are included amongst those fungi which grow and sporulate on wood surfaces producing green and black stains (Scheffar, 1973). A. ustus along with other fungal species comprised 92% of the isolates found colonizing pine wood stakes over a period of twelve weeks. A. niger and A. ustus being the prevelent species during the second and third week respectively of colonization (Merrill and French, 1966). The fungal isolates occured with equal frequency from heartwood and sapwood. Aspergillus species including A. niger

and A. fumigatus has been reported amongst the isolates from wood even in the presence of wood preservatives (kaarick, 1974). A. ochraceus and A. niger were reported amongst the causal organisms of soft rot of cooling tower chamber, the later species also being implicated as causing weight losses in beech and scot pine wood (Eaton, 1976).

Aspergillus species are involved in biodeterioration in the paper industry. A. niger and A. fumigatus are among the microbial isolates obtained from certain moist wood pulps and have been implicated in slime formation in paper processing (Hughes, 1968). A. flavus, A. terreus, A. versicolar and the A. glaucus are amongst the organisms which cause spoilage due to their growth on paper board - some of which grow at relatively low water activities. Aspergillus species are also among the organism reported to penetrate the paper and muslin wrappings of sterile hospital pack which apart from causing wasteful spoilage could also present a health hazard (Standard et al, 1973).

Biodeterioration problems arise at all stages during processing of leather, from the raw hides to the finished leather products (Orlita, 1968). Although vegetable tanning liquors contain a range of nutrients, the tannin inhibit-microbial growth. However initial tanning of hides is usually carried out in weaker suspender liquors which are readily infected by microorganisms. Aspergillus flavus and A. niger which have been grown in 10% tannic acid, are included with

those fungi which cause deterioration of tanning liquors by destroying the tannins (Turner, 1967). A ochraceus and A. wentii have been found to be responsible for causing damage to drying leather (Orlita, 1968). However the worst damage caused by Aspergillus species results from their growth especially in tropical conditions on finished leather products in particular those containing a rich carbohydrate supply - A. flavus, A. oryzae, A. fumigatus, A. glaucus, A. niger, A. ochraceus, A. repens, A. versicolar and A. wentii have been found to be involved (Orlita, 1968). Fungal growth on leather may cause discolouration - A. niger has been known to be responsible for producing grey spots on leather. Although fungal growths on leather is thought not to cause strength loss of the leather, it can result in chemical changes by hydrolysing oils and greese to produce fatty acids. Breakdown of oils and greese alters the physical properties of the leather by making it hard and less pliable.

Most paint biodeterioration problems are due to fungal growth causing visual spoilage and physical damage to water-thinned and solvent-thinned paint films (Ross et al, 1968). The biodeterioration of paint films depends not only on the paint constituents - which may be nutritive, antimicrobial or affect moisture absorption, but also on the type of substrate to which they are applied, and dirt accumulation on their surface (Ross, 1963; Ross et al, 1968). Aspergillus species including A. flavus and A. niger have been isolated from paint

films (O'neil and Skinner, 1966). A. niger utilizes linseed oil which has been used as constitutent of some solvent thinned paints (Ross, 1963).

Under certain environmental conditions natural rubber vulcanizates are susceptible to microbiological deterioration where as most synthetic rubber vulcanizates appear resistant under same conditions (Cundell and Mulcock, 1972). Although Aspergillus species have been reported to grow and cause staining in some instances on diluted latex, crepe, smoked sheet, other materials used in rubber formulations, and rubber products (Cundell and Mulcock, 1972; Zyska et al, 1972), it has been noted that the fungi utilize non-rubber constitutents of the rubber or are opportunists on dirt contamination of the rubber (Heap and Morrell, 1968). It was also reported that A. niger and A. fumigatus found growing on rubber were unable to decompose pure rubber hydrocarbon (Cundell and Mulcock, 1972).

The fungal deterioration of plastics may be due to two causes - disfigurement due to superficial microbial growth using the plastic as an attachment surface and utilising dirt as nutrient; or damage due to assimilatory and dissiminatory microbial action (Mills, 1974). Dissimilatory damage may be due to staining pigments produced by microorganisms actively growing on the plastic or its dirt contaminants. Aspergillus awamori and A. oryzae have been reported to cause stains (Kestel'man et al, 1972). Assimilation of plastic constitutents causes mechanical changes in the plastic apart

from facilitating disfiguring microbial growth. A. niger, A. flavus, A. versicolar, A. amsteldomi, A. oryzae and A. awamori either in mixed or pure culture have been grown or shown to have caused mechanical damage to various plastics (Kestel'man et al, 1972; Klausmeir, 1972; Lazar, 1975).

Although glass itself does not provide nutrient for fungal growth, the optical lenses in various instruments including cameras, binoculars, microscopes, gunsights and surveying instruments are often found to be optically impared due to fungal growth especially in humid conditions (Eggins and Allsopp, 1975). The fungi utilise dirt or leather wax or the vegetable pigments of black lacquer on other areas of the optical instruments in close proximity to the glass lenses, as nutrient sources to grow adventitiously on the glass lenses. Superficial fungal growth can easily be cleaned off; however if such growth is allowed to remain it can cause permanent damage by etching the glass surface due to corresive metabolic products. Among fungal species found growing in such situations are species of Aspergillus including A. fumigatus, A. versicola and the A. glaucus group.

Aspergillus species contaminating various preparations including talcs and ointments have been reported (Bowman et al, 1972). A. niger, A. tamarii and A. flavus are amongst microorganisms which cause spoilage of pharmaceutical emulsions by causing emulsion breakdown or production of acid, odours, unpleasant flavours or discolouration, apart from

utilising the oil or emulgents as nutrients and producing unsightly growth (Beveridge, 1975). A. niger has also been reported to attack a large variety of cosmetics and pharmaceutical products over a wide pH range and having a high resistance to antimicrobial agents (Tenebaum, 1973), though its response to inhibitory process has been shown to be affected by its growth temperature (McRobbie and Parker, 1973).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 ISOLATION AND IDENTIFICATION OF ASPERGILLUS NIGER STRAINS FROM THE ENVIRONMENT

3.1.1 Sample Collection

Various foods (yam, cassava, bread), and fruits (orange, pineapple, mango, pear, tomato) were purchased from various markets in Zaria. Soil samples were collected from the environment (under trees).

3.1.2 Isolation of Aspergillus niger strains

Black mouldy growth which are typical of A. niger on rotten food and fruits were transfered on to potato dextrose agar (PDA) plates. For fresh food and fruits, samples were incubated in a moist chamber at room temperature (25°C±1) until growth occured and the growths were transferred to fresh PDA plates. 10g each of the soil samples was added into separate medical bottle containing 90ml sterile distilled water and mixed by shaking vigorously. Serial dilution of the soil suspensions were prepared and 0.1ml aliquots of the dilutions plated on PDA plates. All the plates were incubated at room temperature for 24-48 hours. Typical colonies of A. niger were isolated from the plates and the presumptive isolates were transferred to PDA slants for storage and subsequent use.

3.1.3 Identification of Isolates

The presumptive isolates were identified following the guide given by Raper and Fennel (1965). Scrapings of the pure isolates were taken from the slants and teased on clean grease-free slides and stained with methylene blue solution. The slides were then covered with clean grease-free cover slips and examined under the microscope for the following characteristics.

(i) Mycelium: - Compact to fairly loose, white to faintly yellow basal mycelium

(ii) Conidal head: Typically large and black to deep brownish-black and globose at first then radiate.

(iii) Conidiophore: Smooth, comparatively thick or in brownish shades

(iv) Vesicle: small and globose

(v) Sterigmata: In two series - primary and secondary

(vi) Conidia: Typically globose at maturity, brownish with walls comparatively heavy and irregularly roughened or showing low to fairly prominent ridges and discontinous echinulations.

3.2 SCREENING OF A. NIGER STRAINS FOR AMYLASE PRODUCTION

The method of Hankin and Anagnostakis (1975) was used. the ability to degrade starch was used as the criterion for

determination of ability to produce amylolytic enzymes. The medium used was Nutrient agar containing 0.2% soluble starch, pH 6. Serial dilutions of spores of each strain was made using sterile distilled water and 0.1ml aliquorts of the appropriate dilutions were plated on separate plates. After 3 days of incubation the plates were flooded with an iodine solution (KI,30g; I_2,3g; distilled water 1 litre) and a yellow zone around a colony in an otherwise blue background indicated amylolytic activity. The yellow zones around 5 colonies in each plate were measured and their averages determined. Two strains CA-3 and OR-5 showing the widest zones were selected for further studies).

3.3 CULTIVATION OF THE SELECTED STRAINS FOR AMYLASE PRODUCTION

The two selected strains CA-3 and OR-5 were grown in submerged culture in shake flask for amylase production using either soluble starch (control), corn bran ('Dusa'), yam peel or banaba peel as the substrate.

3.3.1 Preparation of test substrates

Freshly harvested yam tubers (*Discorea retundata*) and fresh bananas (*Musa paradiciaca*) were thoroughly washed with tap water and peels were carefully removed without cutting into pulp. After washing with distilled water, the peels were dried in an oven at 80°C for 48 hours and then ground into fine powder. The powder was dried to constant weight in an

oven at 60°C. Corn bran ('Dusa') obtained from clean and wholesome grains was treated in a similar manner. The corn bran, yam and banana peels powder were stored in a dessicator until required.

3.3.2 Culture Method and culture Filterate Preparation

Spores of 48 hours old cultures of A. niger CA-3 and OR-5 were harvested by washing the slants with 10ml of sterile distilled water. An aliquort of 1.0ml of spores suspension was used to inoculate 200ml of growth medium containing $NaNo_3$ (3.0g); K_2HPO_4 (1.0g); $MgSO_4$ (0.5g); Kcl (0.5g); $Feso_4$ (0.01g) and 20g of either soluble starch, corn bran ('Dusa'), yam peel, or banana peel powder per litre. The culutres were incubated at 29.0±1°C on rotary shaker (Lab-line instrument Inc. U.S.A) at 80 rpm. At intervals of 24h, 20ml was taken aseptically from the shake cultures and suction filtered through dried No.1 whatman filter paper to obtain the culture filterate. The mycelia were dried to constant weight and the weight recorded as a measure of growth.

3.3.3 Determination of amylase activity

Amylase activity was measured using the method employed by Adams and Deploey (1976). One ml of the culture filterate was incubated with 1.0ml of a 1% soluble starch (M&B) solution containing M/150 Nacl and M/150 phosphate buffer pH 6.9 at 40°C for three minutes. At the end of the incubation period

the reaction was stopped by addition of 2.0ml of a solution containing 1.0g of 3,5-Dinitrosalicylicacid, 20ml of 2N NaoH and 30g of Rochelle salt per 100ml of distilled water. The mixture was heated for five minutes in a boiling water bath after which 20ml of distilled water were added and the absorbance at 540nm measured against a reagent blank in a WPAS101 spectrophotometer (Linton Cambridge, U.K). The amount of reducing sugars released by amylase action on starch was derived from a standard curve previously calibrated with glucose as standard. An amylase activity unit was defined as the amount of enzyme catalysing the formation of 10µg of glucose per three minutes at 40°C.

3.3.4 Determination of Residual Starch

Residual starch was determined using the method employed by Federico (1984). An aliquort of 0.2ml of each of the samples, prepared by mixing 1ml of culture filterate with 0.6ml of 0.1M phosphate buffer (pH 7.2), 2ml of 0.5M Nacl, 0.4ml 1M Hcl and 0.2ml H_2O, was added to 1ml 1M Hcl, 0.5ml iodine reagent and distilled water to make the volume 100ml. The absorbance was read at 620nm on a WPA-S101 spectrophotometer (Linton Cambridge, U.K.). The concentration of the residual starch was determined from a standard curve previously calibrated using standard starch concentrations.

3.4 pH DETERMINATION

The pH of the shake cultures was determined using a pH meter (PYE UNICAM MODEL 294 MK 4).

3.5 EFFECTS OF DIFFERENT CONCENTRATIONS OF YAM PEEL ON GROWTH AND AMYLASE PRODUCTION BY A. NIGER CA-3

Different concentrations of yam peel powder ranging from 0.5 to 4% were used in the fermentation medium to determine their effect on growth and amylase production by A niger CA-3.

3.6 EFFECTS OF pH ON GROWTH AND AMYLASE PRODUCTION BY A NIGER CA-3 GROWN ON YAM PEEL

Buffered basal medium containing yam peel powder were used to determined the effect of different pH values on growth and amylase production by A. niger CA-3.

3.7 EFFECTS OF TEMPERATURE ON GROWTH AND AMYLASE PRODUCTION BY A. NIGER CA-3 GROWN ON YAM PEEL

Cultures of A. niger CA-3 were incubated at 20, 25, 30, 35, 40 and 45°C to determine their effect on growth and amylase production.

CHAPTER FOUR

4.0 RESULTS

Aspergillus niger strains identified by methods and procedures employed in this study were isolated from all the samples analysed viz: yam, cassava, bread, orange, mango, pineapple, pear, tomato and soils. Five strains of A. niger were obtained from yam samples. Cassava, mango and pineapple samples yielded four strains each. Bread, tomato and orange yielded one, five and six strains respectively. Pear and soil samples yielded eight strains. In all, forty five strains of A. niger were isolated (Table 1).

4.1 SCREENING OF ISOLATES FOR AMYLASE PRODUCTION

The forty five strains isolated were screened for amylolytic activity. Table 2 shows the result of this experiment. The amylolytic capability of the isolates varied widely as shown by the means (averages) of the sizes of the yellow zones around the colonies of the strains. Two strains of Aspergillus niger CA-3 and OR-5 were considered to be the best amylase producers: the means (averages) of the size of starch hydrolysis produced by each were 16.6mm and 16.8mm respectively.

4.2 PRODUCTION OF AMYLASE BY ASPERGILLUS NIGER CA-3 AND OR-5 IN SHAKE FLASKS CONTAINING THE DIFFERENT SUBSTRATES VIZ: SOLUBLE STARCH, CORNBRAN (`DUSA') YAM, AND BANANA PEELS

4.2.1 Soluble Starch

Figure 1, shows the effect of soluble starch on growth and amylase production by Aspergillus niger CA-3. The organism grews rapidly up to 24 hours and then less rapidly up to 120 hours. Subsequently the growth of the organism declined up 168 hours. Amylase production by this strain followed a similar pattern with the maximum level of amylase (24 units/ml) being produced at 48 hours and subsequently declined. Starch concentration in the culture medium declined with the increase in growth and amylase activity and was lowest (0.7%) at 120 hours. Subsequently it remained relatively constant up to 168 hours. The pH value of the culture medium decreased up to 96 hours after which it increased up to 120 hours and then declined.

Figure 2, shows the effect of soluble starch on growth and amylase production by A. niger OR-5. The organism under went rapid growth up to 24 hours. Growth slighly increased at 48 hours, then decrease at 72 hours and then increased at 120 hours. Subsequently growth decreased at 144 hours and remained stationary up to 168 hours. Amylase production rose steadily at 96 hours and then remained at constant level (36 units/ml) up to 144 hours and then declined. Starch concentration in the culture medium declined with increase in growth and amylase activity and was lowest (1.2%) at 144 hours. Subsequently the

starch concentration remained constant up to 168 hours. The pH value of the culture medium slightly increased at 24 hours and then decreased at 48 hours. The pH increased again at 96 hours and then declined at 120 hours. Subsequently the pH remained fairly constant up to 168 hours.

TABLE 1: OCCURENCE OF ASPERGILLUS NIGER IN THE SAMPLES ANALYSED

Source of Samples	No. of Samples analysed	No(%) of Samples yielding *A. niger*
Yam	15	5(33)
Cassava	20	4(20)
Bread	4	1(25)
Orange	20	6(30)
Mango	16	4(25)
Pineapple	8	4(50)
Pear	18	8(44)
Tomato	25	5(20)
Soils	10	8(80)
Total	136	45(33)

TABLE 2: SCREENING OF ASPERGILLUS NIGER STRAINS FOR AMYLASE PRODUCTION

Strain Code	Mean(mm) yellow zones Around five colonies	Strain Code	Mean(mm)yellow zones around five colonies
BR1	4.2	S2.1	2.6
CA1	2.8	S3	2.6
CA2	10.6	S4	14.8
CA3	16.6	S5	2.8
CA4	12.8	S6.1	2.8
MG1	2.4	S8	5.4
MG2	5.6	TM1	5.8
MG3	2.2	TM2	3.4
MG4	2.0	TM3	6.4
OR1	11.0	TM4	12.4
OR2	5.4	TM6	3.4
OR3	3.6	YM1	11.2
OR4	2.4	YM2	3.8
OR5	16.8	YM3	5.0
OR6	2.6	YM4	8.2
PA1	13.2	YM5	5.4
PA3	8.6		
PA5	6.2		
PA6	10.0		
PR1	9.0		
PR2	2.4		
PR3	7.4		
PR4.1	6.8		
PR4.2	7.2		
PR5	2.8		
PR6	5.8		
PR8	4.2		
S1.1	9.8		
S1.2	3.6		

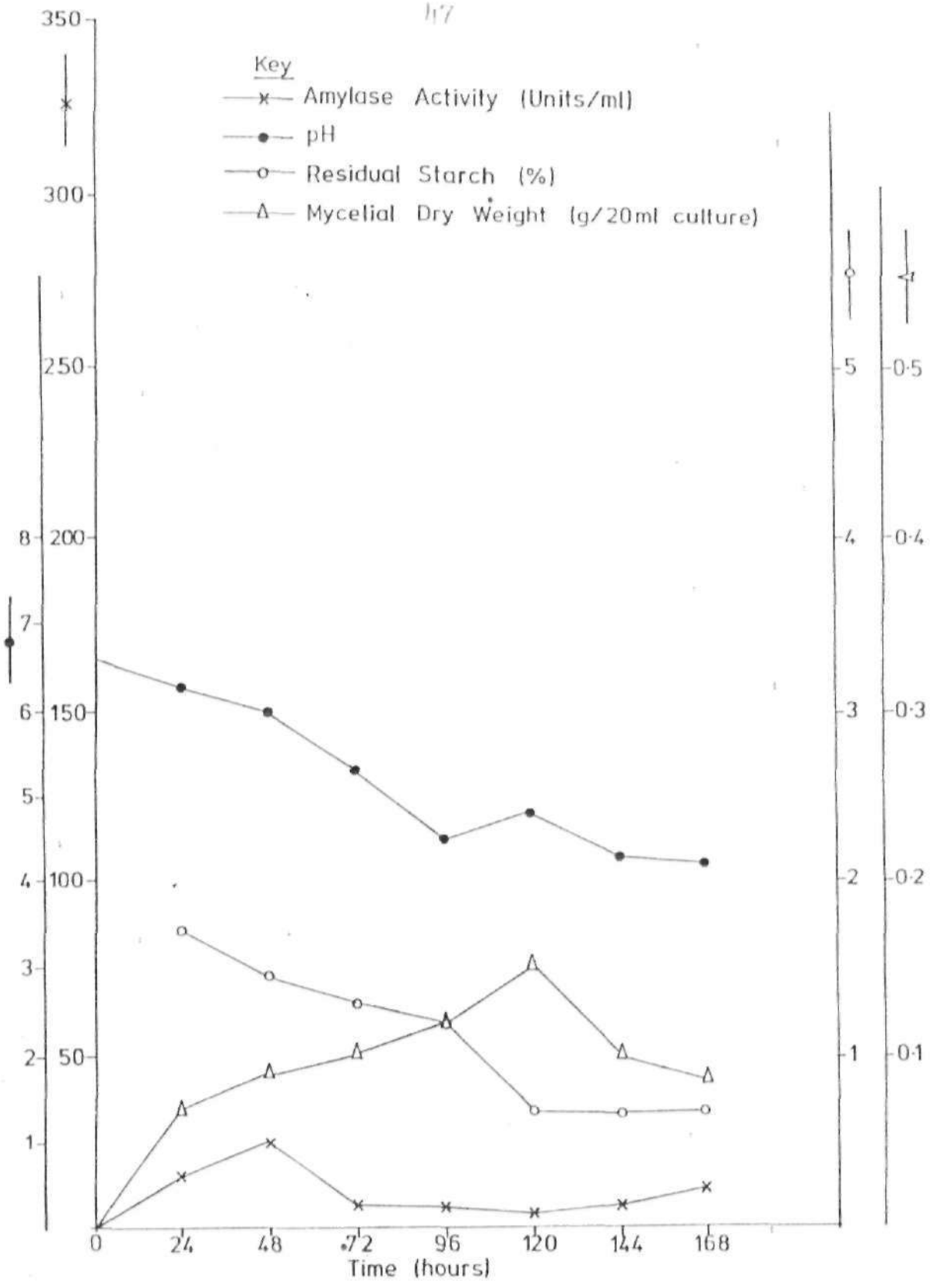


FIG. 1 EFFECT OF SOLUBLE STARCH ON GROWTH AND AMYLASE PRODUCTION BY ASPEGILLUS NIGER CA-3

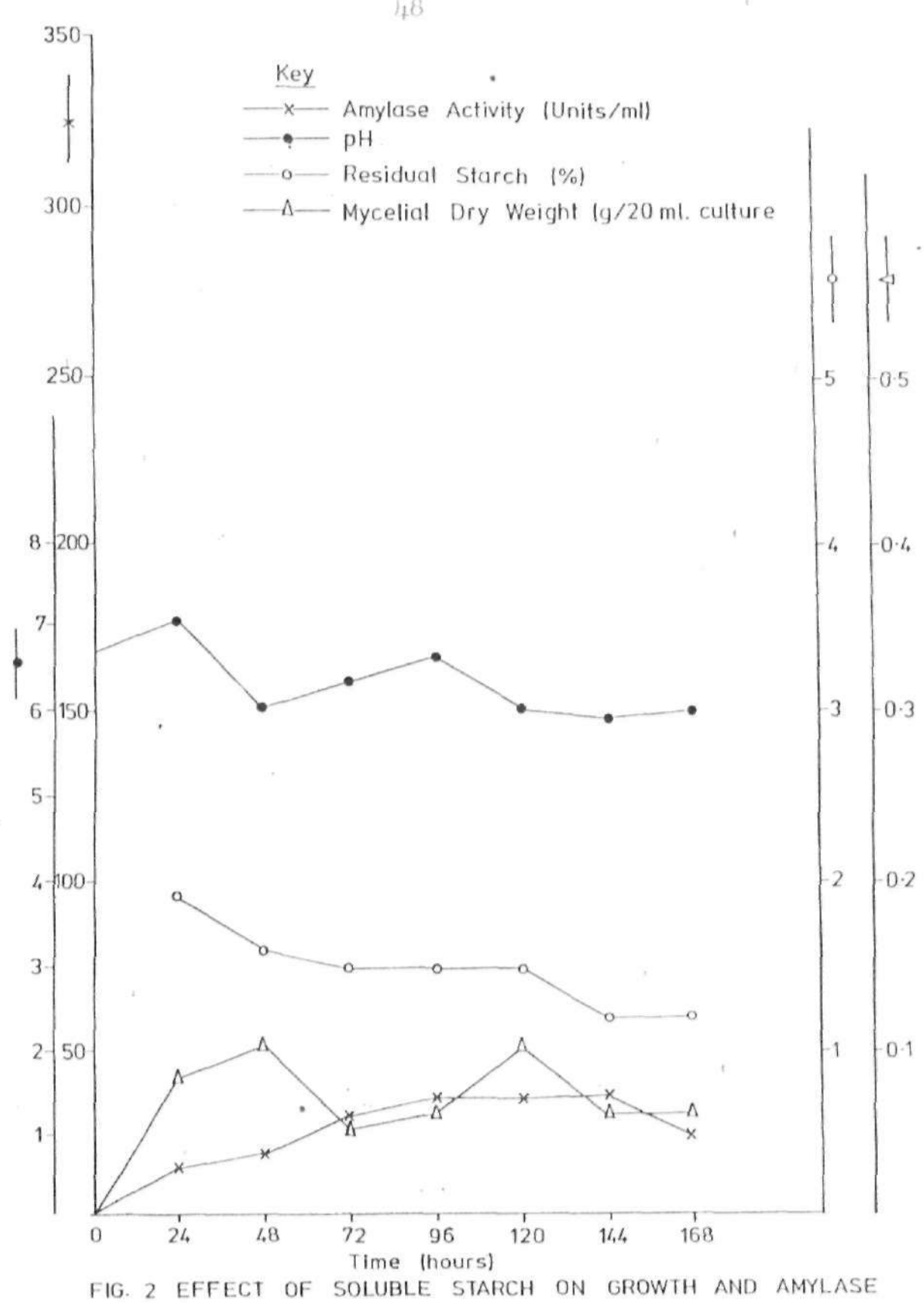


FIG. 2 EFFECT OF SOLUBLE STARCH ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER OR—5

4.2.2 Corn bran ('Dusa')

Figure 3, shows the effect of corn bran ('Dusa') on growth and amylase production by Aspergillus niger CA-3. The organism grew rapidly at 24 hours and then remained constant up to 48 hours. Subsequently growth increased at 96 hours and then decreased at 120 hours. The growth slighly increased again at 144 hours and then declined up to the end of the experiment at 168 hours. Amylase activity appeared to follow a similar pattern with the maximum level of amylase (27 units/ml) being produced at 72 hours. Amylase production then declined at 96 hours and then remained fairly constant up to 168 hours. The residual starch concentration in the growth medium decreased with increase in growth and amylase activity and was lowest (0.4%) at 144 hours. The starch concentration then remained constant up to 168 hours. The pH value of the growth medium declined up to 96 hours and thereafter increased up to 168 hours.

Figure 4, shows the effect of corn bran ('Dusa') on growth and amylase production by A. niger OR-5. The growth of the organism increased rapidly at 24 hours and then gradually at 72 hours. Subsequently the growth declined at 144 hours and remained constant up to 168 hours. The curve of amylase activity followed a similar pattern with that of growth. The maximum level of amylase (66 units/ml) was produced at 72 hours, it then declined at 144 hours and remained constant up to 168 hours. The residual starch concentration in the culture

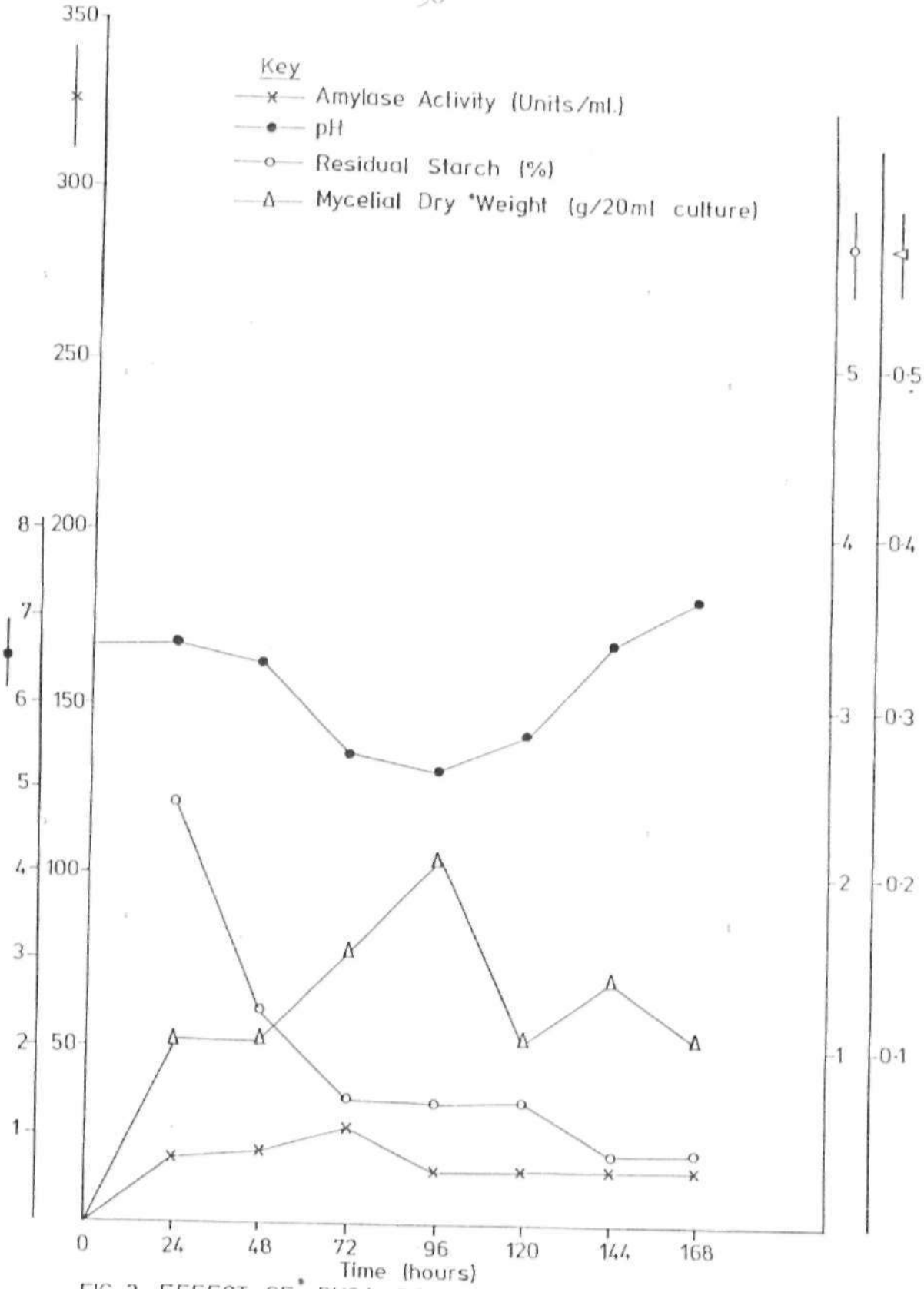


FIG. 3 EFFECT OF DUSA POWDER ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA–3

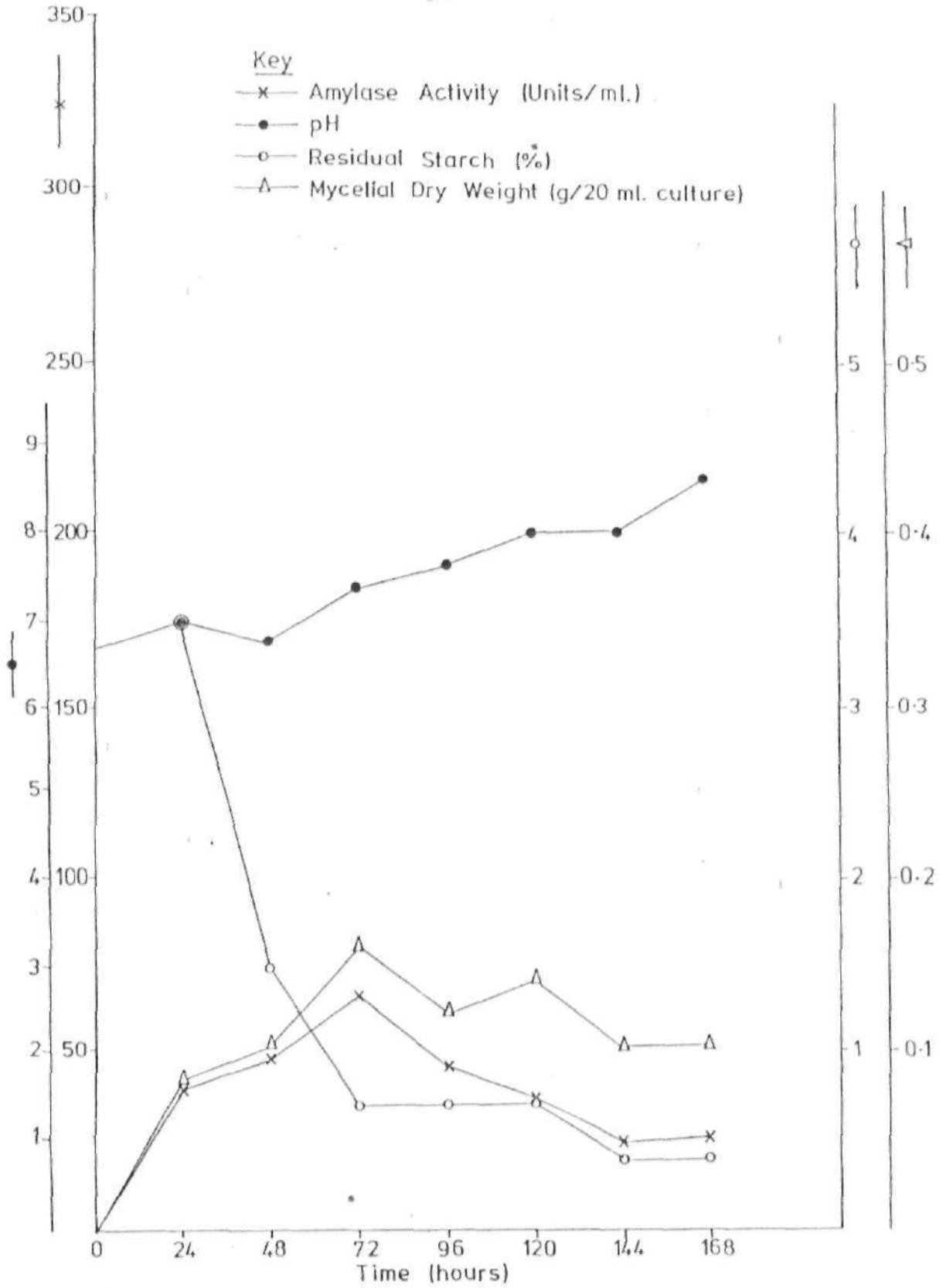


FIG. 4 EFFECT OF DUSA POWDER ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER OR-5

medium decreased with increase in growth and amylase activity and was lowest (0.4%) at 144 hours. Subsequently the starch concentration remained constant up to 168 hours. The pH value of the culture medium slightly increased at 24 hours and then decreased at 48 hours. The pH value then gradually increased up to the end of the experiment at 168 hours.

4.2.3 Yam Peel

Figure 5, shows the effect of yam peel on growth and amylase production by Aspergillus niger CA-3. The growth of the organism increased at 72 hours and then decreased gradually up to the end of the experiment at 168 hours. The curve of amylase activity in the culture medium followed a similar pattern with the growth curve. Amylase production was rapid during the logarithmic phase of growth with maximum level of amylase (297 units/ml) being produced at 72 hours and subsequently declined up to 168 hours. Residual starch concentration in the culture medium decreased with increase in growth and amylase activity, and was lowest (0.4%) at 120 hours. Subsequently, the starch concentration remained constant up to 168 hours. The pH value of the culture medium slightly increased at 24 hours and then decreased. Subsequently, the pH value increased again at 72 hours after which there was a sharp drop which coincided with the rapid production of amylase.

Figure 6, shows the effect of yam peel on growth and amylase production by A. niger OR-5. The organism under went

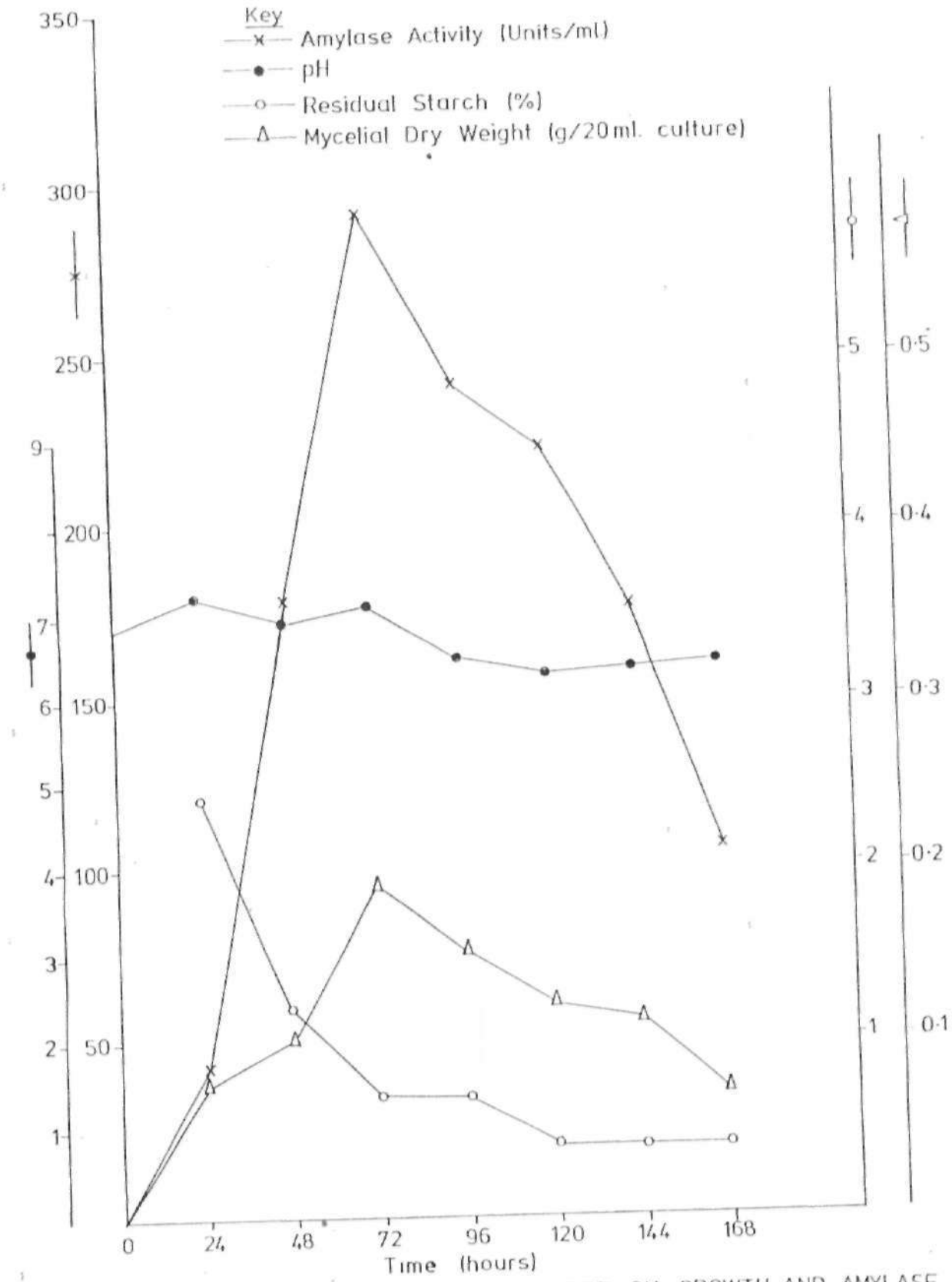


FIG. 5 EFFECT OF YAM PEEL POWDER ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA–3

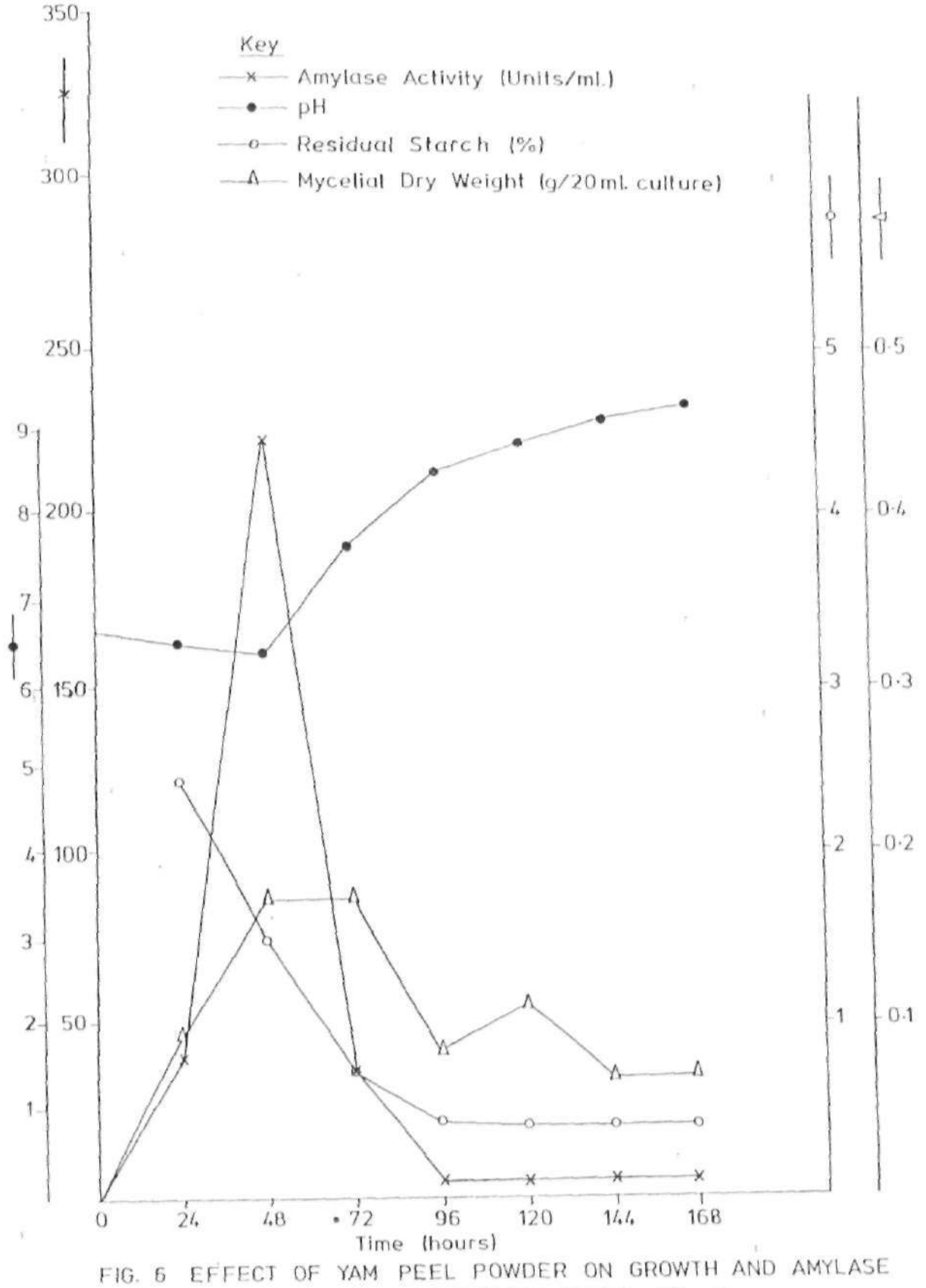


FIG. 6 EFFECT OF YAM PEEL POWDER ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER OR – 5

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a rapid growth at 48 hours after which the growth remained stationary up to 72 hours. Subsequently the growth declined at 144 hours and remained faily constant up to 168 hours. Amylase activity curve also followed a similar pattern with the maximum level of amylase (222 units/ml) being produced at 48 hours. Subsequently amylase production dropped rapidly at 96 hours and remained constant up to 168 hours. The residual starch concentration in the culture medium declined with the increase in growth and amylase activity and was lowest (0.4%) at 96 hours. Subsequently, the starch concentration remained constant up to 168 hours. The pH value of the culutre medium slightly decreased at 48 hours and then increased up to 168 hours.

4.2.4 Banana Peel

Figure 7, shows the effect of Banana peel on growth and amylase production by Aspergillus niger CA-3. The growth of the organism increased at 120 hours and then decreased at 144 hours. Subsequently the growth remained constant up to 168 hours. Amylase activity rose steadly reaching a maximum (150 units/ml) at 48 hours. Subsequently amylase production declined up to the end of the experiment at 168 hours. Residual starch concentration in the culture medium decreased with increase in growth and amylase activity and was lowest (0.4%) at 120 hours. The starch concentration then remained constant up to 168 hours. The pH value of the culture medium slightly decreased at 24 hours and then increased at 48 hours.

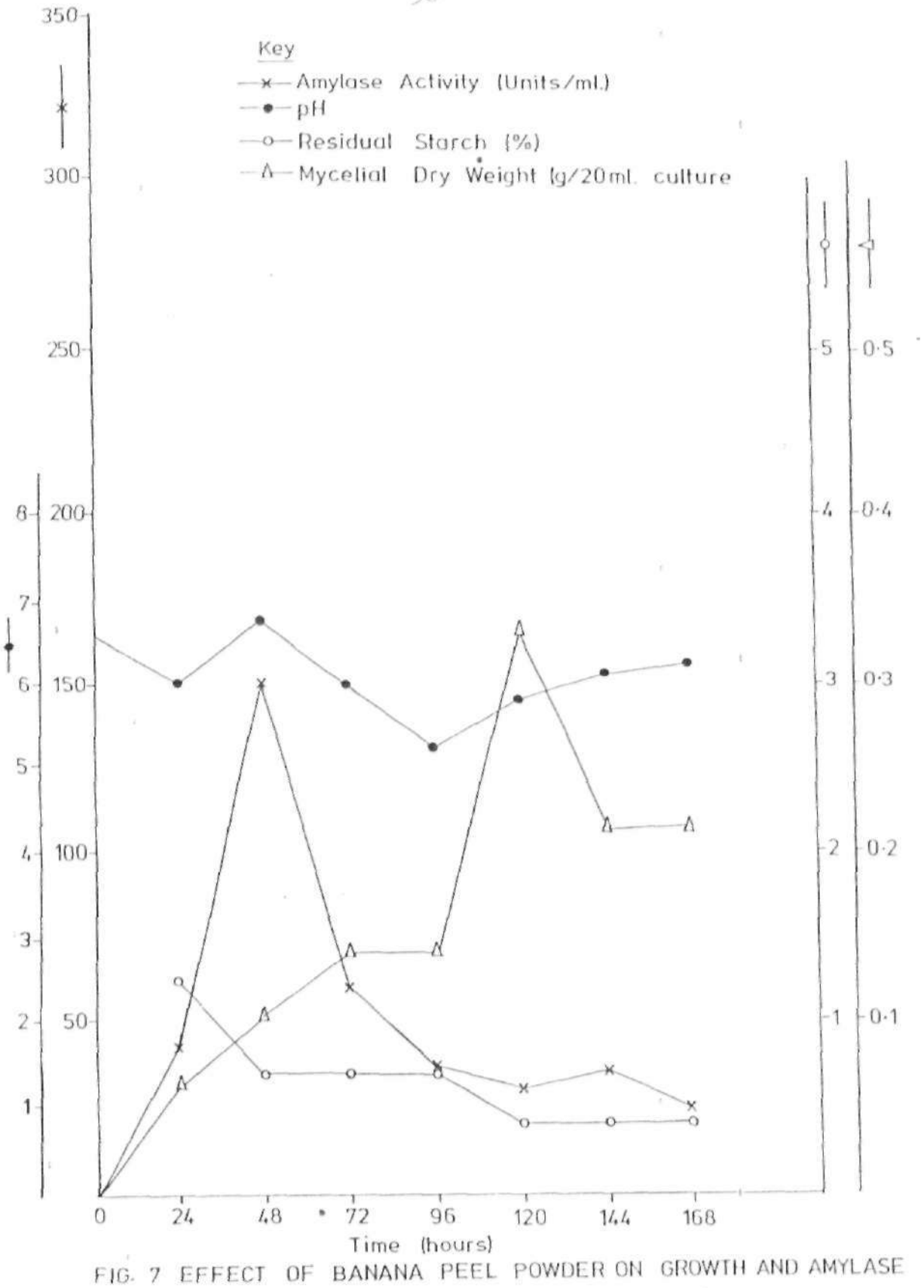


FIG. 7 EFFECT OF BANANA PEEL POWDER ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA-3

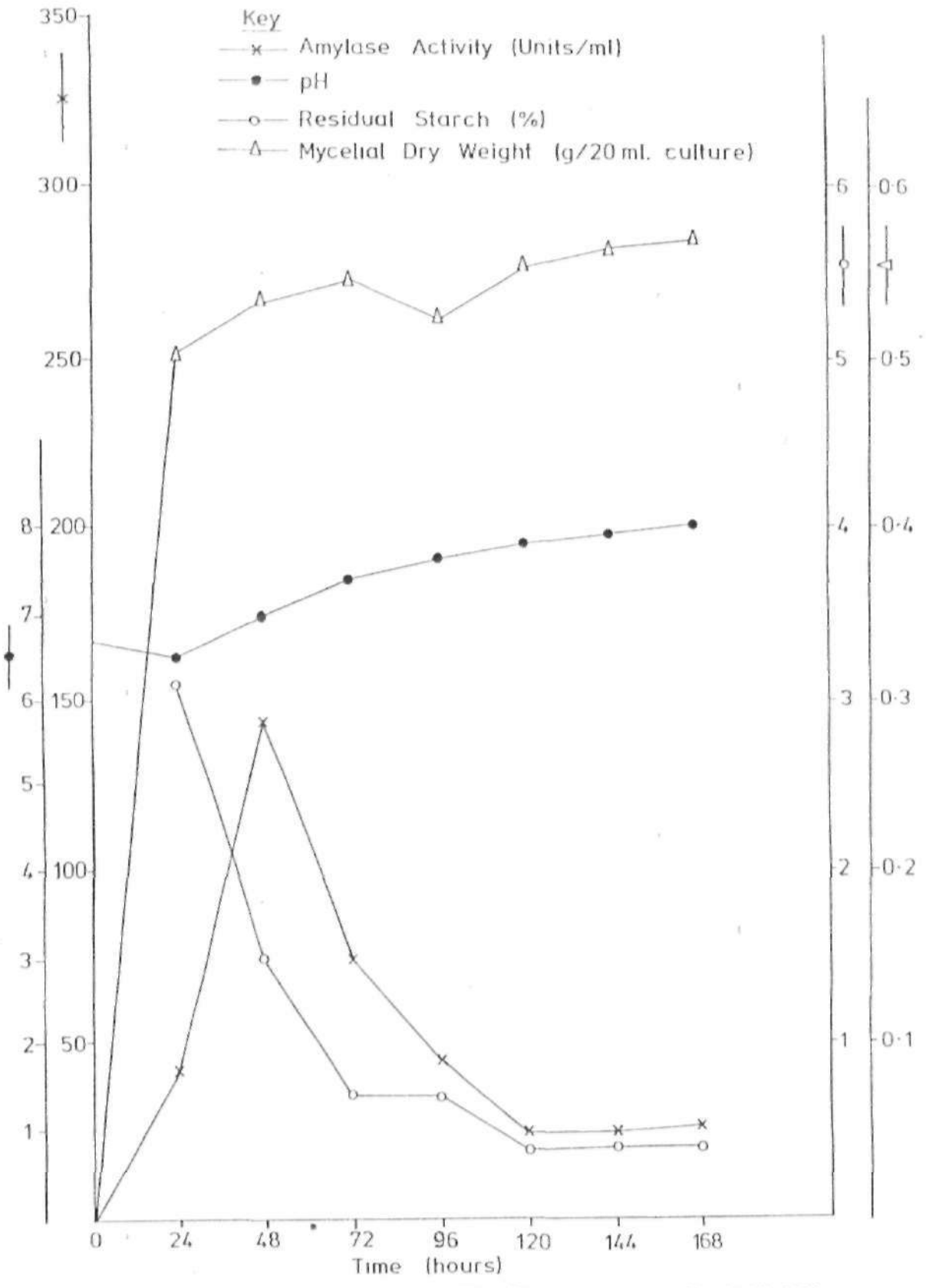


FIG. 8 EFFECT OF BANANA PEEL ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER OR – 5

Subsequently, the pH value declined again at 96 hours and then increased up to 168 hours.

Figure 8, shows the effect of Banana peel on growth and amylase production by A. niger OR-5. The organism grew rapidly at 24 hours and then gradually at 72 hours. Subsequently, the growth declined at 96 hours and then increased up to 168 hours. Amylase activity increased steadly reaching a maximum (144 units/ml) at 48 hours. Amylase production then declined at 120 hours, and subsequently remained fairly constant up to 168 hours. Residual starch concentration in the culture medium decreased with increase in growth and amylase activity, and was lowest (0.4%) at 120 hours. The starch concentration then remained constant up to 168 hours. The pH value of the culture medium slightly decreased at 24 hours, and then gradually increased up to 168 hours.

4.3 EFFECTS OF ENVIRONMENTAL FACTORS ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA-3 GROWN ON YAM PEEL POWDER

4.3.1 Effects of Yam peel concentration on growth and amylase production by A. niger CA-3

Table 3, shows the effects of different concentrations of yam peel powder (0.5% to 4%) in the growth medium on amylase production by Aspergillus niger CA-3. The inoculated flasks were incubated for 72 hours which was shown previously to be the optimum period for amylase production by this strain. The amylase activity appeared to increase with increase of yam peel concentration in the medium up to 3.5%.

TABLE 3: EFFECTS OF YAM PEEL POWDER CONCENTRATIONS ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA-3

Concentration (%)	Amylase Activity (unit/ml)	Mycelialdry weight (g/50ml culture)	Final pH
0.5	25	0.14	5.8
1.0	75	0.18	6.0
1.5	156	0.29	6.2
2.0	295	0.32	6.4
2.5	324	0.35	7.0
3.0	340	0.5	6.8
3.5	365	0.65	6.0
4.0	342	0.60	6.0

Initial pH = 6.8

Mycelial dry weight and final pH of the growth medium increased or decreased consistently with the increase in yam peel concentration in the growth medium (Table 3) above 3.5% yam peel concentration amylase activity declined.

4.3.2 Effects of different pH values on growth and amylase production by A. niger CA-3

Table 4, shows the effects of different pH values on amylase production by A. niger CA-3. The optimum yam peel concentration (3.5%) determined from the previous experiment was used as the concentration of yam peel powder in the growth medium. The pH values of culture media in different flasks were adjusted between 3 and 8 prior to inoculation of A niger CA-3. the organism produced the highest level of amylase (295 units/ml) when the initial pH was 7.0. The final pH value of the growth medium and growth (mycelial dry weight) increased as the initial pH value increased. The corresponding final pH of 7.2 appeared to be most favourable for the maintenance of highest level of growth. At an initial pH of 8.0, conditions in the culture medium seems to be less favourable for amylase production than at pH 7.0.

4.3.3 Effects of Varying Temperature on growth and amylase production by A. niger CA-3

Table 5, shows the effects of varying temperatures on amylase production by A niger CA-3. Relatively high yields of the enzyme were obtained from the organism at temperatures

ranging from 25 to 30°C. Above this range of temperature, amylase activity declined. The pattern of the enzyme activities at the various temperatures appeared to resemble that of fungal growth (mycelial dry weight).

TABLE 4: EFFECTS OF DIFFERENT pH VALUES ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA-3 GROWN ON YAM PEEL POWDER

Intial pH	Final pH	Amylase Activity (units/ml)	Mycelial dry weight (g/50ml culture)
3	5.2	40	0.35
4	6.1	70	0.42
5	6.5	128	0.45
6	7.1	228	0.48
7	7.2	295	0.55
8	6.9	210	0.44

TABLE 5: EFFECTS VARYING TEMPERATURES ON GROWTH AND AMYLASE PRODUCTION BY ASPERGILLUS NIGER CA-3 GROWN ON YAM PEEL POWDER

Temperature (°C)	Amylase Activity (units/ml)	Mycelial dry weight (g/50ml culture)	Final pH
20	52	0.36	6.6
25	140	0.45	6.4
30	288	0.52	6.3
35	165	0.46	5.6
40	120	0.40	5.4
45	45	0.32	5.7

Initial pH = 6.8

CHAPTER FIVE

DISCUSSION

All the sources sampled for isolation of Aspergillus. niger yielded one or more isolates. This shows that the organism is ubiquitious in nature. A total of forty five isolates were obtained from all the samples analysed.

Out of the forty five isolates of A.niger, CA-3 and OR-5 were selected for further studies since they appear to be the most active amylase producers. However, it was noted that amylase producing potentials of A. niger strains could vary considerably. This was shown by the relative wide range (2.0mm to 16.8mm) of means (averages) of the zones of amylase activity observed around colonies of the forty five isolates when their respective starch agar plates were flooded with iodine (Table 2).

The two selected strains of A. niger CA-3 and OR-5) produced extracellular amylase in basal medium containing either 2% (w/v) soluble starch, corn bran (Dusa), yam peel or Banana peel powder as the sole carbon source. In most of the cultures the highest amylase activity was obtined during the late stationary phase of growth (Fig.1 to 8). The production of high amounts of the enzyme during stationary phase may be in response to depletion of carbon source. Induction of enzyme in response to limiting substrate in growth medium have been demonstrated in some organisms (Friedrick, 1982). On soluble starch, the highest amylase activity for A. niger CA-3 was 24

units/ml, while it was 27, 297 and 150 units/ml when grown on 'Dusa' (Cornbran), yam and Banana peels powder respectively (Fig. 1,3,5 and 7). Similarly, the highest amylase activities in culture filtrates of A. niger OR-5 grown on soluble starch, 'Dusa' (Cornbran), yam and Banana peels powder were 36, 66, 222 and 144 units/ml respectively (Fig. 2,4,6 and 8). Therefore the highest amylase activites for both strains were obtained when grown on yam peel powder. This agrees with similar findings of Sani et al, (1992) who reported that A. niger and A. flavus gave highest amylase activities when grown on cassava peel powder which is also from a root tuber. Recently Achi and Njoku-obi (1992) reported that Bacillus alvei gave higher amylase activity when grown on yam starch than on soluble starch. It can also be seen in this study that 'Dusa' (cornbrain) and Banana peel induce higher amylase activities than soluble starch.

The ability of yam peel, banana peel and 'Dusa' to cause increased accumulation of extracellular amylase in the culture medium may be due to the presence of other compounds, such as non-starch carbohydrates, proteins, aminoacids and other compounds. This is in contrast to commercial soluble starch which contains only starch (Sani et al, 1992). Further more Saito and Yamamato (1975) have speculated that soluble starch which was used as the carbon source in their standard medium may not be a good inducer in nature because the starch molucule is too large to enter the cell. It has also been

shown that higher yields of amylase can be obtained on media based on naturally occuring materials such as maize, wheat and barley, rather than artificially defined media (Burbidge and Collier, 1968; Nyiri, 1971). Achi and Njoku-obi (1992) obtained higher yields of amylase by Bacillus alvei using sorghum, rice, corn, yam, cassava and potato starch. Hang and Woodams (1977) obtained high level production of α-amylase and amyloglucosidase by Aspergillus foetidus using baked bean waste as carbon source. Therefore the ability of yam peel; Banana peel and 'Dusa' to support high amylase production as other materials reported may be because they are also naturally occurring materials.

The optimum concentration of yam peel powder needed for amylase production by Aspergillus niger CA-3 was 3.5%. This concentration is in agreement with other studies. Saito and yamamoto (1975) stated that the commercial production of amylase is accompanied with high concentration of starch. It was noted that the yield of amylase increased consistently with increase in initial yam peel concentration until the optimum yam peel concentration was reached. Beyond the optimum concentration there was a decline in the level of amylase produced by the organism. It was also observed that the growth of the organism was also high when the optimum yam peel concentration was used in the growth medium. This can be due to the fact that the high enzyme level lead to the production from yam peel sufficiently high levels of metabolisable sugars

which sustained more viable cells.

The effects of different pH values on amylase production by A. niger CA-3 were different as it was the case for yam peel concentrations. The organism produced the highest yield of amylase at pH 7.0 (Table 4) contrary to the report of Manning and Campbell (1961) that optimum pH values for amylase generally occur between pH 4.8 and 6.5, amylase production by A. niger CA-3 in this study was low at pH less than 6.0 compared to what it was at pH 6.0 to 7.0 (Table 4). However, amylase enzymes when supplied with full compliments of calcium ions are usually stable to extremes of pH values (Fogarty and Kelly, 1980). The initial optimum pH seems to favour enzyme production through its influence on the final pH and on the growth of the organism.

The optimum temperature for growth and amylase production by A. niger CA-3 when grown on yam peel powder was 30°C (Table 5). The final pH in the growth medium of the organism at this optimal temperature supported good growth of the organism. In general, amylase production by A. niger CA-3 is dependent on carbon source and its concentration, prevailing pH and temperature of the medium.

In conclusion, it has been shown in this study that yam peel, banana peel and 'Dusa' (Cornbran) are better carbon substrates than commercial soluble starch for amylase production by the two strains of Aspergillus niger investigated. Therefore these waste materials may serve as

economic carbon substrates for the production of amylase for industrial use.

Further studies could be carried out to determine the type of amylase produced using the most inducing local substrate and whether the amylase can be used to hydrolyse yam starch and other starchy materials for subsequent fermentation by yeast for the production of commercially desirable products.

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APPENDIX A

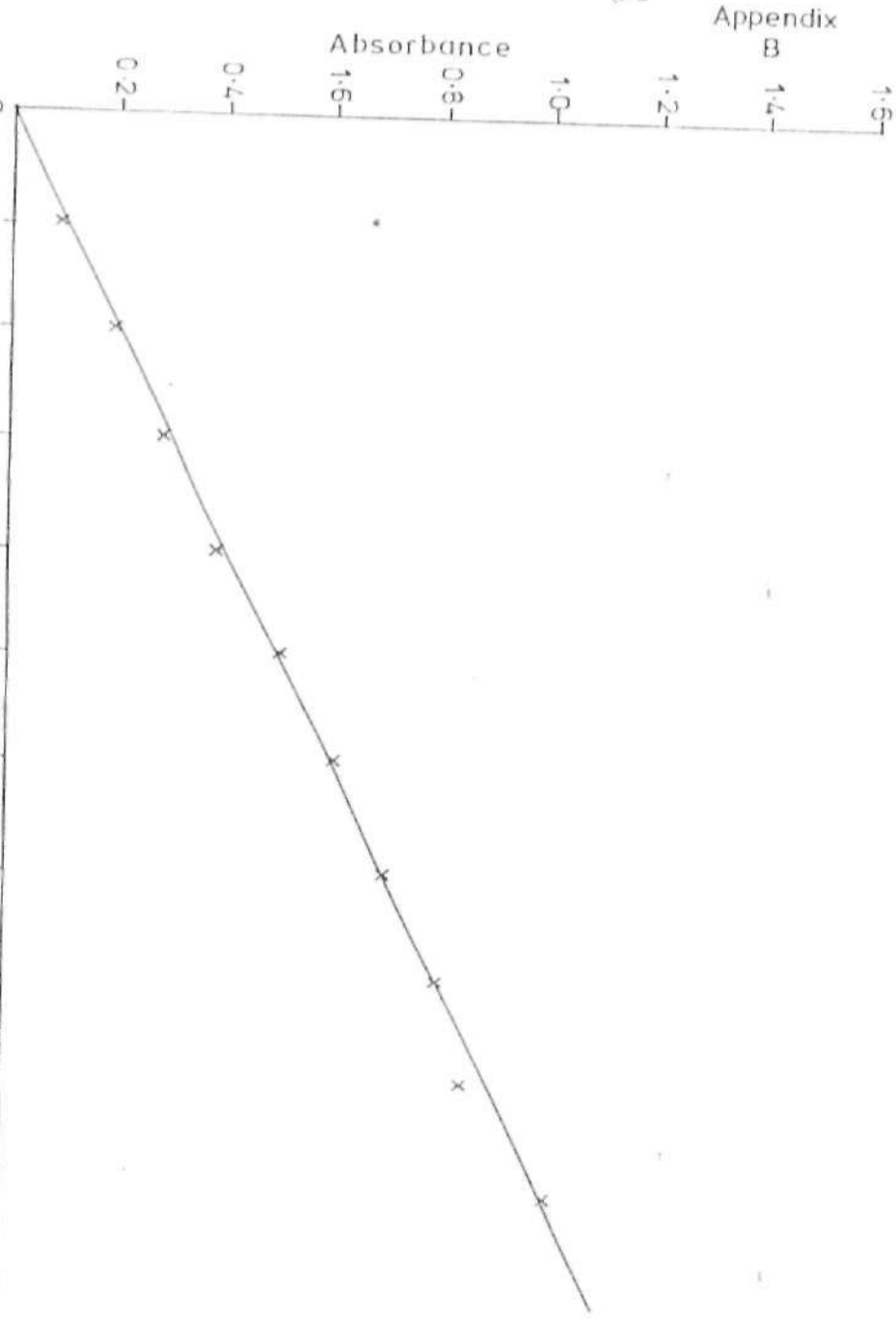
SCREENING OF ASPERGILLUS NIGER STRAINS FOR AMYLASE PRODUCTION

Yellow Zones (mm) around five colonies

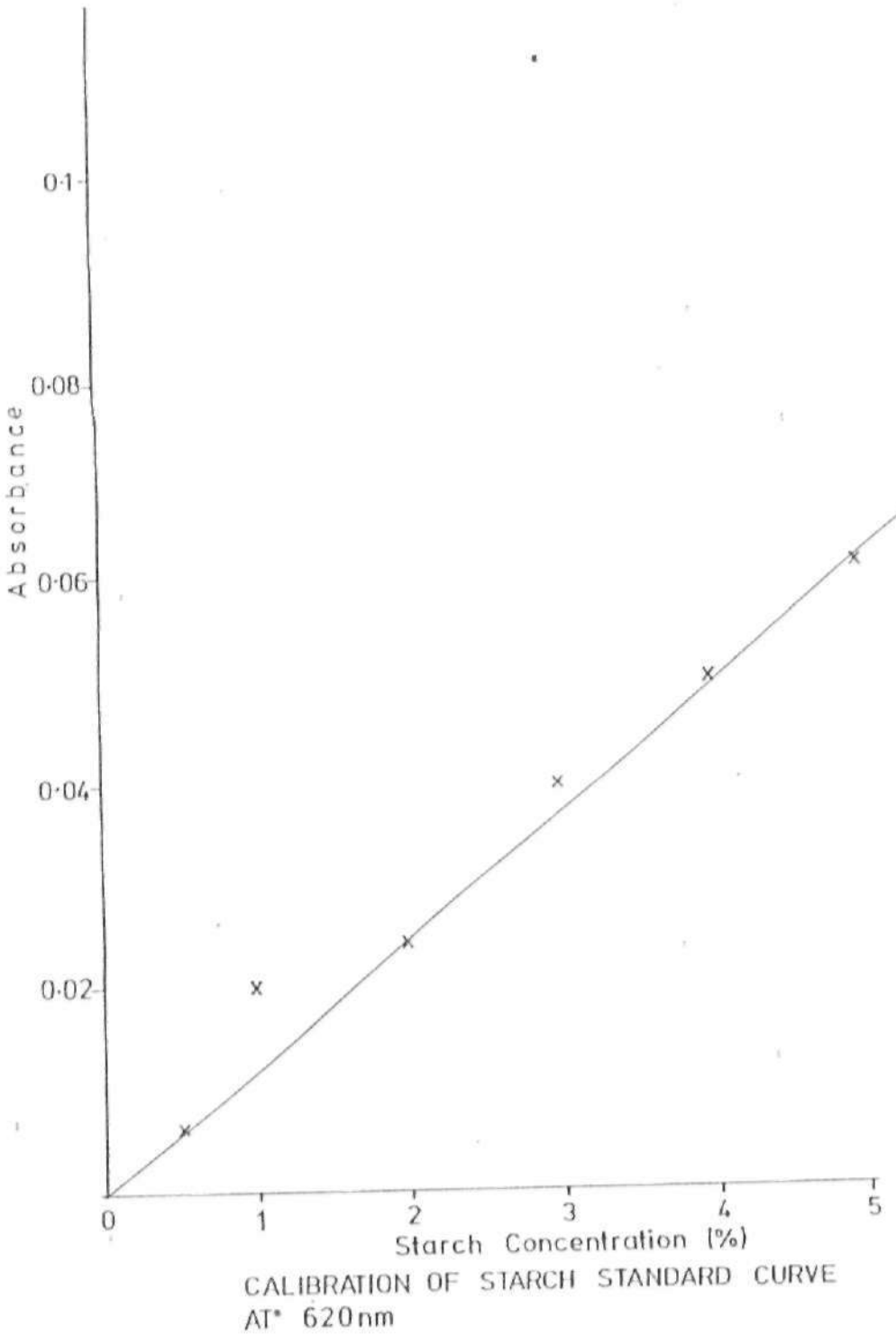
Strain Code	1	2	3	4	5	Mean(mm)
BR1	5.0	3.0	3.0	5.0	5.0	4.2
CA1	3.0	3.0	2.0	2.0	4.0	2.8
CA2	10.0	10.0	11.0	11.0	11.0	10.6
CA3	16.0	17.0	17.0	16.0	17.0	16.6
CA4	15.0	11.0	12.0	13.0	13.0	12.8
MG1	2.0	2.0	2.0	3.0	3.0	2.4
MG2	6.0	5.0	5.0	6.0	6.0	5.6
MG3	3.0	2.0	2.0	2.0	2.0	2.2
MG4	2.0	2.0	2.0	2.0	2.0	2.0
OR1	11.0	11.0	11.0	10.0	12.0	11.0
OR2	5.0	5.0	7.0	6.0	4.0	5.4
OR3	3.0	4.0	3.0	4.0	4.0	3.6
OR4	2.0	2.0	2.0	3.0	3.0	2.4
OR5	17.0	16.0	16.0	17.0	18.0	16.8
OR6	3.0	3.0	3.0	2.0	2.0	2.6
PA1	14.0	12.0	14.0	14.0	12.0	13.2
PA3	9.0	9.0	8.0	8.0	9.0	8.6
PA5	8.0	5.0	6.0	6.0	6.0	6.2
PA6	11.0	12.0	7.0	10.0	10.0	10.0
PR1	11.0	9.0	9.0	9.0	10.0	9.0
PR2	2.0	2.0	3.0	3.0	2.0	2.4

Strain Code	1	2	3	4	5	Mean(mm)
PR3	7.0	8.0	7.0	9.0	6.0	7.4
PR4.1	8.0	7.0	6.0	7.0	6.0	6.8
PR4.2	8.0	7.0	7.0	6.0	8.0	7.2
PR5	2.0	2.0	2.0	3.0	5.0	2.8
PR6	5.0	6.0	6.0	6.0	6.0	5.8
PR8	4.0	5.0	4.0	4.0	4.0	4.2
S1.1	11.0	9.0	9.0	10.0	10.0	9.8
S1.2	3.0	4.0	4.0	4.0	3.0	3.6
S2.1	4.0	2.0	3.0	2.0	2.0	2.6
S3	3.0	3.0	2.0	2.0	3.0	2.6
S4	15.0	15.0	16.0	14.0	14.0	14.8
S5	4.0	3.0	2.0	3.0	2.0	2.8
S6.1	3.0	3.0	2.0	3.0	3.0	2.8
S8	5.0	5.0	6.0	5.0	6.0	5.4
TM1	5.0	6.0	7.0	6.0	5.0	5.8
TM2	4.0	3.0	3.0	3.0	4.0	3.4
TM3	7.0	5.0	7.0	7.0	6.0	6.4
TM4	13.0	12.0	13.0	13.0	11.0	12.4
TM6	3.0	3.0	3.0	4.0	4.0	3.4
YM1	11.0	11.0	13.0	10.0	11.0	11.2
YM2	4.0	5.0	3.0	3.0	4.0	3.8
YM3	6.0	4.0	4.0	5.0	6.0	5.0
YM4	7.0	9.0	8.0	8.0	9.0	8.2
YM5	6.0	4.0	7.0	5.0	5.0	5.4

Appendix B



CALIBRATION OF GLUCOSE STANDARD CURVE USING DINITROSALICYLIC ACID METHOD AT 540 nm



CALIBRATION OF STARCH STANDARD CURVE
AT* 620nm