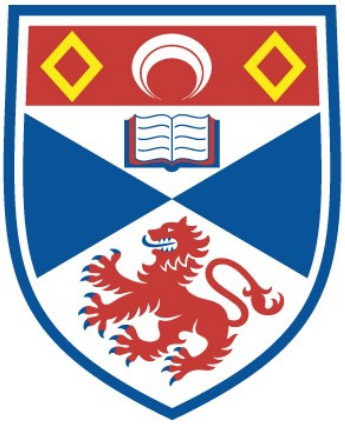
AMYLOLYTIC ENZYME PRODUCTION BY IMMOBILISED CELLS OF 'ASPERGILLUS NIGER'

Wladimir Gusmao do Nascimento Costa

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1984

AMYLOLYTIC ENZYME PRODUCTION BY IMMOBILISED CELLS OF ASPERGILLUS NIGER

A thesis presented by

WLADEMIR GUSMAO DO NASCIMENTO COSTA

to the University of St. Andrews

in application for the degree of

Doctor of Philosophy

Biochemistry Department,

The University,

St. Andrews

July,1983.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

AMYLOLYTIC ENZYME PRODUCTION BY IMMOBILISED CELLS OF *ASPERGILLUS NIGER*

Thesis presented by WLADEMIR GUSMAO DO NASCIMENTO COSTA to the University of St. Andrews in application for the degree of Doctor of Philosophy.

Department of Biochemistry - July, 1983.

A strain of *Aspergillus niger*, isolated from rotting cassava tubers, has been immobilised in beads of calcium alginate (1 percent, w/v) and used to produce amylolytic enzymes.

The optimal operating conditions (cell concentration and gel composition of the immobilised system, temperature of incubation, initial pH of the medium) have been investigated.

Amylolytic enzyme production was associated with the cell growth within the calcium alginate beads. The growth pattern of *Aspergillus niger* cells trapped in the calcium alginate gel has been studied. An early phase of exponential growth followed by a phase of decelerating growth rate was observed. The explanation for this growth pattern has been discussed.

By operating the immobilised cell system under optimised conditions a higher level of amylolytic enzyme production than that obtained with a free cell system was achieved. A better operational stability for the immobilised system was demonstrated.

The continuous production of amylolytic enzymes by the immobilised cells of *Aspergillus niger* has been investigated. Using a nitrogen-limited medium, it was possible to maintain a relatively high enzyme productivity during a 12 day-period of continuous operation.

The thermostability, storage stability, pH and temperature activity profiles of the crude enzyme broth from the immobilised cell culture, and their comparison with these characteristics for enzyme broth from a free cell culture, have been studied.

The conversion of raw (uncooked) cassava starch to glucose syrup and, in the presence of yeast cells, its further fermentation to ethanol, have been investigated using culture liquid from immobilised cells. Also, the immobilised cell system has been employed as a possible treatment system for starch-containing wastewater.

CERTIFICATE

I hereby certify that Wlademir Gusmão do Nascimento Costa has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of Ordinance General No. 12 of the Resolution of the University Court 1967, No. 1, and that she is qualified to submit the Accompanying thesis for the degree of Doctor of Philosophy.

DECLARATION

I hereby declare that this thesis is based on work carried out by me, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, University of St. Andrews, under the direction of Dr W. M. Ledingham.

ACADEMIC RECORD

I graduated with the degree of Doctor of Medicine in 1973 from the Federal University of Alagoas, Maceio, Brazil, and with the degree of Master of Science (Biochemistry) in 1977 from the Federal University of Pernambuco, Recife, Brazil.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October 1980.

ACKNOWLEDGEMENT

I should like to express my gratitude to Dr. W. M. Ledingham for his advice during this work, and to my husband for his incentive.

I am also grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and to the Universidade Federal de Alagoas for financial support.

CONTENTS

1 INTRODUCTION

1.1 Fungal amylolytic enzymes

Fungal production of amylolytic enzymes has been recognised for a long time. In the oriental countries these enzymes are traditionally used for saccharification of rice starch in the production of alcoholic beverages.

In 1894, in the United States, Takamine reported his findings on the production of amylolytic enzymes, using a surface culture of Aspergillus oryzae ("takadiastase") and, subsequent to this, the production of these enzymes was largely developed in the occidental countries.

Today amylolytic enzymes are being used in many commercial applications such as food manufacturing, textile processing and the conversion of starch to fermentable sugars in the brewing and alcohol industries.

From an industrial viewpoint the most important amylolytic enzymes derived from filamentous fungi occur in the groups of alpha-amylases and amyloglucosidases. These enzymes effect the hydrolysis of alpha-1,4- and/or alpha-1,6-glucosidic linkages. Both fungal alpha-amylase and amyloglucosidase are extracellular enzymes, i.e., they are liberated into the medium after their formation.

Fungal starch-degrading enzymes have been reviewed by a number of authors: Banks et al. (1967); Aunstrup (1977); Blain (1975); Shah (1979); Fogarty and Kelly (1979). The following sections, principally based on these publications, describe some characteristics of fungal amylolytic enzymes, such as chemical and physical properties, and mechanism of action. Also, aspects of their biosynthesis, production and application are presented.

1.1.1 Characteristics of the fungal amylolytic enzymes

1.1.1.1 Alpha-amylases

Fungal alpha-amylases (EC 3.2.1.1) belong to the class of amylolytic enzymes known as dextrinogenic amylases or endoamylases. These enzymes cause endo-cleavage of alpha-1,4-glucosidic linkages, in a random fashion, of both amylose and amylopectin molecule components of starch. The result is the rapid decrease of viscosity and

fragmentation of the glucans to lower molecular weight dextrins and oligosaccharides. The enzymes do not attack the branching-point (alpha-1,6-linkages) in the amylopectin molecule. The final products of exhaustive alpha-amylase action on starch are maltose and low molecular weight oligosaccharides containing glucose units linked by alpha-1,4, and alpha-1,6-glucosidic bonds, and smaller amounts of glucose. The alpha-amylases are water-soluble glycoproteins having calcium ions associated with their molecules. The calcium ions do not participate in the formation of the enzyme-substrate complex but maintain the optimum conformation for the maximum activity; without them, the enzyme molecule is relatively unstable and becomes susceptible to attack by proteinases. The molecular weight of alpha-amylases is about 50,000. The optimum pH of the Aspergillus oryzae enzyme is 4.8-5.8; some Aspergillus niger strains produce an additional alpha-amylase which is stable at pH 2.0 or lower.

1.1.1.2 Amyloglucosidases

Amyloglucosidases (EC 3.2.1.3) catalyse the cleavage of alpha-1,4-glucosidic linkages, removing successive units of glucose from the non-reducing ends of the polysaccharide chain as well as cleaving the alpha-1,6-glucosidic linkages in the amylopectin molecule (Pazur and Ando, 1959, 1960). The rate of decrease in viscosity is slower during the amyloglucosidase action than during the alpha-amylase action. The hydrolysis rate decreases as the chain

length decreases. Beta-glucose is the sole final product of the action of amyloglucosidase on starch. The purified amyloglucosidase from Aspergillus niger contains two isoenzymes having slightly different physical properties (Pazur et al., 1971). Amyloglucosidases are glycoproteins of molecular weights of about 60,000 to 100,000 and contain 13-18 percent of carbohydrates. The optimum pH is 4.2-4.5 and the optimum temperature for starch hydrolysis is 50 - 60°C, varying with factors such as source, and degree of purity.

Table 1.1.1 presents some of the important characteristics of fungal alpha-amylases and amyloglucosidases.

1.1.2 Biosynthetic aspects of fungal amylolytic enzymes

1.1.2.1 Biosynthesis and secretion

Microbial enzyme production is under genetic control and four types of genes termed regulator, promotor, operator and structural genes are thought to be involved (Demain, 1972). The first step of enzyme synthesis is the formation of mRNA which has complemental base sequence to one of the DNA strands, by DNA-depend RNA polymerase. The second step (translation) is the synthesis of polypeptide chain from amino acids carried by tRNA according to the base sequence of mRNA on

	alpha-amylase	amyloglucosidase
hydrolyse alpha-1,4 glucosidic bonds	yes	yes
hydrolyse alpha-1,6 glucosidic bonds	no	yes
ability to by-pass alpha-1,6 branch points	yes	bonds cleaved
configuration of C_1 of product	alpha	beta
mechanism of substrate attack	endo	exo
viscosity reduction	fast	slow
production of reducing sugars	slow	fast
iodine staining power	decreased quickly	decreased slowly

Table 1.1.1. Characteristics of alpha-amylases and amyloglucosidases.

surface of ribosomes. Generally one mRNA strand is bound to a couple of ribosomes forming polysomes. Extracellular enzymes are exclusively synthesised on the rough endoplasmatic reticulum, i.e., membrane-bound polysomes.

During the translation of the mRNA, the amino-terminal portion of the nascent protein begins to emerge from a protective cleft (tunnel) through the larger ribosome subunit. This nascent protein is postulated to have an amino acid sequence that allows it to bind to mobile receptors in the membrane of the membrane-bound polyribosomes. The nascent protein would then begin to transverse the membrane. Soon after the amino-terminal end (signal peptide sequence) has emerged from membrane into the luminal space of the rough endoplasmic reticulum, a protease (signal peptidase) removes the signal peptide sequence from the amino-terminal end. The following peptide chains are secreted outside of the membrane, forming mature proteins (Shinmyo, 1980; Ramaley, 1979).

1.1.2.2 Kinetics of amylolytic enzyme biosynthesis

Knowledge of rates of fermentation processes are desirable for process design and optimisation. However, mathematical modelling of enzyme formation is difficult due to the complexity of the biosynthetic process. The prediction of product formation is also complicated by the changes in the relative quantities of cell components which occur throughout the fermentation. A model for

fungal growth and enzyme formation should consider such component changes and also should include the relevant aspects of genetic control of biosynthetic processes.

A model for the kinetics of fungal amylolytic enzymes formation has been proposed by Brown and Fitzpatrick (1979). The model is based on the assumption that:

i) the rate-limiting step of the enzyme synthesis corresponds to mRNA formation and the specific rate of enzyme production (K_{15}) is proportional to the quantity of mRNA per cell;

ii) the mRNA content of the cell is proportional to the concentration of nucleic acid (C_g);

iii) alpha-amylase excreted by the cell is considered to be constitutive, i.e., synthesized in substantial concentration under all conditions of growth, but repressed by the rate of metabolism due to shortage of cyclic 3',5'-adenosine monophosphate (cAMP) or its complex with a receptor protein (crp) at the promotor gene site; a term Q was defined as an active fraction of the promotor gene sites that contain the cAMP/crp;

iv) as the mRNA for fungal-amylase is relatively stable an exponential decay term (K_{19}) could be included.

The following hypothetical relationship was proposed:

$$\frac{dC_a}{dt} = K_{15}Q\{dC_g/dt + C_g.\exp[-K_{19}(t - t_o)]\}$$

where, C_a is the concentration of enzyme at the time t, and t_o is the time which the concentration of substrate approaches such a value that it affects the operation of the mRNA renewal system.

This model has been successfully used to predict the production of amylolytic enzyme by Aspergillus oryzae.

1.1.3 Production

1.1.3.1 Organisms

Many fungi are known to produce amylolytic enzymes; in particular some strains of the genus Aspergillus. Fungal alpha-amylases are usually prepared from Aspergillus oryzae and Aspergillus niger. The organisms currently used for amyloglucosidase production include Aspergillus niger, Aspergillus phoenicus (a sub-species of the

species A. niger), Rhizopus niveus and Rhizopus delemar.

The table 1.1.3.1 presents a list of the organisms currently used for amylolytic enzyme production.

Selection of mutant strains of fungi with enhanced enzyme yields has become an important area of research in recent years. The technique of artificial mutation basically consists in treating spores with a mutagenic agent (ionizing and ultraviolet radiation, and chemical mutagens) and isolation of the high-yielding enzyme strains. The methodology of mutant induction has been outlined by Adler (1971) and presented in more detail by Calam (1970). The selection of isolates of a higher amyloglucosidase-producing strain of A. niger was obtained by using ultraviolet light (Corn Products Co., 1962); also isolates of Aspergillus cinnamoneus, exhibiting a six-fold increase in production of acid-stable alpha-amylase, have been reported (Kurushima et al., 1972). Although the technique of DNA-recombination has been elegantly used in the academic vein, its application with regard to amylolytic enzyme production has been less successful (Thorbek and Eplov, 1974).

enzyme	source
alpha-amylase	Aspergillus awamori
	A. batatae
	A. candidus
	A. niger
	A. oryzae
	A. terricola
	A. usamii
amyloglucosidase	Aspergillus awamori
	A. batatae
	A. niger
	A. oryzae
	A. foetidus
	A. phoenicus
	A. usamii
	Rhizopus delemar
	R. javanicus
	R. niveus
	Rhizopus sp.

Table 1.1.3.1. Some organisms used for amylolytic enzyme production.

1.1.3.2 Cultivation techniques

1.1.3.2.1 Solid surface cultures

Fungal amylolytic enzymes have been traditionally produced with the use of solid cultures (Koji and bran processes). In general, solid cultures are conducted on sterilised moistened bran in rotating drums or in trays. After inoculation with moulds the cultures are kept in large rooms where the moisture of the air, the temperature and the aeration are carefully controlled. After 30-40 hours of growth the mould bran is removed from the incubator and dried. The final product can be sold in this crude form or after further purification.

1.1.3.2.2 Submerged cultures

More recently, the use of submerged cultures for production of amylolytic enzymes by selected strains of Aspergillus niger and related species has received increasing attention. The submerged culture method of producing amylolytic enzymes has definite advantages in relation to surface cultures such as easier control of environmental factors (pH, temperature, etc). These advantages are more prominent when the final product is employed directly without concentration or purification as, for example, in the alcoholic

fermentation of grain and in the manufacture of glucose syrups. Submerged cultures usually utilise stainless-steel fermenters of 1,000-30,000 gallon capacity, with an air sparger at the bottom and a central agitator. The temperature is controlled by a water-jacket or a coil system. The fermentation is complete when the maximum amount of enzymes have been produced.

1.1.3.2.3 Medium formulation

Many authors have related results of studies on mould nutrition to amylolytic enzyme production (Banks et al., 1967).

A satisfactory medium for amylolytic enzyme production should have nutrients providing energy, carbon and nitrogen sources including special growth requirements, such as essential amino acids. However, a correct balance of nutrients for enzyme production is necessary because the medium supporting the best growth is not necessarily the one on which the maximum amount of enzyme is produced.

Enzyme production also depends on the presence of an inducer in the medium. An inducer is generally the substrate of the enzyme or closely related compounds, i.e., starch, glycogen or maltose (Tikhomirova, 1958; Smirkova, 1961). The enzyme synthesis may also be repressed by a component of the medium, the strongest repression being seen in medium containing glucose and lactose (Pal et al., 1980).

Economy is very important in medium formulation. Typically, raw materials account for 60-80% of the variable costs of an enzyme fermentation process. Much development work is directed toward the replacement of costly ingredients with components available in large quantities at low cost. Media prepared from agricultural by-products including malt, wheat bran, corn steep liquor and soybean flour, were found to be an excellent substrate for proliferation of Aspergillus niger and amyloglucosidase production (Sinkar and Lewis, 1980).

1.1.3.3 Enzyme recovery

Mostly frequently the amylolytic enzymes are obtained from the filtered broth, or in the case of solid surface cultures from the aqueous extract of the bran, by precipitation techniques. Water miscible solvents or strong salt solutions are most commonly used for the enzyme precipitation. The precipitates tend to be sticky and difficult to handle so inert carriers, such as Kieselguhr, are added to correct this. After precipitation, the filter cake may be washed free of salts and then, tray-dried and ground or spray-dried to a powder.

Various stabilizers are used to minimize enzyme lost during the processing and these include sodium chloride, propylene glycol, calcium salts and, sometimes, sulphites if the enzyme is prone to oxidation.

1.1.4 Commercial applications

1.1.4.1 Breadmaking

One important commercial application of alpha-amylases is in the baking industry, where the enzymes are used in the supplementation of flours which are weak in amylolytic activity; this is common particularly in those countries where, due to harvesting conditions, ungerminated grains are used. The enzymes promote the hydrolysis of the flour starch and produce sugars for subsequent fermentation by the baker's yeast. Fungal alpha-amylases are superior to the bacterial and cereal enzymes for this purpose because their lower thermostability avoids overdextrinization of the gelatinized starch at the baking stage and, therefore, produces higher quality texture.

1.1.4.2 Production of glucose and sugar syrups

Fungal amylolytic enzymes are also used to produce syrups of a high concentration of glucose, maltose and of higher oligosaccharides in varying proportions. The extent of starch degradation is often expressed in DE (Dextrose Equivalent) which is the content of reducing

sugars calculated as glucose and expressed as the percentage of the total solids. Such syrups have been prepared, on an industrial scale, from corn maize grain and root starches such as sweet potato. Park and Papini (1970) and Lages and Tannenbaum (1978) developed a laboratory scale process for the conversion of cassava starch and cassava meal ("farinha de mandioca") into a very concentrated glucose syrup (DE 98-100%).

The usual procedure in the industrial production of glucose syrups is to carry out a preliminary acid hydrolysis before the enzyme step, but methods using only enzymes for both liquefaction and saccharification steps are being preferred due to their higher conversion efficiency; in these methods, thermostable bacterial alpha-amylase is normally used in the liquefaction step.

Amyloglucosidases and alpha-amylases have also been immobilised into several carriers by entrapment (Butlerworth et al., 1970; Stavenger, 1971; Beck and Rase, 1973), adsorption (Krasnobajew and Boniger, 1975; Bachler et al., 1970; Smiley, 1971; Chen and Tsao, 1976, 1977), ion exchange and covalent bonding (Weetal and Havenwala, 1972) and then used for glucose syrups production; it remains to be seen whether this technique attains commercial usage.

1.1.4.3 Alcohol production and brewing

In western countries, the cereal alpha- and beta-amylases have been traditionally utilised as the saccharifying agent in the alcohol and beverage industries, but in Asian countries fungal enzymes have been extensively used for this purpose for a long time.

The effectiveness of the utilisation of fungal amylolytic enzymes in the production of alcohol and spirits was demonstrated on a laboratory and industrial scale by several investigators (Takamine, 1914; Miyazaki, 1932; Le Mense et al., 1947, and 1949; Hanson et al., 1955; Sadir, 1966; Harrison and Rowell, 1970; Stentebjerg-Olesen, 1971).

Processes using the fungal enzymes are become increasingly accepted in western countries, particularly in places such as Brazil, where the conditions are not favourable to malt preparation and good malt is usually sold at a high price. Work at the Instituto de Tecnologia de Alimentos de Campinas, São Paulo, using submerged cultures of Aspergillus niger for the saccharification of cassava starch for ethanol production showed that the fungal-enzyme process presents several advantages over the cereal-malt process (Araujo Filho, 1977):

i) Higher alcohol yields: the use of barley malt has resulted in alcohol yields of 70-74% of the maximum theoretical value while cassava mashes converted by submerged fungal cultures of Aspergillus niger resulted in alcohol yields up to 90% of the theoretical maximum yield;

ii) lower operating costs;

iii) simple substrates can be used for the propagation of the mould;

iv)faster hydrolysis rates.

1.1.4.4 Pharmaceutical uses

Amylases are the active component in different pharmaceutical preparations used in the treatment of deficiency of the exocrine pancreas, amylaceous dyspepsia, cystic fibrosis, etc. By catalysing the hydrolysis of starch these enzymes also create in the jejunum-ileum favourable conditions for Lactobacillus growth of which the antagonistic properties to pathogenic intestinal microorganisms are well known.

In recent years, pharmaceutical industries have focused much attention on fungal amylases because of their possible role in the replacement of hog pancreas alpha-amylases. Fungal amylases do offer various advantages over the amylases derived from animals, not only because they are cheaper but also most resistant to acid denaturation (Shah, 1979).

1.2 Rheology of mycelial fermentation broths

When fungi grow in submerged cultures, the mycelium forms a dense mass of branched interwoven hyphae (the average of diameter of the hyphae lies in the range 2 - 5 u). The branched mycelial network forms a three-dimensional structure which imparts rigidity to the suspension. Due to such morphology of the hyphal growth, and to the high level of biomass reached in the fermenter, mycelial suspension is usually very viscous (in the order of several thousands of centipoises) and has a non-newtonian rheological behaviour (Pace, 1980; Metz, 1976, Banks, 1977).

Non-newtonian fluids, such as filamentous fungi cultures, do not possess a true viscosity but only an apparent viscosity which is not a constant, being dependent upon the shear rate. Different shear rates exist in different parts of the fermenter, the maximum shear rate being in the immediate vicinity of the impeller. It has, in fact, been shown that the shear rate decreases exponentially with the distance from the impeller tip (Otto, 1957; Taylor, 1955). Since the shear rate varies within the fermenter, the apparent viscosity, being dependent upon it, will likewise vary.

These characteristics lead to a number of problems that decrease the productivity of a fermentation process. These problems, which have been described in detail by Metz (1976), are:

i) problems with mass transfer of nutrients, oxygen in particular due to its low solubility in water;

ii) oxygen concentrations are non uniform and higher close to the impeller; when the mycelia leave the impeller region, partial starvation takes place very easily and eventually this will cause autolysis.

As previously established, the two factors which determine the rheological properties of a fungal culture broth are cell concentration and hyphal morphology. Cell concentration is a simple parameter to control, but decrease in cell concentration leads to a decrease in product yield (Solomons, 1977). Kossen and Metz (1976) and Randall and Solomons (1977) have shown that the possibility to create a less viscous mycelial suspension by influencing the morphology of hyphae by mechanical means is of little practical value; a substantial decrease in hyphal length, in order to obtain the desirable mixing and transfer properties, requires an enormous increase in energy input, and consequently in operation costs.

An alternative way of creating less viscous mycelial suspensions is possible by growing the mould cells confined within inert beads by immobilisation techniques (Gbewonyo and Wang, 1983). Under such circumstances the culture fluid is of lower viscosity and tends to approach newtonian behavior and, consequently, relieve the limitations of gas-liquid mass transfer rate on fermentation. Thus, several advantages over the traditional process (free cell culture) can be

predicted from this hypothesis:

(i) the beads would be capable of sustaining cell growth up to higher biomass concentrations than attainable in the conventional freely suspended mycelial cultures, and consequently faster reaction rates;

ii) the immobilised cells can be repeatedly used since they are easily separable at the end of a process;

iii) the immobilised cells could be deployed in continuous operation reactors.

1.3 Methods for cell immobilisation

Microbial attachment to solid surfaces is a natural phenomenon: immersed stones, soil, internal walls of pipes and dental plaques are some examples of solid ecosystems colonized by immobilised microorganism.

Cell immobilisation for the production of useful compounds is a fairly old technology (for example, Schutzenbach, in 1823, employed microbial cells immobilised on wood shavings for the conversion of ethanol to acetic acid) but it is only in recent years that it has received increased interest (Abbot, 1977; Messing, 1980).

Several authors have surveyed immobilised cell technology: Chibata (1978), Abbot (1977), Messing (1980), Durand and Navarro (1978), Jack and Zajic ((1977) and Kolot (1980). These reviews are comprehensive, covering the methods for cell immobilisation and with emphasis on the activity remaining after the immobilisation process, and the advantages and disadvantages of this technology as compared with more conventional processes.

There are three basic categories of methods for the immobilisation of microbial cells.

The first method is based on ionic binding of the cells to an ion-exchange carrier. The cell activity is usually not affected in this method of immobilisation but the interaction between the cell and carrier can be influenced by the cell metabolism reactions and leakage of cells may easily occur.

Microbial cells can also be immobilised by covalent cross-linking between the cell surface and an activated inorganic support with a bi- or multifunctional reagent. This technique generally gives very stable preparations but the cell activity is usually affected.

The third method of microbial cell immobilisation consists of physical entrapping of the microbial cells in polymeric matrices such as agar, gelatine, polyacrylamide, collagen, calcium alginate and k-carrageenan. This is the method that has been most extensively investigated so far. Usually it is a very effective process and does not interfere with the cell activity.

1.4 Immobilisation of fungal cells for fermentation processes

Filamentous organisms have been immobilised on inert carriers for several purposes. Some examples are listed below:

A U.S. patent issued in 1976 describes an immobilised cell process to isomerise glucose. A Streptomyces sp. is aggregated by cross-linking the cells with tetrazotized benzidine. The cross-linking reaction occurs with little loss of glucose isomerase activity and cross-linked cells are readily recovered by centrifugation (Lartique and Weetall, 1976).

Aspergillus niger cells attached to collagen were used for citric acid synthesis by Venkatasubramanian and Vieth (1979). The preparation exhibited good activity retention (48.4%) in relation to free-cells system. Half-life of the biocatalyst was 138 h.

Suzuki and Karube (1979) carried out studies with Penicillium chrysogenum immobilised in polyacrylamide gel for the production of penicillin G from glucose. The reusability of the immobilised cells and washed cells was examined. The activity of penicillin production by washed cells decreased with repeated use; on the other hand, the activity of the immobilised cells increased initially and decreased gradually thereafter.

Cochet *et al.* (1979) studied starch degradation by *Aspergillus niger* immobilised in ceramic. They reported an increase of metabolic activity of the mycelium with immobilisation.

Matteau and Sadler (1982) reported immobilisation of *Trichoderma sp.* in calcium alginate beads for use in continuous hydrolysis of cellobiose and salicin. Maximal activities achieved were between 70 and 90 umoles substrate reacted per minute per litre of bead volume. Immobilised mycelial-associated beta-glucosidase activity was shown to have a half-life higher than 1000 h when operating continuously at 50°C.

Frein *et al.* (1982) studied continuous cellulase production by *Trichoderma resei* immobilised on 4% k-carrageenan beads. The average cellulase productivity obtained surpassed those of a conventional single stage continuous culture and compared well with results obtained in two stages continuous culture. Carbon and nitrogen requirements of the immobilised system were reduced in relation to those of conventional continuous culture.

1.5 Aims of this thesis

In general terms, the aim of this thesis is the investigation of the use of immobilised cells of *Aspergillus niger* for the production of amylolytic enzymes. In order to fulfil this objective, the following points were investigated:

i) optimisation of the operating conditions for the enzyme production;

ii) growth of the cells confined within the support;

iii) comparison of the production of amylolytic enzymes by free and immobilised cells;

iv) potential applications of the immobilised cell system.

2 MATERIAL AND METHODS

2.1 Chemicals

4-nitrophenyl-alpha-D-glucopyranose, albumin (bovine), sodium alginate, fungal alpha-amylase (E.C. 3.2.1.1) type X-A amyloglucosidase (E.C. 3.2.1.3) grade IV and amylose azure were obtained from Sigma London Chemical Co. Ltd. (England). Ethanol and isopropanol were provided by BDH Chemicals Ltd. (Poole, England). Cassava starch was obtained from Laing National Ltd. (Manchester, England). Soluble starch was provided by May & Baker Ltd. (Dagenham, England). All other chemical used were of analar grade.

2.2 Organism and media

A strain of *Aspergillus niger* isolated in our laboratory from rotting cassava tubers was used in this study.

The basic composition of the media used were the following:

i) medium M-1: cassava starch, 0.5 g/100 ml;
mycological peptone, 0.2 g/100 ml;
agar, 1.5 g/100 ml;
tap water.

ii) medium M-2: cassava starch, 1.0 g/100 ml
mycological peptone, 0.2 g/100 ml;
tap water.

Fermenter vessels and media were sterilised by autoclaving at 15 lb/in^2 for 30 minutes.

2.3 Cultivation procedure and immobilisation method

Stock cultures were kept as a frozen spore suspension. Spores were transferred from the stock culture to surface culture in Petri plates containing 15 ml of medium M-1. The plates were incubated at 30°C for 7 days to bring about sporulation. The spores were washed out from the culture with sterile distilled water and the number of conidia was determined with a Thoma counting chamber. The final conidial suspension was adjusted to contain 2×10^8 conidia per ml.

10 ml of this conidial suspension were used to inoculate 800 ml of medium M-2 in a 2 l vessel. A few small glass beads were added to prevent the formation of filamentous strands of mycelia. The culture was allowed to develop at 40°C in a thermostatted water bath with a magnetic stirrer (800 - 900 rpm).

The cells were harvested in the logarithmic phase of the growth by filtration using a Buchner funnel with filter paper Whatman Grade number 1, and washed with water. The filtered mycelia were squeezed dry by pressing firmly between sheets of filter paper until no more moisture could be extracted and then weighed.

12 g of this mycelial pad were mixed with 120 ml of 1% (w/v) sodium alginate solution. This mixture was peristaltically pumped dropwise into a 0.1 M calcium chloride solution. The slurry was extruded as discrete drops to form small beads (radium of about 1.5 mm). The so formed calcium alginate beads were stored at 4°C in 0.1 M calcium chloride solution until used.

2.4 Fermentation experiments

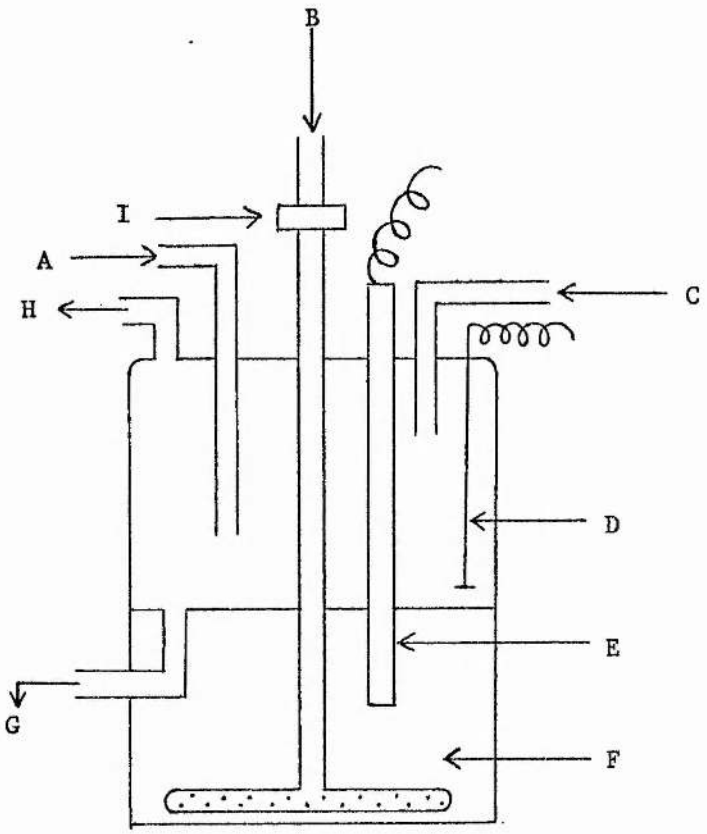
2.4.1 Shake flask cultures

Batch experiments were carried out, unless specified otherwise, in 250 ml conical flask containing 50 ml of medium M-2 and 2 g (wet weight) of immobilised or washed mycelia of Aspergillus niger, on a New Brunswick rotatory shaker operating at 200 rev/min, at 40°C. Samples of beads and liquid aliquots were periodically removed for analysis.

2.4.2 Continuous operation experiments

Continuous experiments were carried out using a fluidized-bed fermenter (FBF) shown in figure 2.4.2. The working volume of the FBF was 450 ml. 10 g (wet weight) of immobilised mycelia of Aspergillus niger was fluidized by aerating the fermenter at a flow rate of 450 $ml.min^{-1}$. Temperature was maintained at 40°C via a contact thermometer/150 W heating lamp system. Foaming was controlled by automatic addition of silicone antifoam compound using an anti-foaming controller unit. The sterilised medium was continuously fed at a flow

rate of 18 ml/h to the fermenter using a precalibrated peristaltic pump. This feed rate corresponded to a retention time of 25 h. The effluent was withdrawn at a constant rate from the reactor by an overflow device. Samples of beads and liquid aliquots were aseptically withdrawn periodically during the experiments for analysis.



A - fresh medium feed
B - air inlet
C - anti-foam inlet
D - foam sensor
E - contact thermometer
F - beads
G - effluent
H - air outlet
I - air filter

Fig. 2.4.2. Fluidized-bed fermenter. Total volume = 1 l; working volume = 450 ml; diameter = 10 cm.

2.5 Microscopic observation of the immobilised cells

Gel beads with immobilised cells of Aspergillus niger were fixed, for microscopic observation, with 2% glutaraldehyde in sucrose buffer at pH 7.4 for 24 h. After dehydration in a graded ethanol series, the beads were embedded in Araldite. 0.5 μm sections were prepared with a glass knife blade, stained with 0.5% toluidine blue for 20 sec, at 60°C, rinsed with distilled water and dry mounted on styrene.

Photographs were taken in a large fluorescent Zeiss Microscope. This procedure gave a good preservation of natural gel structure.

2.6 Determination of the mean density and mean radius of beads

the mean volume of the beads was determined on the basis of displaced volume of distilled water, using a picnometer. The weight of the picnometer was determined first when empty, secondly filled with distilled water, thirdly containing 20 beads, and finally filled with water plus 20 beads. If the following are the results of weighing:

weight of the bottle empty	P
weight of the bottle full of water	W
weight of the bottle and beads	B
weight of the bottle with beads and water	R

then, the apparent weight of the displaced water, which occupies the same volume as the beads is given by:

$$W' = W + B - R - P$$

thus,

$$\text{mean volume of a bead} = W'/20$$

The mean radius of the beads was determined based upon the mean volume measured as described above.

Knowing the dry weight of mycelia in a bead (DW) and the mean volume (V) of the beads, the mean density could be calculated according to the following definition:

$$\text{mean density of a bead} = DW/V$$

2.7 Analytical methods

2.7.1 Evaluation of mycelial growth

2.7.1.1 Free-mycelia cultures

The growth of free mycelia in submerged cultures was followed by turbidity measurements, as described by Trinci (1972).

The turbidities of appropriate dilutions (culture medium used as diluent) of a dense suspension of organism were measured in a spectrophotometer SP 600 Series 2 (Unicam Instruments, Cambridge, England) at 540 nm; measurements were made on triplicate tubes.

A sample of the original suspension was filtered and washed in the same way as with the immobilisation procedure described in section 2.3. The mycelial pad was then dried to a constant weight at 105^{o}C in weighing bottles of known weight and its dry-weight calculated.

A calibration plot of culture turbidity against organism dry-weight was prepared (fig. 2.7.1.1). There was a linear correlation between the turbidity measurements and the organism dry weight from 0 to 0.710.

2.7.1.2 Immobilised-mycelia cultures

The mycelial growth of the immobilised microorganism was estimated by determination of the dry weight of mycelia in the beads. The total solids contents of each flask was harvested by filtration, and washed with distilled water. The dry weight of total solids (cell and gel) was obtained after drying at 105^{o}C for 24 h. The dry cell weight is given as the difference between the final weight of the total solids content of each flask and the initial weight of gel at the begining of the experiment.

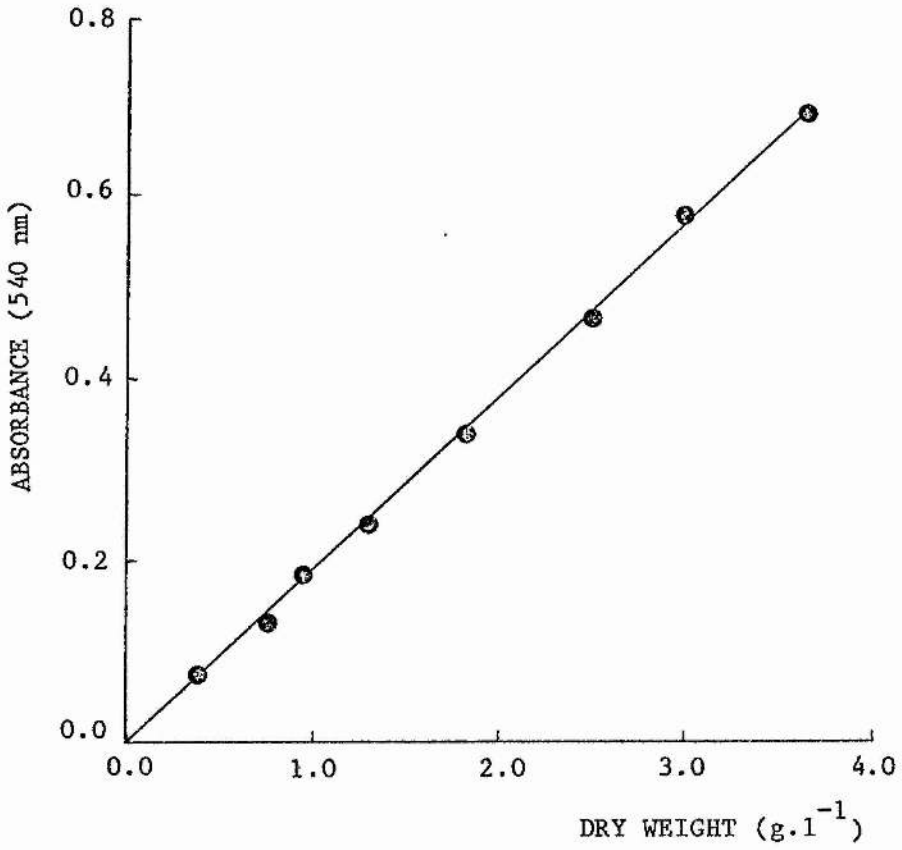


Fig. 2.7.1.1. Calibration plot of culture turbidity against mycelial dry-weight/l.

2.7.2 Determination of starch concentrations

The absorbance of the colour ("blue value") produced in aqueous solution of starch upon the addition of tri-iodide ion was used as the basis of the method of determination of starch concentrations utilised in this study.

4.8 ml of water were added to 0.1 ml of a freshly prepared solution of soluble starch containing between 0 and 5 mg/ml, followed by the addition of 0.1 ml of a solution containing 1.27 mg/ml of iodine and 3 mg/ml of potassium iodide (this solution was prepared from the stock solution containing 12.7 mg/ml of iodine and 30 mg/ml of potassium iodide). Absorbances were measured immediately after colour development in a spectrophotometer SP 600 Series 2 (Unicam Instruments, Cambridge, England), at 590 nm. A calibration plot was prepared of "blue value" against the starch concentration (fig. 2.7.2).

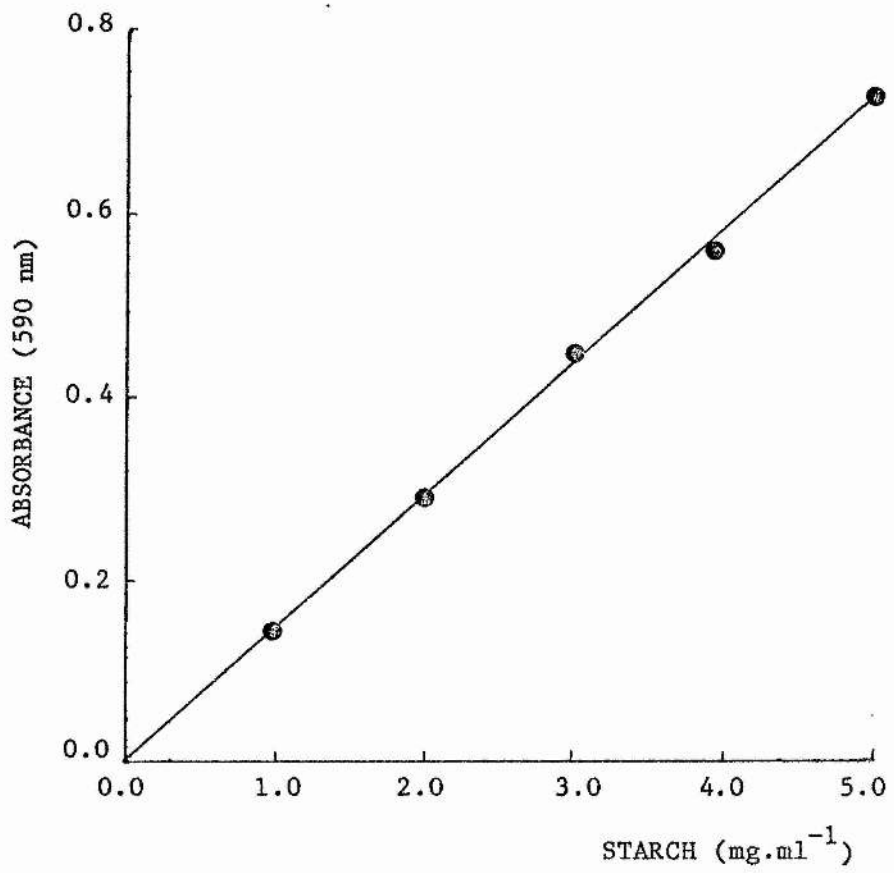


Fig. 2.7.2. Calibration plot for determination of starch concentration.

2.7.3 Determination of reducing sugar concentrations.

The 3,5-dinitrosalicylic acid (DNSA) method was utilised to determine reducing sugar concentrations according to Bruner (1964).

Reagents:

i)Standard solution of glucose - 10 mg/ml;

ii) DNSA reagent - 20 g of 3,5-dinitrosalicylic acid was dissolved in 700 ml of 1.0 N sodium hydroxide solution; the solution was diluted to 1 l with distilled water and filtered. The reagent was stored in polyethylene bottles to minimize the cumulative effects of exposure to air and evaporation.

2 ml of solution to be assayed was added to 2 ml of chilled DNSA reagent. The sample was heated for 5 min in boiling water and then cooled in an ice and water bath. The reaction mixture was diluted with 8 ml of distilled water and the absorbance measured at 540 nm using a spectrophotometer SP 600 Series 2 (Unicam Instruments, Cambridge, England). A calibration plot (fig. 2.7.3) established with glucose (0.2 to 1.0 mg in 2 ml of water) was used to convert the spectrophotometer readings into mg/ml of glucose.

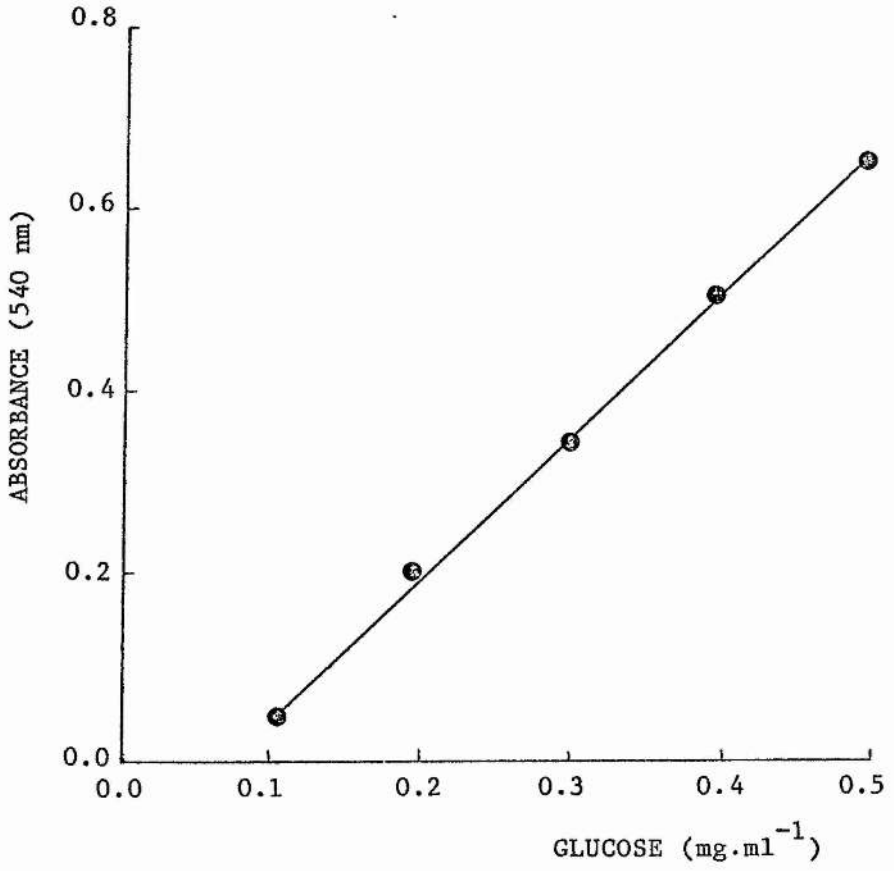


Fig. 2.7.3. Calibration plot for determination of reducing sugar concentration by DNSA method.

2.7.4 Determination of amylolytic activity

Amylolytic activity was estimated by measuring the rate of release of reducing sugar equivalents from soluble starch.

The reaction mixture, containing 0.5 ml of 1 g/100 ml soluble starch in 0.01 M acetate buffer, pH 4.2, and 0.5 ml of the culture liquid was incubated at 50°C for 10 min. Reducing sugars were estimated by DNSA method and expressed as glucose equivalents (section 2.7.3).

One unit of amylolytic activity was defined as the amount of enzyme which released 0.1 mg of glucose per minute under the conditions of assay.

2.7.5 Assay of amyloglucosidase activity

The substrate used for amyloglucosidase activity determinations was 4-nitrophenyl-alpha-D-glucopyranoside (NPG) which is not attacked by alpha-amylase. The hydrolysis product was p-nitrophenol which absorbs between 360 and 420 nm in basic medium (Leisola et al., 1980).

The reaction mixture contained 0.5 ml of the enzyme preparation (10 - 50 µg of enzyme) and 0.5 ml of the substrate solution (2 mg NPG/ml in 0.01 M acetate, pH 4.2). Reagents were preheated to 50°C, enzyme was added and the reaction was allowed to proceed for 15 min. The reaction stopped by addition of 10 ml of 1% (w/v) Na_2CO_3 and the absorbances were read at 400 nm. The calibration plot for amyloglucosidase activity is presented in fig. 2.7.5.

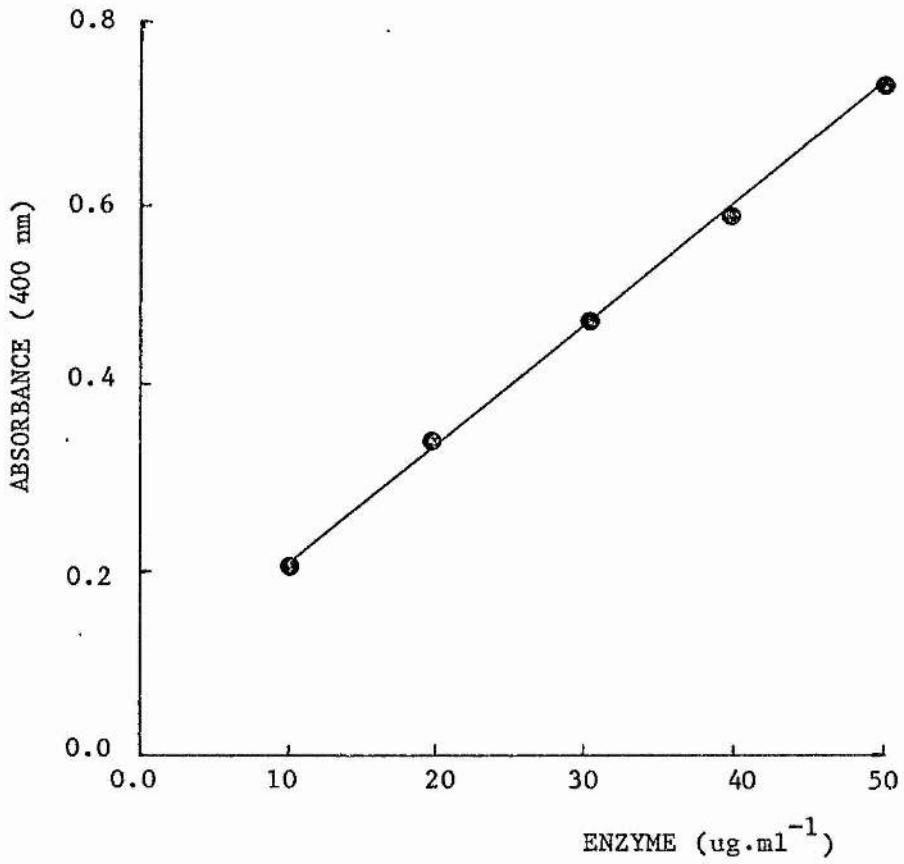


Fig. 2.7.5. Calibration plot for determination of amyloglucosidase activity.

2.7.6 Assay of alpha-amylase activity

The specific assay of alpha-amylase was measured as the release of coloured product from amylose azure which is independent of amyloglucosidase activity (Carvalho, 1974).

0.2% (w/v) amylose azure B grade was suspended in 0.1 M sodium acetate/acetic acid buffer, pH 5.5, containing 0.1% (w/v) bovine serum albumin and heated in a boiling water bath for 5 min. 4.9 ml of this suspension (pre-heated at 30°C, under stirring) and 0.1 ml of the enzymic preparation (containing 50 - 400 µg of enzyme) were incubated for 60 min. The reaction was stopped by addition of acetic acid. After centrifugation (600 x g/5 min), the absorbance of the supernatants was read at 595 nm against a blank in which water replaced the enzyme. The calibration plot for alpha-amylase activity is presented in fig. 2.7.6.

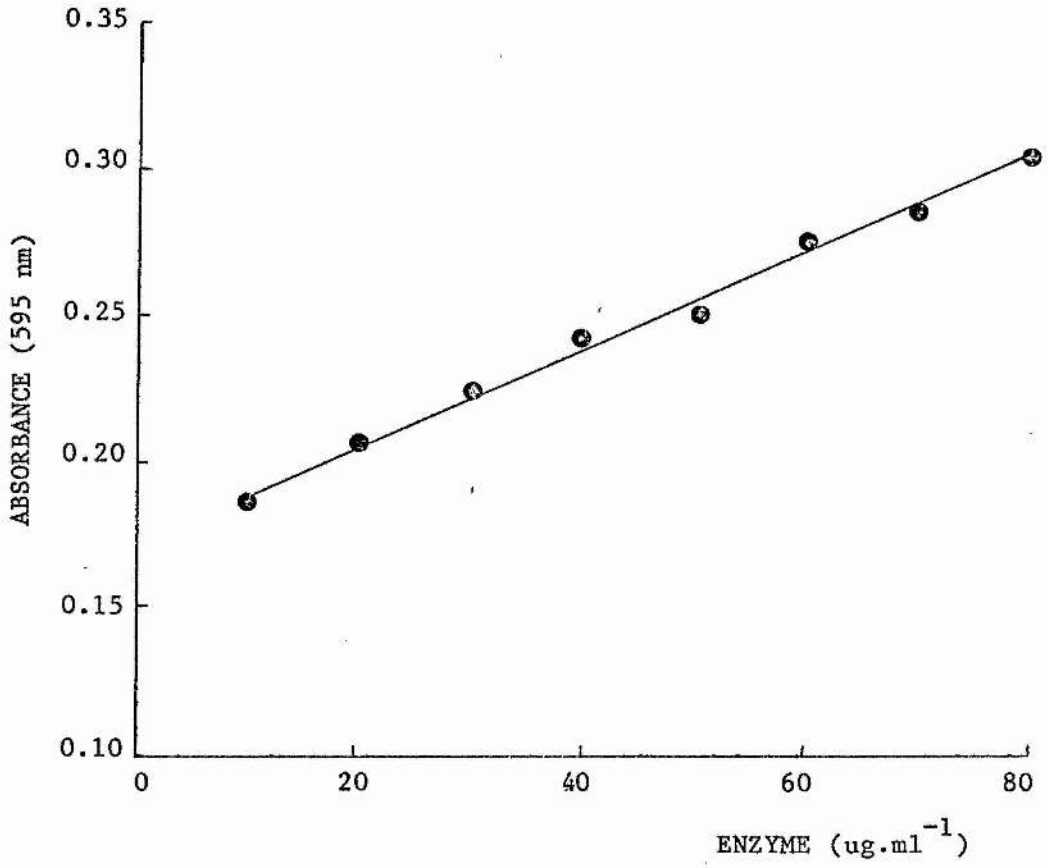


Fig. 2.7.6. Calibration plot for determination of alpha-amylase activity.

2.7.7 Determination of ethanol concentration

Gas-liquid chromatographic procedure was used to measure ethanol concentrations. Assays were performed on a Pye series 104 gas chromatograph (Unicam Instruments, Cambridge, England). The column was packed with Porapak Q 80-100 mesh (Waters Associates, Inc., Milford, Mass., USA) and was maintained at 200°C. The carrier gas was nitrogen at a flow-rate of 37 ml per min. The flame-ionization detector was operated at 250°C. Isopropanol was used as an internal standard. Aqueous standards were prepared containing ethanol in various concentrations from 1 to 9 mg per 1 mg of isopropanol. For each standard, the ethanol/isopropanol peak-height ratio was calculated and plotted against the respective concentrations of ethanol. Fig. 2.7.7 presents the calibration curve obtained.

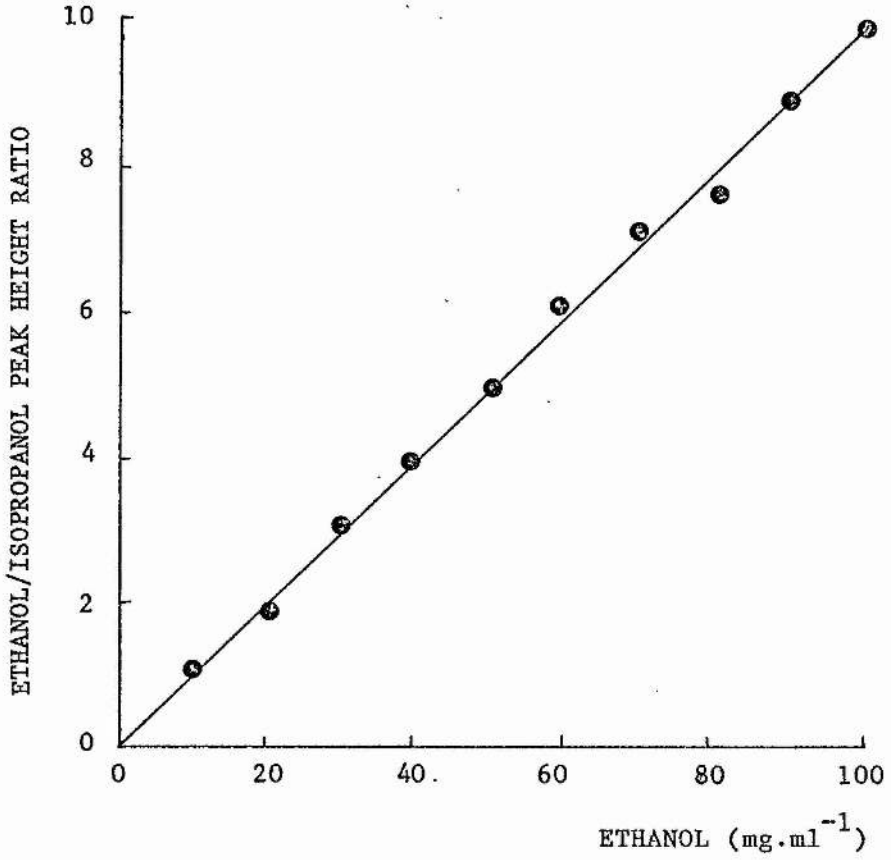


Fig. 2.7.7. Calibration plot for ethanol concentration measurements.

2.7.8 Determination of specific oxygen uptake rates

The specific oxygen uptake rates of immobilised cells were determined at 40°C using a Yellow Springs Oxygen Electrode System, and expressed in terms of mmoles.g^{-1}.h^{-1}. The reaction vessel was surrounded by a thermostatted water jacket. The electrode output was used to drive an AR 45 chart recorder. The reaction vessel (5 ml) was set up containing medium M-2. The medium was saturated with air and the immobilised cells were transferred from the shake flask into the vessel, which was then fitted with the electrode probe. An oxygen concentration of 0.21 mM in air-saturated medium at 40°C was the basis for calculating the oxygen demand. The dry weight of the cells used in the test was then determined (section 2.7.1.2) to provide the specific oxygen uptake rate.

3 RESULTS AND DISCUSSION

3.1 Optimisation studies

The following sections describe the results of experiments carried out with the aim of optimising amylolytic enzyme production by the immobilised cells of *Aspergillus niger*.

Unless specified otherwise, 2 g (wet weight) of immobilised cells were used to inoculate 50 ml of medium M-2 in 250 ml conical flasks and incubated in a rotatory shaker. Further experimental details for each experiment are given in the specific sections.

3.1.1 Cell loading

In order to find the maximum cell loading to be used in the immobilised system, different concentrations of fungal cells were added to a 1% (w/v) sodium alginate solution. The alginate-cell suspension obtained, loaded at various cell concentrations from 5 to 20% (wet weight/v), were used to produce 3-mm beads as described in section 2.3. It was observed that with concentrations above 10%, the alginate-mycelia slurry was too viscous to form beads when extruded. Therefore, it was decided to use a 10% (wet weight/v) cell loading for the subsequent experiments.

3.1.2 Influence of gel composition on enzyme synthesis

The influence of sodium alginate and calcium chloride concentrations utilised for the preparation of the immobilised Aspergillus niger beads on the production of amylolytic enzymes was examined under standard cultivation conditions described in section 2.4.1. The results are shown in table 3.1.2.

The ability of the beads to maintain their physical form is directly related to the sodium alginate and calcium chloride concentrations used for the preparation of the beads. When sodium alginate concentration less than 1% or calcium chloride concentration less than 0.1 M was used the beads did not gel properly and the beads obtained were easily disrupted within a few hours of incubation. With an increase in alginate or calcium chloride the beads become more stable in regard to their mechanical strength. On the other hand, a decrease in the amylolytic activity produced by the immobilised cells was obtained when the alginate concentration used to prepare the beads was increased from 1 to 2% (w/v); it was also found that the enzymic activity of the system decreased with calcium chloride concentration higher than 0.1 M. An explanation for these results is that, with an increase in alginate or calcium chloride concentration, there is greater resistance to diffusion of substrate into and product out of the beads (Cheetham et al., 1979).

concentration	amylolytic activity (U/50 ml)
sodium alginate (%, w/v)*	
1.0	140
1.5	132
2.0	130
calcium chloride (M)**	
0.10	140
0.15	130
0.20	126

* using 0.1 M calcium chloride
** using 1% (w/v) sodium alginate

Table 3.1.2. Effect of sodium alginate and calcium chloride concentrations used to prepare the immobilised A.niger system on the production of amylolytic enzymes, measured after 24 hours of cultivation.

3.1.3 Effect of incubation temperature

The immobilised cells were grown in the standard medium M-2 in shake flask cultures at 200 rev/min at different temperatures ranging from 30 to 50°C.

The results presented in fig. 3.1.3 reveal that the amylolytic enzyme production was higher when the cells were incubated at 40°C. This temperature is higher than the operating temperature generally used for industrial production of amylolytic enzymes in submerged cultures (25 - 28°C) (Banks et al., 1967).

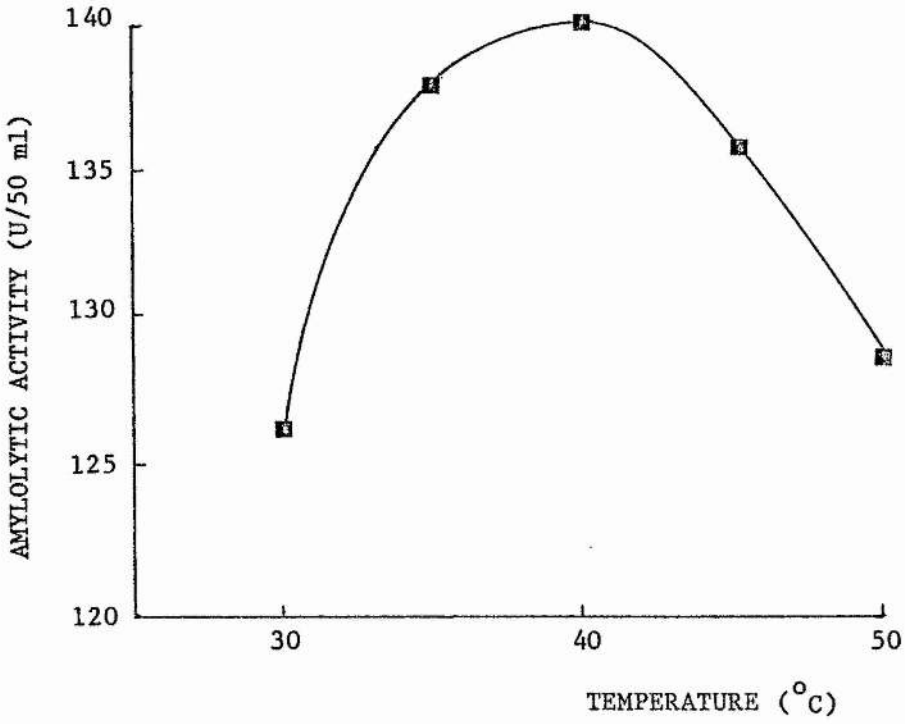


Fig. 3.1.3. Total amylolytic activity produced by immobilised A. niger at different temperatures, measured after 24 h of cultivation.

3.1.4 Effect of the initial pH of the medium

The effect of the initial pH of the medium on the enzyme production by immobilised cells of Aspergillus niger was examined. These experiments were carried out in similar conditions to those in the effect of temperature experiments, with the incubation temperature maintained at 40°C, and the initial pH of the medium ranging from 3.5 to 7.0.

The results are shown in fig. 3.1.4.

Variation of the initial pH of the production medium demonstrated that amylolytic enzyme synthesis was higher between pH 5.0 and 5.5. At pH values that were higher or lower than this range, a sharp decline in enzymic yield was observed. Similar ranges of optimum initial pH of the medium for amylolytic enzyme production by Aspergillus niger have been reported in earlier studies (Adams et al., 1947; Le Mense et al., 1949).

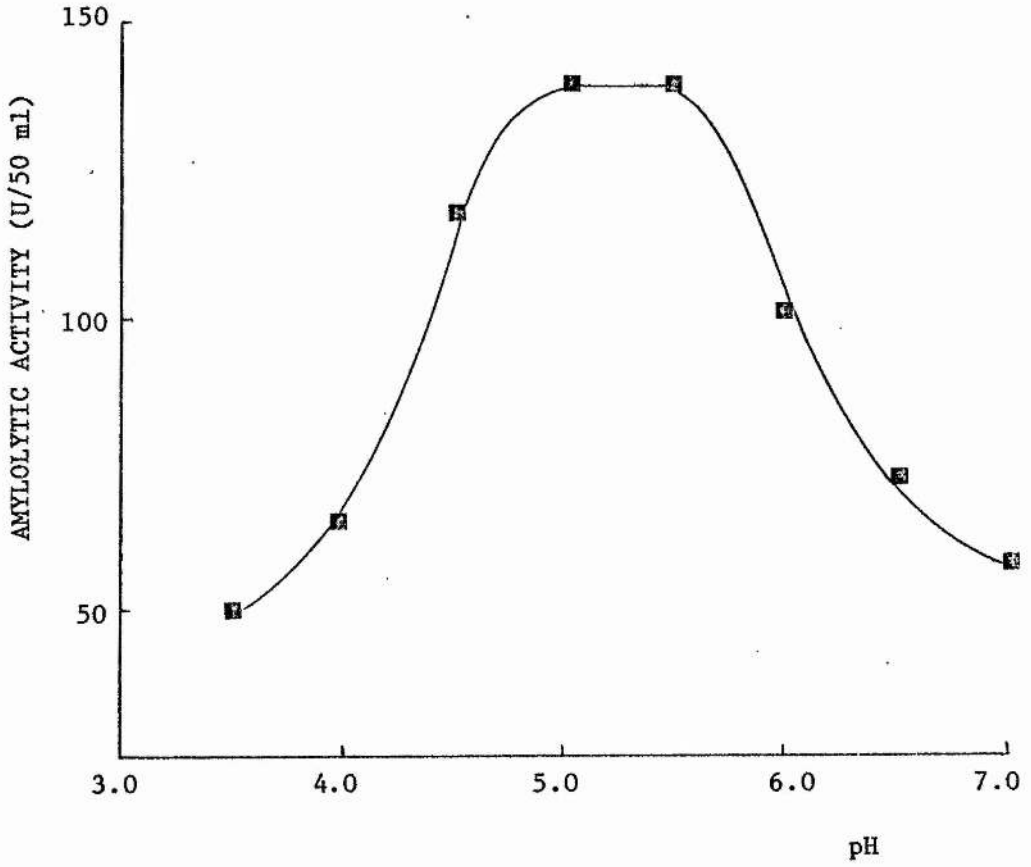


Fig. 3.1.4. Total amylolytic activity produced by immobilised A. niger at different initial pH, measured after 24 h of cultivation.

3.1.5 Effect of aeration rate

The influence of aeration in fermenter flask upon enzyme synthesis was examined to be sure that the shaking speed of 200 rev/min used in previous experiments provided sufficient aeration for maximal enzyme production. The experiments were carried out in 250-ml conical flask containing 50 ml of the production medium M-2 (initial pH 5.5), at 40°C, incubated with 2 g (wet weight) of the immobilised cells, under various shaking speeds on a rotatory shaker: 0, 100, 200 and 300 rev/min. Table 3.1.5 presents the results of these experiments.

It was found that only an insignificant amount of amylolytic activity was produced when the system was incubated without agitation; therefore, aeration is required for the amylolytic enzyme synthesis by the immobilised cells. However, the level of amylolytic activity after 6 and 24 h of incubation was independent of aeration rate (100, 200 and 300 rev/min). One possible reason for this behavior is that, due to the segregation between the microbial and fluid phase and consequent low viscosity of the culture liquid, the immobilised cell system can reach efficient oxygen transfer rates and exhibit full activity even at low aeration rates.

rate of stirring (rev/min)	amylolytic activity (U/50 ml) 6 hours	24 hours
0	5	5
100	90	140
200	95	140
300	95	135

Table 3.1.5. Effect of rate of stirring on the production of amylolytic enzymes produced by immobilised Aspergillus niger measured after 6 and 24 hours of cultivation.

3.1.6 Effect of the initial starch concentration

For these studies, the standard fermentation medium M-2 was used, with a starch concentration varying from 0.5 to 2.0%. The other experimental conditions were similar to those described in the influence of the aeration experiments.

The results of these experiments are shown in table 3.1.6.

An increase in the initial starch concentration from 0.5 to 2.0 g/100 ml in fermentation medium was found to increase the maximum enzymic activity, but the highest enzyme yield based upon the initial starch concentration in the medium was found with 0.5 and 1.0 g/100 ml.

starch concentration (g/100 ml)	amylolytic activity (U/50 ml)	enzymes yield (U/g)
0.5	70	280
1.0	140	280
1.5	150	200
2.0	160	160

table 3.1.6. Effect of initial starch concentration in the medium on the production of amylolytic enzymes produced by A. niger, measured after 24 hours of cultivation.

3.1.7 Influence of nitrogen source

The influence of different nitrogen sources was investigated by replacing peptone in fermentation medium M-2 by urea, ammonium sulphate and ammonium nitrate. The experiments were performed as described in the preceding sections (40°C; initial pH 5.5; 200 rev/min). All the nitrogen sources were tried at a concentration of 0.3 g of nitrogen per litre.

The results depicted in table 3.1.7 show that the difference in enzymic yield was only marginal for peptone, ammonium nitrate and ammonium sulphate. However, urea decreased the enzymic yield to about 50% in relation to the other three nitrogen sources screened.

nitrogen source	amylolytic activity (U/50 ml)
peptone	144
sodium nitrate	132
ammonium sulphate	140
urea	77

Table 3.1.7. Effect of different nitrogen sources (0.3 g N/l) on the production of amylolytic enzymes produced by immobilised A. niger, measured after 24 hours of cultivation.

3.1.8 Conclusions

Based on the results described in sections 3.1.1 to 3.1.7, the following parameters were chosen to proceed with the cultivation and with the immobilisation of *Aspergillus niger* in calcium alginate gel for amylolytic enzyme production.

i) cell concentration in the cell-sodium alginate slurry for preparation of the beads = 10% (wet weight/v).

ii) sodium alginate and calcium chloride concentrations used in immobilisation procedure = 1% (w/v) and 0.1 M, respectively.

iii) cultivation temperature = 40°C.

iv) initial pH of the medium = 5.5.

v) aeration rate = 200 rev/min.

vi) starch concentration in the medium = 1 g/100 ml.

vii) nitrogen source =peptone (0.2 g/100 ml).

3.2 Growth of immobilised fungal cells

This part of the work deals with experiments to investigate the growth of immobilised cells of *Aspergillus niger*.

The growth of the organism was studied in 250-ml conical flasks containing 50 ml of medium M-2 (pH 5.5) incubated at 200 rev/min in a rotatory shaker at 40°C. 2 g (wet weight) of the immobilised cells were used as inoculum. The mean diameter of the beads at the time of inoculation was 2,846 μm. Beads were removed periodically for determination of diameter, density and dry weight as described in sections 2.6 and 2.7.1.2, respectively.

3.2.1 Microscopic examination on immobilised cells

Microscopic examination of entrapped fungal cells in calcium alginate gel was performed. Section 2.5 describes the procedure followed. Fig. 3.2.1 (magnification 155 x) shows the appearance of immobilised cells immediately after immobilisation (fig. 3.2.1.a), and also after 6, 12 and 24 h of cultivation (fig. 3.2.1.b, c and d). The number of cells has clearly increased after incubation in reaction medium supporting the prediction derived from our starting hypothesis that the cultures confined to beads would be capable of sustaining cell growth up to high biomass concentrations.

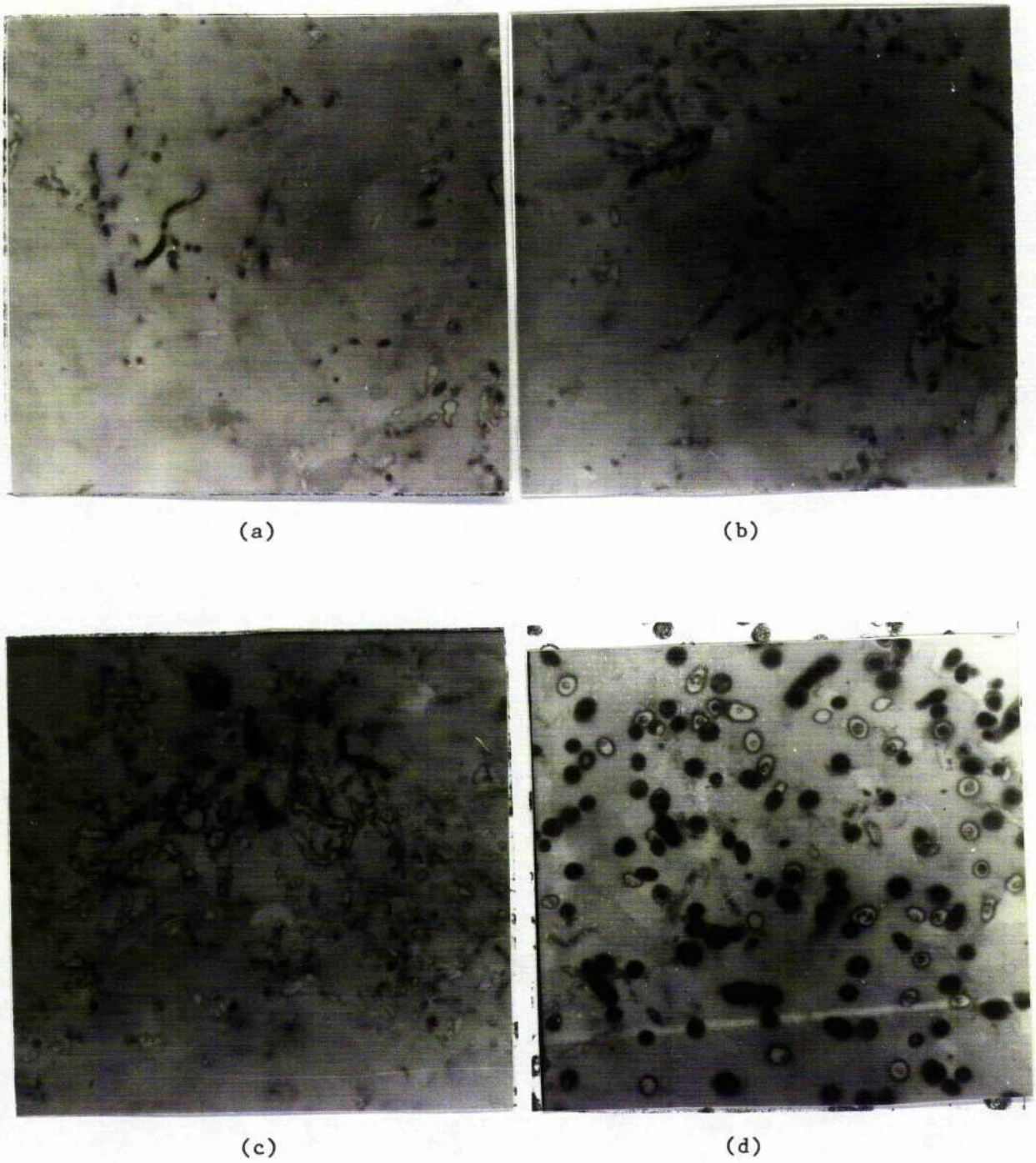


Fig. 3.2.1. Photograph of immobilised cells: immediately after immobilisation (a); after 6 h (b); after 12 h (c) and after 24 h (d) of cultivation. Magnification was 155 x.

3.2.2 Growth of immobilised and free cells of Aspergillus niger

Free and immobilised cells of Aspergillus niger, collected from the same culture sample, were cultivated as described in section 2.4.1. Samples were removed periodially for determination of biomass as described in section 2.7.1.

After 24 h of incubation (fig. 3.2.2), the biomass in the immobilised system reached 8.8 mg dry wt/ml and the concentration of free cells reached 7.0 mg dry wt/ml. The final concentration of the entrapped cells was, therefore, 1.26 fold than that of freely grown cells.

It could be assumed the following reasons for the better growth observed in the immobilised system:

The immobilisation facilitates nutrient and oxygen diffusion into the cells by altering the rheological properties of the suspending medium. Because the cells are agglomerated in the gel beads, fluid viscosity is lower than in free cell suspension. And, lower viscosity contributes to better mixing and mass transfer properties in the immobilised cell system and consequently to a more efficient nutrient availability.

Another factor may also enhance mass transfer in the immobilised cell system. Solid suports, in dilute media, may concentrate nutrients near the surface of the gel. The fungal cells entrapped in the gel are exposed, in their microenvironment, to higher nutrient concentration than exist in the bulk solution. Thus, higher cell population is possible (Abbot, 1977).

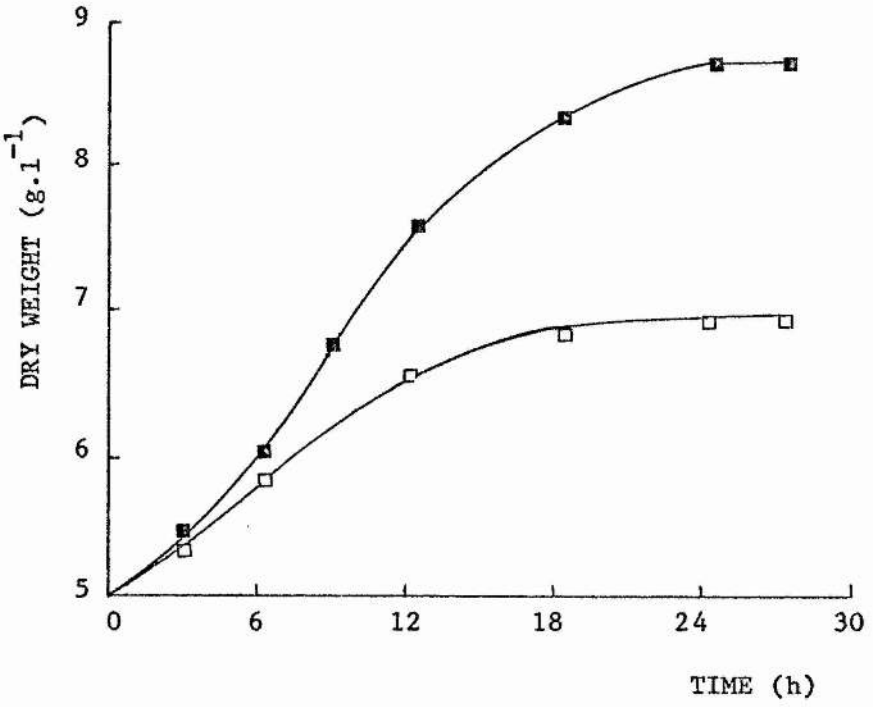


Fig. 3.2.2. Time profile of mycelial growth in shake flask cultures for free (□) and immobilised (■) cells cultures.

3.2.3 Growth pattern

The growth of fungi in the form of pellets has received wide attention in recent literature (Metz and Kossen, 1977; Witaker and Long, 1973; Suijdam et al, 1982). However, there is no consensus about which growth kinetics are best applied to pellets.

Certain workers (Emerson, 1950; Trinci, 1970) have found that the growth of fungi in the form of pellets can be represented by a cube root relation between cell concentration and the time of growth; this "cube root growth" is described by the equation (Pirt, 1966):

$$M^{1/3} = kt + M_o^{1/3} \qquad (1)$$

where M is the dry weight of mycelium per ml at time t, k is a constant, and M_o is the dry weight of mycelium per ml at time zero.

On the other hand, Choudhary and Pirt (1965) and Gillie (1968) found that pellets of fungi grown according to the exponential law, that is

$$M = M_o(\exp)^{kt} \qquad (2)$$

where M is mass of mycelium/ml at time t and k is a constant called specific growth rate.

Pirt (1966) suggested that the two types of growth kinetics may be explained in terms of different textures of pellets and the way the hyphae develop inside the pellets. Pellets with a texture sufficiently loose to permit oxygen and other nutrients in the medium to penetrate and supply the inner mycelia, and hence all hyphae being able to contribute to the pellet growth, would grow exponentially until either lack of internal space or lack of nutrients limits growth.

On the other hand, pellets composed of densely packed hyphae consist of an outer thin layer of growing cells and an inner mass which is prevented from growing by lack of nutrient. This is likely to occur because the pellet mass is proportional to the cube of its radius, whilst the surface (through which the substrates must diffuse) increases only with the square of the radius. After a while only a peripheral shell of the pellet would have sufficient substrate to maintain exponential growth.

If M is the mass of organism in a pellet, then the growth rate of the pellet is:

$$dM/dt = kM \qquad (3)$$

and, if r is the radius of the pellet and w is the width of the peripheral growth zone:

$$dr/dt = wk \qquad (4)$$

or

$$r = kwt + r_o \qquad (5)$$

If p is the pellet density, then

$$M = 4\pi r^3 p/3 \qquad (6)$$

substituting for r in equation 5 , it gives for the growth in mass of a pellet:

$$M^{1/3} = (3/4\pi p)^{-1/3} kwt + M_o^{1/3} \qquad (7)$$

Thus, the growth of dense mycelial pellets is predicted to follow a cube root growth pattern.

Morphological observation of section of pellets (Foster, 1949) and studies of the respiratory activity inside mycelial pellets (Yoshida et al., 1968) support Pirt's theory about the growth of mycelial fungi in form of pellets.

Logarithmic plot of growth curve (fig.3.2.3.a) of the immobilised cells of Aspergillus niger showed that the organism increased in dry weight at an exponential initial phase of growth, followed by a phase of declining growth rate.

This initial phase of growth gave a specific growth rate of 0.039 h^{-1}. This value is not significantly different of the specific growth rate of the organism (0.040 h^{-1}; calculated from fig. 3.2.3.b) when grown in free-cell cultures starting from spores, at the same experimental conditions (described in section 2.3). These results are consistent with an exponential growth unlike "cubic" growth occurring for the immobilised Aspergillus niger as prepared in the present investigation. In fact, the specific growth rate of the fungi in free-cell culture, emerging from spores, presumably represents its maximum value for the conditions. The specific growth rate of the organism entrapped in beads would be expected to decrease in respect to that of the free organism if growth was "cubic". Trinci (1970) reported that the specific growth rates of Penicillium chrysogenun and Aspergillus nidulans pellets growing, according to the cubic root law, were 66% and 43%, respectively, of the supposed maximum specific growth rates of those organisms.

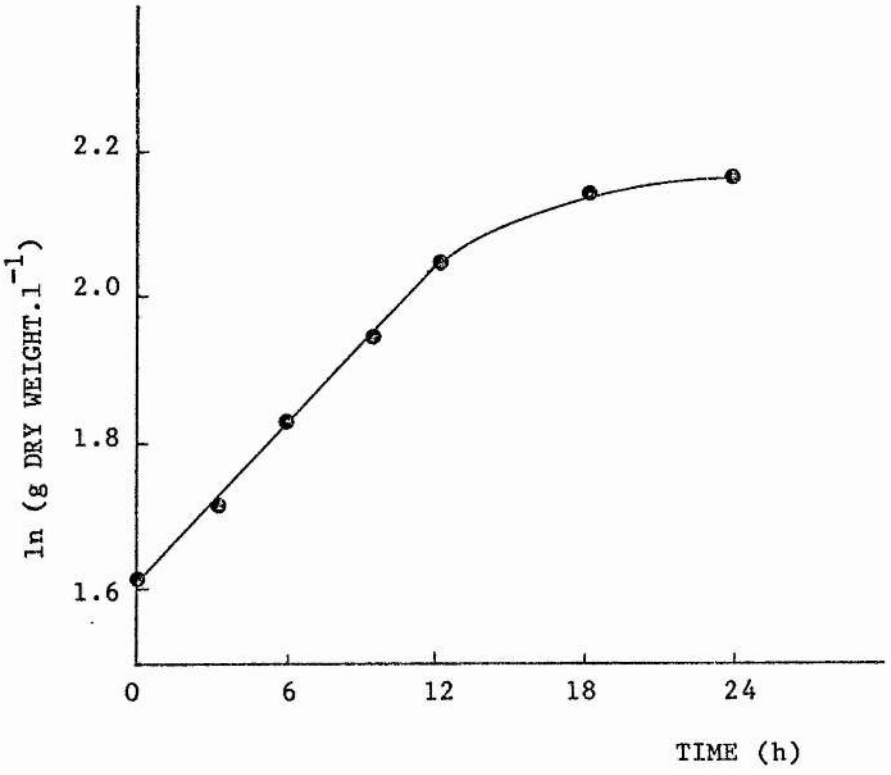


Fig. 3.2.3.a. Changes in the logarithm of mycelial dry-weight/l with time after inoculation in a culture of immobilised cells of A. niger.

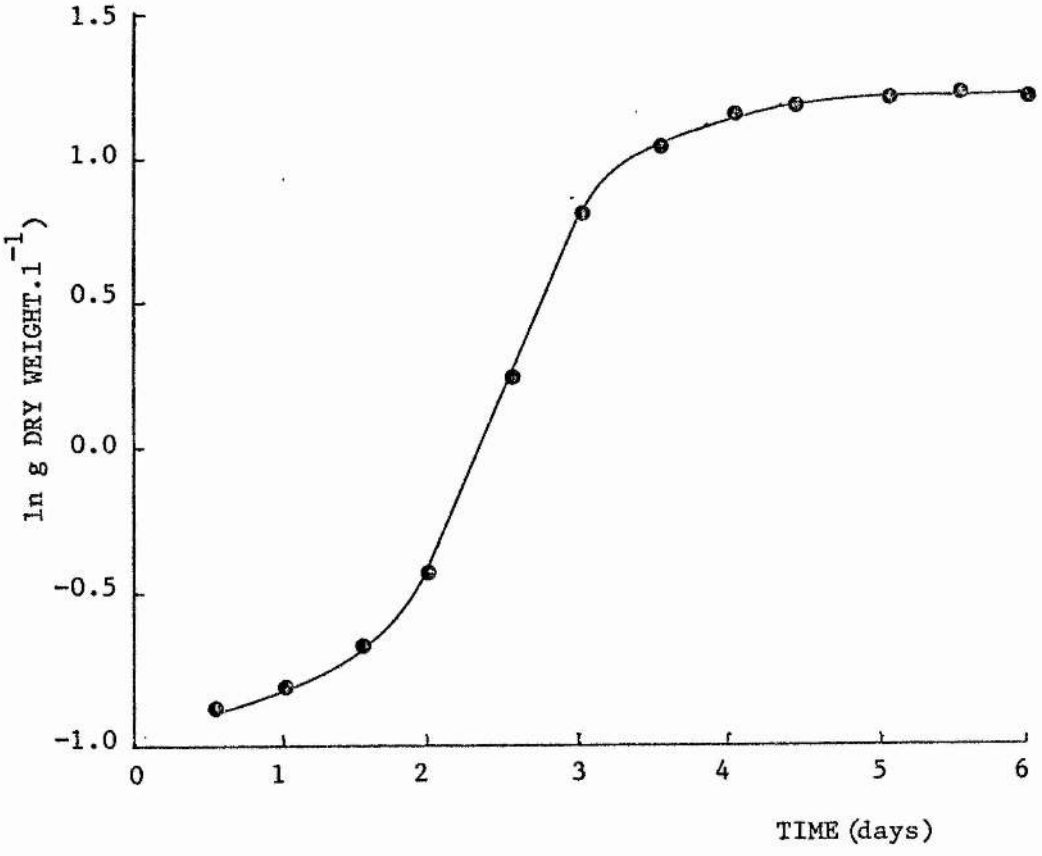


Fig. 3.2.3.b. Changes in the logarithm of mycelial dry-weight/l with time after inoculation in a batch culture of A. niger.

3.2.4 Variation of the bead radius during growth

Further characterization of growth of the immobilised cells was carried out by examining the changes in size of the mycelial particles during the fermentation time.

The mean radius of the beads at the time of inoculation was 1,423 μm. The beads initially increased in size at a constant radial growth rate of 6.25 μm per hour until the end of the exponential growth phase. At this stage the mean radius of the beads was about 1,500 μm. Then, the radial growth rate slowed to 3.75 μm per hour corresponding to the second growth phase (fig 3.2.4).

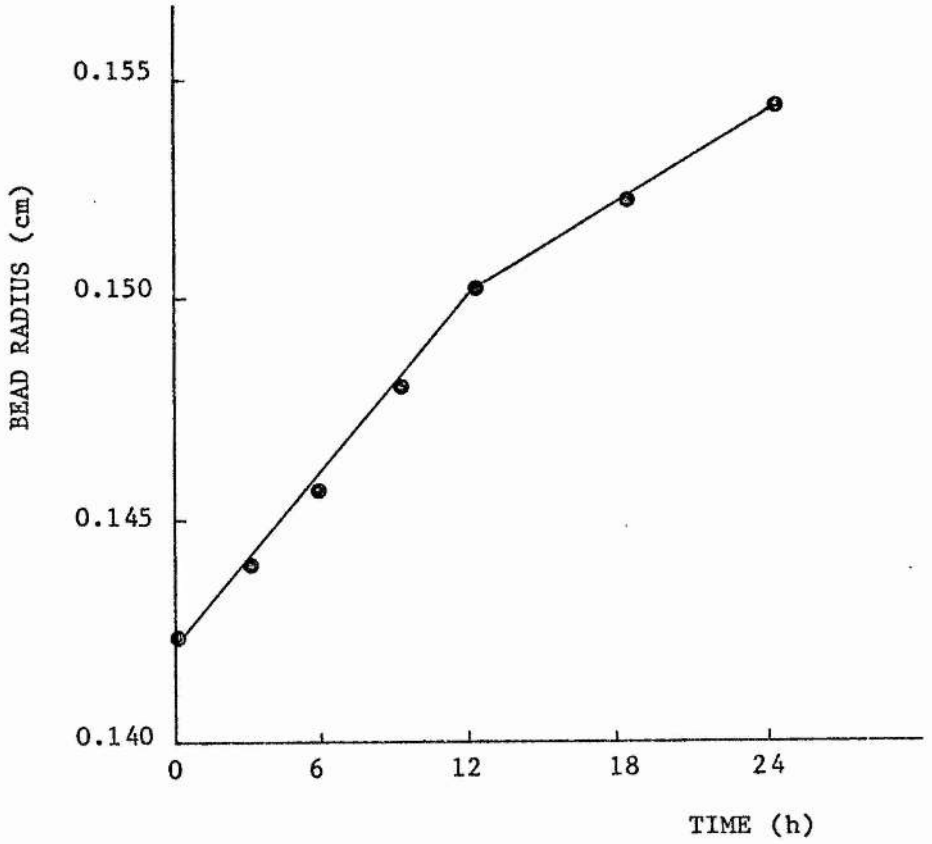


Fig. 3.2.4. Increase in radius of immobilised A. niger beads with the time after inoculation in a batch culture.

3.2.5 Density of the beads

Measurement of the density of the pellets is a key factor in the design of a pellet process and can be used as an indirect indication of the way in which the hyphae develop (morphogenesis) inside the pellets (Metz, 1976; Suijdam, 1980).

Metz (1976) suggested two alternative models to describe the density of mycelial pellets as a function of their radius during a fermentation process. The first model assumes that no branching of hyphae takes place inside the pellet, thus only the length of hyphae increases. This model predicts a decrease in pellet density with increasing pellet radius. The second model is based upon branching of hypha in such a way that the number of growing tips per unit hyphal length stays constant and predicts an increasing pellet density with pellet radius. Based on his own results and on those reported by Yano et al. (1961) and Kobayashi and Suzuki (1972), in which the pellet density decreased with increasing pellet diameter, Metz concluded that the hyphae in a pellet do not grow in a way that agrees with a constant hyphal growth unit model. This is in contrast with the growth of filamentous mycelium where Metz actually did find a constant hyphal growth unit.

The mean density of the beads entrapping the immobilised mycelia of Aspergillus niger used in this study was plotted against the bead radius in fig. 3.2.5. The results show an increasing bead density with bead radius. Therefore, the immobilised mycelia-calcium alginate beads behaved as could be expected if hyphal growth with branching occurred, which may be ascribed to the lattice structure in calcium alginate gel which permits a loose packing of hyphae into void space.

The fig. 3.2.5 also shows that, when the beads exceeded the radius of 1,500 μm (mean density higher than 69 kg/m^3) the density of the beads tended to keep constant with the increasing radius. As this corresponds to the time in which the growth rate declines the change in growth rate seems to occur due to mycelial compression within the beads and consequent limitation of nutrient diffusion into the beads.

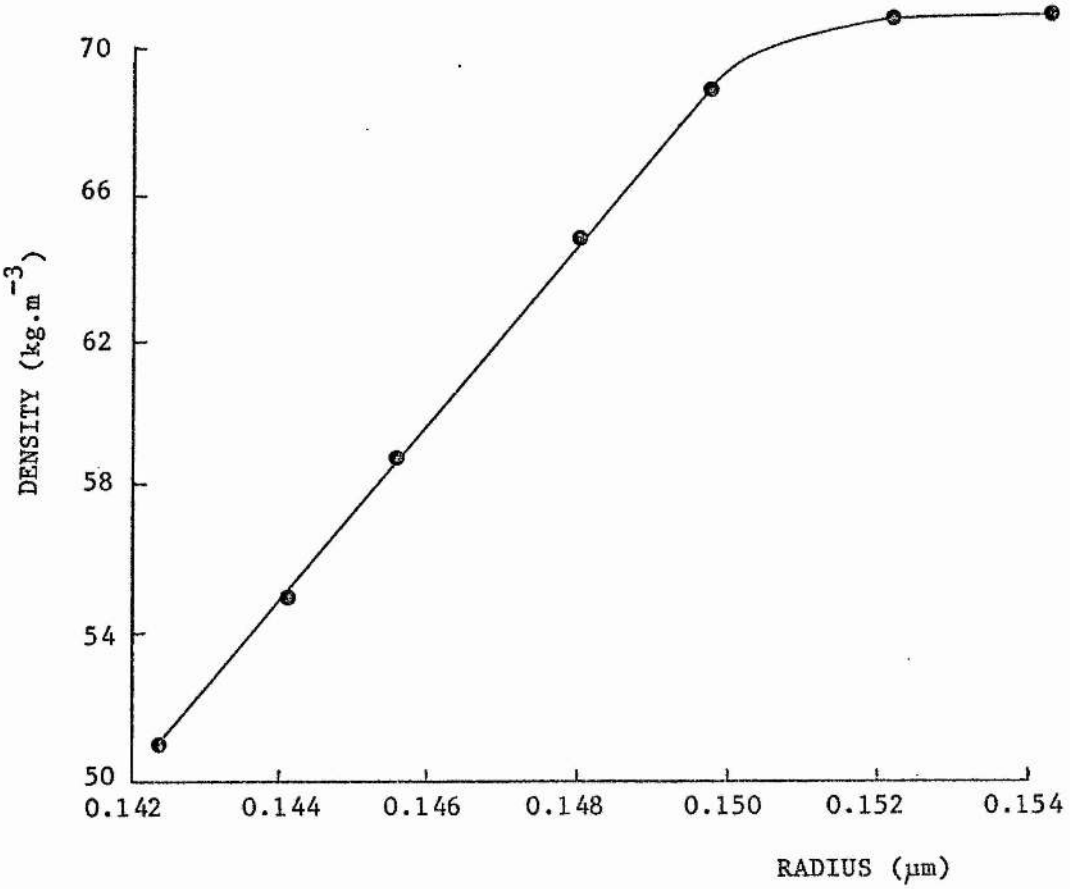


Fig. 3.2.5. Density of the beads of immobilised A. niger vs. radius.

3.2.6 Peripheral growth zone of the beads

In the phase of limited hyphal growth the beads were presumably composed, as suggested by Pirt (1966), of a peripheral growing zone and an inner mass unable to grow because of lack of nutrients. The value for the width of growth zone contributing to radial extension of the beads may be calculated from the equation proposed by Pirt (1966) to describe the pellet growth:

$$r = wkt + r_o$$

or

$$w = (r-r_o)/t \times 1/k$$

where $(r-r_o)/t$ corresponds to the radial growth rate during the slow growth phase and k is the specific growth rate of the organism.

The proportion of the peripheral growing zone contributing to the radial extension of the beads (w), determined by using this equation, was 96 μm. This value is only somewhat higher than the theoretical value found by Pirt (1966) for w with oxygen as the growth-limiting nutrient (77 μm). This result, together with the fact that glucose was still plentiful at the stage of change from exponential to declining growth rate (see section 3.3.2), suggests that oxygen could be the growth-limiting nutrient in the second growth phase of the immobilised cell system.

3.3 Production of amylolytic enzymes by Aspergillus niger

In this part, some experiments were undertaken with a view to studying the effect of the immobilisation procedure on the synthesis and on some of the catalytic properties of amylolytic enzymes produced by the strain of Aspergillus niger used in this work.

3.3.1 Amylolytic enzyme production by free and immobilised cells.

Enzyme production was studied in 250-ml conical flasks containing 50 ml of medium M-2 (pH 5.5) incubated at 40°C, in a rotatory shaker at 200 rev/min. 2 g (wet weight) of cells were used as inoculum. Periodically, samples of the fermentation broth were collected for assay of enzyme activity. Inocula were obtained (see section 2.3) from the same original culture sample; consequently, it was assumed that they were physiologically identical.

The time course of amylolytic enzyme synthesis by free and immobilised cells are shown in fig. 3.3.1. Amylolytic activity gradually increased and reached maximum level after 24 h cultivation in both systems.

It is evident that the immobilisation in calcium alginate did not prejudice the synthesis of amylolytic enzymes by Aspergillus niger. As a matter of fact, the entrapped cells exhibited higher activity than the free cells. The averaged volumetric enzyme productivity, obtained over the entire period of fermentation (U/50 ml.h), was 16.7% higher in the immobilised cell system; note if the comparison is made based on maximal productivity rate it becomes even more favourable for the immobilised cell system, i.e., 33% higher.

In addition to productivities, it is also necessary to examine the concentrations of the product in both cases, as the titer value is crucial with regard to enzyme isolation and purification. Data from fig. 3.3.1 indicate that the immobilised cells yielded a final amount of amylolytic activity (140 U/50 ml) higher than that obtained in the free cell culture (120 U/50 ml). The higher enzyme level produced by the immobilised system may be a consequence of the higher biomass level reached, as seen in the section 3.2.3.

Further, the final free-cell culture was too viscous and collection of the washed cells from the reaction medium was difficult (vacuum filtration had be used). On the other hand, the immobilised cell system presented a relatively low viscosity and the beads were

easily separated from the liquid phase. Beads remained intact for the duration of the experiment and no leakage of the cells was observed. These aspects can be regard as an important advantage for the immobilised fungi process. The relatively lower viscosity of the immobilised system considerably enhances the mixing and mass transfer properties of the reaction medium which would lead to saving in agitation and aeration costs, and the easier separation of the biomass from broth could means considerable saving in energy costs for recovery step process.

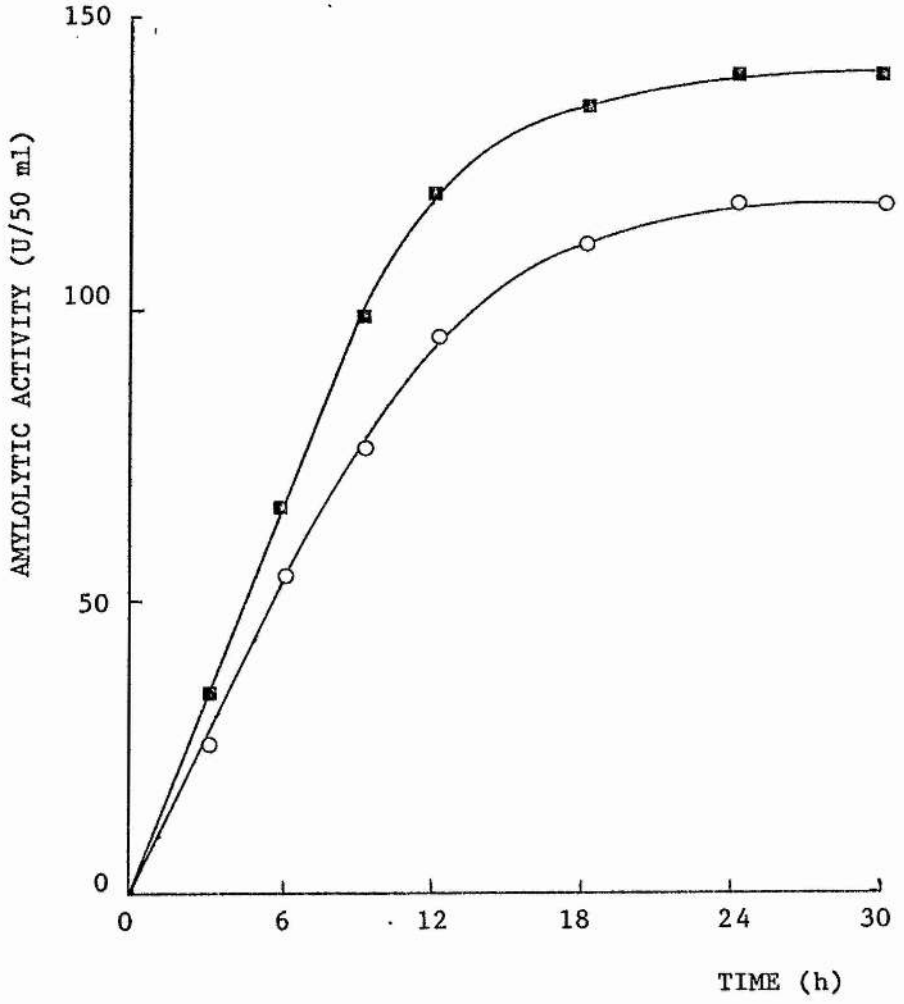


Fig. 3.3.1. Time course of the amylolytic enzyme production by free (○) and immobilised (■) cells of A. niger.

3.3.2 Fermentation pattern of enzyme production

Fig. 3.3.2.a shows that the amylolytic enzyme production by the immobilised cells of Aspergillus niger presents a growth-associated production pattern. Enzyme synthesis proceeded simultaneously with cell growth, the enzyme activity in culture liquid increasing proportionally to the increase of biomass. Maximum amylolytic activity was concomitant with the maximum biomass concentration (about 24 h) and the fastest rate of enzyme production occurred during the phase of exponential growth. The enzyme production stopped after 24 h of fermentation; at this stage, the respiratory activity of the immobilised cells dropped rapidly suggesting loss of cellular viability which may be related to glucose depletion in the culture fluid (fig. 3.3.2.b). In fact, during the first nine hours of cultivation, glucose accumulated to the relative high level of 47% (4.7 mg of glucose/ml) of the original starch concentration; after this, glucose disappeared gradually from cultivation medium and it was completely utilised within 18 h (fig. 3.3.2.b). Since a relatively high concentration of glucose was present in the medium during the production of enzymes, it seems that catabolic repression (by glucose) of the enzyme synthesis was absent. The pH of the medium dropped from 5.5 to 3.0 suggesting synthesis of organic acids during glucose metabolism (fig. 3.3.2.b).

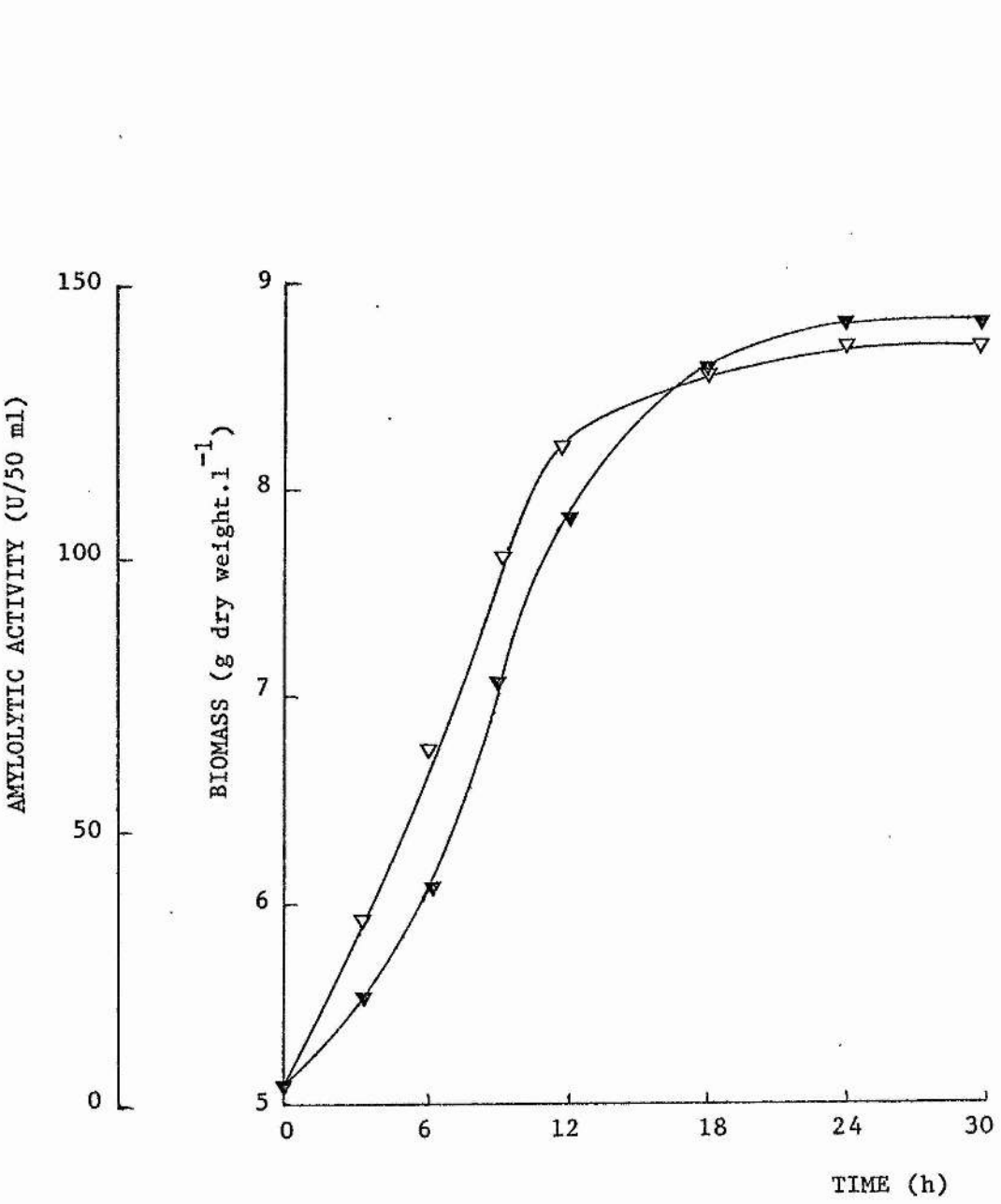


Fig. 3.3.2.a. Growth (▼) and amylolytic-producing activity (▽) of immobilised cells of A. niger.

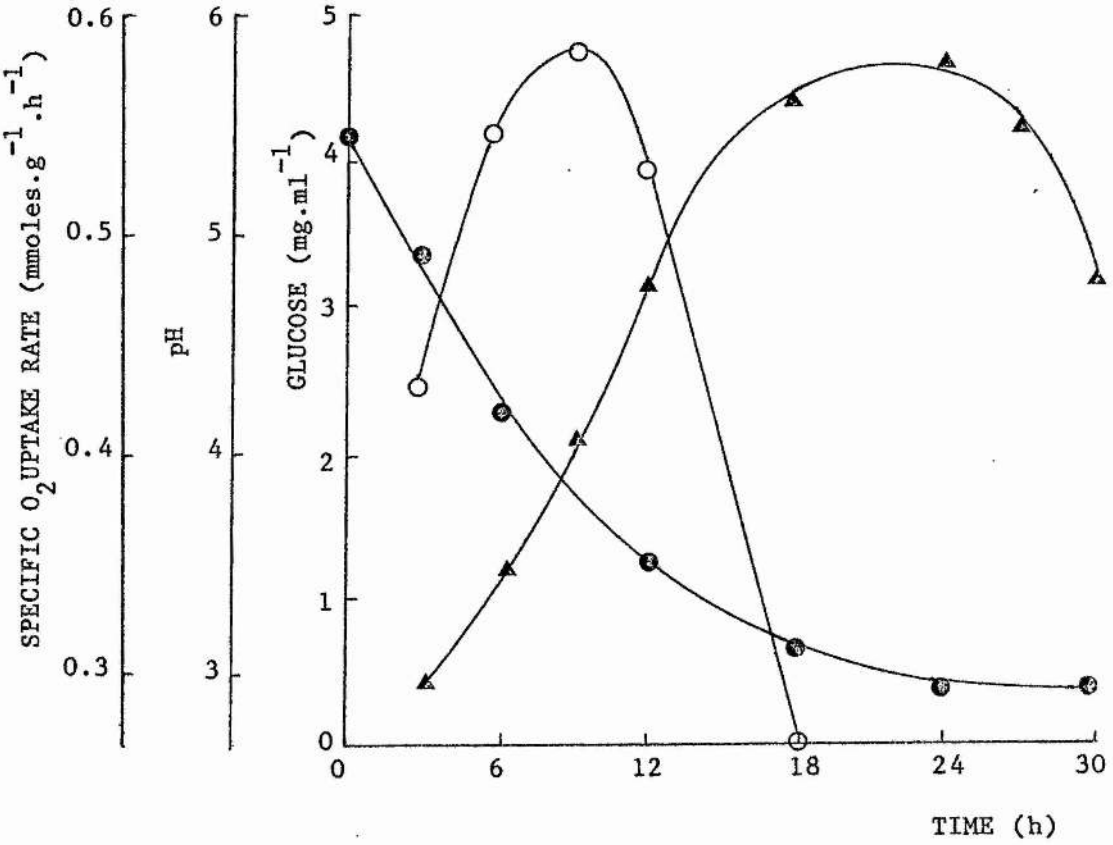


Fig. 3.3.2.b. Time course of glucose concentration (○), pH variation (●) and respiratory activity of the immobilised A. niger (▲) during incubation.

3.3.3 Operational stability of immobilised and free cell system

For operational stability experiments 2 g (wet weight) of cells were incubated in 250-ml conical flasks containing 50 ml of medium M-2 (pH 5.5) at 40°C in a rotatory shaker at 200 rev/min. After 24-hours cultivation the liquid phase of the culture was drained off and replaced by fresh medium, and its amylolytic activity was determined. Beyond the third cycle, the replacement of the medium was made every 12 hours because analysis of glucose content of the medium revealed that it had completely disappeared between 6- and 9-hours fermentation. Results are shown in fig. 3.3.3.a.

The enzyme producing activity of immobilised cells was retained up to ten runs and then the experiment was interrupted because disruption of the beads was observed. Also, increase of the size of the beads was evident, presumably due to growth of the cells in the gel (fig. 3.3.3.b). The growth of the immobilised mycelia during the several cycles offers an obvious explanation for the increase of production of the amylolytic enzymes.

On the other hand, the amylolytic activity of free mycelia decreased gradually with sucessive cycles. In this system, the retention of activity was, presumably, prejudiced by a severe oxygen limitation due to the very viscous suspension produced. In fact, it has been reported that the oxygen mass transfer coefficient could be reduced by 90% due to the presence of Aspergillus niger mycelium at a concentration of 2% (dry weight/v)(Brierley and Steel, 1958). Moreover, separation of free mycelia from the liquid phase was very difficult and sporulation was clearly evident at the fourth cycle.

Thus, the higher operational stability obtained with the immobilised cell system over the free cell system should be related to the modification of the nature of the broth due to confining growth of mycelial cells within the beads, which provided far more favourable conditions for mass transfer. As a result the onset of dissolved oxygen limitation is delayed and the growth of the culture is prolonged to reach higher biomass and productivities.

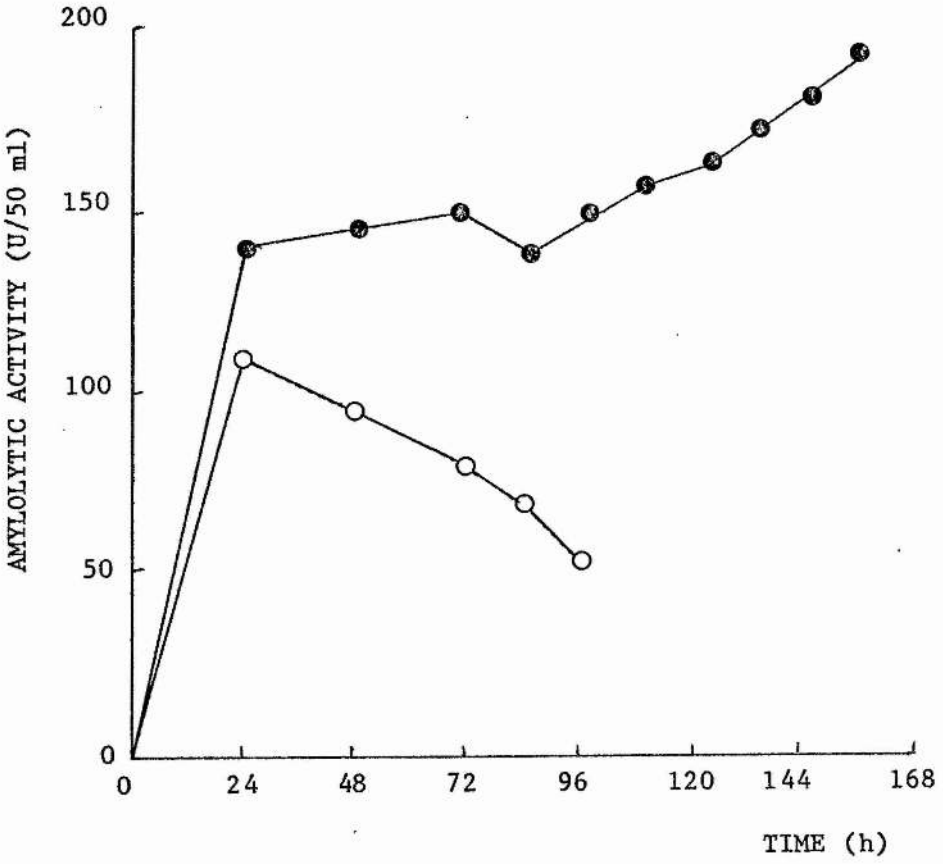


Fig. 3.3.3.a. Production on amylolytic enzymes by free (○) and immobilised (●) cells of A. niger during repeated incubations.

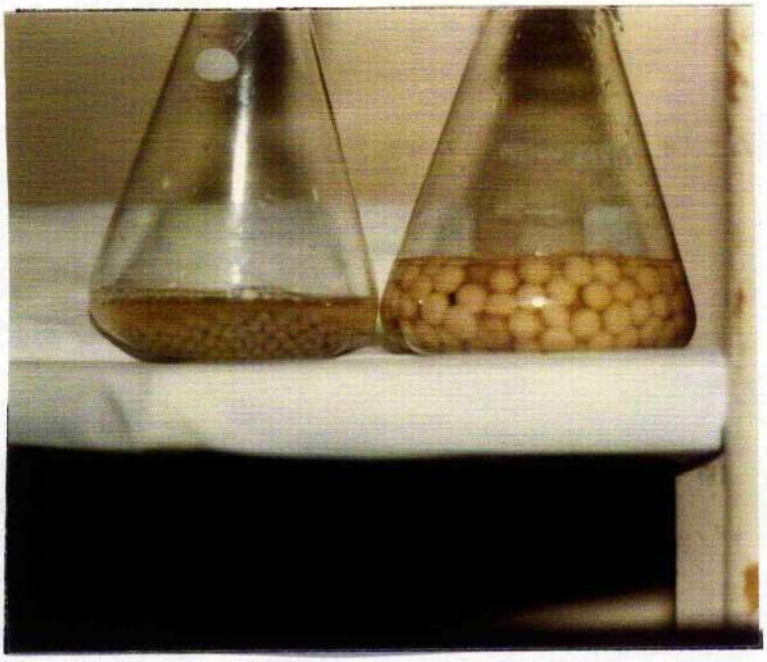


Fig. 3.3.3.b. Size of immobilised A. niger beads after 24 h (1st. cycle) and after 156 h (10th. cycle).

3.3.4 Continuous production of amylolytic enzymes

Shake flask cultures revealed that the synthesis of amylolytic enzymes by immobilised cells of Aspergillus niger is a growth-associated (section 3.3.2) and strictly aerobic process (section 3.1.5), which are characteristics that strongly rule out the possibility of use as a fixed-bed operation fermenter. Therefore, the experimental apparatus described in section 2.4.2 was constructed to be used in a fluidized-bed operation manner. Magnetic stirring could not be used to prevent bead disruption and a high air flow rate (1 vvm)was necessary to provide aeration of the whole bed. The sterile feed was held in a 20 l container and replaced as necessary.

The mean retention time was determined on basis of the working volume:

$$\text{retention time} = V/F$$

where V is the working volume (ml) and F is the volume of entering medium per time unit (ml/h).

Amylolytic enzymic productivities were defined on basis of the residence time as:

$$P = E/\text{retention time}$$

where P is the amylolytic enzymic productivity (U/ml.h) and E is the

enzymic activity (U/ml).

Initially, a suitable medium for continuous production of amylolytic enzymes by the growing immobilised mycelia was investigated (fig. 3.3.4.a and 3.3.4.b).

By continuously feeding with the complete medium M-2, the mycelia grew in the gel beads and after 2 days the particles reached a weight of 4.3 mg (dry weight) per bead. The beads, at this stage, were of an excessively large size and they tended to grow together forming aggregates and, consequently, the fluidization of the bed virtually ceased. The amount of enzyme produced was proportional to the increase in biomass.

An experiment in shake flasks culture (table 3.3.4) showed that cell growth within the gel could be restrained by using a nitrogen-limited medium. This possibility of cell growth control in the FBF, aimed at restricting the biomass level to one which still permitted free movement of the beads, was investigated.

To test the effect of a complete nitrogen starvation, after 12 hours of continuous operation in a unlimited medium (medium M-2) the fed solution was replaced for another medium consisting of medium M-2 without peptone. The cells in the gel could not grow in this medium completely deprived of nitrogen. Moreover, as soon the replacement of the medium was done the production of amylolytic enzymes dropped dramatically.

Next, the concentration of peptone in medium M-2 was deliberately limited to 0.02 g/100 ml. This was then used to feed the FBF after 12 hours of continuous operation with the complete medium. The result presented a slow growth rate, significantly reduced in relation to that of the experiments with complete medium. The system produced increasing amounts of amylolytic enzymes over a period of 12 days. Productivities were maintained higher than 0.14 U/ml.h beyond the third day of continuous operation (fig. 3.3.4.c). At the end of the 12th day the size of the bead was again excessively large and the bed fluidization very difficult. The experiment was then interrupted.

These results indicate that a limited-nitrogen medium, which could still permit cell growing, can be used for continuous production of amylolytic enzymes by immobilised Aspergillus niger.

A fluidized-bed fermenter appears to be an attractive proposition for the continuous production of amylolytic enzymes by immobilised fungi. The use of such apparatus leads to possibilities for achieving good heat transfer, mass transfer and mixing characteristics. In these experiments, a relatively high productivity was achieved; the immobilised cells did not suffer from washout and the viscosity of the fluid phase was kept at a low value . Further developments in this system, however, are necessary to control of the particle size due to the mycelial growth, and so to achieve a steady biomass hold-up in the fermenter compatible with good mechanic fluidization.

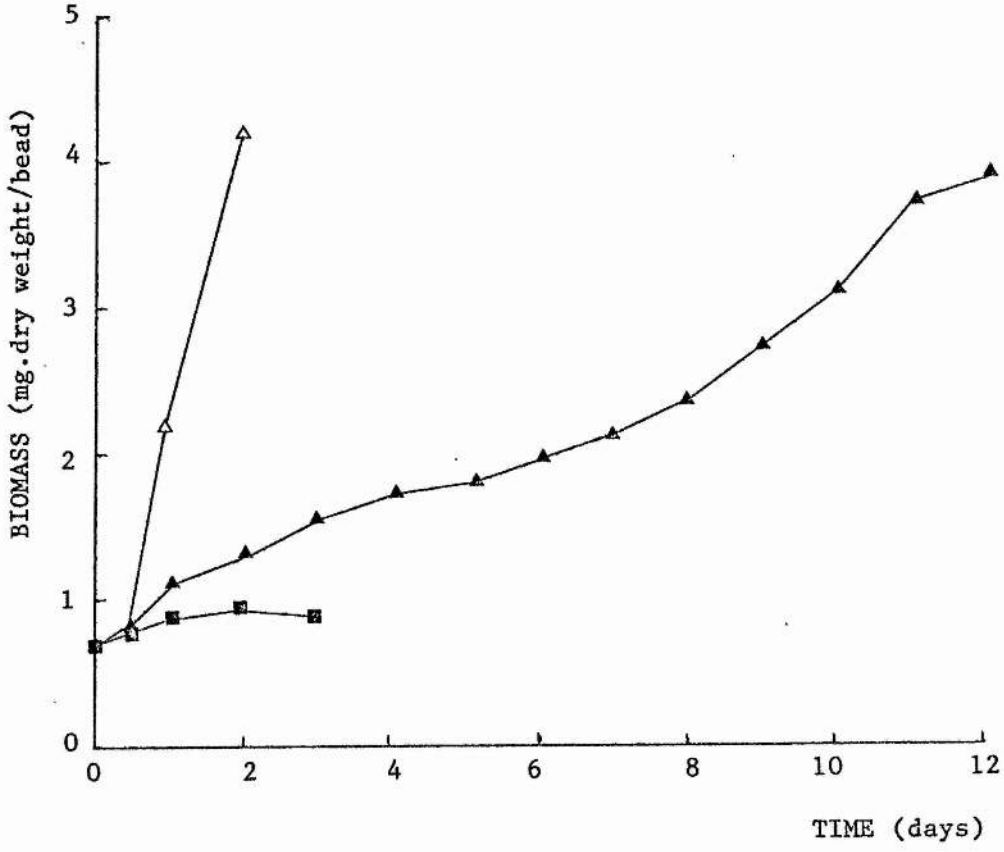


Fig. 3.3.4.a. Growth of immobilised A. niger cells in the FBF. The fermentation was carried out using medium M-2, except for peptone: (△) 0.2% peptone; (▲) 0.02% peptone; (■) 0% peptone.

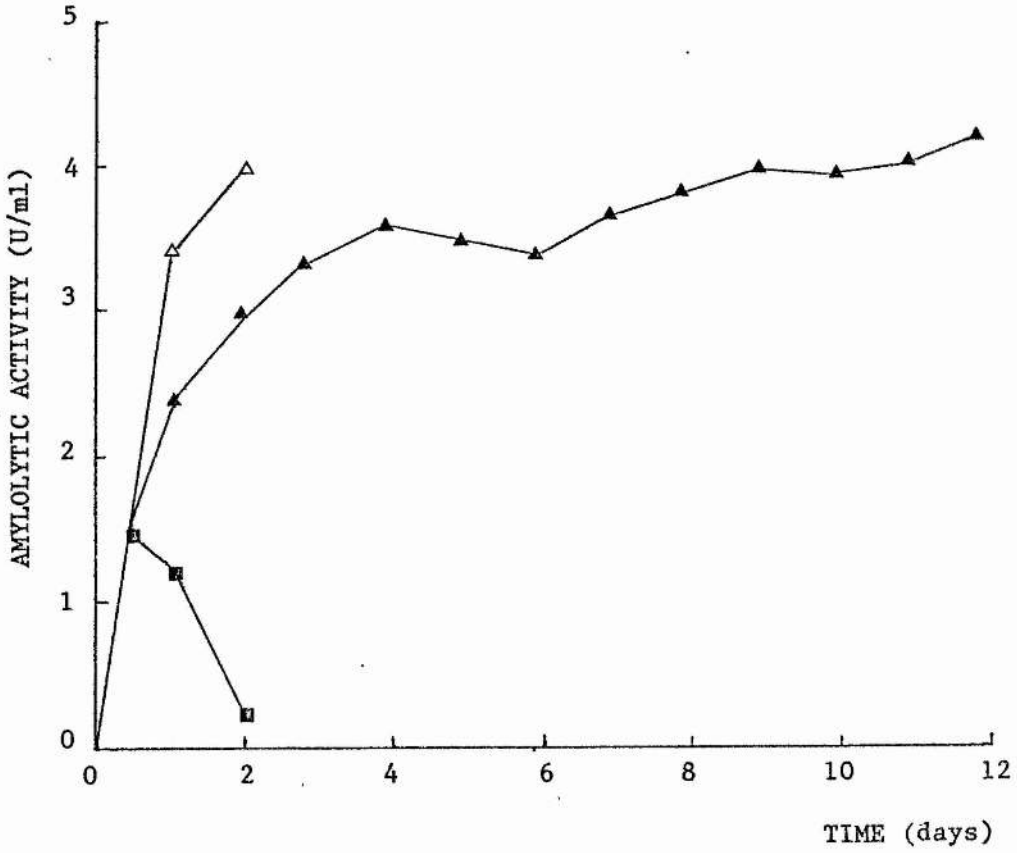


Fig. 3.3.4.b. Production of amylolytic enzymes by immobilised A. niger in FBF. The fermentation was carried out using medium M-2, except for peptone: (△) 0.2% peptone; (▲) 0.02% peptone; (■) 0% peptone.

peptone in medium (g/100 ml)	gain in biomass after 24 h (mg dry weight/beads)
0.00	0.90
0.05	0.98
0.10	1.06
0.20	1.10

Table 3.3.4. Effect of nitrogen concentration in the medium on growth of immobilised A. niger. Experiments were conducted in 250 ml conical flask containing 50 ml of medium (starch, 1 g/100 ml and peptone in tap water) inoculated with 2 g wet weight of immobilised cells and incubated at 40°C in a rotatory shaker at 200 rev/min.

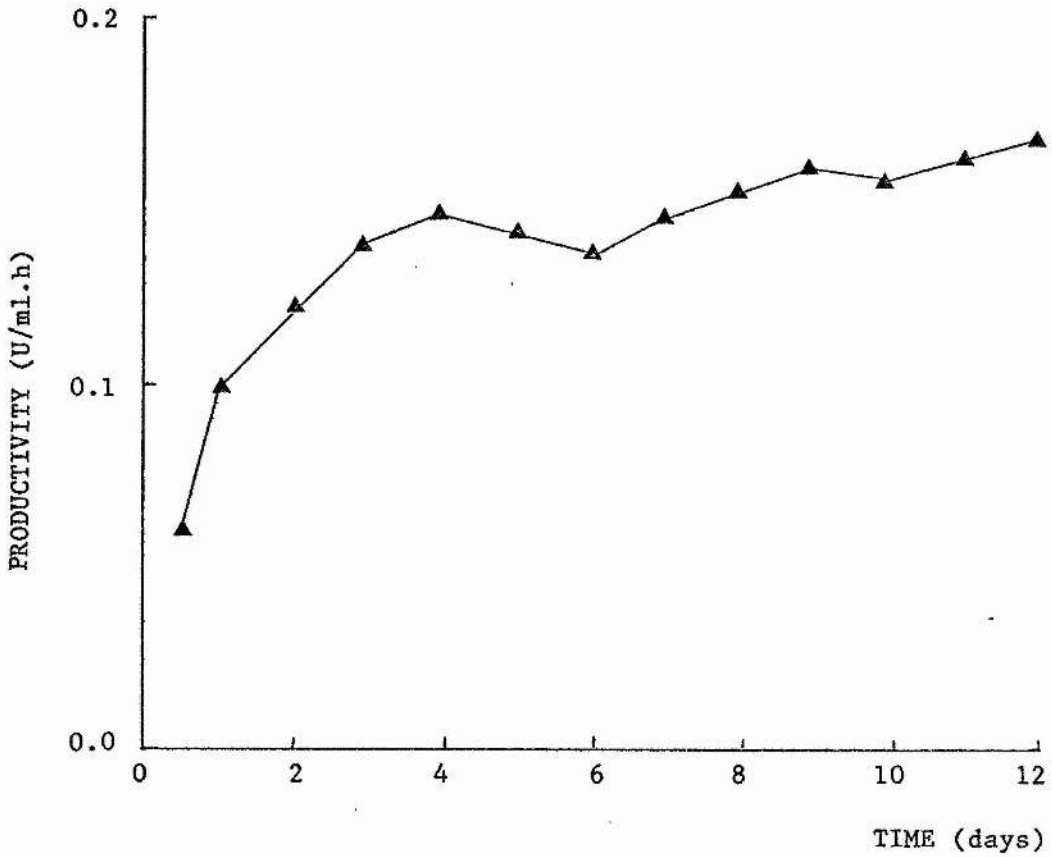


Fig. 3.3.4.c. Enzyme productivities in the FBF. The experiment was performed with medium M-2 containing 0.02% peptone. Productivities were calculated on basis of the residence time (25 h).

3.3.5 Enzyme properties

Numerous articles have been published on the properties of fungal amylolytic enzymes produced in differents cultures (Alazard and Raimbault, 1981; Pazur and Ando, 1959; Lineback et al., 1969; Freedberg et al., 1975 and Banks et al., 1967) but no data is available concerning the properties of these enzymes produced in immobilised cell cultures. Our studies will be limited to some properties of crude enzyme preparations which are relevant to their industrial production and application. It was necessary to determine some properties of the enzyme broth produced in free-cell culture with a view to compare with those of the enzymes produced in immobilised-cell culture.

Experimental conditions for enzyme production were described in section 2.4.1. Further experimental details will be given in the specific sections.

3.3.5.1 Enzyme thermostability

Samples of the fermentation broth were maintained for 60 minutes at various temperatures between 40 and 80°C, and residual amylolytic activity was assayed to determine the degree of inactivation. The residual enzyme activity is expressed as a percentage of the activity of the original preparation.

The results are shown in fig. 3.3.5.1.

These results showed that the enzymes from immobilised-cell cultures were more resistant towards thermaldenaturation than those from free-cell cultures.

The differences in thermostability observed in the enzyme preparations produced by free and immobilised cells could be due to differences in the enzyme content, i.e., ratio alpha-amylase/amyloglucosidase present in the broths. In order to investigate this hypothesis, it was necessary to determine these enzyme activities separately using specific substrates as described in section 2.7.5 and 2.7.6. The results shown in the table 3.3.5.1 indicate a similar enzyme composition for the amylolytic broths produced by both free and immobilised cells of Aspergillus niger.

Another possibility is that differences in the enzyme structure itself account for the different thermostabilities observed in the enzyme preparations from free and immobilised cell cultures (Alazard and Raimbault, 1981). However, further studies with the purified enzyme fractions are necessary to clear up this point.

Concentration of crude enzyme preparations is one of the common step processes in industrial production of amylolytic enzymes and often the enzyme preparation is inadvertently exposed to high temperatures, thus the higher thermotolerance of the enzymes from immobilised cell cultures represents an important advantage with regard to enzymes from free cell cultures.

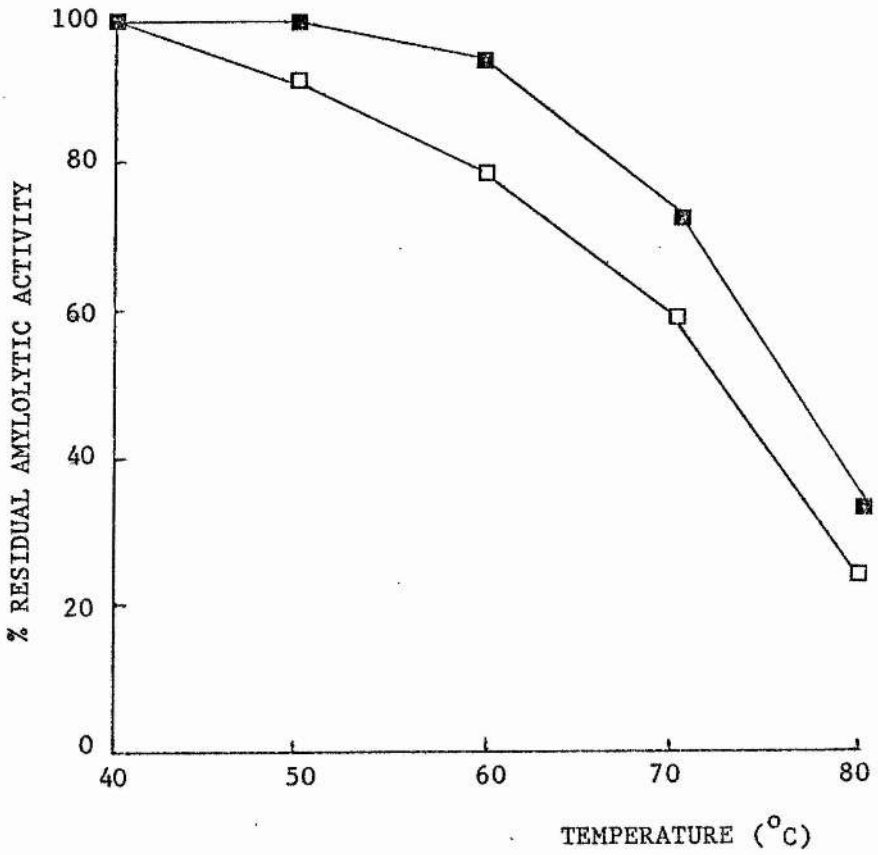


Fig. 3.3.5.1. Thermostability of the amyloiytic broth produced by free (□) and immobilised (■) mycelia of A. niger.

	free cells	immobilised cells
alpha-amylase (AA)	17 $ug.ml^{-1}$	20 $ug.ml^{-1}$
amyloglucosidase (AG)	13 $ug.ml^{-1}$	15 $ug.ml^{-1}$
AA/AG	1.3	1.3

Table 3.3.5.1. Amylolytic enzyme composition in the broth produced by free and immobilised cells of Aspergillus niger.

3.3.5.2 Storage stability of the amylolytic broth

The storage stability of the amylolytic enzymes was examined in the culture broths produced by immobilised and free cells of the Aspergillus niger strain used in this study. The broths (pH = 3.0) were directly cooled from 40°C (fermentation temperature) to 4°C and maintained at this temperature. Samples were periodically removed for analysis of the amylolytic activity. Residual activity was expressed as percentage of the initial activity.

The results presented in table 3.3.5.2 show that enzymes produced under both conditions of fermentation were very stable, more than 90% of enzymic activity being retained for 15 days of storage at 4°C. Similar results have been reported by Sinkar and Lewis (1980) for amylolytic enzymes from free cell culture of Aspergillus niger.

The above results represent an important factor if the enzyme preparation is to be used in a form of a culture filtrate. In this case, thorough cooling of the broth and strict hygienic measures are the most important methods in the control of loss of activity and infections. With enzyme preparations to be used in food processing, storage at low temperature is inevitable because addition of preservatives is normally unacceptable (furthermore, many preservatives may affect the biological treatment of wastewater from the recovery plant). The low pH of the fermentation liquor obtained (3.0) should have contributed to prevent infection of the filtrate.

Period (days)	% amylolytic activity	
	free	immobilised
0	100	100
1	100	100
2	100	100
5	98	99
10	96	98
15	94	96

Table 3.3.5.2. Storage stability of the amylolytic enzymes at 4°C produced by free and immobilised cells of Aspergillus niger.

3.3.5.3 Optimal pH and temperature for amylolytic activity

Optimum pH for amylolytic activity was studied by varying the pH of the acetate buffer in reaction mixture, under the standard assay conditions (as described in section 2.7.4). Enzymes produced from immobilised- and free-cell cultures had a 4.0 to 4.4 range of optimal activity (fig. 3.3.5.3.a).

In analysing temperature effects, observations were carried out with reaction mixtures incubated at differents temperatures from 30 to 60°C, under the standard assay conditions. Results in fig. 3.3.5.3.b. indicate an optimum temperature for enzymic activity at 50°C for the enzymes produced in both free- and immobilised-cell culture.

The values cited above are in the range of values quoted in the literature for optimal pH and temperature for amyloglucosidases produced by A. niger (Pazur and Ando, 1959, Lineback et al., 1969; Freedberg et al., 1975).

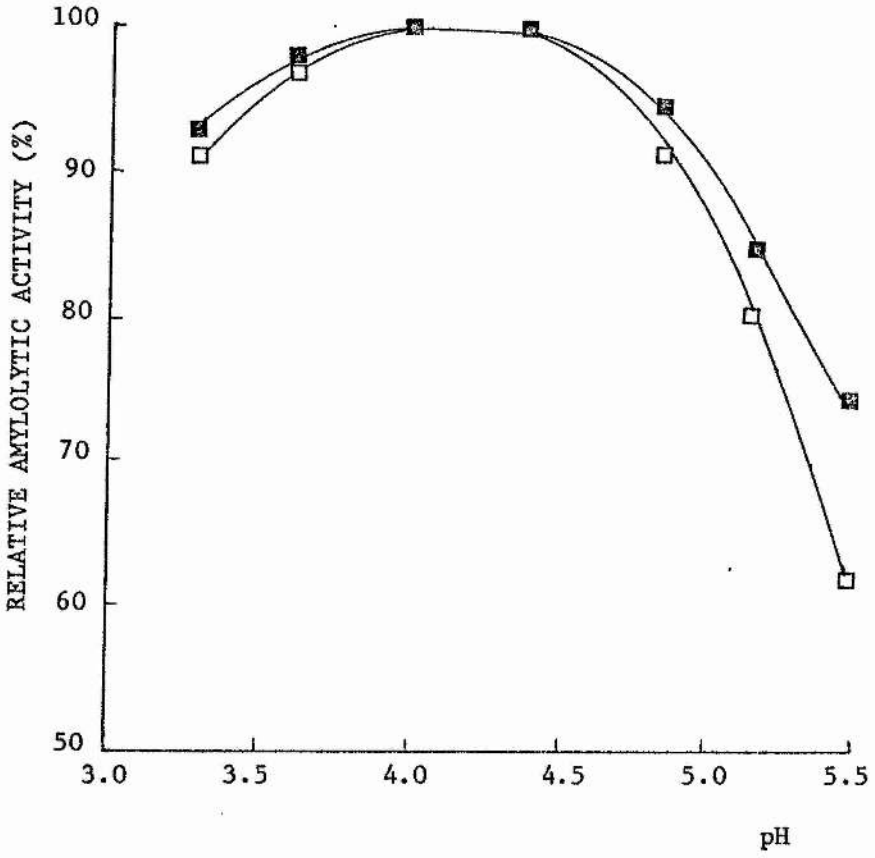


Fig. 3.3.5.3.a. Effect of pH on amylolytic activity of the fermentative broth from free (□) and immobilised (■) mycelia of A. niger.

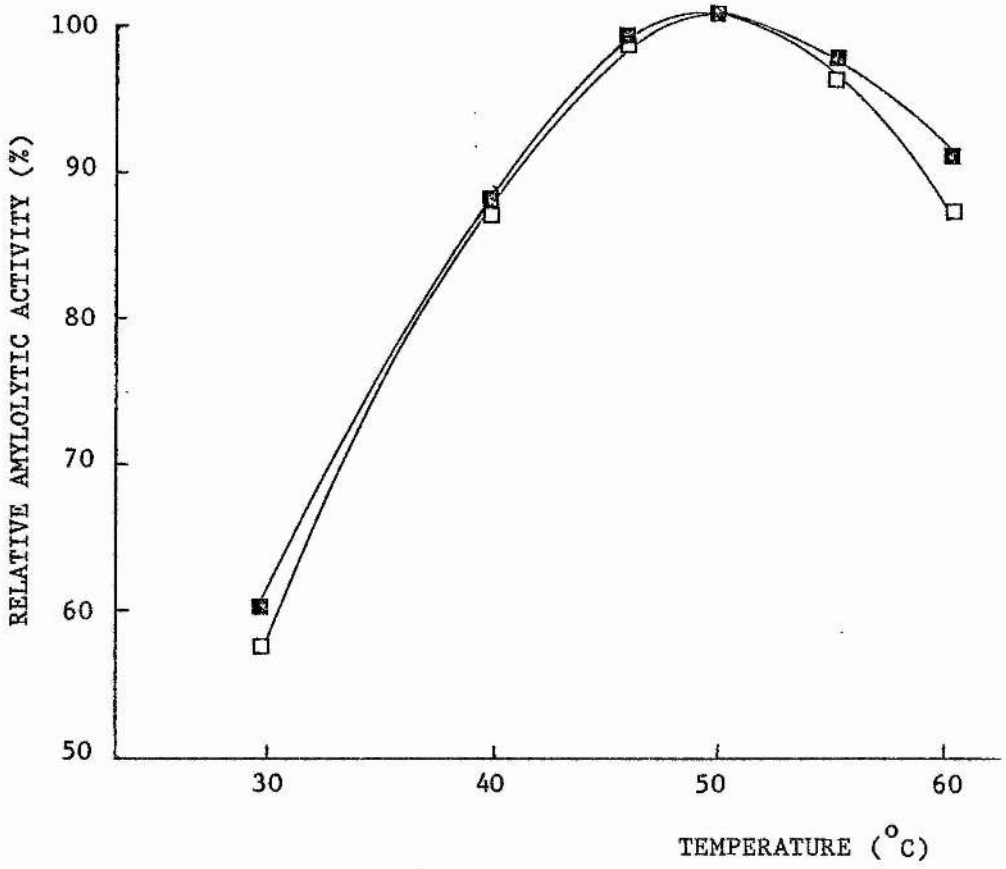


Fig. 3.3.5.3.b. Effect of temperature on amylolytic activity of the fermentative broth from free (□) and immobilised (■) A. niger.

3.4 Potential uses

This part discusses some possible applications (with emphasis on some current problems existing in Brazil) of the crude enzyme preparation obtained in the immobilised Aspergillus niger cultures used in this study.

3.4.1 Saccharification and fermentation of raw cassava starch

The Brazilian authorities instituted in 1975 a large scale programme aimed at partial replacement of imported crude oil by ethanol produced from carbohydrate crops. One abundant carbohydrate resource in Brazil is cassava, a root rich in starch, which grows well on marginal soils with minimal fertiliser and rainfall requirements.

The economic feasibility of ethanol production from cassava is highly dependent on the cooking step, prior to enzymic hydrolysis, that requires extra energy input. Ueda et al., (1981) found that cassava starch mashes can be effectively converted to ethanol without

cooking (thus the cost of energy consumption can be saved) in a single-step process which combines a fungal saccharifying agent and yeast fermentation.

In this study, it was attempted to process the fermentation of raw cassava starch to ethanol, without cooking, by using the enzyme broth produced by the immobilised cells of Aspergillus niger simultaneously with bakers' yeast fermentation.

Dried chopped cassava roots were pounded in a mortar vessel with a pestle; the ground cassava contained 65% (w/w) starch. 5 g of ground cassava roots were mixed with 50 ml of the culture filtrate collected at 24 h cultivation (140 units) in a 250-ml flask. The resulting suspension was adjusted to pH 3.5, then 0.5 g of compressed yeast was added. The flask was kept at 30°C, with stirring. Samples were collected every 12 h for analysis. Ethanol contents were determined by gas-liquid chromatographic procedure (section 2.7.7), reducing sugar content was determined by DNSA method (section 2.7.3) and starch concentration was estimated by the "blue value" method (section 2.7.2).

Fig. 3.4.1.a shows the time course of ethanol fermentation for the mixture of the enzyme broth, yeast and cassava mash without cooking. During the early period of fermentation (24 h), there was an accumulation of reducing sugars; following this initial period, the sugar concentration declined and fermentation was complete after 5 days. At this stage the final ethanol concentration was 30 g/l and yield of ethanol was 90% (w/w) based on theoretical maximum conversion

of starch to glucose. When the cultivation period was continued up to 6 days, no increase in ethanol yield was observed. Similar ethanol yields were reported by Park and Rivera (1982) using a mould bran amyloglucosidase preparation as the saccharifying agent.

These results indicate the feasibility of simultaneous saccharification and fermentation of raw cassava using the filtrate of an immobilised cells of Aspergillus niger culture. However, because the optimum temperature for the amylolytic enzymes (section 3.3.5.3) is appreciably higher than those for yeast fermentation (30°C), the rate of saccharification should become rate-limiting with increase in total fermentation time. A comparison of relative speeds of hydrolysis was made between uncooked raw cassava starch at 30°C and 50°C. It is apparent from the data shown in fig. 3.4.1.b that there is no significant difference in the degree of hydrolysis after 5 days, however the rate of saccharification of uncooked cassava starch at 30°C was appreciably lower. By assuming a correlation between rate of starch hydrolysis by the enzymes and rate of ethanol fermentation by the yeast, it is apparent that the saccharification was the rate-limiting step in the simultaneous saccharification/fermentation process.

Data presented in fig. 3.4.1.b also show that raw cassava starch can be used for the production of glucose syrup with a 95% yield (based on the initial starch concentration), after 2 days of reaction at 50°C; this syrup could be used in several food industries as a sugar additive after nonconvertible material has been removed.

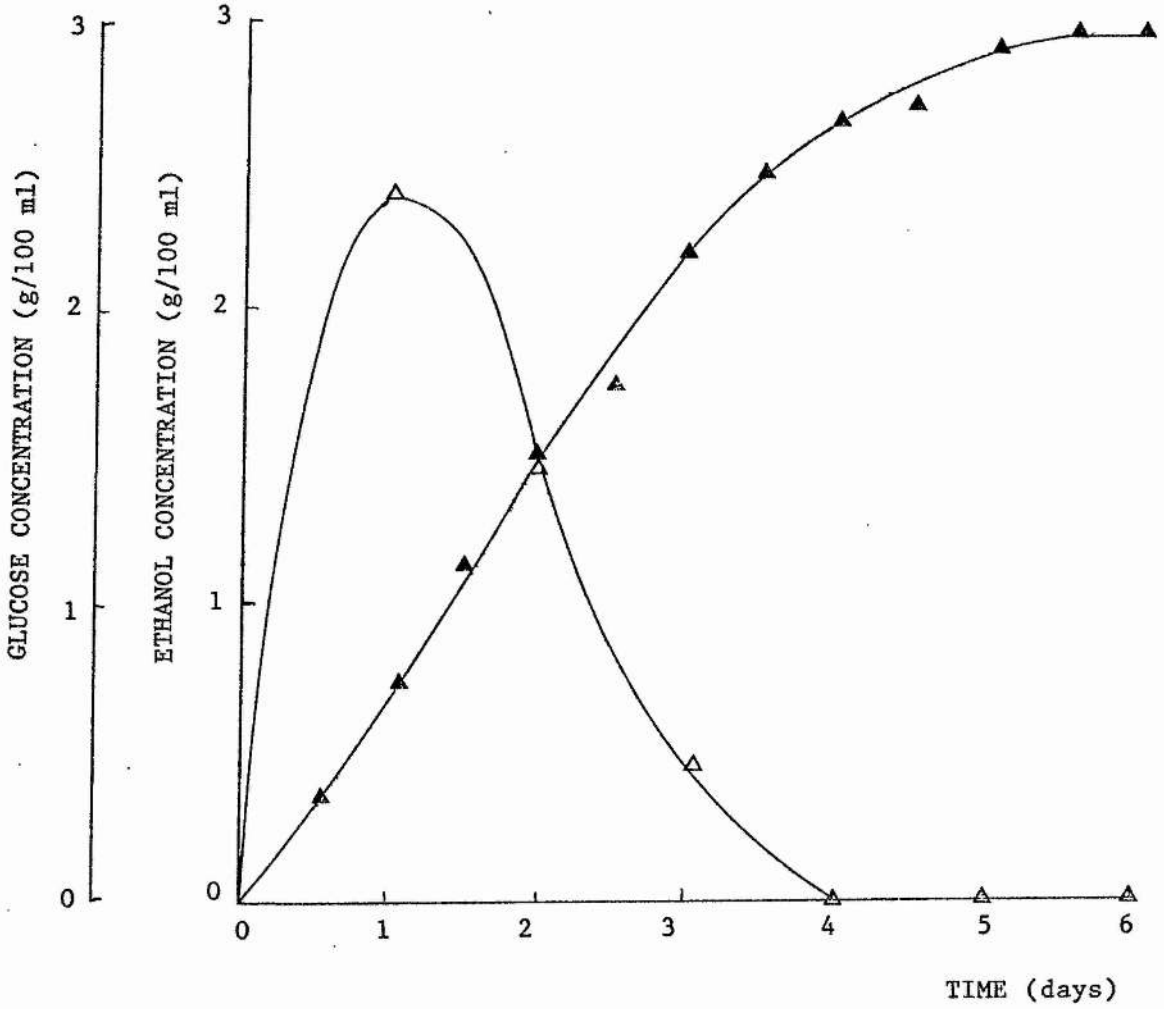


Fig. 3.4.1.a. Time course of fermentation of raw cassava starch to ethanol by a mixture of the amylolytic broth produced by immobilised A. niger and Saccharomyces cerevisiae: (△) reducing sugars; (▲) ethanol.

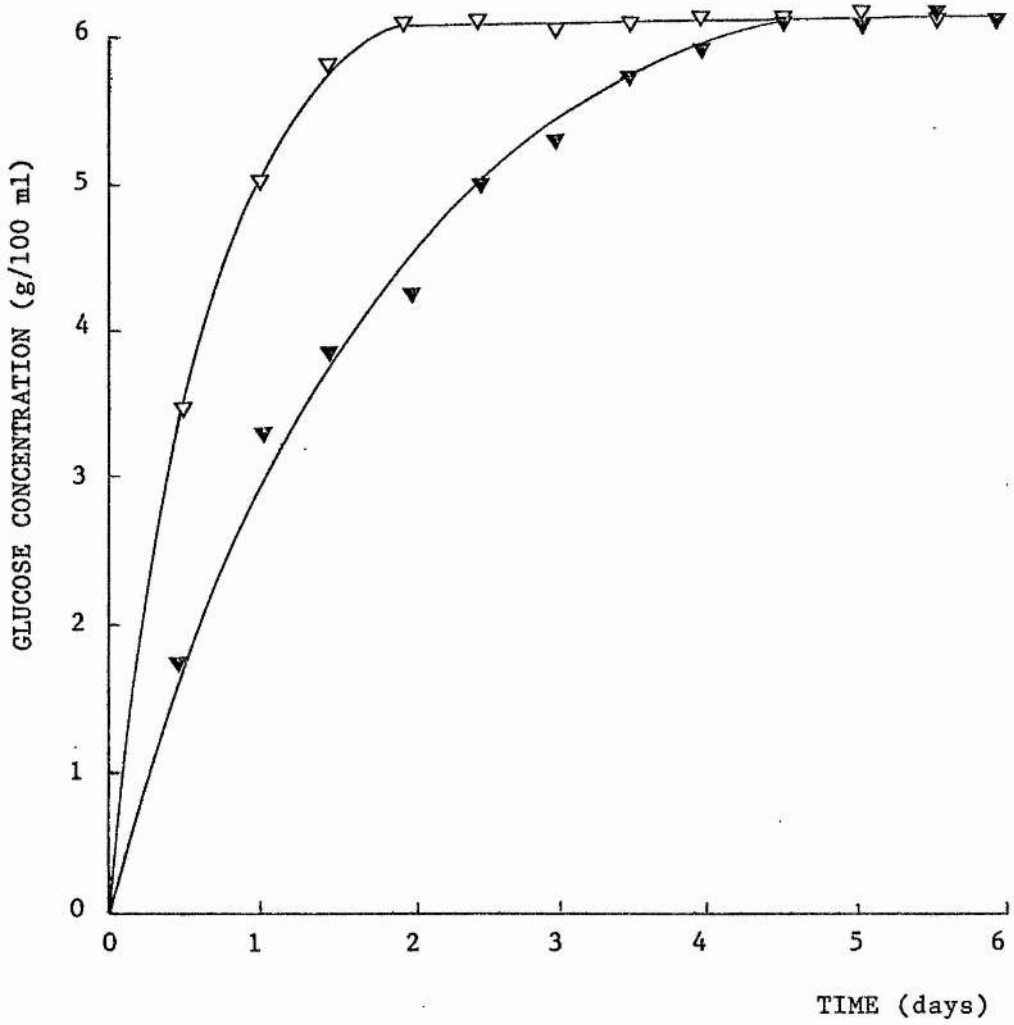


Fig. 3.4.1.b. Time course of hydrolysis of raw cassava starch at 30 (▼) and 50°C (▽) by the amylolytic broth produced by immobilised A. niger.

3.4.2 Treatment of industrial cassava wastewater

Industrial cassava wastewater has a highly polluting strength (of about 74,000 mg/l dissolved oxygen demand in 5 days ($B.O.D._5$) according to the measurements made by Ledingham et al., 1980. In Brazil, large amounts of this aqueous residue are produced in cassava processing for production of starches and meals, in factories widely spread throughout the country (100 - 300 l per ton of processed cassava).

At present, the treatment of these wastewaters is to remove the insoluble material by a sedimentation process, then the supernatant is discharged in watercourses. Because this discharged liquid still has a strongly polluting power, due in part to the soluble starch which can not be removed by sedimentation (Ledingham, et al., 1980), severe environmental pollution can occur.

The use of microorganism to degrade the starch in wastewaters has been previously suggested (Lamo and Menezes, 1979; Garcia et al., 1980). An immobilised cell reactor could offer an attractive solution for the treatment of starchy wastewater. In order to test this, the removal of a biochemical oxygen demand from an artificial wastewater (starch 1 g/100 ml, nitrogen 0.3 g/l and mineral traces, $B.O.D._5$ = 5,148 mg/l O_2) during immobilised Aspergillus niger cultivation was investigated.

Oxygen demand over a 5-day period ($B.O.D._5$) at $30^{\circ}C$ was estimated using a Yellow Spring oxygen electrode system, calibrated for dissolved oxygen concentration over the range 0-6.57 mg/l. A reduction of about 50% of the $B.O.D._5$ was found after 5 hours of incubation.

Therefore, the treatment of starchy wastewaters by an immobilised fungi system seems to be feasible with regard to biochemical oxygen demand.

3.4.3 Conclusions

The results discussed in the two above sections showed that immobilised Aspergillus niger systems are suitable for some industrial applications. The enzyme broth can be used straightforwardly for cassava processing to ethanol and glucose production, even the costly step of cooking of the mash can be eliminated.

The growing immobilised-cell system could also be used for wastewater treatment in cassava processing factories. It is thought that further studies on these systems would result in simple, practical and useful technologies particularly important for countries, like Brazil, where the present developing strategy is mainly based on agro-industrial processes.

4 FINAL CONCLUSION

Fungal mycelia immobilisation can be effectively used for industrial production of amylolytic enzymes. Enzyme yields and productivity comparable with those obtained in filamentous culture can be reached. Moreover, the immobilised system permits lower culture viscosity, the practical value of which is reflected in less energy input, which is required for aeration and mixing of the fermentation system.

Further studies, such as the employment of different substrates, carriers and organisms, would improve even further the performance of an immobilised cell system in relation to a free cell system.

5 REFERENCES

1. Abbot, B. J. (1977). Immobilised Cells. In Annual Reports of Fermentation Processes, vol. 1, 205, Academic Press, New York.

2. Adams, S. L., Balankura, B., Andreasen, A. A. and Stark, W. H. (1947) Ind. Eng. Chem., 39, 1615.

3. Adler, H. I. (1971). In Radiation and radioisotopes for in dustrial microorganisms; pp. 241. International Atomic Energy, Vienna.

4. Alazard, D. and Raimbault, M. (1981). Eur. J. Appl. Microbiol. Biotechnol., 12, 113.

5. Araujo Filho, A. A. (1977) Obtencao de alcool anidro a partir de mandioca, Banco do Nordeste do Brasil ed. pp. 40, Fortaleza, Ceara, Brasil.

6. Aunstrup, K. (1977). In Annual Reports on Fermentation Processes (D. Perlman and G. T. Tsao, eds.) Vol I, pp. 181, Academic Press, New York.

7. Bachler, M. S., Strandberg, G. W. and Smiley, K. L. (1970) Biotechnol. Bioeng., 12,85.

8. Banks, G. T., Binns, F. and Cutcliffe, R. L. (1967). Recent developments in the production and industrial applications of amylolytic enzymes derived from filamentous fungi. In Progress in Industrial Microbiology, vol 6, pp. 95. Ed. D. J. D. Hockenhull.

9. Banks, G. T. (1977). In Enzyme and Fermentation Biotechnology. vol. 1, pp. 72. Ellis Horwood Ltd. Publisher, Chichester, England.

10. Beck, S. R. and Rase, H. F. (1973). IEC Prod. Res. Dev., 12, 260.

Blain, J. A. (1975). Industrial Enzyme Production. In The ...mentous Fungi, vol 1, pp. 193. Ed. J. E. Smith and D. R. ...award Arnold (Publishers) Ltd., London.

12. Brierley, M. R. and Steel, R. (1959). J. Appl. Microbiol., 7, 57.

13. Brown, D. E. and Fitzpatrick, S. W. (1979). Biotecnology Letters, 1, 3.

14. Bruner, R. L. (1964). Determination of Reducing Value. In Methods in Carbohydrate Chemistry, vol. 4, pp. 67. Ed. R. L. Whistler, Academic Press, New York and London.

15. Butlerworth, T. A., Wang, D. I. C. and Sinkey, A. J. (1970). Biotechnol. Bioeng., 12, 615.

16. Calam, C. T. (1970). In Methods in Microbiology, vol. 3 A; pp. 435. Academic Press, London.

17. Carvalho Jr., L. B. (1964). Ph. D. Thesis, pp. 30. St. Andrews University, St. Andrews, Scotland.

18. Cheetham, P. S. J., Blunt, K. W. and Bucke, C. (1979). Biotechnol. Bioeng., 21, 2155.

19. Chen, L. F. and Tsao, G. T. (1976), Biotechnol. Bioeng., 18, 1507.

20. Chen, L. F. and Tsao, G. T. (1977). Biotechnol. Bioeng., 19, 1463.

21. Chibata, I. (1979). Kemia-Kemi, 6(12), 705.

22. Choudhari, Q. and Pirt, S. J. (1965). J. Gen. Microbiol., 41, 99.

23. Cochet, N., Marcipar, A. and Lebeault, J-M. (2379). Colloque Soc. Fr. Microbiol. Compiegne, pp. 211.

24. Corn Product Co. (1962). British Patent 904,423.

25. Demain, A. L. (1972). Biotechnol. Bioeng. Symp., 3, 21.

26. Durand, G. and Navarro, J. M. (1978). Process. Biochem., 13, 14.

27. Emerson, S. (1950). J. Bacteriol., 60, 225.

28. Fogarty, W. M. and Kelly, C. T. (1979). Starch-degrading enzymes of microbial origin. In Progress in Industrial Microbiology, 15, 87.

29. Foster, J. W. (1949). Chemical activity of fungi, Academic Press, New York and London.

30. Freedberg, I. M., Levin, Y., Kay, C. M., Mc Cubbin, W. D. and Katchalsky-Katzir, E. (1975). Biochim. Biophys. Acta, 391, 361.

31. Frein, E. M., Montenecourt, B. S. and Eveleigh, D. E. (1982). Biotechnol. Letters, 4, 287.

32. Garcia, C. O., Waki, T., Suga, K and Ichikawa, K. (1980). Annual Reports of ICME, vol. 3, pp. 83. Edited by H. Taguchi, Osaka, Japan.

33. Gbewonyo, K. and Wang, D. I. C. (1983). Biotechnol. Bioeng., 25, 967.

34. Gillie, O. J. (1968). J. Gen. Microbiol. 51, 179.

35. Hanson, A. M., Bailey, T. A., Malzahn, R. C. and Corman, J.

(1955). J. Agr. Food Chem., 3, 866.

36. Harrison, J. G. and Rowsell, J. E. (1970). Process Biochemistry, 6(4), 29.

37. Jack, T. R. and Zajic, J. E. (1977). Adv. Biochem. Eng., 5, 125.

38. Kobayashi, H. and Suzuki, H. (1972). J. Ferment. Technol., 50, 625.

39. Kolot, F. B. (1980). Develop. Industr. Microbiol., 21, 295.

40. Kossen, N. W. F. and Metz, B. (1976). Fifth International Fermentation Symposium, Berlin, Abstract 4.22, pp.78.

41. Krasnobajew, V. and Boniger, R. (1975). Chimia, 29, 123.

42. Kurushima, M., Fugii, T. and Kitahara, K. (1972). Agric. Biol. Chem., 36, A 26.

43. Lages, A. C. A. and Tannenbaum, S. R. (1978). J. Food Science, 43, 1012.

44. Lamo, P. R. and Menezes, T. J. B. (1979). Colet. Inst. Tecnol. Aliment., 10, 1.

45. Lartique, D. J. and Weethal, H. (1976). U.S. Patent 3,939,041.

46. Ledingham, W. M., Lima Filho, J. L. and Lins, M. B. (1980). Arq. Biol. Tecnol., 23, 299.

47. Leisola, M., Ojlamo, H., Kuppinar, V., Linko, M. and Virkunnen, J. (1980). Enzyme Microb. Technol., 2, 121.

48. Le Mense, E. H., Corman, J., Van Lanen, J. M. and Langlykke, A. F. (1947). J. Bacteriol., 54, 149.

49. Le Mense, E. H., Sohns, V. F., Corman, J., Blom, R. H., Van Lanen, J. M. and Langlykke, A. F. (1949). Ind. Eng. Chem., 41, 100.

50. Lineback, D. R., Russell, I. J. and Rasmussen, C. (1969). Arch. Biochem. Biophys., 134, 539.

51. Matteau, P. P. and Sadler, J. N. (1982). Biotechnol. Letters, 4, 15.

52. Metz, B. (1976) Ph.D. Thesis, Delft University of Technology, The Netherlands.

53. Metz, B. and Kossen, N. W. F. (1977). Biotechnol. Bioeng., 19, 781.

54. Messing, R. A. (1980). Annual Reports on Fermentation Processes, 4, 105, Academic Press Inc., New York, London.

55. Miyazaki, S. (1932). Japanese patent 110,127.

56. Otto, R. E. (1957). Ph. D. Thesis, University of Delaware, Newark, USA.

57. Pace, G. W. (1980). Rheology of mycelial fermentation broth. In Fungal Biotechnology, pp. 95, ed. J. E. Smith, D. R. Berry, and Kristiansen, Academic Press Inc. (London) Ltd.

58. Pal, A., Roy, A. and Das, A. (1980). Mycologia, 72, 1134.

59. Park, Y. K. and Papini, R. S. (1970). Rev. Brasil. Tecnol., 1, 13.

60. Park, Y. K. and Rivera, B. C. (1982). Biotechnol. Bioeng., 24, 495.

61. Pazur, J. H. and Ando, T. (1959). J. Biol. Chem., 234, 1966.

62. Pazur, J. H. and Ando, T. (1960). J. Biol. Chem., 235, 297.

63. Pazur, J. H., Knull, H. R. and Cepure, A. (1971). Carbohyd. Res., 20, 83.

64. Pirt, S. J. (1966). Proc. Roy. Soc., 166, 369.

65. Pirt, S. J. (1974). Principles of Microbe and Cell Cultivation, Blackwell Scientific, Cambridge, England.

66. Ramaley, R. F. (1979). In Adv. Appl. Microbiol., vol. 25, pp. 37. (D. Perlman), Academic Press, New York.

67. Randall, A. J. and Solomons, G. L. (1977). In Fermenter design and fungal growth; In Fungal Biotechnology, pp. 55, ed. J. E. Smith, D. R. Berry and B. Kristiansen, Academic Press Inc. (London) Ltd.

68. Sadir, R. (1966). Boletim do C.T.P.T.A., 6, 5.

69. Shah, R. N. (1979). Farmaceutisch Tijdschfrift voor Belgie, 56, 415.

70. Shinmyo, A. (1980). In Annual Reports of ICME, vol. 3, pp. 433, edited by H. Taguchi, Osaka University, Japan.

71. Sinkar, V. P. and Lewis, N. F. (1980). J. Food. Biochem., 4, 159.

72. Smiley, K. L. (1971). Chem. Eng. Prog., 67, 30.

73. Smirkova, L. S. (1957). Mikrobiologyia, 26, 45.

74. Solomons, G. L. (1980). In Fungal Biotechnology, pp. 55, ed. J. E. Smith, D. R. Berry and B. Kristiansen, Academic Press Inc. (London) Ltd.,

75. Stavenger, P. L. (1971). Chem. Eng. Prog., 67, 30.

76. Stentebjerg-Olesen, B. (171). Process Biochemistry, 6, 29..

77. Suijdam, J. C. (1980). Ph. D. Thesis, Delft University, of Technology, The Netherlands.

78. Suijdam, J. C., Hols, H and Kossen, N. W. F. (1982). Biotechnol. Bioeng., 24, 177.

79. Suzuki, S. and Karube, I. (1979). A.C.S. Symposium Series, 106, 59.

80. Takamine, J. (1914). Ind. Eng. Chem., 6, 824.

81. Taylor, J. S. (1955) M. Ch. E. Thesis, University of Delaware, Newark, USA.

82. Thorbek, L. and Eplov, P. (1974). J. Appl. Bacteriol., 37, 549.

83. Tikhomirova, V. I. (1958). Mikrobiologyia, 30, 229.

84. Trinci, A. P. J. (1970). Arch. Mikrobiol., 73, 353.

85. Trinci, A. P. J. (1972). Trans. Br. Mycol. Soc., 58, 467.

86. Ueda, S., Zenin, C. T., Monteiro, D. A. and Park, Y. K. (1981). Biotechnol. Bioeng., 23, 291.

87. Vekatasubramanian, K. and Vieth, W. R. (1979). In Progress in Industrial Microbiol., 15, 61.

88. Weetall, H. H. and Havawala, N. B. (1972). Chimia, 29, 123.

89. Whitaker, A. and Long, P. A. (1973). Process Biochemistry, 11, 27.

90. Yano, T., Kodama, T. and Yamada, K. (1961). Agric. Biol. Chem., 25, 580.

91. Yoshida, T., Taguchi, H. and Teramoto, S. (1968). J. Ferment. Technol., 46, 119.