A BIOCHEMICAL STUDY OF FUNGAL CELL EXTRACTS

by

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A

THESIS

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SUMMARY

The production of microbial protein has been suggested as one of the means of alleviating the world protein deficiency. This work attempts to contribute to our knowledge of the production and processing of fungal proteins. It is shown that fungal biomass can be produced easily in aerobic tower fermenters. Several fungi were grown and the Fungi Imperfecti found to be the most suitable. The protein content of the biomass produced ranged from 34% to 58%.

A significant portion of the mycelial protein could be solubilised by an enzymatic extraction. This involved drying and grinding the mycelium and subsequently extracting the mycelium in an aqueous suspension with pepsin under acidic conditions and moderate temperatures. Neutralised extracts on evaporation to a semi-solid consistency gave tasty meaty-flavoured products. Several fungal extracts were prepared and found to have a range of flavours.

Amino acid analyses of the various fungal extracts showed them to be rich in the essential amino acids. In particular the extracts are rich in lysine which would make them ideal protein supplements to the diets of people in underdeveloped countries.

Feeding trials were carried out on mice fed on a diet supplemented with 10% Aspergillus niger extract. The supplementation had a beneficial effect and the mice showed weight gains greater than those of mice fed on the unsupplemented diet. Serological and histological studies showed no adverse effects. Mice fed on the extract bred normally. The mice survived a 15-day period of 50% supplementation with no serious adverse effects.

It is also shown that fungal extracts are good fermentation adjuncts suggesting a means of recycling much of the nitrogenous constituents of fermentation media and minimising on effluent problems.

An economic evaluation shows that fungal extracts can be produced at a competitive price compared to other protein hydrolysates.

DECLARATION

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

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CERTIFICATE

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

.....R. Alagaratnam.....

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HISTORICAL INTRODUCTION

THE NEED FOR NEW SOURCES OF FOOD

The phenomenal increase in world population has been pointed out on numerous occasions. It took man a million years to attain a population of 1,000 million. The second 1,000 million was added in 100 years and the third in twenty more. At present trends the world population is expected to reach 6,000 million by the year 2000.

One of the major problems arising from this population explosion is the need to feed this vast increase in numbers. The possibility of world wide food shortage conceived by Robert Malthus in the eighteenth century is being recognised by a growing segment of the population. Vast new areas, especially in the temperate regions, have been opened up for cultivation. Nevertheless, the increases in the area tilled have not kept pace with the population increase (Table I).

TABLE I

Approximate numbers of individuals living on the earth at various times

(Gray, 1966)

Year	Approximate Population	Average number of acres of arable land/ person	Average decrease in acres of arable land/person/ decade
8,000 B.C.	10,000,000	2625	-
1 A.D.	250,000,000	100	3.15
1650 A.D.	500,000,000	52	1.29
1798 A.D.	750,000,000	35	3.40
1850 A.D.	1,000,000,000	26	1.80
1945 A.D.	2,000,000,000	13	2.88
1962 A.D.	3,000,000,000	9	2.35
2002 A.D.	6,000,000,000	4	-

It must be admitted that tremendous increases in agricultural efficiencies have been attained. Between 1800 and 1900 the number of man-hours required to produce 100 bushels of wheat dropped from 373 to 47. New corn hybrids have boosted yields from 22 bushels per acre to as much as 125 bushels per acre (Gilbert and Robinson, 1957). These methods of increasing food production and productivity are not limitless and there is a fallacy in the assumption of ever-increasing yields. In some highly advanced countries the law of diminishing returns is already in operation. Other methods of producing food need to be developed and are being developed.

Consideration of the food problem solely from the calorific standpoint tends to be misleading. Gray (1962) has suggested that there is every indication that food calorie quantities far in excess of the needs of the human population can be produced easily on this earth for a great many centuries to come. The fallacy of this will be immediately apparent when it is realised that to be in a reasonable state of 'well-being' man must ingest a well-balanced diet. The main constituents of man's diet have long been recognised as carbohydrates, fats, proteins, salts and vitamins. The preponderance of each of these is dependent on availability as well as on the feeding habits of the particular community. Studies over the last few decades indicate variations and fluctuations over a period of time in the preponderance of the main dietary components.

Carbohydrates are still the main energy source in the diet though there is a trend to diminishing intake of carbohydrates. Eighty years ago, carbohydrates contributed two-thirds of the calories of the average British diet, but the current contribution is only about half. In some developing countries carbohydrates are still the main energy yielding constituent of the diet and may contribute more than four-fifths of the energy.

Fats constitute another energy source in the diet. Although some fat is present in all human diets, the amount present may be very low. There is a tendency for the fat content in the diet of a country to increase in the later stages of development of the country; whereas eighty years ago fats supplied 7 per cent of the calories of the British diet, today they supply 18 per cent. Fats account for 40 per cent of the calories of the average diet in the U.S.A.

Proteins are the third main constituent of the diet and the protein plays many different roles in the body. In addition to the protein required for growth, protein is required for maintenance and subsistence and for the synthesis of special body proteins such as enzymes and certain hormones. Protein needs are increased in certain physiological and pathological conditions, for example during pregnancy, lactation and convalescence.

There is widespread disagreement on the protein needs of man, partly because the need depends on a number of factors including age, physiological activity and state of health and partly because different methods of expressing protein value are used (see Bender, 1969).

In practice the best diets seem to contain between 15 per cent and 25 per cent protein. A joint Food and Agricultural Organisation/World Health Organisation Expert Group in 1965 estimated that 0.7 g of protein per kilogramme body weight per day would meet the needs of most people. The Medical Research Council recommends 0.75 g to 1.4 g proteins per kilogramme body weight per day.

It must be emphasised that the protein requirement of man is not for proteins <u>per se</u> but for specific amounts of specific amino acids. Proteins are broken down to their constituent twenty or more amino acids prior to absorption in the alimentary tract. Some of the amino acids can be synthesised in the human body from other amino acids and nitrogen-containing compounds in a scale sufficient to meet the body's needs.

Those that are not synthesised on a sufficient scale are termed essential amino acids and these need to be supplied in the diet.

The amino acid requirements of man have been studied by Rose and his co-workers and a review of their work has been published (Rose, Wixom, Lockhart and Lambert, 1955). It has been established that eight amino acids (L-Tryptophan, L-Phenylalanine, L-Lysine, L-Threonine, L-Methionine, L-Leucine, L-Isoleucine and L-Valine) are essential for adult humans. Table II taken from the review indicates tentative quantitative requirements of amino acids for nitrogen balance maintenance in adults. The safe intake of the amino acids has been set at twice the minimum daily needs to allow for individual variations in human response.

The quantitative needs of amino acids for adults and children would be expected to be vastly different, since the adult is in nitrogen equilibrium and the infant is in a marked positive balance. Holt and Snyderman (1961) have reviewed their studies on the amino acid requirements of infants. Table III summarises the data on the requirements of essential amino acids in infants, children, adult males and adult females.

It is generally found that of the eight essential amino acids, three are more often found to be limiting in human diets: lysine, threonine and tryptophan. Table IV summarises the limiting amino acids in various cereal proteins.

Protein malnutrition exists among populations that consume a substantial portion of their calorific intake as cereals: amino acid supplementation should improve the quality of the dietary protein to the point where protein malnutrition will not occur to a significant extent.

Views on the relative merits of animal proteins and plant proteins are changing. Thus, Aylward (1963) states: "The earlier simplified picture that animal proteins were to be regarded as 'first class' and that plant proteins were necessarily inferior has now been in a large measure discarded as a result of our new investigations; it is now known

TABLE II

Summary of Amino Acid Requirements of Man

(Rose, Wixom, Lockhart and Lambeth, 1955)

All values were determined with diets containing the eight essential amino acids and sufficient extra nitrogen to permit the synthesis of the non-essentials.

Amino Acid	No. of Quantitative Experiments.	Range of Requirements observed	Value proposed tentatively as minimum	Value which is definitely a safe intake	No. of subjects maintained in N balance on safe intake or less
		g per day	g per day	g per day	
L-Tryptophan	3▲	0.15 - 0.25	0.25	0.50	42
L-Phenylalanine	6	0.80 - 1.10■	1.10	2.20	32
L-Lysine	6	0.40 - 0.80	0.80	1.60	37●
L-Threonine	3△	0.30 - 0.50	0.50	1.00	29
L-Methionine	6	0.80 - 1.10□	1.10	2.20	23
L-Leucine	5	0.50 - 1.10	1.10	2.20	18
L-Isoleucine	4	0.65 - 0.70	0.70	1.40	17
L-Valine	5	0.40 - 0.80	0.80	1.60	33

▲ Fifteen other young men were maintained in nitrogen balance on daily intake of 0.20 g though their exact minimal needs were not established. Of the forty-two subjects maintained on the safe level of intake, thirty-three received 0.30 g daily or less.

■ These values were obtained with diets which were devoid of tyrosine. In two experiments, the presence of tyrosine in the food was shown to spare the phenylalanine requirement to the extent of 70 - 75 per cent.

● Ten of these individuals received daily intakes of 0.80 g or less.

△ In addition to these three subjects, four young men received rations containing 0.60 g of L-threonine daily and sixteen others received doses of 0.80 g daily. No attempt was made to determine the exact minimal requirements of these twenty individuals but all were on positive balance on the doses indicated.

□ These values were determined with cystine-free diets. In three experiments, the presence of cystine was found to exert a sparing effect of 80 to 89 per cent upon the minimal methionine needs of the subjects.

TABLE III

Estimated Minimal Essential Amino Acid Requirements

(West, Todd, Mason and Van Bruggen, 1966)

Amino Acid	Infants mg/kg	Children mg/day	Adult male mg/day	Adult female mg/day
Histidine	34	-	-	-
Isoleucine	119	1000	700	450
Leucine	150	1500	1000	620
Lysine	103	1600	800	500
Methionine	45▲	800	1100,200■	350
Total Sulphur AA	-	-	1100,1010	550
Phenylalanine	90●	800△	1100,300□	220
Total aromatic AA	-	-	1100,1400	1120
Threonine	87	1000	500	305
Tryptophan	22	250	250	157
Valine	105	900	800	650

▲ In the presence of cystine

■ With 810 mg of cystine, the methionine requirement was 200 mg.

● In the presence of tyrosine.

△ No cystine supplied.

□ With 1100 mg of tyrosine, the phenylalanine requirement was 300 mg.

TABLE IV

Limiting amino acids in cereal (Jansen, 1970)

Cereal	1st. limiting	2nd. limiting
Rice	lysine	threonine
Wheat	lysine	threonine
Corn	lysine	tryptophan
Sorghum	lysine	threonine
Millet	lysine	threonine
Teff	lysine	threonine

that many plant proteins (including those of yeasts and other micro-organisms) in appropriate mixtures with one another or with small quantities of animal protein may have a high biological value".

A proper assessment of the world food problem would show that carbohydrates are available in excess of our dietary requirements. They are obtained almost entirely from vegetable matter of which there is theoretically an inexhaustible supply. It will also be seen that fats, or the potential to produce fats, are in more than adequate supply to meet our dietary requirements.

There is, however, a short supply of proteins. Increases in food production have been most notable in carbohydrate-rich crops. Even in the cultivation of crops which contain protein the tendency has been to select strains with higher carbohydrate content to meet the calorific needs. The nett result has been that the carbon-to-nitrogen ratio in the diet of man has been constantly rising. Increased supplementation of food with proteins is required to restore a healthy balance.

The seriousness of the protein deficit has been recognised. Pirie (1969) estimated that there was a protein deficit of about 20,000,000 tons in 1969. Borgstrom (1964) has made an extensive analysis of the food problem of the human biosphere and its biological and chemical limitations. One of the suggestions he made was the exploitation of the potential of micro-organisms to produce protein-rich food.

In the Report of the Advisory Committee on the Application of Science and Technology to the Economic and Social Council of the United Nations (May 25, 1967) entitled "Increasing the Production and Use of Edible Protein" the suggestion was made that "...protein from single cells offers the best hope for major new protein supplies independent of agricultural land use".

THE CASE FOR MICROBIAL FEED AND FOOD.

The most apparent advantage of using micro-organisms as a feed and food source is the rapid rate of growth of most micro-organisms. Thus, Thaysen (1943) has calculated that a 10 hundredweight bullock synthesises 0.9 pounds of protein every 24 hours; under ideal conditions 10 hundredweight of torula yeast (Torulopsis utilis) would in the same time produce 51 tons of protein. Table V from Humphrey (1969) compares the mass doubling times of micro-organisms and higher organisms. Under optimum conditions, bacteria and yeasts can double their mass five hundred times faster than most agricultural crops and one thousand to five thousand times faster than most farm animals.

The attractiveness of rapid mass doubling is further emphasised when it is realised that far smaller land areas are required for the production of microbial food. Humphrey (1969) has calculated that if yeast were produced, utilising present technology, for producing 10% of the world's food needs (this would fulfil 50% of the world's protein needs) only an area of about one-half square mile would be required for culture tanks.

A less obvious advantage of microbial feed and food is the fact that micro-organisms are more efficient than livestock in the production of protein. Gilbert and Robinson (1957) have estimated that the efficiency of protein production from a given quantity of carbohydrate varies from 4 per cent in beef to 20 per cent in pork. By contrast fungi can achieve up to 65 per cent conversion.

Factory processes are easier to control and there are not so many vicissitudes as in individual agriculture. Fencl (1969) has illustrated the increased efficiency of factory production of feed and food. One worker in a torula yeast plant produces about 50 tons of dry protein per year. By contrast, in very intensive agricultural production (average production of milk equals 20 litres per day; one attendant per 30 cows) the production per worker is about 6 tons of dry protein for the same time. The value of yeast protein is almost the same as that of milk protein.

TABLE V

Mass Doubling Times (Humphrey, 1969)

Maximum rates are being compared.

Organism	Time for One Doubling in Mass
Bacteria and Yeast	20 - 120 minutes
Mould and Algae	2 - 6 hours
Grass and some plants	1 - 2 weeks
Chickens	2 - 4 weeks
Hogs	4 - 6 weeks
Cattle	1 - 2 months
People	3 - 6 months

The production of microbial feed and food would also be economical because a wide range of substrates may be utilised. Wood wastes and pulp, whey and molasses have been used as substrates. Vast quantities of food materials that are currently discarded because they are not in a form suitable for human consumption may be utilised as a substrate for microbial growth. In theory any material that yields more than 3 per cent fermentable carbohydrate without any toxic products could be used as a substrate (Gilbert and Robinson, 1957).

Cellulose is one of the most abundant materials on earth. Though the natural process of cellulosic decay involves microorganisms, there are no commercial undertakings which utilise cellulose as a substrate for microorganisms. Cellulose is at present converted into sugars by chemical means.

Notable among the industrial processes which have been developed for converting wood waste to sugars are those of Scholler and Bergius. The Scholler process employs dilute acid, elevated temperatures and steam under pressure. The wood does not have to be dried and no attempt is made to recover the acid. In the Bergius process, dried shredded wood is hydrolysed with 40% hydrochloric acid at room temperature. The

bulk of the residual acid is recovered by distillation under vacuum. Both these processes produce fermentable sugars from wood chips making them available for utilisation by the yeast Torulopsis utilis. The efficiency of the Scholler process is said to be 14 kg yeast obtained from 100 kg dry wood used. Approximately two-thirds of the nitrogen (added as ammonium hydroxide) is recovered as yeast protein (Robinson, 1952).

Prescott and Dunn (1959) have reviewed the chemical methods available for cellulose saccharification. Because of the numerous problems (quantity and cost of acid required, material of construction and product recovery) involved with chemical means of cellulose saccharification, the direct microbiological utilisation of cellulose would seem preferable. Considerable research has been done in this direction and we seem to be closer today to the accomplishment of enzymic saccharification of cellulose in a manner comparable to that achieved by glucoamylase in the conversion of starch to glucose (Ghose, 1969).

In some industries the utilisation of the wastes for the production of microbial feed and food would have an added economic advantage of reducing the Biological Oxygen Demand of the effluent thus saving in the sewage treatment of the effluent. The propagation of yeast on spent sulphite liquor from pulp and paper mills is undertaken principally for this reason with the yeast as a marketable by-product which pays for the treatment cost. Yields of 10 - 12 kg dry yeast have been obtained from one cubic metre of waste sulphite liquor (Foster, 1949).

The microbiological utilisation of spent sulphite liquor centres on the carbohydrate; no known biological method effectively removes the lignosulphonates. However, Tono, Tani and Ono (1968) have described the adsorption of lignin and clarification of lignin-containing agricultural and industrial wastes by moulds. They used various species of Aspergillus and Penicillium with small amounts (0.05 per cent) of added sugars.

In the light of the success achieved with the utilisation of spent sulphite liquor, investigations of the potential utilisation of waste carbohydrates from the food industry should be investigated.

Microbial feed and food also appear attractive in that they could be used to supplement the diet to obviate dietary deficiencies. Where dietary protein deficiencies exist, micro-organisms with a high protein content could be utilised to reduce the carbon-to-nitrogen ratio of the diet. By careful screening it will be possible to obtain micro-organisms rich in the essential amino acids. Two or three micro-organisms which have complementary amino acid profiles could be blended to give a product of high biological value.

Alternatively, genetic experiments for protein improvement can be readily undertaken. Indeed it is the ease with which microbial cells can be manipulated genetically to control the amino acid profile of their protoplasmic proteins that gives many investigators hope in the promise of this protein source (Humphrey, 1970).

The technological expertise for the large-scale production of microbial biomass already exists and no major advance is necessary for the large-scale production of microbial foods. The advances in fermentation technology which came about with the development of the antibiotics industry could be used with little or no modification.

In particular, the possibilities of continuous fermentation offer further promises in the utilisation of micro-organisms for the production of feed and food. In a discussion on the relative merits of batch or continuous fermentation, Butterworth (1966) stated "It seems fairly clear that there is a lot to be gained by adopting continuous fermentation as the manufacturing method for such simple growth-associated products as yeast cells or alcohol".

Another advantage of microbial feed and food production is that it is not dependent on suitable agricultural and climatic conditions. Growth is not limited by surface or soil types. Heterotrophic organisms such as fungi do not need sunlight and fungal food production can be carried out continuously utilising cheap carbohydrate sources and inorganic nitrogen. It seems likely that micro-organisms may become a very important source of food substances.

Reviews on microbial feed and food have been given by Thatcher (1954), Gilbert and Robinson (1957), Stokes (1958), Rose (1961), Bunker (1963), Mateles and Tannenbaum (1968a), Lipinsky, Kinne and Litchfield (1969), Ghose (1969), Snyder (1970) and Vilenchich and Akhtar (1971) and should be consulted for details. The book edited by Mateles and Tannenbaum (1968b) should also be consulted.

MICROBIAL FEED AND FOOD PREVIOUSLY PRODUCED.

It is not always realised that many tons of microbial feed and food in the form of yeast are being produced in many parts of the world. The idea of growing yeast as a food for direct human consumption occurred in 1910 to Delbrück and his co-workers at the Institut für Gärungsgewerbe in Berlin (Peppler, 1970). Accurate values of current production are not available. A survey by Bunker (1964) showed that the global production of food and feed yeasts was about 208,000 tons in 1964. For the same year Peppler (1967) quotes an estimate of over 310,250 tons being made up of 144,250 tons of Baker's Yeast and 166,000 tons of Dried Yeast.

According to Lindegren (1966) yeast is sold in two forms (i) Live yeast in a compressed cake or in various stages of desiccation, for baker's use or (ii) Dried killed yeast for human and animal nutrition. For many years live yeast was sold for human consumption. In 1948 it was shown that instead of adding to the B-vitamins available to the mammalian system, live yeast cells depleted the B-vitamins in the body because the living yeast cells sequestered the available B-vitamins and produced a condition of avitaminosis by excreting the intact live cells with the faeces.

The yeast most often used is the torula yeast which is known under a variety of names (Torula utilis, Torulopsis utilis, Candida utilis, Cryptococcus utilis). Frazier (1958) states that there are two varieties which are frequently used: Cryptococcus utilis var. major which has larger cells than the wild strain and Cryptococcus utilis var. thermophila which grows at an optimum temperature of 36 - 39°C as opposed to 32 - 34°C of the wild strain. The attractiveness of the larger cell strain lies in increased recovery efficiency. Thaysen and Morris (1943) showed that when Torulopsis utilis is treated with camphor, a giant strain of the organism is obtained. Before treatment, the nominal size of Torulopsis utilis was 3.7 μ x 7μ, but after treatment, a giant strain measuring

4.8 μ x 8.9 μ was obtained. This corresponded to a volume increase from $318\mu^3$ to $644\mu^3$. Bunker (1963) maintains that the larger cell strain was an unstable mutant which rapidly reverted to type. He also stated that higher temperatures appeared to depress the yield though Inskeep, Wiley, Holderby and Hughes (1951) were reported to be using a temperature of 37° at the Wisconsin Food Yeast factories.

During World War II surplus sugar in the form of cane juice or molasses in the West Indies was used as an energy source for yeast by which ammonium salts could be converted into protein (Pyke, 1970). Torula utilis var major, capable of growing at a temperature of 37 - 38°C was used. The sterilised molasses, sugar juice or solution of raw sugar was run continuously into a series of ten vessels, appropriate amounts of caustic soda, ammonium sulphate and ammonium superphosphate were added and the whole aerated through a simple arrangement of ceramic bubblers. The final liquor containing the yeast was continuously drawn off and the yeast cells separated on a centrifuge, washed and dried on heated rollers.

The popularity of the torula yeast is probably due to the fact that it can utilise pentoses as well as hexoses - a property which is important where the raw material is sulphite waste liquor from the paper pulp industry. Another factor is that the torula yeast is less exacting in its nitrogen requirements and is able to utilise urea as well as nitrates.

Mueller and Walden (1970) have given a brief review of the microbiological utilisation of spent sulphite liquor. The concentration of carbohydrates in spent sulphite liquor is 2 - 4 per cent and represents 20 - 30 per cent of the dissolved solids. Candida utilis and Monilia murmanica are the most effective yeasts. Some plants utilise all the sugars of spent sulphite liquor for biomass production, others convert the hexoses to ethanol by Saccharomyces cerevisiae, then distil off the alcohol and grow Candida utilis on the remaining pentose fraction.

Large quantities of Brewer's yeast are obtained as a by-product of

the Brewing Industry. In these the bitter taste due to hops must be removed prior to utilisation as feed or food. The washings can be accomplished in separate mixing tanks or in on-line mixers. These involve centrifugation steps which will generally increase the overall cost of the recovery process. Lindquist (1953) has found that top yeast (Saccharomyces cerevisiae) is richer in protein than bottom yeast (Saccharomyces carlsbergensis) and that the protein of the former is more readily extractable.

Hydrocarbon fermentation by yeasts has recently been widely discussed. A concise review of the production of microbial protein from petroleum has been given by Humphrey (1970). One billion tons of crude oil are utilised in the world. Of this crude oil approximately 10% could be converted to cell mass.

The yeast generally preferred is Candida lipolytica, though Candida intermedia and Candida tropicalis have also been used. The latter yeast is sometimes preferred because of its lower lipid content and greater ease of recovery. In the USSR some microbial feed has been made from Candida rugosa. This species cross adapts well to carbohydrates and thus suggests a speedy means of converting a petroleum-based process to one using molasses.

Cell growth occurs mainly at the interphase between oil and water phases. Oxygen is distributed to the cells via contact with all three phases - oil, water and gas. The yeast utilises the straight chain hydrocarbons in preference to the branched-chain hydrocarbons thus increasing the pour-point of the unaffected oil.

The yield of cell mass per unit of substrate is very high when yeasts are grown on hydrocarbons. In many cases yields approximate 100 per cent or even higher. This is accounted for by the low oxidation level of the hydrocarbon as compared to the cell material. However, the cells require a much greater amount of oxygen per unit of cell produced than do cells grown on more oxygenated substrates. Furthermore, as a result of this oxidation,

the heat produced per pound of cells by the hydrocarbon processes is more than twice that for carbohydrate grown cells (Guenther, 1965). The greater oxygen requirement and increased heat production tend to offset to a considerable extent the savings realised from the use of the relatively cheaper hydrocarbon substrate.

Toxicity problems may arise in hydrocarbon derived cells because of incomplete removal of residual oil. The cells must be solvent extracted to remove residual oil which could cause taste, odour and toxicity problems. To simplify the separation and purification problems, the yeast could be grown under conditions where the transfer rate of hydrocarbon is limiting. Such a condition would require careful control of the rate of addition and dispersion of hydrocarbon. Douros, Naslund and McCoy (1968) obtained a patent for the incorporation of an antibiotic during the biosynthesis of protein from hydrocarbon. The addition of streptomycin increased the amino acids and improved the sulphur-amino acid score.

Table VI summarises the reports of yeast production from hydrocarbons.

Powell and Robe (1964) have described a venture which yields foods and feeds from the propagation of Saccharomyces fragilis in whey collected as a by-product during cheese manufacture.

Other yeasts which have been produced commercially on a small scale or which have been studied as potential food producers include Candida arborea (=Monilia candida), Candida tropicalis, C. pulcherrima, C.reukaufii, Hansenula anomala, H. suaveolens, Trichosporon pullulans, Mycotorula lipolytica, Zygosaccharomyces lactis and Pichia polymorpha.

Excellent reviews on yeast technology have been given by Prescott and Dunn (1959) and Peppler (1967). More recent reviews are to be found in a book edited by Rose and Harrison (1970).

The direct consumption of algae for feed and food is negligible though the consumption of seaweeds, especially in Japan, has been known for many years. Clement, Giddey and Menzi (1967) have noted that the alga Spirulina maxima is a traditional part of the diet in Chad. Tannenbaum and Mateles (1968) record that this alga is scooped up as a green slime from brackish

TABLE VI

Reports of yeast production from hydrocarbons (Humphrey, 1970)

1. U.S.S.R.	At least 3 plants. Fermenters ranging from 300-850 m^3. Output of 1,500 - 5,000 tons per annum. 1969 production was 10,000 tons. Goal of 1,000,000 tons per annum. Utilising Ukrainian crude oil fraction.
2. Czechoslovakia	Construction of pilot plant approved. Eventual full scale plant will have 100,000 tons per annum capacity.
3. Grangemouth, Scotland.	Pilot plant operating for several years by B.P. Uses purified n-alkane substrate.
4. Lavera, France.	Pilot plant operating for several years by B.P. Uses 250 - 400°C gas oil. Capacity 225 tons/annum. Construction started for plant with 16,000 tons per annum capacity.
5. Chi-yee, Taiwan.	Chinese Petroleum Co. have operated a 15,000 litre fermenter for 2 years, with a capacity for producing 700 tons per annum.
6. Jorhat, India.	Pilot plant installed. 350 tons per annum.
7. Baroda, India.	Pilot plant installed.
8. Red China	Large plant operating on Mongolian crude oil south of Shanghai.
9. Japan.	Mitsubishi Chemical plan a 2,000 tons per annum pilot plant at Hokkaido. Kyowa Hakko Kogyo plan a 40,000 tons per annum plant at Bofu. Kanegafuchi Chemical are constructing a 60,000 tons per annum plant at Takasago.

waters and dried in the sun to form a cake known locally as dihe. A soup is made from these cakes. The protein content and amino acid pattern of this food are relatively favourable. Furthermore, Spirulina maxima has a rather inoffensive taste which makes it preferable to the unpleasant and sharp taste of Chlorella, an organism commonly used in laboratory studies of food production from algae.

Moulds have not been widely produced for food. Frazier (1958) records that the Germans in World War II produced a mould food from Geotrichum candidum (Oospora lactis, Oidium lactis). Gilbert and Robinson (1957) record that waste sulphite liquor has been fermented by this mould. The dried powder obtained from this fermentation served as a protein

supplement in the manufacture of sausages in Germany but because of the lack of certain nutritional qualities, use of this mould was discontinued.

In later attempts to grow fungus mycelium for food the first consideration was to grow mushrooms which have edible fruiting bodies. Litchfield (1967) has reviewed work done on the submerged culture of mushroom mycelia. Although many species of mushrooms have been grown successfully in mycelial form in the laboratory, only Agaricus spp. and Morchella spp. have been cultivated in large scale pilot plant fermenters. The commercial production of Morchella spp. by the Szuecs process was initiated in the U.S.A. in 1963. In this process (Szuecs, 1956, 1958) pellets of mushroom mycelium are produced aerobically in submerged culture. Though Morchella esculenta was the most favourable, the patents permitted a wider range of mushrooms from the family Helvellaceae.

THE POTENTIAL USE OF BACTERIA IN THE PRODUCTION OF FEED AND FOOD.

The potential production of feeds and foods by bacteria has been reviewed by Thatcher (1954), Bunker (1963) and Ogur (1966). The principal attraction appears to be the very high growth rates and the fact that many species contain appreciable amounts of the sulphur containing amino acids. Roberts (1953) showed that Escherichia coli grown on a well-aerated medium gave a very good protein supplement for rats and chicks. Anderson, Slinger and Pepper (1953) while studying the effects of antibiotics in chick nutrition noted improved growth when E.coli was fed and suggested that diets based on E.coli protein for other farm animals could be evolved with little difficulty. Mondolfo and Hounie (1951) have shown that the methionine and cystine deficiency noted in yeasts does not occur with bacteria. Calloway and Kumar (1969) suggested Hydrogenomonas eutropha as a bacterial source of animal feed. Its amino acid pattern resembles casein and its biological value is as good as casein. The digestibility of whole boiled and sonically ruptured bacterial cells was only slightly lower than that of casein. Bunker (1969) however reports that one lot of H.eutropha fed to human volunteers was found to induce vomiting and diarrhoea. Further work on this organism is said to have been abandoned. (F.K.E.Imrie, personal communication).

It seems reasonable to suggest that the deficient amino acids in yeasts can be supplemented from bacterial sources (Thatcher, 1954). Indeed, Graham, Gibson, Klemmer and Naylor (1953) have suggested a symbiotic mixture of yeasts and lactic acid bacteria, as a means of increasing the yields of biomass from whey. Rose (1961) has pointed out that the cultivation of rumen bacteria might prove useful as these organisms can utilise both cellulose and hemicellulose.

A thermophilic strain of Bacillus stearothermophilus has been isolated in the Department of Nutrition and Food Science at the Massachusetts Institute of Technology. This strain when grown on n-alkane at 57°C, will produce a protein containing up to 2.7% methionine. (Mateles, Baruah and Tannenbaum, 1967).

A possible disadvantage of growing bacteria for feed and food is that rapidly growing cells would have a high nucleic acid content. Since the nucleic acid content is dependent on the growth rate, considerable manipulation is possible. It is not yet clearly established whether intact nucleic acids can be digested by humans and, if so, whether they contribute to an unacceptably high level of blood uric acid (Tannenbaum and Mateles, 1968). It must also be remembered that in fast-growing bacterial cells up to 15 - 20 per cent of the reported protein nitrogen may be nucleic acid nitrogen (Humphrey, 1970).

THE POTENTIAL USE OF ALGAE IN THE PRODUCTION OF FEED AND FOOD.

Much research has been done on the possibility of the economic utilisation of algae for food production. Theoretically, autotrophic organisms are ideal for the production of food as they require only carbon dioxide and no carbohydrates. The organism most often used in the laboratory studies of algal food production is Chlorella spp. Gaucher, Benoit and Bialecki (1960) demonstrated that Chlorella spp. could grow as well in low (0.5%) carbon dioxide concentrations as in higher concentrations. Of late the feasibility of cultivating algae in space ships to utilise the waste carbon dioxide and replenish oxygen and food has been increasingly quoted. Gafford and Richardson (1960) and Pirie (1961) have pointed out the problems and difficulties involved.

At the present stage of development the growing of algae for food seems to be economically out of the question. Some of the problems involved include the enormous surface areas required and the need for adequate illumination. Indications of high yield seen in laboratory scale work have not been borne out in large scale experiments. The problem of bacterial and protozoal infection is mentioned in several papers. There will also be

the need to concentrate large amounts of carbon dioxide. Nicol (1956) considers that the question of nitrogen supply has been glossed over.

In this respect, the cultivation of nitrogen-fixing blue-green algae or Cyanophyta may offer a solution though these are more difficult to work with than ordinary green algae (Bunker, 1968). As blue-green algae grow more rapidly in an alkaline medium, bicarbonate may be used in the medium instead of a stream of carbon dioxide.

A great disadvantage of algae is their poor consumer acceptance because of the colour and bitter taste. Generally algal protein content is very low and a high protein product is difficult to obtain. In addition the biological value of algal protein is low (Humphrey, 1970).

Reviews on algal food production have been given by Rose (1961), Bunker (1963), Verduin and Schmid (1966) and Vincent (1969).

THE POTENTIAL USE OF FUNGI IN THE PRODUCTION OF FEED AND FOOD.

Of all the potential microbial feed and food producers, the fungi (including yeasts and mushrooms) are probably the most widely studied. A comparison of reports of composition and nutritive properties of fungi shows a rather confused picture. This may be partly attributed to the fact that many types of fungi were reported under the same headings with little or no regard for species variations. Values of cell contents were often made on the fresh weight basis though the moisture content varied from less than 1% in woody fungi to more than 90% in fleshy ones. It has been suggested that when values of cell contents are calculated on a dry weight basis there will be considerably less variation.

Traditionally the protein values have been obtained by determining the total nitrogen by a Kjeldahl method and multiplying this value by a factor of 6.25 on the assumption that the nitrogen content of proteins is 16%. Such a method could lead to error when applied to fungi. Clutterbuck, Lovell and Raistrick (1932) isolated an alkali-soluble protein from Penicillium chrysogenum and after purification found it to contain 12.75% nitrogen. Gorcica, Peterson and Steenbock (1934) isolated another alkali-soluble protein from Aspergillus fischeri. It consisted of two fractions, one of which was insoluble in acid and contained 11.8% nitrogen of which 22.8% was basic nitrogen and 60.2% monoamino nitrogen. The other fraction was soluble in acid and contained 12.3% nitrogen of which 38.0% was basic nitrogen and only 36.5% monoamino nitrogen. Bohonos, Woolley and Peterson (1942) isolated an unautolysable protein from Aspergillus sydowi. It contained 11.3% nitrogen and was resistant to hydrolysis by various proteolytic enzymes. Fitzpatrick, Esselen and Weir (1946) found that the purified protein of Agaricus campestris fruiting bodies contained only 11.79% nitrogen.

An even more serious error could be introduced by the fact that a significant amount of the Kjeldahl-reactive nitrogen may be present in non-protein cell constituents such as chitin, amino sugars, purine and

pyrimidine bases, nitrogen containing lipids, coenzymes and special synthetic products such as penicillin and gliotoxin (Foster, 1949). Blumenthal and Roseman (1957) studied 25 fungi and found the chitin content to vary from 2.6% (Neurospora crassa) to 26.2% (Aspergillus parasiticus). Behr (1930) demonstrated that with Aspergillus niger the chitin content varied markedly with pH and age of culture. The purine and pyrimidine bases are major constituents of nucleic acids and the nucleic acid content can vary from 5% to 20% of the dry weight of the cells (Mateles and Tannenbaum, 1968a). A more specific protein determination would seem to be desirable. Indeed, Pande, Tewari and Krishnan (1961) and Sudenko (1968) have suggested the use of the biuret reaction to determine the true protein value of fungi and yeasts.

Mateles and Tannenbaum (1968a) recommend the estimation of protein content by the sum of the individual amino acids though even this is liable to errors introduced by amino acids contained in unnatural peptides or cell wall material. The amino acids in the cell wall material may not be available through normal digestion.

Another possibility to be borne in mind is that certain amino acids may be present as the D-isomer. This can lead to erroneous values of amino acid content unless appropriate microbiological assays are used. The presence of D-amino acids is likely to be deleterious. D-alanine and D-aspartic acid have been shown to have a growth inhibiting effect in the chick (Sugahara, Morimoto, Kobayashi and Ariyoshi, 1967). D-alanine is a poorer source of non-essential nitrogen than L-alanine in the rat (Brinbaum, Winitz and Greenstein, 1957). However, if the cell wall is indigestible, as has been suggested, the presence of D-amino acids would not pose a problem since they are located exclusively in this cell structure.

Although mushrooms had formed part of man's diet for centuries, the idea of using other fungi for food or food supplements only arose in this century. Delbruk's idea of producing yeasts for food has been mentioned. Pringsheim and Lichtenstein (1920) enriched straw fodder with the protein

of moulds. This was done by treating the straw with inorganic nitrogen and inoculating with non-pathogenic Aspergillus spp.

The protein content of moulds varies within extremely wide limits between different organisms and between different cultural conditions for any one organism (Foster, 1949). Thus Waksman and Lomanitz (1925) found that Zygorhynchus molleri and Trichoderma koningi had 6.7 and 6.5 per cent nitrogen when grown on 1% glutamic acid medium but when 2% glucose was also present the values were lowered to 4.7 and 5.3% respectively.

One of the first studies on the potential utilisation of moulds for human food was done by Takata (1929). In his studies using Aspergillus oryzae he found that the mould contained about 38 per cent protein. The following amino acids were reported to be synthesised: arginine, histidine, lysine, cystine, tyrosine and tryptophan. Takata found that though the mycelium permitted growth of albino rats and was high in vitamin B, it was deficient as a protein source.

Clutterbuck, Lovell and Raistrick (1932) isolated a protein fraction from Penicillium notatum and Penicillium chrysogenum which, upon hydrolysis with enzymes and acids was found to contain melanin, arginine, histidine, cystine and lysine. They pointed out that the mould protein appeared to resemble fairly closely a typical alkali-soluble leaf protein.

Adriano and Cruz (1933) showed that 23 common Philippine mushrooms had a high nutritive value with regards to protein. The average protein content was found to be 28.35 per cent. By comparison five imported species of mushrooms had an average protein content of 17.72 per cent.

Skinner, Peterson and Steenbock (1933) studied the nutritive value of mould mycelium. Mycelia of Aspergillus fischeri and A. sydowi as the sole nitrogen source cannot support growth or provide for the maintenance of young rats. However, addition of mould nitrogen to small amounts of yeast or casein prolongs life and stimulates initial growth indicating lack of toxicity. Replacing half the mould mycelium by an equal amount of starch retarded the growth of young rats kept on good rations, while addition of mould mycelium to a ration of low nitrogen exerts a demonstrable

favourable effect.

Skinner (1934) examined the value of mould proteins in diets and established the synthesis of the aromatic amino acids tryptophane and tyrosine from inorganic nitrogen by Aspergillus niger, A. oryzae, A.terreus, Trichoderma konigi, Zygorhinchus moelleri and Penicillium flavo-glaucum. When used as a source of protein on a 9% level in an otherwise complete diet, the dried mycelium of P.flavo-glaucum allowed only slight growth in young rats. On an 18% level the growth was much better. He also showed that cystine was present in limiting quantities.

Pruess, Eichinger and Peterson (1934) found that the crude protein content (N x 6.25) of a number of Aspergillus and Penicillium species grown on a glucose-inorganic salts medium ranged from 13.7 per cent to 43.7 per cent. The protein content of these same strains when grown on a glucose-malt sprout medium was considerably lower and ranged from 12.5 per cent to 36.3 per cent. Aspergillus nidulans contained 25.6 per cent protein when grown on inorganic salts medium but only 13.1 per cent when harvested from malt sprout medium.

Gorcica, Peterson and Steenbock (1934) made one of the more thorough studies of the nitrogen-containing fractions of Aspergillus fischeri. Water extracts yielded no protein. The main portion of the protein of the mycelium was extracted with 1% potassium hydroxide for two days at room temperature.

Gorcica, Peterson and Steenbock (1935) found that when mycelium of Aspergillus sydowi was fed as a sole protein source to rats it was insufficient to promote more than a slight amount of growth or to sustain life in young rats for a period longer than 7 - 9 weeks. Very good growth was obtained when the basal ration was supplemented with $2\frac{1}{2}\%$ or 5% levels of proteins of whole wheat or corn gluten feed. Supplementation with casein, skim-milk proteins, egg white or yeast protein also resulted in good growth though supplementation with gelatin did not improve the growth-promoting power of the mould protein.

Woolley and Peterson (1937a, 1937b) isolated the following 13 amino acids from Aspergillus sydowi mycelium: aspartic acid, glutamic acid, tyrosine, leucine, isoleucine, proline, valine, serine, threonine, tryptophane, arginine, lysine and histidine. A considerable percentage of the nitrogen of the mycelium was contained in these pure compounds. With the exception of arginine these were all isolated from well autolysed material. The presence of phenylalanine, glycine, alanine and hydroxyproline was not detected though they may have been destroyed during autolysis.

Hilpert, Friesen and Rossee (1937) demonstrated that the composition of the mycelium could be altered rather substantially by different concentrations of nitrogen source. In addition to lowering the nitrogen content of the mycelium the low-nitrogen medium caused a reduction in the total carbon content of the mycelium.

Woolley, Berger, Peterson and Steenbock (1938) reported that the mycelium of Aspergillus sydowi was toxic to rats when it comprised 50% of the diet and supplied all the protein. They suggested that this was not the result of an amino acid deficiency but was due to the presence of a toxic substance in the mould. The effect of this toxic substance could be overcome by supplementing with various protein complexes such as liver or casein. Alternatively, the toxic substance could be adsorbed by an inorganic reagent.

Steinberg and Bowling (1939) produced data on the nitrogen utilisation of Aspergillus niger in relation to sugar concentrations in media with the same initial content of ammonium nitrate nitrogen and otherwise identical. However, the nitrogen removed from the medium in these cases was much the same, regardless of the sugar concentration and the differential in cell material synthesised. An interesting finding was that even where the nitrogen content of medium was the limiting factor the organism did not absorb all the available nitrogen from the medium. In general, only 73 per cent to 90 per cent of the nitrogen originally present was consumed though it may well be that the 10 per cent to 27 per cent apparently

unabsorbed was actually assimilated and excreted again (Foster, 1949).

A need for supplementing mould protein was also reported by Skinner and Muller (1940). In their studies with Aspergillus nidulans, A. oryzae, Penicillium flavo-glaucum, P. roqueforti and Geotrichum lactis and two unidentified species of Penicillium they found deficiencies of cystine and methionine.

Peukert (1940) drew attention to the possible use of mould mycelium as a source of protein fodder. He stated that the mycelium formed an appetising source of protein and that in the dry state it was brittle and easily pulverised.

Fink and Schmidt (1941) obtained a patent for a biological process for the production of protein-rich food. Amylolytic and cytolytic fungi were grown on loose substrata containing starch and/or sugar to which are added nitrogenous materials.

Mangold, Stotz and Columbus (1941) carried out experiments on the digestibility and biological protein value of the fungus-protein flakes of Fink in ruminants and swine. They reported that the dry substance of the flakes contained 24.55 per cent crude protein. This was 64.53 per cent digestible in swine and 70.16 per cent in sheep. The biological value of digestible crude protein for maintenance of swine was 94.9 per cent and for growth 79.0 per cent.

Anderson and Fellers (1942) found that rats receiving mushrooms (Agaricus campestris) as a sole source of protein in their diet not only survived the 6-week test period but made a gain in weight equivalent to 30 per cent of that attained by rats on a casein positive control diet. When 20 per cent of the casein was replaced by mushroom protein a growth rate equal to 84 per cent of that of the control group was obtained. The conclusion made was that the protein present in mushrooms is able to support life and promote growth though not so efficiently as with casein. At about the same time Lintzel (1942) studied the value of proteins of mushroom in human nutrition. He concluded that these proteins were

qualitatively comparable to meat and vegetable protein in nutritional value.

Interest in the production of mould food showed an upsurge during the war years. Bremer (1943) drew attention to the importance of producing fungal protein. Vinson, Cerecedo, Mull and Nord (1945) found that some Fusarium spp. were better than brewer's yeast as a source of protein. Fusarium lini mycelium was satisfactory for growth, reproduction and lactation in mice when supplemented with adequate amounts of thiamine. F. graminearum mycelium, however, was satisfactory only if supplemented with multiple B vitamins. Stokes and Gunness (1946) found all the essential amino acids in the mycelia of Rhizopus nigricans, Aspergillus niger and Penicillium notatum and suggested that there was no reason to believe that this would not be true for other moulds and other amino acids. They also pointed out that the amino acid composition of a microorganism is qualitatively and quantitatively a stable characteristic of the cell under fixed growth conditions but that the amino acid content varies slightly among different strains and under different environmental conditions.

Nikische (1951) found that Oidium lactis was a good protein source for dogs in a 10-week study in which 30 g dried material were given per day.

Robinson (1952) quotes an interview with Professor Lembke by interviewers investigating German industry following World War II. Professor Lembke had stated that he had used several strains of fungi in human feeding. Food preparations were made by him from strains of Fusarium, Candida, Oidium, Endomyces and Rhizopus. The general health of the people to whom these moulds were fed was better than that of the control population on a war-time diet. Professor Lembke suggested that his strain of Fusarium was probably the most satisfactory. The East Munich Dairy Station was reported by him to be growing Fusarium on whey. The strains of Fusarium and Rhizopus used by him were high in cystine, methionine and glutathione. It was also reported that in human feeding experiments, the fungi caused diarrhoea if they were not completely autolysed by heating.

The war also stimulated an interest in the production of food and feed

from yeasts. Braude (1942) cited 113 papers in a review of the use of yeast as fodder for cattle. Comprehensive reviews of literature on the nutritive value of yeasts and moulds were also given by Toursel (1942), Thaysen (1943), Fresenius (1943), Carter and Phillips (1944), Loesecke (1946) and Johnson (1947). The general conclusion drawn was that dried yeast is a valuable foodstuff usually being rich in most essential amino acids and in B vitamins.

Sure (1946) fed brewer's yeast (strain K) and four strains of cultured food yeast as a sole source of proteins and B vitamins to rats and found that all of them produced excellent growth, reproduction and lactation over four generations.

However, Brunner (1949) found that the amounts of cystine in six different types of yeast were too low to allow their use as a sole source of protein. He suggested that cystine deficiency was only significant when methionine was also deficient. At about the same time, Schormuller (1949) reported that there were only slight amounts of cystine in yeasts and in mycelial protein.

Harris, Hajny and Johnson (1951) carried out rat feeding trials with six different species of yeast grown on wood hydroysate and compared them with sulphite yeast, brewers yeast, Fusarium lini, soybean meal and casein. They found that the addition of methionine to all the food yeasts, Fusarium lini and soya meal was necessary in order to provide the same dietetic efficiency as casein.

Deficiencies of the sulphur-containing amino acids were not noticed by Chiao and Peterson (1953) in their examination of 20 yeasts selected from 10 genera. The methionine content on a dry weight basis ranged from 0.17 per cent in Endomycopsis fibuliger to 1 per cent in Rhodotorula gracilis; the cystine content ranged from 0.19 per cent in Dabaryomyces matruchoti to 0.58 per cent in Hansenula saturans.

Tsien, Johnson and Liener (1957) have pointed out that too much emphasis must not be placed on amino acid analyses. They state "It

is well recognised that the amino acid analysis of foodstuffs may not be a reliable index of the nutritive quality of its protein. The biological availability of lysine in particular may diverge appreciably from its analytical value in a number of proteins". They showed for example that there were greater growth responses on lysine-deficient diets supplemented with 15 - 20% yeasts than could be accounted for simply on the basis of the lysine so provided.

Recent research in yeast technology has been directed towards utilising other yeasts for feed and food. Hipolito, Domingo and Sarte (1965) investigated a high protein yeast Rhodotorula pilimanae (Hendrick and Barke) isolated from Philippine strawberry fruit. The yeast was grown on coconut water supplemented with dextrose, inorganic nitrogen and minerals. Maximum yields of 51 per cent based on assimilated sugar were obtained in 60 hours. The yeast contains about 50 per cent protein and appreciable amounts of B vitamins. It had an attractive colour and appetising flavour and aroma.

The introduction of the 'symba-yeast process' promises new potentialities in yeast technology (Tveit, 1966) especially where cheap starch is available and nitrogen is not too expensive. In this process Candida utilis is grown together with Endomycopsis fibuliger. The latter yeast secretes amylases extracellularly making the starch available to the former.

Witkowski (1968) used Monilia murmannica in his investigations of the amino acid content of food yeast produced by fermentation of beech wood hydrolysates. The amino acid content was similar to that of other fodder yeasts and all the essential amino acids were present. Samtsevich and Zalashko (1969) investigated the simultaneous cultivation of protein and carotenoid yeast on whey. In their experiments Candida yeast which is devoid of carotene was grown on a 1:1 ratio with Rhodotorula gracilis or Sporobolomyces roseus. The combined cultures produced approximately the same biomass as cultures of protein yeasts grown separately. No suppression of one type of yeast by another was observed in combined cultures.

Much progress has also been made towards producing feed and food of mould origin. In the years following World War II research was directed towards producing higher fungi in submerged culture. Thus, Humfeld (1948) described the production of mushroom mycelium (Agaricus campestris) in submerged culture, on fruit and vegetable waste. Under these conditions only the mycelium and not the fruiting body or mushrooms is formed. The mycelium, however, has a typical mushroom flavour and can be used as a flavouring material.

The mycelium of another species, Agaricus blazei,has been grown successfully under submerged conditions, on orange juice and citrus press water and synthetic media (Block, Stearns, Stephens and McCandless, 1953). It contained 32.5 per cent protein, on a dry basis, compared to 43 per cent in the fruiting body growing wild. Analysis of hydrolysates of the mycelium indicated the presence of at least fourteen amino acids. The mycelium was also rich in B vitamins. The mycelium when prepared as a food, lacked the true mushroom flavour; however, because of its bland taste the authors suggested that it might be useful for pharmaceutical concentrates of B vitamin and amino acids.

Sugihara and Humfeld (1954) grew twenty species of mushrooms in submerged culture. Of these only Agaricus campestris and Lepiota rhacodes had pleasant flavours.

Jennison, Newcomb and Henderson (1955) made a quantitative comparative study of the growth and nutrition of 42 species of brown-rot and white-rot Basidiomycetes in submerged culture in synthetic media, with particular reference to utilisation of known nitrogen compounds. The average maximum mycelial dry weight was 102.1 mg/70 ml culture for brown rots and 123.0 mg/70 ml culture for white rots. In a subsequent paper, Jennison, Richberg and Krikzens (1957) examined the nutritive composition of these wood-rotting Basidiomycetes when grown in submerged culture. The average protein content of the 17 fungi studied was 30.6 per cent after a 7-day growth. A study of the vitamin contents was also made. The

authors concluded that no one species was 'best' in all-round nutritive composition. Feeding of the uncooked mycelia to mice and guinea pigs indicated that none of the 17 species caused any acute toxicity.

Chastukhin, Goncharova and Golubchina (1957) described their experiments in which 22 moulds were screened as to their ability to grow on 'treacle liquor' from alcohol-producing plants. Basidiomycetes and Phycomycetes were very slow growing whereas representatives of the genera Aspergillus, Penicillium and Fusarium grew well. The yields of biomass considerably exceeded that of yeast when grown on the wastes of hydrolysing and sulphite alcohol-manufacturing plants. The high yields seemed to indicate that the fungi were able to use other organic substances besides sugars. The crude protein value ranged from 20% to 30%. From data obtained the authors concluded that fungi grown on treacle liquor were quite suitable for use as fodder protein.

The National Dairy Products Corporation of New York was assigned a patent (Stimpson and Young, 1957) for a method of increasing the protein content of milk by aerobically propagating a yeast or mould in an aqueous nutrient medium based upon a milk product. The moulds suggested included Aspergillus niger, A. flavus, A. flavipes, A. sydowi, A. flavus oryzae, Fusarium lactis, Penicillium notatum and P. chrysogenum. The patent also claimed methods of releasing the cell substances by heating, plasmolysis, treatment with ultrasonic waves or by freezing and then thawing.

Reusser, Spencer and Sallans (1957) and Anderson and Jackson (1958) reviewed the occurrence of the essential amino acids in microbial protein. Robinson and Davidson (1959) reviewed the progress that had been made in the large-scale growth of higher fungi.

Reusser, Spencer and Sallans (1958a) examined 10 strains of mushrooms grown in submerged culture. High nitrogen concentrations in the media favoured protein production and reduced nitrogen concentrations induced fat formation. High yields of dry matter and protein were obtained on molasses medium, while yields on waste sulphite liquor were slightly lower.

These workers selected Tricholoma nudum for further studies as a source of microbiological protein (Reusser, Spencer and Sallans, 1958b). They concluded that it appeared to be a potentially valuable source of food and fodder protein. It had about the same content of protein as fodder yeast, and contained approximately the same concentration of essential amino acids. Recovery of the mycelium, by filtration rather than by centrifugation was easier than with yeast. The flavour and aroma of the dried mycelium were observed to be distinctive and pleasant.

Eddy (1958) cultured 28 edible mushrooms (Ascomycetes and Basidiomycetes) and concluded that though edible materials of good food value could be produced by the submerged cultivation of higher fungi, in very few instances were mushroom-like flavour or odour obtained. The production of a full mushroom flavour appeared to be connected in some way with sporophore production.

Cirillo, Hardwick and Seeley (1960) were issued a U.S.patent for a fermentation process for producing edible mushroom mycelium from waste sulphite liquors. Highest yields were obtained with Tricholoma nudum and Collybia velutipes.

Litchfield and Overbeck (1963) reported their experiments on submerged culture growth of three species of morel mushrooms using cheese whey, pumpkin canning waste and corn canning waste as substrates. A carbon to nitrogen ratio of 8:1 gave higher yields as did the addition of 0.5% potassium dihydrogen phosphate. Morchella hortensis gave highest yields of biomass and its mycelium had a protein content of 35%.

Litchfield, Vely and Overbeck (1963) studied the nutrient content of morel mushroom mycelium grown in submerged culture in a glucose-ammonium phosphate - corn steep liquor medium. The dried mycelia of three species were subjected to proximate analyses and amino acid determinations. The dry samples contained 22.8 - 51.0% of protein and 2.18 - 7.55% of fat depending upon the species. Amino acid contents were similar to those reported in the literature of other fungi.

Gray (1962) suggested growing fungi to meet the problem of protein deficit. Because of their great numbers, their ubiquity and their rapidity of growth it was decided at the beginning of his programme to restrict the effort to a consideration of the moulds of the Fungi Imperfecti. Gray, Och and Abou-El-Seoud (1964) screened 175 isolates with respect to their economic coefficients, using a shake-flask method with crude glucose as the carbon source and ammonium nitrate as the inorganic nitrogen source. (Economic coefficient was defined as the unit weight of carbohydrate required to produce one unit weight of dried fungus tissue). The authors concluded that about half the isolates had economic coefficients which warranted immediate further study as potential protein sources.

In the next step of the programme different means of reducing the cost of production were explored. To overcome the high cost or scarcity of large quantities of fresh water in some areas Gray, Pinto and Pathak (1963) substituted sea water for fresh water. This was found to favour the growth of many of the fungi tested. However in a few there was a marked reduction in growth. The beneficial effect could not be ascribed to sodium chloride or phosphate ions but may be due to the magnesium ions in sea water.

The use of protected fermentations to reduce the cost of production was found to be successful with some of the fungi tested (Gray and Abou-El-Seoud, 1966). This relied on the lowering of the initial pH of the medium so that the cost of sterilisation of the medium was avoided. Gray and Staff (1967) examined the calorific value of the mycelia of 100 fungal isolates. The average heat of combustion of the fungi examined was found to be 4.431 Kcal/gm of mycelium.

Another programme to investigate protein formation by strains of wood-destroying Basidiomycetes was initiated by Falina, Yakimov and Maslova (1961). Initial work was done on surface cultures and it was found that Hydnum septentrionale had the largest concentration of protein (9 gm/litre) of the strains studied. Falina, Maslova, Yakimov, Andreeva

and Alekseeva (1965) examined 5 cultures of Basidiomycetes and found that the highest protein amino acid content occurred in young mycelia. 15 amino acids were detected in the five cultures examined. Maximum yields of biomass was obtained from Tricholoma fulvum. Notwithstanding the widely held belief that higher fungi are slower-growing, Falina, Maslova and Yakimov (1965) found that some Basidiomycetes grown in submerged culture yielded maximum weights of mycelia in 72 hours. 16 amino acids were detected by paper chromatography following alkaline hydrolysis of the mycelia. The lysine and methionine contents were higher than those of tryptophan in all the species studied.

An interesting finding was made by Falina, Maslova and Andreeva (1966) in their investigations of the biosynthesis of some essential amino acids of Basidiomycetes. Highest contents of methionine and tryptophan were found in mycelial hydrolysates after 24 - 36 hours growth but highest lysine was found after 36 - 60 hours growth. This may have to be taken into account in the production of a 'well-balanced' feed or food. Falina, Maslova, Mattison, Yakimov, Andreeva and Alekseeva (1966) examined 20 strains of Hymenomycetes and obtained maximum yields of 40 - 50% based on added carbohydrate. The maximum mycelial weights ranged from 8 gm/litre to 20 gm/litre and the mycelium composed of 25 - 40% amino acids. The Basidial fungi were found to contain more methionine and less lysine than forage yeast.

Wakita (1962) investigated the carbohydrates and amino acids from 63 species of 40 genera of mushrooms. He found that values ranged from 0.76% for non reducing sugars, 0.4 - 2.6% for reducing sugars, 1.0 - 4.3% for amino nitrogen and 0.01 - 0.7% for free amino nitrogen. No free amino acid was commonly detected in all mushrooms. Glutamic acid, alanine and aspartic acid were detected in the majority of mushrooms while tyrosine, lysine, α-aminobutyric acid and proline were detected in few. Szyniczak (1962) investigated the amino acid composition of edible mushroom proteins by paper chromatography of the hydrolysates. Similar studies of the nutritive value of mushrooms were made by Kaler and Bichevaya (1964)

and Bano, Ahmed and Shrivastava (1964).

Frank (1964) examined 64 samples of dried mycelia of Oospora lactis for feed value and found them to contain an average of 40.7% crude protein. The organism was grown on sulphite waste from a paper factory which used pine as raw material. Falanghe, Smith and Rackis (1964) studied the production of fungal mycelial proteins in submerged culture on soybean whey. They found that Tricholoma nudum and Boletus indecisus gave the greatest rate of growth and production of protein and the best utilisation of soybean whey solids.

The production of feed protein by cultivating mycelial fungi in spent liquors containing oligosaccharides has been reported by Semushina and Monakhova (1965). These oligosaccharides are not utilised by yeast without preliminary hydrolysis. They examined several fungi and concluded that Aspergillus luchuensis was the best for industrial use yielding 48 - 65% biomass based on carbohydrates. The growth rate was less than that of yeasts and the cells contained less protein (35 - 45%).

Mycelial fungi frequently grow on the yeast-growing media of the hydrolysis and sulphite pulp industries. This was investigated by Rodionova, Kryuchkova, Korotchenko and Frolkina (1967) who found that the most common contaminants were from Oospora spp. and Trichosporon spp. Others included species from Dematium spp., Fusarium spp., Spicaria spp. Penicillium spp. and Aspergillus spp. The growth rate of Oospora spp. and Trichosporon spp. were approximately the same as that of Candida yeast. The mycelia contained all the essential amino acids and produced vitamins of the B complex group. Szudzinska (1968) reviewed the fungal fermentation of wood and pulp industry wastes to produce fodder proteins.

The use of mycelial wastes from industrial fermentations has been suggested (Pathak and Seshadri, 1965; Zdzislaw, 1967; Aidinyan, 1967; Kovats, 1969; Yano, Horii and Ozaki, 1969).

Zakordonets (1965) investigated the effects of nitrogen sources on the biosynthesis of free amino acids and fungal growth. Of the 7 ammonium salts, 5 nitrates, 1 nitrite and 14 amino acids studied, the ammonium salts, especially the citrate, gave best growth. In a similar investigation of carbon sources, Zakordonets and Elans'ka (1965) found that maximum growth of mycelia occurred in a media based on glucose as a carbon source. Zakordonets (1968) obtained 17 amino acids by acid hydrolysis of Penicillium expansum, Fusarium moniliformi and Aspergillus ustus. Though there were quantitative differences in the amino acids, the particular amino acids did not change with mycelial development.

More recently, Vessey and Toth (1969) have obtained a patent for the production of protein-enriched fodder and a mushroom-like product useful for human consumption. In their process rice enriched with malt was incubated with Pleurotus ostreatus for two weeks to give a product where the protein content was increased by more than 50%. Wang, Ruttle and Hesseltine (1968) improved the protein quality of wheat and soybeans by fermentation with Rhizopus oligosporus. The growth of rats fed fermented wheat improved significantly over those fed unfermented wheat. This was partly attributed to an increase in the availability of lysine.

A similar process for the protein enrichment of cassava was reported by Brook, Stanton and Wallbridge (1969). A variety of organisms were tested and the experiments were mainly with Rhizopus oligosporus and R. stolonifer. In solid fermentations, protein levels of as high as 3% were obtained, representing a 6 - 7 fold increase compared to that of the original cassava material. The deep culture fermentation of a suspension of cassava flour with added nitrogen and other nutrients was also investigated. The most promising organisms were Heterocephalum aurantiacum, Cladosporium cladosporioides, Mucor racemosus and a Rhizopus spp.

Atacador-Ramos, Palo, Villadolid and Cruz (to be published) studied the submerged culture production of banana mushroom (Volvariella volvacea)

mycelium as a source of protein, B-vitamins and food flavour. On a medium of 4% sucrose and low concentrations of minerals, a maximum yield of 2.4 g dried mycelia per 200 ml. medium was obtained after 5 days at 28° - 32°C. By substituting coconut water in the basic medium and adding sucrose to give a sugar concentration of 4% the yield was increased 2.35 times and the time reduced to 3 days. The mycelium contained 45.3% proteins as well as B-vitamins. Food recipes prepared from the mycelium had the very desirable mushroom flavour.

Coppock (1970) has discussed the recent work of Professor A. Spicer on fungal food of Penicillium notatum on a substrate of starch and ammonium salts. The product is estimated to contain 50 - 55% protein and can be grown with the balance of amino acids being appropriate to human requirements. Drying of the product is apparently important and at 100°C half the nutritive value of the product can be destroyed.

The potential value of fungi in feed and food production is not confined to a consideration of their protein content. Fungi have also been grown for their fat content. It has been estimated that one acre of concrete-enclosed pond used for the propagation of fungi and algae could produce fat equivalent to that yielded by 25 acres of a vegetable oil crop (Robinson, 1952). Germany used microorganisms for fat production during both world wars.

As far as is known, all fungi are capable of synthesising fats but some have received especial attention with respect to this capacity. The fungi most commonly studied are species of Penicilium and Aspergillus. Information of the fat contents of fungi has been collected by Prescott and Dunn (1959). The fat contents ranged from 1% to 50%. In general it is found that the relative amounts of fat and protein synthesised were dependent on the amount of nitrogen in the medium. As available nitrogen became limiting growth rates were greatly reduced and the major synthetic

activity changed to the formation of fats which appeared as globules within the older cells of the culture.

Two processes were developed for the production of fat using fungi. Fink, Haeseler and Schmidt (1937) described a process using Oospora lactis growing on milk whey. The organism was especially resistant to contamination and thus lent itself to the shallow pan process which was found to be superior. A sugar concentration of 4% to 6% was satisfactory and maximum yields of fat were obtained in five days. The fat coefficient (grammes of fat produced per 100 grammes of substrate carbohydrate) was in the order of 12 to 14. Rippel (1940) had calculated the maximum fat coefficient to be expected from any organism. This calculation was made on the basis of energy relations and he predicted a maximum fat coefficient of 15.

Interest in Oospora lactis waned on account of its inability to be grown satisfactorily in submerged culture. Damm (1944) developed a successful submerged process which gave superior results. Ascomycetes and Phycomycetes of the genera ~~Rhizopus,~~ Mucor, Rhizopus and Gibberella were satisfactory. The medium was rich in carbon sources and had a pH between 2.4 and 6.8. Fat production was associated with the production of chlamydospores. The aeration rate appeared to be critical for fat formation though in some cases fat yields were increased by a subsequent quiescent period without aeration. During this period the protein content decreased. The properties of the fat produced in the degeneration stage were practically identical with those of the reserve stock of fat formed during the first stage. Best results were obtained with Mortierella pusilla where a fat content of up to 59.6% of the dry mycelial mass was obtained.

Extensive studies have been made on fat production by yeasts. Microbiological fat production was pioneered by Lindner and the famous Endomyces vernalis process was developed by him and his associates during World War I. This process has been reviewed by Fink, Haehn and Hoerburger (1937).

Of the large number of yeasts studied for fat production Rhodotorula gracilis (=Rhodotorula glutinis) seems to be the most promising (Pan, Andreason and Kolachov, 1948). It is able to grow readily in submerged culture, forms fat rapidly with a high fat coefficient and is easily recovered from the propagation medium. Enebo, Anderson and Lundin (1946) achieved lipid contents of 50% to 60% with this organism when it was subjected to conditions of nitrogen starvation. Lundin (1950) has reviewed the possible industrial development of fat production by Rhodotorula gracilis.

Foster (1949) has reviewed the early work on fungal lipids. Reviews on microbial fat production have been given by Woodbine (1959), Prescott and Dunn (1959) and Gray (1959).

Microorganisms would not be considered as a main source of carbohydrates though there would be some calorific contribution by the carbohydrate fraction in microbial feeds and food. Values of the carbon content of mould mycelia range from 45% to 55%, the organism and cultural conditions deciding the value in any one case. Under appropriate conditions, principally luxury carbohydrate nutrition, a fungus may produce more than its own weight of some simple or complex carbohydrate and this synthesis may be caused to fluctuate within wide limits without impairment to the organism (Foster, 1949).

A large number of chemically diverse carbohydrates have been isolated from fungi. Mannitol occurs commonly in many higher fleshy fungi (Foster, 1949). Trehalose occurs abundantly in fungi and may displace mannitol entirely as the major sugar (Birch, 1970). Glucose, fructose and sucrose are seldom found in the cell although they are readily utilised by most fungi (Hawker, 1950).

The polysaccharides of fungi include starches, glycogen, hemicelluloses and also more difficultly hydrolysable cellulosic lignin-like materials (Stokes, 1958). The most common reserve carbohydrate in fungi is glycogen. True starch is seldom found, although it has been shown that it, or a closely related substance giving the typical blue colour with iodine and hydrolysing to glucose is readily produced by certain species of Penicillium

and Aspergillus. This is sometimes termed 'mould starch'.

Other polysaccharides hydrolysing to glucose and giving red or purple colours with iodine are common. A glucose polysaccharide, known as mycodextran, which gives no colour with iodine is produced by Penicillium expansum.

Mycogalactan, which hydrolyses to galactose, is produced by Aspergillus niger from glucose. Various fungal polysaccharides built up of mixtures of glucose, mannose or galactose molecules have been described but their structures have not been worked out.

GROWTH OF FUNGI

INTRODUCTION

In view of the foregoing discussion it was decided that further studies should be carried out on the production and utilisation of fungi as feeds and foods. It is anticipated that fungi, because of their ability to grow rapidly in a wide variety of carbohydrate substrates giving high biomass yields with relatively good protein contents would be a suitable source of protein that could be produced at a price competitive to those of existing protein sources.

In terms of production costs fungi have an advantage over yeasts and bacteria in that recovery costs would be far lower. These latter organisms are unicellular and their recovery entails centrifugation which is an expensive unit operation. Washing these cells involves resuspension and recentrifugation which further adds to production costs. In contrast, the fungi because of their filamentous nature may be easily recovered by filtration through meshes or string filters. The high porosity of the mycelial mat so obtained would permit its washing with a spray of water without the addition of a filter aid.

The fungi were grown in submerged liquid culture so as to obtain a more economical use of the substrate. Such a culture also minimised the formation of spores which are not desirable in feeds and foods. Furthermore, the chances of mycotoxins being produced are minimal in submerged culture.

The fungi were grown in aerobic tower fermenters, again with a view to reducing production costs. In an aerobic tower fermenter there are no impellers to bring about agitation. Agitation is obtained by the flow of air through the medium. This is an advantage over conventional stirred tank fermenters where the power input required for agitation may account for a significant proportion of the cost of running the fermenters.

MATERIALS AND METHODS

Organisms:

The particulars of the fungi grown for this study are shown in Table VII. The fungi were subcultured at intervals of approximately three months. On subculturing, the fungi were grown in universal bottles at 29°C for one week and thereafter kept at 4°C.

Preparation of inocula:

The fungi were subcultured on agar slopes in 4 fluid ounce flat bottles prior to preparation of inocula. The medium used for this was identical to that used for maintaining the fungus. Various types of inocula were used.

(i) Barley Flasks.

100 g. barley grains were steeped in 50 ml. tap water for about 12 hours in a 1-litre conical flask. 10 ml. glycerol and 0.5 g asparagine were added and the flask autoclaved at 15 p.s.i. for 45 minutes. The spores were removed from the agar slopes with a small quantity of sterile 0.05% Tween 80 and added aseptically to the cooled barley flask. The flask was incubated at 29°C and shaken periodically to break up clumps. For inoculation the spores were removed with sterile 0.05% Tween 80.

(ii) Liquid surface mat.

500 ml. quantities of the growth medium were autoclaved in 2-litre flat bottom flasks at 15 p.s.i. for 15 minutes. On cooling these were inoculated with a mycelial/spore suspension obtained by shaking up a small quantity of sterile 0.05% Tween 80 with fungus grown on agar slopes in flat bottles. The flasks were incubated at 29°C without agitation for 9 days after which time a surface mat was obtained. For inoculation the surface mat and media were aseptically transferred into a sterile Waring blender and macerated for 30 seconds. The suspension so obtained was used as the inoculum.

(iii) Solid surface culture.

This was used for non-sporulating fungi. The fungi were grown at 29°C in Petri dishes using a medium identical to that used for growing the fungus except that the medium was solidified with 2% w/v agar. For inoculation

TABLE VII

Particulars of fungi used

Fungus	Source	Collection Number	Medium for Maintaining
1. Aspergillus luchuensis	Commonwealth Mycological Institute●	83356	5% MEA□
2. Aspergillus niger	ex Dr. R.N.Greenshields collection	[redacted]	5% MEA
3. Aspergillus oryzae	Commonwealth Mycological Institute	44242	PDA▽
4. Aspergillus sydowi	Commonwealth Mycological Institute	94162	PDA
5. Cladosporium cladosporioides	Commonwealth Mycological Institute	59473	PDA
6. Fusarium oxysporum	Commonwealth Mycological Institute	113138	PDA
7. Heterocephalum aurantiacum	Commonwealth Mycological Institute	131684	PDA
8. Lenzites trabea	Forest Products Research Laboratory■	108E	5% MEA
9. Mucor racemosus	Biodeterioration Information Centre▼	[redacted]	5% MEA
10. Penicillium chrysogenum	Commonwealth Mycological Institute	37767	5% MEA
11. Penicillium notatum	Commonwealth Mycological Institute	17968	5% MEA
12. Polyporus anceps	Forest Products Research Laboratory	70A	5% MEA
13. Rhizopus oligosporus	Biodeterioration Information Centre	[redacted]	PDA
14. Rhizopus oryzae○	Commonwealth Mycological Institute	40564	PDA

Key on following page

● Full address: Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey.

■ Full address: Forest Products Research Laboratory, Princes Risborough, Aylesbury, Bucks.

▼ Full address: Biodeterioration Information Centre, University of Aston in Birmingham.

□ 5% Malt Extract Agar

▽ Potato Dextrose Agar

○ Obtained under Licences HH10501/2 and HH10501/7 from Ministry of Agriculture, Fisheries and Food.

the slopes were aseptically transferred into a sterile Waring blender and macerated for 30 seconds with a small quantity of sterile water. The suspension so obtained was used as the inoculum.

Raw materials used in media.

(i) Molasses

Beet molasses were obtained from the British Sugar Corporation refinery at Kidderminster. Three different batches were used and the analyses of these were as follows:

	Batch I	Batch II	Batch III
Percentage solids▼	80.2	82.5	80.5
Percentage reducing sugars●	50.8	51.6	52.1
Percentage nitrogen■	1.01	1.2	1.09
pH of 10% w/v solution	6.8	6.8	6.7

▼ Determined by drying to constant weight at 105°C

● Determined by method of Lane and Eynon (1923) after hydrolysing with concentrated hydrochloric acid at 90°C for approximately one minute

■ Determined by the Kjeldahl Method. The catalyst used for digestion was a 160:40:1 mixture of potassium sulphate, copper sulphate and sodium selenate. The indicator was a mixture of equal volumes of 0.2% Methyl Red and 0.1% Methylene Blue in absolute alcohol.

(ii) Malt

The malt used was concentrated malt extract supplied by EDME Ltd. Analyses carried out by the suppliers showed 73.7% total sugars, 0.756% total nitrogen and 20.3% water.

(iii) Chemical reagents

The chemicals were of laboratory grade.

Composition of Media

Two different media were used.

(i) Molasses medium

Beet molasses		12.5% w/v
Ammonium sulphate		1.0% w/v
Ammonium nitrate		1.0% w/v
Diammonium phosphate		1.0% w/v
Water	to	100.0%

The pH was adjusted to 5.5 with concentrated hydrochloric acid prior to sterilisation.

The calculated carbon to nitrogen (C:N) ratio of this medium was 3.5:1 assuming 50% sucrose in the molasses as the sole carbon source.

(ii) Malt Extract Medium

Concentrated Malt Extract		10.0% w/v
Ammonium sulphate		1.0% w/v
Ammonium nitrate		1.0% w/v
Diammonium phosphate		1.0% w/v
Water	to	100.0%

The pH was adjusted to 5.5 with concentrated hydrochloric acid prior to sterilisation.

The calculated C:N ratio of the medium was 3.8:1 assuming the total sugars of malt to be glucose and ignoring the nitrogen found in malt.

Aerobic Tower Fermenters

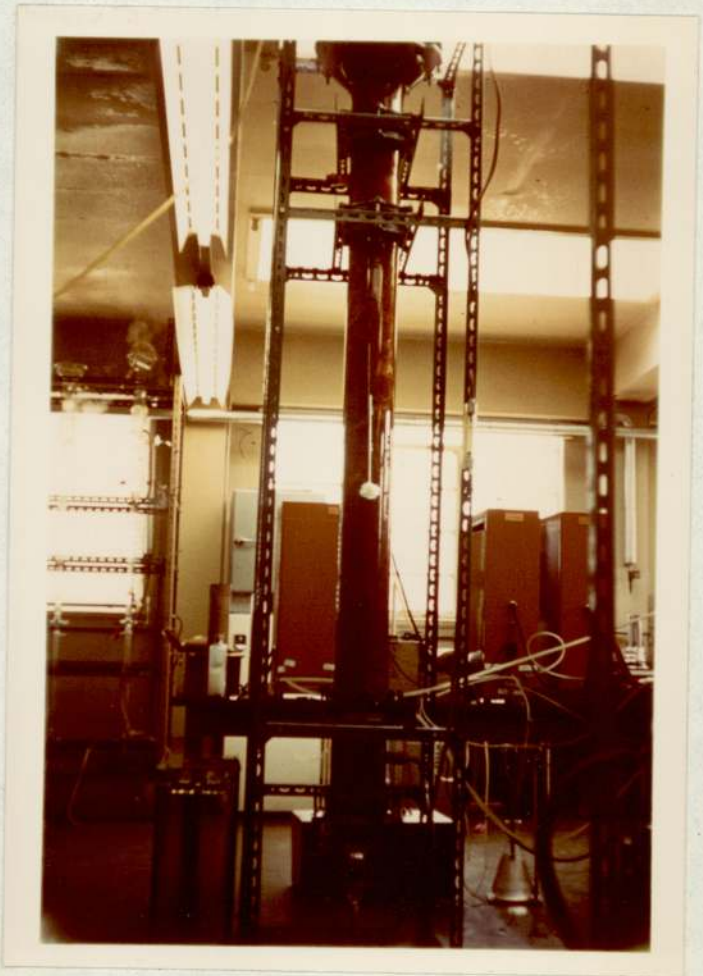
Glass tower fermenters of two different sizes were used.

(i) 50 litre Tower Fermenter (Plate I)

This consisted of a vertical narrow cylindrical portion (6" x 84") which had a capacity of approximately 42 litres and a top expansion chamber having diameter 12" and height 15". The expansion chamber served to control foaming. Sterile air was introduced through a point at the base of the tower and distributed by a sintered glass disc (3.5" diameter, porosity 2 having mean pore size 25μ - 28μ) attached across the cone

Plate 1

50 litre Aerobic Tower Fermenter



at the bottom of the stem portion. There was a sampling point about 12" above the glass sinter.

(ii) 4-litre Tower Fermenters (Plate II)

This consisted of a 6.5 cm x 150 cm cylindrical portion only with no expansion chamber. The actual capacity was 5 litres so that with a working volume of 4 litres there was adequate room for foam control. The glass sinter at the bottom was 4 cm. across and had a porosity of 2 (mean pore size 25μ - 28μ). The sampling point was 34 cm. above the sinter.

Sterile air supply.

The air supply was sterilised by passing through a cotton wool filter connected in line with a Biotec laboratory scale glass fibre paper filter. The quantity of air supplied was metered with Gapmeters^{R} (G.A.Platon Ltd.)

Antifoam.

Silcolapse 437^{R} (I.C.I.Ltd.) was used. This contains 30% silicone as methyl polysiloxane and was diluted by the addition of 2 parts of water to 1 part of antifoam emulsion before sterilising by autoclaving. It was confirmed by the manufacturers that the constituents and emulsifiers were non-ionic and should not interfere with ion-exchange resins in amino acid analysers.

Temperature control.

The fungi were grown at temperatures between 25°C and 27°C. Temperature control was attained by means of thermostatically controlled element wraps on the stem portions of the tower fermenters.

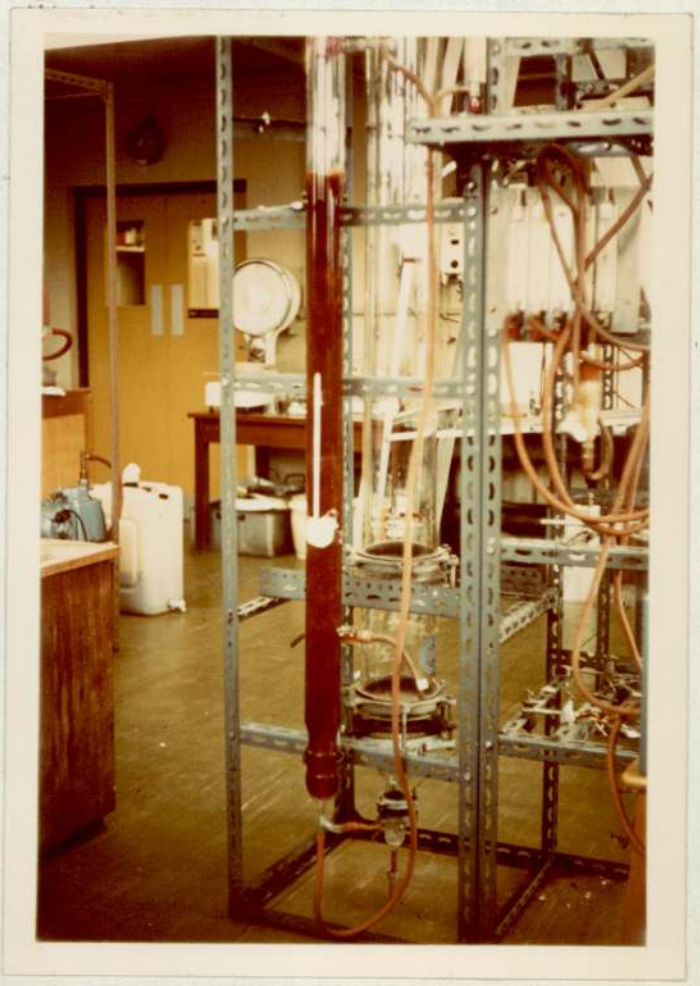
Procedure for use of tower fermenters

The tower fermenters were sterilised by free steaming for 24 hours prior to filling.

Medium for the 50 litre towers was prepared with hot water and mixed with a Silverson mixer until dissolved. The medium was pumped through three flask pasteurisers and recycled back to the media-making

Plate II

4 litre Aerobic Tower Fermenter



vessel until a temperature of 93 - 95°C was attained. It was held at this temperature for one hour before it was pumped into the tower. Sterile air was pumped into the tower to maintain a positive pressure in the fermenter and to prevent backflow through the sinter.

For the 4-litre towers the medium was prepared in 2-litre lots and sterilised by autoclaving at 15 p.s.i. for 15 minutes. The sterile medium was poured into the tower.

The tower contents were cooled to 35°C before inoculation. The culture was stirred by air blown through the sinter. Foaming was controlled by the use of minimal amounts of antifoam. The 50-litre fermenters were aerated at a rate of approximately 25 litres of air per minute. The corresponding figure for the 4-litre fermenters was 4 litres.

A 100-ml. sample of medium was taken for analysis just prior to inoculation. 100 ml. samples were taken periodically after growth had started and the wet and dry weights of the mycelium determined. At the point of maximum yield the mycelium was harvested by filtering through Quadruple Extra Heavy Quality Grit Gauze (Mesh No.40).

One 500 ml. portion of mycelial broth was filtered separately to record the final yield and the volume occupied by the mycelium. The filtrate was used for analysis. The mycelium obtained was washed and dried.

Analytical Methods

(i) Dry weights - determined by drying to a constant weight at 105°C.

(ii) Yield - expressed as grammes per cent, i.e. dry weight in grammes of mycelium obtained from 100 ml. of mycelial broth.

(iii) Sugar content - determined by method of Lane and Eynon (1923) as previously described.

(iv) Nitrogen content - determined by Kjeldahl method as previously described.

(v) Protein content - Kjeldahl nitrogen x 6.25

(vi) Conversion efficiency - this was equal to

$$\frac{\text{Mycelial Dry Weight}}{\text{Initial total sugars} - \text{Residual sugars}} \times 100$$

allowance being made for the volume occupied by the mycelium when calculating residual sugars.

RESULTS AND DISCUSSION

The growth characteristics of the various fungi in tower fermenters were found to be as follows:

(i) Aspergillus luchuensis

	Run I	Run II
Medium	molasses	molasses
Scale	50 litres	4 litres
Inoculum	barley flask	barley flask
Morphological form	diffuse mycelium see Plate III	pellets approx. 1mm in diameter
Time for maximum yield	112 hours	36 hours
Yield	1.62%	1.10%
Protein content	42.7%	42.15%
Conversion efficiency	30.5%	57.5%

Observations: The spores germinated readily in 10 - 12 hours. The mycelium was brown in colour and sporulated readily on exposure to air.

(ii) Aspergillus niger

Medium	molasses
Scale	50 litres
Inoculum	barley flask
Morphological form	pellets approx. 2 mm. in diameter

Time for maximum yield	90 hours
Yield	1.2%
Protein content	37.0%
Conversion efficiency	36.5%

Observations: The spores germinated at approximately 20 hours. A major portion of the biomass was produced in the first 60 hours.

(iii) Aspergillus oryzae

Medium	molasses
Scale	50 litres
Inoculum	liquid surface mat
Morphological form	Pellets approximately 2 mm. in diameter. The hyphae at the outer regions of the pellets bore swellings which were probably chlamydospores (Plate IV)
Time for maximum yield	120 hours
Yield	0.87%
Protein content	34.0%
Conversion efficiency	22.0%

Observations: The strain used did not sporulate and hence a liquid surface mat inoculum was used. Growth was not rapid, the yield at 72 hours being 0.65% and at 96 hours being 0.85%.

(iv) Aspergillus sydowi

	Run I	Run II
Medium	molasses	molasses
Scale	4 litres	4 litres
Inoculum	barley flask	spores from flat bottle
Morphological form	diffuse mycelium	elongated pellets approximately 1mm x 2mm
Time for maximum yield	40 hours	48 hours
Yield	0.75%	0.63%

Plate III

Diffuse mycelial growth of Aspergillus luchuensis

Magnification: X720

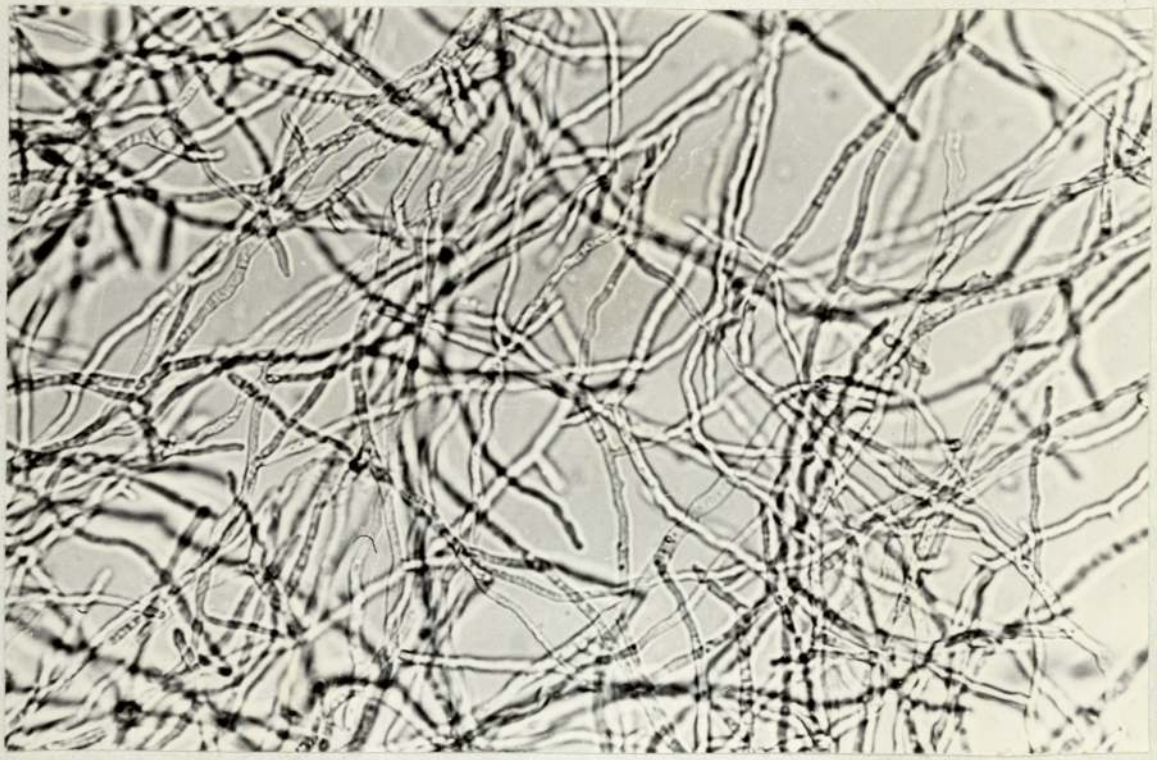


Plate IV

Pellets of Aspergillus oryzae

Magnification: X36

	Run I	Run II
Protein content	43.5%	47.0%
Conversion efficiency	41.5%	36.5%

Observations: Germination was slow unless very large inoculum levels were used. Consequently a number of runs were infected by yeasts at the pre-germination stage.

(v) Cladosporium cladosporoides

Medium	molasses
Scale	4 litres
Inoculum	barley flask
Morphological form	pellets approximately 2 mm. in diameter
Time for maximum yield	30 hours
Yield	0.72%
Protein content	36.5%
Conversion efficiency	38.4%

Observations: The spores germinated rapidly in 6 - 8 hours. Growth was very rapid at about 18 hours and a doubling time of 2 hours was noted at one sampling. The yield at 24 hours was 0.66%. The hyphae were very thin walled.

(vi) Fusarium oxysporum

	Run I	Run II
Medium	molasses	molasses
Scale	4 litres	4 litres
Inoculum	barley flask	barley flask
Morphological form	diffuse mycelium	diffuse mycelium
Time for maximum yield	42 hours	39 hours
Yield	1.29%	1.2%
Protein content	52.1%	57.8%
Conversion efficiency	47.5%	48.2%

Observations: The strain used did not sporulate readily nor form a surface mat on liquid culture. The inoculum from the barley flask

was predominantly mycelial. The mycelium grown was extremely fine and was not retained by the gauze. The mycelial broth was filtered through filter paper and the wet mycelium obtained had a crumbly consistency.

(vii) Heterocephalum aurantiacum

Medium	Molasses
Scale	4 litres
Inoculum	barley flask
Morphological form	elongated pellets 1 mm x 2 mm
Time for maximum yield	44 hours
Yield	0.61%
Protein content	38.5%
Conversion efficiency	37.5%

Observations: Germination and growth in the early stages were very slow and two runs were unsuccessful due to bacterial infections. In one of these it was noticed that the addition of 1 mg thiamine hydrochloride per litre speeded up growth.

(viii) Lenzites trabea

Medium	Malt
Scale	4 litres
Inoculum	solid surface culture
Morphological form	diffuse mycelium tending to aggregate the strands
Time for maximum yield	96 hours
Yield	0.67%
Protein content	53.2%
Conversion efficiency	25.2%

Observations: This organism could not be grown on molasses medium even when supplemented with thiamine hydrochloride and yeast extract. It did not produce a surface mat on liquid culture. The hyphae obtained in submerged growth were very thick walled.

(ix) Mucor racemosus

Medium	molasses
Scale	50 litres
Inoculum	barley flask
Morphological form	diffuse mycelium
Time for maximum yield	90 hours
Yield	0.94%
Protein content	43.4%
Conversion efficiency	32.0%

Observation: Germination and initial growth were very rapid. The yield at 60 hours was 0.74%, and at 78 hours was 0.90%. Sporulation occurred profusely where the mycelium was exposed to air.

(x) Penicillium chrysogenum

Medium	molasses
Scale	50 litres
Inoculum	barley flask
Morphological form	diffuse mycelium
Time for maximum yield	72 hours
Yield	1.2%
Protein content	36.0%
Conversion efficiency	39.5%

Observations: Spore germination was very slow. Actual growth started after 30 hours but vegetative growth was extremely rapid.

(xi) Penicillium notatum

Medium	molasses
Scale	50 litres
Inoculum	barley flask
Morphological form	diffuse mycelium
Time for maximum yield	96 hours
Yield	1.2%
Protein content	39.2%
Conversion efficiency	29.6%

Observations: On several occasions spores of this organism failed to germinate. The cause for this was not determined.

(xii) Polyporus anceps

Medium	malt
Scale	4 litres
Inoculum	solid surface culture
Morphological form	light diffuse pellet
Time for maximum yield	96 hours
Yield	0.65%
Protein content	48.6%
Conversion efficiency	21.5%

Observations: This organism could not be grown on molasses medium even when supplemented with thiamine hydrochloride and yeast extract. It did not produce a surface mat on liquid culture. There was a prolonged lag phase of about 40 hours before any growth was observed in submerged culture. The hyphae were very thick walled.

(xiii) Rhizopus oligosporus

Medium	molasses
Scale	50 litres
Inoculum	barley flask
Morphological form	diffuse mycelium
Time for maximum yield	76 hours
Yield	0.65%
Protein content	39.2%
Conversion efficiency	29.6%

Observations: Spores germinated in 8 - 10 hours and initial growth was very fast. The yield at 48 hours was 0.5%. There was a tendency to sporulate readily.

(xiv) Rhizopus oryzae

Medium	molasses
Scale	4 litres
Inoculum	barley flask
Morphological form	pellets approximately 1 mm. in diameter
Time for maximum yield	42 hours
Yield	0.72%
Protein	38.6%
Conversion efficiency	36.4%

Observations: The strain used did not sporulate readily and the inoculum from the barley flask was predominantly mycelial.

These results show that fungal biomass can be produced very easily in aerobic tower fermenters. If fungal biomass is to be produced as a feed or food it must be produced very cheaply and the aerobic tower fermenter lends itself to such a cheap process on account of its simplicity and ease of construction and operation.

Cheap and large aerobic tower fermenters of up to 1,000 litre capacity have been successfully operated and there is no reason to believe that this is the upper limit. Operating a tower fermenter with its fewer moving parts is relatively easy and cheap compared to the traditional stirred tank fermenters. In particular, the absence of a stirrer should result in a significant reduction in running costs and hence on production costs.

There is no published data on fungal biomass production in stirred tank fermenters. However, there is no reason to believe that the yields obtained in aerobic towers are much lower than those normally found in stirred tank fermenters. It is suggested that even if biomass yields are shown to be slightly lower in tower fermenters the overall economics of producing fungal biomass in aerobic tower fermenters might still be preferable. The cost of a product is dependent on (i) cost of raw material, (ii) yield and (iii) production costs. When one is dealing with a cheap raw material it might be possible to sacrifice a higher yield if compensatory savings can be made on production costs.

It should be pointed out that the growth of filamentous microorganisms in tower fermenters is the subject of a British patent (Ross and Wilkin, 1968). The primary aim in this patent was the continuous fermentation with filamentous microorganisms for the production of metabolites though the production of fungal biomass was not precluded.

It appears that the Fungi Imperfecti are the best fungi for biomass production. Best yields were obtained with Aspergillus luchuensis, A. niger, Penicillium chrysogenum and P. notatum, though the latter two fungi

occasionally did not germinate easily. Cladosporium cladosporioides showed a very fast growth rate but the maximum yield was lower. With fungi that germinated easily and grew quickly there was a far lower tendency towards infections. Fusarium oxysporum grew quickly and gave a high yield with a high protein content. However, no extensive work was done with this organism since serious separation problems were envisaged on account of its fine mycelium.

The Phycomycetes (Mucor racemosus, Rhizopus oligosporus and R. oryzae) grew very quickly, but generally the yields were low. Another disadvantage with Phycomycetes was that there was a tendency to sporulate profusely. There was considerable difficulty in growing Basidiomycetes in aerobic tower fermenters. There were prolonged lag times during which infections could set in. Even in the growth phase the doubling times were prolonged so that unless great precautions were taken regarding sterility there was a grave risk of infections. None of the five Basidiomycetes selected could be grown on a molasses medium and only two could be grown on a malt medium to give yields comparable to those given by Phycomycetes.

The fungi were found to grow in the diffuse mycelial form or, more commonly, as small pellets with dense centres. Ross and Wilkin (1968) differentiate these from true pellets with a hollow centre and describe the form in tower fermenters as 'particulate aeratable form' indicating that the transfer of oxygen to the centre of these forms was not limiting. Whether a fungus grew in diffuse form or in pellets seemed to depend on several factors including inoculum size, type of inoculum (mycelial or spores) and mode of preparation of inoculum.

The protein content was found to vary from 34% (Aspergillus oryzae) to 57.8% (Fusarium oxysporum). Though there was great variability between different organisms, the variability for any one organism in different conditions was less. Presumably, the protein content of a fungus can be altered within limits by altering the media and cultural conditions. In fast growing organisms a fair proportion of the nitrogen

may be in the form of nucleic acid nitrogen and give a false picture of protein content. Worgan (1971) reports that in fast growing fungi the actual protein content is reduced.

Too much emphasis should not be placed on the conversion efficiencies found, in view of the fact that the growth times were vastly different. In several of the fungi, a greater part of the biomass was laid down in the first half of the growth time. In the second half of the growth time little extra biomass was laid down but a considerable amount of sugar was being utilised for the metabolic activities of the existing biomass. Thus it is found that where growth times were prolonged the conversion efficiencies were lower. There are further objections to the validity and accuracy of the conversion efficiencies calculated. The determination of reducing sugars in molasses by the method of Lane and Eynon (1923) assumes that sucrose was the sole reducing sugar. The method itself is subject to errors when calcium is present (Eynon and Lane, 1923). Perhaps a more serious objection would be that no allowance was made for the utilisation of non-sugar carbon in the medium. Agarwal and Peterson (1949) found that non-sugar carbon compounds of molasses contribute materially to the yield of yeasts. Utilisation of non-sugar carbon increased with increased yields and reached 21% with Saccharomyces cerevisiae, 37% with Torula utilis and 35% with Candida arborea. Fungi are not so exacting as yeasts in their carbon requirements and it is possible that non-sugar carbon contributed significantly to the yields.

Three Basidiomycetes (Poria monticola, Lentinus lepidus and Fommes annosus) could not be grown in aerobic tower fermenters on either molasses medium or malt medium. Growth did not occur even on supplementation with thiamine hydrochloride or yeast extract. Geotrichum candidum was found to grow in the yeast form and further work on this organism was not attempted.

PREPARATION OF FUNGAL EXTRACTS

INTRODUCTION: PROCESSING FUNGAL FOOD

Microbial food must look like food, taste like food and be eaten before it can have any impact on world food problems. For these reasons the processing of microbial proteins is of significance (Tannenbaum, 1968). Table VIII summarises the reasons for processing microbial protein.

TABLE VIII

Rationale for Processing Microbial Protein
(Tannenbaum, 1968)

I. Cell Viability
   - A. Undesirable intestinal flora
   - B. Competition for nutrients
   - C. Inactivation of enzymes

II. Distribution and Storage
   - A. Optimise energy output
   - B. Determine product properties
   - C. Packaging requirements

III. Tailoring product properties
   - A. Flavour
   - B. Colour
   - C. Physical properties

IV. Improvement of Nutritional Value
   - A. Digestibility
   - B. Biological value
   - C. Toxicity

In the case of fungi, feeding with intact cells may be of limited nutritional value in view of the resistance of the cell wall to digestion. Various reports have been published on the digestibility of fungi. Skinner, Peterson and Steenbock (1933) reported that the proteins of Aspergillus fischeri were 68% digestible and those of A. sydowi were 69% digestible. Lintzel (1942) reported that in human feeding 72 - 83% of mushroom protein was digestible.

One approach to improved nutritional value of fungal proteins would be to separate the soluble protoplasmic protein from the cell wall as has been suggested by a number of workers (Humphrey, 1969; Wang, 1969; Tannenbaum, 1968). Tannenbaum and Miller (1967) found that the digestibility and biological value of Bacillus megaterium increased after fragmentation of the cells. Mitsuda, Yasumoto and Nakamura (1969) showed that whereas the digestibility of Torula yeast was 65% the digestibility of the cell contents was 95%.

The various methods that have been used for the release of cell components include: autolysis, plasmolysis, hydrolysis, enzymatic, chemical and mechanical. The first three procedures are commonly used for the preparation of commercial yeast extract (Acraman, 1966) but there have been few reports on enzymatic, chemical or mechanical methods of releasing cell components.

Preliminary work with fungal cell extracts showed that an enzymatic treatment gave a protein concentrate with highly desirable organoleptic properties which might be suitable for human consumption as a protein supplement. Investigations were carried out on methods of optimising extraction and analyses of the fungal cell extracts so obtained.

The following experiments were conducted:

(1) Extraction without enzymes

(2) Extraction with pepsin

(3) Extraction with papain

(4) Effect of particle size on extraction

(5) Effect of temperature on extraction

(6) Effect of enzyme concentration on extraction

(7) Effect of concentration of suspended mycelium on extraction

MATERIALS AND METHODS

Mycelia

Two types of mycelia were used in these investigations.

(i) Aspergillus niger M1

The organism was grown in a tower fermenter on a medium of beet molasses containing 4% total sugars. The medium was supplemented with ammonium sulphate to give a C:N ratio of 10:1. The mycelium was harvested in the form of pellets (approximately 2 mm in diameter) after 40 hours growth. It was spin-dried, washed once with warm water and spin-dried again. The spin-dried mycelium was dried on trays in an oven for 16 hours. Analysis of the dried mycelium by the Kjeldahl method showed a nitrogen content of 5.86% which represented an assumed protein content of 36.6%.

(ii) Penicillium chrysogenum

The mycelium was obtained from Glaxo Laboratories Limited, Ulverston as fermentation dried residues from antibiotic production. The mycelium had been killed by autoclaving, dried and ground. Analysis by the Kjeldahl method showed a nitrogen content of 5.06% which represented an assumed protein content of 31.61%.

Chemicals and Enzymes

The chemicals were laboratory grade reagents. The enzymes were obtained from Koch Light Ltd. The pepsin used was a 1:2500 powder and the papain was a pure powder.

(1) Extraction without enzymes

80 g of Aspergillus niger M1 mycelium was ground in a mortar and suspended in 1200 ml. of 0.1% potassium sorbate. The suspension was maintained at 40°C with agitation. After allowing 2 hours for the mycelium to swell more 0.1% potassium sorbate was added to bring the volume up to 1600 ml so as to give an initial concentration of 5% w/v suspended solids. At 6-hour intervals 100 ml. samples were removed for analysis. The

samples were filtered through a Buchner and the residue washed till the washings were colourless. The filtrate was evaporated under vacuum at 70°C and dried to constant weight at 105°C. The extract was taken up in distilled water and made up to 25 ml. for further analysis.

(2) Extraction with pepsin

80 g of Aspergillus niger M1 mycelium was treated as in the previous experiment and suspended in 1200 ml of 0.1% potassium sorbate. The pH was adjusted to 3.0 with a 1:1 mixture of concentrated hydrochloric acid and glacial acetic acid. 1.6 g of pepsin was added and the suspension maintained at 40°C with agitation. After 2 hours a volume adjustment was made with 0.1% potassium sorbate as in the previous experiment. Samples were taken and treated as in the previous experiment.

(3) Extraction with papain

80 g of Aspergillus niger M1 mycelium was extracted as in the previous experiment except that the pH was adjusted to 6.0 and 1.6 g of papain powder added together with 1.6 g disodium ethylene diamine tetra-acetic acid.

(4) Effect of particle size on extraction

Aspergillus niger M1 mycelium was ground in a mortar and the resultant powder graded by sieving. 50 g portions of different particle size fractions were subjected to 24 hour pepsin extraction in 1000 ml suspensions and with 0.1% pepsin. The temperature was maintained at 40°C and the agitation was kept approximately equal for the different extractions. The weights of extracts obtained with the different fractions were noted.

(5) Effect of temperature on extraction

Penicillium chrysogenum powdered mycelium was sieved and only powder which passed through a No.60 sieve (below 500 microns) was used. One litre batches of 10% w/v mycelial suspensions were extracted in the presence of 0.1% pepsin at 40°C, 50°C, 60°C and 70°C. The agitation was kept approximately equal for the different extractions. 100 ml. samples were taken at 3-hour intervals and the dry weights of the

extracts determined. Total nitrogen determinations were also carried out on the extracts.

(6) Effect of enzyme concentration on extraction

Extractions were carried out as in the previous experiment except that the temperatures were kept constant at 40°C and the pepsin concentration adjusted to 0.05%, 0.1%, 0.15% and 0.2%. Only one 100 ml sample was taken at the end of 24 hours. Extract yields and total nitrogen contents were determined.

(7) Effect of concentration of suspended mycelium on extraction

Extractions were carried out as in the previous experiment with the temperature kept constant at 40°C and using 0.1% pepsin but with the initial concentrations of suspended mycelium adjusted to 5% w/v, 10% w/v, 15% w/v and 20% w/v in the different extractions. Two 100 ml. samples were taken at the end of 24 hours. One sample was filtered in a Buchner under vacuum from a filter pump. The volume was recorded and the filtrate evaporated to dryness. The other sample was filtered and the residue washed with 40 ml. of distilled water and filtered dry again. The filtrate and washings were combined and evaporated to dryness.

RESULTS AND DISCUSSION

The results of the extraction experiments are shown below.

TABLE IX

Extraction of Aspergillus niger mycelium without enzymes

5% w/v mycelial suspension. Temperature 40°C

Time (hours)	pH	Residual solids	Solids extracted ■	Nitrogen in extract	Protein in extract	Soluble N Total N%
0	6.50	5%	0	-	-	-
6	6.50	4.12%	21.0%	6.28%	39.25%	22 51
18	6.55	3.86%	25.0%	6.27%	39.19%	26.75
24	6.45	3.82%	28.5%	6.31%	39.44%	30.68
30	6.55	3.56%	31.0%	6.36%	39.75%	33.65
42	5.70	3.51%	34.0%	6.36%	39.75%	36.89
48	5.50	3.46%	34.5%	6.26%	39.13%	36.86
54	5.30	3.42%	36.0%	6.28%	39.25%	38.58
66	5.30	3.30%	36.0%	6.26%	39.13%	35.46

■ Allowance made for potassium sorbate

TABLE X

Extraction of Aspergillus niger mycelium with pepsin

Temperature: 40°C; 5% w/v mycelial suspension; 0.1% pepsin

The pH was adjusted to 3.0 with 16.5 ml. of 1:1 concentrated hydrochloric acid and glacial acetic acid.

Time (hours)	pH	Residual solids	Solids extracted ■	Nitrogen in extract	Protein in extract	Soluble N Total N%
0	3.0	5%	0	-	-	-
6	3.4	4.15%	24.5%	7.69%	48.06%	32.15
18	3.4	2.95%	44.5%	7.64%	47.75%	58.00
24	3.4	2.85%	46.0%	7.83%	48.93%	61.47
30	3.45	2.60%	46.5%	7.92%	49.50%	62.85
42	3.55	2.85%	47.0%	7.88%	49.25%	63.20
48	3.55	2.95%	47.0%	7.82%	48.88%	62.71
54	3.65	2.90%	48.5%	7.63%	47.68%	63.17
66	3.65	2.93%	47.0%	7.77%	48.56%	62.32

■ Allowance made for potassium sorbate and pepsin

TABLE XI

Extraction of Aspergillus niger mycelium with papain

Temperature: 40°C; 5% w/v mycelial suspension; 0.1% papain and 0.1% disodium EDTA

The pH was adjusted to 6.0 with 3.2 ml of 1:1 concentrated hydrochloric acid and glacial acetic acid.

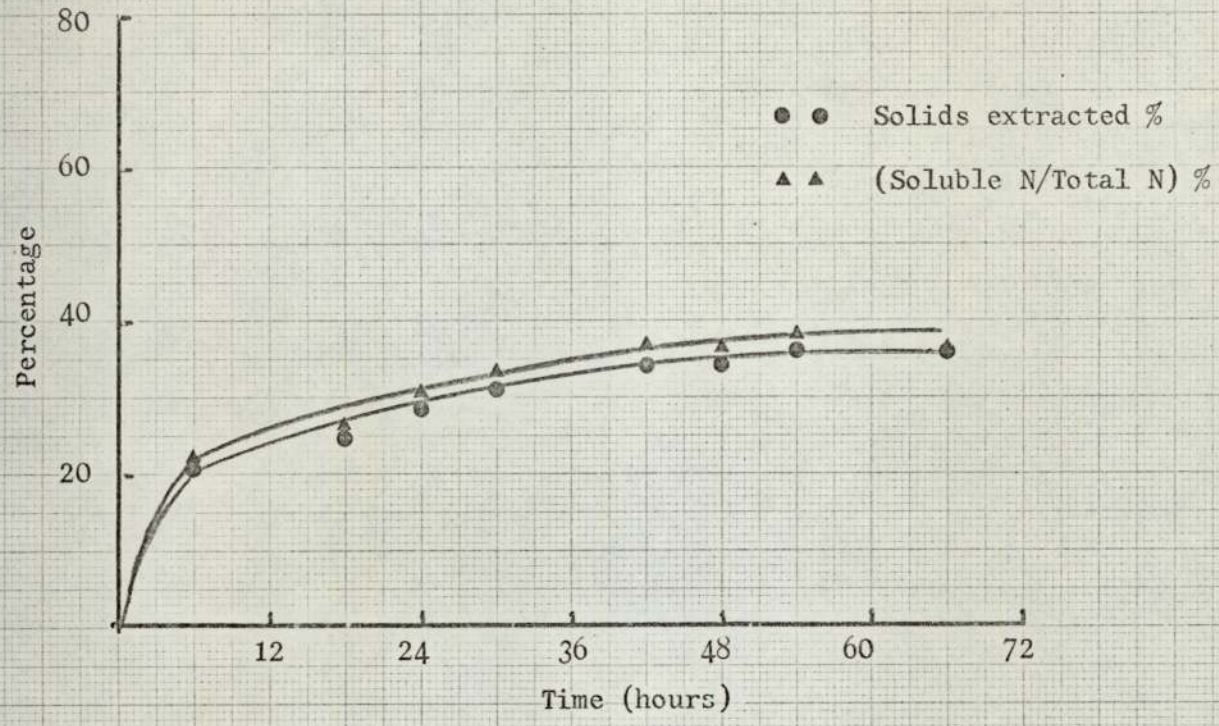
Time (hours)	pH	Residual solids	Solids extracted■	Nitrogen in extract	Protein in extract	Soluble N Total N%
0	6.0	5.0%	0	-	-	-
6	6.4	2.74%	52.5%	4.89%	30.56%	43.81
12	6.4	2.62%	52.0%	4.91%	30.79%	43.57
24	6.3	2.67%	52.5%	5.17%	32.34%	46.31
30	6.1	2.75%	52.5%	5.20%	32.50%	46.59
36	6.2	2.72%	52.0%	5.22%	32.63%	46.31
48	6.2	2.62%	51.5%	5.38%	33.62%	47.29
54	6.3	2.70%	52.0%	5.21%	32.60%	46.23
60	6.2	2.75%	52.0%	5.19%	32.44%	46.04

■ Allowance made for potassium sorbate, papain and disodium EDTA

Graph I

Extraction without enzymes

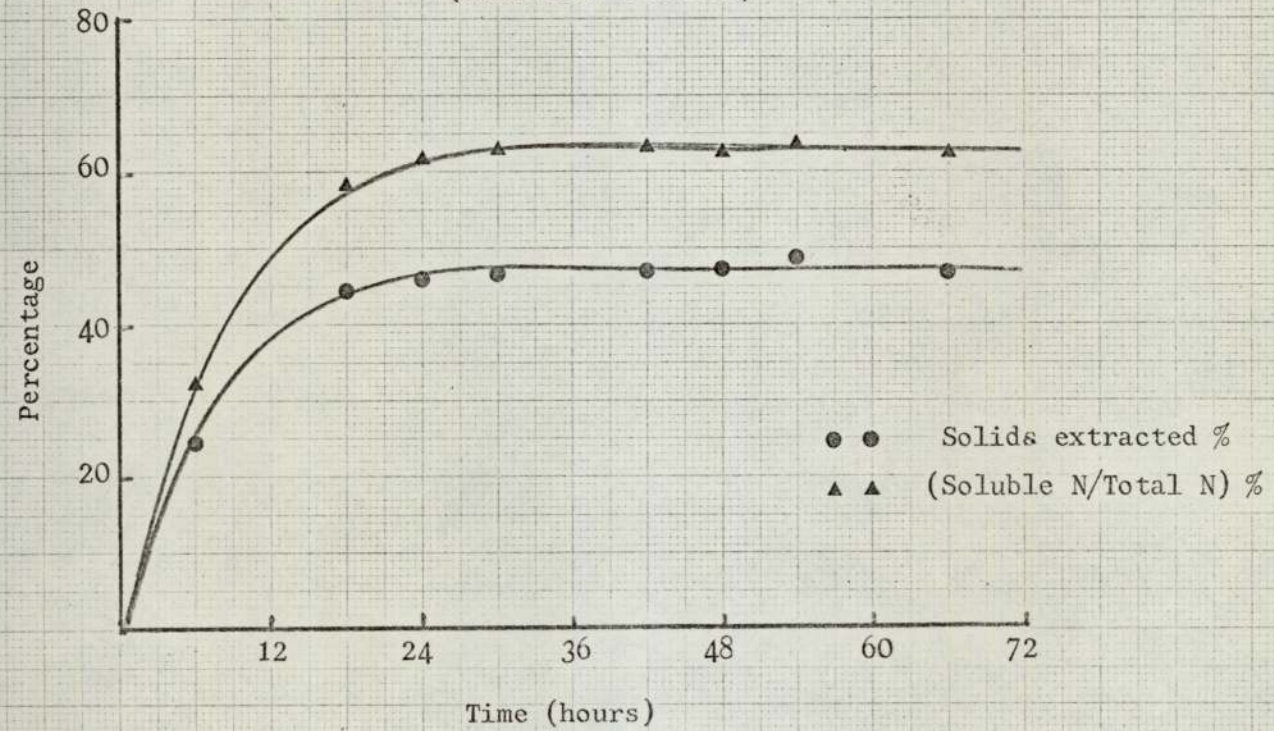
(Data from Table IX)



Graph II

Extraction with pepsin

(Data from Table X)



Graph III

Extraction with papain

(Data from Table XI)

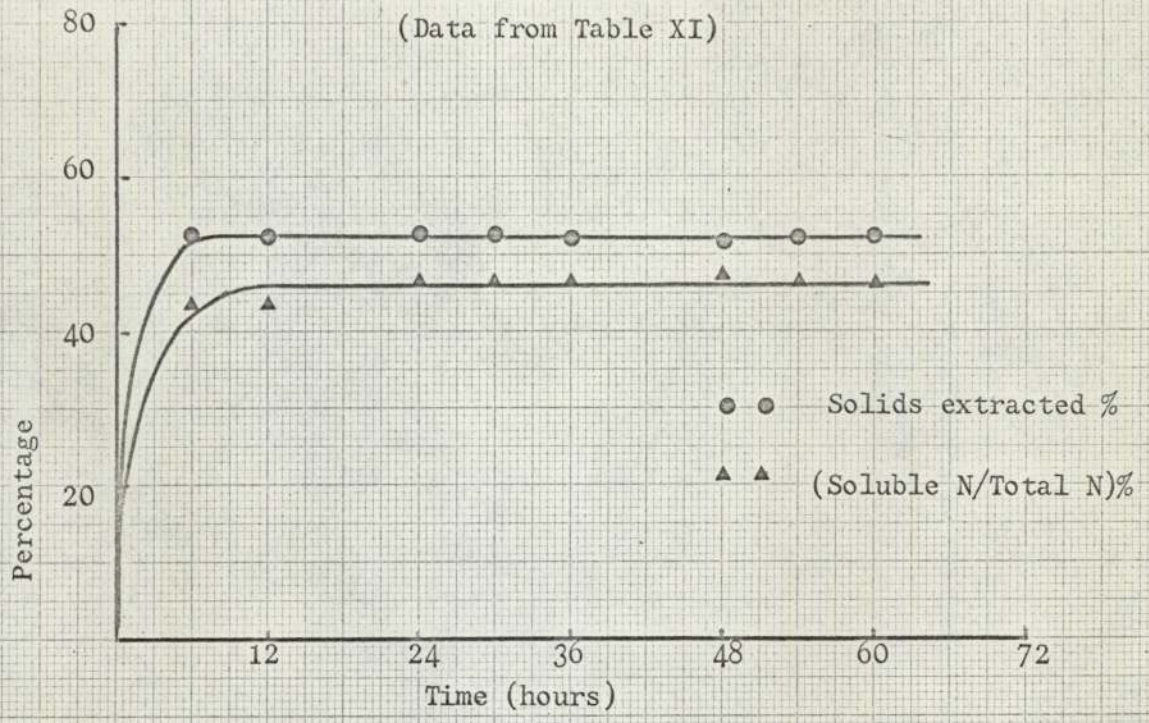


Fig.I

Effect of particle size on extraction of powdered Aspergillus niger

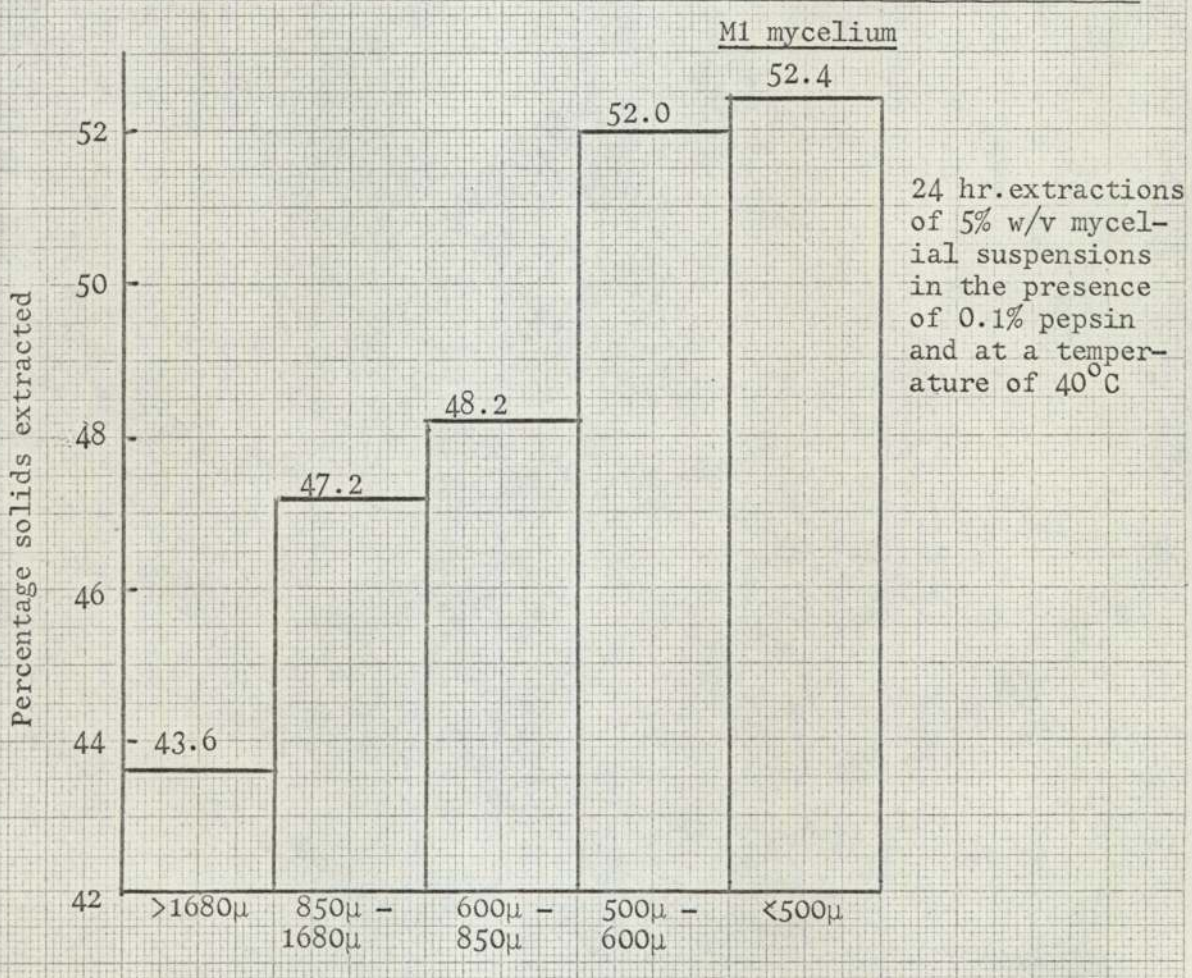


TABLE XII

Effect of temperature on the extraction of Penicillium chrysogenum mycelium

10% w/v suspension of P.chrysogenum mycelium powder, particle size below 500μ. 24 hour extraction of 0.1% pepsin.

Time (hours)	40°C		50°C		60°C		70°C	
	Yield	Protein in extract	Yield	Protein in extract	Yield	Protein in extract	Yield	Protein in extract
	%	%	%	%	%	%	%	%
3	22.6	29.7	27.6	38.2	28.9	42.4	31.2	42.5
6	24.7	33.1	29.7	41.6	34.2	45.4	32.2	43.2
9	27.8	35.3	30.9	39.9	37.4	44.3	32.0	42.6
12	28.6	35.0	31.4	-	38.1	46.0	32.2	-
15	29.2	-	32.4	40.7	37.9	45.1	32.4	-
18	29.6	-	32.8	-	38.3	-	32.0	-
21	29.0	-	32.3	40.0	38.4	-	31.8	-
24	29.4	35.5	32.8	40.4	38.0	45.9	31.2	42.7

TABLE XIII

Effect of concentration of pepsin on the extraction of Penicillium chrysogenum mycelium

10% w/v suspension of P.chrysogenum mycelium powder, particle size below 500μ. 24 hour extraction at 40°C

Concentration of pepsin % w/v	Extract Yield %	Protein in extract %
0.05	25.8	28.2
0.10	30.1	35.2
0.15	32.2	36.1
0.20	34.3	37.2

Graph IV

Effect of temperature on the extraction of Penicillium chrysogenum mycelium

(Data from Table XII)

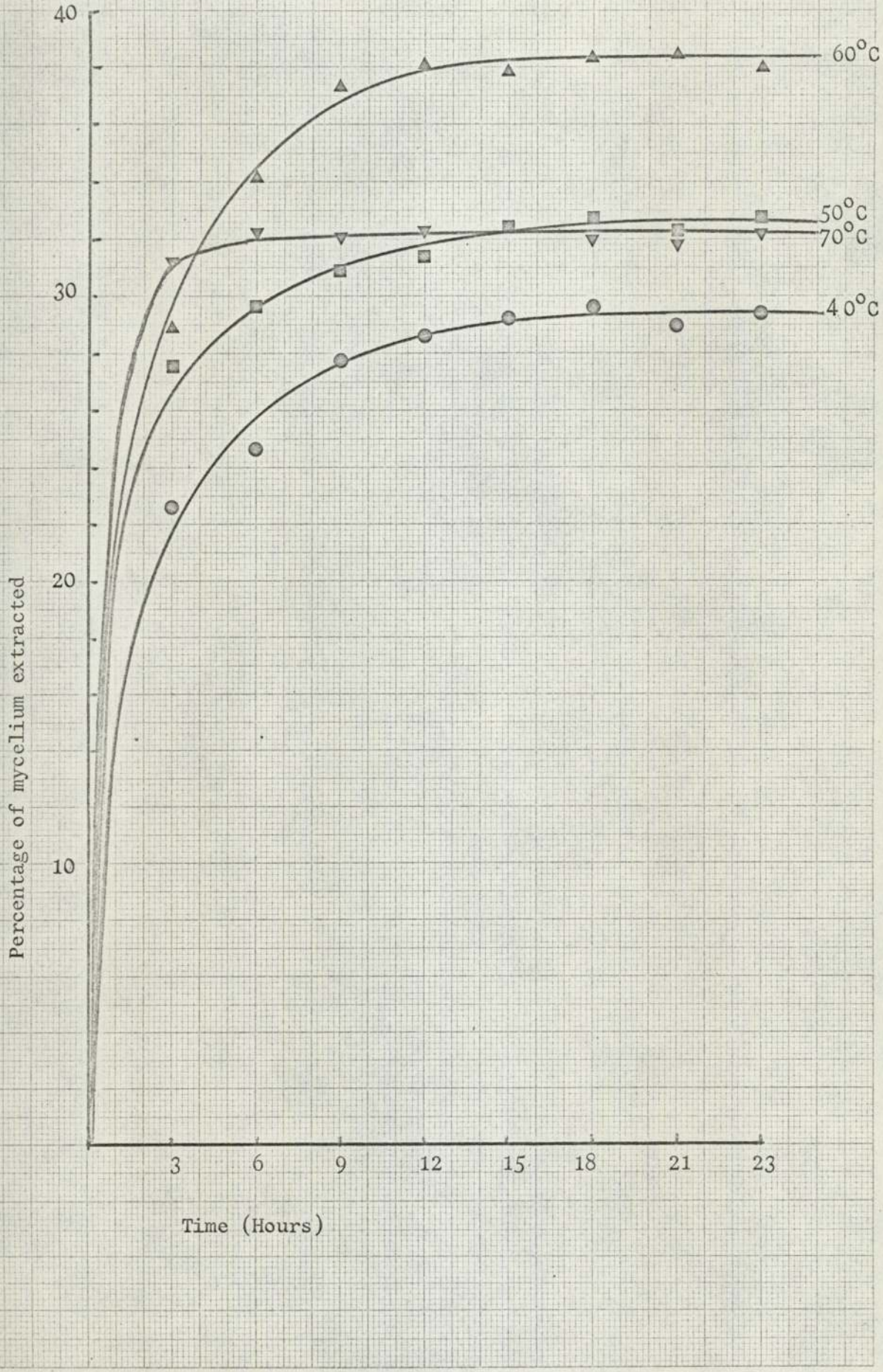


TABLE XIV

Effect of concentration of suspended mycelium on the extraction of Penicillium chrysogenum mycelium

Powdered mycelium of particle size below 500μ.

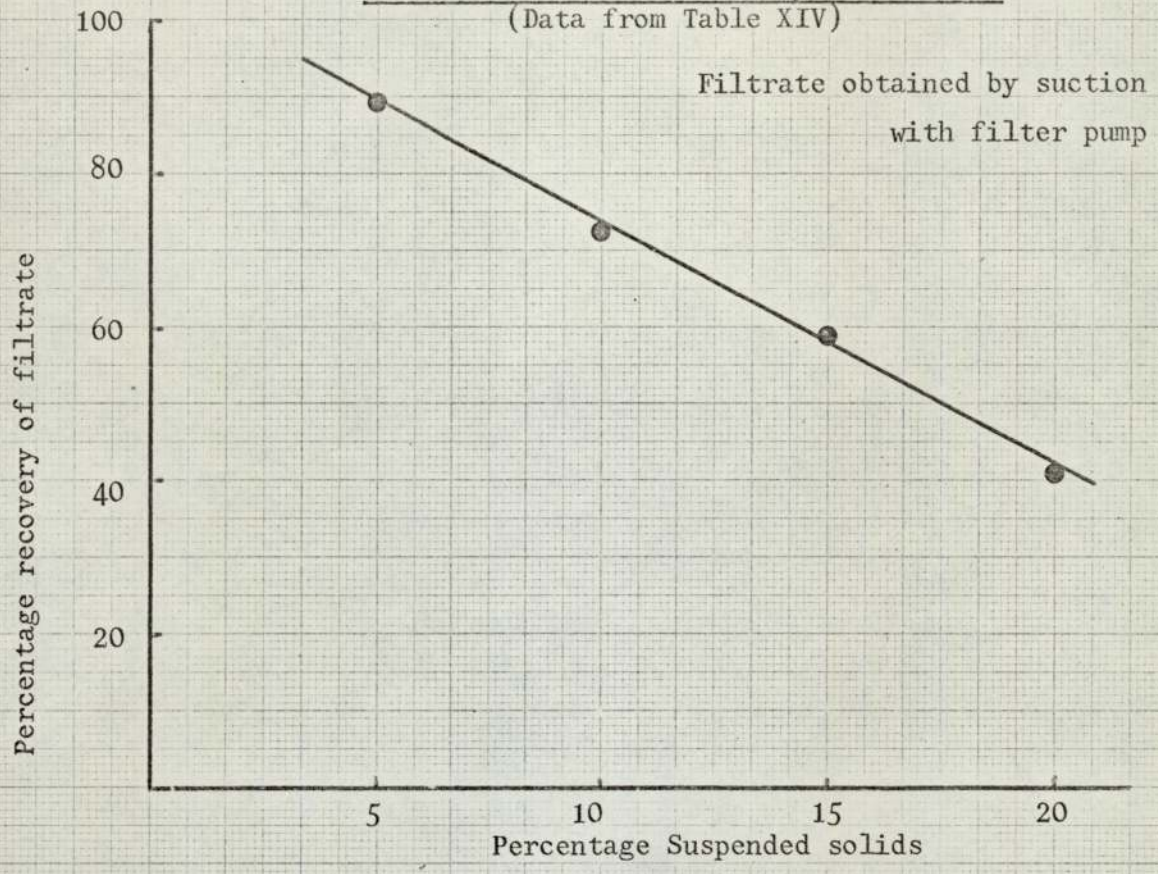
24 hour extractions at 40°C with 0.1% pepsin

	5% solids	10% solids	15% solids	20% solids
Volume of filtrate per 100 ml. sample	88 ml	74 ml	58 ml	42 ml
Dry weight of extract	1.85g	2.16g	2.45g	2.21g
Concentration of extract (mg/ml)	21.02	29.19	42.07	52.62
Percentage of mycelium recovered in filtrate	37	21.6	16.3	11.05
Dry weight of extract together with washing	2.29g	3.22g	3.55g	3.88g
Percentage of mycelium recovered in soluble form	45.8	32.2	23.3	19.4
Volume of liquid taken up by mycelium	12 ml	26 ml	42 ml	58 ml
Volume of liquid taken up per gram of suspended mycelium	2.4 ml	2.6 ml	2.8 ml	2.9 ml
Protein content of dry extract	35.6%	36.1%	35.8%	36.2%

Graph V

Effect of solids on recovery of filtrate

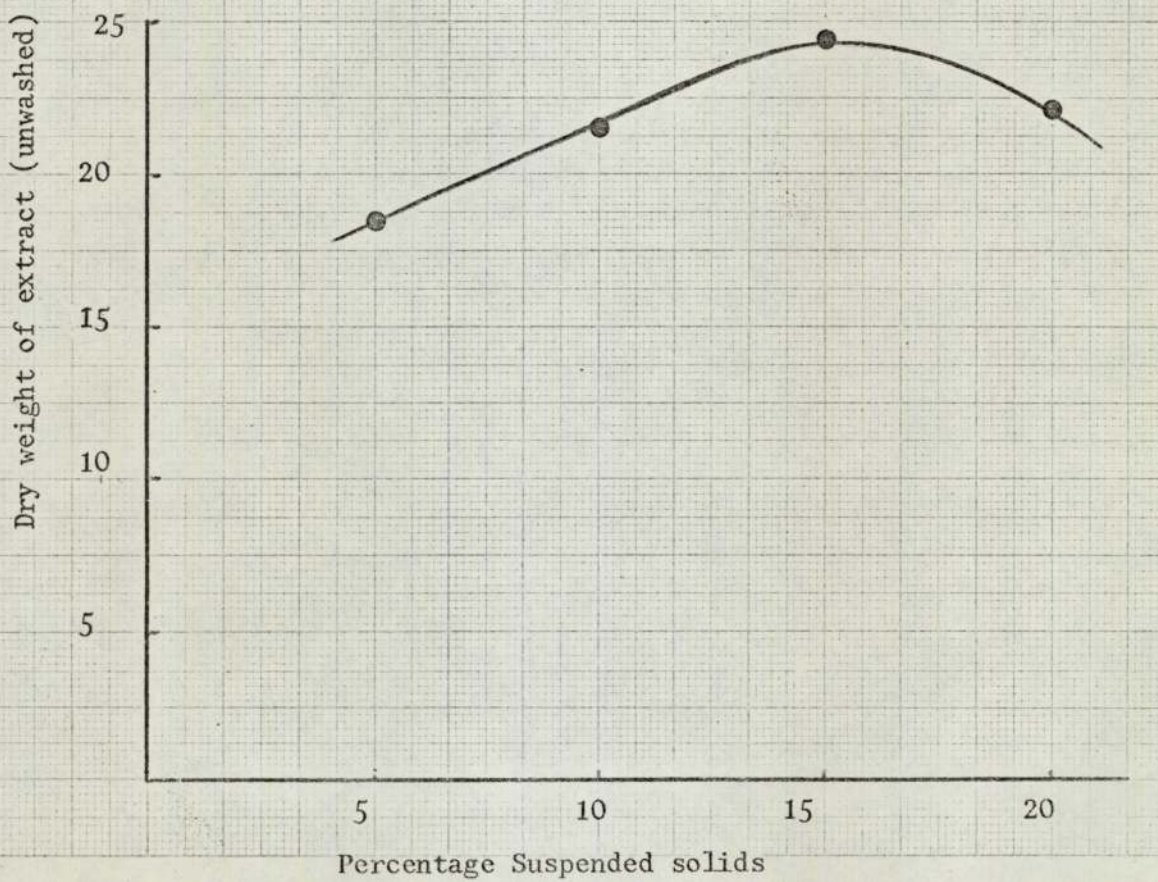
(Data from Table XIV)



Graph VI

Effect of solids on yield of extract

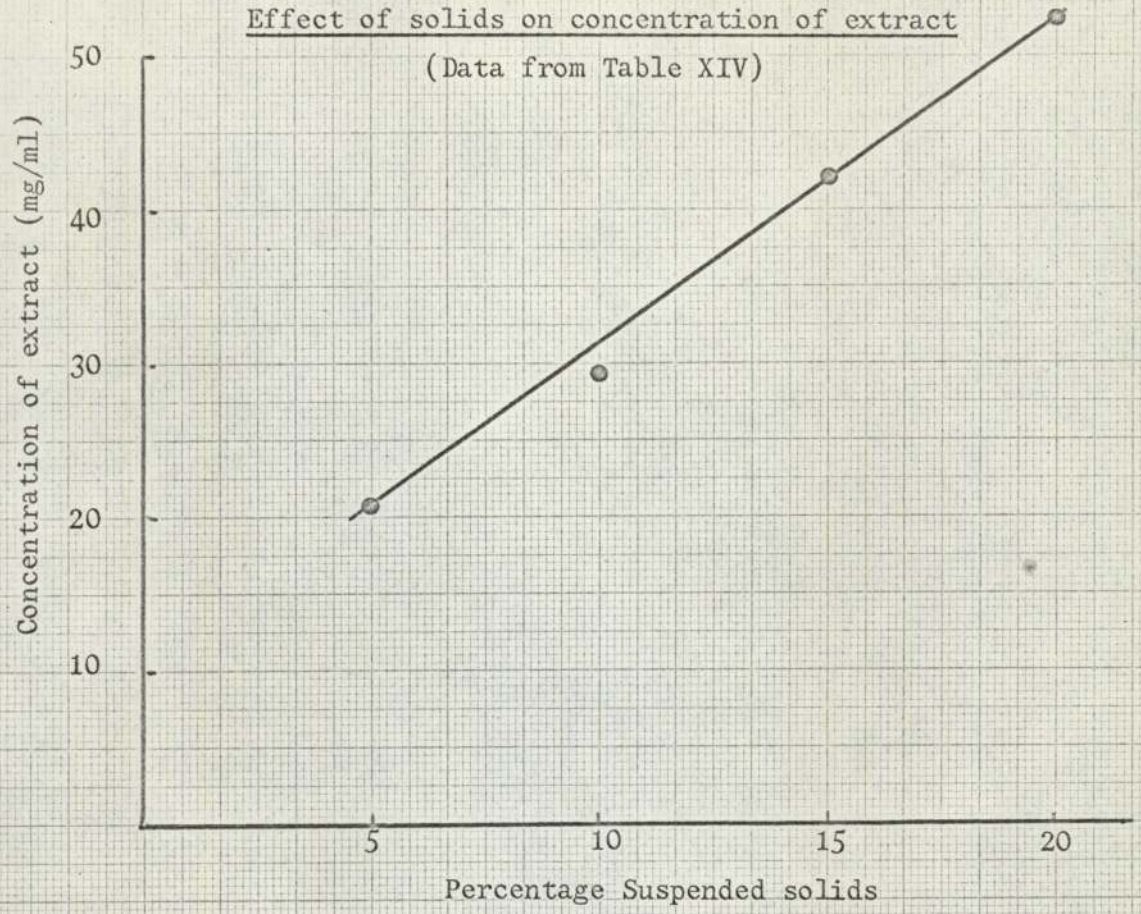
(Data from Table XIV)



Graph VII

Effect of solids on concentration of extract

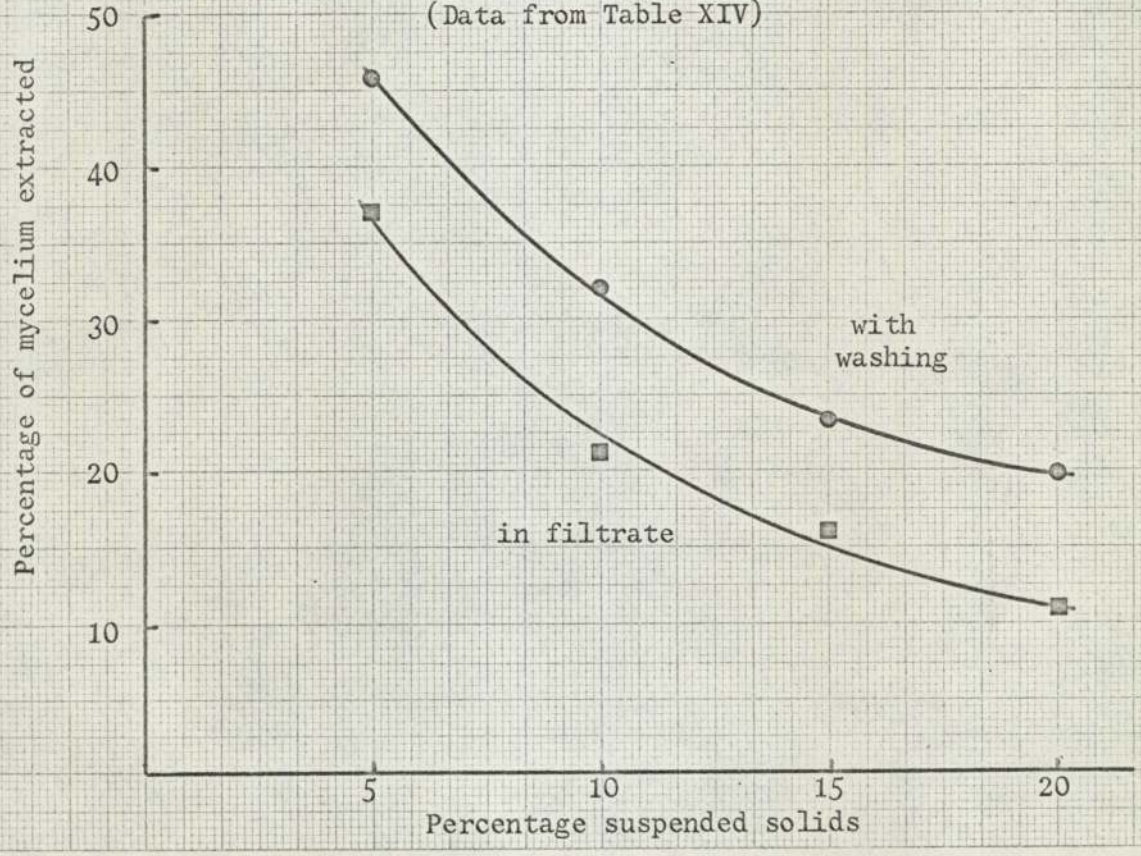
(Data from Table XIV)



Graph VIII

Effect of solids on extraction efficiency

(Data from Table XIV)



Though the use of enzymes has been explored with reference to the release of components of microbial cells there are little or no data on yield or rate (Tannenbaum, 1968). These experiments demonstrate quite clearly the value of enzymatic extraction of fungal mycelia.

EXTRACTION WITHOUT ENZYMES

As a control the extraction of Aspergillus niger mycelium without the use of enzymes was studied. This was essentially an autolytic extraction at neutral pH and at 40°C. 28.5% of the mycelium was extracted in 24 hours and a maximum solubilisation of 36.0% was obtained in 54 hours. 35.5% of the mycelial nitrogen was solubilised (Table IX; Graph I). The extract was a clear, light brown liquid which upon concentration gave a dark brown paste with fine organoleptic properties and a meaty taste. A similar improvement of flavour was also noted by Humfeld (1952) in his studies on the submerged production of mushroom mycelium. In his patent he claimed improvement of flavour by continued aeration and agitation of the culture after growth had ceased and all of the carbohydrate had been consumed. Alternatively, autolysis could be brought about by ageing the fully grown mycelium in a separate vessel at 20 - 35°C for 12 - 72 hours.

Only a slight increase in acidity was observed during the autolysis of A.niger with the pH decreasing from 6.5 to 5.3. There was no breakdown of the cell wall and the hyphae retained their form (Plate V). This was contradictory to the findings of Behr (1930). In his studies on the autolysis of A. niger he found that rapid destruction of mycelium and complete destruction of chitin occurred, at neutral autolysis at a pH of approximately 6.5. This is rather surprising as it is well known that the fungal cell wall is extremely resistant to breakdown. Throughout these studies, there was no instance where a fungal cell wall was completely broken down to give a fluidised state. It has been shown however that fungal cell walls may be lysed by certain bacteria and actinomycetes. An autolysing mycelial suspension is an ideal growth medium for other microorganisms and it is possible that Behr's reported breakdown of mycelium may have been brought about by contamination with a fungus-lysing microorganism.

It was found very early in this work that the measurement of residual solids as a criterion of extraction was very unreliable due to the difficulty of obtaining a truly representative sample of the suspended mycelium. Hence all data on yields were obtained from that portion of the mycelium that had gone into solution.

An examination of literature reports of fungal autolysis shows that the degree of autolysis and loss of mycelial nitrogen can vary within very wide limits. Dox and Maynard (1912) studied the autolysis of Penicillium expansum, P. camemberti and A. niger grown in a medium where all the nitrogen was in the form of ammonium tartrate. On autolysis, A. niger restored only about 75% of the assimilated nitrogen principally in the form of ammonia. It was suggested that the remaining 25% was present in spores in some chitin-like substance or glucosamine complex which did not undergo autolytic change. The findings of Behr (1930) have been mentioned. He also reported that the autolysis of A. niger in acid media with ammonium sulphate as nitrogen source occurs at pH 1 with relatively small loss in mycelial weight.

Woolley and Peterson (1937a) found that the most advantageous way of solubilising the nitrogen of Aspergillus sydowi mycelium was to allow the ground mycelium to autolyse in the presence of thymol for 3 or 4 days, at the end of which time the amount of nitrogen in solution as well as the amino nitrogen was at a maximum. When fresh mycelium was allowed to autolyse at pH 7 as much as 30% of the nitrogen was converted into ammonia. By drying the fresh mycelium at room temperature before carrying out the autolysis the production of ammonia would be reduced by about 50%. In their extraction process 44% of the mycelial mass and 63% of the mycelial nitrogen was solubilised, the mycelium having been grown on glucose-inorganic salts medium.

It appears that not all the protein nitrogen is solubilised during autolysis. Bohonos, Woolley and Peterson (1942) reported an unautolysable protein from Aspergillus sydowi. This protein occurred to

an extent of 0.8% in the mycelium and was resistant to many proteolytic enzymes.

Emiliani and Ucha de Davie (1962) carried out induced autolysis of Aspergillus phoenicis (A. niger group). Non-sporulating mycelium, after developing on a synthetic medium composed of sucrose and inorganic salts and washed with water suffered a strong autolysis under the following conditions (i) in contact with a solution of certain compounds such as 1% acetic acid, (ii) in anaerobiosis and (iii) at a temperature of 48°C. Under these conditions the hydrolysing enzymes maintain their activity for a time and the mycelium loses about 50% of its own weight. The simple compounds formed diffuse through the cell wall which keeps its shape.

Lahoz, Reyes and Beltra (1966) initiated a study on the chemical changes in the mycelium of Aspergillus flavus during autolysis. The organism was grown on Modified Raulin-Thom medium. Autolysis set in after 9 days incubation and by the 117th day the mycelium lost 73.9% of its maximum initial dry weight. The pH remained in the neighbourhood of 7 throughout the whole period. The rate of autolysis was very slow. During the first three days the weight of mycelium per sample decreased from 1.0066g to 0.8493 g. The total nitrogen in the dry mycelium decreased from 49.3 to 39.9 mg per flask.

A. flavus when grown on a medium containing nitrate as a nitrogen source gave alkaline autolysis. With a combined action of a continuous flow of air and mechanical agitation up to 85% of the mycelial mass was solubilised in 24 days with a corresponding solubilisation of 82% of the mycelial nitrogen (Lahoz and Gonzalez Ibeas, 1968). However, the loss of mycelial mass after two days autolysis was only about 14%.

A loss in mycelial weight of only 14.6% was observed by Lahoz, Reyes, Beltra and Gorcia-Tapia (1967) after 150 days autolysis of Aspergillus terreus in an acid medium.

By comparison with the work of Lahoz et al. it is shown in our work that a far faster rate of solubilisation of mycelial mass and nitrogen can be achieved. Autolysis is essentially a proteolytic process making use of the endogenous proteolytic enzymes to yield simpler molecules which would then be leached out of the cells. It is suggested that drying the mycelium and grinding it disrupts the mycelium to some extent so that the outflow of materials out of the cell proceeds more easily. Plates VI and VII illustrate the effect of grinding the mycelium. It is interesting to note that our yields approximated those of Woolley and Peterson (1937a) who subjected their Aspergillus sydowi mycelium to the same treatment.

The rate and extent of autolysis would depend on the interplay of many factors: the species and strain, the conditions under which the microorganism was grown and the conditions under which autolysis was taking place. It is reasonable to assume that autolysis would be maximum when strains with a high endogenous proteolytic activity are grown in conditions which favour production of proteolytic enzymes and are subsequently autolysed under conditions optimal for the action of these proteolytic enzymes.

A similar variability in rate and extent of solubilisation is seen during yeast autolysis (Joslyn, 1955; Joslyn and Vosti, 1955). Hough and Maddox (1970) showed that with Saccharomyces carlsbergensis grown in an all-malt wort and autolysed at 45°C in water at pH 6.5 less than 15% was extracted after 6 hours and the total levelled off at approximately 20%. By contrast, Zazirnaya, Mal'tser, Melet'ev and Chistyakova (1968) obtained a 90% extraction with a brewer's yeast autolysed at pH 5.6 and at 20°C for 144 hours. Extraction rates during the commercial production of yeast extract are said to be in the region of 60% (B.Smith, personal communication).

Hough and Maddox (1970) also suggested that autolysis occurs to a greater extent in yeasts which have been grown on a medium containing protein as these yeasts would have greater proteolytic activity.

Plate V

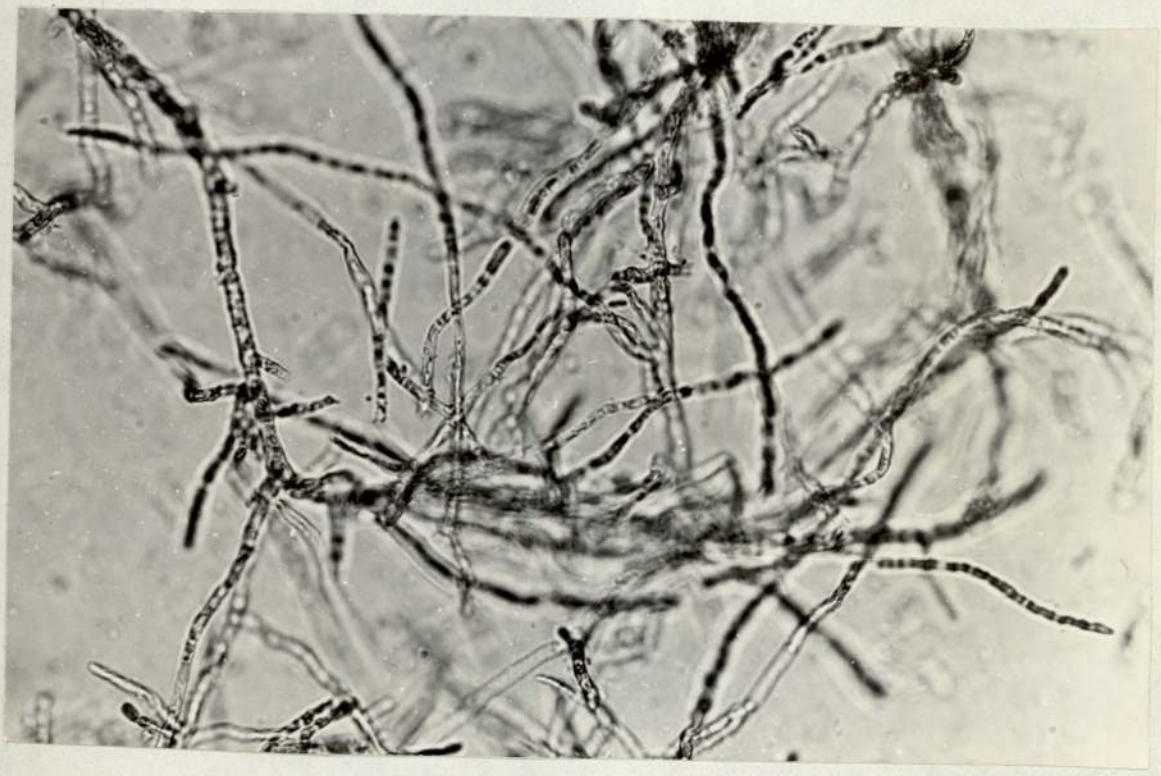
Aspergillus niger mycelium after autolysis

Magnification: X720

Plate VI

Intact Aspergillus niger mycelium

Magnification: X284



Furthermore, since some growth may occur concomitantly with autolysis, solubilisation would be higher if autolysis were carried out in the presence of small amounts of growth inhibitors.

EXTRACTION WITH PEPSIN

Extraction of Aspergillus niger mycelium with pepsin at 40°C showed that almost half the mycelium could be solubilised in as short a time as 24 hours (Table XX, Graph II). The resulting extract was rich in protein containing almost 50% protein (Kjeldahl N X 6.25) on the dry weight basis. On neutralising the soluble extract to pH 5.8 and concentrating under reduced pressure a dark brown pasty product was obtained which had a very meaty flavour and pleasant 'mouth feel'. This product was more acceptable than that obtained by autolysis alone.

Over 63% of the mycelial nitrogen was solubilised by this process. This figure agrees closely with reported values of the digestibility of fungal proteins and indicates that much if not all of the available protein is extracted in this way. It is very likely that the extraction is the result of a combined action by endogenous proteolytic enzymes (as autolysis) and the added pepsin. The mechanical rupturing of the mycelium during grinding would render the mycelial contents accessible to the action of pepsin.

Extracts obtained by the method outlined above were tested with biuret reagent and found to give only a slight colouration at 546 nm. This would seem to indicate that most of the protein nitrogen which was solubilised had been broken down to the constituent amino acids or to ammonia. The amino acid analyses of three different fungal extracts did not alter qualitatively after acid hydrolysis except for the breakdown of tryptophan and cystine.

It is also interesting to note that the nitrogen content of the extract does not vary with the time of extraction (Table X) showing that there was no preferential release of nitrogen at any stage of the extraction.

It is suggested that the initial rapid solubilisation of the

Plate VII

Aspergillus niger mycelium subjected to grinding

Magnification: X284

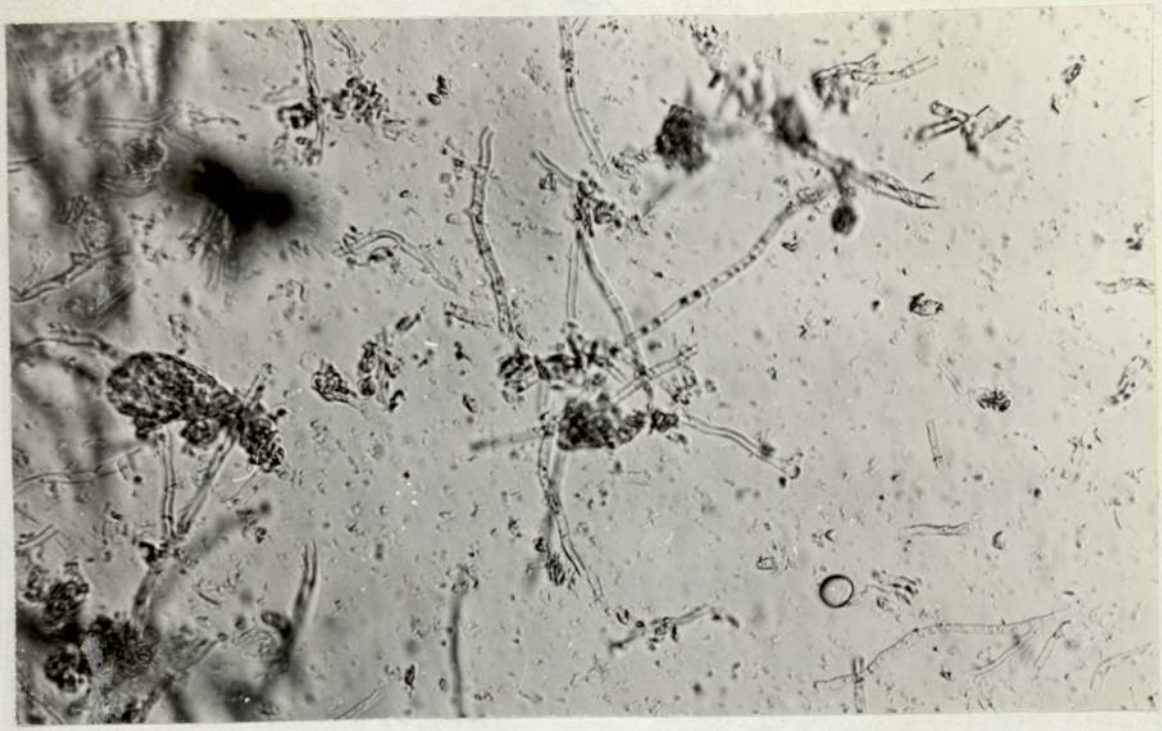
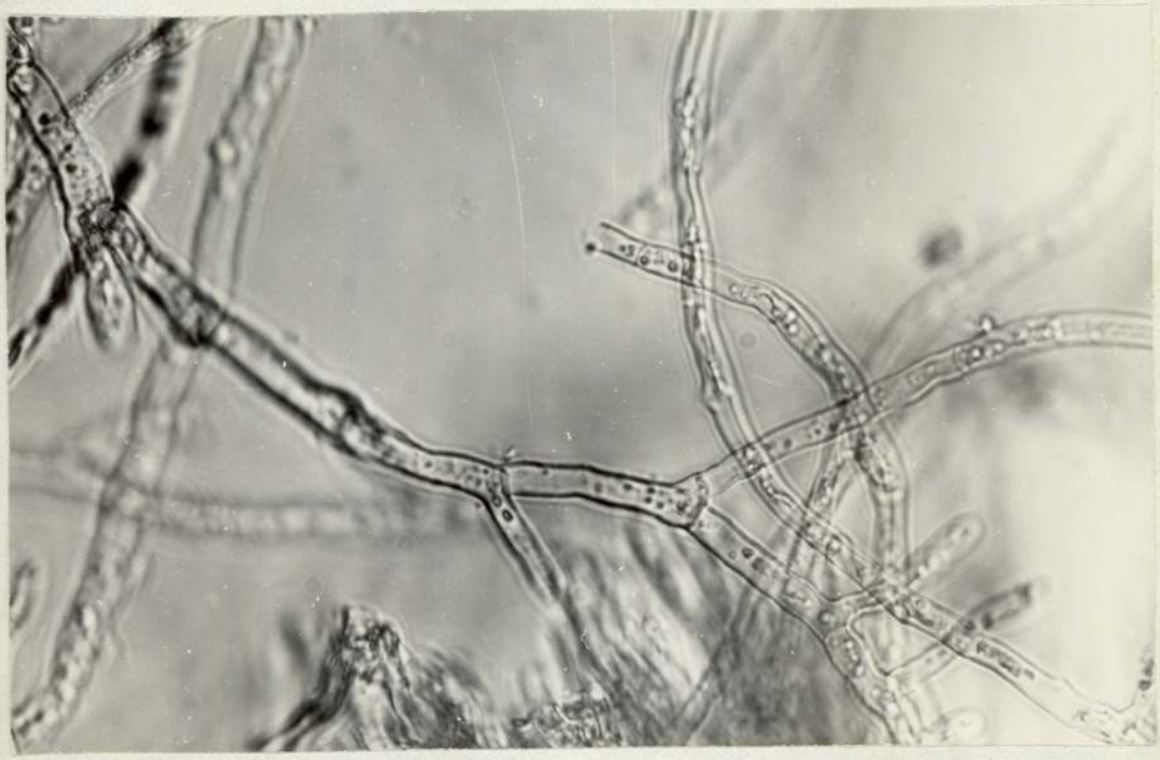


Plate VIII

Aspergillus niger mycelium after pepsin extraction

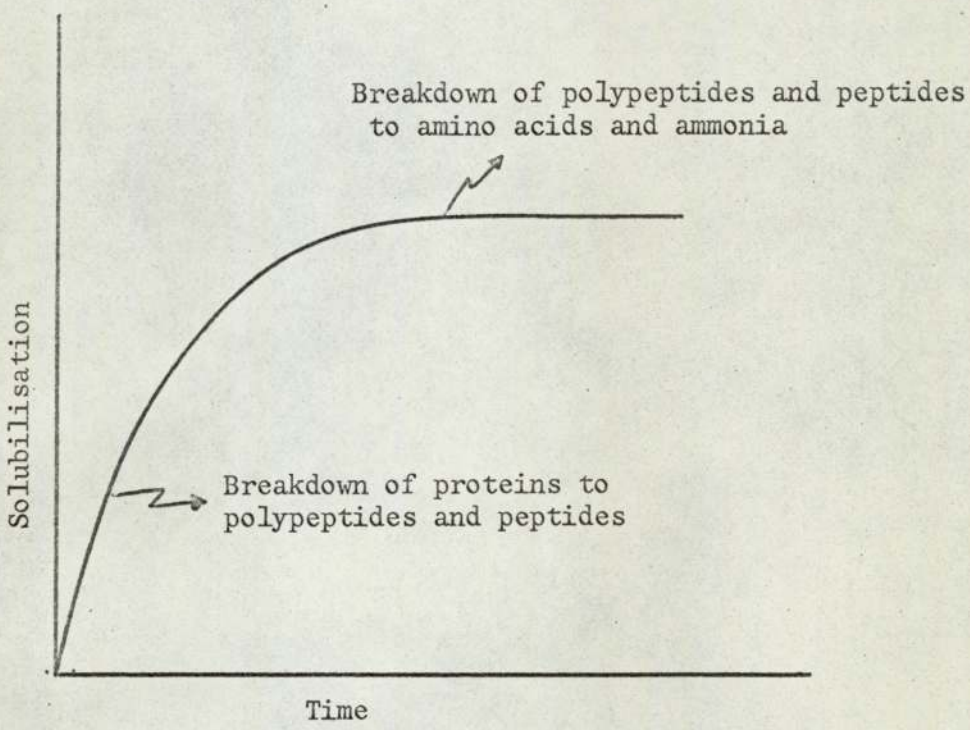
Magnification: X720



mycelium includes the breakdown of protein to soluble moieties such as polypeptides and peptides. The rupturing of the mycelium facilitates the outward diffusion of these moieties. These polypeptides and peptides are hydrolysed in the later stages of the extraction to yield amino acids together with some ammonia as breakdown product. Fig.II summarises the possible sequences.

Fig. II

Breakdown of proteins during extraction



It was noted that even after the mycelium had lost 46% of its mass and 63% of its nitrogen the cell wall still retained its form (Plate VIII). This illustrates the unreliability of looking at the gut contents as an indication of digestibility when fungi are fed in the diet.

Of the 37% nitrogen remaining in the cell wall a fair amount would be present as chitin. The precise chemistry of fungal cell walls is not known perhaps because of the difficulty in obtaining material uncontaminated with cytoplasm. The presence of proteins as an integral part of the cell wall has rarely been established (Aronson, 1965).

Crook and Johnson (1962) carried out qualitative analysis of mechanically isolated fungal cell walls from selected species of fungi. The cell walls contained only 2 - 5% amino acids and these were typical amino acids found in protein hydrolysates. Applegarth (1967) calculated the protein content of Penicillium notatum cell wall to be 8.9%. Grisaro, Sharon and Barkai-Golan (1968) found only 5.1% and 1.25% protein respectively in the purified cell walls of P.digitatum and P.italicum. Novaes-Lidieu and Jimenez-Martinez (1969) examined the cell walls of two species of Phycomycetes, Phytophthora hevea and Pythinon butleri and found the protein contents to be 6% and 11% respectively. Pepsin did not release nitrogenous material from the cell walls and these workers suggested that the cell wall protein was probably indispensable to cell wall integrity.

EXTRACTION WITH PAPAIN

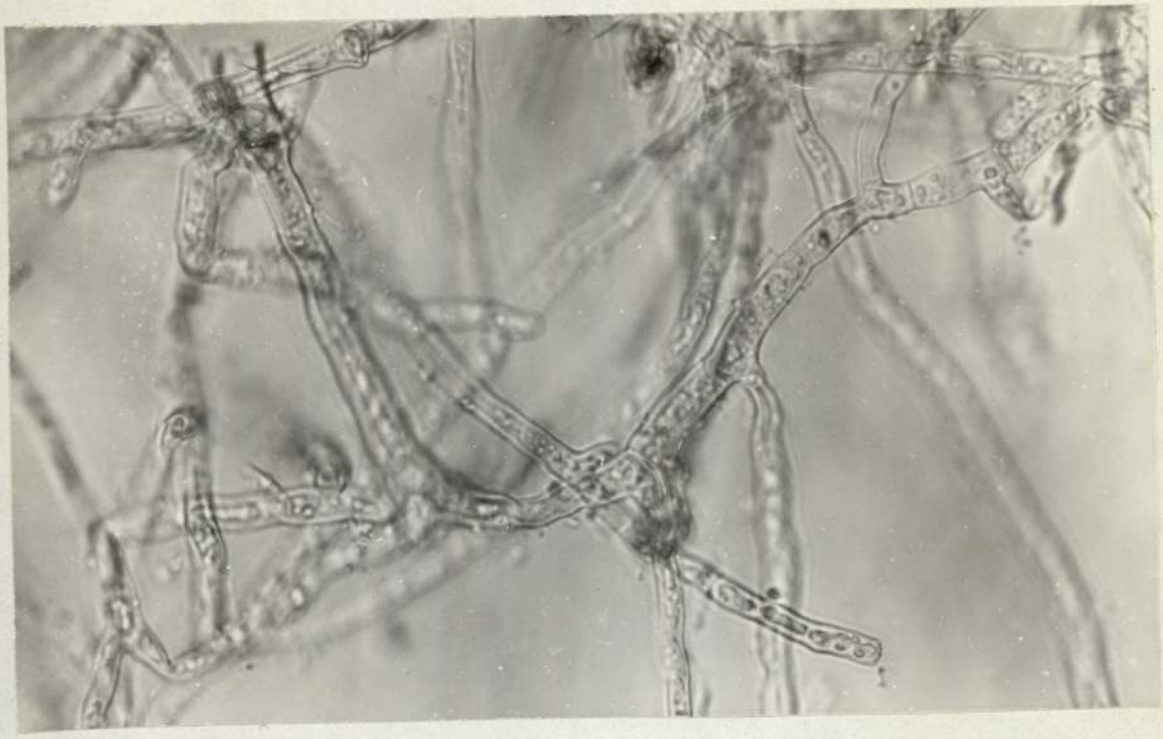
When papain was used to extract mycelium of Aspergillus niger a very rapid solubilisation of just over half the mycelial mass occurred in 6 hours (Table XI; Graph III). However, much of the soluble material was not proteinaceous as the extract contained only about 30% protein (Kjeldahl N x 6.25) on the dry weight basis. This was further reflected in the fact that only 46% of the mycelial nitrogen was solubilised. Papain is very susceptible to inhibitors and it appears that in a product like fungal mycelium these inhibitors are present in large amounts. It was impracticable to add larger amounts of disodium ethylene diamine tetraacetate to overcome the effects of these inhibitors.

The extract obtained with papain had rather poor organoleptic properties. It had a sour odour and taste and on concentration a light brown amorphous powder precipitated out. Again it was noted that there was no breakdown of the cell walls or fluidisation of the mycelium (Plate IX). This was contrary to the claims made in the patents issued to Weaver (1963,1965) for methods of liberating the contents of microbial cells by contacting with papain. The first patent claimed that papain caused a rapid (1 hour) thinning of a suspension of 200g Penicillium chrysogenum in 300 ml. of culture filtrate. Periodic addition of fresh

Plate IX

Aspergillus niger mycelium after papain extraction

Magnification: X720



mycelium resulted in the complete fluidisation of the cells and loss of cell structure. In our investigations several experiments showed that this did not occur with dried powdered mycelium of Aspergillus niger or Penicillium chrysogenum suspended in water. It should be noted that lytic enzymes secreted by some bacteria and actinomycetes bring about complete fluidisation of other microorganisms including fungi (Carenberg and Heden, 1970). Several patents have been issued for the use of such lytic enzymes to release the contents of microorganisms. (AB.Schaffenburger Zellstoff Werke, 1956; Kyowa Hakko Kogyo Co.Ltd., 1968; Udagawa, Oka and Kohata, 1969)

A French patent was issued to Corteel (1968) for the production of proteins from microorganisms by an enzymatic treatment. This involved successively treating the cells of microorganisms with a protease such as pepsin, trypsin, bromelein, ficin or papain followed by treatment with cellulase and lipase. 25 Kg of a 15% suspension of Candida tropicalis treated in this way gave 2.25 kg of a product containing 11% nitrogen. In comparison with our results, such a process is rather cumbersome especially in view of the fact that only the protein fraction is sought. No serious study was made of alkaline proteolytic extraction. In preliminary studies using trypsin at a pH of 9 a slight odour of ammonia was detected. It was also reported by Behr (1930) that the ammonia fraction is high in alkaline autolysis and may be a consequence of the fact that the deaminase and deamidase of A. niger are active at a pH range of 6 - 10 (Schmalfuss and Mothes, 1930). Alkaline extraction was also not advisable because of detrimental effects of alkaline treatment on proteins in so far as it causes a decrease in nutritive value due to the formation of derivatives which are poorly absorbed (De Groot and Slump,1969).

EFFECT OF PARTICLE SIZE ON EXTRACTION

It was suspected that the high yields obtained on enzymatic extraction were partly due to the disruption of hyphae during grinding. This was found to be true in experiments where powdered mycelium of different size fractions were used (Fig.I). Solubilisation was not much

greater when grinding was carried out to give particle sizes below 500 microns in diameter and due to practical considerations powdered mycelium passing through a No.60 sieve (below 500μ) was used for all extractions. In the commercial production of mould extracts it might be thought that the preliminary operations of drying and powdering the mycelium might be expensive unit operations that would add to the cost of the product. This is not necessarily true in view of the increased yields. The mycelium from antibiotic production in the Glaxo Laboratories at Ulverston is ring dried to facilitate subsequent handling problems. Examination of the ring dried powder shows a fairly high degree of mycelial disruption so that both drying and size reduction can be achieved in a single relatively cheap process as in ring drying.

EFFECT OF TEMPERATURE ON EXTRACTION

Highest yields were obtained with extractions carried out at 60°C (Table XII; Graph IV) and the maximum yield was obtained in 12 hours. It was noted that the yield in this experiment with Penicillium chrysogenum was lower than that obtained previously with Aspergillus niger. Several different factors might have contributed to this lower yield. A 10% mycelial suspension was used instead of a 5% suspension and subsequent experiments will show that the extraction efficiency falls off with increasing concentration of suspended mycelium. The mycelium was obtained as a by-product in antibiotic production and would have consisted of relatively older cells which would contain less protein (Vartiovaara, 1938). The medium would have had a higher C:N ratio so that a higher proportion of unextractable fat may have been present and given the residue a slightly waxy texture. Microscopic examination of the mycelial powder showed slight contamination with filter aid and residual calcium carbonate from the medium (confirmed by effervescence on the addition of acid) which would contribute to lower apparent extractability values. A slight bacterial infection with cocci was also noted in the mycelium and such an infection at an early stage would have the effect of reducing the soluble portion of the mycelium. Finally, these

experiments were carried out using autoclaved mycelium. The extraction was therefore due entirely to the added pepsin and there was no solubilisation brought about by endogenous proteolytic enzymes.

Perhaps the most interesting feature of the temperature experiments was the finding that the higher yields at high temperature extractions corresponded with higher protein contents in the extracts, i.e. there was a preferential release of protein at higher temperatures instead of a 'more of the same' phenomenon. Thus if one seeks a high protein product it would be advisable to carry out extractions at high temperatures in the region of 60°C. High temperature extractions were not carried out in this project for fear of destroying any vitamins present.

EFFECT OF ENZYME CONCENTRATION ON EXTRACTION

In experiments to optimise the enzyme concentration to be used, it was found that there was an increased yield with increasing concentrations of enzyme (Table XIII). Again, there was a corresponding increase in the protein content of the extract. From economic considerations it was decided to limit the enzyme concentration to 0.1%, though an economic evaluation in commercial scale production might justify the use of higher concentrations of enzyme.

EFFECT OF CONCENTRATION OF SUSPENDED MYCELIUM ON EXTRACTION

The earlier experiments led us to believe that the yield of extract would depend, amongst other things, on the quantity of mycelium suspended in the extracting fluid. Increasing the concentration of suspended mycelium in the extracting fluid should result in a more concentrated extract. Since a large part of the cost of producing fungal extracts is anticipated to lie in the evaporation of the extracts to a semi-solid consistency, experiments were conducted to determine the optimum concentration of mycelium to be suspended during the extraction process.

As anticipated, the concentration of the extract increased with the concentration of the suspended mycelium (Table XIV, Graph VII). It should appear that the commercial production of fungal extracts

should be carried out with high concentrations of suspended solids. However, there is a marked decrease in the volume of extract that could be obtained and this tended to cancel the effect of increasing concentration (Table XIV; Graph V). In this experiment maximum yields were obtained with 15% w/v suspended solids (Table XIV; Graph VI). The recovery of filtrate would depend very much on the type of equipment used to separate the residual solids and a centrifuge would presumably be more efficient in this respect. The generalisation to be made is that with higher concentrations of suspended solids more water is taken up in wetting the mycelium and less is available for the extraction process. Hence a smaller volume of a more concentrated extract is obtained.

There would be some disadvantage with using very high concentrations of suspended solids. Efficient separation would be difficult if centrifuges were to be used. The suspension becomes very thick and a lot of power would be required to stir it. Perhaps the most serious disadvantage would be the decrease in efficiency of extraction of mycelium. In the commercial production of fungal extracts it would affect the amount of mycelium to be used.

It is interesting to note that there was an inverse relationship between the cell density and the extraction of cell contents. Vosti and Joslyn (1954) found that cell density had no effect on the degree of autolysis of baker's yeast.

Washing the residue after the initial filtration increased the yield significantly (Table XIV; Graph VIII) and could be practised with advantage on the commercial scale. Rather than pool the washings with the filtrate and increase the bulk of liquid to be evaporated it would be preferable to use the washings to suspend the next lot of mycelium in.

Method used to produce fungal cell extracts for this study.

The fungi were grown in tower fermenters as described in the previous ~~chapter~~ section. Washed mycelium was spread out on trays and dried at approximately 45°C. The dried mycelium was ground in a mortar till all the particles were below 500µ. The powder was suspended in sufficient water to give a 5% w/v suspension and the pH adjusted to 3.0 with 1:1 concentrated hydrochloric acid and glacial acetic acid. 0.1% pepsin was added and the suspension extracted with agitation at 45°C for 24 hr. After this period the undigested residue was removed by filtration and washed. The filtrate and washings were pooled and further clarified by passing through a bed of washed kieselguhr. An aliquot portion of the clarified filtrate was evaporated to dryness to find the yield which was expressed as a percentage of the dried mycelium that was solubilised. The remainder of the filtrate was adjusted to pH 5.9 with sodium hydroxide and evaporated under vacuum till a product containing approximately 60 - 70% solids was obtained. 10% w/v sodium chloride was added to some extracts to enhance the flavour. Table XV summarises the yields and tastes of the extracts obtained with the various fungi.

In addition to those of the fungi grown in tower fermenters, three more fungal extracts were prepared in the manner described. One was of Armillaria mellea mushroom collected from rotting timber. An extract was produced from mycelium of Gibberella fujikuroi obtained from I.C.I.Ltd. as a by-product of gibberellin production. Finally, an extract was prepared from the fermentation dried residues of Penicillium chrysogenum obtained from Glaxo Laboratories as a by-product of penicillin production.

TABLE XV

Yields and characteristics of various fungal cell extracts

Fungus	Yield of extract %	Characteristics of extract
1. Armillaria mellea	22.0	Had a very earthy taste. There was a tendency to aggregate into clumps and the extract did not spread evenly
2. Aspergillus luchuensis	63.1	A very pleasant flavour. Taste tests by a food manufacturer showed this to be identical to that of commercially sold mixtures of yeast extract and vegetable protein hydrolysate
3. Aspergillus niger	52.1	Pleasant flavour. There was no 'after taste' with this extract.
4. Aspergillus oryzae	54.1	A very pleasant flavour. Dilute solutions of this extract tasted very much like commercially available soysauce
5. Aspergillus sydowi	46.4	The extract was slightly paler. Had a sharp acidic taste with a slight aftertaste
6. Cladosporium cladosporioides	56.2	Had a pleasant flavour
7. Fusarium oxysporum	62.5	Had a very meaty flavour with no aftertaste
8. Gibberella fujikuroi (ex I.C.I.Ltd.)	28.4	The extract had a pale-golden colour which tended to darken slightly on prolonged storage. The flavour was very favourable.
9. Heterocephalum aurantiacum	64.5	Had a pleasant slightly meaty flavour
10. Lenzites trabea	37.0	Had a slightly woody taste
11. Mucor racemosus	43.7	Had a faint 'fish-paste' taste
12. Penicillium chrysogenum	55.6	Had a pleasant meat-like flavour resembling commercially available meat flavours.
13. Penicillium chrysogenum (ex Glaxo Laboratories)	40.2	Bland taste, slightly sour. A slight metallic aftertaste could be detected
14. Penicillium notatum	55.5	Had a very pleasant flavour with no aftertaste
15. Polyporus anceps	32.7	Had a pleasant taste with no aftertaste
16. Rhizopus oligosporus	49.5	Had a pleasant 'fish-paste' taste and odour.
17. Rhizopus oryzae	59.6	Had a very pleasant taste and no aftertaste

AMINO ACID ANALYSES

INTRODUCTION

The nutritional value of a protein is generally considered to be directly dependent on its amino acid composition and on its digestibility. In the case of fungal cell extracts, digestibility will not be a problem and the amino acid composition will be of prime importance. In particular, the proportion of essential amino acids will determine the nutritional value of the extracts.

Literature reports of the amino acid composition of fungal proteins are sparse. In several cases the values quoted were only semi-quantitative having been obtained by paper chromatographic methods. It was decided that more precise determinations of the amino acid compositions of fungal cell extracts could be carried out with ion-exchange resin chromatography followed by an automated colorimetric determination of the separated fractions.

The amino acid composition of microorganisms is reported in the literature in several different ways, the three predominant methods of experession being (i) per cent of dry weight, (ii) per cent of protein and (iii) amino acid nitrogen as per cent of total nitrogen. Obviously, no one expression is applicable to all needs. The method of expression used in this work is one that is commonly used by manufacturers of yeast extract; the individual amino acids are expressed as a percentage of the total amino acids. The moisture contents, total nitrogen contents and α-amino nitrogen contents of the extracts are given so that appropriate conversions can be made where necessary.

MATERIALS AND METHODS

The amino acid analyses were carried out on an EEL 194 Amino Acid Analyser which operates on the principle described by Spackman, Stein and Moore (1958).

Ion-exchange resin columns

Basic amino acids: One 10 cm x 0.9 cm column

Neutral and acidic amino acids: Two 80 cm x 0.9 cm columns

Column temperature: 55°C ± 0.1°C for all columns

Stationary phase:

"Amberlite" Chromatographic Grade CG-120 sulphonated polystyrene resin, 8% cross-linked, graded for size by EEL. Resin B1 for basic amino acids and B2 for neutral and acidic amino acids.

Mobile phase:

Citrate buffers were used. The composition of the buffers is given in Table XVI. 3 ml. of 50% BRIJ-35 solution was added to each litre of buffer solution before use in the instrument. In addition 5 ml/litre of thiodiglycol solution was added to the pH 3.25 and 4.25 buffers. 10 ml/litre of thiodiglycol was added to the pH 2.2 buffer. All reagents used were of Analytical grade.

TABLE XVI

Composition of Buffers used

Buffer (pH)	Accuracy (pH)	Sodium conc. (N)	Citric Acid (g)	NaOH 97% (g)	HCl conc (ml)	Phenol (g)	Final volume (L)
2.2	±0.01	0.20	21	8.4	16	0.4	1
3.25	±0.01	0.20	840	330	426	40	40
4.25	±0.02	0.20	940	330	188	40	40
5.28	±0.02	0.35	982	576	272	40	40

Ninhydrin reagent:

The ninhydrin reagent was prepared under nitrogen by mixing 40 g ninhydrin and 0.8 g stannous chloride in a mixture of 1.5 litres methyl cellosolve and 0.5 litre of 4M sodium acetate buffer. The 4M sodium acetate buffer was prepared by dissolving 544 g sodium acetate trihydrate and 100 ml glacial acetic acid in sufficient distilled water to give 1 litre of buffer. The pH was 5.5±0.03 and was adjusted if necessary.

Application of samples:

The samples were made up in pH 2.2 buffer. The sample volume was 1-2 ml and the sample strengths were adjusted by dilution to give accurate readings within the scale, when 1 ml of sample was added.

Flow rates:

Buffer pump: 90 ml per hour

Ninhydrin pump: 45 ml per hour

At these flow rates the analysis time for basic amino acids was 1 hour and for neutral and acidic amino acids 3 hours. The neutral and acidic amino acids were separated with pH 3.25 buffer for 105 min. followed by pH 4.25 buffer.

Reaction bath temperature: 100°C - boiling water bath.

Reaction time: Approximately 9 minutes.

Photometer: 20 mm pathlength, twin channel.

Measurement at 440 nm for proline and 570 nm for all the other amino acids.

Recording: Two-point recorder, one for each photocell.

Absorption plotted against time.

Chart speed of 12" per hour.

Plates X and XI illustrate typical basic and neutral/acidic amino acid recordings.

Standardisation: The amino acid analyses were standardised against a standard Amino Acid Mixture supplied by EEL. The standard contained one micromole per ml (±0.1%) of seventeen amino acids (except proline, 2 μmole per ml.) in pH 2.2 buffer. The standard was applied to the column after 1:10 dilution with pH 2.2 buffer.

Method of measuring peaks: Peak heights were measured by the height/width method. The area of each peak was integrated by measuring its height, in terms of absorption values, by its width, in terms of plotted points, at half that height.

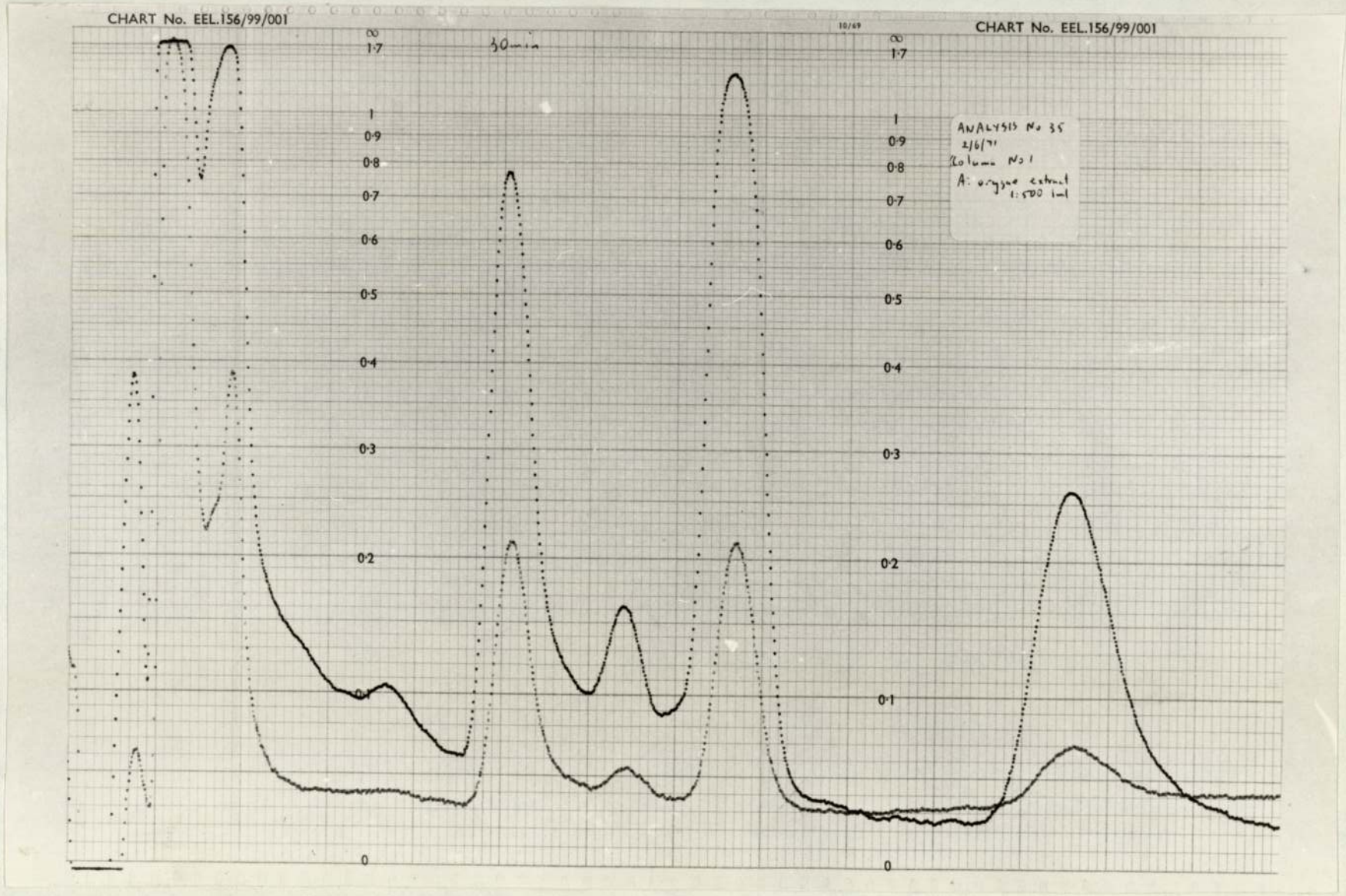


Plate X - Basic amino acid chromatogram of Aspergillus oryzae extract

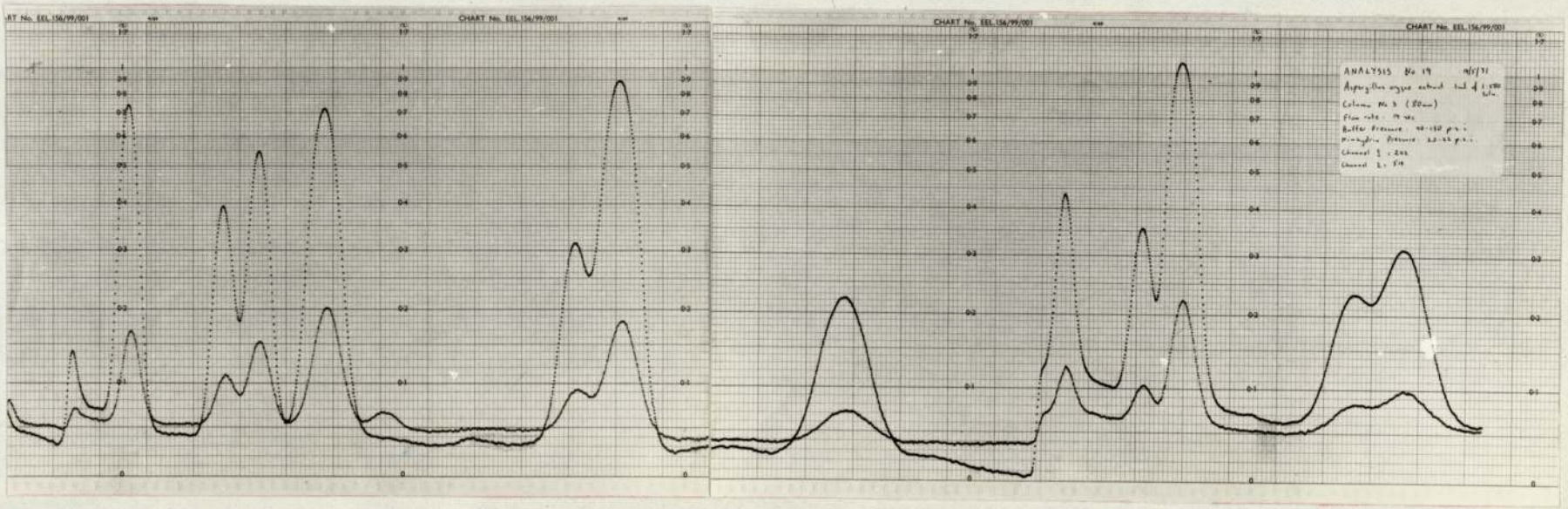


Plate XI - Neutral and acidic amino acid chromatogram of Aspergillus oryzae extract

RESULTS AND DISCUSSION

Table XVII

Amino acid composition of Armillaria mellea extract

Moisture content of extract: 64.3%

Total nitrogen content of dry extract: 6.8%

α-Amino nitrogen content of dry extract: 4.1%

Amino Acid	%	Amino Acid	%
Alanine	31.8	LYSINE	3.8
Arginine	Nil	METHIONINE	2.9
Aspartic Acid	3.2	PHENYLALANINE	4.5
Cysteine	Trace	Proline	1.7
Glutamic Acid	1.1	Serine	1.0
Glycine	9.6	THREONINE	8.3
Histidine	0.5	TRYPTOPHAN	1.0
ISOLEUCINE	8.9	Tyrosine	Nil
LEUCINE	13.5	VALINE	8.1

Table XVIII

Amino acid composition of Aspergillus luchuensis extract

Moisture content of extract: 52.5%

Total nitrogen content of dry extract: 7.9%

α-Amino nitrogen content of dry extract: 5.6%

Amino Acid	%	Amino Acid	%
Alanine	12.0	LYSINE	12.2
Arginine	5.0	METHIONINE	0.9
Aspartic Acid	1.8	PHENYLALANINE	4.6
Cysteine	Nil	Proline	0.7
Glutamic Acid	18.4	Serine	3.3
Glycine	0.7	THREONINE	9.6
Histidine	2.8	TRYPTOPHAN	Trace
ISOLEUCINE	8.0	Tyrosine	4.0
LEUCINE	9.9	VALINE	6.0

Table XIX

Amino acid composition of Aspergillus niger extract

Moisture content of extract: 59%

Total nitrogen content of dry extract: 7.8%

α-Amino nitrogen content of dry extract: 5.3%

Amino Acid	%	Amino Acid	%
Alanine	17.5	LYSINE	15.4
Arginine	14.5	METHIONINE	2.4
Aspartic Acid	5.4	PHENYLALANINE	1.2
Cysteine	Nil	Proline	0.8
Glutamic Acid	12.5	Serine	7.2
Glycine	0.7	THREONINE	4.9
Histidine	4.7	TRYPTOPHAN	Nil
ISOLEUCINE	2.2	Tyrosine	1.2
LEUCINE	5.2	VALINE	4.2

Table XX

Amino acid composition of Aspergillus oryzae extract

Moisture content of extract: 37.5

Total nitrogen content of dry extract: 7.6%

α-Amino nitrogen content of dry extract: 4.9%

Amino Acid	%	Amino Acid	%
Alanine	12.0	LYSINE	10.2
Arginine	7.4	METHIONINE	4.4
Aspartic Acid	6.3	PHENYLALANINE	7.4
Cysteine	Nil	Proline	0.2
Glutamic Acid	14.1	Serine	3.8
Glycine	1.3	THREONINE	3.7
Histidine	0.8	TRYPTOPHAN	0.5
ISOLEUCINE	3.4	Tyrosine	4.8
LEUCINE	15.0	VALINE	4.7

Table XXI

Amino acid composition of Aspergillus sydowi extract

Moisture content of extract: 58.0%

Total nitrogen content of dry extract: 7.3%

α-Amino nitrogen content of dry extract: 4.8%

Amino Acid	%	Amino Acid	%
Alanine	15.6	LYSINE	11.2
Arginine	6.2	METHIONINE	1.6
Aspartic Acid	4.3	PHENYLALANINE	5.2
Cysteine	Nil	Proline	0.7
Glutamic Acid	19.6	Serine	3.4
Glycine	1.1	THREONINE	3.8
Histidine	1.9	TRYPTOPHAN	Nil
ISOLEUCINE	6.2	Tyrosine	4.2
LEUCINE	9.8	VALINE	5.1

Table XXII

Amino acid composition of Cladosporium cladosporioides extract

Moisture content of extract: 56%

Total nitrogen content of dry extract: 7.3%

α-Amino nitrogen content of dry extract: 5.4%

Amino Acid	%	Amino Acid	%
Alanine	17.2	LYSINE	12.5
Arginine	3.5	METHIONINE	2.9
Aspartic Acid	7.6	PHENYLALANINE	5.6
Cysteine	Nil	Proline	1.0
Glutamic Acid	8.4	Serine	1.4
Glycine	5.4	THREONINE	2.9
Histidine	1.3	TRYPTOPHAN	Trace
ISOLEUCINE	6.2	Tyrosine	4.5
LEUCINE	13.6	VALINE	5.9

Table XXIII

Amino acid composition of Fusarium oxysporum extract

Moisture content of extract: 39%

Total nitrogen content of dry extract: 7.7%

α-Amino nitrogen content of dry extract: 5.2%

Amino Acid	%	Amino Acid	%
Alanine	64.7	LYSINE	2.8
Arginine	0.5	METHIONINE	0.6
Aspartic Acid	3.4	PHENYLALANINE	3.1
Cysteine	Nil	Proline	0.1
Glutamic Acid	13.2	Serine	0.4
Glycine	0.1	THREONINE	2.6
Histidine	Trace	TRYPTOPHAN	Nil
ISOLEUCINE	0.6	Tyrosine	1.6
LEUCINE	5.1	VALINE	1.0

Table XXIV

Amino acid composition of Gibberella fujikuroi extract

Moisture content of extract: 30.0%

Total nitrogen content of dry extract: 6.9%

α-Amino nitrogen content of dry extract: 4.4%

Amino Acid	%	Amino Acid	%
Alanine	28.2	LYSINE	10.6
Arginine	Nil	METHIONINE	1.9
Aspartic Acid	3.2	PHENYLALANINE	3.5
Cysteine	Nil	Proline	0.8
Glutamic Acid	8.0	Serine	1.1
Glycine	17.3	THREONINE	5.3
Histidine	2.0	TRYPTOPHAN	1.2
ISOLEUCINE	4.0	Tyrosine	Nil
LEUCINE	8.8	VALINE	4.0

Table XXV

Amino acid composition of Heterocephalum aurantiacum extract

Moisture content of extract: 58%

Total nitrogen content of dry extract: 7.5%

α-Amino nitrogen content of dry extract: 5.0%

Amino Acid	%	Amino Acid	%
Alanine	18.5	LYSINE	12.1
Arginine	5.1	METHIONINE	3.2
Aspartic Acid	3.3	PHENYLALANINE	5.4
Cysteine	Nil	Proline	1.1
Glutamic Acid	14.0	Serine	1.7
Glycine	1.9	THREONINE	5.2
Histidine	2.1	TRYPTOPHAN	Nil
ISOLEUCINE	4.7	Tyrosine	4.7
LEUCINE	10.8	VALINE	6.0

Table XXVI

Amino acid composition of Lenzites trabea extract

Moisture content of extract: 55%

Total nitrogen content of dry extract: 7.5%

α-Amino nitrogen content of dry extract: 4.6%

Amino Acid	%	Amino Acid	%
Alanine	15.0	LYSINE	10.2
Arginine	5.1	METHIONINE	1.6
Aspartic Acid	6.2	PHENYLALANINE	5.6
Cysteine	Trace	Proline	0.8
Glutamic Acid	13.6	Serine	3.4
Glycine	5.2	THREONINE	5.3
Histidine	2.2	TRYPTOPHAN	Nil
ISOLEUCINE	5.3	Tyrosine	4.5
LEUCINE	11.2	VALINE	4.7

Table XXVII

Amino acid composition of Mucor racemosus extract

Moisture content of extract: 39%

Total nitrogen content of dry extract: 7.1%

α-Amino nitrogen content of dry extract: 4.8%

Amino Acid	%	Amino Acid	%
Alanine	19.6	LYSINE	9.8
Arginine	4.6	METHIONINE	1.9
Aspartic Acid	5.7	PHENYLALANINE	5.2
Cysteine	nil	Proline	0.9
Glutamic Acid	13.8	Serine	1.9
Glycine	3.1	THREONINE	4.2
Histidine	2.6	TRYPTOPHAN	Trace
ISOLEUCINE	5.9	Tyrosine	4.2
LEUCINE	11.1	VALINE	5.4

Table XXVIII

Amino acid composition of Penicillium chrysogenum extract

Moisture content of extract: 34.2%

Total nitrogen content of dry extract: 7.6%

α-Amino nitrogen content of dry extract: 5.6%

Amino Acid	%	Amino Acid	%
Alanine	20.1	LYSINE	7.6
Arginine	6.2	METHIONINE	1.6
Aspartic Acid	5.0	PHENYLALANINE	5.7
Cysteine	Trace	Proline	0.6
Glutamic Acid	18.7	Serine	4.1
Glycine	3.1	THREONINE	3.9
Histidine	1.4	TRYPTOPHAN	Trace
ISOLEUCINE	3.0	Tyrosine	4.5
LEUCINE	9.6	VALINE	4.7

Table XXIX

Amino acid composition of Penicillium chrysogenum extract - Mycelium obtained from Glaxo Laboratories.

Moisture content of extract: 31.2%

Total nitrogen content of dry extract: 7.5%

α-Amino nitrogen content of dry extract: 5.0%

Amino Acid	%	Amino Acid	%
Alanine	26.7	LYSINE	6.3
Arginine	8.2	METHIONINE	7.6
Aspartic Acid	5.9	PHENYLALANINE	6.2
Cysteine	Trace	Proline	0.7
Glutamic Acid	5.0	Serine	3.6
Glycine	1.7	THREONINE	3.6
Histidine	2.6	TRYPTOPHAN	Nil
ISOLEUCINE	3.3	Tyrosine	1.8
LEUCINE	11.5	VALINE	5.2

Table XXX

Amino acid composition of Penicillium notatum extract

Moisture content of extract: 36.1%

Total nitrogen content of dry extract: 7.6%

α-Amino nitrogen content of dry extract: 5.4%

Amino Acid	%	Amino Acid	%
Alanine	12.1	LYSINE	8.6
Arginine	6.4	METHIONINE	3.4
Aspartic Acid	7.1	PHENYLALANINE	4.6
Cysteine	Trace	Proline	0.8
Glutamic Acid	11.8	Serine	7.1
Glycine	3.4	THREONINE	5.9
Histidine	2.5	TRYPTOPHAN	0.5
ISOLEUCINE	5.9	Tyrosine	3.6
LEUCINE	10.8	VALINE	5.4

Table XXX1

Amino acid composition of Polyporus anceps extract

Moisture content of extract: 64.1%

Total nitrogen content of dry extract: 7.4%

α-Amino nitrogen content of dry extract: 5.6%

Amino Acid	%	Amino Acid	%
Alanine	20.2	LYSINE	10.2
Arginine	4.6	METHIONINE	2.6
Aspartic Acid	5.2	PHENYLALANINE	5.2
Cysteine	Nil	Proline	1.3
Glutamic Acid	13.2	Serine	2.1
Glycine	4.2	THREONINE	4.1
Histidine	2.6	TRYPTOPHAN	Trace
ISOLEUCINE	5.2	Tyrosine	4.2
LEUCINE	9.9	VALINE	5.1

Table XXXII

Amino acid composition of Rhizopus oligosporus extract

Moisture content of extract: 41.2%

Total nitrogen content of dry extract: 7.7%

α-Amino nitrogen content of dry extract: 5.7%

Amino Acid	%	Amino Acid	%
Alanine	9.5	LYSINE	8.5
Arginine	7.3	METHIONINE	3.6
Aspartic Acid	6.3	PHENYLALANINE	4.2
Cysteine	Trace	Proline	0.8
Glutamic Acid	12.5	Serine	6.9
Glycine	4.3	THREONINE	5.9
Histidine	3.7	TRYPTOPHAN	Trace
ISOLEUCINE	7.4	Tyrosine	3.0
LEUCINE	10.5	VALINE	5.4

Table XXXIII

Amino acid composition of Rhizopus oryzae extract

Moisture content of extract: 65.4%

Total nitrogen content of dry extract: 6.8%

α-Amino nitrogen content of dry extract: 4.7%

Amino Acid	%	Amino Acid	%
Alanine	10.1	LYSINE	9.6
Arginine	6.5	METHIONINE	3.0
Aspartic Acid	6.1	PHENYLALANINE	4.7
Cysteine	Nil	Proline	1.1
Glutamic Acid	12.1	Serine	7.6
Glycine	3.9	THREONINE	5.6
Histidine	2.6	TRYPTOPHAN	Nil
ISOLEUCINE	6.4	Tyrosine	4.2
LEUCINE	11.2	VALINE	5.1

A summary of the amino acid composition in fungal extracts is shown in Table XXXIV. A similarity in patterns was noted in the extracts: alanine, leucine, lysine and glutamic acid occurred in large quantities. Low values were recorded for histidine, methionine, proline and serine. The other amino acids showed intermediate values.

The differences in taxonomic groupings were not reflected in the amino acid composition and the broad division mentioned in the previous paragraph held for fungi in the Phycomycetes, Basidiomycetes and Fungi Imperfecti. A similar conclusion was arrived at by Close (1960) in his studies on the free amino acids of some fungi. However, he noted that the amino acid composition was similar to that of higher plants. Bender (1963) states that generally animal proteins are limited by methionine (with cystine) and plant proteins are limited by lysine. It was noteworthy that fungal extracts were rich in lysine though slightly low in methionine.

It will be noticed that fungal extracts are especially rich in essential amino acids. Table XXXV compares the occurrence of essential amino acids in fungal extracts with the Food and Agricultural Organisation suggestion of an ideal reference protein. Fungal extracts fall short of the reference in only tryptophan and the sulphur amino acids.

Table XXXIV

Amino acid compositions of fungal extracts and yeast extracts

(Expressed as percentage of total amino acids)

	Fungal extracts ■		Yeast extracts ▲	
	Mean	Range	Mean	Range
Alanine	17.9	9.5 - 31.8	8.1	7.2 - 9.7
Arginine	5.7	0 - 14.5	1.2	1.0 -.1.7
Aspartic Acid	5.2	1.8 - 7.6	10.0	8.6 -11.3
Glutamic Acid	12.3	1.1 - 19.6	15.6	11.5 -20.0
Glycine	4.2	0.7 - 17.3	6.6	5.5 - 7.9
Histidine	2.3	0.5 - 4.7	1.6	1.2 - 2.3
ISOLEUCINE	5.4	2.2 - 8.9	5.1	4.2 - 6.5
LEUCINE	10.8	5.2 - 15.0	6.8	5.8 - 7.7
LYSINE	9.9	3.8 - 15.4	6.5	5.4 - 7.2
METHIONINE	2.7	0.9 - 7.6	1.0	0.8 - 1.5
PHENYLALANINE	4.9	1.2 - 7.4	4.9	3.8 - 6.6
Proline	0.9	0.2 - 1.7	5.6	4.5 - 7.4
Serine	3.7	1.0 - 7.6	5.1	4.5 - 5.6
THREONINE	5.1	2.9 - 9.6	3.6	0.5 - 5.8
TRYPTOPHAN		0.5 - 1.2 ●	1.8	1.7 - 2.1
Tyrosine	3.3	0 - 4.8	3.3	2.7 - 4.1
VALINE	5.3	4.0 - 8.1	7.0	6.0 - 8.2

■ Sixteen extracts considered. Fusarium oxysporum extract was omitted.

▲ Three grades of yeast extract marketted by Bovril Group, Burton-on-Trent. Data supplied by manufacturers.

● Measurable quantities found in only a few of the extracts.

Table XXXV

Essential amino acids in fungal extracts

Amino acid	% in Fungal Extract (mean)	% in FAO Reference Protein	
Leucine	10.8	4.8	
Isoleucine	5.4	4.2	
Valine	5.3	4.2	
Lysine	9.9	4.2	
Methionine	2.7	2.2	4.2
Cystine	0	2.0	
Phenylalanine	4.9	2.8	
Threonine	5.1	2.8	
Tyrosine	3.3	2.8	
Tryptophan	Trace	1.4	

(Tables XVII – XXX IV)

These results suggest that fungal extracts would be an ideal protein supplement to the diets of people on a protein deficient diet. In particular the high lysine content is very encouraging since lysine is the limiting amino acid in the diets of most people in underdeveloped countries where cereals are the main source of dietary protein.

It was noted that the extracts were uniformly low in cysteine and tryptophan; they were completely absent in many extracts. The presence of occasional trace amounts would seem to indicate that these amino acids are present in the fungus but are destroyed during the processing. Arginine and tyrosine were absent in Gibberella fujikuroi extract and Armillaria mellea extract (Tables XVII and XXIV). No other instances of the complete absence of an individual amino acid were noted.

Fusarium oxysporum extract was found to have a very high alanine content, in the region of 64% (Table XXIII). This was by far the highest value for any single amino acid and led us to suspect that the strain used was a mutant. Consequently Fusarium oxysporum extract was not considered

in computing the mean amino acid compositions of fungal extracts. Another noteworthy exception was the high value of glycine in Gibberella fujikuroi extract. (Table XXIV)

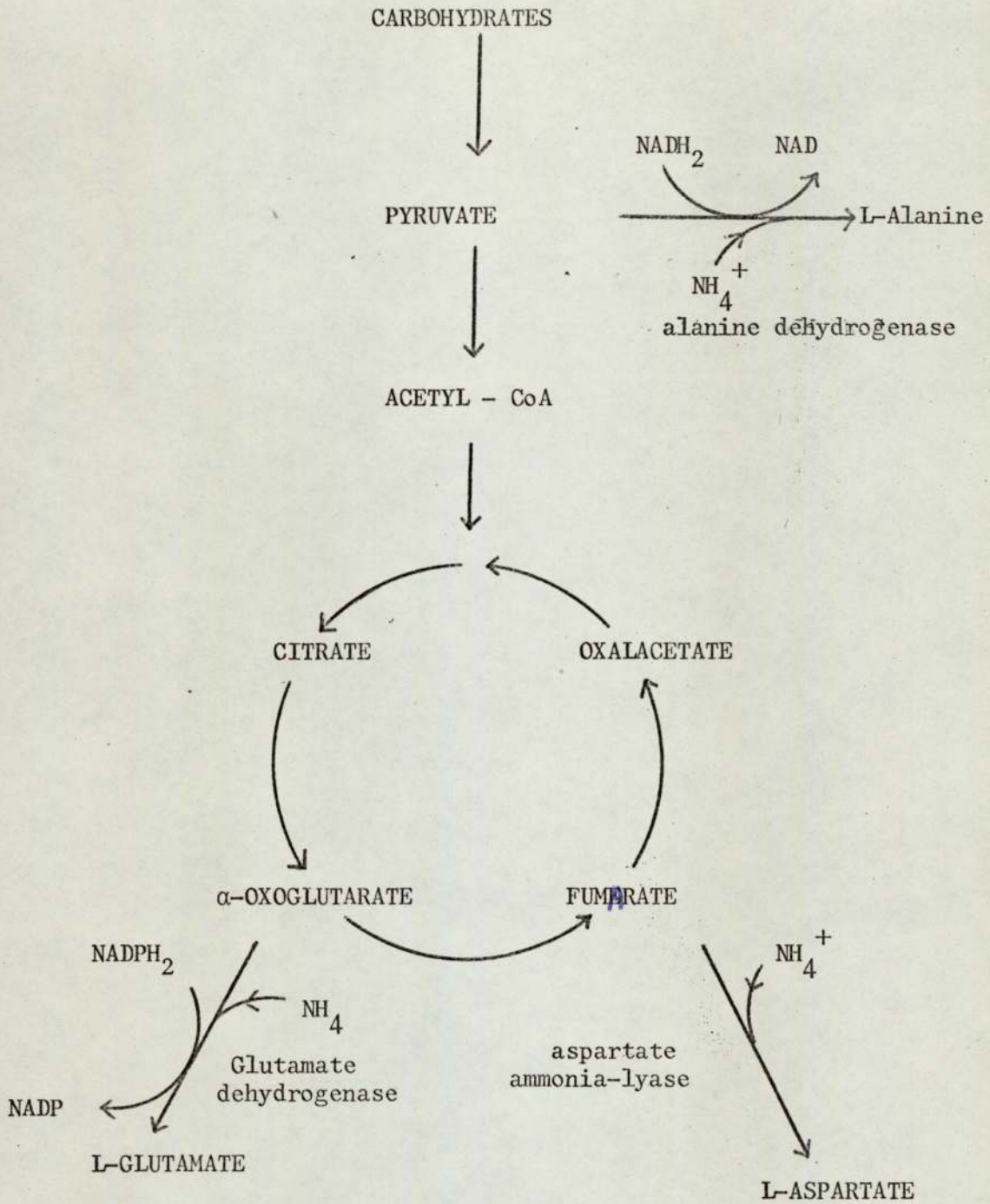
Amino acid analyses were carried out on one extract (Penicillium chrysogenum ex Glaxo Laboratories Ltd.) (Table XXIX) at various intervals during the time of extraction and after acid hydrolysis of the extract. In all instances there was no marked change in the amino acid composition showing that the proteolysis was non-specific to the various amino acids.

An examination of the amino acid compositions yields some interesting clues to the manner of incorporation of ammonia into the amino acid pool. Fig. III summarises the three different reactions by which microorganisms can incorporate ammonia into organic molecules to give amino acids (Mortenson, 1962).

It is seen that fungal extracts have a high content of glutamic acid and alanine but relatively low contents of aspartic acid. This would indicate that fungi incorporate ammonia by either one or both of the alanine dehydrogenase or glutamate dehydrogenase systems. Similar high values of alanine and glutamic acid in fungi were reported by Holden (1962), Das Gupta and Nandi (1956) and Bent and Morton (1964). The cultural conditions and species seem to determine whether alanine or glutamic acid predominate. Kinoshita, Udaka and Shimono (1957) showed that the yield of glutamic acid was affected by the composition of the medium. Dorokhov and Konovalov (1969) found that glutamic acid (and glutamine) formed 62 - 73% of the total amino acids of Aspergillus oryzae and A. flavus. In our work using inorganic nitrogen it was found that alanine was produced in greater quantities than glutamic acid. We suspect that where the nitrogen source is inorganic, the ammonia is incorporated via the alanine dehydrogenase system. Where the nitrogen source is inorganic the glutamic acid content predominates. In this connection it should be noted that the molasses used in the medium contained about 1% nitrogen much of which would be organic nitrogen.

Fig. III

Incorporation of ammonia by microorganisms



In our investigations it was noted that some of the extracts had a distinct soy-sauce flavour and suggested that our process could be adapted for the commercial production of soy-sauce. Baens-Arcega (1970) reports that soy-sauce manufacture consists essentially of growing fungi (principally Aspergillus oryzae) on soybeans and then flooding with brine for prolonged periods. Table XXXVI shows the results of a free amino acids analysis carried out on a commercial grade of soy-sauce.

TABLE XXXVI

Free amino acids of a commercial soy-sauce

Amino Acid	%	Amino Acid	%
Alanine	5.5	Lysine	12.4
Arginine	10.0	Methionine	2.2
Aspartic Acid	10.3	Phenylalanine	3.9
Cysteine	Trace	Proline	0.9
Glutamic Acid	20.4	Serine	5.1
Glycine	4.5	Threonine	3.1
Histidine	3.8	Tryptophan	1.4
Isoleucine	3.9	Tyrosine	1.1
Leucine	8.3	Valine	3.0

The high glutamic acid content seems to re-inforce our theory that glutamic acid contents are high when the substrate contains organic nitrogen. It seems reasonable to suggest that if Aspergillus oryzae were to be grown on a suitable medium and extracted by our process, a product resembling soy-sauce could be obtained.

FEEDING TRIALS

INTRODUCTION

The exact figure for the biological availability of an individual amino acid cannot be properly obtained and the theoretical calculation for the protein quality of a feed or food is difficult. Biological tests succeeding the chemical analyses are recommended.

A potential new source of food must, of course, be evaluated for safety. Before any new food such as fungal cell extracts may be offered for human use, proper assurances of the safety and quality of the food manufactured by the suggested process must be obtained.

Fungal cell extracts would be consumed as food additives rather than as major items of the diet. In the United Kingdom, the government departments concerned with the control of food additives are the Ministry of Agriculture, Fisheries and Food and the Ministry of Health. The administrative procedures to be followed for submissions on food additives and on methods of toxicity testing are set out in a memorandum issued by the Ministry of Agriculture, Fisheries and Food (1965).

Usually a comprehensive series of laboratory tests with animals is necessary. Details will be required on the following:

(i) Composition, purity and commercial usage;

(ii) Acute toxicity, both oral and parenteral, for rats and mice, and at least one other animal species preferably non-rodent;

(iii) Short-term studies, from feeding tests at several dietary levels, on rats or mice, together with at least one other animal species, preferably non-rodent; in the case of rats and mice this should amount to 90 days and in the case of other animals to 10% of their normal span of life.

(iv) Long-term studies including carcinogenicity, from tests following the guide issued by the Ministry of Health's Panel on Carcinogenic Risks in Food Additives and Contaminents. These tests should also be used to detect any long-term toxicity not apparent in the short-term studies; and should extend over the whole of the animal's life. It is also desirable to submit evidence of effects of fertility.

(v) The metabolism, including where appropriate its effect on enzyme activity.

(vi) The effect on man, whenever evidence can be obtained from existing sources.

It can be seen that the officially suggested requirements are fairly extensive. Estimates for the cost of such a test programme range from £30,000 to £60,000. The ensuing feeding trials carried out with mice are only of a very preliminary nature and are, at best, only a guide to the advisability of embarking on an expensive and complete feeding programme.

MATERIALS AND METHODS

Mice:

Eighty freshly weaned (21 day old) white mice strain T.O were obtained from A. Tuck & Son, Laboratory Animal Breeding Station, Rayleigh, Essex. These were made up of forty males and forty females.

Feed:

The feeds used were obtained from the Bugbrook Mills of Heygate and Sons Ltd. Two different feeds were obtained.

(i) Diet VA (low protein diet) containing 10.0% protein, 2.0% oil and 9.0% fibre

(ii) Diet 41B (normal breeding diet) containing 15.0% protein, 2.5% oil and 5.5% fibre.

The feeds were obtained in the form of pellets which were ground to a coarse powder using an 8" C & N Laboratory Mill with a 3 mm. mesh.

Preparation of extract for feeding:

12.5 Kg of wet Aspergillus niger mycelium (representing 2680 g dry mycelium) was suspended in 20 litres of water and homogenised for 30 min. with a Silverson homogeniser (model CX) fitted with a micro-mesh. Microscopic examination showed that a fairly high degree of hyphal disruption was attained. The suspension was made up to 50 litres with water and the pH was adjusted to 3.0 with 1:1 concentrated hydrochloric acid and glacial acetic acid. 50 g of pepsin was added and the mycelium extracted for

24 hours at 45°C. The temperature was maintained by thermostatically controlled heating tapes wrapped around the outside of the stainless steel extraction vessel. Agitation was obtained by a Silverson mixer (model AXR) coupled to a time switch to be on for 15 seconds in every minute. The unextracted residue was removed by centrifuging through an Alfa-Laval continuous centrifuge. 42 litres of extract were obtained and concentrated in vacuo to 5 litres in a climbing film evaporator at not more than 45°C. The pH was adjusted to 5.9 with 40% sodium hydroxide and further concentrated in a rotary evaporator. The final yield was 2640 g of extract at 45.75% solids giving an overall yield of 45.1% on dry material. The protein content (Kjeldahl nitrogen x 6.25) of the extract was 42.2% of the dry material and its amino acid profile was practically identical to that of Aspergillus niger extract produced on a small scale.

Preparation of experimental diet:

The experimental diet was made up in lots by mixing 450 g powdered Diet VA and 109.2 g Aspergillus niger extract; this would correspond to a 10% supplementation on the dry basis. The mixture was allowed to dry at room temperature befcre being fed to the mice.

Preparation of 10% protein control diet:

500 g of powdered Diet VA was mixed with 59.2 ml of water and allowed to dry at room temperature. The granular consistency obtained was similar to that of the experimental diet.

Preparation of 15% protein control diet:

This was prepared as for the low protein control diet except that powdered Diet 41B was used.

Design of the experimental programme:

Three groups of 15 mice each were caged separately and fed on the experimental diet, 10% protein control diet and 15% protein control diet respectively. 25 other mice were caged separately and fed on the experimental diet. The remaining mice were killed at the start of the experiment for analyses. The selection was made at random with approximately equal numbers of males and females.

Of the 25 mice on the experimental diet two males and two females were reserved for breeding experiments. Two were killed for analyses on days 0, 3, 6, 9, 12, 15 and 18. Four were killed on the 35th day for serological analyses. The remaining three were transferred to a diet containing 50% Aspergillus niger extract and 50% Diet VA. After fifteen days on this diet the mice were killed for analyses.

The weights of the three groups of 15 mice were taken every three days. After 21 days a statistical evaluation of the weight gains was made. Five mice from each group were killed on the 21st. day for analysis of free amino acids in plasma. Of the remaining ten, two were killed on days 21, 24, 27, 30 and 33 for analyses. The livers, kidneys and hearts of the final two mice in each group were subjected to histological examination.

It will be noticed that no control mice were killed in the first 21 days. The rationale for this was that any serological changes brought about by differences in diet would be so small at the early part of the experiment that large numbers of mice would have to be killed before any statistically significant differences over and above the normal biological variation can be demonstrated. Serological differences (if any) would be more pronounced at the later stages of the experiment and it was hoped that the killing of controls at this later stage would show any significant differences with the smaller number of mice available.

Experimental conditions:

Individual mice were identified by colour coded markings on their tails. The cages were 41 cm. by 25 cm. by 14 cm. with about 3 cm. of sawdust at the bottom. Each cage was supplied with a water bottle containing 250 ml of water. The temperature of the room fluctuated between 20° and 25°. The food was served in small plastic plates and the mice were fed twice daily, in the morning and evening.

The mice were fed at a rate of 4 g per mouse per feed initially increasing to 5 g per mouse per feed on the third day and subsequently to 6 g per mouse per feed on the ninth day. Any leftover feed was discarded.

The mice were weighed every three days before feeding and were transferred to clean cages after weighing. Observations were also made on the behaviour of the mice, acceptability of the diets and quantity of food and water consumed.

Killing mice for analyses:

The mice were selected at random, starved for 6 hours, weighed and anaesthetised with ether. The thoracic cavity was opened up and blood drawn directly from the heart by means of a syringe. The blood from two mice was pooled and centrifuged and the serum obtained for serological analyses.

The livers of the individual mice were removed, washed under running water and dried with blotting paper. The livers were weighed and Kjeldahl nitrogen determinations carried out on them.

Determination of Serum Total Protein:

Serum Total Protein was determined colorimetrically by the Biuret Method. The reagents used were supplied by Boehringer Mannheim GMBH. 0.1 ml of serum was added to 5.0 ml of a solution of 0.1M sodium hydroxide, 16 mM potassium sodium tartrate, 15 mM potassium iodide and 6 mM copper sulphate. A reagent blank was set up without the serum. A sample blank of 0.1 ml serum and 5.0 ml of a solution of 0.1M sodium hydroxide and 16 mM potassium sodium tartrate was also made up. The solutions were allowed to stand at room temperature for 30 minutes and the optical densities measured at 546 nm in silica cuvettes with 1 cm light path. The optical density of the sample was measured against the reagent blank. The optical density of the sample blank was measured against water and the resulting optical density subtracted from the optical density of the sample. The total protein was read off from a calibration curve prepared using serial dilutions of a standard suspension of 6 g protein in 100 ml. (Graph IX).

Determination of Serum Urea:

Serum urea was measured colorimetrically after hydrolysis with urease to form ammonium carbonate.

$$\text{Urea} + 2\,H_2O \rightarrow (NH_4)_2CO_3$$

The ammonium ions produced react with phenol and hypochlorite forming a blue dye whose intensity measured at 546 nm is proportional to the urea concentration.

The reagents for this determination were supplied by Boehringer Mannheim GMBH. 0.1 ml of serum was mixed with 0.9 ml of physiological saline and 0.2 ml of this dilution was mixed with 0.1 ml of a suspension of 2 mg/ml of urease in 0.05M phosphate buffer, pH 6.5. A standard was set up with the urease suspension and 0.2 ml of a solution of 3 mg urea per 100 ml. A blank was set up with urease suspension alone. The test tubes were covered with Parafilm^{R} (Gallenkamp) and allowed to stand in a water bath at 37^{o}C for 15 minutes. 5 ml of a solution of 0.106M phenol and 0.17 mM sodium nitroprusside and 5 ml of a solution of 11 mM sodium hypochlorite and 0.125N sodium hydroxide were added to each test tube. The contents were mixed immediately after addition and allowed to stand in a water bath at 37^{o}C for 30 minutes. The optical densities of the sample and standard were read against the blank at 546 nm in silica cuvettes with 1 cm light path.

Determination of Serum Leucine Aminopeptidase (LAP):

LAP splits L-Leucine-p-nitranilide into Leucine and p-nitraniline. The amount of p-nitraniline set free in a given time is proportional to the amount of LAP present and on account of its yellow colour can be measured at 405 nm.

The reagents used were supplied by Boehringer Mannheim GMBH. A mixture of 3.0 ml of 50 mM phosphate buffer pH 7.2 and 0.1 ml of 25 mM leucine-p-nitranilide was incubated in a water bath at 25^{o}C for 5 minutes. 0.1 ml of serum was added, mixed and poured into 1 cm silica cuvettes. The optical density E_1 was taken, the cuvette allowed to stand at 25^{o}C for exactly 30 minutes and the optical density E_2 determined.

For calculation, $E_{405nm} = E_2 - E_1$

The non-enzymic hydrolysis of L-leucine-p-nitranilide was determined by using water instead of the sample. The resulting optical density was subtracted from that using the sample.

A Unit (U) is that amount of enzyme which converts 1 µmole of substrate in 1 minute at 25°C. The extinction coefficient of p-nitraniline at 405 nm is 9.9 cm^2/µmole. Therefore with a test volume of 3.2 ml a sample volume of 0.1 ml and a reaction time of 30 minutes,

$$E_{405nm} \quad X\ 108 \quad = mU/ml\ serum$$

Determination of Serum Isocitrate Dehydrogenase (ICDH) :

The enzyme ICDH catalyses the hydrogen transfer reaction

Isocitrate + NADP $\rightleftharpoons$ α-oxoglutarate + CO_2 + NADPH + H^+

which has an equilibrium well on the side of α-oxoglutarate and NADPH. The ICDH activity is determined from the rate of increase of absorption at 366 nm due to NADPH produced by this reaction.

The reagents used were supplied by Boehringer Mannheim GMBH. 0.50ml of fresh serum was mixed with 2.50 ml of a solution of 4.6 mM DL-isocitrate and 52 nM sodium chloride in 0.1M triethanolamine buffer pH 7.5. The mixture was allowed to stand for 5 minutes in a water bath at 25°C. 0.10 ml of a solution of 9.1 mM NADP and 0.12M manganese sulphate was added, mixed and poured into a cuvette. The extinction was read at 1 minute intervals for 4 minutes and the average difference in extinction (E_{360}nm/minute) calculated.

One Unit (U) is that quantity of enzyme which transforms 1 µmole of substrate in 1 minute at 25°C. The extinction coefficient of NADPH at 366 nm is 3.3 cm^2/µmole. Therefore with a test volume of 3.10 ml, a sample volume of 0.50 ml $E_{366\ nm}$/minute X 1880 = mU/ml serum.

Determination of Serum Glutamic-Oxalacetic Transaminase (GOT):

The enzyme GOT catalyses the equilibrium reaction glutamate + oxaloacetate $\rightleftharpoons$ aspartate + oxoglutarate.

The GOT activity is determined by measuring the rate of increase of oxaloacetate in the above reaction. This increase is determined in the coupled indicator reaction catalysed by malate dehydrogenase (MDH)

oxaloacetate + NADH + H^+ $\longrightarrow$ malate + NAD^+

formation
NADH is a measure of activity and can be determined by means of its absorption at 366 nm.

The reagents used were supplied by Boehringer Mannheim GMBH. 0.50 ml of fresh serum was mixed in a test tube with 3.0 ml of a solution of 40 mM L-aspartate in 0.1Mphosphate buffer, pH 7.4, 0.05 ml of a solution of 12 mM NADH and 0.05 ml of a solution containing 0.25 mg each of MDH and LDH/ml. The mixture was allowed to stand in a water bath at 25°C for approximately 5 minutes. 0.10 ml of a solution of 0.25M α-oxoglutarate was added, mixed, poured into a 1 cm silica cuvette and the optical density read at 366 nm. Three additional readings were taken at 1 minute intervals and the mean optical density difference per minute (E_{366nm}/minute) calculated.

One Unit (U) is that quantity of enzyme which transforms 1 μmole of substrate in 1 minute at 25°C. The extinction coefficient of NADH at 366 nm is 3.3 cm^2/μmole. Hence with a test volume of 3.70 ml. and a sample volume of 0.50 ml, E_{366nm}/minute X 2240 = mU/ml serum.

Determination of Serum Glutamic-Pyruvic Transaminase (GPT):

The enzyme GPT catalyses the equilibrium reaction glutamate + pyruvate ⟶ alanine + α-oxoglutarate.

The GPT activity is determined by measuring the rate of increase of pyruvate in the above reaction. This increase is determined in the coupled indicator reaction catalysed by lactate dehydrogenase (LDH)

pyruvate + NADH + H^+ ⟶ lactate + NAD^+

NADH is a measure of activity and can be determined by means of its absorption at 366 nm.

The reagents used were supplied by Boehringer Mannheim GMBH. 0.50 ml of fresh serum was mixed in a test tube with 3.0 ml of a solution of 0.08M DL-alanine in 0.1M phosphate buffer pH 7.4, 0.05 ml of a solution of 12 mM NADH and 0.05 ml of a solution of 0.25 mg LDH/ml. The mixture was allowed to stand in a water bath at 25°C for approximately 5 minutes. 0.10 ml of a solution of 0.25M α-oxoglutarate was added, mixed, poured into a 1 cm silica cuvette and the optical density read at 366 nm. The mean optical density difference (E_{366nm}/minute) was determined as in the GOT determinations.

As defined in the determination of serum GOT, E_{366nm}/minute x 2240 = mU/ml serum.

Electrophoresis of serum proteins:

Electrophoresis was carried out on 25 mm x 160 mm cellulose acetate strips (Cellagram^{R}, Shandon). The buffer used was Oxoid Barbitone Acetate Buffer for Electrophoresis with ionic strength 0.1 and pH 8.6. Before use the strips were soaked in buffer for 5 minutes and the excess buffer blotted out. 0.001 ml of serum was streaked across the centre of the strip by means of a micropipette. A small amount of saturated bromophenol blue was used to tag the albumin fraction. Electrophoresis was carried out over a period of 5 hours with a voltage difference of 250 volts and a current flow of 8 mU. At the end of the electrophoresis the protein bands were fixed with 5% aqueous trichloroacetic acid for 5 minutes and stained for 5 minutes with 0.2% Ponceau S (B.D.H) in 3% aqueous trichloroacetic acid. The excess stain was washed out with several changes of 5% aqueous trichloroacetic acid until the background was completely white and the last rinse showed no traces of the stain. The strips were dried in a hot air oven for 20 minutes at 95°C.

Free Amino Acids in Plasma:

The free amino acids in plasma was determined for the mice killed at the start of the experiment and for the mice on the three different diets killed on the twenty first day. A heparinised syringe was used to draw the blood. The pooled blood was centrifuged and the clear plasma obtained. 2.0 ml of plasma was deproteinised by mixing with 8 ml of 25%.trichloroacetic acid and centrifuging. The clear supernatant was withdrawn by a syringe and made up to 10 ml with water. 1 ml portions of this were used for amino acid analyses.

RESULTS AND DISCUSSION

Table XXXVII

Body weights, Liver weights and Liver nitrogen contents of mice fed on the experimental diet

Day of Experiment	Mouse Number	Sex	Weight (g)	Liver Weight (g)	Liver weight per 100g body weight (g)	Nitrogen content of liver (g%)	Liver nitrogen per 100g body weight (mg/100g)
0	1	M	10.3	0.53	5.15	2.55	131.3
0	2	M	13.0	0.64	4.90	2.35	115.1
3	3	F	13.4	0.62	4.60	2.23	102.6
3	4	M	15.0	0.81	5.40	2.36	127.4
6	5	F	15.2	0.85	5.60	2.50	140.0
6	6	M	12.0	0.57	4.75	2.82	134.0
9	7	F	15.6	0.85	5.45	2.20	120.0
9	8	M	14.0	0.72	5.15	2.36	121.5
12	9	M	19.2	1.01	5.25	2.57	135.0
12	10	F	16.4	0.86	5.25	2.69	141.0
15	11	M	22.4	1.27	5.65	2.75	155.4
15	12	F	18.5	0.99	5.35	2.80	149.8
18	13	M	21.6	1.15	5.30	2.77	146.8
18	14	F	20.2	1.02	5.05	2.84	143.4

Graph IX

Calibration curve for serum total protein

Biuret method, silica cuvettes 1 cm light path 546 nm.,20-25°C

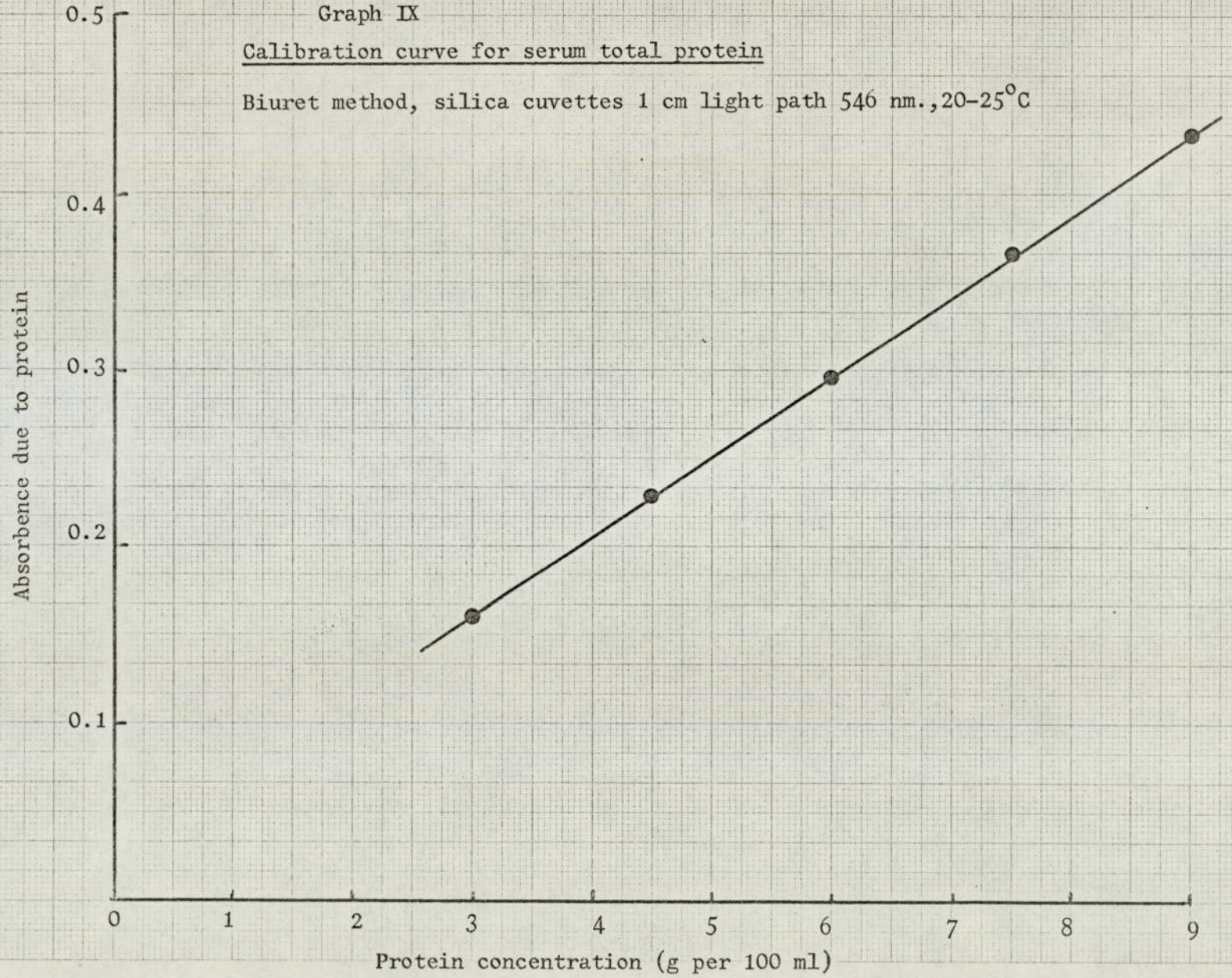


TABLE XXXVIII

Total serum protein of mice on experimental diet

Serum pooled from two mice

Values obtained from calibration curve - Graph IX

Day of Experiment	Absorbence of test (A)	Absorbence of Solution 1 (B)	A - B	Reagent Blank (C)	(A-B)-C	Total serum protein (g/100 ml)
0	0.240	0.042	0.208	0.040	0.168	3.17
3	0.308	0.045	0.263	0.070	0.193	3.75
6	0.295	0.043	0.252	0.065	0.187	3.60
9	0.320	0.048	0.272	0.048	0.224	4.45
12	0.275	0.038	0.237	0.025	0.212	4.15
15	0.320	0.045	0.275	0.015	0.260	5.15
18	0.283	0.042	0.241	0.025	0.216	4.20

TABLE XXXIX

Serum urea of mice on experimental diet

Serum pooled from two mice

Diluted 1:10 with physiological saline before testing

Day of Experiment	Absorbence of Sample	Absorbence of Standard	Serum urea (mg/100 ml)
0	0.250	0.130	57.70
3	0.185	0.190	39.60
6	0.245	0.135	54.45
9	0.195	0.130	45.00
12	0.175	0.135	38.90
15	0.205	0.140	43.90
18	0.190	0.140	40.70

Table XL

Weights (in grammes) of mice fed on 10% protein control diet

Mouse Number	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Sex	F	M	F	F	F	F	F	M	M	M	M	M	F	F	F
Day 0	11.5	9.4	9.5	10.4	11.4	11.1	10.5	9.7	9.0	13.5	9.7	11.7	9.9	10.1	10.4
Day 3	12.4	10.4	11.6	10.4	13.7	11.9	14.2	10.7	9.9	15.5	11.0	13.3	11.1	11.9	11.9
Day 6	14.9	13.1	14.7	13.2	16.8	15.4	17.3	13.1	12.3	18.8	14.6	16.1	14.9	15.9	15.3
Day 9	17.0	13.6	16.1	14.9	17.3	17.0	19.1	15.1	14.4	20.6	17.4	19.5	15.7	18.1	18.0
Day 13	20.9	16.2	18.3	17.4	19.2	19.7	20.8	17.4	17.9	23.4	22.1	24.2	18.2	20.8	21.2
Day 15	21.4	16.9	18.6	17.6	20.5	19.9	22.0	19.0	18.2	24.1	23.2	25.4	18.8	21.9	21.5
Day 18	22.0	19.6	19.5	18.1	20.9	20.6	22.6	19.9	18.8	25.1	24.3	25.9	19.4	22.8	23.2
Day 21	22.9	21.3	20.4	19.1	21.1	21.2	23.3	21.4	19.0	25.7	25.7	27.1	20.5	24.0	23.4

Table XLI

Weights (in grammes) of mice fed on experimental diet

Mouse Number	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
Sex	M	F	M	F	F	M	M	M	M	F	M	F	F	M	M
Day 0	11.4	10.8	10.0	11.2	10.5	11.1	8.5	9.9	11.3	11.4	11.2	11.5	10.4	12.5	12.3
Day 3	13.4	11.6	11.4	12.9	11.7	12.1	9.9	10.4	13.0	13.2	13.0	14.7	11.4	15.7	16.0
Day 6	16.1	14.7	14.4	14.5	15.1	15.0	12.5	11.8	16.0	16.9	15.6	18.0	13.2	18.2	18.7
Day 9	18.4	16.9	17.0	16.1	16.9	17.0	14.5	14.2	18.1	18.0	17.5	19.7	15.1	20.5	21.0
Day 13	20.7	19.9	18.7	19.5	20.6	19.5	18.8	18.8	21.9	20.7	23.6	23.3	19.4	24.6	25.1
Day 15	21.4	20.5	18.9	20.0	21.2	19.9	19.8	20.0	22.9	21.2	24.1	23.7	21.2	25.2	26.0
Day 18	21.9	21.2	19.1	21.0	22.4	21.9	22.0	22.1	25.6	22.1	24.5	24.1	23.5	27.1	27.1
Day 21	22.3	21.8	19.7	21.8	22.8	22.1	24.0	24.2	26.5	22.8	26.1	24.5	25.1	27.6	28.5

Table XLII

Weights (in grammes) of mice fed on 15% protein control diet

Mouse Number	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
Sex	M	F	F	M	M	M	F	F	F	F	M	M	F	F	F
Day 0	11.3	10.5	9.7	9.7	9.7	11.4	9.6	10.6	10.1	11.1	11.3	10.2	9.8	9.5	9.6
Day 3	16.1	13.4	13.0	12.2	13.3	14.1	12.0	13.0	12.7	13.9	16.4	14.2	12.7	14.4	13.8
Day 6	21.0	17.5	16.7	15.4	16.4	17.4	13.6	16.1	16.0	17.7	21.4	17.9	15.1	16.6	17.5
Day 9	24.2	19.6	19.6	16.5	18.2	18.7	15.0	18.3	16.7	19.5	24.7	21.2	18.0	18.0	23.0
Day 13	29.5	24.5	23.0	19.5	19.8	22.2	17.4	22.1	19.1	23.4	28.5	27.0	21.3	19.9	25.9
Day 15	29.8	24.8	23.4	19.8	19.9	22.2	17.6	22.6	19.4	23.7	29.5	27.4	21.6	20.1	26.0
Day 18	30.0	25.6	24.2	20.2	21.1	22.2	17.8	23.3	19.8	24.9	30.4	28.4	22.5	20.9	27.1
Day 21	30.6	26.0	25.5	20.9	22.1	22.5	19.2	24.1	20.3	25.6	31.6	30.0	23.1	21.9	28.3

Graph X

Mean Weights of mice fed on different diets

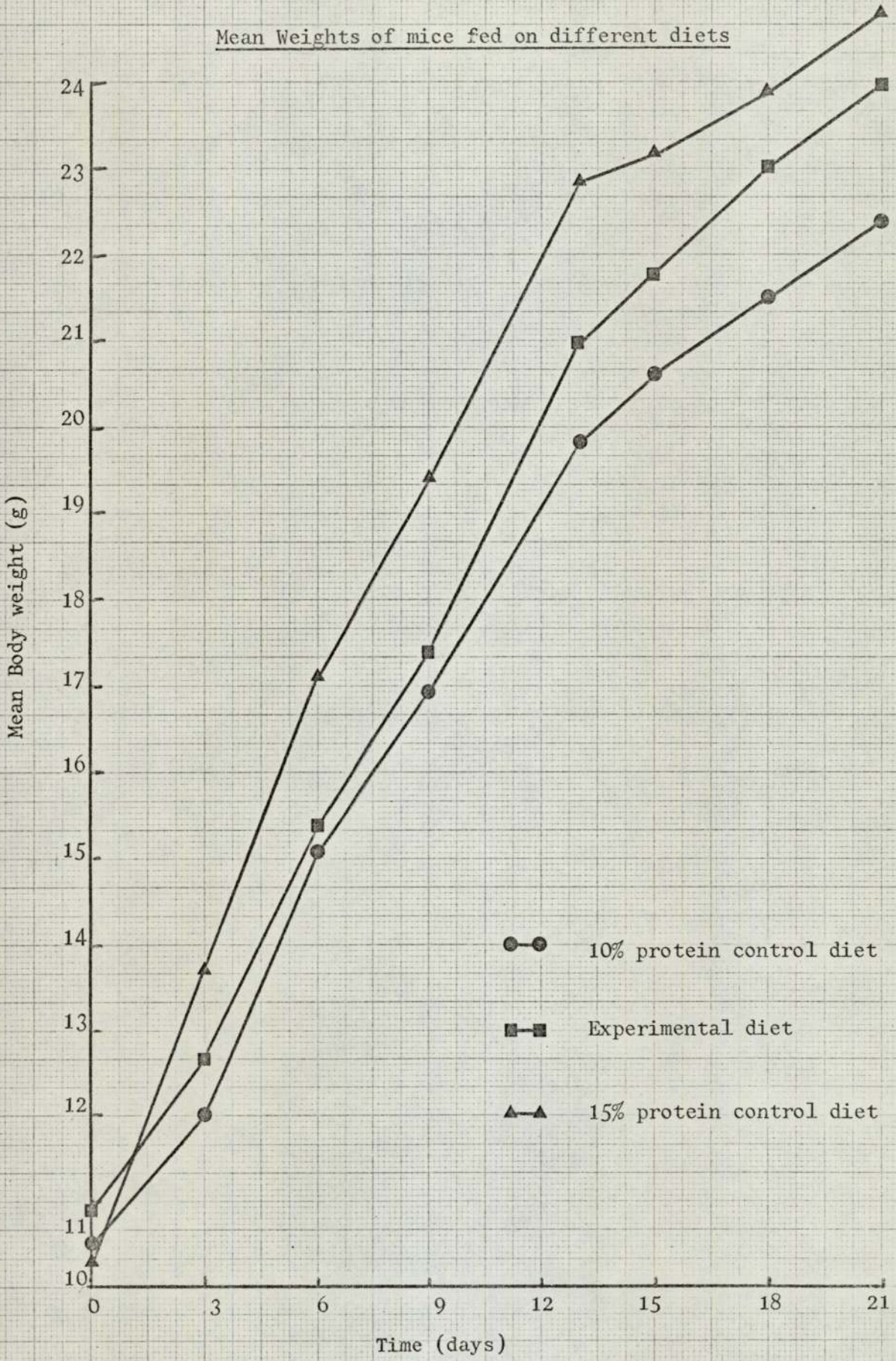


Table XLIII

Mean weights with standard deviations (in grammes) of mice fed on various diets

Day	10% protein control diet		Experimental diet		15% protein control diet	
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
0	10.5	1.1	10.9	1.0	10.2	0.7
3	12.0	1.5	12.7	1.7	13.7	1.2
6	15.1	1.7	15.3	2.0	17.1	2.0
9	16.9	1.9	17.4	2.0	19.4	2.8
13	19.8	2.3	21.0	2.1	22.8	3.5
15	20.6	2.4	21.7	2.1	23.2	3.7
18	21.5	2.4	23.0	2.2	23.9	3.8
21	22.4	2.4	24.0	2.4	24.8	3.9

A t test was carried out on the weights of the three groups of mice on day 21. This was done on an Olivetti 101 Programma computer. The t value for the weights of the 10% protein control and experimental was 1.773. By reference to statistical tables there was a probability of 0.1 that the weight differences were due solely to sampling (Probability = 0.1 for t value of 1.76 for 14 degrees of freedom).

Similarly the probability of the experimental and 15% protein control being different due to sampling was more than 0.1 (t value 0.6684). The corresponding probability for the 10% and 15% protein controls was 0.1 (t value 1.9965).

Table XLIV

Mice fed on 10% protein control diet

Day of Experiment	Mouse Number	Sex	Weight (g)	Wt.of Liver (g)	Liver Nitrogen %	Liver wt / Body wt %	Liver N / Body wt (mg/100g)	Analyses of serum			
								Total protein (g/100 ml)	Urea (mg/100ml)	LAP (mU/ml)	ICDH (mU/ml
21	26	F	22.9	1.21	2.66	5.30	141.0	4.20	30.0	1.1	1.8
21	27	M	20.7	1.13	2.57	5.45	140.0				
24	28	F	20.6	0.97	2.62	4.70	123.1	5.10	47.5	4.1	1.2
24	29	F	19.8	1.03	2.84	5.20	147.7				
27	30	F	23.6	1.05	2.55	4.45	113.5	5.00	45.0	6.5	nil
27	31	F	23.7	1.04	2.64	4.40	116.2				
30	32	F	25.8	1.44	2.65	5.60	148.4	4.80	35.0	4.3	nil
30	33	M	27.7	1.58	2.72	5.70	155.0				
33	34	M	21.1	1.02	2.50	4.80	120.0	5.10	35.5	4.9	0.1
33	35	M	29.0	1.63	2.67	5.60	149.5				

Table XLV

Mice fed on Experimental Diet

Day of Experiment	Mouse Number	Sex	Weight (g)	Wt.of Liver (g)	Liver Nitrogen %	Liver wt / Body wt %	Liver N / Body wt (mg/100g)	Analyses of serum			
								Total protein (g/100 ml)	Urea (mg/100ml)	LAP (mU/ml)	ICDH (mU/ml
21	41	M	22.0	1.03	2.62	4.70	123.1	4.60	45.0	1.1	1.8
21	42	F	21.7	1.35	2.59	6.22	161.1				
24	43	M	19.8	1.27	2.67	6.41	171.1	4.80	49.0	nil	1.0
24	44	F	21.9	1.07	2.84	4.89	138.9				
27	45	F	23.6	1.18	2.59	5.00	129.5	5.00	46.0	6.3	nil
27	46	M	24.1	1.31	2.62	5.44	142.5				
30	47	M	26.2	1.25	2.81	4.77	134.0	5.15	40.0	3.2	0.2
30	48	M	27.4	1.47	2.61	5.36	139.9				
33	49	M	29.0	1.58	2.55	5.45	139.0	5.00	42.5	4.6	nil
33	50	F	24.7	1.36	2.72	5.50	149.6				

Table XLVI

Mice fed on 15% protein control diet

Day of Experiment	Mouse Number	Sex	Weight (g)	Wt.of Liver (g)	Liver Nitrogen %	Liver wt / Body wt %	Liver N / Body wt (mg/100g)	Analyses of serum			
								Total protein (g/100 ml)	Urea (mg/100ml)	LAP (mU/ml)	ICDH (mU/ml
21	56	M	28.2	1.82	2.71	6.45	174.8	4.90	42.0	1.1	1.6
21	57	F	24.1	1.56	2.64	6.47	170.8				
24	58	F	26.3	1.35	2.59	5.13	132.8	5.10	37.9	0.2	1.1
24	59	M	21.7	1.02	2.85	4.70	133.9				
27	60	M	26.1	1.30	2.82	4.98	140.4	4.95	37.5	5.2	0.1
27	61	M	25.4	1.10	2.71	4.33	117.3				
30	62	F	21.9	1.25	2.64	5.71	150.7	5.10	37.5	3.2	1.0
30	63	F	26.9	1.48	2.67	5.50	146.8				
33	64	F	24.5	1.31	2.59	5.35	138.5	5.00	39.5	3.7	nil
33	65	F	29.5	1.62	2.71	5.49	148.8				

Table XLVII

Concentration of amino acids in the serum of mice fed on 10% protein control diet

(Values given in micromoles per litre)

Serum Total Protein: 5.20 g/100 ml	
Essential amino acids	
Isoleucine	128
Leucine	292
Lysine	412
Methionine	49
Phenylalanine	106
Threonine	96
Tyrosine■	62
Valine	245
Total	1390
Non-essential amino acids	
Alanine	596
Arginine	156
Aspartic Acid	125
Glutamic Acid	226
Glycine	520
Serine	312
Total	1935
Ratio of non-essential to essential amino acids:	1.39
Ratio of phenylalanine to tyrosine:	1.71

■ Tyrosine is theoretically not an essential amino acid but since it can only be formed from phenylalanine by hydroxylation it was included in this group. (Whitehead and Dean, 1964)

Table XLVIII

Concentration of amino acids in the serum of mice fed on the experimental diet

(Values given in micromoles per litre)

Serum Total Protein: 5.25 g/100 ml		
Essential amino acids		
Isoleucine		152
Leucine		242
Lysine		436
Methionine		64
Phenylalanine		104
Threonine		70
Tyrosine■		72
Valine		326
	Total	1466
Non-essential amino acids		
Alanine		606
Arginine		152
Aspartic Acid		112
Glutamic Acid		168
Glycine		620
Serine		312
	Total	1970
Ratio of non-essential to essential amino acids: 1.34		
Ratio of phenylalanine to tyrosine: 1.44		

■ Tyrosine is theoretically not an essential amino acid but since it can only be formed from phenylalanine by hydroxylation it was included in this group. (Whitehead and Dean, 1964)

Table XLIX

Concentration of amino acids in the serum of mice fed on 15% protein control diet

(Values given in micromoles per litre)

Serum Total Protein: 5.15 g/100 ml	
Essential amino acids	
Isoleucine	176
Leucine	272
Lysine	428
Methionine	52
Phenylalanine	86
Threonine	72
Tyrosine■	51
Valine	300
Total	1437
Non-essential amino acids	
Alanine	620
Arginine	138
Aspartic Acid	108
Glutamic Acid	240
Glycine	588
Serine	346
Total	2040
Ratio of non-essential to essential amino acids:	1.42
Ratio of phenylalanine to tyrosine:	1.68

■ Tyrosine is theoretically not an essential amino acid but since it can only be formed from phenylalanine by hydroxylation it was included in this group. (Whitehead and Dean, 1964)

Table L

Serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase in mice fed on different diets.

(Expressed as milliUnits per millilitre)

Age of mice and diet	G O T	G P T
Day 24 of experiment		
10% protein control	8.6	
Experimental	9.2	
15% protein control	6.4	
Day 27 of experiment		
10% protein control		7.4
Experimental		6.4
15% protein control		4.7
Day 30 of experiment		
10% protein control	7.4	
Experimental	10.6	
15% protein control	9.2	
Day 33 of experiment		
10% protein control		9.6
Experimental		6.4
15% protein control		8.2

Table LI

Weights (in grammes) of mice fed on diet supplemented with 50% Aspergillus niger extract.

Mice transferred to 50% supplementation diet after 35 days on 10% supplementation diet.

Mouse Number	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
15	26.3	26.9	29.5	31.1	32.4	32.8
16	19.1	18.9	22.1	22.1	22.5	23.1
17	28.5	28.4	29.4	28.6	28.8	29.3

Table LII

Water consumption by mice fed on the different diets

(Expressed as millilitres consumed per mouse per day)

Diet	Day of Experiment			
	7	14	21	35
10% protein control	5.7	5.3	4.4	-
Experimental	8.8	8.0	7.4	-
15% protein control	4.4	4.4	4.0	-
50% supplementation	-	-	-	15.0

The foregoing results taken as a whole would indicate that the addition of Aspergillus niger extract to the diet of mice produced beneficial effects. All the mice on the experimental diet remained healthy and gained weight normally (Tables XXXVII, XLI; Graph X). The addition of 10% Aspergillus niger extract (on a dry weight basis) to a 10% protein diet increased the protein content of the diet to an estimated 12.7% as only about two-thirds of the nitrogen in the extract was protein nitrogen. The weight gains by the mice on this diet were intermediate between the weight gains of mice on 10% protein and 15% protein diets respectively (Tables XL, XLII; Graph X). This would seem to indicate that the quality of the protein in Aspergillus niger extract is as good as that of the proteins normally found in the diet. Extensive nitrogen-balance studies would have to be made before any figure can be derived for the nett-protein utilisation of the proteins of Aspergillus niger extract.

The mice on the experimental diet all looked healthy and were somewhat more active than the mice on the control diets. There was no difference in the density of hair growth of mice on experimental or control diets. The experimental diet was very acceptable to the mice and in most instances it was the experimental diet which was consumed in largest quantities. It was also observed that the mice on the experimental diet consumed more water. This could be attributed to the fact that the experimental diet had a high salt content, the salt being obtained during the neutralisation of the extract to pH 5.9.

The total serum protein was of the same order for the mice fed on the different diets and lay in the range 4.20 to 5.15 g per 100 ml. serum (Tables XXXVIII, XLIV, XLV, XLVI). This was normal for mice. Burns and de Lannoy (1966) in their compendium of normal blood values of laboratory animals quoted a range of 3.35 - 9.07 g per 100 ml. for random sexed albino mice with a mean of 6.27 g per 100 ml. The total serum protein seemed to increase with age at the start of the experiment but this could not be proved statistically (Table XXXVIII).

The serum urea concentration was slightly higher than in the mice fed on the control diets (Tables XLIV, XLV, XLVI). However, this was far from pathological and the observed range of 40.0 - 49.0 mg per 100 ml was well within the normal range quoted by Burns and de Lannoy (1966) to be 18.9 - 66.2 mg per 100 ml. Eggum (1970) has suggested the use of blood urea measurement as a technique for assessing protein quality. There is an inverse relation between the blood urea content and the biological value of the diet which is sufficiently accurate to provide a useful method for the prediction of protein quality from measurement of urea levels. It is almost certain, however, that the high serum urea levels of mice fed on the experimental diet does not reflect on an inferior protein quality. It is more likely a consequence of the high levels of ammonia introduced into the diet in the extract.

Kosterlitz (1944) suggested that the sensitivity with which the nitrogen content of the liver responds to different dietary proteins may be used as the basis of a method for determining the nutritive value of proteins. The procedure used here for determining the nitrogen content of the liver is adapted from the method suggested by Henry, Kosterlitz and Quenouille (1953). For practical purposes the preliminary depletion period suggested by these authors was not enforced. However, it was interesting to note that the liver nitrogen contents (mg. per 100 g.body weight) of mice fed on the experimental diet was in the range 123 - 171 mg (Table XLV). By comparison mice fed on 10% protein diets and 15% protein diets had liver nitrogen contents in the ranges 113.5 - 155 mg and 117 - 175 mg. (Tables XLIV, XLVI).

No abnormal values were detected in the determinations of the various enzymes in the serum (Tables XLIV, XLV, XLVI). The leucine aminopeptidase activity in serum is useful in the diagnosis of liver and bile duct diseases. The observed values all lay within the normal value for serum which is up to 22 mU per ml. Extremely low or zero values were observed for isocitrate dehydrogenase. The diagnostic value of isocitrate dehydrogenase

lies in the diagnosis of liver diseases. Elevated values of serum glutamic-oxalacetic transaminase (GOT) occurs in liver and heart diseases Serum glutamic-pyruvic transaminase (GPT) levels are increased during liver disease. The observed values for GOT and GPT were all within the normal which is up to 12 mU per ml. (Table L).

Holt, Snyderman, Norton, Roitman and Finch (1963) showed that there was a distortion in the plasma amino acids in conditions of kwashiorkor. Whitehead and Dean (1964) suggested a means of quantifying protein malnutrition based on this finding. A ratio of non-essential amino acids to essential amino acids would increase in cases of protein malnutrition. Kihlberg (1970) suggested that the evaluation of protein quality can be considerably shortened by studying the changes in the free amino acids of plasma of laboratory animals. The conditions of the experiment have to be carefully controlled so as not to reflect the level of recent protein intake rather than protein depletion. In our experiments the mice were starved for 6 hours prior to killing. The ratios of non-essential to essential amino acids were of the same order for all three groups of mice. (Tables XLVII, XLVIII, XLIX). Presumably significant differences can only be shown in conditions of extreme protein malnutrition.

Electrophoresis of the serum protein showed no abnormalities. The serum albumin band was clear and distinct in the sera of mice on the three diets. There was no increase in serum γ-globulin. These indicated that all the mice were healthy and were not suffering from malnutrition in any form.

Histological examinations of the livers, kidneys and hearts of the mice killed on the 33rd. day showed no abnormalities. Plates XII to XX illustrate the appearance of the tissues. No gross abnormalities were observed during any of the disections.

The mice on the experimental diet bred normally. There was a litter of two (one male and one female) on the fifty-fifth day. The other female had a litter of three on the sixtieth day. These were

made up of one male and two females. One of the females died (probably of suffocation) a few hours after birth. The first mouse had another litter of nine on the eightieth day. There were six females and three males. All the mice were taken off the experiment on the ninety-fifth day as supplies of fungal extracts had run out.

Mice fed on a diet supplemented with 50% Aspergillus niger extract continued to show weight gains (Table LI). However, the mice were far less active than mice fed on the control diets. A marked decrease in food consumption was noted. There was a very marked increase in water consumption (Table LII); this was almost certainly due to the high salt content of the diet. The mean serum protein level was 5.2g/100 ml and the serum urea was 90 mg/100 ml. The latter figure was far above the normal quoted by Burns and de Lannoy (1966). It was not possible to ascertain if there was any kidney damage. The high value might have been due to high intake of ammonium ions. Both serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase levels were elevated showing levels of 36 mU/ml and 54 mU/ml respectively. This apparently unfavourable serological pattern was inconsistent with the weight gains. Toxicity testing with high levels of supplementation of fungal extracts in the diet will be complicated by the difficulty in distinguishing between true toxicity due to the extract from adverse effects caused by the high salt content produced in the diet.

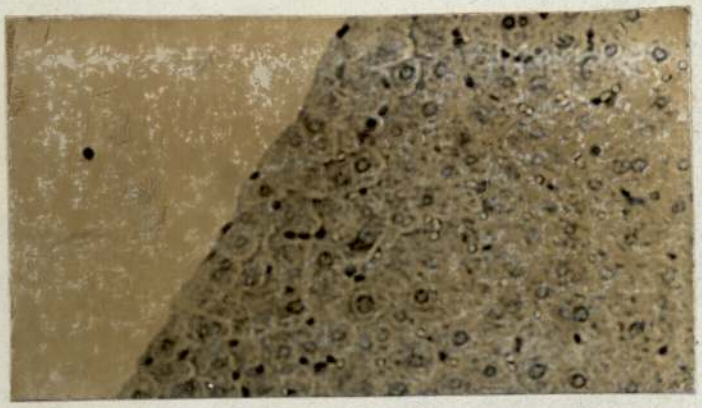


Plate XII

Section of liver of mouse
fed on 10% protein control diet.
Fixed in Bouins
Stained with Haematoxylin and
Eosin.
Magnification: X260

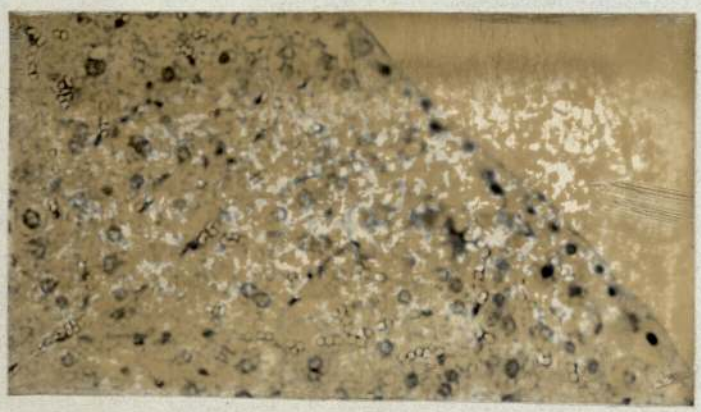


Plate XIII

Section of liver of mouse
fed on experimental diet.
Fixed in Bouins
Stained with Haematoxylin and
Eosin.
Magnification: X260

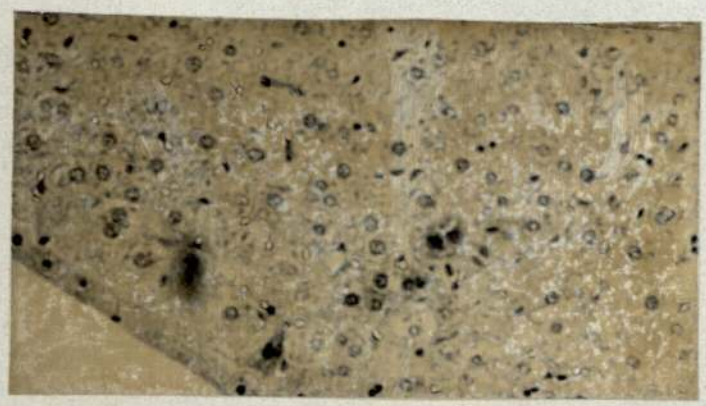
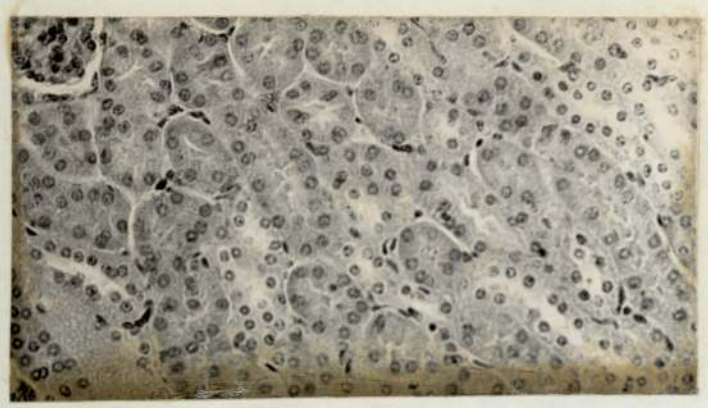


Plate XIV

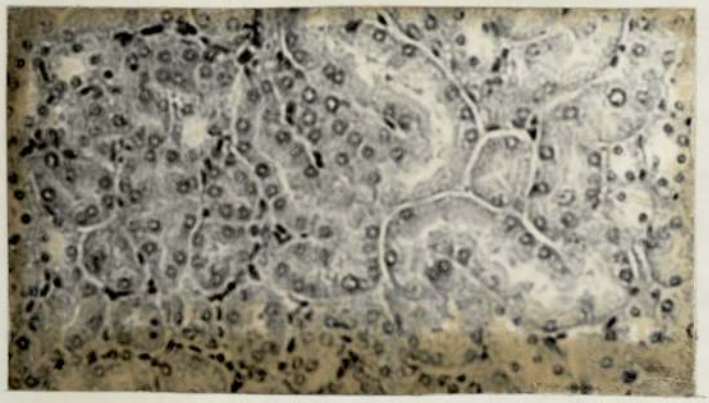
Section of liver of mouse
fed on 15% protein control diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

Plate XV



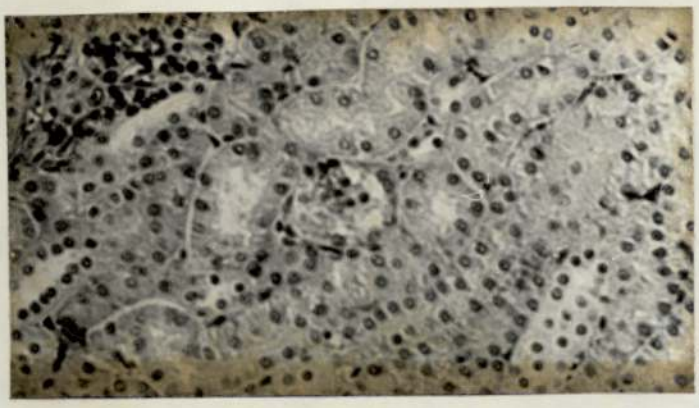
Section of kidney of mouse
fed on 10% protein control diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

Plate XVI



Section of kidney of mouse
fed on Experimental diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

Plate XVII



Section of kidney of mouse
fed on 15% protein control diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

Plate XVIII

Section of heart of mouse
fed on 10% protein control diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

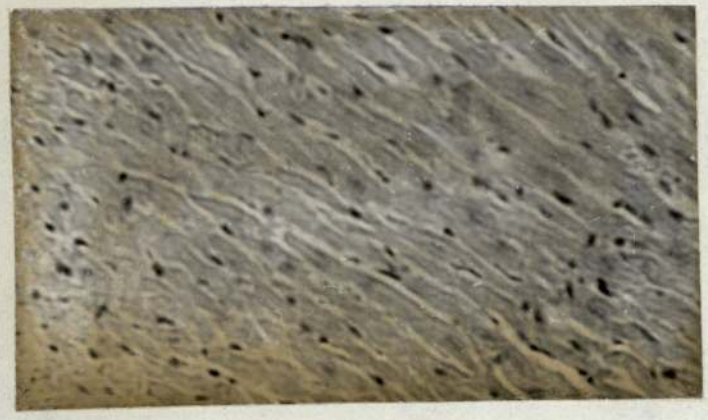


Plate XIX

Section of heart of mouse
fed on experimental diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

Plate XX

Section of heart of mouse
fed on 15% protein control diet.
Fixed in Bouins.
Stained with Haematoxylin and
Eosin.
Magnification: X260

USE OF FUNGAL EXTRACTS AS A FERMENTATION ADJUNCT

INTRODUCTION

A large amount of fungal mycelium is produced as a by-product in the fermentation industry. A sizeable penicillin plant would produce over 1000 tons of dry mycelium a year. One outlet for this could be to dry, autoclave and grind the mycelium to produce fermentation dried residues which can then be sold as cattle feed. The processing cost for the production of fermentation dried residues is estimated to be £26.00 per ton which makes the product competitive with other feeds in terms of cost. However, in view of the Swann Report (Report by the Secretary of State for Social Services et al.,1969) it is doubtful if the use of mycelium from the antibiotic industry as feedstuffs could be continued because of the possibility of residual antibiotics being present.

Some fermentation industries pump their waste mycelium into the sea; measures are being taken to stop this practice because of the pollution problems created. Another alternative would be to incinerate the mycelium. This again is an expensive process, the cost of which is also estimated to be about £26.00 a ton. The effluent problems of the fermentation industry will be reduced if some means of recycling the media constituents can be brought about. In this respect, the use of fungal cell extracts as fermentation adjuncts can result in the recycling of a significant amount of the nitrogenous constituents of the medium.

The quantity of nitrogen source added to a medium is variable but it is common practice to add sufficient of the nitrogen source to give a carbon to nitrogen ratio of 10:1 in the medium. The nature of the nitrogen source plays a very important part in the course of a fermentation. Most fungi are autotrophic for nitrogen and some fungi grow better in a medium containing inorganic nitrogen as opposed to organic nitrogen (Yusef and Allam, 1967). In most industrial fermentations, however, organic nitrogen is preferred. This could be either in the form of simple organic molecules such as urea, glycine,

asparagine or glutamic acid or as complex organic nitrogen compounds as in corn steep liquor, soya bean meal, distillers solubles, pharmamedia or yeast extract. About 400 tons of yeast extract are used annually by the fermentation industry in the United Kingdom (D.A.Gilbert, personal communication). It is believed that fungal cell extracts could be used similarly as a suitable nitrogen source in the fermentation industry. Some experiments were carried out on the growth of filamentous fungi on media supplemented with a fungal cell extract and comparisons made with growth in media supplemented with other nitrogen sources such as ammonium sulphate or yeast extract.

MATERIALS AND METHODS

Preparation of extract:

The mycelium used was that of Penicillium chrysogenum obtained from Glaxo Laboratories, Ulverston. The batch used for this extraction was dried and ground but not autoclaved. Almost all of the powder was below 500μ in size and the powder was used without further grinding or size grading. Analysis of the mycelium by the Kjeldahl method showed a nitrogen content of 4.84% which represented an ~~assumed~~ calculated protein content of 30.25%.

Extraction was carried out in the manner previously described using 8 kg batches of mycelial powder in 80 litres, i.e. 10% w/v suspensions. 0.1% pepsin was used and the extractions were carried out at 45°C with periodic agitation by a Silverson mixer (Model AXR) for 15 seconds in every minute. At the end of 24 hours the undigested residue was separated by centrifuging through an Alfa-Laval continuous centrifuge at 7000 r.p.m. The average recovery from the 80 litre batches of suspensions was 52 litres of extract containing 3.1% solids, i.e. 20.2% solubilisation of the mycelium. The mycelial residue was not washed for further recovery. 20 litre batches of extract were sterilised by autoclaving in steel drums and stored in a cool place prior to concentration.

400 litres of sterile extract (3.1% solids) were evaporated to a product containing approximately 45% solids. (Note: This evaporation was done using a plate evaporator at the A.P.V.Company Ltd., Manor Royal, Crawley. There was some scaling up on the plates. The scale was easily removed by phosphoric acid and analysis of a dried sample of the scale showed only 0.47% nitrogen indicating that the scale was non-proteinaceous. Due to the large losses in the plates and pipes, only 16 litres of extract containing approximately 45% solids could be obtained). A grey amorphous precipitate was deposited in the extract. This was separated by settling and decantation. A sample of the precipitate was washed and dried and on analysis showed a nitrogen content of only 0.21%. The clear supernatant was neutralised to pH 5.9 with 40% sodium hydroxide and evaporated to give a product containing about 65% solids. Analysis of the extract showed a nitrogen content of 7.4% on the dry material. The extract had a very metallic taste which was probably due to prolonged contact with metal at acidic pH.

Preparation of media:

All the three media used contained 5% w/v sucrose as the carbon source. This was equivalent to 2.1% C. Sufficient amounts of the nitrogen sources were added to each media so as to give a nitrogen content of 0.21%. 1% ammonium sulphate was added to the first; the second contained 3.5% of a yeast extract preparation sold commercially as Yeatex^{R} (Bovril Group, Burton-on-Trent) and the third contained 4.54% of a sample of Penicillium chrysogenum extract prepared as previously described. In addition each medium contained trace elements made up of 0.2% w/v disodium phosphate, 0.01% w/v ferrous sulphate, 0.01% w/v magnesium sulphate, 0.01% w/v zinc sulphate and 0.01% w/v potassium chloride. The pH was adjusted to 5.9 with concentrated hydrochloric acid.

Growth of Aspergillus niger:

2.5 litre quantities of each medium were prepared and sterilised by autoclaving at 15 p.s.i. for 15 minutes. Aspergillus niger was grown in each of the three media in 4 litre tower fermenters. The aeration rate was 2.5 litres of air per minute and the growth temperature was maintained at 27°C. The inoculum consisted of a 7-day spore inoculum grown on 5% malt extract agar and removed with a small quantity of sterile 0.05% Tween 80. The inoculum size was standardised as far as possible. 100 ml samples were taken periodically and the yield of dry mycelium determined.

Analyses of mycelia obtained:

The mycelium obtained from the different media was separated, washed and dried. Extracts were prepared as described on page 85 and these extracts were subjected to amino acid analyses.

RESULTS AND DISCUSSION

The growth characteristics of Aspergillus niger in the three media are shown in Tables LIII, LIV and LV and illustrated in Graph XI.

Table LIII

Growth of Aspergillus niger in medium supplemented with ammonium sulphate as nitrogen source

Time (hours)	Yield (g per cent)	pH of medium
0	-	5.9
40	0.26	3.0
44	0.32	2.75
48	0.40	2.60
56	0.49	2.50
64	0.62	2.45
68	0.68	2.40
72	0.70	2.40
84	0.72	2.35

Table LIV

Growth of Aspergillus niger in medium supplemented with yeast extract as a nitrogen source

Time (hours)	Yield (g per cent)	pH of medium
0	-	5.9
18	0.45	5.10
21	0.54	4.70
36	0.76	4.60
40	0.84	4.55
52	1.06	4.40
66	1.09	4.30

Table LV

Growth of Aspergillus niger in medium supplemented with Penicillium chrysogenum extract as a nitrogen source

Time (hours)	Yield (g per cent)	pH of medium
0	-	5.90
19	0.33	5.00
21	0.41	4.90
23	0.46	4.80
30	0.64	4.50
41	0.96	4.40
45	1.07	4.30
62	1.14	4.10

Graph XI

Growth of Aspergillus niger on media supplemented with various nitrogen sources

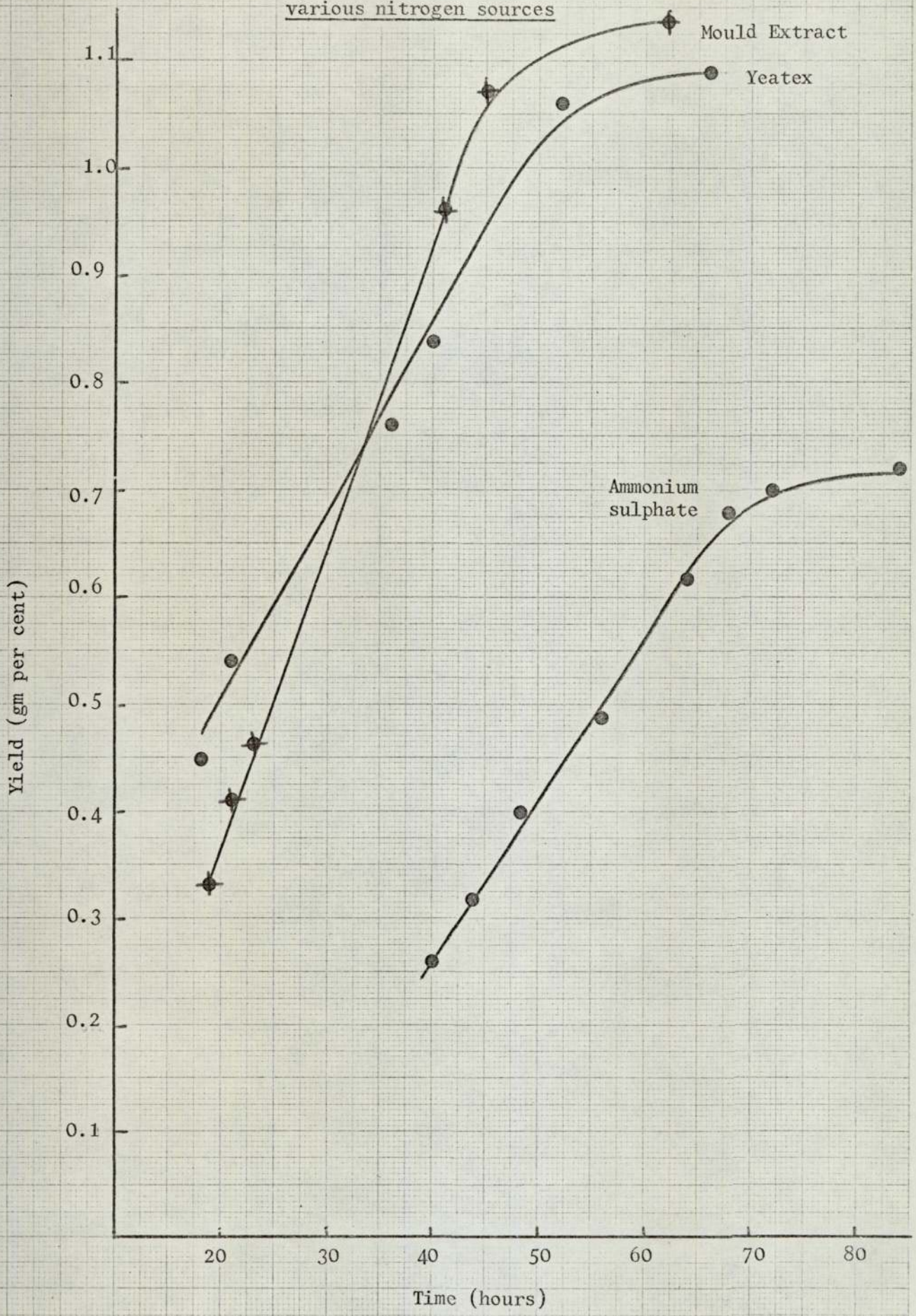


Table LVI

Characteristics of Aspergillus niger grown on media supplemented with various nitrogen sources

Characteristic	Nitrogen souce		
	Ammonium sulphate	Yeast extract	Fungal extract
1. Germination time■	32 hours	12 hours	16 hours
2. Doubling time	20 hours	17.5 "	10.5 "
3. Time to reach maximum biomass	80 hours	66 hours	62 hours
4. Yield at maximum biomass	0.72 g%	1.09 g%	1.14 g%
5. Protein content	36.4%	37.6%	39.8%
6. Conversion efficiency	27.6%	41.6%	42.2%
7. Percentage of mycelium solubilised on extraction	54%	53%	59%

■ Time at which presence of mycelium in the tower fermenter was detectable with the naked eye.

Table LVII

Amino acid composition of extract of Aspergillus niger grown on medium supplemented with ammonium sulphate

Amino acid	%	Amino acid	%
Alanine	22.4	Lysine	15.3
Arginine	15.6	Methionine	2.7
Aspartic Acid	5.3	Phenylalanine	1.2
Cysteine	Trace	Proline	0.4
Glutamic Acid	8.7	Serine	6.4
Glycine	1.6	Threonine	5.1
Histidine	4.4	Tryptophan	Trace
Isoleucine	1.5	Tyrosine	0.9
Leucine	4.7	Valine	3.7

Table LVIII

Amino acid composition of extract of Aspergillus niger grown on medium supplemented with yeast extract

Amino acid	%	Amino acid	%
Alanine	18.6	Lysine	16.1
Arginine	13.9	Methionine	3.3
Aspartic acid	5.2	Phenylalanine	1.0
Cysteine	Trace	Proline	1.5
Glutamic acid	9.5	Serine	7.5
Glycine	0.8	Threonine	5.2
Histidine	4.5	Tryptophan	Trace
Isoleucine	2.1	Tyrosine	1.0
Leucine	4.6	Valine	5.1

Table LIX

Amino acid composition of extract of Aspergillus niger grown on medium supplemented with Penicillium chrysogenum extract.

Amino acid	%	Amino acid	%
Alanine	12.4	Lysine	13.9
Arginine	14.0	Methionine	1.9
Aspartic acid	5.3	Phenylalanine	1.1
Cysteine	Trace	Proline	0.7
Glutamic acid	25.8	Serine	6.7
Glycine	0.5	Threonine	4.2
Histidine	4.6	Tryptophan	Trace
Isoleucine	1.6	Tyrosine	0.7
Leucine	3.6	Valine	3.0

Though the use of mycelial extracts as a growth medium for micro-organisms has been suggested before, the emphasis has been on the use of extracts for growing bacteria. Yoshida and Ishimaru (1951) suggested an aqueous extract of Basidiomycetes. Acid hydrolysates of mycelia were suggested by Kulkarni and Vishvanathan (1958) and Kirsanov (1969 A search of the literature showed that, with the possible exception of the patents issued to Weaver (1953, 1955) there are no suggestions for the re-cycling of waste mycelial constituents in the fermentation industry. We have found that the Weaver process is less efficient in solubilising mycelial nitrogen. What is suggested here is an economic way of recycling a significant proportion of the waste mycelial nitrogen.

Aspergillus niger was found to have a very good growth rate when the medium was supplemented with a fungal extract (Tables LIII, LV; Graph XI). The growth rate was faster and the yield of biomass obtained was greater than when the medium was supplemented with yeast extract (Table LIV, Graph XI). The protein content of the mycelium obtained was greater (Table LVI). This confirmed the findings of Kleber, Aurich and Neumann (1968) that a good assimilable nitrogen source improved the protein content of micro-organisms in contrast to a nitrogen source in which growth was poorer.

It can be seen that higher growth rates will be economically favourable in the fermentation industry. In most industrial fermentation processes product formation is not growth associated and faster growth rates would speed up this non-productive phase.

Theoretically a fungus should grow best in a medium where the nitrogen source contains its own amino acid complement. This presupposes that the individual amino acids are absorbed with equal ease. However, Jones and Pierce (1964) discovered that brewers' yeast absorbed different amino acids from the wort at different speeds. Amino acids could be classified according to the order of their removal from wort. Glutamic acid and aspartic acid were absorbed immediately while alanine and ammonia were absorbed only after a considerable lag. It would be interesting to make comparative studies on the rate of uptake of individual amino acids by fungi growing in their own extracts.

A very interesting finding emerged when an attempt was made to correlate the amino acid composition of the fungus with the amino acid composition of the medium. Jones, Power and Pierce (1965) found that alanine was a major constituent of yeast amino acid pool even at a stage when alanine was not being absorbed from the fermentation wort. We find that alanine is the major constituent of the amino acid pool of Aspergillus niger when the nitrogen source is inorganic nitrogen (Table LVII); this is consistent with the suggestion that inorganic nitrogen is incorporated in a reaction catalysed by alanine dehydrogenase:

$$\text{pyruvate} + NADH_2 + NH_4^+ \rightleftharpoons \text{L-alanine} + NAD + H_2O + H^+$$

However, when the nitrogen source was a fungal extract, the amino acid pool contained less alanine and more glutamic acid, (Table LIX). The decrease in alanine would be consistent with the suggestion that the alanine dehydrogenase system was not operative when organic nitrogen was supplied. The increase in glutamic acid would partly be a consequence of easier absorption.

A further interesting observation was made in attempts to correlate the amounts of alanine and glutamic acid supplied in the medium with the amounts found in the mycelium. The fungal extract used contained approximately 25% alanine and 5% glutamic acid in its complement of amino acids. Thus 100 ml. of media would have contained 0.3 g and 0.06 g of alanine and glutamic acid respectively (100 ml $\equiv$ 4.5 g extract $\equiv$ 3.0 g dry extract $\equiv$ 1.2 g protein). The biomass yield from 100 ml was 1 g containing 40% protein. This protein contained 12.5% alanine and 25% glutamic acid. The biomass produced from 100 ml. of medium thus contained 0.05 g alanine and 0.1 g glutamic acid. The discrepancy in the case of alanine is probably due to incomplete absorption. The increase in glutamic acid points to changes in the order of importance in the various metabolic pathways when a complete nitrogen source like fungal extracts was supplied. Glutamic acid is central to a number of cellular transamination reactions and this could be the reason for the increase in glutamic acid when the medium contained a mixture of amino acids. It should also be noted that, with the exception of a slight decrease in the case of glycine, the percentages of the other amino acids in the amino acid pool were remarkably unchanged.

The alanine -> glutamic acid shift was not so marked when (Table LVIII) Aspergillus niger was grown in a medium containing yeast extract, in spite of the fact that the yeast extract contained more glutamic acid than alanine. This leads us to suspect that the age of the fungus plays a part as well. It will be a useful exercise to grow fungi on various protein hydrolysates to study the rate of uptake of individual amino

acids and to correlate this with the amino acid pool of the mycelium at corresponding times.

COMMERCIAL EVALUATION OF FUNGAL EXTRACTS

The economics of producing fungal extracts would vary greatly in different parts of the world. Because of some commercial interest in the United Kingdom it was decided to make a preliminary evaluation of the economics of producing fungal extracts from fermentation dried residues. The extracts were to be used as a fermentation adjunct.

Plant capacity:

The plant envisaged should be capable of handling about 500 tons of dried mycelium a year. Fig. IV shows the flow diagram of a suitable plant. Working on a 50-week year, the plant should handle 10 tons per week. On a 5-day week it should handle 2 tons a day. It would be more efficient to use two extraction vessels capable of handling one ton each.

The weekly capacity of 10 tons mycelium would yield 4 tons dry extract or 6 tons extract at 66% solids.

Plant requirements:

(1) Storage space - Since the extraction will be done on site at the antibiotic plant there will be little problem with storage space and handling.

(2) Extraction vessels - These will have to withstand pH 3.0 at 50°C. It would seem that wooden vessels will be preferable.

(3) Stirring of extraction vessels - The power requirements for this would be minimal. Large paddle-type stirrers moving slowly at about 1 revolution per minute would be suitable.

(4) Heating of extraction vessels - Supplied by same steam generator that is used for evaporator.

(5) Centrifuge - This would have to be of special material to withstand pH 3.0.

(6) Neutralising - This can be done in the holding tank of the evaporator.

(7) Evaporation - The extract would contain about 3% solids to be evaporated to 66% solids. This would necessitate a double or triple pass in a plate evaporator.

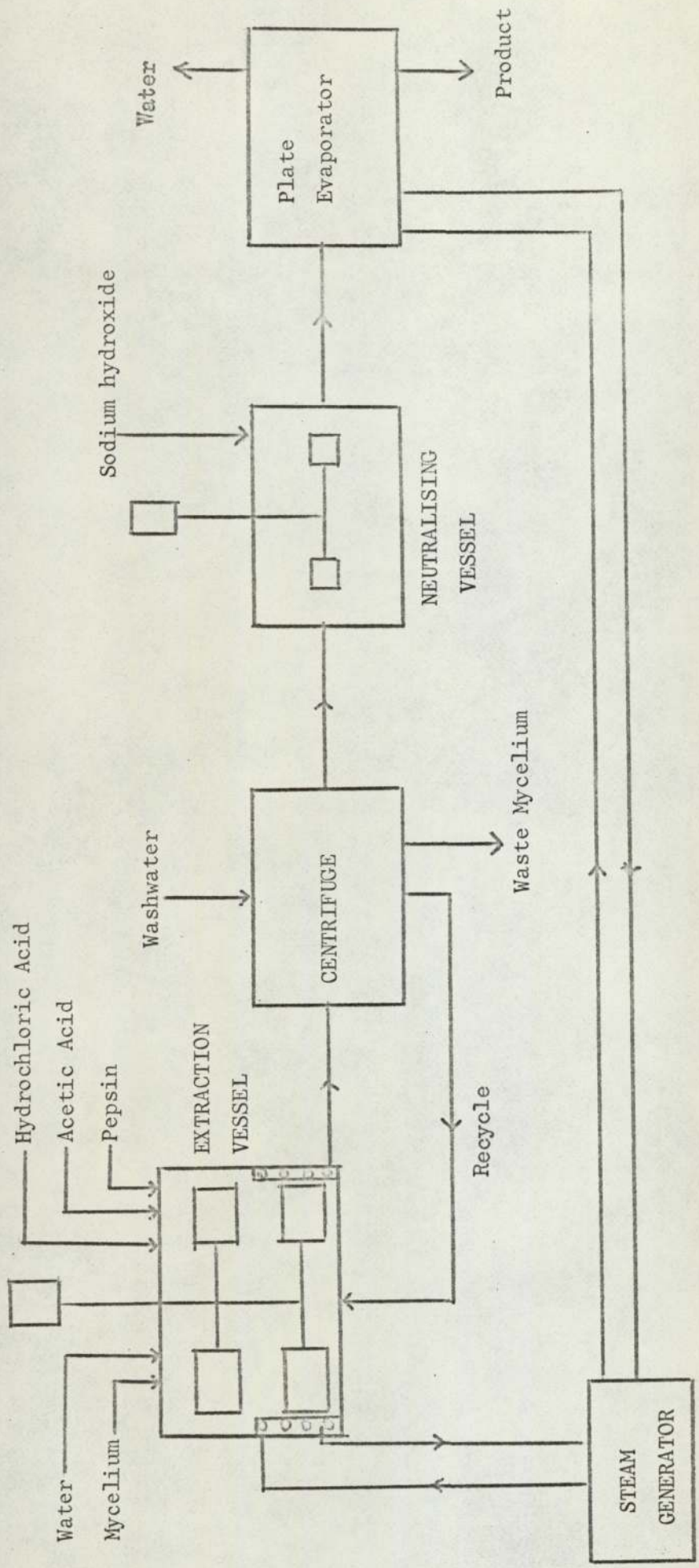


Fig. IV. FLOW CHART OF PLANT TO PRODUCE FUNGAL EXTRACT

Raw Material Requirements:

1. Mycelium - 10 tons at £26.00 per ton.
2. Pepsin - Working at a 10% suspension, 10 tons of mycelium will be suspended in 100,000 litres requiring 100 kg.

   Quotes for pepsin were obtained at 89p per kg.
3. Hydrochloric Acid, Acetic Acid and Sodium Hydroxide - Commercial grades may be used.
4. Water - A large portion of the water obtained from evaporation can be recycled into the system.

Operating Costs:

The quotes for this were extremely variable. As a rough guide the values quoted by Wang (1968) were used.

Labour Costs:

The plant could be operated with two labourers.

Effluent Disposal:

The major by-product will be the 6 tons of waste mycelium. This extracted mycelium will have a lower Biological Oxygen Demand and its disposal problem will be less. Investigations are being made as to the possibility of utilising this by-product.

Weekly estimate of production cost:

Raw Materials:		
	Mycelium	£260.00
	Pepsin	89.00
	Hydrochloric Acid, Acetic Acid, Sodium Hydroxide.	40.00
Operating Costs:		
	Agitation	20.00
	Heating	40.00
	Centrifuging	90.00
	Evaporating	130.00
Capital Investment		130.00
Labour		40.00
		£839.00

The production cost of 6000 kg. of extract is thus approximately £840.00, i.e. the cost of the extract will be approximately 14p per kg.

This preliminary evaluation shows the fungal extracts can be produced at prices very competitive to those of yeast extracts or protein hydrolysates.

CONCLUSION

The introduction of a novel foodstuff such as fungal extracts will involve many problems. The technological problem may be very much more than imagined and may be overshadowed by economic and social considerations. The question of whether or not a microbial food product can be successfully introduced into human diets can only be resolved by a consideration of the elements of cost, nutritive value and acceptability.

It would appear that technological problems of fungal protein production are virtually non-existent. Large quantities of fungal biomass can be produced rapidly by simple techniques. A very wide variety of substrates, especially materials that are lost to the human food chain, may be utilised. It has been shown that very tasty, highly digestible products with excellent nutritive value with regard to protein can be produced from fungal mycelia.

Cost factors would play a critical part in the introduction of a novel foodstuff. The critical cost factors in the process may be summarised as follows (i) raw materials cost; (ii) capital investment; (iii) processing costs, (iv) quality control; (v) market development and (vi) marketing costs. It must be borne in mind that economic consideration will vary considerably with geographical distribution.

We believe that a more long term view should be taken of the economics of microbial protein production. The economics of today might show them to be unfavourable in comparison to conventional protein sources but the economic situation is bound to change with the widening protein gap in the world. The world protein shortage is so serious that every possible method of helping to eliminate this shortage must be worked at. Even today there are certain specialised elements of the human population whose protein needs are so intense and critical that these are not going to be met by normal market processes. There is a strong case for believing that the economics of tomorrow may well favour microbial proteins.

A salient difference between microbial protein and conventional

protein sources is not the price of the products but the quantity that can be produced (Enebo, 1970). A simple calculation would show that the cost of microbial proteins is of minor importance in regard to the quantity needed by any individual. The cost of the protein supplement cannot be a deciding factor per se. Of vital importance for the protein situation in the world is, firstly, that sufficient protein is produced and distributed to cover requirements and secondly, that the consumers can be persuaded to enrich their diet with this extra protein. It is for the production of a sufficient quantity of protein that microbial protein is of greatest importance.

Marketing problems will have to be considered in any economic analysis. Fungal extracts may be used as a formula feed, formulated into food products or sold directly to the consumer. Although direct sale to the consumer may seem most desirable to a producer of fungal extracts, it involves a host of difficult problems, including development of appropriate recipes for use in dishes indigenous to the culture and prevention of misuse of the product. A large-scale distribution system might have to be developed. Sale to a manufacturer of formulated food products has numerous advantages including (i) the extract can be marketed in a pretested beneficial form; (ii) an established market organisation can be used and (iii) many of the product introduction risks can be reduced. On the other hand food processers have access to many protein concentrates and are unlikely to use a microbial source unless it provides performance at a more attractive price than its competitors.

In the final analysis, sociological and psychological factors are going to decide the acceptance of microbial proteins. Fears of toxicity will have to be dispelled by rigorous safety evaluation programmes. Oser (1970) has reviewed the factors to be considered in such an evaluation. The great flood of recent reports of mycotoxins might well raise fears regarding the safety of fungal products. A

perusal of the reviews on mycotoxins by Borker, Insalata, Levi and Witzeman (1966), Wright (1968) and Wogan and Mateles (1968) would put this in a better perspective. The number of fungi which have been proved to synthesise substances toxic to man and/or animals represents such a small percentage of the total number of species that to categorise fungi in general as poisonous is unwarranted. Of the thousands of species of fungi only eleven species (in six genera) have been definitely implicated in the production of toxic substances (Wogan, 1966). Gray (1970) states that with the sole exception of toxic alimentary aleukia, there is little convincing evidence to connect fungal toxins with various human ailments. So long as adequate safety evaluations are undertaken there is no justification for fears of toxicity with fungal foods.

Perhaps the greatest obstacle will be the psychological barrier to the acceptance of microbial food. More and more it is becoming apparent that there is a need for a better interplay among the food scientists, nutritionists and the behavioural scientists (Goldblith, 1970). This interplay and recognition of the role of what the behavioural scientist can offer to the success of the development of products of this type is fundamental to the development of a novel food product. And, equally well, there must be a mutual recognition and appreciation by the behavioural scientist of the advances in food science and technology which may offer means of producing products tailored to local needs, utilising raw materials and based on the societal habits of the target population.

There is nothing new in the consumption of fungi by man, either by design or accident; it must have been going on for millenia rather than centuries. The Presidential address by Dr. C.W.Hesseltine (1965) to the Mycological Society of America dealt with the production of fermented foods by fungi. Almost all the examples of the extensive supplementary list of fungus food fermentations originated from people of Asia, Africa or South America. It is a sad comment on the

irony of the situation that these are the very people plagued by protein malnutrition.

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APPENDIX I

THE PRODUCTION OF EXTRACTS FROM CELLS

Complete specification of U.K.Patent Application No.44714/69 by Dr. R.N.Greenshields, assignor to Greenshields Inventions Co.Ltd.

Complete specification filed on 19th May 1971

Patent Applications are also pending in Japan, Germany, Singapore, France and United States.

This invention relates to the production of extracts from cells, and particularly though not exclusively, to the production of edible extracts.

It is known to produce edible extracts from yeasts, e.g. from Saccharomyces cerevisiae, S. carlsbergensis and S. fragilis, a typical extract being sold for example under the Registered Trade Mark "Marmite". For many years the yeasts were obtained as by-products from industrial fermentation processes such as brewing, and were relatively inexpensive. The situation has now changed, however, and yeasts are in short supply due to a rapidly growing demand. Attempts have been made to grow yeasts specifically for the purpose of making extracts but the cost of yeasts made in this way is relatively high, for yeasts are somewhat fastidious in their nutritional requirements and require relatively expensive media in which to grow.

The object of the invention is to provide cell extracts which may be comparable with yeast extracts and which can often be made more simply and at less expense.

From one of its broadest aspects the present invention consists in a process for making an extract from a mould which is at least substantially free from spores, which process includes the steps of causing lysis of the constituent cells of the mould and separating the resultant extract from the cell debris, any undigested cell material and any other gross solids.

From another aspect the present invention consists in an extract made by the process outlined in the last preceding paragraph.

Extracts in accordance with the present invention are normally rich in amino acids, vitamins, factors (that is enzymes and nucleic acids) and co-factors (that is co-enzymes, vitamins and mineral salts). They are therefore valuable as supplements for human or animal food-stuffs. In general they have characteristic "vegetable" or "meaty" flavours and may therefore be used as flavour additives. Alternatively, extracts in accordance with the invention may be used as nutrients for micro-organisms.

It will be appreciated that terms such as yeast and mould though widely used are somewhat indeterminate. Yeasts may be defined as "fungi which do not produce distinct aerial, asexual spores (conidia) and which spend at least part of the vegetative cycle as individual, single cells". (The Life of Yeasts by H.J.Phaff, M.W.Miller and E.M. Mrak, Harvard University Press, 1966). The yeasts are not all classified together, however and although most occur in the class Ascomycetes others occur in the classes Basidiomycetes and Deuteromycetes. For the purposes of this specification moulds are defined as fungi which are characterised by their growth in the vegetative state as filamentous mycelia; nearly all moulds produce well-defined sporophytes on the air-surface which generate spores. Here again moulds are not restricted to any particular families or classes in the Fungi although certain families may contain more of the characteristic types than others. In general it has been found that moulds in the class Fungi Imperfecti or Deuteromycetes are particularly suitable for use in accordance with the invention. Processes in accordance with the invention may employ a mixture of two or more moulds.

The spore formation in moulds is often associated with unpleasant "musty" or "mouldy" smells and tastes so that little consideration has previously been given to the use of moulds as sources of edible products. Further, some moulds are poisonous, though frequently the poisonous principle resides with the spores. The extracts embodying the present invention are generally free from spores, though if these are initially present they are present in small quantities only and can be removed from the extracts.

The growth of the moulds is preferably carried out under conditions such that few or no spores are present. Nevertheless it is within the scope of the invention to employ moulds grown under conditions such that spores are present in significant quantities. The spores are then severed from the remainder of the mould before the extract is made.

In order to avoid as far as possible the formation of spores when the moulds are being grown the moulds are preferably grown submerged in a nutrient-bearing liquid through which oxygen or a gas such as air which includes oxygen is passed. As described in greater detail below, the nutrient may comprise an extract embodying the present invention. The mould-growing process may be a batch process or may be a continuous or semi-continuous process in which the nutrient-bearing liquid is fed into the lower part of a vessel in which the mould is grown, and some of the mould is continuously or intermittently withdrawn.

Continuous processes for growing moulds are described in patent No.1,133,875 though there the object of growing the moulds is to remove and use products produced by the moulds but external to the cells, such products being normally referred to as metabolites.

Moulds are grown industrially to produce a variety of metabolites, notably to produce a range of antibiotics such as penicillins. Hitherto the moulds themselves have been discarded as waste products, but it is envisaged that they could well be used in carrying out processes embodying the present invention. Experiments conducted with such moulds show that they can yield useful edible extracts.

Alternatively, the moulds may be grown specifically for the purpose of making extracts in accordance with the invention. It is believed that the growth of certain moulds could well be carried out at considerably less expense than the growth of yeasts due to the facts that the growth conditions need be less critical than they are with yeasts, and that nutrients can be used which are less expensive than those used for yeasts.

When the mould has been grown by submerged culture it may be in pellet form or in free form, this depending on the morphology of the particular mould concerned. The mould is preferably separated from the nutrient liquid in which it was grown, as by filtration, and is preferably washed. It may then be resuspended in a relatively small amount of water or other liquid, which liquid preferably comprises washings from the filtered residue after extraction. The amount of liquid used need only be sufficient to yield a free-flowing suspension, though as will be described below, the yield of extract in a given time as well as the maximum yield of extract depends on the amount of liquid used.

In a modified process the mould is not resuspended directly after separation from the nutrient liquid but is dried, as for example in a ring-drier, and powdered, the powder then being suspended as outlined in the last preceding paragraph.

The suspension preferably contains between about 5 and about 20 gms of mould per 100 ml. of liquid.

If the mould has not been dried and powdered the suspension is preferably subjected to physical treatment which breaks the cell walls of the mould. For example the suspension may be treated in a mixer-blender which shears the cells walls.

The physical conditions are preferably adjusted so that they are favourable to lysis. This is effected by the various hydrolytic enzymes normally present within the cells. The most important reaction is the hydrolysis, by the proteolytic enzymes, of the proteins to form peptides and amino acids. In addition other reactions occur; for example the carbohydrases hydrolyse the carbohydrates and the lipases hydrolyse the lipids. Where the mould has been dried and powdered the enzymes may well have been destroyed. It is then necessary to add appropriate enzymes to the mixture. Even when enzymes from the moulds are already present in the mixture it may be desirable to add further enzymes, particularly proteolytic enzymes.

Pepsin is the preferred enzyme, and as this operates in acid conditions the pH of the mixture is preferably adjusted to a value between about 4.5 and about 3.0. This may conveniently be effected by the addition of concentrated hydrochloric acid or by the addition of a mixture of concentrated hydrochloric acid and galcial acetic acid. Citric acid, either alone or with hydrochloric acid or acetic acid or both may also be employed. It has been found that the use of acetic acid tends to increase the yield significantly. It is generally preferred, however, to reduce the pH below a value obtainable with acetic acid alone, and for this reason it is preferred to use acetic acid and a strong acid such as hydrochloric acid. Trypsin may be used as an alternative to pepsin, but as it operates in alkaline conditions it is necessary to adjust the pH accordingly. As it is desirable for the resulting product to be acid, it is preferred to use enzymes which require acid conditions.

The choice of enzymes will depend on the particular mould used, the acidity of the extract (although, as indicated above, this may be adjusted to suit the enzyme) and the extent of autolysis. The most suitable quantity of enzymes can best be determined by experiment, but it would normally be less than 0.5 gms. per 100 ml. of suspension. If necessary the enzyme may be dispersed with a small quantity of wetting agent.

It is found that the enzymes also largely remove any "musty" or "mouldy" smells although the reason for this is not understood. As indicated above such smells are normally associated with spores, and although the presence of spores is avoided as far as possible it is found in practice that a small quantity of spores is usually present.

Lysis is preferably allowed to continue for a period of between about six hours and about seventy two hours. Throughout this period the mixture is preferably maintained at a temperature of not less than about 40° C, but not more than about 60° C as the enzymes are normally rendered ineffective above that temperature.

With certain moulds sporulation tends to occur during lysis. To reduce this tendency the mixture is preferably kept apart from air, either by being confined in a closed vessel which is filled with the mixture or by being maintained in an inert gas such as carbon dioxide.

In preferred methods the extract is separated from the cell debris, undigested cell material and any other gross solids by a centrifuging process. In addition any remaining "musty" or "mouldy" smell can be removed at this stage by the addition of activated charcoal and subsequent filtration of the extract. The residue may be washed with water, and as previously mentioned the washings may be used to make a further suspension of mould for treatment.

The extract is preferably pasteurised or sterilised to prevent spoiling, for it will be appreciated that it constitutes an excellent nutrient for micro-organisms.

The extract may be used without further treatment as a nutrient for the growth of micro-organisms. The extracts often contain a high proportion of amino acids, particularly lysine, which make them particularly valuable for this purpose. Where the growth medium is a carbohydrate such as molasses the amount of extract employed is preferably such as to give a carbon to nitrogen ratio of approximately ten to one. The extract may well be used in place of ammonium sulphate which has been used hitherto.

Where the extract is to be used as a nitrogen and vitamin supplement or as a flavour additive for foodstuffs, further treatment is preferably carried out.

Nucleotides, such as monosodium glutamate, may be added.

Sodium chloride is preferably added to provide microbiological stabilisation and potentiation of the flavour.

The colour of the extract may be adjusted by the addition of colouring agents such as caramel.

The pH of the extract may also be adjusted, for it has been found, rather surprisingly that the pH of the extract has a profound effect on the taste of the extract. A typical extract made in accordance with the invention may be relatively acid and have a pH of as little as 3.6. Such an extract is naturally rather tart to taste but is also found to have a generally "vegetable" flavour. As the pH is increased the flavour becomes progressively more "meaty" until a pH of 6.0 or 7.0 is reached. To increase the acidity it is preferred to add hydrochloric acid or acetic acid or citric acid or a mixture of two or all of them. To decrease the acidity it is preferred to add sodium hydroxide.

Finally the extract may be evaporated under vacuum or can be spray-dried, pan-dried or freeze-dried to produce a liquid or dry final product as desired.

Although it is normally desirable to add sodium chloride before the extract is used, this can be added by the user. The extract may therefore have had no salt, or less than the desired quantity of salt, added to it when it is prepared for sale or use.

The extracts described above may be used much in the way that yeast extracts are used at present.

The following examples illustrate the invention. In these examples the term percentage extract is used to denote the ratio of the dry weight of the extract, before any additives have been added to it, to the dry weight of mould from which that extract was prepared, expressed as a percentage. In the examples certain substances are added in quantities expressed in terms of % w/v; this is used to denote the number of grams of substance per 100 ml. of liquid or mixture to which it is added.

Example 1

An extract was prepared from Penicillium notatum. The mould had been grown by submerged aerated culture and was substantially spore-free. The mycelium was relatively thick-walled, and being septate tended to break at the septa when homogenised. These factors may account for the relatively low yield of the extract.

3.2 kg. of wet mycelium was suspended in distilled water to give 10% w/v solids. The dry weight was determined from a small sample of wet mycelium and was found to be 600 gms. The suspension was homogenised in a Silverson mixer-blender for a period of between 5 and 15 minutes, the cells being sheared and mixed by this treatment. The pH was adjusted to 4.5 by the addition of citric acid. Sufficient bovine pepsin was added to give a concentration of 0.25% w/v. Before being added it was dispersed in a small amount of a 0.05% solution of a wetting agent called Tween 80.

The mixture was placed in a large aspirator immersed in a water bath at 50° to 55°C and the suspension was stirred continuously for three days while lysis occurred.

The undigested solid residue was removed from the resultant extract by centrifuging at 8000 r.p.m. for 10 minutes. Further clarification of the extract was achieved by filtering it through a bed of kieselguhr which had previously been washed with a fair quantity of water to remove any odour.

The filtrate was concentrated in a climbing film evaporator at a temperature not exceeding 45°C and the extract was recycled until it attained a viscous consistency. The wet weight of the concentrate was 225 gms.

The total solids content of the extract was determined by measuring the specific gravity of a sample of the concentrated solution. This was about 75 gms so that the percentage extract was 12.5%.

The concentrated solution was divided into two portions. One portion was evaporated on a rotary film evaporator in a water bath at 60°C under reduced pressure. The solution was concentrated until the resultant extract had a solids content of approximately 75%. This extract was suitable for use as a nutrient for fermentation processes.

The pH of the other portion was adjusted to 5.9 by the addition of a normal solution of sodium hydroxide. Sufficient salt was added to give a final salt concentration of 10% by weight. As before, liquid was evaporated on a rotary film evaporator in a water bath at 60°C under reduced pressure. Again, the solution was concentrated until the

resultant extract had a solids content of approximately 75%. This extract was suitable for use as a supplement or flavour additive for foodstuffs. The taste was found to be somewhat sour, but this was probably due to the use of citric acid.

Example 2.

An extract was prepared from Aspergillus oryzae in much the same manner as that used in Example 1, but concentrated hydrochloric acid was used instead of citric acid. The mycelium was thin-walled and tended to grow in pellets. It was disrupted to a large extent. 2.3 kgs. of wet mycelium were employed, the dry weight being 470 gms. The wet weight of the extract was about 200 gms. and the dry weight about 70 gms. The percentage extract was therefore about 15%. The extract had a very favourable flavour.

Example 3.

An extract was prepared from Giberella fujikuroi which is a waste product from the commercial manufacture of giberellins. The method was much the same as that used in Example 1 but concentrated hydrochloric acid was used instead of citric acid. The hyphae of the mycelium were extremely fine and fragmented easily. 4.7 kgs. of wet mycelium were employed, the dry weight being 700 gms. The wet weight of the extract was 280 gms and the dry weight was 120 gms. The percentage extract was therefore 17.1%. The extract had a pale golden-yellow colour and a very favourable flavour.

Example 4.

An extract was prepared from Aspergillus luchuensis in much the same manner as that used in Example 1, but concentrated hydrochloric acid was used instead of citric acid. The mycelium was thin-walled and easily disrupted, but on homogenisation a very viscous suspension was obtained which was difficult to stir. This probably accounted for the low yield. There was also a tendency for the mycelium to sporulate profusely during lysis. 4.1 kgs. of wet mycelium were employed, the dry weight being 800 gms. The wet weight of the extract was 500 gms. and the dry weight was 110 gms. The percentage extract was therefore 13.8%. The extract had a pleasant flavour but tended to be granular in consistency.

Example 5.

An extract was prepared from Penicillium chrysogenum in much the same manner as that used in Example 1, but concentrated hydrochloric acid was used instead of citric acid. The mycelium was thick-walled and was particularly resistant to homogenisation. This probably accounted for the low yield. 3.8 kgs. of wet mycelium were employed, the dry weight being 600 gms. The wet weight of the extract was 620 gms. and the dry weight was 62 gms. The percentage extract was therefore 10%. The extract had a weaker flavour than those produced by the methods described in Examples 1 to 4.

Example 6.

An extract was prepared from Aspergillus niger. The mycelium was ring-dried and was ground to a powder. The powder was sieved and only particles below a size of 500 microns were used. The powder was suspended in water at a rate of 5% w/v and the pH was adjusted to 3.0 by the addition of a mixture of glacial acetic acid and concentrated hydrochloric acid in a ratio of 1 to 1 v/v. The temperature was raised to 40°C and bovine pepsin was added at a rate of 0.1% w/v. The mixture was then stirred for 24 hours. The resultant mixture was centrifuged at 8000 r.p.m. for 10 minutes and the residue washed in a quantity of water half the volume of the liquid previously extracted, and the residue washings were added to the product.

The extract was clarified by being filtered through a bed of kieselguhr which had previously been well washed with water. The extract was then concentrated in vacuo at a temperature not exceeding 45°C. The extract could be used in this form or could be further treated in the manner outlined in Example 1 to give it a suitable flavour. The percentage extract was 46%.

Similar experiments were carried out with a view to assessing the effects of washing the solids separated by centrifuging and the effects of changes in the suspension rates. The results of these comparative experiments are as follows:-

Table I

Suspension rates	Percentage extract including those from washings of solids	Percentage extract not including those from washings of solids
5% w/v	46%	37%
10% w/v	32%	21%
15% w/v	23%	16%
20% w/v	19%	11%

The differences between the values in the columns are the percentage extract retained by the solids when they have been centrifuged and which can be separated from the solids by washing.

Although the maximum yield is obtained with a low suspension rate it may well be preferable in practice to operate at a higher suspension rate as use of a low suspension rate leads to the need for evaporating a much larger quantity of water than must be evaporated when using a higher rate. Thus a convenient rate is often between 15% w/v and 20% w/v.

In order further to reduce the quantity of water to be evaporated when the residue washings are to be used it is convenient to use the washings to form further suspension of powdered dry mycelium as described above.

Example 7.

An extract was prepared from Aspergillus niger. The mycelium was air-dried at approximately 60°C for 18 hours and was then ground and sieved as in Example 6. The remainder of the process was the same as that described in Example 6 and comparative experiments were carried out to assess the variation of percentage extract with time while using a 5% w/v suspension rate and the effect of pepsin used at 0.1% w/v and papain used at the same rate. The results are as follows:-

Table II

	Percentage extract in:			Equivalent Protein
	6 hrs.	24 hrs.	48 hrs.	
With pepsin	24.5%	46.0%	47.0%	47.4%
With papain	52.5%	52.5%	51.5%	32.3%
Without enzyme	21.0%	28.5%	34.5%	39.2%

The equivalent protein is the percentage of nitrogen in the extract solids, as determined by the Kjeldahl method, multiplied by 6.25 and is thus a measure of the protein content. It will be observed that although the percentage extract obtained when using pepsin is higher than that obtained when using papain the equivalent protein is lower. In general, therefore, pepsin is to be preferred to papain.

Example 8.

Further extracts were prepared from Aspergillus niger by the method used in Example 6, but using a 10% w/v suspension rate. As before pepsin was used at a rate of 0.1% w/v. The suspensions were maintained for 24 hours at different temperatures and the results were as follows:-

Table III

	Temperature		
	40°C	50°C	60°C
Percentage Extract	28.0%	32.5%	38.5%

Although the percentage extracts rose with increasing temperature it is impracticable to raise the temperature much above 60°C as the enzymes rapidly become denatured.

1. A process for making an extract from a mould which is at least substantially free from spores, which process includes the steps of causing lysis of the constituent cells of the mould and separating the resultant extract from the cell debris, any undigested cell material and any other gross solids.

2. An extract for use either as a food-additive or as a nutrient for micro-organisms, comprising material which remains after the lysis of the constituent cells of a mould which is substantially free from spores, and the removal of the cell debris, any undigested cell material and any other gross solids.

APPENDIX II

GROWING MOULDS ON MOULD EXTRACTS

Provisional specification of U.K.Patent Application No. 44783/71 by Greenshields Inventions Co.Ltd.

Inventors: R.N.Greenshields and R. Alagaratnam.

Provisional specification filed on 25th September 1971.

This invention relates to the growing of micro-organisms, and in particular to the growing of moulds for the manufacture of mould extracts.

Dr. R.N.Greenshields, one of the inventors of the present invention, previously made an invention (hereinafter called the earlier invention) relating to the production of extracts from cells, and filed a patent application for that invention (hereinafter called the earlier application) which was given the number 44714/69.

The earlier invention provides a process for making an extract from a mould which is at least substantially free from spores, which process includes the steps of causing lysis of the constituent cells of the mould and separating the resultant extract from the cell debris, any undigested cell material and any other gross solids. The earlier invention also provides an extract made by that process. Such an extract will hereinafter be referred to as a mould extract.

In the earlier application moulds are defined as fungi which are characterised by their growth in the vegetative state of filamentous mycelia, and it is remarked that nearly all moulds produce well-defined sporophytes on the air-surface which generates spores. It is pointed out that moulds are not restricted to any particular families or classes in the Fungi although certain families may contain more of the characteristic types than others. Further the earlier application states that in general it has been found that moulds in the class Fungi Imperfecti or Deuteromycetes are particularly suitable for use in accordance with the earlier invention. Processes in accordance with the earlier invention may employ a mixture of two or more moulds.

The definition of moulds and the other statements in the last preceding paragraph relating to the earlier invention are also applicable to the present invention.

The complete specification of the earlier application states that mould extracts may be used as nutrients for micro-organisms and that the extracts often contain a high proportion of amino acids, particularly lysine, which make them particularly valuable for this purpose. It also states that the extract may well be used in place of ammonium sulphate which has been used hitherto.

The present invention stems in part from the discovery that there are advantages in growing moulds themselves on or in growth media incorporating mould extracts as nutrients.

From one aspect, therefore, the present invention consists in a method of growing mould characterised in that the nutrient in the growth medium comprises at least one mould extract.

From another aspect the present invention consists in mould made by the method outlined in the last preceding paragraph.

When this method is used it is found that in general the maximum yield of mould is greater than those obtained with similar conventional methods differing from the method of the present invention only in the use of comparable amount conventional nutrients such as yeast extracts or ammonium sulphate. Further, in general, the rate of growth is greater than those obtained with those conventional methods, and the maximum yield is obtained sooner than with those methods.

The moulds are preferably grown on or in media incorporating an extract from a mould of the same kind, that is of the same species and variety, but this is not essential to the working of the present invention. Further, it is to be understood that a mould or more than one mould may be grown or in a medium incorporating the extracts of two or more different kinds of moulds. Yet again the growth medium may contain, in addition to one or more mould extracts, other nutrients such as those previously employed, for example yeast extract and ammonium sulphate.

The moulds obtained by this new method may be used for any appropriate purposes and may for example be used as foodstuffs, for humans or animals, in the form usually referred to as "whole moulds". Alternatively, they may be used as a source of mould extracts formed as by a process in accordance with the earlier invention. Such extracts may be used as supplements for foodstuffs or as nutrients for the growth of yet further moulds or other micro-organisms. It has been observed that the yield of extract is in general greater than that obtained from a similar mould grown with a conventional nutrient. This may be due to an increase in the proportion of the mould available for extraction, or due to the fact that the material in the mould capable of being extracted is in a form enabling it to be extracted more readily than the comparable material can be extracted from a mould grown with a conventional nutrient.

From a further aspect, therefore, the invention provides a mould extract (as hereinbefore defined) made from a mould in accordance with that aspect of the present invention outlined in the last preceding paragraph but three.

Another feature of moulds and mould extracts in accordance with the aforementioned aspects of the present invention, is that their protein content is generally greater than that of comparable moulds and mould extracts previously known. This makes them particularly valuable as foodstuffs and supplements for foodstuffs.

In particular the glutamic acid content of the moulds and mould extracts is generally increased. As this has a "meaty" flavour the moulds and extracts are generally more palatable than the comparable moulds and extracts previously produced. Further, the alanine content is generally lower than normal, and in particular it is generally very markedly lower than is the case when a mould is grown on or in a medium incorporating an inorganic source of nitrogen such as ammonium sulphate. Similarly the glycine content is often rather lower than normal.

From another aspect the present invention consists in a mould such that a mould extract (as hereinbefore defined) made from that mould contains more glutamic acid than alanine.

It has also been discovered that a comparable increase in glutamic acid content can be achieved by growing a mould on any suitable medium (whether incorporating any mould extract or not) to which glutamic acid is added.

The invention thus includes within its scope a process for making a mould of the kind outlined in the last preceding paragraph but one, characterised in that glutamic acid has been added to the growth medium in an appropriate strength.

The ratio of the alanine content to the glutamic acid content can be determined by chromatographic analysis.

An example of a method embodying the invention will now be described with reference to the accompanying drawing which is a graph showing the rate of growth of a mould in media differing fron one another in their nutrients.

The mould concerned, Aspergillus niger, was grown in an aqueous medium in a vessel of elongated shape having its axis vertical. Near the bottom of the vessel was a sintered glass disc, and below the disc was an inlet for air. Air was introduced at a rate such that a volume of air equal to the volume of liquid medium was passed into the vessel each minute. The medium was maintained at 27°C.

The medium comprises 5 gms of sucrose per 100 ml water. Trace materials were also included as follows:-

Sodium phosphate	0.2% w/v
Ferrous sulphate	0.01% w/v
Magnesium sulphate	0.01% w/v
Zinc sulphate	0.01% w/v
Potassium chloride	0.01% w/v

A nutrient containing nitrogen was also added. In one experiment the nutrient was ammonium sulphate, in a second it was a proprietary yeast extract sold under the name "Yeatex", and in a third it was a mould extract of Penicillium chrysogenum. In each instance the amount of nutrient added was such that the ratio of carbon (in the sucrose) to nitrogen (in the nutrient) was ten to one.

At the beginning of each experiment (zero on the time scale) a small quantity of mould spores was introduced into the vessel. Samples were extracted at intervals and the yield of mould was measured in terms of the dry weight of mould in gms. per 100 ml. of medium containing the mould.

The results of the experiments are shown in the accompanying graph. This shows that the maximum yield was greatest and the rate of growth of the mould was greatest when the nutrient consisted of the mould extract. Further, the high yield, with mould extract, was obtained more quickly than with the other nutrients.

When extracts were made from the resulting moulds and were subjected to chromatographic analysis it was found that the glutamic acid content was greater than the alanine content in the case of the mould grown with the mould extract nutrient, but not in the other two cases.

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APPENDIX III

THE PRODUCTION OF FUNGAL BIOMASS IN AEROBIC TOWER FERMENTERS

by R.N.Greenshields, G.G. Morris, B. Daunter,

R. Alagaratnam and F.K.E.Imrie

Paper presented at the First International Mycological Congress, Exeter, September 1971.

THE PRODUCTION OF FUNGAL BIOMASS IN AEROBIC TOWER FERMENTERS

by R.N.Greenshields, G.G. Morris, B. Daunter, R. Alagaratnam and F.K.E.Imrie.

Department of Biological Sciences, The University of Aston in Birmingham, Gosta Green, Birmingham.4.

as presented at the First International Mycological Congress, Exeter, September 1971.

1. The tower or tubular fermenter can be described as an elongated non-mechanically stirred fermenter with an aspect ratio (height to diameter ratio) of at least 6:1 on the tubular section or 10:1 overall through which there is a uni-directional flow of medium or gases[1]. It can be used for batch, semi-continuous and continuous fermentations and can be classified using Herberts' criteria[2] of continuous systems, as a partially closed heterogenous tubular system. This was clearly demonstrated when this type of fermenter and its associated system was successfully used for the continuous production of ale and lager beer on laboratory[3,4], pilot[5] and commercial scales[6,7,8]. Such application including that when applied to the similar fermentations of cider, wine, whiskey-wash and vinegar charging-wort are growth-associated metabolite fermentations involving yeast under anaerobic or partially aerobic conditions. Its application in a fully aerobic situation has obviously been considered. Initial work has indicated that the aerobic conversion of ethanol to acetic acid by acetic acid bacteria in vinegar manufacture is feasible and continuous runs have been obtained on a laboratory scale[8,9,10,11]. Moreover, the production of yeast biomass in a flocculent condition is also possible on batch and semi-continuous systems and have been accomplished on laboratory, pilot and commercial scale[8,12]. This process is also being considered for non-flocculent yeast in a continuous system.

The work now presented here is the extension of this application to the filamentous fungi, particularly for the production of biomass - the production of fungal metabolites has been considered[8] but is the subject of further study and will not be dealt with here. The fermenter design[9] is shown in Diagram I. This does not materially differ from that used for anaerobic fermentations[8] except that (i) a means for aeration is provided at the bottom of the tower, (ii) the expansion chamber and thus the air/fob space is larger, (iii) some means is provided to prevent foaming and (iv) if continuous, the overflow and separator is of a different design.

The experimental fermenter vessels were made of glass, high density polythene, polypropylene or stainless steel and were sterilised both chemically (available chlorine) and by steam (up to 5 p.s.i). The sinters were made of glass, P.T.F.E., sintered stainless steel and occupied the whole fermenter tube cross-section and thus effectively held up the fermentation liquid.[8] For larger vessels a simpler aerator was used made of stainless steel and which sat on the bottom of the fermenter.

Four sizes of fermenter were used:-

(a) Experimental tubes 1 to 5 litre. 1'0" to 4'0" x 3" diameter.

(b) Laboratory scale (small) 10 to 20 litre. 4'0" to 8'0" x 4" diameter

(c) Laboratory scale (large) 50 litre 12'0" x 6" diameter.

and (d) Pilot scale 500 to 1000 litre 15'0" to 25'0" x 1'0" diameter.

2. The fungi were grown in the tower fermenters on two media - one based on a supplemented aqueous extract of carob bean (Ceratonia siliqua) and the other based on supplemented molasses.

Normally appropriate quantities of nitrogen sources were added in the form of inorganic ammonium salts or as glutamic acid to give the required Carbon : Nitrogen ratios.

Some preselection of the fungi was made using necessary criteria to ensure the most economic and suitable biomass production. This involved:

(i) A primary selection based on literature survey using the following criteria:

(i) Protein content

(ii) Protein extract

(iii) Toxicity

(iv) Growth rates

(v) Calorific value

(vi) CHO conversion efficiency

followed by:

(ii) A primary screening using race plates from which growth rates were ascertained,

then (iii) A secondary selection using experimental tubular fermenters.

Out of some forty fungi selected, the Fungi imperfecti appeared to grow best. - Table I.

The Basidiomycetes were slower growing and although several were tried the results were not promising. It was possible that the nutritional requirements were not satisfied by the media used. The results are shown in Table 2 together with those typical of the Phycomycetes tested. The phycomycetes grew easily, but biomass yields were too low for further consideration of economic biomass production.

3. It was decided to make a detailed study of the batch growth of Aspergillus niger in various sizes of tower fermenter to ascertain its growth kinetics in terms of (a) parameters which prescribe the fermenter, and (b) parameters which prescribe the medium in an attempt to establish the optimum for economic biomass production in terms of growth rate, yield, protein content and carbohydrate conversion efficiency.

(a) Parameters which prescribe the Fermenter.

(i) Aeration rate

Preliminary experiments in laboratory scale towers (10 litre) were performed to determine optimum conditions of aeration and agitation relative to the volume of air passed through the fermenter. In addition the optimum height to diameter ratio was calculated from this data. In tower fermenters oxygen transfer and agitation is caused by the air itself. Within the limits of the fermenters used, oxygen transfer efficiency and agitation increased linearly with aeration rate. Oxygen transfer rates were determined using the sulphite oxidation method of Cooper and co-workers which measured the maximum rate of solution when dissolved oxygen concentration is very nearly zero.

Graph 1 showed that an aeration rate of between 1.5 to 2 volumes of air per volume of liquid are near the optimum for oxygen transfer in this fermenter.

It should be noted that in laboratory scale fermenters it is impractical to use this optimum aeration rate and values of just below 1 vol/vol/min are generally used.

(ii) Aspect ratio

The aspect ratio of the laboratory scale tower (10 litre, 9 cm. diamete was changed by varying the height of the liquid and hence its volume, whilst keeping the aeration rate per volume of liquid constant. Graph 2 shows that using these conditions, it can be seen that an aspect ratio of 16:1 appears to be optimal for a tower diameter of approximately 9.0 cm.

(iii) Foaming

It has been observed that during actual fermentation runs the addition of an antifoam agent had a deleterious effect on the dissolved oxygen concentration within the fermenter[11]. It caused coalescence of the air bubbles making mass transfer of oxygen into solution far less effective. The 'antifoam effect' was greater with greater mould concentrations, a stage being reached where its effect was irreversible. For this reason, a mechanical foam breaker was fitted into the expansion chamber of the fermenter, obviating the use of antifoam. This resulted in a 75%

increase in the dry weight.

(b) Parameters which Prescribe the Medium

Under submerged conditions the morphology of A.niger varies from diffuse growth through a range of pellet sizes up to several millimetres in diameter[14]. Control of a particular morphology was found to be advantageous. The tower fermenter facilitated this control, unlike a stirred fermenter, it presents relatively little shear force to disrupt the colony form. The morphological form of the organism used was selected by calculating (1) Respiratory demands and requirements; (2) Viscosity of the fermentation broth and (3) Oxygen transfer into the fermentation broth.

The mould morphology within the tower fermenter is dictated largely by the form and size of the inoculum used, secondly by the degree of agitation a few hours after seeding the fermenter and thirdly by the method of preparation of the inoculum.

Graph 3 shows the relationship between the size of pellet and the spore inoculum used.

The respiratory demands and requirements of the organism are at present being studied. It must be ascertained whether the oxygen is breaking down carbohydrate to be channelled into biomass production alone or whether there is an accumulation of metabolites. It is suggested therefore that a particular morphology may be optimal for both oxygen consumption and carbohydrate utilisation. Graph 4 shows the decline in oxygen uptake per gram of mould in relation to the size of the colonies. By computing the maximum growth rates together with oxygen consumption for the organism an optimum figure for pellet size may be calculated for particular circumstances and particular fermentation vessels.

Experiments concerning the viscosity of a fermentation broth have shown a relationship between the degree of viscosity and the size of pellet formed. It can be seen from Graph 5 that there will be less energy required to agitate a broth containing descrete pellets as there is for a diffuse mycelial culture. It is also true to say that a

viscous broth causes a greater tendency towards coalescence of air bubbles, thus creating a situation where it might be possible to aerate a broth containing pellets more efficiently than it is to aerate a broth with filamentous organism.

The optimum media conditions and constituents for the production of cell biomass and protein content were determined experimentally for A. niger, the basis of the media being beet molasses which was supplemented where necessary by ammonium sulphate as a nitrogen source and sodium dihydrogen phosphate as the source of phosphate.

(i) Carbohydrate concentration:

It was found that A. niger exhibited a wide tolerance to sugar concentration. Graph 6 shows that there is no marked change in growth rate between 5 and 12 g%. The apparent optimum lay between 7 and 8 g% However, due solely to economic consideration a slightly lower concentration might be advantageous when considering water and effluent factors. The protein concentration of the organism did not vary significantly over this optimum range of carbohydrate concentration.

(ii) Carbon-Nitrogen ratios:

Supplementation of a 5 g% carbohydrate media by various quantities of ammonium sulphate was made in order to vary the C:N ratio. Graph 7 illustrates the change in growth rates and protein contents during these experiments. The optimum growth rate lay between 7:1 and 13:1 whilst the optimum protein content is at 6:1. At higher C:N ratios however a great deal of fat was seen to be deposited in the mycelium.

(iii) Temperature:

The range of temperature studied was 20 to 40°C. Graph 8 shows that there is an optimum value for growth rate between 27°C and 33°C for the A. niger. Of economic importance is the fact that at elevated temperatures the fungal metabolism is increased, giving rise to a greater utilisation of carbohydrate. However, this may not necessarily be channelled into biomass production.

This study gives an indication of the behaviour of A.niger in a tower fermenter but can only provide a useful guide to the behaviour of any other filamentous fungus in such a vessel. It should also be pointed out that the medium is also unique in this respect and the use of molasses medium for this study can only give a guide to other organism on other media.

Scale. As previously indicated aerobic tower fermenters were constructed from laboratory up to pilot scale. Preliminary experiments have shown that the findings in the 5 litre laboratory scale tower fermenters were applicable on a 10x scale-up in fermenter size (i.e. volume) to 50 litre and again on a further 10 or 20x scale-up in fermenter size to 500 and 1000 litre. Moreover the design of such fermenter vessels could be simplified enabling cheap polypropylene fermenters to be used which gave high concentrations of mould biomass economically. If, therefore, fungal biomass is required for whatever purposes, food, protein, biochemicals or enzymes, in developing countries, then a cheap fermenter is required. The tower fermenter is such a fermenter because of its simplicity of design and construction, its advantageous kinetics and its flexibility.

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DIAGRAM 1

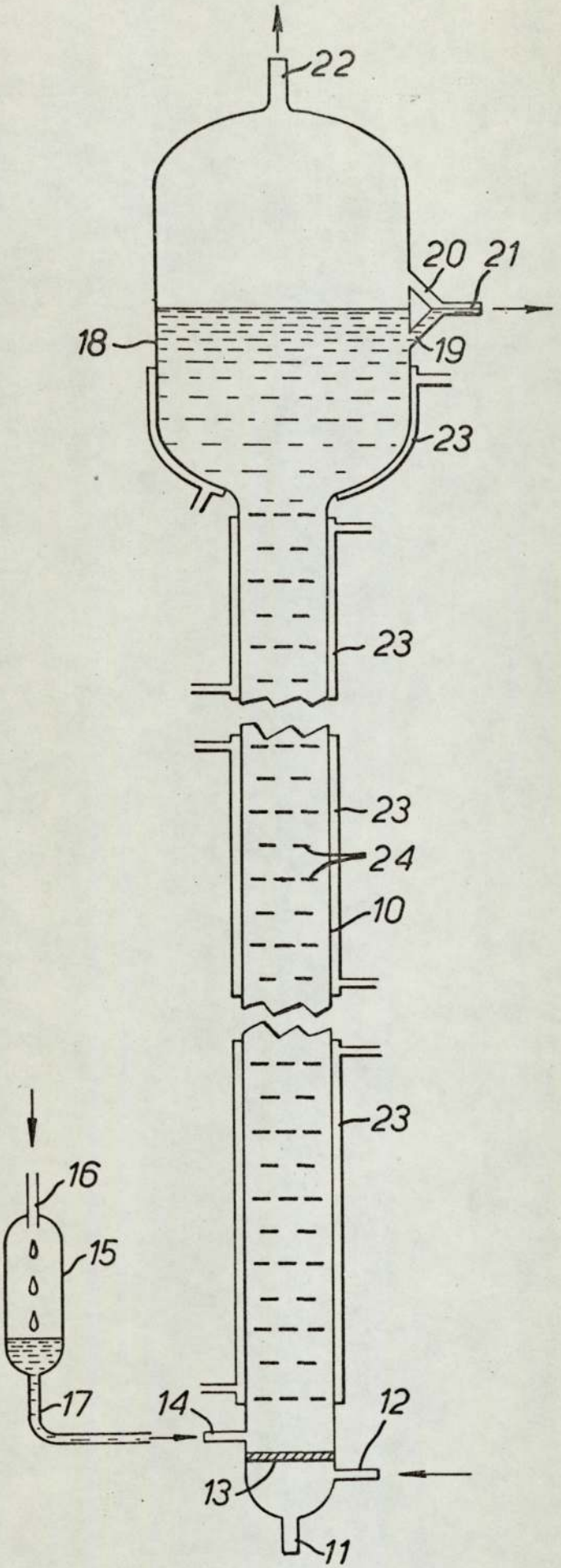


TABLE 1 - GROWTH OF FUNGI IMPERFECTI IN TOWER FERMENTERS

Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
1. *Aspergillus luchuensis*	(RA)	Molasses	50	112	1.62	42.7
2. *Aspergillus niger*	(RA)	Molasses	50	90	1.20	37
3. *Aspergillus oryzae*	(RA)	Molasses	50	120	0.87	34.0
4. *Aspergillus sydowi*	(RA)	Molasses	5	40	0.75	43.5
5. *Cladosporium cladosporoides*	(BD)	Carob	1	42	1.0	30 - 36
6. *Cladosporium cladosporoides*	(RA)	Molasses	5	30	0.72	36.5
7. *Cochliobolus sativus*	(BD)	Carob	1	64.8	0.51	35.35
8. *Fusarium oxysporum*	(RA)	Molasses	5	42	1.29	52.1
9. *Heterocephalum aurantiacum*	(RA)	Molasses	5	44	0.61	38.5
10. *Mycogone spp.*	(BD)	Molasses	1	41.9	0.70	45.9
11. *Penicillium chrysogenum*	(RA)	Molasses	50	72	1.2	36.0
12. *Penicillium notatum*	(RA)	Molasses	50	96	1.2	39.2
13. *Spicaria elegans*	(BD)	Carob	1	40	1.0	30

TABLE 2 - GROWTH OF BASIDIOMYCETES IN TOWER FERMENTERS

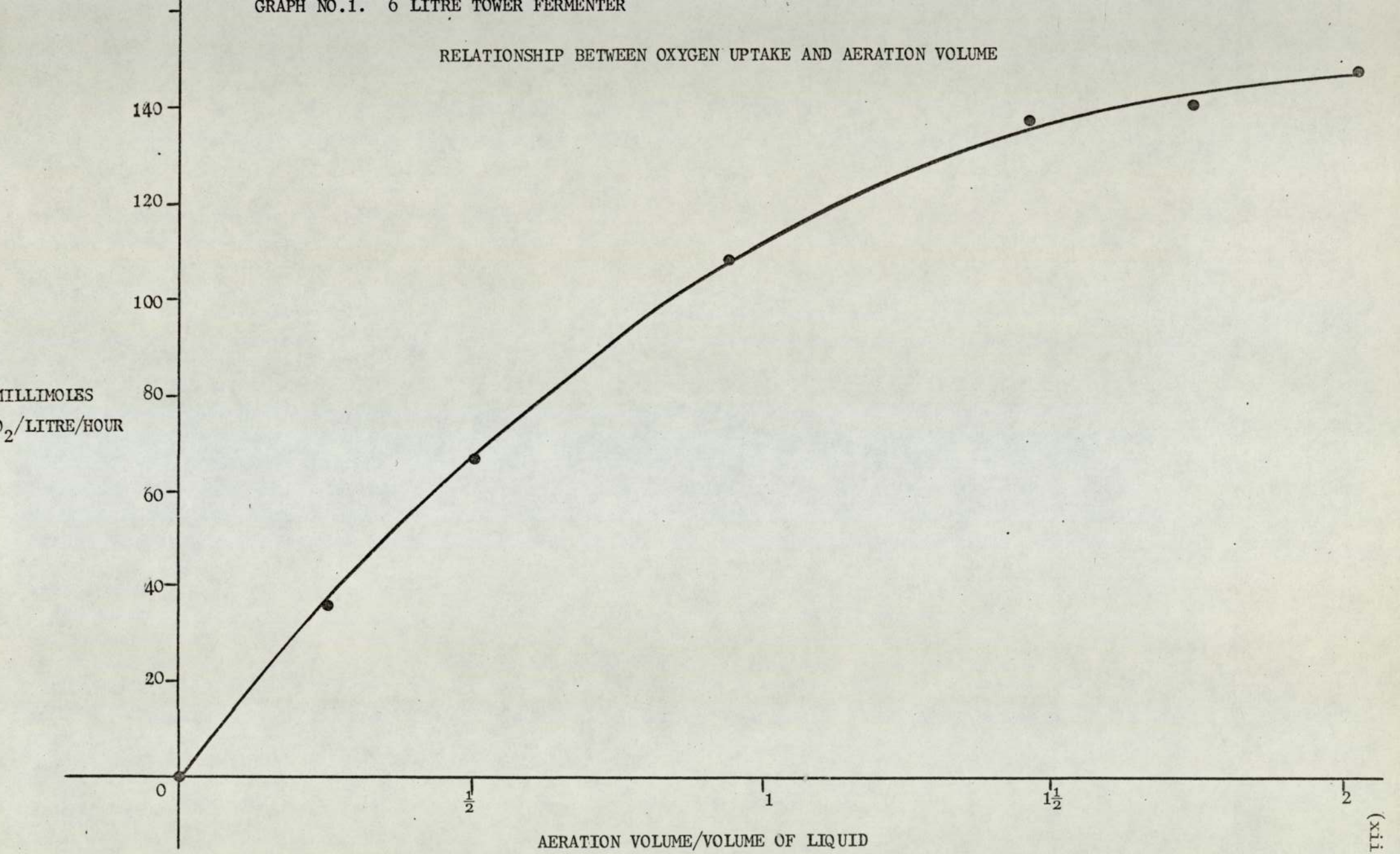
	Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
1.	*Lenzites trabea*	(RA)	Molasses	2	96	0.67	53.2
2.	*Polyporus tulipferus*	(BD)	Carob	1	121	0.58	44.01
3.	*Poria latemarginata*	(BD)	Carob	1	121	0.55	51.75

GROWTH OF PHYCOMYCETES IN TOWER FERMENTERS

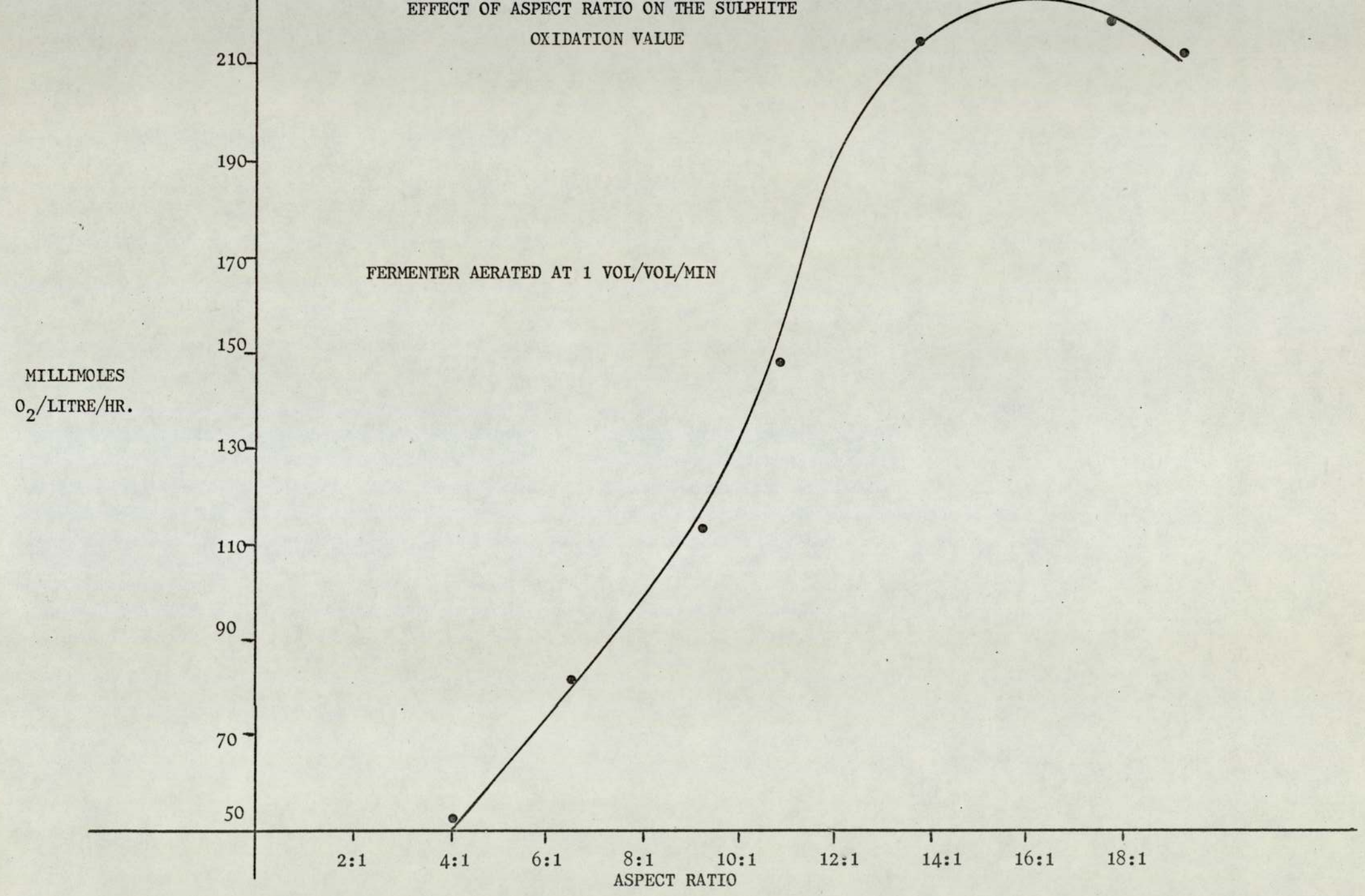
	Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
1.	*Mucor racemosus*	(RA)	Molasses	50	90	0.94	43.4
2.	*Rhizopus oligosporus*	(RA)	Molasses	50	76	0.65	39.2
3.	*Rhizopus oryzae*	(RA)	Molasses	5	42	0.72	38.6

GRAPH NO.1. 6 LITRE TOWER FERMENTER

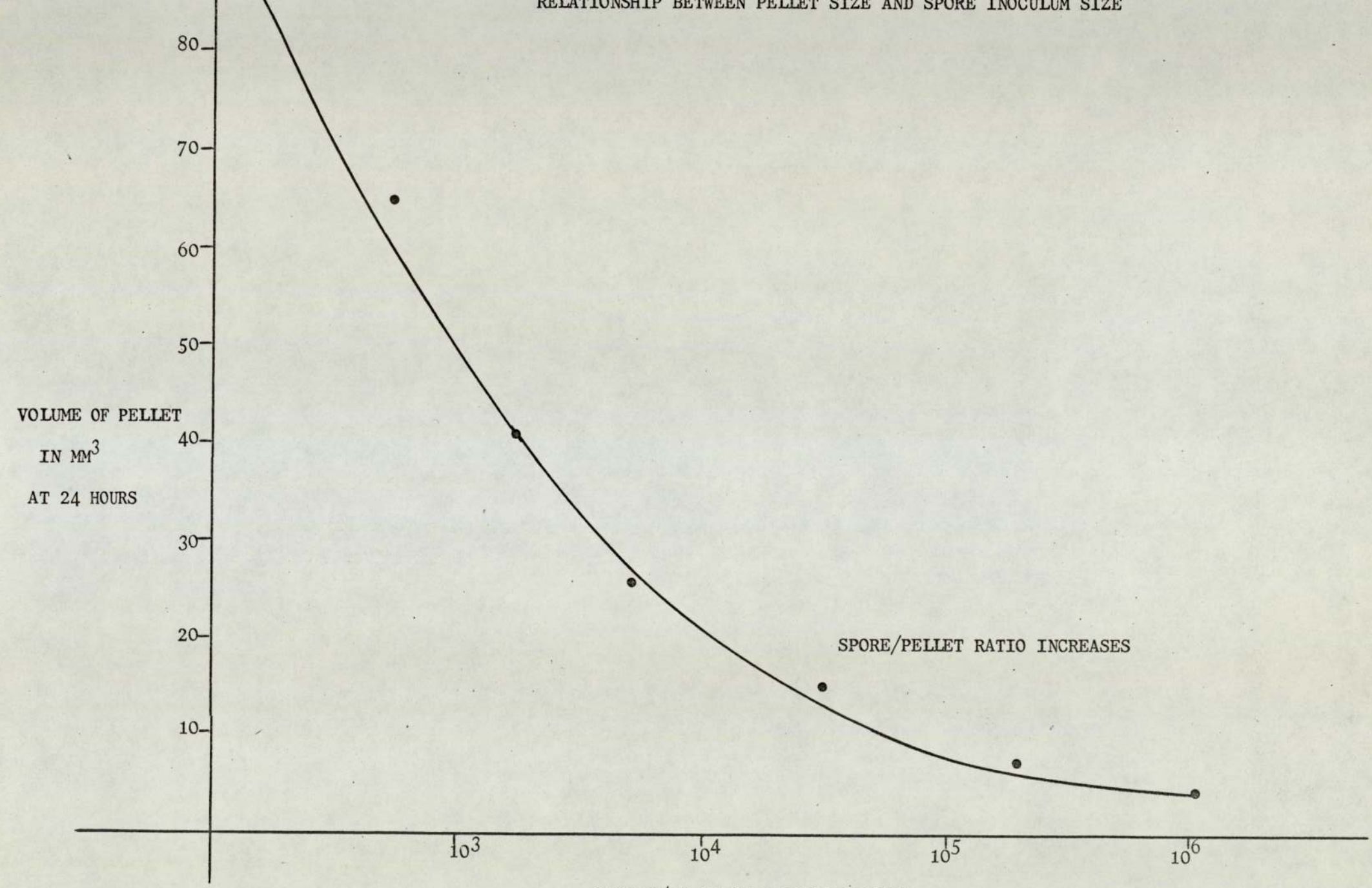
RELATIONSHIP BETWEEN OXYGEN UPTAKE AND AERATION VOLUME



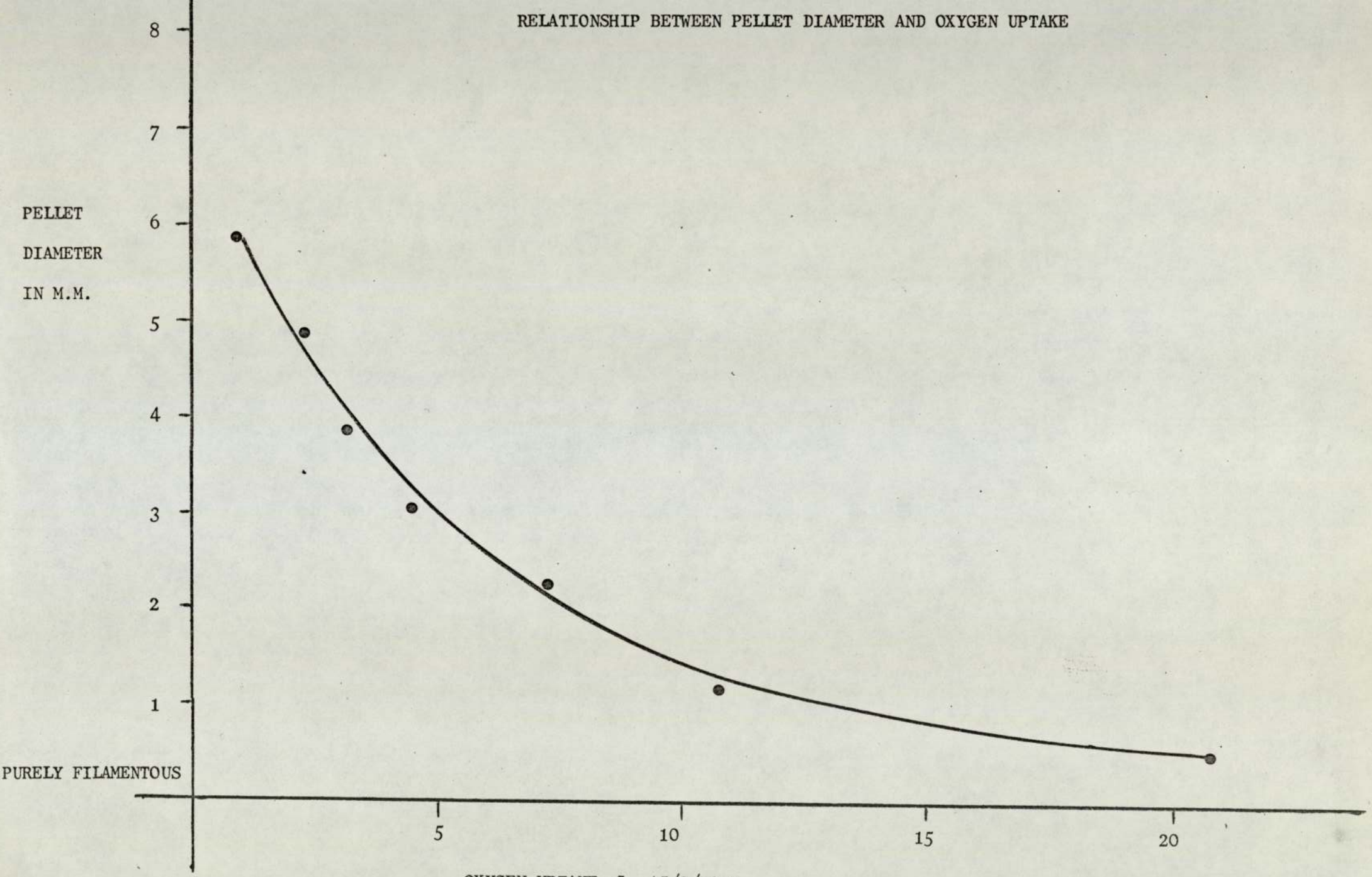
EFFECT OF ASPECT RATIO ON THE SULPHITE OXIDATION VALUE



RELATIONSHIP BETWEEN PELLET SIZE AND SPORE INOCULUM SIZE

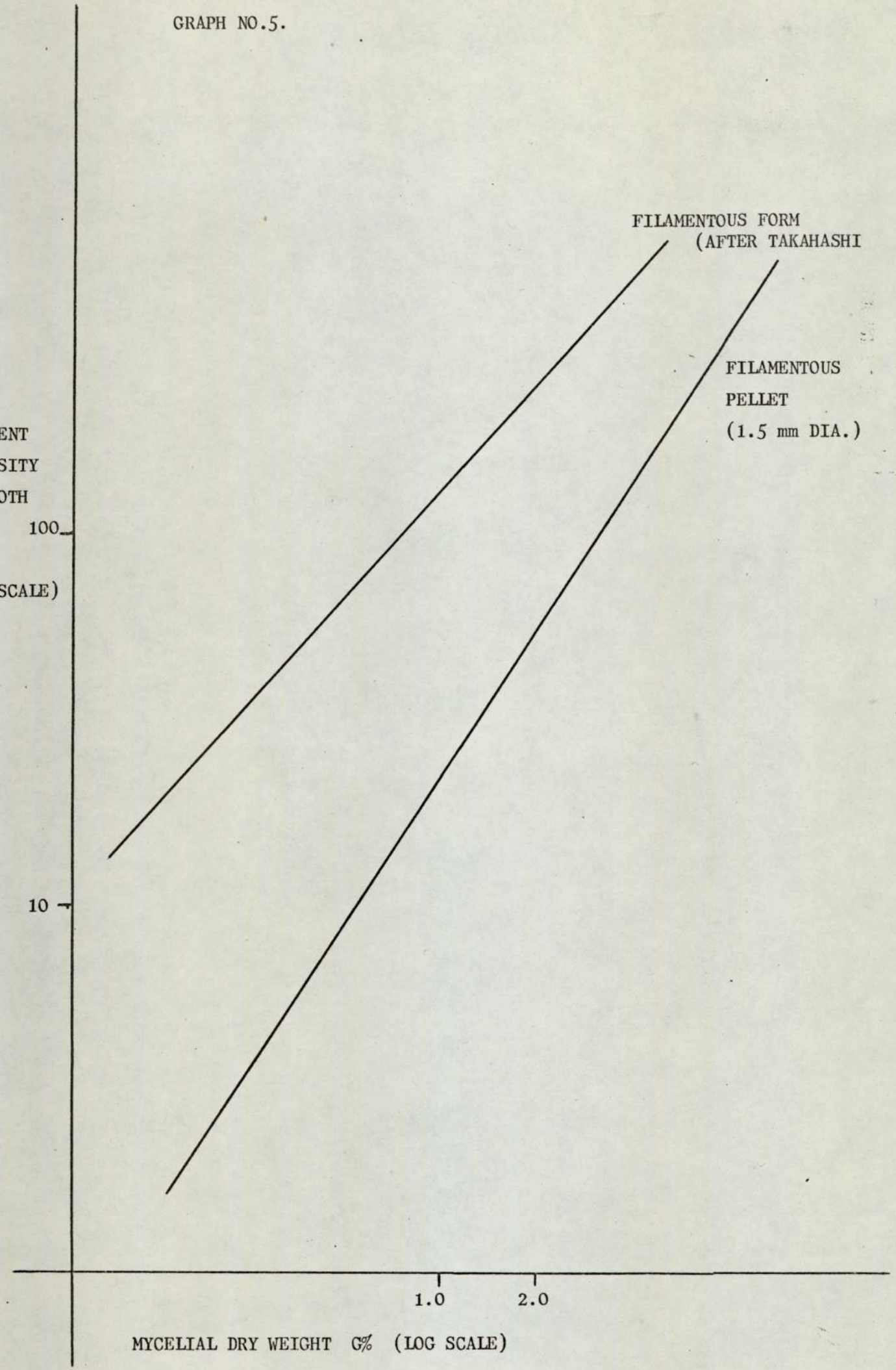


RELATIONSHIP BETWEEN PELLET DIAMETER AND OXYGEN UPTAKE



(xv)

GRAPH NO.5.


FILAMENTOUS FORM
(AFTER TAKAHASHI
FILAMENTOUS
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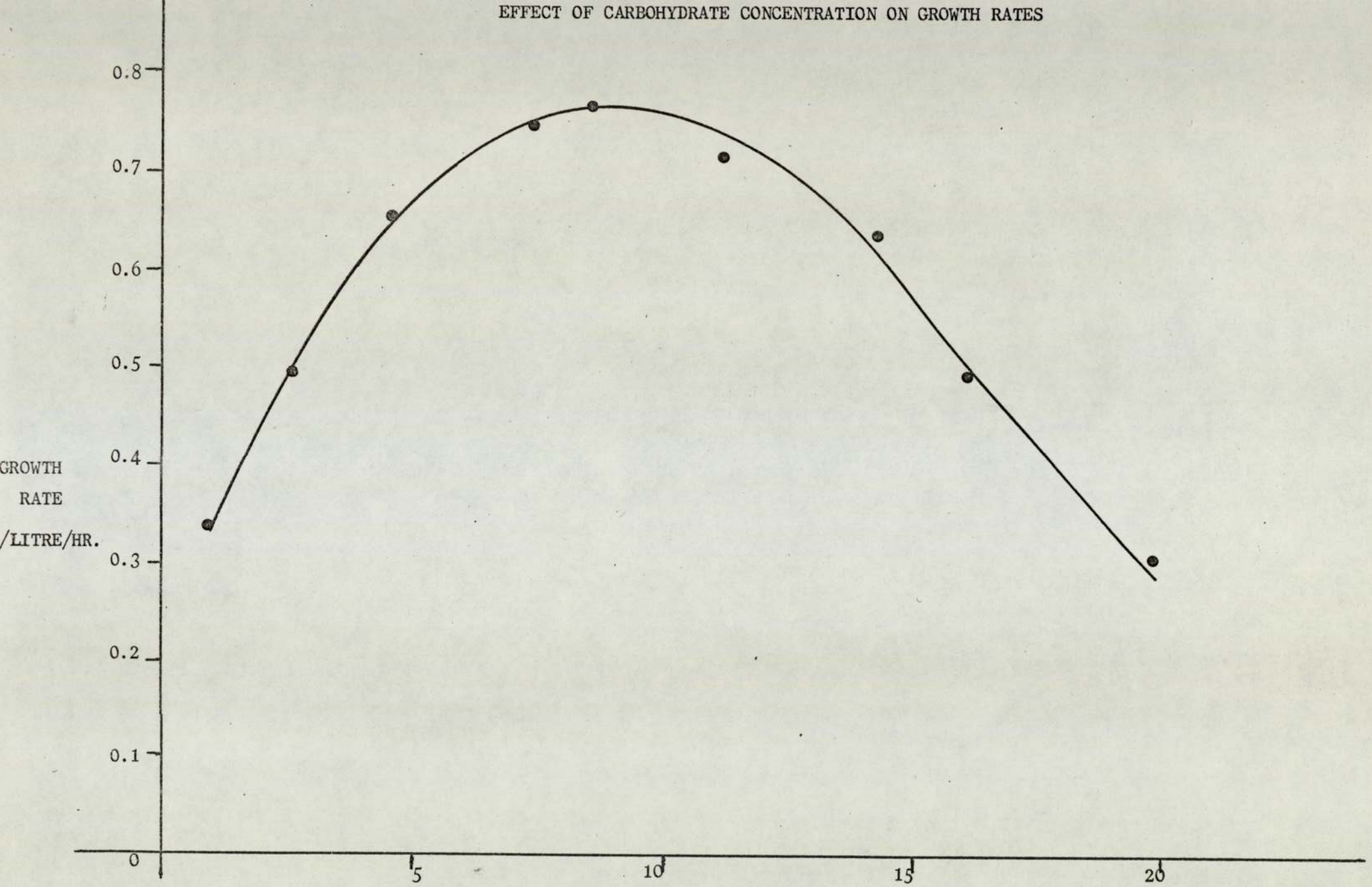
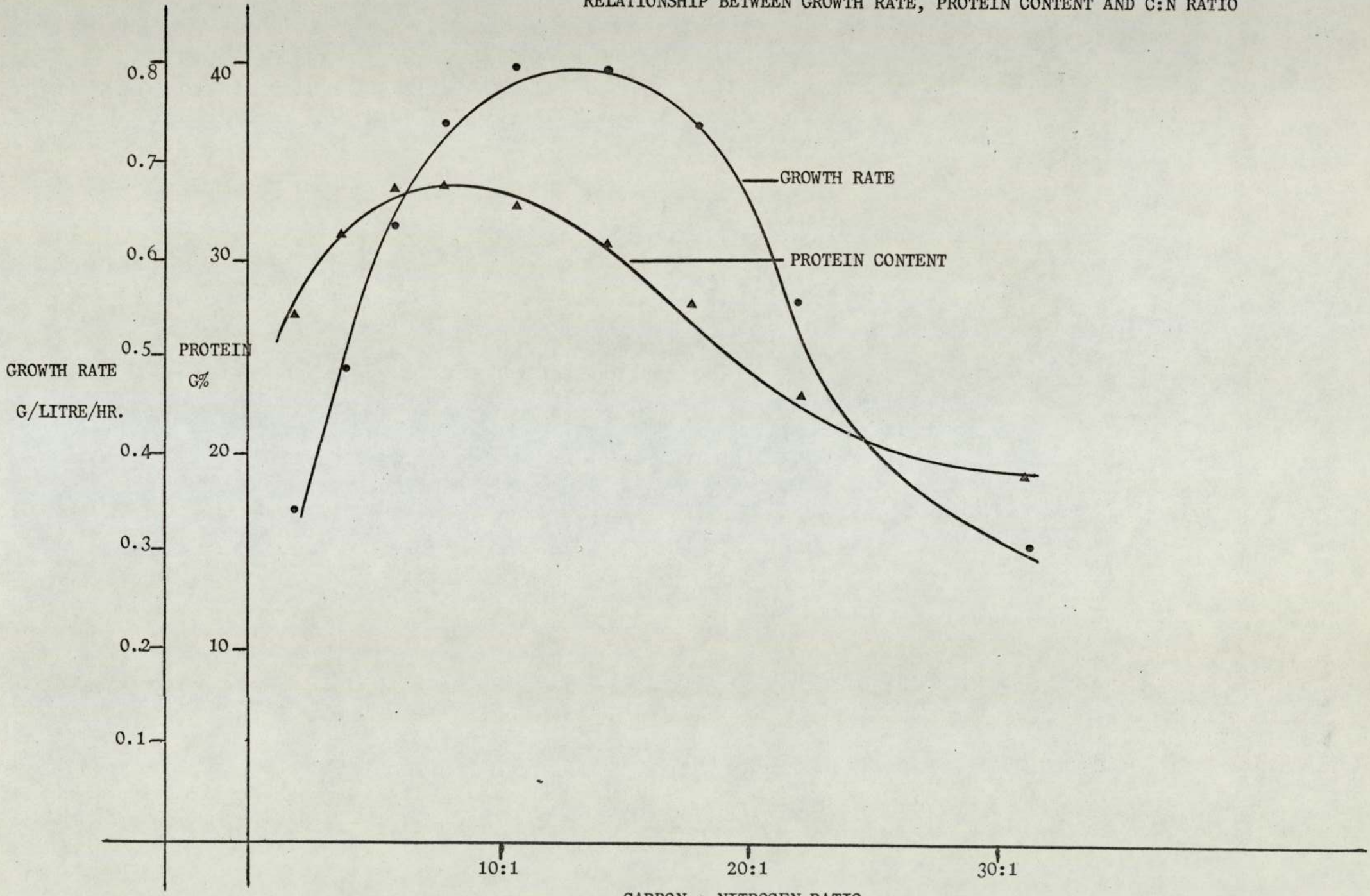
ENT
SITY
OTH
100
SCALE)
10
1.0
2.0
MYCELIAL DRY WEIGHT G% (LOG SCALE)

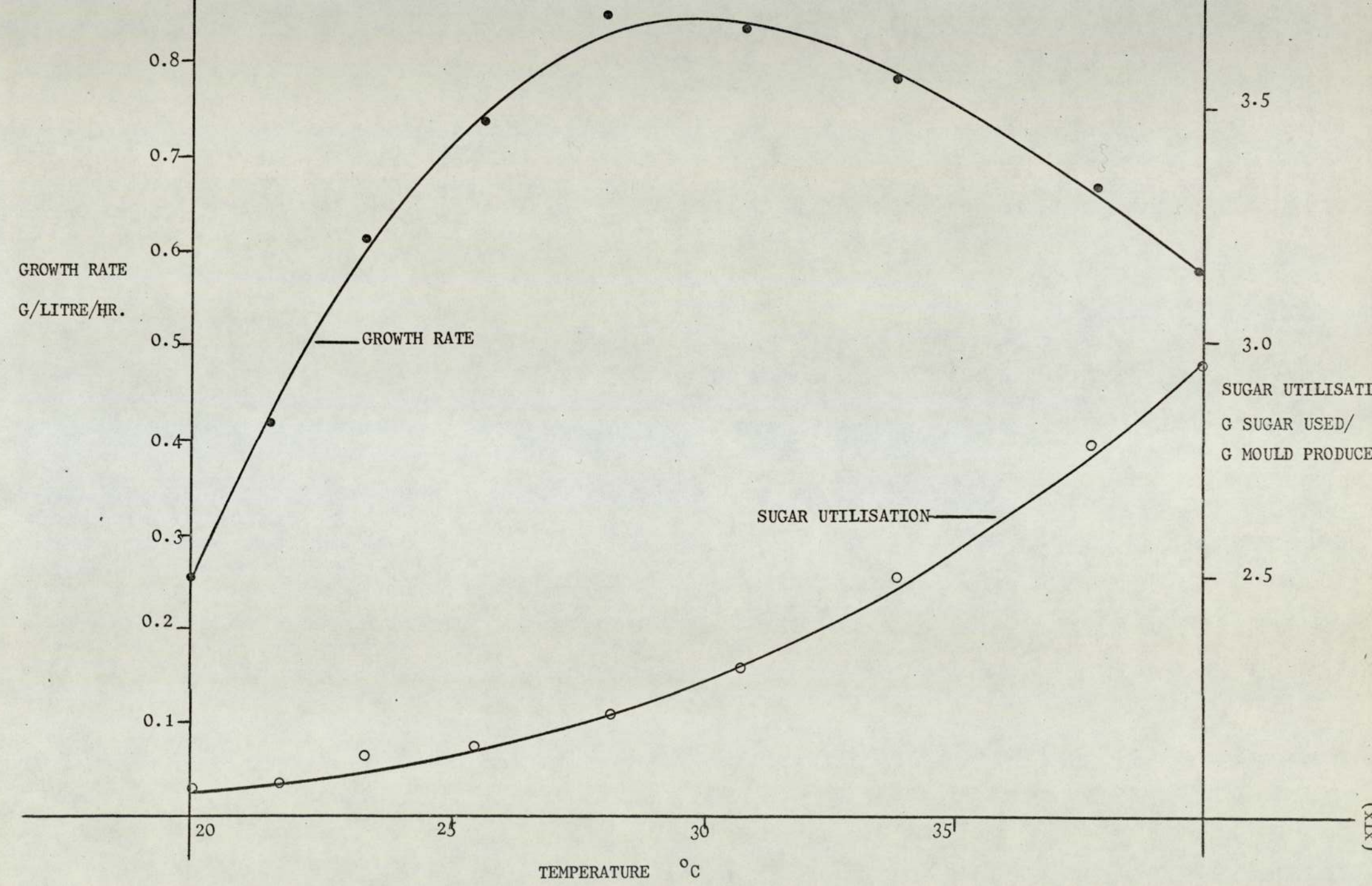
EFFECT OF CARBOHYDRATE CONCENTRATION ON GROWTH RATES



RELATIONSHIP BETWEEN GROWTH RATE, PROTEIN CONTENT AND C:N RATIO



(XVII.1)


0.8
0.7
0.6
0.5
0.4
0.3
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0.1
GROWTH RATE
G/LITRE/HR.
GROWTH RATE
3.5
3.0
2.5
SUGAR UTILISATI
G SUGAR USED/
G MOULD PRODUCE
SUGAR UTILISATION
20
25
30
35
TEMPERATURE °C

APPENDIX IV

Abstract of paper presented at the First International Mycological Congress, Exeter, September 1971.

THE PRODUCTION OF FUNGAL BIOMASS IN AEROBIC TOWER FERMENTERS

by R.N.Greenshields, G.G.Morris, B. Daunter, R. Alagaratnam and F.K.E.Imrie

The tower fermenter has been successfully used for partially aerobic fermentation on laboratory, pilot and commercial scales, for the continuous production of beer. Its application in a fully aerobic situation has been considered for bacteria and yeast. This work represents the extension of this application to the fungi.

Various fungi were grown in aerobic tower fermenters on two media (supplemented molasses medium and a supplemented aqueous extract of carob bean (Ceratonia siliqua) medium). Some preselection of the fungi was made using the necessary criteria to ensure the most economic and suitable biomass production. Out of some forty fungi tested, the Fungi Imperfecti appeared to grow best (Aspergillus niger, A.luchuensis, Penicillium notatum, Mycogone sp., Spicaria elegans, Cladosporium cladosporoides), whilst of the lower fungi tested Rhizopus oligosporus and Mucor racemosus grew easily but yields of biomass were low. Few Basidiomycetes could be grown and of these Poria latemarginata and Polyporus tulipferus were variable and the biomass yields low.

A detailed study has been made of the batch growth of an A.niger in aerobic tower fermenters to determine its growth kinetics in terms of parameters which prescribe the medium and parameters which prescribe the fermenter. The optimum of these parameters was established for economic biomass production in terms of growth rate, yield, protein content and carbohydrate conversion efficiency. Consideration of scale has been made using towers from one to 1,000 litres capacity and comparison made to stirred tank fermenters.

This work forms part of a United Nations Development Project, Global III, Carob Pilot Plant for Cyprus in conjunction with Tate and Lyle Ltd. and their research contracts with The University of Aston in Birmingham.

APPENDIX V

Abstract of paper to be presented at the Fourth International Fermentation Symposium, Kyoto, Japan, March 1972.

THE GROWTH AND MORPHOLOGY OF MICRO-ORGANISMS IN TOWER FERMENTERS

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Industrially important yeasts, moulds and bacteria have been cultured in tower fermenters under aerobic or micro-aerophilic conditions on several media and their morphological form and growth characteristics studied. Most microorganisms grew in the form of aggregates rather than diffuse growth. Single-celled or chain forming organisms formed flocs or compact colonies whilst filamentous types gave various forms of pellets. Saccharomyces spp. in continuous culture could be divided into three types (I) non-flocculent, (II) flocculent dependent on medium concentration and dilution rate and (III) flocculent independent of medium and dilution rate. In aerobic situations II and III grew as compact erythrocyte-shaped colonies (0.01 to 1.0 cm.dia.) whilst in micro-aerophilic conditions II gave light powdery flocs (0.3 cm.dia.40%) and III gave heavy sticky flocs (1.0 cm dia.25% free space). Bacterial cultures were normally diffuse and non-flocculent but some showed loose irregular flocs with diameters up to 1.0 cm. Filamentous fungi usually formed pellets which were either compact or loose depending on culture conditions and reflecting the morphology of the mycelium.

It is possible to describe some of the morphological development of micro-organisms in tower fermenters in semi-quantitative terms. Such calculations show that the control of morphological form allowed by a tower fermenter provides flexibility in determining growth rate, biomass concentration, mass transfer of oxygen and metabolites. Commercial fermentations have been used to study this concept and comparisons made with the traditional stirred tank systems.