PRODUCTION AND UTILISATION OF PECTOLYTIC ENZYMES FROM SOME AGRICULTURAL WASTES

BY

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CERTIFICATION

THIS IS TO CERTIFY THAT THE THESIS -

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SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES UNIVERSITY OF LAGOS FOR THE AWARD OF THE DEGREE OF

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IS A RECORD OF ORIGINAL RESEARCH CARRIED OUT BY

TIMOTHY AYANGBILE AYANLEYE

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CERTIFICATION

We certify that the work embodied in this thesis for the degree of Doctor of Philosophy (Botany) has been carried out by Timothy Ayangbile Ayanleye.

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DEDICATION

This work is dedicated to the memory of my late father, Mr. Elijah Ayangbile Ayanleye; and my mother Mrs. Maria Ogunbola Ayanleye.

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ABSTRACT

Twenty-one fungal strains - five foreign, sixteen local isolates were screened qualitatively and quantitatively for pectinase production. Six potent strains among them were further studied to determine their nutritional requirements. The best two strains were Aspergillus niger (from Mysore, India) and Aspergillus repens (from Agege, Lagos). For mycelial growth and pectinase production, the optimum incubation period in static liquid media at ambient temperatures was 5 days and incubation at 24 ± 2°C was found to be optimum for both strains; moreover, A. repens performed equally well when incubated at 35 ± 1°C thereby indicating perfect ecological adaptation to the generally warm local environment of Lagos.

Three locally available agro-industrial wastes - wheat bran, rice bran and peanut shell were analysed and studied for pectinase production as semi-solid media. They all contained water extractable nutrients such as reducing sugars (8 - 12mg/g); protein (1 - 2mg/g); starch (2 - 537mg/g); and pectin (0.4 - 6.1%). The effect of acid concentration (0 - 0.3M HCl solutions) as diluent on pectinase production by the media was found to be very critical. Generally, 0.05 - 0.1M HCl solutions were adequate in all cases resulting in maximum enzyme production in 1 to 3 days while lower or higher acidity drastically reduced the yield. Inoculation experiments with A. repens showed that 1ml spore suspension per 10g dry medium (about 2.2×10^5 spores per ml) was adequate for rice bran and peanut shell; whereas

10mls inoculum per 10g dry medium was best for wheat bran. For A. niger with about 4.7×10^5 spores per ml, 1ml spore suspension per 10g dry medium was adequate for each of the three media.

Investigations on the effect of some nutrients on pectinase production showed that for A. repens, generally, soya flour (Glycine max) (whole and defatted) and the mixture of grapefruit peel (Citrus decumana), sucrose and soya flour increased pectinase production in the three media whereas plantain peel (Musa sapientum) and sucrose reduced it. For A. niger, plantain peel, glucose and sodium nitrate greatly reduced pectinase production in all three media. Studies on the effect of media depth (2 - 10cm) on enzyme production showed that for A. repens grown on wheat bran, 2cm was the best, with rice bran 2 to 10cm all gave equally good results; with peanut shell 2 - 4cm was quite good. For A. niger; 2 - 4cm was good in wheat bran; 2 - 6cm in rice bran and 2 - 10cm in peanut shell.

The culture filtrates of both Aspergillus strains grown on the three semi-solid media contained mainly endo- and exo-polygalacturonases and relatively small amounts of pectinesterase and polymethylgalacturonate lyase. Apart from pectinases, they also had significant activities of cellulases, amylases and acid protease. Generally, the most significant activity in each enzyme group was the viscosity reducing action.

Aspergillus repens produced endo-polymethylgalacturonase with optimum pH range 4 - 5.5 and optimum temperature range

40 - 50°C. At 80°C the enzyme still retained about 22% activity even after 1 hour and at 90°C the enzyme was denatured within 5 minutes. Aspergillus niger enzyme had [illegible] optimum pH values - 4.5 — 5.5; the former showed higher enzyme activity (81%) than the latter (74%). The optimum temperature was 40°C. At 70°C (pH 4.5) about 82% of the enzyme activity was lost in 15 minutes and complete denaturation of the enzyme occurred between 35 and 40 minutes of incubation. Aspergillus niger enzyme was more thermolabile than A. repens enzyme. The best substrate concentration for A. repens enzyme activity was 0.75% pectin (citrus pectin, 7.7% methoxyl content) while that of A. niger was 0.5% pectin.

Aspergillus repens enzyme filtrate could be stored at ambient temperatures at pH 3 - 4 and in refrigerator (10 ± 2°C) at pH 5 - 6 without loss in activity for at least a month. Aspergillus niger enzyme could also be stored at pH 3 at ambient temperature and pH 3 - 6 in refrigerator for at least a month without loss in activity. Preliminary toxicological screening (chemical and biological studies) of the crude enzyme concentrates obtained from the two Aspergillus strains did not indicate presence of any toxins. The enzyme utilisation studies carried out showed that both enzymes were quite effective in macerating and depectinising banana and guava. They were also effective in producing concentrated orange juice which did not gell when stored at room temperature and in the freezer.

INTRODUCTION AND LITERATURE REVIEW

1.1. Industrial Enzymes

Almost without exceptions, the chemical changes brought about by the cells of all living creatures - animals, plants, and microorganisms - are mediated by appropriate catalysts. These catalysts are enzymes - the protein biocatalysts produced by living cells. Fortunately, most enzymes can be separated readily from the cells that produce them and can perform their catalytic activities entirely apart from the cells. A considerable number of enzyme preparations have found important applications both in research and in industry.

Very considerable quantities of enzymes are currently produced commercially from animal and plant sources, but for both technical and economic reasons microbial enzymes are increasingly important. There is a definite limitation in the supply to us of animal enzymes since they are a by-product of the meat - packing industry. On the other hand, plant enzymes are only limited by available acreage and labour though subject to climatic, seasonal, and weather variations. Productive capacity for microbial enzymes may be expanded without limit to meet all demands. Developments in the production of microbial enzymes have assured potentially unlimited supplies and also have made available enzyme systems which cannot be readily obtained from higher plant and animal sources.

The practical use of enzymes to accomplish reactions apart from the cell goes back into antiquity, long before the

existence, nature, or functions of enzymes were understood. Malt for starch conversion to sugar for brewing; stomach mucosa for clotting milk in cheese making; papaya juice for tenderizing meat, and dung for bating hides in leather making are examples of ancient uses of enzymes. Later, crude enzyme preparations extracted from animal and plant tissues such as pancreas, stomach mucosa, malt, and papaya fruit found applications in the textile, leather, food, beverage, and other industries.

After the biocatalytic enzymes responsible for the action of the crude preparations became recognised and understood, a search began for better, less expensive and more readily available sources of similar enzymes. The development and continual improvement of methods for large scale production of enzymes have resulted in a sizeable number of commercial enzyme products and industrial uses of them. A comprehensive list of such enzymes was given by Underkofler (1976).

1.2. Agricultural wastes as potential raw materials for industrial enzymes production

In recent years, bioconversion and utilisation of agro-industrial wastes into useful products has been receiving increasing attention both in the developed and the developing countries of the world. This is because majority of these wastes contain substantial amounts of biodegradable matter, which when allowed to decompose, produce noxious gases which pollute the environment thereby compounding the problems of

environmental sanitation. Furthermore, the management (including disposal) of agro-industrial wastes is often expensive. Moreover, all these wastes and residues are potential sources for the generation of new wealth, particularly in the developing countries, if the appropriate technologies for their bioconversion can be developed.

Some of the agro-industrial wastes are potentially useful in compounding industrial media where they can serve as cheap sources of nutrients for microbial growth and biosynthesis of useful products such as enzymes (Okafor, 1987a).

The output of major agricultural commodities has greatly increased in recent years in Nigeria. This is partly due to the scarcity of foreign exchange for the importation of many agro-allied products; and also due to the greatly increased government incentives and investments in the agricultural sector. A natural consequence of this is the corresponding increase in agro-industrial wastes being generated.

The agro-industrial wastes of particular interest to this research programme are by-products from three major agricultural commodities: wheat, rice and groundnuts. According to the Economic and Financial Review (1987), provisional estimates for the output of groundnut, rice and wheat were (in metric tonnes) 607,000; 297,000 and 139,000 respectively. The by-products from milling operations of wheat and rice are generally refered to as wheat bran and rice bran respectively, although in actual practice, they are

mixtures of bran and husk. The initial by-product of groundnut processing (shelling) is the groundnut shell.

Wheat bran, rice bran and groundnut shell constitute about 30 percent (dry weight basis) of raw wheat, paddy rice and unshelled groundnut. According to the provisional estimates for 1987, they amounted to the following (in tonnes) groundnut shell 182,100; rice bran 89,100 and wheat bran 41,700.

Practically all the wheat bran produced in Nigeria is consumed by the livestock feeds industry where it is used as an ingredient in livestock feeds formulation (especially in poultry rations). At present, the quantity of wheat bran available in the country is not enough to meet the demand of the poultry feed industry. In the developed countries and some developing ones such as India, where there is abundant supply of wheat bran, it is an important raw material in the fermentation industries that produce industrial enzymes.

Rice bran is at present an underutilised raw material (UNIDO, 1985) inspite of the fact that it contains edible oil, high quality protein, high proportion of vitamins, trace elements and other nutrients. Because of its high fibre content, it is commonly burnt in Nigeria to heat boilers, etc. Groundnut shell is also used similarly due to its very high fibre content.

The demand for industrially useful enzymes and their products has steadily increased in Nigeria in line with the rapid industrialisation of the country in recent times. All the enzymes and enzyme-derived products in use in Nigeria are imported resulting in expenditure of large amounts of foreign

exchange annually. This trend could be reversed if we can make use of locally available raw materials to produce the enzymes biologically from our industrial wastes.

1.3. Some problems of industrial enzyme technology transfer

Information on the production of enzymes of industrial importance is scanty, confusing, sometimes even contradictory and protected by patent rights (Aunstrup, 1979; Okafor, 1987b). Patents are purchased at highly exhorbitant costs and even when this is done, there is additionally the need to carry out further research to adapt the technology to local conditions; for example, microorganisms that produce beer from barley do not act precisely in the same way when given sorghum as a substrate and hence the quality of beer is different. This difference can cause serious problems since the organism (a yeast) is not a local isolate. Therefore it is necessary that the transfer of fermentation technology from the developed to the developing countries such as Nigeria, should be modified to have as much local inputs as possible, if such technology will really be beneficial. Such technology will then become quite appropriate since it is relevant to local needs and capabilities.

1.4. Potentials of pectolytic enzymes production and utilisation in Nigeria

Pectolytic enzymes are a group of industrially important enzymes which catalyse the hydrolysis of pectin - an important

component of plant cell wall. This property makes them particularly useful in food industries. They are needed in the following processes (Fogarty and Kelly, 1983):-

(1) Production of jellies, jam and marmalades.

(2) Production of liquid fruit, ready-to-serve beverages.

(3) Production of fruit juice concentrates.

(4) Fermentation of coffee and cocoa beans.

(5) Recovery of oils from citrus fruits and pulp of olives.

(6) Clarification of fruit juices and wines.

(7) Preparation of potato and carrot purres in baby food production.

(8) Processing of cellulosic materials to cattle feeds.

(9) Production of galacturonic acid - a precursor of ascorbic acid (Vitamin C).

Pectinases are produced by a number of microorganisms, notable among them are the fungi. By growing these fungi on agro-industrial wastes, the enzymes can be cheaply produced. However, there is a serious restriction in the search for productive strains. This stems from the fact that the enzymes of only a few microorganisms take the stamp of approval for use in the food industry. This is probably the main reason why commercial pectinases are all derived from Aspergillus species, mainly from A. niger (Rombouts and Pilnik, 1980).

Citrus fruits such as oranges, lime and grapefruit as well as other pectinaceous fruits such as guava, pineapple, bananas, pawpaw, mangoes and carrots, abound in Nigeria (see Appendix 1). It is common knowledge that a sizeable

proportion of harvested fruits perish annually due to inadequate transportation, storage and processing facilities. Moreover, the annual import bill for fruit products such as jams, marmalades, jellies, juices and wines have always been on the increase until recently when in the 1989 budget of the Federal Republic of Nigeria, the Federal Government banned the importation of wine, fruits and fruit juices.

According to the Nigeria Trade Summary (1987), almost N10 million was spent on the importation of fruit-derived products as listed below.

TABLE 1. Import statistics of fruit-derived products in 1987.

Commodity	Quantity	Value (N)
Jams, marmalades, jellies, etc.	74,682kgs	281,743
Fruit juices (unfermented)	736,849 litres	1,375,076
Non-alcoholic beverages	459,590 litres	455,990
		2,112,809
Wines		
Vermouth and other wines flavored	748,928 litres	1,014,821
Wine, still	770,767 litres	2,139,540
Wine, sparkling	1,473,121 litres	4,418,251
		7,572,612
Other fermented fruit juices	51,689 litres	155,069
	Grand Total	9,840,490

Over ₦1.5 million was spent on importing fruit juices in 1987 while several tonnes of harvested fruits perished in Nigeria. Wine import bill (over ₦7 million) accounted for about 77 percent of the total value of all the fruit-derived products brought into the country that year.

The export bill of fruits and fruit-derived products, from Nigeria, during the same year, 1987, is equally revealing. The total export bill was over ₦3 million. It consisted of the following:-

TABLE 2. Export statistics of fruit-derived products in 1987.

Commodity	Quantity	Value (₦)
Oranges, tangerines, mandarines	78,231kg	107,522
Citrus fruits, others	977,101kg	1,522,615
		1,630,137
Fruits, Dried	1,077,635kg	1,125,333
Fruits, Preserved	1,500kg	7,483
		1,132,816
Non-alcoholic beverages	127,030litres	133,162
Wines		
Wine, still	113,300litres	453,202
Wine, sparkling	1,400litres	700
		453,902
Other fermented fruit juices	1,006litres	3,412
Grand Total		3,353,429

About 50 percent of the total export in 1987 consisted of raw fruits. Another 34 percent consisted of dried, temporarily preserved and sugar-preserved fruits. The wines (still and sparkling) made up about 14 percent of total export bill.

According to the Federal Office of Statistics (1983), the number of modern agricultural holdings for citrus fruits (oranges) in Nigeria in 1980/81 was 42 with total planted area of 358 hectares with the production of 759 tonnes of fruits. The number of holdings increased in 1981/82 to 52, but the total planted area decreased to 337 hectares with a production of 525 tonnes of fruits. The current ban on the importation of fruits and fruit products, coupled with the current government policy of local sourcing of industrial raw materials, will greatly stimulate fruit cultivation and processing in Nigeria. Indeed, Nigeria has the potentials to become a great exporter of fruit-derived products if the appropriate technology for fruit storage and processing can be developed locally. Pectolytic enzymes have a vital role to play in fruit processing.

Fruits rich in pectin (e.g. banana, pawpaw, guava and tomatoes) when over-ripe are often wasted since their organoleptic properties are often unattractive such that they are no longer palatable. Pectinaceous fruits whether just ripe or over-ripe can be liquefied using pectolytic enzymes. These fruit juices can then be concentrated and preserved for long periods. The concentrates can at any time (particularly during the off-season of the particular fruits) be reconstituted into refreshing

fruit juices with pure natural flavours.

Fruit drinks with natural flavours having little or no chemical addition are preferred to those "fruity" drinks with synthetic flavours, since the former have practically no risks of carcinogenic effects on consumers. Several countries are now legislating against un-natural or synthetic drinks and many chemical additives.

The production of fruit juices from pectinaceous fruits in Nigeria has great advantages. There will be less wastage of these fruits. The full benefits of the nutritional values of the fruits will be made availabe to the people. More employment opportunities will be created. The agricultural sector producing these fruits will be greatly stimulated and finally, some foreign exchange for the country will not only be conserved but more can be earned by exporting the enzyme-derived products.

Similar benefits as mentioned above will be obtained by using pectolytic enzymes in the fermentation of coffee and cocoa beans; recovery of oils from citrus fruits and olive pulp; clarification of wines and the preparation of potato and carrot purees for baby food production in Nigeria.

1.5. Pectic Polysaccharides

Pectic substances are a group of complex acidic polysaccharides which occur in varying amounts in all higher plant tissues and are found in the intercellular spaces, i.e. the

middle lamella. A number of reviews of pectic polysaccharides have been published (Doesburg, 1965; Pilnik and Voragen, 1970; Francis and Bell, 1975).

Because of the considerable confusion which existed in naming of pectic substances, a committee of the American Chemical Society defined these complex substances as follows (Kertesz et al., 1944);

Pectic substances is a group designation for those complex colloidal carbohydrates which occur in or are prepared from plants and contain a large proportion of anhydro-galacturonic acid units. The carboxyl groups of polygalacturonic acid may be partly esterified by methyl groups or partly or totally neutralised by one or more bases.

Protopectin is the name given to the water-insoluble parent pectic substance which occurs in plants, and from which pectic substances are produced.

Pectinic acids is the term used to designate colloidal polygalacturonic acids containing more than a small proportion of methyl ester groups. Pectinic acids under suitable conditions are capable of forming gels with sugar and acid; if the methoxyl content is low then gel formation may take place with certain ions. The salts of pectinic acids are either normal or acid salts of pectin acids.

Pectin or Pectins designates those water-soluble pectinic acids of varying methyl ester content and degree of neutralisation which are capable of forming gels with sugar and acid under suitable conditions.

Pectic acid is the name applied to pectic substances composed of colloidal polygalacturonic acid and is essentially free of methyl ester groups. The salts of pectic acids are either normal or acid pectates.

1.5.1. Structure and Composition

Pectic polysaccharides are polygalacturonides with other non-uronides bound to the unbranched chain of α-1,4 galacturonic acid units. The galacturonic acid units have the C_1 conformation and thus the linkages between these units are of the axial-axial type and the polymer has therefore, a screw axis with a tendency to coiling. In addition to D-galacturonic acid the following sugars have been isolated and characterised from pectic polysaccharides: L-rhamnose, L-arabinose, D-galactose, D-xylose and L-fucose.

The carboxylic acid groups of the galacturonic acid residues are partially esterified with methanol and the methoxyl content varies with the source. When all the carboxyl groups in polygalacturonic acid are esterified the methoxyl content is 16.32%, i.e. the degree of esterification is 100%. Some of the hydroxyl groups on C_2 or C_3 may be acetylated. Values from 0.18% to 2.5% have been reported. The acetyl groups are important in that they affect gelling properties.

1.5.2. Occurrence and Properties

The pectic substances are located in the middle lamella, i.e. the intercellular cementing layer, and in the primary cell walls of plants. Synthesis occurs during early stages of growth

when the area of the cell wall is increasing. Lignified tissue contains only small quantities of pectic materials compared with young actively growing plant tissue which is particularly high in pectin.

The texture of vegetables and fruits is greatly influenced by the amount and nature of pectin present. For example, most of the pectic material in unripe fruits is present as protopectin which is transformed into soluble pectin during maturation and ripening. This transformation leads to softening of the fruit on ripening and the relationship between these processes is of major economic importance. Softening can be counteracted in the case of many fruits and vegetables by addition of calcium salts. The pectin content of some foodstuffs is shown in Table 3(Fogarty and Kelly, 1983).

Pectic polysaccharides are soluble in water and other solvents, e.g. dimethylsulphoxide and hot glycerol, and are insoluble in most organic solvents. The ease of dissolution in water decreases with increasing chain length. Aqueous solutions of pectins are highly viscous and the viscosity is dependent on molecular weight and is governed by the degree of esterification, ionic strength, pH, temperature and concentration.

Pectic polysaccharides may be precipitated by water-miscible organic solvents, by water-soluble basic polymers and by polyvalent cations - depending on the degree of esterification. They may also be precipitated by quarternary detergents and

TABLE 3. Pectin content of some foodstuffs.

Source	Pectin (%)
Grapes	0.2 - 1.0
Apples	0.5 - 1.6
Grapefruit	1.6 - 4.5
Lemons	3.0 - 4.0
Lemon seeds	6.0
Lemon rind	32.0
Lemon pulp	25.0
Turnip	10.0
Sugar beet pulp	30.0
Pineapple orange peel	20.0
Pineapple orange membrane	29.0
Pineapple orange juice sac	16.0

proteins. Pectates are also precipitated by monovalent cations. Pectins are rendered more sensitive to calcium by amidation or by specific arrangements of acid and ester groups.

Acids hydrolyse both ester and glycosidic bonds, depending upon the temperature. At low temperature, hydrolysis of ester bonds only takes place and little degradation of the polymer occurs. Higher temperatures accelerate the depolymerization of the polysaccharides. This property is exploited in the preparation of low methoxyl pectins. Acidic treatments lower the level of non-uronic saccharides, e.g. arabans, in pectins, because of their greater acid sensitivity. Extensive or strong acid treatments effect complete degradation with formation of CO_2, furfural and a number of other breakdown products.

The methyl ester groups are easily saponified by dilute alkali at low temperatures without depolymerization. However, degradation of the polymer is greatly increased by rise in temperature; such alkali sensitivity is unique, as polysaccharides are generally alkali resistant. The degradation under these conditions is caused by a B-elimination cleavage of the linkages. This type of degradation also takes place by heating the polysaccharide in a buffer at pH 6.0 - 7.0. Pectin solutions, therefore, cannot be sterilized at neutral pH values without effecting considerable degradation.

Association between molecules takes place below pH 4.0 and viscosity is, therefore, increased and precipitation occurs. The most unique and outstanding physical property of pectins is

their ability to form gels with sugar and acid. Pectin constitutes the essential raw material in the jam making industry, it is the essential ingredient which allows jam to set. Both high and low methoxyl pectins can, under controlled conditions, form gels (Table 4). When sugar-free or low-sugar jellies are required, advantage can be taken of the gel formation occurring when calcium is added under controlled conditions to pectinic acid. Pectinic acid is often added to foods and beverages, particularly in diets for diabetics, where sugar is replaced by non-nutritive sweeteners.

In the pharmaceutical industry, the various pectin preparations serve many different functions. By its colloidal properties, it acts as a lubricant between the food and the intestinal wall, and promotes normal peristalsis without irritation. In fact, it is a standard addition to baby foods. Pectin is effective in diarrhoea and dysentery treatment. Pectic substances have also been reported to prolong drug action and lower cholesterol levels. In blood therapy it has been found to have haemostatic and antifibrinolytic effects.

1.5.3. Commercial Pectins

Commercial pectins are standardized products. The major technical application for them is in the manufacture of jellies, jams and marmalades. Mechanical evaluation of test jellies is determined either as the modulus of elasticity or the breaking strength. Different lots of pectin are normally blended and

TABLE 4. Types of Commercial Pectin.

Type of Pectin	Methoxyl content (%)	Galacturonic acid as free acid (%)	Substances required for gelation
Low methoxyl	5.0 - 6.5	60 - 65	Calcium ions
High methoxyl slow set	8.75 - 10.5	35 - 45	Acid, sugar
High methoxyl rapid set	11.4 - 13.0	20 - 30	Acid, sugar

dextrose is added as a diluting agent in order to produce a product of fixed grade. Certain buffer salts, e.g. sodium citrate, are sometimes added as stabilizers and to control setting time.

1.6. Pectic Enzymes

Pectolysis is an important phenomenon associated with many biological processes in which plant material is involved, including elongation of cells and growth of plants, ripening of fruits and abscission of leaves. Pectic enzymes are also produced by bacteria, fungi, yeasts, insects, nematodes and protozoa. Reviews of pectic enzymes include the following: Fogarty and Ward (1974); Rexova-Benkova and Markovic (1976); Rombouts and Pilnik (1980); and Fogarty and Kelly (1983).

Over the years, several terms have been applied to refer to the enzymes that hydrolyse pectin. Such terms include the following: pectinase and pectic, pectolytic and pectinolytic enzymes. They are still used interchangeably up to date.

1.6.1. Classification of Pectic Enzymes

Pectic enzymes are classified into two main groups, namely deesterifying enzymes (pectinesterases) and chain-splitting enzymes (depolymerases).

Pectinesterase (EC 3.1.1.11) is a carboxyl ester hydrolase, its systematic name is pectin pectyl-hydrolase. It deesterifies pectin, producing methanol and pectic acid.

The depolymerizing enzymes are characterised under three headings:

1. whether pectin, pectic acid or oligo-D-galacturonates is the preferred substrate;
2. whether they act by transeliminative cleavage or hydrolysis; and
3. whether degradation is random (endo) or end-wise (exo).

In the light of recent discoveries these enzymes have been reclassified as shown in Table 5. This classification conforms to a report of the Enzyme Commission on Nomenclature (Enzyme Nomenclature, 1973).

Figure 1 illustrates fragment of pectin molecule with points of attack of pectic enzymes.

1.6.2. Pectinesterases

Pectinesterases are formed by higher plants, numerous fungi and some yeasts and bacteria. Particularly well studied are some plant pectinesterases, but knowledge about fungal, bacterial and yeast pectinesterases is rather limited. Multiple molecular forms and isoenzymes of pectinesterase have been found in a number of fruits, as well as in certain fungi. The molecular and kinetic characteristics of the better known enzymes are summarised in Table 6 (Rombouts and Pilnik, 1980).

Pectinesterases may be assayed by different methods (Rexova-Benkova and Markovic, 1976). Usually, activity is measured by continuously recorded titration of the free carboxyl groups which are produced from the pectin substrate.

TABLE 5. Classification of pectinolytic enzymes.

A. ESTERASE

Pectinesterase, PMGE, EC 3.1.1.11, de-esterifies pectin to pectic acid by removal of methoxyl residues.

Systematic name (S.N.): Pectin pectyl-hydrolase.

Recommended name (R.N.): Polymethyl-galacturonate esterase.

B. DEPOLYMERASES

(a) Acting on pectin

(b) Polymethylgalacturonase (PMG)

Endo-PMG hydrolyses pectin in a random fashion.

(S.N.): Poly(methoxylgalactosiduronate) glycanohydrolase.

(R.N.): Endopolymethylgalacturonase.

(c) Polymethylgalacturonate lyase (PMGL).

Endo-PMGL, EC 4.2.2.10, causes random cleavage in pectin by a transelimination process.

(S.N.): Poly(methoxylgalactosiduronate) endlyase.

(R.N.): Endopolymethylgalacturonate lyase (Endo-pectin lyase).

(d) Acting on pectic acid (polygalacturonic acid)

(i) Polygalacturonase (PG)

(a) Endo-PG, EC 3.2.1.15, hydrolyses pectic acid in a random fashio

(S.N.): Poly(1,4-α-D-galactosiduronate) glycanohydrolase.

(R.N.): Endopolygalacturonase.

(b) Exo-PG-1, EC 3.2.1.67, hydrolyses pectic acid releasing D-galacturonate, i.e. hydrolyses successive bonds.

(S.N.): Poly(1,4-α-D-galactosiduronate) galacturonohydrolase.

(R.N.): Exopolygalacturonase.

TABLE 5 (Contd.)

(c) Exo-PG-2, EC 3.2.1.82, hydrolyses pectic acid from non-reducing end releasing digalacturonate, i.e. hydrolyses alternate bonds.

(S.N.): Poly(1,4-α-D-galactosiduronate) digalacturono-hydrolase.

(R.N.): Exopolydigalacturonase.

ii. Polygalacturonate lyase (PGL).

(a) Endo-PGL, EC 4.2.2.2, causes random cleavage in pectic acid by a transelimination process.

(S.N.): Poly(1,4-α-D-galactosiduronate) endolase.

(R.N.): Endopolygalacturonate lyase (endopectate lyase).

(b) Exo-PGL, EC 4.2.2.9, causes sequential cleavage in pectic acid by a transelimination process.

(S.N.): Poly(1,4-α-D-galactosiduronate) exolyase.

(R.N.): Exopolygalacturonate lyase (exopectate lyase).

(e) Acting on Oligo-D-galactosiduronates

i. Oligogalacturonase (OG).

OG hydrolyses oligo-D-galactosiduronate.

(S.N.): Oligo-D-galactosiduronate hydrolase.

(R.N.): Oligogalacturonase.

ii. Oligogalacturonate lyase (OGL).

OGL, EC 4.2.2.6, causes cleavage of oligo-D-galactosiduronate by a transelimination process.

(S.N.): Oligo-D-galactosiduronate lyase.

(R.N.): Oligogalacturonate lyase.

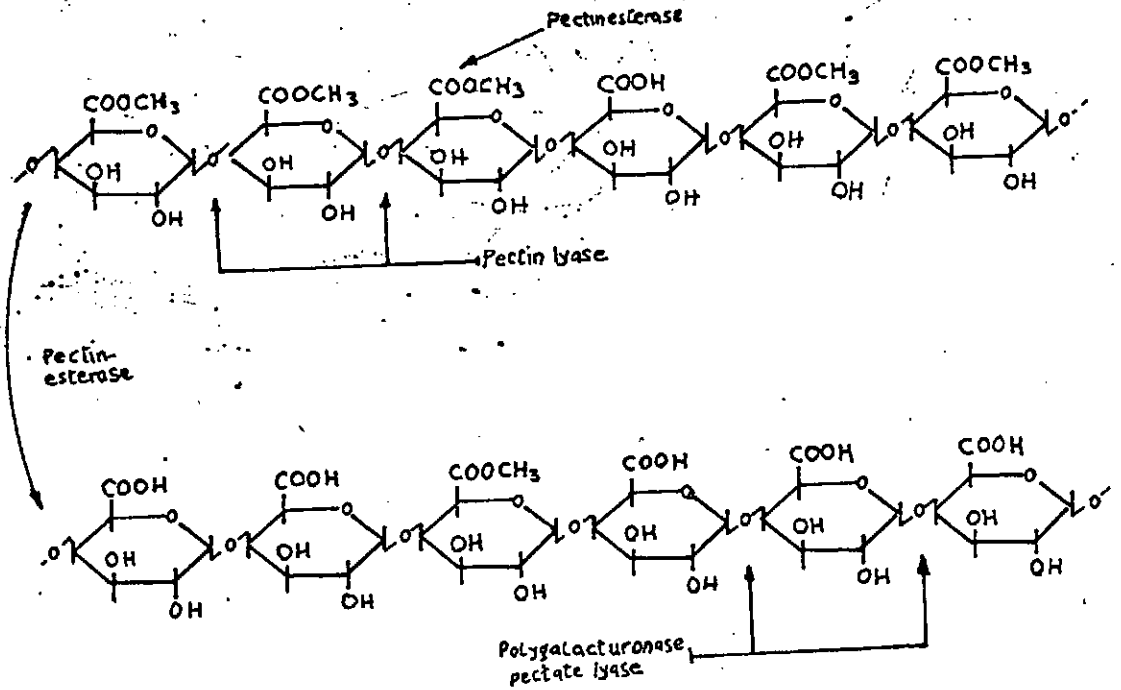


FIG I Fragment of pectin molecule with points of attack of pectic enzymes

TABLE 6. Properties of some plant and microbial pectinesterases.

Source of Enzyme	Molecular weight	Iso-electric point	Specific activity (units/mg of protein)	Optimum pH value	K_m value for pectin (mg/ml)
FRUITS					
Banana I	30,000	8.9	457	6.0	
II	30,000	9.4	529	6.0	
Orange (Citrus natsudaidai)			2,200	8.0	2.3
Orange (Citrus sinensis) I	36,200	10.0	694	7.6	0.083
II	36,200	11.0	762	8.0	0.0046
Plums (Prunus salicina)			25	7.5	0.1
Tomato	27,500		1,150	6-9	0.74
	26,300	8.4			
	27,800		724	8.0	2.4
FUNGI					
Acrocylindrium sp.				7.5	0.7
Coniothyrium diplodiella				4.8	
				4.8	
Corticium rolfsii	37,000			3.5	
Fusarium oxysporum	35,000		203	7.0	
*Aspergillus niger				4.5	
BACTERIA					
Clostridium multifermentans	400,000		48	9.0	0.74

* From Rexovabe-Benkova and Markovic (1976).

A sensitive and accurate method consists of conversion of methanol into volatile methyl nitrile which is then sampled by the head-space technique and analysed by gas-liquid chromatography (Bartolome and Hoff, 1972).

Pectinesterase from plants and fungi have a high specificity towards the methyl ester of pectic acid. Below a degree of polymerization of about 10, the rate of hydrolysis by orange pectinesterase decreases with decreasing chain length of the substrate and drops to zero for the trimethyl ester of the trimer. The Michaelis constant (K_m value) of orange pectinesterase decreases markedly with the degree of esterification of the substrate. The preferred points of attack on a pectin molecule are probably methyl ester groups adjacent to free carboxyl groups. However, on very highly methylated pectin, about half of the pectinesterase activity may be initiated at the reducing end of the molecules.

Enzymatic de-esterification of pectin proceeds linearly along the molecule, by a so-called single-chain mechanism. In this way, blocks of free carboxyl groups are formed which make the pectin extremely calcium-sensitive. Usually enzymic de-esterification of pectin does not go to completion, but levels off at about 10 percent esterification.

The activity of plant pectinesterases is markedly influenced by divalent and monovalent cations. They are also completely inhibited by pectate (end-product inhibition). Oligogalacturonates with a degree of polymerization of 8 and higher are effective inhibitors.

1.6.3. Endopolygalacturonases

Endopolygalacturonases are produced by numerous plant-pathogenic and saprophytic fungi and bacteria, and by some yeasts. Also, they are formed in higher plants, especially in soft fruits. Recently, quite a number of endopolygalacturonases, mainly from fungi, have been thoroughly purified. Their molecular and kinetic properties are compiled in Table 7. Multiple molecular forms and isoenzymes are produced by many of the organisms examined. Most of these enzymes have a molecular weight of 30,000 to 35,000 and a glycoprotein nature has been established for several of them. With the rather narrow range of molecular weights and K_m values, striking differences exist in specific activities, which reflect a great variation in turnover numbers (molecular activities) with these enzymes.

Endopolygalacturonases are specific for polygalacturonate; the rate and degree of hydrolysis of pectins decrease rapidly with increasing degree of esterification. The same is true for glycyl esters of pectate. The activity on oligogalacturonates decreases with decreasing degree of polymerization. Digalacturonate is not hydrolysed, and some of the enzymes do not attack the trimer (Rexova-Benkova and Markovic, 1976). Pectates of which the secondary hydroxyl groups at C-2 and C-3 are partially acetylated are still degraded with the same maximum velocity, but the values of the apparent Michaelis constant are increased and the degradation limits are decreased (Rexova-Benkova *et al.*, 1977).

TABLE 7. Properties of some plant and microbial endopolygalacturonases.

Source of Enzyme	Molecular weight	Iso-electric point	Specific activity (units/mg of protein)	Optimum pH value	K_m value for pectate (mg/ml)
FRUITS					
Tomato	52,000		47	4.5	2.7
	84,000			4.5	
	44,000			5.0	
FUNGI					
Aspergillus niger I		3.8		4.0	
II				4.0	
III		4.5		5.5	1.7
Aspergillus niger I	35,000		81	4.1	
II	85,000		44	3.8	
Aspergillus niger	46,000		75	5.0	0.54
Aspergillus japonicus	35,500		1,362	4.5	
Botrytis cinerea	69,000		2,049	4.0	1.2
Fusarium oxysporum I	37,000	7.0	194	5.0	
II	37,000	7.0	148	5.0	0.54
Rhizoctonia fragariae I	36,000	6.8	1,866	5.0	0.80
II	36,000	7.1	1,845	5.0	0.75
Rhizopus arrhizus	30,300		92	5.0	0.54
Trichoderma koningii I	32,000	6.41		5.0	0.80
II	32,000	6.57		5.0	0.85
Verticillium albo-atrum	30,000		2,075	6.5	1.5
YEASTS					
Kluyveromyces fragilis			168	4.4	
BACTERIA					
Erwinia carotovora			362	5.3	
Pseudomonas cepacia			125	4.5	

Endopolygalacturonases may be assayed by measuring the rate of increase of reducing groups or the decrease in viscosity of the substrate solution. Several methods are available for measuring the increase in reducing groups (Rexova-Benkova and Markovic, 1976). Viscosimetry is a very sensitive assay method for endopolygalacturonases. With these enzymes, a 50% drop in specific viscosity of a pectate solution corresponds to hydrolysis of only a few percent of the glycosidic bonds. (For different enzymes, this percentage may vary even under carefully standardized test conditions) (Endo, 1964a, b, c; Pressey and Avants, 1973). It is likely that these variations reflect differences in action pattern of the enzymes. An endo-enzyme may hydrolyse (randomly) one bond in a single enzyme-substrate encounter, followed by complete dissociation of enzyme and products (multichain attack). In the case of single chain, multiple attack, a single random hydrolytic scission is followed by a number of non-random attacks on one of the products, resulting in liberation of oligogalacturonates. The endopolygalacturonase of Kluyveromyces fragilis shows the first type of action pattern. This enzyme hydrolyses pectate through a series of higher oligogalacturonates, which it subsequently hydrolyses until finally digalacturonate and monogalacturonate accumulate (Phaff, 1966). An example of an enzyme showing the latter action pattern is the polygalacturonase of Colletotrichum lindemuthianum. The enzyme is truly an endopolygalacturonase, but the initial products, as well as the

final products of pectate hydrolysis, are predominantly trigalacturonate and digalacturonate (English et al., 1972).

Endopolygalacturonases also show considerable differences in action patterns on oligogalacturonases. These differences are determined by the nature of the active site of the enzymes, but more specifically by the size of the substrate-binding site and the position of the catalytic groups.

The catalytically reactive groups of Aspergillus niger endopolygalacturonases are a histidine group and a carboxylate group (Rexova-Benkova and Slezarik, 1970; Cooke et al., 1976; Rexova-Benkova and Marckova, 1978).

1.6.4. Exopolygalacturonases

Exopolygalacturonases are found in higher plants, in the intestinal tracts of a number of insects, in fungi and in some bacteria. They are usually assayed by measuring reducing groups of liberated galacturonate monomers.

There are a number of reports on exopolygalacturonases of plants including carrots (Pressey and Avants, 1975a) and citrus fruits (Riov, 1975). These plant enzymes prefer moderately high to high molecular weight pectates, which they attack at the non-reducing end liberating monogalacturonate. Oligogalacturonates and even digalacturonate are also degraded. The enzymes have optimum pH values of about 5.0; they are stimulated by calcium ions and hydrolyse their substrates by a multichain mechanism (Pressey and Avants, 1973b; 1975b) but the hydrolysis does not proceed to completion.

The activity of insect exopolygalacturonases is also proportional to the chain length of the pectate substrate. Monomers are liberated and the enzymes attack at the reducing end (Foglietti et al., 1971).

Many intracellular microbial exopolygalacturonases have a preference for oligogalacturonates. Exopolygalacturonases of fungi have optima from pH 4 to 6 and produce monogalacturonic acid as the main end-product while the bacterial enzyme of Erwinia aroideae has a pH optimum of 7.2 and produces digalacturonic acid as the main end-product (Fogarty and Kelly, 1983).

The exopolygalacturonase of Aspergillus niger hydrolysed both saturated and unsaturated galacturonic acid substrates. Galacturonic acid caused competitive enzyme inhibition when either saturated or unsaturated digalacturonic acid was used as substrate. The two substrates also competitively inhibited each other's hydrolysis (Fogarty and Kelly, 1983). The extracellular exopolygalacturonase of A. niger has no clear preference with regard to substrate chain length (Heinrichova and Rexova-Benkova, 1976).

1.6.5. Endopectate lyases

Endopectate lyases are produced by various groups of bacteria and by some plant pathogenic fungi. All of these enzymes have very high optimum pH values and an absolute requirement of calcium ions for their activity. The β-eliminative attack of these enzymes on their substrates results in formation of products with a double bond between C-4 and C-5. Conjugation of

the double bond with the carboxyl group at C-5 brings about absorption with a maximum at 235nm. Pectate lyases are therefore assayed most conveniently with a recording ultraviolet spectrophotometer.

Generally, pectates are very good substrates for endopectate lyases but for enzymes from two Arthrobacter strains and from Bacillus polymyxa, the best substrates are pectins with degrees of esterification of 21, 44 and 26% esterification respectively (Fogarty and Kelly, 1983). Highly substituted (1 → 4)-α-D-galacturonans, such as gum tragacanth and gum karaya, are not degraded (Rombouts et al., 1978) (Table 8).

Atallah and Nagel (1977) showed that the true substrate for lyase activity is the calcium salt of the substrate, while the free anion is a competitive inhibitor.

With endopectate lyases, the viscosity of the substrate solution decreases rapidly with respect to the number of glycosidic bonds broken. Differences exist among various enzymes, and with a single enzyme under various experimental conditions such as temperature and pH value (Rombouts and Pilnik, 1980). These differences point towards a multiple attack mechanism, just as with endopolygalacturonases.

Activity of endopectate lyases decreases with decreasing chain length of oligogalacturonate substrates. More specifically, the Michaelis constant increases with decreasing chain length, whereas the maximum velocity, under conditions of substrate saturation, remains fairly constant (Nagel and Wilson, 1970; Atallah and Nagel, 1977).

TABLE 8. Properties of some endopectate lyases.

Source of Enzyme	Molecular weight	Iso-electric point	Specific activity (units/mg of protein)	Optimum pH value	K_m value for pectate (mg/ml)
BACTERIA					
Bacillus polymyxa				8.3-9.6	0.056-0.0065
Bacillus subtilis	33,000	9.85		8.5	
Erwinia aroideae	37,000			9.1	
Erwinia carotovora			90	8.5	
Erwinia chrysanthemi	30,000-36,000	9.4-4.6		9.8-8.2	
Erwinia chrysanthemi		9.4	320	9.0	
Erwinia rabifaciens	41,000	6.25	450	9.5	5.0
Pseudomonas fluorescens	42,300	10.3	956	9.4	0.10
Streptomyces fradiae			176	9.1	
Xanthomonas campestris			1,050	9.5	
FUNGI					
Cephelosporium sp.			364	9.9	0.018
Hypomyces solani	32,400-42,000	10.2-10.5		8.5	

1.6.6. Exopectate lyases

Exopectate lyases attack pectate preferentially, having a greater affinity for pectate than pectins, and polymethyl-galacturonate-methylglycoside is not attacked at all. They liberate unsaturated oligogalacturonic acid from the reducing-end of the substrate chain and the smallest substrate they can hydrolyse is the trimer. Optimum pH values in the range 8.0 - 9.5 have been reported for these enzymes.

Exopectate lyase is produced by Clostridium multifermentans, Erwinia dissolvens, Streptomyces nitrosporeus and Fusarium culmorum (Rombouts and Pilnik, 1980).

1.6.7. Endopectin lyases

Endopectin lyases are produced almost exclusively by fungi with only a few bacterial endopectin lyases reported - a soft-rot Pseudomonad (Rombouts and Pilnik, 1980); a strain of E. carotovora (Almengor-Hecht and Bull, 1978), and one of E. aroideae (Kamimiya et al., 1972; 1974) (Table 9).

The method of choice for assaying these enzymes is again the measurement of the increase in absorbance at 235nm. Highly esterified pectins are the best substrates, while pectate, pectic acid amide and the glycyl ester of pectate are not degraded (Fogarty and Kelly, 1983). Most pectin lyases are markedly stimulated by calcium and other cations, the stimulation being dependent on pH value and the degree of esterification of the substrate.

TABLE 9. Properties of some endopectin lyases.

Source of Enzyme	Molecular weight	Iso-electric point	Specific activity (units/mg of protein)	Optimum pH value	K_m value for pectate (mg/ml)
FUNGI					
Alternaria mali I	28,000		176	8.7	
II	31,000		577	8.2	
Aspergillus fonsecaeus			19	5.2	
Aspergillus japonicus	32,000	7.7	355	6.0	
Aspergillus niger		3.5	24	5.2	
Aspergillus niger		3.5		5.9	2.2
Aspergillus niger I	35,400	3.65	17	6.0	5.0
Aspergillus niger II	33,100	3.75	44	6.0	0.9
Aspergillus sojae	32,000		77	5.5	
Dothidea ribesia	31,200	8.9		8.4	3.2
BACTERIA					
Erwinia aroideae	30,000		400	8.1	

Pectin lyases are endo-enzymes that cause a rapid drop in viscosity of the substrate solution with respect to the number of glycosidic bonds broken. Activity decreases rapidly with the chain length of methyl oligogalacturonates.

1.6.8. Synthesis of pectic enzymes in Aspergillus species

Aspergillus niger is an effective producer of pectinesterase, polygalacturonase and pectin lyase. Tuttobello and Mill (1961) reported very good enzyme production in a liquid medium of groundnut flour extract with 2% sucrose and 2% pectin, the enzymes being produced in a stage when the sucrose concentration in the medium dropped rapidly. A synergistic effect of pectin and lactose on synthesis of pectic enzymes by A. niger was also described by Feniksova and Moldabaeva (1967). Likewise, Nyeste and Hollo (1963) had found that the best media were those with a mixed carbohydrate composition (pectin and either glucose or starch). Vasu (1967) showed that the pectic enzymes produced by A. niger remain partly associated with the mycelium, and that secretion of these enzymes is favoured by the presence of pectic substances in the culture medium. Multiple molecular forms of different pectic enzymes occurred, some of them being produced inducibly, others constitutively (Vasu, 1974). Extensive studies were made on the kinetics of pectic enzyme formation by A. awamori and A. foetidus in media containing sucrose or a mixed carbon source (Zetelaki-Horvath and Bekassy-Molnar, 1973; Zetelaki, 1976).

Polygalacturonase production by an adenine-requiring mutant of A. niger was inducible and subject to catabolite repression

(Shinmyo et al., 1978). Glucose, fructose, or intermediates of glycolysis, but not tricarboxylic acid-cycle intermediates, repressed enzyme synthesis. Catabolite repression by glucose occurred quickly and almost completely. As the corresponding messenger RNA was rather stable, it was suggested that catabolite repression of polygalacturonase formation occurred at the translation level. Little is known about enzyme synthesis in the many other pectolytic Aspergillus species (Edstrom and Phaff, 1964; Sreekantiah et al., 1975).

Qadeer and Iqbal (1985) studied the biosynthesis of pectinases by Aspergillus foetidus grown by the surface culture method using wheat bran as solid substrate. They found that wheat bran was ideal providing all nutrients essential for enzyme formation. Blieva and Rodionova (1987) found multiple forms of pectin lyase, polygalacturonase and pectin ethylesterase in in filtrate of culture fluid of immobilized Aspergillus awamori.

1.6.9. Industrial Production of Pectic Enzymes

In the food and related industries major importance is now being attached to use of enzymes in upgrading quality, by-product utilization, increasing yields of extractive processes, product stabilization and improvement of flavour. The enzymes normally used in fruit and vegetable processing are pectinases and cellulases.

Moulds are primarily used for the production of pectinases on a commercial scale, and in particular the enzymes are produced from species of the genus Aspergillus (Nyiri, 1968, 1969).

Suitable organisms include strains of A. niger, A. wentii, A. oryzae and Rhizopus species (Fogarty and Kelly, 1983). The characteristics of enzymes from these sources makes them ideally suited to fruit juice and related technologies, where they find many applications. Mould pectic enzymes used in commercial processes normally contain a mixture of pectinolytic enzymes. Economics of commercial production may be enhanced by selection of more productive mutants which are not subject to catabolite repression or synthesize large quantities of enzyme without the necessity of an inducer. Pectinases are not produced commercially from bacteria, although highly productive constitutive strains producing polygalacturonate lyase are known (Ward and Fogarty, 1974; Chesson and Codner, 1978).

Aspects of the production of microbial enzymes have been described in a number of patents and scientific literature (Beckhorn, 1960; Miura and Endo, 1962; Beckhorn et al., 1965; Aunstrup et al., 1979; Ishii et al., 1980). The procedures adopted by the various manufacturers vary to a degree. However, there are only two methods of production - the semi-solid and the submerged liquid culture techniques (Fogarty and Ward, 1974).

The first step in the manufacture of any commercial microbial enzyme is the selection of an organism that produces the desired enzyme in good yield. Selection usually results from screening hundreds of species and strains for their ability to produce the desired enzyme. The selected strain may be improved by mutation. The organism is maintained as a pure strain under rigidly controlled conditions. These methods include cultivation

on agar slopes and storage as soil and Lyophilized cultures. In order to maintain uniformity in enzyme production, detailed examination of the culture is carried out at regular intervals, to ensure its freedom from contaminants and variants.

In order to optimize production, parameters affecting enzyme synthesis and elaboration must be investigated. Although it is well known that optimum conditions may vary for each organism and each enzyme, certain factors have been established as being most significant in influencing overall enzyme yield (Fogarty and Ward, 1974).

1.6.9.1. Submerged fermentation

The design of a balanced fermentation medium is critical to the production of high enzyme yields. Thus, in the submerged liquid culture process, the liquid nutrient medium is made up of a number of components: The exact formulations developed by the different manufacturers are kept confidential. The medium is composed of mixtures of carbohydrates (glucose, molasses, corn syrup, starch, starch hydrolysate, milled cereal products, e.g. grits, wheat bran, etc.); nitrogenous materials (ammonium salts, corn steep liquor, distillers solubles, yeast extracts, gelatin, casein); and minerals. A medium prepared from the above ingredients is suitable for the production of a range of constitutive enzymes. Most of the commercial pectinases are inducible enzymes and substrates high in pectin must, therefore, be added to the medium to stimulate enzyme production.

It has been shown in laboratory studies that very high levels of pectic enzymes may be obtained in media containing mixed carbon sources, e.g. glucose and pectin, sucrose and pectin or lactose and pectin (Tuttobello and Mill, 1961; Vasu, 1967; Feniksova and Moldabaeva, 1967). It appears that in addition to its inducing properties pectin also enhances release of pectic enzymes into the fermentation liquor (Vasu, 1967; Nyiri, 1968). Pectin as such is not used in media formulation but materials high in pectin are included, e.g. beet pulp, citrus peel or apple pomace (Zetelaki, 1976). Highest pectic enzyme production is achieved when the pH of the fermentation liquor has fallen to approximately 3.5 (Nyiri, 1968).

1.6.9.2. Semi-solid fermentation

In the use of the semi-solid culture technique for pectinase manufacture, the medium is prepared from wheat bran and a number of other ingredients (Beckhorn et al., 1965), somewhat similar to those listed above. They are mixed with water, again according to a specific formulation, to a consistency which is free of running liquid.

Two types of production systems are used for semi-solid media - in one case large closed chambers are fitted with perforated trays, containing layers of the moist bran medium; in the other case, long horizontal rotating drums (several feet in diameter) are half-filled with medium. After inoculation with a spore suspension, air is circulated through the systems.

Temperature is maintained in the chambers with cold air cooling systems, and in the rotating drum system by water flowing over the cylinder. With both systems the time required for enzyme production varies from three to seven days.

1.6.9.3. Extraction and recovery

When the pectinolytic enzymes are produced in submerged culture, no extraction process is required other than to separate the cell mass by filtration or centrifugation following the addition of preservatives and/or filter aids. With semi-solid media, the enzymes may be extracted using a countercurrent extraction system with water and some preservatives. This method provides a relatively clear solution. Alternatively, the semi-solid mass may be dried at low temperature. This dried material may be used as a crude commercial preparation or it may be stored and the enzyme extracted subsequently.

The clear enzyme solution prepared by the methods outlined above may be concentrated in vacuo or alternatively salt (e.g. ammonium sulphate or, sodium chloride) or organic solvents (e.g. isopropanol, acetone, methanol or ethanol) may be used to precipitate the enzyme mixture. Following precipitation, the enzyme cake is centrifuged or filtered and then dried at low temperatures or spray-dried. Subsequently, it is ground to a particular particle size and used to prepare commercial enzyme formulations.

The resulting powder is either sold as a concentrate on the basis of its activity or diluted with various agents to give

a standard activity. Examples of diluents include the following: gelatin, casein, glucose, sucrose, lactose, starch and salts. Diatomaceous earth may be used in certain conditions, e.g. where the product is filtered after enzyme treatment. Buffer and other salts are used in some cases to maintain suitable pH conditions, for enzyme activity and stability.

The semi-solid processes are still used for enzyme production in Japan (Fogarty and Kelly, 1983). Both the rotary drum and submerged fermentation techniques are used in Europe. The submerged culture process is more flexible, easier to control with respect to pH, aeration, temperature, etc. and in addition, sterile operating conditions are more easily maintained (Fogarty and Kelly, 1983). However, it is interesting to note that pectinesterase is more readily produced by the surface culture technique (Ishii et al., 1980).

1.6.10. Application of pectinases

Although pectic enzymes play a major role in destruction and rotting of fruits and vegetables (Byrde, 1982)(Table 10), they also have considerable commercial applications in disintegration of plant tissues, particularly in fruit and vegetable processing and manufacturing industries (Table 11).

TABLE 10. Spoilage of fresh fruits and vegetables by pectinolytic microorganisms.

	Product	Microorganism	Enzymes involved
1.	Apples	Penicillium expansum	1. Endopolygalacturonase
			2. Endopectin lyase
		Pezicula malicorticis	Various pectic enzymes
		Sclerotinia fructigena	1. Endopectin lyases
			2. Endopolygalacturonase
2.	Apricots	Rhizopus arrhizus	Endopolygalacturonase
		Rhizopus stolonifer	Polygalacturonase
3.	Citrus	Geotrichum candidum	Endopolygalacturonase
		Penicillium italicum	Endopectin lyase
4.	Grapes	Botrytis cinerea	Various pectic enzymes
5.	Peaches	Monilia fructicola	Various pectic enzymes
6.	Plums	Monilia spp.	Endopolygalacturonase
7.	Potatoes	Clostridium spp.	Various pectic enzymes
		Fusarium roseum	Various pectic enzymes
		Pseudomonas spp.	Endopectate lyase
8.	Strawberries	Rhizoctonia fragariae	Endopolygalacturonase
9.	Tomatoes	Botrytis cinerea	Pectic and other enzymes

Source: Fogarty and Kelly (1983).

TABLE 11. Industrial pectinases and their applications.

	Trade Name	Manufacturer	Applications
1.	Clarizyme	Societe Rapidase France	Extraction and clarification of fruit juices and grape musts.
2.	Irgazyme M-10	Ciba-Geigy AG Switzerland	Maceration of vegetables and fruits.
3.	Klerzyme	G.B. Fermentation Ind. Inc. USA	Extraction and clarification of fruit juices.
4.	MKC-Pectinase	Miles Kali-Chemie GmbH, West Germany	Used in fruit juice and wine technology.
5.	Panzyme	C.H. Boehringer Sohn, West Germany	Extraction and clarification of fruit juices and grape musts.
6.	Pectinex	Swiss Ferment Co. Ltd., Switzerland	Extraction and clarification of fruit juices and grape musts.
7.	Pectinol products	Rohm GmbH., West Germany	Extraction and clarification of fruit juices and grape musts.
8.	Pectolyase	Kikkoman Shoyu Co. Ltd., Japan	Extraction and clarification of fruit juices.
9.	Pektolase	Grindstedvaerket A/S, Denmark	Extraction and clarification of fruit juices.
10.	Rohament P	Rohm GmbH., West Germany	Maceration of fruits and vegetables.
11.	Spark-1	Miles Laboratories Inc., USA	Fruit juice and wine technology.
12.	Ultrazym 100	Ciba-Geigy AG Switzerland	Extraction and clarification of fruit juices and grape musts.
13.	Ultrazym SE 604	Ciba-Geigy AG Switzerland	Olive oil extraction.

Source: Fogarty and Kelly (1983).

The application of enzymes in this manner has a number of distinct advantages. These include:

(1) increased yield of juice and solids from plant material;

(2) reduction in viscosity of concentrates; and

(3) modification and solubilization of pectic structures to effect sedimentation and clarification of juices.

In the preparation of fruit juice concentrates and extracts, difficulties are encountered in filtration and clarification.

These problems are primarily caused by pectic polysaccharides which act as suspending agents for pulp and other substances and cause cloudiness. The particles causing turbidity may be separated by settling or filtration after treatment with pectic enzymes. In the processing of crushed fruits pectic enzymes increase the efficiency of juice extraction, thus bringing about an increase in yield. Furthermore, reduction in viscosity and destruction of suspending power considerably increases the rate of clearing and therefore filtration. Crude preparations containing a variety of pectic enzymes are traditionally used in these extraction processes (see Table 11). World production of fruit juices has risen to over 2,950 million litres per annum, of which 20% is manufactured in a concentrated form.

1.6.10.1. Fruit processing

Fruit juices are generally extracted by a process of pressing and this is facilitated by enzymic treatment of the pulp which results in partial hydrolysis of the pectin (De Vos and Pilnik, 1973).

Another use of microbial pectinases is in maceration, whereby they are used to produce single cells from the fruit or vegetable tissue by hydrolysis of the intracellular pectin. This process may be used in the production of homogeneous purees for baby foods and nectar bases. In these processes, a better quality of end-product is achieved by using pectic enzymes than by using mechanical methods (Fogarty and Kelly, 1983).

Total liquefaction of fruits and vegetables can be achieved by using both pectic enzymes and cellulase in the enzymic treatment of the pulp.

Another important application of pectinases involves a combination of thermal-mechanical comminution and enzyme action to obtain highly turbid low-viscosity clouding agents from citrus peel. A great demand for these products exists in the soft drinks industry (Rombouts and Pilnik, 1980).

1.6.10.2. Clarification of fruit juices

Clarification of fruit juices is the oldest process using pectic enzymes and it is still the largest market for these enzymes. They are used mainly in apple, pear and grape juices. Freshly pressed juices are viscous and turbid. The use of pectinolytic enzymes results in a rapid drop in viscosity and a reduction of the turbidity. Cloudy particles aggregate and settle out. It is then possible to obtain a clear juice by filtration or centrifugation. Efficient depectinisation ensures stability of clarified juices towards haze formation (Richard, 1974). The

production of high Brix concentrates is possible only if the pectin is first degraded.

1.6.10.3. Wine clarification

The application of pectic enzymes is particularly favoured in the production of red wines that are fermented on the skins. Pectic enzymes from Penicillium notatum, Aspergillus niger and and Botrytis cinerea are particularly useful.

The comparatively low content of pectin in most fruits restricts the quantity of methanol that may be formed in the juice or fermented wine. Addition of pectinolytic enzymes during juice extraction may cause an increase in the level of methanol. Some caution must be exercised during distillation of brandies from such wines.

1.6.10.4. Miscellaneous applications

Low-methoxyl pectins for calcium pectinase gels may be prepared, by partial acid or alkaline hydrolysis of the methyl ester bonds in pectin and also by use of a fungal pectinesterase which is free of polygalacturonase activity. The polygalacturonase is inactivated by urea treatment of the enzyme preparation (Smythe et al., 1952). The pH optimum of the fungal pectinesterase is within the pH range of normal fruit juices and may, therefore, be used to produce a gel in a solution of pectin in a fruit juice, in the presence of calcium and sugar concentrations in excess of 25 - 30%. Tomato pectinesterase is used to produce a pectin gel at a neutral pH, e.g. in milk products (Calesnick et al., 1950).

Pectin enzymes may also be applied in disrupting gels in order to assist recovery of oils. Citrus oil emulsions from peel slurries can be broken by treatment with pectinolytic enzymes. Yields of citrus oils from lemons and oranges can be increased considerably by this technique (Platt and Poston, 1962).

1.7. Rice Bran

The annual production of rice bran in the world is approximately 40 million tonnes. Out of this 6.8 million tonnes consists of edible oil, a similar quantity consists of high quality protein, and there is a high proportion of vitamins, trace elements and other nutrients. Inspite of this, rice bran is at present an under-utilised by-product (UNIDO, 1985).

Rice (Oryza sativa) is undoubtedly one of the world's basic human food items. It is by far the most important cereal food in South-East Asia and the Far East and is fast gaining in popularity in Africa and Latin America. Paddy is the raw material for the rice milling industry where the production of white rice goes hand in hand with the production of its by-product: rice bran. Sor far, however, rice bran, with its rich oil, protein, mineral and vitamin content, has not been given the attention it deserves as an extremely valuable secondary raw material for the production of vegetable oil and protein human food or animal feed.

Chang and Bardenas (1965) described the morphology and varietal characteristics of the rice plant and grain. During the threshing process, the grain normally becomes separated from the pedicel at the abscission layer. In some varieties, however, the

separation can occur when the pedicel itself is broken. When the grain is husked, the caryopsis is uncovered. It has elongated or rounded oval-shaped fruit, largely composed of seed, with ridges on the surface, corresponding to the ribs of the lemma and palea. The external layer of the caryopsis is composed of the pericarp, which envelopes and protects the seed, to which it is closely attached. Immediately below the pericarp is the seed-coat, or tegmen; then come the aleurone layer and the starchy endosperm. The germ is lodged in a hollow, in the lower abdominal region of the grain, adhering to the endosperm. It is covered by the aleurone layer, the seed-coat, the pericarp and finally, the lemma.

The morphology of the grain, together with other characteristics (geographical distribution, morphology of the plant, sterility of hybrids and serological reaction), has served as a basis for classifying cultivated rice into three subspecies: indica, japonica and javanica. The grain of the first is thin and rather flattened, that of the second is short and rounded and that of the last is wide and thick (Chang and Bardenas, 1965).

Bran is the by-product obtained by removing the outer layers from brown rice to whiten the kernel. It consists mostly of pericarp, tegmen, aleurone, whole germ and crushed germ and starchy endosperm, in the form of dust and small fragments; it includes varying amounts of scraps of husk and impurities. When the term is used to distinguish "bran" from "white bran", it refers to the by-product obtained in the first (undefined) stages of whitening. It is defined by FAO as "a by-product of rice milling, consisting

of the outer bran layers of the grain together with part of the germ" (UNIDO, 1985). White bran is defined as "a by-product of rice milling, consisting of the inner bran layers together with part of the germ and a small proportion of the starchy interior". However, commercial bran and the bran layers in the grain are two entirely distinct products.

In commercial bran produced from raw rice, more than 15 different types of discrete particles have been identified. The simple discrete particles are fragments of the flowering glumes, sterile glumes, pedicel, pericarp, starchy endosperm, germ (also whole) and fibres.

1.7.1. The milling process and production of bran

The basic stages in the processing of rice are:

(a) cleaning

(b) husk removal

(c) whitening, and

(d) grading.

Cleaning: This is carried out at the mill to remove foreign matter - straw, soil, stones, twine, metallic particles and foreign seeds. The machines used at this stage are, the scalping machine, the separator sieve, the vibratory grading sieve (plansifter), the gravity separator or densimetric table and the disc separator, may or may not combine suction, sieving and gravity separation. At a later stage, the impurities are separated by means of a magnetic separator.

Husk removal: A husker is used to remove the husk (about 20% of the grain) from the caryopsis. The machines most often used are the disc husker and the rubber-roll husker. The discharge from the husker is a mixture of brown rice, husk, bran, fines, powder, chalky and green grains and also unhusked grains - the huskers are regulated so that they husk about 95% of the grains in a single pass.

Both the disc and the rubber-roll husker produce what is called "husker bran". This consists basically of fragments of husk and may include a small portion of pericarp, germ, rachilla, small fragments of endosperm, powder and soil. The disc husker is rougher and it tears away a considerable part of the germ. It usually produces from 1 - 2.5% of bran in relation to paddy rice. The rubber-roll husker if carefully regulated, does hardly any damage to the pericarp and rarely separates the germ from the caryopsis.

Whitening: The brown rice passes from the paddy separator to the whitening machines to eliminate the bran from the grain and to whiten the rice. The machines in use operate either by abrasion or by friction.

Polishing: In some mills the whitening process goes on to the polishing stage. In some polishers, but strictly speaking polishers are quite different. Their object is to eliminate from the grain that has already been whitened the small particles of flour that adhere to the surface so that the rice acquires

a glossy satin-like appearance. At the same time a new fraction of bran is collected.

Final steps: In most countries, as far as the production of bran is concerned, polishing is the final stage of rice processing. Although, each stream or fraction of bran is generally produced separately, the streams are all combined before being marketed. The rice that is discharged is a mixture of whole and broken grains - they must be separated and graded before marketing.

Categories of mills: The complexity of the methods used in the processing of rice varies from one country or region to another and according to particular circumstances. Installations for rice milling are classified into four categories:

(a) subsistence-level;

(b) rustic;

(c) quasi-commercial; and

(d) export.

The most simple form of mechanical processing is that typical of the rustic category. It employs a single machine (the "huller") involving only two conversions; the processed rice (with a variable quantity of fines) and a by-product consisting of a mixture of ground husk, ground rice, bran and all kinds of impurities, such as straw, soil, stones, etc.

1.7.2. Factors determining the properties of bran

The factors that affect the properties of industrially produced bran are classified into two:

(a) rice related factors;

(b) factors related to the milling process.

A. Rice-related factors:

1. Anatomo-morphological

(a) Size and shape of the grain

(b) Anatomical layers.

2. Mechanical

(a) Resistance of the grain to abrasion and friction

(b) Resistance of the grain to breakage.

(c) Ease of separation of the germ.

3. Chemical

(a) Average chemical composition

(b) Distribution of constituents

(c) Defective grains.

4. Soundness

(a) Physical contaminants

(b) Chemical contaminants

(c) Biological contaminants.

B. Process-related factors:

1. Preliminary operations

(a) Cleaning

(b) Grading.

2. Milling stages; single-stage versus multi-stage.

3. Machinery and operational conditions

   (a) Hullers

   (b) Paddy separators

   (c) Destoners

   (d) Whitening machines

   (e) Grading machines.

1.7.2.1. Sanitary condition of the bran

Physical and chemical contaminants, microorganisms and insects all affect the sanitary condition of the grain. It is customary for rice to be stored for long periods, with the accompanying risk of deterioration caused by the action of insects and microorganisms. The use of chemical compounds to protect the harvest is widespread. Although most of the contaminants are removed along with the husk, the bran is not necessarily free of them.

The usual microbial flora of rice include those capable of producing mycotoxins. It has been shown that a high proportion (60 to 80% by weight) of the toxins in the grain pass into the bran during milling. The resulting bran contains 10 times as much toxin as milled rice (Schroeder et al., 1968).

1.7.2.2. Types of machines and processing conditions (Table 12).

TABLE 12. Average composition of bran from different types of hulling machines.

Component	Disc-type Huller	Rubber-roll Huller
	%	
Moisture	9.2 - 13.5%	11.7
Protein	8.1 - 11.6%	3.8
Fat	6.5 - 10.4%	0.8
Fibre	14.8 - 22.6%	41.5
Ash	11.2 - 20.4%	13.2
Nitrogen-free extract	31.0 - 40.3%	28.9

Sources: Leonzio (1965) and Primo et al. (1970).

1.7.3. Stabilization of rice bran

"Stabilization" is the treatment of rice bran to prevent or limit its deterioration during storage. Since both harmful and valuable constituents are present simultaneously in the bran, stabilization implies that the harmful components must be destroyed or controlled and valuable components must be protected and preserved.

Harmful components in the bran include enzymes (lipases), microorganisms, insects, toxins, and growth inhibitors, and adulterants and impurities. The valuable ones include the oil, proteins and B-vitamins (thiamine and tocopherols). Stabilization methods employ heat, radiation and chemical compounds. Heat is the commonly used method for stabilization.

1.7.4. By-ppoducts of rice milling and their uses

The average yields of by-products obtained in the U.S.A. from 100 parts of paddy rice are 20 parts of hulls, 8.5 parts of rice bran and 2 parts of polishings (Kent, 1975). Where the rice milling industry is well established these products are obtained separately, but in more primitive milling processes the "rice bran" may be grossly contaminated with hulls and even with low grade broken rice (brewers' rice). The inclusion of hulls in rice bran lowers its feeding value because of the high fibre and silica contents of the hulls and their low digestibility. The Association of American Food Control Officials has adopted the following standards for rice bran: not less than 11% of crude

protein, not less than 10% of crude fat, and not more than 15% of fibre. The maximum content of rice hulls in rice "bran", in order to attain this standard, is about 20% (Table 13).

Rice bran oil: Rice bran has a high oil content (10 - 15%). When the bran is separated from the kernel, hydrolytic action by the enzyme lipase begins to split the fat to free fatty acids and glycerol, and oxidation of the fat by peroxidases may also occur. Peroxides are undesirable in feedstuffs because they destroy the fat-soluble vitamins and also cause digestive disturbances. The oil from rice bran is of higher value than the bran and is used for salad oil and cooking oil. The Rice Growers' Association of California have established plants at Sacramento and at Houston, Texas, for the extraction of oil from rice bran. When refined, bleached and deodorised it has twice the stability of comparable commonly used vegetable fats. The nutritive value of the extracted bran is enhanced because the protein content is somewhat increased.

Rice hulls: Rice hulls yield about 22% of ash, of which 95% is silica, and most of the remainder lime and potash.

Uses for rice hulls include roughage for cattle feed, chicken litter, ammoniated for fertilizers, filter aid, burnt for floor sweepings, fuel, abrasives, binder for pelleted feeds, insulating material, filler for building materials, binders and absorbents for pesticides, explosives and other materials. Rice hulls are also used in the commercial manufacture of furfural, although their pentosan content is lower than that of oat hulls or maize cobs.

TABLE 13. Chemical composition of milling by-products from rice.

Material	Moisture %	Protein %	Fat %	Ash %	Fibre %	Carbohydrate %
Hulls	12.0	1.0	0.3	24.0	30.0	30.0
Bran	10.0	12.7	16.9	10.1	5.1	45.2
Polishings	8.7	11.4	8.8	5.3	2.0	63.7

Source: Kent (1975).

Uses for rice hull ash include a source of high grade silica, manufacture of building blocks, absorbent, soil conditioner, carrier for pesticides, filter for insulating materials. (Refer to Appendix Tables V to IX for more data on proximate composition and vitamin contents of rice).

1.8. Wheat Bran

The weight of flour produced per 100 parts of wheat milled is known as the flour yield or percentage extraction rate. The wheat grain contains about 82% of the white starchy endosperm which is required for white flour, but it is hardly possible to separate it exactly from the 18% of bran, aleurone and germ and thereby obtain a white flour of 82% extraction. The mechanical limitations of the milling process are such that in practice 75% is about the limit of white flour extraction, further increase of extraction darken the colour of the flour through inclusion of a proportion of bran, aleurone and germ.

1.8.1. The milling process and production of wheat bran

In order to separate the endosperm from the bran and germ and reduce the endosperm to flour finess, a particular form of grinding has been developed which is a combination of shearing, scraping and crushing, achieved by roller mills, and which exploits the differences in mechanical properties between the endosperm, bran and germ. It is essential to minimize the production of fine particles of bran, and this basic requirement

is responsible for the complex arrangement of the modern flour-milling system and for the particular design of the specialized machinery used in it and also for the conditioning processes required.

There are four basic processes involved in wheat milling - conditioning, grinding, sieving and purifying.

Conditioning of Wheat: The objectives for wheat conditioning are primarily to improve the physical state of the grain for milling, and sometimes to improve the baking quality of the milled flour. The process of conditioning involves adjustment of the average moisture content of the wheat and of the distribution of moisture throughout each wheat grain, and may include the heating and cooling of the grain for definite periods of time; in order to obtain the desired moisture content and distribution. When in this optimum condition the bran is toughened and made less brittle, thereby improving separability of the endosperm from the bran, and the endosperm is made more friable, thereby reducing the amount of power required to grind it. Moreover, when the bran is adequately toughened the flour is less contaminated with bran splinters and is thus whiter in colour and yields a smaller amount of ash upon incineration.

Grinding: This involves fragmenting the grain or its parts, with some dissociation of the anatomical parts of the grain from one another.

Sieving: This is the separation of mixtures of particles of differing particle size into fractions of narrower particle size range. Names of particular sieving processes include:
scalping: Sieving to separate the break stock (the coarsest particles) from the remainder of a break grind;
dusting, bolting, dressing: Sieving flour from coarser particles;
grading: classifying mixtures of semolina, middlings and dunst into fractions of restricted particle size range.

Purifying: This is the separation of mixtures of bran and endosperm particles, according to terminal velocity, by means of air currents.

Gradual reduction process: The modern rollermilling process for making flour is described as a "gradual reduction process" because the grain and its parts are broken down in a succession of relatively gentle grinding stages rather than by one extremely severe grinding stage, as in the now superseded process of stonemilling. No attempt is made to achieve complete separation of endosperm from germ and bran in a single grinding stage; the severity of grinding is carefully adjusted so that only the required amount of endosperm fragmentation and bran cleaning occurs at each stage.

The reduced endosperm is known as flour; the germ, bran and residual adhering endosperm are obtained as a by-product (wheatfeed in Britain, millfeed in the U.S.A.) used primarily in animal feeding (Tables 14 and 15).

Table 14. Proportions of bran, germ and endosperm in flours of various extraction rates.

	Rate of Extraction (%)		
	100	85	80
Bran	12	3.4	1.4
Germ	2.5	1.9	1.6
Endosperm	85.5	79.7	77.0

Source: Kent (1975).

TABLE 15. Composition of flour and milling offals at various extraction rates.

Material	Yield %	Protein %	Oil %	Ash %	Fibre %	Vitamin B_1 ug/g	Nicotinic Acid ug/g
Flour:							
85% Extraction	85	12.5	1.5	0.92	0.33	3.42	-
80% "	80.5	12.0	1.4	0.72	0.20	2.67	19
70% "	70	11.4	1.2	0.44	0.10	0.7	10
Fine wheat feed:							
85% Extraction	10	12.6	4.7	5.1	10.6	6.0	-
80% "	12.5	14.3	4.7	4.7	8.4	10.4	191
70% "	20	15.4	4.7	3.5	5.2	14.0	113
Bran:							
85% Extraction	5	11.1	3.7	6.1	13.5	4.6	-
80% "	7	12.4	3.9	5.9	11.1	5.0	302
70% "	10	13.0	3.5	5.1	8.9	6.0	232

Source: Kent (1975).

1.9. Peanut Shell

Peanuts (Arachis hypogaea) also known as groundnuts, monkey-nut and earth-nuts is an annual herb grown in tropical and subtropical climatic regions and warmer parts of temperate regions. The varieties fall into two depending on their characteristics:

(1) Bushy bunched type which matures in 3 - 4 months;

(2) Runner or spreading type which matures in 4 - 6 months.

Some intermediate hybrids exist. The bunch type contains kernels that average 65 - 75% of the whole nut. The major peanut producing countries are India, China, Nigeria, Senegal and the United States of America. The major proportion of peanut is used for production of vegetable oil, also a substantial amount of shelled peanuts are used for direct human consumption.

1.9.1. Pre-processing of peanuts

Pre-processing of peanuts is in the following order (UNIDO, 1984) harvesting, field drying, shelling, bagging and storage.

Shelling: This is the process of removing the kernels from the pods. It is also refered to as "decorticating". This is usually carried out on the farm just before the farmer sells his produce for the following two reasons:

(1) kernels do not store as well as nuts in the shell, and

(2) peanuts in the shell are 50% heavier than kernels alone and are therefore costlier to transport.

On small holdings, peanuts are shelled manually. But now a number of simple hand operated decorticators are now available. Furthermore, shellers are available for various scales of production and they are powered by various means (manual, diesel engines, electric motors).

1.9.2. Chemical composition and uses of peanut shells (Table 16).

TABLE 16. Chemical composition of groundnut and its by-products.

Feeding-stuffs	Dry Matter	% of Dry matter				
		Protein	Oil	Crude Fibre	N-free Extracts	Ash
Groundnut (undecorticated)	92.88	21.66	39.09	21.14	15.39	2.65
Groundnut (Decorticated)	95.10	27.20	50.92	9.61	9.48	2.64
Groundnut cake	90.36	51.41	10.16	4.64	28.28	3.81
Testa (Red skin) of Groundnut	90.70	14.00	5.00	12.40	65.30	3.30
Groundnut husk	82.31	4.88	0.62	68.36	18.75	3.68

Source: Oyenuga (1968).

Peanut shells are also refered to as peanut husk or peanut hulls. The husk from the undecorticated nut comprises between 20 and 30 percent of the fruit, according to the variety. It consists mainly of crude fibre with very low digestibility (Oyenuga, 1968). It contains, however, about 3% of phosphoric acid, 6% of lime and 9% of potash. It is therefore useful as a fertilizer. It is sometimes used as litter for cattle or as an absorbent medium in making cattle food. Attempts have been made to use it in industry in the production of alcohol and acetone by fermentation, and as an insulating material. It is also used for furfural, fuel and sweeping compounds (Duke, 1981).

MATERIALS AND METHODS

2.1. Isolation and Identification of Potent strains of fungi with Pectolytic activity

2.1.1. Culture collection

Organisms were obtained from infected and decaying vegetable materials such as oranges, bananas, lime, etc. by the culture collection methods of Booth (1971). Some organisms were obtained from the culture bank of the Federal Institute of Industrial Research, Oshodi (see Appendix X for details).

The required organisms were separated by plating on 2% Czapek Dox agar in which the sucrose had been replaced by 2% pectin. Organisms that grew well here were inoculated on potato dextrose agar slants and incubated for 7 days at room temperature before storing in refrigerator at 8 - 10°C.

2.1.2. Rapid screening of cultures for pectolytic activity by the method of Tuttobello and Mill (1961)

The cultures were screened rapidly for pectolytic activity by inoculating them into test tubes containing 9ml of 5% solution of pectin in a 2% aqueous extract of soyabean meal at pH 4.2.

2.1.3. Quantitative analysis of pectinase of culture fluids

The methods by Sreekantiah et al. (1975), Olutiola and Akintunde (1979) and Aziz et al. (1985) were used. The screening medium used was modified Czapek Dox medium (1976) with the following composition (g/litre): $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCL, 0.5; Sucrose, 20; Pectin, 20; 100ml of the medium was

dispensed into special heat resistant white plastic bottles (500ml capacity) with screw caps. They were autoclaved at 15 psi for 20 minutes, cooled and pH adjusted to 4 before inoculation with fungal spores. They were incubated (static) at room temperature for 7 days. The mycelial mats were filtered using Whatman No. 1 filter papers with suction pump.

The pH of the culture filtrate was measured using a Crison digital pH/MV meter. The protein content was determined by the method of Lowry et al. (1951) (Fig. 2).

2.1.3.1. Endo PG/PMG activity EC. 3.2.1.15

The viscometric assay method used was as described by Cappellini (1966). The activity of the enzyme was expressed as percentage reduction in viscosity of 1% pectin or pectic acid solution at pH 4.0 at 30°C after 30 minutes incubation.

2.1.3.2. Exo PG/PMG activity EC. 3.2.1.67

The method of Owens et al. (1952) was used. One unit of activity is that which releases 1 millimole of reducing sugar per minute from a 0.5% solution of sodium pectate at pH 4.0 (Fig. 3).

2.1.3.3. Pectinesterase PE or PME EC. 3.1.1.11

The method of Archer (1979) was used. The activity was expressed as microequivalents of carboxyl groups liberated per minute per ml of enzyme.

STANDARD PROTEIN CURVE LOWRY

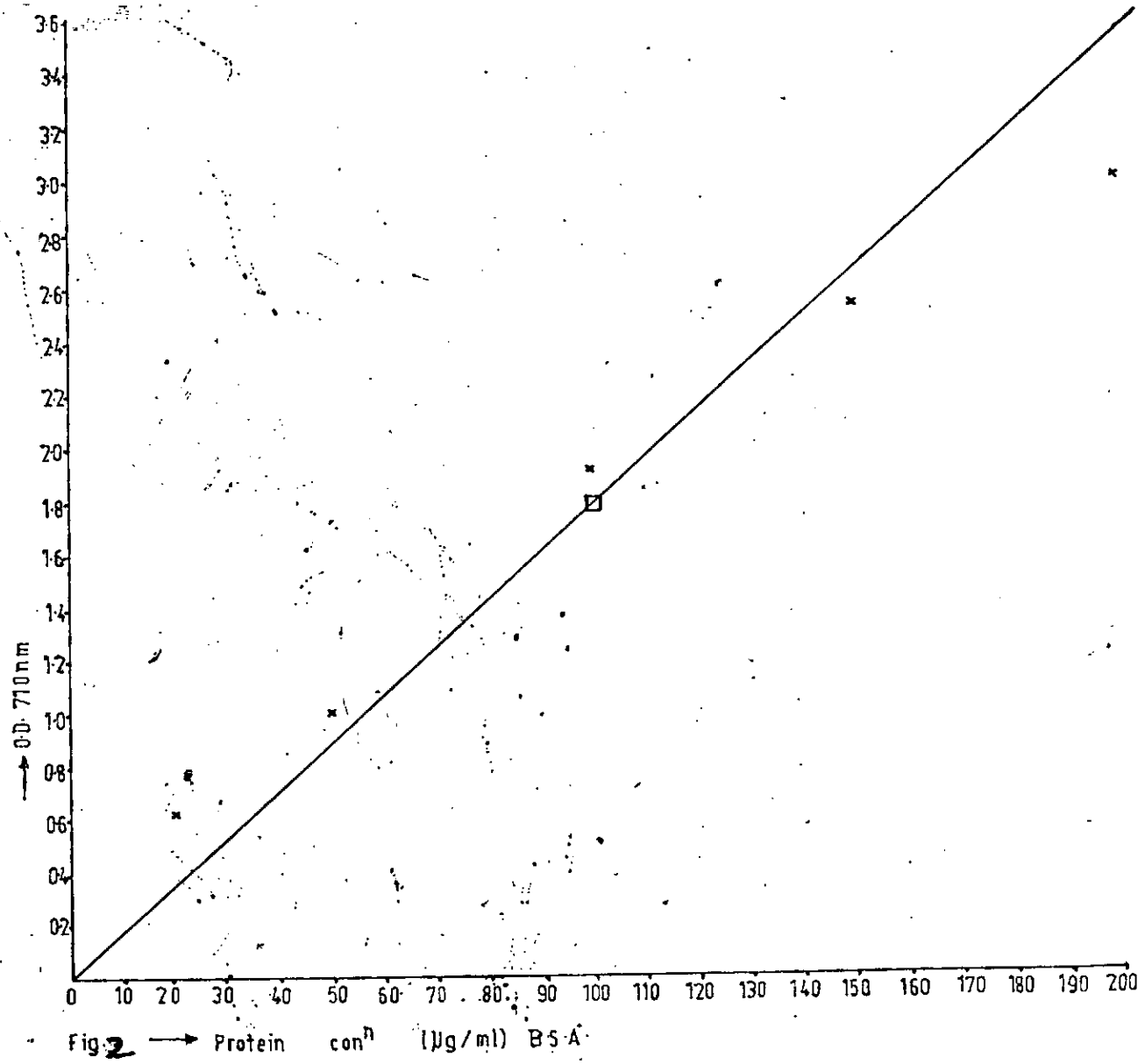


Fig 2

STANDARD EXO – PG ACTIVITY CURVE

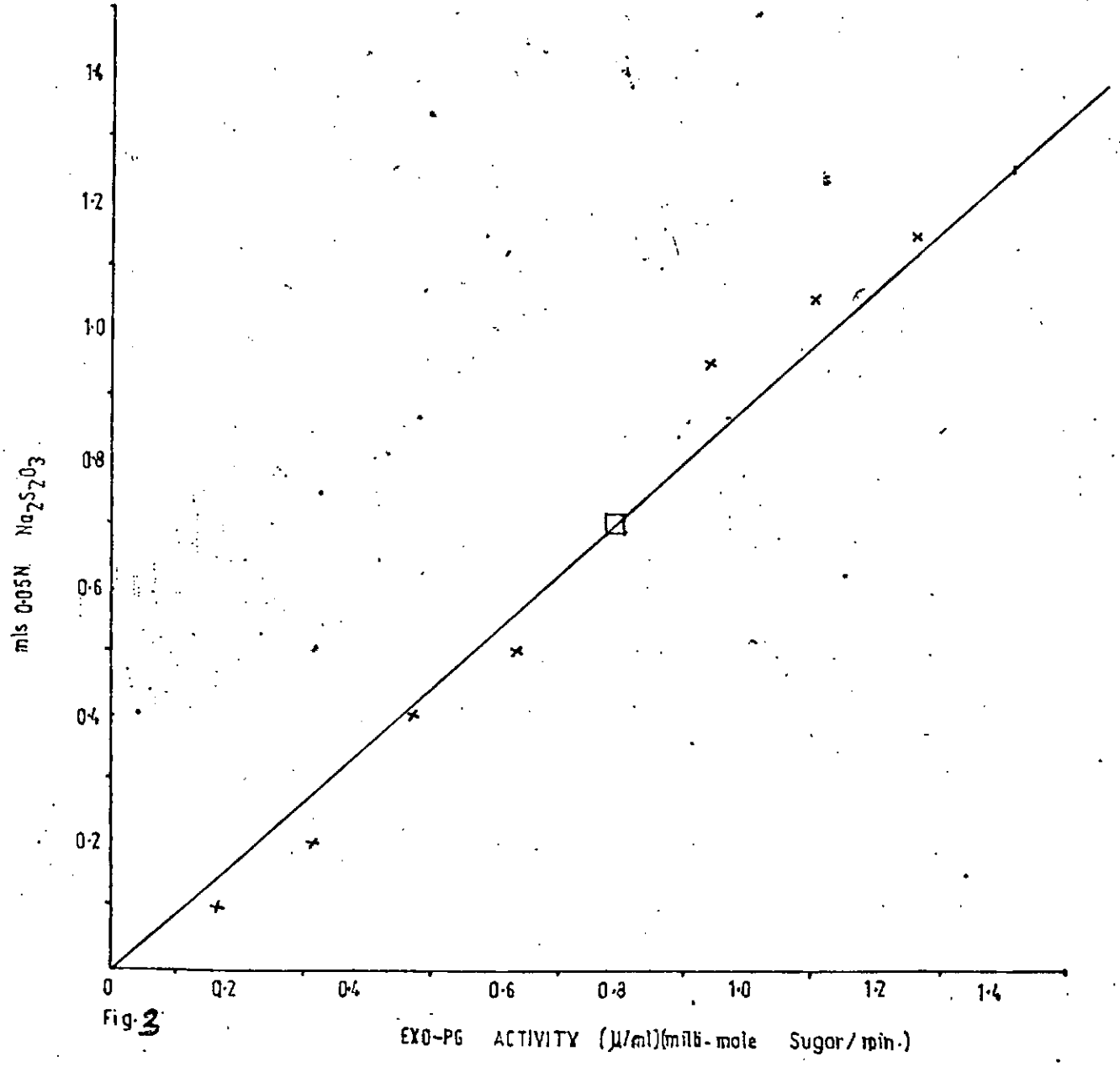


Fig. 3

2.1.3.4. Pectate or pectin lyase PGL, PMGL EC. 4.2.2.2.; 4.2.2.10

The method described by Sreekantiah et al. (1975) was used. A unit of activity is defined as the amount of enzyme catalysing an increase in absorbance at 235nm of the reaction mixture by 0.555 per minute at pH 5.2.

2.1.4. Identification of fungal strains (according to the methods of Onions 1981)

This involves studies of the general morphology, cytology and physiology of all the cultures for their generic classification. Furthermore, the specific identification of one of the Aspergillus strains was done.

2.2. Nutritional Studies of Selected Strains

The basic medium was modified Czapek Dox liquid medium as described earlier in Section 2.1.3.

2.2.1. Determination of minimum time for maximum pectinase production in six static cultures grown in modified Czapek Dox liquid medium at ambient temperatures

Six fungal cultures were selected from the previous experiments for this experiment. Inoculation was with 1ml of spore suspension from 5 day old cultures grown on potato dextrose agar in petri dishes. The spores from each petri dish were suspended in 30ml water. The experiment was maintained for 7 days with samples taken each day for analysis.

2.2.2. Effect of carbohydrate sources on pectinase production of 3 fungal strains

The basal medium was as described earlier (2.1.3) but without pectin and sucrose. The effects of the following were tested on the pectinase production.

1.	Fructose	20g/litre	2%
2.	Glucose	"	2%
3.	Sucrose	"	2%
4.	Starch	"	2%
5.	Pectin	10g/litre	1%
6.	Cellulose	20 "	2%

7. 2% Sucrose + 1% Pectin

8. 2% Sucrose + 1% Pectin + 0.35% Vitamin B-complex.

(For the composition of the Vitamin B-complex, see Appendix II).

The cultures used were No. 1(A); 2(B); and 20(F). The incubation period was 5 days. The experiment was in duplicate for each nutrient. On the 5th day, the pH, protein, Endo-PMG activity and dry mycelia weight were determined.

2.2.3. Effect of nitrogen sources on pectinase production of two fungal strains (F) A. niger and (B) A. repens

The basal medium was modified Czapek Dox medium as described earlier (2.1.3) but without sodium nitrate. The pectin content was 10g/litre. The effects of the following nutrients were investigated:

1.	Sodium Nitrate	2.5g/l
2.	Urea	0.75 "
3.	Peptone	2.5 "
4.	Gelatin	2.5 "
5.	Casein	2.5 "
*6.	Defatted soya flour	2.5 "
*7.	Whole soya flour	2.5 "
8.	Defatted Groundnut flour	2.5 "
**9.	Groundnut cake (35% fat content)	2.5 "
10.	Control - (No nitrogen).	

The cultures used for this study were (F) Aspergillus niger and (B) Aspergillus repens. The inoculum was 470,000 spores per ml for A. niger and 220,000 spores per ml for A. repens. The incubation period was 5 days. The experiment was in duplicate for each nutrient. The pH, protein, Endo-PMG activity and mycelia dry weight were determined on the 5th day.

* See Appendix III for the composition of the soya flours used.

** See Appendix IV for composition.

2.2.4. Effect of Groundnut cake as carbon and nitrogen sources for pectinase production by A. niger and A. repens

The basal medium consisted of the following: (g/litre) K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5.

The effects of the following were investigated.

1.	Groundnut cake	1%
2.	Groundnut cake	2%
3.	Groundnut cake	3%

4.	Groundnut cake	2% +	Pectin	1%
5.	Groundnut cake	2%	"	2%
6.	Groundnut cake	2%	"	3%
7.	Groundnut cake	1%	"	1%
8.	Groundnut cake	2%	"	2% + B-complex.

The Vitamin B-complex composition was as reported earlier. The same procedure was used as reported for previous experiment (2.2.3.).

2.2.5. Effect of temperature on the growth and pectinase production of A. niger and A. repens

The medium used was modified Czapek medium as reported for 2.1.3, but the pectin was 10g/l. Four sets of 15 bottles (each with 100ml medium and 1ml spore inoculum) were used for each of the fungal strains.

One set was incubated at 35 ± 1°C another at 24 ± 1°C and another at 20 ± 1°C in a refrigerated incubator and the fourth at ambient temperatures (22° - 26°C) in the laboratory. Incubation period was seven days.

Triplicate samples for each fungal strain were removed each day from each set on day 3, 4, 5, 6 and 7 of incubation. Each sample was analysed for pH, Endo-PMG activity and protein. The mycelial dry weight of one of the samples was determined for each day.

2.3. Pectinase Production Studies Using Some Locally available Agro-industrial Wastes

Experiments were conducted to screen three locally available agro-industrial wastes for pectinase production according to the

methods of Aziz et al. (1985).

2.3.1. Analysis of wheat bran, rice bran and peanut shell

(a) Proximate analysis for protein, fat, fibre and ash were carried out according to AOAC Methods (1980).

(b) The cold and hot water extracts of wheat bran, rice bran and peanut shell were analysed for pH, reducing sugar, protein, starch and pectin (Ranganna, 1977).

Preparation of Extracts: Twenty grammes of each of wheat bran, rice bran and peanut shell was added to 200ml distilled water. For cold extract, it was made to stand at room temperature for 1 hour, then overnight at 10°C in a refrigerator. For hot water extract, it was autoclaved for 1 hour at 15 psi, cooled and filtered.

2.3.2. Determination of effect of diluent (distilled water and hydrochloric acid 0.01M - 0.3M) on pectinase production by A. niger and A. repens

Ten grammes of each agro-industrial waste wheat bran (WB), rice bran (RB) and peanut shell (PS) was weighed into white heat resistant plastic bottles of 500ml capacity with screw cap. Enough diluent was added just to saturate the bran or peanut shell. The bottles were autoclaved at 15 psi for 1 hour, cooled to room temperature and inoculated with 1ml spore suspension of culture. They were incubated (static) at room temperature for 5 days.

One bottle was removed each day and extracted with distilled water to yield 100ml extract. The extract was analysed for pH, Endo-PMG activity, protein and reducing sugar.

2.3.3. Determination of effect of inoculum on pectinase production by A. niger and A. repens grown on wheat bran, rice bran and peanut shell

Mature colonies of A. niger and A. repens, 1 week old cultures in petri dishes, were used for this experiment. The spore count for A. niger was 470,000 per ml and for A. repens 220,000 per ml. Three spore inoculum sizes studied were 1ml, 10ml and 20ml. The media were prepared with hydrochloric acid 0.05M as diluent and inoculated as in previous experiment (2.3.2). They were all incubated at ambient room temperatures. Samples were removed each day for three days and analysed for pH, Endo-PMG activity and protein.

2.3.4. Determination of effect of nutrient enrichment of wheat bran, rice bran and peanut shell on pectinase production by A. niger and A. repens

The basic media consisted of 10g of each WB, RB and PS- moistened with 0.05M HCl in the ratio 1:2 for WB and RB and 1:3 for PS.

The nutrients investigated were grapefruit peel powder (Citrus decumana var. bigardia; 6.4% pectin), plantain peel powder (Musa sapientum; 6.1% pectin), sucrose, glucose, soluble starch, sodium nitrate, soya bean flour (Glycine max) (whole) and defatted.

In each case, 10g of the basic medium was mixed with 2g of nutrient before addition of diluent. The control consisted of only the basic medium moistened with the diluent. Only one set of media had a combination of three nutrients - grapefruit peel, sucrose and soya bean flour.

The sterilization, inoculation and incubation procedures were as described for previous experiments. After 72 hours incubation, the media were extracted with distilled water to give 100ml of crude enzyme extract in each case. They were analysed for pH, protein and Endo-PMG activity.

2.3.5. Determination of effect of media depth on pectinase production by A. niger and A. repens grown on wheat bran, rice bran and peanut shell

About 200g (in duplicate) of each of WB, RB and PS were moistened with 0.05M HCl. They were sterilized in autoclave at 15 psi for 1 hour. When cool, one set of media was inoculated with A. niger (F) and the second set with A. repens (B). The inoculated media were introduced with sterile spatula into sterilized and pre-marked test tubes arranged in duplicate to give media depths of 2, 4, 6, 8 and 10cms. The test tubes were similar with diameter 2.2cm and length 15cm. Five duplicate tubes were used for each set WBB, RBB, PSB, WBF, RBF and PSF. The tubes were plugged with sterile cotton wool and incubated at ambient temperatures for 3 days. The media were extracted with distilled water in the ratio of 1cm media depth to 10ml water.

The extracts were analysed for pH, protein and Endo-PMG activity.

2.3.6. Enzyme composition of crude filtrates of A. niger and A. repens grown on wheat bran, rice bran and peanut shell

Quantitative analysis of pectinase in the filtrates as described earlier (2.1.3) was done. The filtrates were also assayed

for glucoamylase, α-amylase, proteases and cellulases.

Glucoamylase and α-amylase assay were as described by Ramachandran et al. (1979). One unit of glucoamylase activity releases 1mg of glucose at 60°C and pH 4.2 in 1 hour. One unit of α-amylase activity hydrolyses 1mg starch per ml, per minute at 40°C and pH 4.8. The activity of α-amylase was also monitored viscometrically. The reaction mixture consisted of 1ml enzyme and 5ml 1% starch solution at pH 4 and incubated at 40°C for 10 minutes. One millilitre of 2N HCl was added to stop the reaction.

The acid protease activity was determined by the method of Keay and Wildi (1970). One unit of activity releases one microgramme of tyrosine in 10 minutes at 38°C and pH 2.5.

The C_1-cellulase activity (Filter Paper Assay) was carried out as described by Mandels et al. (1976). One international unit (I.U) of activity releases one micromole of glucose per minute at 50°C and pH 4.8. The C_x-(B-glucanase) activity was determined using carboxy methyl cellulose. The reducing sugars released were determined as for filter paper assay. The C_x-cellulase activity was also monitored viscometrically as reported by Olutiola (1978).

2.4. Crude enzyme Concentration, Characterisation and Toxicological Evaluation

2.4.1. Crude enzyme filtrate concentration

The crude enzyme filtrate was obtained by extracting the wet mouldy bran with distilled water such that 10g (dry weight)

of bran was extracted to get 100ml of enzyme filtrate. Then this was concentrated ten times using a rotary evaporator at room temperature under vacuum to get 10ml of enzyme filtrate.

2.4.2. Characterisation of Endo-polymethyl galacturonase of A. niger and A. repens

Experiments were carried out to study the following characteristics of Endo-PMG-activity:

(a) Optimum pH

(b) Optimum temperature

(c) Time course of enzyme activity

(d) Heat stability of enzyme

(e) Substrate concentration effects on enzyme activity.

2.4.2.1. Optimum pH determination

The pH range investigated was 2.5 to 6.0. Citrate-phosphate buffers used were prepared according to the method of Gomori (1955).

One millilitre of the enzyme, 2ml of the buffer and 3ml of the substrate (1% citrus pectin) were mixed together in a test tube incubated at 30°C. The experiment was in duplicate for each buffer. After 30 minutes, the viscosity was determined in an Ostwald viscometer.

The control samples were prepared in duplicate as above but before addition of substrate, the mixture of enzyme and buffer was placed in boiling water for 15 minutes. After boiling, the substrate was added and incubated at 30°C. The viscosity was determined.

A graph of percentage fall in viscosity, (A) was plotted against pH.

2.4.2.2. Optimum temperature determination

The temperatures investigated in this experiment were 20°, 30°, 40°, 50°, 60°, 70° and 80°C. Duplicate test tubes for B and F (A. repens and A. niger respectively) containing 1ml enzyme, 2ml buffer (pH 4.0) and 3ml substrate (1% citrus pectin) were incubated at each of the above temperatures for 30 minutes. At the end of incubation, the tubes at 20°C were warmed to 30°C before viscosity determination. Those at higher temperatures were cooled in ice bath and then warmed to 30°C before viscosity determination.

A graph of fall in viscosity was plotted against temperature.

2.4.2.3. Determination of time-course of enzyme activity

(1) Experiment to determine the Endo-PMG activity of (B) A. repens at 40° and 80°C and pH 4.0

Fourteen test tubes were incubated at 40°C and another set of 14 tubes were incubated at 80°C. Each tube had 1ml enzyme, 2ml buffer (pH 4.0) and 3ml substrate (1% citrus pectin). Duplicate samples were removed at 10 minute intervals for 1 hour. The tubes were shaken for a few seconds in an ice bath and then in water bath at 30°C before the viscosity was determined in an Ostwald viscometer.

The control consisted of duplicate tubes with 1ml enzyme and 2ml buffer. These were placed in boiling water for 15 minutes. They were cooled to 30°C and the 3ml substrate added before the viscosity was determined.

A graph of change in relative viscosity was plotted against time.

(ii) Experiment to determine the Endo-PMG activity of (F) *A. niger* at 40°C and 60°C and pH 4.5

The procedure was as in previous experiment but the pH was 4.5 and the second set of tubes were incubated at 60°C.

2.4.2.4. Heat stability studies

(i) Heat stability studies of Endo-PMG activity of (B) *A. repens* at pH 4.0 and 90°C

Test tubes containing 1ml of enzyme and 2ml of buffer (pH 4.0) were incubated at 90°C. At 5 minute intervals, two tubes were removed and cooled in ice bath for a few minutes and then warmed at 30°C before the substrate (3ml of 1% citrus pectin) was addéd. It was incubated at 30°C for 30 minutes before viscosity determination.

The control consisted of 1ml enzyme and 2ml buffer in duplicate. They were boiled for 15 minutes. After cooling to 30°C, 3ml of the substrate was added and then it was incubated at 30°C for 30 minutes before viscosity determination.

(ii) Heat stability studies of Endo-PMG activity of (F) *A. niger* at pH 4.5 and 70°C

The procedure was as reported for previous experiment but the temperature was 70°C. A graph of residual enzyme activity was

plotted against time in minutes.

2.4.2.5. Experiment to study the substrate concentration effect on Endo-PMG activities of A. repens (B) and A. niger (F)

The following concentrations of citrus pectin were used for this study: 0.5%. 1.0%, 1.5%, 2.0%, and 2.5%. The pH was adjusted to 4.0 for B and 4.5 for F.

For each concentration, 12 test tubes were incubated at 40°C. Each contained 1ml of enzyme and 2ml buffer. Three millilitres substrate was added to each at the same time. At 10 minutes intervals, duplicate tubes were removed for viscosity determination.

The control test tubes had 1ml enzyme and 2ml buffer. These were plugged with cotton wool and placed in boiling water bath for 15 minutes. After cooling to room temperature, 3ml substrate was added and viscosity determined.

Graphs of change in relative viscosity against time were plotted.

2.4.3. Storage stability studies

2.4.3.1. Effect of pH on storage stability of Endo-PMG activities of A. repens and A. niger at ambient temperatures (24 - 28°C) and in refrigerator at 10 ± 2°C

Procedure:

The pH investigated were 3, 4, 5 and 6. About 500ml of enzyme filtrate was prepared for each fungal strain. This was divided into four (120ml each) and the pH of each was adjusted

and 3ml of appropriate citrate-phosphate buffer added. Fourty millilitres of this was added to each of 3 sample bottles. The procedure was repeated for each pH. They were incubated at room temperature. Samples were analysed for pH, protein and Endo-PMG activity at zero time and each week for four consecutive weeks.

The above procedure was repeated for cold storage in the refrigerator at 10 ± 2°C.

2.4.4. Toxicological studies

The chemical and biological assays were according to the methods of Cole (1984).

2.4.4.1. Chemical screening methods for mycotoxins

Aspergillus repens and A. niger were grown on rice bran as in previous experiments. After 7 days of growth, the cultures were extracted according to the methods of Gorst-Allman and Steyn (1979) (Appendix XI). The rice bran control experiment was prepared as in previous experiment but without inoculating it. It was extracted as above on the same day.

Aspergillus repens and A. niger were grown for 7 days in yeast extract 2% plus sucrose 15% broth (YES) and also in Czapek dox broth (CZ) and extracted according to the methods of Scott et al. (1970).

The absorbance of each extract above was determined in an SP30 spectrophotometer at 366 and 254nm.

The above extracts were also separated by thin layer chromatography and the plates were sprayed with anisaldehyde spray reagent.

Another set of experiments was conducted where only the enzyme filtrates were extracted and examined as above.

2.4.4.2. Quick biological screening method for mycotoxins

The biological screening methods were according to Ngaha (1985) and Essien (1988). Five hundred and forty millilitres of enzyme filtrate was prepared from 540g of 1 week old mouldy rice bran that was inoculated with A. repens. The enzyme filtrate was extracted as in previous experiment (Section 2.4.4.1.). The chloroform layer was evaporated to dryness. The residue was dissolved in 35ml saline solution as reported by Ngaha (1985).

The above procedure was also carried out for 1 week old mouldy rice bran that was inoculated with A. niger.

Twenty Wistar albino rats (5 weeks old) were prepared for this experiment (see Appendix XII for rat weights, liver and kidney weights). Five rats were used for uninoculated rice bran control extract and another five for normal control. Five rats in one cage were used for A. repens extract and the remaining five for A. niger extract. The normal control group was injected subcutaneously with 1ml saline solution (9g/l) for each rat each day for 5 consecutive days. Their urine was collected each day by absorbing it with filter paper which was used to line the special collecting trough under the cage. The A. niger and A. repens test groups were injected similarly with their respective extracts. The uninoculated rice bran control group was treated similarly.

The rats were sacrificed on the sixth day and their blood collected. Some key diagnostic enzymes were assayed in the urine, serum, liver and kidney. These were glutamate oxaloacetate transaminase (GOT), and glutamate (pyruvate transaminase (GPT).

2.5. Enzyme Utilisation Studies

The methods of Viquez et al. (1981) were used. For these experiments, enzyme concentrates prepared as reported earlier (2.4.1.) were used. The enzyme concentrate of A. repens used had 2.38mg/ml protein while that of A. niger had 1.9mg/ml. The fruits used for these studies were banana (Musa sapientum), carrots (Dancus carota), guava (Psidium guajava) and oranges (Citrus sinensis). The effects of adding various amounts of enzyme to the pulps of banana (just ripe and fully ripe), carrots (with and without pH adjustment) and guava (fully ripe) were studied. The parameters investigated were:

(1) pH,

(2) volume of juice,

(3) the dissolved solids content (D.S)

(4) relative viscosity,

(5) optical density at 580nm, and

(6) depectinisation test.

For carrots and guava, 20g of pulp was used while 10g of pulp was used for banana experiments. The experiments were in duplicate for each enzyme concentration studied. In each experiment, the pulp and enzyme were incubated at 40°C for 2 hours.

Juice concentration experiments were carried out using fresh orange juice filtered with a fine cloth. Concentration of control was done at room temperature under reduced pressure using a rotavapor without addition of any enzyme. The dissolved solids content was determined before and after concentration using a hand refractometer.

Procedure for the test was as follows - 50ml of the fresh orange juice at 40°C was introduced into the round bottom flask. 0.5ml enzyme concentrate was added, and the mixture incubated at 40°C for 1 hour before concentrating it with the rotavapor. The dissolved solids content was measured and the volume of the concentrated juice. The concentrate was transferred into a specimen bottle for observation for 2 days before storing at 0 - 4°C. The experiment was repeated using 1ml of enzyme concentrate.

RESULTS

3.1. Selection of Potent Fungal Strains for Pectolytic Enzyme Production

A total of twenty-one fungal strains were collected for screening to obtain potent strains with pectolytic activity. Seven of them were Rhizopus strains while thirteen were Aspergillus strains and one was Geotrichum candidum. More information on the cultures is given in Appendix X.

After the organisms were separated by plating on Czapek agar, an experiment was conducted for their rapid screening for pectolytic activity. The results are in Table 17. Noticeable changes in the media were observed from day 2. The total media depth was 35mm. The media in the control tubes remained translucent with suspended particles throughout the experiment. The data in Table 17 show media depth measured from mycelia mat at the top of the tube. The media in tube Nos. 2 (A. repens), 13 (Aspergillus), 17 (Aspergillus) and 19 (A. carbonarius) became very clear while others remained translucent although without suspended particles.

In order to carry out a quantitative analysis of the various pectolytic enzymes produced by each strain, the cultures were grown in modified Czapek's liquid medium. Two Rhizopus strains - culture Nos. 3 and 4 did not grow under the experimental conditions (static incubation at room temperature for 7 days). The results of the endo- and exo-polygalacturonase enzymes with sodium pectate and apple pectin as substrates, produced by the cultures are given in Tables 18 and 19. The pH of all the

TABLE 17. Rapid screening of cultures for pectolytic activity.

No.	Culture	Extent of clear zone ± 2mm						
		DAY	DAYS					
		1	2	3	4	5	6	7
1	Rhizopus	0	10	18	T	T	T	T
2	Aspergillus repens	0	10	20	20	20	20	35
3	Rhizopus	0	11	T	T	T	T	T
4	Rhizopus	0	10	18	T	T	T	T
5	Aspergillus	0	10	T	T	T	T	T
6	Aspergillus	0	9	18	T	T	T	T
7	Aspergillus	0	10	16	T	T	T	T
8	Aspergillus	0	10	16	T	T	T	T
9	Aspergillus	0	10	15	T	T	T	T
10	Rhizopus	0	10	20	T	T	T	T
11	Aspergillus	0	10	18	T	T	T	T
12	Aspergillus	0	12	15	T	T	T	T
13	Aspergillus	0	8	15	20	20	35	35
14	Rhizopus	0	10	T	T	T	T	T
15	Rhizopus	0	10	18	T	T	T	T
16	Rhizopus	0	10	15	T	T	T	T
17	Aspergillus	0	10	20	T	T	35	35
18	Aspergillus	0	10	16	T	T	T	T
19	Aspergillus carbonarius	0	9	15	15	15	35	35
20	Aspergillus niger	0	8	15	T	T	T	T
21	Geotrichum candidum	0	10	10	T	T	T	T

Note: On Day 1, all were translucent with suspended particles.

T = Translucent throughout but without suspended particles.

TABLE 18. Quantitative analysis of pectinase of culture fluids.

No.	Culture	pH	Protein ug/ml	Action on Sodium Pectate				Action on Apple Pectin			
				ENDO-PG		EXO-PG		ENDO-PG		EXO-PG	
				A	± S.D	Units/ml	± S.D	A	± S.D	Units/ml	± S.D
1	Rhizopus	3.10	52	63	± 5.5	0.88	± 0.16	57	± 5.9	0.79	± 0.16
2	Aspergillus repens	2.40	46	35	5.7	0.60	0.03	57	5.9	0.56	0.16
5	Aspergillus	2.85	64	10	5.0	0.82	0.02	19	3.9	2.34	0.08
6	Aspergillus	2.80	73	42	7.9	0.60	0.22	22	3.8	0.56	0.08
7	Aspergillus	2.85	71	14	7.7	0.82	0.08	13	3.5	0.84	0.02
8	Aspergillus	3.00	71	10	5.8	1.88	0.03	16	3.3	0.64	0.12
9	Aspergillus	2.90	73	6	4.3	0.54	0.16	24	3.1	0.75	0.04
10	Rhizopus	2.80	77	6	4.3	0.60	0.22	36	2.9	0.70	0.04
11	Aspergillus	2.85	81	6	6.2	0.66	0.04	9	3.3	1.78	0.20
12	Aspergillus	2.80	73	44	5.9	0.58	0.03	60	2.4	0.82	0.02
13	Aspergillus	3.00	72	44	5.9	0.42	0.09	55	2.5	0.56	0.12
14	Rhizopus	3.20	55	44	5.9	0.58	0.04	54	2.5	0.56	0.02
15	Rhizopus	2.85	62	44	5.9	0.51	0.08	71	2.4	0.51	0.04
16	Rhizopus	2.90	72	44	5.9	0.22	0.02	44	2.6	0.42	0.02
17	Aspergillus	3.30	60	83	4.6	0.81	0.03	83	2.4	0.66	0.02
18	Aspergillus	2.20	69	24	6.1	0.75	0.12	14	3.7	0.62	0.04
19	Aspergillus carbonarius	2.70	76	24	6.1	0.64	0.09	44	2.6	0.60	0.08
20	Aspergillus niger	2.90	67	56	5.6	0.47	0.12	81	2.3	0.62	0.20
21	Geotrichum candidum	3.30	76	0	0	0.92	0.03	22	2.9	0.51	0.12

A = Percentage reduction in viscosity.

$A = \frac{T_o - T_t}{T_o - T_w} \times 100$. Where T_o = Time (sec.) for control; T_t = Time for test; T_w = Time for water.

S.D = Standard Deviation.

TABLE 19. Polymethyl esterase (PME) pectate lyase (PGL) and pectin lyase (PMGL) activities of nine culture fluids.

No. (from table 17)	Culture	PME Micro-equiv./ min./ml	PGL OD_{235}/min., (milli-units)	PMGL OD_{235}/min. (milli-units)
1	Rhizopus	1.067	0.71	0.85
2	Aspergillus repens	3.067	0.73	0.83
8	Aspergillus	1.633	0.73	0.79
11	Aspergillus	1.833	0.69	0.79
13	Aspergillus	1.600	0.74	0.74
15	Rhizopus	1.767	0.74	0.74
17	Aspergillus	0.933	0.76	0.75
19	Aspergillus carbonarius	2.000	0.73	0.68
20	Aspergillus niger	1.533	0.73	0.71

cultures ranged between 2.2 - 3.3. The results indicated the following cultures to have the highest Endo-PG activity against sodium pectate - culture No. 17 Aspergillus (A = 83); No. 1 Rhizopus (63); and No. 20 A. niger (56). Highest Endo-PG activity against apple pectin was shown by the following: culture No. 17 Aspergillus (83), No. 20 A. niger (81), No. 15 Rhizopus (71), No. 2 A. repens (57).

Furthermore, quantitative determinations of the polymethyl esterase (PME), pectate lyase (PGL) and pectin lyase (PMGL) activities of nine cultures were carried out. The cultures were Nos. 1, 2, 8, 11, 13, 15, 17, 19 and 20. The results are in Table 19. Aspergillus repens and A. carbonarius had the highest PME activities, whereas no striking differences in the activities of PGL and PMGL existed among the cultures.

3.2. Identification of the most potent local fungal isolate

General morphology of the fungus leads to a group diagnosis as follows:

> Conidia bluish-green in young colonies, becoming dull green to brownish green with the colour partially masked by reddish brown pigment encrusting on Czapek Dox agar. The stalks are thin walled and smooth, vesicles globose, phialides born directly on the vesicles covering the whole of it. This description leads to the Aspergillus glaucus group. About 4 or so species belong to this group.

3.2.1. Specific Identification

More detailed morphological studies led to the identification of the specific member of a group series as follows:-

Hyphae were stolon-like at edges of the colonies. The surface was dirty green with yellowish tint. The reverse of a mature culture dish (about 1 week old) on Czapek Dox Agar was brownish red. Very pale yellowish green on PDA. The conidial heads were large, the conidia were smooth or very nearly so. The fungus was said to be of the A. glaucus series and in particular Aspergillus repens.

3.3. Nutritional Studies of Potent Strains

The most economical strain to be finally selected among the potent strains must not require expensive nutrients and excessive energy input for pectinase production; therefore experiments were designed to investigate the nutritional and temperature requirements of the potent strains. The first vital information needed before the nutritional investigations could proceed was the minimum time required for maximum pectinase production by the potent strains. Table 20 shows the results of the experiment to find out the minimum time required for maximum pectinase production by each of the six potent strains - culture Nos. 1, 2, 13, 15, 17 and 20. The data in Table 20 was plotted in Fig. 4 to show the relationship between percentage reduction in viscosity (A) of Endo-PMG activity and growth time in days of the strains.

TABLE 20. Determination of minimum time for maximum pectinase production in six static cultures grown in modified Czapek Dox liquid medium at ambient temperatures.

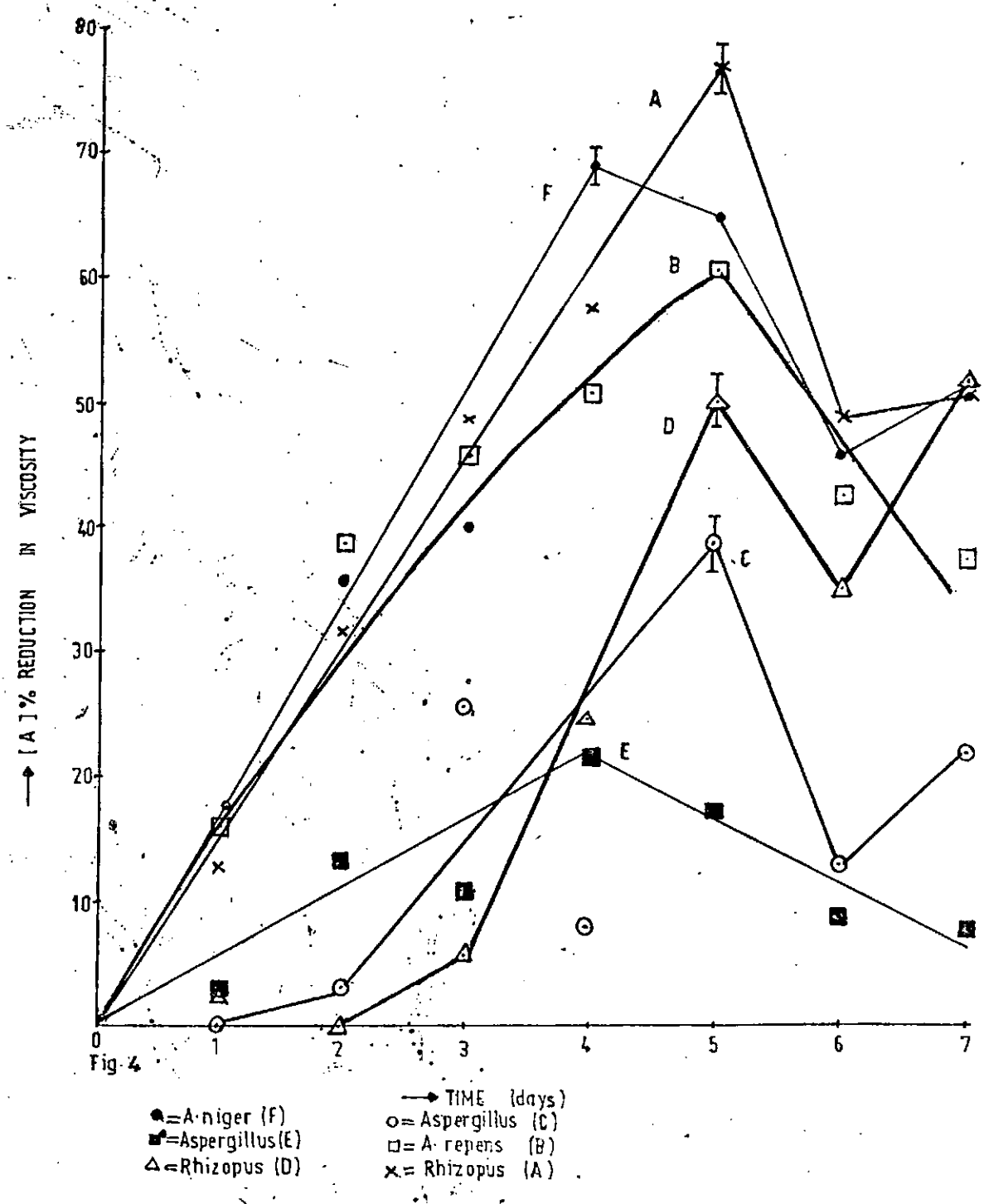
	Culture	DAY 1			DAY 2			DAY 3			DAY 4			DAY 5			DAY 6			DAY 7		
		A	SD	df	A	SD	df	A	SD	df	A	SD	df	A	SD	df	A	SD	df	A	SD	df
1	Rhizopus	13.2	3.7	11	31.8	6.5	9	49.0	5.5	11	58.0	8.4	12	77.3	4.1	8	49.1	3.4	7	50.7	5.5	7
2	Aspergillus repens	16.0	5.3	10	38.7	2.7	9	45.8	2.9	11	50.8	4.0	14	61.0	1.8	8	42.9	2.6	10	37.7	5.0	8
13	Aspergillus	0	0	10	0	0	12	25.5	2.7	10	8.0	5.4	11	39.0	6.0	9	13.2	3.2	7	22.0	4.4	7
15	Rhizopus	0	0	11	0	0	12	6.3	6.8	9	24.9	8.7	7	50.5	4.2	7	35.4	1.6	10	52.2	1.3	7
17	Aspergillus	0	0	11	13.5	5.6	13	10.9	2.2	8	22.3	6.3	9	17.6	5.2	7	9.0	5.8	11	7.7	4.1	7
20	Aspergillus niger	17.9	5.7	11	35.8	2.9	9	39.6	9	10	68.8	1.3	7	64.9	3.3	8	46.0	2.1	11	51.9	1.5	6

A = % reduction in viscosity

SD = standard deviation

df = degrees of freedom.

PERCENTAGE REDUCTION IN VISCOSITY [A] AGAINST TIME (DAYS)



Fig·4

Peak enzyme activity was on the 5th day for culture No. 1, Rhizopus (A); No. 2, A. repens (B); No. 15, Rhizopus (D); and No. 13, Aspergillus (C). Peak activity was on the 4th day for culture No. 17, Aspergillus (E) and finally it was 4 to 5th day for culture No. 20, A. niger (F). The best 3 strains from this experiment were Rhizopus (A), A. niger (F) and A. repens (B). They were studied further for the effect of carbohydrate sources on pectinase production. The results of this study are in Table 21. Although the mycelia weights of the three strains were similar (0.02 - 0.53g), the endo-PMG and protein contents of the filtrates were different. The results were generally poor for endo-PMG activities of Rhizopus (0 - 24%). The highest enzyme activity for A. niger was from a mixture of sucrose, pectin and vitamin B-complex (85%), corresponding value was 35% for same mixture for A. repens. The highest enzyme activity for A. repens was from glucose (54%) and sucrose (51%) for A. niger. Vitamin B-complex greatly increased enzyme activity on A. niger ($P<0.001$) and Rhizopus ($P<0.001$) but it had no effect on A. repens. The protein content of filtrates from A. repens was in all cases much higher than from the other two strains.

The two Aspergillus strains were selected for further studies. The next investigation was on the effect of nitrogen sources on pectinase production by A. niger and A. repens. The results are in Table 22. From the results, endo-PMG activities of A. repens were generally low (4 - 39%) compared to those of A. niger (38 - 81%). Although both grew fairly well

TABLE 21. Effect of Carbohydrate sources on pectinase production of three fungal strains.

Nutrient		Initial pH	Aspergillus niger					Rhizopus					Aspergillus repens				
			Final pH	Endo-PMG A	*SEM	Protein ug/ml	Mycelia wt(gms)	Final pH	Endo-PMG A	*SEM	Protein ug/ml	Mycelia wt(gms)	Final pH	Endo-PMG A	*SEM	Protein ug/ml	Mycelia wt(gms)
Fructose	2%	4.05	5.59	59	1.2	60	0.301	2.50	13	1.0	6	0.337	2.71	43	0.5	126	0.271
Glucose	2%	4.04	5.22	34	1.0	7	0.222	2.89	11	0.8	3	0.285	3.41	54	0.9	32	0.158
Sucrose	2%	3.95	4.38	53	0.8	7	0.222	2.84	4	1.0	1	0.305	2.96	51	0.5	88	0.165
Starch	2%	4.03	6.64	29	3.4	1	0.115	3.21	11	0.3	1	0.184	2.64	17	1.5	25	0.179
Pectin	1%	3.94	4.54	32	1.9	4	0.044	4.41	15	1.1	7	0.051	4.31	46	0.4	32	0.037
Cellulose	2%	3.97	4.32	6	1.2	0	0.082	3.70	0	0	0	0.021	4.03	11	0.7	3	0.092
Sucrose + Pectin	2% + 1%	3.93	4.62	62	2.3	10	0.510	3.75	8	1.1	6	0.486	3.47	34	0.5	119	0.515
Sucrose + 1% Pectin + $B_{com.}$	2% +	3.93	4.35	85	1.1	25	0.483	3.91	24	1.5	20	0.522	3.38	35	0.7	82	0.533

*SEM = Standard Error of the mean

n = 12.

TABLE 22. Effect of Nitrogen sources on pectinase production of two fungal strains *A. niger* (F) and *A. repens* (B).

NUTRIENT	*Aspergillus niger* (F)					*Aspergillus repens* (B)				
	pH	Endo-PMG A	*SEM	Protein (ug/ml)	Mycelia dry wt. (gms)	pH	Endo-PMG A	*SEM	Protein (ug/ml)	Mycelia dry wt. (gms)
Sodium Nitrate 2.5g/l	4.81	49.3	±0.9	100	0.360	4.14	36.3	±2.1	143	0.235
Urea 0.75g/l	3.12	75.0	1.0	106	0.488	2.64	38.6	0.7	145	0.420
Peptone 2.5g/l	3.53	73.8	0.9	172	0.493	2.55	37.3	2.3	198	0.422
Gelatin 2.5g/l	3.67	62.1	0.8	163	0.158	3.18	9.4	1.2	175	0.247
Casein 2.5g/l	3.42	58.0	2.4	125	0.437	3.62	4.8	0.7	163	0.195
Defatted soya flour 2.5g/l	3.53	73.3	1.5	153	0.291	3.62	4.5	0.8	190	0.271
Whole soya flour 2.5g/l	3.63	71.6	1.3	125	0.262	3.59	12.7	1.0	215	0.291
Defatted Groundnut flour 2.5g/l	3.53	68.7	1.5	112	0.362	3.22	27.0	2.7	215	0.334
Groundnut cake 2.5g/l	3.68	81.2	0.9	157	0.261	3.62	21.8	0.7	183	0.261
Control (No Nitrogen)	3.44	37.5	1.0	157	0.090	3.28	13.7	0.3	132	0.184

*SEM = Standard Error of the mean

n = 12.

under the experimental conditions, but enzyme filtrates of A. repens had more protein. For A. niger, groundnut cake gave the highest enzyme activity (81%) while sodium nitrate gave the lowest (49%); apart from control. For A. repens urea gave the highest enzyme activity (39%) while casein and defatted soya flour gave the lowest, 4.8% and 4.5% respectively. Generally, A. repens showed lack of ability to utilise complex proteins for pectinase production whereas A. niger showed ability to utilise both simple and compl x nitrogen sources for pectinase production.

Further experiments were conducted to find out the effect of using groundnut cake as both carbon and nitrogen sources for pectinase production by A. niger and A. repens. The results are in Table 23. It showed similar trend with previous experiment for complex nitrogen sources. However, the addition of Vitamin B-complex to groundnut cake (2%) and pectin (2%) very greatly decreased enzyme production ($P < 0.001$) by both A. niger and A. repens.

The effect of the following temperatures 35 $\pm$ 1°; 24 $\pm$ 1°; 20 $\pm$ 1°C and ambient 22 - 26°C on the growth and pectinase production of A. niger and A. repens was investigated. The results are presented in Table 24. The graphs of endo-PMG activity, protein, pH and mycelia weight were plotted against time (days) for both A. repens and A. niger side by side for easy comparison purposes. These are illustrated in Figs. 5, 6, 7 and 8. Generally, in all cases, increase in mycelia dry weight was small up to day 4 or 5. Between day 5 and 6, there

TABLE 23. Effect of Groundnut Cake+ as Carbon and Nitrogen source for pectinase production by A. niger and A. repens.

Nutrient	Aspergillus niger					Aspergillus repens				
	pH	PMG A	± *SEM	Protein (ug/ml)	Mycelial Dry wt.(gms)	pH	PMG A	± *SEM	Protein (ug/ml)	Mycelial Dry wt. (gms)
Groundnut Cake 1%	4.36	69.5	±2.9	12	0.191	4.46	14.7	±1.5	25	0.063
Groundnut Cake 2%	4.46	71.0	1.5	29	0.089	4.16	27.2	3.0	50	0.091
Groundnut Cake 3%	4.79	80.1	0.4	46	0.132	4.00	36.3	4.0	56	0.123
Groundnut Cake 2% + Pectin 1%	4.72	80.1	1.5	30	0.132	3.70	16.5	1.3	34	0.130
Groundnut Cake 2% + Pectin 2%	4.74	82.9	1.5	41	0.156	3.50	19.2	2.6	55	0.133
Groundnut Cake 2% + Pectin 3%	4.33	76.1	1.5	47	0.131	3.64	21.6	1.6	59	0.148
Groundnut Cake 1% + Pectin 1%	4.44	77.0	1.4	20	0.077	3.56	13.9	2.8	28	0.075
Groundnut Cake 2% + Pectin 2% + **Vitamin B complex	4.73	69.8	2.9	73	0.260	3.61	6.7	1.8	83	0.316

*SEM = Standard Error of the mean

n = 12

+See Appendix Table IV for composition.

**Vitamin B complex 3.5g/l. See Appendix Table II for composition.

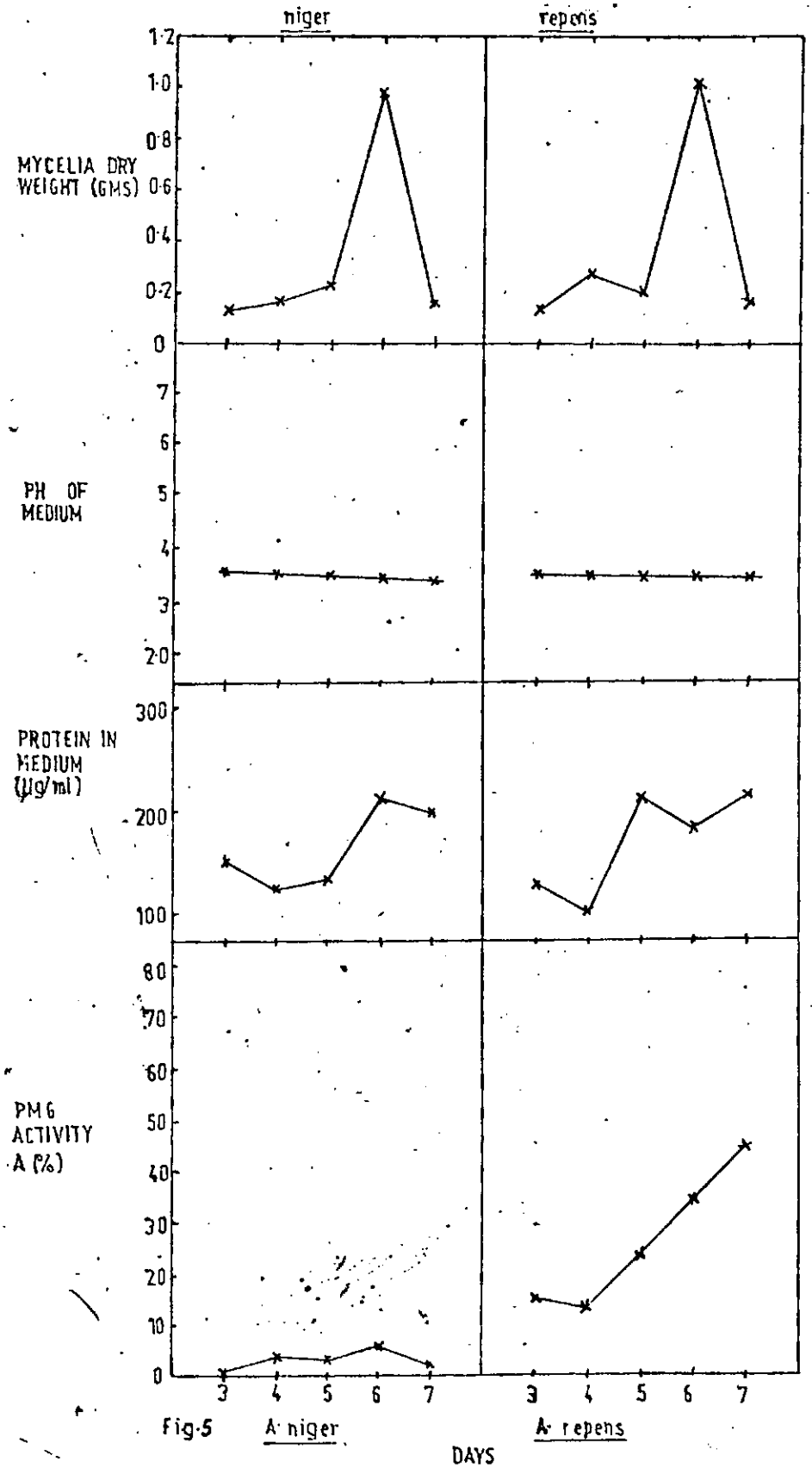
TABLE 24. Effect of temperature on the growth and pectinase production of A. niger and A. repens.

Temperature °C	A %	Protein ug/ml	pH	Mycelia wt(gms)
DAY 3 A. niger				
35	8.7	156	4.55	0.2771
24	11.5	152	3.62	0.2703
20	1.1	151	3.57	0.1153
Ambient	2.8	154	3.61	0.2338
DAY 3 A. repens				
35	41.4	147	2.99	0.2634
24	25.7	134	3.49	0.2357
20	15.3	132	3.59	0.1258
Ambient	30.0	159	3.45	0.1357
DAY 4 A. niger				
35	6.6	131	4.66	0.3444
24	6.8	140	3.79	0.3337
20	3.7	125	3.56	0.1561
Ambient	4.4	128	3.66	0.2106
DAY 4 A. repens				
35	42.1	126	2.71	0.2556
24	39.0	105	3.05	0.2671
20	13.5	104	3.49	0.1198
Ambient	37.0	123	3.06	0.1813
DAY 5 A. niger				
35	0.0	139	5.36	0.3556
24	12.8	115	3.83	0.4346
20	3.4	165	3.55	0.2248
Ambient	19.3	147	3.70	0.3451

TABLE 24 (Contd.)

Temperature °C	A %	Protein ug/ml	pH	Mycelia wt(gms)
DAY 5 *A. repens*				
35	59.6	161	2.29	0.4567
24	62.6	137	2.78	0.4549
20	22.5	134	3.48	0.2006
Ambient	58.9	170	2.96	0.3158
DAY 6 *A. niger*				
35	0.0	192	5.73	0.9132
24	33.6	188	3.76	1.3716
20	6.0	214	3.49	0.9762
Ambient	20.8	192	3.77	1.3452
DAY 6 *A. repens*				
35	69.2	214	2.21	1.3961
24	68.5	180	2.43	1.4814
20	36.2	186	3.48	1.0161
Ambient	68.8	204	2.79	1.3557
DAY 7 *A. niger*				
35	0.0	164	6.50	0.3867
24	18.4	152	3.76	0.6601
20	2.1	198	3.44	0.1475
Ambient	22.9	198	2.97	0.2829
DAY 7 *A. repens*				
35	58.7	264	2.17	0.4812
24	58.7	244	2.24	0.4952
20	44.4	212	3.50	0.1552
Ambient	54.7	238	3.75	0.3310

20 ± 1°c


niger
repens
MYCELIA DRY WEIGHT (GMS)
1·2
1·0
0·8
0·6
0·4
0·2
0
PH OF MEDIUM
7
6
5
4
3
2·0
PROTEIN IN MEDIUM (µg/ml)
300
200
100
PMG ACTIVITY A (%)
80
70
60
50
40
30
20
10
0
3
4
5
6
7
A. niger
A. repens
DAYS

Fig·5

AMBIENT TEMP 22 – 26 °c

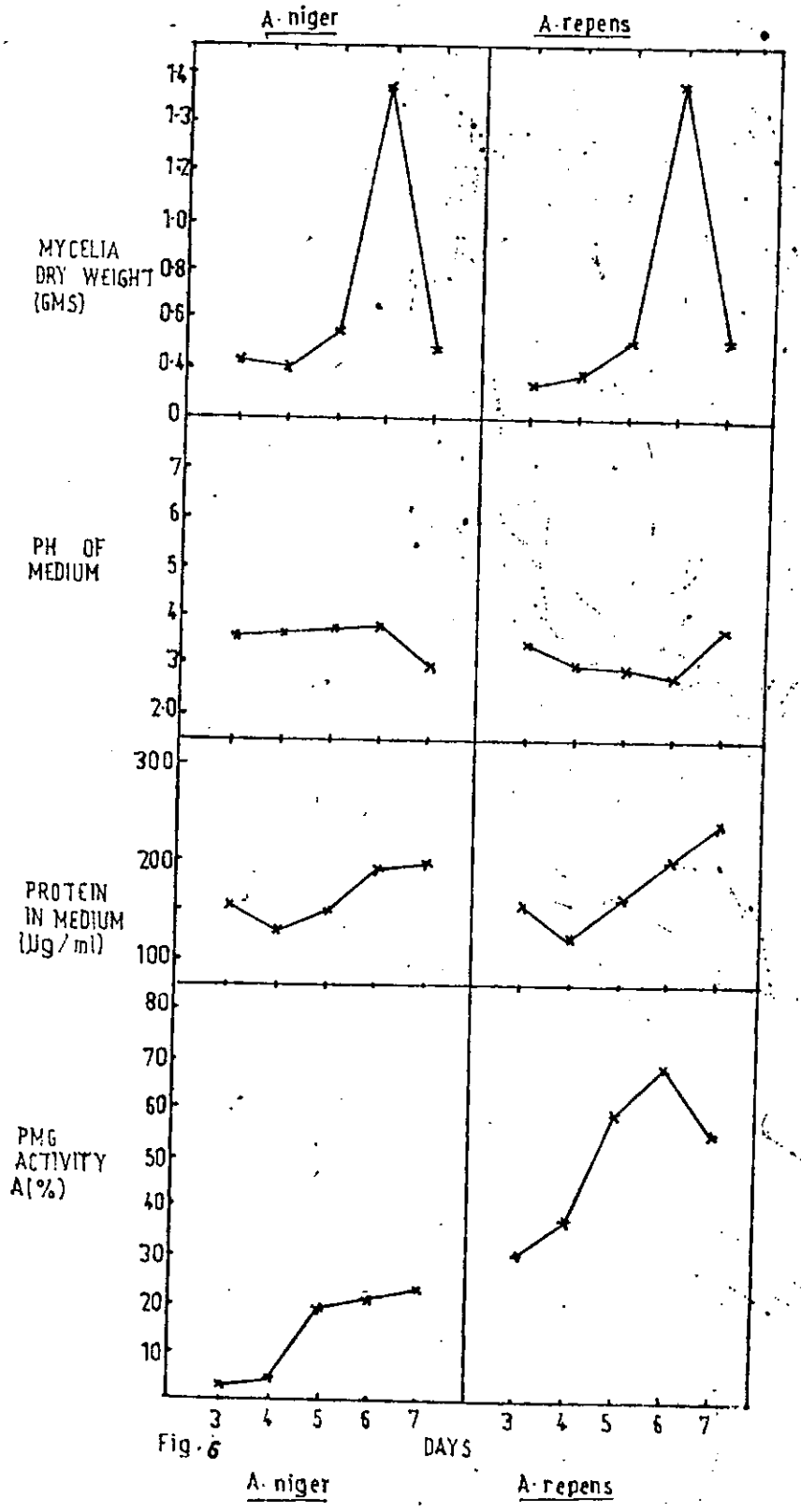
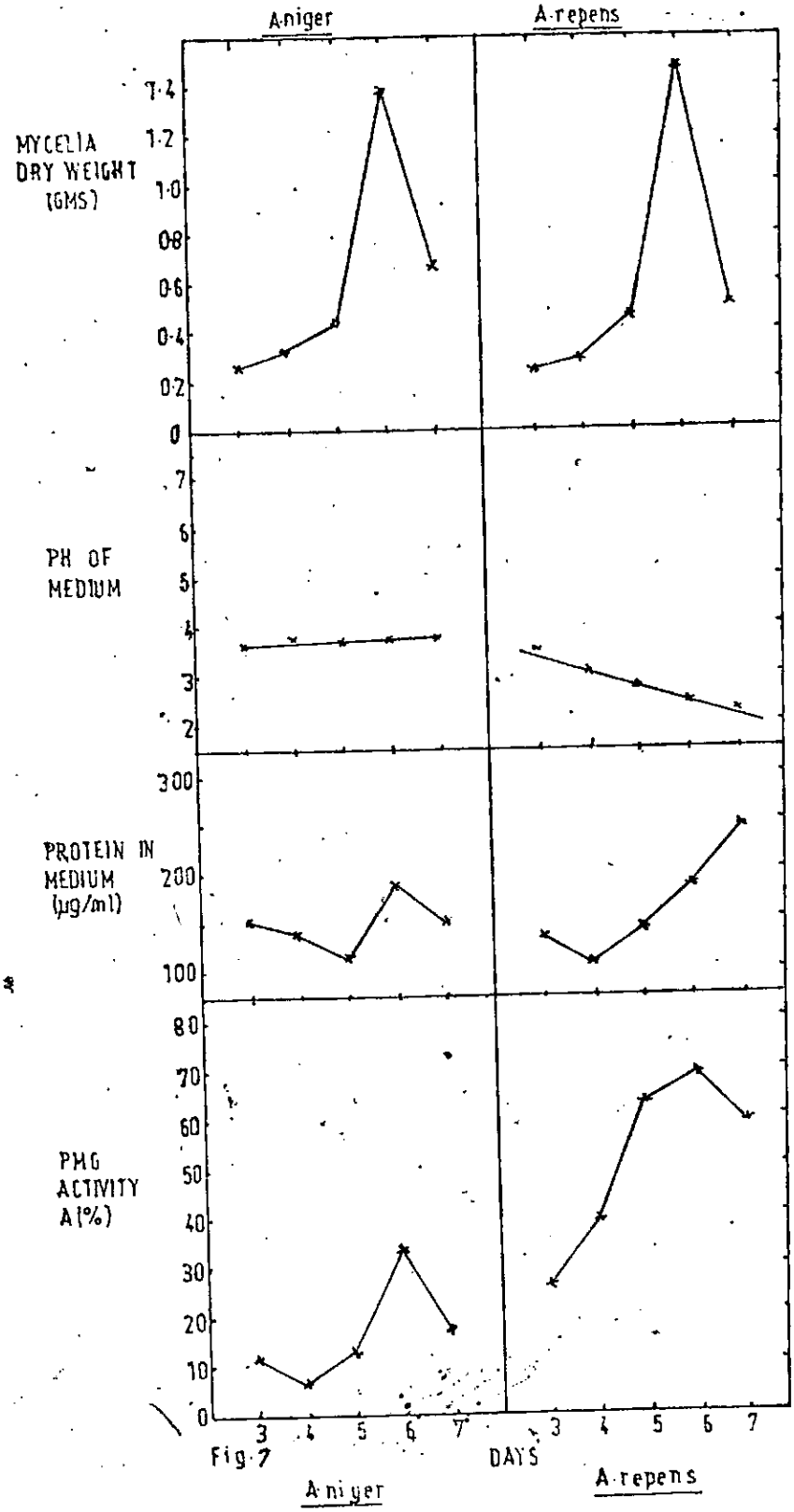


Fig. 6

24 ± 1°


A.niger
A.repens
MYCELIA DRY WEIGHT (GMS)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
PH OF MEDIUM
7
6
5
4
3
2
PROTEIN IN MEDIUM (µg/ml)
300
200
100
PHG ACTIVITY A(%)
80
70
60
50
40
30
20
10
0
3 4 5 6 7
DAYS
3 4 5 6 7
A.niger
A.repens

Fig·7

35 ± 1°c

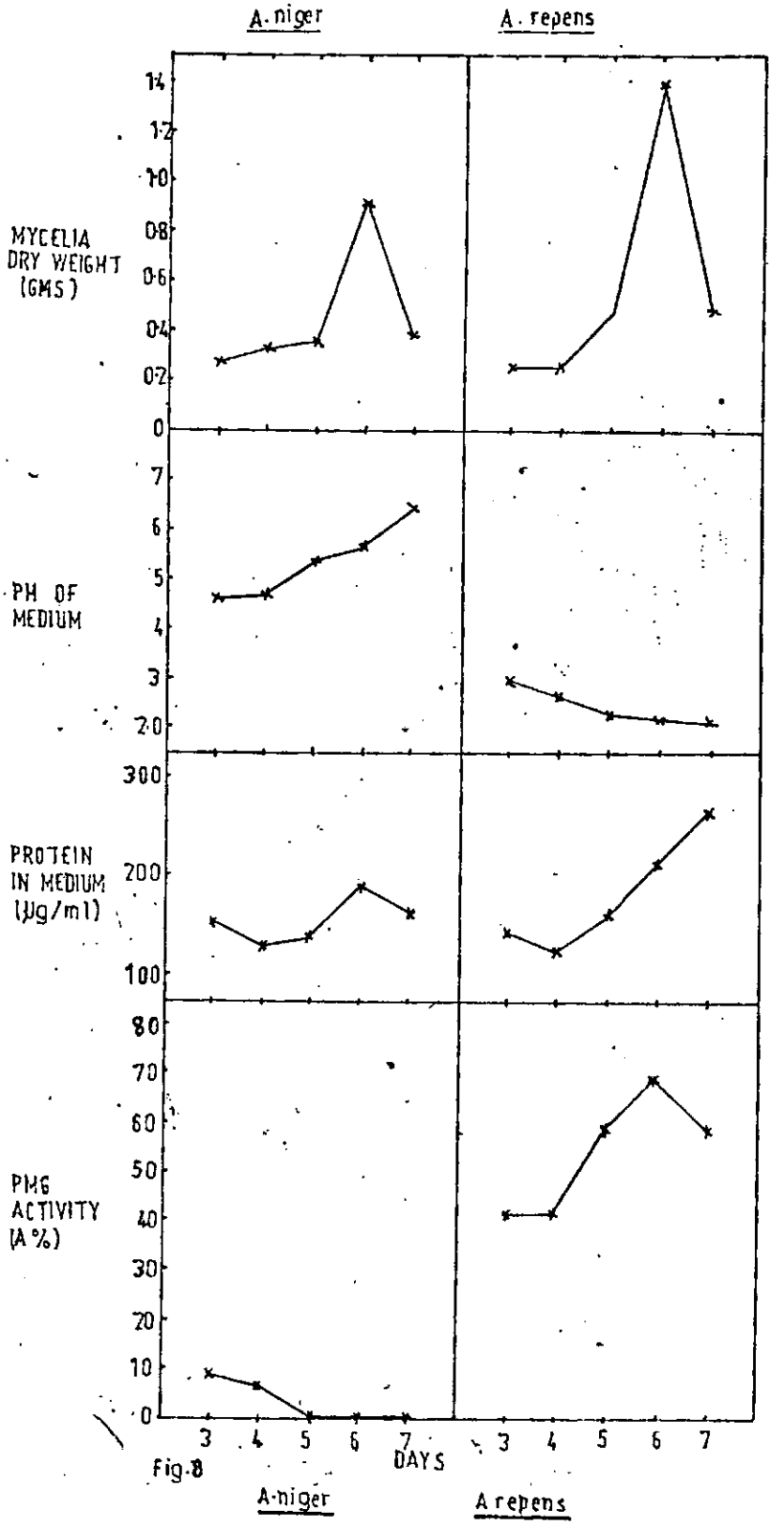

A. niger
A. repens
MYCELIA DRY WEIGHT (GMS)
PH OF MEDIUM
PROTEIN IN MEDIUM (µg/ml)
PMG ACTIVITY (A%)
DAYS
A·niger
A repens

Fig.8

was sharp increase in mycelia weight getting to a climax on day 6. This was followed by a sharp decrease in mycelia weight between day 6 and 7; by day 7, it had fallen back to where it was on day 5. Aspergillus niger produced very little endo-PMG (less than 10% activity) at 35° and 20°C. This was a marked contrast to the performance of A. repens which had enzyme activities 40 - 70% at 35°C and 10 - 45% at 20°C. For A. niger, at 35°C, the protein in the medium decreased slightly from day 3 to 4 and remained constant till day 5 before it rose sharply to the maximum (about 200ug/ml) on day 6; then it fell in day 7 to about 160ug/ml. Meanwhile, the pH profile was the highest (4.5 to 6.5) in all the experiments. For A. repens, the protein level decreased slightly from day 3 to 4 after which there was steady rise up to day 7 to about 260ug/ml, the highest in this experiment. Meanwhile, there was more or less constant decrease in pH from day 3 to 7 (pH 3 to 2). At 20°C incubation, the protein and pH profiles were similar for both Aspergillus strains. However, for A. repens, there was a sharp increase in protein level from day 4 to 5 corresponding to sharp increase in enzyme activity during the same period. The sharp increase in protein activity for A. niger was between day 5 and 6 corresponding to period of maximum increase in mycelia weight.

All the curves for A. niger at 24°C are essentially similar to those obtained when it was incubated at ambient temperatures, except for some differences between day 6 and 7.

At 24°C both the enzyme and protein profiles followed a similar pattern with the mycelia dry weight. The maximum protein (188ug/ml) and enzyme activity (34%) were on day 6 coinciding with time of maximum mycelia weight. The pH was practically constant throughout. At ambient temperatures, the protein and enzyme activity continued to rise between day 4 and 7 whereas the pH dropped between day 6 and 7. The maximum enzyme activity (23%) and protein (200ug/ml) were on day 7.

For A. repens, all the curves at 24°C incubation and at ambient temperatures were quite similar except for the pH increase between day 6 and 7 at ambient temperatures whereas, at 24°C, the pH maintained a steady decrease. At both temperatures, the maximum enzyme activities, which were the same (70%), occurred on day 6 coinciding with time for maximum mycelia weight. Enzyme activity fell slightly on day 7.

The investigations on the effect of temperature on growth and pectinase production by the two Aspergillus strains showed that although they both produced good amounts of protein in the medium (100 - 170ug/ml); A. repens grew well (1 - 1.4g mycelia weight) and produced pectolytic enzymes in good amounts (45 - 70%) whereas A. niger also grew well (0.9 - 1.4g mycelia weight) but at 35°C and 20°C, little or no pectinase was produced (less than 10%) and at 24°C and ambient temperatures, not much pectinase was produced (less than 35% enzyme activity).

3.4. Pectinase production studies using some locally available Agro-industrial wastes as semi-solid growth media for *Aspergillus niger* and *Aspergillus repens*

Three locally available agro-industrial wastes - wheat bran, rice bran and peanut shell, were analysed to determine their nutrient composition. The results of their proximate composition are in Table 25 while those of their cold and hot water extracts are in Table 26. Wheat bran was particularly rich in carbohydrate (55%) and protein (21%). Rice bran was rich in carbohydrate (29%), fibre (35%) and ash (17%). Peanut shell was particularly rich in fibre (65%) and carbohydrate (12%). Moreover, the cold and hot water (extractable) nutrient composition of these media would be critical for supporting the growth of the *Aspergillus* strains and for pectinase production. From the nutritional studies already carried out in the liquid media, the major nutrients of importance were sugars, proteins, pectin and starch. The reducing sugar content of the media ranged between 8 and 19mg/g. Hot water extraction gave significant increase in amount of reducing sugar extractable from wheat bran but there was no significant difference in sugar extraction by hot water from rice bran and peanut shell. The protein content of extracts ranged from 0.8 to 2.3mg/g without significant differences between cold and hot water values. The starch content of wheat bran extracts was exceptionally high - 537mg/g (cold) and 697mg/g (hot). Those of rice bran (10 - 20mg/g) and peanut shell (2 - 15mg/g) were very low, although in both cases, hot water extraction gave very significant increase in starch extraction. The pectin content of the cold water extract

TABLE 25.— Proximate composition of wheat bran, rice bran and peanut shell.

Parameters	Wheat bran %	Rice bran %	Peanut shell %
Moisture	13.12	7.12	9.81
Protein	21.35	6.71	5.60
Ash	4.18	17.44	3.52
Fibre	4.83	35.12	65.42
Fat	1.78	4.82	4.00
Carbohydrate	54.74	28.77	11.65

TABLE 26. Composition of cold and hot water extracts of wheat bran, rice bran and peanut shell.

Parameters	Cold Water Extract			Hot Water Extract		
	Wheat Bran	Rice Bran	Peanut Shell	Wheat Bran	Rice Bran	Peanut Shell
pH	6.26	5.15	6.05	6.00	5.00	5.60
Reducing sugar (mg/g)	12.38	9.32	8.39	19.42	8.44	8.88
Protein (mg/g)	2.34	0.80	1.29	2.04	1.44	1.26
Starch (mg/g)	537	10	2	697	20	15
Pectin (%)	6.1	1.2	0.4	2.8	1.1	1.2

of wheat bran was particularly high (6%); those of the other two ranged from 0.4% to 2.8%. Hot water extraction gave rise to very significant increase in extractable pectin from peanut shell - 0.4% (cold) to 1.2% (hot). Pectin content of hot water extract was the same with that of cold water extract for rice bran but for wheat bran, hot water extraction reduced the extractable pectin considerably from 6.1% (cold) to 2.8% (hot).

Two vital information are necessary in order to use the three semi-solid media efficiently for pectinase production. These are:

(1) the most adequate diluent needed, and

(2) the optimum time necessary for maximum pectinase production by each fungal strain when grown on each of the three media.

Therefore experiments were carried out to determine the effect of diluent (0 - 0.3M hydrochloric acid) on pectinase production by A. repens grown on wheat bran, rice bran and peanut shell. Similar experiments were carried out for pectinase production studies using A. niger. The results are in Tables 27 and 28. They were plotted in graphs of Endo-PMG activity (A %/; pH; protein (ug/ml) and reducing sugar (mg/ml) against time (days) in Figs. 9 - 15. Aspergillus repens and A. niger curves were plotted side by side for easy comparison purposes.

When distilled water was the diluent, the maximum endo-PMG activity produced by A. repens was 57% on day 2 from rice bran and 33% on day 3 from wheat bran. No enzyme was produced from peanut shell. Aspergillus niger did better; within 24 hours it

TABLE 27. Effect of diluent on pectinase production by Aspergillus repens grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Diluent	Semi-Solid Medium	DAY 0 pH	DAY 0 Endo-PMG A	DAY 0 Protein ug/ml	DAY 0 Red Sugar mg/ml	DAY 1 pH	DAY 1 Endo-PMG A	DAY 1 Protein ug/ml	DAY 1 Red Sugar mg/ml
Distilled Water	WB	6.56	0	56	0.63	5.39	0	58	1.40
	RB	5.73	0	164	0.43	5.62	12	140	1.66
	PS	5.58	0	102	0	6.57	0	76	0
0.01M HCl	WB	5.66	0	56	1.6	4.91	19.5	72	1.79
	RB	5.31	0	192	0.79	4.82	9.9	150	1.66
	PS	4.76	0	106	0	6.05	20.8	70	0
0.05M HCl	WB	5.02	0	194	1.57	4.29	70.7	242	1.23
	RB	4.43	0	318	0.53	4.53	60.0	292	1.06
	PS	5.05	0	158	0.25	5.24	58.5	114	0.53
0.10M HCl	WB	2.98	0	152	3.15	2.88	55.1	158	10.85
	RB	2.11	0	204	2.44	3.03	61.7	184	2.39
	PS	2.97	0	96	1.53	2.14	0	78	1.79
0.15M HCl	WB	3.57	0	226	5.87	3.18	46.2	214	15.21
	RB	2.64	0	298	7.73	2.55	0	250	6.20
	PS	1.97	0	170	4.21	1.82	0	158	6.28
0.20M HCl	WB	2.07	0	208	11.0	2.00	26.7	220	5.57
	RB	1.46	0	236	7.72	1.94	0	244	8.21
	PS	1.90	0	96	2.50	1.46	0	90	3.81
0.30M HCl	WB	1.64	0	222	11.78	1.65	0	234	10.74
	RB	1.20	0	214	8.32	1.41	0	232	9.86
	PS	1.40	0	96	3.87	1.20	0	102	5.68

TABLE 27 (Contd.)

Diluent	Semi-Solid Medium	DAY 2 pH	Endo-PMG A	Protein ug/ml	Red Sugar mg/ml	DAY 3 pH	Endo-PMG A	Protein ug/ml	Red Sugar mg/ml
Distilled Water	WB	5.78	7.5	96	1.25	4.07	32.9	78	9.64
	RB	4.92	57.3	152	0.71	5.14	47.7	84	2.23
	PS	6.54	0	90	0	6.94	0	64	0
0.01M HCl	WB	4.08	39.0	82	2.94	4.04	53.7	73	6.89
	RB	4.68	75.9	142	1.91	5.06	67.9	70	2.04
	PS	5.82	24.3	74	0	6.47	0	43	0.34
0.05M HCl	WB	2.79	67.6	296	3.20	3.88	82.1	336	3.02
	RB	4.46	86.0	208	0.69	5.11	81.6	216	0.73
	PS	5.19	62.6	128	0.59	5.57	53.5	134	0.69
0.10M HCl	WB	3.20	61.8	162	1.95	3.63	61.6	96	9.42
	RB	2.95	83.4	166	2.04	3.44	89.0	94	1.53
	PS	2.20	39.9	58	0	2.89	49.5	35	0
0.15M HCl	WB	2.77	55.9	240	16.42	2.83	69.6	208	9.16
	RB	2.44	26.8	276	4.44	2.60	37.2	248	6.02
	PS	1.86	8.4	162	5.44	1.92	0	138	3.44
0.20M HCl	WB	2.14	31.7	210	5.85	2.66	6.9	98	12.72
	RB	1.85	23.8	242	7.66	2.38	0	118	7.66
	PS	1.45	0	92	2.61	2.07	0	52	3.48
0.30M HCl	WB	1.64	0	200	12.28	2.11	0	103	13.3
	RB	1.44	7.2	234	8.98	1.95	0	102	10.9
	PS	1.37	0	98	0	1.75	0	52	4.47

TABLE 27 (Contd.)

Diluent	Semi-Solid Medium	DAY 4 pH	DAY 4 Endo-PMG A	DAY 4 Protein ug/ml	DAY 4 Red Sugar mg/ml	DAY 5 pH	DAY 5 Endo-PMG A	DAY 5 Protein ug/ml	DAY 5 Red Sugar mg/ml
Distilled Water	WB	5.02	29.3	102	0	3.19	31.9	105	5.08
	RB	6.15	10.7	112	12.50	5.33	0	103	1.15
	PS	8.52	0	59	0	7.43	0	76	0.53
0.01M HCl	WB	4.47	49.3	106	7.66	3.80	37.8	104	5.46
	RB	5.93	48.9	102	1.05	5.12	31.3	93	0
	PS	7.77	37.1	531	0.53	7.46	41.5	69	0
0.05M HCl	WB	4.45	78.7	346	3.06	5.93	52.6	306	0.82
	RB	5.29	80.3	212	0.84	5.26	80.3	200	0.12
	PS	5.86	18.0	132	0.17	6.36	0	138	0.31
0.10M HCl	WB	4.20	56.3	115	10.85	3.47	42.9	120	10.68
	RB	4.18	80.3	109	7.61	3.32	44.9	105	1.47
	PS	3.75	0	69	3.21	3.10	29.5	58	3.65
0.15M HCl	WB	3.14	74.5	210	5.54	3.31	78.8	346	10.04
	RB	2.77	88.4	238	0.86	1.93	22.4	204	5.54
	PS	1.88	10.5	136	3.57	1.98	7.2	274	4.11
0.20M HCl	WB	3.58	47.9	135	11.89	2.77	3.0	135	10.02
	RB	3.23	0	131	5.63	2.37	0	153	5.02
	PS	2.72	25	73	4.36	2.05	0	96	0
0.30M HCl	WB	2.86	0	128	12.50	2.08	0	130	12.66
	RB	2.70	0	128	10.30	1.94	0	141	8.49
	PS	2.47	9.1	67	0.43	1.74	0	79	5.02

TABLE 28. Effect of diluent on pectinase production by Aspergillus niger grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Diluent	Semi-Solid Medium	DAY 0 pH	DAY 0 Endo-PMG A	DAY 0 Protein ug/ml	DAY 0 Red Sugar mg/ml	DAY 1 pH	DAY 1 Endo-PMG A	DAY 1 Protein ug/ml	DAY 1 Red Sugar mg/ml
Distilled Water	WB	6.13	0	55	1.35	5.44	24.6	186	3.06
	RB	5.16	0	175	0.91	5.04	69.5	290	1.46
	PS	5.82	0	147	0.84	6.66	14.2	138	0.80
0.01M HCl	WB	5.85	0	56	1.20	5.26	34.1	204	1.61
	RB	5.05	0	171	0.87	5.07	60.5	296	0.86
	PS	5.59	0	129	0.76	6.33	21.5	140	0.35
0.05M HCl	WB	5.21	0	56	1.17	4.73	46.8	226	2.25
	RB	4.60	0	170	1.04	4.54	65.7	102	0.80
	PS	4.64	0	112	0.84	5.11	79.6	234	1.38
0.10M HCl	WB	4.62	0	72	1.40	4.30	72.3	230	3.40
	RB	4.05	0	179	1.00	4.02	92.5	276	1.50
	PS	3.66	0	107	0.84	4.07	50.6	84	0.40
0.15M HCl	WB	3.57	0	226	5.87	3.09	62.7	194	5.98
	RB	2.64	0	298	7.73	2.56	9.3	234	6.02
	PS	1.97	0	170	4.21	1.83	0	176	4.44
0.20M HCl	WB	3.27	0	125	3.11	3.15	62.7	340	4.21
	RB	3.09	0	180	1.20	2.95	47.0	352	2.49
	PS	2.53	0	131	1.30	2.42	11.6	160	1.17
0.30M HCl	WB	2.82	0	158	9.40	2.52	15.8	322	4.27
	RB	2.60	0	188	2.45	2.44	4.9	286	4.25
	PS	2.10	0	141	1.77	2.04	11.3	274	3.79

TABLE 28 (Contd.)

Diluent	Semi-Solid Medium	DAY 2 pH	DAY 2 Endo-PMG A	DAY 2 Protein ug/ml	DAY 2 Red Sugar mg/ml	DAY 3 pH	DAY 3 Endo-PMG A	DAY 3 Protein ug/ml	DAY 3 Red Sugar mg/ml
Distilled	WB	5.0	34.5	260	3.92	5.13	34.7	282	1.81
Water	RB	5.15	78.7	220	2.87	5.55	79.8	228	0.15
	PS	7.41	0	164	1.46	7.59	0	160	0.74
0.01M	WB	5.13	36.5	284	3.02	5.03	28.8	282	2.32
HCl	RB	5.18	66.4	228	2.14	5.49	81.8	214	0.76
	PS	7.35	0	148	1.46	7.67	0	162	0.79
0.05M	WB	4.09	68.6	272	5.09	3.86	85.0	276	3.13
HCl	RB	5.03	85.4	192	2.51	5.25	89.0	214	0.82
	PS	5.75	55.1	124	2.07	6.17	58.1	134	0.15
0.10M	WB	4.20	76.9	264	4.76	4.02	87.0	296	3.11
HCl	RB	4.32	90.0	236	2.65	4.70	91.9	238	0.13
	PS	4.64	60.9	102	2.10	5.04	90.0	114	0.69
0.15M	WB	2.95	83.7	220	13.67	2.90	88.2	216	7.62
HCl	RB	2.60	5.5	284	8.15	2.62	11.9	246	1.91
	PS	1.60	0	178	4.11	1.94	0	162	0.67
0.20M	WB	3.25	75.3	366	8.43	3.20	77.7	332	3.50
HCl	RB	2.95	85.6	276	3.48	3.35	91.8	280	0.91
	PS	2.40	18.3	170	2.21	2.96	79.8	124	0.04
0.30M	WB	2.61	34.5	310	8.54	2.64	45.8	362	12.9
HCl	RB	2.39	0	340	7.22	2.31	11.2	278	3.13
	PS	1.96	0	314	4.51	1.97	0	262	2.51

TABLE 28 (Contd.)

Diluent	Semi-Solid Medium	DAY 4 pH	DAY 4 Endo-PMG A	DAY 4 Protein ug/ml	DAY 4 Red Sugar mg/ml	DAY 5 pH	DAY 5 Endo-PMG A	DAY 5 Protein ug/ml	DAY 5 Red Sugar mg/ml
Distilled Water	WB	4.04	74.0	322	7.39	4.79	83.5	254	3.11
	RB	5.72	72.3	252	0.91	5.86	76.1	212	0.74
	PS	7.21	0	186	0.91	8.02	7.3	168	0.42
0.01M HCl	WB	4.13	83.3	260	2.88	4.82	97.5	230	2.65
	RB	5.64	81.4	252	0.59	5.96	91.1	196	0.71
	PS	7.15	0	170	0.76	7.97	0	168	0.56
0.05M HCl	WB	4.23	87.6	332	9.86	4.68	90.6	258	3.06
	RB	5.54	88.6	206	0.84	5.72	97.5	190	0.86
	PS	6.07	58.5	136	0.82	6.49	80.5	156	0.64
0.10M HCl	WB	4.36	88.5	302	7.61	5.66	111.8	252	2.21
	RB	4.95	95.7	228	0.98	5.32	101.1	212	0.64
	PS	5.08	95.7	122	0.84	5.30	100.0	110	0.59
0.15M HCl	WB	3.22	90.2	220	4.22	3.53	93	324	2.79
	RB	2.62	2.5	234	2.49	2.52	2.4	346	4.66
	PS	1.92	3.9	160	1.25	1.90	1.4	282	4.77
0.20M HCl	WB	3.33	80.0	360	9.09	3.34	91.0	292	3.13
	RB	3.36	92.5	268	0.56	3.42	104.3	220	0.78
	PS	2.90	84.9	116	0.82	2.94	90.5	98	0.64
0.30M HCl	WB	2.88	51.8	340	11.84	2.66	75.1	300	9.7
	RB	2.44	30.8	376	6.62	2.32	3.0	308	1.61
	PS	2.08	7.0	262	2.36	1.93	11.7	244	2.01

DILUENT DISTILLED WATER

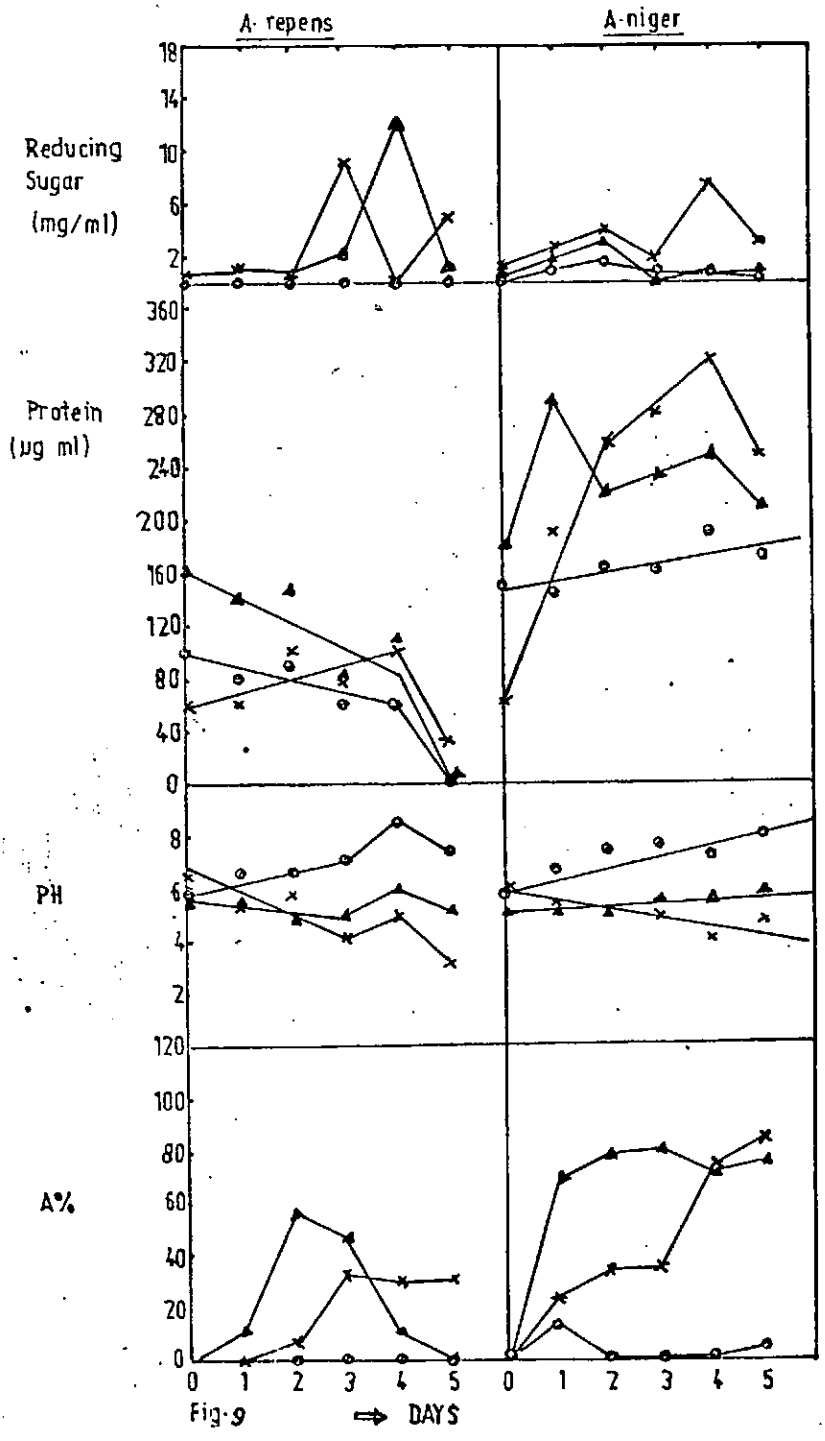
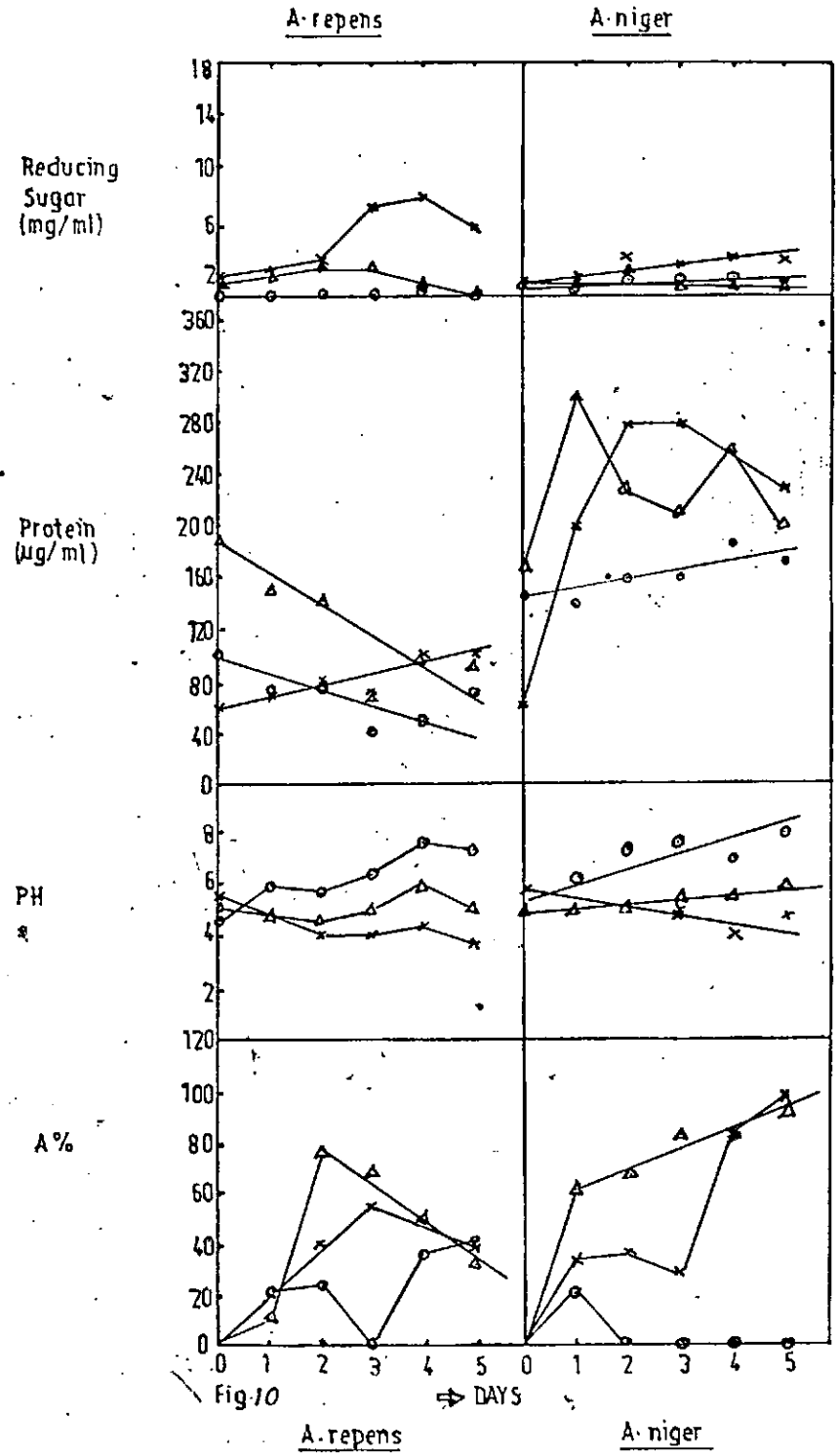


Fig. 9

DILUENT 0·01M



Fig·10

DILUENT 0.05M HCL

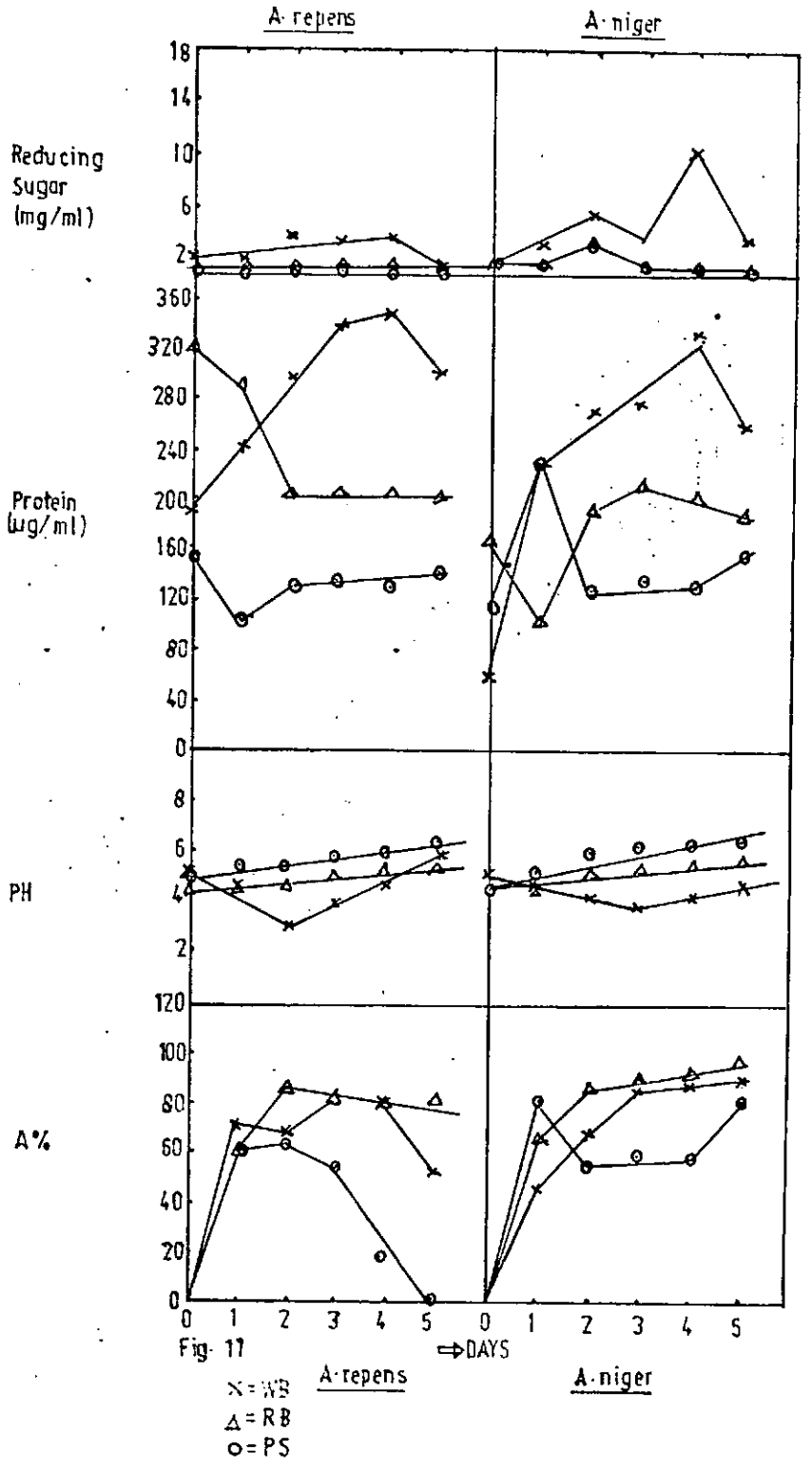


Fig. 11

DILUENT 0·1M HCL

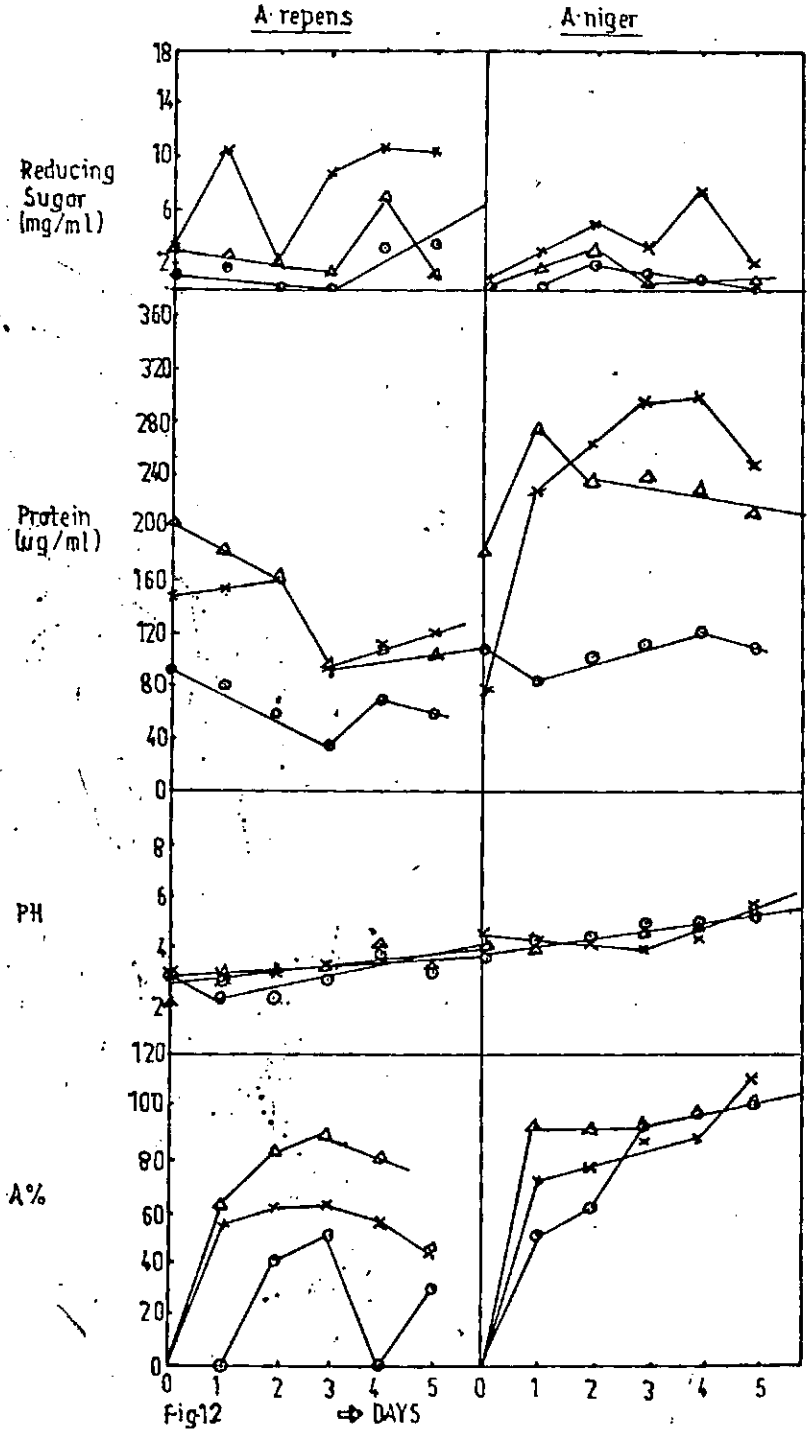


Fig 12

DILUENT 0·15 M HCL

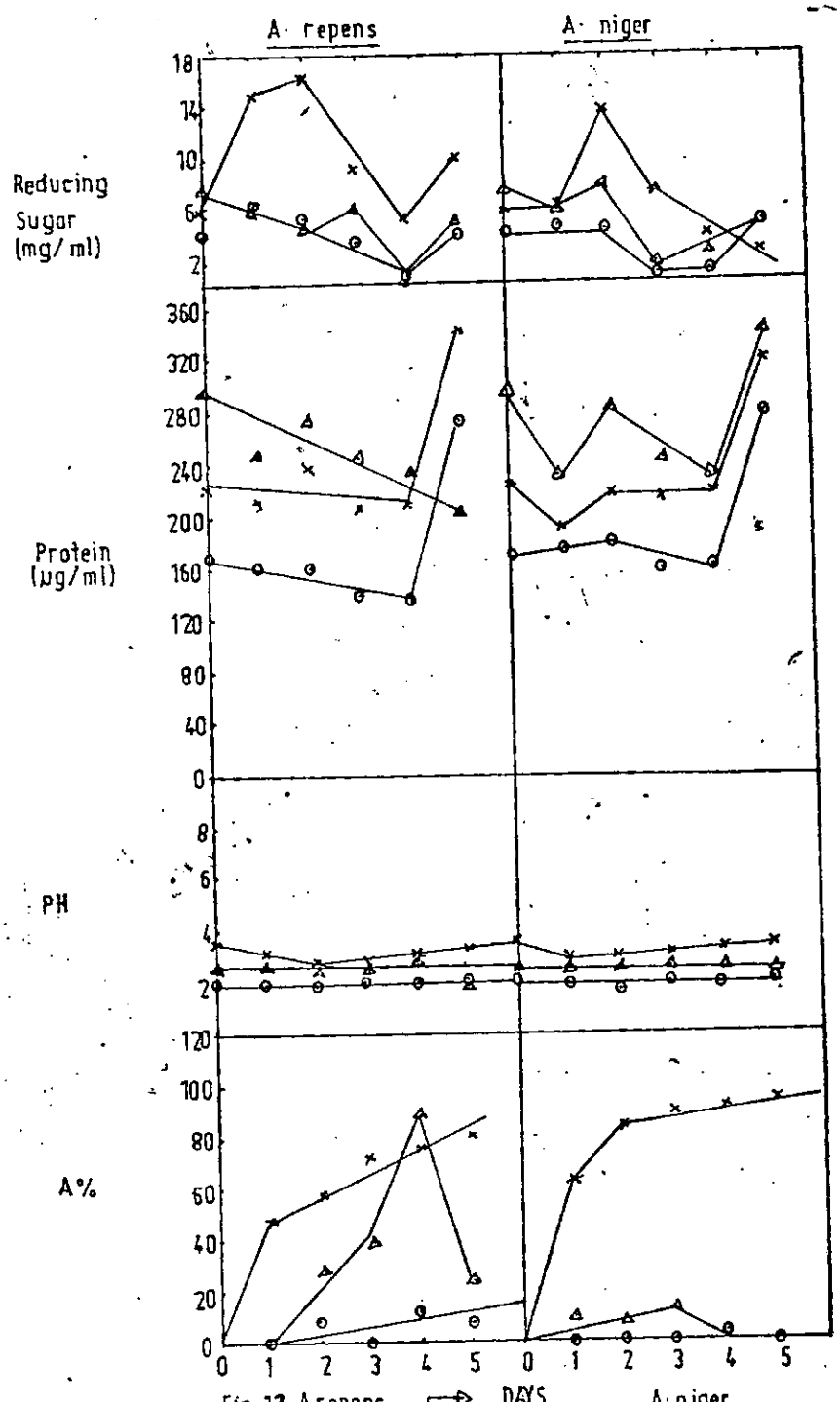


Fig 13, A·repens ⟹ DAYS A·niger

× = WB
△ = RB
○ = PS

DILUENT 0·2 M HCl

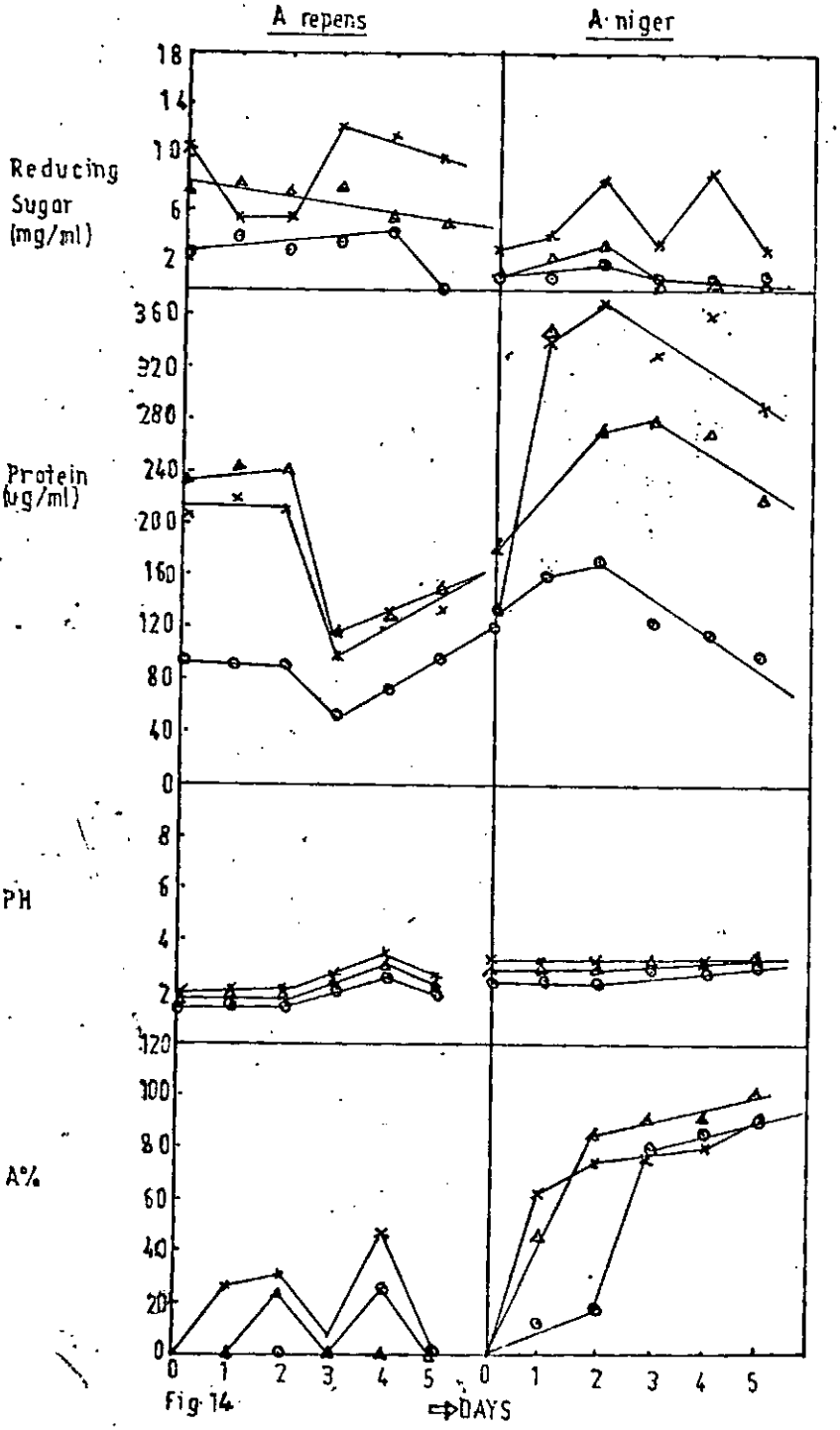


Fig 14

DILUENT 0·3M HCL

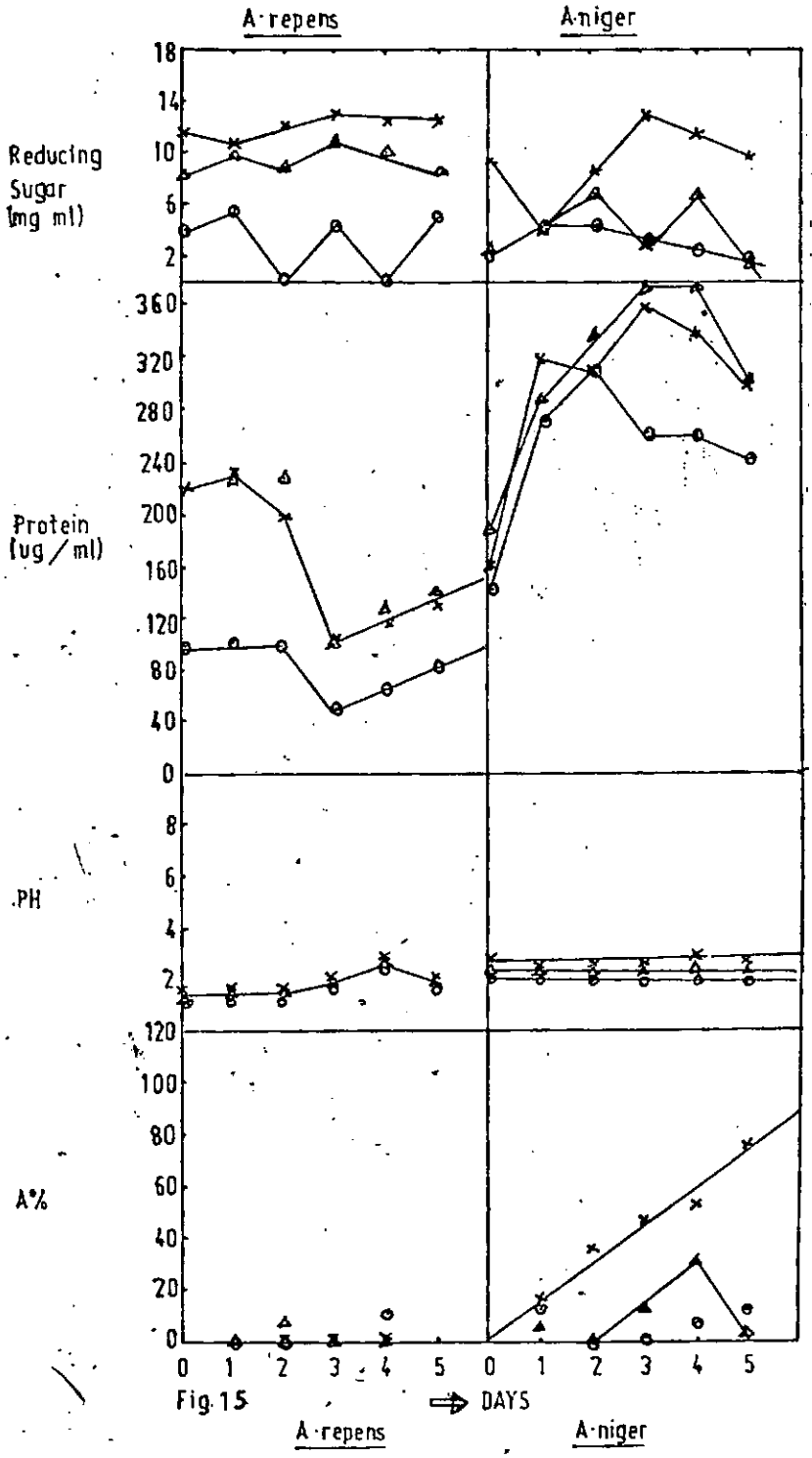


Fig. 15

produced 70% enzyme activity in rice bran rising to 80% on day 2 and 3, remaining above 70% on day 4 and 5. In wheat bran, the activity was 25% on day 1, rising to 35% on day 2 and 3, then rising sharply to 74% on day 4 and 84% on day 5. Little or no enzyme was produced in peanut shell (14% in day 1). The pH profiles of the two strains were similar. The enzyme extracts from rice and wheat brans were below pH 6 while those from peanut shell were between pH 6 and 8. For A. repens, the protein content of extracts from rice bran and peanut shell decreased with time while it generally increased for wheat bran. For A. niger, the protein content generally increased with time. The reducing sugar content of all the media were generally low. For A. repens, there was a sharp peak on day 3 and a smaller one on day 5 for wheat bran, these coincided with enzyme peak on day 3 - 5. Rice bran also had a sharp peak on day 4. This coincided with fall in enzyme activity from peak of day 3. For A. niger, the reducing sugars from the three media had similar profile except for a small peak on day 4 for wheat bran.

When 0.01M hydrochloric acid was used as diluent, the curves were generally similar to that of distilled water as diluent. The main difference was the enzyme activity (20 - 40%) appearing in peanut shell for A. repens which did not produce any enzyme with distilled water. With 0.05M hydrochloric acid as diluent, the best enzyme production was achieved in all the media by both Aspergillus strains. For A. repens, enzyme activity was 60 - 70% in day 1 for all media. This level was exceeded in rice bran in day 2 (86%); subsequently about 80% activity was maintained on

days 3 - 5. In wheat bran, the level rose from 70% in day 1 and 2 to 80% in day 3 and 4 before it fell to 50% in day 5. In peanut shell, the enzyme level fell from 60% in day 1 and 2 to 54% in day 3 and then sharply to below 20% in day 4 and finally to zero in day 5. For A. niger in day 1, enzyme activity rose sharply to 80% in peanut shell, it dropped to 55% in day 2 and maintained that level till day 4 before it rose to 80% again in day 5. The enzyme activity level in rice bran in day 1 was 66%, it rose to 85% in day 2 and continued a gradual rise to 98% in day 5. The enzyme activity level in wheat bran in day 1 was 47% it rose to 69% in day 2 and then 85% in day 3 and it eventually rose to 91% in day 5. The pH profiles of extracts of the three media produced by both Aspergillus strains were identical. The reducing sugar contents of all the extracts were very low as in previous experiments. The protein content of extracts of the media produced by both Aspergillus strains were also similar.

With 0.1M hydrochloric acid as diluent, the enzyme activity curves of both Aspergillus strains were essentially identical with the previous experiment (0.05M HCl), however, there were some notable differences. For A. repens, peanut shell showed no enzyme activity between day 0 and 1; peak activity were day 2 (40%), and day 3 (50%); then activity dropped to zero on day 4 before rising to 30% again on day 5. For A. niger, enzyme activity in peanut shell in day 1 was 50% but it rose to 61% in day 2, 90% in day 3, 96% in day 4 and finally 100% in day 5. In rice bran, enzyme activity rose sharply to 93% and maintained this level throughout and even rising slightly higher to 101% in day 5. In wheat bran,

enzyme activity rose to 72% in day 1, it continued to rise steadily to 89% in day 4 and finally to 112% in day 5. This particular diluent gave the best result for all the three media in this experiment. The pH profiles of both strains were similar to those of previous experiment (0.05M HCl). The same was true for protein content of extracts except for the fall in protein profile of wheat bran for A. repens. The reducing sugar profiles for A. repens indicated generally high reducing sugar (10mg/ml) in wheat bran extracts.

With 0.15M hydrochloric acid as diluent, there was little or no enzyme activity in peanut shell extracts for both Aspergillus strains. For A. repens, enzyme activity rose to 50% in day 1 for wheat bran. It then rose gradually to 80% in day 5. In rice bran, enzyme activity rose from 0% in day 1 to 90% in day 4 and then dropped sharply to 20% in day 5. For A. niger, wheat bran was the only one favoured for enzyme production with this diluent. Enzyme activity rose from 63% in day 1 to 84% in day 2, then it rose gradually to 93% in day 5. The profiles of pH, protein and reducing sugar were similar for both Aspergillus strains. The reducing sugar content of all extracts were higher than in previous experiments. The reducing sugar from wheat bran was the highest, increasing to 15 - 16mg/ml in day 1 - 2 for A. repens and 14mg/ml in day 2 for A. niger.

With 0.2M hydrochloric acid as diluent, only A. niger showed much enzyme activity in the three media. In day 1, there was 45% activity in rice bran rising to 90% in day 2 and then to 104% in day 5. Activity in wheat bran was 60% rising to 75% in day 2

from then it rose steadily to 91% in day 5. With 0.3M hydrochloric acid as diluent, there was no significant enzyme activity in the three media inoculated with A. repens; whereas with A. niger, only wheat bran showed significant enzyme activity. The activity rose steadily from 16% in day 1 to 75% in day 5.

From these results, it is clear that the most adequate diluent was 0.05 - 0.1M hydrochloric acid; and the optimum time necessary for maximum pectinase production was 1 - 3 days.

After the basic conditions necessary for pectinase production in wheat bran, rice bran and peanut shell as semi-solid media were established, further experiments were conducted to investigate other factors which could affect pectinase production from the three media. Such other factors investigated were -

(1) effect of inoculum size;

(2) effect of nutrient enrichment, and

(3) effect of media depth.

The results of these investigations are in Tables 29 - 34. The results of the effect of inoculum on pectinase production by A. repens showed that for rice bran, there were no significant differences in enzyme activities among the three inoculum sizes (1, 10 and 20mls spore suspension) tested. In the case of wheat bran, 10mls spore suspension (about 220,000 spores/ml) gave the best result; it was significantly higher than the result for 1ml inoculum ($P < 0.001$). For peanut shell, 1ml inoculum gave a significantly higher enzyme activity ($P < 0.001$) than the other two inoculum sizes. For A. niger experiments, all the inoculum sizes

TABLE 29. Effect of inoculum on pectinase production by Aspergillus repens grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Media	Inoculum (mls)	DAY 1				DAY 2				DAY 3			
		Endo-PMG				Endo-PMG				Endo-PMG			
		pH	A	±SEM	Protein ug/ml	pH	A	±SEM	Protein ug/ml	pH	A	±SEM	Protein ug/ml
WB	1	4.85	0	±0.5	222	5.20	37.3	0.5	294	4.92	57.4	0.8	388
	10	4.80	49.3	0.4	260	5.19	27.5	1.2	296	5.21	64.0	1.0	396
	20	4.76	45.4	0.4	244	5.10	43.8	0.5	316	4.80	54.0	0.4	370
RB	1	4.63	38.8	0.6	272	5.50	61.4	1.7	286	5.47	73.2	1.1	304
	10	4.47	20.0	1.1	236	5.25	51.2	1.5	324	5.40	70.0	0.4	348
	20	4.55	24.4	0.9	202	5.00	60.3	0.5	310	5.12	72.1	0.3	364
PS	1	4.82	48.1	0.9	148	5.28	51.1	1.3	180	5.36	72.9	0.6	226
	10	4.42	24.4	0.9	136	4.73	37.0	0.4	180	4.86	54.7	0.6	164
	20	4.20	36.7	6.2	152	5.17	53.8	0.6	186	4.86	56.8	0.7	218

TABLE 30. Effect of inoculum on pectinase production by Aspergillus niger grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Media	Inoculum (mls)	DAY 1 Endo-PMG pH	A	±SEM	Protein ug/ml	DAY 2 Endo-PMG pH	A	±SEM	Protein ug/ml	DAY 3 Endo-PMG pH	A	±SEM	Protein ug/ml
WB	1	4.97	47.2	0.4	206	5.20	56.9	0.3	322	4.81	79.6	0.3	372
	10	5.07	56.5	0.4	160	5.18	60.6	0.3	340	4.86	80.0	0.8	364
	20	4.99	56.8	1.0	198	5.11	71.5	0.3	318	4.80	70.6	0.2	370
RB	1	4.76	68.8	0.7	204	5.75	67.7	0.2	258	5.41	88.4	0.6	288
	10	4.59	74.3	0.9	216	5.27	74.2	0.6	280	5.45	88.0	0.7	346
	20	4.58	48.7	1.1	204	5.25	74.2	0.2	318	5.20	85.1	0.7	348
PS	1	4.77	62.7	0.5	140	5.11	64.6	0.3	192	5.12	80.0	0.8	364
	10	4.10	37.6	0.3	142	4.80	59.4	0.9	170	4.84	78.9	0.4	152
	20	4.28	43.1	0.3	146	5.42	71.9	0.1	170	4.88	84.2	1.1	148

Inoculum of A. repens was 220,000 spores per ml.

" " A. niger was 470,000 " " "

SEM = Standard Error of the mean. df = 7.

TABLE 31. Effect of nutrient enrichment on pectinase production by Aspergillus repens grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Media	Nutrient	pH	Protein ug/ml	PMG A	PMG ±SEM*	ΔA (X–Control)	P<
WHEAT BRAN	Control	3.68	198	52.5	0.7		
	Grapefruit peel	3.76	212	63.3	1.0	+ 10.8	0.001
	Plantain peel	4.42	206	40.6	0.8	- 11.9	0.001
	Sucrose	3.64	212	49.6	0.7	- 2.9	0.02
	Glucose	4.25	206	63.4	1.6	+ 10.9	0.001
	Soluble starch	4.24	210	67.2	2.0	+ 14.7	0.001
	Sodium nitrate	4.91	208	43.3	6.2	- 9.2	0.20
	Soya flour	4.38	212	76.5	0.9	+ 24.0	0.001
	Defatted soya flour	4.18	212	76.2	0.1	+ 23.7	0.001
	G.S.S.	3.91	216	68.0	0.6	+ 15.5	0.001
RICE BRAN	Control	4.46	238	59.2	0.6		
	Grapefruit peel	3.60	330	82.8	0.7	+ 23.6	0.001
	Plantain peel	4.55	320	54.4	1.0	- 4.8	0.01
	Sucrose	3.56	332	36.7	0.4	- 22.5	0.001
	Glucose	3.58	320	40.8	0.5	- 18.4	0.001
	Soluble starch	3.29	320	42.3	1.3	- 16.9	0.001
	Sodium nitrate	4.77	284	40.7	0.4	- 18.5	0.001
	Soya flour	4.72	328	73.8	0.6	+ 14.6	0.001
	Defatted soya flour	4.52	322	68.7	0.4	+ 9.5	0.001
	G.S.S.	3.90	380	66.4	0.5	+ 7.2	0.001
PEANUT SHELL	Control	4.85	154	52.4	0.5		
	Grapefruit peel	4.80	240	50.9	0.8	- 1.5	0.20
	Plantain peel	4.10	238	31.8	0.4	- 20.6	0.001
	Sucrose	3.21	218	14.9	1.0	- 37.5	0.001
	Glucose	3.25	170	16.9	0.8	- 35.5	0.001
	Soluble starch	2.54	194	13.5	0.4	- 38.9	0.001
	Sodium nitrate	4.31	164	60.1	0.8	+ 7.7	0.001
	Soya flour	5.29	240	72.6	0.6	+ 20.2	0.001
	Defatted soya flour	5.94	258	59.0	2.1	+ 6.6	0.01
	G.S.S.	3.76	334	55.1	1.2	+ 2.7	0.001

*SEM = Standard Error of the mean. df = 7

ΔA = difference between control (A) and test

G.S.S = Grapefruit + Sucrose + Soya flour.

TABLE 32. Effect of nutrient enrichment on pectinase production by Aspergillus niger grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Media	Nutrient	pH	Protein ug/ml	PMG A	PMG ±SEM	Δ A (X-Control)	P <
WHEAT BRAN	Control	2.73	306	84.9	0.3		
	Grapefruit peel	3.54	410	83.3	0.9	- 1.6	-
	Plantain peel	4.85	358	77.2	0.9	- 7.7	0.001
	Sucrose	3.61	358	85.8	1.5	+ 0.9	-
	Glucose	3.64	346	83.2	0.7	- 1.7	0.05
	Soluble starch	4.08	204	88.8	0.5	+ 3.9	0.001
	Sodium nitrate	4.84	308	80.7	0.5	- 4.2	0.001
	Soya flour	4.19	332	87.2	0.4	+ 2.3	0.001
	Defatted soya flour	4.30	328	79.9	0.3	- 5.0	0.001
	G.S.S.	3.50	408	86.3	0.1	+ 1.4	0.001
RICE BRAN	Control	5.25	214	85.2	1.1		
	Grapefruit peel	5.17	230	84.5	0.3	- 0.7	-
	Plantain peel	5.99	222	67.2	0.2	- 18.0	0.001
	Sucrose	4.05	226	81.3	0.6	- 3.9	0.01
	Glucose	4.00	218	74.1	0.5	- 11.1	0.001
	Soluble starch	4.50	244	83.2	0.1	- 2.0	-
	Sodium nitrate	4.88	262	64.8	1.0	- 20.4	0.001
	Soya flour	5.47	272	73.1	0.9	- 12.1	0.001
	Defatted soya flour	6.47	258	71.1	0.5	- 14.1	0.001
	G.S.S.	4.13	280	86.3	0.3	+ 1.1	-
PEANUT SHELL	Control	6.16	168	78.2	0.7		
	Grapefruit peel	6.06	222	86.4	1.5	+ 8.2	0.001
	Plantain peel	6.77	246	43.4	1.6	- 34.8	0.001
	Sucrose	4.59	220	85.5	1.2	+ 7.3	0.001
	Glucose	4.46	186	48.3	2.6	- 29.9	0.001
	Soluble starch	4.18	184	38.1	2.6	- 40.1	0.001
	Sodium nitrate	5.62	186	48.5	1.7	- 29.7	0.001
	Soya flour	5.89	216	89.4	0.7	+ 11.2	0.001
	Defatted soya flour	6.59	238	78.6	1.0	+ 0.4	-
	G.S.S.	5.02	224	67.1	1.0	- 11.1	0.001

TABLE 33. Effect of media depth on pectinase production by Aspergillus repens grown on wheat bran; rice bran and peanut shell.

Media	Media Depth (cm)	pH	Protein ug/ml	Endo-PMG A	Endo-PMG ±SEM*	A as % of *Reference	A per ug protein per ml of enzyme
WHEAT BRAN	2	4.75	234	66.9	1.1	100.0	0.286
	4	4.77	176	57.0	0.7	85.2	0.324
	6	4.53	220	28.4	5.7	42.5	0.129
	8	4.74	268	59.5	0.4	88.9	0.222
	10	4.53	274	55.1	1.2	82.4	0.201
RICE BRAN	2	5.23	118	77.0	0.5	100.0	0.653
	4	4.94	182	78.8	0.7	102.3	0.433
	6	4.88	190	79.2	0.2	102.9	0.417
	8	4.66	232	75.1	0.5	97.5	0.324
	10	4.48	248	74.1	0.3	96.2	0.299
PEANUT SHELL	2	4.41	142	60.4	0.4	100.0	0.425
	4	3.96	66	61.1	2.4	101.2	0.926
	6	3.72	80	48.6	0.6	80.5	0.608
	8	3.62	98	44.6	0.4	73.8	0.455
	10	3.61	108	35.8	1.5	59.3	0.331

*SEM = Standard Error of the Mean. df = 7.

* Reference was the enzyme activity at 2cm media depth.

TABLE 34. Effect of media depth on pectinase production by Aspergillus niger grown on wheat bran, rice bran and peanut shell.

Media	Media Depth (cm)	pH	Protein ug/ml	Endo-PMG A	±SEM*	A as % of Reference	A per ug protein per ml of enzyme
WHEAT BRAN	2	4.61	188	73.5	1.1	100.0	0.391
	4	4.65	228	85.7	0.5	116.6	0.376
	6	4.43	246	44.1	0.7	60.0	0.179
	8	4.06	184	62.0	0.6	84.4	0.337
	10	4.18	264	62.1	1.1	84.5	0.235
RICE BRAN	2	5.56	106	75.3	0.1	100.0	0.710
	4	4.73	222	93.5	0.5	124.2	0.421
	6	4.36	270	75.0	0.5	99.6	0.278
	8	3.80	80	47.2	0.9	62.7	0.590
	10	3.83	84	40.6	1.3	53.9	0.483
PEANUT SHELL	2	4.56	54	57.9	0.9	100.0	1.072
	4	4.05	60	60.1	1.7	103.8	1.002
	6	3.90	76	47.7	2.6	82.4	0.628
	8	4.55	242	72.3	0.5	124.9	0.299
	10	4.27	286	67.4	0.9	116.4	0.236

*SEM = Standard Error of the Mean. df = 7.

* Reference was the enzyme activity at 2cm media depth.

gave similar results on each medium; this indicated that 1ml spore inoculum (about 470,000 spores) was adequate for 10g dry material of each medium.

The effects of the following nutrients -

(1) grapefruit peel

(2) plantain peel

(3) sucrose

(4) glucose

(5) soluble starch

(6) sodium nitrate

(7) soya bean flour

(8) defatted soya flour, and

(9) a mixture of grapefruit peel, sucrose and soya flour, were investigated on the pectinase production from wheat bran, rice bran and peanut shell by A. repens and A. niger. The results are in Tables 31 and 32. For A. repens, all of the following - soya flour, defatted soya flour and the mixture of grapefruit peel, sucrose and soya flour, gave significantly higher endo-PMG activity over the control ($P < 0.001$) in all the three media. Conversely, plantain peel and sucrose gave significantly reduced ($P < 0.05 - 0.001$) enzyme activity in all the three media. In wheat bran, glucose and soluble starch also greatly increased enzyme activity ($P < 0.001$) while sodium nitrate only slightly decreased enzyme activity ($P < 0.20$). In rice bran, sucrose, glucose, soluble starch and sodium nitrate greatly decreased enzyme activity ($P < 0.001$). In peanut shell, sucrose, glucose and soluble starch also greatly decreased enzyme activity ($P < 0.001$) whereas sodium nitrate greatly increased enzyme activity ($P < 0.001$).

For the nutrient effects on pectinase production in the three media by A. niger, plantain peel, glucose and sodium nitrate greatly reduced enzyme activity ($P < 0.05 - 0.001$) in all three media. Grapefruit peel, sucrose and soya flour greatly increased enzyme activity in peanut shell ($P < 0.001$) whereas soluble starch and the mixture of grapefruit peel, sucrose and soya flour greatly reduced enzyme activity ($P < 0.001$). Generally in rice bran none of the nutrients produced any significant increase in enzyme activity; rather they decreased the activity. In wheat bran, soluble starch, soya flour and the mixture of grapefruit peel, sucrose and soya flour produced significant increases in enzyme activity ($P < 0.001$) while defatted soya flour significantly reduced enzyme activity ($P < 0.001$).

The results of the investigations of the effect of media depth (2 - 10cms) on enzyme production by the two Aspergillus strains grown on wheat bran, rice bran and peanut shell are in Tables 33 and 34. For A. repens; in rice bran, enzyme activity at all levels were very close (74 - 79%). The specific enzyme activity (in micro-units) was highest at 2cm level. The specific activity fell with increase in media depth although the enzyme activity did not change as much. At 10cm level, enzyme activity was 96% of that at 2cm level. In peanut shell, there was no difference in enzyme activity between the 2cm and 4cm level; but at media depth greater than 4cm, there were drastic reductions in enzyme activity. The highest specific activity was at the 4cm level. In wheat bran, the enzyme activity was highest at 2cm level but the highest specific activity was at the 4cm level. The 6cm level

had the lowest enzyme activity. For A. niger, in wheat bran, again the 6cm level had the lowest enzyme activity (as for A. repens); highest activity was at the 4cm level. The highest specific activity was at 2cm level although there was no significant difference in specific activity between the 2cm and 4cm levels. In rice bran, 4cm level had the highest enzyme activity while the 2cm level had the highest specific activity. In peanut shell, the 6cm level had the least enzyme activity while the 8cm level had the highest but the 2cm level had the highest specific enzyme activity. Both the 8cm and 10cm levels had higher enzyme activities than the lower levels (2 - 6cm).

3.5. Enzyme composition of culture filtrates of A. repens and A. niger grown on wheat bran, rice bran and peanut shell

The culture filtrates of A. niger and A. repens from wheat bran, rice bran and peanut shell were likely to contain other enzymes apart from endo-PMG; therefore quantitative analysis of the filtrates was carried out to determine the pectinases, cellulases, amylases and acid protease present. The pectinases assayed were -

(1) endopolymethylgalacturonase (endo-PMG);

(2) exo-polymethylgalacturonase (exo-PMG);

(3) polymethylgalacturonate esterase (PME) or pectin esterase; and

(4) polymethylgalacturonate lyase (PMGL) or pectin lyase.

The cellulases assayed were

(1) C_1-cellulase;

(2) C_x-cellulase (saccharifying) and

(3) C_x-cellulase (viscosity reducing). The amylases assayed were

(1) Amyloglucosidase (AMG);

(2) α-amylase (starch consuming); and

(3) α-amylase (viscosity reducing).

The results are in Table 35. The enzyme filtrates from all th three media from both Aspergillus strains had high activities of endo-PMG (56 - 78%) and C_x-cellulase (viscosity reducing) (87 - 95%). They also had fair amounts of acid protease activity (39 - 131ug tyrosine per ml); and exo-PMG activity (0.4 - 1.8 milli-mole sugar/min.) and small amounts of α-amylase (viscosity reducing) (13 - 39%). Only A. repens produced a fair amount of amyloglucosidase in rice bran (67 units/ml) and peanut shell (52 units/ml). Both strains produced negligible amounts of polymethylgalacturonate lyase (0 - 0.1 milli units/ml) and pectin esterase (0 - 2.6 micro equivalents/ml). Both strains also produced very little C_1- cellulase (0 - 0.4 IU. ml^{-1}) and C_x-cellulase (saccharifying) (0.1 - 1 IU. ml^{-1}).

TABLE 35. Enzyme composition of culture filtrates of Aspergillus repens and Aspergillus niger grown on wheat bran (WB), rice bran (RB) and peanut shell (PS).

Culture	Media	PECTINASES				CELLULASES			AMYLASES			PROTEASE
		Endo-PMG A	Exo-PMG milli-mole sugar/min.	PME Micro-equiv.	PMGL milli-units per ml	C_1 IU ml^{-1}	C_x Saccharifying IU ml^{-1}	C_x Viscosity reducing %	AMG Units/ml	α-Amylase starch consuming mg/min.	α-Amylase viscosity reducing %	Acid protease ug tyrosine per ml
Aspergillus repens	WB	66.5	0.45	0.1	0.01	0.185	0.334	88.8	8.5	0.49	12.8	39
	RB	76.8	0.79	1.8	0.00	0.296	0.740	95.1	67.0	0.92	38.8	79
	PS	56.3	1.82	2.6	0.48	0.185	0.482	95.0	52.0	0.61	31.4	79
Aspergillus niger	WB	72.5	1.59	2.3	0.04	0.185	0.408	94.8	7.0	1.2	24.5	89
	RB	75.2	1.02	1.8	0.09	0.426	1.000	94.4	12.0	0.31	14.4	131
	PS	77.5	0.79	0.0	0.13	0.019	0.186	87.7	6.0	0.00	13.8	43

3.6. Characterisation of Endo-polymethylgalacturonase of A. niger and A. repens

Aspergillus repens and A. niger were grown on rice bran. The crude enzyme filtrates obtained were used for the characterisation studies of their endopolymethylgalacturonase enzymes. The studies included determinations of the effects of pH and temperature on their activities; determination of the time-course of A. repens endo-PMG activities at 40° and 80°C at pH 4.0; and that of A. niger at 40° and 60°C at pH 4.5. Also investigated was the heat stability of A. repens enzyme at 90°C at pH 4.0; and that of A. niger at 70°C and pH 4.5. Lastly, the substrate concentration effect on enzyme activity was also investigated. The results are in Tables 36 - 42. The data were plotted in Figures 16 - 21. From Fig. 16, optimum pH for endo-PMG of A. repens was a range of pH 4 - 5.5. For A. niger, it was pH 4.5 — 5.5; at pH 4.5, the activity was 81% while it was lower (74%) at pH 5.5. From Fig. 17, optimum temperature for A. repens was a range 40° - 50°C while that for A. niger was 40°C. For A. repens, at 20° and 30°C, there was no change in enzyme activity and at 80°C there was still a significant amount of activity (20%). For A. niger, there was a drastic fall in enzyme activity between 60°C (74%) and 70°C (0%). From Fig. 18, the maximum activity of A. repens enzyme at 40°C was achieved in 40 minutes. At 80°C, the enzyme still retained 22% activity even after 1 hour. From Fig. 19, maximum activity of A. niger enzyme was

reached after 40 minutes at 40°C. At 60°C, the same result was achieved in 30 minutes; peak enzyme activity was still maintained at this temperature after 1 hour. Heat stability studies of A. repens endo-PMG at 90°C and pH 4.0 showed that all enzyme activity was destroyed within 5 minutes of incubation at this temperature and pH. From Fig. 20, heat stability studies of A. niger enzyme at 70°C and pH 4.5 showed a rapid decrease in activity to about 18% residual activity in 15 minutes. This residual activity was maintained for 10 minutes without change after which there was rapid decrease in residual activity till all was lost between 35 - 40 minutes of duration of heat treatment. Figure 21 shows the substrate concentration effect on A. repens and A. niger at 40°C and pH 4.0 and 4.5 respectively. It shows that for A. repens, maximum change in viscosity of all the substrate concentrations studied (0.25 - 1.25% pectin) occurred in about 40 minutes after incubation whereas for A. niger, this was about 50 minutes. Moreover, in about 1 hour after incubation, the change in viscosity was generally the same (82 - 94%) for all the concentrations studied and for both Aspergillus strains.

TABLE 36. Optimum pH determination of Endo-PMG activities of *A. repens* and *A. niger*.

A. repens		*A. niger*	
pH	Activity (A)	pH	Activity (A)'
2.5	37.5	2.5	43.8
3.0	56.1	3.0	46.5
3.5	59.1	3.5	57.7
4.0	78.7	4.0	68.7
4.5	74.1	4.5	81.3
5.0	77.9	5.0	66.5
5.5	75.0	5.5	74.1
6.0	64.3	6.0	68.3

TABLE 37. Optimum temperature determination of Endo-PMG activities of A. repens and A. niger.

A. repens		A. niger	
Temperature °C	Activity A	Temperature °C	Activity A
20	84.0	20	83.3
30	84.2	30	90.6
40	95.9	40	95.3
50	96.3	50	90.1
60	72.1	60	74.1
70	35.3	70	0.0
80	20.4	80	0.0

TABLE 38. Determination of Time-Course of Endo-PMG activity of A. repens at 40° and 80°C at pH 4.0.

Time (Minutes)	Endo-PMG Activity (A) 40°C	80°C
0	0	0
10	60.4	9.8
20	75.5	14.5
30	84.3	18.9
40	86.6	22.1
50	87.3	22.1
60	88.9	22.3

TABLE 39. Determination of Time-Course of Endo-PMG activity of A. niger at 40° and 60°C at pH 4.5.

Time (Minutes)	Endo-PMG Activity (A) 40°C	60°C
0	0	0
10	68.8	77.8
20	81.2	83.8
30	89.8	91.2
40	91.7	91.6
50	93.5	90.5
60	93.5	92.8

3.6.1. Heat stability studies of Endo-PMG activity of A. repens at 90°C and pH 4.0

Within 5 minutes of incubation at 90°C, 100% of the enzyme activity was lost.

TABLE 40. Heat stability studies of Endo-PMG activity of A. niger at 70°C and pH 4.5.

Time (mins)	Residual Enzyme Activity (A)
5	38.2
10	32.1
15	17.9
20	18.9
25	18.9
30	12.3
35	3.8
40	0.0
45	0.0
50	0.0
55	0.0
60	0.0

TABLE 41. Determination of substrate concentration effect on Endo-PMG activity of A. repens.

Substrate Concentration g/100ml	Time (mins.) and change in viscosity (A)					
	10	20	30	40	50	60
	A	A	A	A	A	A
0.25	68.9	79.5	78.7	80.5	80.5	83.2
0.5	60.4	75.5	84.3	86.6	87.3	88.9
0.75	58.9	82.2	85.0	88.8	89.9	89.5
1.0	56.1	76.0	80.4	84.6	86.0	86.7
1.25	57.2	73.0	82.0	85.4	87.8	88.5

TABLE 42. Determination of substrate concentration effect on Endo-PMG activity of A. niger.

Substrate Concentration g/100ml	Time (mins.) and change in viscosity (A)					
	10	20	30	40	50	60'
	A	A	A	A	A	A
0.25	74.5	63.8	69.7	78.8	74.3	76.1
0.5	68.8	81.2	89.8	91.7	93.5	93.5
0.75	50.0	79.4	82.2	79.7	82.2	85.2
1.0	49.1	69.9	79.9	78.3	81.3	84.1
1.25	30.9	62.9	68.2	76.9	73.9	82.4

EFFECT OF PH ON ENDO - PMG ACTIVITY OF A. niger AND A. repens AT 30°C

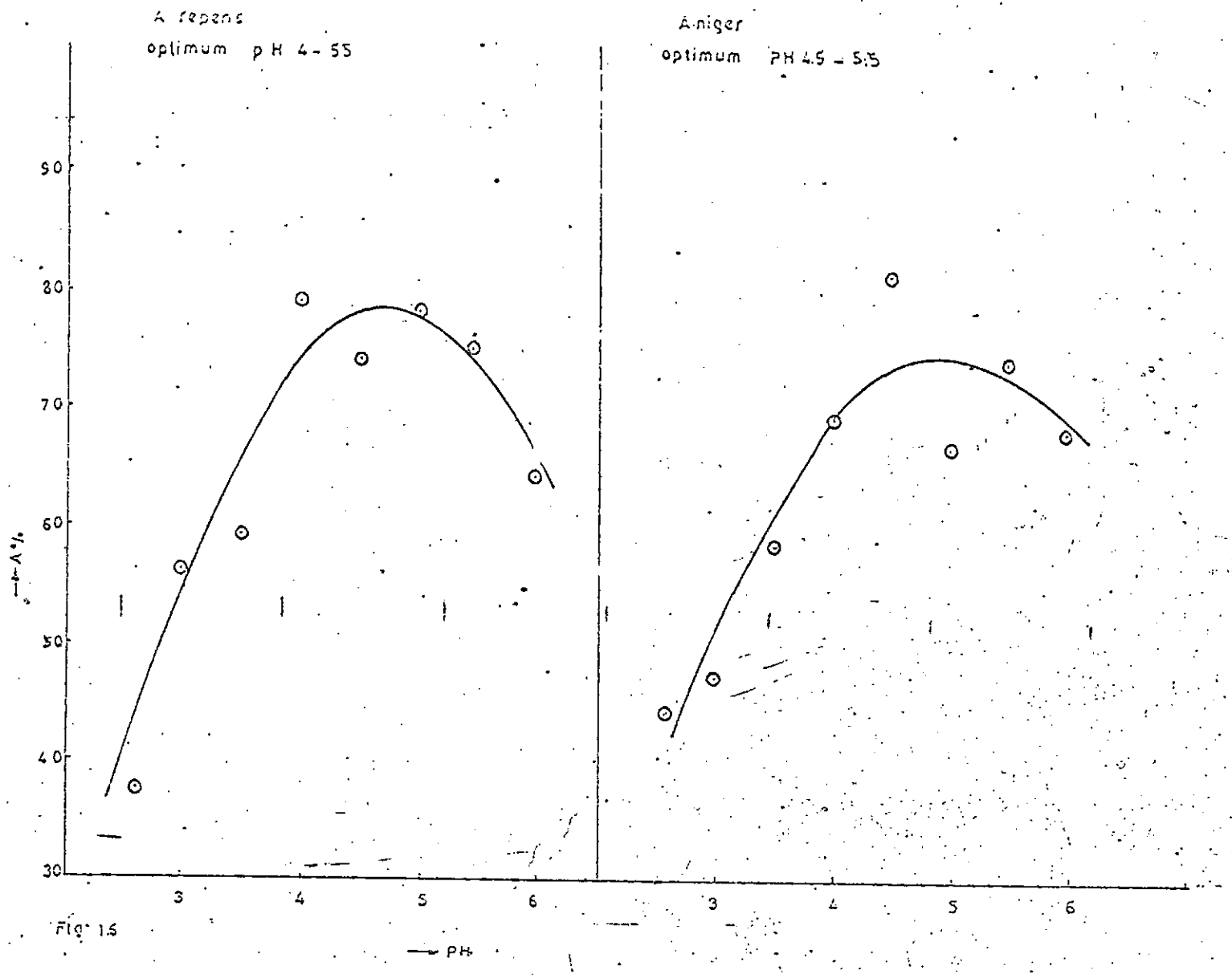


Fig. 1.6

EFFECT OF TEMPERATURE ON ENDO-PMG ACTIVITY OF A. repens & A. niger AT PH 4.0

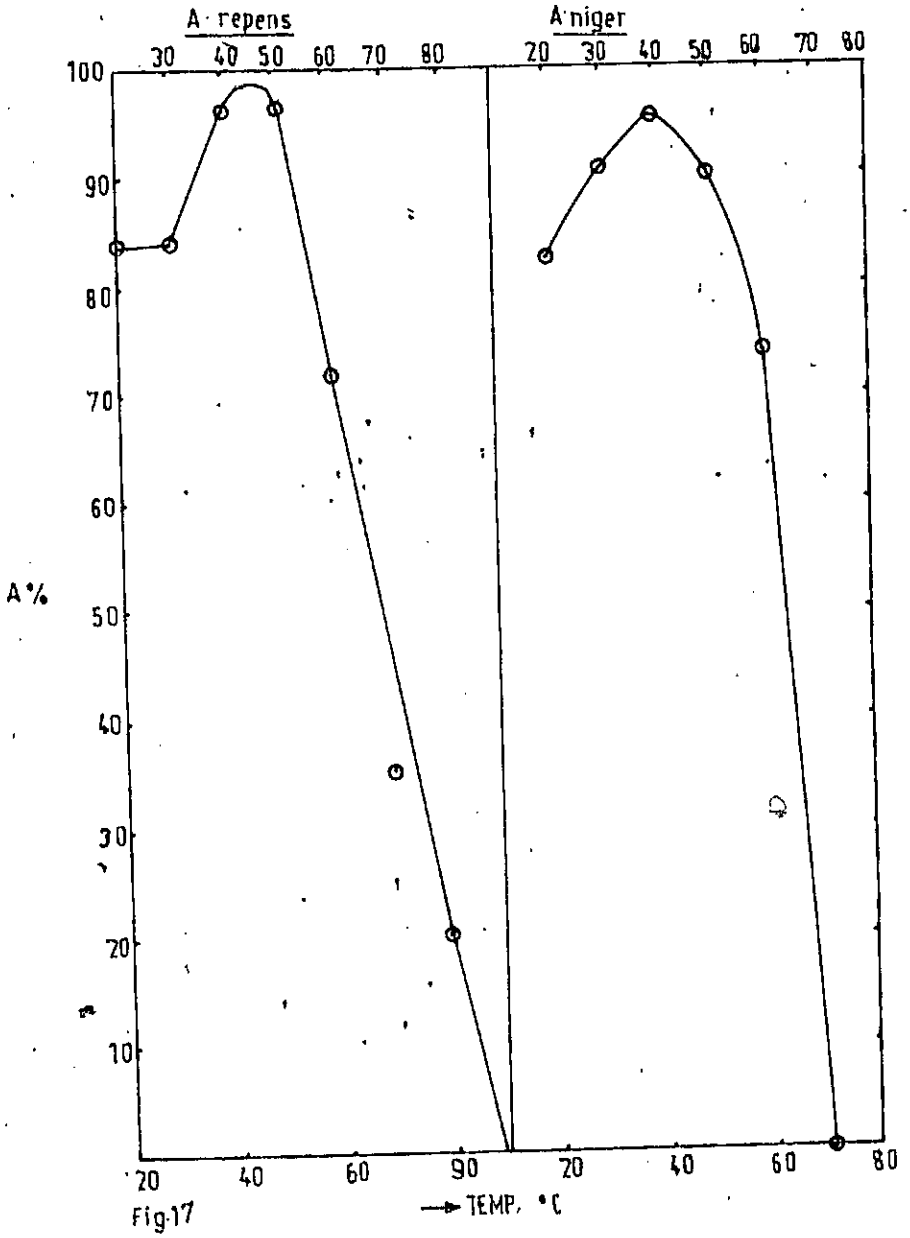


Fig-17

TIME COURSE OF ENDO – PG ACTIVITY OF A. repens AT 40°C AND 80°C AT PH 4.0

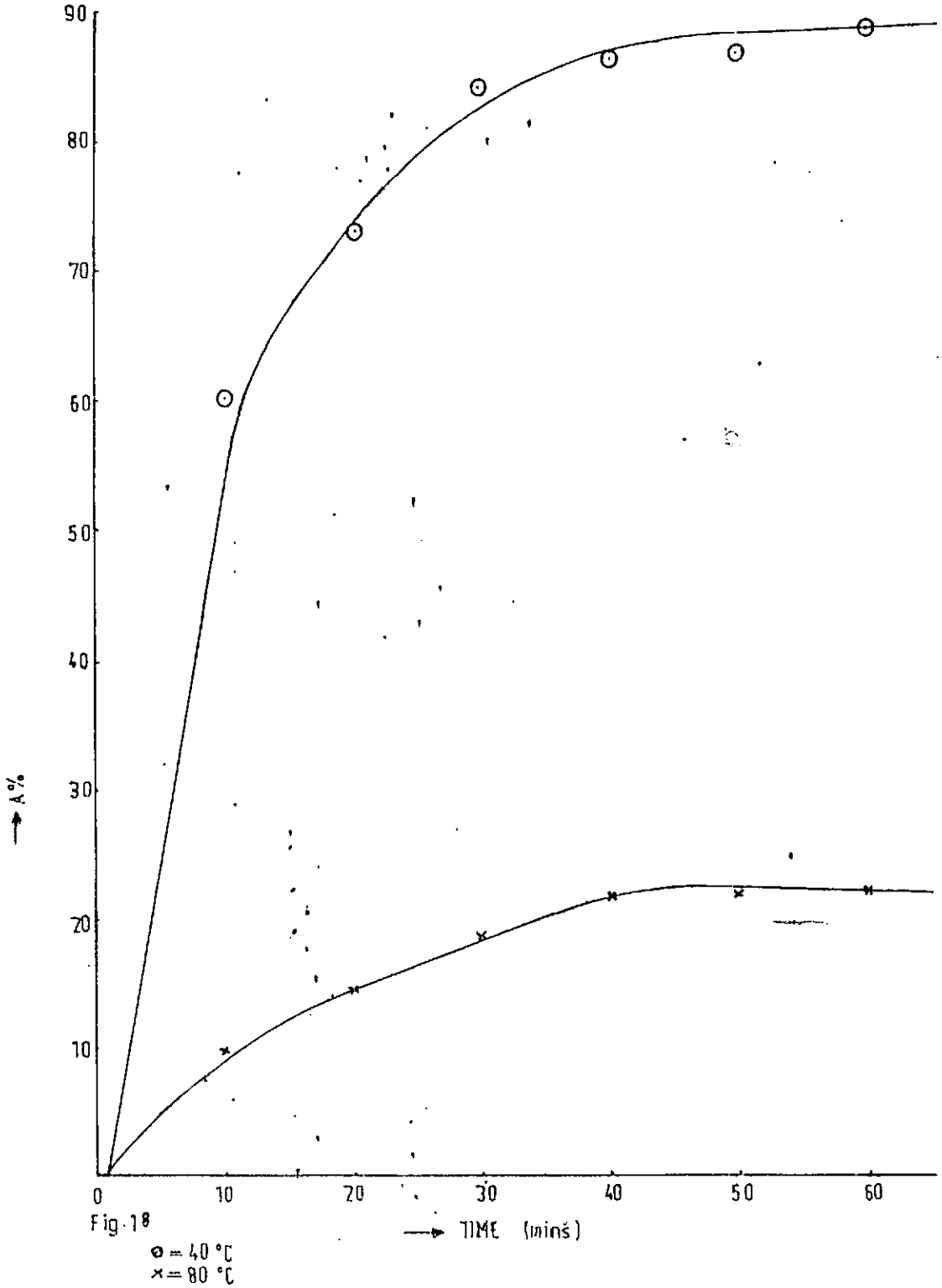


Fig. 18

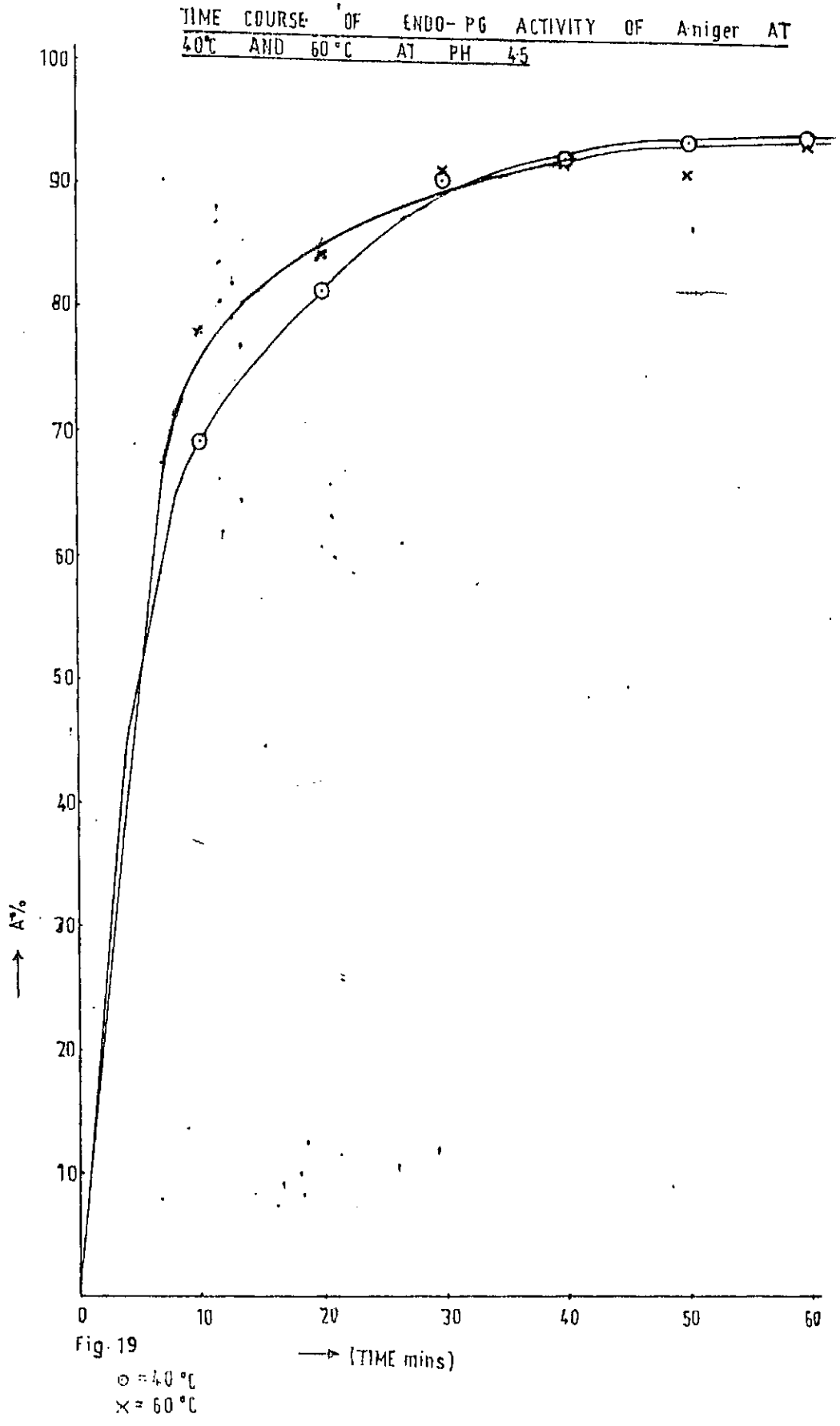

TIME COURSE OF ENDO-PG ACTIVITY OF A.niger AT 40°C AND 60°C AT PH 4.5
100
90
80
70
60
50
40
30
20
10
A%
0
10
20
30
40
50
60
(TIME mins)
⊙ = 40 °C
× = 60 °C

Fig. 19

HEAT STABILITY OF ENDO - PMG OF A.niger AT 70 °C AND PH 4.5

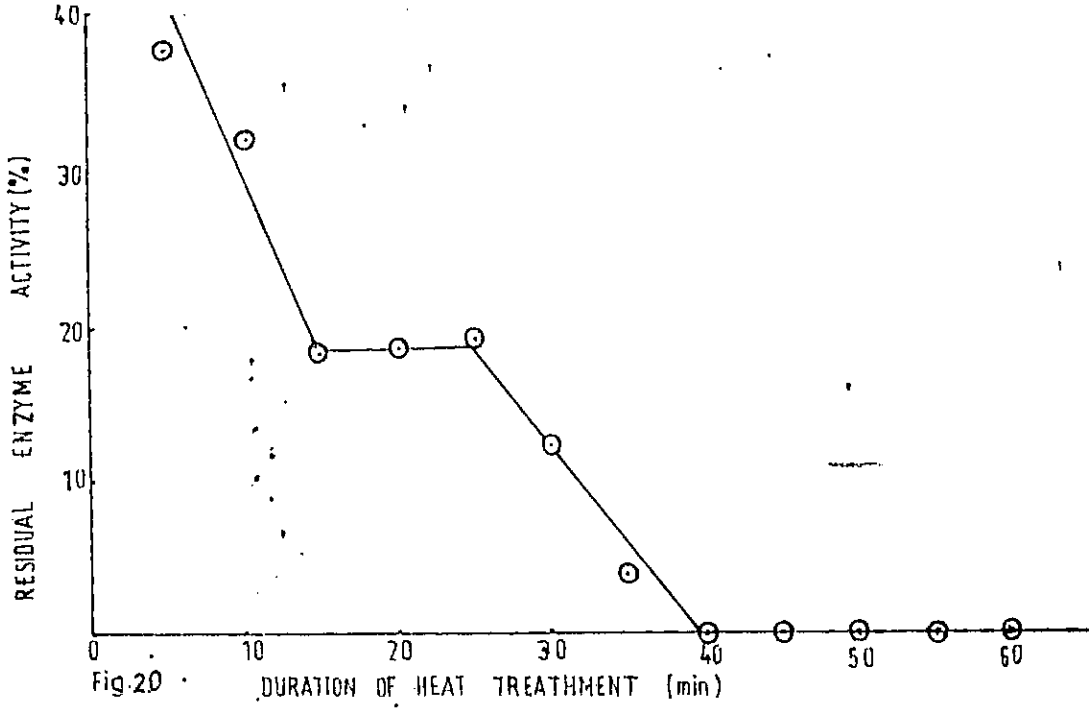


Fig.20

SUBSTRATE CONCENTRATION EFFECT AT 40°C OF ENDO-PMG ACTIVITY OF A. repens & A.niger AT PH 4.0 AND 4.5 RESPECTIVELY

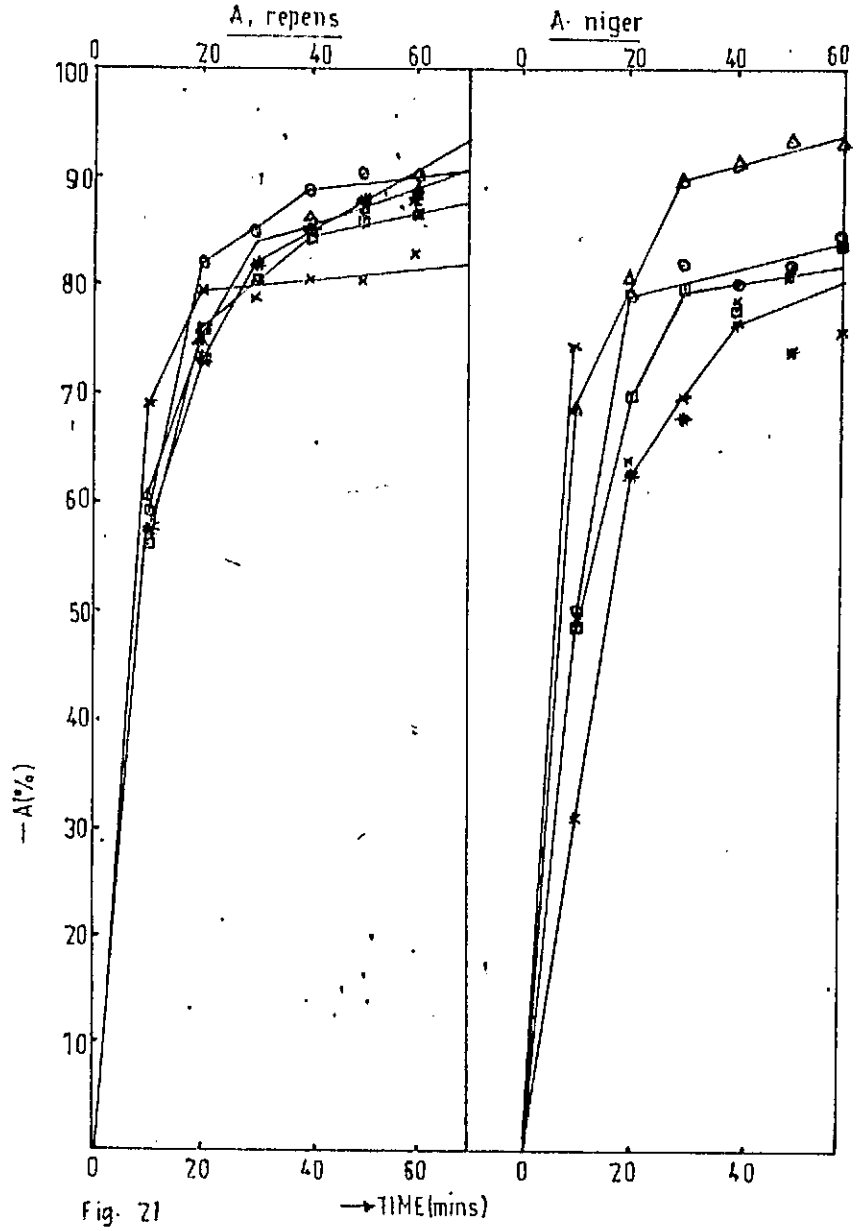


Fig. 21

× = 0·25% pectin conc.
△ = 0·50% pectin conc.
○ = 0·75% pectin conc.
□ = 1·0% pectin conc.
* = 1·25% pectin conc.

3.7. Storage stability studies of Endo-PMG of A. repens and A. niger at ambient temperatures (26 ± 2°C) and in refrigerator at 10 ± 2°C

The effect of pH on storage stability of A. repens and A. niger endo-PMG enzymes at ambient temperatures (26 ± 2°C) and at 10 ± 2°C was investigated for 4 weeks duration. The results are in Tables 43 and 44. Storage at ambient temperatures at pH 3 - 4 did not cause any significant decrease in enzyme activity for A. repensfor 4 weeks; whereas there was highly significant loss in activity at pH 5 - 6 even within one week of storage. For A. niger at ambient temperatures, there was no significant decrease in enzyme activity at pH 3 for 4 weeks, whereas at pH 4, 16% of original activity was lost within one week of storage and after two weeks about 31% of original activity was lost. For A. repens, storage at 10 ± 2°C at pH 5 - 6 for 4 weeks did not cause any significant decrease in enzyme activity; whereas at pH 3, about 36% of original activity was lost in 1 week of storage. At pH 4, there was also about 23% loss in enzyme activity within 1 week of storage. However, there was significant drop in pH within 1 week in samples stored at pH 3 and 4 where the pH dropped to 2.56 and 3.50 respectively. For A. niger, storage at 10 ± 2°C did not cause any significant decrease in enzyme activity in all the samples stored for 4 weeks.

TABLE 43. Effect of pH on storage stability of Endo-PMG activities of *A. repens* and *A. niger* at ambient temperatures (26 ± 2°C).

Time Weeks	0-Time pH	*A. repens*				*A. niger*			
		pH	A	±SEM*	Protein ug/ml	pH	A	±SEM*	Protein ug/ml
0	3.00	3.00	79.6	0.5	194	3.00	83.2	0.8	196
	4.02	4.02	79.6	0.5	194	4.02	83.2	0.8	196
	5.05	5.05	79.6	0.5	194	5.05	83.2	0.8	196
	6.03	6.03	79.6	0.5	194	6.03	83.2	0.8	196
1	3.00	3.31	83.0	1.1	178	3.10	79.7	1.5	192
	4.02	4.40	75.5	0.9	188	4.30	69.5	0.7	196
	5.05	5.10	25.5	0.5	196	5.30	65.7	0.9	190
	6.03	5.80	28.5	0.5	198	6.30	39.9	2.0	194
2	3.00	3.52	75.6	0.8	184	3.17	77.5	2.0	188
	4.02	4.55	77.1	0.5	182	4.60	57.6	1.1	188
	5.05	5.44	20.5	0.3	190	5.70	45.6	0.8	188
	6.03	5.88	19.8	1.1	198	6.50	14.4	3.0	168
3	3.00	4.03	75.2	1.0	180	3.24	80.6	1.7	188
	4.02	4.87	76.7	0.9	169	5.20	59.3	2.1	188
	5.05	6.07	20.8	1.1	190	6.09	34.3	0.9	186
	6.03	6.09	27.5	3.0	196	6.70	6.2	3.3	168
4	3.00	4.38	73.9	1.5	200	3.35	79.9	2.1	222
	4.02	4.95	74.2	1.1	208	5.47	47.6	3.0	218
	5.05	6.41	9.2	2.0	236	6.49	16.6	3.5	220
	6.03	6.11	5.3	2.5	244	6.97	3.4	3.3	176

*SEM = Standard Error of the Mean. n = 12.

TABLE 44. Effect of pH on storage stability of Endo-PMG activities of *A. repens* and *A. niger* when stored in refrigerator at 10 ± 2°C.

Time Weeks	0-Time pH	*A. repens* pH	*A. repens* A	*A. repens* ±SEM*	*A. repens* Protein ug/ml	*A. niger* pH	*A. niger* A	*A. niger* ±SEM*	*A. niger* Protein ug/ml
0	3.05	3.05	56.2	1.0	238	3.05	69.5	0.5	190
	4.02	4.02	56.2	1.0	238	4.02	69.5	0.5	190
	5.01	5.01	56.2	1.0	238	5.01	69.5	0.5	190
	6.05	6.05	56.2	1.0	238	6.05	69.5	0.5	190
1	3.05	2.56	35.9	0.9	208	3.13	70.3	0.1	180
	4.02	3.50	43.4	0.5	214	4.29	71.1	0.2	178
	5.01	4.89	55.1	0.8	218	4.81	67.5	0.3	196
	6.05	5.41	57.2	0.2	218	5.46	68.0	0.2	188
2	3.05	2.73	43.7	1.1	214	3.12	64.4	0.5	188
	4.02	3.45	57.6	0.9	208	5.03	64.6	0.3	176
	5.01	5.36	51.7	2.1	206	5.32	65.7	0.6	184
	6.05	5.20	50.0	1.5	210	4.90	68.2	0.1	174
3	3.05	2.50	43.1	1.3	278	3.04	65.4	0.2	200
	4.02	3.40	52.1	09	270	4.63	72.0	0.1	210
	5.01	4.78	51.2	0.7	276	5.06	71.4	0.1	208
	6.05	5.58	44.1	2.5	274	5.70	67.9	0.1	200
4	3.05	2.49	46.9	0.8	120	3.25	66.1	0.3	206
	4.02	3.47	54.6	2.1	134	4.81	71.3	0.1	168
	5.01	4.85	55.6	0.3	128	5.27	69.2	0.1	178
	6.05	5.75	55.5	0.4	126	6.05	68.1	0.1	172

*SEM = Standard Error of the Mean. n = 12.

3.8. Toxicological screening of A. repens and A. niger cultures and their filtrates

The toxicological investigations carried out consisted of three parts -

(1) measurement of the absorbance (at 366nm and 254nm) of extracts of the cultures and enzyme filtrates;

(2) chromatographic separation of extracts of crude enzyme filtrates, and

(3) biological examination of extracts of the enzyme filtrates - diagnostic enzymological studies involving the assay of key diagnostic enzymes - glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) of rat serum; liver, kidney and urine.

The daily urinary protein excreted during the experiment was also determined. From Table 45, the absorbance of extracts of cultures of A. niger and A. repens grown on rice bran were in all cases at both wavelengths (366nm and 254nm) less than that of the rice bran control extract. Similarly, the absorbance of extracts of cultures grown on yeast extract plus sucrose broth and Czapek Dox broth were less than those of rice bran control extracts. In the case of extracts of crude enzyme filtrates, the absorbance of the neutral extracts of A. niger and A. repens were significantly higher than the rice bran control neutral extracts. From Table 46, the results of chromatographic separation of extracts of crude enzyme filtrates of both Aspergillus strains shows that there was no mycotoxin

TABLE 45. Absorbance of extracts at 366nm and 254nm of cultures and crude enzyme filtrates of *A. niger* and *A. repens*.

(a) Absorbance of extracts of cultures grown on rice bran:

Sample	Optical Density 366nm	254nm
Rice bran control - A	1.912	0.986
Rice bran - N	1.995	2.182
Aspergillus niger - A	0.551	0.757
A. niger - N	1.972	1.113
A. repens - A	0.724	0.798
A. repens - N	1.963	1.798

(A - Acidic extract; N - Neutral extract).

(b) Absorbance of extracts of cultures grown on yeast extract plus sucrose broth (YES) and Czapek Dox broth (CZ):

Sample	Optical Density 366nm	254nm
A. niger - YES	1.819	0.858
A. repens - YES	1.195	0.779
**A. *niger* - CZ	0.179	0.453
A. repens - CZ	1.355	1.073

(c) Absorbance of extracts of crude enzyme filtrates of *A. niger* and *A. repens* grown on rice bran:

Sample	Optical Density 366nm	254nm
Control Extract - A	0.413	1.039
Control Extract - N	0.526	1.097
A. niger - A	0.364	0.996
A. niger - N	1.378*	1.255*
A. repens - A	0.570*	1.036
A. repens - N	1.339*	1.168*

* Significantly higher than control

** *A. niger* did not grow in Czapek Dox broth.

TABLE 46. Chromatographic separation of extracts of crude enzyme filtrates of A. niger and A. repens grown on rice bran.

Extract	SPOT Appearance	AFTER SEPARATION			AFTER SPRAYING	
		*R_f	APPEARANCE			
			Visible light	UV-light	Visible light	UV-light
Rice bran - A	pale yellow	1.0	Yellow	Yellowish-green	Blue	Blue
Rice bran - N	dark brown	1.0	Brownish-yellow	"	"	"
A. repens - A	pale yellow	1.0	Yellow	"	"	"
A. repens - N	yellow	1.0	creamy	"	"	"
A. niger - A	pale yellow	1.0	yellow	"	"	"
A. niger - N	orange	1.0	Brownish-yellow	"	"	"

*R_f - Relative front

A - Acidic extract

N - Neutral extract.

in any of the extracts - all the pigments moved with the solvent front (R_f = 1.0) and spraying with the anisaldehyde reagent failed to confirm presence of any mycotoxin.

The results of the biological investigations of the extracts are in Tables 47 - 51. Table 47 shows the result of the rot serum enzymes - SGOT and SGPT. The rice bran control group, A. niger group and A. repens group had no statistically significant difference in SGOT and SGPT from the normal control group. Table 48 shows the result of the liver enzymes - LGOT and LGPT. Only LGOT of A. niger group was significantly higher than the normal control group ($P < 0.05$). For the kidney enzymes - KGOT and KGPT, the results in Table 49 shows that there was no significant differences among the groups. The results of the daily urinary enzymes excretion - UGOT and UGPT are in Table 50. The values are in units of enzyme excreted per hour. Statistical analysis of the data shows that the UGOT enzymes excreted by A. niger and A. repens groups were significantly higher than the normal control group ($P < 0.01$). This difference gave rise to a significantly higher UGOT to UGPT ratio for A. repens group ($P < 0.001$). The results of the daily urinary protein excretion is given in Table 51. It shows that there were no significant differences in protein excretion among the groups. More detailed results are in Appendix XIII.

TABLE 47. Rat serum enzymes - Glutamate oxaloacetate transaminase (SGOT) and Glutamate pyruvate transaminase (SGPT).

Rat group	SGOT *u/ml	SGPT *u/ml	S $\frac{GOT}{GPT}$
Normal control	145 ± 10	55 ± 10	2.64 ± 0.34
Rice bran control	135 ± 10	50 ± 7	2.70 ± 0.07
A. niger	130 ± 15	45 ± 15	2.89 ± 0.85
A. repens	135 ± 10	50 ± 10	2.70 ± 0.36

* Reitman-Frankel Units/ml serum.

TABLE 48. Liver enzymes - LGOT and LGPT.

Rat group	LGOT •U/mg	LGPT •U/mg	L $\frac{GOT}{GPT}$
Normal control	871 ± 76	640 ± 252	1.68 ± 1.04
Rice bran control	985 ± 372	565 ± 324	2.06 ± 0.82
A. niger	1113 ± 157[3]	810 ± 188	1.42 ± 0.28
A. repens	1122 ± 581	757 ± 277	1.42 ± 0.27

• Reitman-Frankel Units/mg protein

[3] Significantly different from normal control ($P < 0.05$).

TABLE 49. Kidney enzymes - KGOT and KGPT.

Rat group	KGOT U/mg	KGPT U/mg	K $\frac{GOT}{GPT}$
Normal control	863 ± 185	506 ± 89	1.71 ± 0.28
Rice bran control	818 ± 361	451 ± 183	1.80 ± 0.29
A. niger	925 ± 100	447 ± 41	2.08 ± 0.31
A. repens	976 ± 258	596 ± 114	1.65 ± 0.40

TABLE 50. **Urine enzymes** - UGOT and UGPT daily urinary excretion of UGOT and UGPT following extract injections.

Days after 1st Injection	Normal Control			Rice Bran Control			A. niger			A. repens		
	UGOT u/hr	UGPT u/hr	U $\frac{GOT}{GPT}$	UGOT u/hr	UGPT u/hr	U $\frac{GOT}{GPT}$	UGOT u/hr	UGPT u/hr	U $\frac{GOT}{GPT}$	UGOT u/hr	UGPT u/hr	U $\frac{GOT}{GPT}$
1	46	180	0.26	138	180	0.77	155	6	25.83	187	176	1.06
2	150	259	0.58	21	115	0.18	208	6	34.67	161	170	0.95
3	63	173	0.36	104	187	0.56	190	102	1.86	158	178	0.89
4	62	154	0.39	79	165	0.48	375	250	1.50	167	179	0.93
5	69	158	0.44	94	357	0.26	268	188	1.43	141	204	0.69
6	32	134	0.24	63	88	0.72	150	108	1.39	143	158	0.91
$\bar{X}$	64	176	0.38	83	182	0.50	224[2]	110	11.11	160[2]	178	0.91[1]
$\pm$ S.D.	45	44	0.12	40	94	0.24	85	86	15.09	17	15	0.12

1 highly significantly different from normal control ($P < 0.001$)

2 significantly different from normal control ($P < 0.01$).

TABLE 51. Daily urinary protein excretion following daily extract injections of rats.

DAYS after 1st Injection	Urinary Protein mg/24 hrs.			
	Normal control	Rice Bran control	*A. niger*	*A. repens*
1	3.6	4.8	5.3	4.0
2	4.3	3.9	5.1	3.6
3	2.8	2.9	4.6	2.8
4	4.3	4.4	5.7	4.8
5	4.0	3.8	5.2	5.5
6	3.4	3.4	2.7	4.2
$\bar{X} \pm$ S.D	3.73 $\pm$ 0.59	3.87$\pm$0.68	4.77$\pm$1.07	4.15$\pm$0.94

3.9. Enzyme utilisation studies

The enzyme utilisation studies were carried out with concentrates of enzyme filtrates from A. repens and A. niger. The investigations were in two main parts. The first part consisted of investigations of the effect of 0 - 2mls of enzyme concentrate on the pulps of banana, carrot, guava and orange juice. The second part was the study of the effect of the enzyme concentrates on orange juice concentration. The results are in Tables 52 - 58. The results of the effect of the enzyme concentrates on green banana that was just ripe is in Table 52. It shows that for A. repens, 0.4 - 0.8mls enzyme gave the best result - 16^{o} - 17^{o} Be (dissolved solids contents), 7.6mls net juice volume and little or no cloudiness in the juice (from slightly positive to completely negative depectinisation test result). For A. niger, the best result was produced by 0.8 - 1.2mls enzyme which produced 18 - 18.5^{o} Be; 6.9 - 7.5mls net juice volume, and little or no cloudiness in the juice. The net juice volume (6.9 - 7.6mls) produced by 0.4 - 0.8mls A. repens enzyme and 0.8 - 1.2mls A. niger enzyme amounted to 116 - 138% increase over the control juice volume (3.2mls). There was no significant change in relative viscosity. Table 53 shows the result of the effect of the enzymes on very ripe (yellow) bananas. The best result for A. repens was produced by 1.2mls of enzyme concentrate, it gave the highest net juice yield (5.8mls) which was 71% increase over the control (3.4mls). It completely depectinised

the juice and reduced its viscosity by 64%. For A. niger, the best result was produced by 2.0mls enzyme. It produced 6.6mls net juice volume which was 94% increase over the control volume and it reduced the viscosity by 65%. The results of the effect of the enzymes on juice extraction from carrots with and without pH adjustment are presented in Tables 54 and 55. Generally, without pH adjustment, the enzymes made the juice more cloudy and no significant increase in juice volume was produced, the maximum increase being about 16%. With pH adjustment, there was some increase in dissolved solids content (about 27%). Generally, the enzymes made the juice less cloddy. The results of the effect of the enzymes on fully ripe guava are in Table 56. For A. repens, the best result was produced by 1.2 - 2mls of enzyme which caused 36 - 42% increase in volume of juice over the control. It also greatly reduced the cloudiness and caused a decrease of 85 - 92% in viscosity. For A. niger, 0.8ml enzyme produced the greatest increase (50%) in juice volume over the control. It also caused about 90% reduction in viscosity though it did not decrease the cloudiness of the juice. The results of the effect of the enzymes on orange juice are in Table 57. For both A. repens and A. niger enzymes, the effects were on the cloudiness and viscosity. For A. repens 0.8 - 2mls enzyme gave the best result. There was about 30% reduction in viscosity with little or no cloudiness. For A. niger, 0.4 - 1.2mls enzyme gave the best result, there was also about 30% reduction in viscosity with little or no

cloudiness in the juice. Table 58 shows the results of the orange juice concentration and storage experiments. The control was concentrated from 50mls to 6mls. The dissolved solids content increased from 11° Be to 50° Be, but it gelled overnight at room temperature. The A. repens enzyme (0.5 and 1ml) produced 5mls concentrate (58° Be) from 50mls orange juice (11° Be). The concentrates did not gell at room temperature for three days before it was transfered to a freezer. It did not gell also in the freezer. Aspergillus niger enzyme (0.5ml and 1ml) produced similar effects as for A. repens. It produced 5mls of concentrate (62° - 63° Be) from 50mls orange juice (11° Be). The concentrates did not gell at room temperature for three days before it was transfered to a freezer. It did not gell in the freezer.

TABLE 52. Effect of Endo-PMG from A. repens and A. niger on juice extraction from just ripe (green) bananas.

Enzyme Source	Enzyme (mls)	pH of pulp	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	5.01	4.92	15.5	3.2	3.2	+++	1.000	1.528
	0.1	5.49	4.74	16.0	6.0	5.9	++	1.099	1.177
	0.4	5.35	4.40	16.0	8.0	7.6	+	1.086	0.937
	0.8	5.14	4.20	17.0	8.4	7.6	-	1.046	1.500
	1.2	5.67	4.34	14.5	8.5	7.3	-	1.009	1.370
	1.6	5.15	4.63	15.0	8.5	6.9	-	1.039	1.436
	2.0	4.85	4.42	16.5	9.5	7.5	-	1.052	1.492
A. niger	0.0	5.01	4.92	15.5	3.2	3.2	+++	1.000	1.528
	0.1	5.03	3.96	16.5	6.1	6.0	+++	1.017	1.153
	0.4	4.57	3.84	15.0	8.1	7.7	++	0.966	1.222
	0.8	4.80	3.74	18.0	7.7	6.9	+	0.978	1.306
	1.2	4.79	3.73	18.5	8.7	7.5	-	0.996	1.426
	1.6	4.62	3.65	15.5	8.6	7.0	+	0.948	1.530
	2.0	4.75	3.91	17.0	8.3	6.3	-	0.984	1.564

Note: Values are averages of duplicate samples

Net juice volume is total juice volume minus enzyme volume

Depec test is depectinisation test - 1 part of juice mixed with 2 parts of ethanol acidified with HCl (5% conc. HCl v/v with 95% ethanol).

TABLE 53. Effect of Endo-PMG from A. repens and A. niger on juice extraction from very ripe (yellow) bananas.

Enzyme Source	Enzyme (mls)	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	5.11	21	3.4	3.4	+++	1.000	1.339
	0.1	5.04	21	4.0	3.9	++	0.530	1.155
	0.4	4.70	21.5	4.6	4.2	++	0.402	1.056
	0.8	4.76	21	6.0	5.2	++	0.367	0.927
	1.2	5.04	19	7.0	5.8	-	0.358	0.926
	1.6	5.19	18	6.7	5.1	-	0.347	1.145
	2.0	4.86	20	7.3	5.3	-	0.361	1.040
A. niger	0.0	5.11	21	3.4	3.4	+++	1.000	1.339
	0.1	5.03	21	4.9	4.8	+++	0.460	1.235
	0.4	4.54	20	5.25	4.85	++	0.387	1.072
	0.8	4.47	20	5.9	5.1	-	0.366	0.838
	1.2	4.71	20	6.1	4.9	-	0.364	0.892
	1.6	4.72	17	7.5	5.9	-	0.335	0.863
	2.0	4.52	17	8.6	6.6	-	0.350	0.823

TABLE 54. Effect of Endo-PMG from A. repens and A. niger on juice extraction from carrots without pH adjustment.

Enzyme Source	Enzyme (mls)	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	5.32	8	13.3	13.3	+	1.000	2.065
	0.1	5.0	8	14.0	13.9	++	0.980	2.400
	0.4	5.17	8	15.0	14.6	+++	0.990	2.457
	0.8	5.40	8	14.5	13.7	+	0.980	2.760
	1.2	5.46	8	15.0	13.8	++	0.990	2.973
	1.6	5.10	8	17.0	15.4	++	1.000	2.728
	2.0	5.20	8	16.0	14.0	+	1.000	2.783
A. niger	0.0	5.32	8	13.3	13.3	+	0.980	2.276
	0.1	5.46	8	14.5	14.4	++	0.975	2.386
	0.4	6.33	8	14.0	13.6	+++	0.980	2.454
	0.8	5.48	8	15.0	14.2	+++	0.990	2.468
	1.2	5.63	8	16.0	14.8	++	0.990	2.634
	1.6	5.49	8	15.0	13.4	+	0.980	2.747
	2.0	5.56	8	17.0	15.0	+	0.960	2.937

TABLE 55. Effect of Endo-PMG from A. repens and A. niger on juice extraction from carrots with pH adjustment.

Enzyme Source	Enzyme (mls)	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	4.27	5.5	15.75	15.75	-	1.000	1.733
	0.1	4.11	7	16.0	15.9	+	1.000	1.781
	0.4	4.15	7	17.5	17.1	+	1.033	1.743
	0.8	3.72	7	18.0	17.2	++	1.044	1.873
	1.2	4.19	7	17.0	15.8	-	1.022	1.745
	1.6	4.34	7	18.0	16.4	-	1.027	1.815
	2.0	4.35	7	16.0	14.0	-	1.000	1.794
A. niger	0.0	4.27	5.5	15.75	15.75	+	1.000	1.733
	0.1	4.50	8	16.0	15.9	+	1.022	1.779
	0.4	4.41	7	17.0	16.6	+	1.033	1.795
	0.8	4.45	7	17.0	16.2	+	1.044	1.834
	1.2	4.73	7	16.0	14.8	-	1.022	1.808
	1.6	4.85	7	19.0	17.4	-	1.022	1.832
	2.0	4.90	7	18.0	16.0	++	1.092	1.942

Note: pH of carrot pulp was adjusted to 4.00 for A. repens enzyme
" " " " " " " 4.50 " A. niger "

TABLE 56. Effect of Endo-PMG from A. repens and A. niger on juice extraction from fully ripe guava with pH adjustment.

Enzyme Source	Enzyme (mls)	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	4.94	6.5	9.0	9.0	+++	1.000	1.736
	0.1	4.59	7.0	11.3	11.2	+++	0.205	1.626
	0.4	5.06	7.0	11.7	11.3	++	0.306	1.522
	0.8	5.48	7.0	14.3	13.5	++	0.210	1.536
	*1.2	5.49	7.0	15.3	14.1	-	0.080	1.415
	1.6	4.98	6.0	16.5	14.9	+	0.146	1.440
	2.0	5.23	5.0	17.5	15.5	+	0.174	1.461
A. niger	0.0	4.94	6.5	9.0	9.0	+++	1.000	1.736
	0.1	4.25	6.0	10.3	10.2	+++	0.379	1.684
	0.4	4.63	6.5	12.7	12.3	+++	0.404	1.650
	*0.8	4.50	7.0	14.3	13.5	+++	0.108	1.529
	1.2	4.00	6.5	13.8	12.6	+++	0.158	1.655
	1.6	4.56	6.0	14.1	12.5	+++	0.220	1.671
	2.0	4.45	6.0	14.5	12.5	++	0.239	1.727

pH of the guava pulp was 2.86

*Samples did not gel when stored in freezer (all others gelled).

TABLE 57. Effect of Endo-PMG from A. repens and A. niger on orange juice.

Enzyme Source	Enzyme (mls)	pH of juice	DS °Be	JUICE VOLUME (mls) Total	Net	DePec test	Relative viscosity	OD at 580nm
A. repens	0.0	3.89	11.0	10	10	+++	1.000	2.113
	0.1	3.90	11.0	10.1	10	+++	0.717	2.046
	0.4	3.60	11.0	10.4	10	+++	0.691	2.162
	0.8	3.90	11.0	10.8	10	+	0.691	2.092
	1.2	3.55	11.0	11.2	10	-	0.681	2.468
	1.6	3.64	11.0	11.6	10	++	0.678	2.334
	2.0	3.54	11.0	12.0	10	+	0.697	2.548
A. niger	0.0	3.89	11.0	10.0	10	+++	1.000	2.113
	0.1	3.04	11.0	10.1	10	+++	0.717	1.898
	0.4	3.60	11.0	10.4	10	+	0.704	2.045
	0.8	3.66	11.0	10.8	10	-	0.711	2.133
	1.2	3.64	11.0	11.2	10	+	0.674	3.914
	1.6	3.65	10.5	11.6	10	++	0.664	2.554
	2.0	3.67	10.0	12.0	10	++	0.658	2.259

TABLE 58. Effect of Endo-PMG from A. repens and A. niger on orange juice concentration and storage.

Enzyme Source	Enzyme added (mls)	Orange juice volume (mls)	Orange juice concentrate volume (mls)	DS ^{o}Be	Remarks
Control	0.0	50	6-7	50	It gelled overnight at room temperature
A. repens	0.5	50	5-6	58	It did not gel at room temperature and in freezer.
	1.0	50	5-6	58	It did not gel at room temperature and in freezer.
A. niger	0.5	50	5-6	62	It did not gel at room temperature and in freezer.
	1.0	50	5-6	63	It did not gel at room temperature and in freezer.

DISCUSSION

This discussion centers on the following:

(1) Selection of two potent Aspergillus strains for pectolytic enzymes production.

(2) Studies of three locally available agro-industrial wastes - wheat bran, rice bran and peanut shell as growth media for pectinase production.

(3) Characteristics of the endo-polymethylgalacturonases produced.

(4) Storage stability studies.

(5) Preliminary toxicological evaluation of the enzyme concentrates.

(6) Some enzyme utilisation studies.

The screening method used in this work was a combination of the cup-plate and pectin clarification techniques as used by Tuttobello and Mill (1961). All the twenty-one fungal strains screened had pectolytic activity but four of them (all were Aspergillus species) performed best; nevertheless, further experiments carried out to determine the quantitative amounts of endo-polygalacturonase (endo-PG) and exo-polygalacturonase (exo-PG) enzymes led to the choice of six strains for further screening.

Although quite a number of publications have discussed the general procedures for producing microbial enzymes (Wallerstein, 1939; Hoogerheide, 1954; Underkofler, 1954, 1966a,

1966b; Beckhorn, 1960; Arima, 1964; Beckhorn et al., 1965), the specific details of current manufacturing processes have not been disclosed. The success of a microbial enzyme manufacturer depends on the selected cultures, the exact composition of media, and the cultural conditions. In order to maintain competitive positions, most manufacturers hold this information as confidential unless patented. Even in patents only laboratory or relatively small-scale;examples are usually cited (Underkofler, 1976), and specific conditions for large-scale production may be quite different from laboratory operations. A first step in the development and improvement of an industrial production process for pectic enzymes is the search for productive and safe (non-toxic) microbial strains.

Screening for production of specific pectic enzymes may be done with the cup-plate technique, originally described for pectic enzymes and other polysaccharide-degrading enzymes by Dingle et al. (1953). An attractive alternative is to rely on simple performance tests, for instance clarification of apple juice in a test tube, a filter test with homogenised fruit tissue, or a maceration test with potato discs (Endo and Miura, 1961; Arima et al., 1964). Endo and Miura (1961) screened two hundred and fifty strains of moulds using the fruit juice clarification technique. Forty-four of them were found to have the action of clarifying fruit juice; out of those, seven strains - Coniothyrium diplodiella, Agaricus capentris, Botrytis cinerea, Penicillium citrinum, Sclerotinia libertiana, Carpenteles javanicus and Aspergillus niger were

chosen as producers of effective pectolytic enzymes, and C. diplodiella proved the most active of all in clarifying fruit juice and hydrolysing pectin or pectic acid. Endo and Miura (1961), Tuttobello and Mill (1961), all based their judgement of the selection of the most potent strain only on the initial screening experiment for pectolytic activity. In the work reported here, the selection of the most potent strain was not limited to the results of the initial screening experiments alone. The criteria used for the selection of the best strain included the determination of the nutritional requirements of the strains so as to select the most potent strain that would also have the advantage of not requiring the most expensive nutrients. Therefore the process used for the selection was a series of experiments in which the strains that performed best were retained at each stage.

Generally, either of two procedures have been applied by various investigators to the study of microbial growth and enzyme production. The procedures are:

(1) the shake flask fermentation technique by which all growth experiments are done on a rotary shaker at about 240 rpm (e.g. Tuttobello and Mill, 1961; Cappellini, 1966; Sreekantiah et al., 1975), and

(2) the static fermentation technique by which the cultures are incubated without shaking (e.g. Ayers et al., 1966; Alabi and Naqvi, 1977; Olutiola and Akintunde, 1979).

The procedure used in the work reported here was the static fermentation technique. Shaking provides unlimited supply of oxygen to the culture. In static cultures, the amount of medium present in the flask is such that the depth is shallow so that even without shaking, the culture would still have adequate oxygen supply. Alabi and Naqvi (1977) used 50ml medium in 150ml Erlenmeyer flask with 2cm medium depth; Olutiola and Akintunde (1979) used 40ml medium in 100ml flask with 2.5cm medium depth. In this work, 100ml medium was used in 500ml special plastic bottle; the medium depth was 3cm.

All microbiologists are aware that microorganisms multiply by cell division and that there are five well-recognised phases during incubation of an organism inoculated into a favourable growth medium. The phases are:

(1) Lag phase

(2) Logarithmic or exponential growth phase

(3) Stationary phase

(4) Declining or death phase, and

(5) Survival phase.

Lag phase: No increase in cell numbers is evident at this phase. The length of lag observed when a fresh medium is inoculated depends on the changes in nutrient composition/ concentration, the age and size of the inoculum. Physiologically, the cells are very active and are synthesising new protoplasm. They may be defficient in enzymes or coenzymes in the new environment which must first be synthesized in amounts required

for optimal operation of the chemical machinery of the cell. Time for adjustment around each cell may be required. Multiple lag phases may sometimes be observed when the medium contains multiple carbon sources. This phenomenon, known as diauxic growth, is caused by a shift in metabolic patterns in the midst of growth. After one carbon substrate is exhausted, the cell must divert its energies from growth to "retool" for the new carbon supply. A possible explanation for this phenomenon is catabolite repression. By using inoculum medium of the same composition as used in the production fermentor and employing large inocula of actively growing seed cultures, lag phase in plant fermentors may usually be completely eliminated.

At the end of the lag phase, each organism divides. However, since not all organisms complete the lag period simultaneously, there is a gradual increase in the population until the end of this period, when all the cells are capable of dividing at regular intervals.

Logarithmic or exponential phase: During this phase, the generation time is the shortest; also the cells are most sensitive to both physical and chemical agents. All cells are relatively uniform in size and have similarly vigorous metabolic activity. The log of the number of cells plotted against time results in a straight line. During this phase, only a single parameter μ (or t_d) is required to characterise the population. For this reason, the magnitude of the specific growth rate μ is widely used to describe the influence of the cell's environment on its

performance. A functional relationship between the specific growth rate μ_i and an essential compound's concentration was proposed by Monod in 1942. It had the same form as the standard rate equation for enzyme catalysed reactions with a single substrate. The Monod equation states that

$$\mu_i = \frac{\mu_{max} \quad C_i}{K_i + C_i}$$

Here μ_{max} is the maximum growth rate achievable when $C_i \gg K_i$ and the concentrations of all other essential nutrients is unchanged. K_i is that value of the concentration of nutrient i where the specific growth rate has half its maximum value; roughly speaking, it is the division between the lower concentration range, where μ_i is strongly (linearly) dependent on C_i, and the higher range, where μ_i becomes independent of C_i.

Since the nutrient level is often initially high in a batch medium ($C_i \gg K_i$), a true exponential specific growth rate will be observed in early stages of nutrient consumption. As C_i approaches K_i, the value of μ_i diminishes and departure from the original exponential law will be predicted. Since K_i is often quite small, however, the stationary phase is often reached before $C_i = K_i$. In a batch system where growth is sufficiently slow and the population is not too large, balanced growth is a reasonable approximation during the exponential growth phase. The equation describes experimental findings under such conditions quite reasonably. The equation may break down if growth is rapid. Other related forms of specific growth

rate dependence have been proposed which in particular instances give better fits to experimental data such as substrate or product inhibition, etc. However, the past history, age, distribution of cells, and other structural information may affect μ as well. Verlhurst in 1844 and Pearl and Reed in 1920 (Bailey and Ollis, 1977) contributed to a theory which included an inhibiting factor to population growth - it gave rise to a sigmoidal logistic curve leading to a stationary population showing that the production rate of a toxin is proportional to the population growth rate. The log phase tapers off in a gradual fashion represented by the transition through a curve.

The Stationary phase: Deviations from exponential growth eventually arise when some significant variables such as nutrient level or toxin concentration achieves a value which can no longer support maximum growth rate. Many cells stop dividing and some die. Often the dead cells lyse and the carbohydrates and amino acids and other compounds freed from the lysed cells are then used as nutrients by the remaining living members of the population. Such cannibalistic events help maintain the population size during the stationary phase. The population remains constant for a time perhaps as a result of complete cessation of division or the balancing of reproduction rate by an equivalent death rate, but by and large it is the period of adaptation to the new environment.

The Death phase: During this phase, cell division still continues to occur but at an ever-decreasing rate, and the number of new cells being formed is far outnumbered by the number of cells dying off. This phase also shows a logarithmic relationship since cells die exponentially.

The Survival phase: Here, cell division ceases completely so that no new cells are being formed. The extremely hostile environment induces a survival reaction from the microorganism. This may be in the form of spore or cyst formation.

There is great variability in the growth phase during which enzyme accumulation occurs, depending upon the particular organism and enzyme. A desired enzyme may appear mainly during any phase except the lag phase. In some cases an extracellular enzyme may begin to appear during the early part of the logarithmic phase and continue to increase in amount during the later stages. In the work reported here, the minimum time required for maximum pectinase production in static submerged culture fermentation was found to be 4 to 5 days for the six fungal strains chosen (Fig. 4). Sreekautiah et al. (1975) and Tuttobello and Mill (1961) found that 5 days of shake flask fermentation was required for maximum pectinase production by Aspergillus carbonarius and Aspergillus niger respectively.

The effect of various carbohydrate sources on pectinase production was determined for three fungal strains - A. repens, A. niger and a Rhizopus species. Although all the carbohydrates

tested gave rise to similar growth patterns (e.g. mycelial weights) in the three fungal strains, the effect on the endo-polygalacturonase production varied significantly. The enzyme filtrates of A. niger and Rhizopus had similarly low protein content whereas A. repens had generally much higher protein content. At the conditions of these experiments, it was clear that the following carbohydrates - fructose (2%), glucose (2%), starch (2%), pectin (1%) and even the combination of sucrose (2%) and pectin (1%) had no inducing effect on the endo-polygalacturonase production by the Rhizopus strain. It produced small amounts of enzyme constitutively, but the addition of vitamin B-complex to the medium (sucrose - 2% plus pectin - 1%) doubled enzyme production. The increase in enzyme formation probably resulted from increased metabolism of the cells rather than by induction. The composition of the fermentation media had a decisive effect on the quantity of the enzyme produced by the Aspergillus strains. The synthesis of endo-polygalacturonase even in the absence of pectic substances shows that they are constitutive although the carbohydrates had varying effects on the amount of the enzyme produced by each strain. Fructose and sucrose were the best inducers of the enzyme in A. niger whereas glucose and sucrose were the best inducers for A. repens enzyme. The addition of pectin (1%) to sucrose (2%) markedly increased the enzyme production by A. niger but it decreased enzyme production by A. repens. The polysaccharides (starch and cellulose) produced the least amounts of enzyme in the two Aspergillus strains.

Vitamin B-complex addition to the media gave rise to greatly increased enzyme synthesis in A. niger whereas it had no effect on enzyme production in A. repens. The effect of the various carbohydrates on the pH of the cultures was also very significant. For A. niger, the pH increased with all the carbohydrates tested; whereas there was general reduction in pH for Rhizopus and A. repens cultures. Stability or decrease in pH during fermentation would obviate the need for pH control to prevent contamination and consequently there would be a great reduction in cost of production if A. repens or the Rhizopus strain would be used for commercial pectinase production.

Tuttobello and Mill (1961) found a certain synergism of pectin and sucrose which led to the highest pectolytic activity being obtained from A. niger when the two carbohydrates were present in similar concentrations. They also found that the efficacy of carbohydrates other than sucrose varied and that in shake flask experiments, some differences were found between the different sugars but these virtually disappeared with cultures in stirred fermentors. However, they also found that polysaccharides were markedly less effective than the mono- and disaccharides tested. Although they did not investigate the effect of nitrogen sources on enzyme production but they suggested that changes in other factors such as nitrogen source might lead to changes in the optimum carbohydrate proportions required for pectinase production.

The investigations of the effect of nitrogen sources on pectinase production were carried out on A. niger and A. repens in

static submerged culture fermentation. Organic nitrogen sources were better inducers of endo-polygalacturonase in A. niger cultures whereas inorganic nitrogen sources were better inducers in A. repens cultures. The highest enzyme synthesis in A. niger was in groundnut cake medium whereas sodium nitrate, urea and peptone media gave the highest enzyme synthesis in A. repens. Aspergillus repens produced endo-polygalacturonase in trace amounts in casein and defatted soya bean flour media. This suggests that it is unable to hydrolyse casein and defatted soya flour in submerged fermentation. It indicates its inability to produce the required proteinase enzymes. When groundnut cake was used as the carbon and nitrogen sources in the submerged cultures, A. niger produced high amounts of enzyme whereas A. repens did not produce much even in media containing as much as 3% pectin; however, there was corresponding increase in enzyme production with increase in groundnut cake concentration in medium. Vitamin B-complex addition to the media caused significant reduction in enzyme production in both A. niger and A. repens cultures when groundnut cake was used as the sole carbon and nitrogen source.

A study of the effect of temperature 35 ± 1°C; 24 ± 1°C; 20 ± 1°C and ambient 24 ± 2°C on the growth and pectinase production of A. niger and A. repens in submerged cultivation showed that both fungal strains are mesophiles. Generally, they grew well at all the temperatures investigated (20 - 35°C) except for A. niger which did not grow well at 35°C. The best temperature for growth and pectinase production by both cultures turned out to be

24°C. Aspergillus repens grew equally well and produced same amounts of pectinase at 35°C, and proved to be superior to A. niger in enzyme production and pH profile at all the temperatures investigated as it maintained high endo-polygalacturonase synthesis and low pH values throughout. In both Aspergillus strains, at 24°C, pectinase production appeared with the onset of the logarithmic growth phase and increased with cell growth. The peak of enzyme synthesis coincided with the peak of cell growth. Subsequently, enzyme synthesis decreased with decrease in cell mass. By growing well and producing good amounts of pectinase at 24 - 35°C, A. repens has trully proved to be ecologically well adapted to the generally warm sub-tropical (nearly equatorial) climate of the west coast of Africa, particularly Nigeria. This is indeed not surprising since it was isolated in Lagos which is about latitude 6° North of the equator, whereas the A. niger strain was isolated in Mysore, India which is about latitude 13° North of the equator. Ability to tolerate moderately high temperatures by A. repens would be of great economic advantage in commercial scale pectinase production as it would obviate the need for excessive cooling of fermentors. Also of economic advantage is the fact that the use of A. repens for commercial pectinase production would not require much pH control as the media normally become more acidic thereby preventing bacterial contamination.

Prior to World War II all commercial microbial enzymes were produced by surface culture methods. Submerged culture has now come into extensive use. However, several fungal enzyme products

continue to be produced by the original Takamine moldy bran or koji process, in which the mold is cultivated on the surface of a solid substrate, moist wheat bran (Underkoflar, 1976). There are advantages and disadvantages to each of the semi-solid surface and submerged culture methods. The surface culture process has advantages in simplicity of operation, low power requirements, and freedom from contamination problems, but with the disadvantages of needing much space and hand labour. The submerged culture method has advantages, of low labour and space requirements, uniformity, and ease of control; its disadvantages are high power requirements and occasional serious contamination problems. Whether the surface or submerged culture method is employed for a particular enzyme is determined mainly by relative yields and convenience, although sometimes plant equipment availability and enzyme application are also factors.

Three locally available agro-industrial wastes - wheat bran, rice bran and peanut shell were investigated for pectinase production as semi-solid substrates. They all contained water soluble nutrients - sugar, protein, starch and pectin - which supported the growth of A. niger and A. repens for pectinase production. Wheat bran has been the traditional solid substrate used in the production of many industrial enzymes such as amylases, proteases, pectinases and cellulases. The proximate composition and particularly the results of analysis of the cold and hot water extracts of rice bran and peanut shell suggested that they could

be of comparable value to wheat bran as solid substrates for industrial enzymes production. Rice bran and peanut shell contained more fibre and fat than wheat bran; rice bran had more ash than wheat bran but wheat bran had more protein and carbohydrates than both rice bran and peanut shell. Wheat bran had at least twice as much cold or hot water soluble nutrients than either rice bran or peanut shell. Endo and Miura (1961) screened 250 strains of fungi for an organism which would produce pectinolytic activity to clarify apple juice, when grown on commercially available refined defatted rice bran and refined wheat bran media. A strain of Coniothyrium diplodiella was selected. There has not been any reports in scientific literature on any attempts to use peanut shell as substrate for any industrial fermentations.

The effect of hydrochloric acid (0 - 0.3M) as diluent on pectinase production by A. niger and A. repens when grown on wheat bran, rice bran and peanut shell as semi-solid media was investigated. Generally rice bran gave the best results for enzyme production followed by wheat bran and then peanut shell. The best diluent for the two Aspergillus strains was 0.05 - 0.1M hydrochloric acid and the optimum time was 2 - 3 days. The pH profiles for both strains were similar but there was a general increase in pH values for A. niger with 0.1M acid as diluent. This was similar to the results obtained in submerged liquid culture experiments. There was no apparently meaningful relationship between the enzyme production and the reducing sugars and protein content of the media. Although when 0.2M acid was used as diluent,

A. niger produced pectinase in good amounts in all the media but this could prove unreliable and difficult on a commercial scale since the results of 0.15M and 0.3M acid were poor. Qadeer and Iqbal (1985) studied solid substrate fermentation for pectinases by Aspergillus foetidus. They found that 0.1M hydrochloric acid as diluent gave optimum results of both pectinesterase and polygalacturonase. Maximum enzyme production was obtained 48 hrs after incubation at 30°C.

In most instances, extracellular pectinase production by microorganisms has been investigated in submerged culture experiments. Brooks and Reid (1955) isolated an exopolygalacturonase from the complex of pectic enzymes produced by Aspergillus foetidus, grown on solid bran media. This enzyme was not present in submerged cultures.

Spore concentrations of 2.2×10^5 per ml of A. repens and 4.7×10^5 per ml of A. niger were found to be adequate to give maximum enzyme yield per 10g (dry weight) solid medium without a long initial growth lag of each fungus.

Grapefruit peel, Citrus decumana var bigardia, (6.4% pectin content) and soya flour, Glycine max, (whole and defatted) greatly increased pectinase production by A. repens in wheat bran and rice bran. In peanut shell, grapefruit peel had no effect on the enzyme yield but soya flour increased enzyme production - whole soya flour gave greater yield than the defatted flour. Glucose and soluble starch increased enzyme yield from wheat bran. Sodium nitrate increased enzyme yield from peanut shell but caused

a decrease in rice bran and wheat bran. Plantain peel, Musa sapientum (6.1% pectin content) reduced enzyme yield by A. repens and A. niger in all the three semi-solid media. Sucrose decreased enzyme yield in rice bran and peanut shell. Sucrose did not change the enzyme yield significantly in wheat bran. It will be misleading to compare these results of surface culture experiments with those of submerged culture experiments discussed earlier due to two main reasons.

Firstly, the media used in the submerged culture experiments were synthetic chemically defined with only one pure carbon or nitrogen source in each medium whereas the semi-solid media were naturally mixed carbon and nitrogen sources where several inducers and repressors may be present. Secondly, for reasons that are still not yet well explained (Wiseman, 1979), semi-solid fermentation with its high aeration, low water contentration and extremely large surface area, enhanced rapid formation of many enzymes in contrast to the well known multiphase growth in submerged fermentation. Enzyme synthesis is faster and less susceptible to catabolite repression in semi-solid fermentation than in the submerged liquid fermentation.

The results of nutrient enrichment experiments of wheat and rice brans, and peanut shell for pectinase production by A. niger showed that grapefruit peel, sucrose and soya flour (whole) increased enzyme production in peanut shell only whereas plantain peel reduced enzyme production in the three media.

Soluble starch caused decrease in enzyme production in peanut shell. Sodium nitrate and glucose caused decrease in enzyme production in rice bran and peanut shell. It is clear that these rather unpredictable patterns in enzyme synthesis by the fungal strains is responsible for the highly confidential manner the different manufacturers keep the formulations they develop (Underkofler, 1976; Fogarty and Kelly, 1983). From the nutrient enrichment results, two general remarks can be made - the first is that grapefruit peel and soya flour (whole) induced pectinase production in the three semi-solid media (wheat and rice brans, and peanut shell) by A. niger and A. repens; secondly, plantain peel repressed pectinase production in the three media by the two fungal strains.

The results of the effect of media depth on pectinase production by A. repens grown on wheat and rice brans, and peanut shell showed that in rice bran at 2 - 10cm depth, enzyme activity was practically the same although the specific activity decreased with increase in depth. In peanut shell, 2 and 4cm depths showed similar enzyme activity but at 4cm depth, the specific activity was greater. In wheat bran the highest enzyme activity was at the 2cm depth while the lowest was at 6cm depth; however, the enzyme activities at 8cm and 10cm were the same with that at 4cm. The effect of media depth on pectinase production by A. niger showed that in wheat bran 4cm depth gave the highest enzyme yield while in rice bran a depth of up to 6cm was as good as 2cm in enzyme yield. For peanut shell, 6cm depth gave the

lowest enzyme yield but at 8 and 10cm depth enzyme yield was even higher than at 2cm and 4cm levels.

Media depth is very critical in solid substrate fermentation as it would determine the economic feasibility of such a venture for large scale production. Qadeer and Iqbal (1985) studied the effect of varying concentrations of wheat bran (10 - 60g per 1 litre conical flask which gave rise to 0.4 - 2.5cm depths) on pectinesterase and exo-polygalacturonase production by Aspergillus foetidus. They found that the production of both enzymes was maximum at 0.4cm depth and decided that this would not be economically feasible since it would require very large areas. The two fungal strains - A.niger and A. repens studied in this work showed great prospects of doing extremely well if cultivated in any of the three solid media; wheat bran, rice bran and peanut shell.

Because pectic enzymes are produced on complex media with natural substrates, the commercial enzymes normally contain significant activities of other enzymes such as cellulases, hemi-cellulases, proteases, and others. When A. niger and A. repens were grown on wheat bran, rice bran and peanut shell, the enzyme composition of their culture filtrates were analysed for pectinases, cellulases, amylases, and acid protease content. They all contained little saccharogenic activity but highly significant amounts of viscosity reducing enzymes - endopoly-galacturonase, cx-cellulase, α-amylase and also acid protease.

All commercial pectic enzyme preparations are fungal; all those marketed in the United States are from Aspergillus niger and virtually all those imported are made by surface culture on wheat bran or other solid media. Production in the United States is shifting to submerged fermentation with very high energy (agitation) and oxygen level requirements (Kirk-Othmer, 1980). Pectic substances are essentially chains of anhydrogalacturonate residues, with varying amounts of methyl ester linkages. These chains are attacked at random by polymethylgalacturonate lyase and are thus reduced to short pieces of 6 to 8 methoxy anhydrogalacturonate residues which cannot be attacked further by the enzyme, therefore the viscosity drops rapidly and monomeric units are not produced. Polygalacturonase breaks the chain into monomers, but only breaks down the polymer after hydrolysis of the methyl ester. Thus polygalacturonase in combination with pectin esterase breaks pectic substances down completely to galacturonic acid and methanol. In the absence of pectin esterase and polymethylgalacturonate lyase, polygalacturonase partially hydrolyses pectic substances. In fact, it acts on the insoluble protopectin to solubilise it and, hence, polygalacturonase alone can be used to increase viscosity and stabilise the cloudiness in fruit and vegetable juices. Alone, polygalacturonase is also used to separate plant cells which are held together by protopectin.

Comparison of different commercial pectic enzyme preparations shows major differences in the content of polygalacturonase, pectinesterase, polygalacturonate lyase, cellulase, hemi-cellulase

and protease. Some are virtually devoid of polygalacturonate lyase, others almost devoid of polygalacturonase. Yet they can function interchangeably because pectic enzymes are evaluated under actual use conditions based on the amount of enzyme for a given price, not on comparison of units. Pectic enzyme preparations vary in price from about 2 to more than 200 U.S. dollars per kilogram (Kirk-Othmer, 1980). Yet these preparations are competitive on a cost-performance basis.

The endo-polymethylgalacturonase produced by A. repens when grown on rice bran as semi-solid medium was characterised by its action on citrus pectin (77.4% galacturonic acid and 7.7% methoxyl content). The optimum pH for action was 4 - 5.5; optimum temperature was 40 - 50°C. At 20 - 30°C there was no change in activity, at 60°C about 70% of the activity remained; at 70°C about 33% of the activity was retained and at 80°C about 20% of activity was left. At 40°C and pH 4.0, there was exponential decrease in viscosity for the first 10 minutes. Subsequently, there was slower rate of decrease in viscosity till maximum fall in viscosity occurred after 40 - 60 minutes incubation. At 90°C all the enzyme was destroyed within 5 minutes. In the case of endo-polymethylgalacturonase produced by A. niger, two sharp optimum pH values of 4.5 and 5.5 were obtained. There was greater enzyme activity at pH 4.5 than pH 5.5. The optimum temperature was 40°C (pH 4.0). At 30°C and 50°C, enzyme activities were similar (90%) falling to about 75% at 60°C; but at 70°C no enzyme activity was retained. The time-course of enzyme activity

at 40°C (pH 4.5) was similar to that at 60°C (pH 4.5). This means that at the lower optimum pH 4.5, the enzyme was equally active at both 40 and 60°C. At 70°C (pH 4.5), about 65% of enzyme activity was lost in 10 minutes and after about 35 minutes of incubation, all enzyme activity was lost. The heat stability studies again show that A. repens enzymes are more heat tolerant than those of A. niger. This result and those earlier discussed about the mycelia growth, enzyme production and pH profile at various temperatures (20 - 35°C) further shows that A. repens is more adapted to warmer conditions than A. niger. The substrate concentration effect (0.25 - 1.25% pectin) on endo-polymethyl-galacturonase activities of A. repens and A. niger showed that 0.75% and 0.5% citrus pectin concentrations respectively, were most suitable although the enzyme activities were good at all the substrate concentrations.

Sreekantiah et al. (1975) studied pectinolytic enzymes produced by Aspergillus carbonarius and found that pH 3.5 - 4.0 and 40°C were optimum for viscosity reducing activity. They also found that at 40°C the enzyme was quite stable but rather labile at 50°C - as much as 50% activity was lost in 15 minutes at 50°C and complete inactivation of the enzyme was observed within 5 minutes at 70°C.

Enzymes are proteins and are therefore subject to spoilage on storage if proper care is not taken. The shelf life of enzymes can be prolonged in several ways, e.g. removal of microorganisms; asepsis or keeping out microorganisms; cold

storage/freezing; freeze drying and use of chemical preservatives. Only rarely is a single method effective, and usually two or more of the above methods are combined. Storage stability studies of A. niger and A. repens enzymes in liquid form at ambient temperatures and at $10^{\circ} \pm 2^{\circ}C$ were carried out for four weeks. The effect of storing the enzymes at various pH values (3 - 6) was determined. Aspergillus repens enzyme at pH 3 - 4 was quite stable at room temperatures. Aspergillus niger enzyme was stable at room temperatures when stored at pH 3.0. Aspergillus repens enzymes stored well at $10^{\circ} \pm 2^{\circ}C$ at pH 5 - 6 whereas A. niger enzymes stored well at pH 3 - 6. Sreekantiah et al. (1975) found no loss in enzyme activity of Aspergillus carbonarius when it was stored for 30 days at room temperature (20 - $30^{\circ}C$ in Mysore, India) and pH 3.5 to 4.5. The concentrated enzyme solution (2% protein and pH 3.5 - 4.0) could be stored without much loss in activity for one year at room temperature (Sreekantiah et al., 1969).

In the U.S.A. the Food and Drug Administration (FDA) controls all uses of enzymes in foods, pharmaceuticals, etc. U.S food law provides for the approval of enzyme preparations "generally regarded as safe" (GRAS) by experts qualified by scientific training and experience to evaluate such products. The FDA officially recognises the Food Chemicals Codex which is a series of food grade quality specifications for substances added to foods. In 1974, the first supplement to the second edition of the Codex was published. It contained a general

description of and specifications for enzymes commonly used in foods in the United States. Assay methods were also included. In general, this supplement conforms to the report of the Joint FAO/WHO Experts Committee on Food Additives published in 1972 with subsequent publication of reports in 1975 and 1978. This last publication (WHO, 1978), an unofficial opinion of the Joint Experts Committee portends the future and likely reflects the position of the FDA (Kirk-Othmar, 1980). It states among others, that from the safety point of view the presence of potentially harmful contaminants and by-products is of common concern when microorganisms are used for the production of enzymes since mutations might occur that could lead to the emergence of new, potentially toxic products and that it is now felt that chemical and microbiological specifications and the biological control of strains of microorganisms used to produce these food enzymes are of the utmost importance in assuring the safety of these materials. It also states that:

> "Enzymes derived from microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods. These products are regarded as foods and consequently accepted, provided satisfactory chemical and microbiological specifications can be established".
>
> "Enzymes derived from non-pathogenic microorganisms commonly found as contaminants of foods. These materials are not considered as foods. The committee considers it

necessary to establish chemical and microbiological specifications and to conduct short-term toxicity experiments to ensure the absence of toxicity. Each preparation must be evaluated individually and an average daily intake (ADI) must be established".

"Enzymes derived from microorganisms that are less well known. These materials also require chemical and microbiological specifications and more extensive toxicological studies, including a long-term study in a rodent species".

The Aspergillus glaucus group, with A. repens as an important species, is often involved in food spoilage. They are among the most commonly occurring and most destructive of all moulds - they require less moisture for spore germination and growth. They are commonly found in pure culture on jams which contain only a little less than the safe percentage of sugar, they grow on textiles which contain very little more moisture than the amount usually considered to be normal for any particular material, they also attack tobacco. For A. repens enzymes, it is necessary to conduct short-term toxicity studies to ensure the absence of toxicity.

In solid substrate fermentation, toxins could arise from two main sources - the solid substrate itself (wheat and rice brans and peanut shell) and the metabolic products of the microorganism. The sanitary condition of the substrate is

therefore very important. Toxicological studies (chemical and biological assays) were carried out on rice bran, A. niger and A. repens cultures and their crude enzyme concentrates. The absorbance measurements and the chromatographic separation of the extracts did not indicate presence of any mycotoxins. Inspite of the extremely high dose level of extract injections per rat per day (1ml extract injection per rat per day was equivalent to 3 litres orange juice per rat per day or 36 litres orange juice per kilogram body weight of rat per day), statistical analysis of the results of the transaminases-glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) - of the blood serum, liver, kidney and urine of the Wistar Albino rats did not indicate the presence of any mycotoxins. Both GOT and GPT are normally present in most tissues and are found in large quantities in the myocardium, skeletal muscle, brain, liver and kidney. Necrosis in any of these tissues will result in the liberation of large quantities of these enzymes into the serum.

Ngaha (1985) studied biochemical changes in rat urine and tissues treated with five consecutive daily doses of Ochratoxin A (10mg/kg body weight). He found that urine volume and urinary proteins were raised, so were other urinary enzymes such as muramidase, alkaline and acid phosphatases, lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH); but kidney phosphatases, LDH and GDH were correspondingly reduced. Liver LDH was reduced

while serum LDH was raised. Chauhan *et al.* (1986) also studied some biochemical changes in rat vital organs - liver, brain, kidney - caused by feeding a diet infested with *Penicillium rubrum* for 30 days. They determined the haemoglobin, total proteins and transaminases in the blood and tissues and found that the fungus rendered the food toxic - resulting in liver and kidney damage. Essien (1988) studied the antihepatotoxicity of *Garcinia kola* extracts on paracetamol toxicity in Wistar albino rats available locally (in Lagos, Nigeria). The control rats had 135 $\pm$ 5 units/ml SGOT (serum GOT) and 42 $\pm$ 5 SGPT. The paracetamol treated group had SGOT 2,000 $\pm$ 100 units/ml and SGPT 1,600 $\pm$ 50 units/ml. *Garcinia kola* extracts caused amelioration in the toxic effect of paracetamol as evidenced by the reduction in serum transaminases to 1,600 $\pm$ 100 units/ml SGOT and 1,150 $\pm$ 50 units/ml SGPT. In the work reported here, the Wistar albino rats used were from the same source with those used by Essien (1988) and the results of the serum transaminases were not significantly different from those of his control rats. Essien did not examine the liver enzymes. In this study only the liver enzyme - LGOT for *A. niger* extract was significantly higher ($P < 0.05$) than the normal control, but since the LGPT was not significantly higher than the control LGPT and the blood serum SGPT was normal therefore liver necrosis was not indicated especially as the urinary protein excretion was normal in all cases. The results of the urinary excretion of UGOT and UGPT

showed significantly higher ($P < 0.01$) excretion of UGOT for both Aspergillus strains. This result is not reliable due to the fact that the urine and the faeces of the rats could not be separated perfectly. Although it would still be necessary to carry out further toxicological studies that will involve assessment of more parameters such as muramidase, LDH, GDH, phosphatases, amylases, etc; the results of this preliminary investigation suggests that Aspergillus strains - A. niger and A. repens could serve as good sources of food grade pectinase enzymes in future.

Commercial pectic enzyme preparations contain mainly pectinesterase (PE,), polygalacturonase (PG) and pectin lyase (PL). They can therefore fulfil their technical function of degradation of highly esterified pectic substances by two pathways, i.e. deesterification by PE and hydrolysis by PG or direct depolymerisation by eliminative splitting of the glycosidic linkages. Special preparations with mainly PG activity or PL activity are also on the market. Apart from pectolytic activities, all commercial pectinases contain varying amounts of other enzymes e.g. cellulases, amylases, etc. It was estimated that enzyme production for food uses in the western world amounted to 45 million US dollars per annum (Beck and Scott, 1974), one fourth of which related to pectic enzymes (Pilnik and Rombouts, 1979).

The principal uses of pectic enzymes are:-

(1) Production of fruit juices and fruit-juice products.

(2) Production of wines.

(3) Fermentation of coffee and cocoa beans.

(4) Rehydration of dehydrated foods.

(5) Production of galacturonic acid and low-methoxyl pectin.

(6) Recovery and stabilisation of citrus oil.

Freshly pressed fruit juices contain colloidal material that keeps dispersed solids in suspension; most of it is pectin. In citrus and tomato juices, where the high viscosity is a required advantage, the pectic enzymes of the juice are destroyed by pasteurisation in order to maintain the high viscosity. In other fruit juices such as apple and grape juices, a clear flowing liquid is required where the suspended material settles quickly. For such juices a commercial mixture of the pectic enzyme is usually added to the fruit during or after crushing; this also increases the yield. During wine production, the methanol content of the enzyme-treated juices is higher than for wines made from untreated juices; however, it is still well below the permissible level. For instance, an untreated red wine may contain 0.019% (190 ppm) methanol, and the enzyme-treated wine 0.023% (230 ppm) (Kirk-Othmer, 1980).

The two crude enzymes produced in this work were studied for application in processing some fruits. The crude enzymes of A. niger and A. repens were very effective in macerating banana, depectinising the juice and drastically reducing its viscosity. The crude enzymes were not effective on raw carrots but probably precooking the carrot would enhance enzyme action.

The action of both enzymes on fully ripe guava increased juice yield and greatly reduced the viscosity; A. repens enzyme was very effective in depectinising guava juice but A. niger enzyme was not. The crude enzymes of both Aspergillus strains were effective in depectinising orange juice and reducing its viscosity. Orange juice concentrates (58 - 63° Be) which did not gell on storage at room temperature and in the freezer were successfully produced by enzymes from both strains. These studies show that A. niger and A. repens are two fungal strains with a great potential for use in commercial pectinase production in Nigeria.

This work shows that three locally available agro-industrial wastes - wheat bran, rice bran and peanut shell can be used as media for commercial pectinase production. Aspergillus niger A. repens are two potent fungal strains which may be grown on the media and the enzymes can be produced locally, simply and cheaply in tray fermentors with little or no energy (agitation) requirements. Aspergillus repens, a local isolate was found to be ecologically well adapted to the Nigerian environment, there is therefore little fear of the dangers of mutations that could lead to the emergence of new and potentially toxic products. The need to fully utilise these findings for fruit processing to meet local demands cannot be more appropriate than now that the Federal Government has banned the importation of all fruits and fruit products into Nigeria. There is now a very favourable

atmosphere for local utilisation of fruit processing technology which is either locally developed or adapted with local research inputs such as the ones presented in the work reported here. The industrial application of the findings reported here can have the following advantages in Nigeria -

(1) These agro-industrial wastes - wheat bran, rice bran and peanut shell - especially the last two, would become raw materials for the production of more valuable industrial products such as pectic enzymes.

(2) The local production of pectolytic enzymes using local know-how will conserve much needed foreign exchange and even generate more when the enzymes and the enzyme-derived products are exported.

(3) More employment will be generated locally.

(4) The common problems of colossal fruit wastes during the glut seasons will be reduced; in fact, it can be totally prevented since local processing will result in greater capacity utilisation of fresh fruits; moreover, even over-ripe fruits such as bananas, guava, etc. can be liquefied by pectinases and used to make refreshing fruit drinks.

(5) Fresh fruit drinks can be made available throughout the year resulting in better health of the populace.

(6) Nigeria can be self-sufficient in fruit processing technology.

Thus, there is no doubt whatsoever that local production of these enzymes will enhance small-scale industries that are basedon local food crops. However, it would be necessary to carry out further toxicological studies that will involve the assessment of more parameters such as muramidase, lactate dehydrogenase, glucose dehydrogenase, alkaline and acid phosphatases, amylases, etc. - enzymes in the tissues that will indicate the metabolic status of food reserves being degraded.

It is also important to determine the effectiveness of the enzymes in wine clarification as this particular aspect of pectic enzyme utilisation is becoming more important in Nigeria.

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APPENDIX 1

NUMBER OF HOLDINGS, AREA PLANTED, PRODUCTION AND YIELD PER HECTARE AND TYPE OF CROPS - . ECONOMIC AND FOOD CROPS.

Crops	YEAR: 1981/82				YEAR: 1980/81			
	No.	A (hectares)	P (tonnes)	Y/H (Kgs)	No.	A (hectares)	P (tonnes)	Y/H (Kgs)
ECONOMIC:								
Groundnut	296	11,251	7,740	688	262	7,577	2,826	373
Citrus	52	337	525	1,558	42	358	759	2,120
Mango	20	233	504	2,163	17	121	199	1,645
Guava	20	166	173	1,042	20	162	13	80
Pineapple	17	26	49	1,885	15	22	36	1,636
FOOD:								
Rice	302	19,615	30,735	1,567	322	11,310	15,451	1,366
Banana	43	882	2,111	2,393	45	895	2,182	2,438

No. - Number of holdings reported; A - Area planted (hectares);

P - Production (tonnes); Y/H - Yield per Hectare (kilogram).

APPENDIX II

COMPOSITION OF VITAMIN B-COMPLEX (DUMEX LTD. DENMARK)

Vitamin B-1	100mg/litre
B-2	50 "
B-6	50 "
Nicotinamide	500 "
Calcium pantothenate	30 "
Vitamin B-12	10ug/litre

APPENDIX III

PROXIMATE COMPOSITION OF SOYA BEANS USED IN NUTRITIONAL STUDIES 2.2.3.

	Whole	Defatted
Protein	42.9%	48.9%
Fat	24.3%	2.5%
Fibre	3.5%	3.4%
Ash	5.7%	7.9%
Carbohydrate	23.6%	37.3%

(Carbohydrate by difference)

APPENDIX IV

PROXIMATE COMPOSITION OF GROUNDNUT CAKE USED IN NUTRITIONAL STUDIES 2.2.4.

Protein	34%
Fat	35%
Fibre	2.8%
Ash	4.8%
Carbohydrate	23.4%

(Carbohydrate by difference).

APPENDIX V

*PROPORTION OF PARTS IN WHEAT AND RICE (%)

	Pericarp (Hull + Testa)	Aleurone	Starchy Endosperm	Germ	
				Embryo	Scutellum
Wheat:					
Thatcher -	8.2	6.7	81.5	1.6	2.0
Vilmorin 27 -	8.0	7.0	82.5	1.0	1.5
Argentinian -	9.5	6.4	81.4	1.3	1.4
Egyptian -	7.4	6.7	84.1	1.3	1.5
Rice:					
Whole grain 20	4.8		73.0	2.2	
Kernel:					
Indian -	7.0		90.7	0.9	1.4
Egyptian -	5.0		91.7	3.3	

*Data from Kent (1975).

APPENDIX VI

*PROXIMATE COMPOSITION OF WHEAT AND RICE (%)

(Dry Matter Basis)

	**Protein	Fat	Soluble Carbohydrate	Crude Fibre	Mineral Matter
Wheat:					
Manitoba	16.0	2.9	74.1	2.6	1.8
English	10.5	2.6	78.6	2.5	1.8
Mixed grist	15.0	2.1	78.6	2.4	1.9
Rice:					
Paddy	9.1	2.2	71.2	10.2	7.2
Brown	11.0	2.7	83.2	1.2	1.8
Polished	9.8	0.5	88.9	0.3	0.6

*Data from Kent (1975)

**Protein N x 5.7 for wheat; N x 5.95 for rice.

APPENDIX VII

*VITAMIN CONTENTS OF WHEAT AND RICE (ug/g)

Vitamin	Wheat		Rice
	Manitoba	English	(Brown)
Vitamin B_1	4	2.9	4.0
Riboflavin (B_2)	1.2	1.1	0.6
Nicotinic acid	70	50	53
Pantothenic acid	10-15		17
Biotin	0.1		0.1
Pyridoxin (B_6)	5		10.3
Folic acid	0.5		0.6
Choline	1,000		900
Inositol	2,500		-
P-Aminobenzoic acid	1		-
Vitamin B_{12}	0.001-0.002		-

*Data from Kent (1975).

APPENDIX VIII

*DISTRIBUTION OF NICOTINIC ACID AND VITAMIN B_1 IN WHEAT AND RICE

Part of grain	Wheat English (Vilmorin 27)	Rice Indian	Wheat English	Rice Indian
Pericarp, testa and Hyaline	4	5	33	34
Aleurone layer	82	80.5		
Endosperm	12	12.3	3	8
Embryo	1	0.6	2	11
Scutellum	1	1.6	62	47

*Source of Data: Kent (1975).

APPENDIX IX

*DISTRIBUTION OF B-VITAMINS IN THE WHEAT GRAIN

Part of Grain	(Variety: Vilomarin 27) Vitamin B1	(Variety: Thatcher) Nicotinic acid	Riboflavin	Pyrid-oxin	Pantothenic acid
	(a) Concentration in the parts of the grain (ug/g)				
Pericarp, testa and hyaline	0.6	25.7	1.0	6.0	7.8
Aleurone layer	16.5	741	10	36	45.1
Endosperm	0.13	8.5	0.7	0.3	3.9
Embryo	8.4	38.5	13.8	21.1	17.1
Scutellum	156	38.2	12.7	23.2	14.1
Whole grain	3.75	59.3	1.8	4.3	7.8
	(b) Distribution among the parts as % of total in grain				
Pericapr, testa and hyaline	1.0	4	5	12	9
Aleurone layer	32	82	37	61	41
Endosperm	3	12	32	6	43
Embryo	2	1	12	9	3
Scutellum	62	1	14	12	4

*Source of Data: Kent (1975).

APPENDIX X

CULTURE COLLECTION - DESCRIPTION OF MATERIAL

Culture No.	Substratum	Locality of Isolation	Date Collected or Isolated	Collector or Isolator	Identification
1	Lime fruit	Lagos-Agege	Aug. 1986	T.A. Ayanleye	Rhizopus
2	"	"	"	"	Aspergillus repens
3	"	"	"	"	Rhizopus
4	"	"	"	"	"
5	Orange peel	Lagos-Agege	Dec. 1986	"	Aspergillus
6	"	"	"	"	"
7	"	"	"	"	"
8	"	"	"	"	"
9	"	"	"	"	"
10	Plantain fruit	Lagos-Agege	1987	"	Rhizopus
11	-	India-Mysore	1980	C.F.T.R.I.	Aspergillus
12	Plantain fruit	Lagos-Agege	1987	T.A. Ayanleye	"
13	Lime fruit	Lagos-Agege	"	"	"
14	Pawpaw fruit	Lagos-Agege	"	"	Rhizopus
15	Tempeh	Indonesia	1986	F.I.I.R.O.	R. digosporus
16	-	Indonesia	"	"	R. oryzae
17	Decaying plant	Lagos-Agege	1987	T.A. Ayanleye	Aspergillus
18	Dust	Lagos-Oshodi	"	"	"
19	-	India-Mysore	1984	C.F.T.R.I.	A. carbonarius
20	Cellulose	India-Mysore	1980	"	A. niger
21	-	Lagos-Oshodi	1987	F.I.I.R.O.	Geotrichum candidum

Note: CFTRI - Central Food Technological Research Institute, Mysore, India.

FIIRO - Federal Institute of Industrial Research Oshodi, Lagos, Nigeria.

APPENDIX XI

EXTRACTION OF MOULDY RICE BRAN (GORST-ALLMAN AND STEYN, 1979)

The mouldy bran (100g) was extracted in a Waring blender with methanol-chloroform (1:1) (400ml) at 23,000 rpm for 4 x 1 min. The mixture was filtered and the filtrate was evaporated to dryness. The resultant brown residue was partitioned between n-hexane and 90% methanol (1:1) (200ml), the n-hexane layer was discarded and the methanol layer was evaporated to dryness. The brown solid was partitioned between chloroform and water (1:1) (200ml) and the chloroform layer was extracted with saturated sodium hydrogen carbonate solution (3 x 100ml). The chloroform layer was concentrated and contained any of the so-called neutral mycotoxins (viz., aflatoxin B_1, sterigmatocystin, zearalenone, patulin, T_2-toxin, roquefortin, penitrem A, fumitremorgen B and roridin A) that were present initially. The aqueous layer was carefully acidified to pH 2 (with 0.5N HCl) and extracted with chloroform (3 x 100ml). The chloroform extract was concentrated and contained any of the so-called acidic mycotoxins (ochratoxin A, citrinin, α-cyclopiazonic acid and penicillic acid) present.

APPENDIX XII

QUICK BIOLOGICAL SCREENING METHOD FOR MYCOTOXINS-RAT, LIVER AND KIDNEY WEIGHTS

Rat Group	Rat Weight (g)	Liver (g)	Kidneys (g)
A. repens	1. 73.4	4.5	1.8
	2. 58.3	3.2	1.9
	3. 81.2	4.5	2.0
	4. 106.5	3.6	1.5
	5. 96.7	3.7	1.5
A. niger	1. 66.7	3.3	1.5
	2. 77.7	3.75	1.45
	3. 76.7	3.8	1.4
	4. 81.6	3.9	1.65
	5. 106.7	4.5	1.75
Control-I	1. 86.95	3.5	1.75
	2. 98.3	4.05	1.7
	3. 80.7	3.6	1.3
	4. 77.4	3.9	1.35
	5. 99.0	4.9	1.7
Control-II Saline Solution	1. 104.1	5.0	1.8
	2. 86.1	3.6	1.7
	3. 83.1	4.35	1.3
	4. 113.2	5.6	1.7
	5. 105.4	4.5	1.8

Note: Each group had 3 males and 2 female rats.

APPENDIX XIII

DIAGNOSTIC ENZYMOLOGICAL ASSAY

*Reitman-Frankel Standards for Serum Glutamate Oxaloacetate transaminase (SGOT) and Glutamate Pyruvate transaminase (SGPT).

Absorbance 535nm	SGOT Units/ml	SGPT Units/ml	Absorbance	SGOT	SGPT
0.143	1		0.328	178	151
0.149	5	3	0.337	191	162
0.155	8	6	0.347	204	171
0.161	11	9	0.357	218	
0.168	15	12	0.367	232	
0.174	19	16	0.377	246	
0.181	23	20	0.387	262	
0.187	28	25	0.398	278	
0.194	33	35			
0.201	38	35			
0.208	43	40			
0.215	49	45			
0.222	55	51			
0.229	62	57			
0.237	69	64			
0.244	76	71			
0.252	84	79			
0.260	93	87			
0.268	102	94			
0.276	111	101			
0.284	122	110			
0.292	132	118			
0.301	142	127			
0.310	154	135			
0.319	166	143			

*Reitman and Frankel (1957).

Procedure - SGOT:

0.1ml of non-hemolysed serum was mixed with 0.5ml of glutamic-oxaloacetic transaminase substrate and incubated at 37°C for one hour. 0.5ml of 2,4-dinitrophenylhydrazine solution was added and allowed to stand for 15 minutes at room temperature. 5ml of 0.4N sodium hydroxide was added and mixed well and allowed to stand at room temperature for 20 minutes. The absorbance was determined in an SP 30 spectrophotometer at 535 nm using water to set the instrument to zero.

The substrate was a mixture of α-ketoglutaric acid (0.292g/l) and DL-aspartic acid (26.6g/l) in pH 7.4 phosphate buffer (13.97g/l anhydrous K_2HPO_4 and 2.69g/l anhydrous KH_2PO_4).

Procedure for SGPT was same as above except that the substrate contained 17.8g DL-alanine instead of aspartate.

Procedure for liver and kidney -

One gram of fresh tissue was macerated using ice cold mortar and pestle with washed fine sand. This was extracted with 10mls of ice cold phosphate buffer pH 7.4. The rest of the procedure was the same as above.

Procedure for urine -

The urine absorbed in the filter paper was extracted with phosphate buffer (pH 7.4) to give about 50mls solution. The rest of the procedure was the same as above.

LGOT

A. repens		A. niger		Rice Bran Control		Normal Control	
U/ml	U/mg Prot.	U/ml	U/mg Prot.	U/ml	U/mg Prot.	U/ml	U/mg Prot.
640	1524	425	1250	364	674	434	819
634	1921	504	1292	535	1574	475	896
475	579	420	1105	474	1129	615	976
520	929	493	967	380	792	405	779
570	655	380	950	340	756	610	884
$\bar{X}$	1122		1113		985		871
S.D	581		157		372		76

LGPT

360	857	275	809	124	230	410	774
390	1182	395	1013	340	1000	440	830
390	476	355	934	286	681	495	786
390	696	265	520	315	656	115	221
500	575	310	775	115	256	405	587
$\bar{X}$	757		810		565		640
S.D	277		188		324		252

KGOT

A. repens		A. niger		Rice Bran Control		Normal Control	
U/ml	U/mg Prot.	U/ml	U/mg Prot.	U/ml	U/mg Prot.	U/ml	U/mg Prot.
610	1356	440	880	405	723	420	1050
754	1005	490	980	420	1400	435	926
540	964	494	1008	380	844	420	977
515	631	444	987	350	700	240	585
435	926	415	769	240	421	310	775
$\bar{X}$	976		925		818		863
S.D	258		100		361		185

KGPT

275	611	260	520	190	339	195	488
445	593	220	440	230	767	240	511
300	536	205	418	200	444	270	628
290	468	195	433	195	390	155	378
364	774	230	426	180	316	210	525
$\bar{X}$	596		447		451		506
S.D	114		41		183		89