pigeon-liver homogenates stimulated the aerobic and anaerobic rates of reduction of oxidized glutathione to the same degree. At concentrations of oxidized triphosphopyridine nucleotide above 0·11 mM the anaerobic reduction of oxidized glutathione was lower than the aerobic reduction.

4. Citrate, fumarate and glucose 6-phosphate stimulated oxidized glutathione reduction in pigeon-liver homogenates. The addition of oxidized triphosphopyridine nucleotide (0·04 mM) further stimulated the reduction.

5. In pigeon-liver homogenates incubated under 5% carbon dioxide, 5 mM-pyruvate inhibited the anaerobic reduction of oxidized glutathione by about 50% but had no effect aerobically. Higher concentrations (25 mM) of pyruvate inhibited both the aerobic and anaerobic reduction of oxidized glutathione by about 70%. Added fumarate and oxidized triphosphopyridine nucleotide both partially counteracted the inhibition of oxidized glutathione reduction due to pyruvate. Fumarate was more effective in aerobic conditions.

6. The significance of these findings is discussed in relation to the mechanism of maintenance of glutathione in the reduced form in intact tissue.

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The Pectic Enzymes of *Aspergillus niger*

1. THE PRODUCTION OF ACTIVE MIXTURES OF PECTIC ENZYMES

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Aspergillus niger, like many other fungi, produces enzymes which attack pectic substances. It is probably true to say that none of these enzymes has been obtained in a pure state from a filamentous fungus, although Patel & Phaff (1959) produced preparations of a single pectic enzyme from the yeast *Saccharomyces fragilis*. Some work has been published on the mode of action of mould enzymes (Jansen & MacDonnell, 1945; Saito, Yasuji & Marumo, 1954; Ozawa & Okamoto, 1954; Ayres, Dingle, Phipps, Reid & Solomons, 1952; Saito, 1955), but almost invariably crude preparations were used, often obtained from commercial material of unknown provenance; where defined culture methods were employed the activities reported were very low (Saito, 1955) in comparison with those of the commercial preparations.

The entire field of pectic enzymes is extremely confused; almost as many systems of nomenclature exist as authors who have dealt with this subject. A recent review by Demain & Phaff (1957) has brought a welcome clarification and we have adopted their system of nomenclature. Basically this scheme divides pectic enzymes into poly-

methylgalacturonases, which preferentially attack pectin rather than pectic acid, and polygalacturonases, which preferentially attack the latter. Those enzymes which have a terminal mechanism of attack are distinguished by the prefix 'exo' and those with a random mechanism of attack by the prefix 'endo'.

Little information is available on culture methods employed for the commercial production of pectic enzymes. Some of these are apparently produced on solid bran cultures (Swangard, 1947) and the mycelium is often mechanically disintegrated and then extracted to obtain intracellular enzymes. Most laboratory experiments have been made with either surface cultures or shake-flasks (Brooks & Reid, 1955; Saito, 1955; Takehana & Ogura, 1955). Brooks & Reid (1955) reported that *Aspergillus foetidus* produced both endopolygalacturonase and exopolygalacturonase in surface cultures but only endopolygalacturonase in submerged cultures. We have concentrated primarily on the production of the enzymes by submerged growth in stirred fermenters of 10 l. nominal capacity.

Saito (1955) investigated factors affecting the production of pectic enzymes in shake-flask cultures of *A. niger* and reported that endopolygalacturonase (or 'depolymeric polygalacturonase') was constitutive whereas the exopolygalacturonase (or 'galacturonogenic polygalacturonase') was adaptive to the presence of pectic substances. Kontio (1950) reported that the 'pectase' of *A. niger* was adaptive to the presence of pectin.

The present work has had two principal objectives: first, to produce relatively large quantities of crude mixtures of enzymes of high pectolytic activity from a single mould grown under reproducible conditions; this part of the work is described in the present paper; secondly, to separate and purify as far as possible the individual enzymes present in this mixture of known provenance, in order to determine their precise roles in the breakdown of pectin. The next paper describes the purification and properties of endopolygalacturonase.

METHODS AND MATERIALS

Biological techniques

The concentrations of solutions given as percentages refer to w/v throughout.

Selection of strains of micro-organisms. Although the ability to produce pectic enzymes is widespread amongst micro-organisms, it was necessary to screen many of them to obtain highly active strains. Organisms were obtained from a wide variety of sources: from the air, from soil, from grape-must and from beer exposed to the air; special attention was given to infected and decaying vegetable material such as apples, oranges, tangerines, pears and carrots, whose high pectin content might be expected to encourage pectolytic organisms. The majority of the organisms studied were moulds belonging to the genera *Aspergillus, Penicillium, Sclerotinia, Fusarium* and *Mucor*, all of which are well known as producers of pectic enzymes.

Whenever possible, spores were taken directly from the infected material to bean–sucrose agar (Tanner, 1919). Otherwise the required organism was separated by plating on 2% Czapek (1902) agar in which the sucrose had been replaced by 2% pectin. After incubation at 30°, samples of pure culture were transferred to slants of bean–sucrose agar and incubated at 30° for 6–7 days.

Rapid screening of organisms for pectolytic activity was achieved by inoculating them into tubes containing 9 ml. of a 5% solution of pectin in a 2% aqueous extract of groundnut meal, at pH 4·2. In the presence of pectolytic strains the medium rapidly lost its initially high viscosity, and the turbid material in the medium gradually precipitated, leaving a clear zone at the top of the tube. This zone increased in extent with time, and the rate of this increase proved to be a good indication of the pectolytic activity of the organism.

Those strains which proved to be highly pectolytic were further screened by testing the activities of their culture fluids when grown in shake-flasks on the routine sucrose–pectin medium described below; the results of the two screening methods were in good agreement.

Storage of micro-organisms. Cultures of the organisms were preserved on bean–sucrose agar, or as conidia on soil or barley cultures, held at 4°.

Preparation of spore suspensions for inoculation. Spore suspensions were prepared by adding 5 ml. of conidial suspension to 250 ml. Erlenmeyer flasks containing 15 g. of pearl barley moistened with 5 ml. of a sporulation solution (asparagine, 0·1%; glycerol, 3%; Whiffen & Savage, 1947). The flasks were incubated at 30° for a week and then stored at 4°. When required for use, 20 ml. of a sterile 0·01% solution of lauryl sulphate was added followed by 100 ml. of water, and the conidial suspension obtained was passed through a sterile cotton-wool filter. The concentration of the suspension was determined with a Thoma counting chamber.

Shake-flask fermentations. Preliminary experiments were performed in 500 ml. flasks containing 100 ml. of medium agitated in a rotary shaker (Paladino, 1954) at 30°. The flasks were seeded with 1 ml. of a suspension of about 10^7 spores. Fermentations were continued for 5 days at 30°.

Stirred fermenters. Larger-scale fermentations were performed in 10 l. stainless-steel stirred fermenters (Chain, Paladino, Ugolini & Van der Sluis, 1954). After the media within the fermenters were sterilized with steam at atmospheric pressure for 20 min. followed by steam at 110° for 30 min., the fermenters were brought to 30°. The fermenters were fitted with stirrers of 90 mm. diameter, bearing eight vertical blades and rotating at 750 rev./min. Air was admitted at 1 atm. overpressure with a flow rate of 5 l./min.

Sterility tests. Samples withdrawn periodically from the fermenters were tested for microbial contamination by inoculation into liquid and solid media.

Ethanol precipitations. The details of the technique used for ethanol precipitation of the enzyme are described in the

next paper. The precipitate obtained (stage 1, Mill & Tuttobello, 1961) was usually dissolved in ice-cold water in a Waring Blendor, centrifuged and freeze-dried.

Analytical procedures

Evaluation of enzymic activity. No attempt was made at this stage to individualize the various pectic enzymes, but a number of criteria were applied to characterize the ability of the various culture fluids and enzyme preparations to digest pectin.

Changes in the viscosity of pectin solutions were followed in 5 ml. Ostwald viscometers at 30°. A 2·5% solution of pectin was prepared in 1% NaCl and the pH adjusted to 4·0; since considerable difficulty was often encountered in obtaining a clear solution, this was centrifuged and diluted with water so that when 1 ml. was diluted with 4 ml. of 0·2M-acetate buffer, pH 4·0, the resultant mixture had a relative viscosity of 3·5. In use 1 ml. of the 2·5% pectin solution was mixed with 4 ml. of a suitable dilution of the enzyme in the acetate buffer and the fall in viscosity measured. The time required to reduce the relative viscosity by half (i.e. to 1·75) was inversely proportional to the enzyme concentration (Fig. 1). One unit of activity was taken as the amount of enzyme which under the conditions defined reduced the relative viscosity to 1·75 in 20 min. The preparation used for Fig. 1 was, of necessity, simply the crude culture fluid of our earliest preparations. The enzyme content was so low in comparison with other nitrogenous components that neither nitrogen content nor extinction would have much true significance. The solutions used for the test were simply dilutions of the culture fluid obtained in a particular experiment and hence served merely to establish the essential linearity of the assay response without having any absolute significance.

The viscosity-diminishing assay tends to measure preferentially the 'endo' activity of the enzymic mixtures (Demain & Phaff, 1957), so that the preparations obtained were also tested for their ability to release reducing groups from pectin and for the extent to which the pectin was digested. One part of the culture fluid was mixed with 9 parts of a 2% solution of pectin at pH 4; a little toluene was added and the mixture was incubated at 30°. Samples were withdrawn at intervals and assayed by the method of Somogyi (1952).

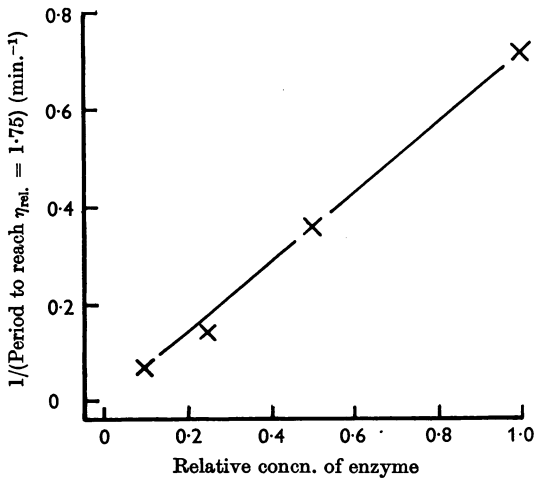

Fig. 1. Activities of various concentrations of pectolytic enzymes in diminishing the viscosity of a solution of pectin from $\eta_{rel.}$ = 3·5 to 1·75, at pH 4·0 and 30°.

Dry weights. Dry weights were determined by filtering 100 ml. of the culture through a layer of cotton wool and gauze, washing the mycelium with water and drying at 100°.

Sucrose consumption. The concentration of sucrose and invert sugar remaining in the culture medium during the fermentation was determined by heating centrifuged samples in the presence of 2N-HCl at 100° for 20 min., followed by the determination of the fructose present by the resorcinol method (Roe, 1934); this technique will of course also measure the fructose present in the culture medium, so that, if there were a preferential utilization of either the glucose or fructose moieties of the sucrose, an erroneous picture of the sugar consumption would be obtained. However, chromatographic studies showed that the concentrations of free glucose and fructose in the culture fluid remained approximately equal throughout the fermentation.

Chromatography. Chromatograms were run on Whatman no. 541 paper with, as solvent, the epiphase of *iso*butyric acid–acetic acid–water (200:1:200) (Mill & Tuttobello, 1961); sugars were revealed by the $AgNO_3$-dip technique (Trevelyan, Procter & Harrison, 1950).

pH *measurements.* These were made with a glass electrode.

Chemicals

Pectin. Apple pectin (Fluka, A. G., Buchs, S. G., Switzerland) with a uronic anhydride content of about 81% (by the method of McCready, Swenson & Maclay, 1946) was used.

Groundnut-meal extract. A 2% suspension of defatted groundnut meal (containing 47–49% of protein and 0·5–1% of lipid) in water was boiled for 30 min. with continuous stirring, cooled, filtered through muslin and adjusted to pH 7·4 with N-NaOH.

Buffers. Buffers were formulated according to Gomori (1955) from analytical-grade reagents.

Antifoam agent. An 8% solution of Alkaterge C (Commercial Solvents Corp., U.S.A.) in seed oil was added in the proportion of 3 ml./l. of medium before sterilization.

RESULTS

Selection of a suitable strain of mould. Of the many organisms screened for their ability to produce pectic enzymes, *A. niger* strain CH, which had been isolated from decaying fruit, was the most active and was used for all the subsequent work.

Effects of the composition of the culture medium. Various media were tried initially in shake-flask cultures. Two types of media were first tested: synthetic media based on those of Czapek (Czapek, 1902), Czapek–Dox (Clutterbuck, Lovell & Raistrick, 1932), Jarvis–Johnson (Jarvis & Johnson, 1947) and Shu–Johnson (Shu & Johnson, 1948),

modified in each case by the replacement of their carbohydrate by 2% of pectin plus 2% of sucrose; and more complex media with organic sources of nitrogen. Five of the latter were employed [the groundnut extract; a 2% suspension of groundnut meal; a 2% suspension of soya meal; a 2% corn-steep liquor; and a nutritive broth (1% of meat extract and 1% of peptone)], and in each case 0·2% of NH_4NO_3 and 0·05% of Na_2SO_4 were added, the pH was adjusted to 7·4 and then 4% of a mixture of equal parts of pectin and sucrose was stirred in. After sterilization the pH of each mixture fell to about 4.

There was a marked difference in the appearances of the cultures produced on the two classes of media. In the synthetic media the growth of the mycelium was scanty and in the form of loose fluffy pellets; in the media with organic sources of nitrogen the growth was very much heavier, being mainly filamentous in those media containing suspensions of the meals, and consisting of large numbers of small, dense and compact pellets in the others.

This morphological difference was accompanied by a similarly marked difference in the activity of the culture fluids measured by the diminution of the viscosity of pectin solutions. Fig. 2 shows the development of this activity during the fermentation. The cultures in the synthetic media all yielded only feeble activity and only the most active (that based on Czapek–Dox medium) is shown in the Figure. It is clear that the medium containing the groundnut-flour extract is superior to the others tested.

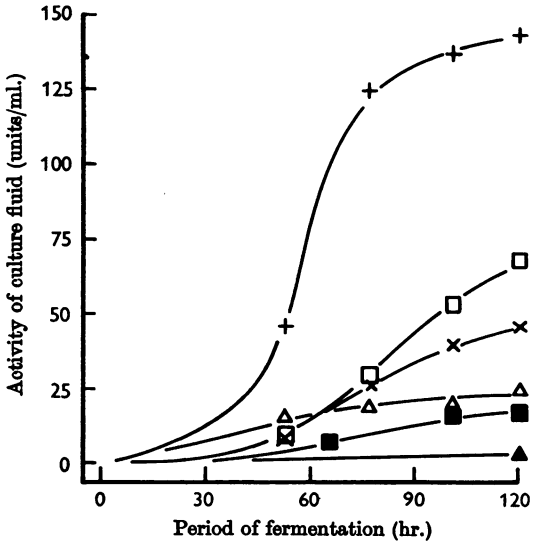

Fig. 2. Development of pectin-viscosity-diminishing activity in shake-flask cultures of *A. niger* grown at 30°. The media were: (1) ▲, Czapek–Dox medium containing 2% of sucrose and 2% of pectin; (2) 2% of sucrose, 2% of pectin, 0·05% of Na_2SO_4 and 0·2% of NH_4NO_3 in: ■, 2% corn-steep; △, 2% soya flour; ×, 2% groundnut-meal suspension; □, nutrient broth; +, groundnut-meal extract.

The effects of variations in the carbohydrate content were tested in a similar series of experiments. In each case the media contained 0·05% of Na_2SO_4 and 0·2% of NH_4NO_3 in the groundnut-flour extract; various carbohydrates were added and the activities obtained after 5-days' growth in shake-flasks are shown in Table 1. Experiments performed in stirred fermenters with the same media gave cultures with much higher activities; the relative efficiencies of the media proved to be very similar to those shown in the shake-flasks, except that galactose was no more effective than sucrose. In view of its low cost the latter was selected for further experiments. Fermentations were performed in tanks with the same basic medium but with variations in the relative amounts of sucrose and pectin used; the total carbohydrate content was maintained at 4%. Table 2 reports the

Table 1. *Effects of changes in the carbohydrate content of the medium on the viscosity-diminishing activity of the culture fluid*

The media contained the carbohydrates together with 0·02% of Na_2SO_4 and 0·5% of NH_4NO_3 in groundnut-flour extract. Fermentations were carried out in shake-flasks for 5 days.

Carbohydrate content of medium (%)	Activity of culture fluid (units/ml.)
None	24
Pectin (2) + starch (3)	28
Pectin (2) + dextrin (3)	88
Pectin (2) + fructose (3)	100
Pectin (2) + sucrose (3)	101
Pectin (2) + glucose (3)	101
Pectin (2) + galactose (3)	159
Pectin (5)	100

Table 2. *Effect of changes in the pectin and sucrose contents of the medium on the viscosity-diminishing activities of the culture fluids*

The media contained the carbohydrates together with 0·02% of Na_2SO_4 and 0·5% of NH_4NO_3 in groundnut-flour extract. Fermentations were carried out in stirred fermenters for 5 days. The extent of digestion of pectin was measured with a 1% soln. of pectin containing 1% of the ethanol-precipitated enzymes.

Carbohydrate content of medium (%)	Activity of culture fluids (units/ml.)	Digestion of pectin (% of complete hydrolysis)
Pectin (4)	90	100
Pectin (3) + sucrose (1)	160	100
Pectin (2) + sucrose (2)	210	95
Pectin (1) + sucrose (3)	125	88
Sucrose (4)	80	85

activities obtained in terms of the pectin-viscosity-diminishing activities of the culture fluids and gives the extent to which a solution of pectin is digested after prolonged incubation with the enzymes obtained from these fluids by ethanol precipitation followed by freeze-drying. Even with the high concentration of enzyme used (1 %), digestion did not go to completion with the enzymes obtained from growth on the media containing little or no pectin; when the original culture fluid was used directly as the enzyme, only some 60 % digestion could be produced with the culture obtained in the absence of pectin. With pectin as the sole carbohydrate source, the culture fluid induced complete hydrolysis of pectin.

The combination of 2 % of sucrose and 2 % of pectin was used as the normal medium.

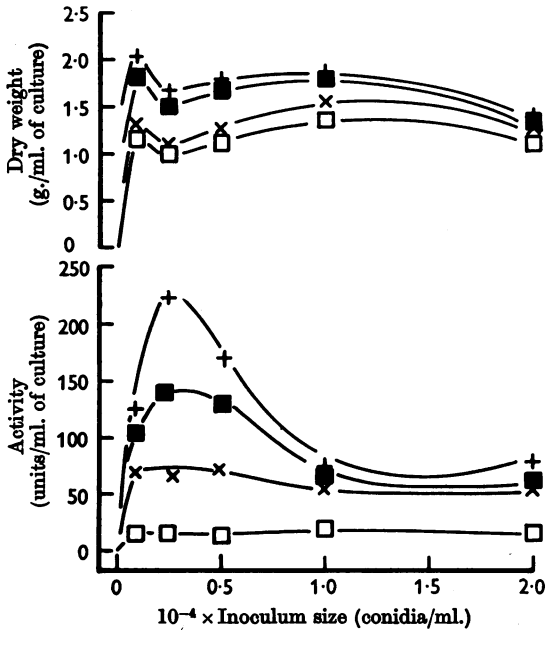

Fig. 3. Effect of inoculum size on the development of pectin-viscosity-diminishing activity and dry weight in 5 l. stirred fermentations in the pectin–sucrose–groundnut-extract medium. Measurements were made after 40 (□), 80 (×), 100 (■) and 120 (+) hr. of growth.

pH during the fermentation. In a typical fermentation in the pectin–sucrose medium, the pH fell slightly from its initial value of about 4 during the first 24 hr. and then remained fairly constant at about 3 for a further 5 days, when it once more rose to about 4. With pectin as the sole carbohydrate present, it rose rather more rapidly, whereas with sucrose alone it often fell below pH 2. Marked changes of pH during the fermentation led to a loss of activity. With the pectin–sucrose

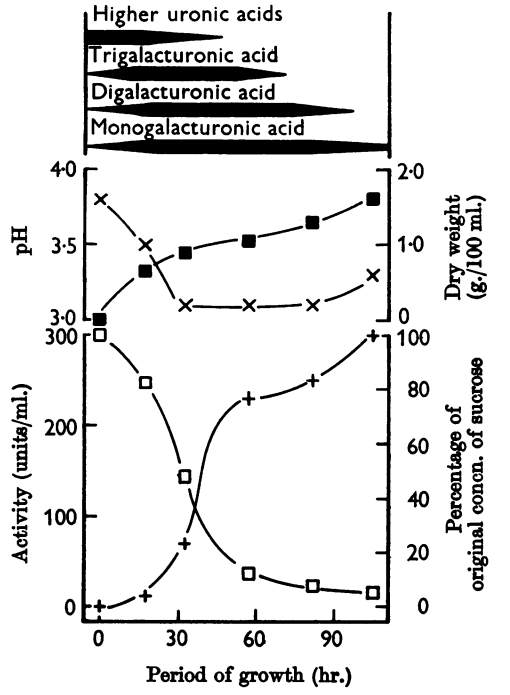

Fig. 4. Progress of a typical fermentation performed as described in the text. +, Pectin-viscosity-diminishing activity; □, relative sucrose concentration; ■, mycelial dry weight; ×, pH. The horizontal bars at the top of the diagram represent the presence in the medium of galacturonic acid polymers derived from the pectin; these uronic acids were detected chromatographically, and the width of the bars is intended to convey a subjective estimate of the relative intensities of the chromatographic spots as judged by eye.

Table 3. *Comparison of the activities of enzymes from different sources*

Enzyme		Viscosity-diminishing activity (units/mg.)
Preparation	Source	
Stage 1 (batch 42)	—	2·5
Pectasin 100-D	Rohm and Haas Co., Philadelphia, 5, Pa., U.S.A.	0·25
Pectinol (soluble, double concn.)		0·01
Pectinase	General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.	0·25
Carterzyme	H. W. Carter and Co. Ltd., Coleford, Glos.	0·67

medium there was no need to correct the pH during the fermentation and this was a further advantage of this combination.

Effects of inoculum size. During these experiments it became clear that the direct inoculation of the fermenters with a suspension of conidia was a more suitable and convenient technique than the use of mycelial inocula; however, the size of the inoculum had a pronounced effect on the activity of the culture obtained. Fig. 3 shows the development of pectin-viscosity-diminishing activity, and changes in dry weight in cultures in 10 l. fermenters inoculated with 2×10^4 to 2×10^5 spores/ml. of medium. It is apparent that an optimum inoculum size exists which under these conditions was 4×10^4 to 5×10^4 spores/ml. of medium.

A typical fermentation. In a 10 l. stirred fermenter were placed 4·8 l. of the groundnut-flour extract containing 0·2 % of NH_4NO_3 and 0·05 % of Na_2SO_3, adjusted to pH 7·4–7·5. A mixture of 100 g. of pectin and 100 g. of sucrose was now added slowly with vigorous stirring to prevent the formation of lumps, followed by 15 ml. of antifoam agent. After being sterilized and cooled, the fermenter was seeded with 2×10^8 spores. It proved to be convenient to stop the fermentation after the fifth day; little increase in activity was obtained with longer periods. The culture was quickly filtered under vacuum through a thin layer of cotton wool or gauze on a wire mesh. The filtrate was preserved at 4° in the presence of a little toluene and as soon as possible purified to stage 1 (Mill & Tuttobello, 1961) and freeze-dried.

Fig. 4 shows the progress of such a fermentation in terms of the production of pectin-viscosity-diminishing activity, the change in pH, the consumption of sucrose and the presence in the culture of those oligogalacturonides, derived from the pectin, which can be detected chromatographically.

Table 3 compares the activity of the final product with that of various commercial preparations. Two to three grams of this product were obtained from each litre of fermentation fluid.

DISCUSSION

The composition of the fermentation medium had a decisive effect on both the nature and quantity of the pectic enzymes produced by *A. niger*. The synthesis of 'endo' enzymes (measured by the viscosity-diminishing assay) even in the absence of pectic substances shows that they are constitutive; the enzymes produced certainly included endopolygalacturonase, but it is not yet possible to say whether endopolymethylgalacturonase is also present. Nevertheless, the addition of pectin to the medium markedly increased the 'endo' activity. It is characteristic of the endopolygalacturonase that the digestion of pectin, or rather of pectic acid, does not go to completion (Demain & Phaff, 1957; Mill & Tuttobello, 1961). The preparations obtained in the presence of sucrose as sole carbon source in fact tended to produce only a limited hydrolysis, whereas those obtained with a pectin-containing medium induced virtually complete hydrolysis; presumably the latter preparations included also the 'exo' enzymes. The use of a mixture of sucrose and pectin as the carbohydrate source was justified for the higher activities obtained with the mixture than with either component alone, for the reduction in cost compared with the use of pectin by itself and for the increased pH stability during the fermentation, which obviated the need for pH control.

The efficacy of carbohydrates other than sucrose varied. In shake-flasks some difference was found between the different sugars, but these virtually disappeared with cultures in stirred fermenters. However, polysaccharides were markedly less effective than the mono- and di-saccharides tested.

Lullar & Johar (1953) reported that the addition of lucerne powder to their media increased the yield of pectic enzymes from *Penicillium notatum*. Various proteinaceous substances have a similar effect with *A. niger*. The greatest effect was found with the boiled extract of groundnut meal, which increased the viscosity-diminishing activities of the cultures by as much as 50-fold in comparison with cultures on the media containing only carbohydrates and salts.

These results are not in agreement with those of Saito (1955) for shake-flask cultures of a strain of *A. niger*. This author traced a general relationship between the amount of carbohydrate present in the culture and the amount of enzyme produced, irrespective of the nature of the carbohydrate. Our experiments, however, have shown merely that the system is exceedingly complex and that the most that can reasonably be expected is to discover empirically a particular set of conditions which will give a high yield of enzyme. The production of the pectic enzymes is probably dependent to a large extent on the general metabolic status of the culture. Under the present conditions there is a certain synergism of pectin and sucrose, which leads to the highest pectolytic activity being obtained when the two carbohydrates are present in similar concentrations. It is probable that changes in other factors such as the nitrogen source would also lead to changes in the optimum carbohydrate proportions.

The production of pectic enzymes is not proportional to the amount of mycelium produced in the culture. The experiment on inoculum size demonstrates that maximum enzymic activity is

obtained with an inoculum size which does not give maximum mycelial dry weight. The pectic enzymes show a marked increase during the third day of growth, and this point is marked also by the virtually complete utilization of the sucrose originally present in the culture and by a discontinuity in the curve of mycelial dry weight.

SUMMARY

1. A method is described for the preparation of a mixture of pectic enzymes with cultures of *Aspergillus niger*.

2. The cultures were produced in stirred fermenters with a medium consisting of 0·05% of Na_2SO_4, 0·2% of NH_4NO_3, 2% of sucrose and 2% of pectin in a boiled extract of groundnut flour. Fermentation was continued for 5–6 days at 30°. The pH of the culture remained between 3 and 4.

3. The preparations induced virtually complete hydrolysis of pectin.

4. Enzymic preparations obtained from the culture fluid by precipitation with ethanol were more active, on a weight for weight basis, than commercially available materials.

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The Pectic Enzymes of *Aspergillus niger*

2. ENDOPOLYGALACTURONASE

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Endopolygalacturonase, as defined by Demain & Phaff (1957), is an enzyme hydrolysing the galacturonosidic links of pectic substances. It acts preferentially on pectic acid rather that on pectin in contrast with endopolymethylgalacturonase; the mechanism of attack is random and does not appear to go to completion, in contrast with exopolygalacturonase with its terminal mechanism of attack. Saito (1955) attempted to purify this enzyme from *Aspergillus niger* under the name of depolymeric galacturonase, but he nowhere expresses the activities of his preparations in terms of their protein content and as no criteria are given for the purity of the final preparation it is not