RAPID DETECTION AND ESTIMATION OF LOW NUMBERS OF SPOILAGE FUNGI

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TO MY MUM (of blessed memory),

MY WIFE - MARY

AND MY SON - KENYOH

SUMMARY

Two methods were established for the detection and estimation of low levels of some of the spoilage fungi which are responsible for severe crop losses during storage under tropical conditions.

The fungi examined produce the enzyme pectinesterase (EC.3.1.1.11) and both methods described, made use of this fact.

A modification of the Most-Probable-Number technique (used for estimating Coliform bacteria in liquid samples) was utilized and this enabled low numbers of *Aspergillus flavus* to be detected and estimated within 18 hours. This method was based on the use of a medium supplemented with pectin in which the pectinesterase produced by the fungus, hydrolyses the ester linkages of the pectin to produce methanol and polygalacturonic acid. This results in pH depression of the medium causing an indicator-dye to change colour. Counts by this method were generally higher than traditional Surface and Pour Plate methods but the method has the advantage of being capable of accommodating large sample volumes.

In the course of the MPN work it was found that the amount of methanol liberated varied with the size of the spore inoculum. A dialysis-cell procedure was developed for the removal of the methanol from the medium followed by its estimation by Gas Liquid Chromatography and this formed the basis of the second method.

A relationship $Y = ce^{-\beta/x}$ was established; where $y = \log_{10}$ of initial spore number/ml OR dry weight of spore material (mg/ml),

x = amount of methanol (µg/ml) released after a fixed incubation time (15 hours) and c, e and β are constants.

Linearization of this function resulted in a straight line relationship between spore numbers/ml of inoculum and methanol production (R = -0.963). When dry weight of spores was plotted against methanol production a better correlation (R = -0.988) was obtained.

Either of these plots when used as a calibration graph enabled the detection and estimation of spores of some spoilage fungi belonging to the *Aspergillus* and *Penicillium* groups in the range 20 - 10^6 spores/ml within 20 hours. The accuracy obtained was comparable to traditional standard plating methods.

The spoilage Yeast strains examined did not obey the above relationship, neither could a generalized equation be produced solely for the yeasts.

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INTRODUCTION

Nigeria was predominantly an agricultural nation prior to 1958 when She started exporting crude petroleum. Until this date, agricultural produce brought Nigeria approximately 96% of Her foreign exchange earnings. Nigerian exports to the United Kingdom alone in the years 1955-58 were £356.3 m of which £348.8 m was agricultural produce (Annual Statement of the Trade of the U.K. with Commonwealth Countries 1955-58). Although emphasis has now shifted to oil and petroleum products, Nigeria still relies a great deal on agricultural products for foreign exchange. Agricultural exports in the years 1973 and 1975 were 23.9% and 10.6% of the total exports to the U.K. A major portion of these exports involved crops such as cocoa beans, groundnuts (peanuts), soybeans and palm products (Annual Statement of Overseas Trade of the U.K. for 1973, 1975).

To understand the problems facing the Produce Inspectorate a short description of each product, its harvesting and storage is necessary:

Palm produce is obtained from *Elaeis guineesis jacq* which bears the fruit as a large, thorny compact bunch. Each fruit consists of a thin exocarp, fleshy mesocarp, a hard endocarp or shell and a hard oily endosperm or seed. The endocarp and endosperm constitute the palm-kernel although only the endosperm which is released from the endocarp by a cracking process is exportable. The endosperm is dried and stored either in large heaps or in bags before use. They are crushed and palm kernel oil is extracted from them. This oil is especially useful in the production of biscuits, ice-cream and biscuit-filling creams where a brittle texture and quick melting properties are required. After extraction of the oil, the produce remaining is very useful poultry and animal feed. Nigeria is the largest supplier of palm kernels to the world market (Coursey et. al. 1963)

Palm oil is obtained from the fleshy mesocarp by a process involving boiling, pressing and skimming. The dark red liquid is stored in large

drums and is used locally as cooking oil. After bleaching to remove the colour it is used extensively in the manufacture of soap and margarine.

Palm oil differs markedly from palm-kernel oil in fatty acid composition. The predominant fatty acid of palm oil is oleic acid (18:1) whilst that of palm kernel oil is lauric acid (12:0).

The cocoa tree, *Theobroma Cocoa*, produces cocoa beans which are economically the most important crop in Nigeria. The mature pod consists of a thick husk containing 30-40 beans embedded in a mucilaginous pulp. The beans vary in shape, size and colour depending on the variety. Key events in the exportation of cocoa beans include fermentation and roasting, the length of fermentation and the degree of roasting being dependent upon the variety used. The major use of cocoa beans is in the manufacture of chocolate and chocolate drinks although they have recently been fermented to produce cocoa wine.

Groundnuts are produced from the plant *Arachis hypogaea* which bears underground fruits. When mature, the harvested fruits are washed free of earth and dried under the sun. The fruit consists of an outer shell surrounding one to four oily seeds. The seeds are dried before being crushed and pressed to obtain groundnut oil which is used for margarine and cooking oil. The pressed cake is used in the biscuit trade and for animal and poultry feed stuffs.

Soybeans are produced by the leguminous plant, *Glycine max L* which bears short pods containing two or three beans. When ripe, the pods explode to release the beans. The size, shape and colour vary depending on the variety and they are frequently dried and stored in concrete bins before being sold. It is used locally as a high protein food but is often extracted to produce soybean oil which is exported for use as cooking oil and making ice cream.

Maize (*Zea mays L*) is worth mentioning as although it is not grown for

export it is a staple food for millions of Nigerians and is an important cash crop for thousands of peasant farmers. It is either eaten fresh or ground into powder for making various types of porridge although it is also frequently fermented to produce alcoholic drinks.

Most of the agricultural produce for export is grown and processed by peasant farmers. They sell their crops to Licensed Buying Agents of the Marketing Boards, often through middlemen. The Marketing Board which is a Government owned Corporation has a monopoly to purchase these crops for export. It controls the appointment of Licensed Buying Agents, the locations of Official Buying Stations and also specifies the minimum and maximum price to be paid for the various grades of produce. The examination and grading is the responsibility of the Produce Inspectorate of the various Ministries of Trade and Industry in the Federation of Nigeria. The ministries frequently have a research laboratory to which disputed or borderline grades of produce are referred for analysis.

Palm oil and oil seeds are graded on their free fatty acid (FFA) content expressed as per cent oleic acid for palm oil and groundnuts, and as per cent lauric acid for palm-kernel oil. Top quality groundnuts and palm produce should not contain more than 3.0% and 4.75% FFA respectively.

The threat to quality during cultivation and harvesting is minimal (Tuite & Christensen 1955, Christensen & Kaufmann 1965). Quality loss (deterioration) is, however, frequently encountered during long periods of storage of groundnuts (Halliday 1968), cocoa beans (Hansen & Keeney, 1970; Oyeniran, 1970), soybeans (Dorworth & Christensen 1968) palm kernels (Cornelius 1966; Idem, 1973; Kuku & Adeniji, 1976) and maize (Ivbijaro et. al. 1979).

Produce is classified as deteriorated if one or more of the following events occurs:-

(a) An alteration of the flavour components in the lipid portions of, for

example, cocoa beans due to an increase in (i) the carbonyl content especially the methyl ketones and (ii) the unsaturated aldehydes especially the 2-enals and 2,4-dienals. These are potent aroma emitting compounds and even small increases in concentration have significant effects on the flavour.

(b) Loss of viability of stored produce rendering it valueless as seed stock. This frequently is a consequence of metabolic heat formed during bulk storage. Such heat which is not easily dissipated results in an overall rise in temperature thus increasing spoilage rates. In extreme cases it may lead to charring and spontaneous combustion.

(c) A decrease in the non-reducing sugars especially sucrose.

(d) A rise in FFA above a threshold value.

(e) A production of musty odours due to oxidative and hydrolytic reactions. Rancidity is caused by the oxidation of polyunsaturated fatty acids especially the non-conjugated ones. Destruction of these essential fatty acids reduces the nutritional value of the produce.

(f) In the case of oil seeds and palm kernel, a discolouration of the endosperm and thus the pressed oil. This is caused by an increased unsaturation of the FFA followed by rancidity. This causes bleaching reactions during processing to become more difficult and expensive.

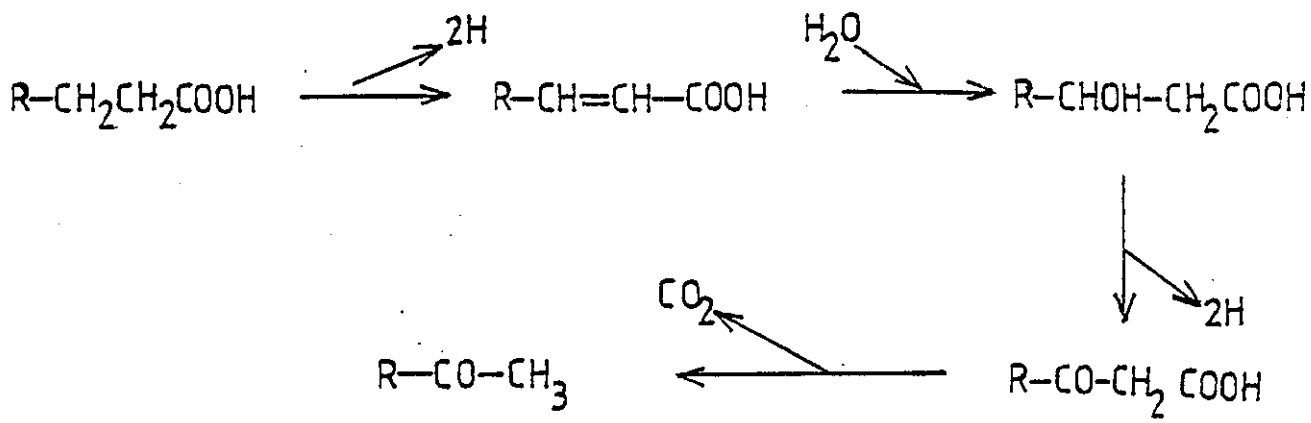
(g) The most important factor is the presence of toxic metabolites especially the aflatoxins which are lethal to animals and a causative agent of liver cancer in humans (Alpert et.al. 1968).

Each of these criteria for deterioration has been attributed either directly or indirectly to invasion by spoilage fungi. Spoilage fungi refers to fungi which although found in a wide range of habitats become prominent in badly stored grains, seed or beans whose moisture content is in

equilibrium with a relative humidity between 70% and 90% (Christensen & Kaufmann 1969). The most important of these fungi are Aspergillus and Penicillium spp.

An increase in FFA has been found in stored produce with fungal lipolysis predominating at moisture contents of > 13% (Glass et.al. 1959; Hutchinson, 1961; Dorworth & Christensen, 1968; Halliday, 1968; Hansen et.al. 1973). Lipolysis causes the hydrolysis of the ester linkages of the triglycerides of fats and oils to form free fatty acids and glycerol via their di- and mono-glycerides.

The formation of methyl ketones with a cocomitant increase in carbonyl values is due to the β-oxidation of free fatty acids followed by decarboxylation. A similar sequence of events leading to methyl ketone formation is found in mould-ripened cheese (Hawke, 1966).



Hansen and Keeney (1970) found that in every case examined in which mould was growing in cocoa beans there was a marked increase in carbonyls and unsaturated aldehydes. The unsaturated aldehydes which are mainly 2-enals and 2,4-dienals are probably formed by the autoxidation of unsaturated fatty acids.

The invasion of seeds by Aspergillus spp. causes loss of viability (Tervet, 1945; Dorworth & Christensen, 1968). Rapid fungal growth causes characteristic 'hot spots' in stored grain. Gillman & Barron (1930) showed that in laboratory tests *Asp. flavus, Asp. niger* and *Asp. fumigatus*

raised the temperature of moist wheat, barley and oats (containing 18% moisture) from 17° to 43°C which reduced viability. Above a certain temperature, heat causes oil to exude from seeds resulting in a loss of oil content and hence a reduction in commercial value.

In addition, spoilage fungi preferentially attack the embryo of seeds causing decay (Golumbic & Laudani, 1966) which invariably reduces the viability of the seeds.

Infected produce is discoloured by the spores and mycelium of the fungus. The FFA formed become unsaturated during fungal growth and this also leads to discolouration and loss of commercial value.

Mould growth also causes the production of n-octanol in hazlenuts which is known to be a major cause of rancidity (Hansen & Keeney, 1970, Grosch & Barthel, 1974).

Many spoilage fungi produce a group of compounds known as the aflatoxins. Probably the most important species is *Asp. flavus* which grows on most stored products and has been reported to produce aflatoxins in barley, maize, wheat, cocoa beans, groundnuts, palm kernels, soybeans and cotton seed (Raymond 1966). This species produces other toxic metabolites as well which include oxalic acid, kojic acid, β-nitropropionic acid and aspergillic acid (Wilson 1966). Other fungi also known to produce aflatoxins include *Asp. parasiticus* (Codner et.al. 1963), *Asp. niger, Asp. ruber, Asp. wentii, Asp. variabile, Penicillium citrinum* (Kulik & Holaday, 1966) and *Pen. puberulum* (Hodges et.al. 1964). Although Hesseltine et.al. (1966) and Yokotsuka et.al. (1966) who together examined 125 strains of *Asp. oryzae* for aflatoxins production found no aflatoxins, they concluded that the very toxic metabolite encountered could be aspergillic acid. Recently El-Hag & Morse (1976) found that variant strains of *Asp. oryzae* unlike the parent strains have an ability to produce aflatoxins.

The physical conditions favouring growth and sporulation in fungi also favour the production of aflatoxins. The temperature required for aflatoxin production in stored agricultural produce has been reported as 13^{0} - 41.5^{0}c with optimum production in the range 20^{0} - 35^{0}c at a relative humidity of 85 - 99% (Diener & Davis, 1967).

It is obvious from the discussion so far that most of the damage caused during storage is due to fungi. Nigerian agricultural exports are highly susceptible to fungal attack due to a number of factors. These are:-

(a) The techniques of harvesting and drying the produce cause considerable contamination by dormant fungal spores which are not detected by the grading techniques available.

(b) Nigeria is a developing nation and as such lacks adequate transportation networks. Thus produce purchased for export by Government agencies in rural areas may remain in storage for up to 12 months before being transported to the docks. At the seaports, produce may remain in warehouses for even longer periods before shipping facilities become available. Modern storage facilities are lacking so that produce may spend these long storage periods in hot, humid, inadequately ventilated conditions which promote rapid fungal metabolism.

(c) The prevailing tropical climate satisfies all the conditions for optimum fungal growth unless elaborate and expensive precautions are taken.

These factors cause inevitable deterioration by the time produce reaches the consumer in Europe and elsewhere. Nigeria consequently suffers severe financial loss because produce purchased from the farmer as top quality is retailed in consumer countries as much lower quality. The Liverpool contract of 1968 for the sale of Nigerian Palm produce states "Any deficiency in the oil content of oil seeds is at the penalty of the vendor and any excess is paid for by the purchaser on the basis of 13% of

the contract price for each 1% under or above a basic level of 49% oil content". Thus fungal 'hot spots' which cause a decrease in oil content during storage and transit are responsible for not only depriving Nigeria of the premium due if the produce had arrived with an oil content above 49% but impose a financial penalty when selling produce with an oil content of less than 49%.

Under the same agreement, oils are sold on the basis of 4.75% FFA (expressed as the molecular weight of the predominant fatty acid) with an allowance of 0.75% of contract price for each 1% under or over the basic rate. As already mentioned fungal lipolysis causes a considerable rise in FFA and hence financial penalties.

These fungi develop from fungal spores which escape detection at the time when produce is purchased from the farmer and examined. Until recently, examination consisted of removing a sample of 25 ozs. and extracting from it all shell, fibre, extraneous matter and all grains suspected of decay after visual examination. These were weighed and expressed as a percentage of the sample weight. First quality produce contained less than 4% of such extraneous and decayed material. This method did not correlate with the FFA tests on the basis of which produce retails in Europe, and neither does it reflect the level of fungal contamination.

The present method of examination makes a determination of FFA. For palm kernels, this is done in the field by the equation, log $F = 0.345 + 0.01D$ (Idem 1973) where F is the percentage FFA expressed as lauric acid and D is the percentage of decayed, mouldy or discoloured kernels of a representative sample for a given consignment. For other produce, and palm kernels in dispute the examination consists of crushing the grains and extracting the oils which are then titrated against a standard potassium hydroxide solution. The FFA is expressed as a percentage using

the molecular weight of the predominant fatty acid. By either method produce with a FFA content of less than 4.75% is first quality and it is assumed that fungal contamination is negligible.

Unfortunately, such an assumption is not justified as produce is frequently heavily contaminated with fungal spores which cannot be detected visually and which have not contributed to the FFA content as they have not germinated and commenced metabolism. It is these spores which under inadequate storage conditions germinate and grow rapidly to cause serious commercial losses by the time produce reaches the consumer.

From the discussion so far, it is obvious that there is great need for rapid and reliable methods of detecting and estimating spoilage fungi especially those present in the dormant spore stage. Such a method should be comparable in accuracy to conventional detection and estimation methods (APHA 1971, 1976) for micro organisms and should also be applicable to a wide range of produce.

The need for rapid and reliable methods of detecting and evaluating microbial contamination has led to a number of new techniques being introduced some of which are now used widely.

Microcalorimetry (Beezer et.al., 1978) measures the metabolic heat output in growing cultures and can be related to the number of bacteria in cultures and clinical specimens of urine within 2 hours.

Many gram-negative bacteria produce endotoxin which can be detected by the very sensitive limulus in-vitro assay (Rojas - Corona et.al., 1969). Jorgensen & Jones (1975) and Ross et. al. (1975) have applied the limulus-lysate assay to detect (within 2 hours) bacteria in urine and cerebro-spinal fluid from cases of meningitis. In both cases the test sample is incubated in a test tube with a pre-standardised lysate at 37^{o}c for 1-2 hours and the test tube is then inverted. The presence of a clot formed by the coagulation reaction of endotoxin and lysate is indicative of the disease.

Radiometry has been used to detect coliforms in milk, milk products (Koga & Tsugo 1970) and in water (Bachrach & Bachrach 1974); to detect low levels (1 - 10^4 cells/ml) of *Salmonella typhimurium* and *Staphylococcus aureus* in foods (Previte 1972) and also to analyse frozen concentrated orange juice (Hatcher et.al., 1977). The method is based on the release of $C^{14}O_2$ from C^{14}-labelled substrates incorporated into the growth medium. Any $C^{14}O_2$ released is detected by counting over a period of 1 - 7 hours of incubation.

Winter (1971) utilized membrane filter techniques to estimate microbial contamination in foods and food processing equipment within 5 hours. Microbial cells were concentrated on a membrane filter which was incubated in a growth medium for 4 hours at 30°C and then heated to 105°C for 5 minutes and stained with Janus green. The dried membrane was then made transparent with immersion oil and examined microscopically.

Guthrie & Reeder (1969) combined membrane filtration and fluorescent antibody techniques to detect and estimate bacteria in water within 5 - 12 hours. This combined the advantages of membrane filtration for concentration and growth of the bacteria and the fluorescent - antibody techniques for rapid identification and counting.

Changes in the electrical impedance of culture media was used for detecting contamination in frozen vegetables (Hardy et.al., 1977) within 5 hours. It consists of passing a small electric current through the culture medium and measuring the rate of change in impedance as the organisms grow and metabolize.

Warren et. al. (1978) used the β-galactosidase assay to estimate faecal coliforms in water. This method is based on the enzymic hydrolysis of O-nitrophenyl β-D galactoside (ONPG) to produce o-nitrophenol which gives a yellow colour with Na_2CO_3 which can be measured spectrophotometrically. An incubation time sufficient to give half maximal absorbance is proportional

to the number of faecal coliforms present.

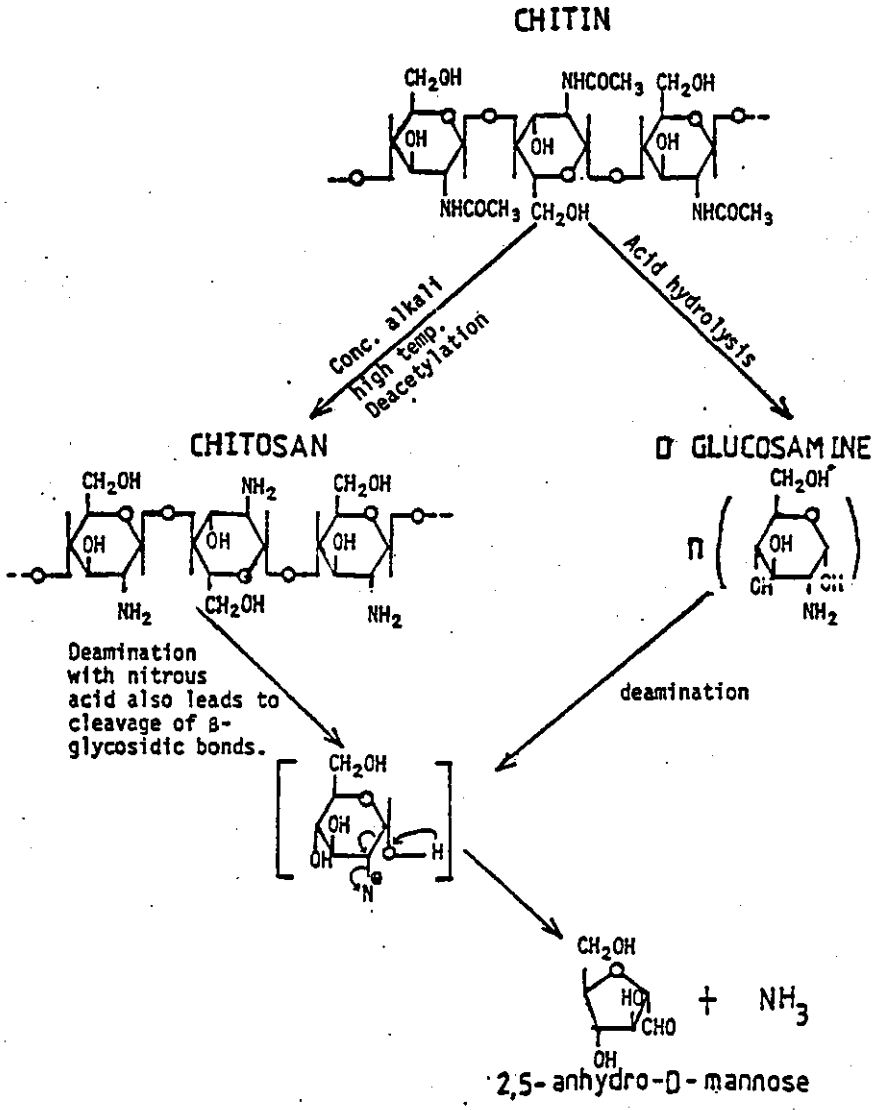
In the enumeration of low levels of microbial contaminations, the most-probable-number (MPN) of organisms in the dilution method has found world-wide acceptance. It was first discussed by McCrady (1915) and has since been reviewed on a number of occasions (Halvorson & Ziegler, 1933[a], 1933[b], 1933[c], 1935 ; Swaroop, 1941; Cochran, 1950; Oblinger & Koburger, 1975). The method allows detection and estimation of bacterial numbers in liquid media without any direct count being necessary. It is used principally for estimating bacterial contamination of water and milk, though it has recently been applied to estimation of bacterial numbers in foods (Brewer et.al., 1977) and soil samples (Rowe et.al., 1977).

The method consists of taking samples from the liquid and inoculating into a suitable culture medium, incubating at appropriate temperature and observing whether any growth of the organism has taken place. Various criteria have been applied to detect growth. Traditionally this involves visually detecting gas bubbles and/or colour change of pH indicator-dye incorporated into the growth media (APHA 1971). To make the method rapid, Munoz & Silverman (1979) have detected growth by measurement of the increase with time in the electrical impedance ratio (R_z) between an inoculated sample tube and an uninoculated sterile reference tube using the relationship $R_z = \frac{z \text{ reference}}{z \text{ reference} + z \text{ sample}}$. By this procedure the analysis time was reduced from 72 to 18 hours.

When all these techniques are examined, it is found that they have been applied mainly to bacterial contamination and relatively little work has been done on spoilage fungi.

Spoilage fungi have been traditionally detected by an examination of the number and type of fungi found on seeds after surface sterilization with sodium hypochlorite (Christensen & Kaufmann, 1969). The method requires 5 - 7 days and is not rapid enough for quality control in the

FIGURE 1



CHITIN HYDROLYSIS

grain industry.

Koburger and Norden (1975) applied the MPN technique in the detection and estimation of yeasts and moulds in foods and compared the results with Standard Surface and Pour Plate methods. They concluded that the MPN procedure for fungi has an application in a food microbiology laboratory. They found it to be at least as efficient in recovery as either the pour or surface plate method, and in some samples it was able to demonstrate growth that was not detected by the pour and surface plate procedures. The problem in their MPN procedure was that it required 5 days for full results to be obtained. This was due to a reliance on the visual detection of mould colonies on the bottom of growth tubes or the use of the Gram stain when media was very turbid due to added food samples.

Chitin a polymer of N-acetyl-D-glucosamine is a consituent of the cell walls of most spoilage fungi. It is not found in seeds free from fungal attack. Golubchuk et. al. (1960) studied chitin levels of wheat with varying degrees of fungal invasion and concluded that measurement of chitin gave promising results in the evaluation of wheat deterioration during storage. Acid hydrolysis of chitin yields glucosamine quantitatively and this can be measured by the method of Elson & Morgan (1933) or the method of Tracey (1952). Alkali hydrolysis deacetylates chitin and when the resulting chitosan is deaminated it is subjected to β-glycosidic bond-cleavage leading eventually to 2,5-anhydro-D-mannose and ammonia. Either of these may be assayed to determine chitin level and hence fungal contamination (see fig. 1).

Arima & Uozumi (1967) analysed chitin as glucosamine by a modification of Elson & Morgan's (1933) method in contaminated rice and koji and found a relationship between the chitin content and mycelial dry weight which gave an approximate indication of the level of fungal contamination.

Donald & Mirocha (1977) analysed chitin in stored soybean and maize. The chitin was deacetylated with alkali to chitosan. This was

hydrolysed with nitrous acid to glucosamine and then 2,5-anhydro-D-mannose which was determined colorimetrically with $FeCl_3$.

Jarvis (1977) estimated mould in tomato products using a modification of Tsuji et.al. (1969).

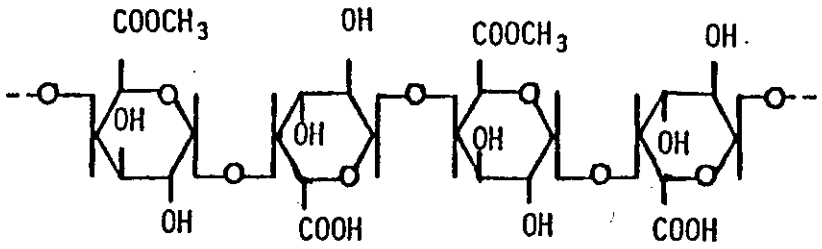
These methods of estimating fungal contamination based on chitin analysis are rapid (4-5 hours) but suffer from several serious drawbacks. High levels of contamination are required to produce sufficient chitin for analysis, and the methods do not distinguish between viable and non-viable fungi nor between dormant spores and actively growing mycelia. Chitin is also a constituent of exo-skeletons of insects, especially the arthropods, which quite often feed on stored produce and the presence of insects would therefore interfere with chitin analysis as an index of fungal contamination. Finally, the many steps involved in converting chitin to detectable substances also mean that the final result is only an approximation and may be subject to considerable errors.

Paliwal & Randhawa (1977) utilized the Bertholot colour reaction used for detecting urease activity in the enterobacteriaceae as a method of detecting yeasts. Urease positive strains could be detected in 30 - 50 minutes and the strength of the reaction was proportional to the number of fungi. However, urease activity is only found in cultures which are at least 4 days old and it is not a common property of spoilage fungi. Besides, as urease activity is also common in the enterobacteria, the test would not be specific to fungi. This might be a considerable problem in crops treated with raw sewage as fertilizer.

Aflatoxin production is common in many spoilage fungi and several sensitive methods exist for the detection and estimation of this compound. These include fluorescence studies (Coomes et.al., 1964) and fluorodensitometry (Pons et. al., 1966). However, aflatoxin production does not commence until cultures are approximately one week old. Also the level of

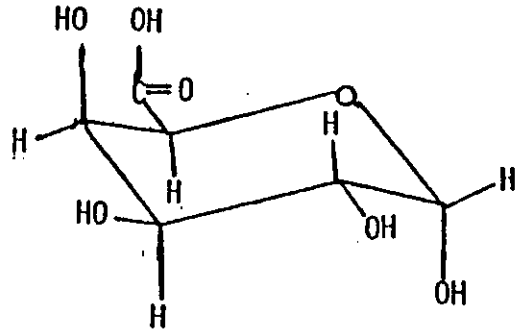
FIGURE 2

(a):



Partially esterified chain of poly-D-galacturonic acid (Pectin)

(b)



α-D-galacturonic acid
CI Conformation

aflatoxin production does not parallel fungal growth as they also metabolize it under certain conditions (Schroeder & Hein, 1967). Therefore, aflatoxin production does not offer a means of rapid detection and estimation of fungal contamination. A further problem is that due to its extreme toxicity, by the time it is detectable, the produce is useless.

Winkler (1973) described a method of determining the fungal contamination of grain which involved the ratio of triglyceride/steroid esters (T/S). The lipids were extracted and analysed by temperature programmed gas chromatography. The method gave good results for heavily infested grains but was not developed further due to the considerable analysis time needed.

Smith (1977) introduced a procedure for the rapid detection of cellulolytic activity in fungi using a dyed cellulose powder as substrate. Cellulolytic fungi uncoupled a blue dye from the substrate during incubation and the free dye could be measured and related to the number of fungi present. Species showing a high cellulolytic activity produce the first detectable effect within 2 days but less active species required 5 - 10 days to release detectable quantities of dye. The method was not investigated further due to the long incubation times needed, and also cellulolytic activity is neither a common nor consistent property amongst spoilage fungi.

Most spoilage fungi produce pectin-degrading enzymes when grown in a medium incorporating pectin (Sreekantial & Johar, 1963; Sreekantial et.al. 1973; Abdel-Fattah & Mabrouk, 1976). Pectin is an esterified polymer of galacturonic acid residues linked by α-1, 4-glycosidic bonds to give an unbranched chain (Fig. 2a). The degree to which pectin is esterified with methanol depends on the source of the pectin. The galacturonic acid has the chair configuration, C1 (Fig. 2b) and therefore the polymer has a screw axis and tends to coil. Other polysaccharides associated with pectin are

Table 1

CLASSIFICATION OF PECTIC DEGRADING ENZYMES (Fogarty & Ward 1974)

ESTERASE

Pectinesterase (PE)(EC. 3.1.1.11) transforms pectin into pectic acid by de-esterification of the methoxyl residues.

DEPOLYMERASES

Acting on Pectin

i. Polymethylgalacturonase (PMG)

(a) Endo-PMG (EC.3.2.1.41), causes random cleavage of α-1,4-glycosidic links in pectin (preferentially highly esterified)

(b) Exo-PMG causes sequential cleavage of α-1,4-glycosidic links of pectin from the non-reducing end of the chain.

ii. Polymethylgalacturonate lyase (PMGL)

(a) Endo-PMGL (EC.4.2.2.3) causes random cleavage of α-1,4-glycosidic links of pectin by transelimination process which results in galacturonide esters with unsaturated bonds between C_4 and C_5 and at the non-reducing end of the fragment formed.

(b) Exo-PMGL causes stepwise breakdown of pectin by transeliminative cleavage.

Acting on Pectic Acid

i. Polygalacturonase (PG)

(a) Endo-PG (EC.3.2.1.15) causes random hydrolysis of α-1,4-glycosidic links in pectic acid (Polygalacturonic acid).

(b) Exo-PG (EC.3.2.1.40) hydrolyses in a sequential fashion α-1,4-glycosidic links in pectic acid.

ii. Polygalacturonate lyase (PGL)

(a) Endo-PGL (EC.4.2.2.1) causes random cleavage of α-1,4-glycosidic bonds in pectic acid by process of transelimination.

(b) Exo-PGL (EC 4.2.2.2) causes sequential cleavage of α-1,4-glycosidic bonds in pectic acid by process of transelimination.

α-1, 4-D polygalacturonide, a highly branched L-araban and a β-1, 4-D galactan (Whistler & Smart, 1953). In addition to galacturonic acid the following sugars have been isolated from pectin, L-rhamnose, L-arabinose, D-galactose, D-xylose and L-fucose (Aspinall & Canas-Rodriguez, 1958; Aspinall & Fanshaw , 1961).

Basically, two types of pectic enzymes exist; pectinesterase (EC 3.1.1.11) which simply removes methoxyl groups from pectin to form methanol and pectic acid, and a range of depolymerizing enzymes (pectinases) which are named individually depending on their mode of action on pectin. Depolymerizing enzymes are differentiated under three headings:-

(a) Whether pectin or pectic acid is the preferred substrate

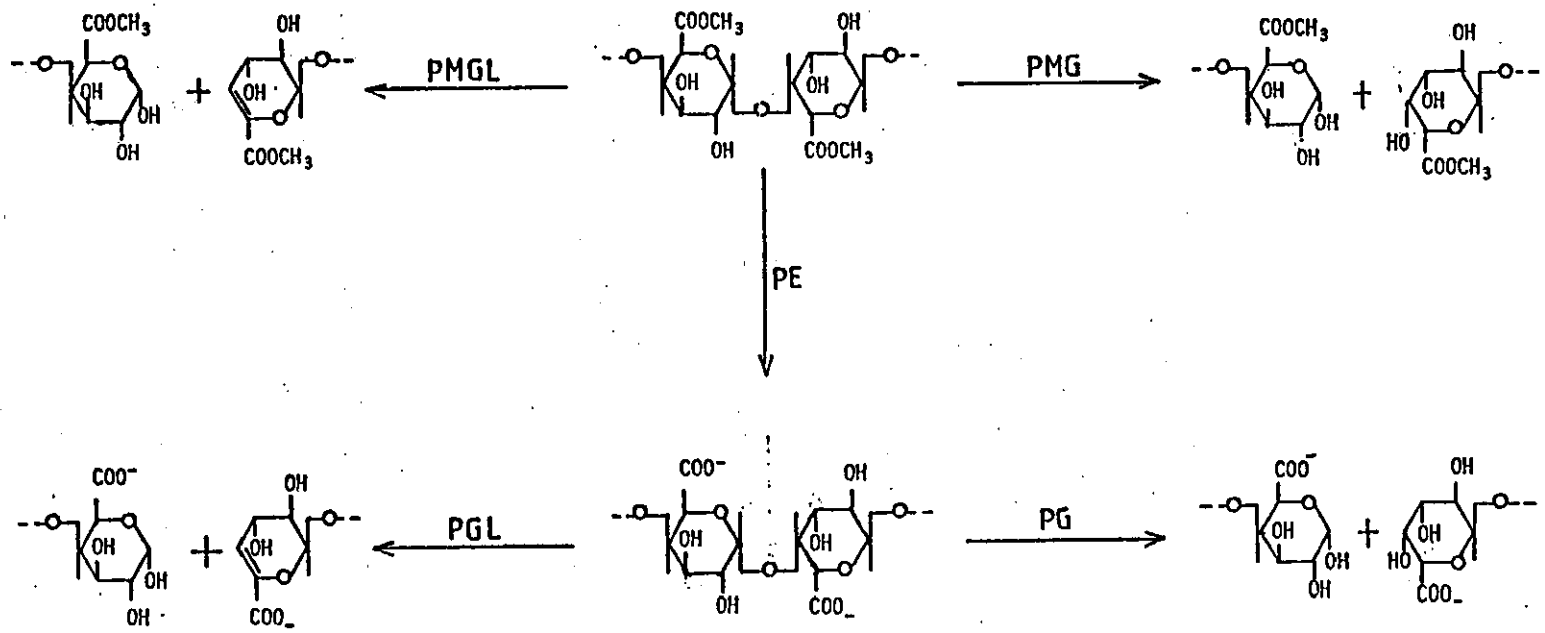
(b) Whether they act by hydrolysis or transelimination

(c) Whether cleavage is random internal (endo) or occurs from the end (exo). The available data is summarized in table 1 and the modes of action are shown in Fig. 3.

Several methods have been used for the determination of pectinase activity. These include a determination of the reducing groups due to the depolymerizing action of the enzyme (Jansen & McDonnell, 1945; Patel & Phaff, (1960; Barash & Khazzam , 1970; Unbehaun & Moore, 1970). Viscosity measurements have also found wide use for measuring pectinase activity. This assay is based on the loss of viscosity of aqueous solutions of pectin or sodium pectate following a decrease in chain length (Mill & Tuttobello, 1961; Yamasaki et. al., 1966; Fielding & Byrde, 1969; Hancock et. al., 1970; Chou et. al., 1970).

There are also several methods for the estimation of pectinesterase, the most common of these being the titrimetric estimation of carboxylic acid groups formed when the ester bond is cleaved (Kertesz & Lavin, 1954; Cole & Wood, 1961). Pectinesterase can also be assayed by measuring the methanol liberated from pectin during the reaction. This may be distilled

FIGURE 3



Modes of action of pectinases

off and oxidized to formaldehyde as described by Holden (1945). Wood & Siddique (1971) determined the methanol as formaldehyde without need for distillation. ^{14}C-labelled methanol released from ^{14}C-labelled pectin has been estimated using scintillation counting (Kauss et. al., 1969). McCready & Reeve (1955) and McComb & McCready (1958) used a cup-plate assay with alkaline hydroxylamine or hydroxamic acid for pectinesterase determination. Gas chromatography has been utilized to determine activity by measuring the methanol liberated (Lee & Wiley, 1970; Krop et. al., 1974).

Pectinesterase is found widely in spoilage fungi (Phaff, 1947; Endo & Miura, 1961; Cole & Wood, 1961; Sreekantiah et. al., 1973; Abdel-Fattah & Mabrouk, 1976) and most of the work of this thesis is based on the measurement of pectinesterase (EC 3.1.1.11) and developing a mathematical relationship between enzyme activity and the number of fungal spores present.

2

MATERIALS & METHODS

2.1 TEST ORGANISMS

The 6 strains of *Aspergillus* and 5 of *Penicillium* used were all obtained from the Commonwealth Mycological Institute, Kew, Surrey. They were *Aspergillus flavus* CMI 39178a, CMI 15959, CMI 86769, *Asp. niger* CMI 31821, *Asp. nidulans* CMI 16643, *Asp. luchuensis Inui* CMI 83356, *Penicillium brevicompactum* CMI 92034, *Pen. expansum* CMI 39761, *Pen. martensii*, CMI 91020 (b), *Pen. lividum* CMI 99648, *Pen. chrysogenum* CMI 26211. The 6 yeast strains used were obtained from the National Collection of Yeast Cultures, Brewing Research Foundation, Nutfield, Redhill, Surrey. These were *Saccharomyces fragilis* NCYC 587, *Cryptococcus albidus* NCYC 445, *Endomycopsis chodati* NCYC 440, *Pichia farinosa* NCYC 386, *Candida pseudotropicalis* and *Hansenula anomala* NCYC 432.

2.2. BUFFER SOLUTIONS

The 1% phosphate buffer, pH 7.0 was prepared by mixing a solution of $Na_2HPO_4.12H_2O$ (3.58 g/litre) with another of $NaH_2PO_4.2H_2O$ (1.56 g/litre) to pH 7.0.

2.3 MEDIA

2.3.1 Maintenance Media (Medium 42)

The *Aspergillus* and *Penicillium* strains were maintained as pure cultures on slopes in McCartney bottles at $4^{o}C$. The medium consisted of Maltose (Fisons Laboratory reagent grade) 38g; Neutralised Soya Peptone 8g; Yeast Extract 2.5g; Malt Extract 2g;

Agar Technical No.3 20g (Oxoid Ltd.). These were dissolved and made up to 1 litre with distilled water. The pH was adjusted to 5.4 before autoclaving.

Yeast strains were also maintained at 4°C as slopes on M.Y.G.P. medium Malt Extract 3g, Yeast Extract 3g, Glucose (Fisons Laboratory reagent grade) 10g; Neutralised Soya Peptone 5g and Agar Technical No. 3 20g; all dissolved and made up to 1 litre with distilled water, the pH before autoclaving was 5.4.

2.3.2 Sporulation Medium

Cultures were grown in the following media to obtain spores.

$K_3PO_4.H_2O$	2.0g
KNO_3	2.0
$CaCl_2$	0.25
Yeast Extract	5.0
Glucose	10.0
Bacto Casamino Acids	5 (Difco Laboratories, Detroit, Michigan).
Metals solution	0.1 ml
Distilled water	1 litre

The metals solution comprised (mg/10 ml)

$MgSO_4$	0.5
$ZnSO_4$	0.02
$FeSO_4$	0.02
$MnSO_4$	0.01
$CuSO_4$	0.05

This medium was adjusted to pH 5.4 before autoclaving at 115°C for 15 minutes.

2.3.3. Assay Media

(a) Sabourand's broth (SB)

4% Glucose

1% Neutralised Soya Peptone (w/v)

(b) Malt Extract Broth (MEB)

Malt Extract	17 g
Peptone	3g
Distilled Water	1 litre

(c) Malt, Yeast, Glucose, Broth (MYGB) (Martin 1954)

Malt Extract	12.75 g
Yeast Extract	5
Glucose	10
Distilled Water	1 litre

(d) Modified Czapek Dox (MCD) (Damodaran & Singh, 1954)

Glucose	40 g
$NaNO_3$	5
KCl	0.5
$MgSO_4.7H_2O$	0.5
$KH_2\ PO_4$	1.0
$FeSO_4.7H_2O$	0.01
Yeast Extract	0.5
Distilled Water	1 litre

(e) Glucose Broth (GB) (Martinelli & Bainbridge, 1974)

Glucose	20
$NaNO_3$	6
$MgSO_4$	0.2
Distilled Water	1 litre

In all the media which they were present, Malt Extract, Yeast Extract and Glucose, were each autoclaved separately and mixed when cool.

2.4 CHEMICALS

2.4.1 Enzyme Preparations and Substrates (Sigma Chemical Co.)

Polygalacturonase (EC.3.2.1.15)

Pectinesterase (EC.3.1.1.11)

Polygalacturonic Acid

D-Galacturonic Acid

2.4.2 pH Indicator dyes

The pH indicator dyes used were prepared by dissolving each in distilled water (4 mg/10 ml) instead of dissolving in ethanol to avoid the ethanol influencing enzyme activities.

The following dyes were used:-

Thymol blue (The General Chemical & Pharmaceutical Co Ltd., Middlesex).

Bromophenol blue (Hopkins & Williams Ltd., Heath, Essex).

Bromocresol green (Fisons Ltd., Loughborough)

Bromocresol purple (Fisons Ltd., Loughborough)

Bromothymol blue (Fisons Ltd., Loughborough)

2.4.3 Supplements to Growth Media

Pectin (Citrus pectin, Rapid Set-Type 104, Bulmer Ltd, Hereford, England)

Chloramphenicol (Sigma Chemical Co.)

Chlortetracycline (Pfizer Ltd., Sandwich)

2.5 FUNGAL SPORES

The sporulation medium was dispensed as 250 ml portions into Roux bottles, autoclaved and cooled. These were then inoculated with pure cultures of the organisms maintained as slopes in McCartney bottles. Incubation was carried out under static conditions at 30°C for 3 weeks. At the end of this period a thick mat of mycelium covered with spores had grown over the surface of the medium in all cases. The spores were prepared by a modification of the procedure described by Dart (1975). The contents of the Roux bottles were violently agitated for 15 minutes to dislodge the spores from the mycelial mass. This was then filtered twice through loose cotton wool plugs into a sterile filtration flask. The filtrate was

centrifuged for 15 minutes at 3500 g the supernatant decanted, and the spores washed with sterile distilled water. The centrifugation, decanting and washing process was repeated until microscopic examination showed the spores to be free of mycelial contamination.

The spores were stored in stock suspensions of about 400 mls volume at $0^{o}C$ until required. To maintain a high percentage viable spores in the suspensions used throughout the work, fresh stock suspensions were prepared every 3 months as described.

Yeast cells were cultivated on 250 ml portions of solid MYGP medium in Roux bottles at $30^{o}C$ for 3 weeks. The cells were harvested by flushing sterile water over the surface of the cell mat. These were similarly washed clean of agar and nutrient material by the centrifugation, decanting and washing process. Cell suspensions were also stored at $0^{o}C$ until required.

2.6 EXPERIMENTAL

2.6.A Most-Probable-Number (MPN) Method of Detection and Estimation

The method was based on the findings of Verbina & Tsytura (1969) that pectolytic enzyme-producing fungi could be rapidly selected by observing the colour change of Methyl Red pH-indicator dye caused by galacturonic acid formed when such fungi grow in media supplemented with pectin. It was considered possible that a combination of this technique and the principle of traditional MPN method of bacterial estimation in water (APHA 1971) could result in the rapid detection and estimation of low numbers of spoilage fungi.

A modification of the "several tubes of each of several dilutions" MPN-method of Halvorson & Ziegler (1933) was used. For

each experiment, 3 effective dilutions of the spore suspension were used. A set of 10 test-tubes (A) each containing the appropriate growth medium was inoculated with a certain dilution of the spore suspension (Suspension A), another set of 10 tubes (B) with the second dilution (Suspension B) and a third set of 10 tubes (C) with the third dilution (Suspension C). The dilution ratio between suspension A and B and C was 100:10:1.

Before use, the spores underwent heat-shock activation by holding each suspension in a water bath at 50°C for 25 minutes (Sussman 1969). The suspensions were then agitated vigorously for 15 minutes to prevent clustering of spores and ensure uniform distribution.

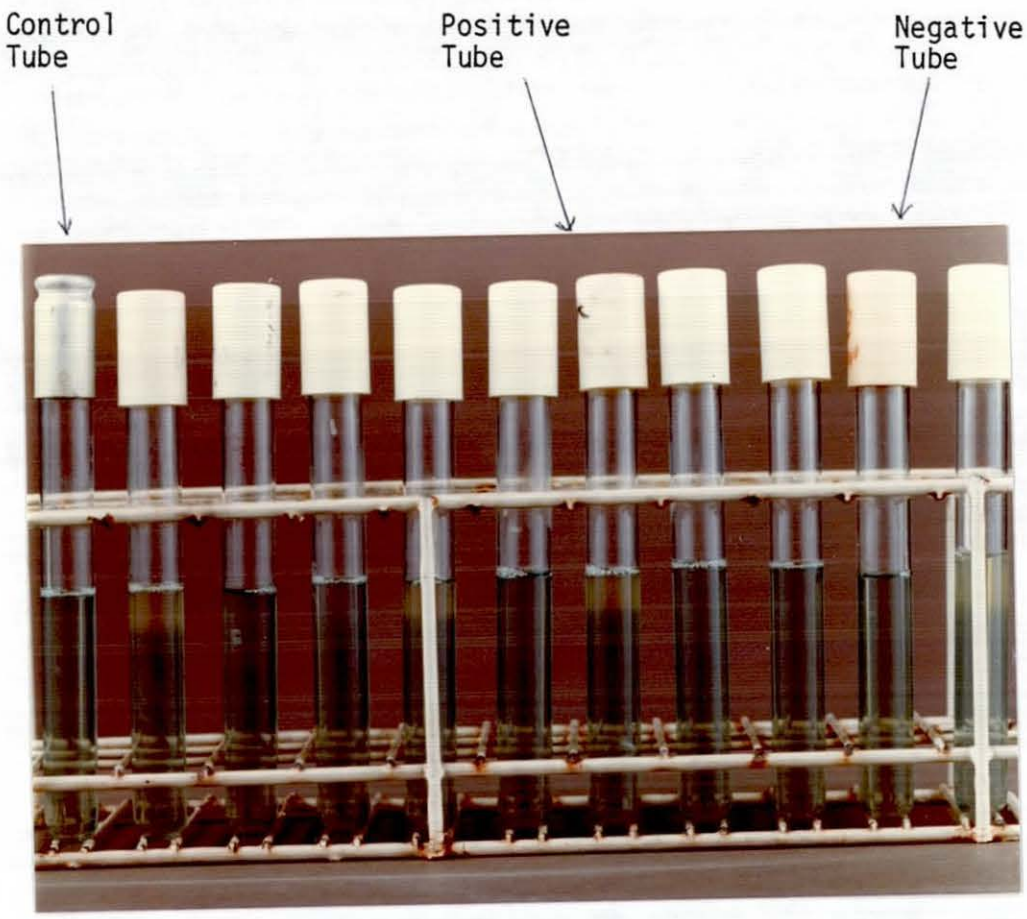
One strain of *Aspergillus (Asp. flavus* CMI 15959) was used in this preliminary work. A 'MASTER' suspension was prepared from the stock and its concentration determined microscopically using the Helber Counting chamber. This 'MASTER' suspension was diluted serially in powers of 10 and the effective dilutions to be used selected according to the guidelines put forward by Cochran (1950).

Each of the 10 tubes of each of the 3 sets (A, B, C) was inoculated with 9 mls of the liquid medium followed by 1 ml of the spore suspension appropriate to that set. An 11th tube for each set that served as control was inoculated with 9 mls of medium and 1 ml of sterile distilled water. Each tube was carefully capped and held in a Whirlimixer to ensure thorough mixing of the spore suspension and medium before incubating at the appropriate temperature.

Several nutritional and environmental factors are know to influence the germination and growth of spores of most fungi. Their influences on these spores, in the establishment of this method, were therefore investigated:

PLATE 1

Sample row of MPN tubes



2.6.A_1 Effect of Media Composition

Each of the 5 Assay Media was tried in turn. Each medium prepared as described, was supplemented with sterile pectin to give a final pectin concentration of 1% (w/v) in the medium. The pH was then adjusted with sterile NaOH or HCl to 5.2. Sterile bromocresol purple indicator dye was introduced, one drop at a time until the medium just assumed the alkaline colour of the dye before dispensing aseptically into the sterile tubes. These were incubated as static cultures at 30°C. and counts of positive tubes were made 6 hourly.

A tube was considered positive upon showing the yellowish acid colour of the indicator dye at the surface of the culture medium by visual comparison with the surface of the culture medium in the control tube (see plate 1). The MPN table of Halvorson & Ziegler (1933c) was consulted to find the most probable number of spores originally present in the spore suspension (Fig. 4).

2.6.A_2 Influence of Peptone Level in the SB Medium

Several media were prepared containing glucose, peptone and pectin. The pectin level in each case was 1%, the glucose level 4% and peptone level varied from 0-5% (w/v). The MPN procedure was carried out and counts of positive tubes were made at 6 hrs. interval for each medium. The results (Fig. 5) were given as the means of at least 5 determinations.

2.6.A_3 Influence of glucose level in the SB Medium

The peptone level as well as the pectin level was constant at 1% (w/v) while the glucose level varied from one medium to the other in the range 0 - 8% (w/v). MPN measurements were carried

out for each medium after every 6 hours of incubation for 72 hours and the results (Fig. 6) are shown as the means of at least 6 determinations.

2.6.A_4 Influence of the Pectin Level in the Sabouraud's Broth

The glucose level was kept constant at 4% and that of peptone at 1% whilst the pectin level varied from one medium to the other in the range of 0 - 10%. MPN measurements were made at every 6 hours of incubation for 72 hours for each pectin level. The results (Fig. 7) are shown as the means of at least 5 determinations.

2.6.A_5 Influence of Agitation of Culture Media

The SB medium used contained 4% (w/v) glucose, 1% (w/v)each of peptone and pectin. The pH of medium after incubation was 5.2 and Bromocresol purple indicator was used. In one experiment the sets of tubes were incubated as static cultures at 30^{o}C while in the second experiment they were incubated at 30^{o}C as shake cultures in a Gallenkamp orbital incubator rotating at 150 r.p.m. MPN measurements were made 6 hourly and the results (Fig. 8) are shown as the means of at least 6 determinations.

2.6.A_6 Influence of Initial pH of Incubation

The glucose - peptone - pectin, 4:1:1, medium (GPP) was used. The pH before inoculating into tubes was adjusted to range from 2 - 9.5. All media at pH 5.5 and above contained 0.01% each of chloramphenicol and chlortetracycline to supress bacterial contamination. Below pH 5.5 bacterial contamination was negligible

(Koburger, 1970). Incubation with agitation at 150 r.p.m. was at 30°C and MPN measurements were made 6 hourly for media at each pH value. The indicator dye for each medium was chosen as indicated below:-

pH range	Indicator
2 - 2.5	Thymol blue
3 - 3.5	Bromophenol blue
4 - 5	Bromocresol green
5.5 - 6.5	Bromocresol purple
7 - 7.5	Bromothymol blue
8 - 9.5	Thymol blue

At the same time 250 ml flasks (in triplicate) which had been inoculated with 90 ml of media and 10 ml of spore suspension were similarly incubated at 30°C with agitation. At 6 hour intervals the pH of each growth medium was taken simultaneously with the pH of a control medium which contained 10 ml of sterile distilled water in place of the spore suspension.

2.6.A_7 Influence of Incubation Temperature

The GPP medium containing 0.01% each of chloramphenicol and chlotetracycline was used. The pH of the medium was adjusted to 7.0, and Bromothymol blue indicator was added before dispensing into tubes. Incubation with agitation was done at temperatures ranging from 20 to 40°C. MPN measurements were made every 6 hours. At each temperature, triplicate 250 ml flasks each containing 90 mls of broth and 10 mls of spore suspension were incubated and changes of pH relative to the control were measured at 6 hour intervals.

2.6.A_8 Influence of Light

The GPP medium with 0.01% each of chloramphenicol and chlortetracycline, pH 7.0 was used. Incubation with agitation was done at 30°C. In one set of experiments the tubes were incubated under total darkness, in the other under normal laboratory conditions and in the third under alternative 3 hours of darkness and 3 hours of normal laboratory conditions. Darkness was achieved by coating the inside of a hard paper carton with a black foil which was then inverted over the tubes. MPN measurements were made for each incubation condition.

2.6.A_9 Comparison of the MPN Method with Standard Surface and Pour Plate Methods

A spore suspension whose concentration was required was diluted appropriately for the MPN method and also for the surface and pour plate methods. Three effective dilutions were used for each method.

Medium 42 was used for both the surface and pour-plate methods. The medium for pour-plates was prepared just before use. Plates on which samples were surface-inoculated were prepared 24 hours before use and left at room temperature to dry.

The plates, in sextuple, for each spore suspension and for each of the surface and pour-plate methods were each inoculated with 1 ml of the appropriate spore dilution and incubated at 30°C (Still Cultures) for 48 hours. Fungal colonies were counted on an Astell colony illuminator with the aid of an electronic colony counter.

MPN tubes were incubated at 30°C with agitation for 18 hours. Positive tubes were counted and the negative tubes confirmed negative by streaking plates. MPN experiments were also done in sextuple.

The analysis was done for all 6 *Aspergillus* strains.

2.6.B INVESTIGATING THE ACID/S RESPONSIBLE FOR COLOUR CHANGE IN INDICATOR-DYE

In order to determine the enzymes involved and their modes of action it was necessary to find out what acid substances in the growth media were causing the indicator-dye to change colour in the MPN tubes.

2.6.B_1 Paper Chromatographic Analysis

The organism was allowed to grow as shake cultures at 30°C for 18 hours. Two media were used: the GPP medium and a glucose (4%) - peptone (1%) medium. Two 250 ml flasks were taken for each medium and while one flask was inoculated with 10 ml spore suspension and 90 ml medium, the other was inoculated with 10 ml sterile distilled water and 90 ml medium. At the end of the incubation period the contents of each flask were centrifuged and the supernatant chromatographed.

Whatman No. 1 paper sheets 25 x 25 cm were spotted with 0.1 ml portions of each supernatant. The diameter of the spots was kept at less than 0.5 cm by the repeated application of less than 5 µl amounts, the drying process being carried out with a hair dryer. Four 1% reference solutions were similarly applied. These were 1% standard solution of D-galacturonic acid, 1% of polygalacturonic acid, 1% of pectin and a composite solution containing each of 1% of pectin, polygalacturonic acid and D-galacturonic acid. Development was by ascending chromatography. Two solvent systems were used separately:

(A) Ethyl acetate - glacial acetic acid - water (2:1:2 ratio) (Demain & Phaff, 1954).

(B) Propanol - formic acid - water (8:1:1 ratio) (Whittaker & Wijesundem, 1951).

The temperature of the developing tank was not controlled, the laboratory temperature, however, averaged about 20 ± 3°C. Chromatography time for solvent system A was 10 hours and for B, 12 hours. At the end of the developing period the paper was dried thoroughly to remove solvent acids from the paper. When dry the papers from solvent A were developed with bromophenol blue (50 mg in 100 ml of 95% ethanol neutralized to pH 6.5 with 0.1M NaoH). Papers for solvent B were developed with alkaline hydroxylamine followed by a mixture of ferric chloride and Hcl.

2.6.B_2 Monitoring the Change in Methanol Content and free D-galacturonic Acid Units in Growth Culture with Time of Incubation

(a) Determination of the degree of Esterification of the Pectin

The degree of esterification is the percentage of uronide carboxyl groups that are esterified with methanol. Determination of this percentage requires measurements of the ester-methoxyl content and assay of the anhydrouronic acid (AUA).

Ester-methoxyl Content

The method of Wood & Siddiqui (1971) was used. It is based on the alkali-hydrolysis of the methoxyl groups of pectin to methanol and oxidising the methanol to formaldehyde which is analysed colormetrically. Oxidation of methanol with acidic permanganate yields formaldehyde. When traces of formaldehyde are added to neutral solutions of acetylacetone

(B) Propanol - formic acid - water (8:1:1 ratio) (Whittaker & Wijesundem, 1951).

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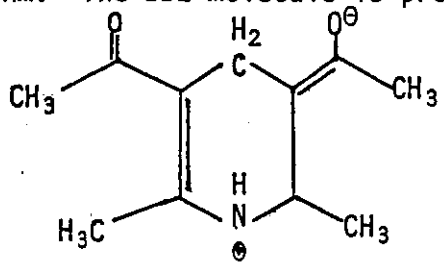
(pentane 2, 4-dione) and ammonium salt, a yellow colour gradually develops owing to the synthesis of diacetyl-dihydrolutidine (3, 5-diacetyl-1, 4-dihydro-2, 6-methyl pyridine) (DDL). Under optimum conditions the molecular extinction in terms of formaldehyde has a maximum at 412 nm. The DDL molecule is probably polar.



A calibration curve of absorbance at 412 nm and methanol concentration (µg/ml) using a UNICAM SP 500 spectrophotometer was made (Figure 14) using methanol standards prepared from absolute methanol (A. R. Merck Methanol, redistilled three times, specific gravity = 0.795).

Pectin solutions of concentrations 50, 100 and 200 µg/ml were used. Hydrolysis of the pectin using NaoH to liberate methanol and the determination of the methanol was as described by Wood and Siddiqui (1971).

(b) Determination of Anhydrouronic Acid (AUA) Content of the Pectin

The method employed has been described by McComb and McCready (1952). It is based on the principle that at 100°C and the experimental conditions described, uronic acid, both in the free state and the polyuronide form, when treated with carbazole, gives a pink colour which has an absorption maxima at 520 nm. The intensity of this colour obeys the Beer-Lambert's Law in the total uronic acid concentration range of 10 - 60 µg/ml.

A calibration curve was prepared using D-galacturonic acid hydrate. The purity of this reagent grade galacturonic acid was checked by titrating 0.5 g with 0.1 NaoH to pH 8.0 using theoretical equivalent weight of the monohydrate i.e. 212. A 0.1% solution of the acid was prepared accurately. Dilutions were made from this in the range 10 - 60 µg/ml and used for the calibration curve (Figure 15).

Pectin solutions of concentrations 10, 20 and 40 µg/ml were used for AUA analysis. Each solution was de-esterified by holding in 0.05M NaoH for 30 minutes at 30°C before being made neutral by same volume of 0.05M Hcl. Three tubes were taken for each solution and the procedure described by McComb and McCready (1952) was followed.

(c) Changes in the Free Galacturonic Acid and Methanol Content of the Culture Media with Time of Incubation

The method used was the modified version of the carbazole reaction of Dische (1950) which can differentiate between free D-galacturonic acid units and polygalacturonic acids. The calibration curve was made from standard D-galacturonic acid solutions in the GPP medium.

90 ml portions of the GPP medium in 250 ml flasks were inoculated with 10 mls of spore suspension. Three effective dilutions of the spore suspension were used. The controls were inoculated with 10 mls of sterile distilled water and incubated at 30°C with shaking. At 3 hourly intervals samples were drawn from each flask and analysed for galacturonic acid by the Dische's (1950) method and for methanol content by the Wood & Siddiqui's (1971) method without the de-esterification

step. This was also carried out for control flasks.

2.6.B_3 INVESTIGATION INTO THE NATURE AND ACTIVITIES OF PECTOLYTIC ENZYMES OF THE FUNGI AT 24th HOUR OF GROWTH

The GPP medium was distributed in 250 ml portions in four 500 ml flasks. Two flasks (test) were each inoculated with 100 mls of spore suspension and the other two flasks (control) inoculated with 100 mls of sterile distilled water. These were incubated at 30°C in the orbital incubator shaking at 150 r.p.m. for 24 hours.

At the end of this period the test and control cultures were separately pooled together and centrifuged. The supernatant was decanted into sterile flasks and the mycelia washed with sterile water and centrifuged. All supernatants of test were pooled together and so were those of control. These were dried to a final volume of 20 mls each. This was used for polygalacturonase (PG) and pectinesterase (PE) assay.

(a) Assay for Polygalacturonase Activity

This was done by measuring the decrease in the viscosity of pectic acid (i.e. pectin solution that has been hydrodrolyzed) with NaoH) buffered to pH 4.0 with phosphate buffer. A polygalacturonase solution (activity = 1.0 unit/ml, where 1 unit activity will liberate 1.0 μ mole of galacturonic acid from polygalacturonic acid per minute at pH 4.0 at 25°C) was prepared from reagent grade polygalacturonase. 1 ml portions of this enzyme solution, 1 ml of the culture filtrate (test) and 1 ml of culture filtrate (control) were separately pipetted

into tubes containing 12 mls each of 0.5% pectic acid solutions and the reaction mixture incubated at 25°C for 20 minutes. Thereafter, 10 mls of the reaction mixture was introduced into a viscomiter held in a glass water bath at 25°C and the time of flow through the capillary of the 10 mls recorded.

The percentage reduction in viscosity was recorded as $\frac{\text{Time of flow of Control - Time of flow of test}}{\text{Time of flow of Control}} \times 100$

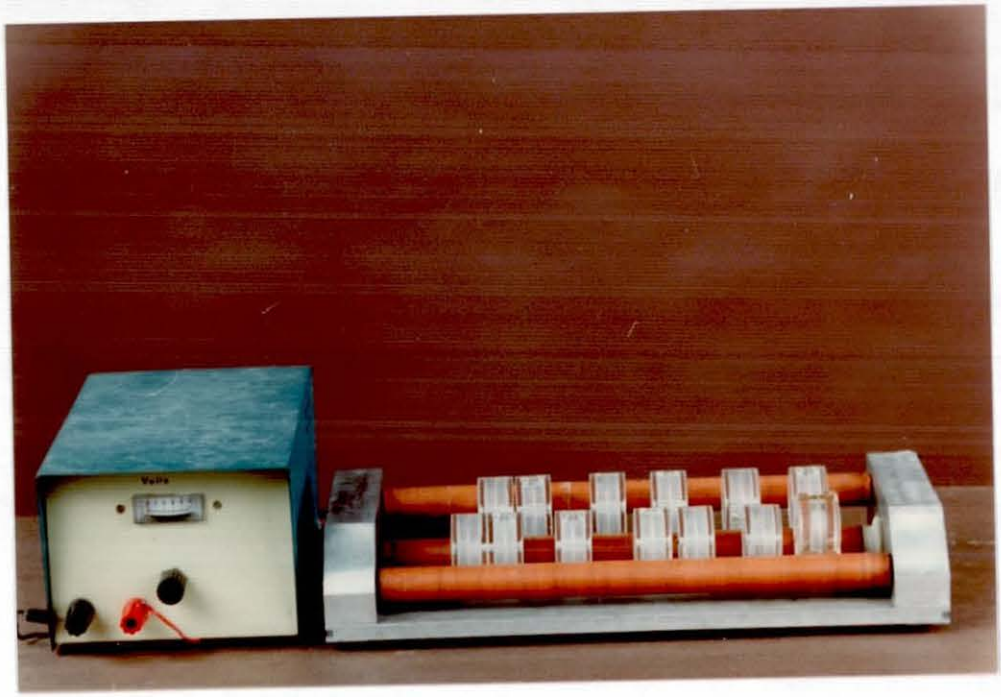
(b) Assay for Pectinesterase Activity

The assay was based on the analysis of methanol released from pectin by the enzyme. Methanol analysis has been described by Wood and Siddique (1971). A solution of reagent grade pectinesterase (1 unit activity/ml) was prepared in phosphate buffer pH 7.5.

1 ml portions of this enzyme solution, 1 ml of the culture filtrate (test) and 1 ml of culture filtrate (control) were separately added to respective tubes containing 5 ml portions of aqueous solution of pectin 1000 µg/ml. The preparations were diluted to 10 ml portions with the phosphate buffer and held at 30°C for 30 minutes at the end of which methanol analysis was carried out on them.

PLATE 4

Revolving power unit for dialysis cells



2.6.C Analysis of Pectinesterase Activities of Germinating Fungal Spores as an Index of Detection and Estimation of Fungal Contaminations

Aspergillus flavus, CMI 15959 and *Aspergillus niger* CMI 31821 were used in the priliminary work.

The assay medium (unless otherwise stated) consisted of 4% glucose, 1% pectin, 1% peptone, 0.01% each of chloramphenicol and chlortetracycline all dissolved in 1% phosphate buffer, pH 7.0. The media was dispensed in 100 ml batches in 500 ml conical flasks. The spore inoculum (50 ml) was added and incubated at 30°C in an orbital incubator shaking at a speed of 150 r.p.m. After the appropriate incubation time, fungal germination and pectinesterase activity were halted by holding the flasks in a water bath at 80°C for 10 minutes. Control flasks were inoculated with 50 ml of sterile distilled water.

The methanol produced was separated from the growth medium by dialysis across a Visking membrane by a modification of the dialysis cell method of Lee & Wiley (1970). The dialysis cell consists of 2 polymethyl methacrylate half cells separated by the Visking membrane. Each half cell has an injection port and a cavity containing up to 5 ml volume. The contacting surfaces were given a light coating of silicone grease to prevent leakage and the front half of each cell was charged with 3 ml of culture. The rear half cell was charged with an equal volume of distilled water and the injection ports were sealed with tightly fitting screws. The cells were rotated on a revolving power unit (Plate 4) at 70 r.p.m. at 30°C for sufficient time to allow the methanol to equilibrate between both compartments. Preliminary work showed

that 4 hours was sufficient time for dialysis.

At equilibrium concentration, the concentration of methanol in front compartment (C_f) = concentration in back compartment (C_b) = ½ concentration of methanol in the culture. Methanol was estimated by gas liquid chromatography on a PYE 104 chromatograph using a flame ionization detector. The column was 180 cm by 6 mm internal diameter and packed with Poropak Q (80 - 100 mesh, Waters Associates, Inc.). The column was operated at a temperature of 110°C with the detector at 140°C using nitrogen (flow rate 20 ml/min.) as carrier gas. A hydrogen and air flame was used; their flow rates were 20 ml/min and 250 ml/min respectively. 10 µl portions were injected at a time.

Methanol from fungal cultures was identified by comparing retention times of peaks with those obtained after GLC analysis of a standard methanol solution (0.05 M). The methanol was determined by peak areas calculated by triangulation (Condal-Bosch 1964). For each test sample injected into the column an equal volume of a standard solution of purified methanol was injected immediately before and after each test sample. Each run lasts approximately 5 minutes. The quantity of methanol in the test sample was then calculated from the formula

$$2\ \frac{A_t.C_s.1000}{A_s.Z} \quad \text{where} \quad \frac{A_t.C_s.1000}{A_s.Z} = C_b$$

and where

A_t = Area of methanol peak of the test run

A_s = Mean of the areas of peaks of standards

Z = Quantity of sample injected (10 µl)

C_s = µg methanol/ml of standard

C_b = µg methanol/ml of content of back compartment of dialysis cell.

1000 converts ml to µl.

2.6.C_1 CONDITIONS FOR OPTIMUM SYNTHESIS AND OPTIMUM ACTIVITY OF PECTINESTERASE BY GERMINATING FUNGAL SPORES.

See Results Section

3

RESULTS

FIGURE 4 EFFECT OF MEDIA COMPOSITION ON THE MPN METHOD

The rate of appearance of positive tubes on all the assay media was compared using the *Asp. flavus* strain, CMI 15959.

■ *Sabouraud's Broth (SB)*

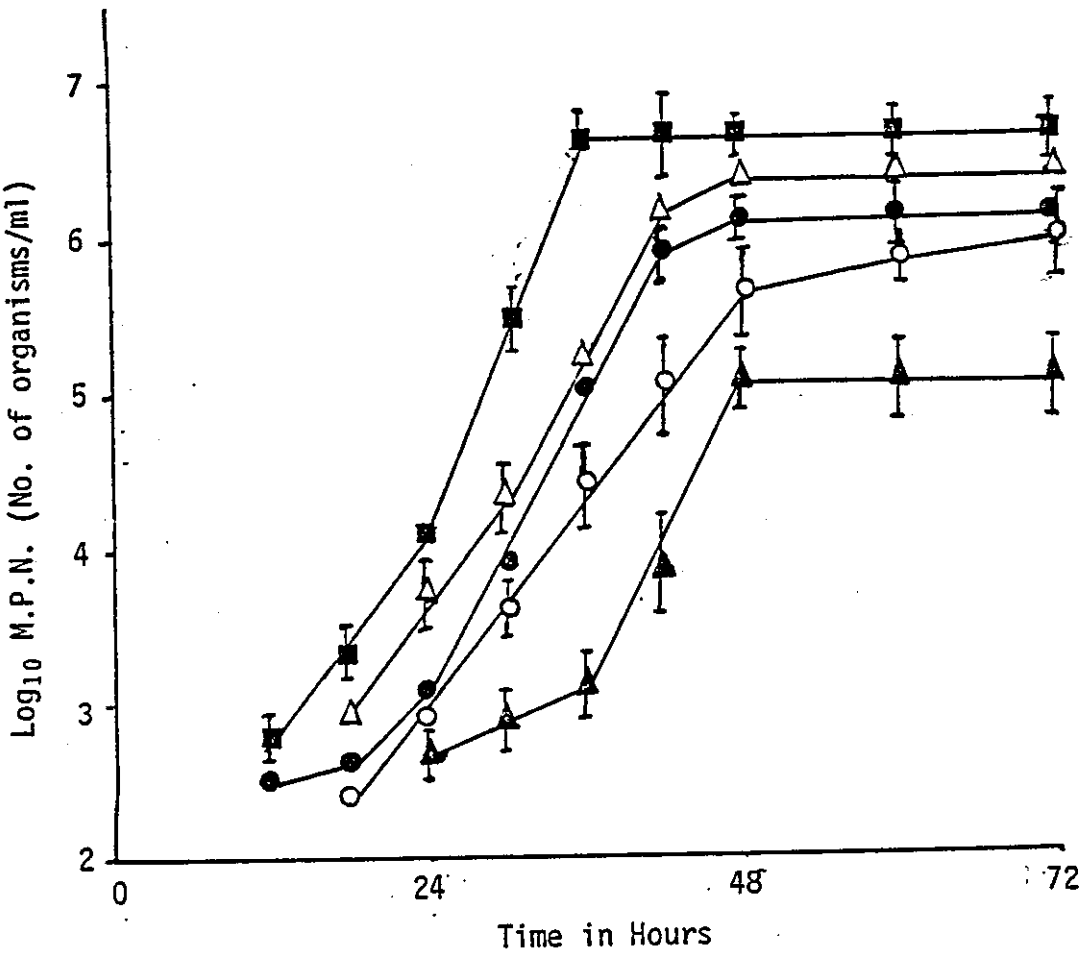
Δ *Glucose Broth (GB)*

▲ *Malt Extract Broth (MEB)*

● *Modified Czapek Dox (MCD)*

0 *Malt, Yeast, Glucose Broth (MYGB)*

Each point is the mean of at least 5 replicates and error bars are standard errors of mean (see appendix 1).


7
6
5
4
3
2
0
24
48
72
Log10 M.P.N. (No. of organisms/ml)
Time in Hours

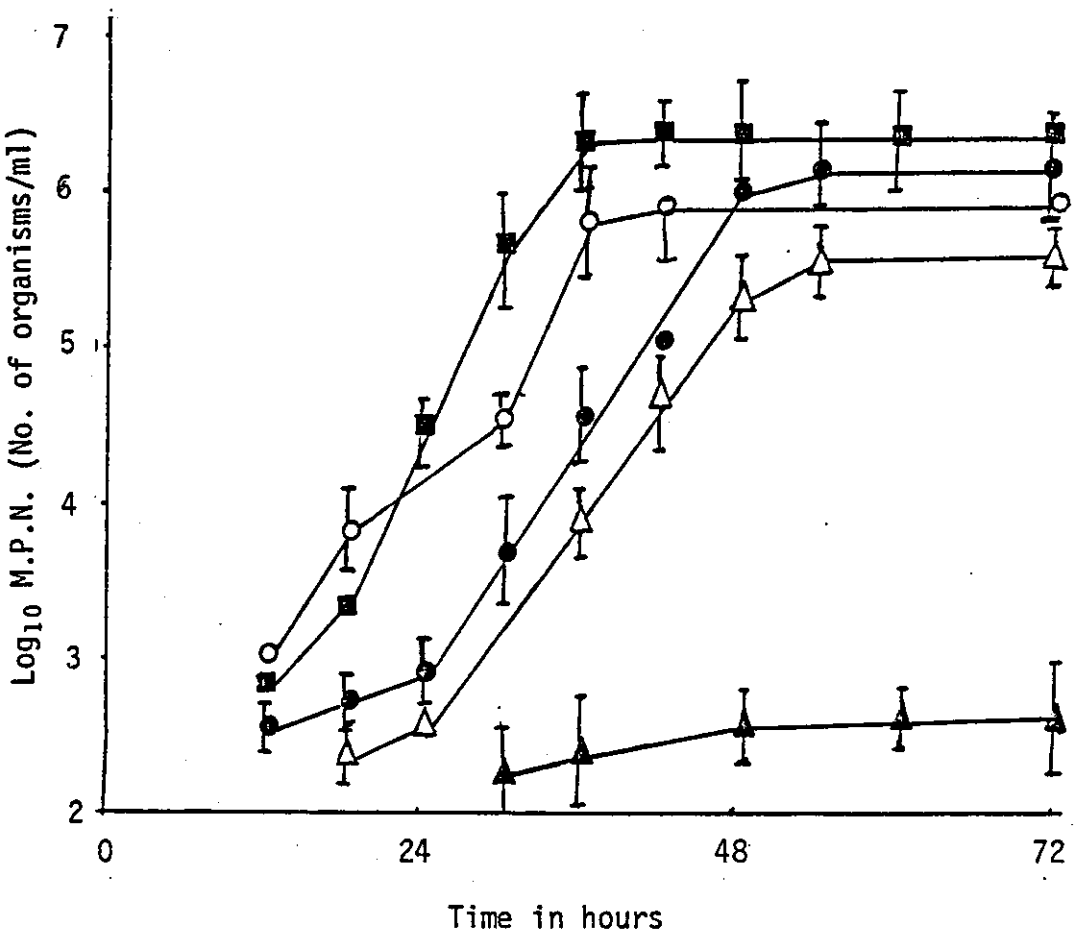

7
6
5
4
3
2
Log10 M.P.N. (No. of organisms/ml)
0
24
48
72
Time in hours

FIGURE 6 INFLUENCE OF THE GLUCOSE LEVEL IN THE SB MEDIUM ON TIME OF RECOVERY OF FULL M.P.N. RESULTS

Positive tubes did not occur in the media with no glucose in it until well after 24 hours incubation time. This would reflect the need for a carbon source in the medium to allow growth of the organism and consequent enzyme activity. MPN values increased and the incubation time for full recovery decreased when the glucose level increased from 2% level through 4% to 6%. The 8% glucose level did not improve the results and 6% was taken as the optimum level for the method. The difference, however, between 4% and 6% was not significant as the error bars crossed each other and for economy purposes the 4% glucose level was preferred in subsequent work.

● 8% level

■ 6.0

○ 4.0

△ 2.0

▲ 0.0

Each point is the mean of 6 replicates and error bars are ± standard errors of the means.

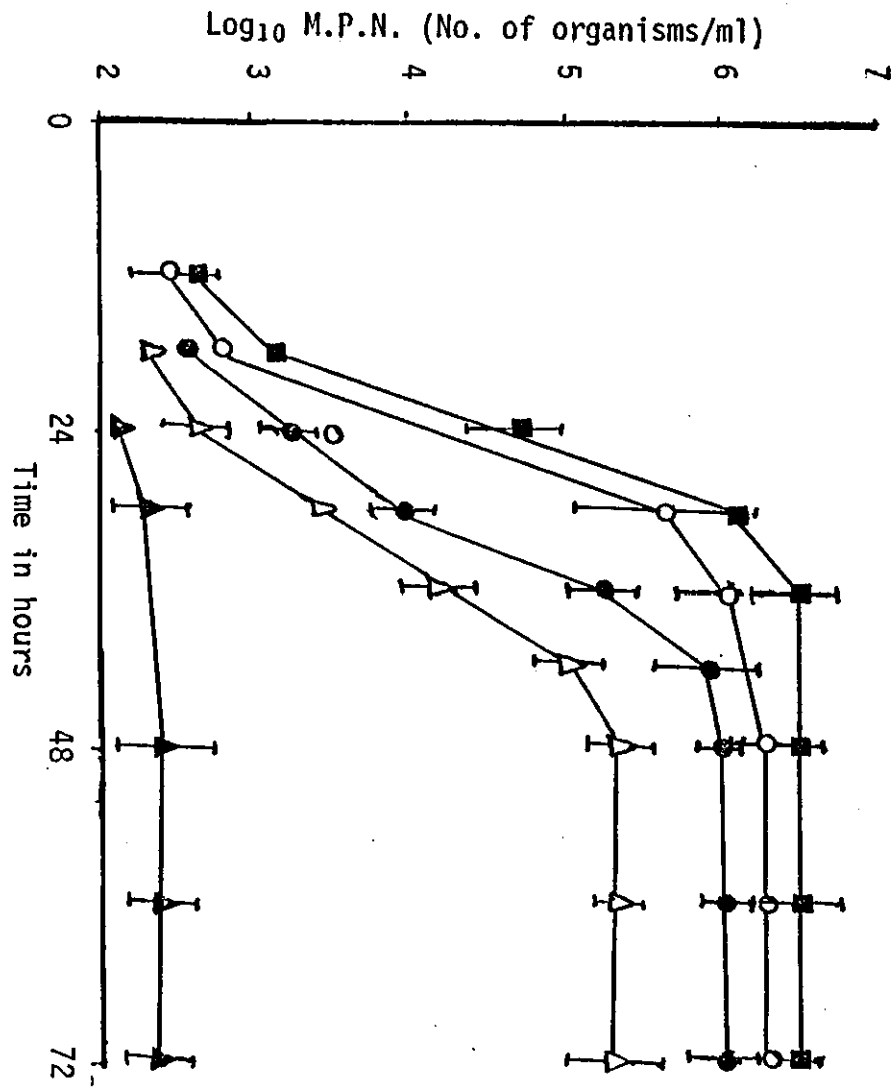
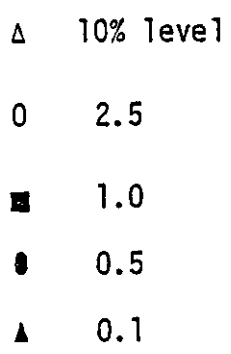

Log10 M.P.N. (No. of organisms/ml)
2
3
4
5
6
7
0
24
48
72
Time in hours

FIGURE 7 INFLUENCE OF PECTIN LEVEL IN THE SB MEDIUM ON TIME OF RECOVERY OF FULL M.P.N. RESULTS

When there was no pectin in the medium a colour change was not observed in any of the tubes even though mycelial growth was visible. This indicates that the acidic substance responsible for colour change of the indicator dye comes only from pectin. As the pectin level increased from 0.1% so did the MPN values up to a value of 2.5% pectin and the maximum results were obtained within 30 hours of incubation for pectin levels of 1.0% and 2.5%. Due to the high viscosity and darker colour of the medium with 10% pectin, positive tubes were difficult to identify and this gave rise to the poorer results for this medium.



There was no positive tube at 0% pectin level

Each point is the mean ± S.D. of at least 5 determinations.

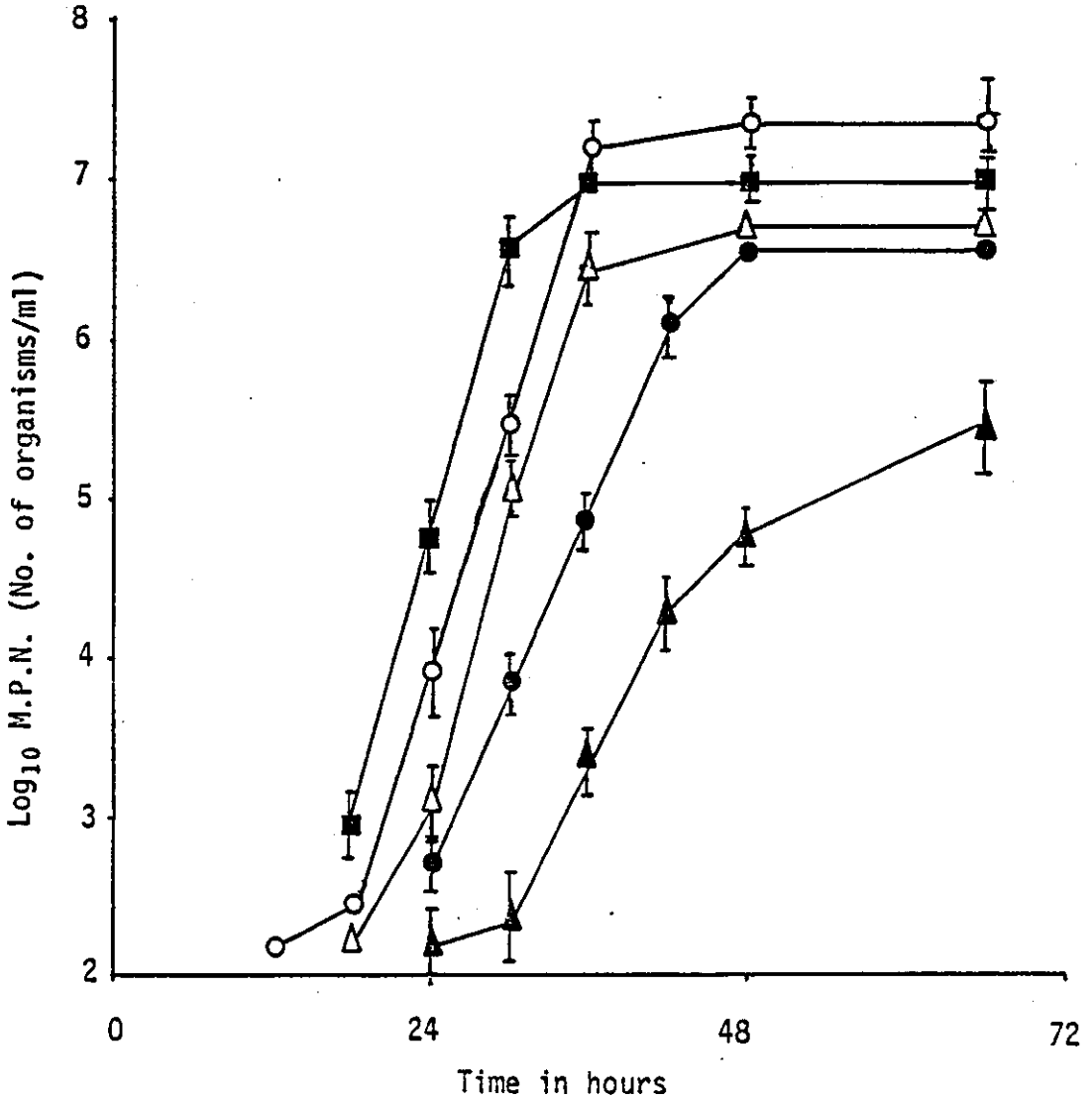

8
7
6
5
4
3
2
Log10 M.P.N. (No. of organisms/ml)
0
24
48
72
Time in hours

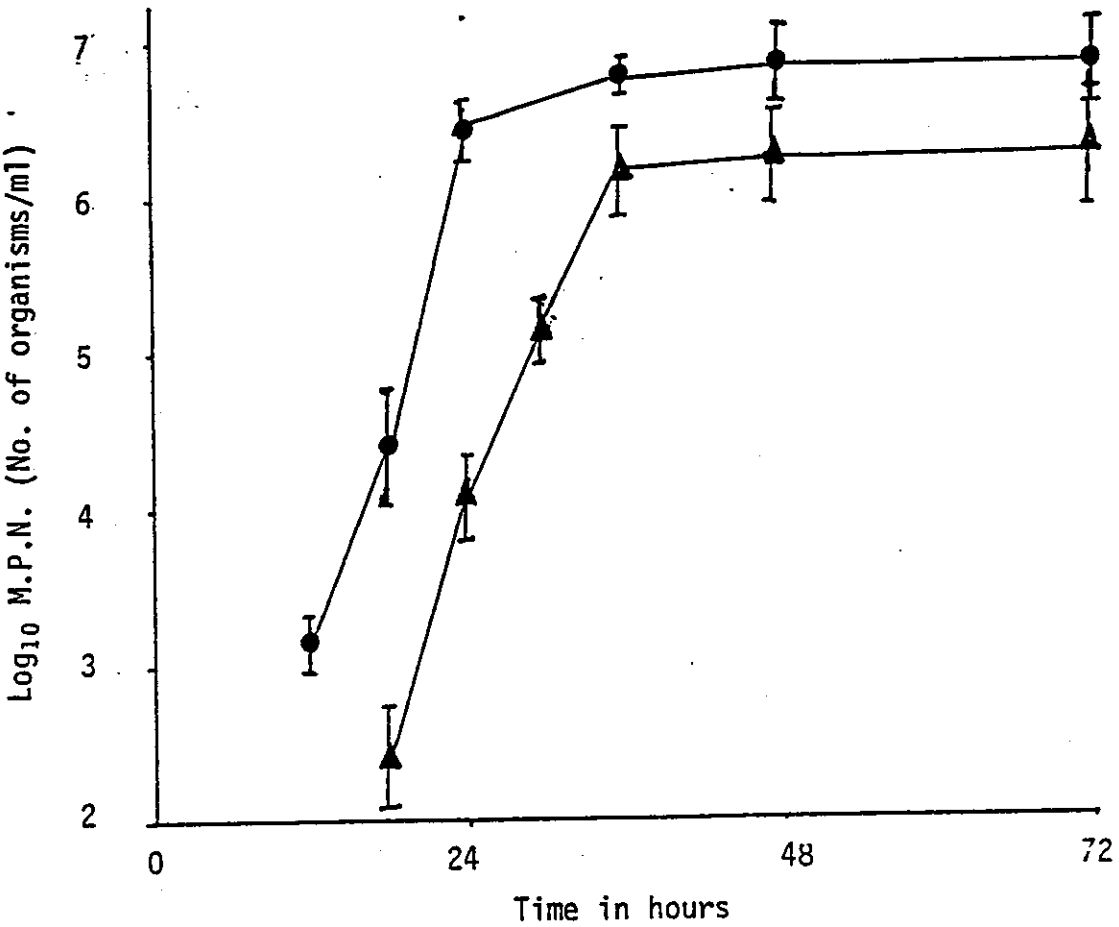
FIGURE 8 EFFECT OF AGITATION ON TIME OF RECOVERY OF FULL MPN RESULTS

The figure shows a clear-cut difference in the incubation time for recovery of full MPN results between shake and static cultures. For the static cultures the first positive results became visible from the 18th hour and rose to 36 hours before levelling out. With the shake culture the first positive results appeared as early as the 12th hour, the number increasing sharply to level out at around the 24th hour. However, there was no significant difference in the final result at P = 0.001. (Calculated T-value = 2.2769). Agitation was used for subsequent work.

● shake culture

▲ surface culture

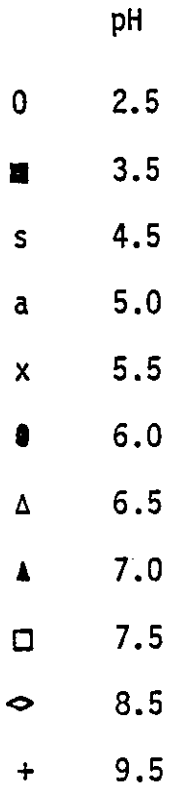
Each point is the mean of 6 replicates and error bars denote ± standard error of means.


7
6
5
4
3
2
Log_{10} M.P.N. (No. of organisms/ml)
0
24
48
72
Time in hours

EFFECT OF INITIAL pH OF INCUBATION

The pH fall in the test media relative to the blank at any given time of incubation was calculated as the difference between the pH of the medium which was inoculated with the spore suspension and the pH of the medium which was inoculated with sterile distilled water. When this difference was plotted as a function of incubation time (Fig. 9) it was seen that the highest values of pH fall occurred in media with pH 7.0 and 7.5 at the 18th and 24th hour of incubation respectively. These peak values progressively decreased and the time to attain them increased in media with more acidic pH values, whilst media with pH values above 7.5 showed very slight differences between blank and test. This was because the pH values of the blank cultures were dropping at almost the same rate as the test cultures. This indicates that the enzyme activity increased with progressive increase in the initial pH of incubation up to pH 7.5. Above this pH, alkali hydrolysis of the pectin competed with enzyme hydrolysis and masked the results. This trend is further exemplified in figure 10 which shows that all tubes became positive as early as the 12th hour of incubation in media with pH's above 7.5. Below this pH the time-pH curve shows the progressive reduction in incubation time to recover maximum MPN values. The higher MPN values above pH 7.5 in the $\log_{10}$ MPN vs pH curve were, therefore, not taken seriously as they were misleading. pH 7.0 was therefore used for subsequent work.

FIGURE 9 THE RATE OF pH FALL IN TEST MEDIA RELATIVE TO BLANK



Each point is the mean of 3 culture flasks, 2 determinations per culture flask, error bars are ± standard deviations.

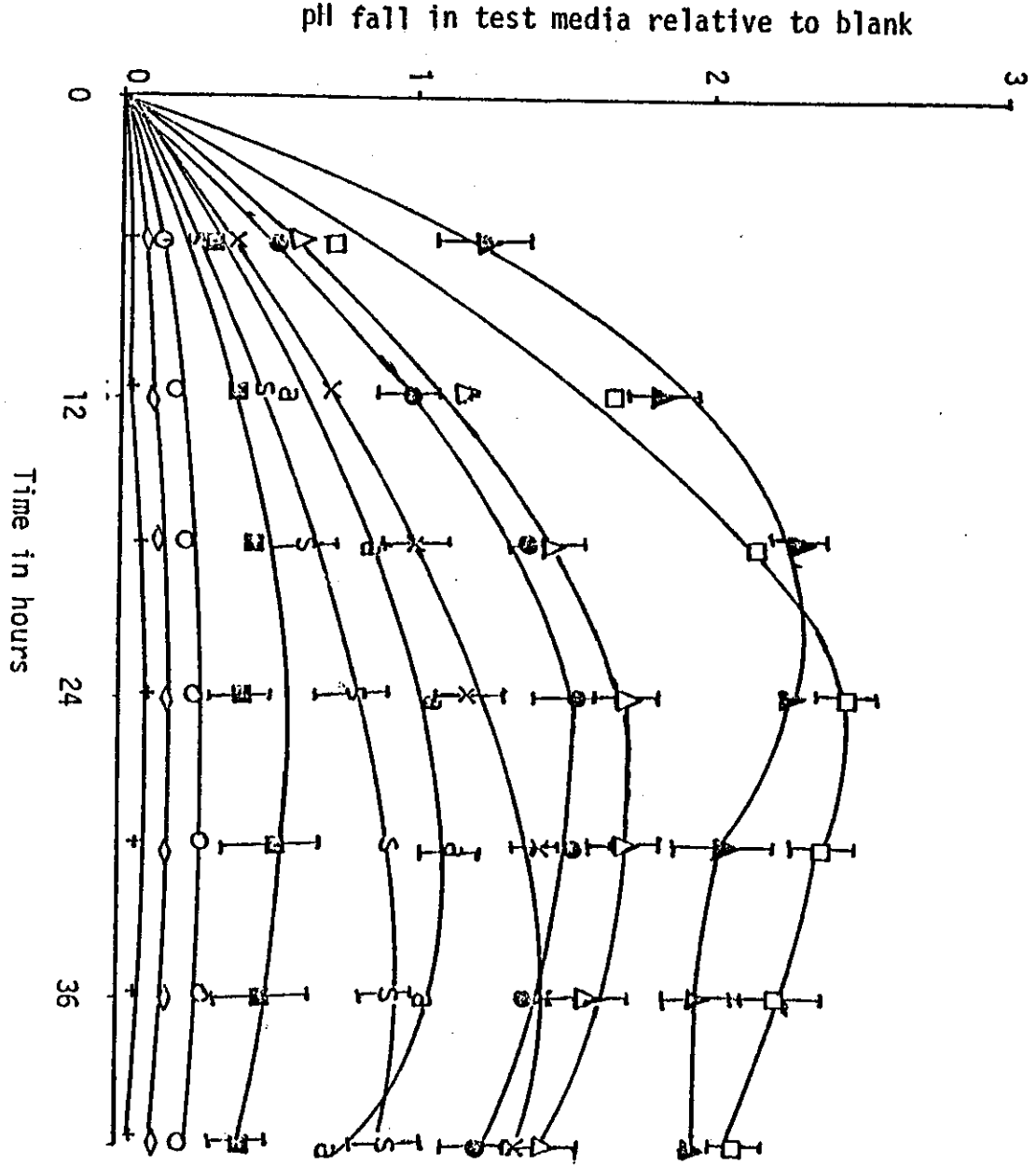

pH fall in test media relative to blank
0
1
2
3
0
12
24
36
Time in hours

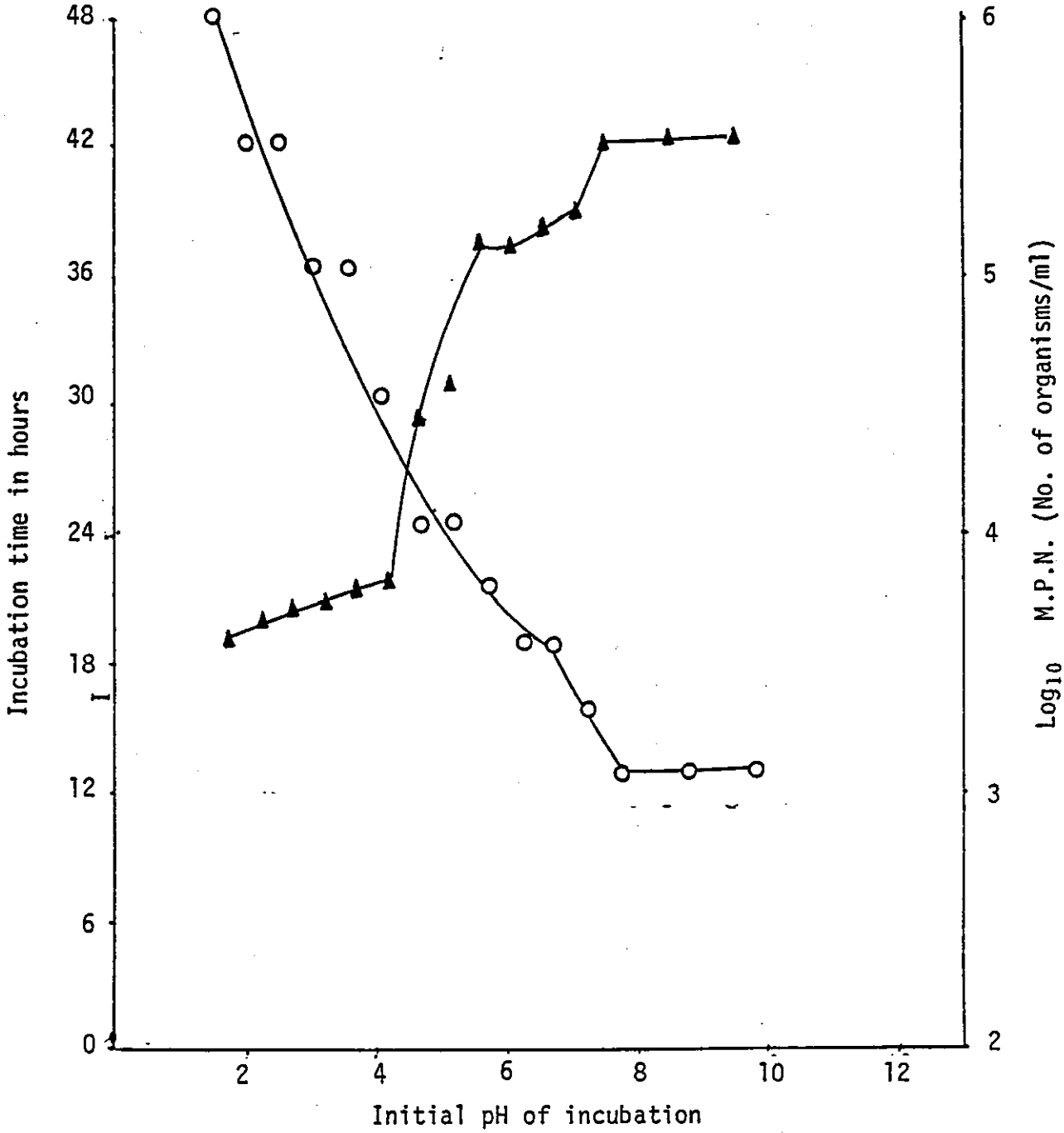
FIGURE 10

Time - pH curve: Effect of initial pH of incubation on time of observation of full MPN results (plateau times in time vs. MPN curves, not shown).

Log_{10} MPN vs. pH curve: Effect of initial pH of incubation on the MPN values at plateau time.

▲▲▲ Log_{10} M.P.N. vs. pH

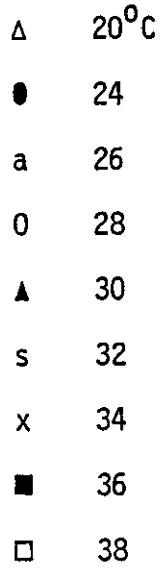
000 Time vs. pH


48
42
36
30
24
18
12
6
0
Incubation time in hours
6
5
4
3
2
Log10 M.P.N. (No. of organisms/ml)
2
4
6
8
10
12
Initial pH of incubation

EFFECT OF INCUBATION TEMPERATURE

The pH fall in test media relative to control was greatest in culture media incubated at temperatures between 28 and 34°C with an optimum at 30°C, and occurred as early as the 18th hour of incubation (Fig. 11). The pH fall above and below this range decreased progressively and the incubation time for the maximum fall to occur at these temperatures also increased progressively. Higher pH falls are indicative of higher pectolytic enzyme activities and result in more easily identifiable positive tubes. This is reflected in figure 12 in which the time-temperature curve shows that maximum MPN results occur around the 18th hour of incubation for cultures at 28 - 34°C with this time increasing above and below this temperature range. MPN values were maximum at this temperature range ($\log_{10}$ MPN vs. temperature curve).

FIGURE 11 RATE OF pH FALL IN TEST MEDIA RELATIVE TO CONTROL



Each point is the mean of 3 culture flasks, 2 determinations per flask, error bars are ± standard deviations.

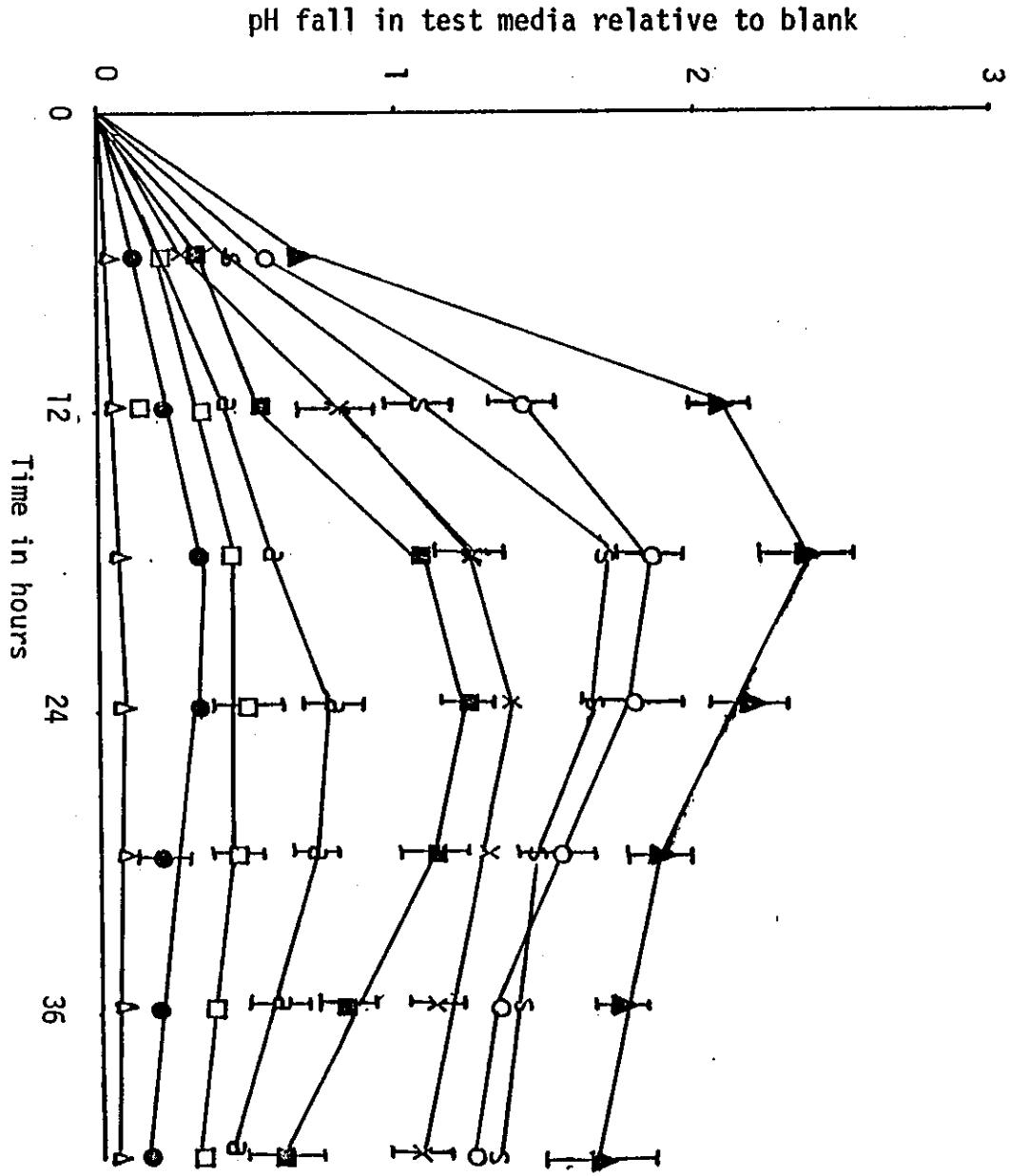

pH fall in test media relative to blank
0
1
2
3
0
12
24
36
Time in hours

FIGURE 12

Time - Temperature Curve: Effect of incubation temperature on time of observation of full MPN results (plateau times in time vs. MPN curves, not shown)

Log_{10} MPN vs. Temperature Curve: Effect of incubation temperature on MPN values at plateau time.

O O Log_{10} M.P.N. vs. Temperature

▲ ▲ Time vs. Temperature

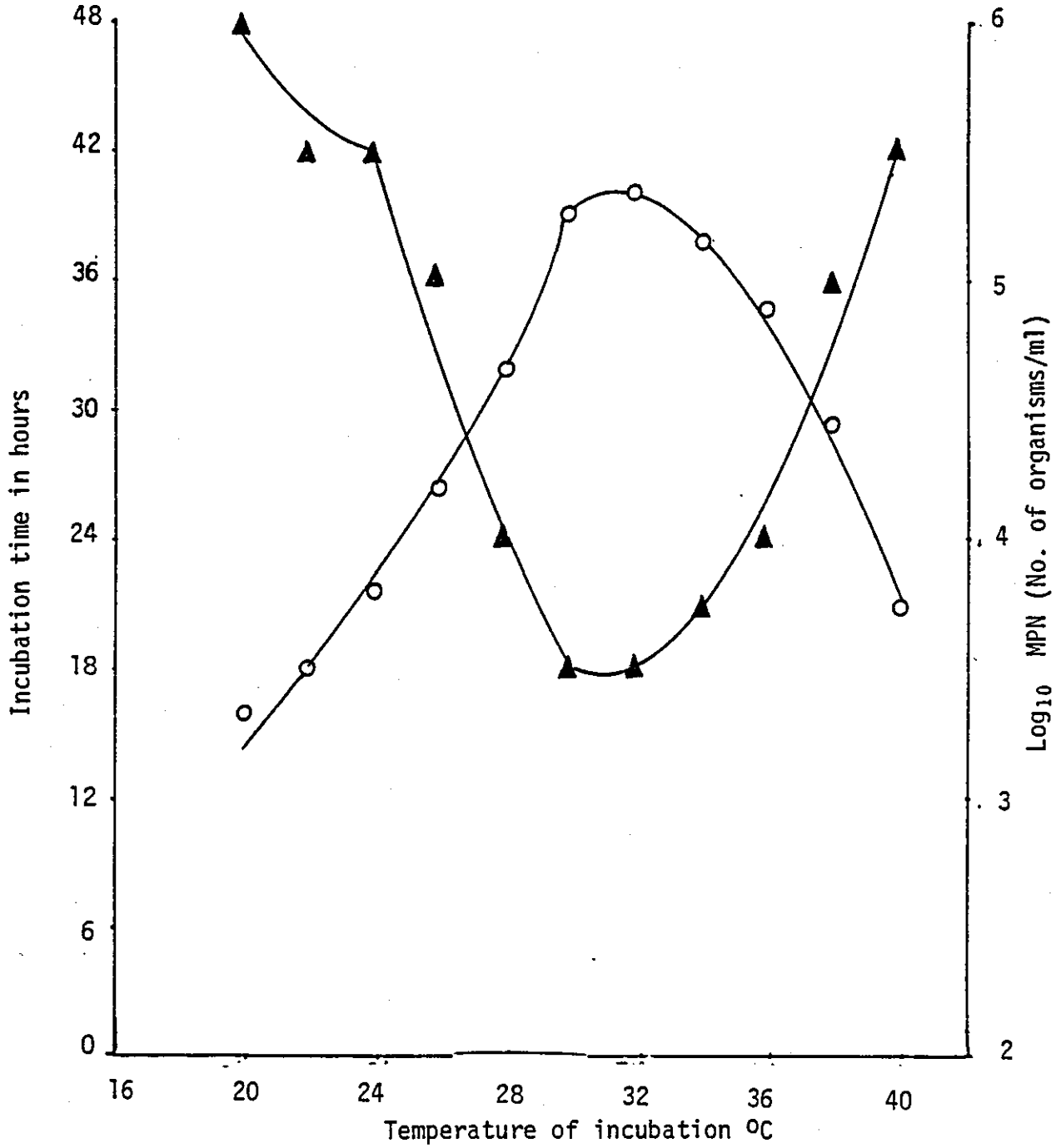

48
42
36
30
24
18
12
6
0
Incubation time in hours
6
5
4
3
2
Log10 MPN (No. of organisms/ml)
16
20
24
28
32
36
40
Temperature of incubation °C

FIGURE 13 EFFECT OF LIGHT ON TIME OF RECOVERY OF FULL MPN RESULTS

Light has been found to stimulate spore germination in some fungi (Gassner - Hoechst, 1954; Gassner & Neimann, 1954) and it is definitely known to stimulate or inhibit mycelial growth depending on the medium employed (Cochrane, 1958; Carlile 1965, 1970). However, no significant effect on analysis time for the MPN method was found. In all 3 cases the curve reached a maximum at about the 18th hour of incubation. Slightly higher MPN values were obtained in tubes maintained in total darkness followed by those in alternative light and darkness. The increases were, however not significant, error bars, especially at the plateau parts of the curves, tend to overlap each other. Normal laboratory conditions were, therefore, preferred for the sake of convenience in subsequent work.

□ Total darkness

0 Alternative light and darkness

▲ Total light

Each point is the mean of 3 cultures, 2 determinations per culture.

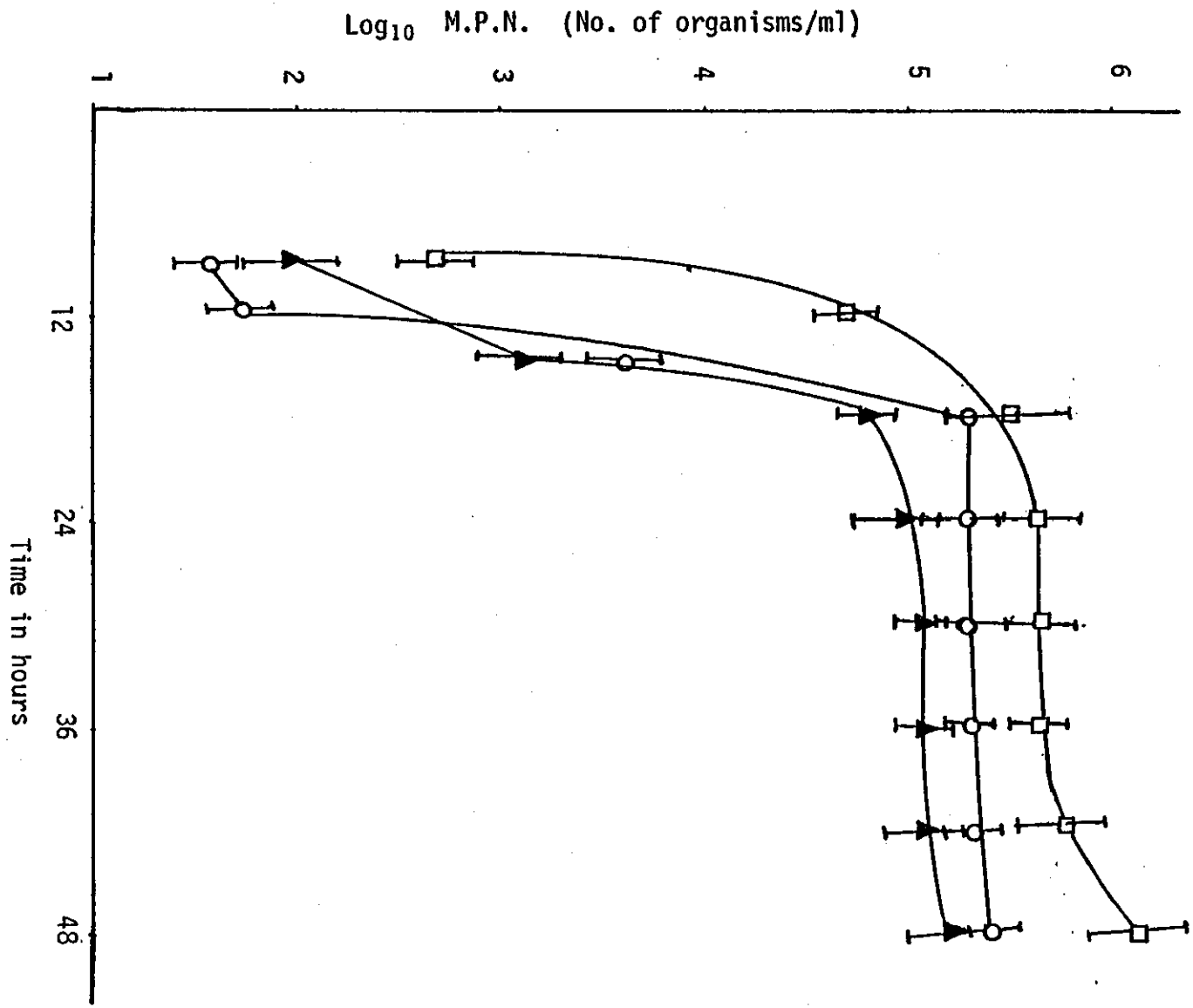

Log10 M.P.N. (No. of organisms/ml)
1
2
3
4
5
6
Time in hours
12
24
36
48

TABLE 2 COMPARISON OF THE MPN METHOD AND STANDARD SURFACE AND POUR PLATE METHODS

Table 2 gives the results of a comparison of the MPN method and standard surface and pour plate methods of all 6 *Aspergillus* strains and a composite suspension of all 6 organisms. Results by all 3 methods are in reasonable agreement. Significance tests show no significant difference in the means by all 3 methods (T-test) and in the variances (F-test) at 0.01 probability level.

ORGANISM	Number of Organisms per ml. Mean ± SD			F-test values		T-test values		Degrees of Freedom
	MPN METHOD(1)	Pour Plate(2)	Surface Plate (3)	(1) and (2)	(1) and (3)	(1) and (2)	(1) and (3)	
A. flavus 39178a	3780 ± 1450	3400 ± 1400	3500 ± 1380	1.073	1.104	.350	.268	5
A. flavus 15959	122000 ± 55000	104000 ± 38000	112000 ± 46000	2.095	1.430	.653	.359	5
A. flavus 86769	11000 ± 2400	11000 ± 3500	11400 ± 3900	.470	.379	.131	.070	5
A. nidulans 16643	86000 ± 3200	80400 ± 2860	83000 ± 3200	1.252	1.000	.300	.117	5
A. niger 31821	850 ± 130	800 ± 110	820 ± 120	1.400	1.174	.644	.272	5
A. luchuensis Inui	3200 ± 370	3030 ± 360	3040 ± 380	1.056	.948	.934	.887	5
Composite suspension of all 6 strains	13500 ± 1800	13000 ± 1700	13000 ± 208	1.121	.749	.459	.192	5

INVESTIGATION OF THE ACID/S RESPONSIBLE FOR COLOUR CHANGE OF THE INDICATOR DYE IN CULTURE MEDIA.

PAPER CHROMATOGRAPHIC ANALYSIS OF CULTURE FILTRATES

Under the conditions described, solvent system A, would separate and identify galacturonic acid units according to the degree of polymerization whilst solvent system B would separate and identify the methyl esters of different uronides.

The chromatogram in A (Plate 2) shows that all test samples that contained pectin had each an acid spot of ca. 2.85 cm from the origin. This compares with the standard polygalacturonic acid spot found at 3.1 cm from the origin (R_f values were not calculated as the solvent front had over-flowed the top of the chromatography paper at the experimental time necessary for the separation of these acids). This spot was also obtained in the 1% pectin solution. However, the yellow acid colour was more intense in the media in which the fungus had grown than either (a) the pectin solution or (b) the media which was inoculated with sterile water. The colour was most intense in the polygalacturonic acid standard. This spot was absent in the media that contained no pectin.

The chromatogram in B (Plate 3) shows a spot each at ca. 2.2 cm from the origin for the pectin-containing samples. These spots which are the methyl esters of uronides could bearly be seen in the medium in which the fungus has grown and were completely absent from the polygalacturonic acid standard, D-galacturonic acid standard and the media without pectin. No spot from any of the test media corresponded to that of standard D-galacturonic acid.

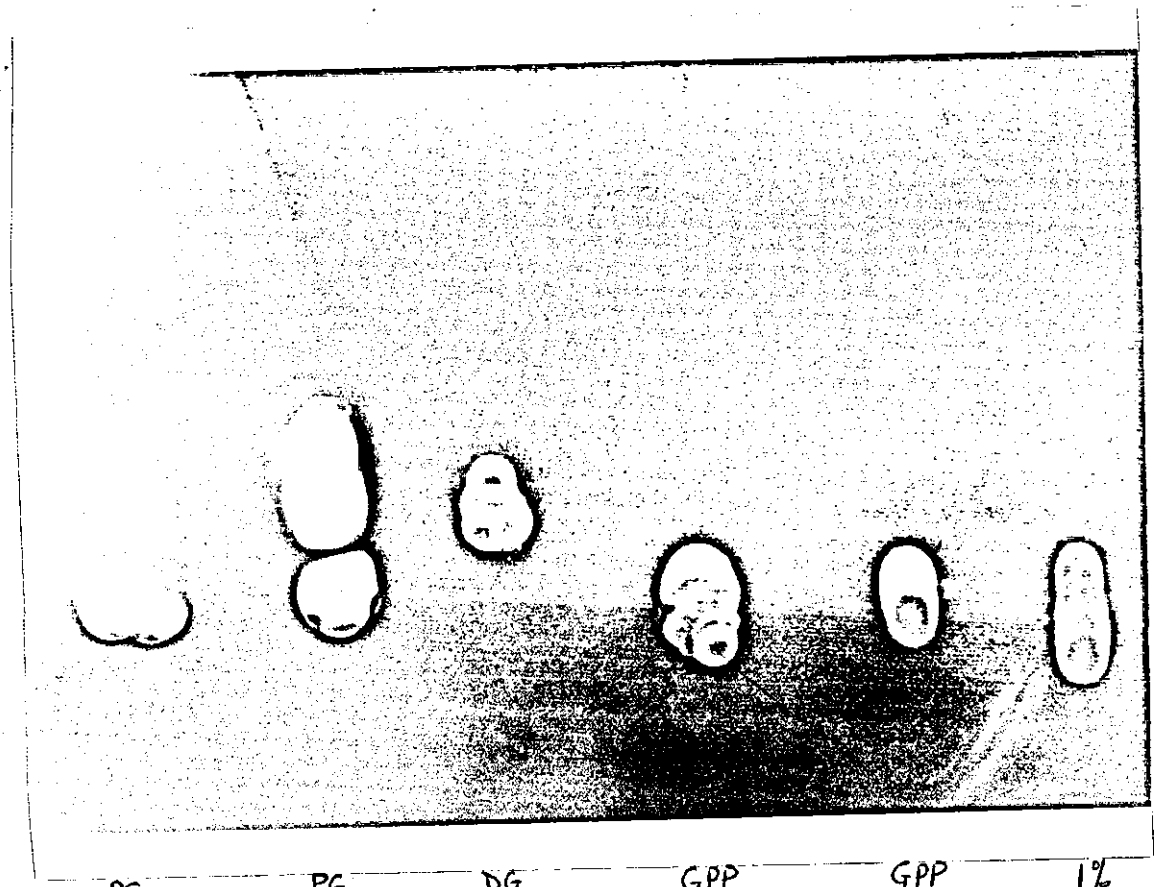


PLATE 2

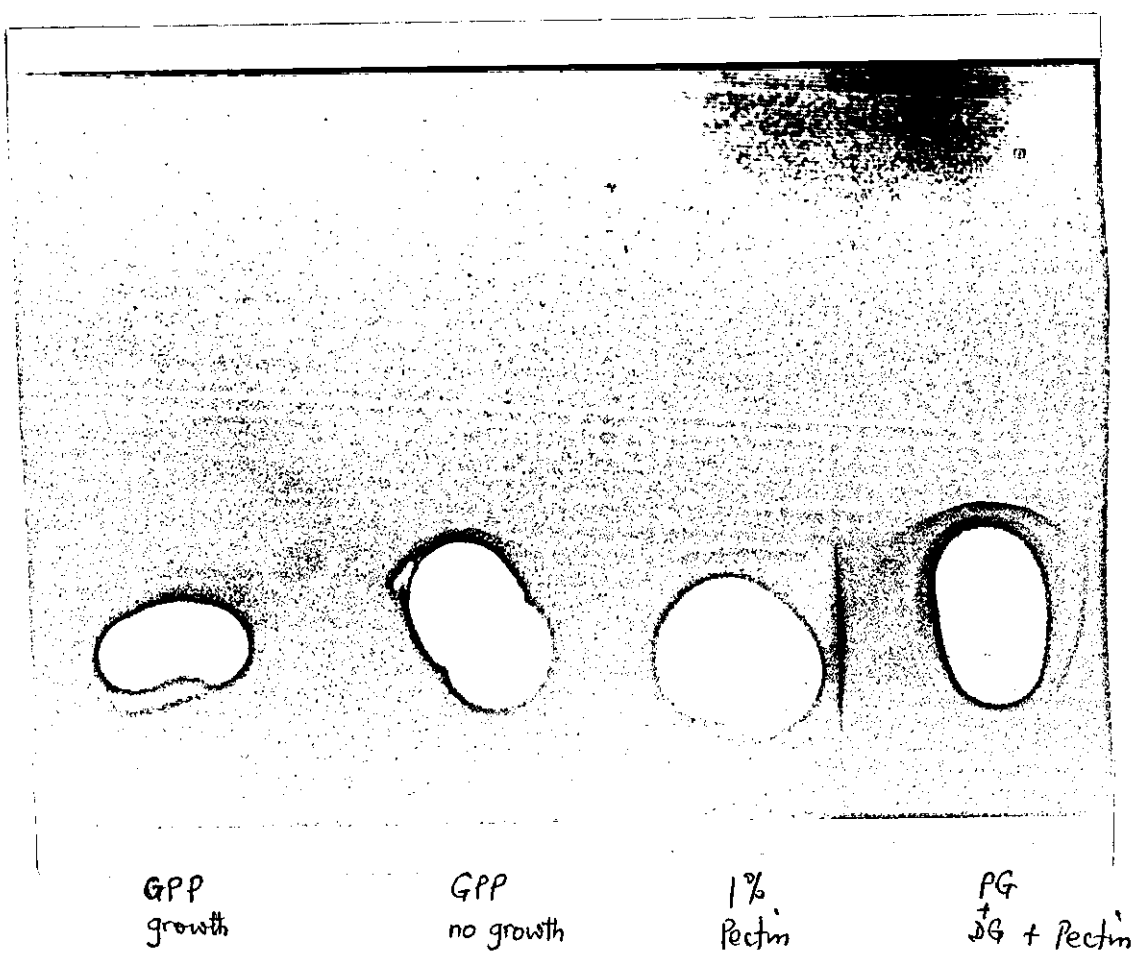
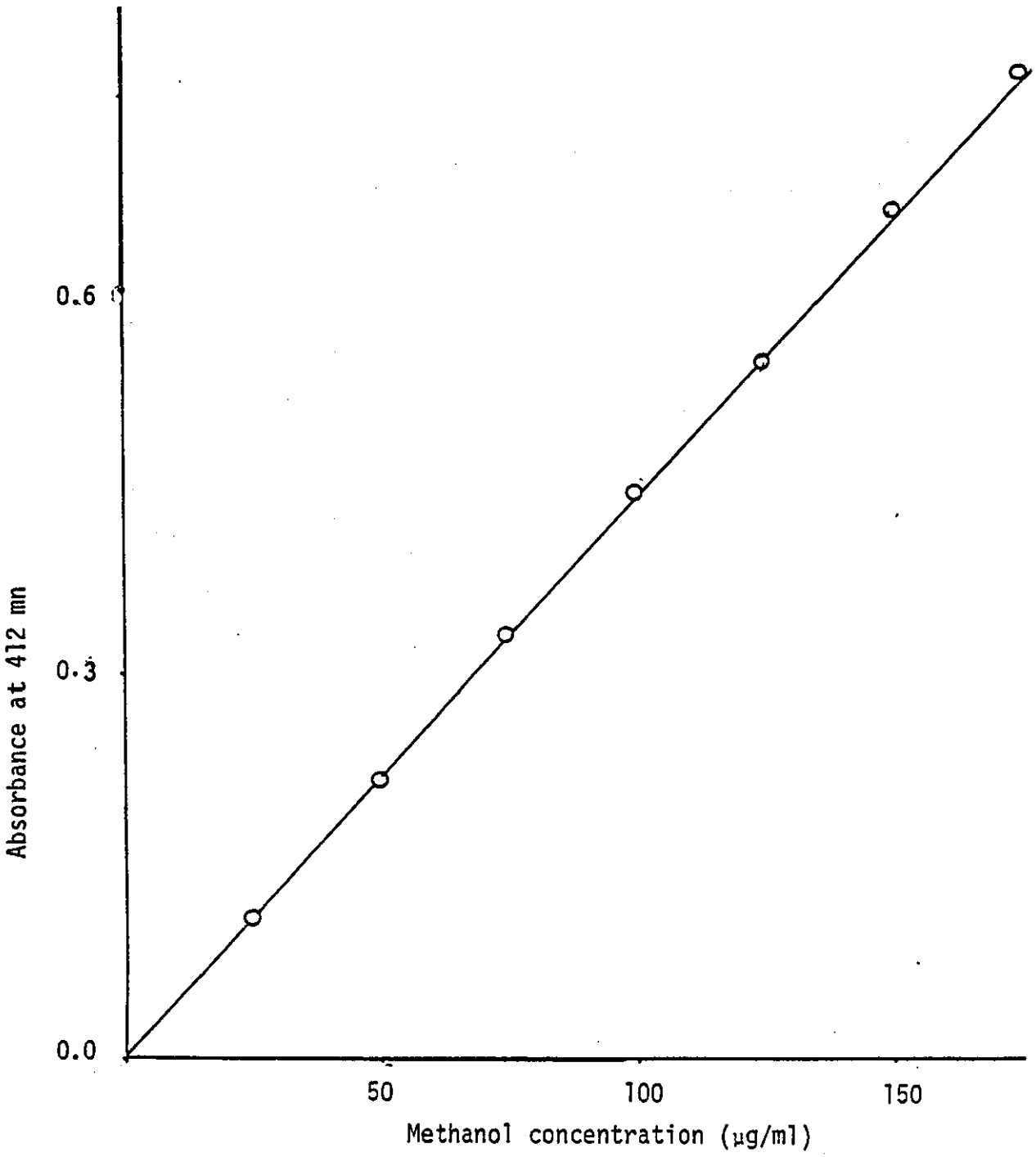


PLATE 3

FIGURE 14 CALIBRATION CURVE FOR METHANOL DETERMINATIONS

Each point is the mean of 5 determinations, largest standard deviations = ± 0.05%


Absorbance at 412 mn
0.6
0.3
0.0
50
100
150
Methanol concentration (µg/ml)

METHOXYL CONTENT OF THE PECTIN

From the calibration graph (Fig.14) the amount of methanol released from the test solutions were as shown in table 3.

Table 3

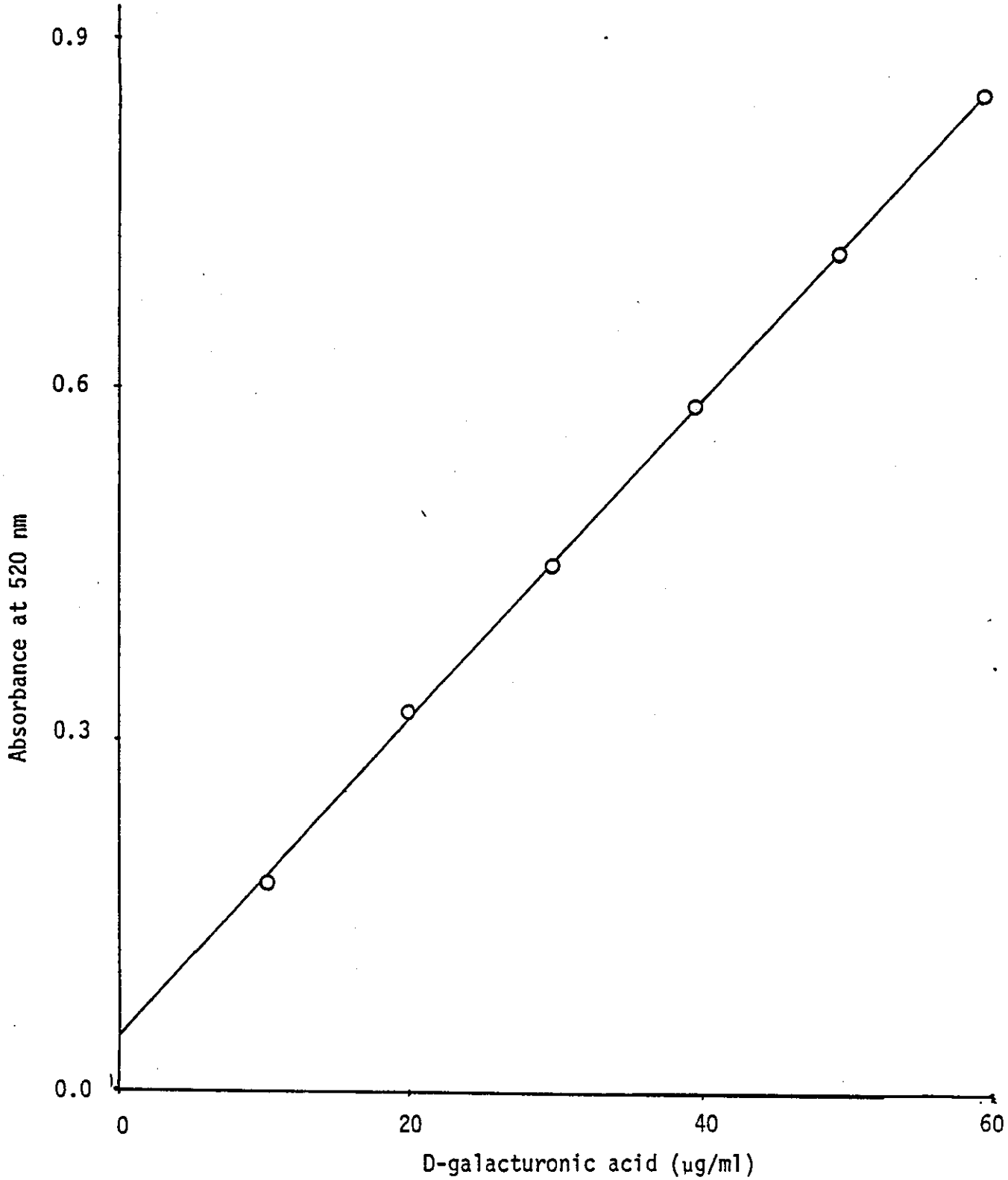
Pectin Concentration (µg/ml)	Amount of Methanol released (µg/ml)	Percentage Methanol (in the Pectin (w/w)
50	4.72	9.44
100	9.25	9.25
200	18.84	9.42

Therefore mean % methanol in the pectin = 9.37.

It follows that 1 ml of GPP medium when completely hydrolysed would yield 937 µg of methanol.

FIGURE 15 CALIBRATION CURVE FOR AUA DETERMINATIONS

Each point is the mean of 6 determinations, largest standard deviations = ± 0.8%


0.9
0.6
0.3
0.0
Absorbance at 520 nm
0
20
40
60
D-galacturonic acid (µg/ml)

From the calibration curve for AUA determinations (Fig. 15)

the AUA content of the 10 µg/ml pectin solution = 7.76 µg/ml

" " " " " 20 µg/ml " " = 15.29 "

" " " " " 40 µg/ml " " = 31.40 "

Therefore AUA percentages (w/w) = 77.6, 76.45 and 78.51 respectively.

Mean value = 77.52

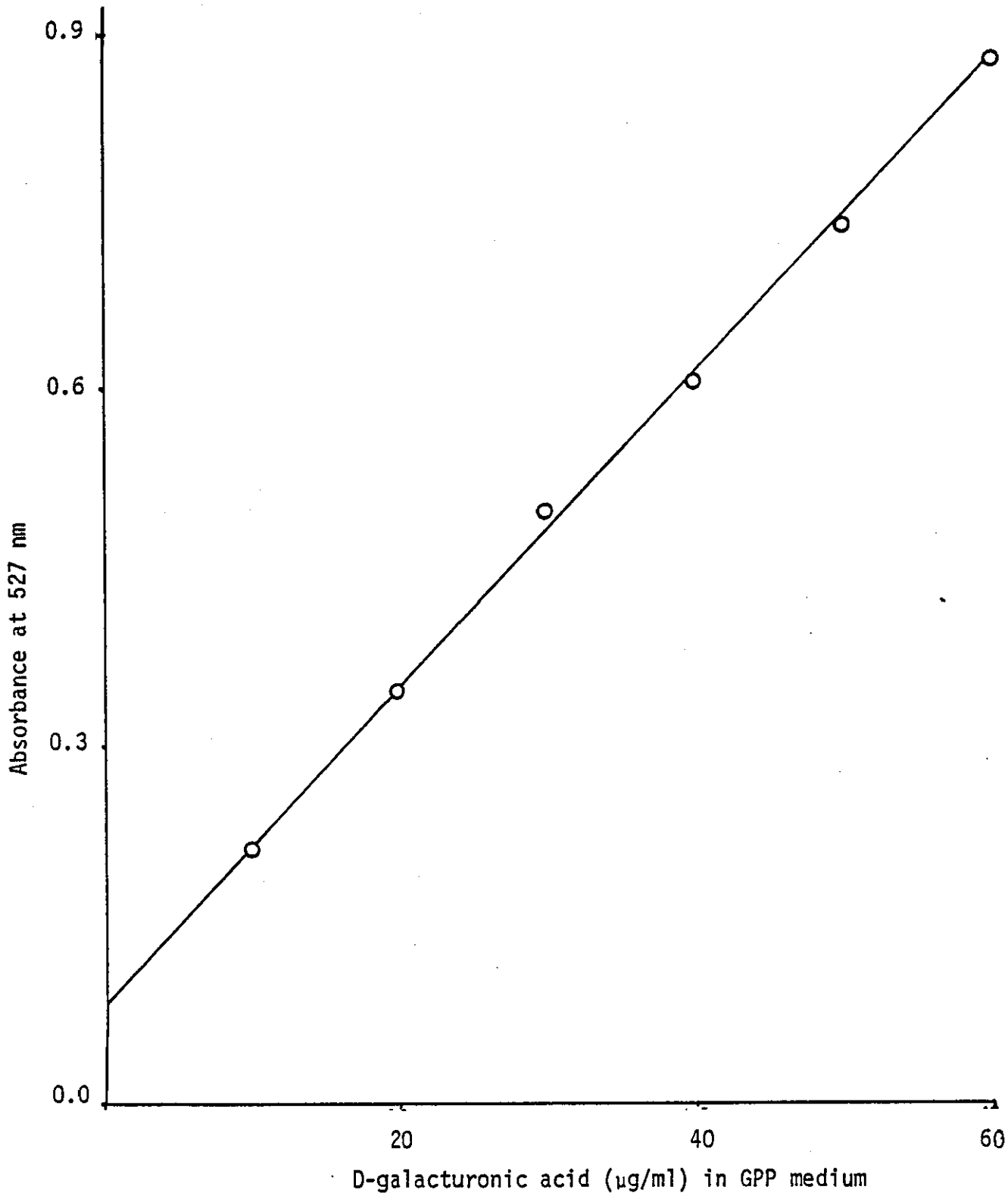
Hence the degree of esterification of the pectin which is calculated from the formula $\frac{176 \times \text{ester-methoxyl content (\% w/w)}}{31 \times \text{AUA content (\% w/w)}} \times 100$

Where 176 is the formula weight of Anhydrogalacturonic acid and 31 is the formula weight of $-OCH_3$ is calculated as $\frac{176 \times 9.37}{31 \times 77.52} \times 100$

$= 68.6\%$

FIGURE 16 CALIBRATION CURVE FOR GALACTURONIC ACID DETERMINATIONS

Each point is the mean of 5 determinations, largest standard deviations = ± 0.1%


0.9
0.6
0.3
0.0
Absorbance at 527 nm
20
40
60
D-galacturonic acid (µg/ml) in GPP medium

ASSAY FOR POLYGALACTURONASE (PG) ACTIVITY

The solution of the commercially available PG contained 1 unit of activity and this was responsible for the reduction in viscosity of 42.87% under the experimental conditions.

Therefore the activity in the test preparation that caused only 0.73% reduction in viscosity (table 4) must be very low and therefore unlikely to be responsible for producing the acid which has caused the colour change of the indicator dye. This means that the spot obtained on the chromatogram is unlikely to be free galacturonic acid.

TABLE 4

SAMPLE	% Reduction in Viscosity
PG solution	42.87 ± 1.22
Culture filtrate	0.73 ± 0.08

Assay for polygalacturonase (PG) activity.

Each value is the mean of 6 determinations.

ASSAY FOR PECTINESTERASE (PE) ACTIVITY

The commercially available PE used contained 1 unit activity per ml. This caused the release of 217.5 µg methanol (table 5) in 30 minutes. Hence the activity of the test preparation which caused the release of 645.33 µg methanol in the same time = 2.97 units/ml.

Hence the activity of the culture filtrate = 2.97 units/ml. Although some of the methanol analysed was present in the culture filtrate itself (the methanol release from the GPP medium by the organism during the 24 hours of incubation) the final PE activity was high enough to justify the effect of PE on GPP medium being responsible for colour change of the pH indicator dye.

Hence within the experimental time of the MPN analysis it is the action of pectic acid in depressing the pH of the media which is responsible for the observed colour change in positive tubes.

TABLE.5

SAMPLE	Methanol released in the experimental time (µg/ml)
PE Solution	217.50
Culture Filtrate	645.33

Assay for Pectinesterase (PE) activity.

Each value is the mean of 6 determinations.

CHANGES IN METHANOL CONTENT OF CULTURE MEDIUM WITH INCUBATION TIME

Figure 17A shows a plot of methanol content of culture media versus incubation time. The methanol content of culture media at any particular time was taken as the difference between the methanol content of the control which was used as blank and that of the test.

With the highest spore concentration methanol could be detected after 9 hours of incubation. Detection times for methanol increased as the spore concentration diminished. For each concentration there was a gradual rise in methanol content of the culture media with time, an indication of increased synthesis of pectinesterase.

CHANGES IN GALACTURONIC ACID CONTENT OF CULTURE MEDIA WITH INCUBATION TIME

Figure 17B is a plot of galacturonic acid content of the culture media versus incubation time. Galacturonic acid content was calculated as the difference between the galacturonic acid content of test media at a given time and that of the control using a calibration graph (Fig. 16). Positive indication of free galacturonic acid in the media did not occur until after 18 hours of incubation even with the highest inoculum size. At the 24th hour of incubation the amount of galacturonic acid content of the test media could be considered negligible. This is indicative of a lack of, or very weak, polygalacturonase activity in the culture media at the 24th hour of incubation and so eliminates the possibility of galacturonic acid taking part in the colour change of the indicator dye.

FIGURE 17

A Relationship between methanol content of culture medium and incubation time.

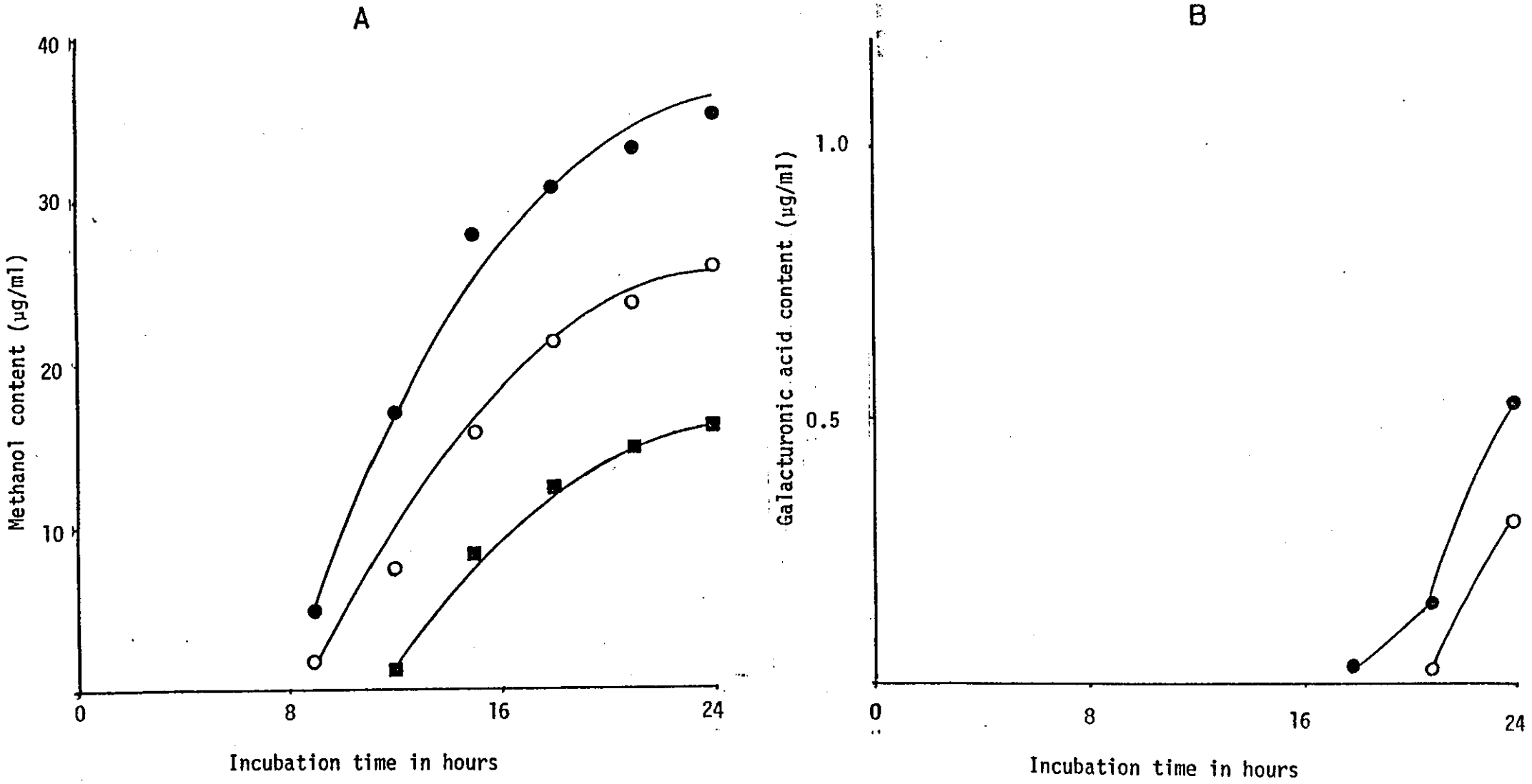
B Relationship between galacturonic acid content of culture medium and incubation time.

● spore suspension A

0 spore suspension B

■ spore suspension C

spore concentrations of A = B X 10 = C X 100


A
B
40
30
20
10
Methanol content (µg/ml)
1.0
0.5
Galacturonic acid content (µg/ml)
0
8
16
24
Incubation time in hours
0
8
16
24
Incubation time in hours

INVESTIGATION OF CONDITIONS FOR OPTIMUM ACTIVITY OF PECTINESTERASE BY GAS LIQUID CHROMATOGRAPHIC METHANOL ANALYSIS

EFFECT OF pH

The sources of the enzyme used were:

i) Reagent grade pectinesterase obtained from *Asp. niger* (Sigma Chem. Co.) a 1% solution (w/v) was used.

ii) Culture dialysate of test *Asp. flavus* CMI 15959

iii) Culture dialysate of test *Asp. niger* CMI 31821

The culture dialysates were obtained thus: spores were grown for 24 hours in the GPP medium. These were individually filtered through glass fibre paper (Whatman GF/A). The filtrate was centrifuged at 5000 g at 4°C for 20 minutes. The supernatant was dialysed against sterile distilled water for 24 hours at 4°C. The dialysis was carried out in acetylated cellophane tubing prepared from visking dialysis tubing as described by Whitaker et. al. (1963). The dialysate was then freeze-dried to concentrate it.

1% pectin in phosphate buffer was prepared at pH values ranging from 4.5 to 8.5. The enzyme and pectin solutions were mixed in the ratio 1:4 (v/v) and incubated at 30°C for 30 minutes. Controls had the same composition except that the enzyme was inactivated at 100°C for 5 minutes before it was added to the substrate. At the end of 30 minutes the reaction was halted by inactivating the enzyme.

On cooling, the methanol released was dialysed and analysed as described earlier.

The protein content of the reaction mixtures at the end of the 30 minutes reaction time was determined by the method of Lowry et. al. (1951) using the calibration curve (Fig. 18) obtained by using standard solutions of serum bovine albumin.

FIGURE 18

CALIBRATION CURVE FOR PROTEIN DETERMINATIONS BY THE METHOD OF LOWRY et.al (1951) STANDARD SOLUTIONS OF SERUM BOVINE ALBUMIN WERE USED

Each point is the mean of 6 determinations, highest standard deviations = 0.06%.

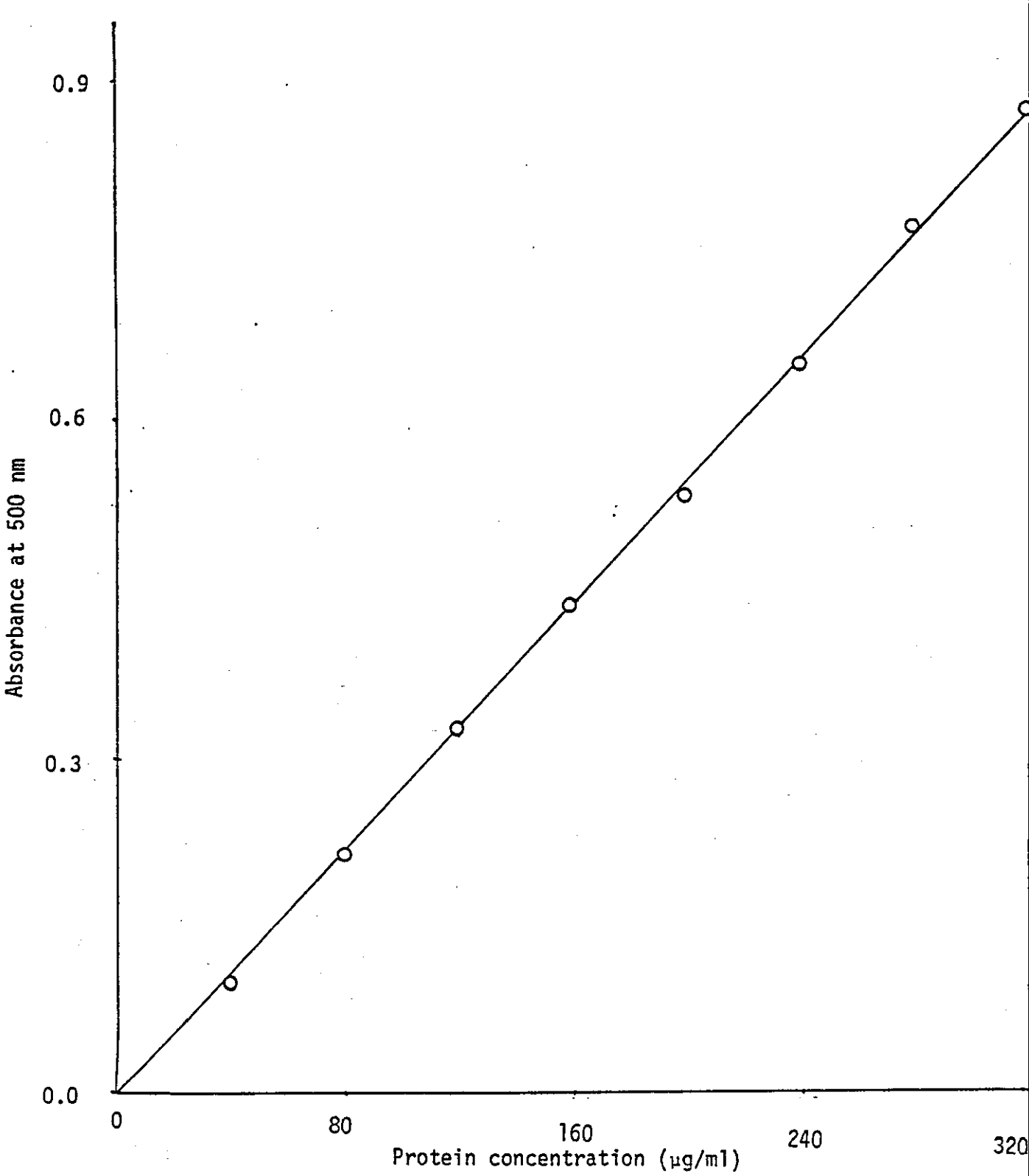

0.9
0.6
0.3
0.0
Absorbance at 500 nm
0
80
160
240
320
Protein concentration (µg/ml)

FIGURE 19

EFFECT OF pH ON SPECIFIC ACTIVITY OF PECTINESTERASE

● culture dialysate *Asp. niger*

■ culture dialysate *Asp. flavus 15959*

▲ commercial PE

Each point is the mean ± S.D. of 5 determinations

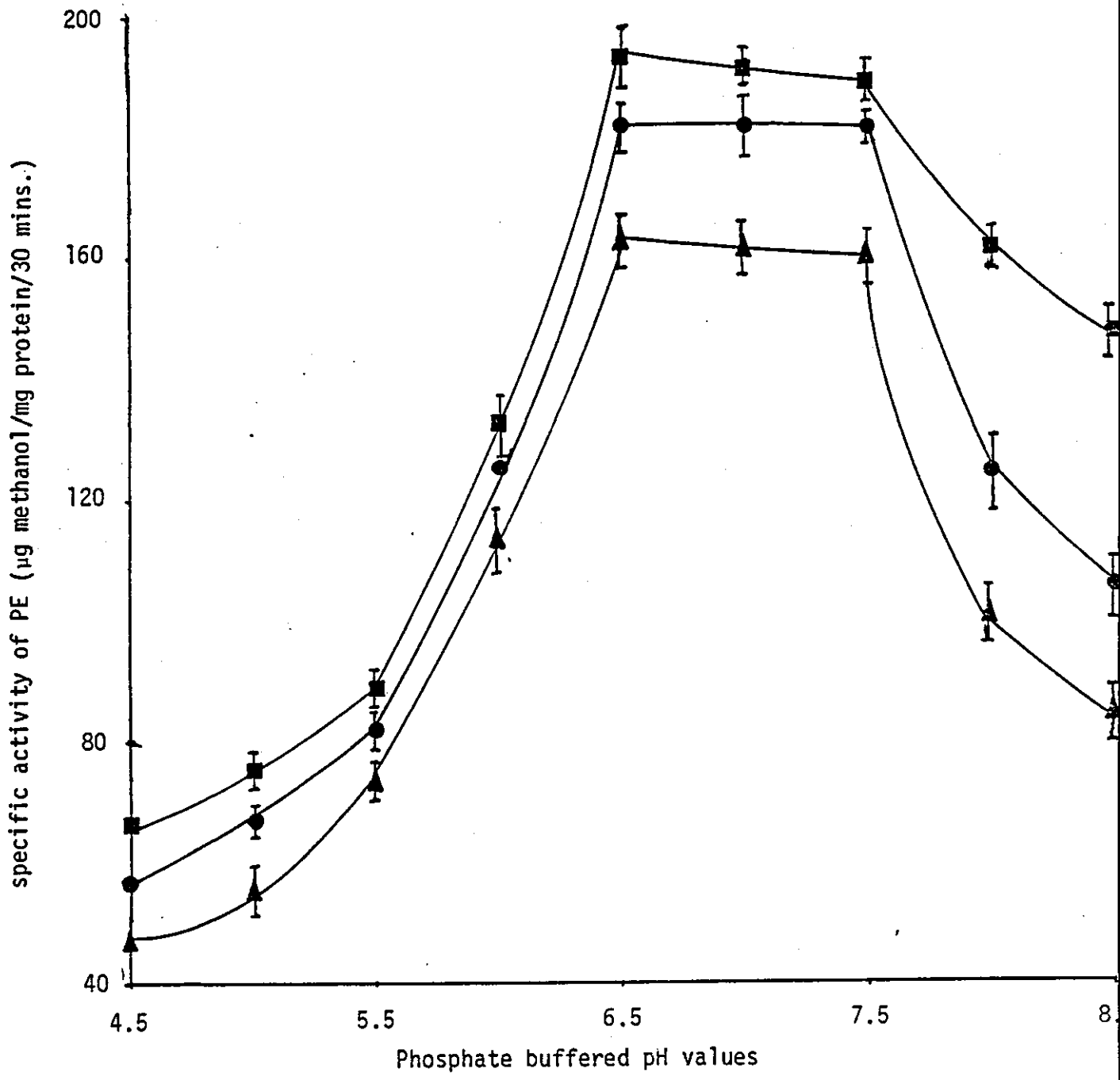

200
160
120
80
40
specific activity of PE (µg methanol/mg protein/30 mins.)
4.5
5.5
6.5
7.5
8
Phosphate buffered pH values

FIGURE 20

EFFECT OF TEMPERATURE ON PE ACTIVITY

Working at phosphate buffered pH 7.0 the reaction mixtures of (a) Commercial PE (1% solution w/v) and 1% pectin and

(b) Culture dialysate of *Asp. niger* and 1% pectin were incubated at various temperatures ranging from 20°C to 85°C for 10 hours and analysed for methanol production.

▲ Commercial PE .

0 *Asp. niger PE*

Each point is the mean of 5 determinations.

No PE activity was observed at 85°C.

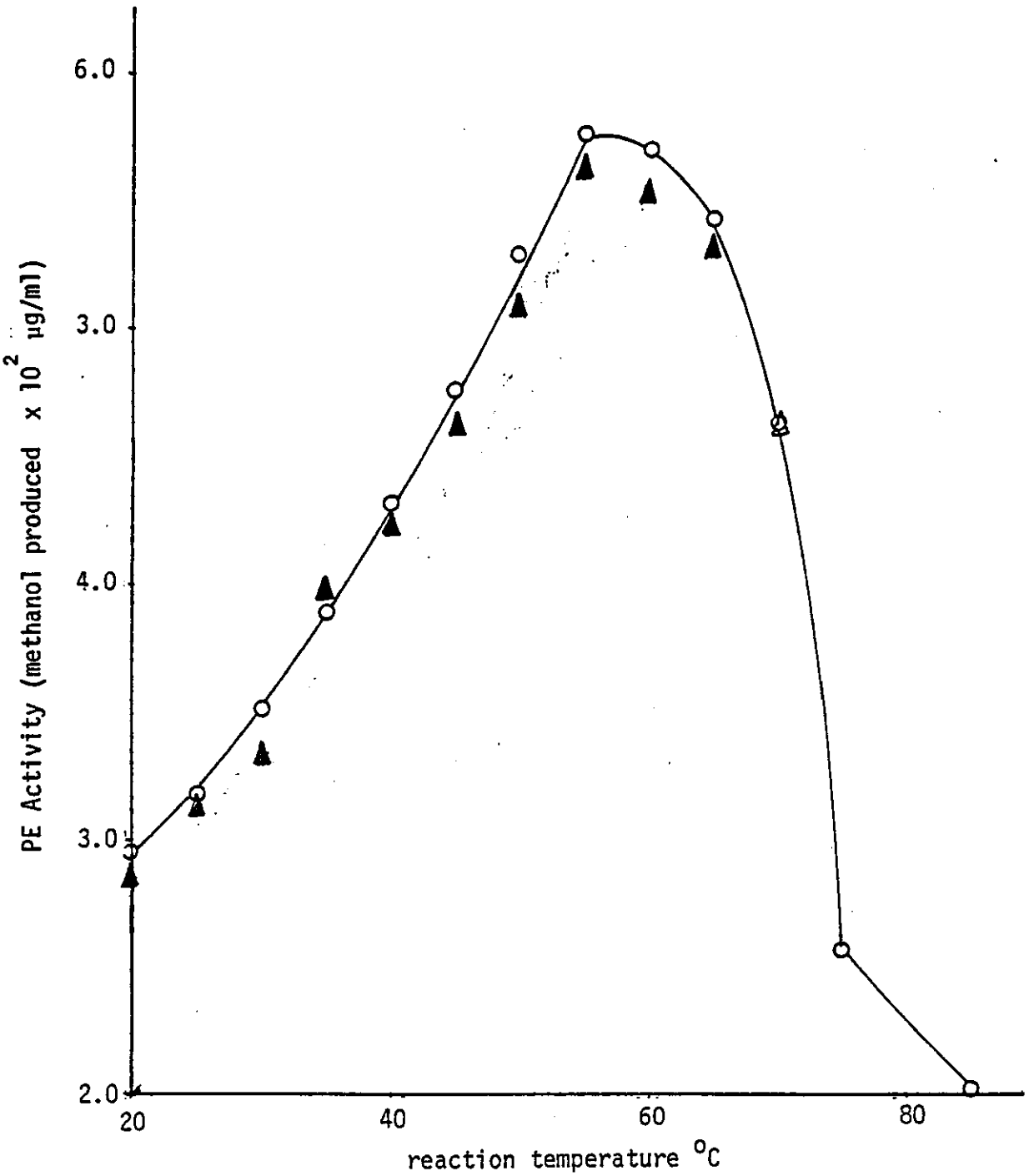

6.0
3.0
4.0
3.0
2.0
20
40
60
80
PE Activity (methanol produced x 10^2 µg/ml)
reaction temperature °C

FIGURE 21

EFFECT OF AGITATION ON ACTIVITY OF PE

The activity of PE de-methylation of pectin was compared on both the dialysis bag under an immobile condition and the dialysis cell under agitated conditions. The commercial PE and the culture dialysate of *Asp. niger* were used on a 1% pectin substrate. Incubation was at 35°C and samples were withdrawn from the reaction mixture at 3 hour intervals for 24 hours and analysed for methanol.

● Culture dialysate *(Asp. niger)* agitated

O Culture dialysate (*Asp. niger)* non-agitated

▲ Commercial PE agitated

Δ Commercial PE non-agitated

Each point is the mean ± S.D. of 5 determinations

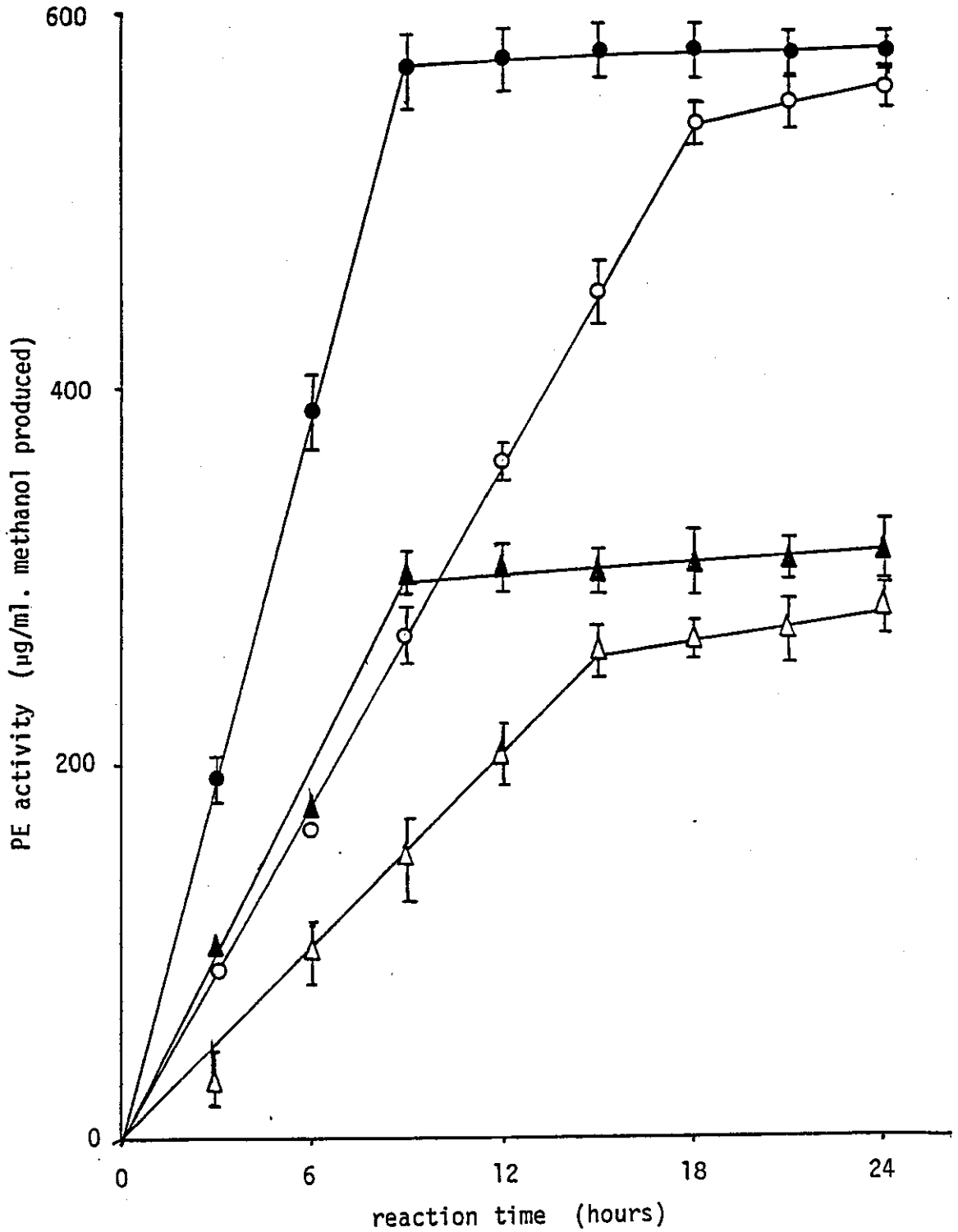

600
400
200
0
PE activity (µg/ml. methanol produced)
0
6
12
18
24
reaction time (hours)

FIGURE 22

RELATIONSHIP BETWEEN INITIAL pH OF INCUBATION AND EXTENT OF MYCELIAL GROWTH OF *ASP. FLAVUS* CMI 15959 SPORES

Two determinations were carried out on each of 3 cultures which were incubated at 30°C with shaking

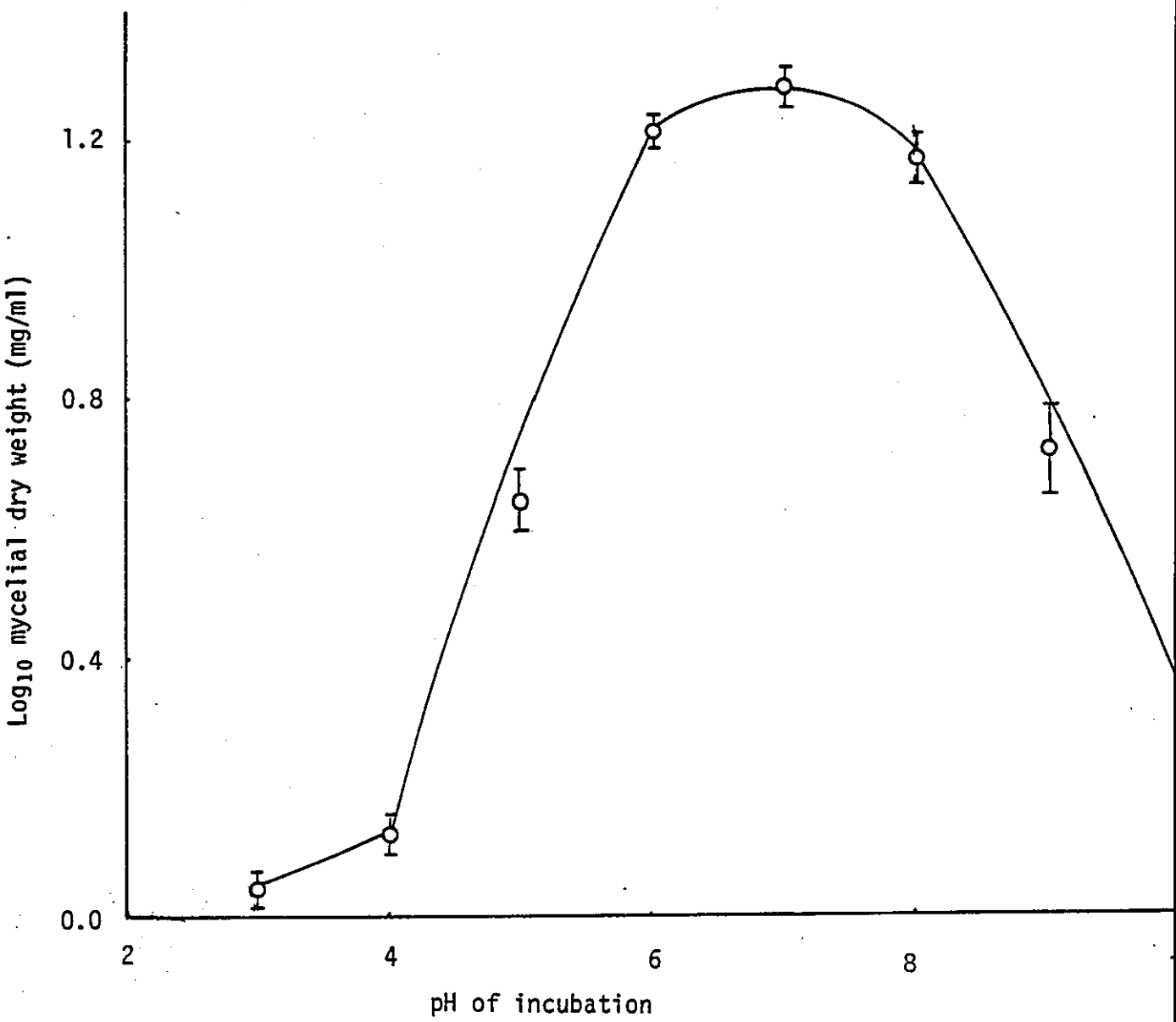

1.2
0.8
0.4
0.0
Log10 mycelial dry weight (mg/ml)
2
4
6
8
pH of incubation

FIGURE 23

RELATIONSHIP BETWEEN INCUBATION TEMPERATURE AND EXTENT OF MYCELIAL GROWTH OF *ASP. FLAVUS* CMI 15959 SPORES

Two determinations were carried out on each of 3 cultures (initial pH 7.0) which were incubated with shaking

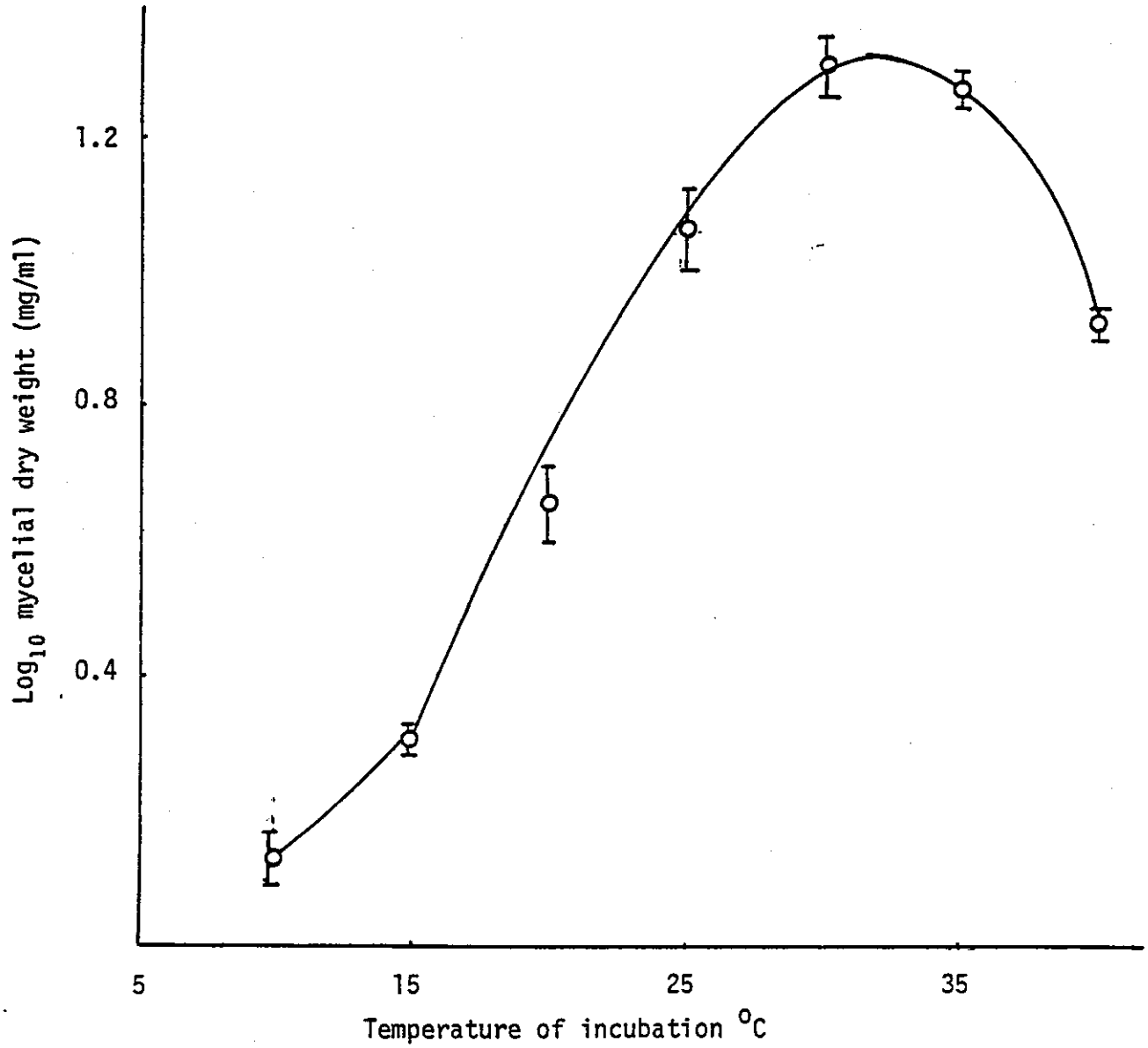
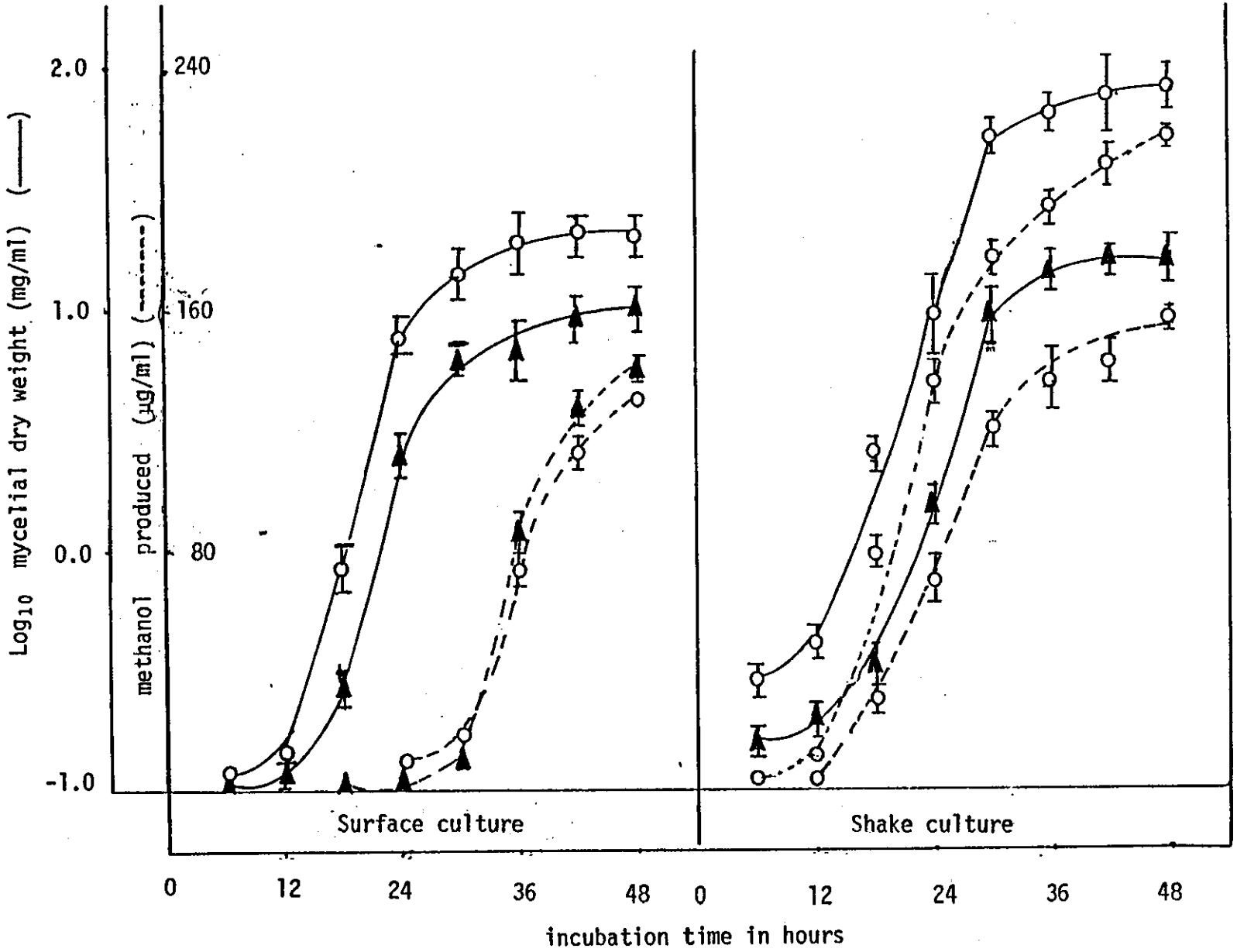

1.2
0.8
0.4
Log10 mycelial dry weight (mg/ml)
5
15
25
35
Temperature of incubation °C

FIGURE 24 EFFECT OF AERATION OF CULTURE MEDIA ON MYCELIAL GROWTH AND METHANOL PRODUCTION

50 ml portions of spore suspension (5 x 10^5 spores/ml) of *Asp. niger* (O) and *Asp. flavus* (▲) CMI 15959 were inoculated into 100 ml of assay medium at 30°C. Three cultures of each organism were shaken at 150 r.p.m. and three cultures were incubated under static conditions. The media were assayed for methanol and dry weight determinations were carried out every six hours up to 48 hours. Two determinations were carried out on each of 3 cultures. Control flasks inoculated with sterile distilled water were treated similarly.

The results show that the level of methanol production followed closely that of mycelial growth. In the shake cultures, methanol was detected just after 6 hours of incubation of *Asp. niger* and 12 hours of *Asp. flavus*. Methanol detection in surface cultures did not occur until after 18 hours of incubation and even then, methanol levels were relatively low. At $\log_{10}$ growth phase there was a linear increase in methanol production with time in both surface and shake cultures. No methanol could be detected in the control flasks.


2.0
1.0
0.0
-1.0
240
160
80
Log10 mycelial dry weight (mg/ml) (——)
methanol produced (μg/ml) (- - - - -)
Surface culture
Shake culture
0
12
24
36
48
incubation time in hours

From figs. 22 and 23, the optimum conditions for germination and growth of the fungi spores, under agitated consitions, are pH 6.5 - 7.5 and temperature 28^{o} - $34^{o}C$. But figs. 19, 20 and 21 show that optimum activity of PE is at pH 6.5 - 7.5, temperature 50^{o} - $65^{o}C$ and with agitation. However, since PE activity followed closely the growth rate of the fungi (fig. 24) and since the conditions for optimum growth of the spores are similar to conditions for higher PE activities of germinating spores encountered in the MPN method, subsequent work in determining PE activities in growing cultures was carried out with agitation at $30^{o}C$ and pH 7.0.

INFLUENCE OF GLUCOSE LEVEL ON MYCELIAL GROWTH AND METHANOL PRODUCTION

The effect of the glucose level present in the medium on mycelial growth and methanol production was determined.

50 ml suspensions of spores (*ca.* 10^5 spores/ml) were inoculated into 100 ml of pectin supplemented medium containing varying levels of glucose in the range 2 - 8%. The cultures were shaken at 30°C for 48 hours and samples were withdrawn at 6 hour intervals. Methanol and dry weight determinations were carried out in duplicate for each of the 3 cultures taken for each glucose level.

The results (fig. 25) show that the mycelial yields in all except *Asp. flavus* 86769 were highest at 6% glucose level but there were no significant differences between these and yields at 4 and 8% levels. *Asp. flavus* 86769 showed its highest mycelial growth at 8% level.

With the decrease in the glucose level, mycelial yields of the various organisms decreased gradually up to 4% then sharply at the 2% level.

Regarding methanol production at 2% level, only *Asp. luchuensis Inui* produced methanol from the 12th hour of incubation. From 4% to 8% level, methanol production followed very closely mycelial growth though level 6% was more favourable for methanol production by *Asp. nidulans* despite higher mycelial yields at 8% level.

An incubation time of 30 hours was chosen arbitrarily and the ratio of methanol produced (µg/ml) to mycelial dry weight (mg/ml) was plotted against glucose levels. The results (Fig. 26) show that there is a wide variation between organisms except at 4% glucose level.

FIGURE 25

INFLUENCE OF GLUCOSE LEVEL ON MYCELIAL GROWTH AND METHANOL PRODUCTION

● *Asp. flavus 15959*

▲ *Asp. flavus 39158a*

□ *Asp. flavus 86769*

Δ *Asp. niger*

0 *Asp. luchuensis Inui*

■ *Asp. nidulans*

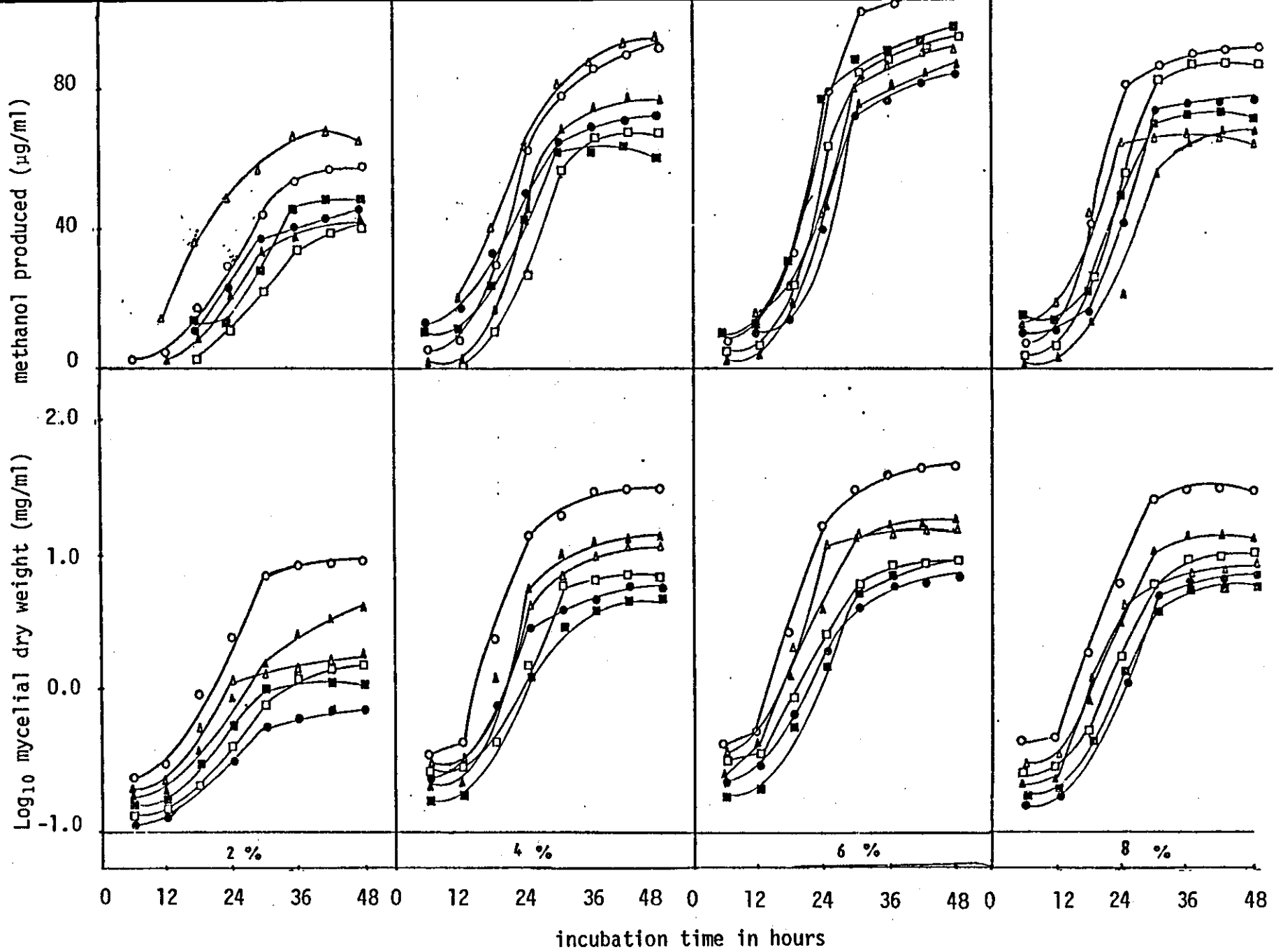

methanol produced (µg/ml)
80
40
0
Log10 mycelial dry weight (mg/ml)
2.0
1.0
0.0
-1.0
2 %
4 %
6 %
8 %
0 12 24 36 48
incubation time in hours

FIGURE 26

Plot of the ratio $\frac{\text{methanol produced } (\mu g/ml)}{\text{mycelial dry weight } (mg/ml)}$ after 30 hours of incubation against glucose level in the medium.

Symbols as defined in fig. 25.

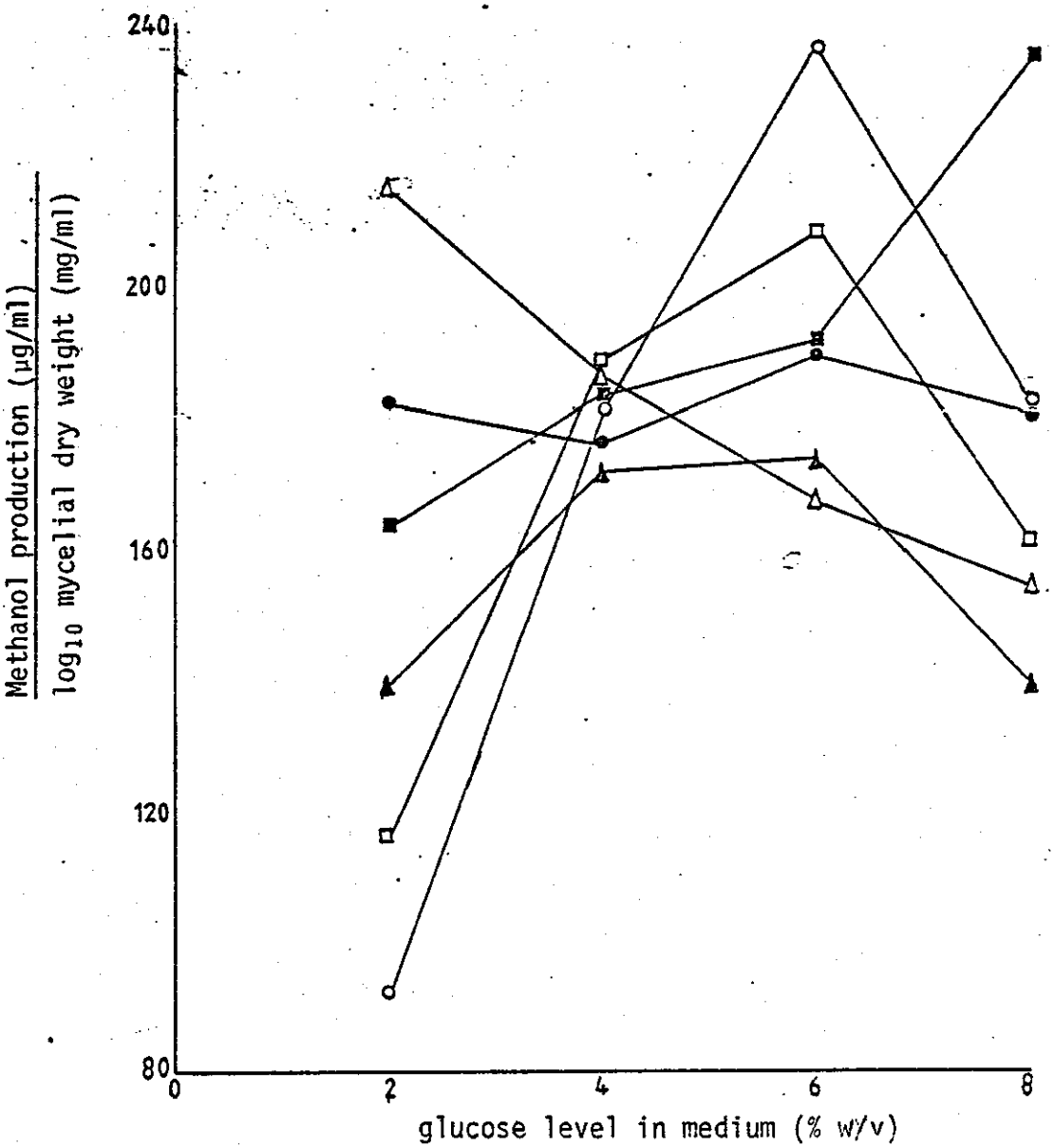

Methanol production (µg/ml)
log10 mycelial dry weight (mg/ml)
240
200
160
120
80
0
2
4
6
8
glucose level in medium (% w/v)

FIGURE 27

INFLUENCE OF PECTIN LEVEL IN CULTURE MEDIUM ON METHANOL PRODUCTION

50 ml of spore suspension of *Asp. niger* (5×10^4 spores/ml) and *Asp. flavus* 15959 (4.5×10^4 spores/ml) were inoculated into 100 ml of assay medium supplemented with pectin ranging from 0.001% to 10%. Cultures were grown at $30^{\circ}C$ for 24 hours with aeration. The results show that methanol production increased rapidly up to 0.5% pectin but then levels off. Methanol could not be detected in control systems containing no pectin.

O *Asp. niger*

▲ *Asp. flavus 15959*

Each point is the mean of 3 cultures, 2 determinations per culture.

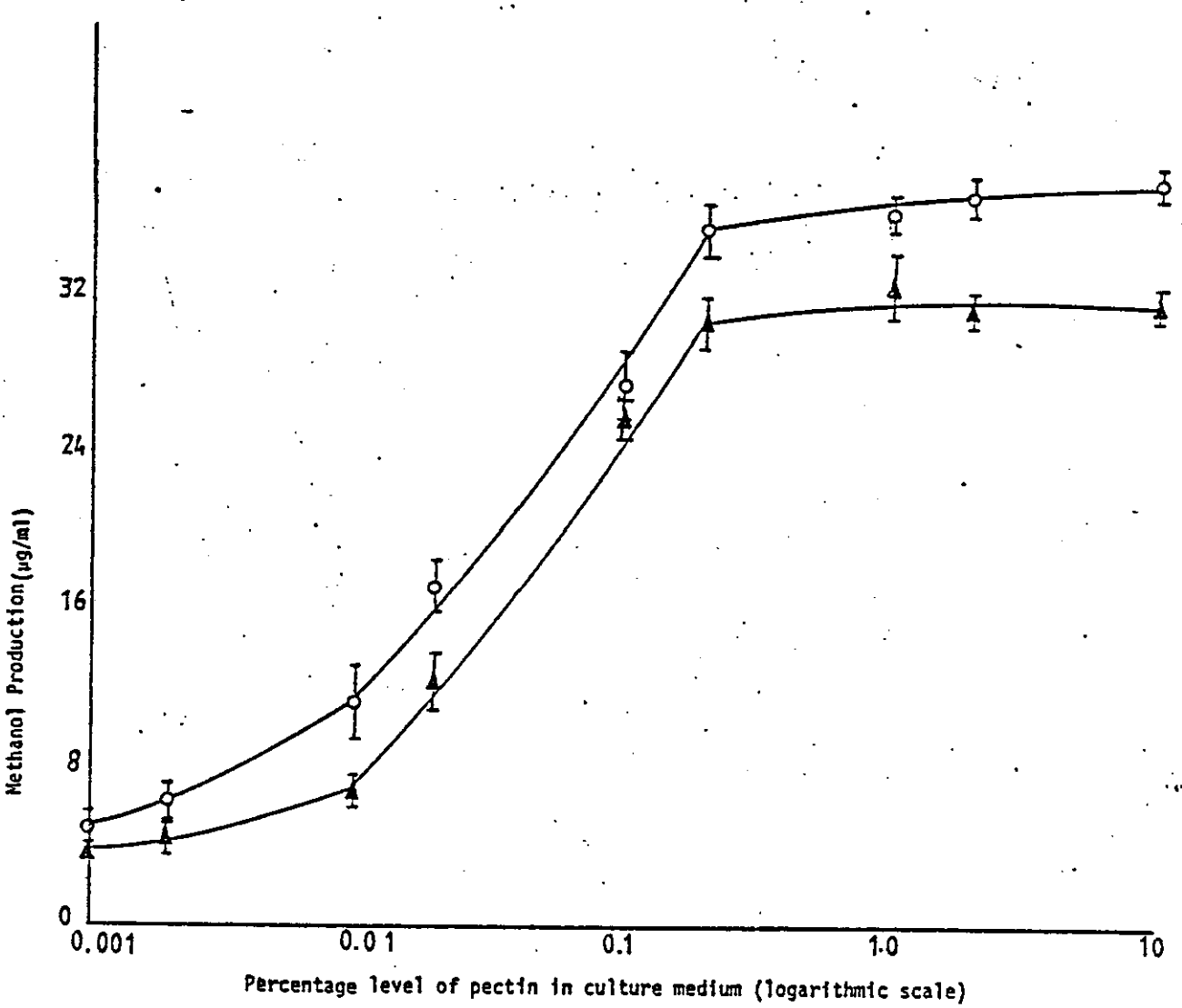

Methanol Production (µg/ml)
32
24
16
8
0
0.001
0.01
0.1
1.0
10
Percentage level of pectin in culture medium (logarithmic scale)

FIGURE 28

TIME COURSE FOR METHANOL PRODUCTION BY *ASP. FLAVUS 15959* (Δ) AND *ASP. NIGER* (0)

100 ml of *Asp. niger* (*ca.* 3 x 10^5 spores/ml) and *Asp. flavus* 15959 (*ca.* 2 x 10^5) were inoculated into 200 ml of the GPP medium. The cultures were incubated at 30^0C with shaking and methanol determinations were made at 6 hours interval.

Each point is the mean of 3 cultures, 2 determinations per culture.

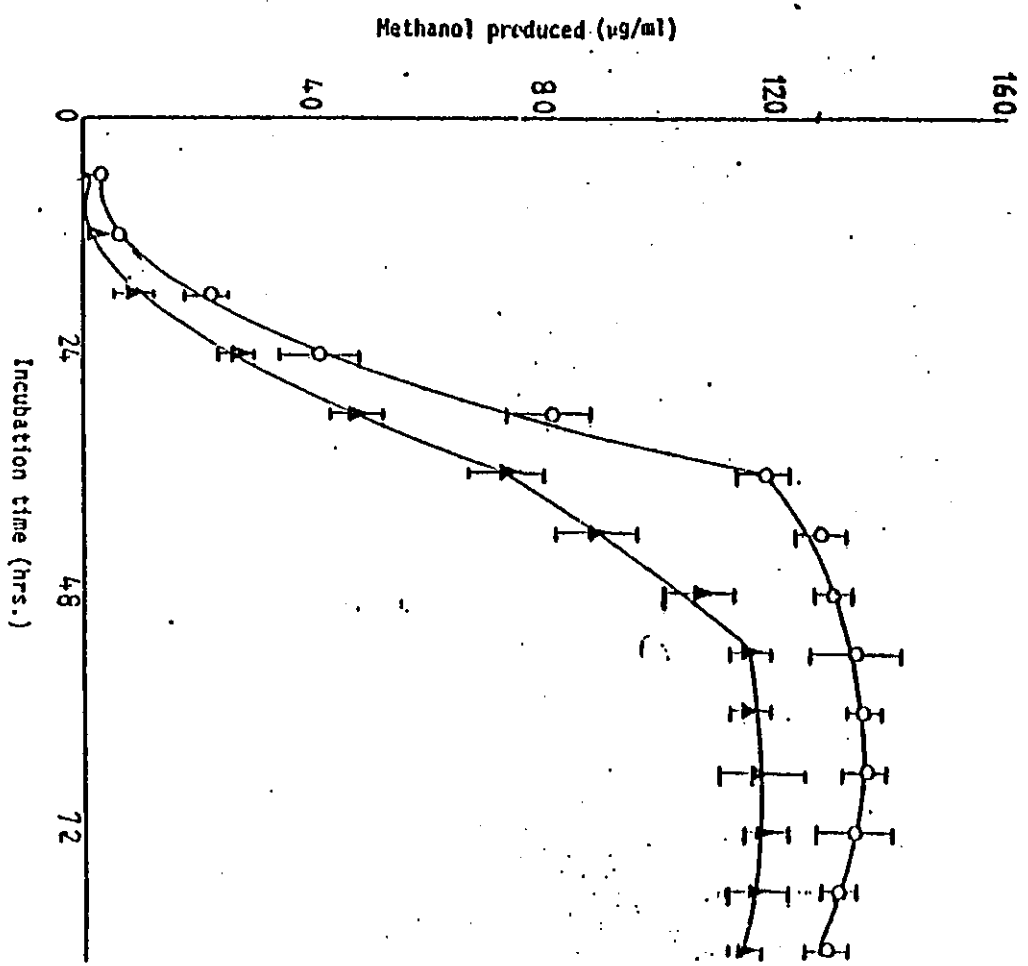

Methanol produced (µg/ml)
0
40
80
120
160
Incubation time (hrs.)
24
48
72

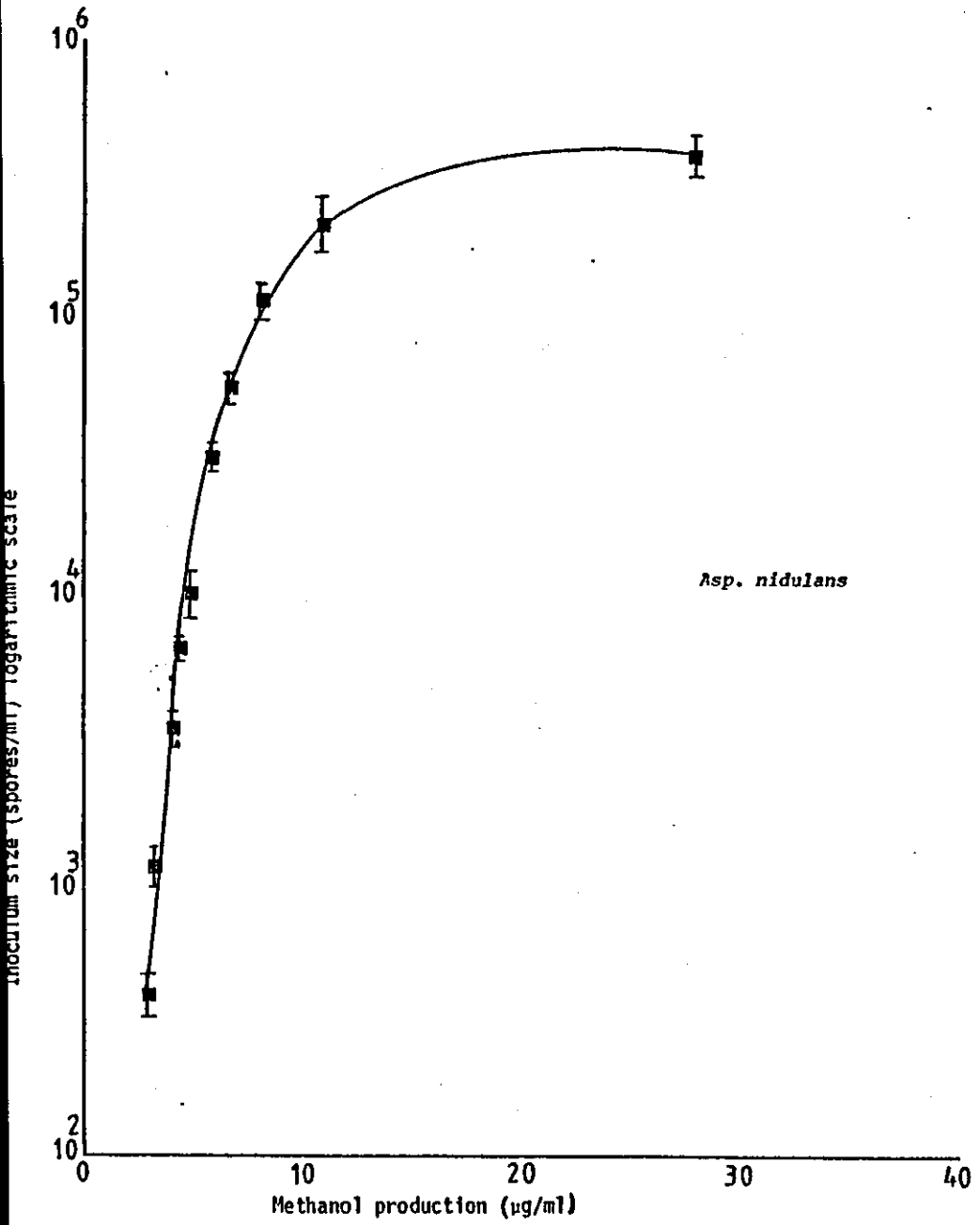
FIGURE 29 a, b, c

INFLUENCE OF VARIATIONS IN INOCULUM SIZE ON METHANOL PRODUCTION

The spore suspension of each of the 6 *Aspergillus* strains was diluted serially. 50 ml of each dilution was inoculated into 100 ml of the GPP medium and incubated with shaking in 500 ml conical flasks at 30°C for 15 hours. Methanol production was then determined for each culture. Individual plots were made for each organism.

Each point is the mean of 3 cultures, 2 determinations per culture.

FIGURE 29a


Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
40
Methanol production (µg/ml)
Asp. nidulans

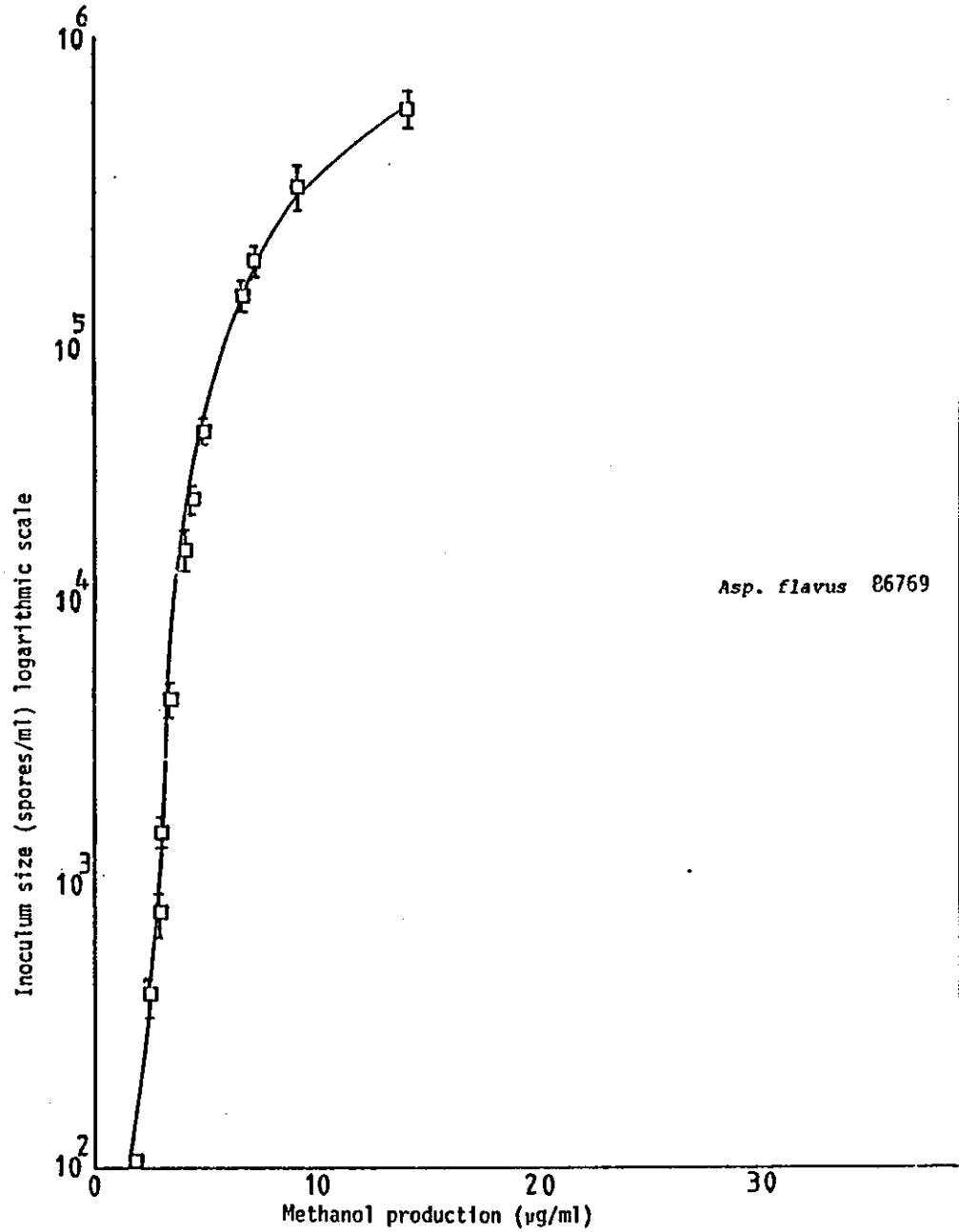
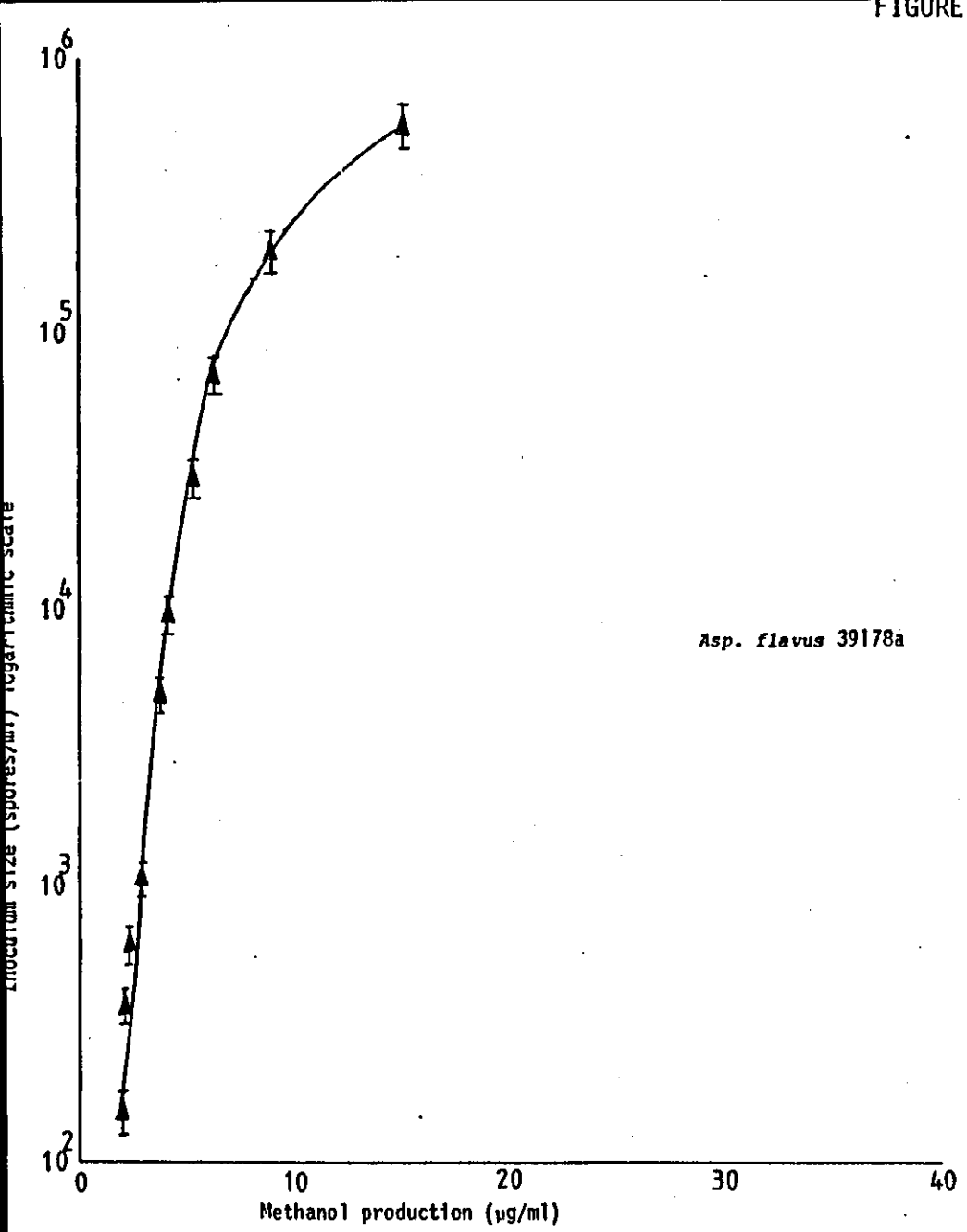

Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
Methanol production (µg/ml)
Asp. flavus 86769

FIGURE 29b


Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
40
Methanol production (µg/ml)
Asp. flavus 39178a

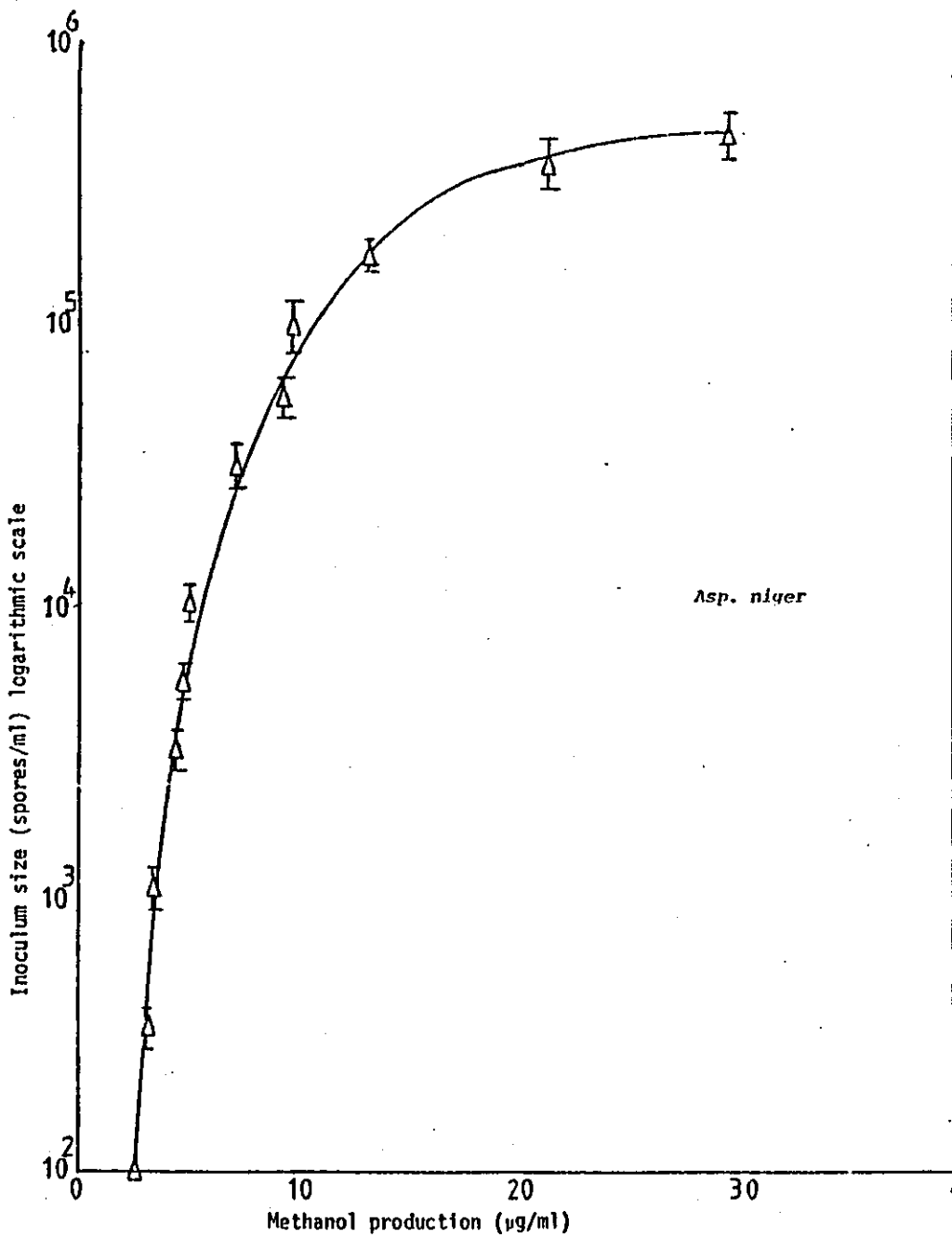
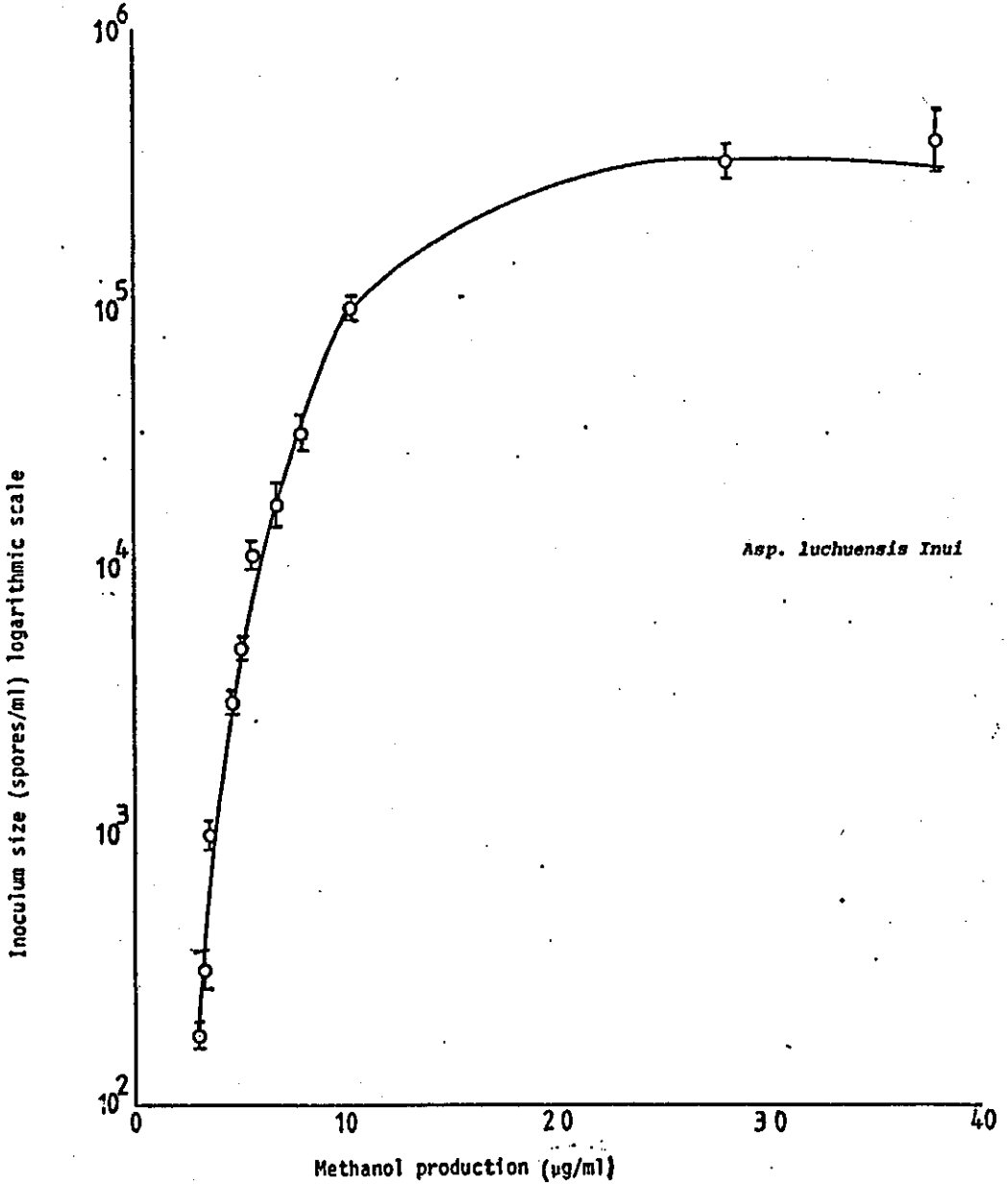
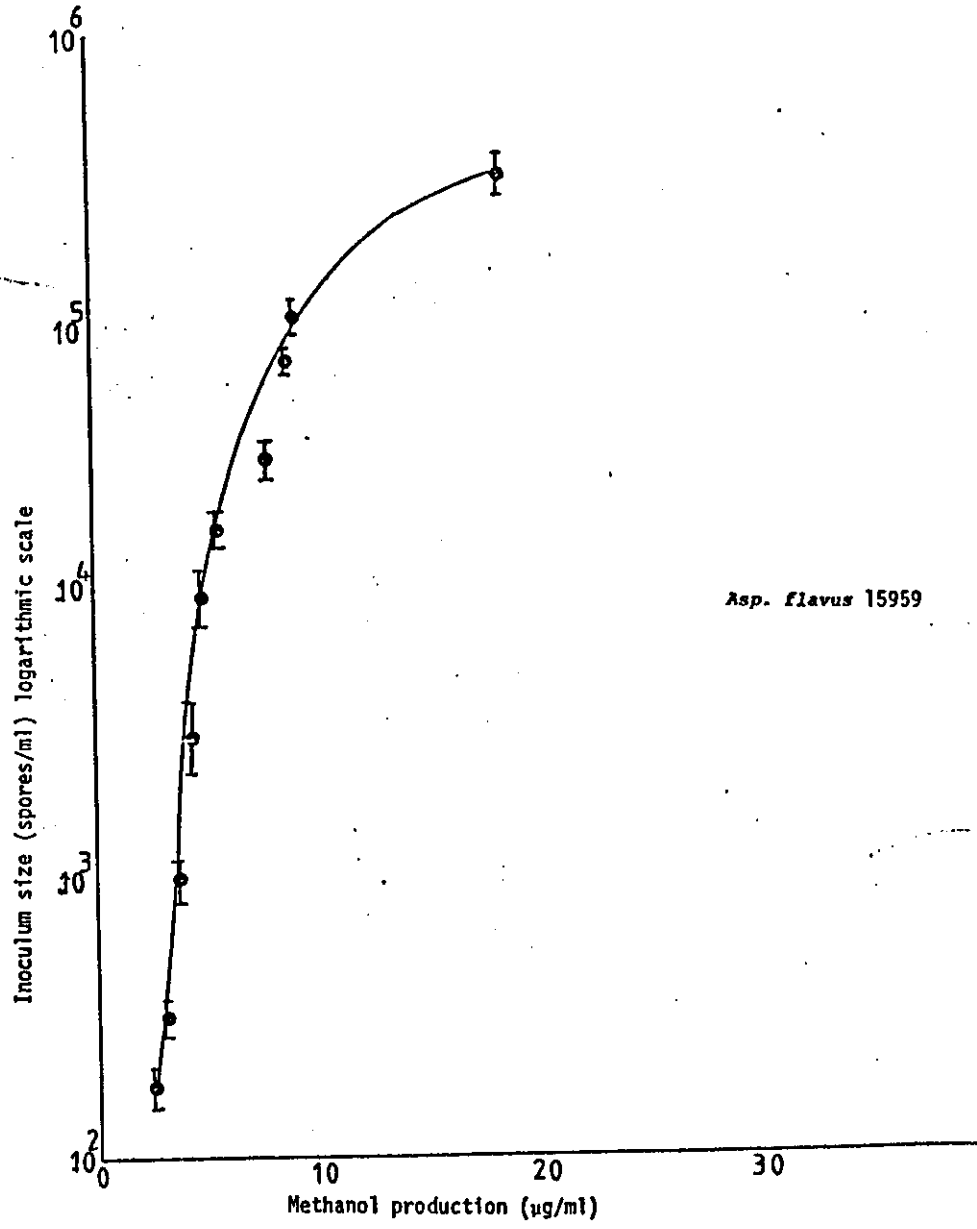

Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
Methanol production (µg/ml)
Asp. niger

FIGURE 29c


Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
40
Methanol production (µg/ml)
Asp. luchuensis Inui


Inoculum size (spores/ml) logarithmic scale
10^6
10^5
10^4
10^3
10^2
0
10
20
30
Methanol production (µg/ml)
Asp. flavus 15959

Suitable mathematical expressions were found to describe the curves in fig. 29 a, b, c, (see page 99) which when transformed, enabled a linear relationship to be established between inoculum size (spore numbers/ml) and the amount of methanol produced for each organism (Fig. 30 and table 6). Each organism exhibited a linear relationship, which was statistically found to be different from the others. To obtain a relationship of linearity that will hold true for all the organisms it was necessary to express methanol production per unit property of the cultures that is known to be a consistent property. Mycelial dry weight was considered appropriate and formed the basis of the next line of investigation.

FIGURE 30

Relationship between the $\log_{10}$ of $\log_{10}$ of inoculum size and the reciprocal of methanol produced by each of the *Aspergillus* strains after 15 hours of incubation.

O *Asp. luchuensis Inui*

▲ *Asp. flavus 39178a*

■ *Asp. nidulans*

● *Asp. flavus 15959*

Δ *Asp. niger*

□ *Asp. flavus 86769*

Each point is the mean of 5 determinations

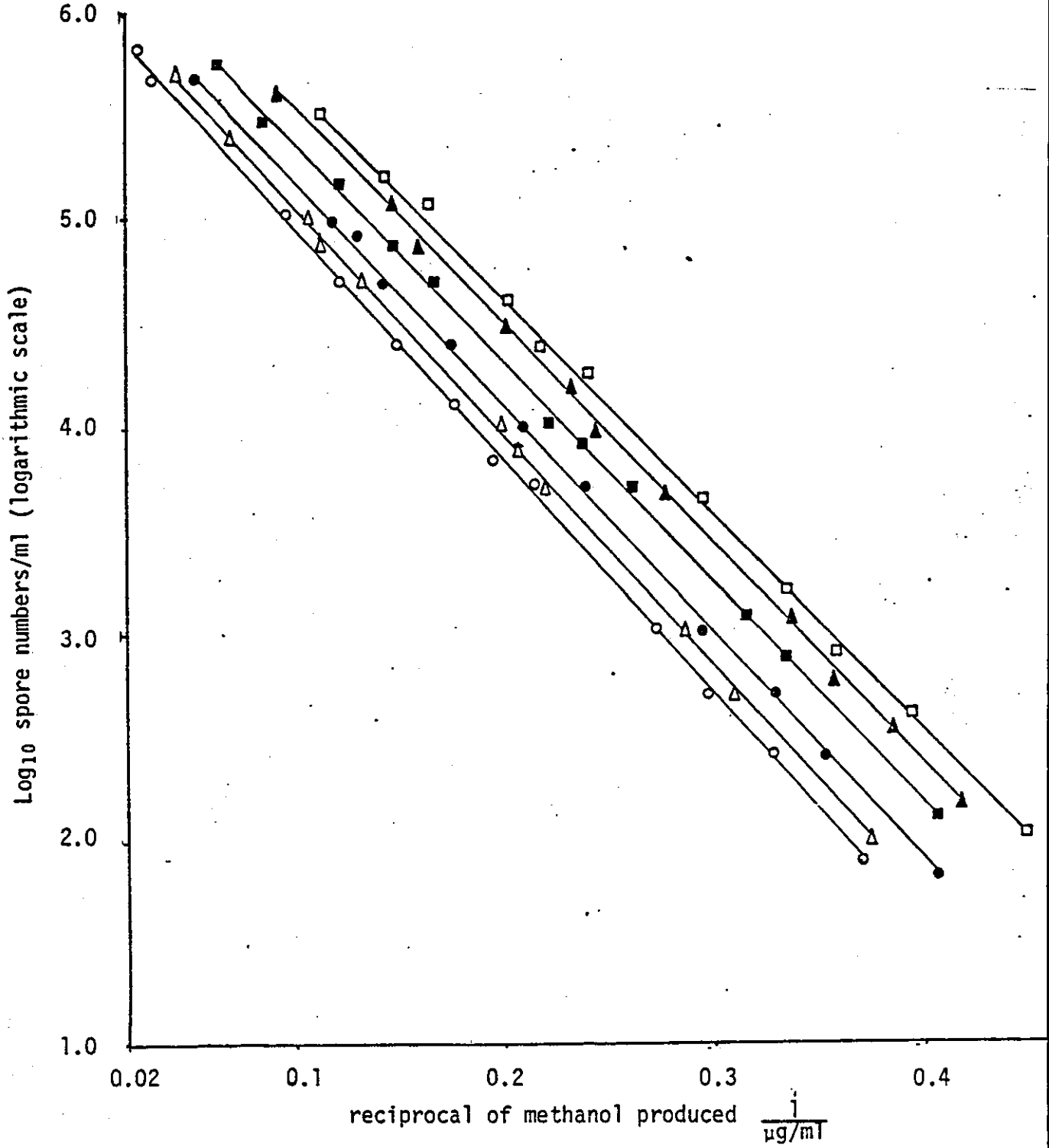

6.0
5.0
4.0
3.0
2.0
1.0
Log10 spore numbers/ml (logarithmic scale)
0.02
0.1
0.2
0.3
0.4
reciprocal of methanol produced 1/µg/ml

TABLE 6

Comparison of results between plots of $\log_{10}$ spore number/ml against $\log_{10}$ methanol production and plots of $\log_{10}$ of $\log_{10}$ spore number/ml against the reciprocal of methanol production.

ORGANISM	Test of the relationship $Y = C + \beta (\log X)$				Test of the relationship $Y = c\, e^{-\beta/X}$			
	Slope	Intercept	Corr. Coeff	Std. Dev.	Slope	Intercept	Corr. Coeff.	Std. Dev.
Asp. flavus 39178a	4.709	0.861	0.898	0.358	-1.228	0.883	-0.946	0.023
Asp. niger	3.963	0.917	0.925	0.338	-1.328	0.842	-0.952	0.021
Asp. flavus 15959	3.625	1.204	0.939	0.493	-1.305	0.851	-0.921	0.026
Asp. luchuensis Inui	2.891	1.615	0.893	0.395	-1.275	0.816	-0.914	0.016
Asp. flavus 86769	5.905	0.307	0.916	0.216	-1.249	0.913	-0.942	0.02
Asp. nidulans	4.287	1.035	0.904	0.290	-1.160	0.853	-0.937	0.011

Influence of Variations in Inoculum size on Methanol Production Per Unit Mycelial Dry Weight (*Aspergillus* strains)

The master suspension for each organism was randomly diluted to give spore suspensions in the range 10 - 7 X 10^5 spores/ml. Each suspension was agitated vigorously in a shaker for 20 minutes to break up clumps. The following procedure was then carried out on each sample:

(a) Total count by the Helber Counting Chamber technique.

(b) Viable counts were determined by surface plate counts using medium 42. Each suspension was counted 15 times.

(c) The dry weight of spores in each suspension was determined.

(d) 50 ml portions of the spore suspension were inoculated aseptically into 100 ml of the GPP medium. The mixture was then shaken in 500 ml conical flasks for 15 hours at $30^{o}C$.

(e) Dry weight measurements were carried out on each culture at 15 hours of incubation, i.e. concurrently with methanol determinations. Methanol production was then described as µg/ml per mg. dry weight of mycelium.

Two curves were prepared for each organism with the information obtained from these experiments: (1) A curve that related spore numbers to methanol production and (2) a curve that related dry weight of spore inoculum to methanol production. From the total count and viable count of each spore suspension, the percentage viability was calculated and used to calculate the dry weight of spore material due to viable spores in each suspension. Since methanol production is considered to be a function of the viable spores, the viable figures were used in the cell plots.

Figures 31a,b,c, give the plots for the individual organisms using the relationship between dry weight of spore inoculum and methanol production. The correlation coefficient for each curve (R values) was highly significant. Plots were also made for the individual organisms using the relationship between viable spore numbers in the inoculum and methanol production.

The slopes, intercepts and correlation coefficients for each set of results are shown in table 7. For each relationship the 6 regression lines of each set of plots were compared by the principle of "Comparison of Several Regression Lines" (Wilsdon 1934)(see page 126) and found to be estimates of the same theoretical line.

This was confirmed by the complete plots of the mean values of all six organisms taken after calculating the mean values for either spore dry weight/ml or viable spore number/ml. The results shown in Figures 32 and 33 are of straight lines with correlation coefficients which are highly significant.

Figures 32 and 33 represent standard calibration curves given by the formulae

$$\text{(Fig. 32)}\quad \log_{10}(\text{dry wt. mg/ml}) = 1.078 - 6.73\tfrac{1}{X} \quad \ldots\ldots\ldots (1)$$

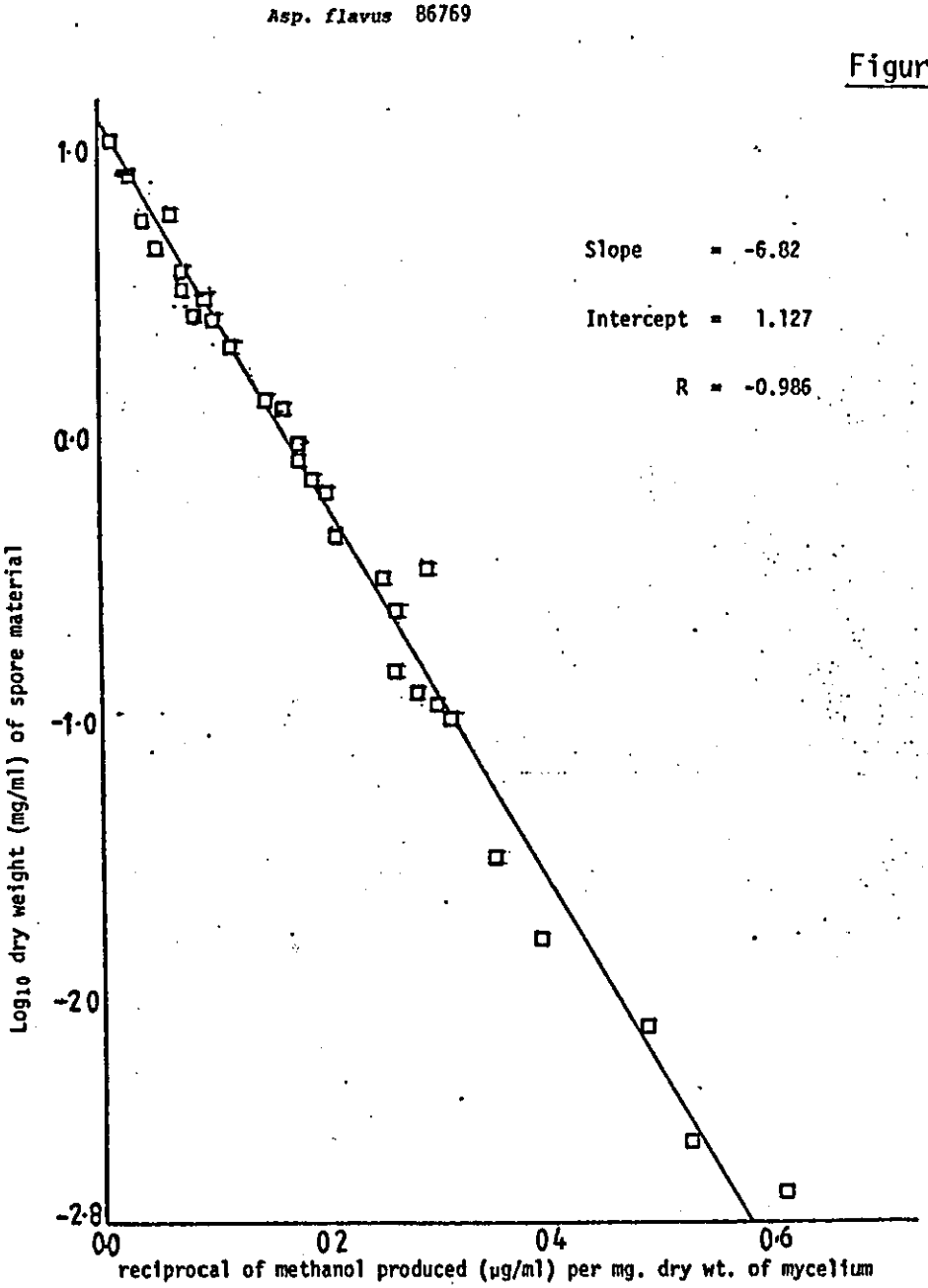
$$\text{(and Fig. 33)}\quad \log_{10} \text{ of } \log_{10}(\text{viable spore number/ml}) = 0.706 - 0.739X \ldots (2)$$

FIGURE 31a, b, c

INFLUENCE OF VARIATIONS IN INOCULUM SIZE ON METHANOL PRODUCTION PER UNIT MYCELIAL DRY WEIGHT (*ASPERGILLUS* STRAINS).

Each point is the mean of 6 determinations

Figure 31a


Asp. flavus 86769
Slope = -6.82
Intercept = 1.127
R = -0.986
1·0
0·0
-1·0
-2·0
-2·8
0·0
0·2
0·4
0·6
Log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

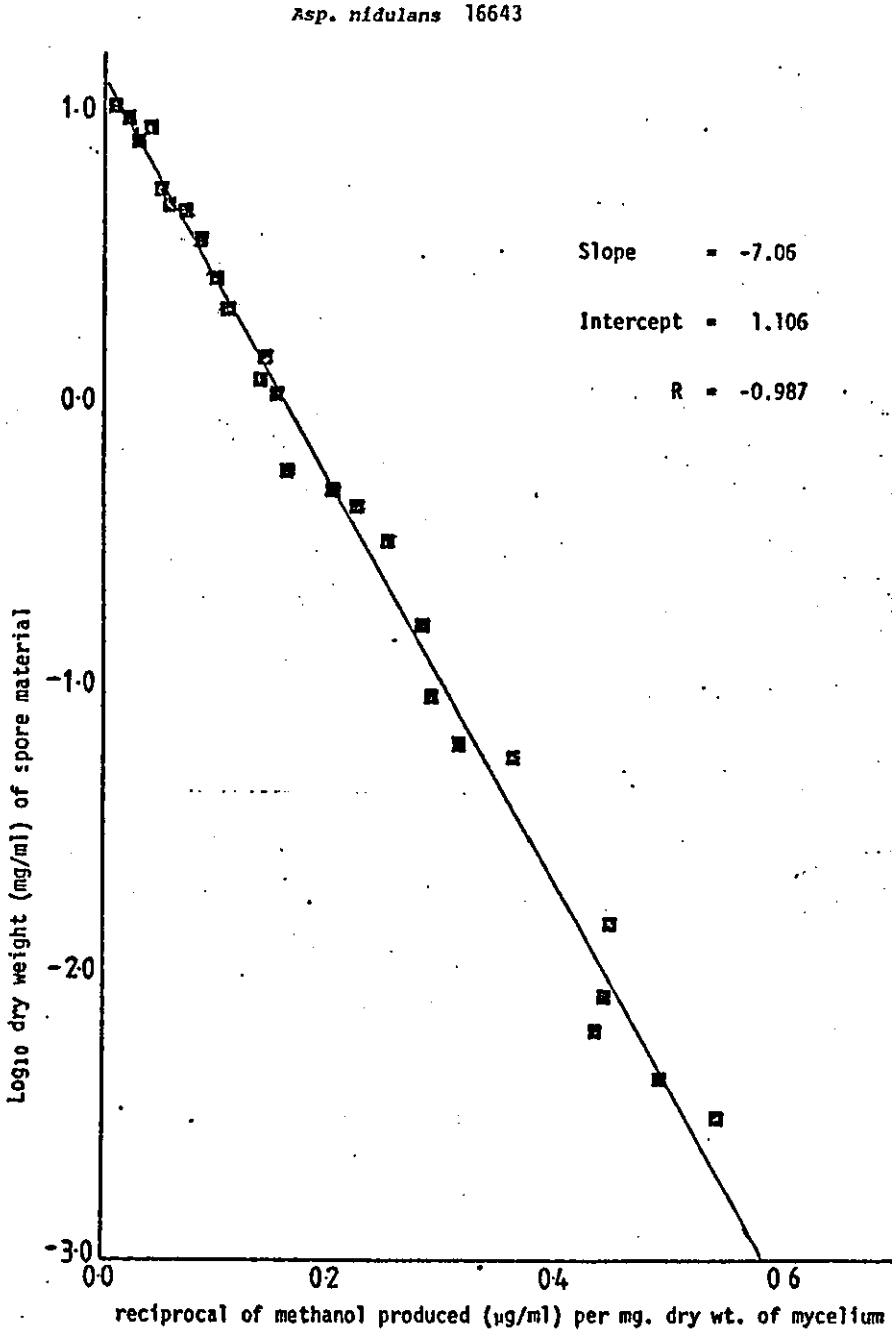
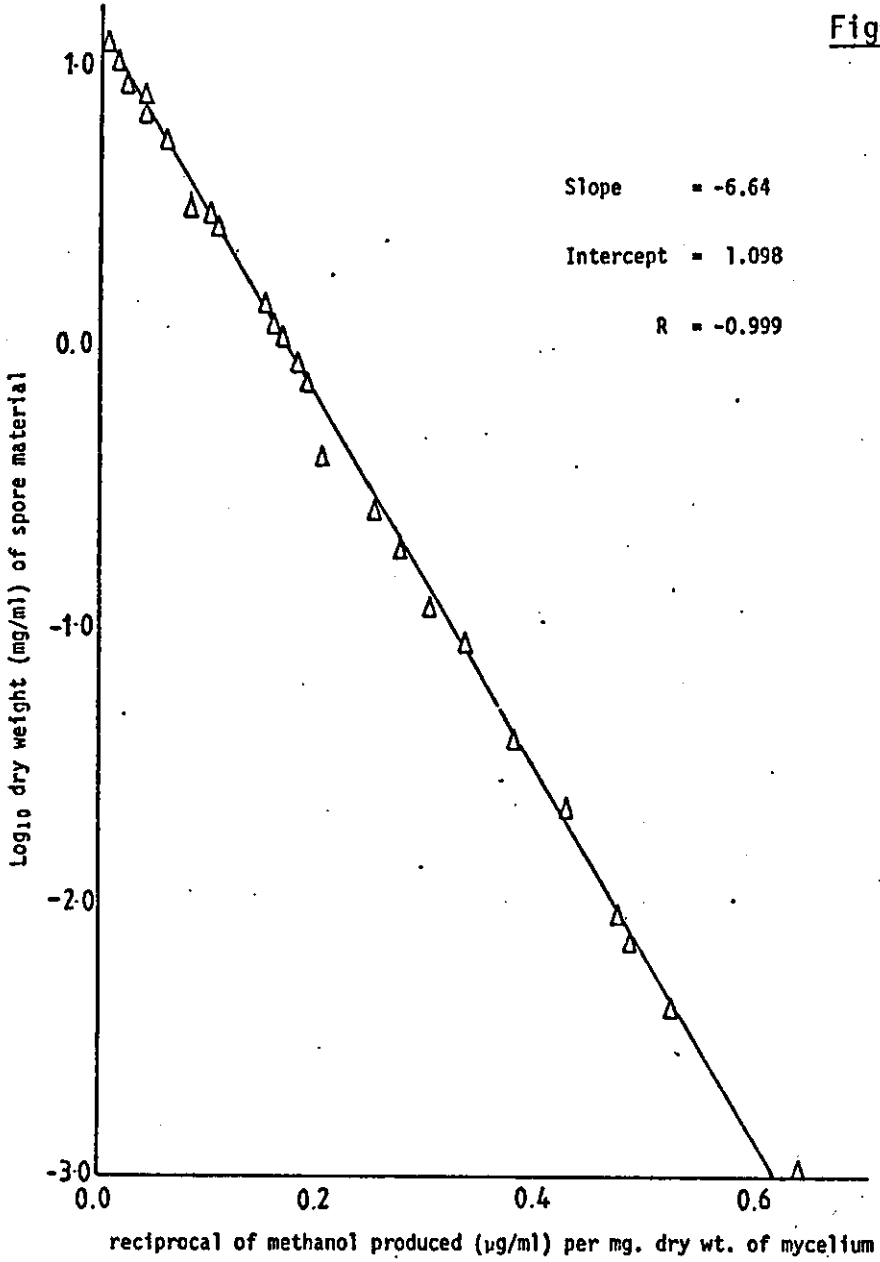
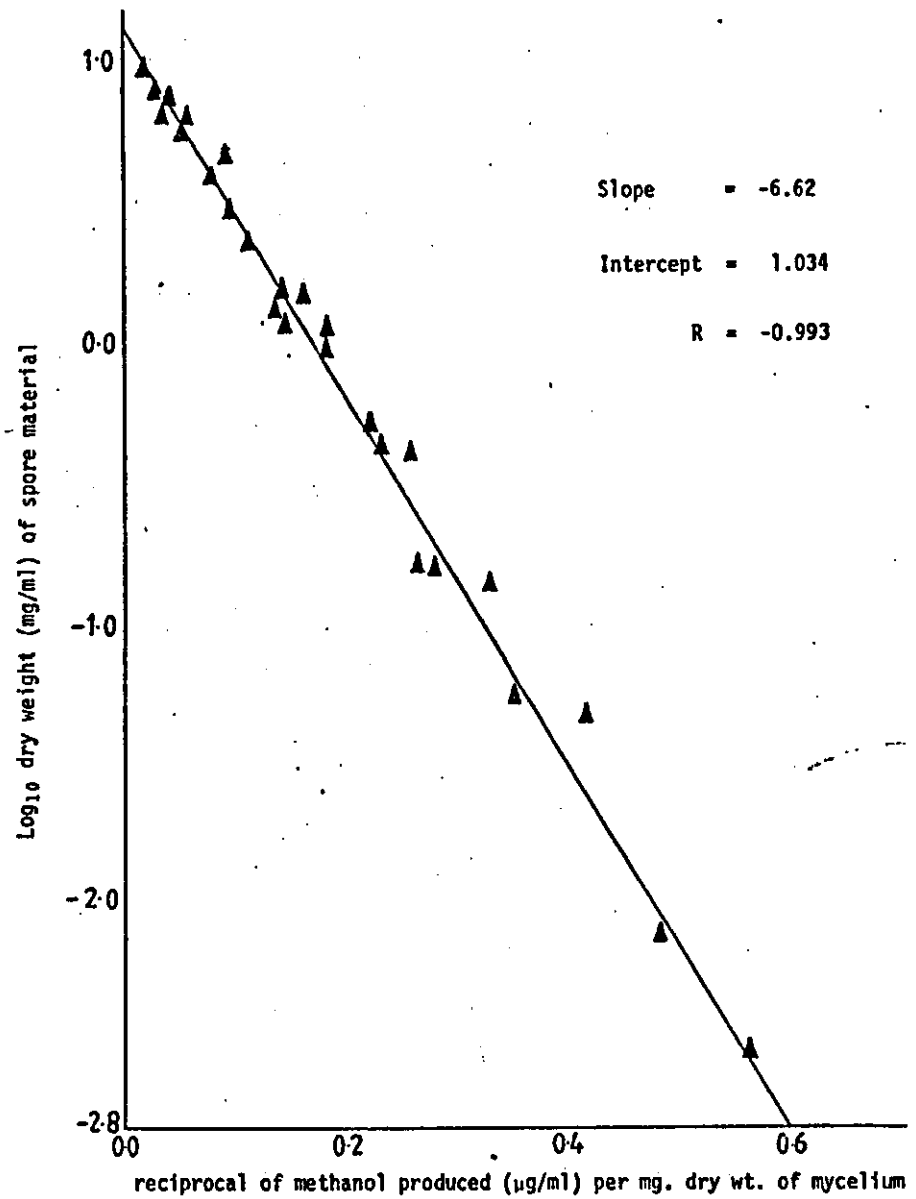

Asp. nidulans 16643
Slope = -7.06
Intercept = 1.106
R = -0.987
1·0
0·0
-1·0
-2·0
-3·0
0·0
0·2
0·4
0·6
Log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

Figure 31b

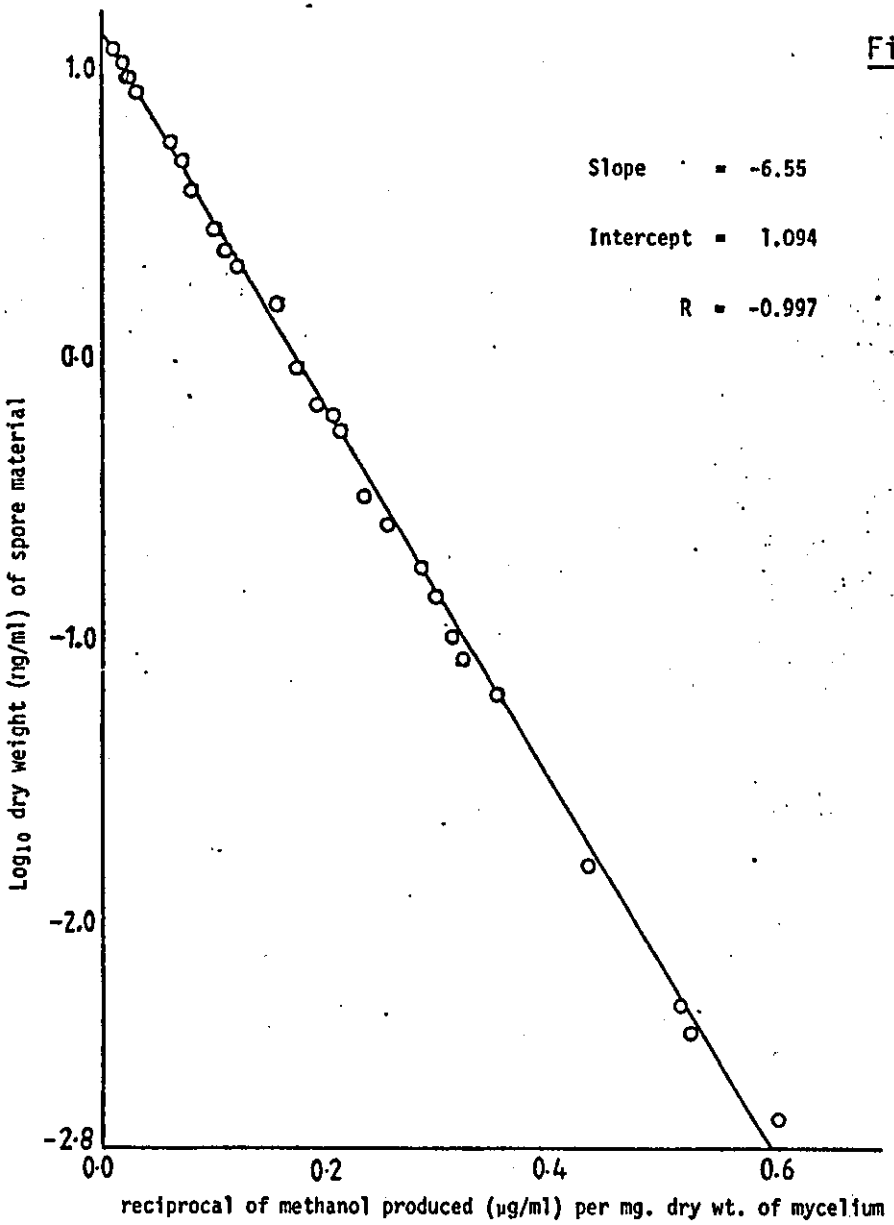


Asp. flavus 39178a



Asp. luchuensis Inui 83356

Figure 31C



Asp. flavus 15959

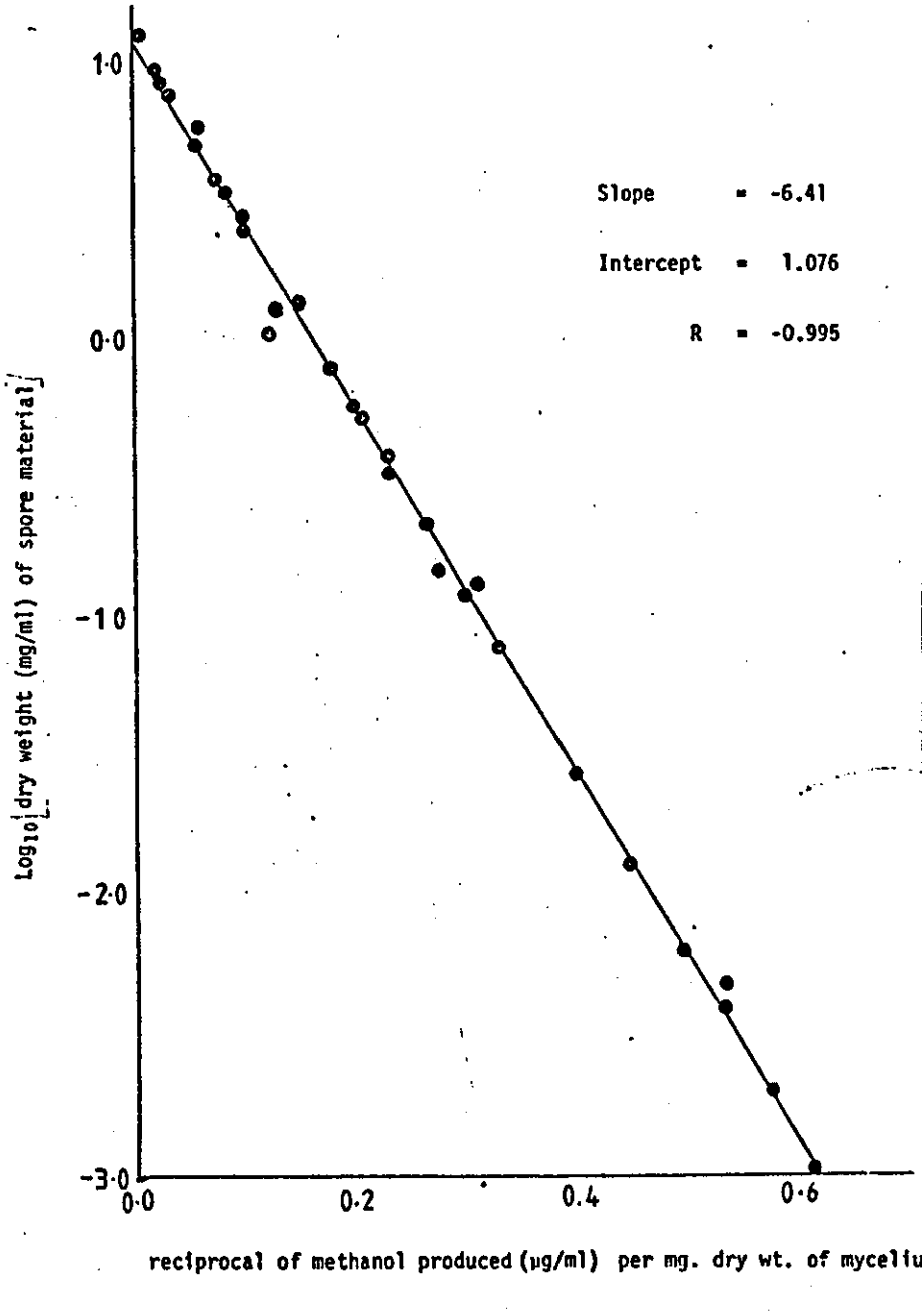


TABLE 7

Comparison of results between the $\log_{10}$ dry weight vs. reciprocal of methanol production and $\log_{10}$ of $\log_{10}$ spore number/ml vs. reciprocal of methanol production.

ORGANISM	$\log_{10}$ dry wt. Reciprocal Methanol			$\log_{10}$ of $\log_{10}$ spore numbers/ml. vs. reciprocal methanol		
	Slope	Intercept	Corr.Coeff	Slope	Intercept	Corr.Coeff.
Asp. niger	-6.64	1.098	0.999	-0.725	0.706	-0.968
Asp. luchuensis Inui	-6.55	1.094	-0.997	-0.712	0.704	-0.984
Asp. nidulans	-7.06	1.106	-0.987	-0.745	0.710	-0.973
Asp. flavus 39178a	-6.62	1.034	-0.993	-0.741	0.701	-0.975
Asp. flavus 86769	-6.82	1.127	-0.986	-0.733	0.703	-0.979
Asp. flavus 15959	-6.41	1.076	-0.995	-0.721	0.704	-0.976
All 6 organisms	-6.727	1.078	-0.988	-0.739	0.706	-0.963

FIGURE 32

COMPOSITE GRAPH FOR ALL 6 *ASPERGILLUS* STRAINS

A standard curve plotted between $\log_{10}$ (dry wt. of *Aspergillus* spore material) (y) and the reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium after 15 hours of incubation (x).

$y = 1.078 - 6.73x$; Coefficient of correlation (R) $= -0.988$.

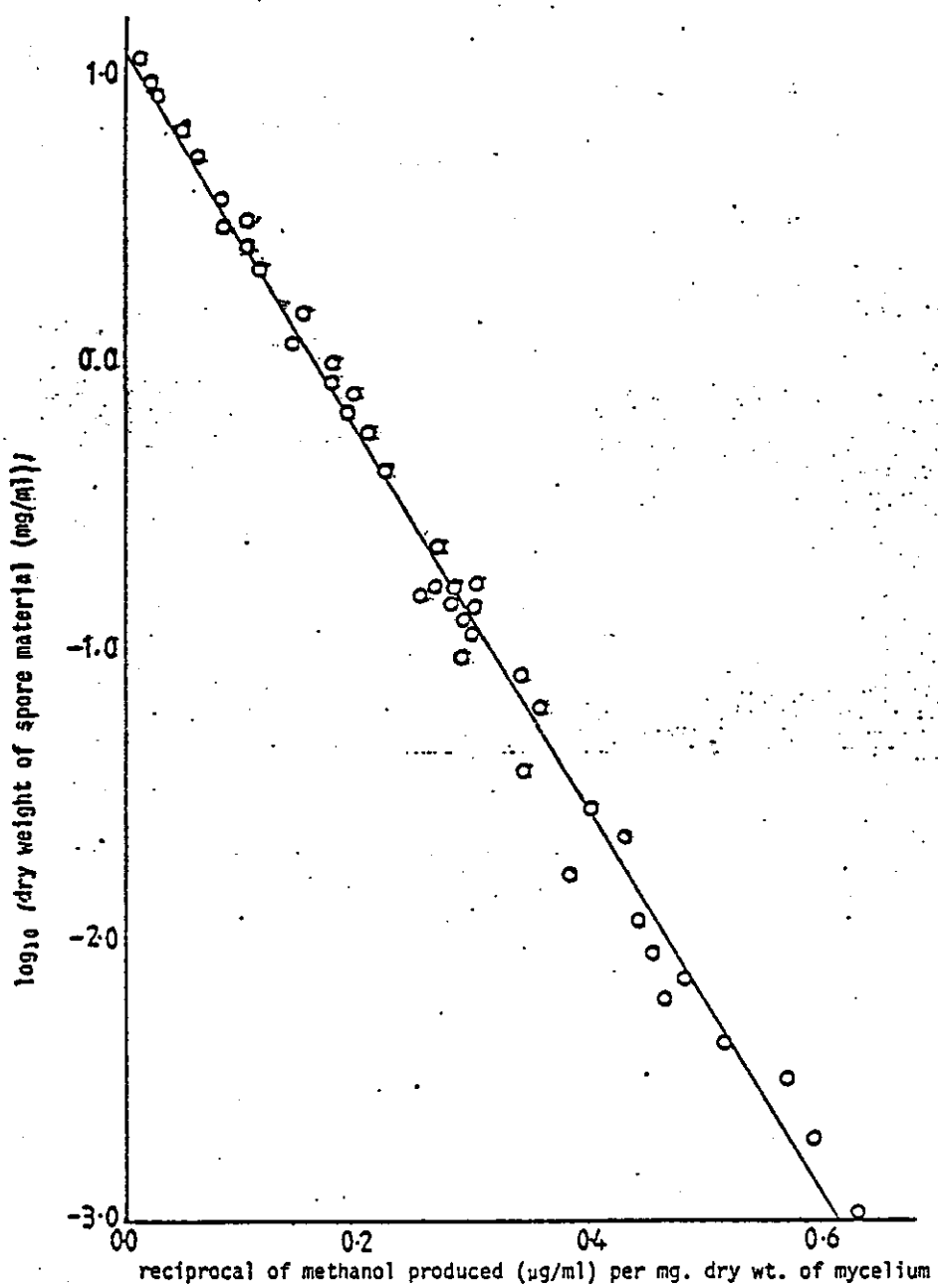

1·0
0·0
-1·0
-2·0
-3·0
log10 (dry weight of spore material (mg/ml))
0·0
0·2
0·4
0·6
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

FIGURE 32A

CALIBRATION CURVE FOR DETERMINING SPORE NUMBERS FROM DRY WT. OF VIABLE SPORES OF *ASPERGILLUS* STRAINS

A calibration curve plotted between $\log_{10}$ dry weight of spore material (mg/ml) (y) and $\log_{10}$ spore number/ml (x)

$y = 0.915X - 3.912$; Coefficient of Correlation (R) = 0.996

Each point is the mean of 5 determinations

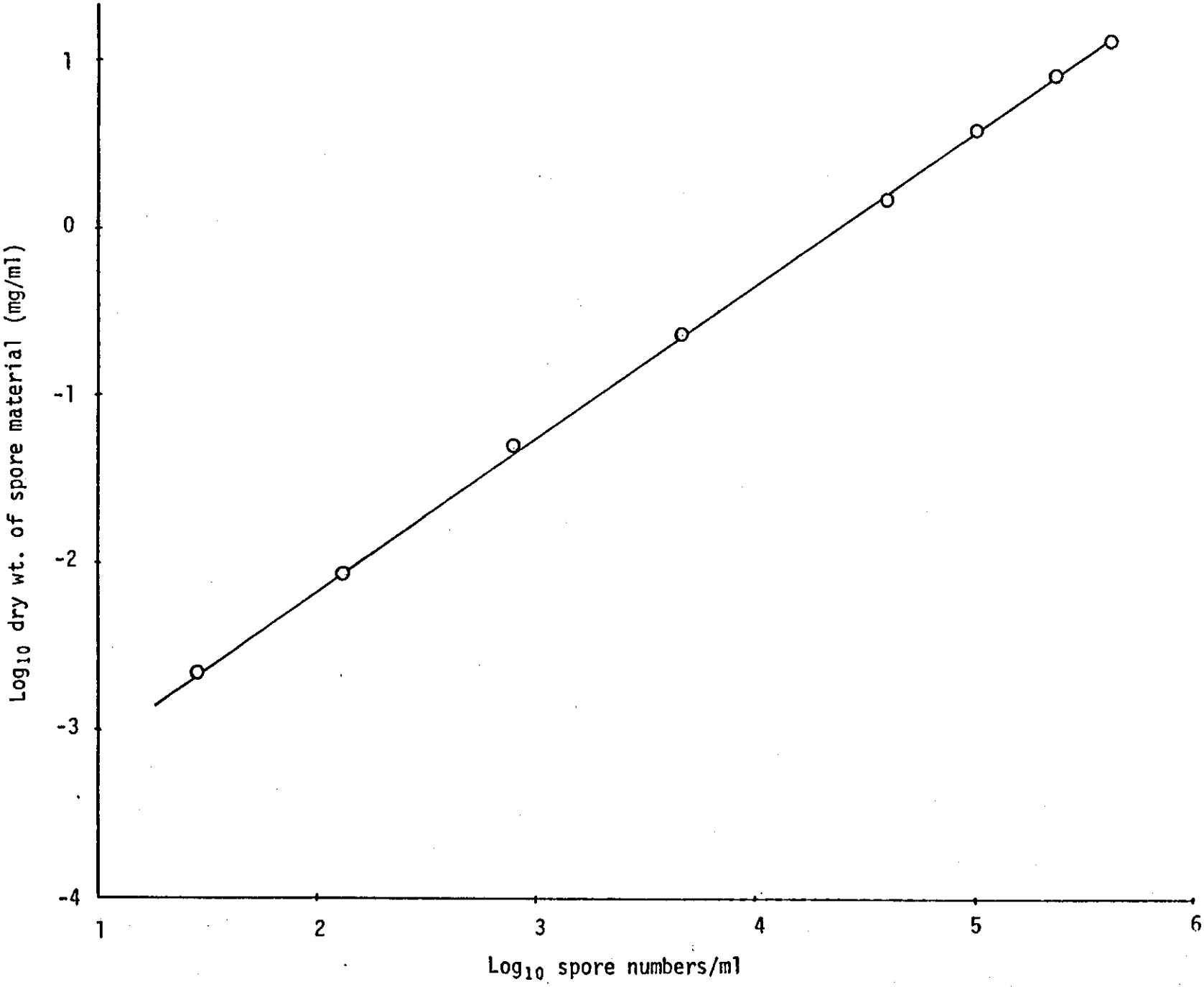
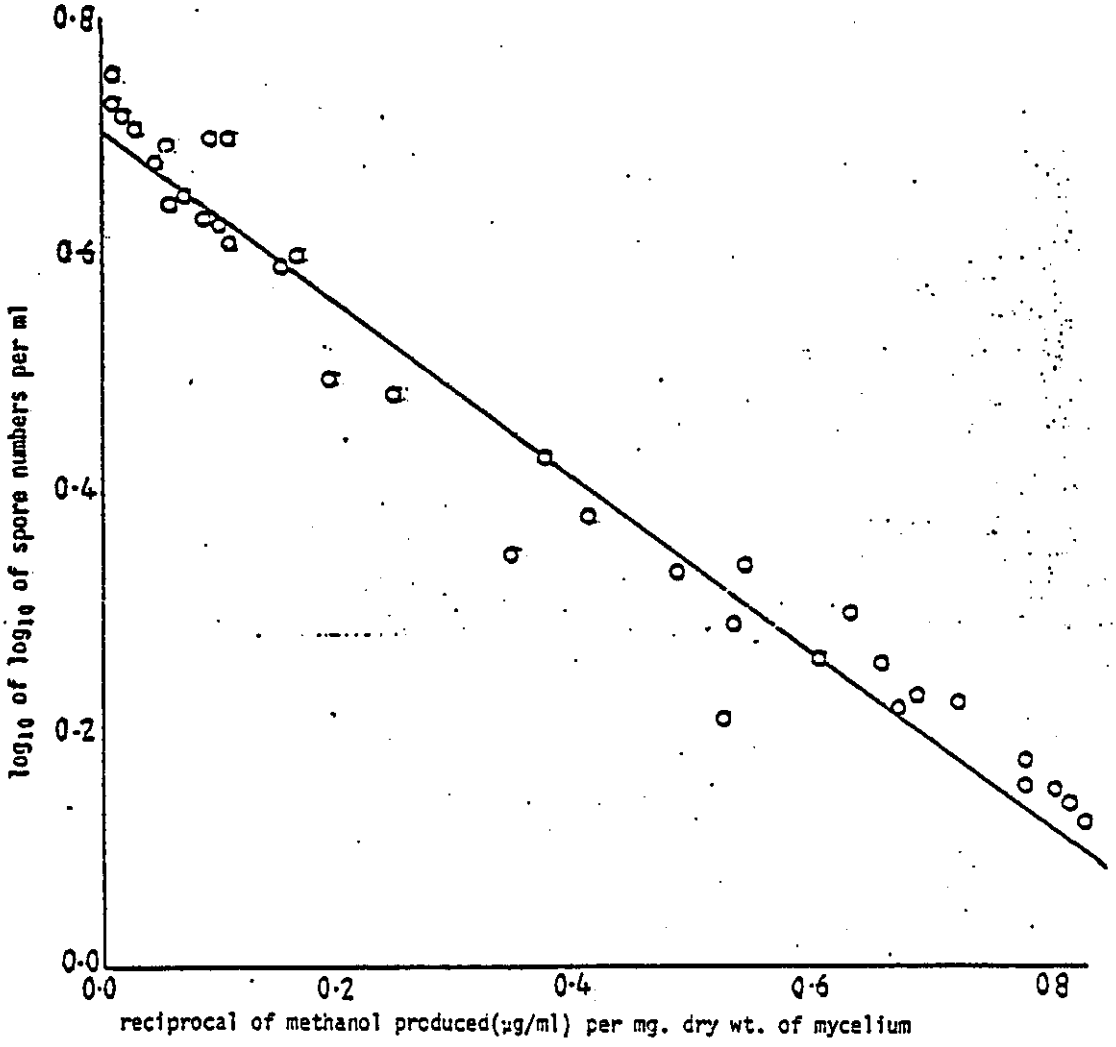

1
0
-1
-2
-3
-4
1
2
3
4
5
6
Log10 dry wt. of spore material (mg/ml)
Log10 spore numbers/ml

FIGURE 33

COMPOSITE GRAPH FOR ALL 6 *ASPERGILLUS* STRAINS

A standard curve plotted between $\log_{10}$ of $\log_{10}$ of spore number per ml. (y) and the reciprocal of methanol produced (µg/ml) per mg. dry weight of mycelium after 15 hours of incubation (x).

$y = 0.706 - 0.739X$; Coefficient of Correlation (R) = -0.963


0·8
0·6
0·4
0·2
0·0
0·0
0·2
0·4
0·6
0·8
log10 of log10 of spore numbers per ml
reciprocal of methanol produced(µg/ml) per mg. dry wt. of mycelium

Test of the Methanol Method for Estimating Unknown Concentrations of Spore Suspensions

The suitability of either of the above expressions in determining the concentration of a spore suspension was tested as follows: Two suspensions of each of the six organisms were prepared by random dilutions. In addition two suspensions were prepared by mixing spores of all six organisms at random. Viable counts were then made of each suspension using the following methods:

(a) Surface plate count

(b) Use of expression (1)

(c) Use of expression (2)

When using expression (1) a calibration curve of viable spore number/ml and dry weight due to viable spores was necessary. (Fig. 32A). The results are shown in table 8.

TABLE 8

Comparison of Estimates of Spore Numbers in Spore Suspensions by the Surface Plate Method and the Use of Expression (1) and (2)

ORGANISM		Spore numbers/ml Mean ± S.D.			F-value between surface plate and use of expression (1)	F-value between surface plate and use of expression (2)	T-value* between surface plate and use of expression (1)	T-value* between surface plate and use of expression (2)	degrees of freedom
		Surface Plate Method	Use of expression (1)	Use of expression (2)					
Asp. niger	1	190 ± 47	205 ± 53	312 ± 104	.8425	.2179	.5651	2.9965	7
	2	4800 ± 420	5041 ± 480	4606 ± 608	.7667	.4772	.9424	.7438	6
Asp. nidulans	1	840 ± 152	915 ± 186	1243 ± 331	.6714	.2124	.8663	3.312	8
	2	570000 ± 56000	562500 ± 62250	510700 ± 46175	.8205	1.4911	.6250	3.075	10
Asp. luchuensis Inui	1	8600 ± 1100	9182 ± 1504	12248 ± 4338	.5610	.0675	.7423	2.2468	7
	2	38000 ± 6400	36815 ± 5775	42253 ± 6280	1.2536	1.0527	.6086	1.0395	7
Asp. flavus	1	950 ± 350	1123 ± 372	1386 ± 544	.8853	.4144	.8765	1.7665	6
	2	1100 ± 270	1002 ± 274	1295 ± 308	.9581	.7579	.8619	1.4616	9
Asp. flavus	1	11000 ± 2700	10217 ± 2729	.9711 3063	1.0411	.8262	.6697	.9768	7
	2	600 ± 130	689 ± 160	847 187	.7297	.5289	1.3876	2.4832	10
Asp. flavus	1	180 ± 100	270 ± 99	264 119	1.1262	.6966	.6872	1.7512	9
	2	77000 ± 13800	91115 ± 8851	105056 24630	2.4159	.3120	2.1269	2.4437	5
Composite mixture for all 6 organisms	1	47000 ± 11600	57915 ± 12777	64103 12193	.8201	.9004	1.9966	2.1537	9
	2	990 ± 320	1195 ± 308	1632 546	1.0631	.3371	1.3310	1.7108	7

* not significantly different at P <0.001

Influence of Variations in Inoculum Size on Methanol Production per Unit Mycelial dry weight (*Penicillium* Strains)

The above procedure was repeated using the *Penicillium* strains. Figure 34a and 34b show individual plots of 4 of the 5 *Pencillium* strains which show highly significant correlation coefficients between the inoculum size and methanol production when expression (1) was used. *Pencillium martensii*, however, was found to behave differently under the same experimental conditions. GLC analysis of culture dialysate gave two peaks which were identified as methanol and ethanol respectively. When inoculum size was plotted against methanol production (Figure 34c) the correlation was very poor indeed.

Like the *Aspergillus* strains, the *Pencillium* curves were statistically found to belong to the same theoretical straight line when results of *Penicillium martensii* were omitted as was confirmed by a plot of the values for the 4 straight lines taken together (Fig. 35). Figure 35 was used as a calibration curve in conjunction with figure 35a to estimate spore numbers in *Pencillium* suspensions and compared with estimates by Standard Surface plate method (table 9). Here again the results were in reasonable agreement.

Finally, all mean values for *Aspergillus* strains (fig. 32) and those for the *Penicillium* strains without *Pen.martensii* (fig. 35) were pooled together, their means taken and the values fed into the computer for a plot of $\log_{10}$ dry weight of spores vs. reciprocal of methanol produced. Figure 36 shows a good straight line correlation for these values. An attempt was made to use figure 36 in conjunction with figure 36a as a calibration curve to estimate mixed spore populations of *Penicillum* and *Aspergillus*.

The spore suspensions of the various organisms were mixed randomly as shown in table 10 without any particular attention to the concentration of individual organisms in the mixtures. Each mixture was diluted to give 3 effective dilutions. The spore concentration of each of these dilutions was determined by both the surface plate method and the methanol method. The results are given in table 10 as the means of 6 determinations.

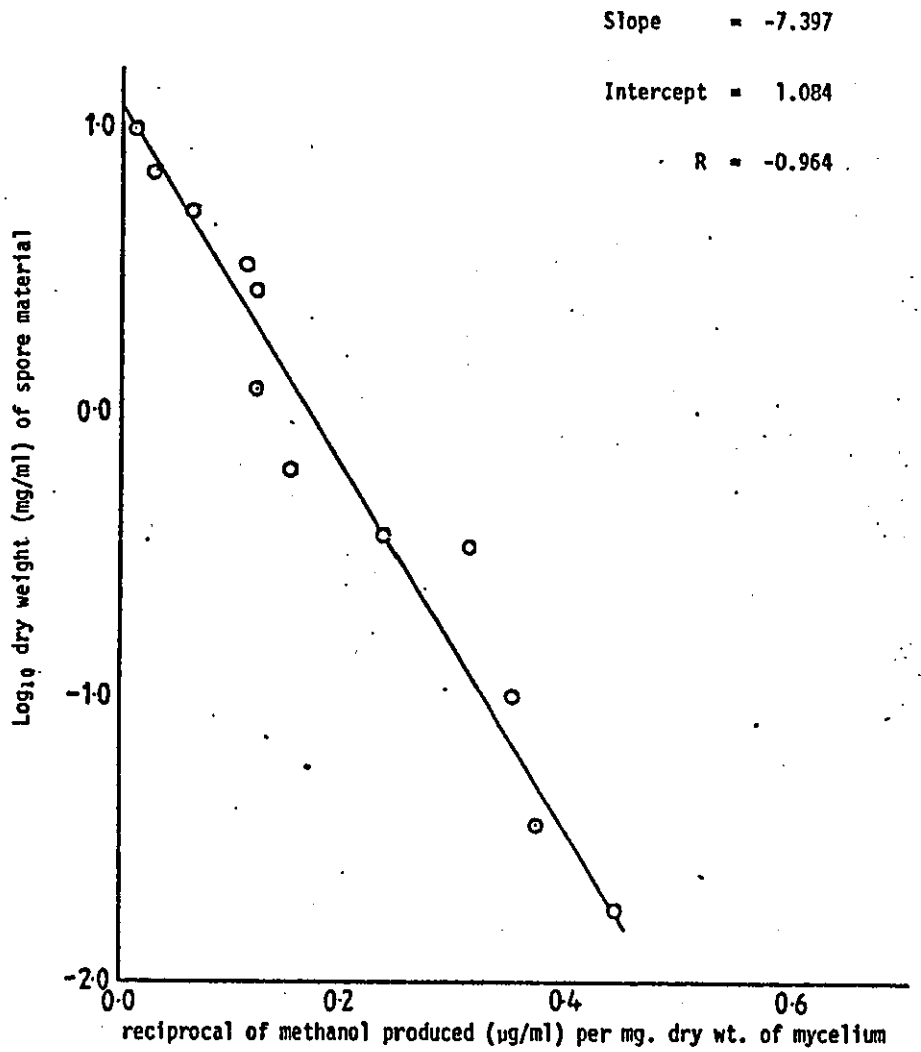
FIGURE 34a, b, c

INFLUENCE OF VARIATIONS IN INOCULUM SIZE ON METHANOL PRODUCTION PER UNIT MYCELIAL DRY WEIGHT (*PENICILLIUM* STRAINS)

Each point is the mean of 5 determinations

Pen. expansum 39761

FIGURE 34a



Pen. chrysogenum 26211

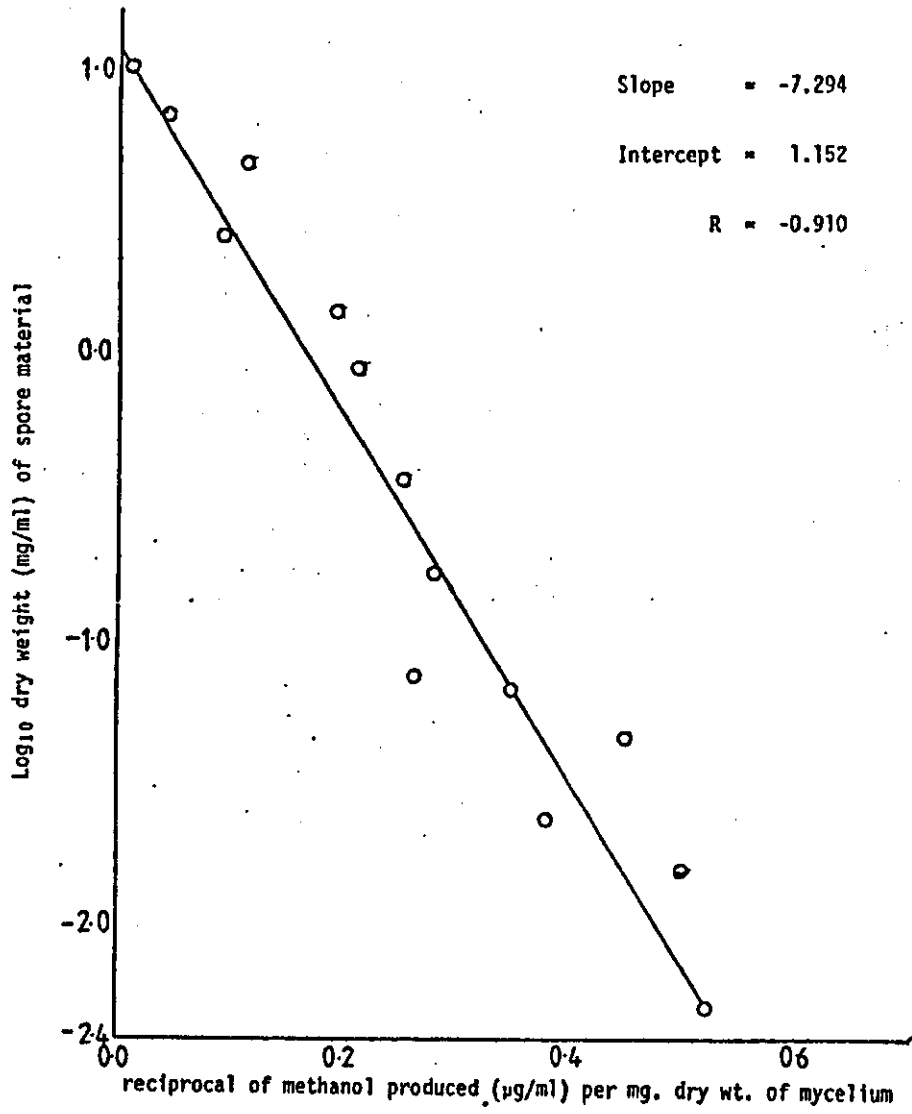
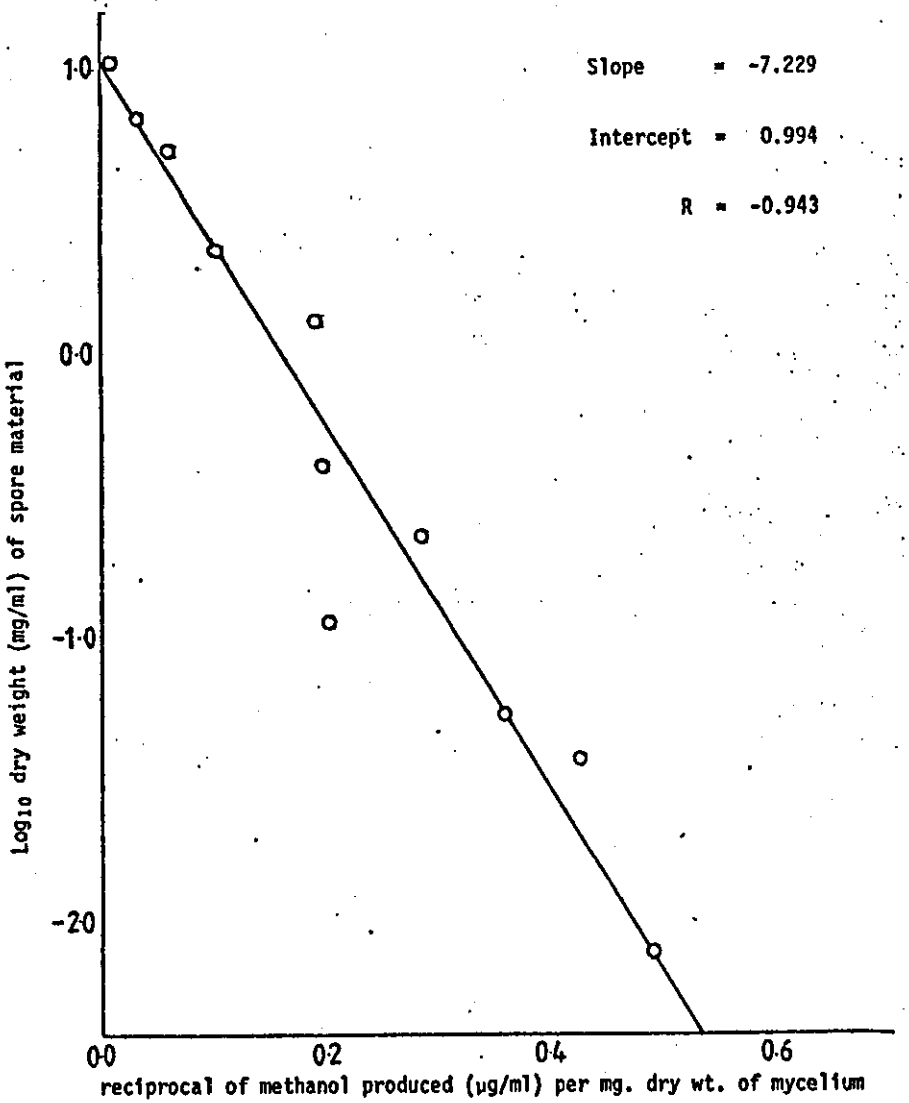


FIGURE 34b

Pen. lividum 99648



Pen. bevicompactum 92034

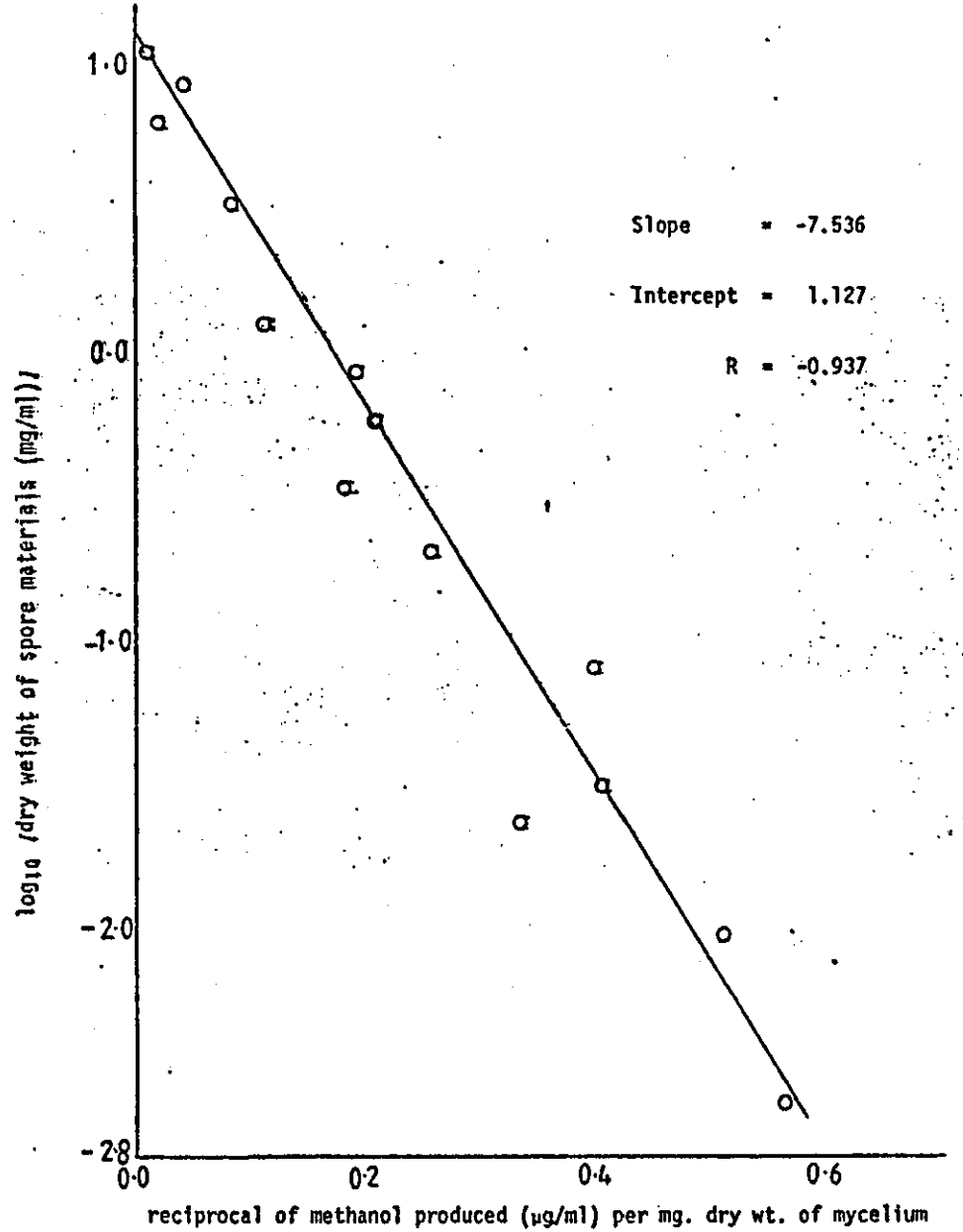


FIGURE 34C

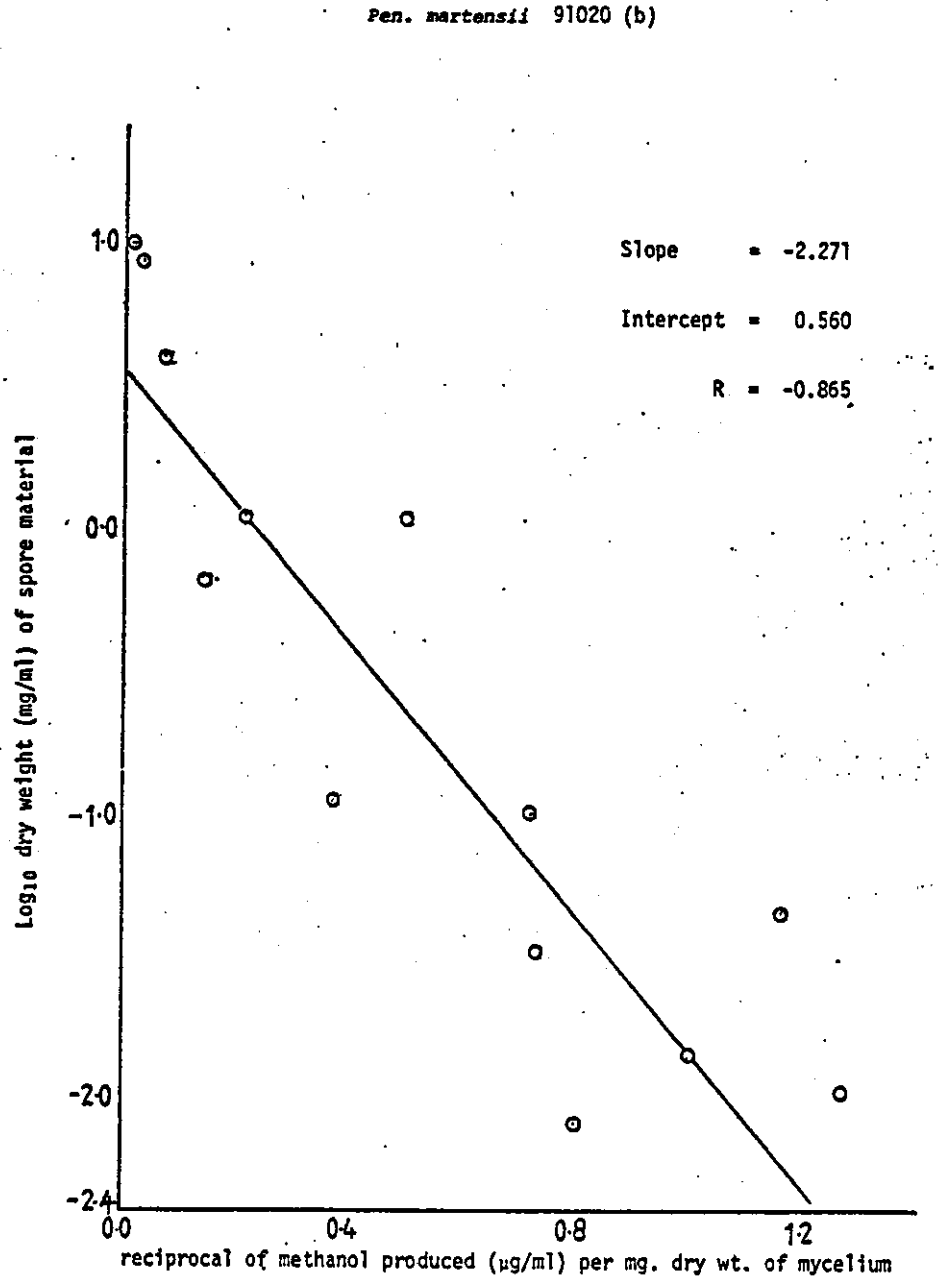

Pen. martensii 91020 (b)
Slope = -2.271
Intercept = 0.560
R = -0.865
1·0
0·0
-1·0
-2·0
-2·4
0·0
0·4
0·8
1·2
Log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

FIGURE 35

COMPOSITE GRAPH FOR ALL *PENICILLIUM* STRAINS WITHOUT *PEN. MARTENSII*

A standard curve plotted between $\log_{10}$ dry weight of *Penicillium* spore material (y) and the reciprocal of methanol produced (µg/ml) per mg. dry weight of mycelium after 15 hours of incubation (x).

$y = 1.086 - 7.33X$; Coefficient of correlation (R) = -0.935

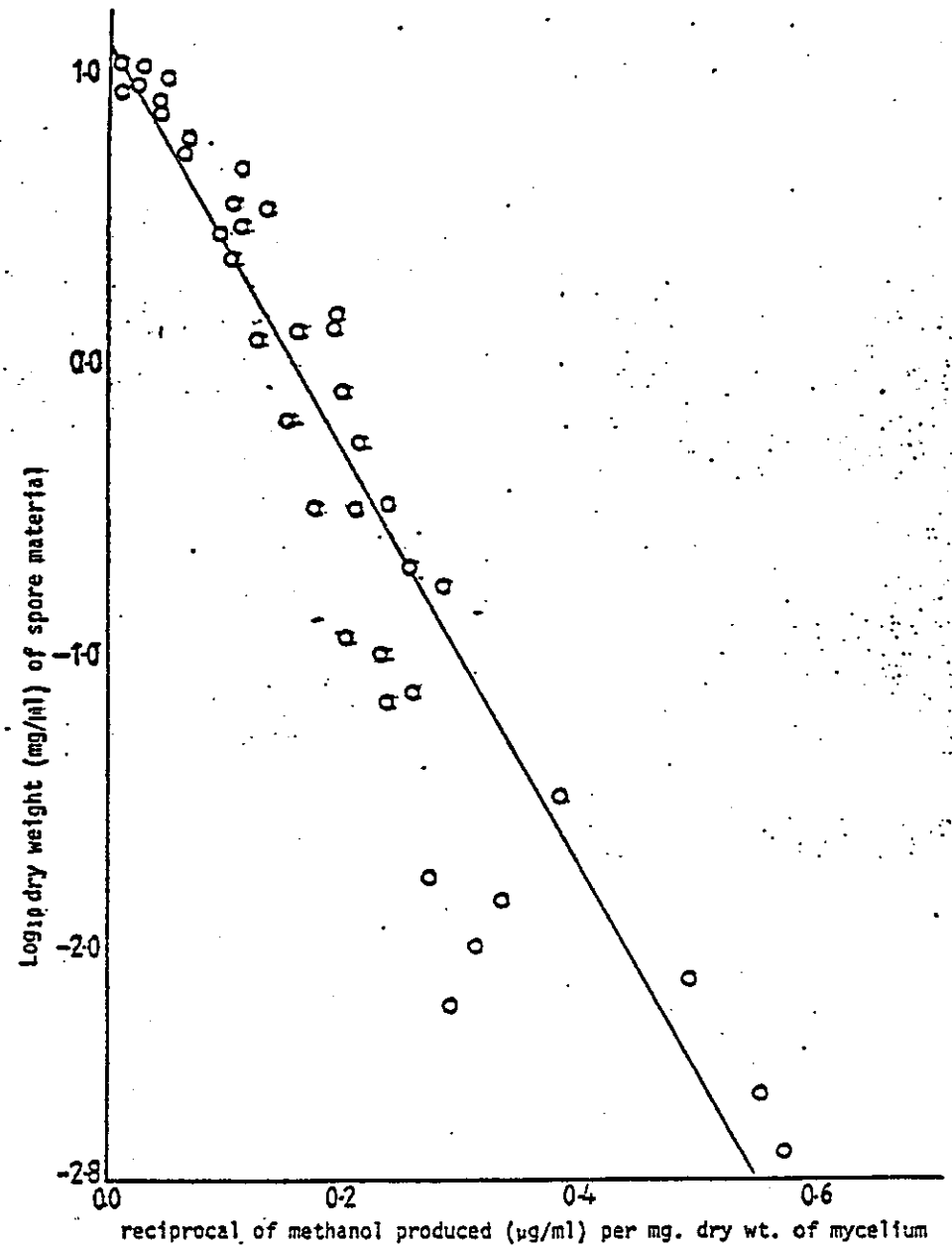

1·0
0·0
−1·0
−2·0
−2·8
0·0
0·2
0·4
0·6
Log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

FIGURE 35A

CALIBRATION CURVE FOR DETERMINING SPORE NUMBERS FROM DRY WT. OF VIABLE SPORES OF *PENCILLIUM* STRAINS

A calibration curve plotted between $\log_{10}$ dry weight of spore material (mg/ml) (y) and $\log_{10}$ spore number/ml (x).

$y = 0.806X - 3.576$; Coefficient of Correlation (R) = 0.999

Each point is the mean of 6 determinations

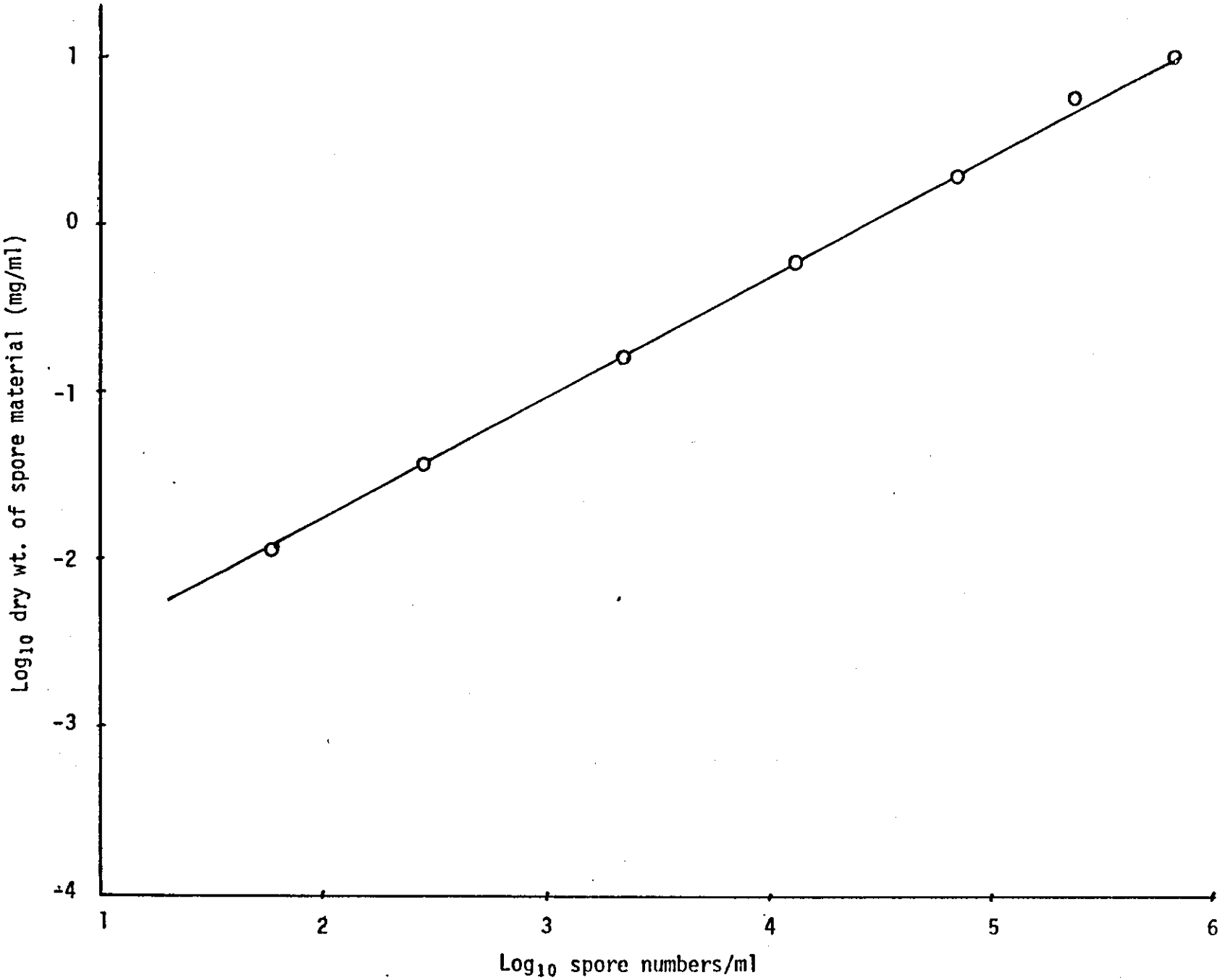

1
0
-1
-2
-3
-4
1
2
3
4
5
6
Log10 dry wt. of spore material (mg/ml)
Log10 spore numbers/ml

TABLE 9

ORGANISM	Spore numbers/ml ± SD		F-test values	T-test values	Degrees of freedom
	Methanol Method	Surface Plate Method			
Pen. lividum	12616 ± 1414	13100 ± 1200	1.260	0.614	5
Pen. brevicompactum	947 ± 109	970 ± 107	1.046	0.429	5
Pen. expansum	63741 ± 2385	63500 ± 2080	1.308	0.176	5
Pen. chrysogenum	4946 ± 246	5000 ± 150	2.538	0.520	5
Composite mixture of all 4 organisms	14263 ± 1500	13700 ± 1500	0.992	0.620	5

Comparison of estimates of spore numbers in unknown spore suspensions of *Penicillium* strains by Surface Plate method and the Methanol method.

FIGURE 36

COMPOSITE GRAPH FOR ALL 6 *ASPERGILLUS* STRAINS AND ALL *PENICILLIUM* STRAINS EXCLUDING *PEN. MARTENSII*

A calibration curve plotted between $\log_{10}$ dry weight of *Aspergillus* and *pencillium* spore material (y) and the reciprocal of methanol produced (µg/ml) per mg. dry weight of mycelium after 15 hours of incubation (x).

$y = 1.079 - 6.86X$; Coefficient of correlation (R) = -0.963

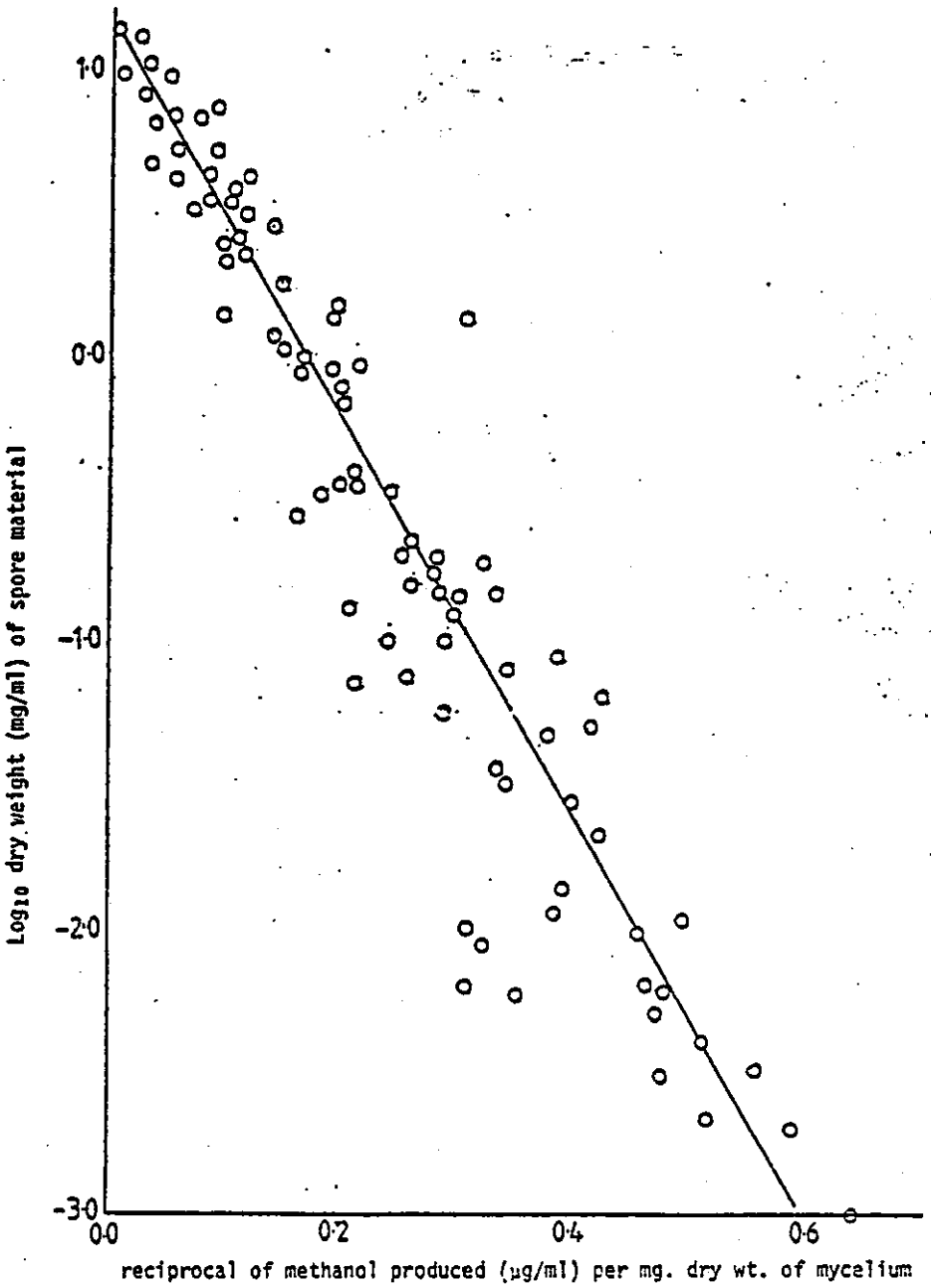

1·0
0·0
-1·0
-2·0
-3·0
0·0
0·2
0·4
0·6
Log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

FIGURE 36A

CALIBRATION CURVE FOR DETERMINING SPORE NUMBERS FROM DRY WT. OF VIABLE SPORES OF MIXED SUSPENSIONS OF *PENCILLIUM* AND *ASPERGILLUS* STRAINS

A calibration curve plotted between $\log_{10}$ dry weight of spore material (mg/ml) (y) and $\log_{10}$ spore number/ml (x)

$y = 0.865X - 3.763$; Coefficient of Correlation (R) = 0.994

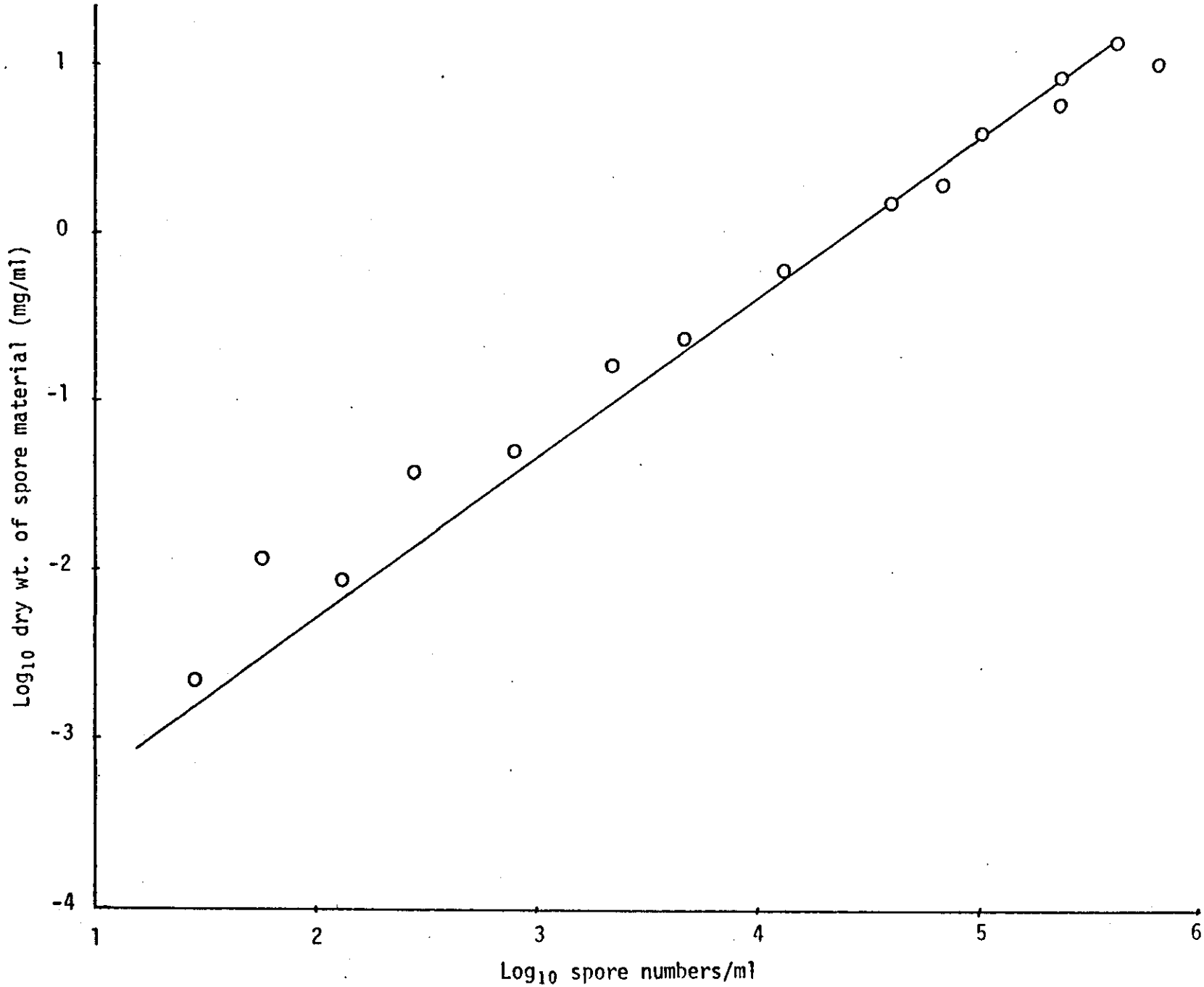

1
0
-1
-2
-3
-4
Log10 dry wt. of spore material (mg/ml)
1
2
3
4
5
6
Log10 spore numbers/ml

TABLE 10

Comparison of estimates of spore numbers in mixed cultures of *Aspergillus* and *Penicillium* strains by the Surface Plate Method and the Methanol Method

Mixed Spore Populations		Number of spores/ml Mean ± S.D.		F-test values	T-test values	Degrees of Freedom
Composition of mixture	Suspension number	By the Surface Plate method	By the methanol method			
Asp. flavus 15959	1	240 ± 14	240 ± 22	0.3771	0.0773	5
and	2	1440 ± 39	1514 ± 90	0.1835	1.7568	5
Pen. chrysogenum	3	15700 ± 220	15402 ± 305	0.5146	2.0069	5
All 6 *Aspergillus*	1	142 ± 14	150 ± 16	0.7021	0.9212	5
strains plus	2	1100 ± 31	1094 ± 103	0.0927	0.1438	5
Pen. chrysogenum	3	57400 ± 190	57662 ± 658	0.0847	0.9351	5
Pen. expansum *Pen. chrysogenum*	1	288 ± 11	282 ± 12	0.773	0.7879	5
Pen. lividum *Pen. brevicompactum*	2	2490 ± 90	2471 ± 48	0.4640	3.6956	5
Plus *Asp. flavus* 15959	3	32500 ± 220	32665 ± 400	0.3071	0.7022	5
The 4 *Penicillia*	1	104 ± 14	101 ± 17	0.7561	0.2325	5
plus all 6	2	5100 ± 50	5071 ± 47	1.3101	0.1841	5
Aspergilli	3	74200 ± 330	41158 ± 9694	0.0012	8.3552	5

Influence of Variations in Inoculum Size on Methanol Production per Unit Mycelial Dry Weight (Yeast Strains)

The above procedure was repeated using the six available yeast strains. Figures 37a, b, c, are the individual plots for the strains that released methanol from the medium under the experimental conditions. These curves are highly different from each other and none of them could be said to be a straight line graph (correlation coefficients not significant).

FIGURE 37a, b, c

INFLUENCE OF VARIATIONS IN INOCULUM SIZE ON METHANOL PRODUCTION PER UNIT MYCELIAL DRY WEIGHT (YEAST SPECIES).

Figure 37a

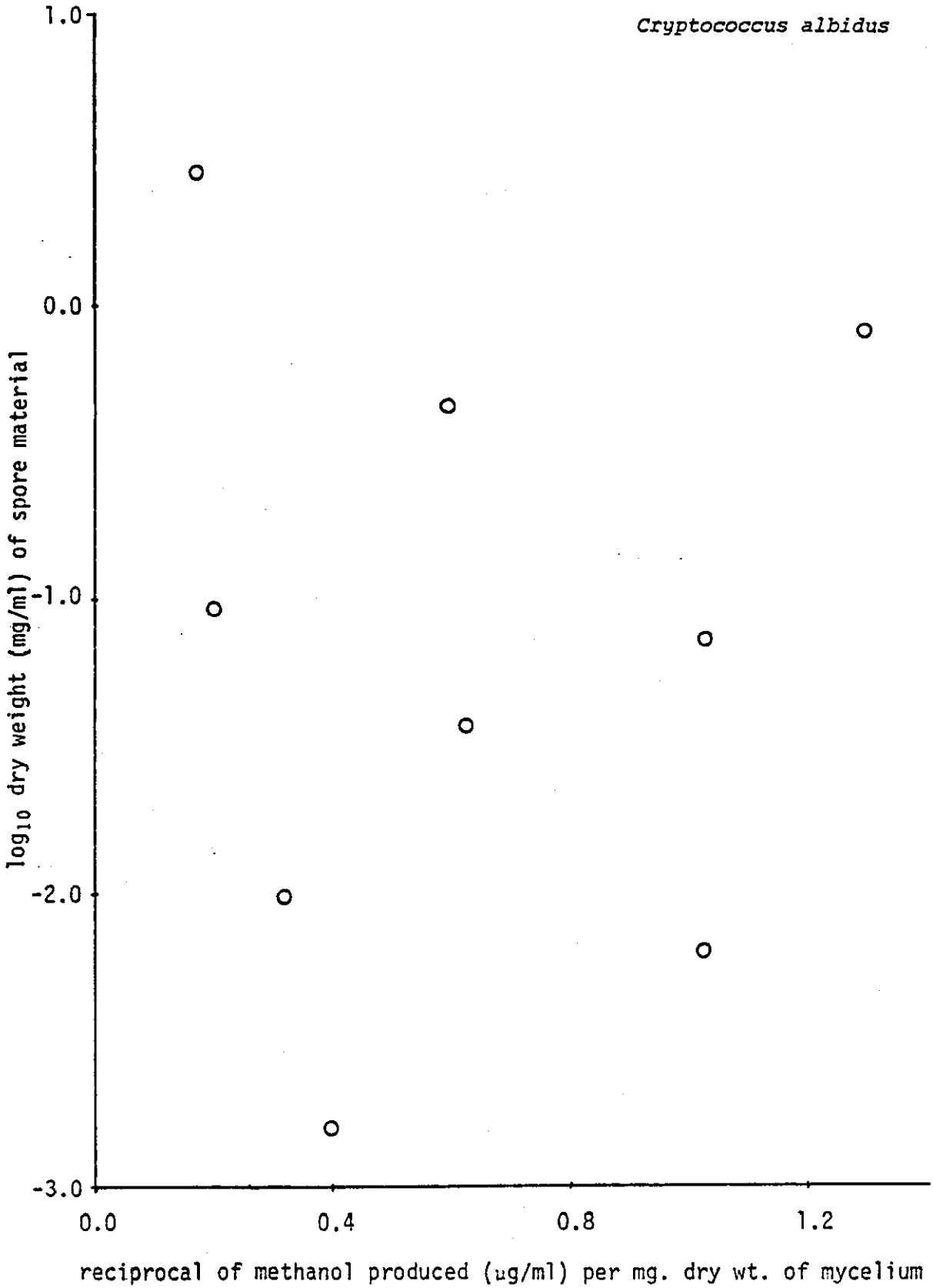

Cryptococcus albidus
1.0
0.0
-1.0
-2.0
-3.0
$\log_{10}$ dry weight (mg/ml) of spore material
0.0
0.4
0.8
1.2
reciprocal of methanol produced (ug/ml) per mg. dry wt. of mycelium

Figure 37b

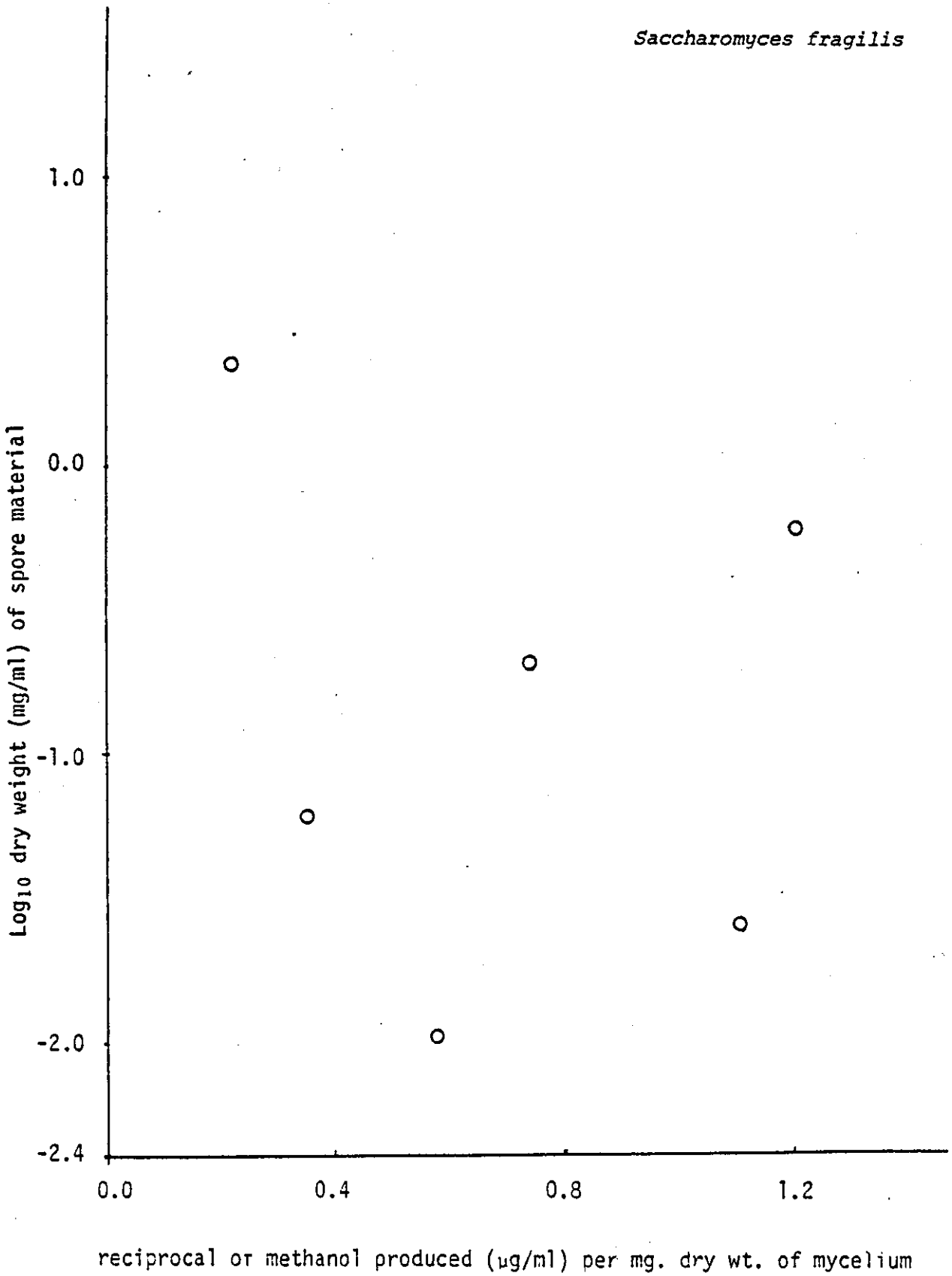
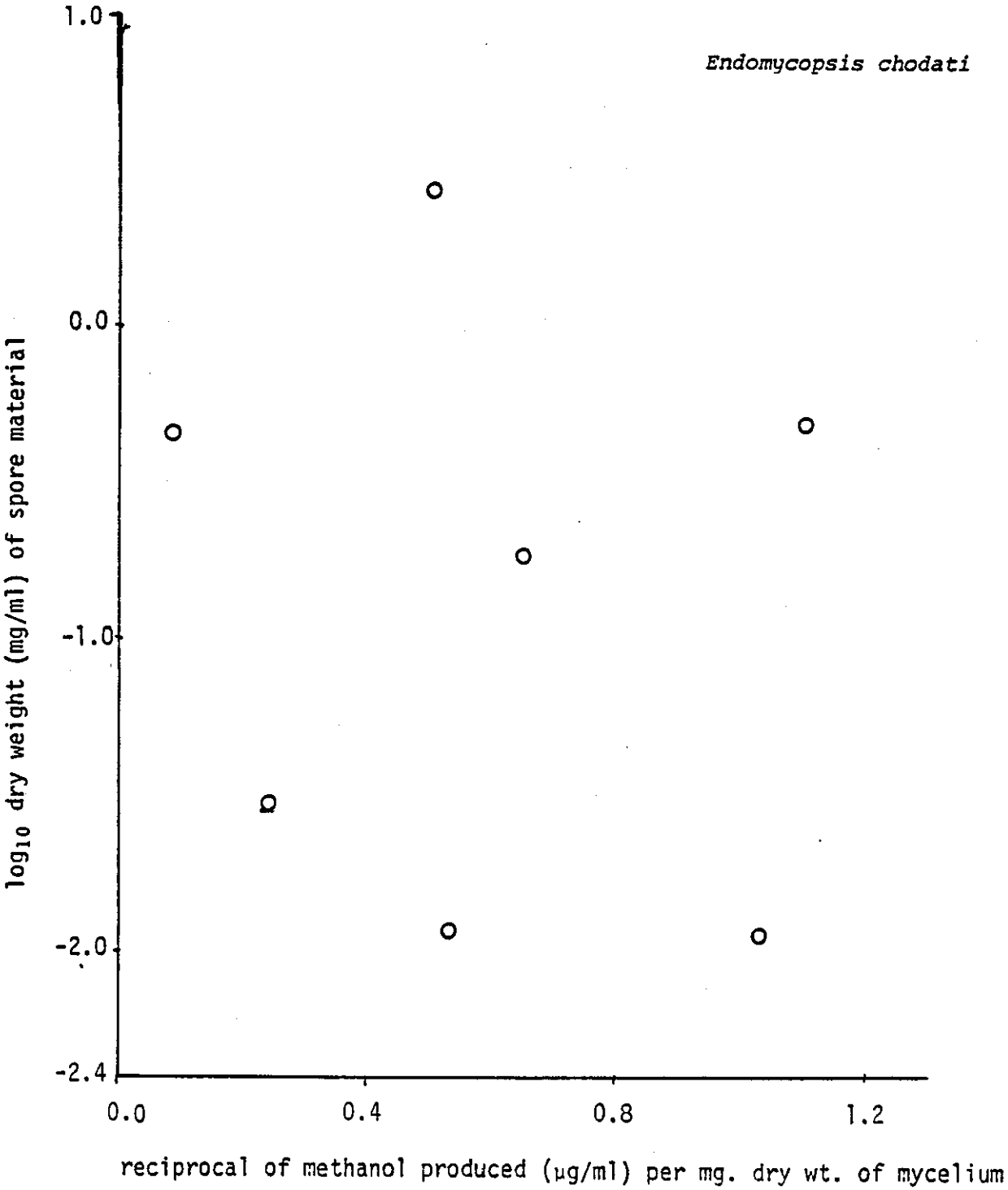

Saccharomyces fragilis
1.0
0.0
-1.0
-2.0
-2.4
0.0
0.4
0.8
1.2
Log10 dry weight (mg/ml) of spore material
reciprocal or methanol produced (µg/ml) per mg. dry wt. of mycelium

Figure 37c


Endomycopsis chodati
1.0
0.0
-1.0
-2.0
-2.4
0.0
0.4
0.8
1.2
log10 dry weight (mg/ml) of spore material
reciprocal of methanol produced (µg/ml) per mg. dry wt. of mycelium

4

DISCUSSION

The presence of spoilage fungi in grain and cereals represent a major problem for industries producing or using these commodities. Due to the toxic nature of some of the fungal metabolites that may be produced, rapid analysis for the purpose of quality control is obviously highly desirable.

The significant words in the title of this thesis are "rapid" and "low numbers". If the concentration of spoilage fungi in stored produce is high enough then their detection, identification to generic level and determination of total number per unit of produce presents few problems especially if the time factor is not a serious consideration. However, by the time these tests had been carried out the produce would not be fit for consumption.

A number of authors (Golubchuk et. al., 1960; Arima & Uozumi, 1967; Ride & Drysdale, 1971; Ride & Drysdale, 1972; Donald & Mirocha, 1977; Jarvis, 1977) have discussed the rapid detection and estimation of fungi using chitin analysis. However, this method, although rapid, suffers from several serious drawbacks: It requires a high level of contamination, it is an inaccurate measure of fungal numbers, it does not distinguish between viable and non-viable fungi, nor does it distinguish between dormant spores and actively growing mycelium and even trace contamination with insect bodies can cause serious discrepancies to arise. Estimation of low levels of fungi by conventional slow methods taking 5 - 10 days have also been described (Bottomley et. al., 1952; Christensen, 1951; Christensen & Drescher, 1954). As already mentioned these are of little use in a modern analytical quality control laboratory. Very little work has appeared in the literature on the rapid detection and estimation of low levels of fungi.

Christensen (1951) showed that fungi were present on both the interior and exterior of infected seeds. Spoilage fungi beneath the pericarp were present as actively growing mycelia whilst externally, fungi were present as dormant spores. The external dormant spores were much more numerous than the internal mycelia . Although Christensen (1951) did not establish any relationship between the level of contamination, either interior or exterior and the rate of deterioration, it is obvious that the dormant spores constitute a considerable hazard to the produce under poor storage conditions. Any damage caused to produce by the active mycelia internally can easily be detected by a rapid chitin analysis, but the potential ability of the spores to cause damage only becomes obvious after the spores have germinated and penetrated the grain.

The work reported in this thesis with pure spore suspensions has produced two methods which show promise for the rapid detection and estimation of low numbers of dormant spores on the surface of stored grains and seeds.

The first of these is the Most-Probable-Number (MPN) method. Results shown in plate 1, table and Fig. 34 a,b,c indicate that under the experimental conditions described, spores of the genera *Aspergillus* and *Penicillium* germinate and produce the enzyme pectinesterase (EC 3.1.1.11.) within 18 hours of incubation. The activity of this pectinesterase is sufficient to hydrolyse methoxyl groups of pectin to carboxyl groups in an amount sufficient to lower the pH of the culture medium sufficiently to cause a visual colour change. By an application of conventional MPN techniques it is possible to estimate fungal properties in liquid samples.

Recognizing the bias inherent in the MPN method (Oblinger & Koburger, 1975) it is understandable why counts were higher in the MPN procedure than either the Surface Plate or Pour Plate method (table 2). Furthermore, in the MPN tubes under agitated conditions it is only the spores at the

surface of the medium which benefit from the aeration process whilst those within the medium are not aerated at the same rate. This could well influence the results and lead to high standard deviations. However, statistical tests show that the results are not significantly different from one method to the other.

However, since MPN measurements are statistical approximations and considering the simplicity of the above procedure and the fact that results are obtained after only 18 hours of incubation as opposed to the 48 hours of the conventional methods it would be reasonable to conclude that the method holds promise in the rapid detection of low levels of spoilage fungi.

Additional advantages are that the method is cheap and does not require very high levels of technician expertise, both of which are major advantages for developing countries.

The method was tried on *Aspergillus* strains alone but there is every reason to believe that it would hold true for the *Penicillium* strains as well, since it was found in the methanol method that *Penicillium* PE production and activity resembled that of *Aspergillus*.

The second method (the methanol method) involves using methanol production from pectin - supplemented culture medium by germinating and growing fungi spores and estimating this by gas chromatography. The major advantage of this method is that it enables estimation of spore numbers over a wide range without prior dilutions having to be made.

The results shown in figure 29 could be described by one or more of several mathematical functions notably:-

$y = Ce^{-\beta/x}$ ..... (1) and $y = c + (\beta \log_{10} x)$ .....(2)

(Daniel & Wood 1971). where $y = \log_{10}$ of initial spore number/ml

x = the amount of methanol released at the end of the incubation period (µg/ml)

and c, e and β are constants.

For the purpose of curve fitting (1) may be transformed to

$$\log_{10} y = \log_{10} c - \beta \log_{10} e \frac{1}{x} \quad \ldots\ldots\ldots \text{(A)}$$

and (2) transformed to $y = c + \beta(\log_{10} x) \ldots\ldots$ (B)

(Daniel & Wood, 1971). Expression (A) means that a plot of $\log_{10} y$ against $\frac{1}{x}$ should be a straight line from which the parameters $\log_{10} c$ and $\beta \log_{10} e$ can be calculated. Similarly expression (B) means that a plot of y against $\log_{10} x$ should be a straight line from which the constants c and β can be duduced.

In general the principle of obtaining a linear relationship by transforming a non-linear one may be formulated as follows:-

If the points (x_i, y_{iv}) when transformed to $[g(x_i), f(y_{iv})]$ are grouped about a straight line in such a manner that f (y) is normally distributed with mean value $M\{\frac{f(y)}{g(x)}\} = C + \beta [g(x) - \overline{g(x)}]$ and variance $v\{\frac{f(y)}{g(x)}\} = \sigma^2$ where M{x} stands for mean of x values and v{x} is variance in x values then the theory of linear regression may be applied to the transformed observations.

The results for each organism were considered separately, these were pooled together and the mean taken. The results were then fed into a computer for a plot of (A) $\log_{10}$ of $\log_{10}$ initial spore numbers/ml vs. the reciprocal of the methanol produced (µg/ml) and (B) $\log_{10}$ of initial spore numbers/ml vs. $\log_{10}$ of the methanol produced (µg/ml).

In either case the results for each organism gave points that seem to be scattered, about a straight line. The slopes, intercepts and correlation coefficients for each set of results are shown in table 6. Therefore, the theory of linear regression was applied thus:

For hypothesis(A)and taking the case of *Asp. niger* alone,

$$M\left\{\frac{\log_{10}\text{ of }\log_{10} Y}{1/x}\right\} = C + \beta\left(\frac{1}{x} - \frac{\overline{1}}{x}\right)$$

and $$V\left\{\frac{\log_{10}\text{ of }\log_{10}}{1/x}\right\} = \sigma^2$$

resulted in $\log_{10}$ of $\log_{10} y = 0.84 - \frac{1.33}{x}$

and $S^2 = 0.01097$

Comparison of variation within sets $S_1^2 = 0.01084$ and the variation of the means about the regression line $S_2^2 = 0.0099$. Therefore the variance ratio, $V^2 = \frac{S_2^2}{S_1^2} = 0.913$. This is not significant and the hypothesis regarding linearity cannot be rejected.

For hypothesis(B)and again taking the case of *Asp. niger* alone

$$M\left\{\frac{\log_{10} y}{\log_{10} x}\right\} = C + \beta\left(\log_{10} x - \overline{\log_{10} x}\right)$$

and $$V\left\{\frac{\log_{10} y}{\log_{10} x}\right\} = \sigma^2$$

resulted in $\log_{10} y = 0.92 - 3.96 \log x$ and $S^2 = 0.0807$

Comparison of the variation within sets $S_1^2 = 0.0802$ and the variation of the means about the regression line, $S_2^2 = 0.087$ demonstrates too, that the hypothesis regarding linearity cannot be rejected on the basis of this test as $v^2 = 1.0898$ is not significant.

It is therefore seen that it is impossible to determine which of these two relationships is the preferred one on the basis of statistical analysis alone. Under the experimental conditions, an increasing spore concentration results in an increasing level of methanol being liberated into

the culture medium to an upper limit which is reached when the spore concentration becomes infinitely large. The curve must therefore possess an upper horizontal asymptote. Also as there is no methanol produced when sterile water is used as inoculum (spore concentration = 0) it follows that the curve should pass through the origin of both axes. Only function (1) seems to satisfy these conditions and so expression (A) is preferred. This preference is supported by the figures in table 6 which show that the correlation coefficients obtained for plots using expression (A) ranged from 0.914 to 0.952 with standard deviations ranging from 0.024 to 0.017. These are closer to perfect linearity than those for expression (B) which ranged from 0.893 to 0.939 for correlation coefficients and 0.493 to 0.216 for standard deviations.

An attempt to plot the results for each organism on the same graph sheet using the preferred relationship of $\log_{10}$ of $\log_{10}$ of spore numbers/ml vs. reciprocal of methanol produced, resulted in 6 different almost parallel lines. That is by the principle of comparison of several regression lines (Wilson, 1934) all the lines had more or less identical slopes and differed only in their intercepts (Figure 31).

The various sets of figures in table 6 for slope and intercept for the *Aspergillus* series of experiments were fed into the computer for standard deviations analysis. The standard deviation of the slopes for (B) relationship is much too high relative to the mean i.e. 4.2298 ± 1.0265. This indicates non-uniformity in the slopes for the various organisms in using this relationship. The non-uniformity is even worse for the intercept, 0.9897 ± 0.4310. On the basis of this the mathematical relationship(B)was rejected.

Using expression (A) the slopes were more or less identical i.e. the standard deviation was relatively low at -1.2576 ± 0.060. At 95% confidence the slope for all 6 curves was -1.2576 ± 0.063. The intercept

cannot be said to be identical even though at the 95% confidence it is 0.8598 ± 0.0355 for the 6 lines. This value is the intercept on the y-axis which is $\log_{10}$ of $\log_{10}$. When this is converted to spore numbers the intercept at 95% confidence varies from 7.2×10^5 to 4.7×10^4. The explanation could be that methanol production by the various strains would be a consistent property if it were related to a more constant character of the organisms in the culture flasks, e.g. relating it to protein content or to mycelial dry weight.

The results of the experiments which described methanol production as methanol produced per mg dry weight of mycelium at 15 hours of incubation (table 7 and figs. 31a, b, c) shows that the straight line correlation between inoculum size and methanol production improve considerably for the individual organisms. When the organisms were taken together and plots made for all 6 organisms between (1) dry weights of spore inoculum and methanol production and (2) between spore numbers and methanol production the correlation coefficients of -0.988 and -0.963 respectively were highly significant (Figures 32 and 33). This enabled two linear relationships to be obtained thus:

$y = 1.078 - 6.73X$ where y is $\log_{10}$ dry weight of spore material (mg/ml)

and $y = 0.706 - 0.739X$ where y is $\log_{10}$ of $\log_{10}$ spore numbers/ml

and x in both cases is the reciprocal of methanol production. Once the level of methanol has been established, these relationships could be used for estimating unknown spore suspensions. The results obtained from such estimations were in good agreement with those of standard surface plate method (table 8).

The repeated determination of spore concentration in spore suspensions takes 48 hours and is tedious for the preparation of calibration curves. Moreover, the counts are not very consistent and so the relationship was applied to a more accurate property of the spores (dry weight of spore material). Comparison of fig. 32 and 33 and the figures in table 8 show an

improved correlation with the relationship which makes use of dry weights of spore material. Although the additional task of preparing a calibration curve to convert dry weight of spore material to spore numbers was needed in this method, this was compensated for because once the curve was prepared, it served the purpose for the rest of the work and the improved correlation meant more accurate assessment of spore populations. This was confirmed in table 9 where values using the dry weight expression were much closer to surface plate values than those using the spore number expression. However, lower levels of fungi could be determined using the spore number expression than by use of the dry weight expression. This was due to the lack of sensitivity in dry weight measurements when dealing with very low numbers of fungal spores. Using expression (2) as few as 20 spores/ml could be estimated within the experimental time whilst (1) required > 50 spores/ml for effective determination of dry weight.

The upper limit of estimations can be fixed at 7×10^5 spores/ml as above this figure, the relationship tends towards curvilinear due to the extremely low values for the reciprocal of methanol production.

It should be possible to detect without necessarily estimating spore suspension with spore levels of 1 spore/ml if a more sensitive gas chromatograph were used.

The relationship also held true for 4 out of the 5 *Penicillium* species examined and as figure 35 and table 9 show, estimates of unknown spore suspensions were in reasonable agreement with standard surface plate method. The one *Penicillium* strain which deviated from the rest produced ethanol as well as methanol under the experimental conditions. The values for methanol production were highly inconsistent as shown in figure 34C. Ethanol has been reported to have an inhibitory effect on the activity of PE (Mikeladze et. al. 1975) and could explain why this particular strain of *Penicillium* failed to obey the established relationship. The source of the

ethanol produced by *Pen. martensii* was not investigated.

When the results of the *Aspergillus* strains (fig. 32) and those for *Penicillium* without *Pen. martensii* (fig. 35) were pooled together into a single calibration curve (fig. 36) and used to estimate spore populations of mixed suspensions of *Penicillium* and *Aspergillus* it was found (table 11) that for the mixtures examined, spore numbers as determined by the methanol method agreed closely with those determined by the standard surface plate method except at the high level for the suspension that contained all 6 strains of *Aspergillus* and all 4 strains of *Penicillium* where significance tests show a marked decrease in spore numbers by the methanol method than by the surface plate method. Although no emphasis was placed on the number of spores of each strain in any particular suspension it could be that at this high spore concentration of all ten strains one or more strains produced a substance which was inhibitory to the germination of the others or to the PE activity of the culture thereby producing a low result. No similar effect was observed in the low concentration range. ($1 - 5 \times 10^3$ spores/ml) of mixtures containing spores of all ten strains. This discrepancy was not investigated further due to lack of time but it would obviously be an ideal topic for further research.

The yeast strains *Sacc. fragilis, Cryptococcus albidus* and *Endomycopsis chodati* also released methanol from pectin but they did not obey the relationship determined from *Aspergillus* and *Penicillium*, neither could a generalized equation be produced solely for the yeasts (Fig. 37a, b, c). In addition three species of the yeasts investigated *(Pichia farinosa, Candida pseudotropicalis* and *Hausenula anomala*) did not produce methanol under the experimental conditions. However, various authors (Del Prado and Christensen 1952) have suggested that yeasts are not a serious factor in deterioration of stored grain although they are an important

factor in the deterioration of fruits and fresh vegetables.

One major advantage of both methods is that as the majority of *Penicillia* and *Aspergilli* obey the relationship determined, it is not necessary to grow the organisms for a sufficiently long time to determine which genus or species is present. The methods could be related to a worst prediction system, for example, the assumption could be made that all species present are toxigenic and produce could be passed or condemned on this basis.

The methods are simple, accurate and rapid and although more work is required, this thesis could form a useful basis for produce control in the grain industry.

The further work required could be based on the following lines:

1) An improvement in the sensitivity and accuracy of the methods in the lower range.

2) A determination of whether other *Aspergilli* and *Penicillia* also produce the same relationship or whether there are a large number of 'rogue' species such as *Pen. martensii*.

3) An investigation to determine whether there is any interference by bacterial species present.

4) An investigation to determine whether there is any interference by pectic substances present in produce.

5) A determination of the basis for the discrepancy observed when very high levels of mixed spore populations are used.

5

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6

APPENDIX

1. STATISTICAL TREATMENT OF DATA

1. Calculations of Standard Error of mean, S.E ($\bar{x}$), of Most-Probable-Numbers were based on the formula $\text{S.E.}(\bar{x}) = \frac{\sigma}{\sqrt{n}}$

where σ was the Standard Deviation (S.D) of the population mean, μ and n was number of determinations. σ was not calculated from μ (estimated no. of organisms/ml) as the distribution of μ was very skew (Cochran 1950) but calculated from $\log_{10}\mu$ which was more nearly symmetrical

Hence σ was taken as $0.58\sqrt{\frac{\log_{10} a}{p}}$

where a was the dilution ratio and p the number of tubes per dilution. In this case, a = 10 and p = 10

$$\text{Therefore } \sigma = \frac{0.58}{3.16}$$

$$\text{and} \quad \text{S.E}(\bar{x}) = \pm \frac{0.18}{\sqrt{n}}$$

2. Tests of significance on all MPN values were based on the equation $\frac{\log d_1 - \log d_2}{0.58\sqrt{\frac{\log a_1}{n_1} + \frac{\log a_2}{n_2}}}$ and referring to the appropriate probability tables.

where d_1 is the most probable number of organisms/ml in Case 1

d_2 " " " " " " " " " in Case 2

a_1 dilution ratio in Case 1

a_2 " " " Case 2

n_1 number of tubes in each dilution in Case 1

n_2 " " " " " " " Case 2

In our case where $a_1 = a_2 = 10$

and $n_1 = n_2 = 10$

the expression became $$\frac{\log d_1 - \log d_2}{0.58\sqrt{\frac{1}{10} + \frac{1}{10}}}$$

$$= \frac{\log d_1 - \log d_2}{0.26}$$

3. Sample Mean

The mean of the values $x_1, x_2, x_3, \ldots\ldots, x_n$ was denoted by $\bar{x}$

where $\bar{x} = \frac{x_1 + x_2 + x_3 + \ldots\ldots x_n}{n}$

i.e. $\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$

4. Variance

The variance was taken as the mean square difference of the observed values from the mean, and was denoted by σ^2

Hence the variance of $x_1, x_2, \ldots\ldots x_i \ldots\ldots x_n$ was given by

$$\sigma^2 = \frac{\Sigma(x_i - \bar{x})^2}{n}$$

5. Standard Deviation (S.D)

The standard deviation is the positive square root of the variance

i.e. $\sqrt{\sigma^2} = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n}}$

6. F-test

The F-test compares variability between sets of data to find out if they are different from each other or whether they are about the same.

First the null hypothesis was adopted that the samples were drawn from populations with the same variance

i.e. $H_0 \equiv \sigma_1^2 = \sigma_2^2$

F was calculated as $\frac{\text{larger variance estimate}}{\text{smaller variance estimate}}$

$$= \frac{\sigma_1^2}{\sigma_2^2}$$

Tables have been drawn up giving the value of F which will be exceeded with stated probability for various degrees of freedom. The value of F was therefore determined from the tables based on $\phi_1 = (n_1 - 1)$ and $\phi_2 = (n_2 - 1)$ degrees of freedom, ϕ and ϕ corresponding to the chosen level of significance. If the calculated value of F was greater than this, then the probability of H_0 being true was less than the risk involved in wrongly rejecting it. Therefore it was asserted that the samples were not drawn from populations with equal variances and that $\sigma_1^2 > \sigma_2^2$.

7. T-test

The T-test compares the means of two samples, i.e., whether two samples with means $\bar{x}_1$, $\bar{x}_2$ respectively could have originated from the same population.

First the null hypothesis was adopted that the population from which the sample was drawn was coincident with a known population, T was then calculated from the equation

$$T = \frac{\bar{x} - \mu_0}{\sigma/\sqrt{n}}$$

where μ_0 was the mean of the known population.

Tables give values of T for various degrees of freedom, which will be exceeded in absolute value, with stated probability.

If the value of T from the degrees of freedom and the probability level was less than the calculated value from formula then the hypothesis was rejected and a difference was inserted.

8. Least square method of fitting a straight line to data points

For data points of x and y

the point $(\bar{x}, \bar{y})$ lies on the straight line and the equation of the line could be written as

$$y - \bar{y} = b(x - \bar{x})$$

or

$$y = \bar{y} + b(x - \bar{x})$$

where b is the slope of the line and calculated from the equation

$$b = \frac{\sum_{i=1}^{n} x_i y_i - \frac{1}{n} \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{\sum_{i=1}^{n} x_i^2 - \frac{1}{n} \left(\sum_{i=1}^{n} x_i\right)^2}$$

9. Correlation Coefficient

The covariance of two random variations x and y is defined as the expected value of the product of the deviations of x and y from their expected values:

$$\text{Cov}\{x, y\} = M\{(x - E)(y - \mu)\}$$

where $E = M\{x\}$ and $\mu = M\{y\}$

$M\{\ \}$ denotes "mean of"

The covariance of the two standardised variables is called CORRELATION COEFFICIENT of (x, y) denoted by R and is given by

$$R = \frac{\text{Cov}\{x, y\}}{\sqrt{V\{x\}\ V\{y\}}}$$

where $V\{\ \}$ denotes "variance of"

If $R = 0$, then x and y are uncorrelated and this implies that

$$\text{Cov}\{x,y\} = 0$$

For a perfect correlation $R = 1$

10. Comparison of Two Regression Lines

Two sets of observational data which have each formed the basis of a regression analysis can be expressed as equations of two regression lines

$$y^1 = \bar{y}_1 + b_1 (x - \bar{x}_1)$$

and $$y^2 = \bar{y}_2 + b_2 (x - \bar{x}_2)$$

From each set of observations there are thus 3 quantities $(\bar{y}_1; b_1, \sigma_1^2)$ and $(\bar{y}_2, b_2 \ \sigma_2^2)$ where σ^2 = variance which form estimates of the corresponding values $(\alpha_1, \beta_1, S_1^2)$ and $(\alpha_2, \beta_2, S_1^2)$ in the two populations.

The identity of the 2 population is tested by comparing the estimates. The hypothesis that $S_1^2 = S_2^2$ is tested by means of the variance ratio $v^2 = \frac{\sigma_1^2}{\sigma_2^2}$. If this test does not reveal a significant difference between the 2 variances, the slopes of the 2 regression lines are then compared by means of the T-test.

T is calculated from

$$T = \frac{b_1 - b_2}{S\sqrt{\frac{1}{SSD_{x_1}} + \frac{1}{SSD_{x_2}}}}$$ based on $N_1 + N_2 - 4$ degrees of freedom.

where b_1 and b_2 are the slopes of the two lines

$$S = \sqrt{\frac{(N_1 - 2)\ \sigma_1^2 + (N_2 - 2)\ \sigma_2^2}{N_1 + N_2 - 4}}$$

N is Σ number of points on the regression line.

and SSD_x is the Sum of Squares of deviations from the mean of x values.

If T is not significant the 2 regression lines are considered parallel.

The equations for two theoretical regression lines with the same slope are

$$y^1 = \alpha_1 + \beta (x - \bar{x}_1) = \alpha_1 - \beta\bar{x}_1 + \beta x$$

$$y^2 = \alpha_2 + \beta (x - \bar{x}_2) = \alpha_2 - \beta\bar{x}_2 + \beta x$$

If the two constant terms are equal, i.e.,

$$\alpha_1 - \beta\bar{x}_1 = \alpha_2 - \beta\bar{x}_2$$

or $$\frac{\alpha_1 - \alpha_2}{\bar{x}_1 - \bar{x}_2} = \beta$$

then the lines are identical.

An estimate of $\frac{(\alpha_1 - \alpha_2)}{(\bar{x}_1 - \bar{x}_2)}$ is calculated from

$$\hat{b} = \frac{\bar{y}_1 - \bar{y}_2}{\bar{x}_1 - \bar{x}_2}$$

$\hat{b}$ is based on the variation between sets and denotes the slope of the straight line which connects the points $(\bar{x}_1\ \bar{y}_1)$ and $(\bar{x}_2\ \bar{y}_2)$.

11. Comparison of Several regression lines

If there are m sets of observations then the m^{th} regression line has the equation

$$y^{(m)} = \bar{y}_m + b_m (x - \bar{x}_m)$$

3 quantities are then computed for each set of data $(\bar{y}_m, b_m, \sigma_m^2)$ $m = 1, 2, \ldots\ldots m,$ which denote estimates of the corresponding values $(\alpha_m \beta_m S_m^2)$ $m = 1, 2, \ldots\ldots m$ in the m populations.

As an estimate of S^2

$$\sigma_1^2 = \frac{f_1 S_{11}^2 + \ldots\ldots\ldots + f_m S_m^2}{f_1 + \ldots\ldots\ldots + f_m}$$

$$= \frac{SSD_{y_1/x_1} + \ldots\ldots + SSD_{y_m/x_m}}{N_1 + \ldots\ldots + N_m - 2_m}$$

f is degrees of freedom

The variance σ_1^2 denotes the variation about the m regression lines, i.e., the variation "within sets".

The parallelism of the regression lines, i.e., the hypothesis $\beta_1 = \beta_2 = \ldots\ldots = \beta_m = \beta$, is tested by comparing the variation between the slopes $b_1, b_2 \ldots\ldots\ldots b_m$ and the variation within sets i.e., $\sigma_2^2 = \frac{1}{m-1} \sum_1^m (b_1 - \bar{b})^2 SSD_x$ (variation between slopes) is compared with the variance σ_1^2 (the variation within sets) by calculating the variance ratio $v^2 = \frac{\sigma_2^2}{\sigma_1^2}$.

If the variation between the slopes is not significantly larger than the variation within sets, the 2 variances are combined to a common estimate of S^2 with

$$(\sum_{1}^{m} N - 2m) + (m - 1) = \sum_{1}^{m} N - m - 1 \text{ degrees of freedom}$$

If the hypothesis $\beta_1 = \beta_2 = ... = \beta_m = \beta$ is correct the m theoretical lines may be written

$$y^{(\mu)} = \alpha_\mu + \beta (x - \bar{x}_\mu) = \alpha_\mu - \beta\bar{x}_\mu + \beta x$$

where $\mu = 1, 2, ..., m$

If these lines are identical, the following relation

$$\alpha_1 = \beta\bar{x}_1 = \alpha_2 - \beta\bar{x}_2 = ... = \alpha_m - \beta\bar{x}_m$$

must hold good, i.e., the m points $(\bar{x}_1, \alpha_1)$, $(\bar{x}_2, \alpha_2)$ $.....(\bar{x}_m, \alpha_m)$ must be situated on a straight line with slope β. The equation of this line is $y = \bar{\alpha} + \beta(x - \bar{x})$ where $\bar{\alpha}$ and $\bar{x}$ denote the weighted means of $\alpha_1, \alpha_2, \ldots \alpha_m$ and $\bar{x}_1, \bar{x}_2, ..., \bar{x}_m$ respectively.

The identity of the m (parallel) regression lines is therefore tested by calculating the regression line for the m points $(\bar{x}_1, \bar{y}_1)$, $(\bar{x}_2, \bar{y}_2)$, $....., (\bar{x}_m, \bar{y}_m)$ and testing its linearity. The equation of the regression line for the means becomes

$$y = \bar{y} + \hat{b} (x - \bar{x})$$

where $\bar{y}$ denotes the weighted mean of $\bar{y}_1, \bar{y}_2, ..., \bar{y}_m$ and the slope $\hat{b}$ is calculated from

$$\hat{b} = \frac{SPD_{\bar{x}\,\bar{y}}}{SSD_{\bar{x}}} = \frac{\sum_{\mu=1}^{m} N\mu\,(\bar{x}_\mu - \bar{x})\,y_\mu}{\sum_{\mu=1}^{m} N\mu\,(\bar{x}_\mu - \bar{x})^2}$$

The variation about this line is expressed by the variance

$$\sigma_3^2 = \frac{1}{m-2} \sum_{\mu=1}^{m} N\mu \, (\bar{x}_\mu - \bar{\bar{x}})^2$$

and the linearity of this line is tested by the variance ratio

$$v^2 = \frac{\sigma_3^2}{\sigma_1^2}$$

The hypothesis that the m regression lines are identical is therefore examined by calculating the quantity

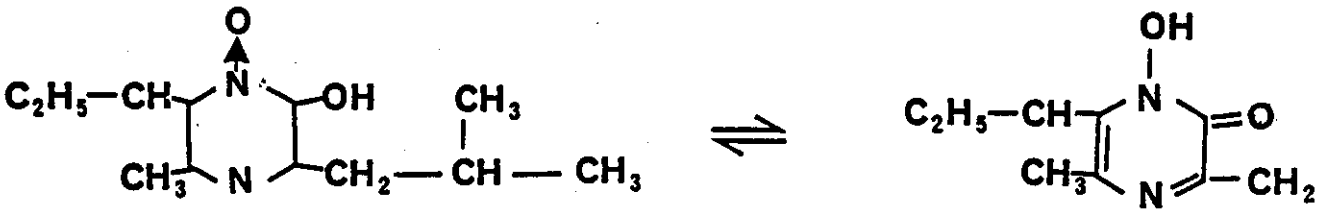
$$T = \frac{\hat{b} - \bar{b}}{\sigma_0 \sqrt{\frac{1}{SSD_{\bar{x}}} + \frac{1}{\Sigma SSD_{x\mu}}}}$$

Where σ_o^2 denotes the variance calculated from σ_1^2, σ_2^2 and σ_3^2

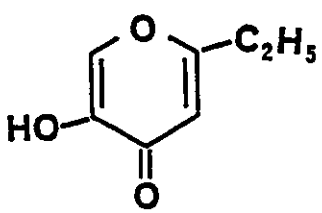
If $\hat{b}$ and $\bar{b}$ do not differ significantly the m empirical regression lines may be considered to be estimates of the same theoretical regression line.

2. SOME MYCOTOXINS PRODUCED BY SPOILAGE FUNGI

1. Aspergillic acid



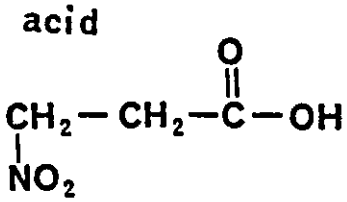
2. Kojic acid



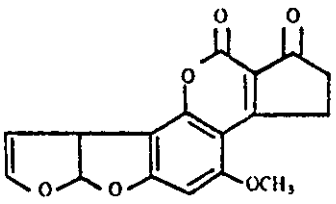
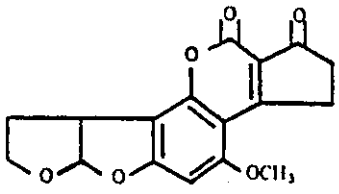
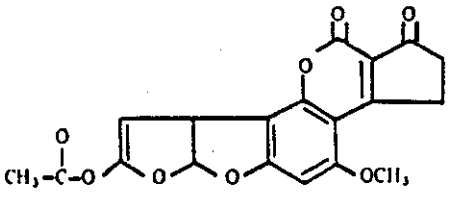
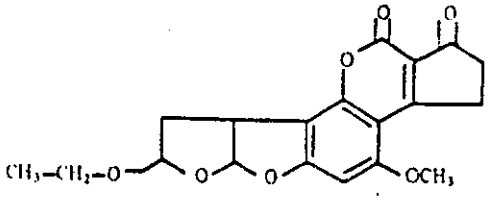
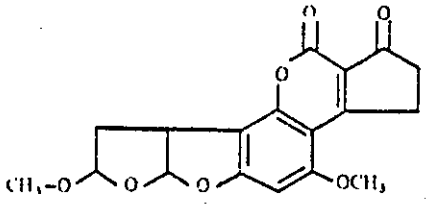
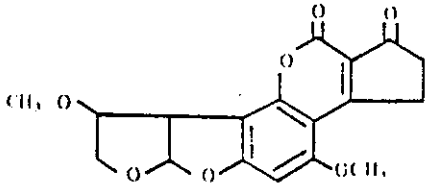
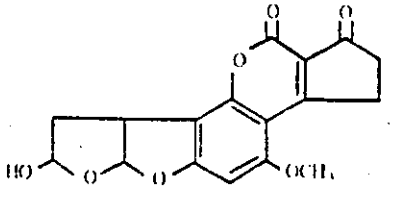
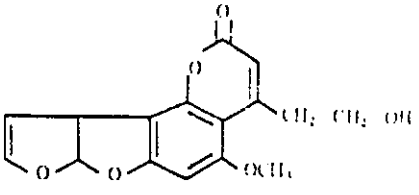
3. Oxalic acid

$$(COOH)_2 \cdot 2H_2O$$

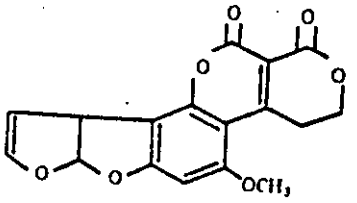
4. β-nitropropionic acid



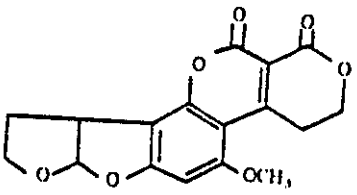
5. Aflatoxins

Name	Structure
Aflatoxin B_1	
Aflatoxin B_2	
1-acetoxy-Aflatoxin B_2	
1-ethoxy-Aflatoxin B_2	
1-methoxy-Aflatoxin B_2	
2-methoxy-Aflatoxin B_2	
Aflatoxin B_{2a}	
Aflatoxin B_1	

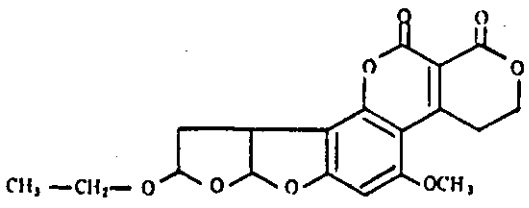
Aflatoxin G_1



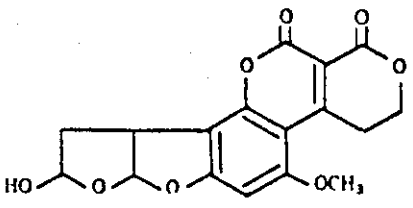
Aflatoxin G_2



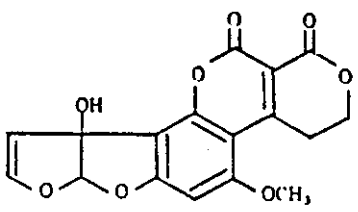
2-ethoxy-Aflatoxin G_2



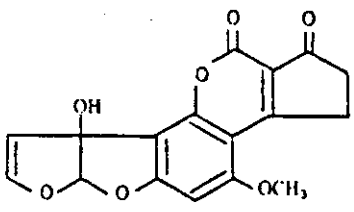
Aflatoxin G_{2a}



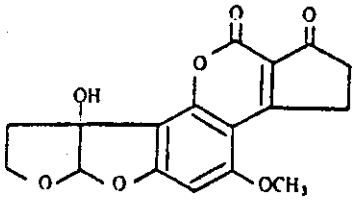
Aflatoxin G-M_1



Aflatoxin M_1



Aflatoxin M_2



Aflatoxin P_1