THE BIOSORPTION OF PARTICULATES AND METAL IONS BY FUNGAL MYCELIUM.

by

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To my parents and friends

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SUMMARY

Particulate adsorption by *Mucor flavus* and *Neurospora crassa* is a physical property of the cell wall, independent of both cellular metabolism and the production of extracellular polymers. Initial attractive forces responsible for particulate adsorption by *N. crassa* are mainly electrostatic in nature and this mechanism probably holds for *M. flavus* also. The outer glucan layer of the cell wall of *N. crassa*, although able to adsorb particulates, was not as efficient as the underlying protein layer at particle adsorption. Young, growing mycelium generally adsorbs the largest amount of particles, due to the continued production of adsorption sites and the entrapment of particulates by hyphae. Factors increasing adsorption include nutrient starvation of mycelium and incubation with low concentrations of magnesium ions. Relatively high concentrations of mercury and copper ions decrease adsorption perhaps due to the precipitation and adsorption of the corresponding metal sulphides on the mycelial surface thereby effectively physically interfering with further particulate adsorption. Optimum conditions for adsorption are a temperature of 25^0C and a slightly acid pH value. Also, small particles are more readily adsorbed than large particles.

Mucor flavus can adsorb clays and this ability may be used to treat industrial effluents which contain large amounts of clay minerals. An acid pH and a temperature of 25^0C are optimum conditions for clay adsorption by the fungus.

Low concentrations of montmorillonite and kaolinite increase biomass production by *Aspergillus niger* in submerged culture, due to the inhibition of pellet formation by the fungus. The clays cause *A. niger* to grow in a more filamentous

form and presumably would affect other fungi in a similar way. The use of clays to control fungal morphology may be important in several industrial fermentations.

Low concentrations of the fungicide thiram stimulated the growth of *Aspergillus niger* in the presence of montmorillonite.

Immobilization of fungi by magnetic means is possible due to their ability to adsorb magnetite. This method could also be used to remove fungi from fermentation media as an alternative to filtration or centrifugation. Even though older hyphae of *Penicillium chrysogenum* are unable to adsorb magnetite, this fungus can still be magnetically immobilized if it is grown from a spore suspension in the presence of magnetite. Either the spores or young hyphae of this fungus adsorb magnetite, producing pellets with magnetic properties. Magnetite adsorption occurs optimally at a temperature of 25^0C and is constant over a wide range of pH values. Waste mycelium of *A. niger* from the surface fermentation method of citric acid production can be magnetically removed from solution after adsorbing magnetite. Dilute solutions of sodium hydroxide and sodium bicarbonate desorbed magnetite attached to mycelial surfaces.

Silver is accumulated selectively by *A. niger* waste mycelium produced by the surface fermentation method of citric acid production. The process is rapid, maximum uptake occurring twenty minutes after initial exposure of the mycelium to a silver solution. Silver accumulation by the mycelium is relatively insensitive to changes in pH and temperature, a slight decrease in uptake only occurring at a temperature of 80^0C. Dilute solutions of H_2SO_4 and HNO_3 desorb silver from the mycelial surface. However, this process is relatively inefficient and more effective desorbents need to be found to make the silver accumulation process economically viable.

1. FACTORS AFFECTING AND MECHANISMS INVOLVED IN PARTICULATE ADSORPTION BY FUNGI.

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INTRODUCTION: MICROBIAL ADHESION TO SURFACES.

Solid surfaces in the natural environment are often coated with a thin film of microorganisms of which bacteria are the most numerous. The term "surface" here is used to describe not only solid-gas or solid-liquid interfaces but also gas-liquid and liquid-liquid interfaces which are probably equally important in relation to microbial growth in the environment.

Most microorganisms probably spend at least a part of their life-cycle associated with an interface, particularly solid surfaces. Important ecological and economical implications result from the adhesion of microorganisms to surfaces. For example, it has been argued that microorganisms show the greatest metabolic activity when associated with inert particulates and therefore have an important function in the environment (Wardell *et al.*, 1983). Significant interest has also been shown in the areas of biodeterioration, biocatalysis and medical microbiology due to biofilm production in relation to microbial adhesion. The term biofilm is used to define the discrete aggregation of organisms, in particular microrganisms, at an interface (Hamilton, 1987). An increase in our understanding of interface-microbe interactions will inevitably lead to:

(1) A greater understanding of the reasons for microbial adhesion in the environment

(2) Ways of increasing or maintaining biofilm production for biocatalysis purposes; and

(3) Ways of decreasing biofilm production for the reduction of problems caused by microorganisms in (a) the biodeteriation of commercial products and process equipment and (b) dental caries.

Ecological apects of microbial adhesion to surfaces.

Adhered microbes or biofilms occur in the environment wherever a surface is available for growth. Examples include: the surface of stones and particulate matter in dilute aqueous environments, soil and sediment particles, leaves, roots and germinating seeds of plants, dental plaque and intestinal and rumen epithelial tissues in animals. The activities of these biofilms appear of fundamental importance in nature, where the number of organisms per cm^2 of surface normally exceeds that in $1cm^3$ of flowing water by a factor of at least 200 (Hamilton, 1987). Given the apparent ubiquitous nature of microbial adhesion the conclusion must be drawn that cell attachnment confers some kind of ecological advantage over the free-living condition.

Many natural habitats are oligotrophic, that is, the concentration of nutrients available for growth are very low. For example, natural aquatic environments are nutritionally limited, due to the dilution and dispersion of available nutrients occuring through diffusion and water currents (Jannasch, 1958). However, ions and various macromolecules can be adsorbed at inert surfaces (Marshall, 1980, Zobell, 1943) leading to the concentration of nutrients. It will obviously be beneficial for any organisms growing in an oligotrophic habitat to be able to attach to such a surface and make use of the relatively high concentrations of nutrients present. One of the first demonstrations of such an advantage was shown, in a now historical piece of work, by Zobell (1943). He observed that the growth of bacteria in an oligotrophic environment could be stimulated by the inclusion of various glass surfaces. An important outcome of this work was the realization that the beneficial

effect of solid surfaces is usually evident only in very dilute nutrient solutions. The following are further examples of surfaces stimulating microbial activity:

(1) The growth of *Escherischia coli* in glucose-peptone water at glucose concentrations of less than 25mg l^{-1} only occurred in the presence of glass beads (Heukelekian & Heller, 1940).

(2) The growth of *Bacillus subtilis* in dilute media occurred only in the presence of chitin particles (Jannasch, 1958).

(3) Enteric bacteria adsorbed to glass surfaces were found to be metabolically more active than organisms in free suspension (Hendricks, 1974).

(4) A two-fold increase in the growth rate of a *Pseudomonas* sp. was observed in the presence of a glass surface (Ellwood *et al.*, 1982).

However, a number of studies have observed that microbial adhesion to surfaces may not always increase metabolic activity and in some cases may actually decrease it. Azam and Cho (1987) cite a number of such examples including an experiment demonstrating that, in ocean profiles, free-living and attached bacteria exhibited comparable growth rates. Gordon *et al.*, (1983) found that the respiratory activity of a periphytic marine bacterium, *Vibrio alginolyticus*, decreased when attached to hydroxyapatite. Two papers published by Hattori & Furusaka (1960, 1961) show that oxygen consumption by *Escherischia coli* and *Azotobacter agile* was inhibited when the cells were attached to an anion-exchange resin.

If particles are sites of nutrient enrichment, how can the above results showing a reduction in metabolic activity be explained? Hattori & Furusaki (1960, 1961) attribute the observed decrease in oxygen consumption by *E. coli* and *A. agile*

to calculated pH differences between the surface microenvironment and the bulk solution. Gordon *et al.*, (1983) mention several factors including oxygen availability, trace metal availability and pH as possible causes of the metabolic inhibition observed in their experiments. Obviously diverse factors are involved in these observations and results obtained are bound to differ due to use of different growth media, organisms and surfaces in experiments.

The effect of nutrient concentration at surfaces must be of more benefit to copiotrophic microorganisms, which need relatively high levels of nutrients, than it is to oligotrophs, which can grow in conditions of extremely low nutrient avalability. It follows therefore, that copiotrophic organisms should show greater adhesive abilities than oligotrophs.

Kjelleberg *et al.*, (1982, 1983) studied two copiotrophic bacteria, a *Vibrio* sp. strain DW1 and a *Pseudomonas* strain P9, which both show a pattern of cell fragmentation when nutrient supply is decreased. Small metabolically competent cells or "dwarf cells" are produced as a result of this fragmentation process and these dwarfs are able to attach to surfaces and grow to normal size on the surface-accumulated nutrients. The increased ability of these bacteria to attach to surfaces would seem to correlate with the observations that adhesion only confers an increase in metabolic activity or growth at nutrient concentrations below a certain value (Zobell, 1943, Atkinson & Fowler, 1974).

The ability of microorganisms to adhere to surfaces is also necessary if they are to remain in a particular habitat. Microbial growth in the oral cavity provides a clear illustration of this. Saliva contains, on average, 10^8 microorganisms ml^{-1}, largely derived from surfaces of the oral tissues. The flow rate of saliva through the mouth produces an effective dilution rate greater than 3.0 l h^{-1}. In order for these organisms to stay in saliva their doubling times would have to be less than 20 minutes. The division rate of oral bacteria *in vivo* is not known with certainty, but values of less than 1 to 2 hours have yet to be reported. Thus, unless organisms

adhere to a surface, saliva flow and swallowing would rapidly result in their removal (wash-out) from the mouth (Ellwood *et al.*, 1982). Similar arguments could be proposed for the growth of bacteria in a flowing stream or perhaps in soil following heavy rainfall.

Biotechnological aspects of microbial adhesion to solid surfaces.

The ability of microorganisms to adhere to surfaces has been exploited successfully for commercial purposes for many years in the form of biofilm fermenters. One of the earliest examples of the use of biofilms is in the vinegar manufacturing industry. The "quick" process was invented in 1823 by Scheutzenbach and used wooden vats with perforated bottoms. These vats were packed with wood shavings on which microbial films were allowed to develop. Alcohol solutions were trickled through the vats and were oxidized to acetic acid by the microbial films on the shavings. Presently, several industrial fermentations are carried out using microbial film fermenters, examples of which are shown in Table 1.1. More recent examples of proposed biotechnological uses of microbial surface adhesion are given in Table 3.1 (Chapter 3).

Biofilm reactors are widely used for the purpose of water purification, where microbes are allowed to grow on an inert support such as gravel. These reactors are known as trickle (or percolating) filters. More advanced forms of effluent treatment using biofilms are being proposed; examples of these include:

(1) The growth of microorganisms on a large rotating disc used as a secondary treatment device for municipal sewage (Borchardt, 1971).

(2) Anaerobic methods of effluent treatment using biofilms. The advantages of lower energy costs and low sludge production for anaerobic wastewater treatment processes have recently come to light, mainly because the

Table 1.1 Industrial fermentations using microbial films (Atkinson, 1979)

Process	Biological Wastewater Treatment	
	Trickling filter	Rotating Disc
Objective	Biological oxidation of industrial and domestic effluent.	
General characters	Non-aseptic microbial growth occurs in a packed bed. Waste-water distributed intermittently over the packing. Aerobic; packing supported on a grid structure, enhancing aeration by natural convection.	Microbial growth on discs rotating in a vertical plane, the disc dipping into wastewater. Microbial growth is alternately in contact with nutrients and air.
Control of film thickness	Self-regulating. Thick film sloughs off or is consumed by insects or worms.	Excess is sloughed from discs.

Process	"Quick" vinegar Process.	Animal Tissue Culture.
Objective	Oxidation of alcohol by acetic acid bacteria.	Growth of animal cells in a surface layer for the culture of viruses.
General characters	Similar in principle to the trickling filter, but with forced aeration. Wine or other feed liquor recirculated over beech-wood chips or similar packing. Batch process (4-5 days).	Animal tissue minced and reduced to single cells by enzyme action. Cells adhere to surfaces provide and grow as a film in the presence of a suitable medium. Used for virus culture. Strictly aseptic.
Control of film thickness	Low growth rates and high substrate conversion. Bacterial film accumulates and packing discarded after several years.	Use of appropriate media.

disadvantage of low rates of degradation using anaerobic treatments has been overcome by allowing organisms to grow as a biofilm on fine suspended particles such as sand. Biofilm production overcomes slow treatment rates by (a) increasing the biomass concentration in the bioreactor and (b) causing retention of the organisms present (Denac & Dunn, 1988).

An interesting use of microbial biofilm has been developed by the Homestake Mine in Lead, South Dakota, U.S.A. (Whitlock & Mudder, 1985). In this process, mutant strains of bacteria are grown on large discs called Rotating Biological Contactors and the biomass effectively removes all toxic parameters from cyanidation wastewaters. During the biological treatment cyanide, thiocyanate, ammonia and toxic metals are removed; the metals of concern being nickel, copper, lead and zinc. The selection of the biological process was made because:

(1) Treatment efficiency equalled or generally exceeded all other tested methods

(2) Toxicity tests on the biologically treated effluent gave this method of effluent treatment a substantially better rating than other forms of chemical treatment

(3) Biological treatment is less subject to upset due to changes in chemicals or other factors and recoveries from upset do not substantially effect the water quality of the effluent as would large chemical additions that may be needed to deal with such an emergency

(4) The effluent produced by living systems is highly compatible with living systems in the receiving stream and

(5) Plant construction costs and daily operational costs are substantially lower than for chemical systems.

Microbial surface adhesion is also important in the mining industry. Bacteria are used in a variety of hydrometallurgical processes that involve oxidation reactions. Examples include the heap leaching of low grade copper and uranium ores (Hutchins *et al.*, 1986). Microorganisms implicated in the process are chemoautotrophic bacteria such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. Some proposed mechanisms by which microbes influence the oxidation of sulphide ores are summarized in Table 1.2., and attachment is a pre-requisite for some of these mechanisms.

Mechanisms of microbial adhesion to surfaces.

This subject has been extensively reviewed (Wardell, 1983, Lipps & Jessup, 1979, Rogers, 1979). These reviews are devoted in the main to bacteria which can be regarded as particles which have a negative surface charge and therefore their behaviour can be readily related to that of colloidal solutions. As a result, many of the theories describing colloid behaviour have been applied to bacterial systems.

There are three stages in the adhesion of microorganisms to a surface:

(1) The deposition of the organism onto the surface (adsorption step)

(2) The permanent attachment to the surface. This often involves polymers acting as bridges between the two surfaces

(3) Colonization of the surface by growth of the organism.

Table 1.2 Mechanisms of bacterial oxidation of sulphide ores. (Ash 1979)

Sulphide ores – overall reaction
$MS + 2O_2 \rightarrow MSO_4$
Mechanism 1 (Direct, requires attached bacteria)
$MS_{solid} + 2O_2 \xrightarrow{\text{bacteria}} M^{2+} + SO_4^{2-}$
Mechanism 2 (indirect, requires presence of iron)
$MS + 8Fe^{3+} + 4H_2O \rightarrow M^{2+} + SO_4^{2-} + 8Fe^{2+} + 8H^+$
Mechanism 3 (sulphur oxidation, requires attached bacteria)
$MS_{solid} + {}^1/_2O_2 + 2H^+ \rightarrow M^{2+} + S^0 + H_2O$
Mechanism 4 (corrosion cell, promoted by attached bacteria)
$MS_{solid} \rightarrow M^{2+} + S^0 + 2e^-$
$2e^- + 2H^+ + {}^1/_2 O_2 \rightarrow H_2O$

Studies have been carried out on the deposition of organisms to a wide range of surfaces including glass, wire, polystyrene and hydroxyapatite. In the environment however, surfaces exposed to to aqueous solutions will rapidly adsorb any polymers present. This means that data on the deposition of bacteria onto clean surfaces is difficult to relate to natural systems.

The major surface forces that operate in particle deposition are:

(a) London-Van der Waals forces

(b) Double-layer electrostatic interactions and

(c) Bridging interactions.

Once a particle has been brought into close proximity to a surface, surface particle interactions determine whether or not the particle is captured. The best described treatment of the interaction of small particles at close separation distances is attributable to the Derjaguin and Landau and the Verwey and Overbeek (D.L.V.O.) theory of colloid stability (Lipps & Jessup, 1979). The D.L.V.O. theory provides a useful guide to the interpretation of data on adhesion but care should be taken when considering bacterial systems as many of the key parameters required to calculate key functions cannot be accurately determined. Physical parameters such as temperature, surface charge and solution pH, the latter two being connected, are all involved in the theory and affect particle deposition to surfaces. In addition to D.L.V.O. interactions other parameters affecting the attachment of microorganisms to solid surfaces include (Brierley and Lanza, 1985):

(1) Chemotaxis

(2) Ability of the organism to produce polymeric fibrils (Rogers, 1979)

(3) Wettability of the substrate (Dexter *et al.*, 1975)

(4) Time allowed for microbial attachment

(5) Growth phase of the culture

(6) Nutrient concentrations (Fletcher, 1977)

(7) Polymer interactions and

(8) Cell surface hydrophobicity (Stenstrom, 1989).

Thus, in general, as bacteria approach a surface electrostatic and Van der Waals attractive forces can hold a cell for a short time (according to D.L.V.O. theory). During this period cell surface polymers are able to either adsorb non-specifically to the surface or interact specifically with complementary polymers adsorbed to or forming a part of the surface (polymer bridging). This increases the possibility of attachment until enough links or bridges have formed to irreversibly hold the cell. Further attachment could occur by the synthesis of insoluble macromolecules, such as the mutan produced by *Streptococcus mutans*. Growth of the attached cells would lead to microcolonies and eventually to film formation.

Fungal adhesion to solid surfaces.

Unfortunately, the attachment of filamentous fungi to solid surfaces has not received the same attention as that devoted to bacteria. This is probably for several reasons:

(1) The theories of colloid behaviour can be more easily applied to bacterial suspensions

(2) Bacteria have been considered more important in biofilm production (they are predominant in biofilms) and are responsible for processes such as biocorrosion and dental decay

(3) The role of bacterial adhesion in ecological and biotechnological processes has been recognised and their importance in these areas has led to many studies on bacterial adhesion mechanisms.

However, recent advances in fungal biotechnology and ecology, involving the ability of fungi to adsorb particulates and attach to surfaces, seem certain to increase the interest in studies on fungal adhesion.

Ecological aspects of fungal adhesion.

The role of filamentous fungi in binding soil particles into aggregates by adhesion as well as physical enmeshment is recognized (Aspiras *et al.*, 1971, Sutton & Sheppard, 1976, Fletcher *et al.*, 1980). Many fungi, in particular yeasts, are able to produce extracellular polymers (Brierley & Lanza, 1985) which could possibly play a role in surface adhesion. The aggregation of soil particles by fungi increases in importance when related to the observed growth of fungi in very low nutrient conditions (Parkinson *et al.*, 1989). The growth of fungi in soils lacking large amounts of available carbon should be possible and therefore soil particle aggregation by fungi will occur even in these nutrient deficient soils. Tiessen & Stewart (1988) observed that fungi formed larger, elongated aggregates of soil mineral components than bacteria. These aggregates contained a mixture of organic and mineral materials, measuring one tenth to several millimetres across, and may

perform an important role in the overall aggregation and structure formation of soils (Tisdall & Oades, 1982). In addition, Lynch (1981) has shown that the cell homogenate of a fungus, *Mucor hiemalis*, was able to promote the aggregation of soil particles. Consideration of the above factors demonstrates the extreme importance of fungi in soil aggregation.

Aquatic hyphomycetes grow following attachment to submerged plant detritus in streams and rivers (Sridhar & Kaveriappa, 1987), and a number of mechanisms appear to be involved in the attachment of marine fungi to surfaces. In addition to initial impaction and entrapment, the production of appendages by ascospores has an important role in the establishment of marine fungi on surfaces. For example, attachment may occur through the production of hair-like appendages by the spores (Jones & Moss, 1978), or by the production of mucilaginous fibrillar pads (Jones *et al.*, 1980). In aquatic hyphomycetes, both the germinating spores and hyphae developing from them, are surrounded by a polysaccharide sheath which appear to promote adhesion to a surface (Wardell *et al.*, 1983). Mucilaginous sheaths produced by hyphae of fungal parasites are thought to attach hyphae to leaf surfaces. For example in *Helminthosporium maydis*, a sheath was observed to spread out from around the germ tube to form a thin film over the surrounding leaf surface (Potter *et al.*, 1980)

The adhesive properties of fungi also involve them in the formation of desert varnishes. These are usually referred to as a coating of ferromanganese oxides and clays that develops on rock surfaces in arid to semi-arid regions. However, varnishes found in different regions vary and some forms may lack clays, or be rich in iron but poor in manganese. Taylor-George *et al.*, (1983), suggest that the primary steps of varnish formation are caused by microcolonial fungi establishing themselves on rock substrates, presumably by adhesion, and then accumulating wind deposited clay and other mineral particles on their surface.

The attachment of fungi to insoluble substrates has been noted and it has been suggested that hyphal contact may be necessary for metabolism or

transformation of these substrates. Substrate contact will be especially necessary if organisms are unable to excrete the enzymes required for degradation or transformation of the substrate. *Phanerochaete chrysosporium* exhibited physical association with tree bark during growth (Daugalis & Bone, 1978) a process which may be an important consideration in the growth and cellulase production by this organism. Binder & Ghose (1978) noted that *Trichoderma viride* adsorbed cellulose onto its hyphal surface during growth, even though the fungus is able to produce an extracellular cellulase enzyme. As a result of a series of experiments they suggested that contact of the fungal hyphae with the insoluble substrate was necessary for the high production of cellulase. Close mycelial association of *Aspergillus flavus* with an insoluble lignin compound has been observed during the breakdown of this substrate (Betts *et al.*, 1987). Erosion of the compound was noted in the vicinity of attached mycelia. Paszczynski *et al.* (1986) found that degradation of 2,6-dimethoxyphenol by *P. chrysosporium* resulted in the formation of the dimer, tetramethoxy-p-dibenzoquinone which they showed was attached to the mycelial surface. It was suggested that contact may be necessary for the reaction to occur and that this may be explained by the presence of a peroxidase enzyme attached to the hyphae.

The above discussion provides more evidence for the need of substrate contact with fungal mycelium for substrate metabolism if the enzymes necessary for product synthesis are cell bound.

Biotechnological aspects of fungal adhesion to surfaces.

The ability of fungi to adhere to surfaces has been used for biotechnological purposes as it is an effective and economical way of immobilizing fungal biomass. A wide variety of materials and fungi are used and the technique of surface immobilization avoids any treatments with harsh chemicals that are sometimes necessary with other immobilization methods. Examples of surface-immobilized

fungal biomass are given in Table 3.2 (Chapter 3). The table also shows the varied uses of fungi for biotechnological purposes.

Interest in fungal immobilization has led to studies on the mechanisms of fungal adhesion to surfaces, since it is hoped that a greater understanding of the mechanisms involved will inevitably lead to the use of more suitable supports and growth conditions. Presently the factors which are thought to affect fungal attachment to and growth on surfaces include:

(1) Electrostatic attraction (Mozes *et al.*, 1987)

(2) Cell surface hydrophobicity (Mozes *et al.*, 1987)

(3) The pore sizes of carriers used for immobilization (Messing *et al.*, 1979).

The production of extracellular polysaccharides, previously suggested to play a role in surface adhesion, by fungi, could also have biotechnological uses. In particular, the polysaccharide pullulan produced by the yeast, *Aureobasidium pullulans*, has patented applications including the flocculation of suspended clay slimes from hydrometallurgical processes such as the extraction of uranium, potash and aluminium (Zajic & LeDuy, 1973).

The first study of particulate adsorption by fungi was made by Williams (1918) who reported the adsorption of colloidal gold by fungal mycelium. Since then the ability of fungi to adsorb particulates has received little attention, apart from work on clay adsorption by fungal mycelium in the early eighties by Brierley *et al.* (1981), and also studies made by Wainwright *et al.* (1986). These authors suggested that the phenomenon might be used industrially to adsorb particulate waste from effluents, or to remove precious particulates from suspension. The applicability of particulate adsorption to waste treatment was emphasized by an investigation by Randol International (Michaelis, 1985). This environmental consultancy firm

encountered numerous smaller effluent treatment needs where 100-1000m^3 water per day was treated and where effluent metal removal was not achieved primarily because the clarification step failed to adequately remove suspended precipitates.

Clays, particularly when colloidal in nature, are difficult to remove from suspension and natural settling may involve a period of a decade or more. The large scale extraction of phosphates in the U.S.A. provides a good example of where large amounts of clay suspensions, which are difficult to de-water, are produced (Brierley & Lanza, 1985). Fungal particulate adsorption could be used to treat both of the above environmental problems. The ability of fungi to adsorb clays from suspension and the effects of clays on fungal growth are discussed in Chapter 2.

This chapter is directed towards the investigation of factors affecting particulate adsorption by fungi in an attempt to determine the mechanisms involved in the process. Hopefully, this information should shed some light as to the commercial viability of the adsorption phenomenon. A range of fungi were tested for their adsorption abilities and in particular studies were made on the cell wall of *Neurospora crassa* to determine the involvement of various hyphal wall components in particulate adsorption. The adsorption of a wide range of particulates was also examined.

MATERIALS AND METHODS.

Effect of different carbon concentrations in the growth medium on the subsequent adsorption of zinc dust by *Mucor flavus*.

Mycelial discs (11mm diameter) of *M. flavus* were added to Raistrick's medium (Raistrick & Vincent, 1948) (100ml, pH 6.8) in Erlenmeyer flasks (250ml), containing either 0.1, 1, 5, 10 or 20% carbon (w/v) as sucrose. Flasks were shaken at 25^0C for 7 days at 150 r.p.m. The fungus grew in the form of large mycelial clumps which were harvested by filtration through Whatman No.1 filter paper. Fresh mycelium (2.5g) from each different carbon concentration was then added to sterile distilled water (50ml) amended with steam sterilized zinc dust (0.2g). The zinc dust was sterilized by steaming for 60 minutes on three successive days. Flasks were shaken in triplicate at 25^0C, for 24 hours at 150 r.p.m. to cause contact of the mycelium with the particulate zinc. After this time the contents of the flasks were filtered through pre-dried, pre-weighed Whatman No.1 filter paper and any zinc loosely adhered to the mycelium was washed onto the filter using distilled water. The mycelium plus adsorbed zinc was removed and the filter containing unadsorbed zinc dried to constant weight (50^0C). The amount of particulate matter adsorbed was then determined by appropriate subtraction.

Effect of different nitrogen and phosphorus concentrations in the growth medium on the subsequent adsorption of zinc dust by *M. flavus*.

Mucor flavus was grown in Raistrick's medium (100ml, pH 4.0) containing 10, 100, 1000 or 10 000 µg N or P ml^{-1} for 7 days at 25^0C and 150 r.p.m. Fresh mycelium (1g) from each different nitrogen and phosphorus concentration was then transferred to sterile distilled water (50ml) containing steam sterilized zinc dust (0.2g) and the flasks shaken in triplicate for 24 hours at 25^0C and 150 r.p.m. The

amount of zinc dust adsorbed by the mycelium from each different growth medium was then determined as previously described.

Effect of different carbon sources supplied in the growth medium on the adsorption of elemental sulphur (S^0) by *M. flavus*.

Mucor flavus was grown in Raistrick's medium (100ml, pH 6.8) in Erlenmeyer flasks (250ml) containing either galactose, glucose, lactose, fructose, mannose or sucrose (1% w/v carbon) for 7 days at 25^0C and 150 r.p.m. Fresh mycelium (3g) was transferred to sterile distilled water (100ml) amended with steam sterilized S^0 (0.75g) and shaken at 150 r.p.m. for 24 hours at 25^0C. The amount of sulphur adsorbed was then determined.

Comparison of sulphur adsorption by *M. flavus* in Czapek Dox liquid medium and distilled water.

Mucor flavus was grown in Czapek Dox medium (100ml) in Erlenmeyer flasks (250ml) for 7 days at 25^0C and 150 r.p.m. Fresh mycelium (5g) was added to either sterile distilled water (100ml) or Czapek Dox medium (100ml) both containing steam sterilized S^0 (1g). Sufficient flasks were set up in triplicate to compare the adsorption of S^0 by nutrient supplemented mycelium to that of mycelium in distilled water over an incubation period of 5 days at 25^0C and 150 r.p.m.

Comparison of sulphur adsorption by *M. flavus* in distilled water containing various carbon sources with adsorption in distilled water alone.

Mucor flavus was grown in Czapek Dox liquid medium (100ml) for 7 days at 25^0C and 150 r.p.m. Fresh mycelium (3g) was transferred to either sterile distilled

water (100ml) or sterile solutions of sucrose, glucose, galactose and mannose (100ml, 1% w/v sugar) amended with steam sterilized sulphur (0.5g). Flasks were shaken at 150 r.p.m. for 24 hours at 25^0C. After this time sulphur adsorption by carbon supplemented mycelium was compared with that of mycelium in distilled water.

Ability of various fungi to adsorb sulphur.

Mucor flavus, *Neurospora crassa*, *Penicillium chrysogenum*, *Aspergillus niger*, *Aspergillus repens* and *Botrytis cinerea* were grown in Czapek Dox medium (100ml) for 7 days at 25^0C and 150 r.p.m. *Thermomucor indicae-seudaticae* was grown in malt extract broth for 7 days at 37^0C and 150 r.p.m. Fungi which grew as pellets were harvested from the growth medium by filtration through sterilized nylon mesh filters (1mm diameter mesh). Fungi which grew as mycelial clumps wre harvested as described previously. Fresh mycelium (5g) of each fungus was transferred to sterile distilled water (100ml) amended with sulphur (1g) and shaken for 24 hours at 25^0C (except *T. indicae-seudaticae* which was incubated at 37^0C) and 150 r.p.m. The amount of sulphur adsorbed by pellet forming fungi was determined by filtering the flask contents through a nylon mesh filter which trapped the mycelial pellets but allowed unadsorbed sulphur to pass through. Any loosely adhered sulphur was washed off using distilled water and added to the filtrate already collected. The amount of unadsorbed sulphur was then determined by filtration. Sulphur adsorption by fungi growing as mycelial clumps was determined as previosly described.

Effect of addition of sorbose to growth medium on the subsequent adsorption of sulphur by *Neurospora crassa*.

Neurospora crassa was grown for 7 days in either Czapek Dox medium supplemented with 2% (w/v) sorbose (100ml), or in Czapek Dox medium alone (100ml). The adsorption of sulphur (1g) from sterile distilled water (100ml) by sorbose grown mycelium (5g) was compared with that of mycelium (5g) grown in Czapek Dox alone. Flasks were shaken in triplicate for 24 hours at 25^0C at 150 r.p.m. to allow sulphur adsorption to occur.

Effect of age of mycelium grown in Czapek Dox medium on sulphur adsorption.

Mucor flavus, *Neurospora crassa*, *Aspergillus niger* and *Penicillium chrysogenum* wre grown in Czapek Dox medium (100ml) for 5, 10, 15 or 25 days at 25^0C and 150 r.p.m. Fresh mycelium (5g) of each different fungus and age was added to sterile, distilled water (100ml) containing sterile sulphur (1g) and the amount of sulphur adsorbed by mycelium determined.

Effect of age of *M. flavus* mycelium grown in carbon-limiting medium on sulphur adsorption.

Mucor flavus was grown in Raistrick's medium (100ml, pH 6.8) containing sucrose (0.15% w/v carbon) for 5, 10, 15, 21 and 28 days at 25^0C and 150 r.p.m. Mycelium (4g) of each different age was added to sterile, distilled water (100ml) amended with sterile sulphur (1g) and mixtures shaken (24 hours, 25^0C, 150 r.p.m.) to allow sulphur adsorption to occur. The amount of sulphur adsorbed by different aged mycelium was then calculated.

Effect of mild acid and mild alkali treatment on sulphur adsorption by 7 day and 28 day old mycelium of *M. flavus*.

Mucor flavus grown in Czapek Dox medium (100ml) for 7 and 28 days was collected and then shaken in solutions (100ml) of H_2SO_4 (0.1M) and NaOH (0.1M) for 30 minutes at 25^0C and 150 r.p.m. Mycelium was removed from these solutions and washed twice with sterile, distilled water. Biomass (5g) of each age and treatment was transferred to sterile distilled water (100ml) containing sterile sulphur (1g). Mixtures were shaken (25^0C, 150 r.p.m., 24 hours) and the amount of sulphur adsorbed determined.

Scanning electron microscopy (SEM) studies.

The following samples of *M. flavus* were examined by SEM:

(1) Seven and 28 day old mycelium grown in Czapek Dox medium

(2) Twenty eight day old mycelium grown in carbon limiting medium

(3) Twenty eight day old mycelium grown in Czapek Dox medium and washed in H_2SO_4 and

(4) Twenty eight day old mycelium grown in Czapek Dox medium and washed in NaOH.

Fungal samples were prepared for SEM work as follows:

Primary fixation: 2% paraformaldehyde plus 2.5 % glutaraldehyde in 0.1M sodium cacodylate at 4^0C.

Buffer wash: overnight and 3 half hour washes in 10% sucrose in 0.1M sodium cacodylate at 4^0C.

Secondary fixation: in 2% osmium tetroxide for 1 hour at room temperature.

Dehydration in acetone for 15 minutes in steps of 30, 50, 75, 95 and 100% acetone.

Drying : critically point dried using carbon dioxide.

Coating: sputter coated with gold.

Specimens were examined using a Philip's S600 SEM.

Effect of fungal starvation on adsorption of sulphur.

Mucor flavus and *N. crassa* were grown in Czapek Dox medium for 7 days and the biomass of each fungus harvested and divided into two halves. One half of the biomass was transferred to Erlenmeyer flasks (250ml) containing sterile distilled water (150ml) and incubated without shaking for 7 days at 25^0C. Starved mycelium (5g) was then transferred to sterile distilled water (100ml) containing sterile sulphur (1g). Adsorption of sulphur by this mycelium was then compared to adsorption by the other half of the mycelium which was left to incubate at 25^0C, without shaking, under non-starvation conditions (ie; left in Czapek Dox medium for 7 days).

Ability of killed mycelium to adsorb particulates.

Mucor flavus was grown in Czapek Dox medium for 7 days and fresh mycelium (5g) was incubated in KCN (0.01M, 100ml); or (2.5g) in solutions (50ml) of carbonylcyanide n-chlorophenyl hydrazone (CCCP) (50 µM); or cycloheximide (100µg ml^{-1}). Mycelium was shaken (25^0C, 150 r.p.m.) for 8 hours in the presence of the inhibitors after which time sterile sulphur (1g to 100ml, 0.5g to 50ml) was added to the flasks to determine the adsorptive abilities of the inhibited mycelium. The effect of the various inhibitors (at the concentrations used) on fungal growth was compared in a separate experiment using non-inhibited mycelium as a control.

Inhibitors were added after 2 days growth in Czapek Dox medium and total biomass production by inhibited and non-inhibited mycelium was determined after 7 days.

Effect of pH on sulphur adsorption by various fungi.

Mucor flavus, *A. niger*, *P. chrysogenum* and *N. crassa* were grown in Czapek Dox medium for 7 days. Mycelium of *M. flavus* (2.5g) and mycelium of the other fungi (3g) were transferred to a range of sterile solutions (100ml, pH 2-11) containing steam sterililized sulphur (0.5g). The solution pH was made by adding either dilute sodium hydroxide or dilute sulphuric acid as required to distilled water. All flasks were shaken in triplicate (24 hours, 25^0C, 150 r.p.m.) and the amount of sulphur adsorbed at each pH value determined.

Effect of pH on adsorption of activated carbon by *M. flavus*.

Mucor flavus was grown in Czapek Dox medium for 7 days. Fresh mycelium (2g) was transferred to a range of sterile solutions (50ml, pH 3-9) amended with 0.5g steam sterilized activated carbon. All flasks were shaken (48 hours, 25^0C, 150 r.p.m.) and the amount of activated carbon adsorbed at the different pH values determined.

Effect of pH on adsorption of zinc dust by *M. flavus*.

Fresh mycelium (2g) of *M. flavus* grown in Czapek Dox liquid medium for 7 days was transferred to a range of sterile solutions (50ml, pH 2-9) amended with sterile zinc dust (0.2g). Flasks were shaken in triplicate (48 hours, 25^0C, 150 r.p.m.) and the amount of zinc adsorbed at different pH values determined.

The effect of temperature on sulphur adsorption by *M. flavus*.

Mucor flavus was grown for 7 days in Czapek Dox medium. Mycelium (5g) was transferred to sterile distilled water (100ml) amended with sterile sulphur (1g). Before addition of mycelium flasks were shaken at experimental temperatures for 2 hours to allow solution temperatures to reach the experimental values used. Flasks were incubated in triplicate for 24 hours at 4, 25, 30 and 37^{0}C and 150 r.p.m. The amount of sulphur adsorbed at each different temperature was then determined.

Effect of particle size on adsorption of rock potash by *N. crassa*,

Rock potash was sieved and subsequently sorted into various particulate sizes (0-0.251, 0.251-0.295, 0.295-1.0, 1.0-1.4, 1.4-2.0 mm diameter). All samples of particulates used were washed with distilled water. This was especially necessary for the larger particulates as they had a large amount of very fine particles attached to their surface. The rock potash was then dried at 37^{0}C until constant weight was achieved. Particulates were sterilized by steaming for 60 minutes on 3 successive days. *Neurospora crassa* was grown in Czapek Dox medium for 7 days. Fresh mycelium (4g) was transferred to sterile distilled water (100ml) amended with different sizes of rock potash (0.75g of each size). Mixtures were shaken (24 hours, 25^{0}C, 150 r.p.m.) and the amount of different sized particulates adsorbed determined.

Effect of metal ions on sulphur adsorption by *M. flavus*,

Mucor flavus was grown for 7 days in Czapek Dox medium. Fresh mycelium (3g) was transferred to sterile distilled water (100ml) or sterile solutions (100ml) of 5, 15 and 100µg ml^{-1} of either Cu^{2+} ($CuSO_4.5H_2O$), Hg^{2+} ($HgCl_2$), Mg^{2+} ($MgSO_4.4H_2O$) or Mn^{2+} ($MnSO_4.4H_2O$), all containing sterile sulphur (0.5g).

These mixtures were shaken (24 hours, 25^0C, 150 r.p.m.) and the effect of metal ions on sulphur adsorption determined.

Adsorption of zinc dust by *M. flavus* in the presence of a toxic amount of mercury and varying thiosulphate concentrations.

Mucor flavus was grown in Czapek Dox medium (100ml) containing either 0, 10, 100, 1000 or 5000µg S-$S_2O_3^{2-}$ ml^{-1} (sodium thiosulphate) and 100µg Hg^{2+} ml^{-1} ($HgCl_2$) for 7 days at 25^0C and 150 r.p.m. After this time sterile zinc dust (0.5g) was added to each flask and the flasks shaken for a further 24 hours to allow zinc adsorption to occur. A biomass determination after 7 days growth was also made using separate flasks containing the same amounts of mercury and thiosulphate ions. No zinc was added to these flasks.

Specificity of particulate adsorption.

Aspergillus niger was grown in Czapek Dox medium (100ml) for 4 days. Mycelium (3g) was harvested by filtration through a sterilized nylon mesh and transferred to sterile distilled water (100ml) containing the following variations of steam sterilized particulates:

a) Zinc (0.25 and 0.5g)

b) Copper (0.25 and 0.5g)

c) Iron (0.25 and 0.5g)

d) Zinc (0.25g) and iron (0.25g)

e) Copper (0.25g) and iron (0.25g).

These mixtures were shaken at 150 r.p.m. for 24 hours at 25^0C. Fungal pellets plus adsorbed materials were then removed by filtration through a nylon mesh net. A magnet was placed by the side of the flask containing mixtures of unadsorbed particulates and all the iron present was held magnetically at the side of the flask.

This allowed the non-magnetic zinc or copper to be decanted off and their weight determined. Subsequently, the iron left in the flask was decanted.

The pH of solutions containing particulate iron were determined and all iron containing solutions were subjected to atomic absorption spectroscopy using a Perkin Elmer 460 Atomic Absorption Spectrophotometer.

Effect of culture age on adsorption of coal dust by *A. niger*.

A spore suspension (0.5ml containing 1.3×10^7 spores ml^{-1}) of *A. niger* was added to Czapek Dox medium (100ml) and grown for 5, 10, 15 and 25 days at 25^0C and 150 r.p.m. Fresh mycelium (3g) was added to sterile distilled water (100ml) containing coal dust (0.5g). The coal dust had been ground into a fine powder and sterilized before use. Flasks were shaken (24 hours, 25^0C, 150 r.p.m.) and the amount of coal dust adsorbed by different ages of mycelium determined.

Effect of mild acid and mild alkali treatment of *N. crassa* mycelium on the adsorption of sulphur.

Neurospora crassa was grown in Czapek Dox medium (100ml) for four days. Mycelium (5g) was transferred to solutions (100ml) of either H_2SO_4 (0.5N) or NaOH (0.5N) and the mixtures shaken at 150 r.p.m. for 24 hours at 25^0C. After this time mycelium was removed from the solutions and washed with sterile distilled water. Control mycelium was prepared by growing *N. crassa* in Czapek Dox medium (100ml) for 5 days. Both test and control mycelium (5g) were added to sterile distilled water (100ml) amended with sterile sulphur (1g) and shaken for 24 hours at 25^0C and 150 r.p.m. The adsorption capacity of alkali- washed and acid -washed mycelium was then compared with that of the control.

Cell wall studies.

Neurospora crassa was used in all cell wall studies. Three methods of cell wall breakage were tried:

(a) sonication (b) French Pressure Cell (c) Treatment in a Braun MSK Cell Homogenizer. Of the three methods, the Braun Homogenizer proved to be the most effective at cell wall breakage and was an easy and quick method to use.

The following types of *N. crassa* mycelium were used for cell wall isolation and subsequent chemical analysis:

(1) Four day old

(2) acid -washed

(3) alkali-washed

(4) Nutrient starved and

(5) Twelve day old.

All biomass produced was grown as described previously.

Cell wall isolation method.

Mycelium of *N. crassa*, prepared as above, was harvested by filtration and washed well with ice-cold distilled water. The mycelium (60g fresh weight) was then homogenized in cold distilled water (50ml) using an MSE Atomix Omnimixer at half speed. The total homogenization time was 5 minutes but after every 30 seconds treatment the Omnimixer was shut down for one minute and placed in the cold (4^0C) to prevent overheating of the mycelium which would lead to the activation of cell wall degrading enzymes. The homogenized mycelium was then ground using a glass cylinder taking care to keep the temperature below 10^0C. The resulting mycelial suspension was then washed at least three times in cold distilled water (until the supernatent became clear) to remove excess cytoplasmic contaminants.

The mycelium was collected in between washings by centrifugation using an MSE Mistral 6L centrifuge (6 x 750ml head, 4000 r.p.m., 15 minutes, 5^0C). The mycelial pellet was resuspended in a measured amount of cold distilled water (approximately 0.2g dry weight mycelium ml^{-1} water) and added to the glass container of the Braun Homogenizer until the container was full. The glass container had previously been kept cool (4^0C) and also filled half full with glass beads (0.45-0.5mm diameter). This mixture was then treated in the Braun Homogenizer, the temperature being kept below 10^0C by short blasts of liquid carbon dioxide every 5 seconds. After this treatment, the glass beads were allowed to settle to the bottom of the flask and the mycelial suspension decanted off. The extent of cell breakage was checked using a microscope to determine the necessity of further treatment in the Braun Homogenizer. The cell walls were washed in cold distilled water and collected by centrifugation using an MSE Mistral 6L centrifuge (12 x 100ml angle head, 4000 r.p.m., 15 minutes, 5^0C). This washing procedure was repeated until the supernatant became clear. The wall pellet was then resuspended in a cold solution (4^0C, 1% w/v) of sodium dodecyl sulphate (SDS) and stirred magnetically overnight in the cold (4^0C). Treatment with SDS removes any membrane proteins that may be attached to the cell walls. The mixture was then centrifuged (MSE Mistral 6L centrifuge, 12 x 100ml angle head, 4000 r.p.m., 5^0C, 15 minutes) to collect the hyphal walls and the pellet washed in cold distilled water. The walls were washed until all traces of SDS were removed from the supernatent. Cell wall isolation was then considered complete and the walls were frozen in liquid nitrogen prior to lyophilization. Freeze-dried samples were stored at -20^0C until required for chemical analysis.

The cell walls of three separate batches of each different type of *N. crassa* biomass were isolated in this way.

<u>Protein content of cell walls.</u>

Samples of cell wall (20mg and 30mg) were analysed for protein content using the Biuret method of protein estimation (Stickland, 1951). (See Appendix)

Total sugar content of cell walls.

Cell wall samples (25mg amd 50mg) were hydrolysed in HCl (3N, 2ml) for 3 hours at 100^0C. This solution was cooled and neutralized with NaOH (3N) (Bisaria *et al.*, 1986). Precipitate was removed by centrifugation in a bench centrifuge (4500 r.p.m. for 10 minutes). The supernatant (0.1ml) was diluted in distilled water (9.9ml) and then this solution tested for sugar content using the established phenol-sulphuric acid method (Dubois *et al.*, 1956). (See Appendix).
The concentration and amount of phenol used was 5% (w/v) and 1ml respectively.

Is particulate adsorption by *N. crassa* due to hydrophobic or electrostatic interactions?

(a) Determination of the hydrophobicity of isolated N. crassa cell walls.

Four day old, 12 day old, nutrient-starved, acid-washed and alkali-washed *N. crassa* cell walls, isolated as previously described, were assessed for their hydrophobic characteristics. The method used was a modification of that used by Bar-Or & Shilo (1988).

Samples of cell wall (100mg or 200mg) were added to distilled water (20ml) and the suspension dispersed by a brief sonication treatment. Any clumped cell walls were allowed to settle and the supernatent containing well dispersed cell walls poured off and used in the following hydrophobicity test. Cell wall suspension (4ml) was added to a small separating funnel containing xylene (1ml). The mixture was

shaken vigorously for 5 minutes and then allowed to settle for 15 minutes. The water layer (4ml) was run off and centrifuged (4500 r.p.m. for 10 minutes) to collect the cell walls. The pellet obtained was then assayed for protein content using the Biuret method. The amount of protein was correlated to the amount of cell wall present in the aqueous layer. Controls were treated in exactly the same way except that xylene was omitted from the separating funnel.

(b) The effect of Mg^{2+} ions on the adsorption of negatively charged, hydrophilic glass beads by N. crassa.

Neurospora crassa was grown in Czapek Dox medium (100ml) for 4 days. Mycelium (2g) was transferred to solutions (100ml) of either sterile distilled water or distilled water containing Mg^{2+} (15µg Mg^{2+} ml^{-1}) supplied as $MgSO_4.7H_2O$. Both solutions contained glass microcarrier beads (0.2g, 150-210µm diameter, density = 1.04 g ml^{-1}). The mixtures were shaken (24 hours, 150 r.p.m., 25^0C) and the amount of microcarrier beads adsorbed with and without the presence of Mg^{2+} ions was determined.

RESULTS AND DISCUSSION.

Effect of different carbon, nitrogen and phosphorus concentrations in the growth medium on the subsequent adsorption of zinc dust by *M. flavus*.

A range of carbon, nitrogen and phosphorus concentrations had no influence on the ability of the fungus to adsorb zinc dust (Fig 1.1). Organisms produce extracellular polymers under a variety of conditions including carbon excess nitrogen limitation and in media containing a high carbon:nitrogen ratio (Brierley & Lanza, 1985). All these conditions were employed to induce polymer production by *M. flavus* and therefore enhance particulate adsorption by the fungus. However, as mentioned previously, no increase in adsorption occurred under all the nutrient concentrations used. This suggests that extracellular polymers are unlikely to be involved in particulate adsorption by *M. flavus*.

Effect of different carbon sources supplied in the growth medium on the adsorption of sulphur by *M. flavus*.

The type of carbon source used to grow *M. flavus* had no significant effect on its subsequent ability to adsorb sulphur from solution (Fig. 1.2). No growth occurred in the presence of lactose presumably because the fungus lacks B-galactosidase which is necessary for the breakdown of lactose into its constituent sugars.

Comparison of adsorption of sulphur by *M. flavus* in Czapek Dox medium and distilled water.

When the fungus was transferred to fresh Czapek Dox medium containing sulphur an increased rate of adsorption was found compared to the control, where

the fungus was transferred to sterile, distilled water containing the element (Fig. 1.3). This continued removal of sulphur from solution was related to the continued growth of the fungus in Czapek Dox medium, leading to conditions where sulphur particles were not only adsorbed, but entrapped by growing hyphae. Growing hyphae will also continually produce new sites for adsorption, suggesting that active fungal growth should be encouraged in systems developed on an industrial scale to employ the particle adsorption abilities of fungal mycelium.

Comparison of sulphur adsorption by *M. flavus* in distilled water containing various carbon sources with distilled water alone.

The presence of different carbon sources had no effect on the ability of *M. flavus* to adsorb sulphur (Fig. 1.4).

Ability of various fungi to adsorb sulphur.

Figure 1.5 shows that not all fungi can adsorb sulphur to the same extent. *N. crassa* and *M. flavus* have significantly higher adsorption abilities than the other fungi tested. The differential adsorption reflects differences in cell wall chemistry and mode of growth. Both *N. crassa* and *M. flavus* tend to grow as large mycelial clumps, while all the other fungi grow in pellet form. The larger mycelial clumps adsorbed the larger particles of sulphur present, the opposite being observed with the pellet forming fungi. Sulphur adsorption by *T. indicae-seudaticae* was tested at 37^0C and this is probably responsible for the low amount of sulphur adsorbed by this fungus.

Effect of addition of sorbose to the growth medium on the subsequent adsorption of sulphur by *N. crassa*.

Figure 1.1.

Effect of growth in Raistricks' medium containing varying carbon, nitrogen and phosphorus concentrations on the subsequent adsorption of zinc dust by the fungus *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D.)

Fig. 1.1.

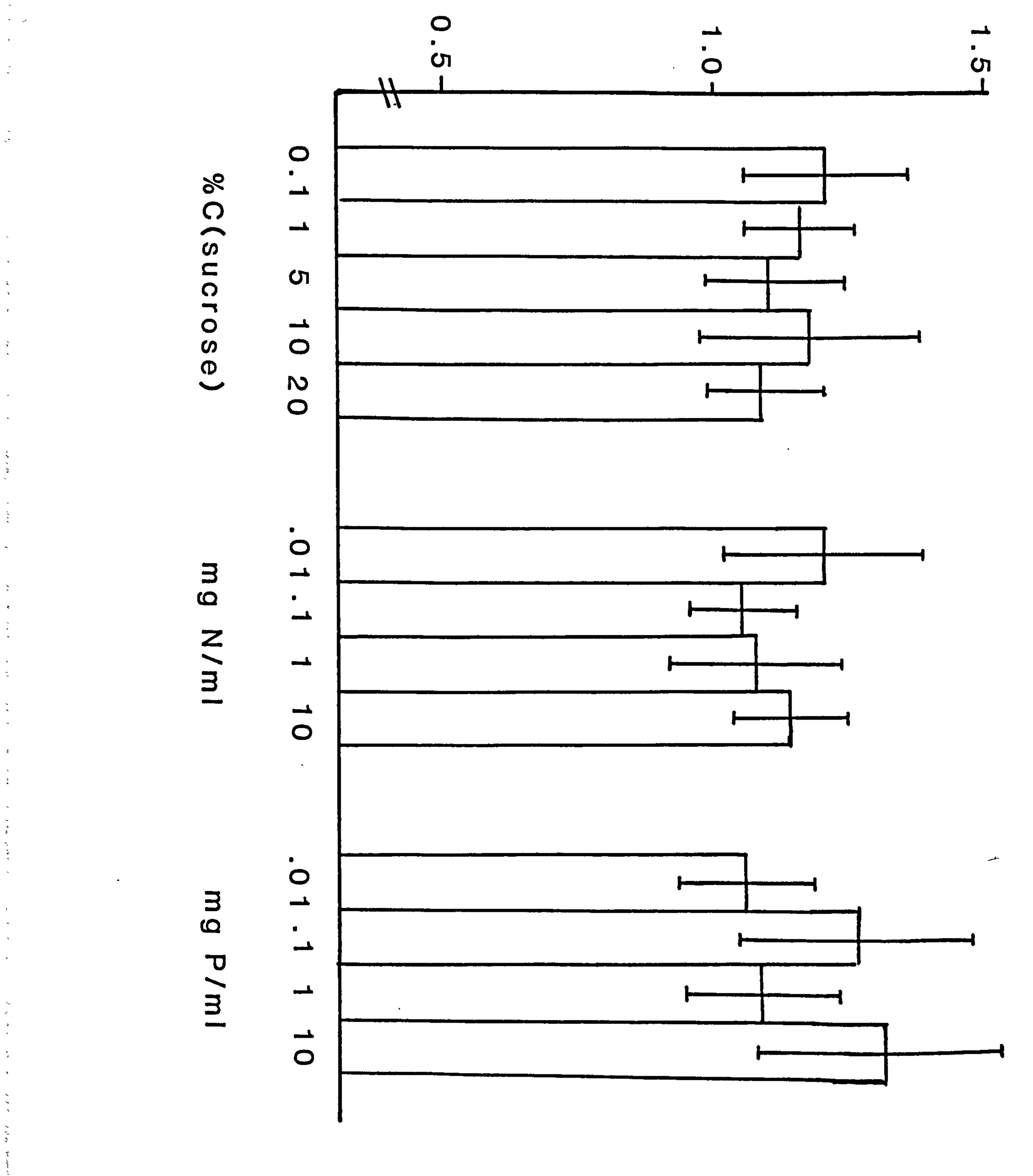

g Zn adsorbed/ g dry weight
0.5
1.0
1.5
%C(sucrose)
0.1 1 5 10 20
mg N/ml
.01 .1 1 10
mg P/ml
.01 .1 1 10

Figure 1.2.

Effect of growth in Raistricks' medium containing different carbon sources (1% w/v carbon) on sulphur adsorption by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D.)

Figure 1.3.

Comparison of sulphur adsorption by *Mucor flavus* in Czapek Dox liquid medium and sterile distilled water.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in sulphur adsorption over the control which was incubated in sterile distilled water only, $p < 0.05$)

●—● : Distilled water

△—△ : Czapek Dox Liquid medium

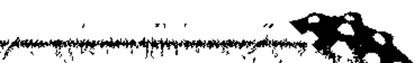


Fig.1.2.

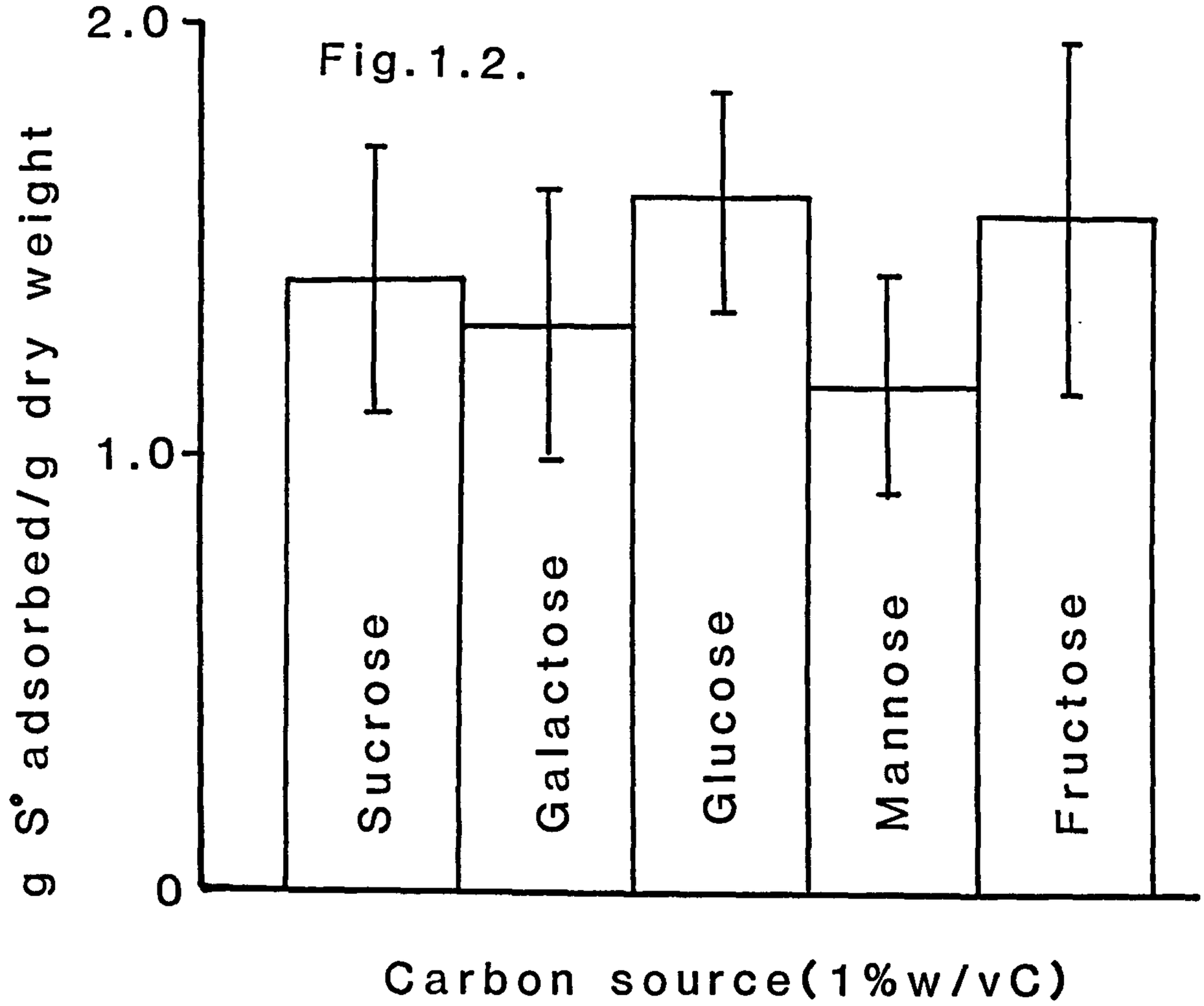

2.0
1.0
0
g S° adsorbed/g dry weight
Sucrose
Galactose
Glucose
Mannose
Fructose
Carbon source(1%w/vC)

Fig.1.3.

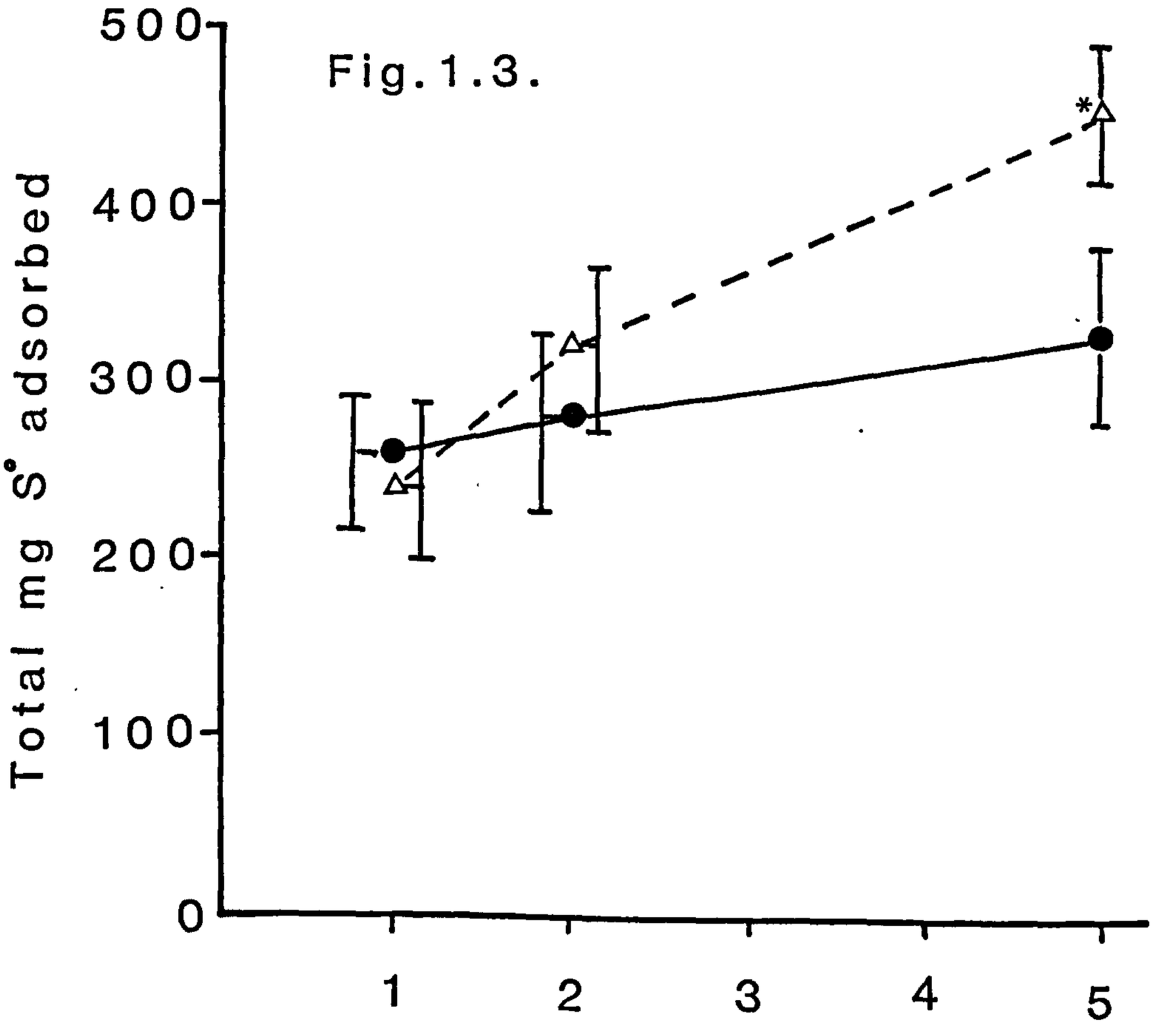

500
400
300
200
100
0
Total mg S° adsorbed
*
1
2
3
4
5
Days incubation

Figure 1.4.

Comparison of sulphur adsorption by *Mucor flavus* in distilled water supplemented with various carbon sources (1% w/v carbon) to sulphur adsorption by the fungus in distilled water alone.

(Means of triplicates $^{+}/_{-}$ S.D.)

Figure 1.5.

Ability of several fungi to adsorb sulphur.

(Means of triplicates $^{+}/_{-}$ S.D.)

1 : *Mucor flavus*

2 : *Neurospora crassa*

3 : *Aspergillus niger*

4 : *Penicillium chrysogenum*

5 : *Aspergillus flavus*

6 : *Aspergillus repens*

7 : *Botrytis cinerea*

8 : *Thermomucor indicae-seudaticae*

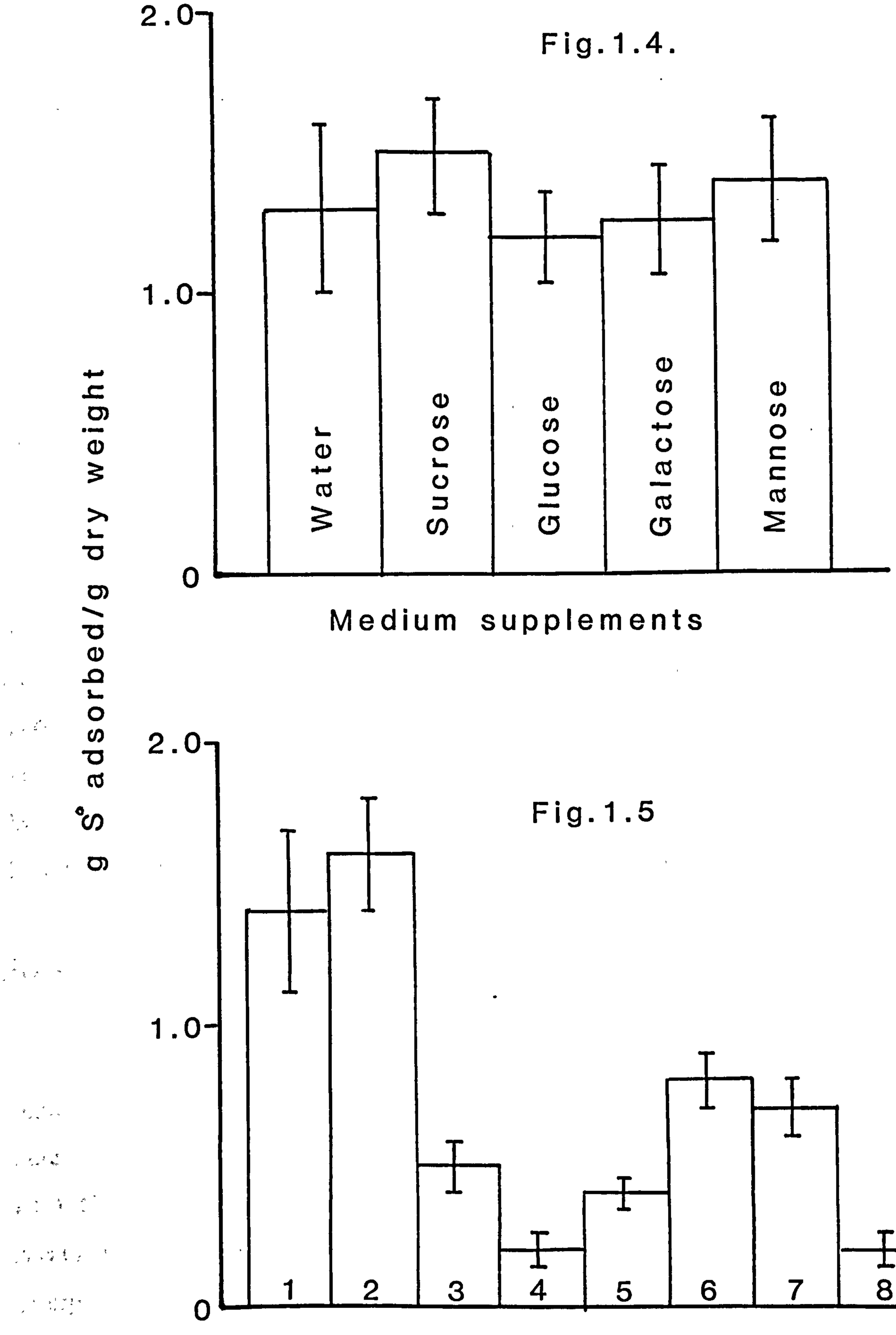

2.0
1.0
0
g S° adsorbed/g dry weight
Water
Sucrose
Glucose
Galactose
Mannose
Medium supplements

Fig.1.4.

2.0
1.0
0
g S° adsorbed/g dry weight
1
2
3
4
5
6
7
8
Fungal species

Fig.1.5

*

This statement is incorrect. Melanization of fungal walls has been shown to increase the number of adsorption sites available for metal ion adsorption, and would presumably increase the number of particulate binding sites. Also, the fungi used in the experiment are not thought to produce melanins.

Sorbose is known to cause a change in the cell wall structure of *N. crassa* and also other fungi (Crocker & Tatum, 1968, Bisaria *et al.*, 1986). This change in cell wall structure may have led to an increase in adsorption but as the results show (Fig. 1.6), such an increase did not occur.

Effect of age of mycelium grown in Czapek Dox medium on sulphur adsorption.

All the fungi examined, except *N. crassa*, showed a decrease in particulate adsorption with age (Fig. 1.7), a result which indicates that a change in wall structure occurs as mycelium ages, or that adsorption sites are saturated with age related products. Melanization of fungal walls occurs with age, a process which probably results in a reduction of available adsorption sites on the cell wall.* Scanning electron micrographs of 28 day old mycelium grown in Czapek Dox medium (carbon rich) showed the presence of crystals on the mycelial and spore surfaces (Figs. 1.8 to 1.10) whereas the surface of 7 day old hyphae appeared perfectly smooth (Figs. 1.11 and 1.12). These crystals, which were presumably produced as a result of growth in rich carbon media, may have decreased adsorption by covering up wall adsorption sites. Therefore, growth of the fungus in carbon limiting medium was tried as a method to prevent the production of these crystals.

Effect of age of *M. flavus* grown in carbon-limiting medium on sulphur adsorption.

Adsorption decreased with age of mycelium grown in carbon-limiting conditions (Fig. 1.13). Scanning electron micrographs show that there no crystals were present on 28 day old mycelium grown under these conditions (Figs. 1.14 and 1.15). These results suggest that the crystals appearing during growth of the fungus in carbon rich medium are not entirely responsible for the observed decrease in adsorption. Melanization of the fungal walls or some other chemical alteration

seems more likely to be the main cause of the decrease in adsorption observed with fungal age.

Effect of mild acid and mild alkali treatment on sulphur adsorption by 7 day and 28 day old mycelium of *M. flavus*.

Mild acid or alkali treatment did not significantly increase the adsorptive ability of 28 day old mycelium grown in rich carbon medium. Untreated mycelium adsorbed 49.4 ($^{+}$/$_{-}$10) mg of sulphur, while acid treated mycelium adsorbed 42.6 ($^{+}$/$_{-}$12) mg and alkali treated mycelium adsorbed 52.0 ($^{+}$/$_{-}$10) mg. Scanning electron micrographs showed that there are no crystals present on the surface of 28 day old mycelium after acid or alkali treatment (Figs. 1.16 to 1.19). These results suggest that the crystals are not responsible for decreased adsorption in older mycelium, but that a change in cell wall composition, such as melanization, is responsible. The crystals could probably be removed by even milder treatments such as washing in distilled water.

Effect of fungal starvation on sulphur adsorption.

The rate of sulphur adsorption doubled when mycelium of *M. flavus* was transferred from Czapek Dox medium to sterile distilled water prior to being exposed to sulphur particulates (Fig. 1.20). Similar results were obtained with *N. crassa* (Fig. 1.20) although the increase in sulphur adsorption was not as high. The results suggest that under starvation conditions, the fungal cell wall undergoes changes which expose or lead to the production of an increased number of adsorption sites: or that there is an increase in surface area as the mycelium undergoes limited breakdown. Starvation of *Saccharomyces cerevisiae* allowed the organism to bind to a glass support (Van Haecht *et al.*, 1984). The starvation pre-treatment of the yeast induced a modification of the cell wall and modified the

Figure 1.6.

Effect of addition of sorbose (2% w/v) to Czapek Dox growth medium on the subsequent adsorption of sulphur by

Neurospora crassa.

(Means of triplicates $^{+}/_{-}$ S.D.)

T : mycelium grown in Czapek Dox medium supplemented with sorbose

C : mycelium grown in Czapek Dox medium alone

Figure 1.7.

Effect of age of several fungi grown in Czapek Dox liquid medium on their ability to adsorb sulphur.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in sulphur adsorption compared with that adsorbed by 4 day old mycelium, $p < 0.05$)

●—● : *Neurospora crassa*

△—△ : *Mucor flavus*

■—■ : *Aspergillus niger*

◇—◇ : *Penicillium chrysogenum*

Fig.1.6.

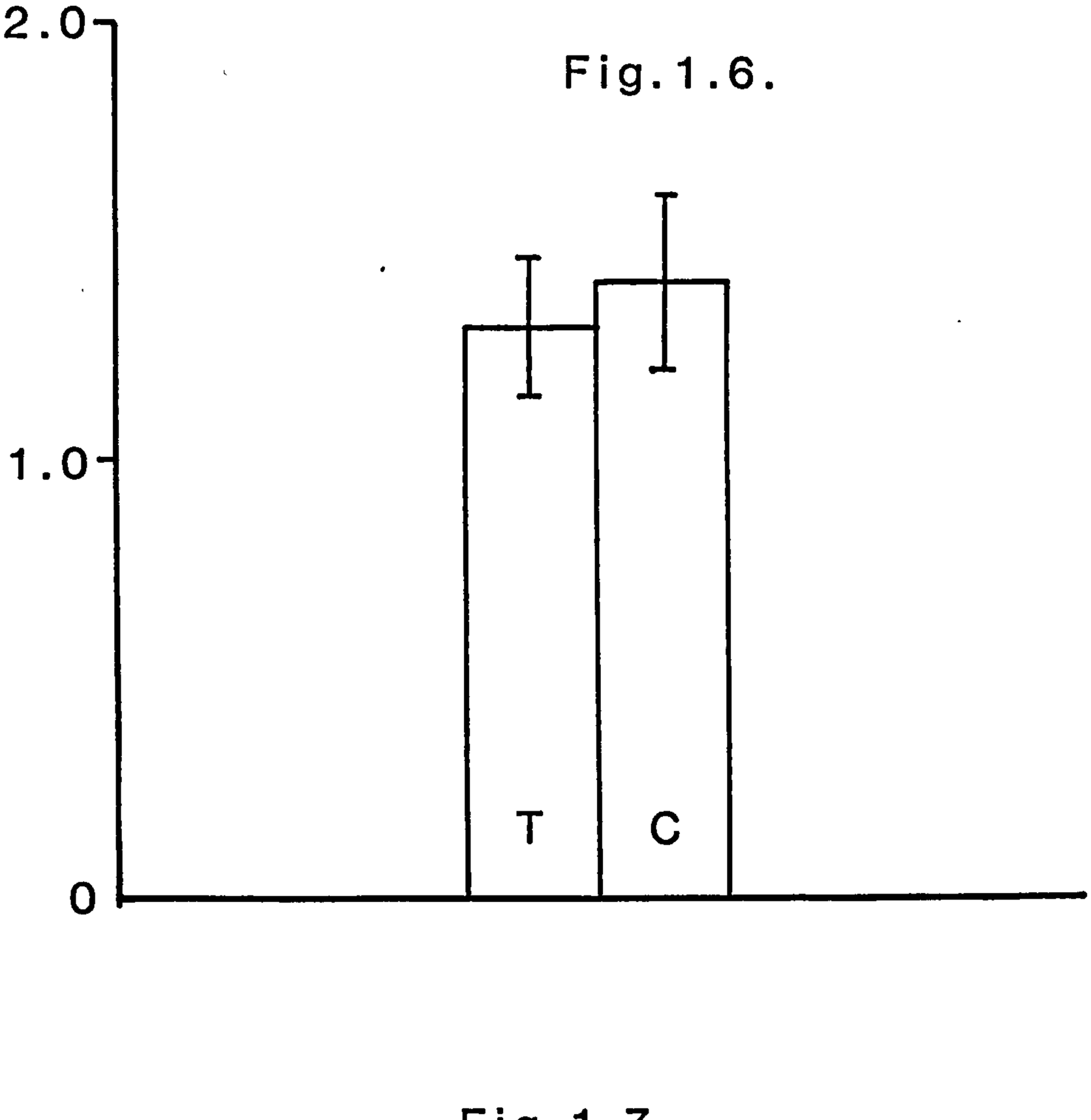

2.0
1.0
0
T
C
g S° adsorbed /g dry weight

Fig.1.7.

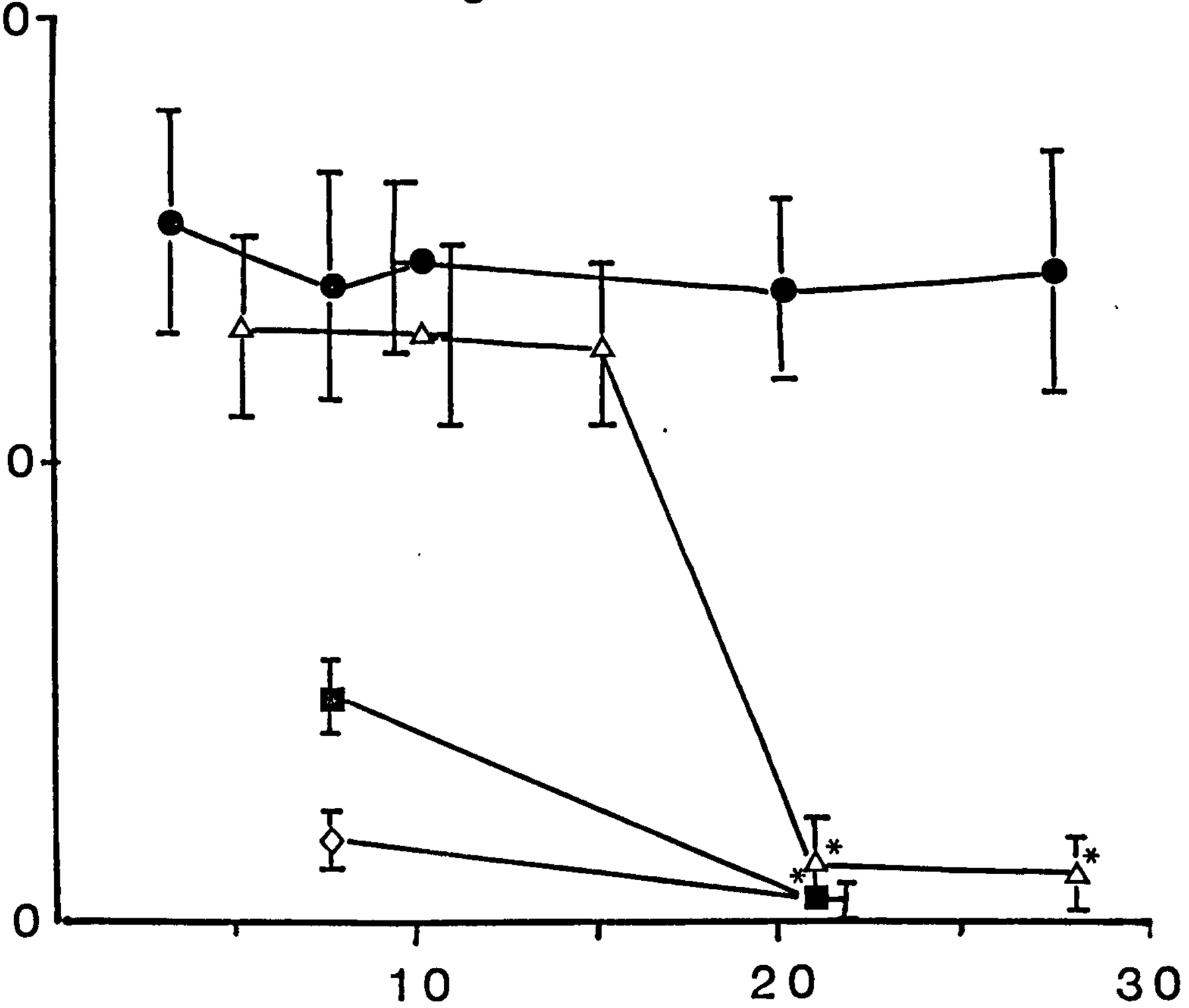

2.0
1.0
0
10
20
30
g S° adsorbed /g dry weight
Mycelial age(days)

Figure 1.8.

Scanning electron micrograph of a 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 2000)

FIG.1.8.

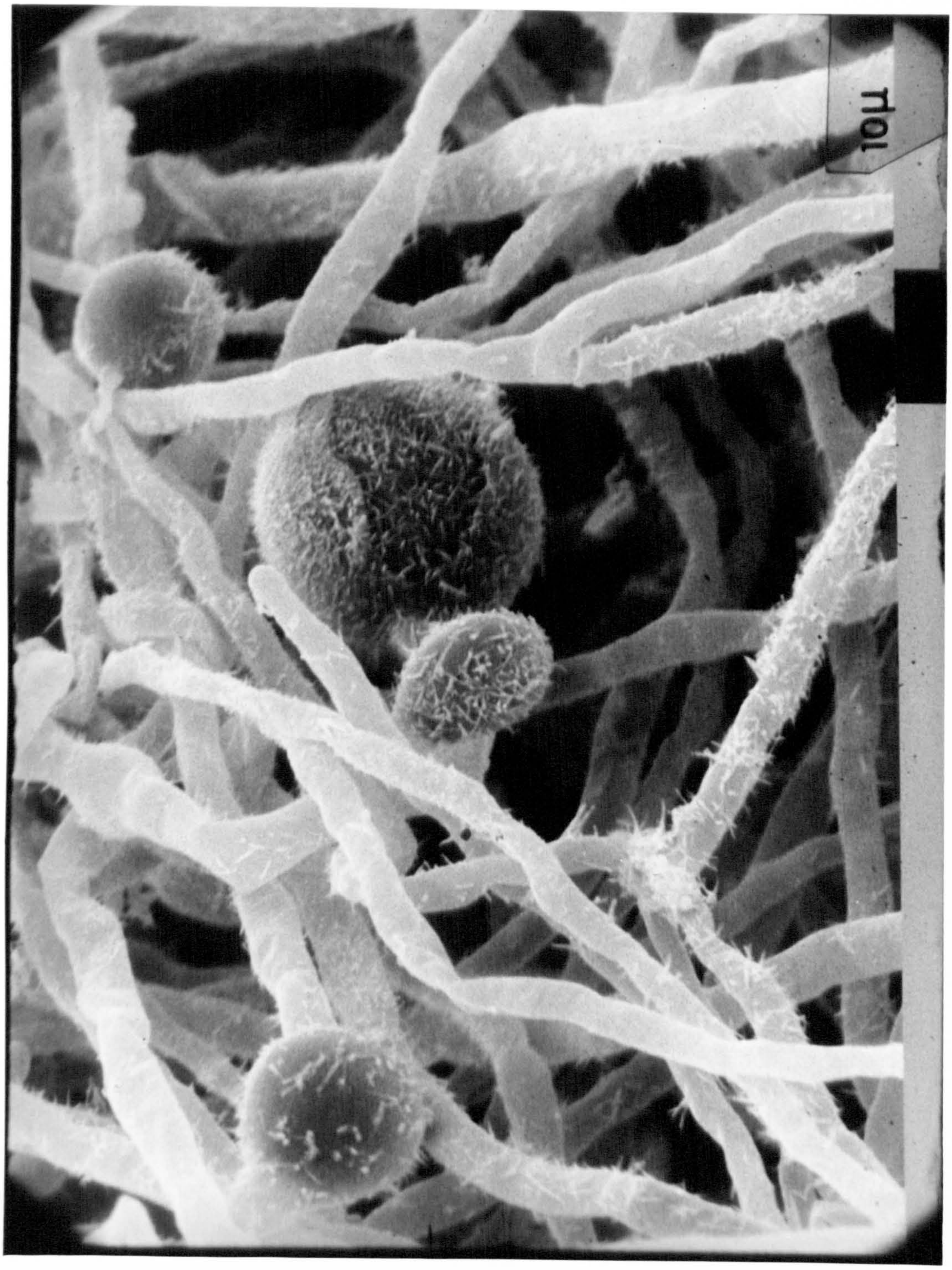


Figure 1.9.

Scanning electron micrograph of a 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 5000)

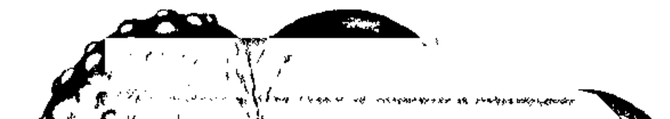


FIG. 1.9.

Figure 1.10.

Scanning electron micrograph of a 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 5000)

FIG.1.10.

Figure 1.11.

Scanning electron micrograph of a seven day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 500)

FIG.1.11.

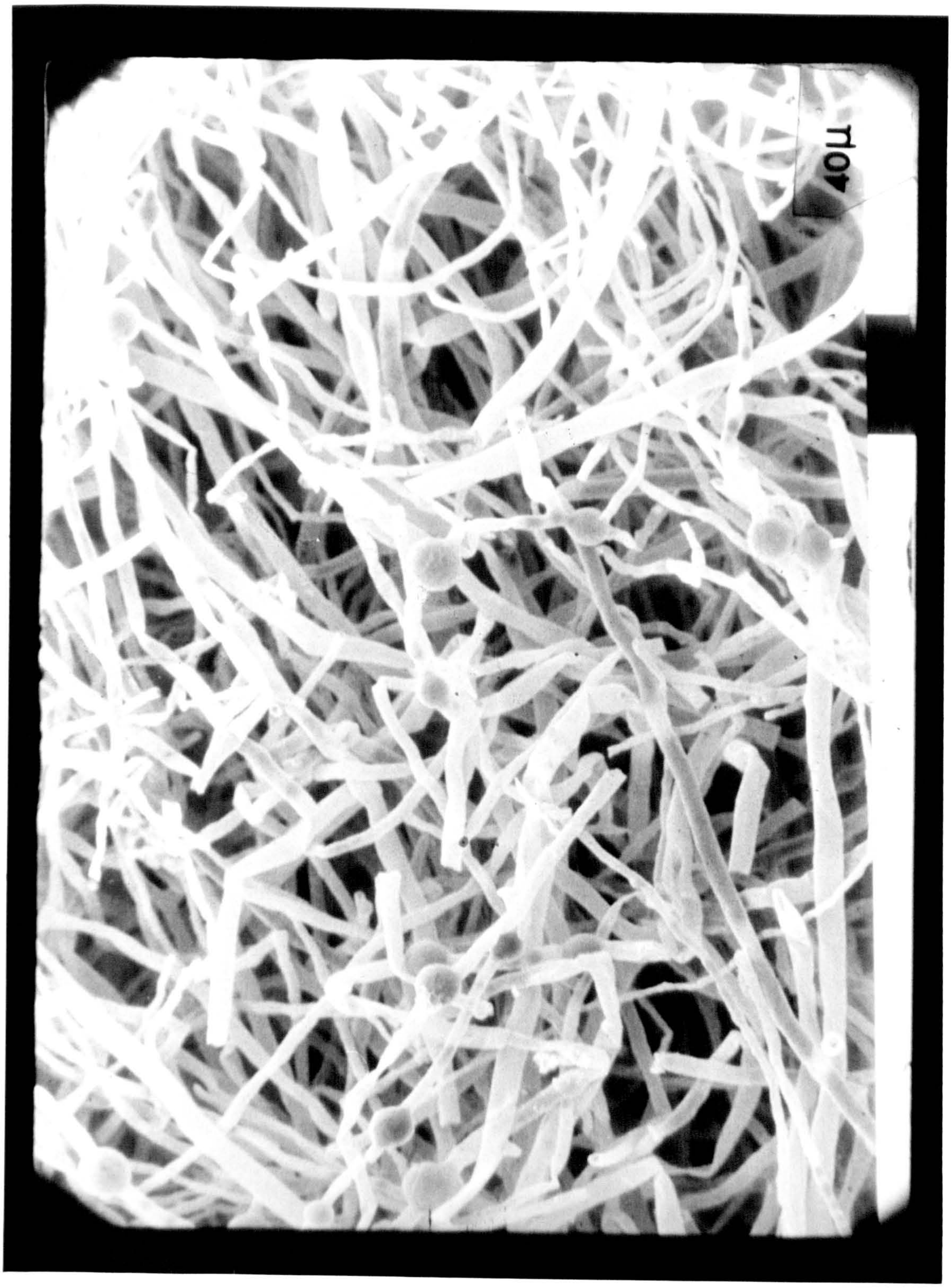


Figure 1,12.

Scanning electron micrograph of a seven day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 2000)

FIG.1.12.

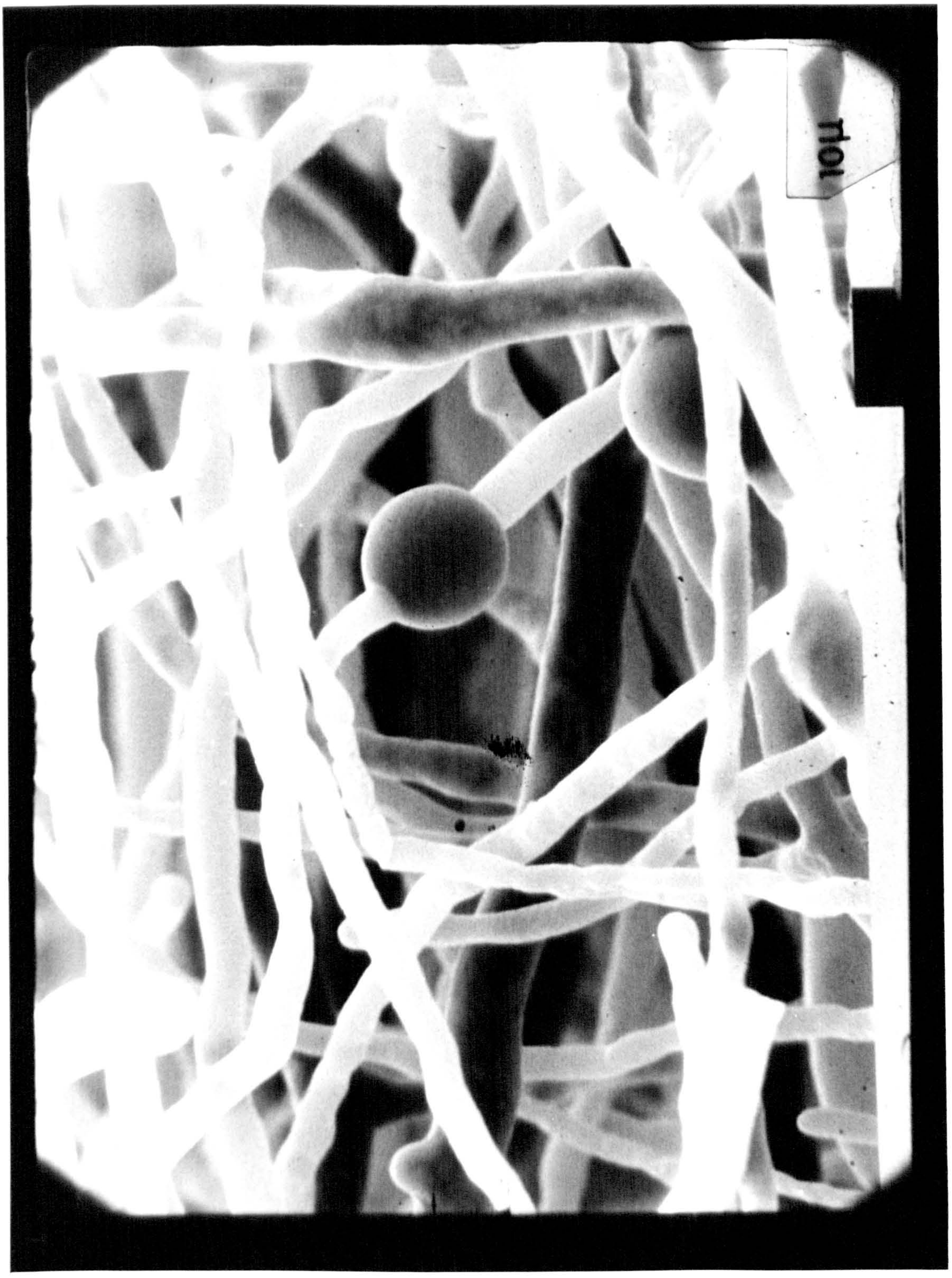


Figure 1.13.

Effect of age of *Mucor flavus* grown in Raistricks' medium (under carbon-limiting conditions) on the ability of the organism to adsorb sulphur.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in sulphur adsorption compared with the amount adsorbed by a four day old culture, $p < 0.05$)

Fig.1.13.

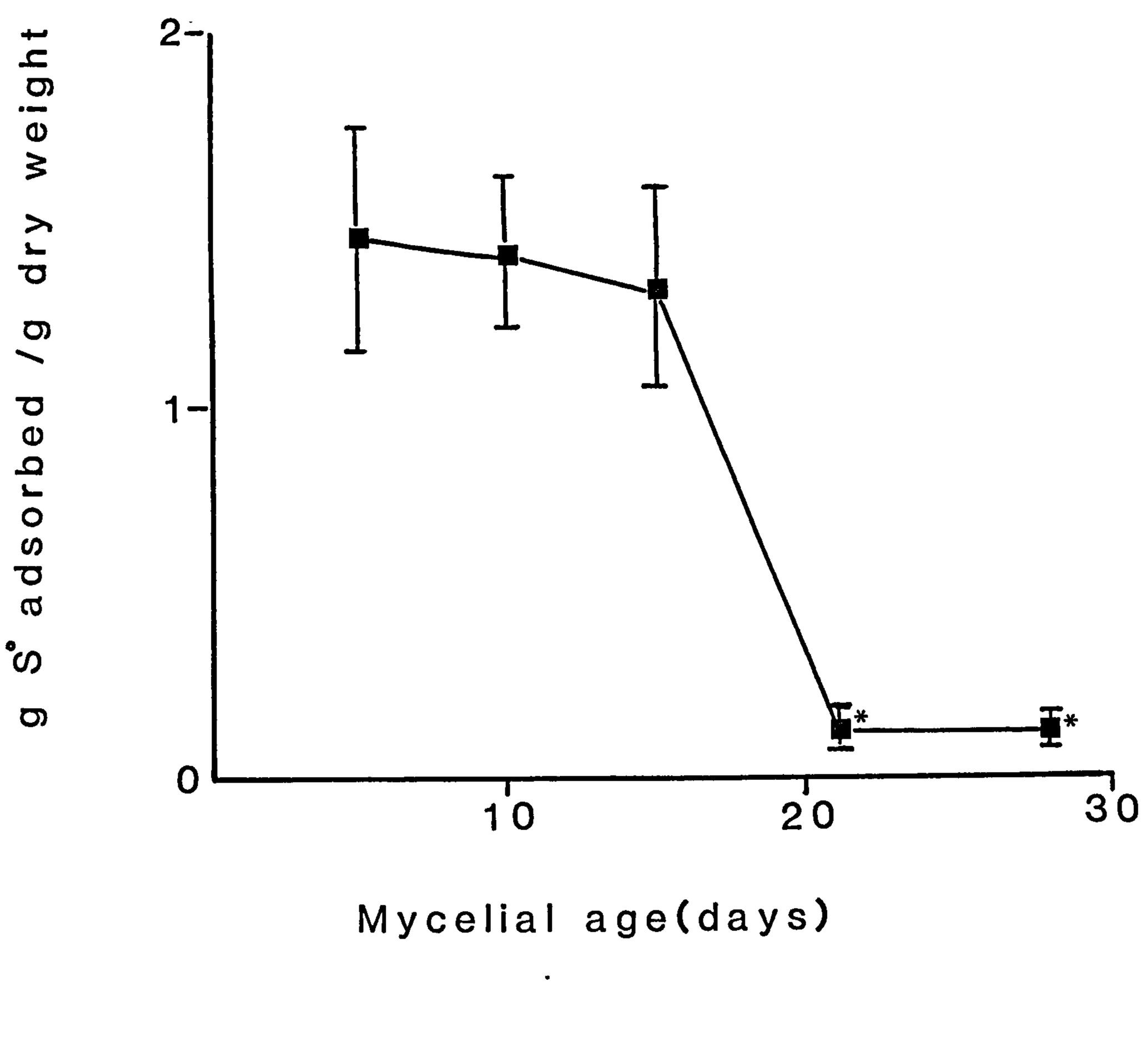


Figure 1.14.

Scanning electron micrograph of a 28 day old culture of *Mucor flavus* grown in Raistricks' medium under carbon-limiting conditions.

(Magnification x 500)

FIG.1.14.

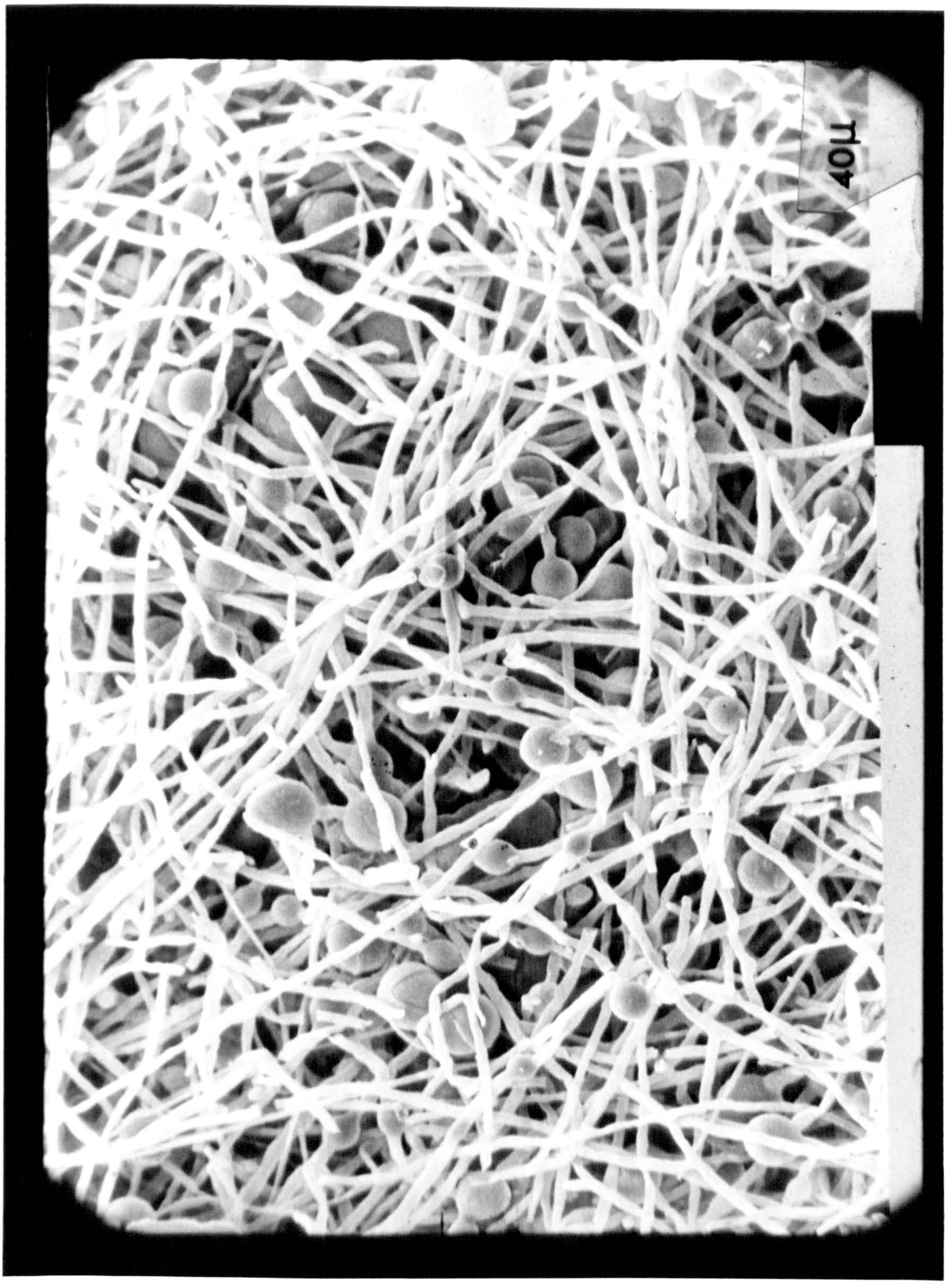


Figure 1.15.

Scanning electron micrograph of a 28 day old culture of *Mucor flavus* grown in Raistricks' medium under carbon-limiting conditions.

(Magnification x 2000)

FIG.1.15.

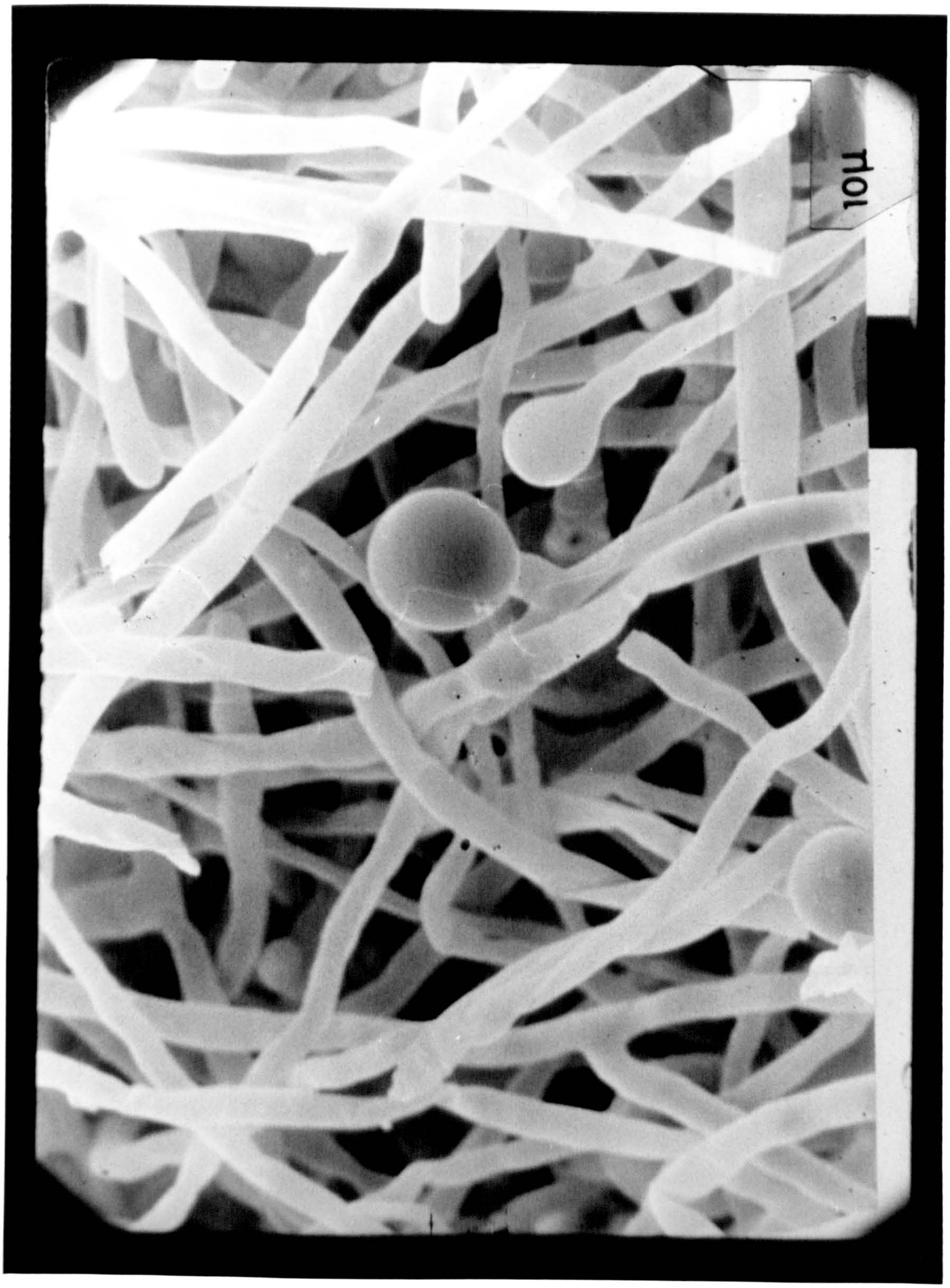


Figure 1.16.

Scanning electron micrograph of an acid-washed 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 1000)

FIG.1.16.

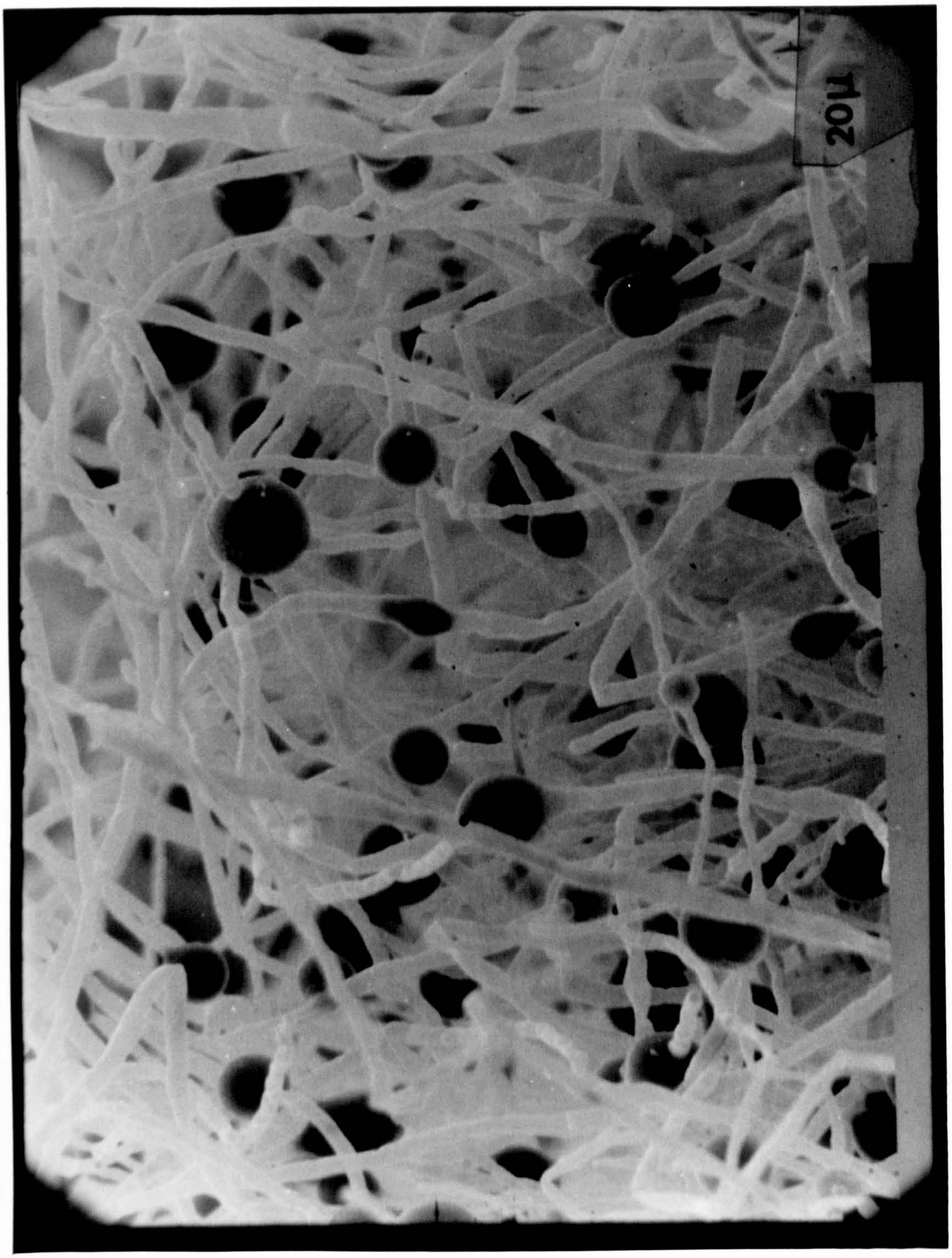


Figure 1.17.

Scanning electron micrograph of an acid-washed 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 2000)

FIG.1.17.

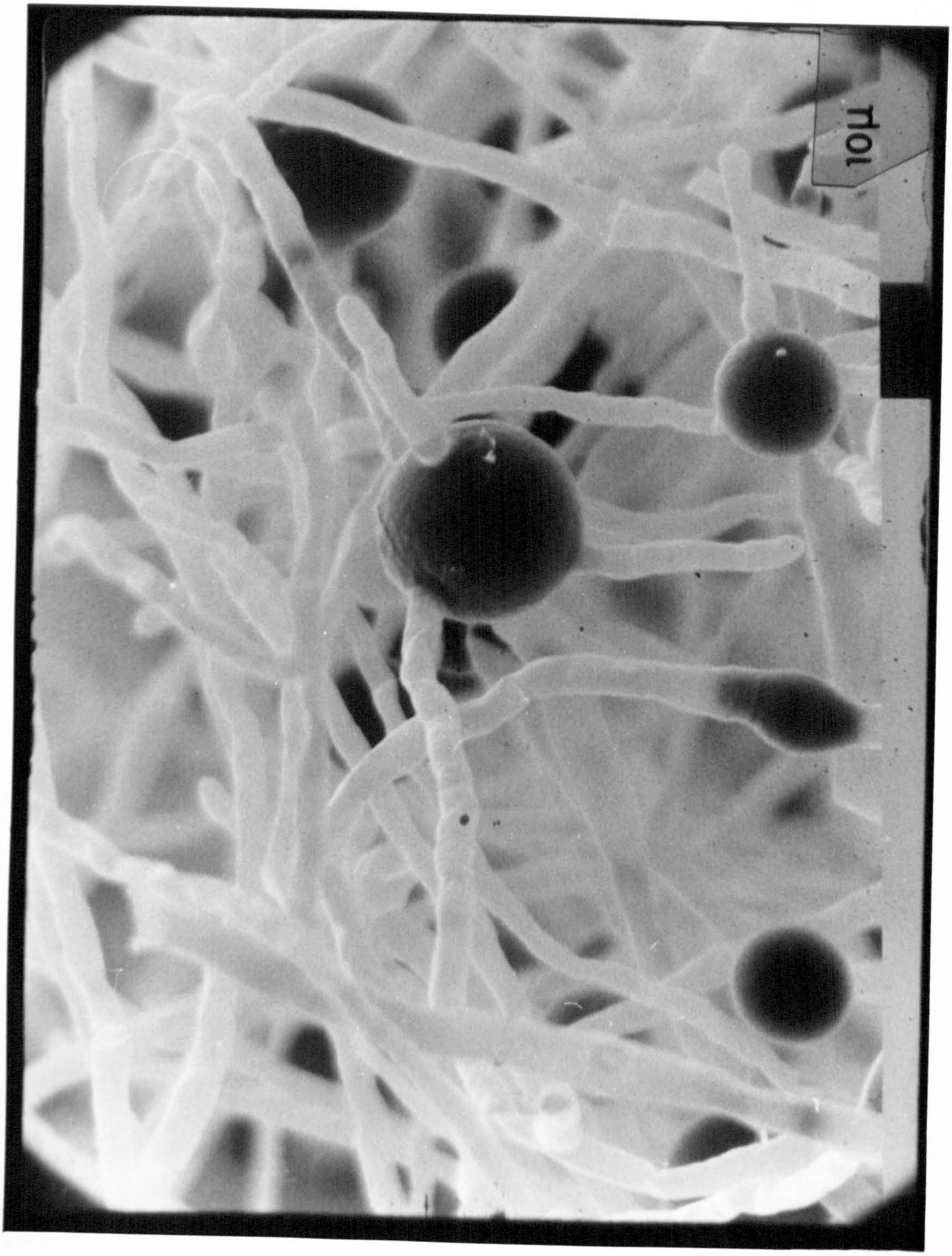


Figure 1.18.

Scanning electron micrograph of an alkali-washed 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 2000)

FIG.1.18.

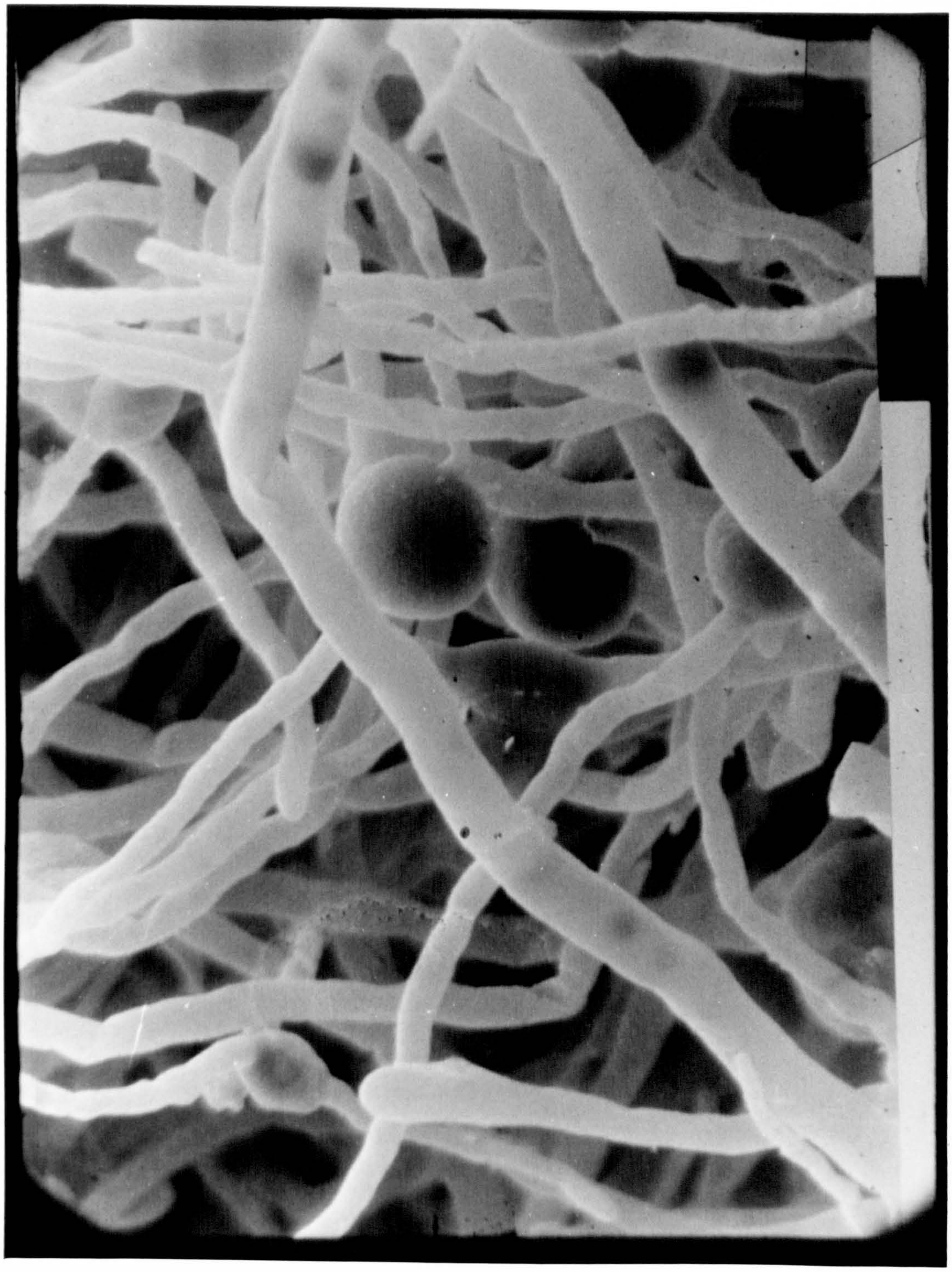


Figure 1.19.

Scanning electron micrograph of an alkali-washed 28 day old culture of *Mucor flavus* grown in Czapek Dox liquid medium.

(Magnification x 500)

FIG.1.19.

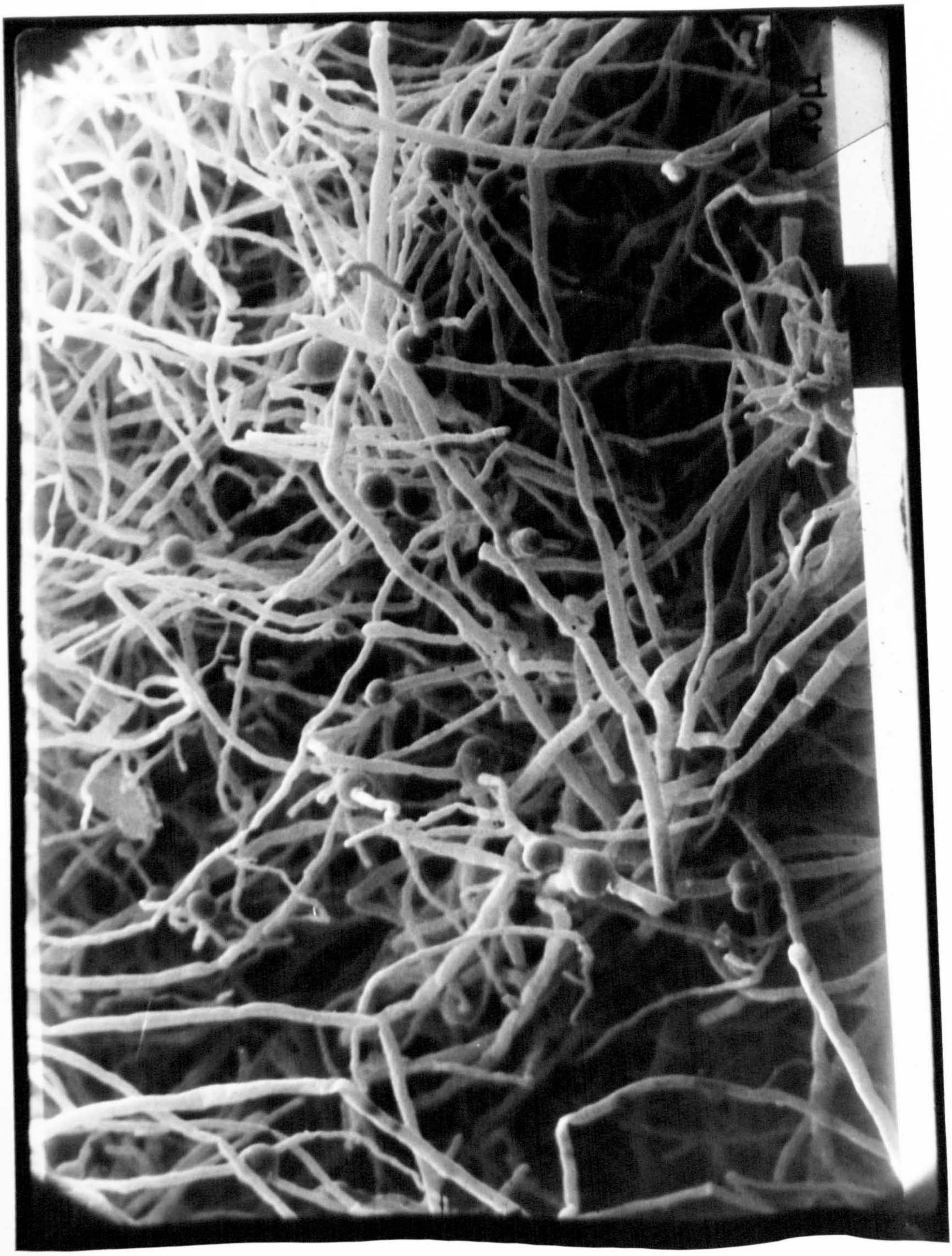


Figure 1.20.

Effect of nutrient starvation on sulphur adsorption by *Mucor flavus* and *Neurospora crassa*.

Means of triplicates $^{+}/_{-}$ S.D. * indicates significant increase in sulphur adsorption due to nutrient starvation, $p < 0.05$)

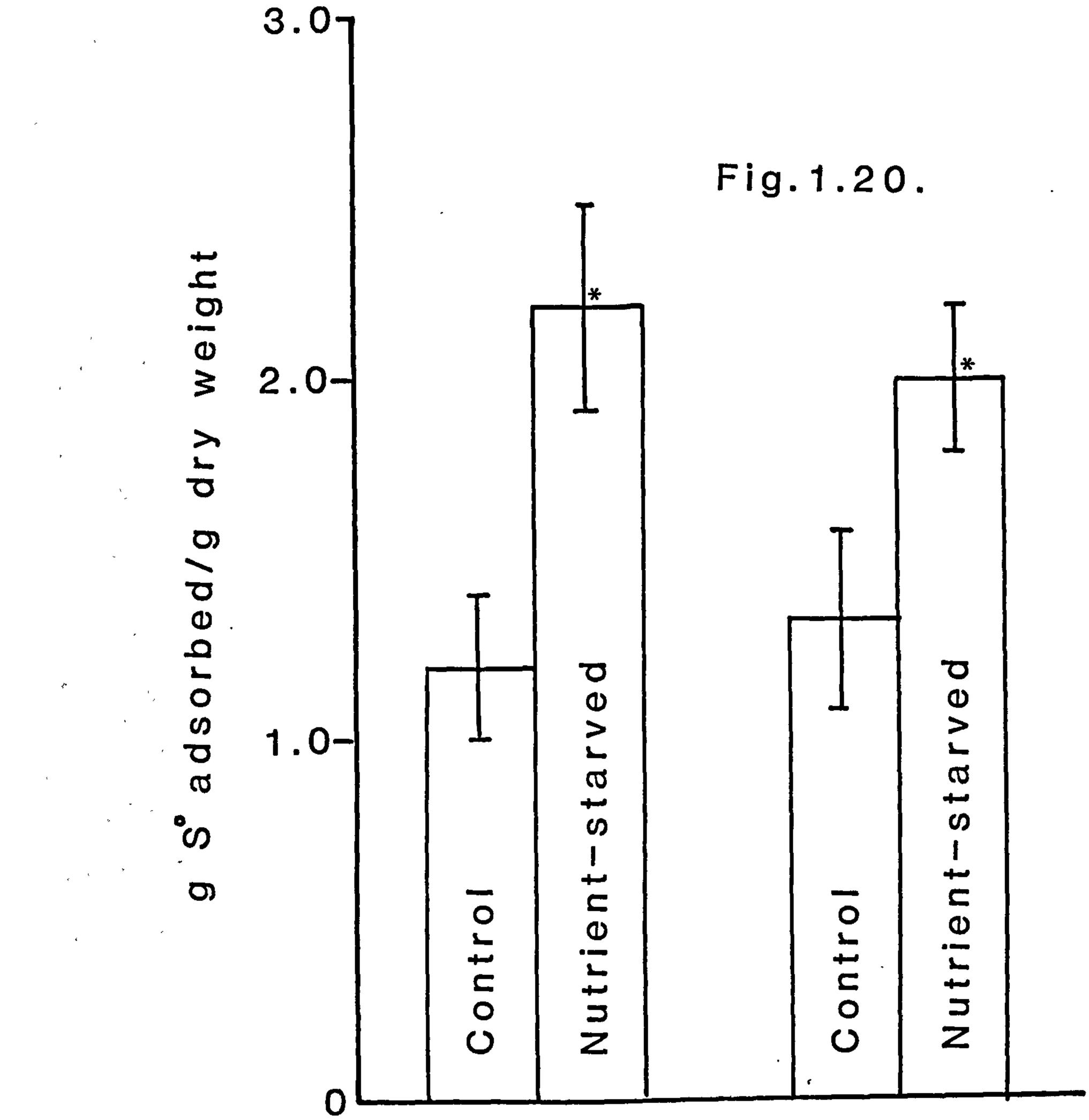

3.0
2.0
1.0
0
g S° adsorbed/g dry weight
Control
Nutrient-starved
Control
Nutrient-starved
Mucor flavus
Neurospora crassa

Fig.1.20.

composition of the medium by releasing substances which reduced cell-cell and cell-support electrostatic repulsions. Both factors were considered important in relation to the adhesion of negatively charged yeast cells to negatively charged glass.

Ability of killed mycelium to adsorb particulates.

The growth inhibitors CCCP, KCN and cycloheximide did not affect the rate of sulphur adsorption by *M. flavus* (Fig. 1.21) implying that the adsorption of particulates is initially a physical process and is independant of cellular metabolism. The toxins used in demonstrating the physical nature of particulate adsorption may not have been effective in inhibiting the growth of *M. flavus*. Therefore, it could have been argued that particulate adsorption had not been fully demonstrated to be a purely physical process. The information presented in Fig. 1.22. proves that the inhibitors completely prevented further growth of an active two day old culture and also validates the conclusion of particulate adsorption being a physical process. The ability of dead mycelium to adsorb particulates improves the chances of using fungi to adsorb particulates in industrial wastes which are likely to inhibit fungal growth.

Effect of pH on sulphur adsorption by various fungi.

N. crassa and *A.niger* tended to adsorb larger amounts of sulphur at lower pH values, while sulphur adsorption by *P. chrysogenum* and *M. flavus* was constant over the pH range tested (Fig. 1.23). Final pH values recorded were different to initial pH values such that the final pH range was generally from 2 to 9. Even so, particulate adsorption occurred over a wide range of pH values. Increased sulphur adsorption by *N. crassa* and *A. niger* at low pH probably reflects a difference in surface charge on the fungal cell wall surface and/or on the particulate sulphur, resulting in increased electrostatic attraction. Increased electrostatic attraction

*

The effect of temperature on particulate adsorption is interesting. The results obtained suggest that fungal metabolism may be involved in the adhesion phenomenon. Adsorption is highest at around 30°C. This is the temperature at which M. flavus is most metabolically active and will therefore be actively excreting compounds and various ions (eg. hydrogen ions) which are capable of changing the pH of the area surrounding the fungal cell. This metabolic activity may be affecting particulate adsorption. Mucor flavus will be less active at 4°C and 37°C and will not affect the pH of the surrounding environment to the same extent.

The inhibitors used to determine the ability of killed mycelium to adsorb particulates stopped biomass production by the fungus but they possibly allowed other metabolic activities to occur which could have altered the pH of the medium surrounding the cell. This factor may have been responsible for adsorption occurring in the presence of the inhibitors.

would increase the possibility of the two surfaces coming into contact and also enhance the DLVO interactions involved in particulate adhesion.

Effect of pH on adsorption of activated carbon by *M. flavus*.

As with sulphur, adsorption of activated carbon by *M. flavus* remained constant over the pH range used (Fig. 1.24).

Effect of pH on the adsorption of zinc dust by *M. flavus*.

Unfortunately, zinc adsorption at acidic pH values could not be determined due to dissolution of the compound during incubation. Zinc adsorption decreased at more alkaline pH values (Fig. 1.25).

The effect of temperature on sulphur adsorption by *M. flavus*.

Maximum sulphur adsorption occurred at 25^0C, while only relatively small amounts were adsorbed at 4^0C (Fig. 1.26). Sulphur adsorption also decreased at 37^0C. Decreased adsorption at low temperatures could be explained by the following:

(1) An increase in viscosity of a fungal surface polymer

(2) Higher temperatures (within limits) favour chemisorption and certain types of physical adsorption. Therefore if fungal particulate adsorption is initially determined by physicochemical adsorption, then the expected result would be one of decreased adsorption at lower temperatures

(3) Temperature may affect the physiology of the fungus in some way, which in turn affects particulate adsorption.

Bacterial adhesion to polystyrene decreased at low temperatures (Fletcher, 1977). Reasons similar to those above were given to explain the decreased adhesion.

Figure 1.21.

Ability of *Mucor flavus*, killed by different inhibitors, to adsorb sulphur.

(Means of triplicates $^{+}/_{-}$ S.D.)

a : Live mycelium (control)

b : Cycloheximide treated mycelium

c : CCCP treated mycelium

d : Cyanide treated mycelium

Figure 1.22.

Effect of growth inhibitors on biomass production by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D. * indicates significant decrease in biomass production compared to that obtained from a seven day old culture grown without inhibitors, $p < 0.05$)

a : Biomass produced after 7 days growth in the absence of growth inhibitors

b : Biomass produced after two days growth in the absence of growth inhibitors

c : Biomass produced after 7 days growth in the presence of cycloheximide (inhibitor added two days after inoculation)

d : Biomass produced after 7 days growth in the presence of CCCP (inhibitor added two days after inoculation)

e : Biomass produced after 7 days growth in the presence of potassium cyanide (inhibitor added two days after inoculation)

Fig.1.21.

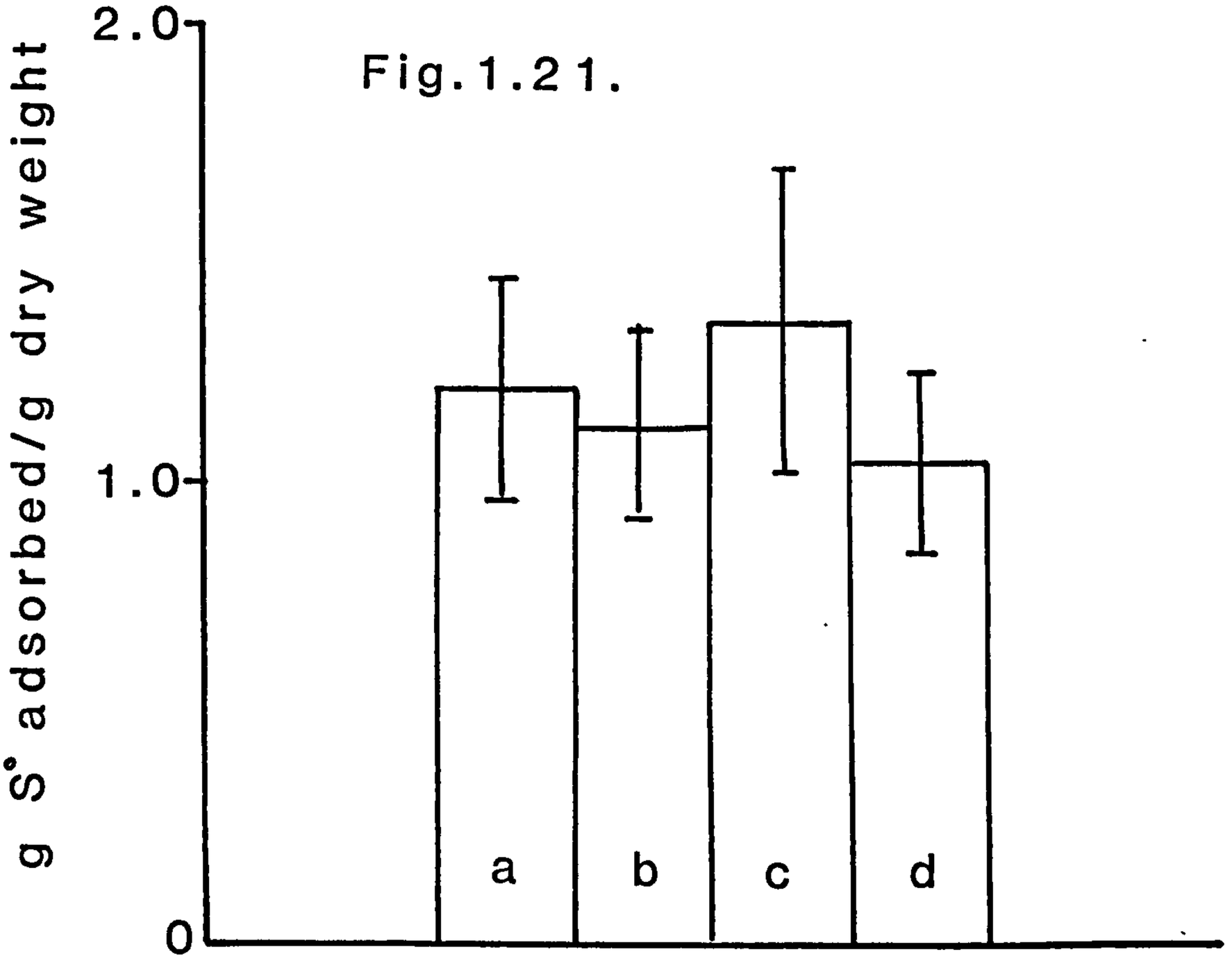


Fig.1.22.

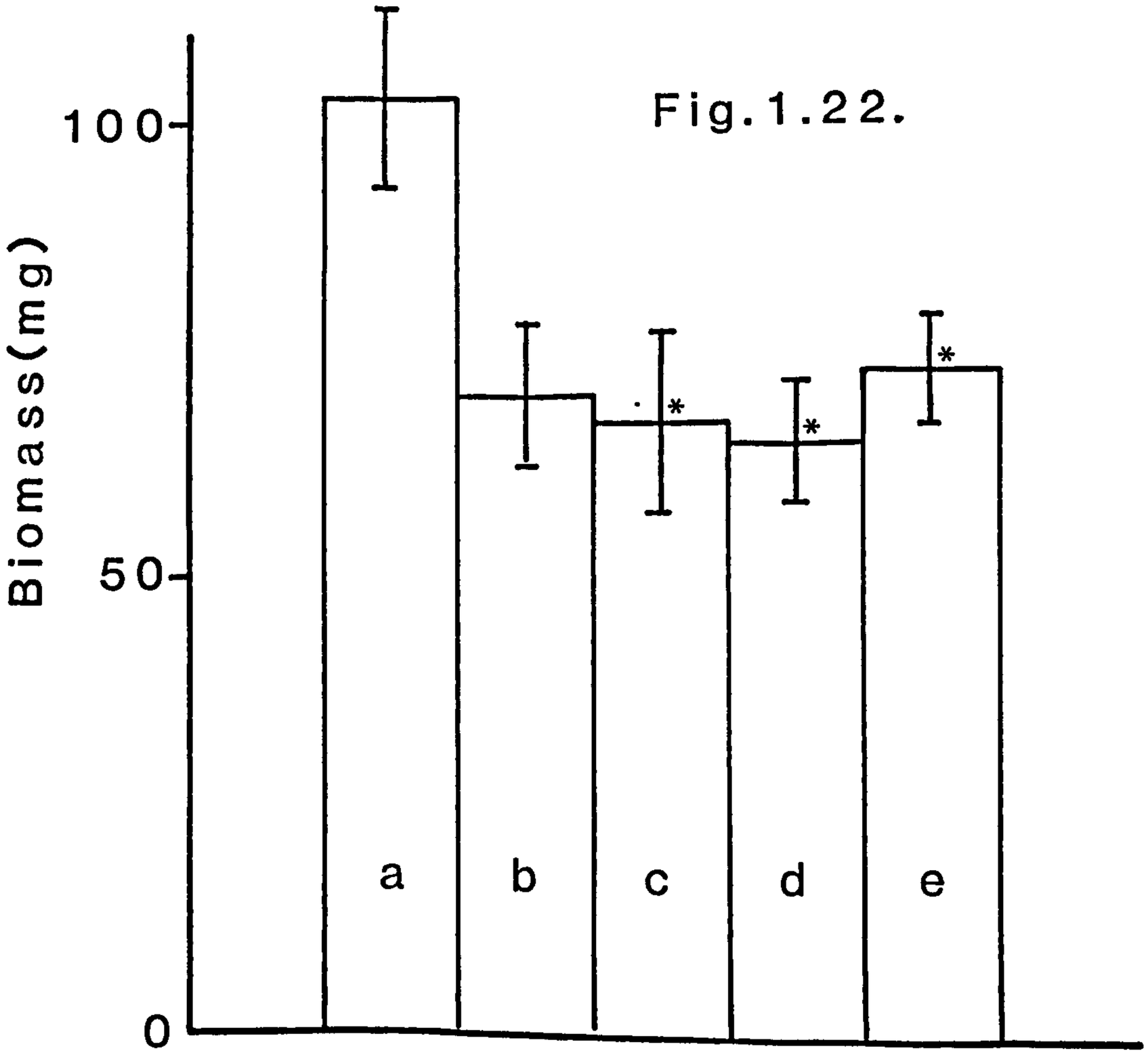


Fig. 1.23.

Effect of pH on sulphur adsorption by several fungi.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in sulphur adsorption compared with the value obtained at pH 7, $p < 0.05$: ** significant increase in sulphur adsorption compared with the value obtained at pH 7, $p < 0.05$)

a : *Mucor flavus*

b : *Neurospora crassa*

c : *Penicillium chrysogenum*

d : *Aspergillus niger*

Fig.1.23.

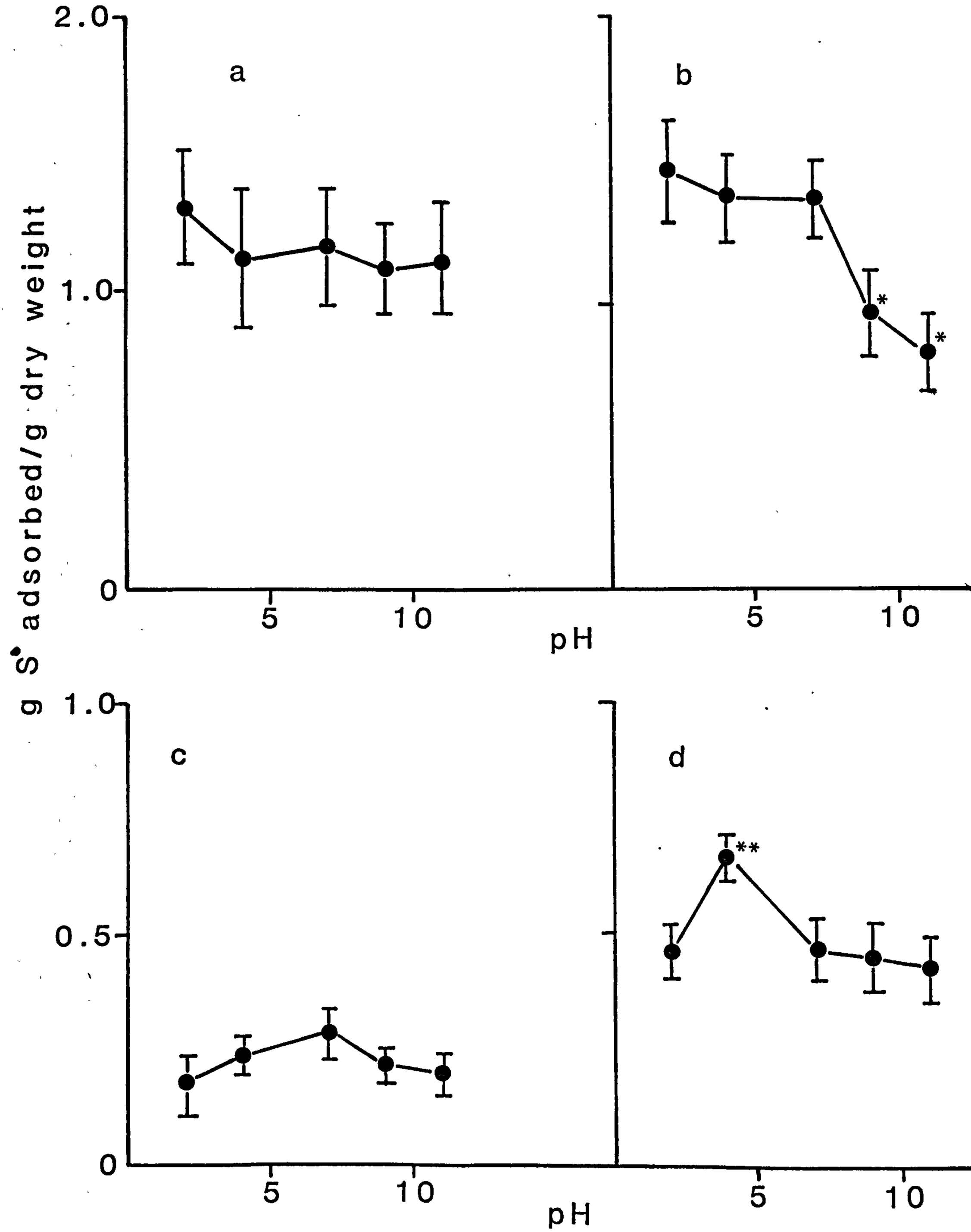


Figure 1.24.

Effect of pH on adsorption of activated carbon by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D.)

Figure 1.25.

Effect of pH on adsorption of zinc dust by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in zinc adsorption compared with the value obtained at pH 7, $p < 0.05$)

Fig.1.24.

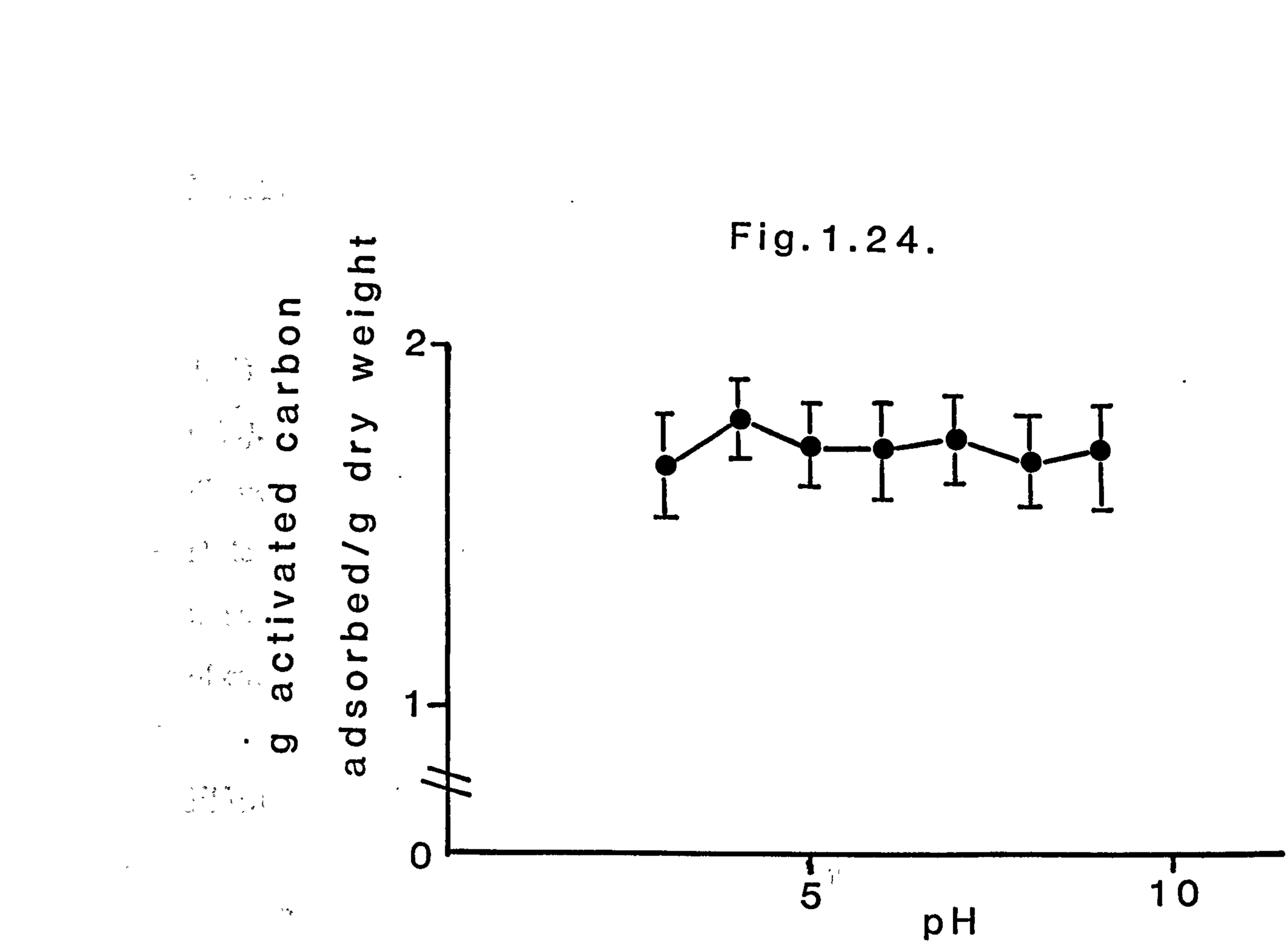
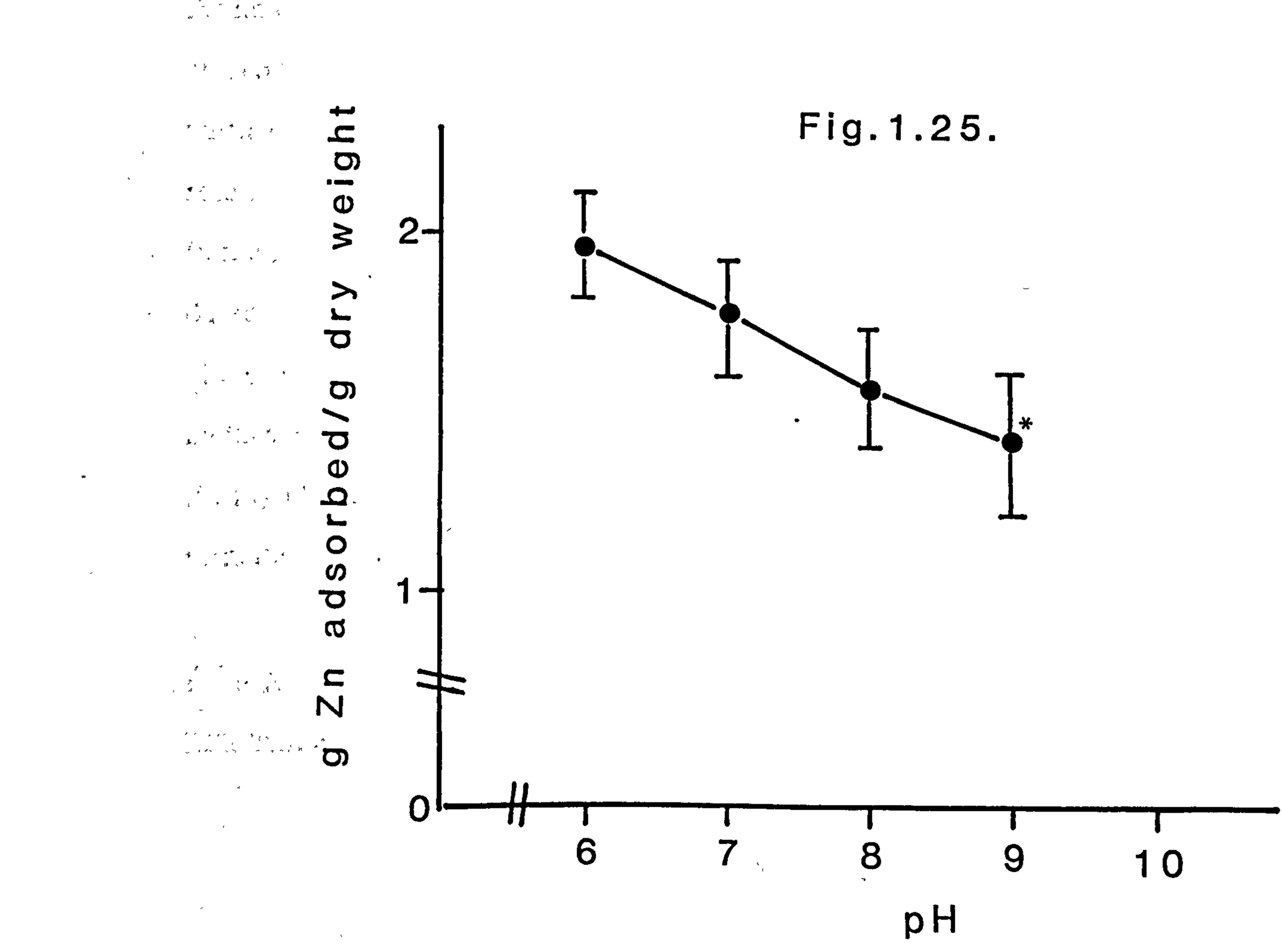


Fig.1.25.



Effect of particle size on adsorption of rock potash by *N. crassa*.

A decrease in particulate size resulted in an increase in adsorption (Fig. 1.27). Particles above 0.251mm in diameter were not adsorbed to any great extent (8%), whereas 95% adsorption was observed with particles below 0.251mm in diameter. Smaller particulates will be more subject to electrostatic and other molecular forces than will larger particles and it follows that the smaller a particle the more easily it will be attracted and adsorbed to the fungal surface. The density of compounds must also play a significant role in particulate adsorption.

Effect of metal ions on sulphur adsorption by *M. flavus*.

The presence of both Cu^{2+} and Hg^{2+} in the adsorption medium reduced the amount of sulphur adsorbed by *M. flavus*, while Mn^{2+} had no effect and Mg^{2+}, at a concentration of 15µg ml^{-1} significantly stimulated adsorption (Fig. 1.28). Since particulates are likely to occur together with metal ions in industrial effluents, these results have an obvious bearing on the practical use of the adsorption phenomenon. In the presence of Cu^{2+} and Hg^{2+} the surface of *M. flavus* became noticably darker in colour (brown) and it was thought that this may be due to the deposition of the corresponding metal sulphides on the mycelial surface. The presence of these metal sulphides presumably physically interferes with the adsorption of sulphur by the mycelium. Magnesium could have increased adsorption possibly by the formation of ionic bond bridges between the fungal cell wall and the sulphur.

Adsorption of zinc dust by *M.flavus* when subjected to toxic amounts of mercury and varying thiosulphate concentrations.

Thiosulphate has been reported to reduce metal toxicity towards fungi by acting as a complexing agent (Wainwright & Grayston, 1983). The biomass produced with mercury present increases when a sufficient amount of thiosulphate ($100\mu g\ S\text{-}S_2O_3^{2-}\ ml^{-1}$)is added to the growth medium (Fig. 1.29). The biomass increase also results in a corresponding increase in zinc adsorption (Fig. 1.30) which suggests that the thiosulphate also overcomes the inhibitory effect of mercury on particulate adsorption mentioned previously. However, at high thiosulphate concentrations ($5000\mu g\ S\text{-}S_2O_3^{2-}\ ml^{-1}$) particulate adsorption decreases perhaps due to an increase in medium pH (Fig.1.30). Thiosulphate would allow growth of fungi to occur in otherwise toxic effluent and this would increase particulate adsorption due to mycelial entrapment and production of new adsorption sites.

Specificity of particulate adsorption.

Of the three metal dusts used, zinc was adsorbed most effectively by *A. niger*, copper was adsorbed to a lesser extent and iron is adsorbed the least (Table 1.3). Incubation of mycelium with zinc and iron particulates resulted in reduced adsorption of iron when compared to a control containing iron only (Table 1.3). The amount of zinc adsorbed was also reduced when compared to a control. No reduction in the amounts of copper and iron adsorbed by mycelium was observed when the two were incubated together (Table 1.3). The results indicate that particulate adsorption is not specific but probably depends on size and surface charge of particles involved.

A yellow coloration was noticed when mycelium was incubated in the presence of iron particulates. It was decided to analyse all iron containing media for the presence of solubilized iron. *A. niger* was able to solubilize iron to a large extent over a period of 4 hours when incubated with iron only (Table 1.4). The iron solubilization seemed to be correlated with a marked reduction in medium pH (Table 1.4). Zinc inhibited this solubilization and also kept the medium pH at quite

Figure 1.26.

Effect of temperature on sulphur adsorption by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in sulphur adsorption compared with the value obtained at 25^{0}C, $p < 0.05$)

Figure 1.27.

Effect of particle size on the adsorption of rock potash by *Neurospora crassa*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in particulate adsorption compared with the value obtained with particles between 2 and 1.4 mm in diameter, $p < 0.05$)

1 : Between 2 and 1.4 mm diameter

2 : Between 1.4 and 1.0 mm in diameter

3 : Between 1.0 and 0.295 mm in diameter

4 : Between 0.295 and 0.251 mm in diameter

5 : Less than 0.251 mm in diameter

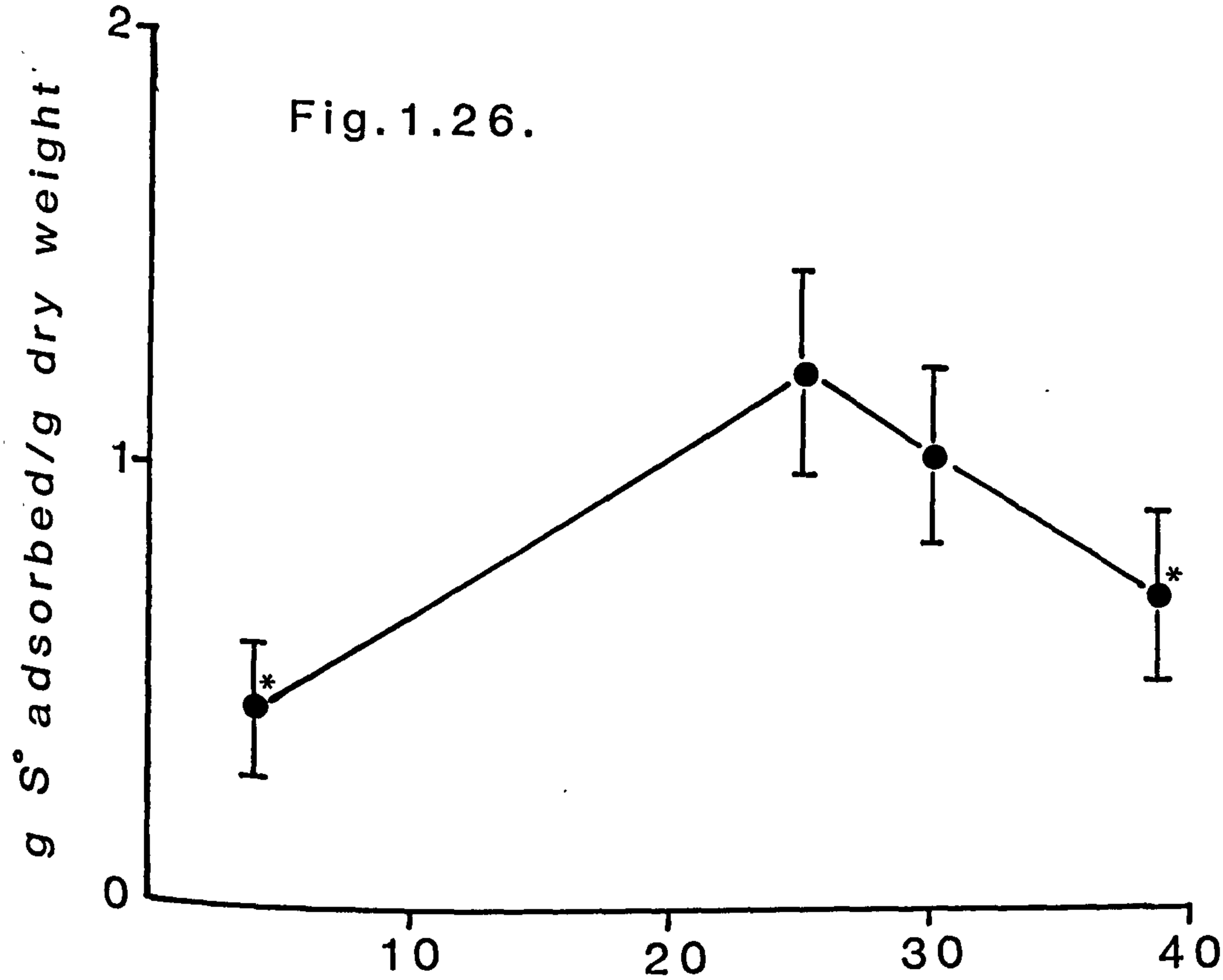


Fig.1.26.

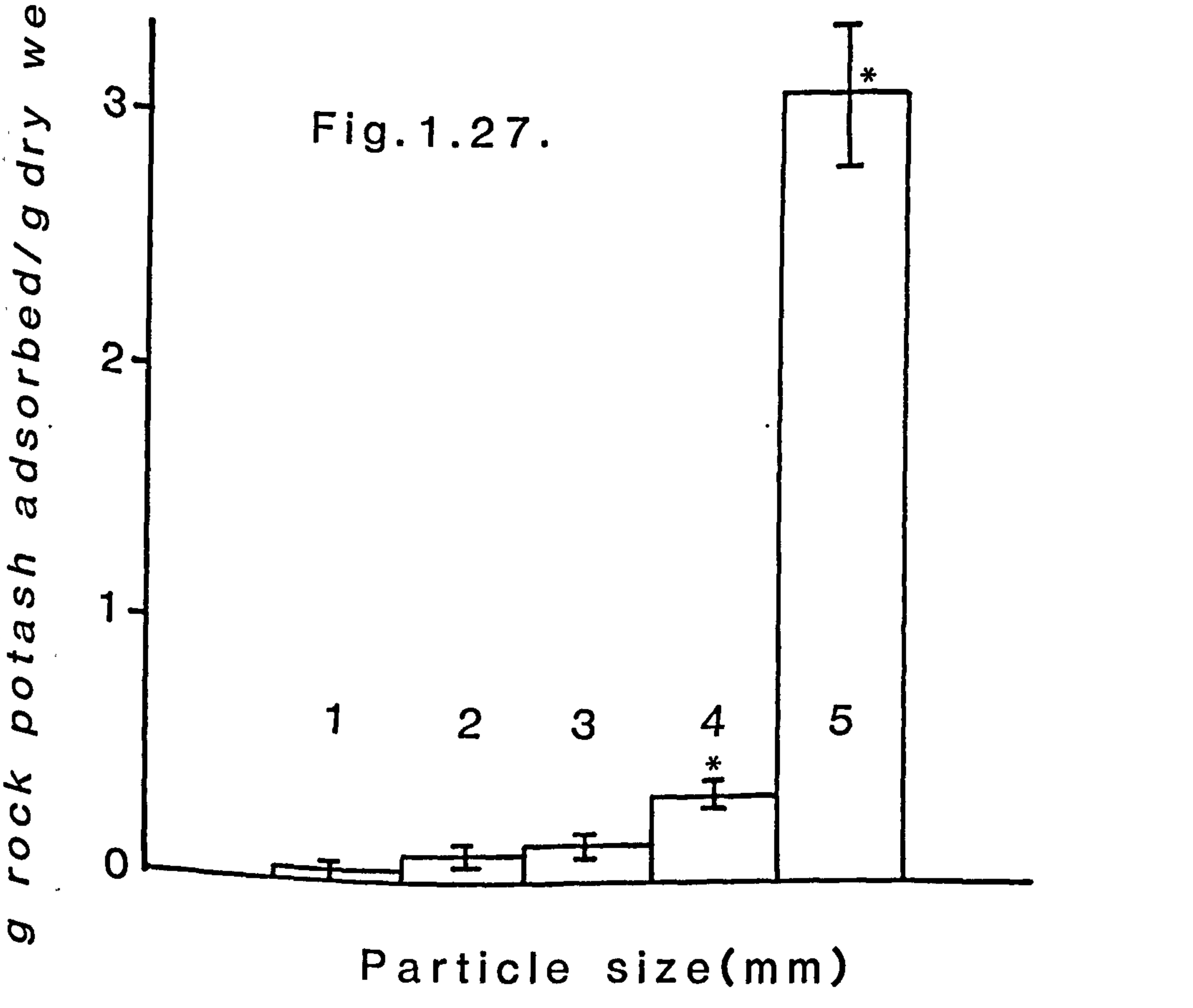


Fig.1.27.

Figure 1.28.

Effect of different metal ions on sulphur adsorption by *Mucor flavus*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in sulphur adsorption compared with the control (incubated in sterile distilled water), $p < 0.05$: ** significant increase in sulphur adsorption compared with the control (incubated in sterile distilled water, $p < 0.05$)

Fig.1.28.

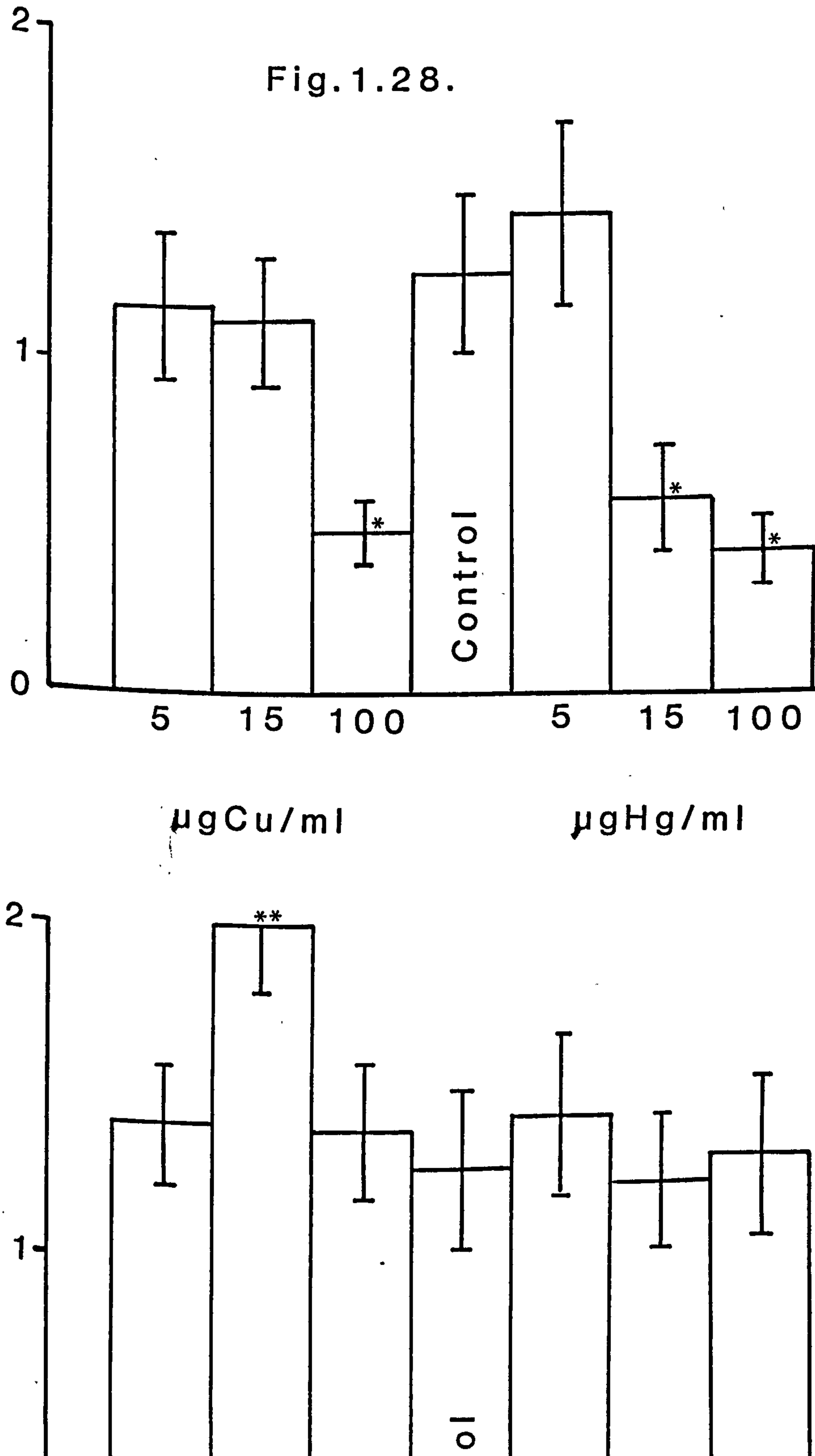

2
1
0
*
Control
5 15 100
µgCu/ml
µgHg/ml
µgMg/ml
µgMn/ml
S°adsorbed/g dry weight

Figure 1.29.

Protection of *Mucor flavus* from the toxic effects of mercury by the addition of sodium thiosulphate to the growth medium.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in biomass production, $p < 0.05$) (100µgHg/ml)

Figure 1.30.

Adsorption of zinc by *Mucor flavus* in the presence of toxic concentrations of mercury and increasing amounts of thiosulphate.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in zinc adsorption, $p < 0.05$; ** significant increase in filtrate pH, $p < 0.05$)

●—● mg zinc adsorbed

△—△ Filtrate pH

Fig.1.29.

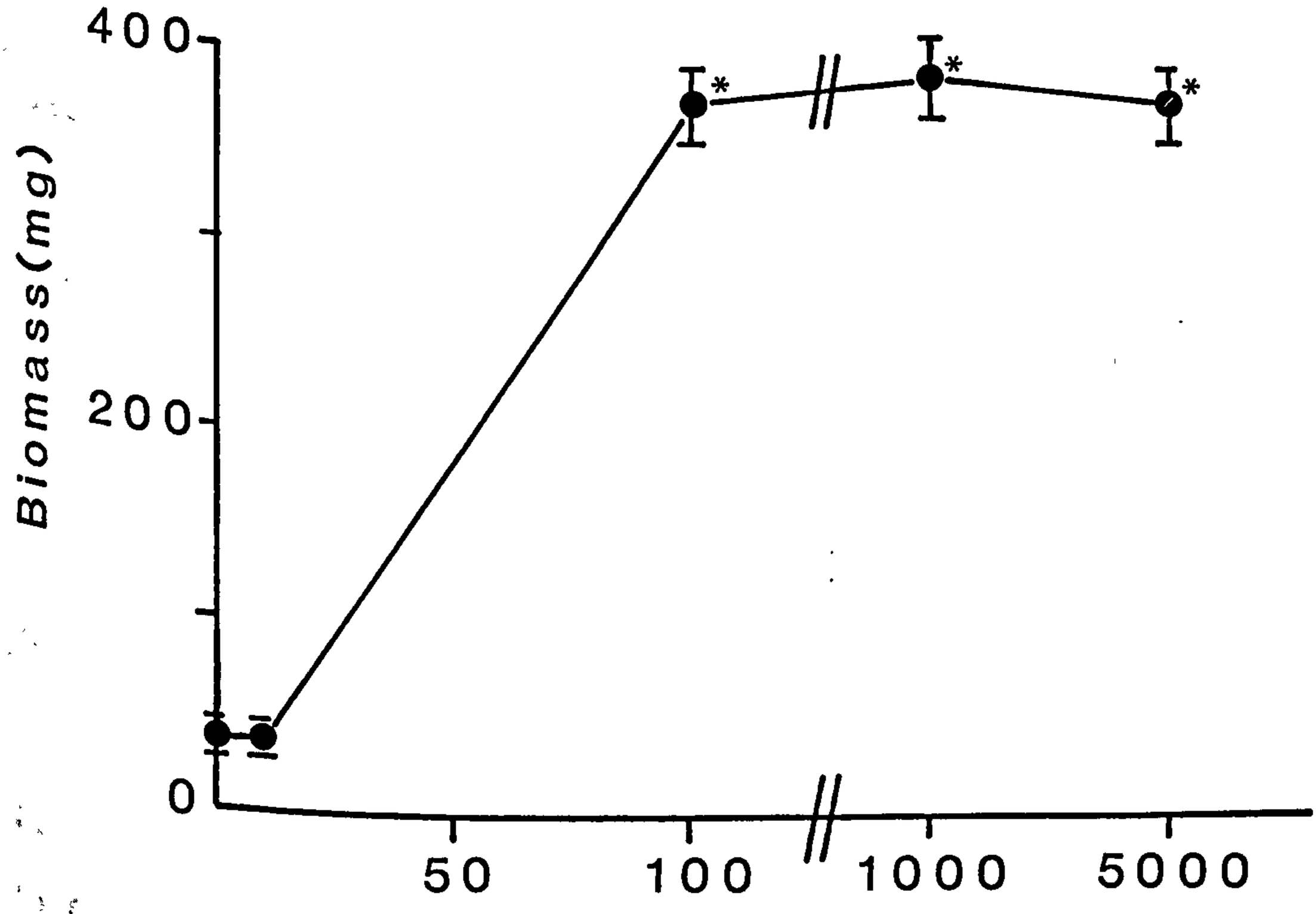


Fig.1.30.

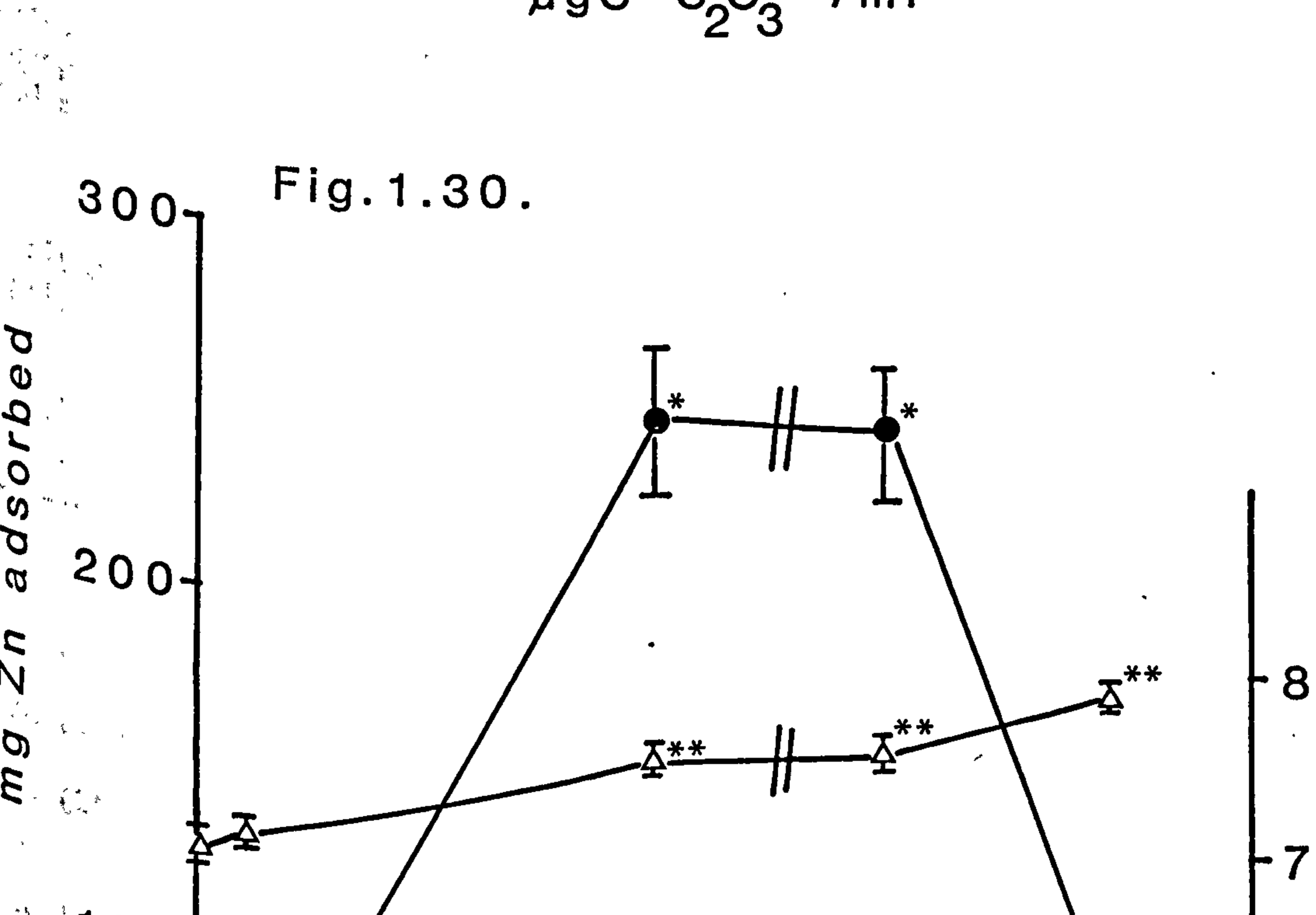


Table 1.3 Specificity of particulate adsorption by *Aspergillus niger*

Metal dust (mg)	Amount of metal adsorbed (mg) Cu	Fe	Zn
Cu (250)	102 ± 4	-	-
Cu (500)	183 ± 14	-	-
Fe (250)	-	79 ± 20	-
Fe (500)	-	78 ± 13	-
Zn (250)	-	-	151 ± 14
Zn (500)	-	-	211 ± 5
Zn (250) + Fe (250)	-	58.2 ± 14	*124 ± 4
Cu (250) + Fe (250)	96 ± 4	69 ± 26	-

(Means of triplicates ± S.D. *Significant decrease in metal adsorption compared to the control value obtained when the metal was incubated alone with mycelium, ($p < 0.05$).

Table 1.4 Solubilization of particulate iron by *Aspergillus niger*. Effect of the presence of copper and zinc dusts on solubilization.

Metal dust (mg)	μg Fe solubilized ml^{-1} filtrate	pH of filtrate
Fe (250)	105 ± 11	4.5 ± 0.1
Fe (500)	108 ± 5	4.7 ± 0.1
Fe (250) + Zn (250)	*1 ± 0.3	**6.4 ± 0.1
Fe (250) + Cu (250)	*63 ± 10	**5.4 ± 0.1
Controls (lacking fungus)		
Fe (250)	0.43 ± 0.03	6.1 ± 0.1
Fe (500)	0.52 ± 0.12	6.3 ± 0.1

(Means of triplicates ± S.D.
*significant decrease in metal solubilization, $p < 0.05$
**significant increase in filtrate pH, $p < 0.05$)

a high value. Copper also inhibited iron solubilization but to a much lesser extent than did zinc (Table 1.4). It seems that contact with the particulate iron was necessary to induce solubilization and the presence of other particulates inhibited this contact. As iron solubilization also appeared to be related to acid production by *A. niger* it is possible that the copper and zinc particulates prevented acid production in some way.

Effect of culture age on adsorption of coal dust by *A. niger*.

A. niger adsorbed coal dust, its ability to do so decreasing with increasing culture age (Fig. 1.31).

Effect of mild acid and mild alkali treatment of *N. crassa* mycelium on the adsorption of sulphur.

Mild acid treatment significantly increased sulphur adsorption by *N. crassa*, while alkali treatment significantly decreased sulphur adsorption (Fig. 1.32). Obviously these treatments must alter the cell wall structure in some way to have these effects. To further understand why the different treatments altered particulate adsorption acid and alkali treated mycelium were used in subsequent cell wall studies.

Cell wall studies.

N. crassa was used in these studies as its cell wall has been extensively studied and its structure and molecular composition well known (Potgieter & Alexander, 1965 Hunsley & Burnett, 1970). The cell wall of *N. crassa* was also relatively easy to isolate in the quantities needed for chemical analysis.

The results obtained for total sugar and total protein contents of 4 day old cell walls (Table 1.5) are consistent with previous findings of 14% (w/v) protein (Manocha & Ross-Calvin, 1967, Hunsley & Burnett, 1970) and 40.8% (w/v) sugar (Potgieter & Alexander, 1965). These results meant that the isolation method adopted was satisfactory and could be used in further isolation experiments.

Analysis of acid-treated cell walls revealed a decrease in total sugar content whereas the protein content remained stable (Table 1.5). To understand the significance of these results a detailed look at the wall structure of *N. crassa* is necesary (Fig. 1.33). The outer glucan (polymer of glucose) layer is accountable for almost all the sugar present in the cell wall. The only other area in which sugars are present in the cell wall of *N. crassa* is the glycoprotein reticulum. Therefore a decrease in sugar content implies loss of the glucan layer. As the different layers of the cell wall are not entirely separate, but tend to grade into one another, removal of the glucan exposes the underlying glycoprotein reticulum and easily removed protein layers. As acid treatment resulted in increased particulate adsorption the implications are that these underlying layers have greater adsorptive abilities than the glucan layer.

Alkali treatment caused a decrease in both total protein and total sugar content in the cell wall (Table 1.5), although the decrease in sugar content was lower than that caused by acid treatment. Alkali treatment is known to solubilize the glycoprotein reticulum (Mahadevan & Tatum, 1965) and as the reticulum is partly made up of glucan, loss of this structure will be responsible for some of the decrease in total sugar content. Sodium hydroxide has been used to extract proteins from the cell wall of *N. crassa* (Wrathall & Tatum, 1973) and my results indicate that approximately half of the wall protein is removed by treatment in NaOH (0.5N) for 24 hours at 25^0C. Removal of the glycoprotein reticulum and the easily removed protein layer probably caused a loss of some of the attached glucan layer which explains the rest of the decrease in total sugar obtained. The components of the wall left, i.e. ; some glucan, discrete protein and chitin layers appear to have low

Figure 1.31.

Effect of culture age on the adsorption of coal dust by *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in coal dust adsorption compared with the amount adsorbed by a 4 day old culture of *A. niger*, $p < 0.05$)

Figure 1.32.

Effect of acid and alkali treatment of *Neurospora crassa* on the organisms ability to adsorb sulphur.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in sulphur adsorption compared with the control, $p < 0.05$: ** significant decrease in sulphur adsorption compared to the control, $p < 0.05$)

a : Untreated mycelium (Control)

b : Alkali treated mycelium

c : Acid treated mycelium

Fig.1.31.

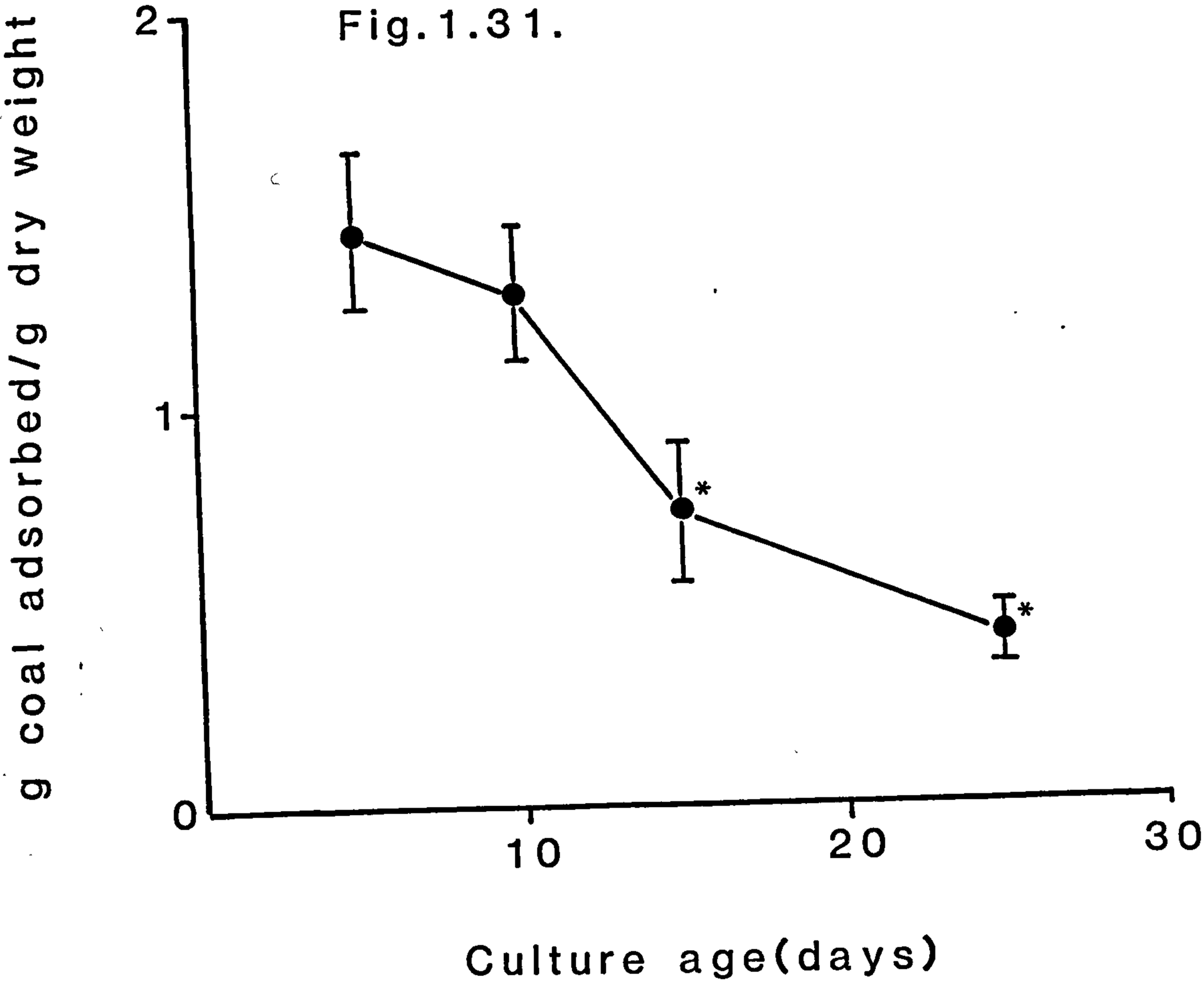


Fig.1.32.

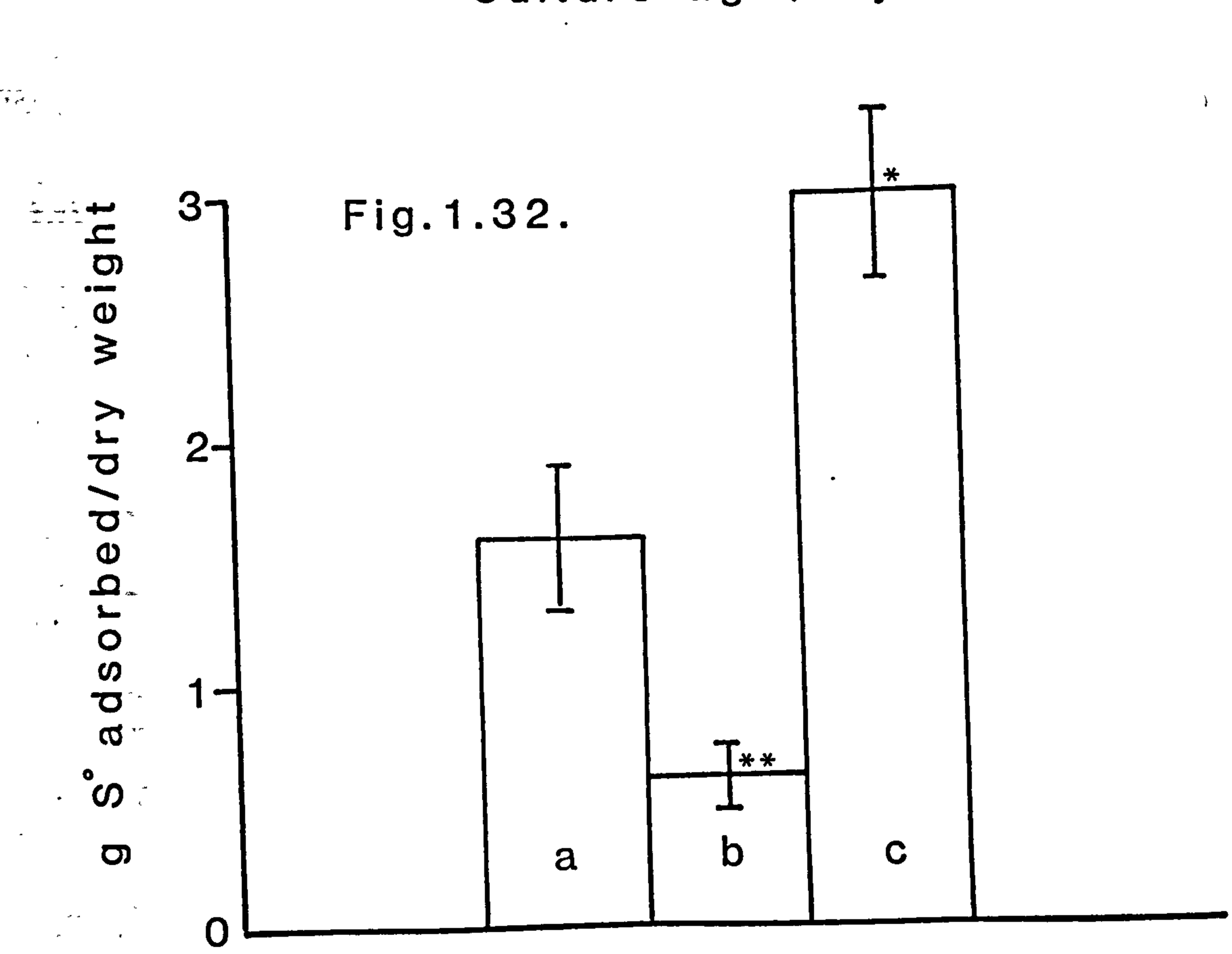
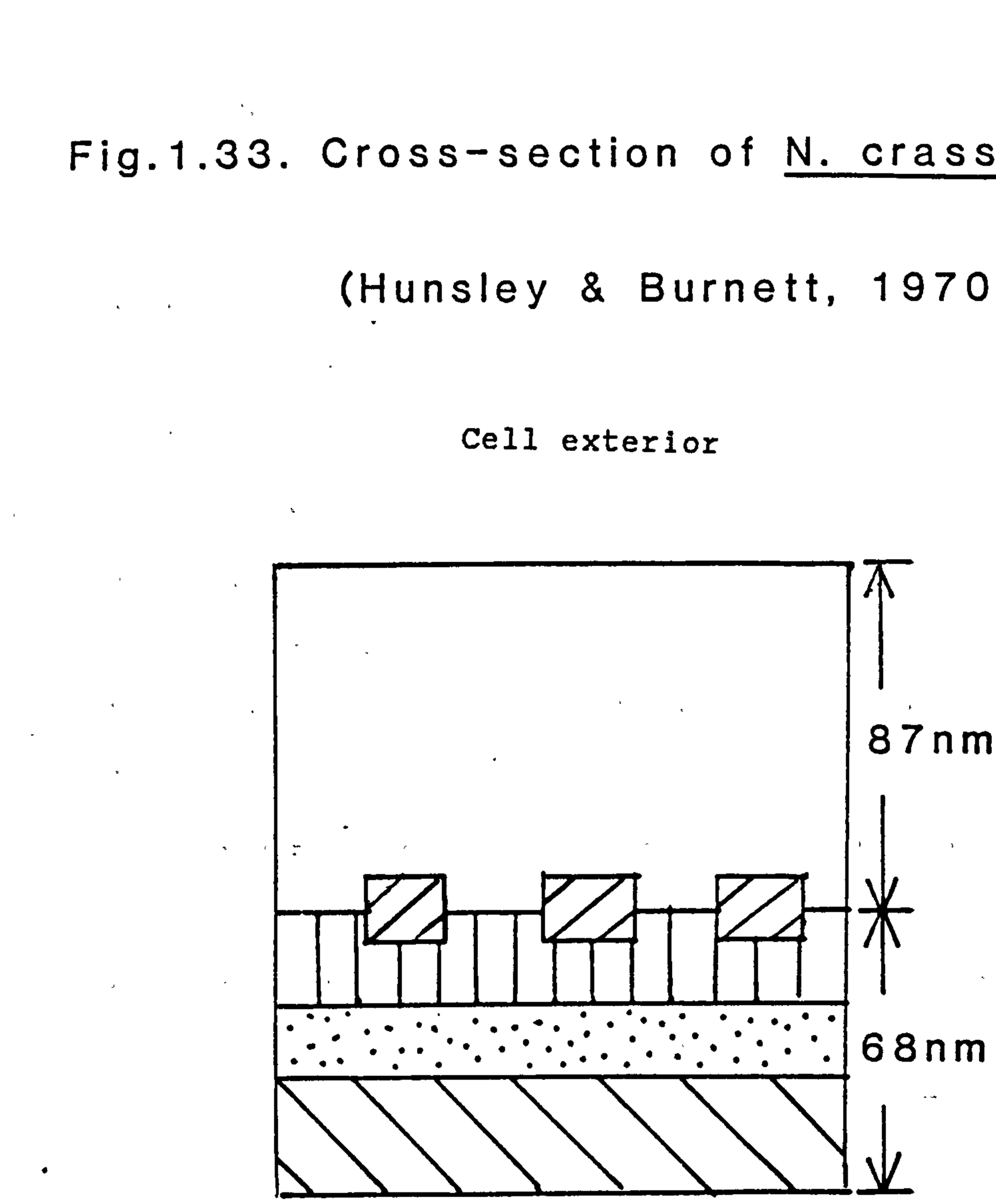


Fig.1.33. Cross-section of N. crassa cell wall

(Hunsley & Burnett, 1970)



adsorption capabilities as alkali treatment resulted in decreased sulphur adsorption. The proteins lost may have also contributed to the overall surface charge of the mycelium.

In standing culture, *N. crassa* exhibited a high degree of adsorption at the hyphal tips. Research by Hunsley & Kay (1976) suggests that the hyphal tips are composed of chitin overlaid with a glucan/protein complex which becomes overlaid with glucan as the hyphae mature (Fig. 1.34). From the results obtained by acid treatment of mycelium this overlaid protein layer appears to exhibit high adsorption capacities, and this would explain the increased adsorption observed at the hyphal tips. Alkali treatment would also remove the protein from the tips which would cause decreased particulate adsorption.

Nutrient starvation of mycelium did not change the total amounts of sugar or protein present in the cell wall (Table 1.5). This does not necessarily mean that no change in cell wall composition occurred. Substances adsorbed to the cell wall from the growth medium may for example, have been lost during the starvation treatment resulting in a change in mycelial surface charge. Starvation of yeast apparently caused a change in surface charge allowing the organism to bind onto a negatively charged glass surface (Van Haecht, 1984).

However, no conclusions can be drawn from the results of chemical analysis of isolated nutrient-starved cell walls of *N. crassa*.

<u>Is particulate adsorption by *N. crassa* due to hydrophobic or electrostatic interactions?</u>

The main forces affecting fungal adhesion to surfaces are considered to be electrostatic and hydrophobic interactions (Mozes *et al.*, 1987).

Cell wall suspensions of *N. crassa* when mixed with xylene showed no hydrophobic characteristics, that is, they remained in the water layer and did not mix at all with the hydrophobic xylene (Table 1.6). However, walls were noticed to

collect at the interface between the two layers, but were run off when the aqueous layer was collected. *N. crassa* adsorbed very few negatively charged hydrophilic microcarrier glass beads from distilled water (Fig. 1.35). Adsorption of the microcarriers increased significantly when Mg^{2+} ions were present in the adsorption medium (Fig.1.35). Presumably the glass beads remained hydrophilic and the Mg^{2+} ions acted as an ionic bridge between the two negatively charged surfaces allowing adsorption to occur. These results imply that particulate adsorption by *N. crassa* is mainly due to forces which are electrostatic in nature.

Figure 1.34.

Hyphal tip of *Neurospora crassa* (Hunsley & Kay, 1976).

Amorphous covering of the apex containing a glucan-peptide-galactosamine complex

Inner region of the apical wall containing chitin microfibrils

Figure 1.35.

Adsorption of negatively charged, hydrophilic, glass microcarrier beads by *Neurospora crassa* with and without the presence of Mg^{2+} ions.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in the adsorption of the microcarriers due to the presence of Mg^{2+} ions, $p < 0.05$)

Table 1.5 Total protein and total sugar concentrations (% dry weight) of the cell wall of *Neurospora crassa* after various treatments

	% (w/w) protein	% (w/w) sugar
4 day old mycelium	16.5 ± 1.75	41.67 ± 2.04
Alkali-washed	*7.58 ± 0.63	*32.42 ± 1.62
Acid-washed	17.35 ± 0.95	*27.87 ± 2.65
Nutrient-starved	17.08 ± 0.63	39.55 ± 4.16
12 day old mycelium	17.83 ± 0.88	40.34 ± 4.23

(Means of triplicates ± S.D.
*Indicates significant difference from values obtained from 4 day old mycelium, $p < 0.05$).

Table 1.6 Hydrophobicity of differently treated, isolated cell walls of *Neurospora crassa*.

Cell wall type	Amount of cell wall (mg) present per 4 ml aqueous layer.	
	Test	Control
4 day old	12.0 ± 2.2	11.1 ± 1.4
12 day old	16.8 ± 0.7	14.9 ± 2.0
Nutrient-starved	11.7 ± 1.6	13.2 ± 2.5
Acid-washed	24.6 ± 1.8	20.9 ± 2.9
Alkali-washed	32.1 ± 3.8	32.1 ± 2.3

(Means of triplicates ± S.D.)
Tests were mixed with xylene plus distilled water. Controls were mixed with distilled water alone. Isolated cell walls exhibited no hydrophobic characteristics.

CONCLUSIONS.

The results of this study suggest that particulate adsorption by *N. crassa* and *M. flavus* is a physical property of the cell wall, which occurs independently of cellular metabolism. However, certain fungi produce large amounts of extracellular polymers and in these cases the polymers will be responsible for particulate adsorption. The initial attractive forces in particulate adhesion by *N. crassa* are mainly electrostatic in nature and this is also probably true for *M. flavus* and *A. niger*. Cell wall studies of *N. crassa* indicate that the outer glucan layer of the hyphae, although able to adsorb, is not as efficient as the underlying protein layers at particle adsorption. Particles are generally adsorbed more rapidly by young, growing mycelium due to the continued production of adsorption sites, the presence of a larger number of hyphal tips and particle entrapment by hyphae. Adsorption increased in the presence of magnesium ions, but decreased in the presence of copper and mercury ions. The decrease in adsorption caused by mercury ions could be overcome by the addition of thiosulphate to the medium. Optimum particulate adsorption would occur at a temperature of 25^0C within a pH range of 3 to 9. Possible industrial applications of particulate adsorption by fungi will be discussed in the following chapters.

2. ECOLOGICAL AND BIOTECHNOLOGICAL IMPLICATIONS OF CLAY-FUNGUS INTERACTIONS.

2. ECOLOGICAL AND BIOTECHNOLOGICAL IMPLICATIONS OF CLAY-FUNGUS INTERACTIONS.

INTRODUCTION.

The ecological significance of clays.

Clays generally constitute a large proportion of soil solids. A considerable amount of the surface activity of soils results from the large surface area presented by clays. By definition, clays are finely divided particles. In a cubic metre of clay, each particle having a diameter of 2µm, the total surface area would be about 3 x 10^6 m^2 (Nedwell & Gray, 1987). This surface, which is in contact with the soil water, contains many chemically reactive groups interacting with one another as well as with molecules in the water film. The principal reactive units on clay surfaces are siloxane ditrigonal cavities, inorganic hydroxyl groups and a variety of organic components of humic substances which are themselves complexed with clays. Since these humic materials are capable of binding protons and immobilizing soil enzymes, they make an important contribution to the surface chemistry of soils.

The siloxane ditrigonal cavities of clays can act as electron donors and interact with neutral dipolar molecules such as water. An excess negative charge can be generated in these cavities if an isomorphous replacement of trivalent aluminium (present in the underlying layer of the clay molecule) with divalent iron or magnesium has occurred. Such a replacement is present in montmorillonite and allows the clay to complex cations. Isomorphous replacement of tetravalent silicon (which is present in the siloxane ditrigonal cavity) generates a stronger excess negative charge allowing clays, such as vermiculite, to form even stronger complexes

with cations and dipolar molecules. Inorganic hydroxyl groups are the most abundant reactive groups, but their properties vary depending upon the atoms with which they are coordinated. They are capable of complexing hydroxide anions, H^+ ions, oxyanions, such as HPO_4^{2-}, and metal cations.

Clays can adsorb soluble organic compounds present in the soil solution in a variety of ways including cation exchange, protonation, anion exchange, water bridging, cation bridging, ligand exchange, hydrogen bonding and van der Waals interactions (Greenland, 1971). The organic compounds themselves are capable of binding protons.

The reactive groups on clay surfaces all have profound effects on the water and its solutes surrounding the clay particles. Adsorbed water extends for up to 3nm around kaolinite, 5nm around vermiculite and perhaps up to 10nm around montmorillonite particles (Nedwell & Gray, 1987). The adsorbed water has different solvent properties to the bulk liquid away from the surface and there is enhancement of complex formation between dissolved materials and exchangeable cations and the siloxane ditrigonal cavity.

The ability of clays to attract and therefore concentrate numerous organic cations, which are present in soils, is likely to influence microbial growth in soils. The adsorbed organic compounds will act as potential substrates for, or inhibitors of microbial growth. In contrast, anionic organics generally tend to be repelled from colloid surfaces. This will presumably make the acquisition and uptake of these compounds difficult for the microorganisms which use them as growth substrates. The consequences of organic substrate sorption for an adjacent microbial cell will depend on the strength of cation retention at the colloid surface; how much, and what part of the molecule, is involved in sorption and how much is exposed; as well as the ability of the microbial species to remove and assimilate adsorbed substrate.

The accumulation of H^+ at clay surfaces will give rise to a surface pH which may be 2 to 3 units more acid than that of the aqueous bulk phase barely 100nm away (McLaren & Skujins, 1968). The magnitude of this pH effect will be accentuated by microbial metabolism and proton release. The consequences of this specific microenvironment could include:

(1) The setting up of microenvironment pH gradients which may influence proton motive force and transport processes.

(2) The solubilization of some inorganic nutrients and the precipitation or adsorption of others.

(3) Different rates of enzyme-substrate interaction at acidic surfaces compared with those distant from that surface.

(4) Changes in the composition and ionogenic properties of microbial cell walls.

Microorganisms also possess fixed reactive groups on their surfaces including COO^- groups (Rogers, 1979). The degree of attraction between particle and organism depends on a balance between electrostatic repulsion, electrostatic attraction and van der Waals attractive forces. The degree of repulsion between surfaces decreases with increasing electrolyte concentration and, as soils dry out, the chances of adsorption will increase so that soil dwelling microorganisms tend to become coated with clay particles. Examples of clays attaching to microorganisms include adsorption of sodium bentonite particles to *Bacillus subtilis* (Lahav, 1962) and the orientation of clay particles sorbed onto bacteria (Marshall, 1969).

In conclusion, microbes, organic substrates and metabolites, and inorganic ions tend to accumulate at charged clay surfaces rather than being freely diffusable in the aqueous phase. The most influential particles are those clays that expand upon hydration to reveal an extensive, highly charged internal surface area.

Influence of clay minerals on bacteria.

Montmorillonite can increase the respiration rates of a wide range of bacteria growing *in vitro*. One such effect is a shortening in the lag phase of growth (Stotzky & Rem, 1966) which is thought to result from the ability of clay to act as a buffering agent, thereby maintaining pH levels at values adequate for sustained growth. Montmorillonite also protects bacteria against the effects of hypertonic osmotic pressures. Kaolinite can also stimulate bacterial respiration, but to a lesser extent than montmorillonite. Bacterial stimulation by montmorillonite was found not to be related to its ablity to supply inorganic nutrients, as both kaolinite and montmorillonite served equally well as a source of minerals for bacterial nutrition suggesting that other unknown mechanisms are involved in the stimulation of bacterial activity. Further studies by Stotzky (1966) demonstrated that bacterial respiration rates were related to the cation exchange capacity (CEC) of the clay particles, but not to particle size. Respiration increased with an increase in CEC up to a threshold value. A relationship between surface area and respiration was also demonstrated, but the influence of specific surface on bacterial activity was not unequivocal as several measurements of specific surface were not independent of the CEC of the particles. Large amounts of montmorillonite were found to be inhibitory to some species of bacteria grown in pure culture presumably because of restricted gas exchange (Stotzky, 1966).

Gadd & Griffiths (1978) demonstrated that clays could protect bacteria from the toxic effects of cadmium. The greater the CEC of clay particles, the greater the amount of cadmium adsorbed, resulting in a decrease in metal toxicity.

Conclusions drawn from these studies are that montmorillonite-like minerals appear dominant in influencing microbial activity and ecology in soil and that this

influence appears to be due to the presence of readily accessible exchange sites on these clay minerals.

Amendment of soils with clay particles, in particular montmorillonite, has considerable influence on the fate of both native and introduced bacteria. For example, in a field experiment, survival of *Rhizobium leguminosarum* biovar *trifolii* in sandy soil, was shown to be greatly improved by the addition of montmorillonite (Marshall & Roberts, 1963). Later work showed that the addition of powdered montmorillonite to a sandy soil protected *R. leguminosarum* from the effects of desiccation (Busby & Marshall, 1977). The clay minerals montmorillonite and kaolinite are able to protect *Rhizobium* sp. against the antagonistic effects of toxin producing fungi in soil by actually adsorbing the toxin (Habte & Barrion, 1984). It was also suggested that bentonite clay partially protected rhizobial cells introduced into loamy sand against predation by protozoa (Heynan *et al.*, 1988). This effect probably resulted from the formation of micro-niches in which bacteria could not be reached by the grazing protozoa. Further examples of clays affecting antagonistic microbial behaviour include the inhibition by montmorillonite of host-parasite interaction between *Escherichia coli* and a specific bacteriophage, an effect which was dependant upon clay particle size (Roper & Marshall, 1978). More recently the influence of the clay minerals, montmorillonite and vermiculite on the suppression of tobacco black root rot, by the biocontrol agent *Pseudomonas fluorescens* CHAO, has been examined (Stutz *et al.*, 1989). Tobacco plants were found to show more disease symptoms when grown in pure montmorillonite or illite soils than when grown in vermiculite. This suggests that *P. fluorescens* CHAO was unable to protect the plants from the action of *Thielaviopsis basicola*, the causal agent of the disease, in the presence of montmorillonite or illite. However, the exact effect of clays on the growth of *T. basicola* and *P. fluorescens* was not demonstrated.

Marshman & Marshall (1981a) studied the growth of bacteria on pure proteins adsorbed on clay minerals. This work demonstrated the complexity of substrate-clay-cell interactions in relation to microbial growth in soils. Their results were best explained by assuming that the protein was bound at two sites; at one, the protein was available to bacteria, at the other it was not. These sites did not appear to coincide with internal and external lattice surfaces. In a later paper, Marshman & and Marshall (1981b) suggested that clays such as montmorillonite could affect microbial growth through interactions with the organisms, their substrates, individual enzymes and growth factors, an effect which would increase markedly the complexity of nutrient flow to organisms. Dashman & Stotzky (1986) have also shown that complexes formed between different amino acids and peptides and montmorillonite are differentially available to microorganisms. The affinities of permeases for different amino acids are some 100-10,000 times greater than the affinities of clays for the same amino acids, so these are utilised readily; unfortunately information on peptides was lacking. The authors suggested that the yield of energy from the intracellular metabolism of some amino acids might be less than the energy required to remove the substrate from clay and transport it into the cell. Thus cysteine was not utilised when bound to montmorillonite or kaolinite whereas proline and arginine were. The requirement for energy to be expended to remove materials from clays may thus be an important consideration in affecting the relative amounts of energy available for growth and maintenance.

Influence of clay minerals on fungi.

The respiration of mycelial homogenates of twenty seven fungi, representing four different classes, was generally not affected by clays at concentrations below

2% (w/v), regardless of the initial pH of the systems or whether simple or complex media were used (Stotzky & Rem, 1967). The buffering ability of clay minerals was apparently not a critical factor in the growth of fungi, which are generally more tolerant to low pH than are bacteria. With higher clay concentrations, however, the metabolic activity of most species was markedly inhibited. This inhibition occurred with montmorillonite at concentrations above 2% (w/v) and with kaolinite, essentially at concentrations above 40% (w/v), and was greater as the metabolic activity of the mycelium increased. Comparable concentrations of other clay minerals (and similar particles) resulted in degrees of inhibition intermediate between those caused by montmorillonite and kaolinite (Stotzky, 1967). Low concentrations of clays stimulated the respiration of some fungi, namely *Cunninghamella echinulata* and *Sordaria fimicola* but only in certain growth media. The inhibition did not result from limitations in carbon substrates, but was related to the viscosity of the systems, which presumably influenced the rate of oxygen diffusion. The viscosity per unit weight of clay was increased more in montmorillonite, than in kaolinite systems, reflecting the structure and physicochemical proerties of these clay minerals; kaolinite is a non-swelling clay, whereas montmorillonite swells enormously by trapping water between its lattices. As gas exchange occurs more slowly in a gel structure than in a particulate suspension, respiration was inhibited more by montmorillonite than kaolinite at comparable or even greater concentrations. Although differences in water structures of the clays (caused by the clay trapping water within it's lattices) may have been involved, this aspect was probably of minor importance as the systems were continuously shaken and the water structure thereby continuously disrupted (Stotzky & Rem, 1967).

The effects of clay minerals on germination of fungal spores were less pronounced than on mycelial respiration, but increasing concentrations of

montmorillonite (but not of kaolinite and other particles), reduced rates of germination of most species (Santoro & Stotzky, 1967). However after 12 hours incubation no differences in spore germination (measured by spore swelling) were apparent in media with or without montmorillonite. The inhibitory effects of montmorillonite became more pronounced after the spores had germinated, presumably because oxygen diffusion was impaired and oxygen availability became more limiting to mycelial metabolism than to spore germination.

As clay-water systems comparable to those studied are not usually found in nature, the relevance of these observations to the influence of clay minerals on fungi in natural habitats is unclear. However, it may be possible to make some comparisons from the observed results. When natural microenvironments lose water, the clay:water ratio increases and a gel-like condition may result in the presence of montmorillonite clays, causing an impairment of gas exchange and inhibition of fungal development. This situation would not occur in the absence of swelling clay minerals and cycles of drying and wetting might be less detrimental to fungi in such habitats. Indeed, it would then be expected that the presence or absence of certain clays would affect the contents of the microbial soil population depending upon the abilities of different organisms to cope with different microenvironments.

The presence of montmorillonite clay minerals in soil is assumed to restrict the geographic distribution of *Fusarium* wilt diseases (Stotzky & Martin, 1963) and the distribution of the fungal pathogen, *Histoplasma capsulatum*, is thought to depend on the clay mineral composition of the soil (Stotzky & Post, 1967). In the latter case, the absence of montmorillonite was highly correlated with the presence of *H. capsulatum* in soil.,a relation that was not apparent with other types of clay minerals. Further studies on the effect of montmorillonite on the growth of *H. capsulatum* revealed that the clay, even at low concentrations, markedly reduced

fungal respiration rates (Lavie & Stotzky, 1986). Kaolinite and attapulgite also reduced respiration of the fungal pathogen. In both cases the reduction was less than that caused by montmorillonite. An increase in viscosity (which could impair movements of oxygen) caused by clays was not responsible for the reduction in respiration and the clays did not interfere with the availability of nutrients. Instead, scanning electron microscope studies revealed that clay particles were tightly bound to the hyphae, suggesting that the clays reduced the rate of respiration of *H. capsulatum* by adhering to the mycelial surface and thereby interfering with the movement of nutrients, metabolites and gases across the mycelial wall. However, the adhesion of clays did not explain the differences in the amount of reduction in respiration caused by the same maximum concentration of each clay, as the amounts of montmorillonite and kaolinite bound to hyphae appeared to be similar. This suggests that montmorillonite may also affect the respiration of the fungus by other, as yet, unknown mechanisms.

The effects of bentonite clay on the interaction between the fungal pathogen *Gaeumannomyces graminis* var. *tritici* and two bacterial pathogens was also studied (Campbell & Ephgrave, 1983). Clay increased the growth rate of *G. graminis*, an effect that was possibly to due the change in water availability caused by the clay. The effectiveness of one of the bacterial culture filtrates in restricting fungal growth was reduced by bentonite, though antagonism was maintained in the presence of bacterial cells. This suggests that the clay protected the fungus by adsorbing some of the toxins produced by the bacterium.

The above report is not alone in showing the stimulation of fungal activity by clay minerals as a stimulation of the metabolism of the fungus, *Penicillium frequentans*, by montmorillonite has also been observed (McCormick & Wolf, 1979).

Montmorillonite and kaolinite were found to protect several species of filamentous fungi from the inhibitory effects of cadmium (Babich & Stotzky, 1977a,

1977b) when grown in pure culture. The protective ability of the clays was again correlated with their cation exchange capacity (CEC). The greater the CEC, the greater the adsorbance of cadmium by the exchange complex and the greater the protection. Montmorillonite afforded greater protection against cadmium than did kaolinite and this was correlated with its higher CEC. A later study by Babich & Stotzky (1977b) showed that fungi could tolerate higher concentrations of cadmium when grown in soil than when grown in laboratory media, indicating that soil reduced the toxic effects of cadmium. In soil amended with clay minerals, montmorillonite provided partial or total protection against fungistatic effects of cadmium, whereas additions of kaolinite provided little or no protection.

The above discussion reveals the complexity of microbial interactions with clays. However, the precise cause of any observed effect is rarely revealed although many plausible explanations have been provided to explain the results (Table 2.1). Table 2.1 also reveals that a large number of the stimulation and inhibitory effects may be due to modifications of extracellular enzyme-substrate interactions which leads to another aspect of the effect of clays on the soil micro-environment.

Clay-enzyme interactions.

Enzymes in soil can be separated into a number of categories according to their location within the soil micro-environment (Burns, 1986):

(1) Enzymes associated with living, metabolically active cells.

(2) Enzymes associated with viable but non-proliferating cells such as resting vegetative cells, bacterial endospores, fungal spores, protozoan cysts and even plant seeds.

(3) Enzymes attached to entire dead cells or cell debris.

Table 2.1 Influence of soil clays on microbial activity (Burns, 1986)

Colloid surface phenomenon	Effect on substrate decay and/or microbial growth (relative to that in absence of clay)
Juxtaposes microbe (or enzyme) and substrate	Stimulation
Orients enzyme beneficially relative to substrate	Stimulation
Functions as buffer during metabolism	Stimulation
Retains water film	Stimulation
Adsorbs inhibitory metabolite	Stimulation
Concentrates inorganic nutrient	Stimulation
Supplies inorganic micronutrient	Stimulation
Protects microbe from predator	Stimulation
Inactivates phage	Stimulation
Adsorbs microbe (or enzyme distant from substrate	Inhibition
Intercalates substrate ∴ inaccessible to microbe	Inhibition
Inactivates enzyme due to structural changes	Inhibition
Masks active site of enzyme	Inhibition
Increases viscosity ∴ retards O_2 diffusion	Inhibition

(4) Enzymes which are more or less permanently immobilized on soil clay and humic colloids.

Expandable clays have a high affinity for enzymes although this is not always synonymous with the retention of catalytic ability. Enzymes associated with soil humates retain their activity for long periods.

The adsorption of proteins to clay surfaces and the subsequent protection of the adsorbate from decomposition have been known for many years (Ensminger & Gieseking, 1942). However the involvement of an enzymes active sites, or changes in tertiary structure, during binding to clay surfaces will reduce or eliminate the enzymes activity. That is, the protein may be strongly held at and protected by the clay, but it no longer displays activity. Most studies (Makboul & Ottow, 1979; Ross, 1983) confirm that enzyme activities are reduced upon adsorption to clays and usually highly adsorptive, expandable clays, such as montmorillonite, have a more marked effect than kaolinite (Table 2.2). However, there are exceptions to this. Makboul & Ottow (1979), for example, showed that V_{max} values of alkaline phosphatase actually increased in the presence of Ca^{2+} - montmorillonite. Also the way an enzyme is adsorbed to a clay surface is an important parameter involved in the loss of enzymic activity.

In conclusion, microbial activity in soil is strongly influenced by clays which attract organic substrates, metabolites, inorganic ions and water films to their surfaces. Thus, clays similar to montmorillonite, assume an importance far in excess of their percentage contribution to the total soil mass. Also extracellular enzymes (together with proteins, peptides and amino acids) are rapidly sorbed at clay surfaces generally resulting in a partial or total loss of activity depending on the mechanism and site of adsorption to the clay.

Table 2.2 Influence of clay minerals on the activity of enzymes (Burns, 1986)

Clay mineral	Invertase	α-Amylase %	β-amylase
Allophane	20	57	12
Muscovite	33	96	1
Illite	9	27	1
Montmorillonite	0	4	1
Kaolinite	45	1	0

Results are expressed as % of original activity.

Biotechnological applications of clay-fungus interactions

Clay suspensions, particularly when colloidal, are difficult to remove from solution and natural settling may involve a period of a decade or more. The large scale extraction of phosphates in the U.S.A. and the beneficiation of uranium, potash and aluminium provide good examples of where large amounts of clays which are difficult to de-water are produced (Brierley & Lanza, 1985).

Microbiological processes have been applied for the removal of suspended clays from aqueous suspensions, the processes essentially consisting of two types (Brierley *et al.*, 1981):

(1) Biologically produced polymers for flocculation of colloidal particles.

(2) Adsorption of particulates by filamentous fungi.

In relation to the first method of clay removal from suspensions, a polymer known as pullulan, produced by *Aureobasidium pullulans*, has been patented for the flocculation of clay-slimes resulting from the beneficiation of uranium, potash and aluminium (Brierley & Lanza, 1985).

A method involving the adsorption of clays by fungal mycelium has been reported by Brierley *et al.* (1981). Here spores of the fungus, *Cladosporium cladosporioides* adsorbed clays to form a clay-fungus pellet. The addition of sugars and yeast extract to the water allowed the spores to germinate and the germlings increased the rate of clay adsorption. It is likely that fungal mycelium, having a net negative charge, will attract the positively charged edge of clay particles, the electrostatic attraction resulting in clay adsorption. Hydrogen bonding occurring between the β-glucan on the outer surface of the fungal cell wall and waters of

hydration on the clay particle has also been suggested as a mechanism of fungal clay adsorption (Lavie & Stotzky, 1986). Unfortunately, spores of *C. cladosporioides* were found to be incapable of adsorbing non-sterile clays, a fact which obviously limits their use on an industrial scale (Brierley & Lanza, 1981).

To date, despite the intensive research, an effective, economical biological process for the treatment of colloidal clay wastes has not been found.

In this chapter, ecological and biotechnological aspects of the effects of the clay minerals, montmorillonite and kaolinite on the growth of *Aspergillus niger* in liquid culture are discussed. In particular, biomass production and resistance to pesticides are examined .

The ability of *Mucor flavus* to adsorb clay particles from suspension and factors affecting this adsorption are determined. Studies on the use of *A. niger* mycelial waste, from the industrial production of citric acid, to either flocculate or adsorb clays are also discussed.

MATERIALS AND METHODS.

Effect of addition of various concentrations of either kaolinite or montmorillonite to Czapek Dox liquid medium on biomass and acid production by *Aspergillus niger*.

Aspergillus niger spore suspension (1×10^7 spores ml^{-1}) was added to Czapek Dox medium (100ml) in Erlenmeyer flasks (250ml) containing the following amounts of montmorillonite: 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 4.0 (% w/v). The montmorillonite was sterilized by steaming for 1 hour on three successive days. Clay concentrations were provided in triplicate and all flasks were shaken (150 r.p.m., 25^0C) for 7 days. After this time flask contents were decanted into pre-dried (55^0C), pre-weighed centrifuge tubes and centrifuged (MSE Mistral 6L, 12 x 100ml angle head, 5000 r.p.m. for 15 minutes) to collect the added clay and the fungal biomass produced. The supernatant was poured off and it's acid content determined as follows: Three drops of phenolpthalein indicator solution were added to a supernatant sample (10ml). NaOH (1N) was then added dropwise until the solution turned pink. The amount of NaOH needed to achieve this end-point was noted and this value was multiplied by 7 to give the g acid per litre of supernatant. The clay-fungus pellet was dried (55^0C) to constant weight and weighed. The fungal biomass produced was calculated by subtracting the initial amount of clay added from the total dry weight of mycelium plus clay.

The effect of kaolinite on biomass and acid production by *A. niger* was determined in the same manner.

Light microscopy studies on the effect of montmorillonite on the growth of *A. niger*.

Aspergillus niger spore suspension (1.5×10^7 spores ml^{-1}, 5.0ml) was added to either Czapek Dox medium alone (25ml) or Czapek Dox medium (25ml) amended with sterile montmorillonite (0.5% w/v) in Universal bottles. Bottles were shaken (150 r.p.m., 25^0C) and samples (3ml) removed aseptically at 0, 6, 12, 18, 24 and 36 hours after inoculation. Samples were kept at 4^0C until ready for examination by light microscopy using a Leitz Dialux 22 microscope. Photographs were taken of each sample.

Ability of montmorillonite and kaolinite to adsorb citric acid.

Citric acid (0.5g) was added to Czapek Dox liquid medium (100ml) containing either 0, 0.05, 0.1, 0.25, 0.5. 1.0 and 4.0 (% w/v) of kaolinite or montmorillonite. All solutions were sterilized by autoclaving and then shaken (25^0C, 150 r.p.m.) for 7 days, after which time the flask contents were centrifuged (MSE Mistral 6l, 12 x 100ml angle head, 5000 r.p.m. for 15 minutes) to separate the clays from the medium. The supernatant was analysed for total acid content to determine the amount of acid removed from solution by varying concentrations of the different clays.

Effect of montmorillonite on the growth of *A. niger* in Raistrick's medium containing various carbon concentrations.

Aspergillus niger spore suspension (1.0×10^7 spores ml^{-1}, 0.25ml) was added to Raistrick's medium (100ml, pH 3.5) containing 0.1, 0.25, 0.5 and 3.0 % carbon (w/v) as sucrose. Montmorillonite (0.3g) was added to an identical set of flasks

containing the same amounts of medium and carbon. Flasks were shaken (150 r.p.m., 25^0C) for 7 days after which time flask contents were centrifuged (MSE Mistral 6L, 12 x 100ml, 5000 r.p.m. for 15 minutes) to collect the mycelium and added clays. The biomass produced at each different carbon concentration, with and without clays, was determined. The pH values of the different supernatant solutions was also recorded.

Does montmorillonite protect *A. niger* from the fungicides Thiram and Dicloran ?

Aspergillus niger spore suspension (1.0×10^7 spores ml^{-1}, 0.25ml) was added to either Czapek Dox liquid medium alone (100ml) or Czapek Dox amended with sterile montmorillonite (0.5% w/v). Flasks were shaken (150 r.p.m., 25^0C) for 2 days after which time either 0, 0.01, 0.025 or 0.05g Thiram or Dicloran were added to the flasks. The mixtures were shaken for a further 7 days and the biomass produced in, and the filtrate pH of each flask was determined.

Clay adsorption by *Mucor flavus*,

Mycelium (3g wet weight) of *M. flavus* grown in Czapek Dox medium (150 r.p.m. 25^0C) for 7 days was exposed to various clays (sterilized by steaming for 1 hour on 3 successive occasions) to determine the factors which influence clay adsorption. Adsorption of montmorillonite, kaolinite and a natural clay was determined by shaking (150 r.p.m.) the mycelium in the clay suspension, removing the mycelial plug, washing it gently with distilled water and centrifuging (MSE Mistral 6L, 12 x 100ml angle head, 5000 r.p.m. for 15 minutes) the washings plus flask contents in pre-dried (55^0C), pre-weighed centrifuge tubes. The supernatant

was removed and the clay dried at 55^0C and then weighed. The amount of clay adsorbed by the mycelium was then determined.

Factors affecting clay adsorption by *M. flavus*,

Length of exposure to clays.

Mucor flavus was incubated with montmorillonite and kaolinite (0.2g of each clay) for 21, 42 and 165 hours at 25^0C and the amount of clay adsorbed after each different time determined.

Initial amount of natural clay added.

Mucor flavus was incubated (25^0C, 150 r.p.m.) with 0.1, 0.2, 0.3 and 0.4 g of a natural clay for 24 hours after which time the amount of clay adsorbed was determined.

Effect of temperature on clay adsorption.

Mucor flavus was incubated (24 hours, 150 r.p.m.) with a natural clay (0.2g) at 5, 15 and 25^0C and the amount of clay adsorbed at the different temperatures determined.

Effect of pH on clay adsorption.

Mucor flavus was incubated (24 hours, 150 r.p.m.) with natural clay (0.2g) at the following pH values: 2.4, 3.8, 5.4 and 7.8 (made by adding dilute H_2SO_4 to

distilled water). The amount of clay adsorbed at the different pH values was determined.

Adsorption of non-sterilized clay.

Mucor flavus was incubated (25^0C, 150 r.p.m.) with either steam sterilized natural clay (0.2g) or non-sterilized natural clay (0.2g). The amounts of sterile and non-sterile clays adsorbed were determined.

Ability of *Aspergillus niger* waste mycelium, produced from the surface fermentation method of citric acid production, to adsorb clays.

Surface fermentation waste (5g fresh weight) was added to sterile distilled water (100ml) amended with either montmorillonite (0.5g) or kaolinite (0.5g). The mixtures were shaken (25^0C, 150 r.p.m.) for 24 hours after which time the waste mycelium was removed from the flask by filtration using a double layer of nylon mesh (1mm diameter mesh) filter. The filtrate was centrifuged (MSE Mistral 6L, 12 x 100ml angle head, 5000 r.p.m. for 15 minutes) in pre-dried (50^0C), pre-weighed centrifuge tubes and the supernatant poured off. The clays were dried (50^0C) and the amounts of different clays adsorbed by the waste mycelium determined.

Ability of soluble products obtained from waste mycelium produced by the surface fermentation and deep fermentation methods of citric acid production, to flocculate clays. (Montmorillonite and kaolinite).

Both types of mycelial waste (100g fresh weight) were shaken (150 r.p.m., 25^0C) in sterile distilled water (500ml) for 24 hours. The mixtures were centrifuged

and the supernatants (after adjustment to pH 3.5 with dilute sulphuric acid) were filter sterilized (0.45µm pore size) and stored at 4^0C until needed. Supernatant (6ml) was added to capped sterilized test-tubes (6") containing sterile suspensions of either montmorillonite or kaolinite (1ml of a solution containing 0.35g clay in 20ml distilled water). Control tubes contained sterile distilled water (6ml) and clay suspension (1ml) and also the supernatants (6ml) obtained from both deep and surface produced mycelial waste. All solutions were mixed (using a whirlimixer), shaken (150 r.p.m., 25^0C) for 16 hours and then left to stand (6 hours) to allow settling to occur. The top 3ml of solution from each test-tube was removed (1ml at a time) taking care not to disturb the contents and each 1ml layer tested for turbidity at 500nm using a Cecil Instruments CE 303 grating spectrophotometer. The turbidity of the suspension was compared to a standard curve, prepared earlier, of the turbidity of known amounts of clays in distilled water. This allowed for the amount of clay in each 1ml of suspension to be determined. The turbidity of the supernatants alone were recorded and subtracted from the total turbidity readings.

The ability of supernatant from surface-produced mycelium to flocculate montmorillonite was also determined by whirlimixing only for 15 seconds and then allowing the clays to settle for 6 hours. The same supernatant was boiled for 10 minutes and tested for its ability to flocculate montmorillonite after whirlimixing for 15 seconds and settling for 6 hours.

<u>Ability of citric acid to flocculate montmorillonite.</u>

Citric acid (6ml) of the following concentrations (M): 0.125, 0.25, 0.5, or 1.0 were added to test-tubes (6") containing 1ml of montmorillonite suspension (0.35g clay in 20ml distilled water). Control tubes contained sterile distilled water (6ml) acidified to pH 2.2 using H_2SO_4 (0.5M). Each tube was mixed (using a whirlimixer)

for 15 seconds and allowed to stand for 6 hours. The turbidity of the top three, 1ml, layers was then determined as previously described.

RESULTS AND DISCUSSION.

Effect of addition of various concentrations of either kaolinite or montmorillonite to Czapek Dox medium on biomass and acid production by *Aspergillus niger*.

Effect of kaolinite:

A large increase in biomass production was observed with increasing concentrations of kaolinite in the growth medium (Fig. 2.1). As biomass production increased, acid production (calculated as g acid l^{-1} per g biomass produced) decreased (Fig. 2.1), probably because the carbon present was used by the fungus to produce biomass rather than acid.

Effect of montmorillonite:

Montmorillonite caused a large increase in biomass production up to a concentration of 1% (w/v) (Fig. 2.2). At 4% (w/v), montmorillonite reduced biomass production by *A. niger* to a level equivalent to that produced in the absence of the clay. Unfortunately, an accurate determination of biomass could not be made at this clay concentration as the fungus produced an extracellular polymer which made it impossible to separate the clays or mycelium from the supernatant after centrifugation. Centrifugation speeds of 12 000 r.p.m. for 30 minutes could not separate the clay from the viscous polymer. Filtration of the polymer through a 0.45µm pore filter was also unsuccessful as clay particles passed through the filter. Only an estimate of biomass was possible but it was clear that only a small amount of mycelium was produced. As with kaolinite, acid production per gram dry weight

mycelium decreased with increasing montmorillonite concentration (Fig. 2.2). Acid produced by *A. niger* at 4% (w/v) montmorillonite could not be determined.

and electron microscopy

Light microscopy studies on the effect of montmorillonite on the growth of *A. niger*,

Table 2.3 gives a summary of the effect of montmorillonite on mycelial growth of *A. niger* as observed by light microscopy. Aggregation of spores occurred almost immediately (Fig. 2.3) although spores still occurred separately while in the spore suspension (Fig. 2.4). Without clay, six hours after inoculation, spore germination had started and a large amount of aggregation was observed. With clay, after six hours, the germ tubes appeared slightly less developed, less spores had germinated and the spores occurred either singularly or in pairs. Twelve hours after inoculation, without clay, the formation of fungal pellets was noticed (Figs. 2.5 to 2.7), but after the same time interval with clay, germ tubes had extended, and no pellet formation was seen. The initial rate of germ tube formation and hyphal extension appeared much slower when the fungus was grown with montmorillonite (Figs. 2.8 & 2.9). Clays interacted closely both with spores and hyphae throughout the experiment. After 18 hours incubation without montmorillonite, pellet formation was well underway (Fig. 2.10), but with clay, still very little hyphal aggregation occured and hyphae began to extend to a much greater extent than hyphae grown alone (Figs. 2.11 to 2.13). Twenty four hours after inoculation, without clay, fungal pellets were too large to photograph well, and an extreme difference in morphology was noted in cultures of the same age incubated with clay. Loose pellets began to form in these 24 hour cultures and very long hyphae were produced (Figs. 2.14 to 2.16). Pellets formed thirty six hours after inoculation, with clay, but were much looser in nature than the pellets formed in the absence of montmorillonite. Hyphal growth was extremely extensive (Figs. 2.17 & 2.18) and

Figure 2.1.

Effect of addition of kaolinite to the growth medium on biomass and acid production by *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in biomass compared to control incubated without kaolinite, $p < 0.05$: ** significant decrease in acid production compared to control incubated without kaolinite, $p < 0.05$)

■—■ Biomass

●—● Total acid production

Figure 2.2.

Effect of addition of montmorillonite to the growth medium on biomass and total acid production by *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in biomass compared with the control incubated without kaolinite, $p < 0.05$: ** significant decrease in acid production compared with the control incubated without montmorillonite, $p < 0.05$)

■—■ Biomass

●—● Total acid production

Fig.2.1.

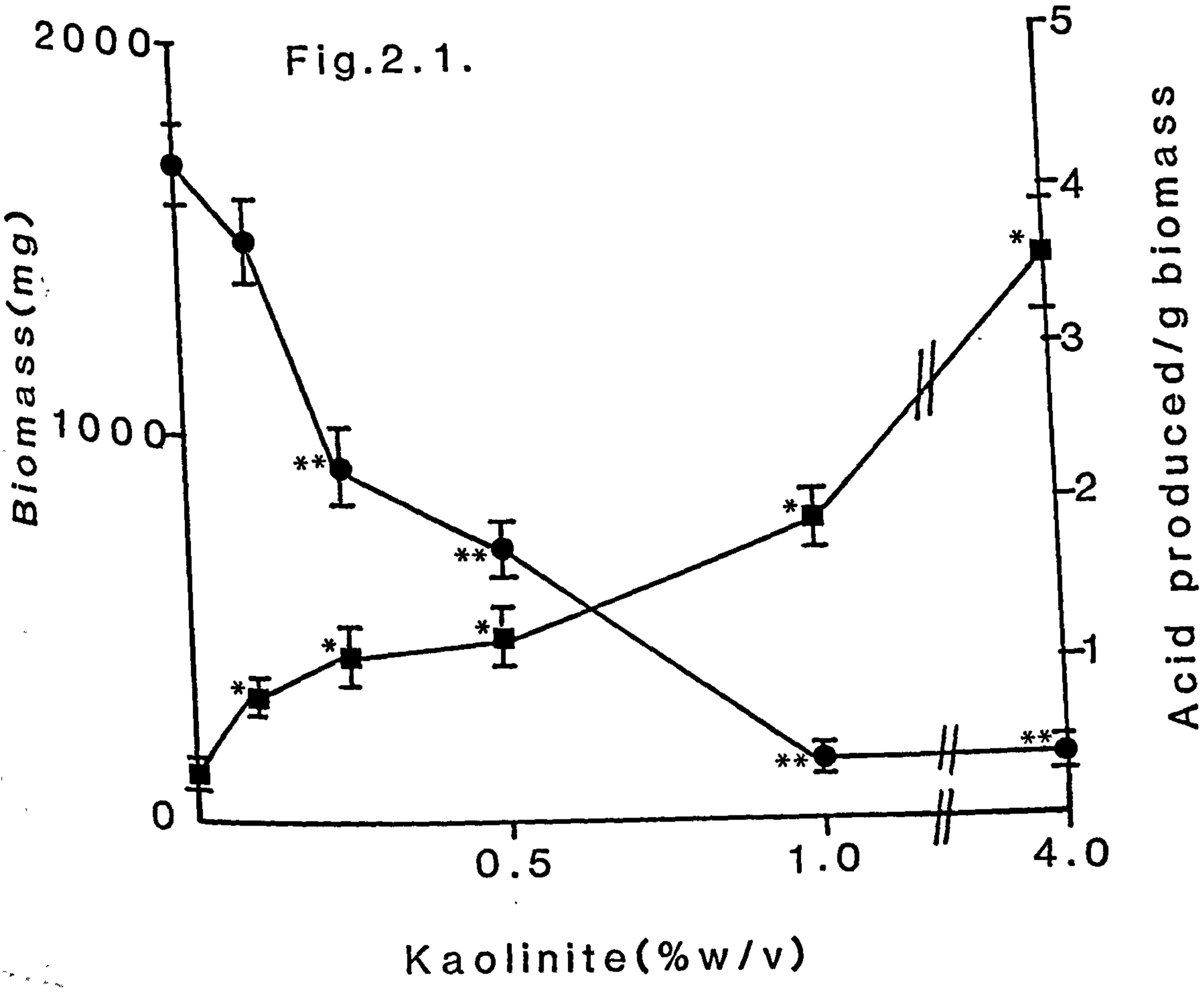

2000
1000
0
Biomass(mg)
5
4
3
2
1
Acid produced/g biomass
0.5
1.0
4.0
Kaolinite(%w/v)

Fig.2.2.

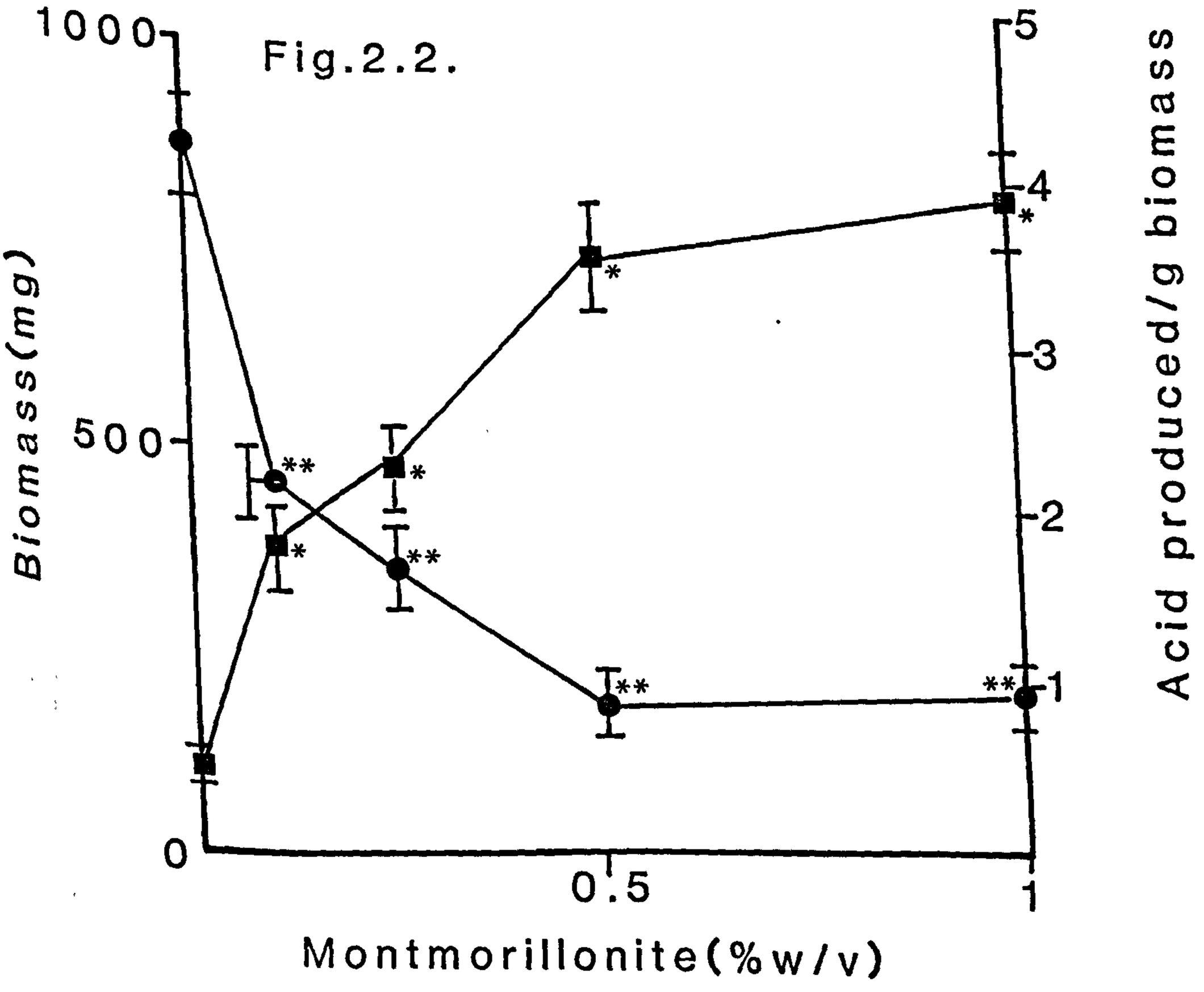

1000
500
0
Biomass(mg)
5
4
3
2
1
Acid produced/g biomass
0.5
1
Montmorillonite(%w/v)

Table 2.3 Effect of montmorillonite on spore germination, hyphal extension and pellet formation by *Aspergillus niger*.

Incubation time (hrs)	Without montmorillonite	With montmorillonite (0.5% w/v)
0	Spores mostly clumped	-
6	Spores germinated and clumped together	Spores separate, no germination
12	Pellets beginning to form	Spores germinating no aggregation
24	Pellets formed, hyphae are short in length. Relatively few spores have germinated	Very long hyphal extension. Loose pellets beginning to form and most of the spores present have germinated.

Figure 2.3.

Light micrograph of a spore suspension (1.7×10^6 spores ml^{-1}) of *Aspergillus niger*.

(Final magnification x 320)

Figure 2.4.

As Fig.2.3. (Final magnification x 320)

FIG. 2.3.

FIG. 2.4.

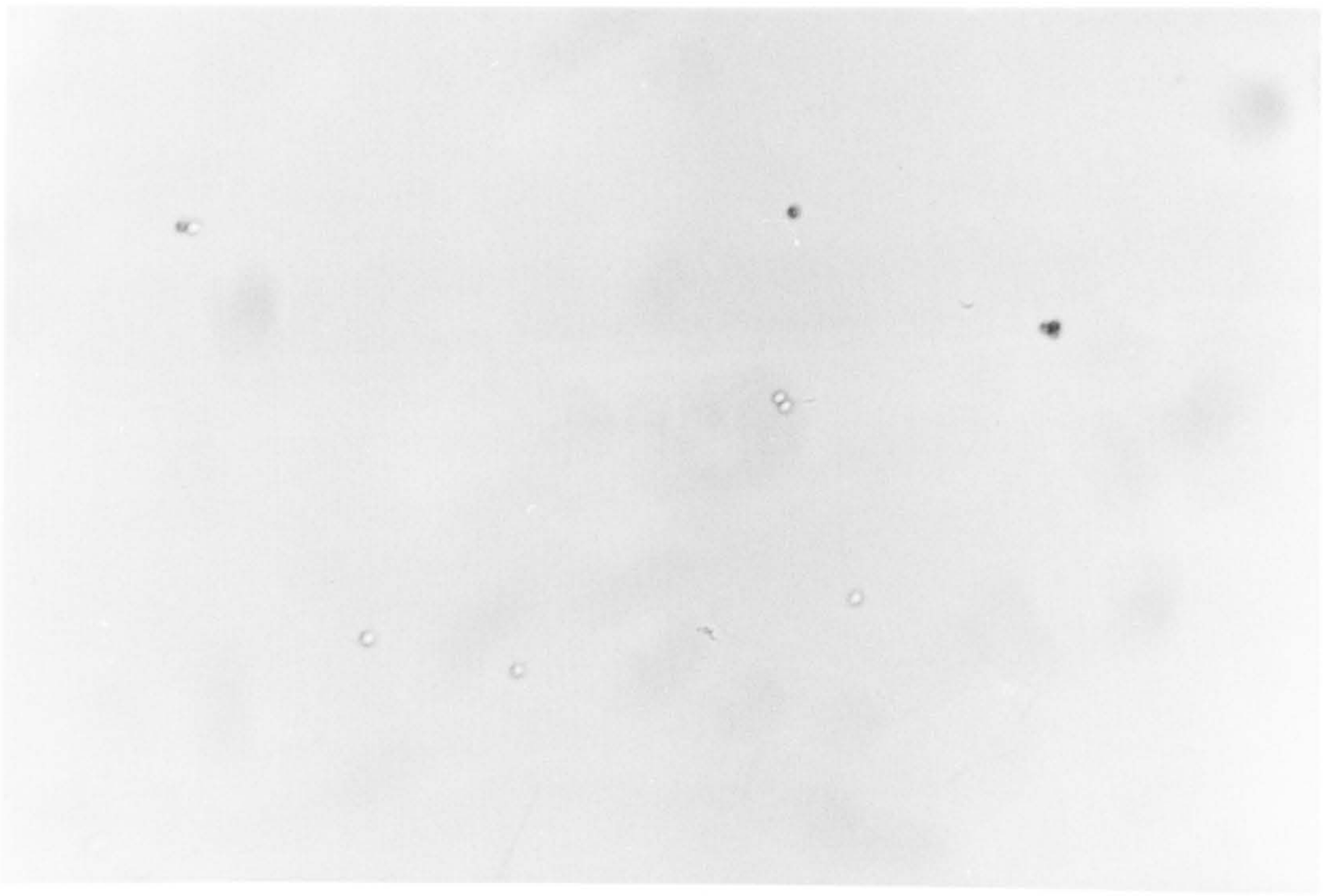


Figure 2.5.

Light micrograph of a culture of *Aspergillus niger* grown in Czapek Dox liquid medium alone. Twelve hours after inoculation with a spore suspension.

(Final magnification x 128)

Figure 2.6.

As Fig.2.5. (Final magnification x 128)

FIG. 2.5.

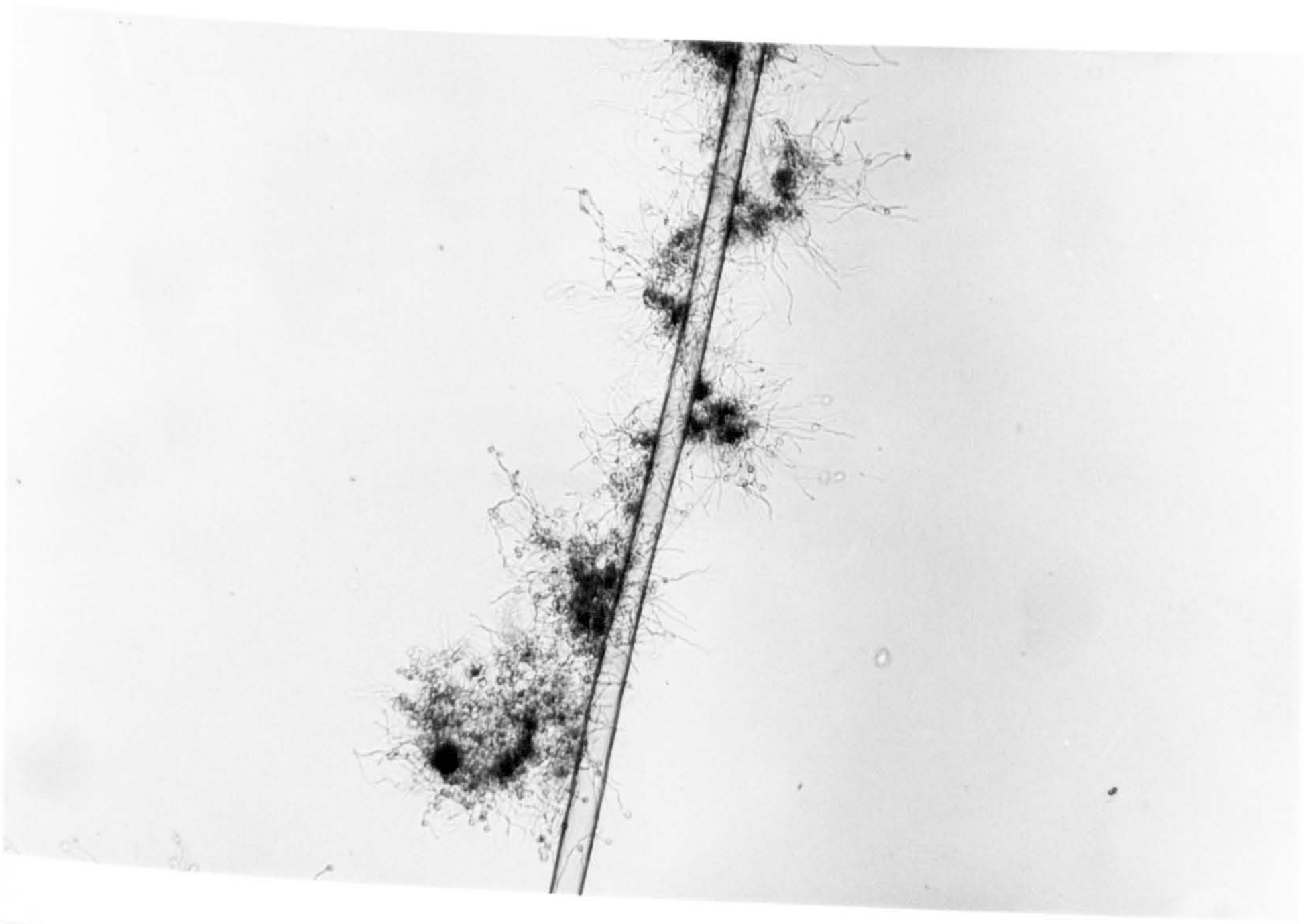


FIG. 2.6.

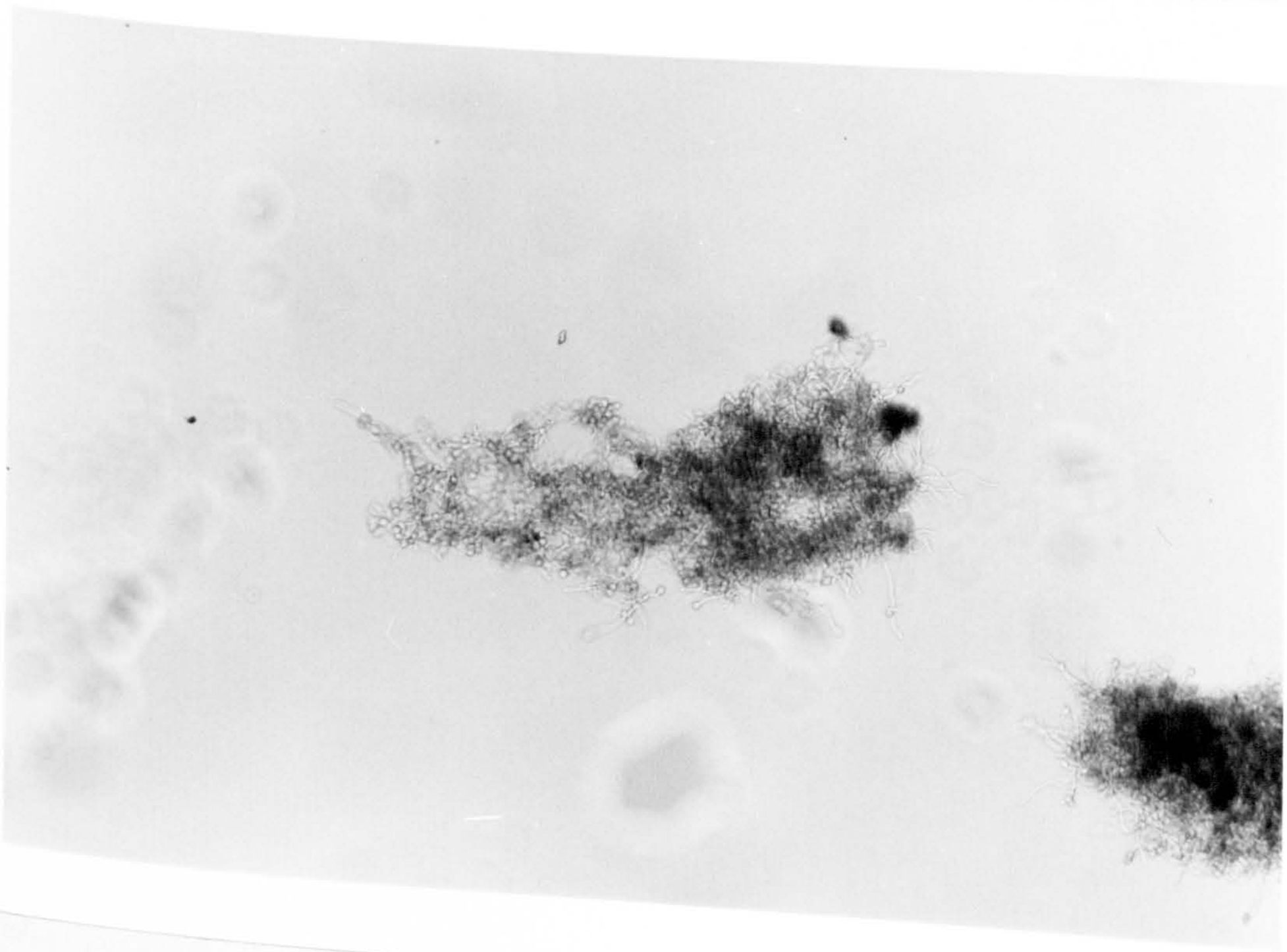


Figure 2.7.

As Fig. 2.5. (Final magnification x 320)

Figure 2.8.

Light micrograph of a culture of *Aspergillus niger* grown in Czapek Dox liquid medium supplemented with montmorillonite (0.5% w/v). Twelve hours after inoculation with a spore suspension.

(Final magnification x 512)

FIG. 2.7.

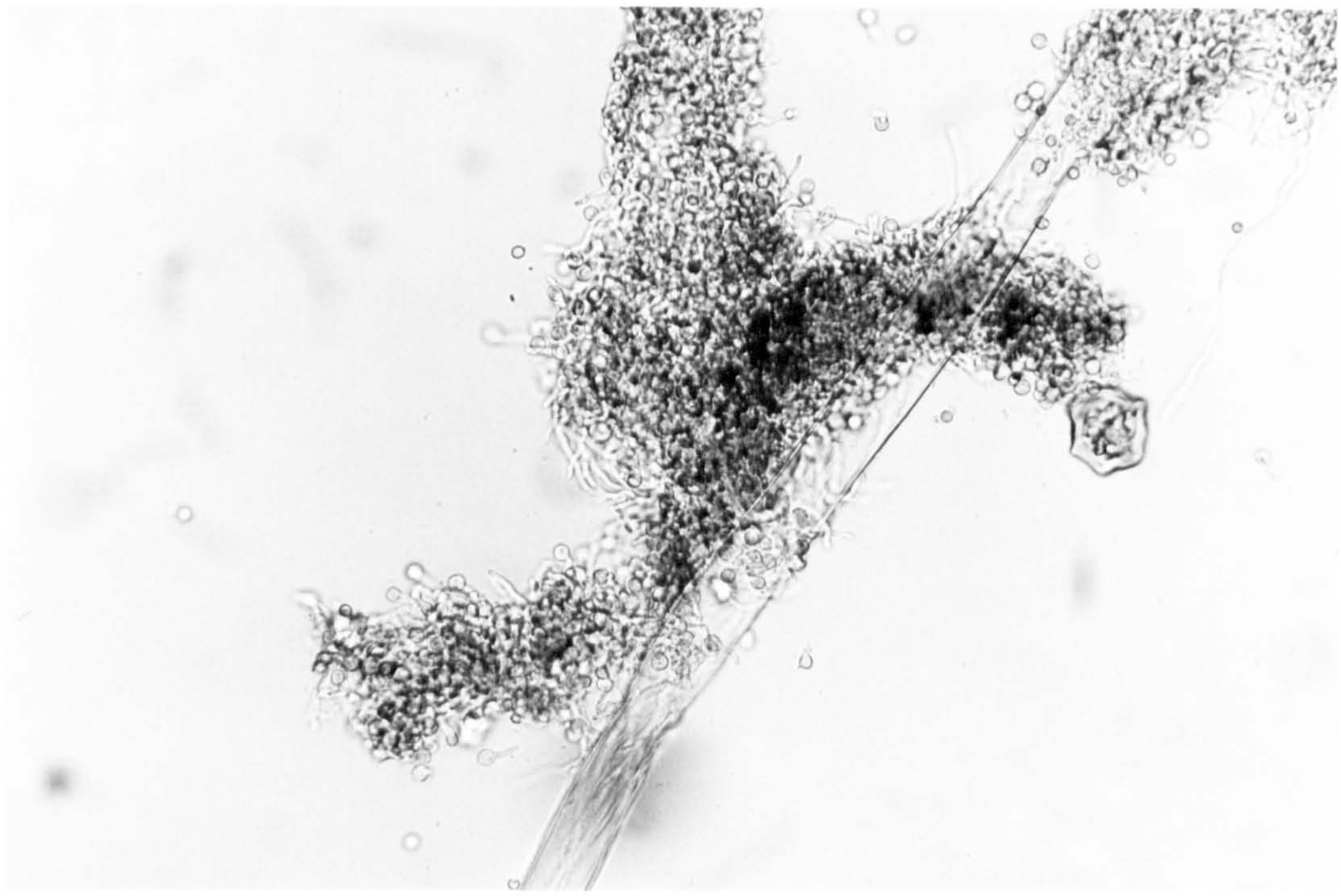


FIG. 2.8.

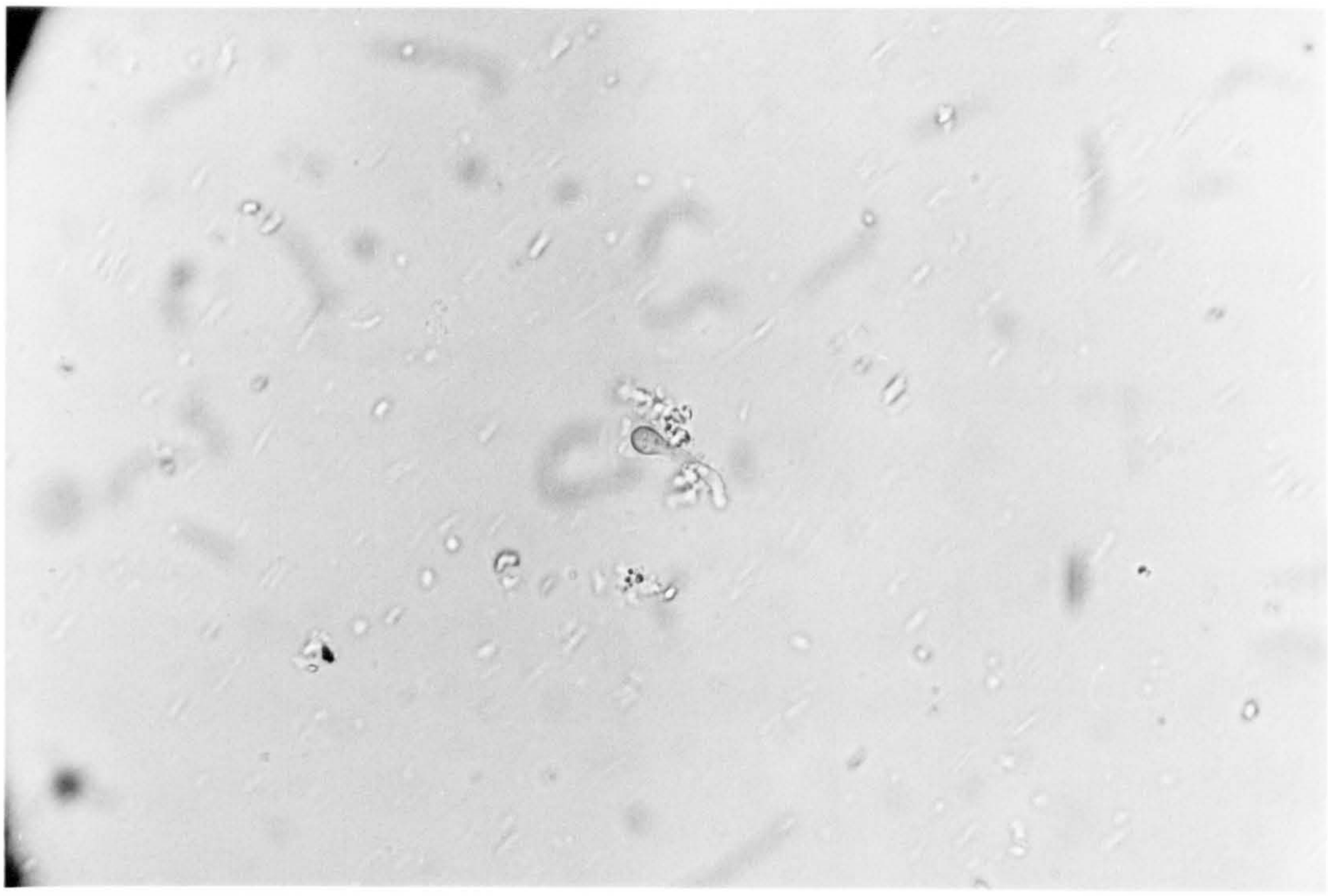


Figure 2.9.

As Fig. 2.8. (Final magnification x 320)

Figure 2.10.

Light micrograph of *Aspergillus niger* grown in Czapek Dox medium alone. Eighteen hours after inoculation with a spore suspension.

(Final magnification x 128)

FIG. 2.9.

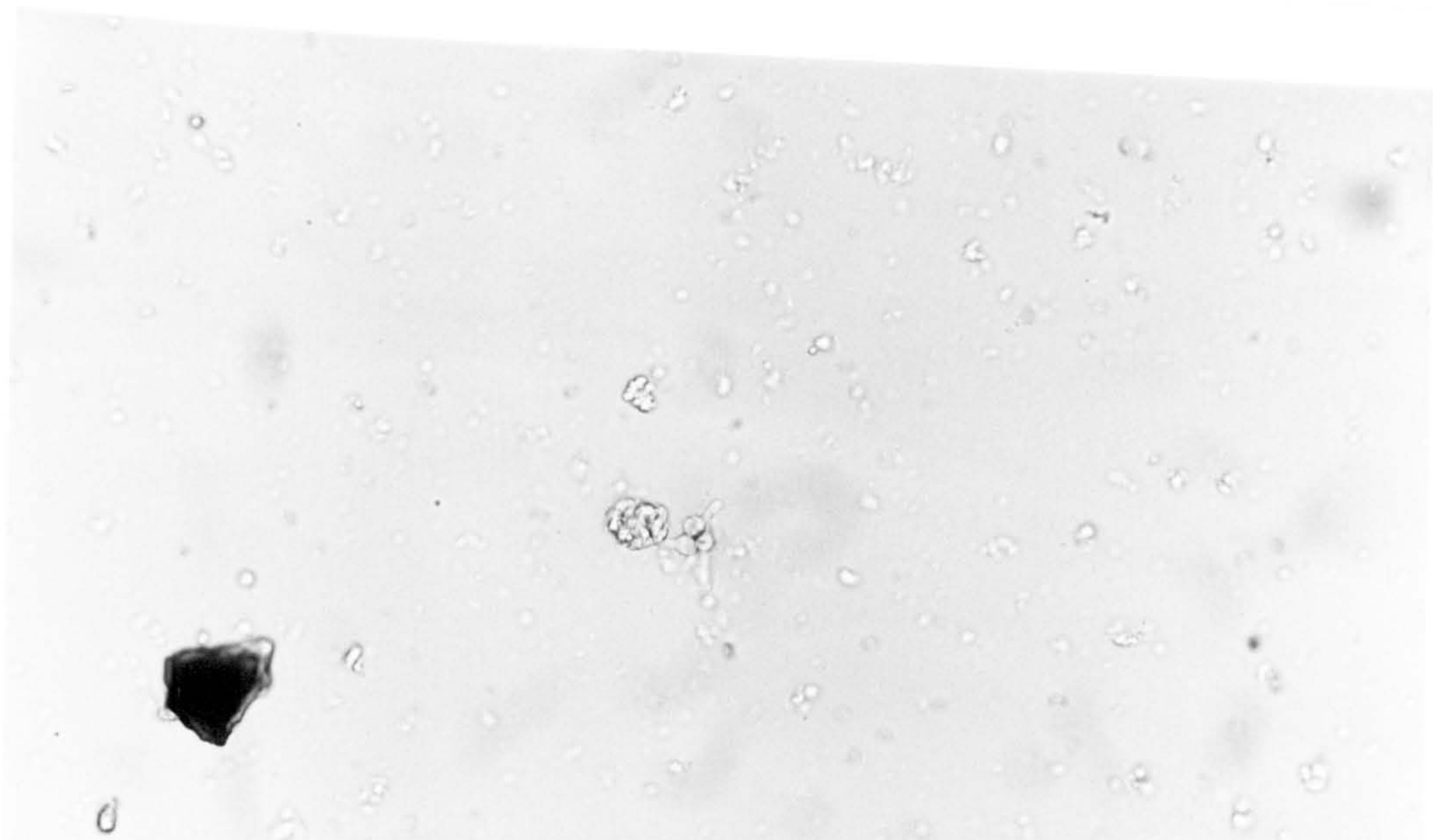


FIG. 2.10.

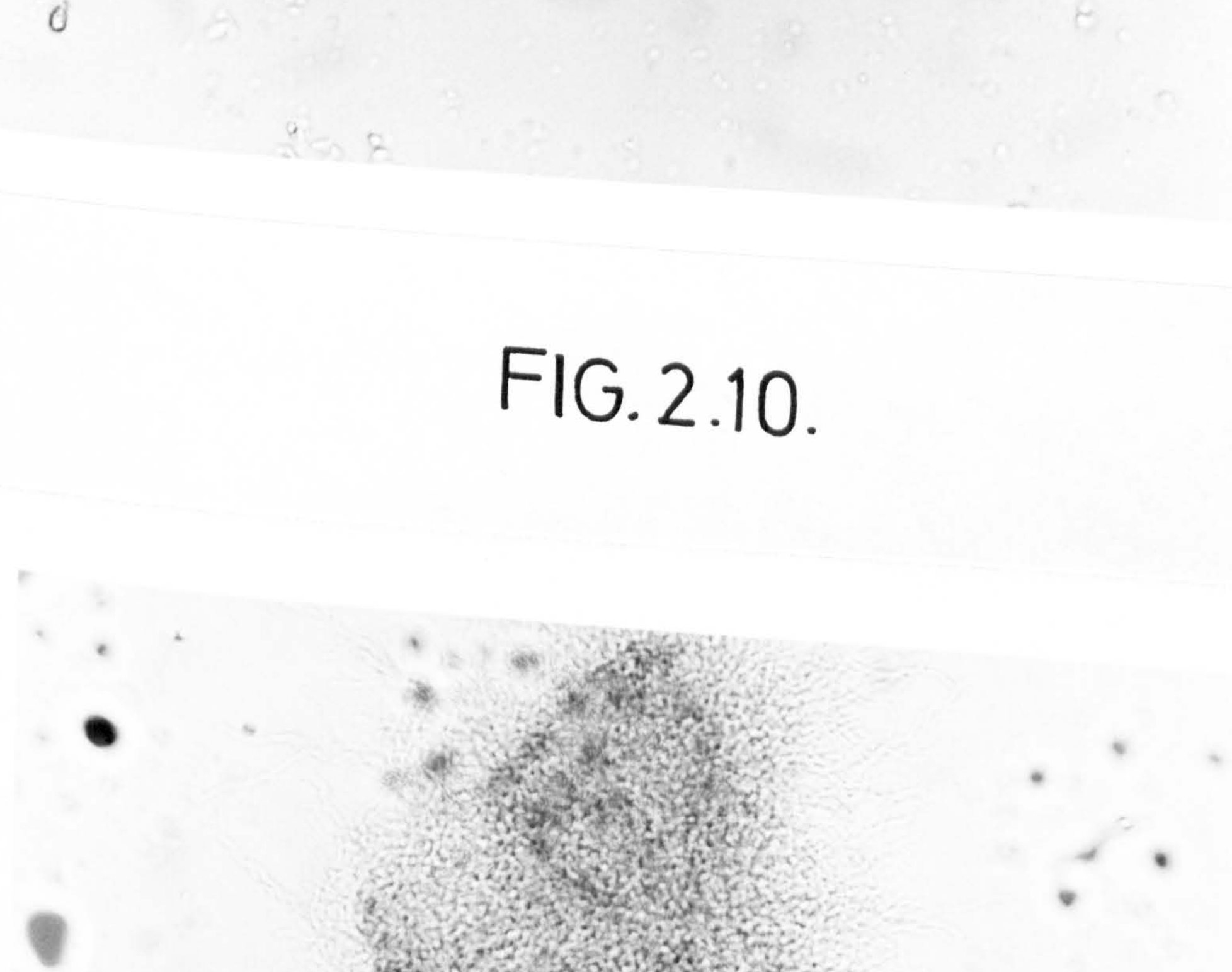


Figure 2.11.

Light micrograph of *Aspergillus niger* grown in Czapek Dox liquid medium containing montmorillonite (0.5% w/v). Eighteen hours after inoculation with a spore suspension.

(Final magnification x 512)

Figure 2.12.

As Fig. 2.11. (Final magnification x 128)

FIG. 2.11.

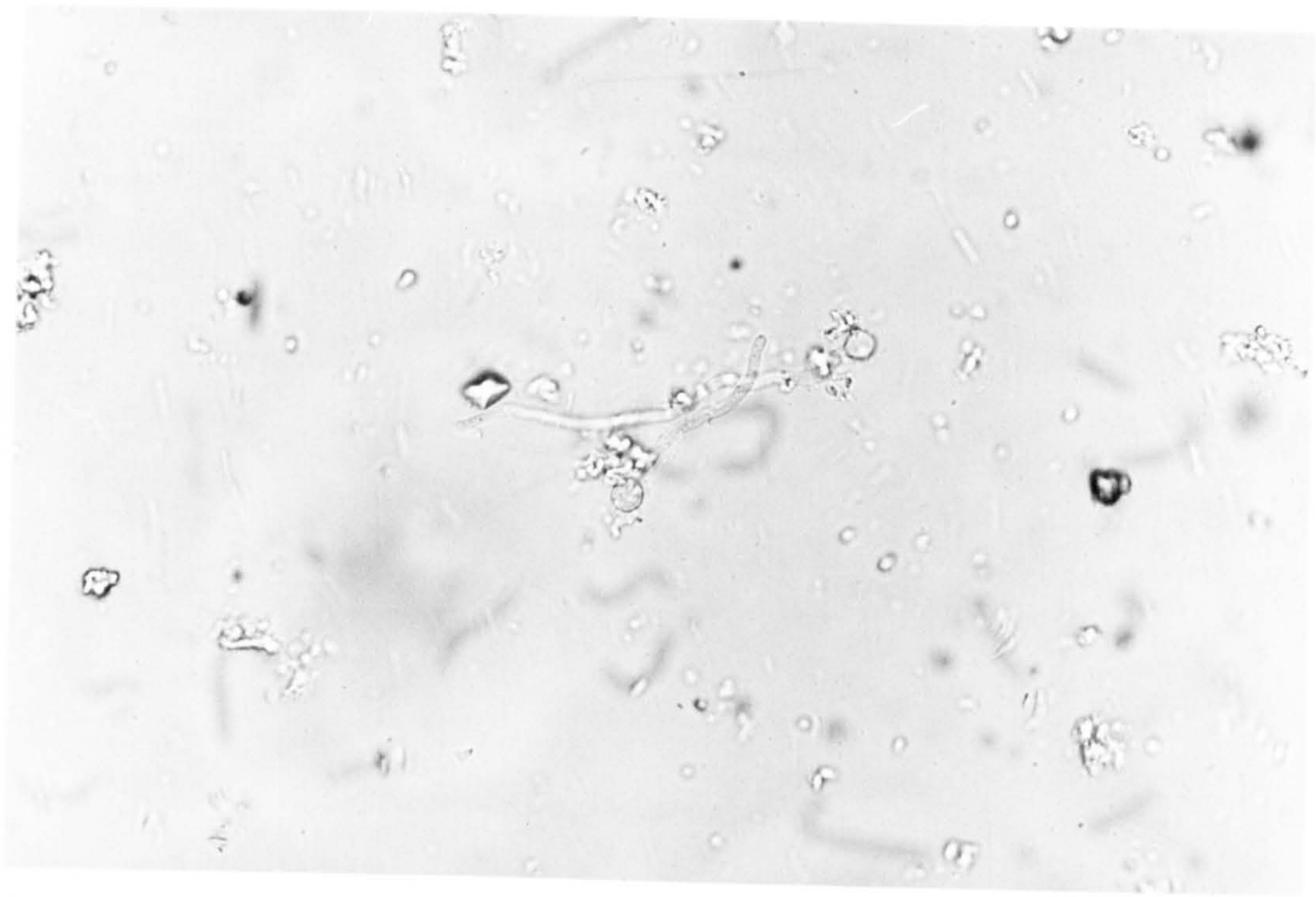


FIG. 2.12.

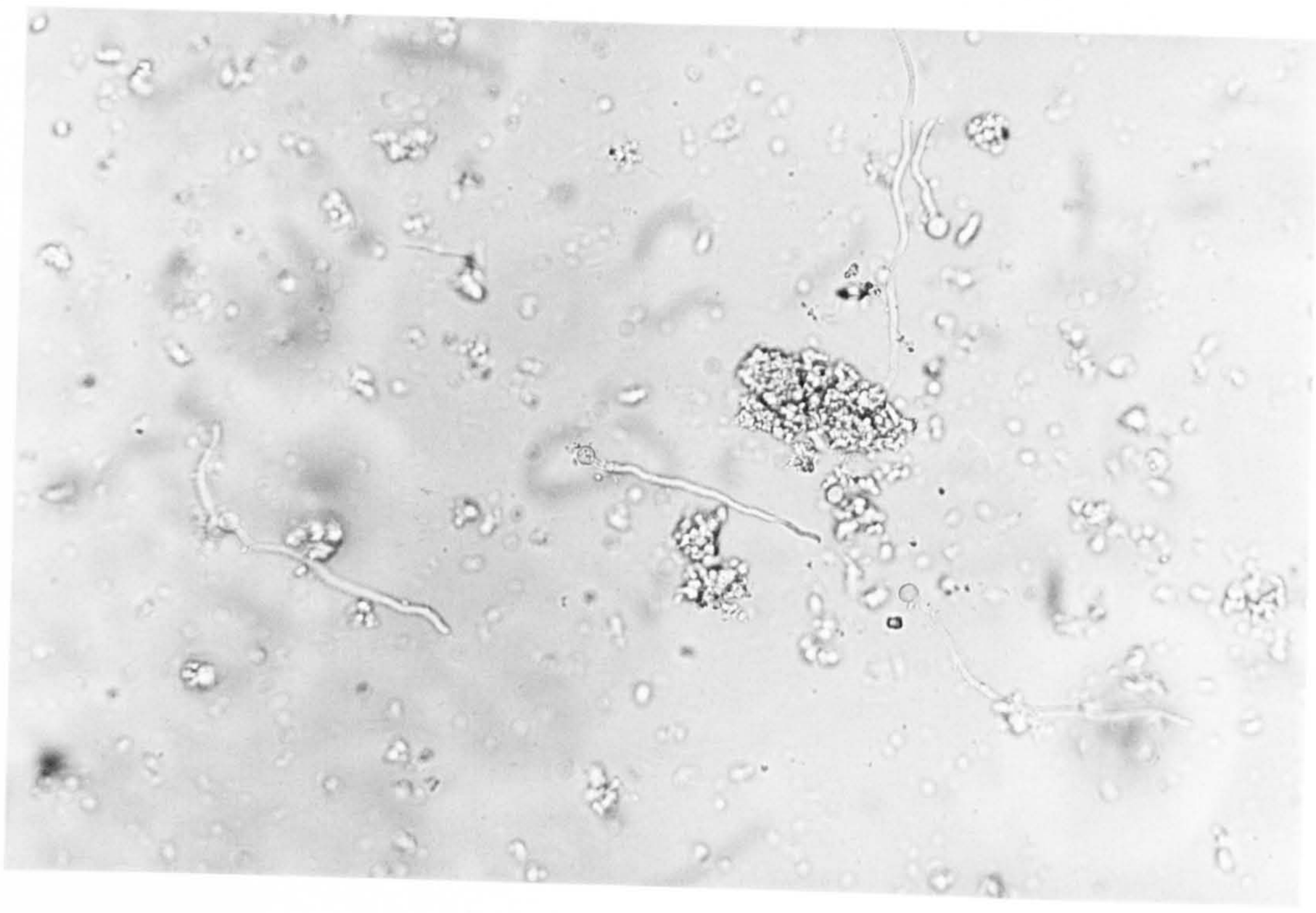


Figure 2.13.

As Fig. 2.11. (Final magnification x 512)

Figure 2.14.

Light micrograph of *Aspergillus niger* grown in Czapek Dox liquid medium supplemented with montmorillonite (0.5% w/v). Twenty four hours after inoculation with a spore suspension.

(Final magnification x 128)

FIG. 2.13.

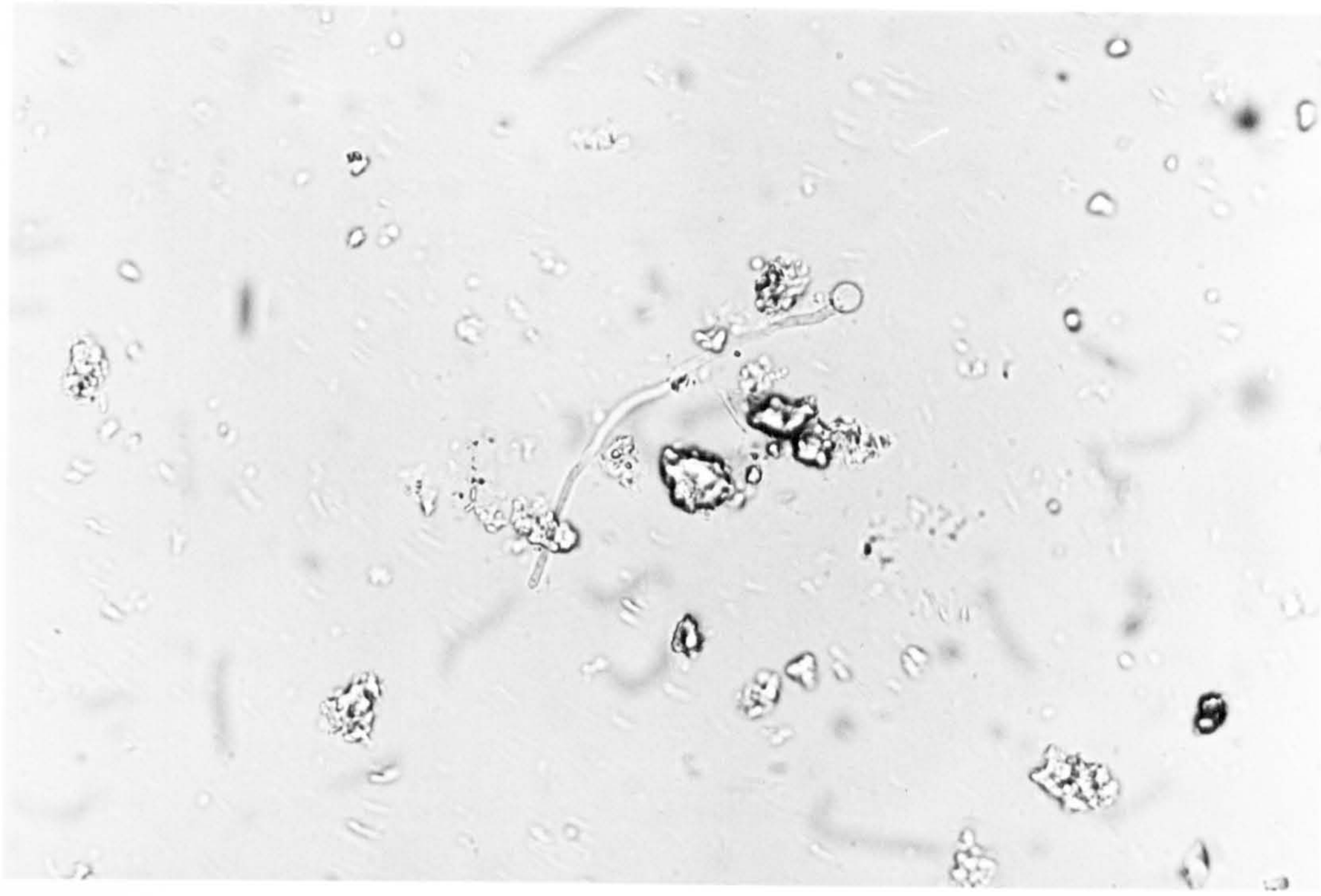


FIG. 2.14.

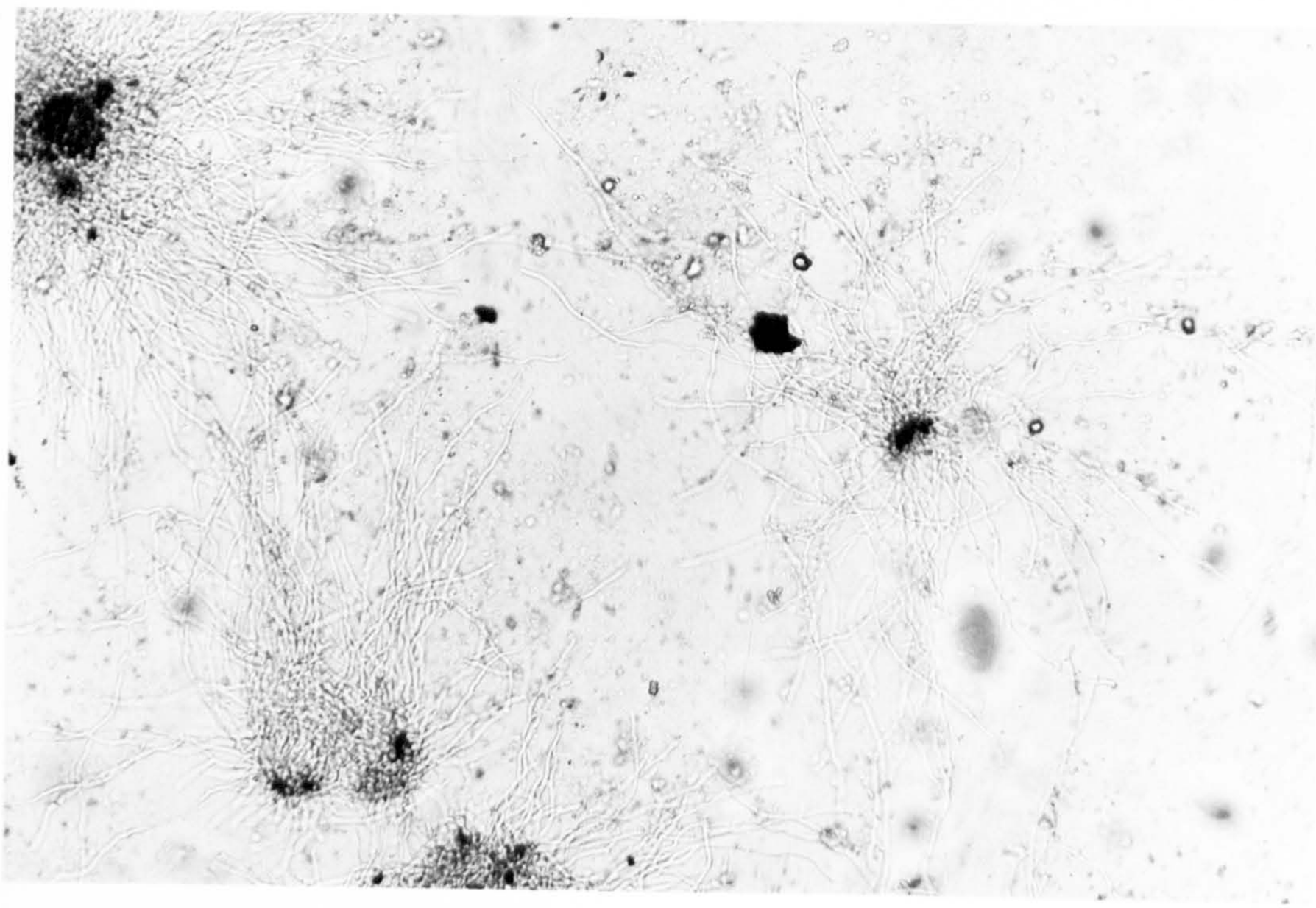


Figure 2.15.

As Fig.2.14. (Final magnification x 128)

Figure 2.16.

As Fig.2.14. (Final magnification x320)

FIG. 2.15.

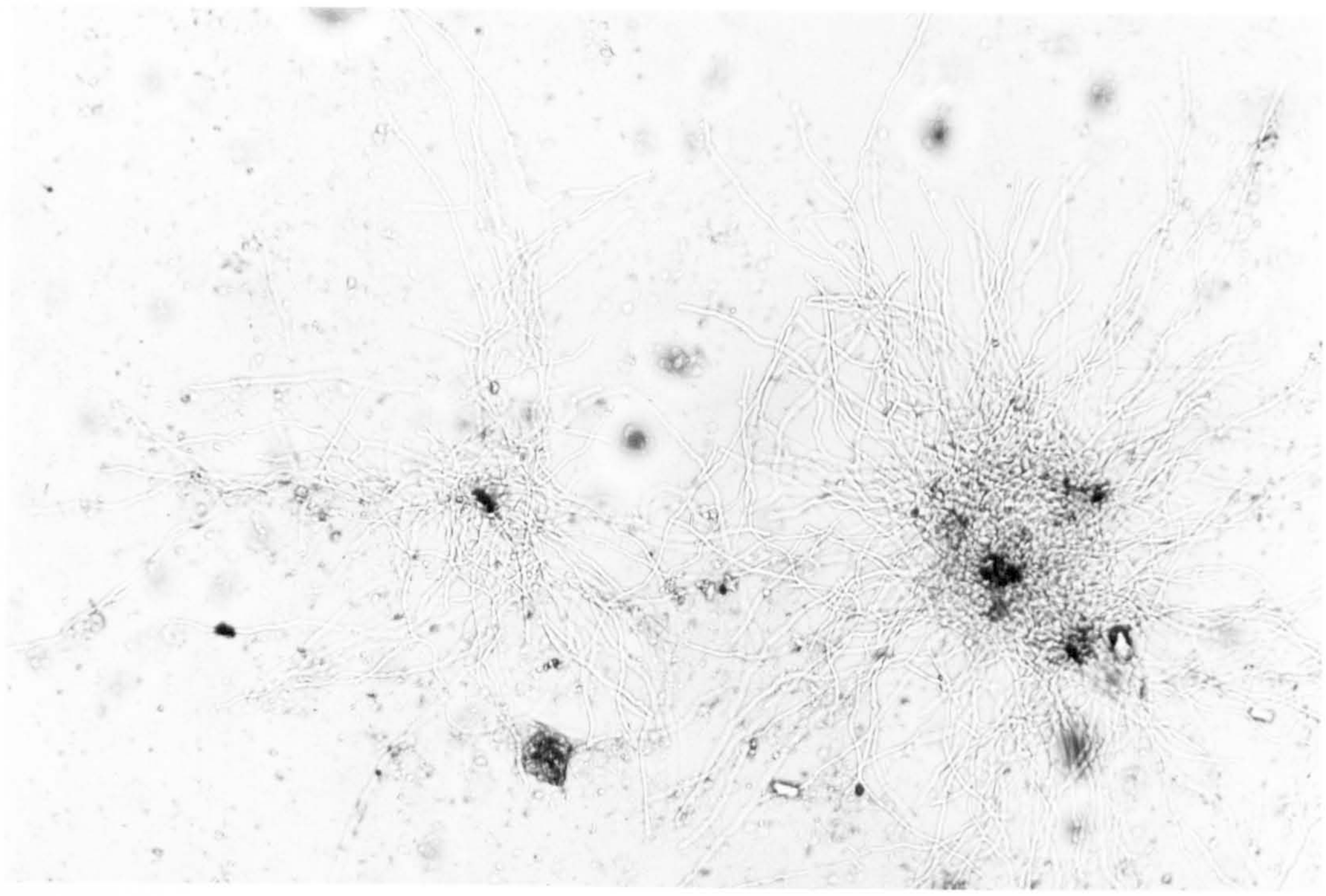


FIG. 2.16.

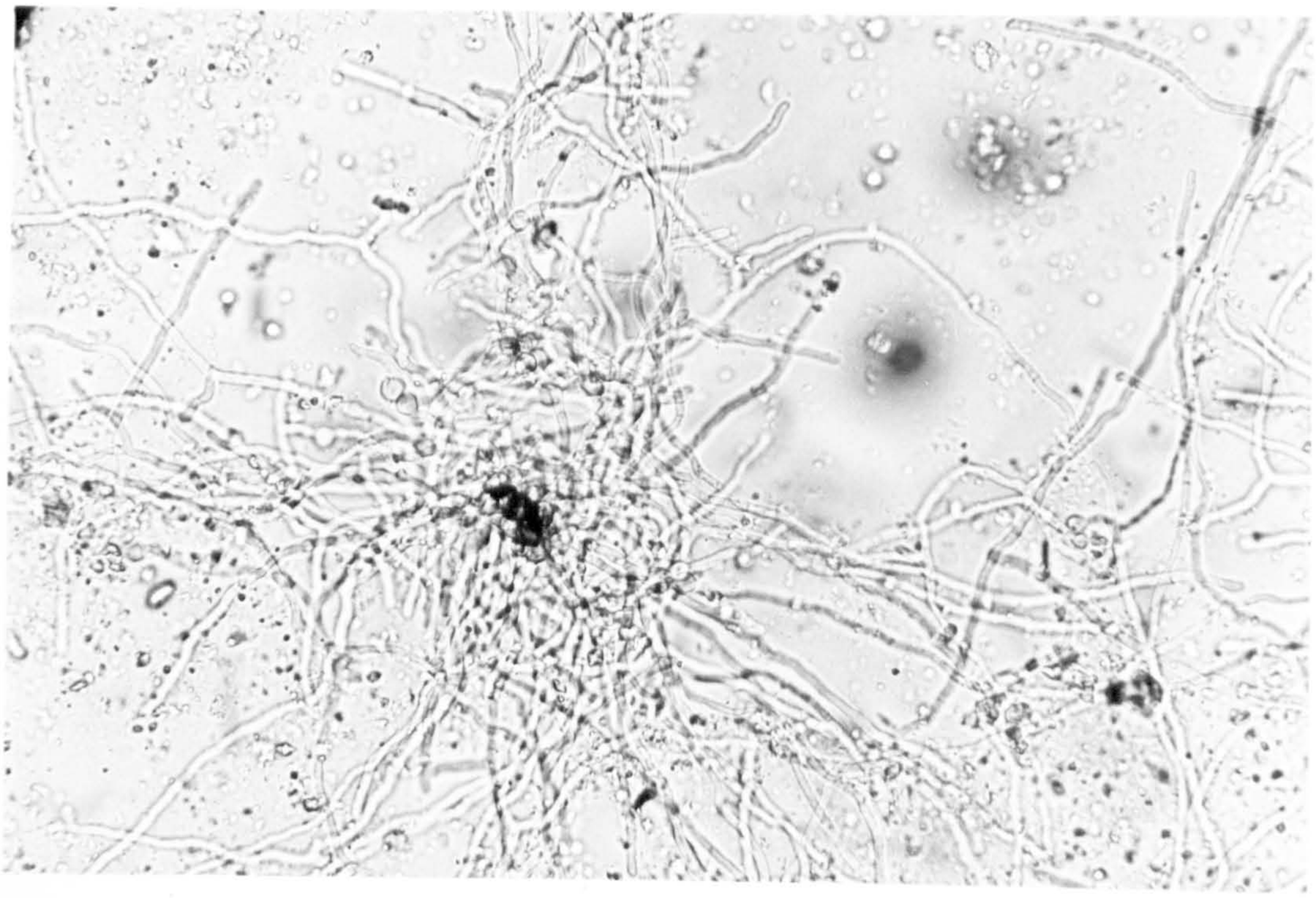


Figure 2.17.

Light micrograph of *Aspergillus niger* grown in Czapek Dox liquid medium supplemented with montmorillonite (0.5% w/v). Thirty six hours after inoculation with a spore suspension.

(Final magnification x 128)

Figure 2.18.

As Fig.2.17. (Final magnification x 128)

FIG. 2.17.

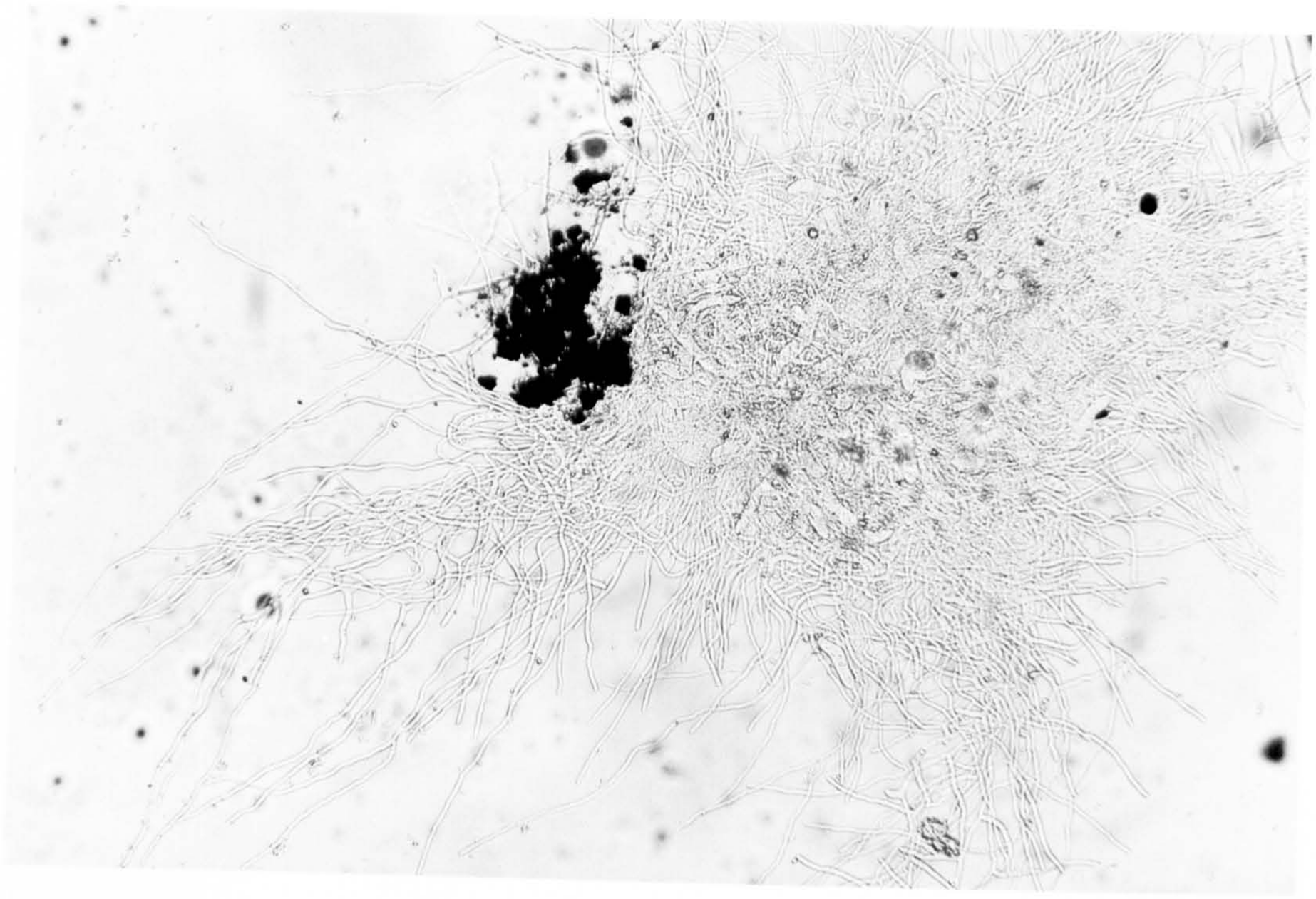
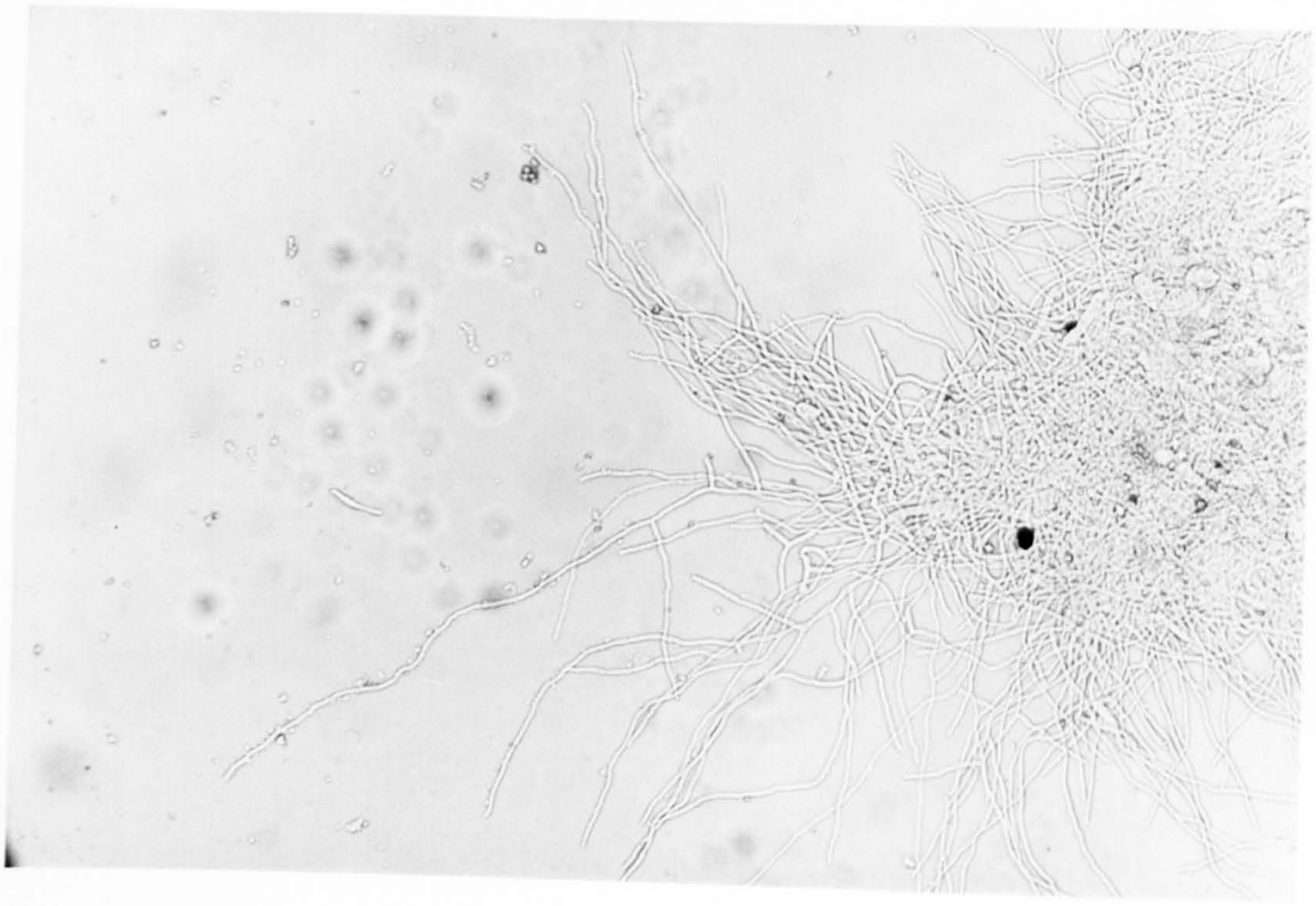


FIG. 2.18.



clay particles were adsorbed onto the hyphal surface (Fig. 2.19). The adsorption of clays to the hyphal surface is also clearly shown in scanning electron micrographs (SEM) of *A. niger* grown with montmorillonite (Figs. 2.20 to 2.22). Figures 2.23 and 2.24 (SEM), of cultures grown in the absence of montmorillonite, clearly show pellet formation and demonstrate the difference between the two differently treated mycelium. Photographs of cultures grown with and without kaolinite (Figs. 2.25 and 2.26) show the effect of the clay on fungal morphology. The clay appears not to prevent pellet formation entirely but the pellets are more numerous, are of different sizes and are more filamentous in nature, in comparison to the morphology of the fungus grown in the absence of clay. Very large pellets were formed at high kaolinite concentrations (4% w/v) (Fig. 2.26). *A. niger* is able to remove all kaolinite, up to a concentration of 1% (w/v), from suspension. A similar effect on pellet formation was caused by montmorillonite except that the clay was only completely removed from solution at concentrations below 1% (w/v) and little growth is observed at montmorillonite concentrations of 4% (w/v).

Most fungi, when subjected to gentle and continuous agitation, will show a tendency to form pellets during growth. This pellet form of growth has been considered the "normal" morphology in submerged culture since it was observed in 150 species by Burkholder & Sinnot (1945). However, since these original observations, a number of different factors have been found to affect fungal morphology in culture.

Pellet formation by *A. niger* has been particularly well studied. Galbraith & Smith (1969) observed that pellet development occurred when germinating spores trapped each other to form a nucleus around which the hyphae developed. In the conditions in which pellets formed, *A. niger* spores began to aggregate at a very early stage in germination, when the cultures consisted of a mixture of unswollen and

Figure 2.19.

As Fig.2.16. Demonstration of close association of montmorillonite particles with the hyphal surface.

(Final magnification x 320)

FIG. 2.19.

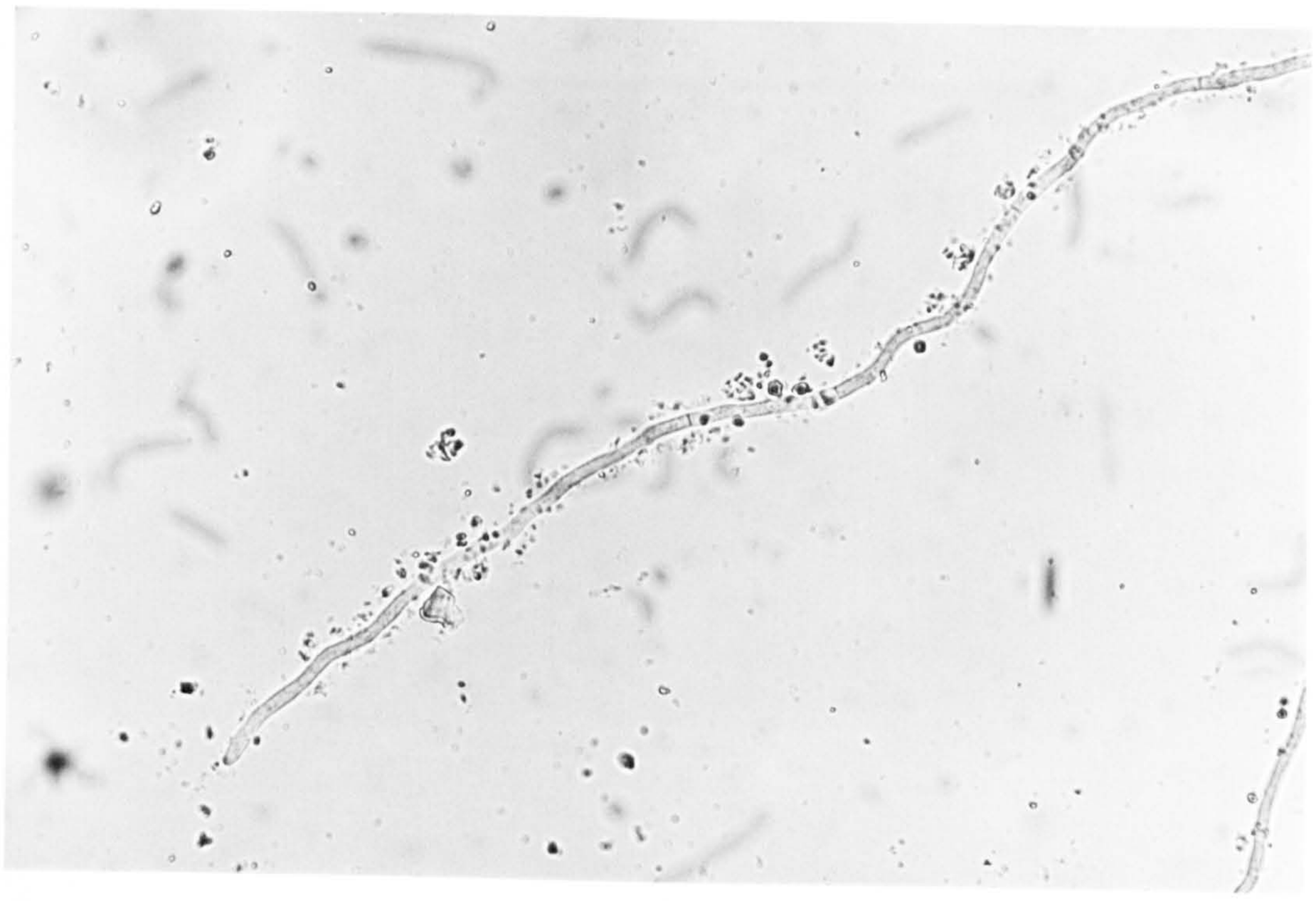


Figure 2.20.

Scanning electron micrograph of a six hour old culture of *Aspergillus niger* grown in Czapek Dox liquid medium supplemented with montmorillonite (0.05% w/v). Micrograph demonstrates close association of clay particles with the fungal hyphae. (Magnification x 2000)

FIG. 2.20.

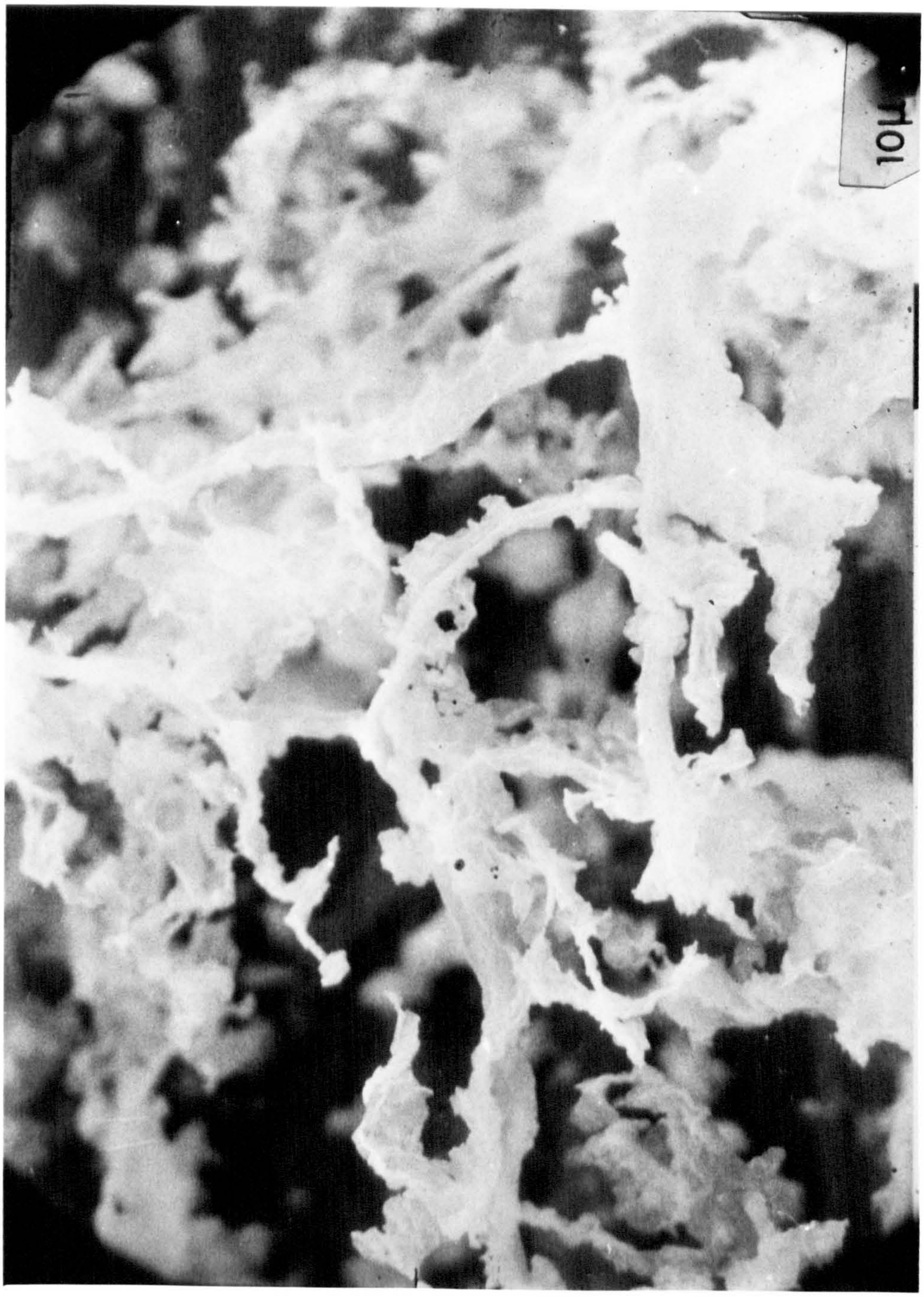


Figure 2.21,

As Fig.2.20. (Magnification x 2000)

FIG. 2.21.

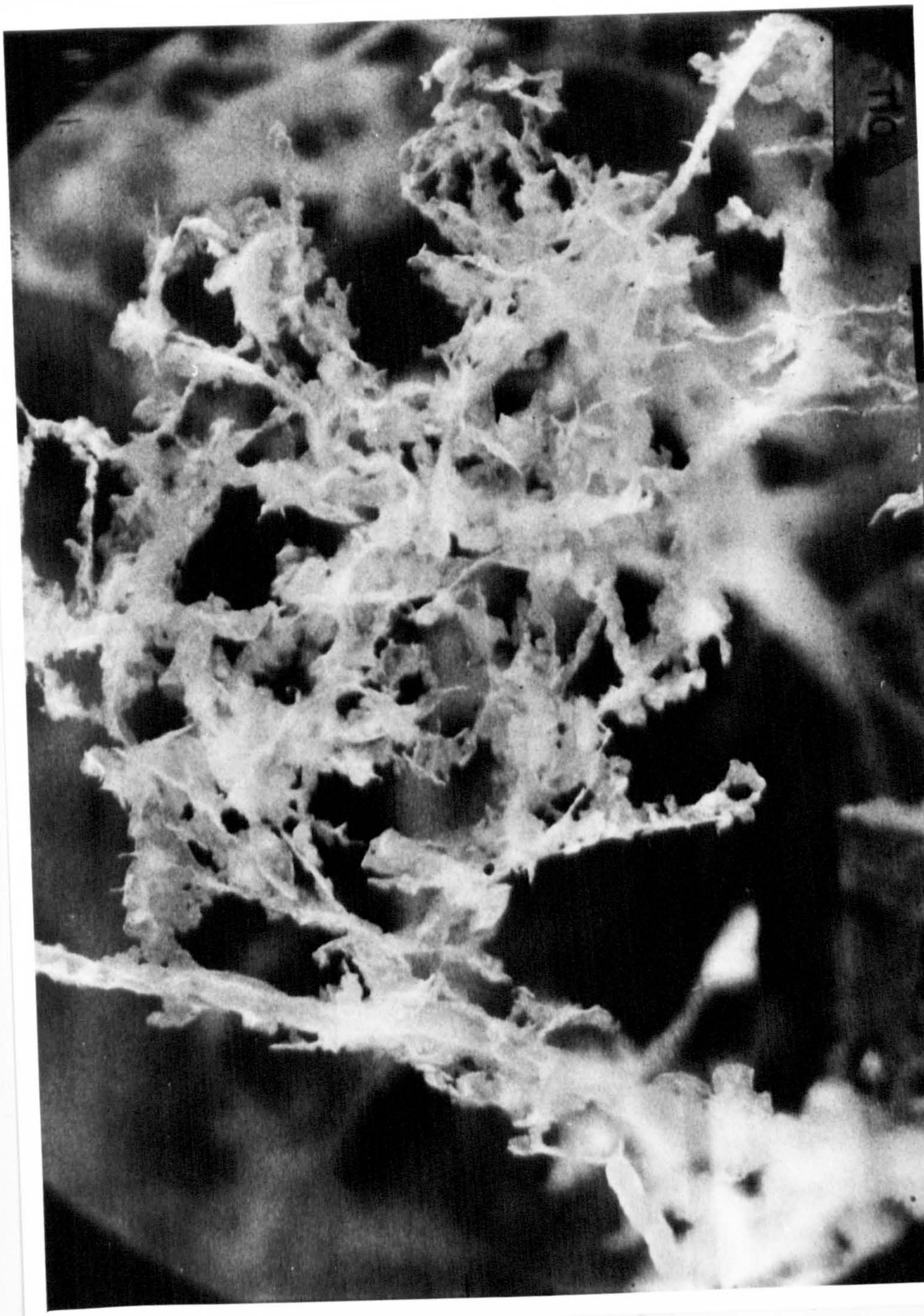


Figure 2.22.

As Fig.2.20. (Magnification x 2000)

FIG. 2.22.

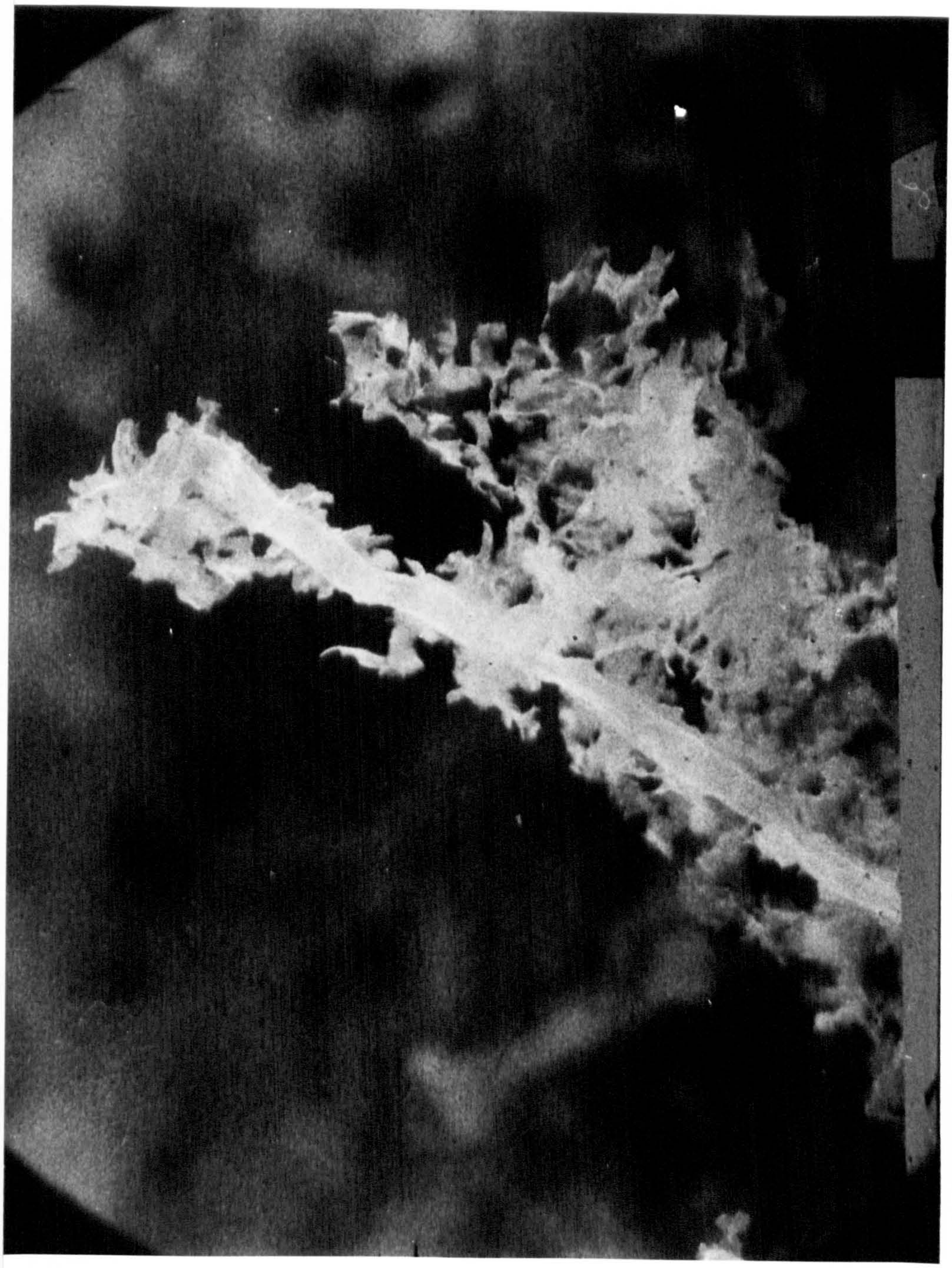


Figure 2.23.

Scanning electron micrograph of a twelve hour old culture of *Aspergillus niger* grown from a spore suspension in Czapek Dox liquid medium.

(Magnification x 1000)

FIG. 2.23.

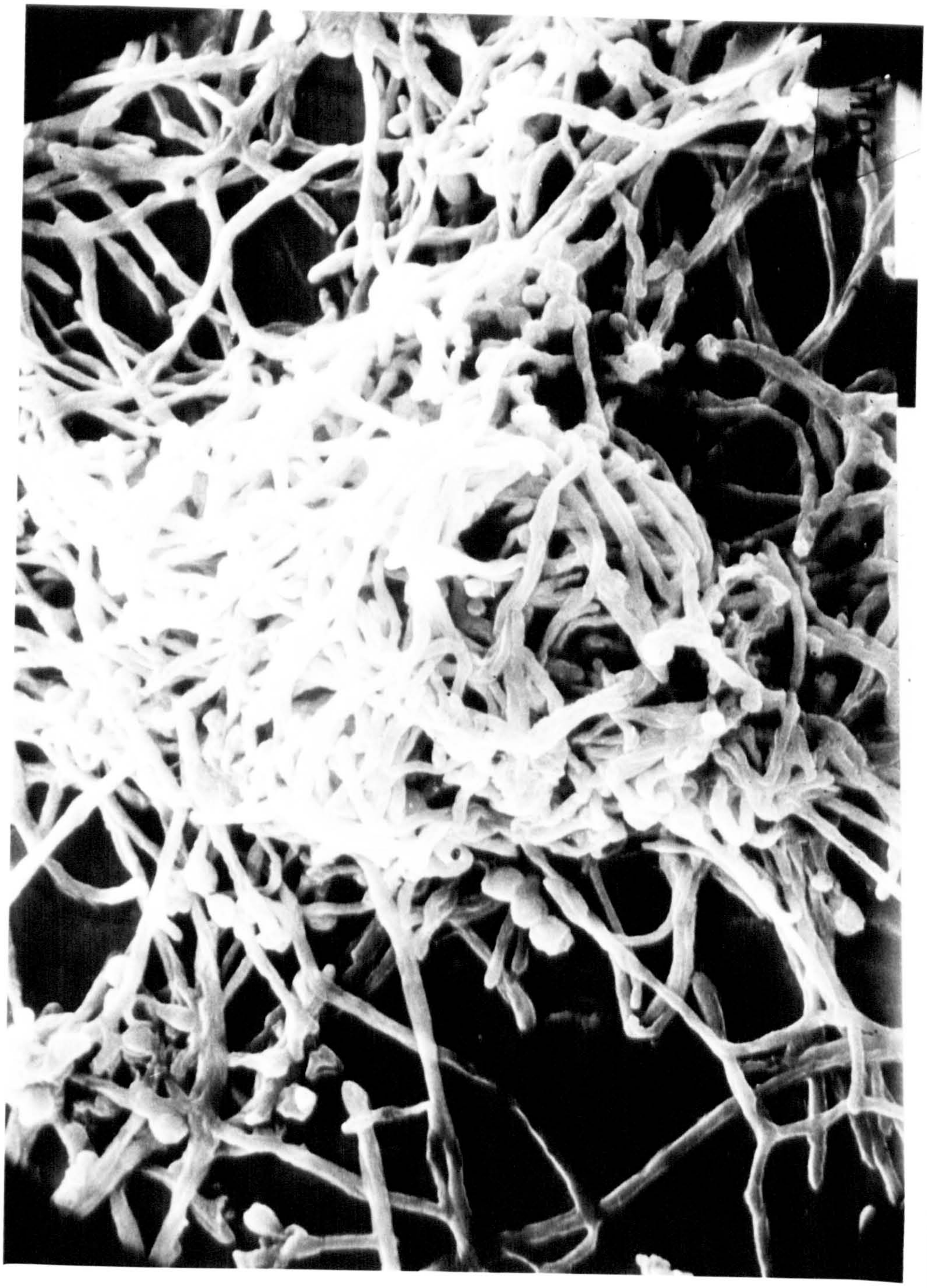


Figure 2.24,

As Fig.2.23. (Magnification x 500)

FIG. 2.24.

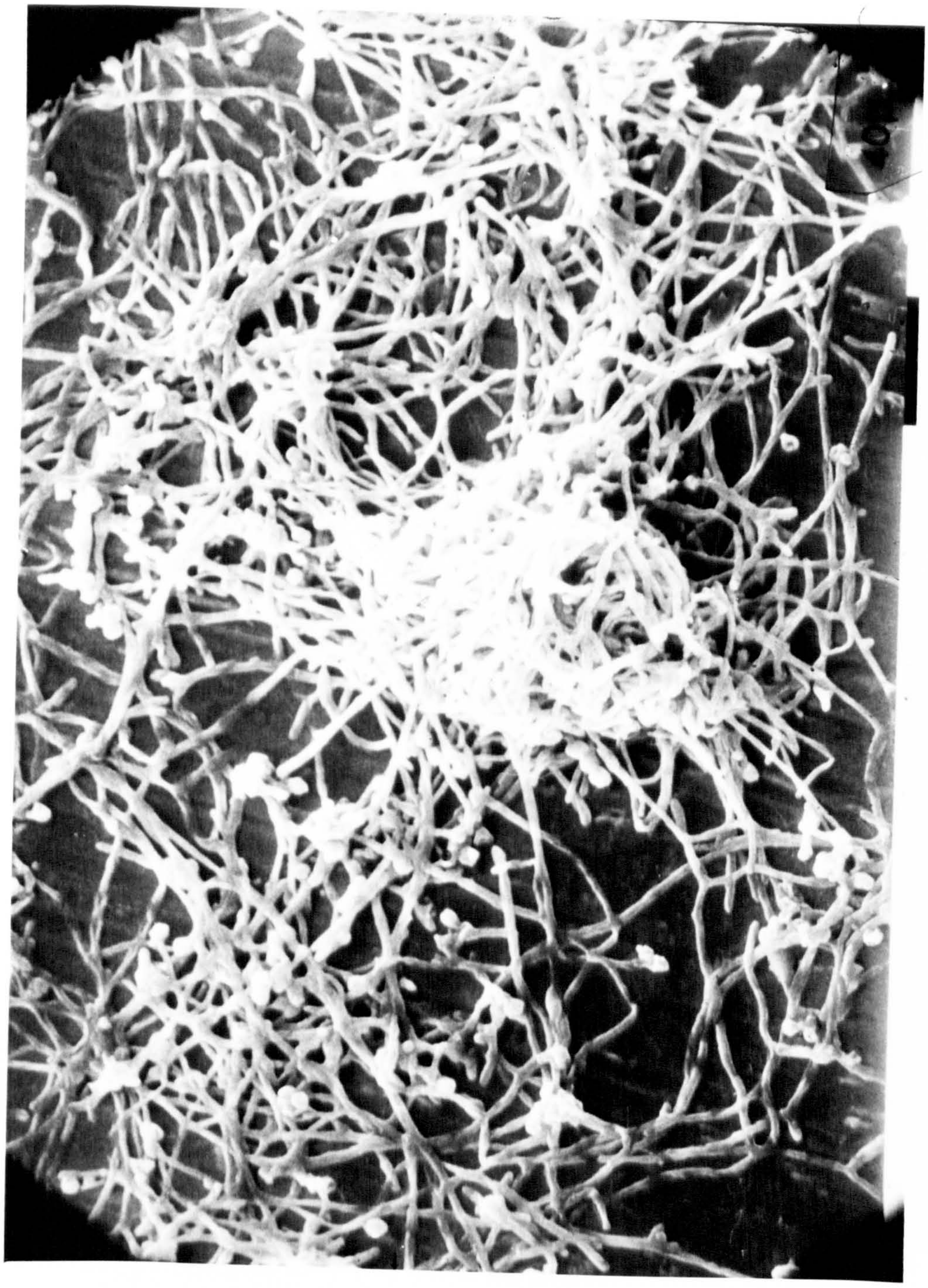


Figure 2.25.

Photograph of the effect of varying concentrations of kaolinite on morphology and biomass production by *Aspergillus niger*.

(Numbers represent the concentration of kaolinite (w/v) added to the growth medium

Figure 2.26.

As Fig.2.25.

FIG. 2.25.

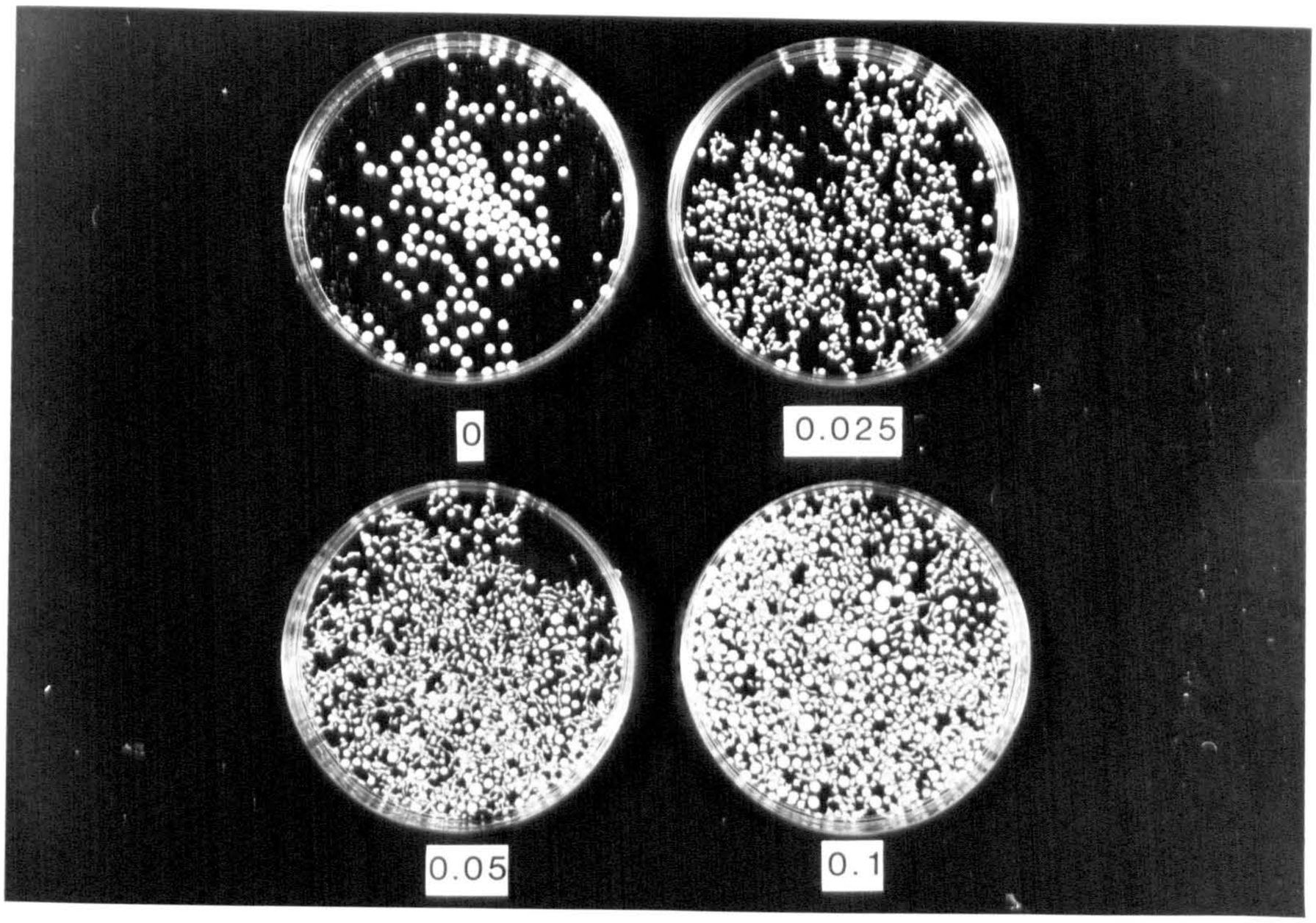
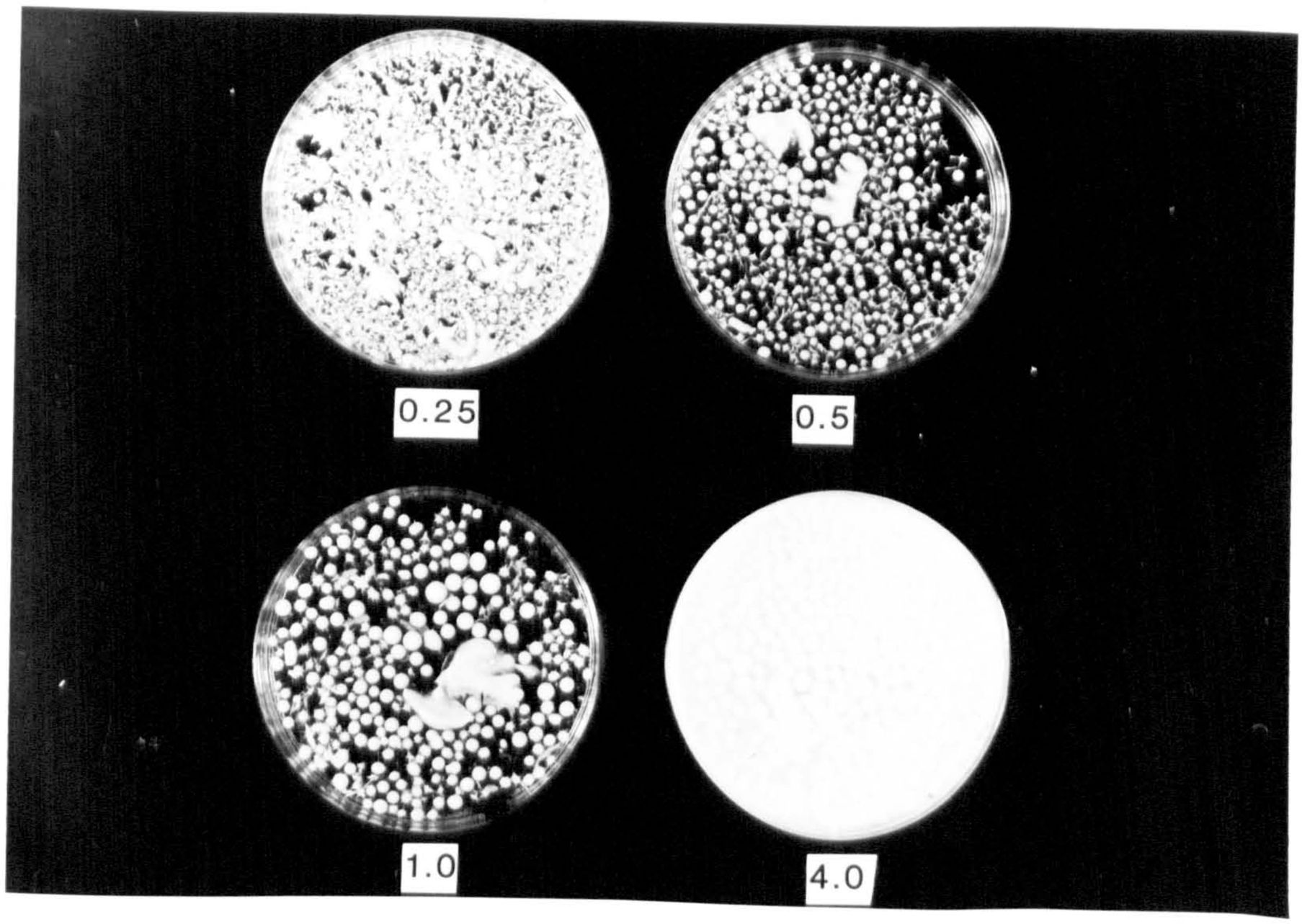


FIG. 2.26.



swollen spores with or without germ tubes. These observations are very similar to the ones reported here (Fig. 2.3). Spore aggregation is considered to be the first stage of pellet formation (Whittaker & Long, 1973). It is also thought that spores germinate at different rates. Those germinating first produce germ tubes and hyphal branches which become interlocked and trap other ungerminated and germinated spores to form the nucleus of a larger pellet (Burkholder & Sinnot, 1945; Trinci, 1970). The interlocking of hyphal branches and germ tubes is considered to be the second stage of fungal pellet formation.

My observations suggest that clays interfere with normal pellet production by *A. niger*. Possibly agglutinated spores are physically broken away from one another by contact with clays during agitation. This process would inhibit the first step of pellet formation. Binding of clays to the fungal spores or young hyphae would prevent the second step of pellet formation as the montmorillonite clay particles bound on a hyphal or spore surface would repel other clay particles bound to another part of the fungal surface. This would obviously limit hyphae to hyphae, spore to spore and hyphae to spore interactions. However, once the hyphae have extended far enough then it seems that physical contact and therefore agglutination between different hyphae occurs; this is seen in older cultures of *A. niger* grown in the presence of clays.

A large number of other factors affect the morphology of *A. niger* grown in submerged culture. For example, a significant change in morphology is often observed when the composition of the growth medium is altered. *A. niger*, in synthetic media, grew as loose fluffy pellets, but when grown in organic media, the fungus grew either in the form of small, dense, compact pellets, or was filamentous (Tuttobello & Mill, 1961). The effects of trace elements on the morphology of *A. niger* have been investigated (Whittaker & Long, 1973). In particular, manganese, at concentrations above 2ppb, has been found to change the morphology of *A. niger*

from its usual pelleted form to a filamentous form. Additional trace elements having morphological effects include iron and zinc (Whitaker & Long, 1973). Other factors influencing morphology are pH (Steel *et al.*, 1954; Galbraith & Smith, 1969), inoculum size and aeration (Whitaker & Long, 1973).

Polymers such as Junlon and Carbopol have been used to alter the growth form of *A. niger*. Carbopol, an anionic carboxymethylene polymer, increased the growth rate and the rate of amylase production by *A. niger*. The polymer also caused dispersed growth of the fungus (Elmayergi *et al.*, 1973). The effects of Carbopol were examined with clusters of spores and mould pellets (Elmayergi *et al.*, 1973). In the first case, it appeared that the ionized carboxyl groups of Carbopol induced electrostatic repulsion amongst the spores thus initiating dispersed growth and increasing the area of contact between the fungus and the nutrient medium. This effect was thought to enhance fungal growth and appears very similar to my proposals on the promotion of dispersed fungal growth caused by clays. In the second case, the added Carbopol formed a thin film attached to the surface of the pellets which seemed to be responsible for an increased rate of potassium transport, and hence, higher fermentation yields. The polymer effect was much less pronounced on preformed pellets than on spore suspensions.

Junlon also causes filamentous growth of *A. niger* and has been suggested to be of use in making turbidity estimations of fungal growth rather than using the more laborious dry weight method (Trinci, 1983).

The reasons why enhanced growth occurs in the presence of polymers in the growth medium appears relevant to an understanding of the effects of clays on the growth of *A. niger*. Increased biomass production by clays was probably due to the increased area of mycelial surface in contact with the nutrient medium which would allow the fungus to remain in the exponential growth phase for a longer period. Dispersed growth is known to be much more efficient than pelleted growth, as pellet

interiors are often anaerobic (Huang & Bungay, 1973; Phillips, 1966). The clays also allowed more spores to germinate and generally more pellets were produced.

Addition of divalent cations to media resulted in pelleted growth, perhaps by overcoming electrostatic repulsion between spores and hyphae (Byrne & Ward, 1987). Polymers reversed this effect possibly by attracting these ions away from the biomass and forming meta-stable chelates with them (Byrne & Ward, 1987; Elmayergi *et al.*, 1973). Clays are known to bind metal cations (Gadd & Griffiths, 1978) and again may have a similar effect to polymer additives on promoting filamentous growth of fungi.

My results, of increased biomass production by *A. niger*, in the presence of clays are in stark contrast to those of Stotzky & Rem (1967). These authors used homogenized mycelium and not spore suspensions in their experiments. Presumably, the homogenized mycelium would not aggregate and form pellets, thereby avoiding any of the oxygen inhibition effects observed during pelleted fungal growth. As the effects of clays on the growth of *A. niger* from a spore suspension appear mainly due to the prevention of compact pelleted growth, the results obtained by Stotzky & Rem (1967) are not entirely unexpected.

The ability to control fungal morphology in industrial fermentations is important as morphology type can influence microbial growth and and product formation. Filamentous growth is favoured for fumaric acid production by *Rhizopus arrhizus* (Metz & Kossen, 1977) and also for pectic enzyme production by *A. niger* (Calam, 1976). Both filamentous and pelleted growth have been used for penicillin production (Konig *et al.*, 1982; Metz & Kossen, 1977) whereas pelleted growth is preferred for production of citric acid and itaconic acid by *Aspergillus* species (Whitaker & Long, 1973). However, in a well agitated vessel pellet formation by *A. niger* may not be necessary for citric acid production (Berry, 1988). My results demonstrate that the more filamentous form of growth by *A. niger* caused by

montmorillonite and kaolinite decreased the efficiency of citric acid production by the fungus.

Ability of montmorillonite and kaolinite to adsorb citric acid.

Only montmorillonite at a concentration of 4% (w/v) was found to significantly reduce the level of citrate in Czapek Dox medium. Kaolinite did not adsorb citrate at all (Table 2.4). This means that the amounts of citric acid calculated to be produced by *A. niger* in varying concentrations of montmorillonite and kaolinite are correct. The reduction in citric acid concentration caused by 4% (w/v) montmorillonite did not matter as this value could not be determined due to polymer production by the fungus.

Effect of montmorillonite on the growth of *A. niger* in Raistrick's medium containing varying carbon concentrations.

Biomass production by *A. niger* was only stimulated in the presence of clay at carbon concentrations of 0.5% (w/v) and above (Fig. 2.27b) when compared with that produced in the absence of clay (Fig. 2.27a). The pH of the medium was affected by the presence of clay, being much higher in value up to carbon concentrations of 0.5% (w/v) (Fig. 2.27b). This may have been due to either decreased citric acid production by *A. niger* or to the buffering abilities of montmorillonite. The results suggest that fungal growth stimulation by montmorillonite only occurs in media rich in carbon or, in other words, in medium containing enough carbon to prolong exponential growth of the fungus.

Does montmorillonite protect *A. niger* from the fungicides Dicloran and Thiram ?

Dicloran prevented fungal growth at all concentrations used with and without the presence of montmorillonite (Figs. 2.28a and 2.28b). Thiram, at 0.025 and 0.05g per 100ml medium, prevented fungal growth, as determined by biomass production, in the presence and absence of clay (Figs. 2.29a and 2.29b). However, the fungicide did not prevent a reduction in medium pH occurring from pH 6.8 (initial pH of Czapek Dox liquid medium) to values around pH 2.5 (Figs. 2.30a and 2.30b). This suggests that the fungus was still metabolically active, but instead of producing biomass, produced acid instead. The fungal growth occurring in the three flasks at a thiram concentration of 0.01g per 100ml medium was completely different as indicated by the high standard deviation obtained (Fig. 2.29a). In one flask, biomass production was completely inhibited, in the second flask, biomass production was intermediate, and in the final flask, biomass was produced in amounts equivalent to the control where no fungicide was present. With clay and Thiram, at a concentration of 0.01g per 100ml, present, biomass production was stimulated (Fig. 2.29b) and the pH value of the growth medium was significantly decreased (Fig.2.30b). The growth stimulation obtained with low fungicide concentration and montmorillonite could be important in determining the amount of fungicides which should be applied to soils. The amount of fungicide needed to prevent fungal growth may be higher in soils with higher clay contents.

Clay adsorption by *Mucor flavus*.

Mucor flavus was able to adsorb montmorillonite, kaolinite and natural clay from suspension. Montmorillonite was adsorbed more efficiently than kaolinite (Table 2.5), with 66% of the montmorillonite being removed from suspension in the

Table 2.4 Ability of montmorillonite and kaolinite to adsorb citric acid

Clay mineral (% w/v)	% citrate removed from medium by increasing concentrations of montmorillonite and kaolinite.	
	Montmorillonite	Kaolinite
0	0	0
0.1	0	0
0.25	0	0
0.5	2 ± 1	0
1.0	5 ± 1	0
4.0	21 ± 2	0

(Means of triplicates ± S.D.)

Table 2.5 Adsorption of kaolinite and montmorillonite (0.2g)

Clay type	Exposure time (h)	% w/w clay adsorbed
Kaolinite	21	25.8 ± 9.7
	42	32.9 ± 9.9
	165	30.7 ± 2.7
Montmorillonite	21	66.3 ± 2.2
	42	71.2 ± 1.0
	165	76.1 ± 1.6

(Means of triplicates ± S.D.)

Figure 2.27.

Effect of montmorillonite (0.5% w/v) on the growth of *Aspergillus niger* in Raistricks' medium containing varying carbon concentrations.

a : Raistricks' medium alone (Control) .

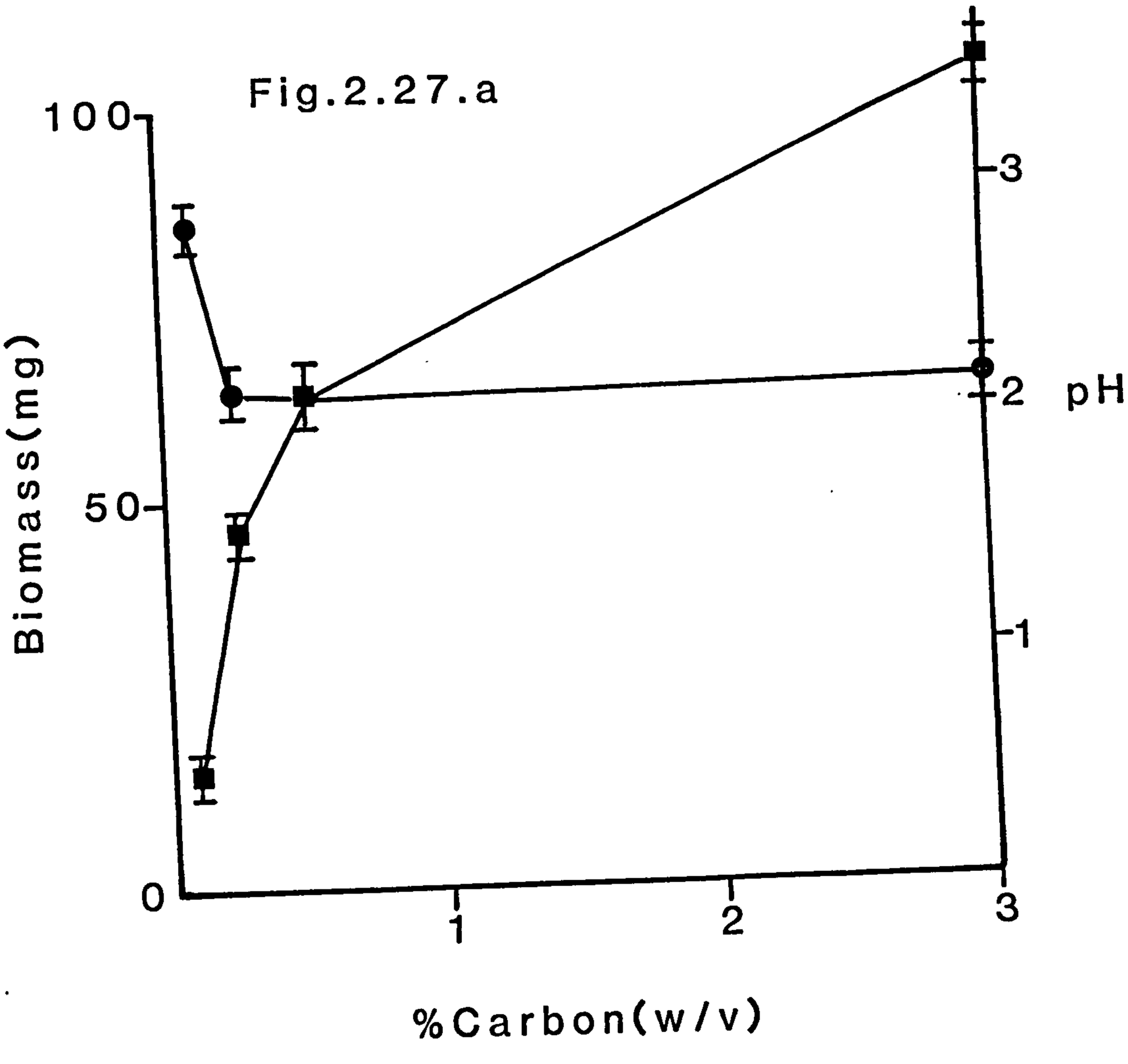
b : Raistricks' medium supplemented with montmorillonite.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in biomass compared with the control incubated without montmorillonite, $p < 0.05$: ** significant increase in medium pH compared with the control incubated without montmorillonite, $p < 0.05$)

■ — ■ Biomass

● — ● Medium pH

Fig.2.27.a


100
50
0
Biomass(mg)
1
2
3
%Carbon(w/v)
3
2
1
pH

b

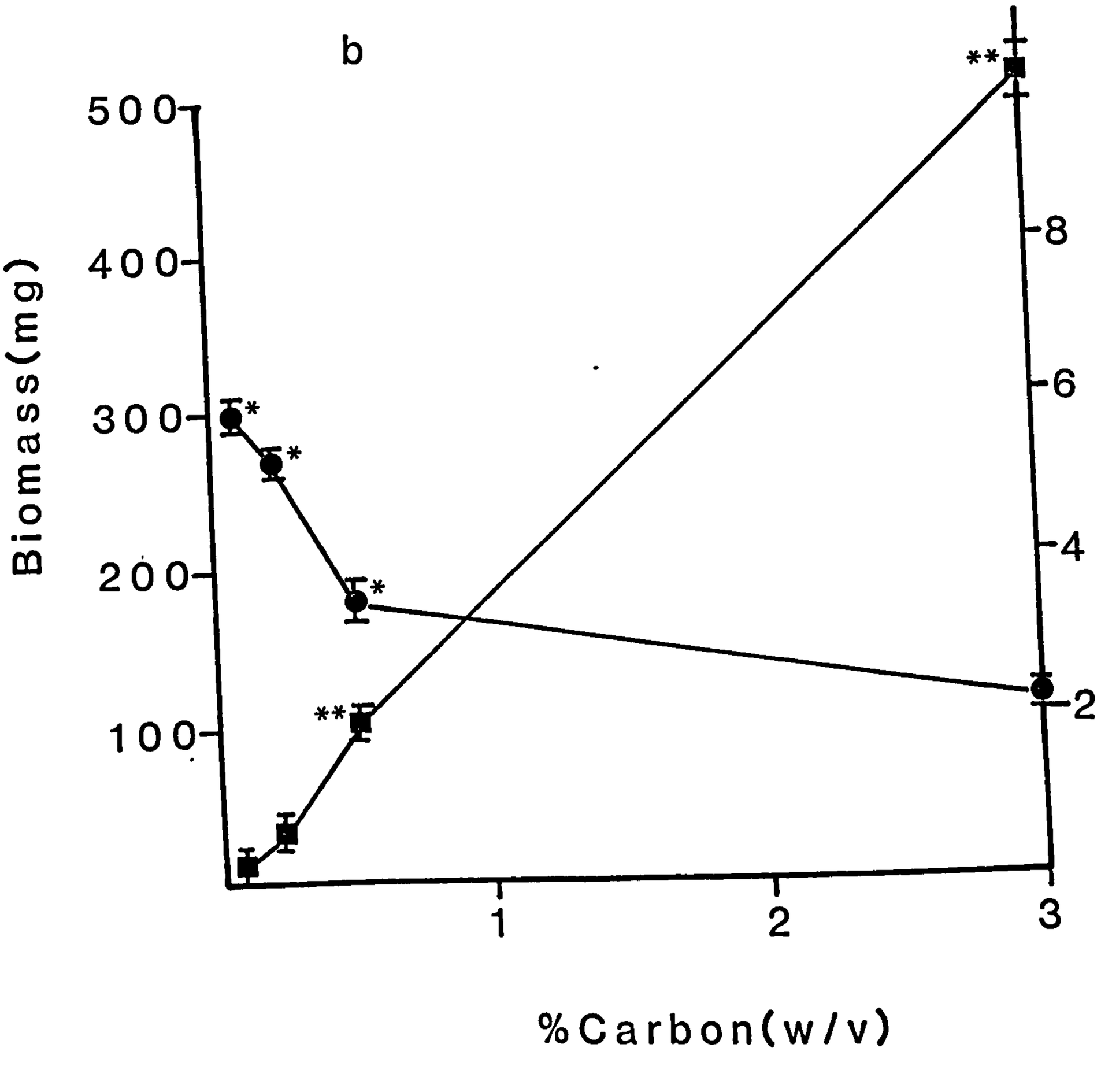

500
400
300
200
100
Biomass(mg)
*
*
*
**
**
1
2
3
%Carbon(w/v)
8
6
4
2
pH

Figure 2.28.

Effect of dicloran on the growth of *Aspergillus niger* a) in Czapek Dox liquid medium and b) in Czapek Dox medium supplemented with montmorillonite (0.5%).

(Means of triplicates $^{+}/_{-}$ S.D.)

1 : Biomass produced after two days growth (no dicloran added)

2 : Biomass produced after seven days growth (no dicloran added)

3 : Biomass produced after seven days growth (0.01g dicloran added after two days)

4 : Biomass produced after seven days growth (0.025g dicloran added after two days)

5 : Biomass produced after seven days growth (0.05g dicloran added after two days)

Figure 2.29.

Effect of thiram on the growth of *Aspergillus niger* a) in Czapek Dox liquid medium alone and b) in Czapek Dox medium supplemented with montmorillonite (0.05% w/v).

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in biomass compared with that produced after seven days growth in the absence of thiram, $p < 0.05$)

1 : Biomass produced after two days growth (no thiram added)

2 : Biomass produced after seven days growth (no thiram added)

3 : Biomass produced after seven days growth (0.01g thiram added after two days)

4 : Biomass produced after seven days growth (0.025g thiram added after two days)

5 : Biomass produced after seven days growth (0.05g thiram added after two days)

Fig.2.28.

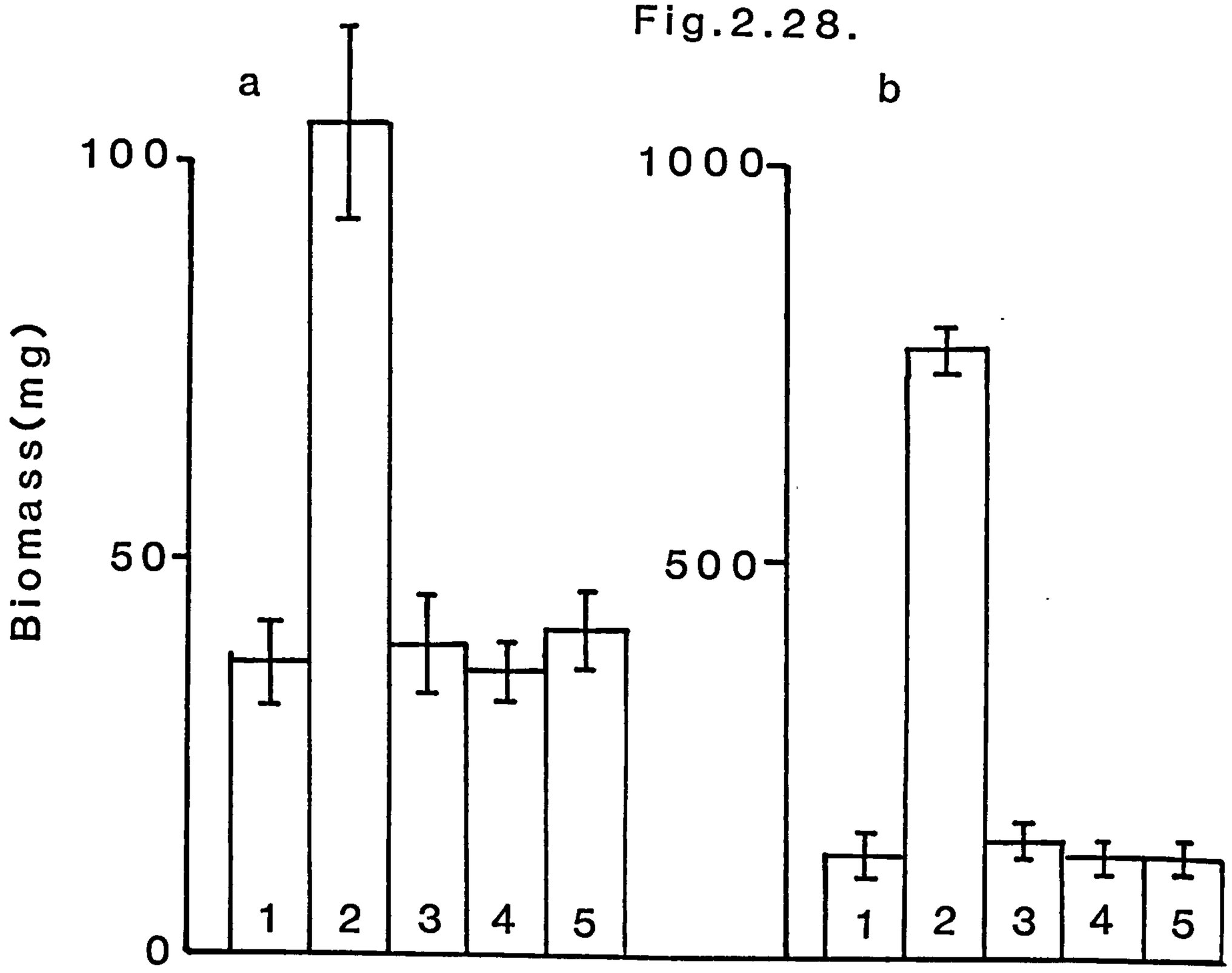


Fig.2.29.

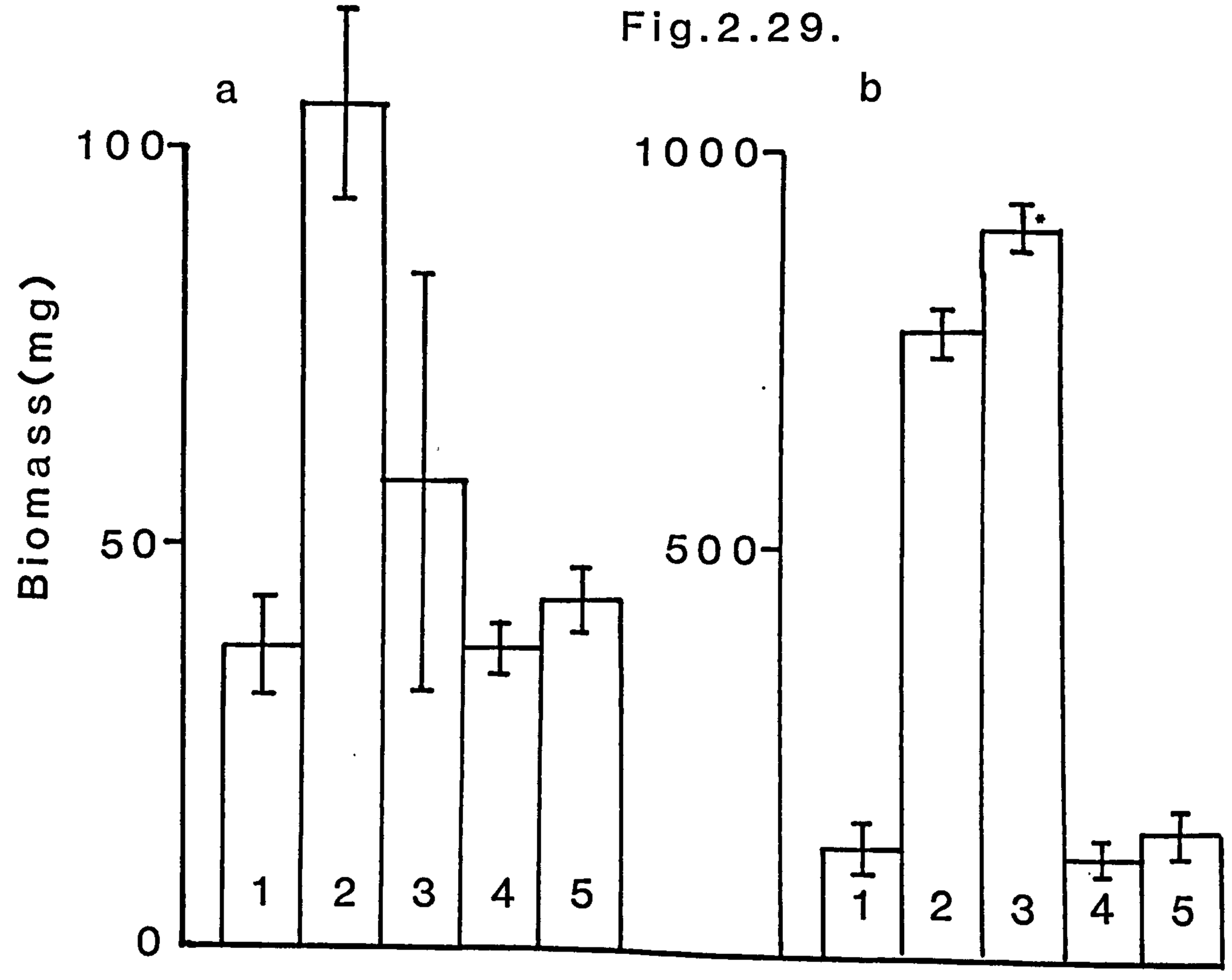
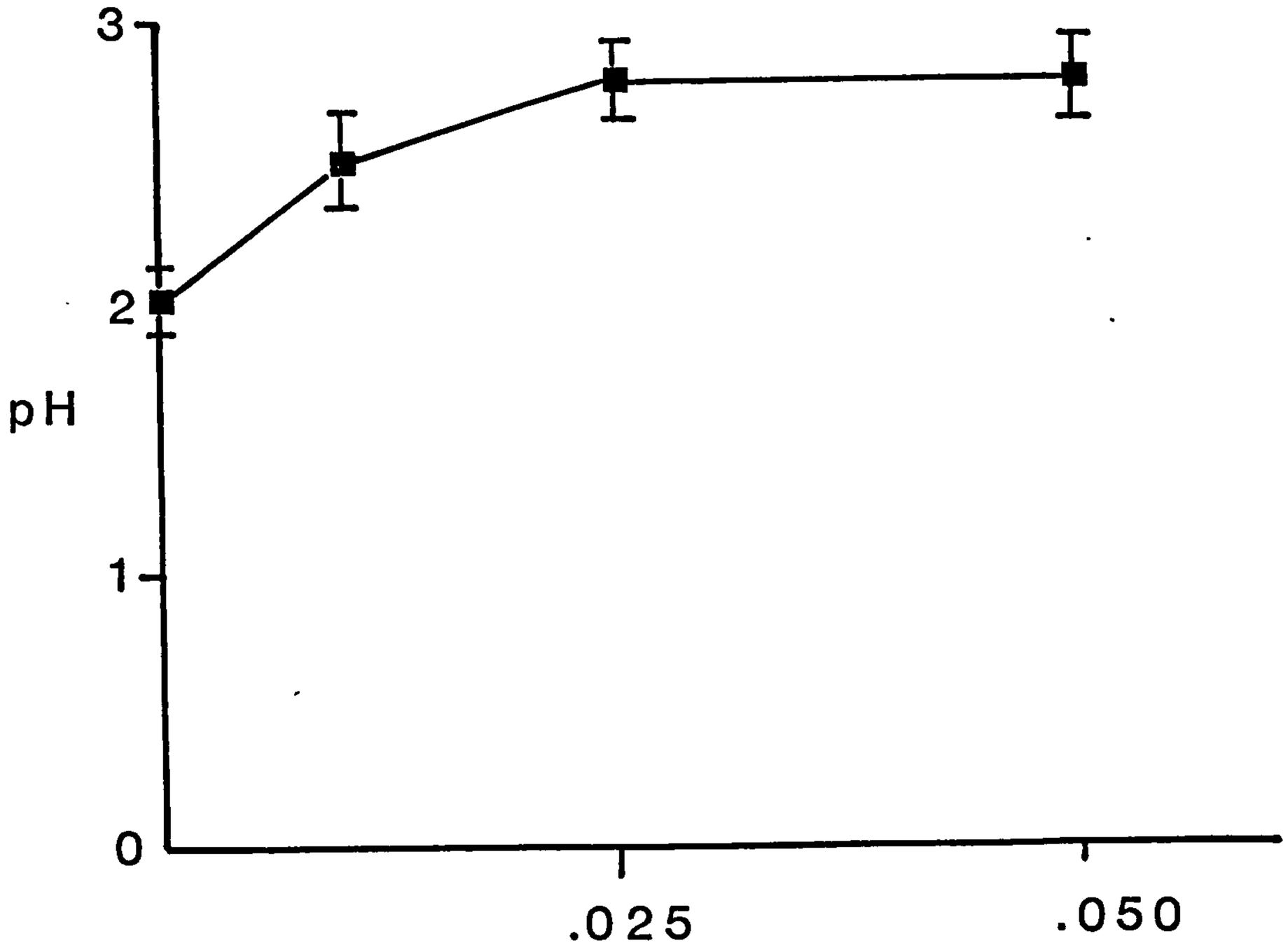


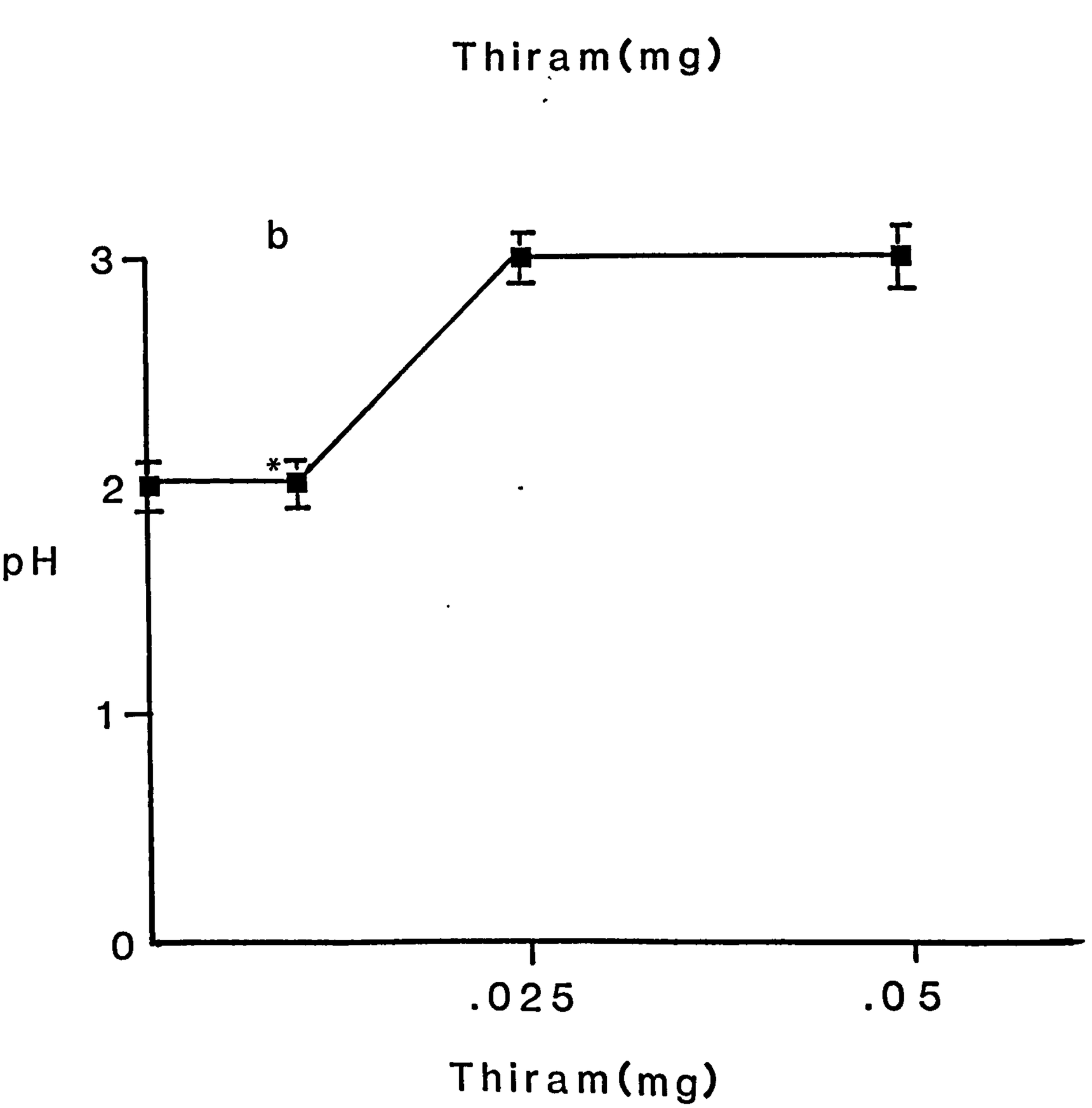
Figure 2.30.

Effect of thiram on the final pH value of the growth medium produced by *Aspergillus niger* incubated in a) Czapek Dox liquid medium alone and b) Czapek Dox medium supplemented with montmorillonite (0.05% w/v).

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in medium pH compared with the control containing thiram but no montmorillonite, $p < 0.05$)

Fig.2.30.a





first 21 hours, compared to a value of 25.8% for kaolinite over the same period. A further 10% increase in montmorillonite adsorption occurred over a 165 hour incubation period while increases in in kaolinite adsorption over the same time reached around 5%. Large amounts of clay adsorption occurred over a range of added clay weights, but the amount adsorbed tended to decrease with increasing proportion of clay to mycelium (Table 2.6), presumably as a result of a decrease in availability of adsorption sites. Steam sterilization of the natural clay did not influence the degree to which it was adsorbed, since 62.2% of the added sterilized clay was removed from suspension compared to 65.9% of non-sterilized clay (Table 2.6). The adsorption of natural clay decreased at temperatures below 25^0C. (Table 2.6). Natural clay adsorption decreased with increasing pH, with maximum adsorption occurring at pH 2.4 (Table 2.6). The increase in clay adsorption at lower pH values may be due to the positively charged areas on the clay becoming more positive at these values (Bashan, 1989). This could result in increased electrostatic attraction between the mycelial surface and the clay particle.

It may prove possible to grow *M. flavus* in clay wastes as long as suitable nutrients were added to the wastes to allow fungal growth. Clays might also stimulate fungal growth therefore enhancing clay removal by *M. flavus*.

Ability of *Aspergillus niger* waste mycelium, obtained from the surface fermentation method of citric acid production, to adsorb clays.

A. niger waste mycelium was unable to adsorb the clays, montmorillonite and kaolinite perhaps because of the age of the mycelium.

Ability of a soluble product, obtained from *A. niger* waste mycelium, (from both surface and deep produced fermentation methods of citric acid production) to flocculate clays,

Table 2.7 shows that after 16 hours incubation the supernatant obtained from surface-produced mycelium flocculated montmorillonite causing the clay to settle significantly quicker than the control. Table 2.8 shows that after only 15 seconds mixing and 6 hours settling the same supernatant flocculated montmorillonite again causing it to settle more quickly than the control. The supernatant obtained from mycelium produced by deep fermentation did not enhance clay settling at all (Table 2.7). The settling time of kaolinite was not decreased by either of the supernatants (Table 2.7).

Ability of citric acid to flocculate montmorillonite,

The lowest concentration of citric acid (0.125M) used was capable of decreasing the settling time of montmorillonite (Table 2.9). The high concentrations of citric acid were unable to flocculate the clay. The result suggests that citric acid which will be present in the supernatant from surface-grown mycelium is responsible for the clay flocculation observed. Citric acid, at low concentrations only, must lead to the formation of inter-particle bridging between the different clay particles causing them to flocculate and therefore settle more quickly. The exact mechanism of clay flocculation by citric acid was not determined.

Table 2.6 Effect of amount of clay, pH and sterilization on the adsorption of natural clay by *M. flavus* (3g, wet weight)

Amount of clay added (g)	% w/w clay adsorbed
0.1	82.7 ± 2.4
0.2	78.5 ± 2.2
0.3	*68.4 ± 5.2
0.4	*71.2 ± 1.0
pH (0.2g clay)	
2.4	84.6 ± 2.8
3.8	81.6 ± 5.6
5.4	*64.6 ± 12.1
7.8	*65.8 ± 4.9
sterilized (0.2g clay)	62.2 ± 4.8
non-sterilized (0.2g clay)	65.9 ± 6.3
Temperature (°C) (0.2g clay)	
5	*36.9 ± 2.4
15	58.8 ± 2.4
25	65.9 ± 4.9

(Means of triplicates ± S.D. *Significant decrease in clay adsorption, $p < 0.05$).

Table 2.7 Ability of *Aspergillus niger* waste mycelium, obtained from the surface and deep fermentation methods of citric acid production, to flocculate montmorillonite and kaolinite
(16 hours incubation at 25°C and 6 hours settling time)

mg clay ml^{-1} suspension

	Kaolinite			Montmorillonite		
	Top layer	2nd layer	3rd layer	Top layer	2nd layer	3rd layer
Control (clays alone)	.135 ±.010	.216 ±.038	.336 ±.026	.871 ±.056	1.581 ±.062	1.833 ±.093
Surface-produced waste (Test)	.120 ±.022	.172 ±.033	.318 ±.078	*.099 ±.031	*.135 ±.041	*.198 ±.082
Deep-produced waste (Test)	.113 ±.011	.224 ±.022	.351 ±.026	1.482 ±.156	2.219 ±.062	2.839 ±.166

(Means of triplicates ± S.D. *Decrease in amount of clay in the layer when compared to the control, $p < 0.05$).

Table 2.8 Ability of surface-produced *Aspergillus niger* waste mycelium to flocculate montmorillonite
(15 seconds mixing, 6 hours settling)

mg montmorillonite ml^{-1} suspension

	Top layer	2nd layer	3rd layer
Control (Montmorillonite alone)	.793 ± 0.038	1.642 ± 0.080	1.922 ± 0.091
Test (Surface waste plus montmorillonite)	*.279 ± 0.056	*.252 ± 0.082	*.288 ± 0.003

(Means of triplicates ± S.D. *Significant decrease in the amount of clay in the layer when compared to the control, $p < 0.05$).

Table 2.9 Ability of different concentrations of citric acid to flocculate montmorillonite
(15 seconds mixing, 4 hours settling)

Citric acid concentration (M)	mg montmorillonite ml^{-1} Top layer	2nd layer	3rd layer
Control (pH 2.2) montomorillonite alone	2.515 ± 0.165	3.683 ± 0.112	3.935 ± 0.235
0.125 M	*0.198 ± 0.031	*0.270 ± 0.054	*0.269 ± 0.027
0.25M	2.839 ± 0.347	3.647 ± 0.082	3.863 ± 0.189
0.5M	2.965 ± 0.270	3.557 ± 0.216	3.810 ± 0.165
1.0M	2.677 ± 0.136	3.504 ± 0.162	3.557 ± 0.285

(Means of triplicates ± S.D. *significant difference from control value, $p < 0.05$).

CONCLUSIONS.

Low concentrations of the clay minerals, montmorillonite and kaolinite, can increase biomass production by *A. niger* in rich carbon media. This biomass increase appears to mainly result from the inhibition of spore and hyphal aggregation, steps which are important in fungal pellet formation. This inhibition of compact pellet formation protects the fungus from the anaerobic conditions formed inside pellets and allows exponential growth to continue for a longer time. The biomass increase is accompanied by a decrease in acid production by *A. niger*. Clays might therefore be used as an economical alternative to polymers, in controlling fungal morphology in important industrial fermentations.

The stimulatory effect on the growth of *A. niger* by the fungicide Thiram in the presence of montmorillonite, may be important in determining the amounts of fungicide needed to be applied to different soils.

A. niger waste mycelium from citric acid production was unable to adsorb clays. Citric acid, at low concentrations flocculated montmorillonite probably by some kind of inter-particle bridging mechanism.

Clay adsorption by *M. flavus* is a temperature-dependent process but adsorption still occurs at 5-15^{0}C. a range of temperatures likely to occur in clay-waste lagoons and in the environment generally. Clay adsorption also occurs over a wide pH range although maximal at low pH values suggesting that neutral to alkaline clay suspensions may be profitably acidified before fungal material is added. *M. flavus* was able to adsorb sterile and non-sterile clays to an equivalent extent. It would be unnecessary to go to the prohibitive expense of sterilizing clays before they could be removed from suspension by fungi.

3. FUNGAL IMMOBILIZATION BY MAGNETITE AND SURFACE ADSORPTION.

3. FUNGAL IMMOBILIZATION BY MAGNETITE AND SURFACE ADSORPTION.

INTRODUCTION.

In Chapter 1 a wide range of particulate compounds were shown to be adsorbed by an equally wide range of fungi. Magnetite was one of these compounds and as a result of the adsorption of this material the mycelium became susceptible to a magnetic field. This phenomenon might be useful in industry since it would allow fungi to be a) immobilized onto a metal surface by magnetic attraction, b) moved within an industrial plant under the influence of a magnetic field, or finally c) removed from solution by magnets, a process which might be more effective and more cost efficient than either centrifugation or filtration.

Previous biological applications of magnetite.

Enzyme immobilization.

Microbial enzyme reactions have long been used by man in various fermentation processes, such as brewing and food production. These processes involve the use of enzymes which are intact inside living microbial cells, an approach which has a number of drawbacks (Prescott & Dunn, 1982):

(1) A high proportion of substrate may be converted to biomass

(2) Optimum conditions for growth and product formation may diverge

(3) Conversion rate to desired product may be poor

(4) Side reactions may occur

(5) Separation of desired product from the fermentation may be difficult.

Most of these problems can be overcome by the isolation and purification of enzymes. Recent developments in protein and genetic engineering have markedly increased the interest in the utilization of enzymes for industrial processes.

Batch processes have been conventionally used to mediate enzyme reactions. However, the recovery of the active enzyme from the reaction mixture is technically very difficult and the enzyme and other proteins present usually have to be removed by denaturation achieved by pH adjustment or heat treatment. This procedure isolates the product but fails to provide an economical use of the enzyme, largely because the remaining active enzyme is discarded after each batch reaction.

To overcome these difficulties the technique of "enzyme immobilization" was developed. The following advantages can be expected by the use of immobilized enzymes (Chibata & Tosa, 1983):

(1) Stability of enzymes is improved

(2) A catalyst can be tailor-made for specific use

(3) Enzymes can be re-used

(4) Continuous operation becomes practical

(5) Reactions require less space

(6) Better control of reaction is possible

(7) Higher purity and yield of products may be obtained

(8) Resources can be conserved and pollution minimized.

Grubhofer & Schleith are credited with the first attempt to immobilize enzymes for commercial applications. The authors immobilized enzymes like carboxypeptidase, diastase, pepsin and ribonuclease to a diazotized polyaminopolystyrene resin (Chibata & Tosa, 1983). Since this work, studies on immobilized enzymes developed rapidly in the United States, Europe and Japan until in 1969 the industrialization of the continuous optical resolution of DL-amino acids using immobilized aminoacylase was achieved (Chibata & Tosa, 1983). This was the world's first industrial application of an immobilized enzyme. Aminoacylase is a fungal enzyme derived from *Aspergillus* species. Other current applications of immobilized enzymes in industry include:

a) The isomerization of glucose to fructose by glucose isomerase

b) Production of 6-amino penicillanic acid by penicillin amidase and

c) Hydrolysis of lactose by B-galactosidase.

A variety of methods have been developed to immobilize enzymes and numerous compounds have been used as immobilization materials (Prescott & Dunn 1982). The general attachment methods used in enzyme immobilization are as follows:

(1) Physical adsorption to a solid phase

(2) Covalent bonding to a solid phase

(3) Covalent bonding to soluble polymers

(4) Cross-linking with bifunctional reagents, and

(5) Inclusion in a gel phase.

Immobilization of enzymes by adsorption at solid surfaces offers the advantage of extreme simplicity. No harmful chemicals are involved in the process and as a result no modification of the enzyme occurs. This is important as any such modification to the enzyme could cause a loss of enzymic activity. However, the binding of enzymes is reversible and desorption of the enzyme from its' support will occur in the presence of the enzymes' substrate or in a solution of increased ionic strength. Commonly used adsorbents are carbon, cellulose, clays, metal oxides and ion-exchange resins such as DEAE-Sephadex.

Covalent coupling of an enzyme to a carrier material is achieved by methods used in peptide and protein chemistry. Covalent bond attachments have the advantage of being stable in the presence of enzyme substrate molecules and are not reversed by solutions of increased ionic strength or pH. However, the chemical reaction necessary for enzyme immobilization by covalent bonding could block the active site of the enzyme. This would render the enzyme inactive.

Enzymes can be polymerized by cross-linking with low molecular weight bifunctional agents. A three dimensional network of enzyme molecules is formed when the reaction is carried out in the absence of a support. This method can result in a considerable loss of activity. Most often enzymes are cross-linked after adsorption onto a suitable carrier. Glutaraldehyde and diazobenzidene are commonly used for this purpose.

The last type of immobilization, inclusion in gels, has the advantage of relatively mild reaction conditions. Other advantages of this method include retention of the enzyme in its' native state and a reduction in the risk of blocking the enzymes' active site. However, retardation of the enzymic reaction due to poor substrate/product diffusion and loss of the enzyme due to differential distribution of pore sizes are major drawbacks to this particular approach to enzyme

immobilization. Recent advances such as the use of calcium alginate gels are beginning to overcome these drawbacks.

Immobilization of an enzyme may cause a significant change in its' properties. These differences may be due to:

(1) the physical and chemical nature of the carrier used,

(2) the chemical and/or conformational changes in enzyme structure, and

(3) the "heterogeneous nature" of the catalysis reaction caused by immobilization.

A very important attribute of enzyme immobilization is that the activity of the enzyme can be retained for long periods of time provided that the enzyme is subjected to suitable storage conditions. Once immobilized, enzymes may also become more stable to heat and pH changes, these properties depending on the nature of the carrier surface to which the enzyme is bound. Melrose (1971) compared the activities of 50 immobilized enzymes with their soluble forms. He found 30 more stable and 8 less stable than their soluble forms; 12 showed no difference from the free systems. The reasons for increased stability are unclear. Possible explanations include, prevention of conformational inactivation and shielding of active groups on the enzyme from reactive groups in solution.

The use of immobilized enzymes is now expanding into different areas besides synthetic chemical reactions, including chemical and clinical analysis, medicine, food processing and in the determination of reaction mechanisms.

<u>Use of magnetite in enzyme immobilization.</u>

The main reason for using magnetite for enzyme immobilization is the compounds obvious susceptibility to a magnetic field. Enzymes immobilized onto magnetite will be recovered easily from reaction mixtures or process streams. One of the first reports on the use of magnetite as an enzyme carrier stated that "the normal techniques for retention of an immobilized enzyme in a reactor or recovery from a product stream are only used where the particle size of the immobilized enzyme is substantially larger than that of the colloids present. The use of magnetic support materials may overcome this" (Robinson *et al.*, 1973).

Since this report a variety of methods have been used to attach enzymes to magnetite. Van Leemputten (1974) immobilized trypsin by cross-linking with glutaraldehyde onto magnetite and found the enzyme to be more heat stable. In the same study invertase was cross-linked on magnetite again using glutaraldehyde as the linking agent. However, no particular advantages were achieved using this method of immobilization for invertase. Matsunaga & Kamiya (1987) isolated pure magnetite particles from magnetotactic bacteria and immobilized the enzymes glucose oxidase and uricase directly onto the magnetite. They found that the glucose oxidase activity was forty times that of the enzyme immobilized on artificial magnetite or Zn-ferrite particles. The enzyme also retained its' activity when re-used five times. Sambamurthy (1987) successfully covalently coupled amyloglucosidase on magnetite using a silane-glutaraldehyde method. This procedure gave higher activities of the enzyme than with other methods tried with immobilization to magnetite.

The above work demonstrates that immobilization of enzymes to magnetite particles is possible but unfortunately most of the enzymes immobilized in this way showed activities lower than the free enzyme. More recent work however appears to have overcome these initial problems. Garcia *et al.*, (1989) in an initial series of experiments immobilized cellulase directly onto magnetite. Cellulase is useful in the

hydrolysis of cotton gin trash for the production of fermentable sugars. The cellulase was coupled to silanized iron oxide particles by linking amino groups of the enzyme to the support particles using glutaraldehyde or by linking carboxyl groups using carbodiimide. Enzymes coupled by amino groups demonstrated a six-fold increase in activity over coupling by carboxyl groups. After multiple recovery and re-use the activity stabilized at ca. 35% of the initial activity. Again these activities were poor and were thought to be the result of steric hindrance and lack of freedom of enzyme movement. Garcia *et al.*, (1989) tried to overcome these difficulties by placing a high molecular weight ligand between the enzyme and the support thus allowing the enzyme greater freedom of movement in the hope of obtaining higher enzyme activities. Two polymers, polyethylene glycol (PEG) and polyvinyl alcohol were used for this purpose. PEG was found to be the most suitable ligand and indeed increased the activity of cellulase in a continuous reactor and increased the half-life of the enzyme. This method of enzyme immobilization to magnetite seems to be particularly useful and has become the subject of a patent (Inada *et al.*, 1988). In this particular case a PEG derivative was activated with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide and lipase was attached. The polymer-enzyme conjugate was then mixed with $FeCl_2$ and $FeCl_3$ to obtain a magnetic polymer-enzyme conjugate. The conjugate could be dispersed in benzene and aqueous solvents and completely recovered within five minutes using a magnetic field. In a similar study by Mihama *et al.*, (1988) discuss the use of a co-polymer of maleic acid and PEG in the magnetic immobilization of lipase. Here high enzymatic activity was reported together with no loss of enzyme activity on recovery from solution.

Possible medical applications of enzymes immobilized to magnetite.

Patients with thrombosis or embolism are treated with the enzyme urokinase (White & Barlow, 1973). The enzyme has a short half-life and is susceptible towards it's inhibitor. This means that the enzyme must be administered in large doses and the therapy is therefore accompanied with risks of unnecessary bleeding and other side effects. A method which could achieve effective localization of the drug would improve the treatment considerably and would also reduce the amount of enzyme needed in the treatment. Inada *et al.*, (1987) showed that urokinase could be immobilized onto magnetite using PEG and that in *in vitro* studies the resulting magnetized enzyme could achieve effective localized fibrinolysis. Furthermore enzymes chemically modified by the binding of PEG to their surface showed a reduction in immunoreactivity. In a more complex experiment (Yoshimoto *et al.*, 1988), magnetic urokinase was produced and apparatus set up to examine the lysis of a fibrin clot in continuously circulating plasma. The fibrin clot almost disappeared within 380 minutes when magnetic urokinase was used and a magnet applied externally to the clot. In contrast no fibrinolysis occurred when native urokinase was used. Magnetic urokinase was also found to be more stable than native urokinase. The authors intend to test the magnetic urokinase further in *in vivo* studies. Although these preliminary results appear promising the use of magnetite in further medical studies may be hazardous as magnetite dust, when applied intratracheally, can lead to an unexpectedly high lung tumour incidence of 69% in rats (Pott *et al.*, 1987).

<u>Immobilization of microbial cells.</u>

Immobilized microbial cells can be defined by substituting the word enzymes for microbial cells in the definition given for immobilized enzymes. The definition

then becomes "microbial cells physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously."

Immobilized microbial cells are used instead of immobilized enzymes when:

(1) Enzymes are intracellular

(2) Enzymes extracted from cells are unstable during and after immobilization

(3) The microbe contains no interfering enzymes, or any interfering enzymes are readily inactivated or removed

(4) The substrates and products are not high molecular weight compounds.

In the above cases, the following advantages of immobilized microbial cells may be expected:

(1) Processes for extraction and purification of enzyme are not necessary

(2) Yield of enzyme activity on immobilization is high

(3) Operational stability is generally high

(4) Cost of enzyme is low

(5) Application for multistep enzyme reaction is possible.

Immobilized microbial cells may be growing, resting or dead; but the enzyme activities are kept in the active state.

As with enzyme immobilization there are several methods available to obtain whole cell immobilization. These include:

(1) Physical cross-linking (flocculation)

(2) Covalent cross-linking

(3) Adsorption onto insoluble matrices

(4) Covalent binding to insoluble matrices

(5) Physical entrapment in porous materials

(6) Encapsulation.

Of these methods the most widely used for the preparation of both immobilized cells and enzymes is encapsulation within gels, polyacrylamide and calcium alginate gels being favoured. As can be seen immobilization methods for both enzymes and cells are very similar. Comprehensive reviews of methods of immobilization of microbial cells and applications of immobilized cells exist (Chibata & Wingard, 1983).

To date only three processes based on immobilized cells are operative on an industrial scale:

(1) Production of L-aspartic acid using immobilized dead cells of *Escherichia coli*

(2) Production of L-malic acid using immobilized dead cells of *Brevibacterium ammoniagenes*

(3) Production of prednisolone using living immobilized cells of *Curvularia lunata* and *Corynebacterium simplex.*

All three processes are based on the entrapment of cells in polyacrylamide gels. The limited number of industrial applications in contrast to the relatively large

Table 3.1. Examples of living cells immobilized by adsorption to surfaces

Carrier	Cells	Application	Reference
Anthracite	*Pseudomonas* sp	Degradation of phenol	Scott & Hancher (1976)
Coal Particles	Waste-treatment bacteria	Biological denitrification	Scott & Hancher (1976) Ngian & Martin (1980)
DEAE-Sephadex	Primary and finite life-span cell strains	Growth of cells on microcarriers and production of poliomyelitis virus	Van Wezel (1967)
DEAE-Sephadex	Cell-line 316 (skin cells)	Production of interferon	Giard et al., (1979)
Wood chips	*Saccharomyces cerevisiae*	Ethanol production	Gencer and Mutharasan (1983)
Wood chips	*Saccharomyces lipolytica*	Citrate production	Briffand and Engasser (1979)
Glass	*Anabaena cylindrica*	Hydrogen production	Lambert et al., (1979).
Glass	*Aspergillus niger*	Biomass production	Messing and Opperman (1979)
Glass	*Penicillium chrysogenum*	Biomass production	Messing and Opperman (1979)
Glass	*Citrobacter sp.*	Cadmium adsorption	Macaskie et al., (1987)
Stainless steel	*Aspergillus foetoidus*	Production of citric acid	Atkinson et al., (1979)
Stainless steel	*Streptomyces griseus*	Production of streptomycin	Atkinson et al., (1979)
Stainless steel	*Trichoderma reesei*	Cellulase production	Webb et al., (1986)
Polyester	*Sporotrichum cellulophilum*	Cellulase production	Tamada et al., (1986)
Polyester	*Trichoderma reesei*	Cellulase production	Duff (1988)
Celite	*Streptomyces cattleya*	Thienamycin production	Arcuri et al., (1983)
Celite	*Penicillium chrysogenum*	Penicillin production	Gbewongo et al., (1983)

number of published reports (Kennedy & Cabral, 1984) result from adverse economic factors arising from the high costs of carriers and reagents involved in the immobilization procedure. Obviously immobilization methods which involve only physical adsorption of the cells to a carrier or *vice versa* would dramatically alleviate these costs.

The adhesion of microbial cells to surfaces has long been recognized to be of importance in natural environments (see Chapter 1). This "natural adhesion" has been utilized in the immobilization of various types of microbial cells (See Table 3.1.) This surface adsorption method of immobilization is based on linking cells directly to water-insoluble carriers. Cell adsorption is thought to be mainly due to electrostatic interactions between the microbial cell surface and the carrier. The main advantage of natural adhesion is that the process is relatively mild and allows good retention of cell viability and enzymatic activity. However, desorption of cells from the carrier surface can occur under certain conditions. Factors which affect the strength of cell attachment include cell wall composition, cell age, various physico-chemical surface properties of the carrier (including surface area) and also the pH and ionic strength of the solution in which the cells are suspended (Kolot, 1981). Kolot (1981) examined the immobilization of *Penicillium chrysogenum* on a variety of inorganic supports including fritted glass, cordierite and zirconia ceramic. The latter material had the highest biomass accumulation and also had a greater negative charge than the other materials. As the microbial cell surface also carries a net negative charge the adsorption of the fungus to this zirconia-coated ceramic seems somewhat surprising. Kolot (1981) explained the result by suggesting that direct linkages were formed between zirconium hydroxide and amino groups at the cell surface.

Several types of rotating biological film reactors, generally referred to as rotating biological contactors, have been developed for waste water treatment (see

Chapter 1). The reactors rely upon the natural adhesion of microrganisms to surfaces for film formation. This rotating disc principle has been used in the design of a rotating disc fermenter system for experimental studies on the characteristics of fungal film growth. The design of such a reactor has been patented (Blain *et al.*, 1981) and suggested uses include waste water treatment. Rotating films of the white rot fungus *Phanerochaete chrysosporium* have been used for the treatment of kraft bleach plant effluent (Anderson, 1983). The effluents, because of their colour, can present a substantial water pollution problem. The colour is mainly caused by fragments of lignin which, because of their resistance to biological attack, pass through biological treatment systems largely undegraded. The chemical technologies developed for reducing the colour levels are expensive and the use of immobilized *P. chrysosporium* to treat the effluents is an interesting, low-cost method.

Unlike the large amount of work with enzymes, little use has been made of the ability of microorganisms to bind magnetite particles for the purpose of cell immobilization. In this chapter the ability of fungi to adsorb magnetite particles to their surface is investigated and an attempt to assess this ability in relation to fungal cell immobilization and recovery from solution is made. The importance of fungi in industrial processes (Table 3.2) makes fungal immobilization by magnetite (and also immobilization by surface adsorption) a particularly worthwhile study. Also biomass recovery using magnetite could prove to be more efficient and cost effective than the existing methods of centrifugation and filtration. The use of magnetite to immobilize fungal biomass is the subject of a patent (Kiyoshi, 1976). Here cells of *Penicillium spiculisporum* were mixed with magnetite particles in a magnetic field. The magnetic field was changed in order to control the immobilization strength, catalytic activity and immobilization yield. The yield of spiculisporic acid produced by the immobilized fungus (22.5 to 33.1%) was comparable with that of an non-immobilized control culture.

Table 3.2 Examples of commercial applications of fungi and their products (Berry, 1988)

Substance	Species	Use
Antibiotics and other bioactive compounds		
Penicillins	*Penicillium chrysogenum* *Pencillium notatum*	Antibiotics
Cephalosporins	*Cephalosporium acremonium*	Antibiotics
Griseofulvin	*Penicillium urticae*	Antibiotic
Ergot alkaloids	*Claviceps purpurea*	Uterine contractant
Enzymes		
α-Amylase	*Aspergillus oryzae* *Aspergillus flavus* *Saccharomycopsis fibuligera*	Food and beverage industry
Amyloglucosidase	*Aspergillus niger* *Aspergillus oryzae* *Aspergillus foetidus* *Aspergillus awamori* *Rhizopus sp.*	Food and beverage industry
Asparaginase	*Aspergillus niger* *Pencillium camembertii*	Pharmaceutical industry
Cellulase	*Aspergillus niger* *Trichoderma viride*	Food industry
Glucose oxidase	*Aspergillus niger* *Penicillium chrysogenum*	Food industry
β-Galactosidase	*Aspergillus niger* *Aspergillus foetidus* *Saccharomyces fragilis*	Food and dairy industry
Invertase	*Saccharomyces cerevisiae*	Food industry
Lipase	*Aspergillus oryzae*	Food and dairy industry
Nucleases	*Penicillium citrinum*	Food (flavour)
Pectinase	*Aspergillus niger*	Food industry
Proteinase	*Aspergillus oryzae* *Mucor meihei* *Mucor pusillus*	Food and dairy industry (cheese)

Table 3.2 - contd.

Substance	Species	Use
Biomass		
	Saccharomyces cerevisiae	Food (baker's yeast)
	Fusarium graminearum	Food (protein)
	Candida utilis	Animal feed
	Agaricus bisporus	Food
	Volvariella volvacea	Food
	Lentinula edodes	Food
	Paecilomyces sp.	Animal Feed
	Saccharomycopsis fibuligera	Animal Feed
	Beauvaria bassiana	Biological control
	Verticillium lecanii	Biological control
	Mycorrhizal Basidiomycetes	Forestry
Biotransformation		
	Cunninghamella blakesleeana	Cortisone manufacture
Organic acids		
Citric acid	*Aspergillus niger*	Food and pharmaceutical
	Candida lipolytica	industry
Fumaric acid	*Rhizopus spp.*	Food industry
Cluconic acid	*Aspergillus niger*	Food and pharmaceutical industry (acidulant)
Itaconic acid	*Aspergillus terreus*	Chemical industry (plastics)
Others		
Ethanol	*Saccharomyces cerevisiae* *Schizosaccharomyces pombe*	Beverages fuel alcohol
Pullulan	*Aureobasidum pullulans*	Food (packaging)
y-Linoleic acid	Not disclosed	Food
Genetically engineered products		
Interferon	*Saccharomyces cerevisiae*	
Serum albumin	*Saccharomyces cerevisiae*	
Vaccines	*Saccharomyces cerevisiae*	

A main disadvantage to the adsorption method of immobilization, in terms of preparation stability, arises from the rate of desorption of cells from the surface of the support (Marcipar *et al.*, 1979). This problem is especially severe when changes in pH or ionic strength occur. This would mean that magnetite could become desorbed from the cell surface and immobilization or recovery by magnetic means made impossible. Another form of immobilization would then become necessary.

Magnetite can also be immobilized within calcium alginate promoting the formation of magnetic beads (Larsson & Mosbach, 1979). Entrapment of cells within calcium alginate is a popular method of immobilization as only nontoxic compounds are used in method. The non-denaturing properties of the alginate and the resistance of the pellets to carbon dioxide generation within the entrapped cells make this method useful for multienzyme conversions, such as ethanol production (Kierstan & Bucke, 1977). Co-immobilization of cells and magnetite within calcium alginate would obviously enhance possibilities of efficient biomass recovery and reuse. The instability of calcium alginate beads in media containing calcium chelating agents, such as phosphates, and certain cations such as Mg^{2+} can be overcome by treating the gel with polyamines (Birnbaum *et al.*, 1981). Recent examples of microorganisms immobilized in alginate gels include:

(1) *Aspergillus awamori* and *Saccharomyces cerevisiae* (co-immobilized) for the production of ethanol from starch (Kurosawa *et al.*, 1989)

(2) *Bacillus megaterium*, *Aeromonas hydrophila* and *Pseudomonas marinoglutinosa* for the solubilization of fish meat proteins (Venugopal *et al.*, 1989).

(3) *Rhizopus oryzae* for the production of L(+)-lactic acid (Hang *et al.*, 1989).

(4) *Pseudomonas sp. B13* cells for the dehalogenation of 3-Chlorobenzoate (Sahasrabudhe *et al.*, 1988).

(5) *Trichosporon pullulans* for the hydrolysis of cellobiose (Adami *et al.*, 1988).

This frequent use of alginate gels has prompted a study of the properties of alginates derived from different sources (Martinsen *et al.*, 1989) and work directed towards removing phenolic compounds which are responsible for a loss in the viscosity of alginates has also been deemed necessary (Skjaek-Braek *et al.*, 1989). Also sensitive cells such as animal cells (Tompkins *et al.*, 1988) and protoplasts (Scheurich *et al.*, 1980) have been successfully immobilized in calcium alginate gels.

Co-immobilization of glucose oxidase, catalase and magnetite into acrylamide beads has been successfully achieved in the continuous oxidation of glucose (Sada *et al.*, 1983). Electromagnets were used to prevent the beads being removed from the bioreactor. Acrylamide gels are another widely used form of cell immobilization and the use of this gel demonstrates the versatility of magnetite for use in biotechnological processes.

Wastewater treatment using magnetite.

Magnetite has been succesfully used for a number of years in wastewater treatment. The treatment is known as" The Sirofloc Process" and utilizes the ability of magnetite to remove turbidity and colour from effluents (Kolarik *et al.*, 1983). The magnetite particles adsorb contaminants in the water and are recovered magnetically. The particles can be re-used after a short treatment in an alkali solution which desorbs any adsorbed compounds. Alkali treatment also increases the isoelectric point of magnetite so that it has a positive surface charge up to a pH value of 8.5. Before alkali treatment magnetite only has a positive surface charge up to a pH value of 6.5 (Macrae & Evans, 1983). This phenomenon results in the pre-

treatment of magnetite with alkali to produce "alkali activated magnetite" which is then used in wastewater treatment (Kolarik, 1983).

Before the establishment of the Sirofloc Process, magnetite and related magnetic compounds had been studied for their ability to adsorb viral particles to their surface. The first of these was a report on the use of haematite to remove influenza virus from solution (Warren *et al.*, 1966). Rao *et al.*, (1968) attempted to use columns packed with magnetic iron oxides to remove enterovirus from water. Unfortunately this early work was unsuccessful due to the clogging of the columns by suspended solids present. A later report by Bitton *et al.*, (1976) attempted to determine factors which affected the adsorption of poliovirus to magnetite in water and wastewater. They found that pH was an important factor in adsorption, viral adsorption to magnetite being steady at pH values from 5 to 9, but dropping at values below 5. Trivalent cations, eg. Al^{3+}, were effective at increasing poliovirus removal from solution. Magnetite was also found to remove algae from lakewater (Bitton *et al.*, 1975). Algae were recovered most effectively from lakes with a pH value of approximately 7, 94% removal being achieved. Lakes with a higher pH displayed a lower rate of algal removal of 60%. Algae are known to contribute to the odour, taste, turbidity and deterioration of water supplies so their removal from such supplies is important. Micro-algae are also grown commercially on a vast scale in large outdoor production ponds. For example *Dunaliella spp.* are grown as a food source in aquaculture and also as a commercial source of substances such as B-carotene and glycerol (Rich, 1978). A major cost in this micro-algal production is the actual harvesting of the algae. A number of methods have been developed for this purpose including centrifugation, sedimentation, filtration and flocculation (Mohn, 1988). However, the efficiency and cost-effectiveness of these methods needs to be improved and the ability of algal cells to adsorb magnetite may well be useful in the harvesting process.

The commercial establishment of the Sirofloc process led to further studies on the interactions of microorganisms with magnetite. Various species of bacteria including *Escherichia coli*, *Streptococcus faecalis* and *Pseudomonas stutzeri* were found to adsorb to magnetite particles, adsorption decreasing at high pH values (pH 11) and being stimulated by low concentrations of the divalent cations, Mg^{2+} and Ca^{2+} (Macrae & Evans, 1983). A subsequent study by Macrae & Evans (1984) led to the proposed use of magnetite to remove bacteria from wastewater. A further development of this approach involved the use of bacteria adsorbed to magnetite to remove pesticides from water (Macrae, 1985). *Rhodopseudomonas sphaeroides*, *Alcaligenes eutrophus* and *Saccharomyces cerevisiae* while adsorbed to magnetite particles were utilized to determine their abilities to remove the pesticides lindane, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) from solution (Macrae, 1985). Magnetite alone was found to remove lindane from solution and activated carbon was more efficient than microbial cells at the adsorption of pesticides.

The possiblity of virus removal from water during the Sirofloc Process was investigated by Atherton & Bell (1983a). Suspended materials, such as clay and other components of sewage effluent, were found to interfere with the adsorption of the bacteriophage MS2 to magnetite. This interference could be overcome by the addition of a strongly basic, highly charged cationic molecule of a moderate chain length (polyelectrolyte A8101) to the solution. The adsorbed virus was also found to be disrupted at pH 10, the viral components being released into solution, whereas the free virus, at the same pH, was able to retain it's infectivity (Atherton & Bell, 1983b).

Despite the ability of many types of microrganisms to adsorb magnetite, little attention has been directed towards the possible applications of magnetite

adsorption by fungi. The research in this chapter examines the capability of two industrially important fungi, *Aspergillus niger* (laboratory culture) and *Penicillium chrysogenum*, to adsorb magnetite and attempts to determine factors which affect adsorption. Magnetite adsorption by *A. niger* waste mycelium from the industrial production of citric acid was also studied in relation to possible industrial applications of the phenomenon. Two types of mycelial waste were studied:

(1) Surface fermentation waste and

(2) Deep fermentation waste.

Adsorption of magnetite would be useful in industry as it would allow fungi to be:

(a) immobilized by magnetic attraction

(b) moved within an industrial plant under the influence of a magnetic field, or

(c) removed from solution by magnetic means; a process which might be more effective and cost efficient than either centrifugation or filtration.

The ability of fungi to grow on thread, nylon mesh and a synthetic foam is discussed. In particular, the effect of adding varying amounts of spore suspensions of different fungi on fungal immobilization to a nylon mesh carrier is examined. The rate of sulphur oxidation by *Aspergillus niger* immobilized on foam was compared to that of a non-immobilized culture to determine the effects of immobilization on metabolic activity.

MATERIALS AND METHODS.

MAGNETITE ADSORPTION BY FUNGI.

Effect of length of exposure to magnetite and age of mycelium on magnetite uptake by laboratory grown *Aspergillus niger*.

A spore suspension of *A. niger* (0.5ml containing 1.5×10^7 spores ml^{-1}) was added to Czapek Dox liquid medium (100ml) in Erlenmeyer flasks (250ml) and allowed to grow for 4, 7 and 28 days at 25^0C and 150 r.p.m. A sample of mycelium (4g wet weight) of each age was then transferred to Erlenmeyer flasks (250ml) each containing magnetite (550mg) in sterile distilled water (100ml). The magnetite had previously been ball-milled in distilled water for two hours to provide a fine suspension and then autoclaved (120^0C for 20 minutes) before being added as a suspension to the flasks. The mycelium was then incubated with the magnetite suspension for 0.25, 1, 5 and 24 hours respectively at 25^0C and 150 r.p.m. The amount of magnetite adsorbed after each period of exposure and by each different age of mycelium was determined by filtering the whole mixture through a nylon mesh (1mm diameter mesh size) which effectively removed the fungal pellets from solution. The pellets were then washed to remove any loosely adhering magnetite and the washings added to the rest of the filtrate. This filtrate containing non-adsorbed magnetite was then filtered through pre-dried (50^0C), pre-weighed Whatman No.1 filter paper and then dried to constant weight at 50^0C. The amount of magnetite adsorbed by the mycelium was determined by subtracting the amount

of magnetite on the filter paper (ie. non-adsorbed magnetite) away from the initial amount of magnetite added.

Magnetite adsorption by *Aspergillus niger* waste mycelium grown by the surface fermentation method of citric acid production.

Surface fermentation waste (4g wet weight) was added to sterile distilled water (100ml) containing sterilized magnetite (550mg) and shaken for 0.25, 1, 5 and 24h at 25^0C and 150 r.p.m. The amount of magnetite adsorbed was determined as above.

Magnetic removal of homogenized laboratory grown mycelium and homogenized fermentation waste from solution.

Surface and deep fermentation waste, 4d and 28d old laboratory grown *A niger* were homogenized in sterile distilled water using an MSE Atomix Omnimixer at half speed for 5 minutes. The mycelium was harvested by centrifugation at 4000 r.p.m. for 10 minutes, resuspended in distilled water and centrifuged again. This washing process was repeated a further 3 times. A sample (3g wet weight) of each different type of mycelium was added to sterile distilled water (100ml) in Erlenmeyer flasks (250ml) containing magnetite (350mg). The flasks were then shaken in triplicate at 150 r.p.m. for 24h at 25^0C. Mycelium which had adsorbed magnetite was removed by placing a strong bar magnet at the side of the flask. The amount of mycelium which did not adsorb magnetite was then

determined by pouring the rest of the filtrate onto a pre-dried (50^0C), pre-weighed Whatman No.1 filter paper and drying at 50^0C to constant weight.

Effect of pH and temperature on magnetite adsorption by laboratory grown *A. niger*,

Biomass production:

A spore suspension (0.5ml containing 1.5×10^7 spores ml^{-1}) of *A. niger* was added to Czapek Dox liquid medium (100ml) in a Erlenmeyer flask (250ml). The flasks were shaken at 25^0C for four days at 150 r.p.m. Pellets of approximately 3mm diameter were produced and these were collected by filtration through sterilized nylon mesh (mesh diameter : 1mm) filters and washed with sterilized distilled water. Mycelium (2g fresh weight) was then transferred to sterile distilled water (100ml) amended with sterile magnetite (350mg) and the flasks were shaken in triplicate at 4, 20, 25, 30 and 37^0C for 24 hours at 150 r.p.m. Before the addition of mycelium all flasks were incubated for four hours to adjust flask contents to the experimental temperatures used.

Also mycelium (2g fresh weight) was added to sterile solutions (100ml) of pH values 1, 3, 5, 7, 9 and 11, amended with 350mg magnetite. Solution pH was adjusted by adding either H_2SO_4 or NaOH to sterile distilled water. Flasks were incubated in triplicate at 25^0C at 150 r.p.m. for 24 hours. Magnetite adsorbed at different temperatures and pH values was determined as above by removing the mycelial pellets and calculating the amount of magnetite present in the filtrate.

Effect of formaldehyde treatment on magnetite adsorption by *A. niger*,

Seven day old *A. niger* biomass (5g wet weight) produced as described above was transferred to either sterile distilled water (100ml) or to 10% (w/v) formaldehyde solution (100ml). Both solutions contained magnetite (550mg) and were contained in Erlenmeyer flasks (250ml). The flasks were shaken in triplicate at 25^0C for 24 hours at 150 r.p.m. Magnetite adsorbed by the mycelium from the different treatments was determined by filtration and appropriate subtraction.

Effect of protease treatment on magnetite uptake by *A. niger*.

Seven day old *A. niger* produced as above was filtered from the growth medium using sterilized nylon mesh (mesh diameter : 1mm) filters and washed extensively with sterile distilled water. Samples of mycelium (5g wet weight) were added to a sterile Tris buffer solution (30ml, pH 7.5) containing either active protease (3mg) or autoclaved (inactive) enzyme (3mg). The mixtures were shaken at 37^0C for 48 hours at 100 r.p.m. The pellets were then filtered using nylon mesh and after washing with sterile distilled water were added (5g wet weight) to sterile distilled water (100ml) amended with magnetite (550mg). The amount of magnetite adsorbed by active and inactive protease treated mycelium was then determined by filtration and subtraction.

Desorption of magnetite from surface fermentation waste.

The mycelial waste was first washed thoroughly using sterile distilled water to prevent contamination during the experiment. Mycelium (5g fresh weight) was then added to sterile distilled water (100ml) amended with magnetite (450mg). This mixture was incubated at 25^0C for 24 hours at 150 r.p.m., filtered as previously described and the amount of magnetite adsorbed determined. The waste on which magnetite was adsorbed was then added to either sterilized distilled water (100ml) or sterile solutions (100ml) of Na_2CO_3 (0.1M), $CaCl_2$ (0.1M), EDTA (0.1M), H_2SO_4 (0.5M) or NaOH (0.5M) in Erlenmeyer flasks (250ml). The flasks were then shaken in triplicate at 25^0C for 24 hours at 150 r.p.m. and the contents filtered to determine the amount of magnetite desorbed by incubation in the various solutions. This adsorption/desorption step was then repeated followed by a final adsorption step.

Desorption of magnetite from laboratory grown *A. niger.*

Four day old mycelium (5g fresh weight) grown as described previously was transferred into sterile distilled water (100ml) containing magnetite (450mg) in Erlenmeyer flasks (250ml) and shaken for either 24 hours or 4 hours at 25^0C and 150 r.p.m. After these times mycelium was removed from the flasks and treated as described above in the desorption of magnetite from surface fermentation waste. However, the mycelium was only subjected to one desorption step and to one further adsorption step.

Adsorption of magnetite by *Penicillium chrysogenum* biomass.

A spore suspension (0.5ml containing 1.33×10^7 spores ml^{-1}) obtained from a two week old culture of *P. chrysogenum*, was added to Czapek Dox liquid medium

(100ml) in Erlenmeyer flasks (250ml). After seven days the mycelial pellets were removed from solution using nylon mesh filters and mycelium (4g fresh weight) transferred to sterile distilled water (100ml) amended with magnetite (550mg) in Erlenmeyer flasks (250ml). The flasks were incubated in triplicate at 25^0C for 24 hours at 150 r.p.m. After this time the amount of magnetite adsorbed was determined as previously described.

The effect of magnetite on the growth of *P. chrysogenum*.

The above spore suspension (0.5ml) was also added to either Czapek Dox liquid medium alone (100ml) or to Czapek Dox liquid medium (100ml) containing magnetite (100mg). All media were contained in Erlenmeyer flasks (250ml) and shaken at 25^0C for 4 days at 150 r.p.m. After this time the amount of biomass produced with and without magnetite, the amount of magnetite adsorbed and the pH of the culture filtrates were determined. Also fungal pellets grown in Czapek Dox liquid medium containing magnetite were transferred into fresh growth medium and grown for a further 4 days in the absence of magnetite. The pellets magnetic properties were determined after 2 and 4 days growth in this medium by subjecting them to an external magnetic field.

Scanning electron microscope studies were made of the surface and interior of 4 day old fungal pellets grown in the presence of magnetite.

Magnetite adsorption by *Aspergillus flavus*.

A spore suspension(0.5ml containing 1.2×10^7spores ml^{-1}) of *A. flavus* was added to of Czapek Dox liquid medium (100ml) in an Erlenmeyer flask (250ml) and incubated at 25^0C for 4 days at 150 r.p.m. The fungal pellets were removed from the

growth medium as described previously and mycelium (4g fresh weight) added to sterile distilled water (100ml) containing magnetite (550mg). The mixture was then shaken at 25^0C for 24 hours at 150 r.p.m. The amount of magnetite adsorbed was determined as previously described.

Adsorption of magnetite by bacteria, yeast and algae.

The ability of magnetite to remove the following microorganisms from solution was studied:

(1) *Escherichia coli* (6 hour old culture grown in nutrient broth)

(2) *Dunaliella salina* (2 week old culture grown in medium described by Hajibagheri *et al.*, (1986).

(3) *Saccharomyces cerevisiae* (4 day old culture grown in YEPD medium).

Magnetite (0.5ml of a 350mg/ml sterile suspension) was added to each organism in their individual growth media (100ml) and the flasks shaken at 25^0C for 24 hours at 150 r.p.m. To determine the ability of the biomass to adsorb magnetite a strong permanent magnet was placed against the side of the flask at hourly intervals over an 8 hour period and then again after 24 hours.

SURFACE IMMOBILIZATION OF FUNGI.

Ability of fungi to grow on thread.

Spore suspensions (1.0ml containing 1.5 x 10^7 spores ml^{-1}) of *Aspergillus niger*, *Aspergillus flavus* and *Mucor flavus* were added to Czapek Dox medium (100ml) in Erlenmeyer flasks (250ml). All the flasks were fitted with a length of cotton thread, such that the cotton wool bung supported one end of the thread while the other end dipped into the growth medium. Flasks were shaken (25^0C, 150 r.p.m.) for 4 days to allow fungal growth to occur. After this time sterile zinc dust (0.1g) was added to the flasks containing *A. niger* and *M. flavus* to check zinc adsorption by the immobilized fungi. Photographs were taken of fungal growth on thread.

Specific attachment of various fungi to a nylon mesh carrier material.

Spore suspensions of *Aspergillus niger*, *Mucor flavus*, *Penicillium chrysogenum*, *Thermomucor indicae-seudaticae* and *Neurospora crassa* were prepared and all adjusted to a final concentration of 1.5 x 10^7 spores ml^{-1}. Spore determinations were made using a Helber slide counting chamber. Varying amounts (0.1, 0.25, 0.5 and 1.0ml) of each fungal spore suspension were added to Czapek Dox medium (100ml) containing a preweighed, sterilized square of nylon mesh (3cm^2, 1mm diameter mesh). Flasks were shaken (25^0C, 150 r.p.m.) for 5 days after which time the nylon mesh plus adsorbed mycelium was removed from the flask, dried (50^0C) and weighed. The unadsorbed biomass was filtered through a pre-dried, pre-weighed Whatman No.1 filter paper, dried (50^0C) and also weighed. The specific attachment of each fungus was determined by dividing the amount of biomass attached to the mesh by the total amount of biomass produced. *Thermomucor indicae-seudaticae* was treated in the same manner as the other fungi except that it was grown in malt extract broth (100ml) at 37^0C.

Effect of growth on synthetic foam on acid production and thiosulphate oxidation by *A. niger*.

A spore suspension (1×10^7 spores ml^{-1}, 0.5ml) of *A. niger* was added to either Czapek Dox medium (100ml) alone or Czapek Dox medium (100ml) containing synthetic foam pieces (either 10 x 8 x 8mm or 5 x 5 x 5mm in size). The flasks also contained sodium thiosulphate (500µg $S_2O_3^{2-}$ ml^{-1}). Control flasks were set up to determine the natural oxidation rates of thiosulphate with and without the presence of foam pieces. Flasks were shaken (25^0C, 150 r.p.m.) for either 10 and 20 days or 6 and 20 days (depending on the size of foam pieces used) after which times the sulphate content of the flask filtrates were tested turbidimetrically for sulphate content (Hesse, 1971) as was pH. (See Appendix)

RESULTS AND DISCUSSION.

MAGNETITE ADSORPTION BY FUNGI.

Effect of length of exposure to magnetite and age of mycelium on magnetite uptake by laboratory grown *A. niger*.

Young fungal mycelium was found to be far more efficient at adsorbing magnetite than old mycelium (Fig.3.1). The decrease in adsorption with age probably relates to a change in fungal cell wall structure, since melanization of fungal walls occurs with age, a process which probably causes a reduction in adsorption sites on the cell wall. The 28 day old pellets also exhibited differential adsorption abilities, i.e. certain parts of the fungus were able to adsorb magnetite equally effectively whether old or young, while other parts were not. The amount of magnetite adsorbed was found to increase with increasing length of exposure to magnetite (Fig. 3.1).

Magnetite adsorption by surface fermentation waste.

Mycelial waste adsorbed magnetite from solution but not as effectively as did laboratory grown mycelium (Fig. 3.1). The amount adsorbed again increased with length of exposure. In this experiment the citric acid fermentation waste was added in an unbroken mass, so the fact that it adsorbed less magnetite than the laboratory-grown mycelium was probably due to its' smaller surface area..

* The greater adsorptive capacity of the homogenized mycelium allowed much greater removal of mycelium from the medium when it was subjected to a magnetic field (Fig. 3.2).

Magnetic removal of homogenized laboratory grown mycelium and homogenized fermentation waste from solution.

Homogenization of surface fermentation waste greatly improved its' adsorptive capacity, allowing it to remove almost as much ~~myceliun~~ magnetite from solution as did laboratory grown mycelium.* In a separate experiment carried out using exactly the same methods, 52% of the mycelial waste produced from the deep fermentation production of citric acid was also removed from solution showing that this particular type of mycelium was also capable of magnetite adsorption. Homogenization of the surface fermentation waste presumably increased the amount of surface area available for adsorption, hence increasing its adsorptive capacity. This finding has important industrial and environmental implications, since citric acid waste mycelium is homogenized prior to its' disposal into rivers. It is therefore commercially available in a form which is efficient at adsorbing particulates, either from river water or when applied to proposed biotechnological processes.

Effect of pH and temperature on magnetite adsorption by laboratory grown *A. niger*.

A. niger adsorbed magnetite over a wide range of pH values (Fig. 3.3), adsorption being constant from pH 3 to pH 9. However, at pH values of 1 and 11 magnetite adsorption decreased dramatically. The decrease in adsorption at these pH values is probably related to differences in surface electrostatic charges and chemical alteration or removal of cell wall components involved the adsorption process. These results are similar to effects of pH on magnetite adsorption observed in other microorganisms. Bacterial adsorption to magnetite decreased at pH 11

(Macrae & Evans, 1983) and the removal of algae from lakewater also decreased with increasing pH (Bitton *et al.*, 1975). The authors of both papers do not however, discuss the effect of very low pH values on microbial attachment to magnetite. Adsorption of the bacterium, *Pseudomonas ovalis*, to an anion exchange resin was also found to be dependent on pH. At pH values below 3 very little adsorption occurred, while at pH 3.2, maximum adsorption was observed. Above pH values of 3.2 adsorption decreased to a certain level, after which the amount of bacterial adhesion to the resin remained relatively constant.

Microorganisms are generally assumed to have an overall negative surface charge. A study by Mozes *et al.*, (1987) demonstrated that three organisms *Moniella pollinis*, *Saccharomyces cerevisiae* and *Acetobacter aceti* all had negatively charged surface at pH values ranging from around 3.5 to 7. In the same paper, the fungus *M. pollinis* was shown to have an isoelectric point of pH 3.1. It can be reasonably assumed therefore, that *A. niger* will have similar surface characteristics to this fungus and will therefore exhibit different surface charges at different pH values. As organisms possess a positive charge below their isoelectric point (Atkinson & Fowler, 1974) *A. niger* will have a positive surface charge at pH 1 and therefore magnetite should not adsorb to the hyphal surface as it too will be positively charged at this pH. Similarly, at pH values of above 8.5, both the surface of magnetite and *A. niger* will be negatively charged. This again suggests that because of electrostatic repulsion magnetite adsorption will not occur at high pH values. My results would appear to substantiate this generalization. Also, as was reported earlier in this thesis, alkali washing decreased particulate adsorption by *N. crassa* and the effect correlated to a decrease in cell wall protein content. A high pH could have a similar effect on the cell wall of *A. niger* bringing about a decrease in magnetite adsorption.

Temperature had a profound effect on the ability of *A. niger* to adsorb magnetite (Fig. 3.4). Adsorption was optimal at 30^0C and decreased at

temperatures above and below this value, as was observed for the adsorption of elemental sulphur by *M. flavus* (Chapter 1). The explanations given in Chapter 1 for the effect of temperature on particulate adsorption apply here also. The decrease in adsorption at high and low temperatures was not due to different pH values of the culture medium as medium pH only varied slightly and is constant over the range of values observed in the experiment (Fig. 3.3).

Effect of formaldehyde treatment on magnetite adsorption by *A. niger*.

Formaldehyde decreased magnetite adsorption by *A. niger* (Fig. 3.5). Formaldehyde is used as a chemical fixative and is known to interact with proteins in the cell wall of microorganisms (Beveridge *et al.*, 1978). The binding of formaldehyde to cell walls is thought to suppress positive charges and can increase the amounts of metal cation bound to the cell wall (Strandberg *et al.*, 1981). Since magnetite has a positive surface charge, and formaldehyde appears to increase the overall negative charge on the mycelial surface, then formaldehyde treatment would be expected to increase adhesion. As mentioned previously the opposite result was obtained. However, work by Pumpel & Schinner (1986) on silver accumulation by a hyphomycete found that dead mycelium, killed using formaldehyde, accumulated approximately the same quantities of silver as live, untreated mycelium. No increase in metal accumulation was observed and so the work demonstrated that formaldehyde has varying effects on metal accumulation by fungi. In the reported work, the fungal mycelium is first killed using formaldehyde and then metal uptake is measured in a formaldehyde free solution. In my research, magnetite uptake was studied in the presence of formaldehyde which could have effected the adsorption process, due to the low pH of the formaldehyde solution. In addition the fungal

Figure 3.1.

Effect of length of exposure to magnetite and age of mycelium on magnetite uptake by laboratory grown *Aspergillus niger*. Effect of length of exposure to magnetite on magnetite uptake by surface produced citric acid fermentation waste.

(Means of triplicates $^{+}/_{-}$ S.D.)

●—● Four day old *A. niger* (laboratory produced)

△—△ Seven day old *A. niger* (laboratory produced)

▲—▲ Twenty eight day old *A. niger* (laboratory produced)

■—■ Surface produced fermentation waste

Figure 3.2.

Removal of different types of homogenized mycelium from suspension by means of magnetic attraction after pre-incubation of the mycelium with a magnetite suspension.

(Means of triplicates $^{+}/_{-}$ S.D.)

a : Four day old *A. niger* (laboratory produced)

b : Twenty eight day old *A. niger* (laboratory produced)

c : Surfaced produced fermentation waste

Fig.3.1.

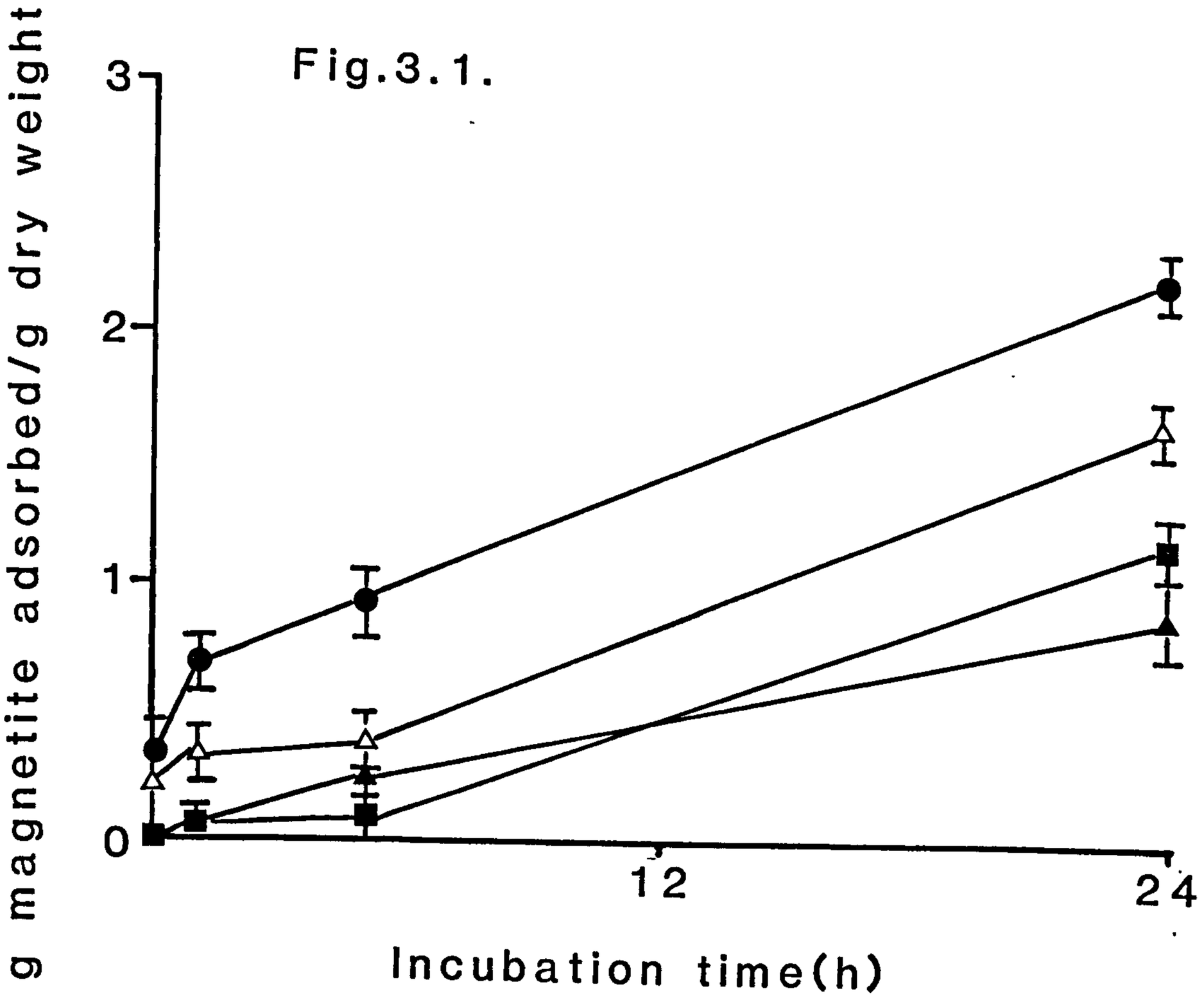

g magnetite adsorbed/g dry weight
3
2
1
0
12
24
Incubation time(h)

Fig.3.2.

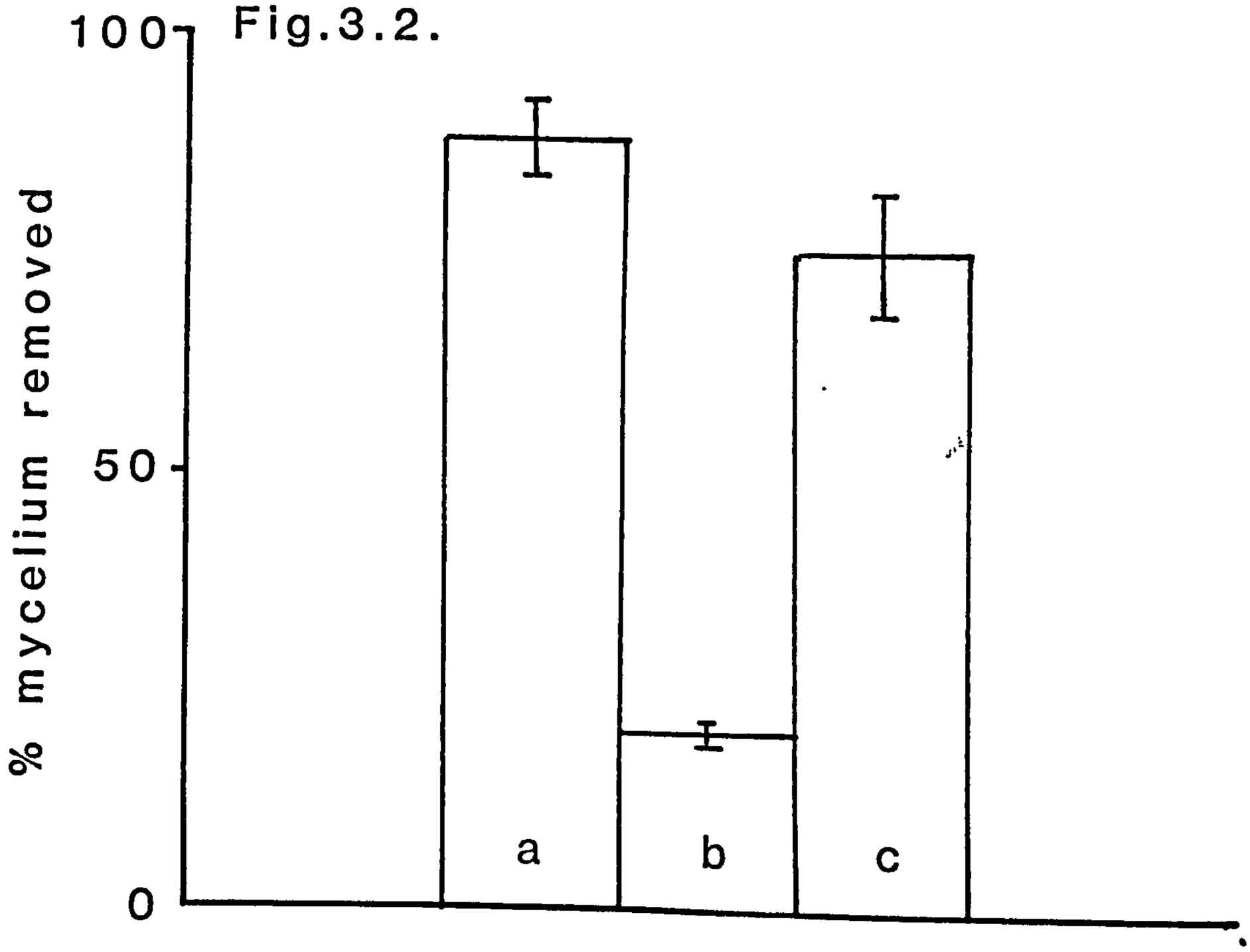

% mycelium removed
100
50
0
a
b
c

Figure 3.3.

Effect of pH on magnetite adsorption by laboratory produced *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * Significant decrease in mmagnetite adsorption compared with the value obtained at pH 7, $p < 0.05$)

Figure 3.4.

Effect of temperature on magnetite adsorption by laboratory produced *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in magnetite adsorption compared to the value obtained at 25^{0}C, $p < 0.05$)

Figure 3.3.

Effect of pH on magnetite adsorption by laboratory produced *Aspergillus niger.*

(Means of triplicates $^{+}/_{-}$ S.D. * Significant decrease in mmagnetite adsorption compared with the value obtained at pH 7, $p < 0.05$)

Figure 3.4.

Effect of temperature on magnetite adsorption by laboratory produced *Aspergillus niger.*

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in magnetite adsorption compared to the value obtained at 25^{0}C, $p < 0.05$)

Fig.3.3.

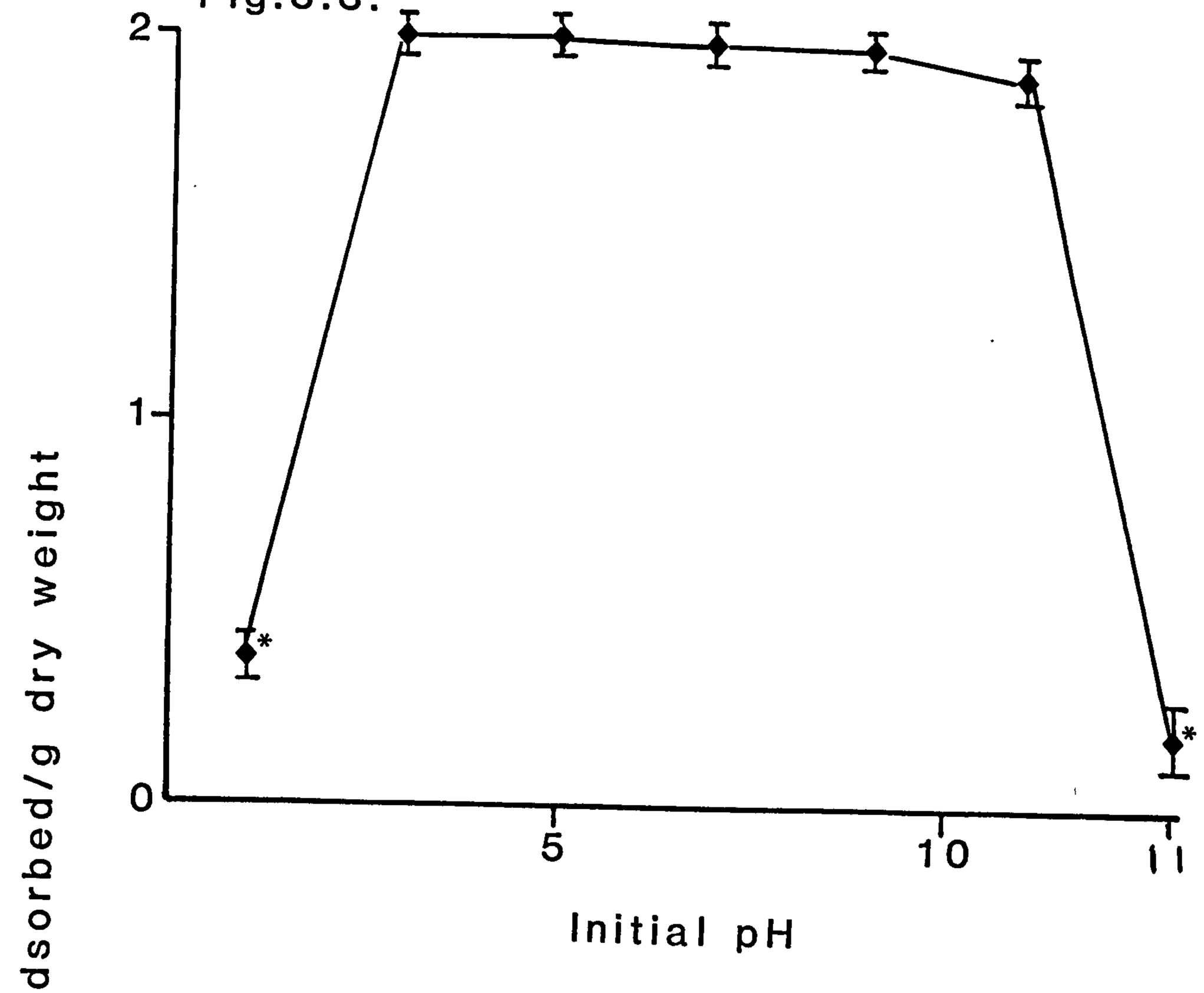
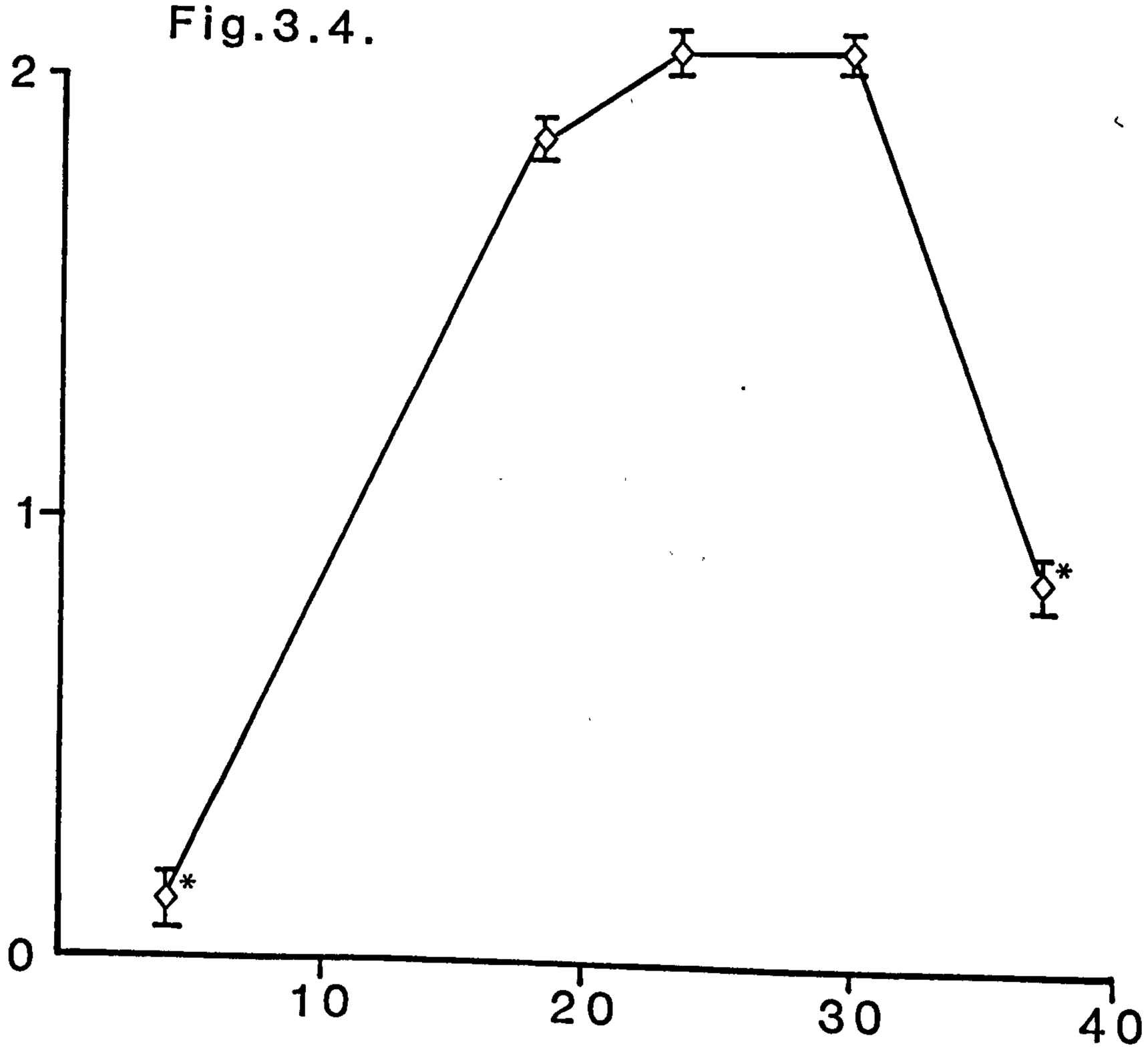

2
1
0
5
10
11
Initial pH
g magnetite adsorbed/g dry weight

Fig.3.4.


2
1
0
10
20
30
40
Temperature (°C)

pellets were observed to "flatten" when incubated with formaldehyde, an effect which might have reduced the surface area of treated mycelium available for magnetite adsorption.

Effect of protease treatment on magnetite adsorption by *A. niger*.

It was noticed that in standing culture fungi tended to exhibit a high degree of particulate adsorption at the hyphal tips. Hyphal tips are thought to consist mainly of protein and previous results in cell wall analysis (Chapter 1) indicate that cell wall proteins play a role in particulate adsorption. Protease treated mycelium should therefore adsorb less particulate matter than untreated mycelium due to the degradation of these proteins. However, both control mycelium, incubated in buffer with inactive enzyme, and test mycelium, incubated in buffer with active enzyme, adsorbed magnetite to the same extent (Fig. 3.6). Also, the amount of magnetite adsorbed by the control and test mycelium was significantly less than that adsorbed by the same amount and age of mycelium grown in Czapek Dox medium alone (Fig. 3.6).

Incubation at 37^0C could have had an effect on wall structure as pellets treated in this way exhibited a flattening similar to that noticed in the presence of formaldehyde. As mentioned previously this effect would decrease the amount of cell wall surface available for adsorption. The higher temperatures could also have activated wall-degrading enzymes in the control mycelium which may have brought about a reduction in some of the the wall components necessary for adsorption. The results are inconclusive as to the contribution of cell wall protein to magnetite adsorption. However, it is probable that the outer glucan layer is able to adsorb magnetite and also that the wall protein, if exposed at the hyphal tips for example, is

Figure 3.5.

Effect of formaldehyde treatment on magnetite adsorption by *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in magnetite adsorption compared with the untreated control, $p < 0.05$)

Control : Untreated mycelium

Test : Formaldehyde treated mycelium

Figure 3.6.

Effect of protease treatment on magnetite adsorption by *Aspergillus niger*.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in magnetite adsorption compared with untreated mycelium, $p < 0.05$)

a : Untreated mycelium

b : Mycelium incubated with autoclaved (inactive) protease

c : Mycelium incubated with active protease

Fig.3.5.

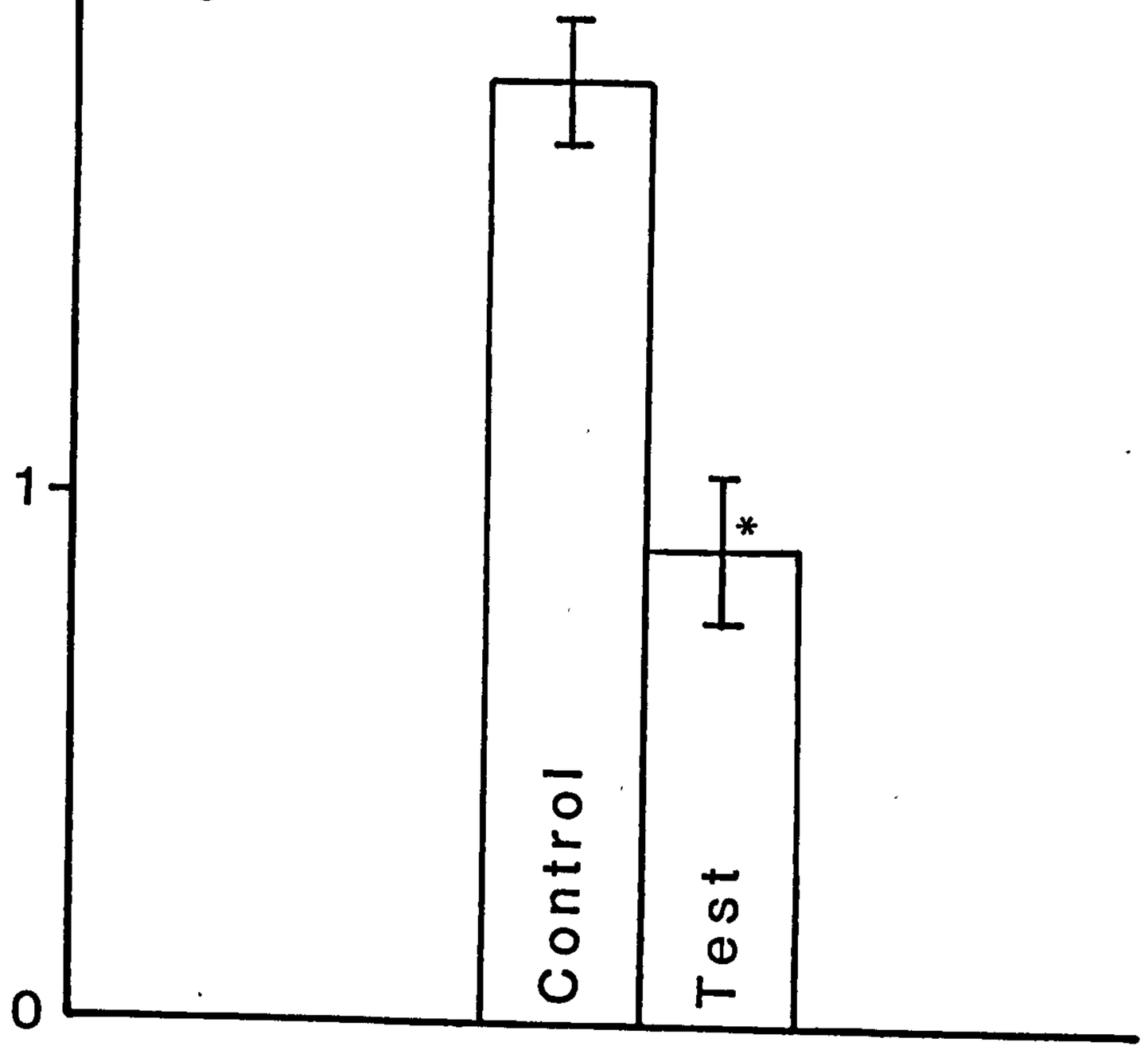
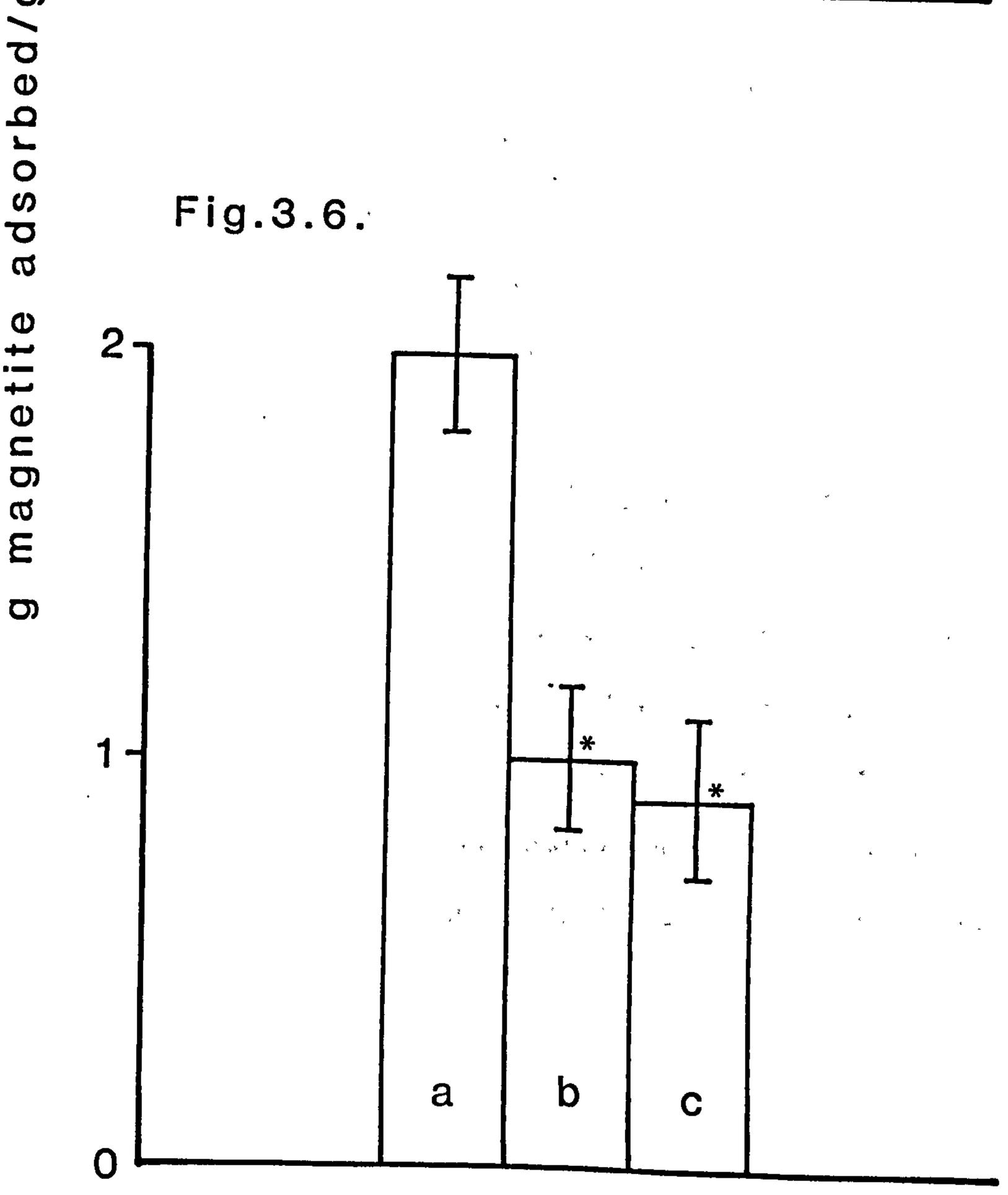


Fig.3.6.



capable of adsorption, a view that is confirmed by the finding that both proteins and polysaccharides can bind to magnetite. (Horisburger, 1976).

Desorption of magnetite from surface fermentation waste.

Dilute solutions of Na_2CO_3, H_2SO_4 and NaOH proved the most effective desorbents of magnetite from the mycelial surface with up to 70% of adsorbed magnetite being removed by NaOH (Fig. 3.7). The other solutions used did not remove any more magnetite from the waste than did distilled water (25% removal). When the mycelium was subjected to a second adsorption a significant increase in magnetite uptake was noticed with waste (Fig. 3.8) after desorption with solutions of H_2O, Na_2CO_3, $CaCl_2$ and EDTA (compared with the first adsorption). Mycelium previously desorbed with H_2SO_4 and NaOH exhibited a decreased ability to adsorb magnetite (Fig. 3.8). The second desorption caused a magnetite removal of around 40% with all the solutions used (Fig. 3.7). In the case of H_2O, Na_2CO_3, $CaCl_2$ and EDTA a final adsorption step resulted in mycelia having an adsorption ability equivalent to that of untreated mycelium (Fig. 3.8). Mycelium treated with H_2SO_4 and NaOH showed a further decrease in magnetite adsorption with the NaOH treated mycelium exhibiting the least adsorption (7%).

Sulphuric acid and NaOH probably decreased magnetite adsorption due a reduction in wall components responsible for the adsorption phenomenon, and perhaps due to the carry-over of acidity or alkalinity from the desorption to adsorption tests. Low or high pH values decrease magnetite adsorption and also seem to be able to cause removal of adsorbed magnetite from the surface of fungal mycelium.

Table 3.3 shows the total amount of magnetite adsorbed by the fermentation waste over the three adsorption steps. Sulphuric acid and NaOH significantly decreased the total adsorbed compared with the other solutions.

Table 3.4 shows that the total amount of magnetite desorbed by all the various treatments was around the same value with the exception of Na_2CO_3 which desorbed a significantly larger amount of magnetite from the mycelial surface than the other solutions tested. The data in Table 3.4 also shows that the percentage of magnetite desorbed after two adsorption steps was greatest with Na_2CO_3 and NaOH.

Desorption of magnetite from laboratory grown *A. niger*.

After 24 hours incubation in magnetite only a trace amount of magnetite was desorbed from the surface of *A. niger* by all the solutions tested for their desorption abilities (Fig. 3.9). This appears to suggest that magnetite was strongly bound to the fungus, a process which seems to have become irreversible. After 4 hours incubation in magnetite solution a much higher percentage of magnetite was removed from the mycelium by the desorption solutions, the best desorbants being the solutions H_2SO_4 and NaOH (Fig. 3.10). However the amount of magnetite desorbed was still much lower than previously obtained with the surface fermentation waste. Even though not all the magnetite had been desorbed from the mycelial surface (fungal pellets were still black in colour), when subjected to a second adsorption step the fungus exhibited a remarkable ability to adsorb further magnetite particles to it's surface (Fig. 3.11). This means that magnetite on the fungal surface was able to bind other magnetite particles, perhaps because the fungus was able to change the surface properties of bound magnetite in some way,

Figure 3.7.

Desorption of magnetite from *Aspergillus niger* waste mycelium obtained from the surface fermentation method of citric acid production.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in magnetite desorption compared with the amount desorbed using distilled water, $p < 0.05$)

Desorption solutions

1 : H_2O

2 : 0.1M Na_2CO_3

3 : 0.1M $CaCl_2$

4 : 0.1M EDTA

5 : 0.1M H_2SO_4

6 : 0.1M NaOH

Fig.3.7.

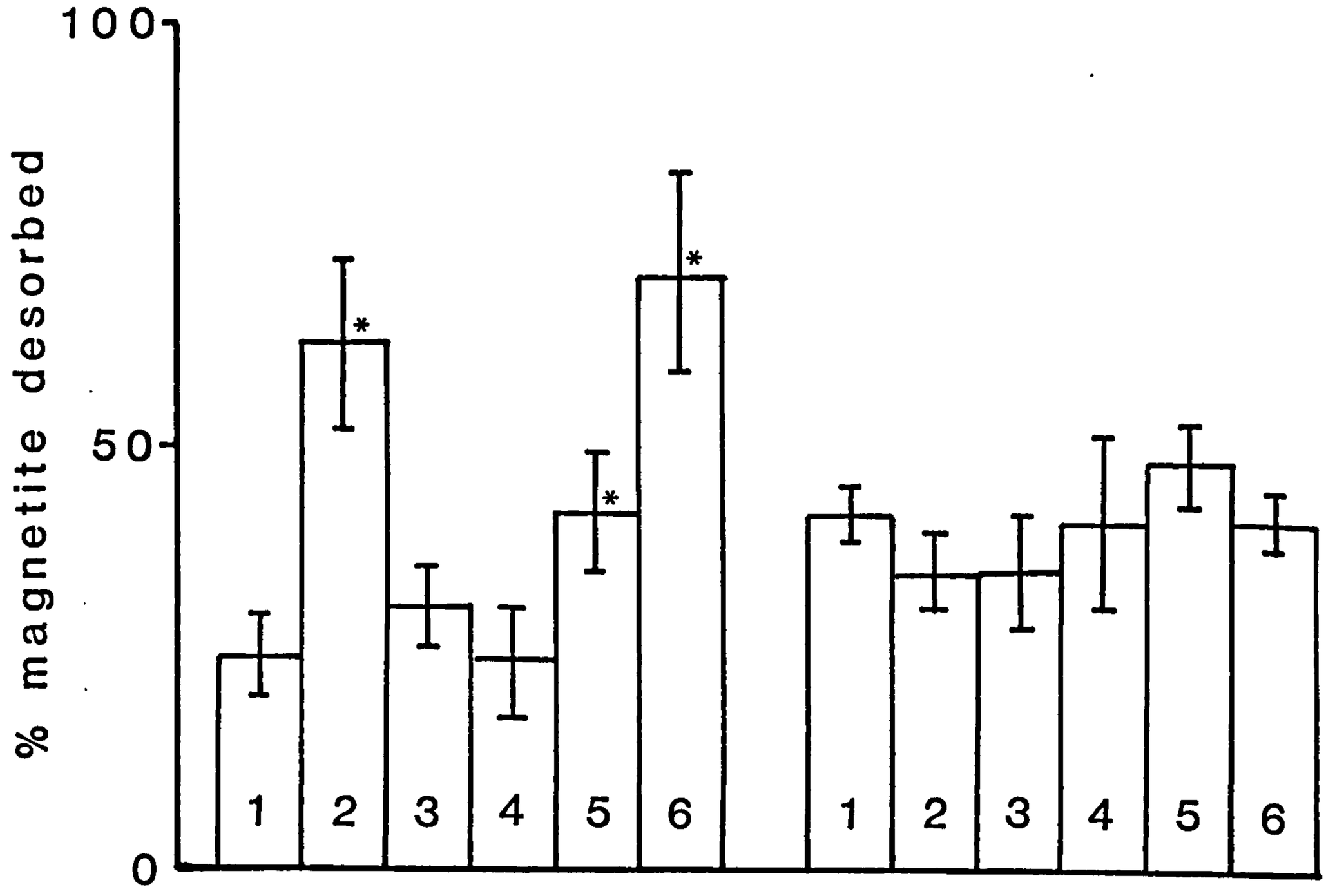

100
50
0
% magnetite desorbed
1
2
3
4
5
6
*
1st Desorption
2nd Desorption

Table 3.3 Total amount of magnetite adsorbed by Surface fermentation waste

Treatment	Magnetite adsorbed (mg)
H_2O	317.4 ± 9.6
Na_2CO_3	287.0 ± 28.5
$CaCl_2$	308.9 ± 44.9
EDTA	307.9 ± 47.6
H_2SO4	*217.1 ± 33.9
NaOH	*172.5 ± 34.9

(Means of triplicates ± S.D.
*Significant decrease in magnetite adsorption compared to control value using H_2O, $p < 0.05$).

Table 3.4 Total amount of magnetite desorbed by various treatments

Treatment	Total magnetite desorbed (mg)	% desorbed (% of 1st 2 adsorptions)
H_2O	87.3 ± 11.6	35.8 ± 4.2
Na_2CO_3	*106.9 ± 8.0	*50.0 ± 5.7
$CaCl_2$	70.5 ± 10.7	32.7 ± 5.7
EDTA	68.5 ± 14.5	33.1 ± 11.0
H_2SO4	70.3 ± 10.0	43.8 ± 7.5
NaOH	72.0 ± 9.8	*56.5 ± 10.8

(Means of triplicates ± S.D.)
(*indicates significant difference from control value using H_2O, $p < 0.05$)

Figure 3.8.

Repeated uptake of magnetite by *Aspergillus niger* surface produced fermentation waste after desorption treatment.

(Means of triplicates $^{+}/_{-}$ S.D. ** significant increase in magnetite adsorption compared with the amount adsorbed after the first adsorption step, $p < 0.05$; * significant decrease in magnetite adsorption compared with the amount adsorbed after the first adsorption step, $p < 0.05$)

1 : First adsorption step

2 : Second adsorption step (After initial desorption cycle)

3 : Third adsorption step (After second desorption cycle)

Fig.3.8.

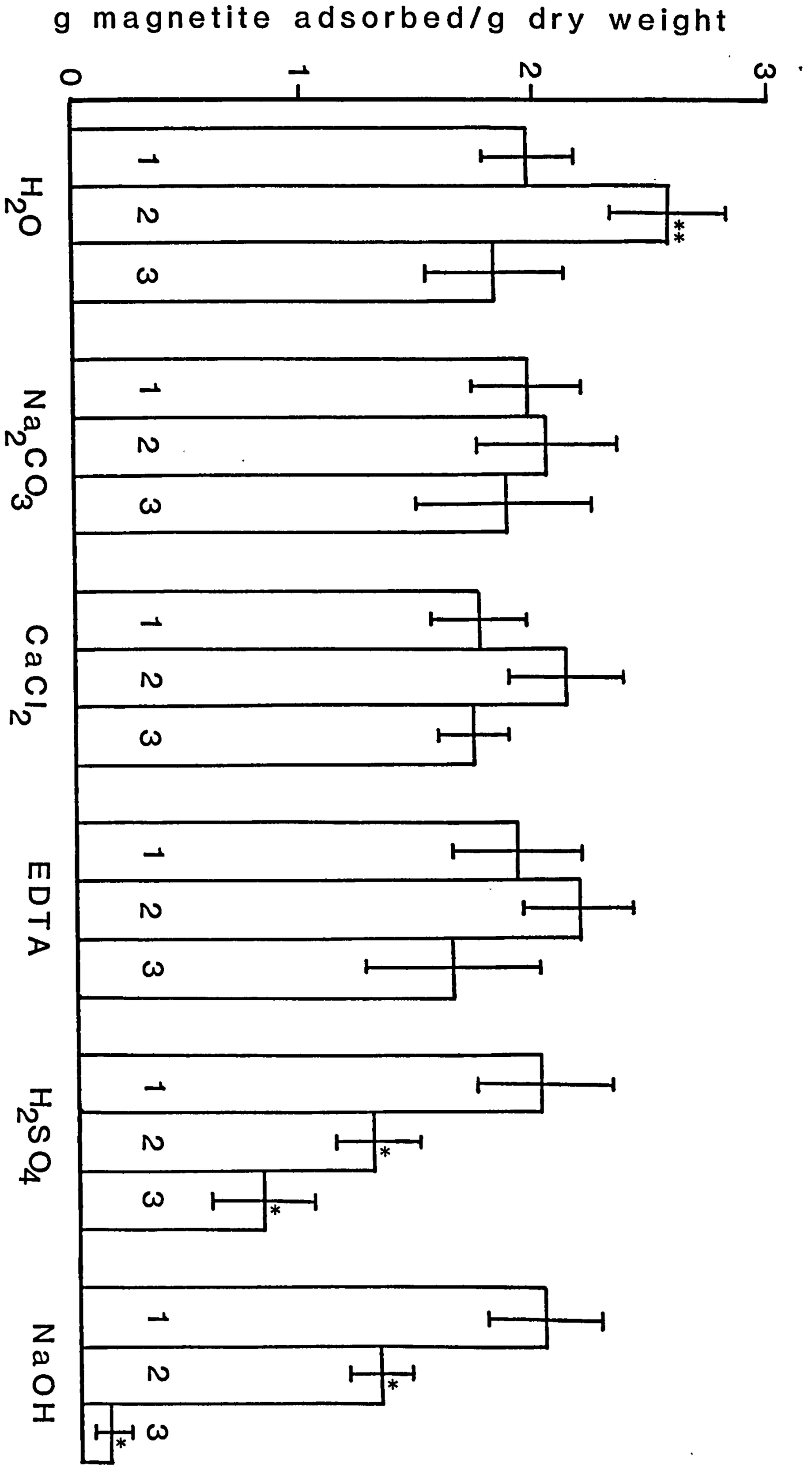

g magnetite adsorbed/g dry weight
0
1
2
3
H_2O
Na_2CO_3
$CaCl_2$
EDTA
H_2SO_4
NaOH
1
2
3
**
*

Figure 3.9.

Desorption of magnetite from laboratory produced *Aspergillus niger*. Incubated for twenty four hours with a magnetite suspension.

(Means of triplicates $^{+}/_{-}$ S.D.)

Desorbant solutions (for Figs. 3.9 & 3.10)

a : H_2O

b : Na_2CO_3 (0.1M)

c : $CaCl_2$ (0.1M)

d : EDTA (0.1M)

e : H_2SO_4 (0.5M)

f : NaOH (0.5M)

Figure 3.10.

Desorption of magnetite from laboratory produced *Aspergillus niger*. Incubated for four hours with magnetite.

(Means of triplicates $^{+}/_{-}$ S.D. * significant increase in magnetite desorbed compared with the control using distilled water as a desorbant, $p < 0.05$)

Fig.3.9.

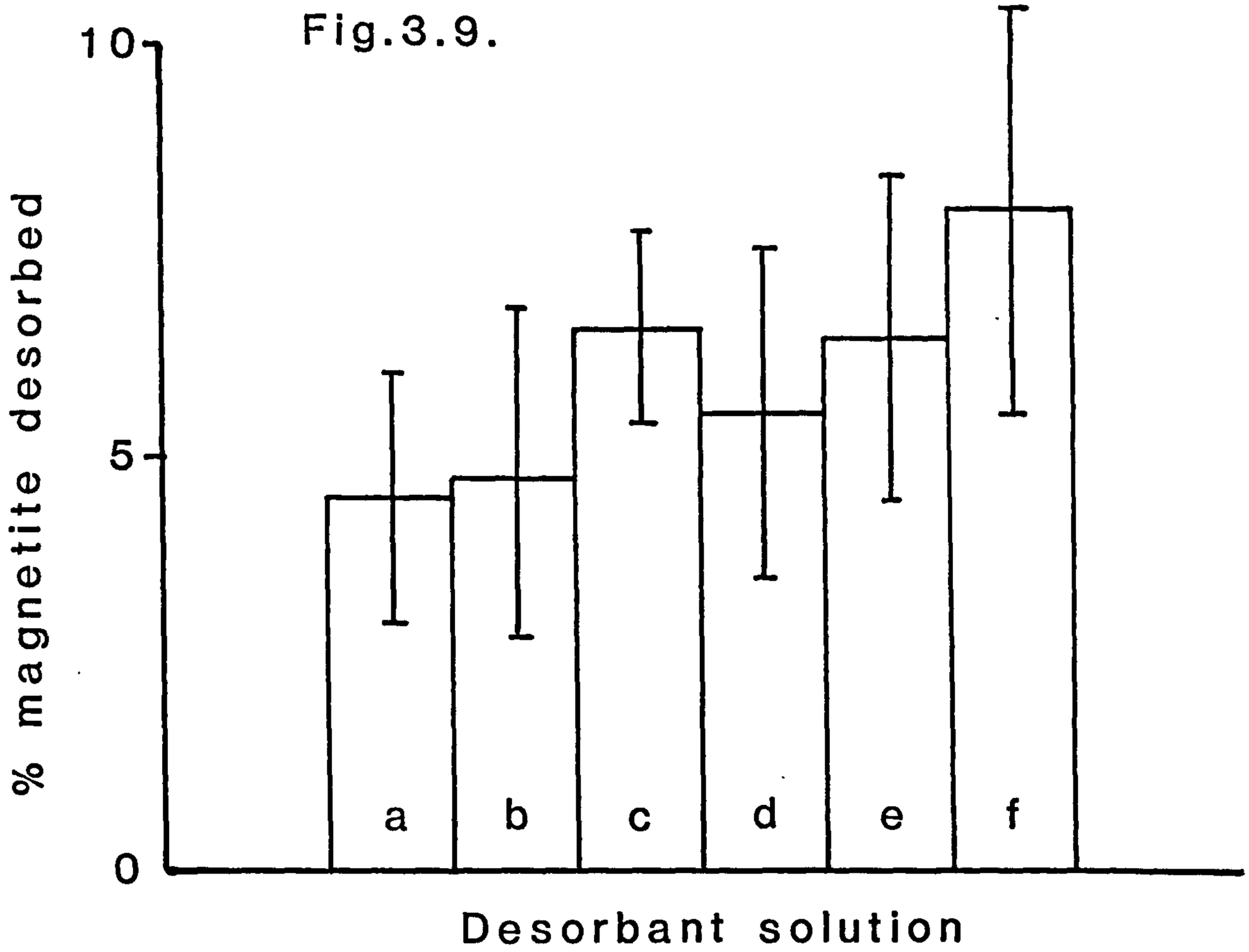


Fig.3.10.

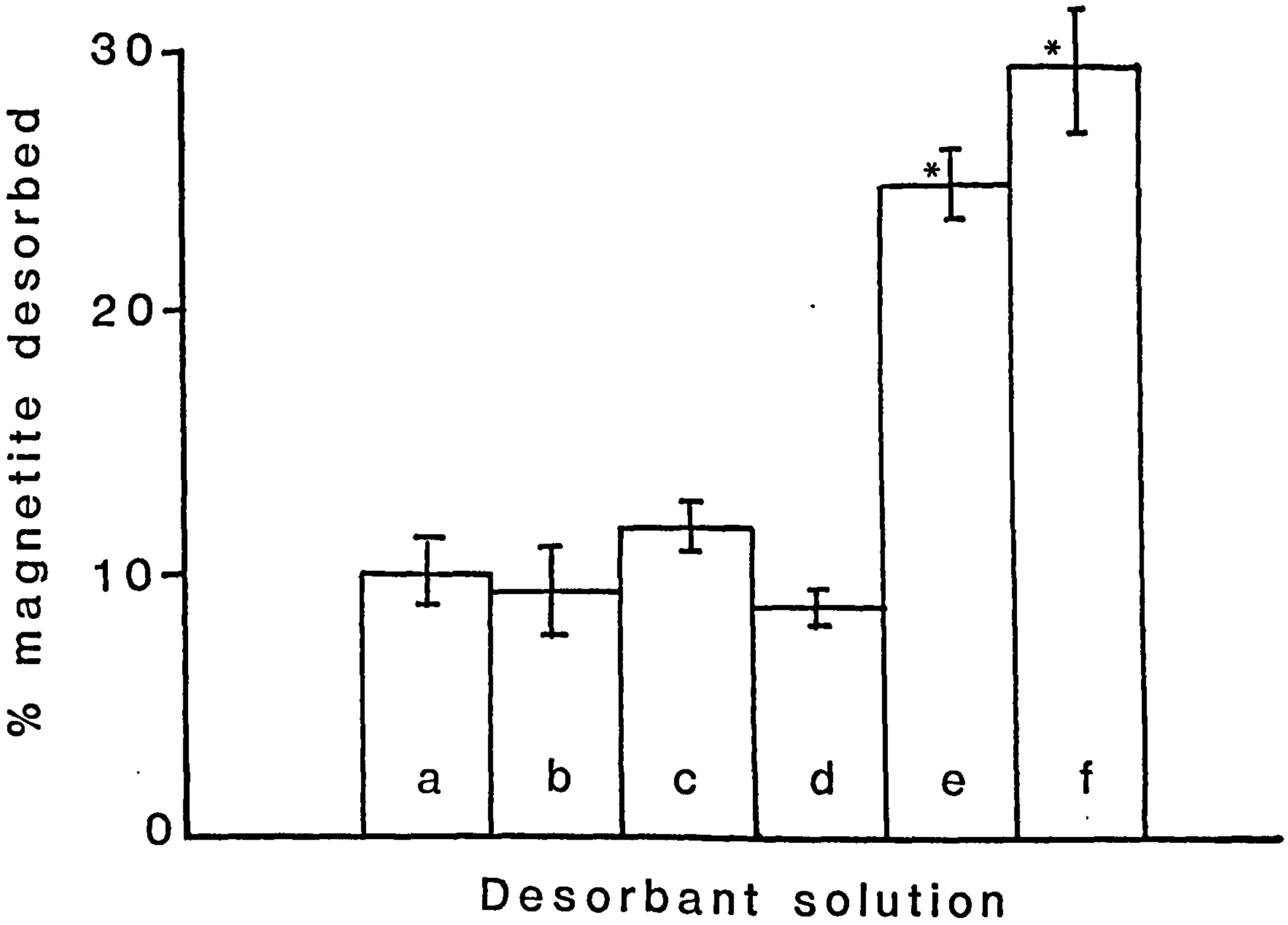


Figure 3.11.

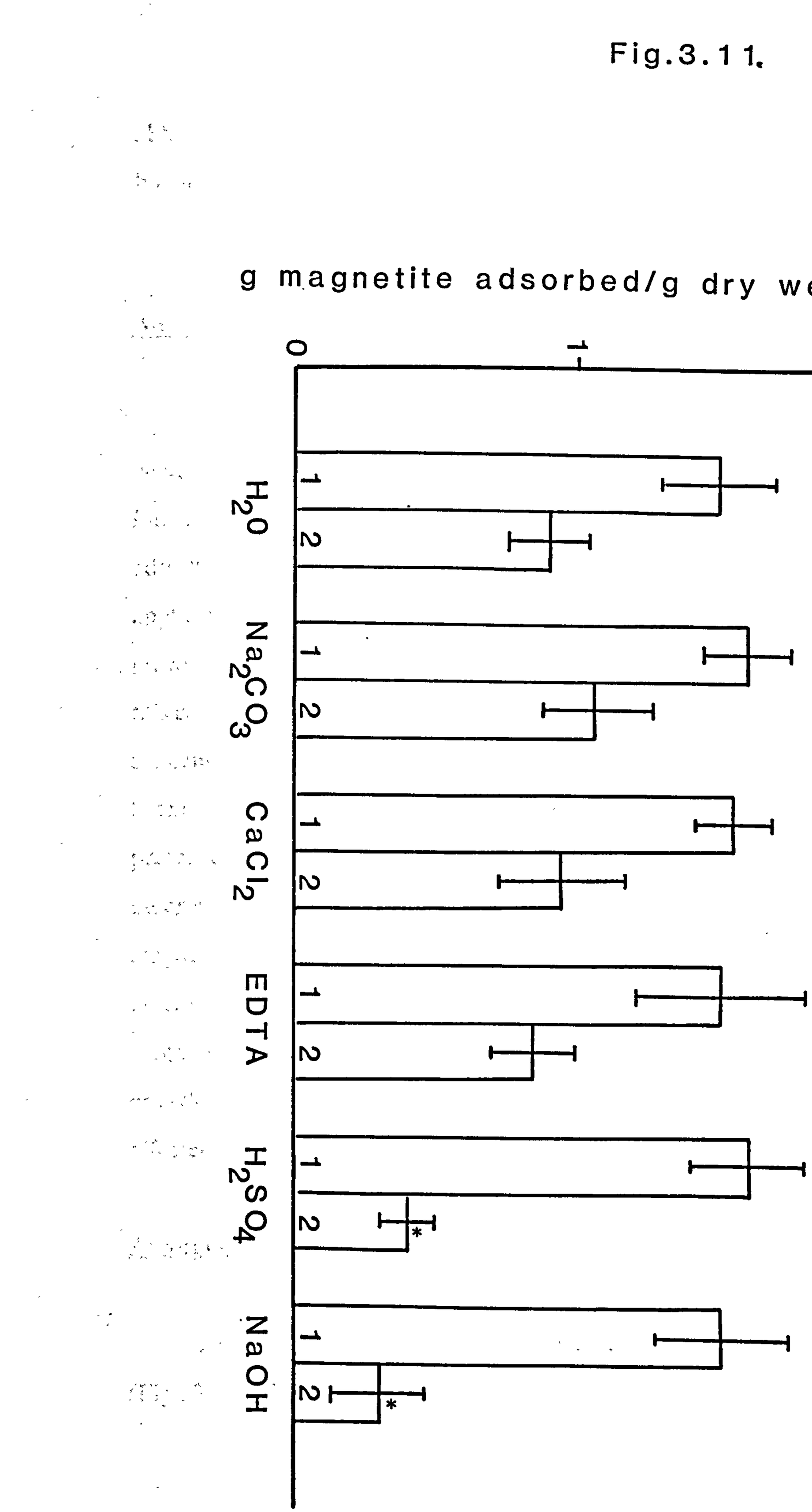
Repeated magnetite adsorption by laboratory produced *Aspergillus niger*. Four hours incubation with magnetite.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in magnetite adsorption compared with the amount adsorbed during the second adsorption step where H_2O was used as the control desorbant solution, $p < 0.05$)

1 : First adsorption step

2 : Second adsorption step

Fig.3.11.


g magnetite adsorbed/g dry weight
0
1
2
H_2O
Na_2CO_3
$CaCl_2$
EDTA
H_2SO_4
NaOH
1
2
*

although the desorption solutions may have affected the surface properties of the bound magnetite.

Adsorption of magnetite by *P. chrysogenum* biomass.

Four day old mycelium of *P. chrysogenum* showed very poor adsorption characteristics compared to the other fungi tested (Figs. 3.12 and 3.13). As the fungus is widely used in penicillin production the fact that it is inefficient at adsorbing magnetite to its surface appears to reduce the potential industrial application of this phenomenon. However, this fungus became magnetic when grown in the presence of magnetite from a spore suspension (Figs. 3.14 to 3.19). In this case, young biomass adsorbed magnetite and the fungus continued to grow, remaining magnetic by virtue of retaining magnetite at the centre of the pellet. Scanning electron micrographs of the surface of a fungal pellet grown in the presence of magnetite (Fig. 3.20) show that very little magnetite is adsorbed to the older hyphae. In contrast, the hyphae in the pellet interior exhibit a high degree of magnetite adsorption (Figs. 3.21 and 3.22). By using this approach, it should be possible to make pellets of this commercially important fungus responsive to a magnetic field. The presence of magnetite in the growth medium did not inhibit the growth of *P. chrysogenum* (Fig. 3.23). The pH of the culture filtrate was also not affected by the presence of magnetite (Fig. 3.23).

Adsorption of magnetite by *A. flavus*.

Aspergillus flavus was able to adsorb magnetite to the same extent as A. niger (Figs. 3.24 to 3.27).

Figure 3.12.

A four day old culture of *Penicillium chrysogenum* after twenty four hours incubation with a magnetite suspension.

Figure 3.13.

As Fig.3.12 but in the presence of a magnetic field.

FIG. 3.12.

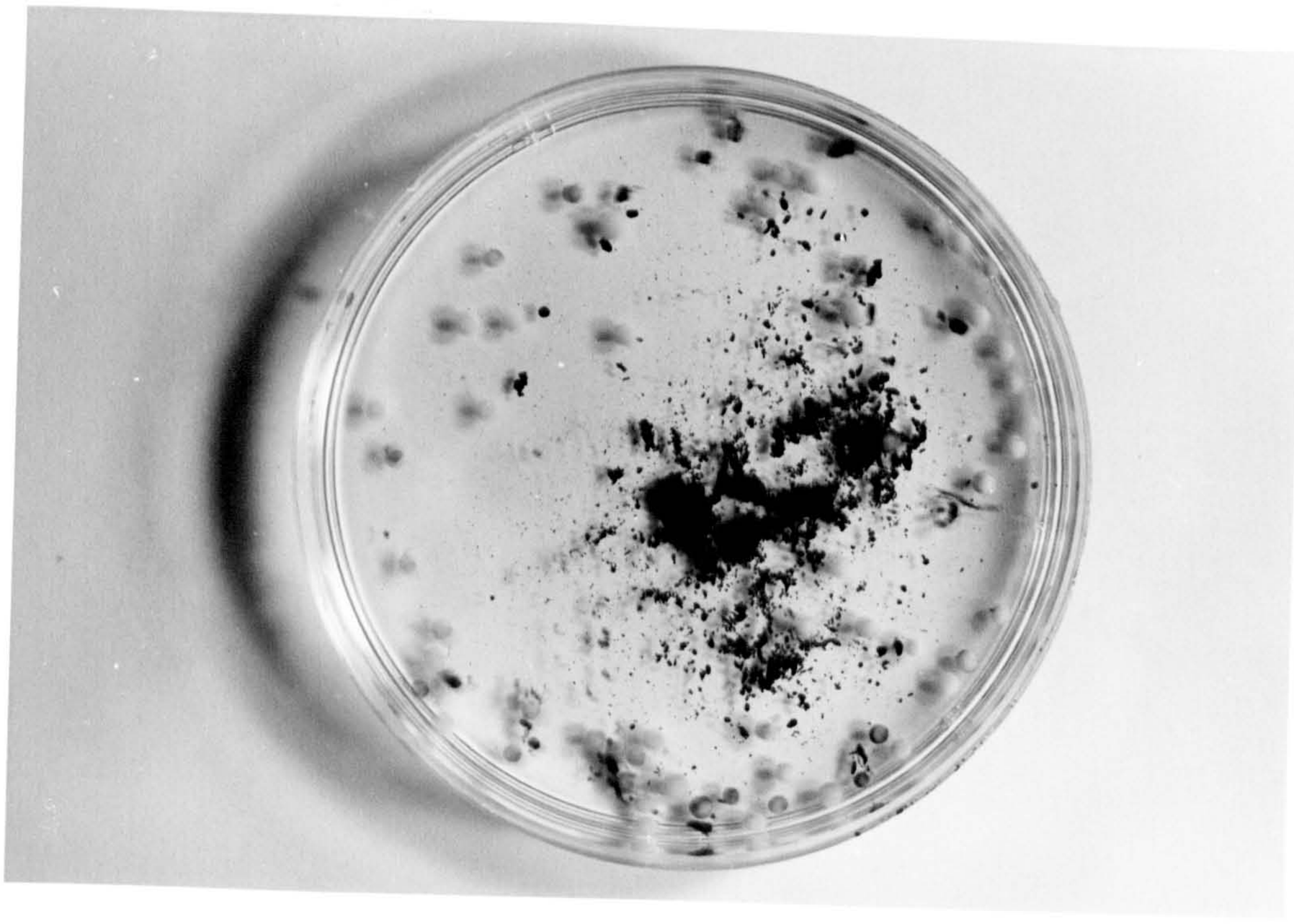


FIG. 3.13.

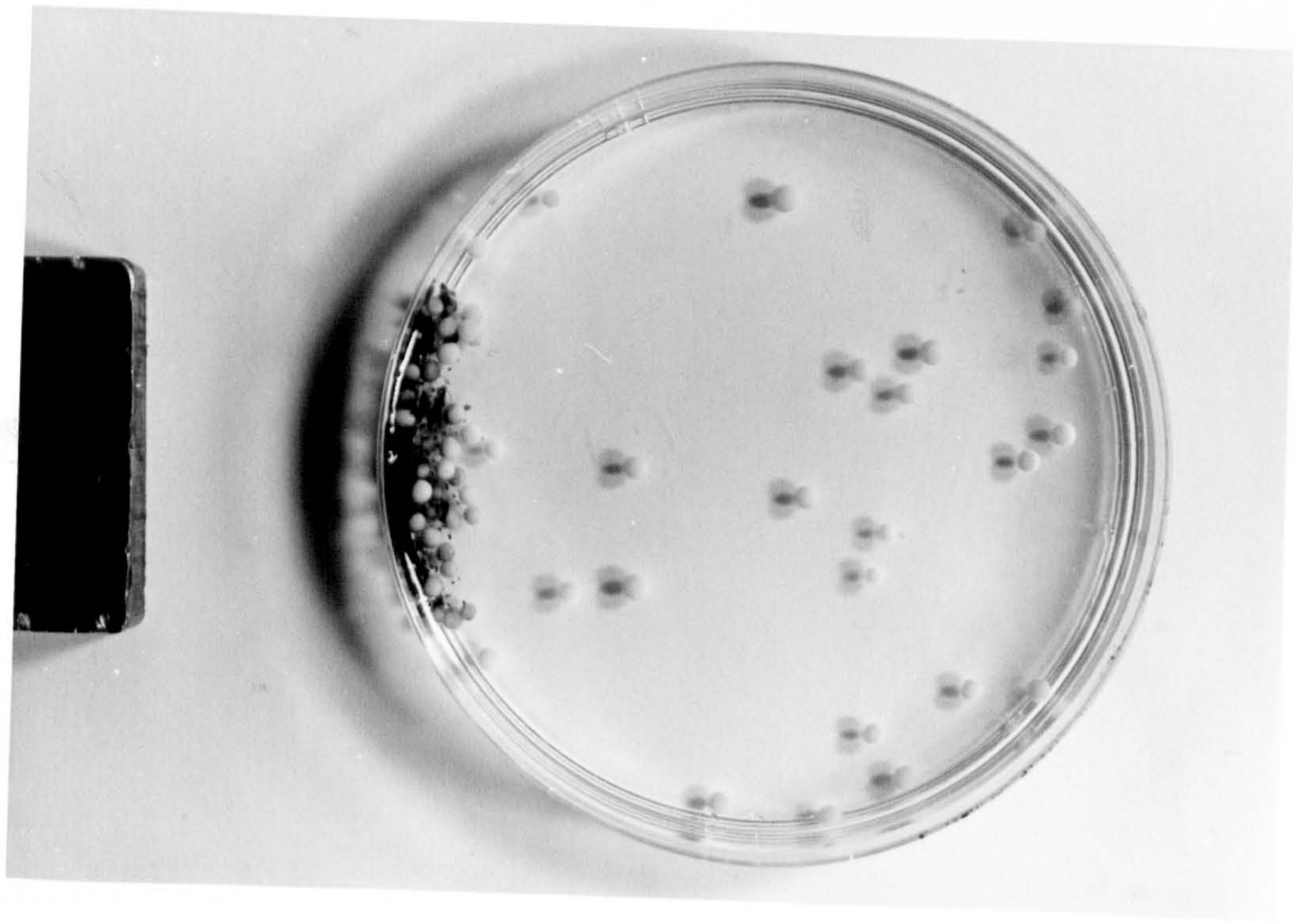


Figure 3.16.

A six day old culture of *Penicillium chrysogenum* grown with magnetite for four days and then grown for a further two days in the absence of magnetite.

Figure 3.17.

As Fig.3.16. but in the presence of a magnetic field.

FIG. 3.16.

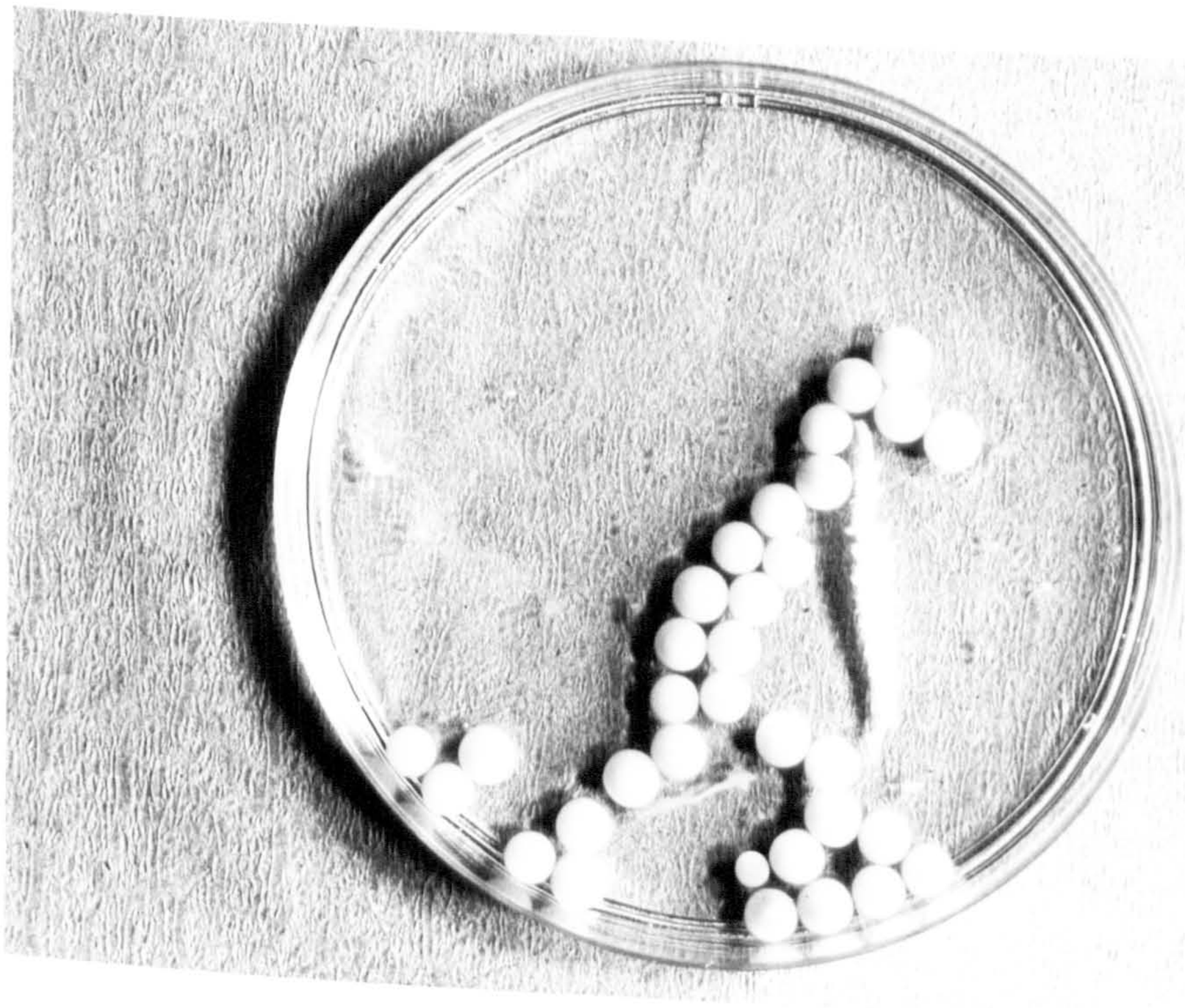


FIG. 3.17.

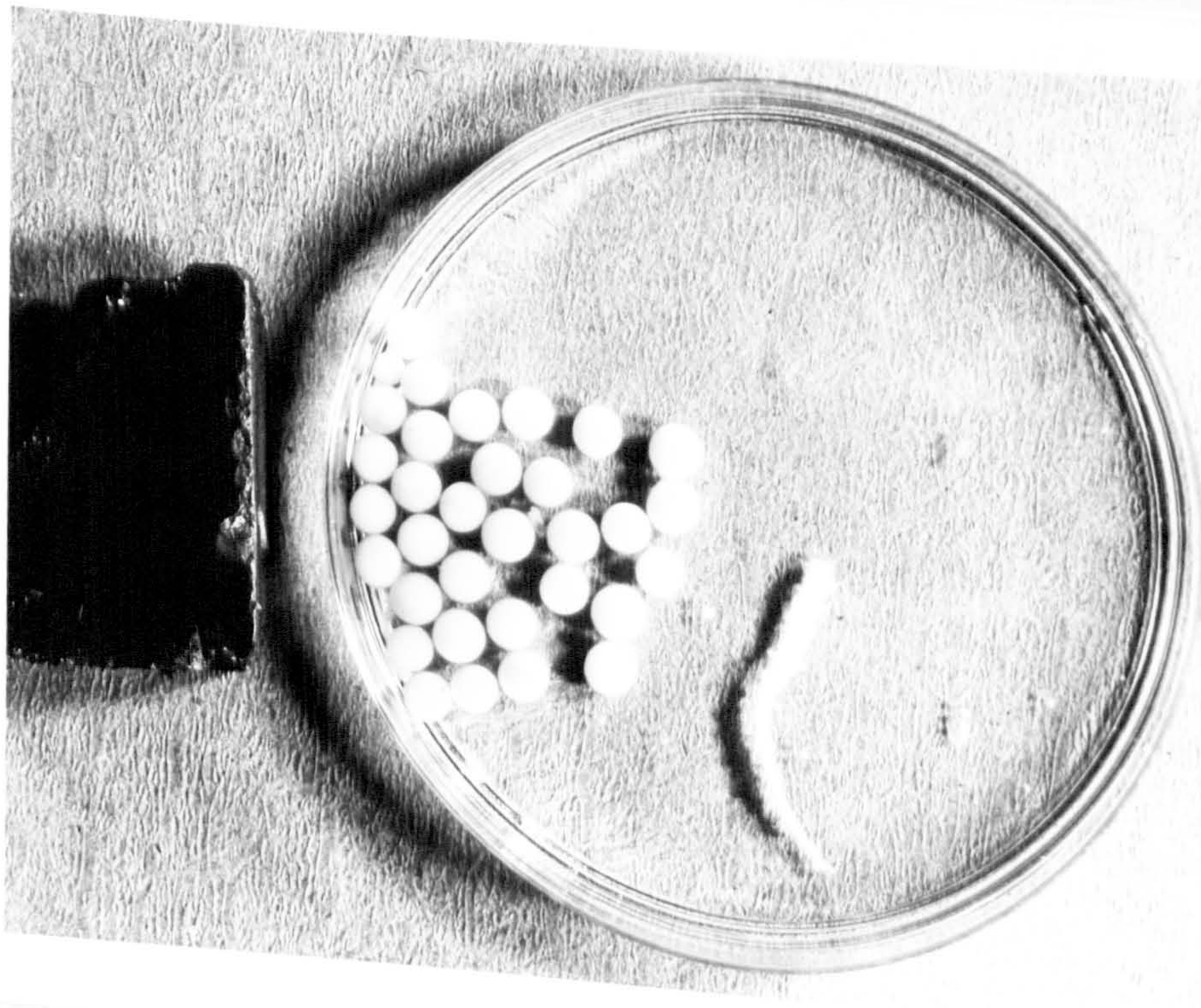


Figure 3.18.

An eight day old culture of *Penicillium chrysogenum* grown with magnetite for four days and then grown for a further four days in the absence of magnetite.

Figure 3.19.

As Fig.3.18. but in the presence of a magnetic field.

FIG. 3.18.

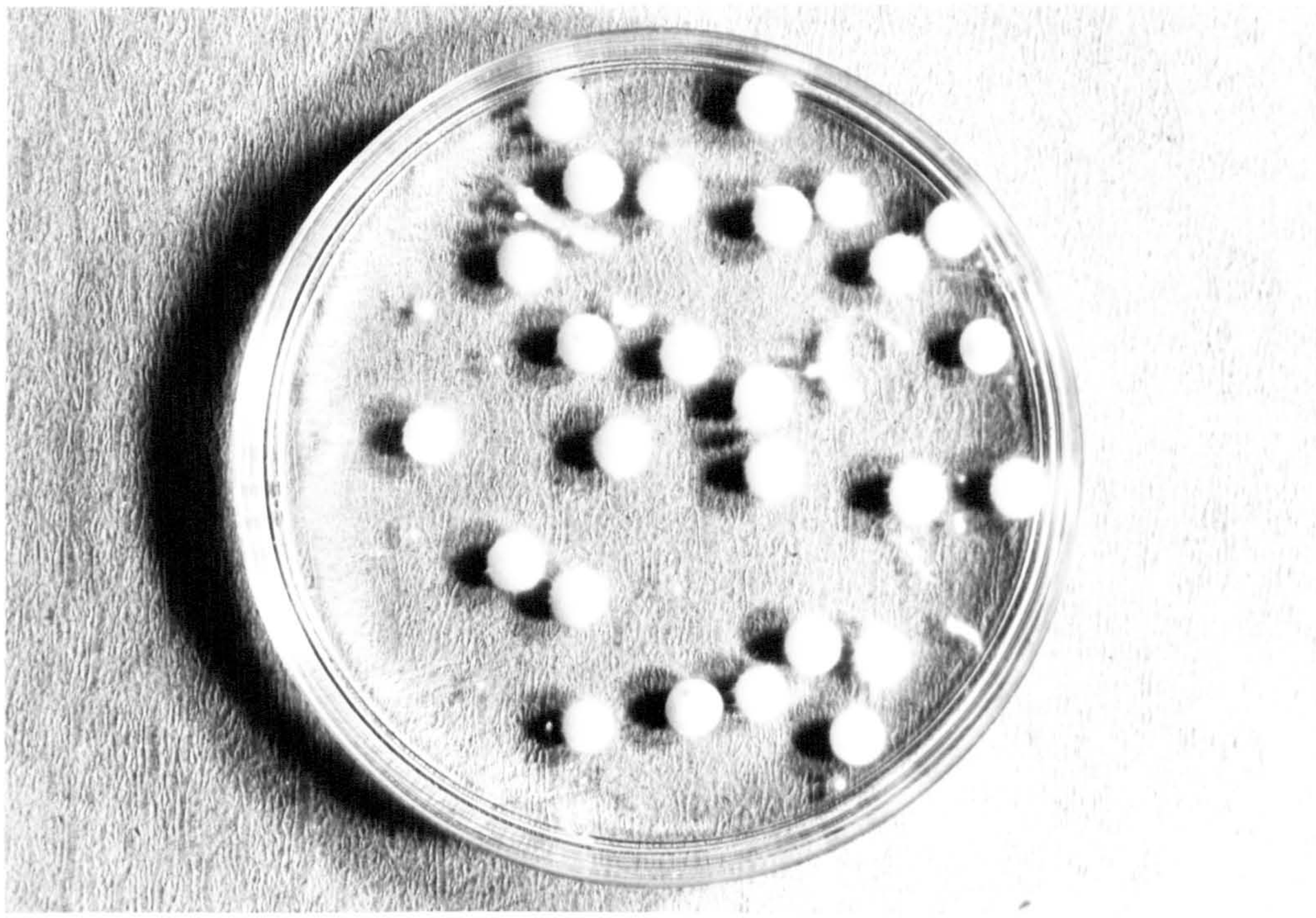


FIG. 3.19.

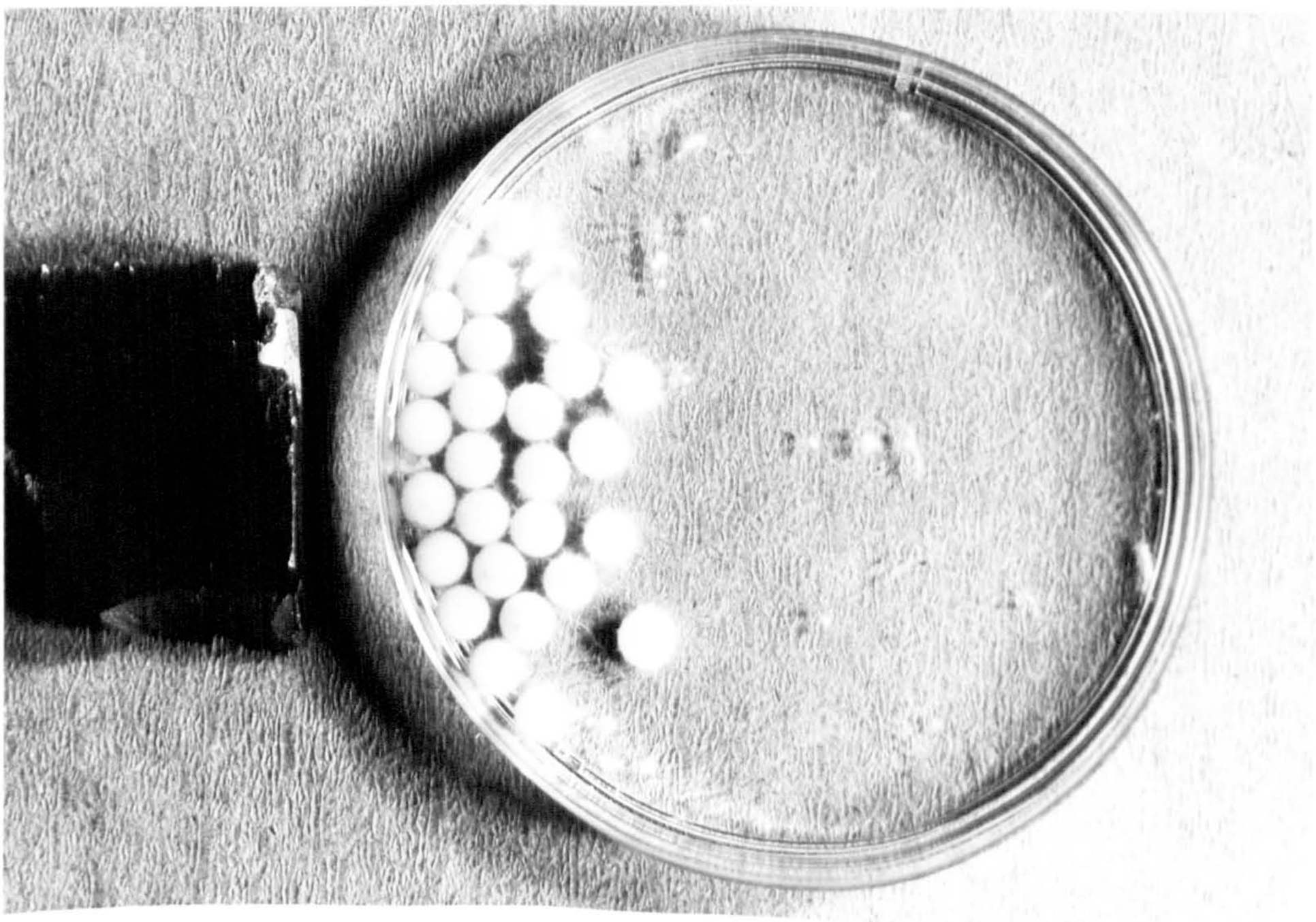


Figure 3.20.

Scanning electron micrograph of the surface of a four day old fungal pellet of *P. chrysogenum* incubated with a magnetite suspension for twenty four hours. (Magnification x 1000)

FIG. 3.20.

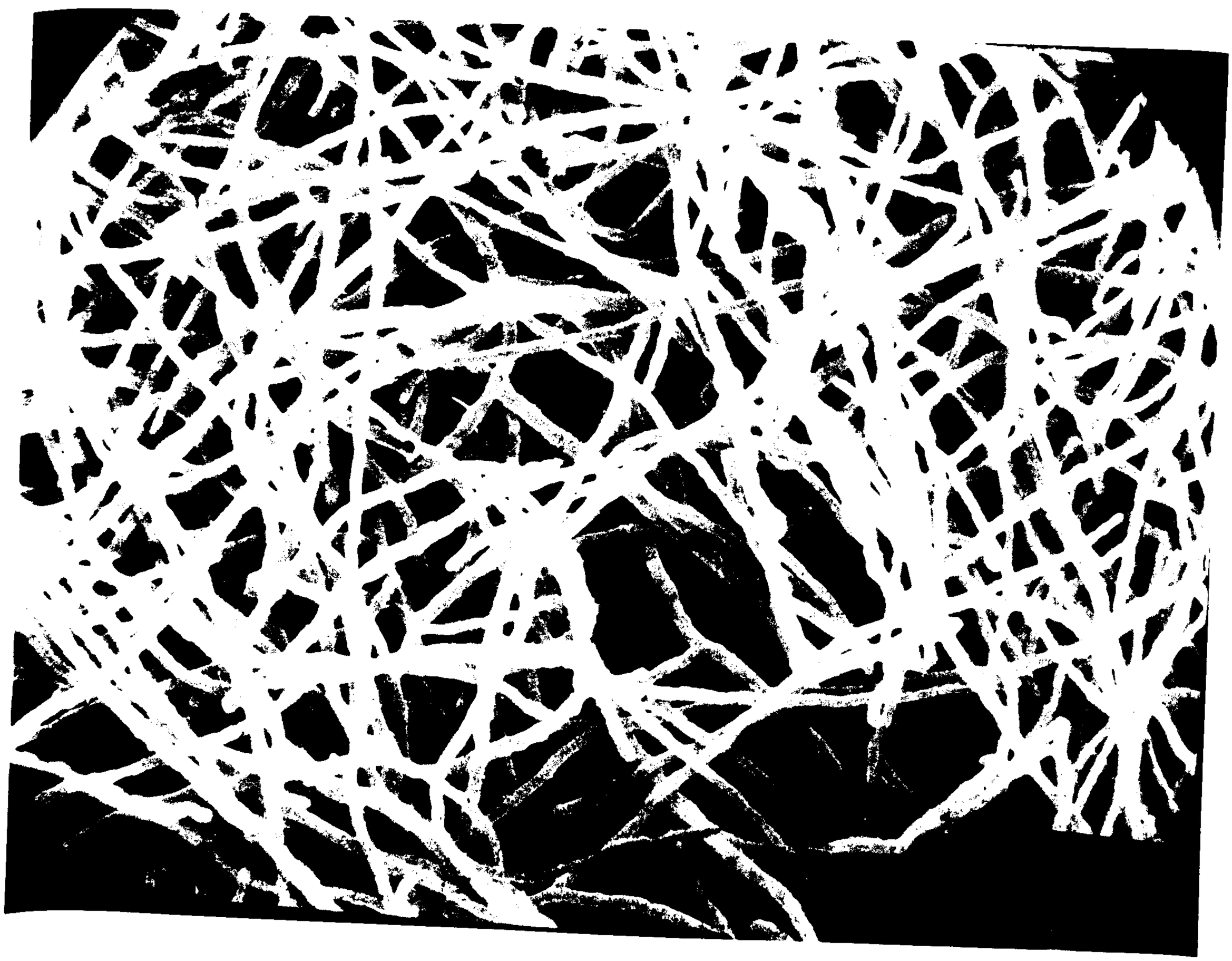


Figure 3.21.

Scanning electron micrograph of the interior of a four day old pellet of *P. chrysogenum* grown in the presence of magnetite from a spore suspension.

(Magnification x 5000)

Figure 3.22.

As Fig.3.21. (Magnification x 2000)

FIG. 3.21.

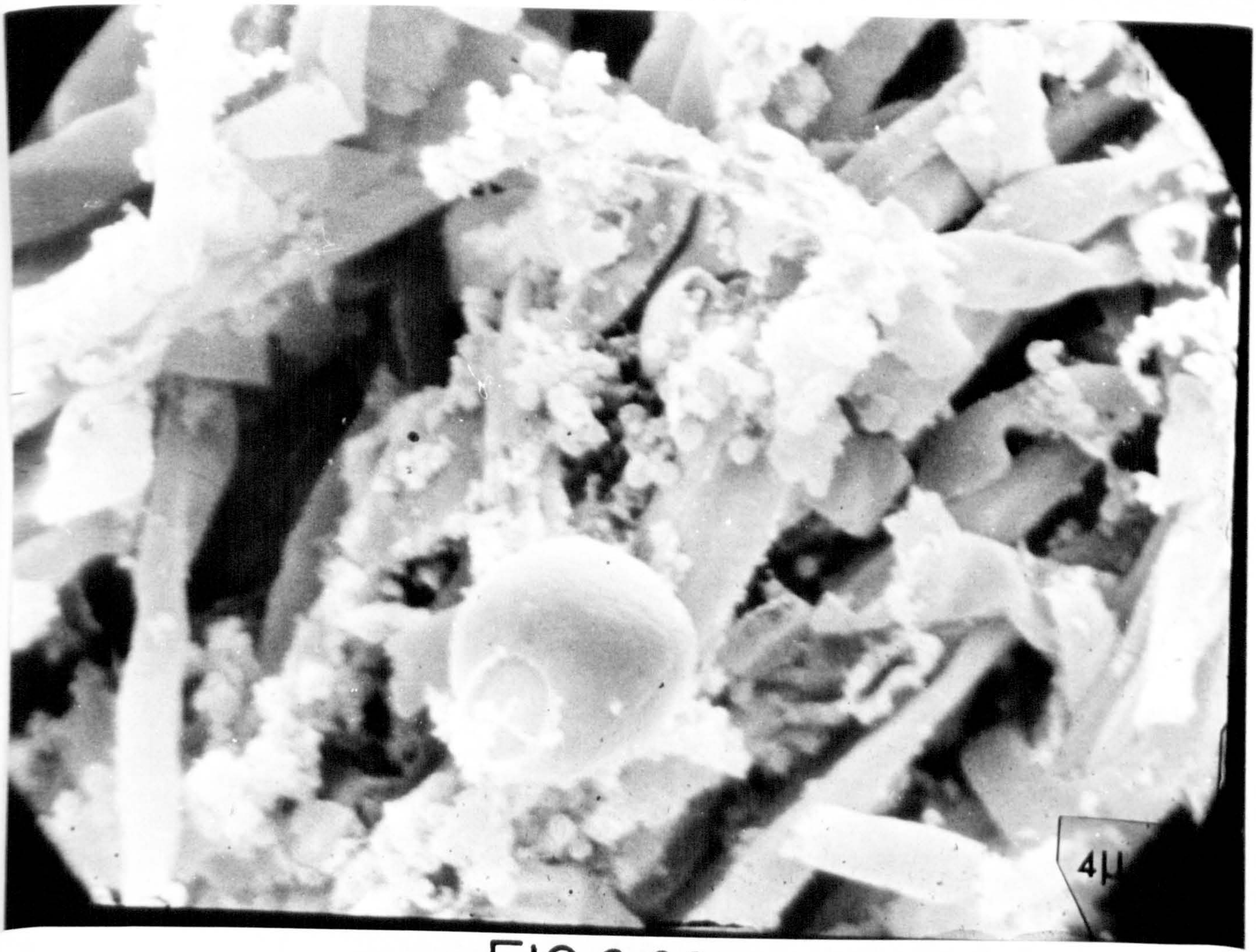


FIG 3.22.

FIG.3.22.

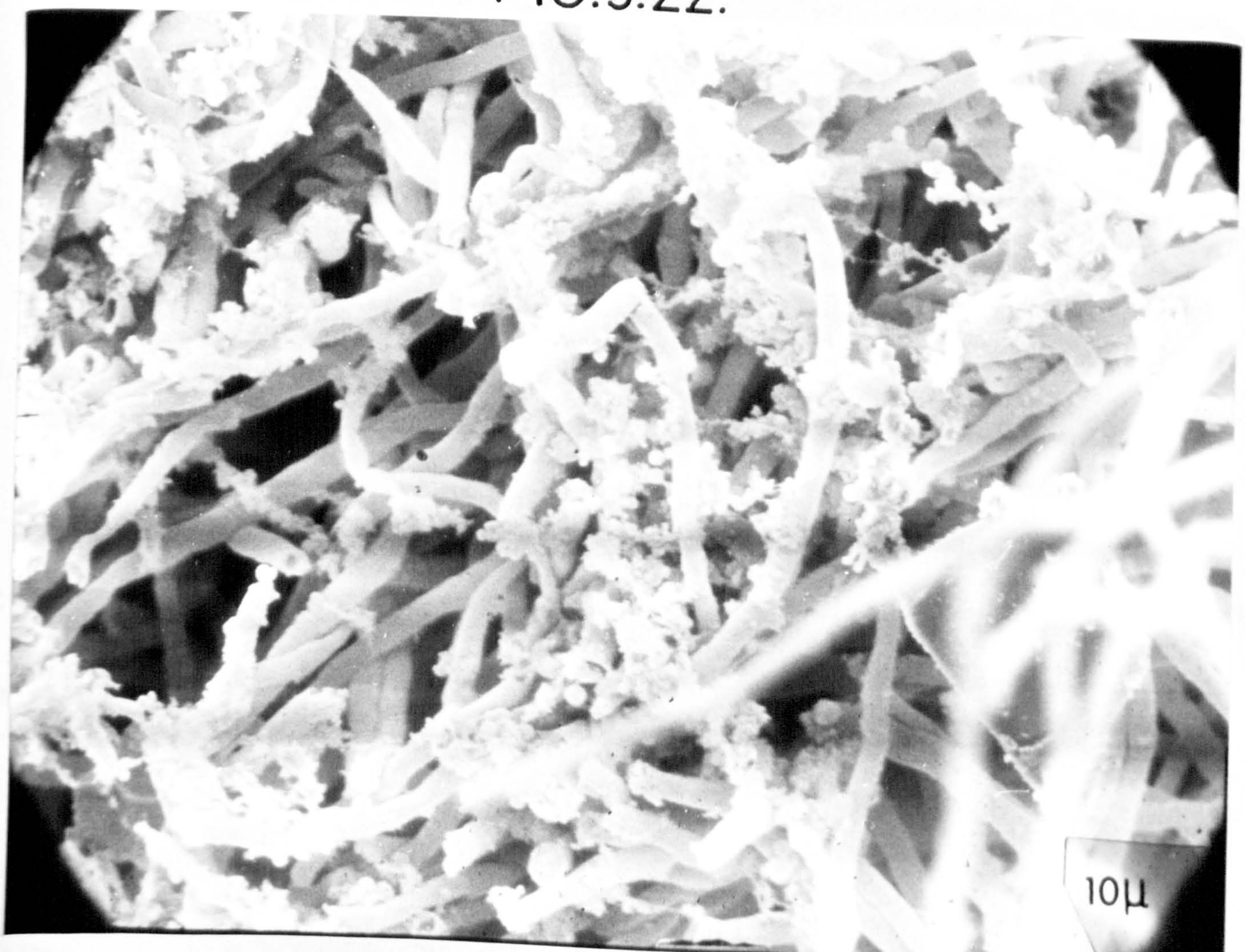


Figure 3.23.

The effect of magnetite on a) biomass production by, and b) the pH value of the growth medium of *Penicillium chrysogenum*.

(Means of triplicates $^{+}/_{-}$ S.D.)

Fig.3.23.

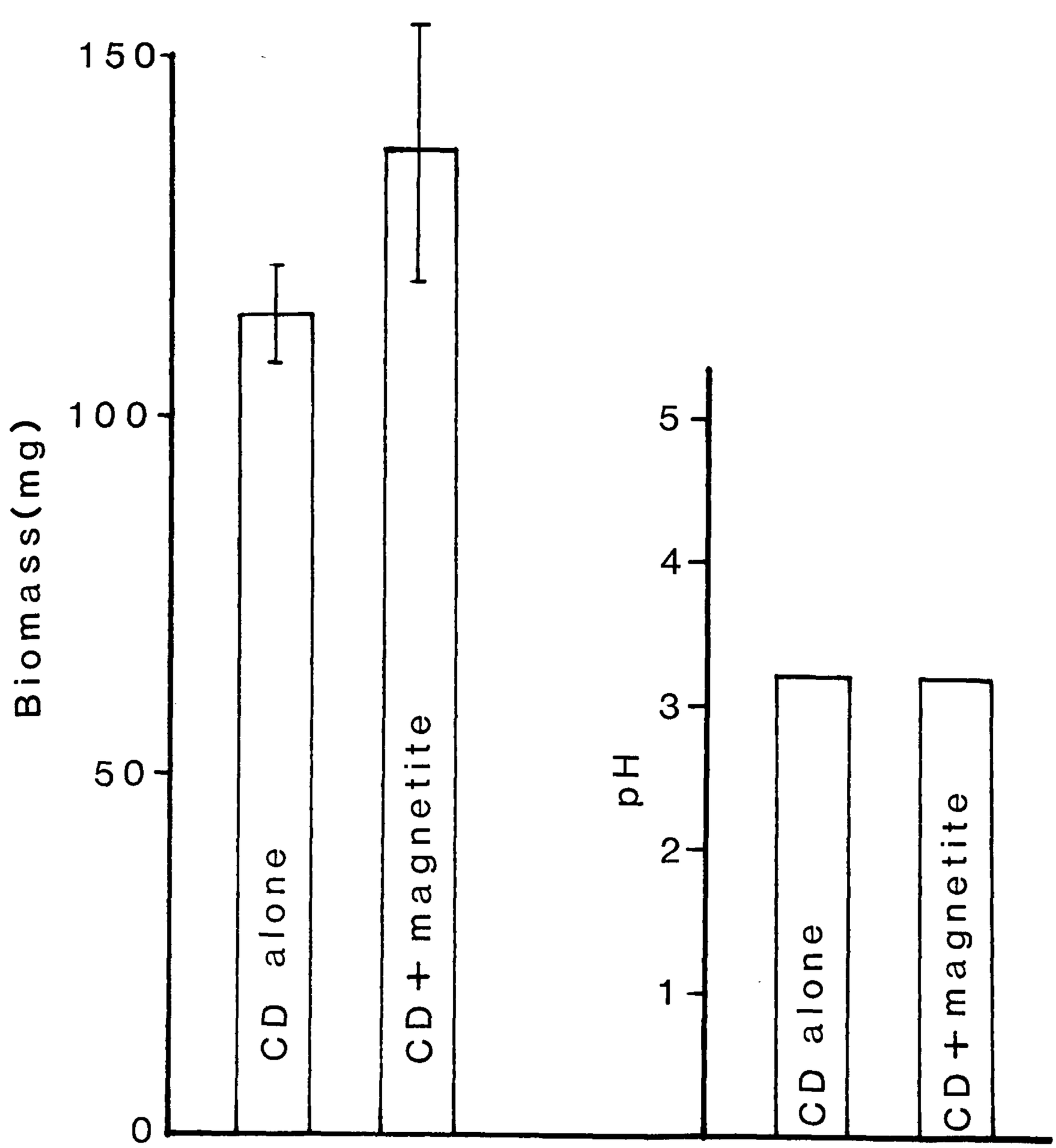

Biomass(mg)
150
100
50
0
CD alone
CD + magnetite
pH
5
4
3
2
1
0
CD alone
CD + magnetite

Figure 3.24.

A four day old culture of *A. niger* incubated for twenty four hours with a magnetite suspension.

Figure 3.25.

As Fig.3.24. but in the presence of a magnetic field.

FIG. 3.24.

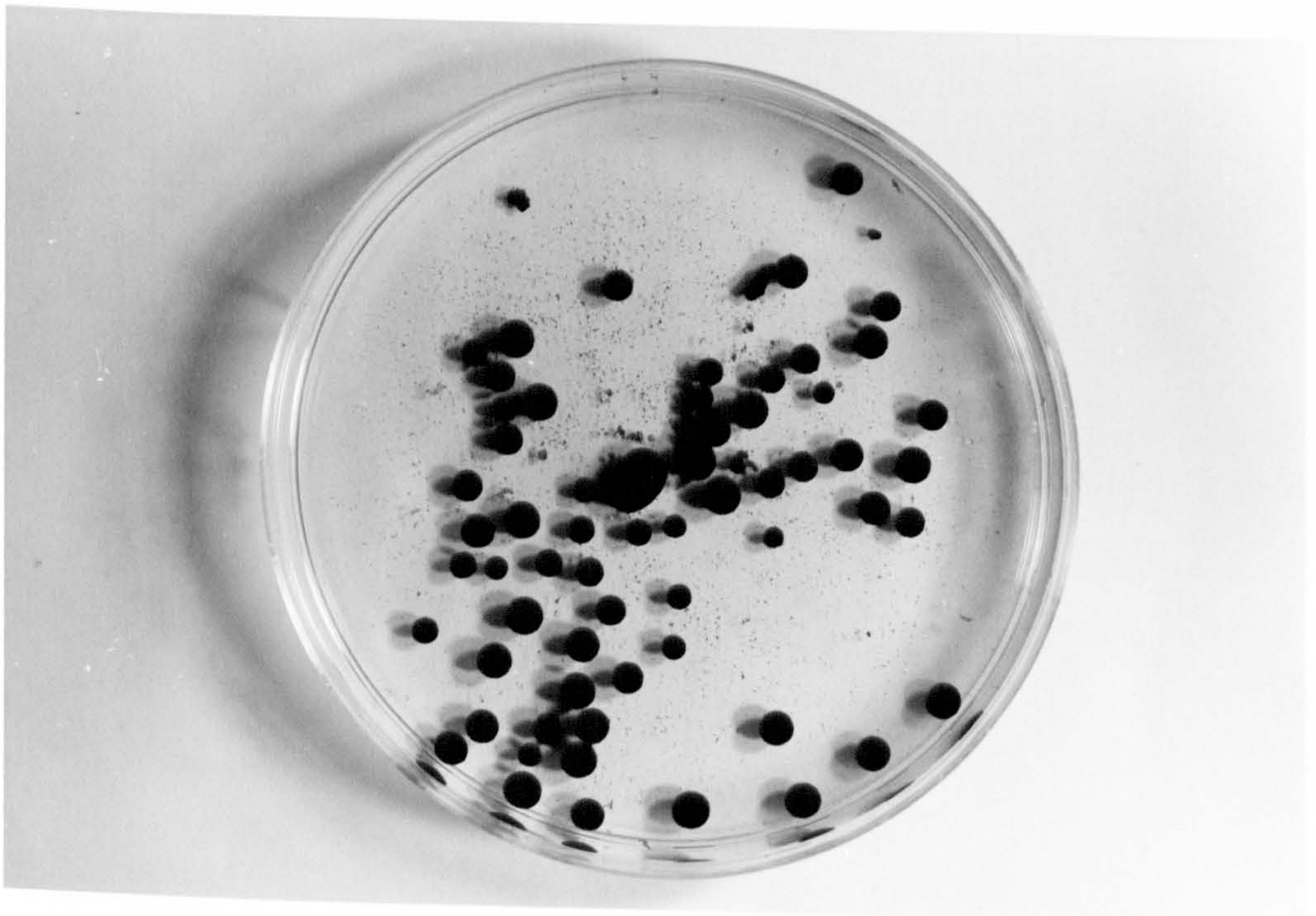


FIG. 3.25.

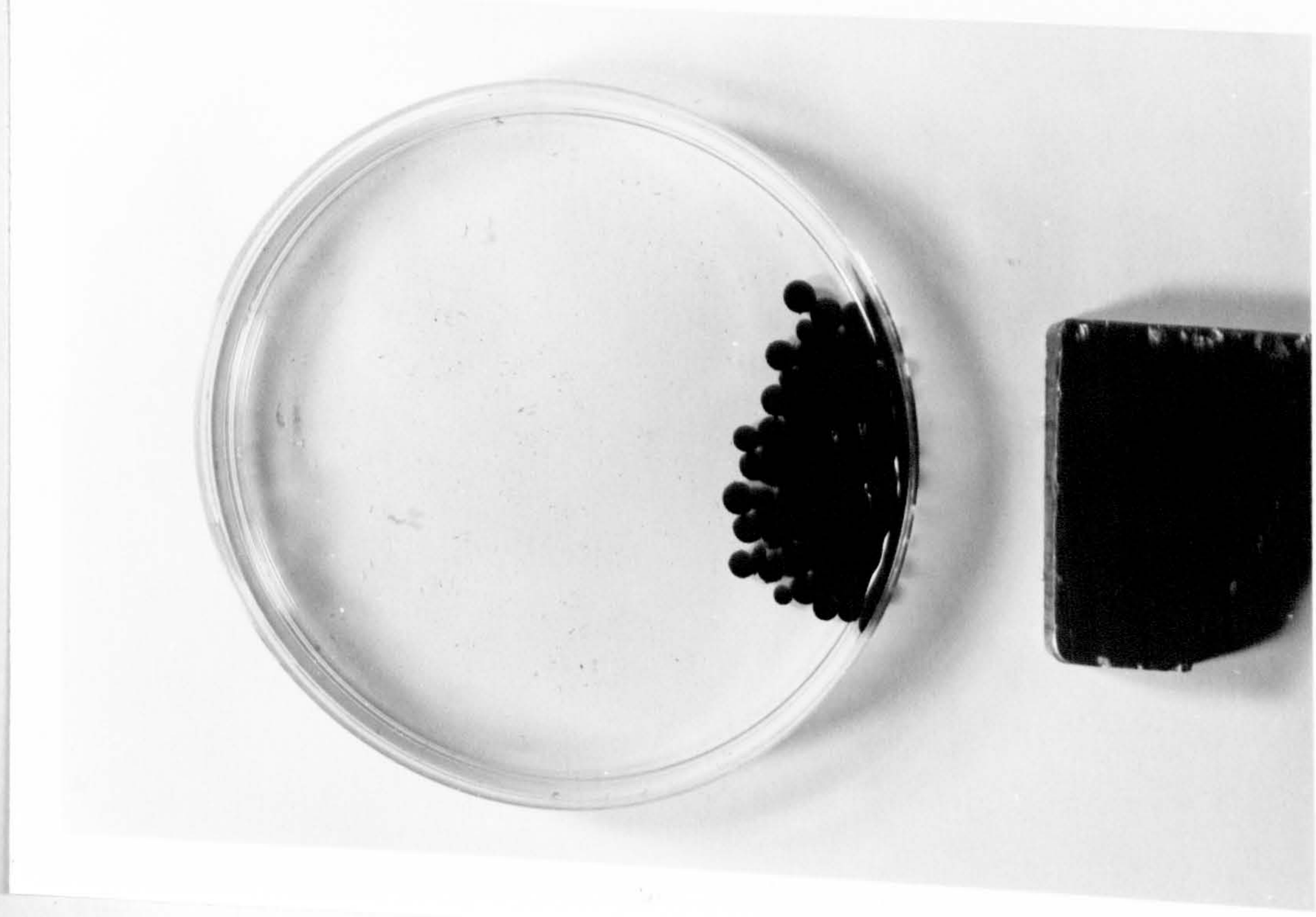


Figure 3.26.

Four day old culture of *Aspergillus flavus* incubated for twenty four hours in a magnetite suspension.

Figure 3.27.

As Fig.3.26. but in the presence of a magnetic field.

FIG. 3.26.

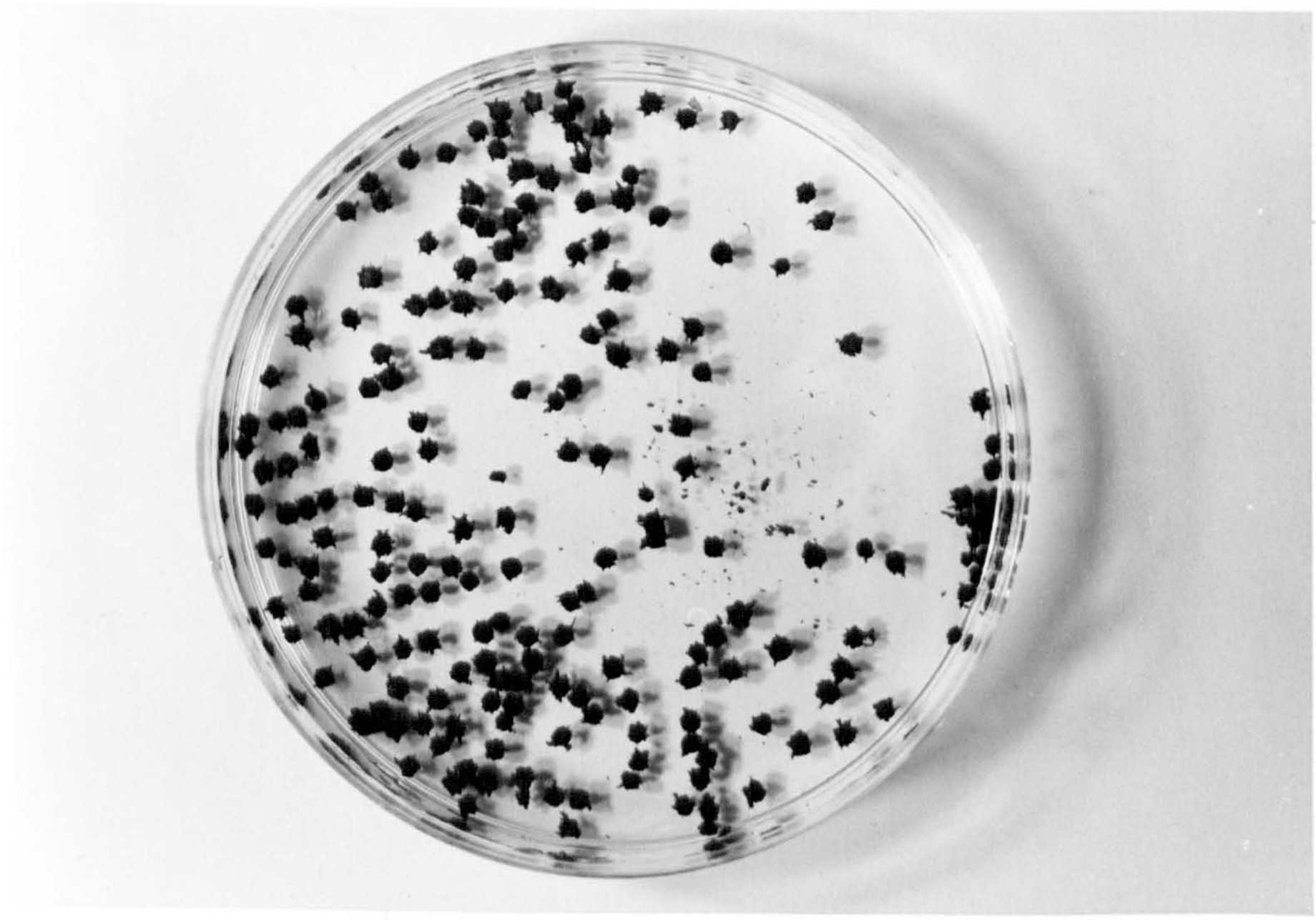
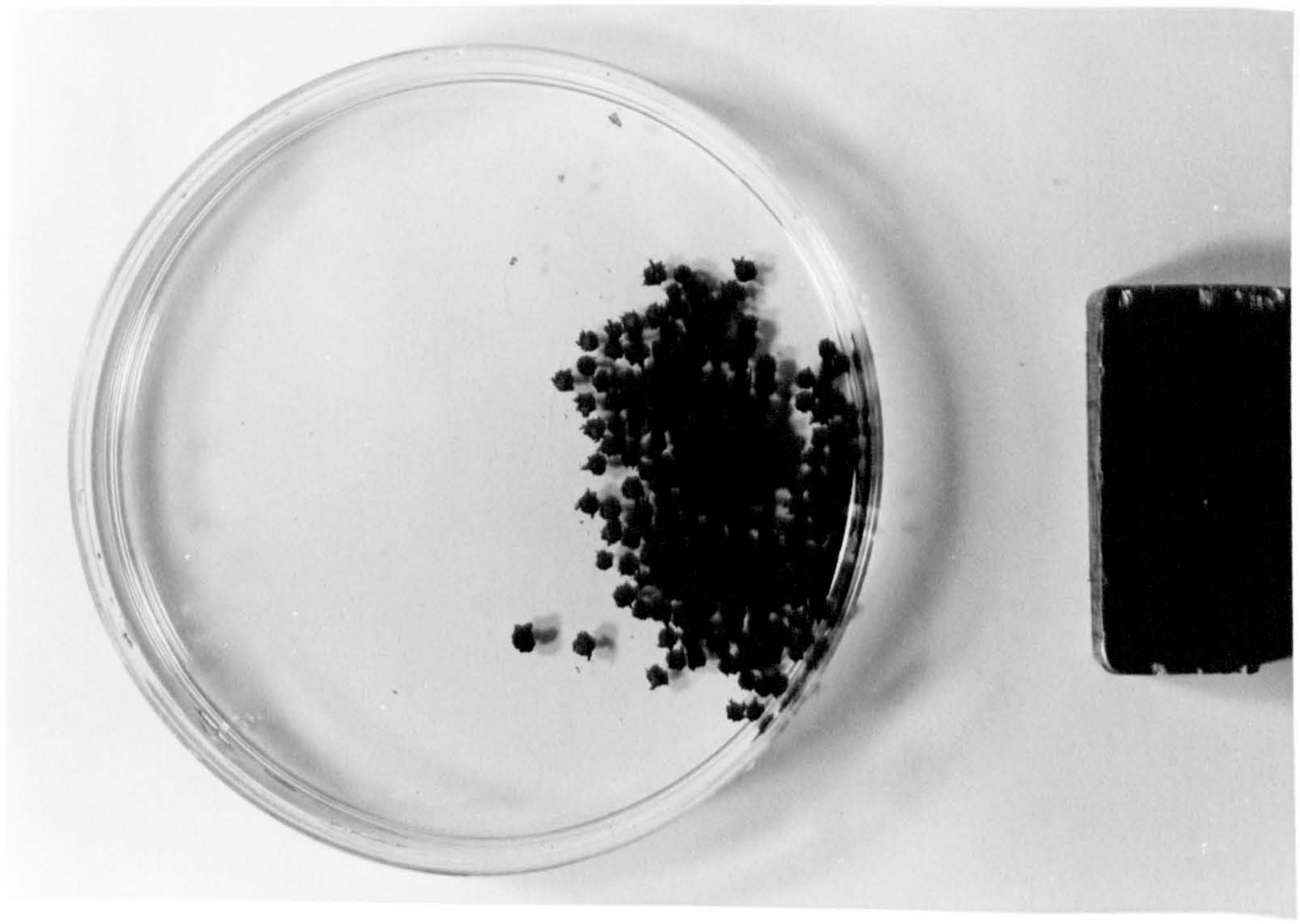


FIG. 3.27.



Adsorption of magnetite by bacteria, yeast and algae.

Under the conditions employed in the experiments the bacterium (*E. coli*), the alga (*D. salina*) and the yeast (*S. cerevisiae*) did not adsorb to magnetite and were not influenced by a magnetic field. However, previous studies have shown that bacteria (Macrae & Evans, 1983), algae (Bitton *et al.*, 1975) and viruses Bitton *et al.*, 1976) can adsorb to magnetite and the phenomenon has been used as a means of removing these organisms from waste-waters. In my experiments magnetite did not remove the organisms from their growth media probably because of the high cell densities present and perhaps because of a high medium pH. Magnetite loses its positive surface charge at pH values above it's isoelectric point (pH 6.5) and therefore the negatively charged microbial cells will not readily adhere to magnetite particles present in media with a high pH. Media constituents also may have interfered with magnetite adsorption by the cells.

SURFACE IMMOBILIZATION OF FUNGI.

Ability of fungi to grow on thread.

Aspergillus niger, *A. flavus* and *M. flavus* were all able to grow on thread (Fig 3.28). Fig 3.28 also shows zinc adsorbed to the surface of immobilized *A. niger* and *M. flavus*. All three fungi showed a preference for growth on the thread surface. This form of fungal growth provides a large surface area which may have several useful applications. For example, fungi grown on cords may be used to adsorb particulates or metal ions from solution; their higher surface area being utilized to obtain more

efficient adsorption of these substances. Fungal filament fermenters have already been experimentally used for waste water treatment (Blain *et al.*, 1981) and the treatment of kraft bleach plant effluent (Anderson, 1983).

Specific attachment of various fungi to a nylon mesh carrier material.

All the fungi tested exhibited an ability to attach to the nylon mesh and therefore become immobilized (Fig. 3.29). *Mucor flavus* shows the highest specific adsorption value and *P.chrysogenum* the lowest specific adsorption value of the fungi tested. Advantages of this type of fungal immobilization include improved reactor stability and less biomass present in the crude extracts of culture medium (Duff, 1988). These factors are important in the production of industrially important enzymes, citric acid and antibiotics. Also most secondary metabolites, such as antibiotics, are produced when cells cease growing and immobilization prevents washout of cells from a continuous culture reactor hence more of the valuable end product is obtained (Webb, 1986). *Penicillium chrysogenum* biomass increases as the initial amount of spore suspension added to the growth medium is increased (Table 3.5). The increase in biomass production by *P. chrysogenum* seems to cause a decrease in the ability of the fungus to adsorb to the nylon carrier material as indicated by the decrease in it's specific adsorption value (Fig. 3.29). None of the other fungi show a biomass increase corresponding to an increase in the amount of spore inoculum (Table 3.5), or a decrease in specific adsorption values (Fig. 3.29). The medium pH of *A. niger* drops from an average of 3.1 to 2.6 as the amount of spore inoculum is increased (Table 3.6) despite the fact that biomass production by the fungus did not increase. This effect may be due to a change in pellet size of *A. niger* corresponding to changes in amounts of spore inoculum used, as the morphology of *A. niger* is known to be important in citric acid production. Pellets

were formed by fungal biomass which did not attach to the nylon mesh carrier material. The medium pH of *M. flavus* also decreased with an increase in the amount of spore inoculum used (Table 3.6). With 0.1ml of spore suspension, *A. niger* formed pellets that were 4mm in diameter, whereas 1.0ml of the same spore suspension caused the production of pellets that were 2.0mm in diameter. A similar effect, that is; a decrease in pellet size with increasing inoculum size, was observed with *P. chrysogenum*. No change in the morphology of the other fungi used in the experiment was noted. The size of inoculum used has previously been connected with a change in pellet morphology (Whitaker & Long, 1973).

Effect of growth on synthetic foam on acid production and thiosulphate oxidation by *A. niger*.

Immobilization of *A. niger* on different sizes of synthetic foam does not alter the rate of thiosulphate oxidation by the fungus when compared to a non-immobilized culture (Table 3.7). This suggests that immobilization of the fungus in this way is a procedure which does not affect metabolic processes. However, the medium pH of immobilized *A. niger* is much higher than that of non-immobilized fungus (Table 3.8). The difference is decreased when the fungus is grown on smaller foam pieces but the change is still significant (Table 3.8). This pH difference indicates that the metabolic activity of immobilized fungus is altered in some way, the difference probably being due to a morphological change between the two cultures. Growth of the fungus on the foam was more filamentous in nature than ordinary pellet growth and the pellet form of growth is generally accepted to be more effective than the mycelial form for citric acid production (Whitaker & Long, 1973).

Figure 3.28.

Attachment and growth of several species of fungi on cotton thread.

A : *Aspergillus niger*

B : *Aspergillus flavus*

C : *Mucor flavus*

D : *M. flavus* plus adsorbed zinc dust

E : *A. flavus* plus adsorbed zinc dust

FIG. 3.28.

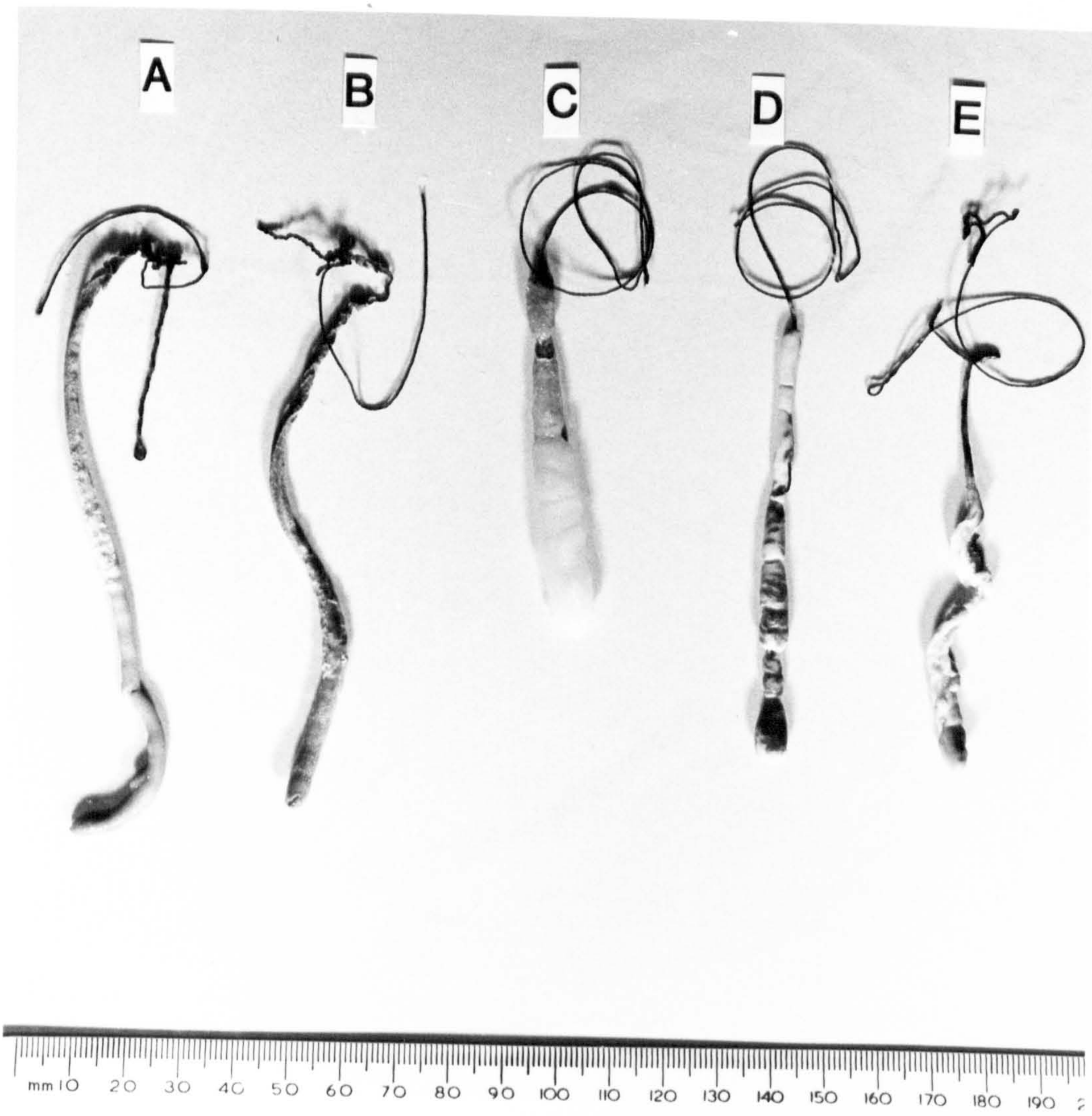

A
B
C
D
E
mm 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190

Figure 3.29.

Adsorption of various fungi to a nylon mesh support.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in the specific adsorption value compared with the value obtained with 0.1ml spore suspension, $p < 0.05$)

Specific adsorption = The amount of biomass attached to the nylon mesh / the total amount of biomass produced.

a : 0.1ml spore suspension

b : 0.25ml spore suspension

c : 0.5ml spore suspension

d : 1.0ml spore suspension

Fig.3.29.

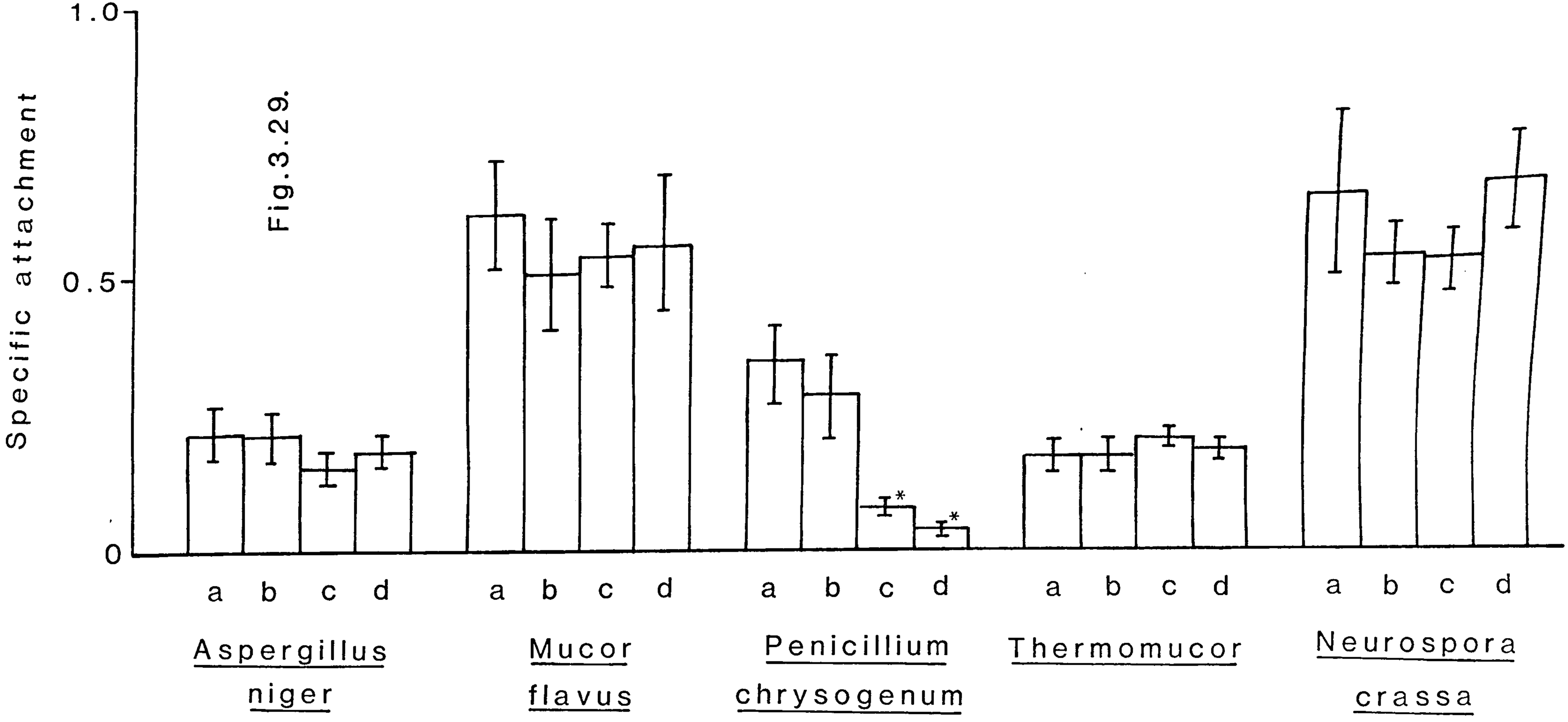

Specific attachment
1.0
0.5
0
a b c d
a b c d
a b c d
a b c d
a b c d
*
*
Aspergillus niger
Mucor flavus
Penicillium chrysogenum
Thermomucor
Neurospora crassa

Table 3.5 Effect of increasing the amount of spore inoculum used on biomass production by several fungi

	Biomass produced (mg dry weight)			
Amount of spore inoculum used (ml)	0.1	0.25	0.5	1.0
Aspergillus niger	219.8 ± 40.6	257.5 ± 27.9	239.1 ± 45.7	253.9 ± 17.4
Mucor flavus	193.1 ± 16.6	189.7 ± 22.0	245.5 ± 56.9	247.6 ± 53.9
Thermomucor indicae-seudaticae	491.2 ± 29.5	564.3 ± 40.1	426.5 ± 10.3	452.0 ± 15.9
Neurospora crassa	222.6 ± 32.7	244.0 ± 13.0	229.9 ± 20.4	255.9 ± 15.7
Penicillium chrysogenum	202.6 ± 75.9	188.5 ± 50.3	287.9 ± 53.6	*372.9 ± 65.1

(Means of triplicates ± S.D.)
(*indicates significant biomass increase over that produced by 0.1 ml spore inoculum, $p < 0.05$).

Table 3.6 Effect of increasing spore inoculum on the final pH value of the growth medium of several fungi

	Final pH value			
Amount of spore inoculum used(m)	0.1	0.25	0.5	1.0
Aspergillus niger	3.1 ± 0.2	3.1 ± 0.1	2.8 ± 0.1	*2.6 ± 0.1
Mucor flavus	7.0 ± 0.1	*6.3 ± 0.2	*6.4 ± 0.4	*6.3 ± 0.2
Penicillium chrysogenum	3.8 ± 0.1	3.9 ± 0.1	3.8 ± 0.3	3.6 ± 0.2
Thermomucor indicae-seudaticae	5.7 ± 0.2	6.1 ± 0.2	5.6 ± 0.2	5.9 ± 0.2
Neurospora crassa	6.3 ± 0.1	6.1 ± 0.3	6.2 ± 0.2	6.3 ± 0.2

(Means of triplicates ± S.D. *significant decrease in medium pH due to increase in spore inoculum, $p < 0.05$).

Table 3.7 Effect of immobilization of *Aspergillus niger* on synthetic foam on the organisms ability to oxidize thiosulphate

	μg S-SO_4^{2-} ml filtrate	
	10 days incubation	20 days incubation
Foam (10 x 8 x 8 mm) plus *A. niger*	275 ± 19	340 ± 17
Foam (5 x 5 x 5 mm) plus *A. niger*	246 ± 20	350 ± 22
A. niger alone	254 ± 12	300 ± 36

(Means of triplicates ± S.D. Results were corrected by determining the natural rates of thiosulphate oxidation with and without the presence of synthetic foam. The foam was found to have no significant effect on natural rates of thiosulphate oxidation).

Table 3.8 Effect of immobilization of *Aspergillus niger* on synthetic foam on the final pH value of the organims growth medium

	Final pH	
	10 days incubation	20 days incubation
Foam (10 x 8 x 8 mm) plus *A. niger*	*2.2 ± 0.1	*2.1 ± 0.1
Foam (5 x 5 x 5 mm) plus *A. niger*	*2.2 ± 0.1	*1.9 ± 0.1
A. niger alone	1.5 ± 0.1	1.5 ± 0.1

(Means of triplicates ± S.D. *Significant increase in pH due to immobilization on synthetic foam, $p < 0.05$).

CONCLUSIONS.

Magnetite adsorption by fungi.

The results of these studies show that fungal mycelium can adsorb magnetite with the result that it becomes susceptible to the influence of a magnetic field. Under appropriate conditions, the adsorbed magnetite can then be removed from the fungal biomass. As was pointed out in the Introduction the industrial applications of this ability are widespread and include:

(1) Immobilization of magnetic susceptible mycelium or mycelial pellets

(2) Removal of fungal biomass from solution in preference to filtration or centrifugation or

(3) Movement of fungal biomass through an industrial plant under the influence of a magnetic field.

In all such cases the magnetic field could be provided by either permanent or electro-magnets.

Care must be taken however, in the use of magnetite in the immobilization or recovery of *A. niger* biomass for the purpose of citric acid production. *A. niger* has been shown to leach iron from magnetite (Ribeiro *et al.*, 1976) and iron has been reported to affect citric acid production by activating the enzyme, isocitrate dehydrogenase, which metabolizes citric acid to isocitric acid (Berry, 1988). This would mean that citrate production would be decreased by the increased amount of iron available in the growth medium. This increase in iron could be overcome by increasing the amount of ferrocyanide presently used in the chelation of heavy metals during citric acid fermentation (Berry, 1988).

Of particular interest in relation to the possible industrial use of magnetic susceptible mycelium is the finding that spores or young mycelium can adsorb magnetite forming young mycelial pellets with magnetic properties which are retained as the pellets increase in size. As a result, the fungus need only be exposed to to magnetite for a short period during initial pellet formation to acquire magnetic properties, so that the magnetite need not be free in the culture medium during the later period of product formation by the fungus. This may overcome the ability of *A. niger* to leach iron from magnetite as much less magnetite will be present during the actual production of citric acid and also the inability of older *P. chrysogenum* biomass to adsorb magnetite can be ignored.

Surface immobilization of fungi.

These studies suggest that immobilization of fungi using their natural adhesive properties is an effective and mild method. Some physiological properties, such as acid production by *A. niger*, may be changed during the immobilization process. Surface immobilization may be used for secondary metabolite production, waste water treatment or particulate and metal ion adsorption by fungal mycelium. Immobilization on synthetic foam or thread would be especially useful for particulate or metal ion adsorption by fungi as this form of immobilization greatly increases the surface area available for adsorption. The initial amount of spore inoculum used may be important in obtaining efficient surface immobilization of certain fungi.

4. METAL ION ACCUMULATION BY *ASPERGILLUS NIGER* WASTE MYCELIUM OBTAINED FROM THE INDUSTRIAL PRODUCTION OF CITRIC ACID.

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INTRODUCTION.

RECOVERY OF METALS BY MICROORGANISMS

Metal wastes, from mining, metal refining and manufacturing, represent a loss of non-renewable resources and also present a significant pollution problem. The recovery of these "lost" metals would be invaluable in reducing environmental contamination and would also lead to the recycling of these economically important resources. Recent international agreements, such as the EEC directive on the Discharge of Dangerous Substances, have come into operation with the specific aim of preventing aquatic pollution by controlling the discharge of metals into the environment (Townsley *et al.*, 1985).

Conventional techniques for metal recovery from effluents, such as ion exchange and electrolysis, are likely to become increasingly expensive and also inefficient when the new metal pollution limits are introduced. These factors have markedly increased the interest in the natural capacity of many microorganisms to accumulate metal ions from solution. The concentration of metals from solution by microbial biomass is termed biosorption.

Several mechanisms are known to be involved in metal uptake by microorganisms. These include (Volesky, 1986):

(1) particulate ingestion or entrapment by flagella or extracellular filaments

(2) active transport of ions

(3) ion exchange

(4) complexation

(5) adsorption, and

(6) inorganic precipitation.

The first two mechanisms are carried out by living cells, whilst the remaining mechanisms have been described for both living and dead microorganisms, as well as cell debris (Shumate *et al.*, 1978; Tsezos & Volesky, 1981; Beveridge & Murray, 1980). As a result either non-viable microbial biomass (via passive mechanisms of metal uptake) or viable microbial species (via both active and passive mechanisms of metal uptake) can be used for the recovery of metals from solution.

Active processes involved in metal accumulation by living microorganisms.

An active, mixed community of bacteria containing *Pseudomonas maltophila*, *Staphylococcus aureus* and a coryneform organism has been shown to accumulate over 300mg Ag g^{-1} biomass (dry weight) at a rate of 21mg Ag^{+} h^{-1} (g biomass)$^{-1}$ (Charley & Bull, 1979). *Pseudomonas maltophila* was primarily responsible for the resistance to the silver. The microbial consortium was suggested to be of potential use in the recovery of silver from industrial effluents and waste materials.

Sulphate-reducing bacteria, such as *Desulphotomaculum* species, *Desulphovibrio* species and *Desulphomonas pigra*, produce hydrogen sulphide which can react with metal cations to produce insoluble metal sulphides. This use of this precipitation reaction has been proposed for the removal of heavy metals from industrial and mining effluents (Hutchins *et al.*, 1986). The mechanism is also primarily responsible for the removal of heavy metals observed from a series of lakes and streams receiving sewage and mining effluents (Hutchins *et al.*, 1986).In

such cases, the sewage enhances the growth of aerobic, heterotrophic bacteria which results in oxygen depletion. The decrease in the oxygen content of the water subsequently promotes the growth of sulphate-reducing bacteria, which produce hydrogen sulphide that precipitates any metals present. Sewage also provides nutrients for the growth of algae leading to further metal removal through sorption onto biomass and subsequent sedimentation of the metal-biomass complex. Algae, on decaying, can enhance further the activity of sulphate reducing bacteria, hence increasing the amounts of metal removed from the waste-waters. Such a system has been proposed as a method of secondary recovery of heavy metals as well as effluent treatment.

The removal of metals, such as selenium and uranium, from a New Mexico uranium mine effluent has been achieved by microbial processes (Michaelis, 1985). Part of the treatment process involves passage of the effluent through a series of three consecutive "algal" ponds. Filamentous algae, such as *Spirogyra* and *Oscillatoria* and benthic species, such as *Chara*, were involved in the metal removal process. Other organisms involved included the sulphate reducing bacteria, *Desulphovibrio* and *Desulphotomaculum*, which were also thought to play a role in the removal of uranium, selenium and possibly molybdenum from the same waste-waters. In this study, there was no significant reduction of uranium or molybdenum concentrations in solution between the influent to the algal ponds and the final effluent. Although the concentrations of both metals in the pond sediments were considerably greater than the concentrations in the water, the amounts of metal removed were not sufficient to be reflected in the water concentrations. It is clear therefore that it becomes necessary to assess the water volume which passes through the system. It was concluded that additions of phosphate and nitrogen containing fertilizers would accelerate the replacement of *Chara* with more productive, annual green planktonic algae and cyanobacteria (Michaelis *et al.*, 1985). This, they hoped would increase

the amount of metals removed and would also provide more organic substrate for the sulphur-reducing bacteria involved in the metal removal process.

The reduction of metals by living microbial cells, resulting in the metal ion being reduced to the free or metallic state, is yet another mechanism whereby microorganisms might be used to recover metals. By far the most well characterized system of metal reduction is that of mercury (Summers & Lewis, 1973), a process which is controlled by a set of genes collectively known as the *mer* operon (Jackson & Summers, 1982). Other metals which are known to be microbially reduced are iron (Silverman & Erlich, 1964), selenium, tellurium, vanadium, arsenic and molybdenum (Woolfolk & Whiteley, 1962). To date, however no commercial metal-reclamation processes are in use which employ the reductive activities of intact, microbial cells.

Commercial processes for heavy metal removal from effluents involving living cells.

A full scale, attached growth, aerobic, biological treatment process to effectively remove all toxic parameters from cyanidation waste-waters has been developed at the Homestake Mine in Lead, South Dakota (Whitlock & Mudder, 1986). The process involves the growth of mutant strains of bacteria on large, corrugated plastic discs which rotate slowly in the mine effluent. The bacteria simultaneously degrade cyanide, thiocyanate and ammonia. The various heavy metals present in the effluent are effectively removed by sorption onto the biomass film present on the disc surfaces. Metal-loaded biomass eventually detaches from the discs and is recovered for controlled disposal.

Algae and bacteria have been effectively used to treat effluents from lead/zinc smelting and refining operation in the mid-western United States

(Hutchins *et al.*, 1976). Heavy metals are sorbed by the organisms which populate an artificial switchback (meander) channel receiving the effluent. The treated water is then able to be discharged into natural waterways.

Active mechanisms involved in metal recovery by non-growing (resting) cells.

Resting cells of a *Citrobacter* species, immobilized in polyacrylamide gel, have been used to remove cadmium from solution (Michel *et al.*, 1986). Cadmium is highly toxic to living organisms (Vallee & Ulmer, 1972) so it's removal from effluents is therefore highly desirable. The mechanism of metal uptake depends on the activity of a surface-located phosphatase induced during cell growth in medium containing glycerol-2-phosphate as the sole phosphorus source (Macaskie & Dean, 1984). The enzyme liberates inorganic phosphate which leads to the precipitation of the insoluble metal phosphate on the cell surface. The process is therefore dependent upon the presence of a suitable phosphate donor. An interesting use of this technique was developed by Watson & Ellwood (1987) who grew two microorganisms, *Candida utilis* and *Bacillus subtilis*, on glycerol-3-phosphate to induce a phosphatase activity in the organisms. The cells were then incubated with glycerol-3-phosphate in the presence of uranyl ions and the cells subsequently picked up significant amounts of the metal from solution. Uranyl ions are strongly paramagnetic so the coated organisms behaved like a paramagnetic material in a magnetic field. The cells were then collected by high gradient magnetic separation, a technique developed in the mineral processing industry for the collection of weakly magnetic particles of colloidal size (Watson, 1979). The process allowed the concentration of metal ions by factors of up to 10^7. Proposed uses for the process include the removal of radionuclides from solutions generated by the nuclear

industry and the removal of other toxic metals, such as cobalt and mercury from waste-waters.

The above processes all employ active processes to recover metals. However, waste-waters are generally highly variable in nature, containing substances toxic to microbial growth and inhibitory to enzymatic processes. The widely fluctuating and very low pH values of industrial effluents also inhibits active processes of metal recovery. Therefore, the use of active microbial populations in metal reclamation is useful only in either non-toxic effluents, or in effluents which have been treated to allow the processes to occur.

Physico-chemical processes of metal recovery by microbial cells.

Metal recovery by microbial physico-chemical processes usually involves the use of dead cells. Accumulation mechanisms involve chelation, adsorption, ion-exchange, complexation and/or microprecipitation of metals (Kuyucak & Volesky, 1988a). The process is relatively rapid and can be reversible (Kuyucak & Volesky, 1988b; Galun *et al.*, 1983). Metals are accumulated usually as a consequence of the anionic net charge of the cell surface (Hutchins *et al.*, 1986). Cell walls of certain types of microbial biomass also offer particularly abundant metal-binding groups such as carboxylate, hydroxyl, sulphate, phosphate and amino groups (Kuyucak & Volesky, 1988a). Many Gram negative bacteria excrete complex polysaccharides that form discrete capsules or loose aggregates around the cell. These polysaccharides, which exhibit a net anionic character, can accumulate metal ions by electrostatic attraction. Often dead cells exhibit higher metal-uptake capacities than living biomass (Kuyucak & Volesky, 1988b). Also, non-living biomass is not subject to the toxic effects of metals or low pH values of certain effluents.

The bacterium, *Zoogloea ramigera* strain 115, produces large amounts of extracellular polysaccharide in media with a carbon-to-nitrogen ratio larger than 10:1 (Norberg & Enfors, 1982). The exopolysaccharide behaves like a polyelectrolyte and shows a strong affinity for metal ions (Friedman & Dugan, 1968). Metal ions sorbed by the *Zoogloea* polymer/biomass include copper, cadmium and uranium. Acid treatment of the metal-loaded material results in a rapid desorption of the metals, a process which can be repeated several times without any significant loss of activity. The biomass exhibits different optimum pH sorption values for different metals and this creates the potential for selective recovery of metals from complex solutions. For example, non-living cells of *Zoogloea ramigera* have been used for the removal of chromium and cadmium from waste-water (Sag & Kutsai, 1989). Chromium exhibited maximum uptake at pH 2, while cadmium uptake was highest at pH 6.

Fungi have been tested widely for their metal binding capacities. In particular, waste mycelium of *Rhizopus arrhizus*, originating from industrial fermentation, has been used for the accumulation of radium and thorium (Tsezos & Volesky, 1981). Non-viable biomass of this organism has a uranium and thorium uptake capacity of around 170mg g^{-1} dry weight, which is more than double the capactiy of IRA-400, a common anion- exchange resin used for the accumulation of uranium. Uptake of the metals by the fungus also exceeded that of a commercially available activated carbon (Filtrasorb 400). The mechanisms of uptake of the two metals by *R. arrhizus* biomass has been extensively studied and they appear to be slightly different. For uranium, three modes of uptake are apparently involved. First, uranium coordinates to the amine nitrogen with participation of a free radical of a hydroxyl group of the chitin component of the cell wall (Tsezos & Volesky, 1982a). This mechanism accounts for an accumulation of 6mg U g^{-1} cells. Secondly, the complexed uranium acts as a nucleation site for

accumulation of additional uranium. These two processes account for the rapid accumulation of 66% of the total metal-loading capacity of the organism. The third process is much slower than the other two, only reaching equilibrium after thirty minutes, and involves the hydrolysis and subsequent precipitation of uranyl hydroxide on the cell wall. Uranium uptake by *R. arrhizus* biomass is optimal at pH 4 and was also reduced at this pH by the presence of iron and zinc ions. In contrast to uranium uptake, only two processes are involved in thorium accumulation by *R. arrhizus* (Tsezos & Volesky, 1982b). Process A of the proposed mechanism involves the formation of a coordination complex between thorium and the nitrogen of the cell wall chitin. This is similar to the formation of the uranium-chitin complex and accounts for less than 8mg Th g^{-1} cells. Process B of the proposed thorium biosorption mechanism involves the adsorption of hydrolysed thorium ions by the outer layers of the fungal cell wall. This second process contributes the major part (approximately 95%) of the overall thorium biosorptive uptake observed (170mg g^{-1} biomass). Thorium uptake by the fungus was again found to be maximal at pH 4, but was not inhibited significantly by the presence of either Fe^{2+} or Zn^{2+}.

The effective use of biosorbents for some processes will require that the metals can be removed in order to recycle the biosorbant and/or recover the bound metal. Non-viable biomass of *R. arrhizus* can be used to recover uranium, which can subsequently be eluted using a bicarbonate solution (Tsezos, 1984).

Penicillium chrysogenum and activated sludge microflora have been used for the biosorption of radium (Tsezos & Keller, 1983). In particular, the adsorption of radium by *P. chrysogenum* exceeded the capacity of conventional adsorbants by a factor of almost fourteen. The fungus, *Penicillium digitatum*, has been used for uranium accummulation. A solution of EDTA was used to extract uranium from the metal-loaded mycelium (Galun *et al.*, 1983) and it was found that after metal extraction the capacity of the biomass to adsorb uranium was doubled. However, the

efficiency of metal extraction by EDTA was poor (61%), when compared to extraction by $NaHCO_3$ (94%) and $(NH_4)_2CO_3$ (97%). The presence of iron was shown to inhibit uranium uptake by *P. digitatum* (Galun et al., 1984). The biosorption of radionuclides by fungal biomass suggests that these biosorbents may be used in the treatment of liquid nuclear waste.

Anions, such as EDTA and SO_4^{2-}, are able to decrease the amounts of certain metal ions sorbed by *R. arrhizus* biomass (Tobin *et al.*, 1987). The study revealed that the presence of ligands in solution can dramatically decrease metal uptake by fungal biomass.

Fungi have been studied for their ability to remove other metals, as well as radionuclides, from solution. Heat-killed mycelium of *Penicillium spinulosum* had a nine-fold increased affinity for copper over that observed for living mycelium (Townsley *et al.*, 1986). In the same study, the ability of *P. spinulosum* and *Aspergillus niger* to adsorb gold was also investigated. Also, non-living biomass of *Saccharomyces cerevisiae* and *R. arrhizus* exhibited higher metal-uptake capacity than living biomass for the uptake of copper, zinc and cadmium (Kuyucak & Volesky, 1988b).

Metal recovery by algal biomass.

Chlorella vulgaris, a common green alga, can accumulate gold from solution (Greene *et al.*, 1986). The amount of gold bound by the organism corresponded to 10% of the dry weight of the algal cell. Under certain conditions, cyanide and thiourea complexes of gold were strongly bound by the algae. Thiourea was found to be effective in stripping bound gold from the biomass.

Algal biomass of *Sargassum natans* and *Ascophyllum nodosum* out-performed ion-exchange resins in sequestering respectively gold and cobalt from solutions

(Kuyucak & Volesky, 1988b). The gold deposited on *S. natans* biomass was eluted by a mixture of 0.1M thiourea and 0.02M ferric ammonium sulphate solution at pH 5. Sequestered cobalt was very effectively stripped from the biomass of *A. nodosum* using a solution of 0.1M $CaCl_2$ at pH 3. More recent work on cobalt uptake by *A. nodosum* has demonstrated that the metal accumulation mechanism is principally one of ion-exchange (Kuyucak & Volesky, 1989).

Commercial processes for metal recovery involving the use of non-living biomass.

Advanced Mineral Technologies, Inc. (AMT) based in the United States has developed a wastewater treatment and metal recovery technology (Brierley et al., 1985). The process is known commercially as AMT-BIOCLAIM and the core of the technology is based on a granulated, non-living biosorbent prepared from a microbial species. The granules have a high capacity for metal cations (86mg Ag g^{-1}, 214mg Cd g^{-1}, 152mg Cu g^{-1}, 601mg Pb g^{-1} and 137 mg Zn g^{-1}) with efficient removal (> 99%) from dilute (10-100mg metal l^{-1}) metal solutions. The metal recovery granules also accumulate gold (394 mg g^{-1}) from gold cyanide solutions. The granules are stable and can be employed in either fixed-bed canisters or fluid-bed reactor systems for the treatment of wastewaters and metal recovery. The granules can be recycled following stripping of the metals. Advantages of the AMT-BIOCLAIM system include:

(1) Effluent metal concentrations meet, and are often lower than, the strictest regulations.

(2) The process is implemented with simple and proven fluidized-bed engineering technology at low capital cost.

(3) The system offers a low operating cost.

(4) The technology effectively responds to shock loads of metal.

(5) AMT-BIOCLAIM is particularly effective at removing pollutant metals at low concentrations (e.g. < 50 ppm).

(6) The pollutant metals are converted to a metal product, eliminating the cost and liability of toxic sludge disposal.

However, microbial-based processes for metals reclamation have not yet been widely used. One of the main reasons is the inability of microbial systems to deal with the large quantities of wastewaters produced by industry. The instability of microbial preparations in the harsh conditions of industrial wastes also presents a problem. Also, the cost of setting up a new plant for the production of biomass especially for metal accumulation is high and reduces the economic viability of such a system. Recent advances in the technology of microbial metal recovery may overcome these problems.

The ability of fungal biomass to accumulate thorium from solution under conditions of pH and chemical composition encountered in process waste streams has been determined (Gadd & White, 1989). Fungi used in the study included *Aspergillus niger*, *Penicillium chrysogenum* and *Saccharomyces cerevisiae*, all of which were found capable of accumulating the radionuclide under the conditions imposed. To cope with the large amounts of mine or wastewaters involved in metal recovery systems a method for the continuous recovery of uranium from biologically leached solutions using immobilized biomass of *R. arrhizus* has been devised (Tsezos *et al.*, 1989). The technique involves the use of sodium bicarbonate as an eluate.

Large amounts of biomass are available as by-products of commercial enterprises which exploit the characteristics of certain fungi. These include the antibiotic and organic acids industries, and organisations which are involved in enzyme production from fungi and in polymer production for the pharmaceutical

and food industries. The present system of dumping biomass will soon become unacceptable and alternative methods of disposal have been sought. The use of biomass for animal feedstuff has certain problems associated with it, as approval has to be obtained for microbial feedstocks and extensive washing is required which can be costly (Townsley et al., 1985). Biomass could also be used as a fertilizer but possible effects of unwanted metabolites have to be considered. The recovery of metals from wastewaters would provide an alternative use of this waste fungal biomass. The economic viability of fungal metal recovery processes would be greatly improved if waste biomass was used for this purpose.

The work described in this chapter was devoted to determining the ability of *Aspergillus niger* waste mycelium, produced by the industrial surface and deep fermentation methods of citric acid production, to adsorb various metals from solution. The two different types of mycelium were investigated for metal accumulation as they differ both morphologically and in their mode of production. The surface produced mycelium appeared as a brown, solid mass with a lighter coloured, convoluted undersurface. Spores were also present with the biomass. This type of biomass is recovered by being "scooped" from the surface of the growth medium. The mycelium produced by the deep fermentation method was obtained as a blue coloured mat as this mycelium is incubated with ferrocyanide (blue) during growth and is collected by filtration, which causes its' mat-like appearence. This mycelium was more filamentous in nature than the surface produced mycelium. Both types of fungal waste (produced by Sturges Biochemicals Ltd., Selby, North Yorkshire) are at present homogenized and dumped into a local river. Thirty tons of waste mycelium is dumped in this way each week and the factory is set to increase production so that, in the near future, sixty tons of mycelium will be produced.

MATERIALS AND METHODS.

Ability of *Aspergillus niger* waste mycelium (surface-produced) to remove various metal ions from solution.

Fermentation waste (50g in 100ml sterile, distilled water) was homogenized for five minutes in an MSE Omni-mixer at half-speed. The mycelium was collected by centrifugation (4000 r.p.m. for 10 minutes) and washed several times with sterile distilled water to remove any nutrient medium that the fungus was originally grown in. The mycelium was collected after each wash by centrifugation. Mycelium (3g fresh weight or 0.4272g dry weight) was added to Erlenmeyer flasks (250ml) containing distilled water (100ml) amended with either 50µg Cu, Cd, Co or Ag ml^{-1}. The pH values of the solutions were not adjusted. The metal salts used were $CuCl_2.2H_2O$, $Cd(NO_3)_2.4H_2O$, $CoCl_2.2H_2O$ and $AgNO_3$. Control flasks (without added mycelium) were set up to determine any non-specific metal adsorption occurring in the flasks. All flasks were shaken (150 r.p.m., 25^0C) for three hours. The contents of each flask were centrifuged (4000 r.p.m. for 10 minutes) and the supernatant poured off for subsequent metal analysis using a Perkin-Elmer 460 atomic absorption spectrophotometer. As a control for metal analysis purposes, mycelial waste (3g) was shaken (150 r.p.m., 25^0C) in distilled water (50ml) only for three hours. The flask contents were centrifuged and the supernatant used to make up solutions containing known amounts of silver. The amount of metal determined to be present in these solutions was compared to that of commercially available standards using atomic absorption spectroscopy (AAS). Any interference caused by this supernatant was corrected for to obtain corrected final readings.

Effect of pH on silver accumulation by *A. niger* mycelial waste (surface-produced).

Samples of distilled water (50ml) containing 20µg Ag ml-1 were adjusted to either pH 1, 2, 3, 4 or 5 using nitric acid (4M). Buffers were not used to maintain pH as their chemical constituents may have affected metal accumulation due to the formation of metal complexes. Waste mycelium (1g fresh weight) was added each flask and the mixtures shaken (150 r.p.m., 25^0C) for two hours. Flask contents were centrifuged (4000r.p.m. for 10 minutes) and the supernatant analysed for silver content by AAS. The final pH value of the supernatant was recorded. Control flasks without mycelium were included to determine any non-specific metal adsorption that occurred at the different pH values. Another set of control flasks containing mycelial waste (1g) and distilled water (50ml) only, were adjusted to the range of pH values used in the experiment. The flasks were shaken (150 r.p.m., 25^0C) for two hours after which time the contents of the flasks were filtered using Sartorius membrane filters (0.45µm pore size) and the filtrate used to make up solutions containing known amounts of silver. These solutions were analysed by AAS to check if the filtrate interfered with silver analysis. Any changes were corrected for to give the final readings presented in the results.

Effect of length of incubation on silver accumulation by *A. niger* waste mycelium (surface-produced)

Homogenized mycelium (5g) was added to distilled water (500ml) containing 20µg Ag ml^{-1} (as $AgNO_3$) , adjusted to pH 4.0 using nitric acid (4M). Before addition of mycelium the silver solution was shaken (150 r.p.m.) for 2 hours to allow it to reach the experimental temperature of 25^0C. The mixture was then shaken (150 r.p.m., 25^0C) for three hours. Samples (10ml) were taken at 1, 5, 10, 20, 60, 120

and 180 minutes after addition of the mycelium and were filtered through a Sartorius membrane filter (0.45µm pore size). The filtrate was analysed for silver content by AAS. The whole experiment was repeated three times to check the reproducibility of the results.

Effect of temperature on silver accumulation by *A. niger* waste mycelium (surface-produced).

Homogenized mycelium (1g) was added to distilled water (100ml) in an Erlenmeyer flask (250ml) containing 20µg Ag ml^{-1} (as $AgNO_3$). All solutions used were adjusted to pH 4 and either heated or cooled to the desired experimental temperatures before adding the mycelial waste. The mycelial waste/Ag solution was stirred magnetically using a BTL magnetic stirrer (maximum speed) and also heated by the same apparatus. All mixtures were stirred for three hours in triplicate at the following temperatures: 4, 25, 30, 37, 50 and 80^0C to determine the effect of temperature on metal uptake. Solutions were filtered through a Sartorius membrane filter (0.45µm pore size) and the filtrate analysed for metal content using a Perkin Elmer 460 atomic absorption spectrophotometer. An increase in filtrate colour was observed at the higher temperatures used in the experiment. This difference in filtrate colour may have altered the amount of silver detected in the filtrate by atomic absorption spectroscopy (AAS) and so controls were deemed necessary to check this. Mycelium (1g) was incubated for three hours in distilled water only (100ml) at the temperatures used in the experiment and were filtered using Sartorius membrane filters. The filtrates were used to make up standards of silver solutions which were subsequently analysed by AAS and compared with commercially available standards. Any change in silver concentration, as determined by AAS, caused by the filtrates was corrected for in the final results.

Effect of presence of Fe^{2+} and Cu^{2+} on silver accumulation by *A. niger* waste mycelium (surface produced).

Homogenized mycelium (1g) was added to distilled water (100ml) containing either 20µg Ag ml^{-1} only, 20µg Ag ml^{-1} and 20µg Cu ml^{-1} or 20µg Ag ml^{-1} and 20ug Fe ml^{-1}. The metal salts used were $AgNO_3$, $Cu(NO_3)_2.3H_2O$ and $Fe(NO_3)_3.9H_2O$. All solutions were adjusted to pH 4 with nitric acid (4M) before addition of the mycelium. Flasks were shaken (150 r.p.m., 25^0C) for two hours after which time they were filtered through Sartorius membrane filters (0.45µm pore size). The filtrates were analysed for silver content by AAS. Controls contained the above combinations of metals with no mycelium present. The filtrates of control flasks were also analysed for silver content.

Desorption of silver from *A. niger* mycelial waste (surface produced).

Homogenized mycelium (1g) was added to distilled water (100ml) containing 20µg Ag ml^{-1}, adjusted to pH 4 with nitric acid (4M). This mixture was shaken (150 r.p.m., 25^0C) for three hours. The flask contents were filtered through a Sartorius membrane filter (0.45um pore size) and the mycelium plus adsorbed silver collected. Three samples of this mycelium were digested using concentrated sulphuric acid and hydrogen peroxide (see Appendix) and the silver content of the mycelium determined. Samples (1g) of metal-laden mycelium were then added to solutions (100ml) of either H_2SO_4 (0.1 and 0.5M), HNO_3 (0.1 and 0.5M) or distilled water alone and shaken (150 r.p.m., 25^0C) for three hours. Mycelium was then collected by filtration through Sartorius membrane filters (0.45µm pore size) and digested using a mixture of concentrated sulphuric acid and hydrogen peroxide

(see Appendix) so that its metal content could be analysed by AAS. The amount of silver in acid-treated mycelium was compared to that of non-acid treated, metal-laden mycelium to determine the amount of metal desorbed from the mycelium.

Ability of *A. niger* waste mycelium (produced by the deep fermentation method of citric acid production) to accumulate silver from solution.

Waste mycelium (50g in 100ml sterile, distilled water) were homogenized in an MSE Atomix Omnimixer at half speed for 5 minutes. The mycelium was collected by centrifugation (4000 r.p.m. for 15 minutes) and washed with sterile distilled water three times to remove any excess of the nutrient medium that the fungus was originally grown in. After each wash the biomass was collected by centrifugation. The homogenized mycelium (1g) was added to distilled water (100ml) containing 20µg Ag ml^{-1}, adjusted to pH 4 with nitric acid (4M) before the addition of the mycelial waste. This mixture was shaken (150 r.p.m., 25^0C) for three hours, after which time the flask contents were filtered through a Sartorius membrane filter (0.45µm pore size). The filtrate was subsequently analysed for silver content by AAS to determine the amount of silver accumulated by the waste mycelium.

RESULTS AND DISCUSSION.

Ability of *A. niger* waste mycelium (surface produced) to remove various metal ions from solution.

The waste mycelium showed differential accumulation of the metals used in the experiment. (Fig. 4.1). Of the four metals, silver was most efficiently removed from solution (99% removal). Cadmium was accumulated by the mycelium to a lesser extent (38% removal), while the accumulation capacity of the biomass for copper (15% removal) and cobalt (10% removal) proved relatively low. This seemingly selective silver accumulation (sorption) by the waste *A. niger* biomass correlates with recent work concerning metal accumulation by 32 species of fungi (Pighi *et al.*, 1989). The fungi were tested for their ability to accumulate the metals copper, cadmium, nickel, silver and lead from aqueous solutions and silver was accumulated selectively by all but one of the strains tested. Copper was accumulated in higher amounts than cadmium by the *Aspergillus* species. These results contrast with those obtained for *A. niger* mycelial waste as this biomass showed a preference for cadmium over copper accumulation (Fig. 4.1). Other studies on silver accumulation by fungi have found that *Rhizopus arrhizus* biomass can accumulate a maximum of 50mg Ag g^{-1} dry weight (Tobin et al., 1984) and that a *Phoma* species (Pumpel & Schinner, 1986) had a maximal capacity of 20mg Ag g^{-1} dry weight. The amount of silver accumulated by *A. niger* waste mycelium was calculated to be 11.59mg g^{-1} dry weight which is less than that observed in the previous studies. Storage of a *Phoma* species PT 35 at 4^0C caused a decrease in silver accumulation capacity of the fungus (Pighi *et al.*, 1989). The *A. niger* biomass used in these experiments was stored at 4^0C for two weeks before being tested for its metal

accumulation capacities and it is possible that this storage period decreased the accumulation abilities of the mycelium. Metal uptake by young *A. niger* waste mycelium may be very high indeed. Silver recovery from solution by the waste mycelium was considered significant enough to carry on with studies on factors which affected accumulation of the metal.

Effect of pH on silver accumulation by A. niger waste mycelium (surface produced).

Silver accumulation by the mycelium remained static over the ranges of pH used (Fig. 4.2). These results contrast with those of Pumpel & Schinner (1986) who found that a *Phoma* species showed the highest silver accumulation capacity at neutral pH. The waste biomass was not incubated with an excess of silver in the medium and it exhibited almost 100% metal removal at all pH values. The effect of pH on metal accumulation may only be observed when a fungus is inhibited with an excess of metal, as in the study made on silver accumulation by *Phoma* (Pumpel & Schinner, 1986).

Effect of length of incubation on silver accumulation by *A. niger* waste mycelium (surface produced).

Accumulation of metal began practically immediately after initial contact of the fungus with the metal solution (Fig. 4.3). Accumulation reached a maximum value 60 minutes after addition of the biomass to the reaction mixture. A very similar time pattern of silver accumulation was observed with *Phoma* strain PT 35 (Pumpel & Schinner, 1986).

Effect of temperature on silver accumulation by *A. niger* waste mycelium (surface produced).

Silver accumulation was steady up to a temperature of 50^0C (Fig. 4.4). At 80^0C a significant drop in metal accumulation was observed. The amount of silver accumulated dropped from a value of around 12mg Ag g-1 dry weight from 4 to 50^0C, to approximately 9mg Ag g^{-1} dry weight at 80^0C. However, silver accumulation can be assumed to be relatively constant over a wide range of temperatures. Pumpel & Schinner (1986) also found a decrease in silver accumulation at temperatures above 50^0C during their study on *Phoma* strain PT 35. Temperature has varying effects on the uptake of metals by fungi. For example, copper uptake by *Penicillium spinulosum* was directly proportional to the incubation temperature (Townsley *et al*., 1985). However, temperatures above 45^0C were not examined. A study by Kuyucak & Volesky (1988b) found that the accumulation of metals by a wide variety of biomass samples (including *Aspergillus* species) was not considerably affected by temperatures up to 40^0C, but temperatures of 80^0C and above significantly inhibited metal uptake. These results are similar to my own. Clearly, different uptake mechanisms are involved for different metals and different fungal species. The present strain of *A. niger* used for citric acid production by Sturges Ltd. will have originated from a mutation programme and may be very different to those strains used in other studies. The method of biomass production, such as surface type or submerged type growth may alter the fungal characteristics for metal uptake. Also the medium used for fungal growth may contain many compounds which will bind to the fungal cell wall and possibly inhibit metal accumulation. It appears that, for *A. niger* waste mycelium, high temperatures have some kind of impact on the metal-binding sites of the cell wall, thereby reducing the uptake capacity of the mycelium. The relative insensitivity of the waste mycelium to

Figure 4.1.

Accumulation of different metals by *Aspergillus niger* waste mycelium produced by the surface fermentation method of citric acid production.

(Means of triplicates $^{+}/_{-}$ S.D.)

Figure 4.2.

Effect of initial pH on silver accumulation by surface produced *A. niger* waste mycelium.

(Means of triplicates $^{+}/_{-}$ S.D.)

Fig.4.1.

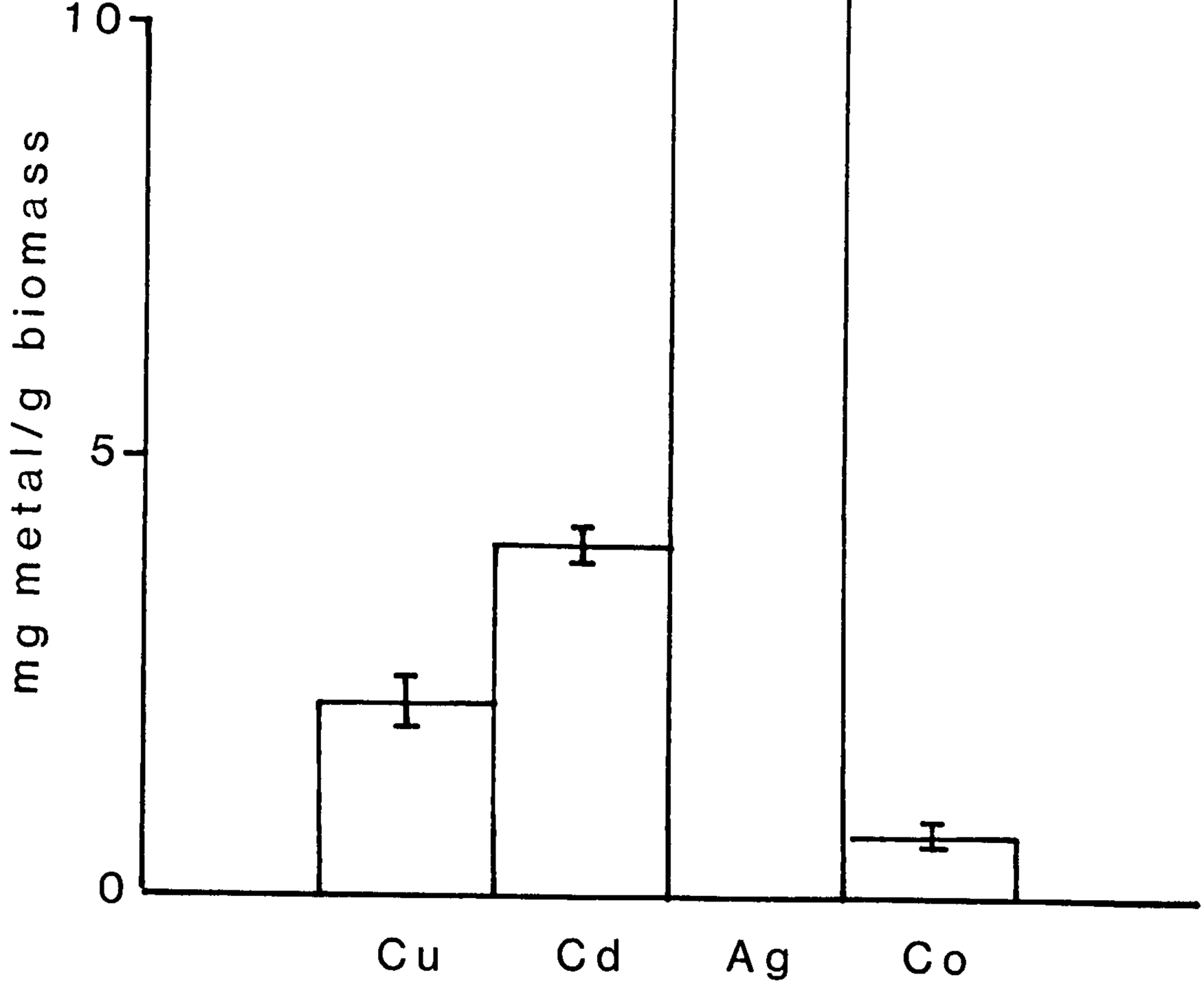

10
5
0
mg metal/g biomass
Cu
Cd
Ag
Co

Fig.4.2.

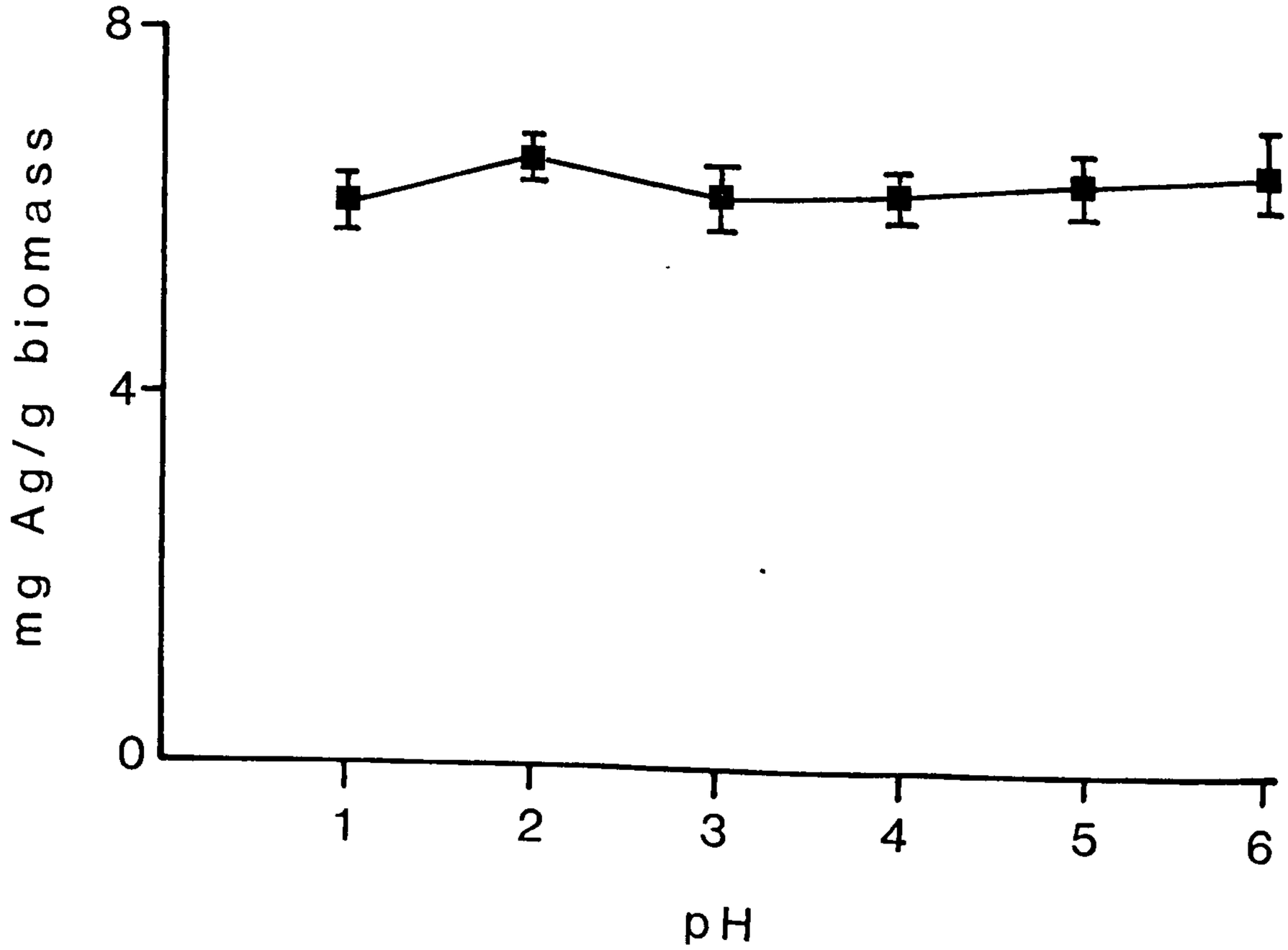

8
4
0
mg Ag/g biomass
1
2
3
4
5
6
pH

Figure 4.3.

Effect of time on silver accumulation by surface produced *A. niger* waste mycelium.

(Means of triplicates $^{+}/_{-}$ S.D.)

Figure 4.4.

Effect of temperature on silver accumulation by surface produced *A. niger* waste mycelium.

(Means of triplicates $^{+}/_{-}$ S.D. * significant decrease in silver accumulation compared with the value obtained at 25^{0}C, $p < 0.05$)

Fig.4.3.

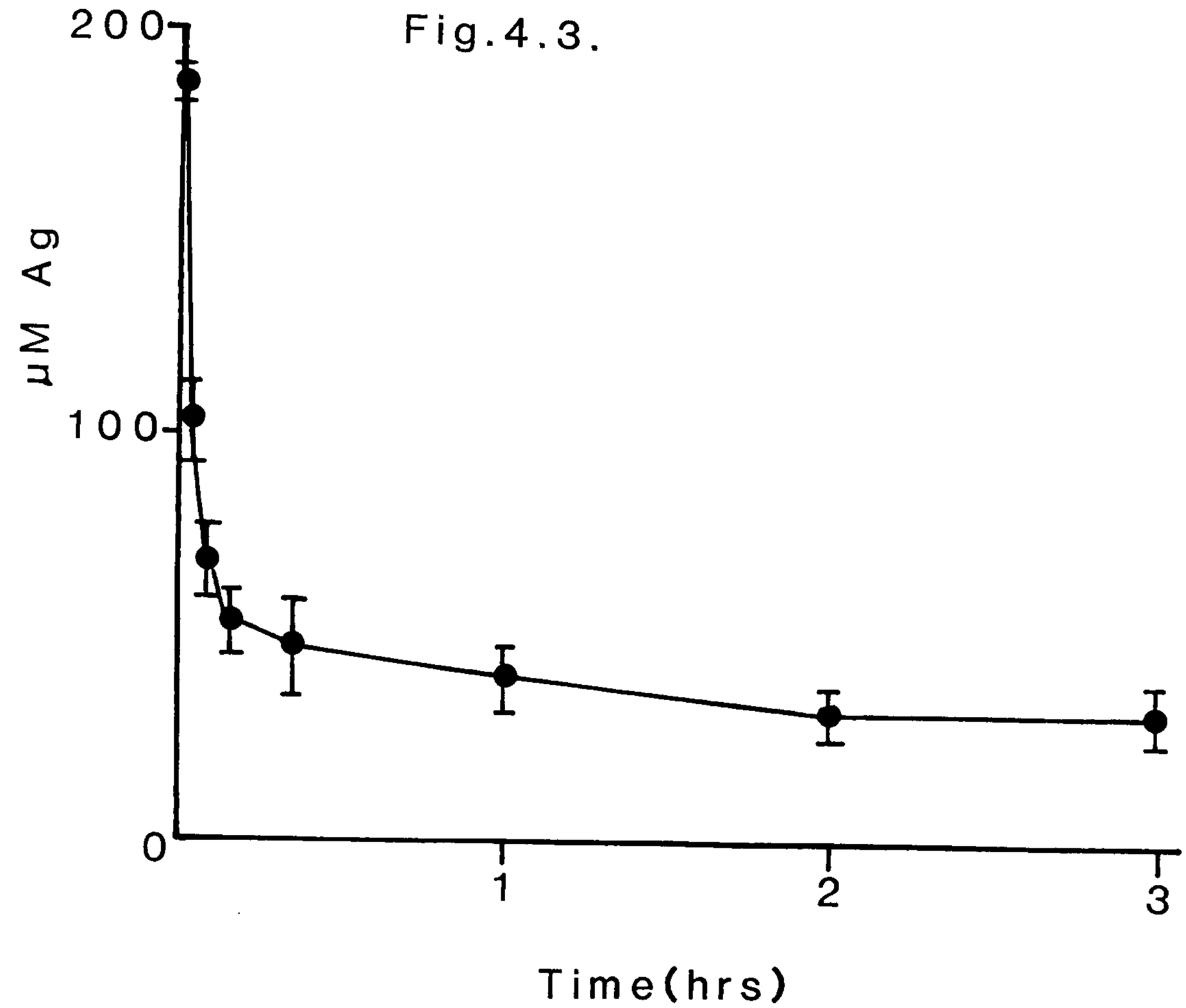
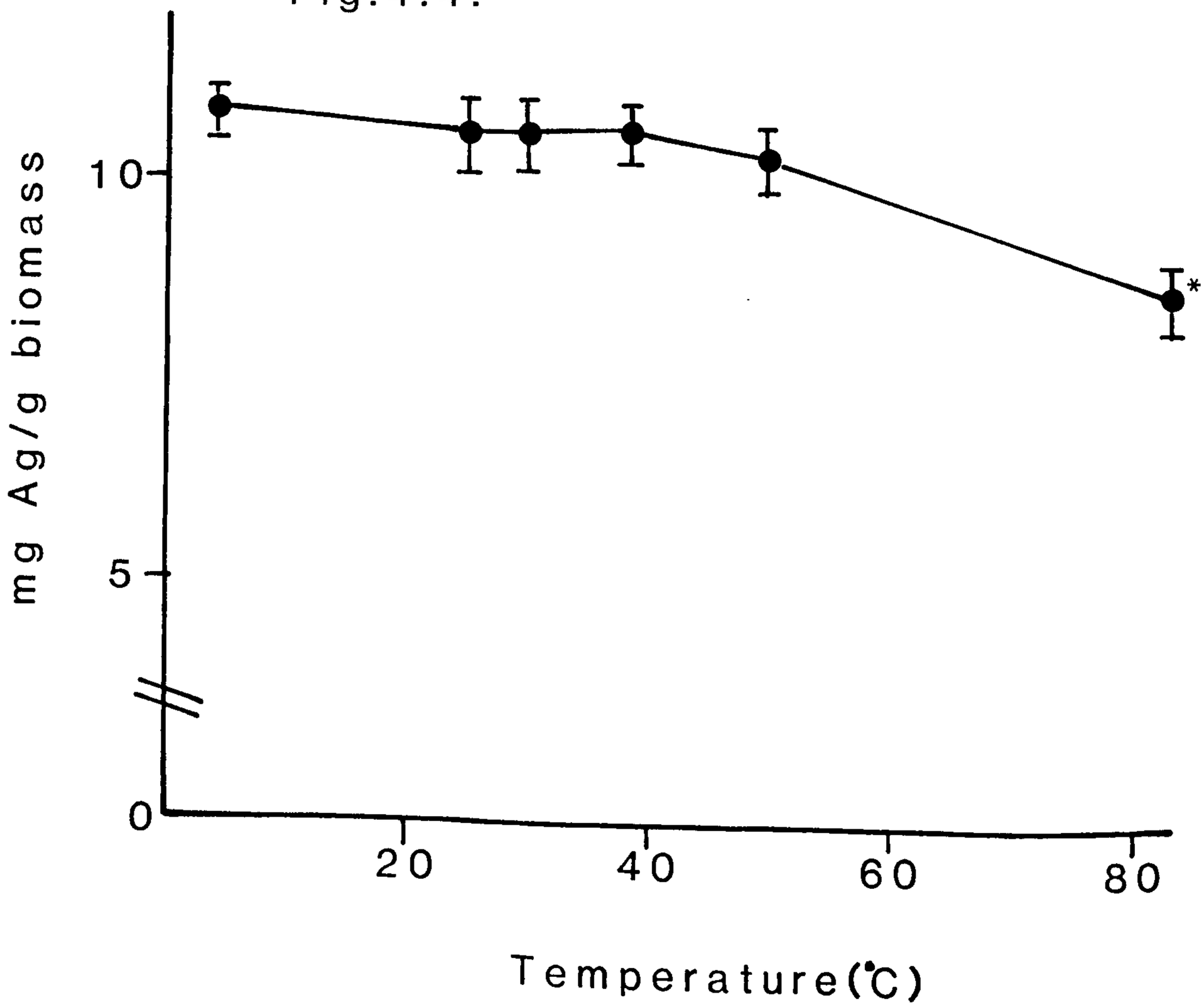

200
100
0
µM Ag
1
2
3
Time(hrs)

Fig.4.4.


10
5
0
mg Ag/g biomass
*
20
40
60
80
Temperature(°C)

extreme temperatures may be particularly useful when considering industrial applications of the metal uptake phenomenon.

Effect of the presence of Fe^{2+} and Cu^{2+} on silver accumulation by *A. niger* mycelial waste (surface produced).

The presence of Fe^{2+} did not inhibit the uptake of silver by the waste mycelium (Table 4.1) at the metal concentrations used in the experiment. Ferrous ion inhibits the uptake of uranium by both *Penicillium digitatum* (Galun *et al.*, 1984) and *Rhizopus arrhizus* (Tsezos & Volesky, 1982a), but has no effect on thorium accumulation by *R. arrhizus* (Tsezos & Volesky, 1982b). The different effect of Fe^{2+} on the uptake of metal ions is indicative of different mechanisms of metal accumulation. As iron (as salts and soluble complexes) is abundant in both geological and and technological environments (Galun *et al.*, 1984) it can be expected to be amongst one of the more common metal elements found in both natural and industrial water. The metals inability to affect silver accumulation by *A. niger* waste mycelium has obvious bearings on the industrial use of the mycelium for metal removal from effluents.

Copper, as Cu^{2+}, only slightly inhibited silver uptake by the waste mycelium (Table 4.1). This result again demonstrates the apparent selective silver uptake ability of this mycelial waste.

Desorption of silver from *A. niger* mycelial waste (surface produced).

Both sulphuric and nitric acids desorbed silver from the mycelium (Table 4.2). However, the amounts desorbed were much less than than other workers have achieved. Nitric acid was slightly more effective than sulphuric acid at silver

removal, removing around 40% as compared to 35% removal achieved by sulphuric acid. Desorption of cobalt from *Ascophyllum nodosum* biomass using a dilute acid solution of $CaCl_2$ was extremely effective, almost all of the sequestered metal being removed in this way (Kuyucak & Volesky, 1988b). Also, up to 99% removal of uranium bound to mycelium of *Penicillium digitatum* has been achieved using alkali carbonate solutions (Galun *et al.*, 1983). Clearly desorption of metals from biomass can be achieved under the right conditions. Alkali carbonates were not used for desorption of silver from mycelial waste as they would have formed an insoluble precipitate with the silver. More efficient metal removal may be achieved by decreasing the contact time of the waste with the silver solution and increasing the time mycelium, with bound silver, is incubated with the acid solutions. Clearly other methods of silver desorption are needed to be examined. As the mycelium is in abundant supply from citric acid production, the desorption of metal from the biomass may not be necessary to make the process economically viable. Ashing of the mycelial waste to recover the bound silver may provide an attractive alternative.

Ability of *A. niger* waste mycelium (produced by the deep fermentation method of citric acid production) to accumulate silver from solution.

This type of waste mycelium removed less silver from solution (3.8mg g^{-1} biomass $^{+}/_{-}$ 0.1) than the mycelium produced by the surface fermentation method of citric acid production (11.9mg g^{-1} biomass $^{+}/_{-}$ 0.5). Perhaps the silver accumulation capacity of the mycelium was affected by storage at $4^{0}C$ for two weeks. The two types of mycelium are grown differently and this may result in the difference in metal uptake observed. The two types of mycelia are very different morphologically and this morphological difference may result from a difference in cell wall structure and content. These possible chemical differences may result in

Table 4.1. Effect of the presence of Fe^{2+} and Cu^{2+} on silver accumulation by *A. niger* mycelial waste (surface-produced).

	mg Ag/g dry weight
Mycelium + Ag	11.9 ± 0.5
Mycelium + Ag + Cu	*11.1 ± 0.1
Mycelium + Ag + Fe	11.5 ± 0.1

(Means of triplicates ± S.D. *significant decrease in silver accumulated compared to control, $p < 0.05$).

Table 4.2 Desorption of silver from *A. niger* mycelial waste (surface-produced)

	mg Ag/g dry weight	% Ag desorbed
Original Silver content of mycelial waste	11.3 ± 0.5	-
Silver content after Desorption: Control (H_2O)	10.8 ± 0.9	-
0.1M H_2SO_4	*7.2 ± 0.4	36
0.5M H_2SO_4	*7.6 ± 0.7	33
0.1M HNO_3	*6.3 ± 0.5	44
0.5M HNO_3	*6.9 ± 0.6	39

(Means of triplicates ± S.D. *significant decrease in metal content of mycelium caused by desorption treatment, $p < 0.05$).

differential metal accumulation. The deep fermentation produced mycelium appears blue due to its treatment with ferrocyanide used as a metal complexing agent in the fermentation process. It is possible that any excess ferrocyanide present in the mixture used to test for silver accumulation complexed silver and inhibited its uptake by the waste mycelium. Whatever caused the decrease in metal accumulation it is clear that the use of surface produced mycelial waste is preferable for silver recovery from solution.

CONCLUSIONS.

The use of mycelial waste from the surface fermentation method of citric acid production seems to have promise for use in silver reclamation from industrial effluents or natural waters. Advantages of the use of this mycelium for silver accumulation include:

(1) Apparent specific selectivity for the silver (Ag^+) ion.

(2) Relative insensitivity to extremes of pH and temperature values.

(3) Rapid silver accumulation.

(4) The mycelium is a waste product and therefore effectively costs nothing to produce.

(5) Large quantities of biomass are available for use.

At present an efficient method for metal desorption from this waste has not been found. This may limit the proposed use of the waste mycelium. More work is needed to find an efficient desorption method. The ability of the biomass to accumulate silver complexed with thiosulphate, acetic acid, or cyanide needs to be demonstrated, as silver is usually complexed with these materials in processing streams and when present as a pollutant (Hutchins *et al.*, 1986). The lower silver accumulation by mycelium produced by the deep fermentation method of citric acid production may indicate that the metal uptake ability of the mycelium is affected by the silver cyanide complex. However, as mentioned above, this lower metal uptake may be due to other differences between the two mycelium caused by differences in their methods of production.

5. APPENDIX.

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1) Chemical analyses

a) Colorimetric analysis of the total protein content of isolated *Neurospora crassa* cell walls. (Stickland, 1951)

Sodium hydroxide (6N, 0.5ml) was added to a lyophilized cell wall sample (20mg) and heated at 100^0C for five minutes. The samples were cooled and a 2.5% (w/v) $CuSO_4.5H_2O$ solution (1ml) was added. The samples were mixed (using a whirlimixer) and left to stand for five minutes after which time they were centrifuged at maximum speed in a bench centrifuge for 10 minutes. The optical density of the supernatant solution was read at 540nm against a distilled water blank. The protein content of the test samples was determined by reference to a standard curve (0 - 5mg protein ml^{-1}) prepared from Bovine Serum Albumin.

b) Colorimetric analysis of the total sugar content of isolated *Neurospora crassa* cell walls. (Dubois *et al.*, 1956)

A cell wall sample (25 or 50mg) was hydrolysed in hydrochloric acid (3N, 2ml) for three hours at 100^0C, cooled and then neutralized with sodium hydroxide (3N). The suspension was centrifuged at maximum speed in a bench centrifuge for ten minutes and a sample of supernatant removed (0.1ml) which was subsequently diluted in 9.9ml distilled water (1 in 100 dilution). A sample (1ml) of this diluted solution was tested for sugar content in the following way. Phenol solution (5% w/w in water, 1ml) was added to the test sample and mixed well. Concentrated H_2SO_4

(5ml) was added rapidly onto the liquid surface the resultant solution being mixed at once and then left to stand for ten minutes. After this time the tubes were cooled to 25^0C in water for 15 minutes. The optical density of the test samples were determined at 480nm. The total sugar content of the samples was determined by reference to a standard curve (0 - 0.072 mg glucose ml^{-1} or 0 - 0.4 µM glucose ml^{-1}) prepared using glucose. A reagent blank was prepared using distilled water (1ml) plus the chemical reagents used in the assay

c) Turbidimetric analysis of sulphate-S ion. (Hesse, 1971)

To 5ml filtrate in a 25ml volumetric flask, $BaCl_2.2H_2O$ (1g) and gum acacia (0.25% w/v, 2ml) were added and mixed. The volume was made up to 25ml with distilled water and the turbidity resulting from the precipitation of barium sulphate was measured at 470nm. The $SO4^{2-}$-S concentration was determined by reference to a standard curve (0-100µg SO_4^{2-}-S ml^{-1}) prepared from a standard solution of $Na_2SO_4.10H_2O$.

2) Media used for the maintenance and growth of microorganisms.

a) Czapek Dox liquid medium (Oxoid)

Formula	(per litre H_2O)
Sodium nitrate	2.0g
Potassium chloride	0.5g
Magnesium glycerophosphate	0.5g

Ferrous sulphate	0.01g
Potassium sulphate	0.35g
Sucrose	30.0g

pH 6.8 (approx.)

A proprietary formulation (Oxoid) of Czapek Dox liquid medium was routinely used, prepared by suspending 33.4g of the powder in one litre of distilled water. This was mixed well until dissolved and sterilized by autoclaving at 115^0C for 20 minutes.

b) Czapek Dox agar (Oxoid).

Formula	(per litre H_20)
Sodium nitrate	2.0g
Potassium chloride	0.5g
Magnesium glycerophosphate	0.5g
Ferrous sulphate	0.01g
Potassium sulphate	0.35g
Sucrose	30.0g
Agar (Oxoid No.3)	12.0g

pH 6.8 (approx.)

A proprietary formulation (Oxoid) of Czapek Dox agar was routinely prepared by suspending 45.4g of the powder in one litre of distilled water. The medium was mixed well and sterilized by autoclaving at 115^0C for 20 minutes.

c) Malt extract broth (Oxoid).

Formula	(per litre H_2O)
Malt extract	17.0g
Mycological peptone	3.0g

pH 5.4 +/- 0.2

A proprietary formulation (Oxoid) was prepared by suspending 20g of the powder in one litre of distilled water. The suspension was mixed well and sterilized by autoclaving at 115^0C for 10 minutes.

d) Malt extract agar (Oxoid)

Formula	(per litre H_2O)
Malt extract (Oxoid L39)	30.0g
Mycological Peptone (Oxoid L40)	5g
Agar	15g

pH 5.4 +/- 0.2

A proprietary formulation (Oxoid) was prepared by adding 50g of the powder to one litre of distilled water. The suspension was mixed well and sterilized by autoclaving at 115^0C for ten minutes.

e) Raistricks' medium (Raistrick & Vincent, 1948).

Formula	(per litre H_2O)
$NaNO_3$	2.0g
$MgSO_4.7H_2O$*	0.5g
$FeSO_4.7H_2O$**	0.01g
KH_2PO_4	1.0g
Sucrose***	To required amount

pH adjusted to required value using sodium hydroxide (usually pH 6.8).

Media constituents were added separately to one litre of distilled water and dissolved before adding the next constituent. The bulk medium was sterilized by autoclaving at 115^0C for 15 minutes.

* $MgSO_4.7H_2O$ was autoclaved separately to the bulk medium and added aseptically after sterilization.

** $FeSO_4.7H_2O$ was filter sterilized and added aseptically to the sterilized bulk medium.

*** Sucrose at high concentrations (above 1% w/v) tended to caramelize during autoclaving. To prevent this stock sucrose solutions were prepared, filter sterilized and added aseptically to the sterilized bulk medium.

3. Digestion of *Aspergillus niger* waste mycelium produced by the surface fermentation method of citric acid production.

After exposure to a silver solution, the waste mycelium was collected by filtration and dried to constant weight at 50^0C. Concentrated H_2SO_4 (2ml) was added to each sample (approx. 50mg dry weight) and the mixture was warmed gently until the mycelium began to disperse. Hydrogen peroxide (100 vol, 30% H_2O_2) was then added dropwise until the reaction began to slow down. The total amount of hydrogen peroxide added was recorded. This method of digestion produced a clear solution. A sample (1ml) of the solution was added to a 25ml volumetric flask and distilled water then added up to the mark (This dilution brought the silver content of the sample into a suitable range for AAS). All samples were tested for silver content using a Perkin-Elmer 460 atomic absorption spectrophotometer.

Publications resulting from this research:

SINGLETON, I., WAINWRIGHT, M. and EDYVEAN, R.J.V. (1989). Some factors influencing the adsorption of particulates by fungal mycelium. Biorecovery, (In Press).

WAINWRIGHT, M., SINGLETON, I. and EDYVEAN, R. (1987). Use of fungal mycelium to adsorb particulates from solution. In : Biohydrometallurgy, Proceedings of the International Symposium, ed., Norris, P.R. and Kelly, D.P., Warwick, 1987 : 499-502.

Contributions to Symposia:

SINGLETON, I., WAINWRIGHT, M. and EDYVEAN, R. (1987). Adsorption of particulates by fungal mycelium. Eighth International Symposium on Environmental Biogeochemistry, Nancy, France, September, 1987 (Poster presentation).

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