STUDIES ON THE MICROBIOLOGY OF SILICON

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by

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SUMMARY

A study was made of the interactions between the element silicon, mainly as silicic acid, and various microbial processes.

The effect of silicon compounds on fungal growth was determined under both oligotrophic and nutrient-rich (copiotrophic) conditions. Mycelium of *Aspergillus oryzae* was grown from a spore inoculum added to ultra-pure water (upw) containing silicon compounds, but not in upw alone. Growth of other fungi also only occurred in upw when silicon compounds were added. Increased growth of fungi also followed the addition of silicon compounds to Czapek Dox medium. Silicic acid also increased the protein content of fungi grown under such nutrient-rich conditions. The fungi solubilised the insoluble silicon compounds under both oligotrophic and copiotrophic conditions. Silicon was not however, accumulated by fungi as electron-dense hyphal bodies. Addition of silicic acid to nutrient rich media also increased the growth of species of *Streptomyces*, but decreased the chlorophyll content of the alga, *Dunaliella parva;* the growth of two yeasts and the bacteria, *E. coli* and *S. aureus* also was not affected by silicon addition; the observed stimulatory effect therefore appears to be restricted to filamentous microorganisms.

The effect of silicon compounds on various microbial processes was also investigated. Silicic acid stimulated the production of citric acid by *Aspergillus niger*, but decreased nitrification and sulphur oxidation in this fungus. Silicic acid addition also led to a reduction in antibiotic production by species of *Streptomyces*.

Studies were initiated to study the possibility that fungi and bacteria can erode the surface of both bulk and porous silicon wafers. While no such surface erosion was evident, we observed that *E. coli* underwent extensive extreme pleomorphism when growing under starvation conditions for up to 14 days. Such pleomorphism consisted of the formation of bulbous protrusions from the normal rod, dumbbell-shaped cells and long filaments, these were up to 50μ in length (compared to the normal 1-3μ, rods). Such filamentation was clearly caused by the inability of the bacterial cells (rods) to separate on division. The observed bacterial pleomorphism was not however, silicon-specific, as it was also found to occur on titanium and glass surfaces. Such extreme pleomorphism may have important implications in relation to the growth of *E. coli* in low nutrient environments and may influence the bacterium's ability to affect pathogenesis.

While the microbiology of silicon has largely been neglected the results of this thesis show that there is considerable interaction between this element and microbial growth. Future studies should in particular be directed towards determining if silicon can be used as an energy source by microorganisms. Additionally, the observed phenomenon of extreme pleomorphism in *E. coli* is clearly worthy of further study.

CHAPTER ONE

SILICON IN THE BIOSPHERE

1.1 Introduction

1.2 Chemistry of silicon

Silicon (Si) is the second most abundant element on the earth, exceeded only by oxygen, and makes up about 27.7% of the earth's crust. Silicon exists mainly as oxy-compounds, and occurs as both amorphous and crystalline forms, as silicon oxide e.g. sand (silica), quartz, rock crystal, amethyst, agate, flint, jasper and opal. Silicon is also found in minerals such as asbestos, feldspar, clay and mica. Silicon and carbon are members of Group IV of the periodic table. Both elements have similar structures and show similar bonding. Silicon also resembles carbon in a number of closely related compounds. Carbon is the basis of the earth's vegetable and animal life, the central element of organic chemistry, while silicon dominates the inorganic domain of rocks (Pawlenko, 1986). The word silicon derives from the Latin word "silicis" meaning "flint". In contrast to inorganic silicon compounds, there are no naturally occurring organic silicon compounds, all of which have been created in the chemical laboratory (Pawlenko, 1986).

Silicon compounds have the following uses :

- Doped with boron, gallium, phosphorus, or arsenic, etc. silicon compounds can be produced for use in transistors, solar cells, rectifiers, and other electronic solid-state devices.

- Organic-silicon compounds i.e. silicones (e.g. Me_2SiCl_2) are industrially important products of silicon, and are prepared by hydrolysing organic silicon compounds.

- Silica as sand, is a principal ingredient of glass, a material with excellent mechanical, optical, thermal, and electrical properties.
- Silicon compounds are also used to make computer chips, lubricants, concrete and bricks and the silicon-based implants used in medicine.

1.3 Silicon in biology

Most microbiologists never come across the element silicon, probably because it is thought to be largely biologically unreactive, and is not transformed by microorganisms (Wainwright, 1997). Scientific interest in the biological role of silicon dates from the early 1900s when silicon was thought to be involved in the synthesis and structure of the connective tissue (Birchall, 1995). Silicon dioxide (SiO_2) is an integral part of protein matrix, and silica is reported to be an essential component of most living organisms and viruses. Voronkov *et al.*, (1975) note that sponges are a source of silica minerals, and that the silica content of sponges varies widely from 1 to 90 %, hard, rigid sponges have skeletons that consist of crystalline spicules of "cubic opal" or silicic acid of cubic symmetry. Silicon is essential for plant, animals and humans. However, large amounts of silicon are permanently present in the body only of primitive organisms; generally the higher the organism, the larger the ratio of C : Si in the body, ranges from 1:1 in plankton to 5000:1 in mammals, plants being intermediate, with ratios of 100:1 to 500:1. Although around 0.5 g of silicon is ingested per day in the human diet, only 20-30 mg is absorbed through the intestine into the bloodstream. The kidneys are responsible for the maintenance of a constant silicon concentration in the blood (Pawlenko, 1986).

Three types of silicon compounds are found in living organisms:

1- Insoluble silicon polymers (quartz, crystalline and amorphous polysilicon acids). These are not important in small amounts, but lead to lung disease (silicosis) if large quantities are inhaled.

2- Water-soluble inorganic compounds which can easily pass through the cell membrane (ortho- and oligo- silicic acids and their salts). These can be rapidly eliminated from the body.

3- Esters of ortho- and oligo- silicic acids combined with polysaccharides, phospholipids, cholesterol, choline, etc., that are soluble in organic solvents. Silicic acid esters of cholesterol have been isolated from feathers, and galactose silicates can be isolated from the blades of rye (Pawlenko, 1986).

An adequate supply of silicon is essential for the growth of hair and nails in humans, hair, horn and hoofs in mammals, and feathers in birds. Chickens maintained on silicon-free feed have malformed feathers, and their bones remain fragile and thin. For normal growth, chickens require only 0.003% silicon in their food (Carlisle, 1972). Fungi and bacteria can solubilise silicates, a process which may be important in biological weathering of rocks (Duff and Webley, 1963). Silicon compounds also increase bacterial growth and have been implicated in aggravating tubercular infection of the lung in patients suffering from silicosis (Price, 1932).

Silicon is present in biological systems as a silanate, an ether (or ester-like) derivative of silicic acid which may play a role in the structure of glycosaminoglycans and their protein complexes. Connective tissues including that of the aorta, trachea,

tendon, bone, and skin and its appendages contain much of the silicon that is retained in the body, (Seaborn and Nielson, 1993).

Nielsen (1988) found that the dietary silicon intake of humans varies greatly with the amount and proportion of the food of animal (silicon-low) and plant (silicon-high) origin consumed, and the amounts of refined and processed foods in the diet. Normally, refining reduces the silicon content of foods. However, silicate additives have been increasingly used (as anticaking or antifoaming agents) in prepared food and confections. Although this increases total dietary silicon, most of it is not bioavailable.

The silicon content of drinking water, and beverages made thereof, shows geographical variation; the concentration of silicon is high in hard water and low in soft water areas. Silicates from foods such as grains or silica supplements (i.e. silicon dioxide or sodium metasilicate) are not directly absorbed by the body. Such silica and silicates must also first be hydrolyzed in the stomach to form orthosilicic acid, which is the only form of silicon absorbed by the human body. Orthosilicic acid is present in living plants (orthosilicic acid, being the only silicon form that is absorbed by the roots from the soil), although none is left after plant processing (Epstein, 1994).

Silicon is not particularly toxic, but finely divided silicates or silica cause major damage to lungs. As has been mentioned, long term exposure to silicates, such as asbestos, causes severe health problems.

1.4 Microbiology of silicon

Except for studies on the accumulation of the element in diatoms, very little research has been devoted to the microbiology of silicon, and as a result, relatively little is known about the interaction between silicon and microorganisms. To a large extent this reflects the view that silicon is essentially biologically unreactive. While science fiction writers have suggested that silicon may act as an alternative to carbon in life forms on other planets, the study of the microbiology of silicon on this planet has been woefully neglected. It has however, occasionally been suggested that silicon may act as an alternative energy source to carbon for microorganisms. This probability will be discussed in more detail in the following Chapter.

The potential importance of microbial silicon transformations in natural environments has been illustrated by a recent paper by Biddle and Azam (1999), who showed that an assemblage of marine bacteria play an important role in the cycling of silica-rich marine diatoms.

CHAPTER TWO

EFFECT OF SILICON COMPOUNDS ON MICROBIAL GROWTH

2.1 Introduction

As was mentioned in the introduction, relatively little is known about the microbiology of silicon. However, the ability of fungi to grow on nutrient-free silica gel has been reported by Wainwright (1993). Although CO_2 fixation has been implicated in growth under these conditions, it is generally believed that fungi grow oligotrophically by using nutrients absorbed by the silica gel from the atmosphere (Parkinson *et al.*, 1991). It is possible, however, that silica gel itself increases hyphal growth or stimulates fungal spore germination. In some cases silicon can account for up to 10 % of plant dry weight. More recently Epstein (1994) suggested that silicon is involved in plant growth, mineral nutrition and the resistance of plants to fungal disease and herbivores. Plants and microorganisms have been noted for their ability to degrade experimentally polymerised silicon and naturally occurring quartz into the monomeric form (Henderson and Duff, 1965).

Since the 1920s, monomeric silicon compounds have been known to enhance microbial growth. For example, Borell (1922) discovered that potassium silicate increased the yield of *Bacillus tuberculosis*.

Das *et al.* (1992) mentions that the exact role of silicon in bacterial metabolism and growth remains largely unknown, but silicon can possibly provide an alternative or additional energy source to a huge number of simpler forms of life, particularly the *Mycobacteria* and *Nocardiae*, which may scavenge silicon from the media and the environment and utilise it, even in the absence of a carbon source. The possible role of silicon in the energy metabolism in microbial growth was first suggested by Allison

(1968). It has long been known that silicon compounds can stimulate microbial growth. Reynolds (1909), for example, suggested that silicon might replace carbon in some types of microbial metabolism. Price (1932) also showed that the growth rate of *Amoeba proteus* was greatly increased by the addition of sodium silicate. Similarly, Mast and Pace (1937) found that *Chilomonas paramecium* will not grow in inorganic solution lacking silicon and also that silicon stimulated starch production, growth and respiration in this organism. Bacteria, such as *Bacillus licheniformis*, can also accumulate silicon from growth media (Mohanty *et al.*, 1990).

In the 1940s Bigger and Nelson (1941, 1943) grew bacilli in distilled water, lacking any added carbon. They found that no growth occurred unless talc (hydrated magnesium silicate) was added. Such growth promotion by magnesium silicate only occurred when carbon dioxide and ammonium were present. Bigger and Nelson (1941, 1943) proposed that magnesium silicate absorbs carbon dioxide and ammonium gases from the atmosphere, making them available to the coliform bacilli as sources of carbon, for both energy and for growth, as well as nitrogen.

Exley *et al.* (1993) reported that silicon is an essential element for diatom growth, since it is used for build the siliceous frustule surrounding the diatom cell wall. Ogurtsova *et al.*, (1989) note that, in the extraction solution of silicon and iron, micromycetes and heterotrophic bacteria were extremely active. Silicon is also essential in the viral metabolism of DNA and chlorophyll synthesis (Exley *et al.*, 1993).

Silicon, as silicic acid (0.1-0.6 mM), is one of the main constituents of soil solution and can be regarded as a plant nutrient (Epstein, 1994; Birchall, 1995). Lauwera and

Lauwera and Heinen (1974) have also suggested that a silicon cycle operates in the environment, involving microbial transformation between insoluble and soluble forms.

As has been already been mentioned a wide range of bacteria and fungi can solubilize insoluble silicates by producing mineral and organic acids, and chelating agents (Henderson and Duff, 1965). Most of these silicate solubilizers are common soil microorganisms, although a specialized bacterium, *Bacillus mucilaginosus*, has been described by Russian workers. Silicate-dissolving microorganisms have also been used to remove silicon from low-grade mineral raw materials, like bauxite, and to extract valuable metals from silicate and aluminosilicate ores and minerals (Karavaiko *et al.*, 1988)

Much of the early work on the interaction between silicon and bacteria relates to studies on the lung diseases silicosis (a form of pneumoconiosis) and tuberculosis. In the past, silicosis was very common amongst industrial workers (especially coal miners) exposed to dust rich in crystalline silica, but not amorphous silica and silicates. Many silicosis sufferers died from tuberculosis which spread rapidly through the lungs and caused death in a relatively short time. This observation led to studies by the Canadian microbiologist Price (1932), who showed that sodium silicate and silicic acid can in fact stimulate the growth of *Mycobacterium tuberculosis*, and that even small amounts of silicon compounds, notably the easily soluble forms, produce the stimulatory effect. More recently, Yoshino (1990) found that 100 μg silicon ml^{-1} has "a remarkable stimulatory effect on the growth of *Staphylococcus aureus*". He also showed that a high concentration of silicon present in the mucous membrane acts to enhance the growth of *Pseudomonas aeruginosa*. Sufferers from chronic sinusitis

apparently have a high concentration of silicon in their mucous membranes, a fact that led Yoshino (1990) to suggest that this stimulatory effect of silicon on bacteria exacerbates the condition.

The aim of the work reported in this Chapter was to determine,

(1) the effect of silicon compounds on fungal growth,

(2) the ability of fungi to release soluble silicon from insoluble silicon compounds,

(3) the effect of silicon compounds on the growth of;

a) *Streptomyces spp.*,

b) the alga, *Dunaliella parva,*

c) *Escherichia coli* and *Staphylococcus aureus,*

d) the yeast, *Pichia pastoris* and *Saccharomyces cerevisiae.*

2.2 Materials and Methods

2.2.1 The following isolates were used throughout this work:

Fungi : *Aspergillus niger 013, Aspergillus oryzae 002, Aspergillus repens 003, Fusarium oxysporum 006, Penicillium janthinellum 007* and *Penicillium chrysogenum 012.* These isolates were obtained from the Department of Animal and Plant Sciences, University of Sheffield.

Bacteria : **Streptomyces spp* (SAN7), and (SAH1). *Staphylococcus aureus*, (Oxford strain) were obtained from the Medical School, Royal Hallamshire Hospital. Sheffield. *Escherichia coli* (DH5α). (Departmental stock).

Algae : *Dunaliella parva,* strain CCAP 19/9. (Departmental stock).

Yeast : *Pichia pastoris* (X33 w.t.) and *Saccharomyces cerevisiae* (X4003 w.t.) (Departmental stock).

* These isolates were isolated by Al-Garni (1990), from a Saudi Arabian soil, using the dilution plate technique.

2.2.2 The following silicon compounds were used in this investigation:

Silicic acid, silica-colloidal powder, calcium silicate, sodium silicate, talc (hydrated magnesium silicate), silicon nitride, potassium silicate, rock potash, colloidal silicic acid, dimethyldichlorosilane and hexamethyldisilane.

2.2.3 Maintenance of cultures.

Fungal isolates were maintained on the Czapek Dox medium, incubated for 7 days at 25^{0}C, then transferred to a refrigerator at 4^{0}C for two months. These procedures were repeated every two months. For long term maintenance, the isolates were grown in Universal bottles on agar slants.

Streptomyces isolates were maintained on the glucose nitrate medium and date syrup medium using a thick layer of medium, incubated for 5 days at 30^{0}C, then transferred to a refrigerator at 4^{0}C for two months. These procedures were repeated every two months. For long term maintenance, the isolates were grown in Universal bottles on agar slants.

Staphylococcus aureus, were maintained by growing on Mueller - Hinton agar medium using a thick layer of medium, incubated for 24 hours at 37^{0}C, while the same procedure was used for *Escherichia coli* when grown on Nutrient agar medium and L-broth medium.

Yeast isolates were maintained by re-culturing every two months (in YEPD medium), while algal isolate was maintained by re-culturing every two months (in Dunaliella medium).

2.2.4 Effect of silicic acid on fungal growth.

In this experiment, five fungal isolates *Aspergillus niger, Aspergillus oryzae, Aspergillus repens, Fusarium oxysporum,* and *Penicillium janthinellum* were grown on Czapek Dox agar (Oxoid) for 10 days at 25^{0}C. Discs (4mm) were then cut from the leading edge of the colonies using a flame-sterilized cork borer. These were

transferred (1 disc per flask) to unbuffered Czapek Dox liquid medium (100 ml in a 250 ml Erlenmeyer flask) amended with 0.5g, 1g, 1.5g and 2g of silicic acid (Sigma). Silicic acid was contained in sealed dialysis tubing (size 14 mm), or otherwise added directly to the medium. Controls lacking silicic acid, and containing silicic acid but no fungus were also included. The medium was autoclaved at 15 psi for 15 min. Three replicates were used throughout, and all flasks were incubated on a rotary shaker (150 rpm) for 8 days at 25^0C. After 8 days, the contents of the flasks were then filtered (through Whatman No.1 filter paper), and the dry weight was determined. Filtrate pH was determined immediately after filtration using a glass electrode. The soluble silicon content of the medium was determined colorimetrically.

2.2.5 Effect of silicic acid on *Aspergillus oryzae* growth with adjusted buffer.

The effect of variations in medium pH on growth of *A. oryzae* was determined using Sorensen's buffer at 6.8.

2.2.6 Effect of water-washed silicic acid on *A. oryzae* growth.

In a separate experiment, silicic acid was washed, from the surface with tap water (3 times), with a 1/2 hour period between each wash, followed by washing with sterile (upw).

2.2.7 Effect of acid-washed silicic acid on *A. oryzae* growth.

Silicic acid was washed with sterile distilled water (3 times) allowing an 1/2 hour period between each wash. This was followed by washing with 0.1M Hydrochloric acid

2 times, 1 hour was allowed between each wash. Finally, the silica was washed with sterile ultra-pur water (3 times), allowing a 1/2 hour period between each wash.

2.2.8 Analysis of protein content of fungal mycelium.

Aspergillus oryzae, *Aspergillus niger* and *Fusarium oxysporum* were grown in (1000 ml) Erlenmeyer flasks containing nutrient-rich medium (500 ml Czapek Dox liquid). Silicic acid 1% (Sigma) was added into sealed dialysis tubing (size 14 mm). The medium was autoclaved at 15 psi for 20 minutes. Three replicates were used, and all flasks were incubated on a rotary shaker (150 rpm) for 8 days in batch culture at 25^{0}C. After 8 days, the culture was removed, and filtered. Mycelium (dry weight, 0.1 g), (control and treatment) was mixed with NaOH (1M) in test tube which were transferred to hot plates and heated at 90^{0}C for 10 min. After 10 min., the tubes were cooled in ice. The tubes were centrifuged at high speed (4000 rpm) for 10 min. The suspension (0.1 ml) was mixed well with Bradford reagent 3 ml (Sigma). Distilled water (0.1 ml) mixed with Bradford reagent was used as the blank. The tubes were left at room temperature for 5 min., when the optical density was read at 595 nm, and the amount of protein determined by plotting the absorbency of standard solution containing 0-100 μg protein (bovine serum albumin) ml^{-1}.

2.2.9 Determination of growth of *Aspergillus oryzae* as influenced by various silicon compounds.

The effect of the following silicon compounds on the growth of *Aspergillus oryzae* was determined: silicic acid, silica colloidal powder, calcium silicate, sodium silicate,

talc (hydrated magnesium silicate), silicon nitride. Each silicon compound (1.5g) was added directly to the Czapek Dox liquid medium, and whole medium was autoclaved. After inoculation, the flasks were incubated for 7 days at 25^{0}C. Three replicates of each silicon compound were used. The dry weight was then measured.

2.2.10 Investigation of *Aspergillus oryzae* growing with silicic acid using transmission electron microscopy (TEM).

Aspergillus oryzae was examined using transmission electron microscopy (TEM). Mycelium of *A. oryzae* culture, either control or when grown in silicic acid (1 g) were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 3 hours at 4^{0}C. The samples were then washed in sucrose (10%) in 0.1M phosphate buffer. Samples were also washed three times with a 30 min. interval at 4^{0}C.

Secondary fixation was carried out in 2% Osmium tetroxide aqueous for 1 hour at room temperature.

Dehydration was achieved by passing the sample through a graded series of ethanol solution (75%, 95%, and 100%), 15 min. for each concentration. The final ethanol solution (100%) was dried over anhydrous copper sulphate for 15 min. The above steps were then repeated in an intermediate solvent, propylene oxide, for two changes of 15 min. duration each.

Infiltration was achieved by placing the specimens in a 50/50 mixture of propylene oxide/Araldite resin. The specimens were left in this 50/50 mixture overnight at room temperature, and then left in full strength Araldite resin, for 6-8 hours at room

temperature, after which they were embedded in fresh Araldite resin for 48 hours at 60^0C.

The araldite resin consisted of :

CY 212 resin 10 ml

DDSA hardener 10 ml

BDMA accelerator was also added (1 drop 1 ml^{-1} of resin mixture). Ultrathin sections, approximately 70-90 nm thick, were cut using a Reichert Ultracut E ultramicrotome and stained for 15 min. with 3% uranyl acetate in 50% ethanol followed by staining with Reynolds lead citrate for 2 min.

The sections were examined using a Philips CM10 transmission electron microscope at an accelerating voltage of 80Kv. Electron micrographs were recorded on Agfa Scientia, 23D56 EM film.

2.2.11 Determination of dry weight.

Samples were removed from the culture flasks, and the mycelial suspension was filtered through a membrane filter paper (Whatman No.1). The total contents of the flasks were filtered and the dry weight determined after heating at 40^0C overnight. In all cases triplicates were used.

2.2.12 Detection of free silicon.

Soluble silicon in medium was determined colorimetrically by adding the following to 1 ml of filtrate : ammonium molybdate (2 ml, 10 % w/v) ; ascorbic acid (2 ml, 5 % w/v) ; oxalic acid (1 ml, 10 % w/v) ; and HCl (5 ml, 1 : 1 dilution of concentrated

HCl). After 15 min. incubation at room temperature, without shaking, the absorbance of the blue colour was measured spectrophotometrically at 600 nm. The concentration of soluble silicon (as SiO_2) in the filtrate was then determined by reference to a standard curve prepared using EIL standard silicon solution (BDH chemical).

2.2.13 Effect of sodium fluorosilicate on fungal growth.

A range of concentrations (5%, 10%, 25%, 50%, 75% and 100%) of sodium fluorosilicate were added to Czapek Dox medium. All flasks were autoclaved at 15 psi for 15 minutes. *Aspergillus niger* was inoculated into the medium and incubated at 25^0C for 7 days. The contents of the flasks were then filtered (through Whatman No.1 filter paper). All flasks were set up in triplicate.

2.2.14 Effect of dimethyldichlorosilane and hexamethyldisilane on fungal growth

Dimethyldichlorosilane ($C_2H_6C_{l2}Si$) and hexamethyldisilane ($C_6H_{18}OSi_2$) 0.5 ml and 1.5 ml were added, respectively (using a sterile syringe filter 0.45 μm) to autoclaved 250 ml Erlenmeyer flasks containing (100 ml) nutrient rich medium (Czapek Dox liquid medium), *Aspergillus oryzae* and *Aspergillus niger* were grown in above medium for 7 days at 25^0C. At the end of growth period, the dry weight was determined after filtration through Whatman filter paper No.1. The amount of silicon released was determined as described in section 2.2.12. Triplicates were used throughout.

2.2.15 Effect of colloidal silicic acid on fungal growth.

Colloidal silicic acid either 0.5 ml or 1.5 ml were added to (100 ml) Czapek Dox liquid medium in (250 ml) Erlenmeyer flasks. All flasks were set up in triplicates, after autoclaving for 15 minutes. Flasks were then inoculated with *Aspergillus oryzae* or *Aspergillus niger* and incubated at 25^{0}C for 7 days. Dry weight was determined after filtration using Whatman No.1 filter paper. The amount of soluble silicon was then determined.

2.2.16 Effect of silicic acid on the growth of fungi in the presence of a range of concentrations of sucrose.

Here, either *Aspergillus niger* or *Aspergillus oryzae* were grown with one of the following concentrations (1, 5, 10, 50, 75, and 100mM) of sucrose in Czapek Dox (100 ml) medium for 7 days at 25^{0}C. Silicic acid (1.5g) was added to each concentration. Silicic acid was added directly to the medium, or in a dialysis tubing. Triplicates of each concentration were included. At the end of the growth period the supernatant was separated by filtration through Whatman No.1 filter paper.

2.2.17 Effect of silicic acid on the growth of *Streptomyces spp.*

Streptomyces SAN7 and SAH1 were grown in 250 ml Erlenmeyer flask containing (100 ml) of date syrup liquid medium containing either 0.5g, 1g, 1.5g or 2g of silicic acid (Sigma) added in sealed dialysis tubing (size 14 mm). In a separate set, the same amounts were also added directly to the medium; which was then autoclaved at 15 psi for 15 minutes. The pH was then adjusted to 7.2 with Tris buffer. Three replicates were

used for each isolate. All flasks were incubated on a rotary shaker (150 rpm) for 5 days in batch culture at 30^{0}C. After 5 days the contents of the flasks were filtered and the biomass determined. Any soluble silicon released from the silicic acid was also determined.

2.2.18 Analysis of protein content of *Streptomyces* mycelium.

The following *Streptomyces* isolates H1 and N7, were grown in (1000 ml) Erlenmeyer flasks containing date syrup liquid medium (500 ml). Silicic acid 1% (Sigma) was added into sealed dialysis tubing (size 14 mm). The medium was autoclaved at 15 psi for 20 minutes. Three replicates were used, and all flasks were incubated on a rotary shaker (150 rpm) for 8 days in batch culture at 25^{0}C. After 8 days, the culture was removed, and filtered. For details of analysis of the protein content see procedure in section (2.2.8).

2.2.19 Effect of silicic acid on growth of the algae *Dunaliella parva.*

Dunaliella parva was grown in Dunaliella medium in Erlenmeyer flasks (250 ml) containing (100 ml), the medium was buffered with Tris buffer to adjust the pH to 6.3 and 7.8. Silicic acid (0.5g and 1.5g) was added directly to the medium. All flasks were autoclaved at 15 psi for 15 minutes. Three replicates were used, and all flasks were incubated under fluorescent light at 25^{0}C for 7 days. The chlorophyll content was then measured.

2.2.19.1 Determination of chlorophyll.

Aliquots (2 X 5ml) were taken from each algal culture to be tested and transferred to 5 inch test tubes. The samples were centrifuged at 3000 x g for 10 minutes. The supernatant was immediately discarded and the pellet re-suspended in 1ml of distilled water; acetone (4 ml, 100%) was then added to each tube, which was left for 5 min away from direct sunlight. The tubes were then centrifuged for 5 minutes at 3000 x g. After confirmation that any white precipitate had collected in the pellet. The supernatant was next poured into glass cuvettes. The samples were measure at O.D. 645nm and O.D. 663nm for each sample against an acetone (80%) blank.

The chlorophyll content was determined as follows:

Mean O.D. 645nm x 202

+

Mean O.D. 663nm x 80.2 = Y

Y/2 = μg chlorophyll in 5 ml (divide this figure by 5 to calculate to the value per ml).

2.2.20 Effect of silicic acid on bacterial growth.

Escherichia coli and *Staphylococcus aureus* were grown in (250 ml) Erlenmeyer flasks containing L-broth medium (100 ml). Silicic acid (0.5g or 1.5g) was then added to the medium. The medium was buffered with Tris buffer to pH 7.0. All flasks were autoclaved at 15 psi for 15 minutes. Three replicates were used, and all flasks were incubated at 37^0C. A growth curve was obtained by measuring the optical density at 660nm every 90 minutes for 9 hours, and also after 24 hours.

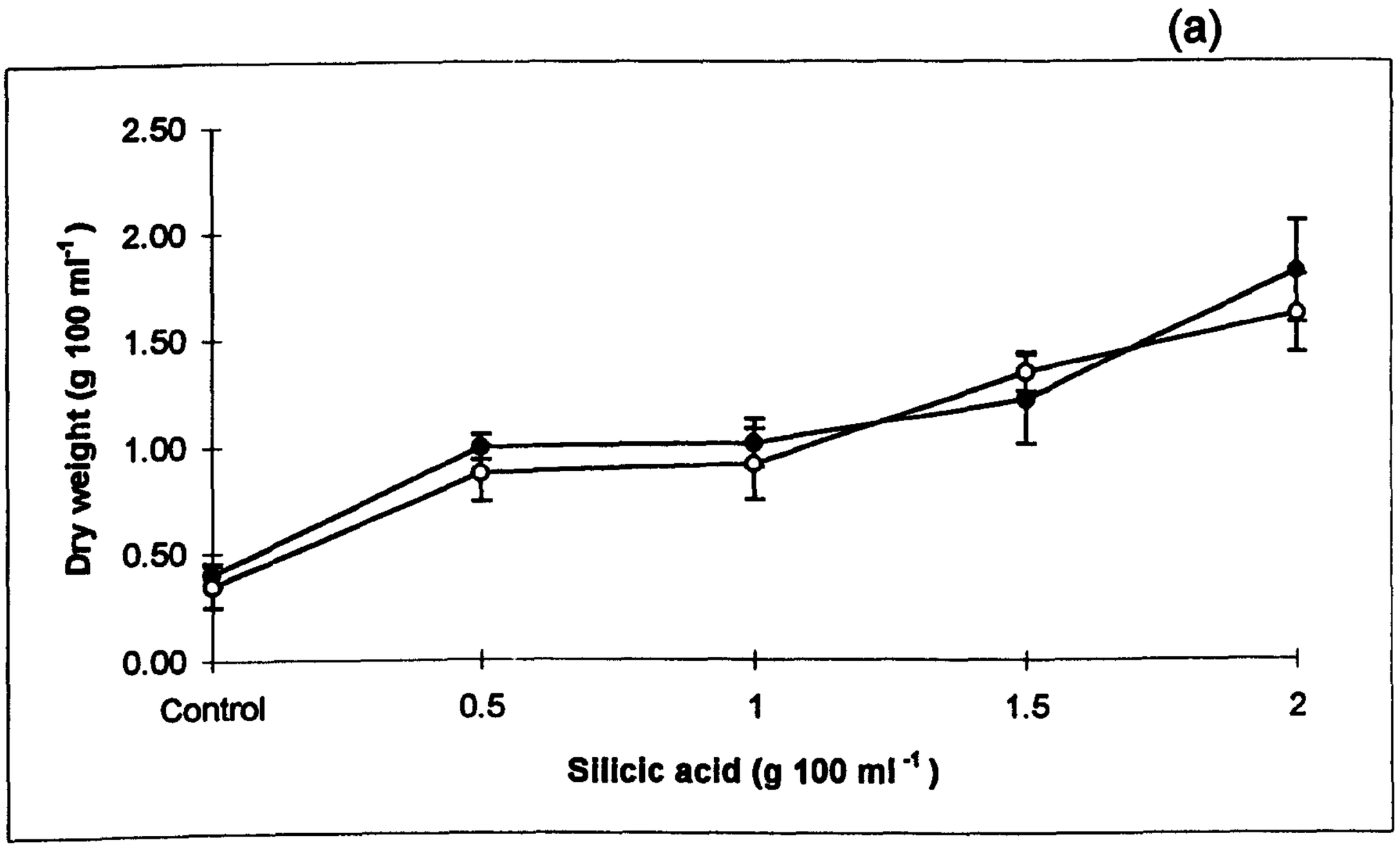
2.2.21 Effect of silicic acid on yeast growth.

The yeasts, *Pichia pastoris* (X33 w.t.) and *Saccharomyces cerevisiae* (X4003 w.t.) were grown on yeast extract peptone dextrose (YEPD) medium for 24 hours at 30^0C in (250 ml) Erlenmeyer flasks containing either 0.5g or 1.5g of silicic acid, added directly to the medium. The medium was buffered with Tris buffer to pH 6.8. All flasks were autoclaved at 15 psi for 15 minutes. Triplicates were used. A growth curve was obtained by measuring the optical density of growth every 90 minutes for 9 hours, and also after 24 hours at 600nm.

2.3 Results and Discussion

2.3.1 Effect of silicic acid on fungal growth.

Data presented in Figs. 2.1a, 2.2a, 2.3a, 2.4a and 2.5a show that the addition of various concentrations of silicic acid (0.5g, 1g, 1.5g and 2g) to Czapek Dox liquid medium led to an increase in the growth (biomass) of *Aspergillus niger, Aspergillus oryzae, Aspergillus repens, Fusarium oxysporum* and *Penicillium janthinellum* over the 8 days incubation period. Biomass increased with increasing amount of added silicic acid. Negligible amounts of silicic acid were solubilised in the absence of fungal inoculum. Since in these experiments, some of the silicic acid was adsorbed onto the surface of the growing mycelium, the measured biomass was larger than the real biomass. However, not all of the silicic acid was removed from solution by the above fungi, even when the lowest amount of silicic acid (0.5% w/v) was added. This suggests that the observed biomass increase, resulting from the addition of increasing amounts of added silicic acid, was not solely due to adsorption of silicic acid to the mycelium. In order to determine if direct contact between the silicic acid and fungus was necessary for the observed growth increase (and increase in solubilization of silicon), when silicic acid was added to flasks in sealed dialysis tubing, Figs. 2.1a, 2.2a, 2.3a, 2.4a and 2.5a show that an increase of biomass continued to occur under these conditions.



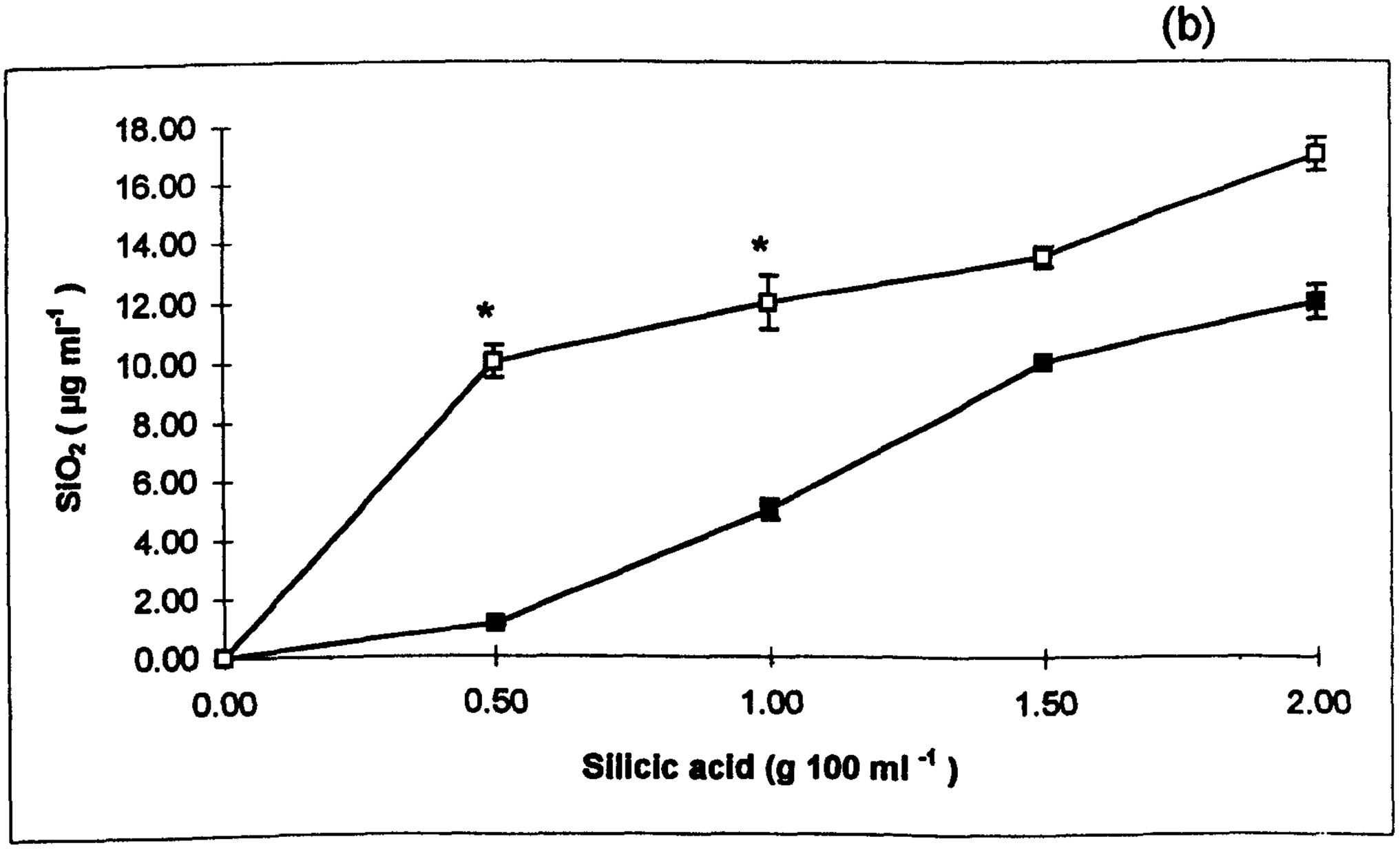


Figure 2.1 Effect of silicic acid on the growth of *A. niger* in Czapek Dox liquid medium, for 7 days at 25°C, (a), release of soluble silicon from silicic acid by *A. niger* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ±Standard error. * Significant difference, $P < 0.05$.

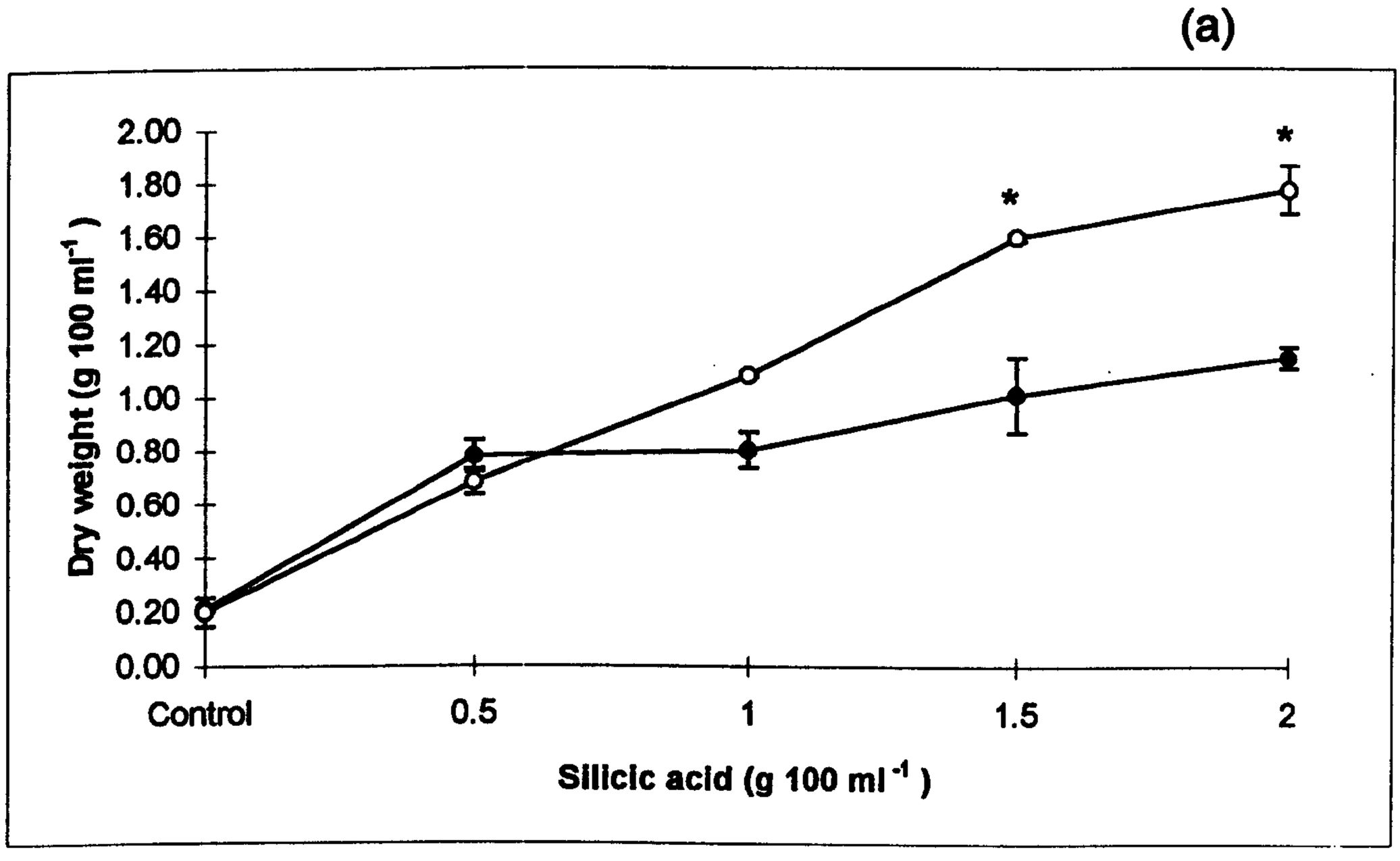
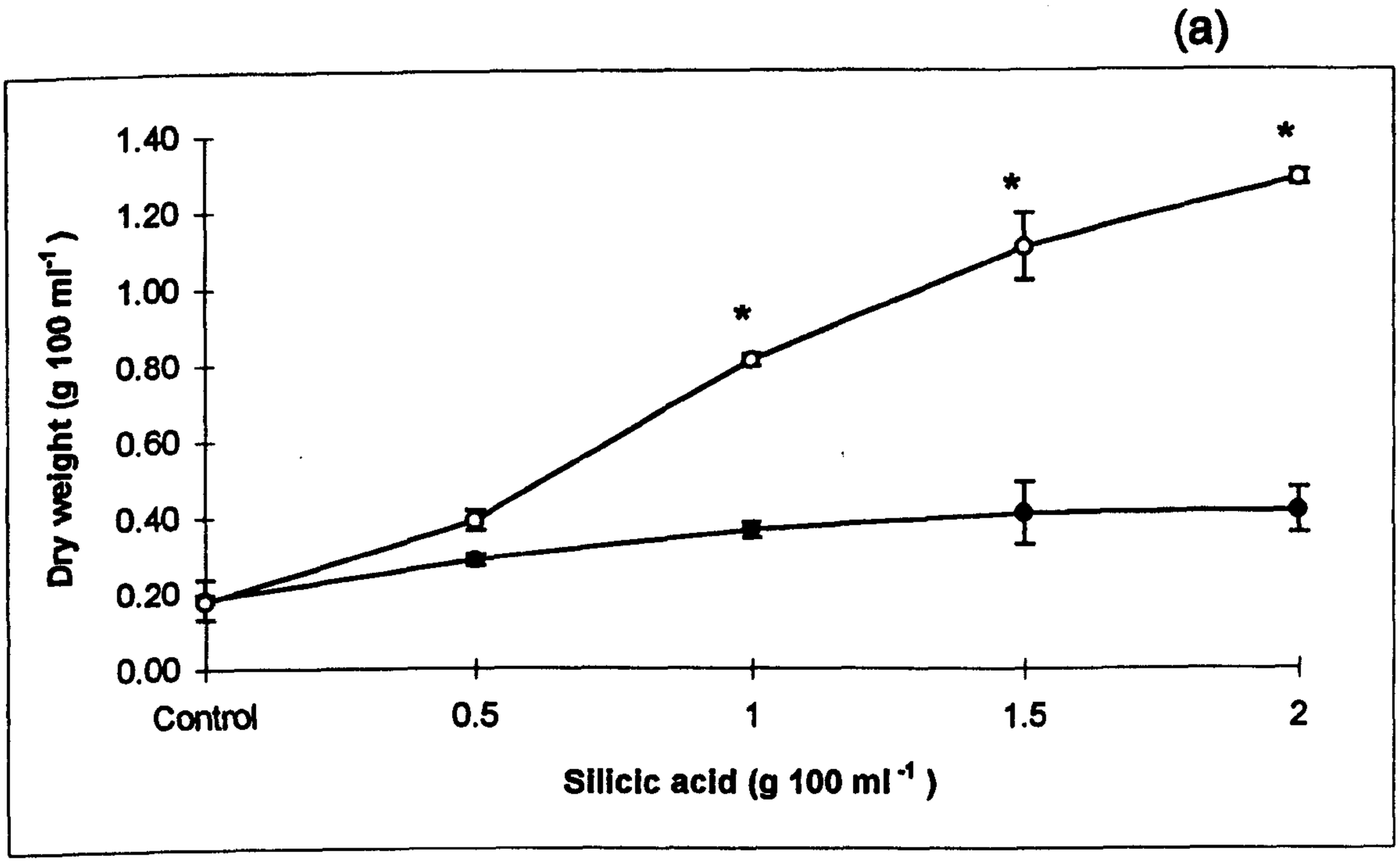


Figure 2.2 Effect of silicic acid on the growth of *A. oryzae* in Czapek Dox liquid medium, for 7 days at 25°C, (a), release of soluble silicon from silicic acid by *A. oryzae* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ±Standard error. * Significant difference, $P < 0.05$.



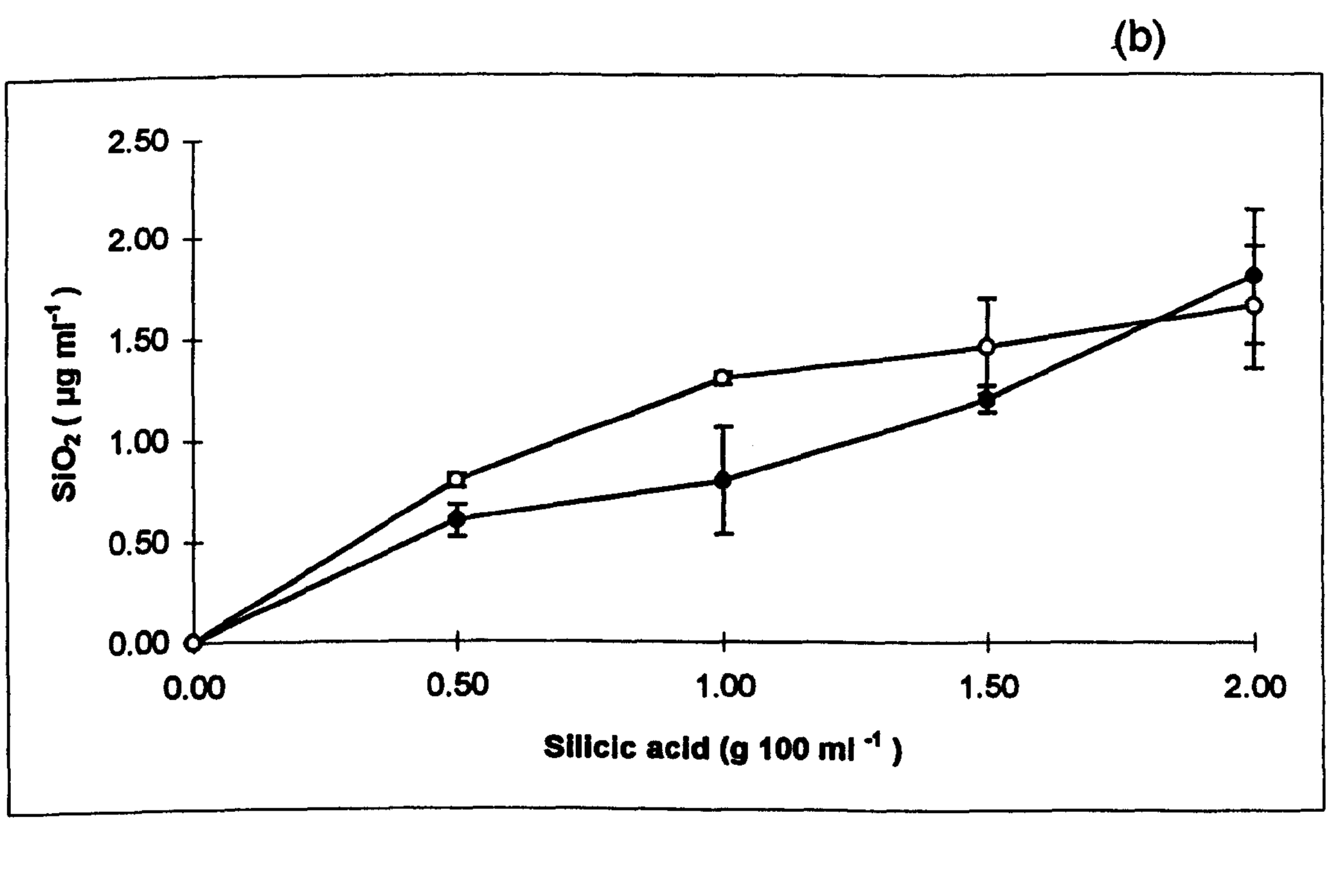


Figure 2.3 Effect of silicic acid on the growth of *A. repens* in Czapek Dox liquid medium, for 7 days at 25^{0}C, (a), release of soluble silicon from silicic acid by *A. repens* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ±Standard error. * Significant difference, $P < 0.05$.

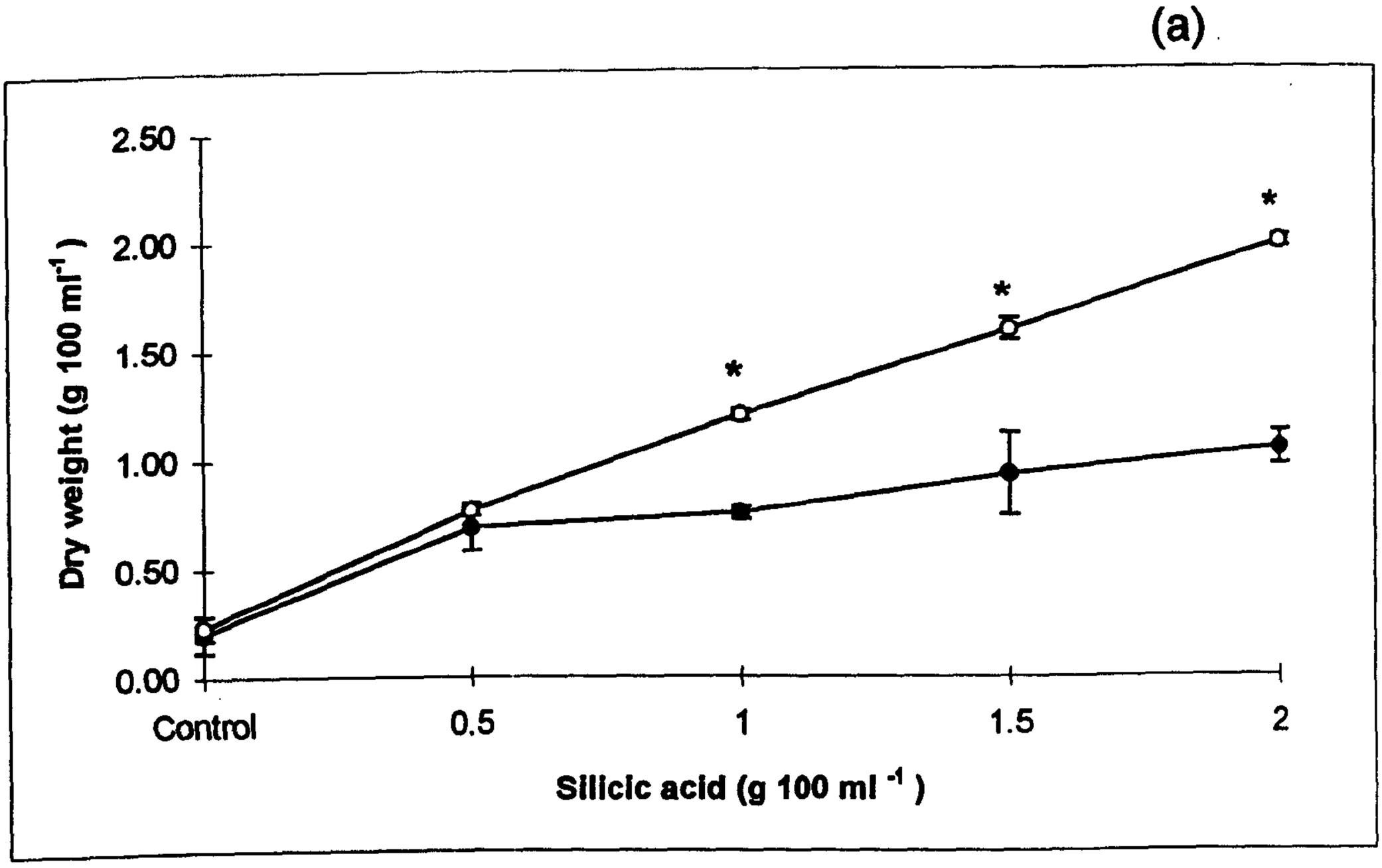
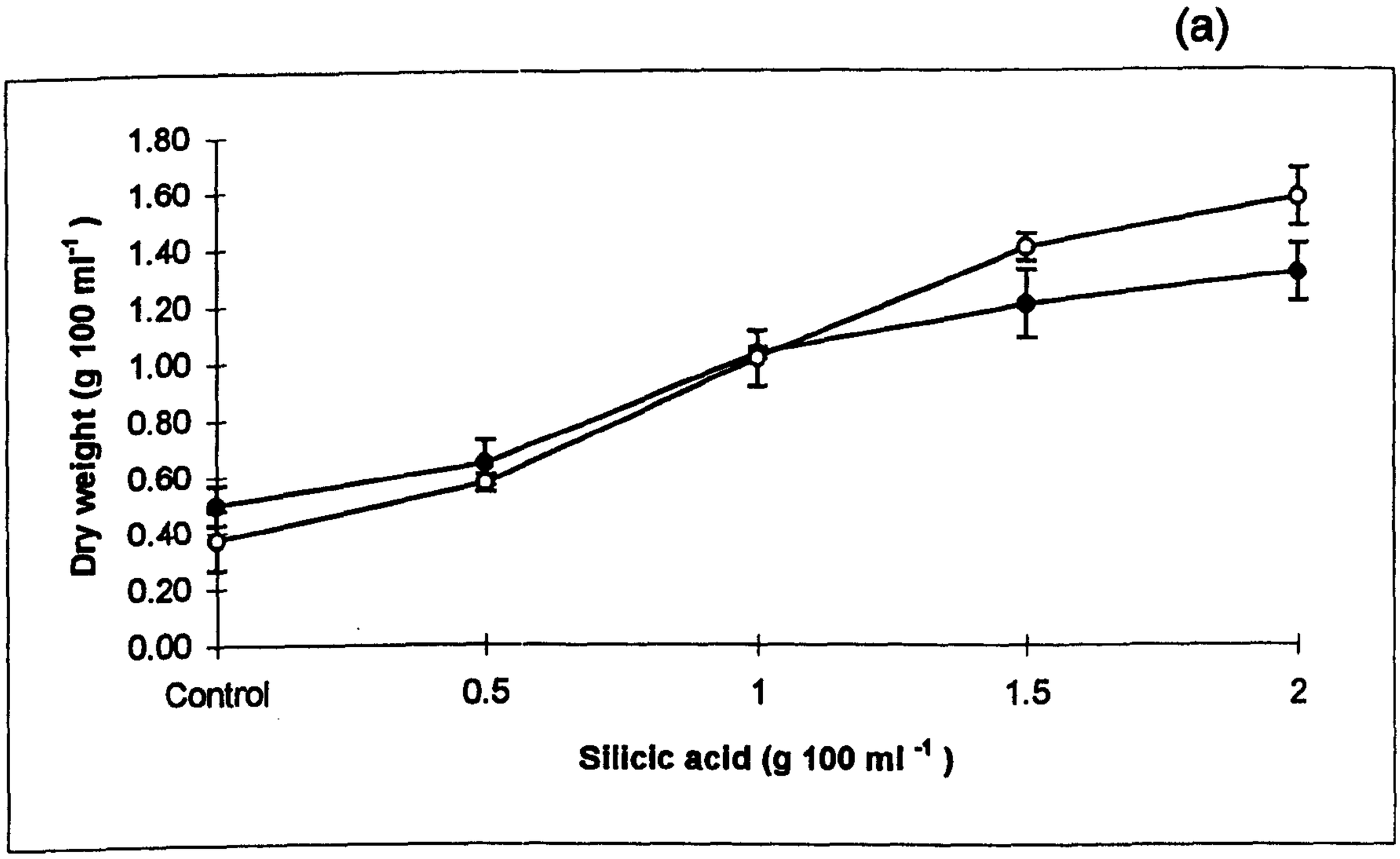


Figure 2.4 Effect of silicic acid on the growth of *F. oxysporum* in Czapek Dox liquid medium, for 7 days at 25^{0}C, (a), release of soluble silicon from silicic acid by *F. oxysporum* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.



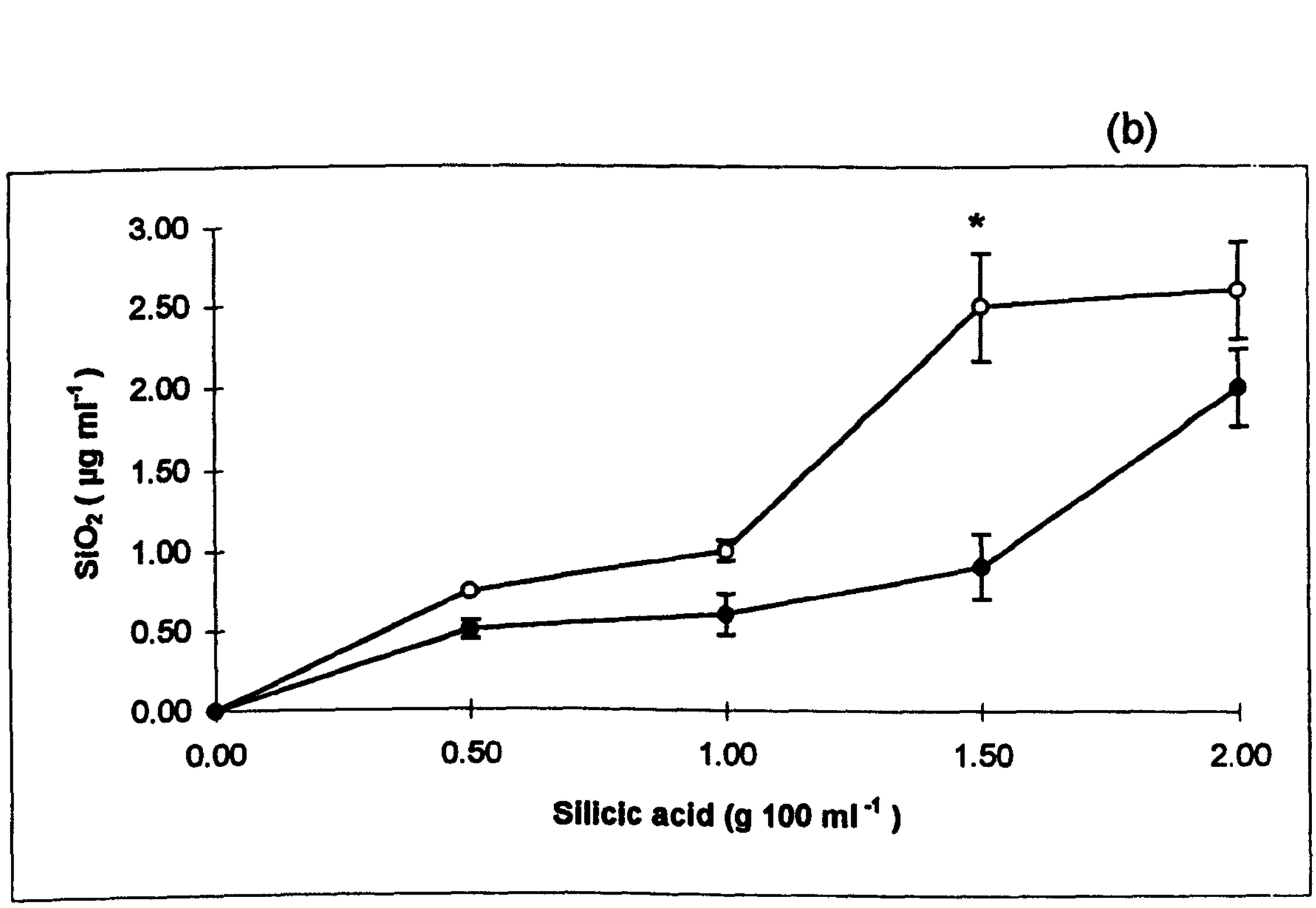


Figure 2.5 Effect of silicic acid on the growth of *P. janthinellum* in Czapek Dox liquid medium, for 7 days at 25°C, (a), release of soluble silicon from silicic acid by *P. janthinellum* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

Figs. 2.1b, 2.2b, 2.3b, 2.4b and 2.5b show that the concentration of soluble silicon, also increased with increasing weight of added silicic acid. The pH of the liquid medium was measured before and after growth (Table 1). There was generally an increase in acidity (decrease in pH) in media in which fungi grew. This was particularly marked in the case of *A. niger*, where the pH fell from 6.8-2.1, as citric acid was produced. Decreases in medium pH were seen independent of whether dialysis tubing was included or omitted.

2.3.2 Effect of silicic acid on the growth of *A. oryzae* with adjusted buffer.

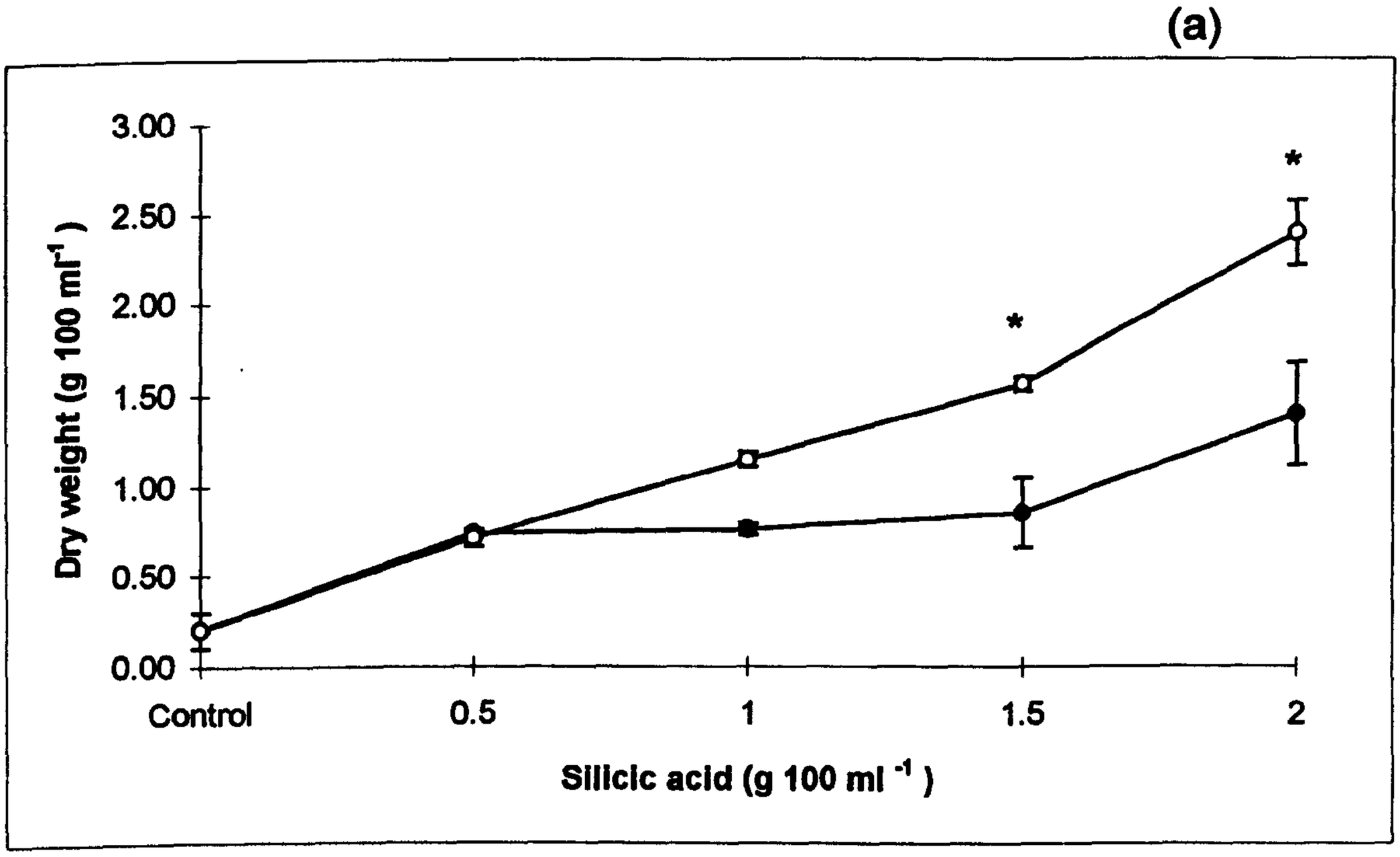
It could be argued that the increased biomass produced with increasing silicic acid concentration was due to pH effects resulting from the addition of differing amounts of silicic acid. However, when the medium was buffered to pH 6.8 (using Sorensen`s buffer) the same trends seen above were observed. Fig. 2.6a shows that variations in the pH of the medium, resulting from the addition of silicic acid, were not responsible for the observed growth increase, since similar increases in the growth of *A. oryzae* and the release of soluble silicon occurred in buffered medium (Fig. 2.6b).

2.3.3 Effect of water-washed silicic acid on the growth of *A. oryzae*.

Data presented in Figs. 2.7a, b show that an increase in the growth of *Aspergillus oryzae* and the release of soluble silicon occurring in buffered medium (pH 6.8), when silicic acid (water-washed) was added to the Czapek Dox medium. This shows that the observed increases in growth and silicon release were not due to the growth stimulating effects of contaminants present in the silicic acid.

Table 1 Change of the pH of the growth medium during the growth of different microorganisms in media containing silicic acid.
Means of three replicates.

Microorganism	pH plus dialysis tubing		pH minus dialysis tubing	
	Initial	**Final**	**Initial**	**Final**
Aspergillus oryzae	6.8	6.1	6.8	5.2
Aspergillus repens	6.8	5.7	6.8	5.7
Aspergillus niger	6.8	2.1	6.8	2.0
Fusarium oxysporum	6.8	6.7	6.8	6.2
Penicillium janthinellum	6.8	6.2	6.8	4.1
Streptomyces (SAH1)	7.2	6.6	7.2	6.5
Streptomyces (SAN7)	7.2	6.7	7.2	6.5
Dunaliella parva	----	----	6.3	6.1
Dunaliella parva	----	----	7.8	7.7
Escherichia coli	----	----	7.0	6.7
Staphylococcus aureus	----	----	7.0	6.8



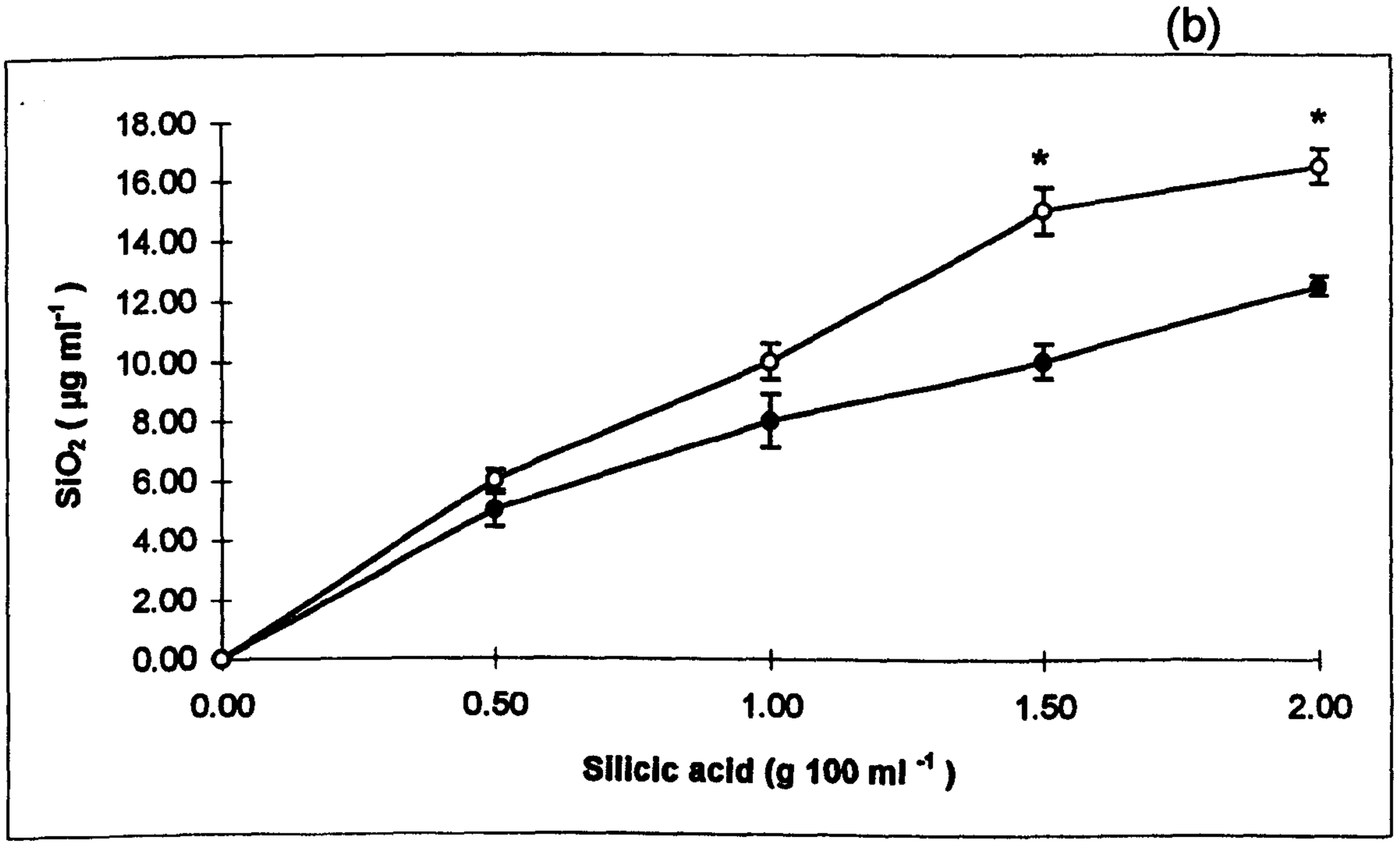
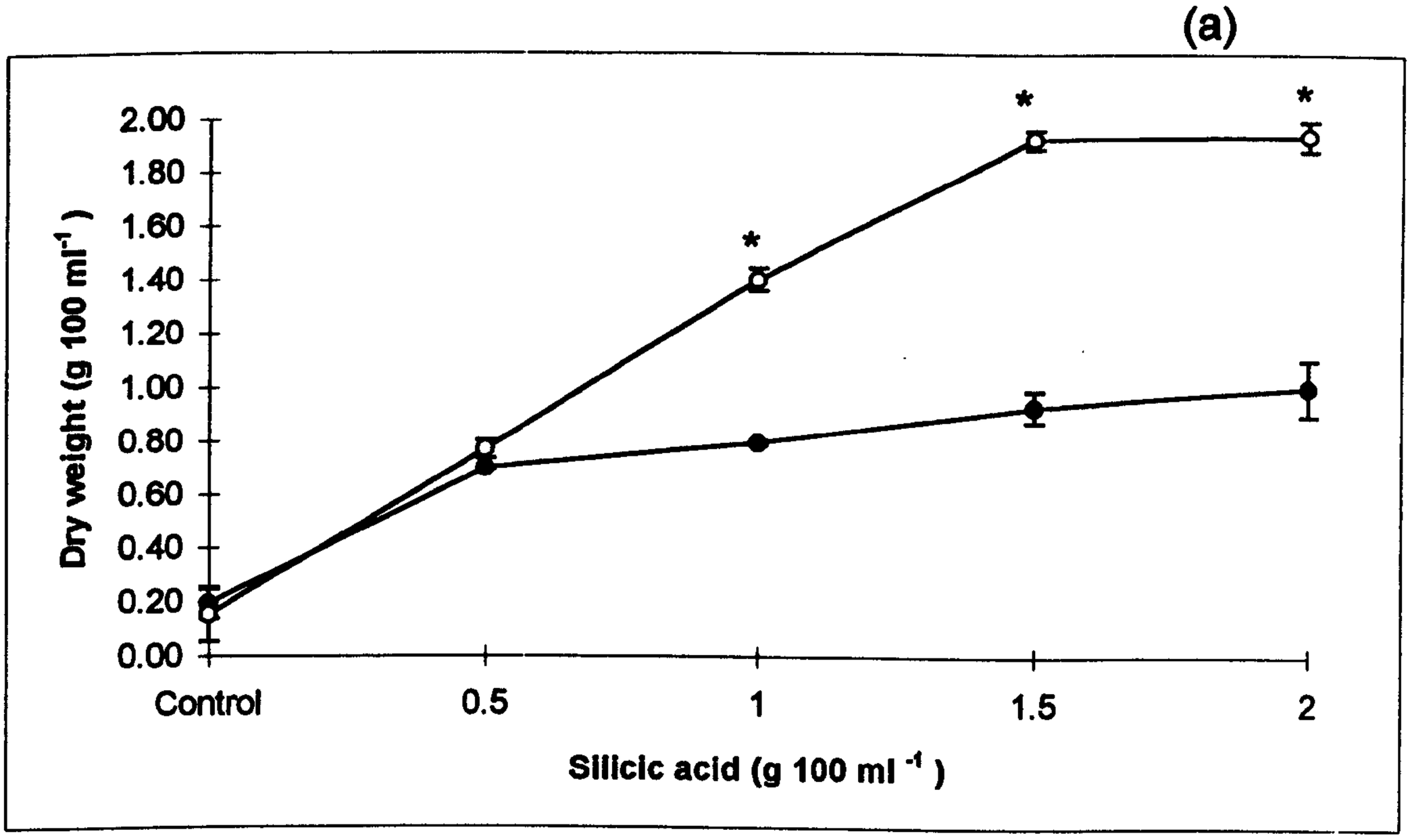


Figure 2.6 Effect of silicic acid on the growth of *A. oryzae* in buffered (6.8) Czapek Dox liquid medium, for 7 days at 25°C, (a), release of soluble silicon from silicic acid by *A. oryzae* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.



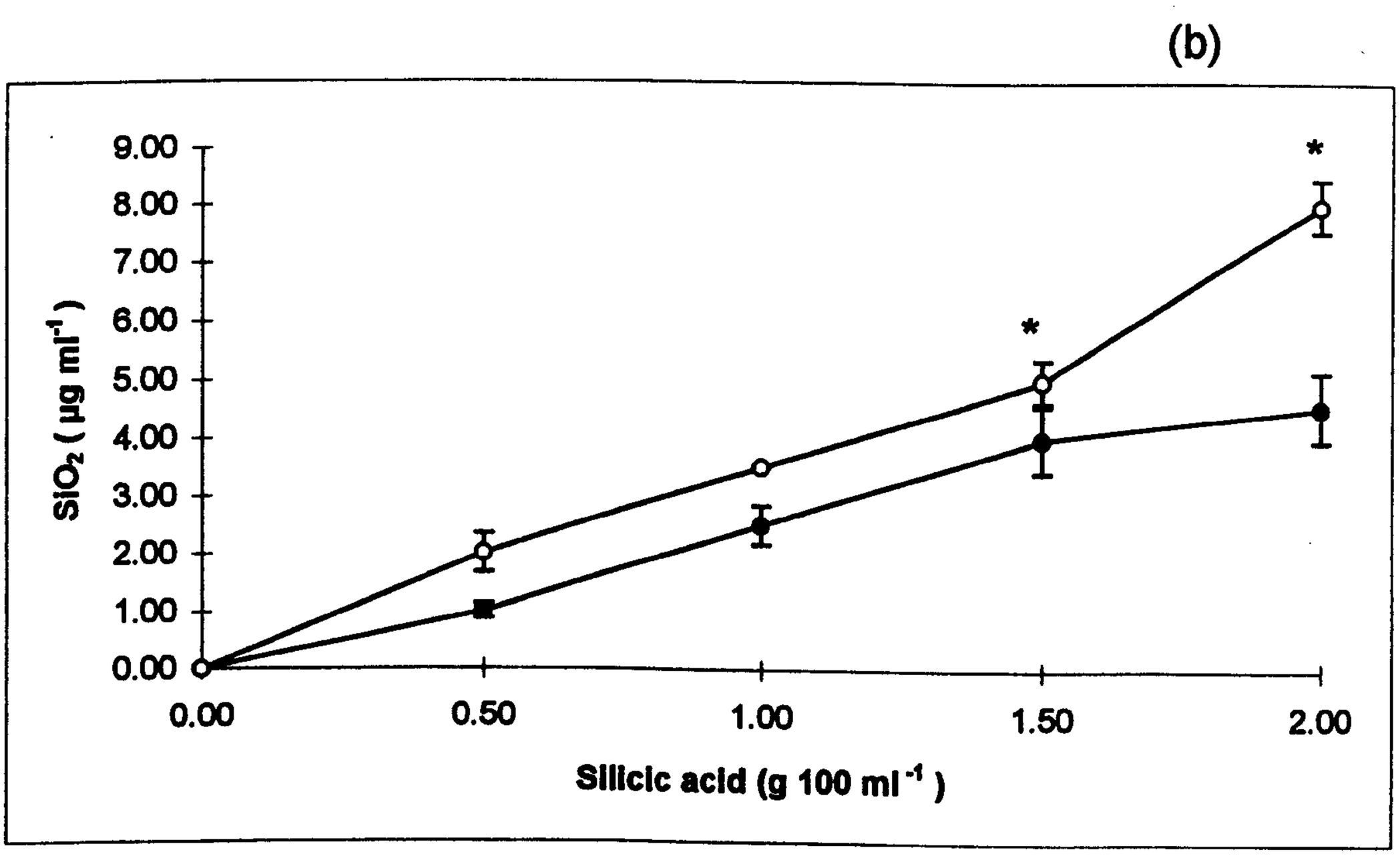


Figure 2.7 Effect of silicic acid (water-washed) on the growth of *A. oryzae* in Czapek Dox buffered (pH 6.8) liquid medium, (a), release of soluble silicon from silicic acid by *A. oryzae* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

2.3.4 Effect of acid-washed silicic acid on the growth of *A. oryzae.*

Similar increases in the growth of *A. oryzae* and the release of soluble silicon occurred in buffered medium (pH 6.8), when silicic acid (HCl-washed) was added to the medium (Figs. 2.8a, b). This again shows that the observed increase in growth and silicon release were not due to the growth stimulating effects of trace elements present in the silicic acid.

2.3.5 Determination of the effect of various silicon compounds on the growth of *Aspergillus oryzae.*

A range of silicon compounds stimulated the growth of *A. oryzae* when added to Czapek Dox medium (Fig. 2.9). However, in the case of sodium silicate, the observed increase was small and statistically insignificant. Why sodium silicate should be exceptional in this respect is not clear. However, like the other compounds it was not completely soluble in the medium, so the lack of growth stimulation was not due to the presence of insoluble particulates.

2.3.6 Investigation of *A. oryzae* using transmission electron microscopy (TEM).

The TEM studies show that there was no obvious accumulation of electron dense material, either the fungal cell-surface, or within the cytoplasm, thereby showing that silicon was not accumulated in the elemental form (Fig. 2.10a, b).

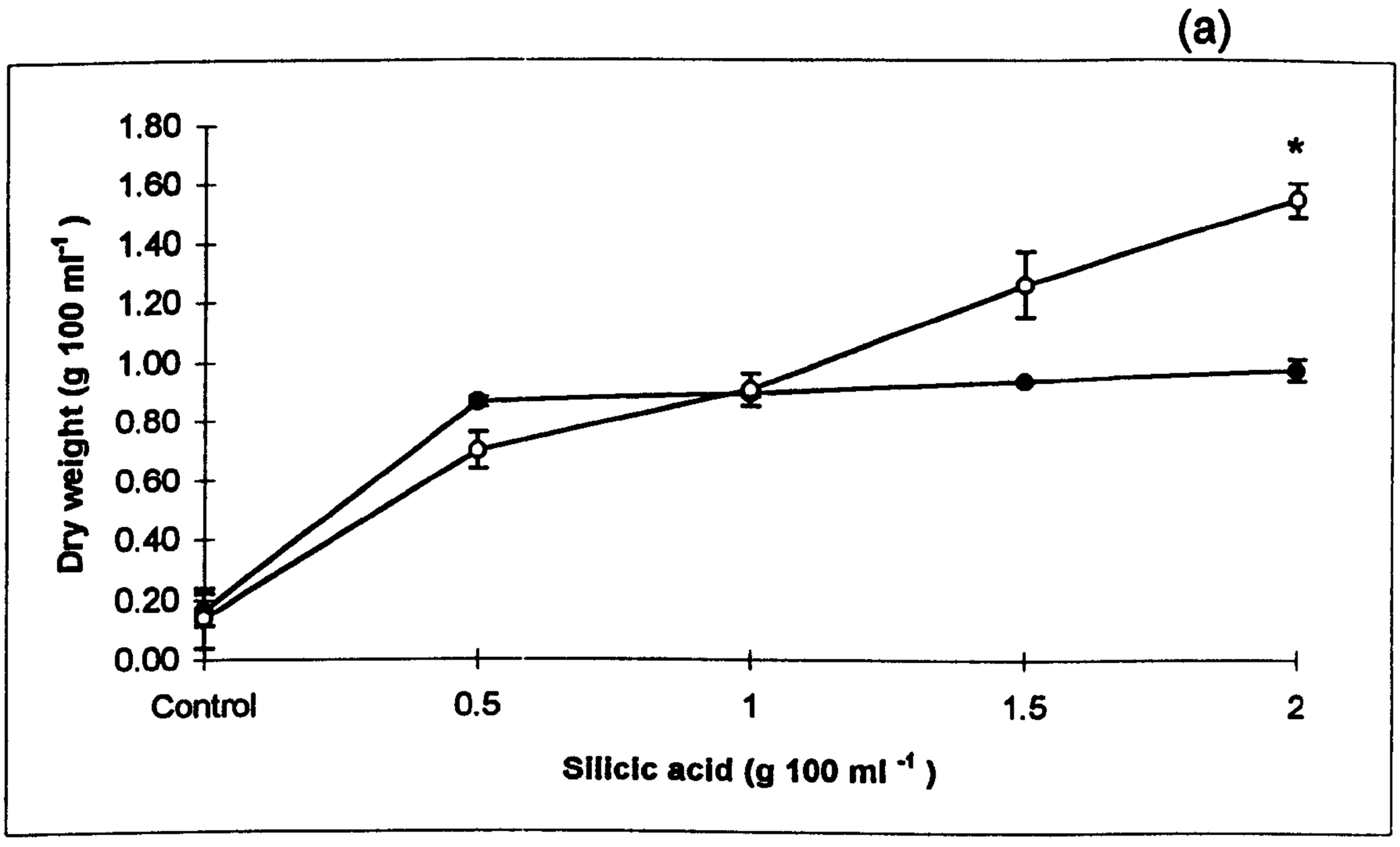


Figure 2.8 Effect of silicic acid (HCl-washed) on the growth of *A. oryzae* in Czapek Dox buffered (pH 6.8) liquid medium, (a), release of soluble silicon from silicic acid by *A. oryzae* (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

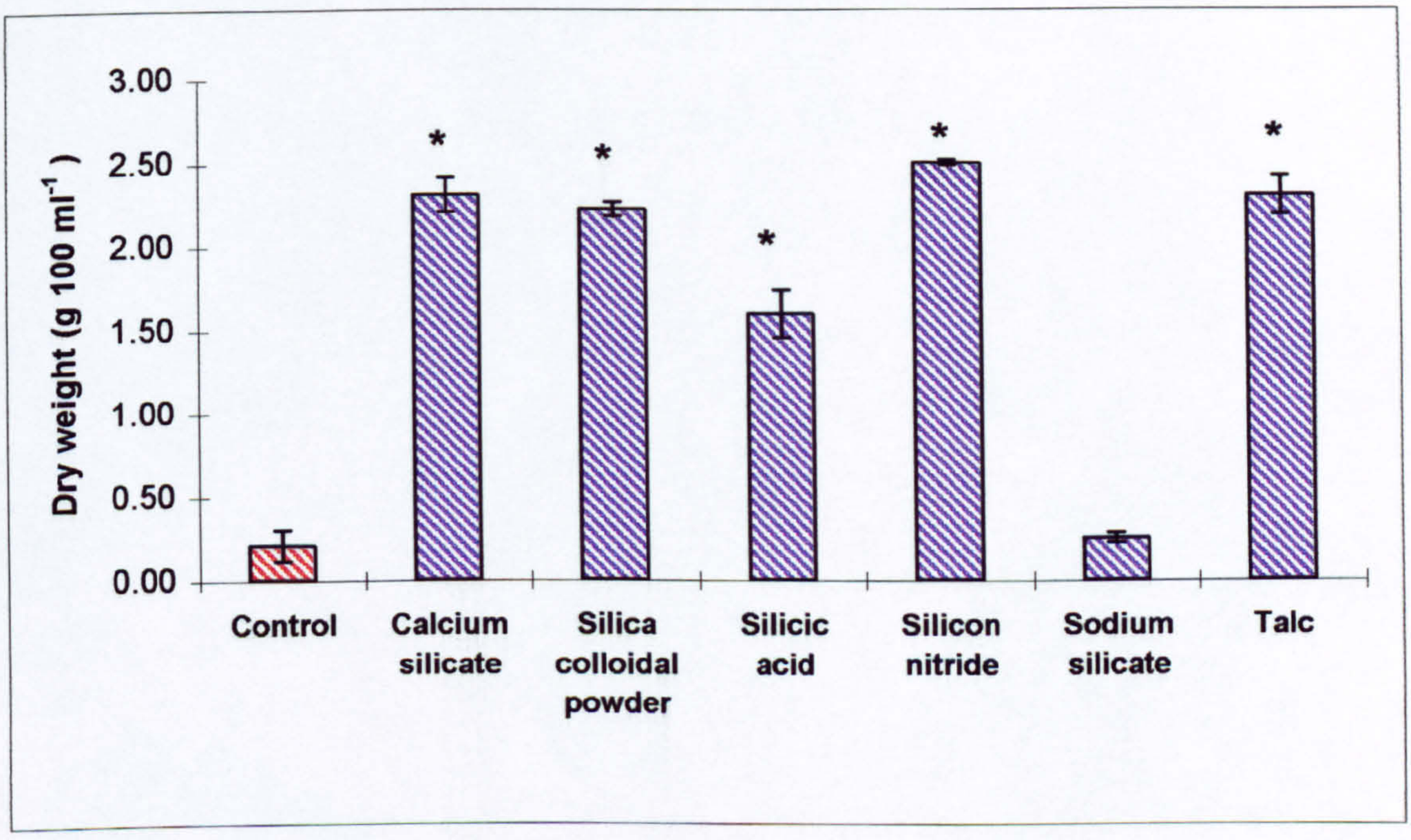
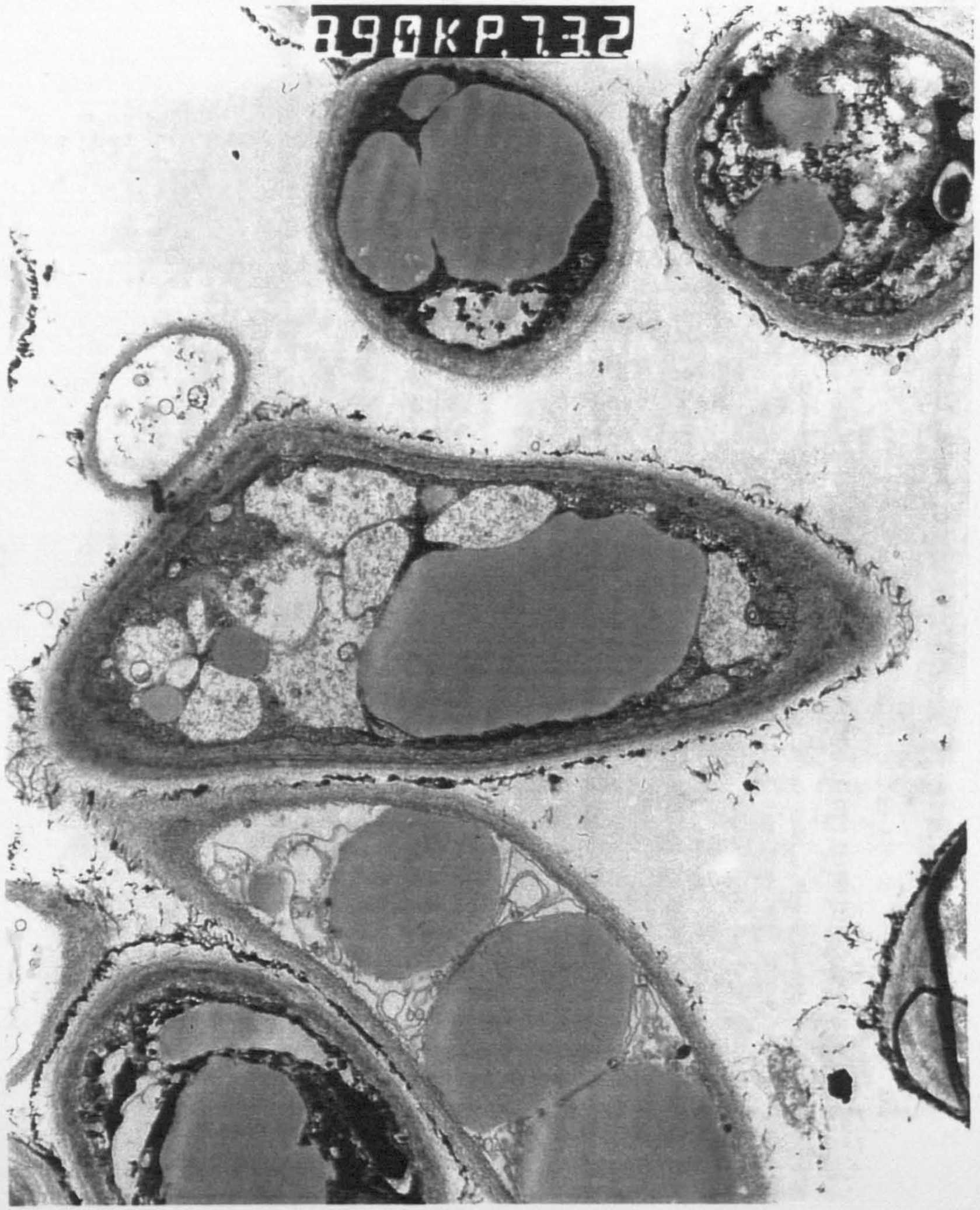


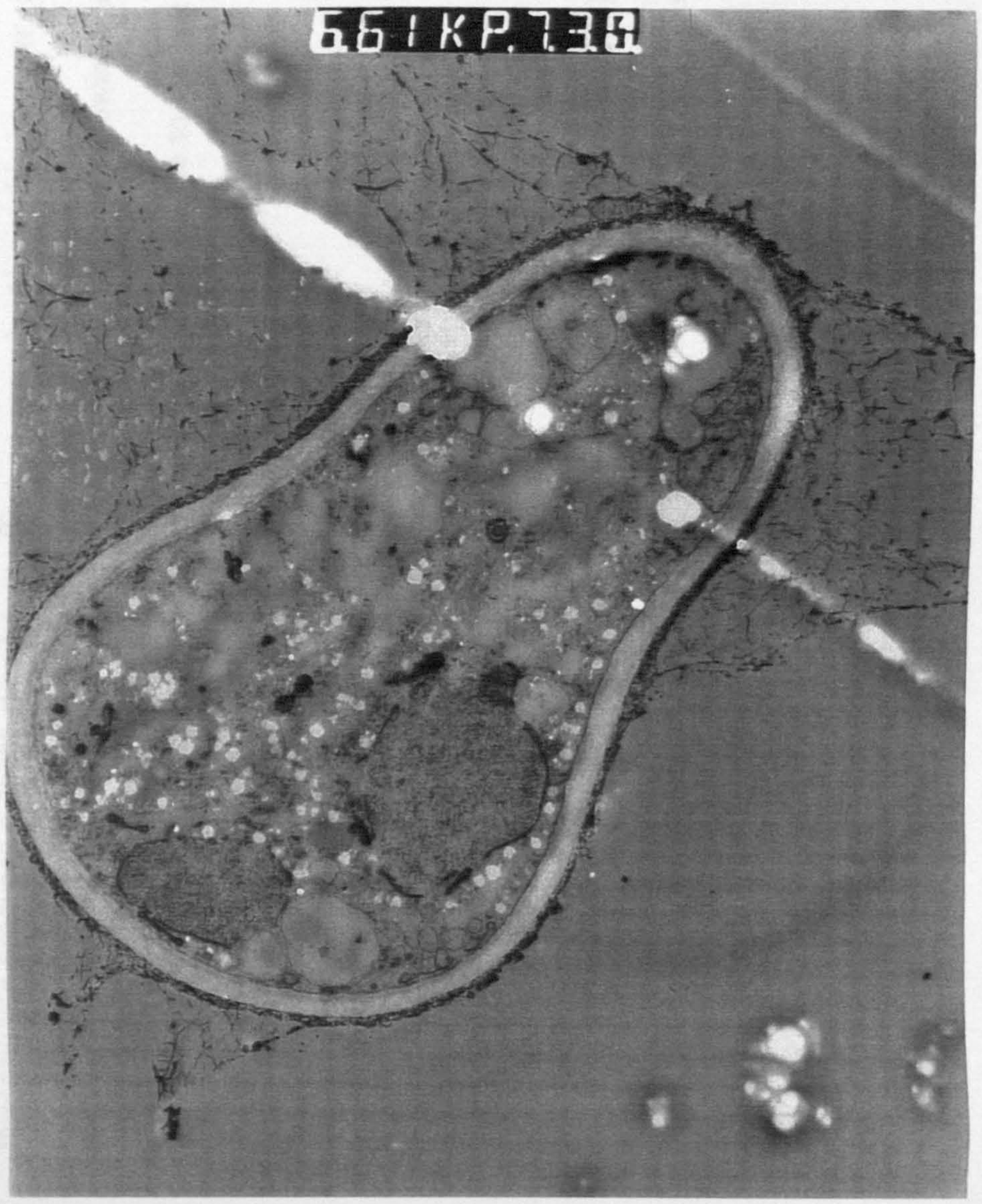
Figure 2.9 Effect of various silicon compounds on the growth of *Aspergillus oryzae* in unbuffered Czapek Dox liquid medium, at 25^{0}C for 7days (All increases except for sodium silicate) were *significant, $P < 0.05$. Means of triplicates

Figure 2.10 Transmission electron micrograph showing *Aspergillus oryzae* grown with silicic acid (1g) (A), and without silicic acid (B).

A



B



2.3.7 Analysis of protein content of fungal mycelium.

Data given in Fig. 2.11 shows that the addition of silicic acid to medium statistically increased the protein content of *Aspergillus oryzae* and *Fusarium oxysporum*, but led to slight (insignificant) increase in the protein content of *Aspergillus niger*. These results show that in the case of the first two fungi, the observed increases in dry weight (Figs. 2.1a, 2.4a), following the addition of silicic acid, correlated with an increase in protein production. It is not clear why *A. niger* should be exceptional in this respect.

2.3.8 Effect of sodium fluorosilicate on fungal growth.

Data presented in Fig. 2.12 shows the effect of varying concentrations of sodium fluorosilicate on the growth of *Aspergillus niger*. Below additions of 25 ml, sodium fluorosilicate produced a slight stimulatory effect on growth. Above this however, a marked decrease in growth occurred; growth was completely inhibited following the addition of 75 ml 100 ml $^{-1}$ sodium fluorosilicate; an effect no doubt due to the anti-fungal effect of the F^{-} component of the compound.

2.3.9 Effect of dimethyldichlorosilane and hexamethyldisilane on the growth of *Aspergillus oryzae* and *Aspergillus niger*.

Dimethyldichlorosilane decreased the growth of *A. oryzae* and *A. niger* (Fig. 213a) when added at 0.5 and 1.5 ml 100 ml $^{-1}$. However, this decreased growth was associated, in both fungi, with an increase in soluble SiO_2. (Fig. 2.13b). The addition

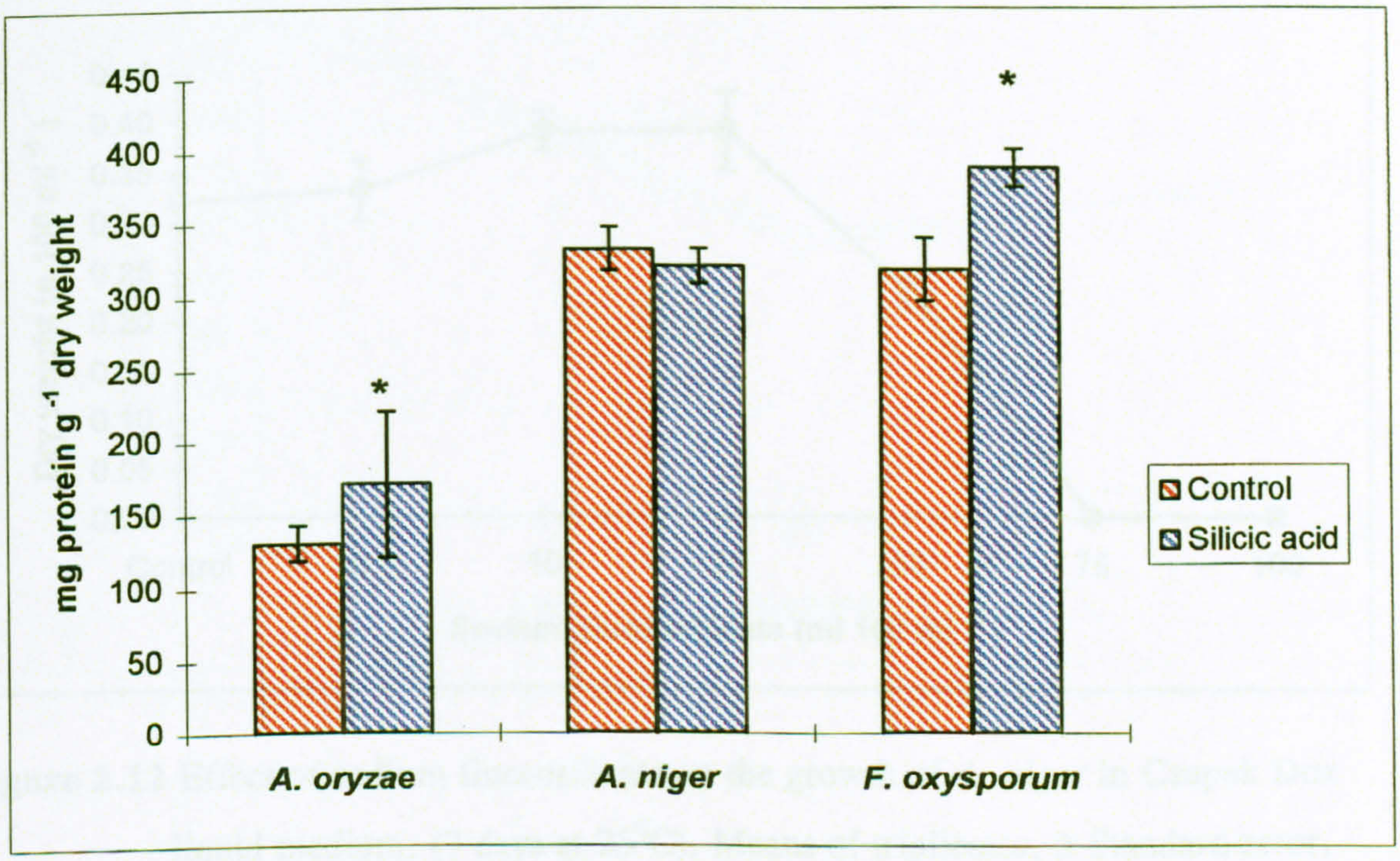


Figure 2.11 Analysis of the protein content of *Aspergillus oryzae*, *Aspergillus niger* and *Fusarium oxysporum* grown on Czapek Dox liquid medium containing silicic acid (1g added to the medium in dialysis tubing), for 7 days at 25^0C. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

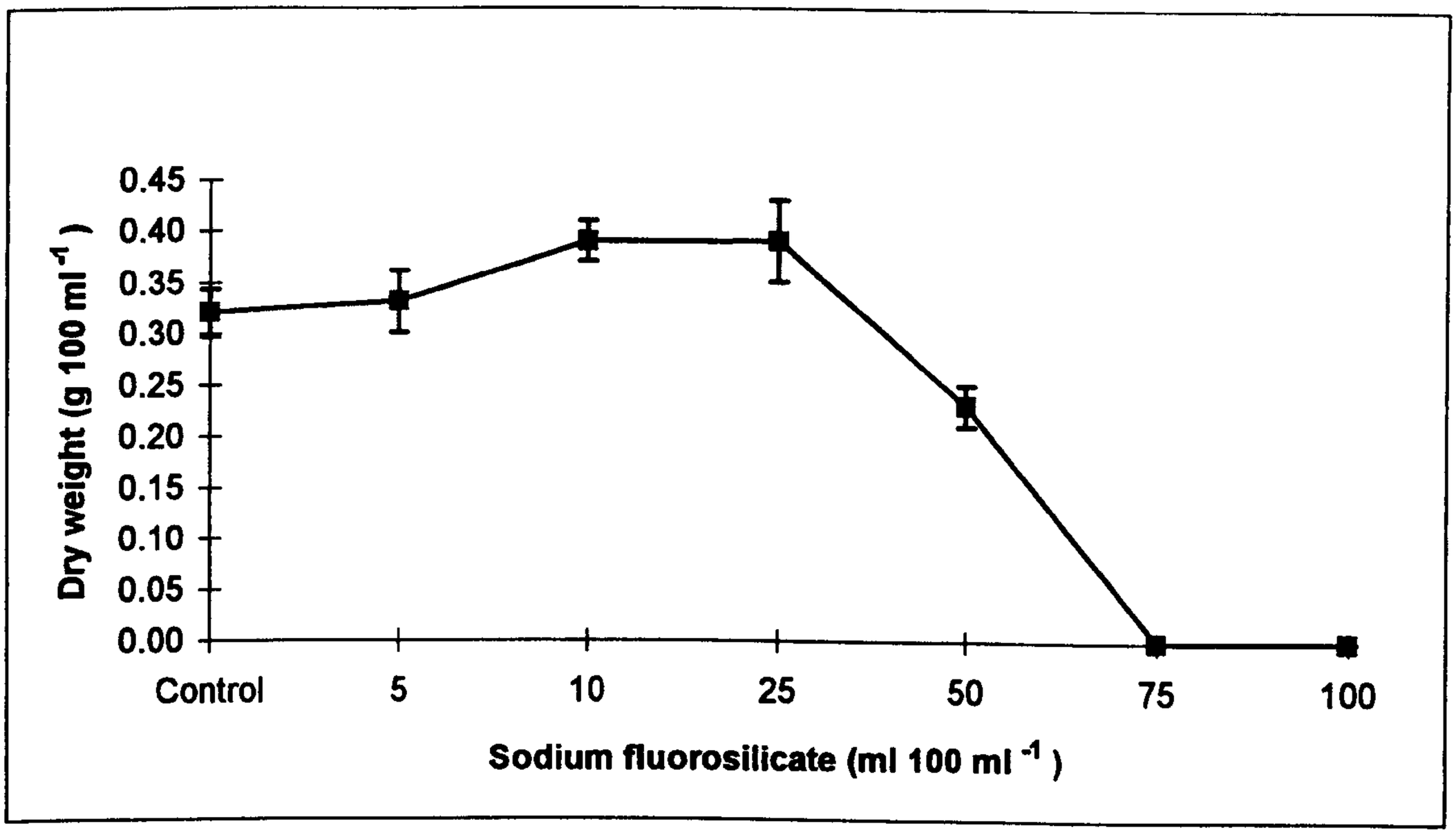
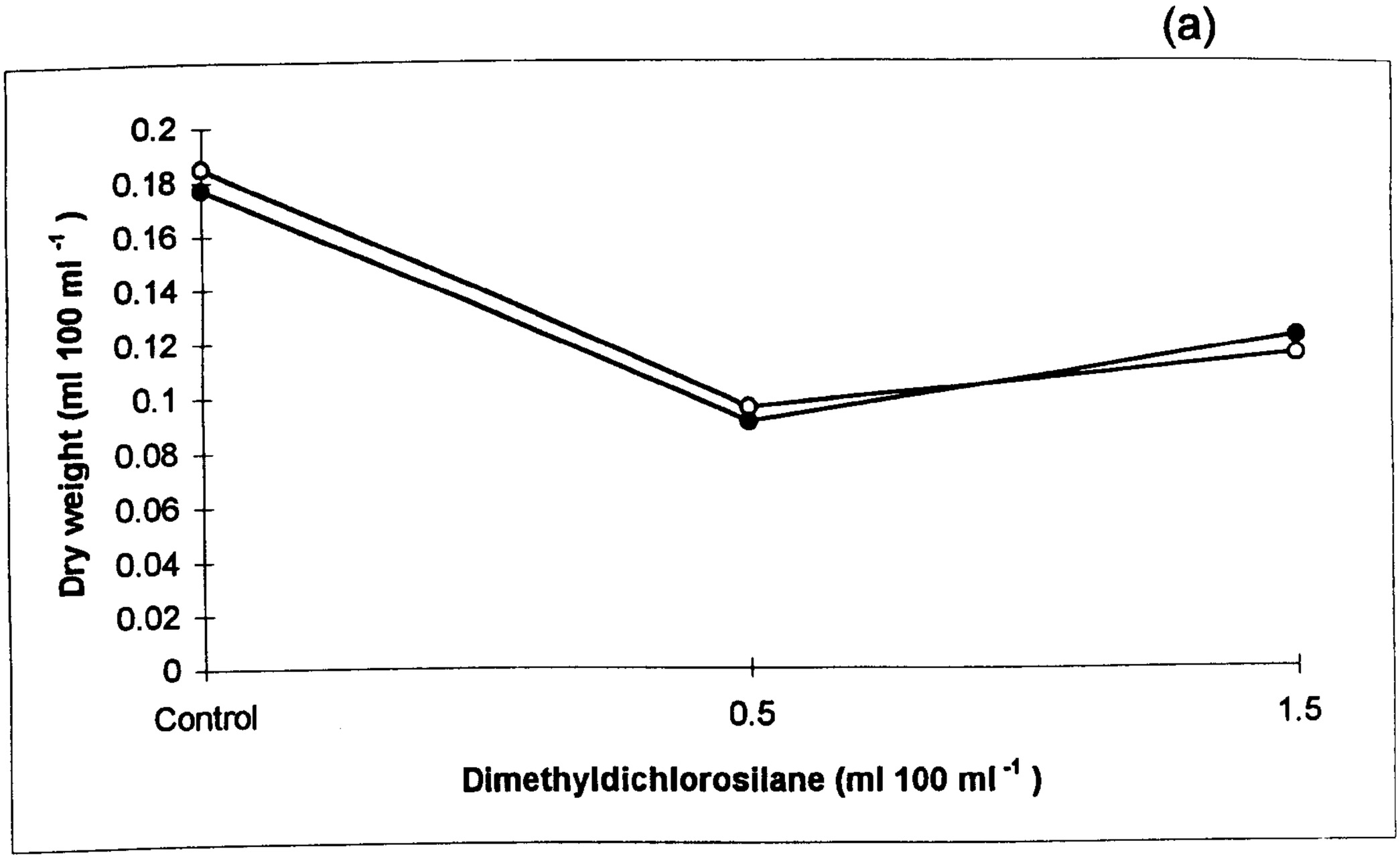


Figure 2.12 Effect of sodium fluorosilicate on the growth of *A. niger* in Czapek Dox liquid medium, (7 days at 25^{0}C). Means of triplicates, ± Standard error.



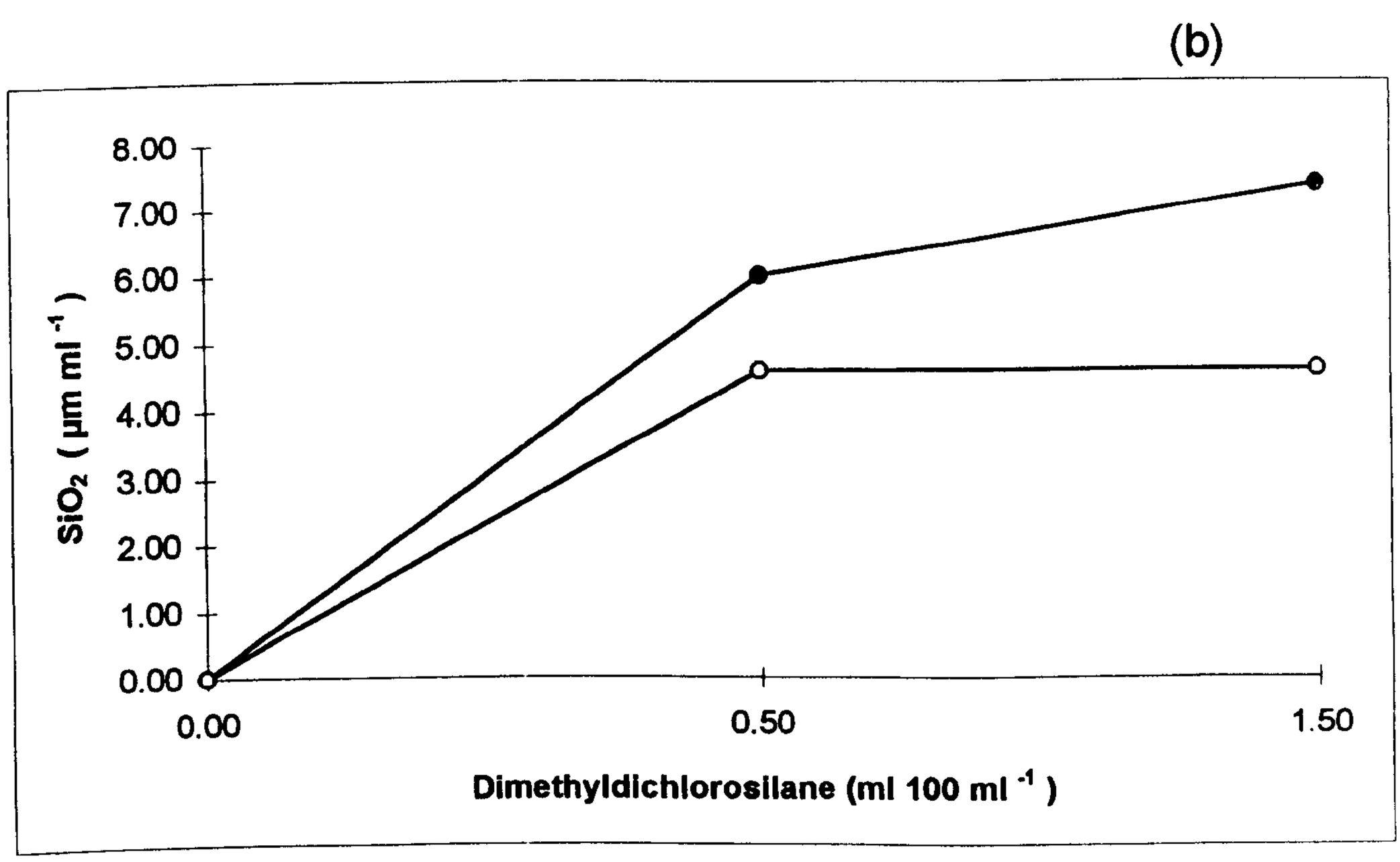


Figure 2.13 Effect of dimethyldichlorosilane (0.5ml and 1.5ml) on the growth of *A. oryzae* ●—● and *A. niger* O—O in Czapek Dox liquid medium, for 7 days at 25^{0}C, (a), release of soluble silicon from dimethyldichlorosilane by *A. oryzae* ●—● and *A. niger* O—O (b), Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

of hexamethyldisilane at both concentration had no effect on biomass of *A. oryzae* and *A. niger* (Fig. 2.14a). However, a marked increase in SiO_2 in the medium occurred when the fungi were grown in the media containing 0.5 and 1.5 ml 100 ml $^{-1}$ of hexamethyldisilane. Fig 2.14b shows that a further increase in the concentration of the organic silicon compounds to 1.5 ml 100 ml $^{-1}$ of medium did not however, lead to a concomitant increase in SiO_2.

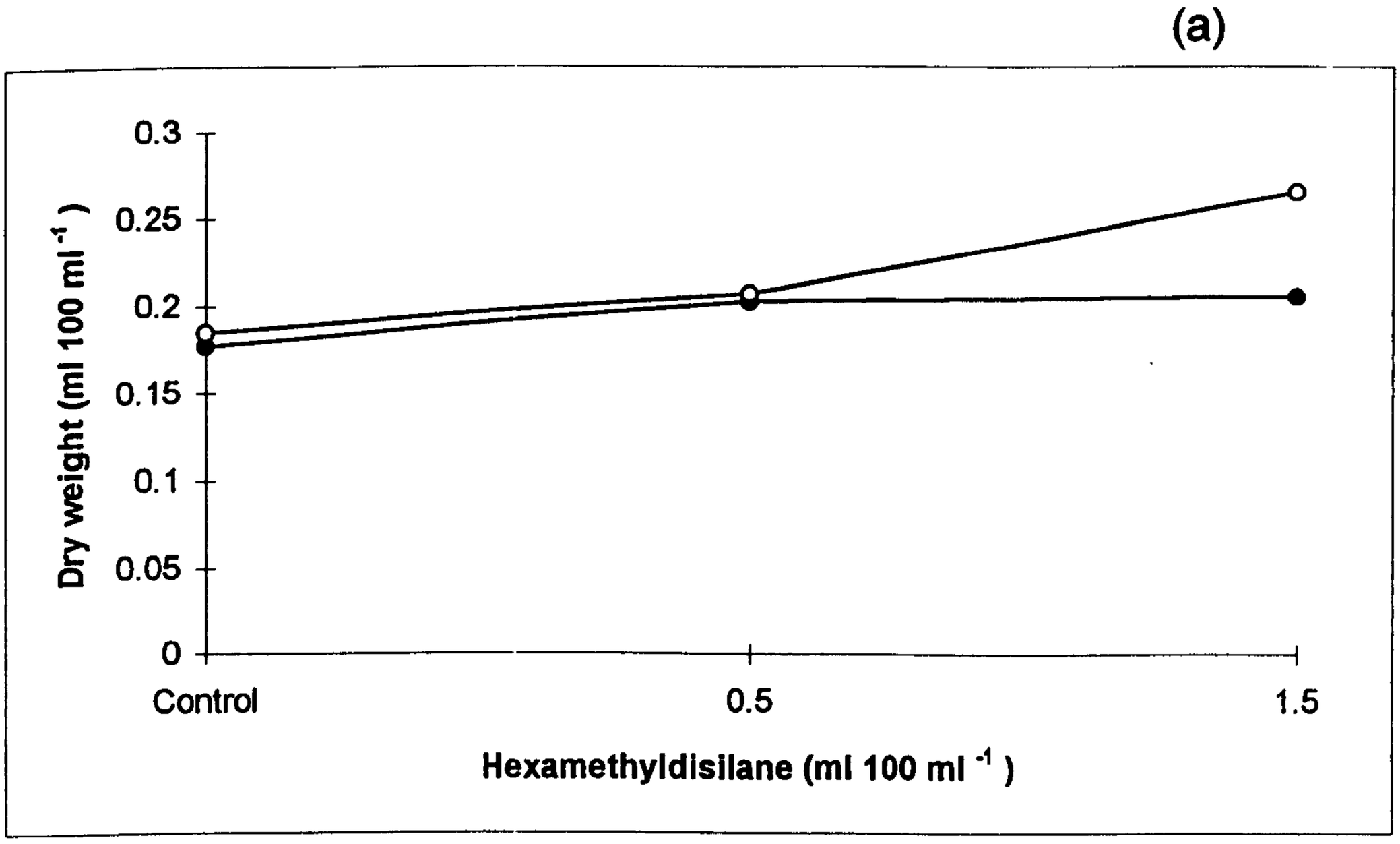
2.3.10 Effect of colloidal silicic acid on fungal growth.

The addition of colloidal silicic acid slightly stimulated the growth of *A. oryzae* and *A. niger* (Fig. 2.15a), but these increases were not statistically significant. The presence of the fungi however, lead to a marked increase in the concentration of SiO_2 in the medium (Fig. 2.15b).

2.3.11 Effect of silicic acid on the growth of fungi in the presence of a range of concentration of sucrose.

Here, either *Aspergillus oryzae* or *Aspergillus niger* were grown with one of the following concentrations of sucrose (1, 5, 10, 50, 75, and 100mM) in Czapek Dox liquid medium for 7 days at 25^0C. Silicic acid (1.5g) was added to each concentration. Data presented in Fig. 2.16 show that the biomass increased only slightly in media with increasing concentrations of added sucrose.

Silicon solubilization also did not increase markedly as the amount of sucrose added increased, while at 100mM sucrose, a decrease in free silicon concentration was observed when silicic acid was directly added to the medium.



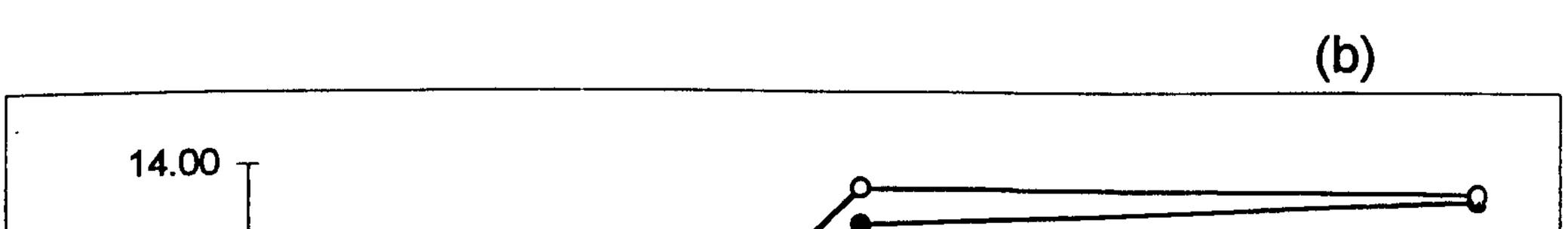


Figure 2.14 Effect of hexamethyldisilane (0.5ml and 1.5ml) on the growth of *A. oryzae* ●—● and *A. niger* O—O in Czapek Dox liquid medium, for 7 days at 25^{0}C, (a), release of soluble silicon from hexamethyldisilane by *A. oryzae* ●—● and *A. niger* O—O (b), Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

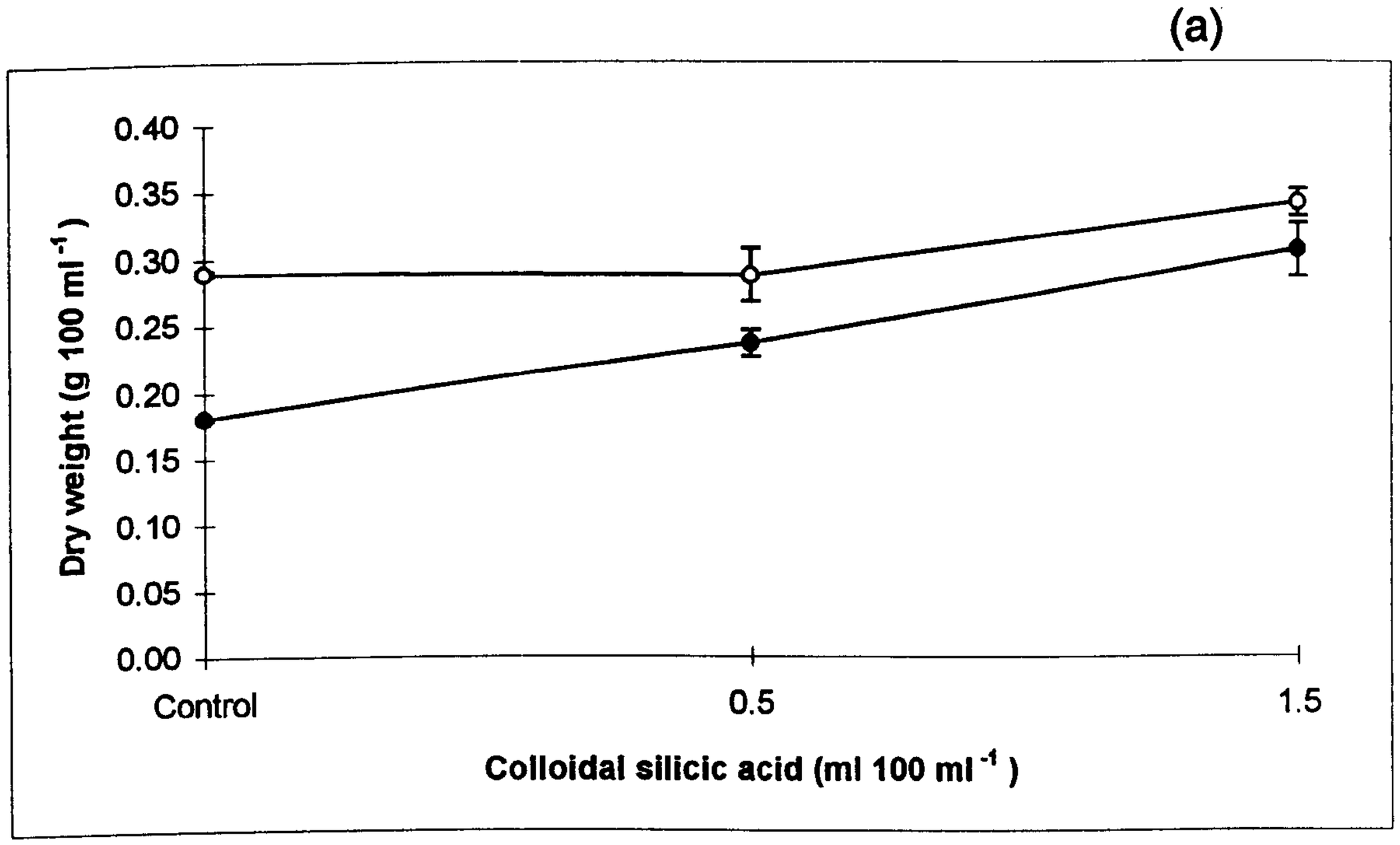
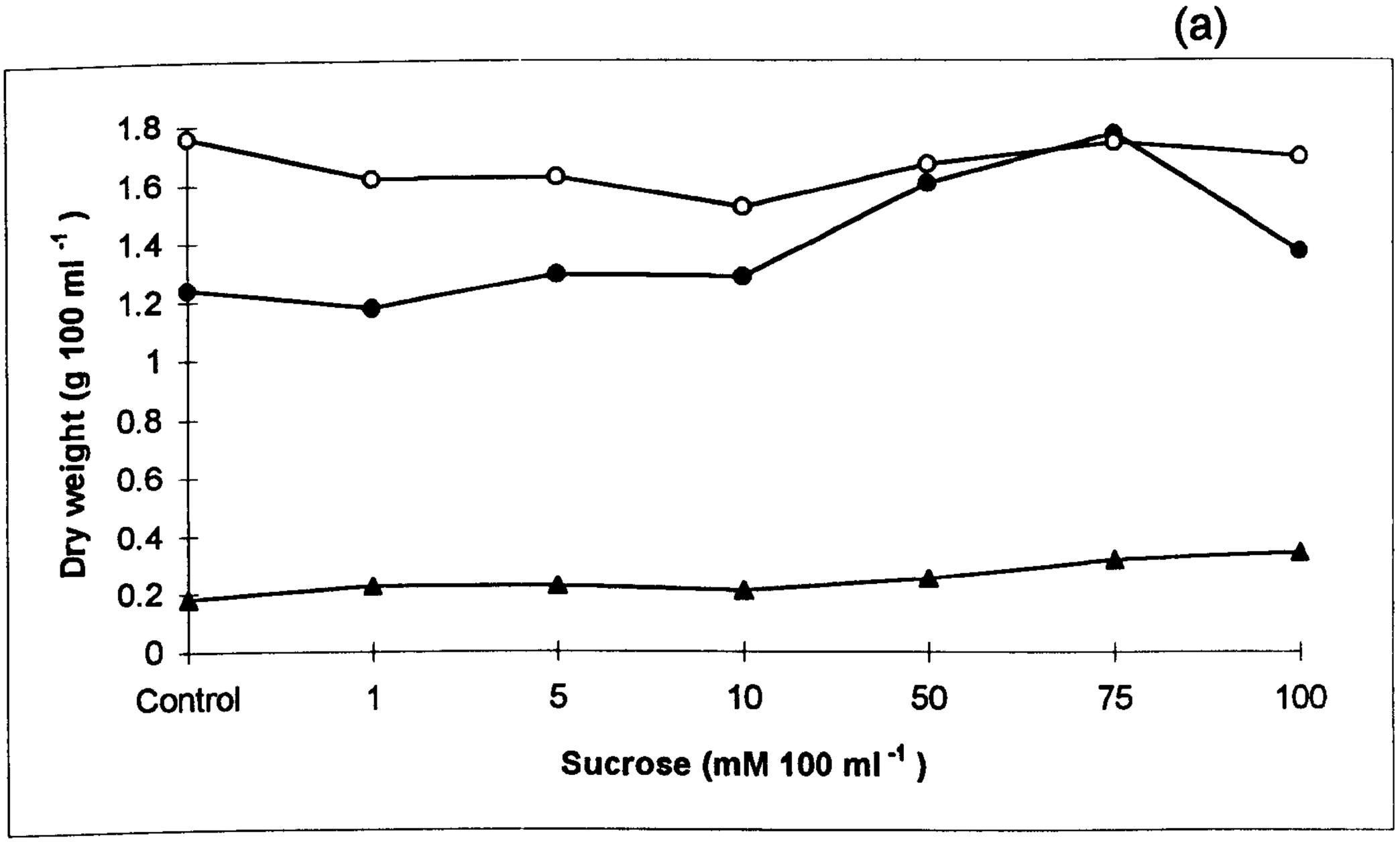


Figure 2.15 Effect of colloidal silicic acid (0.5ml and 1.5ml) on the growth of *A. oryzae* ●—● and *A. niger* O—O in Czapek Dox liquid medium (a), release of soluble silicon from colloidal silicic acid by *A. oryzae* ●—● and *A. niger* O—O (b), Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.



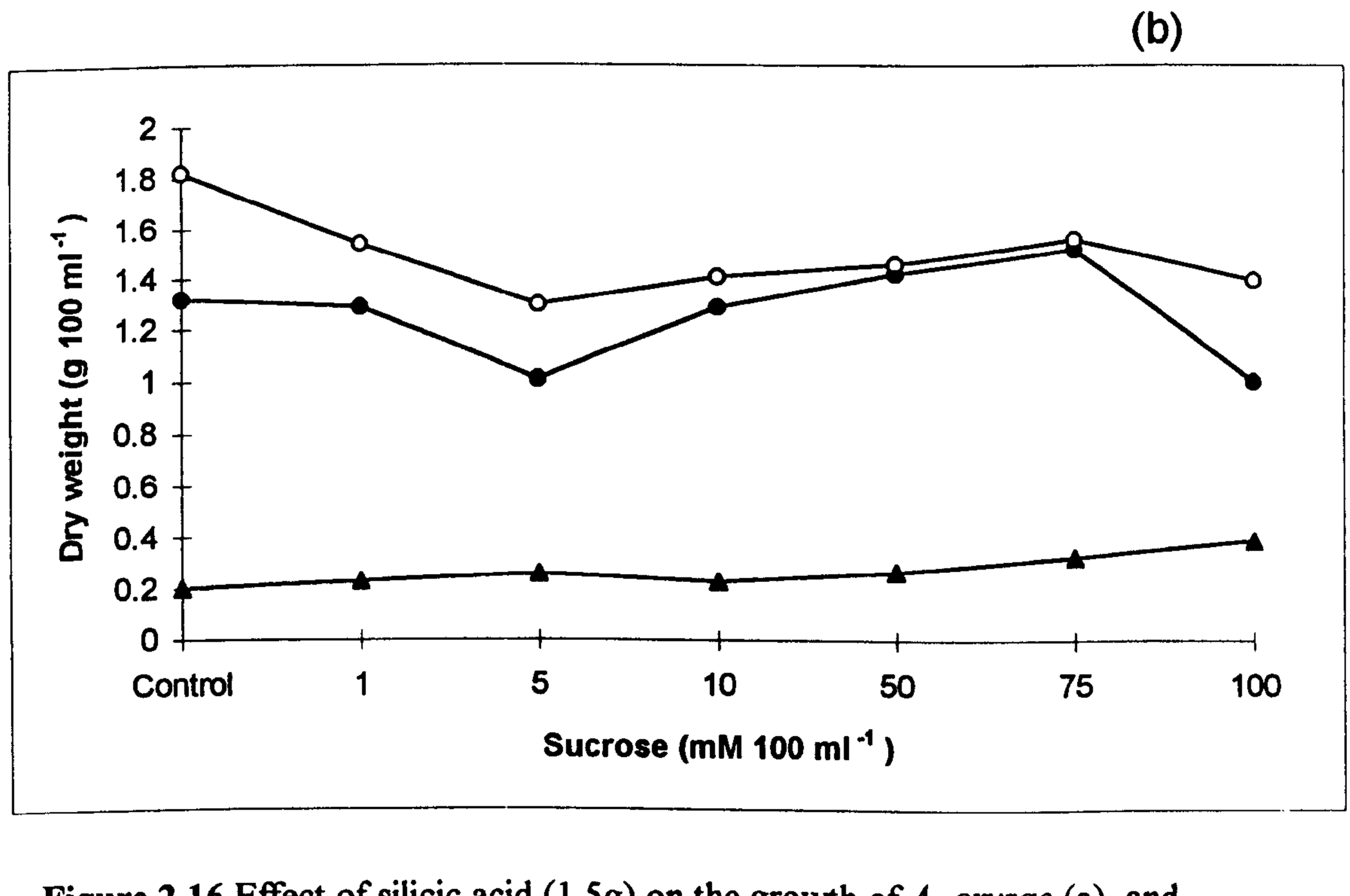
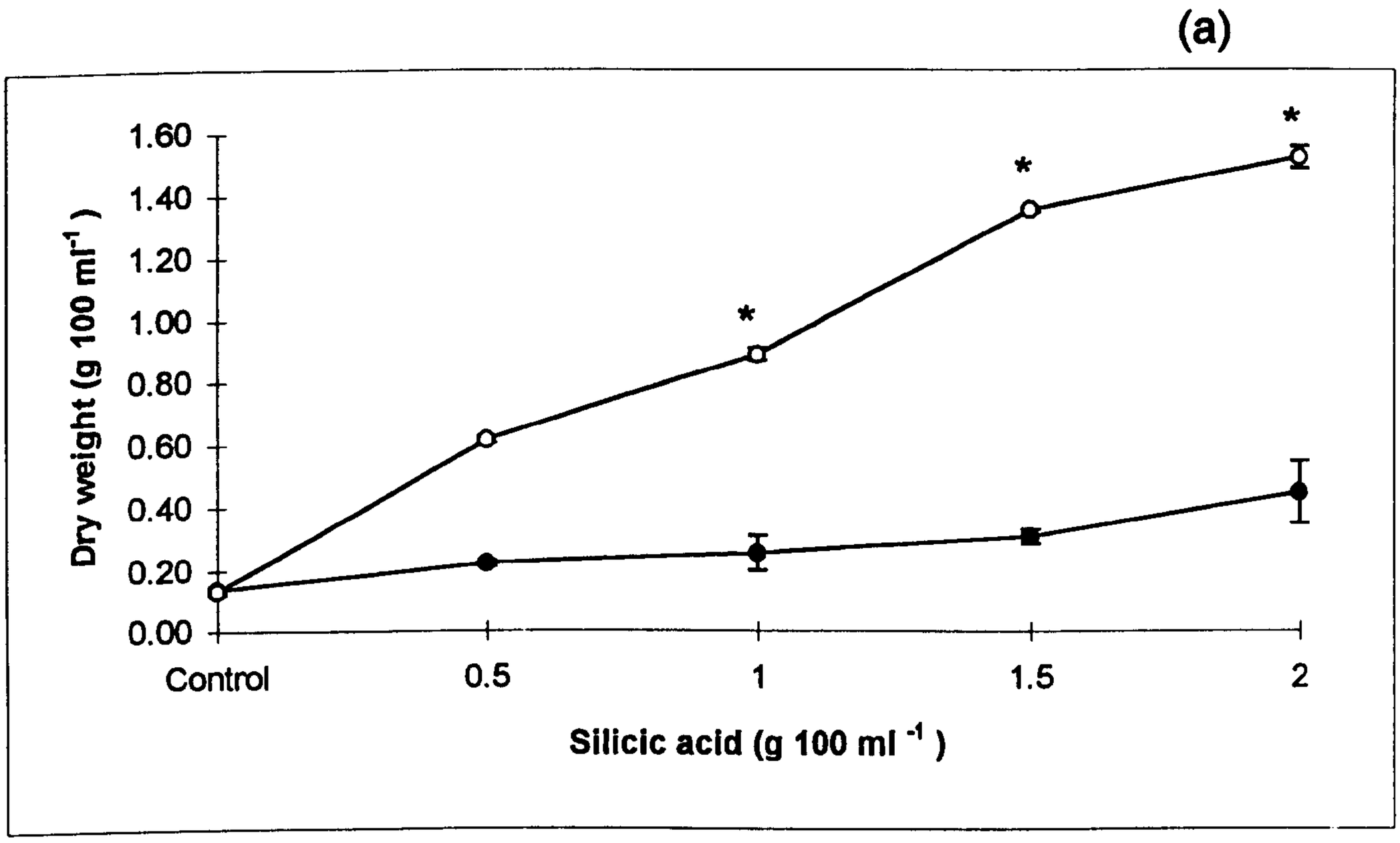


Figure 2.16 Effect of silicic acid (1.5g) on the growth of *A. oryzae* (a), and *A. niger* (b), grown in Czapek Dox liquid medium, containing a range of sucrose concentrations (mM), silicic acid in tubing ●—●; silicic acid added directly to medium O—O, while ▲—▲ control (no silicic acid). Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

These results are somewhat surprising, since it was assumed that an increase in the sucrose concentration of the medium would lead to an increase in fungal growth and resultant silicon solubilisation. This apparent anomaly can be explained by the fact that the C:N ratio of the medium was out of the optimal balance of 10:1, and that there was therefore insufficient nitrogen present for optimal fungal growth.

2.3.12 Effect of silicic acid on the growth of *Streptomyces spp.*

Data presented in Figs. 2.17a and 2.18a show that addition of a range of amounts of silicic acid (0.5g, 1g, 1.5g and 2g) to buffered (pH 7.2) date syrup liquid medium led to an increase in the growth (biomass) of *Streptomyces* isolates (H1 and N7) over the 5 days incubation period. Biomass and amount of silicon released (Figs. 2.17b and 2.18b) increased with increasing amount of added silicic acid. Negligible amounts of silicic acid were solubilised in the absence of *Streptomyces* inoculum. Since some of the silicic acid was adsorbed onto the surface of the growing mycelium, the measured biomass was larger than the real biomass. Silicic acid in the solution was not removed, even when the lowest amount of silicic acid (0.5% w/v) was added. This suggests that the biomass increase resulting from the addition of increasing amounts of added silicic acid was not solely due to adsorption of silicic acid to the mycelium. In order to determine if direct contact between the silicic acid and *Streptomyces* isolates was necessary for the observed growth increases, and increase in solubilization of silicon, silicic acid was added to flasks in sealed dialysis tubing. Figs. 2.17a and 2.18a show that an increase of biomass continued to occur under these conditions.



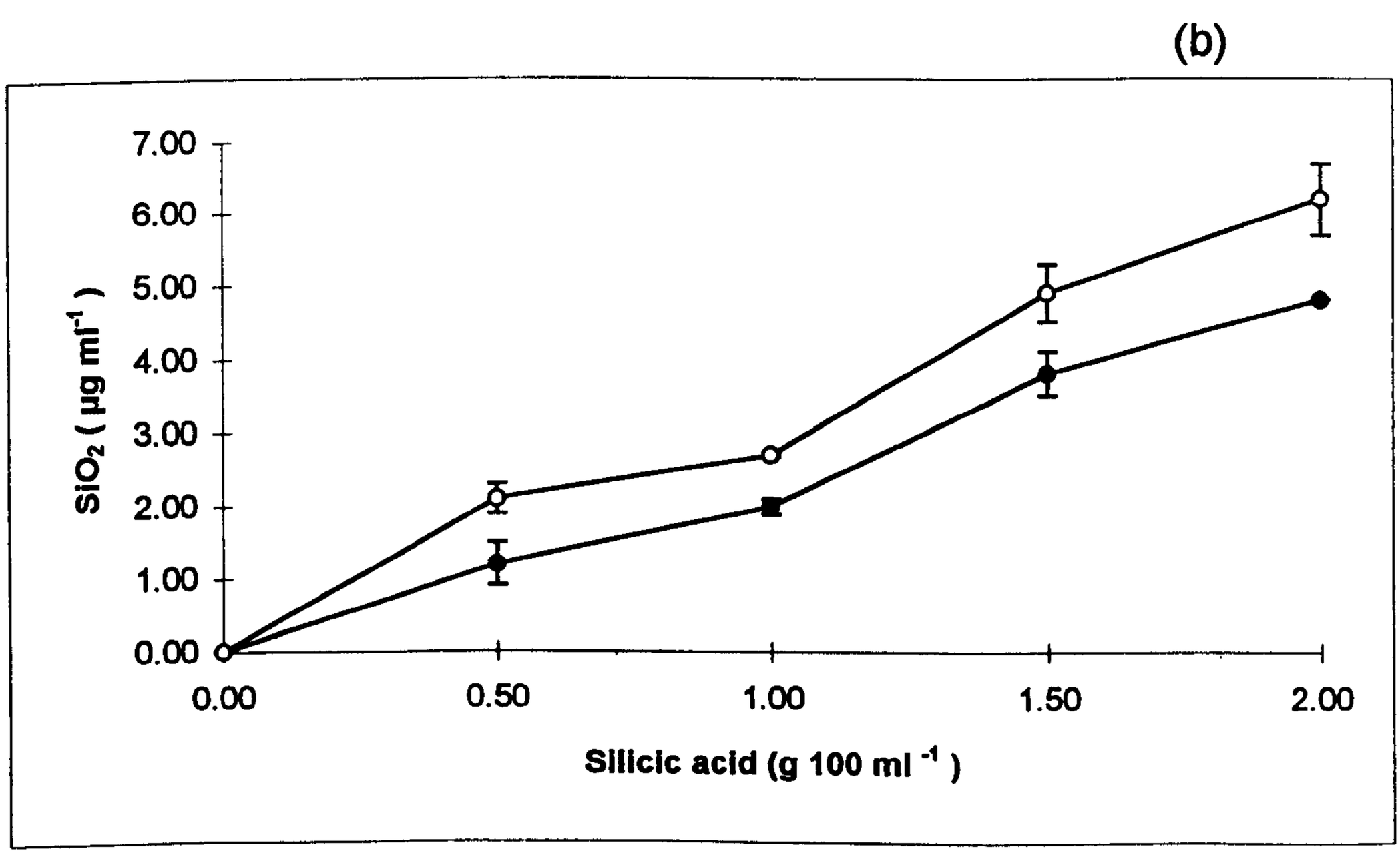
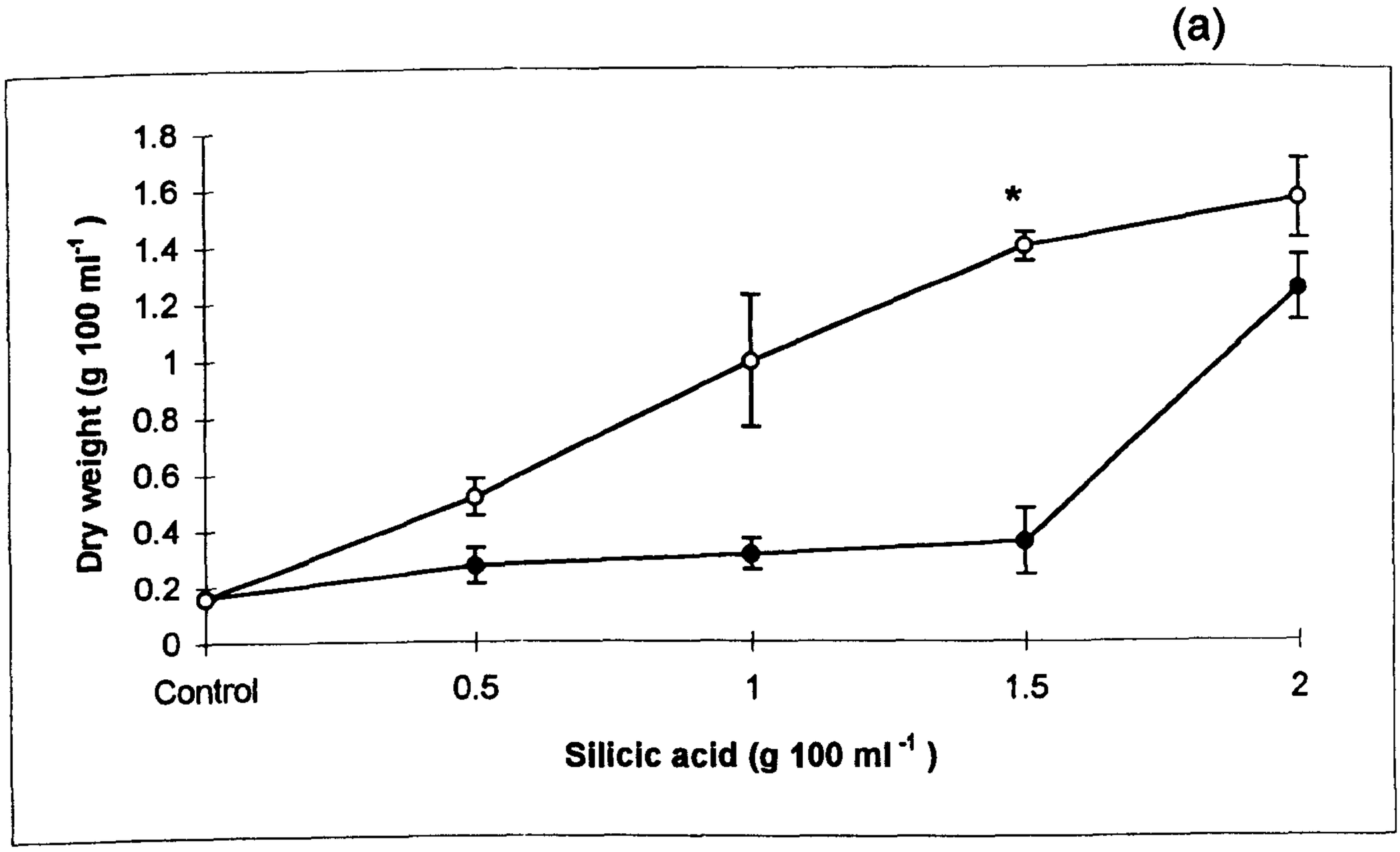


Figure 2.17 Effect of silicic acid on the growth of *Streptomyces* H1 in buffered date syrup liquid medium (pH 7.2), for 5 days at 30^{0}C (a), release of soluble silicon from silicic acid by *Streptomyces* H1 (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.



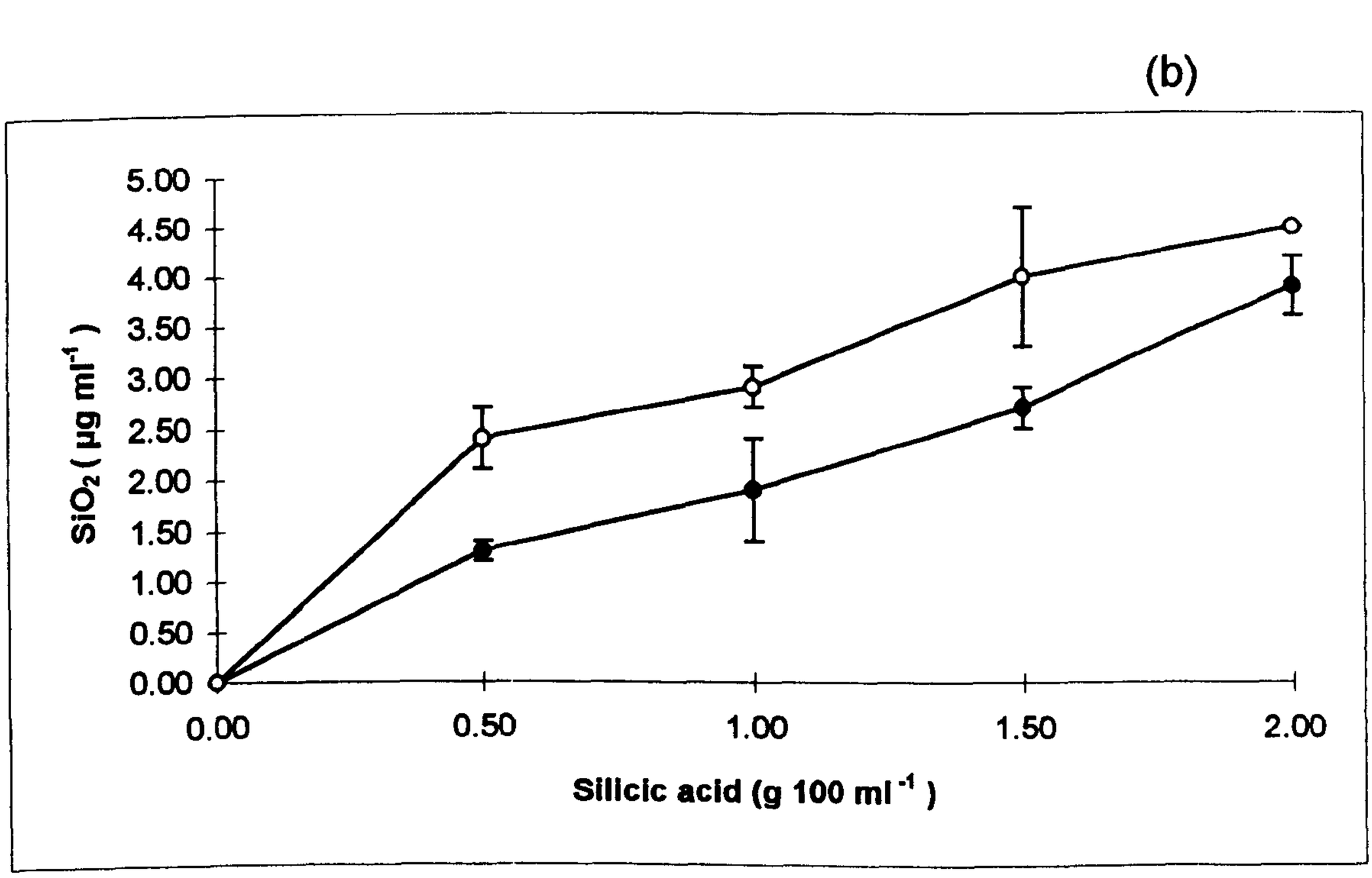


Figure 2.18 Effect of silicic acid on the growth of *Streptomyces* N7 in buffered date syrup liquid medium (pH 7.2), for 5 days at 30^0C (a), release of soluble silicon from silicic acid by *Streptomyces* N7 (b), O—O silicic added directly to medium; ●—● silicic acid added to medium in dialysis tubing. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

The pH of *Streptomyces* isolates in liquid medium was measured before and after growth. Data in (Table 1) show that only a small decrease in pH resulted from growth.

2.3.13 Analysis of protein content of *Streptomyces spp.*

The protein content of both *Streptomyces* isolates increased in the presence of silicic acid, in the case of the N7 strain significantly so ($P = 0.05$). These results show that the stimulatory effects of silicic acid on the growth of *Streptomyces spp.*, was associated with an increase in protein content and was not completely due to adsorption of silicic acid particles (Fig. 2.19).

2.3.14 Effect of silicic acid on growth of the algae *Dunaliella parva*

Data given in Fig. 2.20 show that the addition of silicic acid at 0.5g and 1.5g 100 ml $^{-1}$ led to decreases in the chlorophyll content of *D. parva* at both pH 6.3 (Fig. 2.20a) and pH 7.8 (Fig. 2.20b). The pH of algae in liquid medium was measured before and after growth, and no marked changes in pH were observed (Table 1).

2.3.15 Effect of silicic acid on bacterial growth.

Escherichia coli and *Staphylococcus aureus* were grown in L-broth buffered (pH 7.0) medium overnight at 37^0C. Figs. 2.21a, 2.21b show that the addition of silicic acid (0.5g and 1.5g) did not markedly affect the growth of both bacteria.

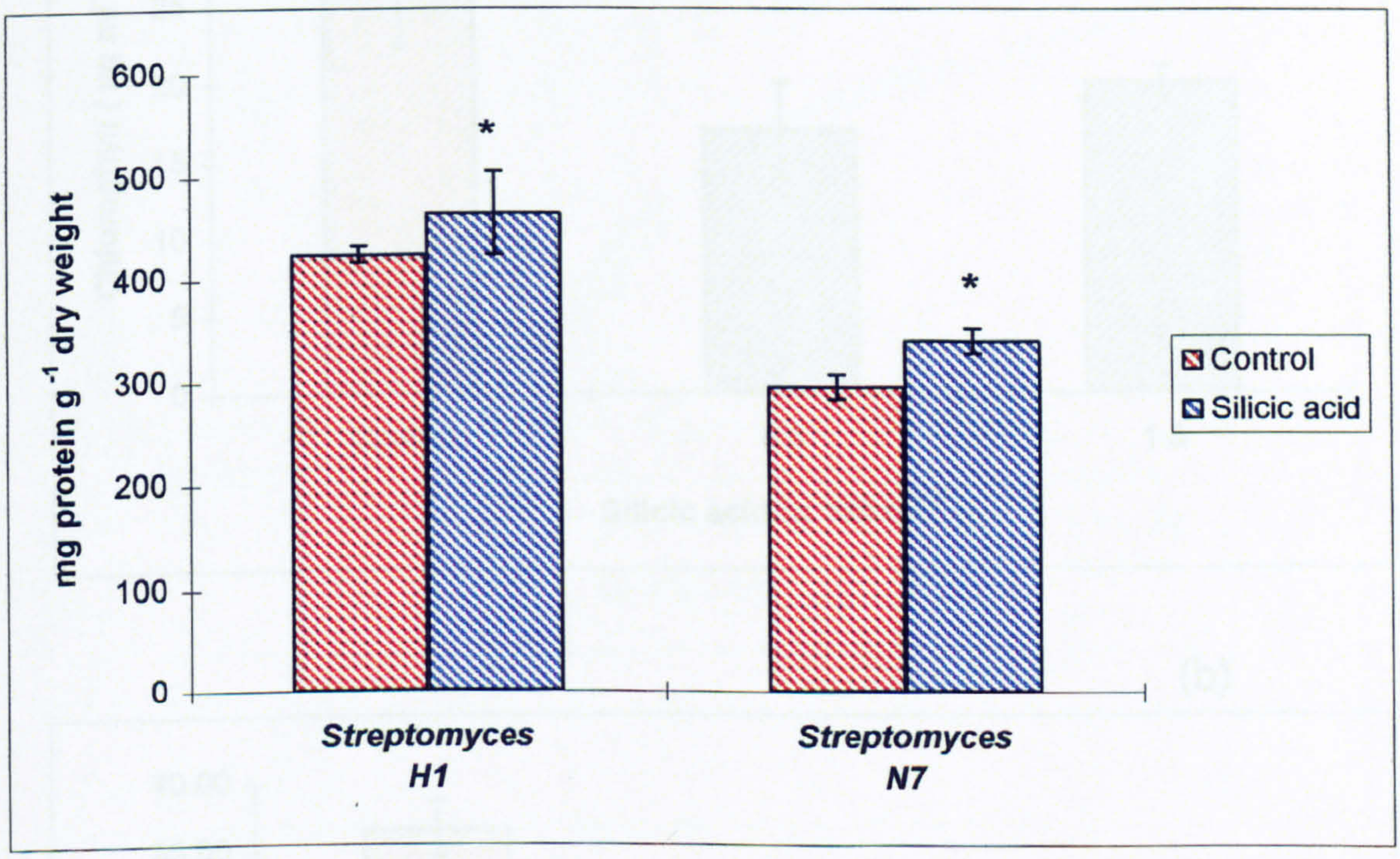
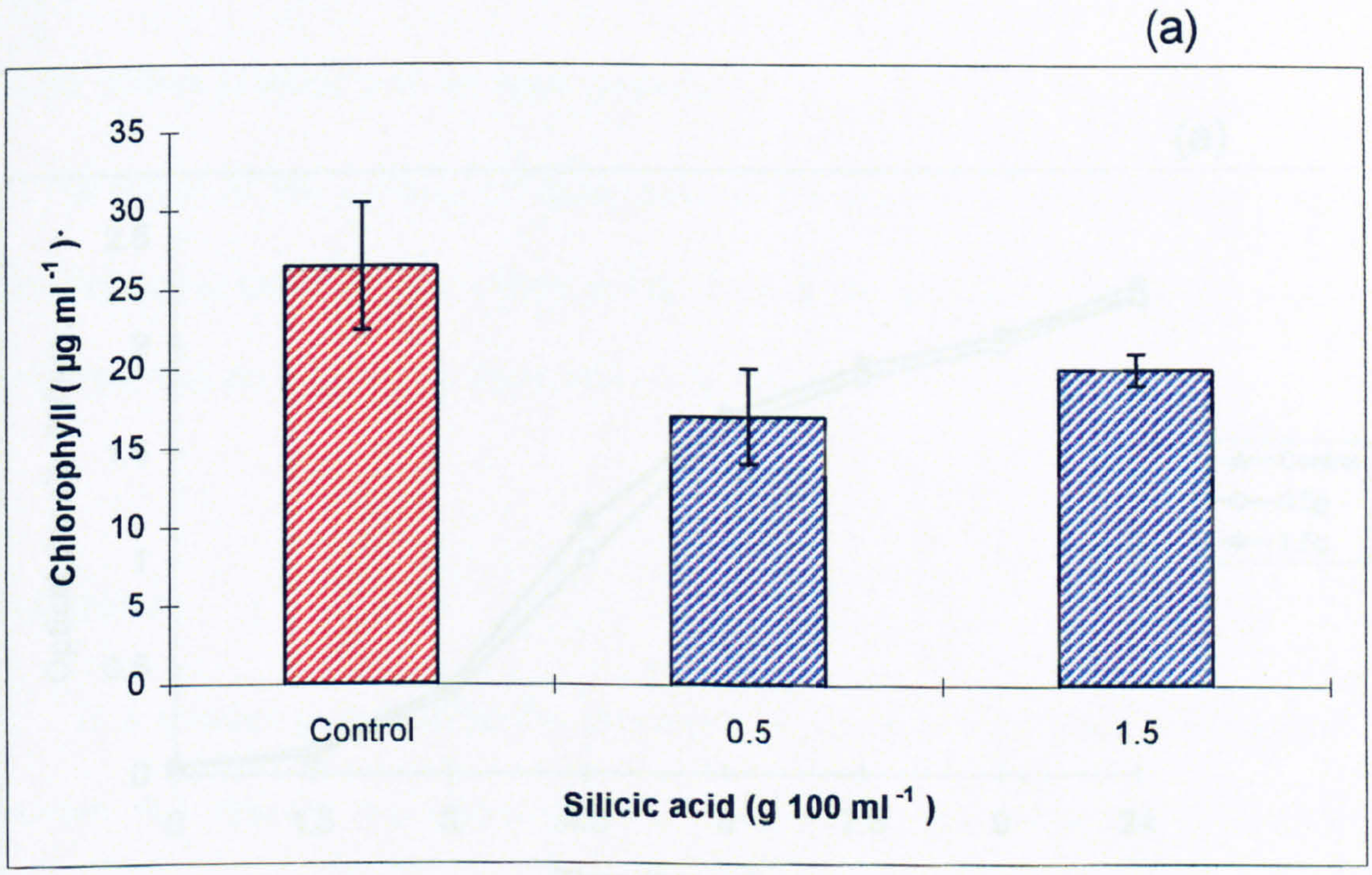


Figure 2.19 Analysis of the protein content of *Streptomyces* isolates H1 and N7 grown in date syrup liquid medium containing silicic acid (1g added to the medium in dialysis tubing), for 5 days at 30^0C. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.



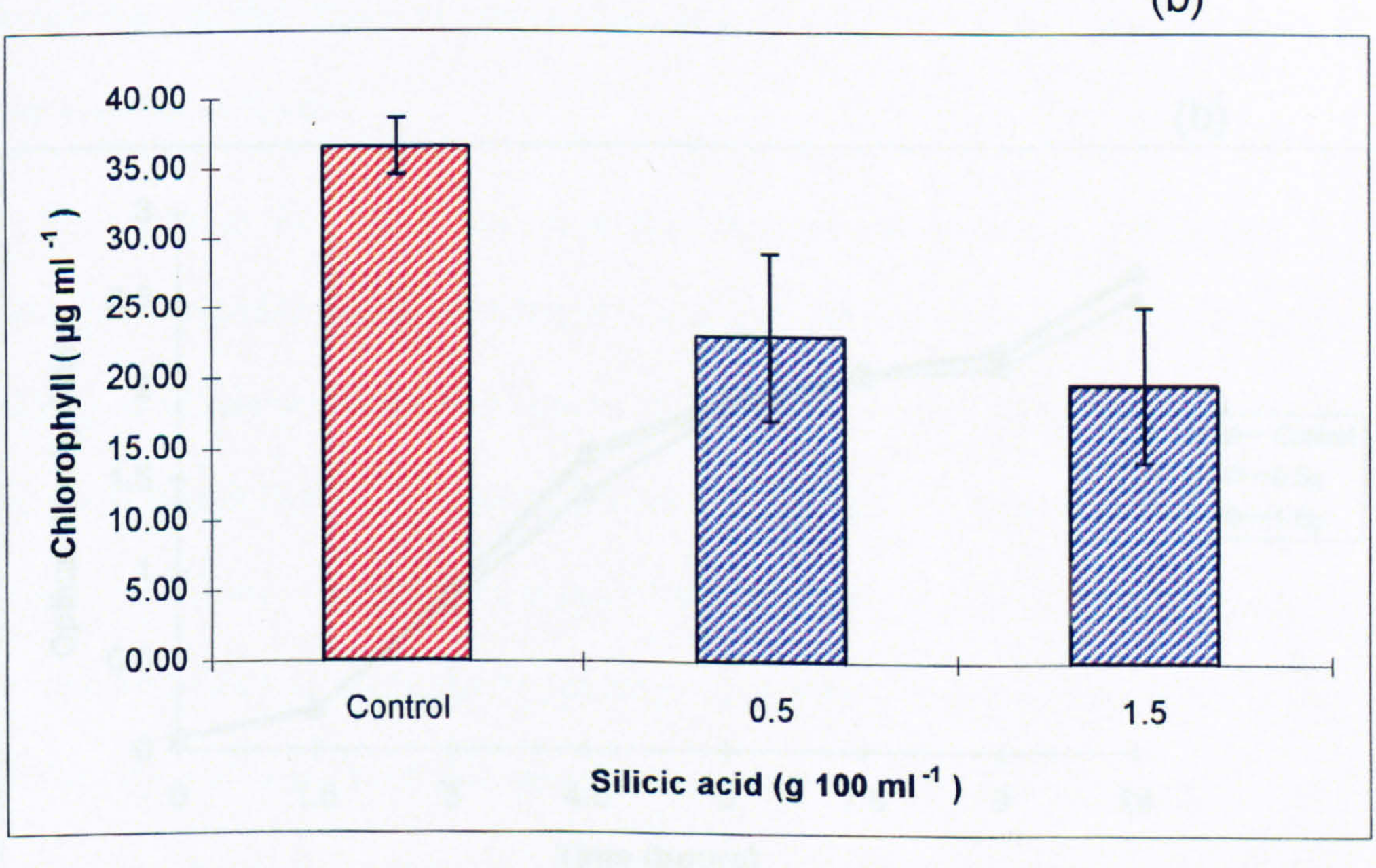
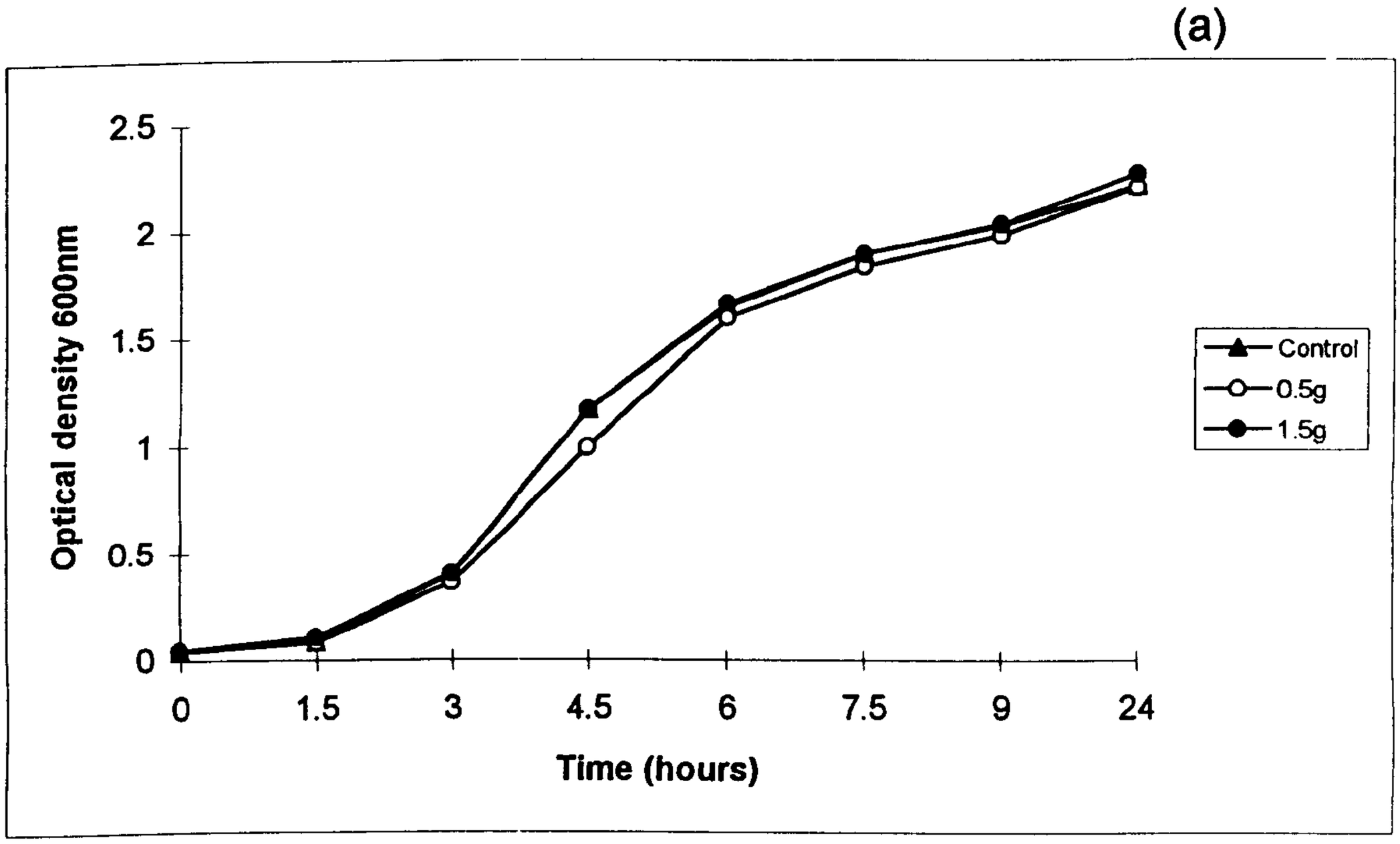


Figure 2.20 Effect of silicic acid (0.5g and 1.5g, added directly to medium) on the growth of *Dunaliella parva* in buffered liquid medium at 6.3 pH (a), and pH 7.8 (b), for 7 days at 25^{0}C. Means of triplicates, ± Standard error.



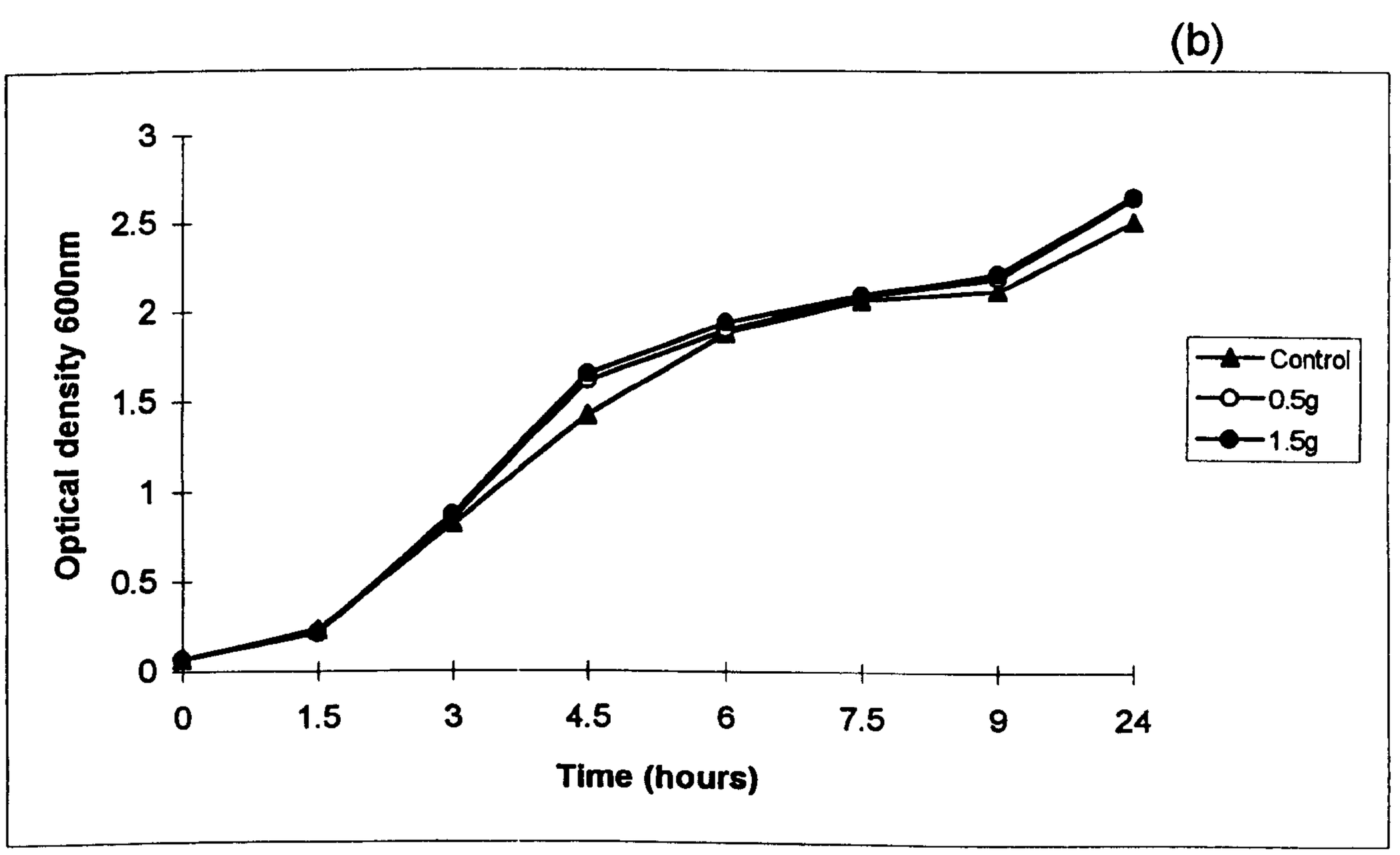


Figure 2.21 Effect of silicic acid (0.5g and 1.5g, added directly to medium) on the growth of *Escherichia coli* (a), and *Staphylococcus aureus* (b), in buffered (pH 7.0) L-broth medium, overnight at 37^{0}C. Means of triplicates, ± Standard error.

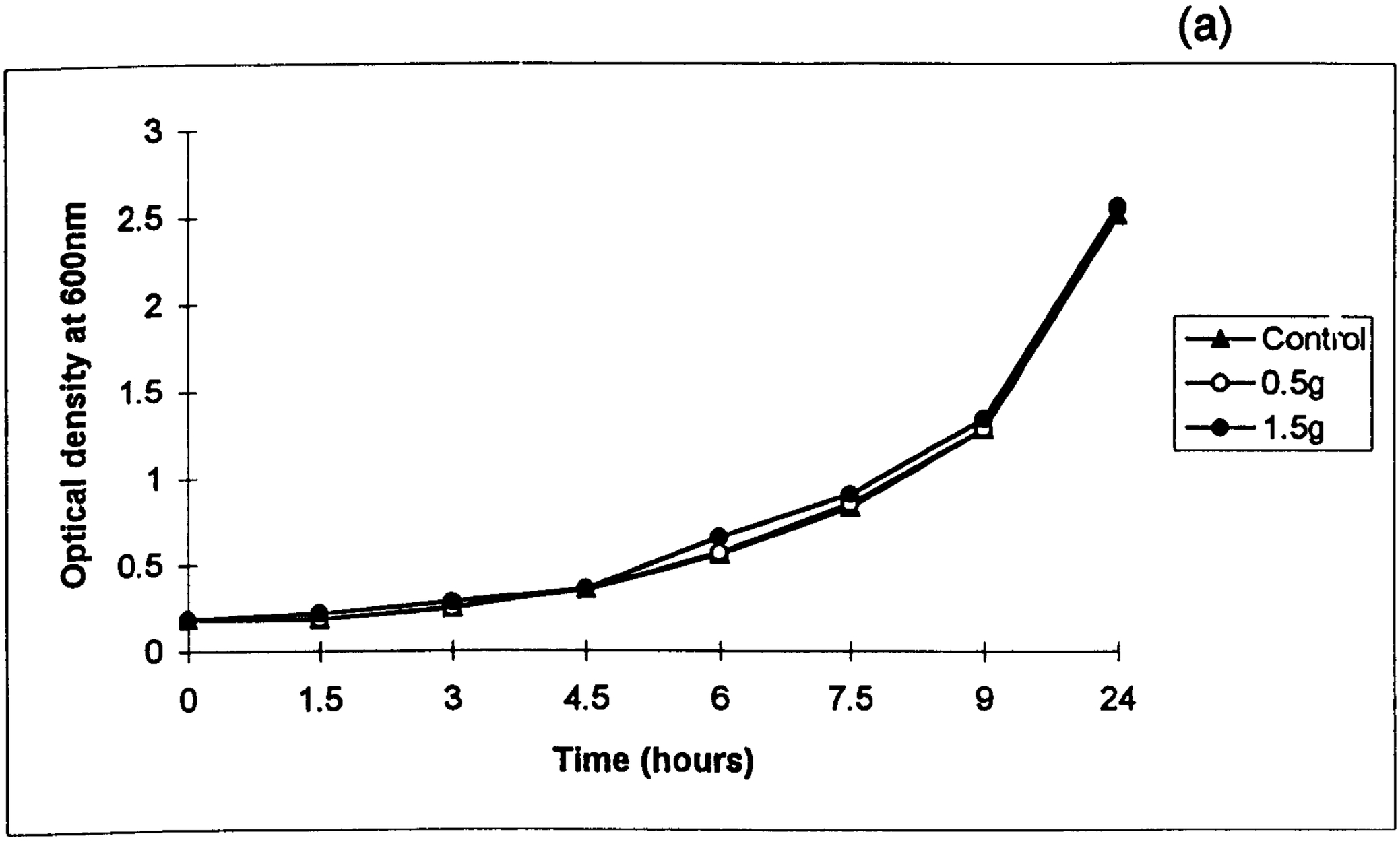
2.3.16 Effect of silicic acid on yeast growth.

The effect of the addition of silicic acid on the growth of *Pichia pastoris* and *Saccharomyces cerevisiae* are shown in Fig. 2.22a,b the growth of *P. pastoris* and *S. cerevisiae* was not affected by silicic acid (0.5g 100 ml $^{-1}$).

Conclusions

It is surprising, considering the abundance of silicon in soils, that the interaction between this element and microorganisms have been largely neglected. The main reason, is of course, that it is generally considered to be biologically unreactive, this, despite the fact that silicon occurs in plants (e.g. rice and *Equisetum*), diatoms and the skeletal parts of animals.

The results of the present study show that fungi are able to both solubilize, partially and totally, insoluble silicon compounds and adsorb the free silicon. Such solublization is likely to be relevant in the rhizosphere of plants that accumulate silicon. As a result, one might expect free-living fungi (and other microorganisms), particularly mycorrhizal fungi to participate in making silicon available to the roots of these plants. Although no attempt was made to study the enzymology or general biochemistry involved in this solubilization, it was found that direct contact between insoluble silicon compounds and the fungus was not necessary. The fact that such solubilization occurred when the fungus was grown in a sealed dialysis tubing suggests the involvement of low molecular weight acids and chelating agents, rather than high molecular weight enzymes.



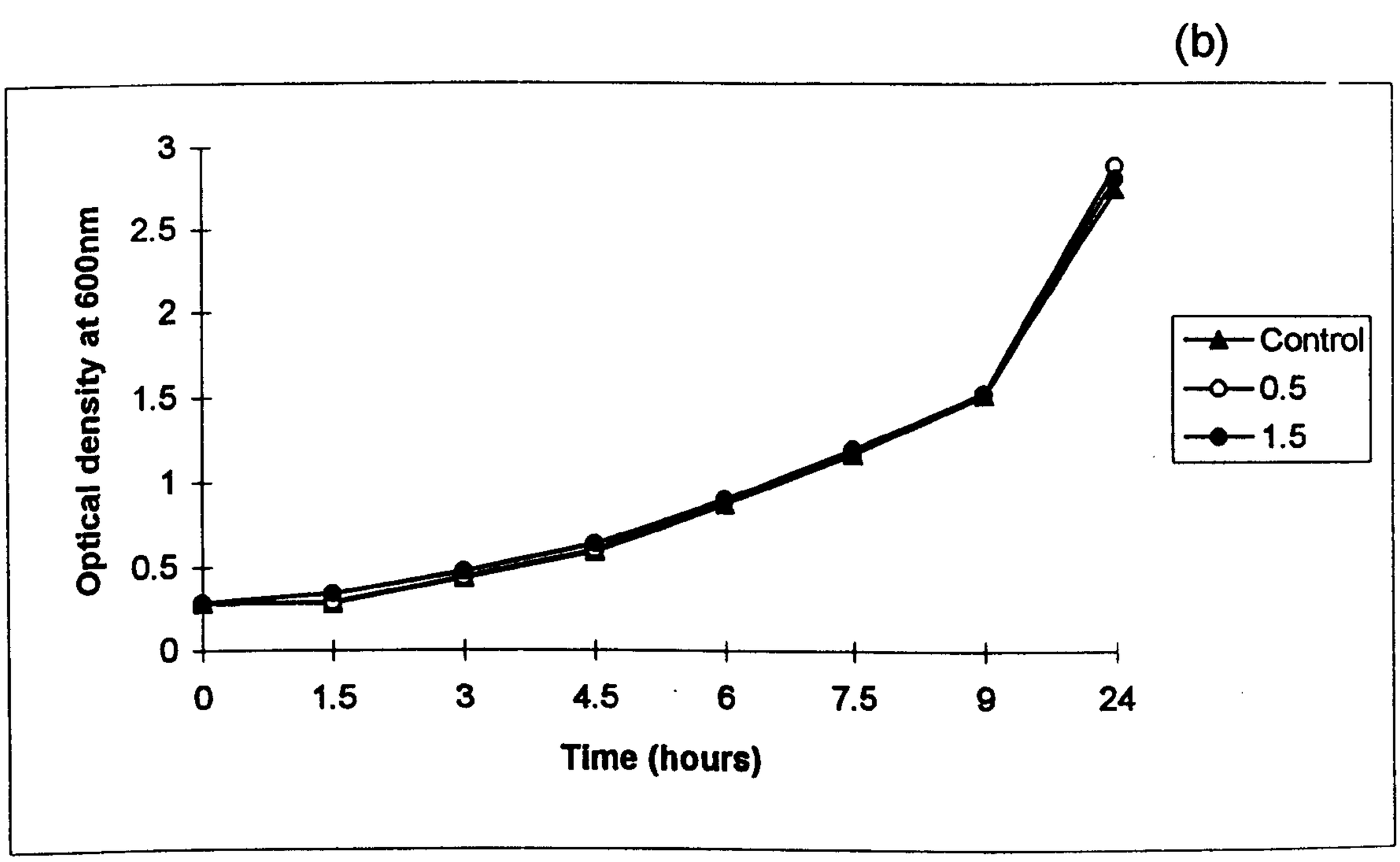


Figure 2.22 Effect of silicic acid (0.5g and 1.5g, added directly to medium) on the growth (optical density) of *Pichia pastoris* (a), and *Saccharomyces cerevisae* (b), in buffered (YEPD) liquid medium (pH 6.8), for 24 hours at 30°C. Means of triplicates, ± Standard error.

result from the solubilization process. The exact mechanism of this stimulation has yet to be determined.

The growth of *Streptomyces spp.* was also stimulated by the addition of silicon compounds to nutrient-rich media. As with fungi, this growth stimulation was evident both in terms of an increase in biomass (dry weight) and protein content. In contrast silicic acid had no effect on growth of *Escherichia coli* and *Staphylococcus aureus*, and the yeast, *Pichia pastoris* and *Saccharomyces cervisiae*, and decreased the chlorophyll content of *Dunaliella parva.* These results suggest that the stimulatory effect of silicon compounds is restricted to filamentous microorganisms, i.e. fungi and species of *Streptomyces.*

growth obviously indicates the likely involvement of acids, such as citric acid, in the solubilization process. On the other hand the fact that the solubilization reaction still occurred when the medium was buffered to pH 6.8, suggests a role for chelating agent(s) rather than acids.

The growth of the fungi in nutrient-rich media investigated increased in the presence of silicon, both in terms of biomass (dry weight) and protein content. The fact that there was an increase in the latter shows that observed dry weight increases were not due solely to adsorption of silicon particles to the mycelium surface. Electron microscope studies tended to confirm this. The lack of adsorption of silicon particles is surprising, since it is well known that fungal mycelium has a marked propensity for particles (Wainwright *et al.*, 1986). It was also perhaps surprising that silicon was not deposited within the fungal cell in the form of electron dense bodies. Such bodies are for example, commonly seen when fungi grow in medium containing heavy metals (e.g. mercury) (Williams and Pugh, 1975).

Not surprisingly, sodium fluorosilicate did not, unlike other silicon compounds stimulate the growth of *Aspergillus niger*. This fact can be explained on the basis that at the fungus solubilizes the compounds both silicon and fluorine ions are released into the medium; the latter proving toxic to the fungus when sodium fluorosilicate was added at concentration exceeding 25 ml 100 ml^{-1} medium. Dimethyldichlorosilane, also inhibited fungal growth, while the other organic compound (hexamethyldisilane) had no effect. Colloidal silicic acid did not stimulate fungal growth, but was readily solubilized. This suggests that the biomass-stimulatory effect of silicic acid did not

CHAPTER THREE

EFFECT OF SILICON COMPOUNDS ON THE GROWTH OF MICROORGANISMS UNDER OLIGOTROPHIC CONDITIONS

3.1 Introduction

As has already been noted, relatively little work has been published on the interaction between silicon and microorganisms. What has been published relates exclusively to the growth of microorganisms in nutrient-rich (so-called copiotrophic) media. However, since most environments are oligotrophic, that is they contain only low concentration of nutrients, such studies on the interaction of silicon and microorganism growing in rich media, bare little relationship to how these organisms grow in nature.

Studies on the effect of silicon on microbial growth under low nutrient condition are partially relevant to the question of whether or not silicon can act as an alternative energy source to carbon, or otherwise enhance the efficiency of carbon utilization. Oligotrophic microorganisms, and their ability to grow both in culture and in the environment, have been extensively studied over the last two decades, although work in this topic appears to be becoming increasingly less fashionable.

3.2 Oligotrophic bacteria

Oligotrophic microorganisms can be broadly defined as organisms that grow on low concentrations of organic substrates. However, there is no generally accepted definition of an oligotrophic bacterium. Kuznetsov *et al.*, (1979) defined oligotrophs as those bacteria isolated on organic media containing 1-15 mg ml $^{-1}$ C, whilst Martin and Macloed (1984), suggested the use of 10 mg ml $^{-1}$. Akagi *et al.*, (1977); Ishida and Kadota (1981), recommended 1 mg ml $^{-1}$, and Poindexter (1981) defined

oligotrophy relative to the nutrient flux of the habitat in which bacteria grow. Oligotrophic habitats having a nutrient flux from near zero to a fraction of milligram of C day $^{-1}$.

Some so-called obligate oligotrophs can grow only on low concentrations of carbon and cannot survive under high nutrient conditions. However, both obligatory oligotrophic bacteria and fungi are relatively rare in nature. Facultative oligotrophs, which can grow at both very low and very high concentrations of carbon, are in contrast, much more common.

3.3 Oligotrophic fungi

The mycelial growth form is particularly advantageous for organisms growing in low nutrient environments as it provides a high surface, volume ratio that favours an increase in nutrient uptake capacity. The thinner the hyphae, the larger the surface area, a fact which explains why the hyphae of oligotrophically growing fungi are so fine.

3.4 Oligocarbotrophy

An oligocarbotroph is a microorganism which a) grows in a carbon deficient environment, and b) can be isolated using low carbon media, and demonstrates relatively high growth rates in culture containing small amounts of organic carbon (Williams, 1985). The ability of some soil bacteria to grow oligocarbotrophic is well documented (Hirsch *et al.*, 1979; Ohta and Hattori, 1983). Many of these bacteria can be defined as being obligate oligocarbotrophs because they are unable to grow in

carbon rich media (Hattori, 1973; Hattori, and Hattori, 1980). Tribe and Mabadeje (1972) showed that certain fungi can germinate and grow on silica gel lacking any added carbon. They assumed that fungi were incapable of growing autotrophically, fixing CO_2, and as a result concluded that fungi must scavenge organic carbon from the atmosphere.

3.5 Oligonitrotrophy

The ability of microorganisms to grow in the absence of nitrogen source is referred to as oligonitrotrophy. Several types of soil microorganisms can grow relatively luxuriantly on N-free media suggesting that they are also fixing-N. However, tests with isotopic nitrogen and acetylene reduction invariably show that most of these so-called "N-fixing ghosts" do not fix N, but instead are very efficient scavengers of traces of fixed nitrogen (Hill and Postgate, 1969). Line and Loutit (1971) isolated five species of oligonitrotrophic bacteria from soils in New Zealand. They were found to be facultatively anaerobic species of *Bacillus circulans*, *B. polymixa, Enterobacter aerogenes, Escherichia intermedia* and species of *Klebsiella.* In Brazilian soils, twenty four *Bacillus sp.* were isolated by Seldin *et al.*, (1983) using N deficient media, eighteen of these bacteria were identified as *B. polmyxa.* The ability of fungi to grow as oligo-nitrotrophs has been reported by Parkinson *et al.*, (1989) who found that some fungi have the ability to grow in silica gel media lacking any added nitrogen. They suggested that the fungi growing in these conditions may scavenge combined nitrogen (as NH_3) from the atmosphere.

Many studies have been made of the oligotrophic bacteria (Akagi *et al.*, 1977; Poindexter 1981; Rochelle *et al.*, 1989). As a result of these studies, it has been established that an oligotrophic bacteria found growing under natural conditions possess a high rate of growth at low concentrations of organic substances.

Dubinina (1977) demonstrated that when the bacteria *Leptothrix pseudoochraceae*, *Siderocapsu eusphaera* and *Metalloogenium personatum* were cultured upon nutrient-rich media, these organisms lysed due to the accumulation of hydrogen peroxide. Catalase, which breaks down hydrogen peroxide, resulted in growth of the microorganisms when added to the media. Furthermore, suppression of the growth of some microorganisms occurs in a nutrient-rich medium. In *Pseudomonus oxalaticus* it has been shown that enzymes involved in active transport of substrates into a cell or that respiration is inhibited by high nutrient concentrations (Dijkuizen and Harder, 1975). The concentration of dissolved assimilated organic substances serves as the major ecological factor responsible for the development of oligotrophic microorganisms, and is generally used as an index of the amount of energy available for heterotrophic bacteria and fungi.

Clark (1967) estimated that 75% of bacteria from some soils do not form spores and questioned how bacteria survive in low nutrient conditions. Oligotrophic bacteria show a marked ability to scavenge nutrients, either from organic material contaminating the medium in which they are growing, or from the atmosphere. It has also been shown that certain bacteria can scavenge sufficient carbon from the laboratory air to allow for limited growth (Geller, 1983). Carbon dioxide seems to act as a nutrient source for oligotrophic, heterotrophic, bacteria. It is scavenged with

other carbon substrates and metabolised heterotrophically, rather than autotrophically. Even though many oligotrophic bacteria can also grow as anaerobes, most oligotrophic bacteria employ aerobic metabolism when growing under oligotrophic conditions. This is because aerobic respiration yields more energy from the dissimulation of organic substrates than does aerobic respiration.

The ability of fungi to grow in media or water to which no carbon substrates have been added has frequently been documented (Castellani, 1939; Stern *et al,* 1956). In the early 1970s, a number of studies were carried out employing more rigorous methodology with regard to maintenance of oligotrophic fungal growth. These included the use of water distilled with $KMnO_4$ and H_2SO_4, membrane filtered distilled water, and analytical grade compounds (Mirocha and Devay, 1971).

A wide variety of fungi have been shown to grow oligocarbotrophically (i.e. in a carbon deficient environment) on silica gel and where no nutrients have been added. Oligotrophy is clearly widely distributed among the fungi and is not restricted to an individual species, genus, or group. However, some fungi, mainly wood-decomposing and mycorrhizal species, do not appear to grow oligotrophically on silica gel (Wainwright, 1993).

Small amounts of biomass are produced when a fungus is grown oligotrophically. Fungi produces very fine hyphae, which form fine mycelial mats (or gossamers). These show numerous anastomoses and float just below the surface of the medium (Wainwright and Grayston, 1988). Gossamers are presumably formed because they provide a large surface area, thereby aiding nutrient scavenging from the atmosphere and nutrient-poor media. Most fungi, including soil isolates, produce spores normally

under low nutrient condition, although not luxuriously, and microcycle condition may occur (Sheehan and Gochenaur, 1984; Dickinson and Bottomley, 1980).

Fungi can grow oligotrophically without the apparent involvement of the lysis and utilization of performed hypha, although such lysis is occasionally seen when fungi grow oligotrophically on silica gel (Tribe and Mabadeje, 1972). Parkinson *et al.*, (1989) found that hypha of *Fusarium oxysporum* growing under these conditions were not subject to lysis, but remained intact and full of cytoplasm from the point of inoculation to the actively growing hyphal tip. Since fungi can be grown oligocarbotrophically on silica gel and then transferred to fresh gel on as many as 20 occasions (Parkinson *et al.*, 1989), it is clear that fungi can grow without relying on the lysis and utilization of performed biomass, but instead utilize exogenous nutrients.

Cytoplasm-free hypha have, however, been reported following fungal growth under low nutrient conditions. Dickinson and Bottomley (1980), for example, showed that when spores of *Alternaria* and *Cladosporium* germinate in a nutrient-free solution, the hyphae that are initially formed become evacuated and remain empty. A recent model of fungal growth by Schnurer and Paustian (1986) similarly predicts that, in soils, fungi grow by producing cytoplasm-free hyphae, along which cytoplasm can be translocated where necessary. This predicted growth pattern is conservative in terms of energy costs because the formation of cytoplasm is energetically less costly that is the production of cell wall.

Mirocha and Devay (1971) suggested that under oligotrophic conditions fungi fix CO_2 from the atmosphere, using energy obtained from the oxidation of atmospheric hydrogen (i.e. by the Knall gas reaction). However, they found no evidence for the

presence of Calvin cycle enzymes. The lack of this enzyme in *F. oxysporum* grown in the apparent absence of nutrients has been confirmed by Parkinson *et al*, (1989). By using stripping autoradiography, these workers also confirmed the findings of Mirocha and Devay (1971) that CO_2 is in some way metabolised by fungi when growing under strict oligocarbotrophic conditions, the label being incorporated into hyphae.

Parkinson *et al.*, (1991) studied the fate of the carbon assimilated by oligotrophically grown *F. oxysporum*. They found that approximately half of the fixed carbon was released into the medium, while 67% of that remaining in the mycelium was present in the protein fraction, i.e. a carbon distribution pattern that is similar to that found when fungi fix CO_2 anaplerotically. Some 0.68% of fungal cell carbon was derived from CO_2 under oligotrophic conditions.

It is now generally assumed that fungi grow under oligotrophic conditions by scavenging nutrients from the atmosphere and fixing CO_2 heterotrophically. As well as acting as a nutrient, CO_2 may have other functions in the fungal cell. Barinova (1961), for example, found that CO_2 speeds up both the swelling and germination of fungal spores and also catalyzes the formation of protein and nucleoprotein polypeptides from amino acids. It has been suggested that fungi may supplement their energy requirements by oxidizing inorganic ions such as ammonium or thiosulphate (Wainwright, 1988). Although such chemolithoheterotrophy is relatively common among heterotrophic bacteria, the ability of fungi to use this growth strategy has not yet been confirmed.

Wainwright and Grayston (1988) provided evidence that fungi use energy gained from thiosulphate oxidation to help the grow oligotrophically, while Jones *et al.*, (1991) found that *F. oxysporum* could oxidize 13% of thiosulphate provided to sulphate under oligotrophic conditions, this being sufficient to provide energy for 25% of the observed growth.

3.6 Oligotrophic growth of microorganisms in soil

Soil is generally regarded as being a low nutrient environment (Hirsch, *et al.*, 1979). Most of the organic material entering the soil has already been partially exploited and as a consequence, readily available substrates have been removed prior to incorporation. Even when there is constant enrichment by energy-yielding substrates, for example around the roots, it has been calculated that carbon inputs are insufficient to maintain the development of large, active microbial communities (Barber and Lynch, 1979). Although, many soil fungi can use the organic materials in the sub-litter soil horizons as carbon sources they do so only very slowly, and may require the presence of other easily available carbon compounds. Most soils are generally considered to contain insufficient carbon to allow the continuous growth of fungi (Lynch, 1982), and in general soil is not regarded as a rich medium for microorganisms being certainly much poorer than the artificially prepared media generally used in a laboratory.

Ko and Lockwood (1967) found that many symbiotic associations between organisms occur in low nutrient environments. In soils, for example, many plants interact with fungi to form some kind of mycorrhizal association. High nutrient

environments show considerably fewer interactions between organisms. It could be inferred from this that low nutrient environments are a prerequisite for such interactions. Various stages of interdependence could be assumed. For example, initially, one organism might live close to a producer and takes advantage of the materials if releases. The next step would be to increase the out flow of the desired nutrients while excess growth of the producer should be prevented. The increased leakiness of the producer should not be unspecific (this would be risky), but should be restricted to one (or a few) kinds of molecules. To make the flow more efficient, the nutrient export should be freed from energy consumption. *Rhizobium*, root nodule actinomycetes, and cyanobacteria may locally influence the host metabolism for the same reasons. (Hirch *et al.*, 1979).

Other unique mechanisms employed by oligotrophs during cell growth include an ability to increase the rate of transport of a nutrient into the cell. This is achieved by synthesizing more of the uptake system, by synthesizing high affinity uptake systems, by alteration of their surface to volume ratio, by being less nutritional fastidious, and by increasing enzyme synthesis or enzyme affinity.

The aim of the work repeated in this Chapter was to determinate the effect of silicon compounds on the growth of :

a) fungi under oligotrophic conditions *in vitro*

b) soil bacteria under aerobic and anaerobic condition.

3.2 Materials and Methods

3.2.1 Effect of silicon compounds on the growth of *A. oryzae* and other fungi under oligotrophic growth condition.

Aspergillus oryzae was grown under oligotrophic condition. This fungus was initially grown on Czapek Dox agar media (Oxoid) at 25^{0}C for 7 days. Mycelium plus spores were carefully removed using a sterile inoculating needle to avoid the transfer of any underlying nutrient-rich medium. Small amounts of this inoculum were then transferred to ultra-pure water (upw, 30 ml) in sterile plastic Petri dishes. The (upw) was obtained from a Millipore RO-4 water filter system and was sterilized by autoclaving at 120^{0}C for 20 min. Although the inoculum size was not strictly controlled, approximately equal amounts of mycelium were transferred on each occasion. Inoculated plates were then amended with silicic acid (0.3g). Control plates were not supplemented. Three control plates and three plates containing silicic acid were inoculated and the experiment was repeated four times, resulting in a total of 12 replicates. All plates were incubated at 25^{0}C for 14 days.

In a separate set of experiments the above protocol was repeated using silicic acid which had been heated in a muffle furnace to remove organic contaminants. Silicic acid was placed in aluminum containers loosely covered with aluminum foil. The cases were then heated at 400^{0}C for 2.5 hours, and allowed to cool to room temperature inside the furnace before transfer to upw.

The above experiments were also repeated using silicic acid which had been heat-treated and then acid-washed to remove inorganic contaminants. After heat treatment, the powder was washed three times with upw for 30 min., followed by two washes with HCl (0.1M) for 1 hour. The powder was then finally washed three times for 30min., in upw and then transferred to upw in sterile Petri dishes.

The above experiments were repeated with *A. oryzae* and the fungi listed in the previous section. The following silicon compounds were added to the medium (0.3g): silica colloidal powder, calcium silicate, sodium silicate, talc (hydrated magnesium silicate), silicon nitride and rock potash

3.2.2 Effect of silicic acid on the growth of various fungi under oligotrophic condition.

The above experiments were repeated using the following fungi. *Aspergillus niger, Aspergillus repens, Fusarium oxysporum, Penicillium chrysogenum* and *Penicillium janthinellum*

3.2.3 Effect of silicic acid on the growth of soil bacteria under oligotrophic condition.

Four different soils were used as the inoculum : a) deciduous woodland soil obtained from underneath beech (*Fagus sylvaticus*), b) coniferous woodland from under Scots Pine (*Pinus sylvestris*), c) soil from beneath fern (bracken) (*Pteridium aquilinum*), and d) an agricultural loam. 1 g soil was added to 100 ml of sterilized ultra-pure water (upw). The ultra-pure water was adjusted with Sorensen's buffer to

pH 6.8. Further dilution (1 ml) of soil suspension were then transferred to 9 ml of sterilize upw. 0.1 ml of each dilution was added to pre-sterilized plastic container containing ;

1) 15 ml of upw only, 2) 15 ml of upw containing *treated silicic acid (0.1g), 3) 15 ml of 1mM potassium nitrate as a nitrogen source. This solution was prepared with sterile ultra-pure water. 4) 15 ml of KNO_3, and treated silicic acid (0.1g).

*Silicic acid was washed (in order to remove any ions exist) with sterile distilled water (3 times allowing 1/2 hour between each wash), followed by a wash with 0.1M hydrochloric acid (2 times allowing 1 hour between each wash), finally washed with sterile, ultra-pure water (3 times allowing 1/2 hour between each wash), then left in 40^0C oven to dry. Washed silicic acid (0.1g) was wrapped in small parcels of foil and heated to 400^0C for 3 hours in a muffle furnace (in order to remove any organic contaminants).

3.2.4 Effect of silicic acid on the growth of soil bacteria under aerobic condition.

All plastic containers were placed in pre-washed (50% H_2SO_4) anaerobic jar, attached to an air pump. Air was passed into the jar via two bubble jars. The first was a third full with 50% sulphuric acid (H_2SO_4) and was used to removed any organic carbon, and the second was a third full with sterile upw (Fig. 3.1). The apparatus was set up and air allowed to filter for 1/2 hour. The anaerobic jar was then closed and incubated at 25^0C and 37^0C for 7 days. Solution (0.1 ml) from each plastic tube solution was then transferred to Petri dishes plates containing Plate Count Agar

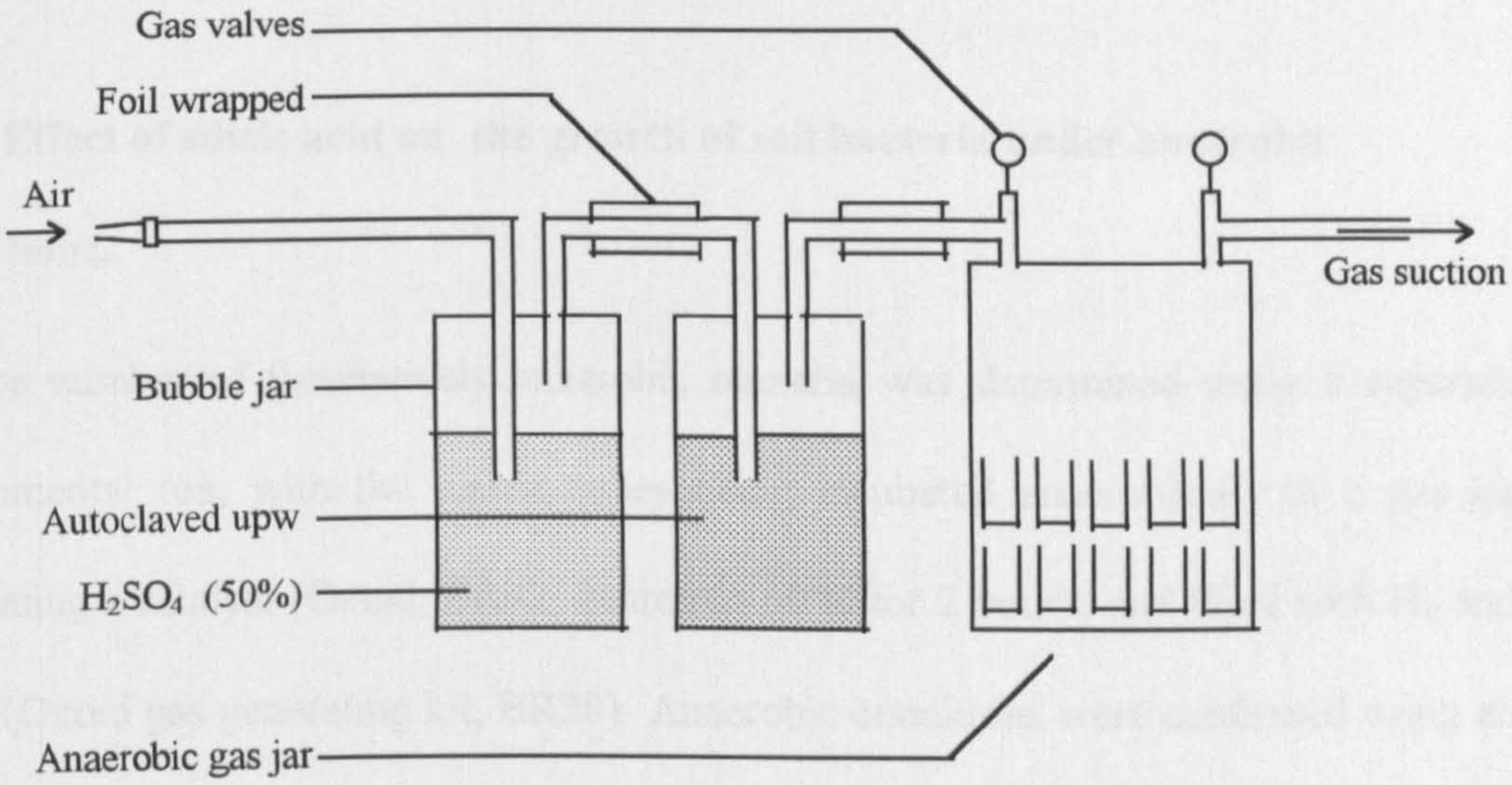


Figure 3.1 Apparatus used to set up an nutrient-free environment, in which the effect of silicic acid on bacterial growth under oligotrophic conditions was determined.

(triplicate). The inoculum was aseptically spread on agar plates surface, and incubated, at 25^0C and 37^0C for 4 days.

3.2.5 Effect of silicic acid on the growth of soil bacteria under anaerobic conditions.

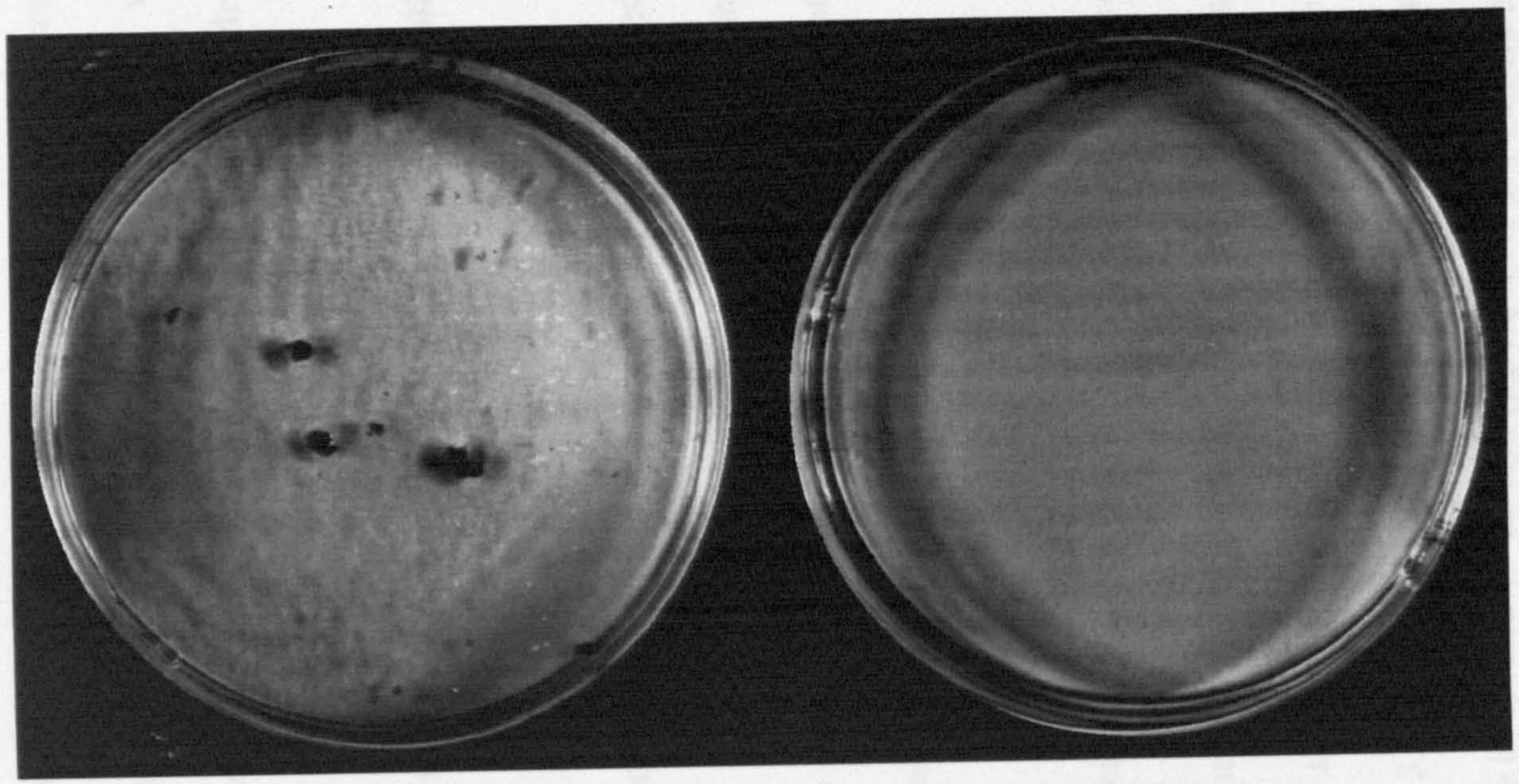
The number of facultatively anaerobic bacteria was determined using a separate experimental run, with the plastic tubes being incubated anaerobically in a gas jar containing a catalyst (Oxoid, BR42, heated at 60^0C for 2 hours) and filled with H_2 and CO_2, (Oxoid gas generating kit, BR38). Anaerobic conditions were confirmed using an indicator (Oxoid, BR55). The gas jar was incubated at 25^0C and 37^0C for 7 days. Plates were incubated aerobically at 25^0C.

3.3 Results and Discussion

3.3.1 Effect of silicon compounds on the growth of *Aspergillus oryzae* and other fungi under oligotrophic growth conditions.

Aspergillus oryzae consistently failed to grow from a mycelia-spore inoculum when added to upw. However, visible mycelial growth occurred in the presence of silicic acid (Fig. 3.2). Growth occurred in the twelve plates containing silicic acid, but in none of the control plates containing only upw. Particles of silicic acid could clearly be seen attached to the surface of *A. oryzae*. Spot tests, using the reagent for colorimetric determination of silicon, also showed that *A. oryzae* solubilized silicic acid in upw. Growth also occurred when acid washed silicic acid was used, showing that the effect was not due to contamination by inorganic nutrients. Due to limitations of analytical equipment available to us, the biomass, or protein content, of the small amounts of mycelium produced could not be determined, so our results are limited to observations. While *A. oryzae* and the fungi listed in oligotrophic condition failed to grow in upw alone, all grew in upw (all 12 replicates in each case) after the addition of one of the following silicon compounds : calcium silicate ; colloidal silica; talc (hydrated magnesium silicate) and rock potash (untreated, heat-treated and heat-treated plus acid-washed). The above observations show that fungi which cannot grow from spore-mycelium inoculum in upw, grow following the addition of silicic acid and other silicon compounds.

Figure 3.2 Growth of *Aspergillus oryzae* in ultra-pure water (upw) containing silicic acid (A), and absence of growth in upw alone (B).



(A) (B)

3.3.2 Effect of silicic acid on the growth of various fungi under oligotrophic condition.

While *Aspergillus repens, A. oryzae, F. oxysporum*, and *P. janthiellum,* did not grow in upw alone, all grew (12 replicates of each fungus) when silicic acid (untreated, heat-treated and heat-treated plus acid-washed) was added to upw.

3.3.3 Effect of silicic acid on the growth of soil bacteria under oligotrophic condition.

Four different soils were used to produce the inoculum : a) deciduous woodland soil obtained from underneath beneath (*Fagus sylvaticus*), b) coniferous woodland from under Scots Pine (*Pinus sylvestris*), c) soil from beneath fern (bracken) (*Pteridium aquilinum*), and d) an agricultural loam.

3.3.4 Effect of silicic acid on the growth of soil bacteria under aerobic condition.

With the exception of the agricultural soil, bacterial numbers increased when the soil inoculum was added to ultra-pure water (upw) without silicic acid and nitrogen Figs. 3.3 a, b and 3.4 c, d). The number of aerobic bacteria generally increased from the inoculum value by around 10-20 fold (agricultural soil inoculum) in upw. Addition of silicic acid and silicic acid plus nitrogen lead to statistically significant increases in bacterial numbers in upw receiving agricultural and fern soil inocula and non-significant increases resulted from the deciduous and coniferous soil.

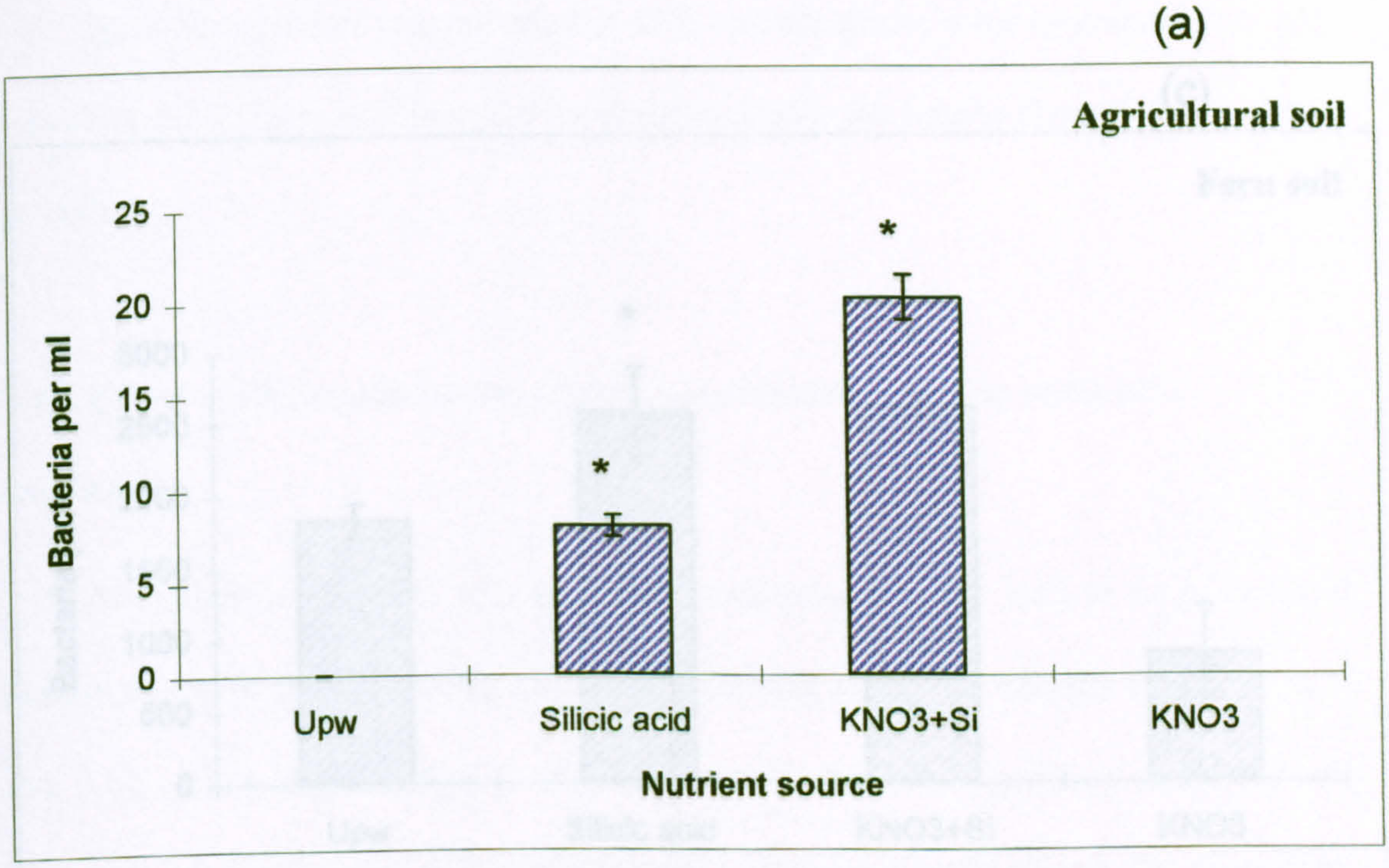
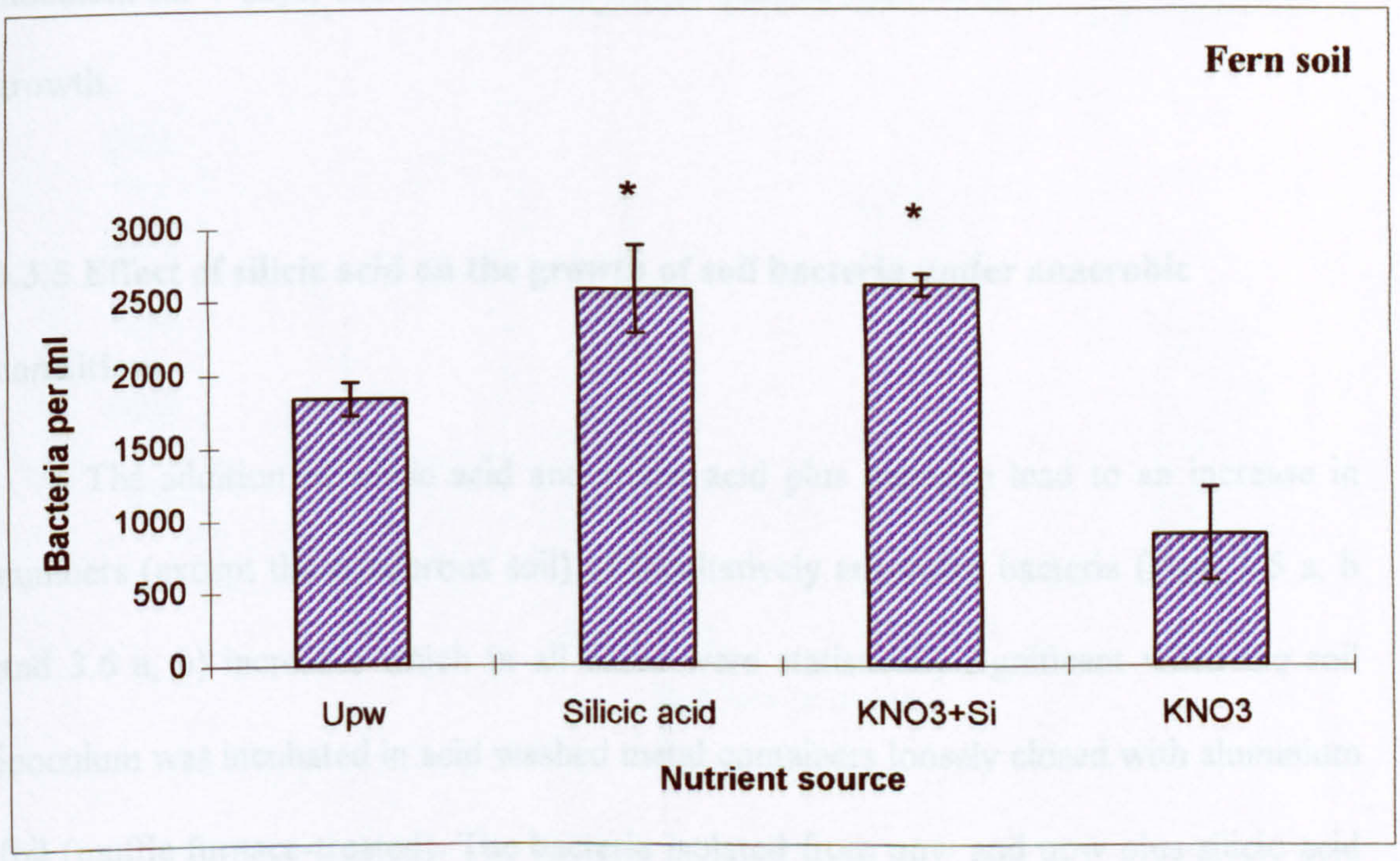


Figure 3.3 Bacterial numbers in ultra-pure water (upw), upw containing silicic acid, upw containing KNO_3 and silicic acid, and upw containing KNO_3 only, after inoculation with aqueous extract of agricultural soil (a), deciduous soil (b). Incubated aerobically at 25^0C for 7 days in plastic tubes. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

(c)



(d)

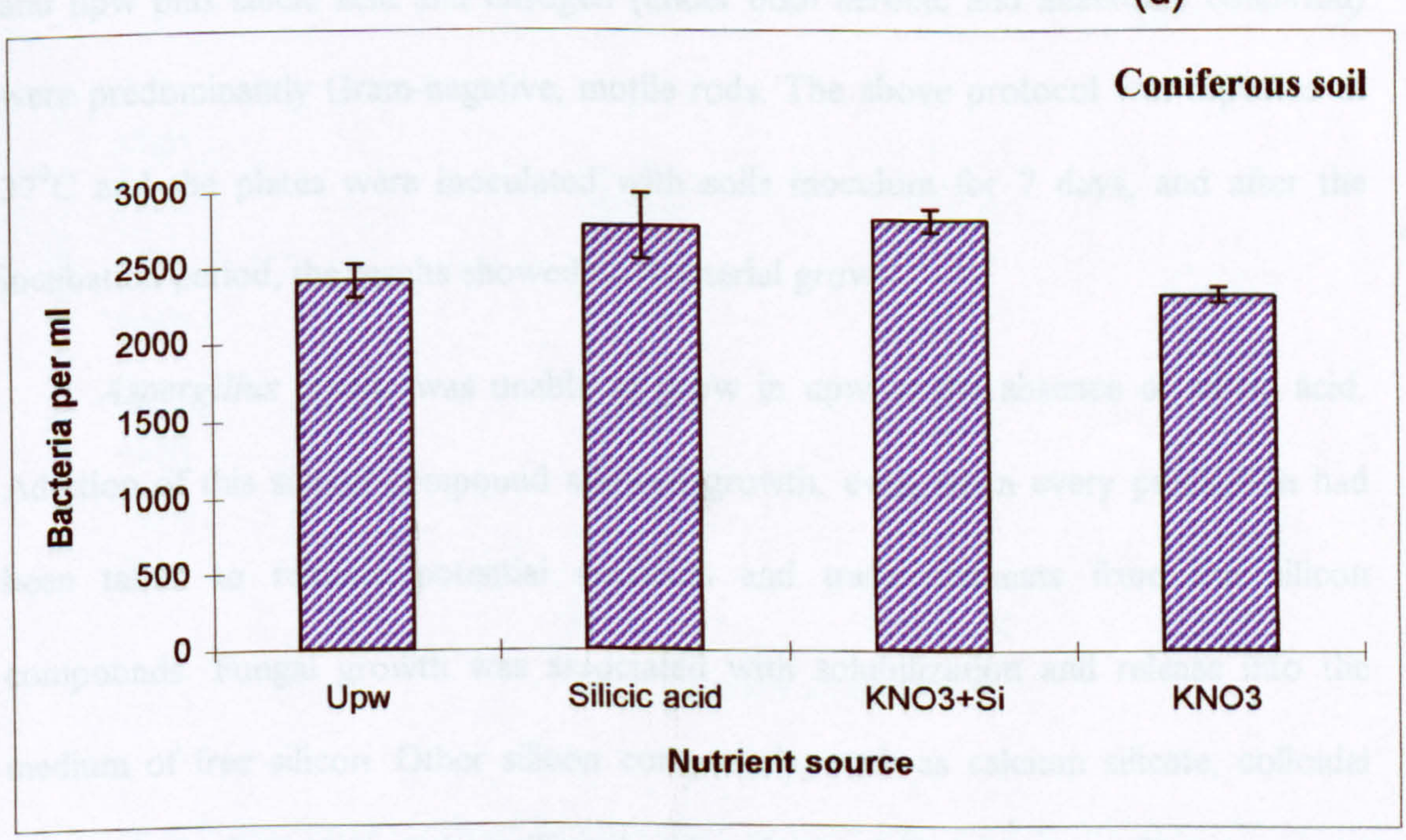


Figure 3.4 Bacterial numbers in ultra-pure water (upw), upw containing silicic acid, upw containing KNO_3 and silicic acid, and upw containing KNO_3 only, after inoculation with aqueous extract of fern soil (a), coniferous soil (b). Incubated aerobically at 25^0C for 7 days in plastic tubes. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

The above protocol was repeated at 37^0C and the plates were inoculated with soils inoculum for 7 days, and after the incubation period, the results showed no bacterial growth.

3.3.5 Effect of silicic acid on the growth of soil bacteria under anaerobic condition.

The addition of silicic acid and silicic acid plus nitrogen lead to an increase in numbers (except the coniferous soil) of facultatively anaerobic bacteria (Figs. 3.5 a, b and 3.6 a, b) increases which in all cases were statistically significant when the soil inoculum was incubated in acid washed metal containers loosely closed with aluminium foil (muffle furnace-treated). The bacteria isolated from upw and upw plus silicic acid and upw plus silicic acid and nitrogen (under both aerobic and anaerobic condition) were predominantly Gram-negative, motile rods. The above protocol was repeated at 37^0C and the plates were inoculated with soils inoculum for 7 days, and after the incubation period, the results showed no bacterial growth.

Aspergillus oryzae was unable to grow in upw in the absence of silicic acid. Addition of this silicon compound allowed growth, even when every precaution had been taken to remove potential nutrients and trace elements from the silicon compounds. Fungal growth was associated with solubilization and release into the medium of free silicon. Other silicon compounds, such as calcium silicate, colloidal silica, talc (hydrated magnesium silicate) and rock potash had the same effect.

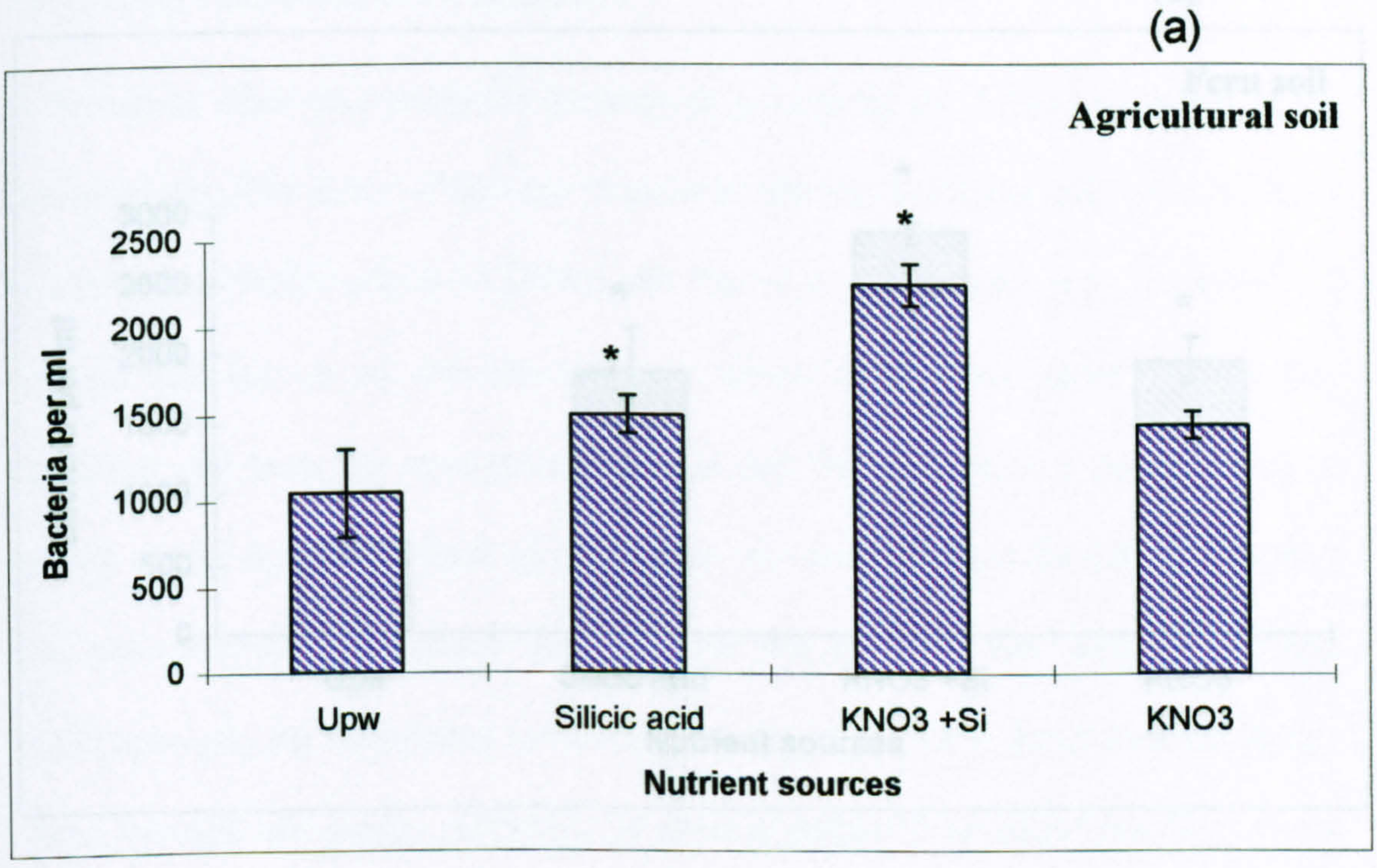


Figure 3.5 Bacterial numbers in ultra-pure water (upw), upw containing silicic acid, upw containing KNO_3 and silicic acid, and upw containing KNO_3 only, after inoculation with aqueous extract of agricultural soil (a), deciduous soil (b). Incubated anaerobically at 25^0C for 7 days in plastic tubes. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

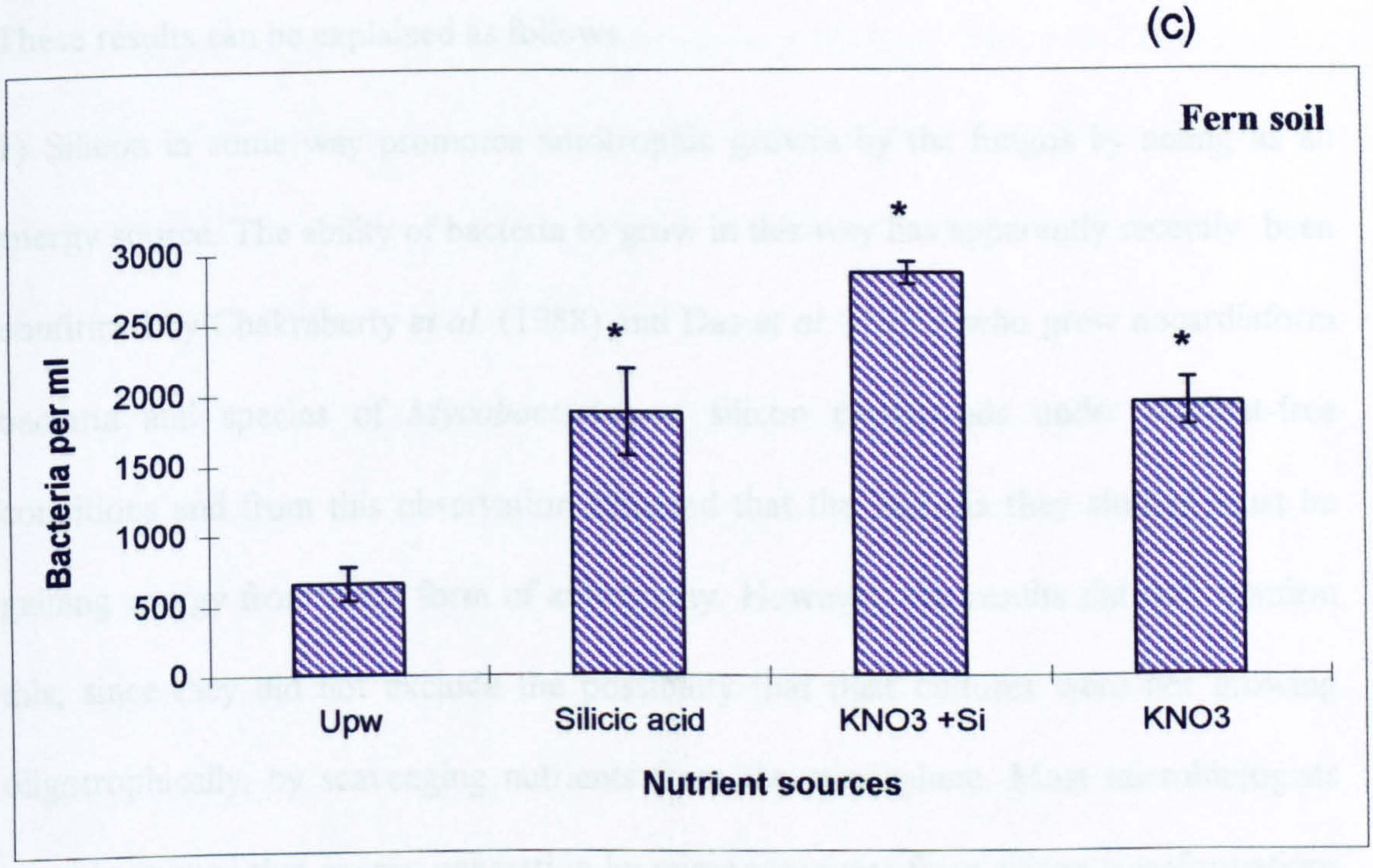


Figure 3.6 Bacterial numbers in ultra-pure water (upw), upw containing silicic acid, upw containing KNO_3 and silicic acid, and upw containing KNO_3 only, after inoculation with aqueous extract of fern soil (a), coniferous soil (b). Incubated anaerobically at 25^0C for 7 days in plastic tubes. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$

These results can be explained as follows :

1) Silicon in some way promotes autotrophic growth by the fungus by acting as an energy source. The ability of bacteria to grow in this way has apparently recently been confirmed by Chakrabarty *et al.* (1988) and Das *et al.* (1992) who grew nocardiaform bacteria and species of *Mycobacteria* on silicon compounds under nutrient-free conditions and from this observation assumed that the bacteria they studied must be gaining energy from some form of autotrophy. However, the results did not confirm this, since they did not exclude the possibility that their cultures were not growing oligotrophically, by scavenging nutrients from the atmosphere. Most microbiologists would assumed that energy generation by microorganisms from silicon transformations is unlikely, if not impossible. However, it should be pointed out that other transformation in nature, notably dinitrogen fixation, appear at first sight to be unlikely if not thermodynamically impossible, yet they occur. One could argue that it is unlikely that, over vast time periods allowed for evolution, microorganisms would have failed to have taken advantage of the abundance of silicon in the biosphere and adapt to using it as a source of energy. However, until the mechanism by which fungi and bacteria might gain energy from silicon transformation is discovered such silicon-based autotrophic growth must remain speculative.

2) Silicon compounds may act as a solid surface "catalyst" bringing together a variety of air-borne nutrients and making them available to microorganisms. The ability of silicon compounds to adsorb gases is well-known, and is used by chemists to concentrate, and thereby react, gases. It is likely therefore that nutrients such as ammonia, organic volatiles and carbon dioxide will be adsorbed from the atmosphere by silicon compounds when added to ultra-pure water and these could then provide

by silicon compounds when added to ultra-pure water and these could then provide nutrients for oligotrophic fungal growth. This likelihood was suggested by Bigger and Nelson (1943) in relation to the stimulatory effect of talc (hydrated magnesium silicate) on bacterial growth in distilled water.

The results show that bacteria can grow in upw and that the addition of silicic acid generally increased the number of both aerobic and facultatively anaerobic bacteria. These results confirm previous reports on the stimulatory effect of silicon compounds on microbial growth. Although every effort was made to remove potential nutrients from the water, silicic acid and atmosphere (by passage of the air supply though sulphuric acid and upw) used in these experiments this did not prevent bacteria from growing in upw alone. It is expected that the passage of air through sulphuric acid and water should have removed most of the airborne organic carbon and ammonia. It seems therefore that, despite the fact that the soil inoculum was well diluted, sufficient nutrients were carried over to support the growth of bacteria; a fact which emphasises the marked ability of bacteria to grow under low nutrient conditions.

It could be argued that the increases in bacterial numbers were supported by nutrients obtained from the plastic bottles, however, the results obtained using metal containers showed that this was not the case.

The stimulatory effect of silicic acid on bacterial numbers under highly oligotrophic conditions could be due to one or more of the following : a) the adsorption by silicic acid of nutrients from solution or atmosphere; b) surface phenomena and c) autotrophic growth, using silicon transformation as an energy source.

Unfortunately we are currently unable to confirm which, if any, of these possibilities operates under the experimental conditions employed. However, the possibility that bacteria obtained energy from transforming silicic acid, although not confirmed by the above experiments, might be worth considering. Bigger and Nelson (1943) suggested that the increase in bacterial growth seen following the addition of talc (hydrated magnesium silicate) was due to their ability to use obtain energy from chemically transforming silicon, fixing CO_2 and using ammonia from the atmosphere as the nitrogen source.

Silicon compounds frequently occur in soils and waters and may stimulate bacterial growth in these, frequently oligotrophic, environments. The stimulatory effect of silicon may also influence the growth of oligotrophic microorganisms in the food and drinks industry (e.g. in bottled waters) as well as in hospitals and industrial environments (Wainwright *et al.*, 1992, 1993).

CHAPTER FOUR

EFFECT OF SILICON COMPOUNDS ON SELECTED MICROBIAL PROCESSES

4.1 Introduction.

The fact that silicon compounds can stimulate the growth of microorganisms under nutrient-rich and oligotrophic growth conditions obviously leads into the question of whether these compounds have an influence on the metabolism of these organisms.

The aim of the work reported here was to determine if silicic acid could influence a) antibiotic production by a species of *Streptomyces*; b) citric acid production by *Aspergillus niger* and the ability of fungi to c) oxidize sulphur and d) nitrify ammonium.

4.2 Antibiotics production by *Streptomyces spp.*

Because their synthesis in not associated with growth, antibiotics belong to a class of microbial products known as secondary metabolites (Calam, 1987).

The use of these compounds as chemotherapeutic agents arose from the discovery that many microorganisms synthesise and excrete organic compounds that are toxic to other microorganisms. In 1928 the British bacteriologist Alexander Fleming observed that the growth of a culture of *Staphylococcus aureus* was inhibited (i.e. lysed) by a contaminating mould which subsequently proved to be a species of *Penicillium*. In 1940 the antibiotic actinomycin was isolated from *Streptomyces antibioticus* as a red crystalline substance. Schatz *et al.*, (1944) discovered another broad spectrum bioactive compounds from *Streptomyces griseus* and called it streptomycin. Four years later chlortetracycline was discovered. This antibiotic also has a broad spectrum of activity against pathogenic Gram positive and negative bacteria.

Since the 1940s actinomycetes have been used to produce two-thirds of the naturally occurring antibiotics including aminoglycosidse, anthracyclines, chloramphenicol, β-lactams, macrolides and tetracyclines (Stanier *et al.*, 1987).

Alexander (1982) reported that 75% of *Streptomyces* isolated can produce antibiotics. Members of the genus *Streptomyces* have became economically important because of the large number of therapeutically useful antibiotics and vitamins they produce (Lechevalier and Lechevalier, 1967). The genus occupies a leading position amongst antibiotic-producing microorganisms (Waksman, 1961), although subsequent reports indicated other genera as a source of new and promising products (Goodfellow, *et al.,* 1988). For example, the nocardicins were are obtained from species of *Nocardia*, and tiacumicins from *Dactylosporangium*, and arizonins from *Actino- arizonaensis*, other macquarimcins were isolated from strain of genus *Micromonospora* (Karwowski, 1986).

Demain *et al.*, (1981) considered that carbon and nitrogen, as well as phosphorus, are important regulators of antibiotic biosynthesis. According to Aharonowitz (1983) antibiotics are derived from intermediates of primary metabolism and accumulate during the late lag phase to stationary phase of growth. Calam (1987) reported that approximately 50% of the carbon input in a typical antibiotic fermentation was respired to carbon dioxide whilst the remainder was required for biomass and antibiotic biosynthesis. Nitrogen is also an essential component of most antibiotics or is required in the biosynthesis of intermediates (Lebrihi *et al.*, 1988).

4.3 Citric acid

Most of a wide rage of biochemicals produced by fungi, are required for growth and metabolism. Citric acid is largely produced by two species of *Aspergillus*, namely *A. niger* and *A. wantii*, although yeasts such as *Saccharomycopsis lipolytica* have also been evaluated for citric acid production on non-sugar substrates. Citric acid is widely used throughout the food industry in the manufacture of soft drinks, effervescent salts and medicines, to silver mirrors, and as an additive in inks (Wainwright, 1987).

Filamentous fungi are well known for their ability to accumulate organic acids in the medium when supplied with large amounts of sugar. The accumulation of citric acid by selected mutants of *A. niger* is probably the best example of a fungal organic acid fermentation (Kubicek, 1987). However, a number of compounds are also produced that are not regarded as essential for growth and these are termed secondary metabolites (Sebek, 1983). Secondary metabolites are produced when the fungus is in the non-growing or stationary phase. Included in this group are a number of compounds which are of great medical importance, such as antibiotics. Alternatively, fungal metabolites can be classified as either general metabolites that are produced by a large number of organisms, or specialised metabolites, produced by restricted number of species. On this basis, citric acid and ethanol, are regarded as general metabolites, whereas pullulan would be regarded as a specialised metabolite (Wainwright, 1987).

Citric acid was formerly produced from citrus fruits, but now nearly 99% of world production is derived from mould fermentation. Today, the annual production of citric acid exceeds of 200,000 tonnes (Wainwright, 1987).

4.4 Sulphur oxidation

The oxidation of reduced S in soil is usually regarded as a microbial process (Wainwright, 1978a), although some nonbiological oxidation of the element can occur.

Burke, *et al.*, (1974) stated that a wide range of microorganisms are able to oxidising sulphur in the environment, including members of the genus *Thiobacillus*, a number of heterotrophs, the photosynthetic sulphur bacteria, and the colourless, filamentous sulphur bacteria. Wainwright, (1978b) commented that in most soils only the thiobacilli, and heterotrophs play an important role in sulphur oxidation, the exceptions being flooded soils where the aquatic bacteria predominate.

The anaerobic photosynthetic sulphur bacteria are responsible for the oxidation of sulphur compounds in the absence of oxygen. There are two groups of photosynthetic sulphur bacteria, the green sulphur bacteria (*Chlorobiniaceae*) and the purple sulphur bacteria (*Chromatiaceae*). Both use light as energy source and H_2S, hydrogen and thiosulphate as inorganic electron donors for CO_2 assimilation (Zinder and Brock, 1978). Actinomyctes have been also reported to be able to oxidize sulphur (Wainwright *et al.*, 1984).

In the first ever major study on fungal S-oxidation by Armstrong, (1921), *Aspergillus niger, Penicillium cyclopium* and *Botrytis cinerea* were shown to oxidise thiosulphate to sulphate, *in vitro*, occasionally forming tetrathionate as intermediate. Wainwright and Killham (1980) subsequently showed that *Fusarium solani* can oxidize elemental sulphur to thiosulphate, tetrathionate and sulphate in liquid culture and when grown in autoclaved soils with the element. The fungi which have shown to

date their capability of sulphur oxidation are mainly soil fungi, but included thermophilous fungi (Wainwright, 1984b), and a marine fungus *Asteriomyces cruciatus* fungi (Wainwright, 1984a).

Killham *et al.*, (1981) reported that sulphur oxidation by *Aureobasidium pullulans* has to be an enzymic process, and it is therefore unlikely that the process is fortuitous and incidental to normal metabolism. However, fungi are regarded as strict heterotrophs and unlikely to gain energy from sulphur oxidation. Armstrong (1921) also reported biomass increases when fungi are grown in thiosulphate. The growth of hyphae of the vesicular arbuscular mycorrhizal fungus *Glomus calledonium* is also stimulated by thiosulphate, metabisulphate, and sulphate (Hepper, 1984).

The following advantages may accrue to fungi as a result of sulphur oxidation; 1) fungi can obtain their nutritional sulphur requirements directly by oxidizing sulphur to sulphate. However, where sulphur is present in the environment, chemical and microbial oxidation give rise to large quantities of free sulphate, so that there is often no need for fungi to oxidize sulphur for nutrition; 2) the fungus could avoid sulphur toxicity by oxidizing the element to sulphate (Tweedy, 1969). Elemental sulphur can be reduced by fungi to H_2S, which is toxic, oxidation of elemental sulphur and H_2S would therefore remove a potential toxic agent. A similar protection mechanism was suggested by Skerman *et al.*, (1975) to explain why *Sphaerotilus natans* and *Beggiatoa sp* oxidize H_2S; 3) thiosulphate and tetrathionate produced during sulphur oxidation can protect fungi from the toxic effect of metals (Wainwright and Grayston, 1988). These ions act as ligands, complexing heavy metals and making them unavailable. 4) Element sulphur acts as a self inhibitor of fungal spore germination by

oxidizing sulphur, fungi would removed such germination inhibitors (Pezet and Pont, 1977).

4.5 Nitrification

Nitrification is the biological oxidation of ammonium ion to nitrite and nitrate (Alexander, 1977). The chemoautotrophic bacteria *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* species are generally responsible for nitrification in the environment and are respectively able to oxidize ammonium to nitrate, and nitrite to nitrate. Schmidt, (1982) mention that two types of nitrification are recognized, chemoautotrophic and heterotrophic. Chemoautotrophic nitrification appears to be dominant in agricultural soil, while heterotrophic nitrification has been particularly implicated in acid soil, although it should be noted that acid tolerant chemoautotrophs also exist. The nitrification bacteria are gram negative, aerobic chemoautotrophs and the bacterial morphology varies from rods and ellipsoids to cocci and spirilla (Alexander, 1977).

Schmidt, (1982) noted that these organisms are typically obligate chemolithoautotrophs oxidizing reduced nitrogen for their energy whilst synthesizing all of their cell constituents from carbon dioxide. In ammonium oxidizing bacteria, ammonia rather than ammonium crosses the cytoplasmic membrane and is oxidized to hydroxylamine (Suzuki, *et al.*, 1974).

$$NH_4^+ + {}^{1/2}O_2 \longrightarrow NH_2OH + H^+$$

The oxidation of hydroxylamine resulting in the production of nitrite and energy.

$$NH_2OH + O_2 \longrightarrow HNO_2 + H_2O$$

The oxidation of nitrite to nitrate occurs by the addition of an oxygen atom from water and not molecular oxygen.

$$NO_2^- + {}^{1/2}O_2 \longrightarrow NO_3$$

Nitrifiers assimilate organic compounds such as amino acids and acetate (Clark and Schmidt, 1967). Schmidt, (1982) reported that the assimilation of organic compounds by ammonium oxidizers occurs only when these organisms are using their specific energy source, i.e. ammonium.

Focht and Verstraete (1977) note that a wide range of heterotrophic microorganisms capable of oxidizing reduced forms of nitrogen, including bacteria (e.g. *Arthrobacter sp., Achromobacter sp*) actinomycetes (e.g. *Nocardia corallina*) and fungi (e.g. *Aspergillus flavus*). The major form from nitrification is nitrite which is produced by heterotrophs of a range of nitrogenous substrates. Heterotrophs fungi have also been reported to be able to produce substantial amounts of nitrate by nitrification, and are considered to be the most numerous and efficient of the heterotrophic nitrifiers (Odu and Adeoye, 1970).

Aleem, (1975) stated that the biochemistry of fungal nitrification has not ben fully elucidated and that it is unclear whether fungi nitrify using an inorganic pathway, with hydroxylamine and nitrite as intermediates, or else use an organic pathway involving the oxidation of an amino or amide to a substituted hydroxylamine followed by oxidation to a nitrose and then to a nitro-compound (Doxtader, 1965).

The aim of the work repeated in this Chapter was to determine the effect of the addition of silicon compounds to media and to study these effects on selected

processes. These were selected for their importance in relation to the environment or commercial, biotechnology process.

The effects of silicon on the following were determined:

a) antibiotic production by *Streptomyces spp.*,

b) citric acid production by *Aspergillus niger*,

c) sulphur oxidation by fungi,

d) nitrification by fungi.

4.2 Materials and Methods

4.2.1 Effect of silicic acid on the antibiotic production by *Streptomyces spp.*

In order to determine the effects of silicic acid on antibiotic production by *Streptomyces* two different diffusion assay methods were used :

4.2.1.1 Disc method.

A disc of agar (8 mm) diameter was aseptically removed from the *Streptomyces* isolates SAH1 and SAN7 culture grown on date syrup medium containing (5 % w/v) silicic acid (silicic acid-free for control medium) for 5 days at 30^0C. The discs were placed on Mueller-Hinton medium plates previously seeded with *Staphylococcus aureus*. The plates were then incubated for 2 days at 37^0C, and any resultant inhibition zone was measured.

4.2.1.2 Cultural filtrate methods.

The *Streptomyces* isolates were grown in 250 ml Erlenmeyer flask for 12 days on date syrup medium (the medium was buffer with Sorensen's buffered at pH 7.2) containing of silicic acid (1.5g) at 30^0C on a rotary shaker; supernatant (5 ml) was removed every 2 days. Paper discs, (dia.,1 cm) were cut from multiple layers of filter paper using a sharp cork borer. The discs were wetted with water prior to autoclaving at 15 psi for 15 minutes, immersed in the filtrates obtained from the two *Streptomyces* isolates, and then transferred to a refrigerator for 10-15 minutes at 4^0C. The discs

were then transferred to petri dishes containing the test bacterium, *Staphylococcus aureus*..

4.2.2 Effect of silicic acid on thiosulphate oxidation.

Aspergillus niger was grown in 250 ml Erlenmeyer flask containing Czapek Dox liquid medium (100 ml), sodium thiosulphate (100 μg ml^{-1}, obtained by filtration through a 0.4 membrane filter) with one of the following range of concentrations of silicic acid (0.5g, 1g, 1.5g, and 2g). Incubation was at 25^0C in shaking culture for 7 days.

4.2.2.1 Turbidimetric analysis of sulphate-S (Hesse, 1971).

Filtrate (5 ml) was transferred to a 25 ml volumetric flask, 1 g of $BaCl_2.2H_2O$ and 2 ml gum acacia (0.25% w/v) were added and mixed. The volume was made up to 25 ml with distilled water then the turbidity resulting from precipitation of barium sulphate was measured at 470nm. The SO_4^{2-}-S concentration was determined by reference to a standard curve (0-100 μg SO_4^{2-}-S ml^{-1}) prepared using a standard solution of $Na_2SO_4.10H_2O$.

4.2.3 Effect of silicic acid on nitrification.

Aspergillus niger was grown in Czapek Dox (100 ml) medium with ammonium sulphate (100 μg ml^{-1}) for 7 days on a rotary shaker at 25^0C. A range of concentrations of silicic acid (0.5g, 1g, 1.5g, and 2g), were added directly to the

medium. At the end of the growth period the supernatant was removed by filtration through a Whatman No.1 filter paper. The dry weight was the measured, and the nitrate concentration determined.

4.2.3.1 Analysis of ammonium, nitrite and nitrate.

(a) Indophenol blue method for determination of ammonium-N.

To filtrate (2 ml) was added, distilled water (7 ml), *phenolate reagent (5 ml), and sodium hypochlorite (5 ml) solution (0.9% v/v active chlorine), and mixed and incubated at 25^0C for 20 minutes in the dark. The intensity of indophenol-blue-ammonium complex was then measured at 630nm. The NH_4^+-N concentration was determined by reference to a standard curve (0-50 μg NH_4^+-N ml^{-1}) prepared from standard solution of $(NH_4)_2SO_4$.

* **Phenolate reagent** : prepared by mixing 20 ml of phenol solution** with 20 ml caustic solution (27% NaOH w/v) and diluting to 100 ml. The reagent was prepared fresh daily.

** **Phenol solution** : prepared by dissolving phenol (62.5 g) in ethanol (25 ml) and adding acetone (18.5 ml) to give a total of 100 ml. The phenol solution was stored in the dark at 4^0C.

(b) Analysis of nitrite-N

Filtrate (2 ml) was added to a 50 ml volumetric flask, distilled water (40 ml) and *diazotising reagent (1 ml) was added and incubated at room temperature for 5 minutes. Coupling reagent** (1 ml) was added and the volume made up to (50 ml) with distilled water. After 20 minutes incubation at room temperature the intensity of the pink colour formed was measured and the amount of nitrite was determined by reference to a standard curve (0-10 μg NO_2^--N ml^{-1}) prepared form standard solution of $NaNO_2$.

* **Diazotising reagent:** 0.5g of sulphanilamide was dissolved in 2.5M HCl (100 ml) and stored in the dark at 4^0C.

** **Coupling reagent :** 0.1 g of N-(1-naphyl)-ethylenediamine hydrochloride was dissolved in 0.1N HCl (100 ml) and stored in dark at 4^0C.

(c) Chromotropic acid method (Simms and Jackson, 1971)

This method was used to determinate of nitrate-N.

To filtrate (3 ml), chromotropic acid reagent (7 ml) was mixed, cooled in cold water and incubated at 40^0C for 45 minutes. The intensity of yellow CAT-NO_3^- complex was then measured at 430 nm.

4.2.4 Effect of silicon compounds on citric acid production by *Aspergillus niger.*

Aspergillus niger was grown in (250 ml) Erlenmeyer flasks containing Czapek Dox liquid (100 ml) medium containing one of the following : silicic acid, sodium silicate and magnesium silicate (0.5g, 1.5g and 2g), added directly to the medium. All flasks were autoclaved at 15 psi for 15 minutes. Three replicates were used, and the flasks were incubated on a rotary shaker (150 rpm) for 8 days in batch culture at 25^{0}C. After 8 days the suspension culture was removed, and filtered. Citric acid was analysed by the Analytical Services, Department of Animal and Plant Sciences, University of Sheffield, using a Phillips PU 4100 High performance liquid chromatography system (HPLC) with UV detection. The conditions were:

- Column: Jones apex C18, 5μm, 4.6 x 250mm
- Eluent: 0.2M phosphoric acid / methanol (ratio 95 : 5)
- Flow rate : 1ml min.
- UV detection at 210nm

Under these conditions citric acid had a retention time of around 6 min.

4.2.5 Effect of silicon nitride on *Aspergillus spp.* and their ability to nitrify this nitride.

Beta silicon nitride (SiN_4), 0.5g and 1.5g, was added directly to Czapek Dox (N-free) (100 ml) liquid medium. Flasks were inoculated with either *Aspergillus oryzae* or *A. niger*, and incubated at 25^{0}C for 7 days in triplicate. Fungal dry weight and soluble silicon were measured. The concentration of ammonium, nitrite and nitrate in the medium was also determined.

4.3 Results and Discussion

4.3.1 Effect of silicic acid on the antibiotic production by *Streptomyces spp*.

4.3.1.1 Disc method.

The amount of antibiotic produced by the *Streptomyces spp*. (presumed to be tetracycline, Kuchari, 1994) was determined by measuring the diameter of the zone against the test bacterium *Staphylococcus aureus*. The results presented in Table 2 show that the silicic acid (1% w/v) had no significant effect on the production of antibiotic by the *Streptomyces* isolate.

4.3.1.2 Cultural filtrate methods

The *Streptomyces* isolates showing antibiotic activity were grown up in date syrup medium containing silicic acid (1.5g) and the inhibition test repeated using the culture filtrate method against the test bacterium *Staphylococcus aureus*. The results show that (with the exception of the 4 days sample of isolate SAH1, where a significant increase was observed) silicic acid addition led to a decrease in antibiotic production by *Streptomyces spp*., a trend which was particularly obvious at the end of the incubation period, when a significant decrease in zone size was observed (Fig. 4.1).

Table 2 Effect of silicic acid on antibiotic production by the *Streptomyces* strains, determined by measuring the zone of growth inhibition (mm) of *Staphylococcus aureus* grown on Muller-Hinton agar (37^0C).

Streptomyces isolates	**Inhibition zone (minus silicic acid)**	**Inhibition zone (plus silicic acid)**
SAH1	27 ± 0.333	25 ± 0.333
SAN7	25 ± 0.888	24 ± 0.577

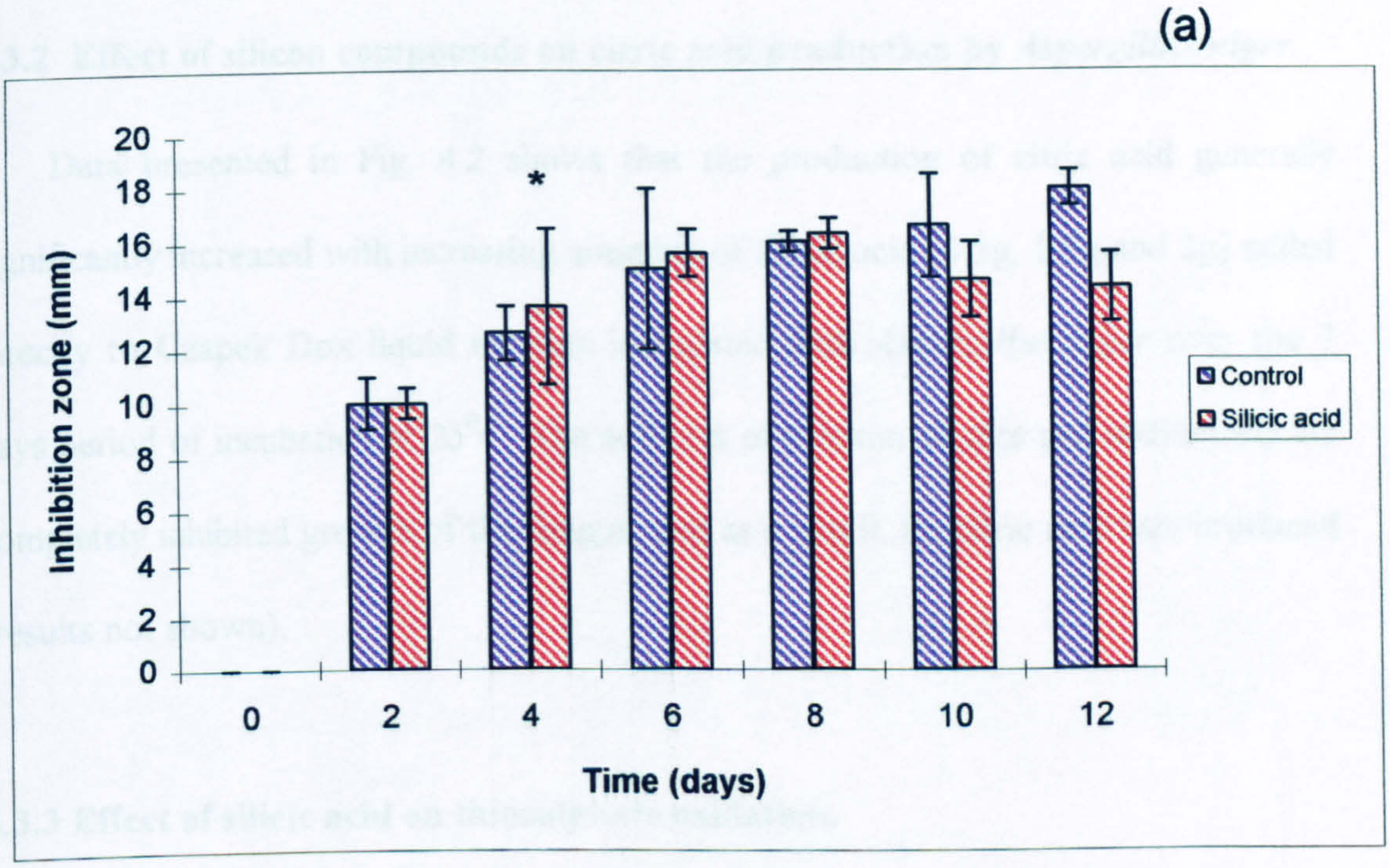


Figure 4.1 Effect of silicic acid (1.5g) on the production of antibiotic (presumed to be tetracycline) by *Streptomyces* isolates SAH1 (a), and SAN7 (b), grown in buffered (pH 7.2) date syrup liquid medium, at 30^0C. Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

4.3.2 Effect of silicon compounds on citric acid production by *Aspergillus niger.*

Data presented in Fig. 4.2 shows that the production of citric acid generally significantly increased with increasing amounts of silicic acid (0.5g, 1.5g and 2g) added directly to Czapek Dox liquid medium inoculated with *Aspergillus niger* over the 7 days period of incubation at 25^0C. The addition of calcium silicate and sodium silicate completely inhibited growth of the fungus, and as a result, no citric acid was produced (results not shown).

4.3.3 Effect of silicic acid on thiosulphate oxidation.

Silicic acid stimulated the growth of *Aspergillus niger* in medium containing thiosulphate (Fig. 4.3a). Despite this, the amount of sulphate present in the medium decreased following the addition of 0.5g silicic acid. However, the addition of increasing amounts of the silicon compounds had no further inhibitory effect on thiosulphate oxidation (Fig.4.3b).

4.3.4 Effect of silicic acid on nitrification.

While the addition of silicic acid stimulated the growth of *Aspergillus niger* (Fig. 4.4a) it decreased the oxidation of ammonium to nitrate (Fig. 4.4b). As with thiosulphate oxidation, increasing amounts of silicic acid did not have a corresponding increased inhibitory effect on the process.

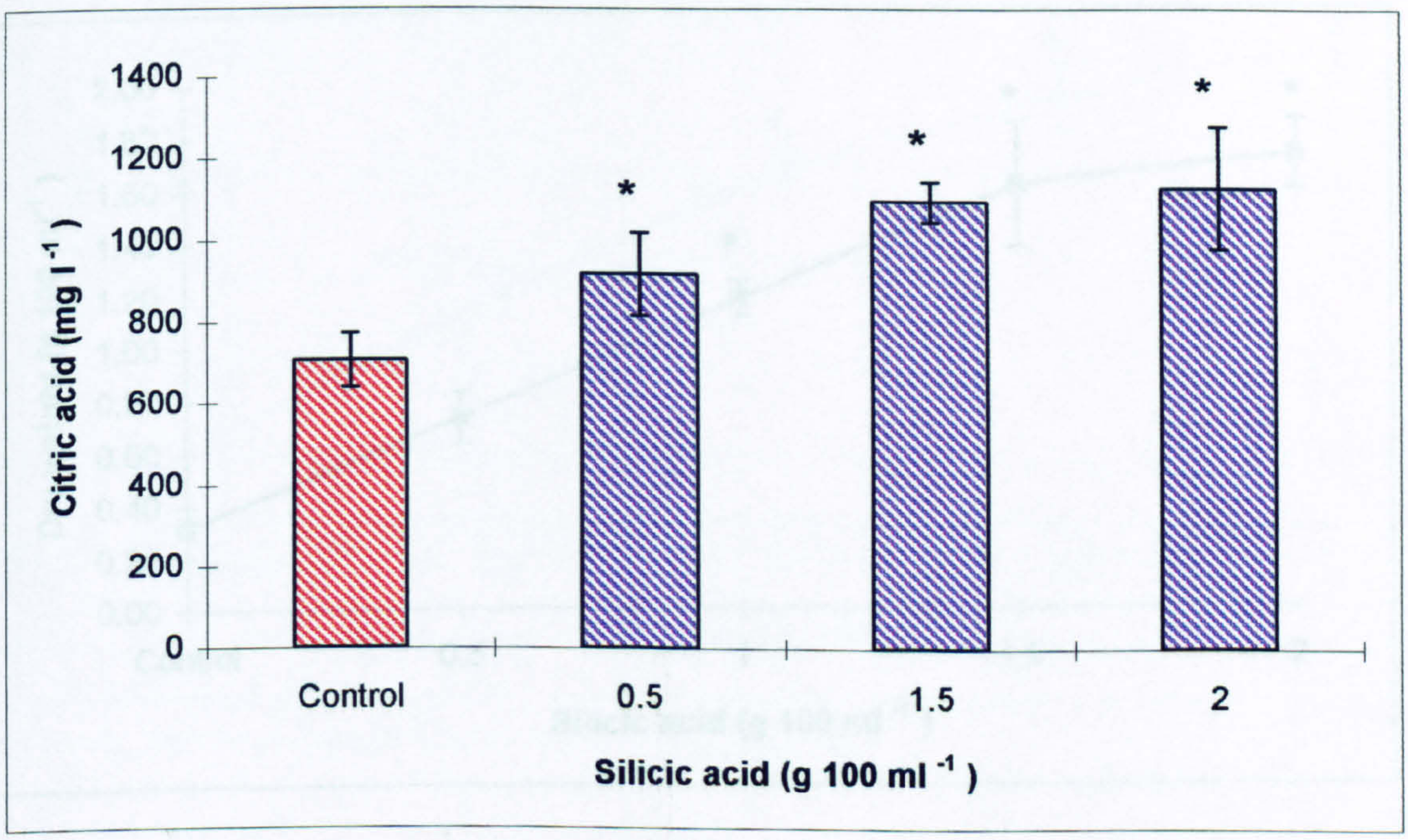
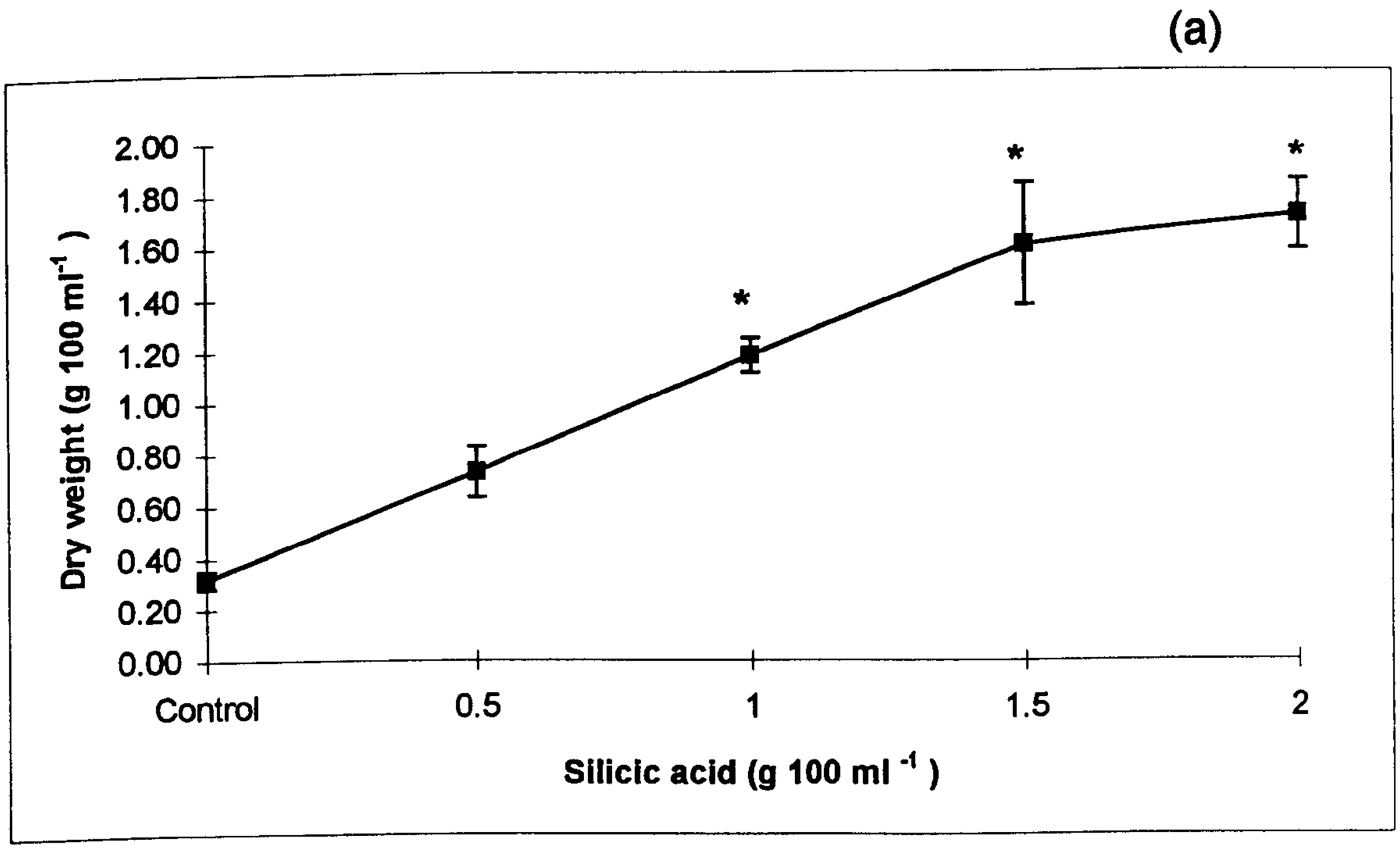


Figure 4.2 Effect of silicic acid (0.5g,1.5g and 2g, added directly to the medium) on the production of citric acid by *A. niger* grown in Czapek Dox liquid medium, for 7 days at 25^0C. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.



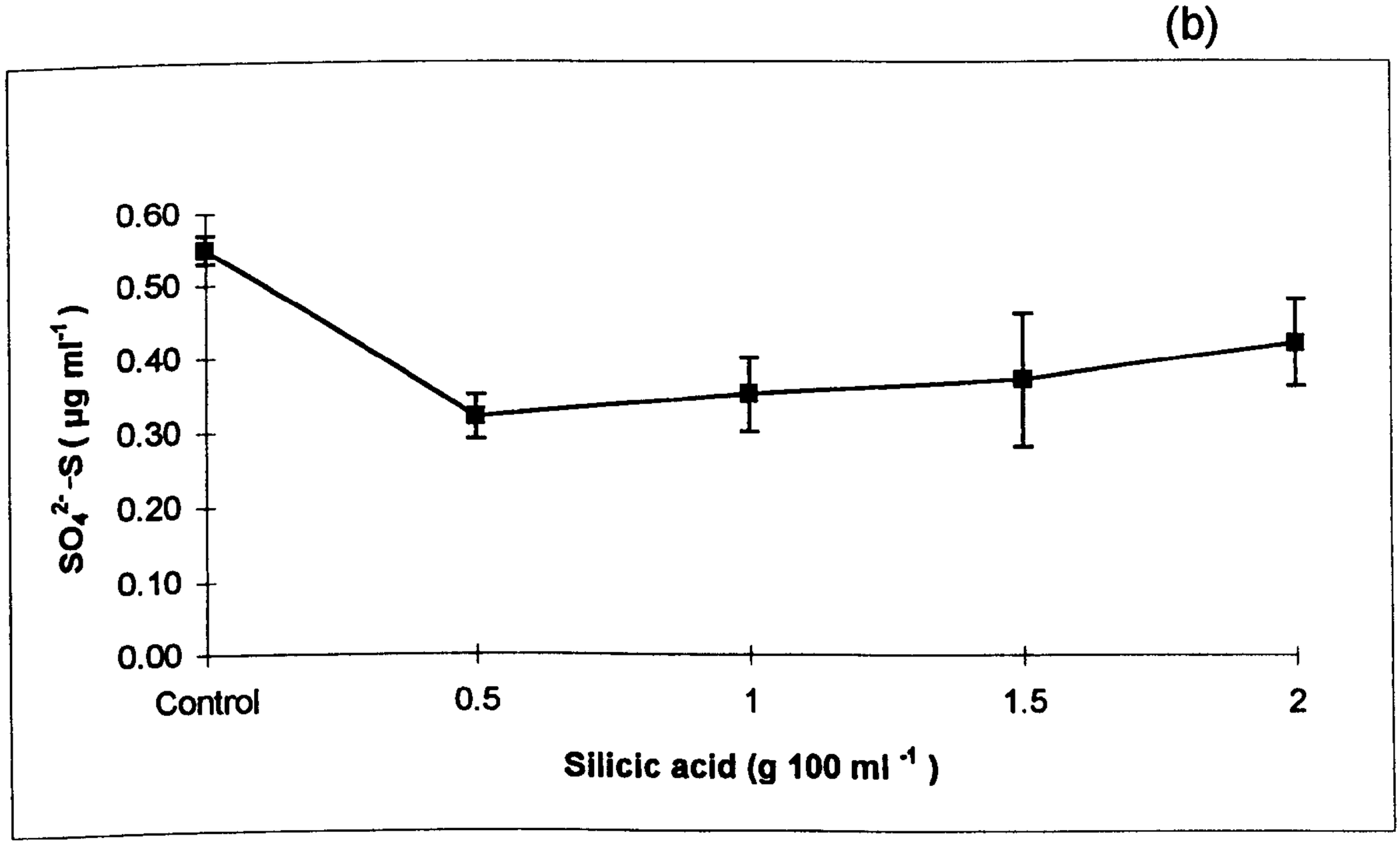
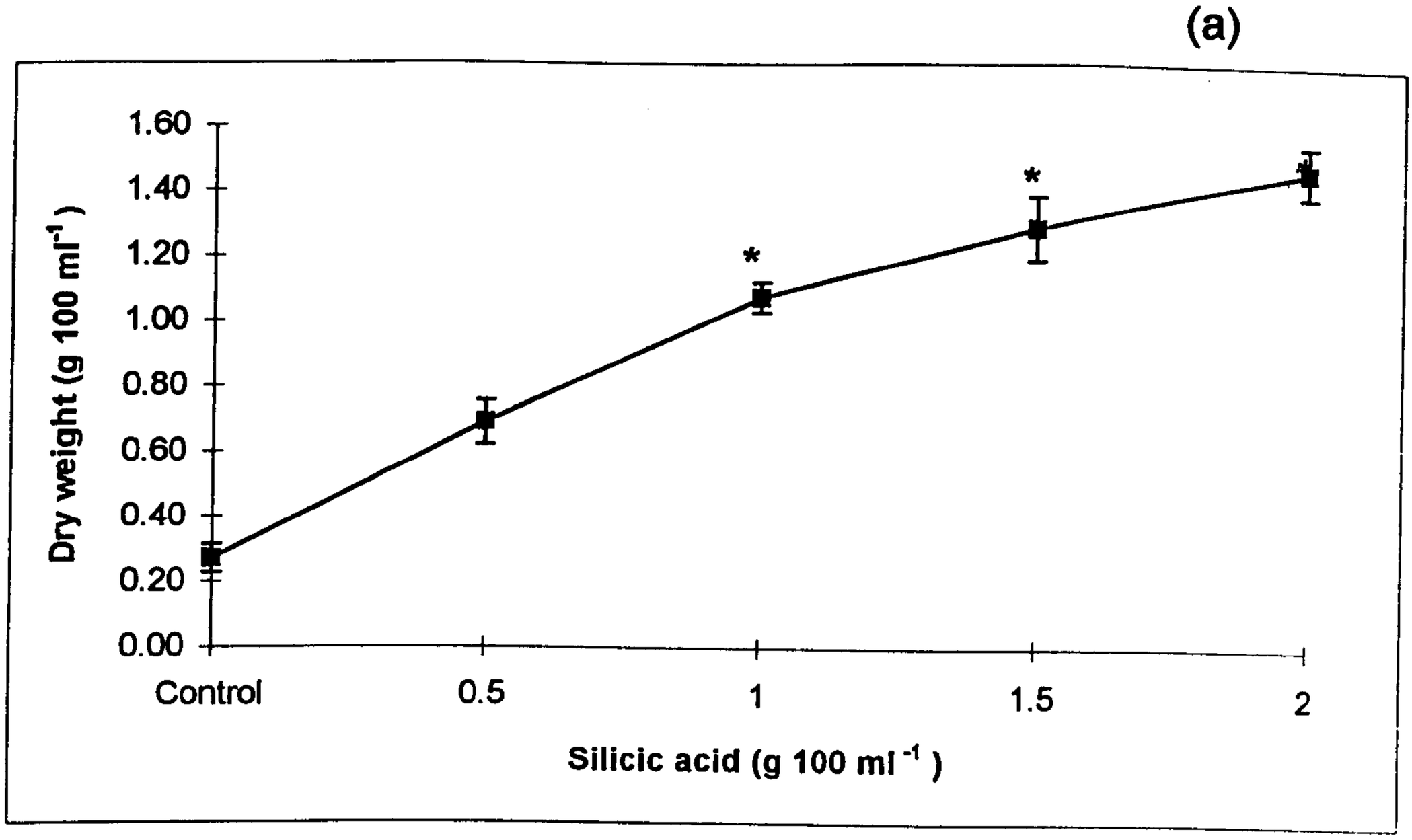


Figure 4.3 Effect of silicic acid on the growth of *A. niger* and thiosulphate oxidation in Czapek Dox liquid medium, containing sodium thiosulphate (100 µg ml $^{-1}$) (a), SO_4^{2-}-S concentration (b). Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.



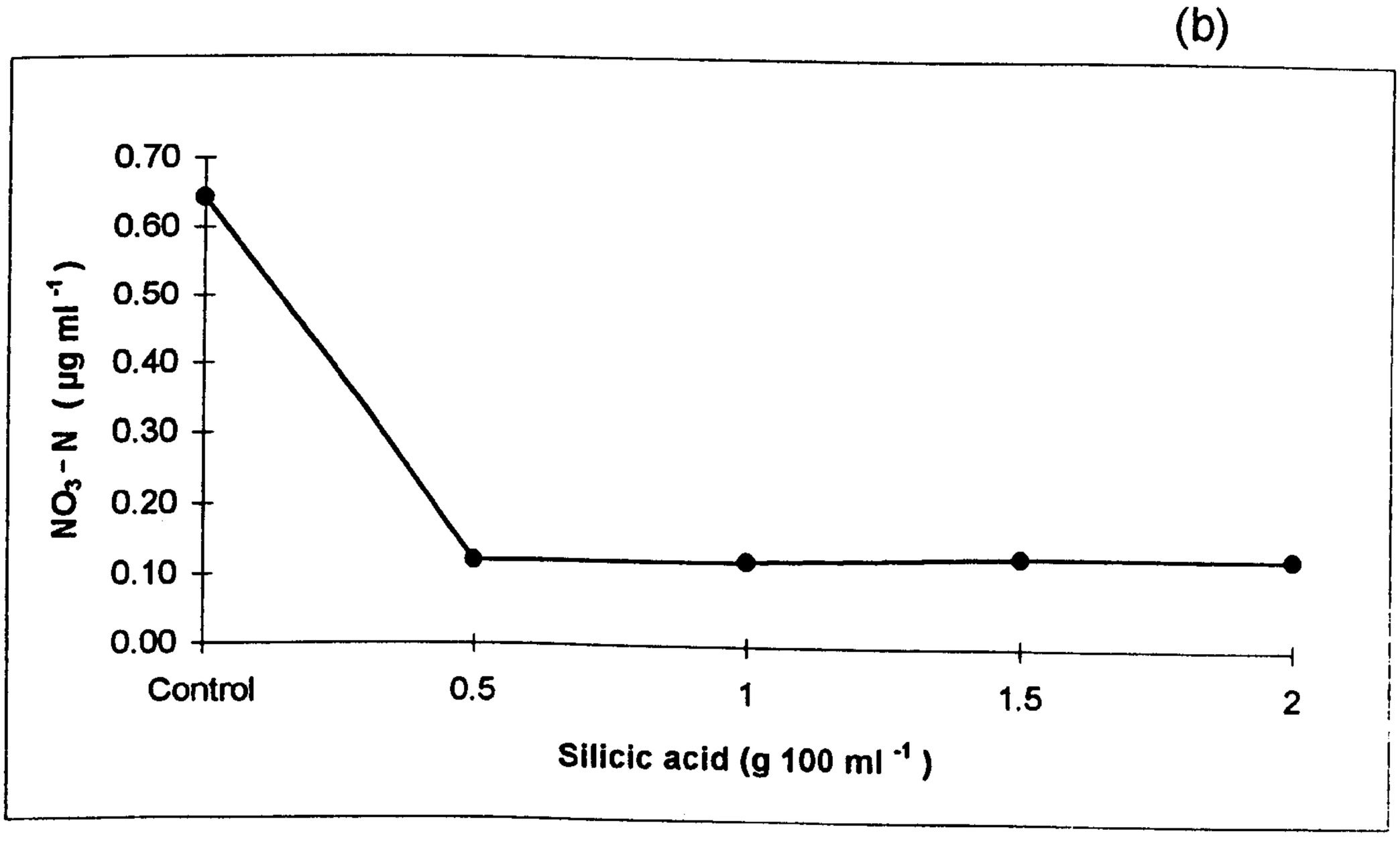


Figure 4.4 Effect of silicic acid (added directly to the medium) on the growth and nitrification by *A. niger* in Czapek Dox liquid medium containing ammonium sulphate (a), concentration of ammonium-N (b). Means of triplicates, ± Standard error. * Significant difference, $P < 0.05$.

4.3.5 Effect of silicon nitride on *Aspergillus spp.* and ability of these fungi to nitrify silicon nitride

Data presented in Fig. 4.5a show that addition of different concentrations of silicon nitride (0.5g and 1.5g) led to increasing of growth (biomass) of *Aspergillus oryzae* or *Aspergillus niger* over the 7 days incubation period, in Czapek Dox liquid medium (nitrogen-free). Biomass increased with increasing amounts of added silicon nitride. Data presented in Fig. 4.5b show that the concentration of soluble silicon, also increased with increasing weight of added silicon nitride. Measurement of ammonium, nitrite and nitrate indicated that no nitrification occurred.

Conclusions

Results presented in Chapters 2 and 3 show that silicon compounds stimulate the growth of both fungi and species of *Streptomyces*, the aim of the research described in this Chapter was to determine whether such growth increases might have a stimulatory effect on certain biochemical process mediated by these organisms which are of biotechnological or environmental significance.

Since silicon compounds stimulate the growth of *Streptomyces* it could be expected that they might also increase the production of antibiotics. The results presented here however, show that antibiotic production (probably tetracycline) was not increased by the addition of silicon compounds to a medium in which an antibiotic-producing *Streptomyces* species was growing. As a result, the addition of silicon compounds to media used in the commercial production of this antibiotic would not be justified.

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Conclusions

Results presented in chapters 1-3 show that silicon compounds stimulate the growth of both fungi and species of *Streptomyces*, the aim of the research described in this Chapter was to determine whether such growth increases might have a stimulatory effect on certain biochemical process mediated by these organisms which are of biotechnological or environmental significance.

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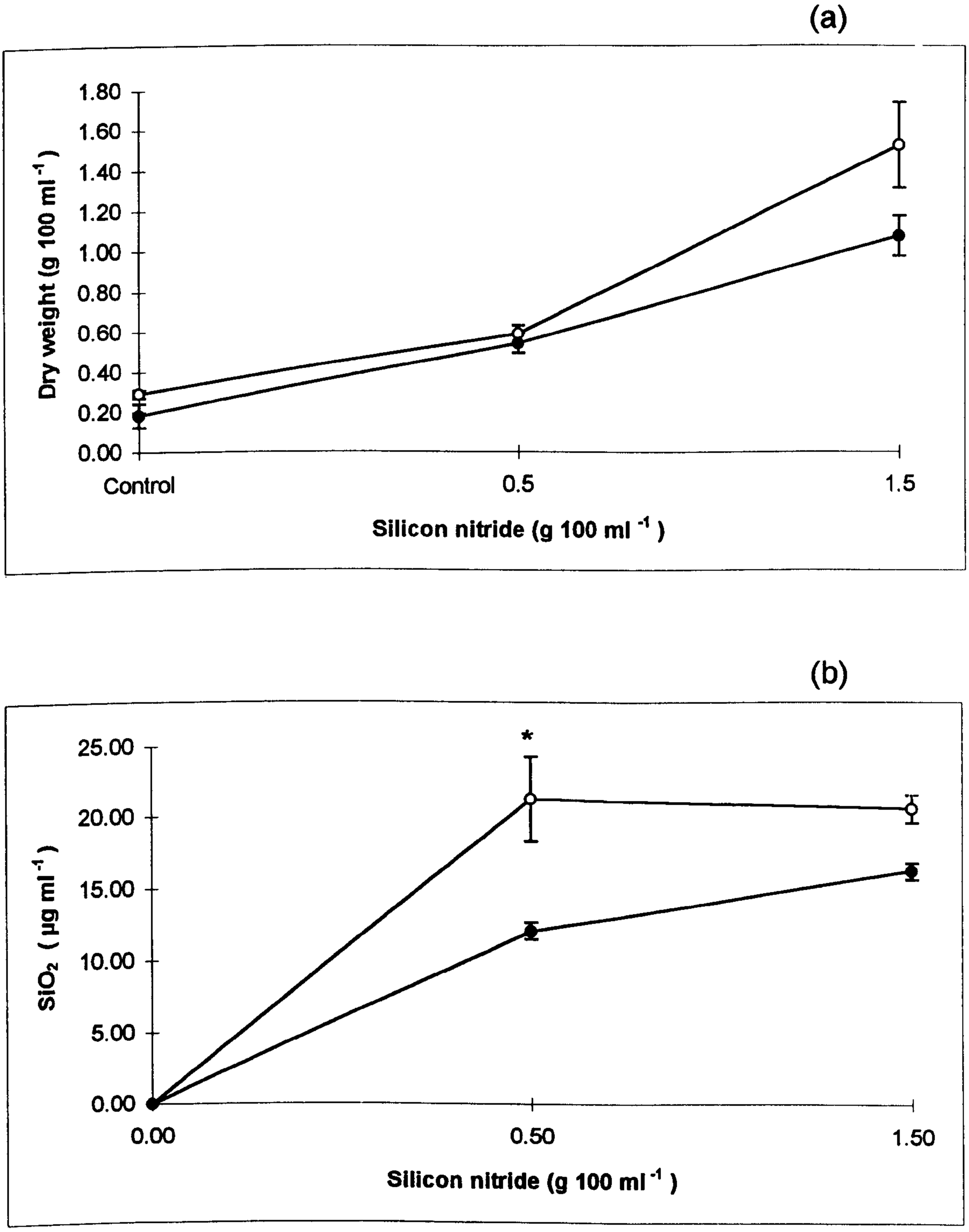


Figure 4.5 Fungal nitrification (No evidence was found for any nitrification of silicon nitride, data not shown) of silicon nitride and its effect on (a) the growth of *A. oryzae* ●—● and *A. niger* O—O in Czapek Dox liquid (N-free), (b), release of soluble silicon from silicon nitride by *A. oryzae* ●—● and *A. niger* O—O. Means of triplicates, ± Standard error. *Significant difference, $P < 0.05$.

This may not however, be true of other antibiotics (e.g. penicillin) produced by other organisms. In contrast, the production of citric acid by *Aspergillus niger* was significantly stimulated by the addition of silicic acid. The mechanism for this stimulation in citric acid production is not clear, but if it can be repeated during the commercial production of this industrially important compound, then it may have a positive influence on the economics of citric acid production.

Studies on the effect of silicic acid on fungal nitrification and S-oxidation were initiated to test the possibly that silicon present in the environment might stimulate these processes. However, the results showed that silicic acid in fact inhibited both the oxidation by fungi of thiosulphate to sulphate and ammonium to nitrate.

Relatively little is known about the biochemistry of both fungal nitrification and S-oxidation. However, both processes have been shown to be enzymic (Killham *et al.*, 1981, Wainwright, 1988). Presumably therefore, silicic acid in some way inhibits the enzymes involved in these two oxidative process, (e.g. by protein denaturation). Alternatively, the inhibitory effects may have resulted from changes induced by silicic acid on the availability of initial substrates and intermediates; for example, as the result of surface adsorption, and or precipitation. Clearly further work is needed to determine the reactions involved in these two oxidative processes before the inhibitory effect of silicic acid can be explained.

CHAPTER FIVE

EXTREME PELOMORPHISM EXHIBITED BY *ESCHERICHIA COLI* GROWN UNDER STARVATION CONDITIONS ON SILICON WAFER AND OTHER SURFACES

5.1 Introduction

5.2 Historical and more recent examples of extreme bacterial pleomorphism

During the first 40 years of this century bacteriologists were involved in a now long-forgotten controversy concerning the question of whether or not bacteria exhibit extreme pleomorphism and go through complex life cycles. The term *pleomorphism* was used to refer to the supposed ability of bacteria to change shape dramatically, or to exist in a number of extreme morphological forms. Bacteria were thought capable of changing from a single coccoid to complex filamentous forms and *vice versa*. Instead of, reproducing by single division, bacteria were thought to undergo complex life cycles involving single cells, spore, filaments, and ultra-filterable forms.

The debate split microbiologists into two opposing schools: the monomorphists and the pleomorphists. Although, the monomorphists finally triumphed, modern reports exist apparently showing that bacteria exhibit extreme morphological variations and undergo complex life cycles.

Nearly all modern microbiologists are monomorphists and accept that, except from minor variations, each bacterial cell is derived from a previously existing cell of practically the same size and shape. Cocci generally give rise to cocci, and rods to rods.

The monomorphist view, stressed by Virchow, Cohn and Koch, is that by binary fission most bacteria divide transversely to produce two new cells which eventually achieve the same size and morphology of the original. Similarly, a single spore germinates to give rise to a vegetative cell essentially the same as the cell from which the spore

originated. Exceptions to this rule are accepted in certain so-called higher bacteria, including some actinomycetes. Simple bacteria, on the other hand, are generally regarded as showing only occasional, slight morphological variation. This view of bacterial morphology and growth is so apparently obvious that we rarely bother to think about it. Despite this, there are a small number of scientists who continue to provide evidence which, they claim, supports the pleomorphist heresy.

5.3 The historical literature on extreme pleomorphism and the bacterial growth cycle.

The original pleomorphists were particularly active from around 1900-1930. This basic belief was that even common bacteria show complex life cycles which often include a (frequently pathogenic) filterable, or hidden phase (Almquist, 1922). Some even suggested that bacteria are merely rudimentary components of the fungal life cycle. The principal proponents of pleomorphism, such as Almquist, Bergstrand, Hort, Lohnis, Mellon, and Enderlein, have largely been forgotten. However, even renowned microbiologists like Ferdinand Cohn published evidence in support of extreme pleomorphism. Similarly, the eminent American bacteriologist, Theobald Smith, isolated a bacterium which apparently occurred in three forms: a bacillus, a coccus with an endospore or arthrospore, and a conglomeration mixture of all three (Smith, 1919).

The most thorough account of the pleomorphist case is given in the monograph by Lohnis entitled *Studies upon the Life Cycle of Bacteria* (Lohnis, 1921). If the weight of proffered evidence alone had been the sole criterion used to establish proof, then this book on its own would "probably have given" the pleomorphists victory.

By 1928, in an article on morphology published in the monograph *The Newer Knowledge of Bacteriology and Immunology,* Clark could state that "bacteria, even amongst the Eubacteriales, do at times reproduce, by means other than equal fission seems to me to be definitely proved" (Clarke, 1928). He quotes the work of Hort, who showed that under adverse conditions, colon-typhoid bacteria reproduce by budding, by producing Y-shaped and large aberrant forms and deeply staining granules which can be filterable (Hort, 1917 and 1920). Hort (1920) went on to describe how these irregular bodies reproduced actively and so were not examples of so-called involution forms. This term was used by the monomorphists to suggest that what the pleomorphists were seeing was merely a collection of freakish, unreproduceable forms produced by old cells. These were invariably sterile, incapable of taking up a stain, and were produced in old cultures by localised cell-wall lysis. However, such unusual forms could also be seen in young cultures (Hort, 1917 and 1920). Alexander Fleming (1915) also described how one of his four-day-old cultures of an anaerobic *Streptococcus* changed from its usual chains of cocci to a variety of strain shapes which he regarded as being involution forms.

Lohnis concluded that all bacteria live alternatively in first an organised and then an amorphous state (Lohnis, 1921). The latter he called the "symplastic state", because at this point the living matter enclosed in separate cells apparently undergoes a thorough mixing, followed by the complete disintegration of cell wall, to form a non-stainable symplasm. Lohnis also suggested that direct union between two or more cells may occur by the process which he termed "conjugation", and that all bacteria multiply not only by fission, but by the formation of so-called "gonidia". These were sometimes seen to grow directly into full-sized cells, or to go through a symplasm stage. Such

gonidia were either produced by partial or complete dissolution of the cell wall or developed while still united to the mother cell. Some of the gonidia were also filterable. Lohnis' main conclusion was that the life cycle of each bacterial species comprises several sub-cycles showing wide morphological and physiological variations, all being connected together by a symplastic stage.

Wade and Manalang (1920), even stated that *Bacillus influenzae* (then thought to be the cause of influenza) could occasionally abandon it usual bacillary form, produce conidiophores, and grow as a "frank fungus". In the same year, the Swedish microbiologist Bergstrand (1920) similarly concluded that bacteria are really *Fungi imperfecti*, a view also held by Melon (1920).

Descriptions of pleomorphism in bacteria were often associated with bacteria isolated from tumors. There is an extensive literature implicating bacteria and other non-virus microorganisms in the etiology of cancer, many of which were said to be highly pleomorphic (Wainwright, 1995). The best examples are provided by the work of Young (1921), Glover (1930) and Gruner (1935), the latter author even named his isolate *Cryptomyces pleomorpha*. Both Young (1921) and Glover (1930) provided illustrations showing complex life cycles, representing the passage of their cancer germs through a variety of stages including spores, bacilli, amorphous forms, and filamentous stages.

Not surprisingly, the monomorphists criticised the apparently absurd claims made by the pleomorphists. The most common criticism was that the pleomorphists exhibited poor technique, their work obviously resulting from contamination. Secondly, the

pleomorphists were said to have merely arranged whatever they saw, either contaminants or the products of aging cells, into convenient life cycles.

Winogradsky, (1949) perhaps not surprisingly, was severely critical of the pleomorphists, but nevertheless suggested that "The observations may be correct, but the interpretation given to the diverse forms observed cannot be taken seriously". Perhaps the most impartial historical analyses of pleomorphism are given by Handley and Henrici, with the first chapter of the latter's book providing a particularly useful introduction to the history of pleomorphism (Hadley, 1927; Henrici, 1928). Although he was essentially critical of the concept of extreme pleomorphism, Henrici (1928) did not dismiss it as readily as many of his contemporaries. He stated that "bacteria do change their morphologic type and within very wide limits; and with this change may go at times important physiological modifications". Henrici particularly objected to the criticism that extreme pleomorphism always resulted from contamination. Henrici (1928), finally came to essentially the same conclusion arrived at by Winogradsky, namely that "In undertaking a critical analysis of this work [of the pleomorphists] one cannot find fault *so much with the actual data* as to the logic followed in erecting the hypothesis.

The modern monomorphist microbiologist, can easily dismiss this historical literature as being merely the result of contaminated cultures. while recognising that some of the early studies were undoubtedly flawed, Wuerthele-Caspe and Alexander-Jackson, (1970) summed up the pleomorphist counter-argument as follows: "the faults of the enthusiastic early workers were certainly no greater than the errors both of commission and omission made later on by some of the monomorphists whose views today

dominate our textbooks". Young (1921) similarly defended the pleomorphist's case in a, short yet, comprehensive review which concludes the following quote:

"Is all this (the evidence which he cities in support of pleomorphism) and a hundred and one similar observations by other careful workers merely a tissue of a self-deceptions originating in an exuberant imagination and on faulty technique? Is it not rather one of those great facts that usher in a new era?"

Although, no such new era followed, modern microbiologists will doubtless be surprised to discover that papers continue to appear in support of extreme pleomorphism.

Examples of pleomorphism continued to be reported with surprising regularity throughout the 1920s and 1930s, but died out by about 1940. Textbooks on bacteriology nevertheless still gave token support to extreme pleomorphism even as late as the 1960s Frobisher (1949), and Lamanna and Mallette (1965). During this period, work on L-forms appeared to substantiate some of the claims made by pleomorphists. Hieneberger-Nobel (1951), for example, suggested that L-forms correlated with the symplasm.

Bacterial conjugation, an idea that had been scoffed at by many monomorphists, was now taken seriously. Previously, Lohnis (1921) had been mocked when he had claimed that he, and numerous other workers including Potthoft, had observed conjugation tubes connecting two bacterial cells (Thornton, 1930).

Reports of the existence of limited pleomorphism continue to appear somewhat infrequently in the modern literature. Wood and Kelly, (1993) for example, recently showed that the morphology of a species of *Thiobacillus* varied in response to

environmental conditions, while limited pleomorphism in *Bradyrhizobium* was reported by Reding and Lepo (1989) to be induced by dicarboxylate. While claims for such limited pleomorphism offend no one, modern reports of extreme bacterial pleomorphism are likely to criticised or merely ignored. The association between cancer etiology and bacteria continues to be the source of many of the claims made by modern pleomorphists. For example, an amazing series of papers linking extremely pleomorphic bacteria and cancer was reported in 1970 in a symposium in the *Annals of the New York Academy of Sciences.* the first of these papers, by Wuetherle-Caspe, *et al.* (1970) reported the isolation of a specific type of highly pleomorphic microorganism found consistently in human and animal cancers. Due to its remarkable pleomorphism, the organism was described as an "unclassified mystery", but was apparently capable of resembling micrococci, diphtheroids, bacilli, fungi, viruses, and host cell inclusions. Of particular interest was the reported appearance of an L-form, symplasm stage. The following quote from this paper could just as easily have come from the historical literature.

"The virus-like bodies present in tumour and culture filtrates can evolve after one or more months into larger mycoplasma-like L forms, and thence to frankly bacterial rods and filaments. Polar or peritrichous flagella can develop under favourable conditions, and motile rods exhibiting a tumbling appearance. Under certain conditions unfavourable to the organisms, large ovoid bodies and still larger cysts from which spore forms develop. When conditions again become favourable, small bodies bud off from the chromatin ring lining the cyst, and filaments also may sprout from the rim. The small bodies, often acid fast, lengthen out into rods and filaments."

This work and the approach to cancer management which has been developed from it have come in for considerable criticism. In particular it has been suggested that the so-called cancer germ involved is not new, but merely a strain of *Staphylococcus epidermidis.*

White (1990) also suggests that within cancerous cells exists "a non-septic, or non-virulent cell-wall deficient or conidial like micrococcus". Similarly intriguing recent papers reporting a complex life cycle in bacteria were written by Pease (1970) and Pease and Tallack (1990). They state that for over a century there have been reports of a widespread, possibly universal, endoparasitism in humans caused by a bacterium capable of passing through a complex life cycle. Not surprisingly, this reported complexity has made this organism difficult to study and sceptics have yet to be convinced of its validity. The organism which Pease and Tallack consider to be a silent but potentially important pathogen was also reported to be associated with cancer by Alexander-Jackson (1966) and by Wuerthele-Caspe and Alexander-Jackson, (1970); they also suggest that the Rous sarcoma virus is a stage in the life cycle of a bacterium. A number of workers have isolated cell-wall deficient bacteria from material containing the Rous sarcoma virus, as well as the Bittner virus and Shope's Papilloma virus (Macomber, 1990). Alexander-Jackson (1966) even claims to have repeatedly grown cell-wall deficient bacteria from the blood of chickens infected with Rous sarcoma. Macomber (1990) apparently stated the obvious when he said that it goes against common sense to suppose that a virus can turn into a bacterium. Instead, he suggested that it is more likely that viruses can be incorporated into cell-wall-less bacteria associated with the virus. He then emphasised the importance of clarifying the exact

relationship between cancer-associated cell-wall deficient bacteria and the oncogenic retroviruses.

Pleomorphic cell-wall deficient bacteria have also been associated with rheumatoid arthritis. Mattman, (1986) for example, has claimed that a bacterium of this type, which apparently causes this disease in chickens, can revert in culture to *Proprionibacterium acne*.

Conclusion

Most modern microbiologists, being monomorphists, would probably assume that the examples of bacterial life cycles and extreme pleomorphism given here are merely the result of a mixture of wild speculation and contaminated cultures (this was the view taken by Frobisher, who coined the word *oligomorphism* to describe the more readily acceptable examples, which clearly exist, of limited pleomorphism Frobisher, 1949). However, most of the microbiologists who have reported examples of extreme pleomorphism went to considerable lengths to demonstrate the purity of their cultures. It is also worth remembering that they often spent more time, much more than most modern microbiologists do, just looking, at bacteria. Similarly, they were generally more practised in the art of microscopy than are most modern microbiologist. On the other hand, Holman and Carson (1935) showed that the work described by at least one researcher, who claimed to have demonstrated extreme bacterial pleomorphism, resulted from faulty bacteriological technique.

Microbiologists of the past had no preconceived ideas about the nature of bacteria, and all possibilities were open to investigation. Of course, they lacked our

technological sophistication; in particular, they knew nothing of the molecular approaches, which might be profitably used to study some of their apparently wild claims.

The literature on extreme pleomorphism remains intriguing, and some aspects of it may be worth re-examining. By merely dismissing it, we may be ignoring something of fundamental importance. This is especially likely since examples of extreme variation in bacterial morphology continue to be linked with various diseases and cancer in animals and humans. The use of molecular techniques should, however, help clarify any lingering uncertainties arising from the historical literature on extreme pleomorphism. Those certain of the phenomenon's validity would however, argue that their claims could be confirmed by simple, if thorough, microscopy.

5.4 Pleomorphism in *E. coli* when grown on silicon and other surfaces

The possibility of implanting silicon, bioactive glasses, and other inert surfaces into the body to fulfill a number of medical applications is being increasingly considered. With such applications in mind, we recently become interested in determining if *Aspergillus oryzae* and *Escherichia coli* can erode bulk and porous silicon. Under standard nutrient conditions *E. coli* generally grows as a rod-shaped cell. However, when growing under starvation conditions on surfaces we observed that some rods exhibited extreme pleomorphism and in particular, turned into long filaments. The formation of filaments by *E. coli* under a variety of growth conditions has been observed previously; for example, it can be induced by the presence of urea (Wilson, 1906), metals (Gibson *et al.*, 1984; Brandi *et al.*, 1989) and in particularly antibiotics

(Lorian and Atkinson, 1975). However, we are not aware that pleomorphic growth by this bacterium has previously been reported during starvation, particularly when growing on silicon and other essentially inert surfaces. Here we report observations on pleomorphism in starved *E. coli,* particularly in relation their possible occurrence in medical environments.

The aim of this work reported in this Chapter was to act on the serendipitous observations on pleomorphic growth by *Escherichia coli* on silicon and other surfaces and study the phenomenon in more detail. Reference is also initially made to the growth of *A. oryzae* on silicon, work which led to the serendipitous observations on extreme pleomorphism in *E. coli.*

5.2 Materials and Methods

5.2.1 Growth of *A. oryzae.*

Aspergillus oryzae was grown on Czapek Dox agar medium for 7 days then transferred to silica gel medium for 20 days at 25^0C. After incubation period (14 days) fungus growth was removed from silica gel and transferred to (non-sterilised) porous silicon.

5.2.2 Growth of *E. coli.*

An ampicillin resistant strain of *Escherichia coli* (strain DH5 α) was grown overnight at 37^0C on L-broth solidified with agar (2%) and containing ampicillin (200 mg ml^{-1}). A loopfull of cells was removed from a resultant colony and transferred to ampicillin-free, L-broth agar and grown overnight as before. Bacteria were then transferred in the same manner from the resultant colonies, care being taken not to remove any underlying medium; finally, they were suspended in sterile ultra-pure water (15 ml) (upw, obtained from a Milli-RO4 water purification system). In some experiments the ampicillin-resistant strain was grown on ampicillin containing medium and the transferred directly to upw without the interim growth on antibiotic-free medium. A wild type strain of the same bacterium was also grown on L-broth agar.

5.2.3 Incubation surfaces.

The following surfaces were used:

Porous silicon- Mesoporous silicon having a Gaussion distribution of pores in the range 2-50nm and porosity of 40-70% void fraction; emits orange light under UV.

Microporous pores less than 2nm width; bioactive, inducing calcification, in SBF (simulated body fluids).

Bulk silicon- The silicon discs (100) orientation single crystal were prepared by "CZ growth" (Walker Chemitronic, Germany); porous silicon discs were electrochemically etched using hydrofluoric acid.

Titanium wafer- Punched out from 99.6% titanium (obtained from Goodfellow Metals Inc.). Abraded to remove surface burs and then chemically etched.

Glass- Microscope slides- 76 x 26mm, 0.8-1.0mm thick (Select Micro Slides).

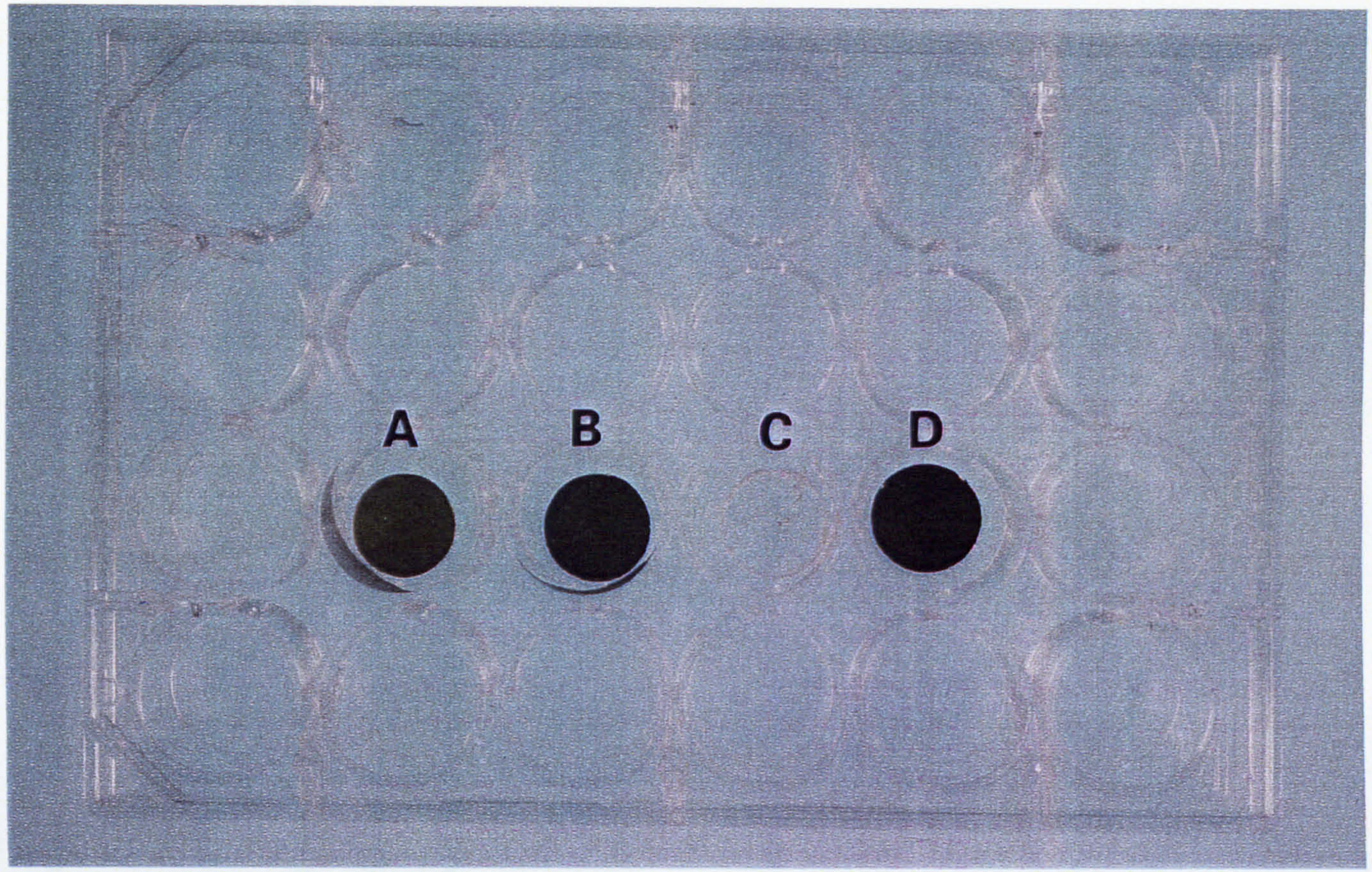
Plastic wafer- Inner surface of sterilised plastic Petri dishes (Sterilin).

5.2.4 Starvation conditions.

In the case of *A. oryzae*, mycelium and spores were transferred to porous silicon wafer (non-sterilised) and moistened every 2 days with sterilise ultra-pure water (upw) at 25^0C.

In the case of *E. coli*, a portion of the above suspension (1 ml) was transferred to the surface of either a) unsterilised bulk or porous silicon wafers b) silicon wafers or titanium wafers sterilised by UV radiation c) plastic wafer UV-sterilised (Fig. 5.1) or d) glass microscope slides (sterilised by autoclaving), the inoculated surfaces were

Figure 5.1 Titanium wafer (A), microporous silicon wafer (B), plastic wafer (C) and bulk silicon wafer (D), on which *Escherichia coli* was grown under starvation conditions


A
B
C
D

then incubated in sterile plastic petri dishes at 37^0C for 14 days. Where UV sterilisation was employed, the wafers were exposed to UV (260 nm) for 1h. Tests, using L-broth agar, confirmed that this treatment effectively sterilised the surfaces of the silicon and titanium wafers. Glass slides were sterilised by autoclaving at 120^0C for 20min.

5.2.5 Scanning electron microscopy.

The samples were splutter coated with 80% platinum and 20% paladium at about 4nm thickness, using a Cressington High Resolution, 208 splutter coater. A JEOL JSM 6400F field emission SEM was used (5kV, incident angle, 30^0C).

5.3 Results and Discussion

5.3.1 The growth of *A. oryzae* under starvation conditions

Figs.5.2 and 5.3 data show *Aspergillus oryzae* growing on a porous silicon wafer. The surface shows no signs of surface biocorrosion, including pitting. Note that in Fig.5.3, bacteria (some showing pleomorphism) can be observed, since the wafer was initially not sterilised it was open to bacterial contamination; a problem that was overcome in subsequent experiments.

When filamentous fungi grow on glass or silica gel, they often bring about pitting of the surface, or in the latter case, cause the gel surface to erode so that the mycelium lies in a surface furrow (Parkinson *et al.*, 1989). This effect is not due to the weight of the mycelium, since a human hair laid on the surface does not cause similar indentations (Barakah, 1992). Such pitting therefore appears to result from the ability for the fungus to produce a chemical agent which dissolves silica gel. These experiments were initiated with the expectation that such pitting would occur on bulk and porous silicon, and our aim was to study such chemical dissolution of the surface of porous and bulk silicon wafers in some detail. Such silicon degradation, either by fungi and bacteria, did not occur, but while conducting these experiments bacterial pleomorphism was observed; this is the main subject of this Chapter.

Considerable historical literature exists (see above) showing that a wide range of bacteria undergo pleomorphism, including filamentation, both when growing *in vitro* as well as in the body (Wainwright, 1997). Critics have suggested that such

Figure 5.2 Scanning electron micrograph showing the growth of hyphae and spores of *Aspergillus oryzae* grown on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions.

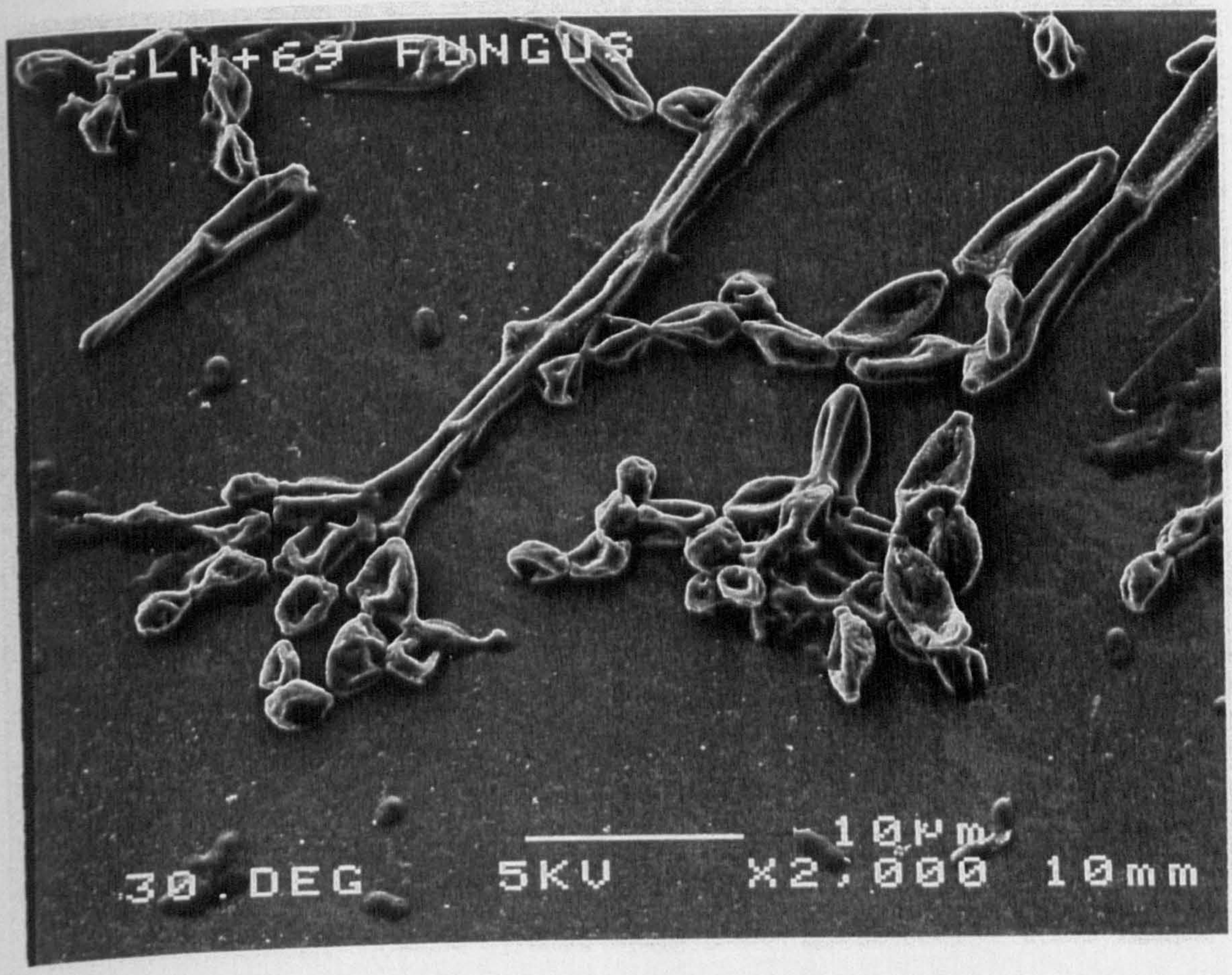
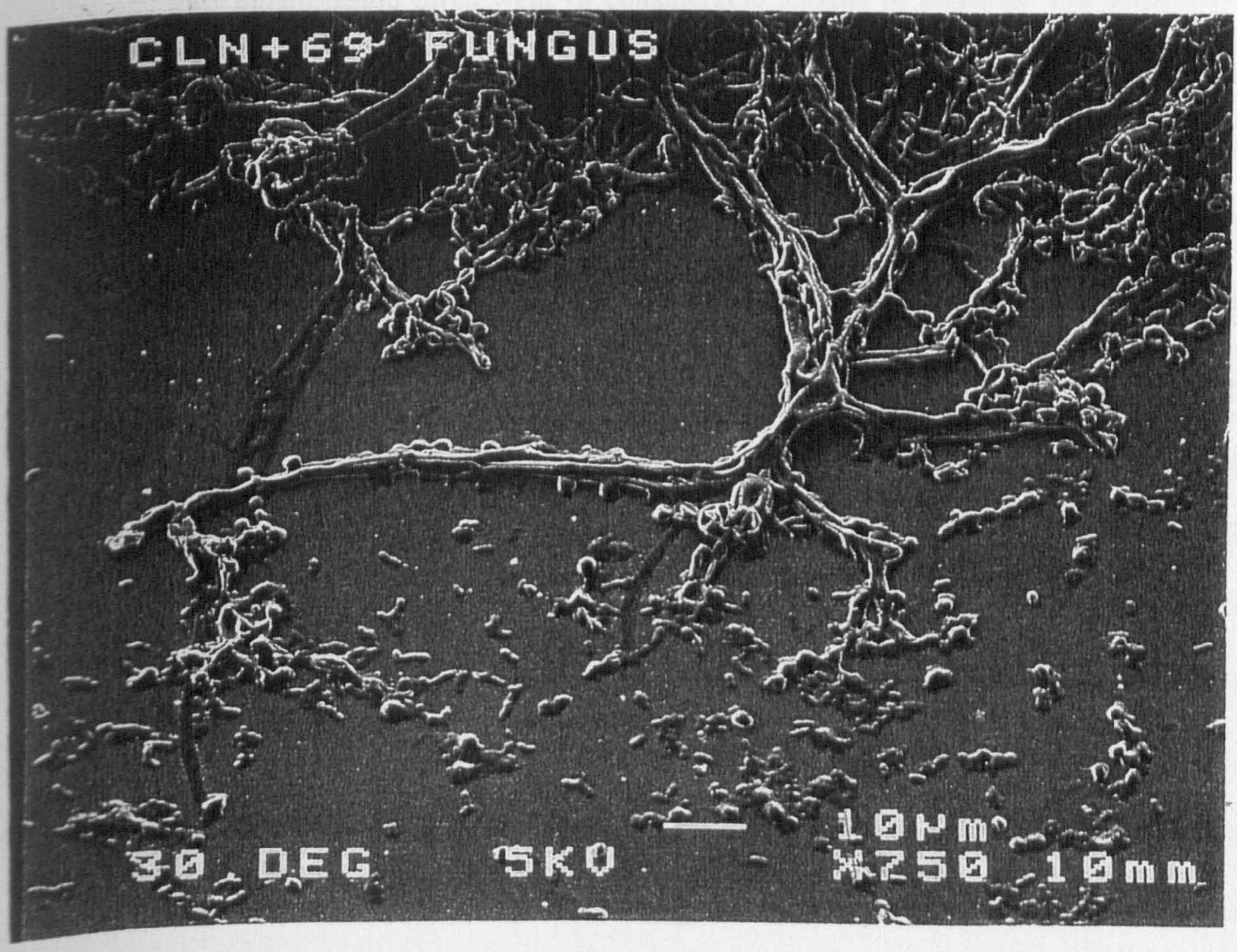

CLN+69 FUNGUS
30 DEG
5KV
X2,000
10µm
10mm

Figure 5.3 Scanning electron micrograph showing the growth of mycelium, hyphae and spores of *Aspergillus oryzae* grown on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions (A). Bacterial contamination, some of the bacterial cells shows pleomorphism (B).

A



B

observations can be explained by contamination, despite the fact that the observers of this phenomenon often went to considerable lengths to eliminate this possibility. To avoid the possibility that a mixed culture was being used in our experiments, an ampicillin-resistant *E. coli* was employed. Prior to starving the cells, this culture was initially grown on ampicillin-containing medium, followed by a period of growth on ampicillin-free medium. In order to avoid the possibility of denaturating the silicon surfaces used here, the initial experiments (in which we made the observation of extreme pleomorphism in starved *E. coli*), the silicon wafers were not sterilised by either heat or chemicals. As a result, it could be argued that the observed pleomorphism was due to the presence of contaminants. However, the wafers were removed from the manufacturer's containers, under sterile conditions, and were subsequently handled using standard sterile techniques. Silicon wafers are made under conditions of extreme cleanliness and when examined under the scanning electron microscope were seen to be completely free of surface contaminants, including microorganisms. However, in order to avoid arguments concerning contamination, in subsequent experiments, the wafers were sterilised using UV light, glass slides were autoclaved and sterile plastic surfaces were used.

5.3.2 Pleomorphism in *E. coli* grown under starvation conditions.

Figs. 5.4, 5.5, 5.6 and 5.7 show *E. coli* growing on (non-sterilised) porous silicon wafer after 14 days. Starvation growth on silicon led to the formation of extreme pleomorphic forms, including filaments (Figs.5.4, 5.5 and 5.7) and dumb-bells (Figs. 5.6). Fig 5.5 shows that the apical cells of the filaments are often swollen.

Figure 5.4 Scanning electron micrograph showing the growth of *Escherichia coli* on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions.

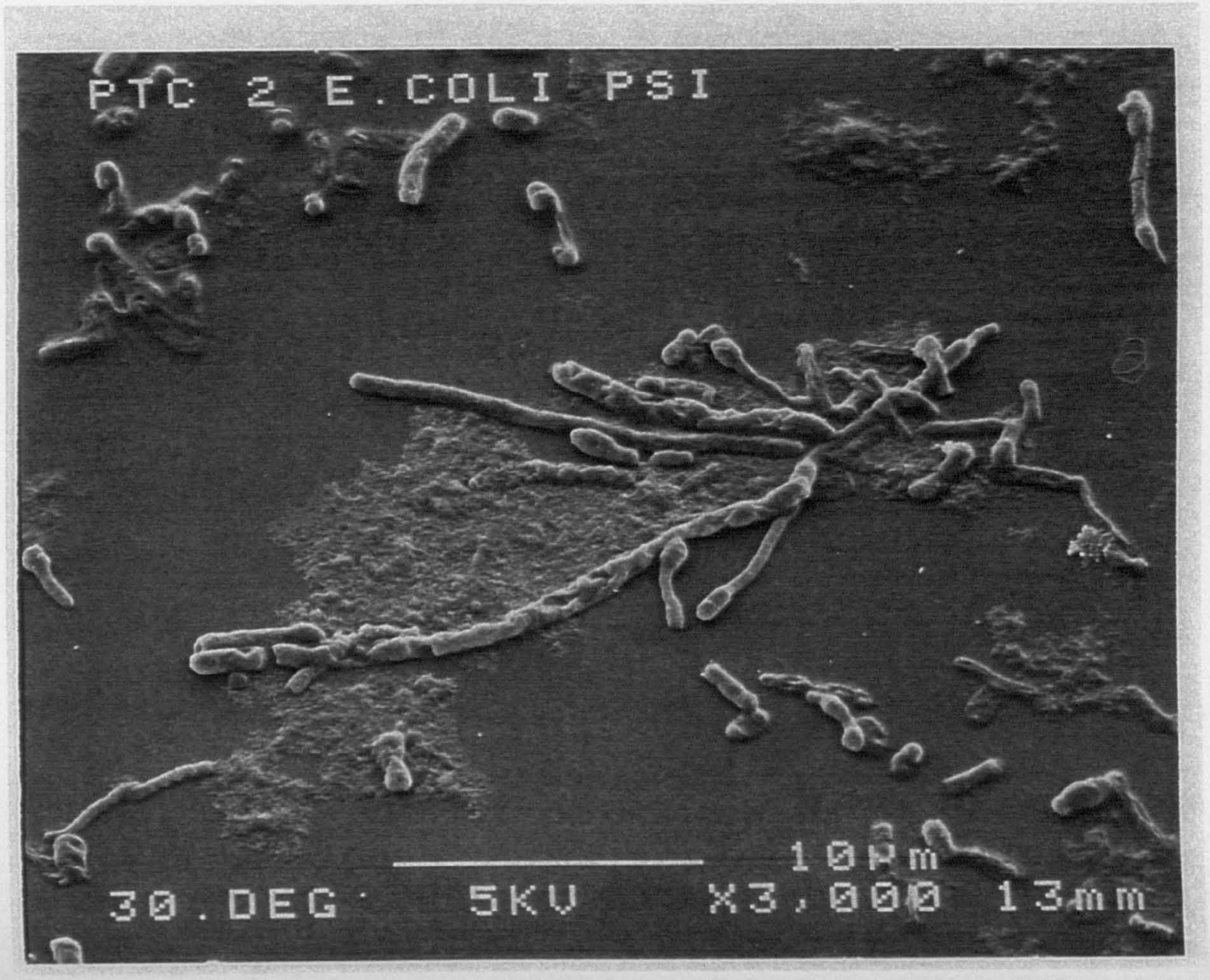

PTC 2 E.COLI PSI
10μm
30.DEG 5KV X3,000 13mm

Figure 5.5 Scanning electron micrograph showing the growth of *Escherichia coli* grown on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions.

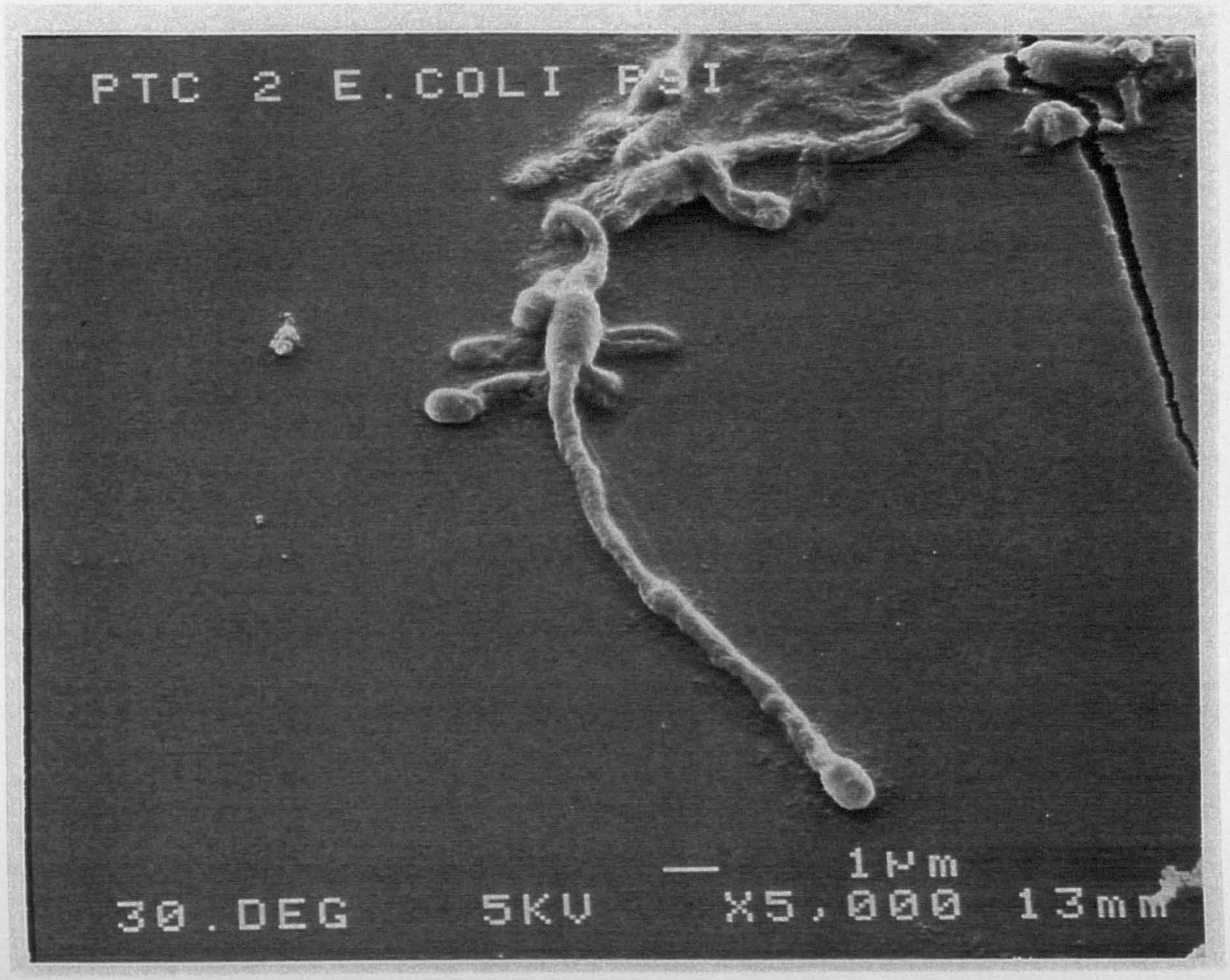

PTC 2 E.COLI PSI
1μm
30.DEG 5KV X5,000 13mm

Figure 5.6 Scanning electron micrograph showing the growth of *Escherichia coli* grown on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions.

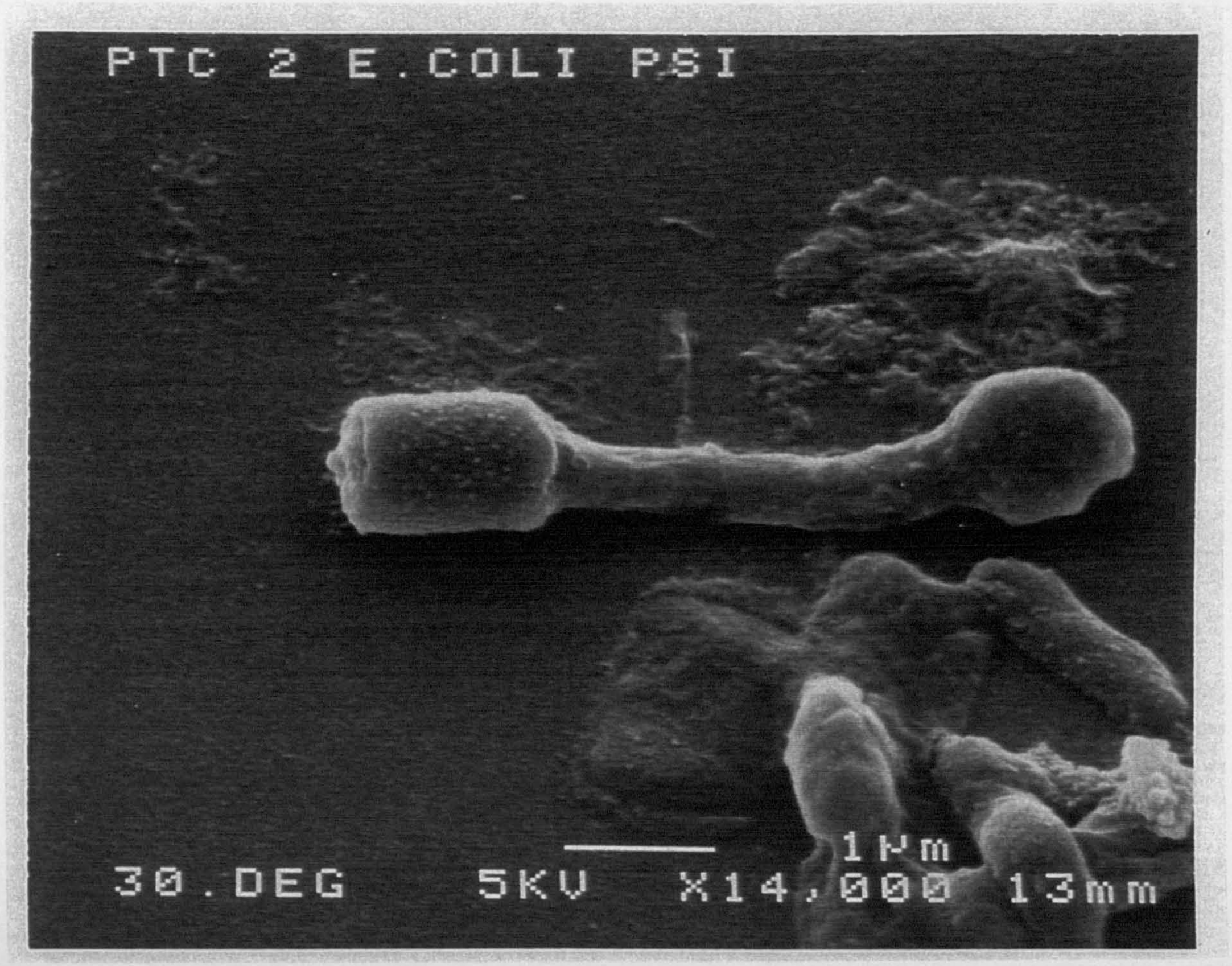

PTC 2 E.COLI PSI
1μm
30.DEG 5KV X14,000 13mm

Figure 5.7 Scanning electron micrograph showing the growth of *Escherichia coli* grown on (non-sterilised) porous silicon wafer for 14 days under oligotrophic conditions.

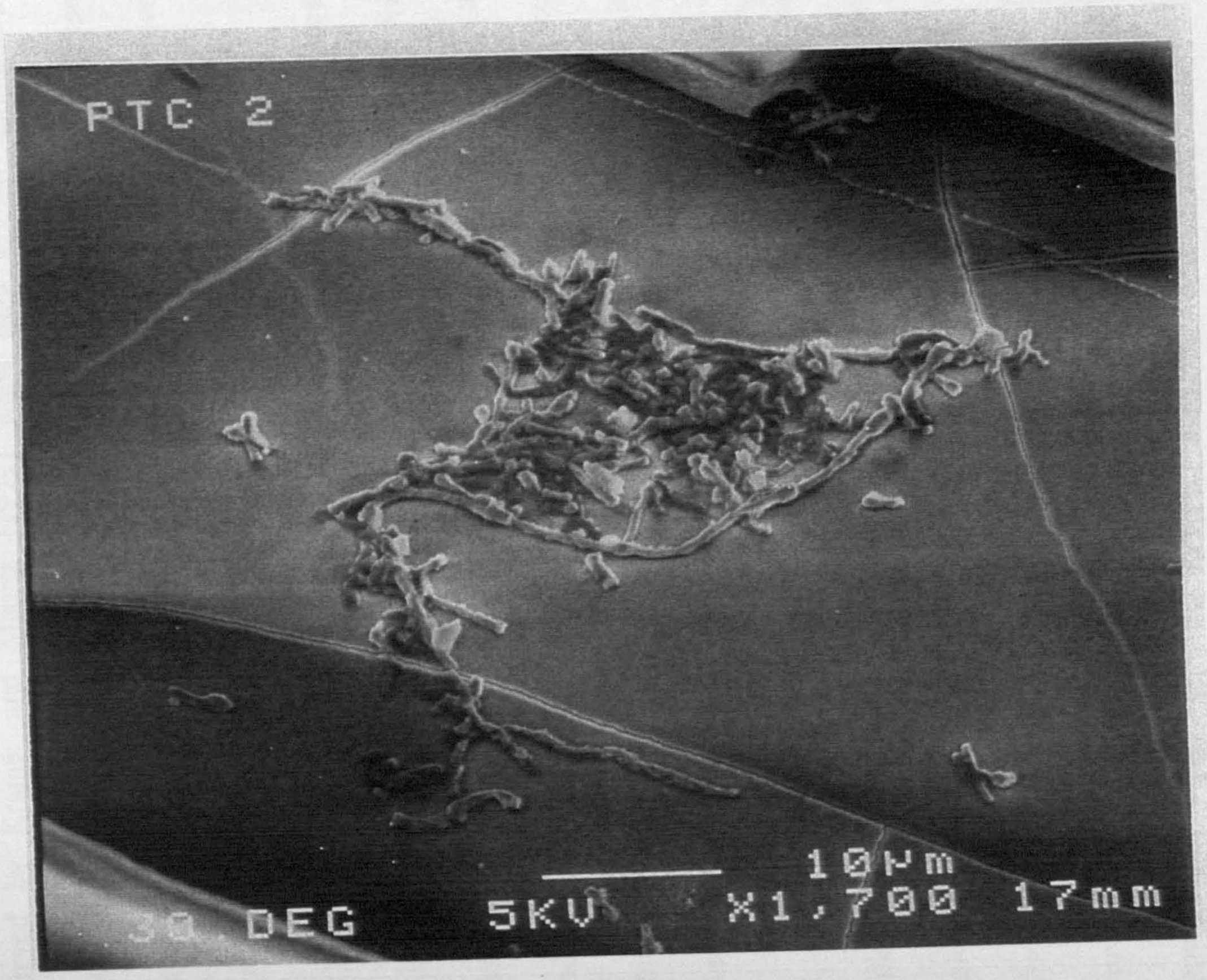

PTC 2
10μm
30 DEG 5KV X1,700 17mm

Fig. 5.8 shows that *E. coli* grown on autoclaved glass (overnight), very limited pleomorphism can occur in non-starved cultures. Similar, rare occurrences of filamentation in control (i.e. in this case, non ampicillin-treated) cultures were also observed by Kramer and Mauriz (1979) when they studied antibiotic-induced filamentation in *E. coli*. It is possible that, in the work reported here, the presence of occasional filaments in non-starved cultures was caused by the introduction of ampicillin in the original culture medium.

Figs. 5.9, 5.10, 5.11 and 5.12 show *E. coli* grown first on ampicillin containing medium and then ampicillin-free medium, followed by starvation on an autoclaved glass slide. Filamentous growth was not seen on day 2 after the imposition of starvation conditions; the presence of bi-cells however, suggest that cell division is already being impaired. Filaments begin to appear 5 days after starvation and continue to be in evidence after 14 days of starvation.

Fig. 5.13 shows that, *E. coli* was grown on UV-sterilised microporous silicon wafer, and after 14 days filamentation appears to result from the lack of separation of individual cells, leading to the production of filaments, reaching a maximum length of approximately 50 μm. It is obvious that not all cells undergo pleomorphism at the same time. Why this should be is not clear, although examples of the same observation appear in the historical (Hort, 1917) and more recent literature (Kramer and Mauriz, 1979).

Fig.5.14 shows that *E. coli* cells started to increase in length as the result of starvation, after 14 days incubation period on bulk UV-sterilised silicon wafer, while Figs. 5.15 and 5.16 show *E. coli* after growth on autoclaved glass slide for 14 days

Figure 5.8 Scanning electron micrograph showing the growth (overnight) of *Escherichia coli* grown on an autoclaved glass slide.

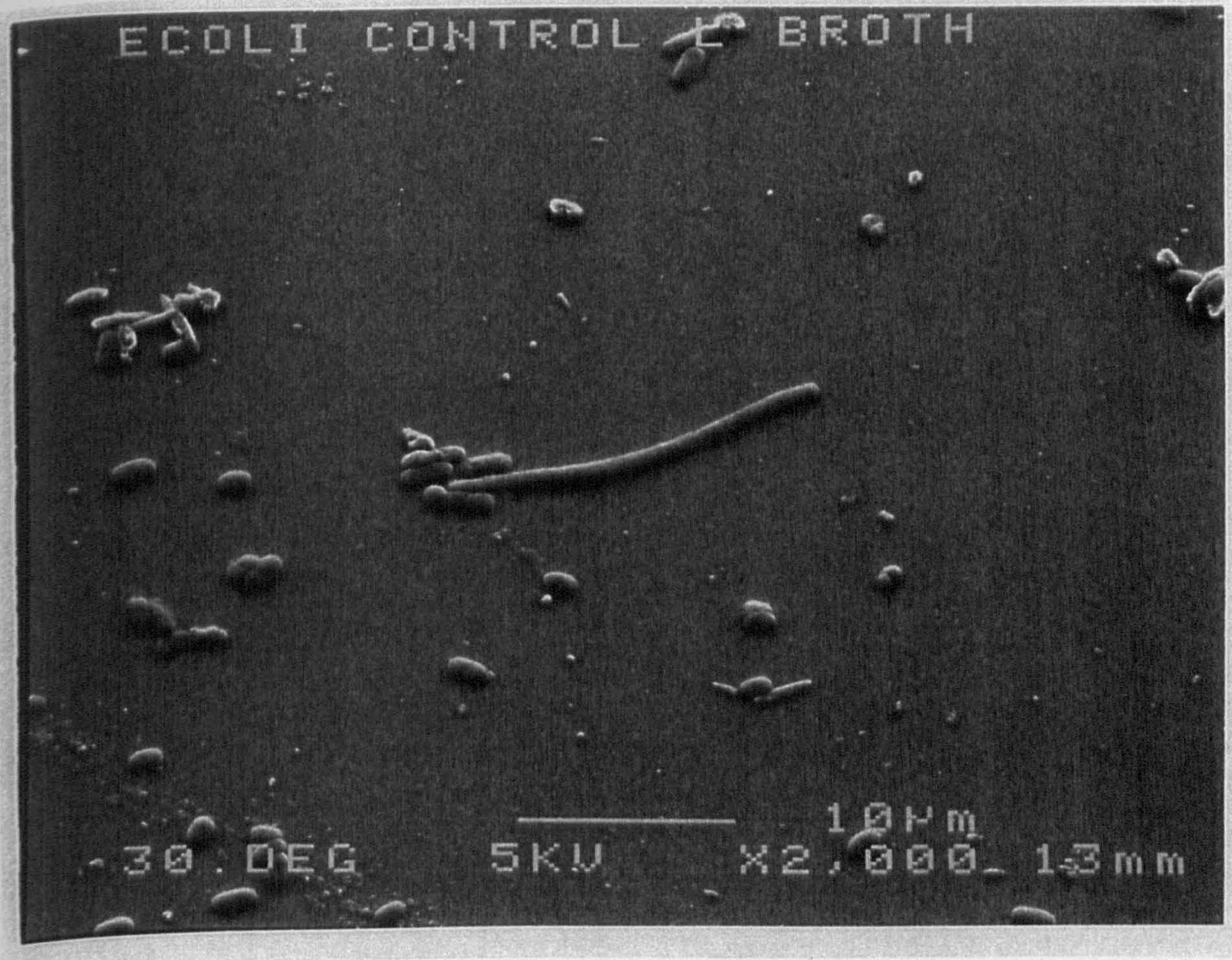

ECOLI CONTROL BROTH
10μm
30.0EG 5KV X2,000 13mm

Figure 5.9 Scanning electron micrograph showing the growth (2 days) of *Escherichia coli* grown on an autoclaved glass slide.

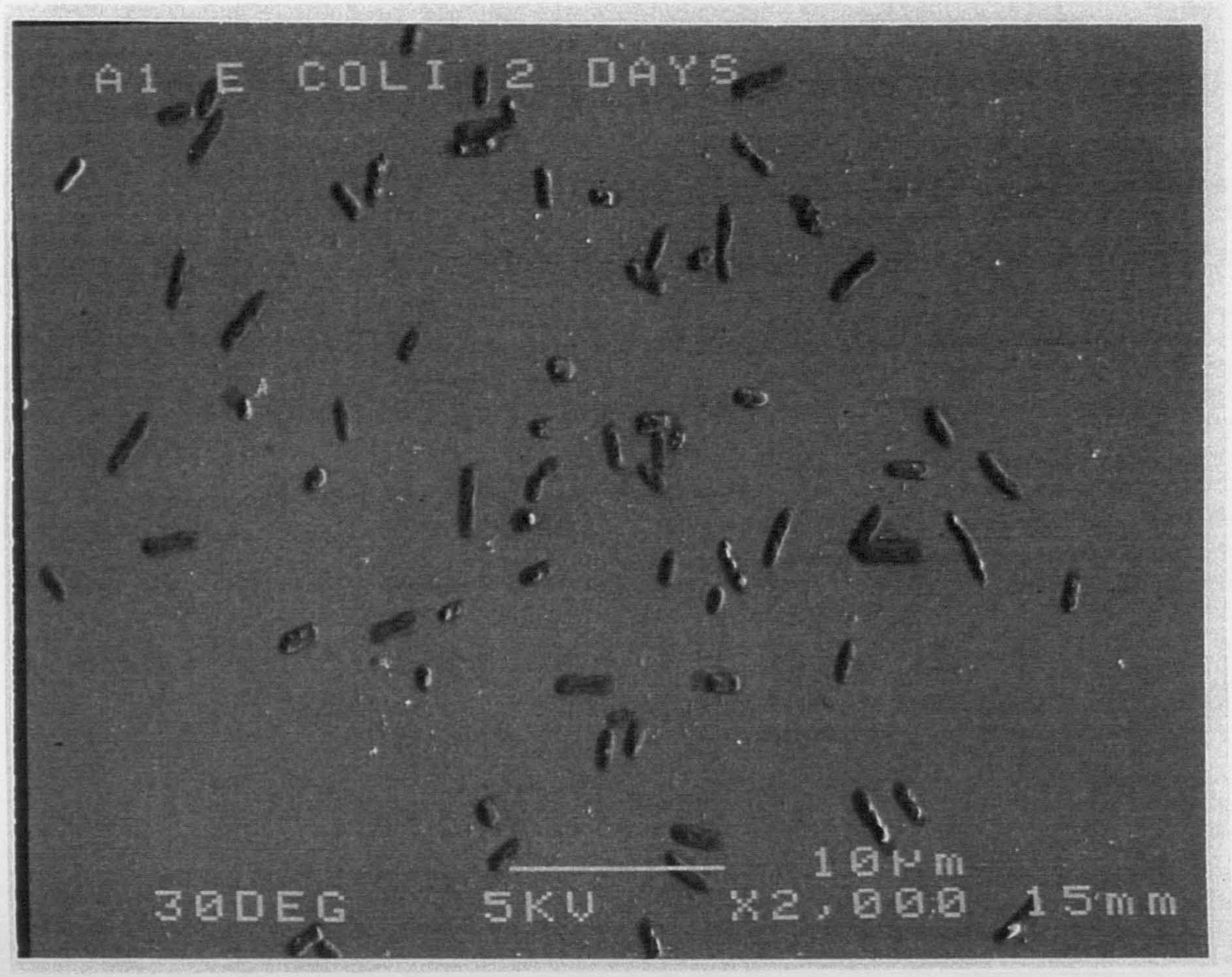

A1 E COLI 2 DAYS
10µm
30DEG 5KV X2,000 15mm

Figure 5.10 Scanning electron micrograph showing the growth (5 days) of *Escherichia coli* grown on an autoclaved glass slide.

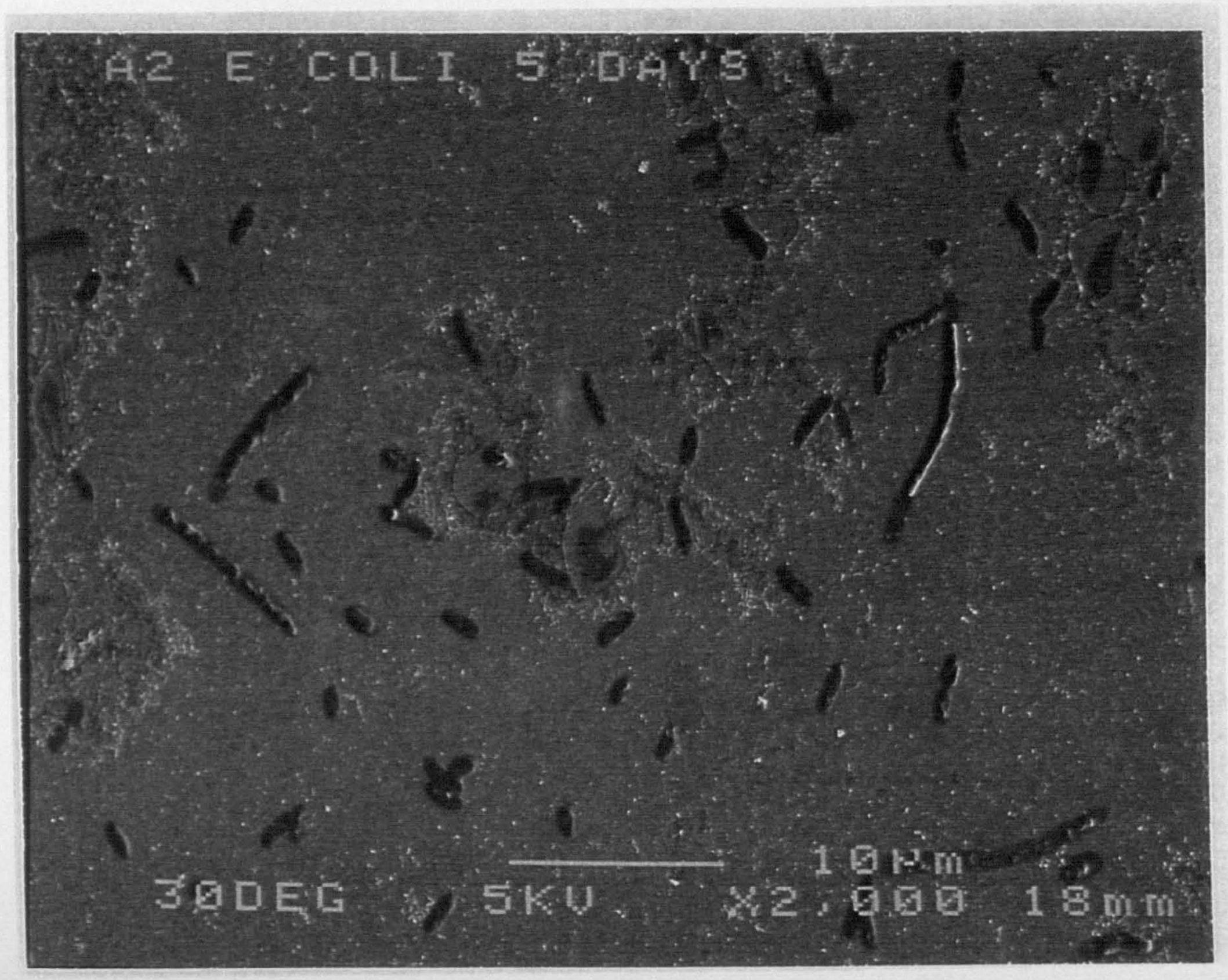

A2 E COLI 5 DAYS
10µm
30DEG 5KV X2,000 18mm

Figure 5.11 Scanning electron micrograph showing the growth (14 days) of *Escherichia coli* grown on an autoclaved glass slide.


A4 E COLI 1A DATS
5KU
X4,000
1μm
14mm

Figure 5.12 Scanning electron micrograph showing the growth (14 days) of *Escherichia coli* grown on an autoclaved glass slide.

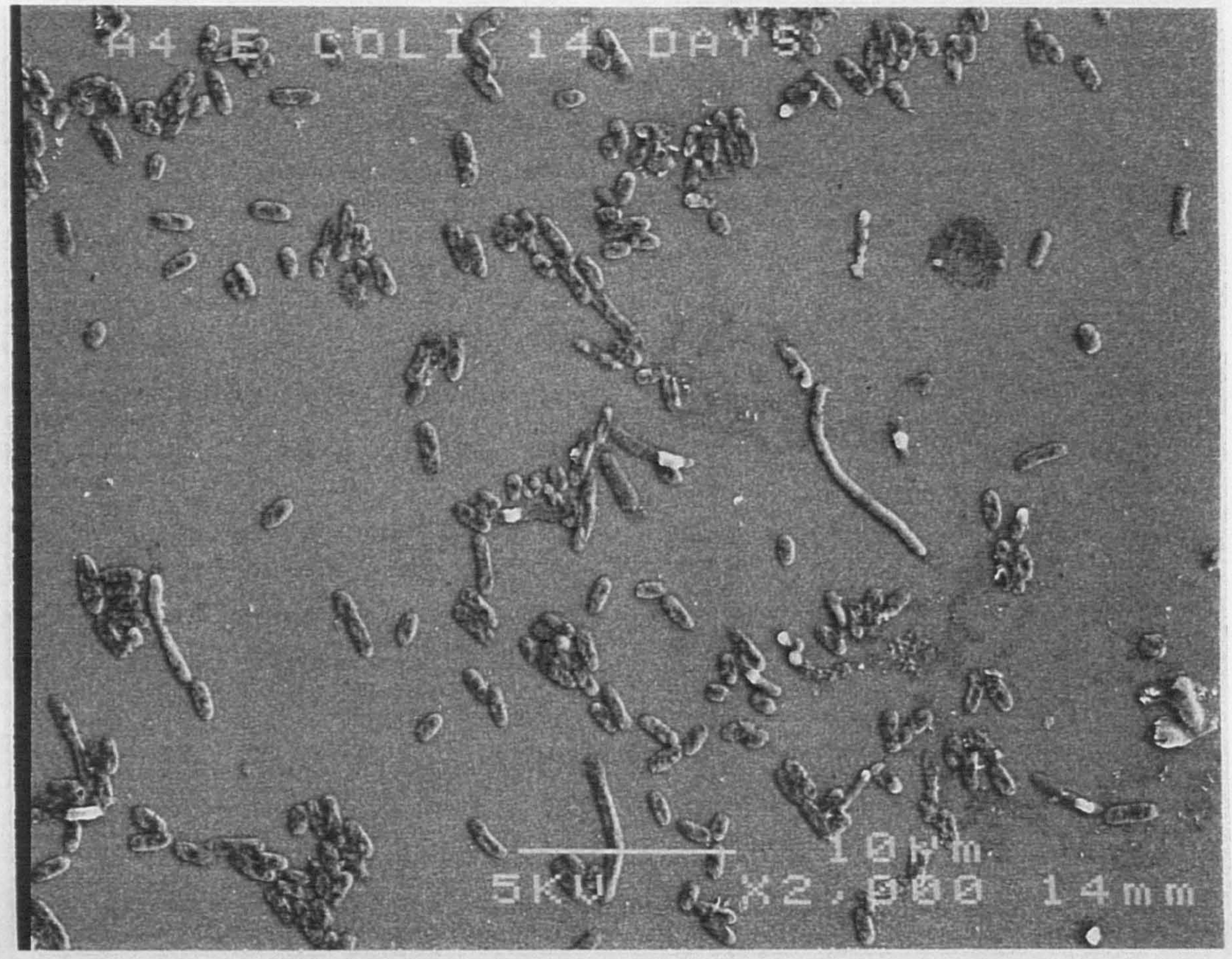

A4 E COLI 14 DAYS
10μm
5KV X2,000 14mm

Figure 5.13 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* grown (14 days) on (UV-sterilised) microporous silicon wafer.

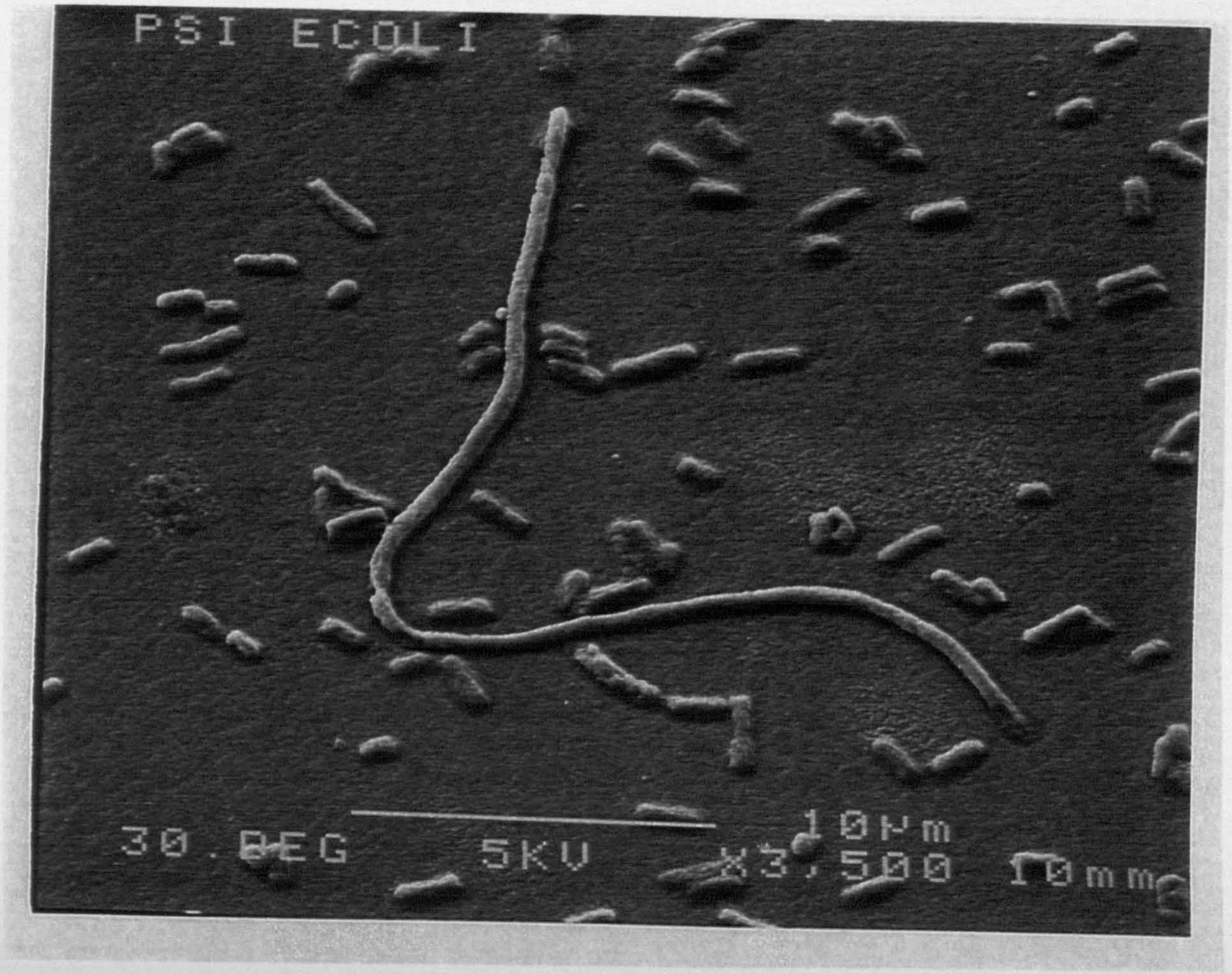

PSI ECOLI
30.0 DEG
5KV
X3,500
10μm
10mm

Figure 5.14 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* grown (14 days) on (UV-sterilised) bulk silicon wafer.

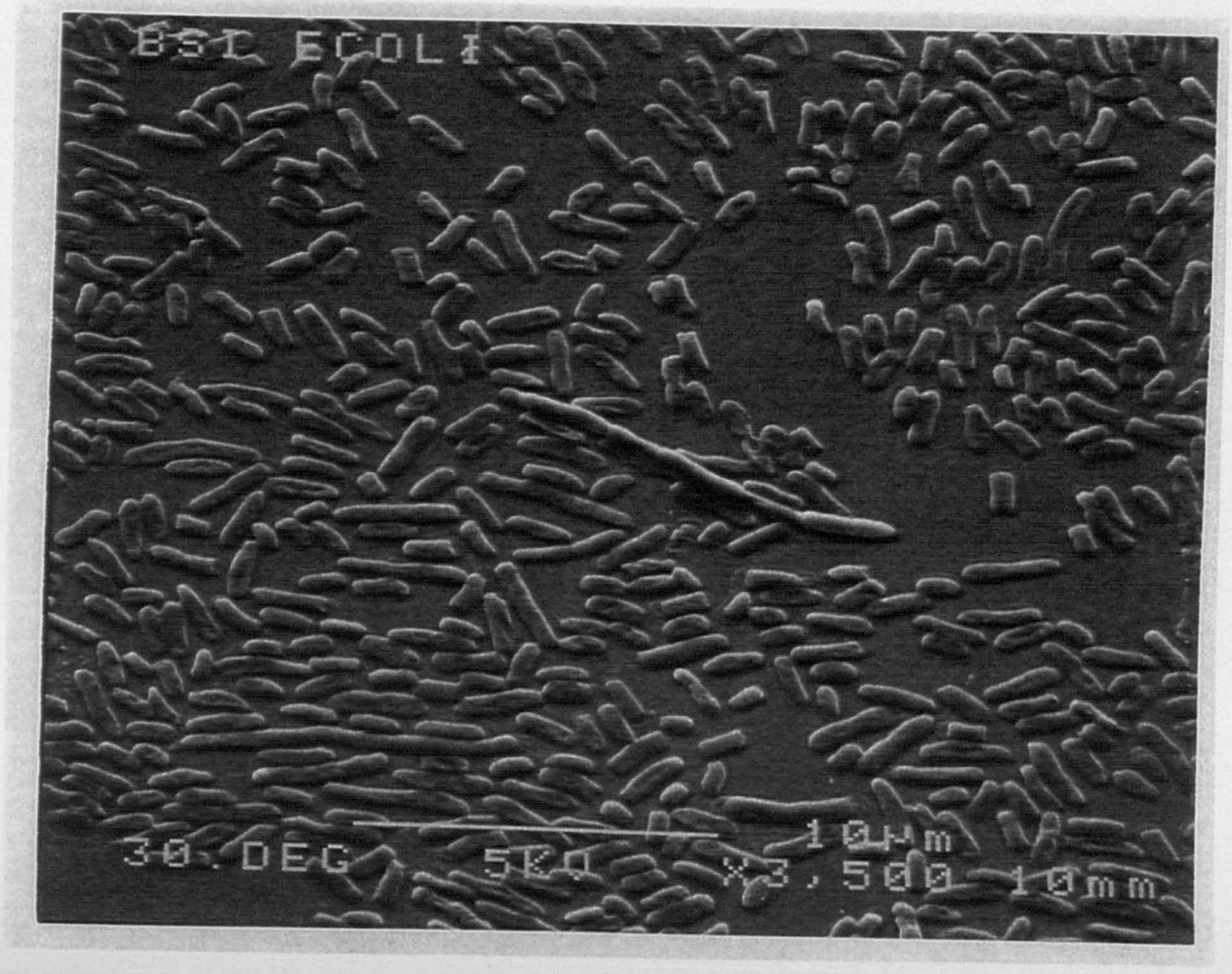

BSI ECOLI
30.0EG 5KV X3,500 10mm
10μm

Figure 5.15 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* (ampicillin-resistant culture) grown (14 days) on an autoclaved glass slide.

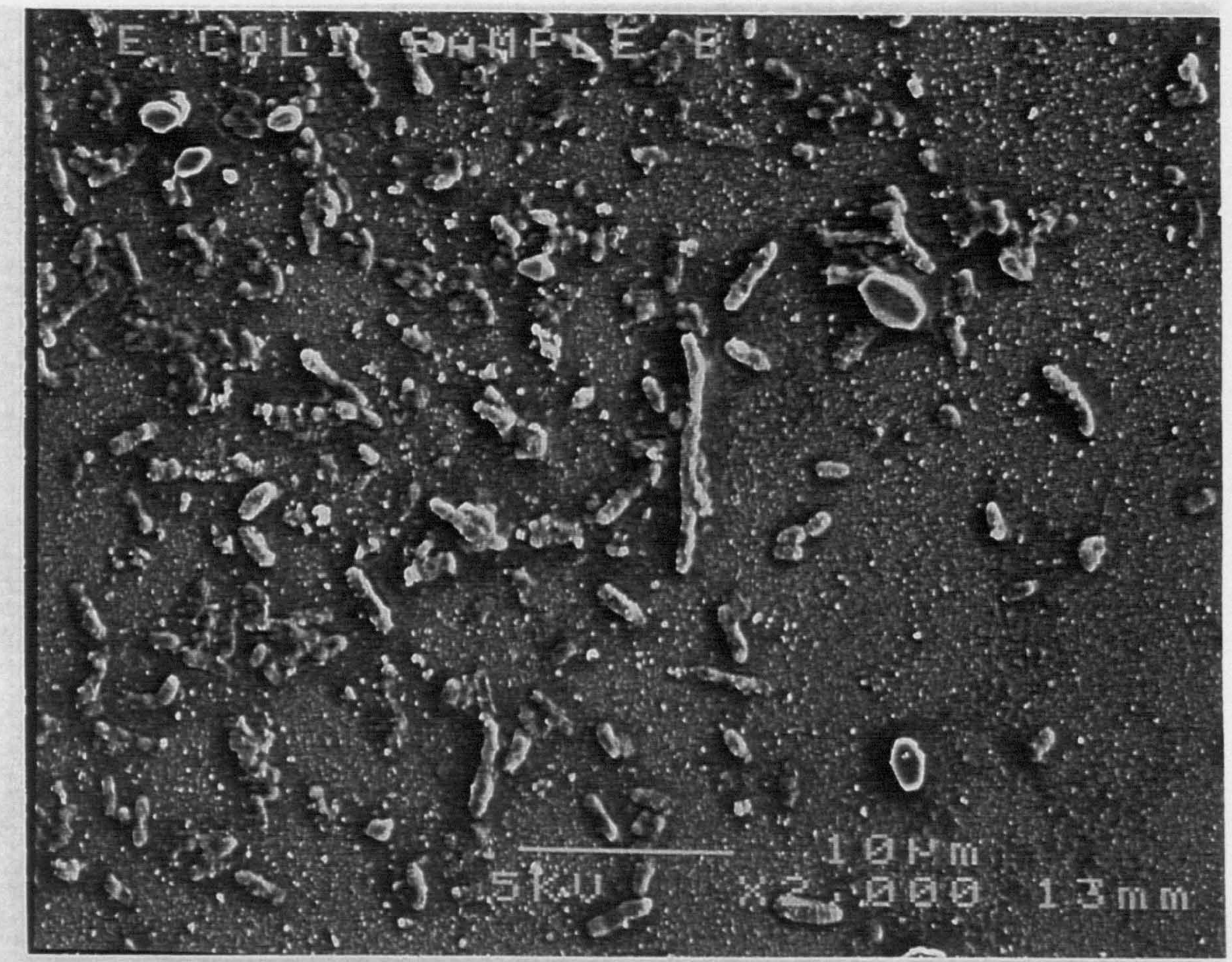
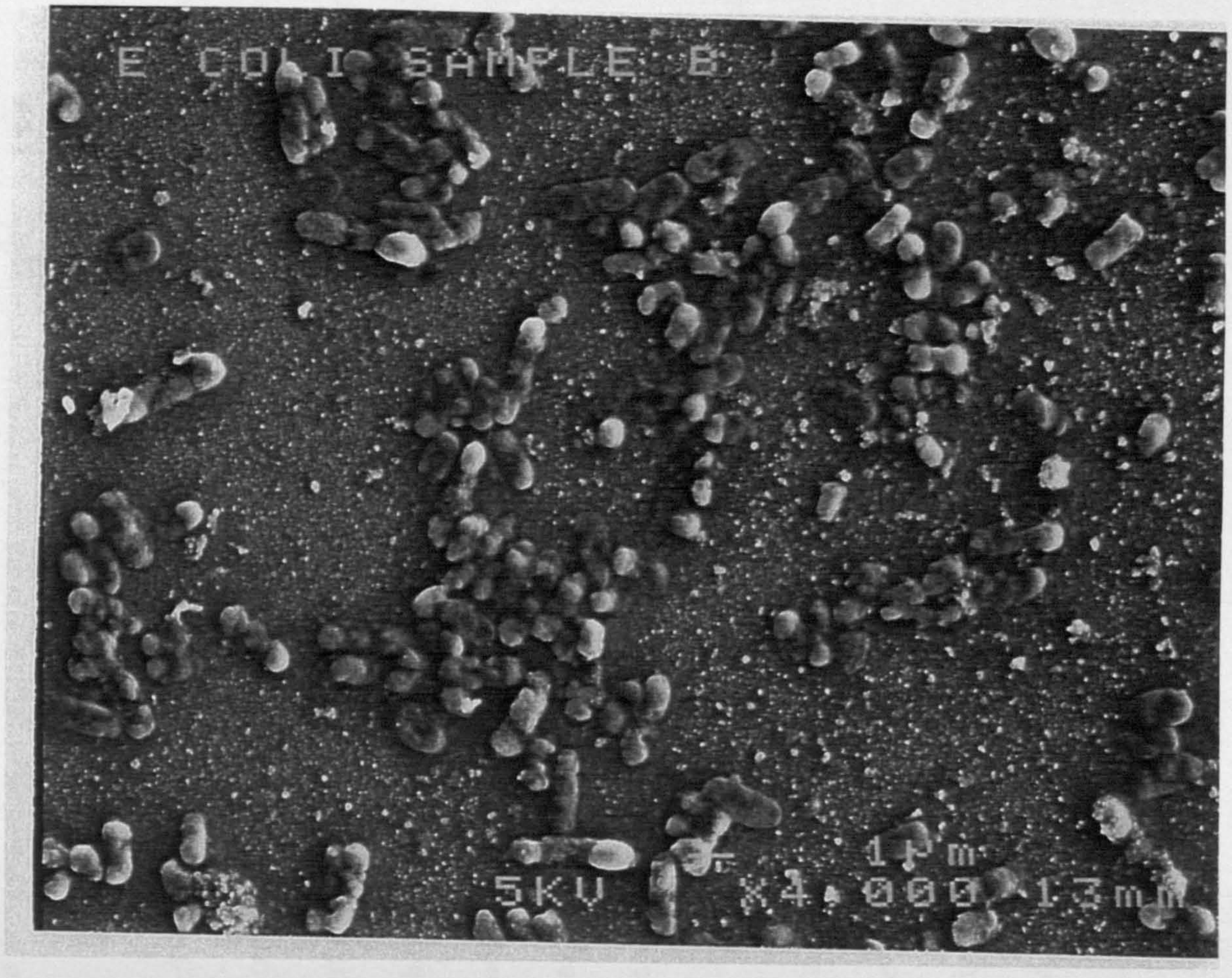

E COLI SAMPLE B
10μm
5KV X2,000 13mm

Figure 5.16 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* (ampicillin-resistant culture) grown (14 days) on an autoclaved glass slide.


E COLI SAMPLE B
5KV
1μm
X4,000 13mm

from culture containing ampicillin; in these electron micrographs, the formation of unbroken chains of cells can clearly be seen. As has already been mentioned, filamentation was also seen when the porous silicon wafers were sterilised by UV light (Figs. 5.13 and 5.14). This confirms that the observed pleomorphism was not due to the presence of microorganisms (as contaminants), other than *E. coli*.

Filamentation also occurred when *E. coli* grew on (a) UV-sterilised titanium surface (Fig. 5.17) and (b) on UV-sterilised plastic surface (Fig. 5.18). These results show that pleomorphism was not induced by silicon or titanium, but occurred on all surfaces studied, independent of their physical nature, or chemical composition. Filamentation was also observed when a wild type *E. coli*, grown on ampicillin-free medium, was starved on sterile glass slides for 14 days (Fig. 5.19); results which show that filamentation is not dependent upon growth on ampicillin-containing medium, nor the presence of the ampicillin resistance genes.

The original aim of the work reported in this Chapter was to determine if fungi and bacteria bring about the corrosion of silicon (bulk and porous) when growing in the absence of nutrients. This work is relevant to the possible biodeterioration of silicon chips and silicon materials used in medicine. Such biocorrosion of silicon was not observed. However, during these studies we noted that when growing under starvation conditions, *E. coli* exhibited diverse and extreme pleomorphism when grown on pure silicon wafers, glass and titanium. It therefore appears to be a function of starvation growth on surface rather than a phenomena, strictly induced by growth on silicon. This observation extends the relevance of the observed phenomena,

Figure 5.17 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* grown (14 days) on (UV-sterilised) a titanium wafer.

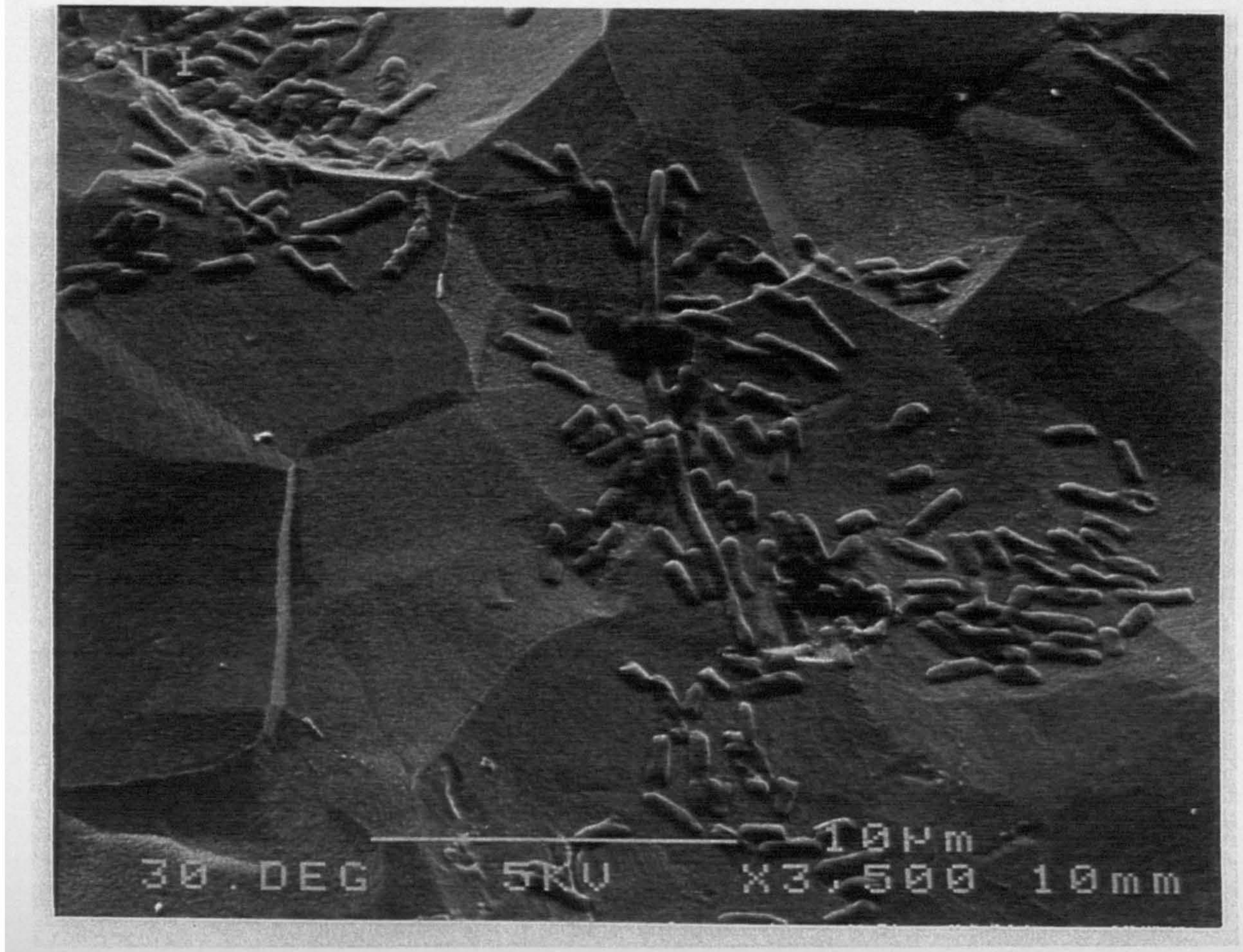
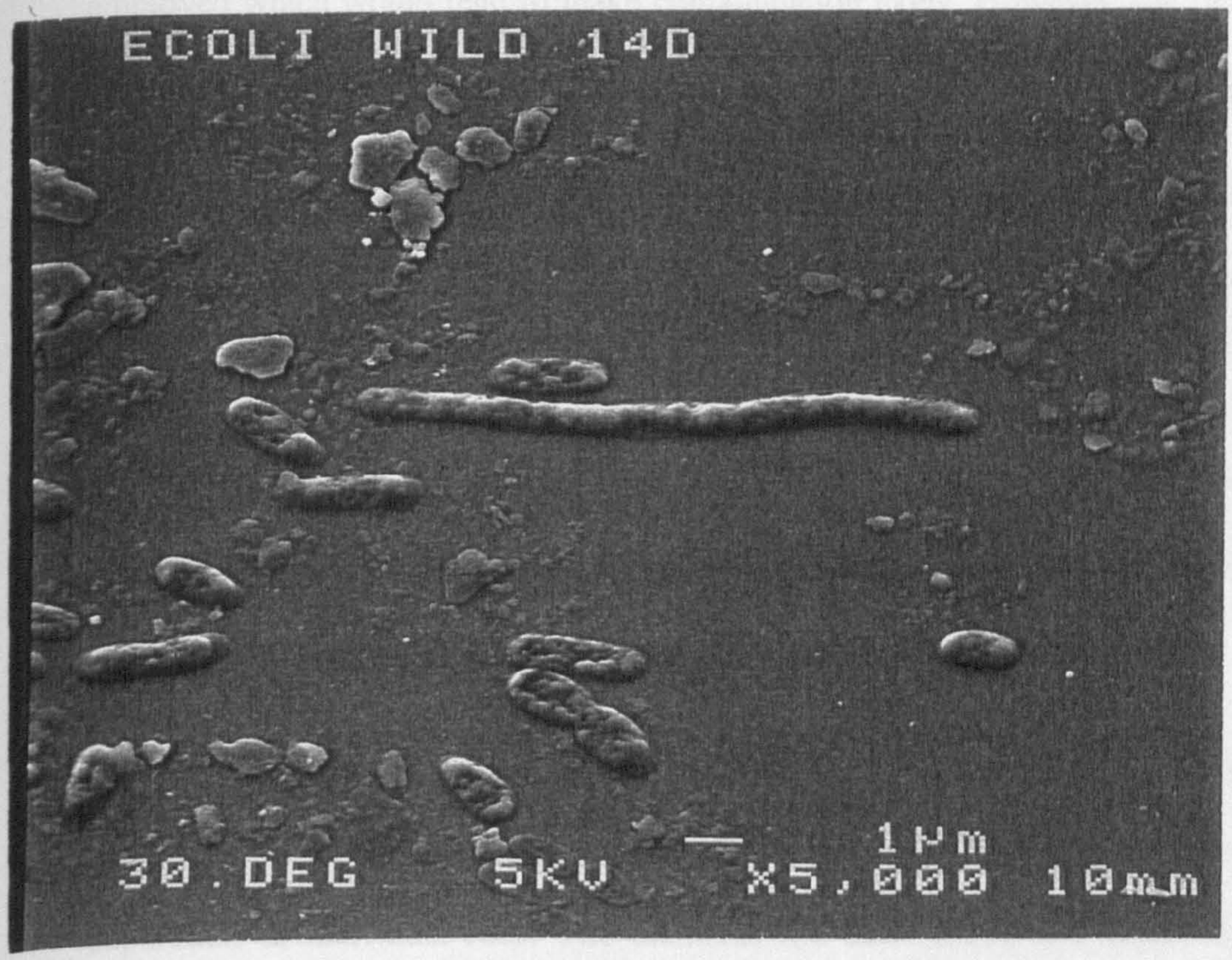

10μm
30.DEG 5KV X3,500 10mm

Figure 5.18 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* grown (14 days) on (UV-sterilised) a plastic wafer.


PL4 BC01
10μm
30° DEG 5KV X3,500 10mm

Figure 5.19 Scanning electron micrograph showing oligotrophic growth of *Escherichia coli* (wild type) grown (14 days) on an autoclaved glass slide.


ECOLI WILD 140
1μm
30.DEG 5KV X5,000 10mm

E. coli will undergo extreme morphological variation when growing, under low nutrient conditions, on most environmental surfaces.

As with other observations of extreme bacterial pleomorphism the results presented here are open to the criticism that the filamentous, and other unusual morphological forms seen mainly represent contamination by microorganisms other than *E. coli*. This is a particularly pertinent criticism because of the long incubation periods (14 days) employed which would tend to make contamination likely. However, the observations appear to represent true morphological variation in *E. coli* for the following reasons :

1) The original culture was an antibiotic-resistance strain, grown on antibiotic-containing media. As a result, it is unlikely that the original culture was contaminated with other, morphologically distinct, bacteria.

2) Sterile technique was maintained throughout the experiment and the fact that the organism was grown in ultra-pure water, rather than a nutrient-rich medium would help select against those organisms requiring large amounts of nutrients.

3) It can be clearly seen from the above electron micrographs that the morphological variation arise from the inoculum. Thus filamentous forms clearly arise from the lack of separation of individual rods. The filaments are also clearly not fungal and do not exhibit spore-forming structures or spores. Such visual indication of the lack of contamination although not apparently "analytical" should not be lightly dismissed.

Filamentation in *E. coli* is generally thought to mediated by the so-called SOS response (Donachie and Robinson, 1987), and has generally been observed in mutants, or in cultures that have in some way been stressed. Damage to DNA via a variety of chemical and physical insults is known to inhibit septation. The non-septated filaments

formed as a result of continued cell growth persist until the DNA damage is repaired; normal division and growth then resumes.

Pleomorphism, including filamentation, in bacteria that have been isolated directly from the body and pleomorphic bacteria have been implicated in pathogenicity and even in cancer aetiology (Wainwright, 1997b). The present observations on the ability of *E. coli* to form a variety of morphological shapes, when growing on surfaces under starvation conditions, is relevant to the survival of this bacterium during growth on glass, plastic and other low nutrient surfaces, for example on hospital walls and the surface of unsterilised equipment. They are also relevant to the growth of *E. coli* in non-sterile water and other low nutrient-liquid environments; it would obviously be of interest to investigate environmental samples, including biofilms, for the presence of pleomorphic forms of *E. coli* and other bacteria. Although pathogens growing in the body are unlikely to be exposed to extreme low-nutrient conditions, such pleomorphism may also be directly relevant to the survival and pathogenesis of *E. coli* when growing on silicon materials, bioactive glass, or plastics following their implantation into the body. Clearly, the growth of *E. coli* as filaments, rather than rod-shaped cells, is likely to have implications in relation to the growth and pathogenicity of this organism.

Suggestions for Future Work

The microbiology of silicon clearly has not received the attention it deserves, and as a result there is relatively little in the way of literature available on the interactions between this element and bacteria and fungi. This lack of literature, while allowing for interesting new observations on silicon microbiology to be uncovered in the work reported here, has conversely made it difficult to relate this work to previous studies. My attempt here to study a wide an area of silicon microbiology as possible has, by necessity, meant that some novel findings have not been studied in as much detail as would have been desirable. This thesis can be regarded therefore as first step in a better understanding of the microbiology of silicon. There is probably a life-times research work remaining to be done on the interactions between silicon and microorganisms and this thesis, by necessity of the time limits imposed, is only a brief snapshot of what might be discovered about silicon microbiology in the future.

Another problem that arose in this work relates to difficulties in explaining how silicon interacted with processes, such as fungal S-oxidation, simply because there is a lack of clear biochemical understanding of such processes; clearly a full understanding of how silicon effects these process necessarily requires a better understanding of the process being influenced.

Two areas of future work of particular importance arise from the work presented here:

1) The question of (a) what causes the observed silicon-based stimulation of microbial growth under oligotrophic conditions and (b) do these results indicate the existence of

a silicon-based form of autotrophy, i.e. do microorganisms gain energy from some form of silicon transformation?. This subject is one of considerable importance and controversy. As has been mentioned, in evolutionary terms it would be surprising if living organisms had not developed some means of using silicon as an energy source, simply because there is so much of it available on the planet. However, the thermodynamics of silicon chemistry would make such energy gain difficult; some would say impossible. While some workers have claimed to have demonstrated silicon-based autotrophy, it is extremely difficult to prove that silicon is the autotrophic source, simply because microorganisms are so efficient at scavenging nutrients even when they are apparently non-existent. It is difficult to see therefore how silicon-based autotrophy could be unequivocally demonstrated.

2) The serendipitous observation of extreme pleomorphism in *E. coli* is potentially of considerable significance and needs to be studied in more detail. From the historical literature, is it is clear that extreme pleomorphism is exhibited by a wide range of bacteria, including many pathogens. By developing unusual morphologies, particularly filaments, microorganisms are likely to be able to invade infected tissues in unusual and possible more efficient ways than are so-called "normal forms". For example, the filamentous forms of *E. coli* observed here would probably be more efficient in penetrating the alveoli of the lung than would for example, the rod-shaped form of this bacterium. Clearly extreme pleomorphism in bacteria is a potentially important phenomenon and needs to be studied in far greater detail.

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Publications Resulting from this Thesis

1) Wainwright, Al-Wajeeh, K. and Grayston, S. J. (1997). Effect of silicic acid and other silicon compounds on fungal growth in oligotrophic and nutrient-rich media. *Mycol. Res.* **101**, 933-938.

2) Wainwright, M., Canham, L. T., Al- Wajeeh, K. and Reeves, C. L. (2000). Pleomorphism (including filamentation) in *Escherichia coli* grown under starvation conditions. *J. Appl. Microbiol.* (Submitted).

7.1 Appendix

7.1.1 Media and solutions used for culturing microorganisms

Several growth medium and solution were used in this study, as either solid agar media or broth.

Medium 1: **Czapek Dox agar (Oxoid)**

Na NO_3	2.00 g
KCl	0.50 g
Magnesium glycerophosphate	0.50 g
Fe $SO_4(H_2O)$	0.01 g
K_2 SO_4	0.35 g
Sucrose	30.0 g
Agar (Oxoid No. 3)	12.0g
Distilled water	1.0 litre

pH adjusted to 6.8

A proprietary formulation (Oxoid) of Czapek Dox agar was routinely used. It was prepared by suspending 45.4 g of the powder in a litre of distilled water. The medium was then dissolved and sterilized by autoclaving at 15 psi for 15 min.

Medium 2: **Czapek Dox liquid medium**

Na NO_3	2.00 g

KCl	0.50 g
Magnesium glycerophosphate	0.50 g
$FeSO_4(H_2O)$	0.01 g
K_2SO_4	0.35 g
Sucrose	30.0 g
Distilled water	1.0 litre

pH adjusted to 6.8

A proprietary formulation (Oxoid) of Czapek Dox agar was routinely used. It was prepared by suspending 33.4 g of the powder in one litre of distilled water. The medium was then dissolved and sterilized by autoclaving at 15 psi for 15 min.

Medium 3: **Glucose nitrate agar**

This medium was used for studying antibiotic production by the *Streptomyces* isolates and had the following composition:

Glucose	20.0 g
KNO_3	1.0 g
$K_2 HPO_4$	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4(H_2O)$	0.01 g

Bacto. Agar (Difco)	20.0 g
Distilled water	1000 ml

pH 7.2

Medium 4: **Date syrup medium**

This medium was used for growing actinomycetes and for investigation of their morphological and cultural characteristics. It had the following composition:

Date syrup	20.0 g
KNO_3	1.0 g
$K_2 HPO_4$	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4(H_2O)$	0.01 g
*Bacto. Agar (Difco)	20.0 g
Tap water	1000 ml

pH 7.2

* Deleted when broth medium was used.

Medium 5: **Mueller - Hinton agar (Oxoid)**

This medium was used for the cultivation of *Staphylococcus aureus*.

Beef hydrolysate infusion	
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml

pH 7.2

Medium 6: **Plate count agar (Oxoid)**

This medium was used for the enumeration of viable organisms.

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0g
Agar	9.0 g
Distilled water	1000 ml

pH 7.0 ± 0.2

Medium 7: **Dunaliella growth medium**

This medium for the growth of halotolerant *Dunaliella* algae.

Stock solution:			**Vol. of solution required**
Magnesium sulphate	2.4M	(591.6g l^{-1})	10ml l^{-1}

Magnesium chloride	**2M**	**(406.6g l^{-1})**	**10ml l^{-1}**
Calcium chloride	**1M**	**(147g l^{-1})**	**10ml l^{-1}**
Sodium nitrate	**4M**	**(340g l^{-1})**	**1.25ml l^{-1}**
Sodium sulphate	**0.5M**	**(71g l^{-1})**	**48ml l^{-1}**
Sodium dihydrogen orthophosphate	**0.1M**	**(12g l^{-1})**	**1ml l^{-1}**
Potassium chloride	**4M**	**(298.4g l^{-1})**	**2.5ml l^{-1}**
Tris-HCL pH 7.6	**1M**	**(121.1g l^{-1})**	**20ml l^{-1}**
Ferric EDTA pH 7.6	**1.5M**	**(0.055g/100ml)**	**1ml l^{-1}**
Sodium chloride	**1.5M**	**(87.66g l^{-1})**	

<u>Supplements (made up in 400ml distilled water):</u>			**1ml l^{-1}**
Boric acid	185mM	(4.576g)	
Manganese chloride	7mM	(0.554g)	
Zinc chloride	0.8mM	(0.043g)	
Cobalt chloride	0.02mM	(4ml of 2mM)	
Copper chloride	0.0002mM	(0.4ml of 0.2mM)	

pH 7.8

<u>Medium 8: Nutrient broth (Oxoid)</u>

Lab-lemco (Oxoid L29)	**1.0 g**

Yeast extract (Oxoid L20)	2.0 g
Peptone (Oxoid L37)	5.0 g
Sodium chloride	5.0 g
***Bacto. Agar (Difco)**	20.0 g
Distilled water	1000 ml

pH 7.4

* Deleted when broth medium was used

A general purpose fluid medium for cultivation of microorganisms that are not exacting in their nutritional requirements.

Medium 9: **Yeast extract peptone dextrose (YEPD)**

Glucose	20g/l
Peptone	20g/l
Yeast extract	10g/l
Oxoid agar No.3	20g/l

pH 6.8

Medium 10: **Silica gel medium (Parkinson *et al.*, 1989)**

A- Salts solution

$KH_2 HPO_4$	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4 (H_2O)$	0.01 g
pdw*	1000 ml

* Purified distilled water :

Pdw was obtained by passing distilled water through a " Milli Q " filtration system (Millipore Corp.)

B- Orthophosphoric acid.

20 ml of orthophosphoric acid in 100 ml of pdw.

C- Potassium silicate

8 g of KOH in 100 ml pdw then added 10 g of silicic acid.

Procedure of preparation medium

Potassium silicate (10 ml) was mixed with 10 ml of the salt solution and 2 ml of Orthophosphoric acid, immediately poured into Petri dishes. This amount of medium is sufficient for one Petri dishe, and the gel sets in about 15 minute. The plates were then left overnight when any water of syneresis was poured off. All constituents were autoclaved at 120^0C for 20 min.

Medium 11: **L-broth**

Very rich media supports growth of a wide spectrum of microorganisms.

Ingredients per litre of distilled water:

Tryptone	10g
Yeast extract	5g
Sodium chloride	10g

7.1.2 Buffer solution

a) Sorensen's buffer ($Na_2 HPO_4$ and $KH_2 PO_4$)

b) Tris (hydroxymethyl) methylamine buffer $NH_2C(CH_2OH)_3$

c) Phosphate buffer ($NaH_2 PO_4$ and $Na_2 HPO_4$)

Effect of silicic acid and other silicon compounds on fungal growth in oligotrophic and nutrient-rich media

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Mycelium grew from a spore-mycelial inoculum of *Aspergillus oryzae* added to ultra-pure water (upw) containing silicon compounds, but did not grow in upw alone. Growth of other fungi also occurred in upw only when silicon compounds were added. Increased growth of *A. oryzae*, and other fungi, also followed the addition of silicic acid and other silicon compounds to Czapek Dox. *Aspergillus oryzae* solubilized silicon compounds in both upw and nutrient-rich media. Although interactions between microorganisms and silicon have been generally neglected, the results show that silicon compounds can increase fungal growth under both oligotrophic and nutrient-rich conditions.

Except for studies on silicon accumulation by diatoms, the microbial metabolism of silicon has been largely ignored. Fungi and bacteria can solubilize insoluble silicates, a process which may be important in the biological weathering of rocks (Duff & Webley, 1963). Silicon compounds also increase bacterial growth and have been implicated in aggravating tubercular infections of the lung in patients suffering from silicosis (Price, 1932). It has also been suggested, although not conclusively proven, that bacteria use silicon-based autotrophy as a source of energy to support CO_2 fixation (Bigger & Nelson, 1943; Chakrabarty *et al.*, 1988; Das *et al.*, 1992).

The ability of fungi to grow on nutrient-free silica gel is now well-established (Wainwright, 1993). While CO_2 fixation has been implicated in growth under these conditions, it is generally believed that fungi grow oligotrophically by using nutrients adsorbed by the silica gel from the atmosphere (Parkinson *et al.*, 1990, 1991). It is possible, however, that silica gel itself increases hyphal growth or stimulates fungal spore germination.

The aim of the work reported here was to determine the effect of silicon compounds on fungi when growing in: (i) upw under conditions in which stringent efforts were made to exclude nutrients and (ii) nutrient-rich Czapek Dox media. While the effects of silicic acid on the growth of *A. oryzae* is emphasized, the effect of a range of silicon compounds on the growth of other fungi was also investigated under both oligotrophic and nutrient-rich conditions.

MATERIALS AND METHODS

Effect of silicic acid on growth of A. oryzae *under oligotrophic growth conditions*

Aspergillus oryzae (Ahlb.) Cohn was obtained from the Sheffield University Animal and Plant Sciences Culture Collection. The fungus was subcultured onto Czapek Dox agar (Oxoid), and incubated at 25 °C for 7 d. Mycelium plus spores were carefully removed using a sterile inoculating needle to avoid the transfer of any of the underlying nutrient-rich medium. Small amounts of this inoculum were then transferred to ultra-pure water (upw, 30 ml) in plastic Petri dishes. The upw was obtained using a Millipore RO-4 water filter system and was sterilized by autoclaving at 120° for 20 min. Although the inoculum size was not strictly controlled, approximately equal amounts of mycelium were transferred on each occasion. Inoculated plates were then amended with silicic acid (0·3 g; Sigma Chemical Company, St Louis). Control plates were not supplemented. Three control plates and three plates containing silicic acid were inoculated and the experiment was repeated four times, resulting in a total of 12 replicates. All plates were incubated at 25° for 14 d.

In a separate set of experiments the above protocol was repeated using silicic acid which had been heated in a muffle furnace to remove organic contaminants. Silicic acid was placed in aluminium containers loosely covered with aluminium foil. The cases were then heated at 400° for 2·5 h and allowed to cool to room temperature inside the furnace before transfer to upw.

The above experiments were also repeated using silicic acid which had been heat-treated and then acid-washed to remove inorganic contaminants. After heat treatment, the powder was washed three times with upw for 30 min, followed by two washes with HCl (0·1 M) for 1 h. The powder was then finally washed three times for 30 min in upw and then transferred to upw in Petri dishes.

Effect of silicic acid on growth of various fungi under oligotrophic conditions

The above experiments were repeated using the following

fungi (obtained from the same collection as *A. oryzae*): *Aspergillus repens* de Bary, *Aspergillus niger* Tiegh., *Fusarium oxysporum* Schltdl., *Penicillium chrysogenum* Thom, *Penicillium janthinellum* Biourge. An asporogenous mutant of *Neurospora crassa* Shear & B. O. Dodge, was also included (culture number 3263, Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City).

Effect of silicon compounds on growth of A. oryzae *and other fungi under oligotrophic conditions*

The above experiments were repeated with *A. oryzae* and the fungi listed in the previous section. The following silicon compounds (Sigma) were added to the medium (0·3 g): calcium silicate, colloidal silica, silicon nitride, sodium silicate and hydrated magnesium silicate (perfume-free talc, from Boots Pharmaceuticals, Nottingham).

Effect of silicic acid on growth of A. oryzae *in nutrient-rich growth conditions*

Aspergillus oryzae was grown on Czapek Dox agar (Oxoid) for 10 d at 25°. Discs (4 mm) were then cut from the leading edge of the colonies using a flame-sterilized cork borer. These were transferred (1 disc per flask) to unbuffered Czapek Dox liquid medium (Oxoid, 100 ml in a 250 ml Erlenmeyer flask), amended with 0·5, 1·0, 1·5 and or 2·0 g of silicic acid. Controls lacking silicic acid, and containing silicic acid but not fungus, were also included. All treatments were incubated for 7 d in triplicate on an orbital shaker (100 throws min^{-1}) at 25°. The contents of the flasks were then filtered through Whatman No. 1 filter paper and the dry weight of the mycelium was determined after drying to constant weight at 40°. Filtrate pH was determined immediately after filtration using a glass electrode. The soluble silicon content of the medium was determined colorimetrically by adding the following to 1 ml of the filtrate: ammonium molybdate (2 ml, 10% v/v); ascorbic acid (2 ml, 5% w/v); oxalic acid (1 ml, 10% w/v); and HCl (5 ml, 1:1 dilution of conc. HCl). After 15 min at room temperature, without shaking, the absorption of the blue colour was measured spectrophotometrically at 600 nm. The concentration of soluble silicon (as SiO_2) in the filtrate was then determined by reference to a standard curve prepared using EIL standard silicon solution (BDH Chemicals, Poole, Dorset). When acid-washed silicic acid was added to nutrient-rich media, the washing procedure described for the oligotrophy experiments was used. The results were analysed for standard error, and significant difference using Student's *t*-test.

Effect of silicic acid on the growth of fungi in Czapek Dox medium

Unbuffered Czapek Dox medium was directly amended with untreated silicic acid (1·5% w/v) and inoculated with one of the following fungi: *Aspergillus niger, A. oryzae, A. repens, P. janthinellum* and *F. oxysporum*. Flasks were set up in triplicate and incubated as above.

Effect of silicon compounds on growth of A. oryzae *in Czapek Dox medium*

Unbuffered Czapek Dox medium was directly amended with one of the silicon compounds listed previously (1·5% w/v) and inoculated with *A. oryzae*. The silicon compounds were not heat-treated or washed. Flasks were set up in triplicate and incubated as above.

Effect of silicic acid contained in dialysis tubing on growth of A. oryzae *under nutrient rich conditions*

The experiment described above in the section growth in nutrient rich conditions was repeated except that the silicic acid was added to unbuffered Czapek Dox medium in a dialysis tubing envelope. This was made by cutting dialysis tubing (3 × 6 cm), inserting the individual silica compounds, and then folding and sealing the ends of the parcel using metal staples. Dialysis tubing envelopes lacking silica compounds were incubated without fungal inoculum as controls. Medium pH, fungal dry weight and soluble silicon concentration were determined as described above.

RESULTS AND DISCUSSION

Effect of silicic acid on growth of A. oryzae *under oligotrophic conditions*

Aspergillus oryzae consistently failed to grow from the myceliaspore inoculum when added to upw. However, visible mycelial growth occurred in the presence of silicic acid (Fig. 1). Growth occurred in all twelve plates containing silicic acid, but in none of the control plates containing only upw. Particles of silicic acid could clearly be seen attached to the surface of *A. oryzae*. Spot tests, using the reagents for colorimetric determination of silicon, also showed that *A. oryzae* solubilized silicic acid in upw. Growth also occurred when acid washed silicic acid was used, showing that the effect was not due to contamination by inorganic nutrients.

Due to limitations of analytical equipment available to us, the biomass, or protein content, of the small amounts of mycelium produced could not be determined, so our results are limited to observation.

Effect of silicic acid on growth of various fungi under oligotrophic conditions

While *Aspergillus repens, A. niger, F. oxysporum,* and *P. janthinellum,* did not grow in upw alone, all grew (12 replicates of each fungus) when silicic acid (untreated, heat-treated, and heat-treated plus acid-washed) was added to upw.

Effect of silicon compounds on growth of A. oryzae *under oligotrophic conditions*

While *A. oryzae* and the fungi listed in oligotrophic conditions failed to grow in upw alone, all grew in upw (all 12 replicates in each case) after the addition of one of the following silicon compounds: calcium silicate; colloidal silica; talc (hydrated

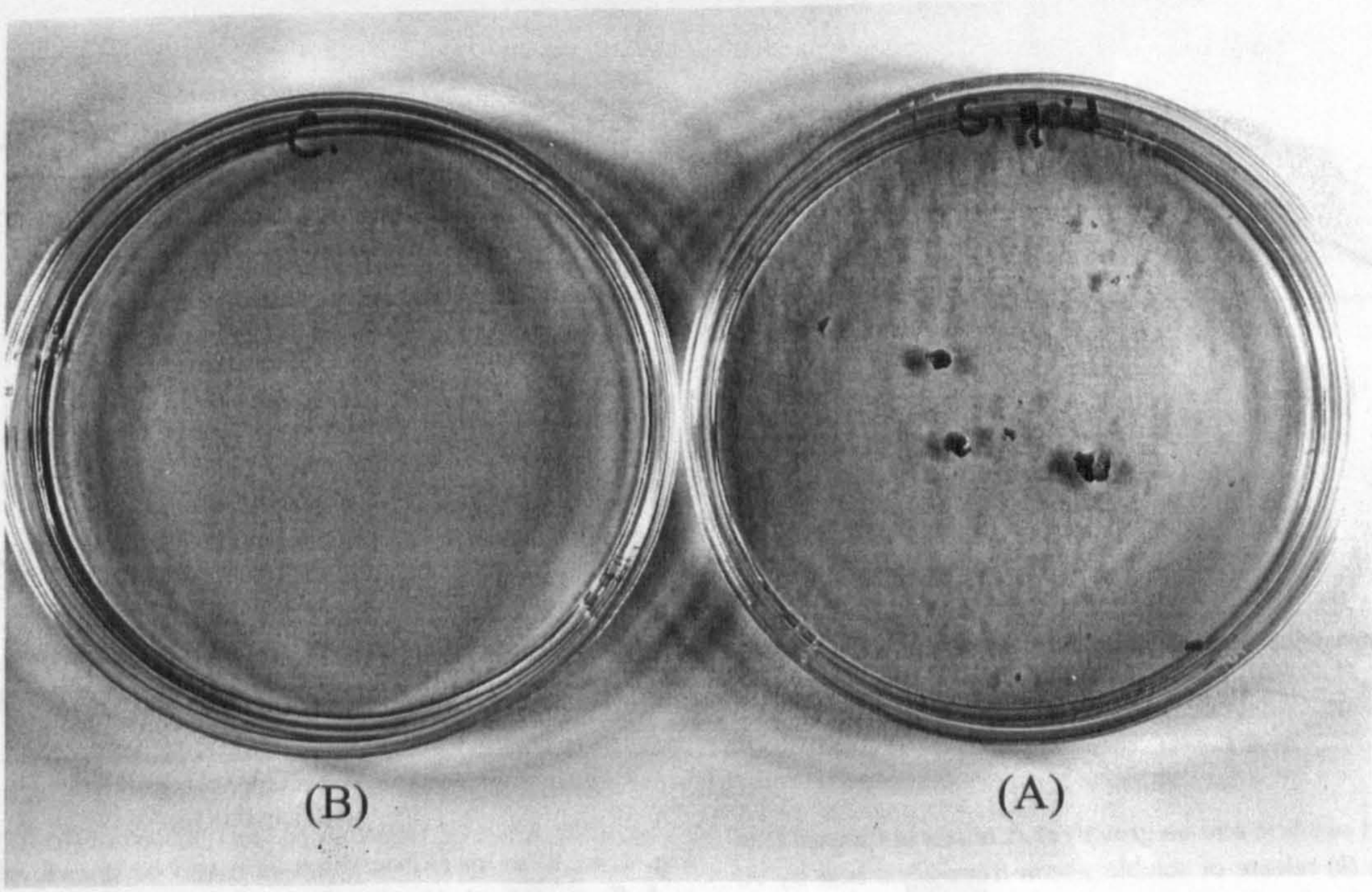

Fig. 1. Growth of *A. oryzae* in upw containing silicic acid (A), and absence of growth in upw alone (B).

magnesium silicate) and rock potash (untreated, heat-treated, and heat-treated plus acid-washed).

The above observations show that fungi which cannot grow from a spore-mycelium inoculum in upw, grow following the addition of silicic acid and other silicon compounds. These observations can be explained as follows. First, by overriding some fungistatic agent, the silicon compounds may have stimulated fungal spore germination. However, when mycelium of an asporogenous mutant of *N. crassa* was used, growth also occurred in upw containing silicic acid (but not in its absence). This shows that silicic acid can promote mycelial growth alone.

A second possibility is that, during storage, silicic acid adsorbed nutrients which then provide the fungus with the necessary growth substrates absent from upw. This is unlikely where heat-treated silicic acid was used, since all organic substrates would have been removed following heating to 400°. Fungal growth continued to occur in the presence of silicic acid and the other silicon compounds following heat treatment and acid-washing. Therefore, the effect of silicon compounds on fungal growth was not due to the presence of contaminating nutrients, including trace elements.

A third possibility is that silicon compounds, which are efficient at adsorbing gases and volatiles, removed combined carbon and nitrogen from the atmosphere, which then acted as nutrient sources for fungal growth.

Finally, the fungi may have used the silicon compounds as an energy source, enabling them to fix CO_2 from the atmosphere. The possibility that fungi can grow autotrophically under oligotrophic conditions (using energy obtained from hydrogen oxidation) was suggested by Mirocha & Devay (1971). Bigger & Nelson (1941, 1943) also suggested that silicon compounds might adsorb ammonia and CO_2 from the atmosphere, thereby allowing bacteria to fix CO_2, using energy obtained from the oxidation of ammonium. Chakrabarty *et al.* (1988) have similarly suggested, although not conclusively proved, that certain bacteria can grow as silicon autotrophs. Although it is generally thought that silicon compounds are biologically unreactive, Allison (1968) stated that there is no theoretical reason why the reaction of Si-Si-Si with oxygen or oxygen compounds could not act as an energy-yielding reaction. However, the possibility that fungi and other microorganisms might use silicon-based autotrophy clearly remains speculative.

Whatever the mechanism involved, it is clear that silicic acid and other silicon-containing compounds, promote fungal growth under oligotrophic conditions, a fact which helps explain the ability of fungi to grow on nutrient-free silica gel. Silicon is not, however, essential for such oligotrophic growth since fungi will also grow on nutrient-free pluronic polyol, a gelling agent which lacks silicon (Wainwright & Grayston, 1988).

Effect of silicic acid on growth of A. oryzae *in nutrient-rich media*

The addition of silicic acid to Czapek Dox liquid medium led to an increase in growth (biomass) of *A. oryzae* over the 7 d incubation period (Fig. 2*a*); with biomass production increasing with increasing amounts of added silicic acid. The biomass increase was associated with increases in the concentration of soluble silicon, which also increased with increasing weight of added silicic acid (Fig. 2*b*). Negligible amounts of silicic acid were solubilised in the absence of fungal inoculum. Since, in these experiments, some of the silicic acid was adsorbed onto the surface of the growing mycelium, the measured biomass was larger than the real biomass. However, not all of the silicic acid was removed from

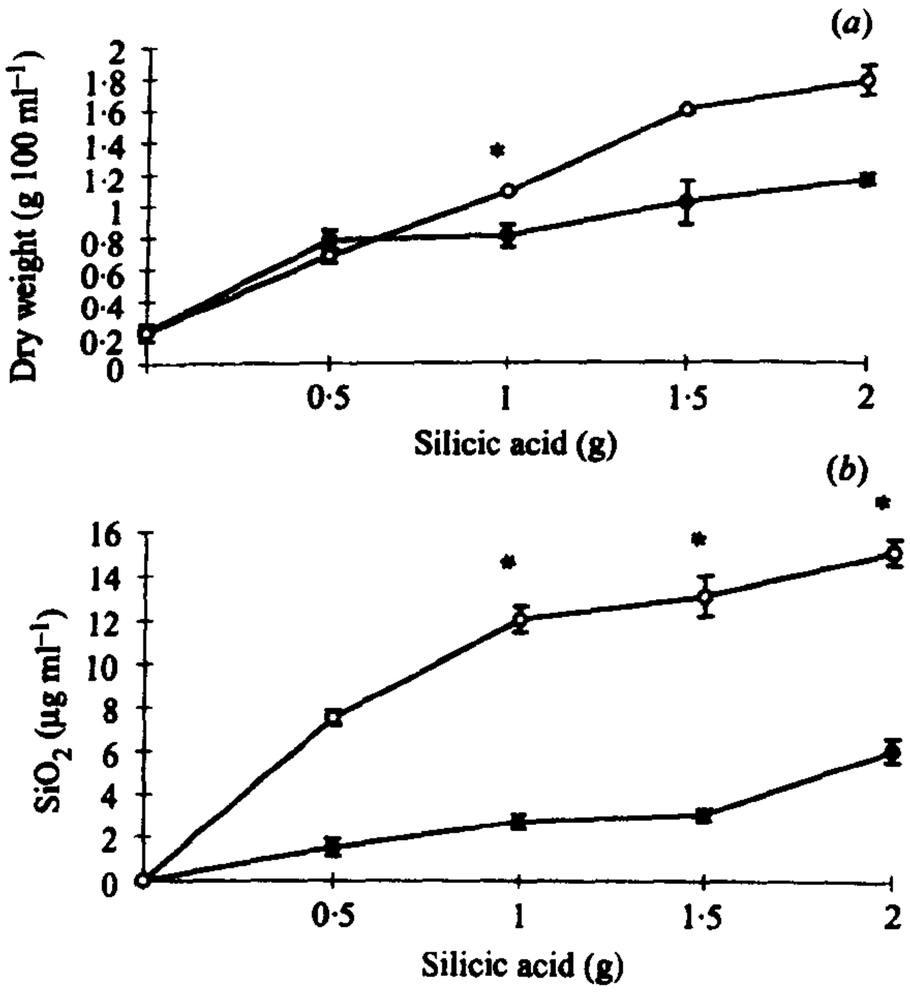

Fig. 2. (*a*) Effect of silicic acid on growth of *A. oryzae* in Czapek Dox liquid medium; (*b*) release of soluble silicon from silicic acid by *A. oryzae*, ○——○ silicic acid added directly to medium; ●——● silicic acid added to medium in dialysis tube. Means of triplicates, ± standard error. *Significant difference, $P < 0{\cdot}05$.

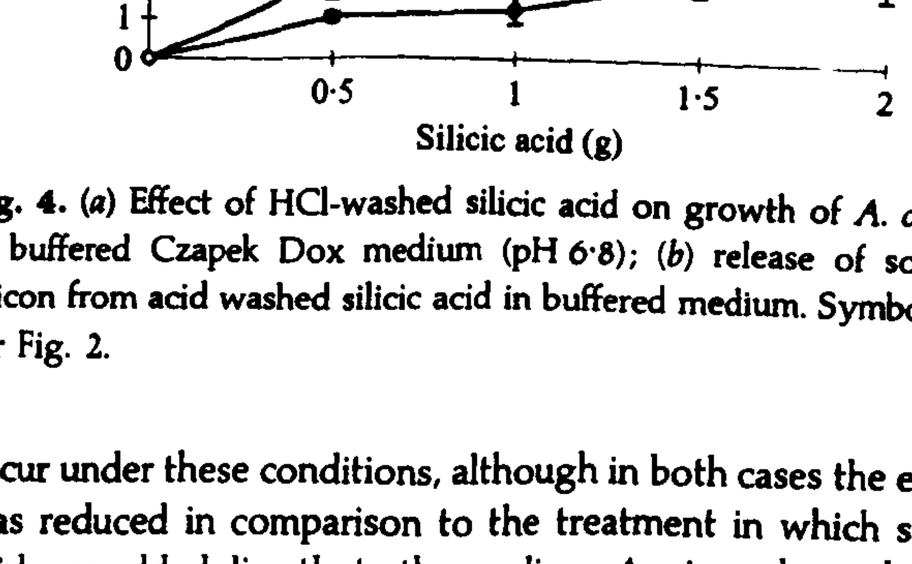

Fig. 4. (*a*) Effect of HCl-washed silicic acid on growth of *A. oryzae* in buffered Czapek Dox medium (pH 6·8); (*b*) release of soluble silicon from acid washed silicic acid in buffered medium. Symbols as for Fig. 2.

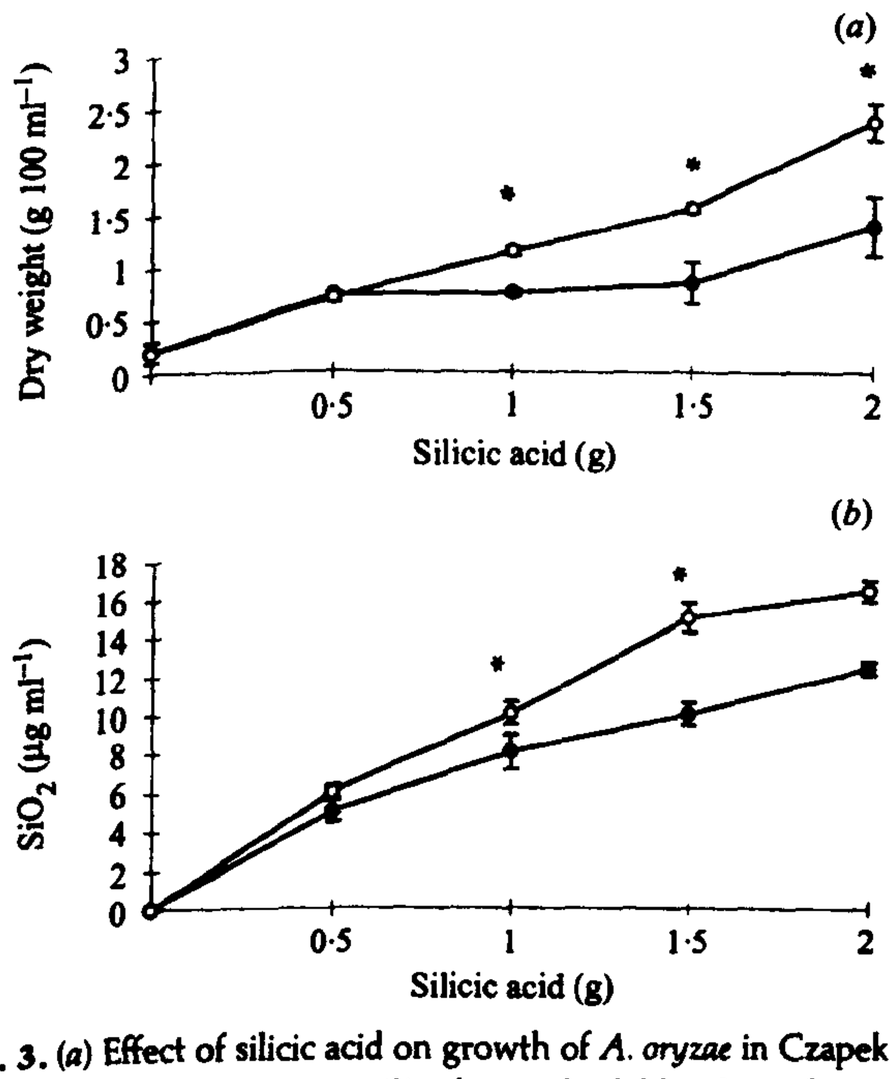

Fig. 3. (*a*) Effect of silicic acid on growth of *A. oryzae* in Czapek Dox medium buffered to pH 6·8; (*b*) release of soluble silicon from silicic acid by *A. oryzae*. Symbols as for Fig. 2.

solution by the *A. oryzae*, even when the lowest amount of silicic acid (0·5% w/v) was added. This suggests that the biomass increase resulting from the addition of increasing amounts of added silicic acid was not solely due to adsorption of silicic acid to the mycelium.

In order to determine if direct contact between the silicic acid and fungus was necessary for the observed growth increase, and increase in solubilization of silicon, silicic acid was added to the flasks in sealed dialysis tubing. Fig. 2 shows that an increase in biomass and soluble silicon continued to occur under these conditions, although in both cases the effect was reduced in comparison to the treatment in which silicic acid was added directly to the medium. Again, only negligible amounts of silicon solubilization occurred in the absence of added fungus. These results show that while *A. oryzae* can solubilize silicic acid, maximum solubilization requires direct contact between the fungus and the particles of silicic acid. These results also re-emphasise that the amount of soluble silicon present in the medium is related to the amount of biomass produced.

It could be argued that the increased biomass produced with increasing silicic acid concentration was due to pH effects resulting from the addition of differing amounts of silicic acid. However, when the medium was buffered to pH 6·8 (using Sørensen's buffer) the same trends seen above were observed (Fig. 3), showing that variations in the pH of the medium, resulting from the addition of silicic acid, were not responsible for the observed growth increases. A similar increase in growth of *A. oryzae* and the release of soluble silicon occurred in buffered medium (pH 6·8), when HCl-washed silicic acid was added to the medium (Fig. 4). This shows that the observed increases in growth and silicon release were not due to the growth stimulating effects of trace elements present in the silicic acid.

A range of silicon compounds stimulated the growth of *A. oryzae* when added to Czapek Dox medium (Fig. 5). However, in the case of sodium silicate, the observed increases were small and statistically insignificant. Why sodium silicate should be exceptional in this respect is not clear. However, like the other compounds it was not completely soluble in the medium, so the lack of growth stimulation was not due to the absence of insoluble particulates.

Silicic acid (untreated, added directly to the medium) also increased the growth of various fungi when growing in un-

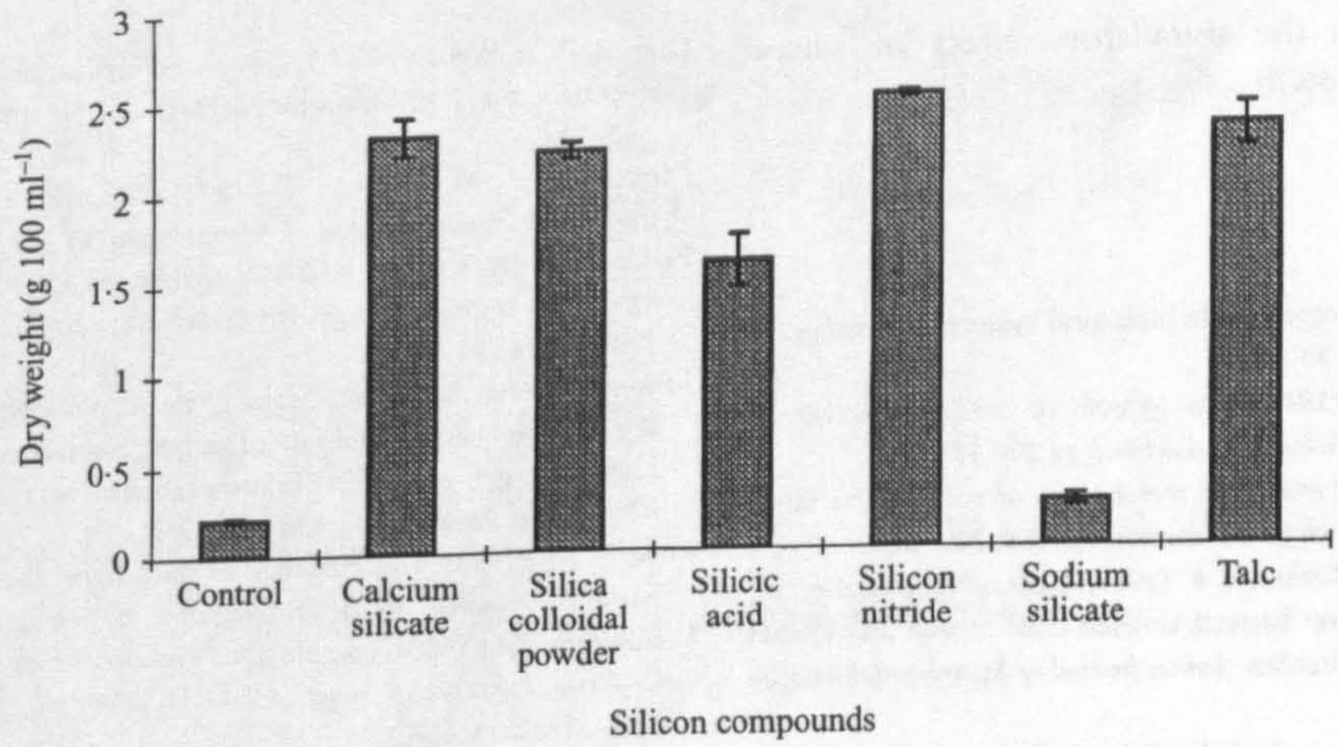

Fig. 5. Effect of various silicon compounds on growth of *A. oryzae* in unbuffered Czapek Dox medium. (All increases, except for sodium silicate, were significant, $P < 0{\cdot}05$).

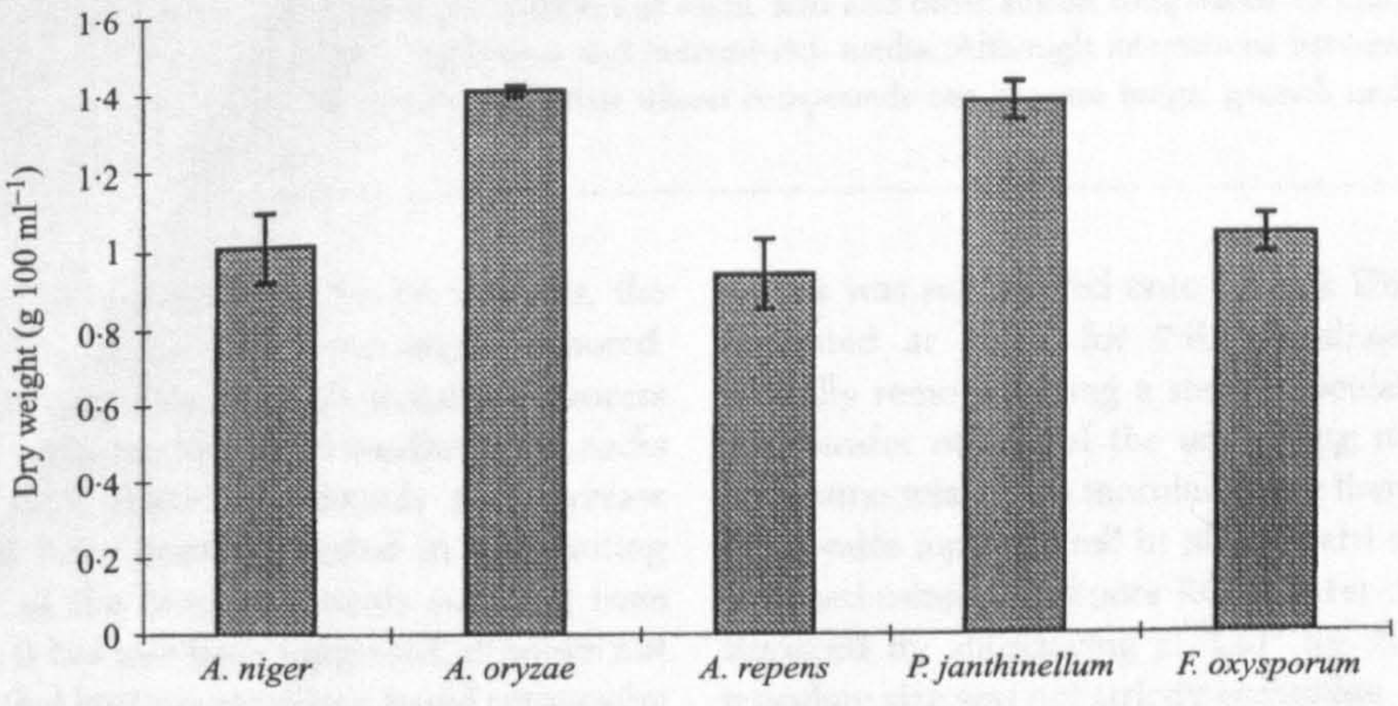

Fig. 6. Effect of silicic acid on growth of fungi in unbuffered Czapek Dox medium, expressed as increase in dry weight after subtraction of the weight of the control for each individual fungus. (All increases were significant at $P < 0{\cdot}05$).

buffered medium, (Fig. 6) so the stimulatory effect was not restricted to *A. oryzae*. (Note that some of the apparent increase in biomass shown in Figs 5 and 6 resulted from adsorption of silicon compounds by the fungi).

While the ability of silicon compounds to stimulate bacterial growth has been reported previously, this appears to be the first report on the ability of silicic acid and other silicon compounds to promote fungal growth under oligotrophic conditions, and increase growth in nutrient-rich media.

Silicon-containing compounds clearly have a stimulatory effect on fungal growth, under both oligotrophic and nutrient-rich conditions. At present we are unable to explain this growth stimulation. One possibility is that the effects are non-specific and merely due to the silicon compounds acting as a solid contact surface; similar growth increases might have resulted from the addition of any, non-silicon containing, particulate material. However, in some of the experiments where nutrient-rich media were employed, increases in the growth of *A. oryzae* occurred in Czapek Dox medium containing silicic acid when dialysis tubing was used to prevent direct contact between silicic acid and the fungus. This suggests that the observed effects on growth were due to some specific biochemical, rather than purely physical, interaction between the particles of silicic acid and the fungus.

While chemo-autotrophic growth, using energy gained from silicon metabolism, may be involved in growth under oligotrophic conditions, it cannot explain why silicon compounds increase both bacterial (Das *et al.*, 1992) and fungal growth in nutrient-rich media.

Silicon is the second most common element on Earth after oxygen and is abundant in soils. Despite the fact that silicon is generally thought to be biologically inert, it is possible that soil microorganisms will have evolved some means of metabolising this element. The presence of silicon in clays and other soil minerals may help explain why soil microorganisms, including fungi, can grow in such an apparently nutrient-poor environment by: (i) using nutrients adsorbed by silicon compounds from the soil atmosphere, or (ii) by using energy derived from silicon metabolism to fix CO_2 chemoautotrophically. *Aspergillus oryzae* is not generally regarded as a soil fungus, but we have also shown that silicon compounds can stimulate the growth of a range of fungi, including common, soil-inhabiting species like *F. oxysporum*.

Further studies are now in progress to determine the

mechanisms involved in the stimulatory effect of silicon compounds on fungal growth.

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