# THE GROWTH OF STREPTOMYCETES AND FUNGI UNDER CONTROLLED CONDITIONS ON THE SURFACE OF COATED MATERIALS

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## SUMMARY

The work presented is an investigation into some factors effecting the commercial testing of cotton textiles. Soil burial is the standard test for microbial biodeterioration of materials and evaluation of biocides used to protect them, but is extremely variable.

Pure culture techniques were employed to evaluate two factors, moisture availability and the action of Streptomycetes (particularly Streptomyces spp.), which might lead to variability.

Present methods for controlling and monitoring relative humidity in the laboratory had disadvantages, and so novel methods for producing and measuring such atmospheres were developed.

A continuous wet/dry air mixing system was designed and constructed to give a controlled humidity environment, allowing fungal and streptomycete colonisation of wheat starch and cellulose substrates to be studied. The apparatus permitted simultaneous production of seven humidities and avoided a decrease in the oxygen-carbon dioxide ratio, giving conditions more akin to those encountered during the service life of a textile.

A dual p.n.p. bismuth-telluride thermocouple hygrometer was devised capable of monitoring the relative humidities of

the air mixing system. Gravity plate airspora isolations of Streptomyces spp. were performed in conjunction with fungal isolations, to assess the potential biodeterioration threat presented by mesophilic Streptomyces spp.

Streptomyces spp. isolated were tested for amylolytic and cellulolytic ability, pH and temperature growth limits.

Moisture requirements of Streptomyces spp. were determined using three methods; air mixing, saturated salt solutions and controlled water activity agar.

Having established the limits within which Streptomyces spp. were capable of starch and cellulose degradation they were tested on starch coated cotton textiles. Sized textiles protected by one of three biocides; sodium pentachlorophenol, tributyltin oxide or salicylanilide, were evaluated under controlled conditions. Estimation of biocide efficiency and Streptomyces spp. tolerance were determined visually and by tensile strength testing of inoculated textile strips.

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C O N T E N T S

Chapter 4 Fungal airspora isolations and the effect of relative humidity on fungal colonisation of starch and cellulose substrates



Chapter 5 Isolation, assessment of growth factors and identification of Streptomyces spp.

APPENDICES

# Chapter 1

## INTRODUCTION

1.1 Introduction

1.2 Biodeterioration test work and principles

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## 1.1 Introduction

The formation of moulds and mildew is possible on most textile fibres. The two essential conditions for the growth of most micro-organisms are warmth and moisture, so that where goods are stored under cool, dry and well ventilated conditions there is little danger of microbial growth. The relative humidity of the atmosphere need not be as high for fungal growth as that required for bacteria, so different parameters exist. Whatever precautions may be taken in a storage warehouse are apt to be nullified by other factors. In the textile industry the type of size or finishing preparation applied to the fabric may act as an ideal culture medium for micro-organisms. Preparations based on starch, for example, will be hygroscopic, favouring colonisation by micro-organisms.

This thesis will consider two factors which have largely been neglected in biological test procedures used to evaluate textile decay:

a) Relative humidity or moisture content of the test environment.

b) The ability of fungi and Streptomycetes to attack preserved and unpreserved starch coated textiles,

and the interrelationship between these two factors, with respect to starch and cellulose substrates.

Before attempting, however, to elucidate on means of accurate estimation and prevention of textile decay, it is

important to put the problem in perspective. Allsopp (1973) has reviewed fully the world problem caused by textile decay, he has outlined the nature of the problem as well as given figures for economic losses incurred, which run into millions of pounds.

Starch is the primary product used in sizing and finishing in the textile industry. A detailed breakdown of the end-uses for starch is difficult to obtain, as a rough guide it would seem that in the U.K. food uses account for 30%, paper 40%, textiles 15% and other uses such as cosmetics and pharmaceuticals 15%, (Selby 1974).

In the textile industry, starch is under attack, from synthetic materials like polyvinyl alcohol, but still holds its own partly due to its competitive price. At the present time starch and starch derived products probably account for over 85% of the total usage of materials for sizing and filling in the textile industry. Although starches do not appear to have made much progress towards improving their performance in weaving man-made fibres for example, no synthetic adhesive is an adequate size for these on its own (Selby 1974).

A considerable number of experiments have been performed to quantify the effects of the physical factors in bio-deterioration including pH, temperature, water activity (R.H.) and reaction to inorganic ions; these have been summarised by Ayerst (1968a). From these physical parameters it is possible,

for example, to set theoretical safety limits for storage environments for textiles. To predict these limits, however, it is necessary to understand how particular conditions of temperature and moisture may arise and be controlled in the environment of the substrate. From this arises the need for knowledge of the interactions of physical condictions both between themselves and with other factors, such as nutrition, halophytic ability and biocide tolerance, on the growth and activity of potential biodeteriogens. However, such information is not readily available for many spoilage organisms such as actinomycetes. Many practical predictions are based on generalisations founded on tests using a limited series of test organisms, thus additional information is required on a wider spectrum of micro-organisms than have at present been investigated.

This project follows those by Malik (1970), Allsopp (1973) and Hollingsworth (1974), which have all investigated factors influencing, and organisms causing cellulose degradation. Each author has mentioned streptomycetes in connection with cellulose degradation particularly at thermophilic temperatures, and possible resistance to biocides. The sponsors of the present research project expressed an interest in determining the role of streptomycetes in textile deterioration following the results of investigations by Allsopp and Hollingsworth which they had previously helped to instigate. Thus, it was decided to elucidate the role of streptomycetes and their comparative importance with the fungi. It was initially thought that the streptomycetes might be capable of growing at low relative humidities and thus playing an extremely important role in biodeterioration.

## 1.2 Biodeterioration test work and principles

The primary requirement of a biodeterioration test is that the results should give an indication of the probable behaviour of the material when exposed in practice to adverse conditions promoting microbial decay. This process is further compounded by the requirement of commercial organisations for accelerated laboratory results. The testing of textiles falls into two major categories, soil burial or inoculation, and pure culture methods. The difficulty arises when assessing the severity of the test, soil burial being extremely thorough, whereas pure culture methods may be too specific and may not be representative of the natural deteriogens.

Hueck van der Plas (1965) has schematised biological stresses in terms of environmental, microbial population and nutritional factors.

Lloyd (1968) stated that soil methods are suitable for speedy commercial tests, imparting a high degree of biological stress to the textile. Because of the wide spectrum of micro-organisms present in soil there is every reason to assume that these species are representative of the species responsible for in service deterioration of textiles. Severe though they are, the nature and interrelationships of the biological stresses in these tests are unknown and a variety of interrelated factors, physical, chemical and biological are involved. At the present time it can not be estimated how accurate the results of these tests are compared with in service situations.

It was stated by Turner (1972) that important factors influencing the quantitative results of soil burial tests were moisture content and packing density of the soil, and that these factors have an interreaction effect. His further experiments showed that other factors remain to be identified, amongst them are; the previous history of the standard soil and the problems associated with the production of sucessive batches of soil of equal degradative activity.

Soil burial trays are frequently allowed to dry out and Barr (1973) noted that Streptomyces spp. were found to be growing on the surface of these trays. It was thought that Streptomyces spp. might be a factor influencing the result of biological tests.

The effect of different moisture contents on the growth and survival of actinomycetes in pure and in mixed cultures in sandy soils has been investigated by Williams et al (1972). It was found that the streptomycetes grew mostly in pore spaces which were humid and air-filled, and growth was reduced in water-logged pores. Thus, the packing of soil trays for biological work can effect the number of Streptomyces spp. isolated. Williams found growth of species was restrained above water tension pF 4.0, but spores of Streptomyces spp. were found to survive for long periods in dry soil, their tolerance of high moisture tensions being greater than that of their own vegetative hyphae or cells of non-sporing bacteria. He found that drying of soil before preparing dilution plates aided significantly the selective

isolation of Streptomyces. The most pronounced trend was the relative increase in the abundance of actinomycetes in soil held at suctions above pF 2.3. At pF 5.6 (equivalent to 75% R.H. at 18°C), numbers of actinomycetes exceeded those of bacteria and fungi. The great peak of fungal activity occurs at pF 1.0 where their vast numbers swamp all other microbial activity, (Griffin 1963).

Meiklejohn (1957) first demonstrated that the proportions of actinomycetes (usually streptomycetes) were increased in dry soil. Williams states that his findings and those of Meiklejohn are probably due to the resistance of Streptomyces spores at high water suctions.

Studies on the ecology of actinomycetes in soil have lead to observations by Mayfield et al (1972), on the form and growth of streptomycetes in soil. They concluded that streptomycetes exist in soil for much of their time as spores, and that periods of active growth were discontinous in space and time making their detection or evaluation by any method a problem.

A series of techniques and experiments were therefore designed to discover whether streptomycetes were capable of growing at low humidities (below 90% R.H.) and to determine the limits for the production of substrate and sporulating aerial mycelium respectively.

In pure culture methods of testing, the factors which may effect the results are numerous:-

a) Sterilization of the specimen and the method used.

b) Variability of the test organisms.

c) The presence of a carbon source in addition to the substrate being tested.

d) Type of inoculation and concentration.

e) Age of organism.

f) Nutrient suitability, pH etc.

g) Incubation temperature.

h) Interreactions between organisms.

These factors have been the subject of cooperative research by the International Biodegration Research Group (I.B.R.G.). The preliminary research work performed by the I.B.R.G. on textiles has been summarised by La Brijn and Kauffman (1971). They discuss the specific effects of the above factors and also record correlation coefficients which have been calculated for interreactions between the factors. The primary factors governing the growth and cellulolytic ability of micro-organisms and the way in which factors interrelate have been described by Malik (1970).

The above factors listed do not, however, mention the importance of a controlled known relative humidity in the test environment and the subsequent moisture content of the test materials. The moisture content of the soil burial environment was stressed by Turner (1972); also by Williams et al (1972) with respect to streptomycetes populations. Moisture determination is carried out on an overall basis and in all probability considerable local variations in

moisture will be recorded in any soil bed, giving rise to local intensification or abatement of microbial attack on materials.

The I.B.R.G. have, however, carried out preliminary experiments to determine the influence of the moisture content of cotton strips before sterilization on the growth and cellulolytic activity of a standard test organism Myrothecium verrucaria, (La Brijn and Kauffman, 1970). They made a comparison between growth evaluated visually and cellulolytic activity (determined by loss of tensile strength) of M. verrucaria on cotton strips, which had been autoclaved containing different amounts of water, - 6, 15, 60, 90 and 200% of dry weight. They stated that, "statistically significant differences were found between cellulolytic activities at different moisture contents, so that it appears that this part of the test proceedures must be more rigorously standardized in future experiments".

## 1.3 Humidity Control and Measurement

Humidity control in pure culture biological experiments is a rather difficult process which may be controlled by three main methods:-

1) Saturated salt solutions.
2) Continuous air-mixing.
3) Controlling solute concentrations of media.

The advantages and disadvantages of these techniques are fully described in chapter 2. The need for a reproducible test apparatus for routine commercial laboratory investigatory work was expressed by the project sponsors. In order to form a constant environment it was necessary to develop a novel continuous flow system based on the air-mixing principle (Smith 1965), (May 1974). In order to monitor the atmosphere produced, the methods of measurement are reviewed and a suggested improved sensing device based on dual thermocouples utilizing Peltier cooling was produced.

The new techniques were required because of six major factors:-

1) Growth over the long duration of time required in biological testing requires an adequate and stable supply of oxygen.
2) Continuous air-mixing can control the ratio between oxygen consumed and carbon dioxide evolved, thus providing a standardised environment for growth.

3) Textile samples were being used which were too large for conventional techniques.

4) Ease of handling and monitoring samples.

5) The test cotton fabrics were coated with starch, which takes up water readily, only continuous air-mixing was capable of reducing the equilibrium period sufficiently to be of use in commercial testing.

6) The test specimen would have an equal moisture content over its entire area, thus avoiding patchy decay of the sample, giving greater standardisation of results.

## 1.4. The role of Streptomyces in biodeterioration

Cotton when it is exposed to conditions of high humidity and warmth is readily attacked by certain micro-organisms. Most of the work reported in the literature deals with the higher orders of fungi. It was the aim of this thesis to try and fill in gaps with respect to the streptomycetes; the genus *Streptomyces* was selected for particular study because their role in biodeterioration had been implied by workers, but actual experimental evidence of their role, if any, was limited.

The actinomycetes are prokaryotic bacteria with elongated cells or filaments (0.5 - 2.0 µm. diam.) usually showing some degree of true branching. They are sensitive to lysozyme for the most part and to the common antibacterial agents. There is a similarity to bacterial flagella when these organelles are present in actinomycetes and the types of cell wall resemble those in bacteria. The actinomycetes are grouped together mainly on morphological grounds but they cannot be satisfactorily distinguished from coryneform genera such as *Arthrobacter* and *Corynebacterium* which also have a tendency to produce branched elements Goodfellow and Cross (1973).

The actinomycetes have been divided by Cross and Goodfellow (1973) into ten families. The family Streptomycetaceae are aerobic actinomycetes forming a

non-fragmenting substrate mycelium which may bear spores, and in most genera a well developed aerial mycelium bearing uniserial chains of arthrospores enclosed within a fibrous sheath. The family Streptomycetaceae is composed of six genera, Chainia, Elytrosporangium, Kitasatoe, Microellobosporia, Streptomyces and Streptoverticillium.

The genus Streptomyces are aerobic actinomycetes with extensive branching substrate and aerial hyphae and type 1 cell wall composition.[1] Fragmentation of substrate mycelium is rare and spores are rarely produced on the substrate hyphae. The aerial mycelium usually bears long chains of spores, these are arthrospores formed by the regular septation of a hyphae enclosed with a fibrous sheath.

The term actinomycetes has been used in the thesis to cover the entire group of organisms, Streptomycetes having been using as a general term meaning the family Streptomycetaceae. Streptomyces refers to the genus and Streptomyces spp. to specific members of the genus Streptomyces.

For sucessful rot-proofing of cotton there is a need for complete understanding of all potential causative micro-organisms and their interactions both between themselves and with their surroundings. The destructive effects of microbial attack on cellulose are well recognised but to date, information on its mechanism has been decidedly non-specific. The causative micro-

[1] Lechevalier and Lechevalier (1967)

organisms of decay particularly among the bacteria and actinomycetes have not been comprehensively recognised and explored.

The role of Streptomyces spp. has been underestimated because of difficulties of isolating in pure culture and of initial visual identification, (early growth of substrate mycelium generally being mistaken for bacterial colonisation); their slow growth rate tends to overrun the limited time scale of commercial tests. The individual contribution of the Streptomyces spp. can, to some extent, best be displayed by using pure culture techniques. However, when relative humidity is being controlled care must be taken to select a suitable technique. A continuous air-mixing system will remove possible aromatic volatile inhibitors of Streptomyces spp. growth.

The general importance of Streptomycetes in biodeterioration has been outlined by Williams (1966). Betrabet et al (1968) were the first workers to recognise that a serious spoilage problem existed in textile storage caused by Streptomyces spp. The ecological pattern emerging from their study showed that cellulolysis in nature is a synergistic process, where fungi and Streptomyces spp. play a major role in bringing about degradation of cellulose, while bacteria with few exceptions, seem to help mainly in the further decomposition of materials by cellulolytic activities

many Streptomycetes can produce colourful pigments which often result in spoilage by staining of fabrics, Waksman (1959).

Betrabet's study was carried out in the tropical Bombay region of India, so a project was developed to see if a similar proponderance of cellulolytic Streptomycetes were present in this country.

Preliminary soil isolation experiments by Malik (1970) showed Streptomycetes to be very common soil micro-organisms, being very frequently isolated at 25, 35 and 50°C. Using a perfusion system employing chromatography paper as a cellulose source Streptomyces spp. were found to be one of the two major colonisers at 35°C. Streptomyces spp. however, were isolated only after two weeks at 50°C, when the substrate was fairly deteriorated.

Malik used a soil enrichment technique combined with an antifungal agent natamycin (2½% suspension 'Pimafucin') to isolate five thermophilic species of actinomycetes from the soil. The cellulolytic ability of these isolates was examined by estimating the weight loss of nutrient perfused cellulose chromatography paper inoculated with these species. After four weeks incubation at 50°C the weight loss caused by Streptomyces spp. varied from 3.5. - 15.6% (one species was non-cellulolytic), the weight loss following an

experimental curve with respect to time. Actinomadura glauca was found to be capable of causing a 33% weight loss over a similar period.

The literature on airbourne mesophilic Streptomyces is very limited, Feinberg (1946), de Vries (1960); so a series of isolations was carried out to gain a rough estimate of the population. The reasons for using airbourne Streptomyces are given in the section on isolation of Streptomyces.

Having isolated the Streptomyces spp. and attempted to identify them the next logical step was to discover the physiological properties of the isolates and to test their amylolytic and cellulolytic abilities.

A series of simple simultaneous tests and isolations were carried out on fungi, principally to give a yardstick against which the Streptomyces spp. findings could be compared. These tests and isolations covered little new ground but acted as a check on experimental techniques; giving a more accurate picture than if experimental results from a literature survey had been used for comparison.

Physical tests using controlled temperature, humidity and pH were utilised so that areas of potential activity in the textile processing and storage cycle could be pinpointed.

The mechanism of biodegradation of cellulose has been reviewed by Selby (1968). The roles of the individual components that can be isolated from the extracellular cellulose systems of certain cellulolytic micro-organisms is not fully understood. Two components have been proposed, the $C_1$ component alone being incapable of attacking highly ordered substrates but, when mixed with the other ($C_X$) components of the cellulase system produces a powerful synergistic action.

Siu and Reese (1953) detected cellulase activity in filtrates of a streptomycete when placed in contact with cotton duck. Norkans and Råsnby (1956) worked on an extracellular enzyme of Streptomyces sp. and concluded that it acted by random scission of the cellulose chain. Reese et al (1969) demonstrated cellotriose and cellobiose accumulation by the enzymatic action of cellulolytic filtrates of Streptomyces sp; further work has been carried out by Enger and Sleeper (1965) and Betrabet and Patel (1969). Betrabet et al (1968) carried out experiments on untreated cotton textile to determine weight loss of the substrate and changes in copper number and alkali solubility to give an estimate of cotton decay.

It was decided to use a more stringent and accurate method for measuring decay, tensile strength testing of standard sized samples, to give an accurate basis for estimating biocide treatment effectiveness.

## 1.5 Uses of starch in the textile industry

The textile samples tested were coated with wheat starch, (1% by weight of fabric). It is the starch surface coating that leads to many of the biodeterioration problems in the textile industry. Wheat starch is able to take up a large amount of moisture and also acts as an ideal easily assimilated carbon source for the invasion of micro-organisms. Microbial attack may be limited entirely to the starch coating, and the micro-organisms may not have the cellulolytic ability to utilise the cotton.

The uses of starch in the textile industry are fully outlined for two reasons:-

a) To emphasise the importance of starch.

b) To pinpoint particular areas in the process where biodeterioration might occur.

Starch is probably the commonest finishing agent for cotton goods. Starch and starch mixtures are used by the textile industry in four general areas:-

a) As a size to strengthen warp yarns and improve their resistance to abrasion during weaving.

b) In finishing, to change the 'hand' and appearance of fabric after it is bleached, dyed or printed (starch in this application is sometimes used as a binder for other materials, such as china clay, or in conjunction with thermosetting resins).

c) In printing, to increase the consistency of printing pastes.

d) As a component in finishes to glaze and polish sewing thread.

Wheat starch was used in a series of experiments to discover physiological properties of the Streptomyces spp. both in starch agar media and as a coating on cotton textile test strips (1% per weight of fabric).

1.5.1 Wheat starch

Wheat starch is sold by the manufacturers in powdered, modified and pregelatinized forms. It has many of the same uses as starches from corn, potato, tapioca or rice; however, its equal or slightly higher cost usually limits the commercial outlets to those which need its superior properties. Wheat starch is considered superior for laundry work, probably because of the large range of granule sizes in the starch. The smaller granules penetrate the fibres of the fabric whereas the larger ones coat the exterior surface.

In some parts of the world where wheat is grown in large quantities flour is the main source of starch. For example in Australia and New Zealand, wheat starch is produced in much larger quantities than maize or sorghum starches.

Commercial wheat starch has a moisture content of about 12% and a protein content of approximately 0.2% (due to impurities). The granules in wheat starch fall into two distinct sizes the smaller being as small as 2$\mu$ in diameter and the larger measuring up to the 30$\mu$ range.

When wheat starch is added to water at room temperature, a slurry or suspension is formed from which the granules quickly settle. When the temperature of the slurry is raised to 50°C the larger granules commence to swell because of the penetration of water through the weakened starch structure. Above 50°C, the smaller granules also begin to swell, although some of the smallest are very resistant to enlargement. At about 65°C, the starch slurry begins to form a paste; when 70°C is reached most of the granular form of starch has disappeared. Thus, the thin slurry becomes a semi-opaque paste and the viscosity is high because of the crowding effect of the swollen granules.

Uses of starch in the textile industry

1. Yarn strengthening

Yarns spun from staple fibres such as cotton are slashed (sized) with starch solutions to improve the strength and abrasion resistance of the yarn and to reduce fuzz of the yarn by cementing the protruding surface fibres to the body of the yarn. The strength of the yarn is increased 10-30% by this action, and the increased stiffness facilitates handling the warp through the loom. To accomplish these actions, size films are applied as a thin coating on the surface of the spun yarn and should penetrate the yarn only far enough to provide satisfactory adhesion. If the size permeates the spun yarn, the yarn will be too stiff. The amount of size added to spun yarns is usually 10-15% of the weight of the yarn. Starch films are moderately easy to remove with enzymes or acids and usually cause little difficulty in wet processing.

"Yarns composed of continuous filaments are slashed by starch. The filament yarns are sized to provide abrasion resistance and also to cement the individual filaments together. The filaments are cemented to prevent the formation of 'fuzz-balls', which occur when a single filament breaks and is pushed back along the body of the yarn. If the filaments are not cemented together, the broken filaments accumulate until they cause sufficient entangling to stop the loom. Cementing the filaments provides a stopping place

for the 'fuzz-balls' because the filament will break at the bonding site. Materials used to size filament yarns thus must penetrate the yarn completely. The amount of size added to filament yarns is 3-5% of the weight of the yarn, and this small amount does not cause excessive yarn stiffness", (Marsh 1957).

Starch size solutions must have several important properties:-

i) Low cost

In most instances, the size is added to improve the efficiency of the weaving operation, and is removed in the first wet-processing operation. Since a large amount of material is used to obtain a temporary change in yarn properties, the cost of material used must be low.

ii) Easy preparation

Size is generally prepared in the mills by relatively unskilled labour. Large amounts of size are used and the properties of sizing solutions must be consistent. For these reasons the size solutions must be easily prepared with minimal supervision.

iii) Uniform viscosity and solids content

The amount of size on the slashed warp yarn must be uniform, not only to ensure uniform weaving performance, but to minimize fluc-

tuation in the weight of the griege fabric. In addition, the penetration of the size into the yarn and, consequently, the adding of the size, are properties affected by the viscosity and solids content of the size solution. The temperature of application of the sizing solution must be kept constant since the viscosity of starch solutions is increased as the temperature decreases.

Sized yarns are dried with hot air or in contact with rotating stream heated cylinders. Size solutions are often held overnight and are occasionally stored during weekend shut-downs. Resistance to microbial action is desirable during these storage periods. Starches are used in preference to flours in sizing because of their improved resistance to biodeterioration. Bacteria are the major problem due to the limited growth time available and these are overcome by adding biocides to the sizing solution. Streptomycetes will not form a problem at this stage of the textile production due to their slow growth rate.

iv) Size films should be resistant to damage by heat and overdrying since it is common practice to dry sized yarns to a moisture content below that at which the yarn is woven. Most mills

prefer overdrying to underdrying as it prevents possible biodeterioration which may occur if the yarn is not sufficiently dried during slashing.

Warp sizes generally contain three ingredients:-

a) film form material, usually starch.

b) film modifiers, such as fats used as softeners, or glycerol used as a humectant.

c) lubricants, e.g. paraffin wax.

all the above ingredients are capable of being attacked by Streptomyces spp., (Williams 1966).

The amount of starch used in sizing to prepare yarns for weaving is much greater than that used in all the other textile operations.

2. Finishing with starch solutions

"Since the finish provided by starch is temporary, its use is confined to inexpensive fabrics or materials such as shade cloth or book bindings that are not washed. Fabrics finished with starch alone are quite brittle and stiff and thus the films contain relatively large amounts of modifying agents".

Starch can also be used in conjunction with thermoplastic or thermosetting resins to obtain a stiffened finish; when used in this manner, it provides a permanent change in the fabric properties.

a) Changes in fabric hand; fabric in the last stage of finishing is immersed in a dilute solution of cooked starch, squeezed to remove excess solution, and dried on steam heated cylinders. Composition of solutions used varies considerably with fabric construction and the properties required. A typical formula for lightweight goods is: 5Kg starch, 10Kg softener, 250 litres of water.

b) Back filling; this is a process of applying a mixture of starches, or of starch and a filler such as talc or china clay to the back of the fabric without obscuring the weave on the face of the fabric. This type of finish increases the stiffness and the opacity of the fabric by filling the interstices of the weave with the starch mixture. Fabrics thus prepared are used in window shades and in bindings for books. A typical formulation for this purpose might be as follows: starch acetate 15Kg, starch 44Kg, sulphonated tallow 22Kg, talc 50Kg in 500 litres of water, (Marsh 1957).

3. Printing pastes and thread glazing

Thickeners are used in printing to give the printing paste the consistency necessary to produce a clean, sharply-defined pattern of colour. Unmodified starches are generally mixed with gum tragecanth. Printing paste used at room temperature gives higher viscosity and may consist of 110Kg starch,

0.5Kg of stearic acid added to 500 litres of boiling water; the dyes being added when the paste is cool.

Thread is also finished using starch, gum and wax giving an improved lustre and reduced friction during sewing.

## 1.6 Prevention of biodeterioration by using biocides

Biodeterioration of textiles may be prevented to a large entent by the controlled use of biocides at the correct point in the manufacturing process. The type of biocides currently used, their concentrations, methods of action and relative effectiveness with respect to soil fungi deterioration of textiles has been reviewed by Allsopp (1973).

A biocide to be effective must show the following characteristics, Selby (1966):

1) it must have a wide toxicity spectrum effective against all prospective microbial deteriogens.

2) the compound must be so formulated that it may be produced and applied easily without toxic effects to the workers, (assuming the relevant safety proceedures are followed).

3) it must not produce unwanted colour in the fabric.

4) compatability with finishes applied to the fabric, such as starch and starch mixes.

5) biocides must not effect the 'handle' of the textile.

6) tensile strength and tear strength of the textile must not be adversely altered.

7) there must be no increased tendancy to actinic degradation caused by the biocide.

8) the formulation must be resistant to leaching in the course of normal usage.

9) competitive cost.

Factor (1) is a point of investigation in this thesis. Whilst all proprietary brands of biocide are thoroughly evaluated against fungal and bacterial attack, there is little or no mention of testing biocides against actinomycetes.

Actinomycetes and specifically Streptomyces are known to be present in large numbers in the soil (though predominantly as uncompetitive spores and mycelial fragments), to be cellulolytic and to be capable of degrading inorganic compounds which other groups of micro-organisms are unable to utilize, Williams (1966).

4 It is postulated that Streptomyces spp. might be capable of one or more of the following effects:

a) Production of metabolites which could act as a stimulus to fungal growth, or conversely have a depressing activity on fungi; though it is thought that these effects may be overstated, taking into account the relative growth rates.

b) Streptomyces spp. could possibly have a detoxifying effect on biocides. Biocides could be inactivated or degrated leading to colonization by more rapidly growing fungi which would overgrow the unobserved initial Streptomyces spp.

c) The possible resistance to fungal biocides of Streptomyces spp. able to deteriorate cellulosic and starch materials.

d) Streptomyces spp. may be responsible for variability in soil burial test results, particularly if soils are allowed to dry out or are kept in atmospheres with R.H.'s below 90%.

The neglect of this group is founded on isolation and identification difficulties; Streptomyces rubrireticuli appears to be the only species used individually in commercial testing, (principally because it produces as distinctive pink staining pigment), (Barr, 1973).

Betrabet et al (1968) showed that Streptomyces spp. could be important in tropical countries, but his textile

samples were unprotected. Thermophilic Streptomyces spp. were isolated by Malik (1970) on model cellulose substrates and their cellulolytic ability estimated. Hollingsworth (1974) has isolated unidentified Streptomyces spp. on standard starch coated textile having biocide protection. His experiments, however, were carried out at thermophilic temperatures and the Streptomyces spp. were obtained from a mixed soil inoculum.

Three commercial biocides were used in this work, tri-butyltin oxide (T.B.T.O.), sodium pentachlorophenol (Na.P.C.P.) and salycilanilide ('Shirlan') and these were evaluated against individual Streptomyces spp. isolated from the air. Both visual assessment and physical estimate the effectiveness of these biocides.

If these biocides were found to allow biodeterioration of the textile to occur this could account for some of the faults found with standard test proceedures.

Test proceedures are notoriously variable in their results and this could be due to using soil with a higher than normal proportion of actinomycetes, (predominantly Streptomyces spp.) possibly caused by the drying out of soil before use. In service failure of textile or unexpected staining could also possibly be due to direct action by Streptomyces spp. or by them acting as potentiators for subsequent fungal or bacterial sucession.

## 1.7 Summary of aims and experimental techniques

The purpose of this project was to investigate the affect of relative humidity on microbial colonisation of starch and cellulose substrates with relevance to commercial testing of coates textile surfaces. Soil burial is the standard test for microbial action on materials and evaluation of the biocides used to protect them.

Many aspects of soil testing, however, remain uncontrollable and so pure culture techniques were adopted to try and pinpoint two factors, moisture availability and the action of Streptomycetes, particularly Streptomyces spp. which might lead to variability in commercial laboratory biological testwork.

It was found that present methods for controlling and monitoring relative humidity in the laboratory had certain disadvantages, and so novel methods for producing and measuring such atmospheres were developed and used.

Continuous wet/dry air mixing based on the principles of May (1974) were modified to form a system suitable for laboratory investigations of the effect of relative humidity on microbial colonisation on cotton textiles.

A dual p.n.p. bismuth-telluride thermocouple device, based on Peltier cooling was proposed to monitor the air mixing system.

A literature survey indicated that actinomycetes, Streptomyces spp. in particular had properties which could make them serious textile deteriogens. Isolations were performed to estimate the size of the Streptomyces spp. population capable of using starch and cellulose substrates.

Experiments on fungi imperfecti were performed to enable comparisons to be made with physiological properties discovered for the Streptomyces spp., particularly with respect to relative humidity.

Having established the limits within which the Streptomyces spp. were capable of starch and cellulose degeneration, it was necessary to test them against the standard methods of textile preservation.

## Chapter 2

# TECHNIQUES FOR PRODUCING A WIDE RANGE OF CONTROLLED RELATIVE HUMIDITIES FOR BIOLOGICAL TESTING

2.1 Introduction

2.2 Saturated salt solutions

2.3 Dry/wet air mixing system

2.4 Scott $A_w$ method

## 2.1 Introduction

The control of atmospheric humidity in the laboratory was required to condition materials to a standard water content to study growth patterns of micro-organisms on starch, cellulose and textiles under reproducible conditions. A controlled relative humidity environment may be obtained in one of three ways:

a) By keeping the substrate in equilibrium with an atmosphere of controlled humidity by employing saturated salt solutions.

b) Continuous airflow systems employing air of known relative humidity obtained by mixing dry and wet air in constant proportions, or by adjusting the temperature of saturation of air prior to raising its temperature to that of the experimental material.

c) Fixing the water content or the solute concentration in the culture substrate.

All three methods were employed in the course of the experimental work. Each system has its advantages and disadvantages and the technique used in any one experiment has to be carefully considered. This chapter will outline the methods used and these will be expanded upon further in the context of individual experiments.

## 2.2 Saturated salt solutions

The traditional and most convenient means of providing standard relative humidities covering a wide range of values is the use of salt solutions, either saturated or unsaturated in small, sealed containers. "Any salt solution at a definite concentration and at a constant temperature, is in equilibrium with a fixed partial vapour pressure of water and hence defines a fixed relative humidity", (Young 1967).

The use of saturated salt solutions to control relative humidity is both a cheap and convenient method. Most reagents are readily available in reasonable purity, are safe to handle and are non-volatile, thus avoiding contamination of the specimen.

Most salt solutions are quite stable, but care should be taken not to use salts near their transition points when new hydrates may be forming, since temperature variations will not give a linear graph. Saturated salt solutions are capable of liberating.or adsorbing fairly large quantities of water without changing the equilibrium relative humidity. The same does not hold for unsaturated solutions, where large changes in water content will appreciably alter the concentration and hence the relative humidity.

The use of saturated salt solutions has been fully reviewed by many authors including A.S.T.M. (1974), Solomon (1951), Wexler and Nasegawa (1954), Robinson and Stokes (1959), Winston and Bates (1960) and Young (1967). The saturated salt solutions used in the following experiments

were compiled from these sources to give a humitity gradient over the range 75 - 100% R.H. The salts used to give this gradient are shown in fig. (1). These salt solutions were used in sealed boiling tubes, the samples being suspended over the salts, Ayerst (1969). It was assumed that, whilst there might be slight fluctuation in temperature and therefore of relative humidity in the tubes during incubation the water activity at the surface of the test material where the micro-organisms were growing would be buffered by adsorption and desorption of water by the strips. The major difficulty with a saturated salt solution in an enclosed chamber is the maintainance of stable oxygen/carbon dioxide ratios with growing cultures over the duration of a biodeterioration test.

Measurement of the relative humidity obtained is a major problem with saturated salt solutions. The small size of the test samples and the tubes meant that one could assume equilibrium to occur fairly quickly (3 days) and that it would differ only very slightly from the value required. This is tested using weighing experiments using both agar strips and wood veneers over the saturated solutions, these gave very significant ($p<0.1$) results for differences between the solutions. These results were then correlated with weight results from strips suspended over glycerin A.S.T.M. (1974). The glycerin could be monitored before and after the experiment using a refractometer.

| Salt | % R.H. at 25 $^{\circ}$ C |
|---|---|
| Water | 100 |
| $CuSO_4 \cdot 5H_2O$ | 97.2 |
| $Pb(NO_3)_2$ | 95.5 |
| $(NH_4)H_2PO_4$ | 92.7 |
| $BaCl_2 \cdot 2H_2O$ | 90.3 |
| $K_2CrO_4$ | 86.5 |
| KCl | 84.2 |
| $(NH_4)_2SO_4$ | 80.2 |
| NaCl | 75.1 |

Fig. (1) Saturated salt solutions used for relative humidity control.

## 2.3 Air mixing system

Shortcomings, and the consequent precautions which need to be observed, when setting up humidity chambers with saturated salt solutions have been reviewed by Martin (1963) and Wexler and Hasegawa (1954).

1) Although saturated salt solutions can, in theory, handle large changes in water content without affecting their equilibrium values, it has been found in practice that where samples absorb moisture appreciably, as is the case with starch and starch mixes, the actual relative humidity over a saturated salt solution remains lower than the true equilibrium value. For example a rate of adsorption as low as 4mg./hour will depress the relative humidity over saturated ammonium nitrate at 20°C (65% R.H.) by 2% even when a circulation of air is maintained (Martin 1963). Thus for a strongly adsorbing material the relative humidity may be depressed by several tens of percent and take a longer period than is probably recognised to reach the true equilibrium value. Equilibration with a mixing system is fast, a matter of hours rather than days and is more easily controlled and measured. The humidity may be measured by means which involve water

vapour exchange (i.e. dew point, or wet and dry thermometry) which themselves affect the humidity being measured in static air, but have little or no effect on continuous flow.

2) Humidity gradients within the sealed chambers are a major problem with saturated salt solutions, where there is no air flow, and dependent on the object being conditioned may result in a 5% R.H. variation within a chamber.

3) For many biological and other purposes it is desirable to be able to study the effect of a range of atmospheric humidities under conditions of continuous air change, which will prevent accumulation of undesirable atmospheric pollutants or give rise to fears that volatile constituents of saturated salt solutions will affect the materials being studied. This factor is extremely important when considering a study of Streptomyces spp., which are commonly recognised as being present in an environment by the 'earthy' smell they give off due to the release of volatile aromatic chemicals into the air. In a confined space with limited airflow these aromatics could build up and possibly have a depressing effect on growth.

4) "An A.S.T.M. recommendation (American Society for

Testing Materials 1951) on air-tight humidity chambers and their loading is that the ratio (volume of air space/surface area of solution) should not be greater than 10in. and that as a general rule (obviously to be modified according to the nature of specimens) the total surface area of specimens should be less than the surface area of the solution", (Martin 1963).

Thus in experiments where agar or textile strips are being conditioned saturated salts are of limited value due to the specimens large surface area and their adsorbing potential.

5) Larger tanks may be used with an air mixing system thus facilitating handling of specimens. Due to faster equilibration times it is possible to open tanks for longer periods than would be possible with saturated salts and also there is greater flexibility in the speed with which different humidities can be obtained.

For experiments using textile strips a system based on continuous mixing of dry and wet air to produce a controlled environment seemed ideal and most nearly fitted the commercial standards required by the sponsors.

The apparatus described uses such a system reduced to its simplest form. It is easily constructed from ordinary

laboratory equipment and it enables a number of different fixed humidities to be maintained simultaneously. The equipment maintains six different levels of relative humidity in containers of about 18 litres capacity all at one constant temperature. Replication of the same equipment would be necessary if it was desired to use more than a single temperature at any one time but the apparatus would remain relatively easy to construct.

The principle of mixing wet and dry air streams to establish a constant relative humidity is not new, but the difficulty of metering air streams accurately has tended to make the apparatus based on it rather complicated for use by non-specialists, especially in biological laboratories. An ingenious mixing valve has been developed by May (1974), this compresses one rubber tube whilst opening another using a single screw. May advises that this is a very effective method for maintaining a constant flow ratio for fairly long periods of time, but requires seperate adjustment and calibration for each air stream. Significant simplifications can be introduced by the use of capillary tube resistances as described by Smith P.R. (1965), for metering and controlling an airflow. The major advantage of capillary tubes was that a desired relative humidity could be pre-set with fair accuracy simply by cutting calculated lengths.

While Smith's apparatus enables the relative humidity of an air stream to be quickly changed, it permits only a

single stream to be controlled at one time. The present need, one which is frequently necessary in biological work, was to maintain a number of air streams at constant humidity over a period of many months.

Principles involved in the design of a controlled environment cabinet have been reviewed by Solvason and Hutcheon (1965). "The provision of the desired conditions within a cabinet has all the engineering problems of air conditioning. The system may be considered in three parts: the enclosure, the conditioning equipment and the control system".

The requirement for simultaneous control of temperature and humidity poses special problems as relative humidity is temperature dependent. When extremely high humidities are produced spatial variations in temperature, including those involved in the conditioning air stream, must be reduced to a low level. Heat gains to the cabinet must be eliminated as far as possible and high rates of air circulation maintained. The ultimate performance of a cabinet depends not only upon the individual components of the system, but also upon the ways in which they interact.

Construction

Fig. (2) shows schematically how each air stream of the equipment works. Air supplied at an approximately constant pressure of 135mm of mercury by a laboratory, vane type, pump (Model RB4, Edwards High Vacuum Ltd.), running

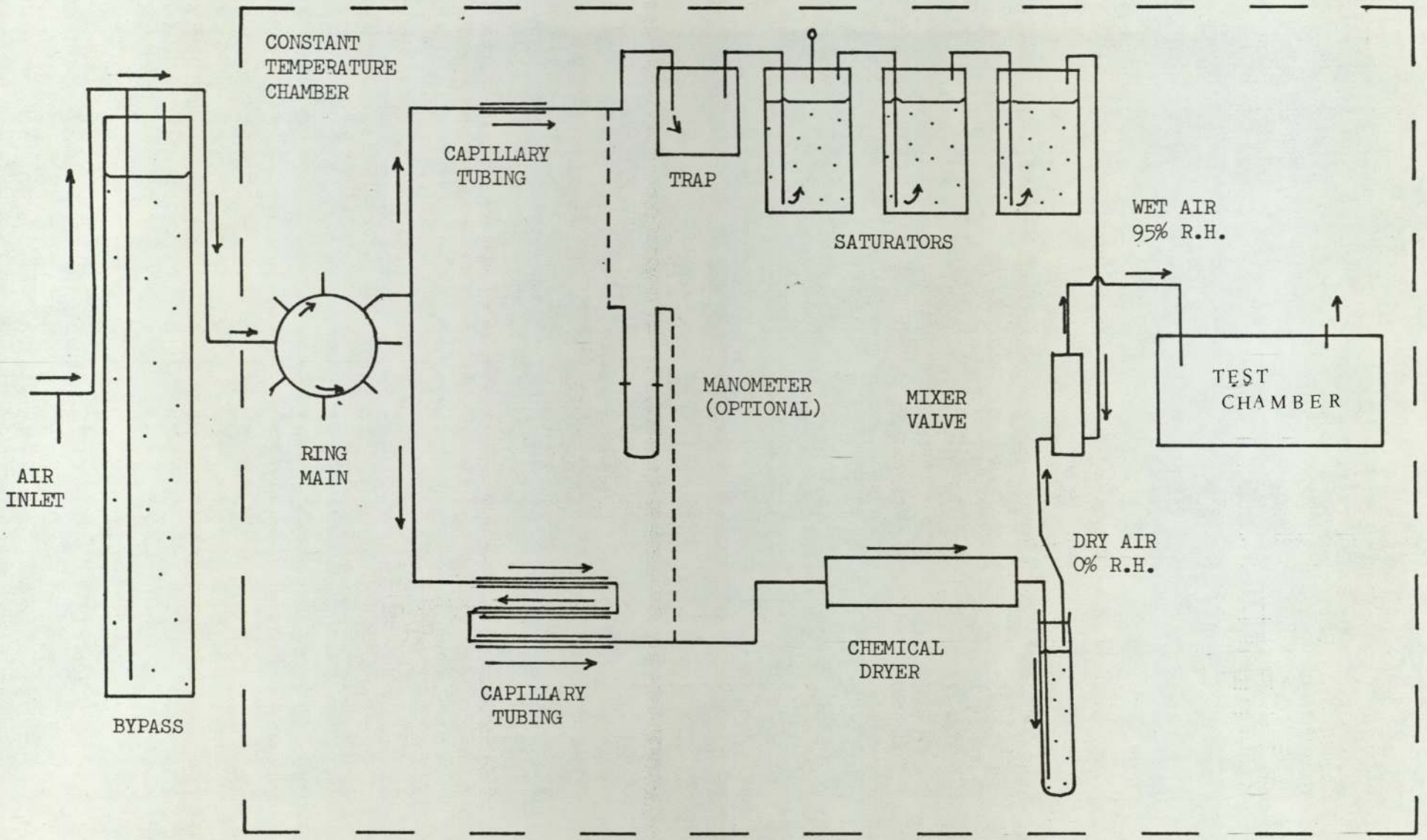



Fig. (2) Schematic flow diagram of continuous wet/dry air mixing system.

continuously and producing a greater volume and pressure than necessary, provided with a teed off bleed tube dipping to a depth of 1.8 metres in water through which excess air bubbled. A ring main made this supply available to up to seven controlled humidity air streams. Although it was desirable to keep the supply pressure approximately constant precise control was not necessary to the proper functioning of the system, hence a bleed tube as described was perfectly adequate.

Each controlled humidity line then proceeded as follows: the air flow was divided into a dry and wet stream and each passed through a capillary tube whose resistance was inversely proportional to the desired rate of air flow. The wet stream was than humidified by passing through three jars of water in series each 150mm deep. The dry stream was dried by passing over self-indicating silica gel (to pass 6-20 per inch mesh). In order that the known ratio of the resistances of the capillaries in the two streams could be interpreted as a known ratio of air volumes it was necessary that the pressure drop across them should be equal. As the back-pressure presented by the saturators was much greater than that presented by the silica gel it was necessary to include in the dry stream a corresponding back pressure; this was conveniently done by bubbling through a depth of anhydrous glycerol 0.795 times the total depth of water in the saturators, i.e. 358 mm. The efficacy of this arrangement could be checked by connecting

a manometer downstream of the two capillary resistances as shown in the diagram. The level of glycerin could then be adjusted until the pressures were equal. In practice this was found to be unnecessary provided that the water level in the saturators was regularly made up to levels marked up on them, and the silica gel regularly replaced so that the glycerin did not become diluted with water vapour.

The two air streams were then brought together in a mixer. This consisted of a piece of copper tube 15mm. in diameter with an axial threaded rod on which were fixed several metal discs, these fitted the copper tube fairly closely but were notched to permit turbulence to the air. This was similar to the mixing device used by Smith. From the mixer, air passed to the experimental chamber which in this case was of transparent polystyrene and volume about 18 litres. It was essential for correct functioning that the saturators and the wet and dry air streams were kept at the same temperature after metering of the air streams would have a very large effect on relative humidity.

In practice it was found easiest to contain the whole of the equipment except the main air supply within a large box kept at constant temperature with the air vigorously stirred. The enclosure was made of insulating board bolted onto a 'Dexion' steel frame, and contained the following temperature control instruments:

a) Thermostat. (bi-metallic sensor)

Sunvic Controls Ltd. Type TS/NC.

Thermostat range $\pm$ 20°C.

b) Thermostat relay switch.

Sunvic Controls Ltd.

Type F102-3.

c) Autoheat Greenhouse Heater Mk.11

Findlay Irvine Ltd. Pencuick nr. Edinburgh.

240v A.C. 1,250 watts.

The greenhouse heater contained a built in thermostat which gave the heater a maximum temperature capability of 68°F. As the experiments were carried out at higher temperatures than this the thermostat and relay switch were added to the circuit. The thermostat in the fan-heater was modified (Personal communication T.A. Oxley) to act as a safeguard, the heater cutting out at 2°C above the required temperature. The thermostat used in conjugation with the relay switch was set at 25°C.

The room containing the apparatus was temperature controlled (Gemini 102 Air Conditioner, Chrysler-Tempair Ltd., Maidstone) at 24°C $\pm$ 0.5°C.

Thus the thermostat temperature control system was an adjusting system covering the range 24-27°C. It was found using a maximum and minimum thermometer that the temperature fluctuation for one weeks operation was negligible (0.5°C).

Seven experimental chambers were used, six controlled as described, the seventh receiving only saturated air and having free water in the bottom to provide 100 per cent relative humidity. The box was 1.5 metres long, 1.2m. high and 1.2m. wide, with slotted wooden shelves 0.65 and 0.15m. from the bottom. This was of generous size to contain seven complete lines.

Performance

The apparatus as described was somewhat crude and was not intended to achieve high precision. The relative humidity in each experimental chamber was measured by means of a wet and dry hygrometer, a technique suitable for moving air conditions but unsuitable for chambers controlled by saturated salt conditions. Having established the initial relative humidities, they were monitored subsequently by an ordinary domestic hygrometer (Diplex 'synthetic' which depended for its action on the expansion and contraction of a strip of synthetic material) permanently enclosed within each chamber. These instruments were calibrated in 1% steps of relative humidity; they were not reliable as absolute indicators but, kept under reasonably constant conditions, they were excellent indicators of small changes. They were read daily; variations did not exceed plus or minus $1\frac{1}{2}$% relative humidity.

This apparatus has been used only for relatively high levels of relative humidity specifically in steps of 5%

from 70% to 95% relative humidity. For this reason it was customary to deal with much lower rates of air flow in the dry air line than in the saturated air line; the dimensions of the capillary resistances indicated in fig. (2) reflect this fact. But there is no reason to doubt that a complete range of relative humidities could be established with the same apparatus. It was found that air was not fully saturated by being bubbled through three bottles of water each 150mm deep; in fact a relative humidity fairly constant at 95% was produced. Vernon and Whitby (1931) stated that, "experiments very soon showed that the simple method of bubbling air through a column of water is definitely inefficient, nevertheless these deficiencies do not seem to be generally appreciated, and it is readily assumed that air may be saturated by this common method". The non-saturation of air was attributed to variation in hydrostatic pressure, the degree of saturation increasing with the total depth of water traversed.

The efficacy of drying in the dry air stream was naturally not perfect and this required some consideration. Still and Cluley (1972) have shown that silica gel is capable of reducing the water content of air to 40 parts per million (by volume) but in practice (laboratory stock from oven at 110°C) probably much higher levels that this are reached before self indicating gel begins to indicate exhaustion. Trusell and Diehl (1963) claimed only 95p.p.m. water vapour, probably because they did not allow sufficient

time for equilibrium to be reached. In practice, probably 100 p.p.m. was the best one could hope for rising to 1000 p.m. before the gel indicated exhaustion. If for a short period a level of 1000 p.p.m. water vapour was reached, this is equivalent to about 3% relative humidity at 25° C.

Vapour pressure of water at 25°C = 23.67 mmm. Hg.

if total pressure is 760 mm. Hg, saturated air will contain $\frac{23.76}{760}$ x 100 per cent by volume of water

= 3.13% water vapour by volume = 100% R.H.

1,000 p.p.m. (=0.1%) by volume = $\frac{100}{31.3}$ = 3.2% R.H.

and 100 p.p.m. water vapour by volume = 0.32% R.H.

The average figure was presumably much less than 3% R.H. and the glycerin could be expected to buffer the changes. Within the limits of accuracy of this apparatus it was reasonable to assume zero relative humidity in the dry stream; in fact it was probably about 1% or less.

Calculation of Capillary Resistance

The size of capillary tube was chosen so as to give a convenient rate of air flow at the chosen air pressure, which was determined by convenience in the height of the water bubbler bypass, minus the unavoidable back pressure imposed by the saturated line. In the system described this pressure difference was 1800 - 450 = 1350 mm. water (= 99.6 mm. mercury).

Using units which are convenient to measure, the appropriate values of capillary resistance may be calculated

as follows:-

Let one unit of resistance pass 1 litre per minute at a pressure difference of 1 metre of water.

If the pressure difference (applied pressure less back pressure of saturators) was P metres of water and the desired flow rate is V litres per minute, the resistance of the double circuit must be $\frac{P}{V}$ units. Donote this value by R and the separate values of the wet and dry stream resistances by R wet and R dry respectively

$$\text{Then } \frac{1}{R} = \frac{1}{R \text{ dry}} + \frac{1}{R \text{ wet}}$$

If it is desired to produce a relative humidity of H% then the rate of flow in the two air streams will be in the ratio $\frac{H}{95} / 1 - \frac{H}{95}$ and this will also be the ratio of the resistances required. Denote this value by K

$$\frac{1}{R} = \frac{1}{KR \text{ wet}} + \frac{1}{R \text{ wet}} = \frac{1 + K}{KR \text{ wet}}$$

$$R \text{ wet} = \frac{R(1 + K)}{K}$$

and substituting for R

$$R \text{ wet} = \frac{\frac{P}{V}(1+K)}{K}$$

and R dry = KR wet

For example, to produce a relative humidity of 75% (assuming a wet stream relative humidity of 95% and a dry stream relative humidity of zero) will require

$$K = \frac{75}{95} / 1 - \frac{75}{95} = \frac{0.789}{0.211} = 3.739$$

For a flow of 2 litres per minute in the equipment where

P = 1.35

$$R \text{ wet} = \frac{\frac{1.35}{2} (1+3.739)}{3.739} = 0.856 \text{ units}$$

and R dry = 3.200 units.

Capillary tube of 0.5 mm. bore diameter produced convenient values of R. Under test, a head of 4 metres of water produced a flow rate of 1.03 litres per minute through a 20c.m. length. Hence one unit of resistance is a length of 51.6mm. Capillary was cut from this stock using 50mm. as the shortest length. With wider bore tubing the danger of blocking would be less (we have not experienced any difficulty from this) but greater lengths per unit would be needed to keep the flow within the limits imposed by our drying and saturated lines. Even with 0.5mm. bore, considerable lengths are needed at extreme humidities. For example, for 90% relative humidity K = 17.867 and the dry line resistance must be about 89cm. long. Similarly, for 5% relative humidity, K = 0.0555 and the wet line resistance must be 90cm. long.

Alternative Designs

Alternative configurations embodying the principle described are varied. One such, which would have some advantages, would utilise a single humidifying line and a single drying line, with appropriate pressure equalisation, serving a series of mixed streams. This would be most convenient if the desired rate of air flow is low. In this case the resistances would be inserted after humidification and drying, instead of before, as in this design. However, it would be very important to ensure that the temperature of the saturation line was exactly equal to, or a little higher than, the temperature of the controlled chamber. If the temperature of the latter falls more than a degree or so below that of the saturator, condensation would occur and it would be necessary to provide a trap to prevent this from occurring in the capillary resistance. An advantage of using a single drying line would be that the desiccant may be preceeded by a cold trap or other crude drying arrangement to remove moisture, thereby reducing the need for frequent change of desiccant. If this was done, the temperature must be made equal to that of the wet stream before the capillary resistance is imposed.

Alternative laboratory desiccants may be used to silica gel and their efficiencies have been thoroughly evaluated by Still and Cluley (1972).

## 2.4 Scott water activity ($A_w$) control technique

Control of $A_w$ in a substrate determines the relative proportions of water and dry matter, so it is also possible to fix $A_w$ by controlling solute concentrations. This technique was used for discovering the growth abilities of Streptomyces spp. under conditions of controlled $A_w$ and is explained in Chapter 6.

Control of $A_w$ in the substrates by means of known solute concentrations was preferred to saturated salt solutions or air-mixing in Streptomyces spp. experiments at $A_w$ above about 0.95 which are the major area of growth for Streptomyces spp. "This follows from the nature of the water sorption isotherm, the rate of change of water contents approaching infinity as $A_w$ approaches 1.00. e.g. from the isotherm given for brain heart infusion given by Scott (1953) it may be deduced that in changing the $A_w$ from 0.78 to 0.79 the water contents increase from about 76 to about 79% of the dry weight. In changing from 0.98 to 0.99 the corresponding increase in water contents is from 1030 to 2080%. Thus from 0.98 to 0.99 $A_w$, a transfer of 1050 parts of water is required to equalise the vapour pressure difference, whereas from 0.78 to 0.79 $A_w$ the same vapour pressure difference is cancelled by the transfer of only 3 parts of water. This is the fundamental reason why equilibration at a high $A_w$ is inconveniently slow", Scott (1957).

The Scott $A_w$ control method has several considerable advantages.

1) It is easy to obtain accuracy at high $A_w$, the error being proportional to $1 - A_w$, and so is ideal for Streptomyces spp. experiments.

2) The $A_w$ can readily be calculated for an agar medium of known composition, and this $A_w$ may be altered by varying the concentration of various solutes, whilst maintaining a constant concentration of nutrients.

3) Solute and solvent effects on the biological system being studied may be distinguished by careful experimentation.

It was essential, however, that there was no exchange of moisture between the agar substrate and the surrounding atmosphere, therefore experiments were performed in lidded petri dishes to cut out water loss from the substrate. A high relative humidity was maintained in the incubator (by means of a water tray) to remove any chance of substrate drying.

Several alternative methods have been used for biological experiments requiring controlled humidities. Pure solutions of known $A_w$ can be prepared and equilibrated with air. Solomom (1951) gives instructions on how to prepare solutions of potassium hydroxide and sulphuric acid

for biological experiments. "The formula relating water activity to concentration is:-

$$A_w = \exp. (-0.018016\ Vm\ \phi)$$

where V is the number of ions generated by each molecule of solute, m is the number of moles of solute per Kg. of water (molal concentration ) and $\phi$ is the molal osmotic coefficient which varies with the solute, its concentration and the temperature. It is a measure of the extent to which the solution departs from the ideal", (Ayerst 1965). The tables in the appendices of Robinson and Stokes (1959) give values of $A_w$ and $\phi$ for many solutes.

The use of solutions such as potassium hydroxide and sulphuric acid have two main advantages:-

a) A precise, evenly graded series of humidities may be obtained and being strongly hygroscopic these materials may be used to produce extremely low humidities.

b) Water activities are nearly constant over a wide range of temperature.

These solutions were considered unsuitable for the present experiments on two counts:-

a) Loss or gain of water results in a change of water activity, causing handling and aeration problems.

b) Acid vapours may have a corrosive effect on the materials being tested.

These problems may be overcome by using glycerin solutions (A.S.T.M. 1974) but this method was impracticable due to the non-availability of a refractometer for regular checking.

# Chapter 3

## THE MEASUREMENT OF RELATIVE HUMIDITY

3.1 Introduction

3.2 Development and construction of an electrical hygrometer based on Peltier cooling.

3.3 Conclusion

## 3.1 Introduction

It was found that during the construction of small constant humidity chambers (both controlled by saturated salt solutions and also air-mixing) a small and accurate hygrometer would be required to monitor the high relative humidities produced. Several possible methods were considered but all were dropped on grounds of size of sensor, inaccuracy at high humidities, need for high ventilation rates or simply expense.

It was decided to build a simple hygrometer to overcome the above problems and the method used was based on that of Doe (1966) using the Peltier cooling effect.

Water vapour is present continuously in our environment but unlike the other components of air it has no fixed proportions; it varies widely dependent on temperature and pressure. The amount of water vapour in air may be specified either by the water content or by the water vapour pressure exerted by it.

"The most accurate method of measuring the humidity of air and other gases still remains the determination of the dewpoint", (Gethersen et al. 1960). Dewpoint is the temperature at which saturation occurs when air is cooled without change in water content. This is a very useful parameter as it specifies temperature and 100% R.H. simultaneously. Changes in temperature above the dewpoint

do not affect the water content, but cooling below the dewpoint removes moisture from the air by condensation on cooler surfaces.

There are numerous possible methods for measuring humidity in a small space which have been fully reviewed by other workers notably Wexler (1957), Hickman (1958), Ayerst (1965), Szulmayer (1969) and Gough (1974).

The wet and dry bulb thermometer was not adopted for continuous monitoring because ventilation and size problems would have made it unsuitable for use in small environment tanks.

It was such practical problems which led to the development of electric hygrometers. These may be defined as instruments for determining the moisture content of air (or any gas) by the measurement of change of resistance with change in humidity, (Wexler, 1957).

Peltier Effect

Prior to 1957 dewpoint methods for measuring relative humidity suffered from three major drawbacks:

1) A degree of uncertainty in precise visual recognition of the point at which dew appeared and disappeared, which made the measurements dependent on the skill of the observer.
2) Difficulties in accurate temperature determination at the moment of dew formation.

3) Maintainance of a contamination-free surface.

At this time dewpoint method had been of resistricted use in the laboratory, as no easy means of cooling to dewpoint had been discovered. But prolonged research in the field of semi-conductors led to the use of a very simple thermoelectric cooling system utilizing the Peltier effect.

In 1834 Peltier discovered a new thermoelectric cooling system.

He found that when a current was passed through a junction between two different conductors connected in series there was adsorption or generation of heat at the junction depending on the direction of the current. This effect was superimposed upon, but quite distinct from the Joule resistance-heating effect usually associated with the passage of electric current. The quantities of heat absorbed or developed per unit time are proportional to the current. If P is the Peltier coefficient (in volts) and i the current (in amperes) the rate of liberation or adsorption of energy at the junction of Pi watts. But an optimum current for cooling will be obtained, as cooling is eventually counteracted by the Joule resistance-heating effect, the Joule effect being proportional to the square of the current. Furthermore, there is also heat transfer from the warm to the cold junction via the Peltier elements themselves.

If, however, the circuit is opened and the junctions are brought to different temperature, there appears across the terminals of the thermocouple so produced, a potential difference that depends on the temperature of the respective junctions. This phenomenon is known as the Seebeck effect and is commonly used for temperature measurement, (Goldsmid 1960).

Suppose now that the junction of a thermocouple was placed in an atmosphere of very humid air, and a current was passed in the direction required to cool it, then if the degree of cooling was sufficient to bring the junction temperature below the dewpoint, moisture will condense on it and the thermocouple will potentially become a delicate 'wet-bulb' thermometer. The dew will re-evaporate on breaking the circuit and a minute electromotive force (E.M.F.) will be generated proportional to the 'wet-bulb' depression associated with the atmosphere surrounding the junction. If a sensitive galvanometer were connected to the thermocouple system it would be possible to calibrate the instrument.

The mathematical theory of cooling by the Peltier effect is fully explained by Spanner (1951), who utilised the phenomenon in the measurement of plant suction pressure. Spanner used wires of bismuth with 5% tin to give a high thermoelectric effect.

The intensive research into semi-conductors led to

renewed interest in Peltier cooling, leading to the production by Gethersen et al. (1957) of an automatic dewpoint hygrometer. Working on similar lines to the experiments of Spanner, Monteith and Owen (1958) evolved a thermocouple for measuring relative humidity in the range 95-100% R.H. They found that at high relative humidities a current of 30mA. through a 38s.w.g. chromel-p/constantan thermocouple produced sufficient cooling to form a film of water on the junction which could then be used as the 'wet-bulb' of a psychrometer. The wet bulb depression could be read to $0.001^{o}C$ and in the range 97-100% R.H. changes of $\pm$ 0.01% could be detected. Monteith's psychrometer was calibrated over sodium chloride solutions; two major modifications having been made to Spanner's design:

i) The fine bismuth and bismuth-tin wires which were brittle and which could not be obtained commercially were replaced by constantan and chromel.

ii) Instead of a ballistic throw which is a function of dewpoint temperature, a steady wet-bulb depression was obtained. This can be more easily read and could be related to a theoretical value.

A more recent method for measuring humidity in a small space was developed by Doe (1966). This method was

developed primarily for the measurement of vapour concentrations close to an evaporating surface in the mass-transfer boundary layer. Doe managed to produce temperatures as low as 12°F below ambient with bismuth; bismuth-tin thermocouple wires 0.0009-0.0012 inches in diameter.

The most serious defect of the 'ballistic-method' of Spanner seemed to lie in the switching delay and the switching resistances which were difficult, if not impossible to keep constant over long periods. Doe overcame these limitations by complex electronic circuitry which alternately cooled and heated the junctions. The junction was set up in a balanced bridge circuit, a sensitive differential amplifier and oscilloscope combination being used to detect the bridge imbalance caused by condensation of moisture onto the junction.

In 1967 Dalton and Rawlins produced a paper entitled "design criteria for Peltier-effect thermocouple psychrometers". In the past, the design of such psychrometers had relied heavily upon characteristics that were determined empirically. The two most common characteristics were the magnitude of cooling current that gives the maximum temperature depression at the wet junction and the heat capacity of the reference junction necessary to maintain its temperature within selected bounds during this cooling. Spanner (1951) developed equations governing

optimum cooling currents, based on a requirement that the ratio of the radii of the wires of the thermocouple be some constant. Similarly a constraint was placed on the length of the thermocouple wires. "In practice these constraints have been impossible to meet, and so it was necessary to put forward equations governing optimum cooling currents and to present a simple method whereby the necessary heat-transfer coefficient can be determined", (Dalton and Rawlins 1967).

## 3.2 Development and construction of an electrical hygrometer based on Peltier cooling

A major deviation from the methods used by Doe was the use of a n- and p-type bismuth-telluride thermocouple. The theory and importance of n- and p-type configurations was fully explained by van Vessem (1955).

In comparing different thermoelectric materials it is rather convenient to deal with the figure of merit Z. The figure of merit for a single material z being defined as

$$z = \alpha^2 \frac{\sigma}{K}$$

where $\alpha$ = Seebeck coefficient

$K$ = thermal conductivity

$\sigma$ = electrical conductivity

Only in exceptional circumstances is the figure of merit Z strictly equal to the mean of the values of z for

the two elements Zp and Zn respectively. In general Z must be regarded as a rather complicated average of Zp and Zn. The use of an individual figure of merit is justified by the fact that nowadays the values of Zp and Zn are never very much different in the thermocouples which are most suitable for thermoelectric applications.

A comparison of the figures of merit which have been obtained with a number of thermoelectric materials was given by Goldsmid (1960). From his tables it could be seen that the highest figures of merit have been achieved using either the compounds of lead with group VI elements, or the V - VI compounds bismuth telluride and antimony telluride. Doe (1966) stated that above 5°F dewpoint depression, his hygrometer failed to register any further increase in dewpoint depression. This was because the maximum temperature drop obtainable with the bismuth, bismuth-tin thermocouple used was about 5°F, so that beyond this point no moisture condensed on the junction. It may have been possible to increase the range of the instrument by using semi-conductor materials, which have a much larger thermoelectric effect. The Peltier coefficient of a n- and p-doped bismuth telluride couple at 0°C is 100-120mV. whereas that of a bismuth, bismuth-tin thermocouple at 0°C is 30mV.

It was decided to build a small compact sensor using bismuth telluride thermocouples. Two thermocouple junctions

were utilised one acting as a standard against which the other could be compared. The electrical circuitry was considerably reduced in complexity as compared with past experiments, and was based on a carefully balanced resistance bridge circuit.

The activity of the thermocouple junctions has been explained in terms of the Peltier cooling theory. An optimum driving current of 0.8Amps was found to cool both thermocouples equally. The two thermocouple junctions were set up as shown diagramatically in fig.(8). One thermocouple (1) was enclosed by a perforated hemisphere and was exposed to the atmosphere to be monitored, the second thermocouple (2) was completely enclosed. Thermocouple (2) acted as a standard, the humidity of the air inside remaining constant.

As a current was passed water condensed on thermocouple (1) as the junction cooled, this caused it to heat up with respect to junction (2); in fact thermocouple (1) remains at a constant temperature (= dewpoint) whilst thermocouple (2) continues to cool. This temperature difference led to a difference in the resistances of the two junctions; the resistance of junction (1) being higher.

The differing resistances of the thermocouples led to an out of balance voltage in the resistance bridge circuit which could be correlated with relative humidity.

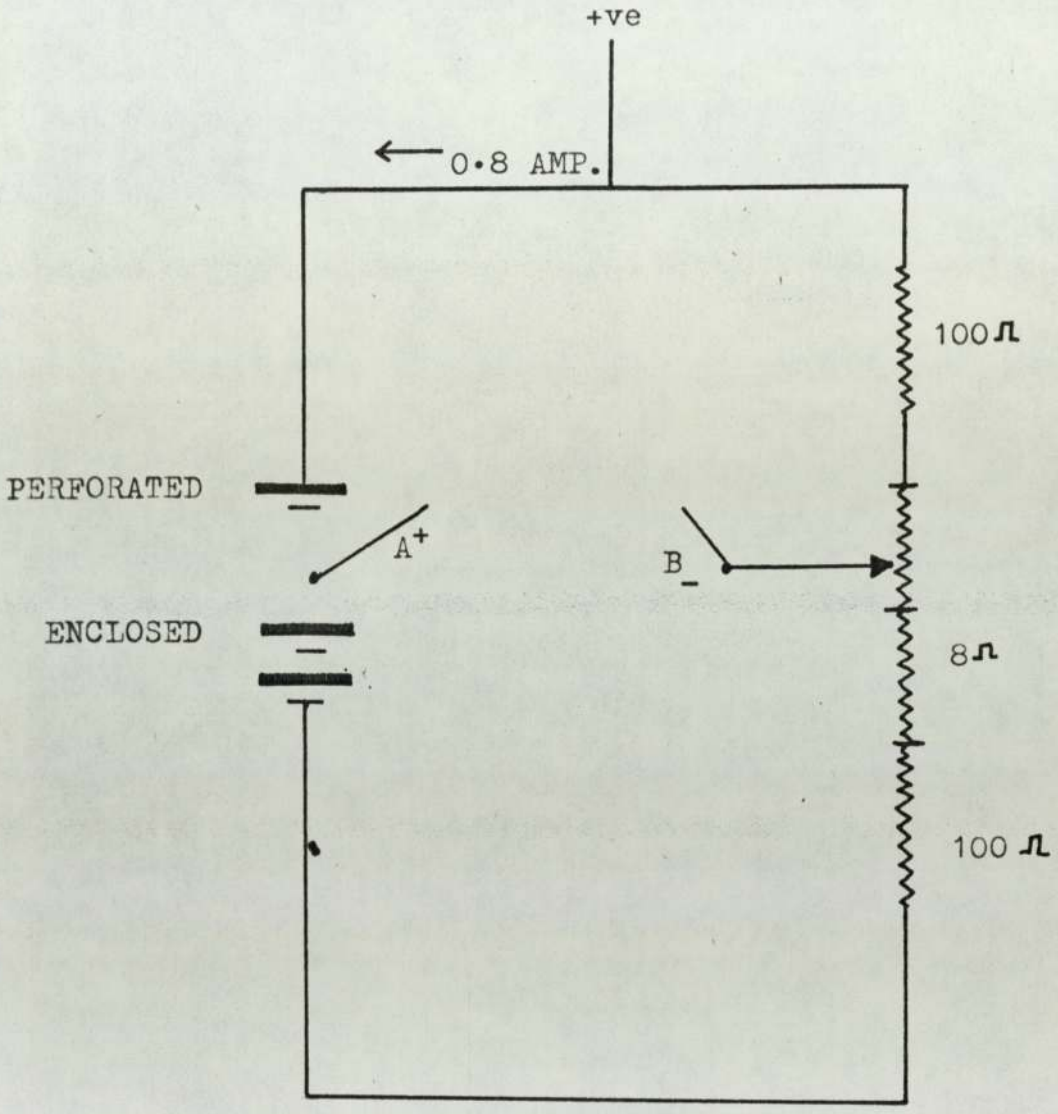



Fig. 3 Representation of Sensor Circuit.

Sensor construction

Bismuth telluride thermocouples of the type required were found to be commercially available only in blocks of forty eight forming components used in the refrigeration industry (supplied by M.C.P. Limited, Wembley). The individual bismuth telluride block p-n-p junctions measured 157 x 60 x 60 ('thou'). In general it is not possible to apply solder directly to thermoelectric materials. For bismuth telluride and its alloys contacts of a sufficiently low electrical resistance can be provided by 'tinning' after electroplating with nickel. Whilst good electrical contacts may be achieved by plating the surface of a semi-conductor, it is difficult to ensure that such contacts are resistant to mechanical strain.

It was necessary to dismantle the commercial product into its basic components to provide the junctions required for the sensor. The blocks were insulated from each other by a thin strip of mica which adhered to the blocks by the use of epoxy resin.

1) The bottom copper contact was removed by quickly and carefully touching with a low wattage soldering iron. Fig. (5).

2) The remaining blocks were carefully aligned on a perfectly plane glass block. With one hand pinning the junctions down with a brass block (to act as a heat-sink) the soldering iron

carefully 'tinned' the required junction plate, whilst a second person using a pair of fine forceps carefully extricated the unrequired block (fig. 5).

3) The thin mica insulating strip was left adhering to the thermocouple block, this was removed by using a razor blade.

4) The block was finally cleaned up using emery paper to remove any surplus epoxy resin.

The block of p-n-p bismuth telluride junctions had a 'cold' and a 'hot' side. By use of a simple test circuit incorporating an avometer the variance in temperature could be found simply by touch.

Formation of hemispheres

The enclosures for the two thermocouple junctions were made out of annealed copper sheet (6 'thou' thick). The hemispheres were fashioned using a mould and ball bearing and a power-press. The hemispheres had the dimensions shown in fig. 7(a).

The outer flange was trimmed and 'tinned' with solder. A central slot was punched in each hemisphere to accomodate the copper leads from the thermocouple junctions. A watchmaker's drill was used to perforate one of the hemispheres using trial and error to find the most suitable number and size of holes. The holes were eventually drilled

Fig. 4 Original arrangement of bismuth telluride blocks.

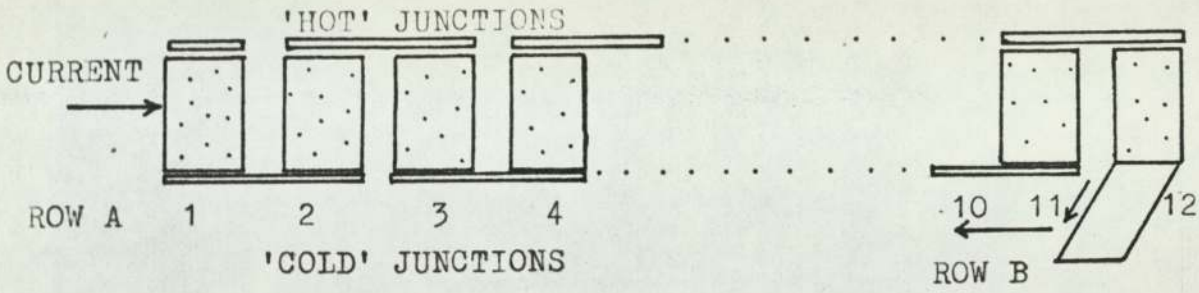



Fig. 5 Final

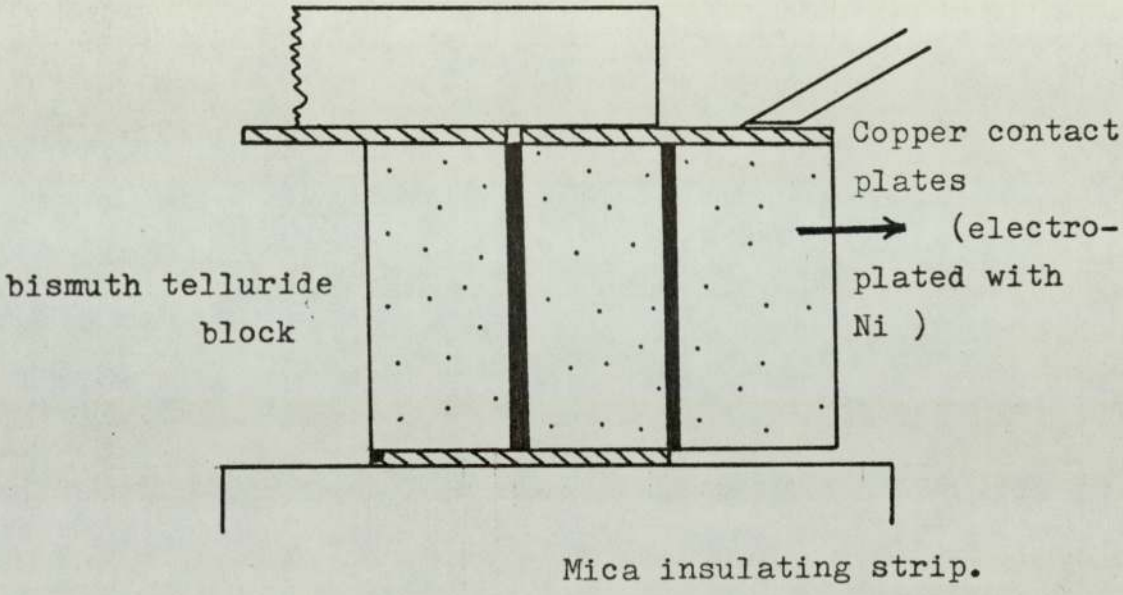
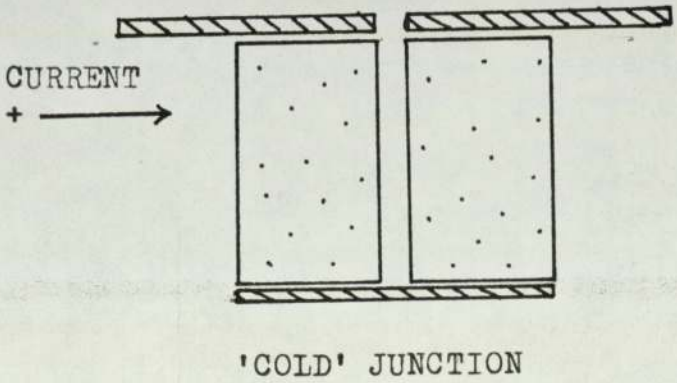



Fig. 6 Final junction for use in sensor.



with a diameter of 22 'thou'; this was found to give adequate diffusion of air into the perforated half of the sensor.

The two hemispheres were separated by a central partition of copper (30 'thou' thick) which acted also as a heat sink to disipate the Joule heating effect and also as an earth.

The partition was 'tinned' as shown in fig. (7b) and the centre was punched to take the thermocouple assembly.

The thermocouple system was assembled as shown in fig. (8). It was constructed on a pegboard to give additional strength and mobility during testing of the circuit. After testing the two hemispheres were carefully slotted and soldered onto the central portion. The gaps where the copper leads entered the hemispheres were carefully sealed with epoxy resin. Wiring and handle formation of the sensor is shown in fig. (7c).

Monitoring Resistance Bridge Circuit

A circuit was devised whereby a very small circuit D.C. imbalance could be monitored. It was decided that a precisely equalisable resistance bridge circuit would be suitable, fig. (9). Owing to the high driving current through the thermocouple a 50 volt transformer coupled with a 'variac' and rectifier was used as a power supply. A

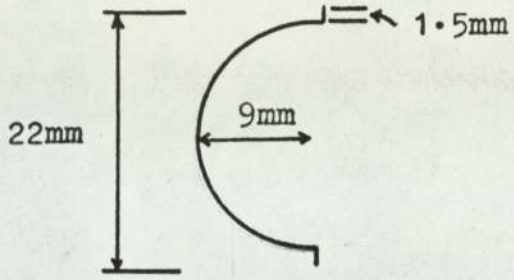



Fig. 7(a)

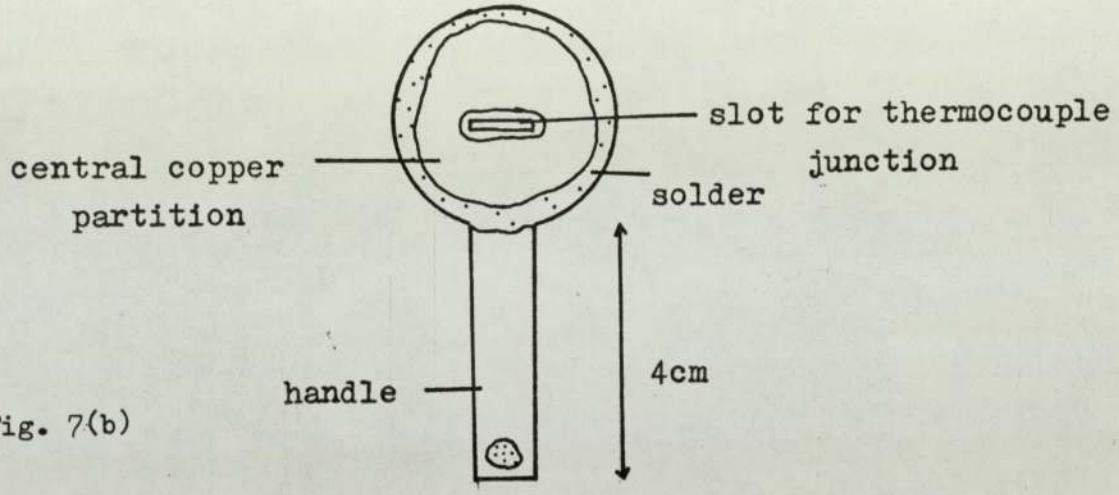



Fig. 7(b)

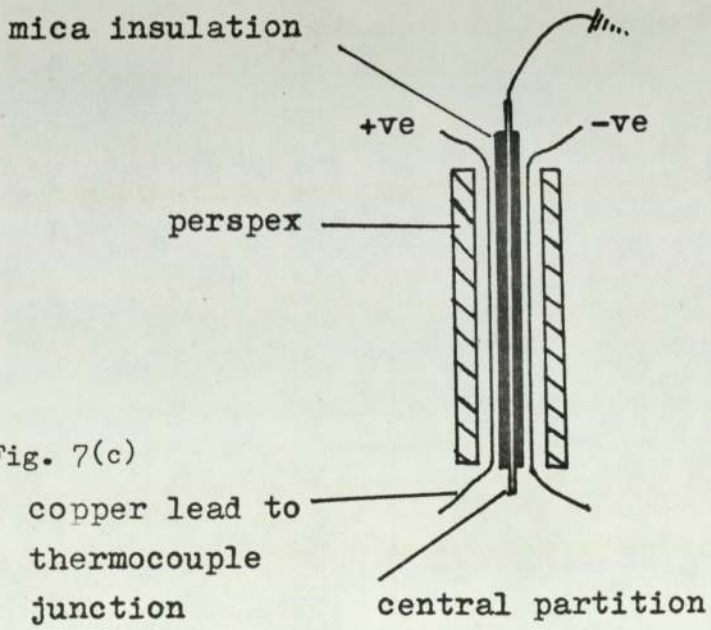



Fig. 7(c)

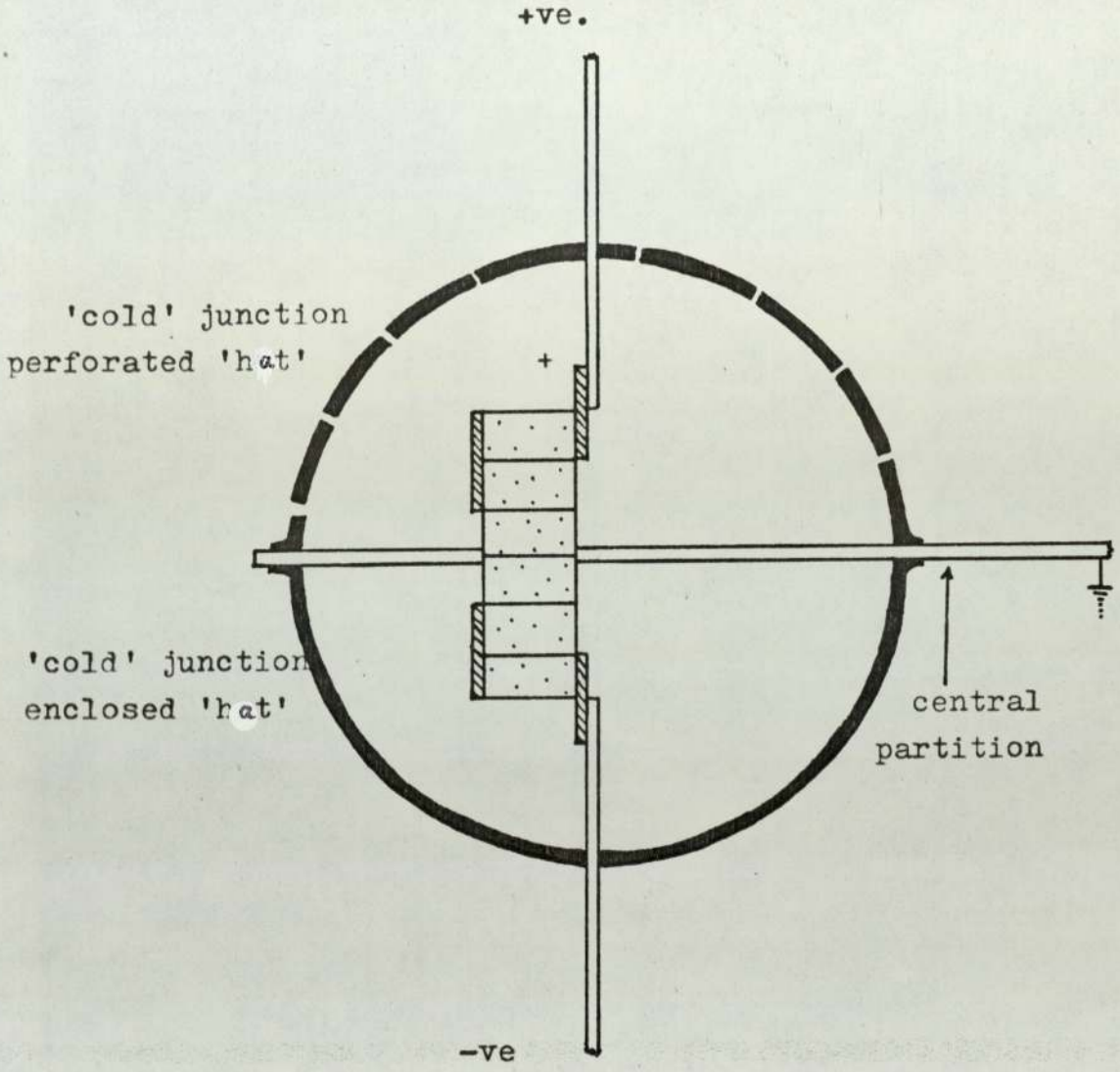



Fig. 8 Bismuth telluride thermocouple sensor.

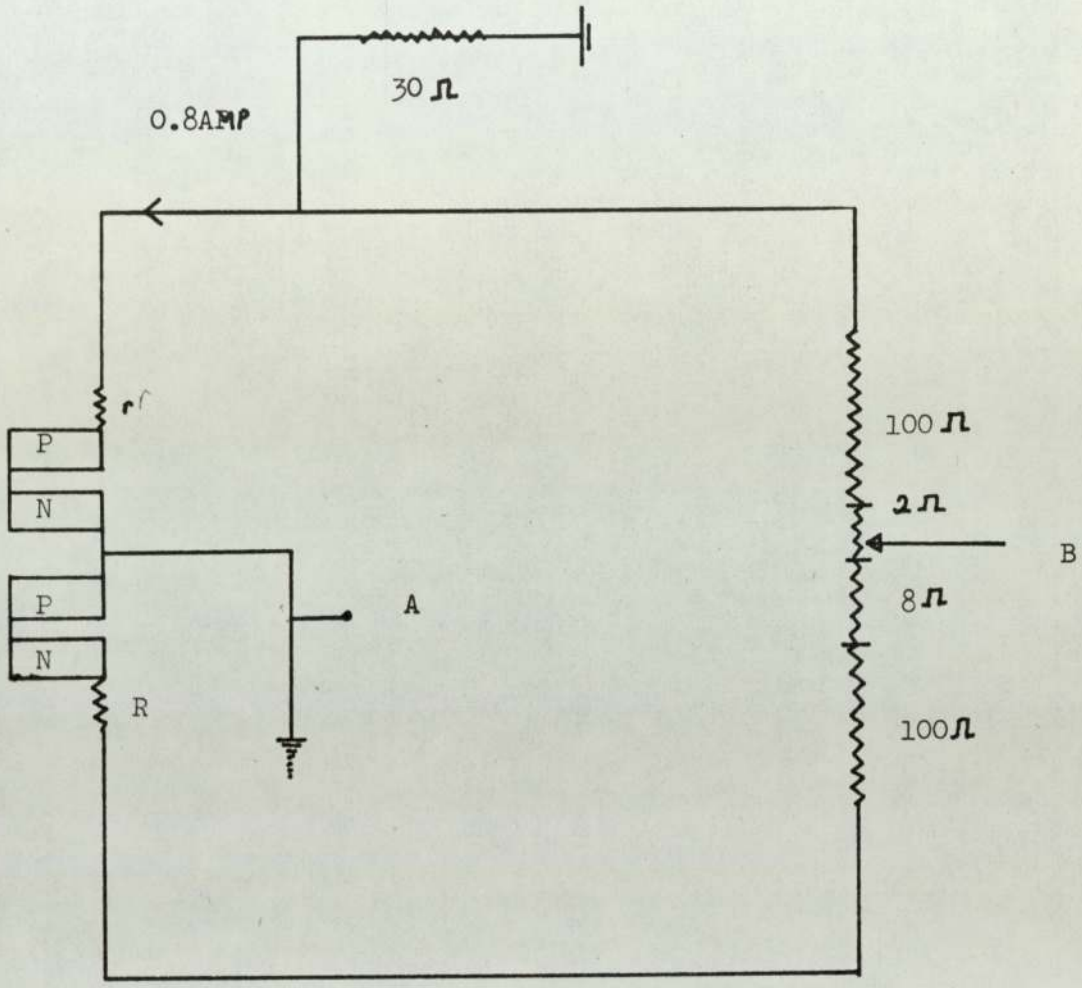



Fig. (9) Resistance Bridge Circuit

30ohm resistance was incorporated into the circuit to protect the thermocouple junctions. The resistances of the two junctions were almost identical, the junction having the slightly higher resistance being encased in the enclosed hemisphere. The two balanced 100ohm resistances were hand-wound using 'Eureka' wire and were precisely equal. A small potentiometer capable of producing a variable 2ohm resistance was incorporated to enable precise balancing of the circuit. The current imbalance in the two halves of the circuit due to the changed resistances of the thermocouple open to the atmosphere was extremely small, so amplification was required fig. (10). It was calculated to give a thousand-fold gain; but the actual gain, as estimated using an oscilloscope, was in the region of 3,000. Thus it was possible to discriminate a sharp imbalance caused by a temperature differential between the two thermocouple junctions, in the order of 1/30th$^{\circ}$ C. The amplifier was housed in a separate box and had an independent battery power source and switch. Wiring from the sensor to the bridge circuit, and from the bridge circuit to the amplifier, was encased in coaxial sheathing to cut out electrical 'noise' and to make assembly easier. The sensor was earthed from its central partition to the outer casing of the amplifier. The amplifier incorporated a 'twiddler' potentiometer to enable the meter to be zeroed.

The small differential currents were thus amplified and read on a sturdy centre-zeroed galvanometer. The galvonometer

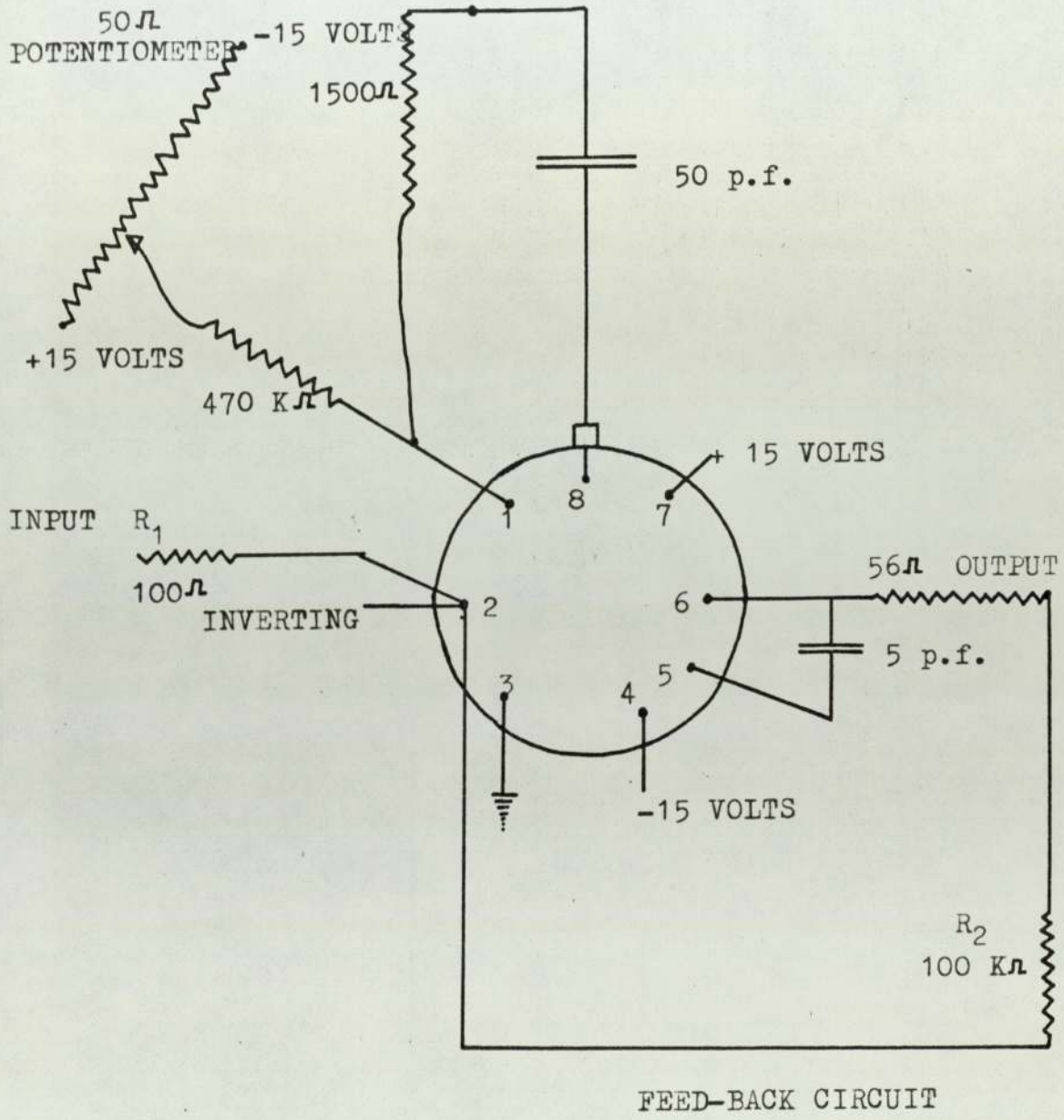



Fig. (10) Amplifier Circuit using μ709c Integrated Circuit

readings could be calibrated by measuring current imbalances produced over saturated salt solutions giving a standard relative humidity.

## 3.3 Conclusion

The dual thermocouple system was capable of discriminating very small temperature changes and was therefore capable of distinguishing very slight relative humidity changes in a confined environment.

There was, however, a problem in the stability of the apparatus caused to a large extent by the considerable amount of electrical apparatus in use in the laboratory. This instability would have required some modification to the basic circuit which would have over-run the available time for the project. As the hygrometer work was progressing the emphasis of the project had been shifting towards the involvement of *Streptomyces spp.* in test-work procedures. The apparatus was designed primarily to work in the range 60 - 10% R.H. allowing fungal growth, using the air-mixing system. The experiment was curtailed at this point; in this form it does, however, present a simplified accurate means of measuring high humidities within a restricted environment.

This resulted in the more laborious method of weight change being adopted to monitor saturated salt solutions; and the wet and dry bulb thermometer plus hair hygrometer being used in the air mixing tanks.

## Chapter 4

# FUNGAL AIRSPORA ISOLATIONS AND THE EFFECT OF RELATIVE HUMIDITY ON FUNGAL COLONISATION OF STARCH AND CELLULOSE SUBSTRATES

4.1 Introduction

4.2 Airspora Isolations - Outdoor Survey

4.2.1. Airspora isolations - Indoors

4.3 Effect of relative humidity on fungal colonisation of starch and cellulose substrates

4.4 Cellulolytic and amylolytic properties of fungi isolated from the air

## 4.1 Introduction

A series of airspora isolations were carried out to discover the dominant fungal species present in Birmingham, capable of colonising starch and cellulose substrates. Tests were performed to ascertain the growth rates of fungi on starch and their ability to degrade cellulose was measured.

These tests were performed for several reasons:

a) To test the performance of the air-mixing system and its ability to provide a sterile testing environment.

b) To give a yardstick against which the amylolytic and cellulolytic abilities of the Streptomyces spp. isolated could be correlated.

c) Pure culture techniques are prone to the vagaries of culture media, fungal strains and incubation conditions, and thus it was thought better to use first hand information rather than rely on past literature.

Actinomycetes have been infrequently recorded in air sampling experiments primarily because of the methods employed, which rely to a large extent on recognising species present on the basis of spore morphology. Observations relating to actinomycetes have been restricted to specific situations such as farm buildings where they were expected to be encountered in large concentrations. Streptomyces spp.

in particular were investigated, because from a survey of available literature it appeared that they could play a significant role in the degradation of textile cellulose and their starch finishes. It was also thought that the streptomycetes, particularly Streptomyces spp. might be capable of growing at lower relative humidities than the dominant fungal colonisers and comparative tests were performed to test this hypothesis using both standard saturated salt solution proceedures and the air mixing system described in Chapter 1. The results obtained for fungal isolate growth at a series of relative humidities are outlined in this chapter. The number of types of fungi isolated from the air and their cellulolytic ability were compared with the list of standard test organisms used by the International Biodeterioration Research Group for textiles to assess the relevance of the species selected to routine textile testing.

A two year survey was carried out on the airspora in Birmingham to estimate the percentage of Streptomyces spp. and to outline the potential deteriogens. Sampling was carried out by exposing Petri dishes containing starch or cellulose agars. Gregory (1974) has outlined the problems concerned with gravity sedimentation of spores onto plates as follows:

Advantages

(1) Plate sampling is convenient as large scale surveys may be carried out.

(2) Only visible propagules are recorded, these can be easily differentiated

after growth. With alternative sampling methods such as the Hirst spore trap, only limited differentiation of spores is attained.

Defects

(1) Sensitivity to particle size

(2) Wind speed effects

(3) Aerodynamic effects

(4) Continuous sampling is impracticable and so diurnal changes of airspora are not revealed.

(5) Sampling is restricted to viable propagules so an overall picture of spore concentrations cannot be obtained.

Many sampling experiments have been carried out on airspora Feinberg et al. (1946), Hyde and Williams (1949), Richards (1956) and Gregory (1961) being examples of isolations. Feinberg, however, is the only worker to have recognised and given a percentage to the streptomycete population in the air, in such surveys. Gregory (1973) has reviewed the whole sphere of aerobiology and his writings are regarded as the standard reference work on the topic.

Isolations indoors were also performed, but are governed by an entirely different set of principles. Outdoor air moves as wind flowing bodily over surfaces and a point near the ground is immersed in a continually flowing system of fresh air. Rooms on the other hand are ventilated and fresh air is assumed to

mix thoroughly with the existing air instead of displacing it bodily. Within a building the temperature of the air may be less changeable than outside and this may lead to characteristic air patterns. Warmer walls will generate an updraught, colder walls a downdraught. Each draught will be balanced by opposite currents in the centre of the building, often moving fast enough to counteract sedimentation under the influence of gravity (Gregory, 1973).

Davies (1960) carried out extensive isolations of fungi from house dust samples, but did not mention Streptomyces spp. as being present.

The air mixing controlled humidity system was employed to determine the minimum relative humidity required by the isolated species for vegetative growth. The relationship between fungal spore germination and growth and relative humidity is one of immense importance in biodeterioration, and has fascinated many workers, Tompkins (1929), Galloway (1935), Snow (1944), Bonner (1948), Block et al. (1961), Davies (1960) and Ayerst (1968 b) being amongst the principal ones. Spo re germination has been the criterion in practically all cases and experiments have invariably been performed under the artificial static air conditions produced by saturated salt or sulphuric acid solutions. Using continuous air mixing and random air sampling in conjunction requires growth and sporulation of the colonising propagules to occur before identification can proceed, therefore these experimental results give limiting R.H.'s for active colonisation. In

different moulds the relationship between spore production and total fungal growth may be very different and at low humidities mycelial growth may occur without spore production. In competitive colonisation on substrates, such as starch and cellulose, in moisture equilibrium with a subsaturated atmosphere, there can be no appreciable diffusion of water-soluble metabolites. The antagonistic effects resulting from the mutual development of a number of fungal species will therefore be greatly diminished, and it must follow that competition between colonising fungi, on a starch film for example, is less severe than that experienced by the same species developing in an agar medium in a Petri dish.

The cellulolytic abilities of fungal deteriogens were estimated using the technique of Rautella and Cowling (1966) who used clearing depth in test tubes of cellulose agar as a measure. They obtained relatively high coefficients of correlation (>0.6) between depth of cellulose clearance, cotton weight and tensile strength loss and carboxy methyl cellulose activity in culture filtrates. Additional screening of fungal cellulolytic ability has been carried out using the same technique at Aston by Malik (1970). The results obtained from the following experiments can be compared to the clearances obtained using isolates of airbourne Streptomyces spp, Chapter 5.

The International Biodegradation Research Group are carrying out pure culture experiments in an attempt to standardize the testing of cotton textiles. They have

employed twelve standard fungi in their test proceedures and it was interesting to see how frequently these species occurred in the airspora and also to see if the Streptomyces spp. should be taken into account in these tests, as their presence had frequently been noted by the sponsors in their routine laboratory test situations.

## 4.2 Airspora Isolations

Isolations were carried out over a period of two years 1972 - 1974 outdoors in Birmingham. Starch and cellulose agar plates were used as the isolating media. Plates were exposed horizontally at a height of 3ft. for two minutes and were then incubated at 25° C for fourteen days. The results given in figs. 11-4 are based on the percentage number of plates colonized by each individual species, the sample size was never less than twenty plates. Using Petri dish sampling means that the resulting percentages can only indicate the species present and no accurate idea of spore concentrations can be obtained from these figures. Quantitative data on spore concentrations would be misleading with such a relatively limited survey as climatic factors play an important part in the presence of spores in the atmosphere.

The results do, however, show several trends which are in agreement with those found in similar surveys.

Cladosporium herbarum was the most common airbourne species, showing a marked mid-summer peak. Aspergillus and Penici llium species were found to occur more frequently between September and May; whereas Botrytis cinerea had an autumnal peak.

Streptomyces spp. were found to occur on a number of plates being as high as 45% in February 1973; this is a far larger figure than found by Feinberg et al. (1946) and makes their absence from other surveys more difficult to comprehend. Streptomyces spp., however, were not isolated during the months of June and July, this may be explained by this period corresponding to the peak spore concentrations of Cladosporium herbarum. Samples during this period would become almost completely colonised by fungal species within 3 days and so nutrients would become depleted before the slower growing Streptomyces spp. could utilize them.

Notable absentees from the species isolated were Epicoccum, Pullularia, Oospora and Stemphylium. Richards (1956) noted that Epicoccum and Pullalaria were more commonly found in rural as opposed to urban environments, and this could be the reason for their non-appearance.

The sampling area was close to a stretch of lawn and so the rather large numbers of plant pathogens including Botrytis cinera, Fusarium roseum, Gliomastix murorum, Helminthosporium sp. and Phoma spp. were not unexpected.

| | 14/2 | 1/4 | 31/4 | 25/5 | 15/6 | 6/7 | 25/7 | 8/8 | 22/8 | 19/9 | 30/9 | 17/10 | 21/11 | 14/12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alternaria sp. | - | - | - | - | - | - | - | 5 | 5 | 5 | - | - | 5 | - |
| Aspergillus fumigatus | 5 | 15 | 5 | 17 | 5 | 10 | - | - | 9 | 5 | - | - | - | - |
| Aspergillus niger | 20 | 10 | 5 | - | - | - | - | - | - | - | - | - | - | - |
| Aspergillus ochraceum | 20 | 20 | - | - | - | - | - | - | - | 5 | - | - | - | - |
| Aspergillus terreus | - | 5 | - | - | - | - | 6 | - | - | - | - | - | - | - |
| Aspergillus versicolor | 20 | 20 | - | - | - | - | - | - | - | 5 | - | - | - | 6 |
| Botrytis cinerea | 15 | 15 | 40 | 50 | 75 | 70 | 50 | 72 | 61 | 74 | 43 | 60 | 53 | 11 |
| Chaetomium globosum | - | - | - | - | - | - | - | - | - | - | - | - | 5 | 6 |
| Cladosporium sp. | 95 | 95 | 95 | 91 | 100 | 100 | 100 | 100 | 100 | 100 | 91 | 65 | 68 | 81 |
| Fusarium roseum | 10 | - | 5 | 5 | 20 | 20 | 20 | 18 | 9 | 11 | 10 | 11 | 20 | 5 |
| Gelasinospora sp. | - | - | - | - | - | - | - | - | - | - | 9 | - | - | - |
| Gilmaniella humicola | - | - | - | - | - | - | - | - | - | 5 | - | - | 5 | - |
| Gliocladium roseum | 5 | 5 | 10 | 12 | 20 | 10 | 12 | - | 22 | - | 13 | - | - | 6 |
| Gliomastix murorum | - | 10 | 15 | - | - | 10 | 37 | 18 | 23 | 5 | 22 | - | - | - |
| Graphium sp. | - | - | - | - | - | - | 6 | - | - | - | 17 | - | 5 | - |
| Helminthosporium sp. | - | - | - | 4 | 15 | - | - | - | 13 | 11 | - | - | 5 | - |

Fig. 11. Airspora isolated outdoors Birmingham 1972-73. Results given as percentage of plates colonised.

| | 14/2 | 1/4 | 31/4 | 25/5 | 15/6 | 6/7 | 25/7 | 8/8 | 22/8 | 19/9 | 30/9 | 17/10 | 21/11 | 14/12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Humicola grisea | - | - | 5 | - | 10 | - | 6 | 9 | 4 | - | - | - | 5 | - |
| Mucor sp. | - | - | - | 8 | 5 | 15 | - | - | - | 5 | 30 | 5 | 5 | - |
| Neurospora sp. | - | - | - | - | - | - | - | - | - | 5 | - | - | - | - |
| Olpitrichum sp. | - | - | - | - | - | - | - | - | - | - | 9 | - | 5 | - |
| Paecilomyces sp. | 10 | 10 | 5 | - | - | - | - | - | - | - | - | - | - | - |
| Pencillium sp. | 65 | 65 | 25 | 12 | - | - | - | - | - | 68 | 26 | 30 | 17 | 21 |
| Phialocephala sp. | - | - | - | - | - | 10 | - | - | - | - | 5 | - | 5 | - |
| Phoma sp. 1 | 20 | 20 | 15 | 28 | 35 | - | 12 | - | 9 | 50 | 39 | 25 | 38 | 16 |
| Phoma sp. 2 | 5 | 5 | - | 8 | 5 | 10 | 6 | - | - | 6 | - | 11 | 17 | - |
| Phoma sp. 3 | - | - | - | - | - | - | - | - | - | - | - | - | 5 | - |
| Sordaria sylvatica | 10 | 10 | 5 | 4 | - | - | - | 9 | - | 5 | 35 | - | - | - |
| Stysanus sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Trichoderma viride | 20 | 10 | - | - | 5 | 5 | 6 | - | 9 | 11 | 9 | - | - | - |
| Trichothecium roseum | 10 | 10 | 15 | - | 10 | 5 | 6 | - | 9 | - | - | - | - | - |
| Zygorynchus moelleri | - | - | - | - | - | - | - | - | - | - | - | - | 11 | - |
| Streptomyces sp. | 35 | 10 | 25 | 28 | * | * | 5 | * | * | 28 | 30 | 11 | 17 | 16 |
| Mycelia sterilia | 10 | 10 | 85 | 87 | 100 | 100 | 100 | 100 | 100 | 74 | 70 | 70 | 89 | 36 |

Fig. 12 Airspora isolated outdoors, Birmingham 1972 - 73 Results given as a percentage of plates colonised.

| | 9/1 | 26/2 | 12/3 | 16/4 | 14/5 | 1/6 | 29/6 | 10/7 | 27/7 | 3/8 | 29/8 | 5/9 | 28/9 | 18/10 | 7/11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alternaria sp. | - | - | 5 | - | - | 10 | - | - | - | - | - | 5 | - | 10 | - |
| Aspergillus fumigatus | 27 | 8 | - | 10 | - | 12 | 5 | - | - | - | - | 5 | 5 | - | - |
| Aspegillus niger | - | - | 5 | - | - | - | - | - | - | - | - | - | - | 5 | - |
| Aspergillus ochraceum | - | - | 10 | - | - | - | - | - | - | - | - | - | - | - | 5 |
| Aspergillus terreus | - | - | - | 5 | - | - | - | - | - | - | - | - | - | - | - |
| Aspergillus versicolor | - | 8 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Botrytis cinerea | - | 18 | - | 40 | 65 | 45 | 50 | 58 | 61 | 75 | 70 | 75 | 70 | 64 | 25 |
| Chaetomium globosum | 11 | 28 | - | - | 5 | - | - | - | - | - | 5 | - | - | 10 | 5 |
| Cladosporium sp. | 17 | 48 | 70 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 95 | 100 | 75 | 60 | 40 |
| Fusarium roseum | 5 | 8 | 10 | 10 | 15 | 15 | 20 | 20 | 20 | 15 | 5 | 20 | 10 | 5 | 5 |
| Gelasinospora sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Gilmaniella humicola | - | - | 5 | - | - | - | - | - | - | - | - | - | - | 5 | - |
| Gliocladium roseum | - | - | 15 | - | 5 | 18 | - | 5 | - | - | 20 | - | 5 | 5 | - |
| Gliomastix murorum | - | - | 5 | - | 17 | - | - | - | - | 15 | 10 | - | - | - | - |
| Graphium sp. | - | - | - | 5 | - | - | - | - | - | - | 20 | - | 10 | - | - |
| Helminthosporium sp. | - | - | - | - | - | - | - | 17 | - | 10 | - | 5 | 5 | - | - |

Fig. 13 Airspora isolated outdoors, Birmingham 1973 - 74 Results given as a percentage of plates colonized

| | 9/1 | 26/2 | 12/3 | 16/4 | 14/5 | 1/6 | 29/6 | 10/7 | 27/7 | 3/8 | 29/8 | 5/9 | 28/9 | 17/10 | 7/11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Humicola grisea | - | 8 | - | 5 | - | - | - | - | - | 5 | - | - | 5 | - | - |
| Mucor sp. | - | - | 5 | - | - | 5 | - | - | - | 5 | - | 30 | 15 | - | - |
| Neurospora sp. | - | 18 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Olpitrichum sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Paecilomyces sp. | - | 8 | - | - | - | - | - | - | - | - | - | - | 15 | 5 | 5 |
| Penicillium sp. | 11 | 57 | 50 | 35 | 10 | 10 | - | - | 10 | - | - | 15 | 45 | 40 | 35 |
| Phialocephala sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Phoma sp. 1 | 5 | 85 | 30 | 5 | - | 30 | - | - | 15 | - | 25 | 45 | 40 | 20 | 20 |
| Phoma sp. 2 | 11 | 69 | - | 5 | - | 5 | 5 | - | - | - | - | 20 | 5 | 5 | - |
| Phoma sp. 3 | - | 28 | - | - | - | - | - | - | - | - | - | 5 | - | - | - |
| Sordaria sylvatica | - | - | 15 | - | 5 | - | - | - | - | - | - | - | 5 | - | 5 |
| Stysanus sp. | 5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Trichoderma viride | - | - | 5 | - | 10 | 5 | - | - | 5 | - | 15 | - | 5 | - | 5 |
| Trichothecium roseum | - | - | - | - | 5 | 10 | 5 | - | 5 | - | - | - | - | - | - |
| Zygorynhus moelleri | - | - | - | 5 | - | - | - | - | - | - | - | - | - | - | - |
| Streptomyces sp. | - | 45 | 30 | 10 | 25 | 20 | • | • | • | • | 5 | 30 | 30 | 5 | 25 |
| Mycelia sterilia | 5 | 45 | 50 | 85 | 100 | 100 | 100 | 100 | 100 | 75 | 70 | 95 | 35 | 50 | 40 |

Fig. 14 Airspora isolated outdoors, Birmingham 1973-74 Results given as a percentage of plates colonized.

Some species having a high isolation percentage from soil were found to be scarce in airspora isolations, this applied particularly to Chaetomium globosum, Mucor spp. and Trichoderma viride. This may reflect to some extent a preferential isolation of certain species by standard soil isolation techniques Waksman (1916), Warcup (1950). This was particularly noticeable with Trichoderma viride, a short experiment using starch films and starch agars seemed to show that the agar had a preferential effect on the isolation of this species.

The I.B.R.G. textile group recommends the use of twelve standard species in pure culture testing of fabrics. It may be seen from fig. (15) that only seven of these species were present in the airspora survey, and of those Aspergillus niger, Chaetomium globosum, Paecilomyces variotti and Trichoderma viride only make up a very small percentage of the spores isolated. Cladosporium herbarum, Botrytis cinera, Phoma sp., Streptomyces spp., Fusarium roseum and Gliocladium roseum, which together with Penicillium spp., were the most frequently isolated outdoor airspora were not included in the I.B.R.G. list of test species. Though as can be seen from the comparison table the I.B.R.G. species tend to be more cellulolytic and thus give a more stringent test. Aspergillus and Penicillium spp. are given heavy weighting by I.B.R.G. as these species are the most competitive at low relative humidities are are certain to form the dominant population of deteriogens at R.H.'s in the range 60 - 85%.

| Species of fungi used in I.B.R.G. testwork | Species most frequently found in two year airspora outdoor isolations | Species most frequently isolated indoors |
|---|---|---|
| Aspergillus niger (3)[x] | Cladosporium herbarum (3) | Cladosporium herbarum (3) |
| Aspergillus amstelodami (-) | Botrytis cinerea (17) | Penicillium spp. (5) |
| Aspergillus flavus (5)[+] | Penicillium spp (5) | Mycelia sterilia (-) |
| Chaetomium globosum (10) | Phoma spp. (5) | Streptomyces spp (0-5) |
| Memnoniella echinata (-) | Streptomyces spp. (0-5) | |
| Myrothecium verrucaria (13)[+] | Mycelia sterilia (-) | |
| Paecilomyces varioti (0) | Gliocladium roseum (9) | |
| Penicillium varioti (-) | | |
| Penicillium brevicompactum (-) | | |
| Penicillium funiculosum (-) | | |
| Stachybotrys atra (-) | | |
| Trichoderma viride (14) | | |

Fig. (15) Comparison of the species used in I.B.R.G. tests with the species most commonly found from airspora isolations.

x Figures in brackets after species denotes their ability to clear cellulose, (measured in mm. by Rautella and Cowling tubes).

(-) No data + Ref. Rautella and Cowling (1966)

4.2.1 Indoor Isolations

A small number of indoor isolations were performed to see if viable Streptomyces spp. were present in the atmosphere. Isolations were again performed using Waksman Starch agar[1] and Cellulose agar ( + Eggins and Pugh salts).[1] These plates were exposed for 2 minutes and incubated at 25° C for fourteen days. The figures given in fig. (16) summarise the colonies found on one sampling series of plates in mid-June.

Being less spores present it was possible to determine if the substrate had a preferential effect on the numbers of Cladosporium herbarum and Penicillium spp. which were isolated. The difference between starch and cellulose media was found to be statistically insignificant ($p > 0.05$).

The range of species isolated was found to be considerably reduced and did not compare with those of Davies (1960) who sampled house dust as opposed to air. He found that if Cladosporium was left out of consideration, (he found negligible colonisation), the dust spora reflected that of the outdoor atmosphere; and concluded that in dust Cladosporium spores lost their ability to germinate. Davies recorded twenty seven species of fungi but these did not include a single Streptomyces sp. The species he most commonly obtained were Penicillium, Phoma, Mycelia sterilia, Pullularia, Mucor, Aspergillus and Stemphylium.

[1] Appendix III

Fig. 16 Fungi isolated indoors

| Species | Colonies | % of total |
|---|---|---|
| Aspergillus fumigatus | 6 | |
| Aspergillus niger | 1 | |
| Botrytis cinerea | 1 plate | |
| Chaetomium globosum | 1 plate | |
| Cladosporium herbarum | 358 | 66.5 |
| Mycelia sterilia | 15 | 2.5 |
| Gliocladium sp. | 2 | |
| Humicola grisea | 3 plates | |
| Mucor sp. | 6 plates | |
| Penicillium spp. | 151 | 28.5 |
| Phoma ap. | 2 plates | |
| Streptomyces spp. | 14 | 2.5 |
| Trichoderma sp. | 1 | |

Davies used the dilution plate technique and was thus able to isolate a far wider range of organisms. Phoma sp. germinated very infrequently at R.H.'s <97.5% and were found to be unable to colonise house dust in competition with other moulds.

## 4.3 Effects of relative humidity on fungal colonisation of starch and cellulose substrates.

A series of isolations were conducted to determine the minimum relative humidity at which fungal species could actively colonise starch and cellulose agar films. Very thin agar plates were poured and these were incubated at 50° C for two days prior to exposure to the outdoor atmosphere, thus a low moisture content film was being used as the substrate.

After 2 minutes exposure the plates were placed in controlled humidity tanks and incubated for 14 days. Fig. (17) shows the number of colonies of Cladosporium herbarum produced on an average of eight replicates after three days incubation at 25° C. From the graph it can be seen that Cladosporium had a minimum relative humidity limit of 84%. The R.H. of the continuous air-mixing tanks was regularly monitored and the silica gel drying agent was replaced daily to avoid significant changes in the atmosphere.

The results show a steady increase in the number of germinating colonies with increase in relative humidity.

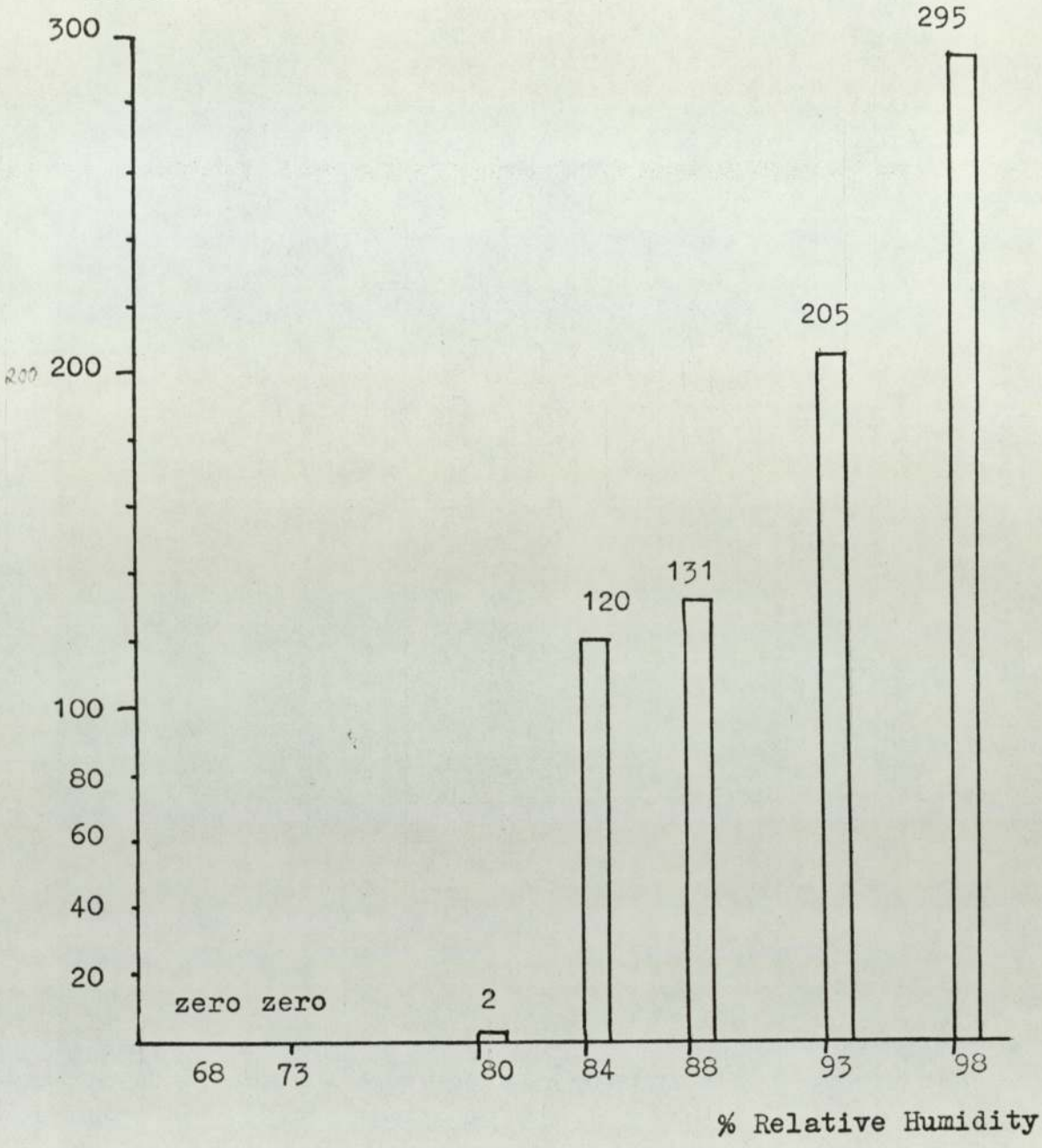



Fig. (17)

Growth of Cladosporium spp. under controlled humidity in continuous air-mixing system.

* Each colony count was an average of eight replicate plates having been exposed for two minutes and incubated at 25°C for 3 days.

The figures compare favourably with those of Snow (1949) and served to demonstrate the ability of the air mixing system to produce the required relative humidity.

It was thought that the excellent germination rate of Cladosporium herbarum at its minimum humidity tolerance level may have been due to the nature of the inoculum. In germination experiments spore suspensions are generally used whereas a natural inoculum may be mycelial in form. The age of the fragments deposited on the exposure plates may allow the species to grow at a low humidity under ideal nutrient conditions.

Results given in figure (18) record the lowest humidities at which the airbourne fungal isolates actively colonised the substrates. A series of twenty tests were performed each having a duration of fourteen days, each test comprising of forty isolation plates.

The plates when exposed in the conditioning tanks were open to the atmosphere of the tank; controls were carefully inspected for contamination. The substrates used were wheat starch agar (+ E and P salts) and 4% cellulose agar (+ E and P salts).

All figures given are for active visual growth producing sporulation, otherwise identification would have been impossible. The majority of species (13 out of 16) were found to be incapable of growth below 85% R.H.

| Minimum relative humidity for active colonisation | Fungal species |
|---|---|
| R.H. 95% | Botrytis cinerea |
| | Gliocladium roseum |
| | Mucor globosum |
| | Trichothecium roseum |
| 90% | Fusarium roseum |
| | Gliomastix murorum |
| | Helminthosporium sp. |
| | Phoma spp. |
| | Streptomyces spp. |
| 85% | Aspergillus fumigatus |
| | Aspergillus niger |
| | Cladosporium herbarum |
| | Trichoderma viride |
| 80% | Aspergillus versicolor |
| | Chaetomium globosum |
| 75% | - |
| 70% | Penicillium spp. |

Fig. (18) Minimum R.H.'s at which active colonisation of starch and cellulose substrates occurred using the continuous air-mixing system to produce the controlled atmosphere.

Only Aspergillus versicolor and Chaetomium globosum both isolated infrequently could grow down to 80% R.H.; the important colonisers at low humidities being Penicillium spp. at 70% R.H.

Streptomyces spp. were able to grow in competition with fungal species to a minimum of 90% R.H. Evidence of growth of Streptomyces spp. colonies was corroborated in two ways, because at 90% R.H. the colonies produced no aerial mycelium. Substrate mycelium was differentiated from that of true bacteria by means of microscopical and staining methods. This was double checked by removing plates to a higher R.H. which allowed mature growth of aerial mycelium on marked colonies.

Germination of fungal spores at controlled relative humidities is a difficult problem to assess but they appear to grow at lower humidities than Streptomyces spp. for instance, because of their superior food stores in some cases, which is important for initial germination. Food storage in fungi is largely in the form of oil droplets, and when fat is completely oxidised rather more than an equal volume of water is produced (Davies 1960).

## 4.4 Cellulolytic and amylolytic properties of fungi isolated from the air

The cellulolytic and amylolytic properties of the fungal isolates were tested using standard techniques. The ability to degrade cellulose was estimated using the method of Rautella and Cowling (1966). Agar plugs of fungal mycelium were placed onto columns of 4% cellulose agar (+ E and P salts) and incubated for 28 days at 25° C. The results obtained are given in fig. (19) and may be compared with the 2-5m.m. clearance zones obtained by Streptomyces spp. (Chapter 5 fig (26) Cladosporium herbarum the most common airbourne spore was shown to have comparitively weak cellulose breakdown properties (3m.m. clearance), as had the Penicillium sp. tested, 5m.m. clearance.

Species obtained infrequently from airspora isolations were not tested for cellulolytic ability.

The amylolytic ability of isolates was simply measured by their growth rate on wheat starch agar, and the results are tabulated in fig (20). All figures refer to percentage plate cover by the colony which resulted from a mycelial plug inoculum, an average being taken from five replicates. Plates were incubated at 25° C and monitored regularly for eight days. All species tested were capable of hydrolysing starch as measured by using Lugol's iodine solution. Starch hydrolysis was found to be directly proportional to the area of growth. Cladosporium herbarum was found to be extremely slow growing and thus showed

Fig. (19) Cellulolytic Ability of Fungal Species Isolated from the Atmosphere, Rautella and Cowling Tubes, E and P Cellulose, Incubated at 25 C for 28 days.

| | Species | Depth of cellulose clearance m.m. |
|---|---|---|
| 1. | Alternaria sp. | 9 |
| 2. | Aspergillus fumigatus | 12 |
| 3. | Aspergillus niger | 3 |
| 4. | Aspergillus ochraceus | 4 |
| 5. | Aspergillus terreus | 13 |
| 6. | Aspergillus versicolor | N.T. |
| 7. | Botrytis cinerea | 17 |
| 8. | Chaetomium globosum | 10 |
| 9. | Cladosporium herbarum | 3 |
| 10. | Fusarium roseum | 10 |
| 11. | Gelasinospora sp. | 8 |
| 12. | Gilmaniella humicola | N.T. |
| 13. | Gliocladium roseum | 9 |
| 14. | Gliomastix murorum | N.T. |
| 15. | Graphium sp. | 7 |
| 16. | Helminthosporium sp. | 11 |
| 17. | Humicola grisea | 8 |
| 18. | Mucor globosum | n/c |
| 19. | Neurospora sp. | N.T. |
| 20. | Olpitrichum sp. | N.T. |
| 21. | Paecilomyces eligans | 7 |
| 22. | Penicillium sp. | 5 |
| 23. | Phialocephala sp. | n/c |
| 24. | Phoma terristris | 5 |
| 25. | Phoma sp. 2 | N.T. |
| 26. | Phoma sp. 3 | N.T. |
| 27. | Sordaria sylvatica | N.T. |
| 28. | Stysanus sp. | 5 |
| 29. | Trichoderma viride | 14 |
| 30. | Trichothecium roseum | N.T. |
| 31. | Zygorynchus moelleri | n/c |

n/c = non cellulolytic N.T. = not tested

Fig. (20) Amylolytic Ability of Fungi on Starch Agar

| Fungi | Percentage plate cover | | | |
|---|---|---|---|---|
| | 68hrs | 138hrs | 164hrs | 188hrs |
| 1. Alternaria sp. | 26 | 67 | 80 | 87 |
| 2. Aspergillus fumigatus | 100 | 100 | 100 | 100 |
| 3. Aspergillus niger | 30 | 100 | 100 | 100 |
| 4. Aspergillus terreus | 27 | 57 | 78 | 89 |
| 5. Aspergillus versicolor | 82 | 100 | 100 | 100 |
| 6. Botrytis cinerea | 25 | 90 | 100 | 100 |
| 7. Chaetomium globosum | 29 | 94 | 100 | 100 |
| 8. Cladosporium sp. | 5 | 10 | 12 | 14 |
| 9. Eurotium repens | 4 | 17 | 35 | 42 |
| 10. Fusarium sp. | 6 | 11 | 13 | 15 |
| 11. Gliocladium roseum | 7 | 18 | 26 | 37 |
| 12. Gliomastix murorum | 7 | Growth became slow and irregular | | |
| 13. Graphium sp. | very slow irregular growth | | | |
| 14. Helminthosporium sp. | 75 | 100 | 100 | 100 |
| 15. Humicola grisea | 7 | 23 | 30 | 41 |
| 16. Penicillium funiculosum | 78 | 100 | 100 | 100 |
| 17. Phoma spp. | growth slow and irregular | | | |
| 18. Sordaria sylvatica | 30 | 65 | 81 | 92 |
| 19. Tricoderma viride | 100 | 100 | 100 | 100 |
| 20. Trichothecium roseum | 6 | 12 | 19 | 29 |

that it was its high spore concentration which posed a biodeterioration problem. In comparison to fungal growth rates on starch that of the Streptomyces is slower than the weakest fungal species.

## Conclusion

The air mixing system described in chapter 2 was found to be a reliable and easily used tool in the determination of the relative humidity requirements of airspora and their growth on cellulose and starch substrates.

The time required to set up an experimental run was considerably less than that required in alternative methods such as saturated salts.

The air flow scheme followed more closely the conditions which would be encountered by a fungus in nature. The staling products of microbial metabolism such as carbon dioxide and volatile compounds were not allowed to build up and thus this system was found to have many of the advantageous features inherent in the perfusion system as described by Eggins et al. (1968).

Streptomyces spp. were isolated in greater numbers and varieties than noted by previous authors. The figures and species of Streptomyces isolated are given in the following chapter.

Chapter 5

# ISOLATION, ASSESSMENT OF GROWTH FACTORS AND IDENTIFICATION OF *STREPTOMYCES* SPP.

5.1 Introduction

5.2 Isolation of *Streptomyces spp.* from the air

5.3 Assessment of growth factors

a) Cellulolytic ability

b) pH

c) Temperature

d) Starch

5.4 Identification of *Streptomyces spp.* isolated

5.5 Discussion and Conclusion

## 5.1 Introduction

Streptomycetes produce specific difficulties when attempts to isolate them are made; this has probably contributed to their neglect in many fields of microbiology. Their rate of radial growth on culture media is lower than that of fungi and their rate of cell production is generally speaking lower than that of bacteria "therefore methods for their isolation must be designed to compensate, at least partially, for their generally poor competetive ability under laboratory conditions," (Williams and Cross 1971).

The actinomycetes are a very sucessful group of bacteria and they may be considered to play a significant role in the breakdown of complex organic compounds. "The actinomycetes appear to have a number of properties which favour them in competition with other microbes and ensure their survival under favourable environmental conditions", (Goodfellow and Cross 1974).

These properties may be summarised as follows:-

(1) They are capable of producing a variety of spores Cross (1970) these spores can be produced after a very short period of vegetative growth (Mayfield et al, 1972) or by the subdivision of the mycelium into fragmentation spores.

(2) The actinomycetes are nutritionally versatile, being able to grow on both rich substrates and those containing a minimum or even apparent lack of nutrients.

(3) The order contains organisms able to attack substrates which are normally resistant to microbial decomposition.

(4) Following active periods of growth they produce a wide variety of secondary metabolites which include antibiotically active compounds which might give them an advantage in certain microsites.

Little quantitative or qualitative work has been performed on airbourne actinomycetes, this is because together with other bacteria they are too small to be efficiently enumerated by standard spore trapping techniques. The Hirst automatic spore trap, Hirst (1952) is efficient only for particles over 3um in diameter. The first reference to frequency of Streptomycetes (presumably Streptomyces spp.) in the atmosphere was made by Feinberg (1946); he found the Streptomyces spp. colonies formed 1% of total colonies from airspora isolations in Chicago. This compared with Botrytis (1.4%), Phoma (0.4%), Trichoderma (0.3%), Chaetomium (0.3%) and Fusarium (0.23%). Thus it can be seen that the proportion of viable Streptomyces spp. propagules was by no means negligible.

Petri dishes of nutrient agar were exposed during flights from 300 - 3,250 metres over Nashville Tennessee, during winter by Wolf (1943), Actinomyces griseolus was isolated twice at 700 and 1,400 metres, and A. phaeochromagenus once at 620M.

Lloyd (1969) found that the number of Streptomycete propagules trapped outdoors depended on the amount of dust in the

air. Less than 20% of propagules occurred as individual spores, the remainder were attached to airbourne soil particles.

Colonies were found to occur on 50% of oatmeal plates (pH 8.5) and 7% of malt agar plates exposed at various locations in Holland (de Vries, 1960).

| | Malt agar | | Alkaline oatmeal agar | |
|---|---|---|---|---|
| | 37°C | 28°C | 37°C | 28°C |
| Indoors | 9/105 | 0/22 | 25/61 | - |
| Outdoors | 2/15 | 0/11 | 11/11 | 3/7 |

It can be seen from these figures that the isolation media is of the utmost importance.

Other work on Streptomyces spp. has centred almost entirely on spore concentrations in farm buildings, Gregory and Lacey (1963), Lacey and Lacey (1964) and have been concerned with thermophilic organisms.

Gregory (1974) stated that, "we now have some knowledge of the occurrence of bacteria, fungi and pollens as components of the outdoor air spora, but there are some groups whose presence is obvious enough, yet about which we have scarcely any quantitative data. Thus I know of no attempts at continuous sampling to assess the concentration of actinomycete spores in the atmosphere".

Why were airbourne Streptomyces spp. chosen for study in preference to isolating from the soil?

(1) Species found from air-sampling are likely to have more relevance to in-use situations where biodeterioration is occurring; as rarely do textiles come into direct contact with soil either during processing or in use. This was a major reason for developing the continuous air stream device as an alternative type of test facility to that of soil testing beds.

(2) It was found easier to isolate pure species from the air, as a greater percentage of colonies would occur from single-spore inoculum.

(3) There is less chance of contamination of isolation plates by fungi and bacteria as their respective numbers are less in air than soil.

(4) Airbourne Streptomyces spp. found in one area are probably representative of a larger area, than are isolates from a single soil isolation which may be specific for that one soil type.

## 5.2 Isolation of Streptomyces spp. from air

Owing to problems caused by fungal overgrowth and their own slow growth rate it was necessary to incorporate a fungal inhibitor into the isolation media for Streptomyces spp. Nystatin was used by Porter et al. (1960) and Williams and Davies (1965); Malik (1968) used natamycin (2½% suspension, 'Pimafucin') to inhibit soil fungi.

'Pimafucin' was used in the present experiments, 25µ/ml was added after the media had been autoclaved. Supression of bacteria on the isolation plates presented a greater problem, as their responses to antibiotics are similar to those of Streptomyces spp. Rose bengal has been used as a standard control for bacteria in media but Ottow (1972) stated that this could have an effect on the numbers of actinomycetes developing on isolation plates. It was found that the numbers of bacteria could be lessened by making sure that a film of water did not form on the agar surface during incubation.

The results of an experiment to show the effects of Pimafucin are shown in fig. (21). Twenty plates of each media were exposed simultaneously for two minutes outdoors and then incubated for fourteen days at 25° C.

Having found a suitable fungal inhibitor it was necessary to find the optimum incubation temperature. Two media were used for isolations, Waksman starch (pH 7.2) and Cellulose agar and Eggins and Pugh salts (pH 6.4), alternative media having greater specificity for Streptomyces spp. could have been used, but these would have had less relevance to the proposed experiments on textile biodeterioration and biocide protection. The optimum temperature for maximum numbers of airspora isolations was found to be 30° C, fig. (22).

Isolations were carried out regularly over a period of one year and the results are summarised in fig. (23).

| CELLULOSE (+E and P) no. colonies | CELLULOSE (+E and P) + PIMAFUCIN 25µ/ml no. colonies | WAKSMAN STARCH no. colonies | WAKSMAN STARCH + PIMAFUCIN 25µ/ml no. colonies |
|---|---|---|---|
| 31 | 54 | 20 | 64 |

Fig. 21 The effect of fungal inhibitor Pimafucin on the numbers of Streptomyces spp. isolated from air, incubated at 30° C.

| Incubation Temperature ° C | Waksman starch no. colonies | Cellulose (+ E and P) no. colonies |
|---|---|---|
| 19 | 20 | 23 |
| 25 | 60 | 56 |
| 30 | 99 | 72 |
| 33 | 79 | 29 |
| 40 | 20 | 23 |

Fig. 22 Effect of incubation temperature and media on colonies of Streptomyces spp. isolated from air.

A minimum of twenty mixed starch and cellulose media plates were exposed outdoors for two minutes at a height of 1 metre above ground level. All tests were carried out at the same time of day in the same location; but were subject to a number of uncontrollable variations caused by the following factors:-

1) relatively small samples, 20-50 plates

2) faults inherent in the gravity sampling method, whereby heavier particles are preferentially isolated.

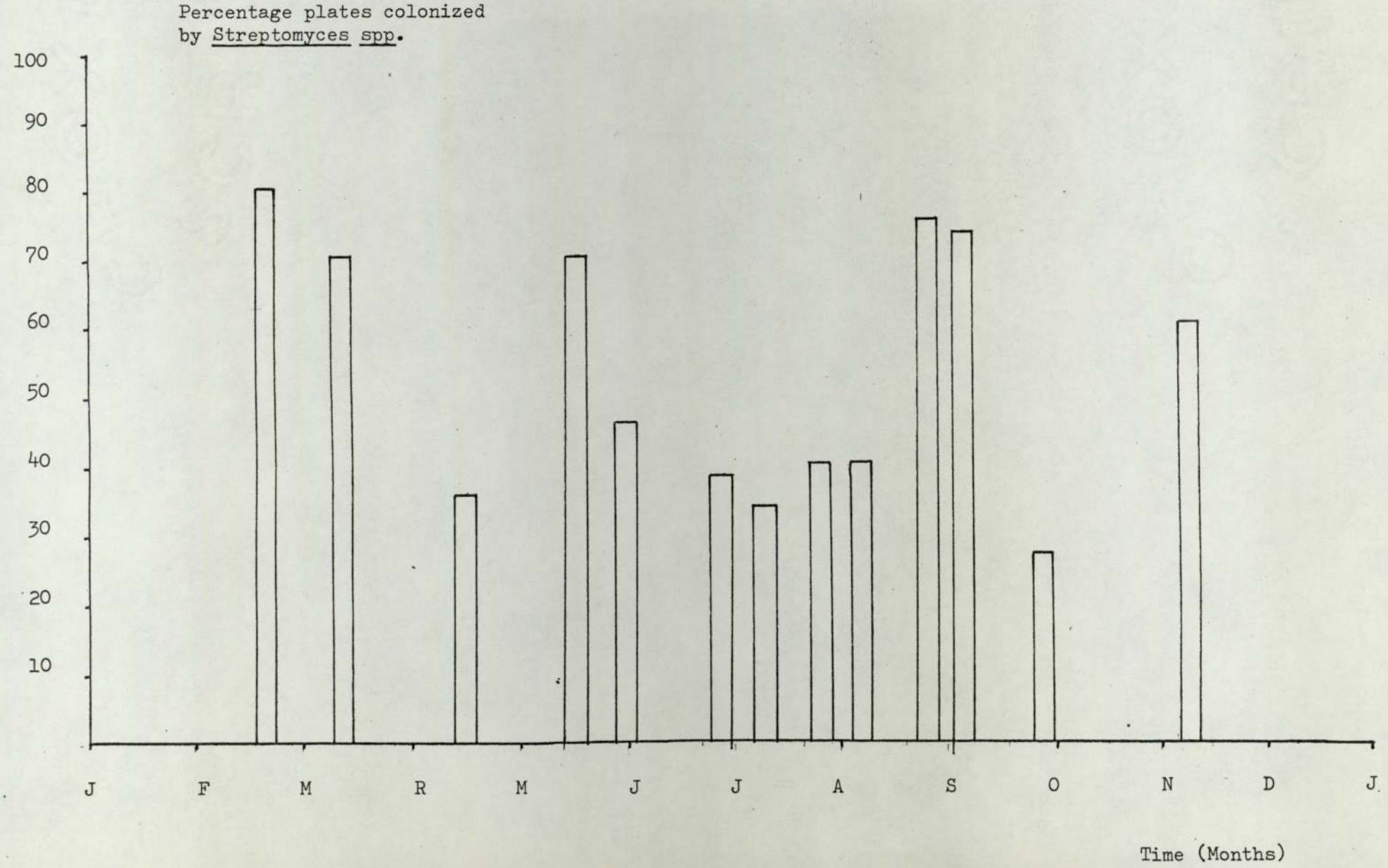
3) Changes in climate at times of sampling
   a) temperature
   b) wind speed
   c) relative humidity

A record was kept of the climatic conditions at the times of sampling, but the number of variables combined with an insufficiently high number of samples meant that no correlation could be made between the number of Streptomyces spp. isolated and and these factors.

It can be seen, however, that the numbers of viable Streptomyces spp. propagules obtained in these isolations is greater than in the isolations reported in chapter 4, though plates were incubated for 14 days at 30° C as opposed to 25° C. The population of airbourne mesophilic Streptomyces spp.is not negligible and deserves far more attention that it has previously received.

Production of aerial mycelium tended to proceed at a faster rate at 30° C (being the optimum temperature for growth of a

Fig. 23 Streptomyces spp. isolated from airspora in 1973.



majority of species isolated), thus the presence of Streptomyces spp. could be distinguished more quickly from those bacteria also colonising the plated which had not been effected by $2\frac{1}{2}\%$ natamycin.

## 5.3 Assessment of growth factors of Streptomyces spp. isolated

A series of tests were carried out for two reasons:

a) to discover growth parameters of the isolates which proved to be important with respect to their ability to degrade cotton textiles.

b) to give additional identification factors which helped to differentiate between isolates having otherwise similar characteristcs.

The factors investigated were:-

i) Cellulolytic ability

ii) pH

iii) Temperature

iv) Amylolytic ability

### a) Cellulolytic ability

This was an extremely important series of tests, not only for demonstrating the wide spectrum of the Streptomyces spp. abilities, but for screening isolates to be used in subsequent textile degradation tests described in Chapter 6.

The common actinomycetes such as streptomycetes, nocardias and micromonosporae, can generally utilize cellulose Reese and Levinson (1952), Chien (1960), Hardisson and Villanueva (1964), Fergus (1969), Ishizawa and Aragi (1970 - cited by Goodfellow and Cross 1974).

The degree to which species were able to utilize cellulose was judged by two independent methods.

a) by visual assessment of growth and the production of clearing zones on cellulose agar (Eggins and Pugh 1962).

b) measurement by Vernier gauge of clearing zones produced in tubes of cellulose agar, (Rautella and Cowling 1966).

Mature fourteen day old cultures of the air isolates were streaked onto plates of cellulose agar. The results obtained are tabulated in figs. (24, 25, 26). Growth was assessed as being at one of two stages, primary mycelial (substrate) or secondary mycelial (aerial) and these two stages were divided subjectively into weak or strong growth.

This subjective visual determination of growth was employed as the standard linear colony measurements used in fungal methods could not be adopted in this instance due to the limited growth of the Streptomyces spp. colonies.

From these results it can be seen that of the fourteen species tested, eight were cellulolytic (capable of clearing cellulose agar) and six were non cellulolytic. Four of the

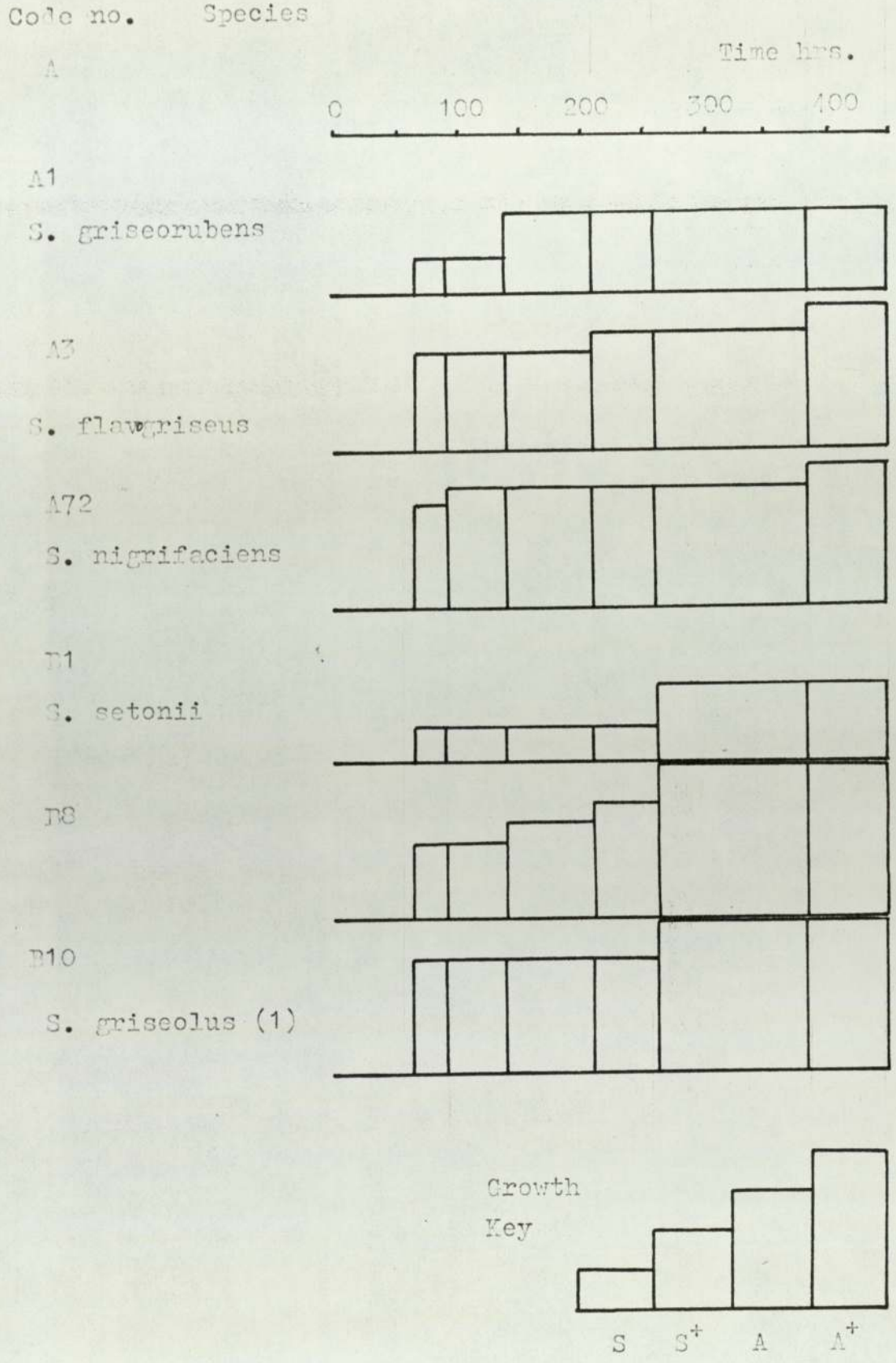



Fig. (24) Visual assessment of growth on Eggins and Pugh cellulose media.

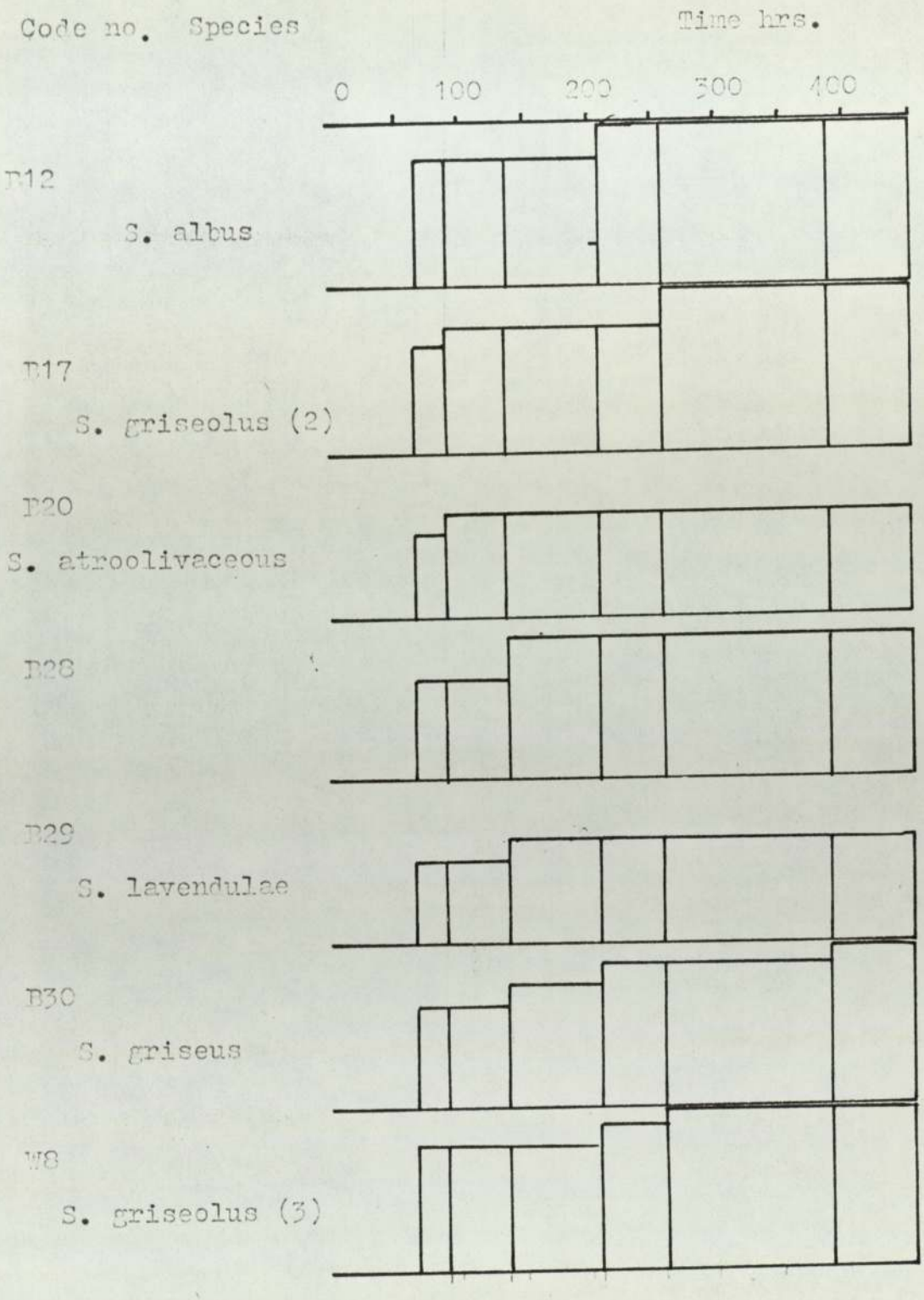



Fig. (25) Visual assessment of growth on Eggins and Pugh cellulose media.

| Code no. of Species | Clearance of cellulose Plate (E and P) | Cellulose Clearance Cowling Tube m.m. |
|---|---|---|
| A1 | V. slow growth - no apparent clearance | - |
| A3 | Growth slow, cellulolytic ability slight | 2.0 |
| A72 | Good growth and clearing | 3.75 |
| B1 | Slight growth, non-cellulolytic | - |
| B8 | Strongly cellulolytic | 4.75 |
| B10 | Cellulose clearance apparent | 4.0 |
| B12 | Non-cellulolytic | - |
| B17 | Strongly cellulolytic | 5.0 |
| B20 | Good growth and cellulose clearance | 2.5 |
| B28 | Good growth, slow cellulose clearance | 3.0 |
| B29 | Slight clearance, production of brown pigmentation | - |
| B30 | Slight cellulose clearance | - |
| R14 | No noticeable cellulose clearance | - |
| W8 | Strong growth, fairly rapid cellulose clearance | 2.5 |

Fig. 26 Cellulolytic ability of Streptomyces spp.

non-cellulolytic species, Streptomyces albus, S. lavendulae, S. griseus and S. rubrireticuli were found to grow and produce aerial mycelium on cellulose agar but were unable to produce clearing zones either on petri plates or in Cowling tubes. S. rubrireticuli was found to produce a dark pink staining of the media and S. lavendulae produced a similar brown pigmentation.

Comparing the clearing depths produced in Cowling tubes shows that the Streptomyces spp. tested were not as cellulolytic as the fungi tested in Chapter 4. This fact agrees with the findings of Fergus (1969) who stated that in general the thermophilic actinomyces were much less capable of degrading insoluble and soluble cellulose than were the fungi. He found only a single species Streptomyces thermoviolaceous that could 'significantly' degrade filter paper and that none of the actinomyces produced detectable Cx enzyme in a medium lacking cellulose.

Degradation of the filter paper (weight loss) was an alternative method by which the cellulolytic abilities of the isolates could have been determined. It was not used in these experiments for two reasons:

i) The difficulty in determining a standard inoculum.

ii) The fact that Streptomyces spp. spores are invariably hydrophobic and the possibly detrimental effect of wetting agents such as 'Tween 80' on subsequent spore germination and development.

b) pH

Streptomyces spp. air isolates were tested over the range pH 5-9, this being the range over which textile biocides are expected to be effective when applied. At pH's in excess of pH9 biocides tend to become increasingly soluble and liable to leaching, at pH's below 5 biocides may have a tenderising effect on fabrics, (Barr 1974).

Species were tested for growth in shake culture flasks containing soluble starch media whose pH was adjusted by N/10 hydrochloric acid or N/10 NaOH as required, the citric acid/phosphate buffer system of McIlvane (1923) was also tried. pH is a commonly used factor in physiological experiments on micro-organisms but is a very difficult factor to control. How can the pH of a culture system be monitored; can micro-organisms change the pH of the environment during the duration of the experiment or are differences in pH between start and end due to purely chemical reactions occurring between the constituents of the culture media? Thus the results for pH growth shown in fig. (31) are only capable of broadly defining the range of growth of the Streptomyces spp. The results could have been made quantitative by weighing the biomass produced by the Streptomyces spp., but this relies on a standard inoculum being used.

Streptomyces are generally unable to grow in soil conditions more acidic than pH 5, activity has, however, been

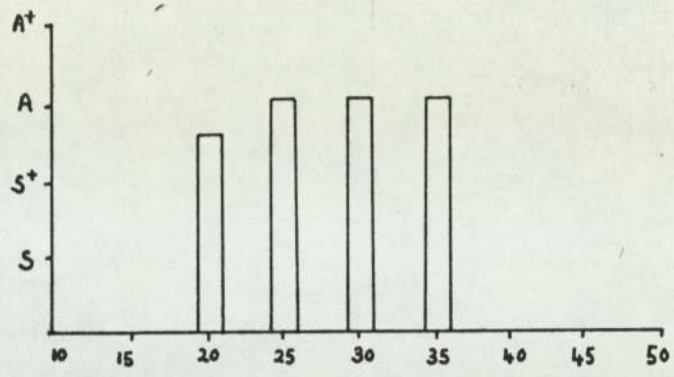
shown in some soils by the recently isolated group of acidophic organisms growing in soil of pH 4-5 (Williams et al, 1971). An investigation by Williams and Mayfield (1971) revealed that the pH close to lysing fungal hyphae was considerably higher because of the liberation of ammonia presumed to be derived from the glucosamine constituents of chitin. Thus Streptomyces spp. were able to grow in these microsites although the bulk of soil formed an unfavourable environment to them.

It may be suggested that Streptomyces spp. are capable of altering the pH of the environment in which they are growing to form a more favourable environment for their growth. Though I have no hard quantitative evidence for this the pH of culture media did tend to become more alkaline during the course of experiments. This would be an interesting line to follow with respect to biocide application as a rise in pH could affect their efficacy to protect textiles if they become liable to an increase in solubility.

c) Temperature

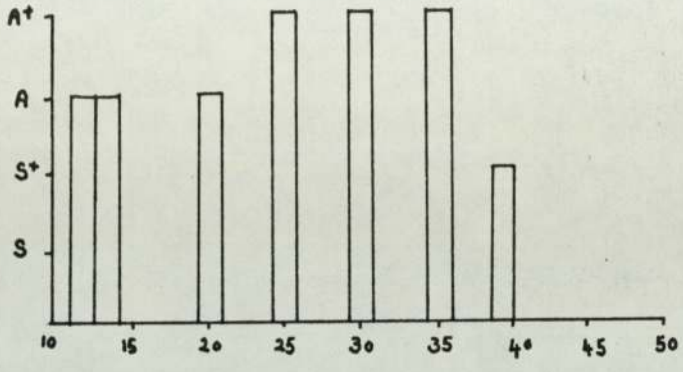
Streptomyces spp. isolates were grown on both starch agar (Waksman 1967) and cellulose agar (Eggins and Pugh 1962) over a range of temperatures from 5-50° C and incubated for twenty one days. The results of individual tests are given in figs. (27, 28, 29, 30). It may be seen that the optimum growth temperature varies from species to species but falls within the range 25-35° C. with the exceptions of B8 and B28. Growth at 11.5° C was found to be common to all species bar Streptomyces griseorubens. Fungal species

A1.



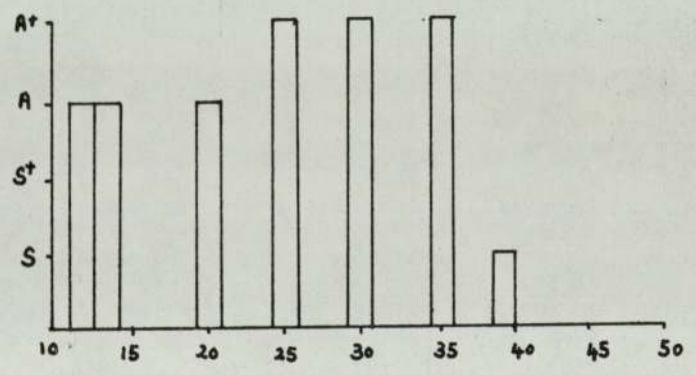

Temp. °C.

A3.



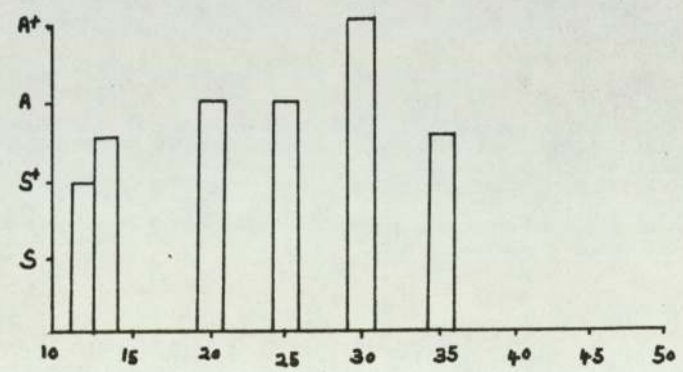

Temp. °C.

A72.




Temp. °C.

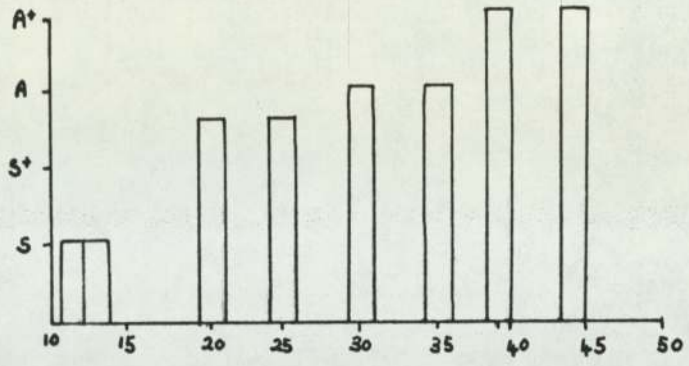
B1.



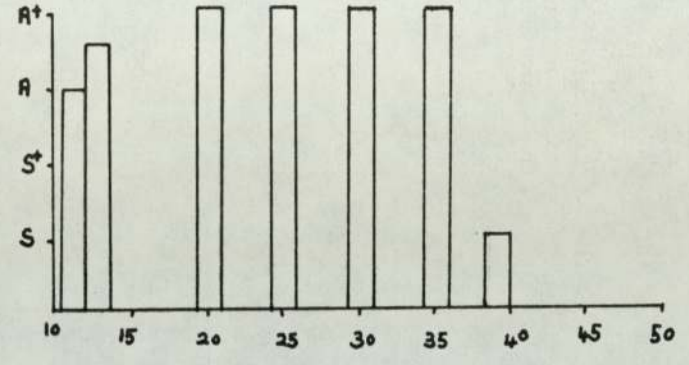

Temp. °C.

Fig. (27) Effect of temperature on Streptomyces spp.

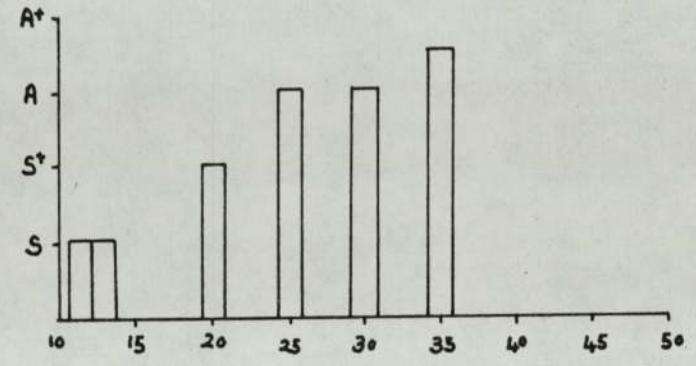
B8.




B10.




B12.




B17.

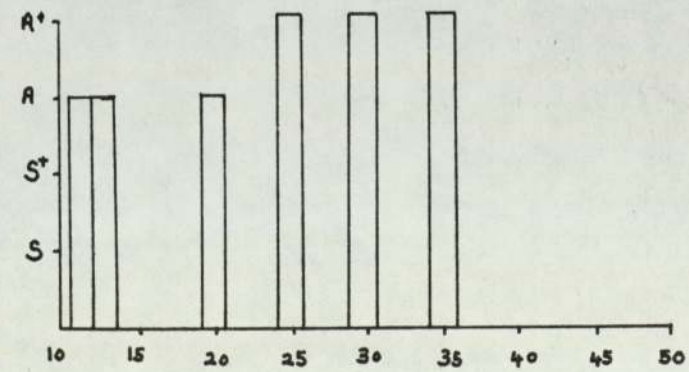
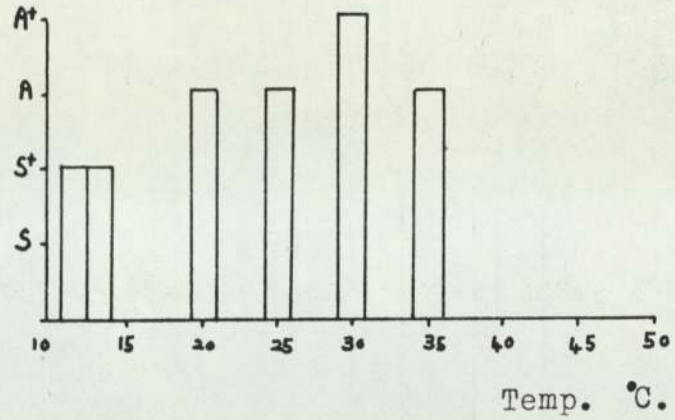
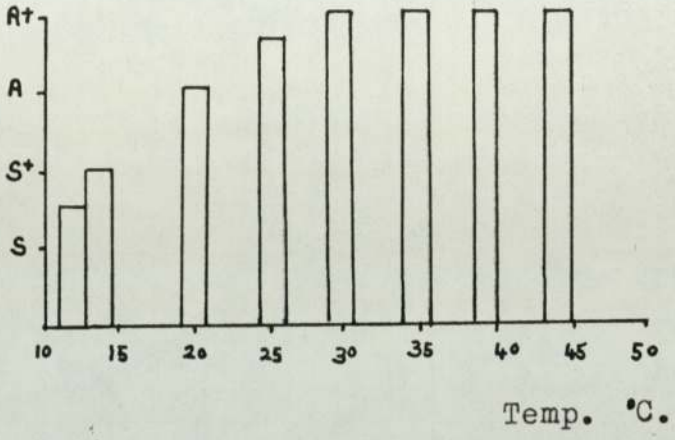



Fig. (28) Effect of temperature on Streptomyces spp.

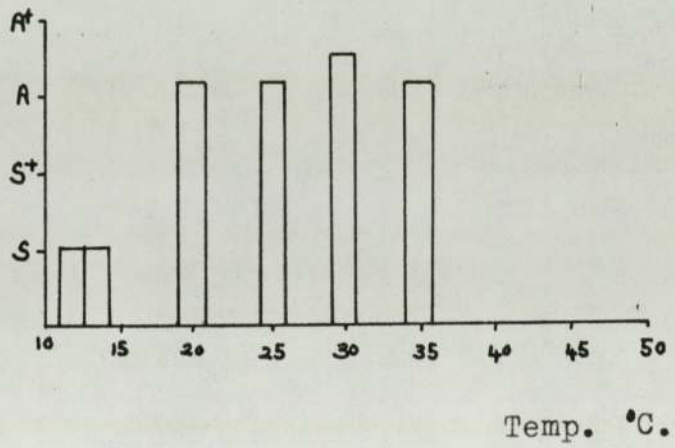
B20.




B28.




B29.




B30.

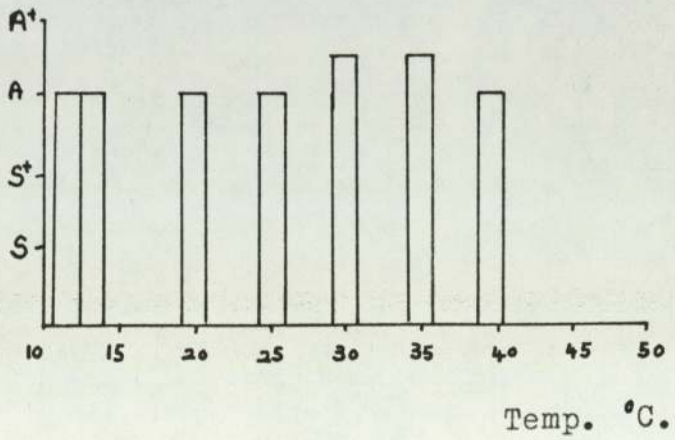



Fig. (29) Effect of temperature on Streptomyces spp.

W8.

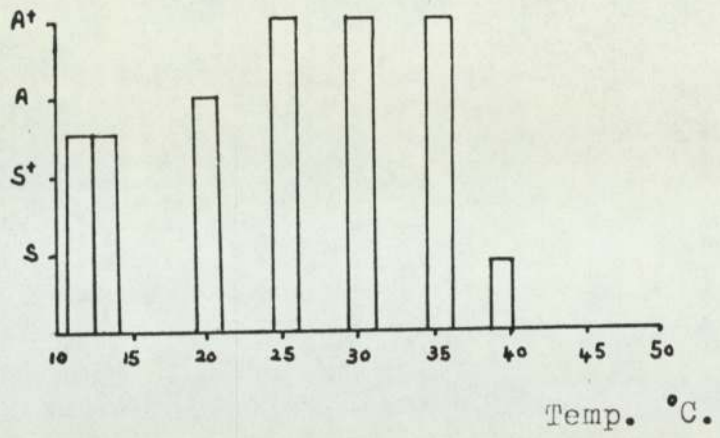



Fig. (30) Effect of temperature on Streptomyces sp.

pH range of species.

| | |
|---|---|
| A1 | 6-9 |
| A3 | 6-9 |
| A72 | 6-9 |
| B1 | 6-9 |
| B8 | 5-9 |
| B10 | 6-8 |
| B12 | 6-9 |
| B17 | 6-8 |
| B20 | 5-8 |
| B28 | 6-9 |
| B29 | 6-8 |
| B30 | 6-9 |
| W8 | 6-9 |

Fig. (31) pH range of species.

commonly isolated from the air will not grow at this low temperature with the exception of a specialized group of organisms the psychrophiles. Streptomyces spp. growth at 11.5° C was limited predominantly to substrate mycelial growth, which on cotton textile would not be visible to the naked eye. However, this could lead to aesthetically displeasing staining of the fabric. Haines (1932) reported that Streptomycete species will grow slowly at temperatures between 5 and 10° C and that certain strains were active at 0° C.

The growth of Streptomyces spp. B8 and B28 were found to cover an extremely wide range, 11.5 - 44° C which made these particularly adaptable micro-organisms and served to demonstrate the extremely flexible physical abilities of the Streptomyces spp. as a whole.

However, it can be said that Streptomyces spp. tested prefered an alkaline environment but were capable of growing in slightly acid (pH 5-6) conditions.

d) Starch

All the Streptomyces spp. isolates were found to be capable of hydrolysing starch, as tested by adding iodine to plates with mature colonies. Pridham and Gottlieb (1948) tested thirteen species and several strains of each species of Streptomyces and found in every case that the isolates produced good growth on starch medium.

## 5.4 Identification of Streptomyces spp isolated from the air

"Identification of Streptomyces spp. is a complicated and somewhat disorganised area of microbiology, and this is a major factor in the neglect of these micro-organisms in the field of biodeterioration. The taxonomy of the actinomycetes constitutes a notorious example of bad systematics. Different taxonomists have given the same organism different names. Other taxonomists have given different organisms the same name. The actinomycete literature is difficult or impossible to follow because we do not know what organisms the authors were studying", (Gottlieb 1960 quoted by Bradley and Bond 1974). It was the upsurge of interest in Streptomyces in the search for new antibiotics in the last two decades that led to a renewed interest in their taxonomy and the setting up of the International Streptomyces Project (I.S.P.).

There are a number of keys of varying usefulness available for the identification of Streptomyces spp; three recent keys which have superseded previous attempts have been used to try to identify the species which were isolated from the air. It is these isolates which were subsequently used for experiments on relative humidity and biocide relationships which could be of importance in biological test proceedures, particularly in burial tests to estimate textile susceptibility to microbiological decay.

The outline scheme of Cross and MacIver (1966) in conjunction with the methods of I.S.P., Shirling and Gottlieb (1966) were used to characterize the species isolated. The characteristics obtained from these morphological and

biochemical tests were then used to key out the isolates Kuster (1972). The resulting prospective species being checked out against the type references supplied by I.S.P., Shirling and Gottlieb (1968a), (1968b), (1969).

Cross and MacIver and I.S.P. both used six major characteristics to describe the appearance and properties of a culture:

(1) Production of a melanin pigment
(2) Carbon compound utilization
(3) Aerial mycelium morphology
(4) Colour of aerial mycelium
(5) Substrate mycelium colour
(6) Surface morphology of spores

Certain small modifications were used in characterization tests due to non-availability of media or equipment and these together with test results on the isolates will be outlined. Isolates were initially typed visually and microscopically and thirteen isolates were used. These isolates appeared to be different species and were estimated to be the most commonly found on the exposed plates. For ease of handling these isolates were sub-cultured onto Waksman starch agar (Appendix) and given a code name.

Melanin pigmentation

Tests were carried out on two types of media:

(a) Peptone-yeast extract iron agar (Tresner and Danga 1958) (Appendix)

(b) Tyrosine agar (Waksman 1967)

Three week old cultures were used, a heavy spore suspension being streaked onto 2 slants of each media. The slants were inspected after 2 and 4 days and compared with uninoculated slants. Cultures producing a greenish-brown to brown to black diffusible pigment or a distinct brown pigment modified by another colour were recorded as giving a positive melanin reaction. The results of isolates investigated are given in fig. (32).

Carbon Utilisation

This test was carried out strictly in accordance with I.S.P. methods which are based on those of Pridham and Gottlieb (1948). Analytical grade reagents only were used in these tests and the following carbon sources were used; no carbon source (negative control), D-glucose (positive control), L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-fructose, Rhamnose and Raffinose. Carbohydrate solutions were sterilized using a bacteriological filter; I-inosotol being fairly insoluble was ether sterilized. Pridham and Gottlieb trace salts were added to the agar before autoclaving. Tests were carried out on universal bottle slopes for convenience; the agar media and trace salts being autoclaved in these. Precise amounts of the carbohydrate solution were then syringed individually into each bottle after autoclaving. Tubes were incubated at 28° C for 16 days and the results are given in fig. (33). The I.S.P. Key was as follows:-

| Code no. of isolate | Mycelium colouration | | Melanin pigmentation | |
|---|---|---|---|---|
| | Aerial | Substrate | Tyrosine | P.Y.I.A. |
| A1 | C2 | G1 | -ve | -ve |
| A3 | C2 | G5 | -ve | -ve |
| A72 | C5 | G2 | -ve | -ve |
| B1 | C2 | G2 | +ve | -ve |
| B8 | C5 | G1 | -ve | -ve |
| B10 | C5 | G1 | +ve | -ve |
| B12 | C2 | G2 | -ve | -ve |
| B17 | C5 | G1 | +ve | -ve |
| B20 | C5 | G1 | -ve | -ve |
| B28 | C5 | G1 | -ve | -ve |
| B29 | C5 | G1 | +ve | +ve |
| B30 | C2 | G1 | +ve | -ve |
| W8 | C5 | G1 | +ve | -ve |

Fig. 32 Mycelium colour and melanin reaction of species isolated (Code according to Cross and MacIver 1966)

i) Strongly positive utilization (++), when growth on tested carbon was equal to or greater than growth on basal medium plus glucose.

ii) Positive utilization (+), when growth on tested carbon was significantly better than on basal medium without carbon, but somewhat less than on glucose.

iii) Utilisation doubtful ($\pm$) when growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than with glucose.

iv) Utilization negative (-) when growth was similar to or less than growth on basal medium without carbon.

Colour determination of aerial and substrate mycelium were made subjectively by three workers independently following the scheme of Cross and MacIver.

Electron microscopy using the technique of Tresner et al. (1961) was used to determine both spore surface morphology and aerial mycelium chain morphology. Preparations for examination were made by a simple spore print technique in which 'Formvar' covered copper grids (200 mesh) were gently pressed onto the sporulating surfaces of the isolates and viewed. Electronmicrographs obtained were compared with pictures of Kurylowicz et al (1971) to determine the spore surface configuration.

Fig. 33 Carbohydrate utilization of Streptomyces spp. isolated from airspora

| CODE NO. OF SPECIES | L-ARABINOSE | D-FRUCTOSE | I-INOSITOL | MANNITOL | RAFFINOSE | L-RHAMNOSE | SUCROSE | D-XYLOSE |
|---|---|---|---|---|---|---|---|---|
| A1 | - | - | - | - | - | - | - | ± |
| A3 | ++ | ++ | - | ++ | ± | ++ | ± | ++ |
| A72 | ± | + | - | + | ± | + | ++ | ++ |
| B1 | ++ | ++ | - | + | + | + | - | + |
| B8 | ± | ± | ± | + | - | ± | ++ | ++ |
| B10 | + | + | - | ++ | - | ++ | - | + |
| B12 | - | + | - | ± | - | - | + | ++ |
| B17 | ++ | + | - | ++ | - | + | ± | ++ |
| B20 | + | + | - | + | + | - | - | ± |
| B28 | - | + | + | + | + | ± | + | ± |
| B29 | ± | - | - | - | - | - | - | - |
| B30 | ++ | ++ | ± | ++ | ± | - | ± | ± |
| R14 | | | | | | | | |
| W8 | ++ | ++ | - | ++ | ± | ± | ± | ++ |

# STREPTOMYCES SPECIES DESCRIPTIONS

A1

Melanin - ve on PYIA and tyrosine
brown pigment produced on Waksman starch, not pH sensitive.

Aerial mycelium griseus (C2)

Substrate mycelium buff (G1)

Carbon utilization Able to utilize glucose only, growth on Waksman starch, slow, non-cellulolytic.

R.H. Minimum 98%

Temperature 20 - 35°C optimum 30°C

pH 6 - 9 optimum pH8

Spore surface Short hairy/spiny Plate (1)

Aerial mycelium morphology Retinaculum apertum (A3)

Streptomyces griseorubens - would have expected shorter chains of spores with less pronounced spines. S. griseorubens would also be expected to use Mannitol, D-fructose, rhamnose and D-xylose.

A3 Streptomyces flavogriseus

Melanin - ve on PYIA and tyrosine

Aerial mycelium griseus (C2)

Substrate mycelium buff + green (G5)

Carbon utilization L-arabinose, D-fructose, Mannitol, Rhamnose, d-xylose, unable to utilize I-inositol, Raffinose or Sucrose. Slight cellulolytic ability.

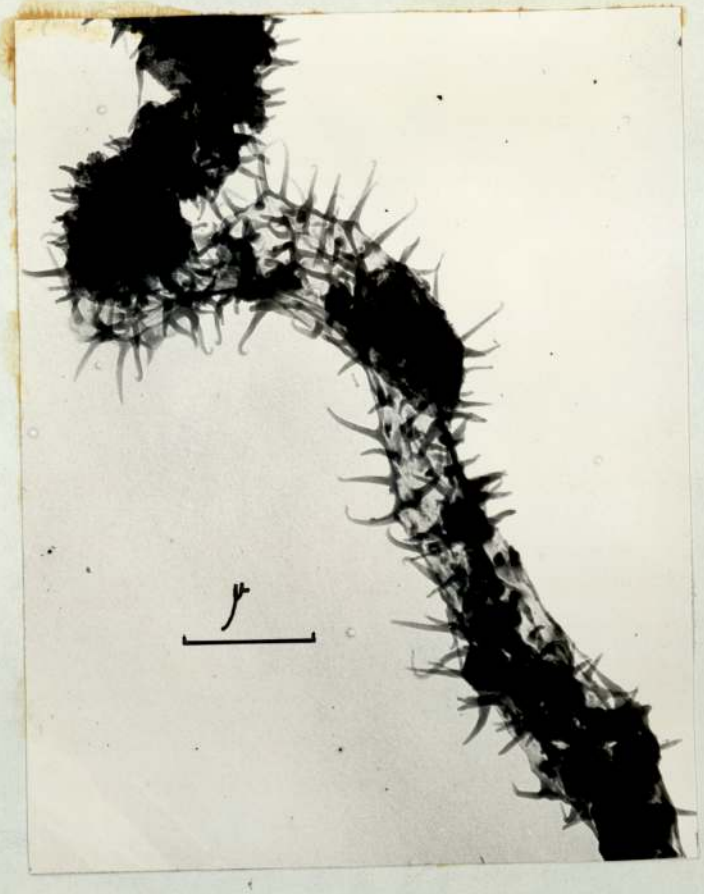


Plate (1)

A1 Streptomyces griseorubens

x 30,000

Showing hairy/spiny spore surface configuration.

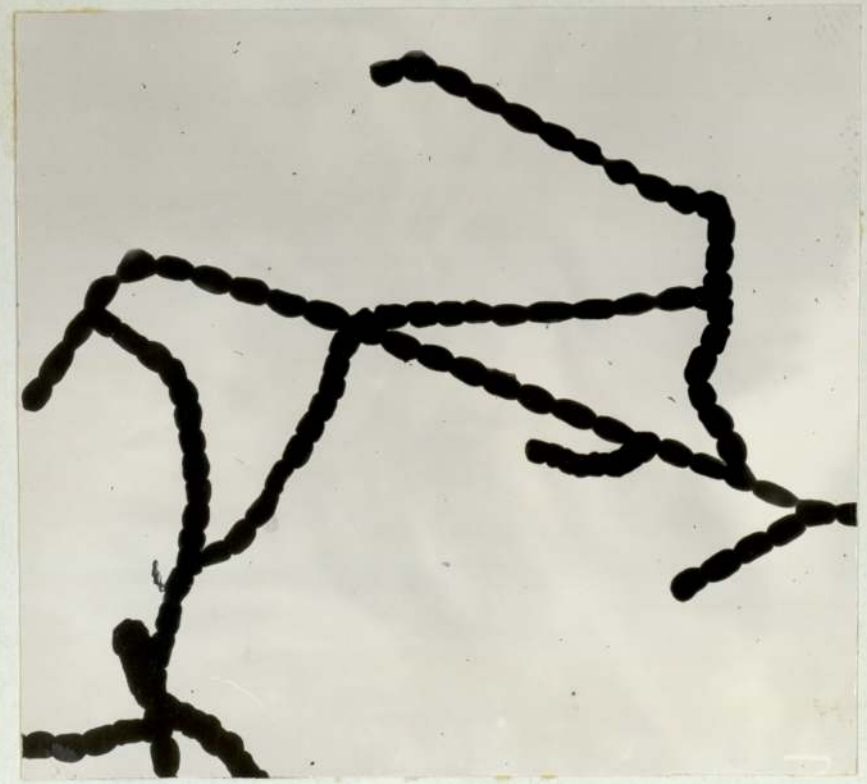


Plate (2)

B20 Streptomyces atroolivaceous

x 7,500

Smooth spore surface.

R.H. Minimum 92%

Temperature 11.5 - 39°C Optimum 25 - 35°C

pH 6 - 9

Spore surface Smooth

Aerial mycelium morphology Flexibilis (A2)

A72 Streptomyces nigrifaciens

Melanin - ve on PYIA and tyrosine

Yellow pigmentation formed on Waksman starch, not pH sensitive.

Aerial mycelium cinereus (C5)

Substrate mycelium buff and yellow (G2)

Carbon utilization unable to utilize L-arabinose, I-inosotol and Raffinose. Cellulolytic.

R.H. Minimum 94%

Temperature 11.5 - 39°C optimum 25 - 35°C

pH 6 - 9

Spore surface Smooth

Aerial mycelium morphology Rectus (A1)

S. nigrifaciens would not be expected to utilize sucrose but should have used L-arabinose

B1 Streptomyces setonii

Melanin Tyrosine +ve PYIA -ve

Aerial mycelium whitish/yellow powdery appearance

Substrate mycelium buff + yellow (G2)

Carbon utilisation Unable to utilize I-inositol and sucrose

Non-cellulolytic

R.H. minimum 94%

Temperature 11.5 - 35°C Optimum 30°C

pH Good growth pH 6 - 9

Spore surface Smooth

Aerial mycelium morphology Rectus (A1)

S setonii is the closest fitting description but two points at variance with this classification:

a) the ability to produce melanoid pigments on Tyrosine media.

b) the utilization of Raffinose.

B8

Melanin -ve on PYIA and Tyrosine

Aerial mycelium cinereus (C5)

Substrate mycelium buff (G1)

Carbon utilization Mannitol, Sucrose and D- xylose only, Cellulolytic

Spore surface Smooth

Aerial mycelium morphology Spira ($A_4$)

RH Minimum 94%

Temperature 11.5 - 44°C Optimum 39 - 44°C

pH Good growth pH 5 - 9

Species B8 did not fit any of the standard I.S.P. descriptions due to its limited utilization of carbon sources.

B10 Streptomyces griseolus Strain 1.

Melanin PYIA -ve, Tyrosine +ve. Yellow pigmentation produced on Waksman Starch media, not pH sensitive.

Aerial mycelium cinereus (C5)

Substrate mycelium buff (G1)

Carbon utilization Unable to utilize I-inositol, Raffinose or Sucrose. Cellulolytic.

Spore surface Smooth

Aerial mycelium morphology Rectus/Flexibilis (A1/A2)

R.H. Minimum 94%

Temperature 11.5 - 39°C optimum 20 - 35°C

pH 6 - 8

Streptomyces griseolus would not be expected to produce melanoid pigments, but the tyrosine media used was that of Waksman (1967) and not the I.S.P. standard media of Shirobu The brown pigment may not in fact have been melanin at all as this result was not confirmed by tests on PYIA media.

B12 Streptomyces albus

Melanin -ve on PYIA and Tyrosine

Aerial mycelium Griseus ($C_2$) - a dirty white colour in appearance.

Substrate mycelium buff + yellow (G2)

Carbon utilization D-fructose, sucrose and D-xylose only Non-cellulolytic.

Spore surface Smooth

Aerial mycelium morphology Flexibilis/spira

R.H. Minimum 94%

Temperature 11.5 - 35°C Optimum 35°C

pH Good growth pH 6 - 9

S. albus according to the I.S.P. type description is capable of utilizing Mannitol, the negative result in this test may be due to the subjective nature of the test, only limited growth being observed on the glucose control media.

B17 Streptomyces griseolus Strain 2

Melanin PYIA -ve Tyrosine +ve. Yellow pigmentation on Waksman Starch media, not pH sensitive.

Aerial mycelium Cinereus (C5)

Substrate mycelium buff (G1)

Carbon utilization Unable to utilize I-inositol, Raffinose or Sucrose. Cellulolytic.

Spore surface Smooth, spore chains short (<10)

Aerial mycelium morphology Rectus/Flexibilis

R.H. Minimum 92%

Temperature 11.5 - 35°C optimum 25 - 35°C

pH Good growth pH 6 - 8

B20 Streptomyces atro olivaceus

Melanin - ve PYIA and Tyrosine

Aerial mycelium Cinereus (C5)

Substrate mycelium buff (G1)

Carbon utilization Unable to utilize I-inositol, L-rhamnose or sucrose. Cellulolytic

Spore surface Smooth

Aerial mycelium morphology Rectus/Flexibilis (A1/A2)

R.H. Minimum 96%

Temperature 11.5 - 35°C Optimum 30°C

pH Good growth pH 5 - 8

This species has been tentatively classified as S. atro olivaceus as this best fits the I.S.P. standard type descriptions, though L-rhamnose should have been utilized as a carbon source.

B28

Melanin -ve PYIA and Tyrosine. Brown pigment formed on Waksman Starch media, not pH sensitive.

Aerial mycelium cinereus (C5), - with reddish tinge.

Substrate mycelium buff (G1)

Carbon utilization Unable to utilize L-arabinose, L-rhamnose or D-xylose. Cellulolytic.

Spore surface Smooth

Aerial mycelium morphology Flexibilis (A2)

R.H. Minimum 94%

Temperature 11.5 - 44°C Optimum 25 - 44°C

pH Good growth pH's 6 - 9

This isolate did not give a good fit with any of the descriptions of I.S.P.

B29 Streptomyces lavendulae

Melanin PYIA +ve, Tyrosine +ve. Brown pigmentation produced on Waksman Starch media, not pH sensitive.

Aerial mycelium Cinereus (C5)

Substrate mycelium buff (G1)

Carbon utilization No utilization of any of tested sugars excepting the glucose control. Slight cellulolytic ability.

Spore surface Smooth

Aerial mycelium morphology Flexibilis (A2)

R.H. Minimum 96%

Temperature 11.5 - 35°C Optimum 30°C

pH Growth range pH 6 - 9

This isolate was provisionally identified as S. lavendulae, all characteristics fit the I.S.P. type description with the exception of the aerial mycelium morphology. S. lavendulae is normally associated with having loops and spirals rather than flexuous mycelium.

B30 Streptomyces griseus

Melanin PYIA -ve, Tyrosine +ve

Aerial mycelium Griseus (C2)

Substrate mycelium buff (G1)

Carbon utilization Able to utilize only L-arabinose, D-fructose and Mannitol efficiently. Non-cellulolytic.

Spore surface Smooth

Aerial mycelium morphology Rectiflexibilis

R.H. Minimum 92%

Temperature 11.5 - 39°C Optimum 30 - 35°C

pH Good growth pH 6 - 9

S. griseus is normally capable of utilizing D-xylose as carbon source but not L-arabinose.

R14 Streptomyces rubrireticuli

This species was not isolated from the air, but was supplied as a test organism by Catomance Limited.

As the organism had previously been typed the test proceedures used for the other isolates were not adopted.

W8 Streptomyces griseolus Strain 3

Melanin PYIA -ve, Tyrosine +ve. Yellow pigmentation was produced on Waksman Starch media, not pH sensitive.

Aerial mycelium cinereus (C5)

Substrate mycelium buff (G11)

Carbon utilization Unable to utilize I-inositol, Raffinose or sucrose. Cellulolytic.

Spore surface Smooth, chains of varying lengths (many short)

Aerial mycelium morphology Rectus (A1)

R.H. Minimum 94%

Temperature 11.5 - 39°C Optimum 25 - 35°C

pH Good growth pH 6 - 9.

## 5.5 Discussion and conclusion

Airbourne Streptomyces spp. were found to form a significant but hitherto underestimated proportion of the airspora. Their numbers compared favourably with commonly isolated species of the fungi imperfecti, which are given far more weight in biodeterioration experiments.

The identification tests for Streptomyces spp. were found to be easy to perform but were extremely time consuming and labour intensive. The major problem concerned evaluation down to species level with the aid of dichotomous key once the main characteristics of an isolate had been observed.

Not all species of Streptomyces are cellulolytic (eight out of fourteen investigated), so it is important to develop a method whereby these species may be screened for activity. Subjective methods such as clearing of ball milled cellulose agar on plates and in Cowling tubes have only limited accuracy; Streptomyces spp. tend to grow slowly and have weak cellulolytic ability as compared to moderately cellulolytic fungi, so tests last longer.

Alternative methods such as weight or tensile strength loss of cellulose filter paper, shake-flask cultures or viscometry could possibly be more accurate in determining cellulolytic ability.

All species isolated were found to grow well over the range pH 6 - 9, which is the pH at which biocides are applied

to textiles. Above pH 9 the solubility of biocides increases and they tend to become more easily leached from the fabric. All species isolated were mesophilic, but many grew over an extended temperature range and so Streptomyces spp. could be expected to occur and grow in all situations employed in textile processing, treatment, storage and usage, provided that sufficient moisture was present.

Moisture is perhaps the dominant factor in the biodeterioration of textiles as it is the least easily controlled. The relationship between relative humidity and Streptomyces spp. growth has thus been dealt with separately in the following chapter.

# Chapter 6

## EFFECT OF RELATIVE HUMIDITY ON GROWTH OF *STREPTOMYCES* Spp

### 6.1 Introduction

6.1.1 Control of substrate $A_w$ by addition of solutes

6.1.2 Comparison of solute and $A_w$ effects

6.1.3 Effect of relative humidity on *Streptomyces* spp. growth

### 6.2 Relative humidity testing

6.2.1 Scott $A_w$ technique

6.2.1.1 Salt tolerance of *Streptomyces* spp.

6.2.2 Ayerst saturated salt solutions technique

6.2.3 Controlled dry/wet air-mixing technique

### 6.3 Conclusion

## 6.1 Introduction

Relative humidity and soil moisture control are two extremely important factors when considering biodeterioration test proceedures. Often soil which has been used in burial tests has an unknown moisture content or has been kept under variable conditions. Although inconsistant results obtained from these tests may be due to many other variables, these two factors are often neglected. Having found that airbourne Streptomyces spp. are present in large numbers and are frequently active cellulose degraders, it was decided to concentrate the study on this group of organisms, as knowledge of the role they play in such tests is scarce. Streptomyces spp. were often found colonising the surface of soil in burial trays which had been allowed to partially dry out (Barr, 1974) these trays would then be wetted and used in the normal manner when required.

Although knowledge of biophysical relationships could cast considerable light on factors affecting survival of cells, particularly under conditions of reduced water availability, comparatively little work has been done on this topic.

The nature of the substrate is usually responsible for the selection of colonisers, so the reaction of airbourne Streptomyces spp. isolates to different relative humidities (or equivalent water activities) was tested. Three methods for producing constant relative humidities were tried and these have been outlined in chapter 2.

The importance of controlling environmental conditions with respect to the prevention of biodeterioration has been reviewed by Ayerst (1968a). He makes four primary points which were used as guidelines in experimenting with the effects of relative humidity on Streptomyces spp.

a) Water activity is the most valid measure of physiological drought in all types of substrate. The ratio of the vapour pressure of the water in a solution or in a hygroscopic material to that of pure water at the same temperature and pressure is a direct measure of the reduction in its chemical potential as compared with that of pure waterand is called the water activity ($A_w$). Relative humidity is equivalent to water activity expressed as a percentage and is usually adopted as a measure of atmospheric water content. Water activity varies only slightly with temperature in most organic materials and in unsaturated solutions and that, unlike osmotic pressure, it is applicable to both soluble and insoluble substrates. In each individual system, the three factors of solute concentration, total amount of water and water activity vary together. Both Scott (1957) and Galloway (1935) concluded that water activity was the major limiting factor of these three for fungal spore germination and growth.

b) The limits and optima of temperature and moisture are stable characteristics of microbial species or of

definite sub-specific groups. Within each group they vary from an average; which is characteristic of the group to only a limited and statistically definable extent. Whenever the moisture limits of a number of isolates of the same species have been studied the variations have been found to be small, (Scott 1953, Christian and Scott 1953, Ayerst 1968). Only individual Streptomyces spp. have been tested, so no concrete predictions can be made as to the behaviour of all Streptomyces spp. through experimental results indicate a useful guide to the effects of $A_w$.

c) The reactions of fungi and bacteria to temperature and to $A_w$ are interdependent. The maximum tolerance to extremes of $A_w$ is exhibited at approximately the optimum temperature and the maximum tolerance to extremes of temperature at approximately the optimum $A_w$.

d) Growth of micro-organisms is possible at a lower $A_w$ on substrates of higher nutritional status. Where direct comparisons have been made between substrates of different nutritional status it has been found that fungal growth appears more rapidly on the material containing a higher concentration or wider range of nutrients, Snow et al (1944). Thus one would expect Streptomyces spp. to grow more readily at low $A_x$'s on starch than cellulose substrates.

### 6.1.1 Control of substrate $A_w$ by the addition of solutes

By controlling the water activity of a substrate it is possible to predetermine the relative proportions of water and dry matter; so consequently it is possible to fix $A_w$ by controlling the solute concentration. This method was employed by Scott (1953) and Christian and Scott (1953) in studying the water relationships of Salmonellae. Scott states that, only rarely has this method been attempted, although a great many workers have studied the effects of various electrolytes and non-electrolytes on the growth of micro-organisms. However, these results have usually been considered only in terms of solute concentration. This is unfortunate as it is likely that many of the effects observed were due to decreased $A_w$ rather than specific inhibition by the solutes or ions concerned.

Heintzeler (1939) looked at the comparative effects of calcium chloride, potassium chloride, sodium chloride and sucrose as solutes to control the $A_w$ in mould growth experiments. Burcik (1950) used broth and agar media whose $A_w$'s were adjusted by sodium chloride, in comparative bacteriological studies. Burcik compared the affect on growth of lowering the $A_w$ by drying a complex medium, with that of lowering it by adding sodium chloride. For the majority of bacteria investigated he obtained somewhat different minima $A_w$ values with the two methods. Furthermore cells did not always have the same minimum Aw values

in salt-adjusted agar.

$A_w$ control has been used in the study of streptomycete physiology by Jagnow (1957) using the methods of Burcik; and Klevenskaya (1960) using sucrose, sodium chloride and sodium sulphate as the solutes.

### 6.1.2 Comparison of solute and $A_w$ effects

When using $A_w$ controlled media as measure of a Streptomyces spp. ability to grow at different relative humidities it is important to distinguish between $A_w$ and solute toxicity effects. Several studies have been carried out by other workers to distinguish between the effects of lowered $A_w$ and specific solute effects on bacteria. It is possible that similar effects might apply to experiments on Streptomyces spp. The inhibitory effects of solutes on bacterial spore germination and subsequent vegetative growth cannot be entirely ascribed to the lowered $A_w$. Different solutes may exert specific effects; work on this subject has been reviewed by Baird-Parker and Freame (1967). In general, spore germination is less affected by solutes that outgrowth to the vegetative cell, and outgrowth is less affected that vegetative cell growth. Corry (1973) has comprehensively reviewed the above problems and summarises current knowledge on the minima $A_w$ limits of growth for a wide range of bacteria and yeasts.

The different physiological actions of ionic and non-ionic solutes have been emphasised by the work of Marquis (1968). He showed that low concentrations of salts, but not of sucrose, caused protons to be released from the walls of Bacillus megaterium, and the walls to contract. These and other bacterial cell walls appear to behave as polyelectrolytes, and their response to salts would naturally be different from their response to sugars.

No solute should be regarded as inert in that its only function is to bind water. If it can attract the H or O atoms of water, it can also attract groups on or in the cell. It may be simplest to remember that high solute concentrations may produce two effects:

a) lowering the water content available to the micro-organism; which may be the most important.
b) a specific action of solute molecules on growth patterns.

Christian and Waltho (1964) measured the internal contents of water, macromolecules, amino acids and several ions in the salt tolerant organism Staphylococcus aureus grown in basal medium ($A_w$ = 0.993) and in media adjusted to lower $A_w$ values by Na Cl additons. Decreasing $A_w$ to 0.90 halved the cell water contents and the concentration of several solutes increased.

It is well known that enzymes are generally more stable if free water is lowered. This suggests that water availability influences the conformation of the enzyme, such as influence might well effect activity. Water participates directly in many biochemical reactions, and if less water were made available such reactions might be inhibited. Very little work has been carried out on the precise sub-cellular and biochemical level of how high solute concentrations react. "At present there is a much clearer knowledge of why certain micro-organisms need salts than why others are inhibited by them, or tolerate them", (Kushner 1971).

### 6.1.3 Effect of relative humidity on *Streptomyces* spp. growth

There has been only a very limited amount of work previously performed on this topic. Jagnow (1957) carried out a series of experiments on Streptomycetes ecology. Using the techniques of Burcik (1950) and using thirteen *Streptomyces spp.* he showed that their spores were able to germinate and grow at relative humidities between 91.5% and 99% R.H. This therefore gives them an intermediate moisture requirement between the bacteria and the fungi. Jagnow's experiments demonstrated that very little mycelial development ocurred below 95% R.H., below this value only vegetative hyphal elongation took place; which he measured microscopically. The species used were; *S. alboflavus* (3 strains), *S. griseus*

(2 strains), S. antibioticus, S. violaceus (2 strains), S. ruberireticuli, S. aureus (4 strains). All species were isolated from woodland soils, and the results were obtained after seven days incubation at 30.5° C.

Klevanskaya (1960) found that streptomycetes were capable of developing high osmotic pressures (limiting 117 atmospheres), which may vary in accordance with environmental factors. Griffin (1972) quotes unpublished data of Wong T.W. and states that, "the growth of soil streptomycetes on agars of different osmotic potentials becomes negligible at -80 bar (approximately 94% R.H.) and is greatly reduced at -40 bar (approximately 97.5% R.H.)".

## 6.2 Relative humidity testing

### 6.2.1 Relationship between water activity ($A_w$) and growth of Streptomyces spp. using Scott (1953) technique

The technique of Scott (1953) was one of three methods employed to discover the relationship between water availability and the growth of Streptomyces spp., and was the most reliable. The theoretical basis of the method had been discussed in section 6.1.1 and the salts used and their quantities are recorded in fig. (34). The concentrations of the salts are expressed in molalities

as, "from a theoretical point of view it is better to express concentration in terms of molality (moles per 1000g of solvent) rather than as molarity (moles per 1000 c.c. of solution) which changes with temperature", Ingram (1957).

Three types of agar media were used:

(i) Nutrient (Oxoid)

(ii) 4% cellulose + Eggins and Pugh salts (1962)

(iii) Starch (Waksman 1967)

To obtain each $A_w$, media was made up separately in 200 ml capacity medical flats; analar grade reagents were used throughout. Bottles were weighed before and after autoclaving (15 lbs for 15 mins) and any weight loss due to evaporation was rectified by adding an equivalent weight of sterile distilled water. In practice it was found that a maximal addition of 5 ml was required to obtain the correct weights.

When large weights of salt were incorporated into the media there was a problem in getting the sodium sulphate to dissolve; this was overcome by shaking and heating. Water activities 0.86 and 0.88 were found to be impossible to obtain in practice as the agar media would not gel completely, forming a rather viscous slurry.

Fig. 34 Molal concentrations of salts required for adjusting medium of $A_w$ 0.999 to various values of $A_w$ at 25°C with a 5:3:2 mixture of NaCl, KCl and $Na_2SO_4$

(Modified from Scott 1953)

| FINAL $A_w$ in MEDIUM | $(1-A_w)$ of SALT MIXTURE | Na Cl (Molal concentration) | Na Cl (gms/200 ml of medium) | KCl (molal concentration) | KCl (gms/200 ml of medium) | $Na_2SO_4$ (molal concentration) | $Na_2SO_4$ (gms/200 ml of medium) |
|---|---|---|---|---|---|---|---|
| 0.990 | 0.009 | 0.1293 | 1.5115 | 0.0776 | 1.1571 | 0.0517 | 1.4687 |
| 0.980 | 0.019 | 0.2789 | 3.2603 | 0.1673 | 2.4947 | 0.1116 | 3.1705 |
| 0.960 | 0.039 | 0.5805 | 6.7860 | 0.3483 | 5.1938 | 0.2322 | 6.5968 |
| 0.940 | 0.059 | 0.869 | 10.1586 | 0.521 | 7.7691 | 0.348 | 9.8866 |
| 0.920 | 0.079 | 1.149 | 13.4318 | 0.690 | 10.2892 | 0.460 | 13.0686 |
| 0.900 | 0.099 | 1.418 | 16.5764 | 0.851 | 12.6901 | 0.567 | 16.1084 |
| 0.880 | 0.119 | 1.663 | 19.4404 | 0.998 | 14.8821 | 0.665 | 18.8916 |
| 0.860 | 0.139 | 1.921 | 22.4564 | 1.153 | 17.1935 | 0.768 | 21.8188 |

Plates were inoculated by finely streaking with spores and mycelium from mature fourteen day old isolates. The plates were incubated at either 25 or 30° C for up to 21 days. Development was monitored microscopically, during the growth of Streptomyces spp. there are distinct phases which may be easily recognised. These are the growth of primary (substrate and colonial) mycelium, production of secondary (aerial) mycelium, sporulation and spore germination (Kalakoutskii and Pouzharitskaja 1973).

The result of tests, on these types of media and in the case of nutrient agar at two temperatures, is recorded in tabular form in Appendix 1.

The notation used has been used in all cases to give an estimate of the growth phase of the Streptomyces spp. at a particular post-inoculation time and $A_w$.

All results tabulated are based on five replicates; there was found to be practically no variation between replicates for any individual Streptomyces spp. or $A_w$ treatment.

In order to determine between salt and $A_w$ effects (discussed in section 6.1.2) individual salts were tested for possible toxicity effects using exactly the same experimental technique. In order that the plates remained at the initial $A_w$ during the growth period the surrounding

atmosphere was kept at about 80-85% R.H. by water trays on the floor of the incubators.

Analysis of Results

The original experimental observations are given in detail in Appendix II. The initial readings took the following form:

Incubation time (hrs)

| $A_w$ \ A3 | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1.00 | $A/A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0.99 | A | $A/A^+$ | $A/A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0.98 | $S^+/A$ | $S^+/A$ | A | A | A | $A/A^+$ | $A/A^+$ |
| 0.96 | - | S | $S^+$ | $S^+$ | $S^+/A$ | $S^+/A$ | $S^+/A$ |
| 0.94 | - | - | - | - | S | S | $S^+$ |
| 0.92 | - | - | - | - | - | - | S |
| 0.90 | - | - | - | - | - | - | - |

Growth of species A3 on starch agar at 30° C

where, S = Distinct growth of substrate mycelium, showing active colonisation to be taking place

$S^+$ = Widespread strong growth of primary substrate mycelium (colonies appear bacteria-like)

A = production of secondary (aerial) mycelium

$A^+$ = Mature colonies showing sporulation and spore germination

The individual readings in the result blocks represent the average of five replicates and thus led to intermediate readings e.g. $S^+$ /A.

The results obtained posed problems in interpretation as there were three quantitative variables, $A_w$, time and Streptomyces spp. whose interactions were measured by subjective visual assesment of growth. The raw data as shown in the above example was therefore converted into a more easily handled numerical form. The following arbitary scoring system was adopted:

S = 1 $S^+$ = 2 A = 3 $A^+$ = 4

Thus the data took on the form:

| $A_w$ | Incubation time hrs. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 48 | 50* | 72 | 100* | 140 | 192 | 200* | 240 | 300* | 336 | 400* | 404 |
| 1.00 | 3.5 | 3.54 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| 0.99 | 3.0 | 3.04 | 3.5 | 3.5 | 3.5 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| 0.98 | 2.5 | 2.5 | 2.5 | 2.7 | 3.0 | 3.0 | 3.0 | 3.0 | 3.31 | 3.5 | 3.5 | 3.5 |
| 0.96 | - | 0.04 | 1.0 | 1.41 | 2.0 | 2.0 | 2.08 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| 0.94 | - | - | - | - | - | - | - | 1.0 | 1.0 | 1.0 | 1.96 | 2.0 |
| 0.92 | - | - | - | - | - | - | - | - | - | - | 0.96 | 1.0 |
| 0.90 | - | - | - | - | - | - | - | - | - | - | - | - |

* Interpolated results

Species A3 grown on starch agar at 30° C.

Similar tables were constructed for each of the three types of media tested, nutrient agar, cellulose agar and

starch agar. The microscopic estimations of growth were taken at slightly different time periods on the three types of nutrient due to the large numbers of replicates. In order to demonstrate trends within the data it was necessary to pool results leaving out one or more of the variables.

To demonstrate the relationship between $A_w$ and time for each individual species the nutrient source factor was ignored. The pooling of results from experiments performed on different substrates also required the time variable to be interpolated as readings had been taken at varying intervals. It was then possible to combine the interpolated results for one species growth on three types of nutrient into a single table. From this table could be plotted graphically the relationship between $A_w$ and incubation time for an individual Streptomyces sp., these are illustrated in figs.(35-40). The graphs are capable of showing only general patterns, the growth scale being based on that of the arbitary scoring system. This system leads to the apparently anomalous situation whereby none of the species tested reached full maturity. This, however, can be explained by the pooling of results from different nutrient sources, each point on the graphs being an average of three readings.

It can be seen that growth follows an exponential curve for 100 hours the growth is relatively rapid showing

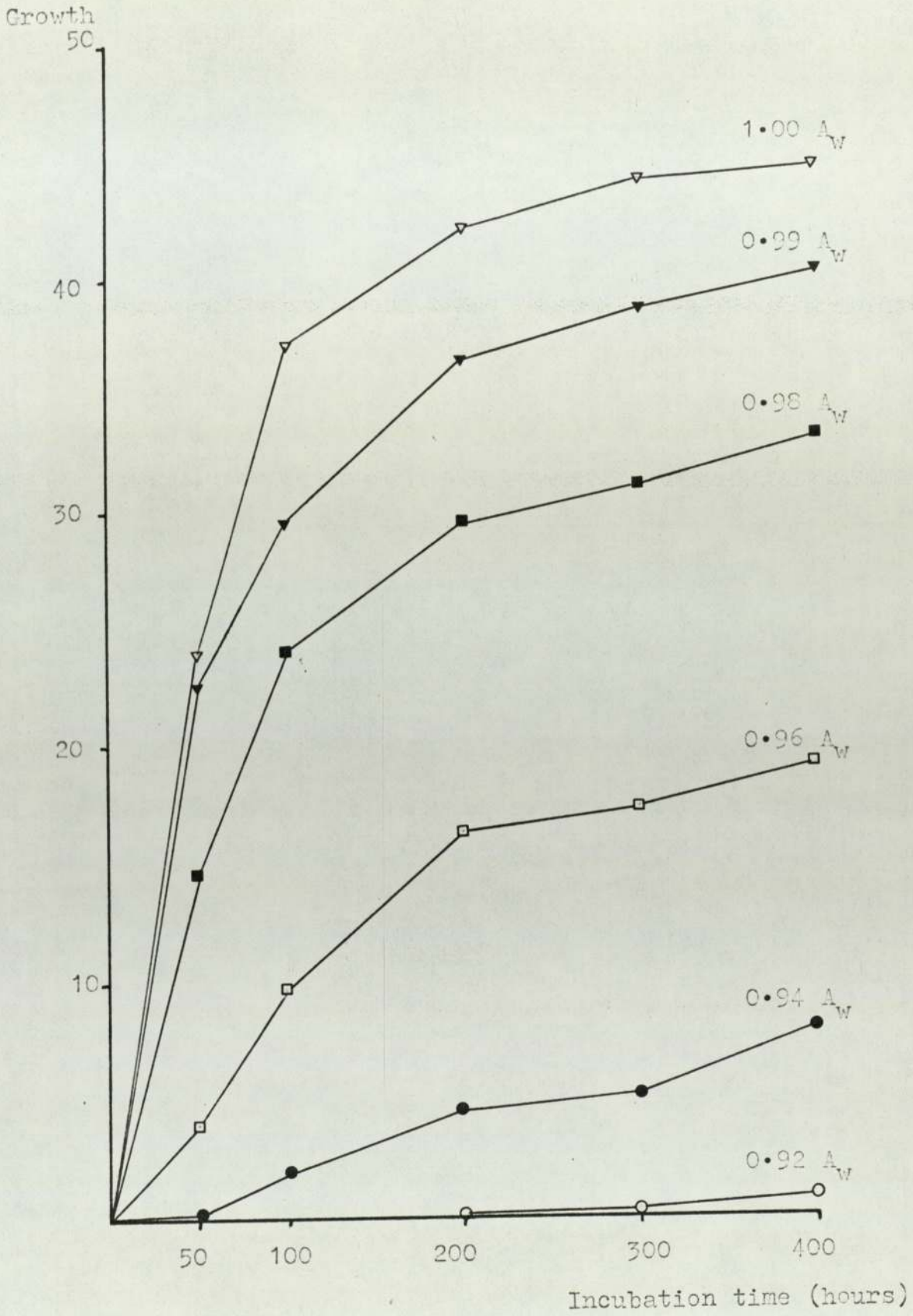



Fig. (35)Pooled growth of all Streptomyces spp. isolates on nutrient, starch and cellulose media with respect to time and $A_w$ at 30 °C.

Fig. (36) Pooled growth of isolates on three types of media

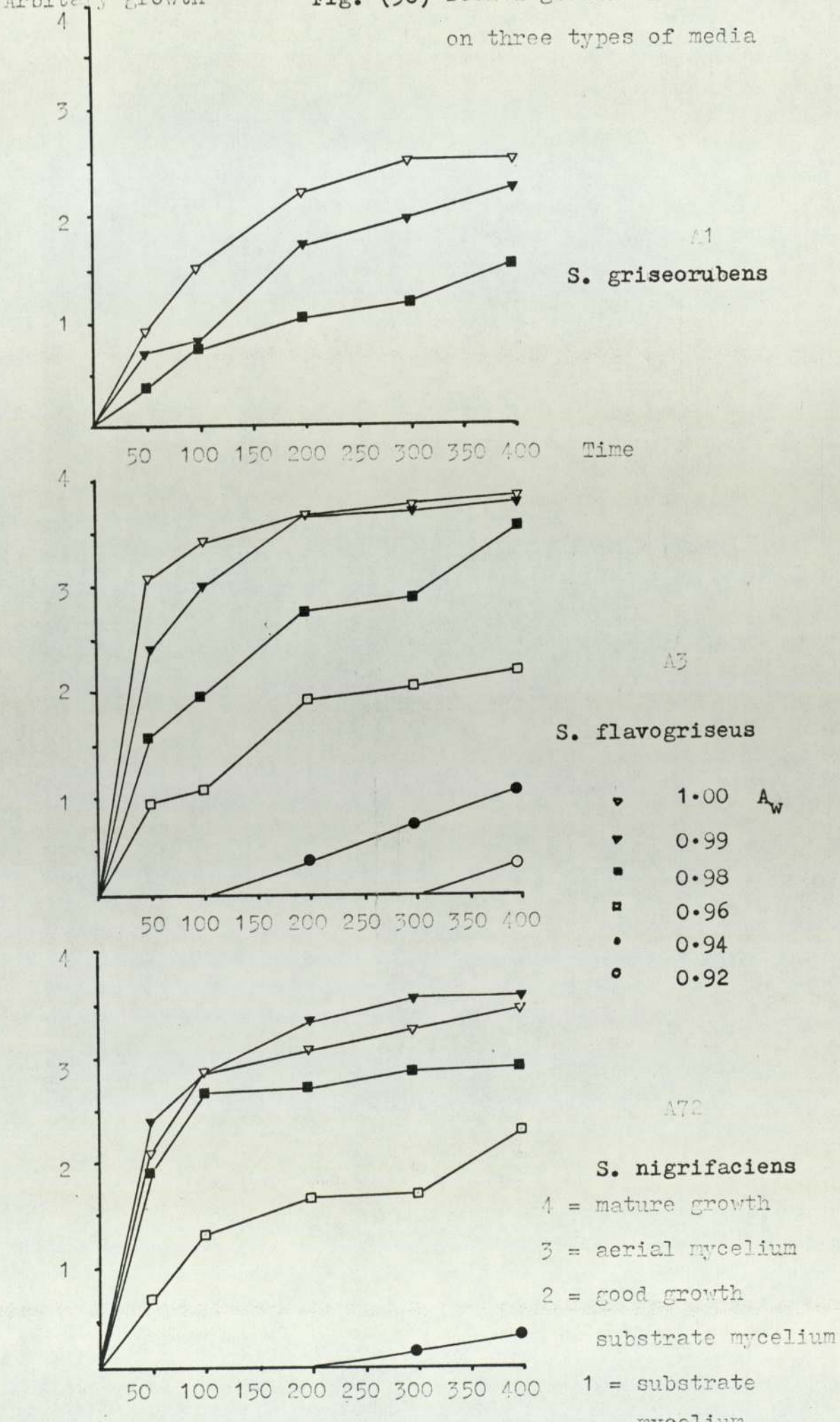
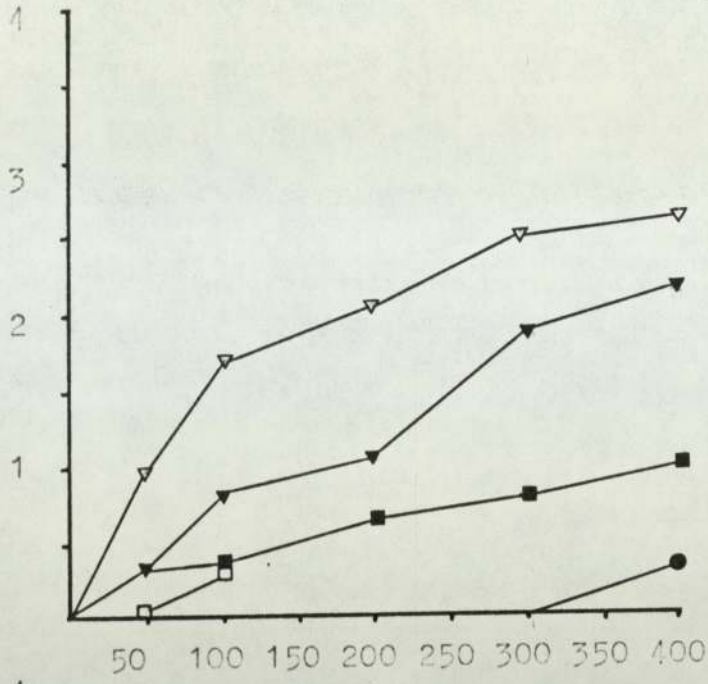
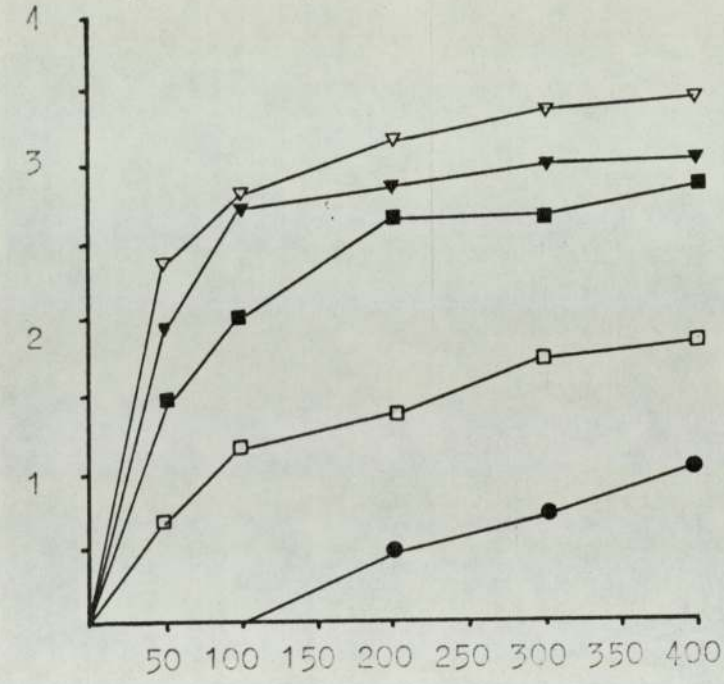


Fig. (37)

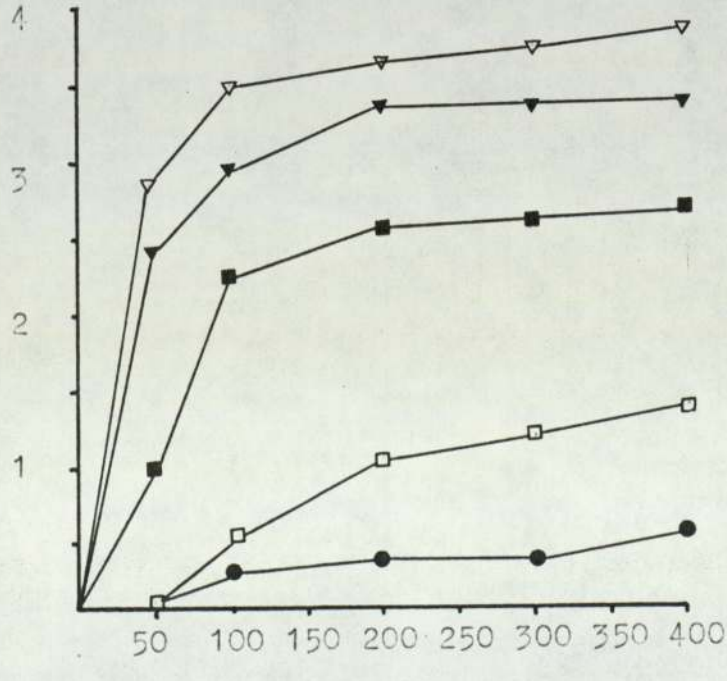



D1

S. setonii



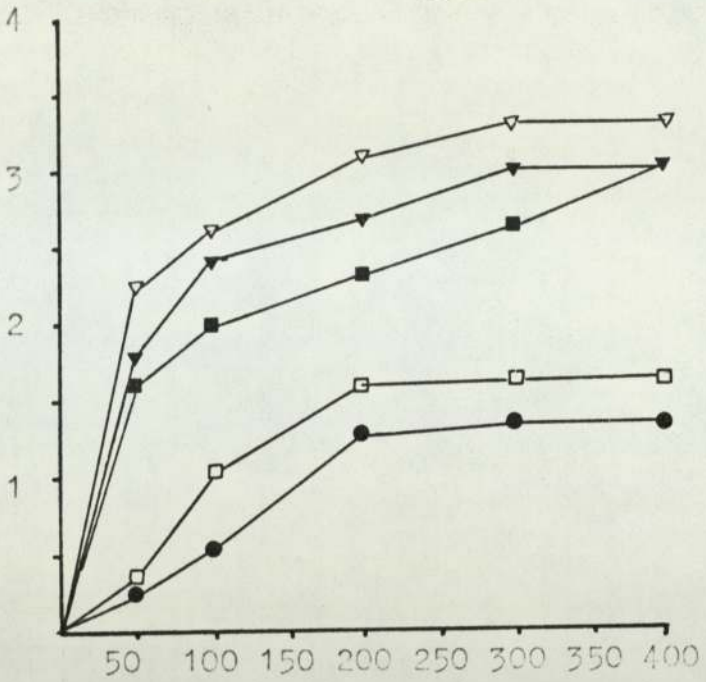

D8




B 10

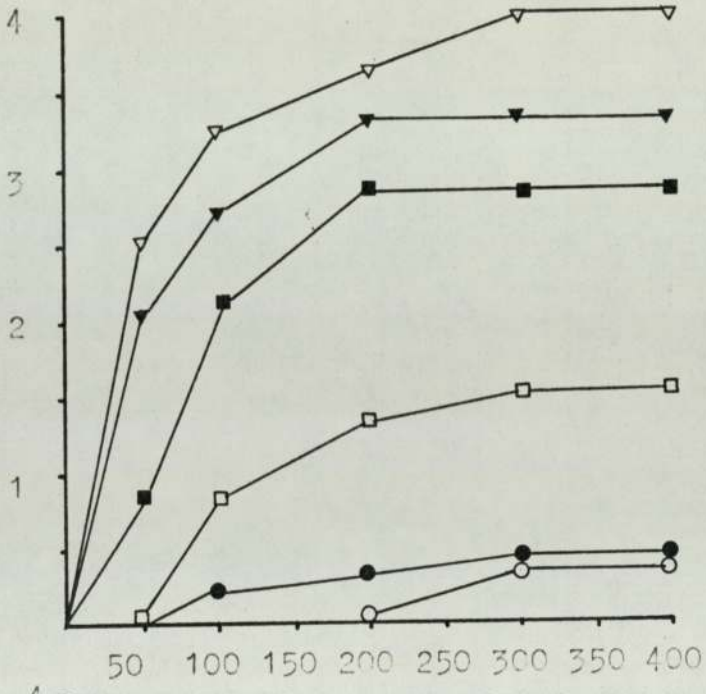
S. griseolus (1)

Fig. (38)



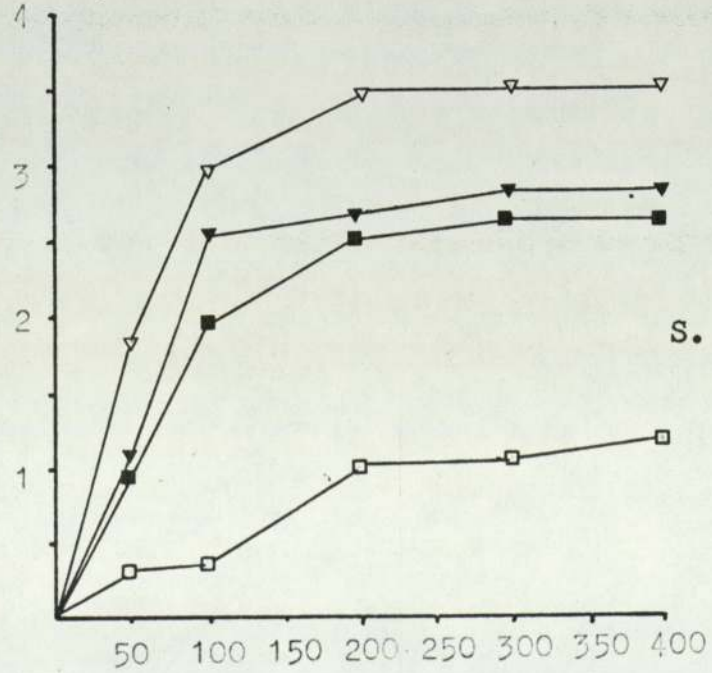

B12

S. albus




B17

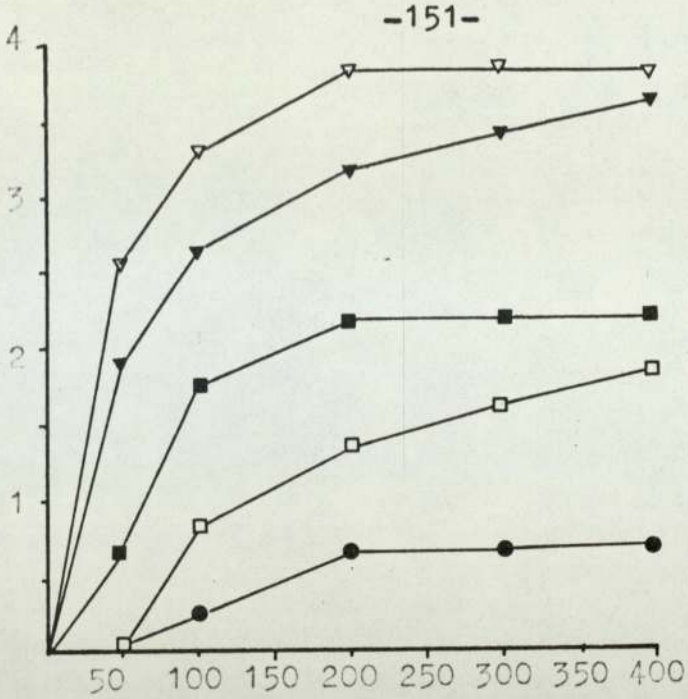
S. griseolus (2)



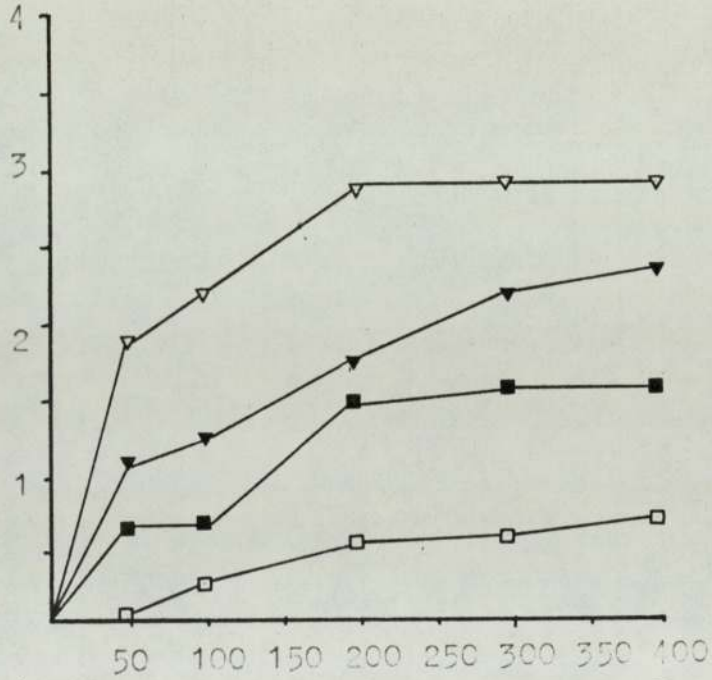

B20

S. atroolivaceous

Fig. (39)

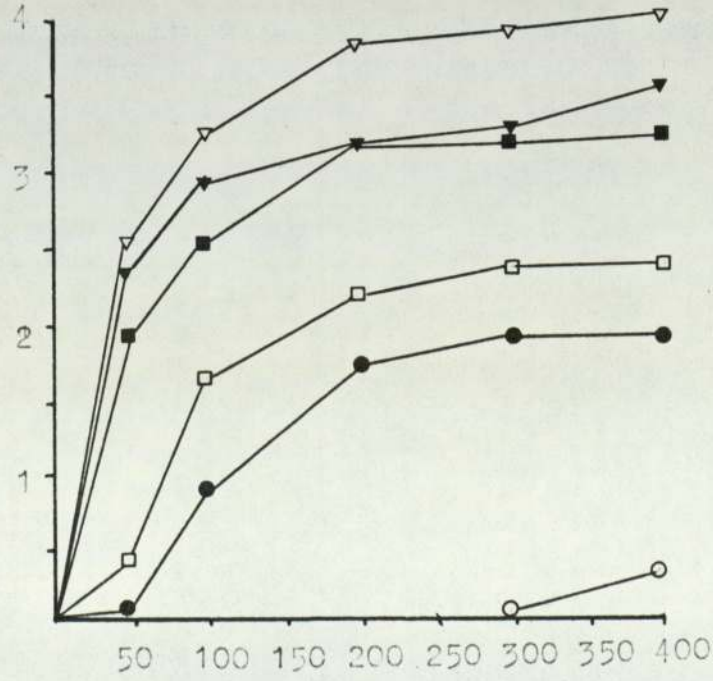



B28



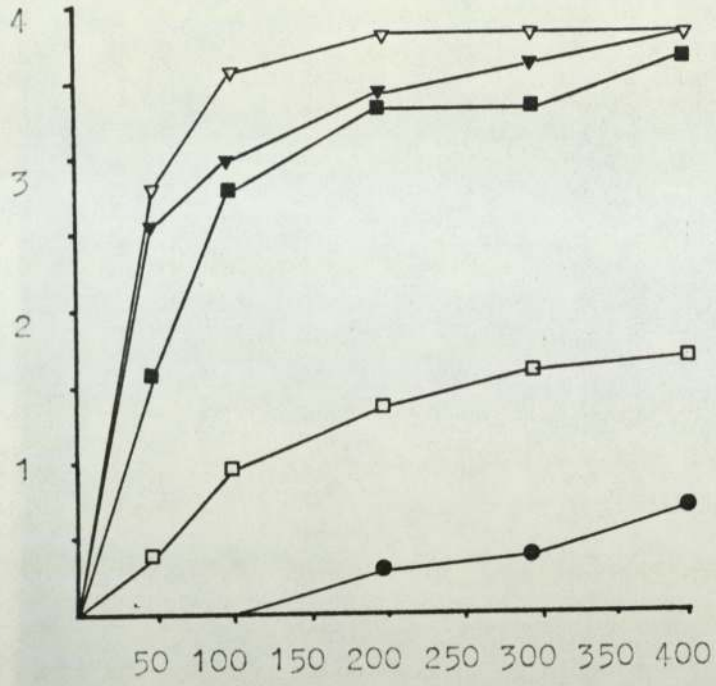

B29

S. lavendulae




B30

S. griseus




W8

S. griseolus (3)

Fig. (40)

formation of substrate mycelium. The graphs then tend to reach a plateau which is caused by two effects:

a) the averaging out of results, aerial mycelial development occuring on some nutrient sources but not on others.

b) some species especially at 100$A_w$ mature within 200 hours and so are incapable of further growth on the arbitary scale.

All species showed good growth at $A_w$'s 1.00, 0.99 and 0.98 and with the exception if A1 (Streptomyces griseorubens) grew at 0.96 $A_w$. At water activities below 0.96 growth was sparse and limited entirely to the production of primary substrate mycelium, no aerial mycelium was formed and hence no sporulation occured. Streptomyces spp. growing at $A_w$'s below 0.96 were indistinguishable to the naked eye from other bacterial colonies; even microscopically they were extremely similar.

In determining the lowest $A_w$ limits of growth for an individual species it is important to know how factors such as temperature, nutrients and pH may affect this limit.

The optimum growth temperatures for the Streptomyces spp. isolates used in these experiments were found to occur between 25-35°C, and so one would not expect there to be significant variations between species grown at 25°c and

30°C on nutrient agar. Three species were found to have increased tolerance at 25°C, two species decreased tolerance, and eight isolates had the same limiting $A_w$.

Ayerst (1968) stated that, "the maximum tolerance to extremes of water adivity is exhibited at approximately the optimum temperature and the maximum tolerance to extremes of temperature at approximately the optimum water activity. There is a tendency in both fungi and bacteria for the optimum water activity for growth to be lower at higher temperature and for the optimum temperature for growth to be higher at lower water activity". There is only limited evidence available of the interdependance of water activity and temperature but experiments by several workers Tomkins (1929), Bonner (1948), Ayerst (1968) support Ayerst's generalisation. There have been a number of investigations into the effect of water activity on the growth of bacteria, but few of the interactions of temperature and water activity. It has been suggested by Ingram (1957) that growth of some bacteria may be possible at higher salt concentrations at lower temperatures, but this has not been substantiated.

There is not a sufficiently large temperature differential in the present experiment to show whether temperature is a major factor in the limiting $A_w$ tolerance of Streptomyces spp.

From the data collected it would appear that nutrients in the $A_w$ controlled media are significant in the $A_w$

tolerance shown by isolates, fig. (41). Three main points emerge:

a) Isolates grown on cellulose media show a greater ability to grow at decreased $A_w$'s than those on starch. Eight species preferred cellulose, Streptomyces flavogriseus and Streptomyces rigrifaciens only grew on lower $A_w$'s on starch. In the case of Streptomyces flavogriseus this was due to its very limited cellulolytic ability.

b) Growth on nutrient agar at low $A_w$'s is slightly better than on starch media, six species prefering nutrient agar to four species showing greater tolerance on starch.

c) Tolerance to $A_w$ control is shown to be equal on cellulose and nutrient agar media for the species isolated. It can therefore be seen that growth occurred at lower $A_w$'s on nutrient agar and cellulose that starch, and thus fit in with Ayerst's generalisation concerning greater tolerance on more nutritious substrates.

It is important to point out, however, that none of the three substrates was shown to alter radically the individual species reaction controlled $A_w$'s.

Ayerst (1968a) noted that the action of specific extracellular enzymes might be inhibited by low water

Fig. 41 Comparison of $A_w$. growth limits on different media

+ indicates greater tolerance to $A_w$ on first media of each pair;
- less tolerance; 0 equal tolerance

a) Cellulose / Starch b) Starch / Nutrient Agar 30°C

| Species | $A_w$. difference | Species | $A_w$ difference |
|---|---|---|---|
| A1 | 0 | A1 | 0 |
| A3 | -0.2 | A3 | +0.4 |
| A72 | -0.2 | A72 | +0.2 |
| B1 | +0.5 | B1 | 0 |
| B8 | +0.5 | B8 | -0.2 |
| B10 | +0.2 | B10 | -0.2 |
| B12 | 0 | B12 | +0.2 |
| B17 | +0.2 | B17 | -0.4 |
| B20 | +0.2 | B20 | -0.2 |
| B28 | +0.2 | B28 | -0.2 |
| B29 | +0.1 | B29 | -0.3 |
| B30 | +0.2 | B30 | 0 |
| W8 | 0 | W8 | +0.2 |

c) Cellulose/Nutrient Agar 30°C d) Nutrient Agar 30 / 25 °C

| | | | |
|---|---|---|---|
| A1 | 0 | A1 | 0 |
| A3 | 0 | A1 | -0.2 |
| A72 | -0.2 | A72 | -0.2 |
| B1 | 0 | B1 | 0 |
| B8 | 0 | B8 | 0 |
| B10 | 0 | B10 | 0 |
| B12 | no reading | B12 | 0 |
| B17 | -0.2 | B17 | +0.2 |
| B20 | 0 | B20 | 0 |
| B28 | 0 | B28 | 0 |
| B29 | -0.2 | B29 | +0.2 |
| B30 | +0.2 | B30 | 0 |
| W8 | +0.2 | W8 | -0.2 |

activity so that breakdown of large molecules e.g. cellulose, to their soluble components would not occur or only very slowly. Growth would then only be possible if nutrients were available in soluble form.

"The reactions to pH resemble those to temperature", quotes Ingram (1957), tolerance by fungi to low $A_w$ being increased near to the optimum pH for growth. The Streptomyces isolates grew well over the range 5 - 9 and so pH was not thought to be an important factor in these experiments.

The limiting $A_w$ for each species on each substrate is shown graphically in fig. (42). The lowest water activity at which growth of substrate or aerial mycelium was noted is given in fig. (43) for the three types of substrate and from this has been determined the minimum overall $A_w$ for production of substrate or aerial mycelium for all the thirteen airbourne Streptomyces spp. isolated.

The limiting $A_w$ for each species on each substrate is shown graphically in fig. (42). The limiting $A_w$ for production of substrate mycelium varied from 0.99 S. setonii (starch substrate) and S. lavendulae (starch) to 0.92 S. flavogriseus (starch), S. griseolus (2) (nutrient 30 C) and S. griseus (cellulose).

The lowest water activity at which growth of substrate or aerial (for nutrient starch and cellulose substrates) was

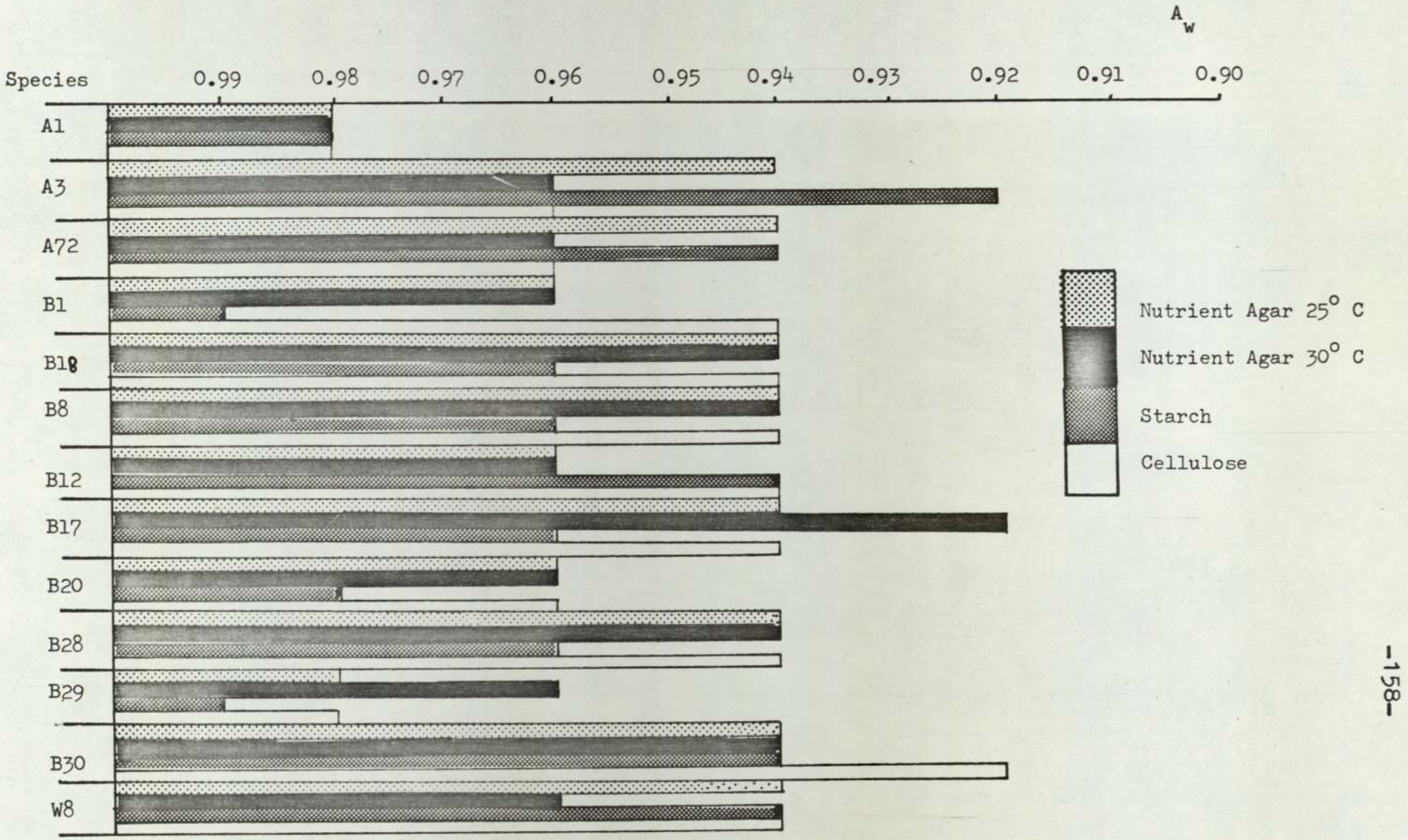



Fig. 42 Growth of Streptomyces spp. on different nutrients with respect to $A_w$

noted in fig. (43). From these results was determined the overall $A_w$ for production of substrate or aerial mycelium for all the thirteen airbourne Streptomyces spp. isolated.

Fig. (44) is an attempt to illustrate the various measurements used for water availability in biological work, and hence allow rough correlation to be made between, say, soil water content available pF and relative humidity.

### 6.1.2.1 Salt tolerance of Streptomyces spp.

It was thought important that solutes used in creating $A_w$ controlled media should be tested for possible toxicity effects. Each Streptomyces sp. isolate was grown on each individual salt used, as opposed to the mixture of salts used in the $A_w$ tests. KCl, $Na_2SO_4$ and NaCl were used in turn, a concentration range of 3, 7 and 10% salt being used. The salts were incorporated into nutrient agar for the tests. The osmotic pressure created in the media by an individual salt concentration could be calculated and correlated with the result produced by the salt mixture needed to produce the same osmotic pressure.

Klevenskaya (1960) showed that his Streptomycete cultures had high salt resistance. All Streptomyces spp. grew well in the presence of 3-7% sodium sulphate and 2-3% sodium chloride. Certain species tolerated considerably higher concentrations of these salts (9-12% $Na_2SO_4$ and 5-7% NaCL). Thus, with respect to salt resistance as well as a capacity to grow on media of high osmotic pressure, the Streptomyces spp. can tolerate a much higher salt concentration than is normally

| Species | | NUTRIENT Min. $A_w$ for substrate mycelial growth | NUTRIENT Min. $A_w$ for aerial mycl. growth | CELLULOSE Min $A_w$ for S | CELLULOSE Min $A_w$ for A | STARCH Min $A_w$ for S | STARCH Min $A_w$ for A | OVERALL Min $A_w$ for S | OVERALL Min $A_w$ for A |
|---|---|---|---|---|---|---|---|---|---|
| S. griseorubens | A1 | 0.98 | - | 0.98 | - | 0.98 | 0.98 | 0.98 | 0.98 |
| S. flavogriseus | A4 | 0.96 | 0.96 | 0.94 | 0.98 | 0.92 | 0.96 | 0.92 | 0.96 |
| S. nigrifaciens | A72 | 0.96 | 0.98 | 0.96 | 0.99 | 0.94 | 0.96 | 0.94 | 0.96 |
| S. setonii | B1 | 0.96 | 1.00 | 0.94 | - | 0.99 | 0.99 | 0.94 | 0.99 |
| | B8 | 0.94 | 0.99 | 0.94 | 0.98 | 0.96 | 0.98 | 0.94 | 0.98 |
| S. griseolus (1) | B10 | 0.94 | 0.96 | 0.96 | 0.99 | 0.96 | 0.98 | 0.94 | 0.96 |
| S. albus | B12 | 0.96 | - | 0.94 | 0.98 | 0.94 | 0.98 | 0.94 | 0.98 |
| S. griseolus (2) | B17 | 0.92 | 0.96 | 0.94 | 1.00 | 0.96 | 0.98 | 0.92 | 0.96 |
| S. atroolivaceous | B20 | 0.96 | 0.98 | 0.96 | 0.98 | 0.98 | 0.98 | 0.96 | 0.98 |
| | B28 | 0.94 | 0.99 | 0.94 | 0.99 | 0.96 | 0.98 | 0.94 | 0.98 |
| S.lavendulae | B29 | 0.96 | 0.98 | 0.98 | 1.00 | 0.99 | 0.99 | 0.96 | 0.98 |
| S. griseus | B30 | 0.94 | 0.98 | 0.92 | 0.96 | 0.94 | 0.94 | 0.92 | 0.94 |
| S. griseolus (3) | W8 | 0.96 | 0.98 | 0.94 | 0.98 | 0.94 | 0.98 | 0.94 | 0.98 |
| Average $A_w$ for all species | | 0.950 | 0.984 | 0.95 | 0.985 | 0.958 | 0.975 | 0.941 | 0.971 |

Fig. (43) Minimum $A_w$'s required for growth of Streptomyces spp. substrate and aerial mycelium on nutrient, starch and cellulose substrates.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RELATIVE HUMIDITY R.H. | 99.9 | 99.0 | 98.0 | 97.0 | 96.0 | 94.0 | 92.0 | 90.0 | 86.0 | 81.0 | 75.0 |
| WATER ACTIVITY | 0.999 | 0.990 | 0.980 | 0.970 | 0.960 | 0.940 | 0.920 | 0.900 | 0.860 | 0.810 | 0.750 |
| WATER SUCTION pF | 3.17 | 4.24 | | 4.74 | | 5.05 | | 5.29 | 5.44 | 5.59 | 5.74 |
| OSMOTIC PRESSURE II ATM | 1.00+ | 13.93 | 27.42 | | 55.38 | 84.02 | 113.02 | 143.3 | | | |
| WATER PRESSURE/ POTENTIAL BAR UNITS | | 13.75 | 27.30 | | 54.60 | 83.00 | 111.9 | 141.5 | | | |

Fig. 44 A comparison of the various terms used for water availability in biological systems.

found in soil. Data on the limiting osmotic pressure obtained by sucrose and various salts showed that the salt toxicity for Streptomycetes is related not only to the osmotic pressure increase, but also to the chemical properties of the salt. Thus, Klevenskaya found that Streptomyces spp. growth was checked at lower osmotic pressure of the media in the presence of increased salt (e.g. NaCl, $Na_2SO_4$ etc.) concentration than on media containing sucrose. Sodium sulphate was found to be the least toxic, sodium chloride the most. Salts that change the pH of a media would be particularly dangerous in this respect e.g. sodium bicarbonate.

Tresner et al (1968) made a survey of the Na Cl tolerance of approximately 1,300 strains of Streptomyces belonging to over 300 species. The normal growth media of these strains were supplemented with a graded series of Na Cl concentrations (4,7, 10 and 13%). They found that only 1.8% of the species could not tolerate Na Cl at 4% level; 26.9% could grow at a maximum of 4%; 49.7% could tolerate a maximum of 7%; 18.8% could grow at a maximum of 10% and only 2.8% could tolerate 13% Na Cl.

The specific effects of inorganic salts on the growth of other groups of micro-organisms has been discussed by Larsen (1963, 1967), Brown (1967) and Ingram and Kitchell (1967).

The maximal tolerance to NaCl, $Na_2SO_4$ and KCl components of Scott's controlling inorganic salts for $A_w$, by the isolated Streptomyces spp. is given in fig. (45). The concentrations of salts may be correlated with osmotic pressure and water activity of the media as shown in fig. (44). The Streptomyces spp. were, in all bar six cases, able to tolerate 7% NaCl; Tresner found that 80% of Streptomyces spp. fell into this category. All isolates were found to tolerate 7% sodium sulphate and 7% potassium chloride (with the exception of B20, S. atroolivaceous). There was insufficient information to make conclusions on salt toxicity as a factor influencing growth. Scott (1957) stated that water activity was the major limiting factor for fungal spore germination and growth in solute controlled media. B1, S. setonii for example, showed that $A_w$ effects of the media overode any possible salt toxicity effect.

Indication of possible solute toxicity may have been gained more effectively by using sugars to control the $A_w$ of the media. Media of the same $A_w$, but controlled by sugar or salts mixture could then have been compared.

### 6.2.2 Ayerst saturated salt solutions technique

The Ayerst technique as described in Chapter 2 was used to test Streptomyces spp. growth at known controlled relative humidities. The following humidities were used 100, 97.2, 95.5, 92.7, 90.3, 96.5 and 84.2% R.H. Agar strips or textile strips were suspended above the

| SPECIES | NaCl | $Na_2SO_4$ | KCl |
|---|---|---|---|
| A1 | 7 | 7 | 7 |
| A3 | 3 | 10 | 10 |
| A72 | 7 | 10 | 7 |
| B1 | 10 | 10 | 10 |
| B8 | 3 | 10 | 10 |
| B10 | 7 | 7 | 7 |
| B12 | 10 | 7 | 7 |
| B17 | 7 | 10 | 7 |
| B20 | 3 | 7 | 3 |
| B28 | 3 | 7 | 7 |
| B29 | 3 | 7 | 7 |
| B30 | 3 | 7 | 7 |
| W8 | 7 | 10 | 10 |

Fig. 45 Maximal tolerance to NaCl, $Na_2SO_4$ and KCl (% concentration) (at the levels tested).

saturated salts. Waksman starch agar was used containing 1ml/250ml agar of 2½% Pimafucin solution to act as a fungal inhibitor (incorporated after autoclaving 15 mins. at 15 p.s.i.). The media was allowed to cool and then poured onto a sterilized Perspex sheet and dried at 50°C for 48 hours, resulting in a thin transparent film. Perspex sheet was used as the starch film was found to adher to the relatively rough surface of glass. The film was cut into strips (1 cm x 5 cm) prior to sterilizing. Sterilization was carried out in a small desicator using propylene oxide (2 ml, 24 hours at 20°C).

The strips were aseptically removed and suspended in tubes above the saturated salt solutions. Tubes were then placed in an incubator at 25°C and left for seven days to ensure that equilibrium had occurred. The strips were rapidly removed and inoculated using spores from mature cultures. The atmosphere of the inoculating cabinet being at a lower relative humidity than the levels at which the strips had been kept in equilibrium. Moisture may therefore have been lost from the strips during inoculation, this would have been slight and the strips would readily return to equilibrium with the saturated salts.

Five tubes were used for each species at each relative humidity. The strips were inspected for growth either visually or microscopically; the technique being simple it was possible to regularly sacrifice strips for

close inspection. Six species were tested in this manner the results being shown in figs. (46,47). The method was not thought to compare in accuracy with the Scott controlled $A_w$ technique because of several factors the major two being:

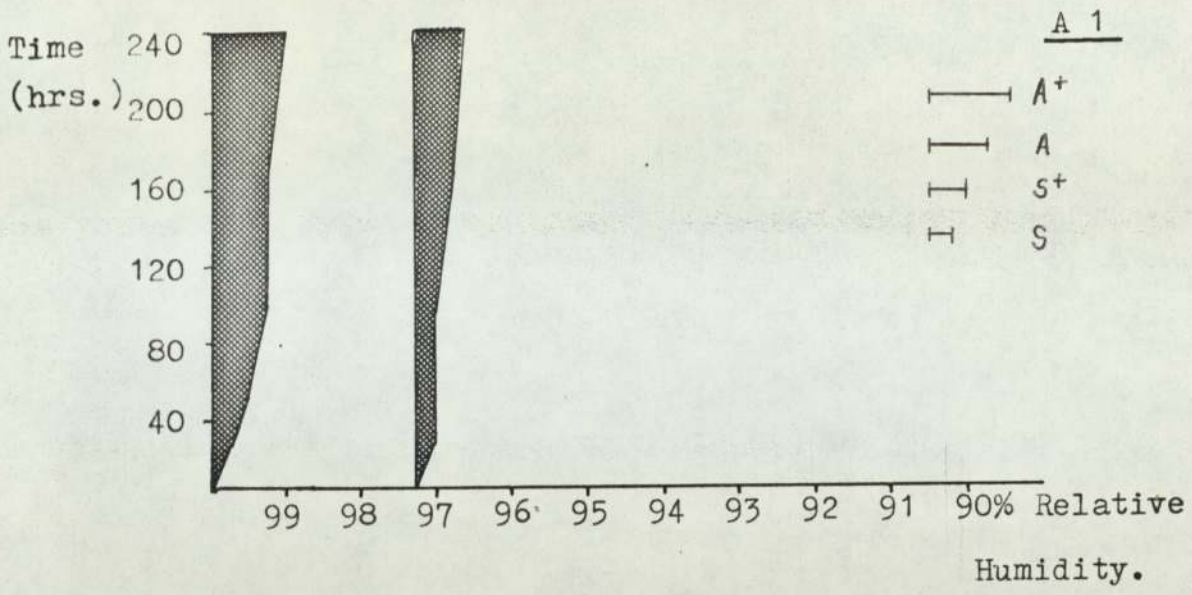
a) gradients of R.H. within the tube

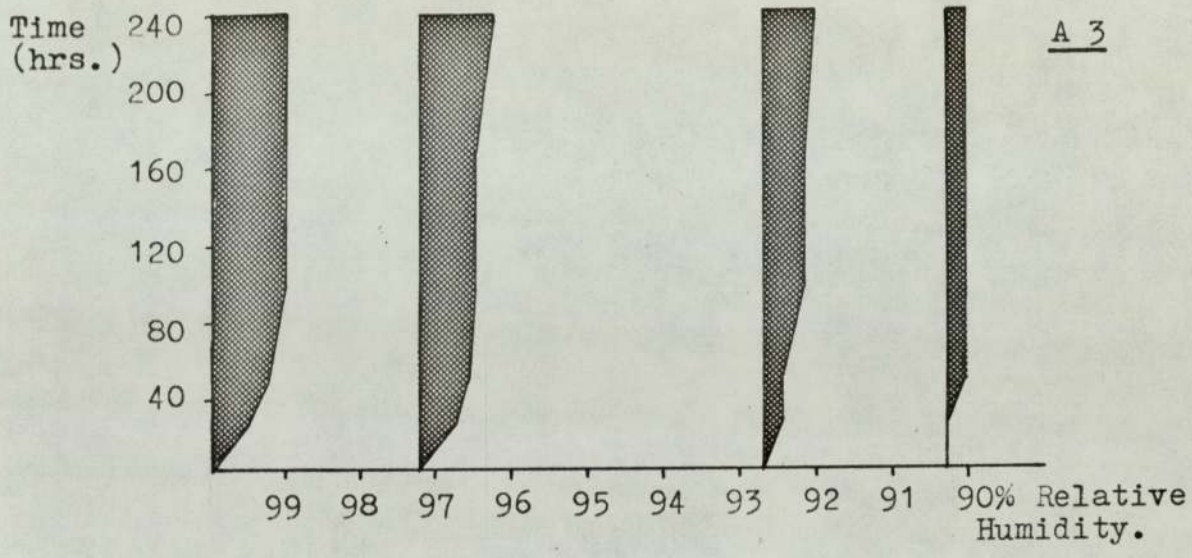
b) a hysteresis effect of the starch film

Figures obtained using this method were in general two points lower for the limiting $A_w$ for Streptomyces spp. growth.

Hysteresis, the different behaviour of water during drying (desorption) and wetting (adsorption) can also apply to soils. This means that there is no one relationship between soil water content and potential and the relationship is further affected by irreversible changes in the relationship of soil particles. The changes are often associated with change in water content; thus soils exhibit hysteresis is the relationship between water content and matric potential.

"The error introduced by hysteresis may be many bars potential, a fact unfortunately not recognised by many workers. The moisture characteristic must be produced by a soil treated in precisely the same way a soil in actual experiments", (Griffin 1972).



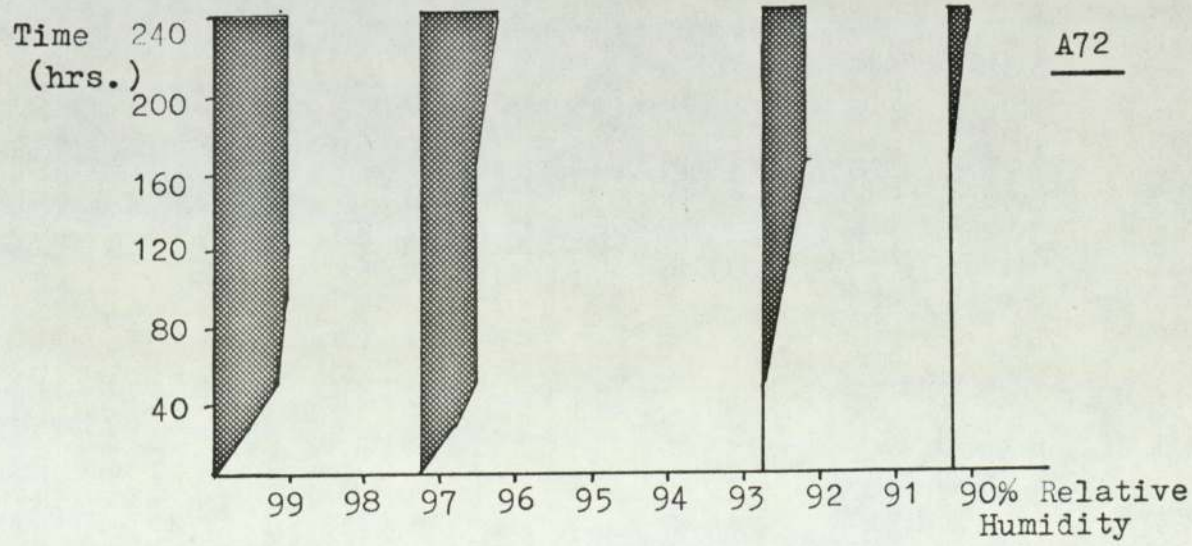
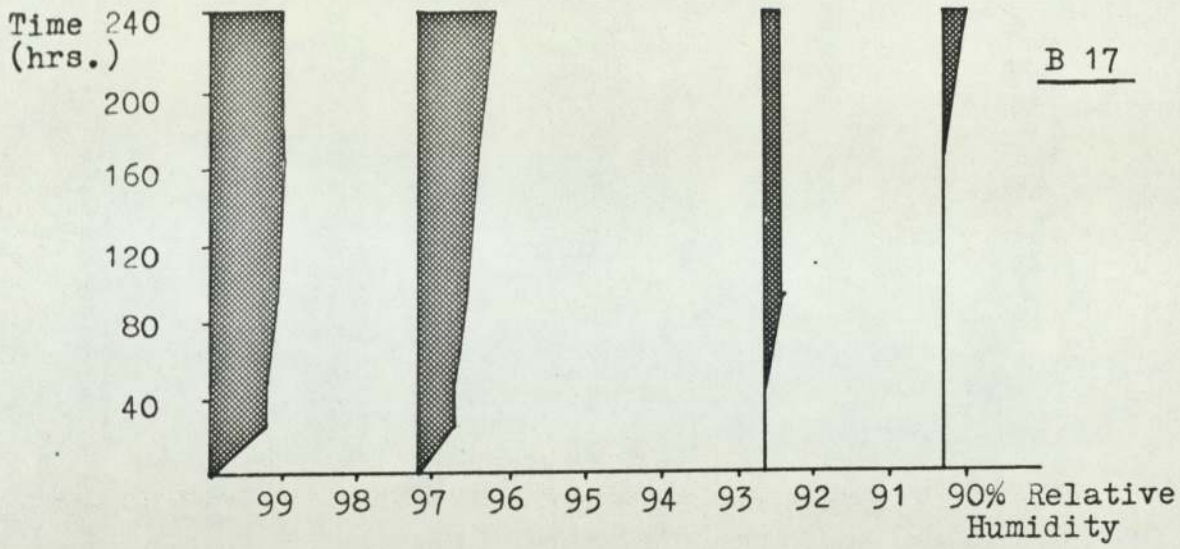
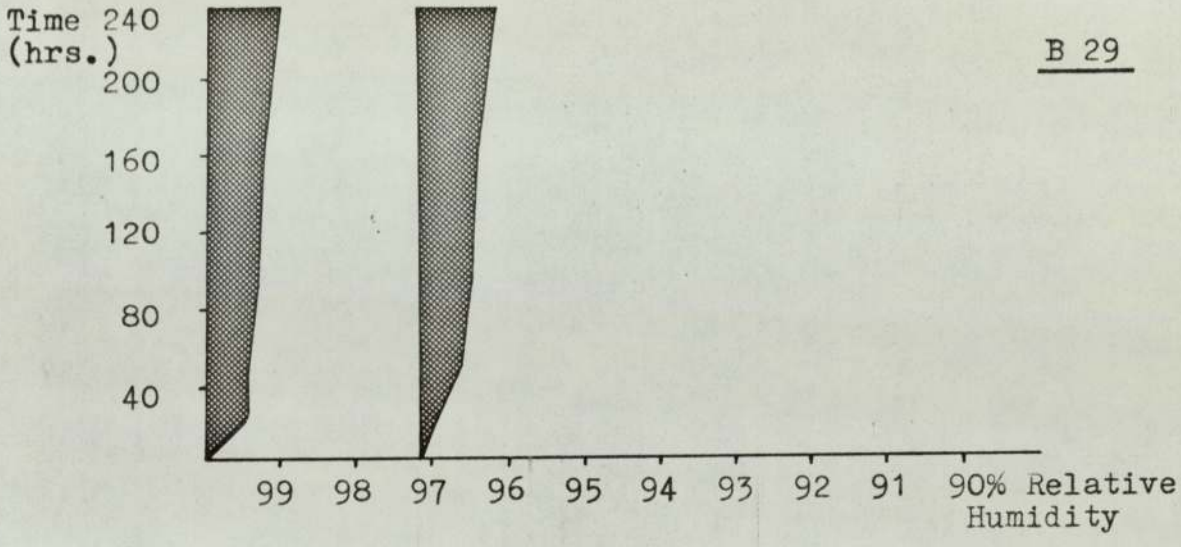




Figure 46 Growth capabilities of *Streptomyces spp.* at known R.H.'s using Ayerst technique of graded saturated salt solutions. ( width of histogram denotes the degree of growth.)



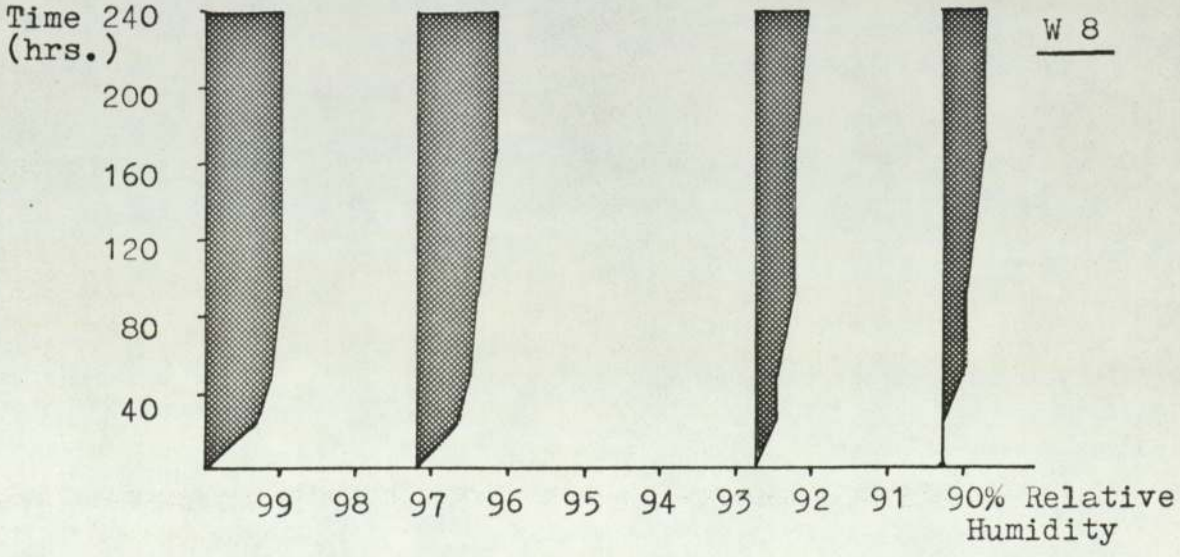




Figure 47 Growth capabilities of *Streptomyces* spp. at known R.H.'s using Ayerst technique of graded saturated salt solutions.

6.2.3 Controlled dry/wet air-mixing technique

This technique afforded a test environment which more nearly equated with natural conditions, the through flow of air removing possible volatile staling products. One of the primary objectives of the study was the development of a test facility giving a realistic environment. The apparatus however, was not sufficiently versatile to carry out precise experiments in the high relative humidity range required for growth of Streptomyces spp.

In experimentation with the air mixing system it was noticed that growth of Streptomycetes (predominently Streptomyces spp.) was occurring at relative humidities of 85% and thus it was decided to investigate that group of organisms. It was subsequently discovered that the relative humidity in the 85% R.H. tank, and relative humidities below, tended to rise during the course of the experiment. This increase in humidity was due to the nature of the self indicating silica gel which was used to remove water from the 'dry' airstream section of the apparatus. The colour change in the silica gel crystals was found to occur gradually, thus not giving a precise indication of moisture present. This led to the desiccant being changed much more frequently in subsequent experiments.

This series of events illustrated the opportunist nature of the Streptomycetes, as soon as there was sufficient water available for growth they could germinate, but if

the conditions reverted their spores were sufficiently adaptable to withstand 'drought' conditions. Thus an unexpected growth of streptomycetes can build up under favourable conditions.

A series of experiments were tried to act as a comparison with results previously obtained by saturated salt solutions and $A_w$ controlled media. The continuous air flow system was efficient when dealing with fungi, some of whose members have a wide range of humidities (60 - 100%) at which they are capable of growing. Streptomyces, however, are limited to R.H.'s in excess of 90% as small changes of R.H. are extremely important at high humidities it was impossible to get an accurate growth profile using only four humidities 85, 90, 95, 100%.

Starch agar films were used to test growth, the films having been dried were allowed to take up water and equilibrate in the tanks prior to inoculation, the results found are shown in fig. (48). The experiment was repeated using loomstate textile and starch finished textile and the results were identical with those for agar strips.

S. flavogriseus and S. nigrifaciens were found to be capable of occasional very limited growth of substrate mycelium at 85% R.H. Growth on the starch films in controlled environment tanks was found to occur at slightly lower humidities than was found on the $A_w$ controlled media. The minimum R.H. allowing growth was found to be on average

| HUMIDITY | SUBSTRATE MYCELIAL GROWTH | AERIAL MYCELIAL GROWTH |
|---|---|---|
| 85% | - | - |
| 90% | A3 S. flavogriseus<br>A72 S. nigrifaciens<br>B8 Streptomyces sp.<br>B17 S. griseolus (2)<br>B28 Streptomyces sp.<br>B30 S. griseus<br>W8 S. griseolus (3) | occasional flecks of aerial mycelium on all seven species |
| 95% | A1 S. griseorubens<br>B1 S. setonii<br>B20 S. atroolivaceous<br>B29 S. lavendulae | All the remaining isolates showed production of aerial mycelium at this R.H. |
| 100% | | All species showed production of mature aerial mycelium |

Fig. 48 Growth of Streptomyces spp. at humidities controlled by the continuous air flow system.

2% lower in the tanks. This lowered limit could possibly be within the range of experimental error but was found to be a constant factor which may have an alternative explanation. Hysteresis involving the starch film may account for the lower humidity limits for growth in a controlled air stream. It has been proposed that air flow has a stimulatory affect on organisms. Spoiling of brazil-nuts in a ship's cargo hold by species of Streptomyces led to an investigation of their cause by Ayerst (Per. comm.). It was thought that humidity and through ventilation conditions in the hold might have been responsible for the presence of the deteriogen. The tolerance of the organism to carbon dioxide was studied by Ayerst, by exposing colonies to atmospheres containing various concentrations of the gas up to 20 per cent. The colonies developed normally in all the experimental atmospheres.

Apparatus was constructed by Ayerst to pass air at a constant velocity (22 metres/min.), but at three humidities 70, 90 and 100% onto an agar substrate inoculated with the Streptomyces sp. at 30° C. Growth was found to be stimulated above all air jets; colonies furthest from the air inlet showing less stimulation. In a second experiment he had petri-dish cultured maintained at varying R.H.'s by means of saturated salt solutions. It was found that still air even at fairly low humidities had no stimulatory effect on the Streptomyces sp. growth. Ayerest concluded from this that it was unlikely that the creation of R.H. gradients was the

mechanism by which aerial mycelial growth was stimulated by air movement. He suggested that the air flow was removing a volatile chemical which inhibited the development of aerial growth.

Static factors may occur in soil inhibiting the growth of Streptomyces spp. and thus their ability to colonise textiles in use may not be fully reproduced by soil burial test proceedures. The continuous air flow system thus enables the Streptomyces spp. to grow in an environment free from possible volatile inhibitors such as proposed in Ayerst's investigation.

## 6.3 Conclusion

The experiments in this chapter have demonstrated several points in relation to determining relative humidity (or $A_w$) limits on growth. These are extremely elusive variants to pinpoint as they are so closely interwoven with other physical and chemical factors. The three methods of controlling relative humidity ($A_w$) served to illustrate this complex nature, as implied by the variations in results obtained by the different techniques. Control of substrate $A_w$ by inorganic salts is the only reliable accurate method of determining individual Streptomyces spp. relationships with water. For rapid, less accurate day to

day testing of substrates the two alternative methods would give a reasonable guideline.

Knowledge of the behaviour of Streptomyces spp. is extremely important in avoiding spoilage of textiles in storage. Prevention of biodeterioration must be based on a thorough understanding of the product, its storage environment and the nature and physiology of potential deteriogens. From the pooled results of all three techniques it was apparent that growth of Streptomyces spp. isolated did not occur below 90% R.H. ($0.90A_w$) on starch or cellulose substrates, and that production of aerial mycelial growth was rare below 95% R.H. Below 95% R.H. the Streptomyces spp. colonies could easily have been mistaken for bacterial colonies and this is a major reason for the lack of interest show in this group in biodeterioration tests. The experiments thus showed that the production of sporulating aerial mycelium was limited to 98% and above in the vast majority of tests. Sporulation did not increase at lower $A_w$'s as one might have expected as a reaction to adverse conditions. The humidity/growth relationships of fungi have been categorised by Hattori (1973):

1. Hygrophiles - Minimum R.H. for growth 95%
2. Mesophiles - Minimum R.H. for growth lies between 90 and 95%. Maximum growth at about 100% but growth shows little reduction at 97 to 98% R.H.

3. Xerophiles - Minimum R.H. for growth lies at or below 90%. Growth shows little reduction until R.H. 95%

Using the above categories all Streptomycetes tested would fall into the hygrophilic or mesophilic classifications.

Streptomyces spp. appear to have a lower competitive ability than the 'sugar' fungi and rapidly growing bacteria. But when the nutrient supply becomes exhausted or when adverse environmental conditions occur some Streptomycetes can survive as spores and have a marginal advantage over some associated fungi. The spores can later germinate and exhibit limited growth when adjacent to autolysing fungal hyphae or more recalcitrant molecules.

Air current experimental conditions represent most nearly the in-use situations encountered by textiles. Gaseous staling products such as $CO_2$ and possibly other aromatic organic volatiles are quickly removed as would be the case in nature. The air current system was found to be insufficiently accurate to determine the ability of Streptomycetes to grow over a range of relative humidities.

The air current system is ideal for the study of Streptomycetes at a single relative humidity say above 86%, but was unsuitable for an investigation requiring a range of relative humidities; the system being far easier to control at humidities below 95% its uses must really be restricted to experiments on fungi.

CHAPTER 7

# Biocide efficiency against *Streptomyces* spp

7.1 Introduction

7.1.1 Textile biocides

7.2 Visual assessment of biocide efficiency

7.3 Tensile strength testing of fabrics inoculated with *Streptomyces spp.*, protected by commercial biocides

7.4 Analysis of biocides

7.5 Conclusion

## 7.1 Introduction

*Streptomyces spp.* formed a significant proportion of the airspora isolated and some species were found to be moderately cellulolytic. The role of *Streptomyces spp.* in biodeterioration particularly with respect to cotton textiles has been outlined in section 1.5. It is because this group of micro-organisms is so difficult to identify, that they have been neglected; only limited estimates of their cellulolytic ability with respect to textiles have been made, Siu and Reese (1953), Betrabet et al. (1968).

The role of biocides in the prevention of biodeterioration has been introduced in section 1.6. However, when new biocides are marketed the tests which they undergo never include the use, as standard organisms, of actinomycetes. Trade literature giving toxicity data never mentions the *Streptomyces spp.*, so the ability of biocides to control them has not been comprehensively tested.

It was the aim of these experiments to:

a) measure physically by tensile strength testing the cellulolytic ability of selected *Streptomyces spp.* on cotton textile strips.

b) test the efficacy of three commercial biocides, both visually and by tensile strength, against attack by *Streptomyces spp.*

7.1.1 Textile biocides

Textile biocides are generally formulated to afford protection against a specific spoilage hazard e.g. biological, actinic, etc., and these preservatives are not necessarily interchangeable. The type of biocide used depends therefore upon the basic nature of the textile and the source of the deterioration expected in its intended use. At the present time no biocide has been discovered which will give complete protection without having any disadvantageous side effects on the textile.

A survey of the commercial products used to protect materials against biological deterioration has been carried out by Hueck van der Plas (1966). The survey is composed of keyed tables containing information on active compounds (arranged in groups of chemically active substances), trade names, fields of application, recommended concentrations and names of producers. Next to wood products, cotton textiles consume a greater quantity of industrial biocides than any other product. In 1959 a potential market of 154 million lbs. of cotton existed in the category of exposure to outdoor weathering and 270 million lbs. was subject to microbial attack but not in outdoor exposure. Estimating that cotton received 1% of its weight as biocides for preservation, Block (1967) calculated approximately 5 million lbs. of biocides per year in the U.S.A. to be the market potential for cotton biocides.

Biocides frequently employed in the protection of textiles have been classified in B.S. 2087 (1971). Three biocides from B.S. 2087 were employed in the following experiments; salicylanilide, tributyl tin oxide and the sodium salt of pentachlorophenol. Salicylanilide has been placed in class A and is recommended primarily for use on scoured or bleached cotton and flax textile in storage, mosquito netting and cotton yarns used in the electrical industry. Tribytyl tin oxide (T.B.T.O.) and Pentachlorophenol (P.C.P.) are both in class B, (biocides with a shorter proven commercial usage). and recommended for the protection of textiles in store and for temporary protection in outside use.

There is a long history of biocidal chemicals employed in the textile industry to prevent spoilage. These include magnesium chloride, zinc chloride, zinc sulphate, barium chloride, phenol cresylic acid, salicylic acid and formaldehyde. The phenolic products suffered from the disadvantage of their odour, whilst salicylic acid was costly and not very effective. The inorganic chlorides caused problems when the goods were heated, when tendering of the fabric might occur, (Marsh 1957).

The modern biocides used in the protection of cotton fibres and consequently also of the starch compounds coating them have been reviewed and investigated by Allsopp (1973) and Hollingsworth (1974). They both showed

that actinomycetes were capable of growing on low concentrations of biocides, though both were primarily working at thermophilic temperatures. Allsopp (1973) tested 'Mystox, L.P.L.', (Pentachlorophenyl laurate, P.C.P.L. produced by Catomance Limited, Welwyn Garden City). P.C.P.L. is an established commercial fungicide, composed of a wide range of fatty acid esters of pentachlorophenol of which the lauryl ester predominates. Isolations were made from soil inoculated, perfused, cotton textile or paper strips, and plated on Eggins and Pugh cellulose agar. The results obtained by Allsopp showed that paper protected by 1% P.C.P.L. supported growth of Thermoactinomyces glauca after 7 days and Thermoactinomyces vulgaris after 14 days, when incubated at 50° C. Filter paper strips protected by 2% P.C.P.L. supported growth of Thermoactinomyces glauca after 21 days and Thermoactinomyces vulgaris after 14 days. Cotton textile with 2% P.C.P.L. showed growth of Th. glauca after 21 days.

Hollingsworth (1974) carried out tests on four biocides:

a) P.C.P.L. (Mystox, L.P.L.)

b) P1, the ammonium sulphate compound of orthophenylphenoxy isopropanol.

c) P2, Orthophenyl phenoxy isopropanol

d) P3, a mixture of pentachlorophenoxy isopropanol and trichlorophenoxy isopropanol in the ratio of 70:30

(produced by Catomance Limited)

The biocides were incorporated into cellulose agar at three concentrations: 1%, 2% and 4%. Plates were soil inoculated and incubated at 50° C. Unidentified actinomycetes were found to grow on all the concentrations of the four biocides employed.

Increased tolerance of actinomycetes to increasing biocide concentration was claimed. This may partially explained by concentration gradients forming in the agar as only P1 was water soluble. Tests were also performed with biocidally protected cotton textile; Aspergillus fumigatus, actinomycetes and bacteria were found to show greater tolerance to all four biocides than any other microorganisms.

None of the streptomycetes tested by Pridham and Gottlieb (1948) were able to utilize phenol or cresol as a carbon source. Kuster (1963) found, however, that phenolase production by Streptomyces spp. was common, phenolic compounds being oxidised to quinoids. The browning of tyrosine containing media, used as an identification test for Streptomyces spp., by melanin formation is caused by a phenol oxidase.

Phenolases as oxidizing enzymes oxidise phenolic compounds with the formation of quinones. It may be suggested that these substances are formed and secreted in order to detoxicate and protect the organisms themselves, rather than as antibiotics against other organisms.

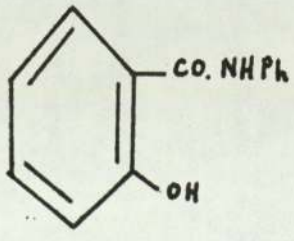
As some Streptomyces spp. possessed phenol oxidases capable of breaking down certain phenolic compounds, it was wondered if a similar enzyme system existed capable of breaking down or particlly detoxifying a phenolic biocide such as sodium pentachlorophenol.

It has been found that biochemical transformations of herbicide-derived anilines may be carried out in culture medium and soil by fungi. Soil Streptomyces were isolated but found to be incapable of producing aniline oxidase (Bordeleau and Bartha 1972), thus showing that salicylanilide was unlikely to be broken down or detoxified by the airbourne Streptomyces spp. isolates.

The general properties of the biocides used and their usage and effectiveness against microbial decay of cotton textiles may be summarised as follows:-

a) Salicylanilide (N-phenylsalicylamide) ('SHIRLAN')

Salicylanilide was one of the first purposely formed biocidal compounds used in the textile industry. It is a ring compound as can be seen from the formula:

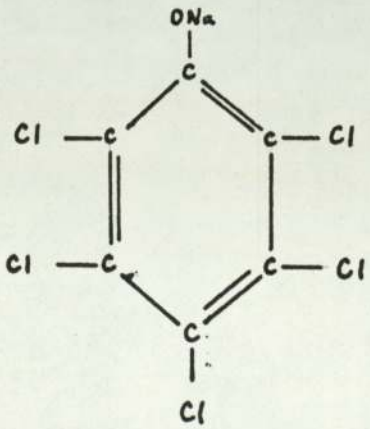


'Shirlan' was developed to replace traditional preservatives such as zinc chloride. Salinylanilide has moderate microbial resistance and should only be used for short term and interior; only limited resistance to actinic degradation and leaching restrict its uses.

Salicylanilide was used for preserving sized cotton yarn (0.06% per weight of fabric). In the finishing of dyed and printed goods, salicylanilide was an effective fungicide for goods which had to be shipped abroad, especially those dyed or printed with Alizarine purples, which are especially susceptible to mildew attack (0.06% per weight of finished fabric). With fabrics such as tent-cloth facing less favourable conditions 0.1 - 0.2% per weight of fabric was used.

Salicylanilide is bacteriostatic at low concentrations and bactericidal at higher concentrations. The biocide is more active against Gram +ve than Gram -ve bacteria, and is most active at acid pH's, the undissociated molecule being the active form (Baird-Parker and Holbrook 1971).

b) Sodium pentachlorophenate (Na.P.C.P.) - Trade name 'Mystox' D



Na.P.C.P. is used widely in the textile industry, being effective as both a bacteriocide and a fungicide, short-term preservation of fabrics may be quickly and cheaply obtained using this biocide. Textiles stored under humid conditions or transported through tropical countries to export destinations are particularly susceptible to spoilage. Long term preservation, however, against decay is better tackled using a pentachlorophenol derivative such as lauryl pentachlorophenate as Na.P.C.P. is slowly decomposed in the presence of U.V. light.

Textile size as mentioned in chapter 1 is particularly prone to microbial attack, especially during storage before use and during storage of sized yarns after beaming, the humid conditions of storage encouraging spoilage. By using a biocide such as Na.P.C.P. sized yarns can be left with a higher moisture content meaning that:

a) Fibres are kept pliable enough for weaving without risk of mould formation.

b) Output of sized yarn can be increased by making more efficient use of yarn-dry equipment.

If a biocide is incorporated into the starch size three advantages ensue:

i) It is not necessary to dry beamed yarns to a 10% moisture content which is the minimum moisture content consistent with the pliability essential for weaving whilst

cutting down on spoilage.

ii) Discoloured yarn is avoided and spoiling of finished textiles is overcome.

iii) Labour costs are reduced because large batches of size can be mixed. There is no need for seperate mixing of small quantities as and when required.

'In a long series of mill trials it was found that the addition of 1 part Na.P.C.P. per 1000 parts of size mixing gave complete protection; no mould growth occurred even when 20% - 30% moisture was left in the size yarns. Since less drying was required, more capacity of the yarn drying cylinders was released for drying sized yarns. A 30% increase in the production of sized warps was obtained, (Monsanto 1972).'

The dosage of Na.P.C.P. required for protection of textile size and yarns varies with the mill environments. 1 part Na.P.C.P./looo parts of size mixing will give adequate protection under normal mill conditions; the optimum concentration being dependent upon the prevailing temperature and humidity conditions. The correct dose of Na.P.C.P. being added to the size paste as a stock solution, before the paste is run into the sow-box of the sizing machine.

The short term preservation of cotton textile using Na.P.C.P. is attained in two ways:

(1) Wet spinning

Where wet spinning is used Na.P.C.P. is added to the water used for wetting the fibres immediately prior to spinning; the manufacturers recommend 0.5Kg of Na.P.C.P. to 1,000 litres of water.

(2) Finishing

Na.P.C.P. is used as a starch preservative and can, therefore be used in conjunction with a starch finish.

Toxicological Data (Monsanto 1972)

Fungi Toxicity of Na.P.C.P. as determined by standard Malt agar petri dish test.

e.g. Chaetomium globosum inhibiting concentration 0.006 (concentration % by wt. of protected article), killing concentration 0.010

Aspergillus niger inhibited 0.008, killed 0.1+

Figures for many wood and textile decaying fungi have been recorded and the greatest concentration of Na.P.C.P. for complete protection was 0.1% per wt.

| Bacteria | p.p.m. causing inhibition |
|---|---|
| Escherichia coli | 60 |
| Chromabacterium sp. | 80 |
| Micrococcus sp. | usually 5 - 40 |

Yeasts Wild yeasts, which cause serious damage in fermentation industries, are controlled by a concentration of 75 - 100 p.p.m. Na.P.C.P.

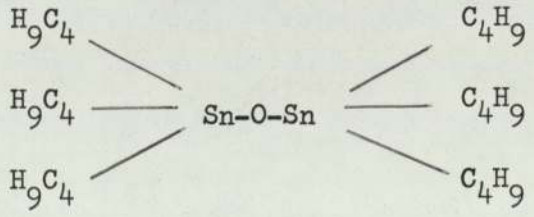
Algae Na.P.C.P. is toxic to a wide range of algae which might for instance occur in a wet-spinning tank, Na.P.C.P. being soluble in water.

| | p.p.m. Na.P.C.P. to kill 25 - 35°C pH7 |
|---|---|
| Diatoms | 5 to 10 |
| Chlorophyceae | 5 to 10 |
| Cyanophyceae | 10 to 20 |

There is no mention, however, in the manufacturer's handbook of any toxicological data with respect to actinomycetes and Streptomyces spp. in particular.

c) Tributyltin oxide (T.B.T.O.)

bis (tri-n-butyltin) oxide is the correct title




Miller (1968) stated that under normal climatic conditions no significant ultra-violet degradation of tributyl tin oxide will occur. Depending upon the techniques of manufacture, T.B.T.O. can contain varying

proportions of dibutyl tin oxide which, even in the presence of a so called stabilizer, will gradually settle out on standing and it is almost certainly this which has been the origin of reports on the instability of T.B.T. to U.V. light.

Information on the technology and commercial applications of T.B.T.O. with respect to its use as a preservative in the textile industry is scanty and ill-defined. "The testing of a textile preservative by natural weathering proceedures is time-consuming and hence recourse has always been to accelerated soil burial tests, even though such conditions are irrelevant to most of the end uses of the preservative treated fabric e.g. tarpaulin, tentage and containers", Miller (1968).

T.B.T.O. is a functional failure as a biocide for textiles when assessed by the soil burial technique, but is claimed to give good protection under conditions of natural weathering. Miller outlines comprehensive testing by outdoor exposure of a series of T.B.T.O. impregnated fabrics (15 oz cotton duch 12 oz terylene core, 5 oz tentage, unboiled flax tow, jute tarpaulin and a boiled line flax) and compared this with conventional pentachlorophenyl laurate (P.C.P.I) treatment.

The following levels of biocide were applied

(a) 2.0 - 2.2% P.C.P.L.

(b) 0.5 - 0.6% T.B.T.O.

(c) 0.1% T.B.T.O.

Pieces of fabric were exposed for 12 months and evaluated in terms of:-

(1) decrease in tensile strength

(2) loss of fungicide

It was found that under normal climatic conditions of weathering a low T.B.T.O. application (0.1%) could give equivalent protection to P.C.P.L. for a wide range of fabrics.

It thus appears that the evaluation of textile preservatives (with particular reference to T.B.T.O.) is dependent upon the method of assessment and the relative importance attached to natural weathering and accelerated soil burial techniques for such assessment.

Chalmers (1967) carried out a survey of the chemistry and applications of organotincompounds. Because the biocidal activity is imparted by the tributyl tin groups a series of tributyltin derivatives have been developed for most physical requirements. Chalmers reports a toxicity test using T.B.T.O. on Escherichia coli; it was found that a standard equimolecular solution of 1 mol. of tin % diluted to 1 in 10 inhibited bacterial growth, but dilution 1 part in 20 did not.

To test whether the bacteriostatic effect was dependent on the type of organic acid combined with the organotin compound, Staphylococcus pyogenes aureus was used as a test organism; results showed the acid portion had no effect on the results. The bacteriostatic effect of organotic

compounds like that of silver, mercury and copper compounds can be neutralised by compounds containing SH- groups, for example cystein.

Where fabrics will need frequent washing, antibacterial and antifungal protection will be given by 0.025%, and 0.25% T.B.T.O. based on weight of fabric.

## 7.2 Visual Assessment of Biocide Efficiency

A series of tests were performed to determine the effectiveness of three biocides commonly used in the prevention of spoilage in the textile industry. The biocides used were 0.5% Na.P.C.P., 0.1% Shirlan and 0.1% and 0.2% T.B.T.O. The biocides were added as a percentage of the weight of starch on the fabric; the starch being 1% by weight of the cotton.

Standard T.N.O. test cloth strips were carefully cut into 1" x 2" pieces for use in the tests. The strips were dampened by spraying with sterile distilled water and placed on the surface of 4% cellulose (+Eggins and Pugh salts) agar plates. Individual inoculants of the Streptomyces spp., isolated from the air were finely streaked over the textile and the surrounding agar. The plates were then incubated at 30° C for 5 days, a pictorial record Plates (5-11) was then taken and the results tabulated in fig. (49).

| | STREPTOMYCETE SPECIES | LOOMSTATE FABRIC | 1% STARCH ON WT. OF FABRIC | 0.5% Na.P.C.P. | 0.1% SHIRLAN | 0.1% T.B.T.O | 0.2% T.B.T.O. |
|---|---|---|---|---|---|---|---|
| S. griseorubens | A1 | S+/A | A | - | - | A | $S^+/A$ |
| S. flavogriseus | A3 | - | $S^+/A$ | - * | - | - | - |
| S. nigrifaciens | A72 | A | A | - * | - | S/S* | $S^+/A$ |
| S. setonii | B1 | S | S | - | - | - | - |
| | B8 | A | A | - | - | $A/A^+$ | $A/A^+$ |
| S. griseolus (1) | B10 | $S^+/A$ | $A/A^+$ | - * | - | S | S |
| S. albus | B12 | - | S | - | - | $S^+$ | - |
| S. griseolus (2) | B17 | A | $A^+$ | - | - | $S^+/A$ | $S^+/A$ |
| S. atroolivaceous | B20 | S | $S^+/A$ | - | - | S | - |
| | B28 | A | $A^+$ | - * | - | $A^+$ | $A/A^+$ |
| S. lavendulae | B29 | A | $A^+$ | - | S | $A/A^+$ | $A/A^+$ |
| S. griseus | B30 | S | $S^+$ | - * | - | $S^+$ | - |
| S. rubrireticuli | R14 | Pink Staining | Pink Staining | - | - | Pink Staining | - |
| S. griseolus (3) | W8 | $A^+$ | $A^+$ | - * | S | $A^+$ | $A/A^+$ |

KEY: S sparse substrate mycelium A sparse aerial mycelium * Inhibition zone
$S^+$ strong substrate mycelium $A^+$ mature aerial mycelium formed

Fig. 49 Growth of Streptomyces spp. on biocide treated cotton textile

The unprotected fabric rapidly became colonised by all species of Streptomyces tested, though S. flavogriseus A3 and S. albus B12 showed no growth on the loomstate cotton. Loomstate fabric is almost entirely pure cellulose, and so the only nutrients available to the species were salts leached from the agar medium. Growth was found to occur more readily on the 1% starch coated fabric than on the loomstate fabric, starch being more easily attacked and assimilated than the pure cellulose fibres.

Streptomyces rubireticuli (R14) caused considerable pink staining of both the loomstate and starch coated fabric, but no growth could be seen on microscopic examination.

Sodium pentachlorophenate (Na.P.C.P) was found to be the most efficient biocide using visual assessment. The concentration used was 0.5% per weight of starch. Na.P.C.P. was found to totally inhibit all species of Streptomyces against which it was tested at this concentration. Na.P.C.P. is slightly water soluble and so a small amount was expected to leach out into the agar media. This fact was borne out by inhibition zones being formed in the agar media surrounding six of the fourteen species. The indicator species S. rubrireticuli showed no growth or staining in the presence of Na.P.C.P.


Plate 3

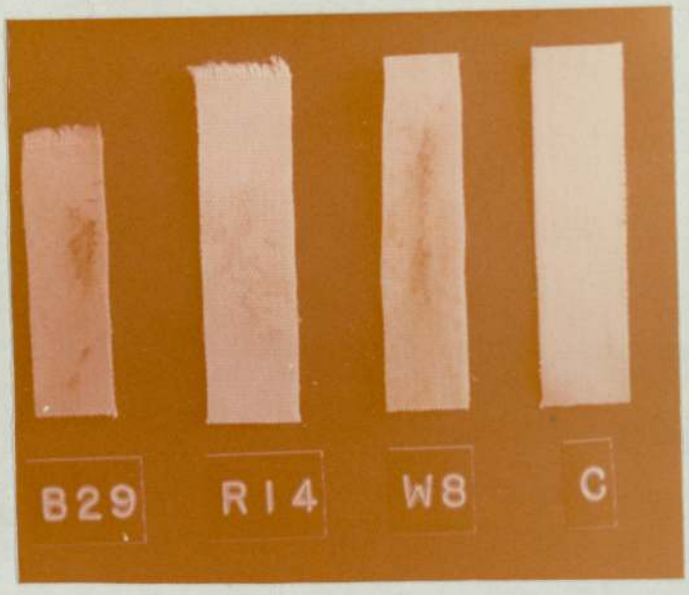



Plate 4

Cotton textile strips coated with 1% (w/w) wheat starch were suspended in Ayerst tubes at 100% R.H. The strips were incubated for 5 days at 30 C and the above staining occurred. No additional nutrients were added, the Streptomyces were growing purely on the starch and cellulose. Pigment formation leading to staining was found to occur most heavily on relatively nutrient deficient substrates and thus sodium nitrite was used as the nitrogen source in the subsequent tensile strength experiments.

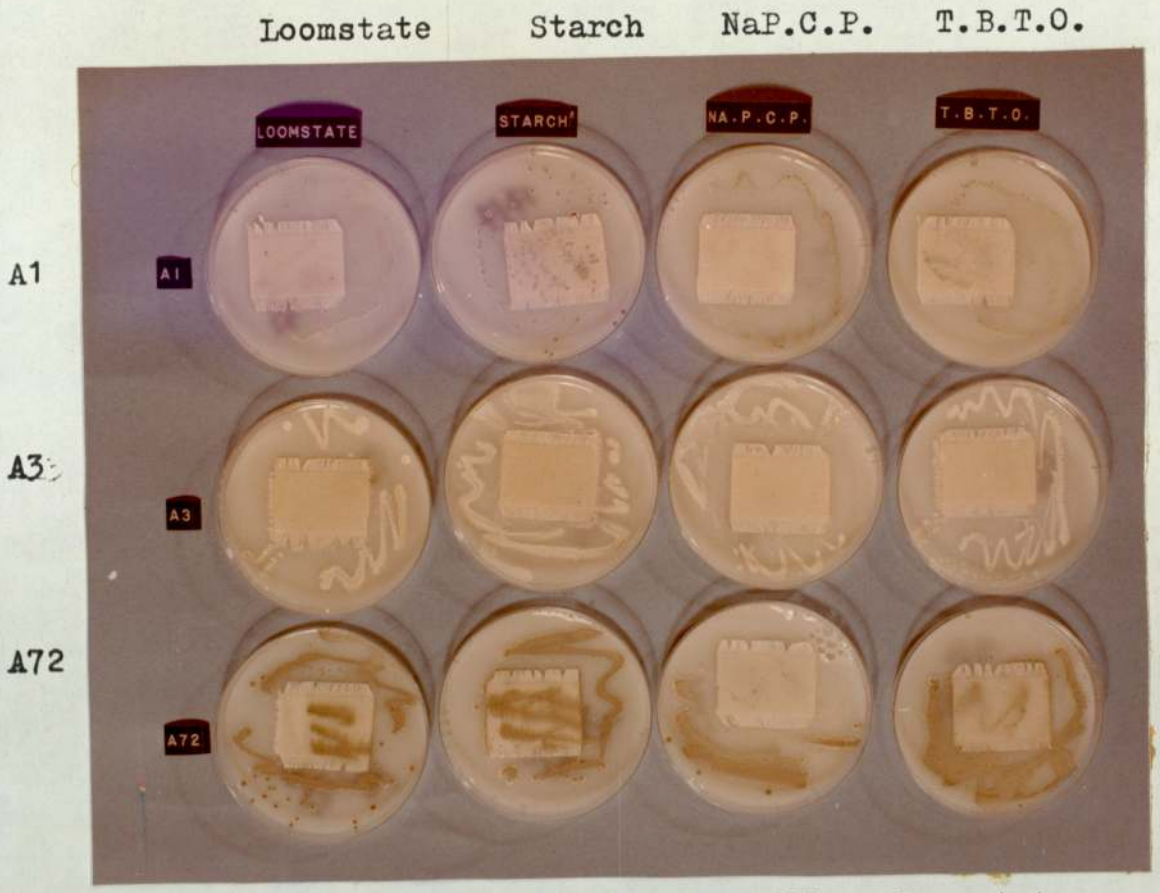



Plate 5. Growth of Streptomyces A1, A3, A72 on loomstate, starch-coated, 0·5% NaP.C.P. and 0·1% T.B.T.O. treated test strips

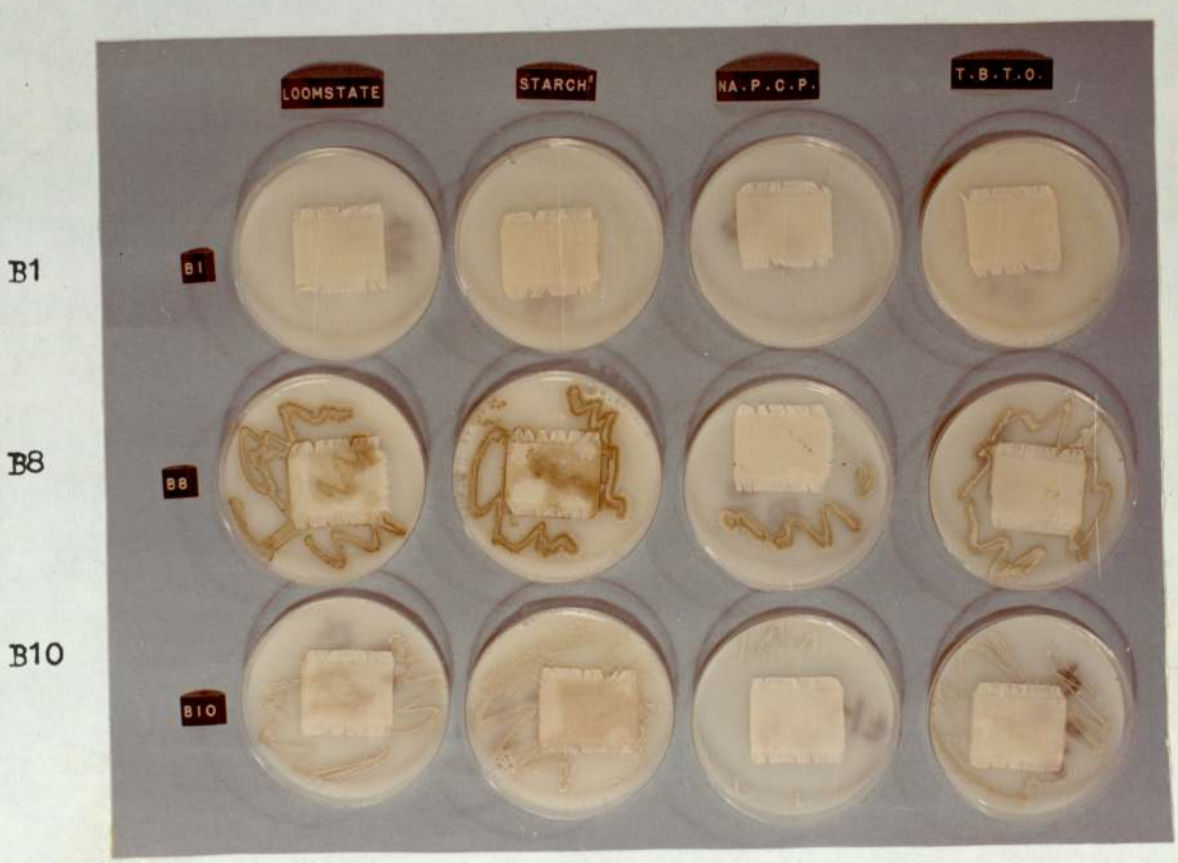



Plate 6. Growth of Streptomyces B1, B8, B10 on loomstate, starch-coated, 0·5% NaP.C.P. and 0·1% T.B.T.O. treated test strips

Loomstate Starch NaP.C.P. T.B.T.O.

B12

B17

B20


Plate 7. Growth of Streptomyces B12, B17, B20 on loomstate, starch-coated, 0·5%NaP.C.P. & 0·1%T.B.T.O. treated test strips.

B28

B29

B30

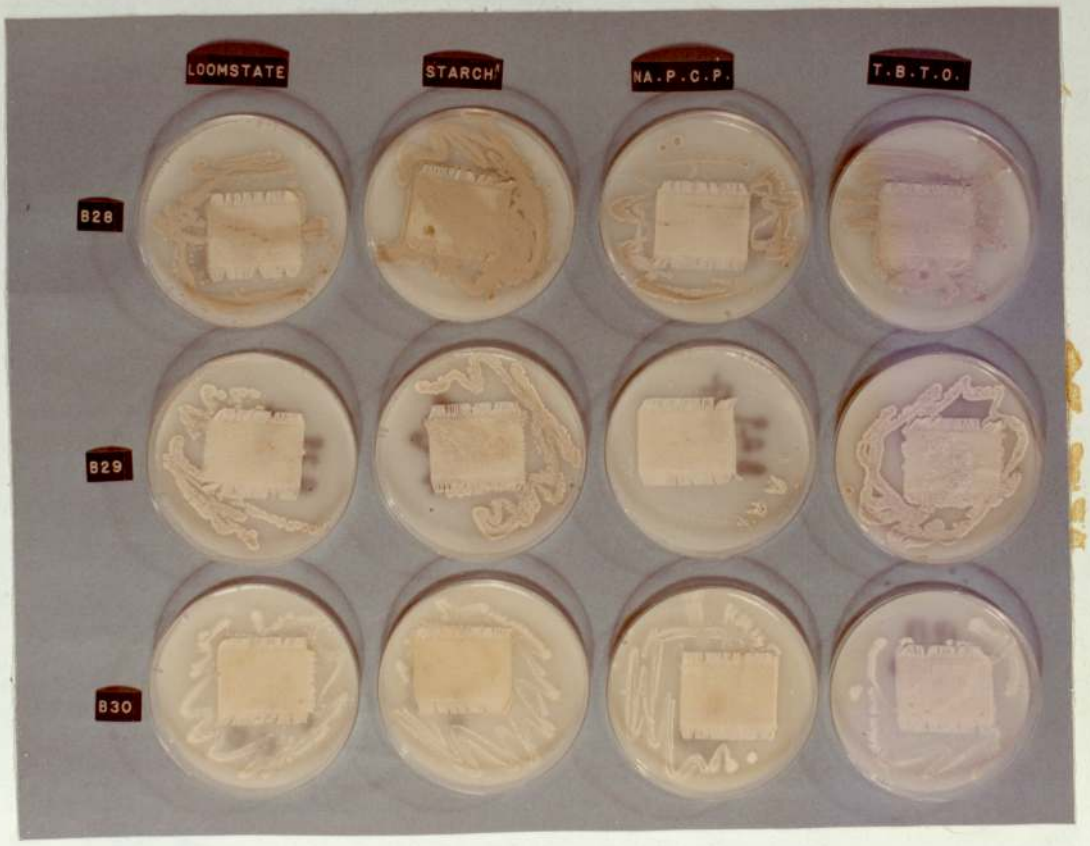



Plate 8. Growth of Streptomyces B28, B29, B30 on loomstate, starch-coated, 0·5%NaP.C.P. & 0·1%T.B.T.O. treated test strips.

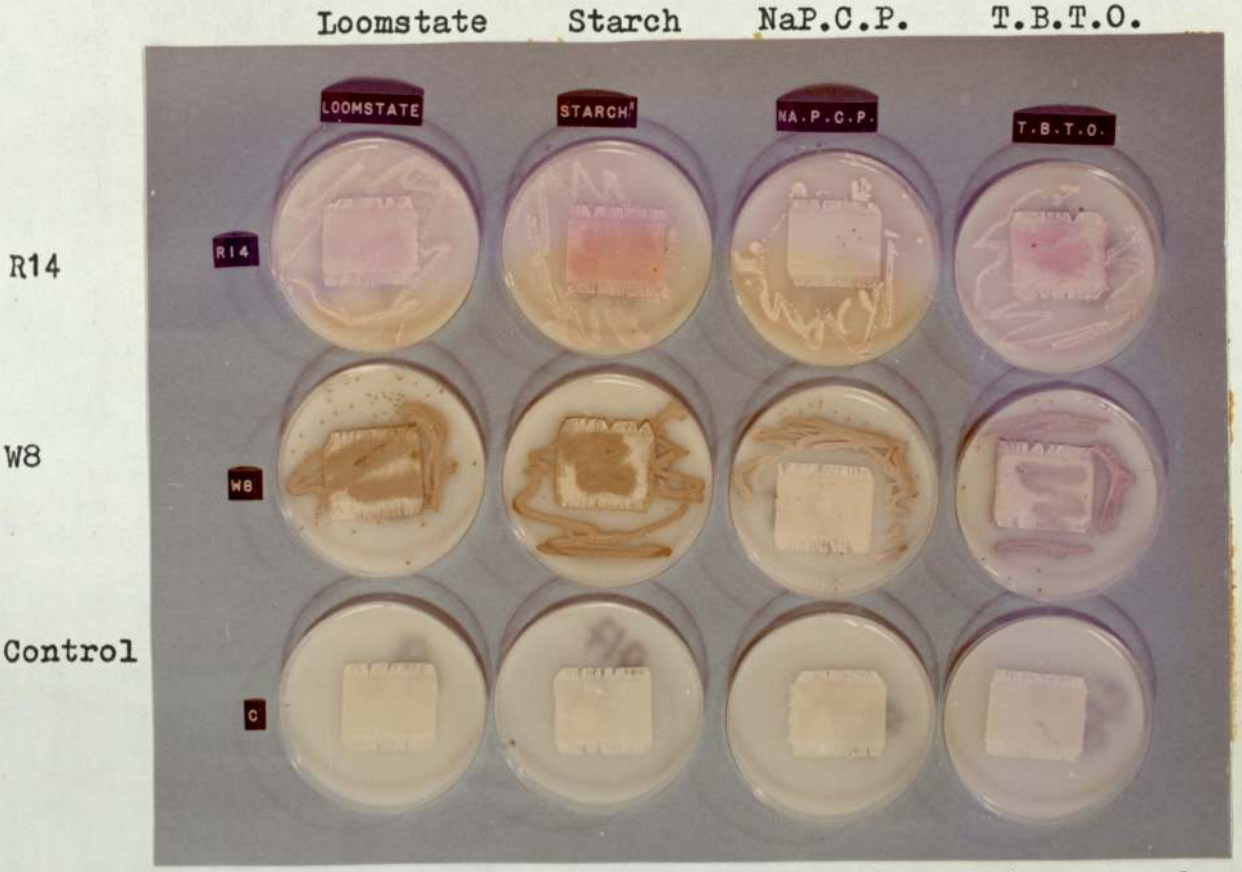



Plate 9. Growth of Streptomyces R14 & W8 on loomstate. starch-coated, 0•5% NaP.C.P. & 0•1% T.B.T.O. treated test strips.

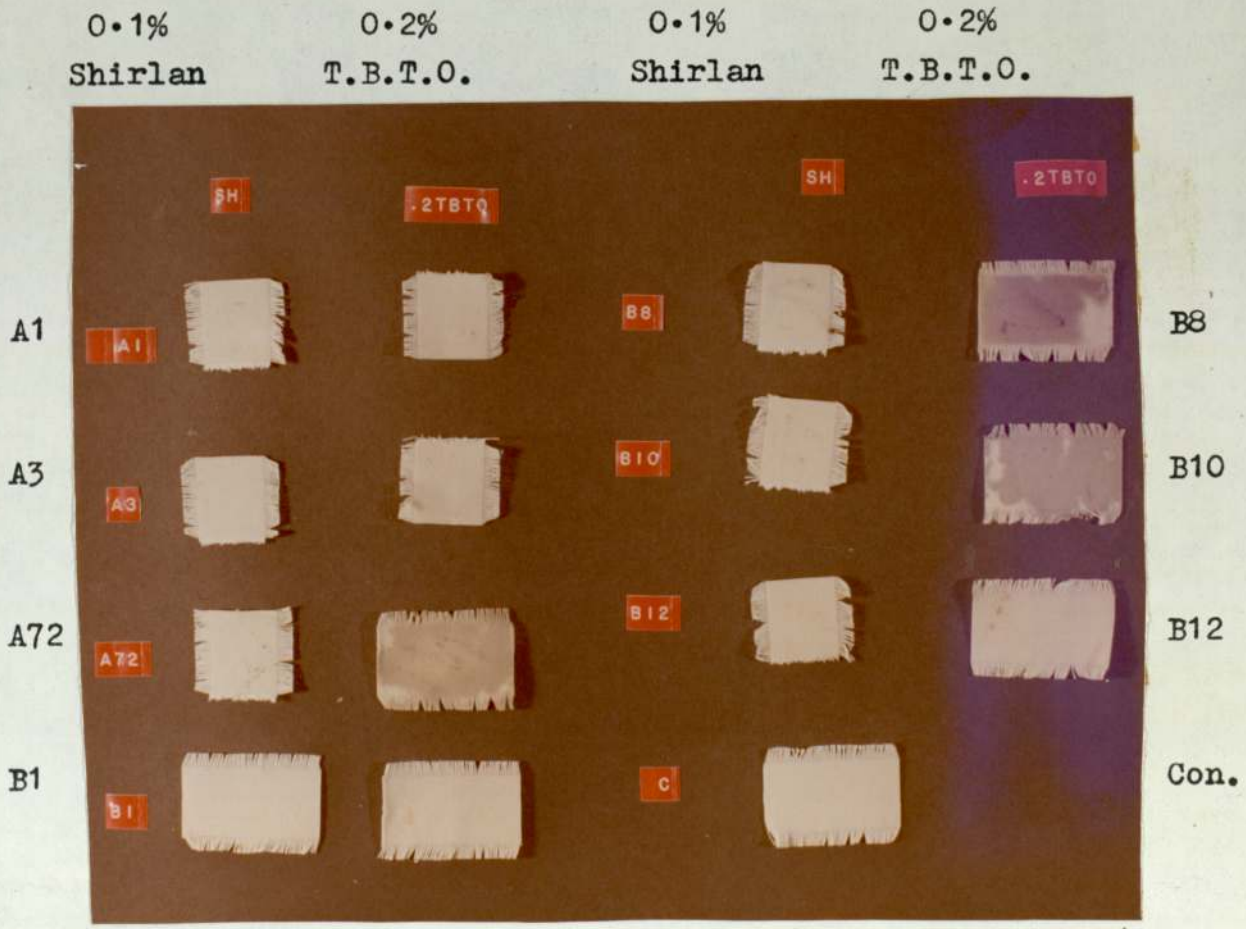



Plate 10. Growth of Streptomyces on 0•1% Shirlan and 0•2% T.B.T.O. treated test strips.

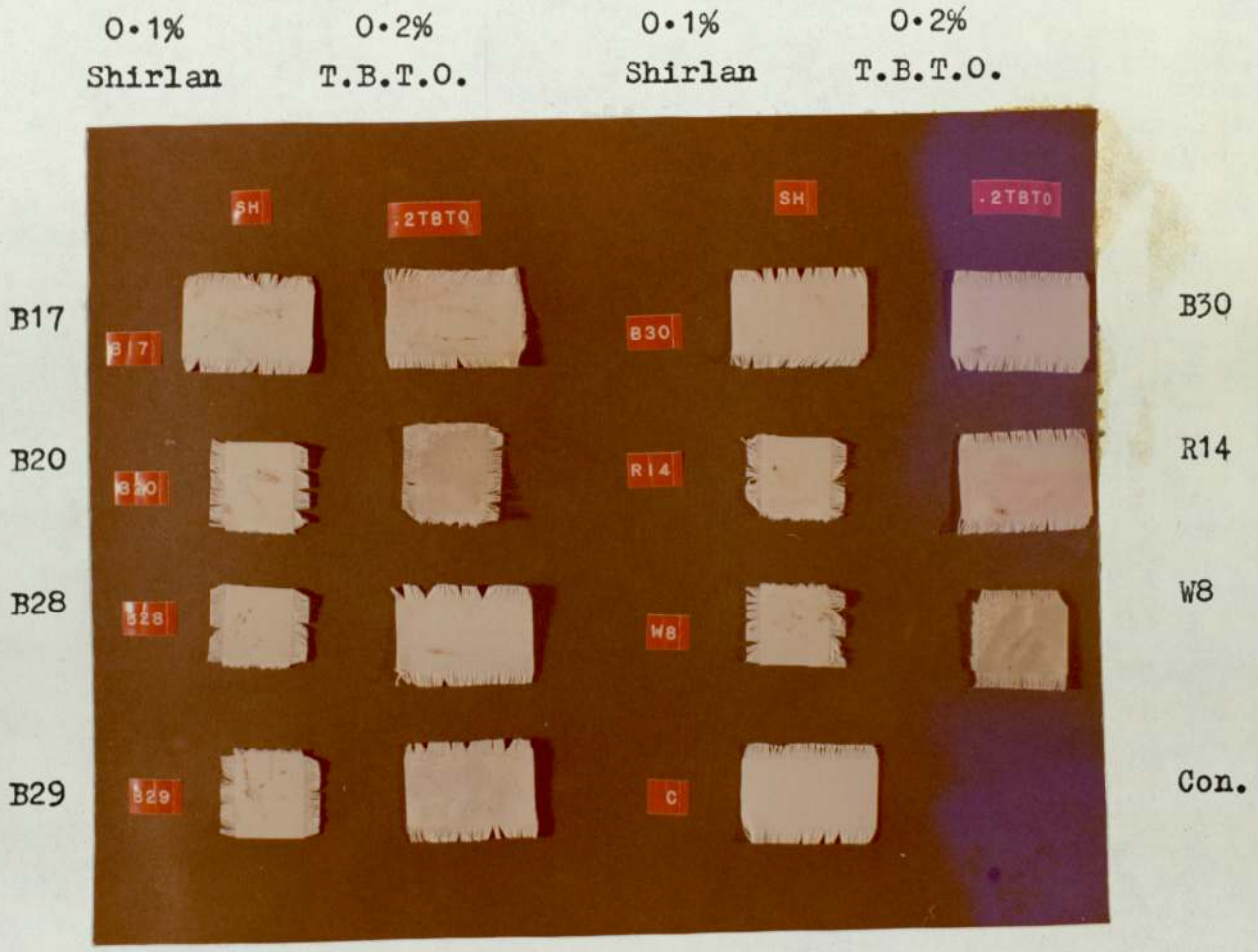



Plate 11. Growth of Streptomyces on 0·1% Shirlan and 0·2% T.B.T.O. treated test strips.

'Shirlan' (Salicylanilide) is a biocide which is no longer in commercial production but has a well documented sucess rate in protection of starch and starch coated textiles, thus it was not expected to be attacked by the Streptomyces spp. A concentration of 0.1% 'Shirlan' was used and growth occurred to a very slight extent by two species, B29 S. lavendulae and W8 S. griseolus (3) which produced very sparse substrate mycelial growth. S. rubrireticuli again failed to grow or produce any pigmentation on the 'Shirlan' protected fabric.

T.B.T.O at 0.1% and 0.2% (w/w starch size), was found to be largely ineffective as a biocide protecting sized cotton textile. T.B.T.O. (0.1%) was found to inhibit the growth of S. flavogriseus and S. setonii but these were only capable of very weak substrate mycelial growth on the loomstate fabric. T.B.T.O (0.2%) inhibited growth of S. albus, S. atroolivaceous, S. griseus and S. rubrireticuli but had little effect on the other Streptomyces spp. tested. Those species capable of growing in the presence of 0.2% T.B.T.O. showed growth of substrate and aerial mycelium comparable to that found on loomstate or starch sized cotton textile. It would thus appear that T.B.T.O. is ineffective as a textile biocide against Streptomyces spp. at the concentrations here employed.

## 7.3 Tensile strength testing of fabrics inoculated with Streptomyces spp. protected by commercial biocides

Standard T.N.O. cotton fabric, employed as a test material by the I.B.R.G. textile group, was inoculated with known cellulolytic Streptomyces spp. isolated from the airspora. Some of the fabrics were protected by commercial biocides of proven fungicidal and biocidal abilities to discover how effective they were against Streptomyces spp. The standard cotton fabric was treated in one of six ways:

1) Control. Loomstate T.N.O. test fabric.
2) Control. 1% B.D.H. Wheat Starch on weight of fabric.
3) 1% Wheat Starch on weight of fabric containing 0.5% Na.P.C.P. (Mystox D.) on weight of starch.
4) 1% Wheat Starch on weight of fabric containing 0.1% Salicylanilide 'Shirlan' on weight of starch.
5) 1% Wheat Starch on weight of fabric containing 0.1% T.B.T.O. on weight of starch.
6) 1% Wheat Starch on weight of fabric containing 0.2% T.B.T. on weight of starch.

The cotton strips were frayed down to a standard 1" width and cut into 6" lengths suitable for breaking on a tensometer. Heavy spore suspensions of the Streptomyces B8, B28, R14 S. rubrireticuli and W8 S. griseolus (3)

were obtained from slopes of mature fourteen-day old cultures on starch agar slopes. Streptomyces spp. spores are hydrophobic so 'Tween 80' and glass beads were used to ensure a homogeneous spore suspension. The spores were suspended in a dilute solution of Waksman Starch inorganic salts and sprayed onto the cotton test strips. The nutrient was added to allow the spores to become quickly established as the strips had not been sterilized. It was thought that autoclaving would cause biocidal changes by either chemical reactions or leaching. Alternative methods of sterilization, e.g. ethylene or propylene oxide fumigation, have yet to be proven to have no side effects with respect to the biocides or the tensile strength of the test strips.

The inoculated strips were suspended in sterile tanks, (the same tanks as used in the air mixing experiments) the humidity of the environment being maintained at a high level by water on the floor of the tank. Strips treated with different biocides were kept in separate tanks; many biocides are volatile and the risk of interference between experiments was thus avoided. The strips were incubated at a temperature of 25° C for a period of 28 days.

Strips were removed from the tanks after 28 days and thoroughly washed to remove all mycelial debris. The samples were air dried for twenty four hours prior to conditioning before breaking on the tensometer. They

were conditioned overnight in a constant humidity room to 65$\pm$ 1% R.H.

The strips were broken individually on a Houndsfield tensometer, breaking strength being measured in pounds, (to the nearest pound).

Large numbers of control strips were used, each having received the same harvesting and conditioning treatment as the inoculated test strips. Careful note was also made of staining and pigment formation on the strips as this is equally as important as losses in tensile strength when considering the efficacy of a biocide and the in service use of the fabric.

After being broken the strips, both test and control, were analysed for respective biocide contents using techniques based on B.S. 2087. These techniques, which are described latter, were to check the initial biocide application and the amount of biocide remaining on the fabric after testing.

The results of the tensile strength tests on cotton strips inoculated with Streptomycetes are shown in fig. (50).

| SPECIES | MEAN TENSILE STRENGTH (lbs) LOOMSTATE | MEAN TENSILE STRENGTH (lbs) STARCH | 0.1% SHIRLAN | 0.5% Na.P.C.P. | 0.1% T.B.T.O. | 0.2% T.B.T.O. |
|---|---|---|---|---|---|---|
| CONTROL | 104.37 | 106.10 | 103.50 | 103.62 | 111.43 | 106.50 |
| B8 | 80.60 | 94.00 | 106.72 | 102.33 | 102.14 | 106.57 |
| B28 | 94.57 | 96.14 | 107.00 | 110.71 | 96.27 | 108.86 |
| S. rubrireticuli R14 | 104.71 | 104.43 | 104.14 | 105.72 | 109.28 | 111.85 |
| S. griseolus(3) W8 | 99.43 | 99.00 | 105.50 | 105.71 | 102.43 | 110.14 |

Fig. 50 Mean tensile strengths (lbs) of strips inoculated with Streptomyces spp.

| SPECIES | LOOMSTATE | STARCH | 0·1% SHIRLAN | 0·5% Na.P.C.P. | 0·1% T.B.T.O. | 0·2% T.B.T.O. |
|---|---|---|---|---|---|---|
| B8 | $22·8^{a}$ | $11·41^{a}$ | - | - | $8·34^{b}$ | - |
| B28 | $9·49^{a}$ | $9·39^{a}$ | - | - | $13·60^{b}$ | brown staining |
| S. rubrireticuli R14 | - | $1·58^{c}$ | - | - | $1·93^{c}$ | pink staining |
| S. griseolus (3) W8 | $4·74^{c}$ | $6·7^{c}$ | - | - | $8·08^{a}$ | patches of yellowing |

Fig. 50(a) Percentage strength losses caused by Streptomyces spp. on untreated and biocidally protected cotton textile strips.

Wilcoxon (Mann-Whitney) two sample test.
a = very significant p 0·01
b = significant p 0·05
c = not significant p 0·05

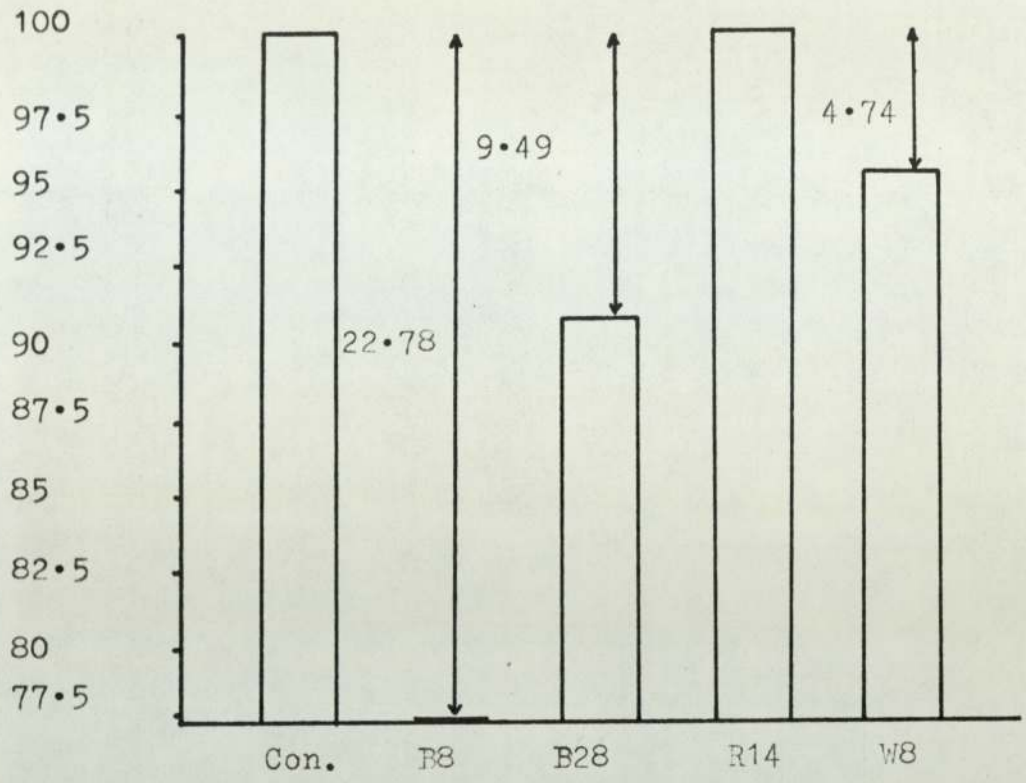



Fig. (51) Percentage tensile strength loss of loomstate fabric.

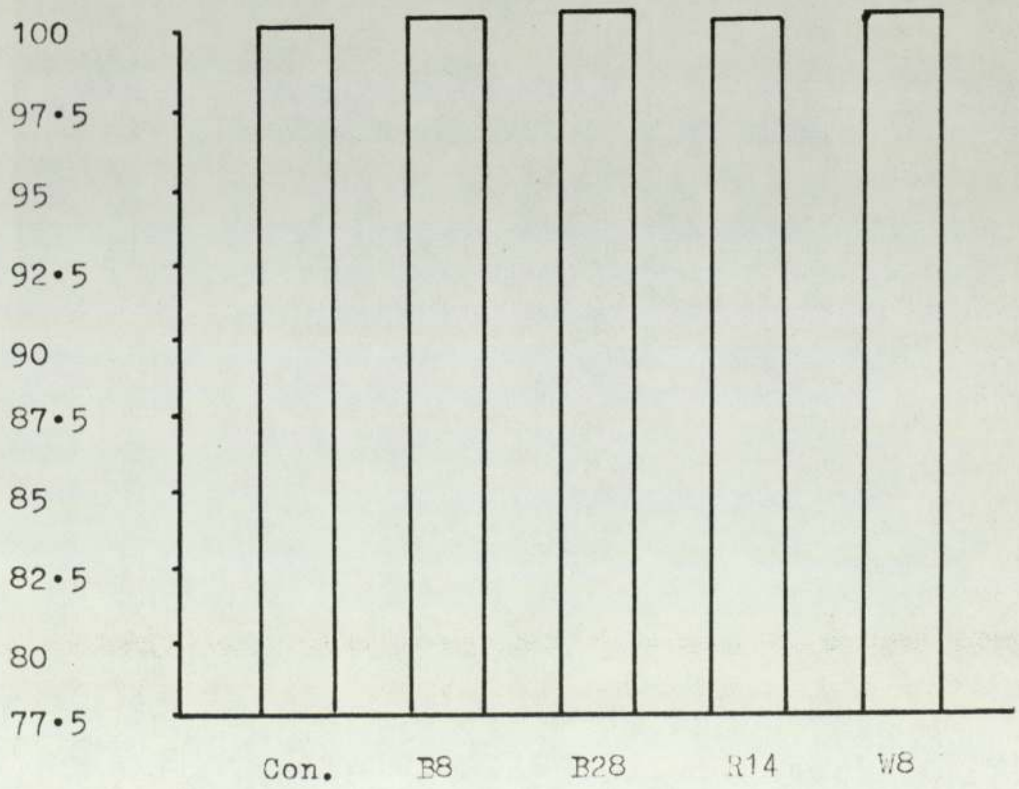



Fig. (52) Percentage tensile strength loss of fabric protected by 0·1% 'Shirlan' on wt. of starch.

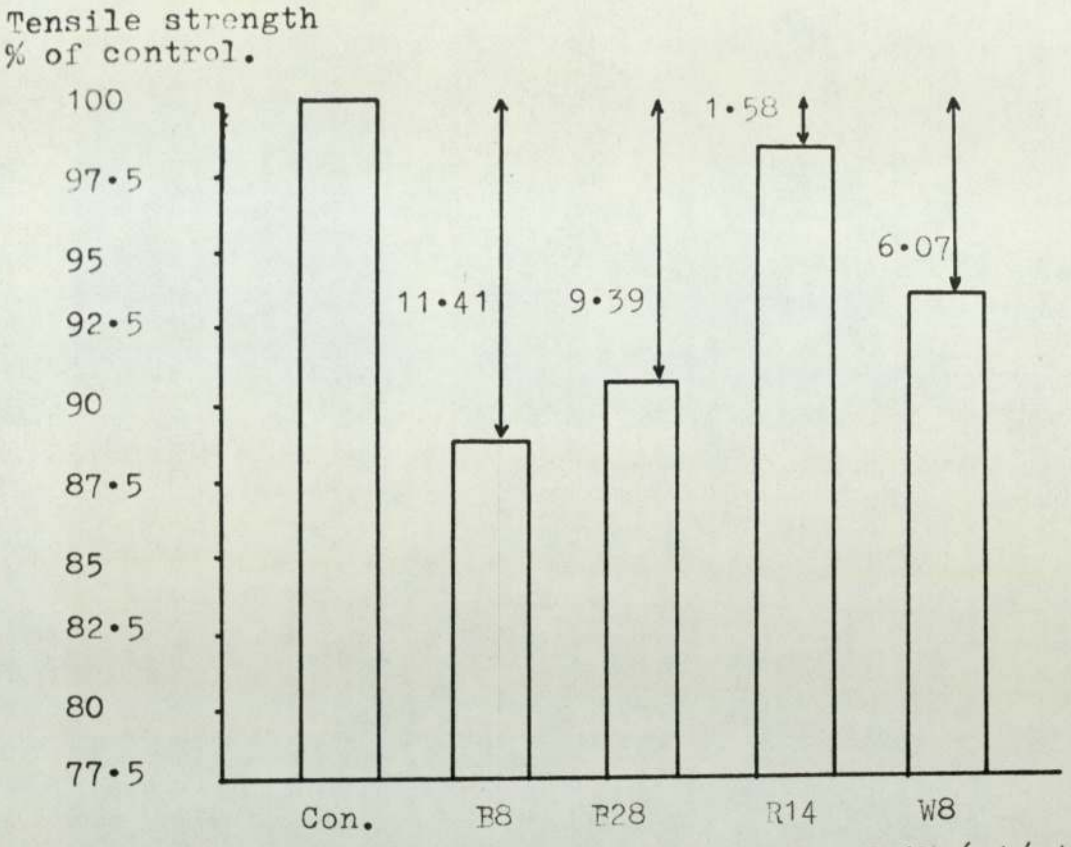



Fig. (53) Percentage tensile strength loss of 1% (wt/wt.) wheat starch coated fabric.

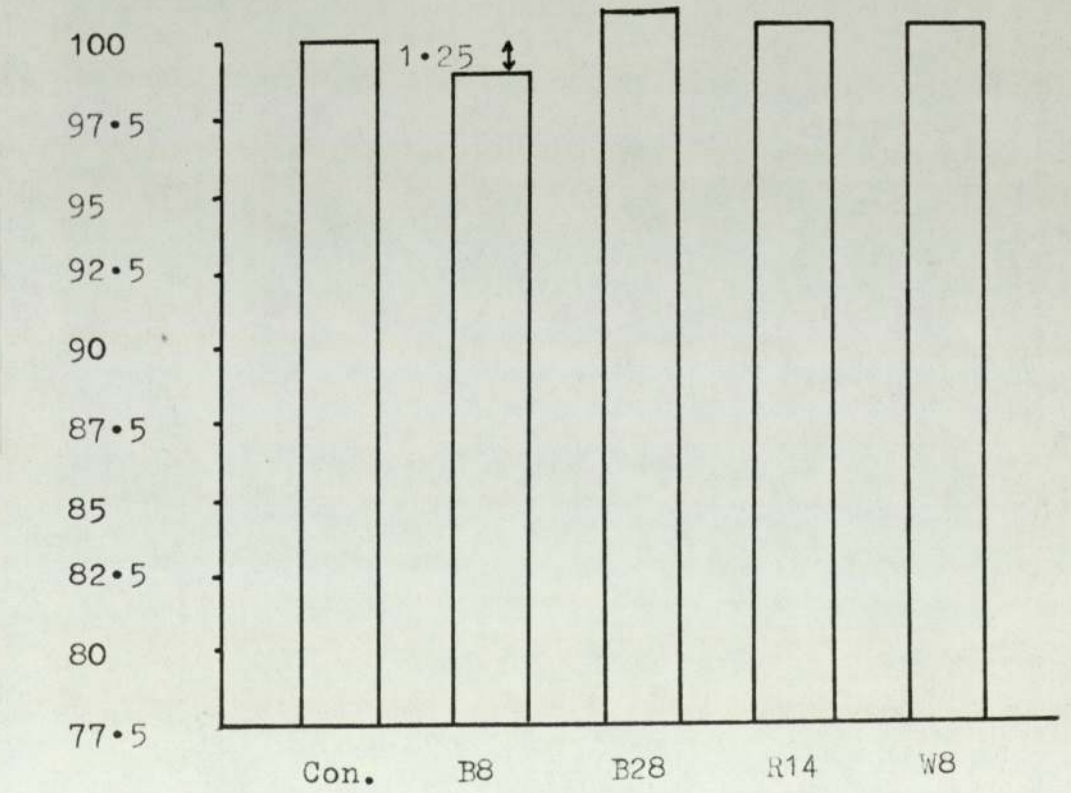



Fig. (54) Percentage tensile strength loss of fabric protected by 0·5% Na. P.C.P. (wt/wt.) of starch.

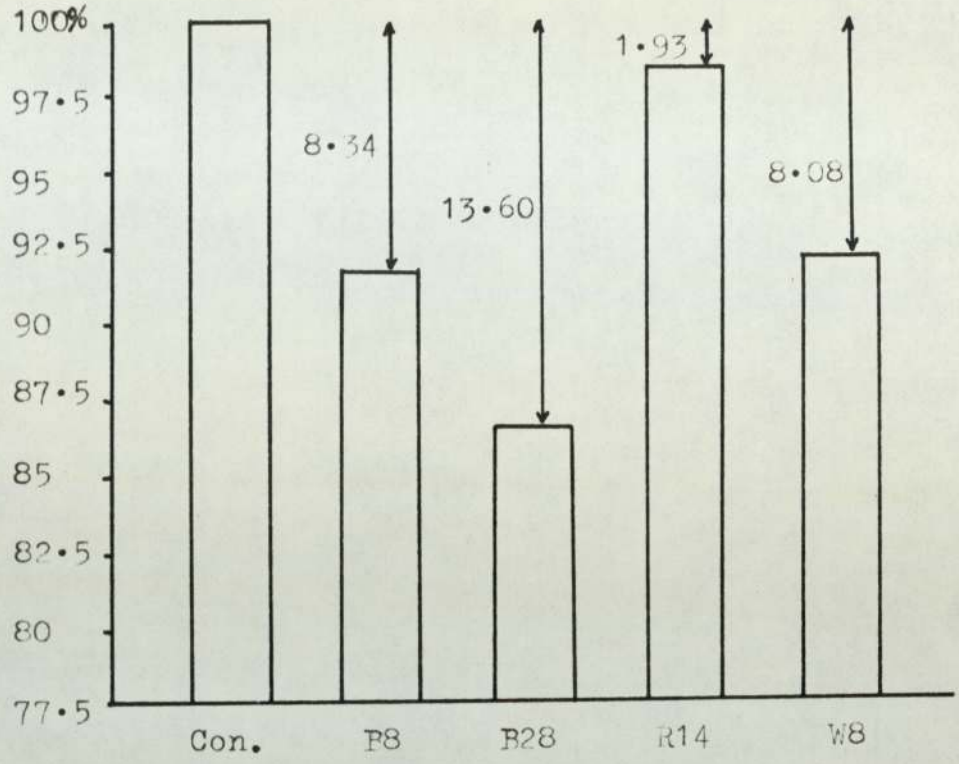



Fig. (55) Percentage tensile strength loss of fabric protected by 0·1% T.B.T.O. on wt. of starch.

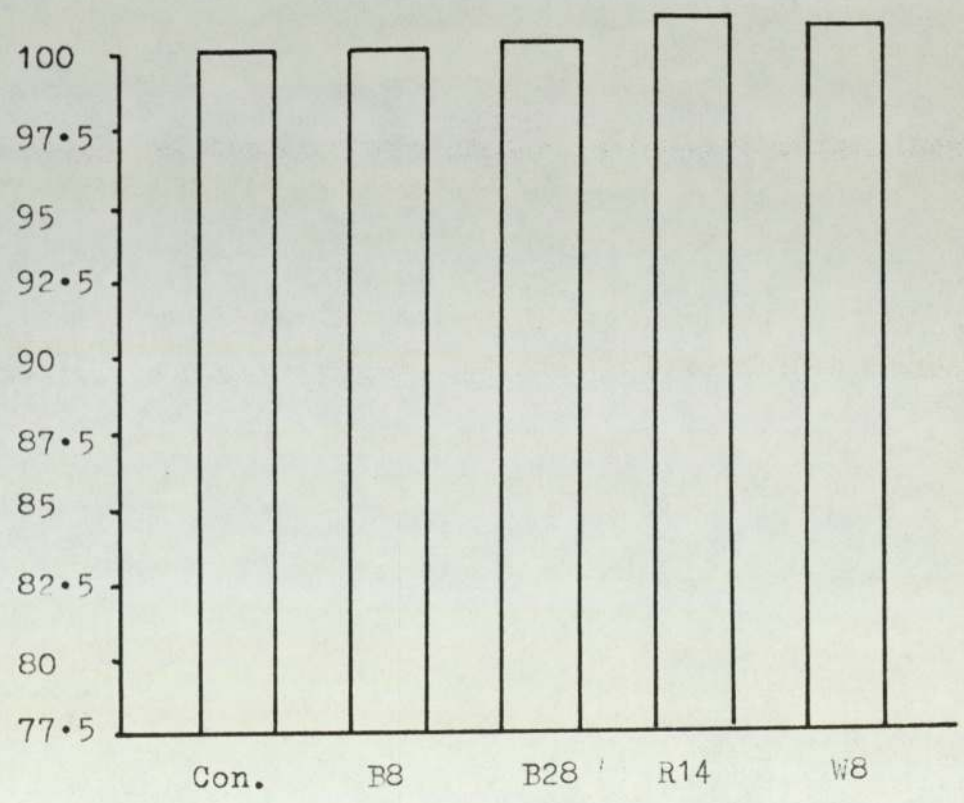



Fig. (56) Percentage tensile strength loss of fabric protected by 0·2% T.B.T.O. on wt. of starch.

## 7.4 Analysis of biocides

Analysis of the content of sodium pentachlorophenate on the cotton textile was found using the copper-pyridine method. The method used was based on the Monsanto commercial testing procedure which forms the basis of B.S.2087 Appendix N (1971), which measures free pentachlorophenol.

Sufficient treated cotton strip was cut up to contain 2-7.5 mg pentachlorophenol, weighed accurately and placed in a 1 litre round-bottomed flask. To the flask were added 300 ml distilled water and 20 ml of concentrated hydrochloric acid. A measured volume of the aqueous pentachlorophenate solution was placed in a 500 ml round-bottomed flask fitted for steam distillation with a splash-head connected, via a water cooled Liebig condenser having a drip-end, to a 500 ml separating funnel. The solution was acidified to Congo red with 40% sulphuric acid and diluted to approximately 300 ml. The mixture was boiled, a 250 ml distillate was collected in the separating funnel. The distillate was extracted using 3 x 15 ml portions of chloroform. The extract was analysed using the copper-pyridine method described later.

The preliminary step in the creation of a calibration curve is the preparation of a standard. NaP.C.P. 2.5 g was dissolved in distilled water containing 10 ml of 10% sodium hydroxide solution and diluted to 1 litre in a

volumetric flask. Further dilution of 100 ml of the above solution to 1 litre with distilled water (also containing 10 ml 10% NaOH) was carried out. This solution contained 0.25 mg of Na.P.C.P. per ml and standards containing 1,2.3,4,5,6,7 and 8 mg were obtained from this.

Each of these aliquots were subjected to acidification and steam distillation as previously described.

Step 2 in the formation of the calibration curve was the addition to the chloroform extract of 5 ml. copper-pyridine reagent (1g. $CuSO_4.5H_2O$ and 10g. pyridine diluted to 100 ml with distilled water and 25 ml water. The mixture was vigorously shaken and the two layers separated, the bottom chloroform layer through a moistened filter paper into a graduated flask. A further 5 ml. chloroform was added to the remaining aqueous layer and shaken. This chloroform layer was allowed to settle out and then used to wash any of the copper-pyridine complex remaining into the flask. The flask solution was made up to 50 ml with chloroform.

The mg. Na.P.C.P. present in the sample was found from the optical density of the sample, and by reference to the calibration curve obtained from the standard aliquots. Knowing the weight of the textile sample the amount of Na.P.C.P. may be calculated and compared with the figure expected and also with the figure after biological testing.

The amount of 'Shirlan' (Salicylanilide) on the cotton textile strips before and after biological testing was determined using the procedure set out in B.S. 2087 Appendix E 1971. The preservative was extracted from the textile by means of a dilute solution of di-sodium tetraborate and the extract was treated with a solution of 2.6-dibromo-p-benzo quinone chlorimine. The blue indophenol colouring matter so formed was absorbed on a disc of filter paper, the shade of which was then compared with the shades of a series of similar discs dyed with the indophenol obtained from solutions containing different amounts of salicylanilide.

The determination of tributyl tin oxide (T.B.T.O.) on textile is rather difficult. The method adopted was that of B.S.2087 Appendix L P71, and is applicable to the determination of total tin content, but is not specific for T.B.T.O. The textile fabric was subjected to wet oxidation, the tin in acid solution was then solvent extracted. The tin was extracted from the organic solvent and the colour developed with catechol violet was measured by a spectro-photometer at a wave-length of 552 n.n.m. This result was then compared with a series of results obtained using standard solutions containing known amounts of tin, and so the amount of T.B.T.O. on the cotton sample could be detected using a calibration graph.

The results of biocide analysis of the broken strips showed B.S. 2087 techniques to have serious limitations when low concentrations (as in this case) were being assayed. For Na.P.C.P. the figure for biocide remaining on the textile after breaking was found to be approximately four times that of the theorectical application. The Shirlan analysis gave a figure of 0.001% biocide/weight of fabric, exactly the theoretical application. Both readings were obtained, however, in the assaying techniques area of maximum error. The analysis show that biocide was definitely present throughout the test proceedures; so it was necessary to assume the figures of theoretical biocide application.

Tributyl tin oxide estimates too were insufficiently accurate to quote with any degree of faith. An alternative method of T.B.T. estimation could be nuclear magnetic resonance (N.M.R.) readings to estimate the amount of tin present on the textile, which is directly proportional to the concentration of T.B.T.O. applied.

Conclusion

Both Na.P.C.P. (0.5% w/w starch size) and 'Shirlan' (0.1% w/w starch size) were found to be completely effective in protecting the starch finished cotton textile against deterioration by Streptomyces spp. No growth was seen visually or significant loss in tensile strength recorded when these two biocides were employed. T.B.T.O. was, however, found to be ineffective as a biocide against the Streptomyces spp. tested. A marked concentration effect was found with T.B.T.O. T.B.T.O (0.1%) treated textile showed a significant tensile strength loss when tested with S. griseolus (3) (W8). B8 and B28 species; 8.08 - 13.60% was recorded. Starch sized cotton textile treated with 0.2% T.B.T.O. was found to prevent tensile strength loss. Widespread staining of fabrics occured however at both concentrations.

The strain of S. rubrireticuli tested was non-cellulolytic, but was found to cause a pink discolouration of the strips when they were protected by both concentrations of T.B.T.O. employed.

Severe limitations in the B.S. 2087 techniques for assaying the quantity of biocide in control and test strips were encountered. The amount of biocide incorporated in the starch finish fell with the area of maximum error of the chemical assay techniques and it was therefore

impossible to state categorically that there was no leaching of biocides during the course of experiments.

As Streptomyces spp. are capable of causing biodeterioration of loomstate, starch finished and T.B.T.O. treated cotton textile it would be prudent to include representatives of such species in any future lists of standard test organisms.

Biocides tested by Allsopp (1973) and Hollingsworth (1974), (pentachlorophenyl laurate, P1, P2 and P3) allowed growth by thermophilic actinomycetes. Cotton textiles are not generally subjected to thermophilic temperatures in storage or service. Thus the above biocides should be tested at mesophilic temperatures (20 - 40° C) with Streptomyces spp. as a check on biocidal efficiency.

# Chapter 8

## 8.1 General Conclusions

## 8.1 General conclusions

The primary aim of the experimental investigation was the production of an alternative means of controlling relative humidity for use in biodeterioration tests. The standard test for resistance of starch finished cotton textiles to microbial decay is the soil burial test. This method has been fully documented in the reports of the I.B.R.G. textile section and is widely used in commerce. Soil beds provide a stringent examination of a textile's resistance but have several commonly recognised drawbacks. Notably products tested rarely come into direct contact with soil during their lifetime, either in use or in storage. Soils are notoriously variable in chemistry, physical attributes, nutritional status and consequently their microbial flora and fauna. Thus it is extremely difficult to correlate investigations carried out in different localities. The moisture content of beds may vary considerably, if fresh soil is not used hysterisis effects may be encountered. Soil which is allowed to dry out prior to use may lead to an imbalance in its natural microflora, the actinomycetes, particularly Streptomyces spp., becoming proportionally more important. A test bed that is in prolonged use may encourage fungistasis giving rise to anomalous results. It would be extremely interesting to investigate the microbiology of such a test bed over a period of use to evaluate its

Streptomycete population. A system of continuous air-mixing was developed to produce controlled humidities enabling textiles to be tested under more natural conditions bearing a direct relationship with their in-use situation.

The apparatus constructed was found to give easily reproducible relative humidities within the range of 70 - 100%, with a maximum error of $\pm 1\frac{1}{2}\%$. Continuous mixing of wet and dry air streams had several advantages over alternative techniques for producing constant atmospheric humidities. Saturated salt solutions were extremely accurate but limiting on the size of sample tested and the equilibrium time required for the humidity to be obtained. Air mixing was readily adaptable, the R.H. being rapidly altered merely by the insertion of different length capillary tubes to control the air-flow rate. A most important characteristic of the air-mixing system was the production of a test environment in which the proportion of carbon dioxide to oxygen would not significantly change during a test period. A through flow of clean air allowed ready removal of gaseous staling products, such as carbon dioxide and volatile organic metabolites capable of inhibiting microbial growth and thus altering the balance of the test environment. Two factors of importance in the testing of textile biodeterioration are:-

a) the test should bear a direct relationship with in service situations encountered by the

coated surface being evaluated.

b) the test must be easily reproducible, the air-mixing system fulfills both these parameters. In respect of removing staling metabolites and microenvironmental stability the apparatus may be compared with the perfusion system of Eggins et al (1968).

The present apparatus could be made increasingly efficient by a reduction in overall size and by simple automation of the desiccant renewal procedure. Experimentation with the system was limited to relative humidities in excess of 70%, as this was the range over which the majority of active microbial growth occurs. The system could, however, be adopted for use in other branches of biology, for example entomology, where a fast inexpensive means of obtaining an accurate controlled environment is frequently required.

The need for a precise hygrometer to monitor relative humidity was realised and a prototype thermocouple hygrometer based on Peltier cooling was developed. This experimentation was curtailed owing to insufficient time, but was found to work in principle. Commercial hygrometers are readily available for measuring relative humidities but they are extremely expensive and tend to be inaccurate at R.H.'s in excess of 80%. The compromise of using conventional

wet and dry bulb thermometers in conjunction with hair hygrometers to measure atmospheric humidities in the test environment tanks was thus employed.

A gravity plate survey of airspora at a site in Aston was performed over a period of two years. Results correlated with surveys carried out elsewhere in Britain showing Cladosporium spp. to be the dominant fungi. The fungi isolated differed on several counts from these utilized by the I.B.R.G. (textiles) and it is proposed that the more dominant species encountered, but not incorporated in the I.B.R.G. scheme, such as Cladosporium herbarum and Botrytis sp. might be worthy of future consideration in test proceedures.

Streptomyces spp. occurred frequently (30 - 40% of plates) on both wide spectrum and natamycin inhibited plates. Streptomyces spp. were isolated throughout the year and were not found to be seasonal in nature, but dependent rather on the prevailing climatic conditions. Eleven distinct species of Streptomyces were isolated and identified using the I.S.P. standard methods. The dominant airbourne species were found to be Streptomyces griseus, S. gris eorubens and S. griseoflavus. Difficulties were encountered, however, in determining species by means of the published keys for the group.

A number of species were found to be cellulolytic using the cellulose agar clearing method of Rautella and

Cowling. The maximum depth of clearing (5m.m.) was less than that produced by most cellulolytic fungi tested (up to 17 m.m. in 28 days at 25° C).

Saturated salt solutions, continuous air-mixing and Scott's $A_w$ controlled inorganic solute agars were all employed to discover the range of $A_w$'s over which the Streptomyces spp. isolates could grow. Of these three methods the technique of Scott (1953) was found to be the most accurate in the high humidity range required by the Streptomyces spp. The species tested had an intermediate moisture requirement between that of the fungi and other bacterial groups. Formation of sporulating aerial mycelium was found to occur only infrequently below 95% R.H. (0.95$A_w$) and vegetative substrate mycelial growth was limited by 92% R.H. Some fungi, mainly Aspergillus spp. and Penicillium spp. were found to be capable of tolerating humidities down to 65% R.H.; though most fungal species require humidities in excess of 90% R.H. to successfully colonise a substrate such as cotton textile. Bacteria rarely grow below 94%R.H. and the majority require 97% R.H. Colonies of Streptomyces spp. not producing aerial mycelium at humidities below 95% were indistinguishable from bacterial colonies with the naked eye and are thus frequently dismissed as such.

Streptomyces spp. isolated were able to grow best in alkaline conditions; no species growing below pH 5. All

Streptomyces spp. obtained from the airspora were mesophilic having a wide temperature growth range. All species with the exception of S. griseorubens were capable of germinating at 11.5° C on a cellulose substrate, a temperature at which many fungi are incapable of growing. Thus the slow growth rate of Streptomyces spp. may not prove a disadvantage at such low temperatures due to the limitations on fungal competition.

Streptomyces spp. B8, B28 and W8 (S. griseolus (3) ) were found to be capable of causing statistically significant losses in the tensile strength of loomstate and starch sized cotton textile test strips. An average tensile strength loss of 22.78% was caused by species B8 over a period of 28 days at 25° C. The tensile strength losses for loomstate and starch sized cotton strips were very similar. The exception being species B8 which caused only 11.41% tensile strength loss of the wheat starch sized textile. The ability to degrade cotton, one of the purest forms of cellulose, indicated that the test Streptomyces spp. possessed both the $C_1$ and $C_x$ cellulose systems. Only four Streptomyces spp. were tested using tensile strength as a criterion of cellulolytic activity. A wider range of species should be investigated in future work to evaluate the Streptomycetes role in textile biodeterioration, as they have been shown to utilize the cotton cellulose and not just the starch size surface coating.

Sodium pentachlorophenol (Na.P.C.P.) and Salicylanilide ('Shirlan') were found to be effective biocides for the protection of loomstate and wheat starch finished textiles against specific Streptomyces degradation. Tributyl tin oxide, however, was found to be ineffective at the concentrations normally employed commercially. T.B.T.O. (0.1% / weight of starch on fabric) was found to allow Streptomyces spp. growth and consequent tensile strength losses by the cellulolytic species. T.B.T.O. (0.2%) prevented tensile strength losses, but did not reduce pigment production and staining of the fabric, thus showing a concentration effect. Further work should be carried out to find the concentration level at which T.B.T.O. becomes effective. The observations of Allsopp (1973) and Hollingsworth (1974) indicated that Streptomyces spp. were tolerant of limited concentrations of other commercially used biocides. It would be interesting to evaluate the Streptomyces spp. isolated against these biocides.

Actinomycetes are not normally thought of as primary colonizers of a substrate. They usually come towards the end of a chain of sucession commencing with faster growing bacterial and fungal species, utilising metabolites which have been produced by these organisms and nutritional sources which they have been unable to utilize.

It may be postulated that the tolerance of some

Streptomyces spp. to certain biocides may allow subsequent colonization of the protected substrate by non-tolerant organisms, thus reversing the general sucessional pattern.

The British Standard (2087) analytical methods for determining biocide concentration on protected textiles was found to be insufficiently accurate in these experiments. The concentration of biocide applied to the textile was found to fall within the area of greatest error for these methods.

It is proposed that future textile test proceedures should incorporate selected Streptomyces spp. as standard test organisms in conjunction with the normally employed fungal test organisms. Streptomyces tested did not have a large effect on the tensile strength of fabrics but may have an important nuisance value. Streptomyces spp. can act as opportunists in the biodeterioration of textiles germinating at temperatures, or under transient moisture conditions which do not favour their stronger fungal competitors. The ability of the Streptomyces spp. to produce disfiguring stains on fabrics may prove to be of greater importance than their cellulolytic ability.

Future experimentation on the relationship between relative humidity, biocide effectiveness and streptomycete and fungal degradation of textiles should form an important role in the planning of commercial textile

evaluation tests. A continuous air-mixing system in harness with a monitoring hygrometer accurate at high humidities is essential for such work in order to define more clearly the factors governing substrate colonisation and to allow greater correlation between experiments carried out in different laboratories.

# APPENDIX 1

a) Key Numbers and Authorities of Streptomyces spp. isolated

| | | |
|---|---|---|
| A1 | Streptomyces griseorubens | Presbrazenskaya, Blinov and Ryabova |
| A3 | Streptomyces flavogriseus | (Duché) Waksman |
| A72 | Streptomyces nigrifaciens | Waksman |
| B1 | Streptomyces setonii | (Millard and Burr) Waksman |
| B8 | Not identified to species level | |
| B10 | Streptomyces griseolus (1) | (Waksman) Waksman and Henrici |
| B12 | Streptomyces albus | (Rossi-Doria) Waksman and Henrici |
| B17 | Streptomyces griseolus (2) | (Waksman) Waksman and Henrici |
| B20 | Streptomyces atroolivaceous | Presbrazenskaya, Blinov and Ryabova |
| B28 | Not identified to species level | |
| B29 | Streptomyces lavendulae | (Waksman and Curtis) Waksman and Henrici |
| B30 | Streptomyces griseus | (Krainsky) Waksman and Henrici |
| R14 | Streptomyces rubrireticuli | Waksman and Henrici |
| | syn. Streptoverticillium rubrireticuli | (Waksman and Henrici) Baldacci |
| W8 | Streptomyces griseolus (3) | (Waksman) Waksman and Henrici |

b) Authorities of fungi isolated

Alternaria alternata (Fries) Keissler

Aspergillus flavus Link ex. Fries

Aspergillus niger Van Tieghem

Aspergillus achraceus Wilhelm

Aspergillus terreus Thom

Aspergillus versicolor (Vuillemin) Tiraboschi

Aureobasidium pullulans (de Bary) Arnaud

Botrytis cinerea Persoon ex. Persoon

Cephalosporium acremonium Corda

Chaetomium globosum Kunze ex Fries

Cladosporium herbarum (Persoon) Link ex S.F. Gray

Doratomyces stemonitis (Persoon ex Fries ) Corda

Epicoccum nigrum Link

Fusarium sp.

Gelasinospora sp.

Gilmaniella humicola Barron

Gliocladium roseum Bainier

Gliomastix murorum (Corda) Hughes

Graphium penicillioides Corda

Helminthosporium sp. Link ex Fries

Humicola grisea Traaen

Memnoniella echinata (Rivolta) Galloway

Mucor hiemalis Wehmeyer

Myrothecium verrucaria (Alb. and Schw.) Ditmar ex Fries

Neurospora crassa Shear and Dodge

Olpitrichum sp. Atkinson

*Paecilomyces varioti* Bainier

*Penicillium funiculosum* Thorn

*Phoma spp.* (Fries) Desmazieres

*Trichoderma viride* Persoon

*Trichothecium roseum* (Persoon) Link

*Sordaria fimicola* (Roberge) Cesoti and de Notaris

*Stachybotrys atra* Corda

*Zygorynchus moelleri* Vuillemin

## APPENDIX II

Results of Streptomyces growth experiments on media of controlled $A_w$

Each of the following result blocks represents the growth of an individual streptomycete species with respect to $A_w$, time and nutrient source. The type of growth which occurred has been signified by a letter as shown in the key, each observation in the blocks is an average of five replicate microscopic examinations.

KEY

S = Distinct growth of substrate mycelium, showing active colonization to be taking place.

S+ = Widespread strong growth of primary substrate mycelium (colonies appear bacteria-like).

A = Production of secondary (aerial) mycelium.

A+ = Mature colonies showing sporulation and spore germination.

\- = No apparent growth.

_____ = Lower limit of substrate mycelial growth.

------ = Lower limit of aerial mycelial growth.

$A_w$ = Water activity in media as controlled by Scott (1953) inorganic salts combinations.

hrs = Incubation time in hours.

Key to numbers and authorities of Streptomyces spp. tested are given in Appendix I.

1.1. Growth of *Streptomyces* spp on $A_w$ controlled Oxoid nutrient agar incubated at 30°C.

A1

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | S | S | S | S | $S^+$ | $S^+$ |
| 0·98 | – | S | S | S | S | S |
| 0·96 | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

A3

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·98 | S | S | A | A | A | A |
| 0·96 | – | $S^+$ | S/A | S/A | S/A | A |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

A72

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | S | $S^+$ | $S^+$ | S/A | S/A | A |
| 0·99 | A | A | A/$A^+$ | A/$A^+$ | A/$A^+$ | A/$A^+$ |
| 0·98 | A | A | A | A | A | A |
| 0·96 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B1

| $A_w$ \ hrs | 40 | 120 | 168 | 228 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | S/A | S/A | A | A | A |
| 0·99 | S | S | S | S | $S^+$ | $S^+$ |
| 0·98 | S | S | S | S | S | S |
| 0·96 | – | S | S | S | S | S |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

Nutrient agar 30 °C cont.

B8

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | A | A | A | A | $A/A^+$ |
| 0·99 | S/A | S/A | S/A | S/A | S/A | A |
| 0·98 | S/S$^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | S | $S^+$ | $S^+$ | $S^+$ |
| 0.92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B10

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·98 | S | A | A | A | A | A |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S | $S^+$ |
| 0.92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B12

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | S | S | S | S | S | S |
| 0·98 | S | S | S | S | S | S |
| 0·96 | – | S | S | S | S | S |
| 0.94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B17

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·98 | S | A | A/$A^+$ | A/$A^+$ | A/$A^+$ | A/$A^+$ |
| 0·96 | – | $S^+$ | $S^+$ | S/A | S/A | S/A |
| 0·94 | – | S | S | S | S | S |
| 0·92 | – | – | – | S | S | S |
| 0·90 | – | – | – | – | – | – |

B20

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | A | A/$A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | S/A | A | A | A | A |
| 0·98 | $S^+$ | S/A | A | A | A | A |
| 0·96 | S | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B28

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | A | A | $A^+$ | $A^+$ |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B29

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | $S^+$ | $S^+$ | $S^+$ | $S^+/A$ | $S^+/A$ | $S^+/A$ |
| 0·98 | S | S | $S^+/A$ | $S^+/A$ | $S^+/A$ | $S^+/A$ |
| 0·96 | – | S | S/$S^+$ | S/$S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B30

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+/A$ | A | A | A | $A^+$ | $A^+$ |
| 0·98 | S | A | A | A | A | A |
| 0·96 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

W8

| $A_w$ \ hrs | 40 | 120 | 168 | 288 | 336 | 456 |
|---|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | A | $A/A^+$ | $A/A^+$ | $A/A^+$ | $A/A^+$ | $A/A^+$ |
| 0·98 | $S^+$ | A | A | A | A | A |
| 0·96 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

Nutrient agar 30 °C.

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1.2. Growth of *Streptomyces sp.* on $A_w$ controlled cellulose agar incubated at 30 °C.

A1

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | – | – | S | S | S | $S^+$ |
| 0·98 | – | – | – | – | – | S |
| 0·96 | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

A3

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | S/A | S/A | A | A | A/$A^+$ |
| 0·99 | $S^+$ | S/A | A | A | A | A |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | S/A |
| 0·96 | – | – | S | S | S | S |
| 0·94 | – | – | S | S | S | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

A72

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | A | A | A | A | A/$A^+$ |
| 0·99 | $S^+$ | S/A | S/A | A | A | A |
| 0·98 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B1

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S | S | S | S | $S^+$ | $S^+$ |
| 0·99 | – | S | S | S | $S^+$ | $S^+$ |
| 0·98 | – | – | – | S | S | $S^+$ |
| 0·96 | – | – | – | S | S | $S^+$ |
| 0·94 | – | – | – | – | – | $S^+$ |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B8

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | S/A | S/A | A | A |
| 0·99 | S | $S^+$ | S/A | S/A | S/A | S/A |
| 0·98 | S | S | $S^+$ | S/A | S/A | S/A |
| 0·96 | – | S | S | S | S | S/$S^+$ |
| 0·94 | – | – | – | – | – | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B10

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | A | A | A | A | A | A/$A^+$ |
| 0·99 | $S^+$ | S/A | S/A | S/A | S/A | S/A |
| 0·98 | S | S | S/$S^+$ | S/$S^+$ | S/$S^+$ | $S^+$ |
| 0·96 | – | – | – | – | – | S |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

Cellulose agar 30 °C.

B12

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | A | A | A | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | S/A | A | A/$A^+$ | A/$A^+$ | $A^+$ | $A^+$ |
| 0·98 | S/A | S/A | S/A | A | A | $A^+$ |
| 0·96 | – | S | S/$S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | $S^+$ | $S^+$ | $S^+$ |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B17

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | A | A | A | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·98 | S/$S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | S | S | S | S | S |
| 0·94 | – | – | – | – | – | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B20

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | S/A | S/A | S/A | S/A | S/A |
| 0·99 | S/$S^+$ | $S^+$ | S/A | S/A | S/A | S/A |
| 0·98 | S | S | $S^+$ | S/A | $S^+$/A | S/A |
| 0·96 | – | – | S | S | S | S/$S^+$ |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B28

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | S/A | A/$A^+$ | A/$A^+$ | A/$A^+$ | A/$A^+$ |
| 0·99 | $S^+$ | $S^+$ | S/A | A | A | A |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | S | S | S | S/$S^+$ | S/A |
| 0·94 | – | – | S | S | S | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B29

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | S/A | S/A | S/A | S/A |
| 0·99 | S/$S^+$ | S/$S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·98 | S | S | S | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

B30

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | S/A | S/A | A | A/$A^+$ | A/$A^+$ | $A^+$ |
| 0·99 | S/A | S/A | A | A | A | A |
| 0·98 | S/A | S/A | A | A | A | A |
| 0·96 | – | S | $S^+$ | S/A | S/A | S/A |
| 0·94 | – | – | S | S | S | S |
| 0·92 | – | – | – | – | – | S |
| 0·90 | – | – | – | – | – | – |

Cellulose agar 30°C.

W8

Cellulose agar 30 °C.

| $A_w$ \ hrs | 66 | 90 | 136 | 208 | 256 | 384 |
|---|---|---|---|---|---|---|
| 1·00 | A | A | A | A/A$^+$ | A/A$^+$ | A/A$^+$ |
| 0·99 | S/A | S/A | A | A/A$^+$ | A/A$^+$ | A/A$^+$ |
| 0·98 | S$^+$ | S/A | S/A | A | A | A |
| 0·96 | – | S | S$^+$ | S$^+$ | S$^+$ | S$^+$ |
| 0·94 | – | – | – | S | S | S |
| 0·92 | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – |

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## 1.3. Growth of *Streptomyces* sp. on $A_w$ controlled starch agar incubated at 30 °C.

A1

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | S | S | S/A | S/A | A/A$^+$ | A/A$^+$ | A/A$^+$ |
| 0·99 | S | S | S$^+$ | S$^+$ | S/A | S/A | S/A |
| 0·98 | S | S | S$^+$ | S$^+$ | S/A | S/A | S/A |
| 0·96 | – | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

A3

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| A | A/A$^+$ | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| S/A | S/A | A | A | A | A/A$^+$ | A/A$^+$ |
| – | S | S$^+$ | S$^+$ | S/A | S/A | S/A |
| – | – | – | – | S | S | S$^+$ |
| – | – | – | – | – | – | S |
| – | – | – | – | – | – | – |

A72

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·99 | S/A$^+$ | A | A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·98 | S | A | A | A | A/A$^+$ | A/A$^+$ | A/A$^+$ |
| 0·96 | – | – | – | S | S | S | A |
| 0·94 | – | – | – | – | – | S | S |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

B1

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| – | S | S/A$^+$ | S/A$^+$ | S/A$^+$ | S/A$^+$ | A |
| – | – | S | S | S$^+$ | S/A$^+$ | S/A$^+$ |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |

B8

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·99 | S/A$^+$ | A/A$^+$ | A/A$^+$ | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·98 | S$^+$ | A | A | A/A$^+$ | A/A$^+$ | A/A$^+$ | A$^+$ |
| 0·96 | – | – | S | S | S$^+$ | S$^+$ | S$^+$ |
| 0·94 | – | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

B10

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| S/A$^+$ | S/A$^+$ | A | A/A$^+$ | A/A$^+$ | A/A$^+$ | A/A$^+$ |
| S | A | A | A | A | A | A |
| – | – | – | S | S | S | S |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |

B12

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | S/A$^+$ | A | A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·99 | S/A$^+$ | A | A/A$^+$ | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| 0·98 | S$^+$ | S/A$^+$ | S/A$^+$ | A | A | A$^+$ | A$^+$ |
| 0·96 | – | S | S$^+$ | S$^+$ | S$^+$ | S$^+$ | S$^+$ |
| 0·94 | – | S | S | S$^+$ | S$^+$ | S$^+$ | S$^+$ |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

B17

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| A | A/A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| S/A$^+$ | A | A$^+$ | A$^+$ | A$^+$ | A$^+$ | A$^+$ |
| – | S | A | A | A | A | A |
| – | – | – | S | S | S | S |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |

Starch agar 30 °C.

B20

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | S | S/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | – | $S^+$ | S/A | S/A | A | A | A |
| 0·98 | – | S | $S^+$ | $S^+$ | S/A | S/A | S/A |
| 0·96 | – | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

B28

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| A | A/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| $S^+$ | A | A | A/A | $A^+$ | $A^+$ | $A^+$ |
| – | S | $S^+$ | S/A | S/A | S/A | S/A |
| – | – | – | S | S | S | S |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |

B29

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | S/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | – | – | – | S | S | S/A | S/A |
| 0·98 | – | – | – | – | – | – | – |
| 0·96 | – | – | – | – | – | – | – |
| 0·94 | – | – | – | – | – | – | – |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

B30

| 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|
| S/A | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| S/A | A | A/A | A/A | A/A | A/A | A/A |
| S/A | S/A | S/A | A/A | A/A | A/A | A/A |
| – | S | S | $S^+$ | S/A | S/A | S/A |
| – | S | S | $S^+$ | S/A | S/A | S/A |
| – | – | – | – | – | – | – |
| – | – | – | – | – | – | – |

W8

| $A_w$ \ hrs | 48 | 72 | 140 | 192 | 240 | 336 | 404 |
|---|---|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | S/A | A/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·98 | S | A | A/A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·96 | – | – | – | – | – | S | S |
| 0·94 | – | – | – | – | – | – | S |
| 0·92 | – | – | – | – | – | – | – |
| 0·90 | – | – | – | – | – | – | – |

Starch agar 30 °C.

## 1.4. Growth of *Streptomyces* sp. on $A_w$ controlled Oxoid nutrient agar at 25 °C.

A1

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | S | S | S | S | S |
| 0·98 | – | S | S | S | S |
| 0·96 | – | – | – | – | – |
| 0·94 | – | – | – | – | – |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

A3

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | A | A | $A^+$ | $A^+$ | $A^+$ |
| 0·98 | $S^+$ | A | A | $A^+$ | $A^+$ |
| 0·96 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

A72

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | A | A | A | A | A |
| 0·99 | A | A | A | A | A |
| 0·98 | S | A | A | A | A |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B1

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+/A$ | $S^+/A$ | $S^+/A$ | $S^+/A$ |
| 0·99 | S | S | S | S | S |
| 0·98 | S | S | S | S | S |
| 0·96 | – | S | S | S | S |
| 0·94 | – | – | – | – | – |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B8

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | A | A | A | A |
| 0·99 | $S^+$ | A | A | A | A |
| 0·98 | S | A | A | A | A |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S/$S^+$ | S/$S^+$ | S/$S^+$ |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B10

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | A/$A^+$ | A/$A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | A | $A^+$ | $A^+$ |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$/A | $S^+$/A |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B12

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·99 | S | S | S | S | S |
| 0·98 | S | S | S | S | S |
| 0·96 | – | S | S | S | S |
| 0·94 | – | – | – | – | – |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B17

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | A | A/$A^+$ | A/$A^+$ | $A^+$ | $A^+$ |
| 0·99 | A | A | A | $A^+$ | $A^+$ |
| 0·98 | $S^+$ | $S^+$/A | A | A | A |
| 0·96 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B20

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | A | A/$A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | A/$A^+$ | $A^+$ | $A^+$ |
| 0·98 | S | A | A | $A^+$ | $A^+$ |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | – | – | – |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B28

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | $S^+$ | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | $S^+$/A | A | A | A |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | – | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

Nutrient agar 25 °C.

B29

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | S | S+/A | S+/A | S+/A | S+/A |
| 0·99 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·98 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·96 | – | – | – | – | – |
| 0·94 | – | – | – | – | – |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

B30

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | A | $A^+$ | $A^+$ | $A^+$ | $A^+$ |
| 0·99 | $S^+$ | A | A | A | A |
| 0·98 | S | A | A | A | A |
| 0·96 | S | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

W8

| $A_w$ \ hrs | 36 | 126 | 178 | 226 | 466 |
|---|---|---|---|---|---|
| 1·00 | A | A/$A^+$ | A/$A^+$ | $A^+$ | $A^+$ |
| 0·99 | A | A | A | $A^+$ | $A^+$ |
| 0·98 | S | A | A | A | A |
| 0·96 | – | $S^+$ | $S^+$ | $S^+$ | $S^+$ |
| 0·94 | – | S | S | S | S |
| 0·92 | – | – | – | – | – |
| 0·90 | – | – | – | – | – |

Nutrient agar 25 °C.

# APPENDIX III

## Media

1) Eggins and Pugh Cellulose Agar (1962)

Ammonium sulphate 0.5g

L-asparagine 0.5g

Potassium dihydrogen phosphate 1g

Potassium chloride 0.5g

Magnesium sulphate 0.2g

Calcium chloride 0.1g

Yeast extract 0.5g

Cellulose (ball -milled) 10g

Agar 18g Distilled water 1 litre

The cellulose source was Whatman Standard grade cellulose powder for chromatography; made up to 4% suspension and ball-milled for 72 hours.

2) Waksman Inorganic Salts/Starch Agar (1967)

Soluble wheat starch 10g

Sodium nitrate 1g

di-Potassium hydrogen phosphate 0.3g

Sodium chloride 0.5g

Magnesium chloride 1g

Agar 15g Distilled Water 1 litre

$Ca\ CO_3$ and $Mg\ SO_4\ 7H_2O$ (1g) may replace the magnesium carbonate. $(NH_4)_2\ SO_4$ (2g) or L-asperagine (0.05g) may be used to replace the nitrate as nitrogen source.

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