Antifungal Activity of some New Zealand Fungal Isolates

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By

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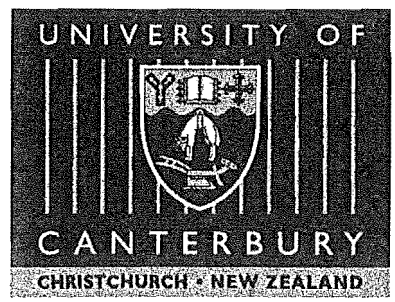

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Abstract

The secondary metabolism of organisms, especially mycelial fungi, is attracting increasing attention since it may produce a reservoir of unique molecules from which therapeutic agents are formed or derived for clinical application

The production of secondary metabolites is not well understood and involves a broad spectrum of metabolic processes that often have little in common. This study screened a number of New Zealand isolates, mostly of the *Arthrinium* genus, and assessed their antifungal activity. The primary screening used for this study consisted of agar diffusion tests with a range of filamentous fungi and yeast. Secondary screening was also started, with active cultures being chemically extracted.

It was found that New Zealand isolates of *Arthrinium phaeospermum* display antifungal activity against yeasts and filamentous fungi, as well as bacteria The teleomorphic state of an *Arthrinium* sp., *Apiospora montagnei,* was found to have activity against the yeast *S. cerevisiae*. In addition, activity against two filamentous fungi by *Apiospora montagnei* was demonstrated.

In the course of this study, a contaminant fungus displaying antifungal activity was isolated and screened. It was found to have antifungal activity against *C. albicans* and filamentous fungi, as well as bacteria.

Chapter I

Introduction

1.1 Background

The secondary metabolism of organisms, especially mycelial fungi, is attracting increasing attention since it may produce a reservoir of unique molecules from which therapeutic agents are formed or derived for clinical application.

In the past decade, there has been a marked increase in the use of aggressive chemotherapy, immunosuppression via drugs, major invasive surgery and the employment of indwelling vascular catheters, as well as the higher incidences of diseases such as AIDS which lead to an immuno-suppressed state. This has led to higher incidences of opportunistic systemic fungal infections, such as candidiasis and aspergillosis (Gold, 1984).

Amphotericin B and flucytosine were have been the only available antifungal agents for the treatment of serious yeast infections. New antifungals, such as fluconazole, are now becoming available and are proving more effective in the treatment of candidiasis and aspergillosis (Gold, 1984) and other fungal diseases. However, antifungal resistance to these agents has already been reported (Odds, 1996). Hence, there is an increasing need for new antifungals with novel modes of action to treat systemic fungal infections.

1.2. The *Arthrinium* genus

The *Arthrinium* genus, proposed by Kunze in 1917, is a member of the subphylum Ascomycotina and is included with the dematiaceous Hyphomycetes. Hyphomycetes are a class of conidial fungi whose conidiophores (specialised hyphae on which conidia are formed) are exposed and never enclosed within a covered conidioma. Fungi that reproduce asexually by way of conidia are known as anamorphic fungi. All the members of the *Arthrinium* genus are anamorphic. *Arthrinium* members are

assigned to the genus based on conidiophore development. *Arthrinium* is characterized by brown or dark brown non-septate conidia, each with a hyaline rim or germ slit, produced on basauxic conidiophores (Samuels *et al.*, 1981). Basauxic conidiophores arise from structures apparently unique to this genus, called conidiophore mother cells (Hughes, 1953).

The *Arthrinium* genus has been discussed by Ellis *et al.* (1951), Hughes (1953), Cooke (1954), Samuels *et al.* (1981), Minter (1985), and most recently by Calvo *et al.* (1994,1995) and Larrondo *et al.* (1994). Many species have been well illustrated, especially by Ellis (1971,1976). However, little is known about the cell ultrastructure of species of the *Arthrinium* genus. Campbell (1975) described the characteristics of the conidiophore mother cell. Cole and Samson (1979) collected and interpreted various cases relating to the basauxic development of the *Arthrinium* conidiophore. Recently, Calvo and Larrondo (1994,1995) described the germination characteristics, nuclei, and mitochondria of various species.

The genus *Arthrinium* contains at least 28 species, most considered to be fundamentally phytoparasites or endophytic(Samuels *et al.*, 1981). However, since the *Arthrinium* genus represents only the asexual phase of reproduction, the teleomorphic (sexual states) must also be considered as part of the group. The problem of a suitable classification for these teleomorphs and anamorphs (together they form a holomorph) is further complicated by the fact that a teleomorph may have more than one anamorph. Moreover, most of the *Arthrinium* anamorphs have no known (or ascribed) teleomorph. All known teleomorphs for the *Arthrinium* genus belong to the genus *Apiospora* Saccardo (Hudson *et al.*, 1976). Some *Apiospora* species also have anamorphs that have been assigned to other genera. For example, *Apiospora setosa* has both an anamorph ascribed to the genus *Cordella* and one ascribed to the genus *Arthrinium*. In this study, members of both the anamorphic states (*Arthrinium*) and teleomorphic states (*Apiospora*) were chosen for study, since secondary metabolite production is not confined to any one particular state.

1.3. Antibiotic metabolites from *Arthrinium*

Ishiyama *et al.* (1975) characterised the physico-chemical properties of a metabolite isolated from *A. phaeospermum* strain T-53, concluding it was 1-β-threo-hydroxyaspartic acid. The same researchers stated that this amino acid has the capability

to inhibit the growth of a wide range of microorganisms, notably *Bacillus subtilis* and other sporulating organisms including *Staphylococcus aureus* and *Mycobacterium smegmatis*. Even *Escherichia coli, Botrytis cinerea, Mucor* sp., yeasts and other Deuteromycetes were inhibited.

Traxler *et al.* (1977) isolated a new family of related compounds from *Papularia sphaerosperma (=Arthrinium phaeospermum)*, termed papulacandins. Five papulacandins were isolated and described chemically, namely papulacandins A, B, C, D and E. Papulacandins are a group of structurally related compounds (see Figure 1.1) displaying antifungal activity, especially against yeasts. The unsaturated side chains have been shown to be essential for activity (Traxler *et al.*, 1987), whilst chemical modifications to the aromatic ring can either increase or decrease *in vivo* potency.

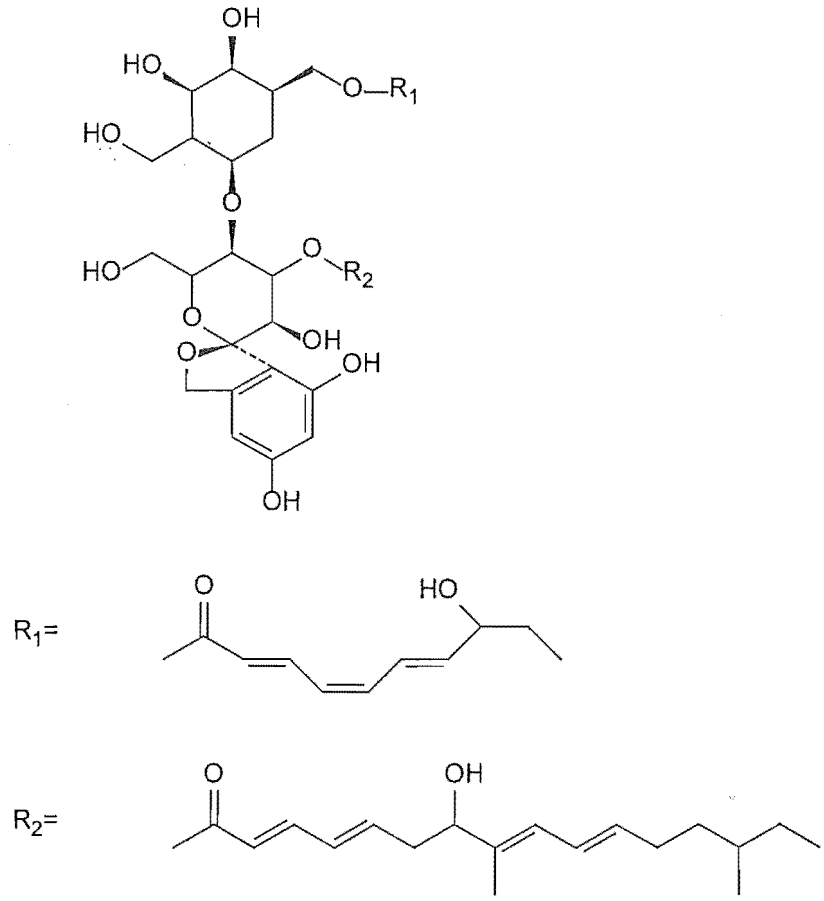


Figure 1.1 Structural formula of papulacandin B. (adapted from Traxler *et al.*, 1977)

Papulacandins inhibit β-(1,3)-glucan synthase activity (Baguley *et al.*, 1979; Perez *et al.*, 1981) resulting in the inhibition of cell wall development, especially in

yeasts, which leads to cell death due to osmotic leakage and rupture. Papulacandins A-E have little or no activity against other fungi, bacteria or protozoa (Traxler *et al.*, 1977).

Antibacterial activity by strains of *A. phaeospermum* was demonstrated by Calvo *et al.* (1982). Calvo *et al.* (1982) also described the antibiotic capability of *Arthrinium aureum*, demonstrating that other members of the genus produced antibiotic and antifungal compound(s). This was confirmed by Larrondo and Calvo (1989, 1990), who demonstrated strains from the *Arthrinium* genus possessed antifungal activity against *Aspergillus niger*. Antifungal activity against filamentous fungi by 14 identified members of the *Arthrinium* genus was confirmed by Larrondo *et al.* (1995), using raw cultured broth extracts.

The teleomorphic state of an *Arthrinium* sp., *Apiospora montagnei,* has been shown to produce a novel secondary metabolite, apiosporamide (Alfatafta *et al.*, 1994). Apiosporamide inhibits the growth of *Ascobolus furfuraceus,* a filamentous fungus, but has no activity against the yeast *Candida albicans*. In addition, it was demonstrated to have inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* (Alfatafta *et al.*, 1994).

1.4. Some other *Arthrinium* metabolites of interest

A new syncytium formation inhibitor, terpestacin (Figure 1.2), has been isolated from a species of *Arthrinium* (Oka *et al.*, 1993). Syncytia are massive bodies consisting of many merged T4 cells, formed during infection by the human immunodeficiency virus (HIV). The giant cells can not survive, and are a major cause of T4 cell death in HIV infection (DeClercq, 1990).

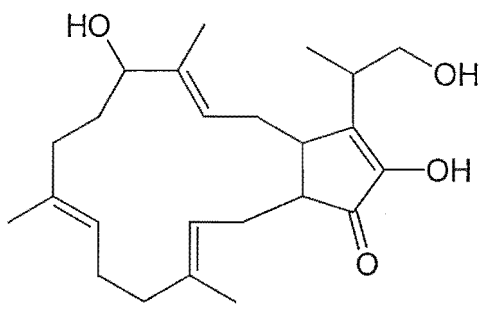


Figure 1.2. Structural formula of Terpestacin. (adapted from Oka, 1993)

3-nitropropionic acid (3-NPA), was shown to be produced by *Arthrinium* cultures isolated from moldy sugarcane (Hu *et al.*, 1986). Neuropathological evidence of *Arthrinium* mycotoxin-induced poisoning was demonstrated by Yitong (1995) using an *in vivo* model, showing conclusively that 3-NPA produced by *Arthrinium* cultures growing on sugarcane was responsible for poisonings of children in China.

1.5. Primary screens

The object of all antimicrobial screens is to provide a definitive answer to the question: Is there something in this sample that warrants detailed investigation? The basic function of the screen is to select, from a large number, those samples that exhibit the desired attributes. The essential elements of a successful primary screen are the ability to examine large numbers of samples quickly and economically, and to provide unambiguous answers. In addition, the screen must be reliable and reproducible. Design of the screen is therefore of paramount importance, for once a sample or organism has returned a "no" answer to the question of warranting further investigation, it is unlikely to be pursued further.

Antimicrobial screens are broadly divided into two classes: *in vivo* and *in vitro*. *In vivo* screens utilize an intact host (e.g., a mouse), the infecting organism and the material to be tested. *In vitro* screens, on the other-hand, usually include a pathogen cultured in liquid or solid medium and the material to be tested. Often it is desirable to use both *in vitro* and *in vivo* screens in the initial phases, as it has been demonstrated that *in vitro* and *in vivo* activity are sometimes poorly correlated (Espinel-Ingroff and Shadomy, 1989). The advantages of using *in vitro* screens are low cost and higher throughput when compared to *in vivo* screens. Hence, *in vitro* screens are frequently used as primary screens.

1.5.1. *In vitro* methods

Most *in vitro* screens that test the sensitivity of a number of fungi to samples thought to contain antifungal compound(s) use methods known as agar diffusion, agar dilution, or broth dilution tests (Espinel-Ingroff and Shadomy, 1989).

1.5.1.1. Broth dilution. Broth dilution involves adding decreasing amounts of sample to tubes, flasks, or microtiter plate wells containing liquid medium

with a defined fungal inoculum. After a specific length of incubation, the medium is examined for fungal growth, which is compared to that of an untreated control.

1.5.1.2. Agar diffusion. Agar diffusion tests measure the inhibition of fungal growth by test compound(s) that diffuse from a single point source. Typically, the fungal inoculum (spores) is seeded into molten agar medium and poured into plates left to set. Samples are then placed at intervals on the surface of the solidified medium. Sample-impregnated assay disks are usually used to this end, although boring holes in the medium and filling the holes with the sample is also used. The plates are incubated and examined for a zone of inhibition. These zones are scored by noting the size of the zone surrounding each disk. Inhibition zones usually indicate the presence of inhibitory or antagonistic compounds, although care has to be taken to ensure that the appropriate positive and negative controls are used. Several concentrations of a sample or one concentration of several samples can be tested against one fungus using this technique. Alternatively, heat sensitive organisms can be spread, using a glass spreader rod, onto the surface of solidified medium and allowed to dry. This is especially useful when yeasts are being used as the test organism. The same methods as those above can be used for testing the samples.

1.5.1.3. Agar dilution. Agar dilution tests use the addition of the sample to the medium, rather than the test organism. A known amount of the sample, or a certain concentration of the sample, is added to the molten agar medium, which is then poured into plates. The test organism is inoculated on the surface of the solidified medium, which is incubated and fungal growth scored. This method is unsuitable when samples contain heat labile compounds, since some active compounds may be inactivated partially or fully.

Primary screens are the first set of "filters" against which samples or compounds will be tested and typically have broad-based targets. Primary screens should be designed to eliminate the generation of false-negatives. For this reason, it is desirable to have a number of tests that together comprise the primary screen.

1.6. Secondary screening

Secondary screens are designed to further "filter" samples or compounds by being more specific and selective. The secondary screen should involve more detailed testing using additional assays and techniques, such as microscopic examination of hanging slides which may give indications as to the mode(s) of action of the active compound(s). The aim of secondary screening is to answer specific questions about the active compound(s). For instance, a secondary screen could be designed to determine the mode of action of the active compound.

For heterogeneous samples, such as the solvent phase of a chemically treated culture broth extract, isolation of the active compound(s) needs to occur. Each of the components has to be isolated and retested against the primary screen to determine which compound(s) are active. Once isolated, in-depth characterisation of the active compound(s) can be undertaken.

The agar diffusion method is reproducible, fast, and gives clear results. It also lacks the difficulties associated with agar dilution and broth dilution tests, such as the problem of heat sensitivity. The test organisms were from the two major fungal groups, since some active compounds affect only filamentous fungi, whilst others affect only yeasts. Resistance of the test organism to the samples also had to be taken into account, so multiple representation of the two groups was needed.

1.7. Aims of Thesis

The aims of the thesis are;

- To screen a number of New Zealand isolates of the *Arthrinium* genus and assess their antifungal activity.

- To optimise the culture conditions and hence optimise antifungal production for the isolates being screened

- To chemically extract the antifungal components from liquid broth cultures

- To confirm antifungal activity results from a number of other workers for New Zealand isolates of the genus *Arthrinium*

- To design a primary screen that selects for fungi displaying antifungal activity.

Chapter II

Materials and Methods

2.1. Initial cultures

Fungal samples were obtained from the culture collections stored at the University of Canterbury and from Landcare Research New Zealand Ltd. in the form of freeze-dried agar plugs (see Table 1 for list of cultures). Each isolate was inoculated onto Potato-Dextrose agar (PDA) and Malt Extract agar (MEA) in 90 mm Petri dishes. Each isolate was cultivated in triplicate and all subcultures were incubated at 20 °C for 4 days. After incubation, contaminated plates were discarded, the remainder used to establish stock cultures (see below).

2.1.1. Long term storage of cultures

Storage cultures for each fungus were established using 1.8 ml cryogenic tubes containing 1 ml of autoclaved 10% glycerol solution (10% by volume dissolved in distilled water). Eight agar plugs, made using a stainless steel tube (internal diameter 6mm), for each culture were placed in a cryogenic tube containing the 10% glycerol solution and labelled with a unique code (see Table 1). The cryogenic tubes were initially placed at –20 °C for 2 hours before being transferred to –80 °C for long-term storage.

2.1.2. Water cultures

Water cultures were made from the initial plates to allow routine sub-culturing. Water cultures were made in sterile Universal bottles and consisted of 10 ml of distilled water and ten individual agar plugs of each culture. Bottles were labelled and stored at 4 °C.

Table 2.1. Cultures codes and source.

Culture	Culture Code	Source Of Culture	-80°C Code
Unclassified	CANU-T51	University of Canterbury Collection	A06
Unclassified	CANU-T365		A05
Unclassified	CANU-T367		A03
Unclassified	CANU-T377		A04
Unclassified	CANU-T789		A01
Unclassified	CANU-T790		A02
Arthrinium arundinus	CANU-T804		A07
Apiospora setosa	6888	Landcare Research	A11
Arthrinium phaeospermum	6966	Landcare Research	A10
Arthrinium phaeospermum	10250	Landcare Research	A08
Apiospora montagnei	10298	Landcare Research	A09

2.2. Optimum temperature for growth

Using a stainless steel tube (inner diameter 6mm), 15 agar plugs were made by pushing the tube into the mycelial mat of a plate culture. The plugs were inoculated onto prepared 90mm Petri dishes containing PDA. The plates were incubated at 10, 15, 20, 25 or 30 °C in triplicate. This process was repeated for each of the 12 cultures. The diameter of the colonies was measured in two directions every 24 hours for 14 days. The second measurement was performed perpendicular to the first.

2.2.1. Growth media determinations

Fungal growth on four different media (MEA, Sauborard Dextrose agar (SDA), Fungal Growth agar (FGA) and Mycological agar (MYA)) was compared to growth on PDA to determine the optimal growth media. Cultured triplicates were incubated at 15, 20, and 25 °C. Growth rates were assessed by measuring the diameter of each colony (using method described above). Two measurements of colony diameter were made every 24 hours for 14 days, with the second measurement performed perpendicular to the first.

2.2.2. Culture plates for initial screening

Using a stainless steel tube (inner diameter 6mm), agar plugs were made by pushing the tube into the mycelial mat of the plate culture. The plugs were inoculated onto prepared 90mm Petri dishes containing either MEA or PDA. This process was repeated for each of the 11 cultures. Cultured duplicates were incubated at 15, 20, 25 and 30 °C for 72 hours and used for initial antifungal screening (see below). Controls consisting of agar plugs taken from uninoculated plates were used.

2.2.3. Initial screening for antifungal activity

Using the plates from the above method (**2.2.2**), *Candida albicans* and *Aspergillus niger* were inoculated directly onto the plates. *C. albicans* was streaked 180° to the mycelial front in four parallel lines, using a sterile platinum loop. The plate was incubated overnight at 25 °C and the antifungal activity against *C. albicans* assessed. Following this 24 hour period, two agar plugs of *A. niger* were placed 30 mm apart on the opposite side of the colony approximately 2 cm from the mycelial front. The plate was incubated for a further 48 hours at 25 °C and assessed for antifungal activity.

2.3. Initial growth in broth

Cultures were further investigated for their ability to grow in broth. Four agar plugs from each culture were introduced to a sterile flask containing 200 ml of either malt extract broth (MEB) or potato dextrose broth (PDB) and incubated at 20 °C and 25 °C. These were then used for section **2.3.1**.

2.3.1. Growth and antifungal activity

To determine the minimum incubation period for the detection of metabolites with inhibitory capacity, 5 ml samples were taken at 48 hour intervals over a 28 day period from each flask prepared as per method **2.3**. Samples were initially filtered through sterile gauze in order to remove mycelium before filtering through a 0.22 μm nitrocellulose Millipore membrane to ensure the sterility of the extract obtained. The extracts were gathered in sterile Universal bottles and activity assays were performed in relation to *C. albicans* and *A. niger,* using the spread and diffusion plate method described in **2.3.3**. Flasks containing uninoculated broths were used as controls.

2.3.2. Effect of aeration on antifungal production

Four agar plugs of each culture were inoculated into 100 ml of MEB contained in separate 250 ml flasks and incubated at ambient temperature (approx. 20 °C). Uninoculated malt extract broths were used as a control. Duplicates were made for all cultures and the control, with one set left to incubate statically and the other shaken on an orbital shaker at 120 r.p.m. 2 ml samples of the broths were removed every 72 hrs for antifungal activity testing. Samples were initially filtered through sterile gauze in order

to remove mycelium before filtering through a 0.22 μm nitrocellulose Millipore membrane to ensure the sterility of the extract obtained. The extracts were gathered in sterile Universal bottles and antifungal activity assays were performed, using the spread plate method and diffusion method described in **2.3.3.**

2.3.3. Diffusion disc testing for antifungal activity

The antifungal activities from samples taken from culture broths were determined by the agar diffusion assay method using nitrocellulose assay discs (6 mm diameter). The test organisms used were *C. albicans* and *A. niger*. Extracellular extracts were obtained by the method described in section **2.3.1.** As the concentration of the inhibitory compound(s) was not known, 10 μl of a broth extract was applied to an assay disc using a sterile Eppendorf pipette tip and allowed to air dry; this procedure being repeated five times. Inoculation of the plates with the test organism occurred in the following ways:

-1ml of a 24 hour culture of *C. albicans* grown in PDB at 25°C, was spread evenly onto PDA plates using a sterilised glass rod and allowed to air dry for 1 hour. This will be referred to as the spread plate method. Impregnated assay discs were placed at the centre of PDA plates previously inoculated with the test organism, with the controls placed halfway between the centre and edge of the plate.

-To make the diffusion plates, 0.5 ml of a conidial suspension (5×10^{-4} to 5×10^{-5} conidia per ml) of *A. niger* was added to 5 ml amounts of 60°C sterile tap water agar. This tap water agar was poured as a layer over PDA plates. This layer was allowed to set and dry. Assay discs were prepared as above and placed on the dry surface of the diffusion plates. Four assay discs, along with a positive, broth and negative control, were placed per diffusion plate.

Inoculated plates were sealed using parafilm and incubated at 20 °C. Plates were observed after 24 hours for a clear zone around the assay disc in the case of *C. albicans*. If a clear zone was present on the yeast spread plates, the diameter of the zone was measured. For *A. niger*, the plates were incubated for 48 hours after application of the assay disc and examined for a zone of inhibition. If there was an inhibition zone, the radius was measured from the edge of the assay discs to the inhibition zone edge. The

positive controls for both *C. albicans* and *A. niger* were assay discs treated with 50 µl of a 100 mg/ml Bravo (Yates) solution, which is a fungicide. Bravo was chosen due to its broad spectrum of antifungal activity, and its non-selective mode of action. A negative control comprising of an assay disc treated with 50 µl of distilled water was also used. In addition, the uninoculated MEB and PDB samples served as a broth control.

2.3.4. Large-scale testing

Culture CANU-T789 and CANU-T790, which had been identified to genus level, were rejected from further testing (see **Chapter V**). Additionally, cultures CANU-T365 and CANU-T377 failed to grow in broth culture and were also rejected from further testing. A flask containing 200ml of MEB was inoculated with six agar plugs of sample culture. One replicate was incubated at 20 °C, the other at 25 °C. Flasks were incubated for at least the period indicated as minimum for antifungal production from the results of method **2.3.1**. After incubation, the broths were filtered as per earlier. The filtrate was gathered in sterile Universal bottles and tested for antifungal activity. A sample of each culture filtrate incubated at 25 °C (except for culture CANU-T365 which had to be re-grown due to contamination) were sent to the Marine Chemistry group at the University of Canterbury for further screening, including an antiviral, antitumor and antimicrobial screen.

Six test organisms were employed, namely *A. niger*, *Botrytis cinerea*, *C. albicans*, *Chaetomium* sp., *Mucor* sp., and *Saccharomyces cerevisiae*. Both *C. albicans* and *S. cerevisiae* were inoculated onto plates using the spread plate method. The assay discs were applied to the spread plates and incubated for 24 hours at 25 °C.

For *A. niger*, *B. cinerea*, *Chaetomium* sp., and *Mucor* sp., agar plugs of the culture were placed in the centre of the plate. These were incubated for 24 hours at 25 °C before five assay discs containing the broth samples were placed on each plate at equal distances from the agar plug and each other. The plates were then incubated for a further 24 hours. A zone of inhibition about the assay disc was used as an indication of antifungal activity. If present, the radius from the edge of the assay disc to the inhibition zone edge was measured. The controls were an uninoculated MEB broth and as a positive control Bravo was used, both prepared as per method **2.3.3.**

2.4. Preparation of broths for extraction

Cultures were incubated in 1 litre square polypropylene bottles, containing 100ml of MEB or PDB. Duplicate cultures were incubated at both 20 °C and 25 °C. Bottles were incubated on their sides to provide a maximum surface area to oxygen ratio. Cultures were incubated for 21 days. Bottles containing 100 ml of uninoculated PDB or MEB were used as controls.

2.4.1. Chemical extraction of compounds from broth

Broths containing the mycelium were placed into 250 ml beakers and blended using an ultra-turrax at high speed for 1 min. The macerated mixture was filtered through a scintillated glass funnel containing a celite pre-filter, attached to a vacuum apparatus. Filtrates were collected in 250 ml conical flasks. Mycelial mats were carefully removed from the celite filter and placed into 250 ml beakers, where 30 ml of ethyl acetate was added. This mixture was incubated at room temperature for 2 min, prior to vacuum filtration. Filtrates for a single sample were pooled into one 250 ml flask. Mycelial mats were washed three times prior to discarding the mat.

Filtrates were tipped into a 1 litre separating funnel and were allowed to stand until the water and solvent phase separated. This was when the solvent phase was clear and the boundary between it and the water phase flat and entire. The water phase was recovered into the 250 ml conical flask. The remaining solvent phase was titered into a 250 ml conical flask. The water phase decanted into the separating funnel, along with 30 ml of ethyl acetate, mixed and allowed to stand until the two phases again separated. The two phases were treated as previously described, with the water phase retained and the water phase extraction process repeated. The spent extracts were discarded.

Approximately 10 g of dried magnesium sulphate ($MgSO_4$) was added to the solvent phase to remove any remaining water. The solvent phase was transferred to a 500 ml round-bottomed flask. Three washes of the conical flask using 5 ml of ethyl acetate were performed and added to the 500 ml flask. The 500 ml round-bottomed flask was then placed in a 35 °C water bath and coupled to a rotary evaporator. Ethyl acetate was evaporated off using this method.

After evaporation, 5 ml of ethyl acetate was added to the round-bottomed flask, mixed, and removed using a Pasteur pipette. This ethyl acetate containing the desired compound(s) was placed in a pre-weighed dry vial, and the process repeated twice more. The vial was dried in a vacuum centrifuge. Once dry, the vial was weighed on an electronic balance and the amount of compound recovered calculated. This process was repeated for each culture, with the resulting vials stored in a dessicater container at 4 °C. Vials were then used for the method described below.

2.4.2. Standardising the concentrations of extracts

A solvent, methanol, was added to each vial in amounts that resulted in a final concentration of 10 mg/ml of wt/volume extracts. These solutions with known concentrations were used for methods **2.4.3-4.4.** In addition, 60 µl units of some extracts were sent to the Marine Chemistry Group at the University of Canterbury for antimicrobial, antiviral, and antitumor activity screening.

2.4.3. Diffusion plates using extracted products

To make the diffusion plates, 0.5 ml of a conidial suspension (5×10^{-4} to 5×10^{-5} conidia per ml) of *A. niger* was added to 5 ml amounts of sterile tap water agar at 60 °C. This tap water agar was poured as a layer over PDA plates. This layer was allowed to set and dry. Then, 50 µl of the solutions from method **4.2** were micropipetted in 10 µl amounts onto sterile nitrocellulose assay discs (6 mm) with the solvent (methanol) allowed to evaporate between transfers. Three discs were placed on the surface of a layered agar plate, along with the appropriate controls (see below), and incubated at 20 °C for 48 hours. The radius of the inhibition zone, from the edge of the assay disc to the inhibition zone edge, was measured if present

As a positive control, the fungicide Bravo (Yates) was diluted (as per manufacturer's instructions) and 50 µl placed on a filter disc and allowed to dry. In addition, a solvent control comprising of a disc treated with 50 µl of methanol and a negative control comprising of an assay disc treated with 50 µl of distilled water were used. The extracted broth control sample also served as a control. These were placed on the layered plate as per the other discs.

2.4.4. Spread plates using extracted products

C. albicans test plates were prepared as described in section **3.3**. Nitrocellulose assay discs (6 mm) were prepared as per section **4.3**. Discs were placed on the surface of the PDA plates inoculated with *C. albicans* and plates incubated at 25 °C for 24 hours. As a positive control, the fungicide Bravo (Yates) was diluted to a concentration of 100 mg/ml and 50 µl placed on a filter disc and allowed to dry. In addition, a solvent control comprising of a disc treated with 50 µl of methanol and a negative control comprising of an assay disc treated with 50 µl of distilled water were used. The extracted broth control sample also served as a control. These were placed on the layered plate as per the other discs.

Chapter III

Results

3.1. Optimum Temperature

Measurements made every 24 hr for 14 days of colonies grown on PDA are presented on the following tables. The range of size for each temperature treatment is from the smallest recorded diameter to the largest recorded diameter. All colonies had an initial diameter of 6mm at day 0, the diameter of the agar plug. A measurement of 85 mm denotes full coverage of the plate. A contaminant fungus with a prominent and extensive inhibition zone was observed on a plate of culture 10250 7 days old. This contaminant fungus was sub-cultured and examined further (see Chapter V).

Table 3.1. Culture CANU-T51 growth on PDA at various temperatures

T51	10°C	15°C	20°C	25°C	30°C
Day 1	7-10 mm	8-11 mm	9-11 mm	10-13 mm	15-17 mm
Day 3	21-24 mm	28-33 mm	38-42 mm	42-47 mm	52-55 mm
Day 5	30-32 mm	42-46 mm	59-67 mm	63-67 mm	61-66 mm
Day 7	40-45 mm	58-65 mm	85 mm	85 mm	71-80 mm
Day 9	57-64 mm	78-85 mm			85 mm
Day 11	72-79 mm	85 mm			
Day 14	85 mm				

Table 3.2. Culture CANU-T365 growth on PDA at various temperatures

T365	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6 mm	7-9 mm	7-9 mm	6 mm
Day 3	7-8 mm	10-12 mm	20-22 mm	20-25 mm	13-15 mm
Day 5	9-11 mm	15-17 mm	24-29 mm	34-40 mm	22-26 mm
Day 7	15-19 mm	23-29 mm	32-38mm	44-51 mm	33-36 mm
Day 9	21-25 mm	34-43 mm	47-56 mm	54-59 mm	43-47 mm
Day 11	32-38 mm	45-52 mm	60-71 mm	63-68 mm	54-59 mm
Day 14	40-53 mm	63-72 mm	85 mm	85 mm	71-80 mm

Table 3.3. Culture CANU-T367 growth on PDA at various temperatures

T367	10°C	15°C	20°C	25°C	30°C
Day 1	6-8 mm	8-10 mm	9-11 mm	11-13 mm	8-10 mm
Day 3	8-12 mm	17-21 mm	27-29 mm	38-47 mm	29-34 mm
Day 5	19-24 mm	37-43 mm	51-62 mm	63-71 mm	55-61 mm
Day 7	34-41 mm	61-69 mm	76-85 mm	85 mm	68-82 mm
Day 9	46-56 mm	85 mm	85 mm		85 mm
Day 11	53-67 mm				
Day 14	67-81 mm				

Table 3.4. Culture CANU-T377 growth on PDA at various temperatures

T377	10°C	15°C	20°C	25°C	30°C
Day 1	8-13 mm	14-24 mm	20-31 mm	19-30 mm	13-25 mm
Day 3	16-25 mm	27-35 mm	32-47 mm	43-58 mm	28-44 mm
Day 5	23-32 mm	42-49mm	60-78 mm	69-85 mm	45-67 mm
Day 7	33-43 mm	68-73 mm	85 mm	85 mm	73-85 mm
Day 9	41-56 mm	81-85 mm			85 mm
Day 11	49-61 mm	85 mm			
Day 14	63-79 mm				

Table 3.5. Culture CANU-T789 growth on PDA at various temperatures

T789	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-7 mm	7-9 mm	7-10 mm	6-9 mm
Day 3	6-7 mm	7-9 mm	11-15 mm	12-14 mm	10-13 mm
Day 5	6-10 mm	12-15 mm	16-22 mm	17-20 mm	13-18 mm
Day 7	7-12 mm	16-20 mm	24-29 mm	22-25 mm	16-24 mm
Day 9	8-14 mm	22-26 mm	35-41 mm	31-34 mm	19-29 mm
Day 11	10-17 mm	24-29 mm	38-48 mm	36-41 mm	23-32 mm
Day 14	19-26 mm	31-38 mm	49-59 mm	45-52 mm	30-39 mm

Table 3.6. Culture CANU-T790 growth on PDA at various temperatures

T790	10°C	15°C	20°C	25°C	30°C
Day 1	7-16 mm	17-30 mm	24-37 mm	18-31mm	15-27 mm
Day 3	18-27 mm	35-45 mm	57-70	51-67 mm	46-59 mm
Day 5	34-47 mm	72-85 mm	76-85 mm	68-85 mm	59-73 mm
Day 7	53-76 mm	85 mm	85 mm	85 mm	85 mm
Day 9	85 mm				
Day 11					
Day 14					

Table 3.7. Culture CANU-T804 growth on PDA at various temperatures

T804	10°C	15°C	20°C	25°C	30°C
Day 1	6-9 mm	7-10 mm	8-13 mm	8-13 mm	7-12 mm
Day 3	7-11 mm	10-14 mm	12-17 mm	11-16 mm	9-16 mm
Day 5	13-19 mm	14-25mm	20-39 mm	19-34 mm	15-31 mm
Day 7	20-29 mm	23-37 mm	35-57 mm	32-45 mm	27-39 mm
Day 9	31-43 mm	38-54 mm	57-81 mm	53-74mm	43-62 mm
Day 11	44-57 mm	56-71 mm	85 mm	73-85 mm	66-85 mm
Day 14	60-76 mm	70-85 mm		85 mm	85 mm

Table 3.8. Culture 6888 growth on PDA at various temperatures

6888	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-10 mm	7-12 mm	7-12 mm	8-11 mm
Day 3	6-9 mm	10-15 mm	10-17 mm	9-19 mm	13-21 mm
Day 5	7-15 mm	15-23 mm	19-27 mm	14-33 mm	22-36 mm
Day 7	11-23 mm	19-30 mm	28-49 mm	27-63 mm	33-52 mm
Day 9	19-35 mm	32-53 mm	49-66 mm	64-85 mm	45-69 mm
Day 11	26-46 mm	46-71 mm	70-85 mm	85 mm	68-85mm
Day 14	43-62 mm	65-85mm	85 mm		85 mm

Table 3.9. Culture 6966 growth on PDA at various temperatures

6966	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-7mm	6-9 mm	7-10 mm	7-12 mm
Day 3	6-8 mm	8-13 mm	18-25 mm	27-35 mm	21-28 mm
Day 5	7-13 mm	27-33 mm	32-41 mm	38-50 mm	29-39 mm
Day 7	10-17 mm	36-49 mm	57-69 mm	55-75 mm	38-50 mm
Day 9	14-23 mm	48-60 mm	68-85 mm	72-85 mm	47-62 mm
Day 11	21-30 mm	59-85 mm	85 mm	85 mm	60-79 mm
Day 14	40-49 mm	85 mm			85 mm

Table 3.10. Culture 10250 growth on PDA at various temps

10250	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-7mm	6-8 mm	8-10 mm	7-11 mm
Day 3	6-8 mm	8-11 mm	20-28 mm	26-32mm	23-27 mm
Day 5	7-11 mm	28-32 mm	35-47 mm	35-48 mm	28-37 mm
Day 7	9-14 mm	38-45 mm	64-77 mm	49-72 mm	36-47 mm
Day 9	10-19 mm	42-55 mm	79-85 mm	69-85 mm	51-67 mm
Day 11	13-26 mm	57-72 mm	85 mm	85 mm	66-85mm
Day 14	20-36 mm	85 mm			85 mm

Table 3.11. Culture 10298 growth on PDA at various temperatures

10298	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-9 mm	11-14 mm	13-19 mm	10-13 mm
Day 3	6-8 mm	7-11 mm	23-27 mm	31-36 mm	19-24 mm
Day 5	7-10 mm	28-33 mm	39-46 mm	41-49 mm	34-43 mm
Day 7	9-15 mm	42-47 mm	55-62 mm	53-66 mm	49-57 mm
Day 9	12-19 mm	53-61 mm	67-85 mm	68-85 mm	59-68 mm
Day 11	14-23 mm	62-78 mm	85 mm	85 mm	70-82 mm
Day 14	20-32 mm	85 mm			85 mm

Growth at 20 °C and 25 °C were considered optimum for all cultures, since these two temperatures produced the fastest growth.

3.1.1. Optimum growth media

Five day old plates of each culture inoculated onto the various media were compared to the 5 day growth of cultures grown on PDA. Variations in growth between the media are expressed in the following ways;

-**Poorer** means the average growth diameter was less than the lowest measurement on PDA.

-**Similar** means the average growth diameter was between the lowest and highest measurements on PDA.

-**Greater** means the average growth diameter was higher than the highest measurement on PDA.

Table 3.12. Growth of cultures on MEA at 3 temperatures

Cultures	15 °C	20 °C	25 °C
CANU-T51	**Greater**	**Greater**	**Greater**
CANU-T365	Poorer	Similar	Similar
CANU-T367	Poorer	**Greater**	**Greater**
CANU-T377	**Greater**	**Greater**	**Greater**
CANU-T789	Poorer	Similar	Similar
CANU-T790	Similar	Similar	**Greater**
CANU-T804	Similar	Similar	Similar
6888	**Greater**	**Greater**	**Greater**
6966	**Greater**	**Greater**	**Greater**
10250	**Greater**	**Greater**	**Greater**
10298	Similar	Similar	Similar

Table 3.13. Growth of cultures on SDA at 3 temperatures

Cultures	15 °C	20 °C	25 °C
CANU-T51	Similar	Similar	**Greater**
CANU-T365	**Greater**	Similar	Similar
CANU-T367	Poorer	Similar	Similar
CANU-T377	Poorer	Poorer	Similar
CANU-T789	**Greater**	Similar	Similar
CANU-T790	**Greater**	**Greater**	Similar
CANU-T804	**Greater**	Similar	Poorer
6888	Similar	Poorer	Poorer
6966	Similar	Poorer	Poorer
10250	Similar	Poorer	Poorer
10298	Similar	Poorer	Poorer

Table 3.14. Growth of cultures on MYA at 3 temperatures

Cultures	15 °C	20 °C	25 °C
CANU-T51	Poorer	Similar	Similar
CANU-T365	Poorer	Poorer	Poorer
CANU-T367	Poorer	Poorer	Similar
CANU-T377	Poorer	Similar	Similar
CANU-T789	Poorer	Poorer	Poorer
CANU-T790	Poorer	Similar	Similar
CANU-T804	Poorer	Similar	Similar
6888	Poorer	Similar	**Greater**
6966	Poorer	Similar	Similar
10250	Poorer	Similar	Similar
10298	Poorer	Similar	Similar

Table 3.15. Growth of cultures on FGA at 3 temperatures

Cultures	15 °C	20 °C	25 °C
CANU-T51	Poorer	Poorer	Poorer
CANU-T365	Poorer	Poorer	Poorer
CANU-T367	Poorer	Poorer	Poorer
CANU-T377	Poorer	Similar	Poorer
CANU-T789	Similar	Poorer	Poorer
CANU-T790	Poorer	Poorer	Poorer
CANU-T804	Similar	Poorer	Poorer
6888	Similar	Poorer	Poorer
6966	**Greater**	Similar	Poorer
10250	Similar	Similar	Poorer
10298	Poorer	Poorer	Poorer

MEA generally produced much faster growth when compared to that produced by PDA. Growth of the known *Arthrinium* and *Apiospora* cultures was generally slower on SDA and FGA. Cultures grown at 15 °C on MYA had poor growth, but cultures showed similar growth at 20 °C and 25 °C. MEA and PDA were selected as the media

to be used for initial antifungal testing since they produced the best growth, especially for *Arthrinium* and *Apiospora* cultures.

3.1.2. Initial antifungal screening

Of the 11 cultures initially screened against *C. albicans*, only CANU-T790, CANU-T789, 6888 and 6966 had observable antifungal activity. Activity was visualised by examining the four parallel streaks of *C. albicans*. If antifungal activity was present, the middle two lines would be shorter (at the end nearest the mycelial front) than the outside two and at no time did any of the four lines meet the mycelial front of the culture.

The culture CANU-T790 was only observed to have activity at 20 °C. Culture 6966 displayed antifungal activity at 20 °C and 25 °C as did 6888. Culture CANU-T789 had observable activity at 15, 20 and 25 °C. No activity was detected for plates incubated at 30 °C. Both treatments for each active culture showed this antifungal activity against *C. albicans*. None of the control plates demonstrated any antifungal activity against *C. albicans.*

Five of the 11 cultures displayed antifungal activity against *A. niger.* Activity was visualised as an inhibited zone of development of the mycelial edges (of *A. niger*) nearest the culture colony being tested. At no time did the two mycelial fronts come in contact if there was observable inhibition.

Those cultures displaying antifungal activity against *A. niger* were CANU-T365, CANU-T789, CANU-T790, CANU-T804 and 6966. Antifungal activity was observed at 15 °C for the cultures CANU-T789 and CANU-T790. All of the cultures with observable antifungal activity displayed it at 20 °C, with cultures CANU-T789 and 6966 showing activity at 25 °C. No activity was detected at 30 °C. Antifungal activity was identical for the second set of plates. None of the control plates demonstrated any antifungal activity against *A. niger.*

3.2 Initial growth in broth

Growth of culture CANU-T365 and CANU-T377 was not observed in either MEB or PDB at 20 °C or 25 °C. Growth of culture CANU-T790 in PDB was also not observed. Cultures CANU-T804 and 6888 produced a red pigmentation of the PDB after 14 days growth, which was much less pronounced in the MEB. CANU-T789 started to produce an ostiole (a type of fungal fruiting body) after 24 days of growth in MEB. No comparable structure was seen in the PDB containing the CANU-T789 culture.

3.2.1. Growth and antifungal activity

Table 3.16. Minimum incubation time for antifungal activity

Culture	PDB at 20 °C	PDB at 25 °C	MEB at 20 °C	MEB at 25 °C
CANU-T51	N/A	N/A	N/A	N/A
CANU-T367	N/A	N/A	N/A	N/A
CANU-T789	20 days	24 days	20 days	22 days
CANU-T790	NG	NG	N/A	16 days
CANU-T804	N/A	N/A	N/A	N/A
6888	18 days	N/A	14 days	18 days
6966	14 days	N/A	10 days	N/A
10250	16 days	N/A	12 days	N/A
10298	N/A	18 days	N/A	20 days
Neg. Control	N/A	N/A	N/A	N/A

N/A= No activity

NG= No growth

3.2.2. Aeration and antifungal production

No growth was observed for the cultures CANU-T365, CANU-T377, or CANU-T789 when incubated as a shaking culture. Additionally, growth of CANU-T365 and CANU-T377 was not observed when incubated statically. Antifungal activity was

detected from cultures 6888, 6966 and 10250 when incubated as shaking cultures. Activity was first detected for cultures 6888 and 10250 on the 15th day of incubation. Activity for culture 6966 was detected on the 9th day of incubation. Antifungal activity for culture 6888 was only observed against *C. albicans* and a radius of inhibition of 2 mm was measured. Inhibition zones were observed for culture 6966 in both the *C. albicans* and *A. niger* screen. A radius of inhibition of 1 mm was measured for the *C. albicans* screen and a radius of inhibition of 3 mm against *A. niger*. Culture 10250 only produced an inhibition zone against *A. niger*, with a radius of inhibition measuring 3 mm. The positive control generated a radius of inhibition measuring 8 mm. The negative controls did not produce an inhibition zone.

Antifungal activity from the static broth samples was also observed. Cultures CANU-T789, 6888, 6966 and 10250 displayed this activity. The sample from CANU-T789 produced a radius of inhibition measuring 6 mm against *C. albicans* from a 21 day sample. Culture 6888 displayed antifungal activity against *C. albicans*, with a radius of inhibition measuring 3 mm observed from a 15 day sample. The 11 day sample from culture 6966 showed activity against *C. albicans* and *A. niger*, with the radius of inhibition measured being 2 mm and 3 mm respectively. Antifungal activity for culture 10250 was confined to *A. niger* from a 12th day sample and a radius of inhibition of 2 mm was measured. The positive control generated a radius of inhibition measuring 10 mm. The negative controls did not produce an inhibition zone.

3.2.3. Large-scale testing

Culture CANU-T789 and CANU-T790, which had been identified to genus level, were rejected from further testing. Also, the failure of culture CANU-T365 or CANU-T377 to grow in broth culture resulted in these two cultures not being used for further testing. The following tables summarise the results obtained. Measurements are of the radius of inhibition by culture filtrate.

Table 3.17. MEB 20 °C filtrates

Culture	*C. albicans*	*S. cerevisiae*	*B. cinerea*	*Chaetomium* sp	*Mucor* sp	*A. niger*
CANU-T51	N/A	N/A	N/A	N/A	N/A	N/A
CANU-T367	N/A	N/A	N/A	N/A	N/A	N/A
CANU-T804	N/A	N/A	N/A	N/A	N/A	N/A
6888	**3 mm**	**2 mm**	N/A	N/A	N/A	N/A
6966	**3 mm**	**4 mm**	N/A	**3 mm**	**3 mm**	**4 mm**
10250	**2 mm**	**1 mm**	**3 mm**	N/A	N/A	2 mm
10298	N/A	N/A	N/A	N/A	N/A	N/A
Pos. Control	**6 mm**	**7 mm**	**8 mm**	**8mm**	**8 mm**	**9 mm**
Neg. Broth control	N/A	N/A	N/A	N/A	N/A	N/A

N/A= No activity

Table 3.18. MEB 25 °C filtrates

Culture	*C. albicans*	*S. cerevisiae*	*B. cinerea*	*Chaetomium* sp	*Mucor* sp	*A. niger*
CANU-T51	N/A	N/A	N/A	N/A	N/A	N/A
CANU-T367	N/A	N/A	N/A	N/A	N/A	N/A
CANU-T804	N/A	N/A	N/A	N/A	N/A	N/A
6888	**3 mm**	**3 mm**	N/A	N/A	N/A	N/A
6966	N/A	N/A	N/A	N/A	N/A	N/A
10250	N/A	N/A	N/A	N/A	N/A	N/A
10298	N/A	**1 mm**	N/A	**2 mm**	**2 mm**	N/A
Pos. Control	**6 mm**	**6 mm**	**9 mm**	**9mm**	**8 mm**	**10 mm**
Neg. Broth control	N/A	N/A	N/A	N/A	N/A	N/A

N/A= No activity

3.2.4. Marine chemistry group results

Six filtered broth extracts (CANU-T51, CANU-T367, CANU-T804, 6888, 6966 and 10250), as well as an uninoculated broth extract control, were sent to the Marine Chemistry group at the University of Canterbury. The extracts were put through antitumor, antimicrobial (see **3.4.**) and antiviral screens. None of the extracts or the control showed any antitumor or antiviral activity. The extract from culture 10250 produced a radius of inhibition of 2 mm against the test organism *Cladosporium*

resinae. This was the only antimicrobial activity detected by the Marine Chemistry group.

3.3. Chemical extraction from broth cultures

Table 3.3 shows the total amount of compounds recovered via solvent extraction from broth cultures.

Table 3.19. Weight of recovered compounds

Culture	Dried weight of recovered compounds	Methanol added to make 10 mg/ml solutions
6888*	21.6 mg	2.16 ml
6888	11.2 mg	1.12 ml
6966*	16.7 mg	1.67 ml
6966	11.4 mg	1.14 ml
10250	5.6 mg	0.56 ml
Control	2.3 mg	0.23 ml

*Culture incubated at 25 °C

The solvent solutions for cultures 6966 and 10250 were pale yellow in colour. Those for culture 6888 were coloured a light red, whilst the solution for the control was clear.

3.3.1. Diffusion plates

Of the solvent extracts tested (see above Table 3.3 for list), the positive control and 3 culture extracts displayed antifungal activity, producing inhibition zones about the assay disc. All treatments of the active solvent extracts produced such zones. The inhibition zone edges were easily distinguished by the mycelium of *A. niger* and were circular. The radius from the assay disc edge to the inhibition zone edge was 9 mm for the positive control in all replications. The solvent extract of culture 6888 incubated at 20 °C produced a radius of inhibition measuring between 3-5 mm. No inhibition zone was observed for the solvent extract of culture 6888 incubated at 25 °C. Both of the solvent extracts of culture 6966, incubated at 20 °C and 25 °C, produced a radius of

inhibition between 6-8 mm, regardless of incubation temperature. The solvent extract from culture 10250 produced a radius of inhibition of 6-7 mm for all treatments. Neither the broth, methanol, nor negative controls produced a clear zone in any of the experimental treatments.

3.3.2. Spread plates results

Of the solvent extracts tested, only the positive control and culture 6966 (incubated at 20 °C) had observable clearing zones. These zones were observed in all treatments of the active solvent extract. The radius from the assay disc edge to the clearing zone edge was 5-6 mm for the positive control in all cases and 2-3 mm for culture 6966 (incubated at 20 °C). Growth of the test organism, *C. albicans,* was vigorous, resulting in a continuous 'lawn' across the agar surface. The clearing zones were easily distinguishable around the assay discs. Neither the broth, methanol, or negative controls had a clearing zone in any of the experimental treatments.

3.4. Summary of the antimicrobial assay

Only one solvent extract was sent to the Marine Chemistry group at the University of Canterbury for antimicrobial screening, due to the small quantities recovered from the broth extraction process. The sample sent was the solvent extract from culture 6966. This was tested against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Trichophyton mentagrophytes*, *Cladosporium resinae* and *Candida albicans*.

The agar dilution method is employed to inoculate the organisms into media and assay disks are prepared by pipetting 60 µl of the sample onto a 6 mm filter paper disk. If a zone of inhibition develops during incubation, the radius of the zone is measured as previously described.

The solvent extract from culture 6966 showed antibacterial and antifungal activity. A zone of inhibition was recorded against *E. coli* (1 mm radius of inhibition), *B. subtilis* (2 mm radius of inhibition), *T. mentagrophytes* (2 mm radius of inhibition) and *Cladosporium resinae* (2 mm radius of inhibition).

Chapter 4

Unknown contaminant

4.1. Description

Characteristics in culture: Colonies grown for 2 weeks at 20°C in dark incubator; MEA: 6.5-8 cm diameter, variable in texture, effused to compact, aerial mycelium scant, tan to brown, non-septate, 2-5 μ thick hyphae; PDA: 6-7 cm diameter, mycelium partly superficial and partly immersed in the substratum, composed of branched and anastomosing, non-septate, white to grey, transparent to translucent, 2-5 μ thick hyphae. A 'cell layer' develops on and within the agar in discrete patches after 5 days incubation, consisting of non-uniform shaped, dark green/black cells.

4.2. Introduction

When sub-culturing for method **2.1.**, a contaminant culture was observed growing on one edge of a PDA plate containing culture 10250. The contaminant colony measured approximately 20 mm across and a radius of inhibition of at least 30 mm was inhibiting the growth of culture 10250. Agar plugs were taken from the growing mycelial edge of the contaminant culture (see method **4.2.1**) and the plate was resealed and returned to incubate at 20 °C. 48 hours later the contaminant was re-examined. A light brown/green layer resembling algae growth had developed on the agar surface. Mycelium still could be observed around the layer and a zone of inhibition was still strongly evident. Plugs of this layer were taken for sub-culturing. In addition, slides were prepared of the layer for examination under the light microscope (see Figure **4.1**)

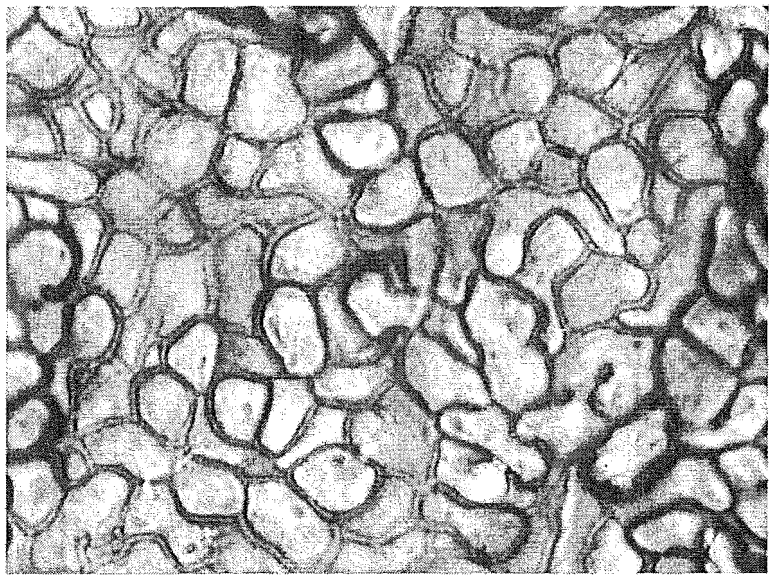


Figure 4.1. Cell layer from culture UNK. 1500x.

Due to the strong antifungal activity displayed by this contaminant culture, it was decided to isolate and include it in this study. The PDA plate it was first observed on had a culture of *Arthrinium phaeospermum* present also, which has no known teleomorph. Hence, it was possible the contaminant was the teleomorphic state of culture 10250.

Methods

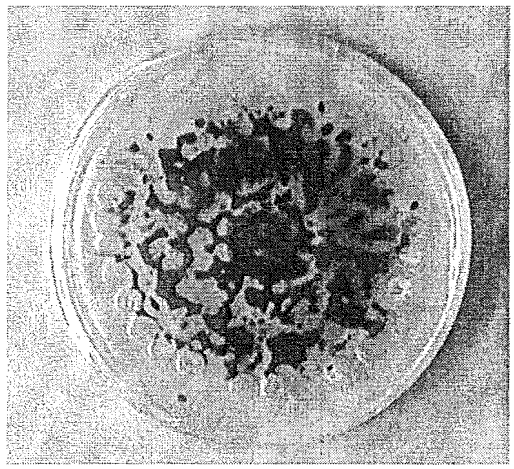
4.3.1. Isolation of unknown culture

Agar plugs were taken from various parts of the contaminant colony, which were subsequently inoculated onto PDA and MEA plates. Plates were incubated at 20°C or 25°C for 72 hours. Agar plugs were then taken from the leading edge of the mycelium of the prepared contaminant culture plates. These were inoculated onto PDA and MEA plates and incubated as above. Colonies closely resembling the original isolate were retained and sub-cultured. In addition, agar plugs taken from the "cell" layer were placed on PDA, MEA and tap water agar plates and incubated at 15, 20 and 25 °C.. Third generation colonies were treated as per methods **2.2-2.4.2**

Results

The contaminant was assigned the code UNK and long-term storage cultures were prepared as per method **2.1.1**. The code A12 was assigned for the long-term storage culture. No growth was observed of subcultures incubated at 25 °C. For this reason, all subsequent subcultures were incubated at 15 °C and 20 °C. Growth closely resembling the original contaminant was observed on all PDA plates incubated at 20 °C. After a period of 5 days, "cell" layers were seen developing on top of and within the PDA (see figure **4.2**).

Figure 4.2. Development of Culture UNK on PDA



4.4.1. Initial growth

Table 4.1. Culture UNK growth on PDA at various temperatures

UNK	10°C	15°C	20°C	25°C	30°C
Day 1	6 mm	6-7 mm	6-8 mm	6 mm	6 mm
Day 3	7-9 mm	9-14 mm	10-15 mm	6 mm	6 mm
Day 5	9-12 mm	13-19 mm	15-23 mm	6 mm	6 mm
Day 7	12-17 mm	18-25 mm	22-31 mm	6-7 mm	6 mm
Day 9	16-24 mm	23-31 mm	27-39 mm	6-8 mm	6 mm
Day 11	21-30 mm	29-42	34-48 mm	7-9 mm	6 mm
Day 14	31-38 mm	44-58 mm	59-67 mm	10-13 mm	6 mm

4.4.2. Growth on other media

MEA was the only growth media that culture UNK grew better on than PDA. Growth was much slower on SDA, and no growth was seen on MYA or FGA until day 3. Because of this, PDA and MEA were chosen over the other media types.

4.4.3. Initial screening for activity

Antifungal activity was visualised by examining the four parallel streaks of *C. albicans*. If antifungal activity was present, the middle two lines were shorter (at the end nearest the mycelial front) than the outside two and at no time did any of the four lines meet the mycelial front of the culture. Culture UNK produced pronounced antifungal activity against *C. albicans*

For the filamentous fungus *Aspergillus niger*, antifungal activity was visualised as a inhibited zone of development of the mycelial edges nearest the culture colony being tested. At no time did the two mycelial fronts come in contact if there was observable inhibition. Culture UNK also displayed strong antifungal activity against *A. niger*.

4.4.4. Initial growth and activity in broth

Culture UNK grew in both PDB and MEB. No coloration of the broth was observed during 28 days of incubation. No growth was observed, or activity detected, for the 25 °C treatments. Antifungal activity was detected after 8 days incubation in PDB and MEB. Zones of inhibition were seen when both *C. albicans* and *A. niger* were the test organisms. The radius of the zones measured 8 mm and 9 mm respectively.

4.4.5. Effect of aeration on antifungal activity

No observable growth or antifungal activity occurred when culture UNK was incubated on a rotary shaker. Activity was detected from the treatment left statically incubating at ambient room temp (approximately 20 °C). The antifungal activity was detected after 9 days and the filtrate produced a radius of inhibition measuring 7 mm

against *C. albicans*. A radius of inhibition of 6 mm was measured for the filtrate when *A. niger* was used.

4.4.6. Large-scale testing

The following tables summarise the results obtained. Measurements are of the radius of inhibition by culture filtrate. No growth was observed for culture UNK at 25 °C.

Table 4.2. MEB 20 °C filtrate

Culture	*C. albicans*	*S. cerevisiae*	*B. cinerea*	*Chaetomium* sp	*Mucor* sp	*A. niger*
UNK	**8 mm**	**6 mm**	**2 mm**	N/A	**4 mm**	**6 mm**
Pos. Control	**6 mm**	**7 mm**	**8 mm**	**8mm**	**8 mm**	**9 mm**
Neg. Broth control	N/A	N/A	N/A	N/A	N/A	N/A

N/A= No activity

4.4.7. Marine chemistry group results

Culture UNK displayed no antiviral or antitumor activity. However, it displayed antimicrobial activity against 3 of the 6 organisms screened. When tested against *C. albicans*, the culture UNK filtrate had a radius of inhibition of 8 mm. The culture UNK filtrate had a radius of inhibition of 4 mm against the filamentous fungus *Cladosporium resinae,* and a radius of inhibition of 1 mm against *Trichophyton mentagrophytes.*

4.4.8. Chemical extracts from broth

66 mg of extract was recovered via the chemical extraction method detailed in method **2.4.1**. This was re-suspended in 6.6 ml of methanol, with a sample being sent to the Marine chemistry group at the University of Canterbury.

4.4.9. Results of extract testing via spread and diffusion plate methods

No activity was detected for the solvent extract of culture UNK. The negative controls also produced no activity. The positive control produced a radius of inhibition measuring 6 mm. No activity was detected in any of the screens employed by the Marine chemistry group at the University of Canterbury.

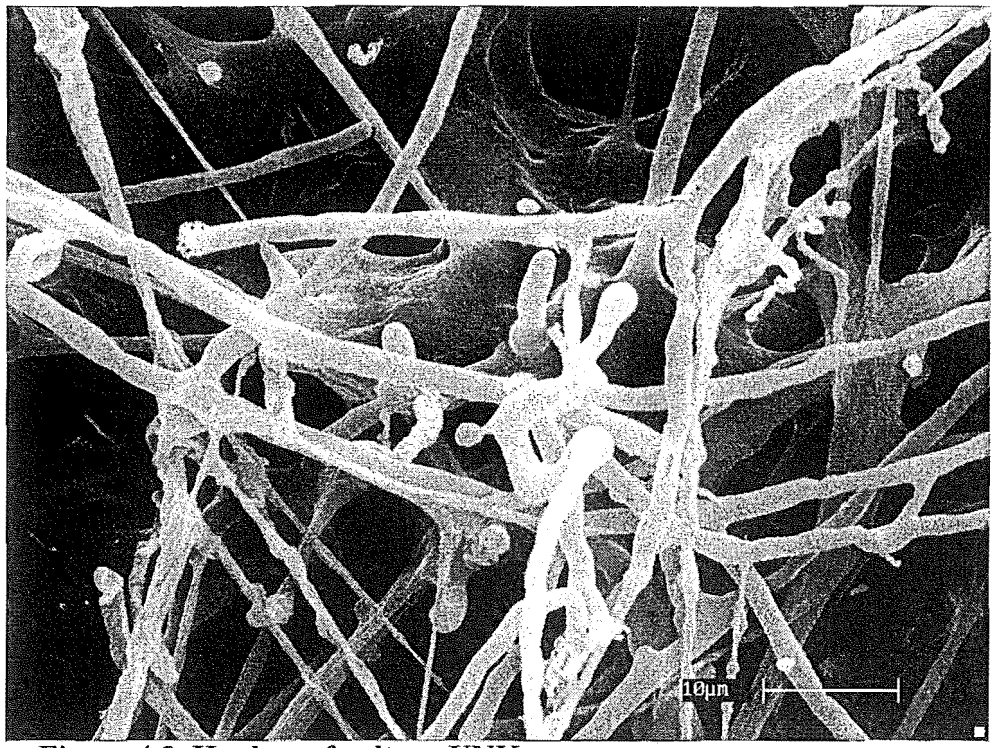
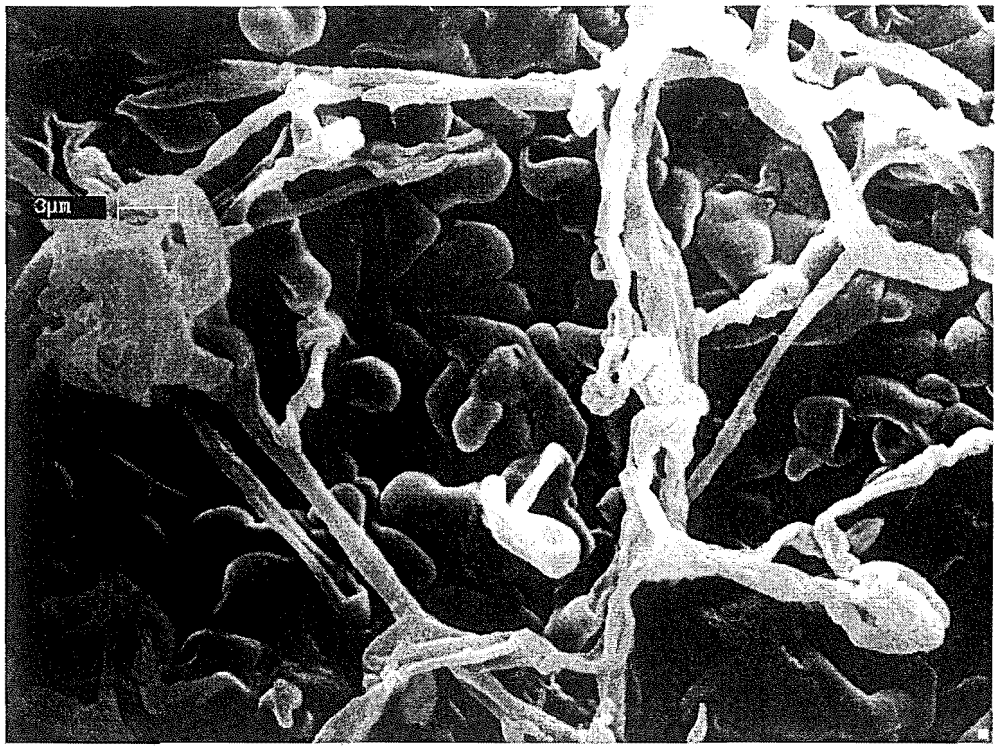


Figure 4.3. Hyphae of culture UNK.

Figure 4.4. Surface of cell layer showing intimate hyphae association.



Chapter V

Discussion

Both MEA and PDA have been shown to enhance the production of secondary metabolites and inhibitory substances (Larrondo *et al.*, 1996) by species of the *Arthrinium* genus. The production of secondary metabolites is not well understood and involves a broad spectrum of metabolic processes that often have little in common. The results obtained for the growth of the cultures on solid media show that PDA and MEA produce the fastest growth at optimum temperature for the various fungi. The temperature results generated when the fungi were grown using PDA as a reference media were essentially duplicated by the MEA temperature results.

The aim of the investigation into optimum temperature was to determine whether temperatures that restrict growth enhance secondary metabolite production, specifically the production of inhibitory substances. It is a generally held belief that secondary metabolite production and antifungal activity are enhanced when normal growth is restricted (Larrondo *et al.*, 1996).

Restricting growth by limiting the temperature of incubation did not enhance antifungal activity of the cultures CANU-T365, CANU-T804, 6888 and 6966. Incubation at 15 °C suppressed antifungal activity for these cultures. Likewise, the higher temperature of incubation (30 °C) also suppressed antifungal activity. Cultures, such as *A. phaeospermum*, showed antifungal activity only when grown at optimal temperatures. Therefore, it was concluded that the optimum temperature for growth was also important when determining the temperature for optimum antifungal activity. All of the cultures that were being studied had optimum temperatures of 20-25 °C. Since temperatures outside this range (15 °C and 30 °C) suppressed antifungal activity, these two temperatures were not used for further incubation of cultures.

Determining antifungal activity by cultures grown on solid media poses a number of problems. Essentially, sampling is laborious, time-consuming and not very reproducible. Difficulties are encountered with non-uniform growth, binding of substances to the agar component of the media and extraction of antifungal compounds from solid media. Culturing of fungi in liquid avoids many of these problems and allows for continuous sampling and culturing of test organisms. This allows activity over time to be assessed, so that the minimum incubation period before antifungal production occurs can be determined. In primary screens this is important, as screening samples after insufficient incubation results in potential antifungal candidates being missed. The main purpose of the primary screen is to identify all antifungal producing organisms for secondary screening. Therefore, it is important to establish liquid cultures early in the primary screen.

The minimum incubation times for the detection of antifungal activity was determined via static liquid culturing. Continuous sampling at regular intervals coupled with an assay to detect the antifungals if present allows the determination of this minimum incubation time. The cultures for this study were sampled at regular intervals and the minimum incubation times determined for those that showed antifungal activity. This information was needed before other factors affecting liquid culturing could be investigated. This information was also useful since it gave an early indication of the sensitivity of the test organism screen for antifungal activity.

Originally the test organism screen for antifungal activity employed an agar plug method than the diffusion plate technique for filamentous fungi. The agar plug technique consisted of a colony of the filamentous fungus *Aspergillus niger* having 6 mm agar plugs taken from the mycelial front. These were inoculated onto prepared PDA plates and incubated for 24 hrs, producing a colony of about 3 cm in the agar plates' centre. Assay discs containing the samples of interest were placed around the colony approximately 30 cm from it and each other. The plate was then incubated for 48 hours and examined for zones of inhibition about the assay discs. This method, however, was discarded due to the following reasons;

- Often the colony of *A. niger* would not grow at a uniform rate, resulting in apparent inhibition zones caused by antifungal activity, when in fact there was no such activity.

- The colony growth was such that at times the mycelial front did not get very close to the assay disc after 48 hrs incubation.

- The colony growth sometimes was such that 48 hrs of incubation resulted in the assay disc being over-run by mycelium. This gave the apparent result of the sample having no antifungal activity at all, when in fact the activity may have been insufficient to produce an inhibition zone that lasted 48 hrs due to factors such as low concentration of compound(s) and the like.

The plate diffusion method addressed these problems and the results gained by the method could be compared with those from the spread plate technique, since they are both similar in effect.

Liquid culture does also have drawbacks. Some fungi will not grow in liquid culture at all, and others do so poorly. However, there are various factors recognised as being limiting to fungi in liquid cultures. One of these is oxygen availability. A low oxygen/surface area of mycelium can be avoided in various ways. One such way is to "shake" the culture whilst incubating. Essentially, the culture is swirled gently on a rotary shaker increasing the surface/oxygen ratio. For this study, aeration was investigated for its effect on antifungal activity of the cultures.

The results for the aerated (shaken) and static liquid cultures generally agreed with each other. Only one culture, CANU-T789, did not grow when incubated on a rotary shaker. However, this was inconsequential to the primary screening, since it produced antifungal activity when incubated statically. Aeration was shown to have little effect on the production of antifungal compounds, with the radius of inhibition (resulting from a concentration of antifungal compound and amount of activity of that compound) comparable between shaken and static cultures. In addition, the minimum incubation times were similar for shaken cultures as for static.

The failure of CANU-T365 and CANU-T377 to grow in liquid cultures meant that they were discontinued from the primary screen. CANU-T365 displayed antifungal activity on solid media against *A. niger*, so it represents the loss from the primary screen of a positive sample. Two other cultures were also discontinued since they had produced spores that allowed for their identification. CANU-T789 produced ostioles (a

reproductive structure) and was identified as belonging to the genus *Xylaria*. CANU-T790 produced pale green spores and was identified as belonging to the genus *Trichoderma*. Testing for antifungal activity against a larger range of fungi represented the last stage of the primary screen. In addition, samples were screened by the Marine Chemistry Group (MCG) at the University of Canterbury for antitumor, antiviral and antimicrobial activity. The MCG did not detect activity in the majority of the samples. Only culture 10250 and UNK showed activity in the MCG screens. The antifungal activity detected for culture UNK was excellent, especially against *C. albicans*. The results generated by our own screening showed many more of the samples had antifungal activity than those detected by the MCG. However, the samples sent to the MCG were filtrates from cultures incubated at 25 °C. These generated a much smaller amount of antifungal activity results than those of the 20 °C filtrates in our screening. Activity against *C. albicans* was shown for culture 6888 from the 25 °C filtrate in our screen, but was not detected by the MCG's *C. albicans* screen. This could be due to a strain difference between the *C. albicans* cultures, or something to do with the sample itself, such as age. The MCG only run screens at set times, so it is possible the samples were one or two days older than the ones we tested.

As the end of the primary screening had been reached, three further cultures that did not display antifungal activity were discontinued. These were cultures CANU-T51, CANU-T367, and CANU-T804. Additionally, 10298 was discontinued since the antifungal activity had not been detected for a number of months. The long-term –80 °C 10298 culture also did not display activity when re-cultured. The remaining 4 cultures were 6888, 6966, 10250 and UNK.

The failure of 10298 to display activity when re-cultured, and its apparent loss of antifungal activity, raises some serious concerns for the reproducibility of this work. Sub-culturing of colonies results in genetic differences over time between the parent colony and 'daughter' colonies. These differences probably arise due to mutations of the fungal DNA, especially in areas of high DNA replication such as the dividing cells at the tip of hyphae. Since sub-cultures are typically made from these areas, the probability that the sub-culture has genetic mutations increases. This effect is amplified over time through successive sub-cultures. To avoid this, it is important to have enough parent material stored too regularly make new sub-cultures. However storage itself, as in the case of 10298, can inactivate or damage the gene(s) necessary for the phenomenon

under study. To avoid this problem, the parent material must be stored in a variety of ways.

Broths produced by the remaining four cultures were chemically extracted. The aim of this extraction was to isolate the active compound(s) from the liquid broth, thereby concentrating the compound(s). By drying the solvent from the isolated compound(s), a known weight of sample could be determined. This sample could be made to a known concentration and tested for antifungal activity. The extracted sample could then be further chemically treated and the compound(s) responsible for antifungal activity purified. Alternatively, purification via the employment of thin-layer chromatography could be pursued.

Only small quantities of compounds were recovered from liquid broths of the 4 cultures. The largest quantity of compound recovered was 66 mg from culture UNK, although this showed no antifungal activity when tested against *C. albicans* and *A. niger*. The small amounts of compounds recovered meant that a limited amount of testing could be performed. The extracts were tested for antifungal activity against *C. albicans* and *A. niger*. There was a sufficient amount of extract from culture 6966 and UNK to allow screening by the MCG. As previously mentioned, no activity was detected for the extract UNK. However, results for extract 6966 show that it had activity against the bacteria *Bacillus subtilis* and *Escherichia coli*, as well as the filamentous fungi in the screen. This demonstrates that chemical extraction and concentration allows much greater accuracy for screening.

Those extracts that demonstrated no antifungal effects, chiefly that from culture UNK, shows that the chemical recovery process can lose the compound(s) of interest, or inactivate them. Only compounds soluble in the solvent phase of the extract are kept, as this phase is considered the more likely phase to contain compound(s) of interest. Any such compounds left in the water phase are discarded with the water phase. In order to try and stop this from happening, different solvents need to be tried, and the water phase of the extract needs to be treated further or at least tested to see if the antifungal compounds still remain in it.

5.2. Conclusions

New Zealand isolates of *A. phaeospermum* display antifungal activity against yeasts and filamentous fungi, as well as bacteria. Additionally, this confirms the results of Larrondo and Calvo (1995,1997) of antibacterial activity and antifungal activity against yeasts by *A. phaeospermum.*

The teleomorphic state of an *Arthrinium* sp., *Apiospora montagnei,* has activity against the yeast *S. cerevisiae.* In addition, activity against two filamentous fungi was demonstrated. Alfatafta *et al.* (1994) has demonstrated activity against a filamentous fungus, but not yeast, before.

Apiospora setosa produces antifungal activity against *C. albicans* and *S. cerevisiae,* as well as displaying good activity against *A. niger.*

Antifungal activity by solvent extracts of *Arthrinium* sp means that the compounds can be recovered by solvent extraction. This allows for the possibility of purification of the active compounds and their eventual identification.

An as yet unidentified fungus, culture UNK, displays excellent activity against yeast and filamentous fungi.

Primary screening a group of samples allows a large number of replicates to be screened and antifungal activity detected. Primary screening also eliminates inactive or undesired samples from the group in the due course of screening.

For this group of cultures, optimum temperature conditions were required for antifungal production to occur. Antifungal activity was the most pronounced when the fastest growth of colonies occurred. This is in contrast with the opinions of others, for example Larrondo *et al.* (1995).

5.3. Suggested future work

Culture UNK should be pursued further and isolation and identification of the active compound(s) undertaken. It also produces interesting colony characteristics and remains unidentified taxonomically.

Bulk broth fermentation could be employed to overcome the small amount of recovered compounds from active cultures. Alternatively, techniques such as TLC and HPLC could be used to pursue the identity of the antifungal compounds.

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