University of Southern Queensland

Discovery and Evaluation of Novel Antimicrobial Agents against Nosocomial Pathogens

A Dissertation Submitted by

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Abstract

High risks and costs are associated with infections caused by antibiotic-resistant organisms. The continued development of antibiotic resistance in pathogenic organisms means that in order to maintain our current standards in health care and disease treatment, new antibiotics need to be sourced. Such was the ultimate goal of this project.

Endophytic fungi, due to their broad diversity, unique habitats and ease of acquisition, have been shown to be an important source of bioactive natural products, including novel antibiotics. Thus, the effort of this project was concentrated on the isolation of endophytic fungi associated predominantly with native plants, and the evaluation of the secondary metabolites they produced.

Tissues were harvested from 61 individual plant hosts representing 15 plant species. Tissue segments were prepared and incubated on two different media formulations. As fungal growths became apparent, they were subcultured in order to obtain pure fungal isolates. A total of 133 fungal cultures were progressively isolated, covering over 100 fungal species.

Ultimately, 77 of these isolates were screened for antimicrobial activity against a panel of 7 bacterial strains. Three isolates subsequently demonstrated repeatable antimicrobial action. They were identified as *Schizophyllum commune*, a species of *Penicillium* and *Sporormiella isomera*, and their bioactivity further evaluated.

During the course of the project, activity originally detected in the *S. isomera* became indiscernable. The *Penicillium* species demonstrated some activity, being able to inhibit the growth of *Staphylococcus aureus* at a dilution of 1:2. It was also found to be mildly toxic to murine fibroblast L929 cells at a dilution factor of between 1:16 and 1:32, depending on the cytotoxicity assay used. Of the three isolates *S. commune* possessed the highest level of activity. A 1:256 dilution inhibited the growth of staphylococci, including an MRSA strain. A 1:32 dilution was bactericidal against *E. coli*. However, while it demonstrated useful antimicrobial activity, its toxicity would prevent its immediate development as such – a dilution factor of 1:1024, the highest dilution tested in the cytotoxicity assay, was capable of killing over 50% of L929 cells.

Further analysis of this isolate's products may reveal multiple products, with varying levels of activity and toxicity, novel modes of action, or novel structures. Separation of the active products could allow for further investigations, and if combinatorial chemistry is utilised, their structures may be manipulated in order to reduce the levels of

toxicity.

As long as bacteria continue to develop resistance to the antibiotics we use to treat their infections, we will need to source new antibiotics. The continued isolation, screening and evaluation of endophytic fungi and their secondary metabolites can only be of benefit to all higher life forms on Earth.

Certification of Dissertation

I certify that the ideas, experimental work, results, and conclusions reported in this thesis are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, at this or any other institution.

Candidate, M. Boddington — Date

ENDORSEMENT

Supervisor, Dr J. Dearnaley — Date

Supervisor, Dr M. Kotiw — Date

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Publications

Publications arising from this research:

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Chapter 1

Introduction

1.1 Antibiotics

In 1929, the bacteriologist Alexander Fleming reported the observation of inhibition of *Staphylococcus aureus* growth by a substance produced by a contaminating mould, identified at the time as *Penicillium rubrum*. Experiments conducted by Fleming indicated the fungus produced a compound that was bactericidal to certain bacteria, whilst also appearing to be non-toxic in animals. While he could see some application for it as a treatment of infection in humans, Fleming suggested this compound, which he called penicillin, would be most useful in the clinical isolation of non-penicillin-sensitive microbes from penicillin-sensitive microbes when present in a mixed clinical culture such as in sputum samples and nasal swabs (Fleming 1929). Small experimental treatments were conducted on human cases using the crude penicillin broth – unsuccessfully by Fleming in 1929, and successfully by Dr C. G. Paine, one of Fleming's former university students. However, these trials were never officially documented (Wainwright & Swan 1986). Chain et al. (1940) suggested the product, produced by the mould now identified as *P. chrysogenum* (also previously known as *P. notatum*), could be used as a chemotherapeutic agent, especially in the potential treatment of gas gangrene. In experiments conducted by Chain et al. (1940), an almost 100% survival rate was achieved when mice infected with penicillin-susceptible organisms were treated with a programme of subcutaneous injections of a penicillin solution. All the infected control mice not treated with penicillin, died. Chain, Florey and Abraham conducted the first therapeutic trials of penicillin in humans during 1941 (Katz 2002).

In the United States, a penicillin solution was first used in 1942 on a patient close to death from a streptococcal blood infection. Within days of treatment with the penicillin, she had virtually fully recovered (Henry 2005, Grossman 2008). After becoming involved in World War II, the US government declared the production of penicillin a priority (Henry 2005). By the fifth month of 1943, a total of 400 million units of penicillin had been produced (Grossman 2008), and the US military commenced large scale use of the drug in North Africa. By 1944, 100 billion units were being produced monthly (Henry 2005), increasing to 650 billion units towards the end of 1945

(Grossman 2008).

Other antibiotic discoveries had also been made by the start of World War II. In 1936, details of the antibiotic properties of the dye Prontosil Rubrum (sulfamidochrysoidine), were reported. In 1932 it was used successfully to save the life of Franklin Roosevelt's son from a severe streptococcal infection (*S. haemolyticus*). As a group, sulphonamides became another antibiotic important in the prevention of infections in open wounds of American soldiers (Stork 2005). In 1944, the first aminoglycoside antibiotic, streptomycin, was discovered (Davis 1987).

The prognosis for patients was greatly enhanced by the discovery of antibiotics (Lowy 2003). For example, prior to their introduction, staphylococcal bacteraemia resulted in the death of 80% (Lowy 2003, Cruickshank & Ferguson 2008) to up to 90% of cases (Nimmo et al. 2007), as metastatic infections developed in most patients. The early use of antibiotics reduced these affects (Lowy 2003).

Until 1957, penicillin was solely produced in fermented culture (Henry 2005, Grossman 2008). Thereafter, a chemist named John C. Sheehan, developed a process to semi-synthesize penicillin. Semi-synthetic derivatives have since been developed which have improved various characteristics of the drug such as the ability to absorb penicillin orally (Henry 2005) or enhance resistance to the enzyme penicillinase, produced by some penicillin-resistant bacteria (Lowy 2003).

Antibiotics function in a number of ways. The penicillins are a member of the β-lactam group of antibiotics, all of which include a β-lactam ring in their structure. They are effective against a wide range of gram-positive and gram-negative bacteria, each antibacterial sub-group having a slightly different spectrum of activity. The β-lactams, which also includes cephems (cephalosporins), penems and monobactams (CLSI 2006), inhibit bacterial cell wall synthesis (Yocum et al. 1980, Yotsuji et al. 1988, CLSI 2006). However, some bacteria produce the enzyme β-lactamase which inhibits the antibiotic. Therefore, to be useful, either the antibiotic needs to be β-lactamase-resistant (Yotsuji et al. 1988), or administered simultaneously with a β-lactamase inhibitor such as clavulanic acid (CLSI 2006). For penicillin to reach its full bactericidal potential, the sensitive-bacteria must be actively multiplying (Lee et al. 1944).

While the sulphonamide group of antibiotics were popular until the 1950's (Stork 2005), today they are currently prescribed predominantly for the treatment of urinary tract infections (CLSI 2006). They are generally bacteriostatic, inhibiting the further growth of bacteria rather than killing the organism (Henry 1944). They act by preventing the formation of folic acid in the bacteria (Alovero et al. 1998, CLSI 2006), and are often used in combination with trimethoprim, which inhibits a second step in the folate pathway (CLSI 2006).

Aminoglycosides bind to the bactrial ribosome, blocking protein synthesis (Davis 1987, CLSI 2006), as do the macrolides, chloramphenicol, clindamycin (Schlünzen et al. 2001, CLSI 2006) and several other single-drug classes (CLSI 2006). Each group performs this task in a unique manner (Schlünzen et al. 2001), and has a differing spectrum of activity (CLSI 2006).

Unfortunately, increasing antibiotic usage has not always had a positive outcome. Super-infections (secondary infections) following antibiotic treatment are common, especially with the use of broad spectrum antibiotics. These antibiotics may not only affect a pathogenic organism. They can also have an adverse effect on the normal flora of the body, and so allow subsequent infection by opportunistic, resistant organisms. In the early years of antibiotic use and resistance, these tended to be organisms that had not been previously encountered (Rantz 1954), undoubtedly creating delays and difficulties in identification and treatment.

However, perhaps the greatest problem has been the development of antibiotic resistance in previously sensitive organisms, a continued predicament foreseen by Fleming in a statement he made in 1945 (Henry 2005).

1.1.1 Antibiotic resistance

The development of antibiotic resistance in *Staphylococcus aureus* is very well documented, and serves as an excellent example of this phenomenon.

Staphylococci resistant to penicillin were actually encountered in hospitals as early as 1942 (Lowy 2003), with the frequency of resistant clinical isolates increasing to 50 to 90% in the mid-fifties (Rantz 1954) to 80% of clinical and community isolates by the late sixties (Lowy 2003). In 1960-61, methicillin was introduced as a new antibiotic to treat penicillin-resistant *S. aureus*. Methicillin-resistant isolates were detected within the year (Lowy 2003, Cruickshank & Ferguson 2008).

Australian isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were detected in the late sixties (Nimmo et al. 2007, Cruickshank & Ferguson 2008), but were usually treatable with non-β-lactams or gentamicin. By 1976, strains resistant to these additional antibiotics were detected, becoming endemic in hospitals in the Eastern states around the end of the 1980's (Nimmo et al. 2007).

Vancomycin subsequently became the treatment of choice for serious cases of MRSA infection (Nimmo et al. 2007). Reports of reduced susceptibility to vancomycin in MRSA followed in 1997 (Lowy 2003, Cosgrove et al. 2004, CLSI 2006). Linezolid is one of the few antibiotics available today for the treatment of multiple-resistant MRSA (mMRSA), and cases of resistance have already been reported (Newman et al. 2003, Nimmo et al. 2007).

The most frequently encountered multiresistant organism today is MRSA (Cruickshank & Ferguson 2008). A study conducted by the Australian Group for Antimicrobial Resistance (AGAR) found that 31.9% of clinical *S. aureus* isolates obtained throughout Australia were MRSAs (Nimmo et al. 2007).

The development of antibiotic resistance has always followed a basic pattern – emergence and detection in hospitals, followed by dissemination into the community (Lowy 2003, Nimmo et al. 2006, 2007, Cruickshank & Ferguson 2008). Consequently, community-

acquired infections caused by antibiotic resistant organisms are on the rise (Nimmo et al. 2006, 2007).

Treatment options for infections caused by antibiotic resistant organisms can be problematic. Alternative chemotherapeutic agents can be more expensive (Cruickshank & Ferguson 2008) and, as suggested by the World Health Organization (WHO) more toxic than the original antibiotic (Anon. 2002*a*), or there may be none readily available (Lowy 2003, Cosgrove et al. 2004).

According to the WHO, development of resistance is a natural process based on evolutionary or selective pressure (Anon. 2002*a*). It starts when there is not a 100% success rate in the elimination of bacteria following an antibiotic treatment, and continues as the antibiotic-resistant bacteria replicate and transfer their resistance genes to the next generation. The process is swift due to the bacteria's propensity for rapid multiplication. However, bacteria also have the ability to transfer resistance genes into other related species through conjugation and on transduction (Anon. 2002*a*, Lowy 2003). Lowy (2003) mentions two incidents involving vancomycin-resistant *S. aureus* (VRSA), where the resistant gene was obtained via conjugal transfer from a vancomycin-resistant *Enterococcus faecalis* (VRE).

Human activity is considered by the WHO to play a major role in the development of antibiotic resistance (Anon. 2002*a*). In health-care settings, issues such as the incorrect use of antibiotics involving insufficient treatment time, dose or potency (Henry 2005); inappropriate use of antibiotics (Lowy 2003, Henry 2005), including the treatment of viral diseases (Henry 2005), incorrectly diagnosed disease or in absence of disease (Anon. 2002*a*); and the use of broad-spectrum antibiotics rather than narrow-spectrum (Rantz 1954, Cruickshank & Ferguson 2008) encourage the selection and survival of antibiotic resistant bacteria. Health-care associated environments are also ideal for the development and spread of antibiotic resistance, due to prolonged (Cosgrove et al. 2004, Nimmo et al. 2007) and widespread antibiotic use and a concentrated population of highly susceptible patients (Cruickshank & Ferguson 2008).

The use of antibiotics in food production has also been linked with the formation of antibiotic resistant organisms (Laich et al. 2002, Lowy 2003). In North America and Europe, antibiotics used for prophylaxis or growth enhancers in animals and poultry accounted for approximately half the tonnage of antimicrobial products (Anon. 2002*a*) They are frequently administered at sub-therapeutic levels, and the antibiotic resistant bacteria can be subsequently spread to humans through the food chain. Antibiotic resistance in the normal flora of the human colon can develop following the ingestion of improperly cured fermented foods containing antibiotic resistant bacteria – their resistance can be transferred to the residing gut bacteria through the process of conjugation (Laich et al. 2002).

In addition to these direct causes, the WHO suggested the spread of antibiotic resistant organisms is enhanced due to urbanisation (Anon. 2002*a*) and international trade and travel (Anon. 2002*a*, Lowy 2003).

1.2 Nosocomial pathogens

Nosocomial pathogens are defined by the Australian Infection Control Association (AICA) as the disease causing bacteria, fungi and viruses found specifically in hospitals, and can cause infections in patients subsequent to admission (Anon. 2005*a*). In terms of infection, the term nosocomial is gradually being replaced with health-care associated, so as to include any infections obtained in an environment in which health care is received, such as aged-care facilities. It also includes infections which do not appear until after discharge, but were incurred as a result of the health care treatment previously received (Anon. 2003). Health-care associated infections are the most frequently encountered complication affecting patients in hospital (Cruickshank & Ferguson 2008).

In 2003, it was roughly estimated that up to 150 000 healthcare associated infections occurred in Australia (Anon. 2003, Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003), a figure which increased to an estimated 200 000 occurrences in 2008 (Cruickshank & Ferguson 2008). The greatest risk of infection occurs in intensive care units, where up to 30% of nosocomial infections develop (Cruickshank & Ferguson 2008).

Surgical sites and the bloodstream are the most frequently affected sites of infection (Cruickshank & Ferguson 2008). According to AICA, up to 13% of surgical patients contract an infection at the surgical site (Anon. 2003). Of all bloodstream infections (bacteraemia), it has been estimated that approximately half are associated with nosocomial infections (Nimmo et al. 2007, Cruickshank & Ferguson 2008), while another third are associated with healthcare procedures (Cruickshank & Ferguson 2008).

Every day spent as a patient in a hospital environment increases the risk of acquiring a nosocomial infection by 6% (Cruickshank & Ferguson 2008).

In Australia, *Clostridium difficile* (Cruickshank & Ferguson 2008) and *Staphylococcus aureus* are the most frequently encountered causes of nosocomial infections (Lowy 2003, Nimmo et al. 2007, Cruickshank & Ferguson 2008). *S. aureus* is the most prevalent cause of infection following coronary artery bypass graft surgery and knee and hip arthroplasty, and is responsible for from 20% (Klevens et al. 2007) to up to 33% of cases of bacteraemia (Cruickshank & Ferguson 2008). In 1989 in the United States, 27% of bacteraemia cases were caused by coagulase-negative *Staphylococcus* species (John & Harvin 2007).

However of greater concern, is the presence of antibiotic resistant organisms. In the United States in 2003, over half of the *S. aureus* bacteraemia cases incurred in the hospital were attributed to methicillin resistant strains (MRSA) (Klevens et al. 2007). Another American study conducted in a single hospital found 31% of cases were caused by multiresistant MRSA (Cruickshank & Ferguson 2008). In the majority of Australian hospitals, both antibiotic-sensitive and methicillin resistant Staphylococci are endemic, with the antibiotic-sensitive strain being more prevalent (Cruickshank & Ferguson 2008). MRSA has been cited as being responsible for 40% of health-care

associated bacteraemia (Nimmo et al. 2007).

Other antibiotic resistant bacterial strains encountered in hospitals include vancomycin resistant enterococci (VRE), multi-resistant Gram negative bacteria (MRGN) (Cruickshank & Ferguson 2008), multiple resistant Acinetobacter, extended-spectrum β-lactamase (ESBL) *Klebsiella pneumoniae* (Anon. 2003, 2005*b*), and reduced vancomycin susceptibility *S. aureus* strains (vancomycin-intermediate *S. aureus* – VISA) (Anon. 2005*c*). While not of current concern in developed countries, multiresistant *Mycobacterium tuberculosis* is an increasing world-wide problem (Cruickshank & Ferguson 2008).

These organisms are spread via contact transmission – via contaminated equipment, from patient to patient, or particularly, on the hands of health care workers (Cosgrove et al. 2004). Many healthcare workers are colonised with antibiotic resistant organisms (Rantz 1954, Cruickshank & Ferguson 2008). While the spread of antibiotic-resistant bacteria into the community, particularly MRSA, is of concern, it currently appears the capacity for person-to-person transmission of these strains within the community is lower than that in health care settings (Nimmo et al. 2006).

While antibiotics can be administered to infected individuals, they may occasionally be of limited value, especially where antibiotic-resistant organisms are involved. However, even with the availability of suitable antibiotics, deaths may still occur (Lowy 2003). New antimicrobials are required, and are being developed (Lowy 2003, John & Harvin 2007), but their use must be managed and reduced in order to slow the development of resistance to the new drugs (Cruickshank & Ferguson 2008).

However, the AICA states that many healthcare associated infections may be prevented by the implementation of infection control programmes (Anon. 2003). Hand washing (Anon. 2005*c*, Cruickshank & Ferguson 2008), and, as suggested by the WHO, using fresh gloves for each patient (Anon. 2002*a*) are two important measures. In the case of MRSA, screening, followed by appropriate management of those patients found to be colonised or infected, is essential (Anon. 2005*c*) though this should be followed in the case of any antibiotic-resistant organism. In 2007, an Australian newspaper reported that a major hospital in Brisbane, Australia, closed 2 medical wards following the positive screening in 21 patients for vancomycin resistant enterococci (VRE). While most of the patients were asymptomatic, the measures were taken to prevent its spread to other areas of the hospital (Anon. 2007).

Surveillance also plays an important role in the control and prevention of healthcare associated infections, (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003, Cruickshank & Ferguson 2008). Information gathered can be used to improve infection control (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003), improve procedures, and improve the response to these issues (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003, Cruickshank & Ferguson 2008).

Currently in Australia, there is no national surveillance system, leading to unreliable, limited information, which ultimately prevents a coordinated response to the problems caused by healthcare associated infections and antimicrobial resistance (Cruickshank

& Ferguson 2008).

Apart from the initial problems created by the infection, healthcare associated infections have a substantial impact in different ways – longer hospital stays, increased pain and suffering, and additional risk of further complications potentially leading to death are faced by infected individuals (Cruickshank & Ferguson 2008); increased workloads and stretched resources in hospitals (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003); increased risk of exposure of antibiotic-resistant organisms to the community (Lowy 2003, Klevens et al. 2007, Cruickshank & Ferguson 2008); and increased financial costs for all involved (Klevens et al. 2007, Nimmo et al. 2007, Cruickshank & Ferguson 2008).

Endocarditis, osteomyelitis and septic arthritis are some of the additional complications incurred by those who develop Staphylococcal bacteraemia (Cruickshank & Ferguson 2008), and 20 to 40% of these people will die (Lowy 2003, Nimmo et al. 2007). When all causative agents of bacteraemia are considered, 17 to 29% of cases will result in death. When infections of surgical sites are also included, this figure increases to 33% (Cruickshank & Ferguson 2008). In 2003, healthcare associated infections were implicated in the deaths of up to 4.6% of those afflicted – approximately 7000 deaths annually (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003).

In 1984, nosocomial infections in Australia resulted in costs of around $180 million. According to AICA, by 2001, these costs had risen substantially, with annual costs of surgical site infections alone up to $268 million (Anon. 2003). In 2007, it was estimated that Staphylococcal bacteraemia costs around $150 million (Nimmo et al. 2007).

Generally, higher risks and costs are associated with infections caused by antibiotic-resistant organisms (Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) 2003, Klevens et al. 2007, Nimmo et al. 2007), particularly those caused by MRSA (Cruickshank & Ferguson 2008). Even in the prevention of infection, such as that mentioned previously in the Brisbane hospital, extra costs were incurred as additional nursing staff undertook the labour intensive cleaning and disinfection of the two affected wards (Anon. 2007).

It is expected that due to Australia's aging population, levels of nosocomial infections will increase – the elderly face reduced immunity levels, higher exposure to antibiotics, more frequent stays in hospital and close contact within aged-care facilities increasing the risk of developing infections (Cruickshank & Ferguson 2008).

1.3 The search for novel antibiotics

Antibiotic discovery primarily relies on the mass screening of suitable products. Initially, the bulk of these products were obtained from natural sources such as fungi, bacteria and plants. The importance of natural-product screening has abated due in

part to difficulties for pharmaceutical companies to secure intellectual property rights (Anon. 2002*b*) and the effort and expense incurred in finding and developing products (Strobel & Daisy 2003). In addition, combinatorial chemistry (Strobel & Daisy 2003) and enhanced target-based biochemical screening processes (Lowy 2003) became the preferred method for developing new antibiotics, as this largely automated process increased the numbers of products which could be synthesised and screened (Newman et al. 2003, Strobel & Daisy 2003, Koehn & Carter 2005).

However, due to the natural diversity in chemical structure (Schulz et al. 2002, Strobel & Daisy 2003, Koehn & Carter 2005) and wide biological spectrum (Anon. 2002*b*), natural products have proved more successful in providing novel substances than combinatorial chemistry (Schulz et al. 2002, Newman et al. 2003, Strobel & Daisy 2003, Newman & Cragg 2007). However, when natural products are used to provide the lead structure, and combinatorial chemistry is used to manipulate and enhance that structure, the results are further improved (Katz 2002, Newman et al. 2003, Strobel & Daisy 2003, Koehn & Carter 2005, Newman & Cragg 2007). The manipulation of penicillin's structure to improve its resistance to various enzymes (Katz 2002, Lowy 2003), its stability (Katz 2002) and its oral bioavailability (Henry 2005) provides a suitable example.

Over an 11 year period, over 62% of the antimicrobial products submitted to the American Food and Drug Association (FDA) for analysis were associated with natural products. More than 77% of these were semi-synthetic forms (Suffredini et al. 2004). A review conducted by Newman & Cragg (2007) revealed that 76.5% of new and approved antibacterials from the time spanning January 1981 to June 2006 were based on natural products, with over 65% of these being semi-synthetic. In 1991, drugs based on natural products constituted almost half of the most popular drugs sold (Cragg et al. 1997).

For approximately 40 years since the identification of penicillin, the discovery of new antimicrobials flourished. The WHO suggests a misplaced belief that treatment of pathogens no longer posed a problem was partially to blame for the decline in further research and development of new antibiotics (Anon. 2002*a*). Costs, differences between *in vitro* and *in vivo* behaviours of antibiotics, and a failure to rapidly develop new efficient products have reduced corporate involvement in the search for novel antibiotics. In 2002, it was suggested by the World Health Organisation that within 10 years there would be no effective treatment available for some infectious diseases (Anon. 2002*a*). Katz (2002) lamented the lack of significant antibiotic discovery in the preceding 20 years. This is compounded by the fact that it can take approximately 12 years and significant costs to develop a new discovery into a clinically available product (Schulz et al. 2002). To maintain the fight against bacterial infection, novel antibiotics need to be sourced as a matter of urgency.

1.3.1 Fungi as a potential source of antibiotics

Fungi have been shown to be one source of a variety of useful natural products (Davis et al. 2008). Important discoveries other than antibiotics have included cyclosporin, an immunosuppressive drug used to prevent rejection of transplanted organs, produced by *Tolypocladium inflatum*; citric, gluconic and itaconic acids from several *Aspergillus* species and alcohol produced by *Saccharomyces cerevisiae* (Hyde 1996).

The anticancer agent paclitaxel (trademarked as Taxol) provides an interesting case. Initially thought to be produced solely in the bark of *Taxus brevifolia* Nutt (Pacific yew tree), it was later found to also be produced by the fungus *Taxomyces andreanae*, isolated from the inner bark of the same tree species. However, it provided an easier, less time-consuming and less costly source of this important drug than from harvesting the bark, or from creating it through artificial means (Katz 2002, Strobel & Daisy 2003).

Since the discovery of penicillin, fungi are possibly best known as a potential source of novel antibiotics. Other fungal-based antibiotic discoveries include Cephalosporin produced by *Cephalosporium* sp., Griseofulvin from *Penicillium griseofulvin* (Hyde 1996) and Usnic acid, produced by a lichen, and used topically in the treatment of human skin diseases (Katz 2002).

By 2002, of the 20 medicines most commonly prescribed, six originated from fungal sources (Schulz et al. 2002).

It has been estimated that between 74 000 (Rodrigues et al. 2000, Hawksworth 2001, Suryanarayanan et al. 2003), to 75 000 (Deacon 2006) to 100 000 (Strobel & Daisy 2003) fungal species have been described, yet this may only account for around 5% of all fungal species in existence (Hyde 1996, Schulz et al. 2002, Deacon 2006). It is conservatively estimated that the total number of species could number as high as 1 million (Strobel & Daisy 2003), or even higher (Rodrigues et al. 2000, Hawksworth 2001, Suryanarayanan et al. 2003). It is difficult to determine a more accurate figure due to differences in sampling methods (Paulus et al. 2006).

Plants provide an easily identifiable habitat for fungi, so a ratio of plant host to fungal species is frequently included in the calculations of total fungal species (Hawksworth 2001). However, the suggested ratios vary from 1:6, which was used for the initial estimate (Hawksworth 2001) to 1:10 used to determine that Australia is estimated to contain 250 000 fungal species (Hyde 1996). Yet work conducted by Hyde (1996) demonstrated a much higher ratio of palm hosts to fungal species than the latter figure. Nonetheless, basing the calculation of fungal species on an estimated 300 000 plant species (Strobel & Daisy 2003) and using the ratio of 1:10 results in an estimate of some 3 million fungal species.

Fungi exist in a diverse range of habitats, including, but not limited to, soil (Schulz et al. 2002), freshwater and marine environments (Hyde 1996, McKenzie 2001), insects (McKenzie 2001, Paulus et al. 2006) and in plants (Arnold et al. 2001, Strobel & Daisy 2003, Arnold & Lutzoni 2007).

Their previous successes in yielding useful natural products, their extensive habitat range and number of species yet to be discovered, imply that fungi will continue to be a promising source of novel antibiotics.

1.3.2 Endophytic Fungi

Endophytic fungi are fungi which for at least a portion of the life cycle, reside within the healthy tissues of plants without causing the plant to display any symptoms or signs of infection (Bills 1996, Petrini 1996, Johnston 1998, Peláez et al. 1998, Fröhlich et al. 2000, Dingle & McGee 2003, Strobel & Daisy 2003, Suryanarayanan et al. 2003, Arnold & Lutzoni 2007). While not all plant species have been surveyed, it is expected that all plant species harbour at least one endophytic fungus (Strobel & Daisy 2003), including the algae and mosses (Arnold et al. 2001). Therefore, a substantial volume of fungal biodiversity can be attributed to the endophytic fungi (Arnold et al. 2001, Suryanarayanan et al. 2003, Arnold & Lutzoni 2007).

It has been suggested the relationship between plant host and fungal endophyte may be symbiotic (Petrini 1996, Dingle & McGee 2003, Strobel & Daisy 2003), mutualistic (Schulz et al. 1995, Petrini 1996, Johnston 1998, Strobel & Daisy 2003, Arnold & Lutzoni 2007) or saprophytic (Bills 1996, Strobel & Daisy 2003). It may also include opportunistic pathogens (Johnston 1998, Strobel & Daisy 2003) involved in a fine balance between fungal virulence and plant defence (Schulz et al. 2002) or well known plant pathogens capable of demonstrating an asymptomatic relationship in a non-susceptible plant species (Schulz et al. 1995, Arnold & Lutzoni 2007). Endophytic fungi can enter a plant host by transfer between individual plants via horizontal transmission, such as airborne delivery or via the seed and so to following generations (vertical transmission) (Bayman et al. 1998, Arnold & Lutzoni 2007). The latter method in particular could enhance the co-evolution of endophytic fungi with its host plant (Bayman et al. 1998).

The role of endophytic fungi is not yet fully understood (Strobel & Daisy 2003). It has been suggested the fungi can assist in the protection of the host from insect predators, disease (Johnston 1998, Schulz et al. 2002, Dingle & McGee 2003, Arnold & Lutzoni 2007) or improve plant growth (Schulz et al. 2002, Dingle & McGee 2003). Studies reported by Arnold & Lutzoni (2007) indicated improved tolerance to drought, heavy metals and salinity in several hundred grass species could be directly attributed to the endophytes present. However, they reported other studies which showed a decrease in photosynthetic efficiency in maize and bananas, and decreased resistance to severe drought.

Due to their broad diversity (Peláez et al. 1998, Strobel & Daisy 2003), unique and varying growth environments (Strobel & Daisy 2003) and relatively easy acquisition (Peláez et al. 1998), endophytic fungi are considered as an important source in the discovery of bioactive natural products (Peláez et al. 1998, Rodrigues et al. 2000, Schulz et al. 2002, Strobel & Daisy 2003), including novel antibiotics. In fact, research conducted by Schulz et al. (2002) revealed fungal endophytes isolated from plants demon-

strated a greater number of antimicrobial inhibitions than pathogenic, epiphytic, algal or soil isolates.

1.4 Project outline

The purpose of this project was the discovery and evaluation of novel antimicrobial agents affective against nosocomial pathogens, and entailed four distinct areas of investigation:

1.4.1 Acquisition of Fungal Isolates

The goal of the first phase of the project was the acquisition of a sufficient number and variety of fungal isolates. While fungi are ubiquitous, it was decided to concentrate efforts on the collection of endophytic fungi from a variety of mostly native plant hosts. Harvested plant tissues were washed, surface sterilised and portions transferred to suitable growth media. As isolates grew, they were subcultured until pure cultures were obtained, cultured onto slants for short term use, and longer term storage preparations made.

1.4.2 Screening for Antimicrobial Activity

The second stage of the project involved the screening of the fungal isolates collected during the first phase, against a panel of selected nosocomial pathogens. Two screening methods were initially trialled, testing the response of *Staphylococcus aureus* and *Escherichia coli* to substances produced in culture by the fungus *Penicillium chrysogenum*. As it was known the substances produced would potentially include penicillin, the two test microbes offered a sensitive and resistant response to the antibiotic. After a suitable screening method was determined, the fungal isolates were progressively subcultured from the slants, and if successfully grown, screened for antimicrobial activity against a broader range of microbes.

1.4.3 Identification of Selected isolates

Fungi which demonstrated antimicrobial activity against one or more of the test bacteria were selected for further study. This firstly required the determination of their scientific classification, so as not to re-evaluate a fungus which had been previously described and assessed. Morphological characteristics, both macroscopic and microscopic, were initially studied, and were sufficient to determine the genus of one of the isolates obtained. Molecular techniques were required to obtain identification of the remaining isolates.

1.4.4 Evaluation of Activity

The final part of the project involved the evaluation of the antimicrobial activity and the assessment of potential toxicity of crude preparations from the selected isolates. The Minimum Inhibitory Concentration (MIC) was determined for the fungal preparations against several of the bacteria used for the initial screening. Once these results were obtained, the Minimum Bactericidal Concentration (MBC) could be determined. Toxic effects of the crude preparations were tested on murine fibroblasts and evaluated using the MTT Cell Viability and Crystal Violet Dye Exclusion (CVDE) assays.

The ultimate goal of this project was the discovery of novel antibiotics. Results from the final part of the project will determine the future direction of any discoveries.

Chapter 2

Acquisition of Fungal Isolates

2.1 Introduction

The goal of the first phase of the project was the acquisition of a sufficient number and variety of fungal isolates for screening, and as such, was considered the largest and most important part of the project. Without a large number of isolates to screen for antimicrobial activity, the chances of finding an isolate with antimicrobial activity would be limited. While fungi are ubiquitous, it was decided to concentrate efforts on the collection of endophytic fungi from a variety of mostly native plant hosts.

Plant samples from two sites were harvested, washed, surface sterilised and sectioned. The sectioned tissues were transferred to one of two types of media and incubated. As hyphae appeared around the edges of the tissue, a portion of hyphae and agar was transferred to a fresh plate, incubated and subcultured until pure growth was obtained. Fungal isolates were placed into storage until required for phase two of the project, the screening for antimicrobial activity.

2.1.1 Strategy

In order to improve the chance of discovering novel antibiotics, novel fungal endophytes need to be studied (Gloer 1997, Schulz et al. 2002, Strobel & Daisy 2003). Therefore, some thought needs to be applied to host selection (Peláez et al. 1998, Strobel & Daisy 2003). In particular, plants restricted to certain areas (Peláez et al. 1998, Strobel & Daisy 2003), surviving in unique environments, or possessing unusual biology or history, are expected to provide an important source of novel fungal endophytes (Strobel & Daisy 2003).

Host source

The greatest diversity in land-dwelling biological species can be found in the worlds tropical and temperate rainforests (Strobel & Daisy 2003). One leaf alone, from a lowland, tropical rainforest played host to 17 endophytic fungi (Arnold & Lutzoni 2007). A high species diversity is associated with the palms of tropical and temperate rainforests (Fröhlich et al. 2000). Studies have shown that forests of both regions have similar rates of host specificity (Arnold & Lutzoni 2007).

However, studies have generally shown that tropical rainforests contain a greater fungal diversity (Hawksworth 2001, Suryanarayanan et al. 2003, Arnold & Lutzoni 2007) and higher incidence of endophytic colonisation (Arnold et al. 2001) than the rainforests of temperate regions. Research cited by Hawksworth (2001) found this was particularly so in the large forests, mountain tops and humid island forests of the tropics. Research conducted by Arnold & Lutzoni (2007) found as the latitude increased, the rate of endophytic fungal colonisation and diversity decreased – almost all leaf sections obtained from a tropical study site bore endophytic isolates, whereas only 1% of arctic leaf sections were productive.

Due to their contribution to fungal diversity, studies into the fungal endophytic assemblages of tropical plants were included in the prediction of total world fungal species (Suryanarayanan et al. 2003).

Regardless of the geographical zone, different forests have demonstrated unique endophytic assemblages (Arnold & Lutzoni 2007), and a great number of fungi are yet to be described (Hyde 1996, Hawksworth 2001, McKenzie 2001, Arnold & Lutzoni 2007). Due to limited resources, increased competition, and increased selection pressure, rainforests are expected to be an important source of diverse, novel fungal endophytes able to produce a range of novel antimicrobially active compounds (Strobel & Daisy 2003). It is expected tropical rainforests in particular, host many of these currently unknown endophytes (Hawksworth 2001, Strobel & Daisy 2003, Arnold & Lutzoni 2007), but unfortunately, the rapid diminishment of the world's rainforests means there will be reduced opportunity to discover them, and the natural products which they can produce (Strobel & Daisy 2003).

Sample selection

Endophytic fungi appear to fall into two populations: one group appearing in many host species (Fröhlich et al. 2000, Suryanarayanan et al. 2003, Strobel & Daisy 2003), the other displaying some degree of host specificity (Bills 1996, Petrini 1996, Peláez et al. 1998, Fröhlich et al. 2000, Arnold et al. 2001, Suryanarayanan et al. 2003, Paulus et al. 2006). Fröhlich et al. (2000) found over half of the fungal endophytes isolated from three Australian palm species came from a single palm species, while only 23% were common to all three palms. A similar pattern was encountered in Brunei, with 46% of isolates demonstrating a preference for one host species and 32% being common to all three palm species studied.

Even species in close proximity can play host to differing endophytic communities. Results related to Petrini (1996) showed out of 70 isolates obtained from both mistletoes and the fir trees on which they grew, less than ten were common to both host species.

In addition to host specificity, there appear to be elements of tissue specificity amongst the endophytic fungi (Petrini 1996, Johnston 1998, Fröhlich et al. 2000, Paulus et al. 2006). Studies on the manuka (*Leptospermum scoparium*) (Johnston 1998) and palms (Fröhlich et al. 2000), showed both hosts were found to have variations in the endophytic assemblages between different leaf tissue types. The petiole in particular has been associated with unique species (Petrini 1996) and greater numbers of isolates (Fröhlich et al. 2000) when compared with other areas of the leaf.

Differences in endophyte assemblages can also occur between identical host species in different environments (Bayman et al. 1998, Johnston 1998, Taylor et al. 1999). In their study on the endophytes of the manuka, Johnston (1998) found major differences between manuka stands established from seedlings raised in a nursery compared with naturally established stands. When the endophytes of the palm *Trachycarpus fortunei* were studied by Taylor et al. (1999), they found similar numbers of taxa, but different species and a poorer population of endophytic fungi in the palm outside its natural range when compared with those obtained from the host in its natural habitat.

Tissue age can also have an impact on endophytic sampling (Taylor et al. 1999), with older leaves generally having a higher population of endophytes than younger leaves (Bills 1996, Johnston 1998, Fröhlich et al. 2000, Arnold et al. 2001, Arnold & Lutzoni 2007). While Fröhlich et al. (2000) found few differences between the taxa encountered in younger compared with older palm fronds, isolation rates were higher in the older tissues.

Other factors can have an influence on the distribution of endophytes, including resource competition (Paulus et al. 2006), host density (Johnston 1998, Arnold et al. 2001, Suryanarayanan et al. 2003), host species (Fröhlich et al. 2000), air quality (Johnston 1998) climactic conditions (Johnston 1998, Arnold et al. 2001, Paulus et al. 2006) and season (Taylor et al. 1999, Arnold et al. 2001, Paulus et al. 2006).

2.1.2 Isolation Techniques

A variety of techniques have been employed in the isolation of endophytic fungi. A summary of some of those techniques is described below.

In all cases examined, an excess of material was collected for processing, with the harvested plant tissues sampled for endophytes within 4 hours (Arnold et al. 2001) to 96 hours (Taylor et al. 1999, Arnold & Lutzoni 2007) after collection. By comparing leaves from the same site plated on the day with those plated 24 hours after collection, Johnston (1998) determined while the numbers of isolates did not fluctuate, the species isolated were affected. In particular, the isolation frequency of the dominant

endophytic species decreased with the delay, while other species were isolated more frequently.

Whole leaves were generally washed thoroughly in running tap water prior to surface sterilisation (Bayman et al. 1998, Arnold et al. 2001, Dingle & McGee 2003, Suryanarayanan et al. 2003, Paulus et al. 2006, Arnold & Lutzoni 2007). For leaves containing latex, Bayman et al. (1998) included approximately 0.05% Tween 80 in the water.

Surface sterilisation of plant tissue appears to be predominantly by the sequential immersion of the tissue in ethanol followed by sodium hypochlorite followed by ethanol. Various concentrations and times were used, and these are summarised in Table 2.1. Other studies have utilised a single treatment in either 5% sodium hypochlorite (Paulus et al. 2006) or 70% ethanol (Strobel & Daisy 2003). Where ethanol was used as the sole agent, it was either allowed to naturally evaporate or the material and ethanol were flamed (Strobel & Daisy 2003).

Table 2.1: Summary of timings of chemical surface sterilisation of plant tissue.

Reference	Ethanol		Sodium hypochlorite		Ethanol	
	Conc.	Time	Conc.	Time	Conc.	Time
Bayman et al. (1998)	75%	1 min	3.4%	10 min	75%	30 sec
	-	-	0.5%	20 min	-	Note[1, 2]
Johnston (1998)	95%	1 min	29.4%	3 min	95%	30 sec[2]
Taylor et al. (1999)	95%	1min	3.25%	10 min	95%	30 sec
Fröhlich et al. (2000)	96%	1 min	3.25%	10 min	96%	30 sec
Guo et al. (2000)	70%	1 min	3.25%	10 min	75%	30 sec[3]
Arnold et al. (2001)	-	-	0.525%	2 min	70%	2 min[2, 3]
Dingle & McGee (2003)	96%	1 min	4.2%	25 min	96%	30 sec
Suryanarayanan et al. (2003)	70%	5 sec	4%	90 sec	-	Note[2]
Arnold & Lutzoni (2007)	95%	10 sec	10%[4]	2 min	70%	2 min[3]

[1] For leaves with a latex content.
[2] Tissues were finally rinsed in sterile water
[3] Tissue was either dried or allowed to dry before continuing
[4] Percentage of chlorine bleach provided

Flat leaves were mostly cut into segments no bigger than $0.5cm^2$ (Arnold et al. 2001, Dingle & McGee 2003, Suryanarayanan et al. 2003, Arnold & Lutzoni 2007), or punched with a hole punch to produce disks (Peláez et al. 1998, Taylor et al. 1999, Guo et al. 2000, Fröhlich et al. 2000). A particulate filtration process was followed by

one research group, where leaf particles measuring between 105 to 210 μm were obtained and plated (Paulus et al. 2003, 2006). Strobel & Daisy (2003) carefully excised and cultured the inner tissues of the leaf, while whole leaves were plated by Johnston (1998). Needle-like leaves (Bayman et al. 1998), and twigs or stems were sliced into small segments (Peláez et al. 1998, Fröhlich et al. 2000).

Over half of the studies examined stated the plant material was sectioned prior to surface sterilisation (Peláez et al. 1998, Taylor et al. 1999, Fröhlich et al. 2000, Guo et al. 2000, Arnold et al. 2001, Dingle & McGee 2003, Suryanarayanan et al. 2003, Arnold & Lutzoni 2007).

Malt Extract Agar (MEA) was the most frequently utilised medium for the initial culturing of endophytic fungi from the plant tissue (Johnston 1998, Taylor et al. 1999, Guo et al. 2000, Arnold et al. 2001, Arnold & Lutzoni 2007), followed by PDA (Fröhlich et al. 2000, Dingle & McGee 2003, Suryanarayanan et al. 2003). Bayman et al. (1998) used a half strength preparation of each medium, while Peláez et al. (1998) and Paulus et al. (2006) used MEA with added yeast extract. The latter also utilised Bandoni's medium, corn meal agar and dichloran rose bengal chloramphenicol agar. Water agar plates were used by Strobel & Daisy (2003). Most media were supplemented with one or more antibiotics to prevent bacterial contamination, and included streptomycin (Bayman et al. 1998, Peláez et al. 1998, Taylor et al. 1999, Fröhlich et al. 2000, Guo et al. 2000), chloramphenicol (Bayman et al. 1998, Suryanarayanan et al. 2003), terramycin (Peláez et al. 1998) and tetracycline (Dingle & McGee 2003). In addition to the antibiotics, 3/4 of the media used by Peláez et al. (1998) contained a fungicide – either benomyl, cyclosporin or cyclohexamide. Low levels of cyclosporin can restrict the radial growth of ascomyctes and prevent sporulation in mucoraceous fungi, without preventing the growth of any species; low levels of benomyl and cyclohexamide will inhibit some species, thus allowing selective growth of others (Bills 1996). Rose bengal was included to slow the growth of rapidily growing fungi in media utilised by Bayman et al. (1998), Taylor et al. (1999) and Guo et al. (2000).

Incubation temperatures ranged from a minimum of 18^{o}C (Johnston 1998) to 26^{o}C (Suryanarayanan et al. 2003), for 3 weeks (Johnston 1998, Arnold et al. 2001, Suryanarayanan et al. 2003, Paulus et al. 2006) to up to one year (Arnold & Lutzoni 2007).

Throughout the incubation period, plates were checked regularly for the appearance of fungal hyphae. Once detected, hyphae were generally transferred to media without supplements, usually PDA (Bayman et al. 1998, Johnston 1998, Peláez et al. 1998, Fröhlich et al. 2000, Dingle & McGee 2003, Strobel & Daisy 2003, Paulus et al. 2006) or MEA (Bayman et al. 1998, Guo et al. 2000, Paulus et al. 2003). To encourage sporulation, additional media were employed, including oatmeal agar (Johnston 1998), or agar incorporating a strip of autoclaved host-plant material (Taylor et al. 1999, Fröhlich et al. 2000, Guo et al. 2000, Paulus et al. 2003). Media used for the latter included MEA with (Taylor et al. 1999) or without antibiotics (Fröhlich et al. 2000), Potato Carrot Agar (Paulus et al. 2003) and PDA, corn meal agar and tap water agar (Guo et al. 2000).

2.2 Materials and Methods

2.2.1 Trialling Isolation Methods

Initially, a brief trial was conducted to determine the best methods for surface sterilisation and sectioning of the plant material. Due to a personal curiosity and extensive availability, the exotic bush *Lantana camara* was selected to provide the test material for the trial.

Prewash

A branch of lantana was cut from a healthy bush, and selected leaves washed by placing them into beakers of clean tap water and stirring. The water in each beaker was replaced twice. Washing lasted approximately 1.5 hours at room temperature. The washed leaves were then transferred to a beaker of sterile reverse osmosis (RO) water in the Biological Safety Cabinet (BSC), where they were held until treated. To determine the impact of the pre-wash step on the population of epiphytic micro-organisms, both surfaces of a washed and an unwashed leaf were gently pressed onto the surface of separate Sabouraud Dextrose Agar (SDA) plates. The leaves were discarded, and the plates incubated in the dark at 25^{o}C for seven days.

To determine the necessity of the pre-wash step, an unwashed leaf was flamed as described below, and a test plate prepared as described above.

Surface sterilisation

Individual pre-washed leaves were submitted to one of two surface sterilisation treatments – an ethanol-bleach-ethanol treatment as described by Dingle & McGee (2003) and flaming as suggested in Bills (1996). A pair of flame sterilised forceps were utilised for all stages of handling the leaves. Both treatments commenced by allowing excess water from the final rinse to drip back into the beaker, before the leaves were placed into a beaker containing 96% ethanol.

The ethanol-bleach-ethanol treatment continued after the leaf was submerged in the alcohol for one minute. It was subsequently transferred to a beaker containing bleach which had been diluted to a concentration providing two percent available chlorine. After 25 minutes, it was transferred to a second beaker containing 96% ethanol, where it remained for 30 seconds before being rinsed in sterile water and held until required.

For the flaming treatment, excess alcohol was allowed to drip back into the container and the leaf passed quickly through the blue flame of a bunsen burner to burn off the alcohol remaining.

Both surfaces of each treated leaf were gently pressed on to the surface of an SDA plate, and the leaf discarded. All plates were incubated in the dark at 25^{o}C for seven days.

Leaf sampling

Several leaves were surface sterilised using the ethanol-bleach-ethanol procedure as described above. Sections of each leaf were then taken using different methods:

Method 1 A sterile scalpel was used to cut a leaf into quarters, each of which was placed on one half of an agar plate

Method 2 A sterile scalpel was used to cut two small squares out of the center of one leaf, and each placed on one quarter of an agar plate.

Method 3 A single hole punch was sterilised by dipping into 95% ethanol, followed by passage through the flame of a bunsen burner to burn off the alcohol. Two holes were punched into the center of one leaf, and the punched-out disks placed on to the other two quarters of the preceding agar plate

Method 4 Two disks from one leaf were obtained using the hole punch. Each disk was placed in a sterile microcentrifuge tube containing 0.5ml sterile water. A sterilised pestle was used to break up the disk, and expose its contents to the water. The entire liquid content of one tube was spread onto a single agar plate. 0.1ml aliquots from the other tube were spread on multiple agar plates.

The leaves and sections were kept in sterile Petri dishes during sectioning. SDA plates which had been previously prepared as per the manufacturer's instructions were used for all tests. All plates were incubated at 25^{o}C for 14 days.

2.2.2 Isolate Collection

Formulations and recipes of all media can be found in Appendix B.

15g/1000ml solutions of streptomycin and tetracycline were prepared by dissolving 0.15g of antibiotic into separate vials containing 10ml RO Water. Both were filter sterilised through a 0.22μm syringe filter into several sterile microcentrifuge tubes. Streptomycin solutions were stored at 4^{o}C and the tetracycline at -20^{o}C.

Four-hundred millilitre volumes of PDA and MEA were prepared and autoclaved according to the manufacturer's instructions. Once cooled to approximately 55^{o}C, the molten agar without antibiotics was poured directly into twenty sterile 90mm Petri dishes and allowed to set. For media containing antibiotics, 400μl of the streptomycin and tetracycline solutions were added and gently mixed into the cooled media before

pouring into plates. All plates were labelled and stored upside down in plastic bags in the refrigerator, and used within one month.

Five-hundred millilitre volumes of RO Water in Schott Bottles were autoclaved on a Fluids cycle at 121^{o}C for fifteen minutes.

Blotting paper strips were placed into autoclavable paper bags and autoclaved on a Porous Cycle at 121^{o}C for fifteen minutes.

Pyrex beakers (250 – 500ml) were topped with foil and autoclaved on a Hard Goods cycle at 121^{o}C for fifteen minutes or autoclaved with either of the other items.

Plant Sample Collection

Two areas local to Toowoomba were selected for collection of plant specimens – a dry sclerophyll forest to the South of the city at Preston, and a dry rainforest at Redwood Park in the North-Eastern suburbs of Toowoomba. A permit was granted from the Queensland Parks and Wildlife Service for collection of plant samples from Redwood Park. Collections at Preston were on private land.

A minimum of three plant specimens of each species were located in the sample area, ensuring some distance was kept between each plant specimen (i.e. they were not next to each other, but were separated by other plants). Secateurs were used to remove a small (but excess) sample of the plant, which was placed immediately into its own plastic or paper bag and labelled. At the completion of collection, the samples were returned to the laboratory for the next stage of the process.

Samples were stored in cool areas or in a refrigerator if they were unable to be processed on the day of collection. A list of plants sampled from each location can be found in Table 2.2.

Plant Sample Sectioning

Initial washing of the plant material was performed in non-sterile conditions. The final wash, and all subsequent steps involved in sectioning were undertaken in the BSC and aseptic techniques followed.

Two to three leaves or smaller samples of twigs, stems and roots were placed into small plastic bags (one per plant sample) and filled with tap water. The bags were then sealed and shaken in order to clean the sample material. The water was replaced twice more, and the agitation repeated. The final prewash was performed in the BSC, where the plant material was removed from the bag and placed into a sterile beaker containing sterile RO water. From this point on, the plant material was handled as aseptically as possible. Having been rinsed in the sterile water, the plant material was finally removed to a sheet of sterile blotting paper and excess water gently removed. At this point of

Table 2.2: Species of plants sampled from each location.

LOCATION	*SCIENTIFIC NAME*	COMMON NAME
Preston	*Acacia fimbriata*	Brisbane Wattle
	Alphitonia excelsa	Red Ash
	Smilax australis	Smilax
	Xanthorrhoea johnsonii	Grass Tree
	Lantana camara	Lantana
Redwood Park	*Alyxia ruscifolia*	Chain fruit
	Aphananthe philippinensis	Rough-leaved Elm
	Austromyrtus bidwillii	Python Tree
	Bridelia leichardtii	Bush Ironwood
	Dendrobium rex	King Orchid
	Elattostachys xylocarpa	White Tamarind
	Hoya australis	Native Hoya
	Mallotus claoxylloides	Green Kamala
	Streblus brunonianus	Whalebone
Cultivated	*Wollemia nobilis*	Wollemi Pine

A small, but excess amount of material was removed from each plant specimen and bagged, before being transported to the laboratory for isolation of endophytic fungi.

the procedure, the first control plate was prepared.

A piece of the washed plant material from the first plant sample of each species was gently pressed to the surface of an antibiotic-free PDA plate, which was then set aside for incubation. The same piece of plant material was then sterilised (as described below), and a second antibiotic-free PDA plate inoculated as for the first control plate. This provided a sample of the epiphytic micro-organisms present on the washed sample, so that the effectiveness of surface sterilisation could be estimated.

After removal of the excess water, the plant material was dipped briefly into 96% ethanol, removed, and excess alcohol allowed to drip back into the container. The alcohol remaining on the surface of the plant material was then flamed off by passing the sample quickly through the blue flame of a bunsen burner, thus sterilising the material's surface. The sterile material was transferred to a sterile Petri dish to allow for ease of handling and for sectioning.

Flamed equipment (dipped into 96% ethanol then passed through blue flame of a bunsen burner and allowed to cool) was used to section the plant material. In the case of flat leaves, a single hole punch was used to punch numerous holes into several leaves (where possible) of each specimen. The pieces punched out were used as the sample

section. For roots, stems and grass-like leaves, a sterile scalpel was utilised, to produce numerous sliced sections. Four to eight sections (depending on number of sections obtained) were aseptically transferred to each of a PDA and MEA plate, both containing antibiotics. Plates were then placed into plastic bags and lightly sealed for ease of handling and incubation.

The bagged plates were incubated at 25^{o}C for up to one month, and routinely examined for evidence of hyphae growth. A small amount of natural light was able to enter the incubator via a small panel in the door. No artificial light was employed.

Endophyte Isolation and Collection

All manipulations of fungal cultures were performed on the bench, using a bunsen burner to provide a small sterile environment. To reduce bacterial contamination, the bench was pre-sprayed with 70% ethanol which was allowed to evaporate immediately before work commenced.

As fungal growth was detected, small sections of agar containing the growing hyphae were removed with a flamed and cooled agar cutter, and placed on fresh, antibiotic-free PDA plates. One plate was used per fungal isolation. The plates were incubated as previously described, and the growth checked every several days. As colony form became distinct, its morphology was compared with the growth obtained on the original plate. Where this was obviously different, a second plate was prepared from the original.

In most cases, the initial endophyte isolation was sufficient to provide a pure culture, and no further subculturing was required. In the few instances where this was not the case, subculturing of each apparent isolate was performed until pure cultures were obtained. In two cases, this required the use of a spread plating technique, as would be performed with bacteria.

Once in pure culture, three methods of storage were employed to minimise the risk of loss of an isolate, and ranged from short (up to 12 months) to longer term storage. All three methods required cubes of agar containing hyphae, which were cut from the pure isolate culture. A flame sterilised scalpel was used to slice at least ten cubes measuring approximately 3 to 5mm square, from an area containing the isolate.

1. PDA Slopes – PDA was prepared as per the manufacturer's instructions, and boiled to dissolve the agar. 14ml of the liquid, cooled to approximately 55^{o}C, was dispensed into a number of wide-mouthed 25ml glass vials. Following sterilisation in the autoclave, the vials were placed on to a board and allowed to set in a sloped position. Once set, a single cube of agar containing hyphae was placed in the centre of the agar slope, and the vial labelled. Inoculated vials were incubated at 25^{o}C and routinely examined for presence of sufficient, pure isolate growth. Once obtained, the vial was transferred to a storage container and stored at 4^{o}C. This form of storage is suitable for short term storage, with fungal viability lasting for up to one year (Smith 1988).

2. Water storage – Cryovials containing 1ml of RO water were sterilised in the autoclave on a Fluids cycle at 121^{o}C for 15 minutes and allowed to cool. Several cubes of agar containing hyphae were added to each cryovial and submerged under the water. Labelled vials were placed into a storage box and placed into a fridge at 4^{o}C. It has been shown by McGinnis et al. (1974) that storage in a similar manner at 25^{o}C has a successful revival rate of at least 92% after 5 years;

3. Cryopreservation – Several cubes of agar containing hyphae were placed into a sterile cryovial, and covered with a sterile, aqueous 25% glycerol solution. Labelled vials were placed into a storage box which was placed into a -70^{o}C freezer. This method can provide long term viability (Bills 1996).

2.3 Results

2.3.1 Trialling Isolation Methods

Pre-wash

As can be seen in Table 2.3, washing the plant material reduced the number of epiphytic organisms on the leaf surface. However, problems were encountered with the material becoming entangled in the magnetic stirring bar, so an alternative method of agitation needed to be developed.

Flaming an unwashed leaf also led to a reduction in surface organisms. However, when compared with the results obtained when flaming is combined with a pre-wash, as seen in Table 2.4, flaming on its own did not provide a suitable surface sterilisation result.

Surface sterilisation

Table 2.4 shows both methods of sterilisation reduced the population of surface organisms. However flaming alcohol off the leaf surface proved to be the more successful of the two, being quicker and more simplified. Occasionally the leaf matter continued to burn after the alcohol had gone, but burnt areas were simply avoided during sampling.

Leaf Sampling

Table 2.5 shows the description of growth obtained from the leaf samples, and an indicator of potential sub-culturing efficiency. The approach using homogenised disks appeared to be unsuccessful, as no useful colony growth was obtained on any of the plates. Fungal growth from the leaf quarters was excessive, reducing the ease of obtaining individual isolates. The larger leaf sections were also more difficult to handle

Table 2.3: Results showing impact of pre-washing leaves on the fungal epiphytic organism population.

TREATMENT	RESULTS
Unwashed	Entire agar surface covered with diverse range of fungal colonies
Washed	Entire agar surface covered, however with a reduced diversity of species compared with the unwashed plate
Unwashed/flamed	Entire agar surface covered with two main moulds and several smaller ones. Reduced number of colonies compared with the washed plate

Test leaves were either washed in several changes of non-sterile tap water, followed by a final rinse in sterile RO water, or left unwashed. Flamed leaves were immersed in ethanol before being passed through the flame of a bunsen burner. Both surfaces of each test leaf were subsequently pressed onto a SDA plate, the leaf discarded, and the plate incubated.

than the smaller disk or square sections. Disks or small leaf squares proved to be the most efficient way of obtaining discreet endophytic colonies. Using the hole punch was much quicker and easier than using the scalpel, and would be the preferred method of providing sections from flat leaves.

Therefore, based on observations and results of the trial, it was decided that plant material collected for isolation of endophytic fungi would be washed and then flamed before being punched with a hole punch where possible, to provide the sections.

2.3.2 Isolate Collection

Control Plates

All washed but pre-sterilised plant samples demonstrated the presence of a large number and diversity of fungal species colonising the external surfaces. Surface sterilisation of leaf samples generally resulted in the destruction of these potential contaminants. However, some apparent epiphytes remained on the root samples after sterilisation.

Flame sterilisation proved to be an effective method for the surface sterilisation of the plant material - the leaves in particular. This can be seen in Figure 2.1(a) in the photograph of the control plates prepared from the *Streblus sp.* leaf sample. The agar plate exposed to the washed but unsterilised leaf became completely covered by growth of fungal epiphytes. However, the plate exposed to the same leaf after surface flaming,

Table 2.4: Results comparing two different surface sterilisation methods applied to pre-washed leaves.

TREATMENT	RESULTS
Ethanol-bleach-ethanol	Bulk of agar surface covered by a single mould species. Two other fungal species evident
Flamed	No growth

The ethanol-bleach-ethanol method involved sequential soaking of the plant material in 96% ethanol, bleach diluted to provide 2% available chlorine, followed by immersion in another volume of 96% ethanol. Flaming involved immersing the material into 96% ethanol, removing the excess, and passing the material through the blue flame of a bunsen burner. Both surfaces of each test leaf were pressed onto a SDA plate, the leaf discarded, and the plate incubated.

Table 2.5: Results of different leaf sampling techniques.

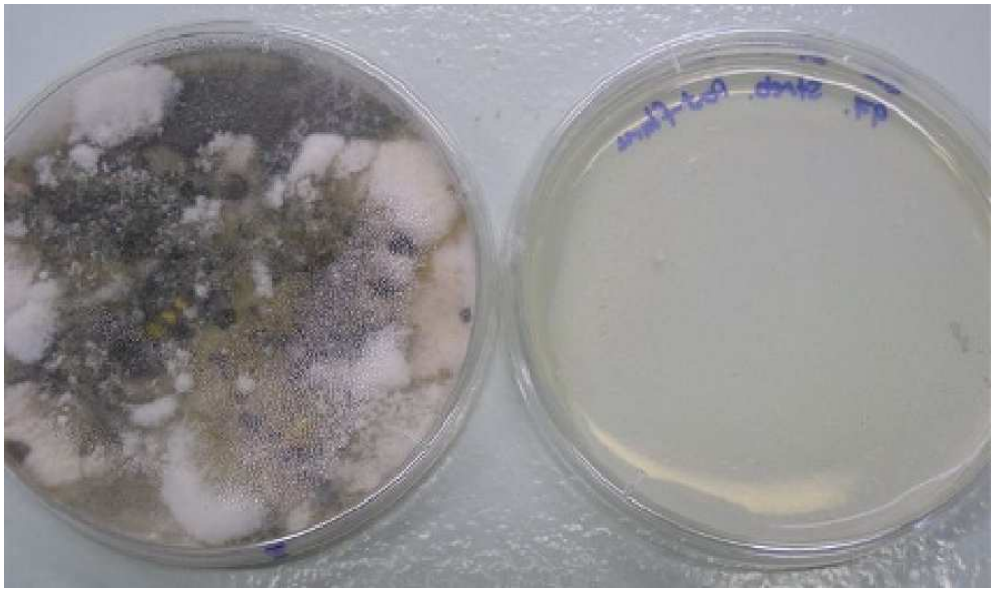
SECTION TYPE	RESULTS
Disks vs. cut squares	Mycelial growth occurred at the edge of one of each section type. Easy to sub-culture
Homogenised disks	One compact yeast-like colony grew from the plate with the 0.5ml aliquot. No growth from the others
Quartered leaf	Entire surface of both plates covered by less than ten fungal species. Difficult to isolate individual colonies

Test leaves were washed and surface sterilised. Disks were punched out of the leaf with a flame-sterilised hole punch. Cuts were made with a sterile scalpel.

grew nothing, which suggested the leaf surface had been effectively sterilised.

Figure 2.1(b)shows the same process but with a root sample from an *Acacia fimbriata*. The washed but unsterilised root remained heavily contaminated, and this was extensively reduced but not eliminated by flame sterilisation. However, in the majority of cases, it was found that epiphytes remaining on the root surface appeared to be yeasts, which were not a focus of this project, and so could be discounted.

Where epiphytic contamination remained possible after flame sterilisation, the morphology of individual fungi was compared with the morphology of the isolates obtained from the sections. Where morphological characteristics were found to be similar, those isolates were considered to be epiphytic contaminants.



(a) Control plates – Leaf

(b) Control plates – Root

Figure 2.1: Examples of Sterilisation Control plates. Both figures show the growth obtained after pressing the washed, but unsterilised plant tissue to the agar surface in the left hand plate, while the plate on the right demonstrates the same tissue after flame sterilisation. The upper set were from *Streblus brunonianus* leaf; the lower set were from *Acacia fimbriata* root.

Sample Plates

Fungi did not grow from all sections plated, while a small number of individual sections yielded two or three isolates. Both yeasts and moulds were recoverable from the sections. However, due to the nature of the project, efforts were concentrated on the endophytic moulds.

Some plates were overwhelmed by growth from one fast growing fungus. This required quick intervention, where unaffected sections were transferred to a fresh plate. However, this did not always lead to a satisfactory result, where either the fast-growing fungus persisted, or no new isolates grew at all.

Endophytic Isolates

Most sub-cultured isolates showed good resemblance to the cultures from the original plates, and in addition, resulted in pure cultures without the need for further subculturing. A total of 133 fungal cultures were grown with over 90% being moulds. Macroscopic examination suggested that over 100 fungal species were represented, including species of *Fusarium, Epicoccum, Aspergillus* and *Penicillium*.

Duplicate isolates tended to be from the same forest location but not from the same plant species. One duplicate fungal species was isolated from both the Preston and Redwood Park sites.

All plant species sampled provided at least one endophyte. Details of the total number of fungal isolates and number of unique isolates obtained per plant host species can be seen in Table 2.6. Within each plant host species, any multiple occurances of isolates only occured within individual plant hosts.

Unfortunately, eleven of the cultures proved difficult to grow sufficiently to enable storage, and so did not become part of the isolate collection. Each isolate was designated a unique identifier, based on the plant sample of origin, and the number of isolates obtained from that sample.

2.4 Discussion

It was determined during the initial trial that agitating the plant material in sequential fixed volumes of water was sufficient to decrease the contaminant load, and thereby increase the efficiency of the surface sterilisation method employed. Similarly, the final part of the pre-wash was performed using sterile water and equipment, so as not to introduce extra contaminants.

Flaming of the plant material appeared to provide a sufficient level of surface sterilisa-

Table 2.6: Summary of Isolates obtained from each plant species.

PLANT NAME	SAMPLE TYPE	PLANTS SAMPLED	SECTIONS PREPARED	ISOLATES OBTAINED	UNIQUE ISOLATES[1]
A. fimbriata	Leaf	5	20	6	6
	Root	3	7	4	4
A. excelsa	Leaf	3	36	4	3
S. australis	Leaf	3	36	2	2
X. johnsonii	Leaf	3	38	9	7
	Trunk	1	10	9	9
L. camara	Root	4	12	17	16
	Leaf	3	32	4	4
A. ruscifolia	Leaf	3	36	3	3
A. philippinensis	Leaf	3	37	1	1
A. bidwillii	Leaf	3	26	4	4
B. leichardtii	Leaf	3	28	13	12
D. rex	Root	9	118	29	22
	Leaf	2	24	0	0
E. xylocarpa	Leaf	3	36	3	3
H. australis	Leaf	3	28	18	13
M. claoxylloides	Leaf	3	36	3	3
S. brunonianus	Leaf	3	36	3	3
W. nobilis	Leaf	1	24	0	0
	Stem	1	9	1	1
	Sap	1	N/A	0	0

SITE	PLANTS SAMPLED	SECTIONS PREPARED	ISOLATES OBTAINED
Preston	25	191	55
Redwood Park	35	405	77
TOTAL (not including Wollemi Pine)	60	596	132
TOTAL (including Pine)	61	629	133

[1] Unique isolates per plant host

The first part of the upper section of the table lists samples obtained from Preston, while the second part lists those collected from Redwood Park. The Wollemi Pine was a cultivated specimen.

tion, though there may have been times where it resulted in over- or under-sterilisation of the plant tissue surface. This can be dependent on the texture and size of the material, and is a risk in common with any surface sterilisation method. However, it was noted that in previous studies many researchers surface sterilised the material after it was sectioned, rather than before, as was done in this project. It is not known what impact this would have on the isolation of endophytic fungi, but may possibly decrease the number of taxa obtained.

The procedures followed here, proved to be successful in the isolation of endophytic fungi. Fifteen plant species from two main sites were selected for sampling, and material was harvested from 61 individual plants. Following surface sterilisation and sampling, 629 tissue segments were placed onto solid media for culturing. 133 isolates were grown, and were predominantly moulds. Cursory morphological comparison suggested over 100 fungal species were represented.

On average, 0.21 isolates were obtained per tissue segment. This figure is quite low when compared with other studies – an average of 0.6 isolates per tissue sample was obtained by Taylor et al. (1999), whilst Fröhlich et al. (2000) obtained an average of 1.44. Many factors would influence the rate of isolates obtained per tissue segment including plant host species, growth conditions and location, treatment of plant tissue, and culturing conditions. Of particular note in the latter, the use of additional supplements in the media may have been of benefit, especially those used to restrict the growth of fast growing fungi.

At least one endophyte was cultured from each plant species, a similar finding to that of Peláez et al. (1998), where all but one plant species yielded an endophytic isolate. Their study examined fewer plant species (9), and fewer individual hosts (45) than this project. 2880 tissue samples were cultured, and produced 152 fungal species, a rate of approximately 0.05 species per sample. This was almost half the rate obtained for this project (0.16 species per sample), but is the closest to this research.

In their study of the endophytic assemblages of two tropical forests, Suryanarayanan et al. (2003) examined 24 plant species. The culture of 3600 tissue segments resulted in the production of 81 species of fungi, a rate of 0.02 species per sample.

A similar rate was obtained by Taylor et al. (1999) and Guo et al. (2000) in their bids to identify all endophytic fungi associated with a single palm-host species. Taylor et al. (1999) obtained 75 fungal species from 3256 leaf samples of *Trachycarpus fortunei*; Guo et al. (2000) cultured 750 tissue samples from *Livistona chinensis*, which yielded 16 fungal species. This work resulted in an average species to sample rate of 0.23 and 0.2 respectively.

Due to the higher level of diversity encountered in this project, multiple isolations of an individual fungal species were quite low. 7.5% of isolate species were found more than once, and were predominantly isolated from the same individual host. Therefore, 92.5% of the fungal species isolated were unique. The study conducted by Fröhlich et al. (2000) found 49.8% of isolates were found once, while only 30.9% of isolates obtained by Suryanarayanan et al. (2003) were unique.

Many aspects in the collection of endophytic fungi could have an impact on the results. These include selection of hosts, host sampling conditions and methods, sample size, tissue segment size, and tissue segment treatment.

Therefore, it is difficult to compare results with other studies due to the differing methods followed. In addition, the ultimate purpose of the individual research will have an impact on the process, from the selection of plant hosts and sites, to the processing of the material, to the subculturing and levels of identification of the resulting isolates.

The aim of this stage of this project was not to determine every endophytic species of a plant host or site, but to obtain a diverse range of fungal species for further study in the search for novel antibiotics. While the number of individual isolates obtained was smaller than other studies, the diversity obtained was larger. Therefore, the procedures followed as described under Materials and Methods, were sufficient for the fulfilment of the original goal.

Chapter 3

Screening for Antimicrobial Activity

3.1 Introduction

Acquisition of endophytic fungi from the 15 plant species sampled yielded a total of 122 isolates for the next phase of the project. This involved the preliminary screening of each fungal isolate for antimicrobial activity against a panel of selected nosocomial pathogens.

Antibiotic activity comes from the production of secondary metabolites by the fungal isolate. To determine the most suitable medium and method to encourage and detect production of secondary metabolites, a trial was conducted. Two screening methods were initially examined, testing the response of *Staphylococcus aureus* and *Escherichia coli* to substances produced in culture by the fungus *Penicillium chrysogenum*. As it was known the substances produced would potentially include penicillin, the two test microbes offered a sensitive and resistant response respectively to the antibiotic.

Using information garnered from the trial, two methods were developed for the screening of the isolates. These involved the culturing of each isolate on a suitable medium and exposing the test bacteria to the secondary metabolites produced in the medium. After incubation, the bacterial cultures were examined for the presence of inhibition. Fungal isolates which demonstrated some activity against any of the test organisms were further examined in the next two stages of this project.

3.1.1 Secondary Metabolites

Secondary metabolites are a by-product of an organism's growth, yet do not appear to be required for its normal growth (Demain 1986, Strobel & Daisy 2003, Deacon 2006). However, while production of secondary metabolites does not appear to be a necessary function (Deacon 2006), particularly when the fungus is grown in pure culture (De-

main 1986, Strobel & Daisy 2003) it is thought that in a natural environment these metabolites may play a specific role (Calvo et al. 2002, Strobel & Daisy 2003). Secondary metabolites would certainly enhance the survival of the fungus (Demain 1986), for example by providing protection from pathogens (Strobel & Daisy 2003). They have also been associated with fungal sporulation, but do not always appear to be an essential component of the process (Calvo et al. 2002). It has also been suggested that during periods of restricted growth, in order to avoid the accumulation of metabolic intermediates and their potentially toxic affects, the fungus converts them via the secondary metabolite pathways into alternative products which may be less toxic, or can be eliminated completely (Deacon 2006). Nonetheless, the purpose of endophytic secondary metabolites remains uncertain (Strobel & Daisy 2003). Examples of secondary metabolites include mycotoxins and pigments (Demain 1986, Calvo et al. 2002).

Secondary metabolites with antimicrobial properties are not produced by all fungi (Demain 1986, Peláez et al. 1998, Rosa et al. 2003, Strobel & Daisy 2003), and those produced may vary depending on the environment to which the fungi have adapted (Schulz et al. 2002, Strobel & Daisy 2003). Great variation has been observed between species (Demain 1986, Schulz et al. 1995) and between strains or isolates of the same species (Peláez et al. 1998, Schulz et al. 1995, 2002, Rosa et al. 2003, Strobel & Daisy 2003). Strobel & Daisy (2003) reported a case where a biologically active compound was produced from a single endophytic fungal isolate out of many of the same species isolated from the same plant. Therefore, in the search for antimicrobially active secondary metabolites, all isolates should be screened regardless of species similarity (Peláez et al. 1998, Rosa et al. 2003).

A number of factors influence the production of secondary metabolites in culture. Growth rate is possibly the most important, as it has been found that secondary metabolites are not produced during rapid growth (Demain 1986, Deacon 2006), and often do not occur until the organism has reached its stationary phase (Demain 1986, Calvo et al. 2002, Deacon 2006). Growth rate can be influenced particularly by medium composition. For example, a rapidly utilised carbon source, such as glucose, encourages rapid growth, and therefore can interfere with the production of secondary metabolites. Production of penicillin and cephalosporin are inhibited where glucose is the sole carbon source. Slowly metabolised sugars, including sucrose and galactose are preferable to the rapidly metabolised sugars such as glucose and maltose (Demain 1986). Early nutritional experiments conducted by Fleming (1929) found penicillin production by *Penicillium chrysogenum* was delayed or inhibited when glucose or saccharose were added to the culture medium.

The limiting factor for fungal growth is usually the nitrogen component of the medium (Trilli et al. 1978, Calvo et al. 2002, Deacon 2006). While secondary metabolite production may not commence until the nitrogen source has been depleted, the choice of nitrogen source can also have a major impact on its production. Ammonia, for instance, can suppress it completely while amino acids offer a better alternative. However, where specific amino acids share elements of the same metabolic pathway as the secondary metabolite of interest, they may inhibit its production by being a part of the feedback control (Demain 1986).

Growth conditions may also be used to encourage the production of secondary metabolites. Incubation of the culture at a temperature that is lower than that for optimal growth, slows the growth rate down, and may also be required for the production of some secondary metabolites (Demain 1986). Levels of aeration also have an impact on secondary metabolism production (Jennison et al. 1956, Demain 1986, Schulz et al. 1995).

Secondary metabolites extracted from endophytes have found uses as antibiotics (Demain 1986, Strobel & Daisy 2003), antivirals, anticancer, antioxidant, insecticidal, anti-diabetic and immunosuppressive agents (Strobel & Daisy 2003).

3.1.2 Screening techniques

Methods for the screening of fungal secondary metabolites for antimicrobial activity appear to be divided into three general approaches:

Approach 1 The fungal isolate was cultured on an agar plate capable of supporting bacterial and fungal growth. When sufficient growth was obtained, the test bacteria was applied to the plate. Plates were incubated, and then observed for evidence of inhibition.

This was the primary screening method used by Emerson et al. (1946) to screen 210 moulds against a panel of 5 bacteria. Two types of media were utilised, and the fungi grown until the colony size was between 20 to 25mm in diameter. Test organisms were grown in broth, and streaked from the edge of the colony to the edge of the plate. Following incubation overnight at 37^oC, the streaks were examined for areas of inhibition.

To screen for the presence of antimicrobial activity in 85 strains of *Pezicula* species, Schulz et al. (1995) cultured the fungi on a medium similar in composition to Nutrient agar with glucose. Once the fungal colonies reached 3.5cm in diameter, the plates were sprayed with a suspension of the test organism in water. After overnight incubation at 37^oC, the plates were examined for zones of inhibition. The same laboratory subsequently utilised this procedure in the screening for antimicrobial activity in over 6000 endophytic isolates (Schulz et al. 2002).

Approach 2 The fungal isolate was cultured in/on media suitable for the cultivation of fungi. When suitable growth was obtained, the medium, or a portion of it, was transfered to media suitable for cultivation of bacteria. The test bacteria were applied, the plates incubated, and observed for inhibition.

Fleming combined broth in which *P. chrysogenum* had grown with agar, and poured the mixture into a furrow previously cut into an agar plate. Once set, test bacteria were streaked from the furrow edge to the edge of the plate, and incubated (Fleming 1929).

Trilli et al. (1978) poured media into plates to a depth of 3mm. Once set, 4mm disks

were cut from the agar with a sterile cork borer, and the disks point inoculated with the fungi. After incubation of up to 5 days, the disks were transferred to Nunc plates containing bacterial medium incorporating the test organism, and the plates incubated overnight. Uninoculated disks were kept in sterile conditions in a humid chamber at 28^{o}C and used within 3 days.

In researching the production of penicillin by various species and strains of *Penicillium*, Laich et al. (2002) grew the fungi on plates of Malt Extract Agar (MEA). After 7 to 10 days of growth at 25^{o}C, 9mm plugs of the colonised agar were transferred onto the surface of Tryptic Soy Agar (TSA) plates. After 48 hours of incubation at 25^{o}C, the plates were overlayed with a mixture of 1% TSA and the penicillin-sensitive test organism, *Micrococcus luteus*. The plates were held at 5^{o}C for 2 hours, and subsequently incubated overnight at 30 ^{o}C.

Approach 3 The fungal isolate was cultured in liquid media suitable for the cultivation of fungi. Any secondary metabolites produced were chemically extracted, and the finished preparation applied to plates inoculated with the test organism. Plates were incubated and observed for inhibition.

Peláez et al. (1998) cultured 187 endophytic fungal isolates in a suitable broth for 3 weeks at 25^{o}C with shaking at 220 rpm. Two millilitres of each culture were mixed with 2ml of 100% methanol, and shaken for 15 minutes. After centrifugation for 15 minutes at 3000 rpm, 2ml of the supernatant was removed and evaporated to 1ml by nitrogen flow, to remove some of the methanol and concentrate any secondary metabolites present. Bacterial test plates were prepared by adding 3.3ml of a bacterial suspension adjusted to an optical density of between 0.22 to 0.35 at 660nm, to 100ml of medium and poured into 24x24cm Nunc plates. Once set, the fungal extracts were applied to the plate surface, and the plates incubated overnight.

In screening basidiomycetes for antimicrobial activity, Rosa et al. (2003) cultured the fungi in 100ml of liquid medium for 9 days at 28^{o}C with shaking at 150 rpm. The cultures were subsequently frozen until the extraction process could proceed. After thawing, the cultures were homogenised and extracted 5 times with 30ml volumes of ethyl acetate. The organic fraction was dried over anhydrous sodium sulphate, and rotary evaporation used to remove the solvent. 10mg/ml stock solutions of the product were prepared in di-methyl sulfoxide (DMSO) and stored at -40^{o}C. Test plates were prepared by spreading test bacteria over the surface of Brain Heart Infusion (BHI) agar plates, and applying 10μl spots of diluted extract (1mg/ml in 5% aqueous DMSO) to the surface. After 24 to 48 hours of incubation, the plates were observed for zones of inhibition.

A similar culture and extraction process was followed by Rodrigues et al. (2000) with selected endophytic isolates harvested from *Spondias mombin*, a Brazilian plant associated with traditional medicine. Extracts were dissolved in methanol, and 10μl added to wells in a 96-well cell culture plate, to which a system of growth media and test organisms was added. The plates were analysed by a pharmaceuticals company as part of a high throughput screening program.

The screening programmes undertaken by the above researchers examined the responses of a range of organisms, including *Bacillus subtilis, S. aureus* and *Pseudomonas aeruginosa* (Emerson et al. 1946, Peláez et al. 1998, Rosa et al. 2003), *E. coli* (Emerson et al. 1946, Schulz et al. 1995, Rodrigues et al. 2000, Rosa et al. 2003), *B. cereus, S. epidermidis, Salmonella typhimurium* (Rosa et al. 2003), *Serratia marcescens* (Peláez et al. 1998), and a number of fastidious bacteria and yeasts (Peláez et al. 1998, Rodrigues et al. 2000, Rosa et al. 2003). These have comprised laboratory and ATCC typed strains.

3.2 Materials and Methods

3.2.1 Trial with known antibiotic producer

Two trials were utilised to determine the best procedure for screening the isolates for production of antimicrobially-active secondary metabolites. A known antibiotic producer, *Penicillium chrysogenum*, was used as the test fungal species against the penicillin-sensitive bacterium *Staphylococcus aureus* (ATCC 25923). *Escherichia coli* (ATCC 25922) was included as a negative (resistant) control. The first trial was based on an ethanol extraction method and examined three areas – the growth medium; growth conditions; and pre-extraction treatment. The second trial focused on the use of direct screening on solid bacterial culture medium.

P. chrysogenum was grown on a PDA plate for seven days at 25^{o}C. PBS (Phosphate Buffered Saline) was prepared by diluting the appropriate volume of GIBCO's 10XPBS to make 200ml, and PBS-Tween prepared by adding 0.02ml of Tween 20 to the PBS. After mixing, a portion of the solution was sterilised by filtration through a 0.2μm filter. A spore suspension was prepared from the *Penicillium* culture by washing the plate surface with 1ml of the sterile PBS-Tween solution. The spore-laden liquid was then transferred to a 2.5ml volume bottle containing another 1ml of the PBS-Tween, and stored at 4^{o}C until required.

Four 100ml volumes of Nutrient broth, two 100ml volumes of Czapek Dox broth and one 100ml full-strength Malt Extract broth were prepared from commercially available stocks, and prepared as per the manufacturer's instructions. A trace metals solution was prepared by dissolving 0.05g zinc sulphate and 0.025g copper sulphate in 5ml of water. Czapek Dox Yeast broth was prepared by adding 0.1ml of the trace metals and 0.5ml of yeast extract to one of the 100ml Czapek Dox broths. A 50% strength Malt Extract broth was also prepared by preparing a 50ml volume as per the manufacturer's instructions, and diluting to 100ml with RO water. All broths were placed into 250ml Erlenmeyer flasks, stoppered with rolled cotton wool, and the tops covered with a square of alfoil.

Saline was prepared by dissolving 1.74g sodium chloride in 200ml reverse osmosis (RO) water. This was divided into two volumes of 20ml and two of 80ml.

Four hundred millilitres of Nutrient, Sensitest, Tryptic Soy, MacConkey and Mannitol Salt Agars were prepared as per the manufacturer's instructions. All media and the saline were autoclaved at 121^{o}C for 15 minutes, and once cooled to 50^{o}C, the agar was poured into 90mm Petri dishes. Formulations of all media can be found in Appendix B.

A dozen Whatman 7cm No. 2 filter papers were placed into a glass Petri dish, and fifty Whatman 13.0mm Antibiotic Assay disks were numbered with pencil and placed into a small glass jar. These hard goods were autoclaved at 121^{o}C for 15 minutes together with eight 250ml Schott bottles and two alfoil-topped 25ml measuring cylinders.

Trial 1

One volume of prepared Nutrient broth was left uninoculated as a negative control; the remaining seven flasks of broth were each inoculated with 50μl of the freshly prepared *Penicillium* spore suspension. All cultures, except for one of the Nutrient broths, were incubated at 25^{o}C with shaking at 100rpm for six days. The remaining flask was incubated as a stationary culture at 25^{o}C for the same period of time. Therefore, a total of eight flasks containing 100ml of a particular broth were incubated for this trial.

At the end of incubation, the contents of one shaken Nutrient broth culture were transferred entirely to a sterile 250ml Schott bottle. The contents from the negative control (broth with no fungal growth) were transferred to a second sterile Schott bottle. The mycelia were filtered out of the six remaining cultures, by vacuum filtration through a sterile No. 2 Whatman filter – a fresh filter being used for each culture. Each filtrate was transferred to a sterile 250ml Schott bottle, and all bottles stored at -20^{o}C.

Culture solutions were progressively thawed overnight at 4^{o}C. The first Nutrient broth culture, which had been frozen without the prior removal of the mycelium, had the mycelium filtered out as described for the other cultures. This mycelial mass was stored at -20^{o}C and freeze dried later as a separate sample. Two millilitres were transferred from each thawed broth solution to a sterile 2ml tube and refrozen. The remaining liquid was transferred to a sterile vacuum flask and refrozen at an approximately 45^o angle and gradually rotated to obtain as small a frozen profile as possible. Once solidified, each flask was transferred to the -70^{o}C freezer overnight to ensure the sample was sufficiently cold for freeze drying. Each sample required approximately 30 hours to freeze dry completely.

Each freeze dried product was transferred to a sterile 10ml McCartney bottle, and 10ml of ethanol added to the freeze dried broths. Three millilitres of ethanol were added to the freeze dried mycelial sample. After thorough mixing, the samples were held at -20^{o}C until testing.

The test bacteria were revived from -70^{o}C storage, and cultured onto TSA plates. After overnight incubation at 37^{o}C, the *E. coli* (ATCC strain 25922) was streaked onto a MacConkey agar plate, and the *S. aureus* (ATCC strain 25923) streaked onto a Man-

nitol Salt Agar plate. Swabs were also used to apply each species to one half of a Sensitest agar plate, and a commercial (Oxoid P10) Penicillin antibiotic disk placed in the middle of each swabbed area. After incubation overnight at 37^{o}C, these plates were used to confirm identity, and resistance and sensitivity to penicillin.

Bacterial suspensions were prepared by swabbing each organism over the surface of a fresh TSA plate and incubated as previously described. Approximately 3ml of sterile saline was applied to the surface of each plate, and the growth dislodged and suspended with a swab. Each bacterial wash was collected into separate sterile bottles, and with additional sterile saline was used to prepare a 50ml bacterial suspension. In order to standardise the inoculum, the final suspension was prepared with an optical density of approximately 0.4 at 660nm against a saline blank.

Bacterial test plates were prepared by adding 8ml of the bacterial suspension to 250ml molten Nutrient Agar (NA) which had been cooled to 50^{o}C. After swirling to mix, 20ml volumes were measured with a sterile 25ml graduated measuring cylinder, and poured into 90mm sterile plastic Petri dishes. Ten plates were prepared for each test bacterium.

Test solutions were removed from storage, and the 2ml broth samples allowed to thaw. Sterile paper disks were spread onto a sterile surface in the BSC. Two disks were prepared for each sample by adding 100μl of test solution to each disk, and leaving to dry. An additional two disks were similarly prepared as a control by adding 100μl of ethanol only.

Flame sterilised forceps were used to place four test disks onto half of the prepared bacterial test plates. A 3mm agar punch was used to create four wells in the remaining plates, and 10μl of test solution added in the same pattern as the disks – two sets of filtrate and ethanol extracted pairs per plate. A P10 penicillin disk was added to one set of plates for comparison. After allowing thirty minutes for absorption of liquid in the wells, the plates were bagged and incubated overnight at 37^{o}C, before being examined for the presence or absence of zones of inhibition. The radius of zones present were measured from the edge of the well or disk to the edge of clear inhibition.

Trial 2

The centre of one Nutrient agar plate was inoculated with a 50μl spot of the *Penicillium* spore suspension, and spread to cover an area of approximately 2.5cm in diameter. After incubation at 25^{o}C for four days, the plate surface was flooded by the *S. aureus* suspension prepared for Trial 1, and incubated overnight at 37^{o}C. The plate was then examined for the presence of inhibition as per the plates from Trial 1.

A second Nutrient agar plate was prepared by inoculating with *Penicillium* spores collected on a swab which was then rolled in the centre of the plate surface to cover an area of approximately 1.5cm in diameter. The plate was incubated for five days at 25^{o}C.

Nutrient broths were used to grow a selection of additional bacteria for screening: methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis, Pseudomonas aeruginosa, Bacillus cereus* and *Serratia marcescens*. Inoculated broths were incubated overnight at 37^{o}C. These additional bacteria were all laboratory strains except for *S. epidermidis* ATCC 14990. All bacteria were readily available, and can cause disease in humans.

After incubation of the fungus, a loopful of each bacterial broth was streaked across the plate from the edge of the fungal growth to the edge of the plate. The plate was then incubated overnight at 37^{o}C, and examined for areas of non-growth of the bacterial streaks.

3.2.2 Fungal Isolate Screening

Using information garnered from the trial, two methods were developed for the screening of the isolates. The first method resulted in the production of an ethanol extract for testing, while the second method utilised direct screening.

Bacterial cultures that were screened included *S. aureus*, MRSA, *S. epidermidis, E. coli, P. aeruginosa, S. marcescens* and *B. cereus*.

PDA was prepared and autoclaved as per the manufacturer's instructions, and 10ml poured into 5cm diameter Petri dishes. Fungal isolates were revived by inoculating the centre of the small PDA plate with a small amount of culture removed from the PDA slant storage. Plates were incubated at 25^{o}C until sufficient growth was produced.

Nutrient Broth was prepared as per the manufacturer's instructions, and 100ml volumes prepared as described in the trial. Sensitest and TSA plates and saline were prepared as previously described.

A flame sterilised scalpel was used to cut small cubes of mycelium from the revived cultures. One square was used to inoculate a flask containing 100ml Nutrient Broth, and incubated with shaking at 100rpm. Another square was placed in the centre of a Sensitest Agar plate. Both cultures were incubated at 25^{o}C for up to one month.

Method 1

The mycelium was removed from the broth culture as described in the trial, and the broth transferred to a 100ml sterile McCartney bottle. Samples were stored at -20^{o}C until they could be freeze dried, as previously described. After freeze drying, 10ml of ethanol was added to the freeze dried product. After swirling to ensure adequate contact with the product, the alcohol was removed to a sterile 15ml McCartney bottle, and 10 ml of sterile water added to the remaining product. Once dissolved, the solution was transferred to another sterile 15ml McCartney bottle. Both solutions were stored at -20^{o}C until tested.

Confluent growth plates of *S. aureus*, MRSA, *S. epidermidis, E. coli, P. aeruginosa* and *S. marcescens* were prepared on Tryptic Soy Agar (TSA). After incubation overnight at 37^{o}C, a suspension with an optical density of 0.5 at 660nm was prepared by washing the plate and diluting in sterile saline. Ten millilitres of sterile glycerol were added to 30ml of each suspension, and the mixture aliquoted into 5ml volumes for storage at -70^{o}C. As bacterial testing progressed, an aliquot of each bacterium was removed from the freezer and allowed to thaw for incorporation into test plates.

Sensitest Agar was prepared by combining 16g of medium powder with 500ml of RO water and boiling to dissolve. Eighty millilitres were dispensed into 100ml McCartney bottles, and autoclaved at 121^{o}C for 15 minutes. After being cooled to 50^{o}C, 4ml of a thawed bacterial suspension was added and mixed before pouring 20ml volumes into sterile Petri dishes. Five plates were prepared per bacterium at a time.

Test disks for filtrate and ethanol extracts were prepared as previously described, allowing sufficient disks to test against all six bacteria. Five dried disks were applied per bacterial plate, and plates incubated overnight at 37^{o}C. Each plate was examined for the absence of bacterial growth surrounding a disk. A positive result was recorded where a clear zone was found. Where growth occurred to the edge of the disk, a negative result was recorded.

A total of five isolates were tested in this manner.

Method 2

Cultures of *S. aureus*, MRSA, *S. epidermidis, E. coli, P. aeruginosa, S. marcescens* and *B. cereus* were grown on TSA plates overnight at 37^{o}C. Once the fungal cultures on Sensitest Agar had sufficient growth, a flame sterilised bacteriological loop was used to streak a small amount of each bacterial species across the plate from the edge of the fungal growth to the edge of the plate. After incubation overnight at 37^{o}C, the growth of each bacterial streak was examined. Where growth did not occur to the fungal edge, the result was recorded as a positive. If the bacterium was able to grow the entire length of the streak, it was recorded as a negative result.

3.3 Results

3.3.1 Trial with known antibiotic producer

Trial 1

As can be seen in the results for Trial 1 in Table 3.1, Nutrient Broth proved to be the most useful medium for encouraging the production of secondary metabolites from *P.*

chrysogenum.

While the Czapek Dox Yeast broth filtrate resulted in the largest zone of inhibition, it was believed that this was due to the trace metals in the medium rather than production of a secondary metabolite. As these were not completely dissolved in the ethanol, their absence from the ethanol extract would allow the bacteria to grow without inhibition, thus explaining the reduction in zone size.

There appeared to be little difference between the stationary and shaken cultures, or whether filtration occurred with or without the mycelium filtered out.

Apart from the unusual result for the Czapek Dox Yeast filtrate, no filtrate produced a zone of inhibition. Concentrating the filtrate by freeze drying and reconstitution in 1/10 the volume improved the result. The use of ethanol as the reconstitution liquid also had the potential to semi purify the solution, as non-alcohol soluble elements were not dissolved in to the solution. However, this would be a problem if any useful secondary metabolites produced by an isolate were not alcohol soluble.

The use of disks further increased the likelihood of positive inhibition detection, as ten times the volume of extract was used when compared with the wells.

Trial 2

The initial plate prepared for Trial 2 proved to perform better than anticipated as the zone of inhibition extended to the extreme edge of the plate. As the bacterial suspension had successfully grown in Trial 1, it was decided the lack of growth by the *Staphylococcus* could only be explained as inhibition by products produced by the fungus. Ultimately, a small number of individual colonies were located at the edge of the agar, confirming that interpretation.

The second plate prepared was much easier to read than the first and it was therefore easier to interpret a result. Two bacterial species clearly did not grow the entire length of the streak, whereas the remaining bacteria did so. The full results can be seen in Table 3.2.

3.3.2 Fungal Isolate Screening

Initially, two screening methods were used to screen the isolates. However, it became apparent that the direct Sensitest plate method would be the better screening choice, as it was quicker and as seen in Table 3.3, appeared to be more sensitive than the ethanol extraction.

Of the 122 isolates in storage, 13 weren't tested as they were macroscopically identified as yeasts. Of the 109 isolates remaining for screening, 27 (24.7%) did not recover from PDA slant storage; 3 (2.8%) did not grow directly on the Sensitest agar and 2 (1.8%)

Table 3.1: Results from Screening Trial 1

BROTH MEDIUM	TREATMENT/ROLE	TEST LIQUID	*E. coli*		*S. aureus*	
			DISK	WELL	DISK	WELL
Nutrient	Negative Control (No inoculant)	Filtrate	-	-	-	-
		Extract	-	-	-	-
Nutrient	Shaken. Filtered before freezing	Filtrate	-	-	-	-
		Extract	-	-	5mm	3mm
Nutrient	Shaken. Filtered after freezing	Filtrate	-	-	-	-
		Extract	-	-	3mm	-
		Mycelium extract	-	-	1mm	1mm
Nutrient	Stationary. Filtered before freezing	Filtrate	-	-	-	-
		Extract	-	-	5mm	4mm
100% Malt Extract	Shaken. Filtered before freezing	Filtrate	-	-	-	-
		Extract	-	-	3mm	-
50% Malt Extract	Shaken. Filtered before freezing	Filtrate	-	-	-	-
		Extract	-	-	-	-
Czapek Dox	Shaken. Filtered before freezing	Filtrate	-	-	-	-
		Extract	-	-	-	-
Czapek Dox Yeast	Shaken. Filtered before freezing	Filtrate	-	-	6mm	3.5mm
		Extract	-	-	1mm	-
N/A	Solvent Control (Ethanol only)	N/A	-	-	-	-
N/A	Positive Control (P10 Disk)	N/A	-	-	9mm	10mm

Measurements were taken from the edge of the disk/well to the edge of inhibition. Where no inhibition was detected, the result was recorded as a negative (-). From these results it was determined the use of Nutrient Broth followed by freeze drying, ethanol extraction and antibiotic disk assay was the preferred method for the screening of antimicrobial activity.

Table 3.2: Results from Screening Trial 2

ORGANISM	RESULT
S. aureus	+
MRSA	-
S. epidermidis	+
E. coli	-
P. aeruginosa	-
S. marcescens	-
B. cereus	-

Following 5 days of growth, streaks of bacteria were made from the *Penicillium chrysogenum* colony to the plate edge. After incubation the streaks were scored for presence of a section of non-growth near the fungus (+ve) or total streak growth (-ve).

grew so quickly and heavily that the bacteria could not be applied to the plate surface. Seventy-seven (70.6%) isolates were successfully screened using the direct Sensitest screening procedure.

Seven isolates demonstrated some level of activity against the selected micro-organisms. However, in the case of four of the isolates, the results were not repeatable. Therefore, the screening process yielded three isolates whose antimicrobial activity would be further investigated. Based on the plant sample of origin, and the number of isolates obtained from that sample, these isolates were designated as numbers 6.5, 12.1 and 129.1. Their activity profile can be seen in Table 3.4

3.4 Discussion

As a rapid screening method, the direct Sensitest plate method was preferred over the ethanol extraction method for two reasons – it was quicker and appeared to be more sensitive. It also required substantially fewer materials, and if it was to be utilised in a small laboratory, less specialised equipment. A summary of the requirements for both methods can be seen in Table 3.5.

However, the direct Sensitest plate method also has its limitations – as seen in the results, not all fungi are capable of growing on the Sensitest medium or alternatively, too much fungal growth prohibits the application of the test bacteria. Results could also be altered by the addition of supplements required for the growth of fastidious organisms; finally, the test is limited to screening against bacteria – the medium is not suitable for fungal pathogens. In the majority of these cases, the ethanol extraction method, or similar, would be required.

Table 3.3: Comparison of results obtained from each screening method.

ISOLATE	SCREEN	*S. aureus*	MRSA	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. marcescens*
6.4	Sensitest	-	-	-	-	-	-
	Extract	-	-	-	-	-	-
6.5	Sensitest	+	+	+	+	-	-
	Extract	+	+	+	+	-	-
7.2	Sensitest	-	-	+	-	-	-
	Extract	-	-	-	-	-	-
12.1	Sensitest	+	+	+	-	-	-
	Extract	-	-	-	-	-	-
19.2	Sensitest	-	-	-	-	-	-
	Extract	-	-	-	-	-	-

While the majority of the results were identical, the Sensitest results obtained for isolates 7.2 and 12.1 would suggest that it was potentially a more sensitive screening method than the ethanol extraction method. A “+ve” indicates inhibition of bacterial growth was detected, while no inhibition is indicated with a “-ve”.

Table 3.4: Antimicrobial activity profile of selected isolates

ISOLATE	*S. aureus*	MRSA	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B. cereus*
6.5	+	+	+	+	-	-	+
12.1	+	+	+	-	-	-	+
129.1	+	+	+	-	-	-	-

Following sufficient growth of the isolate, streaks of bacteria were made from the edge of the fungal colony to the edge of the plate. After incubation overnight at 37^{o}C, the streaks were scored for presence of a section of non-growth near the isolate (“+ve”) or total streak growth (“-ve”). The isolates listed above were selected for further investigation.

Table 3.5: Summary of the main requirements of two screening procedures

	Sensitest plate method	Ethanol extraction method
Time after fungal incubation	Approximately 20 hours	Minimum of 70 hours
Consumables per fungal isolate	Sensitest Agar plate	100ml Nutrient broth Filter paper 10ml ethanol Paper disks (1 per test bacterium) Sensitest Agar plate
Equipment required	Standard incubators	Standard incubators Shaking incubator Filtration equipment Vacuum flasks -40^{o}C freezer Freeze drier

However, neither method guarantees the production of antimicrobially active secondary metabolites.

Sensitest agar and Nutrient broth are media normally reserved for the culturing of bacteria, so it is not surprising that not all the fungal isolates grew successfully on them. However, it is possible the use of this media encourages the production of secondary metabolites in some of the fungi that can be successfully grown. Specifically, as the media is not ideal for standard fungal growth, the growth rate may be reduced – one aspect of secondary metabolite production is that high growth rates can reduce and even halt its occurrence (Demain 1986).

Four percent of the fungal isolates which were successfully screened demonstrated consistent antimicrobial activity against at least one of the seven test organisms. When compared with results obtained from other studies, this appears to be quite low.

Emerson et al. (1946) cultured 210 moulds on two media formulations, and tested for antimicrobial activity against a panel of five bacteria. 56% (117) of the fungi demonstrated some antimicrobial activity against at least one of the test bacteria.

Of the 103 extracts obtained by Rosa et al. (2003), 10% showed activity against at least one of the 12 bacteria tested. However, their research was confined to Basidiomycetes, a single phylum of the Fungal kingdom.

Approximately 22% of the 85 *Pezicula* species isolates tested by Schulz et al. (1995) demonstrated some activity against one of the two bacteria tested. The same screening

procedure was followed by the researchers later when examining over 6000 endophytic fungal isolates. Antibacterial, fungicidal, algicidal or herbicidal activity was detected in 80% of the isolates (Schulz et al. 2002).

Peláez et al. (1998) found that of 187 fungal endophytes tested, 24% demonstrated activity against at least one of the test organisms, which included six bacteria and three yeasts. One third of the active isolates showed activity against both classes of test microorganism.

However, results are difficult to compare due to the differences in screening method. In addition, different results would be expected if the number of test organisms were expanded, different test species or strains were used, if alternative or multiple fungal culture media were employed or if fungi were cultured in alternative conditions. Schulz et al. (1995) found every *Pezicula* strain tested produced the same metabolite when grown on one medium, but growth on a different medium failed to produce the compound at all. In addition, it was found that the secondary metabolites produced by one strain of *Pezicula* when grown in a fermenter culture were different when grown in the same medium but in shaken culture. The difference appeared to be that levels of oxygen saturation and pH were constant in the former culture, whereas they were variable in the latter.

Aspects of this screening process could be improved. As previously mentioned, the Sensitest plate method appeared to be more sensitive than the ethanol extraction method. However, this may actually be as a result of enhanced production of secondary metabolites by the fungus due to the presence of the bacteria. This potential feature of secondary metabolite production is routinely utilised by Kingsland & Barrow (2009), who incorporate an autoclaved suspension of *S. aureus* cells into their fermentaion medium to encourage said production. This possibility was not considered prior to the conduction of this project, and therefore, not included. It would be of interest to compare differences in fungal secondary metabolite production with and without the presence of bacteria, and include this as part of future screening programmes.

While the Sensitest plate method was intended as a rapid screening process, it must be confirmed that it is also an effective screening process. Due to time constraints and initial result comparisons, the Sensitest plate method was utilised for the majority of the isolate screening, rather than the ethanol extraction method. However, this decision was based on the comparison of results involving just five fungal isolates. Since expenses are incured in any type of screening programme, from the initial gathering of the fungal isolates, to their maintenance and screening, there needs to be a maximum chance of success. Therefore, a larger comparison of the two screening methods, and others, needs to be undertaken to determine the optimal procedure.

Chapter 4

Identification of Selected Isolates

4.1 Introduction

Following screening for antimicrobially active isolates, three unique mould species were selected for further analysis. This firstly required the determination of their scientific classification, so as not to re-evaluate a fungus which had been previously described and assessed. Morphological and molecular identification techniques were utilised for this process.

The isolates were grown on PDA until some colony colour developed. A description of the colony's physical appearance was recorded, and the plate photographed. The culture was subsequently used to prepare slides for microscopic examination, where a sample of the fungus was placed onto a slide and stained with lacto-fuchsin stain. Slide preparations were examined under x400 magnification, and microscopic structures described.

Morphological examination was sufficient to determine the identification of one of the isolates to genus level. Molecular analysis was required for the identification of the two remaining isolates.

DNA was extracted from the mycelium of each isolate, and the internal transcribed spacer (ITS) region of their DNA amplified. Following purification and cloning, the DNA was sequenced, and the results compared with other sequences held in the GenBank database in order to determine a likely identification. Phylogenetic relationships were also analysed for these two isolates.

4.1.1 Morphological identification

Macroscopic examination of fungal cultures can provide a description of the colony colour (Guo et al. 2000), margin shape (Bills 1996), texture and growth rate (Bills

1996, Guo et al. 2000), as well as the production of pigmented compound into the culture medium (Bills 1996).

Slides for microscopic examination have been prepared in water (Arenal et al. 2005), lactophenol cotton blue (Guo et al. 2000, Arenal et al. 2005) or in 90% lactic acid, which also renders the slides semi-permanent (Paulus et al. 2003). Prepared slides can also be sealed with nail varnish to improve storage (Guo et al. 2000).

Microscopic examination can be used for the description of hyphae (hyphal pigments (Bills 1996), size, colour, presence/absence of septa), and when present, description of the sporulation structures (Bills 1996, Guo et al. 2000, Yanna & Hyde 2002, Paulus et al. 2003, Arenal et al. 2005), and description of spores (size, shape, texture, production) (Guo et al. 2000, Yanna & Hyde 2002, Paulus et al. 2003, Arenal et al. 2005).

Gross colony characteristics can be used to place isolates into morphologically-similar groups (Bills 1996, Johnston 1998, Peláez et al. 1998), and is especially useful where fungi fail to sporulate (Taylor et al. 1999, Fröhlich et al. 2000, Guo et al. 2000, Arnold & Lutzoni 2007). However, morphological characterisation of fungi has been used to divide isolates into phyla (Arnold & Lutzoni 2007), families (Johnston 1998), genera (Fröhlich et al. 2000) and species (Taylor et al. 1999, Yanna & Hyde 2002, Paulus et al. 2003).

For morphological examination to be used to its full potential, fungi are required to sporulate. However, many fungi do not produce spores in artificial culture, or the time required for them to sporulate may be prohibitive (Bills 1996). Therefore, if identification is required, other methods must be employed (Guo et al. 2000, Healy et al. 2004, Arnold & Lutzoni 2007).

4.1.2 Molecular identification

In the study of the endophytic fungi from the palm host *Livistona chinensis*, Guo et al. (2000) were determined to identify or describe the phylogenetic relationship of every isolate obtained. By manipulating the culture media and growth conditions to encourage sporulation, 83.5% of isolates obtained could be identified by morphological examination. This was comparable with results obtained by Taylor et al. (1999), who were able to identify 87% of the isolates obtained from the palm *Trachycarpus fortunei*; whilst Fröhlich et al. (2000) were only able to identify 56% of the isolates obtained in their study of palms.

In the case of Guo et al. (2000), the remaining 16.5% of isolates were identified using molecular techniques.

Located within each tandem repeat of ribosomal DNA, and flanked by the small subunit 18S and large subunits 5.8S and 28S, lie two non-coding sections – the internal transcribed spacer (ITS) region (Gardes & Bruns 1993). While the subunit genes are highly conserved, ITS rDNA is variable between species, making it useful in molecu-

lar identification (Gardes & Bruns 1993, Guo et al. 2000). In fungi, the combined ITS regions comprise approximately 600 to 800 base pairs (Gardes & Bruns 1993, Arnold & Lutzoni 2007).

Several primers have been designed for amplification of various sections of the ITS-DNA, and several combinations of these primers have been useful in the identification of fungi. ITS1-F is a primer specifically designed for amplification of fungal DNA (Gardes & Bruns 1993), and in combination with a universal primer, ITS4, is highly selective for basidiomycetes and ascomycetes (Gardes & Bruns 1993, Arenal et al. 2005). Guo et al. (2000) combined ITS4 with the primer ITS5, which amplifies the same ITS1-5.8S-ITS2 region as the former combination, but with less fungal specificity.

Following the sequencing of the amplified DNA, the National Centre for Biotechnology Information (NCBI) database on GenBank is searched to locate similar sequences of known fungi to obtain an identification (Guo et al. 2000, Healy et al. 2004, Arenal et al. 2005, Arnold & Lutzoni 2007). The Basic Local Alignment Search Tool (BLAST) on GenBank is generally used to compare and obtain the sequences, which can be further analysed so as to infer phylogenetic relationships (Bills 1996, National Centre for Biotechnology Information 2009).

4.2 Materials and Methods

4.2.1 Morphology

The isolates to be identified were grown on PDA plates until some colony colour developed. At this point, macroscopic examination was recorded. This included general colony size, shape, colour, surface texture, margin shape and growth rate. The plates were photographed, and the culture subsequently used for the preparation of slides for microscopic examination.

Lacto-fuchsin Stain was prepared by adding 0.1 g of Acid fuchsin to 100 ml of concentrated lactic acid, and mixed well to dissolve. The solution was placed in to dropper bottles for easy application to slides.

Three methods were used to prepare slides. The simplest method involved pressing a small amount of sticky tape to the surface of the culture, and then transferring the tape to a microscope slide onto which a drop of stain had been placed.

Slides were also prepared by removing a small amount of the culture with an agar cutter, and emulsifying it in the drop of stain on the slide. A coverslip was placed over the stain and, if necessary, pressed gently onto the slide surface with the handle-end of the cutter.

The third method involved the preparation of slide cultures. The isolates were cultured

on squares of PDA, measuring approximately 1cm by 1cm, which had been cut from the plate and flipped onto the remaining agar surface. There were two such squares prepared on each plate. The upper edge of each square was inoculated with material from the isolate, and an alcohol flamed coverslip was placed on the top. The plates were incubated at 25^{o}C until colour developed in the culture. Slides were finally prepared by placing a drop of the stain on to the middle of a microscope slide, and one of the cultured coverslips placed over it.

All preparations were placed briefly onto a warm heat plate to remove air bubbles and aid stain penetration.

Slides were examined under a compound microscope as they were prepared using x400 magnification. Slides which clearly showed hyphae and any other identifiable structures were sealed with clear nail polish and stored flat in the dark for further observation and photography.

4.2.2 Molecular Identification

LB Broth was prepared as per the manufacturer's instructions, and dispensed into 10ml volumes before autoclaving, along with 100ml volumes of Milli-Q water. Both were stored at room temperature.

Ampicillin solution was prepared by dissolving 250mg ampicillin in 5ml Milli-Q water. After filter sterilisation through a 0.22μl syringe filter, it was dispensed into 1.25ml aliquots. 0.1M IPTG was prepared by dissolving 120mg of IPTG in 5ml Milli-Q water. It was filter sterilised as for the ampicillin, but dispensed into 1ml volumes for storage. A 50mg/ml X-Gal solution was prepared by dissolving 250mg X-Gal in 5ml of molecular grade DMSO, and dispensed into 1 ml volumes. All solutions were stored at -20^{o}C until required.

Four hundred millilitres of LB Agar was prepared as per the manufacturers instructions. After being allowed to cool to 50^{o}C, 400μl of 50mg/ml ampicillin, 1000μl 0.1M IPTG and 500μl 50mg/ml X-Gal solutions (thawed) were aseptically added and mixed before pouring into 90mm Petri dishes. Once set, plates were labelled, bagged and stored upside down in the refrigerator, and used within one month.

All preparations and manipulations were performed in a lamina flow cabinet, until the cloning procedure, where the presence of bacteria necessitated the use of the BSC.

DNA Extraction was performed using a DNeasy Plant Mini Kit (Qiagen, Doncaster, VIC, Australia), which was designed to extract total DNA from up to 100mg of plant or fungal tissues. Ethanol was previously added to the extraction and wash buffer concentrates as per the manufacturer's instructions.

Fungal material was available either on a PDA plate or in Yeast Extract-Sucrose (YES) broth culture. A sterile scalpel blade was used to scrape the fungal growth off each

plate and into a sterile microcentrifuge tube. The amount of agar removed with the growth was limited as much as possible. Due to its small growth area, two plates of isolate 6.5 were used, whereas only one plate was required of isolates 12.1 and 129.1. Alternatively, flamed forceps were used to remove a sufficient amount of growth from the broth culture and placed into sterile 1.5ml microcentrifuge tubes. Each tube was topped up with sterile water, and mixed. This was removed from the fungal mass after centrifugation at 1000rpm for 5 minutes.

A small amount of previously autoclaved fine sand was added to each tube, and the mixture ground with a plastic pestle. To minimise cross-contamination, gloves were changed between samples.

400μl of lysis buffer was added to each tube, followed by 4μl RNase A. Each tube was well vortexed to ensure a lump free mixture was obtained. After incubation, with occasional shaking, at 65^{o}C for 10 minutes, 130μl of precipitation buffer was added, and the mixture incubated on ice for 5 minutes.

After centrifugation at 13,200rpm for 5 minutes, the supernatant was transferred to a shredder spin column held in a collection tube, and centrifuged for 2 minutes at 11,000rpm. The flow through (approximately 450μl) was transferred to a new set of microcentrifuge tubes, and 675μl of extraction buffer added and mixed. 650μl of this mixture was transferred to a DNeasy spin column in a collection tube and centrifuged for 1 minute at 8000rpm. The flow through was discarded, and the centrifugation repeated with the remaining sample. The final flow through and the collection tube were discarded, and the column placed into a fresh 2ml collection tube.

500μl of wash buffer was added to the column, followed by centrifugation for 1 minute at 8000rpm. The flow through was discarded, and 500μl of buffer added to the column again before centrifugation for 2 minutes at 13,200rpm. The column was then transferred to a fresh microcentrifuge tube, ensuring no contact was made between the column and the final flow through liquid.

Elution buffer was heated to 65^{o}C, and 100μl added to the column. After five minutes of incubation at room temperature, the DNA was eluted by centrifugation at 8000rpm for 1 minute. This step was repeated with the column transferred to a fresh microcentrifuge tube before the pre-warmed buffer was added. The final products were then combined for use in PCR.

A PCR master mix was freshly prepared by combining the following proportions of ingredients in sufficient quantity for each duplicate test, a PCR control and small amount for pipette error:

Milli-Q Water	$38\mu l$		$14\mu l$
10x Buffer[1]	$5\mu l$	10xBuffer incorporating $MgCl_2$[2]	2μl
50mM $MgCl_2$[1]	$2.5\mu l$		
10mM dNTP[1]	$1\mu l$		$2\mu l$
ITS1F Primer[3]	$1\mu l$		$0.5\mu l$
ITS4 Primer[4]	$1\mu l$		$0.5\mu l$
Taq DNA polymerase[1]	$0.5\mu l$	*Taq* DNA polymerase[2]	$0.5\mu l$
Total Volume	$49\mu l$		$19.5\mu l$
DNA Volume required	$1\mu l$		$0.5\mu l$

[1] Supplied by Invitrogen Australia, Mt Waverley, VIC, Australia
[2] Supplied by Scientifix, Cheltenham, VIC, Australia
[3] Gardes & Bruns (1993)
[4] White *et al*., (1990), as cited by Gardes & Bruns (1993).

After mixing, the solution was dispensed into sterile thin walled PCR tubes in either 49μl or 19.5μl aliquots, depending on the PCR preparation used. The volume of extracted DNA required was then added to each tube; the control tube received the same volume in sterile distilled water. Amplification was performed over 35 cycles consisting of 95^{o}C for 1 minute; 50^{o}C for 1 minute and 72^{o}C for 1 minute. Final incubation was at 72^{o}C for 10 minutes.

A 2% (w/v) agarose gel was prepared by dissolving the appropriate amount of agarose in TAE Buffer. When cooled to approximately 50^{o}C, a 0.625mg/ml ethidium bromide solution was added at a rate of one drop (25μg ethidium bromide) per 50ml gel. After mixing, the mixture was poured into the selected casting tray, and the comb added. Once set, the comb was removed and the gel placed into the electrophoresis tank. The compartment/s were filled as required with TAE Buffer and/or RO water.

Following PCR, 2μl from each tube was mixed with 0.5μl of running dye, and electrophoresed for 30 minutes at 100V on the freshly prepared gel. After visualisation under UV light, if it was determined there was sufficient PCR product available, it was ready for DNA purification. Where insufficient product was produced, as occurred with isolate 6.5 and 12.1, the PCR was repeated with the initial PCR product.

A DNA Purification Kit (Machery Nagel, Cheltenham, VIC, Australia) was utilised to purify the PCR products for use in cloning. Replicate products were combined, and the volume made up to 100μl with sterile distilled water. 200 μl of binding buffer was added to each tube and well mixed, before transferring the tube contents to a spin column held in a collection tube. After centrifugation for 1 minute at 13,200rpm, the flow through was discarded, and the column returned to the collection tube. Six hundred μl of wash buffer was added to the column, and the centrifugation repeated. After discarding the flow through, remaining traces of wash buffer were removed by centrifugation for 2 minutes at 13,200rpm.

The column was placed into a new microcentrifuge tube, and 30μl of elution buffer

added. After incubation at room temperature for 1 minute, the column and tube were centrifuged for 1 minute at 4000rpm. This produced approximately 12.5 μl of eluate, containing the purified DNA.

The concentration of the purified product was checked by running 2μl volumes of eluate on a 2% (w/v) agarose gel as previously described, and the DNA made available for cloning.

The pGEM®-T Easy Vector System (Promega, Annandale, NSW, Australia) was used for preparation of ligation tubes as follows:

	Samples	Positive	Negative
2x Buffer[1]	$5\mu l$	$5\mu l$	$5\mu l$
Easy vector[2]	$1\mu l$	$1\mu l$	$1\mu l$
PCR Product	$3\mu l$	$-$	$-$
Control insert DNA[2]	$-$	$2\mu l$	$-$
T4 DNA ligase	$1\mu l$	$1\mu l$	$1\mu l$
Milli-Q water	$-$	$1\mu l$	$3\mu l$
Final Volume	$10\mu l$	$10\mu l$	$10\mu l$

[1] Vortexed before use
[2] Centrifuged briefly before use

The ligation was allowed to progress overnight at 4^{o}C.

The following day, 2μl of each ligation mixture was placed into fresh sterile microcentrifuge tubes, and 50μl of *E. coli* competent cells (JM109) added. The mixture was placed on ice for 20 minutes, followed by heat shocking for 90 seconds at 42^{o}C. The tubes were then returned to the ice for a further 2 minutes. 950μl of LB broth was added to each tube, and the tubes transferred to a platform shaker for incubation at 37^{o}C and 150rpm for 1.5 hours. A sufficient number of LB Agar plates containing ampicillin, IPTG and X-Gal were allowed to come to room temperature. Two plates were allowed for each sample transformation, and one plate for each of the controls. 100μl volumes from each tube was spread onto its respective plate, and the surface allowed to dry. Plates were incubated at 37^{o}C until sufficient growth was apparent.

After incubation, the negative control plate was checked for the presence of blue coloured colonies, and the positive for a mixture of blue and white. A white colony from the positive control, and each sample (if present) was streaked on to a fresh plate and incubated overnight at 37^{o}C, to ensure transformation was truly successful.

For bulking of plasmid DNA, ten microlitres of 50mg/ml ampicillin was added to several tubes containing 10ml LB broth. A single colony from each of the latter plates was added to the broth, and the tubes incubated overnight in a 37^{o}C waterbath and shaken at 150rpm.

Mini-prepping of the plasmid DNA was undertaken by Dr John Dearnaley, with an Eppendorf FastPlasmid Mini kit used as per the manufacturer's instructions: 1.5ml of each fresh broth culture was pelleted at 13,000rpm for one minute and the supernatant discarded. 400μl of ice cold lysis solution was added, and the mixture thoroughly vortexed to produce a homogenous solution. Following a three minute incubation at room temperature, the lysate was transferred to a spin column assembly and centrifuged at maximum speed for 30-60 seconds. Centrifugation was repeated after the addition of 400μl of wash buffer, and the waste contents discarded from the collection tube. After another minute of centrifugation at maximum speed, the spin column was transferred to a fresh collection tube. 50μl of elution buffer was added directly to the surface of the spin column membrane, and the DNA eluted by centrifugation at maximum speed for 30-60 seconds. The spin column was discarded, and the DNA stored in the collection tube at -20^{o}C until required for sequencing.

Two microlitres of T7 promoter primer were placed into a number of sterile microcentrifuge tubes, and 8μl of purified plasmid DNA or PCR product added. The tubes were sent to the Australian Genome Research Facility (AGRF) for sequencing. A BLAST search (Altschul et al. 1997) using the National Centre for Biotechnology Information (NCBI) database on GenBank was used to analyse the ITS sequence to locate possible matches. In addition, phylogenetic analysis of isolates 6.5 and 129.1 were performed by comparing their sequences with selected, related sequences, copied from GenBank in FASTA format. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Sequences aligned using Clustal W were used to create a neighbour-joining tree (Saitou & Nei 1987), using the Maximum Composite Likelihood method in order to draw the tree to scale. 1000 replicates were utilised in the bootstrap test (Felsenstein 1985).

4.3 Results

4.3.1 Morphology

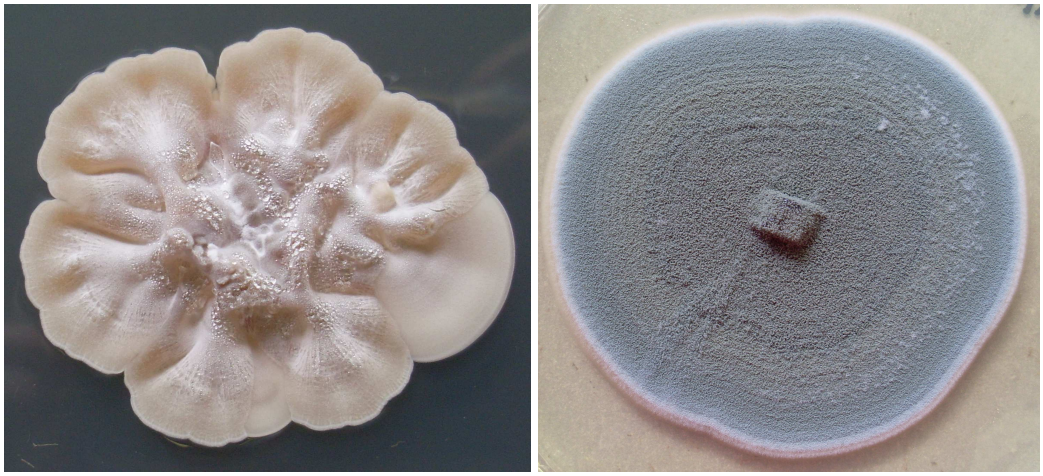
Following sufficient incubation on PDA, results of macroscopic examination of the three isolates was described and recorded.

Isolate 6.5 was slow growing, and would grow to a certain point and not become any larger. It produced a relatively small, heaped and folded colony, which was light tan in colour on both sides. Figure 4.1(a) shows a photograph of the isolate taken after about 30 days incubation.

Figure 4.1(b) shows the colony morphology of isolate 12.1. This was the fastest growing of the three isolates, producing a large flat colony with a blue-green, powdery surface edged with white within seven days. The reverse was dark-orange to a yellow edge and smooth.

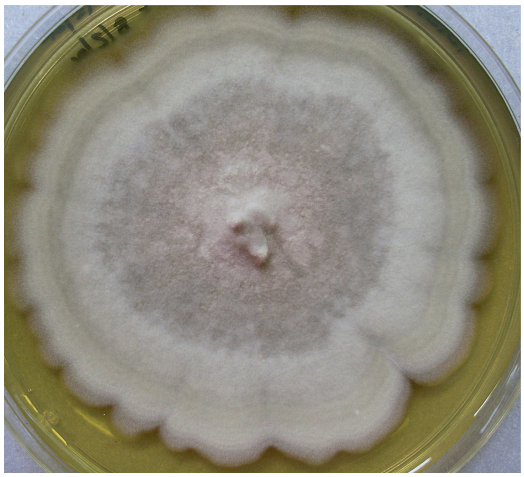
Isolate 129.1 was moderately fast growing, and produced a colony which almost cov-

ered the full surface of the 90mm plate after approximately 2 weeks incubation. It was off-white to tan in colour, and velvety to fluffy in appearance. As can be seen in Figure 4.1(c), a yellow pigment diffused into the agar.



(a) Isolate 6.5 (34mm across)

(b) Isolate 12.1 (55mm across)

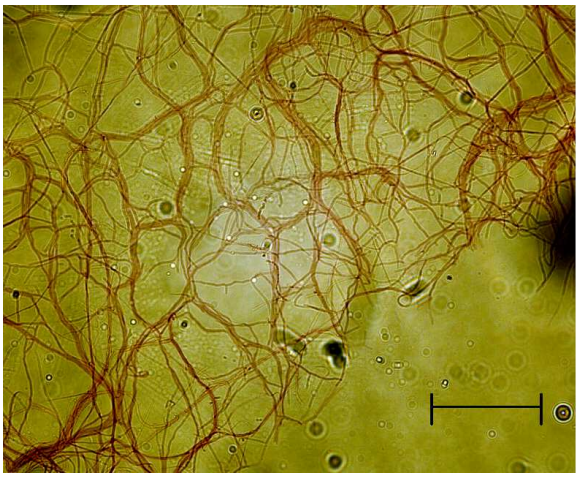


(c) Isolate 129.1 (75mm across)

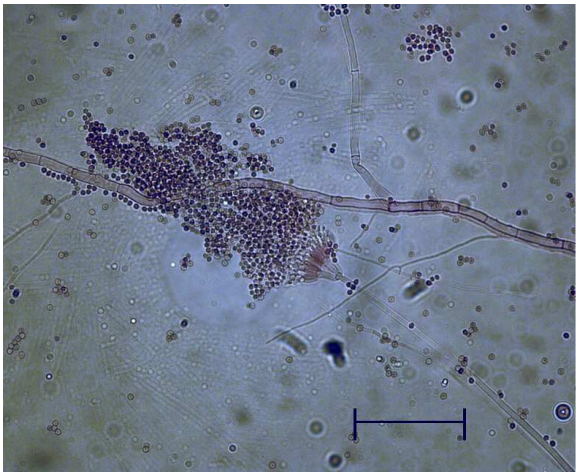
Figure 4.1: Photographs of PDA cultures of each isolate, taken after colour had developed in the colony. Note yellow pigment produced by isolate 129.1, diffused into the medium.

Microscopic morphology of two of the isolates was not definitive in the identification process. In an attempt to obtain sufficient information from microscopic examination to aid in the identification of isolates 6.5 and 129.1, all three methods of slide preparation were used. Figures 4.2(a) and (c) reveal hyphae, but little else. Regardless of the preparation, microscopic examination was inconclusive for both of these isolates.

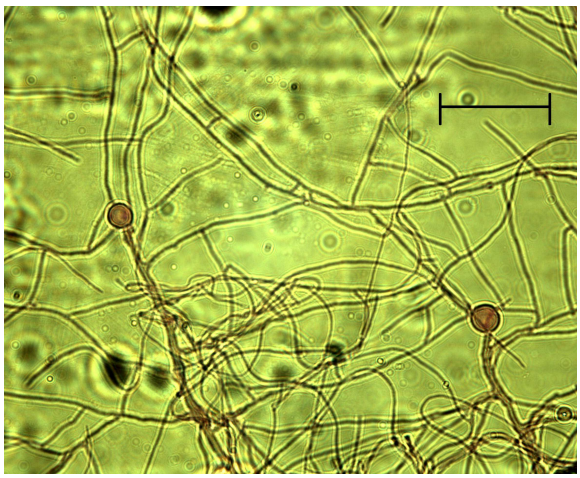
Several microscopic features were readily identified in isolate 12.1 with both the sticky tape slide preparation, and direct culture emulsion techniques. Therefore, a slide culture was not required. As can be seen in Figure 4.2(b), identifiable features include septate hyphae, phialides atop a conidiophore and masses of smooth walled, round conidiospores. These features were consistent with a *Penicillium* sp.



(a) Isolate 6.5



(b) Isolate 12.1



(c) Isolate 129.1

Figure 4.2: Photographs of microscopic preparations of each isolate taken at x400 magnification. Note the septate hyphae and phialides present in the view of isolate 12.1. Bar measurement in all photographs is 5μm

Table 4.1: Closest species results from BLAST sequencing search

ACCESSION CODE	CLOSEST SPECIES MATCH	MAX. IDENTITY (%)	E-VALUE
Isolate 6.5			
EF155505.1	*Schizophyllum commune*	99	0.0
AY605705.1	Basidiomycete sp	98	0.0
AB505421.1	*Schizophyllum sp*	98	0.0
FJ478109.1	*Schizophyllum commune*	97	0.0
AB369910.1	*Schizophyllum commune*	97	0.0
Isolate 129.1			
EU490094.1	Uncultured ascomycete	99	0.0
AY943053.1	*Sporormiella isomera*	89	0.0
EU551184.1	*Sporormiella isomera*	88	0.0
AY510420.1	*Preussia africana*	81	0.0
AY510418.1	*Preussia africana*	81	0.0

Data obtained following BLAST searches of the ITS sequences amplified from isolates 6.5 and 129.1.

4.3.2 Molecular Identification

Difficulties were experienced in obtaining transformants from isolates 6.5 and 12.1. However, as isolate 12.1 was identified microscopically as a *Penicillium* sp., it was decided not to further pursue its identification; the purified PCR product obtained from isolate 6.5 was used for sequencing rather than the preferred, but difficult to obtain, plasmid DNA.

After sequencing, a nucleotide BLAST search (Altschul et al. 1997) was performed. Isolate 6.5 was identified as *Schizophyllum commune* and isolate 129.1 was identified as *Sporormiella isomera*. GenBank accession codes, maximum identity and E-values for the closest species matches as at June 2009 for each isolate are shown in Table 4.1.

These and fourteen additional sequences were obtained from each isolate's BLAST search result for phylogenetic analysis and generation of phylogenetic trees. Figure 4.3 shows the phylogenetic tree obtained for isolate 6.5. Figure 4.4 displays the tree generated for isolate 129.1. Captions for each figure were primarily generated by the "Caption" option available in the MEGA4 software.

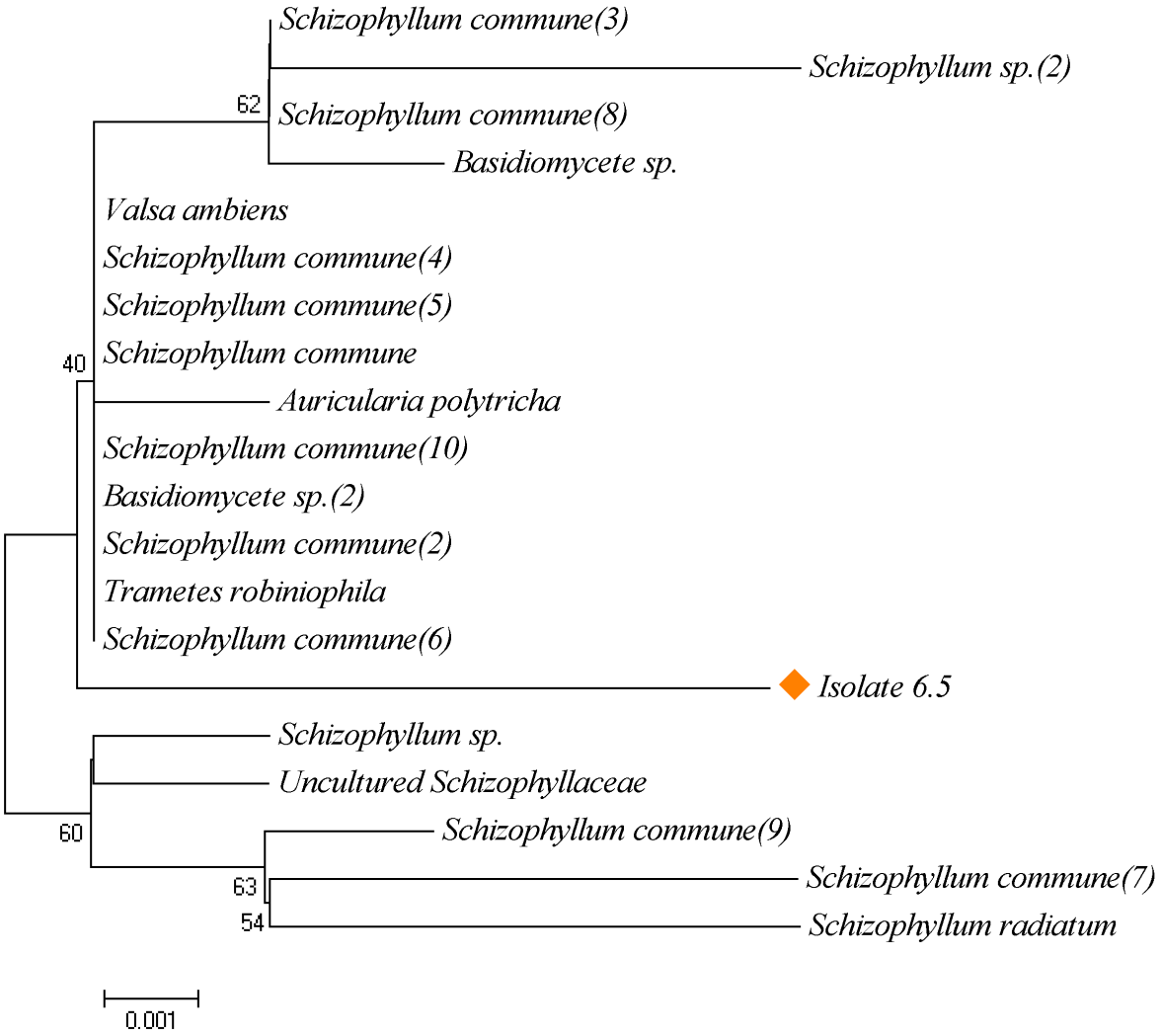


Figure 4.3: Phylogenetic tree of isolate 6.5, showing evolutionary relationships of 20 taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.03921422 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 538 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. Isolate 6.5 clusters with a group predominantly populated with *S. commune* strains

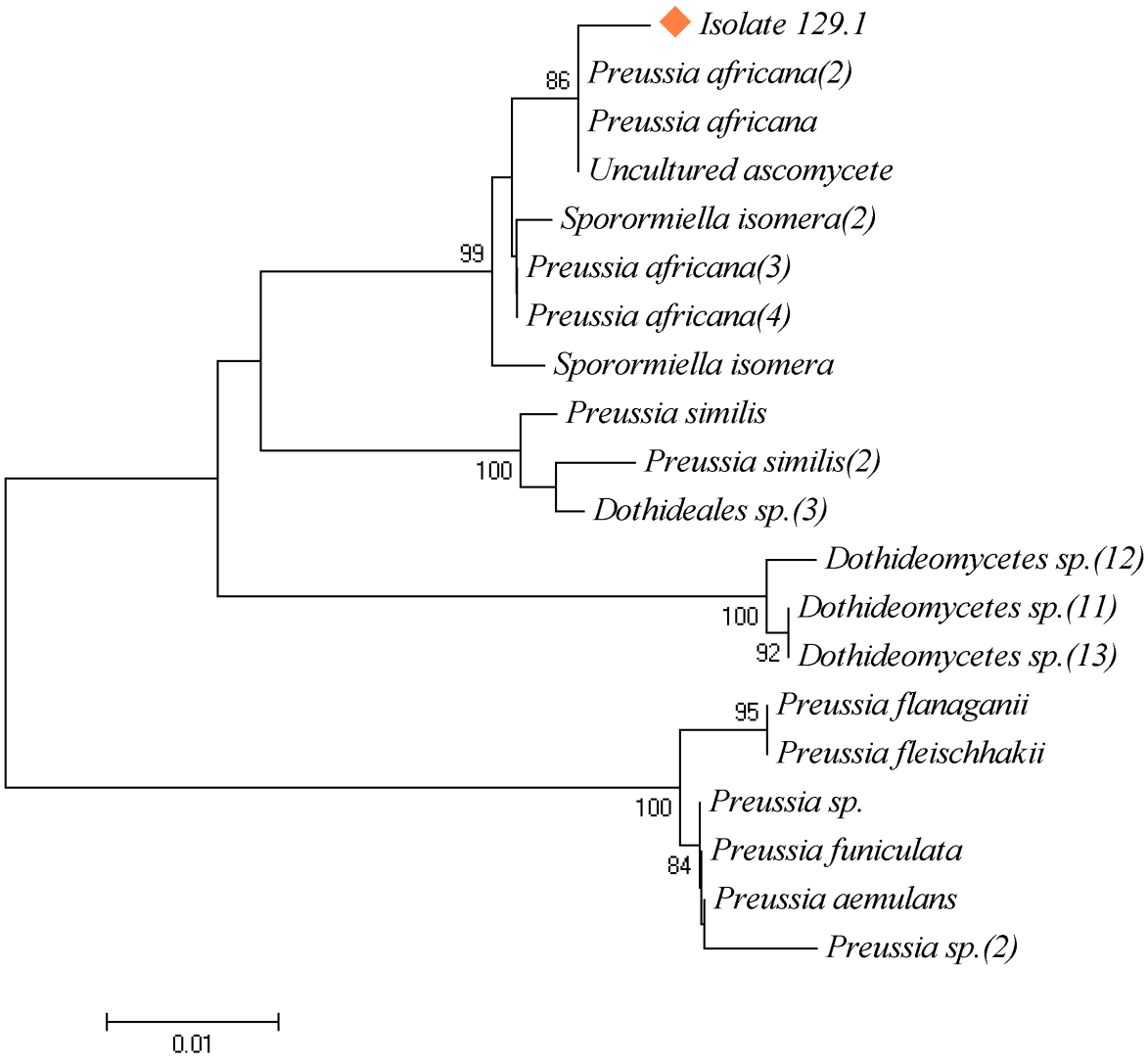


Figure 4.4: Phylogenetic tree of isolate 129.1 showing evolutionary relationships of 20 taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15633813 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 406 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. While isolate 129.1 clusters predominantly with *Preussia africana* and not *Sporormiella*, it is suggested that the two genera names can actually be used synonymously (Arenal et al. 2005).

4.4 Discussion

The three isolates demonstrating repeatable antimicrobial activity were identified as follows:

ISOLATE	IDENTIFICATION	PHYLUM
6.5	*Schizophyllum commune*	Basidiomycota
12.1	*Penicillium* sp	Ascomycota
129.1	*Sporormiella isomera*	Ascomycota

Schizophyllum commune, also known as Split Gill fungus, is generally a saprobe, and usually found on rotting wood. However, it is also known to cause disease in humans, causing sinusitis and bronchopulmonary mycosis particularly in the immunocompromised patient (Ellis et al. 2007).

Saprobes have been classed as epiphytes as well as endophytes (Bills 1996, Guo et al. 2000), and are frequently isolated from the inner tissues of leaves (Bills 1996). While it is possible isolate 6.5 was an epiphytic contaminant of the root material from which it was obtained, it is also feasible that at the time of isolation, it was present *within* the tissue as part of its normal life cycle. Thus, it potentially fulfils the original definition of endophytic fungi as previously described. In addition, it should be considered that hyphal growth from within surface sterilised tissues is sufficient proof of their endophytic status (Bills 1996).

Penicillium species are frequently isolated as endophytes. Species from this genus were the third most frequently isolated endophyte from the bark of the neem tree (*Azadirachta indica*) by Mahesh et al. (2005), making up 11.6% of the total number of isolates. Vega et al. (2006) isolated 13 different species of *Penicillium* from coffee plants (*Coffea* spp.).

Sporormiella and *Preussia* are two genera with a high degree of similarity. Differentiation has previously been based primarily on substrate and the presence/absence of ostiolate ascomata. *Sporormiella* has been associated with a coprophilous habitat, whereas *Preussia* was associated with plants, soil and wood; the former genus produces ostiolate ascomata, whereas the latter does not. However, it has been found that *Sporormiella* is not confined to dung, and has been isolated from other habitats, and production of the ostiolate ascomata is variable, often dependent on the culture conditions. Therefore it has been suggested the two genera names be used synonymously (Arenal et al. 2005). Three species of *Sporormiella* have been previously isolated as endophytes from nine plants studied by Peláez et al. (1998)

Microscopic examination can be used to identify the genus. However, the culture conditions used in this project did not induce sporulation in this isolate. As well as PDA, (Arenal et al. 2005) cultured *Preussia* species on oat meal agar, and utilised a 12 hour near-UV light/daylight cycle for a minimum of 14 days. While the lighting

regime is currently beyond the capability of our laboratory, alternative media may have been sufficient to encourage the sporulation of the isolate.

This project utilised morphological and molecular procedures to identify the three isolates previously determined to demonstrate reliable antimicrobial activity. Difficulties can be associated with each process. To reach its maximum potential, morphological identification relies on the production of sporulating structures. Molecular analysis requires the use of critical procedures, and identification relies on the availability of sequences in the GenBank database from known and correctly identified fungi. However, the methods utilised in this project were sufficient for determining the identification of the three isolates in question.

Chapter 5

Evaluation of Activity

5.1 Introduction

Screening of the endophytic isolates collected in the first stage of this project yielded three isolates possessing antimicrobial activity. The antimicrobial activities of these isolates, identified as a *Penicillium* species (isolate 12.1), *Schizophyllum commune* (isolate 6.5) and *Sporormiella isomera* (isolate 129.1), were further evaluated in the final stage of this project.

Crude preparations of each isolate and the control fungus *Penicillium chrysogenum* were prepared from cultures grown in two different media formulations. After sufficient growth had been obtained, the cultures were filtered, freeze-dried and concentrated by resuspending the freeze-dried product in water with a volume of approximately one tenth of that of the original. A dilution series was generated from each crude preparation, and used in the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against three strains of bacteria.

A second set of dilutions were prepared for use in the determination of the toxicity of the crude product against murine fibroblast cells. Evaluation of toxicity was performed using the MTT Cell Viability and Crystal Violet Dye Exclusion (CVDE) assays.

5.1.1 Sample Preparation

Both Fleming (1929) and Emerson et al. (1946) conducted further antimicrobial analysis directly on fungal culture broths; the former in his standard nutrient medium, and the latter in four different formulations of liquid media. Antimicrobial evaluation was progressively examined by removal of a small portion of the broths after various days of incubation.

Predominant sample preparations undertaken by others in the evaluation of biological activity of fungal secondary metabolites appears to involve the chemical extraction and some level of purification of the products from the culture medium (Wei et al. 1973, Schulz et al. 1995, Pongcharoen et al. 2007, Aly et al. 2008). Following incubation, liquid cultures were filtered and the filtrate chemically extracted, usually with ethyl acetate (Schulz et al. 1995, Healy et al. 2004, Davis 2005, Davis et al. 2005, Aly et al. 2008, Davis et al. 2008), n-butanol (Aly et al. 2008) or ethanol/dichloromethane mixture (Kingsland & Barrow 2009). Mycelial masses have been extracted with ethanol (Michael et al. 2003, Kingsland & Barrow 2009) or methanol (Schulz et al. 1995, Aly et al. 2008). Cultures grown on solid media were blended before being extracted with ethyl acetate (Schulz et al. 1995, Aly et al. 2008). Further purification of compounds included processes such as partitioning, fractionation, evaporation, chromatographic separation and/or dissolution in additional solvents. Thus, multiple compounds could be obtained from each culture, and biological activity determined for each.

Prior to compound extraction, Schulz et al. (1995) were able to increase the initial production of secondary metabolites produced by the *Pezicula* isolates being examined by optimising the culture medium and culture conditions for each strain.

5.1.2 Antimicrobial Activity

Minimum Inhibitory Concentration (MIC)

Fleming (1929) and Emerson et al. (1946) performed quantitative analysis of the antimicrobial activity each had detected in fungi grown in broth culture. Both prepared serial dilutions of the broth in which the fungi had been grown in fresh liquid media, and inoculated each dilution with a set volume of broth containing the test organism. After incubation, the opacity of the broths was examined to determine the highest dilution of fungal broth culture required to prevent growth of the test bacteria. This demonstrates the underlying principle behind the Minimum Inhibitory Concentration (MIC) assay (CLSI 2006).

According to the Clinical and Laboratory Standards Institute (CLSI) standard M7-A7: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Seventh Edition, two methods utilising broth and one method utilising a solid medium can be used in the determination of an agent's MIC (CLSI 2006).

The two broth methods differ mainly in the volume of broth and containment vessels used – 2ml per test tube are required for the macrodilution method, whereas 0.1ml per well in 96 well microdilution plates are required for the microdilution method. A dilution series of the test agent is prepared in the broth, and each dilution inoculated with a volume of standardised bacterial suspension. After incubation, the broths are examined for inhibition of growth as detected by the unaided eye.

The agar dilution method involves adding the test agent directly into the medium, each

plate prepared containing a different concentration of the agent. The plate surface is then point inoculated with multiple test organisms, preferably using an inoculum-replicating device. Up to 36 inocula can be tested on a single plate. After incubation, plates are examined for inhibition of growth.

The MIC is defined as the lowest concentration of an antimicrobial agent required to prevent visible growth of the test organism in a susceptibility test. The true MIC actually lies between the reported MIC value and the next lower agent concentration.

The MIC assay provides a quantitative measure of an antimicrobial agent's *in vitro* activity against a particular bacterial isolate. However, different end points can be obtained each time the test is performed, despite following carefully controlled conditions. Acceptable reproducability of the test is considered to be within one twofold dilution of the actual end point.

Minimum Bactericidal Concentration (MBC)

Fleming further evaluated his crude penicillin broth, by examining its bactericidal properties. A volume of a staphylococcal broth culture was added to tubes containing a series of dilutions of the penicillin broth. At various timed intervals, a measured quantity of the mixture was removed from each tube and spread onto solid medium. Following incubation of the agar plates, Fleming was able to estimate the rate of death in the bacteria, as well as the minimum concentration required to be effective. This remains the basic premise behind the assessment of the killing effect of an antimicrobial agent (CLSI 1999).

According to CSLI guideline M26-A: Methods for Determining Bactericidal Activity of Antimicrobial Agents, two methods can be generally followed in bactericidal assessment: MBC and time-kill.

MBC can be determined via a macrodilution or microdilution method, the methods differing in the volume of broth and containment vessels used – 2ml per tube in borosilicate glass tubes for the former, and 0.1ml per well in 96 well microdilution plates in the latter.

A series of broths containing dilutions of the test agent are inoculated with a volume of a standardised bacterial suspension. After incubation for 24 hours, a sample from each tube or well showing inhibition of bacterial growth is removed and plated so as to perform a colony count after incubation of the plate.

Time-kill analysis is established in a manner similar to the MBC tests, but with a larger volume ($\geq$ 10ml). This allows for optimal growth of the test microorganism and kill rate, as well as reducing the impact of sample removal. At timed intervals over a 24 hour period, a sample is removed, diluted and plated so as to perform a colony count 24 to 48 hours after incubation of the plate. Results obtained can be plotted and a time-kill curve prepared and the MBC determined.

The MBC is defined as the Minimum Bactericidal Concentration (MBC) required to kill 99.9% ($\geq$ 3 $\log_{10}$ drop in CFU/ml) of the final inoculum. Therefore, counts obtained from each test must be compared with a count obtained from the final standardised bacterial inoculum used to establish the test.

The advantage of the MBC over the time-kill analysis, is that it can directly follow the MIC assay – once the MIC is obtained, the cultures showing inhibition can be immediately sampled to obtain the MBC. Time-kill analysis is useful in determining the rate at which killing occurs, and can determine whether it is dependent on agent concentration or contact time.

Generally, bacteriostatic agents are sufficient for the treatment of bacterial diseases – the organism is inhibited while the body's natural defences neutralise the organism. However, in the case of immunocompromised patients, and diseases such as endocarditis and meningitis, bactericidal agents are necessary. These tests can provide an approximation of the effectiveness of an agent's ability to eradicate bacteria, but the results may differ in clinical use (CLSI 1999).

5.1.3 Cell Toxicity

Animals have been frequently utilised in the assessment of the toxicity of bioactive products (Wei et al. 1973, Tominaga et al. 1999, Anon. 2001). Fleming (1929) used a rabbit and a mouse to make initial observations that the filtered penicillin broth was not toxic. This was followed by constant irrigation of large areas of skin and repeated irrigation of the conjuctiva in two human subjects, again with no apparent signs of toxicity. Wei et al. (1973) utilised weanling albino rats in determining the toxicity levels of mycotoxins produced by *Penicillium roqueforti*. Lethality assays involving brine shrimp (*Artemia salina*) have also been used to determine the toxicity of natural products (Healy et al. 2004, Davis 2005). However, alternative methods in toxicity assessment have been developed utilising tissue culture cell viability assays (Tominaga et al. 1999, Anon. 2001).

While for the moment animals will continue to be used in cytotoxicity testing *in vivo*, the use of *in vitro* methods can be used to lower the numbers of animals used by predicting toxic endpoints. A wide variety of cell types are available though rodent- or human-derived cell lines appear to be the most frequently used (Anon. 2001). These include Balb/c 3T3 (mouse embryo) (Tominaga et al. 1999, Anon. 2001), L929 (mouse fibroblast) (Tominaga et al. 1999), NHK (normal human keratinocytes) (Anon. 2001), as well as a variety of tumour cell lines (Tominaga et al. 1999, Aly et al. 2008, Atalla et al. 2008, Davis et al. 2008) such as HeLa (human cervical cancer) (Tominaga et al. 1999, Atalla et al. 2008) and L5178 mouse lymphoma cells (Aly et al. 2008).

A variety of assays are available, and can be used to detect cell proliferation as well as cell viability. Unless a particular compound requires a specific cell type in which to express it's toxicity, no basal cytotoxicity assay can be recommended as the best assay to follow (Anon. 2001). Two of the more frequently encountered tests are the SRB

(sulforhodamine B) anticancer drug screening assay (Pongcharoen et al. 2007, Atalla et al. 2008) and the MTT cell viability assay (Tominaga et al. 1999, Morrison et al. 2001, Aly et al. 2008).

For precise measurement of the end point, 96-well microplates which can be scanned with suitably designed spectrophotometers are preferred (Anon. 2001).

In general, each well is seeded with actively growing cells usually at a rate of $5x10^3$ (Tominaga et al. 1999) to $5x10^4$ (Morrison et al. 2001, Atalla et al. 2008) cells per well. After 24 hours incubation, allowing for cell attachment and growth, the old media is replaced with fresh media incorporating the test agents and the plate reincubated for 24 (Morrison et al. 2001) to 48 hours (Tominaga et al. 1999, Atalla et al. 2008). After the appropriate time of incubation, the specific assay is run.

Final results can be reported as IC_{50} values, which is the concentration of test agent required to inhibit 50% of cell growth (Tominaga et al. 1999, Anon. 2001).

MTT Assay

The MTT assay is one method used to determine cell viability by measuring levels of cellular metabolic activity (Anon. 2001).

When in the presence of metabolically active cells, the tetrazolium salt (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) is reduced by mitochondrial dehydrogenase to produce formazan crystals (Tominaga et al. 1999, Morrison et al. 2001, Anon. 2001).

Following sufficient exposure to the test agents, an MTT solution is added to the contents of each well and the plates reincubated for three (Tominaga et al. 1999) to four hours (Morrison et al. 2001). The well contents are subsequently removed, and the adherent cells lysed to release the formazan crystals which then dissolve in the solvent used. Suitable solvents include DMSO (Tominaga et al. 1999) and 10% sodium dodecyl lauryl sulphate (SDS) in 0.01M HCl (Morrison et al. 2001).

Absorbance of each well is read at a wavelength determined by the solvent used.

CVDE Assay

The CVDE assay is routinely practiced in our laboratory for cytotoxicity assays, such as in determining the impact of TNFα concentrations on L929 cells.

Generally, following exposure to the test agents for a predetermined period of time, the media is removed from the wells and the plates gently washed. This allows dead cells to be washed away while viable cells remain adhered to the well surface. A solution containing crystal violet is added, and the stain allowed to penetrate the adherent cell

layer at room temperature for up to 30 minutes – crystal violet is absorbed by the cells' DNA. Excess stain is removed by gentle washing of the plate, and the plate allowed to dry. One molar acetic acid or other suitable solvent is added to each well to dissolve the crystal violet, and the absorbance read at a wavelength determined by the solvent used (Grekova et al. 2002).

5.1.4 Collaboration

Slant cultures of each isolate were sent to a collaborating laboratory for secondary metabolite analysis and extraction. Small amounts of penicillic acid were extracted from isolate 12.1. Since this product was well known, it was not analysed further.

Small amounts of montagnetol were extracted from isolate 129.1, a compound first extracted from a lichen-associated fungus, *Roccella montagnei* in 1942 (Rao & Seshadri 1942). Approximately 6mg of montagnetol were extracted from the isolate, and provided for analysis of antimicrobial activity.

Difficulties were encountered with isolating active products from isolate 6.5. Insufficient growth of the fungus was obtained for analysis, when the culture protocol as suggested was followed. However, alternative conditions which increased the culture matter prevented formation of the relevant secondary metabolites.

Therefore, several cultures were grown in Yeast Extract Sucrose (YES) as described in the materials and methods below. The mycelium was removed by filtration, and it and the filtrate placed into separate vacuum flasks. After freeze-drying, both flasks were sent to the collaborating laboratory. However, despite demonstrating substantial antimicrobial activity in a rapid test performed on the culture broth, the active substances could not be extracted.

5.2 Materials and Methods

5.2.1 Sample preparation

Four 100ml volumes of Nutrient broth were prepared from commercially available stocks, and prepared as per the manufacturer's instructions. Twenty 100ml volumes of YES (Yeast Extract Sucrose) broth were prepared by dissolving 40g yeast extract, 300g sucrose and 1g magnesium sulphate in 1770ml of reverse osmosis (RO) water. Both broths were dispensed into 100ml volumes into 250ml Erlenmeyer flasks, stoppered with rolled cotton wool, and the tops covered with a square of alfoil. All flasks were autoclaved at 121^{o}C for 15 minutes.

Fresh subcultures of *Penicillium chrysogenum* and isolates 6.5, 12.1 and 129.1 were prepared on fresh PDA plates. Small squares were cut into each culture, and a single

square used to inoculate each Nutrient and YES broths. Due to a variable growth pattern, 16 YES broths were inoculated with isolate 6.5, while one YES culture was prepared for the remaining fungi. One Nutrient broth only was inoculated per fungus.

All broths were initially incubated at 25^{o}C. After sufficient growth had been obtained, the cultures were transferred to 18^{o}C, to decrease the growth rate and hopefully maintain secondary metabolite production. After approximately two weeks they were held at 4^{o}C until the next stage of the process occurred.

The cultures were progressively filtered to remove the mycelium by vacuum filtration through a sterile No. 2 Whatman filter – a fresh filter was used for each culture. The filtrate was transferred to a sterile vacuum flask, which was held at -20^{o}C at a 45^o angle. During the freezing process, the flask was gradually rotated to obtain as small a frozen profile as possible. Once solidified, each flask was transferred to the -70^{o}C freezer overnight to ensure the sample was sufficiently cold for freeze drying. Each sample required approximately 30 hours to freeze dry completely.

Ten millilitres of sterile RO water was added directly to the freeze dried product in the vacuum flask, and the product allowed to redissolve. The crude preparations were stored at -20^{o}C until testing.

To ensure ingredients in the media were not responsible for any biological activity detected, concentrated solutions of Nutrient and YES broth were prepared for use as media controls. 1.3g of Nutrient broth powder was dissolved in 10ml of RO water, and 2g yeast extract, 15g sucrose and 0.05g magnesium sulphate combined with 8.85ml of RO water. Both solutions were autoclaved at 121^{o}C for 15 minutes, and stored at 4^{o}C until required. At 10 times stronger than the normal working concentration, these were equivalent in strength to the culture preparations after freeze-drying. These preparations will be referred to as concentrated media controls, with the medium specified where necessary.

The montagnetol provided by the collaborating laboratory, was resuspended in 0.5ml sterile Milli Q water. This volume was split into two 250μl aliquots, which were made up to two 3mg/ml stock solutions by the addition of 750μl of either bacterial or cell culture medium.

5.2.2 Antimicrobial activity

Minimum Inhibitory Concentration

Where applicable, the Clinical and Laboratory Standards Institute standard M7-A7: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Seventh Edition were followed in the execution of the MIC assay, with additional reference to M100-S18: Performance Standards for Antimicrobial Susceptibility Testing; Eighteenth Informational Supplement (CLSI 2006, 2008). The microdilution broth method was followed.

Two 100ml volumes of Mueller Hinton Broth (MHB) were prepared as per the manufacturer's instructions, and stored at 4^oC. Cation adjustment stocks were prepared by dissolving 0.836g magnesium chloride heptahydrate ($MgCl.7H_2O$) in 10ml RO water, and dissolving 0.368 calcium chloride dihydrate ($CaCl.2H_2O$) in a second 10ml volume of RO water. Both solutions were filter sterilised through a 0.22μm filter into sterile bottles and stored at 4^oC. For use in standardised MIC testing, the broth required adjustment of cations from 3.36mg/L Ca^{++} and 6.531mg/L Mg^{++} (levels as specified on the medium container) to a maximum level of 25mg/L Ca^{++} and 12.5mg/L Mg^{++}. Addition of 0.1ml of either cation adjustment stock increased that cation level by 1mg/L. Thus 0.217ml of cold Ca^{++} adjustment stock and 0.06ml of cold Mg^{++} adjustment stock were added to each cold 100ml volume of MHB with stirring. The cation adjusted Mueller Hinton Broth (CAMHB) was dispensed into 1ml volumes into sterile bottles with a capacity of 2.5ml, and stored at 4^oC until required.

Tryptic Soy Agar (TSA) plates were prepared as previously described, and stored at 4^oC until required

100ml of saline was prepared by dissolving 0.87g sodium chloride (NaCl) in 100ml RO water. This was dispensed into eight 10ml volumes and four 5ml volumes and autoclaved with the media.

A stock solution of 0.2M monobasic phosphate was prepared by dissolving 15.6g of sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$) in 500ml of RO water. A 0.2M dibasic phosphate stock was prepared by dissolving 28.4g of anhydrous di-sodium orthophosphate (Na_2HPO_4) in 1000ml of RO water. Six millilitres of 0.1M phosphate buffer pH 8.0 was prepared by combining 0.16ml of the monobasic stock with 2.84ml of the dibasic stock, and made up to the final volume with RO water. 0.1M phosphate buffer pH 6.0 was prepared in the same manner, combining 2.63ml and 0.34ml of each stock respectively.

Ampicillin stock (5120μg/ml) was prepared by dissolving 25.6mg of ampicillin powder in 2.5ml of 0.1M phosphate buffer pH 8.0 and made up to final volume (5ml) with 0.1M phosphate buffer pH 6.0.

A stock solution of penicillin G, sodium salt was available at 10 000 Units/ml with an activity listed by the manufacturer as 1500-1750 U/mg. This was calculated to be equivalent to 900-1050μg/mg, with the value of 1000μg/mg being selected for ease of calculations. Therefore the stock was determined to be equivalent to 6000μg/ml. To prepare an intermediate stock of 512μg/ml, 0.427ml of the 10000U/ml stock was added to 4.573ml of sterile RO water.

Both antibiotic stocks were stored at -20^oC until required.

A series of 10 doubling dilutions were prepared for each of the freeze-dried Nutrient and YES broth fungal cultures, using 1ml of inoculum and the 1ml volumes of CAMHB. The ampicillin, penicillin and montagnetol stocks were diluted with CAMHB to obtain 128μg/ml working stocks, and a set of 10 doubling dilutions prepared for each as for the cultures.

Nine sterile 96-well cell culture plates were used to establish the MIC assay. Wells were round-based, and each plate was lidded. Columns 1-11 contained the test agents, while column 12 was used for controls. Two adjacent rows were used for each sample test, so four samples were run per plate. Therefore, three plates were required to test all eight culture samples, a known antibiotic sample and the montagnetol. Two staphylococcal strains and an *E. coli* were challenged, so three sets of each plate were prepared. Every well in each plate received 100μl of the relevant sample dilution or control broth, as summarised in Table 5.1.

Table 5.1: Design summary of each MIC plate prepared.

ROW	COLUMNS 1-11	COLUMN 12
	TEST PLATE 1	
A, B	Isolate 6.5 Nutrient broth culture. Dilutions 1:1[1] to 1:1024	CAMHB
C, D	Isolate 6.5 YES broth culture. Dilutions 1:1 to 1:1024	50% NB[2]
E, F	Isolate 12.1 Nutrient broth culture. Dilutions 1:1 to 1:1024	50% YES[2]
G, H	Isolate 12.1 YES broth culture. Dilutions 1:1 to 1:1024	CAMHB
	TEST PLATE 2	
A, B	Isolate 129.1 Nutrient broth culture. Dilutions 1:1 to 1:1024	CAMHB
C, D	Isolate 129.1 YES broth culture. Dilutions 1:1 to 1:1024	100% NB[3]
E, F	*P. chrysogenum* Nutrient broth culture. Dilutions 1:1 to 1:1024	100% YES[3]
G, H	*P. chrysogenum* YES broth culture. Dilutions 1:1 to 1:1024	CAMHB
	TEST PLATE 3	
A, B	Antibiotic[4] Dilutions 128μg/ml to 0.125μg/ml	CAMHB
C, D	Montagnetol Dilutions 128μg/ml to 0.125μg/ml	CAMHB
E, F	Not used for this project	N/A
G, H	Not used for this project	N/A

[1] All 1:1 dilutions were the reconstituted freeze-dried product with no additional dilution
[2] Concentrated Nutrient or YES medium control diluted to 50% with CAMHB
[3] Concentrated Nutrient or YES medium control with no additional dilution
[4] Penicillin used for the two staphylococci and ampicillin for the *E. coli*

Once prepared, the assay plates were placed into plastic bags and stored at -20^{o}C until required, when they were allowed to thaw and come up to room temperature.

Bacteria challenged were *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, and *E. coli* ATCC 25922. After revival from -70^{o}C stock, each organism was subcultured on TSA plates at least three times before use in the test.

The bacteria were cultured on TSA for 20 hours before preparation of test organism suspensions. As each suspension had to be used within 15 minutes of preparation, each bacterial strain was processed to completion before the next one commenced.

Several colonies from the plate were added to 5ml sterile saline until the optical density was within 0.08 to 0.13 when measured at 625nm. 225μl of this suspension was added to 10ml sterile saline and transferred to a multichannel pipette reservoir. A multichannel pipette was used to dispense 20μl of the final suspension into each well in columns 1-11 of each of the three plates. 20μl of the final suspension was added to alternate wells in column 12, so that each medium control pair provided a positive (rows A, C, E, G) and negative (rows B, D, F, H) growth control.

By following the procedure described for preparation of the bacterial suspension, each well theoretically contained a final concentration of approximately 5x10^5 CFU/ml. In order to confirm the final inoculum size, a bacterial count was conducted on well 12A of test plate 1. A 10μl volume was removed from the well and added to 10ml sterile saline. After mixing, 100μl was spread over the surface of a TSA plate and allowed to soak in.

An inoculum purity check was performed by streaking a sample of the inoculum onto a TSA plate.

All plates were placed into plastic bags and incubated at 35^{o}C $\pm$ 2^{o}C for 20 hours. The inoculum purity test plate was checked to ensure each test organism remained a pure culture, and number of colonies on the inoculum size test plates counted. Finally, the MIC assay plates were read by comparing the growth obtained in well 12A with the growth obtained in the remaining wells of the plate. Results were recorded as either positive for growth present or negative for no observable growth.

Minimum Bactericidal Concentration

Guidelines for determining the bactericidal activity of antimicrobial agents (CLSI 1999) were referred to in the development of the procedure to be followed as described below.

The plates utilised for the MIC assay were subsequently used for the determination of the MBC. Following the reading of the MIC plates, wells showing no growth (i.e. demonstrating inhibition) were mixed, 10μl removed and the sample spread across half the surface of a TSA plate. One plate was required per two well samples. Only one set of wells per paired MIC assay were sampled (rows A, C, E and F from each MIC plate). All plates were bagged and incubated at 35^{o}C for 24 to 48 hours.

Following incubation, the numbers of colonies grown on each MBC assay plate were counted. Counts of 11 colonies or greater were recorded as positive for growth, while less than 11 were recorded as growth negative.

5.2.3 Cell Toxicity Assay

The murine fibroblast-like L929 cells were revived from liquid nitrogen storage and cultured in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10%

foetal calf serum at 37^{o}C in a humidified environment containing 5% carbon dioxide. The cells were passaged every 3 days, using TrypLE Express (Gibco) a Trypsin replacement enzyme to dislodge the cells. Cells were passaged three times before being used for the assay.

To prepare the test plates, the cells were resuspended in fresh medium, and a visual count performed using a haemocytometer loaded with a sample comprising a known dilution of cell suspension in 0.4% trypan blue. Following calculation of number of viable cells available, a suspension containing a final concentration of 2x10^5 viable cells/ml was prepared in 100ml of warmed DMEM.

Six, flat-based 96-well tissue culture plates were prepared for the assays. All wells on the outer edge of each plate were filled with 200μl sterile water, and the remaining wells seeded with 100μl of the prepared cell suspension. All plates were incubated overnight at 37^{o}C in a humidified environment containing 5% carbon dioxide.

Based on observations made during the antimicrobial assays, it was decided to only perform the cell toxicity assay on the crude YES broth preparations. In addition, the penicillin used in the antimicrobial assays was included, as well as a set of higher concentrations aimed at eliciting a toxic response from the antibiotic. Therefore, six agents were tested. Where necessary, dilutions tested were adjusted so as to cover the MIC and MBC levels obtained previously for each test agent.

Test agent dilutions were prepared in six V-based 96-well plates – two agents per plate; 3 rows each (B-D and E-G). Two sets of each plate were prepared – one for each assay. 100μl of fresh DMEM was added to all wells in rows B to G. 100μl of the highest test agent concentration was added to the wells containing media in column 3, and additional doubling dilutions prepared in the plate, so that the lowest test dilution was located in column 10. Therefore, only the inner 8 columns were used for test agent dilutions. A smaller dilution series was also prepared of the concentrated YES medium control. This proceeded by adding 100μl of the concentrated preparation to the media in wells 2E-G of the first plate of each assay set. Doubling dilutions were subsequently continued in wells 11B-D of the first plate, followed by wells 2E-G and 11B-D of the second plate, then wells 2E-G and 11B-D of the third plate. No additional agents were added to the remaining wells containing media. A diagram of the overall plate design can be seen in Figure 5.1.

Following the incubation of the cell culture plates, they were progressively processed, to reduce risk of damage to the cell layer. Water from wells 1B-G and 12B-G was removed, and old media carefully removed from the wells containing cells. A multi-channel pipette was then used to transfer the contents of one V-based plate, containing the diluted test agents, directly into the equivalent wells of the cell culture plate, and the plates re-incubated for up to 48 hours.

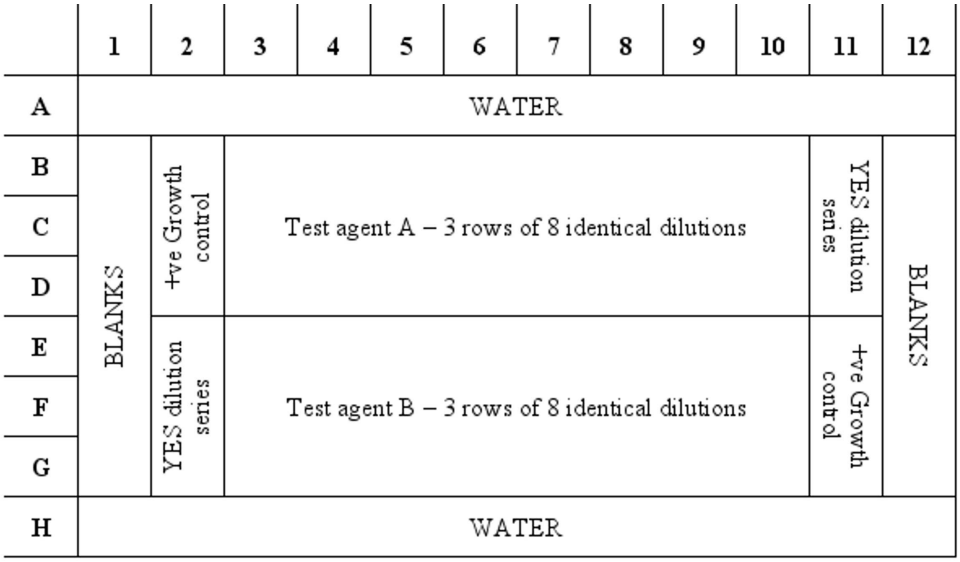


Figure 5.1: General layout of the 96-well plates prepared for the cell toxicity assay. Rows A and H contained water only. Wells for Blanks initially contained water, which was replaced with culture medium during test plate setup. Wells for the +ve growth control contained cells and medium only, while the remaining wells contained cells and medium containing dilutions of either one of the test agents or concentrated YES medium control (YES dilution series)

MTT Assay

Fresh MTT solution was prepared by dissolving 50mg of MTT in 10ml phosphate buffered saline (PBS), filtering through a 0.22μm filter into a sterile bottle, and stored at 4^{o}C until required. Acid propanol was prepared by adding 2ml of 1M hydrochloric acid to 48ml of propan-2-ol.

After incubation for approximately 44 hours, one set of three assay plates was removed from the incubator, and 20μl of MTT solution added to all wells containing media. The plates were placed on an orbital shaker and gently shaken for 5 minutes, before being returned to the incubator for a further 3.5 hours. The contents of each test well were removed, and any formazan crystals formed dissolved with the addition of 100μl acid propanol. After mixing for ten minutes on an orbital shaker, the absorbance of each well was read at 570nm with reference to 630nm.

CVDE Assay

0.2% crystal violet in 20% methanol was prepared by dissolving 0.08g of crystal violet in 8ml of 100% methanol, and diluting to 40ml with MilliQ water. One molar acetic acid was prepared by diluting 3ml of glacial acetic acid in 50ml MilliQ water.

After approximately 48 hours of incubation, the second set of three assay plates were removed from the incubator. The contents of all wells containing media were removed

and replaced with 100μl of 0.2% crystal violet in 20% methanol. The plates were placed on an orbital shaker and allowed to incubate at room temperature with gentle shaking. After 30 minutes, the stain was removed, the plates thoroughly washed with MilliQ water, and then set aside to dry. Any crystal violet which had been absorbed by the living cells was redissolved with 1M acetic acid. After 10 minutes of addition of the acid, the absorbance of each well was read at 540nm with reference to 690nm

Both assay plates were read on an anthos Zenyth 200rt microplate spectrophotometer controlled via a computer with ADAP Basic PC software. This program automatically calculated the difference between the two absorbance measurements obtained for each well. The raw data was exported and analysed with OpenOffice 3.1 calc (spreadsheet).

5.3 Results

The freeze dried products were to be initially dissolved with 10ml ethanol in order to obtain a solution containing antimicrobially active product and reduced levels of media components. However, subsequent to freeze drying it was discovered the products, particularly from the YES broths, formed into a high volume, highly absorbent matrix. An excessive volume of ethanol would have been required to obtain a sufficient volume of test solution. It was therefore decided to dissolve the freeze-dried product from both media formulations in 10ml of water.

Chemical extraction, as previously described, would have been the preferred method for sample preparation. Unfortunately, this level of compound preparation was beyond the time and scope of this project.

5.3.1 Antimicrobial activity

Minimum Inhibitory Concentration

Purity checks performed on the bacterial inocula confirmed the absence of any contamination. Bacterial counts conducted on well 12A of test plate 1 for each organism confirmed the presence of approximately 5x10^5 CFU/ml in the final mixture. Counts obtained ranged from 4.5x10^5 CFU/ml for the *E. coli* to 5.5x10^5 CFU/ml for the MRSA.

Growth obtained in the MIC assay plates appeared as either turbidity of the cell contents, or as a button on the base of the well. Firstly, the concentrated media growth controls were checked to ensure growth had occurred in the wells containing concentrated medium but no test agent. Results were recorded as “+ve” for the presence of growth or “-ve” for its absence, and can be seen in Table 5.2.

These results would suggest the increased concentration of components in the Nutrient broth did not interfere with the MIC assay, whereas the 10 times concentration of

Table 5.2: Results of media and growth controls in MIC determination.

Growth Control	*S. aureus*	MRSA	*E. coli*
CAMHB	+ve	+ve	+ve
100% NB	+ve	+ve	+ve
50% NB	+ve	+ve	+ve
100% YES	-ve	-ve	-ve
50% YES	+ve	+ve	+ve

Results were recorded as "+ve" for the presence of growth or "-ve" for its absence, and are listed for each microorganism tested. Note, when present at a 100% concentration the concentrated YES medium inhibited growth of all bacteria.

components in the YES broth did. Therefore, absence of growth in the 1:1 dilution of the freeze-dried YES broths could not be solely attributed to action by the test agent, and had to be taken into account when reading the assay.

To finally read the MIC plates the growth obtained in well 12A was compared with the growth obtained in the remaining wells of the plate. Results were recorded as either positive or negative for observable growth, and can be seen in Appendix C. The Minimum Inhibitory Concentration for each sample was obtained by recording the lowest dilution factor of the stock concentrate at which no bacterial growth occurred. Where growth was detected in every dilution tested, a negative result for inhibition was recorded. The MIC for each sample against each test organism can be seen in Tables 5.3(a) and (b).

Results obtained for the known antibiotics against the test organisms were compared with expected MIC ranges as listed in the supplement to the standard (CLSI 2008), to ensure the organisms responded appropriately. As can be seen in Table 5.4 results for ampicillin against *E. coli* were within the expected range, while those obtained for the staphylococci were not.

Due to its known resistance to penicillin, this result was expected for the MRSA. However, the other *S. aureus* should have been sensitive to penicillin. This suggested either a problem with the bacterial isolate, or a problem with the dilution of the penicillin. As a penicillin sensitivity test plate had been prepared elsewhere and demonstrated the organism was sensitive to the antibiotic, it was suspected the latter problem was more likely. A fresh penicillin sensitivity plate was prepared, and sensitivity in the bacterium demonstrated. Therefore, the suggestion of a problem with the penicillin dilutions was confirmed.

Unfortunately the montagnetol was not inhibitory at the concentrations tested. Growth occurred in all wells of the Nutrient broth preparation of isolate 129.1, while the single well showing inhibition for each organism against the YES broth culture of isolate 129.1 contained the undiluted crude preparation. As previously discussed under media growth controls, concentrated components of this medium were sufficient to inhibit the growth of the bacteria. Therefore, it was concluded that preparations from isolate

Table 5.3: Results of MIC Assay on known antibiotic, and Isolate Stock Concentrate dilutions against *S. aureus*, MRSA and *E. coli*.

Control Drug	*S. aureus*	MRSA	*E. coli*
Penicillin	0.5μg/ml	32μg/ml	NA
Ampicillin	NA	NA	4μg/ml

(a) Antibiotic controls

Isolate	Medium	*S. aureus*	MRSA	*E. coli*
6.5	NB	1:256	1:256	1:64
	YES	1:256	1:256	1:64
12.1	NB	-ve	-ve	-ve
	YES	1:2	1:2	-ve
129.1	NB	-ve	-ve	-ve
	YES	-ve	-ve	-ve
P. chrysogenum	NB	1:32	1:8	1:1
	YES	1:16	1:32	1:4
Montagnetol		-ve	-ve	-ve

(b) Test agents

96-well plates were prepared with doubling dilutions of the test agents, and the wells inoculated with the test organism. Following incubation, results were recorded as positive or negative for growth, and the minimum inhibitory concentrations for each sample determined. All bacteria were inhibited to some extent by products produced by isolate 6.5 and *P. chrysogenum*, while the YES medium preparation of isolate 12.1 inhibited the staphylococci. Note the differences in MIC outcomes between the two media formulations used to grow the fungi, an indication that medium choice plays an important role in the evaluation of secondary metabolites produced by fungi. No inhibition was detected with isolate 129.1 or montagnetol, and is recorded as "-ve": this indicates that bacterial growth occurred at every dilution tested. "NA" indicated the test solution was not applicable to that test organism.

Table 5.4: Comparison of MIC results obtained for known antibiotic standards against expected ranges provided in CLSI (2008).

ORGANISM	SENSITIVE	RESISTANT	MIC	STATUS
Penicillin				
S. aureus	$\leq 0.12\mu$g/ml	$\geq 0.25\mu$g/ml	0.5μg/ml	Resistant
MRSA			32μg/ml	Resistant
Ampicillin				
E. coli	$\leq 8\mu$g/ml	$\geq 32\mu$g/ml	4μg/ml	Sensitive

129.1 weren't inhibitory.

Low activity was measured against the staphylococci in the YES broth preparation of isolate 12.1, but not in the Nutrient broth preparation. This may mean that low activity of any secondary metabolites produced, augmented the inhibition properties of the concentrated medium to give rise to a "false" positive inhibition. Alternatively, the YES broth may have provided a better medium for encouraging the production of secondary metabolites in this isolate. The latter would also account for differences obtained between the Nutrient broth and YES broth preparations of the *P. chrysogenum*. Isolate 6.5 demonstrated the highest activity of all the isolates, and was active against all three test organisms. However, unlike the others, there appeared to be no difference in activity between the two preparations.

Minimum Bactericidal Concentration

As the MBC assay was not conducted in duplicate, and the final well inoculum in the pre-incubated MIC plates was approximately 5x10^5 CFU/ml, the figure of 11 colonies was documented in the guidelines (CLSI 1999) as the rejection value for the determination of the MBC. Therefore, MBC plate counts of 11 colonies or greater were recorded as positive, while less than 11 were recorded as negative for growth. These full results can be seen in Appendix C. The MBC for each sample was obtained by recording the lowest dilution factor of the stock concentrate which was negative for growth (equivalent to positive for bactericidal activity), and are shown in Tables 5.5(a) and (b).

Suitable MBC result ranges for ampicillin against *E. coli* ATCC 25922 were the only results supplied in the guidelines, and the result obtained in this assay (8μg/ml) fell within the range provided (4-16μg/ml).

When reading the results obtained for the MIC assay, isolate 129.1 showed inhibition only in the lowest dilution factor of the YES broth preparation. As the concentrated broth also inhibited growth at that dilution, it was determined inhibition displayed in these wells was due to the medium rather than products of the isolate. Despite this decision, one of these wells was sampled as part of the MBC assay. Once spread onto the TSA MBC test plate, it was expected that growth would occur, as the concentrated media components would effectively be diluted. This proved to be the case.

The only preparations to provide bactericidal activity were the control fungus *P. chrysogenum* and isolate 6.5 against *E. coli* only. The *P. chrysogenum* Nutrient broth preparation against *S. aureus* and both preparations against *E. coli* aligned with the MIC result – i.e. the minimum inhibitory concentration also proved to be bactericidal. All remaining bactericidal results were achieved at higher concentrations than their respective MIC results.

By examining the MIC and MBC results for each isolate preparation and organism, it appeared that when grown under the conditions and in the media previously described,

Table 5.5: Results of MBC Assay on known antibiotic or Isolate Stock Concentrate dilutions against *S. aureus*, MRSA and *E. coli*.

Control Drug	*S. aureus*	MRSA	*E. coli*
Penicillin	1μg/ml	32μg/ml	NT
Ampicillin	NT	NT	8μg/ml

(a) Antibiotic controls

Isolate	Medium	*S. aureus*	MRSA	*E. coli*
6.5	NB	1:1	-ve	1:32
	YES	-ve	-ve	1:32
12.1	NB	-ve	NT	NT
	YES	-ve	-ve	NT
129.1	NB	NT	NT	NT
	YES	-ve	-ve	-ve
P. chrysogenum	NB	1:32	-ve	1:1
	YES	1:8	1:8	1:4

(b) Test agents

10μl was removed from the negative growth MIC test wells, and spread onto agar plates. After incubation, colony numbers were counted and the minimum bactericidal concentration determined for each sample. Of the isolates, only isolate 6.5 demonstrated any bactericidal activity, especially against *E. coli*. Results for the remaining isolates demonstrated no bactericidal activity, and are recorded as "-ve"; this indicates that 11 colonies or more grew with every MIC well tested. "NT" indicated no dilutions were tested, as growth occured in all the MIC test wells.

isolate 129.1 showed no antimicrobial activity; isolate 12.1 had a minor inhibitory effect on the two staphylococcal species; and isolate 6.5 had an inhibitory effect on all three organisms but no bactericidal effect on the staphylococci. This would suggest the latter's effect on staphylococci was bacteriostatic, but bactericidal against *E. coli*.

What is not known is whether these results are due to the presence of one or more secondary metabolites, or in the case of the latter, due to different metabolites.

5.3.2 Cell Toxicity Assay

Despite its inhibitory effect on bacterial cells, it was decided to perform the cell toxicity assays on the crude YES broth preparations only, as they had generally provided a slightly better result in the antimicrobial assays than the Nutrient broth preparations.

As fewer agent concentrations were able to be tested in the toxicity assay than the

antimicrobial assay, dilutions tested for isolate 6.5 were adjusted so as to cover the MIC and MBC levels obtained previously. Therefore, dilutions covered the range of 1:8 to 1:1024 for isolate 6.5, and 1:2 to 1:256 for the remaining fungal preparations.

Mean values and standard deviation were calculated from the raw data obtained from the readings of each plate. Each test agent's results and those for the positive growth control were adjusted for the blank, and the percent viability calculated by the formula $\frac{OD_{testagent}}{OD_{growthcontrol}}$ x 100. This process was repeated for the YES medium control dilutions. Raw data and calculations can be seen in Appendix D.

Toxicity was determined as the test agent concentration at which 50% of the cells were no longer viable, expressed as the IC_{50} value.

As a reference chemical, penicillin was listed as having an IC_{50} of 5.73mmol/L (Anon. 2001) which is equivalent to 2.04mg/ml. Unfortunately, using the stock penicillin of 6mg/ml did not allow for a large range of high dilutions, and a clear result was not obtained in either assay. However, there was a decrease in the percentage of viable cells at the first dilution tested (3mg/ml) (MTT: 58% viable; CVDE: 70% viable) and the second dilution in the MTT assay (1.5mg/ml: 76% viable). These results may confirm a problem with the penicillin stock.

Results for the YES medium control were analysed first, to determine the presence of any effect caused by the fungal culture medium. As can be seen in Table 5.6, presence of 25% (CVDE) to 12.5% (MTT) of the medium control was sufficient to have a deleterious effect on the cells.

Therefore, results for determination of toxicity in the isolate tests were restricted to those containing less than 12.5% of the medium. These results can be seen in Table 5.7 for isolates 12.1 and 129.1, and Table 5.8 for isolate 6.5.

From these results it was determined that at least one secondary metabolite produced by isolates 6.5 and 12.1 were toxic to L929 cells. In the case of the former, this toxicity covered the entire range of dilutions tested, whilst toxic levels were detected at a dilution factor of 1:16 for isolate 12.1

Results which indicate a viability of greater than 100% can indicate an agent contributes to cell proliferation. However, in this case it is considered that these results highlight the variability inherent in living culture assays.

5.4 Discussion

Because they have already proven to be a successful base for production of antimicrobially active secondary metabolites by the relevant isolates, the media utilised for this part of the project should have been identical to those used in the initial screening, namely Nutrient Broth and Sensitest Agar. While the former was used, at the time it

Table 5.6: Determination of toxic effect on L929 cells as caused by the concentrated YES medium control.

Component Percent	50.00	25.00	12.50	6.25	3.13	1.57
CVDE						
Mean	1.4020	0.5703	1.8700	2.8457	3.1913	3.2590
SD	0.1057	0.0248	1.0830	0.5092	0.0863	0.0229
% Viability	**40.76**	**13.65**	54.59	85.61	96.70	99.46
MTT						
Mean	0.0853	0.0727	0.0987	0.6463	0.6493	0.9130
SD	0.0189	0.0065	0.0287	0.0295	0.1607	0.0806
% Viability	**10.35**	**7.71**	**10.90**	88.62	96.89	118.61

Results show the mean optical density, measured at 570nm with reference at 630nm for MTT results, and 540nm with reference at 690nm for the CVDE assay, standard deviation (SD) and the calculated value for percent viability. Toxicity was determined as the agent concentration at which 50% of the cells were no longer viable. These figures are highlighted in the results in bold. As can be seen in the MTT results, when the L929 cells were exposed to the concentrated YES medium at a concentration of 12.5 percent, almost 90 % were destroyed. This had to be taken into consideration when determining the results of the test agents.

was considered to be too difficult to obtain sufficient extracted test material from the agar. This would have involved the culturing of many plates of each isolate, followed by chemical extraction, filtration and evaporation of excess solvent. As time was limited, it was decided to use an alternative liquid medium. Yeast Extract Sucrose Broth was selected for this purpose. Unfortunately, this meant the suitability of Sensitest Agar as a medium useful in the production of secondary metabolites by fungi could not be verified.

As noted earlier, the freeze dried products were to be initially dissolved with 10ml ethanol in order to obtain a solution containing antimicrobially active product and reduced levels of media components. This proved difficult, as the products, particularly from the YES broths, formed into a high volume, highly absorbent matrix, requiring significantly greater volumes of ethanol to ensure complete dissolution of the product. With sufficient time, this would not have been an issue, as the excess ethanol could have been evaporated to produce the required volume of test sample. The benefits of this process would have been two-fold – the elimination of the toxic components of the YES medium; and the production of an optimal volume/maximum concentration of the test sample for testing. However, sufficient time was not available, hence the decision to dissolve the freeze-dried product from both media formulations in 10ml of water.

The results obtained in the evaluation of bioactivity in the water-based test samples are summarised in Table 5.9.

Table 5.7: Determination of toxic effect caused by the test agents on L929 cells.

Dilution factor	1:16	1:32	1:64	1:128	1:256
ISOLATE 12.1			MTT		
Mean	0.2567	0.3573	0.6120	0.7070	0.6753
SD	0.0199	0.0565	0.1161	0.1260	0.1029
% Viability	**36.16**	51.33	89.70	104.02	99.25
			CVDE		
Mean	1.2727	2.1263	3.1417	3.1810	3.2403
SD	0.0280	0.0903	0.0775	0.0135	0.0384
% Viability	**36.65**	63.78	96.04	97.29	99.17
ISOLATE 129.1			MTT		
Mean	0.6120	0.8097	0.7037	0.7333	0.7757
SD	0.0234	0.0231	0.0506	0.0696	0.0492
% Viability	83.83	111.38	96.61	100.74	106.64
			CVDE		
Mean	2.9700	3.1947	3.2467	3.2880	3.2903
SD	0.0308	0.0344	0.0663	0.0139	0.0150
% Viability	89.64	96.92	98.61	99.95	100.02
P. chrysogenum			MTT		
Mean	0.4833	0.6823	0.7023	0.6423	0.8153
SD	0.0664	0.0561	0.0992	0.0281	0.0306
% Viability	61.03	86.97	89.57	81.75	104.30
			CVDE		
Mean	2.8647	3.1250	3.2527	3.2603	3.2700
SD	0.0912	0.0331	0.0180	0.0468	0.0241
% Viability	86.24	94.52	98.58	98.82	99.13

Results show the mean optical density measured at 570nm with reference at 630nm for MTT results, and 540nm with reference at 690nm for the CVDE assay, standard deviation (SD) and percent viability calculated on the blank-adjusted optical densities by the formula $\frac{OD_{testagent}}{OD_{growthcontrol}}$ x 100. Toxicity was determined as the agent concentration at which 50% of the cells were no longer viable, and is highlighted in bold in the results. This can be seen in the results obtained for isolate 12.1, which demonstrated toxicity to the cells at a dilution of 1:16. No toxicity was detected at these dilutions for the remaining test agents. Results for the first three dilutions tested were not included here, due to the toxic effect caused by the culture medium at those levels.

Table 5.8: Determination of toxic effect of secondary metabolites produced by isolate 6.5 on L929 cells.

Component percent	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
ISOLATE 6.5				MTT			
Mean	0.0177	0.0153	0.0630	0.2887	0.3810	0.2980	0.2547
SD	0.0012	0.0015	0.0139	0.0227	0.0095	0.0092	0.0236
% Viability	0.27	-0.05	6.40	36.91	49.39	38.17	32.31
				CVDE			
Mean	0.4050	0.4533	0.3677	1.1593	1.9530	1.2587	1.0293
SD	0.0243	0.0374	0.0215	0.2176	0.1326	0.0295	0.0627
% Viability	8.46	9.98	7.29	32.13	57.03	35.25	28.05

Results show the mean optical density measured at 570nm with reference at 630nm for MTT results, and 540nm with reference at 690nm for the CVDE assay, standard deviation (SD) and percent viability calculated on the blank-adjusted optical densities by the formula $\frac{OD_{testagent}}{OD_{growthcontrol}}$ x 100. Toxicity was determined as the agent concentration at which 50% of the cells were no longer viable. Note that this is the case in every dilution tested for this isolate

Table 5.9: Summary of results obtained in the evaluation of biological activity of the three main isolates

ISOLATE	6.5	12.1	129.1
IDENTIFICATION	*S. commune*	*Penicillium* sp.	*S. isomera*
MIC - Staphylococci	1:256	1:2	No activity
MIC - *E. coli*	1:64	-ve	No activity
MBC - Staphylococci	1:1	-ve	No activity
MBC - *E. coli*	1:32	-ve	No activity
IC_{50}	$<$1:1024	1:16 (to 1:32)	Little activity
TOXICITY STATUS	Toxic	Some toxicity	No activity

No inhibition against *E. coli* and no bactericidal activities against any test bacterium were displayed by isolate 12.1, and are indicated by a "-ve" in the results. No biological activity was demonstrated by isolate 129.1, while isolate 6.5 demonstrated the highest levels of activity of all agents tested. Toxicity status is an arbitrary assignment given to each isolate in order to give meaning to the IC_{50} result.

Isolate 129.1, *Sporormiella isomera*, appeared to lose its ability to produce antimicrobially active secondary metabolites. This may be a result of repeated subculturing, despite attempts to minimise the number of sequential subcultures, or may be a spontaneous occurance. It is not an unusual phenomenon – others have found the same effect in strains of *Penicillium chrysogenum* (Wainwright & Swan 1986).

Sporormiella species have some history of producing bioactive natural products. Two compounds have been isolated from a strain of *S. minima* by Hatori et al. (2004). These particular compounds demonstrated promise as inhibitors of cholesterol synthesis, and were antifungal against *Candida albicans* and *Aspergillus fumigatus*.

The *Penicillium* species, isolate 12.1, showed some bioactivity, but not as much as the control fungus, *P. chrysogenum*, used in the experiments.

Isolate 6.5, *Schizophyllum commune* demonstrated promising antimicrobial activity, particularly against MRSA, with a dilution of 1:256 inhibiting growth of the organism.

Research conducted by Rosa et al. (2003), concentrated on the screening of basidiomycetes for antimicrobial activity. This included three strains of *S. commune* located in Brazil. A range of bacteria were screened including several common to this project. However, no antimicrobial activity was detected in any of these strains. This could indicate a difference between their *S. commune* strains and the one isolated in this project, or culture medium and growth conditions utilised by the researchers were not effective. Emerson et al. (1946) found great differences in the antibiotic activities obtained from soil-borne fungal isolates when grown in four different formulations of liquid media.

However, the toxicity assay for isolate 6.5 revealed its toxic nature, with the highest dilution tested being capable of killing over 50% of L929 cells. Therefore, while it demonstrated useful antimicrobial activity, its toxicity would prevent its immediate use as such.

Chapter 6

Summary of Results

A total of three fungal isolates were determined to produce secondary metabolites posessing antimicrobial activity, and were therefore subjects of the full range of procedures encountered in this project. A summary of the information and results obtained for each of these isolates is provided on the following pages.

6.1 Isolate 6.5

Identified by molecular techniques as *Schizophyllum commune*
Phylum: Basidiomycota
Isolated from *Lantana camara* root obtained from Preston.

Antimicrobial screening profile:
"+" indicates inhibition observed; "-" indicates no inhibition observed

S. aureus	MRSA	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B. cereus*
+	+	+	+	-	-	+

MIC and MBC:
"-ve" indicates no bactericidal activity observed

	MEDIUM	*S. aureus*	MRSA	*E. coli*
MIC	NB	1:256	1:256	1:64
	YES	1:256	1:256	1:64
MBC	NB	1:1	-ve	1:32
	YES	-ve	-ve	1:32

Antimicrobial status: Bacteriostatic against staphylococci
Bactericidal against *E. coli*

Cytotoxicity:

Assay	MTT	CVDE
IC_{50}	<1:1024	<1:1024
Toxicity status	Toxic	

6.2 Isolate 12.1

Identified by morphological techniques as *Penicillium* sp.
Phylum: Ascomycota
Isolated from *Acacia fimbriata* root obtained from Preston

Antimicrobial screening profile:
"+" indicates inhibition observed; "-" indicates no inhibition observed

S. aureus	MRSA	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B. cereus*
+	+	+	-	-	-	+

MIC and MBC:
"-ve" indicates none of the relevantl activity was observed;
"NT" indicates procedure not tested

	Medium	*S. aureus*	MRSA	*E. coli*
MIC	NB	-ve	-ve	-ve
	YES	1:2	1:2	-ve
MBC	NB	-ve	NT	NT
	YES	-ve	-ve	NT

Antimicrobial status: Bacteriostatic against staphylococci
No activity against *E. coli*

Cytotoxicity:

Assay	MTT	CVDE
IC_{50}	1:16	1:16
Toxicity status	Some toxicity	

6.3 Isolate 129.1

Identified by molecular techniques as *Sporormiella isomera*
Phylum: Ascomycota
Isolated from *Xanthorrhoeae johnsonii* leaf obtained from Preston

Antimicrobial screening profile:
"+" indicates inhibition observed; "-" indicates no inhibition observed

S. aureus	MRSA	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. marcescens*	*B. cereus*
+	+	+	-	-	-	-

MIC and MBC:
"-ve" indicates none of the relevant activity was observed;
"NT" indicates procedure not tested

	Medium	*S. aureus*	MRSA	*E. coli*
MIC	NB	-ve	-ve	-ve
	YES	-ve	-ve	-ve
MBC	NB	NT	NT	NT
	YES	-ve	-ve	-ve

Antimicrobial status: No activity detected

Cytotoxicity:
"-" indicates no toxicity detected

Assay	MTT	CVDE
IC_{50}	-	-
Toxicity status	No activity	

Chapter 7

Conclusion

The purpose of this project was the discovery and evaluation of novel antimicrobial agents affective against nosocomial pathogens, and was divided into four distinct areas of investigation:

Acquisition of Fungal Isolates The goal of the first phase of the project was the acquisition of a sufficient number and variety of fungal isolates. While fungi are ubiquitous, it was decided to concentrate efforts on the collection of endophytic fungi from a variety of mostly native plant hosts. Harvested plant tissues were washed, surface sterilised and portions transferred to suitable growth media. As isolates grew, they were subcultured until pure cultures were obtained, cultured onto slants for short term use, and longer term storage preparations made.

A total of 133 fungal cultures were isolated, with over 90% being moulds. Macroscopic examination suggested that over 100 fungal species were represented, including species of *Fusarium*, *Epicoccum*, *Aspergillus* and *Penicillium*. Almost 92% of these isolates grew sufficiently enough to be incorporated into the isolate collection.

Screening for Antimicrobial Activity The second stage of the project involved the screening of the fungal isolates collected during the first phase against a panel of selected nosocomial pathogens. After a suitable screening method was determined, the fungal isolates were progressively subcultured from the slants, and if successfully grown, screened for antimicrobial activity.

Of the 122 isolates in storage, 13 were not tested as they were macroscopically identified as yeasts. Of the 109 isolates remaining for screening, 27 (24.7%) did not recover from PDA slant storage; 3 (2.8%) did not grow directly on the test medium and 2 (1.8%) grew so quickly and heavily that the bacteria could not be applied to the plate surface. Seventy-seven (70.6%) isolates were successfully screened using the direct Sensitest screening procedure.

Seven isolates demonstrated some level of activity against the selected microorganisms. However, in the case of four of the isolates, the results were not repeatable. Therefore, the screening process yielded three isolates whose an-

timicrobial activity was investigated further. Based on the plant sample of origin, and the number of isolates obtained from that sample, these isolates were designated as numbers 6.5, 12.1 and 129.1.

Identification of Selected isolates The three isolates which demonstrated antimicrobial activity against one or more of the test bacteria required the determination of their scientific classification, so as not to re-evaluate a fungus which had been previously described and assessed. Morphological characteristics, both macroscopic and microscopic, were initially studied, and were sufficient to determine the genus of isolate 12.1 as that of *Penicillium*. Molecular techniques were required to obtain identification of the remaining isolates, which were ultimately identified as *Schizophyllum commune* (isolate 6.5) and *Sporormiella isomera* (isolate 129.1).

Evaluation of Activity The final part of the project involved the evaluation of the antimicrobial activity and the assessment of potential toxicity of crude preparations from the three selected isolates. The MIC and MBC were determined for the fungal preparations against three strains of bacteria. Toxic effects of the crude preparations were tested on murine fibroblasts and evaluated using the MTT Cell Viability and Crystal Violet Dye Exclusion (CVDE) assays. Cytotoxicity results were expressed as the IC_{50}, the concentration at which 50% of the cells were no longer viable.

Following the final part of the project, it was determined that isolates 6.5 and 12.1 could produce secondary metabolites that were capable of demonstrating bacteriostatic activity against staphylococcal species. In addition, products manufactured by isolate 6.5 proved to be bactericidal against *E. coli*. The antimicrobial screening process also indicated both isolates possessed activity against *B. cereus*, but this was not investigated further at this stage.

Initial screening of isolate 129.1 demonstrated antimicrobial activity against staphylococci. However, this activity was not repeated during MIC or MBC evaluation. Numerous reasons may be used to explain this occurance, including spontaneous halt in the production of the relevant secondary metabolite by the fungus; subtle differences in culture conditions; or a requirement for bacterial inducement. Continued evaluation would be required to determine the actual cause.

Any secondary metabolites produced by isolate 129.1, under the culturing methods utilised for bioactivity evaluation, did not display any cytotoxicity against the fibroblast-like L929 cells. Some cytotoxicity was detected with isolate 12.1, while products of isolate 6.5 proved to be highly toxic. The highest dilution tested was able to kill greater than 50% of test cells.

Attempts were made by a collaborating laboratory to analyse and extract the secondary metabolites produced by isolates 6.5, 12.1 and 129.1. Small amounts of penicillic acid were extracted from isolate 12.1. Since the product was well known, it was not analysed further.

Approximately 6mg of monagnetol was extracted from isolate 129.1. This was evaluated for antimicrobial activity, but none was detected at this concentration.

To date, no secondary products have been extracted from isolate 6.5. Continued attempts will be made, as further analysis of the isolate's secondary metabolites may reveal multiple products, with varying levels of activity and toxicity, novel modes of action, or novel structures. Separation of the active products could allow for further investigations, and if combinatorial chemistry is utilised, their structures may be manipulated in order to reduce the levels of toxicity.

All isolates collected over the course of the project remain stored in glycerol at -70^{o}C. Therefore, screening can be repeated with additional bacterial species and alternative screening methods can be employed so as to screen other microorganisms, including pathogenic yeasts and fungi. Not only does this work potentially contribute to the discovery of novel antimicrobial products, but as so few fungi have been described, the identification of the fungi so far isolated could reveal new species, and provide additional information about fungal diversity.

The increasing rate in the development of antibiotic resistance by pathogenic organisms means new antibiotics need to be sourced quickly, so that current standards in health care and disease treatment can be maintained. It has been shown by this study and numerous others, that endophytic fungi can be a valuable source in the search for these new antimicrobials. Their variable hosts, habitats, and history, coupled with the sheer number of undiscovered species, suggests that numerous discoveries are yet to be made.

As long as bacteria continue to develop resistance to the antibiotics we use to treat their infections, we will need to source new antibiotics. The continued isolation, screening and evaluation of endophytic fungi and their secondary metabolites can only be of benefit to all higher life forms on Earth.

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Appendix A

Acronyms

BSC Biological Safety Cabinet

CAMHB Cation adjusted Mueller Hinton Broth

CVDE Crystal Violet Dye Exclusion

DMSO di-methyl sulfoxide

IPTG Isopropyl-β-D-1-thiogalactopyranoside

ITS internal transcribed spacer

LB Luria-Bertoni

MEA Malt Extract Agar

MBC Minimum Bactericidal Concentration

MHB Mueller Hinton Broth

MIC Minimum Inhibitory Concentration

MRSA methicillin-resistant *Staphylococcus aureus*

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide)

NA Nutrient Agar

NB Nutrient Broth

PDA Potato Dextrose Agar

RO reverse osmosis

SDA Sabouraud Dextrose Agar

TSA Tryptic Soy Agar

VRE vancomycin-resistant Enterococci

X-Gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

YES Yeast Extract-Sucrose

Appendix B

Media Formulations

Note: All media autoclaved at 121^{o}C for 15 minutes unless specified All ingredients listed as per litre unless specified

Czapek Dox

Czapek Dox Broth

Supplier:	Difco
Bacto Laboratories, Mt Pritchard, NSW, Australia	
Saccharose	30g
Sodium nitrate ($NaNO_3$)	3g
di-Potassium hydrogen orthophosphate (K_2HPO_4)	1g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5g
Potassium chloride (KCl)	0.5g
Ferric sulphate ($FeSO_4.7H_2O$)	0.01g
35g per 1000ml	

Czapek Yeast Broth

Supplier:	Difco (Base Broth as above)
Yeast Extract	5g
Trace Metals	1ml

Trace Metals Solution:

Zinc sulphate ($ZnSO_4.7H_2O$)	1% w/v
Copper sulfate ($CuSO_4.5H_2O$)	0.5% w/v

Luria-Bertoni

LB Agar

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Casein Peptone Pancreatic Digest	10g
Sodium chloride	5g
Yeast Extract	5g
Agar, bacteriological	15g

35g per 1000ml

LB Broth

Supplier: Difco
Bacto Laboratories, Mt Pritchard, NSW, Australia

Pancreatic Digest of Casein	10g
Yeast Extract	5g
Sodium chloride	10g

25g per 1000ml

Malt Extract

Malt Extract Agar

Supplier: Oxoid
Oxoid Australia, Adelaide, SA, Australia

Malt Extract	30g
Mycological Peptone	5g
Agar	15g

50g per 1000ml

Malt Extract Broth

Supplier: Oxoid
Oxoid Australia, Adelaide, SA, Australia

Malt Extract	17g
Mycological Peptone	3g

20g per 1000ml

Mueller-Hinton Broth

Supplier: Oxoid
Oxoid Australia, Adelaide, SA, Australia

Beef, dehydrated infusion from	300g
Casein hydrolysate	17.5g
Starch	1.5g

21g per 1000ml

Nutrient

Nutrient Agar

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Bacteriological Peptone	5g
Sodium chloride	5g
Yeast Extract	2g
Beef Extract	1g
Agar, bacteriological	13g

26g per 1000ml

Nutrient Broth

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Bacteriological Peptone	5g
Sodium chloride	5g
Yeast Extract	2g
Beef Extract	1g

13g per 1000ml

Potato Dextrose Agar

Supplier: BD Difco
Bacto Laboratories, Mt Pritchard, NSW, Australia

Potato Infusion from 200g potato	4g
Dextrose	20g
Agar	15g

39g per 1000ml

Sabouraud Dextrose Agar

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Mycological Peptone	10g
Glucose	40g
Agar	14g

64g per 1000ml

Sensitest Agar

Supplier: Oxoid
Oxoid Australia, Adelaide, SA, Australia

Hydrolysed casein	11.0g
Peptone	3.0g
Sodium chloride	3.0g
Glucose	2.0g
Starch	1.0g
Buffer salts	3.3g
Nucleoside bases	0.02g
Thiamine	0.00002g
Agar	8.0g

32g per 1000ml

Tryptic Soy

Tryptic Soy Agar

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Casein Peptone Pancreatic Digest	15g
Soy Peptone	5g
Sodium chloride	5g
Agar	13g

38g per 1000ml

Tryptic Soy Broth

Supplier: Amyl Media
Amyl Media, Dandenong, VIC, Australia

Casein Peptone Pancreatic Digest	17g
Soy Peptone	3g
di-potassium hydrogen orthophosphate	2.5g
Sodium chloride	5g
Glucose	2.5g

30g per 1000ml

Yeast Extract-Malt Agar

Supplier: Prepared In-house

Yeast Extract	4g
Malt Extract	10g
Glucose	4g
Agar	20g

per 1000ml

Yeast Extract-Sucrose Broth

Supplier:	Prepared In-house
Yeast Extract	20g
Sucrose	150g
Magnesium Sulfate	0.5g
R.O. Water	885ml

Dissolve and dispense before autoclaving

Appendix C

MIC and MBC Data

The tables on the following pages show the data obtained for evaluation of the MIC and MBC of each test agent against the three test bacteria, as described in Chapter 5. The general layout of each table follows that of the 96 well plates used for the MIC assay. Each well in columns 1 to 11 contained a dilution of a test agent, and a standard volume of test organism. Column 12 contained media with no test agents. “Medium +” were inoculated with the test organism; “Medium -” were not inoculated at all. Following incubation, the plates were scored for presence or absence of bacterial growth. Test wells which had been inoculated with bacteria and showed no growth were plated to determine if the bacteria were inhibited or eliminated.

Eash test agent was tested twice, in adjacent rows. Therefore, in able to refer which MIC wells were sampled to determine the MBC, the MBC results are displayed under each test agent’s paired rows. Wells which were not sampled as part of the MBC assay are indicated with a “/”.

The MIC is defined as the lowest concentration of an antimicrobial agent required to prevent visible growth of the test organism. The true MIC actually lies between the reported MIC value and the next lower agent concentration. The MBC is defined as the Minimum Bactericidal Concentration (MBC) required to kill 99.9% ($\geq$ 3 $\log_{10}$ drop in CFU/ml) of the final inoculum.

Tables C.1(a-c) show data related to E. coli; Tables C.2(a-c)show data related to *S. aureus*, and Tables C.3(a-c) show data related to MRSA.

Table C.1: MIC and MBC data for each test preparation against *E. coli*. For the MIC data,"+" indicates growth of the bacterium; "-" indicates no growth observed. For the MBC data, "+" indicates 11 colonies or greater were counted on the test plates; "-" indicates less than 11 colonies were observed; "/" indicates well contents were not tested as growth had occured in the wells in the MIC test.

	Column	1	2	3	4	5	6	7	8	9	10	11		12
Row	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 6.5	-	-	-	-	-	-	-	+	+	+	+	CAMHB +	+
B	NB	-	-	-	-	-	-	-	+	+	+	+	CAMHB -	-
	MBC	-	-	-	-	-	-	+	/	/	/	/		
C	Isolate 6.5	-	-	-	-	-	-	-	+	+	+	+	1:2 NB +	+
D	YES	-	-	-	-	-	-	-	+	+	+	+	1:2 NB -	-
	MBC	+	-	-	-	-	-	+	/	/	/	/		
E	Isolate 12.1	+	+	+	+	+	+	+	+	+	+	+	1:2 YES +	+
F	NB	+	+	+	+	+	+	+	+	+	+	+	1:2 YES -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		
G	Isolate 12.1	-	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
H	YES	-	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	+	/	/	/	/	/	/	/	/	/	/		

(a) *E. coli*, plate 1

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 129.1	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
B	NB	+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		
C	Isolate 129.1	-	+	+	+	+	+	+	+	+	+	+	1:1 NB +	+
D	YES	-	+	+	+	+	+	+	+	+	+	+	1:1 NB -	-
	MBC	+	/	/	/	/	/	/	/	/	/	/		
E	*P. chrysogenum*	-	+	+	+	+	+	+	+	+	+	+	1:1 YES +	-
F	NB	-	+	+	+	+	+	+	+	+	+	+	1:1 YES -	-
	MBC	-	/	/	/	/	/	/	/	/	/	/		
G	*P. chrysogenum*	-	-	-	+	+	+	+	+	+	+	+	CAMHB +	+
H	YES	-	-	-	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	-	-	-	/	/	/	/	/	/	/	/		

(b) *E. coli*, plate 2

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Conc. μg/ml	128	64	32	16	8	4	2	1	0.5	0.25	0.13	Controls	
A	Ampicillin	-	-	-	-	-	-	+	+	+	+	+	CAMHB +	+
B		-	-	-	-	-	-	+	+	+	+	+	CAMHB -	-
	MBC	-	-	-	-	-	+	/	/	/	/	/		
C	Montagnetol	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
D		+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		

(c) *E. coli*, plate 3

Table C.2: MIC and MBC data for each test preparation against *E. coli*. For the MIC data,"+" indicates growth of the bacterium; "-" indicates no growth observed. For the MBC data, "+" indicates 11 colonies or greater were counted on the test plates; "-" indicates less than 11 colonies were observed; "/" indicates well contents were not tested as growth had occured in the wells in the MIC test.

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 6.5	-	-	-	-	-	-	-	-	-	+	+	CAMHB +	+
B	NB	-	-	-	-	-	-	-	-	-	+	+	CAMHB -	-
	MBC	+	+	+	+	+	+	+	+	+	/	/		
C	Isolate 6.5	-	-	-	-	-	-	-	-	-	+	+	1:2 NB +	+
D	YES	-	-	-	-	-	-	-	-	-	+	+	1:2 NB -	-
	MBC	+	+	+	+	+	+	-	-	+	/	/		
E	Isolate 12.1	+	+	+	+	+	+	+	+	+	+	+	1:2 YES +	+
F	NB	-	+	+	+	+	+	+	+	+	+	+	1:2 YES -	-
	MBC	+	/	/	/	/	/	/	/	/	/	/		
G	Isolate 12.1	-	-	+	+	+	+	+	+	+	+	+	CAMHB +	+
H	YES	-	-	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	+	+	/	/	/	/	/	/	/	/	/		

(a) *S. aureus*, plate 1

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 129.1	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
B	NB	+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		
C	Isolate 129.1	-	+	+	+	+	+	+	+	+	+	+	1:1 NB +	+
D	YES	-	+	+	+	+	+	+	+	+	+	+	1:1 NB -	-
	MBC	+	/	/	/	/	/	/	/	/	/	/		
E	*P. chrysogenum*	-	-	-	-	-	-	+	+	+	+	+	1:1 YES +	-
F	NB	-	-	-	-	-	-	+	+	+	+	+	1:1 YES -	-
	MBC	+	+	+	+	-	-	/	/	/	/	/		
G	*P. chrysogenum*	-	-	-	-	-	+	+	+	+	+	+	CAMHB +	+
H	YES	-	-	-	-	-	+	+	+	+	+	+	CAMHB -	-
	MBC	-	-	-	-	-	/	/	/	/	/	/		

(b) *S. aureus*, plate 2

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Conc. μg/ml	128	64	32	16	8	4	2	1	0.5	0.25	0.13	Controls	
A	Penicillin	-	-	-	-	-	-	-	-	+	+	+	CAMHB +	+
B		-	-	-	-	-	-	-	-	-	+	+	CAMHB -	-
	MBC	-	-	-	-	-	-	-	-	+	/	/		
C	Montagnetol	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
D		+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		

(c) *S. aureus*, plate 3

Table C.3: MIC and MBC data for each test preparation against *E. coli*. For the MIC data,"+" indicates growth of the bacterium; "-" indicates no growth observed. For the MBC data, "+" indicates 11 colonies or greater were counted on the test plates; "-" indicates less than 11 colonies were observed; "/" indicates well contents were not tested as growth had occured in the wells in the MIC test.

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 6.5	-	-	-	-	-	-	-	-	-	+	+	CAMHB +	+
B	NB	-	-	-	-	-	-	-	-	-	+	+	CAMHB -	-
	MBC	+	+	+	+	+	+	+	+	+	/	/		
C	Isolate 6.5	-	-	-	-	-	-	-	-	-	+	+	1:2 NB +	+
D	YES	-	-	-	-	-	-	-	-	-	+	+	1:2 NB -	-
	MBC	+	+	+	+	+	+	-	-	+	/	/		
E	Isolate 12.1	+	+	+	+	+	+	+	+	+	+	+	1:2 YES +	+
F	NB	+	+	+	+	+	+	+	+	+	+	+	1:2 YES -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		
G	Isolate 12.1	-	-	+	+	+	+	+	+	+	+	+	CAMHB +	+
H	YES	-	-	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	+	+	/	/	/	/	/	/	/	/	/		

(a) MRSA, plate 1

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Dilution Factor	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Controls	
A	Isolate 129.1	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
B	NB	+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		
C	Isolate 129.1	-	+	+	+	+	+	+	+	+	+	+	1:1 NB +	+
D	YES	-	+	+	+	+	+	+	+	+	+	+	1:1 NB -	-
	MBC	+	/	/	/	/	/	/	/	/	/	/		
E	*P. chrysogenum*	-	-	-	-	+	+	+	+	+	+	+	1:1 YES +	-
F	NB	-	-	-	-	+	+	+	+	+	+	+	1:1 YES -	-
	MBC	+	+	-	+	/	/	/	/	/	/	/		
G	*P. chrysogenum*	-	-	-	-	-	+	+	+	+	+	+	CAMHB +	+
H	YES	-	-	-	-	-	-	+	+	+	+	+	CAMHB -	-
	MBC	-	-	-	-	+	+	/	/	/	/	/		

(b) MRSA, plate 2

Row	Column	1	2	3	4	5	6	7	8	9	10	11		12
	Conc. μg/ml	128	64	32	16	8	4	2	1	0.5	0.25	0.13	Controls	
A	Penicillin	-	-	-	+	+	+	+	+	+	+	+	CAMHB +	+
B		-	-	-	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	-	-	-	/	/	/	/	/	/	/	/		
C	Montagnetol	+	+	+	+	+	+	+	+	+	+	+	CAMHB +	+
D		+	+	+	+	+	+	+	+	+	+	+	CAMHB -	-
	MBC	/	/	/	/	/	/	/	/	/	/	/		

(c) MRSA, plate 3

Appendix D

Cell Toxicity Data

The tables on the following pages show the data obtained for evaluation of the toxicity of each test agent as detected by two cytotoxicity assays – MTT and CVDE, as described in Chapter 5.

The general layout of each table follows that of the 96 well plates used for the cytotoxicity assays. Reading for rows A and H are not shown, as they contained water only and were not used to generate results. The remaining wells of columns 1 and 12 were for blanks, which contained medium and no cells. Columns 2 and 11 were used for controls, either for positive cell growth or concentrated YES medium dilutions. Growth controls contained medium and cells, whereas the others contained various dilutions of the concentrated YES medium in DMEM and cells. The remaining wells contained cells exposed to test agent dilutions in DMEM. Following incubation and assay procedure, the plates were read in a spectrophotometer at the wavelengths specified.

Eash test agent was tested three times, in adjacent rows. Results displayed in the following tables are grouped as such.

Tables D.1(a-c) show data related to the MTT assay; Tables D.2(a-c)show data obtained from the CVDE assay.

Table D.1: Cell toxicity data obtained for each test preparation using the MTT assay. Figures listed for each row are the optical densities obtained at 570nm with reference to 630nm. The mean and standard deviation were calculated for each group of three results, and the results adjusted for the blank ("x" indicates blank used). The result obtained for the growth control was assumed to be 100%. Percent viability was calculated by the formula $\frac{OD_{testagent}}{OD_{growthcontrol}}$ x 100.

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate 6.5								Controls	
Row	Blank	Growth	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	YES 25%	Blank
B	0.0140	0.7670	0.0380	0.0190	0.0140	0.0710	0.3130	0.3820	0.2880	0.2630	0.0790	0.0160
C	0.0100	0.7390	0.0390	0.0170	0.0150	0.0710	0.2850	0.3900	0.3060	0.2730	0.0660	0.0150
D	0.0170	0.7600	0.0320	0.0170	0.0170	0.0470	0.2680	0.3710	0.3000	0.2280	0.0730	0.0160
Mean	0.0137	0.7553	0.0363	0.0177	0.0153	0.0630	0.2887	0.3810	0.2980	0.2547	0.0727	0.0157
SD	0.0035	0.0146	0.0038	0.0012	0.0015	0.0139	0.0227	0.0095	0.0092	0.0236	0.0065	0.0006
Adjusted		0.7397	0.0207	0.0020	-0.0003	0.0473	0.2730	0.3653	0.2823	0.2390	0.0570	x
%Viability		100.00	2.79	0.27	-0.05	6.40	36.91	49.39	38.17	32.31	7.71	
			Test Dilutions isolate 12.1									
Row	Blank	YES 50%	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Growth	Blank
E	0.0160	0.1060	0.1460	0.0490	0.0480	0.2710	0.4150	0.7310	0.8230	0.7910	0.7850	0.0180
F	0.0090	0.0810	0.1490	0.0520	0.0520	0.2650	0.3550	0.6060	0.7250	0.6410	0.6830	0.0160
G	0.0160	0.0690	0.1140	0.0450	0.0560	0.2340	0.3020	0.4990	0.5730	0.5940	0.5730	0.0160
Mean	0.0137	0.0853	0.1363	0.0487	0.0520	0.2567	0.3573	0.6120	0.7070	0.6753	0.6803	0.0167
SD	0.0040	0.0189	0.0194	0.0035	0.0040	0.0199	0.0565	0.1161	0.1260	0.1029	0.1060	0.0012
Adjusted		0.0687	0.1197	0.0320	0.0353	0.2400	0.3407	0.5953	0.6903	0.6587	0.6637	x
%Viability		10.35	18.03	4.82	5.32	36.16	51.33	89.70	104.02	99.25	100.00	

(a) MTT assay, plate 1

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate 129.1								Controls	
Row	Blank	Growth	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	YES 6.25%	Blank
B	0.0080	0.7430	0.0520	0.0490	0.4280	0.6390	0.8360	0.6650	0.7160	0.8320	0.6750	0.0170
C	0.0120	0.6830	0.0490	0.0440	0.2430	0.5970	0.7930	0.6850	0.6740	0.7540	0.6160	0.0180
D	0.0120	0.7580	0.0490	0.0430	0.2660	0.6000	0.8000	0.7610	0.8100	0.7410	0.6480	0.0220
Mean	0.0107	0.7280	0.0500	0.0453	0.3123	0.6120	0.8097	0.7037	0.7333	0.7757	0.6463	0.0190
SD	0.0023	0.0397	0.0017	0.0032	0.1008	0.0234	0.0231	0.0506	0.0696	0.0492	0.0295	0.0026
Adjusted	x	0.7173	0.0393	0.0347	0.3017	0.6013	0.7990	0.6930	0.7227	0.7650	0.6357	
%Viability		100.00	5.48	4.83	42.05	83.83	111.38	96.61	100.74	106.64	88.62	
			Test Dilutions *P. Chrysogenum*									
Row	Blank	YES 12.5%	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Growth	Blank
E	0.0180	0.0760	0.1310	0.1720	0.1210	0.5150	0.7020	0.7940	0.6390	0.8420	0.7790	0.0180
F	0.0240	0.0890	0.1120	0.1360	0.1230	0.5280	0.7260	0.7160	0.6720	0.8220	0.7530	0.0130
G	0.0140	0.1310	0.0850	0.1140	0.1280	0.4070	0.6190	0.5970	0.6160	0.7820	0.8150	0.0140
Mean	0.0187	0.0987	0.1093	0.1407	0.1240	0.4833	0.6823	0.7023	0.6423	0.8153	0.7823	0.0150
SD	0.0050	0.0287	0.0231	0.0293	0.0036	0.0664	0.0561	0.0992	0.0281	0.0306	0.0311	0.0026
Adjusted		0.0837	0.0943	0.1257	0.1090	0.4683	0.6673	0.6873	0.6273	0.8003	0.7673	x
%Viability		10.90	12.29	16.38	14.21	61.03	86.97	89.57	81.75	104.30	100.00	

(b) MTT assay, plate 2

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate Penicillin – MIC dilutions μg/ml								Controls	
Row	Blank	Growth	32	16	8	4	2	1	0.5	0.25	YES 1.57%	Blank
B	0.0070	0.8510	0.8250	0.7110	0.8330	0.8900	0.9340	0.8940	0.9650	0.8170	0.9620	0.0050
C	0.0080	0.7860	0.7740	0.8030	0.8230	0.7500	0.8690	0.8780	0.8690	0.7970	0.9570	0.0050
D	0.0090	0.6760	0.8810	0.9050	0.7780	0.8350	0.8440	0.7880	0.8750	0.7110	0.8200	0.0080
Mean	0.0080	0.7710	0.8267	0.8063	0.8113	0.8250	0.8823	0.8533	0.9030	0.7750	0.9130	0.0060
SD	0.0010	0.0885	0.0535	0.0970	0.0293	0.0705	0.0465	0.0571	0.0538	0.0563	0.0806	0.0017
Adjusted	x	0.7630	0.8187	0.7983	0.8033	0.8170	0.8743	0.8453	0.8950	0.7670	0.9050	
%Viability		100.00	107.30	104.63	105.29	107.08	114.59	110.79	117.30	100.52	118.61	
			Test Dilutions isolate Penicillin – strong dilutions mg/ml									
Row	Blank	YES 3.13%	3.00	1.50	0.75	0.38	0.19	0.09	0.05	0.02	Growth	Blank
E	0.0110	0.6760	0.3880	0.6920	0.8370	0.8450	0.7950	0.8800	0.7740	0.7160	0.7460	0.0050
F	0.0370	0.7950	0.3510	0.4270	0.7880	0.8200	0.8750	0.8400	0.9860	0.7520	0.7010	0.0070
G	0.0200	0.4770	0.4370	0.4140	0.7850	0.7490	0.7910	0.8020	0.5830	0.6180	0.5630	0.0060
Mean	0.0227	0.6493	0.3920	0.5110	0.8033	0.8047	0.8203	0.8407	0.7810	0.6953	0.6700	0.0060
SD	0.0132	0.1607	0.0431	0.1569	0.0292	0.0498	0.0474	0.0390	0.2016	0.0693	0.0954	0.0010
Adjusted		0.6433	0.3860	0.5050	0.7973	0.7987	0.8143	0.8347	0.7750	0.6893	0.6640	x
%Viability		96.89	58.13	76.05	120.08	120.28	122.64	125.70	116.72	103.82	100.00	

(c) MTT assay, plate 3

Table D.2: Cell toxicity data obtained for each test preparation using the CVDE assay. Figures listed for each row are the optical densities obtained at 540nm with reference to 690nm. The mean and standard deviation were calculated for each group of three results, and the results adjusted for the blank ("x" indicates blank used). The result obtained for the growth control was assumed to be 100%. Percent viability was calculated by the formula $\frac{OD_{testagent}}{OD_{growthcontrol}}$ x 100.

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate 6.5								Controls	
Row	Blank	Growth	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	YES 25%	Blank
B	0.1440	3.2920	0.3450	0.4330	0.4870	0.3780	1.3800	2.0250	1.2250	1.0820	0.5990	0.1390
C	0.1340	3.3590	0.2950	0.3930	0.4600	0.3820	1.1530	1.8000	1.2710	1.0460	0.5560	0.1730
D	0.1280	3.3160	0.2280	0.3890	0.4130	0.3430	0.9450	2.0340	1.2800	0.9600	0.5560	0.1320
Mean	0.1353	3.3223	0.2893	0.4050	0.4533	0.3677	1.1593	1.9530	1.2587	1.0293	0.5703	0.1480
SD	0.0081	0.0339	0.0587	0.0243	0.0374	0.0215	0.2176	0.1326	0.0295	0.0627	0.0248	0.0219
Adjusted	x	3.1870	0.1540	0.2697	0.3180	0.2323	1.0240	1.8177	1.1233	0.8940	0.4350	
% Viability		100.00	4.83	8.46	9.98	7.29	32.13	57.03	35.25	28.05	13.65	
			Test Dilutions isolate 12.1									
Row	Blank	YES 50%	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Growth	Blank
E	0.1190	1.2830	0.9720	0.7660	0.4350	1.2500	2.0320	3.0920	3.1680	3.2250	3.2550	0.1180
F	0.1230	1.4380	0.9980	0.7560	0.4920	1.2640	2.1350	3.1020	3.1950	3.2120	3.2740	0.1200
G	0.1160	1.4850	0.9100	0.5750	0.3780	1.3040	2.2120	3.2310	3.1800	3.2840	3.2700	0.1080
Mean	0.1193	1.4020	0.9600	0.6990	0.4350	1.2727	2.1263	3.1417	3.1810	3.2403	3.2663	0.1153
SD	0.0035	0.1057	0.0452	0.1075	0.0570	0.0280	0.0903	0.0775	0.0135	0.0384	0.0100	0.0064
Adjusted	x	1.2827	0.8407	0.5797	0.3157	1.1533	2.0070	3.0223	3.0617	3.1210	3.1470	
% Viability		40.76	26.71	18.42	10.03	36.65	63.78	96.04	97.29	99.17	100.00	

(a) CVDE assay, plate 1

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate 129.1								Controls	
Row	Blank	Growth	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	YES 6.25%	Blank
B	0.2040	3.2490	0.2800	0.2850	0.9810	2.9420	3.2150	3.2400	3.3040	3.2980	3.1730	0.2400
C	0.2020	3.3330	0.2600	0.2510	0.8540	2.9650	3.2140	3.3160	3.2810	3.3000	3.1050	0.3050
D	0.2080	3.2870	0.2480	0.2860	1.1900	3.0030	3.1550	3.1840	3.2790	3.2730	2.2590	0.1910
Mean	0.2047	3.2897	0.2627	0.2740	1.0083	2.9700	3.1947	3.2467	3.2880	3.2903	2.8457	0.2453
SD	0.0031	0.0421	0.0162	0.0199	0.1697	0.0308	0.0344	0.0663	0.0139	0.0150	0.5092	0.0572
Adjusted	x	3.0850	0.0580	0.0693	0.8037	2.7653	2.9900	3.0420	3.0833	3.0857	2.6410	
% Viability		100.00	1.88	2.25	26.05	89.64	96.92	98.61	99.95	100.02	85.61	
			Test Dilutions *P. Chrysogenum*									
Row	Blank	YES 12.5%	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Growth	Blank
E	0.1420	2.7180	0.2450	0.5750	0.8530	2.8100	3.0960	3.2520	3.2170	3.2930	3.2730	0.2550
F	0.1550	2.2420	0.1180	0.5730	0.7670	2.8140	3.1180	3.2350	3.2540	3.2720	3.2530	0.1980
G	0.1650	0.6500	0.0940	0.6060	0.6940	2.9700	3.1610	3.2710	3.3100	3.2450	3.3660	0.2110
Mean	0.1540	1.8700	0.1523	0.5847	0.7713	2.8647	3.1250	3.2527	3.2603	3.2700	3.2973	0.2213
SD	0.0115	1.0830	0.0811	0.0185	0.0796	0.0912	0.0331	0.0180	0.0468	0.0241	0.0603	0.0299
Adjusted	x	1.7160	-0.0017	0.4307	0.6173	2.7107	2.9710	3.0987	3.1063	3.1160	3.1433	
% Viability		54.59	-0.05	13.70	19.64	86.24	94.52	98.58	98.82	99.13	100.00	

(b) CVDE assay, plate 2

Column	1	2	3	4	5	6	7	8	9	10	11	12
		Controls	Test Dilutions isolate Penicillin – MIC dilutions μ/ml								Controls	
Row	Blank	Growth	32.00	16.00	8.00	4.00	2.00	1.00	0.50	0.25	YES 1.57%	Blank
B	0.1350	3.2790	3.3000	3.3280	3.2580	3.3520	3.2810	3.2800	3.2780	3.2440	3.2330	0.2110
C	0.1320	3.2510	3.3700	3.3050	3.2630	3.2360	3.3270	3.2980	3.3080	3.3040	3.2760	0.2030
D	0.1480	3.2980	3.2820	3.2540	3.3000	3.2820	3.3510	3.2420	3.3110	3.3060	3.2680	0.3330
Mean	0.1383	3.2760	3.3173	3.2957	3.2737	3.2900	3.3197	3.2733	3.2990	3.2847	3.2590	0.2490
SD	0.0085	0.0236	0.0465	0.0379	0.0229	0.0584	0.0356	0.0286	0.0182	0.0352	0.0229	0.0729
Adjusted	x	3.1377	3.1790	3.1573	3.1353	3.1517	3.1813	3.1350	3.1607	3.1463	3.1207	
% Viability		100.00	101.32	100.63	99.93	100.45	101.39	99.92	100.73	100.28	99.46	
			Test Dilutions isolate Penicillin – strong dilutions mg/ml									
Row	Blank	YES 3.13%	3.00	1.50	0.75	0.38	0.19	0.09	0.05	0.02	Growth	Blank
E	0.1860	3.1410	2.3060	3.1270	3.2590	3.2840	3.2640	3.3010	3.2260	3.3270	3.3280	0.2120
F	0.1450	3.1420	2.3990	3.1590	3.2960	3.3500	3.2810	3.3190	3.3240	3.3280	3.2380	0.2370
G	0.1270	3.2910	2.3900	3.1310	3.3430	3.2820	3.2510	3.2980	3.3030	3.2580	3.3190	0.2060
Mean	0.1527	3.1913	2.3650	3.1390	3.2993	3.3053	3.2653	3.3060	3.2843	3.3043	3.2950	0.2183
SD	0.0302	0.0863	0.0513	0.0174	0.0421	0.0387	0.0150	0.0114	0.0516	0.0401	0.0496	0.0164
Adusted	x	3.0387	2.2123	2.9863	3.1467	3.1527	3.1127	3.1533	3.1317	3.1517	3.1423	
% Viability		96.70	70.40	95.04	100.14	100.33	99.06	100.35	99.66	100.30	100.00	

(c) CVDE assay, plate 3