

ISOLATION AND CHARACTERISATION OF BACTERIAL METABOLITES AS POTENTIAL WOOD PRESERVATIVES

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ABSTRACT

The durability of wood has traditionally relied on the use of heavy metals and toxic compounds to deter wood degrading fungi. Conventional wood preservatives such as Chromated Copper Arsenate (CCA) have been widely used to control wood degrade for many decades, however public pressure over perceived environmental and health risks have led to recent restrictions for use in some countries. This issue has focused the need for research towards environmentally friendly (benign) wood preservatives including compounds such as plant extractives and microbial metabolites.

Previously chilli waste has been shown to have moderate antifungal activity against common wood sapstain fungi. Furthermore, *Lactobacillus* sp. isolated from chilli showed synergistic activity with chilli against these fungi. The intention of this study was to further develop this work, by isolating and identifying the range of bacteria from chilli waste, screening for antifungal activity against wood decay fungi then investigating any possible synergy between the isolates and chilli waste.

Initially a quick screening 96-well optical density assay was optimised which allowed the screening of lactic acid bacterial metabolites against wood decay fungi. This technique proved to be comparable to a commonly used growth rate method and has potential as a standard initial screening method in the laboratory.

Seven isolates from chilli juice had an antifungal effect on the wood decay fungus *Oligoporus placenta* and were identified using 16S rRNA phylogenetic

techniques as *Lactobacillus brevis*, *Leuconostoc mesenteroides* subsp. *mesenteroides* (three isolates), *L. mesenteroides* subsp. *cremoris*, *L. pseudomesenteroides* and *Gluconobacter oxydans*. Cell-free supernatants of these isolates were used to treat wood blocks which were then exposed to wood decay fungi. *L. brevis* metabolites showed the greatest inhibition of wood decay and were identified as lactic and acetic acids by high performance liquid chromatography (HPLC) amongst other techniques. The media in which the bacteria were grown also had an antifungal effect on wood decay fungi most likely due to its hygroscopicity increasing wood moisture content to a point inhibitory to decay fungi.

Synergy between the bacterial metabolites and chilli juice was examined using the optical density assay and it was discovered that two bacteria showed complete inhibition of decay fungi when grown in chilli juice. However, when subjected to a wood assay, both *L. brevis* and *L. mesenteroides* subsp. *cremoris* did not give satisfactory control of decay fungi suggesting the main mechanism of fungal inhibition was due to increased moisture content of wood induced by the specific media initially used in this study.

Further research should determine whether the isolated bacteria can be induced to increase metabolite production and if this would improve antifungal activity. Ultimately these bacterial metabolites and chilli combined or alone, may not be suitable for long term protection against wood decay, but may provide a solution to other fungal degradation issues.

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LIST OF ABBREVIATIONS

AAB	Acetic acid bacteria
BLAST	Basic local alignment search tool
CFS	Cell-free supernatant
DNA	Deoxyribonucleic acid
EMC	Equilibrium moisture content
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
MIC	Minimum inhibition concentration
MODA	Microscale optical density assay
MRS	DeMan-Rogosa-Sharpe
OD	Optical density
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SD	Sabourand Dextrose
YM	Yeast Malt

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CHAPTER 1: INTRODUCTION

1.1 BIODETERIORATION AND TREATMENT OF WOOD PRODUCTS

Wood is the largest volume material produced worldwide and is a renewable resource used in a multitude of applications. In New Zealand 90% of plantation forests are made up of *Pinus radiata* D. Don (Ministry of Agriculture and Forestry, 2010) with log and lumber exports of \$2.42 billion dollars for the year ending June 2011 (5.5% of total exports) (Ministry of Agriculture and Forestry, 2011). *Pinus radiata* is a softwood species, which is highly susceptible to biodeterioration by a range of microorganisms including wood decay fungi. Decay fungi will only grow and develop within a fairly limited range of wood moisture contents (20 – 50%) (Butcher, 1974) however, even a minor infection by decay fungi, which attack the cell wall, can cause significant loss of wood strength and render it unsuitable for most structural uses (Hedley et al., 2004). Biodeterioration of wood products can have serious effects economically and socially. There has been much coverage in the media of ‘leaky house syndrome’ which has involved the failure of structural timbers in new houses due to decay brought on by failures in weather-tightness systems.

Wood-inhabiting fungi are traditionally controlled by chemical formulations, which are to varying degrees toxic to people, animals and the environment. The prohibition of traditional formulations such as Chromated Copper Arsenic (CCA) in some countries (USEPA, 2002, CSTEE Scientific Committee on Toxicity, 2003, Housenger, 2003) is being led by the public’s increasing awareness of the environment and the need for change in order to protect the earth for future

generations. With this comes increased concern about the safety of chemicals and hence a worldwide quest is underway to develop environmentally friendly natural preservative options for the protection of timber.

1.2 THE SEARCH FOR NOVEL PRESERVATIVES

Plant extracts have been used for generations as biopreservatives and to treat human infections as traditional medicines in developing countries (Ahmad et al., 1998). Chilli has been used extensively for many years not only for flavour, but also for its preservative and medicinal properties (Govindarajan and Salzer, 1985, Govindarajan and Sathyanarayana, 1991, Chowdhury et al., 1996, Singh and Chittenden, 2008b). For many chilli growers and merchandisers, who are in the business of chilli seed extraction, the remaining juice is a waste product. Our laboratory was approached by a chilli grower to test Rocoto (*Capsicum pubescens*) chilli juice for antifungal activity. Rocoto or Manzano chilli is a perennial chilli with apple shaped fruit and distinctive black seeds. It is a hot variety with a Scoville rating of 50000 – 250000 SHU, compared with 0 SHU for bell peppers and 1040000 SHU for Naga Jolokia (the hottest known chilli). Under laboratory testing it was found that the chilli juice showed moderate antifungal activity against two common sapstain fungi. There was also a notable increase in sapstain inhibition when chilli and *Lactobacillus* sp., isolated from the chilli juice, were combined (Singh and Chittenden, 2008b).

1.3 ANTIFUNGAL METABOLITES OF LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) including *Lactobacillus* sp., are found naturally in food products and have been used for many years as biopreservatives in food

(Schnurer and Magnusson, 2005). A prime example of biopreservation by LAB is the widespread use of the lantibiotic nisin, a post translationally modified bacteriocin derived from the bacterium *Lactococcus lactis*, in the dairy industry (Ross et al., 2002). As well as lantibiotics, biopreservation is achieved by production of other secondary metabolites including organic acids, hydrogen peroxide and other bacteriocins (Corsetti et al., 1998, Schnurer and Magnusson, 2005). Some of these metabolites have been shown to have antifungal activity (Magnusson et al., 2003) but the majority of research has focused on food spoilage organisms, moulds and yeasts (Corsetti et al., 1998, Lavermicocca et al., 2000, Magnusson et al., 2003, De Muynck et al., 2004). A few recent studies however, have shown that *Lactobacilli* isolates had an antifungal effect on some early wood colonising mould and sapstain fungi (Yang and Clausen, 2005, Singh and Chittenden, 2008b). *Bacillus subtilis* has also been shown to produce metabolites with antifungal activity against wood moulds, sapstain and phytopathogenic fungi (Feio et al., 2004, Moita et al., 2005, Caldeira et al., 2006). Humphris et al. (2002) studied the effects of the secondary metabolites of a fungus *Trichoderma* sp. against the dry rot fungus *Serpula lacrymans*. Ejechi (1997) investigated the effect of a combined bacterial and urea treatment on wood biodegradation by decay fungi and showed reduced fungal growth and degradation. However, there is no evidence to date that the efficacy of lactic acid bacterial metabolites have been tested on wood decay fungi.

Production of antifungal (secondary) metabolites by bacteria is linked to environmental conditions and the stage of bacterial growth. Most bacteria will employ basic metabolic functions when grown in nutrient-rich media, however when nutrients are depleted they will start to produce various secondary

metabolites to help with survival (Demain, 1998). In the laboratory, incubation conditions (media, temperature, pH, aeration and agitation) can have a direct effect on the production of secondary metabolites (Cabo et al., 2001, Bizani and Brandelli, 2004, Moita et al., 2005). Moita et al. (2005) found that high pH values favoured the production of metabolites active against mould isolates; however the effect of temperature and aeration was different for different fungi. Thus the effect of environmental variations appears to depend on the metabolite producing bacteria and the target organisms for inhibition.

1.4 TESTING PROCEDURES FOR WOOD PROTECTION

Traditional testing of wood preservatives is a relatively long process with a number of steps to go through to get approval for commercial use. Testing starts with laboratory trials which take many weeks or months to get results and take a great deal of resources and time to complete, making screening of potential new compounds a long and expensive process. Microscale assays which allow screening of many actives at one time and which take less than a week to complete, are a useful tool and have been used in laboratories involved in screening actives for potential medicinal or food preservation uses (Lash et al., 2002). This type of assay also has potential to be used in the search for new wood preservatives (Langvad, 1999, Yang and Clausen, 2005) by cutting screening time and costs, in addition to increasing throughput in the laboratory. The Microscale Optical Density Assay (MODA) (Lash et al., 2002) was specifically developed for screening inhibitory activity of *Lactobacillus* sp. against spoilage and pathogenic bacteria and with slight modification should be suitable for this project. If successful this technique will be incorporated in future screening trials in the

laboratory and the wider usage of this technique in wood preservation research will be developed.

1.5 RESEARCH OBJECTIVE

To investigate the potential effectiveness of antifungal metabolites from lactic acid bacteria, isolated and characterised from chilli waste, against wood decay fungi and thus as sustainable timber preservatives, the following approach was taken:

1. Develop a quick screening methodology to enable the fast identification of antifungal metabolites from bacteria.
2. Isolate and identify bacteria from chilli juice using 16S rRNA phylogenetic techniques.
3. Screen antifungal activity of isolated bacteria, using the methodology developed above along with pure culture wood decay assays.
4. Characterise active compounds in bacterial supernatants using various techniques including HPLC.
5. Evaluate the antifungal performance of secondary metabolite and chilli extract as an integrated preservative system.

An overview of the steps taken into this research are given in Figure 1.1.

MICROSCALE OPTICAL DENSITY ASSAY PROTOCOL DEVELOPMENT

- Optimise time frame for incubation of fungi.
- Determine dilution of cell-free supernatants required for successful results.
- Optimise growth of decay fungi and LAB by trialing different media.
- Compare MODA to *in vitro* growth rate assay.



ISOLATION, IDENTIFICATION AND ANTIFUNGAL SCREENING OF LACTIC ACID BACTERIA FROM CHILLI WASTE

- Isolate bacteria from chilli juice.
- Screen for antifungal activity using MODA developed previously.
- Identify active bacteria using 16S rRNA phylogenetic techniques.
- Screen active bacteria in a wood decay assay to determine efficacy.
- Use HPLC and other methods to determine the nature of antifungal activity.



ANTIFUNGAL EFFECT OF BACTERIAL METABOLITES IN COMBINATION WITH CHILLI JUICE

- Screen bacteria grown in chilli juice at different concentrations for antifungal activity using MODA to establish most suitable treatment for wood assay.
- Perform wood assay on most promising isolates combined with chilli juice.
- Use HPLC to determine differences in metabolite production between bacteria and between different growth media.

Figure 1.1 Overview of research approach.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

This section reviews the current literature around wood preservation treatments and trends in new preservatives against wood decay, the role that natural products, including chilli and bacterial metabolites are currently playing as antifungal treatments and the implications and possibilities of these in the future. It also covers the methodology currently used for determining antifungal activity and the benefits of developing a quick screening methodology for the wood preservation sector.

2.2 CURRENT TRENDS IN WOOD PRESERVATION

Chromated copper arsenate (CCA) has been the most used wood preservative in history (Tazakor Rezai et al., 2011), since it was introduced in the 1930's. Due to concerns about the leachability and possible environmental toxicity of chromium and arsenic, two of the main components of CCA, countries around the world began restricting its use in 2004 (USEPA, 2002). There are a number of approved substitutes for CCA that rely on the use of greater levels of copper to achieve control against fungal and insect degradation including copper azoles and alkaline copper quaternary compounds. These preservatives have relatively low mammalian toxicity, however there is still concern about their affects in aquatic environments (Tazakor Rezai et al., 2011). Therefore, whilst many countries still use CCA with no restrictions, a niche has developed for the production of wood preservatives with both low mammalian and environmental toxicity that are easy to dispose of at the end of service life.

2.2.1 Traditional wood preservatives

Many researchers and chemical companies continue to work at improving traditional copper and borate based preservatives. Copper naturally leaches from wood over time and the purpose of chromium in CCA was to ‘fix’ this copper to the wood structure. In the absence of chromium, much of the recent research involving copper based preservatives revolves around the use of nanoparticles (Matsunaga et al., 2010, Matsunaga et al., 2011, Weitz et al., 2011) and micronized copper (Zhang and Ziobro, 2009, McIntyre, 2010, Ray et al., 2010, Stirling and Morris, 2010, Xue et al., 2010, McIntyre and Freeman, 2011) in an effort to improve copper penetrability into the wood structure and thus make it less available to leaching. The use of silver nanoparticles has also been explored with some success (Tazakor Rezai et al., 2011).

Boron preservatives which have also been used for many years, are highly leachable and much of the current research around this well known preservative involves manipulating the boron to ‘lock’ into the timber either by chemical modification (Li et al., 2010b, Franich et al., 2011) or additives (Mohareb et al., 2009, Yu and Cao, 2009, Mohareb et al., 2010, Tomak et al., 2010, McIntyre and Lake, 2011, Thevenon et al., 2011, Yu and Cao, 2011). It is inevitable that these types of preservatives will continue to play a large part in wood preservation due to proven performance and their approved use in the preservation industry as well as in other industries such as horticulture and agriculture for pest control.

2.2.2 Modern preservation methods and current trends

Wood modification

There are various alternatives to chemical treatment with a great deal of research focused on the use of wood modification techniques as a way to improve wood properties including durability. Wood cell walls can be modified either by chemical or thermal modification or enzymatic treatment (Rep and Pohleven, 2001) with both acetylated and heat treated wood being produced commercially in Europe. Wood acetylation, the most common chemical modification, involves reacting the wood with acetic anhydride, therefore changing wood structure by altering free hydroxyl groups into acetyl groups. This reduces the ability of wood to absorb water and makes it more dimensionally stable and durable against decay (Rowell et al., 2008). Thermal modification which is achieved by immersing wood in high temperature oils (usually vegetable oils), N₂ gas or steam (Westin et al., 2004) also increases stability, water repellency and improves durability (Cooper et al., 2007). A number of studies have shown both chemical and thermal modifications of wood to increase fungal durability (Takahashi et al., 1989, Fojutowski et al., 2009, Skyba et al., 2009, Papadopoulos et al., 2010).

Natural Products

Research into natural compounds is becoming more popular and attractive from a human and environmental health perspective. Singh and Singh (2010) recently undertook a review of natural compounds and their use for wood protection. Four main groups were considered when reviewing natural products; plant extracts and essential oils, waxes and resins from bark, heartwood and heartwood extractives from naturally durable woods and other miscellaneous bio products.

Plant extracts and essential oils

A number of plant extracts and essential oils have been tested against wood degrading fungi and have provided effective control, for example extracts from cinnamon leaf proved effective against decay fungi and termites (Wang et al., 2005, Cheng et al., 2006, Lin et al., 2007, Maoz et al., 2007). Yang and Clausen (2007) and Matan and Matan (2007, 2008) investigated a number of essential oils against mould fungi eg. rosemary, tea tree, thyme, lemon grass, anise, lime, tangerine, cinnamon and clove, and found them to be highly effective. Extracts of the New Zealand native plant Hinau (*Elaeocarpus dentatus*) were tested against decay fungi and found to have antifungal activity (Rickard et al., 2009) and a number of other oils and extracts have been found to inhibit decay fungi ie. cinnamaldehyde (Kartal et al., 2006, Hsu et al., 2007, Cheng et al., 2008, Singh and Chittenden, 2008a), cassia oil, wood tar oil, cinnamic acid (Kartal et al., 2006), eugenol (Hsu et al., 2007, Cheng et al., 2008, Singh and Chittenden, 2008a), cinnamon oil (Li et al., 2008), macadamia shell tar oil (Kartal et al., 2010), basil oil and thyme oil (Jones et al., 2011).

Clausen et al. (2010) also investigated the purified bioactive compounds of some essential oils and found that carvone, citronellol, geraniol, thymol and borneol all inhibited the mould fungi *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma viride*, and that thymol and borneol inhibited the decay fungi *Postia placenta*, *Gloeophyllum trabeum* and *Trametes versicolor*. However, antifungal activity of essential oils has been seen to be varied (Singh and Chittenden, 2008a), fungus dependent in some cases (Kartal et al., 2006) and solvent dependent in others (Li et al., 2008). Some of the oils and extracts are also able to leach out of

the wood when subjected to continuous wetting cycles once again making the timber susceptible to degradation (Singh and Chittenden, 2008a).

Waxes and resins from bark

Bark can be a rich source of antioxidants and antimicrobial substances, and tannins have been used for some time in the adhesive and wood protection industries (Mitchell and Sleeter, 1980, Laks et al., 1988, Lotz and Hollaway, 1988, Lotz, 1993, Singh and Singh, 2010), however tannins also have a problem with not ‘fixing’ into the wood. A number of attempts have been made to improve this including addition of additives such as ferric chloride (Mitchell and Sleeter, 1980) and metallic salts (Laks et al., 1988) with some success. Other compounds such as bio-oils and resins obtained from bark have also been tested for antifungal activity, for example Nakayama et al. (2001) showed that resin from the wood of guayule (*Parthenium argentatum*) provides protection against decay organisms as well as termites and marine borers. Carrillo et al. (2010) tested extracts from the wood and bark of *Condalia hookerii*, *Ebanopsis ebano* and *Helietta parvifolia* against the white rot fungus *T. versicolor* and found that all species showed antifungal activity with *H. parvifolia* having the best activity. Wood particle boards impregnated with *Pinus bruita* bark have also shown decay resistance (Nemli et al., 2006).

Heartwood and heartwood extractives from naturally durable woods

Many timbers around the world, especially heartwood from tropical trees, are naturally durable against decay organisms; it is therefore thought that the extractives from these timbers may impart durability upon other less durable species if they can be impregnated into the wood structure. Numerous studies

have been done on various durable species including *Gmelina arborea*, *Prosopis juliflora*, *Eremophila mitchelli*, white cypress pine, *Taiwania cryptomeroides*, and *Cunninghamia lanceolata*, and many have shown antifungal and/or termiticidal activity (French et al., 1979, Chang et al., 2003, Kawamura et al., 2004, Kawamura et al., 2005, Scown et al., 2009, Sen et al., 2009, Sirmah et al., 2009, Li et al., 2010a). Individual extractives from *Cunninghamia lanceolata* (China-fir) showed little antifungal activity on their own, however the timber itself is well known for its durability, suggesting that a combination of extractives rather than a single compound is important for imparting durability (Li et al., 2010a). In addition to chemical composition, the distribution and amount of extractives is related to heartwood durability and knowledge of this is useful in developing extractive based preservative systems (Singh and Singh, 2010).

Miscellaneous bioproducts

Other bioproducts that have been tested for antifungal activity include chitosan, a product derived from chitin which is produced commercially from crustacean shells (Singh and Singh, 2010). A number of researchers have focused time on this bioactive due to its antimicrobial activity and cost-effectiveness (Kobayashi and Furukawa, 1995, Chittenden et al., 2004, Maoz and Morrell, 2004, Eikenes et al., 2005a, Eikenes et al., 2005b, Torr et al., 2006, Singh et al., 2008, Hussein et al., 2011), however there is a difference in opinion about whether low or high molecular weight chitosan is most effective (Singh and Singh, 2010).

Propolis from bee hives was tested against mould and decay fungi in a soda based formulation and was shown to improve durability of Scot's pine (Jones et al.,

2011), however the soda alone improved the durability class to that similar to the propolis treatment and activity was lost after leaching.

Biological control agents (BCA) and microbial secondary metabolites are another area of interest to the wood preservation industry but have shown variable results. It is thought that the combination of a biological agent with another natural product may enhance the competitive advantage of the BCA and this has been seen to be effective in the laboratory (Singh and Chittenden, 2008b, Chittenden and Singh, 2009), however there has been less satisfactory results in the field and more work is required in this area in order to produce a viable product.

2.3 CHILLI AS AN ANTIFUNGAL AGENT

Chilli peppers (*Capsicum* sp.) are small perennial herbs native to South America (Cichewicz and Thorpe, 1996) and have been around since ancient civilisations, with pepper seeds dating from approximately 7500BC having been found in Mexico (Barceloux, 2008). As well as playing a part in the flavouring of food, chilli was used for medicinal purposes by both Mayans (Cichewicz and Thorpe, 1996) and Native Americans, and as an irritant smoke to deter invaders by the latter (Barceloux, 2008). Chillies were also known for their antimicrobial properties making them useful for preserving food (De et al., 1999, Schulze and Spiteller, 2009). In the early 20th Century Nelson (1919) determined the basis for the pungency of chillies by characterising the presence of vanillyl amides with the major alkaloid responsible for the characteristic irritant effects of chilli being capsaicin (trans-8-methyl-N-vanillyl-6-noneamide) (Barceloux, 2008, Schulze and Spiteller, 2009). It is thought that one of the roles of these capsaicinoids in the wild is to prevent *Fusarium* fungi from infecting chilli seeds as the majority of the

capsaicinoids are found around the seeds rather than the flesh (Tewksbury et al., 2006, Schulze and Spiteller, 2009). Research has shown that capsaicin is responsible for the biological (Chowdhury et al., 1996) and antimicrobial activity of chilli. Both Jones et al. (1997) and Zeyrek and Oguz (2005) reported the inhibition of *Helicobacter pylori* (the major cause of gastric cancer, peptic and gastric ulcers) and other researchers have reported the inhibition of *Escherichia coli*, *Pseudomonas solanacearum* and *Bacillus subtilis* (Molina-Torres et al., 1999) by capsaicin, and *Bacillus cereus*, *B. subtilis*, *Clostridium sporogenes*, *C. tetani* and *Streptococcus pyrogenes* by extracts of capsicum (Cichewicz and Thorpe, 1996). However the same extracts, which were derived from different species of capsicum (*C. annum*, *C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens*) had either no effect on *Candida albicans* or in fact stimulated growth of the yeast and capsaicinoids tested had no activity against any of the microorganisms they tested.

Ribeiro et al. (2007) isolated peptides and Diz et al. (2011) isolated lipid transfer proteins (LTP's) from chilli pepper seeds and found some of them inhibited the growth of pathogenic yeasts *C. albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *Saccharomyces cerevisiae* and the fungus *Colletotrichum lindemuthianum*. Many other researchers have shown extracts and compounds from *Capsicum* spp. to exhibit both antibacterial and antifungal activity against both yeast and mould fungi (Chowdhury et al., 1996, Bowers and Locke, 2000, Anaya-Lopez et al., 2006, Erturk, 2006, Nazzaro et al., 2009).

The majority of research into the antibacterial and antifungal effects of *Capsicum* spp. has concentrated on the medical application of these effects (Cichewicz and Thorpe, 1996, Molina-Torres et al., 1999, Anaya-Lopez et al., 2006, Erturk, 2006, Ribeiro et al., 2007, Nazzaro et al., 2009), most likely due to the use of chilli for medical application in ancient cultures. There have also been various applications for patents to make use of the repellent properties of *Capsicum* spp. against living organisms (Fischer, 1993, Neumann, 2003c, Neumann, 2003b) and antimicrobial properties (Neumann, 2003a). Little research appears to have been done on the antifungal activity of chilli for other purposes and what has been done has shown discrepancy regarding the activity of *Capsicum* spp.. Wilson et al. (1997) showed that *Capsicum* spp. had antifungal activity against *Botrytis cinerea* and Bowers and Locke (2000) reported that a combined treatment of chilli pepper extract and essential oil of mustard effectively controlled *Fusarium* wilt disease in glasshouse soil. Singh and Chittenden (2008b) tested both chilli juice and capsicum oleoresins against wood staining fungi (*Sphaeropsis sapinea* and *Leptographium procerum*) and found that activity varied depending on the fungi, but that both the juice and the oleoresins had a moderate antifungal effect. However, Thyagaraja and Hosono (1996) found that when testing the ability of chilli, cumin, pepper, asafoetida and coriander to control food spoilage mould, only asafoetida showed any real fungal inhibition. There appears to be no information on the antifungal effect of *Capsicum* spp. and in particular chilli extracts on basidiomycete fungi, specifically those which cause wood decay.

2.4 THE USE OF MICROBIAL METABOLITES

Microorganisms produce an amazing array of primary and secondary metabolites which have a major effect on our society including organic acids, antibiotics,

toxins, pigments, antitumor agents, enzyme inhibitors, pheromones, pesticides and growth promoters (Demain, 1998). Antimicrobial metabolites are produced by both bacteria and fungi usually as competitive weapons against other fungi, bacteria, amoebae, insects and plants (Demain and Adrio, 2008) furthermore many of these metabolites can be extremely beneficial to people. The most well known of these is the production of antibiotics from such organisms as *Penicillium* sp. (penicillin) and *Streptomyces* sp. (eg. streptomycin, neomycin and chloramphenicol) and bacteriocins from organisms such as *Lactococcus lactis* (nisin), *Bacillus subtilis* (subtilin) and *Lactobacillus reuteri* (reuterin). Bacteriocins are ribosomally produced peptides or proteins which inhibit closely related bacteria, however some, such as nisin, exhibit a much broader range of activity (Stiles, 1996). The production of these bacteriocins by the lactic acid bacteria (LAB) along with the production of organic acids (mainly lactic and acetic) has been the basis of the biopreservation of food products for many centuries and in recent years much research has been done on the antifungal metabolites of these bacteria with regards to the food industry (Roy et al., 1996, Gourama and Bullerman, 1997, Lavermicocca et al., 2000, Magnusson and Schnürer, 2001, Atanassova et al., 2003, De Muynck et al., 2004, Gerez et al., 2009a, Mojgani et al., 2009, Falguni et al., 2010, Franco et al., 2011, Ndagano et al., 2011).

Moulds and other fungi cause significant losses in the food and feed industry worldwide with an estimate of 5-10% of all losses due to fungal contamination (Pitt and Hocking, 2009), therefore there has been much interest in the use of LAB fungi (which are generally regarded as safe (GRAS)) and their range of antifungal metabolites. Muñoz et al. (2010) investigated two LAB (*Lactobacillus*

fermentum and *Lactobacillus rhamnosus*) for their ability to inhibit a strain of the mycotoxin producing fungi *Aspergillus* and found *L. rhamnosus* to have good antifungal activity when grown at 37°C, however no attempt was made to determine the nature of the antifungal metabolite(s). Corsetti et al. (1998) investigated the antimould activity of a number of *Lactobacillus* species and found *L. sanfrancisco* to have the largest spectrum of activity, inhibiting moulds such as *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp. and *Monilia* sp. (bread moulds). Activity was attributed to a mixture of organic acids including acetic, caproic, formic, propionic butyric and *n*-valeric acids. Magnusson et al. (2003) tested 42 LAB isolates against five species of mould fungi and three yeasts and found that *Lactobacillus coryniformis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus* were the most commonly isolated species with high antifungal activity. Fungal inhibition was attributed to antifungal cyclic peptides in addition to lactic and acetic acids, plus several other compounds suggesting that the antifungal activity had a highly complex nature. Ström et al. (2002) reported the presence of cyclic dipeptides along with phenyl lactic acid as being responsible for the antifungal activity expressed by a *L. plantarum* strain. Mulhialdin et al. (2011) showed the shelf life of bread and other foods to be increased by the addition of *L. fermentum*, *P. pentosaceus*, *Lactobacillus pentosus* and *Lactobacillus paracasi*. Both *L. fermentum* and *P. pentosaceus* were thought to produce proteinaceous antifungal agents. An antifungal compound active against a range of moulds and yeasts isolated from a *L. plantarum* strain from kimchi (a Korean fermented vegetable dish) was identified as delta-dodecalactone (Yang et al., 2011) which produced a favourable flavour indicating that it could be used as both a flavouring compound and a biopreservative. Mauch et al. (2010) screened 129 isolates of LAB for antifungal activity against *Fusarium* spp. isolated from

brewing barley and found *Lactobacillus brevis* to have the highest spectrum of activity which was attributed to both organic acids and proteinaceous compounds.

There have been a number of studies in recent years into the effects of microbial metabolites on wood degrading fungi. A study by van der Waals et al. (2003) on the effects of bioextracts, from incubated forest soil, garden compost and chicken manure, on sapstain fungi showed suppressed fungal growth especially by the forest soil and compost extracts. Autoclaved extracts produced no fungal inhibition suggesting the presence of an active microbial population in the extracts. Volatile secondary metabolites of mould fungi *Trichoderma psuedokoningii*, *T. viride* and *T. aureoviride* affected protein synthesis and mycelial growth in the decay fungus *Serpula lacrymans* (Humphris et al., 2002) with volatiles from *T. aureoviride* having the greatest effect. Protein synthesis and mycelial growth resumed as normal upon removal of the antagonistic fungi. *Bacillus* spp. have been shown to produce antifungal activity against a number of wood contaminant mould and sapstain fungi (Feio et al., 2004, Caldeira et al., 2006), and Ejechi (1997) found that *Bacillus* sp. and *Proteus* sp. combined with urea inhibited the decay fungi *Gloeophyllum sepiarium*, *Gloeophyllum* sp., *Plerotus* sp. and *Trametes* sp.. Zulpa et al. (2003) investigated the potential of the cyanobacteria *Nostoc muscorum*, *N. piscinale* and *Microchaete tenera* to inhibit wood degrading fungi and found that *N. muscorum* and *M. tenera* extracts inhibited the sapstain fungus *Sphaeropsis sapinea*, however *N. muscorum* and *N. piscinale* extracts promoted growth of the wood moulds *Trichoderma boningii* and *T. viride*. Extracellular products of the LAB *Streptococcus termophilus* strongly inhibited all the fungal strains they tested. Other researchers have also seen the strong antifungal effects of LAB metabolites against wood degrading

fungi. Yang and Clausen (2005) studied the effects of cell-free supernatants of *Lactobacillus casei* subsp. *rhamnosus* and *L. acidophilus* on three wood moulds and one wood staining fungi and found that both LAB inhibited fungal growth by 95-100%. Lactic acid and four unknown metabolites were deemed responsible for antifungal activity. Singh and Chittenden (2008b) discovered that when they tested chilli juice in combination with the LAB *L. casei* against the sapstain fungi *S. sapinea* and *Leptographium procerum*, antifungal activity was greatly enhanced than when chilli was tested alone.

2.5 CURRENT METHODOLOGY FOR TESTING ANTIFUNGAL ACTIVITY

There are a number of methods commonly used to investigate the antifungal activity of various compounds whether they are plant extracts, chemicals or metabolites from microorganisms. The wood preservation industry also has standard methods for the testing of potential preservatives which are used worldwide by chemical companies and independent researchers when developing new permanent preservatives for the industry. Such methods involve the impregnation of wood with the potential wood preservative and then subjecting the wood to either laboratory pure culture decay fungi (ONORM, 2004, American Society for Testing and Materials, 2007) or to either above-ground or in-ground exposure (American Wood Protection Association, 1983) in field test sites commonly called 'graveyards'. Laboratory trials can take up to six months (ONORM, 2004) from start to finish to achieve results and field testing takes many years, therefore when screening new compounds for antifungal activity other methods are required which take much less time and can handle many different compounds and permutations at one time.

Many 'quick' screening methods for antifungal activity involve measuring growth of fungi on nutrient medium contained in petri dishes (hereafter referred to as plates). One such method involves supplementing the nutrient medium with the compound to be tested and then inoculating with various fungi in the centre of the plate and measuring the growth after several weeks' incubation (Humphris et al., 2002, Singh and Chittenden, 2008b). This method appears to work well, however we have found in many cases that the concentration of inhibition achieved in this test does not equate to that required when transferred to the more robust wood assay. It also requires a large amount of nutrient media and petri dishes to investigate the minimum inhibition concentration (MIC) of just one compound never mind a series of compounds.

Another common method used in assessing both antibacterial and antifungal activity is the well diffusion method in which the agar is supplemented with conidia or bacteria, wells are cut in the agar and then the compound is added to the well and incubated. The zone of inhibition around the well is measured to quantify the antimicrobial activity (Roy et al., 1996, Corsetti et al., 1998, Falguni et al., 2010, Gerez et al., 2010). A similar method involves the placing of small filter discs impregnated with compound on top of the media instead of cutting wells and measuring the inhibition zone (Cichewicz and Thorpe, 1996, De Muynck et al., 2004, Feio et al., 2004, Erturk, 2006, Nazzaro et al., 2009). These methods both rely on the ability of the compound to diffuse into the media and thus can overlook compounds which diffuse slowly or cannot diffuse in the water based medium (Lash et al., 2002). They also require large amounts of media and consumables and are therefore less cost effective than some other methods.

Some researchers use a method in which fungi are incubated in liquid media along with the compound to be tested. At the end of incubation the media is filtered through pre-weighed filter paper, in order to retain the fungal biomass, then oven dried and weighed to determine differences in antifungal activity (Gourama and Bullerman, 1997, Zulpa et al., 2003, Yang and Clausen, 2005, Singh and Chittenden, 2008b). Lavermicocca et al. (2000) and Caldeira et al. (2006) report on another method which uses liquid media to expose fungi to the compound in question. This method incubates a known concentration of conidia or spores with the compound for a set time period and then plates it on to suitable solid nutrient media. At this point the plates are incubated and colonies are subsequently counted to give a quantitative result. In large scale experiments, when comparing many compounds, these methods can be extremely space demanding and therefore limit the amount that can be achieved at one time (Langvad, 1999).

An increasingly more common method for testing antimicrobial activity is the measurement of microbial growth by measuring optical density (OD) of liquid cultures. Molina-Torres et al. (1999) used this method by incubating compound and microbes in flasks then extracting a small aliquot to be measured for turbidity in a spectrophotometer. More recent studies have reduced the size of the experiment by incubating the liquid cultures in 96 well or 200ul microplates and then reading the OD in a microplate reader (Ström et al., 2002, Magnusson et al., 2003, Levenfors et al., 2004, Anaya-Lopez et al., 2006, Ribeiro et al., 2007, Gerez et al., 2009a, Mauch et al., 2010). The method is suitable for screening inhibitory activity of lactic acid bacteria (Lash et al., 2002) and filamentous fungi (Langvad, 1999). Langvad (1999) determined that in this method OD measurements directly relate to fungal biomass measurements from the previously mentioned method.

2.6 SUMMARY

The wood preservation industry is changing, driven by the public's increasing awareness and demands for safer, less environmentally toxic alternatives to traditional chemicals. This has led to increased research into so called natural products including plant and timber extracts and oils, and other bioproducts including the natural inhibition seen by competing microorganisms which encompasses production of metabolites from both bacteria and fungi.

Chilli extracts are one such plant compound which show promise in this area with many reports of antifungal activity for different extracts from *Capsicum* spp.. Much of this research to date has been based on medicinal application and research is thin when it comes to wood degrading organisms. The use of chilli juice as a wood preservative would resolve some of the issues facing the preservation industry as a non-toxic environmentally friendly alternative to traditional chemicals. As a waste product from seed production it would also be relatively cost effective.

The use of microbial metabolites for human benefit has been happening for many years with the production of substances such as antibiotics among others. Much of the current research into this field is concentrated on the food and medicinal areas given the fact that many of these metabolites are considered safe for general use. Researchers in wood preservation have also begun exploring the potential of these compounds as an alternative to traditional formulations. Again the use of such metabolites either alone or in combination with other natural products could provide a cost effective, natural, non-toxic alternative to traditional chemicals.

In order to screen the multitudes of different extracts, microbial metabolites and combinations thereof, new, faster, more cost effective, time and space saving techniques need to be established as routine in the laboratory. The technique which appears to cover all these parameters is the optical density technique using 96-well plates. A method such as this which can screen a multitude of parameters in a short time period using little space and consumables should be essential in the search for new natural preservatives.

CHAPTER 3: MICROSCALE OPTICAL DENSITY ASSAY PROTOCOL DEVELOPMENT

3.1 INTRODUCTION

A standard method of screening potential new bioactive molecules for use in wood preservation is by measuring fungal growth rates on solid agar amended with active compounds. This type of assay takes time and resources, in addition to limiting the number of compounds screened at one time. Microscale assays which allow screening of many compounds at one time and which can take less than a week to complete, are a useful tool and have been used in laboratories involved in screening actives for potential medicinal or food preservation uses (Langvad, 1999, Lash et al., 2002). Langvad (1999) showed that optical density (or absorbance) can be directly related to the growth of filamentous fungi, therefore a microscale absorbance assay has potential to be used in the search for new wood preservatives (Yang and Clausen, 2005). It also has potential to cut screening time and costs, and increase throughput in the laboratory.

Lactobacillus sp. are well known for their ability to produce secondary metabolites with potent inhibitory activity against a wide range of bacterial species and there is increasing evidence to support their antifungal activity. These metabolites are often screened in laboratories using microscale 96-well plate optical density assays. The objective of this study was to optimise an optical density assay using 96-well microplates to assess the activity of lactic acid bacteria (LAB) metabolites against wood decay fungi. The method was also compared with the standard solid agar growth-rate assay. Furthermore, various nutrient media were used to optimise the growth of LAB and decay fungi.

3.2 MATERIALS AND METHODS

3.2.1 Organisms

Fungi

The following fungi were used as representatives of wood decay fungi, specifically brown rot fungi, which are commonly found in timber framing.

Oligoporus placenta (Fries) Gilbertson & Ryvarden. (NZFR 07/02)

Antrodia xantha (Fries) Ryvarden. (729.1)

Coniophora puteana (Schumacher ex Fries) Karsten. (BAM Ebw.15)

O. placenta and *A. xantha* are both New Zealand isolates obtained from ‘leaky’ buildings in Auckland and used regularly in the Wood Mycology Laboratory at Scion. *C. puteana* is a European standard organism.

Fungal cultures were maintained on 2% malt agar (2% Naturlok malt powder (Danisco) 1.5% Bacteriological agar (Danisco)).

Bacterium

Cultures of *Lactobacillus rhamnosus* (ATCC 7469) were maintained in MRS broth (Oxoid).

3.2.2 Microscale optical density assay (MODA)

Fungal cultures were grown in liquid malt broth: 2% malt powder, 0.5% mycological peptone (Oxoid), at 26°C, 75% RH for a period of up to four weeks (Figure 3.1). Before use, cultures (including liquid media) were homogenised in a

Waring blender for 30 seconds at low speed followed by 30 seconds at high speed. With cultures older than three weeks an equal volume of sterile malt broth was added to dilute the inoculum. A volume of 50 μ l of this inoculum was added to each test well in a sterile 96-well covered cell culture plate (Sarstedt), except for the negative control which contained 50 μ l sterile malt broth plus 100 μ l sterile liquid bacterial media.



Figure 3.1 *Oligoporus placenta* growing in liquid media

Lactobacillus rhamnosus was grown overnight in liquid media at 30°C, 100 rpm. Media used were: de Man-Rogosa-Sharp (MRS) broth, Yeast Malt (YM) broth (1.5% Malt powder, 0.2% yeast extract (Oxoid)), Sabouraud Dextrose (SD) broth (Merck) and MRS – Salts broth (1% mycological peptone, 0.8% beef extract (Difco), 0.4% yeast extract, 2% glucose, 0.1% Tween 80). Resulting cultures were centrifuged at 5100 g for 10 minutes at room temperature and the resulting

supernatant filter sterilised through a 0.22 μm Steritop filter (Millipore). Sterile liquid media was then diluted with supernatant as follows: 4:1, 2:1, 1:1, 1:2, 1:4 and 0:1. An aliquot of 100 μl of each dilution was added to eight micro test wells (containing fungal inoculum) to serve as replicates (Figure 3.2). A positive control contained 50 μl fungal inoculum and 100 μl sterile liquid media was also used.



Figure 3.2 Example of fungi growing in 96-well plate. Translucent wells indicate growing fungi, clear wells indicate no or reduced fungal growth.

Inoculated plates were read at 550 nm on a Multiskan® Spectrum (Thermo Electron Corporation) plate reader to get an absorbance measurement. Plates were wrapped with plastic wrap or parafilm during incubation at 25°C, 75% RH (to prevent evaporation of media) and reread after 4 – 7 days. Change in absorbance was recorded by subtracting the initial reading from the final absorbance.

3.2.3 Solid agar plate growth-rate assay

Sterile agar medium (200 ml) containing 2% malt extract plus 4.5% bacteriological agar (kept at 55°C), was amended with 400 ml of supernatant dilutions as listed above and immediately poured into Petri dishes and allowed to set. Fungal inoculum plugs (5 mm diameter) were cut from the edge of an actively growing culture (2 – 3 weeks old) and transferred to the centre of each Petri dish (plate). The inoculated plates were incubated at 26°C and 75% RH in the dark for 1 – 2 weeks at which time colony diameter was recorded. Colony diameter was measured along two axis perpendicular to each other (Figure 3.3).

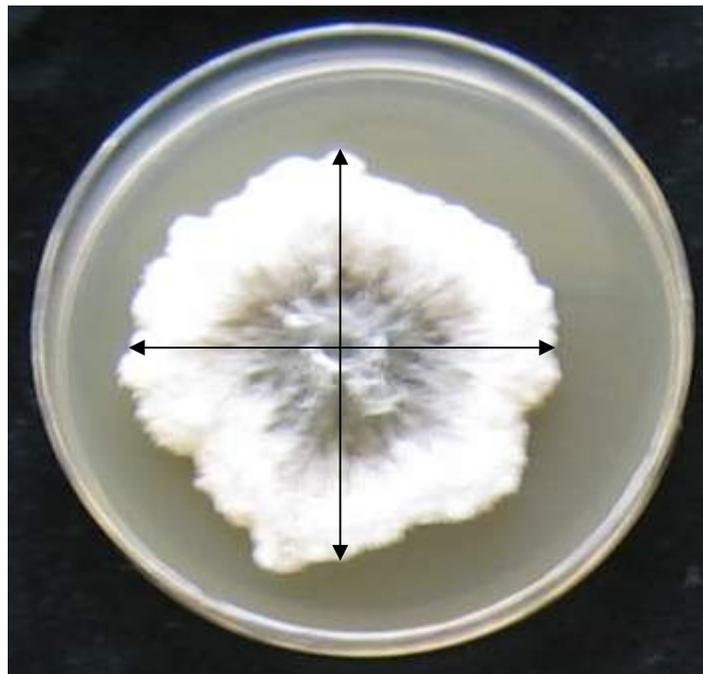


Figure 3.3 Measurement of fungal growth on petri dish containing solid media.

3.3 RESULTS AND DISCUSSION

3.3.1 Antifungal activity of metabolites produced in MRS media

A clear dose response in antifungal activity is demonstrated against *O. placenta* by the cell free supernatant (CFS) produced by *L. rhamnosus* (Figure 3.4). When 100% supernatant (0:1 dilution) was used fungal growth was almost completely inhibited. These results also confirmed that MRS media appeared to mediate the growth of *Lactobacillus* (hence production of antifungal metabolites), as well as supporting fungal growth (Figures 3.4 and 3.5). This result is comparable with previous studies where MRS media has been recommended as a medium for the growth of *Lactobacilli* (Oxoid, 2000, Lash et al., 2002) and was capable of supporting growth of a number of fungal organisms (Yang and Clausen, 2005).

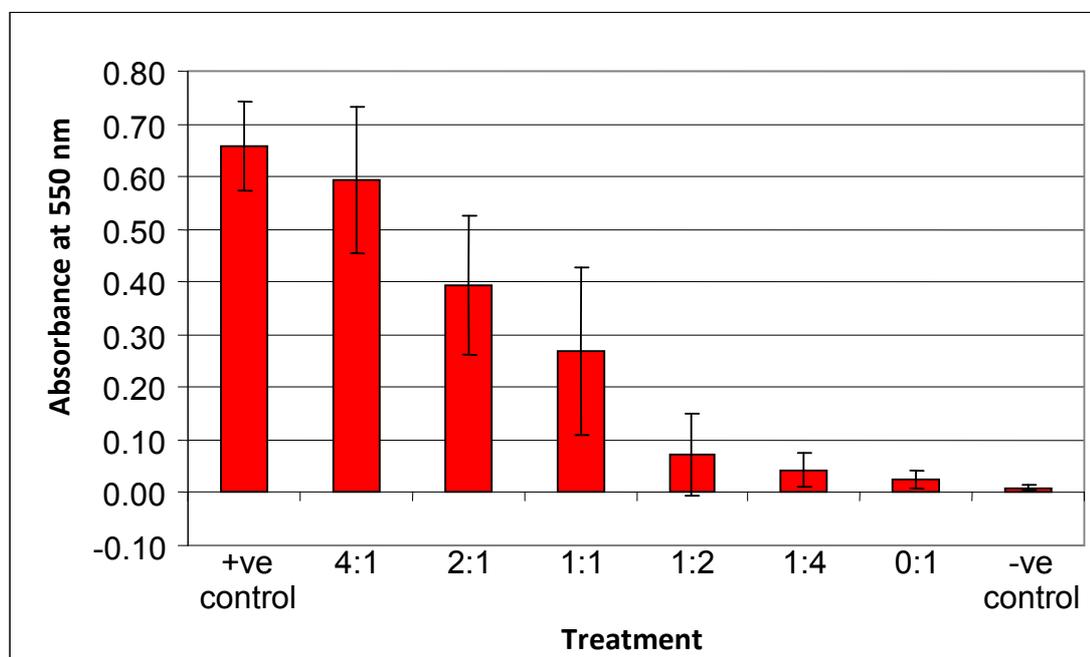


Figure 3.4 Effect of *L. rhamnosus* supernatant on growth of *O. placenta* in MRS media. Error bars indicate the standard deviation of the population.

Antifungal activity of the secondary metabolites of *Lactobacillus* was also observed against *A. xantha* (Figure 3.5) however, unlike *O. placenta*, there was no clear dose response. The *Lactobacillus* supernatant produced a similar result when the growth rate of *O. placenta* was measured on solid media (Figure 3.6), suggesting that the two methods are comparable.

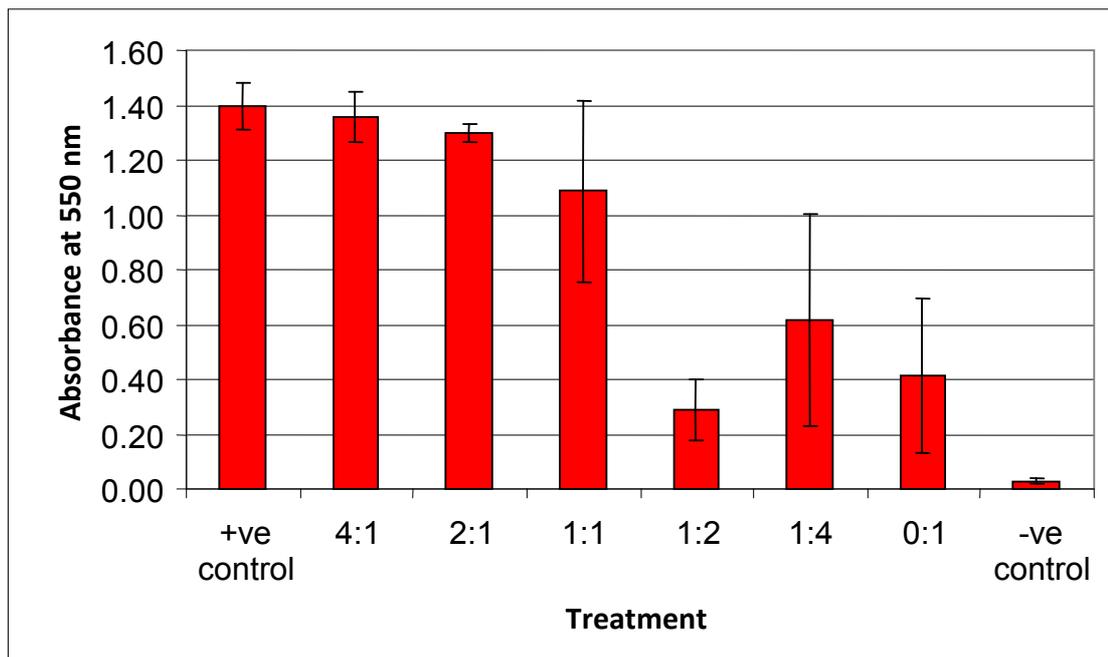


Figure 3.5 Effect of *L. rhamnosus* supernatant on growth of *A. xantha* in MRS media.

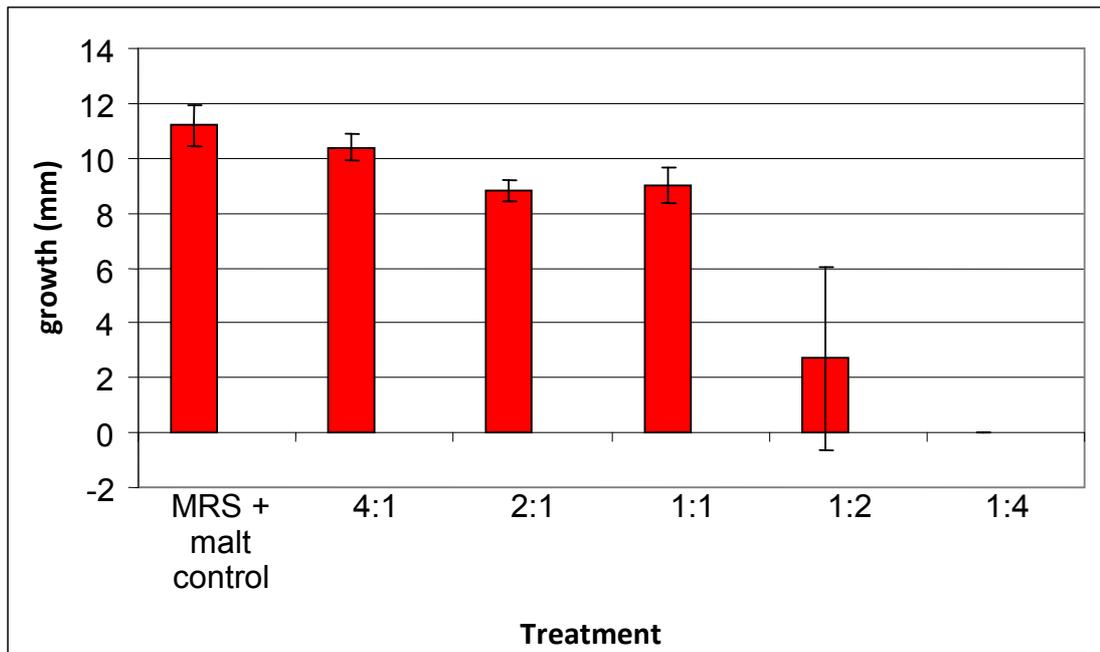


Figure 3.6 Growth of *O. placenta* after 6 days on MRS agar amended with *L. rhamnosus* supernatant dilutions.

It was found that *C. puteana* was unable to grow in MRS liquid medium. Further results showed that *A. xantha* and *C. puteana* would not grow on the solid MRS media. This suggests that the commonly used growth-rate assay (using MRS medium) would not be a useful method in assessing metabolite activity against all fungi.

3.3.2 Other media

Due to the lack of growth of *C. puteana* in MRS broth, the trial was repeated using different media (i.e. MRS media without salts, YM media, and SD media), to find a medium which would support both *Lactobacillus* sp. and the decay fungi. Both fungi and bacteria grew on all three other media though none of these media appeared to stimulate the production of antifungal secondary metabolites (Figure

3.7). This suggests that there is an ingredient (i.e. one or more of the salts) in the MRS media which supports the production of metabolites.

C. puteana did not grow well on the MRS media without salts, and it is unclear whether this is common for this fungus, or whether this is isolate specific to the culture used in this study. Further testing on other isolates of *C. puteana* would need to be carried out to confirm this result.

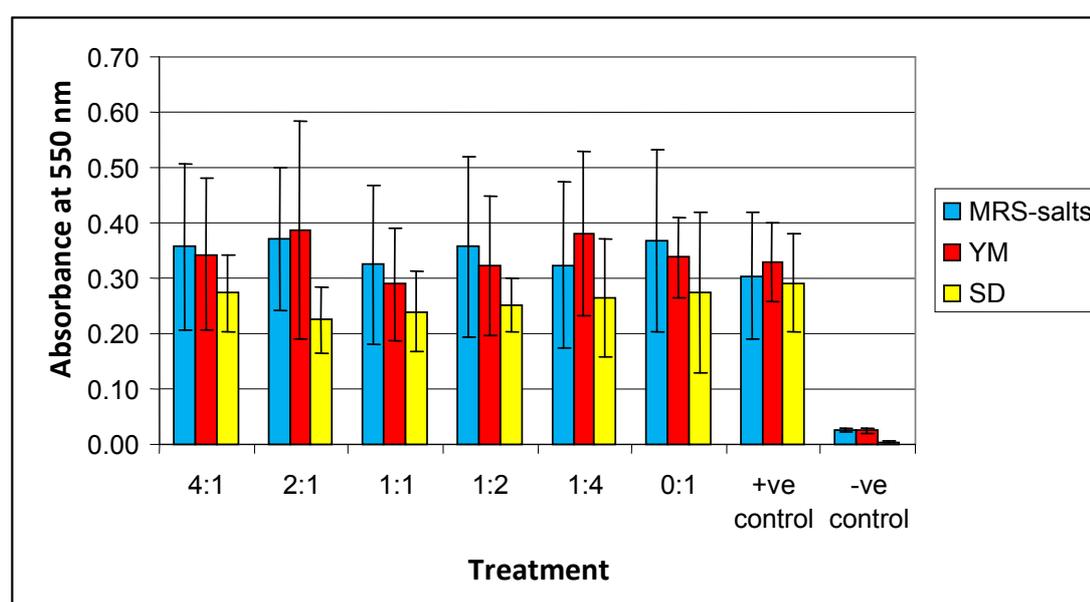


Figure 3.7 Effect of *L. rhamnosus* supernatant on growth of *O. placenta* in MRS-salts, YM and SD media.

3.4 SUMMARY

The correlation between the microscale assay method and the more commonly used growth rate assay, supports the use of the MODA method for screening wood decay fungi against *Lactobacillus* isolates to determine the presence of antifungal metabolites. The preferred media is MRS broth as this is able to support the production of secondary metabolites from *Lactobacillus* sp. and is also capable of supporting the growth of some decay fungi. Given this result it was

decided that all metabolite screening for this project would use the above method to screen for antifungal activity in order to choose the best isolates for further wood assays.

CHAPTER 4: ISOLATION, IDENTIFICATION AND ANTIFUNGAL SCREENING OF LACTIC ACID BACTERIA FROM CHILLI WASTE

4.1 INTRODUCTION

Lactic acid bacteria (LAB) are found naturally in food products and have been used for many years as biopreservatives in food (Schnurer and Magnusson, 2005, Gerez et al., 2009a). A few studies have shown that *Lactobacillus* isolates can have an antifungal effect on some early wood coloniser moulds and sapstain fungi (Yang and Clausen, 2004, Yang and Clausen, 2005) however there is no literature on their possible antifungal effects on wood decay fungi. Previous research has shown chilli waste to have moderate antifungal activity against two common Ascomycetes fungi (*Sphaeropsis sapinea* and *Leptographium procerum*) (Singh and Chittenden, 2008b). There was also a notable increase in fungal inhibition when chilli and *Lactobacillus* sp., isolated from the chilli juice, were combined.

The objective of this study was to isolate LAB from chilli waste and screen isolates against a common wood decay fungus using the microscale optical density assay described in the previous chapter (Chapter 3). Isolates which exhibited pronounced antifungal activity would then be identified using 16S rRNA phylogenetic procedures and subjected to a wood decay assay commonly used in the Wood Mycology Laboratory at Scion. An attempt would then be made to characterise the bacterial metabolites responsible for observed antifungal activity.

Work from this chapter has been published in the Journal of Applied Microbiology and is attached in Appendix IV.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of chilli juice

Rocoto chillies (*Capsicum pubescens*) (Figure 4.1) were obtained from a grower in the eastern Bay of Plenty, New Zealand, then cut in half, seeds and stalk removed, processed in a vegetable and citrus juicer (Elegance, Zip) and the pomace discarded. Chilli juice was refrigerated (4°C) until required.



Figure 4.1 Rocoto chilli (*Capsicum pubescens*) cut and whole chillies.

4.2.2 Isolation of bacteria from chilli

A 1 ml aliquot of raw chilli juice which had been refrigerated for at least 12 months (fresh chilli juice used in previous experiments did not produce bacteria with significant antifungal properties) was inoculated into 100 ml of deMan-Rogosa-Sharp (MRS) broth (Oxoid) and incubated in a shaking (100 rpm) water

bath at 30°C. After 24 hours 100 µl of the resulting culture was spread onto MRS Agar (Oxoid) and plates incubated at 25°C, 75% RH. After 2 days, individual colonies were isolated by streak-planting onto MRS agar. Pure cultures of bacteria from streak-plates were suspended in storage media containing MRS broth plus 15% glycerol in a 1.5 ml cryovial and stored at -80°C.

4.2.3 Identification of isolates

Bacterial isolates were identified using 16S rRNA gene sequencing. DNA was extracted from isolates using a previously described method (Marmur, 1961) (Appendix I). The 16S rRNA gene was PCR amplified using primers F27 (5'- AGAGTTTGATCMCTGGCTCAG-3') and R1492 (5'- TACGGYTACCTTGTTACGACTT -3'). The PCR product was purified using a 5M Quickclean PCR Purification Kit (Genscript Corporation, New Jersey, USA) and sequenced at the Waikato DNA Sequencing Facility (The University of Waikato, Hamilton, New Zealand). To identify the isolate the sequence database of the National Center for Biotechnology Information (NCBI) was searched for 16S rRNA gene sequence similarities using the computer algorithm Basic Local Alignment Search Tool (BLAST) (Aguiar et al., 2004).

4.2.4 Assay for antifungal activity (microscale optical density assay)

Initial screening of bacterial isolates was performed using the microscale optical density assay optimised specifically for this purpose in chapter 3 (O'Callahan et al., 2009). The wood decay fungus *Oligoporus placenta* (Fries) Gilb & Ryvardeen, (strain FR07/02), was selected as the test organism in this assay due to its virulent

nature and common occurrence in New Zealand's leaky buildings. Fungal cultures were grown in liquid malt broth: 2% malt powder, 0.5% mycological peptone (Oxoid), at 26°C, 75% RH for a period of up to four weeks. Inoculum was prepared by homogenising liquid fungal cultures in a Waring blender for 30 seconds at low speed followed by 30 seconds at high speed. A volume of 50 µl of this inoculum was added to test wells in a sterile 96-well covered cell culture plate (Sarstedt).

Lactic acid bacteria isolated from chilli waste were grown overnight in MRS broth at 30°C, 100 rpm. Resulting cultures were centrifuged at 5100 g for 10 minutes at room temperature and the supernatant filter sterilised through a 0.22 µm Steritop filter (Millipore). An aliquot of 100 µl of cell-free supernatant (CFS) was added to eight micro test wells (containing fungal inoculum) to serve as replicates. A positive control which contained 50 µl fungal inoculum and 100 µl sterile MRS broth and a negative control which contained 50 µl sterile malt broth plus 100 µl sterile CFS were also used.

Inoculated plates were read at 600 nm on a Multiskan® Spectrum (Thermo Electron Corporation) plate reader to get an absorbance measurement. Plates were wrapped with plastic wrap during incubation at 25°C, 75% RH (to prevent evaporation of media) and reread after 6 days. Change in absorbance was recorded by subtracting the initial reading from the final absorbance.

4.2.5 Assay for wood decay

Performance against wood decay was assessed using a modified agar/block decay test described below (Sutter, 1978). Bacterial isolate *L. brevis* C11 was tested

against three different incubation regimes to determine the conditions which produced the most antifungal activity and then isolates C15, C16, C17, C18, C19, and C20, were subjected to the conditions deemed best (Table 4.1). *Pinus radiata* ‘Sutter’ blocks (~35 x 35 x 7 mm longitudinal) were treated by vacuum impregnation with solutions as described in Tables 4.1 and 4.2.

Table 4.1 Treatment schedule for *P. radiata* wood blocks with bacterial isolates (experiment 1).

Treatment	
A	24 hour incubation at 30°C LAB culture CFS [C11]
B	24 hour incubation at 30°C LAB culture (not filter sterilised) fixed for one week in a sealed plastic bag at room temperature (~20°C) [C11]
C	1 week incubation at 30°C LAB culture CFS [all isolates]
D	MRS broth control
E	Untreated control

Table 4.2 Treatment schedule for *P. radiata* wood blocks (experiment 2).

Treatment	
A	100% MRS Broth (52 g in 1L H ₂ O)
B	50% MRS Broth
C	25% MRS Broth
D	Untreated control

The test blocks (16 replicates per fungus/treatment combination) were weighed after treatment, then left to dry at ambient room temperature (averaging approximately 20°C). Eight replicate blocks from each treatment combination were subjected to leaching for 14 days. Leaching involved the resaturation of treated blocks with distilled water after which they were placed in nine times their volume of water (distilled). Leaching water was changed every two days. After leaching the blocks were air dried and then all blocks were conditioned to constant weight at 12% equilibrium moisture content (emc) in a room controlled at 20°C and 65% RH. Blocks were then weighed, packaged and sterilised by exposure to ethylene oxide gas. The blocks were then placed aseptically into prepared agar containers containing pure culture decay fungi and incubated for six weeks at 26°C and 75% RH. Test fungi used were the brown rot fungi *Coniophora puteana* (Schumach.) P. Karst. (strain: BAM Ebw 15), *Antrodia xantha* (Fr.) Ryvarden (strain: 729.1) and *Oligoporus placenta* (strain: FR07/02).

Following incubation, the blocks were cleaned, air dried, reconditioned to constant weight at 12% emc and reweighed. Percentage mass loss for each block was calculated as below and means were determined for each fungus/treatment combination.

$$\text{Percentage mass loss} = \frac{(\text{weight before exposure} - \text{mass after exposure})}{\text{weight before exposure}} \times \frac{100}{1}$$

4.2.6 Assay for pH, temperature and proteolytic enzyme effect

To determine the nature of the antifungal substance, CFS from each isolate was altered as below and assessed using the microscale absorbance assay described above.

pH sensitivity

Aliquots of CFS were adjusted to pH 4, 5, 6, 7, 8 and 9 using 1 mol l⁻¹ NaOH or HCl.

Heat resistance

Further aliquots of CFS were heated to 50, 60, 70, 80, 90, 100 and 121°C (autoclave) for 10 minutes.

Effect of proteolytic enzyme

An aliquot of 25 µl of Proteinase K (20 mg ml⁻¹) was added to a 1 ml aliquot of CFS adjusted to pH 7 and incubated at 45°C for 60 minutes. Proteinase K was then inactivated by heating to 100°C for 10 minutes.

4.2.7 HPLC analysis

Organic acids in the *L. brevis* CFS were analysed on an Agilent 1290 HPLC (Agilent, Santa Clara, CA, USA). Samples, 5 µl, of filtered (0.2 µm) CFS were injected onto an Acclaim OA column (5 µm, 4 × 150 mm) (Dionex, Cedar Rapids, IA, USA). Elution was at 1.0 ml min⁻¹, with 100 mmol sodium sulphate (pH 2.65 with methanesulphonic acid) for 3 minutes followed by a gradient from 0 to 45% acetonitrile in 12 minutes. Detection was at 210 nm. Retention times for acetic, lactic and phenyl lactic acids were obtained using standards in water. MRS

medium was spiked with 50 and 100 mmol lactic and acetic acid standards and amounts of these acids present in the MRS-containing samples were estimated by comparison with the spiked chromatograms.

4.2.8 Statistical analysis

One-way analysis of variance (ANOVA) was conducted for the optical density assay to determine significant differences ($P \leq 0.05$) between the antifungal activity of different bacterial isolates and the control, with the null hypothesis being that there was no difference between isolates and the control. Significant differences were also determined in the wood assay between bacterial treatments and the MRS media control to determine if the media accounted for all mass loss observed.

4.3 RESULTS

4.3.1 Screening and identification of bacteria

Eleven potential LAB isolates (C6, C10, C11 and C13 to C20) were obtained using the isolation protocol. All organisms were capable of growing in or on MRS media tentatively identifying them as lactic acid bacteria. Screening trials using the microscale optical density assay (Figure 4.2) identified that organisms C11, and C15 to C20 (inclusive) had a significant ($P < 0.05$) antifungal effect on the decay fungus *O. placenta* with change in absorbance decreasing as fungal growth is inhibited.

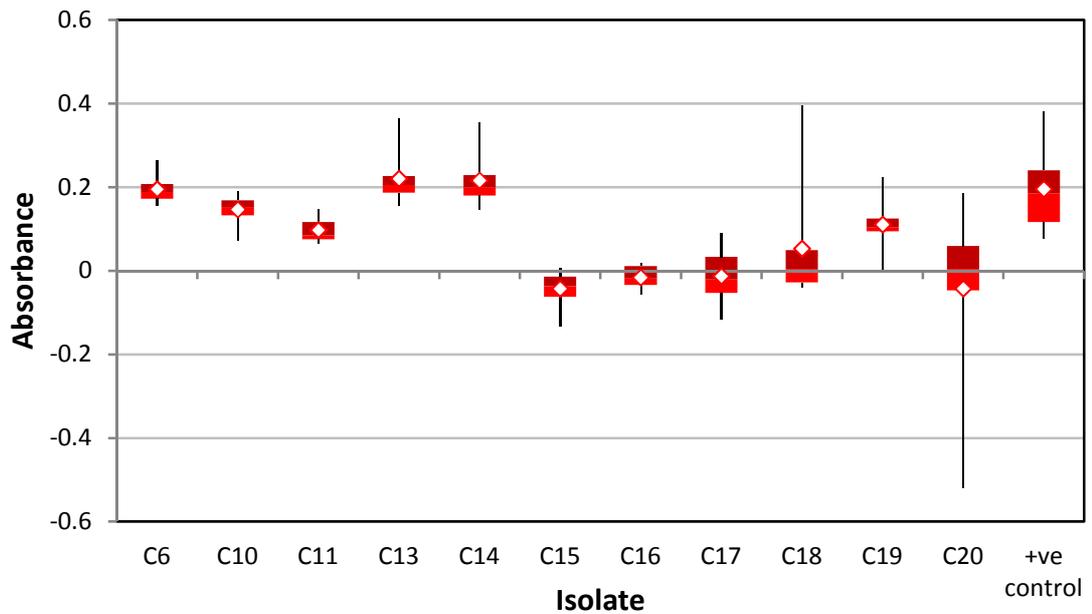


Figure 4.2 Growth of *O. placenta* in 24 hour cell-free supernatants of chilli isolates.

These organisms were subsequently identified using 16S rRNA gene sequencing (Table 4.3) and were used in ensuing wood assay trials. It was identified that the four other isolates (C6, C10, C13 and C14) were not bacterial and therefore were identified using primers ITS1-F and ITS4 which are generally used for identifying yeast and fungal organisms. Identities of these microorganisms can be found in Appendix II along with further details of the identifications below.

Table 4.3 Identification of bacteria from chilli using 16S rRNA gene sequencing and BLAST search of NCBI database.

Isolate number	Identification (closest BLAST match)
C11	<i>Lactobacillus brevis</i>
C15	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
C16	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
C17	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
C18	<i>Gluconobacter oxydans</i>
C19	<i>Leuconostoc pseudomesenteroides</i>
C20	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>

4.3.2 Wood decay assay

Experiment 1

Mass loss results for *L. brevis* C11 treatments (Figure 4.3) demonstrate that all non leached (samples not exposed to water) wood treatments exhibit antifungal activity against the decay fungi in this test, including the control treatment of MRS broth suggesting that one or more ingredients of this media has an ability to suppress fungal growth. This was also seen when developing the microscale absorbance assay method with *A. xantha* and *C. puteana* failing to grow satisfactorily in MRS broth (O’Callahan et al., 2009). Despite that, there was increased activity in the one week incubated treatments of *L. brevis* when exposed to *C. puteana* and *O. placenta* with these treatments performing significantly better ($P < 0.05$) than the MRS control, with the one week CFS treatment having 2.4% mean mass loss compared with 4.8% for MRS when inoculated with *C. puteana* (Figure 4.3B) and a mean negative mass loss compared to the MRS control ($>10\%$ mass loss) when inoculated with *O. placenta* (Figure 4.3C).

Although not significantly different ($P>0.05$) the one week CFS inoculated with *A. xantha* had 3.5% mean mass loss compared to 5.8% for MRS (Figure 4.3A). When the treated blocks were subjected to leaching the wood became as susceptible to decay as the control, more so in some cases. This suggests that the substance inhibiting fungal growth is water soluble and subject to leaching under high moisture contents.

From these results it was determined that incubating the bacterial isolate for one week at 30°C produced the most pronounced antifungal activity. Consequently further wood assays of other bacterial isolates focused mainly on this incubation regime.

All treatments showed significant ($P<0.05$) antifungal activity against the decay fungi they were exposed to (Figure 4.4), but only *L. brevis* C11 and *G. oxydans* C18 performed significantly better than the MRS control when exposed to *C. puteana* ($P<0.05$) (Figure 4.4B). *L. brevis* C11 and *L. pseudomesenteroides* C19 performed significantly better than the MRS control when exposed to *O. placenta* ($P<0.05$) (Figure 4.4C) and only *L. mesenteroides* subsp. *mesenteroides* C17 gave significantly better control against *A. xantha* ($P<0.1$) (Figure 4.4A).

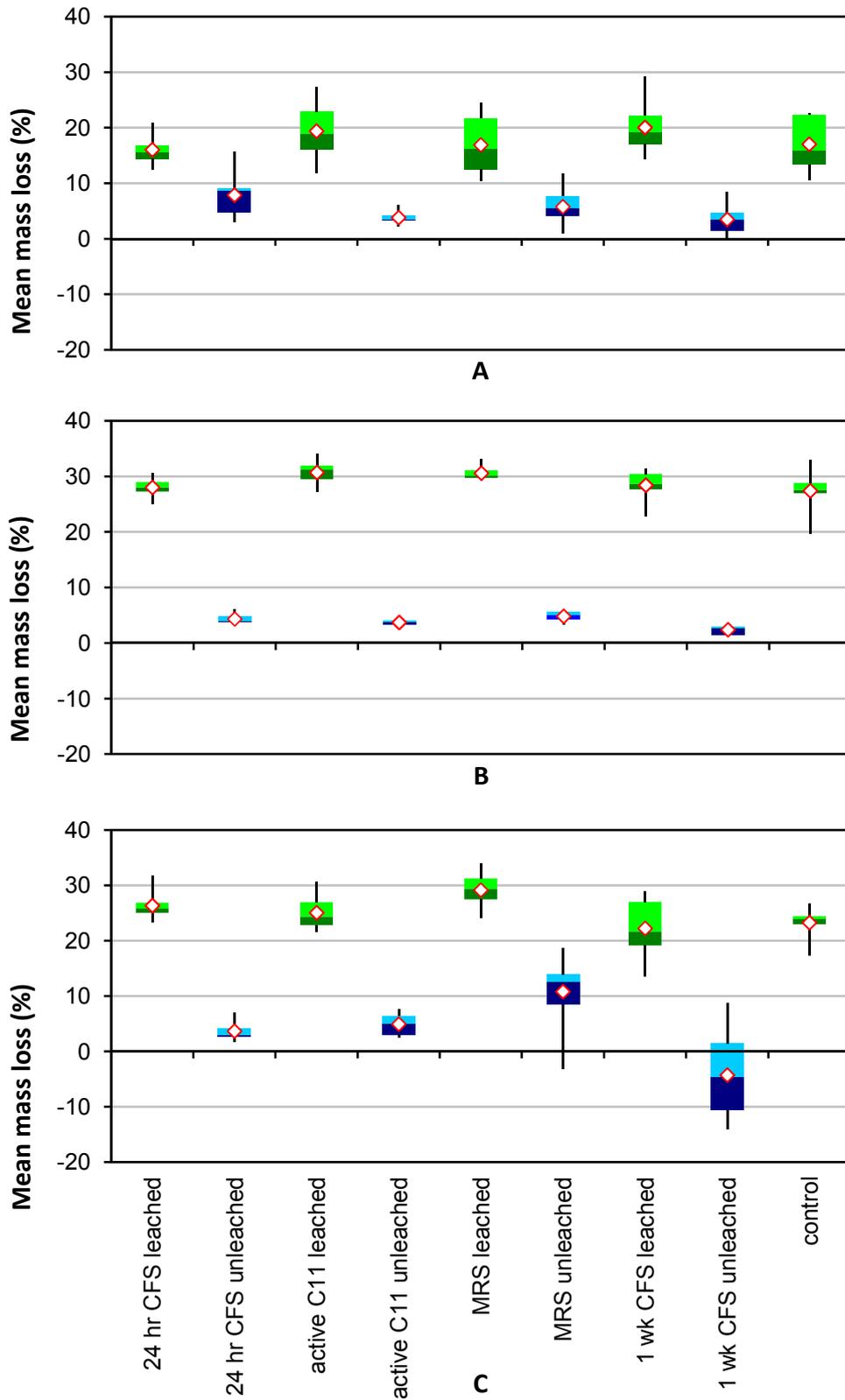


Figure 4.3 Mass loss of *Pinus radiata* blocks treated with *L. brevis* C11 CFS and exposed to wood decay fungus (A) *A. xantha*, (B) *C. puteana* and (C) *O. placenta*.

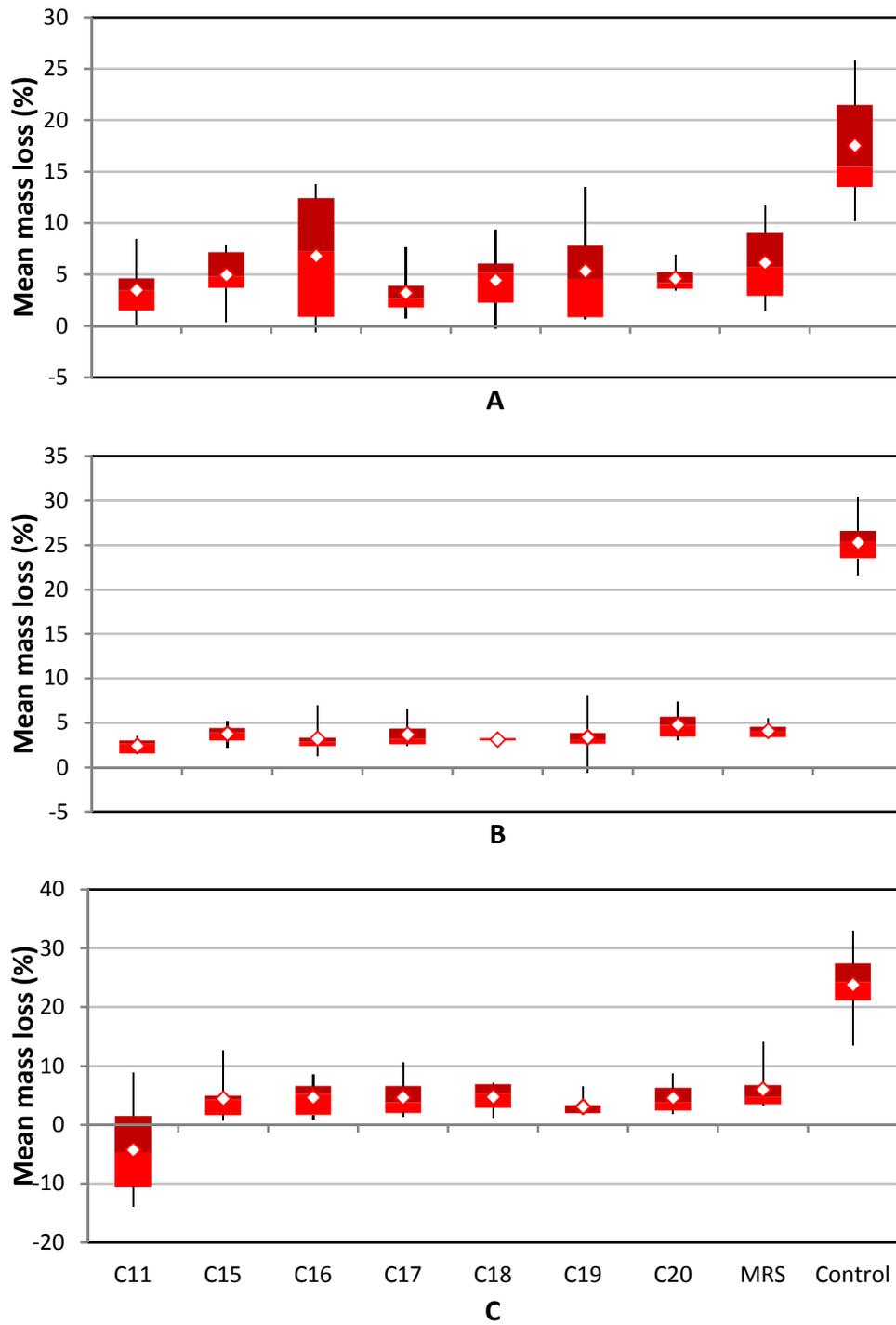


Figure 4.4 Mass loss of wood blocks treated with CFS of various isolates incubated for one week and exposed to (A) *A. xantha*; (B) *C. puteana*; (C) *O. placenta*.

Experiment 2

It was observed when removing unleached wood blocks from the fungal cultures that a number of blocks appeared to have higher than normal moisture contents for this type of trial. A second trial was set up to examine whether the MRS media promoted increased moisture content and subsequently the effect this has on mass loss due to fungal degradation. In this trial there was a clear correlation between the moisture content (which tended to increase as the concentration of media increased) and mass loss (which decreased as concentration increased) (Figure. 4.5).

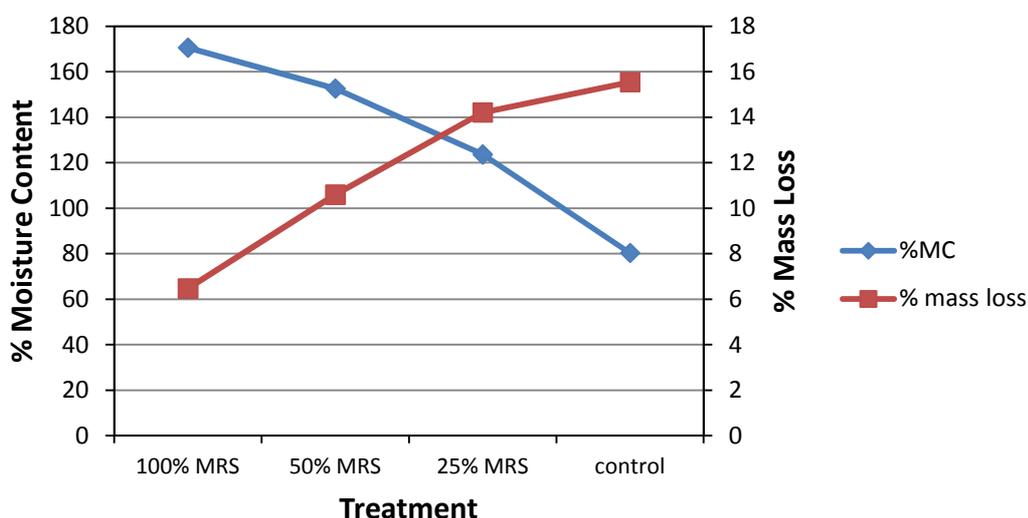


Figure 4.5 Correlation between moisture content and fungal decay due to *A. xantha*.

4.3.3 Characterisation of antifungal metabolites

The Proteinase K and temperature treatments had no effect on the antifungal properties of the *L. brevis* C11 CFS with no fungal growth being observed, indicating that the metabolite was not of a proteinaceous nature. However, antifungal activity decreased with increasing pH (from addition of NaOH) suggesting an acidic nature of the antifungal metabolites (Fig. 4.6A). Similar

patterns were seen for *G. oxydans* C18 (Fig. 4.6B), *L. pseudomesenteroides* C19 (Figure 4.6C) and *L. mesenteroides* subsp. *cremoris* C20 (Figure 4.6D). A different pattern was seen with *L. mesenteroides* subsp. *mesenteroides* isolates C15, C16 and C17 (Figure 4.7). These isolates all showed a marked reduction in antifungal activity at neutral pH 7 and increasing activity as the pH increased or decreased, with complete inhibition at pH 4 and 5 for all isolates and pH 9 for both C15 and C17. The antifungal activity was generally not affected by temperature but began to show signs of decreased activity at 121°C and the addition of proteinase K caused complete loss of antifungal activity. Altogether these results suggest that antifungal activity from these isolates may be due to something other than production of organic acids.

High performance liquid chromatography analysis of the cell-free supernatants (Figures A3.1 – A3.7, Appendix III) showed that acetic and lactic acids were produced by *L. brevis* C11. When compared against MRS media spiked with different volumes of acetic, lactic and phenyl lactic acids, isolate C11 produced slightly greater than 100 mmol of lactic acid and just over 50 mmol of acetic acid. Phenyl lactic acid was not produced, but there were several other small peaks, which could relate to other organic acids that were not identified. *Leuconostoc mesenteroides* subsp. *mesenteroides* C15 produced a similar amount of acetic acid as *L. brevis*. It also produced small amounts of lactic and phenyl lactic acids and other unidentified peaks. *L. mesenteroides* subsp. *mesenteroides* C16 produced small amounts of all three acids and *Leuconostoc mesenteroides* subsp. *cremoris* C20 produced small amounts of both acetic and phenyl lactic acids and a larger amount of lactic acid (similar to *L. brevis* C11).

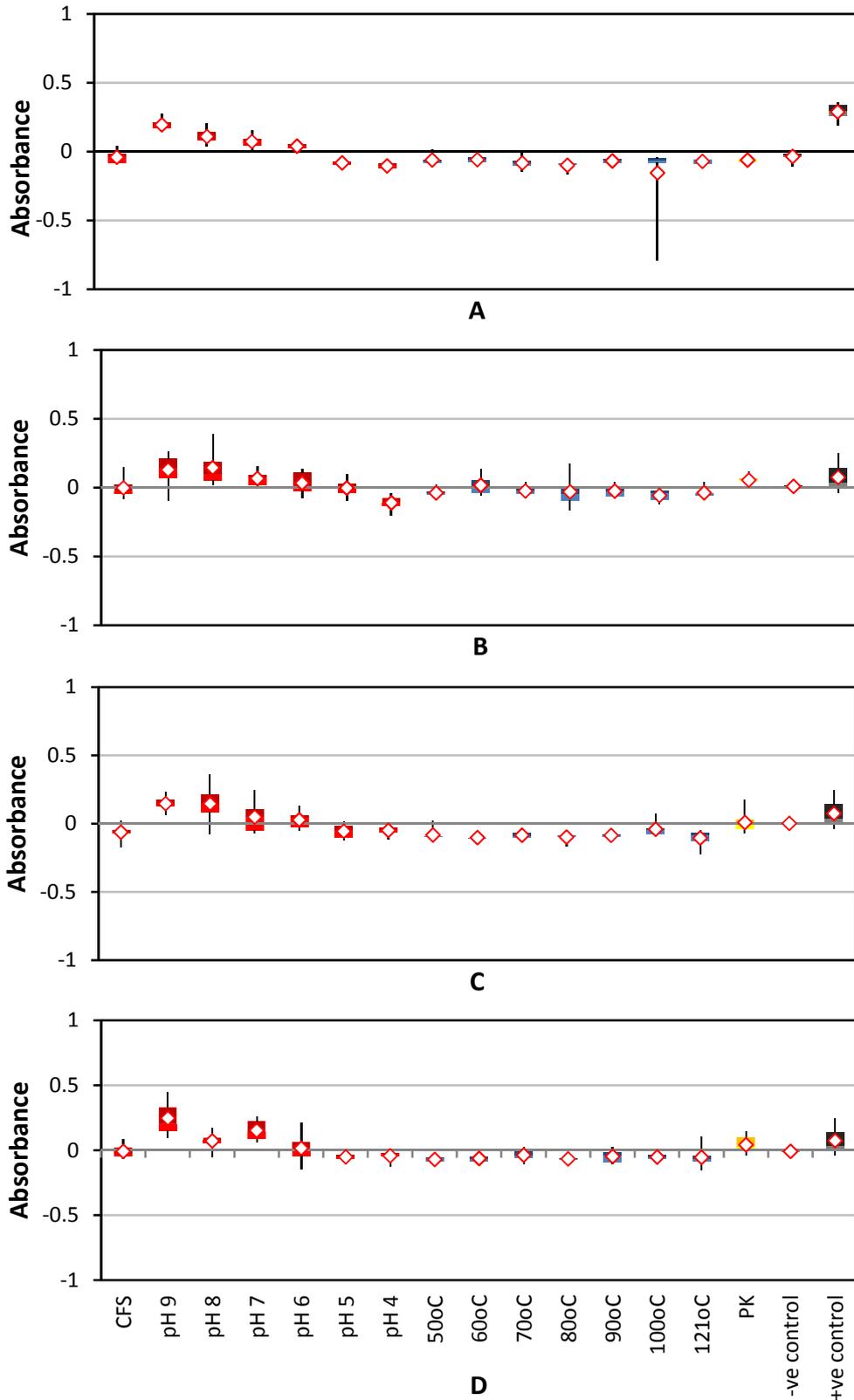


Figure 4.6 Growth of *O. placenta* against CFS treated to different pH and temperature or addition of proteinase K. (A) C11; (B) C18; (C) C19; (D) C20.

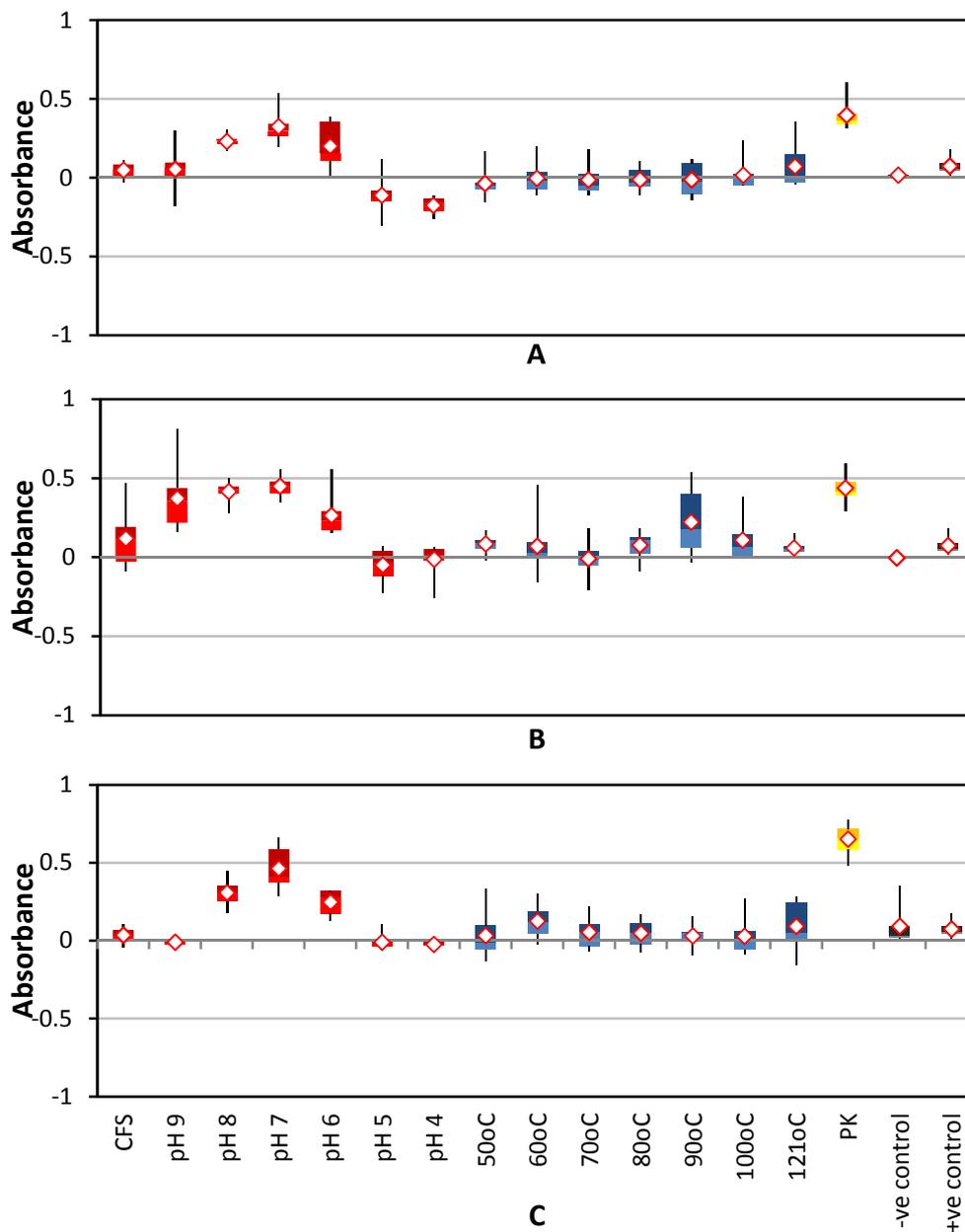


Figure 4.7 Growth of *O. placenta* in CFS treated to different pH and temperature or addition of proteinase K. (A) C15; (B) C16; (C) C17.

Other isolates, C17, C18 and C19 did not appear to produce any organic acids in the samples tested. Given the antifungal activity seen in the MODA assay and the results from pH, temperature and proteinase treatment, it is considered possible that compounds other than organic acids are responsible for the antifungal activity produced by isolate C15, C16 and C17. It is feasible that isolates C18 and C19

also produce other compounds or proteins which may have some antifungal activity.

4.4 DISCUSSION

In this study eleven microbial isolates from chilli waste were evaluated for their antifungal properties against common wood decay fungi. The most promising seven isolates C11 and C15 – C20, were identified as *Lactobacillus brevis*, *Leuconostoc mesenteroides* subsp. *mesenteroides* (three isolates), *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc pseudomesenteroides* and *Gluconobacter oxydans* by 16S rRNA gene sequence analysis and were further analysed for their ability to prevent wood decay caused by basidiomycetes *O. placenta*, *C. puteana* and *A. xantha*. Results from the wood decay assay suggest that a number of the isolates, in particular *L. brevis* C11, produce metabolites which inhibit decay fungi in the wood, with greater inhibition occurring with a longer incubation period of the isolate. However, results also suggest that the medium in which the bacteria were grown also has some inhibitory effect. It is unclear as to why this media would have this effect on the basidiomycetes as many of the MRS media components are common in fungal culture media (Mycological Society of America Mycology Guidebook Committee, 1974, O'Callahan et al., 2009) and similar studies involving mould fungi have not reported any inhibition of fungal growth in MRS media (Yang and Clausen, 2004). It was noticed that during removal of the blocks from exposure in the test vessels, that moisture contents of the wood treated with the media or CFS appeared higher than leached or untreated blocks. It is proposed that the salt content of the media acted to absorb extra moisture from the environment thus making the wood too wet for the fungi to cause decay. Decay fungi, such as those

used in this study, generally attack wood at moisture contents of between approximately 35 and 50% although some fungi can invade timber at much higher moisture contents (Butcher, 1974). Moisture contents at 100% MRS (recommended concentration) reached values of 170% on average in this study.

Despite the inhibitory effect of the media used, wood blocks treated with *L. brevis* C11 supernatant (either cell-free or active, incubated for 1 week) had significantly ($P < 0.05$) less mass loss than their untreated counterparts when exposed to decay fungi *C. puteana* and *O. placenta*. These results suggest that the isolated *L. brevis* culture produced an antifungal substance or substances, which was capable of inhibiting decay fungi. *L. brevis* has been shown to produce antifungal substances of proteinaceous (Falguni et al., 2009) and acidic natures (Gerez et al., 2009b), and sometimes both (Mauch et al., 2010). Gerez et al. (2009a) found two *L. brevis* strains which had antifungal activity against various bread moulds. As with this investigation the antifungal activity was not changed by heating or treatment with Proteinase K, though it was removed by neutralization with NaOH. Antifungal activity was attributed to the production of lactic and acetic acid and the subsequent lowering of pH. However, Mauch et al. (2010) discovered that reduction in antifungal activity from two *L. brevis* strains tested was only achieved after increasing the incubation time of proteinase K to 5 hours instead of 60 minutes. Therefore, there is a possibility that the antifungal substance in this study is proteinaceous, although this is unlikely due to the lack of effect from heating. Falguni et al. (2010) and Mojgani et al. (2009) both reported proteinaceous substances produced by *L. brevis* which were heat stable. *L. brevis* is heterofermentative and produces lactic acid from glucose as well as producing CO₂, ethanol, formic and acetic acids (VanDemark and Batzing, 1987, Battcock

and Azam-Ali, 1998, Madigan et al., 2000b, Todar, 2011). Schnurer and Magnusson (2005) suggested that antifungal activity produced by LAB is likely due to synergistic effects between several active components.

Leuconostoc sp. are also known to produce lactic acid, ethanol and CO₂ (Grigoriev et al., 2011) along with various other compounds such as dextrans, diacetyl, acetoin and hydrogen peroxide (Hemme and Foucaud-Scheunemann, 2004). Studies have also shown the production of bacteriocin-like substances (Stiles, 1994). In this study the production of organic acids (specifically lactic, acetic and phenyl lactic acids) was determined from a number of *Leuconostoc* isolates as the compounds most likely contributing to antifungal activity. However, some isolates showed no or very little production of organic acids yet still showed some measure of activity against *O. placenta* in a quick screening assay. Results from subjecting CFS of these isolates to temperature and pH variations suggest that other compounds may be produced which also have antifungal activity. In the presence of oxygen, such as in this study, hydrogen peroxide can be produced which is known to be inhibitory to some microorganisms (Hemme and Foucaud-Scheunemann, 2004).

Gluconobacter oxydans is a member of the acetic acid bacteria (AAB) and produces acetic acid from ethanol (i.e. vinegar production). Acetic acid bacteria are aerobic organisms and carry out incomplete oxidation of substrates such as the oxidation of glucose to gluconic acid, galactose to galactonic acid and sorbitol to sorbose, a precursor in the formation of ascorbic acid (vitamin C) (Madigan et al., 2000b). In this study *G. oxydans* C18 produced very little in the way of organic acids with only a small amount of acetic acid and one other unidentified peak

when examined by HPLC. The CFS of C18 inhibited fungal growth in the optical density assay and blocks treated with CFS had significantly less decay caused by *C. puteana* compared to the untreated control. There is no evidence in the literature of *G. oxydans* producing proteinaceous antifungal substances and the results from this experiment support the idea that the antifungal effect is due to either the production of organic acids or some unidentified compound.

Production of antifungal metabolites by bacteria is linked to environmental conditions and bacterial growth stages. Most bacteria will employ basic metabolic functions when grown in nutrient-rich media, however when nutrients are depleted they will start to produce various secondary metabolites to help with survival (Demain, 1998). In the laboratory, incubation conditions (media, temperature, pH, aeration and agitation) can have a direct effect on the production of metabolites (Cabo et al., 2001, Bizani and Brandelli, 2004, Moita et al., 2005). Moita et al. (2005) found that high pH values favoured the production of metabolites which were active against mould isolates; however the effect of temperature and aeration was specific to the fungal species. Microorganisms isolated in this study differ in their preferred temperature of incubation with all bacteria being able to grow at 30°C (the incubation temperature in these experiments). However, *L. brevis* has been shown to have optimal production of antifungal substances at 37°C (Falguni et al., 2010), *L. mesenteroides* is said to favour temperatures of 21 – 25°C (Hemme and Foucaud-Scheunemann, 2004) and the optimum temperature for growth of AAB is between 25 – 30°C (Sengun and Karabiyikli, 2011). The effect of environmental variations appears to depend on the metabolite producing bacteria and the target organisms for inhibition. In these experiments basic incubation conditions were employed to produce the bacterial

supernatant. It is proposed that the antifungal effect of all the bacterial supernatants could be improved by optimising incubation conditions.

4.5 SUMMARY

Seven bacterial isolates showing antifungal activity against wood decay fungi were isolated from chilli waste. Isolates were identified using 16S rRNA phylogenetic techniques and were comprised of both lactic and acetic acid bacteria including *Lactobacillus brevis* (LAB), *Leuconostoc mesenteroides* subsp. *mesenteroides* (LAB), *Leuconostoc mesenteroides* subsp. *cremoris* (LAB), *Leuconostoc pseudomesenteroides* (LAB) and *Gluconobacter oxydans* (AAB).

When tested against wood decay by exposing wood blocks impregnated with cell-free supernatants from one week old bacterial cultures, grown in complex media, to pure decay cultures for six weeks, all isolates showed resistance to degradation, but only the CFS of *L. brevis* C11 consistently provided significantly better resistance to decay than the media control.

High performance liquid chromatography results in combination with the effects of pH and temperature on antifungal activity suggested the most likely cause of antifungal activity was due to the production of relatively large amounts of lactic and acetic acids. Other unidentified compounds were also produced which may or may not have contributed to activity.

Given the tendency of the bacterial metabolites in this project to leach out of timber when subjected to wetting, they are unlikely to be considered suitable as a permanent preservative for wood protection when used alone. Nevertheless they

may have potential for use in combined treatments with other benign substances with antifungal properties (Singh and Chittenden, 2008b), especially in situations where timber is unlikely to get wet and this option is investigated further in chapter 5.

CHAPTER 5: ANTIFUNGAL EFFECT OF BACTERIAL METABOLITES IN COMBINATION WITH CHILLI JUICE

5.1 INTRODUCTION

In the previous chapter it was seen that lactic acid bacteria and acetic acid bacteria all were capable of some degree of antifungal activity. However, it was unclear how much of an influence the culture media was in the amount of antifungal activity seen. It was also suggested that antifungal activity could possibly be increased by combining the LAB and AAB with another benign compound such as a plant extract. In this chapter we explore the possibility of combining the isolated bacteria with the chilli juice that they were originally isolated from to produce a potential 'wood preservative'. Chilli extracts have been shown in previous experiments to have antifungal activity (Chowdhury et al., 1996, Bowers and Locke, 2000, Anaya-Lopez et al., 2006, Erturk, 2006, Singh and Chittenden, 2008b, Nazzaro et al., 2009) and the isolated bacteria are known to grow in the chilli juice, therefore if the bacteria could be grown in the chilli juice to produce the same metabolites as when grown in MRS media it is plausible that we may see increased antifungal activity.

A previous study which looked at the synergy between chilli juice and addition of a LAB (Singh and Chittenden, 2008b) suggested that there was a synergistic effect between the two when tested on agar plates against sapstain fungi. In this study the aim was to take this a step further and investigate this possible effect against decay fungi using the bacteria previously isolated (chapter 4) in quick screening methods as well as a wood assay. High performance liquid

chromatography was also performed to compare production of metabolites in the chilli juice with those produced in MRS media.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of chilli juice

Rocoto chillies were obtained and prepared as described previously (chapter 4.2.1). Chilli juice was frozen (-20°C) until required.

5.2.2 Assay for antifungal activity (microscale optical density assay)

Screening of bacterial isolates in combination with chilli at different concentrations was performed using the microscale optical density assay developed in chapter 3 (O’Callahan et al., 2009) and described in chapter 4 (4.2.4).

Experiment 1

Lactobacillus brevis C11 was inoculated into growth media (Table 5.1) then incubated for one week at 30°C, 100 rpm.

Table 5.1: Treatment schedule for MODA (experiment 1).

Treatment	Growth media
A	100% MRS broth
B	50% MRS broth (diluted with distilled water)
C	50% MRS broth + 50% chilli juice
D	50% chilli juice (diluted with distilled water)
E	100% chilli juice
F	100% MRS. Chilli added after filtration to get 50:50

Resulting cultures were centrifuged at 5100 g for 10 minutes at room temperature and the supernatant filter sterilised through a 0.22 µm Steritop filter (Millipore). Positive controls which contained 50 µl fungal inoculum and 100 µl uninoculated growth media as in table 5.1 and negative controls which contained 50 µl sterile malt broth plus 100 µl uninoculated growth media were also included.

Experiment 2

Bacterial cultures C11, C15, C16, C17, C18 and C20 were inoculated into sterile growth media (Table 5.2) then treated as for experiment 1. The isolate C19 would not grow sufficiently in chilli juice and was consequently left out of further experiments.

Table 5.2: Treatment schedule for MODA (experiment 2).

Treatment	Growth media
A	100% MRS (C15 – 18, C20)
B	50% chilli in distilled water (C15 – 18, C20)
C	25% chilli in distilled water (C11, C15 – 18, C20)

5.2.3 Assay for wood decay

Performance against wood decay was assessed using a modified agar/block decay test (Sutter, 1978) as described in chapter 4 (4.2.5). Bacterial isolates *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 were grown in 50% chilli (diluted with distilled water) for one week, centrifuged and filter sterilised. *Pinus radiata* ‘Sutter’ blocks (~35 x 35 x 7 mm longitudinal) were treated by vacuum impregnation with CFS solutions described above. A control treatment of

uninoculated filter sterilised 50% chilli juice and an untreated control were also included in the trial.

5.2.4 HPLC analysis

Organic acids in the cell-free supernatants were analysed on an Agilent 1290 HPLC (Agilent, Santa Clara, CA, USA). Samples, 5 μ l, of filtered (0.2 μ m) CFS were injected onto an Acclaim OA column (5 μ m, 4 \times 150 mm) (Dionex, Cedar Rapids, IA, USA). Elution was at 0.6 ml min⁻¹, with 100 mmol sodium sulphate (pH 2.65 with methanesulphonic acid) for 3 minutes followed by a gradient from 0 to 45% acetonitrile in 12 minutes. Detection was at 210 nm. Retention times for acetic, lactic and phenyl lactic acids were obtained using standards in water.

5.2.5 Statistical analysis

One-way analysis of variance (ANOVA) was conducted for the optical density assay to determine significant differences ($P \leq 0.05$) between the antifungal activity of different bacterial isolates grown in chilli and the controls, with the null hypothesis being that there was no difference between isolates and the controls. Significant differences were also determined in the wood assay between bacterial/chilli treatments and the chilli and untreated controls to determine if there was any significant improvement in wood protection.

5.3 RESULTS

5.3.1 Assay for antifungal activity

Initially, to determine if bacterial isolates would grow in chilli juice and produce antifungal metabolites, *L. brevis* C11 was assessed for activity when grown in

various concentrations of MRS media and/or chilli juice (Figure 5.1). *Lactobacillus brevis* C11 was seen to completely inhibit *O. placenta* at 100% and 50% MRS, 100% and 50% chilli juice and also when chilli juice was added later to a culture of bacteria grown in MRS media. When grown in 50% chilli juice the growth of *L. brevis* appears to have produced metabolites which significantly ($p < 0.01$) enhanced the antifungal activity of 50% chilli juice alone (D +ve control). Given these results it was determined that 50% chilli juice was a suitable medium to grow the bacterial isolates in and a second experiment was set up in which each bacteria was grown in 100% MRS, and 50% and 25% chilli juice (Figure 5.2)

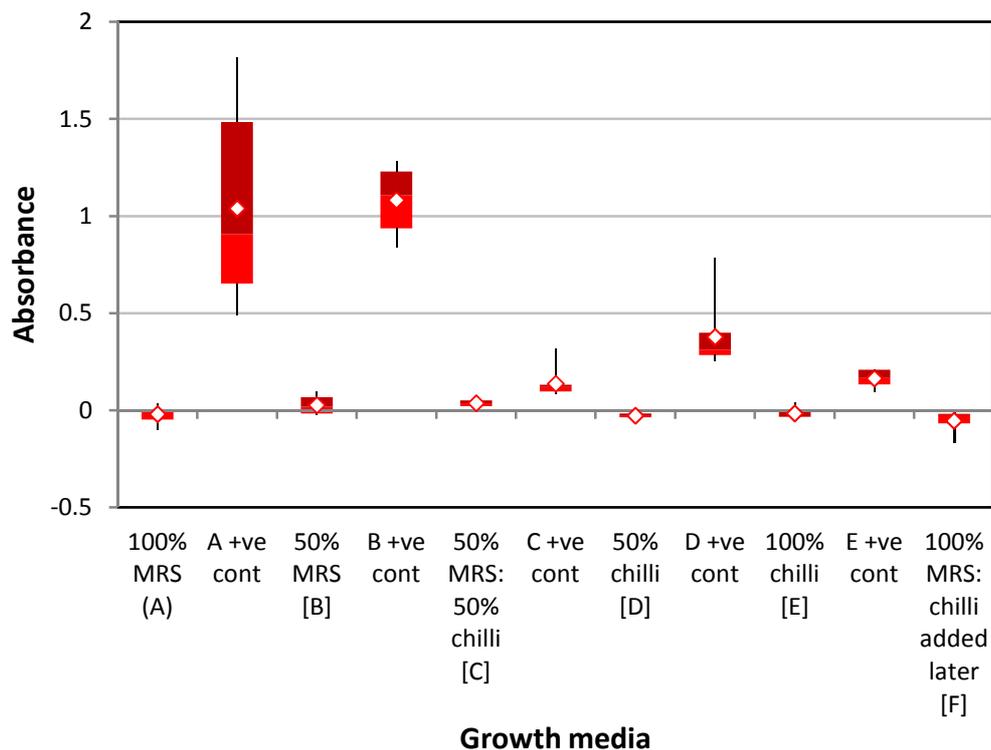


Figure 5.1 Growth of *O. placenta* in one week CFS of *L. brevis* C11 grown in varying concentrations of MRS and chilli growth media (experiment 1).

In Figure 5.2 it can be seen that *L. brevis* C11 and *L. mesenteroides* subsp. *mesenteroides* C20 both provided complete inhibition of *O. placenta* when grown in both MRS and 50% chilli juice and almost complete inhibition when grown in 25% chilli juice. Isolates C15 and C18 showed an improvement in antifungal activity as the concentration of chilli juice was increased with *G. oxydans* C18 giving almost complete inhibition of *O. placenta* when grown in 50% chilli juice, while the remaining isolates had superior antifungal activity when grown in MRS media, although 50% chilli juice provided better activity than 25%. From these results it was determined that isolates C11 and C20 were the most promising to pursue in a wood assay.

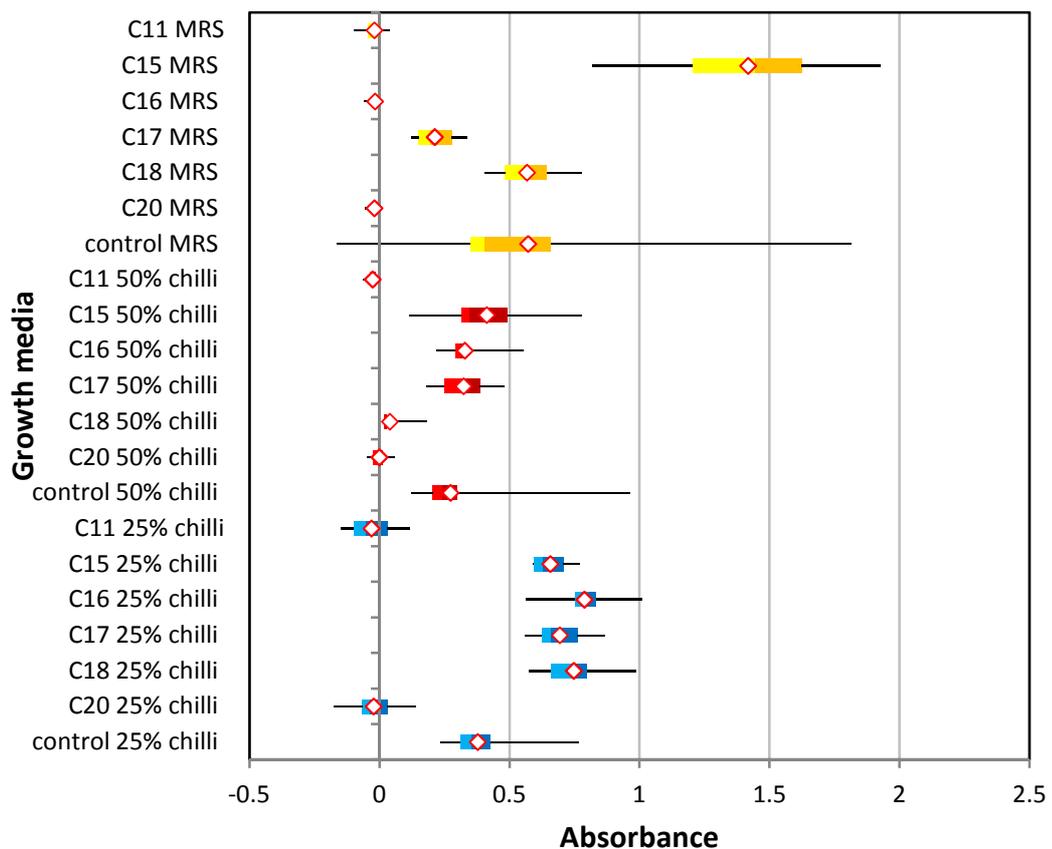


Figure 5.2 Growth of *O. placenta* in one week CFS of bacterial isolates in MRS and chilli growth media (experiment 2).

5.3.2 Assay for wood decay

When wood blocks treated with *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20, both grown in 50% chilli juice and then filtered prior to impregnation, were exposed to wood decay fungi there was virtually no antifungal activity (Figure 5.3). The only treatments being significantly different to the control were both C11 and C20, and the chilli control exposed to *C. puteana* all of which performed significantly worse than the untreated control ($P < 0.05$). When exposed to *O. placenta* the chilli control did perform significantly better than the untreated control ($P < 0.05$) but the treatments containing LAB did not significantly differ from the control. All treatments had mean mass losses of over 10%, all considered not acceptable with 2 - 3% mass loss being the usual bench mark for this type of test.

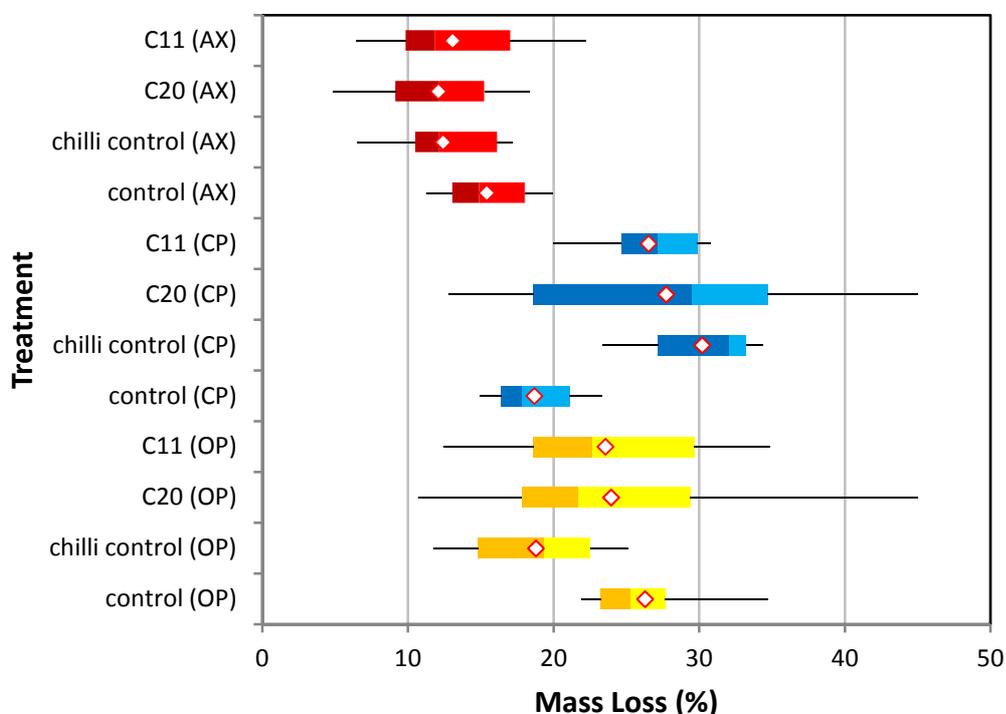


Figure 5.3 Mass loss of wood blocks treated with LAB grown in chilli juice and exposed to decay fungi *A. xantha* (AX), *C. puteana* (CP), and *O. placenta* (OP).

5.3.3 HPLC analysis

When HPLC analyses were done on the CFS's of isolates grown in chilli juice (Figures A3.8 to A3.13, Appendix III) it was seen that *L. mesenteroides* subsp. *mesenteroides* isolates C15 – C17 all produced no lactic or acetic acid. All of these isolates along with C18 produced an unidentified peak at just less than 7 minutes retention time, however given the lack of antifungal activity from C15 - C17 it can be assumed that this compound did not contribute to activity. *Gluconobacter oxydans* C18 did show some activity against fungi when grown in 50% chilli juice and HPLC showed the production of acetic acid for this isolate when grown in 50% but not in 25% chilli juice. Both *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 produced lactic and acetic acids when grown in 50% chilli juice, C20 also produced one other unidentified compound and phenyl lactic acid which may or may not have contributed to the antifungal activity shown by this bacterium.

5.4 DISCUSSION

In this study the possible synergy between chilli juice and bacterial metabolites was investigated using the previously isolated bacteria (chapter 4) which showed the most promising antifungal activity. Initial testing using the microscale optical density assay showed that *L. brevis* C11 was able to be grown in chilli juice and in fact improved the antifungal activity of the juice with its addition. Antifungal activity in 50% chilli juice was similar to that given when the bacteria was grown in MRS media and therefore this concentration along with 25% juice was used to screen the rest of the bacteria for antifungal activity. Of the remaining bacteria only *L. mesenteroides* subsp. *cremoris* C20 showed complete inhibition of the

decay fungus at both 25 and 50% chilli juice with *L. brevis* C11 also showing complete inhibition at both these concentrations. Both of these bacteria produced lactic and acetic acid at both concentrations of juice and it is believed these metabolites are most likely responsible for the antifungal activity shown. *Leuconostoc mesenteroides* subsp. *cremoris* C20 also produced a small amount of phenyl lactic acid, as it did when previously grown in MRS media. *Gluconobacter oxydans* C18 also showed good antifungal activity in 50% juice and produced acetic acid at this concentration. However in 25% juice *G. oxydans* showed no antifungal activity and also produced no acetic acid, therefore it is most likely that acetic acid is the metabolite to which antifungal activity can be attributed.

The remaining bacteria *L. mesenteroides* subsp. *mesenteroides* C15 – C17 all showed no antifungal activity when grown in chilli juice and in fact showed less activity than the chilli juice controls, which suggests that a compound or compounds in the chilli juice that were responsible for antifungal activity may have been metabolised by these bacteria into something that no longer is able to inhibit the decay fungus. Results from HPLC analysis showed an unidentified peak in CFS from isolates C15 – C18, which appears to not have any antifungal activity and no lactic or acetic acid production for isolates C15 – C17. When grown in MRS media (chapter 4), isolates C15 and C16 produced lactic, acetic and phenyl lactic acids and C17 produced no discernible acids and it was determined using pH, temperature and enzymatic tests that other compounds may also be responsible for any antifungal activity produced. It appears that these compounds, if they indeed exist, have not been produced when the *L. mesenteroides* subsp. *mesenteroides* isolates C15 - C17 were grown in chilli juice.

Lactic acid bacteria are renowned for their complex vitamin requirements as they are unable to synthesize all the components of their coenzymes (Madigan et al., 2000a) and *L. mesenteroides* is described as being extremely fastidious (nutritionally demanding) which means it generally requires complex media in which to grow and metabolise. It may be possible that whilst *L. mesenteroides* grows naturally in and initiates the fermentation of many vegetables (eg, sauerkraut (cabbage) and silage (grass)) (VanDemark and Batzing, 1987, Battcock and Azam-Ali, 1998, Hemme and Foucaud-Scheunemann, 2004, Todar, 2011), the chilli juice may not have contained the correct coenzyme containing vitamins to allow these isolates to produce the antifungal metabolites it was able to produce in the complex MRS media. This would explain the loss of activity of these isolates, in contrast *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 were able to produce antifungal metabolites (lactic, acetic and phenyl lactic acids) when grown in the chilli juice as well as the MRS media.

When *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 were grown in larger quantities for treatment of wood blocks for the wood bioassay, similar amounts of lactic, acetic and phenyl lactic acids were produced as in the earlier experiments. However, on wood the CFS of these isolates did not exhibit any discernable antifungal activity even though mean mass loss against *A. xantha* and *O. placenta* was slightly less than the untreated control blocks. When Singh and Chittenden (2008b) assessed the antifungal activity of chilli with LAB against sapstain in a growth rate experiment on nutrient media, they discovered that fungi varied in tolerance to the concentration of chilli juice. They also found that autoclaved chilli juice showed slightly more antifungal activity than filtered chilli juice. In this experiment the chilli juice/LAB CFS was filtered prior to wood

treatment to remove the LAB from the supernatant as it was determined from previous experiments that there was no benefit to leaving the live active bacteria in the treatment (and in fact this tended to lead to contamination by mould fungi). In addition it was decided that autoclaving may affect the bacterial metabolites in some manner and therefore filtering appeared to be the best option. It is possible that this may have removed some of the antifungal components of the chilli in the CFS. Whilst this may have also been the case in the MODA experiments, it may not have been picked up due to the superior antifungal activity seen by isolates C11 and C20 at all concentrations tested. In spite of this it is unlikely that the filtering of the CFS has accounted entirely for the lack of antifungal activity in the wood assay. It is possible that using 100% chilli juice or a concentrated form of capsaicin or other oleoresins and peptides may increase the antifungal activity. Various researchers have shown the antifungal effect of these components of *Capsicum* sp. (De et al., 1999, Anaya-Lopez et al., 2006, Singh and Chittenden, 2008b, Nazzaro et al., 2009, Diz et al., 2011). Results from this experiment suggest that the majority of the antifungal activity seen in the wood assay performed in Chapter 4 was due to the inhibitory action of the MRS media and subsequent increase in moisture content during exposure to the test fungus. It would be of interest to determine whether addition to the chilli juice of some complex vitamins required by LAB would improve the antifungal activity of this bacteria/chilli juice combination on wood.

5.5 SUMMARY

In this study six lactic and acetic acid bacterial isolates which had been isolated from chilli juice (Chapter 4) were assessed for their antifungal activity when grown in chilli juice for one week. The quick screening MODA assay revealed

that both *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 completely inhibited *O. placenta* when grown in 25 and 50% chilli juice and HPLC analysis revealed the production of lactic and acetic acid by both bacteria and phenyl lactic acid by isolate C20. Of the other bacteria examined only *G. oxydans* C18 produced discernible antifungal metabolites in 50% chilli juice (acetic acid) and none of the isolates produced antifungal organic acids in 25% chilli juice.

Isolates C11 and C20 were consequently subjected to a wood assay using 50% chilli juice as the medium for LAB growth. Neither bacteria exhibited significant antifungal activity against wood decay fungi in this assay despite producing similar amounts of metabolites as when tested in the MODA assay. It was therefore concluded that the antifungal activity of these bacteria was partially reliant on the complex MRS media as was seen in Chapter 4.

Further studies should be undertaken to optimise media and incubation conditions to determine whether the isolated lactic acid bacteria have any real potential as antifungal agents for wood protection.

CHAPTER 6: CONCLUSIONS AND RECOMENDATIONS

6.1 CONCLUSIONS

6.1.1 MODA protocol development

In this study it was seen that an assay which relies on optical density measurements to assess fungal growth in microscale 96-well plates is a viable alternative method to the standard growth rate assay on solid nutrient media. The method is simple and easy to set up and allows for the screening of a large number of different plant extracts or CFS from different bacteria or combinations of such products at one time. Results can be obtained within one week, from set up to growth measurement, which then gives an indication of suitable products to pursue further in wood bioassays. Furthermore MODA gives a result which is comparable to results obtained in the growth rate assay, but takes less time and materials and uses much less space.

One possible difficulty with this method is that when testing LAB metabolites, some of the decay fungi tested would not grow in or on the standard media for growth of LAB (which are extremely fastidious and require a complex media full of vitamins and precursors for growth). This could put some limitations on interpreting results from this method. Further development of the assay could include additional fine-tuning of the media to allow for the growth of decay organisms and the growth and metabolite production of LAB. In addition, screening of further decay fungi could be undertaken to find other organisms which can grow happily in the MRS media.

6.1.2 Isolation and antifungal activity of LAB from chilli waste

Isolation of bacteria from chilli waste and screening of bacterial CFS for antifungal activity revealed the presence of several bacteria of interest in this study. The use of 16S rRNA phylogenetic techniques for identification of the relevant bacteria identified the presence of LAB *Lactobacillus brevis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc pseudomesenteroides* as well as AAB *Gluconobacter oxydans*. The metabolites of these bacteria grown in complex media, were subjected to a wood assay in which all isolates showed resistance to fungal decay, however only *L. brevis* provided significantly better antifungal activity than the control.

Results from HPLC in combination with the effects of pH and temperature on antifungal activity, suggested the most likely cause of antifungal activity was due to the production of lactic and acetic acids. Phenyl lactic acid and other unidentified compounds were also produced by some bacteria, which could have contributed to activity.

None of the bacterial metabolites appeared to be resistant to leaching when subjected to wetting, therefore they are unlikely to be considered suitable as a permanent preservative for wood protection when used alone. It is thought that they may have potential for use in combined treatments with other benign substances with antifungal properties and this was investigated further in chapter 5.

6.1.3 Combining bacterial metabolites with chilli juice

When six of the lactic and acetic acid bacterial isolates were assessed for their antifungal activity after being grown in chilli juice for one week, the MODA assay revealed that both *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20 completely inhibited *O. placenta* when grown in 25 and 50% chilli juice, equivalent to results obtained when grown in MRS media. HPLC analysis revealed the production of lactic and acetic acid by both bacteria and phenyl lactic acid by isolate C20. Of the other bacteria examined only *G. oxydans* C18 produced discernible antifungal metabolites in 50% chilli juice (acetic acid) and none of the isolates produced antifungal organic acids in 25% chilli juice.

When isolates C11 and C20 were subjected to a wood assay using 50% chilli juice as the medium for LAB growth, neither of the bacterial/chilli CFS's exhibited significant antifungal activity against wood decay fungi despite producing similar amounts of metabolites as when tested in the MODA assay. It was therefore concluded that the antifungal activity of these bacteria was partially reliant on the complex MRS media which inhibited fungal decay alone and in combination with bacterial metabolites.

Whilst the chilli/bacteria combinations used in this study did not arrest decay in wood, there are many ways in which it may be possible to increase and induce metabolite production and these should be explored to determine whether the bacteria in this study have any potential as antifungal agents for wood protection.

6.2 RECOMMENDATIONS

Whilst the results in this study were not satisfactory for the protection of wood from decay fungi, the bacteria isolated did show some potential as antifungal agents and this could be explored further.

- Lactic acid bacteria can be very fastidious and therefore the bacteria isolated from this study, in particular *L. brevis* C11 and *L. mesenteroides* subsp. *cremoris* C20, should be examined under a wider range of temperature regimes and media to perhaps induce or optimise metabolite production.
- It is often seen in the laboratory that MIC values of compounds tested *in-vitro* are much lower than concentrations required on a wood substrate, therefore further testing of chilli and chilli extracts (at higher concentrations) alone or in combination with bacterial metabolites should be undertaken to determine whether there is any true potential as an antifungal agent for wood protection.
- The isolated bacteria should be tested against a wider range of wood degrading organisms. Whilst ultimately these organisms may not be suitable for protecting against decay, they may be suitable as mouldicides or antisapstain formulations.
- The MODA method optimised in this study shows significant potential for the testing of bacteria from other waste streams. Media could be changed depending on the bacteria of interest.
- With little modification, the MODA method is also suitable for quick screening of various non-volatile plant extracts and other potential antifungal compounds. With modification of the method, volatile compounds could also be tested. The method could become a standard test method in the laboratory for initial screening of large numbers of compounds.

CHAPTER 7: REFERENCES

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APPENDIX I: MEDIA AND DNA METHODOLOGY

A1.1 CULTURE MEDIA FORMULATIONS

A1.1.1 DeMan-Rogosa-Sharpe agar and broth (MRS)

Prepared as per manufacturer's instructions except where specified in the method.

A1.1.2 Malt agar

20 g Malt Powder

15 g Bacteriological agar

1000 ml Distilled water

Autoclave for 20 minutes at 121°C. Pour into sterile petri-dishes once cooled (approximately 40 – 45°C).

A1.1.3 Malt Peptone broth (MP)

2 g Malt powder

0.5 g Mycological peptone

100 ml Distilled water

Autoclave for 20 minutes at 121°C.

A1.1.4 Yeast Malt broth (YM)

1.5 g Malt powder

0.2 g Yeast extract

100 ml Distilled water

Autoclave for 20 minutes at 121°C.

A1.1.5 Sabourand Dextrose broth (SD)

Prepared as per manufacturer's instructions.

A1.1.6 DeMan-Rogosa-Sharpe minus salts (MRS-salts)

1 g Mycological peptone

0.8 g Beef extract

0.4 g Yeast extract

2 g Glucose

0.1 g Tween 80

100 ml Distilled water

Autoclave for 20 minutes at 121°C.

A1.2 DNA EXTRACTION METHOD

1. Grow up 50 ml of bacterial culture overnight.
2. Centrifuge at 5000 rpm for 10 minutes.
3. Decant supernatant.
4. Add 2 ml SET buffer, mix and transfer to microcentrifuge tubes (1 ml).
5. To 1 ml of culture add 50 µl lysozyme solution.
6. Incubate at 37°C for one hour in a water bath.
7. Add 50 µl SDS (20%) and 25 µl Proteinase K to 1 ml culture.
8. Incubate at 55 – 60°C for at least one hour (3-4 hours for maximum digestion).
9. Add an equal volume (1125 µl) of phenol:chloroform:isoamyl alcohol (25:24:1) to tube (may need to split into two tubes), mix.
10. Centrifuge at 5000 rpm for five minutes.
11. Remove top aqueous layer (retain).

12. Add equal volume of chloroform:isoamyl alcohol (24:1), mix.
13. Centrifuge at 5000 rpm for five minutes.
14. Remove and retain aqueous (top) layer.
15. Add 0.1 volumes of 3 mol l⁻¹ sodium acetate and 2 volumes of 95-100% ethanol.
16. Incubate at -20°C for at least one hour or overnight.
17. Centrifuge at 13000 rpm for 15 minutes.
18. Decant supernatant.
19. Resuspend pellet in 200-500 µl 70% ethanol (ice cold).
20. Incubate at -20°C for 10 minutes.
21. Centrifuge at 13000 rpm for 15 minutes.
22. Decant supernatant.
23. Air-dry pellet in lamina flow.
24. Resuspend DNA in TE (10:1) buffer.
25. Quantify DNA.

A1.2.1 DNA Solutions and Buffers

SET Buffer

- | | |
|-------|-------------|
| 20% | Sucrose |
| 50mM | EDTA |
| 50 mM | Tris buffer |

Stock solutions

EDTA (1 M) = dissolve 372.24 g EDTA (Ethylenediaminetetraacetic acid) in deionised water; adjust to pH 8.0 with NaOH before making up to 1 litre.

Tris (1 M) = dissolve 121.14 g tris(hydroxymethyl)aminomethane in deionised water; adjust to pH 8.0 with NaOH before making up to 1 litre.

NaCl (5 M) = dissolve 292.2 g of NaCl in deionised water. Make up to 1 litre.

Sodium acetate (3 M) = dissolve 408.1 g of sodium acetate.3H₂O in 800 ml deionised water. Adjust to pH 7 with acetic acid. Adjust volume to 1 litre.

SDS (20%) = Add 200 g SDS (sodium dodecyl sulphate) to deionised water, heat to 68°C to dissolve. Adjust pH to 7.2 with concentrated HCl. Adjust volume to 1 litre.

TE (10:1) buffer = 10 mM Tris + 1 mM EDTA

Proteinase K = 20 mg Proteinase K in 1 ml TE (10:1) buffer

Lysozyme solution = 5 mg lysozyme in 1 ml TE (10:1) buffer plus 10 mM NaCl.
(10 µl of 5 M NaCl in 5 ml TE)

A1.3 16S RRNA GENE PCR

Primer Sequences

27F 5'-AGAGTTTGATCCTGGCTCAG -3'

1492R 5' -GGTTACCTTGTTACGACTT -3'

Reaction Mix

Sterile water	17.25 µl
PCR buffer 10X	2.5 µl

dNTPs 2 mM	2.5 μ l
27 F 5 μ M	0.5 μ l
1492R 5 μ M	0.5 μ l
DNA	0.5 μ l
<i>Taq</i> DNA Pol 5 U μ l ⁻¹	0.25 μ l

PCR Program

Temperature °C	Time (mins)	Number of cycles
94.0	3:00	1
94.0	1:00	30
55.0	1:00	30
72.0	1:00	30
72.0	5:00	1
4.0	∞	

Electrophoresis

Run out 3 μ l of PCR product with 4 μ l loading dye on a 1.5% agarose mini gel with 2 μ l 1 Kb+ ladder for 60 minutes at 120 V. Stain for 20-30 minutes in ethidium bromide solution, de-stain in running water and visualise under UV illumination.

PCR products sent to Waikato DNA Sequencing Facility for sequencing then run through the NCBI database using a BLAST search for sequence alignments.

APPENDIX II: 16S rRNA BLAST RESULTS

Table A2.1 Overall chilli isolate BLAST matches.

Isolate	Sequence length	BLAST match %	Closest match	
			Species	GenBank No.
C6	550	426/438 (97%)	<i>Candida sake</i> CBS 159	AJ549822.1
C10	435	296/350 (85%)	<i>Candida sp.</i>	HQ115735.1
C11	1107	1055/1085 (97%)	<i>Lactobacillus brevis</i> ATCC 367	NC008497.1
C13	742	723/728 (99%)	<i>Saccharomyces servazzii</i>	D89895.1
C14	420	393/403 (98%)	<i>Pichia fermentans</i> ATCC 10651	GQ458040.1
C15	1093	1044/1069 (98%)	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC8293	NC008531.1
C16	1103	925/970 (95%)	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC8293	NC008531.1
C17	1137	841/909 (93%)	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC8293	NC008531.1
C18	664	604/638 (95%)	<i>Gluconobacter oxydans</i>	NC006677.1
C19	1117	868/977 (89%)	<i>Leuconostoc pseudomesenteroides</i> KCTC 3652	AEOQ01000906.1
C20	1072	1031/1060 (97%)	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> ATCC19254	NZ ACK01000113.1

APPENDIX III: HPLC RESULTS

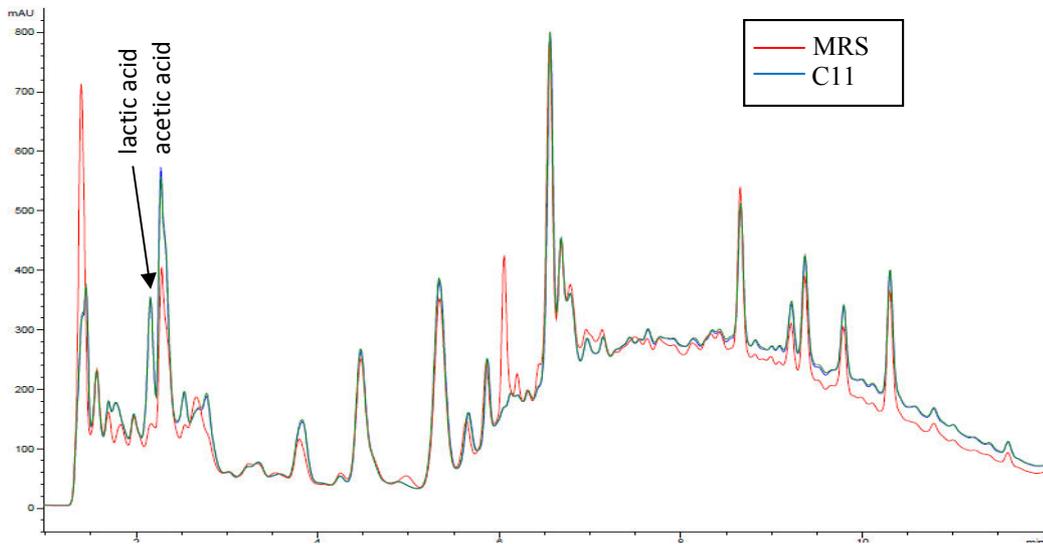


Figure A3.1 HPLC of *Lactobacillus brevis* C11 vs MRS media.

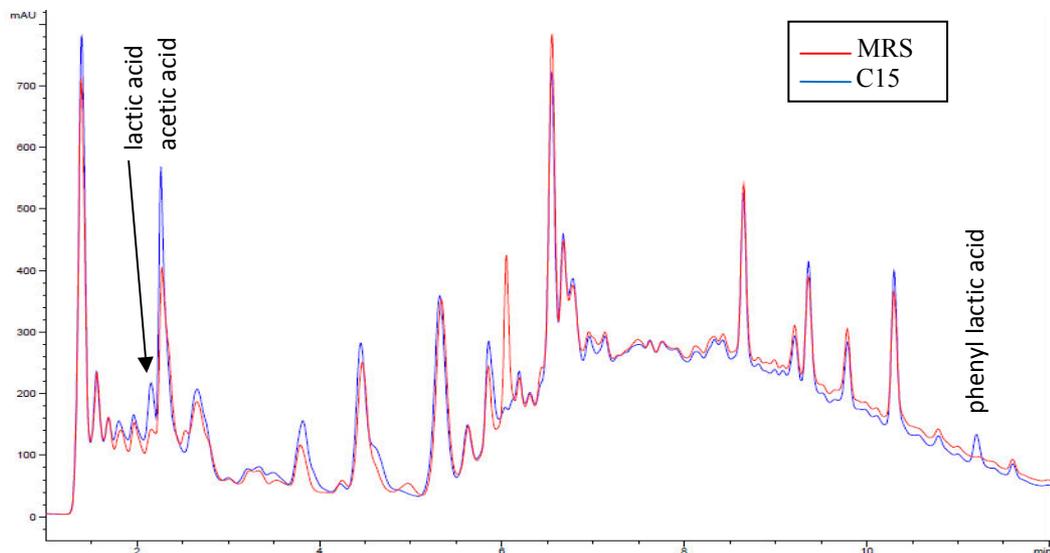


Figure A3.2 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C15 vs MRS media.

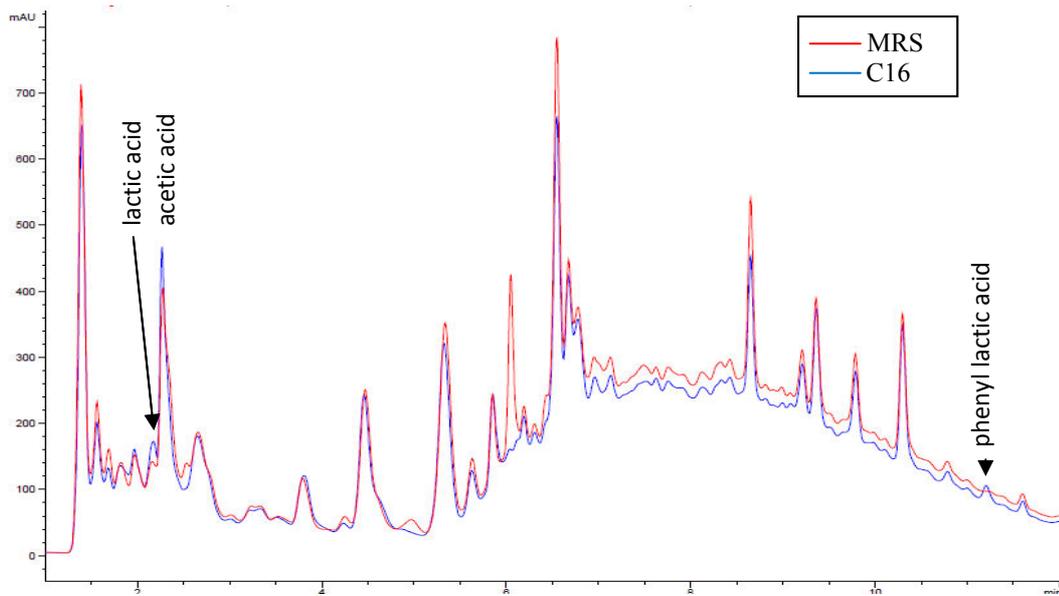


Figure A3.3 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C16 vs MRS media.

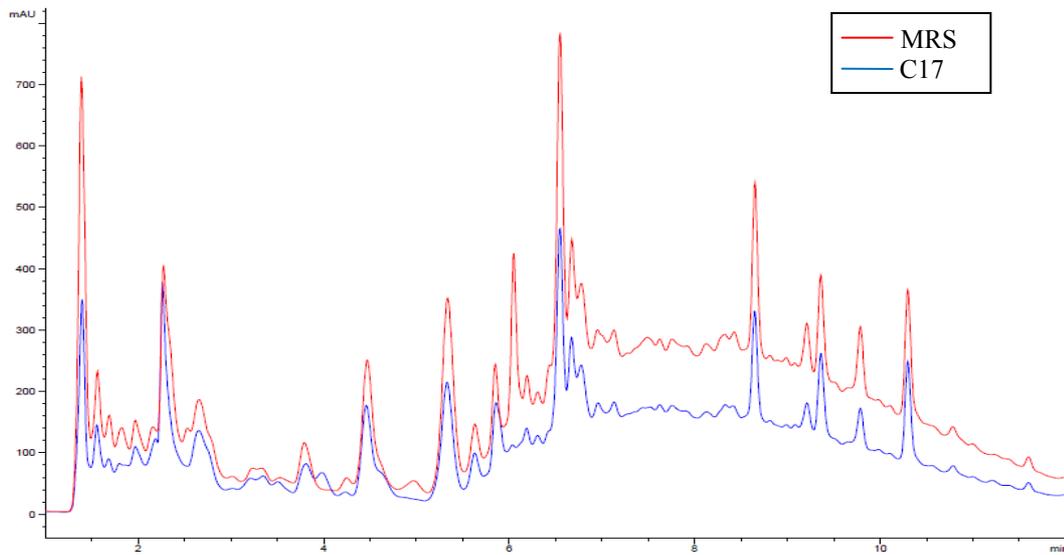


Figure A3.4 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C17 vs MRS media.

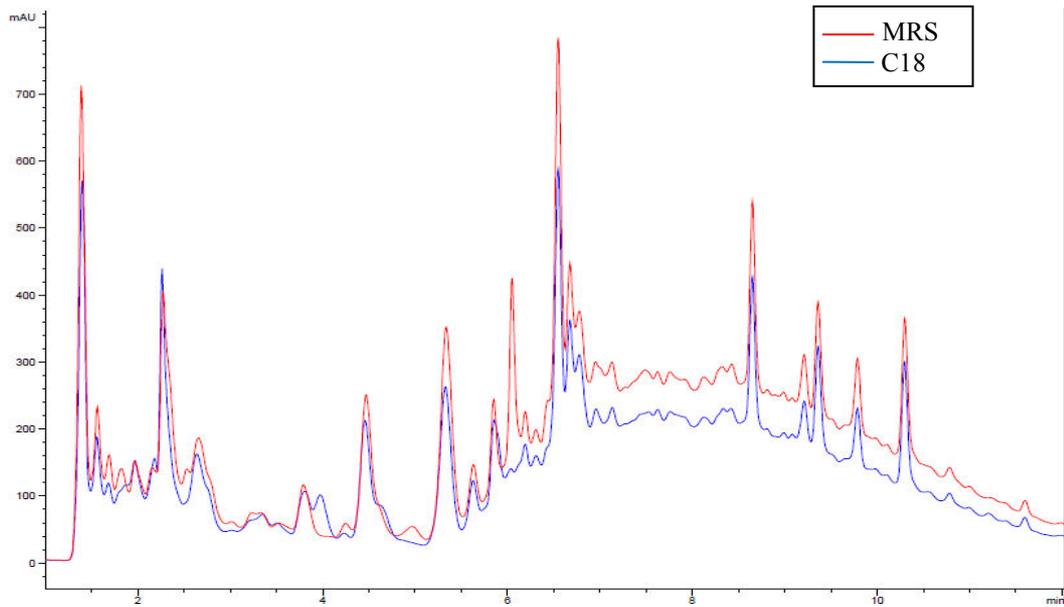


Figure A3.5 HPLC of *Gluconobacter oxydans* C18 vs MRS media.

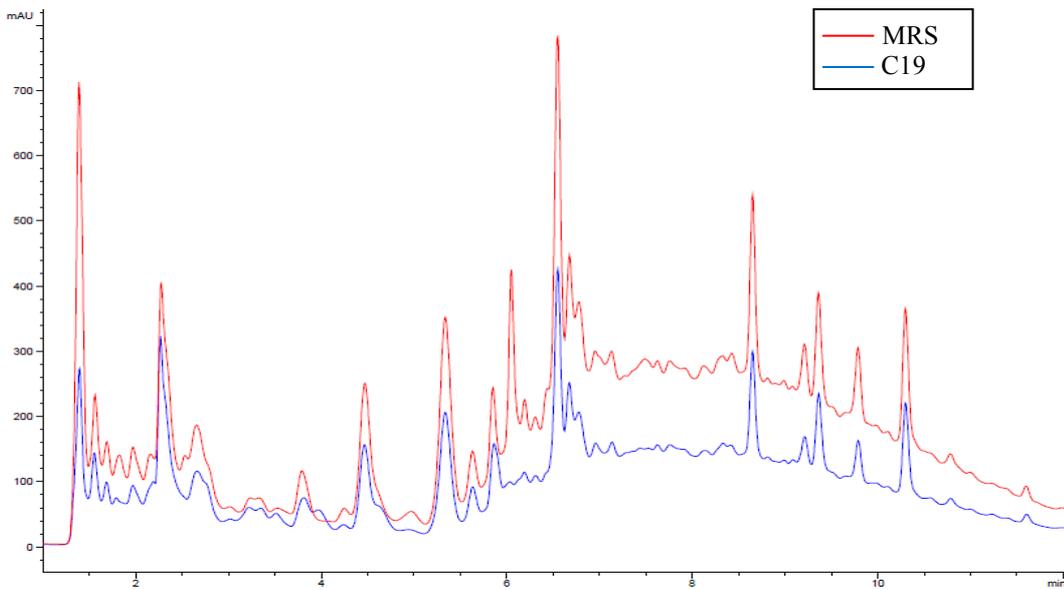


Figure A3.6 HPLC of *Leuconostoc pseudomesenteroides* C19 vs MRS media.

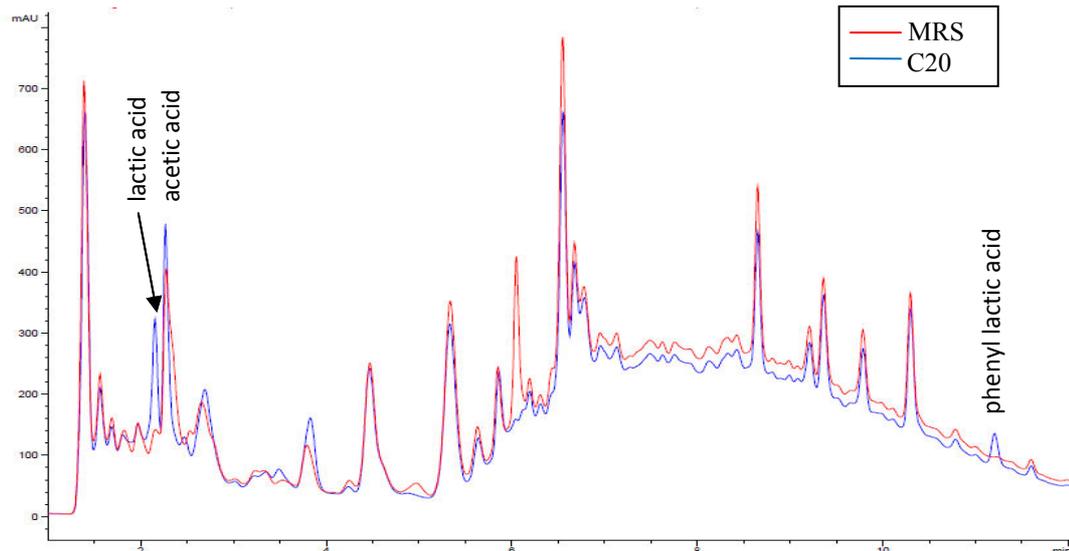


Figure A3.7 HPLC of *Leuconostoc mesenteroides* subsp. *cremoris* C20 vs MRS media.

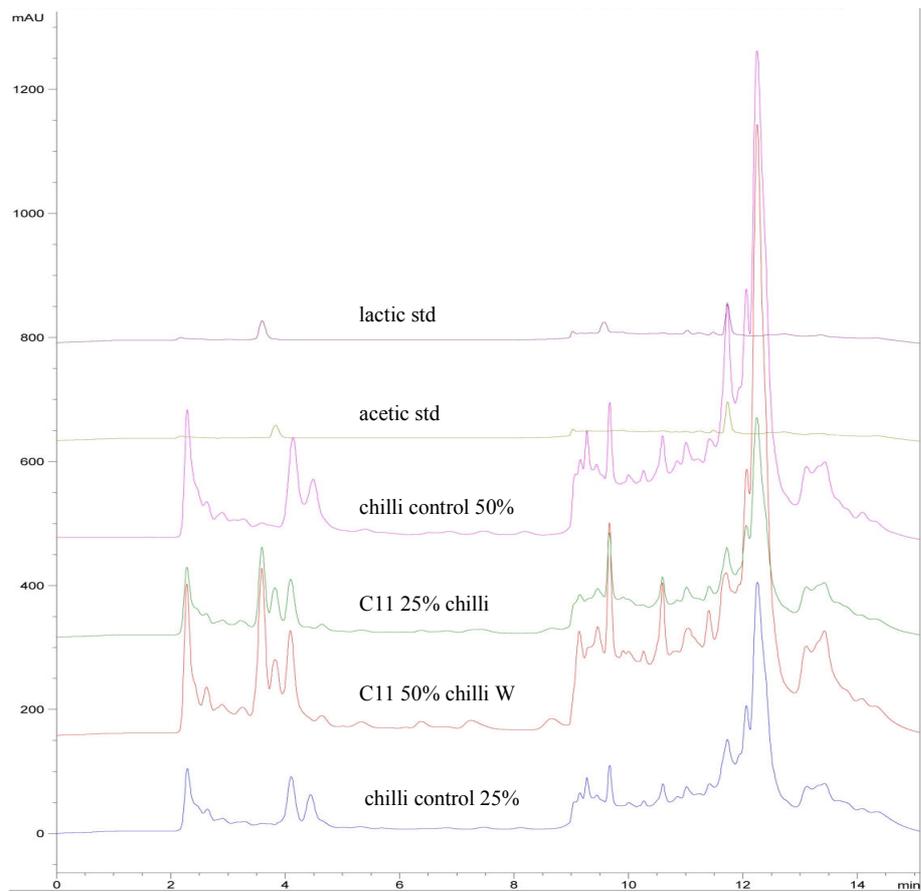


Figure A3.8 HPLC of *Lactobacillus brevis* C11 grown in 25 and 50% chilli juice. (W = wood assay)

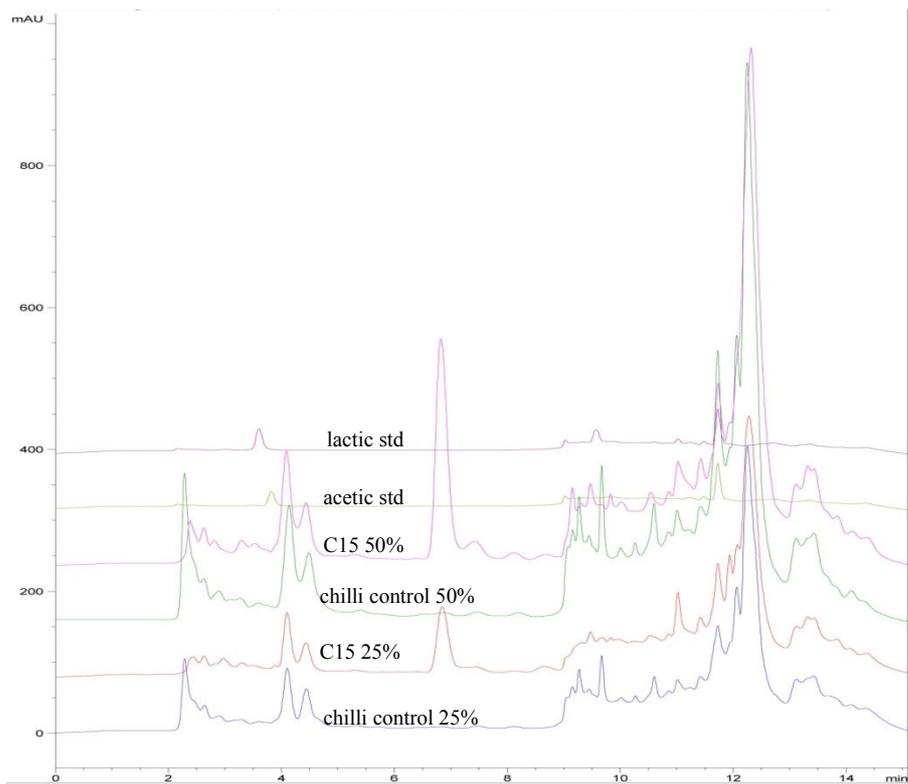


Figure A3.9 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C15 grown in 25 and 50% chilli juice.

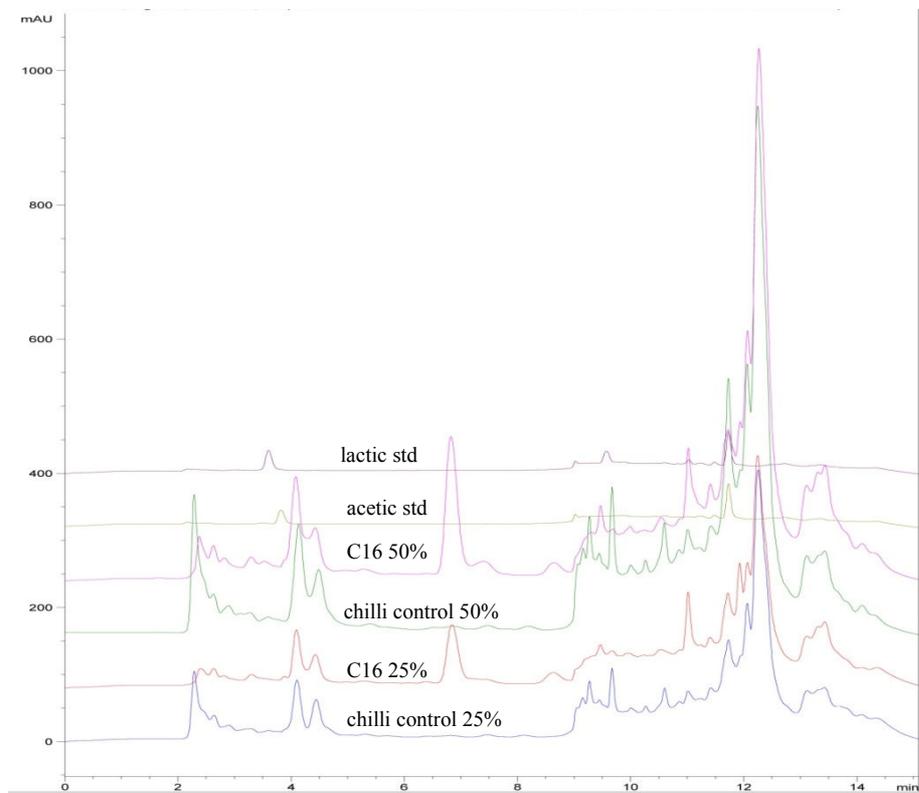


Figure A3.10 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C16 grown in 25 and 50% chilli juice.

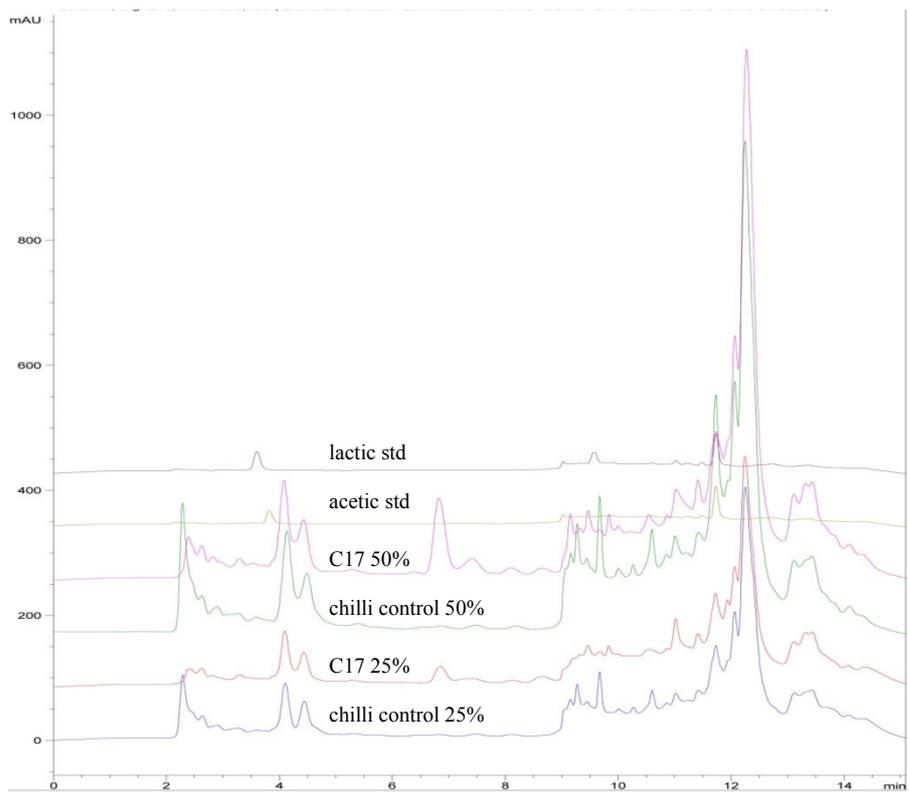


Figure A3.11 HPLC of *Leuconostoc mesenteroides* subsp. *mesenteroides* C17 grown in 25 and 50% chilli juice.

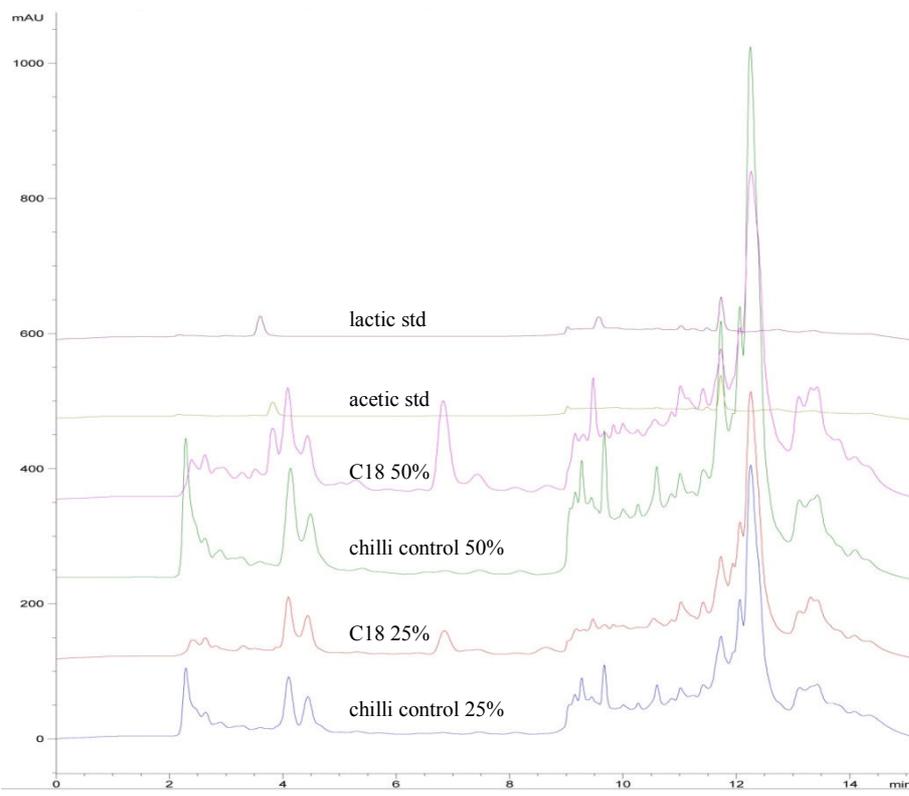


Figure A3.12 HPLC of *Gluconobacter oxydans* C18 grown in 25 and 50% chilli juice.

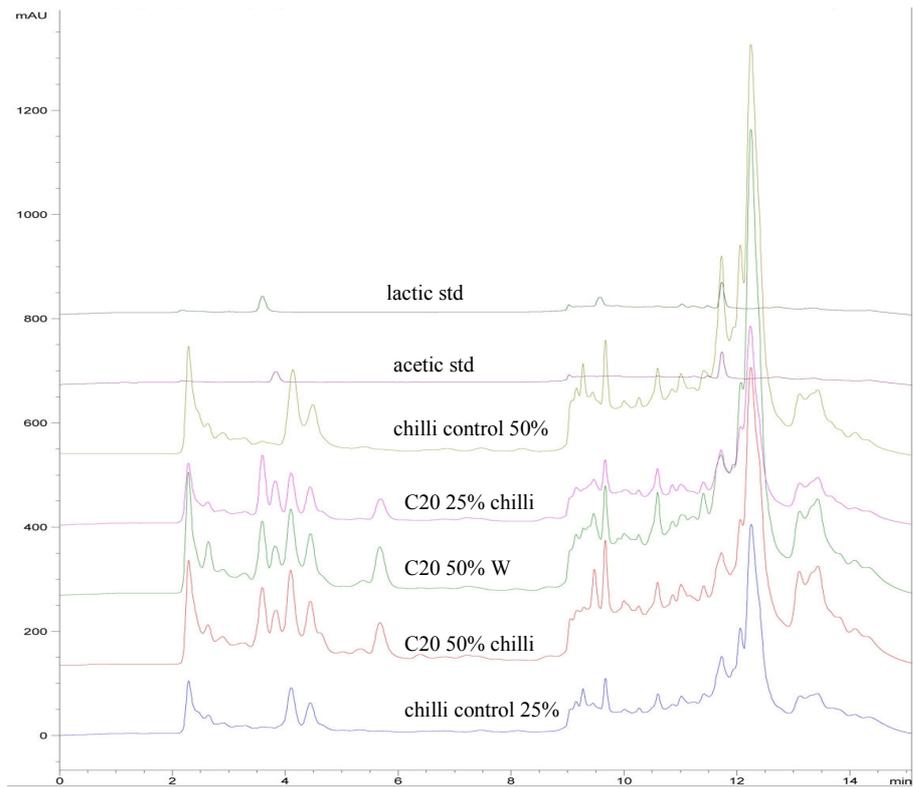


Figure A3.13 HPLC of *Leuconostoc mesenteroides* subsp. *cremoris* C20 grown in 25 and 50% chilli juice. (W = wood assay)

APPENDIX IV: JOURNAL PAPER

The following paper is based on work from this project/thesis and accepted for publishing by the Journal of Applied Microbiology on 29 November 2011.