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ORIGINAL ARTICLE

Evaluation of lactic acid bacterium from chilli waste as a potential antifungal agent for wood products

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Abstract

Aims: The aim of this study was to isolate lactic acid bacteria from chilli waste and evaluate metabolites produced for the ability to arrest wood decay.
Methods and Results: Using an optical density screening method, one bacterium (isolate C11) was identified as having pronounced antifungal properties against *Oligoporus placenta*. This isolate was identified as *Lactobacillus brevis* by 16S rRNA gene sequencing. To determine antifungal activity in wood, *Pinus radiata* blocks were impregnated with *Lact. brevis* [C11] cell-free supernatant and exposed to brown rot fungi *O. placenta*, *Antrodia xantha* and *Coniophora puteana*. The treated timber demonstrated resistance to degradation from all fungi. The antifungal metabolites were heat stable and not affected by proteinase K, but were affected by neutralization with NaOH suggesting the metabolites were of an acidic nature. The presence of lactic and acetic acid was confirmed by HPLC analysis.
Conclusions: *Lactobacillus brevis* [C11] produced acidic metabolites that were able to inhibit the growth of wood decay fungi and subsequent wood decay.
Significance and Impact of the Study: Traditional wood treatments are becoming an environmental issue as the public demands more benign options. The use of lactic acid bacteria which are considered safe for general use is a potential alternative to the conventional heavy metal chemicals currently in use.

Introduction

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; Gerez *et al.* 2009). A prime example of biopreservation by LAB is the widespread use of the lantibiotic nisin, a posttranslationally modified bacteriocin derived from the bacterium *Lactococcus lactis*, in the dairy industry (Ross *et al.* 2002). Biopreservation is gaining increased popularity because of consumer demand (Gerez *et al.* 2010) and is achieved by the production of metabolites including organic acids, fatty acids, hydrogen peroxide and 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; Mauch *et al.* 2010). A few studies have shown that *Lactobacillus* isolates can have an antifungal effect on some early wood colonizer moulds and sapstain fungi (Yang and Clausen 2005). Previous research has shown chilli waste to have moderate antifungal activity against two common Ascomycetes fungi (*Sphaeropsis sapinea* and *Leptographium procerum*) (Singh and Chittenden 2008). There was also a notable increase in fungal inhibition when chilli and *Lactobacillus* sp. isolated from the chilli juice were combined (Singh and Chittenden 2008).

Wood is the largest volume material produced worldwide and is a renewable resource used in a multitude of applications. Softwood species such as *Pinus radiata*, which makes up 90% of plantation forests in New Zealand (Ministry of Agriculture and Forestry 2010), are highly susceptible to biodeterioration by sapstain and 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. In New Zealand, there has been much coverage in the media of 'leaky house syndrome' which has involved the failure of structural timbers in new houses because of decay brought on by failures in weather-tightness systems. Traditionally, biodeterioration by decay fungi has been controlled by chemical formulations that are to varying degrees toxic to people, animals and the environment. The restricted use of traditional formulations such as chromated copper arsenic (CCA) in some countries (USEPA 2002; CSTEE Scientific Committee on Toxicity, E.a.t.E. 2003; Housenger 2003) is being led by the public's increasing awareness of the environment and has focused the need for research into environmentally friendly (benign) wood preservatives.

The aim of this study was to isolate lactic acid bacteria from chilli waste that exhibit a pronounced antifungal effect against common wood decay fungi, to identify those bacteria using 16S rRNA phylogenetic techniques and attempt to characterize the bacterial metabolites responsible for observed antifungal activity.

Materials and Methods

Preparation of chilli juice

Rocoto chillies (*Capsicum pubescens*) 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.

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, Cambridge, UK) and incubated in a shaking (100 rev min^{-1}) water bath at 30°C. After 24 h, 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-plating 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.

Identification of isolates

Bacterial isolates were identified using 16S rRNA gene sequencing. DNA was extracted from isolates using a previously described method (Marmur 1961). 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 Corp., Piscataway, NJ, 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).

Assay for antifungal activity (microscale absorbance assay)

Initial screening of bacterial isolates was performed using a microscale absorbance (optical density) assay, described later, which was developed specifically for this purpose (O'Callahan *et al.* 2009). The wood decay fungus *Oligoporus placenta* (Fries), Gilb & Ryvarden (strain FR07/02), was selected as the test organism in this assay because of its virulent nature and it is a 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% r.h. for a period of up to 4 weeks. Inoculum was prepared by homogenizing the above fungal cultures in a Waring blender for 30 s at low speed followed by 30 s at high speed. An aliquot of 50 μl of this inoculum was added to test wells in a sterile 96-well covered cell culture plate (Sarstedt, Nümbrecht, Germany).

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 min at room temperature, and the supernatant filter sterilized through a 0·22 μm Steritop filter (Millipore, Billerica, MA). 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 Corp., Waltham, MA) plate reader to get an absorbance measurement. Plates were wrapped with plastic wrap during incubation at 25°C, 75% r.h. (to prevent evaporation of media) and reread after 6 days. Change in absorbance was recorded by subtracting the initial reading from the final absorbance.

Assay for wood decay

Performance against wood decay was assessed using a modified agar/block decay test (Sutter 1978). *Lactobacillus brevis* C11 was grown, centrifuged and filter sterilized where appropriate as discussed earlier. *Pinus radiata* 'Sutter' blocks (approx. 35 × 35 × 7 mm longitudinal) were treated by vacuum impregnation with solutions as described in Tables 1 and 2.

The test blocks (16 replicates per fungus/treatment combination) were weighed after treatment and then left to air dry on racks at ambient room temperature (averaging approximately 20°C). Eight of the replicates from each treatment combination were subjected to leaching for 14 days. Leaching involved the resaturation of treated blocks with water after which they were placed in nine times their volume of water. Leaching water was changed every 2 days. After leaching, the blocks were air dried and all blocks were conditioned to constant weight at 12% equilibrium moisture content (emc) in a room controlled at 20°C and 65% r.h. Blocks were then weighed, packaged and sterilized by exposure to ethylene oxide gas after which they were placed aseptically into prepared agar containers and incubated for 6 weeks at 26°C and 75% r.h. 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 *O. placenta* (strain: FR07/02).

Following incubation, 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.

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

Assay for pH, temperature and proteolytic enzyme effect

To determine the nature of the antifungal substance, *Lact. brevis* CFS was altered as below and assessed using the microscale absorbance assay described earlier.

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 min.

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 and incubated at 45°C for 60 min. Proteinase K was then inactivated by heating to 100°C for 10 min.

HPLC analysis

Organic acids in the *Lact. 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 min followed by a gradient from 0 to 45% acetonitrile in 12 min. 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

Table 1 Treatment schedule for *Pinus radiata* wood blocks (trial 1)

	Treatment	pH
A	24 h incubation at 30°C *Lactobacillus brevis* C11 culture CFS	5·17
B	24 h incubation at 30°C *Lact. brevis* C11 culture (not filter sterilized) fixed for 1 week in a sealed plastic bag at room temp	5·17
C	1 week incubation at 30°C *Lact. brevis* C11 culture CFS	4·18
D	MRS broth control	5·71
E	Untreated control	na

CFS, cell-free supernatant; MRS, deMan Rogosa Sharp; na, not applicable.

Table 2 Treatment schedule for *Pinus radiata* wood blocks (trial 2)

	Treatment
A	100% MRS Broth (52 g in 1l H_2O)
B	50% MRS Broth
C	25% MRS Broth
D	Untreated control

MRS-containing samples were estimated by comparison with the spiked chromatograms.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted to determine significant differences ($P \leq 0{\cdot}05$) between the antifungal activity of different bacterial isolates with the null hypothesis being that there was no difference between isolates. Significant differences were also determined in the wood assay between bacterial treatments and the MRS media control to determine whether the media accounted for all mass loss observed.

Results

Screening and identification of bacteria

Three bacterial isolates (C11, C13 and C14) were obtained using the isolation protocol. All bacteria were capable of growing in or on MRS media tentatively identifying them as lactic acid bacteria. Screening trials using the microscale absorbance assay (Fig. 1) identified that all bacteria had some degree of antifungal effect on the decay fungus *O. placenta* with change in absorbance decreasing as fungal growth is inhibited. However, results from both C13 and C14 were not significantly different ($P > 0{\cdot}05$) from the positive control. The bacterium with the most pronounced effect, C11, performed significantly better ($P < 0{\cdot}05$) than the control and the other micro-organisms tested and was subsequently identified using 16S rRNA gene sequencing as having 97% sequence identity to *Lact. brevis* ATCC 367. This bacterium was used in subsequent wood assay trials.

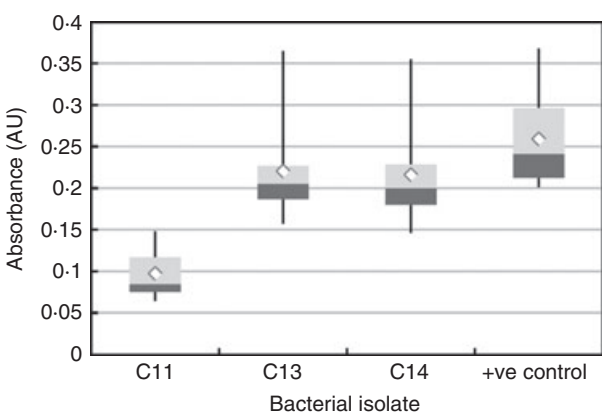

Figure 1 Antifungal effect of isolated bacteria from chilli waste against *Oligoporus placenta*.

Wood decay assay

Mass loss results (Fig. 2) demonstrate that all nonleached (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). However, despite this there was increased activity in the 1-week incubated treatments of *Lact. brevis* when exposed to *C. puteana* and *O. placenta* with these treatments performing significantly better ($P < 0{\cdot}05$) than the MRS control, with the 1 week CFS treatment having 2·4% mean mass loss compared with 4·8% for MRS when inoculated with *C. puteana* (Fig. 2b) and a mean negative mass loss compared to the MRS control (>10% mass loss) when inoculated with

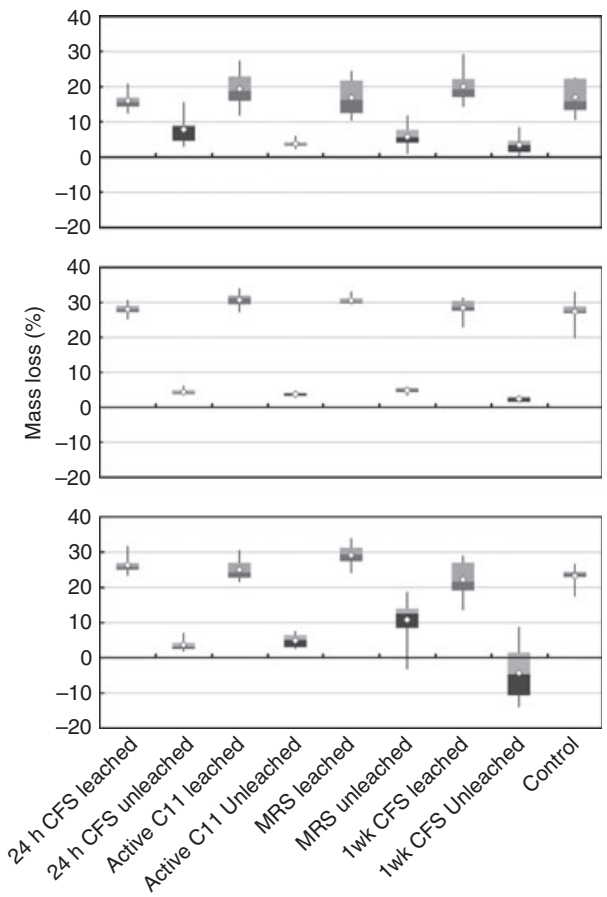

Figure 2 Mass loss of *Pinus radiata* blocks treated with *Lactobacillus brevis* (C11) cell-free supernatant and exposed to wood decay fungus (a) *Antrodia xantha*, (b) *Coniophora puteana* and (c) *Oligoporus placenta*.

O. placenta (Fig. 2c). Although not significantly different ($P > 0{\cdot}05$) the 1 week CFS inoculated with *A. xantha* had 3·5% mean mass loss compared to 5·8% for MRS (Fig. 2a). 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, therefore, subject to leaching under high moisture contents.

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 test. A second experiment was set-up to examine whether the MRS media promoted increased moisture content and subsequently the effect this has on mass loss because of fungal degradation. In this experiment, 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) (Fig. 3).

Characterization of antifungal metabolites

The proteinase K and temperature treatments had no effect on the antifungal properties of the *Lact. brevis* 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).

High-performance liquid chromatography analysis of the CFS showed that acetic and lactic acid were produced by *Lact. 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 (retention time = 2·16 min) and just over 50 mmol of acetic acid (retention time = 2·29 min). Phenyl lactic acid was not produced, but there were several other small peaks, which could relate to other organic acids that were not identified.

Discussion

In this study, three strains of lactic acid bacteria were evaluated for their antifungal properties against common wood decay fungi. The most promising bacterium, isolate C11, was identified as *Lact. brevis* by 16S rRNA gene sequence analysis and was further analysed for its ability to prevent wood decay caused by basidiomycetes *O. placenta, C. puteana* and *A. xantha*. Results from the wood decay assay suggest that *Lact. brevis* [C11] produces metabolites that inhibit decay fungi in the wood, with greater inhibition occurring with a longer incubation period of *Lact. brevis*. However, results also suggest that the medium in which the bacterium was 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 attack wood at moisture contents of between approximately 35 and 50% (Butcher 1974). Moisture contents at 100% MRS (recommended concentration) reached values of 170% on average.

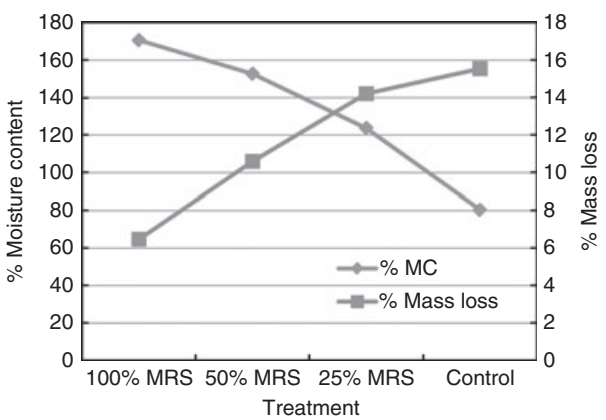

Figure 3 Correlation between moisture content and fungal degrade due to *Antrodia xantha*.

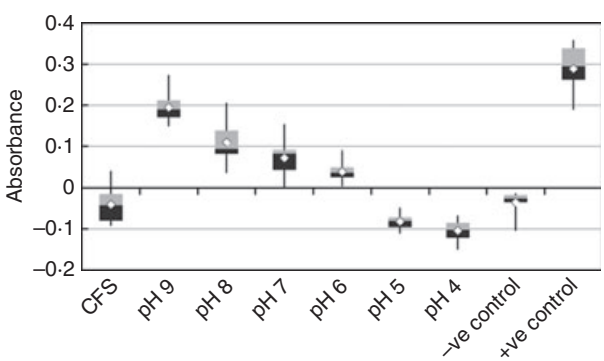

Figure 4 Effect of pH on the antifungal activity of *Lactobacillus brevis* (C11) cell-free supernatant.

Despite the inhibitory effect of the media used, wood blocks treated with *Lact. brevis* C11 supernatant (either cell free or active, incubated for 1 week) had significantly less mass loss ($P < 0{\cdot}05$) than their untreated counterparts when exposed to decay fungi *C. puteana* and *O. placenta*. These results suggest that the isolated *Lact. brevis* culture produced an antifungal substance, which was capable of inhibiting decay. *Lactobacillus brevis* has been shown to produce antifungal substances of proteinaceous (Falguni *et al.* 2009) and acidic natures (Gerez *et al.* 2009), and sometimes both (Mauch *et al.* 2010). Gerez *et al.* (2009) found two *Lact. brevis* strains that had antifungal activity against various bread moulds. As with this investigation, the antifungal activity was not changed by heating or treatment with proteinase K; however, 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 *Lact. brevis* strains tested was only achieved after increasing the incubation time of proteinase K to 5 h instead of 60 mins. Therefore, there is a possibility that the antifungal substance is proteinaceous although this is unlikely due to the lack of effect of heating. Schnurer and Magnusson (2005) suggest that antifungal activity produced by LAB is likely due to synergistic effects between several active components.

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. Thus, the effect of environmental variations appears to depend on the metabolite producing bacteria and the target organisms for inhibition. In this trial, basic incubation conditions were employed to produce the bacterial supernatant. It is proposed that the antifungal effect of the *Lact. brevis* [C11] supernatant could be improved by optimizing incubation conditions.

Given the tendency of the bacterial metabolites in this project to leach out of timber when subjected to wetting, they are unlikely be considered suitable as a permanent preservative for wood protection when used alone. However, they may have potential for use in combined treatments with other benign substances with antifungal properties (Singh and Chittenden 2008), especially in situations where timber is unlikely to get wet, and this option is being investigated further.

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