**Enerbio Ph.D. thesis**

**LIGNIN-DEGRADING ENZYMES FOR BIOFUEL PRODUCTION AND AROMATIC CHEMICAL PRODUCTION**

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**SUMMARY**

A recently published UV-vis assay method for lignin breakdown activity has been used to isolate novel lignin-degrading bacteria. Four *Microbacterium* isolates, two *Micrococcus* isolates, a *Rhodococcus* isolate (all *Actinobacteria*) and two *Ochrobactrum* isolates (*Alphaproteobacteria*) showed activity in the UV-vis assay, although the most active isolate is *Sphingobacterium sp.*, a thermo-tolerant strain in the *Bacteroidetes* family that showed approximately ten-fold higher activity than other lignin-degrading strains.

All isolates are capable of growing on lignin-related aromatic substrates such as biphenyl and vanillic acid as sole carbon sources, and of depolymerizing high molecular weight forms of Kraft lignin.

**INTRODUCTION**

The sustainable production of second-generation bioethanol from renewable feedstocks such as plant biomass could facilitate a reduction in the global dependence on fossil fuels and consequently lower atmospheric CO$_2$ levels. Breakdown of the highly recalcitrant lignin polymer (1) is an energy-intensive pre-treatment process that limits the wide-scale commercialization of lignocellulosic bioethanol (1).

Novel lignin-degrading bacteria may be used as an alternative pre-treatment that would offer several advantages over existing chemical methods including reduced energy requirements, environmentally-friendly processes, enhanced yields and a variety of commercially valuable byproducts from the breakdown of lignin.

Lignin (Fig. 1) is a non-repeating heterogeneous polymer of very high molecular weight that is composed of phenylpropanoid units linked by stable carbon-carbon bonds and hydrolysis-resistant ether linkages.
The combination of these complex features offers high resistance to microbial breakdown.

Fungi and bacteria naturally degrade lignin and several fungal species have been found to produce peroxidase (2) and laccase (3) enzymes that are capable of breaking it down. The advantages of studying lignin-degrading bacteria rather than fungi include easier gene cloning, higher-level protein expression and the potential use of thermotolerant bacteria that are stable at higher temperatures, therefore more adaptable to industrial conditions.

RESULTS

i) Isolation of novel lignin-degrading bacteria

Soil samples from two different geographical regions of the United Kingdom and composted wheat straw were enriched in minimal medium containing lignocellulose from wheat straw as a sole carbon source at 30°C and 45°C respectively. After 3 weeks of incubation aliquots were spread onto agar plates of the same composition and sprayed with nitrated lignin solution (4). The six most yellow colonies from each plate were classed as the most active and fully isolated. The UV-vis assay (4) was subsequently performed on the culture supernatant of each isolate.

Identification of each isolate was performed by analysis of the 16S rRNA gene sequence, and 16S rRNA gene amplification performed by PCR. Nine different strains were isolated, including four Actinobacteria, three Alpha–proteobacteria, a Nocardioform and an uncultured strain from the Bacteroidetes family

- **Actinobacteria**: Microbacterium marinilacus, Microbacterium oxydans, Microbacterium phyllosphaerae, Micrococcus luteus.
- **Alpha-proteobacteria**: Ochrobactrum pseudogrignonense, Ochrobactrum rhizosphaerae, Rhizobiales sp. (thermo-tolerant).
- **Nocardioform**: Rhodococcus erythropolis
- **Bacteroidetes**: Sphingobacterium sp. (thermo-tolerant).

The clustering of the mesophilic isolates, collected from two geographical areas, into the Actinobacteria and the Alphaproteobacteria is consistent with previous observations that the majority of lignin-degrading bacteria cluster into the Actinobacteria, Alphaproteobacteria, and Gammaproteobacteria (5), suggesting that there are metabolic capabilities in these groups that support lignin breakdown. Although there are no previous reports of lignin-degrading Sphingobacteria, Sphingobacterium composti (6) has been discovered in paper mill pulps containing recycled fibres and several strains of Sphingobacterium have been isolated from compost (7). The Sphingobacterium strain isolated in this project is not closely
related to characterized *Sphingobacteria* and is particularly unique in growing aerobically at 45-50 °C and forming filaments, since most *Sphingobacteria* are rod-shaped and anaerobic.

![Figure 2](image)

**Figure 2.** Change in absorbance at 430nm from 0-20 minutes for novel bacterial lignin degraders with 2mM hydrogen peroxide. Controls include *R. jostii RHA1* and *P. putida*, known degraders, and non-degrader *B. subtilis*.

All of the isolates are more active in the UV-vis assay (Fig. 2) than known lignin degraders *Rhodococcus jostii RHA1* and *Pseudomonas putida*. The thermotolerant strain *Sphingobacterium sp.* is approximately 10-fold more active than the mesophiles. It is interesting that in some cases most of the activity is in the absence of hydrogen peroxide, suggesting that the activity is mainly due to laccases rather than peroxidases.

**ii) Biotransformation of Kraft lignin**

In order to examine whether or not *Sphingobacterium sp.* is capable of depolymerizing high molecular weight forms of lignin, a sample of high molecular weight size-fractionated Kraft lignin was incubated with *Sphingobacterium sp.* for 172 hours and monitored by gel filtration HPLC at various time points.

![Figure 3](image)

**Figure 3.** Gel filtration HPLC analysis of the breakdown of high-molecular weight Kraft lignin by *Sphingobacterium sp.* in minimal media at 45 °C. The retention time for the high molecular weight lignin peak at 11 min is indicated with a dashed line.
A peak at retention time 11 min is characteristic of the HPLC analysis for the high molecular weight Kraft lignin (Fig. 3). Following incubation with *Sphingobacterium sp.*, the intensity of the peak decreases vs. time, a reduction of approximately 75 % after 72 hours and disappearance after 192 hours. This reduced intensity correlates with the appearance of a peak at 24 min, which corresponds to de-polymerisation products of a low molecular weight. The appearance of a low intensity peak at 17 min corresponds to the formation of a small amount of medium molecular weight products.

The formation of a peak at 9 min after 72 hours suggests that a higher molecular weight species has also been formed by treatment with *Sphingobacterium sp.*, possibly due to re-polymerisation of the fragmented lignin to yield a higher molecular weight polymer. The decrease in intensity of this peak after 192 hours suggests that the re-polymerised material is broken down with increased growth of *Sphingobacterium sp*.

CONCLUSIONS

The recently reported assay method has proved useful for screening environmental bacteria for lignin breakdown activity. Several novel lignin-degrading bacteria have been isolated, many of which appear to be more active than previously reported lignin degraders and are capable of de-polymerising high molecular weight forms of lignin. Purification of the extracellular enzymes responsible for lignin breakdown has been carried out from the culture supernatant of *Sphingobacterium sp.*, and the application of the strains towards the bioconversion of lignocellulose into bioethanol is currently being studied.

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REFERENCES