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Research paper

Fungal pre-treatment of forestry biomass with a focus on biorefining: A comparison of biomass degradation and enzyme activities by wood rot fungi across three tree species

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ABSTRACT

The fungal enzyme activities and breakdown of wood components may be influenced by the fibrous and structural composition across different wood species. White-rot fungi were inoculated onto wood chips of *F. excelsior*, *A. pseudoplatanus* or *Q. robur* and incubated for 28 days revealing that most fungi appeared to successfully colonize the different types of wood chips. Fibre analysis revealed that the fungi causing the highest mass losses in *F. excelsior* and *A. pseudoplatanus* were those that degraded more cellulose compared with hemicellulose or lignin. Fungal degradation leading to high mass losses of *Q. robur* was more complicated as lignin-degrading activities became more important. The structural composition in terms of the largest vessel sizes only showed an inverse correlation with remaining moisture content and not with mass loss or fibre degradation. These results provide an insight into fungal degradation of wood from three common tree species, and the link between the compositional characteristics of each wood type and the ease of degradation. This could have an impact on future biological pre-treatment strategies and valorisation approaches for forestry residues in integrated biorefineries.

1. Introduction

The utilization of forestry waste products in the advanced (lignocelluosic) biofuels sector, or in the recovery of potentially useful value added speciality chemicals remains challenging due to the highly recalcitrant nature of these residues. This is because of the small internal pore spaces in wood cell walls as a result of the highly cross-linked lignin component and the linear, crystalline nature of the cellulose backbone in lignocellulose materials, which limits the accessibility to enzymes during pre-treatment and prior to downstream bioprocessing. Currently, there are a range of chemical and physical pre-treatment approaches used for different biomass feedstocks, in order to facilitate optimised downstream fermentation for the production of biofuels and platform chemicals, but each have their limitations [1]. Fungal degradation of wood will reduce the structural integrity and increase the porosity of wood allowing easier physical disruption and greater access of chemicals and enzymes further into the wood structure. The application of biological pre-treatments, using edible fungi in particular, should be considered as a sequential strategy in the biomass processing of challenging forest biomass substrates [2].

The cellular construction and chemical composition which form the wood structures varies between different tree species and this has an impact on the utilization of biological pre-treatments, including fungal decomposition [3]. Understanding the relationship between the cellular structure of different wood species and the relative ease of fungal mediated degradation, could therefore have a major impact on future pre-treatment strategies for forestry residues and their valorisation within integrated biorefineries [4]. *Fraxinus excelsior* (ash), *Acer pseudoplatanus* (sycamore) and *Quercus robur* (oak) are tree species commonly found in Europe, yet no study has compared fibre analysis of these species. However, separate values obtained from different studies reveal that cellulose forms a considerable component of most hardwoods, and in these particular tree species constitutes between 38 and 50% [3,5–7]. In some of these studies, the Klason lignin contents reported were 26% for *F. excelsior* and *A. pseudoplatanus* which was higher compared with *Q. robur* which ranged from 22 to 24%. Factors affecting fungal decay of *Q. robur* have shown that moisture content is important and that wood sources from different geographical locations

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seemed to yield similar rates of decay [8].

The vessels are an important anatomical feature which provides wood rot fungi with a conduit into the centre of wood, as demonstrated with *Phellinus flavomarginatus* which has been shown to enter the wood chips of *Eucalyptus grandis* through fibre vessels and pits [9]. Besides providing fungi with access into the wood structure, the vessels facilitate gaseous exchange. Richter and Dallwitz [10] have provided the expected ranges of vessel diameters across different species which are 54–120 μm for *F. excelsior*, 44–65 μm for *A. pseudoplatanus* and 130–290 μm for *Q. robur*, clearly showing that the vessel diameters in *Q. robur* are larger. A previous study also revealed that the vessel diameters of *F. excelsior* and *A. pseudoplatanus* were similar but much less compared with *Q. robur* [11]. *A. pseudoplatanus* differs from *F. excelsior* in that the vessel diameters are similar throughout the seasons and is described as being diffuse porous. In contrast, *F. excelsior* and *Q. robur* are described as ring porous because wider vessels are formed early in the growing season and narrower vessels are formed near the end of the growing season.

The aim of this study was to obtain a comprehensive understanding into the degradation *F. excelsior*, *A. pseudoplatanus* and *Q. robur* by white rot fungi, which are commonly found wood substrates readily found in the European region. Only one study has made a comparison in white rot fungal degradation of several types of wood chips revealing higher enzyme production on one wood species [12]. There was particular emphasis on *Lentinula edodes* (used in Shiitake production) in comparison to other white rot fungi. This was investigated in order to obtain a greater understanding in how this fungus utilizes other wood substrates, compared with its preferred wood substrate, *Q. robur,* and in comparison to other wood rot fungi. *L. edodes* is becoming increasing popular worldwide [13] and could be effectively used in the pre-treatment of woody substrates.

2. Methods

2.1. Preparation of microcosms

Fresh wood from three different tree species were examined: *F. excelsior* (ash), *A. pseudoplatanus* (sycamore) and *Q. robur* (oak) that were coarsely cut into small chips. Glass jars containing wood chips (100 g) were sealed with a screw capped lid, each lid containing a 2.5 cm central hole that was filled with non-absorbent cotton wool that would allow gaseous exchange. The jars were autoclaved at 121 °C and 15 p.s.i for 1 h and autoclaving was determined to have no effect on the moisture content. Duplicate jars were inoculated with an agar square containing one of the fungi growing on 2% malt agar. The fungi used in this study were white rot wood decaying basidiomycete fungi: *Lentinula edodes* (LE), *Phlebiopsis gigantea* (PG), *Ganoderma tsugae* (GT); *Trametes versicolor* (TV1) *Ceriporiopsis subvermispora* D98698 (CS), *Phanerochaete chrysosporium* S596 (PC) and two fungal isolates. The two fungal isolates isolated from an *Q. robur* log in Treborth Botanical Gardens, Bangor, Gwynedd, UK were revealed by DNA sequencing revealed to be highly similar to *Trametes versicolor* (referred to in following text as *T. versicolor* 2) and *Phlebia radiata* (PR), respectively. The glass jars were incubated in the dark in an environmentally controlled room at 22 °C and 65 ± 5% humidity for 4 weeks. This period allowed complete fungal colonisation which occurred from the central inoculation point within the glass jar microcosms and completely colonized the wood, including wood close to the glass vessel walls which was furthest from the inoculation point. After incubation, the glass jars were weighed and were samples removed to determine enzyme activities. The remaining material was oven dried (103 °C for 24 h) to determine dry mass loss, then ball milled prior to Klason lignin analysis and fibre analysis of hemicellulose, cellulose and acid detergent lignin [14].

2.2. Analysis of microcosms and original wood material

The wood chips were air dried for one week and the sizes of the wood chips were determined by rigorously shaking wood chips for 10 min through >3.15 mm, 1.4 mm, 600 μm, 250 μm and 250 μm sieves. The contents collected on each of the sieves were weighed. The sizes of the largest vessels were determined by thinly cutting cross sections of wood and staining with safranin. The thin sections were viewed under a phase contrast microscope and at least 10 of the largest vessels were measured using a micrometer. Fibre analysis was achieved by oven drying wood chips (103 °C for 24 h), ball milling the chips and weighing 0.525 ± 0.025 g into each Ankom bag which was then heat sealed. The hemicellulose and cellulose contents were determined by extractions methods using neutral detergent fibre (NDF) and acid detergent fibre (ADF), respectively as previously described [14]. Lignin was extracted from each wood sample remaining after the ADF extraction by the Klason method. Each filter bag was immersed in 7 ml of 72% (v/v) sulphuric acid for 2 h at 20 °C with periodic agitation to facilitate mixing. The acid solution was diluted to 4% (v/v) with 196 ml distilled water and autoclaved at 121 °C for 1 h, washed thoroughly with hot water ~40 °C, dried at 103 °C for 20 h and weighed. The ash content was determined by heating 1 g of the milled wood in a muffle furnace (4 h at 600 °C). The percentage of ash was subtracted from the percentages of hemicellulose, cellulose and lignin. The percentage of hemicellulose remaining was calculated by subtracting ADF from NDF extracts. The percentage of cellulose remaining was calculated by subtracting the lignin and percentages of ash from the ADF extracts. The percentages of cellulose and lignin were calculated by subtracting the percentages of ash.

The wet mass of wood in the glass jars and the moisture content were determined with samples collected at the start and end of the experiment. The change in moisture content during autoclaving was also taken into account, to calculate the moisture content of the wood at the start of the experiment. At the end of the experiment, each microcosm was weighed and a sample was removed after thoroughly mixing the contents with a spatula in order to determine enzyme activities. The residual wood chips were oven dried to determine moisture content which was calculated on the dry weight basis, i.e. by subtracting the dry mass from the total wet mass and dividing by the total dry mass. The oven dried wood chips were ball milled in order to determine fibre analysis and ash content. The ash content was determined by heating 1 g in a muffle furnace for 4 h. The total quantities of hemicellulose, cellulose and lignin that were degraded by each fungus were calculated by factoring in mass losses.

2.3. Enzyme activities

Enzyme activities were determined from fungal cultures growing in fungal degraded wood (5 g) that was suspended in 1 mM sodium acetate buffer (100 ml) at pH 5 and vigorously blended for 1 min in a Waring Blender. The macerated wood extract was centrifuged (11,337 × g 1 min), 1 ml supernatant was removed, filtered through a 0.2 μm membrane filter (Millipore) and serially diluted 10 fold in 1 mM sodium acetate buffer, pH 5. Laccase activities were determined in 1 M sodium acetate buffer, pH 5 with 0.5 M 2,29-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) and manganese peroxidase activities were determined in 50 mM sodium succinate (pH 4.5), 50 mM sodium lactate (pH 4.5) using 0.1 mM $MnSO_4$, 0.1 mM phenol red and 50 μM hydrogen peroxide and measured in a microplate reader at 610 nm as previously