International Biodeterioration & Biodegradation 120 (2017) 21–35

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Degradation of hardwoods by treatment of white rot fungi and its pyrolysis kinetics studies

Darshan M. Rudakiya, Akshaya Gupte*

N. V. Patel College of Pure and Applied Sciences, Vallabh Vidyanagar, Gujarat 388 120, India

ARTICLE INFO

Article history:
Received 3 August 2016
Received in revised form
28 January 2017
Accepted 3 February 2017
Available online 10 February 2017

Keywords:
Ca oxalate crystal
Delignification
Lignocellulolytic enzymes
White rot fungi
Wood pyrolysis

ABSTRACT

The present work investigated the degradation of two Indian hardwoods (*Pithecellobium dulce* and *Tamarindus indica*) and the activities of lignocellulolytic enzymes by *Pseudolagarobasidium acaciicola* AGST3 and *Tricholoma giganteum* AGDR1. Laccase exhibited the highest ligninolytic activity, followed by manganese peroxidase and lignin peroxidase in both white rot fungi. Accessory enzymes such as aryl alcohol oxidase and cellobiose dehydrogenase showed higher activities till 21 days after incubation and then declined till 30 days. Xylanase and β-glucosidase were also found to be stable in both fungi during the degradation of hardwoods. The cultures, *P. acaciicola* AGST3 and *T. giganteum* AGDR1 accumulated high amounts of oxalic acid and produced weddellite (0.2–9 μm) and whewellite (5–21 μm) crystals, respectively. Comparative decay studies revealed that both the fungi were able to degrade lignin, cellulose and hemicelluloses of two hardwoods efficiently. During the pyrolysis reaction, decrease in onset temperature and activation energy was observed with the fungal treated wood as compared to the untreated wood. The basidiomycete cultures used in the study are efficient lignin degraders and can be applied as a pretreatment for second generation biofuels.

1. Introduction

Currently, woody biomass and waste contribute to 10% of global energy supply. The estimated potential of available biomass is 1.08×10^{11} TOE (tons of oil equivalents), which is almost 10 times higher than current energy demand (Havlík et al., 2011; Taha et al., 2016). The abundant biomass availability, renewability and CO_2 neutrality are major driving forces for utilization of biomass. Moreover, woody biomass has received great attention for a second generation biofuel production in recent times (Skyba et al., 2013). Pyrolysis is a possible thermochemical route, which converts biomass to a large number of chemical compounds that are used for biofuel generation (Kan et al., 2016). The major hindrance for biofuel production is the amount, structure, and composition of lignin present in hardwoods. Pretreatment processes break lignin structure and disrupt the crystalline cellulose, thus providing more accessibility and making pyrolysis reaction cost-effective. Physical and chemical treatments require substantial energy input and chemical addition, respectively. Biological treatment has an advantage of lower energy and chemical use (Masran et al., 2016; Taha et al., 2016). In biological pretreatment, white rot fungi have been proven to degrade wood components as they have the ability and machinery to break down and mineralize lignin into CO_2 (Guerriero et al., 2016; Shirkavand et al., 2016).

White rot fungi secrete an array of ligninolytic enzymes to depolymerize lignin and modify monomeric lignin compounds. Mainly two types of ligninolytic enzymes are involved in lignin degradation; (i) phenol oxidases (mainly laccases) (ii) peroxidases (lignin peroxidase, Mn peroxidase, versatile peroxidase) (Schmidt-Dannert, 2016). In addition, fungi secrete accessory enzymes like cellobiose dehydrogenase (CDH), aryl alcohol oxidase (AAO), glyoxyl oxidase, Cu oxidase, etc. These enzymes provide free radicals and intermediates that help in lignin and polysaccharide degradation (Manavalan et al., 2015). White rot fungi also secrete hydrolytic enzymes, which cause the simultaneous or selective degradation of cellulose and hemicelluloses along with lignin (Zhu et al., 2016).

The ligninolytic action of enzymes secreted by white rot fungi during wood decay is hampered due to their large size. As a result, fungi produce low molecular weight organic acids and free radicals at the initial stage (Plassard and Fransson, 2009). Oxalic acid is the

* Corresponding author. Department of Microbiology, Natubhai V. Patel College of Pure and Applied Sciences, Vallabh Vidyanagar, Gujarat 388 120, India.
E-mail addresses: darshan.rudakiya@hotmail.com (D.M. Rudakiya), akshaya_gupte@hotmail.com (A. Gupte).

http://dx.doi.org/10.1016/j.ibiod.2017.02.004
0964-8305/

predominant organic acid produced by fungi, which drops the pH outside of fungal hyphae. It also provides H_2O_2, which degrades the side chains of hemicelluloses. All these factors provide a stable environment for the ligninolytic enzymes (Mäkelä et al., 2002; Xu et al., 2015). Oxalic acid production can lead to metal immobilization in the form of insoluble mineral oxalate crystals (Guggiari et al., 2011; Harms et al., 2011). The mycogenic oxalate crystals have a significant role in bio-weathering and geo-microbiological processes, which are directly applied in radionuclide leaching, bioremediation, metal recovery and detoxification (Gadd et al., 2014).

The present study is aimed to evaluate certain fungal strategies (enzymatic and oxalic acid) for wood decay of two Indian hardwoods (*Pithecellobium dulce* and *Tamarindus indica*) as well as physico-chemical properties of untreated and treated wood.

2. Materials and methods

2.1. Chemicals and hardwood materials

Oxalic acid, 2, 2-azino-bis (3-ethylbenzthiozoline-6-sulfonic acid) (ABTS), guaiacol, birchwood xylan, 2, 6-dimethoxy phenol (DMP) and veratryl alcohol were procured from Sigma-Aldrich, USA. Sabouraud dextrose broth, Agar-Agar, $CaCO_3$ and other chemicals were purchased from Hi-media Labs, India. All other chemicals used in the present study were of analytical grade and of the highest purity. The sapwood of *P. dulce* and *T. indica* woods were cut individually into small pieces. Further, small pieces of sapwoods were ground and sieved to a uniform particle size (4–5 mm).

2.2. Isolation and screening of fungal strains

White rot fungi used in the present study (*Pseudolagarobasidium acaciicola* AGST3 and *Tricholoma giganteum* AGDR1) were isolated from Anand (22.56°N, 72.92°E) region, Gujarat, India. Fungal samples were sterilized using mercuric chloride (1 g l^{-1}) for 2–3 min followed by washing with distilled water. Sterilized fungal samples were plated on Sabouraud dextrose agar containing streptomycin (25 μg ml^{-1}) and incubated at 30 °C for 12 days and repeatedly transferred until pure cultures were obtained. Pure cultures of fungi were maintained by sub-culturing on Sabouraud dextrose agar with streptomycin (25 μg ml^{-1}) at 30 °C for 15 days. Molecular identification of fungi was achieved by isolating fungal genomic DNA and amplifying ITS4 and ITS5 region.

2.3. Biological pretreatment of hardwood

Five gram oven-dried wood particles (*P. dulce* and *T. indica*) were placed in 250 ml Erlenmeyer flasks and autoclaved at 121 °C, 15 psi for 60 min. Asther's medium was also autoclaved (121 °C, 15 psi for 15 min) in 250 ml Erlenmeyer flask and supplemented (20 ml) in the flasks containing autoclaved hardwood particles to give a final substrate to liquid ratio of 1:4 (w/v) (Asther et al., 1988). Flasks containing moistened wood particles were inoculated with 5 mycelium agar plugs (10 mm diameter) of fungus. All flasks were kept in humidity controlled incubator (Electroquip, India) to maintain 60% relative humidity at 30 °C for up to 30 days under static condition. Myco-wood samples (wood with fungal mycelia) were harvested at a regular interval of 3 days. Estimation of fungal biomass was carried out using the method described by Rodríguez-Couto et al. (2009). A set of wood samples containing Asther's medium without fungal biomass was also kept for 30 days, which was referred as a control for fungal biomass estimation. All experiments were conducted at least in triplicate.

2.4. Ca oxalate crystal production

P. acaciicola AGST3 and *T. giganteum* AGDR1 were tested for their ability to produce Ca oxalate crystals. Initially, Ca oxalate production was examined using microscopic observation of crystal presence in 15 days-old cultures incubated in Sabouraud dextrose agar with and without $CaCO_3$ (5 g l^{-1}). For each fungal species, three agar blocks (5 × 10 × 5 mm) were removed from the outer edge to the central part of hyphal growth. This method allowed to investigate the presence of crystals on younger mycelia (outer edge region) as well as older mycelia (the central part). The agar blocks were washed with deionized water and observed under an optical microscope with a magnification of 400 × (Guggiari et al., 2011). The presence of Ca oxalate crystals were also confirmed on the hardwood by incubating both fungi individually on *P. dulce* wood particles at 30 °C for 20 days. Asther's medium supplemented with 5 g l^{-1} $CaCO_3$ was used as a moistening agent. The presence of Ca oxalate crystals from wood samples was studied using scanning electron microscope (EVO-18 ESEM, Zeiss, Germany).

2.5. Determination of enzyme and oxalic acid

Harvested myco-wood samples were suspended in Na-acetate buffer (100 mM, pH 5.0) and shaken (100 rpm) at 30 °C for 3 h. Extracts were squeezed through a muslin cloth and centrifuged (8000 rpm) for 15 min at 4 °C. The clear supernatant was used for the determination of enzyme activity and oxalic acid.

Laccase (E.C. 1.10.3.2) assay was performed by measuring the time-dependent oxidation of 50 mM ABTS at 420 nm in Na-acetate buffer (100 mM, pH 5.0) (Niku-Paavola et al., 1990). Mn peroxidase (MnP, E.C. 1.11.1.13) activity was determined from A_{469} changes related to the oxidation rate of DMP in the presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$ in Na-tartarate buffer (100 mM, pH 4.5). Final MnP activity was corrected by subtracting Mn independent peroxidase activity obtained at pH 3.25 in the absence of $MnSO_4$ at 469 nm (Martinez et al., 1996). Lignin peroxidase (LiP, E.C. 1.11.1.14) activity was determined by oxidation of guaiacol (1.32 mM) at 436 nm in the presence of 0.1 mM H_2O_2 in Na-tartarate buffer (330 mM, pH 4.0) (Sarkanen et al., 1991). AAO (E.C. 1.1.3.7) activity was detected by the oxidation of veratryl alcohol to veratrylaldehyde in Na-phosphate buffer (100 mM, pH 6.0) at 310 nm (Bourbonnais and Paice, 1988). One unit enzyme activity (U) was defined as the amount of enzyme which leads to the oxidation of 1 μM of substrate per min under standard assay condition. Time dependent reduction of CDH (E.C. 1.1.99.18) activity was determined according to the method described by Harreither et al. (2009) at 520 nm. One unit CDH activity (U) was referred as the amount of enzyme which leads to the reduction of 1 μM of substrate per min under standard assay condition.

Xylanase (EC 3.2.1.8) activity was determined according to Bailey et al. (1992) using birchwood xylan (1 g l^{-1}). Filter paper activity was measured using filter paper as substrate. The release of reducing sugar was measured using di-nitrosalicylic acid method (Ghose, 1987). One unit enzyme is defined as the amount of enzyme that liberates 1 μM of reducing sugar equivalent per ml per min under standard assay condition. The β–glucosidase (E.C. 3.2.1.21) activity was assayed using *p*-nitrophenyl–β–D–glucopyranoside (4 mM pNPG; 50 mM Na-acetate buffer, pH 5.0) at 405 nm (Saha and Bothast, 1996). One unit of enzyme activity expressed as 1 μM of *p*-nitrophenol per minute under standard assay condition.

Quantification of oxalic acid was obtained by HPLC using reverse phase C-18 column (250 mm × 4.6 mm × 5 μm; Waters, UK) (Dutton et al., 1993). The centrifuged extrudates were filtered through 0.45 μm filter. The filtered extrudates were diluted (1:10) with deionized water, injected into C-18 column and spotted on