ASSESSING LIGNOCELLULOLYTIC POTENTIAL OF FUNGAL ISOLATES FOR WOOD DEGRADATION

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ABSTRACT

Mechanical stump removal shows high financial and environmental costs and the stumps and roots remaining after shallow cutting can cause damage to machines and implements used in soil preparation. The natural degradation of stumps and roots is slow, remaining practically unchanged for several years after cutting. In this sense, this work aimed to investigate the potential for accelerating the degradation of remaining stumps by fungi and their secreted enzymes for *in vitro* wood degradation tests. To this end, 28 fungal isolates were induced by wheat bran, pinus sawdust and soy husk, and studied regarding its lignocellulolytic enzyme activities. Wheat bran was the best inducer biomass. *Chrysoporthe cubensis* isolates, *Aspergillus niger* and Im isolate (not yet identified) showed the widest range of cellulolytic and hemicellulolytic activities, while higher laccase levels were produced by *Larssoniporia tropicalis*, *C. cubensis* and *Pycnoporus sanguineus*. *In vitro* degradation tests using Im isolate, *L. tropicalis* and *P. sanguineus* promoted higher mass loss and hardness reduction. Such findings indicate the potential for applying the biotechnological approach using degrading fungi and/or their enzymes as alternatives to accelerate degradation and provide less impact on the area, contributing to greater sustainability of planted forests.

Keywords: Enzyme cocktail. Lignocellulosic biomass. Semi-solid fermentation. Wood degrading fungi.

1 INTRODUCTION

The presence of stumps and remaining roots after harvesting eucalyptus or pine is an obstacle to a new cycle of crop management.[1] Due to the high resistance of their structure, even twenty years after cutting, it is possible to find eucalyptus stumps only partially degraded in some regions of Brazil. The mechanical removal of remaining stumps, which is the currently used strategy, is an economically costly process that requires the deployment of men and large machinery, and results in soil destabilization, compaction, and excessive disturbance, potentially causing surface erosion, reduction of organic matter content, and changes in physical, chemical, and biological characteristics, leading to ecosystem damage.[1,2] For this reason, alternative methods for stump removal are necessary. Previous studies have evaluated the potential application of wood-degrading fungi to accelerate stump degradation.[1-4] However, due to the high susceptibility of these microorganisms to environmental conditions in the field and their dependence on controlled factors such as temperature and humidity, the results have not been consistent in areas with adverse conditions. Since the decomposing action of fungi is due to the lignocellulolytic activity of the enzymes they secrete, this study aims to evaluate the enzymatic potential of different wood-degrading fungi in the degradation of wood test specimens. For this purpose, the enzymatic profile of different fungal isolates was investigated. The most promising profiles were selected and applied for *in vitro* wood degradation tests to access the potential application of fungi as well as their enzymatic cocktails to accelerate stump degradation in field.

2 MATERIAL & METHODS

A total of 28 fungi belonging to the collection of microorganisms from the Forest Pathology and Biochemical Analysis laboratories of the Federal University of Viçosa, including fungi isolated from wood (*Chrysoporthe cubensis*, *Kretzchmaria zonata*, *Scedosporium boydii*, *Talaromyces aculeatus*, *Larssoniporia tropicalis*) and other fungi known as good producers of lignocellulolytic enzymes (*Aspergillus niger* and *Pycnoporus sanguineus*) were investigated. The fungi were activated in Potato-Dextrose-Agar (PDA) medium at 28 °C for 7 days, and the induction of enzymes (endoglucanase, total cellulases, xylanase, laccase, β-glucosidase and β-xylosidase) was evaluated by cultivation in semi-solid medium with wheat bran, pinus sawdust or soy husks as carbon sources. Autoclaved 250 mL Erlenmeyer flasks containing 5 g of biomass and 12 mL of mineral medium plus trace elements were inoculated with 10 discs (5 mm in diameter) of fungal mycelium and incubated for 8 days at 28 °C, in duplicates. Enzymes were extracted with 50 mL of 50 mM sodium acetate buffer, pH 5.0, at 28 °C and 150 rpm for 1 h, and enzyme activities were evaluated at 50 °C as previously described.[5] The potential for degradation of wood by fungi that stood out in terms of enzymatic activities was investigated in *in vitro* degradation tests using 4 different treatments: T1 - fungus-biomass mixture after induction for 8 days at 28 °C; T2 - fungus grown in liquid medium for 8 days at 28 °C and 150 rpm; T3 - enzyme extract from semi-solid cultivation; and T4 - mycelium discs from PDA plates incubated for 8 days at 28 °C. Each treatment was performed in 5 replicates. The eucalyptus wood (*Eucalyptus urophylla*) specimens, measuring 2.5 cm x 2.5 cm x 1.0 cm, were previously dried at 105 °C until constant weight and the mass of each wooden specimen before treatment was recorded. The degradation flasks were prepared with 70 g of soil from a eucalyptus planting area moistened with 15 mL of distilled water and autoclaved for 30 minutes at 121 °C. The flasks, subjected to treatments were kept in an incubation chamber

at 28 °C for 90 days. After this period, the specimens were recovered, cleaned with soft bristle brushes, dried at 105 °C until constant weight to determine mass loss and evaluated for hardness using a universal testing machine.

3 RESULTS & DISCUSSION

Filamentous fungi are known as good producers of hemicellulolytic enzymes since expression is modulated by different cultivation conditions and inducing biomasses, and the enzymes are secreted into the extracellular environment, facilitating their recovery[1]. Between the evaluated biomasses, wheat bran was the best inducer, resulting in higher enzyme activities (Table 1). In general, *Talaromyces aculeatus*, *Chrysoporthe cubensis*, *Aspergillus niger* and Cs 6.1 (not yet identified) stood out in terms of hemicellulolytic activities; *C. cubensis*, *A. niger, P. sanguineus and T. aculeatus* were the best cellulases producers; and *Larssoniporia tropicalis*, *C. cubensis*, *P. sanguineus* and the isolate Im (not yet identified) induced higher laccase activities. *Chysoporthe cubensis* is the phytopathogen reported in Brazil as the main cause of canker disease in commercial plantations of *Eucalyptus* spp..[6] In general, phytopathogenic fungi have a high number of copies of genes that encode CAZymes, which guarantees the extracellular machinery necessary to degrade plant cell wall components during infection[7] and reinforces the wide range of enzymatic activities observed in the *C. cubensis* isolates evaluated (Table 1). *Talaromyces aculeatus* (*Penicillium aculeatum*) are endophytic fungi commonly found associated with soil and decomposing organic matter, but have not been described as phytopathogens.[8] *Aspergillus niger* is a ascomycete widely used to produce commercial cocktails of hemicellulases and cellulases.[9] *Larssoniporia tropicalis* (*Wrightoporia tropicalis*) is a Basidiomycota fungus that causes white rot reported in Angiosperms.[10,11] *Pycnoporus sanguineus* is a white-rot fungus reported as good ligninolytic enzymes such as laccases.[12]

Table 1 Enzyme activities (U.mL^{-1}) of the most promising fungal isolates induced with wheat bran.

Fungi	Xylanase	Laccase	Endoglucanase	FPase	β-glucosidase	β-xylosidase
Peniophora sp.	8.3 ± 0.3	0.47 ± 0.10	0.39 ± 0.06	0.01 ± 0.03	1.15 ± 0.21	0.11 ± 0.03
Annulohypoxylon sp	6.6 ± 0.6	0.01 ± 0.00	0.83 ± 0.06	0.14 ± 0.12	0.49 ± 0.11	0.02 ± 0.01
Scedosporium boydii 66-1	4.3 ± 0.4	nd	0.25 ± 0.28	0.08 ± 0.07	0.07 ± 0.03	0.01 ± 0.00
S. boydii 66-3	4.0 ± 0.4	0.01 ± 0.01	0.48 ± 0.08	0.02 ± 0.05	0.43 ± 0.06	0.01 ± 0.00
Nectriaceae sp.	1.2 ± 0.1	0.01 ± 0.01	0.06 ± 0.09	0.01 ± 0.04	0.06 ± 0.01	0.01 ± 0.03
Larssoniporia tropicalis	2.1 ± 0.4	2.32 ± 0.54	0.60 ± 0.16	0.02 ± 0.04	1.16 ± 0.21	0.04 ± 0.00
S. boydii 66-2	4.4 ± 0.3	nd	0.43 ± 0.04	0.13 ± 0.05	0.45 ± 0.04	nd
Cladosporium cladosporioides	2.3 ± 0.7	nd	0.68 ± 0.32	0.06 ±0.07	0.10 ± 0.01	0.01 ± 0.00
Talaromyces aculeatus	19.8 ± 4.2	0.01 ± 0.01	0.76 ± 0.08	0.22 ± 0.04	2.46 ± 0.59	0.26 ± 0.03
Aspergillus niger	16.9 ± 1.2	nd	1.02 ± 0.04	0.40 ± 0.07	2.21 ± 1.08	nd
Cs 4.1 - Not identified	1.5 ± 0.4	nd	0.26 ± 0.16	0.08 ± 0.12	0.34 ± 0.05	0.11 ± 0.02
Cs 5.1 - Not identified	3.8 ± 0.5	0.20 ± 0.11	0.63 ± 0.06	0.06 ± 0.07	0.66 ± 0.34	0.05 ± 0.01
Cs 6.1 - Not identified	19.8 ± 0.9	0.05 ± 0.00	0.85 ± 0.15	0.03 ± 0.07	0.32 ± 0.05	0.18 ± 0.04
Cs 6.2 - Not identified	2.5 ± 0.4	nd	0.25 ± 0.28	0.06 ±0.07	0.45 ± 0.06	0.14 ± 0.04
Cs 7.1 - Not identified	5.3 ± 1.2	0.06 ± 0.04	0.73 ± 0.03	0.16 ± 0.05	1.14 ± 0.45	0.22 ± 0.09
Cs 8.1 - Not identified	3.0 ± 0.3	nd	0.21 ± 0.10	0.08 ± 0.04	0.21 ± 0.02	0.04 ± 0.00
Im - Not identified	8.0 ± 0.7	0.64 ± 0.10	0.83 ± 0.14	0.16 ± 0.08	0.55 ± 0.10	0.04 ± 0.01
Chrysoporthe cubensis LPF2305	18.1 ± 0.7	0.10 ± 0.02	1.08 ± 0.08	0.25 ± 0.07	2.10 ± 0.19	0.21 ± 0.04
Chrysoporthe sp. LPF2667	2.9 ± 0.5	0.25 ± 0.01	0.17 ± 0.02	0.10 ± 0.03	1.68 ± 0.26	0.06 ± 0.01
C. cubensis LPF2374	17.1 ± 5.3	1.45 ± 0.24	0.52 ± 0.04	0.16 ± 0.09	2.27 ± 0.37	0.19 ± 0.03
Chrysoporthe sp. LPF2432	2.8 ± 0.2	0.29 ± 0.11	0.42 ± 0.23	0.08 ± 0.07	2.08 ± 0.14	0.08 ± 0.01
C. cubensis LPF2432	2.6 ± 0.1	0.48 ± 0.06	0.23 ± 0.02	0.08 ± 0.08	1.84 ± 0.25	0.10 ± 0.01
C. cubensis LPF2470	13.6 ± 1.8	0.11 ± 0.03	0.74 ± 0.30	0.14 ± 0.17	2.73 ± 0.31	0.17 ± 0.01
C. cubensis LPF2497	6.8 ± 1.2	0.85 ± 0.29	0.48 ± 0.12	0.17 ± 0.04	1.50 ± 0.13	0.08 ± 0.02
C. cubensis LPF2583	15.9 ± 1.1	0.14 ± 0.04	0.46 ± 0.30	0.22 ± 0.06	2.10 ± 0.53	0.13 ± 0.02
Kretzschmaria zonata LPF2620	3.2 ± 0.5	nd	1.50 ± 0.09	0.04 ± 0.07	0.40 ± 0.09	0.01 ± 0.00
K. zonata LPF2118	4.1 ± 0.4	0.08 ± 0.01	0.68 ± 0.11	nd	0.25 ± 0.07	0.02 ± 0.01
Pycnoporus sanguineus	1.9 ± 0.3	0.79 ± 0.13	0.33 ± 0.11	0.33 ± 0.19	0.27 ± 0.08	nd

nd- not dettected.

To investigate their ability to degrade wood and the potential to be applied to accelerate the degradation of eucalyptus stumps, the isolates *A. niger*, *C. cubensis* LPF2374, *L. tropicalis*, *P. sanguineus* and the Im isolate were selected to be applied in the wood degradation tests. These isolates showed different profiles, with a more complete enzymatic arsenal in terms of cellulolytic and hemicellulolytic activities, or high levels of laccase. The *C. cubensis* isolates were the ones that stood out the most due to the greatest activities and combined secretion of the largest number of enzymes evaluated, specially the isolate LPF2374.

After incubation time, the different treatments showed visible colonization of wood and soil by fungi. Regarding the treatments, the application of induced fungi by wheat bran (T1) and the mycelium discs (T4) provides the highest levels of wood degradation, followed by enzymes extract (T3) (Table 2). Treatment 2 did not promote significant changes in the mass and hardness of the wood. In this sense, the application of fungi cultivated in solid or semi-solid conditions promoted the best

establishment of selected isolates in the wood specimens and allowed the achievement of higher levels of mass loss and hardness reduction.

Table 2 Relative mass loss and Janka hardness of wooden test specimens subjected to *in vitro* degradation tests. Effect of each treatment was evaluated in relation to control specimens. T1 – fungus-biomass mixture; T2 - fungus grown in liquid medium; T3 - enzyme extract; and T4 – mycelium discs; An + Ps – *A. niger* + *P. sanguineus*, An – *A. niger*, Cc – *C. cubensis* LPF2374, Im – not identified isolate, Lt – *L. tropicalis,* Ps – *P. sanguineus.*

	Relative mass loss (%)				Relative Janka hardness (%)			
Fungi	T1	T2	T3	T4	T1	T2	T3	T4
An+Ps	7.7 ± 6.5	2.0 ± 0.4	1.5 ± 1.6	3.8 ± 3.2	95.4 ± 10.7	101.5 ± 3.7	92.2 ± 1.3	88.7 ± 29.6
An	0.0 ± 0.0	1.6 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	120.4 ± 6.8	100.3 ± 7.7	92.7 ± 10.7	109.8 ± 18.8
Cc	1.5 ± 1.2	2.0 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	101.5 ± 7.0	101.1 ± 3.1	85.2 ± 18.7	89.2 ± 24.0
Im	13.2 ± 4.0	3.5 ± 5.2	0.3 ± 0.3	8.8 ± 4.4	87.2 ± 3.5	100.7 ± 6.5	91.2 ± 1.8	81.7 ± 21.6
Lt	11.8 ± 4.5	0.8 ± 0.8	5.1 ± 3.5	7.6 ± 5.0	85.9 ± 15.2	84.6 ± 18.4	80.1 ± 12.3	66.5 ± 20.3
Ps	6.4 ± 2.3	2.0 ± 1.1	5.5 ± 3.0	0.6 ± 0.5	100.1 ± 9.1	77.1 ± 17.3	79.6 ± 8.3	105.8 ± 19.6

The evaluation of degradation parameters indicated that Im and *L. tropicalis* isolates promoted higher mass loss, especially in treatments 1 and 4, while for T3, the isolates *L. tropicalis* and *P. sanguineus* stood out (Table 2). Hardness tests indicated that these same treatments were those that most altered the wood's resistance, suggesting that isolates Im and Lt promoted higher levels of wood degradation. These isolates, in addition to other cellulolytic and hemicellulolytic activities, stood out in terms of laccase activities. Lignolytic activities are essential for plant biomass degradation since they act in the destructuring of lignin, facilitating the access of cellulases and hemicellulases to the sugars in the plant wall, increasing the levels of hydrolysis. *C. cubensis*, despite its range of enzymes and high impact known in the field, did not promote mass loss or reduction in hardness in the performed tests.

4 CONCLUSION

Stumps and roots remaining in planting areas constitute one of the greatest challenges for forestry companies, requiring new approaches that help to overcome this obstacle. In the present work, lignocellulosic biomasses from eucalyptus wood, wheat bran, and soybean hulls were evaluated for their potential to induce lignocellulolytic enzymes by 28 fungi. Wheat bran was the carbon source that induced the highest enzyme activities. Its composition rich in proteins and minerals contributes to the growth of microorganisms. The main lignocellulolytic activities were obtained for isolates of *C. cubensis*, *T. aculeatus*, *L. tropicalis*, *A. niger*, *P. sanguineus* and the Im isolate (unidentified). *In vitro* degradation tests using the selected isolates indicated that the best laccase producers promoted the greatest mass losses in wooden specimens in association with the highest levels of Janka hardness reduction. These findings reinforce the potential of the isolates evaluated with a view to biological destocking and open views to the prospect of a biotechnological approach to a major obstacle in forestry production.

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