Effect of wood flour as carbon source on cellulases and xylanases production by white-rot-fungi native from Misiones

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ABSTRACT

There is a need to explore lignocellulosic materials to select an adequate substrate for lignocellulolytic enzyme production. Utilization of some residues provides an opportunity to produce high yields of lignocellulolytic enzymes in a simple medium. The aim of the present work was to study the effect of wood flour as a carbon source on the cellulolytic and xylanolytic secretion of white-rot fungi native from Misiones. Fungi were incubated with 5 g Pinus sp. wood flour/L and 5 g Eucalyptus sp. wood flour/L as carbon sources in a reciprocal shaker at 80 rpm and 29ºC for 15 days. Total cellulase, endo-1,4-β-glucanase, β-glucosidase, cellobiohydrolase and endo-1,4-β-xylanase activities were determined in culture supernatants. Tested fungi showed high endo-1,4-β-xylanase activity between 6 and 12 days. Total cellulase showed the highest activity between 12-15 culture days. The test did not show differences among Pycnoporus sanguineus LBM 014, P. sanguineus BAFC 2126, Irpex lacteus BAFC 1171, Irpex sp. LBM 032, Irpex sp. LBM 034 and Lenzites elegans BAFC 2127, showing the highest activity for this group regard the others strains. I. lacteus BAFC 1171 was the strain with major endo-1,4-β-glucanase activity at day 9 (847 U/L). In the case of β-glucosidase and cellobiohydrolase, P. sanguineus BAFC 2126 was the strain with the highest activity between 12-15 culture days (18 U/L), and between 9-15 culture days (39 U/L), respectively. Wood flour proved to be a suitable carbon source to produce hydrolytic enzymes. I. lacteus BAFC 1171 and P. sanguineus BAFC 2126 have potential for cellulase production whereas P. sanguineus LBM 008 is a good endo-1,4-β-xylanase producer.

Indexing terms/Keywords

Cellulase, endo-1,4-β-xylanase, white-rot fungi, wood flour.

Academic Discipline And Sub-Disciplines

Biotechnology.

SUBJECT CLASSIFICATION

Microbiology.

TYPE (METHOD/APPROACH)

Quasi-Experimental.
1. INTRODUCTION

The availability of fossil fuel resources and the increasing energy demand are the main driving forces in the search for alternative energy sources. The large-scale replacement of petroleum fuels by biofuels, such as ethanol from lignocellulosic materials appears to be a powerful approach to meet the growing energy demands [1]. The lignocellulosic material from softwood source contains about 44% cellulose, 21% hemicellulose and 28% lignin [2], while hardwood has 40% cellulose, 17% hemicellulose and 21% lignin [3]. To breakdown polymeric sugars in an environmental friendly process, it is necessary to decrease the cost of cellulases and hemicellulases production, to increase volumetric productivity, to use cheaper substrates and to produce enzymes with high stability [4].

There are many microorganisms able to hydrolyze cellulose and hemicellulose. The description and analysis of new enzymes with distinctive biochemical properties from the rich and vast biodiversity of our region can reveal peculiar and worthy capacities. White-rot fungi have the ability to degrade most of wood components due to their capacity to synthesize hydrolytic extracellular enzymes. Potential applications of lignocellulolytic enzymes in industrial and environmental biotechnology require huge amounts of these enzymes at the lowest cost possible [5]. The enzyme cost is one of the factors determining the economics of a biocatalytic process and it can be reduced with optimum conditions for their production [6-7]. Hence, finding new microbial strains able to produce cellulases and xylanases and increasing the hydrolytic activity of fungal culture extracts is a way to lower the cost of enzymes used in hydrolysis of polymeric sugars to fermentable sugars.

Submerged liquid cultures (SLC) are widely used for enzyme production [8-10]. Synthetic media, such as carboxymethyl cellulose, xylan, cellulose powder and glucose, are usually used for cellulolytic and xylanolytic enzyme production [11-13]. However the cost of raw materials constitutes a large part of the total production cost [14-15]. Hence, there is a need to explore lignocellulosic materials to select an adequate substrate for enzyme synthesis to fully express and correctly evaluate the lignocellulolytic potential of fungi. Utilization of some residues provides an opportunity to produce high yields of lignocellulolytic enzymes in a simple medium [5].

The aim of the present work was to study the effect of wood flour as a carbon source on the cellulolytic and xylanolytic secretion of white-rot fungi native from Misiones.

2. MATERIALS AND METHODS

2.1 Microorganisms

*Coralus versicolor* f. *antarcticus* BAFC 266, *Pycnoporus sanguineus* BAFC 2126, *Lentinus elegans* BAFC 2127, *Trametes villosa* BAFC 2755 and *Irpex lacteus* BAFC 1171 were provided by the Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. *Ganoderma* sp. LB0 001, *Trametes villosa* LBM 002, *Trametes* sp. LBM 003, *Trametes* sp. LBM 004, *Pycnoporus sanguineus* LBM 008, *Trametes* sp. LBM 009, *Trametes villosa* LBM 010, *Lentinus* sp. LBM 011, *Pycnoporus sanguineus* LBM 014, *Schizophyllum* sp. LBM 015, *Trametes elegans* LBM 017, *Trametes villosa* LBM 018, *Trametes* sp. LBM 029, *Trametes* sp. LBM 030, *Trametes* sp. LBM 031, *Irpex* sp. LBM 032, *Trametes* sp. LBM 033, *Irpex* sp. LBM 034 and *Pleurotus sajor-caju* LBM 105 were provided by the Laboratory of Molecular Biotechnology Culture Collection, Institute of Biotechnology of Misiones, Argentina.

2.2 Raw material

Sawdust from *Pinus* sp. and *Eucalyptus* sp. were collected from Valerio Oliva S.A. saw mill. Both materials were air-dried until 10% moisture, ground in a hammer mill and sieved. The *Pinus* sp. wood flour (PWF) and *Eucalyptus* sp. wood flour (EWF) was classified by screening on a 40 mesh sieve. Total solid, extractives and lignin content were determined according to standard protocol NREL/TP-510-42621, NREL/TP-510-42619, NREL/TP-510-42618, respectively [16-18]. Total carbohydrates were measured according to López-Miranda et al. [19].

2.3 Culture conditions

Fungi were first cultured on malt extract agar for 5 days at 29°C. To prepare the pre-inoculum, two 78 mm² agar-plugs from each fungus were cut and transferred to 100 mL Erlenmeyer flasks containing 20 mL of modified Mandels media [12] and 5 g glucose/L, incubated at 29°C in static conditions for 13 days. The pre-inoculum was washed with sterile distilled water and transferred to 250 mL Erlenmeyer flasks containing 60 mL of modified Mandels medium at pH 5 with 5 g PWF/L and 5 g EWF/L as carbon sources. The flasks were placed in a reciprocal shaker at 80 rpm and 29°C for 15 days. At regular intervals of 72 h, samples of culture supernatants were collected and stored at -20°C. Each experiment was performed in duplicate.

2.4 Enzyme assays

The total cellulase (FPA) and endo-1,4-β-glucanase (EGs - EC 3.2.1.6) activities were determined according to International Union of Pure and Applied Chemistry [20]. The β-glucosidase (BGLs – EC 3.2.1.21) activity was determined by assaying the release of p-nitrophenol [21]. The cellobiohydrolase (CBHs – EC 3.2.1.91) activity was determined according to Wood and Bhat [22]. Endo-1,4-β-xylanase (EXs – EC 3.2.1.8) activity was measured in 0.1 mL of culture supernatants in 50 mM acetate buffer (pH 4.8) for 60 min at 50°C using 0.1 mL of 1% (w/v) xylan from beechwood (SIGMA) as substrate. The released reducing sugars were assayed using DNS method [23]. One unit of FPA, EGs, CBHs
and EXs activity was defined as the amount of enzyme required to liberate 1 µmol/mL min of glucose from the particular substrate under the assay conditions. One unit of BGLs was defined as the amount of enzyme required to liberate 1 µmol/mL min of p-nitrophenol under the assay conditions.

2.5 Statistical analysis
Two-way ANOVA with multiple range test was performed using the Statgraphic Centurion program (StatPoint, Inc.).

3. RESULTS
3.1 Compositional analysis
The chemical composition of both PWF and EWF was determined and the results are tabulated in Table 1.

Table 1. Compositional analysis of PWF and EWF.

| Constituent | PWF [%]     | EWF [%]    |
|-------------|-------------|------------|
| Carbohydrates | 65.17±0.74 | 69.9±2.11  |
| Lignin      | 24.45±1.31 | 12.01±0.93 |
| Extractives | 1.82±0.29  | 1.27±0.13  |

3.2 Cellulolytic and xylanolytic activities
The FPA activities are shown in Table 2. These activities were used for ANOVA.

Table 2. Total cellulase activity for each strain grown in wood flour as carbon source.

| Strain                     | Cellulase activity [FPA/L] |
|----------------------------|-----------------------------|
|                            | 0  | 3  | 6  | 9  | 12 | 15 |
| Coriolus versicolor f. antarcticus BAFC 266 | 21±2 | 25±1 | 19±6 | 25±5 | 29±6 | 29±3 |
| Ganoderma sp. LBM 001      | 24±1 | 18±1 | 26±14 | 50±16 | 58±19 | 62±17 |
| Irpex lacteus BAFC 1171    | 25±1 | 45±6 | 71±23 | 65±10 | 47±7  | 73±1 |
| Irpex sp. LBM 032          | 25±3 | 25±2 | 72±33 | 82±25 | 50±15 | 116±21 |
| Irpex sp. LBM 034          | 27±2 | 55±5 | 47±3  | 65±5  | 102±1 | 83±26 |
| Lentinus sp. LBM 011       | 29±4 | 26±1 | 24±1  | 26±3  | 23±4  | 38±18 |
| Lentites elegans BAFC 2127 | 25±1 | 32±16| 40±16 | 84±45 | 111±47| 116±33|
| Pleurotus sajor-caju LBM 105 | 27±4 | 25±1 | 26±13 | 38±14 | 43±16 | 48±18 |
| Pycnoporus sanguineus BAFC 2126 | 22±1 | 29±6 | 78±18 | 71±22 | 73±2  | 40±1 |
| Pycnoporus sanguineus LBM 008 | 29±6 | 30±2 | 21±1  | 38±9  | 39±11 | 47±16 |
| Pycnoporus sanguineus LBM 014 | 26±4 | 73±7 | 43±7  | 42±2  | 57±2  | 56±15 |
| Schizophyllum sp. LBM 015   | 24±2 | 27±3 | 33±11 | 34±11 | 32±10 | 41±13 |
| Trametes elegans LBM 017    | 27±5 | 23±2 | 20±5  | 30±5  | 29±5  | 35±1 |
| Trametes sp. LBM 003        | 21±1 | 28±8 | 20±9  | 33±11 | 36±12 | 39±14 |
| Trametes sp. LBM 004        | 28±1 | 24±1 | 44±17 | 27±1  | 25±1  | 27±2 |
| Trametes sp. LBM 009        | 25±3 | 26±3 | 19±4  | 15±4  | 25±11 | 27±1 |
| Trametes sp.LBM 029         | 24±2 | 27±1 | 23±1  | 21±3  | 26±1  | 25±1 |
| Trametes sp. LBM 030        | 18±1 | 22±1 | 24±2  | 25±1  | 29±1  | 25±3 |
| Trametes sp. LBM 031        | 26±1 | 23±1 | 18±1  | 22±1  | 25±5  | 29±2 |
| Trametes sp. LBM 033        | 25±3 | 21±1 | 26±1  | 23±1  | 28±5  | 25±1 |
| Trametes villosa LBM 002    | 32±4 | 24±1 | 14±1  | 15±1  | 26±1  | 24±1 |
| Trametes villosa LBM 010    | 22±5 | 24±1 | 14±1  | 22±4  | 41±7  | 43±1 |
| Trametes villosa LBM 018    | 22±1 | 23±1 | 20±6  | 24±4  | 24±4  | 24±3 |
| Trametes villosa BAFC 2755  | 27±3 | 25±1 | 14±1  | 13±1  | 22±2  | 21±2 |
The EXs activities are shown in Table 3. Most of the tested fungi showed high EXs activity between 6 and 12 days without significant differences. Twelve of the twenty four strains exhibited the highest EXs activity between 6 and 9 days of incubation. *Trametes* sp. LBM 004 produced 42 U/L on the 6th culture day, *P. sanguineous* LBM 008 41 U/L on the day 9 and *T. elegans* LBM 017 46 U/L on the 12th culture day. These activities were used for ANOVA.

Table 3. Endo-1,4-β-xylanase activity for each strain grown in wood flour as carbon source.

| Strain                      | Endo-1,4-β-xylanase activity [U/L] | Culture days |
|-----------------------------|------------------------------------|--------------|
|                             | 0±0                                | 3±0          |
|                             | 6±6                                | 9±2          |
|                             | 12±4                               | 15±1         |
| *Coriolus versicolor* f. antarcticus BAFC 266 | 0±0                                | 2±0          |
| *Ganoderma* sp. LBM 001     | 3±1                                | 4±1          |
| *Irps lacteus* BAFC 1171    | 10±0                               | 22±1         |
| *Irps* sp. LBM 032          | 0±0                                | 3±0          |
| *Irps* sp. LBM 034          | 3±0                                | 15±1         |
| *Irpex lacteus* BAFC 1171   | 4±1                                | 7±2          |
| *Lenzites elegans* BAFC 2127| 3±1                                | 7±2          |
| *Pleurotus* sajor-caju LBM 105| 3±3                               | 1±1          |
| *Pycnoporus sanguineous* BAFC 2126 | 5±5                               | 32±4         |
| *Pycnoporus sanguineous* LBM 008 | 0±0                               | 34±0         |
| *Pycnoporus sanguineous* LBM 014 | 4±1                               | 5±0          |
| *Schizophyllum* sp. LBM 015  | 3±1                                | 16±4         |
| *Trametes elegans* LBM 017   | 4±1                                | 15±3         |
| *Trametes* sp. LBM 003       | 2±2                                | 15±3         |
| *Trametes* sp. LBM 004       | 3±3                                | 23±1         |
| *Trametes* sp. LBM 009       | 4±4                                | 22±6         |
| *Trametes* sp.LBM 029        | 3±1                                | 4±0          |
| *Trametes* sp. LBM 030       | 4±0                                | 15±3         |
| *Trametes* sp. LBM 031       | 4±1                                | 21±1         |
| *Trametes* sp. LBM 033       | 3±0                                | 0±0          |
| *Trametes villosa* LBM 002   | 3±1                                | 28±2         |
| *Trametes* villosa LBM 010   | 3±3                                | 20±2         |
| *Trametes* villosa LBM 018   | 4±1                                | 10±1         |
| *Trametes* villosa BAFC 2755 | 1±1                                | 9±3          |

Results from ANOVA are given in Table 4. Main effects (Time and Strain) and interaction (Time x Strain) were statistically significant for total cellulases and EXs (p<0.05). Multiple range tests were performed to determine statistical differences between each level of main effects.

Table 4. A) ANOVA for FPA. B) ANOVA for EXs activity.

| Source            | Sum of squares | Gf | Mean square | Ratio-F | P-value |
|-------------------|----------------|----|-------------|---------|---------|
| A Main effects    |                |    |             |         |         |
| A:Time            | 14.7277        | 5  | 2.94555     | 18.24   | 0.0000  |
| B:Strain          | 59.9663        | 23 | 2.60723     | 16.15   | 0.0000  |
| Interaction       |                |    |             |         |         |
| AB                | 44.2764        | 115| 0.385012    | 2.38    | 0.0000  |
| Residues          | 23.2525        | 144| 0.161476    |         |         |
| Total (corrected) | 142.223        | 287|             |         |         |
For FPA, the multiple range tests run for all fungi did not show significant differences between 12 and 15 days, showing the highest activity in this period. Concerning the strain effect, the test did not show significant differences among *P. sanguineus* LBM 014, *P. sanguineus* BAFC 2126, *I. lacteus* BAFC 1171, *Irpex* sp. LBM 032, *Irpex* sp. LBM 034 and *L. elegans* BAFC 2127, showing the highest activity for this group regard the others strains. Regarding cellulose activity results, *Irpex* sp. LBM 032 and *L. elegans* BAFC 2127 exhibited the highest values of activity on the 15th culture day (116 U/L), *P. sanguineus* LBM 014 secreted 73 U/L on the 3rd culture day, *P. sanguineus* BAFC 2126 78 U/L on the 6th culture day, and *Irpex* sp. LBM 034 102 U/L on the 12th culture day.

The strains exhibiting the highest FPA were tested for EGs, BGLs and CBHs (Figure 1). ANOVA test was performed for EGs, BGLs and CBHs. The analysis showed significant differences at 95% for each factor (Strain and Time) and interaction (Strain x Time). The multiple range tests showed that *I. lacteus* BAFC 1171 was the strain with highest EGs activity at 9th day (847 U/L), with statistical differences among the other strains. Regarding BGLs and CBHs, *P. sanguineus* BAFC 2126 was the strain with the highest significant activity between 12-15 culture days (18 U/L), and between 9-15 culture days (39 U/L), respectively.

![Graphs showing enzyme activity](image)

**Figure 1.** Activity percentage of EGs (■), BGLs (□) y CBHs (●) of each strain a function of culture day. 100% activity is the maximum activity value for each enzyme.
4. DISCUSSION

The wood material has about 40-50% cellulose, 25-35% hemicellulose and 20-35% lignin [24]. These results agree with Table 1 and many authors [3, 25-26]. Differences in some of the components may be seasonal or due to species differences.

For an industrial application it is important to obtain high enzyme activity in the shortest possible period of time. P. sanguineus LBM 008 showed the highest activity over a longer period of time (3\textsuperscript{rd} to 15\textsuperscript{th} culture day). This result was lower compared with that reported by Falkoski et al. [27] for P. sanguineus grown on sugarcane bagasse as carbon source. These results agree with Diorio et al. [28] using different lignocellulosic substrates, where EGs and EXs showed lower activity using wood wastes.

Analyzing the bar graphs in Figure 1, it can be seen that BGLs activity curves for P. sanguineus LBM 014 and Irpex sp. LBM 034 displayed a negative skewed behavior both with the maximum at 12\textsuperscript{th} culture day (34 and 8 U/L, respectively), whereas EGs showed a positive skewed behavior, with a maximum for P. sanguineus LBM 014 on the 3\textsuperscript{rd} culture day (562 U/L) and Irpex LBM 034 on the 9\textsuperscript{th} culture day (541 U/L). It must be pointed out that the enzyme activity for Irpex sp. LBM 034 growing on wood flour as substrate was higher than using wood chips as substrate (1 U/L) for 4 to 6 days [29]. CBHs from P. sanguineus LBM 014 reached the maximum value on the 15\textsuperscript{th} culture day (16 U/L). Regarding this strain, each enzyme displayed maximum activity at different times. CBHs from Irpex sp. LBM 034 exhibited two peaks, 33 and 26 U/L on the day 3 and 15, respectively.

Irpex sp. BAFC 1171 and Irpex sp. LBM 032 showed two peaks of EGs, for Irpex sp. BAFC 1171 on the 3\textsuperscript{rd} and 9\textsuperscript{th} days (667 and 847 U/L) and for Irpex sp. LBM 032 on the 6\textsuperscript{th} and 15\textsuperscript{th} days (821 and 762 U/L). CBHs had similar behavior as EGs for Irpex sp. LBM 032 whereas EGs for Irpex sp. BAFC 1171 showed a bell-like behavior with the maximum value at 9\textsuperscript{th} day (42 U/L). Irpex sp. BAFC 1171 exhibited a positive skewed behavior for BGLs with a maximum at 9\textsuperscript{th} day, however Irpex sp. LBM 032 had an incremental pattern, rising slowly until the last day (10 U/L), when the three enzymes had similar and maximum activity.

For P. sanguineus BAFC 2126, EGs secretion pattern had a negative skewed behavior with a maximum at the 12\textsuperscript{th} day (291 U/L) and BGLs showed two peaks, at days 6 and 15 (15 and 17 U/L, respectively). This strain had a normal shape behavior with a maximum for CBHs (129 U/L- day 9). Yoon et al. [30] found similar results for FPA (80 U/L on the 6\textsuperscript{th} culture day), and EGs activity on the 9\textsuperscript{th} culture day (203 U/L for P. sanguineus grown in medium supplied with sugarcane bagasse, but lower BGLs activity (17 U/L) on the 15\textsuperscript{th} culture day. P. sanguineus BAFC 2126 showed higher CBHs activity (129 U/L) compared with L. elegans (76 U/L) and Irpex sp. LBM 032 presented in this and another work [27].

EGs from L. elegans had normal bell shape pattern throughout the time with a peak at 9\textsuperscript{th} culture day (528 U/L), whereas CBHs and BGLs increased gradually until rising the maximum at day 15 (10 U/L for BGLs).

Dynamic knowledge of enzyme is important for efficient biotechnological applications [6]. According to the present results, PWF and EWF proved to be suitable carbon source to produce hydrolytic enzymes. I. lacteus BAFC 1171 and P. sanguineus BAFC 2126 have potential for cellulase production when growing on wood flour as a carbon source, since both fungi not only present filter paper activity, but also produce EGs, BGLs and CBHs. P. sanguineus LBM 008 secreted high EXs activity over an extended period of time. This function is remarkable because some biotechnological application require mixtures of cellulases and hemicellulases for maximum benefits [31]. To accomplish an efficient saccharification process, high activities of EGs, BGLs and CBHs are necessary to degrade cellulose and high EXs activity to degrade hemicellulose, thus increasing yields to obtain bioethanol by fermenting 5 and 6 carbon sugars. Nevertheless, it is still necessary to explore other variables to improve these levels of cellulase and xylanase using wood flour as substrate.

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**Author' biography with Photo**

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