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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.

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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

Coriolus versicolor f. *antarcticus* BAFC 266, *Pycnoporus sanguineus* BAFC 2126, *Lenzites 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. LBM 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, *Schyzophyllum* 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 29ºC. To prepare the pre-inoculum, two 78 mm^2 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 29ºC 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 29ºC 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 50ºC 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]					
	Culture days					
	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
Lenzites 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
Schyzophyllum 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±14	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	3	6	9	12	15
Coriolus versicolor f. *antarcticus* BAFC 266	0±0	2±0	6±6	27±9	5±4	2±0
Ganoderma sp. LBM 001	3±1	4±1	8±3	6±3	3±1	2±1
Irpex lacteus BAFC 1171	10±0	22±1	21±1	21±1	27±3	32±5
Irpex sp. LBM 032	0±0	3±0	5±0	7±1	5±0	3±2
Irpex sp. LBM 034	3±0	15±1	23±1	14±1	22±1	6±1
Lentinus sp. LBM 011	4±1	8±2	17±2	20±3	33±4	11±1
Lenzites elegans BAFC 2127	3±1	7±2	10±6	28±6	21±1	13±1
Pleurotus sajor-caju LBM 105	3±3	1±1	7±4	12±1	13±0	12±1
Pycnoporus sanguineus BAFC 2126	5±5	32±4	35±2	14±1	15±4	9±2
Pycnoporus sanguineus LBM 008	0±0	34±0	37±0	41±1	33±7	37±1
Pycnoporus sanguineus LBM 014	4±1	5±0	5±2	12±2	6±1	8±4
Schyzophyllum sp. LBM 015	3±1	16±4	6±4	8±1	6±1	3±1
Trametes elegans LBM 017	4±1	15±3	25±5	28±6	46±4	10±2
Trametes sp. LBM 003	2±2	15±3	26±3	13±2	19±9	13±2
Trametes sp. LBM 004	3±3	23±1	42±8	24±12	31±8	23±3
Trametes sp. LBM 009	4±4	22±6	16±2	14±2	18±1	24±1
Trametes sp.LBM 029	3±1	4±0	13±5	21±6	7±2	5±1
Trametes sp. LBM 030	4±0	15±3	5±2	4±0	6±6	0±0
Trametes sp. LBM 031	4±1	21±1	13±1	8±1	5±2	4±1
Trametes sp. LBM 033	3±0	0±0	4±0	19±0	20±1	21±4
Trametes villosa LBM 002	3±1	28±2	37±2	34±1	43±5	6±6
Trametes villosa LBM 010	3±3	20±2	15±3	15±1	14±2	2±1
Trametes villosa LBM 018	4±1	10±1	19±6	28±4	31±4	11±3
Trametes villosa BAFC 2755	1±1	9±3	22±9	16±1	17±2	12±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			

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B	Main effects					
	A:Time	8355.98	5	1671.2	84.57	0.0000
	B:Strain	13993.7	23	608.42	30.79	0.0000
	Interaction					
	AB	12422.4	115	108.021	5.47	0.0000
	Residues	2845.5	144	19.7604		
	Total (corrected)	37617.6	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.

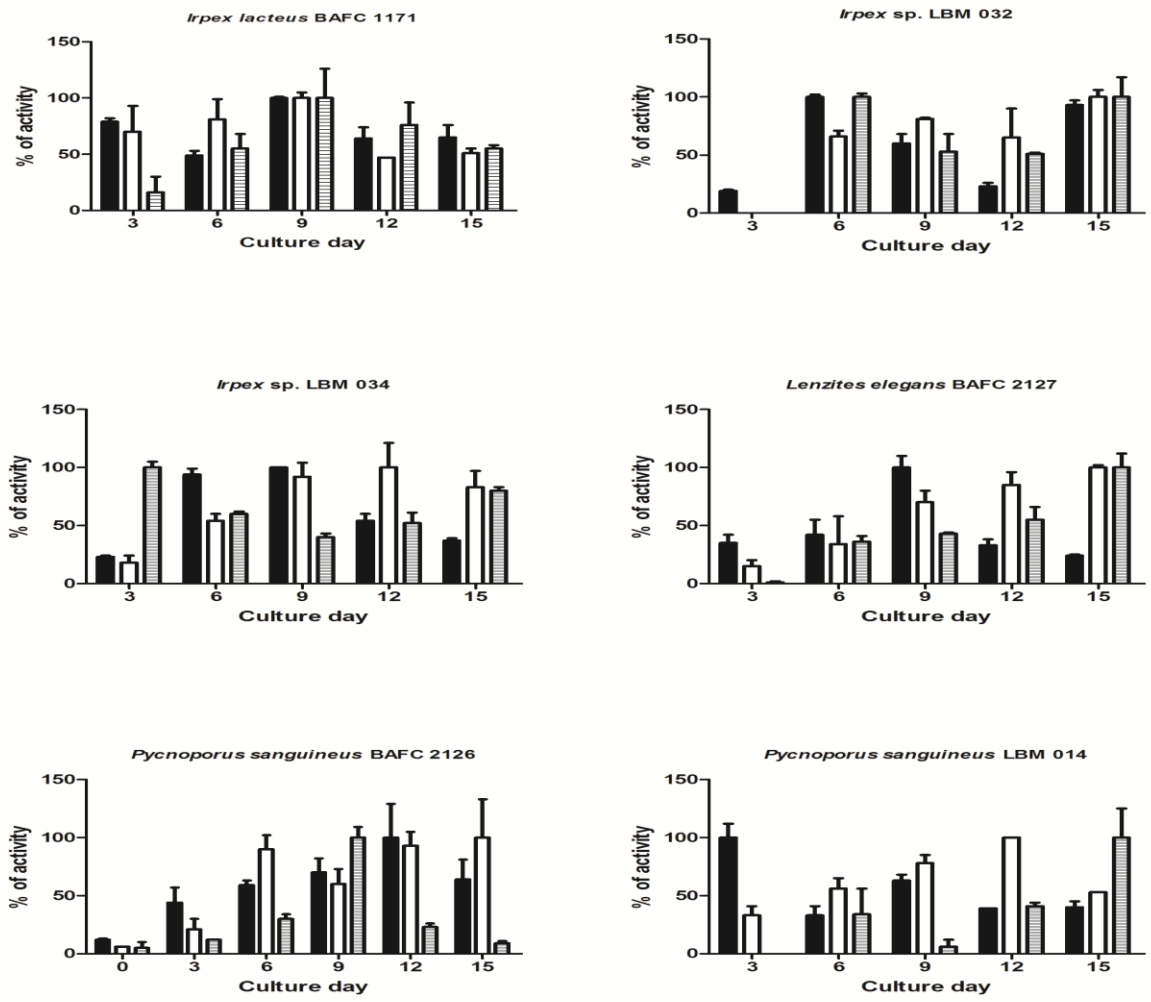

Figure 1. Activity percentage of EGs (■), BGLs (□) y CBHs (▤) of each strain a function of culture day.100% activity is the maximum activity value for each enzyme.

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 (3th to 15th 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 12th 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 3rd culture day (562 U/L) and *Irpex* LBM 034 on the 9th 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 15th 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 3rd and 9th days (667 and 847 U/L) and for *Irpex* sp. LBM 032 on the 6th and 15th days (821 and 762 U/L). CBHs had similar behavior as EGs for *Irpex* sp. LBM 032 whereas *Irpex* sp. BAFC 1171 showed a bell-like behavior with the maximum value at 9th day (42 U/L). *Irpex* sp. BAFC 1171 exhibited a positive skewed behavior for BGLs with a maximum at 9th 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 12th 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 6th culture day), and EGs activity on the 9th culture day (203 U/L) for *P. sanguineous* grown in medium supplied with sugarcane bagasse, but lower BGLs activity (17 U/L) on the 15th culture day. *P. sanguineous* 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 9th 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 bioetanol 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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