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LIGNIN DEGRADATION, LIGNINOLYTIC ENZYMES ACTIVITIES AND EXOPOLYSACCHARIDE PRODUCTION BY *GRIFOLA FRONDOSA* STRAINS CULTIVATED ON OAK SAWDUST

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ABSTRACT

Fourteen strains of *Grifola frondosa* (Dicks.) S. F. Gray, originating from different regions (Asia, Europe and North America) were tested for lignin degradation, ligninolytic enzyme activities, protein accumulation and exopolysaccharide production during 55 days of cultivation on oak sawdust. Lignin degradation varied from 2.6 to7.1 % of dry weight of the oak sawdust substrate among tested strains. The loss of dry matter in all screened fungi varied between 11.7 and 33.0%, and the amount of crude protein in the dry substrate varied between 0.94 to 2.55%. The strain, MBFBL 596, had the highest laccase activity (703.3 U/l), and the maximum peroxidase activity of 22.6 U/l was shown by the strain MBFBL 684. Several tested strains (MBFBL 21, 638 and 662) appeared to be good producers of exopolysaccharides (3.5, 3.5 and 3.2 mg/ml respectively).

Key words: *Grifola frondosa;* exopolysaccharide; laccase; lignin degradation; peroxidase

INTRODUCTION

Grifola frondosa is a white-rot basidiomycete that produces a highly nutritious fruit body used as food in different parts of the world. It has also been reported to contain bioactive metabolites, which exhibit various medicinal properties such as antitumor, antiviral, antioxidant, antidiabetic, immunomodulation (11, 14, 23, 12, 13). Different plant waste material has been used for the cultivation of *G. frondosa* (15). In commercial cultivation, sterilized hardwood sawdust of alder and poplar is often used (16). Chung (7) used sawdust and cotton seed composts, while Xing *et al.* (22) reported cultivation of this fungus on a substrate consisting of beech sawdust, wheat bran and corn meal.

G. frondosa secretes ligninolytic enzymes to degrade the lignocellulose substrate from which it obtains needed nutrients for its growth and development. Extracellular laccase activity was detectable in liquid cultures of *G. frondosa* during the early/middle stages of primary growth (22). Total peroxidase and manganese independent peroxidase were found in brewery waste substrates used in solid-state fermentation involving *G. frondosa* (18). Polysaccharides are secreted during *G. frondosa* cultivation in both liquid and solid substrates used for its cultivation (3, 24).

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G. frondosa is of huge economic importance as a result of its nutritional and medicinal properties. Favorable conditions for growing it exist in the southeastern United States, where oak sawdust is abundant. However, poor yields persist, despite huge supplementation. This situation calls for basic research into substrate degradation and utilization, as well as into how the strains originating from different regions may affect enzyme production and substrate utilization. Therefore, a total of 14 isolates of *G. frondosa,* originating from North America, Europe and Asia were studied for ligninolytic enzymatic activities, lignin degradation rates, and exopolysaccharide production during cultivation on un-supplemented oak sawdust.

MATERIALS AND METHODS

Fungal strains and cultivation

Fourteen strains of *G. frondosa* from the Mushroom Biology and Fungal Biotechnology Laboratory (MBFBL) culture collection at North Carolina A&T State University were used in this study (Table 1). Stock cultures of selected isolates were maintained on potato-dextrose agar at 4°C. The inocula were grown for seven days in 250 mL flask containing 100 mL basal medium (g/l): glucose 10; KH_2PO_4 0.8; NH_4NO_3 2; Na_2HPO_4 0.4; $MgSO_4 \cdot 7H_2O$ 0.5 and yeast extract 2 (pH 6.0). Mycelia were homogenized in a laboratory Warring blender. Solid-state fermentation of oak sawdust with test fungi was conducted in 250 ml Erlenmeyer flasks, containing 5 g milled sawdust, mixed with 20 ml water. The substrate was inoculated with 2 mL homogenate (43-52 mg of mycelia dry weight) and incubated at 23-24°C in the dark. After 15, 25, 35, 45 and 55 days of cultivation, crude extract from the biomass were extracted with 40 ml of sodium acetate buffer (100 mM, pH 5.0) at 4°C, for 2 hours. The extract was filtered through Whatman paper and was used for determining enzyme activities and exopolysaccharide content. The biomass was dried at 60°C until reaching a constant weight. Loss of organic matter was calculated as the percent difference in dry weight between the test substrate and the control (uninoculated substrate). The three replicate flasks per strain that contained samples of the dried substrate were then combined into one sample, milled, and analyzed to determine crude protein and lignin contents.

Enzyme activity assays

Laccase activity was determined by the rate of oxidation of ABTS (2,2'–azinobis(3– ethylbenzothiazoline–6–sulfonic acid)) as a substrate at 420 nm. The reaction mixture (1 ml) contained 50 mM acetate buffer (pH 3.8), 1 mM ABTS, and 100 µl of appropriately diluted culture filtrate (9). Peroxidase activity was assayed by the oxidation of Phenol Red (8). The 1 ml reaction volume contained 450 µl sodium lactate–succinate buffer (pH 4.5), 50 µl 2 mM H_2O_2, 100 µl 3 mM Phenol Red and 400 µl diluted culture filtrate. The reactions were terminated by the addition of 100 µl 2 M NaOH and absorbance was recorded at 610 nm. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µmol substrate per minute.

Lignin and crude protein assay

Lignin components were analyzed by the Van Soest et al. (18) method for dietary fiber. The crude protein in the colonized sawdust substrate was determined by official methods of analysis (1). The conversion factor of total nitrogen to protein in the mushroom samples was 4.38 (4). The above mentioned analyses were conducted at the certified Rumen Fermentation Profiling Lab, West Virginia University, WV.

Exopolysaccharides assay

To measure levels of exopolysaccharides, 1 volume of culture extract (separated from the biomass) was mixed with 4 volumes of absolute ethanol, stirred and left for 24 hours at 4°C. Precipitated polysaccharides were collected by centrifugation at 6000 g for 20 min, dried at 60°C and weighed.

Statistical analysis

SPSS software version 11 (Lead Technologies Inc. 2001) was used to carry out statistical analysis. A one-way analysis of variance was carried out at $\alpha = 0.05$, and Duncan's multiple range test was used to compare the enzyme activities, polysaccharide production and biomass utilization among the fourteen tested strains.

RESULTS AND DISCUSSION

Results of the substrate utilization as measured by dry matter (biomass) loss revealed that most of the *G. frondosa* strains have weak abilities to utilize oak sawdust (Fig. 1). For most of the strains, dry matter decreased steadily between days 25 through day 55 of cultivation. However, the Asian strains (MBFBL 660, 684 and 662) and European strains (MBFBL 637, 638 and 649) showed a significant decrease in substrate weight much earlier (at day 15) during the cultivation period. MBFBL 662, 21 and 34 were found to be the best performing strains in terms of substrate utilization, and were associated with 1.3 - 1.5 times dry matter loss, compared to the control (Table 1). The Asian strains (MBFBL 660 and 684) and Northeast USA strains (MBFBL 598 and 605) produced the lowest rate of substrate utilization. Statistical analysis of dry matter loss results revealed significant differences ($P < 0.05$) among strains tested (not shown in Fig.1). Our findings are consistent with the report of Chen et al. (6), who observed differences among *G. frondosa* strains in biological and physiological characteristics, with different strains showing different mycelia colonization rates on agar plates and in solid-state fermentation in flasks.

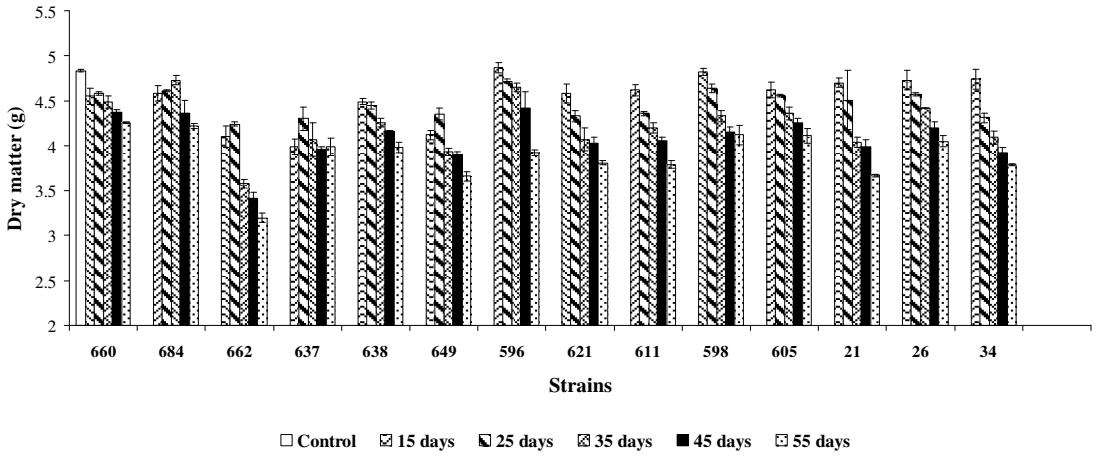

Figure 1. Changes in substrate dry matter during cultivation of *G. frondosa* strains

Table 1. Dry matter loss and lignin content in oak wood sawdust subtrate after cultivation with *Grifola frondosa*

Region of origin	Strain ID	Loss of dry matter	Loss of lignin
Asia	MBFBL 660	10.8	2.6
	MBFBL 684	11.7	2.8
	MBFBL 662	33.0	7.1
Europe	MBFBL 637	17.5	3.2
	MBFBL 638	16.8	3.7
	MBFBL 649	23.4	4.4
Northwest USA	MBFBL 596	18.0	3.8
	MBFBL 621	20.4	3.8
	MBFBL 611	20.6	4.1
Northeast USA	MBFBL 598	13.7	5.3
	MBFBL 605	13.9	5.3
North Carolina (USA)	MBFBL 21	23.2	4.8
	MBFBL 26	15.3	4.4
	MBFBL 34	20.6	6.0

Results are presented as % of substrate dry weight

The lignin content in the uninoculated substrate was 16.4%. At the end of 55 days, the lignin loss ranged from 2.6 to 7.1% (Table 1). The lignin degradation during the first 25 days was low compared to the values obtained after 35 days of cultivation (Fig. 2). The Asian strain, MBFBL 662, showed the highest lignin degradation rate (7.1%), followed by the North East USA strains (MBFBL 598, 605). Arora and Sandhu (2) reported an angiospermic wood sawdust total weight loss of 6% accompanied by a 14% lignin loss during 60 days of incubation with *Pleurotus ostreatus.*

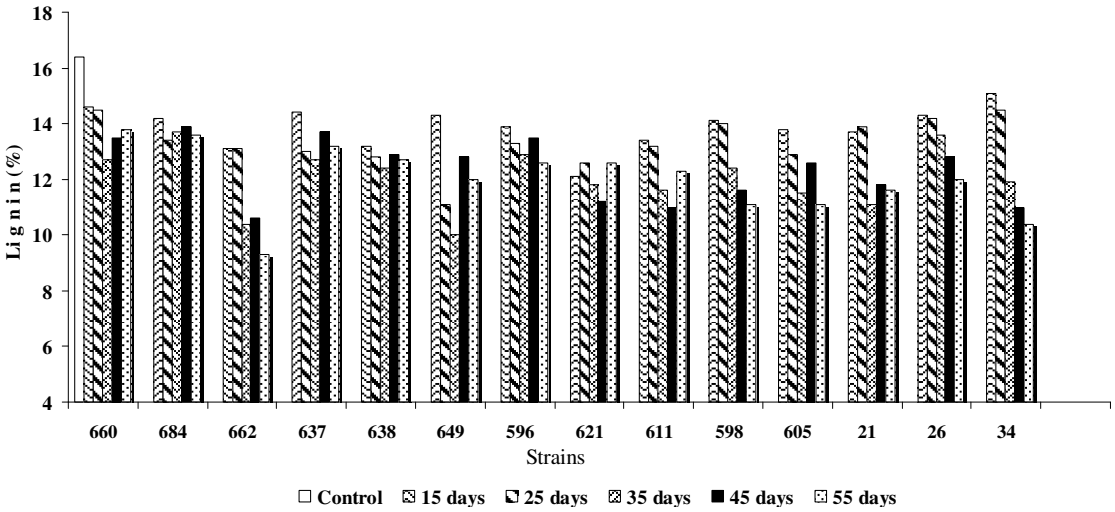

Figure 2. Changes in residual lignin level in dry matter during cultivation of *G. frondosa* strains

Total protein content varied between strains and length of cultivation (Table 2). After 55 days, crude protein content in the substrates increased from 1.48% in the uninoculated substrate to values ranging from 1.85 to 2.55%. Asian strains showed the highest protein accumulation, ranging from 1.98 - 2.55%. Northwest USA strains also showed comparatively high protein content (2.21 - 2.37%). Tabata *et al*. (19) reported a 1.66% substrate protein accumulation during fructification of *G. frondosa* in rice bran supplemented sawdust.

Table 2. Crude protein accumulation in dry matter during cultivation of *G. frondosa* species

Strains	10 days	25 days	35 days	45 days	55 days
MBFBL 660	1.58	2.08	1.97	1.97	2.11
MBFBL 684	1.27	0.94	1.12	1.50	1.98
MBFBL 662	1.72	1.89	2.30	2.42	2.55
MBFBL 637	1.66	1.70	1.75	1.81	1.94
MBFBL 638	1.63	2.03	1.96	1.94	2.00
MBFBL 649	1.96	1.98	2.19	2.18	2.48
MBFBL 596	1.35	1.37	1.75	2.01	2.21
MBFBL 621	1.70	1.77	2.15	2.06	2.25
MBFBL 611	1.74	1.80	2.21	2.03	2.37
MBFBL 598	1.51	1.92	1.69	1.99	1.91
MBFBL 605	1.49	1.61	2.05	2.09	1.85
MBFBL 21	1.72	1.69	2.17	1.97	2.08
MBFBL 26	1.35	1.74	2.11	2.21	1.92
MBFBL 34	1.82	2.25	2.09	2.20	2.40

Results are presented as % of substrate dry weight
Uninoculated substrate consists of 1.48 % crude protein

The data on ligninolytic enzyme activities (Table 3 and 4) shows that all strains produced the highest amount of laccase enzyme on day 15. The strong correlation (0.817), between substrate utilization and laccase activity was obtained only on strain MBFBL 21. The highest laccase activity (703.3 U/l) was recorded for the *G. frondosa* strain MBFBL 596, and the lowest activities were in MBFBL 598 and 605 strains, 13.2 and 10.7 U/l, respectively. Xing et al. (22) showed that during liquid cultivation of *G. frondosa*, laccase activity reached a maximum value of 70 U/l after 52 days. Vikineswary et al. (21) observed degradation of rubberwood sawdust by *Pycnoporus sanguineus*, where maximal laccase productivity reached 5.7 U/g on day 11.

Table 3. Laccase activities among *G. frondosa* strains during 55 days of cultivation

Strains	15 days	25 days	35 days	45 days	55 days
MBFBL 660	50.06 ±1.98d	29.81 ±1.27cde	5.92 ±0.46abc	5.46 ±0.79bcd	3.64 ±0.91cd
MBFBL 684	38.46 ±1.78c	34.36 ±2.17de	36.41 ±2.41d	15.02 ±1.58e	10.92 ±0.79e
MBFBL 662	44.60 ±1.38cd	54.15 ±2.41f	6.37 ±0.46abc	17.29 ±0.91e	5.01 ±0.45d
MBFBL 637	6.37 ±0.60a	30.94 ±1.20cde	3.19 ±0.91ab	2.28 ±0.91ab	1.82 ±0.91abc
MBFBL 638	14.56 ±0.99ab	19.79 ±1.04abc	6.37 ±0.46abc	8.19 ±0.79d	3.64 ±0.46cd
MBFBL 649	10.47 ±0.60ab	38.23 ±1.81e	6.83 ±0.79abc	3.64 ±0.46abc	2.73 ±0.79bc
MBFBL 596	703.30 ±12.43e	688.52 ±12.32g	145.17 ±4.04e	52.33 ±3.28f	0.91 ±0.46ab
MBFBL 621	11.60 ±0.79ab	31.85 ±2.77de	4.55 ±1.98abc	4.55 ±0.45 abd	2.73 ±0.79bc
MBFBL 611	21.39 ±1.21b	8.19 ±0.39a	2.28 ±0.45a	1.82 ±0.45ab	0
MBFBL 598	11.38 ±0.82ab	13.20 ±1.21ab	8.42 ±0.60c	6.83 ±1.37cd	0.46 ±0.46a
MBFBL 605	5.01 ±0.23a	10.69 ±0.60a	1.82 ±0.45a	0.91 ±0.46a	0
MBFBL 21	16.38 ±0.79ab	10.24 ±0.79a	9.10 ±0.46c	7.28 ±0.45cd	2.73 ±0.79bc
MBFBL 26	12.52 ±1.38ab	23.89 ±1.18bcd	7.74 ±1.98bc	2.28 ±0.45ab	0
MBFBL 34	18.20 ±0.60b	9.56 ±0.79a	3.19 ±0.46ab	7.74 ±0.45d	0

Results of laccase activities are presented in U/l
Each value is expressed as mean ± SD (n = 3)
Different letters in each column indicate significant differences at $P < 0.05$

Table 4. Peroxidase activities in *G. frondosa* strains during 55 days of cultivation

Strains	15 days	25 days	35 days	45 days	55 days
MBFBL 660	5.45±0.55d	6.67±0.28g	3.03±0.21a	4.00±0.73a	6.55±0.36bc
MBFBL 684	7.00±0.72e	8.67±0.76h	22.55±0.55h	9.82±0.63e	8.36±0.36de
MBFBL 662	5.39±0.56d	6.61±0.46g	7.45±0.18f	15.18±0.91e	10.06±0.42f
MBFBL 637	3.76±0.37bc	5.58±0.38de	5.09±0.48cde	8.97±0.56de	7.52±0.76cd
MBFBL 638	3.27±0.33b	4.18±0.18ab	3.33±0.10a	4.97±0.21a	6.79±1.87bc
MBFBL 649	4.21±0.41c	5.21±0.10fg	5.88±0.28de	7.03±0.56e	13.33±0.56g
MBFBL 596	4.15±0.19c	6.36±0.18cde	3.88±0.46ab	9.70±0.56bc	5.70±0.56b
MBFBL 621	2.36±0.09a	4.79±0.28bc	5.45±0.48cde	6.79±0.56b	7.15±0.56cd
MBFBL 611	3.64±0.24bc	5.27±0.09i	3.58±0.46a	7.39±1.11bc	4.36±0.63a
MBFBL 598	2.39±0.14a	3.76±0.38a	6.00±0.48e	4.12±0.56a	6.42±0.56bc
MBFBL 605	2.61±0.19a	6.24±0.28fg	5.03±0.28cd	7.39±0.21bc	9.33±0.42ef
MBFBL 21	4.85±0.46d	4.97±0.21cd	5.82±0.58g	10.06±0.76e	7.03±0.92bcd
MBFBL 26	3.36±0.18b	5.88±0.46ef	4.67±0.21bc	6.42±0.56b	9.45±0.73ef
MBFBL 34	2.33±0.10a	5.45±0.48cde	5.45±0.48cde	8.00±0.96cd	5.70±0.42b

Results of peroxidase activities are presented in U/ l
Each value is expressed as mean ± SD (n = 3)
Different letters in each column indicate significant differences at $P < 0.05$

Among the strains, MBFBL 684 appeared to be the best producer of peroxidase (22.6 U/l) at 35 days. Mn-Peroxisade activity, though measured, showed insignificant activity in strains tested (data not shown). The correlation between substrate utilization and peroxidase activities was poor, and the relationship between substrate utilization and enzyme activities is not linear. Kadimaliev *et al*. (10) observed considerably lower laccase activity during 14 days of growing *Lentinus tigrinus* on pine sawdust (2.3 U/g) compared to birch sawdust (20 U/g), while peroxidase activity measured by *o*-dianisidine ranged from 0.6 and 0.65 U/g on birch and pine sawdust, respectively.

G. frondosa MBFBL 21 and 662 produced the highest yields exopolysaccharides (3.5 and 3.2 mg/ml) on day 45 of cultivation (Table 5). A positive correlation between dry matter loss and polysaccharide secretion was obtained only in MBFBL 26. Zhou *et al*. (24) showed a 3.81 mg/ml exopolysaccharide accumulation by *G. frondosa* mycelium in a sucrose-brain medium. Bae *et al*. (3), obtained 7.2 mg/ml exopolysaccharide on day 4 during cultivation of *G. frondosa* in a fermenter.

Table 5. Polysaccharides secretion in *G. frondosa strains* during cultivation on oak wood sawdust

Strains	15 days	25 days	35 days	45 days	55 days
MBFBL 660	1.00±0.10c	1.23±0.06e	0.87±0.06bcd	1.77±0.15ab	1.13±0.06ab
MBFBL 684	0.93±0.06c	1.27±0.06e	1.00±0.10cde	0.87±0.06ab	1.70±0.10e
MBFBL 662	1.30±0.00de	1.10±0.10de	1.47±0.06g	3.23±0.15	3.00±0.60f
MBFBL 637	1.23±0.06de	0.53±0.23a	0.93±0.06bcd	1.77±0.15ab	0.83±0.06a
MBFBL 638	0.97±0.06c	0.80±0.10bc	1.23±0.15f	2.17±0.06ab	1.47±0.12bcde
MBFBL 649	1.50±0.10e	0.93±0.15bcd	0.40±0.10a	2.37±0.06ab	1.20±0.26bc
MBFBL 596	0.23±0.06a	2.83±0.12f	1.43±0.15g	2.67±0.15ab	1.67±0.06de
MBFBL 621	2.23±0.64f	0.53±0.15a	1.17±0.06ef	1.67±0.15ab	1.30±0.10bcd
MBFBL 611	0.70±0.10bc	2.83±0.15f	1.73±0.15h	2.90±0.10ab	1.33±0.06bcde
MBFBL 598	2.67±0.12g	0.83±0.06bc	1.03±0.06de	1.53±0.06ab	1.33±0.06bcde
MBFBL 605	1.07±0.06cd	1.00±0.10cd	0.83±0.12bc	2.93±0.15b	1.50±0.10bcde
MBFBL 21	0.43±0.06ab	3.10±0.10g	1.90±0.10h	3.50±3.90b	1.57±0.15cde
MBFBL 26	0.97±0.06c	0.77±0.06b	1.13±0.12ef	1.97±0.21ab	1.43±0.06bcde
MBFBL 34	1.53±0.25e	0.90±0.10bcd	0.80±0.10b	1.50±0.10ab	1.67±0.15de

Results of polisaccharides presented in mg/ml
Each value is expressed as mean ± SD (n = 3)
Different letters in each column indicate significant differences at $P < 0.05$

It appears that *G. frondosa* is not as hardy a lignin degrader as *Pleurotus spp, Lentinula edodes, Phanerochaete chrysosporium and Ganoderma colossum*, which have been reported to have lignin degradation of 14% on angiospermic wood sawdust (2), 39-60% on *Eucalyptus* sawdust (5), 12% on red oak and 16.7% on white fir (15), respectively. It fruits off living roots of trees as a weak parasite, which does not kill its host quickly (http://botit.botany.wisc.edu/toms_fungi/nov2006.html). From a particular tree in Greensboro NC where *G. frondosa* has been collected continuously for 5 years, each time the fruit body is picked up, latex was seen oozing from the point of collection of the *G. frondosa* fruit body from the oak tree root. It is possible that the photosynthetic system of their host (live oak trees) is exploited, in addition to minimal substrate degradation, to acquire the nutrients that the fungus needs to make fruit bodies in nature; that might explain the relative difficulty in cultivation of this fungus for fruit body production.

The results showed that ligninolytic enzymes production, sawdust substrate degradation and exopolysaccharide production appears to be strain specific and not affected by the origin of strains tested. In general, *G. frondosa* seems to be a weak degrader of sawdust, although it is found associated with oak trees in the wild. Our results have helped us to detect strains that seem to be the best oak substrate degraders, which we are now applying in mass production studies, polysaccharide secretion and breeding to obtain improved strains needed for other biotechnological applications.

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