IV. AGROECOLOGYCAL SYSTEMS

THE COMPARISON OF WHITE-ROT BASIDIOMYCETES LIGNOCELLULOLYTIC POTENTIAL IN WHEAT STRAW SOLID-STATE FERMENTATION

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Abstract: In the present study, a screening of 12 white-rot basidiomycetes has been carried out to evaluate their potential to deconstruct cellulose and lignin in solid-state fermentation (SSF) of wheat straw. The degradation patterns of cellulose and lignin in wheat straw by tested fungi differed significantly. Among them, *Cerrena unicolor*, *Trametes hirsutus* 68, and *Tsalka* 5 were able to remove lignin slightly preferentially and faster than cellulose during 12 days of fermentation and are appropriate candidates for biological pretreatment of wheat straw. Evaluation of the tested fungi cellulases and ligninases activities revealed that the cellulose and lignin degradation not always directly correlates with the production of enzymes.

Key words: white-rot basidiomycetes, wheat straw, cellulose and lignin degradation, cellulase, laccase, manganese peroxidase

1. INTRODUCTION

The white-rot basidiomycetes (WRB) are the most active destructors of lignocellulosic materials in nature [1]. These fungi are capable to degrade wood and other lignin-containing materials without participation of other microbes owing to production of extracellular enzymatic complexes deconstructing the plant polysaccharides and lignin. Their major hydrolytic enzymes are endo-1,4- β -D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (EC 3.2.1.91), and xylanase (EC 3.2.1.8). These fungi also secrete one or more of the three extracellular enzymes that are essential for lignin degradation: lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2). Lignocellulose-degrading enzymes of basidiomycetes are of fundamental importance for efficient bioconversion of plant residues in nature and they are promising for a great variety of biotechnology and environmental applications, including pulp and paper industry, bioremediation, feed and bioenergy production, which require huge amounts of these enzymes at low cost [2-4].

Lignocellulosic materials represent the most abundant renewable energy resource available on earth. Among them, agricultural wastes, especially wheat straw, are the most extended and the cheapest. However, these materials need preliminary pre-treatment to disruption the lignocellulose structure to improve cellulose and hemicellulose accessibility. Nowadays, due to the ability to degrade lignin extensively, WRB have received considerable attention for bio-pretreatment of plant raw materials [5]. Biological pretreatment presents several advantages as being cheaper, safer, less energy-consuming and more environmentally friendly as compared with chemical or physical methods. Several WRB, such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, and *Coriolus versicolor* have been used for biological pre-treatment of various plant residues. In general, only low saccharification degrees (35 - 40%) were obtained when compared to those achieved after physical and chemical pre-treatments [5, 6]. Nevertheless, saccharification degree of 66.4% was obtained after pre-treatment of corn stover with the *Irpex lacteus*, which was about 152.5% higher than the control [7[. Moreover, *Echinodontium taxodii* was used in biological pretreatment of two native woods and significantly increased enzymatic hydrolysis ratios (4.7-fold for hardwood and 6.3-fold for softwood) [8].

Rational search for fungi with selective lignin degradation combined with comprehensive investigation of the physiological mechanisms regulating lignocellulose deconstruction will lead to development of commercially significant technologies for their application in biorefinary. In this study, the lignocellulolytic potential of novel, taxonomically and physiologically distinct basidiomycetes was evaluated in the solid-state fermentation of wheat straw and the role of the lignocellulolytic enzymes in the process is discussed. These enzymes expression, wheat straw cellulose and lignin degradation by selected fungus in response to the nutrient medium supplementation with various concentrations of glycerol was also assessed.

2. MATERIALS AND METHODS

Organisms and inoculum preparation

The following WRB isolated from various regions and ecological niches of Georgia were included in this study: *Cerrena unicolor* 301, *Cyatus striatus* 978, *Fomes fomentarius* 16*, Lenzites betulina* 105, *L.betulina* 158, *Trametes gibbosa* 21, *T. gibbosa* 304, *T. hirsute* 68, *T. versicolor* 28, *T. versicolor* 113, *T. versicolor* 132, *T. versicolor* 159, *P. nebrodersis* 1019, and three unidentified fungi *Tetritskaro* 59, *Terjola*1, *Tsalka* 5.

Fungal inocula were prepared by growing the strains on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 100 ml of the following standard medium (g/l): glucose - 10, NH_4NO_3 - 1, KH_2PO_4 - 0.8, K_2HPO_4 - 0.6, $MgSO_4 \cdot 7\ H_2O$ - 0.5, yeast extract - 2. After 7 days of cultivation, mycelial pellets were homogenized using a Waring laboratory blender.

Cultivation conditions

The solid-state fermentation (SSF) of wheat straw was carried out at 27°C in 100-ml flasks containing 4 g of substrate moistened with 10 ml of the medium (g/l tap water): yeast extract – 10, glycerol - 100, $CuSO_4 \cdot 5\ H_2O$ – 0.5. The initial pH of the medium was adjusted to 5.3 ± 0.1 prior to sterilization. 3 ml of homogenized mycelium was used to inoculate the flasks containing media with lignocellulosic substrate. After 12 and 24 days of fungal growth, 30% of total fermented substrate (by weight) was used for the extracellular enzymes extraction twice with 15 ml of distilled water (total volume 30 ml). The solids were separated by filtration through nylon cloth followed by centrifugation at 14,000 g for 5 min at 4°C. 70% of total fermented substrate was dried at 60°C and used for cellulose and lignin determination.

Determination of cellulose and lignin content

Method is based on a sequential extraction including two refluxing and filtering steps. To remove hemicelluloses, 1 g of fermented straw was placed in 250 ml Erlenmeyer flask, covered by tinfoil hat and treated with 100 ml 0.5 M H_2SO_4 for 1 hour at 95-100°C in a water bath. Content of flask was filtered through tarred Whatman filter paper using Buchner funnel and water jet pump and washed with hot distilled water to neutral pH of effluent water. The filter paper with residue was dried to constant weight at 95°C. Residue containing cellulose and lignin was determined as the difference between the total weight of the filter paper and residue and weight of filter after. Then the dry residue was mixed with 10 ml of 72% H_2SO_4 in 250 ml flask and incubated for 60 min at 30°C on a rotary shaker at 200 rpm. Following digestion residue was transferred into new 250 ml flask and 145 ml of distilled water was poured. The content of flask was diluted up to 4% H_2SO_4 and autoclaved for 40 min at 121°C. Acid-insoluble residue was collected by filtration using Buchner funnel and water jet pump, extensively washed on filter paper with distilled water dried overnight at 95°C. Content of lignin was calculated as difference between dried filter residue and pre-weighed filter paper while loss was considered as cellulose content. The index of ligninolysis was calculated as follows: I_{lig}= lignin loss/cellulose loss + lignin loss

Enzyme assays

The supernatants obtained after biomass separation were analyzed for the enzyme activity. Carboxymethyl cellulase (CMCase) and filter paper (FPA) activities were assayed according to IUPAC recommendations using the low-viscosity carboxy-methyl cellulose (1% w/v) and the Whatman filter paper No.1, respectively, in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [9]. Xylanase activity was determined using birch wood xylan (Roth 7500) (1% w/v) in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [10]. Glucose and xylose standard curves were used to calculate the cellulase and xylanase activities. In all assays the release of reducing sugars was measured using the dinitrosalicylic acid reagent method [11]. One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute.

Laccase activity was determined by monitoring spectrophotometrically the change in absorbance at 420 nm (A_{420}) related to the rate of oxidation of 1 mM 2,2-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 3.8) [12]. Assays were performed in 1-ml cuvettes at room temperature with 50 μl of adequately diluted culture liquid. One unit of activity was defined as the amount of enzyme which leads to the oxidation of 1 μmol of ABTS per minute. Manganese peroxidase (MnP) activity was measured by oxidation of Phenol Red [13]. The 1-ml reaction mixtures were incubated for 1–5 min at the room temperature, the reaction was terminated with 50 μl 4 M NaOH and absorbance was read at 610 nm. One unit of enzyme activity was expressed as the amount of enzyme required to oxidize 1 μmol of Phenol Red in 1 min. Activities in the absence of H_2O_2 were subtracted from the values obtained in its presence to establish the true peroxidase activity.

The experiments were performed at least twice using three replicates. The data presented correspond to mean values with a standard error of less than 15%.

3. RESULTS AND DISCUSSION

Evaluation of WRB lignocellulolytic potential

It is known that the lignocellulose deconstruction is influenced by many biotic and abiotic factors [14]. First of all, it depends on fungal strains peculiarities and their capability to produce high levels of lignocellulose-degrading enzymes and to cause a selective degradation of lignin on the specific plant substrate. Thus, in evaluation of four WRB, *Pleurotus ostreatus* degraded more lignin (41%) than cellulose (17%) while *Phanerochaete chrysosporium* and *Trametes versicolor* deconstructed more cellulose (49 and 51%, respectively) than lignin (21 and 37%, respectively) in the biological pre-treatment of rice straw [5].

Table 1. Degradation of cellulose and lignin during solid-state fermentation of wheat straw

Fungi	Cellulose loss				Lignin loss				I_{lig}	
	mg		%		mg		%			
	Cultivation days									
	12	24	12	24	12	24	12	24	12	24
C. striatus 978	123	377	7.1	21.7	93	129	13.4	18.6	0.43	0.25
C. unicolor 301	182	448	11.5	20.0	200	308	26.5	40.8	0.52	0.41
P. nebrodensis 1019	195	245	11.8	14.9	102	144	14.7	20.0	0.34	0.37
P. eryngii 671	198	342	12.2	20.4	14	72	2.2	11.0	0.07	0.17
T. hirsutus 68	102	471	2.4	27.0	109	149	15.7	17.1	0.52	0.24
T. gibbosa 21	525	766	43.2	57.6	178	268	27.2	41.0	0.25	0.26
T. pubescens 663	248	335	14.3	19.3	23	54	3.3	7.8	0.08	0.14
T. versicolor 86	648	857	38.6	51.1	138	144	21.1	22.0	0.18	0.14
T. versicolor 113	106	373	6.9	17.7	56	128	7.8	10.9	0.35	0.32
Tetritskaro 59	226	712	13.0	40.9	119	212	17.1	30.5	0.34	0.23
Terjola 1	104	497	6.0	28.6	49	212	7.1	30.6	0.32	0.30
Tsalka 5	114	344	6.1	19.8	144	182	20.7	26.2	0.56	0.35

In this study, a screening of twelve WRB was carried out to evaluate cellulose and lignin degradation from wheat straw after 12 and 24 days of SSF. All fungi well colonized the substrate but different degradation patterns were revealed among the studied basidiomycetes (Table 1). By contrast to study [5], some fungi, such as *T. gibbosa* 21 and *T. versicolor* 86 distinguished with exceptionally high degradation of cellulose (766 and 857 mg, respectively, after 24 days of SSF). Although these fungi simultaneously deconstructed high portion of lignin (268 and 144 mg, respectively) the ligninolysis index I_L achieved only of 0.14-0.26 values. *P. eryngii* 671 and *T. pubescens* 663 degraded much lower quantity of polysaccharide (342 and 335 mg, respectively); however, these fungi were not able to actively degrade lignin under the studied culture conditions (72 and 54 mg, respectively), also showing low values of I_L. Only three fungi, *C. unicolor* 301, *T. hirsutus* 68, and *Tsalka* 5 were able to remove lignin slightly preferentially and faster than the carbohydrate component in wheat straw during the first 12 days of SSF providing as high ligninolysis index as 0.52-0.56. Other researchers have also shown different removal of cellulose and lignin by WRB ranging from 5–30% and 2–40% [15-17].

The analysis of date received shows that the degradation patterns of wheat straw by tested fungi differed significantly. One group of fungi, *T. hirsutus* 68, *C. striatus* 978, *T. versicolor* 113, *Terjola* 1, and *Tsalka* 5 during first 12 days of SSF degraded exceptionally small quantities of cellulose (102-123 mg). One may suppose that during this period, the synthesis of cellulases was undergone to catabolite repression because of presence in nutrient medium of easily metabolizable carbon source – glycerol. Subsequently, when glycerol was consumed by these fungi the hydrolases synthesis switched on, degradation of cellulose sharply accelerated and almost 3-5 times more cellulose was degraded during the second 12 days of SSF. The

second group of fungi, such as *T. gibbosa* 21 and *T. versicolor* 86, obviously, wasn't sensible to catabolite repression or glycerol was consumed very rapidly; therefore, these fungi degraded cellulose with maximal rate. Another reason of different degree of polysaccharide hydrolysis could be the medium pH that varied during the SSF from 5.0 to 7.3 depending on the fungus strain (Table 2). Naturally, media pH near 5.0 favored to cellulases catalytic activity and cellulose maximum hydrolysis and loss. Higher pH decreased enzymes activity and, obviously, caused a gradual inactivation of synthesized cellulase providing low degree of polysaccharide degradation.

Evaluation of WRB lignocellulolytic enzyme activity

The difference in the degradation patterns of wheat straw by tested fungi could be attributed to the different enzymatic activity expressed during substrate SSF. In relation with this, it was important to evaluate the capability of the tested fungi to produce the lignocellulolytic enzymes in SSF of wheat straw and to correlate their potential to deconstruct cellulose and lignin with the fungi enzyme activity. Understanding the mechanisms used by WRB to degrade the lignocellulosic materials would be useful for improving the technological process.

Table 2. WRB laccase and MnP activity in solid-state fermentation of wheat straw

Species	pH		Laccase, U l^{-1}		MnP, U l^{-1}	
	Cultivation days					
	12 d	24 d	12 d	24 d	12 d	24 d
C. striatus 978	6.6	6.1	30	270	0	20
C. unicolor 301	5.5	5.5	10580	10420	40	0
P. nebrodensis 1019	6.3	6.3	1720	690	0	0
P. eryngii 671	6.0	6.4	820	610	0	20
T. hirsutus 68	6.7	5.5	370	40	40	40
T. gibbosa 21	5.0	5.1	170	150	0	20
T. pubescens 663	6.8	7.3	100	30	0	0
T. versicolor 86	5.0	5.9	360	250	0	20
T. versicolor 113	5.8	6.5	1610	21780	80	550
Tetritskaro 59	6.1	5.9	3910	1200	20	0
Terjola 1	5.4	5.3	330	210	30	110
Tsalka 5	6.0	5.5	170	90	70	0

The data represented in Tables 1, 2 show that the degradation of wheat straw continued throughout the entire period of SSF, but lignin degradation was not found to be directly correlated with the production of enzymes as has been reported by [18]. Recently, Shrivastava et al. [16] and Sharma and Arora [19] have also observed that the enzyme production profile cannot be correlated well with the degradation of polymer, which showed that fiber degradation not only depends upon the production of enzymes but also regulated by a variety of physiological factors. Indeed, while high laccase activity of *C. unicolor* 301 throughout the fermentation period well correlated with lignin degradation (26.5% and 40.8% after 12 and 24 days of SSF) the maximum laccase and MnP activities obtained in cultivation of *T. versicolor* 113 didn't cause efficient degradation of aromatic polymer (only 7.8% and 10.9%, respectively) (Table 3). By contrast, *T. gibbosa* 21 appeared to be the most efficient lignin destructor from wheat straw (27.2% and 41% after 12 and 24 days of SSF) although this fungus was poor producer of ligninolytic enzymes.

In the evaluation of the selected fungi' hydrolases activities wide variation in enzyme activity was revealed. Namely, the WRB endoglucanase, xylanase, and FP activities varied from 0.1 U ml^{-1} to 8.0 U ml^{-1}, from 0.1 U ml^{-1} to 9.0 U ml^{-1}, and from 0.05 U ml^{-1} to 1.23 U ml^{-1}, respectively (Table 3). In SSF of wheat straw by *C. striatus* 978, *P. nebrodensis* 1019, *T. hirsutus* 68 the degree of cellulose degradation well correlated with these fungi total cellulase activities. However, *T. pubescens* 663, *Terjola* 1 and *Tsalka* 5 expressed high endoglucanase and FP activities after 12 days SSF nevertheless, degraded only 6.0-14.3% cellulose (Table 1). On the contrary, *T. gibbosa* 21 and *T. versicolor* 86 secreted during this period comparatively low enzyme activity degraded 38.6-

43.2% cellulose. These data suggest that plant raw materials deconstruction depends not only on fungus enzyme activity but also on the other factors which should be elucidated in further research.

Table 3. WRB cellulolytic enzyme activity in solid-state fermentation of wheat straw

Species	CMCase, U ml^{-1}		Xylanase, U ml^{-1}		FPA, U ml^{-1}	
	Cultivation days					
	12 d	24 d	12 d	24 d	12 d	24 d
C. striatus 978	0.1	1.3	0.1	0.6	0.05	0.37
C. unicolor 301	1.5	2.2	1.0	1.1	0.26	0.71
P. nebrodensis 1019	0.2	0.1	0.1	0.1	0.05	0.06
P. eryngii 671	0.4	0.4	0.3	0.6	0.14	0.27
T. hirsutus 68	0.6	1.6	0.2	1.7	0.07	0.37
T. gibbosa 21	1.5	1.4	0.9	1.1	0.30	0.32
T. pubescens 663	7.5	0.4	8.5	1.8	0.78	0.26
T. versicolor 86	2.9	5.9	1.2	7.4	0.31	0.86
T. versicolor 113	5.6	8.0	6.6	9.0	0.57	1.23
Tetritskaro 59	1.0	2.5	1.7	1.0	0.27	0.37
Terjola 1	2.5	1.0	3.4	1.6	0.48	0.15
Tsalka 5	3.4	1.6	3.2	1.4	0.50	0.34

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ЛИГНОЦЕЛУЛОЛИТИЧЕН ПОТЕНЦИАЛ НА ДЪРВЕСНИ ГЪБИ ПРИ ТВЪРДОФАЗНА ФЕРМЕНТАЦИЯ НА ПШЕНИЧНА СЛАМА

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Резюме. В настоящата работа се прави сравнителна оценка на потенциала на 12 вида дървесни гъби да разграждат целулоза и лигнин в условия на твърдофазна ферментация.Показано е, че биодеградационната способност на изследваните гъби се различава значително. Сред тях *Carrena unicolor, Trametes hirsutus 86, Tsalka 5* разграждат най-напред лигнина (до 12-я ден) и чак в последствие въздействат върху целулозата. Поради това, те могат да се използват за предварително третиране на пшеничната слама. Сравнителната оценка на целулазната и лигниназна ензимни активности на тестваните гъби показва, че деградацията на целулозата и лигнина не винаги пропорционална корелира с родукцията на съответните ензими.

Ключови думи: дървесни гъби, слама, целулозна и лигнинова биодеградация, целулаза, лаказа, манган пероксидаза

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