

LIGNOCELLULOSIC WASTE BIOREFINERY FOR UNLOCKING THE BIOTECHNOLOGICAL POTENTIAL OF BASIDIOMYCOTA

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Abstract- Screening of fourteen white rot basidiomycetes for lignocellulose-deconstructing enzyme production in the submerged fermentation of mandarin peels revealed promising producers of cellulase, xylanase (*Pycnoporus coccineus* 310 and *Irpex lacteus* 104), and laccase and manganese peroxidase (*C. unicolor* 305). Crystalline cellulose promoted the highest secretion of cellulase, xylanase, and FPA. Among lignocellulosic materials tested, the wheat straw and mandarin peels provided high hydrolases secretion by *P. coccineus* 310 and *I. lacteus* 104, respectively. Supplementation of mandarin peels-based medium with Avicel provided an accelerated development of fungal cultures and stimulated increase of CMC_{ase}, xylanase, and FPA production. The co-cultivation of *P. coccineus* 310 and *I. lacteus* 104 in the presence of mixed substrates caused a synergistic effect on the hydrolases secretion. Maximum laccase activity of *C. unicolor* 305 (88.5 U/mL) was observed in the mandarin peels containing medium while the highest MnP activity was detected in the submerged fermentation of ethanol production residue. Supplementation of mandarin containing medium with trinitrotoluene 1.5-fold increased *C. unicolor* 305 laccase activity whereas xylinidine and veratryl alcohol more than two-fold increased MnP activity as compared with the control medium. Presence of lignocellulosic material in the medium for the inoculum preparation favors subsequent enzyme production.

Keywords – Basidiomycetes, Lignocellulose fermentation, Cellulases, Laccases

1. INTRODUCTION

Agro-industrial wastes from crop cultivation and food processing constitute vast renewable resources for microbial conversion into different value-added products. The white-rot fungal group of Basidiomycetes have a suitable potential to grow on lignocellulosic biomass due to their ability to produce a variety of hydrolytic and oxidative enzymes. Their major hydrolytic enzymes are endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and xylanase [1]. These fungi secrete one or more of extracellular enzymes that are essential for lignin degradation: lignin peroxidase, manganese-dependent peroxidase, and laccase [2]. These lignocellulose-degrading enzymes of wood-rotting basidiomycetes are of fundamental importance for the efficient bioconversion of plant residues and they are promising for a great variety of biotechnological applications including pulp and paper, food, textile and dye industries, cosmetics, agriculture, bioremediation, and analytical biochemistry [1, 2]. The application of lignocellulolytic enzymes in industrial and environmental technologies, including the modern concept of integrated biorefineries [3] requires significant amounts of these enzymes at low cost. Therefore, the increasing demand for lignocellulose-degrading enzymes has intensified the search for fungi having outstanding enzyme activity. Moreover, understanding the growth conditions regulating lignocellulolytic enzyme production is important to achieve high yields of these biocatalysts.

The goal of this study was to evaluate taxonomically different fungi for their capacity to secrete lignocellulolytic enzymes in the submerged fermentation of plant raw materials and to elucidate the physiological mechanisms determining an enhanced production of these enzymes by the most promising strains.

2. MATERIALS AND METHODS

The following white-rot basidiomycetes (WRB) were used in this study: *Cerrena maxima* BCC401, *C. unicolor* BCC305, *Coprinus comatus* BCC220, *Coriolopsis gallica* BCC142, *Fomes fomentarius* BCC39, *Funalia trogii* BCC146, *Ganoderma lucidum* BCC246, *Irpex lacteus* BCC104, *Lenzites betulina* BCC141, *Pycnoporus coccineus* BCC310, *Trametes hirsuta* BCC45, *Trametes ochracea* BCC76, and *Trametes versicolor* BCC13.

Detailed well-adopted media compositions, conditions of plant raw materials submerged fermentation, and enzyme activity assays are published elsewhere [4-6]. Shortly, the submerged cultivation was carried out on the rotary shaker Innova 44 (New Brunswick Scientific, USA) at 160 rpm and 27°C in 250 mL flasks containing 50 mL of medium containing (g/L): KH₂PO₄ – 1.0, MgSO₄·7H₂O – 0.5, peptone – 5.0, yeast extract – 5, crystalline cellulose or/and lignocellulosic materials at various concentrations as a growth substrate. After 5, 7, 10, and 14 days of cultivation, 1 mL of culture was sampled and solids were separated by centrifugation. The supernatants were analyzed for enzyme activities. Endoglucanase (CMC_{ase}) activity was assayed using 1% low-viscosity carboxymethyl cellulose, xylanase activity was determined using 1% birch wood xylan, the total cellulase activity (filter paper activity, FPA) was measured with Whatman filter paper No. 1. One unit of hydrolases

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activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute. Laccase activity was determined as the rate of ABTS oxidation, manganese peroxidase activity was measured by following the formation of a Mn^{3+} -malonate-complex (MnP270) and by oxidation of Phenol Red (MnP610). One unit of oxidases activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

Table 1. Basidiomycetes lignocellulolytic enzymes activity in the submerged fermentation of mandarin peels

Fungi	CMCase (U/mL)	Xylanase (U/mL)	Laccase (U/mL)	MnP270 (U/mL)
<i>Cerrena maxima</i> 401	3.4 \pm 0.4	3.0 \pm 0.5	25.3 \pm 2.1	0.41 \pm 0.03
<i>Cerrena unicolor</i> 305	3.2 \pm 0.6	5.7 \pm 1.3	115.9 \pm 14.0	1.96 \pm 0.22
<i>Coprinus comatus</i> 220	1.4 \pm 0.1	1.0 \pm 0.1	10.6 \pm 1.3	0
<i>Coriolopsis gallica</i> 142	3.0 \pm 0.4	4.6 \pm 0.3	79.9 \pm 10.2	0.37 \pm 0.04
<i>Fomes fomentarius</i> 39	2.8 \pm 0.5	5.5 \pm 0.7	8.1 \pm 1.0	0
<i>Funalia trogii</i> 146	4.9 \pm 0.3	7.2 \pm 0.4	5.2 \pm 0.9	0.09 \pm 0.01
<i>Ganoderma applanatum</i> 258	3.5 \pm 0.8	5.8 \pm 0.6	0.6 \pm 0.1	0
<i>Ganoderma lucidum</i> 246	1.6 \pm 0.2	1.7 \pm 0.2	75.4 \pm 6.8	0
<i>Irpex lacteus</i> 104	14.7 \pm 1.8	17.9 \pm 2.2	0	0
<i>Lenzites betulina</i> 141	4.0 \pm 0.3	5.4 \pm 0.3	6.6 \pm 0.5	0
<i>Pycnoporus coccineus</i> 310	17.6 \pm 1.3	8.5 \pm 1.0	5.1 \pm 0.7	0
<i>Trametes hirsuta</i> 45	3.6 \pm 0.5	4.3 \pm 0.5	2.7 \pm 0.3	0.04 \pm 0.01
<i>Trametes ochracea</i> 76	15.1 \pm 1.3	7.5 \pm 0.3	21.5 \pm 1.9	0.16 \pm 0.02
<i>Trametes versicolor</i> 13	3.5 \pm 0.4	4.2 \pm 0.4	6.2 \pm 0.9	0.07 \pm 0.01

3. EXPERIMENT AND RESULT

Fourteen WRB strains were screened for lignocellulolytic enzyme production in submerged fermentation of mandarin peels (40 g/L). The used lignocellulosic material provided an abundant growth of all fungi in the form of small pellets. However, the tested fungal strains displayed a wide interspecies diversity in their ability to produce lignocellulose-deconstructing enzymes. Among them, *P. coccineus* 310 followed by *T. ochracea* 76 and *I. lacteus* 104 appeared to be the promising producers of endoglucanase (Table 1). However, only *I. lacteus* 104 secreted high xylanase activity. The measurement of fungi laccase activity revealed an outstanding enzyme producer – *C. unicolor* 305; *C. gallica* 142 and *G. lucidum* 246 also secreted an exceptionally high laccase activity. However, no laccase activity was detected in the cultivation of *I. lacteus* 104. In addition to laccase, *C. unicolor* 305 was capable of producing the high MnP activity in cultivation in the mandarin peels-based medium. Other WRB either showed low MnP activities or failed to produce this enzyme in the same cultivation conditions. In this respect, this finding is in an agreement with data obtained by other authors [7].

Table 2. The effect of lignocellulosic growth substrate on *P. coccineus* 310 and *I. lacteus* 104 cellulases production

Growth substrate	CMCase (U/mL)	Xylanase (U/mL)	FPA (U/mL)
<i>P. coccineus</i> 310			
Avicel	51.5 \pm 5.9	66.0 \pm 5.8	5.26 \pm 0.4
Mandarin peels	15.9 \pm 1.7	8.9 \pm 1.0	2.61 \pm 0.3
Wheat bran	7.5 \pm 0.5	11.9 \pm 0.9	1.95 \pm 0.2
Banana peels	7.5 \pm 0.8	10.3 \pm 0.7	1.57 \pm 0.2
Wheat straw	18.1 \pm 1.3	20.8 \pm 1.2	4.86 \pm 0.5
<i>I. lacteus</i> 104			
Avicel	48.7 \pm 3.2	74.0 \pm 6.2	5.04 \pm 0.6
Mandarin peels	15.8 \pm 2.1	19.9 \pm 2.3	2.66 \pm 0.3
Wheat bran	7.0 \pm 0.9	39.8 \pm 3.7	2.99 \pm 0.3
Banana peels	2.3 \pm 0.2	4.0 \pm 0.3	1.10 \pm 0.2
Wheat straw	0.5 \pm 0.1	0.8 \pm 0.1	0.73 \pm 0.1

In the development of fermentation technologies for lignocellulolytic enzyme production, the most important factors are availability and cost of growth substrates. Agro-industrial residues are generally considered as the best substrates to reduce the cost of fermentation [8-10].

P. coccineus 310 and *I. lacteus* 104 which expressed significant cellulase and xylanase activities in screening experiment were selected for the further evaluation of their enzymatic potential in fermentation of several cellulose-containing materials. Undoubtedly, 2% crystalline cellulose was the best growth substrate promoting accumulation of 48.7-51.5 U/mL CMCase, 66.0-74.0 U/mL xylanase and 5.04-5.26 U/mL FPA (Table 2). It is interesting that the wheat straw provided high enzyme activity secretion by *P. coccineus* 310 but it failed to stimulate the hydrolases production by *I. lacteus* 104.

Supplementation of 4% mandarin peels-based medium with 2% Avicel provided an accelerated development of the fungal cultures and stimulated increase of CMCase, xylanase, and FPA production by *P. coccineus* 310 and *I. lacteus* 104 to 61.9, 80.2, and 5.5 U/mL and to 65, 70.3 and 5.7 U/mL, respectively (Fig. 1). Moreover, the co-cultivation of these compatible fungi in the presence of mixed substrates caused a synergistic effect and almost two-fold increased the endoglucanase activity and significantly augmented the xylanase and FPA. The crude enzyme preparation obtained from the mixed culture at a FPA load of 10 U/mL yielded 4 g/L sugars during 24 h hydrolyses of 20 g/L wheat straw.

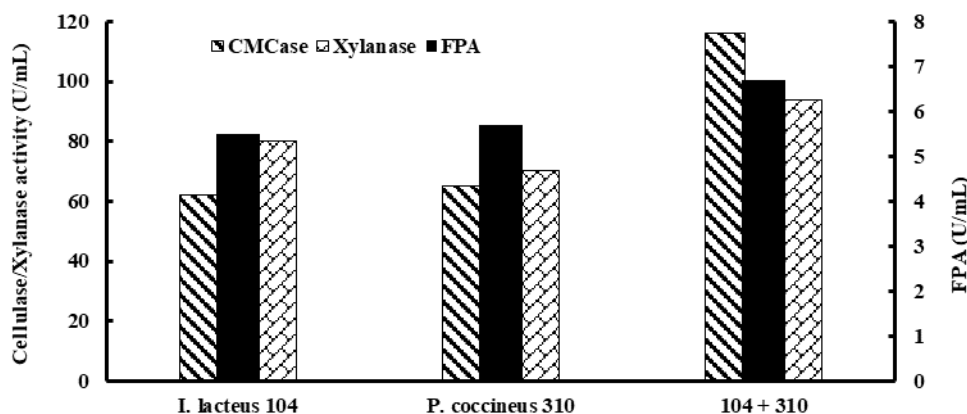


Figure 1. *I. lacteus* 104 and *P. coccineus* 310 mono and dual cultures cellulases and xylanase activities in the fermentation of 4% mandarin peels and 2.0% Avicel.

Table 3. Effect of lignocellulosic substrates on *C. unicolor* 305 ligninolytic enzymes activity

Substrates	Laccase (U/mL)	MnP270 (U/mL)
Banana peels	12.8 ± 1.1	0.49 ± 0.05
Ethanol production residue	66.4 ± 7.4	1.42 ± 0.17
Mandarin peels	88.5 ± 6.9	1.36 ± 0.17
Dog rose	11.2 ± 1.2	0.87 ± 0.11
Pomegranate	19.3 ± 2.7	0.26 ± 0.03
Sawdust	0.9 ± 0.1	0.12 ± 0.02
Wheat bran	60.1 ± 5.3	1.03 ± 0.11
Wheat straw	80.6 ± 6.8	0.32 ± 0.04

Significant dependence of fungi laccase and MnP activities on lignocellulosic growth substrate used was revealed in their submerged fermentation by the best enzyme producer, *C. unicolor* 305 (Table 3). Specifically, laccase activity of this fungus varied from 0.9 U/mL in the medium containing lignified beech wood sawdust to 88.5 U/mL in the mandarin peels containing medium rich in a wide spectrum of aromatic compounds. It is interesting that the lignified wheat straw also favoured laccase production. Evaluation of the fungus MnP activity showed the maximum enzyme activity in the submerged fermentation of ethanol production residue and mandarin peels by *C. unicolor* 305. It is evident that the growth substrate' nature and chemical composition plays a crucial role in the lignin-modifying enzyme expression by the WRB. The obtained results are in good agreement with the data previously obtained for a number of other fungi, which provided evidences that some lignocellulosic materials appear to regulate laccase expression [8-10].

Table 4. Effect of aromatic compounds on *C. unicolor* 305 ligninolytic enzymes activity in the mandarin peels fermentation

Aromatic compounds	Laccase (U/mL)	MnP270 (U/mL)	MnP610 (U/mL)
Control	91.6 ± 7.9	1.79 ± 0.14	1.59 ± 0.12
2,6 DMP	106.1 ± 11.9	2.51 ± 0.25	1.33 ± 0.12
Ferulic acid	93.5 ± 11.3	2.75 ± 0.24	0.85 ± 0.10
Pyrogallol	119.4 ± 15.7	3.20 ± 0.29	1.86 ± 0.22
Veratryl alcohol	97.4 ± 9.1	4.06 ± 0.52	1.83 ± 0.25
Xylidine	118.7 ± 13.0	4.23 ± 0.47	2.01 ± 0.31

TNT	138.7 ± 16.1	3.17 ± 0.45	2.60 ± 0.30
Hydroquinone	62.2 ± 9.3	1.51 ± 0.20	1.21 ± 0.15

Since the submerged fermentation of mandarin peels stimulated laccase and MnP production it was necessary to establish the effect of several individual aromatic compounds on these enzymes production by *C. unicolor* 305. Ferulic acid, pyrogallol, veratryl alcohol, and xyloidine were supplemented to the medium at the concentration of 0.5 mM while trinitrotoluene (TNT) and hydroquinone at the concentration of 0.2 mM. Of these compounds, TNT increased laccase activity by 51% as compared with the control medium whereas hydroquinone significantly inhibited enzyme secretion (Table 4). It should be noted that the existence of a positive synergistic effect of copper and aromatic compounds on the enzyme production cannot be ruled out. Excluding hydroquinone, all other favored Mn-oxidizing MnP production with the highest enzyme activity in the xyloidine and veratryl alcohol supplemented media more than two-fold increasing MnP270 activity as compared with the control medium. With regard to phenol red oxidizing MnP, TNT followed by xyloidine appeared to be the best stimulator of this enzyme secretion. By contrast, ferulic acid two-fold decreased this enzyme activity.

Table 5. Effect of carbon source in inoculum medium on WRB enzyme activity

Inoculum medium	Cultivation medium	Laccase (U/mL)	MnP610 (U/mL)
<i>C. unicolor</i> 303	Glucose	60.8 ± 5.2	0.05 ± 0.01
	MP	167.6 ± 19.5	1.16 ± 0.12
Mandarin peels (MP)	Glucose	316.8 ± 40.7	0.04 ± 0.01
	MP	477.2 ± 50.2	2.01 ± 0.25
<i>C. gallica</i> 142	Glucose	37.0 ± 4.9	0.18 ± 0.02
	MP	75.2 ± 8.0	0.37 ± 0.04
Mandarin peels (MP)	Glucose	63.8 ± 8.4	0.41 ± 0.03
	MP	75.9 ± 10.1	0.25 ± 0.03

Analysis of literature data show that there are still many gaps in our knowledge on physiology of lignin-modifying enzymes production by WRB. In particular, there is no study on laccase and MnP production by WRB in dependence on the composition of media used for maintaining of enzyme producer and for inoculum preparation. At the same time, results in Table 5 show that the *C. unicolor* 303 inoculum preparation in the nutrient medium containing mandarin peels increased the fungus laccase activity from 60.8 to 316.8 U/mL in the subsequent cultivation in the synthetic medium and from 167.6 to 477.2 U/mL in the lignocellulose-containing medium. However, in the case of *C. gallica* 142, inoculum grown in the presence of mandarin peels favored laccase production only in the cultivation in the synthetic medium.

No changes were observed in the MnP activity in the *C. unicolor* 303 cultured in the synthetic medium whereas the enzyme activity almost two fold increased when the inoculum was grown in lignocellulose-based medium. Like laccase activity, two-fold higher MnP activity was revealed when the inoculum grown in the presence of mandarin peels was used for *C. gallica* 142 cultivation in the glucose containing medium.

4. CONCLUSION

The data received 1) show that the tested fungal strains display a wide intra- and interspecies diversity in their ability to produce lignocellulolytic enzymes, 2) highlight the role of lignocellulosic growth substrate in the enzyme activity expression, 3) confirm a regulatory role of aromatic compounds in lignin-modifying enzymes production, 4) evidence that the co-cultivation of two compatible fungi is a promising strategy to enhance the lignocellulose-deconstructing enzyme secretion, 5) indicate that nature of growth substrate in the inoculum preparation affects the biosynthetic potential of WRB.

5. ACKNOWLEDGMENTS

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