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UNDERSTANDING OF FACTORS REGULATING LIGNOCELLULOSE-DECONSTRUCTING ENZYME ACTIVITY

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Abstract- In the submerged fermentation of mandarin pomace, Funalia trogii 146 and Pycnoporus cinnabarinus 811 appeared to be the best cellulase producers while Trametes versicolor 159 and Funalia trogii 146 secreted the highest xylanase activities. The addition of 0.5% glucose to the fungal cultures grown in the presence of mandarin pomace during 4 days resulted in the sharp catabolite repression of cellulase synthesis by F. trogii 146 and P. cinnabarinus 811 but after one day of fermentation the enzyme secretion resumed. The presence of lignocellulosic growth substrate was a prerequisite for the laccase activity expression by G. lucidum 447 and P. cinnabarinus 811. Supplementation of glucose to the mandarin pomace-based medium did not inhibit the fungi laccase activity but decreased the T. versicolor 159 and F. trogii 146 manganese peroxidase activity. Inoculation of young inoculum (middle of logarithmic phase of growth) favored the laccase and MnP production by G. lucidum 447 and T. multicolor 511 while the use of inoculum from the end of logarithmic growth phase provides the highest yields of these enzymes in the cultivation of C. unicolor 305. The highest cellulase activity was revealed when inocula from the late stationary phase were used for the fungi cultivation. Variation of the inoculum rate permits affects the kinetics of lignocellulolytic enzymes accumulation and their yields.
Keywords – Basidiomycetes, Lignocellulolytic enzymes, Lignocellulose fermentation, Carbon source, Inoculum

1. INTRODUCTION

White-rotting basidiomycetes (WRB) are the major decomposers of lignocellulosic materials in several ecosystems and they play a crucial role in the recycling of carbon and other nutrients. Complete degradation of lignocellulosic biomass requires the synergistic action of a large number of oxidative, hydrolytic, and auxiliary enzymes. Their key hydrolytic enzymes are endo-1,4-ß-D-glucanase), exo-1,4-ß-D-glucanase, and xylanase and these fungi secrete one or more of four lignin-modifying enzymes (LME) that are essential for lignin degradation: laccase, lignin peroxidase, manganese-dependent peroxidase, and a versatile peroxidase [1, 2]. These lignocellulose-degrading enzymes of WRB are of fundamental importance for the efficient bioconversion of plant residues and they are promising for a wide variety of biotechnological applications including food, pulp and paper, textile and dye industries, bioremediation, cosmetics, agriculture, and analytical biochemistry [1-3]. Naturally, the application of lignocellulolytic enzymes in industrial and environmental technologies requires significant amounts of these enzymes at a low cost.

Recently, many species of WRB have been studied from both a basic and an applied viewpoint and some of them showed high potential for the production of individual groups of hydrolytic and oxidative enzymes after cultivation conditions optimization [4-7]. Nevertheless, analysis of literature data shows that the current knowledge on physiology and biochemistry of WRB is still limited to realize their biotechnological potential on the industrial level. Moreover, only a few reports are concerned with the simultaneous production of hydrolytic and oxidative enzymes by WRB [8-12]. Furthermore, very little is known about mechanisms up- and down-regulating lignocellulolytic enzyme synthesis. In particular, it is not clear the role of easily metabolizable carbon sources in LME activity expression and why many fungi, such as Pycnoporus coccineus [13], species of genus Ganoderma [14, 15] are not able to produce LME in glucose/glycerol-containing medium. Finally, there is scarce information on enzyme production by WRB depending on the inoculum age/physiological state and rate. Only Songulashvili et al. [14] showed that the substitution of young inoculum (7 days) with the older one (12 days) more than 3-fold increased laccase activity and significantly decreased MnP activity of Ganoderma lucidum. Therefore, this study was focused on solving the above-mentioned fundamental challenges.

2. MATERIALS AND METHODS

Seven WRB from the basidiomycetes culture collection of the Agricultural University of Georgia were used in this study: Cerrena unicolor BCC305, Coriolopsis gallica BCC142, Funalia trogii BCC146, Ganoderma lucidum BCC447, Pycnoporus. cinnabarinus BCC811, Trametes multicolor BCC511, and Trametes versicolor BCC159. Detailed well-adopted media compositions, conditions of plant raw materials submerged fermentation, and enzyme activity assays are published elsewhere [4, 7, 10, 16]. Shortly, the submerged cultivation was carried out on the rotary shaker Innova 44 (New Brunswick Scientific, USA) at 160 rpm and 27oC in 250 mL flasks containing 50 mL of medium containing (g/L): mandarin pomace after the mandarin juice production – 40.0, KH_2PO_4 – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, peptone – 3.0, yeast extract – 3.0, $CuSO_4 \cdot 5H_2O$ –

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0.25. After 5, 8, 11, 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 (CMCase) activity was assayed using 1% low-viscosity carboxymethyl cellulose, xylanase activity was determined using 1% birchwood xylan. One unit of hydrolases 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 Mn3+-malonate-complex (MnP270) and by oxidation of Phenol Red (MnP610). Lignin peroxidase (LiP) activity will be determined spectrophotometrically at 310 nm by the rate of oxidation of 2 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH 3.0) with 0.2 mM hydrogen peroxide. One unit of oxidases activity was defined as the amount of enzyme that oxidized 1 µmoL of substrate per minute.

All experiments were performed twice using three replicates each time. Data presented correspond to the mean values with the standard deviations being less than 15%.

Table 1. Effect of an additional carbon source on the basidiomycetes cellulase and xylanase activities

Carbon source in the nutrient medium	Maximum CMCase activity (U/mL)	Maximum xylanase activity (U/mL)	Media final pH
F. trogii 146			
Without carbon source	0.2 ± 0.035□	0.3 ± 0.0211	8.0 ± 0.1
4% mandarin pomace (MP)	6.7 ± 0.28	24.8 ± 2.111	7.8 ± 0.1
MP + 0.5% glucose	3.3 ± 0.38	31.4 ± 3.78	8.1 ± 0.1
MP + 1.0% glucose	2.9 ± 0.411	37.3 ± 4.214	7.8 ± 0.2
MP + 0.5% glucose on day 4	2.8 ± 0.48	11.2 ± 3.05	8.0 ± 0.1
G. lucidum 447			
Without carbon source	0.5 ± 0.055	0.3 ± 0.035	4.5 ± 0.1
4% MP	1.8 ± 0.25	2.0 ± 0.25	5.8 ± 0.1
MP + 0.5% glucose	1.4 ± 0.38	0.6 ± 0.15	5.2 ± 0.1
MP + 1.0% glucose	2.8 ± 0.611	0.5 ± 0.15	5.4 ± 0.1
MP + 0.5% glucose on day 4	3.7 ± 0.411	1.1 ± 0.15	5.7 ± 0.1
P. cinnabarinus 811			
Without carbon source	0.4 ± 0.025	0.3 ± 0.025	6.9 ± 0.1
4% MP	5.8 ± 0.55	2.1 ± 0.25	7.1 ± 0.1
MP + 0.5% glucose	5.5 ± 0.75	1.8 ± 0.25	7.3 ± 0.1
MP + 1.0% glucose	3.6 ± 0.58	1.7 ± 0.28	7.0 ± 0.1
MP + 0.5% glucose on day 4	5.5 ± 0.95	2.3 ± 0.35	6.2 ± 0.2
T. versicolor 159			
Without carbon source	0.4 ± 0.028	0.5 ± 0.035	7.2 ± 0.1
4% MP	4.9 ± 0.65	25.3 ± 3.15	7.4 ± 0.1
MP + 0.5% glucose	5.7 ± 0.58	24.3 ± 3.311	6.6 ± 0.2
MP + 1.0% glucose	4.6 ± 0.711	29.4 ± 4.111	6.2 ± 0.2
MP + 0.5% glucose on day 4	9.5 ± 1.011	26.7 ± 4.011	6.6 ± 0.2

□ Here and in other Tables, the number indicates the day of maximum enzyme activity.

3. EXPERIMENT AND RESULT

To evaluate the effect of an easily metabolizable carbon source on LME activity, glucose as an additional carbon source was supplemented to the mandarin pomace-based medium (control) at inoculation time and after 4 days of fermentation by the selected basidiomycetes. The medium without carbon source was tested as well. Results in Table 1 show that despite the absence of carbon source and low biomass yields (<1 g/L), all fungi produced the basal level of cellulase and xylanase through the use of peptone and yeast extract. Supplementation of this medium with lignocellulosic material promoted hydrolases synthesis by the tested fungi. Among them, F. trogii 146 followed by P. cinnabarinus 811 appeared to be the best CMCase producers while T. versicolor 159 and F. trogii 146 secreted the highest xylanase activities. Different response of fungi was observed to the supplementation of mandarin pomace-based medium with glucose. In all cultures, very low hydrolases activities were detected after 3 days' growth in the presence of easily metabolizable carbon source, especially at 1% glucose (data not shown). One can assume that like in another study [17], during initial days of fungi cultivation the catabolite repression of cellulase and xylanase synthesis by glucose took place; subsequently, when this carbon source was exhausted, the active enzyme secretion started. It is interesting that addition of 0.5% glucose to the fungal cultures grown in the presence of mandarin pomace during 4 days resulted in the sharp catabolite repression of CMCase synthesis by F. trogii 146 and P. cinnabarinus 811 but after one day of fermentation the enzyme secretion resumed (Fig. 1). By contrast, in the cultivation of G. lucidum 447 and T. versicolor 159, no decrease in enzyme activity was observed. Obviously, in this case, glucose was consumed very rapidly to a concentration not causing the enzyme synthesis repression.

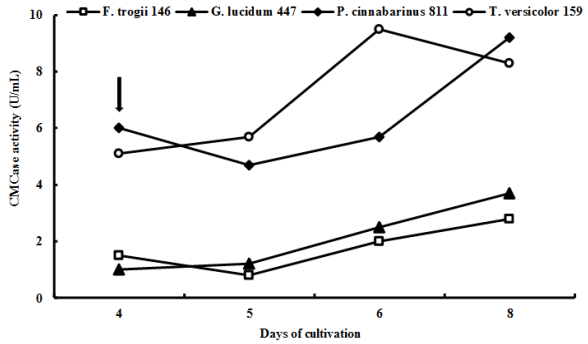

Figure 1. Kinetics of basidiomycetes CMCase accumulation after supplementation of mandarin-based medium with 0.5% glucose on day 4.

Table 2. Effect of an additional carbon source on the basidiomycetes LME activity

Carbon source in the nutrient medium	Laccase (U/mL)	MnP610 (U/mL)	LiP (U/mL)
F. trogii 146			
Without carbon source	2.6 ± 0.211	0.04 ± 0.0111	0
4% MP	10.1 ± 1.211	0.32 ± 0.0311	0.05 ± 0.016
MP + 0.5% glucose	12.1 ± 1.511	0.22 ± 0.0211	0.08 ± 0.016
MP + 1.0% glucose	12.9 ± 1.811	0.25 ± 0.0311	0.06 ± 0.016
MP + 0.5% glucose on day 4	15.2 ± 1.711	0.14 ± 0.0211	0.05 ± 0.016
G. lucidum 447			
Without carbon source	0.2 ± 0.014	0.03 ± 0.014	0
4% MP	74.9 ± 6.311	0.24 ± 0.0311	0.71 ± 0.0911
MP + 0.5% glucose	71.5 ± 8.211	0.33 ± 0.0411	0.69 ± 0.0711
MP + 1.0% glucose	85.4 ± 7.611	0.43 ± 0.0711	0.50 ± 0.0411
MP + 0.5% glucose on day 4	66.3 ± 7.911	0.24 ± 0.0411	0.51 ± 0.0611
P. cinnabarinus 811			
Without carbon source	0.3 ± 0.048	NM□	NM
4% mandarin pomace (MP)	4.2 ± 0.35	NM	NM
MP + 0.5% glucose	6.0 ± 0.68	NM	NM
MP + 1.0% glucose	7.5 ± 0.68	NM	NM
MP + 0.5% glucose on day 4	4.5 ± 0.711	NM	NM
T. versicolor 159			
Without carbon source	1.2 ± 0.18	0.07 ± 0.0111	0
4% MP	7.8 ± 0.98	0.13 ± 0.0111	0.15 ± 0.024
MP + 0.5% glucose	14.1 ± 1.311	0.49 ± 0.0611	0.15 ± 0.024
MP + 1.0% glucose	31.8 ± 3.811	0.55 ± 0.0811	0.19 ± 0.034
MP + 0.5% glucose on day 4	24.5 ± 3.011	0.23 ± 0.048	0.16 ± 0.024

□NM – the activity wasn't measured

The measurement of the LME activities revealed that F. trogii 146 and T. versicolor 159 are capable to synthesize noticeable amounts of laccase in the absence of pure carbon source but the fungi cultivation in the presence of mandarin pomace significantly enhanced the enzyme production (Table 2). Unlike these fungi, cultivation of G. lucidum 447 and P. cinnabarinus 811 in the same medium didn't support laccase production; moreover, the presence of lignocellulosic growth substrate was a prerequisite for the enzyme activity expression. The most important finding of this study is that neither presence of glucose in the initial medium nor its supplementation to the fungal cultures grown in the presence of mandarin pomace during 4 days lead to a decrease of the fungi laccase activity. On the contrary, in some media, especially in the cultivation of T. versicolor 159, addition of glucose to the mandarin pomace-based medium highly increased the laccase yields, possibly, due to better growth of the fungi and the accumulation of greater biomass.

The different effect was revealed in the measurement of the fungi MnP activity. Specifically, in the media with an easily metabolizable carbon source, the MnP activity of F. trogii 146 was rather inhibited as compared with that in the mandarin pomace containing medium, especially taking into account greater biomass of the fungi grown in the presence of glucose (Table 2). At the same time, supplementation of mandarin pomace medium with glucose in the cultivation of T. versicolor 159 significantly favored MnP secretion. Concerning the LiP production, no clear effect of an easily metabolizable carbon source on this enzyme activity secretion was revealed.

Analysis of the results received shows that growth of P. cinnabarinus 811, T. versicolor 159, and especially that of F. trogii 146 accompanied with an increase of the medium pH whereas in the cultivation of G. lucidum 447 significant acidification of the nutrient medium took place (Table 1). It is not ruled out that these changes in media pH affected both enzyme production and activity of already secreted enzymes and this circumstance should be taken into account in the evaluation of WRB enzymatic potential.

Table 3. Effect of inoculum age on the basidiomycetes lignocellulolytic enzyme activity

Inoculum age	Laccase (U/mL)	MnP610 (U/mL)	CMCase (U/mL)	Xylanase (U/mL)
C. unicolor 305				
4 days	57 ± 6.15	0.86 ± 0.0611	1.8 ± 0.27	12.5 ± 1.07
7 days	113 ± 9.711	1.01 ± 0.1211	2.5 ± 0.37	14.1 ± 1.87
14 days	95 ± 9.014	0.26 ± 0.0311	2.7 ± 0.411	12.0 ± 1.611
G. lucidum 447				
4 days	89 ± 6.814	0.28 ± 0.037	1.0 ± 0.17	7.4 ± 0.97
7 days	78 ± 6.414	0.12 ± 0.0211	1.5 ± 0.27	8.6 ± 1.311
14 days	60 ± 7.111	0.14 ± 0.027	1.9 ± 0.211	5.4 ± 0.811
T. multicolor 511				
4 days	24 ± 2.014	0.25 ± 0.0211	2.7 ± 0.27	28.1 ± 3.011
7 days	23 ± 3.211	0.22 ± 0.0211	2.9 ± 0.47	25.5 ± 2.311
14 days	18 ± 2.111	0.06 ± 0.017	6.4 ± 0.714	26.9 ± 2.911

Subsequently, the effect of fungal inoculum age and rate on the target enzyme production was evaluated. Results in Table 3 evidence that 4 days (middle of logarithmic phase of growth) mycelial inoculum is the most appropriate for the laccase and MnP production by G. lucidum 447 and T. multicolor 511 while the 7 days' mycelium (end of logarithmic growth phase) provides the highest yields of these enzymes in the cultivation of C. unicolor 305. Unlike these enzymes, the highest cellulase activity was revealed when the old mycelial inocula were used for the fungi cultivation. Finally, the xylanase production by the three tested fungi only slightly depended on the inoculum physiological state. It is worth noting that achieving individual enzymes' maximum activity also depended on the inoculum physiological state although there is no correlation between the inoculum age and individual enzymes peak activity.

In the subsequent set of experiments, the 7-days inocula were used and the inoculum rate varied from 2.5% to 20%. Although the inoculum rate didn't play a crucial role in the enzyme production by the tested fungi several features can be noted. First, fungal culture development in media with different inoculum rates is species dependent. Thus, the highest MnP270 activity of C. unicolor 305 and T. multicolor 511 was observed when 2.5% inoculum was used while in the cultivation of C. gallica 142 and G. lucidum 447 the highest yields of this enzyme were detected using the greatest amount of inoculum (Table 4). At the same time, C. gallica 142 needs a small amount of inoculum to secrete the highest LiP activity whereas G. lucidum 447 requires an elevated inoculum rate. Second, in some cases, the use of high inoculum concentration accelerates enzyme production and favors the early appearance of laccase and MnP peak activity. Obviously, expression and kinetics of enzyme activity in cultures with different inoculum concentrations depend on individual physiological peculiarities of fungi, such as generation and metabolism rate, lignocellulose-deconstructing enzyme activity and their capability to rapidly hydrolyze lignocellulosic material.

Table 4. Effect of inoculum rate on the basidiomycetes lignocellulolytic enzyme activity

Inoculum rate, %	Laccase (U/mL)	MnP270 (U/mL)	MnP610 (U/mL)	LiP (U/mL)	CMCase (U/mL)	Xylanase (U/mL)
C. unicolor 305						
2.5	109.1±13.010	2.56±0.3910	0.60±0.0410	1.06±0.1310	6.7±0.67	10.6±0.87
5.0	123.2±10.37	2.28±0.2110	0.48±0.0610	0.93±0.1010	6.4±0.47	9.6±1.07
10.0	144.3±8.57	1.98±0.1510	0.50±0.0410	1.17±0.0910	7.2±0.67	8.8±0.97
20.0	157.8±12.77	1.95±0.0810	0.51±0.0310	0.94±0.0810	6.6±0.87	9.8±1.27
C. gallica 142						

2.5	57.9±4.214	0.59±0.0814	0.26±0.0110	0.34±0.0414	4.9±0.310	8.8±0.810
5.0	54.5±3.710	0.59±0.0514	0.29±0.0210	0.22±0.027	4.8±0.410	7.0±0.810
10.0	87.3±8.010	0.46±0.0314	0.44±0.0310	0.17±0.017	5.6±0.710	10.8±1.210
20.0	70.9±9.510	0.73±0.0510	0.44±0.0410	0.20±0.027	3.4±0.410	10.2±1.310
G. lucidum 447						
2.5	64.6±5.910	0.55±0.067	0.10±0.017	0.41±0.0610	2.5±0.37	5.8±0.97
5.0	76.4±4.87	0.59±0.047	0.29±0.027	0.80±0.0910	9.1±1.27	5.7±0.67
10.0	83.0±6.87	0.52±0.057	0.32±0.047	0.89±0.0510	8.2±0.97	5.7±0.47
20.0	74.5±8.27	0.73±0.057	0.25±0.037	0.68±0.0410	6.3±0.57	4.2±0.67
T. multicolor 511						
2.5	21.1±2.314	2.39±0.2110	0.15±0.0110	0.53±0.0410	7.4±0.610	17.0±1.410
5.0	21.7±1.814	2.25±0.1310	0.11±0.0110	0.65±0.0714	7.9±0.510	19.1±1.010
10.0	23.2±2.014	1.97±0.1010	0.09±0.0110	0.76±0.0814	8.9±0.914	20.7±0.910
20.0	22.8±2.710	1.95±0.1410	0.09±0.0110	0.68±0.0614	11.6±1.414	23.6±1.510

4. CONCLUSION

From the information presented here, it is evident that a specific feature of WRB is their extremely wide biodiversity and lack of universal conditions equally good for individual lignocellulolytic enzyme production by different fungi. It is not surprising taking into account that basidiomycetes studied were isolated from very different ecological niches and various lignocellulosic materials. The data received highlight a regulatory role of lignocellulosic material in hydrolases and oxidases production and indicate that the inoculum age and rate affect the cellulases and LME accumulation kinetics and enzyme yield. High laccase and MnP activities of C. unicolor 305 and G. lucidum 447 under high carbon and high nitrogen conditions make them promising candidates for industrial production of these important enzymes.

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