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Lignocellulosic Ezymes of Basidial Fungi-Isolated from Different Ecological Niches of Georgia

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Abstract: Great interest in basidiomycetes for targeted technological treatment of agro-industrial plant substrates are conditioned by their ability to produce lignocellulosic enzymes. Consisting of 29 soil-climatic zones, Georgia (South Caucasus area) represents a unique place for the isolation of microorganisms. In order to study the biochemistry and physiology of wood-degrading basidiomycetes, samples of wood-degrading basidiomycetes were collected from different taxonomic niches of Georgia. 45 strains were obtained as pure cultures and 32 ones – identified. Producers of lignocellulosic enzymes were revealed among test fungi under solid-state and submerged cultivation conditions. Pleurous ostreatus GV10, Pleurotus drynus IN 11 and Fomes fomentarius GK33 were found to be the best producers of cellulosic enzymes using orange and mandarin peels, wheat and Potato straw, wheat bran as substrates, and Ganoderma lucidum GB03 – the best producer of laccase during cultivation on orange and mandarin peels waste. The influence of lignocellulose on the accumulation of the enzymes laccase, xylanase and Filter paper assay was studied.

Keywords: lignocellulosic materials, Basidiomycetes, laccase, cellulase, xylanase.

Introduction

The increasing expansion of agro-industrial activity over the last few years has led to the accumulation of a large quantity of lignocellulosic residues all over the world (Mahesh et al., 2013). Mentioned the process of waste biodegradation should be noted the role of white rot basidial fungi capable of synthesizing hydrolyzing and oxidizing enzymes, which play an important role in the degradation of polysaccharides and lignin of plant biomass, should be especially noted (Huang et al., 2008). Cellulolytic enzymes play the most indispensable and centralized role in the hydrolysis of cellulosic material. Effective enzymatic hydrolysis is one of the major prerequisites in the development of a successful lignocellulosic bio refinery (Dey et al. 2021).

The major obstruction in the biological conversion of lignocelluloses is the physical protection of cellulose by lignin against cellulolytic enzymes (Keller et al., 2003). The different degrees of lignin degradation with respect to other wood components depend on the environmental conditions and the fungal species involved. White-rot fungi have been studied extensively for application in biological pulping and bleaching because they are the only organisms that are able to degrade lignin efficiently (Heinzkill et al., 1998). Fungal laccases have higher redox potential than bacterial or plant laccases and their action seems to be relevant in nature finding also important applications in biotechonology. Thus, fungal laccases are involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation (Thurston, 1994). Therefore, it is necessary to identify new laccases with lower cost but higher activity (Fang et al., 2015). Environmental factors influence the ability of fungi to produce enzymes by high activity and different strains react differently to these conditions. One should thus select the strains capable of producing high concentrations of suitable enzymes and then optimize conditions for enzymes production by the selected organism. It is therefore not surprising that these enzymes yet remains a topic of intense research today.

Method

Microorganisms

Were test of the 45 Basidial fungi strains, collected from different ecological niches of Georgia.

Lignocellulosic substrates

Orange, banana, tea and mandarin peelings, wheat bran, wheat and Potato straw, apples residual, sawdust, bagasse. Lignocellulosic wastes available at the local market and bagasse existent at the international market were applied in studies. All residues were dried at 50oC and milled to dust extent (<1 mm).

Fungal Inoculant

Were prepared by growing the fungi on a rotary shaker at 180 rpm, at 27oC in 500-ml flasks containing 100 ml of synthetic medium with the following composition (g/l): glucose – 15.0; ammonium nitrate – 3.0; yeast extract – 3.0; NaH2PO4 – 0.9; K2HPO4 – 0.3; MgSO4 – 0.5; initial pH was adjusted to 5.7 prior to sterilization. The nutrient media was sterilized at 121ºC for 20 min. After 7-9 days of fungi cultivation, mycelium was inoculated to conduct the SSF and SF of selected lignocellulosic materials. (Tsiklauri et al., 2014)

Cultivation Conditions

Solid-state fermentation (SSF) of selected plants residues was carried out at 27ºC in 100-ml flasks containing 5 g of lignocelluloses substrates moistened with 18 ml of the nutrient medium (g /l): NaNO3 – 2.0; yeast extract – 3.0; NaH2PO4 – 0.9; K2HPO4 – 0.3; MgSO4×7H2O – 0.5; 0.2mM CuSO4×5H2O; final pH 5.8. The flasks were inoculated with 5 ml of mycelial homogenate. After the 7th, 15th and 22nd days of cultivation, the extracellular enzymes were extracted from the whole biomass twice with 25 ml of distilled water (total volume 50 ml). The extract was centrifuged at 10000g for 15 min at 4ºC.The filtrate was used for the determination of biologically active compounds.

Submerged fermentation (SF) of fungi was performed at 27oC on a rotary shaker at 180 rpm in 500-ml flasks containing 100 ml of above-mentioned medium, (g/l): Lignocellulosic substrate – 5%; NaNO3 –3.0; yeast extract – 2.0; NaH2PO4 – 0.9; K2HPO4 – 0.3; MgSO4×7H2O – 0.5; 0.2mM CuSO4×5H2O; Initial pH was adjusted to 5.8. The flask was sterilized at 121ºC for 35 min. The flasks were inoculated with 10 ml of mycelial homogenate. On the 6th and 10th days of cultivation, the culture liquid was centrifuged at 10000g for 15 min at 4oC, and the filtrate was used for the determination of enzyme activities (Tsiklauri et al., 2014).

Enzyme Assays

Xylanase activity was determined by mixing 70 µl appropriately diluted samples with 630 µl of birchwood xylan (Roth 7500) (1% w/v) in 50 mM citrate buffer (pH 5.0) at 50oC for 10 min. Carboxymethyl cellulase

(CMCase) activity was assayed according to IUPAC recommendations by mixing 100 µl appropriately diluted samples with 100 µl of low-viscosity carboxymethyl cellulose (1% w/v) in 50 mM citrate buffer (pH 5.0) at 50oC for 10 min (Ghose, 1987).

Glucose and xylose standard curves were used to calculate cellulase and xylanase activities. In all assays, the release of reducing sugars was measured using the dinitrosalicylic acid reagent method (Bailey et al., 1992)
Filter paper assay for saccharifying cellulase (FPA) activity was assayed using Whatman filter paper №1 according to IUPAC recommendations (Yennamalli et al., 2013). 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 the A420 change related to the rate of oxidation of 1 mM 2,2-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) in 100 mmol sodium tartrate buffer (pH 4.5). Assays were performed in 1 ml spectrophotometric cuvette at 30 ± 1oC with adequately diluted culture liquid. One unit of laccase activity was defined as the amount of enzyme, which leads to the oxidation of 1 mmol of ABTS per minute (Bourbonnais, 1990).

Results and Discussion

Basidiomycetes Enzyme Activity In Solid-State Fermentation (SFF).

SSF is suitable for the production of enzymes by using natural substrates such as agricultural residues because they mimic the conditions under which the fungi grow naturally. The lignin, cellulose ,and hemicelluloses are rich in sugar and promote fungal growth in the fermentor and make the process more economical (Brijwani et al., 2010).

At the initial stage to estimate enzyme production, basidial fungi were screened during their cultivation on the wheat straw. The mentioned substrate is a complex plant waste containing all those polymers (cellulose, hemicellulose, lignin), degradation ability of which possess higher filamentous fungi.45 strains of different genera and species were chosen for testing, At SSF testing of basidiomycetes on the wheat straw substrate, their various potentials were revealed in terms of accumulation both hydrolyzing (CMCase, xylanase, FPA) and oxidizing (laccase) enzymes (Table 1. Abbreviated). Activities of synthesized enzymes were tested on the 7th, 15th, and 22nd days of cultivation, as it is known that enzyme synthesis by microorganisms significantly depends on the period and conditions of cultivation. Pleurotus ostreatus GK10 was found to be the best producer of hydrolyzing enzymes (CMCase, xylanase, FPA). Enzyme activities were 10.4 U/ml, 18.3 U/ml and 0.42 U/ml, respectively. However, Ganoderma sp. GB 03 displayed rather high laccase activity – 45.5 U/ml.

Table 1. Basidiomycetes enzyme activities (U/ml), during in solid-state fermentation of Wheat straw

Fungus	*Laccase*	*CMCase*	*Xylanase*	*FPA*
Fomes fomentarius GK33	0.9±0.2	5.0±0.1	6.9±0.5	0.42±0.05
Fomes sp. KA 35	2.9±0.9	1.0±0.5	4.9±0.7	0.23±0.07
Fomitopsis sp. IK46	2.2±0.4	3.9±0.3	3.3±0.2	0.12±0.05
Ganoderma applanatum IN10	13.4±1.2	2.2±0.9	3.4±1.2	0.15±0.10
Ganoderma sp. GB 03	45.5±2.3	3.0±0.3	2.1±0.7	0.11±0.03
Ganoderma lucidum IG74	10.3±1.3	3.5±0.1	5.3±0.4	0.09±0.02
Ganoderma sp. IN59	5.3±0.7	2.2±0.3	1.5±0.1	0.07±0.02
Lentinus edodes GK97	4.1±0.8	1.3±0.2	0.44±0.1	0.05±0.01
Pleurotus ostreatus IN20	3.5±0.7	5.2±0.5	2.5.±0.6	0.11±0.03
Pleurotus ostreatus GK10	5.1±0.5	10.4±1.2	18.3±1.1	0.42±0.05
Pleurotus drynus IN 11	22.5±3.3	5.4±0.6	8.6±1.0	0.34±0.03
*Trametes sp.*GK 60	22.5±0.8	1.6±0.4	4.0±0.1	0.05±0.01
Sabaduri 16	9.1±1.2	3.1±0.5	3.8±0.1	0.10±0.06
Bordjomi 23	7.2±1.7	3.7±0.4	6.2±0.3	0.28±0.05

Basidiomycetes Enzyme Activity in Submerged Fermentation (SF) on Orange Peels.

As seen in Table 2, all fungi somewhat displayed the ability to synthesize enzymes on the orange peels substrate. Pleurotus ostreatus GK10 was found to be the best producer of hydrolyzing enzymes – CMCase, xylanase and

the FPA with activities 14.4 U/ml, 26.3 U/ml, and 0.82 U/mI, respectively. Enzyme production has been found to be highly dependent on the conditions for fungus cultivation and media. As for laccase, the highest activity (87.5U/mL) showed the strain Ganoderma sp. GB 03. High ability to synthesize laccase also displayed the strains –Ganoderma applanatum IN10, Pleurotus drynus IN 11 and Trametes sp.GK 60. It is noteworthy that fungi in the range of the same genus revealed different activities of enzymes: e.g. Pleurotus drynus IN11 displayed a high ability to accumulate all four test enzymes; Pleurotus ostreatus GK10, representative of the same genus was distinguished by synthesis of hydrolases only and Pleurotus ostreatus IN20 showed low all four test enzymes activity.

Table 2. Basidiomycetes enzyme activities (U/ml), during submerged fermentation on orange peels

Fungus	*Laccase*	*CMCase*	*Xylanase*	*FPA*
Fomes fomentarius GK33	0.9±0.2	7.0±0.2	8.9± 1.1	0.42±0.05
Fomes sp. KA 35	2.9±0.9	2.0±0.5	7.9±0.7	0.23±0.07
Fomitopsis sp. IK46	2.2±0.4	3.9±0.3	4.3±0.2	0.19±0.05
Ganoderma applanatum IN10	30.4±1.2	3.2±0.9	5.4±1.2	0.25±0.10
Ganoderma sp. GB 03	87.5±3.3	4.0±0.6	3.1±0.9	0.17±0.05
Ganoderma lucidum IG74	10.3±1.3	3.5±0.1	5.3±0.4	0.09±0.02
Ganoderma sp. IN59	9.3±0.7	2.2±0.3	1.5±0.1	0.09±0.02
Lentinus edodes GK97	6.1±0.8	1.3±0.2	0.44±0.1	0.05±0.01
Pleurotus ostreatus IN20	3.5±0.7	5.2±0.5	2.5.±0.6	0.11±0.03
Pleurotus ostreatus GK10	5.1±0.5	14.4±2.2	26.3±1.3	0.82±0.08
Pleurotus drynus IN 11	39.5±3.3	10.4±0.4	11.6±1.3	0.34±0.03
*Trametes sp.*GK 60	32.5±0.8	1.6±0.4	4.0±0.1	0.05±0.01
Sabaduri 16	10.9±1.2	3.1±0.5	3.8±0.1	0.20±0.06
Bordjomi 23	8.2±1.7	4.7±0.4	6.2±0.3	0.28±0.05

Effect of the Lignocellulosic Substrates on Fungi Enzymes Activity

For efficient development of microbial technologies, selection of proper plant raw materials, on which synthesis of targeted enzymes occurs more effectively is of main importance for cultivation of fungi (Rosales et al., 2007; Levin et al., 2008). The effect of various lignocellulosic substrates on the accumulation of enzymes by the selected strains basidiomycetes was studied. Basidiomycetes strains of the genus Pleurotus were the best producers of the hydrolyzing enzymes while growing on substrates: mandarin peels, orange peels, wheat bran and tea peels) Ganoderma sp. GB 03 proved to be the best laccase producer while cultivation on almost all tested lignocelluloses with, the highest activity 93.5 U/ ml when grown on mandarin peels (Fig. 1, 2).

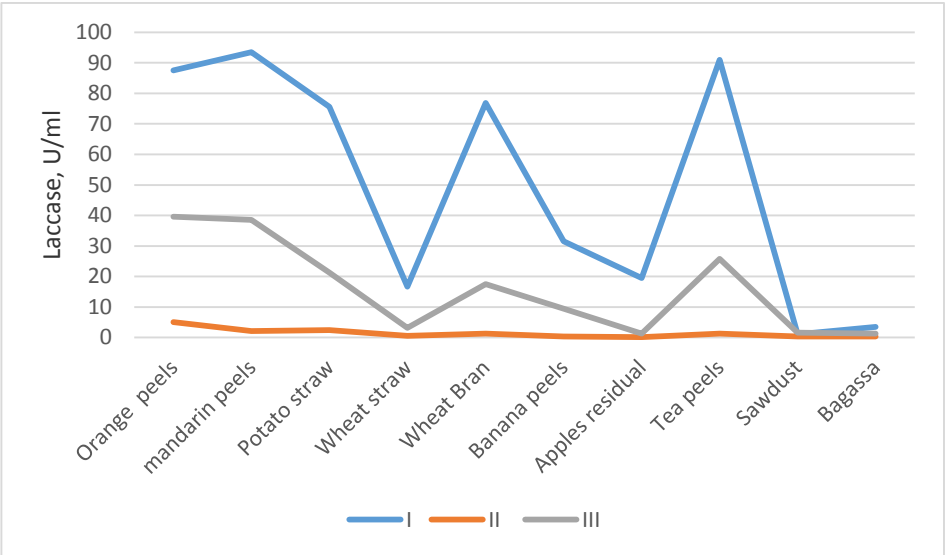

Figure 1. Effect of lignocellulosic substrates on the laccase activity while SF of: I - Ganoderma sp. GB 03; II - P. ostreatus GK10; III- P. drynus IN 11.

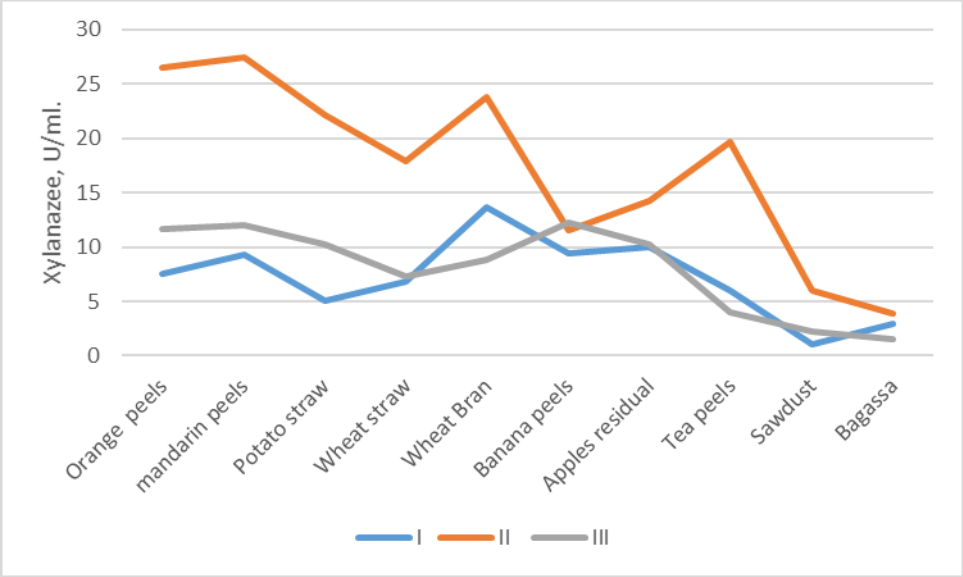

Figure 2. Effect of lignocellulosic substrates on the Xylanase activity while SF of: I- Ganoderma sp. GB 03; II- P. ostreatus GK10; III - P. drynus IN 11.

It should be mentioned that for the accumulation of enzymes, in both types of cultivation, the best was orange and mandarin peel and next came wheat bran and tea peels. Hence, how the process of delignification proceeded in mentioned plant substrates is the issue of further investigations.

Conclusion

The production of ligninolytic enzymes by 45 basidiomycetes species isolated from various geographical and ecological regions of Georgia was investigated under submerged (SF) and solid-state fermentation (SSF) of lignocellulosic materials. Notable intergeneric and intragenic differences were revealed with regard to the extent of oxidases activity. The regulatory role of lignocellulosic materials in laccase and hydrolyzing enzymes secretion by the selected fungi was shown. The studies revealed promising strains of the genera Pleurotus, Ganoderma and Fomes producers of lignocellulose deconstruction enzymes. The best producer of oxidative enzymes is Ganoderma sp. GB03; the best producer of hydrolizing enzymes is Pleurotus ostreatus GK10, Pleurotus drynus N11 and Fomes fomentarius GK33. The best condition for accumulation of laccase for all test strains was SF, when the index of laccase activity was significantly high in comparison of with that of SSF.

Recommendations

Conducted studies showed that the selection of plant biomass for the production of target enzymes is of special importance playing a decisive role in the effective development of technologies. The results obtained indicate that the production of biologically active compounds of basidiomycetes is a regulatory process. subject to extensive regulation and that understanding involved will be important for the development of modern technologies of their production.

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Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPSTEM journal belongs to the authors.

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