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Cerrena unicolor BCC306 - new potent producer of lignin-modifying enzymes

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ABSTRACT

In this study, the nutritional requirements of Cerrena unicolor BCC306 providing enhanced production of lignin-modifying enzymes have been elucidated in the submerged fermentation of plant raw materials. Mandarin peels followed by the wheat bran and pomegranate residues manifold enhanced laccase production. All tested lignocellulosic materials 2-6-fold stimulated secretion of phenol red oxidizing manganese peroxidase (MnP_{610}) as compared with the control medium, only mandarin peels more than 3-fold increased the Mn oxidizing manganese peroxidase (MnP_{270}) activity whereas addition of pomegranate or wheat straw 5- and 11-fold decreased this enzyme activity, respectively. Mandarin peels and pomegranate ensured accumulation of appreciated levels of extracellular lignin peroxidase (LiP). Variation of mandarin peels concentration from 0 to 6% revealed that the higher was the growth substrate concentration the higher was enzyme activity. Supplementation of mandarin peels-based medium with an additional nitrogen source (20 mM N) favored of laccase secretion, none of them increased MnP_{610} activity and only peptone and casein hydrolysate enhanced the MnP_{270} activity. The highest LiP activity was revealed during the fungus cultivation in the medium supplemented with KNO_3 as an additional nitrogen source. Cultivation of C. unicolor 306 in 7 L laboratory fermenter resulted in accumulation of 302.4 U/mL laccase, 4.9 U/mL MnP_{270}, 0.96 U/mL of MnP_{610}, and 0.29 U/mL LiP indicating that this fungus is a good candidate for scaled up production of LME for different biotechnological applications.

Keywords: Basidiomycetes, Cerrena unicolor, Submerged fermentation, Lignin-modifying enzymes, Mandarin peels, Biotechnological applications.

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1. Introduction

Wood-degrading basidiomycetes are the only organisms capable of completely mineralizing lignocellulose, the most abundant recalcitrant renewable material available in the nature due to their ability to synthesize relevant enzymes for breaking down cellulose, hemicellulose, and lignin, a natural aromatic polymer [1]. The white-rot basidiomycetes (WRB) secrete one or more of four extracellular lignin-modifying enzymes (LME) that are essential for lignin degradation: lignin peroxidase (LiP, EC 1.11.1.14), manganese dependent peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC1.11.1.16) and laccase (EC 1.10.3.2). The LME are of fundamental importance for the efficient bioconversion of plant residues and they are prospective for a large number of biotechnological applications in chemical, fuel, pulp and paper, food, textile, dye, and cosmetic industries as well as in agriculture. Moreover, these enzymes are directly involved in the soil and water bioremediation processes degrading various xenobiotic compounds (polycyclic aromatic hydrocarbons, dioxins, explosives, synthetic dyes, micropollutants and many other) [2-4].

It is evident that the growing market satisfaction for LME applications in industrial and environmental technologies and processes require huge amounts of this enzyme at low cost. The main issue delaying wide implementation of LME at industrial scale is their too high cost because of comparatively low enzyme yield in most tested fungi and limited fundamental knowledge on their physiology and biochemistry. Although many recombinant organisms

efficiently overproduce other industrial enzymes, high expression of LME in heterologous systems has not been achieved [5, 6]. Therefore, various approaches and strategies, including search for new enzyme producers, exploitation of cheap plant raw materials as growth substrates, optimization of fermentation media and cultivation conditions, usage of effective inducers, surfactants, development of better bioprocess technologies, etc. [7-9] have been exploited. Nevertheless, analysis of literature data shows that there is an increased demand for fungi with increased growth rate and, at the same time, with an increased enzyme production during the primary metabolism. Moreover, current knowledge on the physiology and biochemistry of WRB is still limited in order to realize their biotechnological potential on industrial level. In particular, very little is known about mechanisms up- and down-regulating oxidative enzyme synthesis in the fermentation of plant raw materials. It is clear that to develop strategies and technologies for LME overproduction a deeper understanding of mechanisms of individual LME synthesis regulation under specific growth conditions is a critical task.

The aim of this study was to elucidate the nutritional requirements of *Cerrena unicolor* BCC306 providing enhanced production of LME by this new potent laccase producer.

2. Materials and Methods

2.1. Organism and inoculum preparation

C. unicolor BCC306 was isolated from the forest of Eastern Georgia and properly deposited in the basidiomycetes' culture collection of the Agricultural University of Georgia. The fungal inoculum was prepared by growing mycelium taken from agar slants on a rotary shaker in 250 mL flasks containing 100 mL of the basal medium (per L): 15 g glucose, 3 g peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract. The inoculum cultivation was performed at 27°C and 150 rpm for 7 days, then the grown fungal biomasses were homogenized in a Waring laboratory blender and used as an inoculum for submerged cultures.

2.2. Lignocellulosic materials

The following available in large amounts in Georgia plant raw materials were tested as growth substrates in order to establish their impact to *C. unicolor* BCC306 enzyme activity: wheat bran, mandarin peels, residue after ethanol production from the wheat grains (EPR), pomegranate, and wheat straw. All plant residues were oven-dried at 50°C and ground to powder in a laboratory mill prior to addition to the nutrient medium.

2.3. Shake-flask cultivation conditions

The submerged cultivation of fungi was conducted in the rotary Innova 44 shakers (New Brunswick Scientific, USA) at 27°C and 160 rpm. The homogenized mycelium (5 mL) was used to inoculate the 250-mL flasks containing 50 mL of the basal medium supplemented with 1.0 mM $CuSO_4 \cdot 5H_2O$. The above-mentioned lignocellulosic materials in an amount of 40 g/L were used as growth substrates instead glucose. Several organic and inorganic nitrogen sources at an equal concentration of 20 mM as nitrogen were tested to optimize LME production by *C. unicolor* BCC306.

The pH of all media was adjusted to 5.0 prior to sterilization to create optimal conditions for lignocellulosic polysaccharide hydrolysis and all submerged cultivations were carried for 10 days. At predetermined time intervals, 1 mL of culture was sampled and solids were separated by centrifugation (Eppendorf 5417R, Hamburg, Germany) at 10,000 *g* for 5 min at 4 °C. The supernatants were analyzed for pH, reducing sugars and enzyme activities.

All experiments were performed twice using three replicates at each time point. All results were expressed as the mean ± SD with only $p \leq 0.05$ considered as statistically significant.

2.4. Cultivation in bioreactor

To scale up the LME production, the cultivation of C. unicolor BCC306 was performed in the 7 L fermenter LILFUS GX (Incheon, South Korea) with three Rushton impellers. The fermenter was filled with 5 L of the optimized medium (per L): 40 g mandarin peels, 1 g KH_2PO_4, 5 g peptone, 3 g yeast extract, 0.5 g $MgSO_4$, 1.0 mM $CuSO_4$ and 3 mL polypropylene glycol 2000 as an antifoam. The fermenter equipped with pH, temperature and pO_2 probes was sterilized (121°C, 40 min) and inoculated with 500 mL of homogenized mycelium. Fermentation was carried out without baffles at 27°C and at the constant airflow rate of 1 v/v/min. The medium pH was controlled at 5.3 during initial 5 days of fermentation to provide optimal conditions for the mandarin peels polysaccharide hydrolysis, then it was increased to 6.2 where the synthesized

enzymes are most stable. The agitation rate was 200 rpm during 3 days, then it was increased to 300 rpm and after 7 days decreased to 200 rpm till the end of fermentation. During the fermentation process, samples were collected daily and immediately analyzed for enzyme activity. After 9 days of fermentation, fungal biomass was separated from culture liquid by successive filtration and centrifugation at 5,400 g for 15 min. Enzyme preparation was isolated from the culture liquid by precipitation with $(NH_4)_2SO_4$ at 70% saturation and the precipitate was dissolved in 0.05 M phosphate buffer (pH 6.0).

2.5. Enzyme activity assay

Laccase activity was determined spectrophotometrically (Camspec M501, Cambridge, UK) at 420 nm as the rate of 0.25 mM ABTS (2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate)) oxidation in 50 mM Na-acetate buffer (pH 3.8) at room temperature [10]. MnP activity was measured at 270 nm by following the formation of a Mn^{3+}-malonate-complex [11] and by oxidation of Phenol Red [12] in the presence of 0.1 mM H_2O_2. LiP activity was 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 [13]. To establish true peroxidase activity, activities in the absence of H_2O_2 were subtracted from the values obtained in the presence of hydrogen peroxide. One unit (U) of LME activity was defined as the amount of enzyme that oxidized 1 μmoL of substrate per minute.

3. Results

3.1. Effect of lignocellulosic growth substrate

To obtain an insight into physiology of the C. unicolor BCC306 LME activities regulation, initially, the effect of selected plant raw materials on the fungus enzyme activity was evaluated. All materials supported equally good fungal growth in form of small pellets. However, the values for individual oxidases differed significantly depending on growth substrates used. Results given in Table 1 indicate that in the submerged cultivation in the absence of lignocellulosic material the fungus secreted significant activities of laccase and MnP but supplementation of this medium with lignocellulosic growth substrates highly enhanced the target enzyme production. In particular, C. unicolor 306 secreted the highest laccase activity in the mandarin peels fermentation followed by the wheat bran and pomegranate residues. Specifically, supplementation of the control medium with mandarin peels caused 31-fold increase in the C. unicolor BCC306 laccase activity. Other tested lignocellulosic materials, including the lignified wheat straw, also provided accumulation of comparatively high laccase activity.

Unlike laccase, the MnP activity significantly varied depending on the growth substrate used. Among them, only mandarin peels supplementation to the basal medium more than 3-fold increased the MnP_{270} activity whereas addition of pomegranate or wheat straw 5- and 11-fold decreased this enzyme activity, respectively. The measurement of phenol red-oxidizing activity revealed that the submerged

Table 1. *Effect of growth substrates on the C. unicolor 306 lignin-modifying enzyme activity*

Growth substrates	Laccase (U/mL)	MnP_{270} (U/mL)	MnP_{610} (U/mL)	LiP (U/mL)
Control	$5.7 \pm 0.3^{(7)}$	$0.69 \pm 0.10^{(4)}$	$0.18 \pm 0.02^{(4)}$	0
EPR	$43.8 \pm 4.2^{(9)}$	$0.62 \pm 0.09^{(7)}$	$0.37 \pm 0.03^{(7)}$	$0.12 \pm 0.02^{(9)}$
Mandarin peels	$179.1 \pm 14.0^{(7)}$	$2.32 \pm 0.30^{(7)}$	$0.93 \pm 0.07^{(4)}$	$0.35 \pm 0.04^{(9)}$
Pomegranate	$103.4 \pm 9.1^{(9)}$	$0.13 \pm 0.01^{(7)}$	$0.57 \pm 0.04^{(7)}$	$0.27 \pm 0.03^{(9)}$
Wheat bran	$108.5 \pm 13.2^{(9)}$	$0.59 \pm 0.05^{(7)}$	$0.88 \pm 0.08^{(4)}$	0
Wheat straw	$40.1 \pm 3.7^{(12)}$	$0.06 \pm 0.01^{(4)}$	$1.09 \pm 0.12^{(7)}$	$0.11 \pm 0.01^{(9)}$

fermentation of all used lignocellulosic materials 2-6-fold stimulated secretion of MnP_{610} as compared with the control medium. Especially, wheat straw appeared to be the most appropriate growth substrate for this enzyme production. Finally, the data received show that C. unicolor BCC306 did not produce LiP in the synthetic medium without lignocellulosic material. Moreover, wheat bran did not provide this enzyme expression. Of plant raw materials used, mandarin peels and pomegranate ensured accumulation of appreciated levels of extracellular LiP.

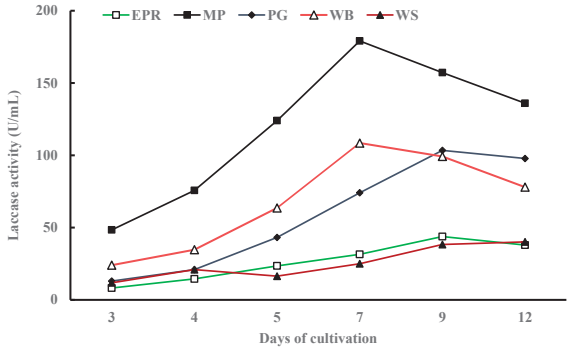

Fig. 1. *Profiles of the C. unicolor 306 laccase activity accumulation in dependence on lignocellulosic growth substrate. EPR – ethanol production residue, MP – mandarin peels, PG – pomegranate, WB – wheat bran, WS – wheat straw.*

Analysis of kinetics of LME accumulation in the submerged fermentation of plant raw materials showed that not only enzyme yield but profiles of enzyme activity depend on the growth substrate used. Thus, in the fermentation of mandarin peels and wheat bran laccase activity rapidly accumulated and reached maximum on the day 7 while in the fungus cultivation in presence of pomegranate and ethanol production residue the laccase peak activity was achieved after 9 days of fermentation (Fig. 1). Moreover, in the fermentation of wheat straw, small laccase activity peak was revealed after 3 days of the cultivation, then it slightly decreased and after that gradually increased till the end of experiment. Kinetics of MnP activity accumulation also depended on lignocellulosic material; however, maximum of this enzyme activity was detected after 4 and 7 days of cultivation (Table 1). At the same time, the period of achieving of LiP activity maximum did not depend on the used growth substrate.

3.2. Effect of mandarin peels concentration

Subsequently, the effect of growth substrate concentration on LME production by C. unicolor 306 was evaluated. The data presented in Table 2 indicate that in the fermentation of mandarin peels the higher was this material concentration the higher was the laccase yield. The highest laccase activity was revealed in the fungus cultivation in media containing 5 and 6% of the growth substrate; however, the peak of the laccase activity was achieved after 7 and 10 days of cultivation, respectively. The same regularities were revealed in the measurement of MnP activities. In particular, C. unicolor 306 MnP_{270} activity gradually increased with elevation of growth substrate concentration from 0 to 6% reaching very high value of 6.56 U/mL after 10 days' fermentation while the maximum of MnP_{270} was achieved in the medium containing 5% of mandarin peels. It worth noting that exploitation of media with high concentration of mandarin peels shifted the maxima of MnP activities to later period of cultivation. The data received show that as compared with laccase and MnP activities expression, C. unicolor 306 is comparatively poor producer of LiP. This enzyme activity gradually increased only with an elevation of mandarin peels concentration to 4%, higher concentrations of the growth substrate negatively affected the enzyme synthesis.

Table 2. *Effect of mandarin peels concentration on the C. unicolor 306 lignin-modifying enzyme activity*

Concentration (%)	Laccase (U/mL)	MnP_{270} (U/mL)	MnP_{610} (U/mL)	LiP (U/mL)
Control	$6.1 \pm 0.5^{(7)}$	$0.54 \pm 0.06^{(4)}$	$0.14 \pm 0.02^{(4)}$	0
2.0	$15.8 \pm 1.4^{(7)}$	$0.66 \pm 0.06^{(7)}$	$0.27 \pm 0.03^{(4)}$	$0.07 \pm 0.01^{(7)}$
3.0	$57.5 \pm 7.3^{(7)}$	$1.88 \pm 0.23^{(4)}$	$0.76 \pm 0.07^{(4)}$	$0.21 \pm 0.03^{(10)}$
4.0	$182.0 \pm 19.8^{(7)}$	$3.13 \pm 0.41^{(4)}$	$0.97 \pm 0.13^{(7)}$	$0.32 \pm 0.03^{(10)}$
5.0	$246.5 \pm 30.2^{(7)}$	$4.69 \pm 0.55^{(7)}$	$1.23 \pm 0.14^{(7)}$	$0.31 \pm 0.04^{(10)}$
6.0	$254.1 \pm 33.7^{(10)}$	$6.56 \pm 0.81^{(10)}$	$1.16 \pm 0.15^{(10)}$	$0.24 \pm 0.03^{(10)}$

Table 3. *Effect of nitrogen sources on the C. unicolor 306 lignin-modifying enzyme activity*

Nitrogen sources	Final pH	Laccase (U/mL)	MnP_{270} (U/mL)	MnP_{610} (U/mL)	LiP (U/mL)
Control	5.0	$138.6 \pm 11.7^{(7)}$	$2.34 \pm 0.26^{(7)}$	$1.09 \pm 0.12^{(7)}$	$0.14 \pm 0.02^{(10)}$
KNO_3	7.0	$230.7 \pm 21.5^{(10)}$	$1.90 \pm 0.25^{(4)}$	$0.78 \pm 0.07^{(4)}$	$0.39 \pm 0.04^{(10)}$
NH_4NO_3	6.0	$229.0 \pm 27.0^{(10)}$	$1.34 \pm 0.20^{(7)}$	$0.47 \pm 0.06^{(4)}$	$0.16 \pm 0.03^{(10)}$
$(NH_4)_2SO_4$	5.7	$238.3 \pm 21.0^{(10)}$	$1.67 \pm 0.21^{(4)}$	$0.57 \pm 0.06^{(4)}$	$0.18 \pm 0.02^{(10)}$
Peptone	6.5	$298.2 \pm 26.3^{(10)}$	$6.46 \pm 0.59^{(7)}$	$1.14 \pm 0.10^{(7)}$	$0.24 \pm 0.03^{(10)}$
Casein hydrolysate	6.6	$278.8 \pm 30.27^{(10)}$	$4.67 \pm 0.50^{(7)}$	$1.10 \pm 0.11^{(7)}$	$0.27 \pm 0.03^{(10)}$

3.3. Effect of nitrogen sources

To optimize the cultivation conditions favoring the target enzyme production effect of an additional nitrogen source was investigated. The data received evidence that the fungus effectively utilize the nitrogen available in the lignocellulosic material and in the yeast extract for the laccase production (Table 3). Specifically, the medium without an additional nitrogen ensured accumulation of 138.6 U/mL laccase, 2.34 U/mL MnP_{270}, 1.09 U/mL MnP_{610}, and 0.14 U/mL LiP. However, all the tested additional sources of nitrogen favored accumulation of laccase in the culture liquid. Among them, organic compounds, peptone and casein hydrolysate appeared especially appropriate for the laccase secretion providing 2-fold increase in C. unicolor 306 laccase activity as compared with the control medium.

The measurement of the MnP activities again showed high productivity of C. unicolor 306 in the control medium and in the media containing inorganic and organic compounds. However, none of them increased MnP_{610} activity and only peptone and casein hydrolysate enhanced the MnP_{270} activity. On the contrary, supplementation of the control medium with several nitrogen containing compounds inhibited the MnP activity expression. Finally, like in the previous experiments, C. unicolor 306 expressed comparatively low lignin peroxidase activity, peptone and casein hydrolysate rather promoted LiP accumulation, but the highest enzyme activity was revealed during the fungus cultivation in the medium supplemented with KNO_3 as an additional nitrogen source.

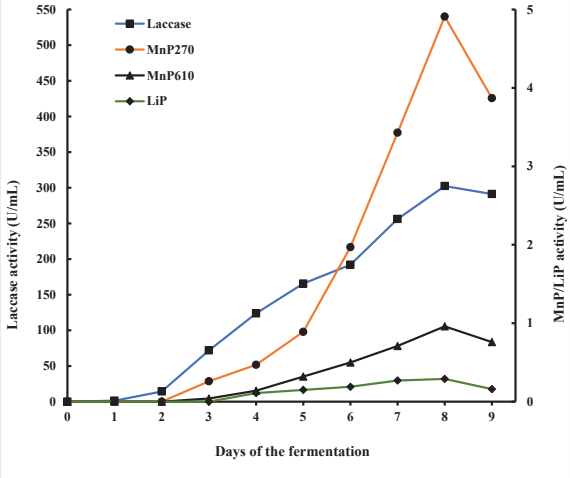

Fig. 2. *Profiles of laccase, MnP, and LiP accumulation in the fermentation of mandarin peels by C. unicolor 306.*

3.4. Scaled up LME production in bioreactor

To scale up the LME production by C. unicolor 306 the fungus cultivation was carried out in a fermenter filled with an optimized medium, taking into account the results obtained in agitated flask cultures. A stirred-tank bioreactor was employed to efficiently produce the target enzyme since it provides better mixing of medium and the fungus growth in form of pellets of desired size. The medium pH was controlled at 5.3 during initial 5 days of fermentation to provide optimal conditions for the mandarin peels polysaccharide hydrolysis and to steadily supply the growing culture with carbon and energy sources, then it was increased to 6.2 where the synthesized enzymes are most stable.

As shown in Figure 2, the presence of C. unicolor 306 laccase activity was detected after the first day of fermentation, with a gradual increase in its activity throughout the cultivation time, reaching the maximum on day eight (302.4 U/mL) followed by slight decrease of the enzyme activity. An appreciable amount of MnP_{270} activity was released by the third day of fermentation with the maximum activity detected on day 8 (4.9 U/mL) followed by the sharp decrease on the next day of cultivation. Low MnP_{610} activity was also detected after three days of fermentation, the enzyme activity consistently increased till day 7 of fermentation, after that it gradually decreased. LiP activity was detected later all other enzymes, after four days of fermentation, reaching its maximum (0.29 U/mL) after eight days of cultivation. The isolated and concentrated (158 mL) enzyme preparation from C. unicolor 306 contained 8625 U/mL of laccase activity, 112 U/mL of MnP_{270} activity, 16 U/mL of MnP_{610} activity, and 4.7 U/mL of LiP activity.

Discussion

Like in earlier studies with C. unicolor strains [7, 14], the recently isolated WRB C. unicolor 306 has proven to be a very promising producer of LME, especially laccase and MnP. Therefore, establishment of optimal cultivation conditions for this industrially important fungus was of high practical value. The utilization of industrial and agricultural wastes or byproducts for LME production is a common approach and an effective way to promote enzyme synthesis and reduce the cost of fermentation [1, 7, 15, 16]. Thus, significant levels of Pleurotus sajar-caju laccase and MnP production was recorded in the mushroom cultivation in media containing oil palm waste followed by banana residues and cotton stalks [Thiribhuvanamala]. Among various types of agro-industrial wastes, orange peel was the best inducer of Pleurotus ostreatus laccase [17]. The results described in this study demonstrate a clear regulatory role and significant stimulation of LME activity by several plant raw materials tested. Among them, mandarin peels containing significant concentrations of soluble carbohydrates and other nutrients for an abundant growth of fungi as well as flavonoids and phenolic compounds [9, 18] ensured simultaneous and exceptional production of laccase, MnP, and LiP by C. unicolor 306. It is interesting that this fungus was capable to secrete significant levels of laccase and especially MnP_{610} activities in the fermentation of wheat straw. This observation is consistent with the results of Munir et al. [15] and Kachlishvili et al. [18]. The most important finding of this study is that C. unicolor 306 is capable to constitutively produce laccase and MnP in the control medium, although it is not excluded these enzymes production occurred due to the availability in the yeast extract of a pool of specific amino acids. On the contrary, C. unicolor 306 does not express LiP activity in the synthetic medium and an availability of lignocellulose in nutrient medium is a prerequisite for this production by the fungus.

Supplementation of all lignocellulosic materials to the control medium manifold improved the fungus laccase activity but with a different extent. It is evident that the growth substrates used have different chemical compositions and differ in aromatic compounds content that may be released to the liquid medium during sterilization and fungal growth. It is also possible that new aromatic compounds appeared during lignocellulose metabolism, enriching the pool of new LME inducers. Obviously, this circumstance is a main reason of significant increase of laccase activity in parallel with gradual increase of mandarin peels concentration. This finding is in good agreement with observations of other researchers. Thus, Zhao et al. [17] showed that the more orange peel was added to the culture of Pleurotus ostreatus, the better was the laccase production by this mushroom.

It is well known that the source and concentration of nitrogen in cultivation media affect LME production [8, 15, 19]. However, some studies have reported increased enzyme activity under non-limiting nitrogen conditions, while others reported opposite results [20, 21]. In this study, supplementation of the control medium with all additional nitrogen sources resulted in increase of C. unicolor 306 laccase activity. It can be assumed that the higher enzyme activity was simply due to a higher biomass production in the media supplemented with nitrogen sources. Nevertheless, unlike laccase, the fungus MnP activity decreased in the presence of inorganic salts added to the control medium at concentration of 20 mM as nitrogen. At the same time, organic nitrogen sources promoted the MnP accumulation. We can assume that these compounds contain aromatic amino acids which cause stimulating effect on the enzyme synthesis. It is worth noting that supplementation of the control medium with organic nitrogen source and KNO_3 favored the increase of the media pH and it is possible that this circumstance

in turn favored the enzyme secretion by the tested fungus. However, this hypothesis need testing**.**

Finally, based on the data obtained in this study and several approaches, LME production by C. unicolor 306 was performed in 7 L laboratory fermenter indicating that these enzymes production with a high yield can be successfully scaled up for different biotechnological applications. Undoubtedly, C. unicolor 306 is a good candidate for these purposes. However, more detailed information on regulation of each individual LME synthesis by this fungal species is required for the development of cost-effective production and application technologies.

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