

I. ENVIRONMENTAL BIOTECHNOLOGY

IMPROVED CELLULASE AND XYLANASE PRODUCTION BY CO-CULTIVATION OF WHITE-ROT BASIDIOMYCETES

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Abstract: Among four white-rot basidiomycetes tested, monocultures of *Irpex lacteus* and *Pycnoporus coccineus* accumulated the highest endoglucanase (53.2 and 61.5 U ml⁻¹), FPA (3.9 and 5.1 U ml⁻¹), and xylanase (58.9 and 58.6 U ml⁻¹) activities in submerged fermentation of crystalline cellulose. Co-cultivation of these two fungi accompanied with their synergism in enzyme production and provided the highest yields of cellulase and xylanase. By contrast, mixed cultivation of *Trametes versicolor* with *P. coccineus* 310 appeared to be unfavourable for cellulases and xylanase production. In co-cultivation of *I. lacteus* and *P. coccineus* it was established that the hydrolases yield depends on the growth substrates, nitrogen source, and their concentrations. The medium containing 2% avicel and 4% mandarin peels along with peptone at concentration of 40 mM provided an abundant growth of fungi and accumulation of 127 U ml⁻¹ CMCase, 6.6 U ml⁻¹ FPA, and 136.2 U ml⁻¹ xylanase activity.

Key words: basidiomycetes, co-cultivation, cellulase, xylanase, submerged fermentation, production optimization

INTRODUCTION

Agro-industrial plant residues are produced worldwide in huge quantities as wastes/by-products of crop cultivation and food processing. This organic waste material is a source of disease, groundwater contamination, toxins, allergens, and environmental pollution. At the same time, these wastes can be converted by chemical, biological and enzymatic processes into value-added products. Among lignocellulose-fermenting organisms, the white-rot basidiomycetes (WRB) are the most efficient decomposers of lignocelluloses since they have a capability to synthesize all relevant hydrolytic and oxidative (lignin-modifying) enzymes required to degrade the lignocellulosic matrix into the low molecular weight compounds [1, 2]. Moreover, unlike the soil fungi, the WRB use lignocellulosic materials without any chemical or physical pre-treatment completely mineralizing them without association with other microbes. This indicates that the WRB possess a complete well equilibrated with individual enzymes wood-deconstructing machinery. The major plant polysaccharide-degrading 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 enzymes are of fundamental importance for the efficient bioconversion of plant residues and they are promising for a great variety of biotechnological applications, including food, textile, pulp and paper industries, agriculture, and bioenergy, in particular, for depolymerisation of the carbohydrate polymers to fermentable sugars [3, 4]. Recently, several species of WRB have been studied in submerged and solid-state fermentation of lignocellulose, of which some have shown high potential for the production of individual

groups of hydrolytic enzymes at the appropriate cultivation conditions [4-8]. Thus, *Armillaria gemina* secreted 146 U endoglucanase/ml, 15 U β-glucosidase/ml, and 1.72 U FPA/ml when the fungus was grown in medium containing rice straw as growth substrate and yeast extract as the best nitrogen source [8]. Moreover, reactions with Celluclast 1.5L and Novozyme 188 supplemented with AgEG were 20% more efficient in the production of reducing sugars than the Celluclast 1.5L and Novozyme 188 alone. *Coprinellus disseminatus* produced 469 U/ml of alkali-thermo-tolerant xylanase along with negligible cellulase activity [7]. Furthermore, the literature data also prove that WRB secrete a full range of hydrolytic enzymes required for the biomass polysaccharides deconstruction [1, 9].

Various approaches and strategies, such as supplementation of nutrient media with food industry wastes/by-products, favourable nitrogen sources at an appropriate concentration have been explored to accelerate the hydrolases synthesis and to increase their yield [6, 8, 10]. Usually, WRB are cultivated as pure mono cultures either in liquid cultures or in solid media; little is known about the WRB physiology in co-fermentation of lignocellulosic residues. Hu et al. [11] reported that high activities of β-glucosidase, α-cellobiohydrolase, β-galactosidase and laccase were produced by *Aspergillus oryzae* in combination with other fungi, in particular with *Phanerochaete chrysosporium*. Co-culturing of *P. chrysosporium* with *Trichoderma reesei* QM6a and *T. reesei* Rut C30 induced the production of cellulolytic proteins and stimulated expression of hemicellulolytic enzymes [12]. However, at present little is known about fungi response during lignocellulose fermentation and there is no study dealing with hydrolases production in basidiomycetes

co-cultivation. Based on the knowledge of physiology of enzyme synthesis by individual fungi, the strategy of co-culture to get technological advantages or to increase microbial productivity could be attractive since it can provide a synergism of fungi with different enzymatic systems in biodegradation of lignocellulosic substrate while a competition for the growth substrate and biotic stress can provoke an accelerated synthesis of the target enzymes.

In this study, mono and dual cultures of four WRB expressing comparatively high cellulase and xylanase activities were investigated under submerged fermentation conditions to establish if these enzymes synthesis up- or down-regulated upon interspecies interaction. Moreover, the *I. lacteus* and *P. coccineus* cellulase and xylanase synthesis was optimized using different growth substrates and nitrogen sources at various concentrations.

MATERIALS AND METHODS

Organisms and inoculums preparation

The following WRB have been used in this study: *Irpex lacteus* 98, *I. lacteus* 104, *Pycnoporus coccineus* 310, *Trametes versicolor* 159. *P. coccineus* 310 (MUCL38527) is from the Belgian Coordinated Collections of Microorganisms/Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL), other fungi have been isolated from the forests of Georgia. All fungi are deposited in the Basidiomycetes Culture Collection of the Agricultural University of Georgia. Fungal inoculums were prepared by growing mycelium on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 100 ml of standard medium with the following composition (g l⁻¹): glucose - 15.0; peptone - 3.0; KH₂PO₄ - 1; MgSO₄ • 7H₂O, 0.5; yeast extract, 3.0. The medium was adjusted to pH 6.0 with 2M NaOH. After seven days of fungal cultivation mycelium pellets were harvested and homogenized with a Waring laboratory blender.

Cultivation conditions

Submerged fermentation of growth substrates was carried out on a rotary shaker at 150 rpm and 27°C in 250-ml flasks containing 50 ml of the following medium (g l⁻¹): crystalline cellulose and/or milled mandarin peels as the fungi growth substrates at various concentrations; peptone - 5.0; KH₂PO₄ - 1.0; MgSO₄ • 7H₂O - 0.5; yeast extract - 5.0. The initial pH of the media was adjusted to 5.8-6.0 prior to sterilization. The flasks were inoculated with 3 ml of mycelial homogenate. When the effect of nitrogen sources on the enzyme production was studied inorganic and organic compounds were used in final

concentration of 20 mM as nitrogen. In all experiments, samples (1 ml) were taken from the flasks after five, eight, eleven, and fourteen days of growth and solids were separated by centrifugation at 14,000 g for five minutes at 4°C. The separated biomasses were dried at 60°C to constant weight. The data presented correspond to mean values, the standard deviation being lower than 20%.

Analytical methods

In study of nitrogen source effect on the enzyme synthesis the fungal biomass was determined by measuring the total nitrogen according to the Kjeldahl method with Nessler reagent. Each sample was pre-boiled in 0.5% of trichloroacetic acid for 15 min to remove non-protein components. True protein content was calculated as total nitrogen multiplied by 4.38.

The supernatants obtained after biomass separation were analyzed for the enzyme activity. Filter paper activity (FPA) for total cellulase activity was measured with Whatman filter paper No. 1 according to IUPAC recommendations [13]. Endoglucanase (CMCase) activity was assayed according to the IUPAC recommendations using 1% low-viscosity carboxymethyl cellulose in 50 mM citrate buffer (pH 5.0) at 50°C for ten minutes [13]. Xylanase activity was determined using 1% birch wood xylan (Roth 7500) in 50 mM citrate buffer (pH 5.0) at 50°C for 10 min [14]. 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 [15]. One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute.

All experiments were performed twice using three to five replicates, each. Data presented correspond to the mean value with the standard deviation being less than 20%.

RESULTS AND DISCUSSION

Effect of co-cultivation

Four WRB expressing comparatively high cellulase and xylanase activities have been selected to improve these enzymes yield. At the first stage, monocultures and their dual cultures have been evaluated for their potential to produce hydrolases in submerged fermentation of crystalline cellulose at concentration of 15 g l⁻¹. The screened fungi showed a good growth in the presence of avicel and they were capable to synthesize the main hydrolytic enzymes participating in lignocellulose polysaccharides

degradation (Table 1). Among them, *P. coccineus* 310 following by *I. lacteus* 104 accumulated the highest endoglucanase, xylanase, and FPA. It is interesting that another strain of *I. lacteus*, namely *I. lacteus* 98, showed 4- and two-fold lower CMCase and xylanase activities, respectively. Nevertheless, co-cultivation of these two strains accompanied with their synergism in enzyme production and provided the highest yields of cellulase and xylanase. In addition, co-cultivation of *I. lacteus* 104 with *P. coccineus* 310 caused significant increase of all measured hydrolytic enzymes. Paired cultivation of *T. versicolor* 159 with *I. lacteus* 98 rather favoured the fungi endoglucanase and xylanase activities as compared with those in monocultures. By contrast, mixed cultivation of *T. versicolor* 159 with *P.*

coccineus 310 appeared to be unfavourable for cellulases and xylanase production. The mechanism of this phenomenon is unclear; one possible explanation is that an antagonistic relationship was established between these fungi or they produced some inhibitors as it was suggested in other studies [16]. However it is worth noting that no growth delay or biomass yield decrease was observed in their co-cultivation. It is possible that the paired fungal culture more efficiently hydrolyzed the growth substrate and accumulated sugars caused a catabolite repression of hydrolases synthesis. However, it is not excluded that simply the competition for the growth substrate causes an accelerated enzyme production.

Table 1. Effect of basidiomycetes co-cultivation on hydrolases activity in the avicel-containing medium

Fungi	Final pH	CMCase, U ml ⁻¹	FPA, U ml ⁻¹	Xylanase, U ml ⁻¹
<i>I. lacteus</i> 98	6.3±0.1	13.6±1.7	2.7±0.4	28.4±5.0
<i>I. lacteus</i> 104	6.5±0.2	53.2±8.2	3.9±0.5	58.9±9.0
<i>P. coccineus</i> 310	7.4±0.2	61.5±8.0	5.1±0.8	58.6±7.8
<i>T. versicolor</i> 159	7.5±0.2	12.6±2.0	2.2±0.3	17.6±2.9
<i>I. lacteus</i> 98+ <i>I. lacteus</i> 104	6.4±0.1	68.6±9.1	6.0±1.1	95.7±15.1
<i>I. lacteus</i> 98+ <i>P. coccineus</i> 310	7.3±0.1	7.3±0.8	2.8±0.4	11.9±2.0
<i>I. lacteus</i> 98+ <i>T. versicolor</i> 159	5.6±0.2	16.7±2.5	3.1±0.4	34.0±5.2
<i>I. lacteus</i> 104+ <i>P. coccineus</i> 310	6.7±0.2	68.7±9.3	5.4±0.8	71.4±14.3
<i>I. lacteus</i> 104+ <i>T. versicolor</i> 159	5.9±0.1	12.8±1.8	2.2±0.2	22.2±3.1
<i>P. coccineus</i> 310+ <i>T. versicolor</i> 159	7.4±0.2	0.6±0.1	1.9±0.3	6.4±1.0

Subsequently, to provide a rapid initial growth of fungi and to further promote the target enzyme synthesis, three fungi have been compared in submerged fermentation of two substrates - crystalline cellulose (15 g l⁻¹) and mandarin peels (40 g l⁻¹). In addition to the cellulose (21%) and hemicellulose (13%), the dried mandarin peels have a significant content of free sugars and organic acids (14%). Indeed, this medium ensured an abundant growth of all WRB and significantly increased cellulase and xylanase activities of *I. lacteus* 104 and *P. coccineus* 310 individual cultures (Table 2). In particular, the total cellulase activities of *I. lacteus* 104 increased more than two-fold as compared with avicel-containing medium. By contrast, neither cellulase activity nor xylanase secretion by *T. versicolor* 159 was enhanced in supplementation of cellulose-based medium with mandarin peels. The results obtained indicate that *I. lacteus* 104 and *P. coccineus* 310 are compatible microbial partners

since co-fermentation of the growth substrates by these fungi greatly increased all enzymes yield. These data are in agreement with results obtained by other authors. Thus, 1.5–2.1-fold enhancement cellulase activity was observed in mixed cultures of *A. niger* and *T. reesei* as compared to their monocultures [17]. The dual cultures of *P. ostreatus* and *T. viride* yielded a fivefold increase in laccase production by *P. ostreatus* [18].

On the contrary, co-cultivation of *I. lacteus* 104 and *T. versicolor* 159 failed to promote the enzyme synthesis. It is likely that these two fungi developed independently and did not affect each other. It is interesting that in the mixed cultivation of *T. versicolor* 159 with *P. coccineus* 310 comparatively low CMCase and xylanase activities were observed; however, this pair gave very high total cellulase activity which can be directly applied for enzymatic saccharification of plant raw materials.

Table 2. Basidiomycetes enzyme activity in the avicel and mandarin peels co-fermentation

Fungi	Final pH	CMCase, U ml ⁻¹	FPA, U ml ⁻¹	Xylanase, U ml ⁻¹
<i>I. lacteus</i> 104	6.9±0.2	70.2±11.0	6.0±1.3	83.2±10.8
<i>P. coccineus</i> 310	6.5±0.2	68.9±13.1	6.3±0.9	65.6±10.1
<i>T. versicolor</i> 159	7.4±0.2	9.8±1.3	1.5±0.2	12.8±1.8
<i>I. lacteus</i> 104+ <i>P. coccineus</i> 310	7.0±0.2	117.8±18.0	6.7±1.0	113.3±18.0
<i>I. lacteus</i> 104+ <i>T. versicolor</i> 159	7.0±0.1	62.2±8.8	4.9±0.8	42.6±6.8
<i>T. versicolor</i> 159+ <i>P. coccineus</i> 310	7.1±0.1	32.3±5.3	10.2±1.6	47.1±6.3

Effect of cultivation conditions on enzyme production by I. lacteus 104 and P. coccineus 310

I. lacteus 104 and *P. coccineus* 310 have been selected for the subsequent co-cultivation experiments as these pair of fungi appeared to be very promising for hydrolases production and their application in biorefinary. Since mixture of mandarin peels and cellulose ensured an efficient secretion of cellulases and xylanase it was mandatory to justify the optimal concentrations and ratio of these growth substrates in the nutrient medium. To establish a role of easily

metabolizable carbon sources in fungal biomass accumulation and enzyme production the fungi co-cultivation was also performed in cellulose and glycerol-containing medium. Moreover, data in Tables 1, 2 indicate that the fungi growth accompanied with significant increase of the medium pH which can affect the enzyme production and inactivate already synthesized enzyme. Therefore, in this set of experiments peptone in nutrient medium was substituted with the physiologically acid salt, ammonium sulphate at concentration of 0.5%.

Table 3. Effect of the growth substrates concentrations on the *I. lacteus* 104 and *P. coccineus* 310 enzyme activity

Growth substrates	Final pH	CMCase, U ml ⁻¹	FPA, U ml ⁻¹	Xylanase, U ml ⁻¹
1.0% avicel	5.5±0.1	42.0±6.3	3.0±0.3	39.8±5.0
1.0% avicel+2% MP	5.6±0.1	57.1±9.7	2.9±0.5	41.2±6.8
1.0% avicel+4% MP	5.3±0.1	83.1±15.7	4.4±0.7	80.0±14.0
2.0% avicel	5.3±0.1	64.9±9.4	5.1±0.6	62.8±8.7
2.0% avicel+2% MP	5.9±0.1	70.9±12.8	5.1±0.8	87.2±15.6
2.0% avicel+4% MP	5.5±0.1	104.0±19.5	5.4±0.9	108.7±15.9
2.0% avicel+0.5% glycerol	5.7±0.1	103.2±17.3	2.6±0.5	76.0±10.2
2.0% avicel+1.0% glycerol	5.7±0.1	36.8±5.0	0.8±0.1	30.2±4.9

In co-cultivation of *I. lacteus* 104 and *P. coccineus* 310 comparatively high activities of cellulases and xylanase were observed in 1% avicel containing medium (Table 3). Supplementation of this medium with 4% mandarin peels favoured the target enzymes secretion increasing endoglucanase and xylanase yields two-fold. Elevation of the cellulose concentration from 1 to 2% positively affected the hydrolases synthesis by the selected fungi. Further increase of all enzymes activity was achieved in supplementation of this medium with mandarin peels; moreover, the higher was the additional growth substrate concentration the higher was the target enzymes yield. It means that this

material contains compounds promoting both an abundant growth of the tested fungi and their biosynthetic activity. Supplementation of the medium containing 2% avicel with 0.5% glycerol rather stimulated xylanase accumulation and increased endoglucanase production by 59%. However, the further elevation of glycerol concentration to 1% significantly decreased the target enzymes secretion obviously because of long-term catabolite repression of hydrolases synthesis.

To optimise the enzyme production by *I. lacteus* 104 and *P. coccineus* 310 the effect of nitrogen source on the fungi growth and cellulases and xylanase production was studied.

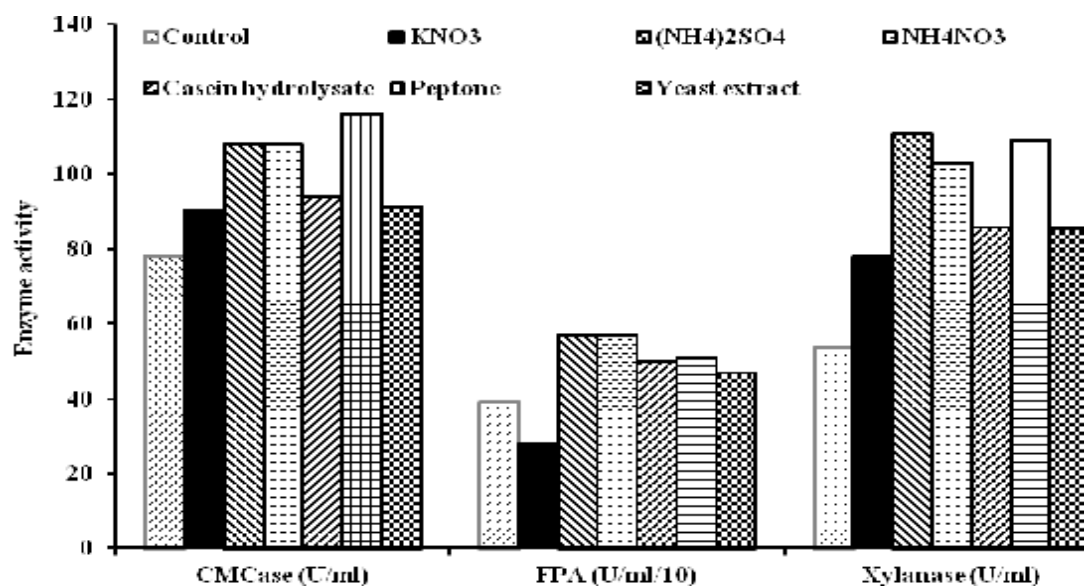


Fig. 1. Effect of nitrogen sources on the *I. lacteus* 104 and *P. coccineus* 310 enzyme activity

The fungi cultivation was performed in the medium containing 2% avicel and 4% mandarin peels as the growth substrate. The data presented in Fig. 1 show that the fungal enzyme activity was increased in presence of all tested nitrogen source as compared with the control without nitrogen. Among them, inorganic compounds, $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3 as well as peptone provided significant increase of cellulase and xylanase activity. However, the measurement of protein content in fermented

products after cultivation in the control medium and media containing KNO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , casein hydrolysate, peptone, and yeast extract showed the following quantities of true protein: 42.6, 49.9, 60.0, 56.3, 58.9, 58.9, 52.2 mg/g fermented product, respectively. It means that the increased cellulase and xylanase yields were achieved due to the higher biomass accumulation in presence of the additional nitrogen source as compared with control medium.

Table 4. Effect of nitrogen source concentration on the *I. lacteus* 104 and *P. coccineus* 310 enzyme activity

Nitrogen, mM	Final pH	Biomass protein, mg g ⁻¹	CMCase		FPA, U ml ⁻¹	Xylanase, U ml ⁻¹
			U ml ⁻¹	U mg ⁻¹		
	(NH ₄) ₂ SO ₄					
0	5.8±0.1	39.5±2.9	71.5±9.6	1.81	3.2±0.4	57.7±8.1
10	5.9±0.1	46.7±3.1	83.6±9.1	1.79	3.7±0.5	76.5±12.0
20	6.2±0.2	58.2±4.0	103.4±15.4	1.78	4.9±0.7	96.4±15.8
40	6.5±0.1	55.0±3.7	108.6±14.6	1.97	6.1±1.0	101.2±15.6
80	7.9±0.2	32.7±3.1	114.8±16.0	3.51	4.9±0.9	100.5±17.2
	Peptone					
10	6.0±0.1	47.5±3.2	89.0±14.2	1.87	5.1±0.8	80.8±9.4
20	6.2±0.1	54.9±4.7	110.4±14.7	2.01	5.9±1.0	112.6±17.2
40	6.2±0.1	41.9±3.3	127.0±17.0	3.03	6.6±1.2	136.2±20.3
80	7.4±0.2	34.8±2.5	123.0±17.3	3.53	5.1±1.0	147.4±22.9

Finally, the effect of nitrogen source concentration on the target enzymes synthesis was studied varying $(\text{NH}_4)_2\text{SO}_4$ and peptone concentration in the nutrient medium from 0 to 80 mM as nitrogen.

The data in Table 4 show that protein content in the fermented biomass increased only with elevation of nitrogen concentration in medium from 0 to 20 mM. Thus, the optimum nitrogen concentration sufficient

for the fungal biomass accumulation is 20 mM. Higher concentrations of inorganic or organic nitrogen source led to delay of fungal growth and decreased the protein content in final biomasses. At the same time, all enzymes activity also correlated with biomass yield when the nitrogen concentration increased from 0 to 20 mM since, for example, the calculated data on the fungi CMCase specific activity have very similar values. However, maximum CMCase, FPA, and xylanase activities were achieved at 40-80 mM nitrogen. Moreover, during growth at these concentrations of nitrogen the fungi showed the highest values of CMCase (and other enzymes) specific activity. In this way, we can conclude that high concentration of nitrogen in nutrient medium delays *I. lacteus* 104 and *P. coccineus* 310 growth but favours the hydrolases activity accumulation. In conclusion, it is likely that during co-cultivation fungi behave in a complex manner and the interaction may be antagonistic or synergistic. Because of the compatibility and synergistic interaction of *I. lacteus* 104 and *P. coccineus* 310, enhanced production of cellulase and xylanase was achieved under co-cultivation condition. The exact process of stimulation remains to be studied further in detail. The understanding of this process encourages the use of co-cultivation of white rot fungi as an improved and promising method for enzyme. In addition, this study showed that the target enzyme production can be further improved by optimization of the nutrient medium through the establishment of the most appropriate carbon and nitrogen sources and their concentrations.

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ПРОМЯНА НА ЦЕЛУЛАЗНАТА И КСИЛАНАЗНА АКТИВНОСТИ ПРИ КУЛТИВИРАНЕ НА ДЪРВЕСНИ БАЗИДИОМИЦЕТИ

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Резюме: Сред тестваните четири базидиомицетни гъби, монокултурите на *Irpex lacteus* *Pycnoporus coccineus* и синтезират най-много ендоглуконазна (53,2 и 61,5 U.ml⁻¹), и ксиланазна (58,9 и 58,6 U.ml⁻¹) активности и по отношение на филтърна хартия (3,9 и 5,1 U.ml⁻¹) при дълбочинно култивиране върху кристална целулоза. При ко-култивирането на тези две гъби, проявеният синергизъм по отношение продукцията на ензими, води до получаване на по-високи добиви на целулаза и ксиланаза. Смесеното култивиране на *Trametes versicolor* с *P.coccineus* 310 не са подходящи за целулазна и ксиланазна продукция. При ко-култивирането на *I. Lacteus* и *P.coccineus* беше установено, че добивите на хидролази зависят от вида и концентрацията на въглеродния и азотен субстрат. Среда съдържаща 2 % целулоза (avicel), 4 % мандаринови кори и 40 mMol пептон позволяват обилен растеж на гъбите и натрупване на 127 U.ml⁻¹ карбоксиметилцелулазна, 6,6 U.ml⁻¹, активност за филтърна хартия и 136,2 U.ml⁻¹ ксиланазна активности.

Ключови думи: базидиомицети, ко-култивиране, целулази, ксиланази, дълбочинна ферментация.

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