Bulgarian Journal of Agricultural Science, 13 (2007), 349-355
National Centre for Agrarian Sciences

Nutritive Medium Dependent Biosynthesis of Extracellular Laccase from *Trichoderma* spp.

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Abstract

KRASTANOV, A. I., V. K. GOCHEV and T. D. GIROVA, 2007. Nutritive medium dependent biosynthesis of extracellular laccase from *Trichoderma* spp. *Bulg. J. Agric. Sci.,*13: 349-355

The effect of nutritive medium composition on laccase production by *Trichoderma viride* and *Trichoderma longibrachiatum* was studied. On the basis of the results obtained it was determined that glucose concentration higher than 20 g/l caused catabolic repression of laccase production The studied phenolic compounds stimulated fungal growth, but only caffeic acid increased laccase production by *T. longibrachiatum*. The highest stimulating effect demonstrated $CuSO_4$ at concentration 10 mg/ml. Maximum laccase activities 3.00 U/ml for *T. viride* and 2.25 U/ml for *T. longibrachiatum* was reached at 96 h of cultivation which correspond to the beginning of stationary phase of strain development.
Key words: laccase, *Trichoderma viride, Trichoderma longibrachiatum*, nutritive medium

Introduction

Laccases (E. C. 1.10.3.2, p-diphenol : dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water. Laccases find wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors (Cuoto and Herrera, 2006). More than 60 fungal strains, belonging to various classes such as *Ascomycetes, Basidiomycetes* and *Deuteromycetes*, have been demonstrated to produce laccase (Gianfreda et al., 1999). The majority of laccases characterized so far have been derived from efficient lignin degraders such as white-rot

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fungi (Eggert et al., 1996; Niku-Paavola et al., 1990). *Trichoderma spp*. also active participates in delignification and biodegradation of lignocellulosic compounds in nature. Nevertheless only a few publications are concern on laccase producing *Trichoderma spp*. (Assavaning et al., 1992, Flegel et al., 1982; Holker et al., 2002). Laccases ere generally produced during the secondary metabolism of different fungi growing on natural substrate or in submerged culture (Gayazov and Rodakiewicz-Nowak, 1996). Laccase production has been found to be highly dependent on the nutritive medium composition (Heinzkill et al., 1998; Xavier et al., 2001) and conditions for cultivation culture (Gayazov and Rodakiewicz-Nowak, 1996).

The aim of present study was to investigate the influence of different carbon sources, and phenolic compounds on laccase biosynthesis by *Trichoderma viride* and *Trichoderma longibrachiatum*.

Materials and Methods

Strains

For the purposes of present study *T. viride* and *T. longibrachiatum* were used as a laccase producer strains. The strains were isolated from soils (Gochev et al., 2006) and deposited in microbial culture collections of Dep."Biotechnology", UFT and Dep. "Biochemistry and Microbiology", "Paisiy Hilendarski" University of Plovdiv. The strains were stored on Potato Dextrose Agar (PDA, Difco).

Fermentation medium and cultivation conditions.

Trichoderma spp. were cultivated on Basal medium of a following composition ($g.l^{-1}$): Glucose 10.0; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; $CaCl_2.2H_2O$ 0.1; $FeSO_4.7H_2O$ 0.005; $(NH_4)_2SO_4$ 0.3; $ZnSO_4.7H_2O$ 0.005; KCl 0.5 and L-Glutamin 0.5; pH 6.0. Glucose (Scharlau), sucrose (Merck), CM-cellulose (Merck); lactose (Sigma) and xylan from oat spelt (Sigma) were examined as sole carbon sources. Laccase production was studied in basal medium supplemented with different phenolic compounds: catechin (Fluca); catechol (Fluca); cafeic acid (Fluca) and chlorogenic acid (Sigma) at concentration 0.286 g/l and $CuSO_4$.

Cultivation was carried out in 300 ml Erlenmayer flasks containing 50 ml basal medium, inoculated with 1% (v/v) vegetative inoculum on a rotary shaker at 220 min^{-1}, 30°C for 96 h.

Enzyme activities assay

Extracellular Laccase activity (LA) was assayed following the method of Ride (1980) in which the increase in absorbance at 530 nm from the oxidation of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydeazine, Sigma) was measured in pH 6.5 phosphate buffer at 30 °C for 10 min. One unit (U) of LA was defined as a change in absorbance of 0.001 min^{-1}.

The biomass quantity was determined gravimetrically after drying at 105°C.

Results and Discussion

Laccase production has been found to be highly dependent on the nutritive medium composition especially type and concentration of carbon source (Gayazov and Rodakiewicz-Nowak, 1996; Heinzkill et al., 1998; Xavier et al., 2001). According to Eggert et al. (1996) the use of excessive concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase

production For these reasons the influence of glucose concentration on the laccase production by *T. viride* and *T. longibrachiatum* in basal medium was studied in the range of 5 to 40 g/l. The results obtained have been presented in Figures 1 and 2. As seen from data (Figures 1 and 2) no correlation between LA and biomass quantity was observed. Maximum LA of 2.25 U/ml for both strains was achieved at 20 g/l glucose. The increase in the amount of glucose in the media more than 20 g/l resulted in a delay of the laccase production. An excess of glucose reduce the production of laccase, because it allows constitutive production of the enzyme, but repress its induction. A simple but effective way to overcome this problem is the use of cellulose or other less degradable substrates as carbon source during cultivation (Egger et al., 1996). Sucrose, CM-cellulose, lactose and xylan were studied as sole carbon sources at concentration 20 g/l. The results obtained have been presented in Figures 3 and 4. As seen from data (Figures 3 and 4) all of the investigated substrates decreased the biomass quantity. The LAs on the basis of glucose, sucrose and CM-cellulose as sole carbon source were almost equal. Xylan caused significant decreasing of biomass quantity and almost total laccase inhibition. Probably xylan induces xylanase production, but repressed LA. On the other hand the lack of xylanase activity os studied strains at the experimental conditions makes xylan unutilized.

Laccases are multi-copper containing enzymes. The addition of low concentrations of copper to the nutritive medium of laccase producing fungi stimulates laccase production (Palmieri et al., 2000). The effect of $CuSO_4$ on laccase production by *T. viride* and *T. longibrachiatum* in basal medium was studied in the range of 1 to 40 mg/l. The results obtained have been presented in Figures 5 and 6. As seen from data (Figures 5 and 6) the highest

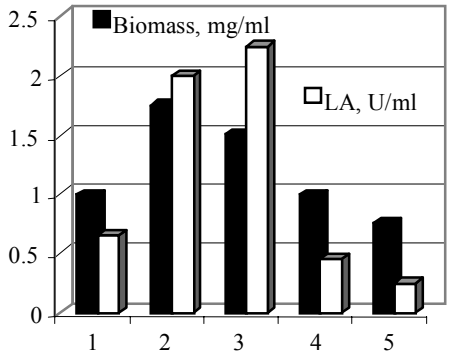

Fig 1. The effect of glucose on extracellular laccase production by *T. viride*

1-5 % glucose , 2-10 % glucose; 3-20 % glucose; 4-30 % glucose and 5-40 % glucose

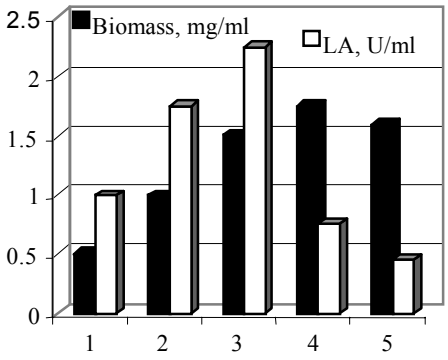

Fig. 2. The effect of glucose on extracellular laccase production by *T. longibrachiatum*

1-5 % glucose , 2-10 % glucose; 3-20 % glucose; 4-30 % glucose and 5-40 % glucose

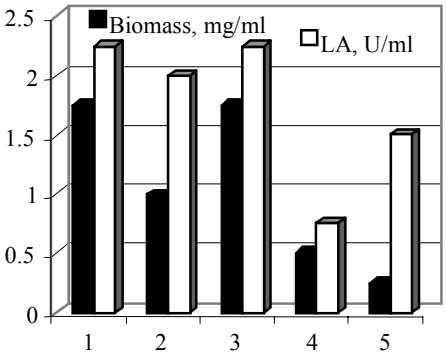

Fig. 3. The effect of carbon sources (20 g/l) on extracellular laccase production by *T. viride:*
1- glucose; 2-sucrose; 3-CM-cellulose; 4-xylan and 5-lactose

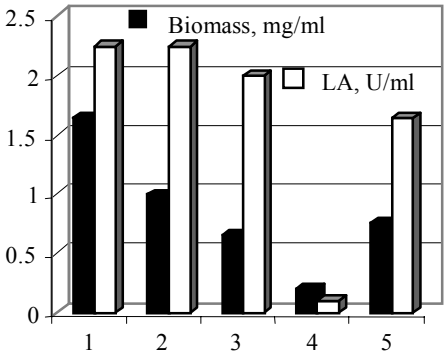

Fig. 4. The effect of carbon sources (20 g/l) on extracellular laccase production by *T. longibrachiatum*
1- glucose; 2-sucrose; 3-CM-cellulose; 4-xylan and 5-lactose

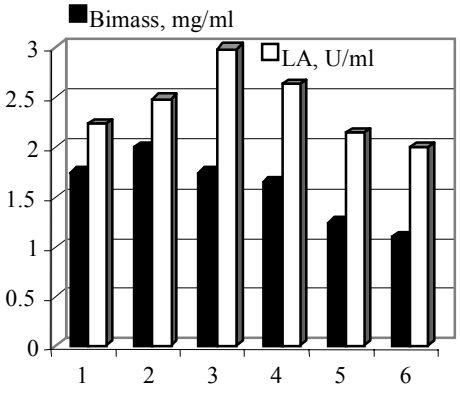

Fig. 5. The effect of $CuSO_4$ on extracellular laccase production by *T. viride*
1-0%, 2-1%; 3-10 %; 4-20%; 4-30 % and 5-40 %

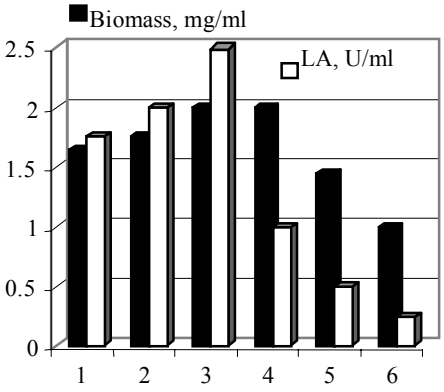

Fig. 6. The effect of $CuSO_4$ on extracellular laccase production by *T. longibrachiatum*
1-0%, 2-1%; 3-10 %; 4-20%; 4-30 % and 5-40 %

LAs was achieved at 10 mg/l $CuSO_4$ – 3.0 U/ml for *T. viride* and 2.5 U/ml for *T. longibrachiatum*.

Laccases were generally produced at low concentrations but higher yields were achieved with addition of various supplements to media. The addition of ethanol and phenolic compounds such as xylidine, lignin, veratryl alcohol is known to increase and induce laccase activity (Xavier et al.,

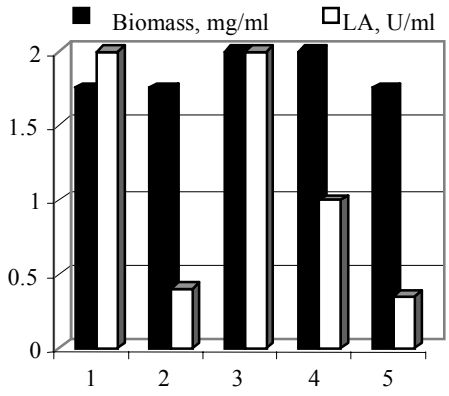

Fig. 7. The effect of phenolic compounds on extracellular laccase production by *T. viride*
1-Control (basal medium); 2-catechin, 3-caffeic acid; 4-chlorogenic acid and 5-catechol

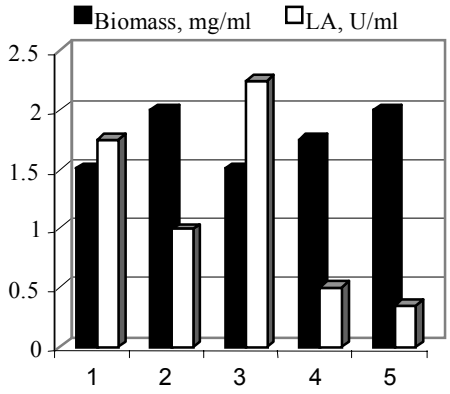

Fig. 8. The effect of phenolic compounds on extracellular laccase production by *T. longibrachiatum*
1-Control (basal medium); 2-catechin, 3-caffeic acid; 4-chlorogenic acid and 5-catechol

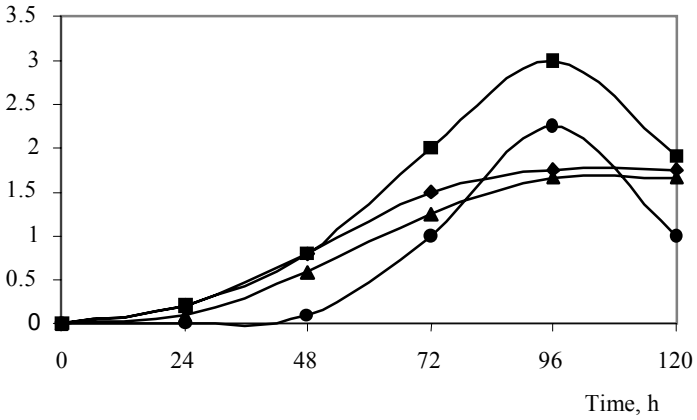

Fig. 9. Laccase production by T. viride and T. longibrachiatum in basal media containing 20 g/l glucose and 10 mg/l $CuSO_4$
LA, U/ml, *T. viride*; Biomass, mg/ml, *T. viride*;
LA, U/ml, *T. longibrachiatum*;
Biomass, mg/ml, *T. longibrachiatum*

2001). Some of these compounds affect the metabolism or growth rate while others, such as ethanol, indirectly trigger laccase production. The effect of catechin, catecho, caffeic and chlorogenic acid on the production of extracellular laccase by

T. viride and *T. longibrachiatum* was determined. The results obtained have been presented in Figures 7 and 8. As seen from data (Figures 7 and 8) all phenolics stimulate the growth, which means that studied *Trichoderma* strains utilize phenolic compounds as additional carbon source. Parallel to growth stimulating effect all phenolic compounds except caffeic asid inhibited laccase production. Only caffeic acid induces the production of extracellular laccase by *T. longibrachiatum* and no effect was observed on laccase production by *T. viride*. Stimulating effect of caffeic acid was weaker than the stimulating effect of CuSO4. No correlation was observed between laccase activity and quantity of biomass.

Dynamics of laccase production by *T. viride* and *T. longibrachiatum* in basal media containing 20 g/l glucose and supplemented with 10 mg/l $CuSO_4$ was determined. The results obtained have been presented in Figure 9. As seen from data the production of laccase by *T. longibrachiatum* in distinction from *T. viride* starts after 48 h after inoculation. Maximum LAs 3.0 U/ml for *T. viride* and 2.25 U/ml for *T. longibrachiatum* was reached at 96 h of cultivation which correspond to the beginning of stationary phase of strain development.

Conclusion

On the basis of the results obtained it was determined that glucose concentration higher than 20 g/l caused catabolic repression of laccase production by *T. viride* and *T. longibrachiatum*. The studied phenolic compounds stimulated fungal growth, but only caffeic acid increased laccase production by *T. longibrachiatum*. The highest stimulating effect demonstrated $CuSO_4$ at concentration 10 mg/ml. Maximum LAs 3.00 U/ml for *T. viride* and 2.5 U/ml for *T. longibrachiatum* was reached at 96 h of cultivation which correspond to the beginning of stationary phase of strain development.

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Received September, 12, 2006; accepted November, 16, 2006.