

Isolation of Laccase Producing *Trichoderma* Spp.

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Abstract

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Based on its dye decolorization activities four *Trichoderma* strains were screened as laccase producers. The strains were identified as *T. atroviride*, *T. longibrachiatum*, *T. reesei*, and *T. viride*. It was determined that it was expedient to screen laccase producing *Trichoderma* strains among cellulase producing strains on the basis of its dye decolorization activity (correlation coefficient between was 0.87). For further studies *T. atroviride*, *T. longibrachiatum* and *T. viride* were screened as prospective laccase producers.

Key words: laccase, *Trichoderma*, isolation, cellulase

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). Laccases are widely distributed in fungi, higher plants, bacteria and

insects. 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 fungi (Eggert et al., 1996 and Niku-Paavola et al., 1990).

Trichoderma spp. also active participate in delignification and biodegradation of cellulose in nature and many strains have been studied extensively as sources of cellulase enzymes for potential commercial hydrolysis of cellulosic materials. Nevertheless only a few publications are

concern on laccase producing *Trichoderma* spp. (As-savaning et al., 1992; Flegel et al., 1982; Holker et al., 2002 and Velazques et al., 2002) Extracellular laccases have been isolated from *Trichoderma atroviride* (Holker et al., 2002), *Trichoderma harzianum* (Holker et al., 2002) and *Trichoderma longibrachiatum* (Velazques et al., 2002). Some *Trichoderma* spp. increased laccase production of white-rot fungi in mixed cultures (Velazques et al., 2002). Thus, it was of interest to know whether *Trichoderma* strains with high cellulase activity possess laccase activity and how these activities correlated.

The aim of present study is to isolate laccase producing *Trichoderma* spp. with a high cellulase activity from soil and to determinate the correlation between both enzyme activities.

Materials and Methods

Sampling procedure

Four soil samples (farming, forest, city surroundings and mountain) were collected from 5-10 cm depth into sterile plastic bags from Plovdiv Province of Bulgaria and its surroundings. Soil samples were used as substrate for isolation of laccase producing *Trichoderma* spp. and were analyzed as soon as possible.

Isolation, primary screening and characteristics of laccase producing *Trichoderma* spp.

Isolation and primary screening of laccase producing *Trichoderma* spp. was performed by dye-decolorizing method (Swamy and Ramsay, 1999). The samples were grounded in a Petri dish and air dried. One gram of dried sample was diluted ten times in sterile physiological solution. The

suspension obtained after vigorous mixing was left to stand for 30 min and 100 μ l of different aqueous dilutions of suspension were inoculated onto the plates containing Crystal Violet Media (CVM) on the following composition (g.l⁻¹): yeast extract 5; (NH₄)₂SO₄ 0.5; KH₂PO₄ 2.66; Na₂HPO₄ 4.32; agar 20; crystal violet 2.5 μ g.ml⁻¹ as a sole carbon source and Triton X-100 1.0. The plates were incubated at 30°C for 30 days. Laccase producing strains were selected on the basis of the laccase activity (LA) measured by using coefficient K_L which reflects the ratio between the diameter of the decolorized zone (D_{DZ}, mm) and the diameter of the respective colony (D_C, mm). Laccase positive colonies showing typical characteristics of *Trichoderma* were selected and then transferred on Potato Dextrose Agar (PDA) (Scharlau) and stored at 4 °C until further examinations.

Cellulase activity (CA) of selected laccase positive strains was determined on agar plates containing nutritive media on the following composition (g.l⁻¹): Carboxy Methyl Cellulose (CMC) (Merck) 10; yeast extract 5; (NH₄)₂SO₄ 0.5; KH₂PO₄ 2.66; Na₂HPO₄ 4.32; agar 20 and Triton X-100 1.0. CA was measured by using the coefficient KC which reflects the ratio between the diameter of the hydrolize zone (D_{HZ}, mm) and the diameter of the respective colony (D_C, mm).

Morphological examinations of the selected strains were carried out on PDA and Cornmeal Dextrose Agar (CMD). The selected *Trichoderma* strains were identified by *Trichoderma* Identification Interactive Key (TIK) (Samuels et al., 2006).

Fermentation medium and cultivation conditions

Selected strains were cultivated on

Basal medium on a following composition (g.l^{-1}): Glucose 10.0; KH_2PO_4 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.005; $(\text{NH}_4)_2\text{SO}_4$ 0.3; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.005; KCl 0.5 and L-Glutamin 0.5; pH 6.0. Cultivation was carried out in 300 ml Erlenmayer flasks containing 30 ml basal medium, 0.5 ml 72 h old vegetative inoculum on a rotary shaker at 220 min^{-1} , 30°C for 96 h.

Enzyme activities assay

Laccase activity (LA) of cultural broth 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} .

Total cellulase activity (TCA) of cultural broth was measured by the most common filter paper assay (FRA) method using Whatman 1 paper as substrate (Ghose, 1987). One U of TCA was defined as the amount of enzyme releasing $1 \mu\text{mol}$ reducing sugars in one minute reaction. The reducing sugars released was measured by 3,5-dinitrosalicylic method (DNS) (Miller, 1959).

Correlation coefficients between K_L and LA, between K_C and TCA and between K_L and K_C of selected strains were determined by standard computer program Excel.

Results and Discussion

Trichoderma species are frequently isolated from forest or agricultural soils at all latitudes. In the case of the solid nutrient media used for isolating fungal strains

from soil samples, the limiting factor is the restriction of the size of the colonies on the agar surface. The use of crystal violet and CMC as substrates of action for laccases and cellulases provided for the rapid visual manifestation of laccase and cellulase positive colonies. The use of Triton X-100 as restricting factor proved very effective under the conditions of experiment because did not affect fungal physiology.

A total of 23 fungal strains were isolated on CVM. By day 4, the extent of mycelia growth on the agar plates was comparable for all cultures whether or not any dye present. Fifteen of the isolated strains can utilize crystal violet as a sole carbon source but only eight strains decolorized the dye. Decolorization began with the formation of clear zone around the colonies. Four of the strains that possess decolorization activity showed morphology typically for genus *Trichoderma* and four strains showed morphology typically for genus *Aspergillus*. On the one hand *Trichoderma* strains actively participated in biodegradation of lignocellulose materials in nature. On the other hand the activity of cellulase enzyme complex in some strains of the genus *Trichoderma* correlated with the size of the hydrolysis zone around the mould colony (Atev et al., 1983). For this reasons screened *Trichoderma* strains were selected for further morphological and physiological examinations. The results obtained of morphological examination have been presented in Table 1. On the basis of the results obtained selected strains were identified by TIK as follows: strain T1 as *T. atroviride*, strain T2 as *T. longibrachiatum*, and strain T3 as *T. reesei*, and strain T4 as *T. viride*.

Cellulase and laccase active participate

Table 1
Morphological characteristics of screened *Trichoderma* strains

Criteria	T1	T2	T3	T4
Conidia				
-shape	globose	ellip-soidal	ellip-soidal	subg-lobose
-length, μm	2.8-3.4	4.9-5.5	4.2-4.8	4.2-4.8
-width, μm	2.7-3.2	2.7-3.2	2.7-3.2	2.7-3.2
- length/width ratio	<1.2	1.2	1.2-1.5	<1.2
-ornamentation	smooth	smooth	smooth	conspicuous warted
-pigmentation	green	grey green	green	grey green
-conidia dry or held in drops of clear liquid	dry	dry	dry	dry
Conidiophore				
-shape	highly intricate	cottony	phialides arising singly over a long distance	highly intricate
-sterile hairs arising from conidiophore	absent	absent	absent	absent
-pustules on CMD	absent	absent	present	absent
Chlamydospore				
	present	present	present	present
Culture				
PDA, colony radius at 25 ° C after 72 h, mm	40	65	55	35
PDA, colony radius at 30 ° C after 72 h, mm	25	55	45	12
PDA, colony radius at 35 ° C after 72 h, mm	<2	>56	38-55	<2
Growth on PDA at 40 ° C after 72 h, mm	no growth	>5	>5	no growth
Strong sweet odor				
	present	absent	absent	present
Most likely species				
	<i>T. atroviride</i>	<i>T. longibrachiatum</i>	<i>T. reesei</i>	<i>T. viride</i>

in biodegradation of lignocellulosic complexes in nature (Cuoto & Herrera, 2006 and Gianfreda et al., 1999). Probably in natural habitats both enzyme activities are a must for *Trichoderma* strains utilizing

lignocellulosic compounds as nutritive substrate. It was found that the use of excessive concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on

Table 2
Laccase and cellulase activity of screened *Trichoderma* strains

Strain	<i>T.atroviride</i>	<i>T.longibrachiatum</i>	<i>Treesei</i>	<i>T.viride</i>
D _C , mm	40	60	50	40
D _{DZ} , mm	50	70	55	60
K _L	1.3	1.2	1.1	1.5
LA, U.ml ⁻¹	1.5	1.7	0.2	2
D _{HZ} , mm	80	120	60	100
K _C	2	2	1.2	2.5
TCA, U.ml ⁻¹	7.6	11.4	4.2	12.4

laccase production and the increase in the amount of glucose in the media resulted in a delay of the laccase production (Eggert et al., 1996). A simple but effective way to overcome this problem is the use of cellulose as carbon source during cultivation (Egger et al., 1996). For this reason from biotechnological point of view is very important to select and use as laccase producers, *Trichoderma* strains with a high cellulase activity.

The obtained results for K_L, K_C, LA, TCA and correlation coefficients for *T. atroviride*, *T. longibrachiatum*, *T. reesei*, and *T. viride* have been presented in Table 2.

T. viride characterized with the highest KL (1.5) and possessed the highest LA (2.0 U.ml⁻¹). *T. reesei* characterized with the lowest K_L (1.1) and possessed the lowest LA (0.2 U.ml⁻¹). Correlation coefficient between K_L and LA was very high (0.99) which means that the chosen method for screening of laccase producing *Trichoderma* strains on the basis of decolorization activity on solid CVM was reliable. Correlation coefficient between KC and TCA was very high (0.91), too. The results obtained for straight correlation between diameters of cellulose hydrolyze zone and TCA confirmed the results pub-

lished by other authors (Atev et al., 1983). Correlation coefficient between K_L and K_C was 0.87 and it was lower than the correlation coefficients between K_L and LA and K_C and TCA, but we decided that for the purposes of primary screening it was high enough. The results obtained demonstrated that it is expedient to screen laccase producing *Trichoderma* strains among cellulase producing strains on the basis of its dye decolorization activity.

Conclusion

On the basis of its dye decolorization activities on solid CVM four *Trichoderma* strains were screened as laccase producers. The strains were identified by TIK as *T. atroviride*, *T. longibrachiatum*, *T. reesei*, and *T. viride*. Correlation coefficient between KL and LA was very high (0.99) which means that the chosen method for screening of laccase producing *Trichoderma* strains was reliable. To overcome the problem with catabolic repression of laccase production by glucose it is expedient to screen laccase producing *Trichoderma* strains among cellulase producing strains on the basis of its dye decolorization activity (correlation coefficient between K_L and K_C was 0.87). For

further studies *T. atroviride*, *T. longibrachiatum* and *T. viride* were screened as prospective laccase producers.

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