SCREENING OF *TRICHODERMA* STRAINS ISOLATED FROM RHIZOSPHERE SAMPLES FOR LACCASE PRODUCTION

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ABSTRACT
In this study we screened formerly isolated *Trichoderma* strains for laccase production on solid media supplemented with two different substrates, ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] or guaiacol. We detected outstandingly strong colour changes in the case of three *Trichoderma* strains in this experiment. The strains were identified based on internal transcribed spacer (ITS) sequence analysis as *T. asperellum* (SZMC 20786 and SZMC 20866) and *T. atroviride* (SZMC 20780). We also investigated the production of laccase enzymes in the case of these *Trichoderma* strains in two types of liquid media. The pH dependence of the secreted laccases was determined in cell free ferment broths at pH 3.5, 4, 5, 5.5, 6 and 6.5 adjusted with 25 mM succinate buffer. Laccase activities from liquid cultures were measured with ABTS as substrate. The results showed that the best laccase producer among the investigated *Trichoderma* strains was *T. atroviride* SZMC 20780 under these conditions. This strain shows the highest laccase enzyme activity on the second day of incubation in a rotary shaker at 25 °C.

Keywords: laccase, *Trichoderma*, ABTS, guaiacol, pH dependence

INTRODUCTION

Laccases (EC 1.10.3.2, p-diphenol:oxygen oxidoreductase) are widely distributed in higher plants, insects and bacteria. However, laccases of fungal origin were also intensively studied. Various fungi over a wide range of taxa are able to produce laccase enzymes. Laccases are also produced by certain members of the filamentous fungal genus *Trichoderma* (*Ascomycota*, *Hypocreales*, *Hypocreaceae*): laccase production was reported from *T. atroviride* and *T. harzianum*, which correlated with the production of the green pigment in conidial spores (HÖLKER ET AL. 2002, SADHASIVAM ET AL. 2008, CHAKROUN ET AL. 2010). Furthermore, researchers demonstrated that soil-derived *T. viride* and *T. reesei* strains also produce laccase enzyme (GOCHEV and KRASTANOV 2007, KRASTANOV ET AL. 2007), however, the species level identification of these isolates has not been confirmed by molecular techniques. There are still only a few publications about *Trichoderma* laccases, although it would be important to know more about the *Trichoderma* strains capable to produce this enzyme, the role of which is also significant in industrial processes such as textile dye decolourization, pulp delignification and detoxification (KIISKINEN ET AL. 2004). Furthermore, laccases oxidize various organic and inorganic compounds such as diphenols, polyphenols, substituted phenols, diamines and aromatic amines with concomitant reduction of molecular oxygen to water (THURSTON, 1994).

The aim of this study was to screen *Trichoderma* strains derived from the thizosphere of vegetables on solid media for laccase production and to further evaluate their laccase activities in the cell-free ferment broths of the potential producers.

MATERIAL AND METHOD

Fungal strains

Trichoderma strains isolated from vegetable rhizosphere samples were used for the purposes of the present study. Forty-six isolates deriving from the Microbiological Collection of the University of Szeged (SZMC) were investigated. The strains were maintained on yeast extract – glucose (YEG) medium, one liter containing 5 g glucose, 5 g KH_2PO_4, 1 g yeast extract and 20 g agar.

Solid media used in the screening

For the detection of laccase-producing *Trichoderma* strains, one liter of solid medium contained 1 g glucose, 1 g yeast extract, 0.2 g Na_2HPO_4, 0.4 g KH_2PO_4 and 20 g agar. Digitonin was applied at a concentration of 4 µg/ml in order to slow down the rapid growth of *Trichoderma* strains. Two different indicator compounds were added to the solid media after autoclaving in order to detect laccase-producing *Trichoderma* strains: 0.5 g/l ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] (AppliChem) or 800 µl/l guaiacol (Sigma) as substrate.

The production of laccases

The production of laccase was examined in two types of liquid media. Malt extract liquid medium contained 50 ml 20 % malt extract, 2.5 g yeast extract and 10 g glucose per liter, alone or supplemented with 80 µl mineral solution (1 g $CaCl_2 \times 2\ H_2O$, 1 g $FeSO_4 \times 7\ H_2O$, 0.1 g $ZnSO_4 \times 7\ H_2O$, 0.16 g $CuSO_4 \times 5\ H_2O$ and 1 g Na_2EDTA per liter). Thirty milliliters of liquid media were inoculated with suspensions of the three examined *Trichoderma* strains containing 2×10^6 conidia. The incubation was carried out at 25 °C in a rotary shaker (180 rpm) for four days. Ferment broth samples were collected at each day of the fermentation period. The activity assays were performed with tenfold dilutions of the cell-free ferment broths. The daily changes of laccase production were examined during this 4-day-period. The experiment was performed in 25 mM succinate buffer at pH 3.5 with the application of 5 mM ABTS as substrate. Optical densities were measured at 436 nm after overnight incubation at 25 °C. Measurements were performed with a SPECTROstar^{Nano} microplate reader (BMG LABTECH).

pH-dependence of laccase activities

The pH-dependence of the secreted laccase activities was studied in cell-free tenfold diluted supernatant at the pH values of 3.5, 4, 5, 5.5, 6 and 6.5 adjusted with 25 mM succinate buffer. Laccase activities were measured as described above, with 5 mM ABTS as substrate.

RESULTS

Screening for laccase production

The laccase production of *Trichoderma* strains isolated from rhizosphere samples was examined. These experiments were performed on solid media with ABTS or guaiacol as substrates for the detection of laccase activities. Strong laccase activities could be detected on solid media in the case of three *Trichoderma* strains. Among these *Trichoderma* strains, two proved to belong to the species *T. asperellum* (SZMC 20786 and SZMC 20866) while one proved to be *T. atroviride* (SZMC 20780). One of the *T. asperellum* strains (SZMC 20866) showed positive reactions both on ABTS and guaiacol as substrates. The other *T. asperellum* strain (SZMC 20786) and the *T. atroviride* strain (SZMC 20780) showed positive reaction only on guaiacol and on ABTS, respectively. Based on these results we selected these three *Trichoderma* strains for further examination of laccase activities.

Laccase production in liquid cultures

The laccase production of these three *Trichoderma* strains was monitored during a cultivation period of four days. Our results showed that laccase production was detectable in the fermentation period when malt extract liquid media were used. Addition of mineral solution to the liquid media decreased the relative laccase enzyme activities. The laccase enzyme production of the tested *Trichoderma* strains reached the maximum level on the second day in malt extract liquid media. In the case of *T. atroviride* SZMC 20780 the relative laccase activity was the highest on the second day (*Figure 1*). On the third day we measured high relative laccase activity in the case of *T. asperellum* SZMC 20786.

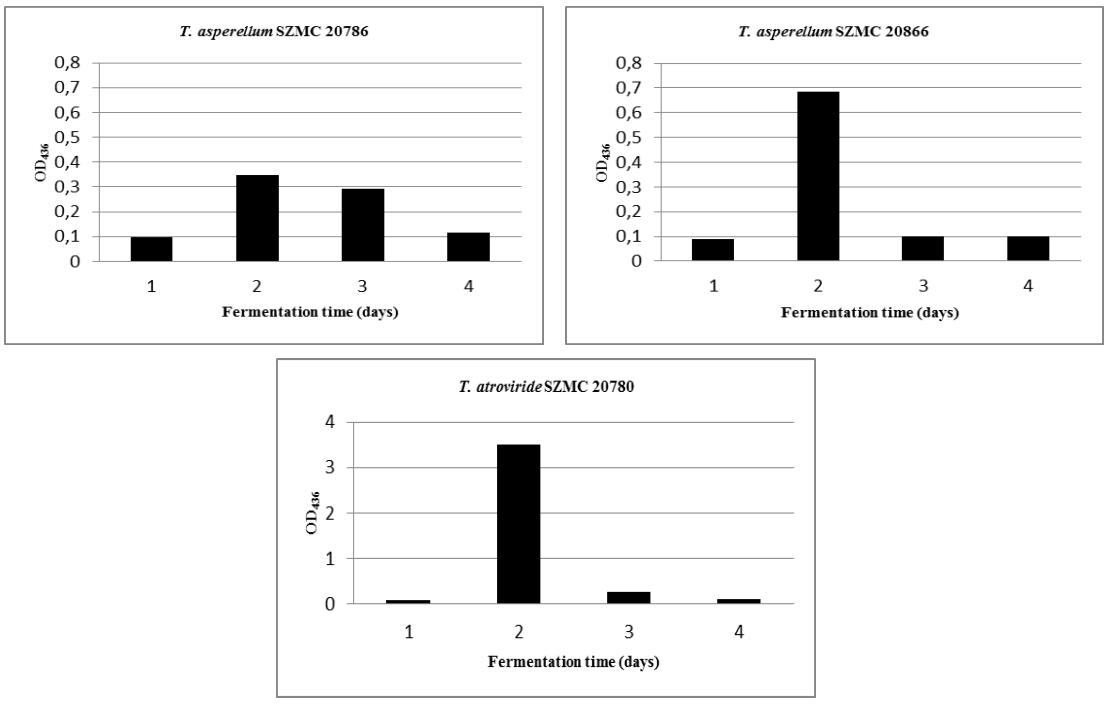

Figure 1. Laccase enzyme activities of the three laccase producer *Trichoderma* strains in malt extract liquid media.

pH-dependence of the laccase activities

As described above, the laccase enzyme activity was high on the 2nd and 3rd day at pH 3.5 in malt extract liquid media. The cell-free ferment broths taken at these two time points

were selected for further studies. Laccase activities were measured at pH 3.5, 4, 5, 5.5, 6 and 6.5.The pH optimum of the laccases produced by the two *T. asperellum* strains (SZMC 20786, SZMC 20866) was pH 4, while in the case of *T. atroviride* SZMC 20780 it was pH 3.5. This strain had the highest relative laccase enzyme activity among the investigated strains. We observed significantly decreased enzyme activities at pH 6.0 and 6.5 in the case of all examined *Trichoderma* strains (*Figure 2*).

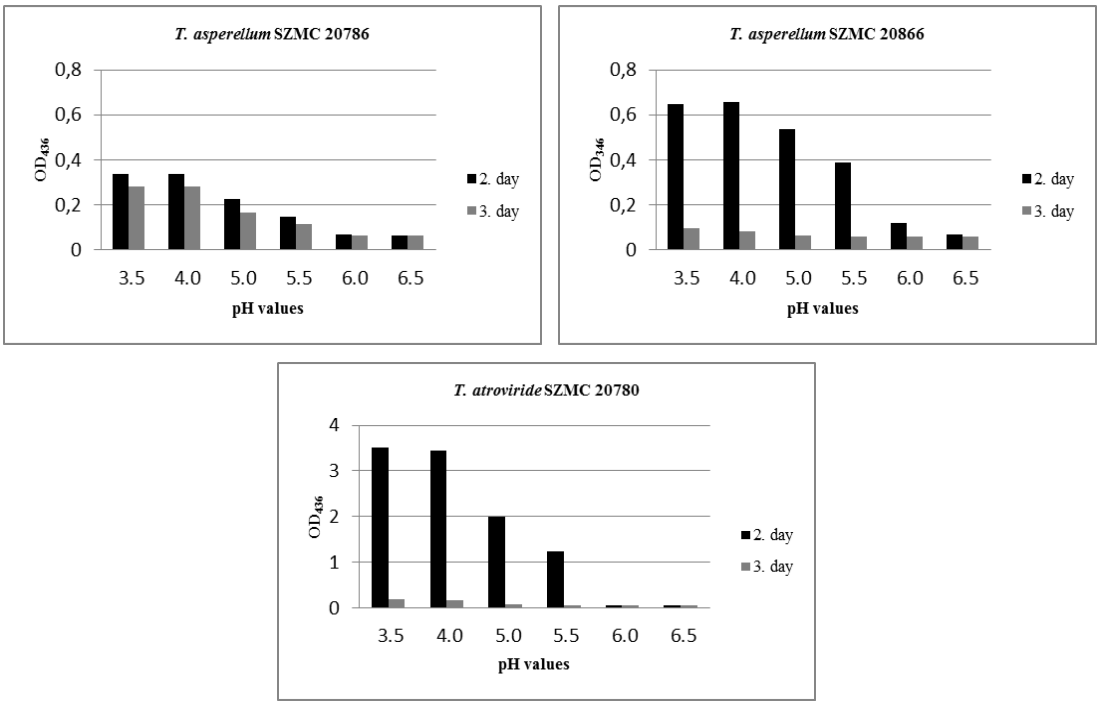

Figure 2. Laccase enzyme activities of the three laccase producer *Trichoderma* strains at different pH values in 25 mM succinate buffer

CONCLUSIONS

Trichoderma strains isolated from vegetable rhizosphere were screened for laccase production. We detected 3 laccase-producing *Trichoderma* strains on solid media which contained two different indicator substrates (ABTS or guaiacol). Two strains were belonging to *T. asperellum* while one was *T. atroviride*. These two substrates proved reliable for laccase activity screening. Laccase production was also examined in malt extract liquid media and the results showed that it reached the maximum level on the 2nd day. The laccase activities were lower when mineral solution was added to the media, suggesting that this solution inhibits laccase activity. HÖLKER ET AL. (2002) reported that the laccase of activity *T. atroviride* appeared on the 7th day. The pH optimum of the laccases produced by the examined *Trichoderma* strains was at pH 3-4. CHAKROUN ET AL. (2010) reported that the pH optimum of a T. atroviride laccase was between 2 and 3.

The laccase-producing *Trichoderma* strains detected during this study will be further characterized and their potential applicability for bioremediation purposes – e.g. for the degradation of xenobiotic pollutants like polycyclic aromatic hydrocarbons – will be evaluated.

ACKNOWLEDGEMENTS

The project is co-financed by the European Union through the Hungary-Serbia IPA Cross-border Co-operation Programme (PHANETRI, HUSRB/1002/214/068).

REFERENCES

CHAKROUN, H., MECHICHI, T., MARTINEZ, M. J., DHOUIB, A., SAYADI, S. (2010): Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds. Process Biochemistry, Volume, *45.* Number 4. pp. 507-513.

GOCHEV, V. K., KRASTANOV, A. I. (2007): Isolation of laccase producing *Trichoderma spp*. Bulgarian Journal of Agricultural Science, Volume *13.* Number 2. pp. 171-176.

HÖLKER, U., DOHSE, J., HÖFER, M. (2002): Extracellular laccases in ascomycetes *Trichoderma atroviride* and *Trichoderma harzianum*. Folia Microbiologica, Volume *47. Number* 4. pp. 423-427.

KIISKINEN, L. L., RÄTTÖ, M., KRUUS, K. (2004): Screening for novel laccase-producing microbes. Journal of Applied Microbiology, Volume *97.* Number 3. pp. 640-646.

KRASTANOV, A. I., GOCHEV, V. K., GIROVA, T. D. (2007): Nutritive medium dependent biosynthesis of extracellular laccase from *Trichoderma spp*. Bulgarian Journal of Agricultural Science, Volume *13*. pp. 349-355.

SADHASIVAM, S., SAVITHA, S., SWAMINATHAN, K., LIN, F. H. (2008). Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochemistry, Volume *43.* Number 7. pp. 736-742.

THURSTON, C. F. (1994): The structure and function of fungal laccases. Microbiology, Volume *140.* Number 1. pp. 19-26.