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Research Article

Studies on Laccase Activity in the Filamentous Fungus *Trichoderma reesei*

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Abstract

Laccase have been mainly studied in wood rot fungal species of the basidiomycetes family especially in white rot fungi. Studies in other fungal families are largely lacking. This study has evaluated laccase activity from *Trichoderma reesei* in catechol based medium. Results showed that laccase enzyme from *T.reesei* was active in acidic pH range and that optimum pH was 4.5. The optimum temperature of laccase from *T. reesei* was also 27^{0}C. Laccase activity in medium containing 10 gL^{-1} catechol was 1.22 U ml^{-1}, which was more than 6 times higher than in medium containing 10 gL^{-1} glucose. Laccase activity of *T.reesei* was also determined in different catechol concentrations. At a concentration of 15 gL^{-1}, laccase activity slightly decreased and the obtained maximum activity was 1.1 Uml^{-1}. Laccase activity of *T. reesei* was found higher than glucose, in the medium containing catechol as carbon source.

Keywords: Laccase activity**,** *Trichoderma reesei*, Catechol
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Trichoderma reesei Küf Mantarında Lakkaz Aktivitesinin İncelenmesi

Özet

Basidiomycetes sınıfında bulunan beyaz çürükçül mantarlar en çok çalışılmış lakkaz üreten mantarlardır. Diğer mantar ailelerinde lakkaz enzimi ile ilgili araştırmalar büyük ölçüde eksiktir. Bu çalışmada, *Trichoderma reesei* küf mantarında ve kateşol içeren ortamda lakkaz aktivitesi incelendi. Deneysel çalışmadan elde edilen sonuçlara göre, *Trichoderma reesei*'de lakkaz enziminin asidik pH'da aktif olduğu gözlendi. Lakkaz enziminin optimum pH'sı 4.5 olarak belirlendi. *Trichoderma reesei*'de lakkaz enziminin optimum sıcaklığı da 27ºC'olarak tespit edildi. 10 gL^{-1} kateşol içeren ortamda, Trichoderma reesei'de lakkaz enziminin aktivitesi 10gL^{-1} glukoz içeren ortam'a göre 6 kat daha fazla bulundu. Lakkaz aktivitesi 1,22 U ml^{-1} olarak belirlendi. *Trichoderma reesei*'de lakkaz aktivitesi farklı kateşol konsantrasyonları için de tespit edildi. 15gL^{-1} kateşol konsantrasyonunda lakkaz aktivitesinin azaldığı gözlendi. 15gL^{-1} kateşol konsantrasyonunda maksimum lakkaz aktivitesi 1,1 UmL^{-1} olarak belirlendi.

Anahtar Kelimeler: Lakkaz aktivitesi, *Trichoderma reesei,* Kateşol

Introduction

Laccase (E.C.1.10.3.2, p-benzenediol: oxygen oxidoreductase) is a multi copper enzyme belonging to the group of blue oxidase that catalyzes the one electron oxidation of a broad range of organic substrates including phenols, polyphenols, anilines, benzenethiols and even certain inorganic compounds, with a concomitant four electron reduction of oxygen to water (Kunamneni et al. 2008; Brijwani et al. 2010; Desai and Nityanand 2011). This makes laccase very useful for wide commercial applications, for instance, in the detoxification of industrial effluents, mostly from the paper and pulp, tex-

tile and petrochemical industries, in polymer synthesis, bioremediation of contaminated soils and wastewater treatment, in wine and beverage stabilization. Laccases are also used as catalysts for the manufacturing of anti-cancer drugs and even as ingredients in cosmetics. Recently, laccase has also been applied to nanobiotechnology Kunamneni et al. (2008). Laccases are widely distributed in fungi, higher plants and also in insects and bacteria. Yoshida first described and extracted laccase from the exudates of Japanese lacquer tree, *Rhus vernicifera* in 1883 (Baldrian 2006; Desai and Nityanand 2011). More than 60 fungal strains, from various classes, such as *Ascomycetes, Deuteromycetes, Basidiomycetes,,* and particullarly many white-rot fungi, have been demostrated that degrade lignin to produce laccase (Gochev and Krastanov 2007; Desai and Nityanand 2011). Laccase have been mainly screened and studied in wood rot fungal species of the basidiomycetes family especially in white rot fungi. Studies for other fungal families are largely lacking. Therefore in this study we have studied laccase activity in *Trichoderma reesei* from another fungal family.

T. reesei is one of the best known cellulolytic organisms having biotechnical importance. It has been extensively studied as biotechnological factory for secreted cellulase enzyme production and used commercially in delignification and biodegradation of cellulose materials in nature. The production of laccase by *Trichoderma* species is very interesting due to combined production of laccases and cellulases, which enlarge the application especially in the degradation of lignocellulosic materials (Rodriguez and Herrara 2006; Gochev and Krastanov 2007; Harman et al. 2012). Only a few publications are concerned on laccase production from Trichoderma spp. The presence of laccase in *Trichoderma atroviridae, Trichoderma harzianum, and Trichoderma longibrachiatum has* been demonstrated respectively by Assavanig et al. (1992), by Hölker et al. (2002), and by Velazquez et al. (2004). It was of new interest to know whether *Trichoderma reesei* with laccase activity could degrade more natural substrates.

In this work the laccase activity of *T.reesei* was evaluated in a culture media containing catechol as a carbon sources. In addition, we compared catechol with glucose for the abilitiy of enhancing laccase activity in *T. reesei*.

Materials And methods

Chemicals

Catechol and glucose were purchased from Sigma (St. Loui MO, USA). Growth medium components were obtained from Merck (Darmstadt, Germany). Other chemicals and reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) unless otherwise indicated.

Fungal culture and culture conditions

The fungal species *Trichoderma reesei* were obtained from Wien (Vienna) Technical University, Applied Biochemistry and Gene Technology Research Center. The fungus was maintained on 4% (w/v) Patateos Dextrose Agar (PDA) at 4 ºC and subcultured every 3-4 weeks. For inoculum preparation fungi were cultured at 28 ºC on slant PDA. After 1 week (seven days), fully grown mycelia mat on an agar plate were used for the cultivation of inoculum. Two of 10x10 pieces of fully grown mycelia were transferred into the 500 ml Erlenmeyer flask including 100 mL of laccase production medium. Cultivation was carried out in an orbital shaker incubator at 27 ºC, 127 rpm for 8 days. Two different laccase-production medium (Table1) were prepared in two solutions, one of which one was a glucose based and the other was catechol based medium (Kahraman and Gurdal 2002; Pazarlıoğlu et al. 2005).

Sampling

2 mL of samples were taken every 24 h from each flask .Samples were centrifuged to remove suspended biomass. Protein concentrations and laccase activities were determined in supernatant.

Laccase assay

Laccase production was assessed by measurement of enzyme oxidation of 2, 2'Azinobis – (3-ethylbenzothiazo-line-6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon = 3.6x\ 10^{-4}\ cm^{-1}\ M^{-1}$). The reaction mixture contained 300 mL of super-

natant with extracellular fluid, 300mL of 1mM ABTS and 100mM sodium acetate buffer (pH 4.5). The reaction mixtures were incubated at 30^0C for 10 minutes and monitored at 420 nm with a spectrophotometer (Agilent Technologies). One unit of enzyme activity is defined as the amount of enzyme that oxidizes one µmol ABTS in 1 min. To calculate laccase activitie following formula was applied, where **V** is total reaction volume; **v** is enzyme volume; d beam path (cm); ε is the molar extinction coefficient of ABTS equal to $3.6x\ 10^{-4}\ cm^{-1}\ M^{-1}$ Pazarlıoğlu et al.(2005).

$$U/L = (V / v.d.\varepsilon)\ (\Delta A.\Delta t)$$

Effect of pH and temperature on laccase activity

The pH optimum for the activity of the laccase was determined by carrying out the laccase assay with ABTS at fixed assay temperature of 25^0C at various pH between 3.5 and 5.5 using sodium acetate buffer (100mM). Optimum temperature for the activity of the laccase was also determined at selected constant pH, between temperatures ranging from 20 to 40^0C. In each case the substrate was preincubated at the required temperature.

Protein content

Protein was determined by the method Bradford with bovine serum albumin as standard Bradford (1976).

Table 1. Standard medium

Component		gL^{-1}
Solution A	**Solution B**	
$NH_4H_2PO_4$	$NH_4H_2PO_4$	1.0
$MgSO_4\ x7H_2O$	$MgSO_4\ x7H_2O$	0.1
$CaCl_2$	$CaCl_2$	0.05
Yeast extract	Yeast extract	0.3
Catechol	Glucose	10

Results And Discussion

Effect of pH and temperature

There are many studies dealing with the production of laccase by white rot fungus and the addition of inducer to stimulate laccase formation. Phenolic compounds were shown in studies to be the most effective inducer in laccase formation. Most of these were phenol, catechol, and guaiacol and ferulic acid. Pazarlıoğlu et al. (2005) Phelolytic microorganisms have the ability to degrade these compounds in microbial cultures. In the literature, there is infomation regarding the use of yeast cultures growing on phenolic compounds, especially growing on catechol, while there is lack of information about the impact of phenolic compounds on the growth of the filamentous fungi. Rigo et al.(2010). Thus, this study focused on both optimizing of pH and the temperature conditions for laccase formation and on the concentration of catechol as carbon source compaired with glucose in *T. reesei*.

The effect of pH and temperature on laccase production is scarce but several authors described that pH between 4.5 and 6.0 is suitable for enzyme production. Thurston. (1994) The optimum temperature for laccase production is between 25^0C and 30^0C. When fungus were cultivated at temperatures higher than 30^0C the activity of enzyme was reduced . Brijvani et al.(2010). We also detected pH and temperature for the highest laccase activity from *T. reesei*. The effect of pH value on the activity of laccase in *T.reesei* was examined in the range 3.5-5.5 as shown in Figure 1.

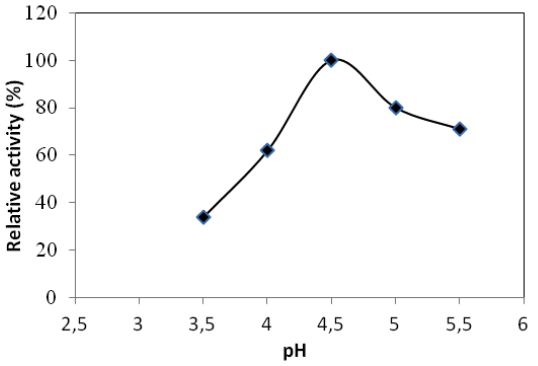

Figure 1. Relative activity (%) of laccase as a function of pH at 25^{0}C

The enzyme appeared to be active in acidic pH range and the optimum pH was 4.5. The temperature effect for laccase in acetate buffer (pH:4.5) is shown in Figure 2. The optimum temperature was also around 27^{0}C. It is exactly the same as early reported.

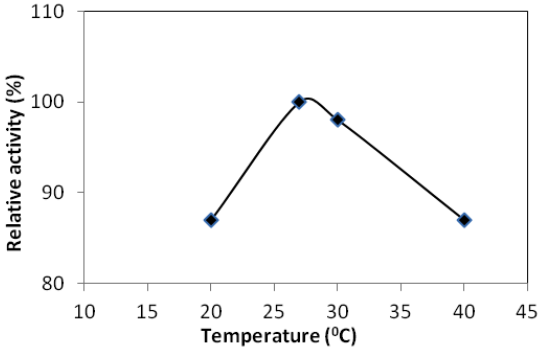

Figure 2. Relative activity (%) of laccase as a function of temperature at pH 4.5

Effect of catechol on laccase activity

In Figure 3, the ability of *T. reesei* using catechol as a carbon source compared to glucose was examined for production of laccase enzyme. According to the literature exceeding concentration of glucose as a carbon source in cultivation has an inhibitory effect on laccase production and increased amount of glucose in the media results in a delay of the laccase production. Eggert et al. (1996). In the growth of 10 g L^{-1} glucose medium, Gochev and Krastanov (2007) have demonstrated that laccase activity in four *Trichoderma strains* were respectively *T.atroviride* (1.5 U mL^{-1}), *T.longibrachiatum* (1.7U mL^{-1}) , *T.viride* (2U mL^{-1}) and *T.reesei* (0.2U mL^{-1}). *T.reesei* have characterized with the lowest laccase activity. Gochev and Krastanov (2007) Different carbon sources could be used for enhancing the activity of laccase in *T. reesei* Brijwani et al. (2010). In this work, *T. reesei* was also cultured in pure culture with catechol as the only carbon sources in laccase production medium. As shown from Figure 3, while the medium containing 10 gL^{-1} glucose was used as a carbon sources, less laccase activity was obtained. As seen n Figure 3, within 3 days maximum obtained laccase activity was 0.18 U ml^{-1}.

However, maximum laccase activity in the culture medium containing 10 gL^{-1} catechol was 1.22 U ml^{-1}. It was more than 6 times higher than in medium containing 10 gL^{-1} glucose.

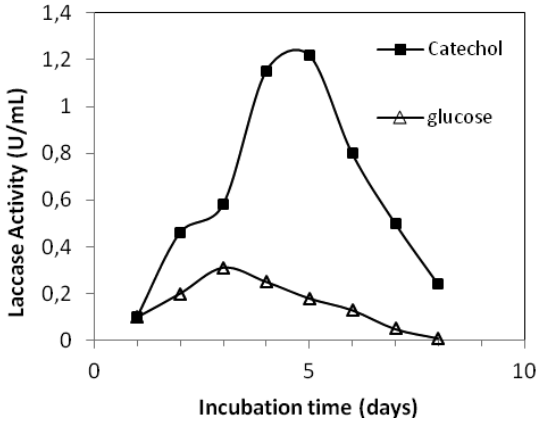

Figure 3. Effect of cathecol on laccase activity. Cells were cultivated at 27 ^{0}C and pH:4.5 for 8 days on rotary shaker at 176 rpm.

The specific activity was 1.9 unit per mg of extracellular protein (Figure 4.). Both activity and specific activity of laccase increased up to the 5-day culture.

The effect of different catechol concentrations (5gL^{-1}, 10 gL^{-1}, 15 gL^{-1}) on laccase activity in 5-day cultures of *T.reesei* is also shown in Figure 5. The highest laccase activity was observed in culture including the catechol concentration of 10 g L^{-1}. Laccase activity in the medium containing 10 g L^{-1} catechol was 1.22 U ml^{-1}. At the catechol concentration of 15 gL^{-1}, laccase activity decreased to 1.1 U ml^{-1}. The maximum laccase activity in different catechol concentrations changed in a narrow range.

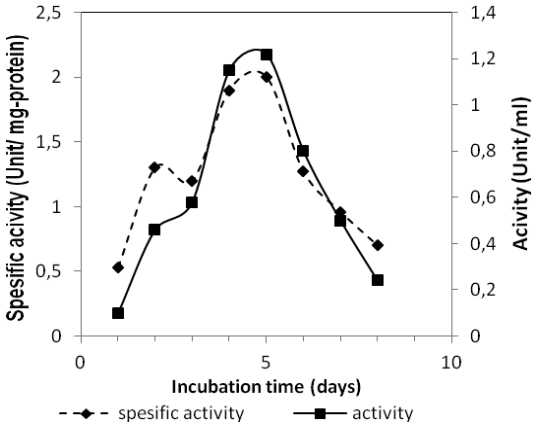

Figure 4. Effect of catechol on laccase activity. Cells were cultivated at 27 ^{0}C and pH:4.5 for 8 days on rotary shaker at 176 rpm.

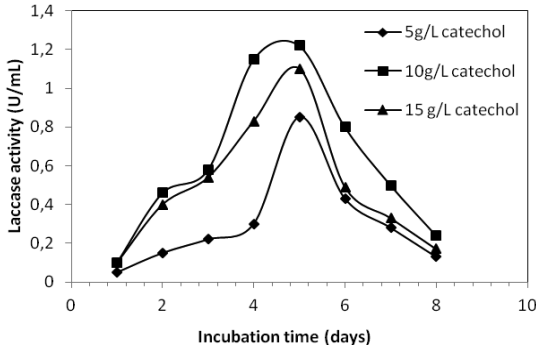

Figure 5. Effect of catechol in a different concentrations on laccase activity. Cells were cultivated at 27 ^{0}C and pH:4,5 for 8 days on rotary shaker at 176 rpm.

Conclusion

It is clear in this study that catechol as a carbon source in the culture medium enhances laccase production in *Trichoderma reesei*. However, after the certain concentration due to ihibition of cell growth, the catechol has reducing effect on the activity of laccase. Last but not least, *Trichoderma reesei* capable of using catechol as the carbon source could make this fungi considerable for bioremediation technologies.

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