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Production of laccase without inducer by *Chaetomium* species isolated from Chettaba forest situated in the East of Algeria

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A positive laccase strain which showed a positive reaction with guaïacol was isolated from Chettaba Forest, which is situated in Constantine, the East of Algeria. It was identified as *Chaetomium* species (Ref 051A) according to the morphological and ribosomal internal transcribed spacer (ITS) DNA genomic sequence analysis. Laccase activity was determined by using 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS) as a substrate. Its highest activity was achieved by using potato dextrose broth (PDB) as a culture medium. Metal ion $CuSO_4$ had no positive effect on laccase production. The laccase activity obtained in submerged fermentation (20 L) was higher than that produced in Erlenmeyer flask (500 ml). The study of pH and temperature effects showed that pH optimum was 5 and 6, and temperature was 35°C. Laccase produced by *Chaetomium* spp. can be used in industrial production.

Key words: *Chaetomium* species, guaïcol, laccase, 2,2-azinobis(3- ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS), submerged fermentation.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a part of a broad group of enzymes called polyphenol oxidases containing copper atoms in the catalytic centre and are usually called multicopper oxidases (Khushal et al., 2010), which are able to oxidise a variety of organic and inorganic compounds, including mono-, di-, and polyphenols, aromatic amines, carboxylic acids, and non-phenolic substrates (Zhixin et al., 2010). Fungal laccases play an important role in plant pathogenesis, pigment production and degradation of lignocellulosic materials (Thurston, 1994; Gianfreda et al., 1999; Sunil et al., 2011). Laccases are widely distributed in higher plants, fungi and insects, but recently, it was found in some bacteria, such as *Streptomyces lavendulae*, *Streptomyces cyaneus*, and *Marinomonas mediterranea* (Thakker et al., 1992; Arias et al., 2003; Jimenez-Juarez et al., 2005). Up to now, fungal laccases were mainly isolated and characterised from ligninolytic basidiomycetes (Baldrian, 2006). A smaller number of laccases was characterised from other fungi, such as

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Ascomycetes and imperfect fungi (Junghanns et al., 2009).

Laccase is important because it oxidizes both the toxic and nontoxic substrates; this enzyme is very specific, ecologically sustainable and a proficient catalyst. It plays an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors (Couto and Herrera, 2006; Faccelo and Cruz, 2008; Shraddha et al., 2011). Recently, laccases have been efficiently applied to nanobiotechnology due to their ability to catalyse electron transfer reactions without additional cofactor (Majolagbe et al., 2012). However, a serious problem often encountered with industrial exploitation of fungal laccases is the low production level by the native hosts. This problem may be overcome by heterologous production in fungal hosts capable of producing high amounts of extracellular enzymes (Sunil et al., 2011). Submerged mode of fermentation has been used intensely for the production of laccase (Shraddha et al., 2011).

The aim of the present paper was to screen and isolate laccase producing fungi from Chettaba Forest's soil samples. The objective of this study was to produce laccase for large scale from the isolated wild strain and its partial characterization.

MATERIALS AND METHODS

Isolation and purification of fungal strains

Soil's sample of botany pine was collected in sterile plastic bags from Chettaba Forest which is situated in Constantine, in the East of Algeria. The isolation of fungal strains was done by employing standard serial dilution method. Soil samples (1 g) were added into 9 ml of physiological water and a serial dilution (10^{-1} to 10^{-6}) was prepared, respectively, then 0.1 ml of each dilution was distributed on potato dextrose agar (PDA) screening medium and incubated at 28°C until apparition of colonies. The fresh fruiting body of each fungus was split by a spatula; a small part of basidiocarp was then picked up and put on PDA Petri dish for 7 days. The PDA screening medium contained potato 20%, glucose 2.0%, and agar 1.5% (Zhixin et al., 2010).

Selection of strains producing laccase

The selection of fungal strains producing laccase was done by following the methods of Budolla et al. (2008) and Thakur and Gupte (2014) with minor modifications. Isolated strains were inoculated on plates containing Olga medium, supplemented with 0.01% guaïacol and incubated at 30°C for 7 days. Laccase activity was visualized on plates by forming reddish brown zones in the medium, caused by laccase which catalyses the oxidative polymerization of guaiacol. The selected strain was identified by Mycotec of Louvain la Neuve (Belgium).

Production of laccase in different media of culture

The method of Das et al. (1997) was used with some modifications. Five discs of potent strain selected previously with 1 cm diameter were inoculated into 500 ml Erlenmeyer flasks, containing 250 ml of three different liquid mediums: Olga g/L (Peptone 3; Glucose 10; KH_2PO_4 0.6; $ZnSO_4$ 0.001; K_2HPO_4 0.4; $FeSO_4$ 0.005; $MnSO_4$ 0.05; $MgSO_4$ 0.5); potato dextrose broth (PDB g/L: Glucose 20; potato extract 200), and ME g/L (Malt extract 20; Peptone 3) at pH 5.5, and kept in incubator shaker at 100 rpm, at 30°C until optimum laccase activity is obtained.

Enzyme essay

After 12 days, fungal mycelium was separated from the broth by filtering it through Whatman No1 filter paper, and then the filtrate was centrifuged at 10000 rpm for 30 min at 4°C.

The culture supernatant was collected and protein concentration was estimated following Folin's method as modified by Lowry et al. (1951) using bovine serum albumin as standard. The colour was changed into blue one, which was measured at 660 nm against the blank (Majolagbe et al., 2012).

Laccase activity was determined spectrophotometrically as described previously by Sunil et al. (2011) with minor modification.

The assay of laccase was based on the oxidation of 2,2-azinobis(3- ethylbenzthiazoline-6-sulfonate) diammonium salt (ABTS) (Sigma) (A420 (ε420, 3.6 × 104 $M^{-1}cm^{-1}$). 100 µl of enzymatic extract was incubated in 900 µl of tartrate acid solution adjusted at pH 4.5 with NaOH 10 N, which contained 10 µl of 10 mM ABTS. The reaction mixture was stopped by adding 50 µl of 50% (w/v) trichloroacetic acid (TCA). Oxidation of ABTS was monitored by spectrophotometer at 420 nm. One unit was defined as the amount of the laccase that oxidized 1 µmol of ABTS substrate per minute.

Effect of metal $CuSO_4$ on laccase activity

It was done by following Zouari et al. (2006) method with minor modification. For laccase production and induction studies, 5 discs of potent strain with 1 cm in diameters were inoculated into 500 ml Erlenmeyer flasks, containing 250 ml of PDB as culture medium, which was supplemented with $CuSO_4\,5H_2O$ (0 - 600 µM) as inducer of laccase.

Production of laccase in submerged medium on fermenter (20 L)

Laccase production was done on fermenter of 20 L. Potent strain was cultivated on PDB, the pH was adjusted and maintained at 5.8 and temperature at 30°C, with agitation of 120 rpm and 100% of dissolved oxygen. At the end of fermentation, the culture was first filtered and centrifuged at 10000 rpm for 30 min at 4°C. The supernatants obtained were stored at 4°C and used as crude enzyme extracts. Laccase's concentration was measured spectrophotometrically each day as explained previously.

Partial characterization of laccase

The optimum of temperature and pH of laccase was determined by following Xia et al. (2014) with minor modification.

The effect of pH on laccase activity was studied by recording the absorbance of enzyme extract catalysed reaction at room temperature after 15 min of incubation using reaction mixture dissolved in buffers over pH range 2.0 to 8.0. Different pH gradients were obtained using sodium acetate buffer (3.0 to 6.0) and sodium phosphate buffer (6.0 to 8.0).

The effect of temperature on laccase activity was determined by recording the absorbance of enzyme extract using reaction mixture

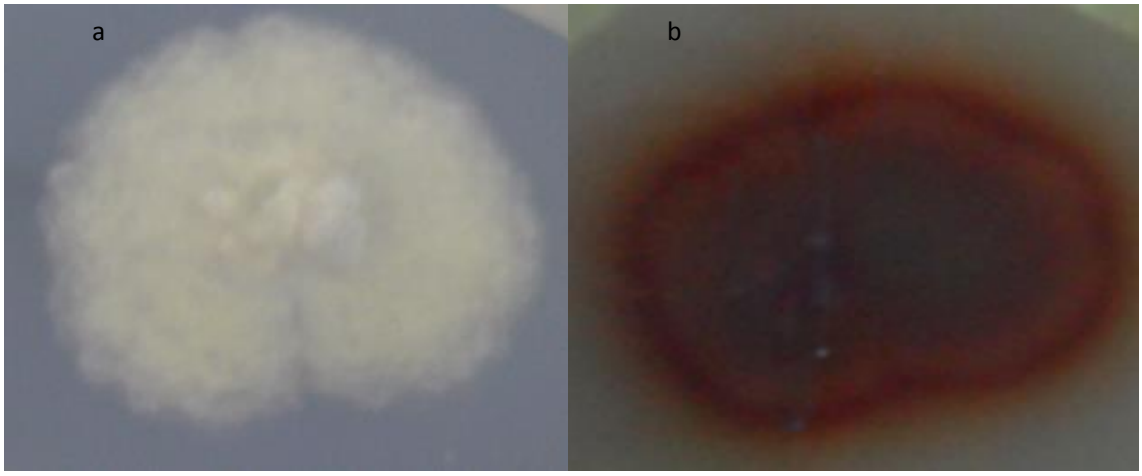

Figure 1. Macroscopic aspect (a) before and (b) after reaction with guaiacol.

incubated for 15 min at pH 5.5 and at temperatures ranging from 15 to 85°C.

The absorbance was recorded spectrophotometrically at 420 nm. The enzyme essay was done as explained previously with ABTS as substrate and the highest laccase activity was taken as 100%.

RESULTS

Isolation and selection of fungal strains

Twelve strains were isolated and purified from samples taken from Chettaba Forest, situated in Constantine, in the East of Algeria.

After 7 days of incubation, 1 fungal strain (P4) had a positive reaction with guaïcol indicating laccase producer (Figure 1). Laccase activity was visualized on plates by forming reddish brown zone in the medium.

The selected strain (P4) did not give spores; this makes the strain identification difficult. However, according to the morphological and ribosomal internal transcribed spacer (ITS) DNA genomic sequence analysis effected by Louvain la neuvemycotec (Belgium); the genus only was known but not the species. It was identified as *Chaetomium* species (Ref 051A).

Production of laccase in different media of culture

Chaetomium spp. was grown and tested on three different mediums; the optimum of laccase activity was obtained after 12 days of incubation, on PDB medium, with 550 U/L of laccase concentration. The use of ME as a medium for the production of laccase gave an important activity when compared with the Olga medium (Figure 2).

Effect of metal $CuSO_4$ on laccase activity

The effect of different $CuSO_4$ concentrations on

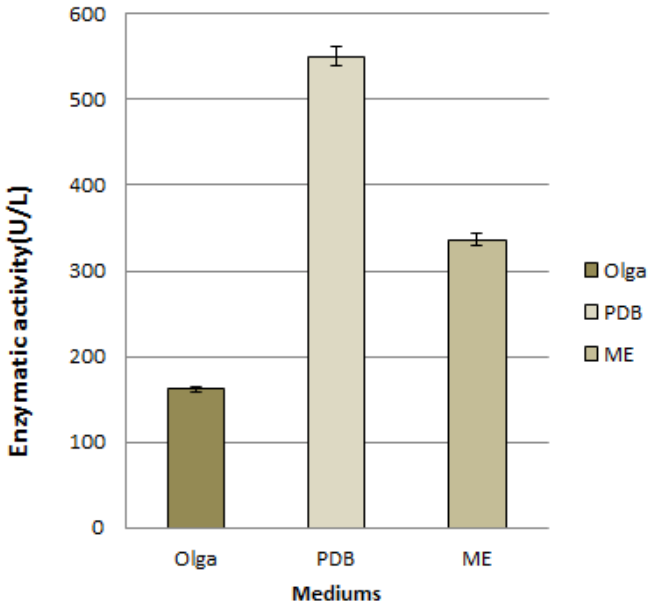

Figure 2. Laccase activity in different meda.

Chaetomium spp. laccase production is as shown in Figure 3. Results show that laccase activity decreases when $CuSO_4$ was added. The maximum laccase activity was obtained without adding any concentration of $CuSO_4$.

Laccase production in submerged fermentation (20 L)

Laccase production increased greatly from the 7th day of incubation and reached the highest laccase activity of 986.63 U/L and protein content of 170 µg/µl at the 12th day (Figure 4); thereafter, the laccase yield dropped rapidly.

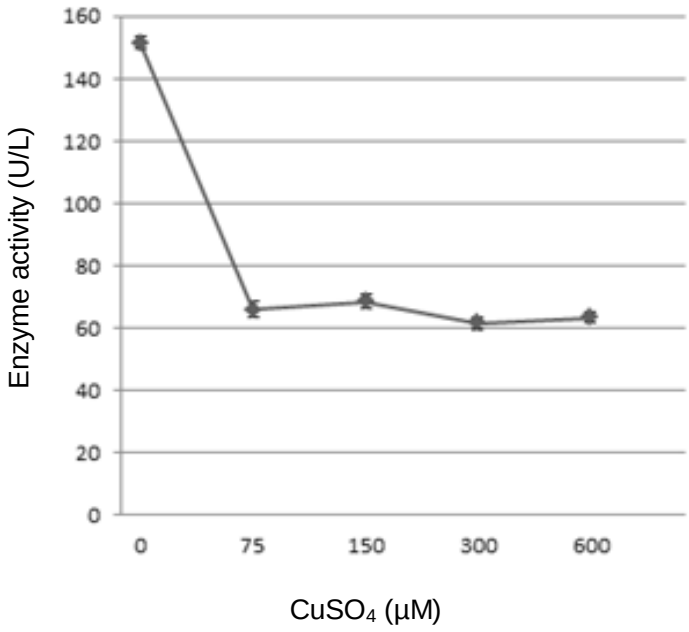

Figure 3. Effect of different concentrations of $CuSO_4$ on laccase activity.

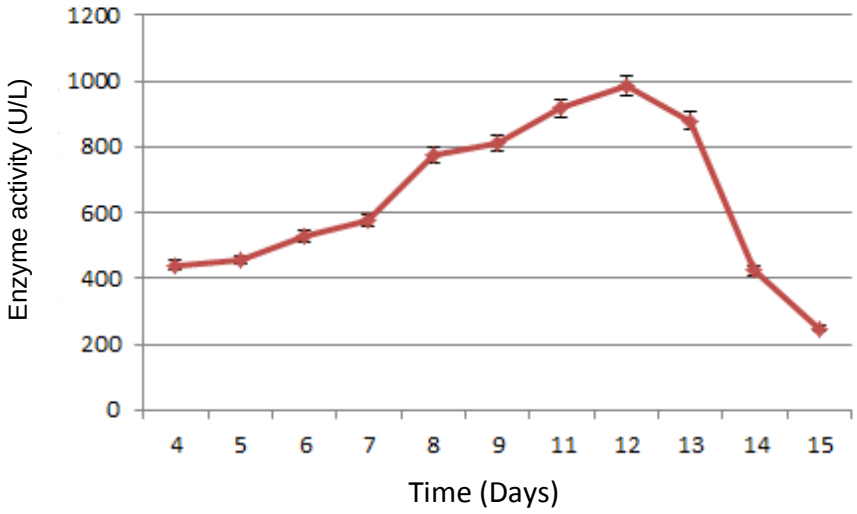

Figure 4. Time course of extracellular laccase activity.

Partial characterization of laccase

The effects of pH and temperature on laccase activity are as shown in Figures 5 and 6. High laccase activity was obtained over a pH range of 5.0 to 6.0. The optimal temperature for laccase activity was 35°C.

DISCUSSION

Laccases were first described in 1883 from the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1983). Since then, several laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and industrial applications (Thurston, 1994; Gianfreda et al., 1999; Xu et al., 2000; Sunil et al., 2011). In this study, a *Chaetomium* spp. (Ref051A) was isolated from Chettaba forest which is situated in Constantine, in the East of Algeria. This strain is able to secret laccase in submerged medium.

El zayat (2008) showed the ability of *Chaetomium globosum* to produce appreciable amounts of laccase

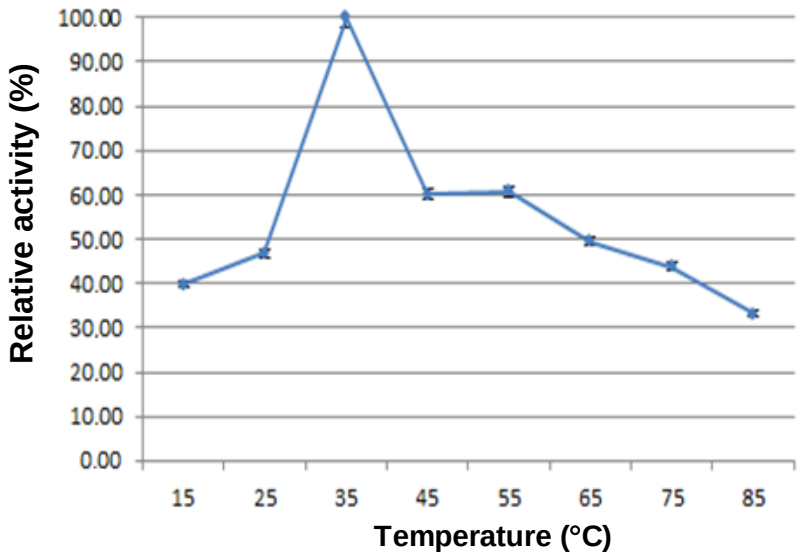

Figure 5. pH optimum with ABTS as substrate.

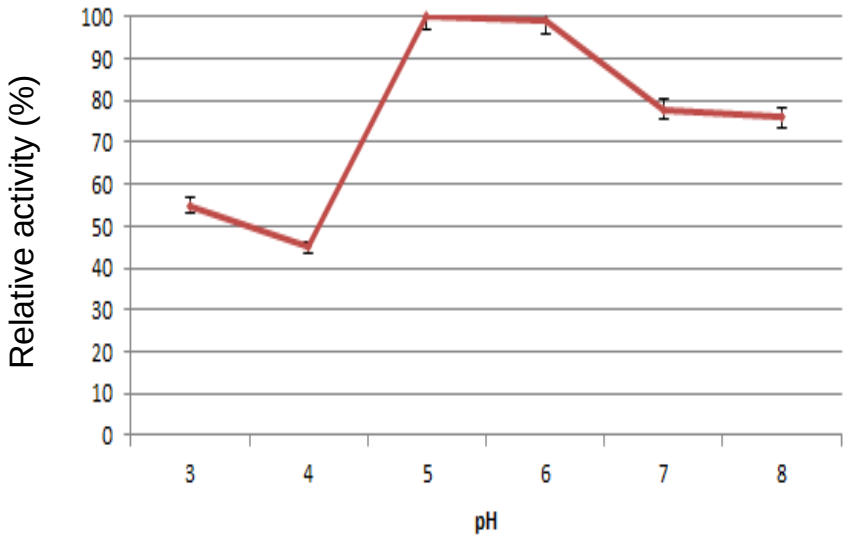

Figure 6. Temperature optimum with ABTS as substrate.

extracellularly. However, Qasemian et al. (2012) confirmed the capacity of *Chaetomium* spp., isolated from a Mediterranean coastal area, to produce an halotolerant laccase.

One of the parameters widely used in the detection of ligninolytic enzymes is the chromogen. In the present study, guaiacol was used as a chromogen. The reddish brown zone surrounding the mycelia of the culture on the plate supplemented with guaiacol was an indication of Bevandamm's reaction (Thakur and Gupte, 2014). Out of the 12 isolates, one isolate showed a positive Bevandamm's reaction for laccase activity. Similar results have also been reported by Viswanath et al. (2008), Patel et al. (2009), Gao et al. (2011) and Thakur and Gupte (2014).

Our results show that the optimum of laccase activity is done by using PDB as a culture medium when compared with Olga and ME, which is similar with the results obtained by Rosales et al. (2002). It can be explained that the extract of potato is used as inducer of production of laccase. However, Buddolla et al. (2008) confirmed that the maximum laccase activity was obtained by using Olga as a culture medium.

The activity of laccase obtained from the isolated strain

without inducer ($CuSO_4$) is more important than that acquired with inducer. Similarly, Sunil et al. (2011) obtained a higher activity without inducer (Tannic acid), though Zouari et al. (2006) showed that the addition of 300 µg of $CuSO_4$ allows the optimum laccase activity to be obtained.

Extracellular laccase activity reached its maximum on the 12^{th} day with 986.63 U/L without inducer, which corroborate with the study of Cordi et al. (2007), but with 40.77 U/L. Dissimilarly, Sunil et al. (2011) showed that the maximum laccase activity was obtained after the 19th day. Therefore, Buddolla et al. (2008) obtained 600 U/L of laccase activity after the 4th day of incubation using PDB as a culture medium.

The effect of temperature is limited in case of laccase production. The optimal temperature differs greatly from one strain to another (Shraddha et al., 2011). Laccase examined in this study had an optimal temperature at 35°C. Generally, the optimal temperature for fungal laccase activity ranged from 30 to 60°C (Nishizawa et al., 1995; Youn et al., 1995).

The optimal of laccase activity was obtained at pH 5 and 6. Generally, the growth of the fungi is ideal at low pH (Sunil et al., 2011). Zhixin et al. (2010) obtained the maximum activity at pH 4.4, which is approximately similar to that realized by Khushal et al. (2010).

Conclusion

The reddish brown zone surrounding the mycelia of the culture on the plate supplemented with guaiacol was an indication of the presence of laccase activity produced by *Chaetomium* spp. The results show that PDB is the suitable medium for laccase production. Partial characterization displays that the maximum laccase activity is obtained over pH range of 5.0 to 6.0 and at 35°C. The laccase activity obtained in submerged fermentation (20 L) is higher than that produced in Erlenmeyer flask (500 ml), which revealed that this strain can be used in industrial production.

Conflict of interests

The authors have not declared any conflict of interest.

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