

LACCASE FROM THE FUNGUS *Simplicillium sp.* GSH 1: EFFECT OF pH AND TEMPERATURE ON ENZYME ACTIVITY AND STABILITY

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Summary

Laccase oxidizes both phenolic and non-phenolic lignin related compounds which makes them very useful for their application to several processes. Laccases have been found to be applicable in textile, food, pulp and paper industries. Laccase can be used in immunoassay, as biosensors as well as biolinkers, degradation of xenobiotics and bio-remediation, organic syntheses, cosmetics and antimicrobials. An extracellular laccase was isolated from the culture filtrate of the ascomycete *Simplicillium sp.* GSH 1. The optimum pH and temperature for the phenolic substrate 2, 6 Dimethoxyphenol (2, 6 DMP) were 4.9 and 55°C, respectively. The enzyme remained stable within an acidic pH range from 3.0 to 6.5 and the thermal stability of the enzyme was also determined and the enzyme remained stable up to 40°C.

Key Words: Laccase, Fungus, Guaiacol, 2, 6-DMP, pH, Temperature and Enzyme stability

Introduction

Laccases are glycoproteins, which are abundant in nature – they have been reported in higher plants and virtually every fungus that has been examined for them. Yoshida described the enzyme of this group for the first time at the end of the 19th century as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (1); more recently, proteins with features typical of laccases have been identified in insects and prokaryotes and algae (2, 3). Though the laccases from different origin are different from each other, they all catalyse polymerization or depolymerization processes. They are an important virulence factor in many fungal diseases as these enzymes can protect fungal pathogens from toxic phytoalexins and tannins (4).

Laccases are found in a wide range of higher plants and fungi (5) and previously some bacterial laccases have also been characterized from *Azospirillum lipoferum* (6), *Bacillus subtilis* (7), *Streptomyces lavendulae* (8), *S. cyaneus* (9) and *Marinomonas mediterranea* (10) and recently some soil algae (3). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (11). The occurrence of laccases in higher plants appears to be far more limited than in fungi (12).

Laccases may also be of great interest in synthetic chemistry in future, where they have been proposed to be applicable for oxidative deprotection (13) and production of complex polymers and medical agents (14, 15). Suberose® (Novo Nordisk A/S, Bagsvaerd, Denmark) is an industrial analogue of laccase has been used in the synthesis of phenolic colourants (16). Laccases are second to none in the application in cosmetic world. Laccase-based hair dyes is one of the example which as compare to other commercially available hair dyes are less irritant and easier to handle. The science behind is just the replacement of irritant H₂O₂ used as an oxidising agent in the dye formulation with laccase (17). More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (18)

In our study, laccase – producing fungus was isolated from various environmental samples using Guaiacol, a chromogenic substrate in agar plates. In addition, the production of laccase by the positive strain was monitored in liquid cultures. The laccase produced in significant amount was preliminarily characterized. pH optima, temperature optima and Laccase stability were studied.

Materials and Methods

Organisms, culture conditions and screening of laccase producers

a. Organism

Isolate *Simplicillium sp.* GSH 1 was selected in a screening procedure involving 21 fungi collected in the soil and water samples and grown on modified malt extract agar (table1) and potato dextrose agar (PDA). The cultures were purified by repeated transfer to fresh agar plates, and grown at 25°C for 9 days and then stored at 4 °C. The isolates were subcultured regularly (19).

Table 1 Modified Malt Extract Medium

Ingredients	gm/L
malt extract agar	45
Peptone	3
Final pH	5.5±0.2 at 37°C

b. Screening

Screening for the presence of laccase activity was done by formation of reddish brown halos (zones) on solid Guaiacol medium (table 2). In addition, 0.01% (w/v) chloramphenicol and chlorotetracycline were added to the media in order to inhibit the growth of bacteria and the pH was adjusted to 5.5 with 9M HCl. The media was autoclaved at 120°C for 30 minutes prior to use (19).

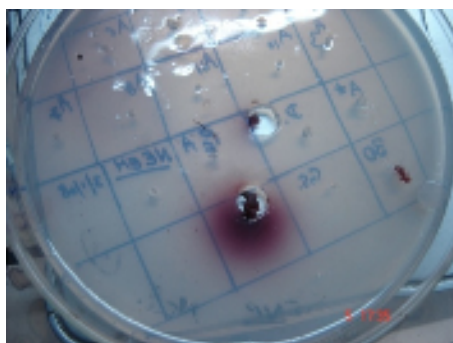


Fig:1 Reddish–brown colonies/ zones apparently due to *Simplicillium sp.* GSH 1 laccase catalyzed polymerization of guaiacol

Table 2 Solid Guaiacol medium

Ingredients	gm/L
Glucose	1
Agar-Agar	15
Yeast Extract	0.5
Guaiacol	20 mM

c. Culture conditions

For production of laccase a flask of 250 mL containing 50 mL of culture medium was inoculated with four mycelial plugs (4 mm diameter) taken from the periphery of a colony grown on PDA at 25°C for 9 days in triplicate on a rotary shaker at 150 rpm. Six day old *Simplicillium sp. GSH 1* was transferred to 20 ml of medium in 100 ml Erlenmeyer flasks (in triplicate). Samples were taken each 24 h after the second day of the fermentation process in the basal medium.

pH and Temperature Dependence

The optimum pH for the laccase was estimated using 2,6 DMP as the substrate in a 100 mM sodium citrate buffer (pH 2.5-6.0) and 100 mM sodium phosphate buffer (pH 6.5-8.0). The effect of pH on the enzyme stability was measured after 1 h of incubation at various pHs at 25°C. The optimum temperature for the laccase was determined by measuring the enzyme activity at various temperatures ranging from 20°C to 90°C in a 100 mM sodium acetate buffer (pH 4.9). The effect of temperature on the enzyme stability was investigated by incubating the enzyme solution for 1 h in a 100 mM sodium acetate buffer (pH 4.9) at various temperatures. After incubation, the remaining activity was determined.

Enzyme Assay

The laccase activity was determined using 2, 6 DMOP as the substrate. The assay mixture contained 10 mM 2,6 DMP, a 100 mM sodium acetate buffer (pH 4.9), and 100- μ l aliquots of an appropriately diluted enzyme solution and 10- μ l sodium azide to terminate the reaction after 3 minutes. The formation of Laccase activity was determined spectrophotometrically by monitoring the conversion of 2 mM 2,6-dimethoxyphenol (DMOP) to 3,5,3',5'-tetramethoxydiphenylquinone (orange/brownish) at 30°C at 468 nm ($\epsilon = 49.6 \text{ mM}^{-1} \text{ cm}^{-1}$), and laccase activity was calculated from the molar extinction coefficient (20).

One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 μ mole of DMOP per minute.

Results

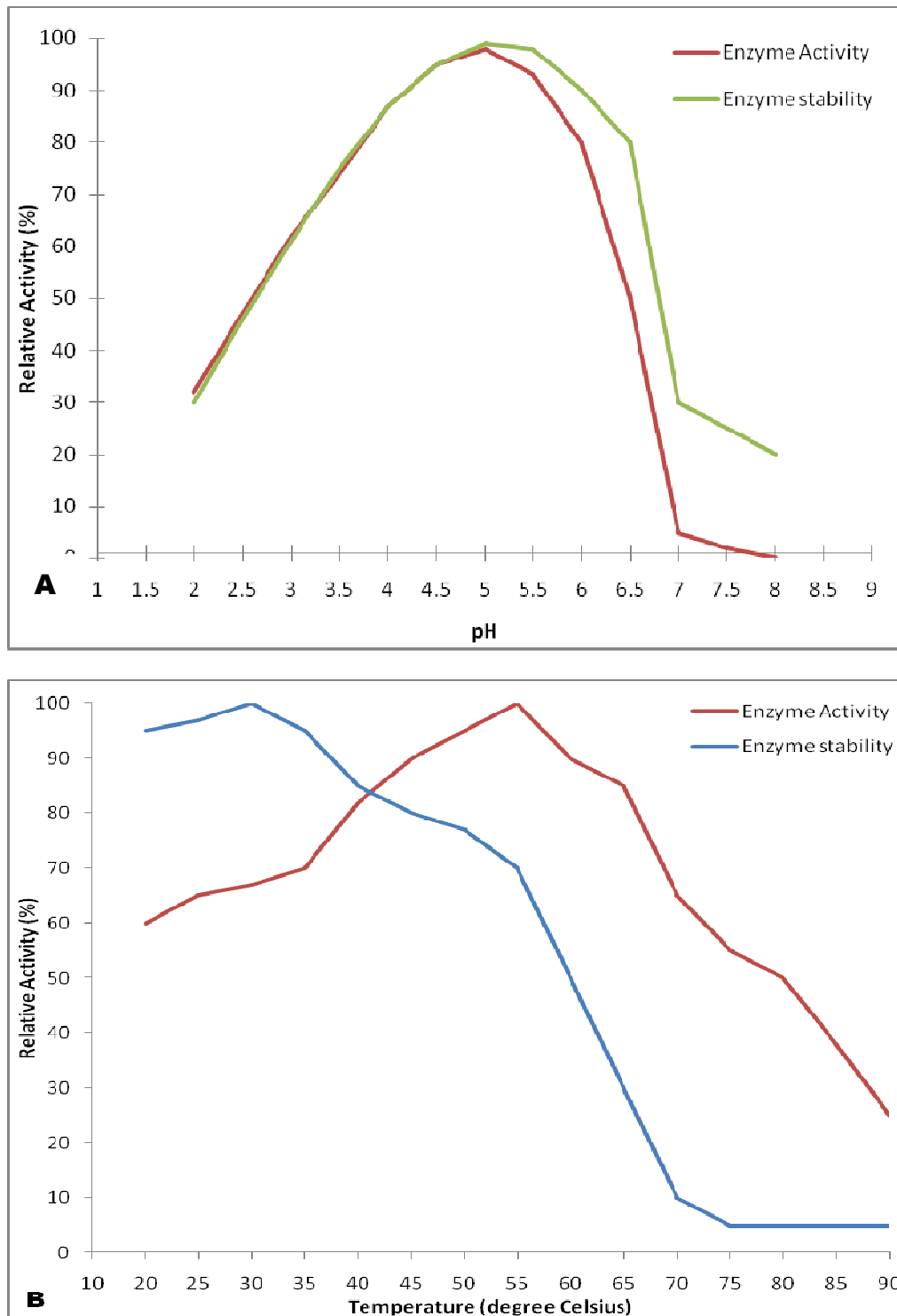


Fig:2 Effect of pH (A) and temperature (B) on the activity of *Simplicillium sp.* GSH 1 Laccase

Effect of pH and Temperature on Laccase Activity and Stability

The effect of pH on the enzyme activity was investigated at pH values ranging from 2.5 to 8.0 with 2,6 DMP as the substrate (Fig.2a). The optimum pH for the enzyme was identified as 4.9, which was consistent with the optimum pH for the laccase *Coriolus zonatus* (21).

Other studies have also reported very low optimal pHs (between 3.0 and 5.7) for fungal laccases, except for the laccase from *Rhizoctonia praticola*, which exhibited a neutral optimal pH with various substrates (22). When the effect of pH on the enzyme stability was examined at 25°C for 1 h, the enzyme remained stable within an acidic pH range from 3.0 to 6.5. Meanwhile, the optimum temperature of the laccase for 2, 6 DMP oxidation was 55°C (Fig.2b) (21), which was higher than the optimum temperatures previously reported for other fungal laccases, ranging from 40°C to 50°C (23-25). The thermal stability of the enzyme was also determined by incubating the enzyme at pH 4.9 for 1 h. The enzyme remained stable up to 40°C, yet the stability decreased rapidly above 55°C, which was similar to the results previously reported for the laccases from *L. edodes* and *A. blazei* (23, 26). However, the enzyme was less stable than the laccases from *Phellinus ribis* and *Trametes sp.* strain AH 28-2, which remain stable at 55°C and 70°C for more than one hour, respectively (25, 27).

Discussion

Therefore, our study found that the *Simplicillium sp.* GSH 1 laccase was very active at a relatively acidic pH and higher temperature when compared with other fungal laccases. Its thermal stability remained till 40°C. In conclusion, the present results showed that the laccase from the Ascomycete *Simplicillium sp.* GSH 1 is a new member of a growing family of laccase enzymes that possess important properties for industrial application. In view of the importance of laccase, the structure and catalytic mechanism of the *Simplicillium sp.* GSH 1 laccase will be studied further.

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