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Biochemical characterization and kinetic studies on a purified yellow laccase from newly isolated *Aureobasidium pullulans* NAC8 obtained from soil containing decayed plant matter

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Abstract The study investigated the biochemical characteristics and kinetic parameters of laccase from a newly isolated *Aureobasidium pullulans* NAC8 obtained from soil containing decay plant litters. This was with a view to identifying the type of laccase and its possible suitability for biotechnological applications.

The fungal strain was identified as *A. pullulans* NAC8 by sequencing of its 5.8S rRNA and adjacent internally transcribed sequences (ITS) 1 and 2. *A. pullulans* NAC8 laccase was purified 2.0-fold with a yield of 59.3% and specific activity of 9.34 μmol/min/mg protein. The kinetic parameters K_M, V_{max}, k_{cat} and k_{cat}/K_M for laccase with guaiacol as substrate were 1.05 ± 0.12 mM, 12.67 ± 0.55 μmol/ml/min, 25.3×10^{-1} s^{-1} and 2.4×10^{3} M^{-1} s^{-1} respectively. Laccase exhibited maximum activity at 45 °C and optimum pH of 4.5. The enzyme showed stability at a temperature range of 45–55 °C after a 2 h incubation. The molecular weight determined on SDS–PAGE was 68.4 kDa. The enzyme was stable at 10% of all organic solvents used but displayed a loss of activity at 50%. 2.5 mM thioglycolic acid (TGA) and 0.05 mM sodium azide inactivated the enzyme. The substrate specificity was guaiacol > catechol > tannic acid > gallic acid. There was no peak observed at 610 nm and the ratio of absorbance at 280 nm and 610 was 26. This suggests a yellow laccase.

The biochemical properties of *A. pullulans* NAC8 yellow laccase makes it potentially useful in several biotechnological applications.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2), multicopper enzymes belonging to the blue oxidases, catalyze the one-electron abstraction from a wide variety of organic

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and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, and metal complexes such as ferrocene, ferrocyanide or iodide, with the concomitant four electron reduction of oxygen to water [1–3]. Laccases are found in plants, insects and bacteria, but the most important sources of this enzyme are fungi. By means of enzymatic catalyzed oxidative reactions, laccase can detoxify phenolic contaminants, such as aromatic amines, to harmless/less harmful products [4]. The suitability of laccases for such processes has been known for some time [5–6]. Lack of substrate specificity introduced laccase as an enzyme able to oxidize a wide range of chemical compounds such as diphenols, polyphenols, diamines, aromatic amines, benzenethiols, and substituted phenols [7–8] as well as different groups of colored pollutants [9–10]. Laccase requires no H_2O_2 for oxidation reaction unlike other oxidases such as peroxidases and these properties make laccase an important enzyme in biodegradation of xenobiotics and phenolic compounds and decolorization of dyes [11–12].

Yellow/white laccases are rarely studied unlike blue laccases. The major difference between yellow and blue laccases is the lack of an absorption band at 610 nm always found in blue laccases. As a matter of fact, yellow laccases are known to catalyze oxidation without the need for mediators and this makes yellow laccases a better biocatalyst than blue laccases [13]. In this study, *Aureobasidium pullulans*, a black-yeast-like fungus, of immense biotechnological application (such as the production of a battery of industrially important enzymes [14], polysaccharide (pullulans) and antimycotic agent, aureobasidin A [15]) was isolated from soil containing decayed plant litters at an unfarmed site (Latitude N 7°31.2006′ and Longitude E 4°31.5797′), in the Department of Botany, Obafemi Awolowo University Campus, Ile-Ife, Nigeria. Since yellow laccases are often less studied with more focus on the blue laccases, this study investigated the yellow laccase elaborated by *A. pullulans* NAC8, which was subsequently purified, biochemically characterized and the catalytic properties determined. Preliminary investigations on the utilization of this enzyme in decolorization of textile dyes and textile waste water effluents have been carried out in our laboratory [16]. The catalytic properties and laccase type of this enzyme from *A. pullulans* NAC8, has not been reported in any literature. The possible biotechnological applications of this yellow laccase such as in biocatalysis and possible utilization in the detoxification of textile dyes makes it necessary to explore its biochemical and catalytic characteristics.

2. Materials and methods

2.1. Materials

2-Methoxyphenol (Guaiacol), veratryl alcohol, tyrosine, EDTA, gallic acid, phenol, catechol, Diethylaminoethyl (DEAE)-Sephadex and chemicals used in gel electrophoresis of the protein samples were obtained from Sigma Chemical Company, St. Louis (USA). Qiagen DNA Mini Kit and ITS 4 and ITS 5 primers were obtained from Qiagen, Valencia, USA. Protein standards were obtained from Bio-Rad, UK. All other reagents such as those for DNA and protein gel electrophoresis were of analytical grade, were used without further purification and were obtained from either Sigma or BDH.

2.2. Methods

2.2.1. Strain isolation and identification

Fungal strain isolated from soil containing decayed plant litters at an unfarmed site (Latitude N 7°31.2006′ and Longitude E 4°31.5797′), in the Department of Botany, Obafemi Awolowo University Campus, Ile-Ife, Nigeria on Malt Extract Agar (MEA) was screened for laccase production on guaiacol amended agar plate. Culture was maintained at 4 °C on MEA agar slants. Morphological identification was carried out by examination of spores, lactophenol in cotton blue test and light microscopy.

2.2.1.1. Maintenance of fungal cultures and inoculum. For DNA extraction a 5 mm mycelial plug was cut from the growing margin of 7-day old 2% MEA culture and inoculated into 50 ml of potato dextrose broth (PDB) in a sterilized 250 ml conical flask. The cultures were left to grow for 3–5 days at 25 °C on a shaker at 150 rpm, or until sufficient growth was apparent. 3 ml was then aseptically transferred to a fresh 50 ml of PDB in another sterile 250 ml conical flask. This was left at 25 °C for 3 days or until sufficient growth was apparent.

2.2.1.2. Genomic deoxyribonucleic acid (DNA) isolation. The molecular identification was carried out at the International Institute of Tropical Agriculture (IITA) Ibadan, Oyo State, Nigeria. Deoxyribonucleic acid (DNA) was extracted according to manufacturer's instructions with a Qiagen DNA Mini Kit (Qiagen, Valencia, CA). DNA samples were analyzed for 18S rRNA gene amplification and products sequenced. To confirm the protocol was successful in extracting genomic DNA, 5 µl from each extraction was mixed with 5 µl of DNA loading buffer (20% sucrose, bromophenol blue) and run on a 1X TBE (5.4 g TRIS-base, 2.75 g Boric acid, 20 ml 0.5 M EDTA, 1000 ml dH_2O, pH 8.0 with 1 M NaOH) 1.2% (w/v) agarose gel pre-stained with ethidium bromide at 100 V for 60 min.

2.2.1.3. Polymerase chain reaction (PCR) reagents and conditions. The reaction mixture for the PCR contained approximately 20 ng template genomic DNA, 1U PCR buffer (0.05 M KCl, 0.01 M Tris HCl pH 9.0, 0.1% Triton-X), 2.5 mM MgCl2, 200 µM dNTPs, 1U of *Taq* DNA polymerase, 400 µM of each primer (forward primer ITS 5-GGAAGTAAAAGTCGTAACAAGG, reverse primer ITS 4-TCCTCCGCTTATTGATATGC and sterile distilled water to make up to 50 µl of reaction mixture.

2.2.1.4. Ribosomal DNA (rDNA) sequencing. The sequencing reaction was performed by the use of a big dye terminator kit from PE/ABI. The primer used for the sequencing reaction was the reverse ITS4 primer. The PCR protocol for big dye terminator sequencing from PCR products requires 80 ng of template DNA (PCR product), 3.2 pmols primer, 4 µl FS big-dye seq. mix and sterile distilled water to 10 µl.

2.2.1.5. Species identification. For the species identification the sequencing results from the PCR reaction were first edited in a sequence editing program, 'CHROMAS' version 1.45, any undetermined bases were corrected given the shape and size