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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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Kinetics;
Biochemical characterization

**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, $25.3 \times 10^{-1}$ $s^{-1}$ and $2.4 \times 10^{3}$ $M^{-1}$ $s^{-1}$ 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

of the peak recorded in the big dye terminator sequencing. The use of the reverse primer for the sequencing reaction required the Chromas file to be reversed in sequence. Species identification was determined by submitting the edited sequences to the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) to perform a BLAST, nucleotide-nucleotide search (BLASTn). The BLASTn search looks for sequence homology between the submitted sequence and those contained within the extensive database. Results are displayed as matched sequence length, percentage homology and an *E*-value, which is the number of hits one can "expect" to see just by chance when searching a database of a particular size.

### 2.2.2. Laccase isolation

The laccase production for *A. pullulans* NAC8 was carried out by inoculating a well grown fungus (agar plugs of 5.0 mm) in a sterilized medium that consists of (g/L): Peptone (3.0), glucose (10), $KH_2PO_4$ (0.6), $ZnSO_4$ (0.001), $K_2HPO_4$ (0.4), $FeSO_4$ (0.0005), $MnSO_4$ (0.05), $MgSO_4$ (0.5). In order to monitor the production of laccase in the liquid culture medium, 1 ml aliquots of the growth medium were withdrawn at regular intervals of 24 h and were centrifuged at 1000 rpm for 1 min and were analyzed for activity of laccase using guaiacol as the substrate by the method provided in the assay section. Extracellular secretion of laccase in the liquid culture medium by the fungi was determined by plotting the enzyme activity of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of three measurements.

### 2.2.3. Assay for laccase activity

Laccase activity was determined using guaiacol (2-methoxyphenol) as substrate. This phenolic compound was oxidized to a more stable phenone cation radical. The concentration of the cation radical responsible for intense reddish brown color can be related to enzyme activity when monitored at 450 nm. The reaction mixture consists of 0.8 ml of acetate buffer (pH 5.0, 10 mM.), 0.1 ml of 10 mM guaiacol and 0.1 ml of the enzyme solution. The reaction medium was incubated at room temperature (25 °C) for 15 min and absorbance of the reaction medium was taken at 450 nm. The extinction coefficient of guaiacol is 12,100 $M^{-1}$ $cm^{-1}$ [16]. One unit of laccase activity is defined as the amount of laccase that catalyzed the oxidation of one micromole of guaiacol per minute [17].

### 2.2.4. Protein concentration determination

The protein concentration was determined by the method of Bradford [18] using bovine serum albumin (BSA) as standard.

### 2.2.5. Purification of laccase on DEAE-Sephadex A-50 ion-exchange chromatography

For purification of laccase, the fungi was grown in 100 ml culture flasks, each containing 50 ml sterilized optimal growth medium. Maximum activity of laccase appeared on the 6th day of inoculation of the fungal mycelia. On the 6th day, all the cultures in the 10 flasks were pooled together; mycelia were removed by filtration through double layers of cheese cloth. The culture filtrate (200 ml) was then lyophilized and the powdered enzyme reconstituted with 10 mM Tris–HCl buffer, pH 7.2. The reconstituted crude enzyme (3 ml) was layered on a 1 cm × 10 cm column of DEAE-Sephadex A-50 which had previously been equilibrated with 10 mM Tris–HCl buffer, pH 7.2. Fractions (2 ml) were collected at a flow rate of 20 ml/h. Laccase activity in the fractions were determined and the protein concentrations monitored at 280 nm. All laccase active fractions were pooled and dialyzed against 80% glycerol in 10 mM acetate buffer, pH 5.0.

### 2.2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of A. pullulans NAC8 laccase

Molecular weight of the enzyme preparation was adjudged using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [19]. The resolving gel was 10% acrylamide in Tris–HCl buffer (pH 8.8) and the staking gel was 8% acrylamide in Tris–HCl buffer (pH 6.8). Gel was run at a constant current of 100 V. Molecular masses of bands were estimated using the Bio-Rad Precision Plus All Blue Protein Standards (BioRad, #161-0373)

### 2.2.7. Spectrum and copper content

The pure enzyme preparation was then scanned at wavelength between 250 and 800 nm in a UV–Visible spectrophotometer (U-2800, Hitachi, Japan). The presence of metal ions such as copper and manganese was determined using atomic absorption spectrophotometry. The metal ions were estimated by atomic absorption spectrophotometry using an air acetylene flame using Alpha-4 ChemTech spectrophotometer. The sample was extensively dialyzed against distilled water to remove any ions present and then subsequently digested with a mixture of $HClO_4$ and $HNO_3$ (1:1) [20].

### 2.2.8. Kinetic parameter determination

Enzyme kinetics of the purified laccase was studied using guaiacol as the substrate. $K_M$ and $V_{max}$ values for the enzyme were determined from the linear regression of double reciprocal plots obtained by varying the final concentration of guaiacol in the reaction medium from 0.1 to 3.0 mM.

### 2.2.9. Effect of pH and temperature

The pH and temperature optima of the enzyme were determined by measuring the initial state velocities of the enzyme-catalyzed reaction in the solutions of varying pH/temperature keeping the other parameter fixed and drawing graphs of steady state velocities versus the variable parameter. All the data points of the velocity measurements were the average of the triplicate measurements.

### 2.2.10. pH stability

The pure enzyme was incubated in several buffers ranging from pH 3 to pH 6 for two (2) h. The residual activity was routinely determined at thirty minute intervals with guaiacol as a substrate.

### 2.2.11. Thermal stability

Thermal stability of the enzyme was tested by incubating 1 ml at a particular temperature for 120 min. The residual activity was plotted against temperature. Also the Arrhenius plot was plotted and the energy of activation ($E_a$) was estimated.

*2.2.12. Effect of salts on laccase activity*

Effect of salts on laccase activity was investigated using the following salts of concentrations of 6.25–50 mM: $FeCl_3$, $MnCl_2$, $MgCl_2$, $NiCl_2$, $CaCl_2$ and $CoCl_2$. The enzyme solutions with the presence of various salts were pre-incubated for 15 min at 25 °C, the substrate was added and the residual laccase activity was assayed. Enzyme solution without the metals was taken as control with 100% activity.

*2.2.13. Inhibition of laccase activity*

The effect of ethylenediamine tetraacetic acid (EDTA) on laccase activity was determined at 0.05, 0.5 and 2.5 mM, the effect of sodium azide on laccase activity was determined at 0.0005, 0.005 and 0.05 mM and the effect of $\beta$-mercaptoethanol on laccase activity was determined at 0.05, 0.5 and 2.5 mM. The effect of L-cysteine on laccase activity was determined at 0.05, 0.5 and 2.5 mM and the effect of SDS on laccase activity was determined at 0.05, 0.5 and 2.5 mM concentrations in the final assay mixture. The residual activities were expressed as a percentage of the activity. Activity in the absence of inhibitors was taken as 100%.

*2.2.14. Substrate specificity*

Several laccase substrates such as tannic acid, catechol, caffeic acid, para hydroxylbenzoic acid, potassium ferrocyanide ($K_4Fe(CN)_6$), gallic acid, phenol, oxalic acid and tyrosine were used. Laccase activity was measured using 100 µl of the enzyme and 100 µl (10 mM) of each substrate in 10 mM acetate buffer pH 5.0. The relative activities were compared with guaiacol, which was taken as 100%.

*2.2.15. Effect of organic solvents on laccase activity*

The influence of organic solvents on laccase activity was carried out using dichloromethane, methanol, acetone ethanol and dimethylsulfoxide. The organic solvents were added directly to the reaction mixture to achieve 10 and 50% final concentration for each organic solvent. The residual activities were measured under standard assay conditions. The activity of laccase on guaiacol in 10 mM acetate buffer, pH 5.0 without organic solvents would be taken as 100%. The percentage residual activities were plotted against the varied concentrations of the organic solvents.

## 3. Results and discussion

The fungal strain, *A. pullulans* NAC8, shared 82% homology with *A. pullulans* HN2.3, 79% homology with *Aureobasidium mansonii* strain ATCC36276 and 100% homology with *A. pullulans* strains P-18, YY7. The phylogenetic tree was drawn using NJPLOT after alignment of the sequences with the Clustal X software (Fig. 1). The sequence was deposited in the gene bank of the NCBI (Accession No: KX023301). The choice of amplifying the 5.8S gene and the adjacent ITS regions 1 and 2 was to determine the phylogenetic position of the strains. ITS regions are highly variable and can only be aligned with confidence when comparing closely related taxa. Recently, laccase production by several strains of *A. pullulans* was carried out by Rich et al. [27].

Laccase from *A. pullulans* was purified using ion-exchange chromatography on DEAE-Sephadex and dialysis of the active pooled fractions against glycerol had a single peak (Fig. 1) of activity was obtained a final yield of 59.3% and a purification fold of 2.0. The summary of a typical purification is shown in Table 1. The enzyme bound to the ion exchanger and hence it is anionic. Laccase from *Gaeumannomyces graminis* had a 4.6% yield and 120-fold purification [21] using a combination of dialysis and ultrafiltration. Laccase from *Bacillus* sp. ADR was purified using DEAE-anion exchanger with 33% yield and 56 purification fold [22]. A distinct band was obtained corresponding to 68.4 kDa (Fig. 2) hence the molecular weight determined on SDS–PAGE was 68.4 kDa. Fungal laccases vary in their molecular weight but most fall within the range of 60–70 kDa [23]. The molecular weight reported for *A. pullulans* laccase varies. Rich et al. [27] reported molecular weights above 70 kDa after deglycosylation with Endo H and even a particular strain, NRRYL-2568 had a molecular weight of 100 kDa after treatment with Endo H. This suggests that the degree of glycosylation affects the molecular weight from several strains of *A. pullulans*.

The concentrated enzyme was yellow in color, the absorption from 250 to 800 nm gave just a single peak at 280 nm (Fig. 4). There was no peak shown at 610 nm. The ratio of absorption at 280 nm ($A_{280}$) to absorption at 610, ($A_{610}$) was 26. This is characteristic of yellow laccases. The ratio of absorption at 280 nm ($A_{280}$) to absorption at 610, ($A_{610}$) was determined to be 26.0. This was higher than (15–20) meant for blue copper laccases. A value of 36.0 was obtained for *Ganoderma fornicatum* laccase [24]. This suggests that *A. pullulans* NAC8 laccase is a yellow laccase rather than a blue one. Yellow laccases are able to catalyze the oxidation of non-phenolic aromatic compounds in the absence of exogenous mediators [25]. The atomic absorption spectrophotometry showed that the ratio of copper to manganese is 3:1. Laccases contain 4 copper atoms termed Cu T1 (where the reducing substrate binds) and trinuclear copper cluster T2/T3 (where oxygen binds and is reduced to water). The three copper atoms can be distinguished using UV/visible and electronic paramagnetic resonance (EPR) spectroscopy [1]. There have been reports of yellow laccases having less than four copper atoms with the copper atoms replaced with manganese or other metals. Telke et al. [22] reported the existence of 2 copper atoms in *Bacillus sp*. ADR laccase. Atomic absorption spectrophotometry indicated that laccase from *A. pullulans* has three copper atoms and one magnesium atom. Palmeiri et al. [26] reported the existence of just a copper atom instead of four in *Pleurotus ostreatus*. Most non blue laccases probably have the fourth copper atom replaced with another metal and it might be responsible for lack of absorption at 610 nm. (See Fig. 4).

The $K_M$ and $V_{max}$ of the purified laccase of *A. pullulans* were 1.05 ± 0.12 mM and 12.67 ± 1.50 µmol/ml/min respectively for guaiacol. The $k_{cat}$ obtained was $25.3 \times 10^{-1}\ s^{-1}$. The catalytic efficiency, $k_{cat}/K_M$ is $2.4 \times 10^{3}\ M^{-1}\ s^{-1}$. The Michealis–Menten plot is shown in Table 5. The enzyme showed optimum activity at 45 °C. The kinetic parameters obtained for guaiacol falls within reported values for *G. fornicatum* laccase with a $K_M$ value of 1.24 mM. The maximum velocity, $V_{max}$, obtained for *A. pullulans* laccase is 12.66 ± 0.55 µmol/ ml/min. Varying values of $K_M$ and $V_{max}$ are reported for laccases depending on organism and substrates [9]. Substrate binding preference varies significantly among fungal laccases. The optimum temperature obtained for *A. pullulans* laccase was 45 °C. This was similar to laccase from sev-


**Figure 1** Phylogenetic relationship based on homology index for *Aureobasidium pullulans* NAC8. YY7 represents *Aureobasidium pullulans* strain YY7, HN2.3 represents *Aureobasidium pullulans* strain HN2.3, NAC8 represent *Aureobasidium pullulans* strain NAC8.

**Table 1** Summary of purification process for *Aureobasidium pullulans* laccase.

| | Total activity (Units/min) | Total protein (mg) | Specific activity (Units/min/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude | 22.04 | 4.68 | 4.71 | 100 | 1 |
| DEAE-Sephadex A-50 | 12.40 | 1.35 | 9.185 | 56.3 | 1.95 |
| Dialysis against 80% glycerol | 13.08 | 1.40 | 9.34 | 59.3 | 2.0 |

Activity and protein concentration were determined after each step. Enzyme activity was determined using 10 mM guaiacol in 30 mM acetate buffer pH 5.0. Protein was determined using Bradford's method.

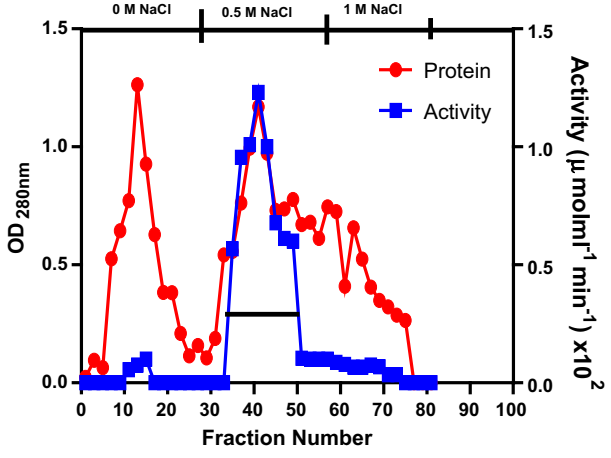


**Figure 2** Ion-exchange chromatography of laccase from *Aureobasidium pullulans* NAC8 on DEAE-Sephadex equilibrated with 10 mM Tris–HCl buffer, pH 7.2. 2 ml fractions were collected at a flow rate of 30 ml/h. Bound proteins were eluted stepwise with 1 M NaCl. —●— protein concentration at 280 nm. —■— laccase activity.

eral strains of *A. pullulans* reported by Rich et al. [27] which had optimal temperature between 50 and 60 °C. The optimal temperature of laccase can differ greatly from one strain to another. Laccase isolated from *Ganoderma lucidum* showed optimum temperature 20–25 °C and was stable between 10 and 50 °C [11]. Laccases isolated from *Marasmius quercophilus* was found to be stable for 1 h at 60 °C and an optimum temperature at 80 °C [11]. The activation energy, Ea obtained for *A. pullulans* NAC8 laccase is 63.4 kJ/K/mol. The Ea obtained for *Coriolopsis floccose* MTCC-1177 laccase is 36.6 kJ/mol/K [5] (see Fig. 5).

Thermal stability studies on the purified enzyme shows that the enzyme is fairly stable at 45–55 °C, as it retained more than 60% of its activity after two hours of incubation at these temperatures (Fig. 6). At increased temperatures ranging from 65 to 75 °C the enzyme lost nearly 80% of its activity after a 2 h incubation. Similar results were obtained for *G.* laccase. It retained nearly 70% of its activity after three hours of incubation at 60 °C [24]. Usually, the initial rate of enzyme activity has been ascribed to increase in kinetic energy which invariably increases the collision between reacting molecules. Sharp decline in the rate of reaction (as seen in this study at 75 °C) could be associated with thermal inactivation of the enzyme. At a critical temperature, an enzyme tends to lose its compact three dimensional structure accompanied by a parallel loss in its activity [28].

The optimum pH 4.5 was obtained for *A. pullulans* HN2.3 laccase (data not included). The enzyme was stable for one hour in all the buffers (ranging from pH 3 to pH 6) used and continued incubation led to a rapid loss in enzyme activity. The pH optima of laccases are highly dependent on the

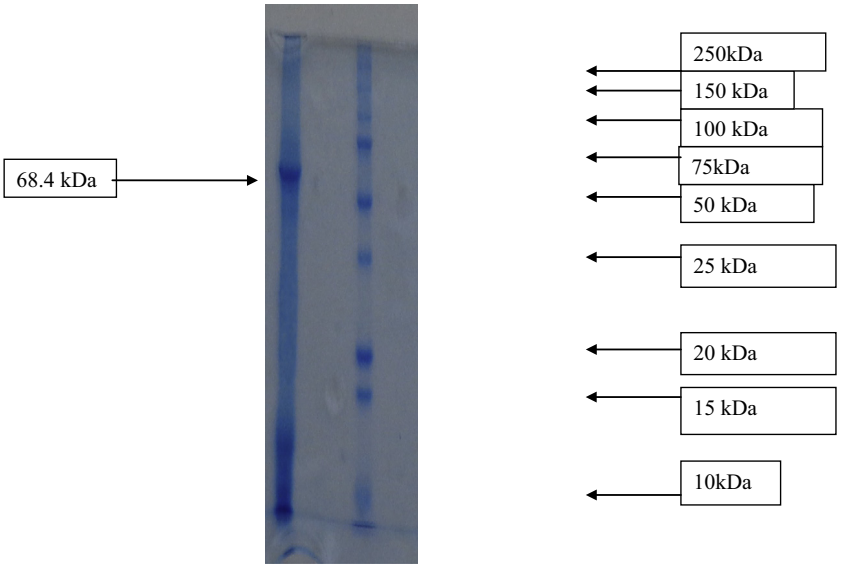


**Figure 3** SDS–PAGE of purified laccase from *Aureobasidium pullulans*.

**Table 2** Effect of several inhibitors on laccase activity.

| Inhibitor | Concentration [mM] | Residual activity [%] |
|---|---|---|
| EDTA | 0.05 | 83.3 ± 4.7 |
| | 0.5 | 71.5 ± 5.7 |
| | 2.5 | 72.4 ± 4.8 |
| SDS | 0.05 | 58.9 ± 1.9 |
| | 0.5 | 34.2 ± 7.9 |
| | 2.5 | 9.0 ± 1.9 |
| Sodium azide | 0.0005 | 80.6 ± 7.2 |
| | 0.005 | 62.0 ± 11.2 |
| | 0.05 | 18.3 ± 6.5 |
| 2-Mercaptoethanol | 0.05 | 70.7 ± 10.7 |
| | 0.5 | 22.6 ± 2.4 |
| | 2.5 | 6.5 ± 1.6 |
| L-Cysteine | 0.05 | 55.9 ± 10.7 |
| | 0.5 | 29.1 ± 7.0 |
| | 2.5 | 6.5 ± 1.9 |
| Thioglycolic acid | 0.05 | 68.0 ± 9.2 |
| | 0.5 | 30.1 ± 2.4 |
| | 2.5 | – |

The residual activities [%] were measured using guaiacol as substrate after adding each inhibitor to the assay mixture to reach the final inhibitor concentrations. The values are presented as mean ± SD of triplicate test.

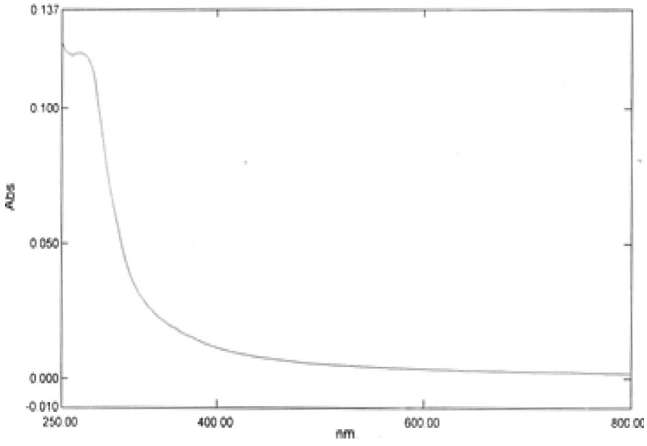


**Figure 4** Wavelengths scan of purified laccase from *Aureobasidium pullulans* NAC8. The wavelength scan was varied from 250 nm to 800 nm.

substrate utilized. For phenols the optimal pH can range from 3 to 7 for fungal laccases. The optimum pH obtained using guaiacol as substrate is 5.0 while the pH is 6.0 with catechol as substrate. Further increase in the pH relatively decreased the activity of the enzyme. The optimum pH obtained for *G. fornicatum* laccase was 2.5, 3.0 and 3.0 with 2,2′-azino-bis (ABTS), 2,6-dimethoxyphenol (DMP) and guaiacol as substrate respectively [24]. The pH obtained for laccase from *Bacillus* sp. ADR were 3.0, 4.0 and 5.0 for o-tolidine, 2,6-dimethoxy phenol (DMP) and guaiacol respectively [22]. The variation may be due to changes to the reaction caused by the substrate, oxygen or the enzyme itself [29].

The degree of inhibition increased in the order TGA > 2-Mercaptoethanol > L-cysteine > SDS > $NaN_3$ > EDTA as shown in Table 2. In general, laccases respond similarly to several inhibitors (thioglycolic acid, azide, diethyldithiocarbamic acid of enzyme activity [30] while ethylenediaminetetraacetic acid (EDTA) to a lesser extent inhibited laccase activity. In this present study sodium azide and thioglycolic acid (a copper chelator) and 2-mercaptoethanol at 2.5 mM concentrations were found to strongly inhibit the enzyme. The enzyme was mildly inhibited by EDTA. Inhibition may be due to binding of type 3 copper, resulting in an interruption of the internal electron transfer and activity inhibition. It can be deduced that copper plays significant roles in regulating the activity of *A. pullulans* laccase. The results obtained here are similar to that obtained by the effects of these inhibitors on laccase activity from *G. fornicatum* [24].

Enzymes for industrial purposes should display a higher degree of stability in organic solvent apart from being

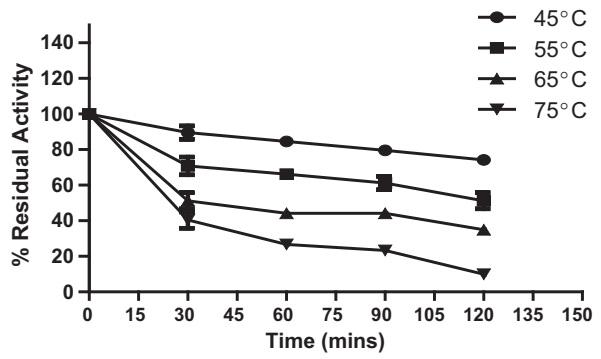


**Figure 5** Heat stability of laccase from *Aureobasidium pullulans*. 1 ml of laccase was incubated at different temperatures [45 °C–75 °C] for 2 h. 100 µl was withdrawn at every 30 min interval and assayed for laccase activity and the residual activity was determined under the standard reaction conditions. The activity at zero time was taken as 100%. The residual activity was plotted against the time of incubation.

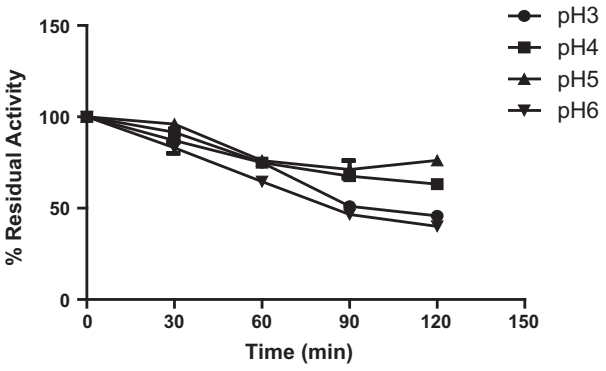


**Figure 6** The pH Stability of *A. pullulans* laccase.

thermostable and pH stable. Laccase was very stable at 10% of DCM, DMSO and acetone and slightly inhibited at the same concentration by ethanol and methanol. At 50% all organic solvent inhibited the enzyme activity as shown in Fig. 7. Laccase was very stable in 10% of all water miscible organic solvents but its activity decreased considerably at 50% final concentration of the organic solvents used. The results obtained were albeit similar to that obtained when for laccase from *G. fornicatum*. It was stable at 10% for all organic solvent but was inhibited by 50% by water miscible organic solvents, acetone and acetonitrile. Although it maintained relative stability in Dimethyl Sulfoxide (DMSO), Dichloromethane (DCM) and methanol [24]. Metal ions play crucial role as inhibitors or enhancers of enzyme activity. Laccase activity was relatively stable with $Mg^{2+}$ but was inhibited at varying degrees by other metals used. The effect of several metals on laccase activity is shown in Table 4. Laccase activity was not significantly inhibited by $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ at 6.25 mM concentrations. Further increase in $Mg^{2+}$ concentrations seems to have no profound inhibition and it's in agreement with reported values for laccase from edible mushroom, *Clitocybe maxima* [31]. $Ni^{2+}$ and $Co^{2+}$ are strong inhibitors at 12.5–50 mM. At least 62–87% loss of activity was observed at these concentrations.

**Table 3** Oxidation of several phenolic and non-phenolic substrates.

| Substrates | Concentration [mM] | Wavelength [$\lambda_{max}$] [nm] | Residual activity [%] ± SD |
|---|---|---|---|
| Guaiacol | 5.0 | 450 | 100 ± 6.3 |
| Catechol | 5.0 | 450 | 85.4 ± 4.1 |
| Tyrosine | 5.0 | 280 | NR |
| Caffeic acid | 5.0 | 420 | 24.3 ± 2.2 |
| Tannic Acid | 5.0 | 458 | 63.1 ± 9.4 |
| 8-Hydroquinolone | 5.0 | 248 | NR |
| Oxalic acid | 5.0 | 450 | 3.7 ± 8.4 |
| Phenol | 5.0 | 370 | NR |
| Gallic Acid | 5.0 | 450 | 13.7 ± 2.2 |
| $K_4[Fe(CN)_6]$ | 5.0 | 420 | 7.54 ± 1.2 |

The oxidation of several substrates was determined with guaiacol oxidation set at 100%. The values shown represent the average from triplicate experiments. Error bars represent the standard deviation.

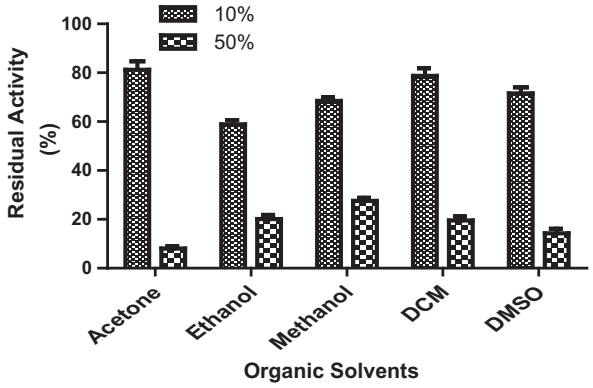


**Figure 7** The organic solvent tolerance of the *Aureobasidium pullulans* laccase. The effect of organic solvents on laccase activity from *A. pullulans* was obtained by varying the final concentration in the reaction medium at 10% and 50%. Control experiments contained no organic solvents. The values shown represent the average from triplicate experiments. Error bars represent the standard deviation.

The oxidation of several phenolic and non-phenolic substrates by laccase is shown in Table 3. The rate of oxidation is Guaiacol > Catechol > Tannic acid > Caffeic acid > Gallic acid > Oxalic acid. There was no reaction with 8-hydroquinolone, phenol, tyrosine and $K_4[Fe(CN)_6]$. Thurston [1] stated that hydroquinone and catechol are good laccase substrates, but that guaiacol and 2,6-dimethoxyphenol (DMP) are often better, but not always. Paraphenylenediamine is a common substrate and syringaldazine is a unique substrate for laccase only. Thus, laccase oxidizes polyphenols, methoxy-substituted phenols, diamines and a vast range of other compounds [1]. *Neurospora crassa* laccase [32] only effectively oxidizes para and ortho-diphenols with the exception of phloroglucinol. In this study, laccase from *A. pullulans* NAC8 was able to oxidize methoxy, di and meta hydroxy substituted phenol compounds [22]. The rate of oxidation of the substrates

**Table 4** Effect of metal ions on laccase activity.

| Metal ion | Relative activity [% of control] | | | 50 mM |
|---|---|---|---|---|
| | 6.25 mM | 12.5 mM | 25.0 mM | |
| $Mg^{2+}$ | 107 ± 2.9 | 87.3 ± 0.9 | 64.6 ± 13.5 | 55.2 ± 5.6 |
| $Mn^{2+}$ | 87.3 ± 4.2 | 54.2 ± 4.2 | 44.5 ± 2.4 | 29.4 ± 1.1 |
| $Ca^{2+}$ | 76.9 ± 18.8 | 54.9 ± 5.5 | 29.7 ± 1.4 | 22.1 ± 5.3 |
| $Ni^{2+}$ | 60.0 ± 12.0 | 46.9 ± 2.8 | 24.9 ± 5.0 | 13.0 ± 1.1 |
| $Co^{2+}$ | 59.1 ± 4.9 | 38.8 ± 3.8 | 29.1 ± 3.3 | 13.0 ± 2.6 |

Assays were carried out in final concentrations of 6.25–50 mM of chloride salts of Magnesium, Manganese, Calcium, Nickel and Cobalt. The relative activity was determined by measuring the laccase activity in the control that contained no chloride salts. The values shown represent the average from triplicate experiments. Error bars represent the standard deviation.

**Table 5** Summary for the kinetic parameters for *A. pullulans* NAC8 laccase using guaiacol as substrate.

| Substrate | $K_M$ (mM) | $V_{max}$ (μmol/ml/min) | $k_{cat}$ ($s^{-1}$) × $10^{-1}$ | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) × $10^3$ |
|---|---|---|---|---|
| Guaiacol | 1.05 ± 0.12 | 12.67 ± 0.55 | 25.3 | 2.40 |

shows laccases not to be a highly specific for a particular substrate. The rate of oxidation is guaiacol > catechol > tannic acid > caffeic acid > gallic acid > oxalic acid. There was no reaction with phenol and tyrosine, this suggests the enzyme purified from *A. pullulans* NAC8 is laccase and not tyrosinase. Also laccase from *A. pullulans* NAC8 was able to oxidize the non-phenolic and non-aromatic substrate, $K_4[Fe(CN)_6]$. This is indicative of laccase's reported potential in utilizing a wide range of substrates. This ability suggests it is capable of detoxification and detoxification of several environmental pollutants such as textile dyes [10].

## 4. Conclusion

This study established the biochemical and kinetic properties of the purified yellow laccase from a newly isolated *A. pullulans* strain NAC8. As earlier established in our previous investigations on this fungi, this strain has enormous potentials in the bioremediation of textile dye waste water. Therefore the results obtained make this enzyme from this strain of *Aureobasidium* a potentially useful target in several biotechnological applications such as in bio catalysis (without the need for mediators) and also have a possible role in bioremediation of textile dye waste water.

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