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Full Paper

Characterization of Laccase from the Fungi *Fusarium* Isolated from Potato Peels Using Carbon and Nitrogen Sources

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Abstract

Laccases (E.C. 1.10.3.2 benzenediol: oxygen oxidoreductase) are an interesting group of N glycosylated multicopper blue oxidase enzymes. They are widely found in fungi, bacteria plants, insects, and lichen. They catalyze the oxidation of various phenolic and non-phenolic compounds, with the concomitant reduction of molecular oxygen to water. Laccase has various applications in industries such as textile dye bleaching, paper, and pulp bleaching, food processing, bioremediation, biodegradation, wood processing, and pharmaceuticals. However, the high cost of production has been a major hindrance to its commercial usage. This study was carried out to investigate the extraction, purification, and characterization of laccase from fungi isolated from potato peels using three different substrates. Extraction was carried out using submerged fermentation, with glucose, lactose, and maltose as the carbon sources and varying nitrogen sources; yeast and ammonium chloride (NH_4Cl) Laccase was also characterized by assessing parameters such as pH, temperature, and protein concentration. Enzyme activity for maltose (yeast), glucose (yeast), glucose (NH_4Cl) and lactose (NH_4Cl) increased from 25^oC -45 ^oC with optimum pH of 6,6,8 and 5 respectively while activity for maltose (NH_4Cl) and lactose(yeast) increased from 25^oC-65^oC with optimum pH at 5 and 8 respectively. This study suggests that increased laccase production from potato peels can be achieved by using maltose, glucose and lactose as carbon sources with NH_4Cl as nitrogen source.

Keywords: *Laccase, guaiacol, potato peels, carbon source, nitrogen source*

Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), a member of enzymes known as the multi-copper containing oxidases, can catalyze oxidation of an array of substrates including mono-,di-, and polyphenols, methoxyphenols, and ascorbate through the simultaneous reduction of oxygen to water [1,2]. Currently laccases are extensively employed in pulping, bleaching, bioremediation, biosensor, food technological uses, and in the treatment of industrial wastewater [3, 4, 5, 6].

The four copper atoms of a typical laccase molecule are divided into type 1 (T1), Type 2 (T2), and binuclear Type 3 (T3) based on unique spectroscopic features. The four copper ions are in the +2 oxidation

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state in the resting enzymes. The T1 and T3 coppers are characterized by absorbance ~600 and ~330nm, respectively, whereas the T2 site lacks strong absorption features. Substrates oxidation occurs at the T1, and electrons are transferred to the T2/T3 trinuclear copper cluster (TNC), where molecular oxygen is reduced to water [7]. Redox potentials of the T1 sites in laccases range from 0.4 to 0.8V; plant and bacterial laccases (e.g., 0.43 and 0.4V for *Rhus vernicifera* and wild-type *Bacillus subtilis* CotA laccases, respectively) typically have potentials on the low end of this range, whereas fungal laccases have higher redox potentials (0.47-0.79V) [7,8]. Laccases may be found in abundance in higher plants, bacteria, fungi, and even insects. *Malus pumila, Brassica oleracea, Brassica rapa, Solanum tuberosum, Pyrus calleryana*, and certain vegetables are among the plant sources [9]. Fungal laccases are considered as the most abundant forms of laccases because more than 60 fungal strains like *Ascomycetes, Deuteromycetes* and white-rot *Basidiomycetes* are excellent sources of laccase [10,11]. Potatoes are one of the most commonly consumed vegetables throughout the world. The global consumption of potatoes as food is shifting from fresh potatoes to value-added processed products such as French fries and chips [12]. Peels are the major byproducts of potato processing industries, which represent a waste disposal problem for the industry concerned [13].

Materials and Method

Materials: Potato peels (collected from Oyingbo market Yaba, Lagos State, Nigeria), $MgSO_4$, $CuSO_4$, K_2SO_4, yeast extract, peptone, NH_4NO_3, NH_4Cl, glucose, lactose, PDA (potato dextrose agar), BSA (Bovine serum albumin), Guaiacol, Chloramphenicol, distilled water, Ethanol, Glycine NaOH buffer, Ferulic acid, Phosphate buffer, Sodium acetate buffer. Other reagents used are of analytical grade.

Isolation of Laccase-producing Organism

A 1g of grinded potato peels sample was measured and added to 10 mL of sterile distilled water and mixed. The suspension was then serially diluted from 10^{-1} to 10^{-5}. 1 mL of each dilution was spread on the surface of Potato dextrose agar which contained 0.02% Chloramphenicol and incubated at 30^{o}C for 7d [14, 15].

Screening of Laccase Producers

The fungal strain was inoculated in potato dextrose agar plate which contained 0.02% Guaiacol and incubated at 30^{o}C for 7d. After incubation, reddish-brown colour zones were observed around the colony since laccase catalyzes the oxidative polymerization of guaiacol [16,14,15]

Macroscopic and Microscopic Examination of Isolated Fungus

The fungal morphology was studied by observing the colony features (color, shape, size, and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue-stained slide with a small portion of the mycelium [17,18].

Production by Submerged Fermentation

Potent fungal strains were cultivated in a seed medium. Mycelia plugs will be transferred into each 250 mL Erlenmeyer flask containing 100 mL seed medium. After cultivation at 30 °C and 120 rpm for 3 d, 3% (v/v) seed culture was inoculated into 50 mL basic nutrient medium and then incubated at 30 °C and 120 rpm for 5 d. Individual carbon sources and nitrogen sources were added to the basal medium. Maltose, glucose, and lactose were tested to examine its effects on laccase production. They were individually added at 2% (w/v) concentration to the basal medium. Two nitrogen sources, ammonium chloride, and yeast extract were individually added at 1% (w/v) concentration to the basic nutrient medium containing the fixed carbon source. Crude enzymes were obtained from supernatants, stored at 4°C, and used for the enzyme assay [19].

Partial Purification of Laccase

After submerged fermentation, the crude fungal culture was filtered through Whatman No 1 filter paper. Ammonium sulfate precipitation was done in an ice bath using the finely grounded ammonium sulfate. The powder was weighed and added slowly to the extract by constant stirring to ensure complete solubility, and the solution was centrifuged at 3000 rpm for 30 min at 4°C. The beaker was then left to stand overnight [14, 15, 20]

Enzyme Assay

Enzyme activity was determined using Guaiacol as the substrate. For this assay, two test tubes were used. 1mL of 10 mM Guaiacol and 3mL of sodium acetate buffer (100 mM, pH 5) were added to each tube. 1mL of culture filtrate was added to one of the tubes to give a final volume of 5 mL and this was used as the test. 1 mL of distilled water was then added to the second test tube, and this was used as the blank. The mixture was incubated at 30°C for 15min, and absorbance was read from 340nm to 470nm using a UV spectrophotometer to determine the best wavelength for enzyme activity.

Enzyme activity was expressed as International Units (IU), where 1 IU is defined as the amount of enzyme required to oxidize 1 Micromole of Guaiacol per minute. The laccase activity in U/ml was calculated using the extinction coefficient of Guaiacol 2.6 $M^{-1}cm^{-1}$ at 340nm by the formula: E.A = $(A * V)$ / $(t * e * v)$

where E.A = Enzyme Activity (U/ml), A = Absorbance at 340nm, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and e = Extinction Coefficient (M-1cm-1) [21].

Characterization of the Enzyme

Enzymes were characterized based on the procedures described by Aruna et al. [14] and Minari and Agho [15] with slight modifications. The effect of temperature on laccase activity was determined by recording the absorbance of enzyme-catalyzed reaction using Guaiacol (10 mM) as substrate dissolved in sodium acetate buffer (10 mM, pH 5.0) incubated at temperatures 25°C, 30°C, 45°C, 55°C, and 65°C. The reaction mixture was incubated for 15mins. The temperature at which the enzyme showed maximum activity was noted as the optimum temperature for the enzyme.

The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalyzed reaction at optimum temperature using Guaiacol (10mM) as substrate dissolved in buffers of different pH (acetate buffer pH 4, pH5, phosphate buffer pH 6, pH7, and tris-HCl buffer pH 8) and was incubated at 25^{o}C for 15 min. Absorbance was recorded at 340 nm [22].

Estimation of Protein Concentration

Working standards 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1mL were pipetted into a series of the test tube and all the tubes were made up to 1 mL using distilled water except the test tube with 1mL which contained only the working standard. Blank containing 1 mL distilled water was also taken. 0.5mL of test solution in was added to a test tube and this was also made up to 1mL with distilled water. 5 mL of alkaline copper reagent (Reagent c) was added to all the test tubes and kept for 10mins at room temperature. Then 0.5 mL of Folins Ciocalteau reagent was added to all the tubes mixed well and incubated at 37^{o}C in the dark for 30 minutes. The blue colour developed was read at 660 nm. A standard graph was plotted with a concentration of BSA on the X-axis and optical activity on the Y-axis [23].

Statistical Analysis

Statistical analysis was done using analysis of variance (ANOVA). The means were determined at 5% confidence levels using Tukey's multiple range tests. All the experiments were performed 3 replicates.

Results

Figure 1a shows whitish creamy coloured fungi growth was observed after 7 days of incubating at 30^{o}C. Laccase-producing fungi were confirmed using guaiacol as an indicator compound. A reddish-brown colored zone was observed around the colonies after incubation at 30^{o}C for 7days as shown in figure 1b. A microscopic view of the fungi growth is shown in figure 2, the cylindrical shape is called phialides with complex branching systems that are morphologically compatible with *Fusarium* species. The positive strains were cultivated by submerged fermentation in a seed medium; a light-yellow colored broth was obtained after 3 days of incubation. The broth was further inoculated in a basal medium containing the maltose, glucose, lactose, yeast extract, and NH_4Cl, a brown-colored broth was obtained with particles after 5days of incubation. A clear solution was obtained after partial purification using ammonium sulfate. Figure 3 shows the absorbance of the enzyme at different wavelengths and it is observed that with the increase in wavelength, the absorbance of the enzyme decreased significantly ($p < 0.05$). The highest absorbance was observed at 340nm while the lowest absorbance was at 470nm.

The effect of temperature on the laccase enzyme activity for each combination of carbon and nitrogen sources is shown in Figure 4. There was an increase ($p < 0.05$) in the enzyme activity of maltose (yeast) from 25°C to 45°C and a further increase in the temperature from 45°C to 65°C resulted in a decrease in the enzyme activity. The enzyme activity for maltose (NH4Cl), showed a significant increase ($p < 0.05$) from 25°C to 65°C, and the maximum activity was at 65°C. For glucose (yeast) and glucose (NH_4Cl), there was a significant increase ($p < 0.05$) in enzyme activity from 25^{o}C to 45^{o}C. From 45^{o}C to 55^{o}C, there was a significant reduction ($p > 0.05$) in enzyme activity and no significant change ($p > 0.05$) when the temperature was further increased to 65^{o}C. Enzyme activity was highest at 45^{o}C and the lowest enzyme

activity was recorded at 25°C while for lactose (yeast), there was an increase in enzyme activity from 25°C to 35°C, it shows a significant reduction (p>0.05) from 35°C to 55°C and increases again from 55°C to 65°C however, in lactose (NH_4Cl) there was the increase in the enzyme activity from 25°C to 45°C, the enzyme activity then decreased from 45°C to 55°C and increases again from 55°C to 65°C. The optimum temperature for lactose (yeast) and lactose (NH_4Cl) was 65°C. Figure 5 shows the effect of pH on the enzyme activity of maltose (yeast) and maltose (NH_4Cl). There was no significant ($p < 0.05$) change in the enzyme activity of maltose (yeast) from pH 4 to5. There was a significant increase ($p < 0.05$) in the enzyme activity from pH 5 to pH 6. There was a significant decrease ($p > 0.05$) in the enzyme activity from pH 6 to pH 7 but no significant reduction between pH 7 to pH 8. The enzyme activity was at its peak at pH 6. For the maltose (NH_4Cl), there was an initial significant increase in the enzyme activity ($p > 0.05$) between pH 4 and pH 5. Significant reduction ($p < 0.05$) was observed between pH 5 to pH 8. The enzyme activity was highest at pH 5, for glucose (yeast) and glucose (NH_4Cl) a significant increase ($p < 0.05$) was observed in enzyme activity from pH 4 to 6. Further, an increase in pH from pH 6 to 8 resulted in a significant reduction ($p > 0.05$) in enzyme activity. Enzyme activity was highest at pH 6 and lowest at pH 8 while for lactose (yeast) there was a significant reduction in enzyme activity from pH4 to pH5, it then increases significantly from pH5 to pH6, but there was no significant reduction (p>0.05) from pH6 to pH7 and it increased again from pH7 to pH8 and lactose (NH_4Cl), the enzyme activity increases from pH4 to pH5, it then decreases from pH5 to pH7 and increased from pH7 to pH8. Determination of the unknown protein concentration of the enzyme is shown in Figure 4. The total yield of the partially purified laccase enzyme was estimated at 59.57µg/ml for maltose (yeast), 181.18ug/ml for maltose (NH_4Cl), 97.01µg/ml for glucose (yeast), 45.90µg/ml for glucose (NH_4Cl), 194.714µg/ml for lactose (yeast) and 503.2µg/ml for lactose (NH_4Cl). Laccase production was highest with the value of 0.210U/ml when glucose and NH_4Cl were used as carbon and nitrogen sources respectively, 0.186U/ml for lactose (NH4Cl), 0.171U/ml for lactose (yeast), 0.162U/ml for glucose (yeast), 0.128U/ml for maltose (NH4Cl) and 0.091U/ml for maltose (yeast).

(A)  (B) 

Figure 1. (A) White mycelia fungi growth after 7 days of incubation of the cultured potato peels. (B) Fungi growth indicated the presence of laccase from potato peels.

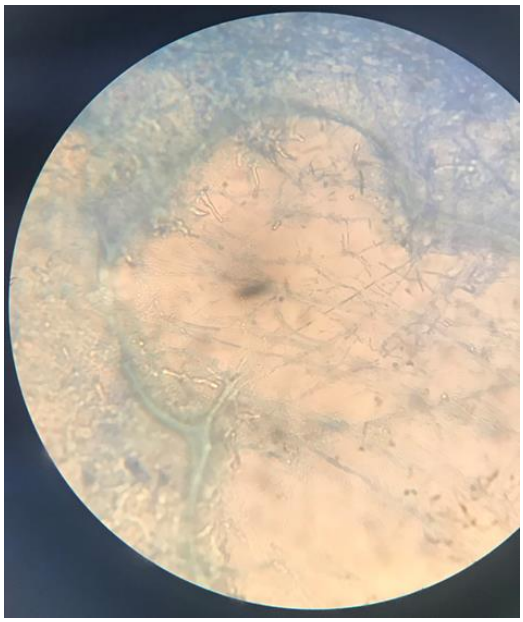
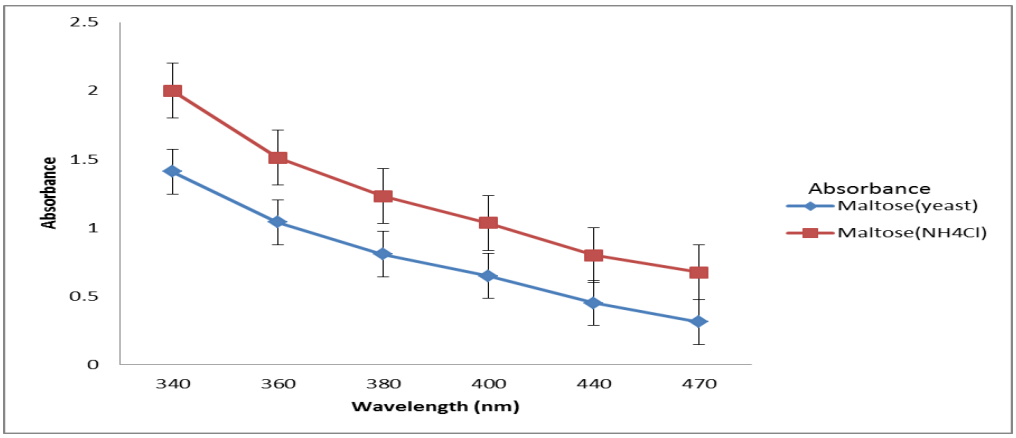
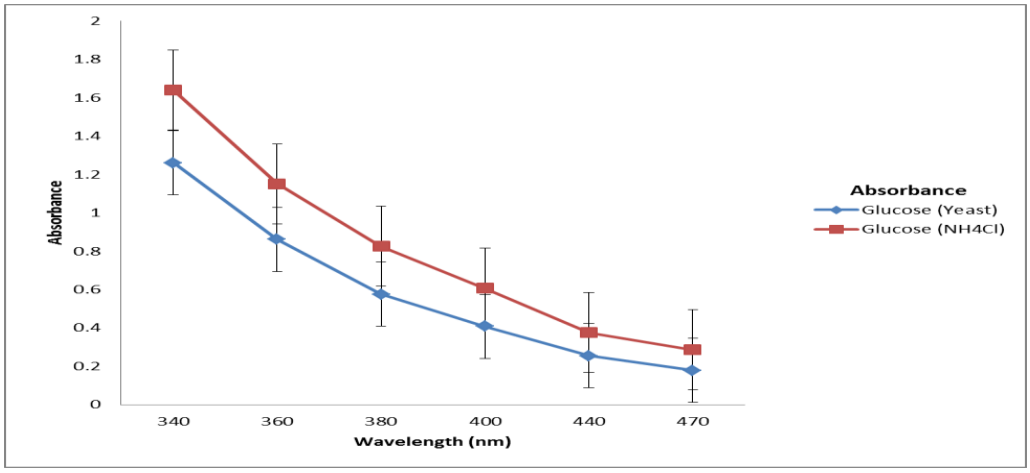


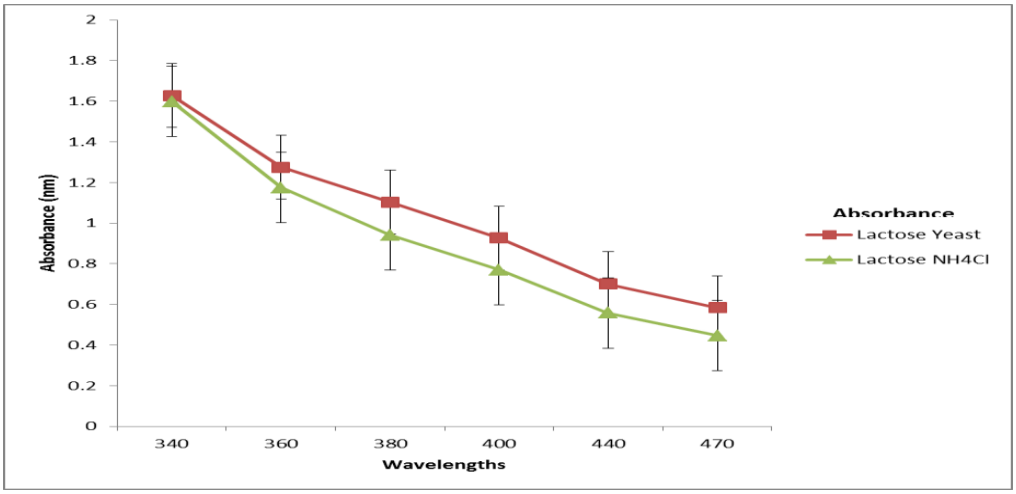
Figure 2. Microscopic image of the characteristics of the fungi that indicated presence of laccase.



(A)

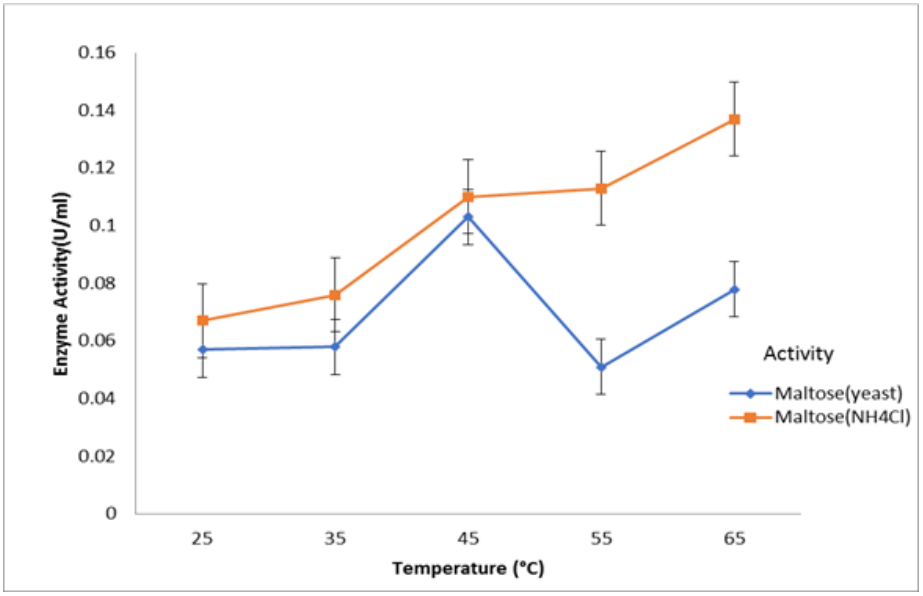


(B)

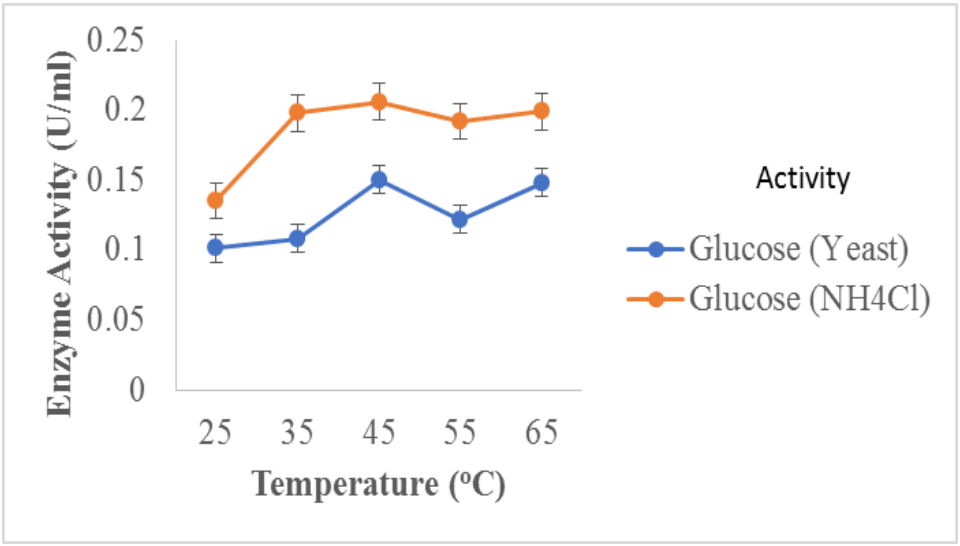


(C)

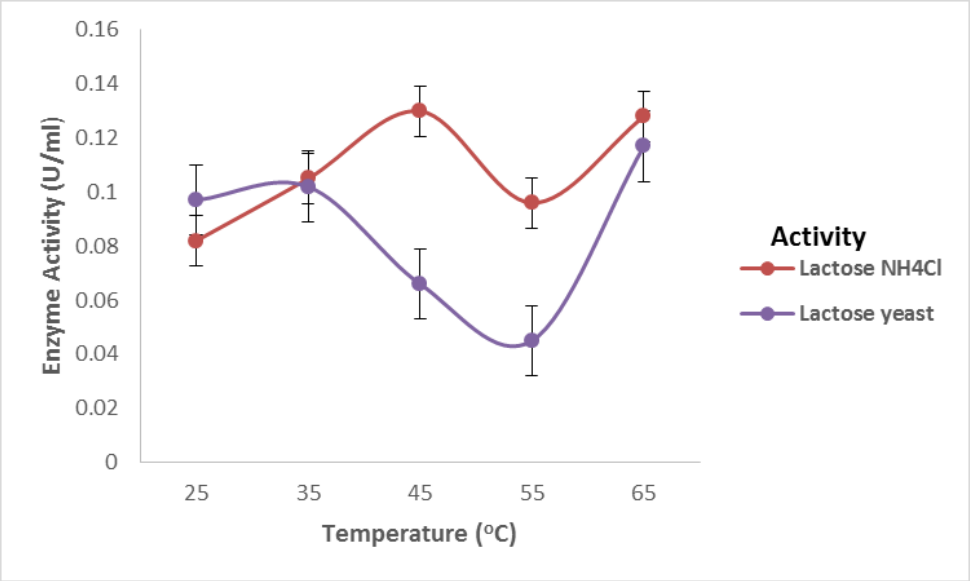
Figure 3. Absorbance of partially purified laccase obtained from the culturing of potato peels with the carbon and nitrogen sources at different wavelengths. **(A**) Maltose (yeast and NH_4Cl). (**B**) Glucose (yeast and NH_4Cl). (**C**) Lactose (yeast and NH_4Cl) .Each plotted value is a mean of three determinations ± SD.


0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Enzyme Activity(U/ml)
25
35
45
55
65
Temperature (°C)
Activity
Maltose(yeast)
Maltose(NH4Cl)

(A)

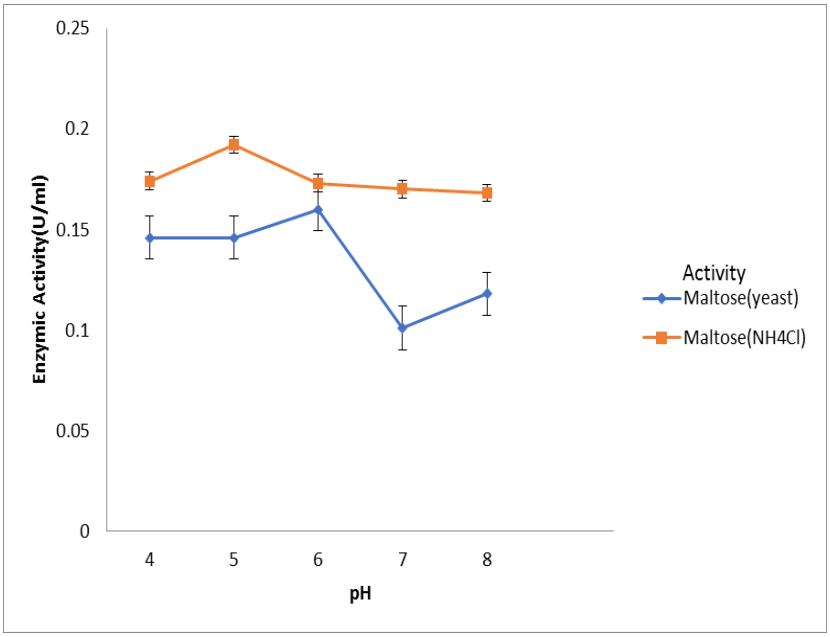

0.25
0.2
0.15
0.1
0.05
0
Enzyme Activity (U/ml)
25
35
45
55
65
Temperature (°C)
Activity
Glucose (Yeast)
Glucose (NH4Cl)

(B)

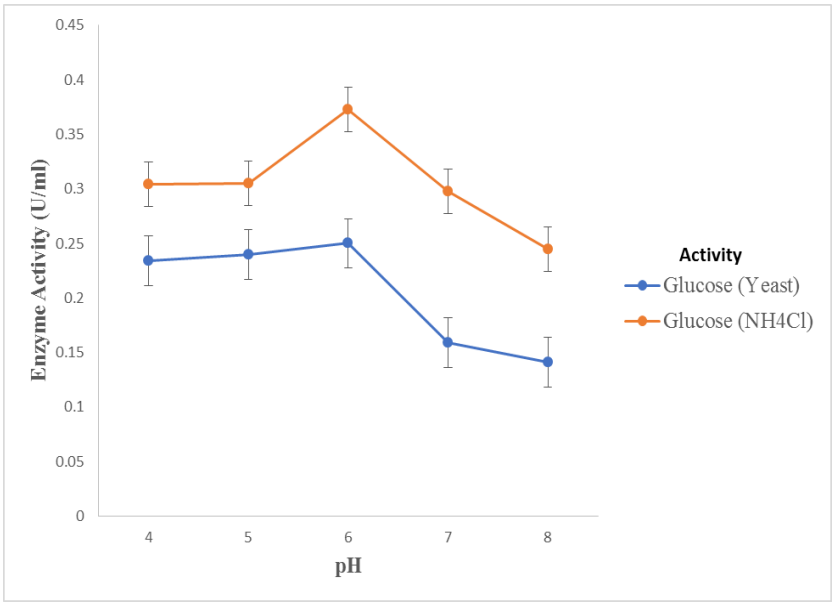


(C)

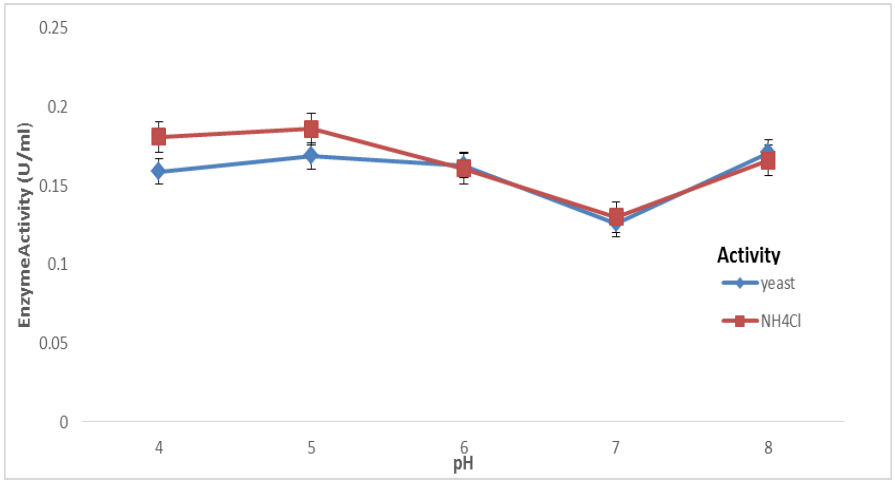
Figure 4. Effect of temperature on the purified laccase enzyme obtained from potato peels using the different carbon and nitrogen sources. Each plotted value is a mean of three determinations ± SD



(A)

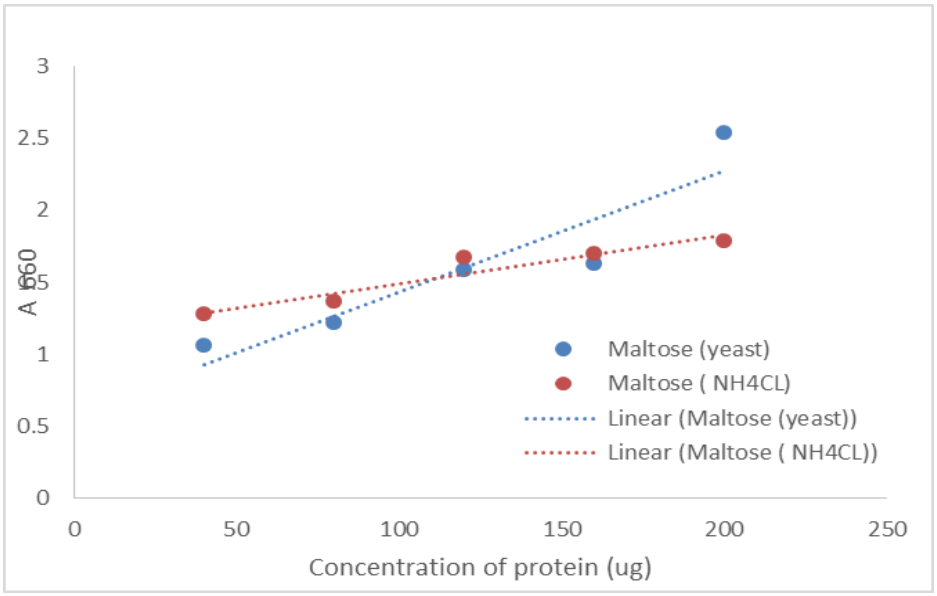


(B)

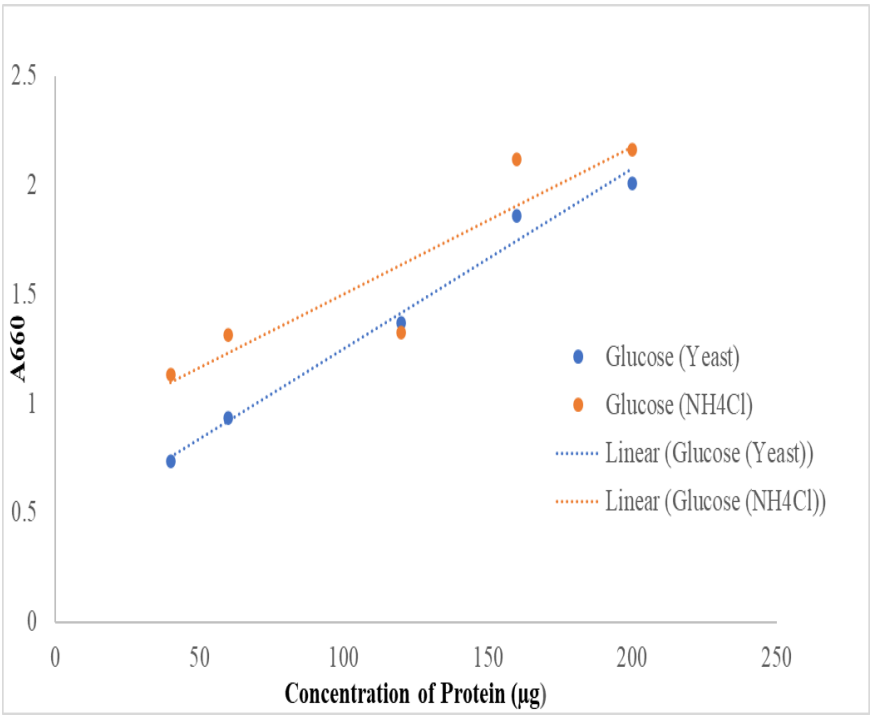


(C)

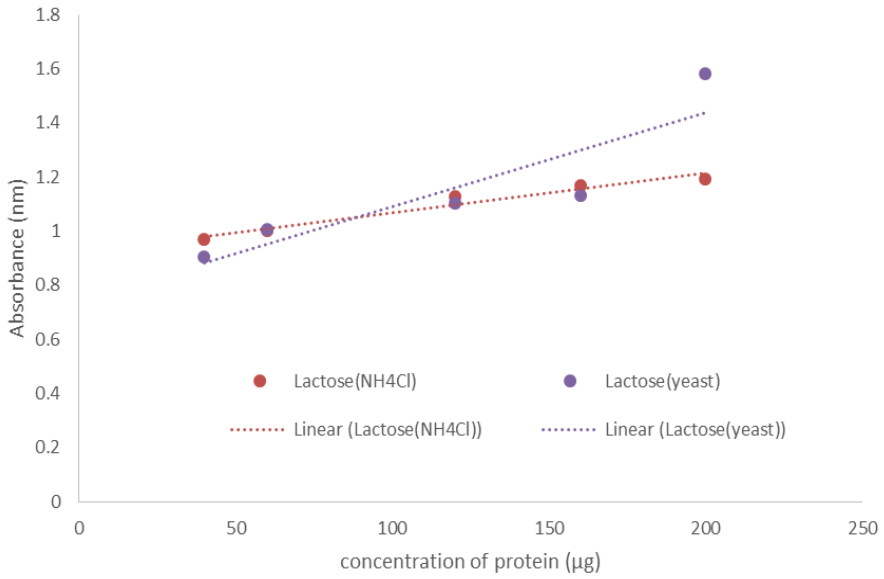
Figure 5. Effect of pH on purified laccase enzyme obtained from potato peels using the different carbon and nitrogen sources. Each plotted value is a mean of three determinations ± SD.


3
2.5
2
1.5
1
0.5
0
A 660
0
50
100
150
200
250
Concentration of protein (ug)
Maltose (yeast)
Maltose (NH4CL)
Linear (Maltose (yeast))
Linear (Maltose (NH4CL))

(A)


2.5
2
1.5
1
0.5
0
A660
0
50
100
150
200
250
Concentration of Protein (µg)
Glucose (Yeast)
Glucose (NH4Cl)
Linear (Glucose (Yeast))
Linear (Glucose (NH4Cl))

(B)



(C)

Figure 6. Determination of unknown protein concentration of laccase enzyme obtained from potato peels using the different carbon and nitrogen sources. Each plotted value is a mean of three determinations ± SD.

Discussion

The fungi growth produced extracellular laccase as indicated by the reddish-brown colour on PDA plate containing guaiacol. The same phenomenon was found in some fungal laccase producers such as *Trichoderma harzianum* [24], *Paraconiothyrium variabile* [25], *Trametes sp. LS-10C* [26], and *Pseudolagarobasidium acaciicola LA 1* [27]. The organism produced white cushions of sporulating filaments and the microscopic observations showed arrangements of microconidia. These colony characteristics and microscopic visualizations were similar to the fungi genus *Fusarium* and the isolated strain was concluded to be a member of the *Fusarium* genus. *Fusarium oxysporum* is one of the *Fusarium* species studied as laccase sources. El-Fakharany et al. [28] identified *Fusarium oxysporum* as a laccase producer. The positive strains were cultivated by submerged fermentation using seed medium and further cultivated in basic nutrient medium for a period and 3 and 5 days consecutively. Nitrogen sources were varied; ammonium chloride and yeast extract were added individually to a fixed carbon source (maltose, glucose and lactose). This is similar to the work carried out by Narkhede, 2014 [29]. After submerged fermentation, the crude enzyme was subjected to partial purification using ammonium sulphate. It was left to sit overnight and a clear solution was obtained. This agrees with that of Aruna *et al.,* 2012 and Minari and Agho, 2018, where a clear solution was obtained after treating the crude enzyme obtained from their study with ammonium sulphate. Ammonium sulphate was chosen for this study because of its availability and relatively cheap cost. The enzyme activity of the purified laccase was determined using

guaiacol as substrate and sodium acetate buffer and is measured in U/ml. Jhadav et al. [21] used a similar method for the enzyme activity.

Common factors have important effect on laccase production, such as carbon sources, nitrogen sources, temperature, pH, and fermentation time [3, 30], some of these factors were primarily studied in this work. The effect of temperature on the enzyme activity is showed in figure 4. Enzyme activity was at its highest at 45°C for the maltose (yeast), glucose (yeast), glucose (NH_4Cl) while at 65°C for maltose (NH_4Cl), lactose (yeast) and lactose (NH_4Cl). These results do not differ from other laccase with optima temperature between 50 and 70°C [32]. Dias et al. [33] reported that the enzyme proved to be fully active between 45 and 70°C. The effect of pH on the enzyme activity is showed in figure 5. Enzyme activity for maltose (yeast), glucose (yeast) and glucose (NH_4Cl) was highest at pH 6. For maltose (NH_4Cl), the enzyme activity was highest in pH 5 while for pH 8 was the optimum for lactose (yeast) and lactose (NH_4Cl). The activity of the enzymes depends strictly on the pH in the assay mixture. The effect of pH of most enzymes follows a bell-shaped curve [34]. Dhakar and Pandey, [9] had optimum pH for laccase between pH 5.5 and pH 7.5. This result agrees with that of Bisswanger (2014) [34] as a bell-shaped profile was observed in the activity of laccase with pH.

The total concentration of protein for maltose (yeast) was estimated to be 59.57μg/ml, maltose (NH_4Cl) was 181.18μg/ml, glucose (yeast) was 45.9μg/ml, glucose (NH_4Cl) was 97μg/ml, lactose (yeast) was 194.714μg/ml and lactose (NH_4Cl) was 503.2μg/ml using Lowry's method. The curve was plotted based on the absorbance values at 660nm in which bovine serum albumin was used as standard. Similar procedures were carried out by Aruna et al. [14] and Minari and Agho [15] where the total protein concentration was 500μg/ml and 1004 μg/ml respectively. The protein concentration gotten in this study is less than those obtained by Aruna *et al*, 2012 and Minari and Agho, 2018 except for lactose (NH_4Cl) that agrees with that of Aruna *et al*, 2012.This may be due to the various carbon and nitrogen sources that were used.

Conclusion

An attempt was made to increase the production of laccase by using three carbon sources, maltose, glucose and lactose and two different nitrogen sources, yeast extract and ammonium chloride (NH_4Cl). As a result of this, the combination of NH_4Cl with each carbon source showed increased production when compared to yeast extract. The parameters assessed in this study (temperature, pH and protein concentration) showed that laccase produced from potato peels compared favorably with laccases that have been characterized previously from other sources. This suggests that laccase from potato peels using maltose, glucose and lactose as carbon sources with NH_4Cl as nitrogen source can bring about increase in laccase production.

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