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Laccase extraction, purification and characterization from potato peels

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ABSTRACT

Laccases are multi-copper enzymes belonging to the group of blue oxidases which oxidise diphenol and use molecular oxygen as an electron acceptor. The enzyme exists widely in plants, fungi and some bacteria. It has widespread applications in textile industries, food industries, pharmaceutical industries and bioremediation. This study was carried out to investigate the extraction, purification and characterization of laccase from potato peels. The sample was screened for laccase production by plate test method using the indicator compound Guaiacol. Extraction was carried out using submerged fermentation while ammonium sulphate was used to purify the crude enzyme. Characterization was carried out using the following parameters: pH, temperature, protein concentration, and molecular weight of the enzyme. Enzyme activity decreased with increase in pH. Activity increased from 15 ^{o}C to 25 ^{o}C and was stable from 25 ^{o}C to 45 ^{o}C and decreased afterwards from 45 ^{o}C to 65 ^{o}C. Protein concentration was high and the molecular weight of the enzyme was in the range of 36-50 kDa. Laccase activity was found to be 0.024 U/ml; optimum temperature was from 25-45 ^{o}C while optimum pH was 4.0. This study revealed that the laccase enzyme characterized from potato peels which is always found as waste materials in our environment compared favourably with laccase enzyme characterized previously from other conventional sources.

Keywords: Laccase; Guaiacol; Potato peels

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are the oldest and most studied enzymatic systems (Williamson, 1994). They are multi-copper enzymes belonging to the group of blue oxidases which oxidizes diphenol and allied substances and use molecular oxygen as an electron acceptor (Desai and Nityanand, 2011). Laccases are the most important components of lignolytic complex of wood-destroying white rot fungi responsible for decomposition (but in some cases also synthesis) of lignin, which is one of the widely distributed natural polymer (Morozova *et al*., 2007). Laccase carries out one –electron oxidation and reduce molecular oxygen to water. The biotechnological importance of this enzyme lies in its ability to oxidize both phenolic and non-phenolic lignin-related compounds (Bourhonnais *et al*., 1990). When the substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or nonenzymatic reactions including; hydration, disproportionation and polymerization (Faccelo and Cruz 2008).

Laccases are attractive, industrially relevant enzymes that can be used for a number of diverse application e.g. biosensors (Freire *et al.,* 2001), pulp bleaching (Call and Mucke, 1997; Balakshin *et al.,* 2001) labeling in immunoassays, (Kuznetsov *et al*., 2001), bioremediation (Mayer and Staples, 2002) and green organic synthesis (Karamyshev *et al*., 2003). These enzymes are also used for pulp delignification, pesticide or insecticide degradation, organic synthesis (Faccelo and Cruz, 2005), waste detoxification, textile dye transformation, food Technological uses and analytical application (Shraddha *et al.,* 2001).

However, in spite of the numerous uses of laccase, its high cost of production has been one of the major hindrances to the commercial usage of it. A need, therefore, arises for a search into the possible ways of obtaining maximum production from a relatively cheap source with minimum

expenditure.

Potatoes are one of the most important staple crops for human consumption together with wheat, rice and corn. Potato peels contain a wide variety of compounds that could be used in foods and also in non-food applications (Andreas and Marleny, 2009). Presently, while fresh potato consumption is decreasing in many countries, more potatoes are currently processed into value-added products to meet the demand especially from fast food and convenience food industries (FAO, 2008). Potatoes are usually peeled during processing, as a consequence, large quantities of peels are generated which represent a severe disposal problem to the industry (Andreas and Marleny, 2009). This work was carried out to evaluate the presence of laccase in potato peels. This was achieved through extraction, purification and characterization of laccase from potato peels.

MATERIALS AND METHODS

Potato peels were collected from a local potato chips company at Abule Ijesha Yaba Lagos, Nigeria. All the reagents were of analytical grade

Isolation of laccase producing organism

1gram of the collected sample was added to 10 ml of sterile distilled water and mixed. This suspension was serially diluted from 10^{-1} to 10^{-5}. 1ml of each dilution was spread on the surface of Potato dextrose agar which contained 0.02% Chloramphenicol incubated at 30°C for 7days (Aruna *et al.*, 2012)

Screening of laccase producers

The fungal strain was inoculated in potato dextrose agar plate which contained 0.02% Guaiacol and incubated at 30°C for 7days. After incubation, the plates were observed for the formation of reddish-brown zones around the colony (Buddolla *et al.*, 2008; Aruna *et al.*, 2012).

Production by submerged fermentation

Potent strains where cultivated in Olga liquid medium. Plugs of mycelia where inoculated into 50mL olga liquid medium and incubated at 37^0C on a rotary shaker for 15 days. After the incubation period, the content of the flask was filtered through Whatman No.1 filter paper and the filtrate was centrifuged at 10,000rpm for 10mins at 4^0C. The supernatant obtained was treated as the enzyme extract for the study (Chawchart *et al.,* 2004; Udayasoorian and Prabu, 2005; and Arul and Shanmugam, 2012).

Partial Purification of laccase

After submerged fermentation, the crude fungal culture was filtered through Whatman No 1 filter paper. Then the protein solution was poured into a beaker with magnetic bar and it was placed on a stirrer at 4°C.

0.6g of ammonium sulphate per millilitre of protein solution was taken. Then the protein solution was stirred and a small portion of ammonium sulphate was added to it and allowed to dissolve before adding next portion. Finally, the beaker was allowed to stand overnight (Patrick et al, 2009).

Enzyme Assay

Enzyme activity was determined using Guaiacol as the substrate. For these assay, two test tubes were used. 1mL of 10 mM Guaiacol and 3mL of sodium acetate buffer (100 mM pH 5) were measured into each tube. 1mL of culture filtrate was added to one of the tubes which was taken as the test to give final reaction volume of 5 mL. 1 mL of distilled water was added to the second test tube which was taken as blank. The mixture was incubated at 30°C for 15 mins and absorbance was read at 340nm using a spectrophotometer. 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^{-1} cm^{-1}) (Jhadav *et al.*, 2009).

Characterization of Enzyme

The effect of temperature on laccase activity was determined by recording the absorbance of enzyme catalysed reaction using Guaiacol (10 mM) as substrate dissolved in sodium acetate buffer (10 mM pH 5.0) incubated at temperature 15°C, 25°C, 35°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 of the enzyme. The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalysed reaction at optimum temperature using Guaiacol (10mM) as as substrate dissolved in buffers of different pH (acetate buffer pH 4, 5,

phosphate buffer pH 6, 7 and tris-HCl buffer pH 8) and incubated at 25 ^{o}C for 15 min and absorbance were recorded at 340 nm (Savitha *et al*., 2011).

Estimation of Protein Concentration

Working standards 0.2mL, 0.4mL, 0.6mL, 0.8mL and 1mL were pipetted into a series of test tube and all the tubes were made up to 1 mL using distilled water except the text tube with 1mL. A blank containing 1 mL distilled water was also taken. 0.5mL of test solution in a test tube was taken 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°C in the dark for 30 minutes. The blue colour developed was read at 660 nm. A standard graph was plotted with concentration of BSA on X-axis and optical activity on Y-axis (Minussi *et al*., 2007)

RESULTS

Whitish creamy coloured growth was observed after 7 days of incubation. The mycelia growth had filamentous appearance with the presence of spores as shown in Plate 1. The screening of laccase production using Guaiacol is shown in Plate 2. A reddish brown colouration was observed on the medium after 7 days of incubation. The positive strains were cultivated by submerged fermentation in Olga liquid medium; a dark green coloured broth was obtained after 12 days of incubation as shown Plate 3. The protein solution was partially purified using ammonium sulphate. It was allowed to stand overnight after which the clear solution shown in Plate 4 was obtained. The effect of temperature on laccase enzyme activity is shown in Figure 1. There was a significant increase ($p < 0.05$) in enzyme activity from 15°C to 25°C. There was no significant change ($p > 0.05$) in enzyme activity from 25°C to 45°C. Further increase in temperature resulted in significant reduction ($p > 0.05$) in enzyme activity from 45°C to 65°C. Figure 2 shows the effect of pH on enzyme activity. A significant reduction ($p < 0.05$) in enzyme activity was observed with increasing pH. Enzyme activity was highest at pH 4. A significant reduction ($p < 0.05$) was observed in activity from pH 4 to pH 6. There was no significant reduction ($p > 0.05$) in the enzyme activity from pH 6 to pH 7 but significant reduction ($p < 0.05$) was observed from pH 7 to pH 8. Determination of the unknown protein concentration of the enzyme is shown in Figure 3. The total yield of the partially purified laccase enzyme was estimated at 1004 µg/ml and 1550 µg/ml for the crude.

Plate 1: White mycelial growth after 7 days of incubation

Plate 2: Screening of laccase production using Guaiacol

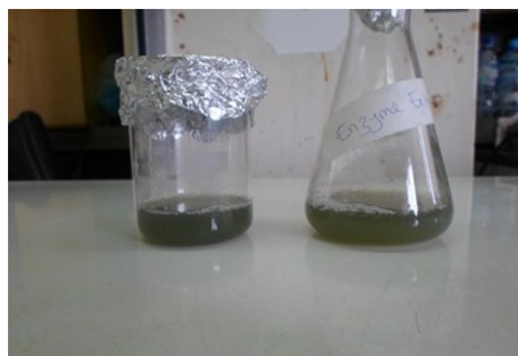

Plate 3: Crude enzyme extract after submerged fermentation

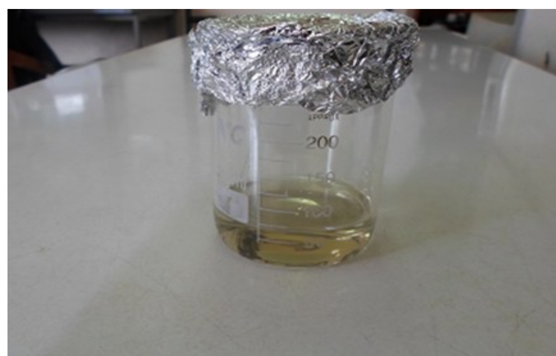

Plate 4: Enzyme extract after partial purification using ammonium sulphate

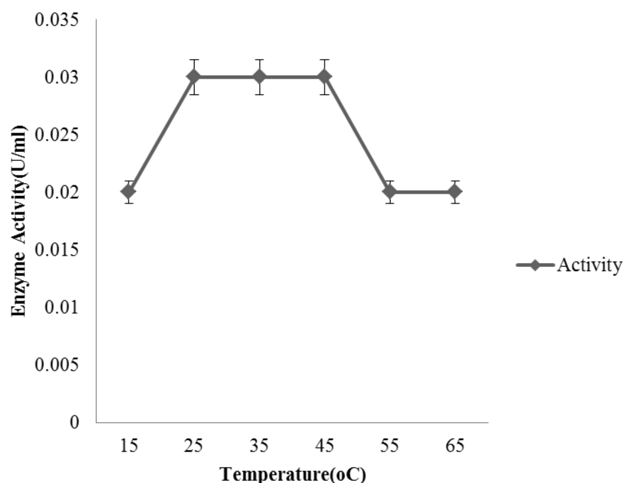

Figure 1: Effect of temperature on purified potato peel laccase activity (Each plotted value is a mean of four determinations ± SD)

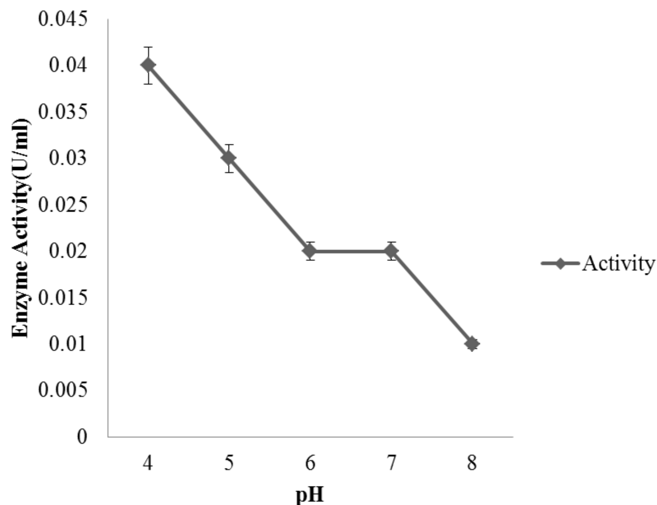

Figure 2: Effect of pH on purified potato peel laccase enzyme activity (Each plotted value is a means four determinations ± SD)

The white mycelia growth observed (Plate 1) after 7 days on incubation indicated the presence of fungi. The organism produced white-creamy cushions of sporulating filaments. This agrees with that of Kiiskinen *et al* (2004) and Aruna *et al*, (2012).

The isolated fungi were screened for laccase production on PDA media supplemented with Guaiacol. It has been reported that laccase catalyses the oxidation of Guaiacol to form reddish-brown colour around the fungi colonies (Rasheeda *et al*, 2012). The reddish-brown colouration observed in this present study (Plate 2) was consistent with that obtained by Rasheeda *et al*. (2012). Also, similar results were obtained by Kumar et al. (2011) and Aruna et al (2012), where they used Guaiacol as a substrate for screening for laccase producers and observed reddish brown colour zones around the colonies on the medium. The positive strains were cultivated by submerged fermentation using Olga liquid medium for 7 days. Glucose was added as a carbon source. After submerged fermentation, the dark coloured broth (crude enzyme) produced (Plate 3) was purified using ammonium sulphate. A clear solution (Plate 4) was obtained after this treatment. Aruna et al. (2012) used a similar method in treating the crude enzyme obtained from their study and also got a clear solution after treating with ammonium sulphate. According to Patrick et al. (2009) several methods have been used to purify the crude enzyme sample. They include: desalting/buffer exchange of protein, anion exchange chromatography and gel filtration chromatography. Since this study aimed at reducing the cost of laccase production, ammonium sulphate was used for the purification of the crude enzyme because according to Aruna et al. (2012), it is less expensive and easy to use.

Enzyme activity was determined after partial purification by using Guaiacol and acetate buffer. Enzyme activity was measured in U/ml which is defined as the amount of enzyme catalysing the production of one micromole of coloured product per minute per ml. Resulting form this assay laccase activity was found to be 0.024U/ml.

The relationship between temperature and enzyme activity is a parameter in determining the purity of the enzyme. Enzyme activity was highest at temperatures 25°C, 35°C and 45°C; this agrees with the report of Han et al. (2005) that

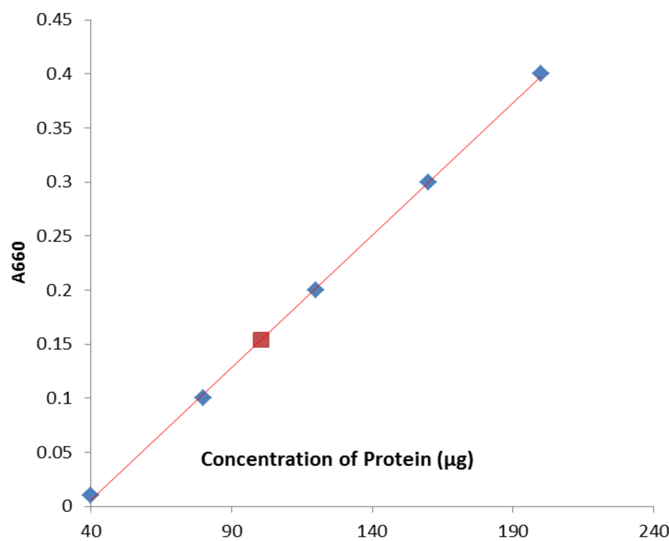

Figure 3: Determination of unknown protein concentration of laccase enzyme

temperature range where the laccase enzyme is active is remarkably wide and activity can be detected from as low as 15°C and optimum temperature is significantly affected by the assay used. Enzyme purity is also dependent on the effect of pH on enzyme activity. The optimum pH value for laccases varied depending on the substrate employed because different substrates cause different reaction for laccases (Palmieri et al., 1997; Savitha et al., 2011). Laccase from this study had an optimum pH of 4. Laccase activity decreased as pH increased. Palmieri et al. (1997) recorded that the normal range of pH for a typical laccase enzyme is 3-5. This result agreed with that of Palmieri *et al*. (1997) but a bell-shaped profile was not observed in the case of laccase activity with pH.

The total concentration of protein was estimated to be 1004 µg/ml for the partially purified enzyme and 1550 µg/ml for the crude enzyme. The standard curve was drawn based upon the optical density values measured at 660 nm in which Bovine serum albumin (BSA) was used as standard. A similar procedure was carried out by Aruna et al (2012) where protein concentration was found to be 500 µg/ml for partially purified laccase obtained from the synthetic medium (medium with glucose as carbon source). The protein concentration of the partially purified laccase for this study was observed to be 1004 µg/ml which is higher than that obtained by Aruna et al (2012).

CONCLUSION

The result from this study has shown not all waste is useless since laccase enzyme was produced from potato peels. The parameters considered in this study (pH, temperature, protein concentration, SDS PAGE profile and substrate concentration) revealed that the laccase enzyme obtained from potato peels compared favourably with laccases that have been characterized previously from other conventional sources. This suggests that laccase from potato peels can be used industrially. Further studies on laccase from potato peels can be carried out using solid-state fermentation and the use of a natural substance such as wheat bran, rice bran and saw dust as a carbon source in the culture medium.

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