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OPTIMIZATION OF LACCASE PRODUCTION BY *CURVULARIA LUNATA* USING MAIZE COB AS SUBSTRATE

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ABSTRACT
Researchers are showing interest in laccase because it is able to degrade several lignocellulosic biomass. It is important to optimize laccase production to achieve higher yield at a lower cost using agricultural wastes. This study was aimed at optimizing the culture conditions for laccase production. Previously isolated and characterized laccase producing *Curvularia lunata* was collected from the laboratory of Microbiology department, ABU, Zaria. Maize cobs which were used as substrate were collected from Seed Processing Unit of Institute for Agricultural Research, Zaria and the proximate composition of the substrate was determined according to AOAC guideline. The optimum fermentation type for laccase production was determined after which the culture conditions for laccase production were optimized. Laccase activity was determination by guaiacol assay. The proximate compositions of the maize cob were found to be Moisture content (3.18%), Crude protein (2.50%), Crude fat (32.20%), Crude fibre (3.15), Ash content (1.88%) and Carbohydrate content (57.09%). Higher laccase activity was recorded under solid state fermentation compared to submerged fermentation. The optimum culture conditions were found to be inoculum size 3 x 5 mm, pH 5, incubation temperature 30 ^{o}C and incubation period of 6 days. The culture conditions for laccase production by *Curvularia lunata* using maize cob as substrate were optimized.

Keywords: laccase, *Curvularia lunata*, optimum, fermentation, maize cob, production

INTRODUCTION

Laccase is an oxidoreductase extracellular enzyme that catalyzes the oxidation of numerous aromatic compounds accompanied by reduction of oxygen to water. It was discovered first in Japanese lacquer tree *Rhus vernicifera* sap and was later characterized as a metal containing oxidase in 1985 by Bertrand (Giardina *et al.*, 2010; Vantamuri and Kaliwal, 2015). Researchers are paying more attention to laccases due to their ability to oxidize lignin and pollutants that are highly resistant to biodegradation (Hernández-Monjaraz *et al.*, 2018). They are used widely in food, cosmetic, chemical and textile industries as well as in environmental bioremediation (Vantamuri and Kaliwal, 2015). For these purposes, laccase must be produced under optimum culture conditions (Mtui, 2012; Thakkar *et al.*, 2020).

Researchers are now showing increased interest in fungal ligninolytic enzymes because of their ability to degrade several lignocellulosic biomass as well as biodegradation-resistant and toxic phenolic compounds (Geethanjali *et al.*, 2020). Ligninases of fungal origin belong to three main groups namely lignin peroxidase, manganese peroxidase and laccase. Laccases are the most studied among the ligninases (Mtui, 2012).

The most important duty of microbiologists and scientists alike is to apply metabolic activity of microorganisms in the biodegradation of chemicals that are toxic, so that bioremediation will be an important prospect in the future (Amutha *et al.*, 2014; Geethanjali *et al.*, 2020). Side by side metabolic enzymes by which microorganisms degrade toxic chemicals are extracted from them and are use in various aspects of the industries and environment. Enzymes of microbial origin especially laccases have a variety of positive applications (Amutha *et al.*, 2014; Afreen *et al*., 2018; Sayyed *et al.*, 2020). Laccases are produced by both fungi and bacteria, however, fungi serve as the major source of laccases. Laccase plays a vital role in making the environment green (Amutha and Abhijit, 2015; Edae and Alemu, 2017).

New methods of producing laccases with a higher enzyme activity at a relatively lower cost are required. This can be achieved by using lignocellulosic agro-industrial wastes as substrate in the production of laccases (Perdani *et al.*, 2020). It is highly essential to optimize the culture conditions in order to achieve an effective laccase production. The diverse metabolic activity of microbes makes it possible for them to use various chemicals (toxic and non-toxic) as source of carbon, nitrogen, phosphorus etc. and after utilization, most toxic chemicals become completely non-toxic (Sinha *et al.*, 2009; Sondhi and Saini, 2019; Thakkar *et al.*, 2020).

MATERIALS AND METHODS

Collection of Maize Cob

Maize cobs were collected from Seed Processing Unit of Institute for Agricultural Research, Zaria. The maize cobs were collected in clean plastic bags, labeled and taken to the Environmental Laboratory, Department of Microbiology, ABU, Zaria where the samples were crushed and grinded. The samples were then stored for further analysis.

Collection of laccase producing *Curvularia lunata*

Laccase producing *Curvularia lunata* (EMBL/GenBank accession number QIE06317.1) isolated previously from soil was collected from the Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria on Potato Dextrose Agar slant and stored at room temperature.

Determination of proximate composition of maize cob

The proximate composition of the substrate was determined at Biochemical Laboratory Unit of Animal Science Department,

Faculty of Agriculture, Ahmadu Bello University, Zaria. The following properties were determined: Moisture content, Ash content, Fibre, Lipids and Protein according to AOAC (2000) guidelines.

Production of Laccase by Submerged and Solid-State Fermentation Using Maize Cob as Substrate

Submerged fermentation

Submerged fermentation medium was prepared by adding 10 g of maize cob to 100 mL of productive liquid medium consisting of: NH_4NO_3, 3 g; $(NH_4)_2HPO_4$, 3 g; MgSO4. $7H_2O$, 0.5 g; NaCl, 0.5 g; $CaCO_3$, 0.5 g; FeSO4.$7H_2O$, 0.35 g and $CuSO_4.5H_2O$, 0.6 g as described by Szabo *et al.* (2015). The pH of the fermentation medium was adjusted to pH 6 and then autoclaved at 121 ºC for 15 minutes. Standard inoculum (5 mm disk of 7 days old laccase producing fungal growth) was inoculated into the prepared productive liquid medium and then incubated at 25 ºC for 7 days. Guaiacol assay was used to determine the laccase activity.

Solid State fermentation

Submerged fermentation medium was prepared by moistening 5 g of maize cob with 15 mL of productive liquid medium (NH_4NO_3, 3 g; $(NH_4)_2HPO_4$, 3 g; MgSO4. $7H_2O$, 0.5 g; NaCl, 0.5 g; $CaCO_3$, 0.5 g; FeSO4.$7H_2O$, 0.35 g and $CuSO_4.5H_2O$, 0.6 g) (Szabo *et al.* 2015). The pH of the medium was adjusted to pH 6 and then autoclaved at 121 ºC for 15 minutes. The sterilized medium was inoculated with one mycelial plug (5 mm in diameter) from 7 days old culture of the laccase producing fungi and incubated at 25 ºC for 7 days (Masutti *et al*., 2012). Guaiacol assay was used to determine the laccase activity.

Extraction of crude enzyme

For solid state fermentation, 50 mL of sodium acetate buffer at pH 4.5 was added to the fermented media and then shaken thoroughly for 2 hours at 100 rpm. This was followed by centrifugation of the flask contents for 5 minutes at 8000 rpm. Supernatant of the filtrate was then used to determine the laccase activity. Laccase activity was assayed in the supernatant after filtration using Whatman filter paper No.1 (Risdianto *et al.,* 2012). For submerged fermentation, following incubation of the production medium for appropriate period, the flask contents were centrifuged for 5 min at 8000 rpm and the supernatant which is an extracellular enzyme was collected. Guaiacol assay was used to determine the laccase activity (Bhuvaneshwari *et al.,* 2015).

Guaiacol assay for laccase activity determination

Determination of laccase activity was carried out as reported by Abd El Monssef *et al.* (2016). The technique involves guaiacol oxidation by laccase which leads to development of reddish brown color. The reaction mixture was prepared using: 2 mM Guaiacol (1 mL), 10 mM Sodium acetate buffer (3 mL) and fungal supernatant as source of enzyme (1 mL). Distilled water (1 mL) was used in place of fungal supernatant for the blank. The mixture was incubated at 30ºC for 15 min and the absorbance was measured using UV spectrophotometer at 450 nm (Abd El Monssef *et al.,* 2016). The Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml was calculated using the formula E.A = A*V/t * e * v. Where E. A = Enzyme activity, A =Absorbance, V =Total volume of mixture (mL), v = volume of enzyme source (mL), t = incubation period, e = guaiacol extinction coefficient (0.6740 µM/cm).

Optimization of Culture Conditions for Laccase Production

Optimization of inoculum size

Different inoculum sizes of 1, 2, 3, and 4 fungal mycelial plugs from 7 day old cultures of laccase producing fungi measuring 5 mm in diameter each were inoculated into four different flasks containing 5 g of maize cob humidified with 15 mL of productive liquid medium (NH_4NO_3, 3 g; $(NH_4)_2HPO_4$, 3 g; MgSO4. $7H_2O$, 0.5 g; NaCl, 0.5 g; $CaCO_3$, 0.5 g; FeSO4.$7H_2O$, 0.35 g and $CuSO_4.5H_2O$, 0.6 g). The flasks were incubated at 25 ºC for 7 days (Abdulredha, 2013). Crude enzyme was extracted from the culture filtrate and the laccase activity was determined using Guaiacol assay method as described above. The inoculum size with the highest laccase activity was recorded as the optimum inoculum size.

Optimization of Medium pH

To optimize the pH of fermentation medium for laccase production, four different flasks (labeled as pH 4, 5, 6 and 7) containing 5 g of maize cob humidified with 15 mL of productive liquid medium were autoclaved and the media in the flasks were adjusted to pH 4, 5, 6 and 7 as appropriate. Each flask was inoculated with the predetermined optimum inoculum size and incubated at 25 ºC for 7 days (Abdulredha, 2013). Crude enzyme was extracted from the culture filtrate and the laccase activity was determined using Guaiacol assay method. The pH of the medium with the highest laccase activity was recorded as the optimum pH for laccase production.

Optimization of incubation temperature

To optimize the incubation temperatures for laccase production, four different flasks (labeled as 25, 30, 35 and 40ºC) containing 5g of maize cob humidified with 15 mL of productive liquid medium were autoclaved and the media was adjusted to the predetermined optimum pH. The flasks were inoculated with the predetermined optimum inoculum size and incubated at 25, 30, 35 and 40 ºC for 7 days as appropriate (Abdulredha, 2013). Crude enzyme was extracted from the culture filtrate and the laccase activity was determined using Guaiacol assay method. The incubation temperature with the highest laccase activity was recorded as the optimum incubation temperature for laccase production.

Optimization of Incubation period

To optimize incubation period for laccase production, the medium prepared using the previously optimized condition and autoclaved was inoculated with the optimized inoculum size and then incubated under optimized incubation temperature for 4, 5, 6, and 7 days. (Abdulredha, 2013). Enzyme was extracted from the culture filtrate and laccase activity was determined using Guaiacol assay method. The incubation period with the highest laccase activity was recorded as the optimum incubation period for laccase production.

RESULTS

Proximate Composition of Maize cob

Result of the proximate composition revealed that the Moisture content, Crude protein, Crude fat, Crude fibre, Ash content and Carbohydrate content of the maize cob were 3.18%, 2.50%, 32.20%, 3.15%, 1.88% and 57.09% as shown in Table 1. Carbohydrate content of the maize cob had the highest composition while ash content had least composition.

Laccase producing fungi

Plate 1a shows the colonial morphology of *Curvularia lunata* on Potato Dextrose Agar after incubation for 7 days. *C. lunata* appeared as brownish black colony with dark brown reverse on PDA. The isolate was further characterized molecularly by ITS rDNA sequence analysis using the FASTA algorithm with the Fungus database from EBI and the sequence was submitted in EMBL GenBank with accession number QIE06317.1. The isolate was found to be 100% similar to *Curvularia* sp. FIESC strain CBS 131009 which belongs to the *Curvularia lunata* species complex. *Curvularia lunata* with dark-purple halo on PDA supplemented with 1 mM ABTS presented on Plate 1b indicates laccase production. The dark halo around *Curvularia lunata* is due to oxidation of ABTS by laccase produced by the fungus.

Effect of Fermentation Type on Laccase production by *Curvularia lunata*.

Efficiency of fermentation type on laccase production by *Curvularia lunata* (pH 6, 25ºC incubation temperature and 7 days incubation period) is presented in Figure 1. Results obtained from this study indicates that solid state fermentation proves to be more efficient fermentation method for the production of laccase using maize cob as substrate. Higher laccase enzyme activity was recorded under solid state fermentation (1.374 U/mL) compared to submerged fermentation (1.286 U/mL). Statistical analysis of the data obtained indicates that solid state fermentation results in significantly higher laccase activity compared to the submerged fermentation method ($p \leq 0.05$).

Table 1: Proximate composition of Maize cob

Parameters	**Proximate Compositions (%)***
Moisture contents	3.18
Crude fat	32.20
Crude protein	2.50
Crude fibre	3.15
Ash content	1.88
Carbohydrate	57.09

*Values are mean of triplicate readings

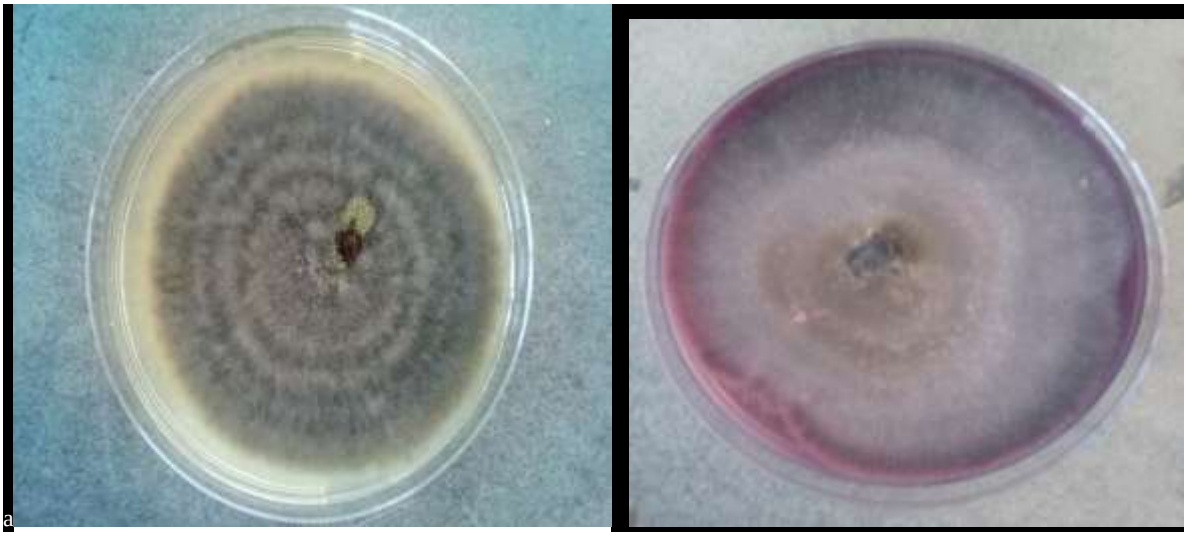

Plate 1a: Colonial morphology of *Curvularia lunata* on Potato Dextrose Agar

Plate 1b: *Curvularia lunata* with dark-purple halo on PDA supplemented with 1 mM ABTS indicating laccase production

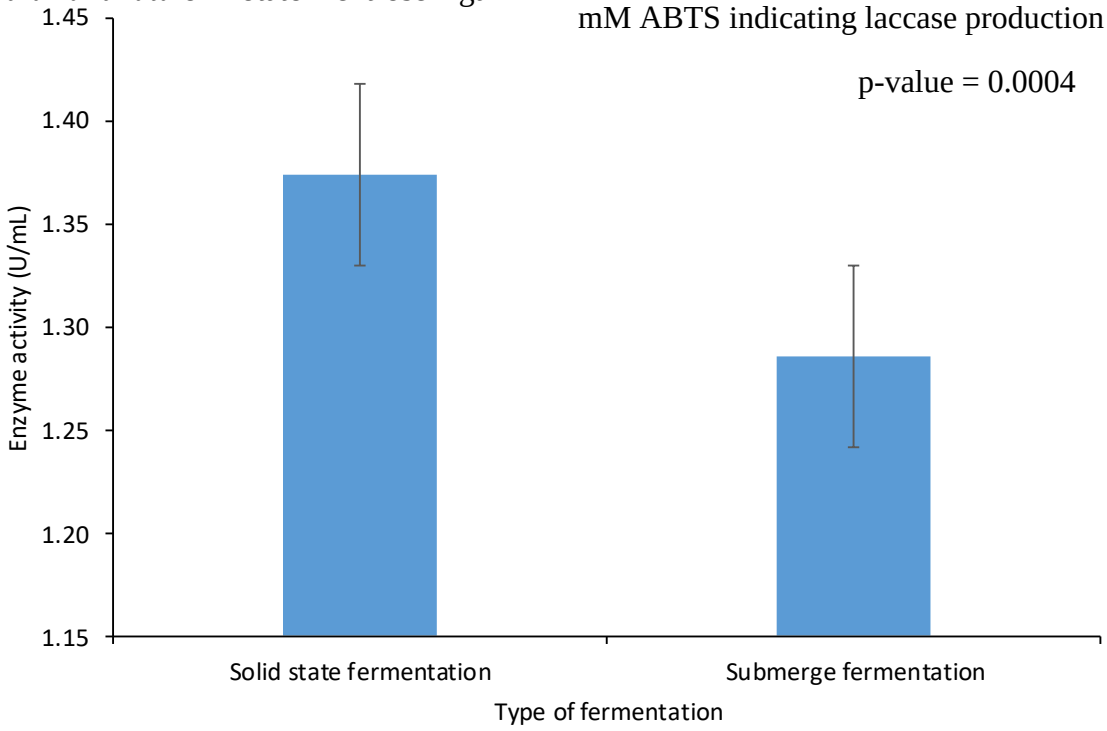

Figure 1: Effect of fermentation type on laccase production by *Curvularia lunata*

Effect of Inoculum Size on Laccase production by *Curvularia lunata*.

The effect of inoculum size on laccase production by *Curvularia lunata* under the same conditions is presented in Figure 2. Inoculum size 3 x 5 mm had the highest laccase enzyme activity (1.326 U/mL) while the least laccase activity was observed at inoculum size 1 x 5 mm (0.932 U/mL). The difference observed in laccase activity was statistically significant ($p \leq 0.05$).

Effect of pH of fermentation medium on laccase production by *Curvularia lunata*

The effect of pH of fermentation medium on laccase production by *Curvularia lunata* under the same conditions is presented in Figure 3. Highest laccase enzyme activity was observed at pH 5 (1.426 U/mL) while the lowest laccase activity was observed at pH 7 (0.753 U/mL). The difference observed in laccase activity was found to be statistically significant at $p \leq 0.05$.

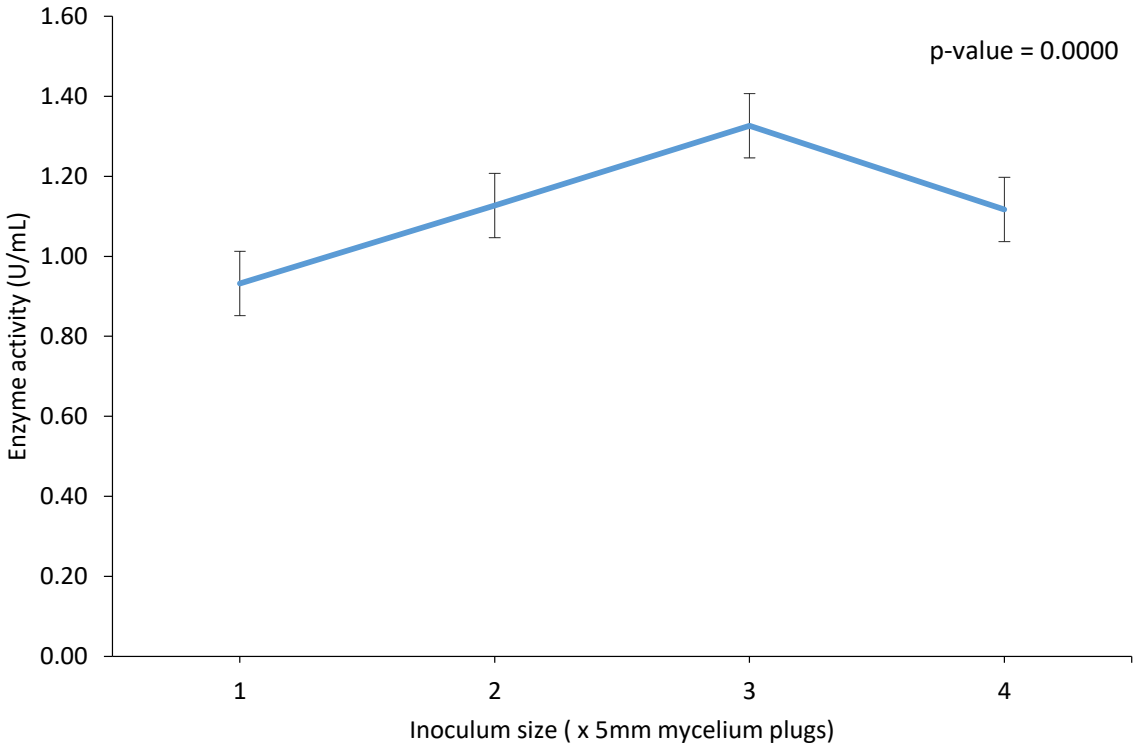

Figure 2: Effect of inoculum size on laccase production by *Curvularia lunata*

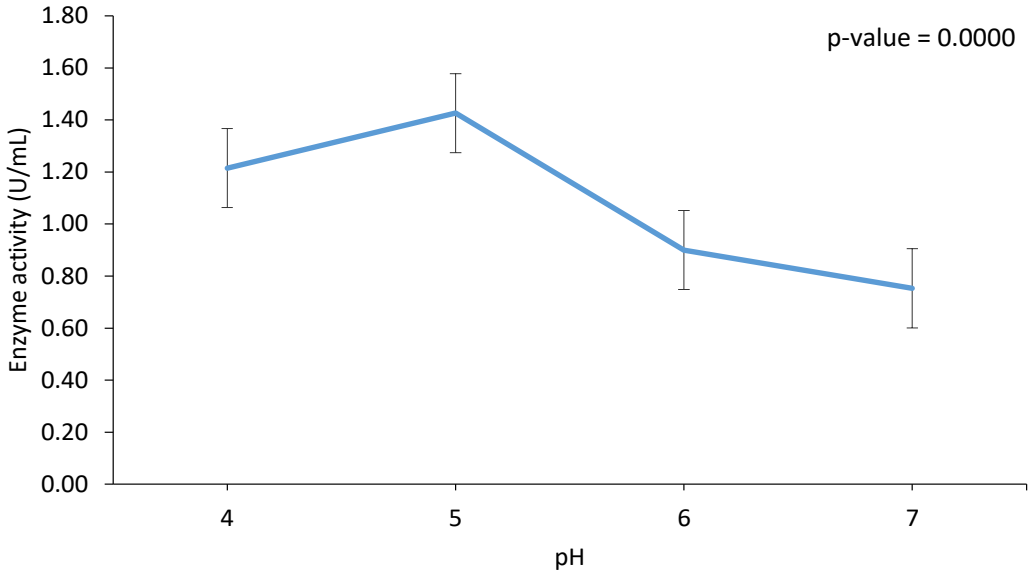

Figure 3: Effect of pH of fermentation medium on laccase production by *Curvularia lunata*

Effect of incubation temperature on laccase production by *Curvularia lunata*

The effect of incubation temperature on laccase production by *Curvularia lunata* under the same conditions is presented in Figure 4. Incubation temperature of 30ºC was recorded to be optimum temperature as the highest laccase activity (1.464 U/mL) was observed at this temperature. The lowest enzyme activity (0.818 U/mL) was recorded at incubation temperature of 25ºC. The difference observed in enzyme activity was found to be statistically significant ($p \leq 0.05$).

Effect of incubation period on laccase production by *Curvularia lunata*

The effect of varying incubation period on production of laccase by *Curvularia lunata* under the same conditions is shown in Figure 5. Highest laccase activity (1.272 U/mL) was observed after 6 days of incubation. The lowest laccase activity (1.129 U/mL) was recorded after 4 days of incubation. The difference observed in enzyme activity was statistically significant at $p \leq 0.05$.

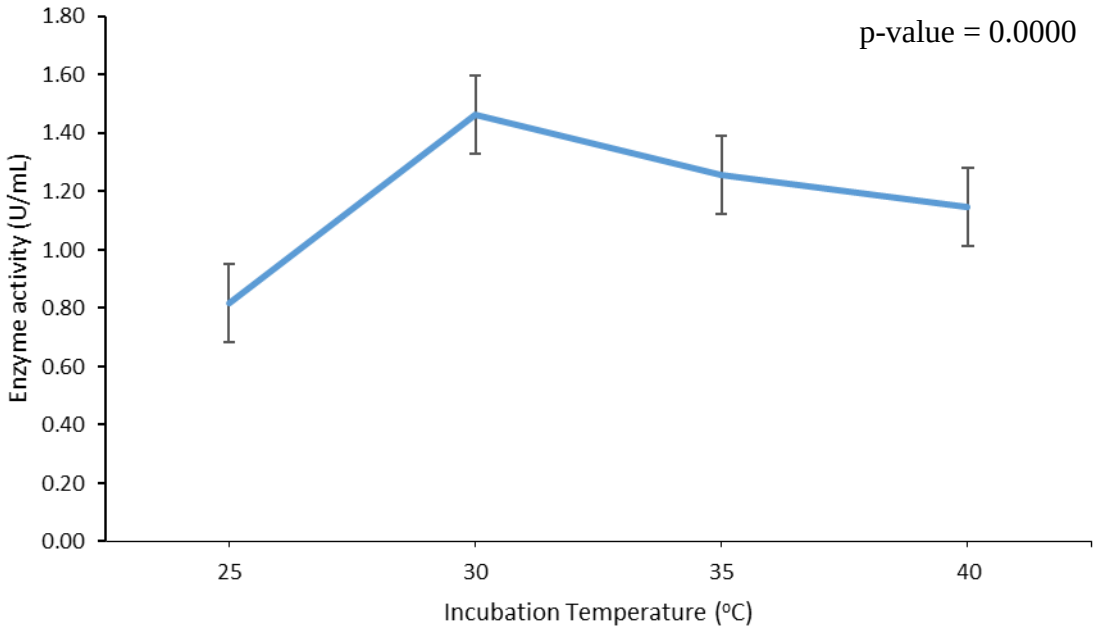

Figure 4: Effect of incubation temperature on production of laccase by *Curvularia lunata*

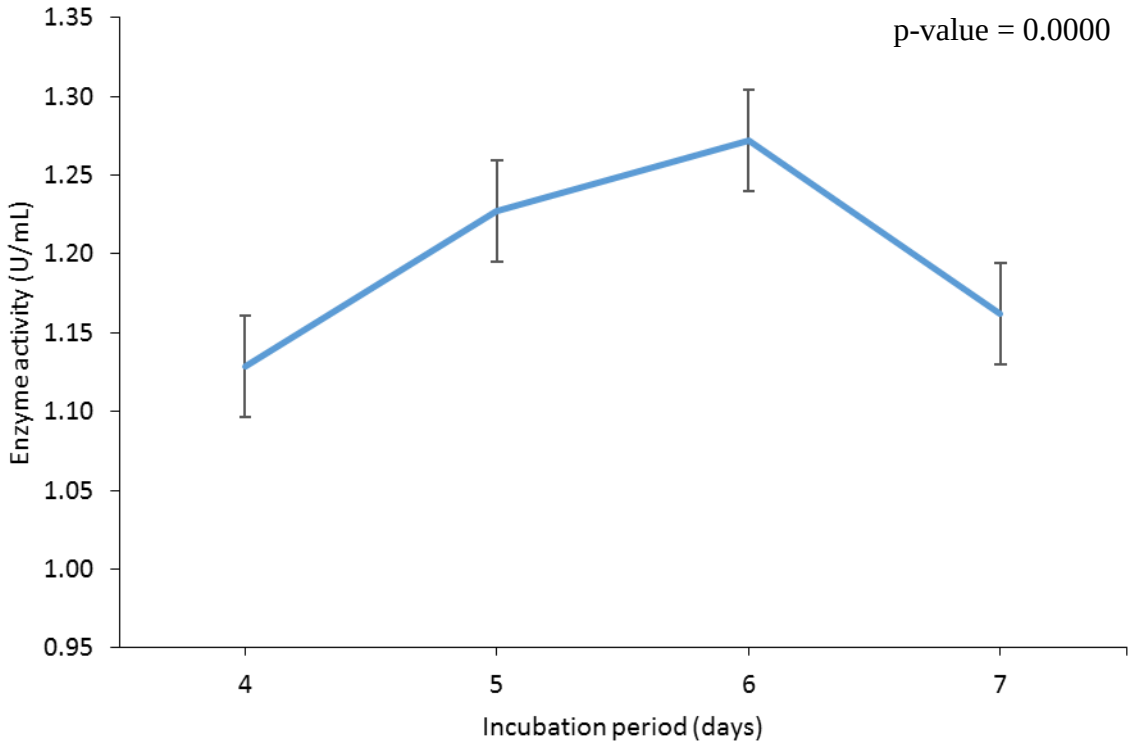

Figure 5: Effect of incubation period on production of laccase by *Curvularia lunata*

DISCUSSION

The result of the proximate analysis revealed that the maize cob was made up of 2.50% of crude protein, 57.09% of carbohydrate and 1.88% of ash. The crude protein content is an indication that the maize cob can serve as a source of nitrogen which is a requirement for efficient fungal growth and expression of enzymes. Presence of fermentable sugars which are also required for efficient fungal growth as well as enzyme production by the test fungi is indicated by the high carbohydrate content in the substrate. In addition to carbon and nitrogen sources, minerals are also required for fungal growth, the presence of these minerals is indicated by the ash content (Madika *et al.*, 2017).

Production of laccase is affected by various factors, such as the species and strain of organism, fermentation type, temperature, pH, moisture content and fermentation media composition (Birhanli and Yeşilada, 2013). *Curvularia lunata* was able to use maize cob as substrate for the production of laccase using both solid state fermentation (1.374 U/mL) and submerged fermentation (1.286 U/mL). This can be linked to the relatively high crude protein content, carbohydrate content and ash content of the maize cob as indicated by the proximate analysis. These proximate contents serve as source of nitrogen, fermentable sugar (carbon source) and minerals respectively for the growth of the fungi and subsequent laccase production. Lower enzyme production observed in submerged fermentation might be as a result of reduction in substrate porosity and oxygen limitation within the substrates due to higher moisture content. This consequently affected the oxygen transfer within the substrate and thus resulting in poor growth and lower enzyme production (Ibrahim *et al.*, 2012). This is also likely due to low enzyme deactivation when adsorbed or immobilized on fermentation substrates under solid state fermentation. The lower laccase production observed in submerged fermentation may as well be due to higher enzyme production and stimulation in solid medium (Desai *et al.*, 2011). This finding is in line with the reports of different researchers: Desai *et al.* (2011), Hong *et al.* (2011), Dos Santos Bazanella *et al.* (2013), Das *et al.* (2015) and Megersa *et al.* (2017) who indicated that laccases and other ligninolytic enzymes are better produced under solid state fermentation compared to submerged fermentation.

The optimum inoculum size for production laccase by *Curvularia lunata* in this study was 3 x 5 mm of mycelium plug (1.326 U/mL). Increased production of laccase with increased inoculum size up to optimum inoculum size followed by gradual decrease was observed. This is likely because at lower inoculum size, fungal growth might not be sufficiently initiated. Higher inoculum size might result in competitive inhibition and faster nutrient depletion, leading to decreased metabolic activity (Bellettini *et al.*, 2019). Similar trend in production of laccase was also reported by other researchers. An increase in laccase production by *Pleurotus ostreatus* and *Neurospora sitophila* with increase in inoculum size up to the optimum inoculum size under solid state fermentation was reported by Saqib *et al.* (2015) and Patel *et al.* (2009) respectively.

Laccase production by fungi depends strongly on pH of the extracellular environment as the pH influences the movement of molecules and components across the cell membrane (Robinson, 2015). The initial pH of the production media may become altered due to production of organic acids during fermentation of substrate, however, the buffering action of agricultural substrates reduces the effect of this pH change on enzyme production (Gomaa, 2013).

The optimum pH for production of laccase was recorded to be pH 5. Laccase production at extreme pH was low, this is likely due to the negative effect of such pH on the stability of membrane, activities of enzyme as well as movement of nutrients required for

microbial growth and development (Edae and Alemu, 2017). This result is similar to that of Abd El Monssef *et al*. (2016) who observed that fungi growth in fermentation medium at pH of 5 produce laccase in excess and Kalra *et al*. (2013) who observed that 4.5-5.5 were the optimum pH for enzyme production. Shraddha *et al.* (2011) reported that the optimum pH for the production of laccase varies based on the substrate used because different laccases reactions are caused by different substrate.

Another important factor that affect fungal growth is temperature of incubation. The optimum incubation temperature for production of laccase by *Curvularia lunata* in this study was 30 ºC. Generally, the range of optimum incubation temperature for the production of laccase lies between 25-30 ºC. This finding is similar to the findings of Megersa *et al.* (2017) who reported 30 ºC as the optimum temperature for laccase production by ligninolytic fungal isolates. It is however in contrast with the report of Abd El Monssef *et al*. (2016) who recorded 35 ºC as the optimum incubation temperature for the production of laccase by *Trichoderma harzianum.* This difference is likely due to the fact that different strains differ in their optimal incubation temperature for laccase production. Fungal growth, metabolite and enzyme production are affected by incubation temperature. Temperature also plays a vital role biological processes development, since it determine denaturation of protein and rate of enzyme production (Risdianto *et al*., 2012). Laccase production at the two extreme temperatures (25 and 40 ºC) were very low. This is likely because temperature cardinality influences the rate of fungal growth and metabolism. Extremely low incubation temperature inhibit fungal growth and metabolism by resulting in enzyme inactivated and hardening of membrane lipoproteins. So also, at extremely high incubation temperature, enzymes and membrane lipoproteins lose their activities because they get denatured (Robinson, 2015).

Highest laccase production by *Curvularia lunata* was observed after 6 days of incubations. Beyond this day, gradual decrease was observed, likely due to exhaustion of nutrients in the fermentation medium (Simoes *et al.,* 2009). This finding agrees with that of Abd El Monssef *et al*. (2016) who also recorded highest laccase production on day 6 of incubation and reduction in laccase production after this optimum incubation period. This is in contrast with the report of Saqib *et al.* (2015), whose optimum incubation period for the production of laccase by *Neurospora sitophila* with corn cob as substrate was 4 days. Laccase is produced as a secondary metabolite during fermentation to prevent self poisoning due to primary compounds accumulation (Chan *et al*., 2016).

CONCLUSIONS

The maize cob was found to be made up of 2.50% crude protein, 57.09% carbohydrates, 32.20% crude fat, 3.15% crude fibre, 3.18% moisture content and 1.88% ash contents. *Curvularia lunata* was able to use maize cob as substrate for laccase production under solid state and submerged fermentation. Highest laccase production by *Curvularia lunata* was observed under solid state fermentation. The optimum culture conditions for laccase production were found to be inoculum size of 3*5mm mycelia plug, pH 5, incubation temperature of 30ºC and 6 days incubation period.

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