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 (Giardi 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; Aafreen 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: (NH4)2SO4, 3 g; (NH4)2HPO4, 3 g; MgSO4. 7H2O, 0.5 g; NaCl, 0.5 g; CaCO3, 0.5 g; FeSO4.7H2O, 0.35 g and CuSO4.5H2O, 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 (NH4NO3, 3 g; (NH4)2HPO4, 3 g; MgSO4. 7H2O, 0.5 g; NaCl, 0.5 g; CaCO3, 0.5 g; FeSO4.7H2O, 0.35 g and CuSO4.5H2O, 0.6 g) as described by 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 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 (NH4NO3, 3 g; (NH4)2HPO4, 3 g; MgSO4.7H2O, 0.5 g; NaCl, 0.5 g; CaCO3, 0.5 g; FeSO4.7H2O, 0.35 g and CuSO4.5H2O, 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 pH 4, 5, 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

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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, 25°C 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 ≤ 0.05).

Table 1: Proximate composition of Maize cob

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Proximate Compositions (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture contents</td>
<td>3.18</td>
</tr>
<tr>
<td>Crude fat</td>
<td>32.20</td>
</tr>
<tr>
<td>Crude protein</td>
<td>2.50</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>3.15</td>
</tr>
<tr>
<td>Ash content</td>
<td>1.88</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>57.09</td>
</tr>
</tbody>
</table>

*Values are mean of triplicate readings
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1.15
1.20
1.25
1.30
1.35
1.40
1.45

Solid state fermentation
Submerge fermentation

Enzyme activity (U/mL)

Type of fermentation

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 ≤ 0.05).

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

p-value = 0.0004
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 ≤ 0.05.
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 30°C 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 25°C. The difference observed in enzyme activity was found to be statistically significant (p ≤ 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 ≤ 0.05.

Figure 4: Effect of incubation temperature 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 Mengersa 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 °C. Generally, the range of optimum incubation temperature for the production of laccase lies between 25-30 °C. This finding is similar to the findings of Megersa et al. (2017) who reported 30 °C 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 °C 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 °C) 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 30°C and 6 days incubation period.

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