Extraction of Good Quality Genomic DNA from Dry woody Mushroom Samples for Molecular Analysis: A Case Study of *Ganoderma lucidum*

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Abstract

Dry and woody mushrooms materials are among the most difficult sources for high quality DNA (deoxyribonucleic acid) extractions. The key to properly use DNA markers to establish reliable identities of samples is based on extracting good quality DNA that is amenable to downstream applications. In this work, attempts were made to extract good quality DNA from dry and woody samples of *G. lucidum* using different modified protocols including kits and conventional methods involving the use of CTAB (Cetyl trimethyl ammonium bromide) and SDS (Sodium dodecyl sulphate) buffers. The extracted DNA was eventually subjected to quality and quantity analyses to ascertain its yield and purity levels. Among the five different protocols used, a carefully modified CTAB protocol produced the best quality DNA with a purity check of 1.78 at OD_{260}/OD_{280} and good yield of about 500ng/µl which is suitable for PCR amplification.

KEYWORDS: DNA Extraction, *Ganoderma lucidum*, Optimization, woody mushroom.

Introduction

The number of biodiversity studies using molecular approaches such as population genetics and molecular systematics, has increased considerably[1, 2]. Typically, studies involving mushroom species have used mycelia as the main sample for DNA extraction. However, production of mycelia from old, dry and woody mushroom samples can be a very laborious task, time consuming and most times not successful which poses a major obstacle to efficient and fast molecular studies.

Different problems can arise when depending on mycelia as the only source for isolating genomic DNA from mushrooms. These include i) unavailability of fresh mushrooms for mycelia production ii) recalcitrant mushrooms samples that are difficult to produce mycelia .iii) using mushroom containing powders (herbal samples) for molecular analysis.

Mushroom tissue culture is not only a time taking process but expensive and cumbersome since fresh samples can only be collected in rainy season and mostly in the tropical rain forests, followed by tissue culture optimization process to produce mycelia. All these common problems could be easily circumvented by DNA directly extracted from dry samples regardless of how old they are, with simple tools in a relatively short time and available any time of the year.

Mushrooms have continued to generate a lot of interests particularly in its consumption as food, in cure of diseases, in biodegradation and as important items of commerce in Nigeria and all over the world[3, 4]. Mushrooms represent a major and yet

largely untapped source of potent pharmaceutical products[5, 6].

Mushrooms belong to the kingdom of Fungi, a group very distinct from plants, animals and bacteria. They are macro fungi with distinctive fruiting bodies and reproductive structures[7, 8]. Higher fungi have been identified as a major source of biologically active natural products, which provide varieties of active secondary metabolites[9, 10]. Mushrooms are widespread in nature and are of great economic importance to man. They are the earliest form of fungi known to mankind with their occurrence dating back to the time of the early man as mushrooms appear in traditional Yoruba art works known as "tie and dye", which are materials of traditional costumes[11]. Many genera of mushrooms are edible and are rich in essential nutrients such as carbohydrates, proteins, vitamins, minerals, fat, fibers and various amino acids[12, 13]. However, some others are poisonous and may claim lives within few hours after consumption.

G. lucidum, a glossy and woody mushroom is a seasonal fungus that grows wildly in the tropical rain forests. It is a widely used medicinal fungus because of its high nutritional and pharmaceutical value. Among cultivated mushrooms, *G. lucidum* is unique in that, its pharmaceutical rather than nutritional value is paramount. A variety of commercial *G. lucidum* products are available in various forms, such as powders, dietary supplements, and tea. These are produced from different parts of the mushroom, including mycelia, spores, and fruit bodies. Due to its high value, there are ongoing molecular studies into this 'wonder' mushroom. Hence a fast, inexpensive and effective method of extracting good quality DNA at any time with both dry and fresh sample is the key to exploring the molecular realities of this fungus.

Materials and methods

Mushroom sample

Dry woody samples of *G. lucidum* were collected and identified at the Industrial Biotechnology and Biodiversity Unit and taken to Molecular Biology Unit, both in Biotechnology and Genetic Engineering Advance Laboratories of Sheda Science and Technology Complex (SHESTCO), Abuja for DNA extraction.

Solutions

Solutions of extraction buffers and other reagents used include: 5%(w/v) SDS (BDH Biochemical England), 100mM Tris (pH 8.0) (BDH Biochemical England) , 50mM EDTA (pH 8.0) (BDH Biochemical England), and 500mM NaCl (Sigma Aldrich GmbH), 5M potassium acetate, 2%(w/v) and 5%(w/v) CTAB (Surechem Products England). Phenol-chloroform-isoamylalcohol (25:24:1), Chloroform: Isoamylalcohol (24:1), 70%(v/v) and 100%(v/v) ethanol, TE buffer consisting of 10mM Tris (pH 8.0) and 1mM EDTA (pH 8.0), TBE buffer consisting of 890mM Trisma base, 890mM Boric acid and 2mM EDTA, DNase free RNase A 10mg/ml, Zymo Research Fungi/Bacterial DNA extraction kit.

Preparation of DNA Extraction Buffers

Extraction buffer 1: GL (DEB-2% CTAB) = 2%(w/v) CTAB extraction buffer for *G. lucidum* DNA extraction. This extraction buffer contained 100mM Tris-HCl, 20mM EDTA, 2% CTAB (w/v), 1% PVP (molecular biology grade BDH Biochemica England), 1.4 M NaCl, 2%(v/v) β-mercaptoethanol (BME) added prior to usage.

*Extraction buffer 2***:** GL (DEB-5% SDS) = 5% (w/v) SDS extraction buffer for *G. lucidum* DNA extraction.
This extraction buffer contained 100mM Tris-HCl, 50mm EDTA, 5% SDS (w/v), 2%(w/v) PVP, 500mMM NaCl, 1%(v/v) BME (BME added prior to usage).
*Extraction buffer 3***:** GL (DEB-1% SDS-PK) = 1 % (w/v) SDS extraction buffer containing proteinase K for *G. lucidum* DNA extraction.
This extraction buffer contained 100mM Tris-HCl (pH 8), 10mM EDTA, 1% SDS (w/v), 2%(w/v) PVP, 1M NaCl, 0.5%(w/v) Proteinase K, 1%(v/v) β-mercaptoethanol (BME added prior to usage).

Optimization process

(A) DNA Extraction Using ZR DNA Extraction Kit For Bacteria /Fungi
Genomic DNA extraction of *G. lucidum* was carried out with Zymo Research (ZR) Bacterial/Fungal DNA extraction mini prep kit, (Cat no 6005, USA) following the instruction manual (www.zymoresearch.com).
(B) DNA Extraction Using Modified SDS Method -1 (with GL DEB-1% SDS-PK solution)
Extraction of genomic DNA from *G. lucidum samples* was carried out according to Sharma *et* al*.,* (2009).
(C) DNA Extraction Using Modified CTAB method -1 (with GL DEB-2% CTAB solution)
Two hundred milligrams (200mg) of ground tissues of *G. lucidum* was transferred into labeled 2ml microfuge tubes followed by addition of 500ul of GL DEB-2%CTAB extraction buffer. It was mixed well and incubated at 65^0C for 60min with intermittent shaking at 10 min interval after which it was cooled to room temperature and 500ul of Chloroform Isoamylalcohol (CIA) was added, shaken well to homogenize and spun at 13000 rpm for 5min. The supernatant was transferred into new microfuge tubes and retreated with CIA twice more. Fifteen micro liters (15µl) of 5M potassium acetate and 500µl of ice cold isopropanol were added and gently mixed before incubating the samples at -20^0C for 60 min. This was followed by spinning at 13,000 rpm for 5 min after which the isopropanol was discarded carefully. Washing with 200µl of ice cold 70 % (v/v) ethanol was done thrice and lastly with absolute ethanol then air drying was carried out carefully. The extracted DNA sample was finally reconstituted with 50µl of low salt TE (Tris- EDTA) buffer.
(D) DNA Extraction Using Modified SDS Method -2 (*with GL/DEB-5% SDS solution*).
Extraction of genomic DNA from *G. lucidum samples* was carried out according to Ihase *et al.,* (2014).
(E) DNA Extraction Using Modified CTAB Method – 2 (with GL DEB-2% CTAB solution)
Two hundred milligrams (200mg) of ground samples of *G. lucidum* was transferred into labeled falcon tubes (1.5ml). Two thousand five hundred micro liters (2500µl) of pre-warmed extraction buffer (GL DEB- 2%CTAB) were added. It was incubated at 65°C in a water bath for 30 min with intermittent mixing; the samples were cooled for 5 min at room temperature and equal volume of Phenol-Chloroform-Isoamylalcohol (PCI) 25:24:1 was added and vortexed for 1 min for proper mixing before dispensing it into 4 different 2ml microfuge tubes (1.25ml each). The samples were then centrifuged at 10,000rpm for 10 min at 40^0C after which the supernatants were transferred into new tubes and 700µl (2/3 volume) of ice cold isopropanaol was added to each and incubated overnight at -20^0C. Centrifugation at 10,000rpm for 10min at 4^0C was carried out after the overnight

incubation followed by washing with 70 % (v/v) ethanol twice and air drying. Resuspension of the samples with 50µl of TE buffer plus 2µl of 10mg/ml of RNase A and 30 min incubation at 37^0C and later precipitation with absolute ethanol was carried out before washing with 70 % (v/v) ethanol and air drying the samples. The samples were finally reconstituted in 50µl of low salt TE (Tris- EDTA) buffer. .

Determination of Quality and Quantity of Isolated Genomic DNA

*By Agarose Gel Electrophoresis***:** Fifty milliliters (50ml) of 0.8% (w/v) of agarose gel were prepared by weighing accurately 0.4g, of agarose powder (BDH Biochemical England) and made up to 50ml with X1 TBE (Trisma base-Boric acid-EDTA) as a solvent. To obtain a homogenous solution, it was heated in a microwave oven for about 1min. In the fume hood, 10,000X (5µl) of GR Green fluorescent dye (Inqaba biotech, South Africa) was added with vigorous shaking and was allowed to cool. It was poured in gel casting tray after cooling down to about 40^0C.

The prepared gel was placed in the electrophoresis tank and x1TBE buffer was added to submerge the gel. The extracted genomic DNA samples (5µl) mixed with 1µl of 6x DNA loading dye (Thermo Scientific, USA) was loaded in the wells of the gel accordingly as well as the step-ladder DNA (Thermo Scientific O' Gene Ruler, 1kb plus) . The set up was covered and connected to the power pack and was run at 55volts for 45 min, after which it was visualized using Alpha Innotech Gel documentation system model 201103 (Taiwan). This process was carried out for each of the extraction methods optimized.

*By UV Spectrophotometry***:** In the spectrophometric analysis, 10µl of DNA sample was diluted to 1000µl with T.E (100X dilution).The spectrophotometer readings were recorded at 260/280 nm using Lab Kit U V-VIS spectrophotometer model ST- U V 755B (Korea). DNA concentration was calculated using OD values at 260 nm using the formula below.

Concentration of DNA (µg/µl) = OD at 260 nm x 50 x Dilution factor.

PCR Amplification

The PCR amplification was carried out using a pair of internal transcribed spacers' primers. The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. The summary of the parameters used is 0.5µM each of the ITS-1 and ITS-4 primers, 2mM $MgCL_2$, 0.2mM deoxynucleoside triphosphate (dNTP) mix, 1.25U of T*aq.* polymerase enzyme and 100ng of template DNA per reaction mix at 95°C for 3min, 35 cycles of 94°C for 1 min, 56°C for 2min 72°C for 2min and 72°C for 10min.

Result and Discussion

Filamentous fungi have rigid cell walls and high polysaccharide contents which poses difficulty during DNA extraction. For this reason, it is scientifically recommended to use extraction methods that enhance breaking of cell walls. Zhou *et al.* (2007**)** indicated that methods like CTAB, SDS-CTAB, SDS methods and benzyl chloride method are only suitable when DNA is extracted from mycelia. However careful modifications of methods involving CTAB and SDS can yield DNA samples with good yield and quality as seen in the table and figures below. The quality and quantity of the

extracted DNA were evaluated by agarose gel electrophoresis and UV spectrophotometry. The results of the extracted DNA, run on a 0.8 % (w/v) agarose gel, stained with GR Green and visualized with UV light is shown in Plates A, B, C, D and E

Table 1.0: Quantity and quality analyses result of genomic DNA extracted from dried sample of *G. lucidum* using ZR Fungal/ Bacteria DNA extraction kit, modified CTAB methods 1 & 2 and modified SDS methods 1 & 2.

Sample No	**Concentration (µg/µl)**	**Absorbance (260nm)**	**Absorbance (280nm)**	**Ratio (260/280nm)**
(A)1		-	-	-
2		-	-	-
(B**)** 1	0.245	0.049	0.035	1.39
2	0.345	0.069	0.050	1.38
(C)**1**	**0.490**	**0.098**	**0.055**	**1.78**
2	**0.695**	**0.139**	**0.079**	**1.76**
(D)1	0.895	0.179	0.142	1.40
2	1.080	0.216	0.154	1.40
(E)1	1.350	0.270	0.194	1.39
2	1.230	0.246	0.178	1.38

*Legend***:**

(A) = DNA extraction with ZR Kit
(B) = *DNA Extraction Using Modified SDS Method -1*
(C) = *DNA Extraction Using Modified CTAB method -1*
(D) = *DNA Extraction Using Modified SDS Method -2*
(E) = DNA extraction using *Modified CTAB* method -*2*
- = Nil

Below is electrophoretogram results of the different optimized processes (A-E) outlined above for genomic DNA extraction from the dry and woody sample.

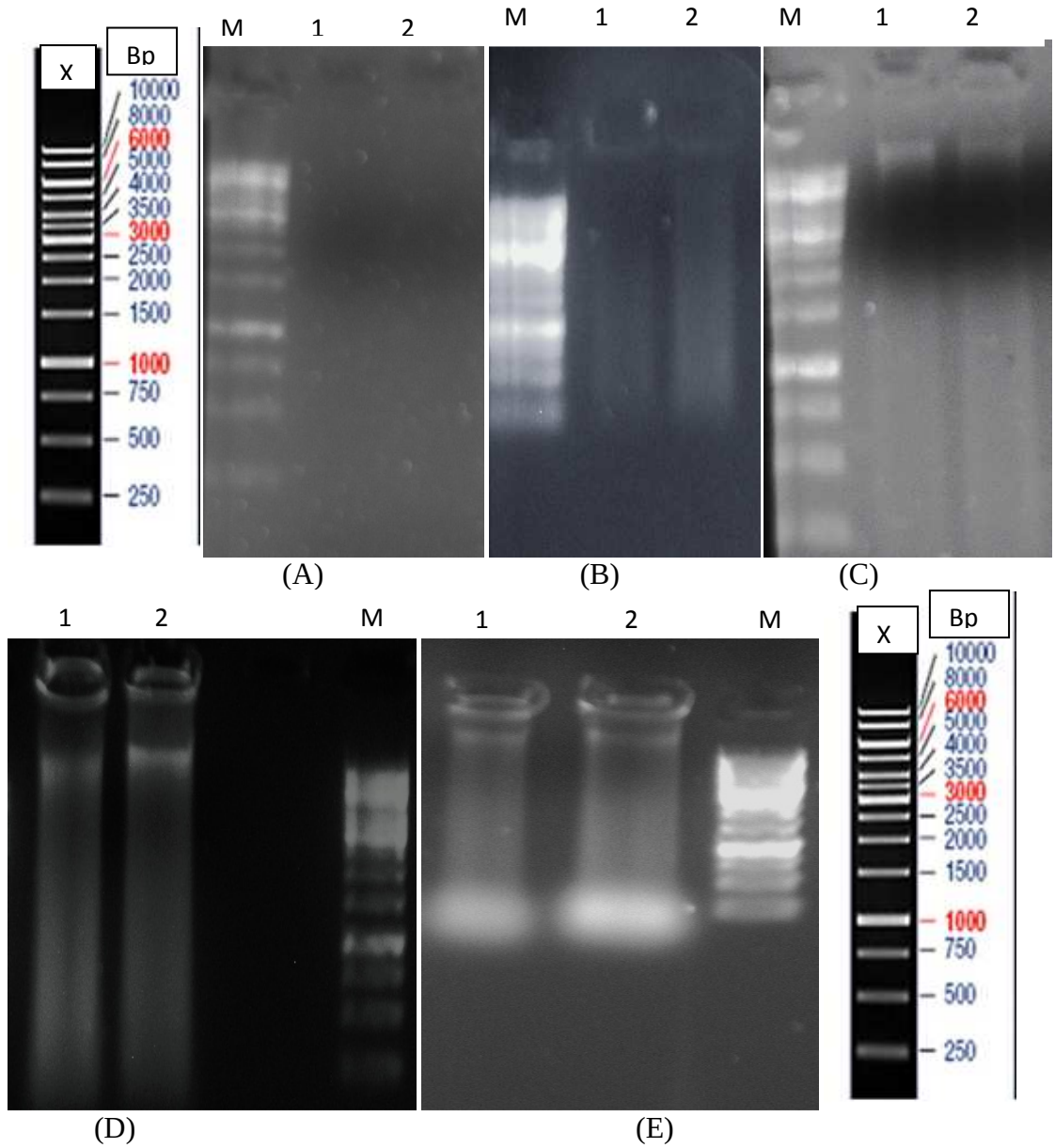

Figure 1 (Plate A-E): Electrophoretogram of genomic DNA extraction from the *G. lucidum* sample using ZR Fungal/ Bacteria DNA extraction kit, CTAB methods 1and 2, SDS methods 1 and 2, ran on 50ml 0.8% agarose gel electrophoresis for 45 min at 55V, then visualized by GR Green staining using a gel documentation system (Alpha Innotech, Taiwan).

Legend:

(A) = DNA extraction with ZR Kit
(B) = *DNA Extraction Using Modified SDS Method -1*
(C) = *DNA Extraction Using Modified CTAB method -1*
(D) = *DNA Extraction Using Modified SDS Method -2*
(E) = DNA extraction using *Modified CTAB* method *-2*

(X) = Step ladder Chart [1Kb O' Gene Ruler product information chart from Thermo Scientific showing bands in base pairs (bp)
(M) = Marker DNA (Step ladder, 1Kb O' Gene Ruler 0.1µg/µl by Thermo Scientific.
(1) = Sample 1.

(2)= Sample 2

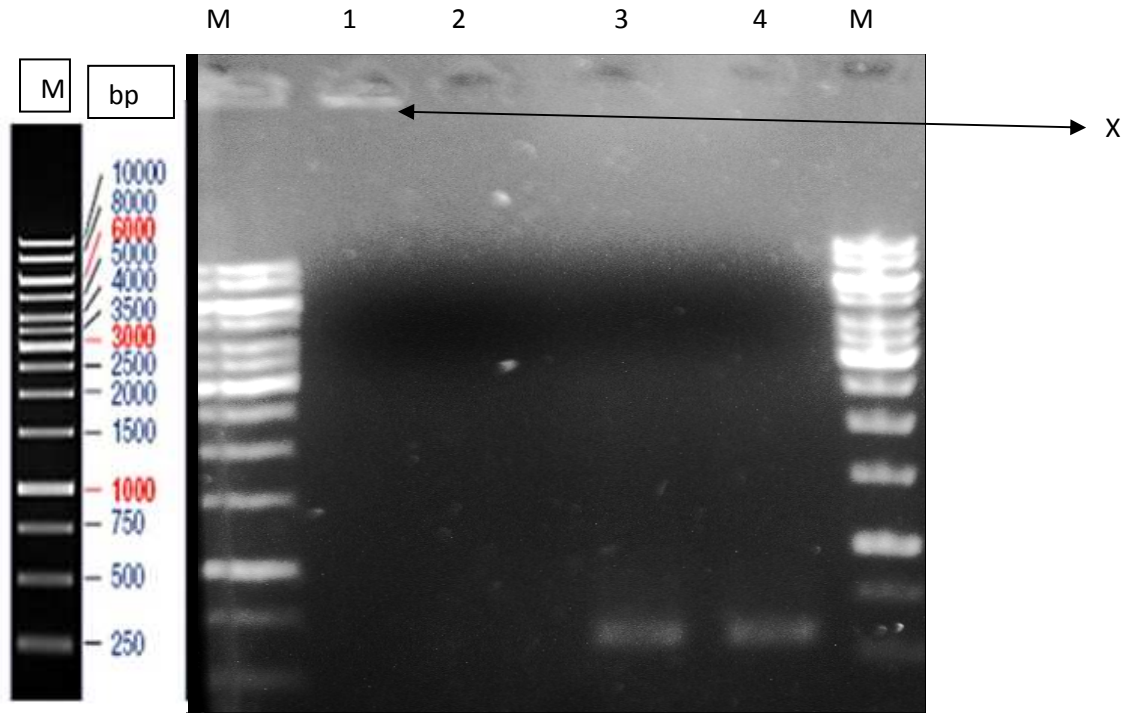

Figure 2 (Plate F): Electrophoretogram of ITS PCR amplification of *G. lucidum* genomic DNA with primer ITS1 and ITS4. M = Molecular weight marker (O' Gene ruler) 1kb. Lane 1: (X)= positive control, Lane 2 = negative control, Lane 3 and 4= PCR product from plate C (modified CTAB Method 1) in figure 1 above.

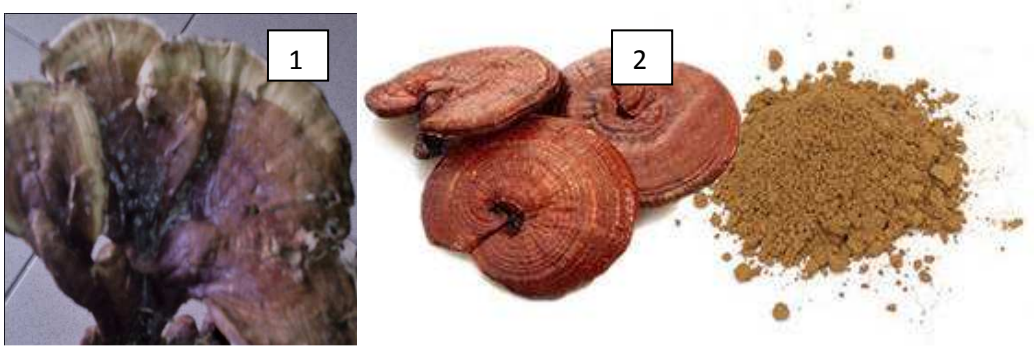

Figure 2.0: Pictures of Ganoderma *lucidum* samples used for the work

Legend: 1= Sample 1 (Ganoderma *lucidum* 1)
2 = Sample 2 (Ganoderma *lucidum* 2)

Conclusion

A good DNA preparation generally exhibits a spectral property of absorbance (A) at A260/A280 = 1.80 ± 0.04 and a reasonable yield of not less than 50ng/µl. Good quality DNA is the basis for molecular based analysis and a DNA sample yield of 490ng with purity level of 1.78 is no doubt suitable

for molecular analyses which was confirmed by its amplification via PCR using ITS primers. After series of optimization of different protocols with careful modifications, extraction using modified CTAB method –2 yielded the best quality DNA sample within the range of 0.490µg/µl - 0.695µl (490ng/µl- 695ng/µl) with purity level of 1.76-1.78 (See **(C)** in table 1.0 and figure 1.0 above) .

In conclusion, a carefully modified protocol involving CTAB is used to extract DNA samples with good yield from dry woody mushrooms materials.

Conflict of Interest.

We hereby declare that there is no conflict of interest.

References

1. Avise JC (2000). Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.
2. Crawford DJ (2000). Plant macromolecular systematics in the past 50 years: one view. *Taxon* 49: 479-490.
3. Egwin, E. C., Elem, R. C., and Egwuche, R. U. (2011). Proximate composition, phytochemical screening and antioxidant activity of ten selected wild edible Nigerian mushroom. *American Journal of Food and Nutrition*; 1:89-94. http://doi:10.5251/abjna.2011.1.2.89.94
4. Jonathan, S. G., Okorie, A. N., Babayemi, O. J., Oyelakin, A. O., Akinfemi, A. (2012). Biodegradation of agricultural wastes (rice straw and sorghum stalk) into substrates of utilizable products using white rot fungus (*Pleurotus florida*). *Nature and Science,*10: 131-137. http://www.sciencepub.net/nature18
5. Gbolagade, J. and Fasidi,I. (2005). Antimicrobial activities of some selected Nigerian mushrooms. *African Journal of Biomedical Sciences*,;8:83-8.
6. Joseph, S., Sabulal, B., George, V., Smina, T. P., and Janardhanan, K. K. (2009). Antioxidant and ant-inflammatory activities of the chloroform extract of *Ganoderma lucidum* found in south India. *Journal of Pharmaceutical science*, 77: 111-121. .
7. Pilz, D., Norvell, L., Danell, E. and Molina, R. (2003): Ecology and management of commercially harvested Chanterelle mushrooms. *Gen. Tech. Rep. PNW-GTR-576. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station*. Pp. 1-16.
8. Prasad, Y. and Wesely, W. E. G. (2008). Antibacterial activity of the bio-multidrug (*Ganoderma lucidum*) on Multidrug resistant Staphylococcus aureus (MRSA). *Advanced Biotech*., 10: 9-16.
9. Jonathan, S.G, Fasidi, I.O. (2003). Antimicrobial activities of two Nigeria edible macro-fungi: Lycoperdon pusilum (Bat. Ex) and *Lycoperdon giganteum* (Pers.) African Journal of Biomedical Research. 6:85–90.
10. Barranco P. G., Ocanas L. G., Cabrera L. V., Carmona M. C. S., Ocanas F. G., Gomez X. S. R., and Rangel R. L. (2010). Evaluation of antioxidant, immune-modulating, cytotoxic and antimicrobial properties of

different strains of Basidiomycetes from Northeastern Mexico. *Journal of Medicinal Plants Research*; 4: 1762- 1769.

11. Okhuoya, J.A.,Akpaja, E.O., Osemwegie, O.O., Oghenekaro, A.O., Ihayere, C. A. (2010). Nigerian Mushrooms: Underutilized Non-Wood Forest Resources. J. Appl. Sci. Environ. Manage. *March*, 2010 Vol. 14(1) 43 – 54.
12. Khatun, S., Islam, A., Cakilcioglu, U., and Chatterjee, N.C. (2012). Research on mushroom as a potential source of Nutraceuticals: A review on Indian perspective. *American Journal of Experimental Agriculture*, 2
13. Jonathan, S. G., Okon, C. B., Oyelakin, A. O., and Oluranti, O. O.(2012) Nutritional values of oyster mushroom (Pleurotus ostreatus) (Jacq. Fr.) Kumm cultivated on different agricultural wastes. *Nature and Science*, 10: 186-191. .
14. Zhou, X., Li, Q.,Zhao, J.,Tang, K., Lin, J., & Yin, Y. (2007). Comparison of rapid DNA extraction methods applied to PCR identification of medicinal 74 mushroom *Ganoderma* species. *Preparative Biochemistry & Biotechnology*, 37, 369-380.
15. Ihase, L.O., Horn,R., Anoliefo, G.O., Eke, C. R., Okwuagwu, C.O and Asemota, O. (2014). Assessment of an Oil palm population from Nigerian Institute for Oil palm Research (NIFOR) for simple sequence repeats(SSR) marker application.
16. Sharma, K., and Kumar, P.L. (2009). Simple and rapid method for extraction of DNA from leaf, tuber and seed tissue for PCR applications. African Journal of Biotechnology.http://dx.doi.org :10.5897/AJB12.2096.