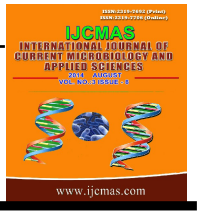
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 115-126**
http://www.ijcmas.com



Original Research Article

Production of *Pleurotus tuber-regium* (Fr.) Sing Agar, chemical composition and microflora associated with sclerotium

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Keywords

Mushroom, Sclerotium, Chemical Composition medium, *Pleurotus tuber-regium* sclerotium agar

A B S T R A C T

Pleurotus tuber-regium is a tropical mushroom whose sclerotium is consumed like many other edible mushrooms for their nutritional value, medicinal properties and for their taste and aroma. Assessment of its chemical composition shows mean carbohydrate (63.8%), protein (12.36%), moisture (19.88%), fat (3.23%) and fiber (0.25%). Potassium, calcium, magnesium, sodium and phosphorous were present in amounts ranging from 0.028 to 0.223 %, while oxalate, tannin, flavonoid, alkaloid and hydrogen cyanide were confirmed to be present. *Pleurotus tuber-regium* sclerotium agar (PTRSA) was evaluated as a complex medium compared with Nutrient agar (NA) and Plate count agar (PCA) for the growth of heterotrophic organisms. Five bacteria and a fungal species (*Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus* and *Candida albican* were accessed. There was no statistical significant difference ($P< 0.05$) in the colony forming unit per ml (cfu/ml) recovery of these heterotrophic organisms diluted $1.8x10^8$ by McFarland standard. The PTRSA function compares favorably with those of NA and PCA and can be standardized for use as a cheap medium for the cultivation of heterotrophic organisms. Evaluation of bacterial and fungal flora associated with the sclerotia shows the presence of species of *Aspergillus*, *Bacillus*, *Pseudomonas*, *Klebsiella*, and *Staphylococci. Pleurotus tuber-regium* is an underutilized fungus whose potentials can be further harnessed for the benefit of man.

Introduction

Mushrooms are popularly consumed as delicacy by Nigerians as in many other countries of the world (Gregori *et al*., 2007; Zhang *et al*., 2010; Gucia *et al*., 2011; Nnorom *et al*., 2013). *Pleurotus tuber-regium* is an edible mushroom and like many other edible mushrooms are consumed for their nutritional value, medicinal properties and for their taste and aroma (Falendysz and Gucia, 2008; Nnorom *et al*., 2013). The sclerotium of *Pleurotus tuber-regium* is a compact mass of hardened fungal mycelium containing food reserves which help the fungi survive environmental extremes (Thorn *et al., 2000*). It is spherical to ovoid in shape and can be quite large measuring up to 30cm or even larger in diameter (Oso, 1997) and could weigh as

much as 5kg (Iwuagwu and Onyekweli, 2002). It is highly nutritious containing good quality proteins and carbohydrates with little fat.

The sclerotium produced by *Pleurotus tuber-regium* has been reported to constitute of about 64.31% WW and 71.21%DW protein, 20.00% WW and 22.15% DW carbohydrate, 2.20% WW and 2.44 DW ash and 2.89% WW and 3.20% DW crude fiber (Ikewuchi and Ikewuchi, 2009). It has also been noted to contain about 18.6% sugar having high concentrations of galactose and low concentrations of glucose and maltose. Extracts of *Pleurotus tuber-regium* sclerotium in combination with some herbs can be used in preparations for cures to headache, stomach ailments, Asthma, High blood pressure and also serve as good anti-tumor, anti-hyperglycemic and anti-hypertensive agents (Zhang *et al.,* 2004, 2006, and 2007).

Pleurotus tuber-regium helps in agricultural waste cycling, waste material that can be used in making compost for the cultivation of *Pleurotus tuber-regium* include rice straw, wheat straw, corn cub, cassava peels, palm wastes, cotton seed hull, peanut hull and sunflower straw. *Pleurotus tuber-regium* as an edible wild mushroom has been found to be a nutritionally rich source of food (Fasidi *et* al., 1993; Onyeike and Ehirim, 2001; Basu *et al.,* 2007), important for bioremediation and waste recycling (Anoliefo *et al*., 2002; Iskheuemhen *et al.,* 2003), as a source of income and with lots of health benefits to mankind (Zhang *et al.,* 2007).

Several studies have investigated the chemical compositions of sclerotium of *Pleurotus tuber-regium* (Onyeike and Ehirim, 2001; Alobo, 2003; Ikewuchi and Ikewuchi, 2009), mineral compositions (Udeh *et al*., 2001; Nnorom *et al*., 2013), phytochemical constituents (Akindahunsi and Oyetayo, 2006; Ikewuchi and Ikewuchi, 2009; Ijeh *et al*., 2009) and medicinal and other values (Zhang *et al*., 2004; Gregori *et al*., 2007). Data are however, scanty on other possible economic applications of *Pleurotus tuber-regium.* Iwuagwu and Onyekweli (2002) investigated the use of Pleurotus *tuber-regium* powder as a tablet disintegrant.

In order to grow and reproduce, microorganisms need nutrients, a source of energy and good environmental conditions (Bridson and Brecker, 1970; Atlas, 2005; Sigma-Aldrich, 2014). The nutrients and energy are often from plants and animals sources. Ramakrishnan *et al.* (2011) utilized degraded chicken feathers in media formulation, Egwari *et al*. (2013) evaluated crayfish charcoal agar and found it a good transport medium for anaerobes. The need for evolving knowledge still exist, the rich chemical composition of slerotium of *Pleurotus tuber-regium* necessitate that it can support the growth of microbes on and around it as associated microfrora.

This study explored the production of complex nutrient medium from the infusion and powder of the sclerotium of *Pleurotus tuber regium* as the only nutrient and carbon source in the formulation of PTRSA. And it also includes the assessment of associated microflora and proximate composition of the sclerotium.

Materials and Methods

Sample collection and treatment:

Twelve (12) samples of fresh *Pleurotus tuber regium* sclerotia were purchased from food vendors in four different markets (Main market, new market, road side market at Orji

and Ihiagwa market) all in Owerri, Imo state, Nigeria. Samples were dully identified at the research institute Umudike, before commencement of analysis.

Ten gram (10g) portion of samples were cut from the surface of the sclerotium using sterile knife, samples were blended in sterile waring blender and homogenized in 90 ml distilled water for 1:10 dilution. Further tenfold dilutions of the sample homogenate to 10^{-6} were achieved. Aliquot 0.1 ml of appropriate dilutions were spread plated in triplicate unto Nutrient agar (NA) for total aerobic plate count (TAPC), MacConkey agar (MA) for coliform count and Potato dextrose agar (PDA) for fungal count. One gram (1 g) samples were inoculated into lactose broth with inverted Durham tube in Macartheny bottles for coliform test. Cultures on NA, MA and lactose broth were incubated for 24 to 48 h at 37°C. PDA was incubated at 29±2°C for 3 to 7 days.

Preparation of media

Three culture media were assessed for the growth of heterotrophic microorganisms. Nutrient agar (NA, Biomark), Plate count agar (PCA, Biomark) and *Pleurotus tuber regium* sclerotium agar (PTRSA, formulated in the laboratory). The NA and PCA were prepared according to manufacturer's instructions. Two forms of PTRSA were prepared. In the first instance, *Pleurotus tuber regium* was blended to fine powder, 20g was infused in 200 ml distilled water. A hundred (100) ml of the infusion was mixed with agar-agar (Oxoid), homogenized at 100°C and autoclaved at 15Ibs pressure (121°C) for 15 minutes. In the second type, 0.75g powdered *Pleurotus tuber regium* was mixed with 1.5 g agar in 100 ml distilled water and then homogenized at 100°C before sterilization at 15Ibs pressure (121°C) for 15 minutes. This second preparation did not support growth of the test organisms.

Preparation of test organisms

Cultures of test organisms obtained from Leeuwenhoek laboratory, Nekede were checked for viability and purity by repeated subculture on NA and Mueler Hinton agar (Oxoid) and were confirmed by some biochemical tests to belong to specified genera. Pure culture of test organisms was cultured overnight in peptone water (Biomark) and stadadized with McFarland turbidity standards to an approximate concentration of $1.5x10^8$ cfu/ml

Media evaluation

Aliquot 1ml of each standardized culture was plated on the test media in triplicate by both the spread plate and pour plate techniques. A loopful of the culture was also made as inoculums and plated out on the media. Plates were incubated at 37°C for 18 to 24 h and examined at the expiration of incubation time for colony count using colony counter (Gallenkamp, England). Counts were expressed as mean colony forming unit (cfu/ml)

Enumeration and identification of microbial isolates from sclerotium

At the expiration of incubation period, culture plates were examined and colonies enumerated using colony counter (Gallenkamp, England). Colonial morphology was observed and recorded. Preliminary identification of bacterial isolates was based on Gram staining, catalase activity, indole, methyl red, Voges proskaur test, motility, citrate utilization, urease production, starch hydrolysis, gelatin liquefaction, coagulase and fermentation of sugars. Further identification of bacterial isolates was based on standard bacteriological procedures (Jolt *et al*., 1994) and the Biomerieux® sa API system.

Fungal isolates were identified based on morphological and microscopic characteristics, pigmentation on media, sugar assimilation tests and with reference to standard identification key and atlas (Tsuneo, 2010). Confirmation for coliform organisms was based on presumptive, confirmatory and completed tests following the description of Speck (1976), Oranusi *et al*. (2004).

Determination of chemical composition

Evaluation for the chemical compositions was achieved by analyzing the sclerotium for its proximate, phytochemicals and mineral compositions.

Proximate Analysis: The standard methods of AOAC (1990), was adopted for all analysis.

Moisture content: Two gram (2g) weights of samples were weighed and oven dried to constant weight at a temperature of 105°C. Percentage moisture content was calculated from difference in weight of initial and final samples.

Ash Content: Aliquot 2g of each of the sample was weighed into crucible, and placed in the muffle furnace at a temperature of 575°C until it is carbonized. Percentage ash content was calculated from difference in weight of sample before and after ignition.

Crude fiber: Two gram (2g) of sample was treated based on AOAC (1990) standard procedure. The loss on incineration was obtained as the mass of the crude fiber, and was used for percentage crude fiber calculation.

Lipid content: Soxhlet extraction of 2g samples was carried out, the defatted samples were carefully removed and the solvent recovered. The flask and oil was oven dried until all the solvent was gone and the content was weighed and percentage lipid content calculated.

Crude Protein: The total nitrogen content of samples was determined by MicroKjedalh method. The protein value was derived from the nitrogen content by multiplying by a factor of 6.25 and further by a hundred for percentage protein composition

Carbohydrate: The total carbohydrate content was determined by difference following the description of Sarkiyayi and Agar (2010). The sum of the percentage moisture, ash, lipid, protein and crude fiber was subtracted from 100%.

Phytochemical analysis

The phytchemical compositions of the samples were determined following standard procedures. The sclerotium samples were analyzed for presence of Alkaloid, Saponin, Flavonoid, Tannin, Hydrogen cyanide and Oxalate respectively. Alkaloid was determined by the alkaline precipitation method Harborne (1998). Saponin was by the method described by Obadoni and Ochuko (2001), while hydrogen cyanide was determined by the method as described by Anhwange *et al.*(2011). The flavonoid values were determined by the method described by Harborne (1998). Oxalate was determined by using the method of Oke (1969) as described by Sarkiyayi and Agar (2010). The method as described by Sarkiyayi and Agar (2010) was followed for the determination of tannin.

Minerals analysis

The mineral elements Potassium, Calcium, Magnesium, Sodium and Phosphorous were

assayed by the use of Atomic Absorption Spectrophotometer (AAS).

Statistical analysis

The counts as obtained on each media type for the triplicate sampling were presented as mean and analyzed with Chi-square test and employing Duncan Multiple Range Test (DMR) to determine level of significance at P= 0.05

Results and Discussion

All the test organisms with the exception of the *streptococcus* spp grew luxuriantly in all the media. The mean counts for recovery of heterotrophic test organisms on the different media are as shown in table 1. The counts obtained at 24 h showed no significant difference between the three media (P=0.05). Fig 1 shows the growth of the test organisms on PTRSA at 18 h and 37°C.

Table 2 present the mean counts of samples of sclerotium grouped into the four different markets of purchase (ABCD). It reveals that all the samples were contaminated with TAPC ranging from $8.0x10^7$ to $3.0x10^{10}$. The fungal counts in all the samples are of the order of 10^6 except for sample D. All the samples had coliform contamination with sample C having the highest count of 10^7. The table also shows that species of *Aspergillus* and *Bacillus* are the major organisms associated to the sclerotium as they are present in all the samples. Other organisms isolated in $\geq$ 50% of samples included species of *Pseudomonas, Klebsiella*, and *Staphylococci*.

Table 3 presents the chemical composition of the sclerotium. It is rich in carbohydrate (63.81%), moisture (19.86%), protein (12.36%) and fat (3.23%). Oxalate and Alkaloid are present at 4.25 and 2.20%, with trace amounts of Saponin. Sodium is present at concentration of 0.22%, calcium 0.18% and potassium 0.13%.

The use of complex media (general purpose media) for culturing of specimen remains the most popular/frequent practice in laboratories, because determination of total viable heterotrophic count helps to assay the level of contamination/bioburden and as such the threshold that signifies significant/concern. This is achieved before other specific organisms are sought for using special media. It is pertinent that a cheap and easy to compound medium for this all important culture step is designed. Many research efforts at designing microbiological culture media (complex and defined) from locally available materials have been reported each with its own limitations and successes (Westeijn and Okafor, 1971; Nzeribe and Gugnani, 1984; Oloke and Famurewa, 1991; Egenu and Njoku, 2006; Laleye *et al*., 2007; Famurewa and David, 2008; Ramakrishnan *et* al., 2011; Ravathie *et al.,* 2012; Amadi and Moneke, 2012; Egwari *et al.,* 2013). Report on the use of *Pleurotus tuber regium* sclerotium for microbiological media formulation has not been documented to the best of our knowledge, this makes this work innovative.

No single medium can totally support the growth of all organisms; there is the need for more innovations in media formulation. Some documented information on media formulation use common and stable food materials that are already in short supply for the food need of the ever increasing human population. This negate the usual claim of "cheap media" formulation by authors because industrial/commercial scale production of such media will have adverse effect on human and animal food supply, thus the media from common and stable food materials are rather expensive and not cheap. The cheapness of media from

Pleurotus tuber regium sclerotium in all respect is innovative. Nutrient agar and Plate count agar are known and established general purpose media for culturing non-fastidious heterotrophic organisms, this study was meant to use them to evaluate the effectiveness of cheap and easy to compound PTRSA for general purpose medium. It was not to evaluate the effectiveness of NA and PCA.

The results reveal that the growth of organisms in PTRSA is comparable with NA and PCA as the differences in total counts cfu/ml were not significant. Further studies are necessary to standardize PTRSA and determine microbial survival dynamics on PTRSA over long period of time. The high microbial counts (TAPC) recorded in this study could be associated to the fact that *Pleurotus tuber-regium* is a saprophyte that grows on dead decaying woods, the sclerotium is harvested from under the soil. The attitude of food vendors leaving the product (whole sclerotium) on the bare floor in market places to attract the attention of potential buyers could have contributed to the high TAPC in the *Pleurotus tuber-regium* sclerotium.

Table.1 Mean count Log_{10} $Cfuml^{-1}$ for recovery of test organisms on the different media

Test organism	Count on media		
	NA	**PCA**	**PTRSA**
S. aureus	8.12±2.08	8.15±2.11	8.00±2.30
E. coli	8.12±1.70	8.08±2.48	8.04±2.20
Salmonella	8.12±2.36	8.04±1.78	8.08±2.48
P. aeruginosa	8.18±3.08	8.15±2.34	8.12±1.73
B. subtilis	8.14±2.45	8.17±3.15	8.02±2.40
Streptococcus	0.60±0.00	1.04±0.00	NG
C. albicans	8.08±2.49	8.08±2.91	8.15±2.84

NA= nutrient agar; **PCA=** plate count agar; **PTRSA=** *Pleurotus tuber regium* sclerotium agar; **NG=**no growth

Table.2 Mean counts Log_{10} $cfug^{-1}$ and microbial isolates from sclerotium of *Pleurotus tuber-regium*

Samples	Total aerobic plate count	Coliform count	Fungal count	Microbial Isolates
A	7.90±2.04	4.60±1.03	6.00±2.01	*Aspergillus spp; Enterobacter spp; Corynebacterium spp; Bacillus spp*
B	9.04±2.49	4.56±1.81	6.60±2.03	*P. earuginosa; Klebsiella spp; Bacillus cereus; Saccharomyces spp; Aspergillus spp*
C	10.48±2.57	7.36±1.95	6.00±1.85	*Aspergillus spp; Rhizopus spp; Staphylococcus aureus; Bacillus spp; Serratia marcescens; Penicellium spp; Escherichia coli*
D	8.42±2.04	3.58±1.63	3.00±1.45	*Aspergillus spp; Klebsiella spp; Pseudomonas spp; Bacillus spp; Staphylococcus spp*

Figure.1 18h growth culture of *E. coli* (A), *C. albican* (B), *B. cereus* (C), *S. aureus* (D), *P. aeruginosa* (F) and *Salmonella* (E) on *Pleurotus tuber regium* sclerotium agar (PTRSA)

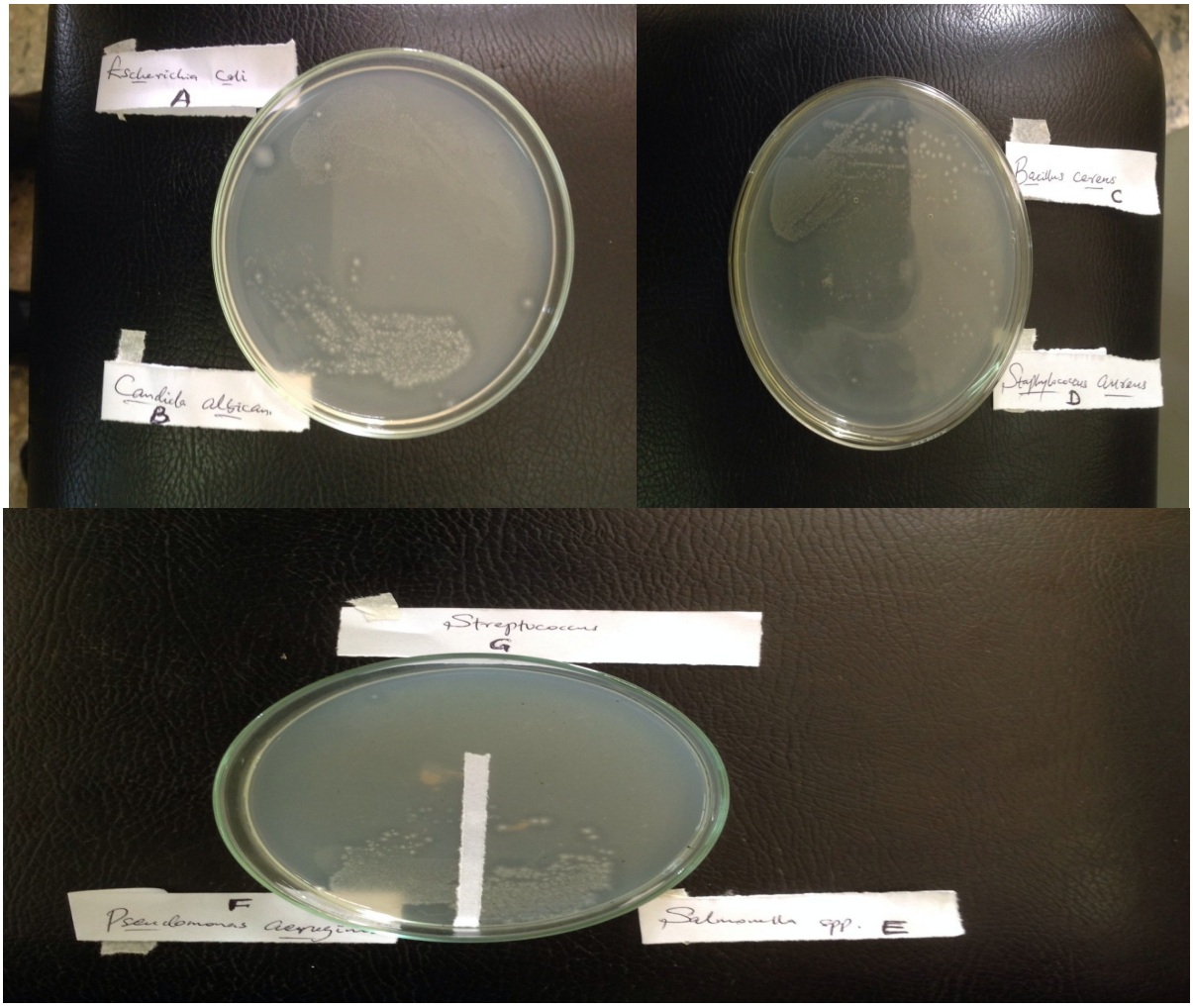

Table.3 Chemical composition of the sclerotium of *pleurotus tuber-regium*

Type of analysis	Components	Percentage composition
Proximate analysis	Ash	0.50
	Fat	3.23
	Fibre	0.25
	Protein	12.36
	Moisture	19.86
	Carbohydrate	63.81
Phytochemical analysis	Oxalate	4.25
	Tannin	0.41
	Saponin	Trace
	Flavonoid	0.70
	Alkaloid	2.20
	Hydrogen cyanide	1.13
Mineral analysis	Potassium	0.13
	Calcium	0.18
	Magnesium	0.03
	Sodium	0.22
	Phosphorous	0.05

The presence of coliforms in all the samples indicates contamination by faecal matter either of man or other animals. The poor handling of these products by food vendors may have led to coliform contaminations. Fungi and bacillus species are spore bearers, their presence could be explained by the hardy nature of microbial spore. *B. cereus*, some species of *Aspergillus* and *Penicillium* are known to produce deleterious toxins of public health importance (Sweeney and Dobson, 1998; Kabak *et al.,* 2006, Oranusi *et al*., 2013), toxins produced on raw materials may ultimately be carried over into the finished product and constitute health hazard to man and livestock.

Similarly species of *serratia*, *klebsiellae* and *staphylococcus* have been implicated in human infections and food related diseases (Mensah *et al.,* 1999; Oranusi *et al.,* 2007), their presence in the sclerotia, coupled with coliforms, calls for concern and for adequate measures to be taken in the processing of *Pleurotus tuber-regium* sclerotium for human consumption.

The chemical compositions of the sclerotium indicate rich moisture and nutritional constituents; it is therefore a good source of food for man. The moisture level and rich chemical compositions also dictates that associated bacteria and fungi will have favourable environment to proliferate, this could have contributed to the high level of associated microflora.

The moisture level and rich nutrient also signify that the sclerotium will easily develop fruit bodies if not dried or stored appropriately as a whole sclerotium. Similarly if blended it can easily grow moldy on storage. The sclerotium was also found to contain low fats (3.23%) which makes it a healthy diet for humans since excessive fats in diet can cause cardiovascular disorders such as atherosclerosis.

The phytochemicals detected in the sclerotium are known to have antioxidant activity and are mainly responsible for the medicinal properties of plants and plant products. However, above certain concentration, some phytochemicals can constitute ant-nutrient.

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