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SUBMERGED LIQUID FERMENTATION OF SOME TANZANIAN BASIDIOMYCETES FOR THE PRODUCTION OF MYCELIAL BIOMASS, EXOPOLYSACCHARIDES AND MYCELIUM PROTEIN USING WASTES PEELS MEDIA

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ABSTRACT

Mycelia from five species of higher fungi of genera Ganoderma, Pleurotus and Laetiporus were used in this study. These were cultured for the investigation of higher exopolysaccharides and mycelia biomass production. Mycelial growths of all species were measured on six different non-defined complex media prepared from waste peels and cattail rhizome; Yam dextrose agar (YADA), cassava dextrose agar (CADA), potato malt extract peptone dextrose agar (PMPDA), sweet potato dextrose agar (SPDA), plantain dextrose agar (PLDA) and cattail rhizome dextrose agar (CATDA). The average mycelial growth rate on the aforementioned solid media ranged between 10 ± 2.6 and 14 ±1.7 mm/day with the highest growth rate obtained from YADA followed by PMPDA while the lowest was recorded from PLDA. The best media based on mycelial growth were selected for further investigation. Therefore, the effect of YAD and PMPD complex media on the submerged mycelia growth and exopolysaccharides, mycelium protein production in five mushrooms was investigated in shake-flask culture at an ambient temperature 30^{o}C±2. The maximum mycelial growth (14g/l) and exopolysaccharides production (540mg/l) were achieved in YAD medium by Pleurotus spp and Pleurotus HK-37, respectively. The crude protein content of mushroom mycelium in YAD medium varied among the mushroom species. The Pleurotus spp mycelium contained 55% crude protein, which was the highest followed by 41% obtained from Pleurotus HK-37 mycelium. The genus Pleurotus amongst the other mushroom genera investigated, should be considered for reasonable production of mycelial biomass, exopolysaccharides and mycelium protein using YAD medium in submerged fermentation. In conclusion, the data obtained in this study provides useful information for further investigation of higher fungi in submerged culture using waste peels complex media.

Keywords: mushroom, mycelia, growth, biomass production, exopolysaccharides, submerged fermentation, waste peels.

INTRODUCTION

Mushrooms are currently of interest because they are rich source of various bioactive natural products (Pokhrel, C.P. and Ohga, S., 2007). They have long been used in folk medicines and health foods and have attracted a great deal of interest in many areas of foods and biopharmaceuticals, and are regarded as effective medicines used to treat various human diseases, such as hepatitis, gastric cancer chances of contamination etc (Kim, S.W., Hwang, H.J., Park, J.P., Cho, Y.J., Song, C.H. and Yun, J.W., 2002). Also mushrooms have become more and more important foodstuffs because of their possible preventive roles against human life-style-related diseases such as hyper-lipidemia and diabetes (Tang, Y-J., Zhu, L-W., Li, H-M. and Li, D-S., 2007). The number of mushroom species on earth is estimated at 140,000. However, about 14,000 named mushroom species are known. Additionally, less than 25 mushroom species are widely exploited as edible and medicinal mushrooms, even few have attained commercial status (Hawksworth, D.L., 2001). This is unfortunate, because mushrooms comprise a vast and yet largely untapped source of potential new pharmaceutical products. In particular, and most importantly for modern medicine, they represent a source of new polysaccharides with antitumor and immuno stimulating properties (Tang, Y-J., Zhu, L-W., Li, H-M. and Li, D-S., 2007). Fungal-delivered polysaccharides especially from edible and medicinal species belonging to basidiomycetes, possesses potential immuno-modulatory, antitumor, hypoglycemic, antibacterial, antiviral, antiparastic effects, they can be used as insecticidal and nematocidal agents (Xiao, J., Dai-xiong, C., Wei-hong, W., Xi-jie, H., Ying, Q. and Liang, Z., 2006).

Basidiomycetes is a class of mushrooms that have macroscopic fruit bodies, also known as basidioma or basidiocarp. These fruit bodies are large enough to be seen with the naked eye, and can be picked by hand. They produce potential useful natural products and novel bioactive compounds. The basidiomycota comprise about 30,000 species, which is 37% of the described species of fungi (Fadenza, M.L., Seviour, R., MCNeil, B. and Harvey, L.M., 2008). Previously medicinal mushroom were either gathered from the wild or cultivated on solid media such as wood rather than submerged culture. Many investigators have tried to cultivate mushrooms on solid artificial media for fruit body formation in order to obtain polysaccharides (Bae, J.T., Sinha, J., Park, J.P., Song, C.H. and Yun, J.W., 2000). However, this method does not guarantee a standardized product, since composition of bioactive substances varies from batch to batch. Furthermore, the cultivation of mushrooms for fruiting bodies production is a long-term process needing from one to several months for the first fruiting bodies to appear,

depending on species and substrate (Pfefferle, C., Theobald, U., Gürtler, H., Fiedler, H.P., 2000). Submerged culture offers potential advantages of faster production for both mycelial biomass and exopolysaccharides in a shorter period of time within a reduced space and lesser chances of contamination (Kim, S.W., Hwang, H.J., Park, J.P., Cho, Y.J., Song, C.H. and Yun, J.W., 2002; Tang, Y-J., Zhu, L-W., Li, H-M. and Li, D-S., 2007). High stability and standardization of mycelium grown in submerged cultures is important not only for producing desired product, but also might be beneficial for producing mushroom-based medicines and nutraceutical that can achieve higher quality standards and safety (Wasser, S.P. and Weis, A.L., 1999). The additional advantage of submerged mycelia culturing are provision of scalable production method and increased yields of biologically active compounds such as protein rich in the essential amino acids and vitamins serving as functional foods compared with the yield of these components in carpophores of the standard fruiting basiodiomycetes (Friel, M.T. and McLoughlin, A.J., 2000). The medicinal properties are due to various cellular components and metabolities of basidiomycetes (Lorenzen, K. and Anke, T., 1998). The majority of active substances are polysaccharides and polysaccharide complexes, active hexose correlated compounds (AHCC), polysaccharide-peptides, nucleosides, triterpenoids, complex starches, and other metabolites (Mizumo, T., 1999; Tang, Y-J., Zhu, L-W., Li, H-M. and Li, D-S., 2007). Several different polysaccharides have been isolated from the fruiting bodies, mycelia, and culture media of various medicinal mushrooms, such as shiitake (*Lentinus edodes* (Berk.) Sing., Tricholomataceae), reishi (*Ganoderma lucidum* (Curt.:Fr.) P. Karst., Ganodermataceae), turkey tail (*Trametes versicolor* (L.:Fr.) Lloyd, Polyporaceae), split gill (*Schizophyllum commune* Fr.:Fr., Schizophyllaceae), mulberry yellow polypore (*Phellinus linteus* (Berk. et Curt.) Teng., Hymenochaetaceae), and chaga or cinder conk (*Inonotus obliquus* (Pers.:Fr.) Pilat, Hymenochaetaceae) *Polyporus tricholoma,* (Mizumo, T., 1999; Wasser, S.P. and Weis, A.L., 1999; Vieira, G.R.T. Liebl, M., Benathar,L., Tavares, B., Paulert, R and Júnior, A.S., 2008). The *Agaricus blazei* Murill is one kind of rare, valuable and edible mushroom, which the original place of production is in south mountain region of Brazil. It is a mushroom which, also contain the highest level of polysaccharide than all the mushrooms so far tested for this important polysaccharide (Masashi, M., Ken-ichiro, M., Hitoshi, I., Mitsuo, K., Hirofumi, T. and Hironobu, T., 1999).

Basidiomycetous fungi mycelial extract usefulness as food and medicine has contributed significantly for human nutrition and health welfare for many centuries world wide (Wasser, S.P. and Weis, A.L., 1999). Numerous higher basidiomycetes are an important source of medicinal substances, applied in the treatment of a wide range of ailments. Ganoderma P. Karst. (Ganodermatales, Basidiomycetes) is a cosmopolitan genus polypore mushrooms found throughout the world on all types of wood, gymnosperms, hard, softwood dicots and palms. The pharmacological effects of the Ganoderma species are attributable mainly to polysaccharides and triterpenoids (Wagner, R., Mitchell,D.A., Sassaki, G.L., Lopez del Almeida Amazonas M.A. and Berovi, M., 2003). The genus Pleurotus (Jacq.:Fr.) P.Kumm. (Pleurotaceae, higher Basidiomycetes) covers a group of ligninotrophic mushrooms that are edible and medicinal (Lewinsohn, D., Wasser, S.P., Reshetnikov, S.V., Hadar,Y., Beharav, A. and Nevo, E., 2005). Various Pleurotus species have been shown to possess medicinal properties exhibited by extracts or isolated compounds from fermentation broth, mycelia and fruiting bodies. In particular, polysaccharides appear to be potent antitumour and immuno-enhancing substances, besides possessing other beneficial activities (Wasser, S.P., 2002; Gregori, A., Vagel, M., and Pohleven, J., 2007). *Laetiporus sulphureus* the common sulphur polypore is a macrofungi basidiomycetes edible and medicinal mushroom, belongs to the Aphyllophorales, Polyporaceae, (Imazeki, R. and Hongo, T., 1998). It is widespread and grows in large clusters on live and dead trunks of deciduous forest, mangrove or coniferous tree or hard wood species and its distribution is of the pan-world distribution grows. It is the most readily recognized among macrofungi due to its striking yellow or orange color (Imazeki and Hongo 1998; Mtui, G. and Masalu, R., 2008; Dzygun L.P., 2008). Many polysaccharides and polysaccahrides-peptide complexes with antitumor, immuno-modulatory, antimicrobial activities and stimulatory effects on insulinoma has been reported recently from *Laetiporus sulphureus* (Ershova, EIu., Tikhonova, O.V., Lur'e, L.M., Efremenkova, O.V., Kamzolkina, O.V., Dudnik, IuV., 2003; Turkoglu, A., Duru, M. E., Mercan, N., Kivrak, I., Gezer, K., 2007; Aziz, T., Mehmet, E.D., Nazime, M., Ibrahim, K. and Kudret, G., 2007; Hwang, H.S., Lee, S.H., Baek,Y.M., Kim, S.W., Jeong, Y.K. and Yun, J.W., 2008). However, critical evaluation of recent advances in submerged liquid cultivation of vegetative or mycelial of those members of higher fungi that produce macroscopic spore bearing structures namely the macrofungi reavealed that this group of organisms has been rather neglected from biotechnological view point, when compared to more widely exploited and better understood microfungi. Also among the sources of bioactive metabolites, macrofungi fungi seem less intensively investigated organisms even though seem to hold an excellent promise for new structures with interesting biological activities for the benefit of human kind (Fadenza, M.L., Seviour, R., MCNeil, B. and Harvey, L.M., 2008).

Basidiomycetes not only can convert the huge lignocellulosic biomass waste into human food, but most remarkably can produce notable mycopharmaceuticals, myconutriceuticals and mycocosmeceuticals. This makes basidiomycetes fungal biotechnology clearly relevant to the requirements of both the developing and developed countries. However, irrespective of such potential the future role of mushrooms will be governed by the economy of production. Medium composition is key to successful growing macrofungi mycelium. Synthetic chemically defined media, complex media and waste

substrates all have been used. It is important to bear in mind that each component of the culture system media has a multiplicity of interconnected effects and so there can be no generalized ideal medium (Fadenza, M.L., Seviour, R., MCNeil, B. and Harvey, L.M., 2008).

Many macrofungi particularly white rot fungi are efficient ligninolytic because they synthesize non specific extracellular enzymes which degrade complex substrates such as lignocellulose into fermentable substrates such as sugars that can be used as starting compounds in the biosynthesis of many bioproducts (Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L. and Howard, S., 2003). Complex sources of nutrients (preferably waste materials) are most often used in large scale cultivation of fungi, since they are cheaper than synthetic media or have to be disposed. In addition, growth of many strains is more abundant in complex media, either because unknown growth substances are present, or because high molecular carbon or nitrogen sources are broken down gradually, so that the concentration of their metabolizable products always remains low sugars and amino acids does not become excessive (Crognale, S., Federici, F., Petruccioli M., 2003). Biomass concetrations are generally lower with synthetic defined media than with complex media (Xiao, J., Dai-xiong, C., Wei-hong, W., Xi-jie, H., Ying, Q. and Liang, Z., 2006; Fan, L., Soccol, A.T., Pandey, A. and Soccol, C.S., 2007). Therefore, various agricultural by products have been used as inexpensive growth substrates for economical production of fungi mycelial species and their metabolites in fermentation by micro and macro fungi (Albuquerque ,M., Streit, F., Esposito, E. and Ninow, J.L., 2008; Okonko,I.O., Adeola, O.T., Adeola, O.T., Aloysius, F.E. and Oluseyi, A., 2009). However, the literature on the utilization of wastes peels and cattail rhizome weed as carbon sources for the production of exopolysaccahrides, mycelial biomass and protein by macrofungi basadiomycetes in submerged fermentation is very meager indeed.

Enormous quantities wastes peels residues from the processing of banana, cassava, potatoes, sweet potatoes, yams are generated throughout the world from processing agricultural materials for foods. For example Tanzania has a potential to generate 700,000 tons of cassava peels per annum estimated as 10% of the wet weight of the root (Obadina, A.O., Oyewole, O.B., Sanni, L.O. and Abiola, S.S., 2006). About 54,000 tons of patato rejects can be generated annually in Tanzania (Guenthner, J.F., 2003). On the other hand, rhizomes (underground stems) of cattail weeds (*Typha* spp) about 70 tons can be estimated per hectare (Mshandete, A.M., 2009). The cattail rhizomes are fairly high in starch content usually listed at about 36 to 46% and contain around 6% to 8% protein (http://Plant-Materials.nrcs.usda.gov). These peels wastes and rhizomes if properly exploited and processed, could save as biobased feedstocks for bioconversion into valuable bioproducts through fermentation processes (Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L. and Howard, S., 2003; Thomsen, M.H., 2005). However, currently they are underutilized and disposed of in the environment leading to pollution problems. Since the cost of carbon substrates for production of bioactive metabolites from macrofungi basidomycetes is central to the success and competitiveness of sustainable production of bioactive production (Okonko, I.O., Adeola, O.T., Adeola, O.T., Aloysius, F.E. and Oluseyi, A., 2009). This study explored the potential application of peels extract (liquid extracted from waste peels of cassava, yams, potato, sweet potato and that obtained from cattail rhizome as a potential fermentation feedstock for production of myceial biomass, exopolysaccaharides (EPS) and mycelial protein by some Tanzanian basidiomycetes of genera Ganoderma, Pleurotus and Laetiporus in submerged fermentation conditions.

MATERIALS AND METHODS

Fungal materials source and culture maintenance

(a) Ganoderma

The fungi were collected at Mlimani campus, University of Dar es Salaam, Tanzania from a dead stump of *Laucaena grauca*. The specimens were identified to be *Ganoderma lucidum* (Curt.:Fr.) P. Karst with the help of macroscopic and microscopic features according to the related literature (Ainsworth, G.C., 1973; Buczacki, S., 1992). The fruiting bodies were brought to the laboratory for tissue culturing as reported by Mshandete, A.M. and Cuff, J., (2008).

(b) *Laetiporus sulphureus*

The mushroom has been collected at Oyster Bay and Mtoni mangrove forests along the Indian Ocean coast in Dar es Salaam, Tanzania. It was identified to be *Laetiporus sulphureus* (Fr.) Murr (Mtui, G. and Masalu, R., 2008). The fungal culture of *L. sulphureus* stored at 4°C in our laboratory at the Department of Molecular Biology and Biotechnology (DMBB), College of Natural and Applied Sciences (CoNAS), University of Dar es Salaam (UDSM), Tanzania was used in this study after subculturing.

(c) Oyster mushrooms, pleurotus species

(i). *Pleurotus flabellatus* (Berk and Br.) Sacc is an indigenous oyster mushroom, which has been found growing on the bark of a dead *Ficus benjamina* tree at the University of Dar es Salaam, Mlimani main campus (Dar es Salaam, Tanzania) (Mshandete, A.M. and Cuff, J., 2008). The preserved isolate of *P. flabellatus* at the DMBB, CoNAS, UDSM was subcultured as already described by Stamets, P., (2000) and was used in the present investigations.

(ii). Pleurotus HK-37

The oyster mushroom Pleurotus HK-37 strain originated from South Africa is among the oyster mushrooms grown in Tanzania (Kivaisi, A.K., 2007). The fruiting bodies of these mushrooms were obtained from the first author's mushroom farm located at Changanyikeni, a village close to the UDSM. These

fruiting bodies were tissue cultured as previously described by Dhouib, A., Hamza, M., Zouari, H., Mechichi, T., H'midi., R., Labat, M., Martínez, M.J., Sayadi, S., (2005).

(iii). Pleurotus species

This oyster mushroom strain was obtained from culture collection of DMBB, CoNAS, UDSM, Tanzania. The strain was cultivated at UDSM to produce the fruiting bodies, which were used for tissue culturing. However, the original source of this strain of oyster mushroom is not known.

All stock fungi cultures were maintained on Oxoid potato dextrose agar (PDA) (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England) in slant and plate by periodical subculturing every one-month. Slants and plates were incubated at ambient temperature, which ranged from 25-30 ^{o}C for 5-7 days and then preserved at 4 ^{o}C in a refrigerator (West Point, WRK 143 G–Tropical, West Point International, Korea).

Sources of food materials and cattail rhizomes used in medium preparation

(a) Agricultural food materials

i. Plantain Green banana (*Musa paradisiaca*, Linn.) for cooking "cooking plantain" (Ndizi Mshale) (Synonym *Pisane lilin*)
ii. Cassava (*Manihot esculenta* Crantz) sweet variety
iii. Potatoes (*Solanum tuberosum* (L) red-skinned variety with white flesh
iv. Sweet potatoes, *Ipomea batatas* (L) white tuber flesh variety
v. Yams (*Dioscorea* spp) predominantly white tuber flesh color mixed with purple patches

These fresh food materials, each were procured from Kariakoo market in Dar es Salaam, Tanzania. Kariakoo market is one of the largest markets in Tanzania and is well known for stocking various fresh agricultural produce. It should be noted that it is difficult to peel potatoes, sweet potatoes, yams so that the skin only is removed without the cortical layer, so the peels for the three aforementioned vegetables refers both the skin and part cortical layer.

(b) Cattails (Typha spp) weeds rhizomes

Cattail rhizomes were obtained from Jangwani wetland in Dar es Salaam City, Tanzania. They were cleaned by water to remove debris, sand and mud. Afterwards the skin of the cattail rhizome was peeled out.

Estimation of peels wastes

Quantification and characterization of green banana, cassava, potatoes, sweet potatoes and yams peels wastes was necessary to make approximations of how much biomass could be available for bioconversion into high value added products such as exopolysaccharides, mycelial biomass and biomass mycelial protein. The peels and rhizome were washed with water to remove sand and debris. Using three replicates of 1kg each of plantain, cassava, potatoes, sweet potatoes and yams. These items were hand peeled using a sharp knife and peels obtained were weighed. The weight of the peels obtained was computed against the original fresh weight of each item to ascertain the percentage of the peels, which can be generated.

Medium extraction from peels and cattail rhizome

The five types of peels and the cattail rhizome were grated using a laboratory blender (Snijders Scientific, Tilburg, Holland, Waring blender, Torrington, CT, USA) into small pieces. About 200g peels each of green banana, cassava, potatoes, sweet potatoes, yam peels and 200g of cattail rhizome were carefully weighed using an Adventurer TM balance (Ohaus Corp. Pine Brook, NJ, USA) out into 800 ml of distilled water. Then the slurry of each item was boiled at 100 ^{o}C in a pan using a Table hot plate (E.G.O. (Elektro-Gerätebau GmbH), Germany) for 20 minutes. The slurry was left to cool to room temperature and was strained through two layers of cheese cloth to extract the liquid. The solid was sun dried for five days and weighed while the filtrate (extract/liquid) was used for medium preparation. The composition of the six medium used in solid media experimented in this study were as follows:

i. Yam dextrose agar (YADA)
Yam peel extract = 200 g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml
ii. Cassava dextrose agar (CADA)
Cassava peel extract = 200 g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml
iii. Potato malt peptone dextrose agar (PMPDA)
Potato peel extract = 200 g
Malt extract =10g
Peptone =1g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml
iv. Sweet potato dextrose agar (SPDA)
Sweet potatoes peel extract = 200 g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml
v. Cattail rhizome dextrose agar (CATDA)
Cattail rhizome peel extract = 200 g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml
vi. Plantain dextrose agar (PLDA)
Plantain peel extract = 200 g
Dextrose = 20g
Agar powder = 20 g
Distilled water = 1000 ml

The chemicals used in solid medium preparation were of analytical grade: Dextrose was procured from (Lamers and Indemans, s-Hertogenbosch, The Netherlands); malt extract was obtained from (Pronadisa, Laboratorios Conda, S.A., Madrid, Spain); peptone and agar were procured from (Fisher Scientific UK Ltd, Bishop Meadow Road, UK). The medium i-vi prepared above, were sterilized in an autoclave (Koninklijke AD Linden JR.BN-Zwijinderect, Holland) at 121 ^{o}C and for 20 minutes. To avoid solidification after sterilization the media was maintained at temperature of 50 ^{o}C in water bath (GLF Gesellschaft für Labortechnik mbH, Burgwedel, Germany). It was then poured in cooled petri dishes presterilized in oven (GallenKamp Sanyo OMT oven, UK) at 180 ^{o}C for three hours under aseptic conditions in a laminar flow cabinet (Envair C-Flow, Envair Ltd, York Avenue, Haslingden, UK). Fifteen ml of the medium poured per plate of 90-mm diameter and allowed to solidify to produce solid used for mycelial growth determination.

Cultivation of mycelia on solid media agar

The inoculum of five mushroom species investigated was grown on PDA for 7 days at 30 ^{o}C. Six complex (undefined) medium i.e. YADA, CADA, PMPDA, SPDA, CATDA and PLDA were evaluated to determine their effect on mycelial linear growth (in mm) for three days. Also in selection of two media which could be used for subsequent experiment in liquid submerged fermentation of the five macrofungi mushroom mycelia investigated. Cultivation on solid media was carried out at an ambient temperature around 30 ^{o}C in Petri dishes (90-mm diameter). The plate dishes (three replicates for each mushroom species) were inoculated with mycelial plugs of 9-mm diameter cut from actively growing mycelia using a sterile cork borer and used as inocula. The plates were sealed with laboratory film (Parafilm "M" Pechiney Plastic packing, Chicago, IL, USA), to prevent drying out and the plates were incubated in darkness. Because the colonies grow in a circular fashion (radial extension), the data were collected daily by measuring the diameter of colony in millimeters as it grew on the Petri dish. The radial extension rates estimated can be assumed equivalent to the growth rate of the mycelia. Thus the linear mycelial growth was followed by measuring radial extension of the mycelium measured, as described by Weitz, J.H., Ballard, A.L., Campbell, C.D. and Killham, K., (2001) with a calliper gauge along two diameters at right angles to one another. The mean mycelial growth diameter was then calculated from the three replicates of each mushroom species investigated. All growth measurements were made at the same time (between 9.00 am and 12.00 Noon) and the colony characteristics were observed daily. Background readings for the five mushroom species were obtained using uninoculated (control) agar plates.

Submerged macrofungi mycelia fermentation

Mycelia biomass and exopolysaccharides production was carried out using two media namely; YADA and PMPDA which gave better mycelia growth than the other four media investigated during cultivation of mycelia on solid media. However, during liquid fermentation agar was not added hence the two media were referred to as Yam Dextrose (YAD) and Potato Malt Peptone Dextrose (PMPD). Fifty ml of each media were transferred in batch bioreactors made up of 250 ml Erlenmeyer flasks narrow neck flange-edged, graduated (Boeco, Germany), plugged with cotton wool and then covered with aluminium foil. The culture media was sterilized by autoclaving at 121 ^{o}C and 1atm for 20 minutes. The two sterilized culture medium YAD and PMPD were cooled to room temperature. The sterile media were inoculated aseptically in a laminar flow cabinet, with seven days old pure culture of the five mushroom species, grown on YADA and PMPDA solid media. Ten 10 plugs of 9-mm diameter of actively growing mycelia of each mushroom were cut using a sterile cork borer were transferred aseptically into batch fermentation bioreactors. In this experiment three replicates batch fermentation bioreactors were used for each five mushroom strain per media used hence a total of 30 bioreactors. In each set of medium there were three batch bioreactors without mycelial inoculum (with medium only) as control. The experiment was conducted at 30 ^{o}C in the aforementioned batch bioreactors by shaking at 100 rpm using a laboratory orbital shaker (Edmund, Bühler, 7400 Tübingen, West Germany). After 15 days of submerged fermentation of the mycelia of the five mushroom species, the EPS, mycelial biomass and mycelium protein production were evaluated using techniques of product recovery and analysis (Wagner, R., Mitchell, D.A., Sassaki, G.L., Lopez Del Almeida Amazonas, M.A. and Berovi, M., 2003).

Analytical methods

Characterization of the raw materials for total solids (TS), moisture content, volatile solids (VS), ash content were done according to standard methods American Public Health Association, (1998). The organic carbon was determined by the rapid dichromate oxidation method previously described by Nelson, D.W. and Sommer, L.E., (1996). All samples were determined in triplicate. Cultured broths were collected from culture shake flask after 15 days submerged fermentation of the five mushroom mycelium, using YAD and PMPD extract medium. The broths were analyzed for EPS, mycelial biomass and mycelial protein. The mycelial biomass was recovered by filtration under suction (Handy Aspirator, Model WP-25, Yamato Scientific Co. Ltd., Tokyo, Japan). To quantify the mycelial biomass the samples were filtered through a preweighed 0.45 µm Whatman®cellulose Nitrate membrane filters (Whatman, GmbH, Dassel, Germany). The biomass was dried until constant weight at 40 ^{o}C (Kim, S.W., Hwang, H.J., Park, J.P., Cho, Y.J., Song, C.H. and Yun, J.W., 2002). Temperature below 50^{o}C was used to avoid the effect of drying temperature of burning the biomass or loss of volatile cell components other than water (Wagner, R., Mitchell, D.A., Sassaki, G.L., Lopez del Almeida Amazonas, M.A. and Berovi, M., 2003). The culture filtrate after membrane filtration was

mixed with 4 times the volume of absolute ethanol 96% (BDH laboratory supplies, Poole, England), stirred vigorously by vortexer (Janke and Kunkel GmbH & Co. KG-IKA-WERK, Staufen, Germany) and kept overnight at 4°C. The precipitated EPS was centrifuged at 10,000 *g* for 20 min using a laboratory centrifuge (Universal 30 RF; Hettich Zentrifugen, Tuttlingen, Germany) and discarding the supernatant (Bae, J.T., Sinha, J., Park, J.P., Song, C.H. and Yun, J.W., 2000). The precipitate of EPS was collected on preweighed membrane filter (0.45 µm) pore size and dried at 80 °C to constant weight and the weight of the polymers was estimated (Nour El-Dein, M.M., El-Fallal, A.A., El-Shahat, A.T. and Hereher, F.E., 2004). In addition, the dried mycelia biomass of each isolates were analysed for their total nitrogen contents by using indophenol-blue method according to Allen, S.E., (1989) using NH_4^+-N as standard. The UV absorbance of the solution was measured using spectrophotometer (Thermo Spectronic Helios β, England) at 660 nm. Protein content was calculated by multiplying the total nitrogen content by the universal factor of 6.25. The organic matter content of the peels and cattail rhizomes was calculated as differences in weight between dry weight (80 °C until a constant weight) and ash weight (550 °C for 4 h) according to Lyimo T.J. Pol, A. and Op den Camp, H.J.M., (2002).

Statistical analysis

All experiments were carried out at least in triplicates to ensure reproducibility and all data were expressed as mean ± S.D. The data for mycelial growth, EPS, mycelial biomass and mycelial protein were subjected to analyses of variance (one-way ANOVA) at the 5% level (significant different at $p<0.05$) using the Statistical Package for Social Sciences (SPSS) Program 15.0. Version (SPSS, 2006).

RESULTS AND DISCUSSIONS

Quantification and composition of peels

Cost of substrates has always been an important factor in production cost during bioconversion processes of organic materials waste into value added products. Thus exploitation of organic biomass such peels wastes as substrates during submerged fermentation culture can be a viable and feasible cost-effective option (Adeniran, A. H. and Abiose, S. H., 2009). Efficient and effective utilization of biological resources is the backbone of the present 21st bioresource based economy "bioeconomy". To this effect bioconversion of organic biomass waste into high value added products necessitates approximation of the bioresource that could be available. This could also guide proper waste management and utilization. The estimation of the relative quantity of peels investigated, that can be generated per year in Tanzania is presented in Table-1. Nevertheless, it should be pointed out that the quantity of peels estimated based; on the food commodity variety investigated, on manual peeling using a knife and on three replicates each of 1kg per food commodity employed. Therefore, it can be stated that the estimates were fair representative of the quantity of peels, which can be generated from the food items investigated. Quantitatively over 1,900,000 tons of peels of the five food commodities can be generated annually. The lowest and highest quantities were obtained from yam and cassava, respectively. This was expected due to differences quantities produced per year. Twenty one percent peels of the total weight of the cassava tuber obtained in this study was within the range of 20-35% peels, which have been reported for hand peeled cassava tubers (Ekundayo, J.A., 1980; Cuzin, N., Farinet, J.L., Segretain C. and Labat, M., 1992). The ratio of cassava peel to pulp of 3.7 obtained in this study compare very favorably with the ratio of 4 reported by Cuzin, N., Farinet, J.L., Segretain, C., and Labat, M. (1992). Our results of 40% plantain peels of the total weight of the plantain fruit were in agreement with the observation of Emaga, T.H., Ronkart, S.N., Robert, C., Wathelet, B. and Paquot, M., (2008). Furthermore, the ratio of plantain fruit pulp to peel fresh weights of 1.5 obtained in present study was within the range of 1.18-2.28 reported previously for the peel of plantain and cooking banana fruits (Burdon J. N., Moore K. G., and Wainwright, H., 1993).

Table-1. Relative quantity of peels waste used for media extraction (Mean values ± SD, n = 3).

Fresh food item	Peels g/1kg fresh weight	% fresh peels waste of the original food item	Pulp : Peel ratio (fresh weight)	Estimated fresh food commodity production tons/year in Tanzania	Source	Estimated fresh peels generation tons/year	Contribution (%) to the total peels waste generated/year
Yam	246 ± 2.25	25	3	10,000	FAO, 1995	2,500	0.13
Cassava	213 ± 13	21	3.7	7,000,000	FAO, 2005	1,470,000	75.68
Sweet potatoes	171 ± 2	17	4.8	970,000	FAO, 2005	164,900	8.49
Potatoes	253 ± 15	25	2.9	260,000	FAO, 2005	65,000	3.34
Plantain	400 ± 9	40	1.5	600,000	FAO, 2005	240,000	12.36
Total				**8,840,000**		**1,942,400**	**100**

Results in (Table-2) show that about 258,000 tons of sun dried solid residues can be obtained after medium extraction, which represent about 13% of the total peels generated annually by the five food commodities (Table-1). Such solid residues through bioprocesses can also provide raw materials for a diversity of other value added products such as enzymes, single cell protein, feeds, worms, biofertilizer, mushrooms etc potentials yet fully to be investigated and exploited in Tanzania (Mtui, G.Y., 2007). If such residues or neglected resources could be transferred into biobased products such animal protein in the form of eggs and meat, protein rich mushrooms, a great problem would be solved helping the people of the developing countries like Tanzania to avoid hunger and alleviate protein deficiency (Vendruscolo, F., Albuquerque, P.M., Streit, F., Esposito, E. and Ninow, J.L., 2008).

Table-2. Proximate quantity of sun dried solid residues after media extraction (Mean ± SD, n = 3).

Peels	**Solid residue after extraction g dry weight/200g fresh weight peels**	**% dry peels solid residues**	**Estimated fresh peels tons/year in Tanzania**	**Estimated dry weight of solid peels residues tons/year**
Yam	28 ± 2	14	2,500	350
Cassava	21 ± 2	11	1,470,000	161,700
Sweet potatoes	30 ± 1	15	164,900	24,735
Potatoes	11 ± 1	6	65,000	3,900
Plantain	56 ± 2	28	240,000	67,200
Cattail rhizome	18 ± 1	9	70* tons/hectare	6.3
Total			**1,942,470**	**257,891**

*Source Mshandete, A.M., (2009).

To date emphasis is on bioconversion of plant wastes, especially agricultural and food wastes into value added products. Fungi are known organic waste decomposers in nature and are generally capable of hydrolyzing complex organic compounds as major source of energy and essential nutrients (Essien, J.P., Akpan, E.J. and Essien, E.P., 2005). The intimate understanding of the composition of the raw material whether it is whole plant or botanical fractions, is essential so that the desired functional elements can be obtained for bioproduct production (Howard, R.L., Abotsi, E., Jansen van Rensburg E.L., Howard, S., 2003). Table-3 shows the proximate composition (% TS) of peels and cattail rhizomes. Significance tests at the 5% level ($P<0.05$) showed that there was a statistically significant difference in the values recorded for chemical constituents. This was not unexpected since the distribution of nutrients resources in plants is allocated differently in plant botanical fractions of various species, even of the same species. Additionally, soils and environmental factors also contribute to the differences in waste peels and cattail rhizomes proximate chemical compositions. The content of TS, moisture, VS, organic carbon, ash and protein of yam, potatoes, sweet potatoes, cassava plantain waste peels and that of cattail rhizomes were similar to that reported in the literature by various investigators on bioconversion of wastes peels and cattail rhizomes into value added products (Cuzin N., Farinet J.L. and Segretain C. and Labat M., 1992; Parawira W., Murto M., Zvauya R. and Mattiasson B., 2004; Essien J.P., Akpan E.J. and Essien E.P., 2005; Mshandete A.M., 2009; Anhwange B.A., Ugye. T.J. and Nyiaatagher T.D., 2009). The high protein content, organic carbon material content (fraction) measured as VS, organic carbon and total organic matter of waste peels and cattail rhizomes is an indication that these bioresources could serve as a possible alternative substrate for microbial growth. Also could support large scale cultivation of fungi for the production of valuable fungal products (Essien J.P., Akpan E.J. and Essien E.P., 2005).

Table-3. Proximate chemical composition of waste peels and cattail rhizomes (Mean ± SD, n = 3).

Peels	Yam	Cassava	Sweet potatoes	Potatoes	Plantain	Cattail rhizome
Parameters						
Total solids (% fresh)	25 ± 2	29 ± 1	32 ± 0.5	19 ± 2	15 ± 1	24±2
Moisture content (%)	75 ± 1	71 ± 0.6	68 ± 0.9	81 ± 1	± 0.8	76 ± 1
Volatile solids (% TS)	94 ± 1	94 ± 0.9	90 ± 1	97 ± 0.8	90 ± 2	82 ± 3
VS/TS ratio	3.76	3.24	2.81	5.1	6	3.4
Total ash (% TS)	8 ± 2	9.6 ± 0.5	8 ± 2	16 ± 0.6	26 ± 1	17 ± 0.9
Organic carbon (% TS)	51 ± 1	50 ± 0.3	51 ± 1	51 ± 0.4	41 ± 0.7	40 ± 2
Total organic matter (% TS)	94 ± 0.2	83 ± 0.9	94 ± 2.5	95 ± 0.6	95 ± 0.5	59± 0.9
Total nitrogen (% TS)	1.4 ± 0.4	0.9 ± 0.2	1.1 ± 0.3	1.3 ± 0.5	1.2 ± 0.5	1.7 ± 1
Crude protein (% TS)	8.8	5.6	6.9	8	7.8	11
Carbon: Nitrogen ratio	36	55	46	39	34	23

Mycelia growth rate on waste peels and cattail rhizome extracts solid medium agar

Variation in colony diameter of different five mushrooms species was presented in Figure-1. The observed mycelial growth rate of *G. lucidum*, *L. sulphureus*, *P. flabellatus*, Pleurotus HK-37 and Pleurotus spp on six different solid medium extracts from waste peels and cattail rhizomes differed significantly at ($p< 0.05$). The average mycelial growth rate on media used in this study ranged between 10±2.6 and 14±1.7 mm/day with the highest and lowest obtained from YADA and PLDA, respectively. The mycelial growth rate were order of YDA > PMPDA > CADA > SPDA > CATDA> PLDA. Comparatively, among the media used in this study, it was indicated that YDA and PMPDA were relatively more suitable media for mycelial growth of the five mushroom species than others. Although PMPDA medium was contained more nitrogen source as peptone and carbon source as malt extract, relatively the best mycelial growth rate was not determined in this medium. On the other hand, with exception of Pleurotus spp, the other four mushrooms grown on PLDA medium showed slowest mycelial growth rate. This could be that plantain peels contained in the medium is known to contain polyphenoloxidase, which leads to the darkening of the medium and is thought to be inhibitory to the mycelial growth. It was interesting to note that *L. sulphureus* isolate showed relatively good mycelial growth in all media. This demonstrated that Laetiporus spp could survive in different (undefined) complex media based on vegetable peels and cattail rhizome. Nevertheless, to the best of our knowledge mycelial growth of the five mushrooms on CATDA is being reported here for the first time. Thus utilization of cattail rhizome in fungal biotechnology needs further research since it's a potential renewable carbon source. A review of the available literature on the information on the use of waste peels and cattail rhizomes extracts used in this study as constituents of solid medium for the Ganoderma, Laetiporus and Pleurotus mushroom mycelial growth is lacking. Therefore, rigorous comparison of the mycelial growth rates of the five mushrooms grown on the six media employed in this study could be only indicative of the trend, which can be obtained. Nevertheless, recently a similar observation has been noted on mycelial growth rate variations of six wild Morchella spp mushrooms grown on four different defined (synthetic) media namely; minimal medium (MO), Hagem media (HM), Potato dextrose agar (PDA), malt extract agar (MEA). PDA and MEA were found to be best media for mycelial growth of Morchella spp, which ranged from 10-18 mm/day (Kalm, E. and Fatih Kalyoncu, F., 2008). Moreover, mycelia of *Pleurotus ostreatus* var *sajor caju*, *Pleurotus ostreatus* var *citydeosus* and *Volvariella volvaceae* mushrooms grown on MEA, PDA and Murashige and Skoog's (MS) culture defined (synthetic) medium has also been reported to exhibit variations in their mycelial growth rates. MEA gave the highest mycelial growth for the three mushrooms studied, which ranged from 7.5-9.2 mm/day (Nasim G., Malik S.H., Bajwa R., Afzal M. and Miam S.W., 2001).

The colony characteristics of all five mushrooms species grown on the six media namely; YADA, PMPDA, CADA, SPDA, CATDA and PLDA differed grossly or slightly among species (data not shown). The mycelium, which, grew on PMPDA, was slightly thinner than that grew on YADA medium. For instance mycelia growth of *L. sulphureus,* Pleurotus spp and *P. flabellatus* on PMPDA were thinner than in YADA. In contrast, mycelia growth of *G. lucidum* and Pleurotus spp and Pleurotus HK-37 were both thicker in PMPDA and YADA media. Some fungal isolates growth was significant thinner on SPDA and CATDA media. It seems that the colony characteristics of mycelium depend on the medium composition, efficacy of biocompounds and type of species (Kim S.W., Hwang H.J., Park J.P., Cho Y.J., Song C.H. and Yun J.W., 2002; Lee S., Bae H., Kim N. and

Hwang S., 2008). From the results, it is evident that some medium support the growth of some strains better than others do, which means that each needs to be studied individually. The medium chosen must promote both mycelial growth and product formation (Papagianni M., 2004). To this effect, in study YAD and PMPD media were used for further investigation of mycelia biomass, exo-biopolymers and protein production in submerged mycelia culture fermentation.

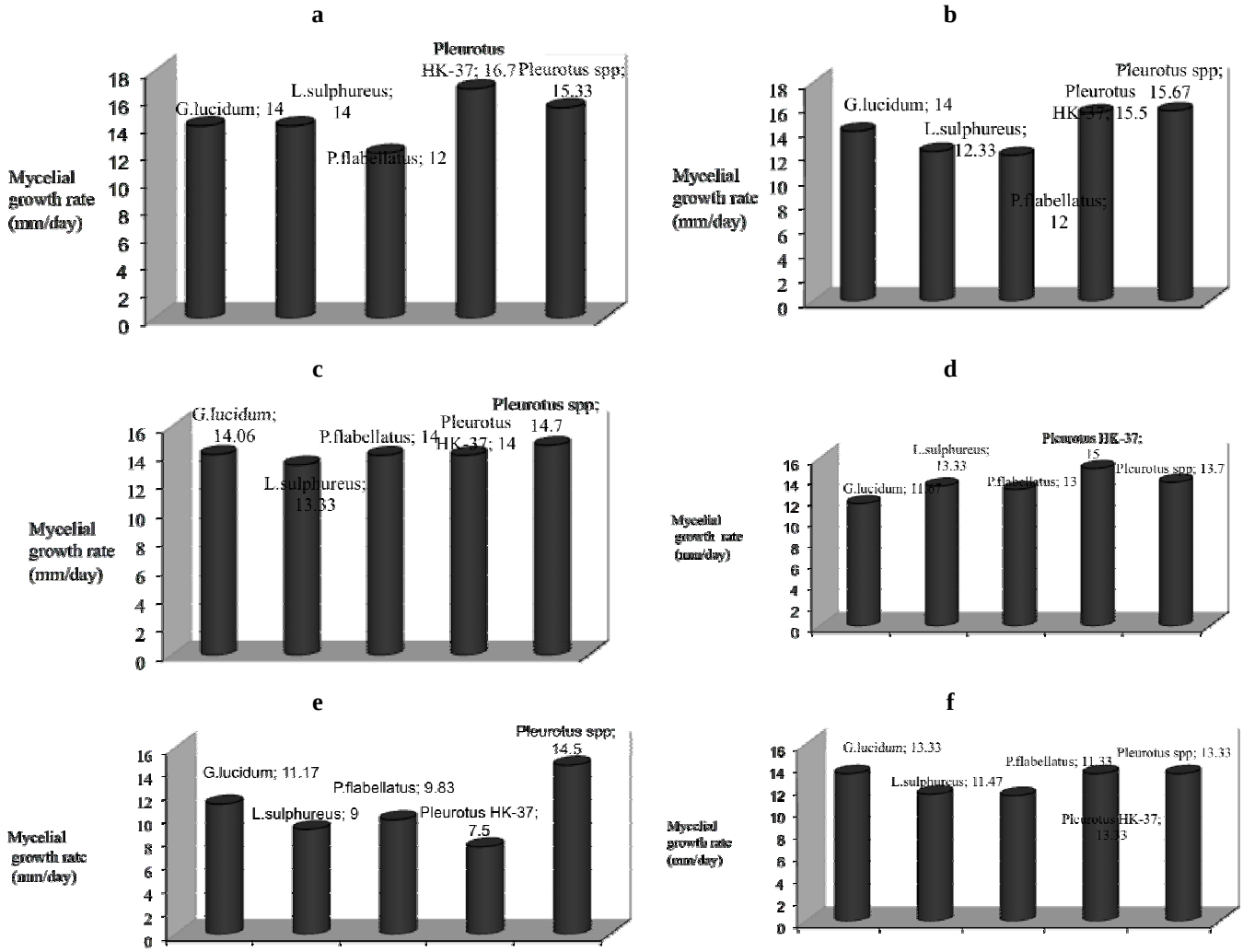

Figure-1. Observed mycelial growth rate of *G. lucidum*, *L. sulphureus*, *P. flabellatus*, Pleurotus HK-37 and Pleurotus spp at 30°C on six different solid medium extracts from waste peels and cattail rhizomes (a) YADA, (b) CADA, (c) PMPDA, (d) SPDA (e) PLDA and (f) CATDA.

Mycelia biomass and exopolysaccharides production in YAD and PMPD media

Figure-2 shows the results of mycelium yield and exopolysaccharides synthesis of five mushrooms in YAD and PMPD media. The data analyzed statistically and showed significant difference at ($p<0.05$). Comparatively, the mycelium and exopolysaccharides yields in YAD medium were relatively higher than those in PMPD medium. The best mycelium yield obtained from YAD ranged from 8-14g/l and were in the order of Pleurotus spp>*L. sulphureus*>Pleurotus HK-37 >*P. flabellatus*>*G. lucidum*. On the other hand, mycelium yield of PMPD ranged from 4.6-10g/l. Mycelium yield obtained from YAD was higher by 29-62% compared to that obtained from PMPD depending on the mycelial dry weight yield data. As shown in Figure-2 Pleurotus HK-37, Pleurotus spp and *P. flabellatus* were found to be good producers of exopolysaccharides in YAD medium with a yield of 380-540mg/l. This was higher by 24-66% compared to that obtained by the other three species in PMPD medium. In contrast, PMPD medium supported good biosynthesis of exopolysaccharides for *G. lucidum* and *L. sulphureus* with a yield of 180 and 340 mg/l, respectively. This was higher by 29 and 44% compared to that obtained from YAD medium for the two species. It was interesting to note that YAD was relatively a suitable medium compared to PMPD medium, even though the latter contained malt extract and organic nitrogen peptone, which are known to increase mycelial growth and exopolysaccharides synthesis in submerged cultures. Precise comparison of the

present results with others investigators on higher fungi submerged fermentation was not possible since there is no published information about mycelial growth and exopolysaccharides production by the five mushrooms on YAD and PMPD complex media. Most of results on exopolysaccharides synthesis and mycelial growth by submerged fermentation of higher fungi mycelia employ synthetic media such as MCM (mushroom complete medium), YM (yeast malt extract) and PMP (potato malt peptone) (Kim S.W., Hwang H.J., Park J.P., Cho Y.J., Song C.H. and Yun J.W., 2002). Mycelium yield (dry weight) ranging from 1 to 7.3 g/l and exopolysaccharides ranged from 379-1,169 mg/l has been reported for *Ganordema lucidum* and Pleurotus species grown on MCM, YM and PMP synthetic media after 15 days submerged fermentation in shake flask culture (Kim S.W., Hwang H.J., Park J.P., Cho Y.J., Song C.H. and Yun J.W., 2002). The maximum mycelium yield 4g/l and exopolysaccharides production 600 mg/l, respectively, were achieved in a medium containing maltose, soy peptone and manganese sulphate for *Laetiporus sulphureus* var. *miniatus* in shake flask culture (Hwang H.S., Lee S.H., Baek Y.M., Kim S.W., Jeong Y.K. and Yun J.W., 2008). The findings on mycelium yield and exopolysaccharides for both YAD and PMPD media, showed that in general, good mycelium yield does not seem to be a determining factors for high production of exopolysaccharides. A similar observation have been reported by other investigators in macrofungal submerged cultures (Kim S.W., Hwang H.J., Park J.P., Cho Y.J., Song C.H. and Yun J.W., 2002; Nour El-Dein M.M., El-Fallal A.A., El-Shahat A.T. and Hereher F.E., 2004; Pokhrel C.P. and Ohga S., 2007; Hwang H.S. Lee S.H., Baek Y.M., Kim S.W. Jeong Y.K. and Yun J.W., 2008).

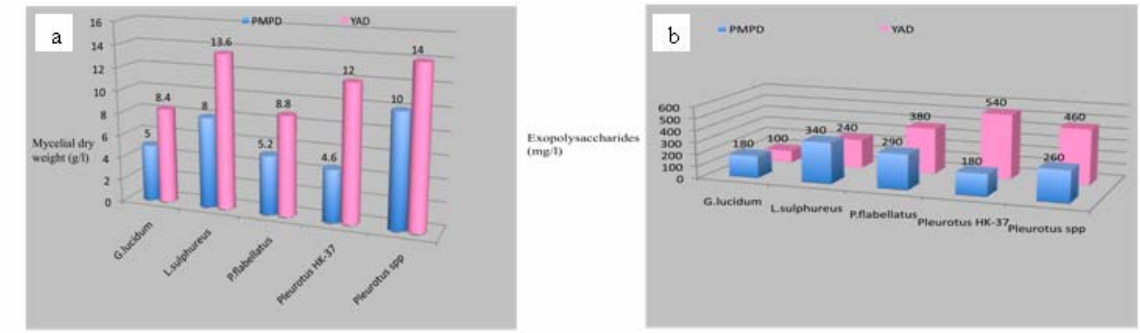

Figure-2. (a) Yield of mycelium (mycelial dry weight) and (b) exopolysaccharides production in shake flask culture of *G. lucidum*, *L. sulphureus*, *P. flabellatus*, Pleurotus HK-37 and Pleurotus spp on YAD and PMPD media after 15 day of submerged fermentation.

Mushroom mycelium as a protein source

Higher fungi grown in submerged culture are capable of producing materials with good nutritive value. Mushroom mycelia are considered as potential source of protein for human food or animal feed (Falanghe H., 1962; Falanghe H., Smith I.A.K. and Rackis J.J., 1964). The results in Table-3 show the mycelial crude protein content in submerged culture of YAD medium for the five mushroom species investigated in this study. The crude protein content of mushroom mycelium ranged from 31 to 55% and varied considerably among the mushroom species. However, there has been no report on the production of mushroom mycelium protein in submerged culture of YAD complex medium by Ganoderma, Laetiporus and Pleurotus mushroom species. Nevertheless, mycelia crude protein ranging from 23 to 61% has been reported for higher fungi such Agaricus, Boletus, Tricholoma, Morchella, Xilaria, Cantharellus and Collybia using soy bean whey and vinasse wastes complex media in submerged culture fermentation under various parameters investigated (Falanghe H., 1962; Falanghe H., Smith I.A.K. and Rackis J.J., 1964). On the other hand, crude protein range of 20-39% was reported for *P. flabellatus* using synthetic medium supplemented with various salts, carbon, nitrogen sources under various parameters studied such as pH, incubation period, varying concentrations of supplements in shake flasks (Srivastava H.C. and Bano Z., 1970). Furthermore, Sugimori T., Oyama Y. and Omichi T., (1971) found that the protein content of *Lentinus edodes* mycelium varied with the carbon source. The one grown on glucose, the mycelium contained 49% crude protein while on ethanol was 58%.

Table-4. Proximate mycelium protein content of five mushrooms grown on YAD (Mean ± SD, n = 3).

Mushroom species	**Crude protein content** (% TS)
G. lucidum	34 ± 0.79
L.sulphureus	39 ± 0.21
P.flabellatus	31 ± 0.28
Pleurotus HK-37	41 ± 0.25
Pleurotus spp	55 ± 0.59

CONCLUSIONS

- This study reports for the first time submerged culture fermentation of Tanzanian basidiomycetes mycelia for production of mycelial biomass, exopolysaccharides and

mycelium protein using non defined wastes peels extract complex media.

- The mycelial growth, mycelial biomass and exopolysaccharides yield and mycelium protein content of mushrooms varied widely with respect to the mushroom species and media employed.
- Among the different media examined, relatively high level mycelial biomass, exopolysaccharides and mycelium protein were achieved in YAD medium.
- The genus Pleurotus amongst the other mushroom genera investigated, should be considered for reasonable production of mycelial biomass, exopolysaccharides and mycelium protein using YAD medium in submerged fermentation.
- It seems generally that the utilization of waste peels as media in mushrooms submerged fermentation may have economic value in waste peels disposal and production of value added products. This may also help to alleviate pollution problems related to waste peels disposal. Therefore, it recommended that intensive research on higher fungi submerged fermentation biotechnology using waste peels be carried out in Tanzania and elsewhere in the world.

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