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Taxonomic studies and optimization of *Lentinus tuberregium* (HM060586) Tamil Nadu, India

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Abstract : The genus *Lentinus* is a white rot fungus, with many taxonomic controversies and it has attracted the attention of many mycologists for many years. Basidiospore shape, size and structure and pileal surface have been used as primary taxonomic character in the identification of *Lentinus* spp. However, high levels of phenotypic plasticity and descriptive key led many taxonomists to explore chemical and molecular methods to distinguish the species of *Lentinus*. Phylogenetic studies were initiated in *Lentinus* during 1990's based on internal transcribed spacer (ITS) and 26SrDNA. Their studies implied the significance of ITS gene in systematic. Growth requirements of *Lentinus tuberregium* (Fr.), was optimized using different carbon, nitrogen, vitamins and amino acids followed by different ratio of C:N showed significant increment on biomass of mycelium with the amendment of dextrose, yeast extract, thiamine and glycine. Different rational supplement of dextrose and yeast extract confirmed the effective mycelia formation with 1:3 and 1:5 ratio.

Key words: Isolation, classical taxonomy, molecular taxonomy, phylogenetic tree, optimization.

Introduction

The vast majority of terrestrial biomass takes the form of wood and other plant tissues. The monumental task of recycling the carbon sequestered in wood falls primarily to basidiomycetes, which include 13,000 described species of mushrooms and related macrofungi. Two principal modes of wood decay are recognized in the basidiomycetes: white rot and brown rot (1,2). White rot fungi degrade both lignin and cellulose (the major components of plant cell walls), leaving the substrate bleached and with a stringy consistency (3). In contrast, brown rot fungi selectively remove cellulose but do not appreciably degrade lignin. After colonization by brown rot fungi, the substrate has a reddish brown color and a soft, crumbly consistency. Brown rot residues are highly resistant to further decomposition and make up a major component of humic soils, especially in temperate and boreal forests (4). The origin and diversification of wood decay mechanisms

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in basidiomycetes has had a large impact on terrestrial ecosystems. In this study, we inferred phylogenetic relationships of basidiomycetes and the historical pattern of transformations between white rot and brown rot modes of wood decay. Using phylogenetic comparative methods, we evaluated previously proposed ecological and genetic correlates of decay modes. The general goal of this study was to understand some of the causal factors that have shaped the evolution of forested ecosystems.

The cost effectiveness of meat and fish people are turning to mushrooms as an alternative source of protein. Local people collect the mushroom in the wild and consumed as food or used as condiments to add to their food. Mushrooms are used extensively, especially by the local people as food item and for medicinal purposes (5,6,7,8,9,10). Inspite of the importance of this mushroom in India it has never been cultivated else where in the world. The mushroom is usually collected in the wild and consumed when a large collection is made, they are either sun-dried or smoked and then stored for longer.

It has been known that macro fungi are used as a valuable food source and traditional medicines since Greek and Roman antiquity (11). Dioscorides, first century Greek physician, knew that *Laricifomes* (*Fomitopsis*) *officinalis* (Vill.) Kotl. & Pouzar (*Fomitopsidaceae*) can be used for treatment of "consumption", a disease now known as tuberculosis (12). It is believed that mushrooms need antibacterial compounds to survive in their natural environment. Antimicrobial compounds could be isolated from many mushroom species and some proved to be of benefit for humans (13). In early studies performed by Anchel, Hervey and Wilkins in 1941, diverse antibiotic activity was detected in basidiocarp or mycelia culture extracts of more than 2000 fungal species (14). Inthe present work, taxonomy and optimization of mycelia growth of *L. tuberregium* were carried out. This will serve as a base or provide information on nutrients that can help in the mycelial growth for *L. tuberregium*.

Taxonomic Distribution

The mode of wood decay has been an important taxonomic character in basidiomycetes, especially in the predominantly wood-decaying polypores and corticioid fungi (crustlike, resupinate forms; 4,15,16,17,18,19). Wood decay mode has generally been regarded as a moderately conservative character and has often been used to differentiate genera. For example, (18) segregated the brown rot gilled mushroom genus *Neolentinus* from *Lentinus*, which is otherwise composed of white rot species. Similarly, the brown rot polypore genus *Antrodia* from the morphologically similar white rot genus *Antrodiella*. White rot is by far the more common form of wood decay in the basidiomycetes,(4) estimated that 1,568 species of wood-decaying basidiomycetes have been described from North America, whereas only 103 species (7%) produce a brown rot. According to (4), 71 species (70%) of the North American brown rot fungi are in the Polyporaceaes, which has long been regarded as an articial taxon (20). According to the dominant morphologybased taxonomies of basidiomycetes (20,21), the remaining brown rot basidiomycetes are classified in six additional families: Coniophoraceae, Corticiaceae, Paxillaceae, Sparassidaceae, Stereaceae, and Tricholomataceae. Except for the Sparassidaceae, which has only two species in North America, each family that contains brown rot species also contains white rot species. Recent molecular studies reviewed by (22) suggest the families that contain brown rot species (Corticiaceae, Paxillaceae, Polyporaceae, Stereaceae, and Tricholomataceae) are polyphyletic. In contrast, (15,16) suggested that brown rot is the plesiomorphic form in the homobasidiomycetes, and that white rot has been repeatedly derived by elaboration of wood decay mechanisms (i.e., gaining the ability to degrade lignin). Most recent authors have supported Gilbertson's view that the brown rot fungi are derived (2,23,24); however, these inferences have not been based on phylogenetic analyses. Indeed, the lack of a broad phylogenetic classification of basidiomycetes has been the primary obstacle to understanding the evolution of decay modes (1). The artificial nature of the Polyporaceae is especially limiting, owing to the concentration of brown rot taxa in this family.

Materials and Methods

Collection and isolation of basidiomycetes

During the months of September - November 2007, the fresh basidiomata were collected from decomposed wood materials in the Keeriparai forest Kanyakumari District, Tamil Nadu., India. A small piece of inner tissue of the fresh basidiomata was aseptically collected using a sterile forceps. Then, it was immediately placed on the surface of the PDA plates and incubated at 25 ± 1° C for 6 days. The pure culture was maintained on PDA slants at 4 ± 1°C and subculture at regular intervals for further studies. (Fig-1)

Fig.1.Natural habitat of *Lentinus tuberregium*

Macroscopic characters

Macroscopic features were followed to study the morphological characters of the isolated Basidiomycete (25). The macroscopic characters such as size, shape and colour of the stipe and pileus; position of the gills; lamella colour attachment, presence and absence of annulus; volva and type of fruit bodies were recorded in the character recording sheet. (26)colour chart was referred to determine the colour of fresh specimen.

Spore print

The colour of the spore print is the most important character for delimiting the families in the order Polyporaceae. The complete opened fresh mushroom was cut at the place of stipe joins with the cap and placed on a black paper and covered with a bell jar. The gills were rested on the paper. The spore impression or print was obtained on the paper after 2-3 h and dried at room temperature. After collecting, the spore print was properly tagged and preserved in polythene bags with naphthalene balls for further studies.

Microscopic characters

Thin sections were prepared from pileus of the dried basidiocarp. These sections were rehydrated with 3% KOH and stained with 2% aqueous phloxine. The stained specimens were observed under light microscope. The size, shape, ornamentation and colour of the spores; size and number of sterigmata of the basidia; colour, size and form of the pleurocystidia and cheilocystidia were observed. Spore measurements such as length and breadth were recorded. Spore range was determined by measuring 50 different matured spores. Mean and standard deviation of fifty spores were determined and identified by standard identification keys. All the observations and measurements of the microscopic structures were made under oil-immersion objective of light microscope. Line diagrams were illustrated with the aid of a mirror type Camera Lucida fixed to a Labomed C × L plus compound microscope.

In the present study, the identification and classification of isolated basidiomata was carried out following (21,27)methods. Moreover, standard monographs were also used for identification. (Fig-2)

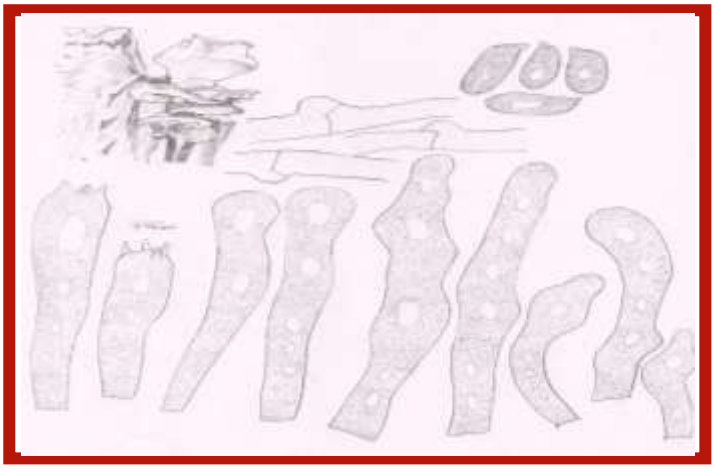

Fig.2.Microscopic characters of *Lentinus tuberregium*

Molecular characteristics of isolated Basidiomata

Mycelial culture

For isolation of DNA, the pure culture of *L.tuberregium* was grown in the Erlenmeyer's flask containing 100 mL of potato dextrose broth (PDB). The mycelial mat was filtered through filter paper and washed with sterile glass distilled water. The excess of water was drained out by pressing the mycelial mat using blotting paper and the mat was lyophilized to dry.

The extraction of DNA from fungal mat was performed by CTAB method. Briefly, 20 mg of freeze dried mycelium was ground to a fine powder under liquid nitrogenwith fine sand in a mortar, the powdered mycelium was placed in a 2 mL microtube with 500 µL TES buffer (100 mM Tris, 10 mM EDTA, 2% SDS, pH 8.0), 50-100 µL of proteinase K and incubated for 30 min (minimum) up to 1 h at 55-60°C with occasional gentle mixing. Salt concentration was adjusted to 1.4 M with 5 M NaCl (140 µL), 1/10 volume of (65 µL) 10% CTAB and incubated for 10 min at 65°C. One volume of Sevag (700 µL) was added, mixed gently, incubated for 30 min at 0°C and centrifuged for 10 min at 4°C at 13,000 rpm. The supernatant was transferred to a 1.5 mL microfuge tube, to this 225 µL of 5M NH_4Ac was added, mixed gently; it was placed on ice for 30 min and centrifuged at 4°C at 13,000 rpm. The supernatant was transferred to a fresh tube, isopropanol (510 µL) was added to precipitate DNA and it was centrifuged immediately for 5 min at 13,000 rpm. In the event of no DNA lumps become visible the sample was placed immediately on ice before centrifugation for 15-30 min. In the event that compact DNA lump is present, DNA was transferred with a hooked Pasteur pipette. The supernatant was aspirated off and the pellet was washed thrice with cold ethanol (70%), pellet was dried and it was dissolved in 50 µL of TE buffer or sterile glass distilled water.

Polymerase Chain Reaction (PCR) amplification of ITS Regions

PCR was performed using standard procedures according to the manufactures protocol. Briefly, the PCR ready mix 25 µL (containing Taq DNA polymerase, assay buffer and dNTP - mix) was taken in a 0.5 mL PCR tube and added with each primer of 2 µL (~200 ng of each). Along with that, 2 µL of template DNA (~100ng) was added, the final volume was adjusted to 50 µL with sterile nuclease free water. An overlay of mineral oil 20 µL was added in the reaction tube to avoid the evaporation. Control reactions without DNA template were also prepared. The amplification of the ITS I and ITS II regions of the fungal DNA was performed using MyGene^{TM} Thermal Cycler - MG96G with a heated lid under the conditions as follows,

1. Initial Denaturation 94°C for 4 min.
2. Denaturation 94°C for 4 min.
3. Amplification

a. 50°C for 1 min.
b. 72°C for 2 min.

PCR steps 2 to 3 were repeated for 30 times.

4. Extension 72°C for 10 min.
5. Stop at 4°C for 1 h.

Agarose gel electrophoresis

Electrophoresis of DNA through agarose was performed as described by (Sambrook and Russell 2001). Agarose (1%) was prepared in 1 × TAE buffer with 1 µL of aqueous ethidium bromide (10 mg mL^{-1}) and electrophoresis was performed in the same buffer at 50 V. Samples containing a tracking dye (ratio 5:1) were loaded into the wells of the agarose gel. The DNA ladder of 1000 bp was also loaded for the comparison of PCR product size and visualized under gel documentation system(28).

Purification of DNA

The PCR product to be purified was electrophoresed on 1% agarose gel as the same method followed in section 2.3.7.3. DNA was visualised by UV trans-illumination and the expected DNA band was excised from the gel using a sterile scalpel and placed into a 1.5 mL microtube. The DNA was then purified using gel extraction kit (Chromous Biotech Pvt. Ltd. Bangalore, India) according to the manufacturer's specifications. (Fig-3)

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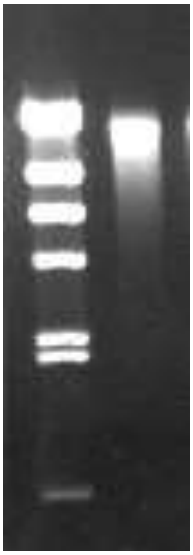

Fig-3. Purification of DNA

DNA Sequencing and construction of phylogenetic tree

The purified PCR product was sequenced at the Synergy Scientific Services, Chennai, India. Sequences were determined by the chain termination method with the use of the DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, UK), using an AVI377 automated DNA sequencer. A typical reaction consisted of 400 ng of rDNA together with 3.2 mol of primer. Primers used for sequencing are ITS 1 and ITS 4 and sequenced in the forward direction. (Fig-4)

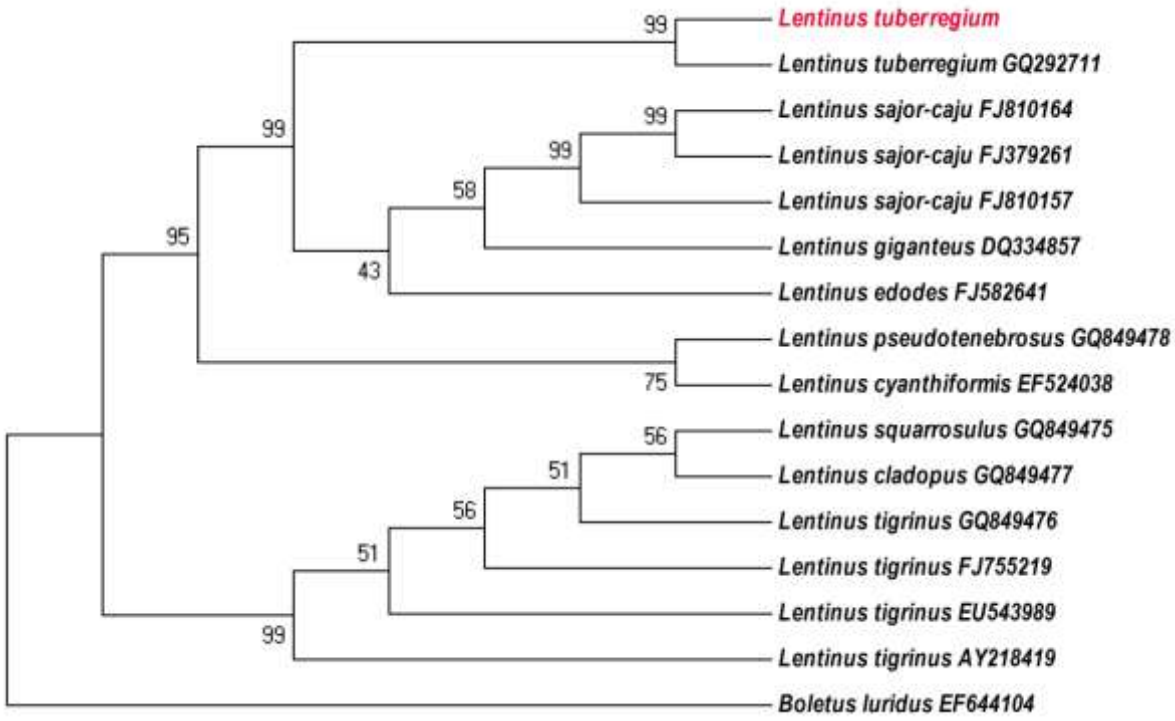

Fig.4.Phylogenetic tree of *Lentinus tuberregium*

Multiple sequence alignment

Multiple sequence alignment (MSA) is an extension of pairwise alignment to incorporate more than two sequences at a time (29). MSA method align all the sequences in a given query set. Multiple alignments are often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionary related. Such alignments are also used to aid in establishing evolutionary relationship by constructing phylogenetic trees. Multiple sequence alignment was done using the programme multalin (http://bioinfo.genetoul.fr/ multalin/multialin .html).

Construction of phylogenetic tree

Sequence data of ITS region was aligned using multalign online programme. Final alignments were visually examined and adjusted manually, phylogenetic analysis was conducted using the program MEGA version 4 (30). The combined ITS I and ITS II data sets were analysed by Maximum Parsimony (MP) analyses. Missing data/gaps were treated as pairwise deletions. A heuristic search was performed using Close Neighbourhood Interchange (CNI) branch swapping on starting trees generated with twenty random addition sequences. A strict consensus tree was derived from the results. The robustness of the internal branches was evaluated by 100 bootstrap replications using a heuristic search.

The mycelial growth was determined by a mycelial dry weight method. The basal medium used in this study was that described by (31,32). The basal medium and supplementary compounds were dissolved in 1 litre of distilled water. 100 ml was dispensed into 250 ml flat bottom flasks and the pH was adjusted to 6 . The mouth of each flask was sealed with cotton wool and covered with aluminium foil paper before sterilization. The media after cooling were then inoculated with 5 mm diameter agar block of 7-9 days old mycelium of the mushroom. The flask was incubated at room temperature for 28 days. Each experiment was replicated three times. The mycelium in each flask was filtered through a pre-weighed 9 cm diameter filter paper, and dried at 85^{o}C for 10 h and recorded the fresh weight.

carbon source

Each five g of six different carbon sources of dextrose, lactose, sucrose, maltose, manitol and starch were amended in the basal medium having the PH of 6. The mycelia disc of 5mm was cut from 9-10 days old mycelia mat, and this blocks were aseptically inoculated in the flasks including the control flask ie basal medium without any carbon source. All the flasks were kept undisturbed for 28 days of incubation. After the incubation period the mycelia mat collected and recorded the results.

Nitrogen source

Two grams of each nitrogen sources such as, sodium nitrate calcium nitrate, ammonium nitrate, peptone, yeast extract , beef extract, and urea were added to the basal medium containing fructose (10g) KH_2PO_4 (0.5 g), $MgSO_4.7H_2O$ (0.5 g), thiamine hydrochloride (500 µg) and made up to 1 litre with distilled water (31). Sterilization, inoculation and assessment of dry weight were carried out as described for the carbon sources above. Basal medium alone was used as control.

Vitamin source

Vitamins were selected to this study such as, biotin, ascorbic acid, thiamine, and tocoferrol. The basal medium was the same for determination of nitrogen sources. Each vitamin was added 500 µg of each to the basal medium and made up to 1 litre. The set up was treated the same way as for carbon, nitrogen and amino acids. Basal medium alone was used as control.

Amino acid source

The amino acids of were selected aspartic acid, cysteine, phenyl alanine, tyrosine, methionine, L-glutamic acid, DL-leucine, histidine, L-leucine, tryptophan, DL-dopa, proline, DL-2-amino butric acid, DL-threonine, Isoleucine, hydroxy proline, glycine, DL-valine, L-cysteine, L-lysine mono hydrochloride, DL-serine, L-ornithine mono hydro chloride, L-arginine monohydro chloride, and DL-alanine. The basal medium was used the same as that of nitrogen source. For each amino acid (500 µg) was added to the basal medium and made up to 1 litre and dispensed into the flasks which were treated as described above. Basal medium alone without amino acid was used as control.

Carbon to nitrogen ratio (C/N)

The basal medium was similar to that used for nitrogen compounds but glucose was varied composition with yeast extract as sources of C/N. Concentration of 0.15 g/litre of dextrose and yeast extract in the basal medium serve as 1:1 ratio (32). Other ratio was prepared proportionately i.e., 1:1, 1:3, 1:5 and 5:1, 3:1.

Results and Discussion

Classical Taxonomy

Basidiome robust, in troops, lignicolous. Pileus 6–12(–18) cm dia., hard, rigid, dry, deeply depressed, infundibuliform, surface pale yellow to yellowish white (4A2–4A1), smooth, velvety at centre, glabrous, fleshy; margin inrolled when young later entire. Lamellae deeply decurrent, yellowish white to creamy (3A2–3A3), thin, upto 2 mm broad, crowded with lamellulae of seven different lengths; edge entire. Stipe central, 5–11.5 × 0.7–1.5 cm, slightly tapering downwards, pale yellow (4A2), cylindric, hard, woody, solid, velvety, rhizoidal, rhizoids upto 3.5–7.3 cm long, arising from the sclerotium. Veil absent. Context hard, thick, pale yellow 5–8 mm dia. at disc, weekly dextrinoid, heterogenous with of dimitic hyphal system with loosely interwoven, frequently branching, non inflated, hyaline, clamped generative hyphae 2.3–5.46 µm dia., thick walled skeletal hyphae upto 8.04 µm dia., pale brown with broad lumen, often consisting of a short to long skeletal elements. Spores 5.74–9.72 × 3.21–4.58 (7.73 ± 0.73 × 3.89 ± 0.61) µm, Q = 1.98, oblong cylindric, hyaline, thin walled with elongated apiculus upto 1.2 µm long, inamyloid. Basidia 19.72–22.18 × 4.6–5.46 µm, narrowly clavate bearing four sterigmata, sterigmata thin, short. Lamella edge sterile with ephemeral cheilocystidia. Cheilocystidia 25-40× 5.1-7.2 µm, cylindric to fusiform, thin walled. Hyphal pegs absent. Pleurocystidia 26–35 × 4.6–5.3 µm, narrow, elongate, hyaline, thin walled. Hymenophoral trama irregular to sub regular 45.27–105.55 µm broad, with thin walled generative hyphae 2–3.8 µm dia., dextrinoid. Subhymenium poorly

developed. Pileal surface more or less trichodermial palisade, 85.54–225.44 µm broad, semierect to erect, tapering, hyaline, thin walled hyphae upto 3.81µm dia., together with narrow generative hyphae. All hyphae having clamp connections.

This specimen has pale yellow infundibuliform pileus, well developed long radicated stipe inserted in the wood substrate, cylindric spores with prominent apiculus and thick trichodermial pileipellis. This specimen shows similarity with *L. tuberregium* in having robust pale yellow basidiocarp, but differs in having tapering long radicated stipe and smaller basidia. It differs from *L. fusipes* (33) in having pale yellow basidiocarp and long radicated stipe, spores some what similar but basidia are much smaller.

Molecular Taxonomy

Isolation of genomic DNA

The DNA was isolated from the mycelia mat of *L.tuberregium* by CTAB method. The resulting DNA was subjected to electrophoresis on 0.8% agarose gel and good yield of DNA was obtained ~200ng / µl with the size of ~ 1500bp.

PCR amplification of ITS regions

The internal transcribe spacer region (ITS 1 and ITS 11) of the isolated DNA was amplified by PCR using the primers ITS 1 and ITS 4. The resulting amplified PCR product showed the band with ~ 659pb in the agarose gel electrophoresis with some impurities. The impurities were removed by agarose gel elution method and the purity was again rechecked by electrophoresis on 1% agarose gel. Molecular techniques are becoming increasingly important in the identification of new fungal species and comparison of genetic relationship between the known and rare fungal species. Various molecular genetics tools have been introduced for the verification of fungi, such as RFLP, RAPD and SSU rDNA and ITS sequence analyses (34). Ribosomal DNA regions are generally informative for species and genera differentiation of fungi (35). The different regions of rDNA evolve at variable rates, making them useful for phylogenetic studies among closely or distantly related organisms. In this study, genetic relationship of *L. tuberregium* VKJM24, was performed using modern molecular techniques including PCR and sequencing of amplified rDNA fragments. This specific region, which contains fungal evolutionary information, was subjected to analysis through PCR investigations, which mainly focused on nucleotide sequences of the ITS located between the nuclear rDNA 18S and 28S subunit genes and made it possible to determine the relationships. This was done for the species of *Ganoderma* (36).

Sequence arrangements of purified PCR product

The purified PCR product was analyzed for sequence arrangements with the primers ITS1 and ITS 4 at forward direction. The sequence results showed that the purified PCR product was 659bp. The sequence data obtained in the present study was deposited in the GenBank database (Maryland, USA) and the accession No. was **HM060586**. The sequence data from the ITS1 and ITS 11 region of L.tuberregium was alligned against sequence data from NCBI GenBank and analyzed using the data analysis progrms Multalign online software. The alligned sequence were again analyzed for evolutionary relationships using the software MEGA version 4. The analyses were performed with available closely related Lentinus taxa from Genbank database. The results obtained were used to estimate the evolutinary relationships between *L.tuberregium* and a wide range of *Lentinus* species.

The ITS region of the nuclear rDNA unit are known to exhibit a high degree of polymorphism between species but are often highly conserved within the species; thus, they contain valuable genetic markers for species identification (37,38). Further, it has been reported that the ITS region is a convenient target region for the molecular identification of mycorrhizal fungi (39) due to variability in length and in nucleotide content among different species. The direct sequencing of the purified PCR product from *Ganoderma* species has been successfully performed. by (36). Considering that, in the present study also the direct sequence of the rDNA (ITS 1, 5.8S, ITS 2) region of the *L. tuberregium,* using ITS 1 and ITS 4 primer set were carried out.

Evolutionary trees

The data was analyzed using Maximum Parsimony method and tree building method. Complete deletion and pairwise deletion was investigated by close neighbourhood interchange (CNI) method. The consensus trees produced by both models had the same topology and only differed in the use statistical support

of internal branches. The strict consensus trees derived using pairwise deletion gave better statistical support than complete deletion. Therefore, pairwise deletion phylogenies are presented in this investigation. The evolutionary trees produced from combined data set of ITS 1 and ITS 11.

To determine the evolutionary relationships of the *Lentinus* spp. at the molecular level and in turn identify the species, a phylogenetic study was performed by the Neighbour Joining (NJ) method. Another phylogenetic method based on DNA sequencing, ITS1-5.8S rDNA-ITS2 sequence analysis, is frequently used for fungal verification (40,41). Although it proved to be powerful in the verification of fungal species among mixed fungal species, it failed to discriminate variants within the strains (42). To approach the phylogenetic classification it is necessary to identify it with legitimate type species (35). The most basal clade consists of a single out group taxa *Boletus luridus* (EF 644104) used to root the tree. *L. sajorcaju* clade forms a separate branching with short branches while *L. tuberregium* with long branches, suggests that it is more distantly related or a higher substitution rate when compared to *L. sajorcaju*. ITS sequences among the members of subgenus *Lentinus* were extremely similar, differing by only few base pairs. To resolve such close relationship within these groups, a combination of more samples from different geographic regions and additional genetic regions could be used.

The results of this study demonstrates that the amplified regions of the ITS contains sufficient variations for the taxonomic problem. Since all the dendrograms were constructed from the same distant matrix, it is not surprising that they are highly congruent. This tree, clearly agrees with (35) except for *L. sajorcaju*. This is revealing that molecular tool is reliable with the classical taxonomy. These results indicate that both are congruent with each other. In this analysis phenetic similarity was used to infer phylogenetic relationship. Because the dendrograms presented in this figure are unrooted, it is not possible to make statements about polarities of evolutionary character or identify monophyletic groups.The mushroom was identified at their molecular level and their gene sequences were submitted to the Genbank, have the accession number of HM060586.

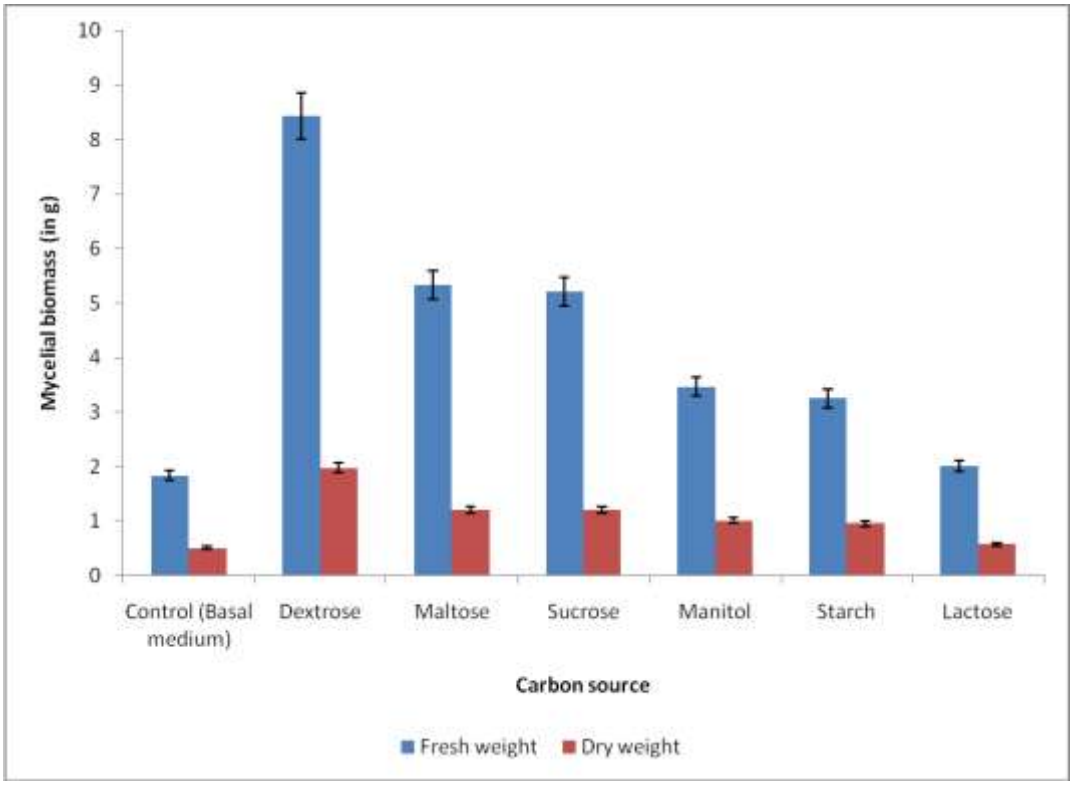

Fig. 5 Growth of *Lentinus tuberregium* on different carbon source.

L. tuberregium shows different preferences for carbon sources for its metabolism(Fig. 5). The ability of an organism to utilize the carbohydrate depends on type of enzyme produced by the organism(43). In this study, dextrose was best source of carbon for this mushroom. This shows that *L.tuber-regium* produces enzymes that utilize dextrose better than any other carbon source. (44) also reported that *Volvoriella volvacea* utilizes glucose and starch better than other carbon sources. (45) also reported that fructose, glucose and

maltose were the most suitable carbon sources for *Auricularia auricular*. (46) reported that the best utilizable carbon sources for *Lentinus subnudus* were fructose, maltose, dextrin and glucose. This study showed that *L.tuber-regium* utilizes dextrose better than maltose, mannitol, lactose and starch. The least carbon sources were lactose. (47) reported that glucose has been good respiratory substrate.

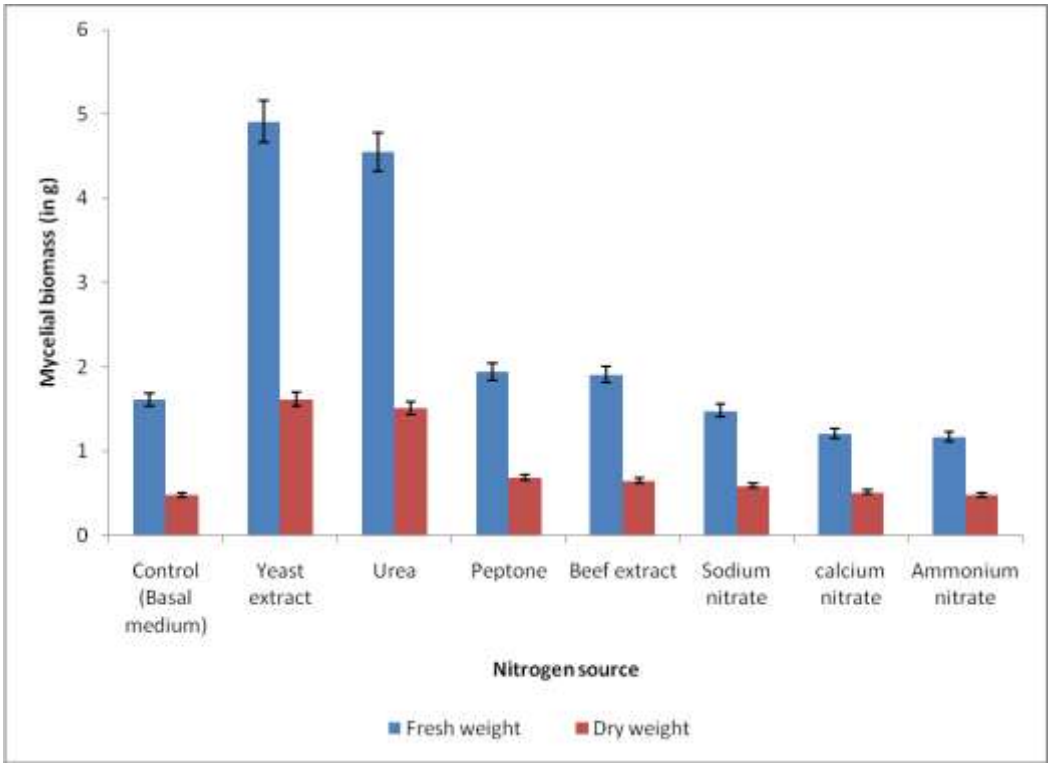

Fig.6 Growth of *Lentinus tuberregium* on different nitrogen source

L.tuber-regium utilises organic nitrogen better than inorganic nitrogen(Fig.6). This observation are agreement with the report of (32) who observed that yeast extract which is a complex nitrogen source sustained the greatest growth of *P. tuberregium*. (48) reported peptone as the best nitrogen source for *L. subnudus*.(49) also reported that *V. volvacea* frequently responds better to organic nitrogen than inorganic nitrogen. (44) reported that the best yield of *Volvoriella* were obtained on media containing peptone or potassium nitrate. In the same vein,(45) reported that organic nitrogen sources such as yeast extract and peptone are the preferred nitrogen sources for *A. auricular*. In this study, *L.tuber-regium* showed preference for organic nitrogen than inorganic nitrogen.

Thiamine proved best among the vitamins followed by biotin and tocoferrol. According to (50) who found that thiamine stimulates mycelial growth of *Cercospora arachidicola* in liquid culture. Also, (45) reported that different vitamins produce different effects on myelial growth within a certain concentration range. (51) reported that combined amino acids stimulate greater growth than single amino acids.The least effective vitamin in this study was ascorbic acid (Fig. 7). (32) who reported that ascorbic acid, folic acid and riboflavin did not support good growth of *P. tuberregium*.

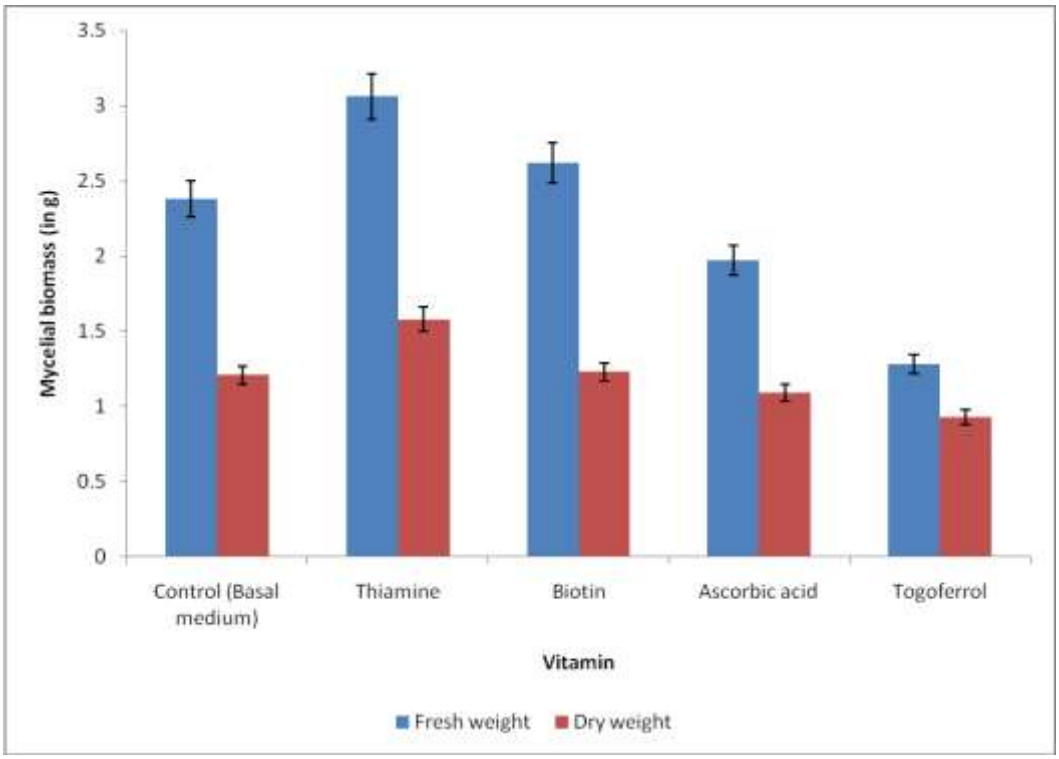

Fig.7 Growth of *Lentinus tuberregium* on different vitamin source

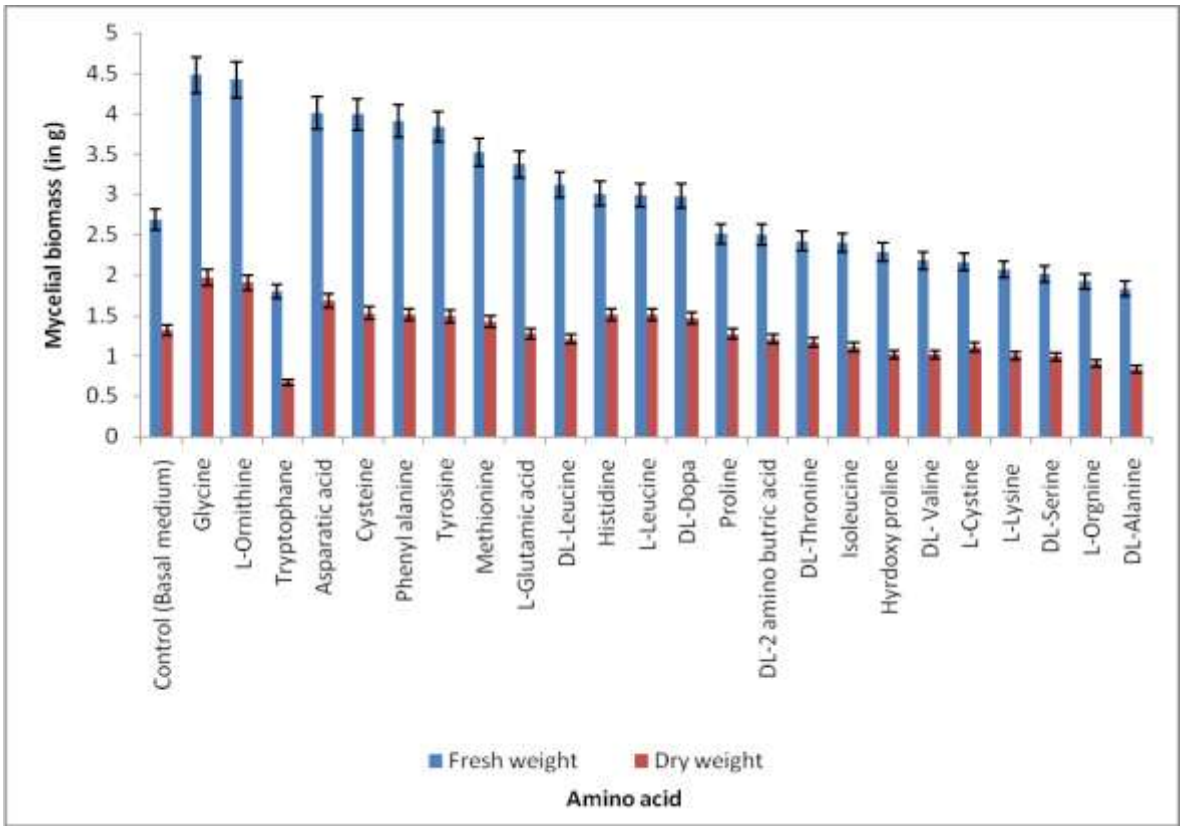

Fig. 8 Growth of *Lentinus tuberregium* on different amino acids source.

Glycine proved to be the best amino acid, this is followed by L-ornithine mono hydrochloride (Fig. 8).(31), reported that asparagine and aspartic acid have been employed in increasing the mycelial growth and

fruit body production in *Agaricus bisporus*. (52) reported that higher and lower concentrations of these amino acids are found to be either ineffective or inhibitory for the mycelial growth of mushrooms.

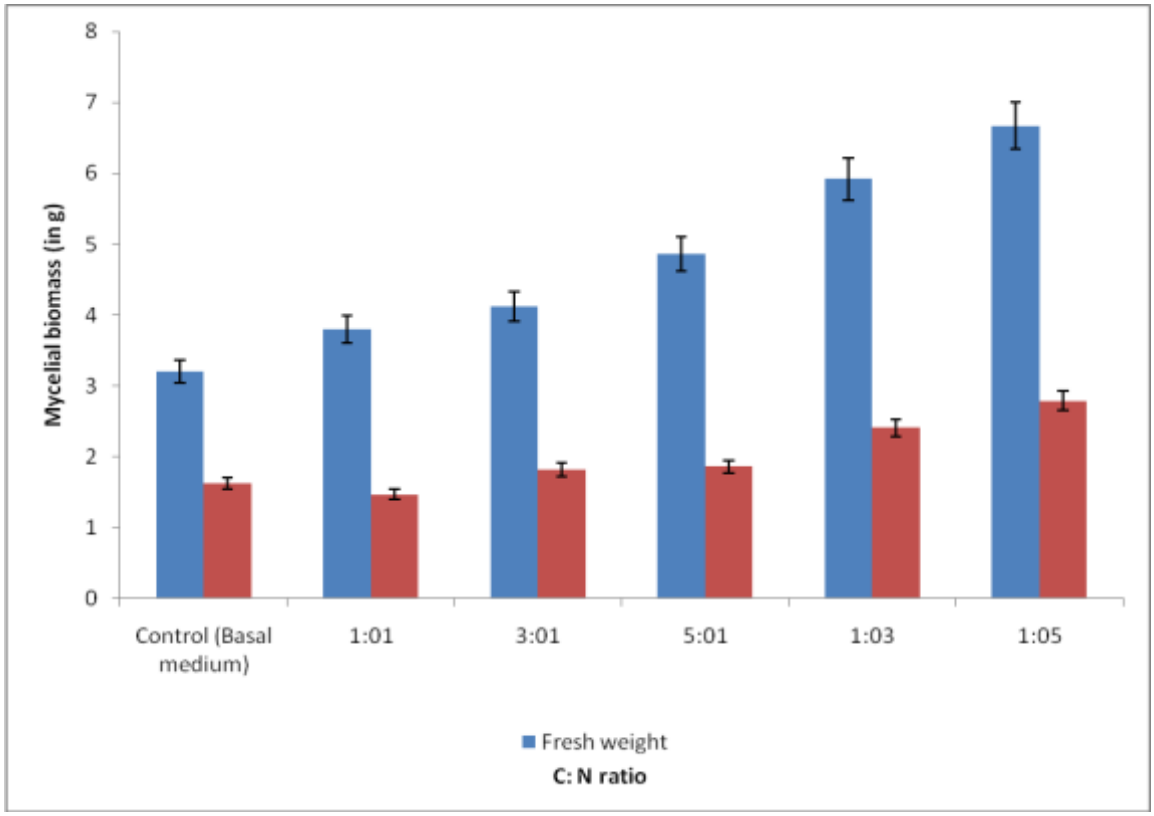

Fig.9. Growth of *Lentinus tuberregium* on different carbon to nitrogen ratio.

(53) reported that the ratio of carbon to nitrogen (C: N) balance in mushroom substrate is very important. A well balanced carbon to nitrogen ratio enhances the growth and development of mushrooms while an imbalance of C: N impedes their growth (54,55). In this study the C: N ratio of 1:3 and 1:5 supported best growth of the mushroom (Fig. 9), growth was reduced above or below this levels. (32) also reported C: N ratio of 1:3 and 1:5 for *P. tuberregium*. According to (53,54), over-supplementation of mushroom substrates with nitrogen and carbohydrates impedes mycelial growth of mushrooms. As the ratio of C: N increased, the mycelial growth of *L. tuberregium* also increased up to a point after which further increase in carbon decreased the mycelial growth. The same was applicable to nitrogen. In this study, the vegetative growth of *L. tuberregium* was greatly improved by carbon, nitrogen, vitamins and amino acids. Carbon to nitrogen of 1:3 and 1:5 was best and however, *L.tuber-regium* can be cultivated on substrates containing C: N ratio of 1:3 or 1:5.

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