# STRAIN EVALUATION AND PRODUCTION TECHNOLOGY OF SHIITAKE MUSHROOM (*Lentinula edodes* (Berk.)Pegler)

**DEEPA RANI C.V**
**(2012-21-114)**

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**COLLEGE OF AGRICULTURE**
**VELLAYANI, THIRUVANANTHAPURAM- 695522**
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## DECLARATION

I hereby declare that this thesis entitled '**Strain evaluation and production technology of Shiitake mushroom (*Lentinula edodes* (Berk.)Pegler)**' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other university or society.

Vellayani,
19 -03-2016

DEEPA RANI C.V
(2012-21-114)

## CERTIFICATE

Certified that this thesis entitled '**Strain evaluation and production technology of Shiitake mushroom (*Lentinula edodes* (Berk.) Pegler)**' is a record of research work done independently by Mrs. Deepa Rani C.V (2012-21-114) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Vellayani,
19 -03-2016

**Dr. Lulu Das**
(Chairperson, Advisory Committee)
Professor
Department of Plant Pathology
College of Agriculture, Vellayani
Thiruvananthapuram - 695 522.

## CERTIFICATE

We, the undersigned members of the advisory committee of Mrs. Deepa Rani C.V (2012-21-114) a candidate for the degree of **Doctor of Philosophy** in Agriculture, agree that this thesis **entitled 'Strain evaluation and production technology of Shiitake mushroom (*Lentinula edodes* (Berk.) Pegler)'** may be submitted by Mrs. Deepa Rani C.V (2012-21-114), in partial fulfilment of the requirement for the degree.

**Dr. Lulu Das**
Professor,
Department of Plant Pathology
College of Agriculture, Vellayani
(Chairman)

19/3/16

Dr. C. Gokulapalan
Professor and Head
Dept. of Plant Pathology
College of Agriculture, Vellayani

19/3/16

Dr. Sumam George
Professor
Dept. of Soil Science and Agricultural Chemistry
College of Agriculture,Vellayani

19.3.16

Dr. V. G. Jayalekshmy
Associate Professor
Dept. of Plant Breeding and Genetics
College of Agriculture,Vellayani

19/3/16

Dr. C. Nirmala
Associate Professor
Dept. of Home Science, College of Agriculture
Vellayani, Thiruvananthapuram

19/3/16

19.3.16

**EXTERNAL EXAMINER**

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*DEEPA RANI C.V.*

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*Dedicated to*

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| | |
|---|---|
| % | per cent |
| $^{O}C$ | Degree Celsius |
| B.E. | Biological efficiency |
| cm | centimeter |
| dia. | diameter |
| h | hours |
| *et al.* | And other co workers |
| Fig. | Figure |
| M | molar |
| g | gram |
| l | litre |
| kg | kilogram |
| mg | milligram |
| ml | millilitre |
| min. | minute |
| ppm | parts per million |
| RH | Relative humidity |
| sp. | species |
| *viz.* | namely |
| wt | weight |
| RAPD | Randomly Amplified Polymorphic DNA |
| PCR | Polymerase Chain Reaction |
| ITS | Internal Transcribed Spacer |
| DAI | Days after inoculation |
| temp. | temperature |
| sec. | Seconds |
| bp | base pair |
| rpm | rotation per minute |
| CD | Critical difference |

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# *Introduction*

# INTRODUCTION

The name Shiitake (shee-tah-key) is derived from the Japanese words 'shii' meaning oak tree and 'take' meaning mushroom and is scientifically known as *Lentinula edodes* (Berk.) Pegler. It is also known as Japanese wood mushroom or Chinese black mushroom or Golden oak mushroom or Black oak mushroom or Oriental mushroom . Shiitake is a white rot fungus which produces brown sporocarps with high medicinal properties. Since shiitake is a temperate mushroom , it is extensively grown in Japan, China and other Asian countries due to the suitability of the climate.

Shiitake mushroom cultivation were probably introduced to Japanese farmers by the Chinese between 1500 and 166 AD. The history of shiitake cultivation dates back to Ming Dynasty where the mushroom was believed to keep people vigorous and young. Since only Emperors and his family could consume the mushroom during that period, shiitake were widely known as the Emperor's food. According to Chinese folk fare, the mushroom is called ' elixir of life' capable of generating stamina, curing colds, improving circulation and preventing premature aging.

Shiitake is a valuable medicinal mushroom having anticancerous properties which enhances immune response due to the presence of a polysaccharide 'lentinan'. Moreover the mushroom is used as anticarcinogenic, anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral, and anti cardiovascular disorders. The mushroom is rich in proteins, fibres, vitamins, minerals and low content of lipid especially cholesterol. Shiitake mushrooms are known for their unique taste and flavor. The aroma component in the mushroom is imparted by alcohols, ketone, sulfides, alkanes, fatty acids *etc*. The characteristic flavor imparting components in the mushroom are matsutakeol (octen-1-ol-3) and ethyl-n-amyl ketone. The components responsible for the delicious flavor are monosodium glutamate, free aminoacids, peptides, organic acids (malic acid, fumaric acid, oxalic acid, lactic acid, glycolic acid) and sugars.

Japan was the world's highest producer of shiitake till 1980 but in 2012, China overcame this with an annual production of 4 million tonnes of shiitake which accounts for more

than 90 per cent of total production ( USDA, 2014). Shiitake ranks third with 17 per cent of total edible mushroom production in the world.

Natural log method is an established and traditional method of cultivation of shiitake which take a very long crop cycle . Therefore attempts were done by the researchers to develop a more efficient and cost effective method for the production of shiitake, where they focused on synthetic sawdust substrates and agriculture wastes like straw, corncobs, grass, coffee pulp, oil palm waste, sugarcane bagasse, coconut husks, tree leaves *etc* as cultivation substrates. In India, shiitake cultivation in both natural and synthetic log method is being standardized in North eastern regions of Meghalaya and middle east regions of Solan, Uttarkhand, Rajasthan *etc* where the climatic conditions suited to the mushroom, prevails.

The climatic conditions of hilly places in Kerala like Ponmudi, Munnar, Wayanad etc. are best suited for the growth of shiitake. Shiitake mushroom can also be cultivated under controlled conditions in plains though it makes the production expensive. An effort has therefore been undertaken to standardize shiitake cultivation in Kerala conditions, thus adding a new species to the artificially cultivated mushroom flora of Kerala.

Based on these facts, the present study was conducted with the major objective to exploit various strains of *Lentinula edodes* for novel production technology by utilizing a range of agrowastes of Kerala .Physiological and molecular studies were conducted for their phylogenetic analysis. Attempts were also made to study shelf life, enzymatic activities and pest and incidence during the cultivation of the mushroom strains. Various recipes using this mushroom were formulated and subjected to organoleptic studies considering its unique exotic flavor.

# *Review of Literature*

# 2. REVIEW OF LITERATURE

Shiitake (*Lentinula edodes*) commonly known as Japanese Black Oak mushroom was first cultivated in China more than 800 years ago (Chang & Miles, 1987). The fruiting bodies are rich in minerals, vitamins, essential amino acids (especially lysine and leucine), had high fibre content but contained less than 10 per cent crude fat (Mizuno *et al.*, 1995; Yang *et al.*, 2002). Besides this, *Lentinula*, a nutritionally valuable mushroom has a protein-bound polysaccharide (lentinan) extracted from its fruiting bodies were found to have clinically-useful immunomodulatory, anti-cancer and anti-viral effects (Chihara, 1993; Mizuno, *et al.* 1995). The great interest in Shiitake's commercialization is due to its unique flavor/ taste, nutritive value and medicinal properties (Sugui *et al.*, 2003; Silva *et al.*, 2007).

Primitive methods of Shiitake (*Lentinula edodes*) cultivation started in the middle of the17$^{th}$ century in Japan. At that time, Shiitake growers gathered logs bearing Shiitake mushrooms and placed them near fresh logs, the bark of which had been cut with a hatchet, allowing airborne spores to infect the new logs. Shiitake cultivation had developed and expanded rapidly since then. In 1943, Dr. Kisaku Mori invented a new inoculation method based on wooden dowels or plugs of colonized mycelia inserted into drilled holes in the logs. Recently, bag cultivation of Shiitake has increased rapidly in conjunction with a decrease in log cultivation (Royse, 2002).

Cultivation of *Lentinula edodes* (Shiitake) was achieved for the first time in India on local sawdust substrates (Kaur and Lakhanpal, 1995). In India, the Directorate of Mushroom Research, Solan, Himachal Pradesh and Indian Institute of Horticulture Research, Bengaluru had developed technology for Shiitake mushroom cultivation. Although Shiitake is a globally well-known cultivated species it is yet to find a place in Indian markets. This is mainly because the cultivation technology of

this mushroom especially on locally available substrates has not been commercially standardized, so far. Hence a concerted effort has been made in the present study to identify locally available strains of *Lentinula edodes* and also to standardize its cultivation in Kerala.

Literature related to collection and identification of *Lentinula* sp., morphological and cultural aspects spawn production technology, cultivation technology and crop management aspects of this species of mushroom are reviewed and presented here. Further, research on nutrient analysis, enzyme activity and RAPD analysis to study the phylogenetic relationship between different strains of *L. edodes* are elaborately reviewed. As mushroom growers continue to use the old name *Lentinus edodes* till 1989, this historical name *Lentinus edodes*, is still kept in consideration while conducting literature searches or other explorations (Chen, 2001). Keeping this in view, distribution and morphological studies of *Lentinus* sp. is also reviewed here to supplement the information pertaining to the present study of *Lentinula edodes*.

## 2.1. SURVEY AND COLLECTION

### *2.1.1. Lentinula sp.*

Boruah *et al.* (1996) surveyed and obtained three edible species of mushrooms from East Khasi Hills of Meghalaya which included *Lentinus edodes*, *Boletus edulis* and *Cantharellus floccosus*. Guzman *et al.* (1997) reported that *Lentinula edodes* was confined mainly to temperate, subtropical and tropical lands of east, south and south East Asia and Australia. Natural local distribution of *Lentinula edodes* was reported by Chiu *et al.* (1999), from remote broad leaved *Fagus longipetiolata* forest in Shaanx Province of China.

Morais *et al.* (2000) reported that *Lentinula* species occurs both in the New World as well as in Asian and Australian countries. According to Birkumar *et al.* (2008), Shiitake cultivation is a potential agro industry for hilly areas of north eastern India including Sikkim, Meghalaya regions rich in forest diversity and coverage. In these regions, *Lentinula edodes* mushroom grows in nature on dead wood of hard wood trees mainly on oak (*Quercus dealbata*), chestnut (*Castanopsis chinensis*), *Elaeocarpus sinensis* , birch (*Betula* sp. ) etc.

Approximately 68 mushroom flora belonging to nineteen genera *i.e. Agaricus* sp., *Lentinula edodes, Amanita* sp., *Mycena* sp., *Macrolepiota, Pleurotus cystidiosus*, *P. djamor, Auricularia* sp., *Calocybe* sp., *Rusella* sp., *Schizophyllum* sp., *Termitomyces* sp., *Polyporus versicolor, Tricholoma giganteum, Volvariella volvaceae, Trametes* sp., *Lycopersicon* sp., and *Ganoderma lucidum* were collected form Anaikatti, Siruvani, Palakkad and Nilgiris regions of Western Ghats (Tamil Nadu) during 2008-10 (Thirubhuvanamala *et al.*, 2013).

### *2.1.2. Lentinus sp.*

Bose (1920) documented *Lentinus connatus* Berk. on dead wood from Howrah district of West Bengal. It was also documented from Dehradun, Maharashtra and Assam (Manjula, 1983; Pegler, 1983). Oso, 1975; Nicholson, 1989; Akpaja *et al.*, 2003 reported that *Lentinus squarrosulus* (Mont.) Singer as one of the common edible mushrooms in the southern parts of Nigeria. Sharma *et al.* (1985) conducted a survey all over Kerala and observed only four species of *Lentinus* namely *Lentinus caespiticola, L. giganteum, L. sajor caju* and *L. squarrosulus.* Joseph *et al.* (1995) and Manimohan *et al.* (2004) documented *Lentinus giganteum* for the first time from India. Survey was conducted by Lakshmanan *et al.* (1997) to exploit microflora which resulted in detection of a new funnel shaped mushroom on cashew wood, identified as *Lentinus connatus*.

From the intensive survey of forests and local markets conducted in Manipur state during 1997-1999, 33 species of wild edible fleshy fungi were collected which were identified as *Lentinus lateritia, L. cladopus, L. tigrinus* and *L. sajor caju* (Singh and Singh, 2000). Among various *Lentinus* sp. collected, *L. cladopus* Lév. are reported to be edible which can be cultivated on pasteurized as well as non pasteurized substrates.

Manimohan *et al.* (2004) reported that total nine species of *Lentinus* have been documented from Kerala *viz., L. caespiticola, L. sajor-caju, L. giganteus, L. squarrosulus, L. dicholamellatus, L. polychrous, L. similis, L. strigosus* and *L. hookerianus.* A new *Lentinus* species of *Lentinus bambusinus,* its habit, distribution and morphology were first reported based on collections made by Kumar and Manimohan (2005) in Kerala.

Pukahuta *et al.* (2006) reported *Lentinus polychrous* Lev. as a popular mushroom seen in the north and north east of Thailand and in Laos. Atri *et al.* (2010) reported a wood decaying basidiomycetes fungus, *Lentinus torulosus* (Pers.: Fr.) Lloyd, for the first time from North West India. Five wild edible *Lentinus* species *viz., Lentinus sajor caju, L. connatus, L. torulosus, L. cladopus* and *L. squarrosulus* were collected from different localities of North West India (Gulati *et al.*, 2011).

Karunarathna, *et al.* (2011) reported a new collection of *Lentinus giganteus* from SriLanka, represented by relatively large fruiting bodies which are phylogenetically and morphologically similar to *Pleurotus giganteus*. Saprobic on buried well rotted wood in forests, *P. giganteus* is widely consumed in Sri Lanka and can be profitably cultivated in Thailand. Karunarathna *et al.* (2011) studied the taxonomy and biodiversity of various *Lentinus* genus in Thailand of which three new species of *Lentinus* like *Lentinus sensustricto, L. roseus* and *L. concentricus* have

been reported. Kumar and Kaviyarasan (2012) reported distribution of *L. tuber-regium* (Fr.) as an indigenous edible medicinal mushroom in Tamil Nadu.

Tongou *et al.* 2013 reported South China as the native of Giant cup mushroom *(Lentinus giganteus)*. A new species of *Lentinus* named *L. alparus* which was distinguished by small, reddish brown, squarrose basidiomycota was reported by Senthilarasu *et al.*, 2013.

Pires *et al.* (2014) reported that among 1079 specimens collected from Araucaria forest, Polyporaceae has greatest number of representatives totaling 307 specimens from four genera *Microporellus, Lentinus, Polyporus* and *Trametes.*

## 2.2. MORPHOLOGICAL STUDIES OF LENTINULA SP. AND LENTINUS SP.

### *2.2.1. Studies on macroscopic and microscopic characters of strains of Lentinula sp.*

Pegler (1975) in his descriptions of *L. edodes* indicated that mushrooms had pileus ranging between 5-15 cm dia., with tawny to dark vinaceous brown colour , which was darker at the centre and often pale at the margin in young specimens. Lamellae was adnexo-adnate with decurrent tooth, whitish, 4-7 mm broad and was moderately crowded. Stipe was central to eccentric, 3-7 cm x 8-15 cm, cylindric to slightly fusoid often compressed with a subbulbous base, solid, surface pale reddish brown with small darker brown squammules below. Veil poorly developed, cortinoid forming a ring zone on the stipe. Hyphae had a slightly thickened wall , branching with clamp connections at the constricted septa. Spore print was pure white. Spores were ovoid to oblong ellipsoid, hyaline, inamyloid, non-dextrinoid, thin walled and smooth with size of 5.0 - 6.5 µm x 3.0 - 3.7 µm. Basidia was 17- 23 x 4-5 µm, narrowly clavate bearing four sterigmata.

Other *Lentinula* sp. include *Lentinula lateritia, Lentinula novaezelandiae, Lentinula boryana* and *Lentinula guarapiensis*.

### *2.2.2. Studies on macroscopic and microscopic characters of native isolates of Lentinus sp.*

Genus *Lentinus* was characterized by xeromorphic tough carpophores having gills with serrated margins (Pegler 1977). Singer (1986) described *Lentinus* sp. with centrally to eccentrically stipitate basidiocarps, tough and coriaceous, dimitic hyphal construction with either skeletal or binding hyphae along with generative hyphae belonging to the Order Polyporales and Family Polyporaceae. Some of the commonly found *Lentinus* sp. include *L. tuber-regium, L. squarrosulus, L. connatus, L. sajor-caju, L. giganteus, L. polychrous, L. tigrinus* etc.

*Lentinus tuber-regium* had a robust pileus of 6-12 cm dia., hard, rigid, deeply depressed, infundibuliform, surface pale yellow to yellowish white and glabrous. Lamellae were deeply decurrent and crowded. Stipe was central, tapering downwards and cylindric in shape. Spores were oblong cylindric , hyaline with size of 5.74-9.72 µm x 3.21 - 4.58 µm (Manjunathan *et al.*, 2011). Kumar and Kaviyarasan (2012) described *Lentinus tuber-regium* as mushroom having robust basidiocarp with pileus of 3-15 cm dia., tough leathery to rigid infundibulliform to deeply depressed surface ochraceous cream color, smooth at centre, velvety due to mass of loosely arranged hyphae, glabrous and fleshy. Lamellae were deeply decurrent, pale ochraceous, cream color and crowded. Stipe was central to eccentric, 2.5-5.0 cm ×1.7-2.5 cm, tapering downwards, solid, hard, woody velvety and pale ochraceous yellow . Basidia were 20.72 -32.18 µm × 4.36 -6.54 µm, narrowly clavate bearing four sterigmata on which hyaline, cylindric thin walled spores of 6.54 - 8.72 µm × 3.27 -3.49 µm were present.

Leon *et al.* (2013) described *Lentinus squarrosulus* as pileus having convex to funnel shaped with wavy margin. Gills were decurrent, crowded with ragged edges. Stipe was central to eccentric with tapering ends with hairs. Pileus was thin, tough, fibrous and white. Spores were narrowly cylindric nonamyloid with size of 6-9.5 µm x 2.5 -3.5 µm.

*Lentinus connatus* was originally described by Berkeley in 1842 from Phillipines which have white pileus when fresh, yellowish white when dried, depressed in the centre to finally obliquely infundibuliform to fang shaped with eccentric stipe. Spores were 5.63-80 µm x 1.60-2.40 µm in size (Lakshmanan *et al.*, 1997).

Thus morphologically, *Lentinula* differs from *Lentinus* with hyphal composition and gill form. *Lentinula* belongs to agaric family Tricholomataceae with monomitic, thick walled hyphae with constricted septa and non decurrent gills whereas *Lentinus* belong to Polyporaceae family with dimitic hyphae with decurrent gills.

***Taxonomy and naming of Lentinula edodes***

The fungus was first described scientifically as *Agaricus edodes* by Miles Joseph Berkeley in 1877. It was placed in the genus *Lentinula* by David Pegler in 1976. The specific epithet *edodes* is derived from the Latin word "edible". The fungus has acquired an extensive synonymy in its taxonomic history.

- *Agaricus edodes* Berk. (1878)
- *Armillaria edodes* (Berk.) Sacc. (1887)
- *Mastoleucomyces edodes* (Berk.) Kuntze (1891)
- *Cortinellus edodes* (Berk.) S.Ito & S.Imai (1938)

- *Lentinus edodes* (Berk.) Singer (1941)
- *Collybia Shiitake* J.Schröt. (1886)
- *Lepiota Shiitake* (J.Schröt.) Nobuj. Tanaka (1889)
- *Cortinellus Shiitake* (J.Schröt.) Henn. (1899)
- *Tricholoma Shiitake* (J.Schröt.) Lloyd (1918)
- *Lentinus Shiitake* (J.Schröt.) Singer (1936)
- *Lentinus tonkinensis* Pat. (1890)
- *Lentinus mellianus* Lohwag (1918)

Shiitake is known by several other names like black forest mushroom, the fragrant mushroom, the flower mushroom, the winter mushroom or the variegated mushroom (Chen, 2001). Based on macro and micro-morphological characteristics as well as other features including DNA analysis, the taxonomic position of *Lentinula edodes* is as follows:-

| | |
|---|---|
| Subphylum | - Basidiomycotina |
| Order | - Agaricales |
| Family | - Tricholomataceae |
| Genus | - *Lentinula* |
| Species | - *edodes* |

## 2.3. MOLECULAR CHARACTERISATION OF PROCURED STRAINS OF LENTINULA EDODES AND LOCAL LENTINUS SP.

### *2.3.1. Lentinula sp.*

Zhang and Molina (1995) reported that Randomly Amplified Polymorphic DNA assay can be used to differentiate strains of *Lentinula edodes* and have potential application in mushroom breeding and strain improvement programmes. Cristina *et al.* (2001) studied the phylogenetic analysis of thirty four *Lentinula edodes* strains

using twenty OPA primers. Among these OPA 01 to OPA 05, OPA 07 to OPA 14, OPA 17 to OPA 20 presented good polymorphism whereas OPA 6, OPA 15 and OPA 16 primers not at all amplified *L.edodes* DNA in RAPD amplification.

Matsumoto *et al.* (2003) suggested that AFLP markers can be used for strain typing of Japanese Shiitake cultures and for estimation of genetic relationships among fifty strains. The cluster analysis revealed that sixteen strains, produced fruiting bodies in middle to low temperature (5-20 °C) and are used for wood log cultivation while that of twenty eight strains produced fruiting bodies during middle to high temperature (10-25 °C) which can be used for wood log and sawdust cultivation.

Molecular markers of r DNA sequencing , RAPD, RFLP and mitochondrial genotypes have been used to discriminate mushroom species of *Volvariella* (Chiu *et al.*, 1995 ) , *Lentinula* (Chiu *et al.*, 1996), *Ganoderma* (Hseu *et al.*, 1996), *Auricularia* (Pei Sheng *et al.*, 1999), *Agaricus* ( Barroso *et al*,. 2000), *Stropharia rugoso-annulata* (Pei Sheng *et al.*, 2003). Genetic diversity of mushrooms has been determined using molecular markers like RAPD (Ravash *et al.*, 2009; Stajic *et al.*, 2005; Staniaszek *et al.*, 2002).

Ivanova *et al.* (2006) modified enzymatic extraction of vertebrate animal tissue. Since mushrooms are filamentous fungi, these methods will enable rapid processing of most fungi using DNA.

Bryn *et al.* (2010) described two DNA extraction protocols of which first method combines DNA absorbing filter paper with a commercial DNA extraction kit. Second method developed by Kwan *et al.* (2011) suggested that understanding the genomics and functional genomics of *Lentinula edodes* is very essential to improve its cultivation and quality. Genome of *Lentinula edodes* monokaryon L54 A using Roche 454 and ABI SOLID was sequenced. For functional genomics molecular

techniques like RNA primed –PCR, SAGE, Long SAGE, EST sequencing and cDNA microarray were used to analyse genes differentially expressed during development.

Zhang *et al.* (2012) differentiated twenty five cultivable strains of *Lentinula edodes* mushroom using thirty five sequence repeat (SSR) molecular markers based on whole genome. Agarwal *et al.* (2013) studied phylogenetic relationship among eleven edible mushrooms using RAPD markers. Out of ten primers used for DNA fingerprinting OPS 5, OPT 5, OPW 2 and OPZ 10 produced clear banding patterns. OPZ 10 gave the best banding pattern hence it can be used for interpretation of the phylogenetic relationship.

Phylogenetic analysis of seven samples of mushrooms *viz.*, King Oyster, Portobello, White beach, Shimeji, Chinese Shimeji, Button mushroom and Oyster mushroom were done by RAPD techniques to know their genetic diversity. Results showed that White beach, Shimeji and Chinese Shimeji showed 100 per cent similarity with each other thus confirming the suitability of RAPD markers for discrimination of mushroom samples (Prasad and Rekha, 2013).

Shivani *et al.* (2013) conducted a study on molecular characterization of seven strains of *Lentinula edodes* using RAPD, ITS sequencing, SDS-PAGE and isozyme analysis. Out of twenty primers used for RAPD analysis, fifteen primers gave distinct amplification products. Maximum similarity coefficient was obtained between Le C and Le I strains. ITS sequences indicated that Le C strain was highly divergent from all the other strains in ITS-1, 5.8 S and ITS-2 region while Le I strain showed high degree of divergence in ITS-1 region only.

Prasad and Agarwal (2013) utilized molecular markers like ISSR and SSR for exploring traits from wild isolates and to expand genetic base of cultivated mushrooms. Eleven commercial mushroom samples including *Lentinula edodes* were used for the study. Among IISR markers tested, only ISSR 4 and ISSR 6 produced

good distinguishable bands. Out of total three SSR markers used for study, only SSR 3 amplified and produced maximum number of bands for all the eleven samples.

Sharma *et al.* (2013) studied molecular characterization by ITS sequencing of six high temperature strains of *L. edodes viz.*, OE-16, OE-22, OE-23, OE-28, OE-38 and OE-388 to distinguish similarity between strains and the results revealed that OE-22, OE-28 and OE-388 were closely related to each other and fall in one group and OE-23 in another group. Strain OE-38 showed 92 per cent similarity to both the groups.

Sharma *et al.* (2014) studied the molecular phylogeny of seven strains of *Lentinula edodes* (Le C, Le I, Le S, OE- 38, OE- 142, OE- 329 and OE- 388) based on RAPD and their ITS regions. Size of polymorphic bands ranged from 100-1000 bp and size of ITS 1-2 and ITS 1- 4 regions varied among the strain from 278 and 575 bp and from 410 to 616 bp respectively. There was high degree of divergence of Le C and Le I strains. Le I , Le C , OE- 38 and Le S were more closer to one another as compared to the other strains.

### *2.3.2. Lentinus sp.*

Molina *et al.* (1992) reported that when restriction polymorphisms in two regions of the ribosomal DNA (rDNA) repeat unit were examined in various strains of *Lentinus, Neolentinus, Pleurotus* and the Shiitake mushroom *Lentinula edodes*. All strains of *Lentinula edodes* consistently exhibited identical restriction profiles that were distinct from the genera *Lentinus, Neolentinus*, and *Pleurotus*. Thus Ribosomal DNA restriction polymorphisms confirms in placement of the strains in separate taxa.

## 2.4. CULTURAL STUDIES OF LENTINULA EDODES

**Isolation and purification**

Culture media YMMBSA (yeast extract, malt extract, multigrain oatmeal, brown sugar, agar), YVMBSA(yeast extract, V-8 vegetable juice, multigrain oatmeal, brown sugar, agar), and YVMSA (yeast extract, V-8 vegetable juice, multigrain oatmeal, sucrose, agar) and broths YVMBS (yeast extract, V-8 vegetable juice, multigrain oatmeal, brown sugar),YVMS (yeast extract, V-8 vegetable juice, sucrose), and MVBS (multigrain oatmeal V-8 vegetable juice brown sugar) were formulated and demonstrated to be excellent media and broths for isolation and growing Shiitake mushrooms (*Lentinula edodes* (Berk.) Pegler) in the laboratory (Pacumbaba and Pacumbaba, Jr., 2010). For isolation of *Lentinus edodes* (Berk.) Sing., inner tissue bits from the basidiocarp, after sterilization with 10 per cent sodium hypochloride recorded maximum tissue growth with least microbial contamination (0.79 per cent) on potato dextrose agar medium (Ramkumar *et al.*, 2011).

### *2.4.1. Effect of different media on the mycelial growth of Lentinula edodes*

Jodon and Royse (1976) recorded maximum mycelial growth of *L. edodes* in potato dextrose agar medium. Furlan *et al.* (1997) reported that mycelial growth rates of *Lentinula edodes* were higher in wheat dextrose agar (WDA) medium than in potato dextrose agar / malt peptone agar. Cultural characterization of *Lentinus edodes* in various solid and liquid media revealed wood extract agar as the best solid medium followed by potato dextrose agar. Glucose asparagine solution proved to be the best liquid medium (Kaur and Lakhanpal, 1999) for the biomass production of *L. edodes*.

Sharma *et al.* (2006) observed good mycelial growth of Malaysian strain of Shiitake mushroom in Brown's medium and Czapek Dox liquid medium. Studies

conducted by Kannan and Eswaran (2010 a), concluded that among the five culture media *viz*., Czapek Dox agar, malt extract agar, yeast extract agar, potato dextrose agar and oat meal agar used to determine the suitable medium for the cultivation of *Lentinula edodes*, maximum mycelial growth was recorded in oat meal agar (90 mm in 9 days) followed by potato dextrose agar medium (90 mm in 11 days).

Pacumbaba and Pacumbaba Jr. (2010) reported that when Shiitake culture was grown in various culture media like YMMBSA (Yeast extract, malt grain, oat meal, brown sugar, agar), YVMBSA (Yeast extract, V-8 vegetable juice, multigrain oat meal, brown sugar, agar), YVMSA (Yeast extract, V-8 vegetable juice, multigrain oat meal, brown sugar) and broths YVMBS, YVMS, MVBS were excellent for growing Shiitake mushroom.

Petre *et al.* (2011) reported that Shiitake mushrooms can be grown on special culture media using liquid nutritive broth whose composition was 15 g cellulose powder, 5 g wheat bran, 3 g malt extract, 0.5 g yeast extract, 0.5 peptone and 0.3 g powder of natural argillaceous materials.

Ramkumar *et al.* (2011) observed that potato dextrose agar medium encouraged the radial mycelial growth of *Lentinula edodes* which covered the plate within 8.6 days. Highest mycelial biomass yields of three strains of *Lentinula edodes* mushrooms were obtained in a medium containing 70 g/ l glucose and 4 g/ l yeast extract (Medany, 2011).

Among the different culture media used namely malt extract agar, potato dextrose agar, yeast extract agar, water agar, teak sawdust agar (teak sawdust extract in PDA) and poplar sawdust agar (poplar sawdust extract in PDA) to evaluate the vegetative growth of *L. edodes*, highest growth rate (8.7 mm/ day) was observed in potato dextrose agar whereas least growth was observed in yeast extract agar medium (1.04 mm/ day) (Puri, 2012).

Out of thirteen culture media evaluated for the vegetative growth of *Lentinula edodes* (Lata and Sharma, 2012), malt extract peptone dextroase agar medium supported maximum radial growth followed by potato dextrose agar. In the same experiment among seventeen broth cultures tested, maximum mycelial dry weight was recorded in Asthana and Hawker's media, followed by malt extract peptone dextrose broth and Walksman's broth.

Petre and Petre (2013) tested *Lentinula edodes* and *Pleurotus ostreatus* with culture media prepared from different fruit wastes like juice and pulps obtained after industrial processing of apples, pears and plums. To study the effect of media formulation on dry mycelial yield of *Lentinula edodes*, potato dextrose broth (PDB) and PDB++ (PDB, yeast extract, malt extract, peptone, $KH_2PO_4$ and $MgSO_4.7H_2O$) media were used and an optimum growth of mycelia was observed in PDB++ medium (Aminuddin *et al.*, 2013).

Quaicoe *et al.* (2014) studied the influence of natural media on growth of three strains of *Lentinula edodes* (Quagu, Le P and Le 75). Out of various media like rice meal agar, wheat meal agar, millet meal agar, sorghum meal agar and potato dextrose agar, the best mycelial growth of the fungus was recorded in sorghum meal agar.

Among different solid and liquid media tested, yeast extract agar medium and glucose peptone medium supported best vegetative growth of *L. connatus* (Atri *et al.*, 2011). Sharma and Atri (2013) studied the cultural behaviour of five wild *Lentinus* species namely *Lentinus sajorcaju, L. torulosus, L. cladopus* and *L. squarrosulus* culture on potato dextrose agar. *L. squarrosulus* (1.8 cm/ day) recorded fastest average growth followed by *L. cladopus* (1.5 cm/ day), *L. sajorcaju* (1.3 cm/ day) and *L. connatus* (0.8 cm/ day).

## 2.5. NUTRITIONAL REQUIREMENTS OF LENTINULA EDODES

Shiitake is a saprophytic white rot fungus, which produces mycelia during its vegetative growing phase. These mycelia absorb nutrients and by its enzyme production, the lignocellulosic substances are decomposed which serve as major carbon source for the mushroom.

**2.5.1. Carbon**: Carbon is the most important required nutrient for Shiitake. It is the major component for the energy source which is provided by organic compounds like sugars, organic acids, starch, cellulose hemicellulose and lignin (Chen, 2004).

### *2.5.1.1. Effect of different carbon sources on the mycelial growth of Lentinula edodes*

Song *et al.* (1987) suggested that glucose was the best carbon source for submerged mycelial cultures of *Lentinula edodes*. Kaur and Lakhanpal (1995) studied the effect of nutrient, element sources, vitamins and growth regulator on vegetative growth of *Lentinus edodes*. This study revealed maximum mycelial growth of the mushroom in glucose followed by fructose and sucrose while minimum mycelial growth was recorded in starch.

According to Khan *et al.* (1995) starch was the best source of carbon for supporting mycelial growth of *Lentinula edodes*. Jung *et al.* (2001) reported that among eight carbon sources like D-fructose, galactose, glucose, sucrose, maltose, starch, mannose and cellulose tested for the mycelial growth of *Lentinula edodes*, sucrose showed the best growth (1.291 g dry weight) followed by D-galactose (1.276 g dry weight) and D- fructose (1.248 g dry weight).

Meng *et al.* (2007) reported that when four carbon sources *viz.*, glucose, maize flour, sorghum flour and sweet potato starch were used for liquid fermentation of

*Lentinula edodes*, sweet potato starch proved to be the best carbon source. Peter and Teodorescu (2008) suggested that among various carbon sources like glucose, maltose, sucrose and xylose used, maltose proved to be the best source in increasing the mycelial growth and fungal biomass synthesis for *Lentinula edodes.* Maltose proved to be the best carbon source showing the highest influence on mycelial and fresh fungal biomass production of *L. edodes* @ 28-35 gper cent (Atri *et al.*, 2011).

Marquez *et al.* (2014) suggested that when four media *viz.*, GYEA media containing glucose, yeast, $KH_2PO_4$, $MgSO_4$, $K_2HPO_4$, $CuSO_4$, $FeSO_4$, $MnSO_4$, $ZnSO_4$ and agar , GYEA + 750 mg of DEHP/L (Di-2 ethylhexyl) phthalate , GYEA + 1200 mg DEHP/L and GYEA + 1500 mg DEHP/L were prepared and inoculated with *Lentinula edodes* and *Pleurotus ostreatus*, there was no catabolic repression (glucose effect), which reveals that DEHP can be used as carbon and energy source for both the mushrooms.

**2.5.2. Nitrogen:** Nitrogen constitutes protoplasm and cell structural elements in Shiitake which was provided by various organic and inorganic nitrogen compounds. Primodia formation and subsequent fruiting body formation are both sensitive to nitrogen concentrations and ideally the nitrogen concentration during these phases should not be over 0.02 per cent (Chen, 2004).

***2.5.2.1. Effect of different nitrogen sources on the mycelial growth of Lentinula edodes***

Ammonium chloride proved to be the best nitrogen source for the production of submerged mycelial cultures of *Lentinula edodes* (Song *et al.*, 1987). Khan *et al.* (1995) reported that best source of nitrogen for growth of *Lentinula edodes* were urea and asparagine. Peptone @ 0.3 per cent increased the mycelial growth of *Lentinula edodes* (Kaur and Lakhanpal, 1995; Balazs *et al.*, 1998).

Seven nitrogen sources *viz.*, calcium nitrate, potassium nitrate, $NH_4NO_3$, $NaNO_3$, tryptone, yeast extract and peptone were tested for best mycelial growth of *L. edodes*. Tryptone proved to be the best (1.109 g dry weight) followed by Ca $(NO_3)_2$ (1.092 g dry weight), $KNO_3$ (1.014 g), yeast extract (0.981 g dry weight) and peptone (0.81 g weight) (Jung *et al.*, 2001).

Out of various nitrogen sources studied, rice bran and malt extract were the most efficient for mycelial growth and fungal biomass production of *Lentinula edodes* (Petre *et al.*, 2004).

Meng *et al.* (2007) suggested that among four nitrogen sources like soyabean flour, wheat bran, peptone and yeast powder used for liquid fermentation of *Lentinula edodes,* wheat bran proved to be the best source. Maximum mycelial growth of *Lentinula edodes* was obtained when yeast extract was added as nitrogen source (Mahmud and Ohmesa, 2008).

Wheat bran was the most efficient on mycelial growing and fungal biomass production of *Lentinula edodes* (35-40 g) when nitrogen sources like wheat bran, peptone, tryptone , malt extract and yeast extract were examined (Atri *et al.*, 2011).

## 2.6. PHYSIOLOGICAL REQUIREMENTS OF LENTINULA EDODES

**2.6.1 Temperature**: Shiitake is a temperate fungus, which requires low temperature and temperature fluctuations for the production of fruiting bodies. When temperature is too low, the nutrient absorption, enzymatic activity and respiration rate will be adversely affected. At high temperature, protein molecules in the enzyme will be denatured and the mycelial viability will be lost. The optimal temperature for Shiitake mycelial growth is 24-27 $^{o}$C (Chen, 2004).

### *2.6.1.1. Effect of different temperature on the mycelial growth of Lentinula edodes*

Song *et al.* (1987) opined that optimum temperature for the mycelial growth of *Lentinula edodes* was 20-25 $^{O}$C. Khan *et al.* (1995) reported that maximum growth of *Lentinula edodes* was recorded at 25 $^{O}$C and the growth was reduced at temperature below 20 $^{O}$C and above 30 $^{O}$C. Balazs *et al.* (1996) claimed that *Lentinula edodes* grew best at a temperature of 23 $^{O}$C.

Cristina *et al.* (2001) assessed the mycelial development of *Lentinula edodes* at different temperature of 16, 25, 28 and 37 $^{O}$C and the results showed that temperatures of 25 $^{O}$C and 28 $^{O}$C suited more for *Lentinula edodes* mycelial growth. The temperature of 37 $^{O}$C was extremely unfavourable whereas 16 $^{O}$C just allowed the development of the fungal growth.

Inglet *et al.* (2006) suggested that maximum mycelial growth of edible mushroom *Lentinula edodes* was obtained at temperature range of 23.6 $^{O}$C. The culture temperature for best activity of *Lentinula edodes* laccase was 25 $^{O}$C as reported by Zhang and Hing (2006).

Lata and Sharma, 2012 reported that 20 $^{O}$C and 25 $^{O}$C proved to be the optimum temperature for the culture growth of *Lentinula edodes.* With further increase or decrease in temperature, there was corresponding decrease in the mycelial growth. Puri (2012) reported that out of the five temperatures tested for the mycelial growth of Shiitake, maximum growth rate (10 and 8.8 mm/ day) was observed at 25 $^{O}$C temperature. Decrease in growth rate was observed by decreasing the temperature below 15 $^{O}$C and increasing the temperature upto 35 $^{O}$C.

Sharma *et al.* (2013) reported that maximum mycelial growth of *Lentinula edodes* strains was at 20 $^{O}$C. Quaicoe *et al.* (2014) investigated on the ability of *Lentinula edodes* to grow at temperature ranging from 16 $^{O}$C to 30 $^{O}$C, and observed that 25 $^{O}$C proved to be the optimal temperature.

**2.6.2. Light:** Light acts as direct and indirect source of energy for Shiitake. It is required for the basidiospore formation and dispersal. Its mycelia grow better in darkness than under direct light (Chen, 2004).

### *2.6.2.1. Effect of different light conditions for the mycelial growth of Lentinula edodes*

Munjal *et al.* (1975) reported that the light requirement for mushroom is not photoperiodic. San Antonio (1981) suggested that for the cultivation of *Lentinus edodes* , cool nights followed by warm days are essential for fruit body formation. For the cultivation of *Lentinus edodes*, light was essential for brown-pigment formation of the mycelia coat and for fruiting-body maturation, but was not required for formation of primordia (Miles and Chang, 1987). Medda *et al.* (2011) reported that diffused light and alternate dark and light conditions showed good result in terms of average yield and biological efficiency of *L. edodes*.

**2.6.3. pH :** Extracellular enzymes function at a specific pH in degrading the substrate and the substrate pH is thus of great importance in Shiitake cultivation. Though the mushroom can grow at a wide range of pH 3-7, they prefer an acidic environment for primordia and fruiting body formation (Chen, 2004).

### *2.6.3.1. Effect of different pH for the mycelial growth of Lentinula edodes*

According to Song *et al.* (1987) pH range of 4.30 to 4.80 was the best for the optimum growth of *Lentinula edodes*. The pH value of five was found most favorable for the growth of *Lentinula edodes* as reported by (Chen, 1988 and Khan *et al.* 1991). *L. edodes* fungus passed from the first development phase (incubation phase) to the second (harvesting phase) at a stabilized pH of 3.30.

Khan *et al.* (1995) claimed pH five as most suitable for the mycelial growth of *Lentinus edodes*. Furlan *et al.* (1997) reported that *Lentinula edodes* maintained high growth rates at a low pH of 4.00. In the invitro studies conducted by Balazs *et al.* (1998), *Lentinula edodes* grew best at pH 6.00.

Kaur and Lakhanpal (1999) observed the mycelial growth of *Lentinus edodes* at different pH levels ranging from 3.50 to 8.50 and concluded that acidic pH of 4.50 supported maximum growth of the fungus.

Singh *et al.* (2000) studied the effect of pH on different edible mushrooms like *Lentinus edodes, Agaricus bisporus, Pleurotus ostreatus, Auricularia polytricha, Morchella esculenta etc.* and found that majority of these mushrooms grew well at a pH of 6.00.

Cristina *et al.* (2001) reported that when mycelial dry weight of most contrasting *Lentinula edodes* strains cultivated in PDA medium was taken at different pH 's of 5, 6, 7 at 25 $^{O}$C for 11 days, the most suitable pH was reported to be 6.00.

The best activity of laccase in *Lentinula edodes* was obtained when pH value was 3.50 (Zhang Yu and Hing Feng, 2006). Maximum mycelial growth rate of *Lentinula edodes* was obtained at pH 4 to 6 as reported by Inglet *et al.* (2006).

Petre and Teodorescu (2008) found 5.00- 5.50 as optimal pH levels for fungal fruit body production of *Lentinula edodes.* The highest mycelial yields of tested strains of *Lentinula edodes* LC 0032, LC 236 and LC 2161 was obtained in pH value of 6.50 (Medany , 2011). Markovic *et al.* (2011) reported that highest weight of mycelial drymass of *Lentinula edodes* was obtained between pH of 3.00 and 3.63.

Lata and Sharma (2012) reported 5.00- 7.50 as the most suitable pH for the growth of various strains of *Lentinula edodes.* Sharma *et al.* (2013) conducted study to optimize pH for six high temperature strains of *Lentinula edodes* like OE-16, OE-22, OE -23, OE-28, OE-38 and OE-388 and the results showed that maximum

mycelial growth was obtained at a pH of 7.00. The optimal pH for mycelial growth of *L. edodes* in potato dextrose broth was pH 6.00 ( Aminuddin *et al.*, 2013).

## 2.7. SPAWN PRODUCTION TECHNOLOGY OF LENTINULA EDODES

Spawn is the vegetative mycelium from a selected mushroom grown on a convenient medium (Klingman, 1950). Shiitake is a saprophytic white rot fungus which feeds on dead hardwood trees in nature. Each basidium under the gill of fruiting bodies produces four basidiospores which sporulate under a certain condition to become a primary mycelium and then a secondary mycelium. Shiitake spawn is a medium colonized by the secondary mycelia. The secondary mycelia will form fruiting bodies eventually.

### *2.7.1. Substrates*

#### *2.7.1.1 Grain substrates*

Song *et al.* (1987) described that number of days to the appearance of first flush of sporophores of *Lentinula edodes* was reduced by 45 days by using liquid spawn instead of cotton seed hull spawn. Schunemann (1988) reported grain spawn @ 5 per cent by weight of substrate can be used for the cultivation of *Lentinula edodes*.

Kirchhoff and Lelley (1991) compared liquid spawn with grain spawn for the inoculation of substrate blocks by top spawning and injection of liquid spawn. Results showed that injected liquid spawn gave higher fruit body yield up to 112 per cent biological efficiency than surface applied grain spawn.

Hiromoto (1991) reported that when sawdust was substituted with a mixture of cereal grain and supplementary nutrients both as substrate for spawn and cultivation of Shiitake mushroom, incubation time was reduced to 3-4 weeks for substrate colonization and a biological efficiency of 325 per cent was obtained.

Kawai *et al.* (1996) compared liquid culture for the production of liquid spawn for cultivating Shiitake on synthetic sawdust substrate with solid spawn. Results showed that initial colonization was faster with solid spawn than with liquid spawn but later carbon dioxide production in liquid spawn was higher and so incubation period for production of fruiting bodies was reduced to 90 days than compared with 120 days of solid spawn.

Terashita *et al.* (1997) reported that storage of *L. edodes* spawn for longer periods (160-200 days) at temperature of 4 $^{O}$C or 15 $^{O}$C decreased the formation of normal fruit bodies. Proteinase activity in mycelia of stored spawn did not differ but amylase β- 1,3 glucanase and trehalase activity decreased upon storage of spawn. According to Stamets (2000 a) rye grains proved to be most suitable, among various cereal grains used for spawn production.

Stamets (2000 b) and Chen (2002) reported that sorghum emerged as an ideal material under Indian conditions for the production of Shiitake spawn. For spawn production of *L. edodes*, ill filled paddy + calcium carbonate (20 g/ kg) significantly enhanced the mycelial growth and basidiocarp formation, followed by silver oak sawdust (Kalaiselvam, 2007 and Ramkumar *et al.*, 2011).

Seven different spawning substrates *viz.*, wheat grain, sorghum grain , paddy grain, sawdust alone, saw dust and paddy straw, saw dust + paddy straw + wheat straw + cotton waste were evaluated for the mycelial development of Shiitake mushroom. Among these wheat grains, sawdust + paddy straw and sorghum grain substrates recorded complete mycelial colonization at 30 days after inoculation (Dewangan *et al.*, 2007).

Bruhn *et al.* (2009) reported that inoculation of Shiitake with sawdust spawn gave significantly higher yield than colonized wooden dowels / pre packaged thimble plug inoculum. According to Ramkumar *et al.* (2010), addition of calcium carbonate enhanced mycelial growth of spawn and basidiocarp formation of *Lentinula edodes*.

Wang *et al.* (2010) used spent bleaching clay (sbc) from oil refining as a substrate for spawn production of Shiitake mushroom. Results showed that the formula with SBC had lower incidence of microbial contamination and had higher mycelial growth speed. The optimal formula was 15 per cent SBC, 78 per cent coarse sawdust, 5 per cent wheat bran, 1 per cent magnesium sulphate and 1 per cent gypsum.

Kannan and Eswaran (2010 b) reported that among various grain substrates like black gram, green gram, ill filled paddy, ragi, samai grain, varagu, wheat and sorghum grains tested for their efficiency in supporting the mycelial growth of *Lentinula edodes*, paddy grains recorded minimum number of spawn run days (11.30), followed by ill filled paddy (11.50) and par boiled wheat (12.10).

Puri (2011) conducted a study using locally available poplar and teak sawdust, sorghum and wheat grains for Shiitake spawn production. Fastest mycelial colonization was obtained in sorghum grains which yielded maximum biological efficiency of 6 to 8 per cent proving sorghum as ideal substrate for Shiitake spawn production.

Liu *et al.* (2012) suggested that liquid nitrogen preservation was the best preservation method of *Lentinula edodes* spawn where the growth rate and enzyme activities (laccase, CMC, xylanase, alpha amylase) were highest. Qiu *et al.* (2012) studied on how *Campotheca acuminate* Decne residue (CA residue) influence the mycelial growth of spawn production in four species of mushrooms like *Pleurotus ostreatus*, *Flammulina, Auricularia* and *Lentinula edodes*. Mycelia of all the mushroom strains grew faster on the substrates containing CA residue than on the substrate with no CA residue and on cotton seed hull based combination of cotton seed hull, 10 per cent sawdust, 15 per cent wheat bran , 1 per cent lime and 1 per cent white sugar.

### *2.7.1.2. Sawdust substrates*

Khan *et al.* (1991) reported that when three types of sawdust from shishum (*Dalbergia sissoo*), kikar (*Acacia arabica*) and poplar (*Populus alba*) amended with wheat bran and lime were used for the Shiitake spawn production, sawdust from *Dalbergia sissoo* proved to be the most suitable substrate. Also all sawdusts amended with cotton waste were found to give optimum results for spawn running. Sawdust spawn medium when supplemented with rice bran which provides three kinds of nutrients (starch, nitrogen and minerals) supported fast growth and prevented contamination by other microorganisms (Lee, 1991).

Fomina *et al.* (1999) demonstrated that oak sawdust (1: 1) with 20 per cent rye bran was the favorable substrate for the spawn mycelium production of *Lentinula edodes*. Combination of sawdust (80 per cent), rice bran (10 per cent) and wheat bran (10 per cent) supported good spawn run of Shiitake, resulting in cheap and best spawn base in term of spawn production (Thirubhuvanamala *et al.*, 2005).

Shiitake mycelium grew well in substrates composed of sawdusts of maple, oak, maple + oak, maple + vermiculite and oak + vermiculite. When these substrates were amended with broths of YVMBS, YVMS/ MVBS, spawn attained growth in 25 to 30 days after inoculation (Pacumbaba and Pacumbaba Jr., 2010).

## 2.8. CULTIVATION TECHNOLOGY OF LENTINULA EDODES

*Lentinula edodes* was traditionally cultivated on hardwood logs where plug spawn or sawdust spawn were introduced into the holes drilled on the surface of hardwood. This method takes a very long time for mushroom production and yields are unpredictable. Therefore its cultivation has been now shifted to synthetic logs where cultivation is mainly done on sawdust of hardwood or broad leaved trees (Royse, 2002). However other agro wastes like straw, corncobs, coffee pulp, coconut

husks, leaves etc. were also been used as cultivation substrates of Shiitake whose review are mentioned below in detail.

### 2.8.1. Evaluation of substrates

#### *2.8.1.1. Lentinula sp.*

Schmidt and Kebernik (1986) reported that *Lentinula edodes* can be inoculated on waste wood of deciduous and coniferous trees like pines and resinous spruces.

Mushrooms like *Flammulina velutipes, Lentinula edodes, Pleurotus ostreatus, Stropharia cubensis* and *Volvariella bombycina* can be successfully cultivated on coffee grounds, which is a waste material produced during the manufacture of coffee powder ( Theilke, 1989).

Wu (1989) described a method for preparing a non sterile, pasteurized, nutrient substrate for growing Shiitake mushrooms. Substrate mixture consisted of vegetable waste, a mineral fertilizer and water which is digested aerobically at a temperature of 18-30 $^{o}$C for 1-9 days. Schmidt (1990) reviewed intensive cultivation of Shiitake (*Lentinula edodes*) on different woody substrates. Artificial log cultivation of *Lentinus edodes* in semi ground room was reported by Hu (1990).

Hu and Song (1990) reported a technique of cultivating *Lentinula edodes* on raw sawdust by means of liquid spawn. Compared with conventional brick spawn cultivation, cycle time decreased by about a half and cost by 19-27 per cent. The biological efficiency (BE) and the production rate of Shiitake have been evaluated using polypropylene plastic bag containing 0.50 kg to 1.50 kg of supplemented oak sawdust (Mata *et al.*, 1990). Cultivation of Shiitake on wood logs, sawdust and wheat straw had been reported by many workers (Suman and Seth, 1982; Sohi and Upadhyay, 1988; Thakur *et al.*, 1992).

According to Delpech and Oliver (1991) Shiitake can be cultivated on wheat straw and chicken feather meal mixture. A mixture of apple pomace and sawdust of *Quercus rubra, Fraxinus americana* and *Acer saccharum* were tested as substrate for production of Shiitake and Oyster mushroom on synthetic logs (Worral and Yang, 1992). Both species produced higher yields on a mixture of equal parts of apple pomace and sawdust than on sawdust alone.

Balazs and Kovacs (1993) proved straw with additives in different proportions as the best substrate for cultivation of *Lentinula edodes*. Here fruiting bodies appeared 60-70 days after spawning and a yield of 20-25 mushroom/ 100 kg wet substrate were obtained. Levanon *et al.* (1993) demonstrated that a mixture of bulk pasteurized wheat straw and cotton straw can be used for the cultivation of Shiitake .

*Lentinula edodes* can be grown on coffee pulp with a biological efficiency of 64.30 per cent (Mata and Hernandez, 1994). Wonchull *et al.* (1996) described breeding of *Lentinula edodes* strains for sawdust based cultivation and effect of various methods to stimulate primordia formation. First flush was stimulated by a cold water (12 $^{o}$C) spray, followed by dipping for 24 h for second and third flushes which yielded about 283 g whereas continuous spray method yielded 245 g.

Ikegaya (1997) reported sawdust as a medium for indoor cultivation of Shiitake. Kawai *et al.* (1997) revealed that synthetic saw dust logs can be used for the production of Shiitake (*Lentinus edodes*).

According to Mata and Savoie (1998), *Lentinula edodes* can be commercially cultivated on wheat straw due to the degradation of soluble phenolic compounds present in it. An experiment was conducted using smoke heated sugi (*Cryptomeria japonica*) and karamatsu (*Larix leptolepis*) sawdust for Shiitake (*Lentinula edodes*) and Hiratake (*Pleurotus ostreatus*) cultivation. Best results were obtained with 3 : 1 and 1 : 1 beech/ softwood and smoke treated sugi sawdust than with nonsmoker treated one's (Yoshizawa *et al.*, 1998).

Ohja (1998) studied the effect of different genotypes on growth and fructification of *Lentinula edodes*. Wide range genotypes produced higher yields while cold weather genotype fruited with high quality mushrooms. Mycelial growth of two strains of *Lentinula edodes* (IE- 40 and IE-105) were evaluated on barley straw, corn cob, corn stover, rice bran, bracts of pineapple crown, coffee husk, sugarcane bagasse and sugarcane leaves. Results showed that both the strains produced highest biological efficiency (130-133 per cent) in sugarcane bagasse followed by 83-98 per cent in sugarcane leaves and 36-37 per cent in bracts of pineapple crown. There was no primordial development on coffee husk substrate (Salmones *et al.*, 1999).

Kovascsne and Kovacs (2000) and Zervakis *et al.* (2001) reported wheat straw as the most suitable substrate for the production of *Lentinus edodes*. Supplementation of sawdust-corn flour substrate with urea or ammonium chloride increased the yield of *Lentinula edodes* (Kalberer, 2000). Urea when added to the substrate caused the failure of primordial development and leads to fruiting body deformation.

In the studies conducted with various *Lentinula* substrates *viz.,* red oak sawdust, composted spruce bark, composted spent mushroom substrate and wheat straw supplemented with varying proportions of wheat grain, wheat bran and limestone, highest yield were obtained in oak and spruce bark combinations whereas straw based substrates showed a negative response with grain supplementation (Kilpatrick *et al.,* 2000). Jung *et al.* (2001) developed a cheap substrate of SFW (sikhae factory waste water) for use of liquid spawn of *L. edodes* , that yielded more fruiting body than grain spawn in sawdust cultivation.

Royse and Sanchez (2001) studied the influence of substrate wood chip particle sizes *viz.*, 2.80 to 4.00 mm, 1.70 to 2.80 mm, 0.85 to 1.70 mm and < 0.85 mm on the biological efficiency of Shiitake mushroom. 93.70 per cent of biological efficiency was obtained in substrate combination of 45 per cent sawdust, 30 per cent millet, 15

per cent wheat bran and 10 per cent rye where particle size ranged between 2.80 mm to 4.00 mm. Grodzinskaya *et al.* (2002) reported that mixture of rice straw and husk with maize cobs, sugarcane bagasse, coconut fibre and coffee wastes can be used as suitable substrates for the fruiting body formation of *Lentinula edodes*.

According to Mata and Salomones (2003) *Lentinula edodes* can be cultivated on pasteurized non-conventional substrates like coffee pulp, sugarcane and wheat straw.

Royse and Sanchez (2003) studied the effect of three calcium carbonate levels on *Lentinula edodes* production and the results revealed that yield and size of mushroom were highest from substrates containing 0.40 per cent and 0.60 per cent calcium carbonate. Comparatively less biological efficiency of 62.30 per cent was obtained from substrates non supplemented with calcium carbonate when compared to 90.60 per cent BE on the substrate supplemented with 0.40 per cent calcium carbonate.

Cavallazzi *et al.* (2004) reported eucalyptus bark based medium as one of the best substrate for *L. edodes* cultivation. Mata and Hernandez (2004) remarked that *L. edodes* can be cultivated on wheat straw which was pasteurized by immersion in water and was heated by residual geothermal vapor. When inoculated with supplemented spawn, biological efficiency of 24.80 per cent to 55.60 per cent was obtained.

*Lentinula edodes* was grown on oak wood sawdust substrates which was supplemented with wheat straw or corn cobs inorder to examine the influence of wastes on mycelial growth and sporophore production (Philippoussis *et al.*, 2004). Higher sporophore yields were obtained on oak wood sawdust supplemented with corn cob in the ratio 1 : 1 and 1 : 2.

Silva *et al.* (2005 a) studied the growth and enzymatic activities of strains of Shiitake mushroom cultivated on eucalyptus residues constituted by ground barks, branches and leaves. Eucalyptus residue mixed with rice bran in 80 : 20 ratio proved ideal for extracellular enzyme production of fungus and also for the production of mycelial biomass.

Inglet *et al.* (2006) suggested that when whey permeate was used as an alternative growth medium for the cultivation of *L.edodes,* addition of lactose @ 40 g/ l at temperature of 23.6 °C and pH 5.00 maximized mycelial growth of mushroom @ 6.41 ± 0.47 mm/ day.

Ozcelik and Peksun (2007) observed hazelnut husk as a new basal ingredient for substrate preparation for the cultivation of Shiitake mushroom. Yield was relatively high when hazelnut husk content in the mixtures were kept below 50 per cent. Villegas (2007) reported 80 per cent wheat grain and 20 per cent eucalyptus saw dust as the best combination of substrates for growth of Shiitake.

When oak woodchips were substituted with substrate formula of 0 per cent, 8 per cent and 16 per cent ground wheat straw and 52 per cent, 44 per cent and 36 per cent oak sawdust respectively for the cultivation of *Lentinula edodes*, mushroom yields were 11 per cent higher in 8 per cent wheat straw + 44 per cent sawdust combination and 19 per cent higher in 16 per cent wheat straw + 36 per cent sawdust combination (Royse and Sanchez, 2007).

Brienzo *et al.* (2007) revealed that *Lentinula edodes* can successfully convert eucalyptus waste, vineyard pruning, barley straw and wheat straw when used as cultivation substrate.

Regular consumption of Shiitake mushroom can be good dietary supplement to fight malnutrition in poor rural population in north east India (Birkumar *et al.*, 2008). Kumar *et al.* (2008) reported the commercial cultivation of Shiitake on sawdust of

oak (*Quercus* sp.), maple birch or any other hard wood or broad leaved trees (toona, mango, rubber etc.). Lee *et al.* (2008) reported that maize processing waste can be utilized as an alternative growth medium for the cultivation of *Lentinus edodes*.

When Shiitake mushroom was cultivated on timber plants like babala, champa, ipil ipil, jack, mango, shisoo and rain tree pinhead initiation occurred significantly earlier on jack sawdust compared to other substrates. Biological efficiency, yield at first harvest and final harvest were also highest on jack substrate (Ashrafuzzaman *et al.*, 2009). No primordia was produced on paddy straw substrate; it gave no yield and took maximum time in completion of mycelium running.

Marino and Abreu (2009) used coconut sawdust supplemented with wheat bran or rice bran @ 0, 5, 20, 30, and 40 per cent as an alternative substrate for the cultivation of *Lentinula.* The growth and vigor of all strains tested, increased with supplementation. A novel improved method for sawdust based cultivation of mushroom, high temperature and water filling treatment of mycelia block was reported by (Yamauchi *et al.*, 2009).

Bruhn and Mihail (2009) revealed that significantly greater weight of *L. edodes* mushroom was produced in sugar maple logs compared with white or northern red oak due to the higher proportion of undiscoloured wood volume in maple logs rather than to bark thickness or log diameter.

Various agro wastes like oak, horn bean, sweet gum, poplar, alder, willow, pine, maple and birch sawdust, cereal straw, corn cobs, sugarcane bagasse, tea waste, sunflower seed hulls, peanut shells, coffee straw and seed hulls can be used alone or in combination with other wastes in Shiitake cultivation ( Curvetto *et al.*, 2002; Rossi *et al.*, 2003).

World wide, the most popular basal ingredient used in synthetic formulations of substrate for the commercial production of *Lentinula* mushrooms was hardwood (oak wood) sawdust supplemented @ 20-30 per cent wheat bran/ rice bran, millet *etc* (Miller and Jong, 1986., Pire *et al.*, 2001).

Three fungal species namely *Ganoderma lucidum, Lentinus edodes* and *Pleurotus ostreatus* were tested to determine their biological potential to grow on substrates of vineyard and winery wastes. *P. ostreatus* was the fastest mushroom culture which yielded in 25-30 days, then *Lentinula edodes* (35-50 days) and finally *Ganoderma lucidum* (50-60 days) (Peter and Teodorescu, 2008).

Ramkumar *et al.* (2010) reported that when ill filled paddy was amended with calcium carbonate, it enhanced the browning of mycelia (16.40 days), basidiocarp formation (51.80 days), yield (360.70 g/ kg) and biological efficiency (36.07 per cent) of *Lentinula edodes* which were better than paddy straw, sugarcane bagasse, silver oak sawdust etc.

According to Ramkumar *et al.* (2011) maximum yield of Shiitake mushroom (360 g) was obtained on silver oak sawdust + calcium carbonate combination as compared to ill filled paddy + corn flour, ill filled paddy + calcium carbonate, paddy straw + calcium carbonate, sugarcane thrash + corn flour/ calcium carbonate, sugarcane thrash, paddy straw, ill filled paddy etc.

Medda *et al.* (2011) studied the effect of different substrates like paddy straw, wheat straw, sugarcane bagasse, mustard straw, paddy hulls, water hyacinth, waste of rice mill, sesbania leaves, dry Azolla, fibreless jute stick dust and sawdust on yield performance of *Pleurotus sajor caju* and *Lentinula edodes*. Significantly higher biological efficiency of 28 per cent was recorded in paddy straw ( *Pleurotus sajor*

*caju)* and 15 per cent biological efficiency in sawdust (*Lentinula edodes)* followed by sugarcane bagasse (24 per cent and 13 per cent BE respectively) and water hyacinth with 12.50 per cent BE.

Puri *et al.* (2011) reported biological efficiency of 3.80 per cent to 24.20 per cent for all sawdust substrates than compared to other agricultural wastes (9.20 per cent to 45.90 per cent). Supplementation of wheat bran @ 10 per cent gave higher yield (80.40 g per 500 g dry substrate) than other supplements in Shiitake cultivation. Peter and Peter (2012) revealed that the winery and apple wastes could be recycled as useful raw materials for mushroom compost preparation of *Pleurotus ostreatus* and *Lentinula edodes* in order to get significant mushroom production. Yamauchi (2012) studied the possibility of sawdust based cultivation technique (FFUP) of *Lentinula edodes* using sugi wood and konare wood where mass production of Shiitake was obtained on sugi wood.

Biological efficiency and yield attributes of Shiitake mushroom studied on various substrates like paddy straw, poplar sawdust, coir pith, teak and sal sawdust alone and in combinations of 1 : 1 as substrates supplemented with wheat bran @ 10 per cent w/w , poplar sawdust and coir pith substrate gave highest yield followed by paddy straw + coir pith combination (Puri and Kumar, 2012).

Martinez-Guerrero *et al.* (2012) cultivated two genotypes of Shiitake using different supplemented formulations like oak sawdust, maize stubble, wheat straw, corn cobs, peanut husk, millet stems from *Tithonia tubaeformis*, cotton waste, wheat kernels, chopped cardboard, wheat bran , rice flour and corn flour along with gypsum, calcium carbonate, magnesium sulphate, urea and thiamine. Among these, 70 per cent sawdust + 10 percent corn cobs + 10 per cent maize stubble, 7 per cent wheat bran and 3 per cent rice meal and 60 per cent oak sawdust + 28.5 per cent corn cobs + 10 per cent maize stubble, 1.5 per cent gypsum, thiamine (100 mg/ kg)

and magnesium sulphate (20 g/ 100 kg) proved best for mycelial growth and fruiting of Shiitake strains.

Puri (2012) studied the effect of different substrates *viz*., sugarcane bagasse, paddy straw, poplar sawdust, coir pith, teak and sal sawdust on growth and fruiting induction of *Lentinula edodes*. Sawdust and paddy straw and coir pith substrates had very good mycelial growth, more number of pinheads were formed and maximum number of fruiting bodies developed with a biological efficiency of 45-52 per cent.

An effort was done to understand the different constituents of agricultural lignocellulosic residues and the impact of different substrates (wheat straw, pulses wastes, banana leaves, cotton wastes, sugarcane bagasse and corn stalks) on mushroom yield, crude protein, crude fibre and ash content of *L. edodes* . Maximum number of fruiting bodies (11 numbers) with biological efficiency of 45.90 per cent was obtained from wheat straw followed by sugarcane bagasse (9 number and 33 per cent biological efficiency) (Puri and Kumar, 2012).

Lee *et al.* (2012) studied the effect of aeration of sawdust cultivation bags on hyphal growth of *Lentinula edodes* and the results indicated that traditional cotton plugs are economically efficient but among them, 15 mm hole lids are the most efficient at maintaining hyphal growth and controlling water loss.

Liu and Zhang (2013) reported that when 52 per cent Italy poplar sawdust and 24 per cent cotton seed hulls were used as main carbon source medium, there was good mycelial growth and the production of fresh Shiitake mushroom was highest with a biological efficiency of 89.40 per cent.

Aguiar *et al.* (2013) used *Protium puncticulatum, Cariniana micrantha* and *Caryocar glabrum* sawdust supplemented with 20 per cent of grinded residues from

the barks of the fruits of *Astrocaryum aculeatum* and *Theobroma grandiflorum* for the cultivation of Shiitake mushroom. Results showed that media prepared with *Protium puncticulatum* extract supplemented with wheat bran was favorable for the fungal development confirming the potential of those residues for mushroom production.

Chen *et al.* (2013) cultivated two strains of *Lentinula edodes* (Q7 and L607) on substrate combinations of 40 per cent cotton, 40 per cent sawdust, 18 per cent wheat bran, 1 per cent gypsum and 1 per cent calcium carbonate. In each case, mycelial growth, biological efficiency, crude protein and polysaccharide content of fruiting bodies were similar to that of conventional substrate of 80 per cent sawdust, 18 per cent wheat bran, 1 per cent gypsum and 1 per cent calcium carbonate.

Wonchull *et al.* (2013) developed a new Shiitake strain named " Chunbaegko" during spring and autumn season and total amount of fruit body production during one generation was 140 kg/ m3 log. According to Wang *et al.* (2013), when sugarcane bagasse (SCB) pretreated by liquid hot water (LHW) was hydrolysed for 120 h with cellulase, enzyme hydrolysed residues of sugarcane bagasse (EHR-SCB) were obtained. This EHR- SCB when mixed with wheat bran and gypsum powder can be used as a substrate for *Lentinula edodes* cultivation.

According to Sharma *et al.* (2013), when *Lentinula edodes* strains were cultivated on wheat straw and sawdust substrate, wheat straw supplemented with 10 per cent wheat bran produced maximum biological efficiency of 66.80 per cent whereas sawdust supplemented with 5 per cent wheat bran gave significantly higher yield of 608 g/ 350 g bag with 81 per cent biological efficiency.

Sales- Campos *et al.* (2013) reported that when *Lentinus edodes* variety (LED 96/13) was evaluated in sawdusts of *Simarouba amera*, *Anacardium giganteum*, *Euterpe precatoria*, *Musa* sp AAB stems , the best performance was obtained in

substrate of *A. giganteum* (13.22 $cm^3$/ day) followed by *S. amera* (11.30 $cm^3$/ day). The most intense rates of vigor was observed in *E. precatoria* substrate supplemented with brans. There was no mycelial growth in banana stem formulation.

Koo *et al.* (2013) investigated the internal and external morphological characteristics of decomposition and browning of oak sawdust medium for ground bed cultivation of *Lentinula edodes.* The results showed that mushrooms fruited on the brown surface in 150 days and not on the blackened medium. Takaki *et al.* (2014) found that, the fruit body formation of mushrooms increased 1.3 to 2 times in total weight when a high voltage of 50-130 KV with a 100 ns pulse was used as an electrical stimulation. Likewise, in the case of *L. edodes* was improved from 160 g to 320 g when voltages of 50 or 100 KV were applied.

Gregori and Pohleveri (2014) investigated on substrate mixtures composed of different proportions of olive oil press cake (OOPC), wheat bran, crushed corn seeds and beech sawdust for the cultivation of *Ganoderma lucidum, Lentinula edodes* and *Grifola frondosa*. Highest biological efficiency of 38 per cent *Lentinula edodes* fruiting bodies was obtained on substrates composed of 0 per cent OOPC, 80 per cent beech sawdust, 2 per gypsum and 18 per cent wheat bran whereas substrate containing 80 per cent OOPC ceased fruiting body formation completely.

Mohan *et al.* (2014) reported that when sawdust of different timber plants which act as carbon sources was used for the growth of *Lentinula edodes* strains, maximum linear mycelial growth was observed in Le C strain followed by Le-5 and OE-329.

Alemu (2015) reported cultivation of *Lentinula edodes* on coffee husk substrate which was an abundantly available solid waste in Dilla town of Ethiopia.

### *2.8.2. Substrate supplementation*

Lizuka and Takeuchi (1978) formulated a ratio of 80 per cent sawdust and 20 per cent bran for the cultivation of *Lentinula edodes* in Asia. Royse (1985) cultivated Shiitake on a mixture of maple and birch (60: 40) sawdust substrate with a 10 per cent supplementation of spring wheat bran and 10 per cent millet which proved to be the best formula for nutritional components. Royse (1985) and Miller and Jong (1987) reported that *Lentinula edodes* can be cultivated on 80 per cent sawdust, 10 per cent bran and 10 per cent wheat/ millet in USA. Schunemann (1988) conducted a study using beechwood or coniferous sawdust either alone or in 1: 1 mixtures with or without adding maize meal (20 per cent), spent brewer's grains (30 per cent) or dry molasses (20 per cent) as supplements for the cultivation of *L. edodes* of these, best result was obtained in beechwood sawdust and dry molasses.

Substrate formula of 84 per cent sawdust, 5 per cent rice bran, 5 per cent wheat bran, 3 per cent soyabean and 3 per cent lime were used for *Lentinula edodes* cultivation in Taiwan (Kalberer, 1987). Royse *et al.* (1990) advocated that starch based supplements like wheat bran (10- 40 per cent dry weight) served as nutrients providing an optimum growing medium for Shiitake. Rinker (1991) studied the effect of substrate temperature treatment, genotype, spawn run time and water immersion on Shiitake mushroom production on sawdust and the results showed that mixture of red and white oak sawdust blended with 10 per cent wheat bran and 10 per cent millet had no significant interaction between genotype and heat treatment. Kirchoff and Lelley (1991) reported that when *Lentinula edodes* was cultivated on substrates blocks of 75 per cent beech wood sawdust supplemented with 25 per cent corn meal, fruiting bodies were obtained 105 days after inoculation.

Diehle and Royse (1991) studied the effect of substrate heat treatment at 100 $^{o}$C, 111 $^{o}$C and 122 $^{o}$C on biological efficiency of *Lentinula edodes*. When mixture of

60 per cent maple sawdust + 40 per cent birch sawdust supplemented with 10 per cent rice bran and 10 per cent white millet bran was preheated at 111 °C, greater sized mushrooms was obtained compared with heat treatment at 122 °C.

Addition of organic and inorganic supplements to the substrate during cultivation is known to influence the yield of various species of mushroom (Fasidi and Kadiri, 1993). Royse (1996) reported sawdust as the most popular basal ingredient along with starch based supplements like rice bran, wheat bran, millet, rye and maize for producing Shiitake. These supplements provide protein, carbohydrates, fat, minerals and vitamins.

Starch based supplements like wheat bran, rice bran, millet, rye and maize powder when added to sawdust served as major nutrients which provide optimum growth medium (Royse, 1997, 2001).

Palamo *et al.* (1998) reported that eucalyptus sawdust alone or in combination with 10 per cent rice bran proved to be the most suitable substrate for production of *Lentinula edodes*. Kalberer (1998) suggested 80 per cent hardwood sawdust supplemented with 20 per cent maize meal ( nitrogen source) and 2 per cent calcium carbonate as the best substrate for higher yields of *L. edodes*.

First report of inducing basidiocarp of Shiitake mushroom on hardwood sawdust amended with YVMBS (Yeast, vegetable juice, multigrain oatmeal, brown sugar) broth in the green house in 1-3 months after spawn block inoculation (Pacumbaba and Pacumbaba Jr., 1999). Fomina *et al.* (1999) proved oak sawdust with 20per cent rye bran as the favorable substrate for the cultivation of *Lentinula edodes*.

Wheat bran, rice bran, millet, rye or corn @ 10- 40 per cent of dry weight can be used as supplements to the main ingredient for Shiitake cultivation (Royse and Schisler, 1986; Ivan *et al.*, 2003). Synthetic logs from the sawdust (800 g) of *Tectona*,

*Terminalia* and *Acasia* woods supplemented with 100 g wooden wool and 100 g pearl millet served as substrates for the cultivation of *Lentinula edodes* (Arya and Arya, 2003). Maximum biological efficiency of 45 per cent was obtained on sawdust without any supplements. Rossi *et al.* (2003) studied the effect of addition of sugarcane molasses along with different proportions of rice bran @ 10, 15, 20, 25, 30, 40 per cent on sugarcane bagasse substrate for the growth of *Lentinula edodes*. Among these, 25 per cent and 30 per cent rice bran induced the highest stimulation of mycelial vigor whereas addition of sugarcane molasses had no influence on mycelial growth.

Permana *et al.* (2004) conducted a study to assess the suitability of milled wheat straw and sugarcane bagasse substrate supplemented with 0 , 5 , 10 and 15 per cent of wheat bran and were inoculated with *Pleurotus ostreatus* and *Lentinula edodes* mycelia. *P. ostreatus* degraded organic matter of the substrate and lignin, faster than *L. edodes*. The invitro digestibility of spent substrate after cultivation of *Lentinula edodes* was 68 per cent.

Shiitake mushroom cultivated on ground corn cob based substrates like corn cob (90 per cent) + rice bran (10 per cent) , corn cob ( 5 per cent) + eucalyptus sawdust (45 per cent ) + 10 per cent rice bran, corn cob (50 per cent) + eucalyptus sawdust (50 per cent) and corn cob (100 per cent) proved that ground corn cobs supplemented with rice bran as a viable alternative substrate (Frederico *et al.*, 2005).

Silva *et al.* (2005 b) and Nikitina *et al.* (2007) reported that eucalyptus residues supplemented with cereal bran supported fast growth of *Lentinus edodes* indicating that mycelium extension was related to the bioavailability of nitrogen. Dewangan *et al.* (2007) reported that out of ten different fruiting substrates like sawdust of Biza wood + rice bran (20 per cent) , teak wood + 22 per cent rice bran, sal wood + rice bran (22 per cent) , mix wood + rice bran (22per cent), mix wood + rice bran (15 per cent) , mix wood + rice bran (20 per cent), teak wood + rice bran (15 per cent), teak

wood + rice bran (20 per cent), mix of all wood + rice bran (15 per cent) and saw dust + paddy straw + rice bran (20 per cent) evaluated for growth and development of *Lentinula edodes* , 94.16 per cent mycelial colonization was observed on sal wood saw dust + rice bran (22 per cent) 80 days after inoculation.

Agarwal (2007), Lalitesh (2009) and Kaur (2010) reported improved mycelial extension rates and higher biological efficiency of *Lentinula edodes* strains on supplementation of wheat straw with wheat bran.

The use of lignocellulosic solid waste supplement can improve the nutrient content in substrate composition substantially to benefit the commercial production of mushrooms (Omoanghe *et al.*, 2009). Regina and Leonardo (2009) evaluated the use of coconut husk sawdust supplemented with wheat bran/ rice bran as an alternative substrate for axenic production of Shiitake mushroom. The growth and vigor of Shiitake strains increased when supplemented with 0 , 5 , 20 , 30 and 40 per cent of wheat bran or rice bran.

According to Sharma and Kumar (2010), the cultivation of *Lentinula* species on sawdust and wheat straw alone or in combination, resulted in more than 90 per cent biological efficiency. Sawdust + 20 per cent wheat bran combination yielded highest biological efficiency of 115 per cent followed by 92 per cent biological efficiency in wheat straw + sawdust (1 : 1 ratio). Cold water treatment for six hours was most effective for the induction of fruiting body formation.

Peralta and Frutis (2010) studied the cultivation of *Lentinus edodes* on wheat straw supplemented with 15 per cent bran, 13 per cent wheat flour and 1 per cent sucrose with an average production of 327.30 g fruiting body with biological efficiency of 84.20 per cent.

Radial mycelial growth of *Lentinula edodes* (Berk.) Pegler strain LE-96/13 was studied in media containing organic residues extract by using substrates like

pineapple crown, *Astrocaryum aculeatum* Meyer peel, *Theobroma grandiflorum* shell, *Musa* sp. peel when supplemented with wheat bran @ 0 , 10 and 20 per cent, the highest mycelial growth of *L. edodes* were found in *Tectona grandiflorum* shell and *A. aculeatum* peel supplemented with 10 per cent wheat bran (Aguiar *et al.*, 2011).

Effect of different substrate supplementation on mycelial growth of *Lentinula edodes* strains (Le 1 to Le 6) and one strain of *Pleurotus eryngii* was studied by Maciel *et al.* (2011) on mixture combination *viz.*, 80 per cent sawdust + 20 per cent wheat bran, 80 per cent sawdust + 12.9 per cent rice bran, 1.94 per cent wheat bran, 1.94 per cent cotton meal, 1.94 per cent coarse cornmeal and 80 per cent sawdust + 20 per cent sugar combinations and the results showed that growth speed was high in Le-5 but had a lower biomass production than Le 1.

*Lentinula edodes* when cultivated on sawdust supplemented with different concentrations of 10 , 15 , 20 , 25 , 30 , 35 and 40 per cent of wheat bran, rice bran, maize powder and their 1 : 1: 1 combination, highest number of fruiting bodies (34.8 / 500 gm packet), highest biological yield (153.3/ 500 g packet) and biological efficiency (76.60 per cent) were obtained from sawdust substrate supplemented with 25 per cent wheat bran (Moonmoon *et al.*, 2011).

According to Sharma *et al.* (2012), substrates like sawdust of *Toona ciliata,* wheat straw, pine needles, wood chips of *Lantana camara* and *Parthenium hysterophorus* alone and in various combinations were screened to find out the suitable substrate for cultivation of *Lentinula edodes*. Maximum yield of 680 g was obtained on the mixture of 60 per cent sawdust and 20 per cent Lantana wood chips supplemented with 20 per cent rice bran. Minimum yield (24 g) was obtained on 50 per cent wheat straw and 50 per cent sawdust combination.

Veena and Pandey (2012) developed a complete cultivation package of *Lentinula edodes* on saw dust supplemented with 20 per cent rice bran and 3 per cent

calcium carbonate. Sharma *et al.* (2012) reported maximum yield of *L. edodes* in the mixture of 60 per cent sawdust, 20 per cent lantana wood chips supplemented with 20 per cent rice bran .

### *2.8.1.2. Lentinus* sp.

Das and Samajpati (1998) reported that when *Lentinus squarrosulus* was cultivated on a medium composed of 100 g rice straw, 260 g rice bran, 13 g sugar and 13 g $CaCO_3$, a biological efficiency of 76.50 per cent was obtained.

When *Lentinus squarrosulus* were cultivated on pararubber sawdust supplemented with different concentration of oil palm kernel meal, maximum of 37.20 per cent biological efficiency was obtained in 950 g sawdust + 15 per cent oil palm kernel meal + 1 per cent Ca ($OH_2$), sixty days after inoculation ( Petcharat and Tongwised, 2003). According to Ayodale *et al.* (2007) when sawdust of seven tree species *viz.*, *Mansonia artissima, Piptadeniastrum africanum, Nesogordonia papaverifera, Combretodendron macrocarpum, Terminalia* sp., *Kheya ivorensis* and *Brachystegia nigerica* were used for the cultivation of *Lentinus squarrosulus*, highest mycelial density was observed in sawdust of *Mansonia artissima* and lowest in *Piptadeniastrum africanum*.

Adejoye *et al.* (2009) reported that when *Lentinus squarrosulus* was cultivated on two different cellulosic wastes supplemented with banana peel and poultry manure, wood waste of *Cordia milleni* supplemented with 5 g or 10 g poultry manure and banana peel induced the widest mycelial extension. Various substrates *viz.*, sawdust + rice bran + calcium sulphate + calcium carbonate, sawdust + rice bran+ maize meal + calcium carbonate, sawdust + maize cobs + calcium carbonate when inoculated with grain spawn of *Lentinus squarrosulus* and amended with oils like groundnut, coconut, palm kernel, butterfat, palm and cotton oils @ 0, 0.007, 0.014, 0.021 and 0.028 ml/ g ,maize and millet spawn induced high carpophore wet weights which was superior to that of wheat spawn (Oghenekaro *et al.*, 2009).

Adesina *et al.* (2011) studied the mycelial growth of *Lentinus squarrosulus* on the leaves and bark of common fruit trees supplemented with 25 per cent rice bran, horse dung, poultry droppings, cow dung, fresh cassava flour and oil palm waste fibre. The best mycelial growth was obtained on leaves of *Spondias mombin* supplemented with rice bran while the highest yield of fruit bodies was obtained on the log of *S. mombin*. Sales-Campos and Andrade (2011) reported the use of wood residues for the cultivation of *Lentinus strigosus* in Amazon. Sawdust of *Simarouba amara, Ochroma piramidale* and *Anacardium giganteum* supplemented with rice and wheat bran resulted in yield of 98, 119 and 177 g/ kg with a biological efficiency of 38 , 48 and 59 per cent respectively.

Leon *et al.* (2013) conducted a study on domestication of two wild species of mushroom *viz., Lentinus squarrosulus* and *Polyporus grammocephalus* using rice straw- sawdust formulation. Biological efficiency of 7.83 per cent was obtained in rice straw sawdust formulation of 100: 40 in *L. squarrosulus* followed by 2.91 per cent biological efficiency in *P. grammocephalus*.

## 2.9. NUTRIENT ANALYSIS

### *2.9.1. Lentinula sp.*

Nutritive value of edible mushrooms like *Lentinula edodes* (Shiitake) and *Pleurotus ostreatus* (Oyster mushroom) have huge protein content, carbohydrate, and mineral salts (Raaska, 1990; Chahal and Hackey, 1990). Dehydrated mushroom contained 23 per cent protein, 65 per cent carbohydrate, 2 per cent lipids and high mineral content (Sayadi and Ellouz, 1993).

Aoyagi *et al.* (1993) reported that carbohydrate, Ca, Cu, Hg and Mn content of *L. edodes* were significantly higher when cultivated on logs than those cultivated on

sawdust substrate. Whereas moisture, protein, ash, K, P and Zn contents were high in on sawdust cultivated mushroom than on logs.

*Lentinula edodes* was a good source of proteins, carbohydrate and mineral elements with beneficial effects on human nutrition (Wasser and Weis, 1994; Mizuno *et al.*, 1995). Singh *et al.* (2003) tested 14 edible mushrooms found in Manipur including *Lentinula edodes* for various nutrients and the results showed that moisture content ranged from 40.07 per cent to 92.92 per cent, crude protein (8.89 per cent - 27.29 per cent), fat (1.5 -8.5 per cent), ash (4.75-21 per cent) and crude fibre (3.25-13 per cent ). Silva *et al.* (2007) reported the mycelium of the mushroom contains high content of proteins, carbohydrates, fibre and vitamins.

Shiitake mushrooms are rich in nutritional value and contain polysaccharides, antioxidants, dietary fibre, ergosterol, minerals, V-B1, B2 and V-C (Beluhan and Ranogajee, 2011).

Caglarirmak (2011) reported mean protein values of *Lentinula edodes*, *Pleurotus ostreatus* and *Pleurotus sajor caju* as 2.61 per cent, 1.76 per cent and 0.92 per cent respectively. The presence of lentinan, β-glucan in Shiitake stimulates the immune system and fight against AIDS and exhibits antitumor activity.

George *et al.* (2014) analyzed three available strains of Shiitake mushrooms for major and minor elements like Na, K, Ca, Mg, Al, Fe, Mn, Cu, Pb and Ni. Results showed that fruiting bodies contained K (13.2- 6.2 g/ kg), P (7.8- 54.5 g / kg), Mg (3.4- 6.5 g/ kg), Ca (179.8- 1698 mg/ kg), Na (191.3- 3448 mg/ kg), Al (15.3 -79.5 mg/ kg). Heavy metals include Fe (44.4-125.1 mg/ kg), Zn (59.3 -283.9 mg/kg) and Cu (13.7 -182.4 mg/ kg).

### *2.9.2. Lentinus sp.*

Nwanze *et al.* 2006 reported the proximate composition of *Lentinus squarrosulus* as crude protein (22.82 per cent), crude fibre (7.64 per cent), ash (7.52

per cent), moisture (2.76 per cent), fat (6.29 per cent) and carbohydrate (60.65 per cent). *Lentinus tuber-regium* contained higher concentrations of carbohydrates (55.80 per cent) ,protein (25 per cent), moisture (9.40 per cent), total ash (4.70 per cent), crude fibre (3.60 per cent), fat (1.60 per cent), potassium (7.53 mg/gm), calcium (2.66 mg/gm), magnesium (2.45 mg/gm), sodium (1.20 mg/gm), iron (0.53 mg/gm), copper (0.11 mg/gm), zinc (0.41 mg/gm) manganese (0.08 mg/gm) and energy (338 kcal) ( Manjunathan and Kaviyarasan, 2010).

## 2.10. ENZYME ACTIVITY

Enzymatic degradation of the substrate was usually associated with phenol oxidase activity of the mushroom (Sermanni *et al.*, 1985). Kannan and Oblisami (1990) reported that polyphenol oxidase activity in *Pleurotus sajor caju* tremendously increased in the initial stages and associated with growth. Krishnamoorthy *et al.* (2005) observed variation in enzyme production *viz.*, polyphenol oxidase, cellulose, laccase depending upon the variation of the substrates.

The increase in enzyme activities during vegetative growth and during mycelial regenerative stage is associated with the energetic requirements for initiating fructification whereby enzymes are secreted to digest the substrate to provide carbon and other nutrients ( Kurt and Buyukalaca, 2010; Hernandez *et al.*, 2011).

## 2.11. SHELF LIFE

Shelf life of mushrooms *viz.*, *Lentinus edodes*, Oyster mushroom, nameko and eniki-take was 14 to 20 days at 1 $^{O}$C , 10 days at 6 $^{O}$C and 2 to 3 days at 20 $^{O}$C (Minamide *et al.*, 1980). Ding (1987) reported that by maintaining a temperature of 20 $^{O}$C and RH of 85-90 per cent satisfactory color will be imparted to Shiitake mushroom.

According to Santana *et al.* (2008) the shelf life of minimally processed Shiitake mushroom was 10 days at 7 $^{O}$C but less than 5 days at 10 $^{O}$C and 3 days at

15 $^{O}$C. Chlorinated sanitizer, followed by fast freezing give the produce acceptable color up to 60 days at 18 $^{O}$C ( Mateus *et al.*, 2009).

Lin *et al.* (2010) studied the effect of different storage temperature and days on color and quality of dried Shiitake mushroom when stored at 0 $^{O}$C and 15 $^{O}$C for seven days and 25 $^{O}$C for three days before drying. Results showed that mushrooms stored at 25 $^{O}$C for three days before drying turned to brown color in dried products while that stored at 0 $^{O}$C for 0-3 days had better quality. The color of pileus and gill, aroma, external preference, total sensory scores of dried mushrooms became worse when storage temperature and days increased.

Ramkumar *et al.* (2010) reported that Shiitake mushroom can be stored up to 10 days in perforated polybags under natural condition and 30 days in refrigerated condition. Dehydrated mushroom can be stored for more than eight months in air tight containers.

Bechara *et al.* (2010) reported microwave oven as an alternative to dry and preserve Shiitake mushroom compared with the traditional method of drying. Humidity can be reduced from 85-95 per cent to 5-20 per cent by this method. Fernandes *et al.* (2012) suggested that gamma, electron beam, and UV irradiation are the potential tools in extending the post-harvest shelf life of fresh mushrooms like *Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus*.

Liu and Xing (2012) described that coating with tea polyphenols @ 0.2 per cent and 0.3 per cent inhibited increase in respiratory rates and firmness was observed in controls treated with distilled water / chitosan alone. Coating fruiting bodies with chitosan containing 0.3 per cent tea polyphenols exhibited a significant fresh keeping effect on *Lentinula edodes.*

Yanjie *et al.* (2013) reported that high oxygen packaging especially with initial 100 per cent oxygen, maintained sensory quality of fresh mushrooms.

## 2.12. ORGANOLEPTIC STUDIES

A study conducted by Desai *et al.* (1991) revealed that consumer acceptability of *Pleurotus sajor-caju* was poor due to the tough texture of the stipe and unattractive colour of the pileus but its flavor was found good. In a comparative study Balakrishnan (1994) showed that *Pleurotus sapidus, P.membranaceous* and *P. petaloides* obtained maximum consumer acceptability with respect to color and flavor. Overall acceptability of these species was significant when compared to the standard species *Pleurotus sajor-caju* and *P. flabellatus* which were found inferior in all the qualities. Das (2011) prepared different dishes using oyster mushroom, button mushroom, Jew's ear mushroom and milky mushroom and obtained maximum consumer acceptability in case of appearance, color, flavor, taste and texture.

Ibrahium and Hegazy (2014) conducted a study to evaluate the effect of partial replacement of wheat flour by different levels (10, 20 and 30 per cent) of mixture of mushroom powder and sweet potato flour at equal rates in biscuit making and the results showed that increase in corporation of mushroom powder in biscuits increased the protein, fibre, ash, Fe, Ca, K, P and amino acid content. Prodhan *et al.* (2015) reported that biscuits can be fortified with protein rich mushroom powder in order to supplement protein in the diet and nutrition.

## 2.13. CROP MANAGEMENT

### *2.13.1. Pests*

Nakai *et al.* (1982) observed presence of rod shaped bacteria in hyphal cells of abnormal fruit bodies of Shiitake mushroom showing browning symptom. Jiang (1986) reported that larvae of sciarid *Phorodonta flaviceps* infest sporophores of edible fungus like *Pleurotus ostreatus, Pleurotus sajor caju, Lentinula edodes* and

*Auricularia auricula* in China. Ohya (1992) reported Coleopteran insect *Dacne japonica* as pest of Shiitake mushroom.

Kim and Hwang (1996) described five insect species and six other animal species as pests of *L. edodes* in Korea Republic. Insect species include *Achorutes armatus, Scaphidium amurense* and mollusc *Philomycus confuse* which caused severe damage to mushroom fruiting body. Both immature and mature bed logs were attacked by *Moechotypa diphysis*.

Han *et al.* (1998) reported *Aphelenchoides composticola* and *Rhabditis* species which caused damage to *L. edodes* in Pinnan County, China. Red rust beetle (*Tribolium castaneum*) a pest of dehydrated mushroom can be disinfested using gamma radiation at lethal dose of 2000 Gy ( Santos *et al.*, 1999).

Barreto *et al.* (2002) reported insect pests of Shiitake as insects from Orders Coleoptera (Bostrichidae, Staphylinidae and Dermestidae), Diptera (Calliphoridae), Hymenoptera (Formicidae) and Lepidoptera (Stenomidae) as infesting Shiitake beds.

Yoshimatsu and Nakata (2003) revealed a fungivorous moth, *Diomea cremate* which caused damage to Shiitake mushroom when cultivated on sawdust. The larvae appeared to consume the thick brown mycelial coat formed on the surface of synthetic logs rather than the fresh fruiting body.

Zorzenon and Potenza (2003) diagnosed *Opogona sacchari* moth as pest of Shiitake cultivation which caused much difficulty to small and medium sized producers. Costa *et al.* (2006) reported Dipteran insects as the major pests of Shiitake grown on Eucalyptus wood logs in Arroido do Padre, Brazil.

Sutou (2009) reported a Dipteran pest (*Chaetosciaara takahashii*) of Shiitake mushroom, which was originally reported on Hachijo Island, Tokyo and was recently recorded in Shizuoka, Miyagi and Iwate Prefectures, Japan.

The new record of two gall midges *Camptomyia* species (*C. corticalis* and *C. heterobia*) that feed on Shiitake mycelium thereby disturbing the formation of fruit bodies was reported by Shin *et al.* (2011). Sueyoshi and Kitajima (2011) first reported ditomyiid flies, *Asioditomyia japonica* as pest of *L. edodes*, and was the first record of such pest reared from an artificial medium.

According to Shin *et al.* (2012) out of seven species of sciarid flies collected from Shiitake mushroom farm in Korea, *Lycoriella ingénue* and *Bradysia difformis* were dominant both on oak bed logs and in artificial sawdust beds. Kitajima *et al.* (2012) observed harmful mushroom fly, *Neoempheria ferruginea* in sawdust based cultivation of Shiitake.

### *2.13.2. Diseases*

Ushiyama and Hashioka (1973) reported two types of filamentous particles of size 15 and 22 nm dia. which were seen in sections of hymenia and stipes of *Lentinula edodes*. According to Liao (1985) *Trichoderma* species caused sawdust contamination which can be controlled by Penmush (Y101) and Tecto-60 at 200 ppm without affecting the cultivation of *Lentinula edodes*.

Markawa *et al.* (1987) reported four species of *Ceratocystis* namely *C. microspore*, *C. monoliformis*, *C. picrea* and *C. tenella* from bedlogs of Shiitake which suppressed mycelial growth in sawdust and oak logs.

The major moulds reported from cultivated *Lentinus* species include *Trichoderma, Myceliophthora, Rhizopus, Penicillium, Monilia* and *Aspergillus* which can be controlled by spraying 20 per cent Bavistin and 70 per cent Topsin-M @ 0.1 - 0.5 per cent (Huang *et al.*, 1988). Jiang *et al.* (1995) observed five different species of *Trichoderma* of which *Trichoderma harzianum* severely affected *Lentinula* cultivation in Fujian province.

Tsuneda *et al.* (1995) reported a bacterial disease caused by *Pseudomonas tolaasii* which exhibited wild browning to severe necrotic cavity symptoms on fruiting bodies along the periphery of attachment area to the stalk.

Black spot disease of *Lentinula eodes* fruiting bodies caused by *Hyphozyma synanamorph* of *Eleutheromyces subulatus* was reported by (Tsuneda *et al.*, 1997). Multiplication of yeast like cells and hyphae of *Hyphozyma* caused blackening and mild lysis of host cap tissues.

*Penicillum* species accounted for 71.40 per cent of total colonies in sawdust based cultivation of *Lentinula edodes* followed by 12.40 per cent mycelia sterilia, 6.70 per cent *Cladosporium* spp, yeast 2.50 per cent and 1.90 per cent *Trichoderma* species (Togashi *et al.*, 1997).

Mata *et al.* (1998) described reduction in the incidence of *Trichoderma* species by substrate supplementation with peat and by activated *Lentinula edodes* inoculum. This improved competitive ability of *Lentinula edodes* and thus decreased the incidence of *Trichoderma* and increased the overall yield of mushrooms.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

## 3.1. SURVEY AND COLLECTION

Genus *Lentinula edodes* (Shiitake) is long thought to be a synonym of *Lentinus edodes* (Singer, 1941). Based on the microscopic analysis of hyphae and spores of Shiitake mushroom Pegler (1975) placed it under the genus *Lentinula*. Molina *et al.* (1992) delineated *Lentinula* from the genus *Lentinus* based on phylogenetic studies.

Keeping in view, these controversies pertaining to affiliations between *Lentinula* sp. and *Lentinus* sp., the preliminary part of the present study was focused mainly on conducting survey to ascertain the distribution of local strains of mushrooms followed by morphological studies and their molecular characterisation to determine phylogenetic relationship between collected isolates of *Lentinula* and *Lentinus* sp.

Survey was conducted during pre and post monsoon showers from May to December in various regions of Thiruvananthapuram, Kollam, Idukki, Wayanad, Pathanamthitta, Kannur and Kasargode districts which were assumed to be probable habitats of the two mushrooms under study. Survey was conducted to collect mushroom sporocarps of *Lentinula* sp. and *Lentinus* sp. The study was undertaken in locations having dense vegetation of trees, in each of the selected districts (Table 1). Observations on the natural occurrence and habitat of the spotted mushrooms were also made and recorded.

Mushroom sporocarps obtained from the surveyed locations were brought to the Plant Pathology lab, College of Agriculture, Vellayani in order to examine the specimens and to isolate the fungal culture by the standard technique. Subsequently morphological studies of collected isolates were also undertaken by

detailed examination of macroscopic and microscopic characters of the sporocarps.

Identified strains of *Lentinula edodes*, procured from GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand and Maharana Pratap University of Agriculture and Technology, Udaipur were included in the study. These strains were serially numbered as LE-1, LE-2, LE-3, LE-4 and LE-5 (GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand) and LE-6 strain (Maharana Pratap University of Agriculture and Technology, Udaipur) and subsequent studies of these reference strains were also conducted along with the collected isolates.

## 3.2. MORPHOLOGICAL STUDIES ON THE ISOLATES OF LENTINULA AND LENTINUS SP.

Morphological studies were conducted to study and identify the macroscopic and microscopic characters of *Lentinula edodes* (LE-1 to LE-6) and *Lentinus* sp.

### *3.2.1. Studies on macroscopic and microscopic characters of strains of Lentinula sp.*

Reference strains of *L. edodes* procured from recognised institutes were examined. Macroscopic studies were conducted based on data sheet as described in Appendix – I. Details such as colour, texture, pileus, stipe, spore print, lamellae and gills were observed.

Spore print was made by cutting and keeping portions of the pileus on plain microscopic slides and also on black paper sheets. A medium mature mushroom sporocarp was selected for obtaining the spore print. The pileus was detached and placed on a piece of paper as gills facing towards the surface of the

paper. A bell jar was placed over this to keep moist and to protect from air currents. This was done in an air conditioned room and after ten hours, the bell jar was removed and the pileus was taken off from the paper to obtain the spore print.

Microscopic studies were done by taking sections of fresh sporocarps of procured strains of *Lentinula* sp. by the standard technique. Thin sections transferred to the glass slide were stained using lactophenol cotton blue ( Appendix II) and examined under microscope (10 X and 40 X), for observing the spores and mycelial characters.

### *3.2.2. Studies on macroscopic and microscopic characters of native isolates of Lentinus* sp.

Isolates closely resembling *Lentinus* sp. in their external features, obtained during the survey were examined and were sorted out. Macroscopic and microscopic characters of the selected sporocarps were then studied in detail for their morphological characterisation.

## 3.3. MOLECULAR CHARACTERISATION OF PROCURED STRAINS OF LENTINULA EDODES AND LOCAL LENTINUS SP.

Molecular characterisation of the reference strains of *Lentinula edodes* from recognised institutes and sporocarps of mushrooms collected during the survey were done inorder to determine their phylogenetic relationship using two methods 1) ITS sequencing and 2) RAPD analysis.

### 3.3.1. DNA Barcoding by partial sequencing of Internal Trascribed Spacer (ITS) region of rDNA of *Lentinula* and *Lentinus* sp.

Six strains of *L. edodes* obtained from different institutes and isolates closely resembling *Lentinus* sp. were subjected to ITS sequencing.

ITS sequencing of the *Lentinula edodes* strains procured and the native isolates obtained were done at Rajiv Gandhi Centre for Biotechnology, Poojappura. The procedure for molecular characterisation was as follows.

### *3.3.1.1. DNA isolation using Nucleo Spin Plant II Kit (Macherey-Nagel)*

About 25 mg of the tissue /mycelium was homogenised using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 was added and vortexed for one minute. Ten microliters of RNase A solution was added and inverted to mix. The homogenate was incubated at 65 $^{O}$C for 10 min. The flow through liquid was collected and the filter was discarded. Four hundred and fifty microliters of buffer PC was added and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for one minute and the flow through liquid was discarded. Four hundred microliter buffer PW1 was added to the column, centrifuged at 11000 x g for one minute and flow through liquid was discarded. Then 700 µl of PW2 was added, centrifuged at 11000 rpm and flow through liquid was discarded. Finally 200 µl of PW2 was added and centrifuged at 11000 rpm for two min. to dry the silica membrane. The column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65 $^{O}$C for five min. The column was then centrifuged at 11000 rpm for one minute to elute the DNA. The eluted DNA was stored at 4 $^{O}$C.

### *3.3.1.2. Agarose Gel Electrophoresis for DNA Quality check*

The quality of the DNA isolated was checked using agarose gel electrophoresis. One µl of 6 X gel-loading buffer (0.25 per cent bromophenol

blue, 30 per cent sucrose in TE buffer pH-8.0) was added to 5 µl of DNA. The samples were loaded to 0.8 per cent agarose gel prepared in 0.5 X TBE (Tris-Borate-EDTA) buffer containing 0.50 µg/ml ethidium bromide. Electrophoresis was performed with 0.5 X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

### *3.3.1.3. PCR Analysis*

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1 X Phire PCR buffer (contains 1.5 Mm MgCl2 ), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phre Hotstart II DNA Polymerase enzyme, 0.1 mg/ ml BSA and 3 per cent DMSO, 0.5 M Betaine, 5 pM of forward and reverse primers.

**Primers used**

| Target | Primer Name | Direction | Sequence (5' → 3') |
|---|---|---|---|
| ITS | ITS-1F | Forward | TCCGTAGGTGAACCTTGCGG |
| | ITS-4R | Reverse | TCCTCCGCTTATTGATATGC |

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

**PCR amplification profile**

**ITS**

98 °C - 30 sec
98 °C - 5 sec
62 °C - 10 sec
72 °C - 15 sec
} 40 cycles
72 °C - 60 sec
4 °C - ∞

### *3.3.1.4. Agarose Gel electrophoresis of PCR products*

The PCR products were checked in 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ ml ethidium bromide. One µl of 6X loading dye was mixed with five µl of PCR products and was loaded and electrophoresis was performed at 75 V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

### *3.3.1.5. ExoSAP-IT Treatment*

ExoSAP-IT (GE Healthcare) consisted of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five µl of PCR product was mixed with two µl of ExoSAP-IT and incubated at 37 $^{O}$C for 15 min followed by enzyme inactivation at 80 $^{O}$C for 15 min.

### *3.3.1.6 . Sequencing using BigDye Terminator v3.1*

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

| | | |
|---|---|---|
| PCR Product (ExoSAP treated) | - | 10-20 ng |
| Primer | | -3.2 pM (either Forward or Reverse) |
| Sequencing Mix | - | 0.28 µl |
| 5x Reaction buffer | - | 1.86 µl |

Sterile distilled water - make up to 10µl

The sequencing PCR temperature profile consisted of a first cycle at 96 $^{O}$C for two min followed by 30 cycles at 96 $^{O}$C for 30 sec, 50 $^{O}$C for 40 sec and 60 $^{O}$C for four min for all the primers.

### *3.3.1.7. Post Sequencing PCR Clean up*

Master mix I of 10 µl milli Q and 2 µl 125 mM EDTA per reaction were made, of this 12 µl of master mix I was added to each reaction containing 10 µl of reaction contents and were properly mixed. Mastermix II of 2 µl of 3 M sodium acetate pH 4.6 and 50 µl of ethanol per reaction were made. 52 µl of master mix II was added to each reaction. Contents were mixed by inverting and incubated at room temperature for 30 min. This was centrifuged at 14,000 rpm for 30 min. The supernatant was decanted and added 100 µl of 70 per cent ethanol. This was centrifuged at 14,000 rpm for 20 min. The supernatant was decanted and 70 per cent ethanol wash was repeated. The supernatant was decanted and the pellet was air-dried.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

### *3.3.1.8. Sequence Analysis*

The sequence quality was checked Sequence Scanner Software Vi (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010). The identity of ITS rDNA conserved region of the mushroom was established by performing a similarity search using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database

and the sequences were matched with existing available database for species confirmation.

#### *3.3.1.9. Phylogenetic Analysis*

The data set based on the ITS-rDNA region of the six strains of *L. edodes* sequences were retrieved from NCBI GenBank database (USA) and compared. Multiple sequence alignment was done using ClustalW2 and phylogenetic analysis through Phylogeny. Fr software (Dereeper *et al.*, 2008). A phylogeny tree was constructed using neighbour-joining (NJ) method. All traits had equal weight and gaps were treated as "missing" values.

### 3.3.2. RAPD analysis of *Lentinula* and *Lentinus* sp.

RAPD profiling of the *Lentinula* and *Lentinus* species were carried out at Department of Plant Breeding and Genetics, College of Agriculture, Vellayani.

RAPD was done using fifteen different RAPD primers *viz.*, OPA-01, OPA-02, OPA-03, OPA-04, OPA-07, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-17, OPA-18 and OPA-19 to find out the phylogenetic relationship between the six strains of *Lentinula edodes* mushroom and the native isolates of *Lentinus* sp. obtained during the survey.

The procedure for isolation of DNA and PCR are elaborated below:

#### *3.3.2.1. Isolation of Genomic DNA*

Isolation of genomic DNA from all the strains of *L. edodes* was carried out as per the method by Regowsky *et al.* (1991). The procedure is as follows:

• Approximately one g of mushroom mycelia was weighed and taken in a clean autoclaved mortar and was crushed by freezing in liquid nitrogen.

• The powder was transferred to 2 ml Eppendorf tube and one ml of extraction buffer [1.00 g SDS (0.1 per cent), 1.576 g Tris HCl (100 mM), 0.584 g sodium chloride (100 mM), 0.37224 g EDTA (10 mM), volume made up to 100 ml with distilled water] was added.

• The tubes were then placed in a water bath with the temperature maintained at 60 $^{O}$C for 30 min after homogenization

• The mixture was then centrifuged at 10,000 rpm at 4 $^{O}$C for 10 min.

• The aqueous phase was collected and 400 µl of phenol:chloroform (25:24) was added and again centrifuged at 10,000 rpm at 4 $^{O}$C for 10 min.

• The supernatant was collected to which 200 µl of chloroform iso-amyl alcohol (24:1) was added and was centrifuged at 10,000 rpm at 4 $^{O}$C for 10 min.

• The aqueous phase was collected and 200 µl of chloroform iso-amyl alcohol (24:1) was added and was centrifuged at 10,000 rpm at 4 $^{O}$C for 5 min.

• The supernatant was collected and 60 µl of 3 M sodium acetate and 600 µl of ice cold iso-propanol were added and was kept overnight at -20 $^{O}$C for precipitation.

• The solution was centrifuged after about 16 h at 12,000 rpm for 10 min and the supernatant was discarded without dislodging the pellet.

• The precipitate was then washed twice using 70 per cent ethanol and dried.

• After drying, the precipitate was dissolved in 100 µl 0.1 x TE buffer [Tris buffer 0.12 g (10 mM), EDTA (0.037 g)] and stored at -20 $^{O}$C

### *3.3.2.2 PCR Reaction*

The reaction was performed in 25 µl reaction mixture containing 3µl template DNA, 16 µl of dNTP mix, 14 µl of 10X buffer, 8µl of $MgCl_2$, 8 µl Taq DNA polymerase, 8 µl of 10 pmoles primer and 116 µl of distilled water.

Amplification was done in a programmable thermocycler (BIORAD) that was programmed as follows:

An initial denaturation of 95 °C for one min followed by 35 cycles of denaturation of 94 °C for ten sec, annealing at 36 °C for one min and extension at 72 °C for two min. The synthesis step of final cycle was extended further by seven min. amplified products were separated by agarose gel electrophoresis using 1.4 per cent gel as detailed earlier which was photographed using gel documentation system (BIORAD, USA).

### *3.3.2.3 Agarose gel electrophoresis*

For carrying out the agarose gel electrophoresis, a horizontal gel electrophoresis unit was used. Agarose (1.4 per cent) was weighed and melted in 1x TE buffer. When the solution cooled to 42-45 °C, ethidium bromide was added at the rate of two µl per 100 ml and the solution was poured to a preset, sealed gel casting tray to a height of 3 mm-5 mm after fixing up of the comb in position.

The assembly was then kept undisturbed for 15-20 min. for solidification that is, gelling of the solution. The adhesive tapes and the comb were then removed and the tray was then submerged in the electrophoresis tank filled with 1x TE buffer in such a way that the buffer covered the gel to a height of one mm.

The required volume of the sample (DNA) and the loading dye (Glycerol (30 per cent) + bromophenol blue) were mixed at a ratio of 5:1 and were loaded into the slots of gel near the negative terminal with the help of a micropipette. The cathode and anode of the electrophoresis unit were connected to the power supply with constant voltage of 60 volts. The power supply was turned out when

the loading dye moved about 3/4th of the gel. The gel was then documented with the help of a gel documentation system (BIORAD).

#### *3.3.2.4. Scoring of DNA bands*

Individual bands were scored as one or zero for their presence or absence of RAPD bands. The bivariate data were analysed to generate Jaccard's similarity coefficient values for each pair wise comparison between accessions were calculated and similarity coefficient matrix was constructed. The matrix was subjected to unweighted pair group method to generate a dendrogram. All the numerical analysis was conducted using the software NTSYS.

## 3.4. CULTURAL STUDIES OF LENTINULA EDODES

The cultural studies of the strains of *L. edodes* were conducted for isolation of pure culture as well as for determining their nutritional requirements and physiological characters.

### 3.4.1. Effect of different media on the mycelial growth of *Lentinula edodes*

The procured strains of *Lentinula edodes* were isolated using standard technique. During the survey, no native isolates of *L. edodes* were obtained and hence the further studies were conducted using six strains procured from various institutions *ie.*, LE-1 to LE-5 strains (GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand) and LE-6 strain (Maharana Pratap University of Agriculture and Technology, Udaipur). Cultural studies were conducted both in solid and liquid media to evaluate the mycelial growth of all the six strains of *Lentinula edodes*.

### *3.4.1.1. Effect of different solid media on the mycelial growth of Lentinula edodes*

The experiments were carried out with all the six strains of *L. edodes* in completely randomised design (CRD) on the following seven different media. The growth of *L. edodes* were evaluated in terms of radial mycelial growth (cm) to find out the best medium for the growth of the fungus.

1) Potato dextrose agar (PDA)
2) Oat meal agar (OMA)
3) Malt extract agar (MEA)
4) Malt extract peptone dextrose agar (MEPDA)
5) Carrot agar (CA)
6) Czapek Dox agar (CDA)
7) Yeast extract agar (YEA)

Design: CRD

Replication: 3

The composition of the media used is given in Appendix III. Each of the medium was prepared and transferred to 250 ml conical flasks at the rate of 150 ml per flask and plugged tightly with cotton plugs. The flasks were sterilized in an autoclave at 15 psi pressure and 121 $^{O}$C for 20 min. The molten media were poured into sterile petriplates and allowed to solidify. The mycelial disc of nine mm dia. from seven day old culture of each strain was inoculated at the centre of the dish. The dishes were properly labelled, sealed with parafilm and incubated at room temperature (28±2$^{o}$C). Three replications were maintained for each treatment. Nature of mycelial growth and colony dia., were recorded for each strain. Observations were taken till the mycelial growth covered the entire petriplate.

### *3.4.1.2. Effect of different liquid media on the mycelial growth of Lentinula edodes*

The different liquid media *viz*., potato dextrose broth, oat meal broth, malt extract broth, malt extract peptone dextrose broth, carrot broth, Czapek Dox broth and yeast extract broth were evaluated to find out the best medium that supported maximum biomass production of all the six strains of *L. edodes*. The composition was same as that of solid media used in the previous experiment except for the omission of agar maintaining three replications for each treatment.

The liquid media were prepared and fifty ml of each medium were dispensed in 100 ml conical flask and autoclaved at 15 psi pressure and 121 $^{O}$C for twenty min. The media were then inoculated aseptically with nine mm culture disc's of each strain obtained from actively growing culture. The flasks were incubated at room temperature. Observations were recorded 25 days after inoculation by filtering the mycelia through Whatman No: 1 filter paper and drying at 60 °C. The dry weights were taken until a constant weight was obtained.

## 3.5. NUTRITIONAL REQUIREMENTS OF LENTINULA EDODES

Nutritional requirements of *L. edodes* with respect to carbon and nitrogen sources were evaluated in the following experiment.

### *3.5.1. Effect of different carbon sources on the mycelial growth and biomass production of Lentinula edodes*

*L. edodes* was grown in media with different carbon sources *viz.,* sucrose, lactose, galactose, mannitol and fructose. These were substituted for dextrose, in the best media screened on 3.4.1.1. The malt extract peptone dextrose media were prepared and sterilized in an autoclave at 15 lbs pressure and 121 $^{O}$C for twenty min. The medium containing dextrose as carbon source also maintained as check. After cooling, the media was poured into sterile petriplates and allowed to

solidify. Seven day old culture disc of nine mm dia. of all the six strains were inoculated at the centre of the dishes that were sealed with parafilm and incubated at room temperature (28 ± 2°C). Three replications were maintained for each treatment. Observations were taken till the mycelial growth of the fungus covered the entire petriplate.

The liquid media with different carbon sources were also prepared, using the same composition as used in previous experiment except for the omission of agar. Fifty ml of sterilised medium in 250 ml conical flasks were then inoculated with nine mm culture disc of the fungal strain and incubated at room temperature (28 ± 2 °C). The mycelial mat was filtered after 25 days and dry weight was taken after drying at 60 °C till a constant weight was obtained.

### *3.5.2. Effect of different nitrogen sources on the mycelial growth and biomass production of Lentinula edodes*

Different forms of nitrogen like ammonium nitrate, ammonium carbonate, ammonium chloride, beef extract and peptone were substituted for sodium nitrate in Czapek Dox medium which was the only nitrogen containing medium that was screened in 3.4.1.1. , so as to give the same per cent of nitrogen in each case. The media were prepared and sterilized in an autoclave at 15 lbs pressure and 121 $^{O}$C for 20 min. After cooling it was poured into sterile petriplates and was allowed to solidify. Seven day old culture disc of nine mm dia. of *L. edodes* were inoculated at the centre of the dishes that were then sealed with parafilm and incubated at room temperature (28 ± 2°C). Three replications were maintained for each treatment. Measurements of radial growth of each strain of *L. edodes* was taken when the mycelial growth of any of the tested strains completely covered the entire petriplate.

The liquid media with different nitrogen sources were prepared, the composition was same as that used in previous experiment except for the omission of agar. Fifty ml of medium was taken in each 100 ml conical flask. After

sterilisation, flasks were inoculated with nine mm culture disc of actively growing culture and incubated at room temperature for 25 days. The mycelial mat was filtered through Whatman No: 1 filter paper and dry weights were taken after drying at 60 °C until constant weight was obtained.

## 3.6. PHYSIOLOGICAL REQUIREMENTS OF LENTINULA EDODES

### *3.6.1. Effect of different temperature on the mycelial growth and biomass production of Lentinula edodes*

Disc of nine mm taken from the actively growing mycelium of six strains of *Lentinula edodes* were inoculated into the sterilised malt extract peptone dextrose agar medium poured on sterile petriplates. The discs were placed at the centre of the medium and incubated at 5, 10, 15, 20, 25 °C and room temperature (28±2°C). These experiments were conducted in three replications. Measurements of radial growth of each strain of *L. edodes* were taken when the mycelial growth of any of the tested strains completely covered the entire petriplate.

Fifty millilitres of malt extract peptone dextrose broth were taken in 100 ml conical flask and autoclaved at 121 °C and 15 lbs pressure for twenty min. The media were then inoculated with nine mm disc of seven day old culture of six strains of *Lentinula edodes* and incubated at 5, 10, 15, 20, 25 and room temperature (28 ± 2 °C). Observations were taken 25 days after inoculation. The mycelial mat was then filtered, dried at 60 °C until constant weights were obtained.

### *3.6.2. Effect of different light conditions on the mycelial growth and biomass production of Lentinula edodes*

Twenty ml each of malt extract peptone dextrose agar medium was poured into sterile petridishes on which culture discs of nine mm taken from the actively

growing mycelium of *Lentinula edodes* were inoculated. The discs were placed at the centre of the medium and incubated under ambient light, fluorescent light and dark conditions. Radial measurements of the fungus was taken till the mycelial growth of the fungus covered the entire petriplate.

Fifty millilitres of malt extract peptone dextrose broth was taken in 100 ml conical flask and autoclaved at 15 lbs pressure and 121 $^{O}$C for twenty min. The medium were then inoculated with nine mm disc of seven day old culture of *Lentinula edodes*. All the six strains were used for the experiment. The flasks were then incubated under ambient light, fluorescent light and dark conditions. After 25 days, the mycelial mat was filtered and dried at 60 °C until constant weights were obtained.

### *3.6.3. Effect of different pH on the mycelial growth and biomass production of Lentinula edodes*

Malt extract peptone dextrose agar medium was used for studying the effect of pH on mycelial growth of different strains of *L. edodes*. The medium was prepared and pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 by adding 0.1 N Hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH). Sterilization was done by autoclaving at 15 lbs pressure and 121 °C for twenty min. After cooling, the media was poured into sterile petriplates and allowed to solidify. All the six strains of *L. edodes* were used for the experiment. Seven day old culture disc of nine mm dia. of all the six strains of *L. edodes* were inoculated at the centre of the dishes that were then sealed with parafilm and incubated at room temperature ( 28 ± 2 °C). Three replications were maintained for each treatment in CRD. Measurements of radial growth of each strain of *L. edodes* was taken when the mycelial growth of any of the tested strains completely covered the entire petriplate.

Malt extract peptone dextrose broth was prepared with different pH concentration as given above. Fifty ml of the media was taken in 100 ml conical flask and autoclaved at 15 lbs pressure and 121 °C for twenty min. The media were then inoculated with nine mm disc of seven day old culture of *Lentinula edodes* strains and incubated at room temperature (28 ± 2 °C) for 25 days. The mycelial mat was filtered, dried at 60 °C until constant weights were obtained.

## 3.7. SPAWN PRODUCTION TECHNOLOGY OF LENTINULA EDODES

*Lentinula edodes* strain (LE-6) that was screened as the best strain due to its vigorous growth, was used for the evaluation of substrates in spawn production technology. Mother spawn of the strain was prepared in paddy grain by inoculating two week old fungal culture grown on malt extract peptone dextrose agar medium. Mass multiplication of the prepared mother spawn was done using different substrates (Sinden, 1934) as follows.

**Treatments:**

I. Grain substrates :

1) Paddy 2) Maize 3) Wheat 4) Ragi 5) Sorghum

II. Non grain substrate

1) Sawdust (hard wood)

Design : CRD

Replication : 3

One month old mother spawn was inoculated in each of the above treatments as described below in order to evaluate their efficacy of these substrates for spawn production of *L. edodes*.

### *3.7.1. Preparation of grain spawn*

The grains were soaked overnight in water. They were then drained and boiled just enough to soften them avoiding the seed rupture. The excess water

was drained off and the grains were spread on a clean silpaulin sheet for drying. The dried grains were mixed thoroughly with calcium carbonate at the rate of 40-50 g/ kg grain and filled in polypropylene bags. The filled bags were sterilized in an autoclave at 121 $^{O}$C and 15 lbs pressure for two h. The bags were inoculated aseptically inside a laminar air flow chamber with pure cultures of *L. edodes* isolates at the rate of one third of the agar pieces colonised by the fungal mycelium cut from a fully covered plate using a sterile inoculation needle and incubated at room temperature (28 ± 2°C). The mycelial growth of fungi were measured and recorded. The spawn thus obtained as mother spawn was used for further spawn production.

### *3.7.2. Preparation of non- grain substrate (sawdust)*

The sawdust spawn was prepared by mixing 99 per cent sawdust and 1 per cent calcium carbonate. The sawdust was thoroughly sieved to remove bigger particles and mixed with water to attain the moisture content to 60 per cent. The substrate mixture was then filled in polypropylene bags and sterilized at 121 $^{O}$C and 15 lbs pressure for two hours in an autoclave. The bags were inoculated as done above and were kept for incubation at room temperature (28 ± 2 °C).

The best spawn substrate was selected based on the minimum time taken for the spawn run and colour and nature of mycelial growth.

## 3.8. CULTIVATION TECHNOLOGY OF LENTINULA EDODES

Cultivation technology of *L. edodes* was standardised after evaluating the substrates and method of cultivation.

### *3.8.1. Evaluation of substrates*

Comparative efficiency of six strains (LE-1 to LE-6) of *Lentinula edodes* for cultivation under Kerala conditions were evaluated. The study was conducted by finding out the biological efficiency of each strain using widely available

cheap agro industrial wastes. Three different substrates were included for standardising the cultivation of mushroom strains. They are

1) Paddy straw
2) Sawdust
3) Banana pseudo stem

Starch based supplements like wheat bran and rice bran were also added to the substrates which served as nutrients to create an optimum growing condition.

Polybag method or artificial log method of cultivation was followed as per Royse (2002).

Beds were laid during October- November, which was the suitable period for Shiitake cultivation in our climatic condition. Substrates like sawdust or paddy straw or banana pseudo stem were prepared by mixing one kg substrate with two per cent calcium carbonate retaining 60-65 per cent moisture. Starch based supplements like rice bran and wheat bran were added at different proportions @ 10 per cent and 20 per cent and tested for evaluation of different strains of *Lentinula edodes*. The experiment was conducted as follows

Design : Completely Randomised Design

Replication: Five

Treatments:

T1 Paddy straw + sawdust + 10 per cent rice bran

T2 Paddy straw + sawdust + 20 per cent rice bran

T3 Sawdust + 10 per cent rice bran

T4 Sawdust + 20 per cent rice bran

T5 Sawdust + 10 per cent wheat bran

T6 Sawdust + 20 per cent wheat bran

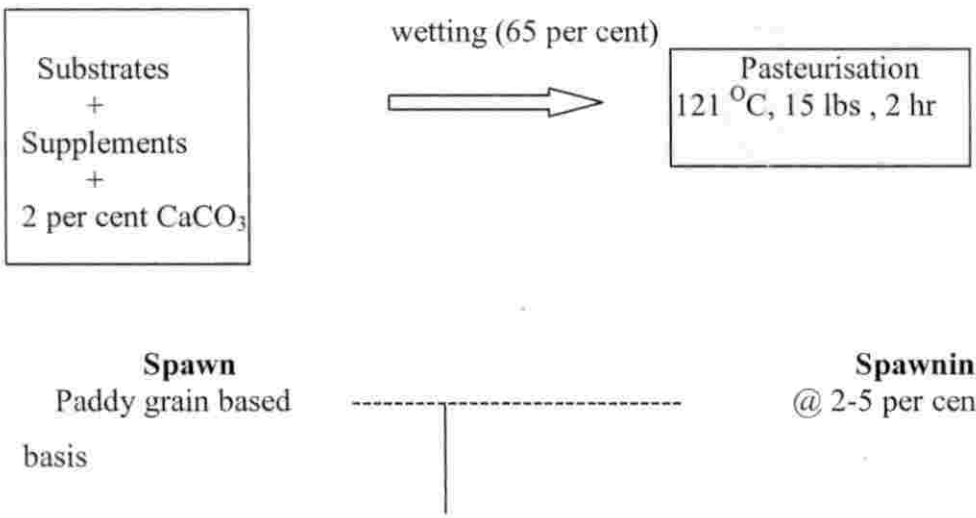
T7 Sawdust alone

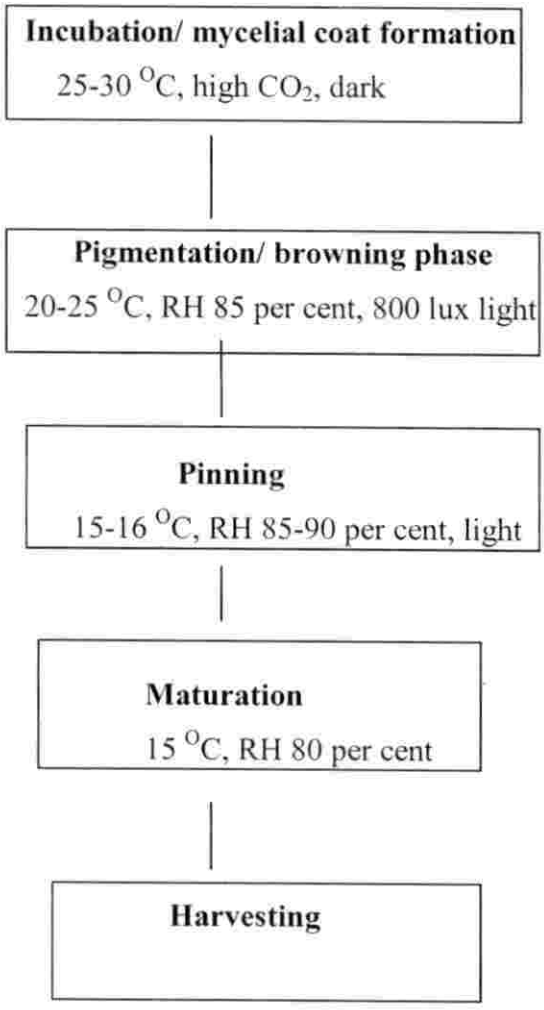
T8 Paddy straw alone

T9 Banana pseudo stem

After inoculating the substrates, the following procedure was adopted as shown in the flow chart. The inoculated beds were placed on racks and incubated at room temperature (28 ± 2 $^{O}$C) under prevalent humid conditions for 30 days. Within this period bump formation and formation of brown exudates occurs after which the beds are transferred to low temperature conditions (20 $^{O}$C). High relative humidity (85-90 per cent) was constantly provided by humidifier as well as by covering the racks with moistened jute sacks. Simultaneously all the beds are opened and incubated for seven days with intermittent watering. With the appearance of brown colour of the beds, they were subjected to physical shock treatment by immersing in cold water (4 $^{O}$C) for 24 h and the process was repeated for seven days. After the last harvest, beds were maintained for three weeks of resting period following which the further initiation of sporocarp was stimulated by repeating the process of physical shock treatment as described above.

**FLOW CHART OF SHIITAKE PRODUCTION BY POLYBAG METHOD**






The best strain as well as the best substrate were selected based on the following criteria

3.8.1.1. Time taken for complete mycelial colonization of the bed substrates:

Period in days from the time of spawn inoculation up to the complete colonisation of the bed substrates by the fungal mycelium by each strain of *L. edodes* was recorded.

3.8.1.2. Time taken for browning of mycelium of mushroom beds in different substrates :

Browning denotes the maturation stage of fungal mycelium which is accompanied by the formation of brown exudates on the bed surface. The period in days for the transformation of the colour of colonised fungal mycelium from white to brown by each strain of *L. edodes,* was recorded.

3.8.1.3. Time taken for initiation of sporocarp of mushroom beds in different substrates :

Initiation of sporocarp occurs after browning stage and observations of the period in days from browning stage to initiation of sporocarp in each strain of *L. edodes*, was recorded.

3.8.1.4. Total number of fruiting bodies in different substrates :

Mushroom sporocarps were harvested when the pileus remains in downward curled condition and the total number of sporocarps harvested at a time was recorded.

3.8.1.5. Average yield on different substrates: Average yield was calculated by adding the total weight of the sporocarps obtained from each harvest divided by the number of harvest.

3.8.1.6. Biological efficiency of each strain was calculated using the formula

$$B.E = \frac{\text{Wet weight of harvested mushrooms}}{\text{Weight of substrate}} \times 100$$

3.8.1.7. Total crop growth period:

The period in days from the time of spawn inoculation stage of the bed till the last harvest of sporocarps from bed of each strain of *L. edodes* laid out in the experiment was counted as the total crop growth period.

The fruiting bodies harvested in the above experiment were then oven dried and preserved for analysis.

**3.8.2. *Lentinus* sp.**

Two native isolates of *Lentinus* sp. (*Lentinus tuber-regium* and *Lentinus connatus)* were also evaluated by the same procedure (3.8.1.) using the best substrate screened for *Lentinula edodes* in order to standardise the cultivation technology of native isolates of *Lentinus* sp. Observations were recorded as mentioned in the cultivation technology of *L. edodes*.

3.8.2.1. Time taken for complete mycelial colonization of the bed substrates
3.8.2.2. Time taken for browning of mycelium of mushroom beds on different substrates
3.8.2.3. Time taken for initiation of sporocarp of mushroom beds on different substrates
3.8.2.4. Total number of fruiting bodies on different substrates
3.8.2.5. Average yield on different substrates
3.8.2.6. Biological efficiency
3.8.2.7. Total crop growth period

3.9. NUTRIENT ANALYSIS

The harvested sporocarps of *L. edodes* were dried and powdered for the analysis of proximate constituents like moisture, crude protein, total ash, carbohydrates, fat, crude fibre, minerals, Vitamin C, minerals like calcium,

magnesium, zinc, manganese, iron, phosphorus, potassium and sodium. The moisture content was analysed on wet weight basis while the rest of the parameters were analysed on dry weight basis. Standard methods were used for the analysis of all the constituents.

### *3.9.1. Estimation of moisture content*

Ten gram sample (W1) was taken in a preweighed crucible and dried in an oven until constant weight was obtained (W2). The difference between the initial and final weight gave the moisture content, which was converted into percentage.

$$\text{Per cent of moisture content} = \frac{W1-W2}{10} \times 100$$

### *3.9.2. Estimation of protein*

Protein content of *Lentinula edodes* was estimated by initial assessment of total nitrogen in the sample by Kjeldahl's method as outlined by Jackson (1973).

The estimation was performed in two stages, namely, digestion and distillation. Initially, 0.3 g of dried mushroom powder was transferred to a 100 ml Kjeldhal flask. Ten ml of concentrated $H_2SO_4$ and 0.2 g to 0.3 g of digestion / catalytic mixture (25 g of $K_2SO_4$ with five g of $CuSO_4$-$5H_2O$ and 0.5 g of metallic selenium powder by grinding in a mortar) was added to this and allowed to stand overnight. This mixture was digested on low flame initially for 10-15 min. until frothing stopped. Then it was digested at high flame for four h till the contents of Kjeldahl's flask became clear or pale green or blue. The flask was cooled and the

contents were transferred to 50 ml volumetric flask and the volume was made upto 50 ml with distilled water.

In the second stage, ten ml of acid digest was transferred to a micro Kjeldahl distillation assembly. 10-15 ml of 40 per cent NaOH was added to it to make the contents distinctly alkaline and the funnel of distillation assembly was washed with small amounts of distilled water three to five times. Before adding NaOH, boric acid-mixed indicator solution was kept ready at the receiving end of condenser outlet so that outlet is dipped in boric acid (about 20 ml of two per cent boric acid-mixed indicator solution taken in conical flask). The distillation was carried out by passing steam into the distillation flask and the colour of boric acid-mixed indicator solution changed from reddish purple to green. The process was continued till all the $NH_3$ released from distillation of sample was trapped. After the distillation, bluish green coloured ammonia trapped boric acid (ammonium tetraborate) was titrated against 0.01 N $H_2SO_4$ till colour changed to purple releasing boric acid with formation of $(NH4)_2SO_4$. A blank without the sample was run to check for contamination and to ensure precision.

Observations and calculations:

$$\text{Per cent N} = \frac{\text{(S-B) x N of } H_2SO_4 \text{ x volume of digest x 100 x 0.014}}{\text{Weight of sample x aliquot used for distillation}}$$

Per cent protein = Per cent N x 6.25

### *3.9.3. Estimation of fat*

The extraction of fat was carried out using Soxhlet extraction apparatus (Moore and Stein, 1948).

Five gm of sample was taken in an extraction thimble and placed in the extractor so that top of the thimble was over the bent siphon tube outside extractor. The extractor was connected to previous weighed extraction flask. Sufficient quantity of petroleum ether was poured into the extractor. The extractor was attached to the condenser with a constant flow of cold water. The sample packet was placed in the butt tubes of the Soxhlet extraction apparatus. The sample was then extracted with petroleum ether for 6 h without interruption by gentle heating. The ether extract was then cooled and was transferred to a preweighed beaker ( W1). The ether present in the extract was evaporated on a steam or water bath till no odour of ether remained. The beaker was cooled to room temperature in a dessicator and weighed. The heating was repeated until constant weight was recorded (W2).

$$\text{Per cent fat content} = \frac{W2-W1}{\text{Weight of the sample (g)}} \times 100$$

### *3.9.4. Estimation of carbohydrates*

Total carbohydrate content was estimated by Anthrone method (Hedge and Hofreiter, 1962).

One hundred mg of mushroom powder was weighed and transferred into boiling tubes. It was hydrolyzed by keeping it in a boiling water bath for three h with five ml of 2.5 N hydrochloric acid, cooled to room temperature and neutralized with sodium carbonate till effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 min. The supernatant was collected and used as an aliquot for analysis. The standards were prepared by taking 0, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and one ml in all the tubes including the sample tubes by adding distilled water. From the supernatant 0.5 ml of aliquot was taken and made upto one ml by adding distilled water. The reaction mixture

containing 0.5 ml of aliquot, 0.5 ml distilled water and four ml of anthrone reagent was added to the tubes and heated for 8 min in a boiling water bath. The reaction mixture was cooled rapidly and colour was read at 630 nm in a spectrophotometer (systronics UV-VIS spectrophotometer 118). The amount of carbohydrate present was calculated from the standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of sample on fresh weight basis.

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

### *3.9.5. Estimation of crude fibre*

Crude fibre content was estimated by the method described by Misra *et al.* (1975).

One gram of filtered dried sample was ground with ether to remove fat. After ether extract the dried sample was boiled with 100 ml of sulphuric acid (1.25 per cent) for 30 min. by adding bumping chips. The digested sample was filtered through a muslin cloth and washed with boiling water until the washings were no longer acidic. The sample was again boiled with 100 ml sodium hydroxide (1.25 per cent) for 30 min. The digested samples were again filtered through a muslin cloth and washed with boiling water. The sample was washed with 25 ml of boiling 1.25 per cent sulphuric acid, 50 ml of water and 25 ml of alcohol. The residue was removed and transferred to preweighed ashing dish ($W_1$). The residue was dried at 130 °C for two hour, cooled in a dessicator and weighed ($W_2$). The residue was further ignited at 600 °C which was cooled and weighed.

$$\text{Per cent of crude fibre} = \frac{\text{Loss in weight}}{\text{Weight of the sample}} \times 100$$

$$\text{Loss in weight} = (W2-W1) - (W3-W1)$$

### *3.9.6. Estimation of ash*

Three gram sample was transferred to a weighted silica dish (W1). It was heated on a Bunsen burner at a low flame and when the substrate charred the dish was transferred to a muffle furnace. It was heated at 500 to 550 °C for about two h till a white ash was obtained. It was then cooled in desiccator and weighed (W2). The difference between two gives the result, which was converted into per cent.

$$\text{Per cent of ash} = \frac{W2-W1}{\text{Weight of the sample}} \times 100$$

### *3.9.7. Estimation of minerals*

Analytical methods followed for mineral estimation

| Sl No | Element | Method | Reference |
|---|---|---|---|
| 1 | N | Microkjeldhal digestion in sulphuric acid distillation. | Jackson (1973) |
| 2 | P | Nitric-perchloric acid digestion (9:3) and colourimetry making use of vanado molybdo phosphoric yellow colour method. | Jackson (1973) |
| 3 | K | Nitric-perchloric acid (9: 3) digestion and flame photometry. | Jackson (1973) |

| | | | |
|---|---|---|---|
| 4 | Ca, Mg | Nitric-perchloric acid (9: 3) digestion and versenate titration with standard EDTA. | Tandon ( 1993) |
| 5 | Fe, Mn, Zn | Nitric-perchloric acid (9: 3) digestion and atomic absorption spectrophotometry. | Lindsay and Norvell ( 1978) |

### *3.9.8. Estimation of Vitamin C*

Five ml of the working standard solution was pipetted out into a 100 ml conical flask and to this, 10 ml of four per cent oxalic acid was added and titrated against the dye (V1 ml). End point was the appearance of pink colour which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. The sample was extracted in four per cent oxalic acid was made upto a known volume (100 ml) and centrifuged. Five ml of this supernatant was pipetted out and 10 ml of four per cent oxalic acid was added and titrated against the dye (V2 ml).

$$\text{Amount of ascorbic acid mg/ 100 g sample} = \frac{0.5 \text{ mg} \times V2 \times 100 \text{ ml}}{V1 \text{ ml} \times 5 \text{ ml} \times \text{wgt of the sample}} \times 100$$

## 3.10. ENZYME ACTIVITY

### *3.10.1. Phenyl alanine ammonia lyase (PAL)*

PAL activity was analysed using the procedure described by Dickerson *et al.* 1984. The enzyme extract was prepared by homogenising one gram mushroom sample in five ml of 0.1 M sodium borate buffer (pH 8.8) containing 0.05 g polyvinyl pyrrolidone using chilled pestle and mortar. The homogenate was

centrifuged at 10,000 rpm for 20 min. at 4 $^{O}$C. The supernatant was used for assessing the enzyme activity. The reaction mixture contained three ml of 0.1 M sodium borate buffer, 0.2 ml enzyme extract and 0.1 ml of 12mM L-phenyl alanine prepared in same buffer. Blank contained three ml buffer and 0.2 ml enzyme extract. Reaction mixture and blank were incubated at 40 $^{O}$C for 30 min. Reaction was stopped by adding 0.2 ml of 3 N HCl and read at 290 nm in a spectrophotometer. Standard curve was prepared using different concentrations of cinnamic acid.

### *3.10.2. Peroxidase (PO)*

Mycelial sample of 200 mg was homogenised in one ml of 0.1 M sodium phosphate buffer pH (6.5) to which a pinch of poly vinyl pyrrolidone was added. Homogenisation was done at 4 $^{O}$C using mortar and pestle. Homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 min. at 4 $^{O}$C. Reaction mixture consisting of one ml of 0.05 M pyrogallol and 50 µl enzyme extract was taken in both reference and sample cuvettes, were mixed and placed in spectrophotometer and reading was taken at 420 nm. The enzyme reaction was started by adding one ml of 1 per cent HCl in sample cuvettes.

$$\text{Peroxidase content} = \frac{150 \text{ sec.} - 30 \text{ sec.} \times 5000}{2 \times 50} \text{ activity/min/g}$$

### *3.10.3. Polyphenol oxidase (PPO)*

200 mg of filter dried mycelium was homogenised in one ml of 0.1 M sodium borate buffer 6.5 pH to which a pinch of poly vinyl pyrrolidone was added. Homogenisation was done at 4 $^{O}$C using mortar and pestle. Homogenate was filtered through muslin cloth and centrifuged at 5000 rpm for 15 min. at 4 $^{O}$C. Supernatant was used as enzyme extract. Reaction mixture contained one ml of

0.1 M sodium borate buffer (pH 6.5) and one ml of 0.01 M catechol. Cuvette containing reaction mixture was placed in spectrophotometer and absorbance was set to zero at 495 nm. Reaction was started by adding one ml of catechol to 50 µl of enzyme extract. Change in absorbance was recorded at 495 nm.

$$\text{Polyphenol oxidase content} = \frac{150 \text{ sec.} - 30 \text{ sec.} \times 5000}{2 \times 50} \text{ activity/min/g}$$

## 3.11. SHELF LIFE

Mushrooms packed in polypropylene covers and cardboard boxes with and without perforation were kept at room temperature and under refrigerated conditions. The weight of mushroom sporocarps were observed each day up to one week. Observations on days taken for keeping quality and signs of spoilage were recorded.

## 3.12. ORGANOLEPTIC STUDIES

*Lentinula edodes* mushroom cultivated were subjected to studies of organoleptic characters like colour and appearance, texture, flavour and taste. Six products were prepared based on various recipes like *Lentinula* cutlets, mushroom masala, soup, mushroom biscuit, mushroom scramble and baji. The quality evaluation of mushroom recipes using raw as well as dehydrated mushroom was done using five point score card. The overall acceptability of cooked mushroom recipes were recorded based on evaluation done by ten judges based on a five point score card. The average ranking was given for each character. Score card values for each character was given in Appendix - IV. The recipes tried were as given below:

**Soup**

| | | |
|---|---|---|
| Shiitake mushrooms | - | 100g (chopped) |
| Shallots | - | 3 g |
| Butter | - | 30 g |
| Ground pepper | - | ¼ tsp |
| Ground cardamom | - | ¼ tsp |
| Corn flour | - | 1 tsp |
| Milk | - | 2 cups |
| Egg | - | 1 no. |
| Salt | - | to taste |

Onions and chopped mushrooms were fried in butter. Corn flour was mixed in two cups of milk, and boiled for 10 min. in shallow pan. Before removing from fire beaten egg white and sprinkle pepper powder, cardamom salt were added to serve hot.

**Cutlets**

| | | |
|---|---|---|
| Shiitake mushrooms | - | 200 g (chopped) |
| Onion finely chopped | - | 1 medium |
| Garlic crushed | - | 3 cloves |
| Ginger crushed | - | a 1" inch piece |
| Green chilli chopped | - | 1 or 2 |
| Garam masala | - | 1 tsp |
| Chilli powder | - | ¼ tsp (optional) |
| Curry leaves chopped | - | Optional |
| Salt | - | to taste |
| Potato boiled, peeled and mashed | - | 1 medium |
| Egg | - | 1 |
| Plain bread crumbs | - | as needed |
| Oil | - | to fry |

Chopped onion was fried in oil for seven to eight minutes. Crushed ginger, garlic and green chillies were added and fried for three to four min. Then chopped mushrooms were added and sauté till the water evaporated (8-10 min.). To this garam masala, salt and curry leaves were added , fried and allowed them to cool.

Mashed potato was then mixed with the cooked mushroom. Small balls were made by keeping it in the centre of palm and gave a gentle press to get it shaped. Dipped in beaten egg, rolled in bread crumbs and deep fried till it was golden brown.

**Masala**

| | | |
|---|---|---|
| Shiitake mushrooms | - | 250 g (chopped) |
| Green chillies | - | 2 (medium size) |
| Onion (big) | - | 2 (100 g) |
| Ginger shredded | - | 1 tsp |
| Garlic | - | 3 flakes |
| Clove | - | 2 |
| Cardamom | - | 2 |
| Cinnamon | - | 2 small piece |
| Chilly powder | - | 1 tsp |
| Pepper powder | - | ½ tsp |
| Turmeric powder | - | a pinch |
| Salt | - | to taste |
| Oil | - | for frying |
| Curry leaves | - | a few |

Onions, ginger, green chillies and curry leaves were fried in oil. Mushrooms were marinated with a paste of garlic, clove, cinnamon, cardamom, chilly powder, pepper powder, turmeric powder and salt. The mixture was added to the pan, sprinkled with water and cooked for ten min. to serve hot.

**Mushroom biscuit**

| | |
|---|---|
| Butter | - 100 g |
| Sugar | - 200 g |
| Mushroom powder | - 100 g |
| Flour | -100 g |
| Nuts | - 50 g |

Butter and sugar was beated till it got fluffy. Then it was gently whisped in the flour and mushroom powder. Nuts were added and placed the mix in the moulded plate and baked in a preheated oven at 175 $^{o}$C for 20 min.

**Baji**

| | |
|---|---|
| Mushroom | - 500 g |
| Bengalgram flour | - 300 g |
| Chilly powder | -3 tsp. |
| Asafoetida powder | - 1 tsp. |
| Oil | -350 ml |
| Salt | - to taste |

A thick paste was made with a mixture of bengal gram flour, water, chilly powder, asafoetida powder and a pinch of salt. Mushrooms were dipped in this batter one by one to get a smooth coating of the paste. Deep fried till it got golden brown in colour.

**Scramble**

| | |
|---|---|
| Shiitake mushroom | - 200 g |
| Coconut grated | - 300 g |
| Green chillies | -3 |
| Turmeric powder | - 1/2 tsp |

| | |
|---|---|
| Mustard seeds | - a few |
| Oil | -20 ml |
| Salt | -to taste |

Grated coconut with turmeric powder, green chillies and salt were seasoned at first and to this cooked mushrooms were added and stirred for five min and served hot.

## 3.13. CROP MANAGEMENT

Pest and disease incidence were observed and noted during the cultivation of different strains of *L. edodes* from the time of spawn run till the maturation and harvest of sporocarp. The beds were monitored periodically for their incidence. The total number of beds damaged as well as nature of damage by pests and diseases were also recorded.

# Results

# 4. RESULTS

The experimental results obtained from the study entitled "Strain evaluation and cultivation technology of Shiitake mushroom (*Lentinula edodes* (Berk.) Pegler) " are presented below.

## 4.1. SURVEY AND COLLECTION

Surveys were conducted in different locations of Thiruvananthapuram, Kollam, Wayanad, Idukki, Pathanamthitta, Malappuram, Kannur and Kasaragod districts to collect the native flora of *Lentinula* sp. that were growing under natural conditions during pre and post monsoon showers of 2012-2015 .

Shiitake (*L. edodes* ) is a temperate mushroom which prefer cool and humid climate for fruiting of the mushroom. During the entire period of survey, mushroom sporocarps having any close resemblance to *Lentinula* sp. were not observed in any of the locations of the various districts explored.

During the course of the survey, fungal sporocarps that were externally similar to *Lentinus* spp. were obtained. These mushrooms were gregarious and lignicolous in habit and obtained from different locations as listed in Table 1. Tree stumps of mango, coconut and cashew were found to be the usual spots for the occurrence of these *Lentinus* species. These wild isolates of mushrooms resembling *Lentinus* sp. that are obtained during the survey were classified into (VLYN 1 to VLYN 13) based on variations in external features.

Isolates (VLYN-1 to VLYN-6) were gregarious in habit and lignicolous in habitat. Isolate (VLYN-7) was solitary and terrestrial in habitat and were found in areas rich in organic matter. Isolates (VLYN-8 to VLYN-10) were gregarious in habit. They were found mainly on lignicolous habitats like tree stumps of jack fruit, mahogany and cashew plants. Mango tree was found to be the host plant of VLYN-11 which was gregarious and lignicolous in habitat. Two isolates (VLYN-12 and

**Table 1. Occurrence of native isolates of *Lentinus* species**

| Sl No. | Species | Isolates | Habit | Habitat | Host plant | Location | Month of collection |
|---|---|---|---|---|---|---|---|
| 1 | *Lentinus squarrosulus* | VLYN-1 | Gregarious | Lignicolous | Mango | Vellayani, Trivandrum | May, 2013 |
| | | VLYN-2 | Gregarious | Lignicolous | Coconut | Balaramapuram, Trivandrum | June, 2013 |
| | | VLYN-3 | Gregarious | Lignicolous | Mango | Aralam, Malappuram | August, 2013 |
| | | VLYN-4 | Solitary | Lignicolous | Mango | Cheemany, Kasargode | August, 2013 |
| | | VLYN-5 | Gregarious | Lignicolous | Coconut | Neyyattinkara, Trivandrum | December, 2013 |
| | | VLYN-6 | Gregarious | Lignicolous | Cashew | Vellayani, Trivandrum | November, 2014 |
| 2 | *Lentinus giganteum* | VLYN-7 | Solitary | Terrestrial | Organic matter | Kalpetta, Wayanad | December, 2014 |
| 3 | *Lentinus tuber-regium* | VLYN-8 | Gregarious | Lignicolous | Jack fruit | Palode, Trivandrum | May, 2015 |
| | | VLYN-9 | Gregarious | Lignicolous | Mahagony | Vellayani, Trivandrum | May, 2015 |
| | | VLYN-10 | Gregarious | Lignicolous | Cashew | Ezhon, Kannur | May, 2015 |
| 4 | *Lentinus connatus* | VLYN-11 | Gregarious | Lignicolous | Mango | Kottiyam, Kollam | May, 2015 |
| 5 | *Lentinus sp.* | VLYN-12 | Solitary | Lignicolous | Mango | Palode, Trivandrum | June, 2015 |
| 6 | *Lentinus sp.* | VLYN-13 | Gregarious | Lignicolous | Coconut | Mangalathukonam, Trivandrum | July, 2015 |

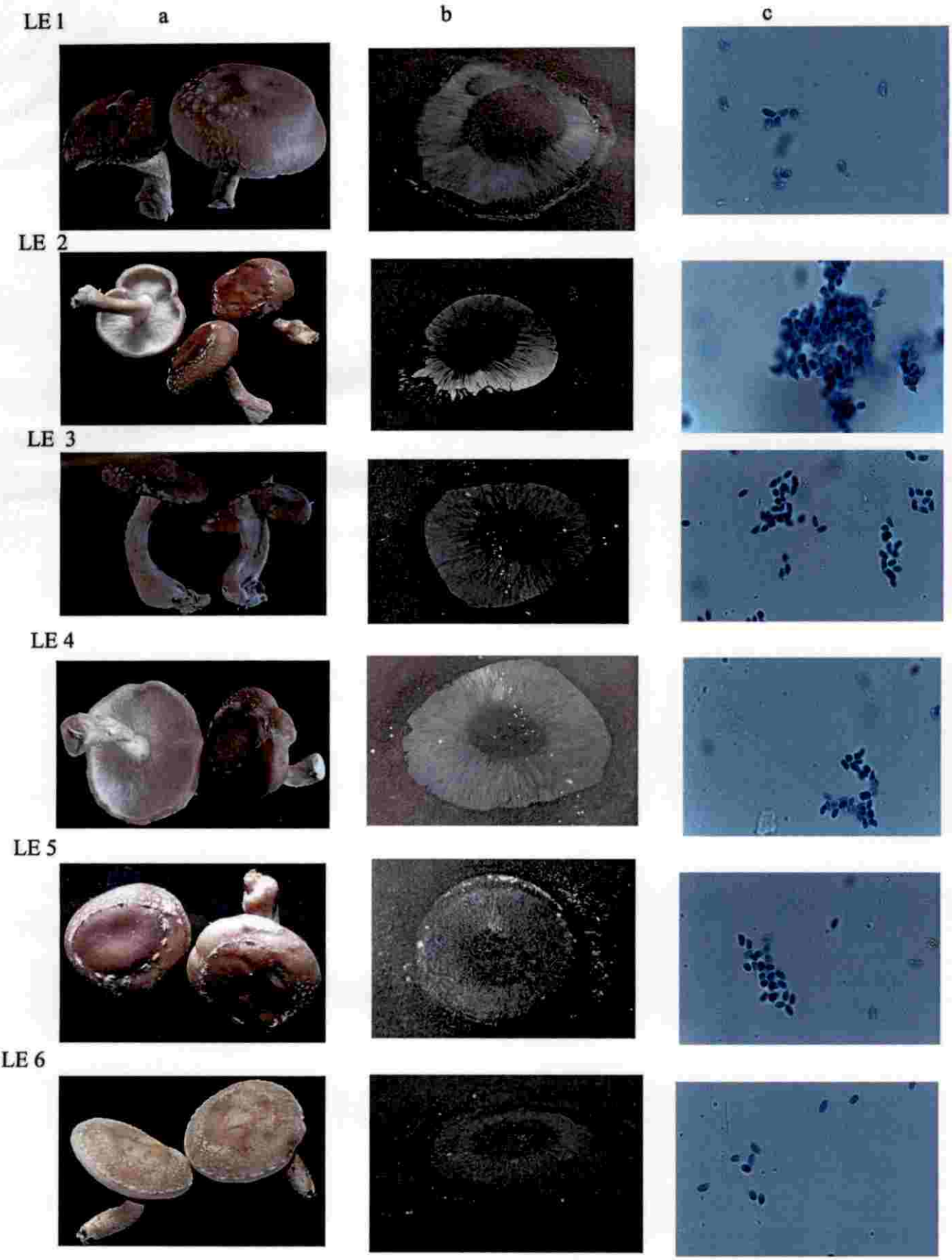



Plate 1: Morphological and microscopic characters of strains of *L. edodus*

a. sporocarp b. spore print c. spores (40 X)

VLYN-13) were solitary and gregarious in habit and were obtained from the host plants mango and coconut, respectively.

## 4.2. MORPHOLOGICAL STUDIES OF LENTINULA SP. AND LENTINUS SP.

Local strains of *Lentinula edodes* were not obtained throughout the period of survey conducted and so subsequent studies related to morphology, phylogeny, cultivation and nutritional aspects were done using the strains procured from recognized institutes, LE1 to LE-5 (GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand) and LE-6 strain (Maharana Pratap University of Agriculture and Technology, Udaipur).

Thirteen isolates resembling *Lentinus* which were obtained during the survey were examined in detail for studying their morphological characters.

Comparative evaluation of the reference strains of *L. edodes* with native isolates of *Lentinus* sp. obtained from the survey, was also made in this study.

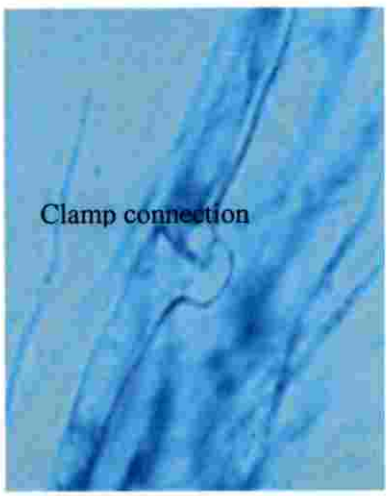
### *4.2.1. Studies on macroscopic and microscopic characters of strains of Lentinula sp.*

Morphology of various strains of *Lentinula edodes* (LE-1 to LE-6) were studied in detail and their descriptions are given below (Table 2).

All the six strains of *L. edodes* (LE-1 to LE-6) had convex shaped pileus with diameter ranging between 6.50 cm to 11.22 cm which were fleshy in texture. Sporocarps of strain LE-6 was golden yellow whereas those of LE-1 to LE-5 were chocolate brown in colour (Plate 1a). Stipe length varied from 4.60 cm to 7.64 cm. Stipe had scaly surface, and was cylindrically shaped with central attachment . The basal part of the stipe was subbulbous in all the strains.

**Table 2. Morphological characters of strains of *Lentinula edodes***

| Strains | Pileus | | | | Stipe | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Shape | Colour | Diameter (cm) | Texture | Length (cm) | Diameter (cm) | Shape | Attachment | Basal part | Surface |
| LE-1 | Convex | Chocolate brown | 6.50 | Fleshy | 4.60 | 1.56 | Cylindrical | Central | Subbulbous | Scaly |
| LE-2 | Convex | Chocolate brown | 10.00 | Fleshy | 7.00 | 1.25 | Cylindrical | Central | Subbulbous | Scaly |
| LE-3 | Convex | Chocolate brown | 9.50 | Fleshy | 6.30 | 1.75 | Cylindrical | Central | Subbulbous | Scaly |
| LE-4 | Convex | Chocolate brown | 11.22 | Fleshy | 5.50 | 1.58 | Cylindrical | Central | Subbulbous | Scaly |
| LE-5 | Convex | Chocolate brown | 10.20 | Fleshy | 6.82 | 1.20 | Cylindrical | Central | Subbulbous | Scaly |
| LE-6 | Convex | Golden yellow | 10.10 | Fleshy | 7.64 | 1.58 | Cylindrical | Central | Subbulbous | Scaly |



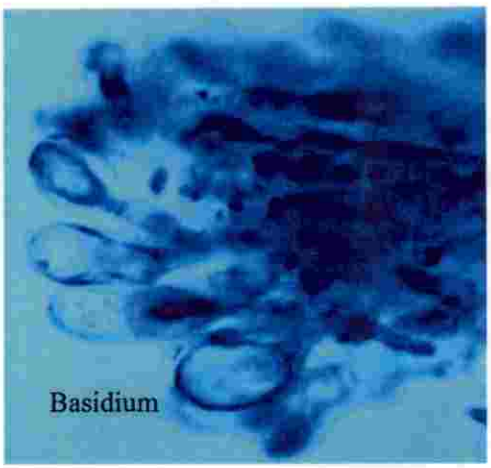





Plate 2: Clamp connection and basidia of *L. edodes*

Gill and spore characters of *L. edodes* strains are depicted in Table 3. Gills of all the strains were freely arranged, soft textured and with smooth margin. Gill number varied from 22 to 28 / cm. Spores were ellipsoidal in shape (Plate 1 b) with size ranging 3.25 - 4.41 µm x 1.96 -2.35 µm and spore print was pure white in colour (Plate 1 c). Spore reaction was cyanophilic for all the six strains of *L. edodes* .The clamp connections and basidium were visible upon microscopic examination (Plate 2).

### *4.2.2. Studies on macroscopic and microscopic characters of native isolates of Lentinus sp.*

The macroscopic and microscopic characters of native isolates of *Lentinus* sp. were also observed and recorded (Table 4 and 5). Characters of the isolates were studied in detail and their descriptions are presented below. The isolates were classified based on the variations in morphological characters and these classified isolates were then identified according to mycokeys .

The isolates (VLYN-1 to VLYN-6) (Class I) were concave shaped, white to creamy white in colour and fibrous textured. The diameter of the pileus ranged from 6.00 cm to 11.66 cm. Stipe was cylindrical, fibrous and centrally attached to the pileus. Gills were crowdly arranged with soft texture and smooth margin (Plate 3). Gill number varied from 26 to 32 / cm. The spores were cylindrically shaped and spore size varied from 4.9-8.0 µm x 2.0-3.1µm. Spore reaction was cyanophilic for all the six isolates. From these morphological studies, the isolates (VLYN-1 to VLYN-6) were identified as *Lentinus squarrosulus* .

The VLYN-7 isolate (Class II) was convex shaped and brown coloured with a fleshy texture. Maximum diameter of 75.00 cm was observed in this species. Stipe was cylindrical with central attachment having stipe length of 61.00 cm and was hardy in texture (Plate 4). Spores were not obtained in this species. Gills were free,

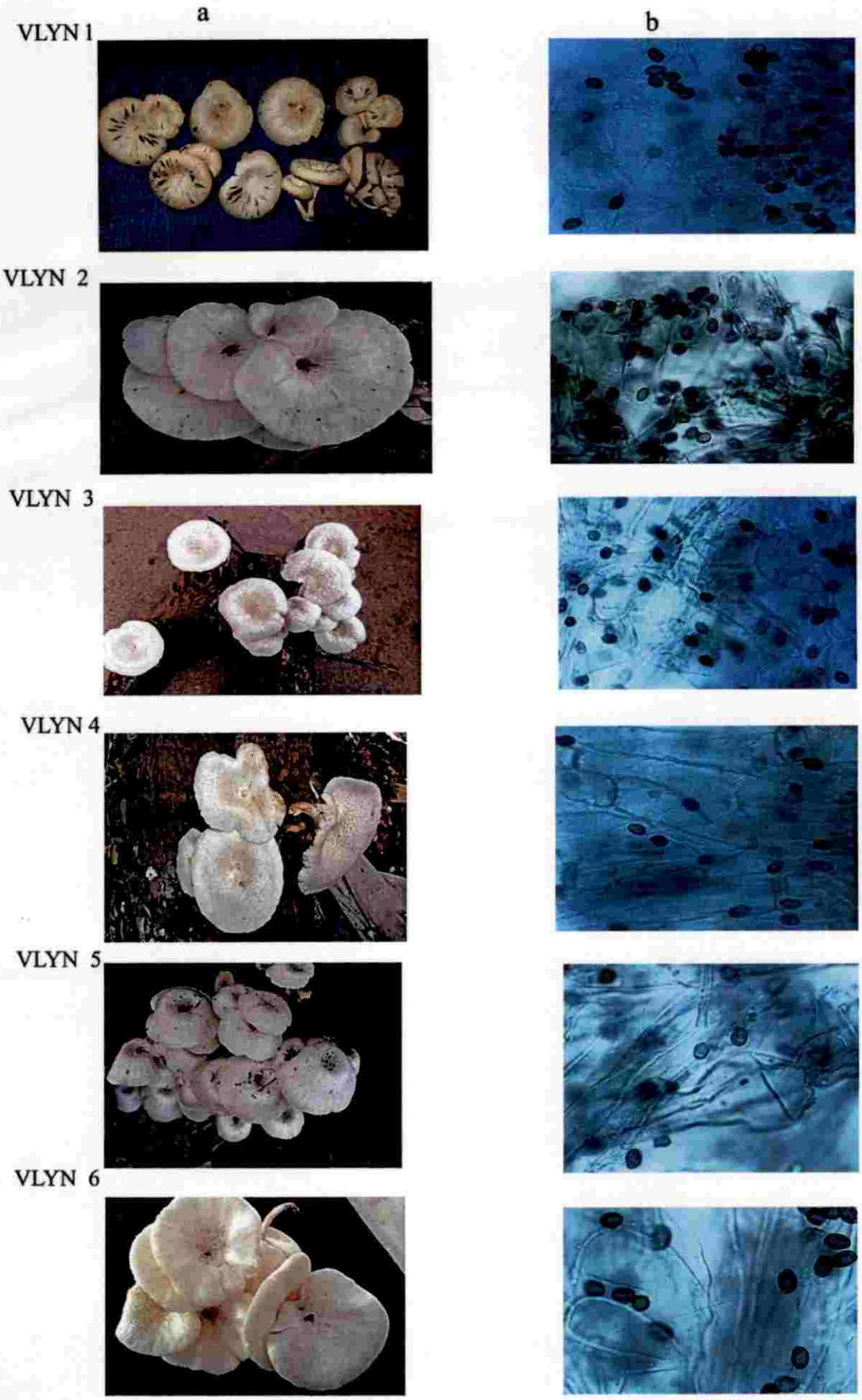



Plate 3: Morphological and microscopic characters of *Lentinus squarrosulus*

VLYN 7

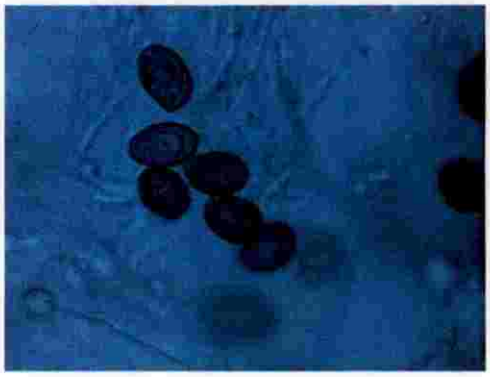
Sporocarp

Stipe and gills

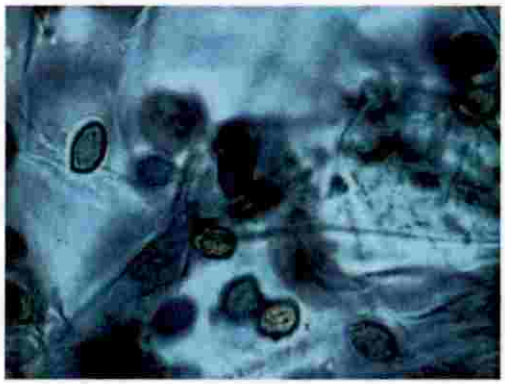
Plate 4 Morphological characters *Lentinus giganteum*

VLYN 8 a

b



VLYN 9



VLYN 10

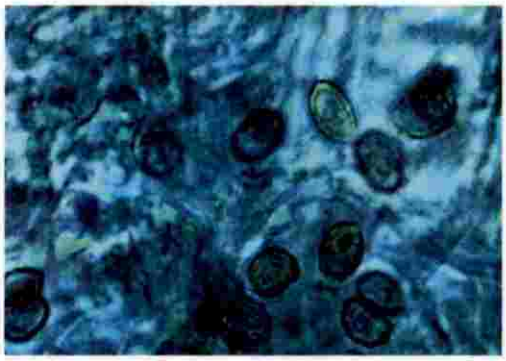
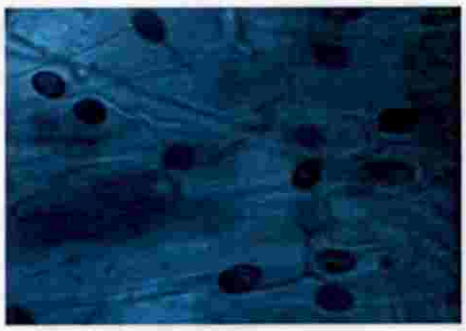


Plate 5: Morphological and microscopic characterss of *Lentinus tuber-regium*

a. Sporocarp b. spores (40 X)

VLYN 11

Sporocarp



Spores (40 X)

Plate 6 : Morphological and microscopic characters of *Lentinus connatus*

a

b.

VLYN 12

VLYN 13

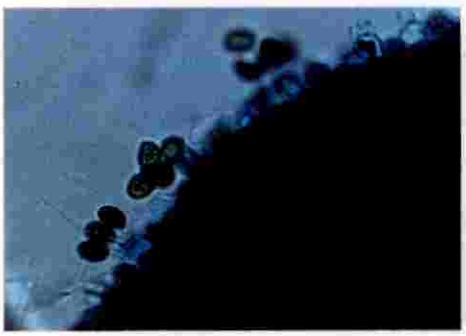


Plate 7 : Morphological and microscopic characters of *Lentinus* sp.

a Sporocarp b Spores (40 X)

**Table 3. Gill and spore characters of strains of *Lentinula edodes***

| Strains | Gills | | | | Spores | | | |
|---|---|---|---|---|---|---|---|---|
| | Arrangement | Texture | Margin | Gills/cm | Spore shape | Spore size (µm) | Spore print | Spore reaction |
| LE-1 | Free | Soft | Smooth | 22 | Ellipsoidal | 3.59 x 2.32 | White | Cyanophilic |
| LE-2 | Free | Soft | Smooth | 28 | Ellipsoidal | 4.41 x 2.35 | White | Cyanophilic |
| LE-3 | Free | Soft | Smooth | 24 | Ellipsoidal | 3.76 x 2.08 | White | Cyanophilic |
| LE-4 | Free | Soft | Smooth | 22 | Ellipsoidal | 3.84 x 2.12 | White | Cyanophilic |
| LE-5 | Free | Soft | Smooth | 26 | Ellipsoidal | 3.59 x 1.96 | White | Cyanophilic |
| LE-6 | Free | Soft | Smooth | 25 | Ellipsoidal | 3.25 x 1.98 | White | Cyanophilic |

**Table 4. Morphological characters of native isolates of *Lentinus* species**

| Species | Classification of isolates | Isolates | Pileus | | | | Stipe | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Shape | Colour | Diameter (cm) | Texture | Length (cm) | Shape | Attachment | Surface |
| *Lentinus squarrosulus* | Class I | VLYN-1 | Concave | White | 11.6 | Fibrous | 5.16 | Cylindrical | Eccentric | Fibrous |
| | | VLYN-2 | Concave | Light yellow | 7.43 | Leathery | 3.90 | Cylindrical | Eccentric | Fibrous |
| | | VLYN-3 | Concave | White | 15.91 | Fibrous | 8.00 | Cylindrical | Eccentric | Fibrous |
| | | VLYN-4 | Concave | White | 14.50 | Fibrous | 7.80 | Cylindrical | Eccentric | Fibrous |
| | | VLYN-5 | Concave | Creamy white | 10.00 | Fibrous | 6.40 | Cylindrical | Eccentric | Fibrous |
| | | VLYN-6 | Concave | Creamy white | 6.00 | Fibrous | 5.91 | Cylindrical | Eccentric | Fibrous |
| *Lentinus giganteum* | Class II | VLYN-7 | Convex | Brown | 75.00 | Fleshy | 61.00 | Cylindrical | Central | Hardy |
| *Lentinus tuber-regium* | Class III | VLYN-8 | Funnel | Cream yellow | 13.30 | Fibrous | 5.80 | Tapering | Eccentric | Fibrous |
| | | VLYN-9 | Funnel | Light yellow | 11.00 | Fibrous | 5.90 | Tapering | Eccentric | Fibrous |
| | | VLYN-10 | Funnel | Cream yellow | 23.90 | Fibrous | 2.12 | Tapering | Eccentric | Fibrous |
| *Lentinus connatus* | Class IV | VLYN-11 | Funnel | Light yellow | 13.20 | Fibrous | 5.60 | Tapering | Eccentric | Fibrous |
| *Lentinus* sp. | Class V | VLYN-12 | Funnel | Dark yellow | 14.50 | Fibrous | 5.00 | Tapering | Central | Fibrous |
| *Lentinus* sp. | Class VI | VLYN-13 | Convex | Yellow | 10.70 | Fleshy | 10.20 | Cylindrical | Central | Fibrous |

**Table 5. Gill and spore characters of native isolates of *Lentinus* species**

| Species | Isolates | Gills | | | | Spores | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Arrangement | Texture | Margin | Gills/cm | Spore shape | Spore size (µm) | Spore print | Spore reaction |
| *Lentinus squarrosulus* | VLYN-1 | Crowded | Soft | Smooth | 26 | Cylindric | 5.0-7.5 x 2.0-3.0 | White | Cyanophilic |
| | VLYN-2 | Crowded | Soft | Smooth | 28 | Cylindric | 4.5-7.2 x 1.8-3.0 | White | Cyanophilic |
| | VLYN-3 | Crowded | Soft | Smooth | 32 | Cylindric | 5.1-7.0 x 1.8-3.1 | White | Cyanophilic |
| | VLYN-4 | Crowded | Soft | Smooth | 29 | Cylindric | 4.9-7.8 x 1.9-2.1 | White | Cyanophilic |
| | VLYN-5 | Crowded | Soft | Smooth | 32 | Cylindric | 5.0-8.0 x 2.1-3.0 | White | Cyanophilic |
| | VLYN-6 | Crowded | Soft | Smooth | 25 | Cylindric | 5.0-7.2 x 1.9-3.0 | White | Cyanophilic |
| *Lentinus giganteum* | VLYN-7 | Free | Soft | Smooth | 21 | -- | -- | -- | --- |
| *Lentinus tuber-regium* | VLYN-8 | Crowded | Hardy | Smooth | 32 | Oblong cylindric | 6.5-8.0 x 4.1-4.9 | White | Cyanophilic |
| | VLYN-9 | Crowded | Hardy | Smooth | 29 | Oblong cylindric | 6.2-8.0 x 2.2-3.0 | White | Cyanophilic |
| | VLYN-10 | Crowded | Hardy | Smooth | 35 | Oblong cylindric | 6.5- 8.0 x 2.1-3.0 | White | Cyanophilic |
| *Lentinus connatus* | VLYN-11 | Crowded | Hardy | Smooth | 22 | Cylindric | 5.8-8.1 x 2.0-2.8 | White | Cyanophilic |
| *Lentinus* sp. | VLYN-12 | Free | Hardy | Smooth | 32 | Cylindric | 3.6-4.2 x 1.9-2.9 | White | Cyanophilic |
| *Lentinus* sp. | VLYN-13 | Free | Soft | Smooth | 15 | Cylindric | 5.3-6.2 x 4.3-5.7 | White | Cyanophilic |

soft textured with 21 number of gills/ cm. Based on these characters the isolate was identified as *Lentinus giganteum*.

The isolates VLYN-8 to VLYN-10 (Class III) were funnel shaped and cream yellow to light yellow in colour. The pileus was fibrous in nature with dia. varying from 11.00 cm -23.90 cm. Stipe was tapering with eccentric attachment to the pileus . The isolates had crowded gills which were hardy with smooth margin. Gill number varied from 29-35 per cm. They had oblong cylindric spores with spore size varying 6.2 - 8.0 µm x 2.1 - 4.9 µm (Plate 5). Spore print was white with cyanophilic spore reaction. The isolates were identified as *Lentinus tuber-regium* based on these morphological characters.

The pileus of VLYN-11 (Class IV) isolate was funnel shaped and was light yellow in colour with fibrous texture with pileus diameter of 13.20 cm. Stipe was tapering with eccentric attachment having a stipe length of 5.60 cm and was fibrous in texture. The isolates had crowded gills which are hardy with smooth margin. Gill number was 22 number / cm. Spores were cylindric with spore size varying 5.8 µm - 8.1 µm x 2.0 µm - 2.8 µm (Plate 6). Spore print was white with cyanophilic spore reaction. The morphological studies identified the isolate as *Lentinus connatus*.

Isolate VLYN-12 (Class V) was funnel shaped with dark yellow pileus with stipe tapering with central attachment. Isolate VLYN-13 (Class IV) had convex shaped with yellow pileus which had a dia. of 10.70 cm with fleshy texture. Stipe was cylindrical with fibrous texture. Both isolates had cylindric spores. The isolates VLYN-12 and VLYN-13 were unidentified on species level based on these morphological characters (Plate 7).

From among the various species of *Lentinus* sp. obtained during the survey, *Lentinus tuber-regium* and *Lentinus connatus* were used for further molecular characterization and cultivation studies to confirm their phylogenetic relationship with the procured cultivable strains of *Lentinula edodes*.

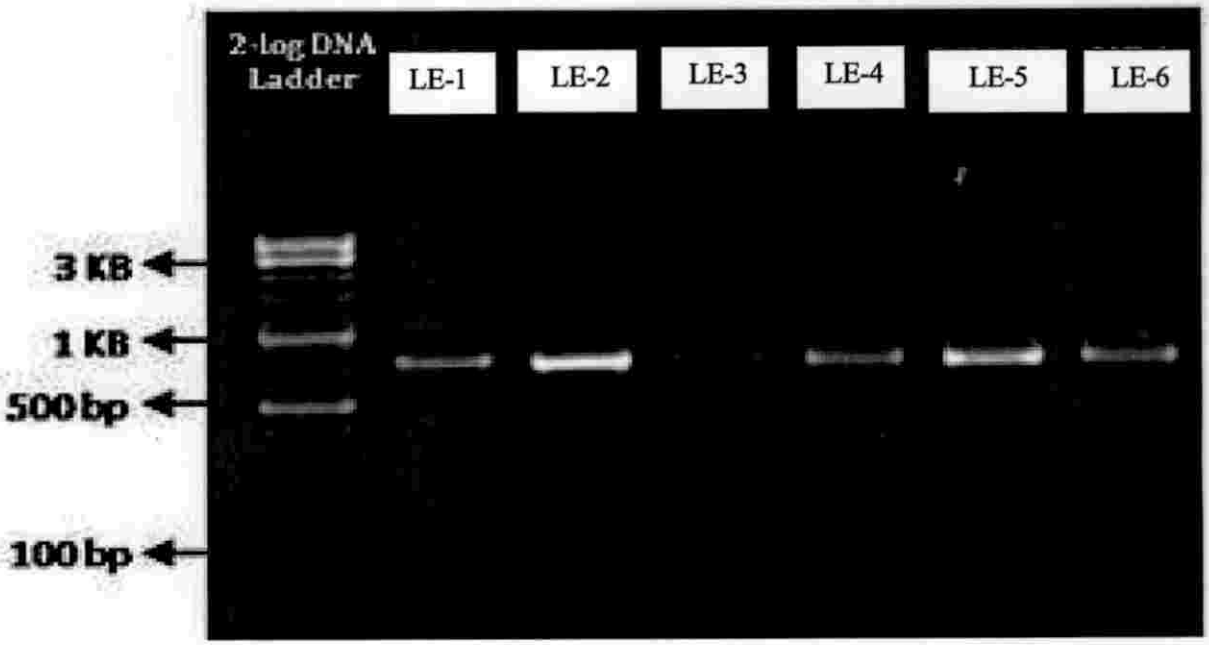



**Plate 8: PCR amplification profile of the different *L. edodes* strains with the universal primers. ITS 1F and ITS-4R**

## 4.3. MOLECULAR CHARACTERISATION OF PROCURED STRAINS OF LENTINULA EDODES AND LOCAL ISOLATES OF LENTINUS SP.

Results of ITS sequencing and RAPD analysis of procured strains of *L. edodes* and the local isolates of *Lentinus* sp. are presented below.

### *4.3.1. DNA Barcoding by partial sequencing of Internal Transcribed Spacer (ITS) region*

***Lentinula* sp**

ITS region refers to the spacer DNA (non coding DNA) situated between small ribosomal RNA (r RNA) and large subunit (r RNA) genes in the chromosome or the corresponding transcribed region in the poly cistronic r RNA precursor transcript. The ITS region is the most widely sequenced DNA region in molecular biology of fungi and has been recommended as the universal fungal barcode sequence. It has typically been most useful for molecular systematics at species level and even between species (to identify geographic races). Hence we had sequenced the ITS regions of the Shiitake strains selected using universal ITS primer for fungi.

ITS regions of the six strains of *Lentinula edodes* were sequenced for determining the phylogenetic relationship between the strains. Amplification of the ITS rDNA region of the six strains of *L. edodes* using ITS- 1F and ITS- 4R primers yielded a PCR product between 700 bp and 800 bp long (Plate 8). Later the PCR amplification was carried out followed by sequencing using the Big-Dye Terminator v3.1 Cycle sequencing kit. The sequence identity was checked using Sequence Scanner Software v1 (Applied Biosystems). The sequences obtained for six different strains of *L. edodes* were as follows.

>LE-1 (Pantnagar)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT
TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC
ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA
TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCCGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

>LE-2(Pantnagar)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT
TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC
ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA

TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCCGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

>LE-3(Pantnagar)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT
TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC
ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA
TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCCGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

>LE-4 (Pantnagar)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT
TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC

ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA
TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCTGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

>LE-5 (Pantnagar)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT
TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC
ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA
TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCTGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

>LE-6 (Udaipur)

TGTTGCTGGCCTTTGGGTATGTGCACATCCTCCTCCGATTTCTATTCATCC
ACCTGTGCACTTTTTGTAGGAGTTCTTTCATCGGGTTTTTGAAGGTGCTCA
TTATGAGTTACTTGAAAAGACTAGTTGACAAGGCTTCTATGTTCTTATAAA
CCATTGAAGTATGTTATAGAATGATCTTGTTATTGGGACTTTATTGACCCT
TTAAACTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCCCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTCTGGTATTCCGGAGGGCAT
GCCTGTTTGAGTGTCATTAAATTCTCAACTTTATAAGTTTTTACTTATTAA
AGCTTGGATGTTGGAGGCTTGCAGGCGTTTGTCAGCTCCTCTTAAATTTAT

TAGTGGGAACCCTGTTTTGTTAGTTCTAACCTTGGTGTGATAATTATCTAC
ATTTTGGTGGAACCTTACAATAATAAAGCTCTATTGGTTTGGGTTGTTGCA
TTTAGTTTGCTCAATCTGTTCTATTCATTGGAGAAAAAGGGAAGTTCCGCT
TTCTAACTGTCTTGATTGACTATATATAACTTAT

Sequence similarity of the six strains of *L. edodes* with the nucleotide sequences in NCBI was searched using BLAST program. The BLAST results showed that the reference strains (LE-1 to LE-6) were 99-100 per cent similar with the known sequences of *L. edodes* available in NCBI database . This confirms that the strains procured from different institutes irrespective of the locality were similar.

A phylogenetic tree was constructed from ITS rDNA region sequences using Neighbour Joining (NT) method. The tree view (Fig 1) of the phylogenetic relationship with six strains of *L. edodes* with already known sequences yielded a dendogram which showed that LE-1, LE-3, LE-4, LE-5 and LE-6 strains were clustered in one group and LE-2 in another cluster.

***Lentinus* sp.**

ITS regions of the native isolates collected during the survey which were morphologically similar to *L. edodes* were sequenced. Sequences of these isolates were compared with available sequences in NCBI database using BLAST programme. The sequences of the native isolates obtained during the survey were as follows.

>SR431-LC-ITS

TTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAGAGTTCGATGATT
ATTGTCTCGTAAAGAGACGACTAGAAGCTGGCCTATAAAAACGCTTCAAC
GGTCGCGGCGTAGACAATTATCACACCGTGAGCCGATCCGCACAGGAACC
AAGCTAATGCATTTAAGAGGAGCCGACTTGACATTGAAGCAAGCCGACA
AGAACCTCCAAGTCCAAGCCTAAAGCAAGTCCCGTTAAGAACCCGTTAGG

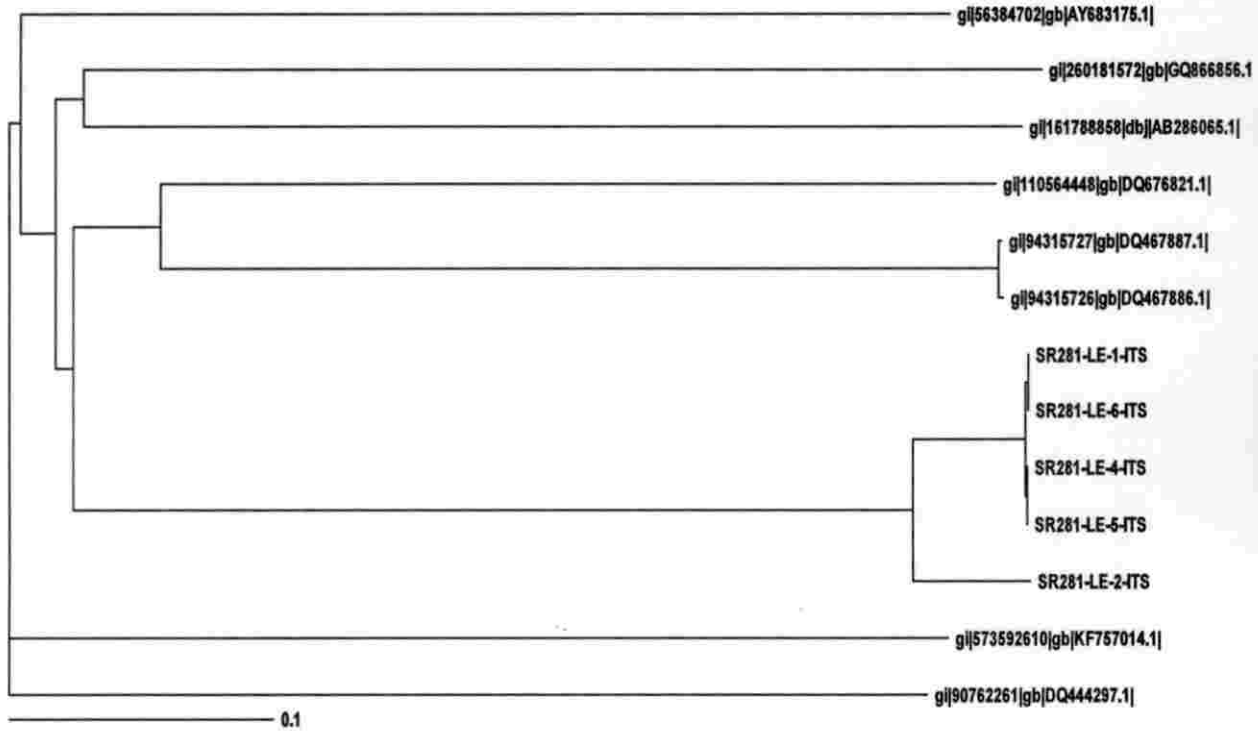



**Fig 1: Dendrogram showing the phylogenetic relationship of the sequences of *L. edodes* strains with already known sequences in NCBI database**

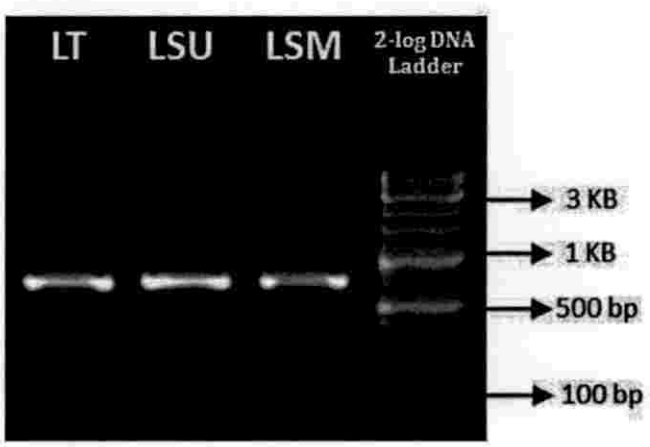



**Plate 9: PCR amplification profile of the native isolates of *Lentinus* sp. with the universal primers, ITS 1F and ITS-4R**

TTGAGAATTTCATGACACTCAAACAGGCATGCTCCTCGGAATACCAAGGA
GCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGAT
CCGTTGCTGAAAGTTGTATATAGATGCGTTACATCGCAATACACATTCTG
ATACTTTATAAGTGTTTGTAGTGAAACGTAGGCCCAGTAACAACTAGGCA
AGAAAAAAGCCCGTGAAGGCCCCTTTTCTCGCTTTCGAAGCTCCTGAAAC
CCACAGTAAGTGCACAGGTGTAGAGTGGATGAGCAGGGCGTGCACATGC
CTCGGAAGGCCAGCTACAACCCGTTTCAAAACTCGATAATGATCCTT

>SR431-LT-ITS

TTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAAATGTTCAATAATT
GTCCGAAGACGATTAGAGAGCTGGACACATTATAGTATTACATGCAACTG
GTGTAGATAATTATCACACCATGCGCAGAGGCAATAGAGTCCTGCTAATG
CATTTAAGGAGAGCTGACTCTGACAAGCCAGCAGCCCCCAACAATCCAAG
CCTATAATGACTTCAACAAAAGCCTTTATAGGTTGAGAATTTAATGACAC
TCAAACAGGCATGCCCCTCGGAATACCAAGGGGCGCAAGGTGCGTTCAA
AGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCG
CTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTGT
ATTAGGTTTATAGGCACATGGCCCAAAGATAAACGACATTCATTAACATA
CATTAGGGTGTGTAAATTAGATCACTGAGAGTCAGCCAGAAGGTATAAGA
ACCTCCCGACATCCAAGTCACTAATGACCTGAGAGATGACTTCAATGATC
TACAAAAAGTGCACAGGTGGTTGAATGGACTGATGAAGCGTGCACATGC
CCCTAGAGGCCAGCAACAGCTTCAAGAGTGAATTCATTAATGATCCTT

Amplification of the ITS rDNA region of two native isolates using ITS- 1F and ITS- 4R primers yielded a PCR product of 800 bp long (Plate 9). The sequence identity was checked using Sequence Scanner Software v1 (Applied Biosystems). The BLAST result showed that the native isolates were 99-100 per cent similar to *Lentinus tuber-regium* and *Lentinus connatus*.

### 4.3.2. RAPD analysis of *Lentinula* and *Lentinus* sp.

Good quality DNA was isolated from six strains of *L. edodes* for RAPD analysis. They were subjected to PCR using 15 random primers from Operon series *viz*., OPA- 01 to OPA -15. Out of these, five primers which gave good polymorphism were selected for the study (Plate 10). Their primer name, sequence, number of amplicons, number of monomorphic amplicons, number of polymorphic amplicons, per cent polymorpism are given in Table 6.

The details of amplicons produced by each primer is given below.

Primer OPA-02 :

This primer produced 5 amplicons within the range of 1.00 kb and 0.5 kb. This primer could produce 4 polymorphic bands. One polymorphic product each was present in LE-5 and LE-6 out of these one was unique for LE-5.

Primer OPA-03 :

Four amplicons within the range of 1 kb to 0.5 kb were produced by this primer. It could produce four amplicons of which 4 were polymorphic. This primer produced specific or unique bands in LE-3 and LE-5 strains.

Primer OPA-04:

The primer produced 3 amplicons within the range of 1 kb to 0.5 kb. All the three amplicons were polymorphic with 100 per cent polymorphism. Unique bands were observed in LE-4 strain.

Primer OPA-07:

Three amplicons were produced by this primer which was all polymorphic. Specific bands were observed in LE-5 strain by this primer.

**Table 6: Base sequence of RAPD primers, number of amplicons and percentage of polymorphism in strains of *L. edodes***

| Primer name | Sequence | No. of amplicons | No. of monomorphic amplicons | No. of polymorphic amplicons | Polymorphism (%) |
|---|---|---|---|---|---|
| OPA-02 | TGCCGAGCTG | 5 | 1 | 4 | 80.0 |
| OPA-03 | AGTCAGCCAC | 4 | 0 | 4 | 100.0 |
| OPA-04 | AATCGGGCTG | 3 | 0 | 3 | 100.0 |
| OPA-07 | GAAACGGGTG | 3 | 0 | 3 | 100.0 |
| OPA-09 | GGGTAACGCC | 3 | 1 | 2 | 66.6 |
| | Total | 8 | 2 | 16 | 386.7 |
| | Average | 3.4 | 0.4 | 3.2 | 77.0 |

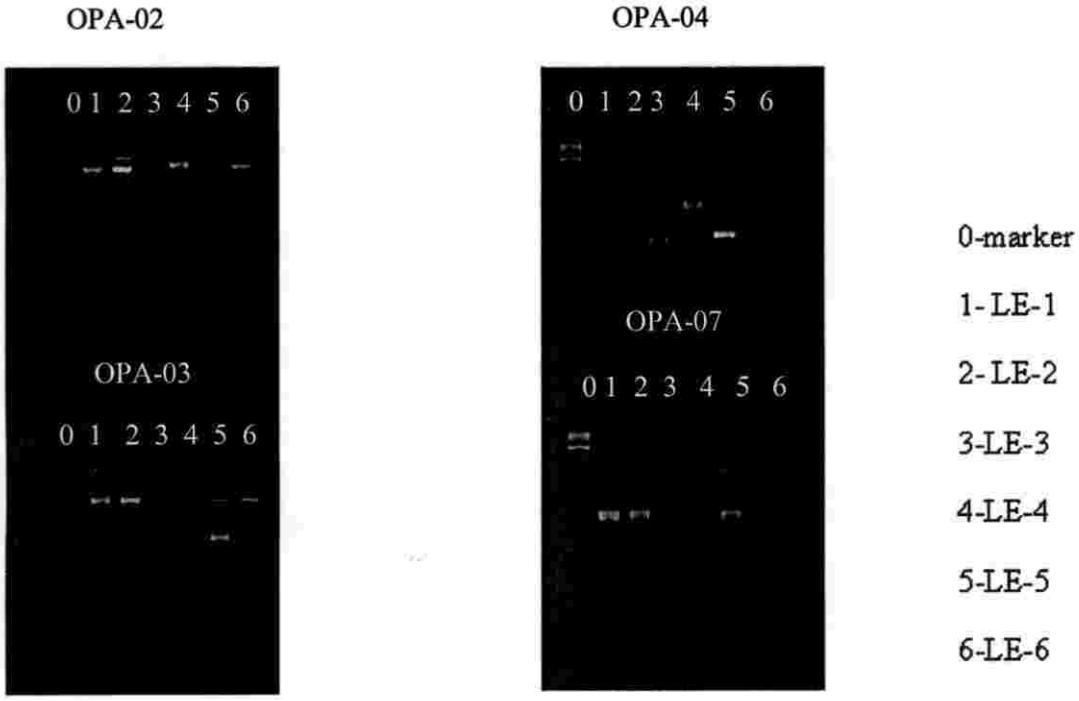



**Plate 10. RAPD profile of *L. edodes* strains using OPA-2, 3, 4,7 primers**

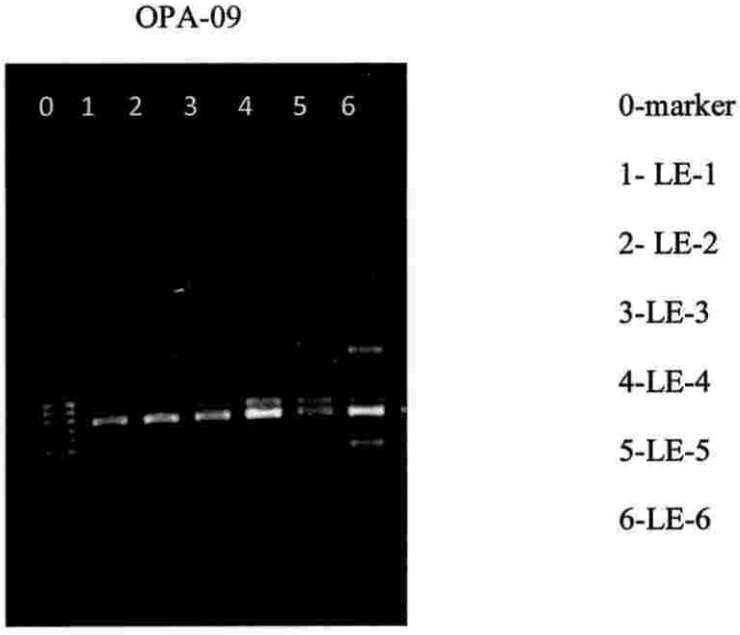



**Plate 11. RAPD profile of *L. edodes* strains using OPA -09 primer**

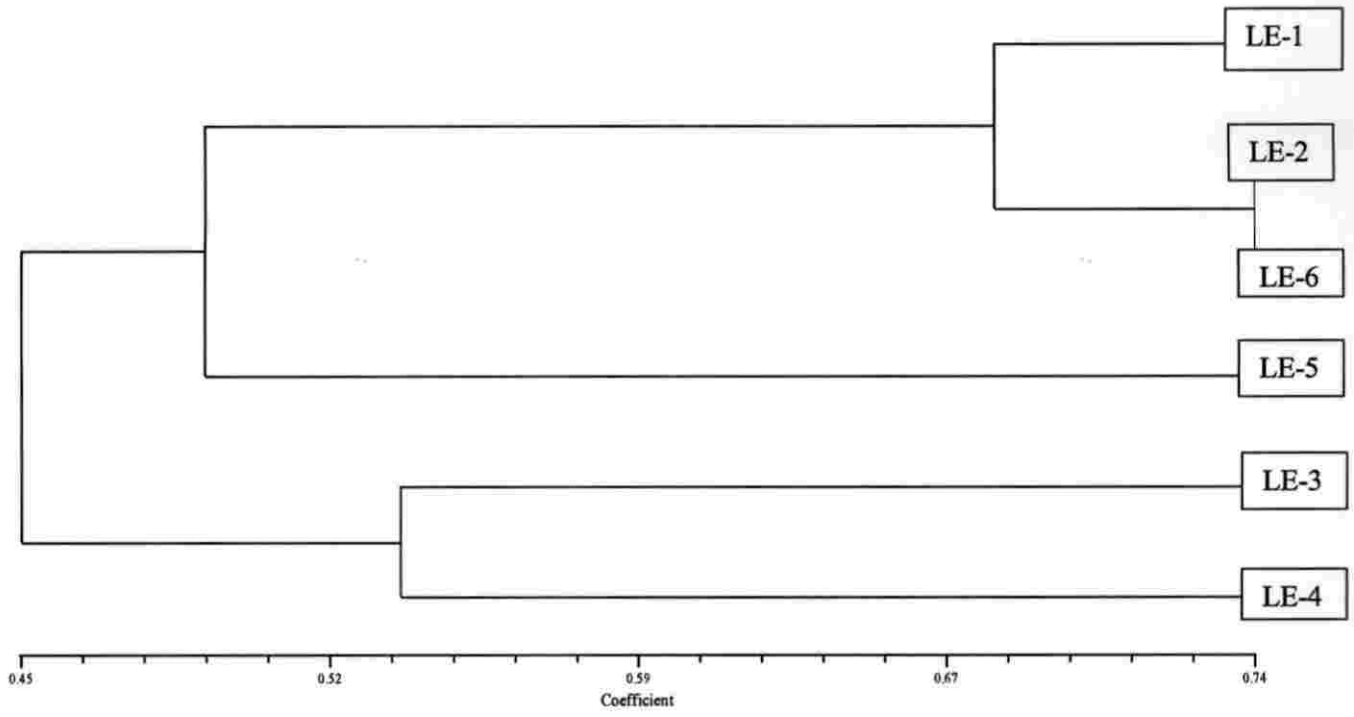



**Fig 2: Dendrogram of *L. edodes* strains based on RAPD profile**

Primer OPA-09 :

The primer produced three amplicons of which two were polymorphic. One unique band was recorded in LE-6 strain (Plate 11).

Similarity matrix calculated on the basis of the results is shown in Table 7. The matrix was subjected to unweighted pair group method analysis to develop a dendrogram (Fig 2). The random primers distinguished the strains into four phylogenetic groups. Phylogenetic tree generated using RAPD primers showed LE-2 and LE-6 strains in one cluster with a maximum similarity of 74 per cent. All the six strains clustered at 45 per cent of which LE-1, 2, 5 and 6 stains are clustered at 50 per cent similarity coefficient whereas LE-1 and LE-2 were clustered at 68 per cent, whereas LE-3 and LE-4 at 53 per cent.

## 4.4. CULTURAL STUDIES OF LENTINULA EDODES

Subsequent studies on nutritional requirements and physiological characters were focused on the strains of *L.edodes* which form the basis of the present investigation.

### 4.4.1. Effect of different media on the mycelial growth of *Lentinula edodes*

The extent and nature of mycelial growth of six strains of *L. edodes* that were evaluated on different solid and liquid media were recorded and presented as below.

#### *4.4.1. Effect of different solid media on the mycelial growth of Lentinula edodes*

The growth of *L. edodes* on petriplate was completed 12 days after inoculation (Plate 12). Among the various media tested, malt extract peptone dextrose agar was found to be the best, inducing maximum radial growth (9.00 cm) for all the strains. Oat meal agar and malt extract agar were found to be statistically on par with MEPDA in supporting their mycelial growth (Table 8).

**Table 7. Matrix of similarity coefficients of strains of *L. edodes***

| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
|---|---|---|---|---|---|---|
| LE-1 | 1.0000000 | | | | | |
| LE-2 | 0.7272727 | 1.0000000 | | | | |
| LE-3 | 0.4814815 | 0.3600000 | 1.0000000 | | | |
| LE-4 | 0.5714286 | 0.3571429 | 0.5384615 | 1.0000000 | | |
| LE-5 | 0.5862069 | 0.4285714 | 0.4000000 | 0.5333333 | 1.0000000 | |
| LE-6 | 0.6250000 | 0.7368421 | 0.4000000 | 0.5000000 | 0.4642857 | 1.0000000 |

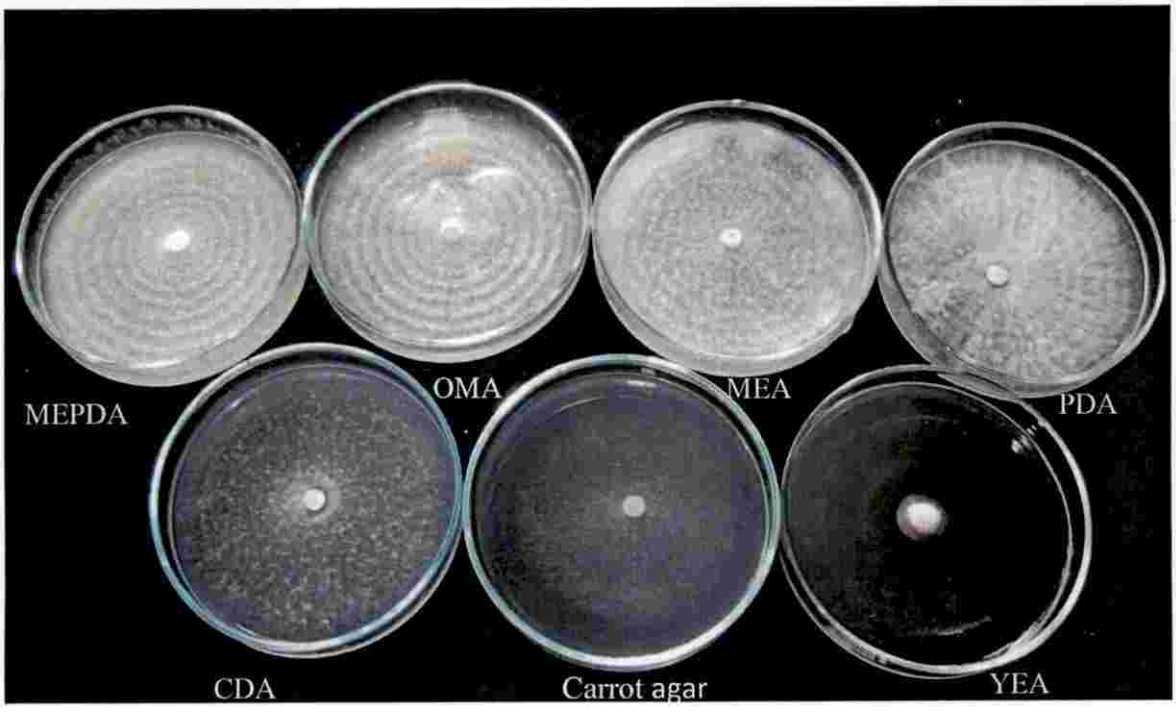



**Plate 12 : Growth of *L. edodes* on different solid media**

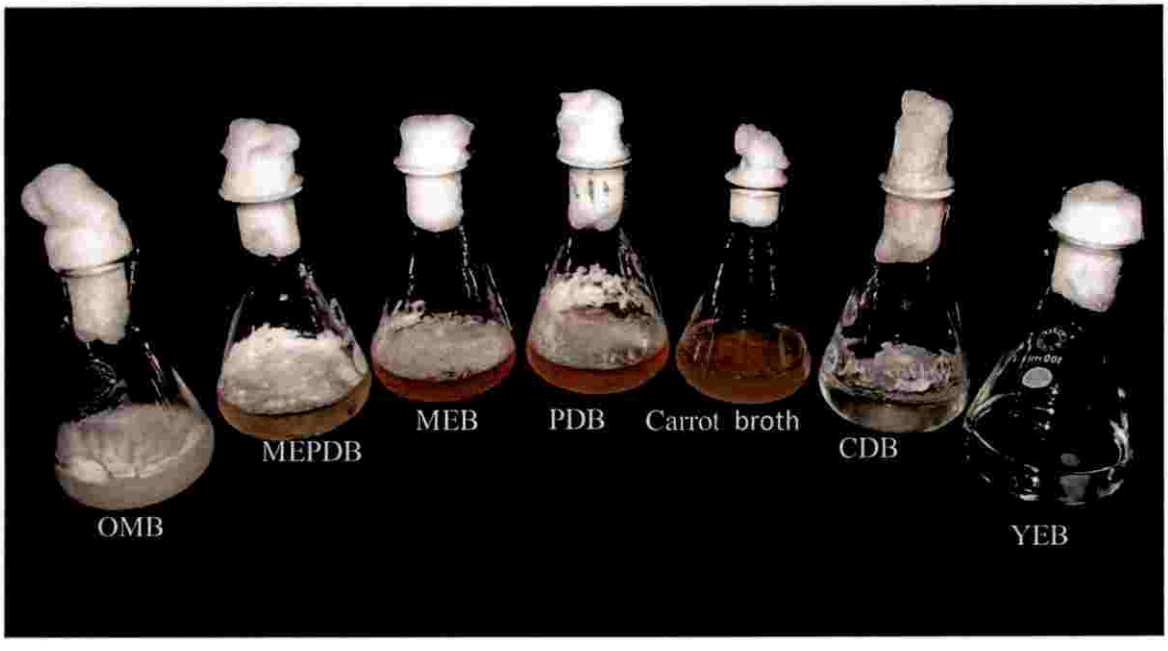



**Plate 13: Growth of *L. edodes* in different liquid media**

From the above studies, it was observed that significantly high radial growth of 9.00 cm was observed in strains LE-1, 2, 3 and 6 on malt extract peptone dextrose agar media twelve days after inoculation. Mycelial growth of LE-4 and LE-5 recorded 8.63 cm and 8.80 cm which were statistically on par. LE-1 also showed highest growth of 9.00 cm on oat meal agar. LE-2 and LE-6 strains showed 9.00 cm growth on malt extract and potato dextrose agar media. On oat meal agar, LE-2,3,4,5 and 6 strains produced 8.80 cm, 8.63 cm, 8.96 cm, 8.62 cm and 8.90 cm of radial growth respectively which was statistically on par with LE-1 (9.00 cm). On malt extract, LE-3 and LE-5 showed mycelial growth of 8.33 cm and 8.53 cm respectively which were on par with LE-1, 2 and 3. Mycelial growth of all strains except LE-1 and LE-2 were significantly low in potato dextrose agar, Czapek Dox and carrot agar. Radial growth of all the strains were least on yeast extract agar.

### *4.4.2. Effect of different liquid media on the mycelial growth of Lentinula edodes*

Biomass production of all the strains of *L. edodes* in liquid media were taken 25 days after inoculation. There was significant difference between each liquid media in influencing biomass production of *L.edodes* (Plate 13).

Maximum biomass production of all the six strains were obtained in oat meal broth ranging from 145 mg/ 50 ml to 405 mg/ 50 ml (Table 9). LE-2 and LE-4 strains produced biomass of 188.46 mg/ 50 ml and 118.66 mg/ 50 ml respectively in potato dextrose broth which were statistically on par with oat meal broth. Strain LE-3 produced biomass of 132.66 mg/ 50 ml on MEPDB, followed by LE-4 (74.40 mg/50 ml), LE-2 (63.80 mg/ 50 ml), LE-1 (48.13 mg/ 50 ml), LE-6 (40.30 mg/ 50 ml) and LE-5 (34.16 mg/ 50 ml). In malt extract broth, LE-2 produced maximum biomass of 71.40 mg/ 50 ml which was followed by 49.93 mg/ 50ml (LE-4), 39.30 mg/ 50 ml (LE-6), 39.06 mg/ 50 ml (LE-3), 31.00 mg/ 50 ml (LE-1) and 18.46 mg/ 50 ml (LE-5). In carrot broth, maximum biomass of 48.16 mg/ 50 ml was produced by LE-2 strain followed by 38.96 mg/ 50 ml (LE-4), 34.66 mg/ 50 ml (LE-3), 30.70 mg/ 50 ml

**Table 8. Growth of *L. edodes* strains on different solid media**

| Media | Radial growth 12 DAI (cm)* | | | | | |
|---|---|---|---|---|---|---|
| | Strains | | | | | |
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| MEPDA | 9.00 [a] | 9.00 [a] | 9.00 a | 8.63 [a] | 8.80 [a] | 9.00 [a] |
| OMA | 9.00 [a] | 8.80 [a] | 8.63 [a] | 8.96 [a] | 8.62 [a] | 8.90 [a] |
| MEA | 9.00 [a] | 9.00 [a] | 8.33 [a] | 8.13 [b] | 8.53 [a] | 9.00 [a] |
| PDA | 8.83 [a] | 9.00 [a] | 8.23 [a] | 6.36 [c] | 8.23 [b] | 8.36 [b] |
| CDA | 6.50 [c] | 8.36 [b] | 5.93 [c] | 5.20 [d] | 6.20 [d] | 6.90 [c] |
| CA | 8.36 [b] | 6.90 [c] | 7.66 [b] | 6.20 [c] | 8.13 [b] | 7.93 [b] |
| YEA | 1.00 [d] | 0.96 [d] | 1.00 [d] | 1.50[e] | 1.56 [e] | 1.43[d] |
| CD (0.05) | 0.23 | 0.25 | 0.43 | 0.28 | 0.36 | 0.31 |

** Average of three replications*

MEPDA- Malt extract peptone dextrose agar, OMA- Oat meal agar

MEA- Malt extract agar, PDA- Potato dextrose agar

CDA- Czapek Dox agar, CA- Carrot agar, YEA- Yeast extract agar

**Table 9. Growth of *L. edodes* strains in different liquid media**

| Media | Mycelial dry weight 25 DAI (mg/ 50 ml) * | | | | | |
|---|---|---|---|---|---|---|
| | Strains | | | | | |
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| MEPDB | 48.13 [c] | 63.80 [bc] | 132.66 [b] | 74.40 [bc] | 34.16 [c] | 40.30 [bc] |
| OMB | 168.23 [a] | 164.66 [a] | 405.50 [a] | 153.36 [a] | 150.33 [a] | 145.76 [a] |
| MEB | 31.00 [cd] | 71.40 [b] | 39.06 [c] | 49.93 [cd] | 18.46 [cd] | 39.30 [bc] |
| PDB | 104.73 [b] | 188.46 [a] | 92.23 [b] | 118.66 [ab] | 117.16 [b] | 52.46 [b] |
| CDB | 4.90 [d] | 4.43 [d] | 6.26 [d] | 3.00 [d] | 3.16 [d] | 4.23 [d] |
| CB | 8.46 [cd] | 48.16 [bc] | 34.66 [c] | 38.96 [cd] | 30.40 [c] | 30.70 [bc] |
| YEB | 14.96 [cd] | 6.33 [c] | 35.90 [c] | 4.83 [d] | 4.03 [d] | 7.50 [c] |
| CD (0.05) | 42.13 | 61.40 | 40.85 | 57.03 | 25.22 | 38.59 |

** Average of three replications*

MEPDB- Malt extract peptone dextrose broth, OMB- Oat meal broth

MEB- Malt extract broth, PDB- Potato dextrose broth

CDB- Czapek Dox broth, CB- Carrot broth, YEB- Yeast extract broth

(LE-6) and 30.40 mg/ 50 ml (LE-5) . Lowest biomass production of *L. edodes* strains were recorded (3.59 mg/ 50 ml to 14.96 mg/ 50 ml) in yeast extract broth.

## 4.5. NUTRITIONAL REQUIREMENTS OF LENTINULA EDODES

Results of the influence of carbon and nitrogen sources on the growth of *L. edodes* are presented below.

### *4.5.1. Effect of different carbon sources on the mycelial growth and biomass production of Lentinula edodes*

Six different carbon sources namely sucrose, lactose, galactose, fructose, dextrose and mannitol were separately substituted in both solid and liquid media and tested for their efficacy in radial mycelial growth and biomass production of *L. edodes.*

*On solid media:*

The best strain LE- 6, and best medium malt extract peptone dextrose agar screened in 4.4.1. were used in the study. Results showed that different carbon sources substituted in MEPDA medium differed significantly in influencing the radial growth of *L. edodes* (Table 10).

Growth of *L. edodes* in different carbon sources indicated the full attainment of growth in petriplates 12 days after inoculation (Plate 14). Dextrose containing medium were significantly superior with radial growth of 9.00 cm which was statistically on par with sucrose (8.93 cm), mannitol (8.90 cm), galactose (8.87 cm) and fructose (8.63 cm). Lactose proved to be the least significant with radial growth of 5.40 cm .

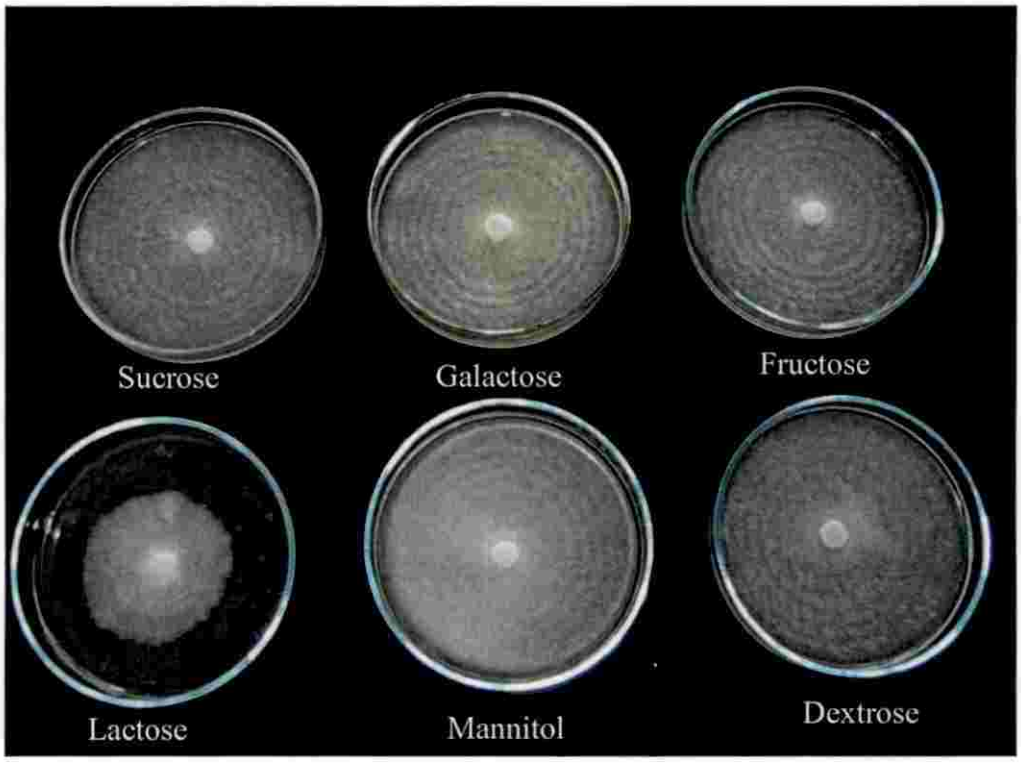



**Plate 14 : Growth of *L.edodes* on solid media amended with different carbon sources**

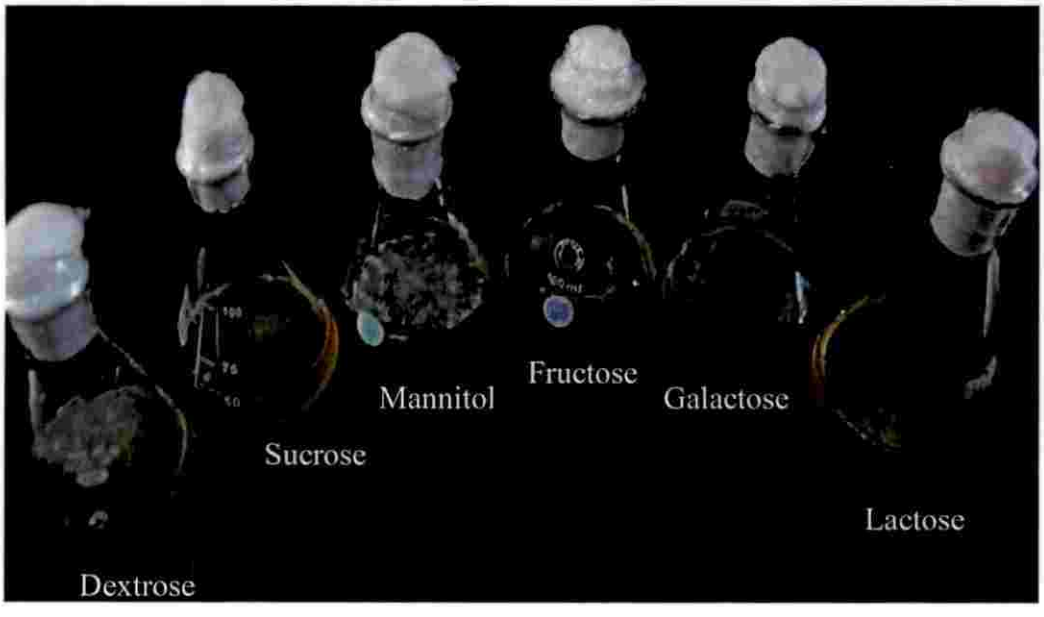



**Plate 15: Growth of *L.edodes* in liquid media amended with different carbon sources**

**Table 10: Growth of *L.edodes* on solid medium amended with different carbon sources**

| Carbon sources | Radial growth 12 DAI (cm)* | |
|---|---|---|
| | 9 days | 12 days |
| Sucrose | 7.80 | 8.93 [a] |
| Lactose | 4.87 | 5.40 [b] |
| Galactose | 7.20 | 8.87 [a] |
| Fructose | 7.36 | 8.63 [a] |
| Dextrose | 7.63 | **9.00** [a] |
| Mannitol | 7.70 | 8.90 [a] |
| CD (0.05 ) | 0.28 | 0.37 |

** Average of three replications*

*In liquid media :*

Different carbon sources substituted in MEPDB differed significantly in influencing the biomass production of *L. edodes*.

Medium amended with mannitol as carbon source were significantly superior with biomass production of 87 mg/ 50 ml of the broth which was statistically on par with fructose (77 mg/ 50 ml) (Plate 15). Medium containing galactose and sucrose produced biomass of 72.27 mg/ 50 ml and 65.20 mg/ 50 ml respectively. Other carbon sources like dextrose and lactose were found to be least significant with biomass production (61.60 mg/50 ml and 58.40 mg/ 50 ml of biomass per 50 ml of broth) (Table 11).

### *4.5.2. Effect of different nitrogen sources on the mycelial growth and biomass production of Lentinula edodes*

Both organic and inorganic nitrogen sources were evaluated by substitution in solid and liquid media( Czapek Dox) and the results are presented below.

*On solid medium*:

Growth of *L. edodes* on different nitrogen sources were tested separately on Czapek Dox medium. Radial growth of *L. edodes* was significantly superior (9.00 cm) in the medium amended with organic nitrogen source, peptone within 14 days after inoculation (Plate 16). This was closely followed by growth on inorganic sources like ammonium chloride (8.57 cm) and sodium nitrate (8.40 cm). Among the other nitrogen sources tested, radial growth on beef extract (8.03 cm) was significantly higher compared to potassium nitrate (7.40 cm). Ammonium carbonate was found be least significant among all others (Table 12).

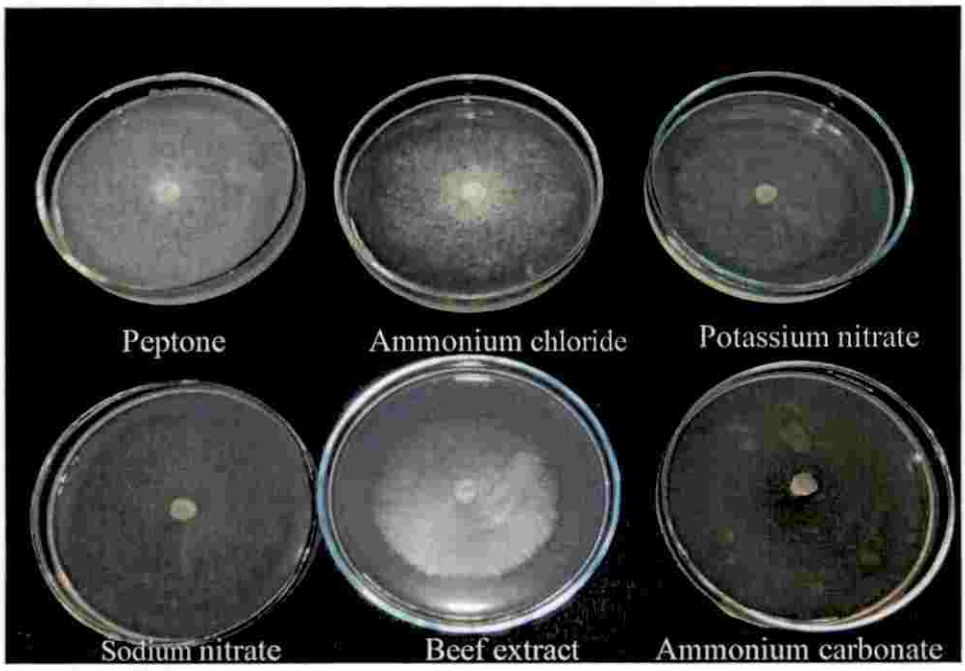



**Plate 16 : Growth of *L.edodes* on solid media amended with different nitrogen sources**

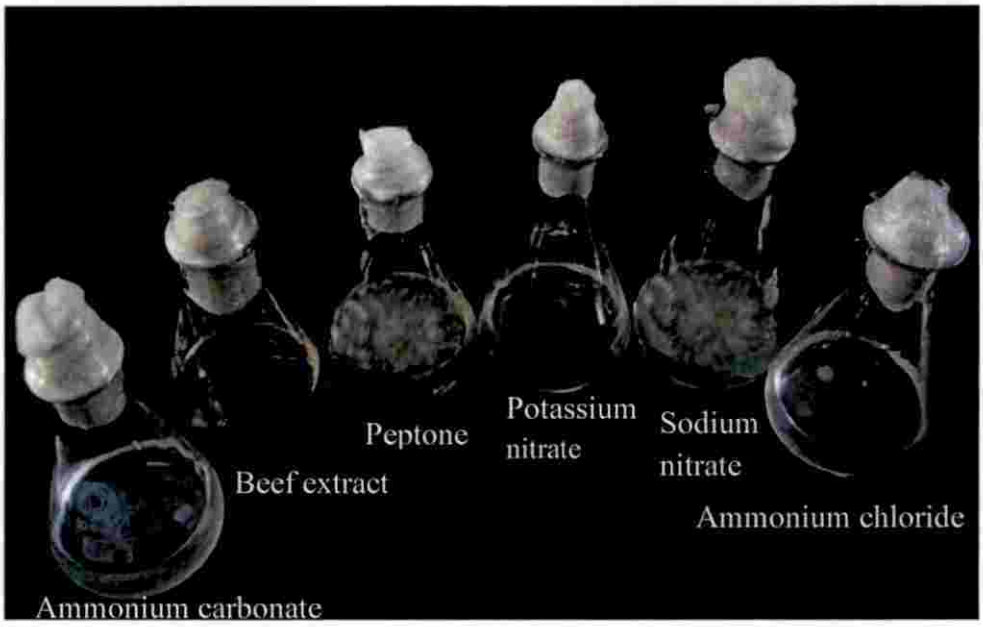



**Plate 17 : Growth of *L.edodes* in liquid media amended with different nitrogen sources**

**Table 11: Growth of *L.edodes* in liquid medium amended with different carbon sources**

| Carbon sources | Mycelial dry weight 25 DAI (mg/ 50 ml)* |
|---|---|
| Sucrose | 65.20 [cd] |
| Lactose | 58.40 [d] |
| Galactose | 72.27 [bc] |
| Fructose | 77.00 [ab] |
| Dextrose | 61.60 [cd] |
| Mannitol | **87.00** [a] |
| CD (0.05 ) | 13.59 |

** Average of three replications*

**Table 12. Growth of *L.edodes* on solid medium amended with different nitrogen sources**

| Nitrogen sources | Radial growth 12 DAI (cm)* | |
|---|---|---|
| | 7 th day | 14 th day |
| Beef extract | 2.86 | 8.03 [c] |
| Ammonium carbonate | 0.00 | 0.00 [e] |
| Potassium nitrate | 3.17 | 7.40 [d] |
| Peptone | 3.56 | **9.00** [a] |
| Sodium nitrate | 3.20 | 8.40 [b] |
| Ammonium chloride | 3.60 | 8.57 [b] |
| CD (0.05 ) | 0.18 | 0.20 |

** Average of three replications*

**Table 13. Growth of *L. edodes* in liquid medium amended with different nitrogen sources**

| Nitrogen sources | Mycelial dry weight 25 DAI (mg/ 50 ml)* |
|---|---|
| Beef extract | 1.21 [b] |
| Ammonium carbonate | 1.03 [b] |
| Potassium nitrate | 1.08 [b] |
| Peptone | 2.10 [a] |
| Sodium nitrate | **2.24** [a] |
| Ammonium chloride | 1.03 [b] |
| CD (0.05 ) | 0.34 |

**Average of three replications*

*In liquid medium*:

Biomass production in medium amended with sodium nitrate as nitrogen source (2.24 mg/ 50 ml) was significantly superior and was on par with peptone (2.10 mg/ 50 ml) (Plate 17). This was followed by biomass production in beef extract( 1.21 mg/ 50 ml) which was on par with biomass production of medium amended with potassium nitrate, ammonium chloride and ammonium carbonate respectively (1.08 mg/ 50 ml,1.03 mg/ 50 ml and 1.03 mg/ 50 ml) (Table 13).

## 4.6. ENVIRONMENTAL REQUIREMENTS OF LENTINULA EDODES

### *4.6.1. Effect of different temperature on the mycelial growth and biomass production of Lentinula edodes*

Results of the influence of varying temperature conditions on the growth of strains of *L. edodes* on solid and liquid (MEPD) medium are presented below.

*On solid medium:*

All strains of *L. edodes* attained significantly higher radial growth (8.63 cm to 9.00 cm) at 20 °C (Plate 18). This was closely followed by growth at 25 °C temperature where LE-6 showed maximum radial growth (8.30 cm) followed by LE-1(8.03 cm), LE-2 (8.17 cm), LE-3 (8.00 cm), LE- 4 (7.60 cm) and LE-5 (7.43 cm). Radial growth was comparatively high at room temperature (28 ±2 $^{O}$C) (5.23 cm to 6.03 cm) when compared with lower temperatures of 15 $^{O}$C and 10 $^{O}$C. Least significant growth was observed at 5 $^{O}$C ( 5.23 cm to 6.03 cm) (Table 14).

*In liquid medium:*

All the strains produced significantly higher biomass production at 20 °C (58.73 mg/ 50 ml to 81.90 mg/ 50 ml) (Plate 19). Significantly superior biomass of 56.90 mg/ 50 ml at 25 $^{O}$C was produced by LE-6 strain , which was followed by LE-5 (55.60 mg/ 50 ml), LE-1 (52.40 mg/ 50 ml), LE-3 (50.23 mg/ 50 ml), LE-2 (46.87

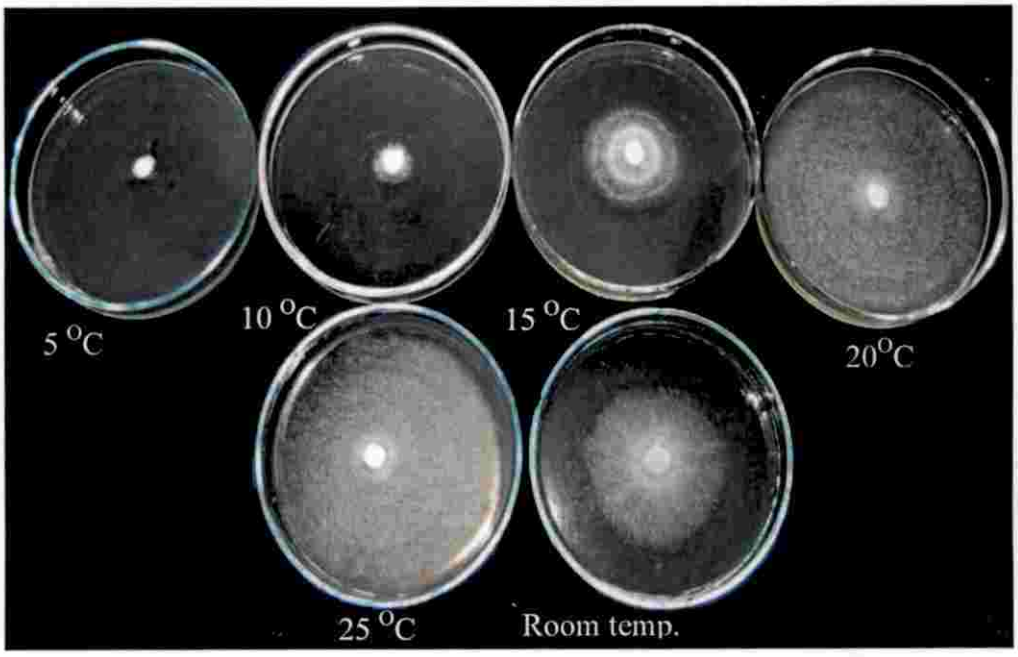



**Plate 18: Growth of *L.edodes* at different temperatures on solid media**

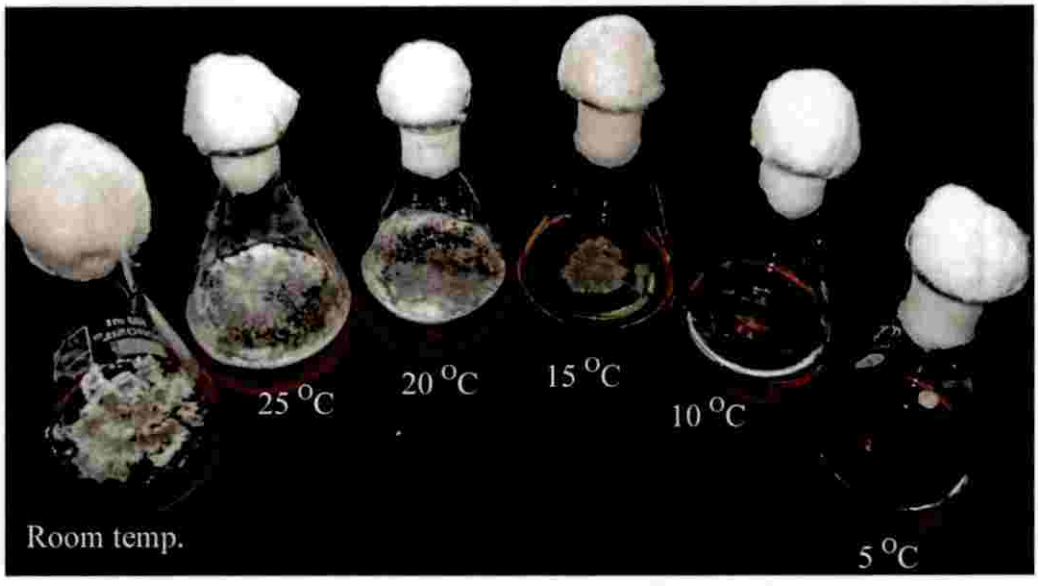



**Plate 19 : Growth of *L.edodes* at different temperatures in liquid media**

**Table 14. Growth of *L. edodes* strains at different temperature on solid media**

| Temperature (°C) | Radial growth 12 DAI (cm)* | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| 5 | 0.90 [f] | 0.90 [d] | 0.90 [e] | 0.90 [e] | 0.90 [f] | 0.90 [f] |
| 10 | 1.50 [e] | 1.30 [d] | 1.50 [e] | 1.26 [e] | 1.70 [e] | 1.77 [e] |
| 15 | 4.20 [d] | 3.73 [c] | 4.03 [d] | 3.96 [d] | 3.70 [d] | 3.97 [d] |
| 20 | 8.76 [a] | 8.73 [a] | 8.63 [a] | 8.67 [a] | 8.70 [a] | 9.00 [a] |
| 25 | 8.03 [b] | 8.17 [b] | 8.00 [b] | 7.60 [b] | 7.43 [b] | 8.30 [b] |
| Room temp. (28± 2 °C) | 5.27 [c] | 5.26 [c] | 5.43 [c] | 5.60 [c] | 5.23 [c] | 6.03 [c] |
| CD(0.05) | 0.37 | 0.40 | 0.60 | 0.58 | 0.42 | 0.27 |

**Average of three replications*

**Table 15. Growth of *L. edodes* at different temperature in liquid media**

| Temperature (°C) | Mycelial dry weight 25 DAI (mg/ 50 ml) * | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| 5 | 4.47 [e] | 2.33 [e] | 3.93 [f] | 3.87 [e] | 2.40 [e] | 4.40 [c] |
| 10 | 9.63 [d] | 5.67 [e] | 8.00 [e] | 5.13 [e] | 4.20 [e] | 5.57 [c] |
| 15 | 14.13 [d] | 13.87 [d] | 11.93 [d] | 13.40 [d] | 11.70 [d] | 17.10 [b] |
| 20 | 62.67 [a] | 81.90 [a] | 78.80 [a] | 74.67 [a] | 65.70 [a] | 58.73 [a] |
| 25 | 52.40 [b] | 46.87 [b] | 50.23 [b] | 43.67 [b] | 55.60 [b] | 56.96 [a] |
| Room temp. (28±2 °C) | 25.00 [c] | 24.23 [c] | 24.17 [c] | 34.67 [c] | 30.93 [c] | 22.66 [c] |
| CD(0.05) | 4.65 | 4.22 | 2.46 | 2.96 | 3.25 | 8.93 |

**Average of three replications*

mg/ 50 ml) and LE-4 ( 43.67 mg/ 50 ml). The biomass production at room temperature (28 ± 2$^{O}$C) ( 22.66 mg/ 50 ml to 34.67 mg/ 50 ml) was significantly high compared to 15 $^{O}$C ( 11.93 mg/50 ml to 17.10 mg/ 50 ml) and 10 $^{O}$C (4.20 mg/ 50 ml to 9.63 mg/ 50 ml). Least significant biomass production was observed at 5 $^{O}$C by all the strains of *Lentinula edodes* (Table 15).

### *4.6.2. Effect of different light conditions on the mycelial growth and biomass production of Lentinula edodes*

Three different ranges of light conditions like ambient light, fluorescent light and darkness were tested in malt extract peptone dextrose medium for their efficacy in the radial growth and biomass production of *L. edodes.* Results of radial growth and biomass production of *L. edodes* under different ranges of light sources are presented below

*On solid medium:*

Significantly superior radial growth of 9.00 cm was observed in ambient light and darkness twelve days after inoculation, which was followed by fluorescent light (8.70 cm) (Table 16) (Plate 20).

*In liquid medium:*

In liquid medium, biomass production (84.93 mg/ 50 ml) of *L. edodes* was significantly high under conditions of total darkness throughout the period of study (25 days after inoculation) and was followed by ambient light producing a biomass of 51.70 mg/ 50 ml . Medium exposed to fluorescent light were least significant with biomass of (26.13 mg/ 50 ml) (Plate 21) (Table 17).

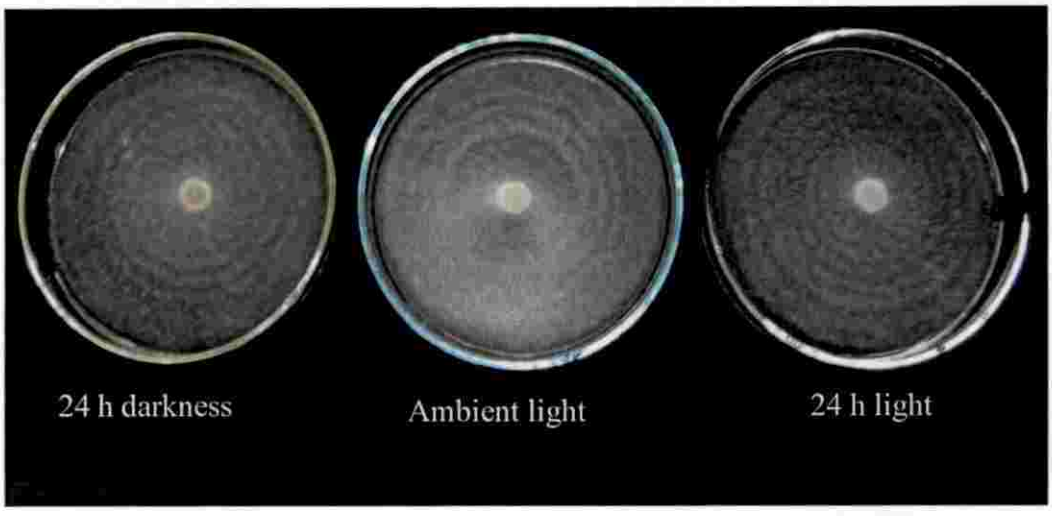



**Plate 20 : Growth of *L.edodes* in different light sources on solid media**

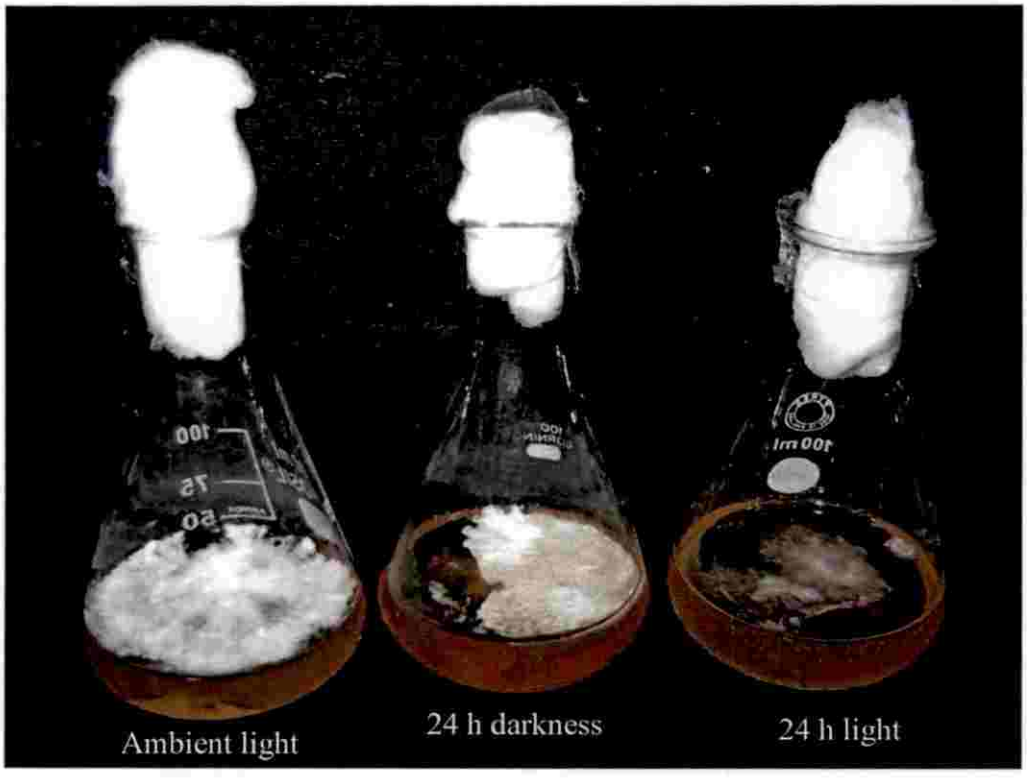



**Plate 21 : Growth of *L.edodes* in different light sources in liquid media**

**Table 16. Growth of *L.edodes* in different light sources on solid media**

| Light sources | Radial growth 12 DAI (cm) * | | |
|---|---|---|---|
| | 7 days | 9 days | 12 days |
| Ambient light ( 400 lux at day time + darkness at night time) | 4.30 | 8.47 | 9.00 [a] |
| Darkness | 4.20 | 8.27 | **9.00** [a] |
| 24 h light (500 lux) | 4.06 | 7.90 | 8.70 [b] |
| CD (0.05) | 0.16 | 0.12 | 0.11 |

**Average of three replications*

**Table 17. Growth of *L. edodes* in different light sources in liquid media**

| Light sources | Mycelial dry weight 25 DAI (mg/ 50 ml) |
|---|---|
| Ambient light<br><br>( 400 lux at day time + darkness at night time) | 51.70 [b] |
| Darkness | **84.93** [a] |
| 24 h light (500 lux) | 26.13 [c] |
| CD (0.05 ) | 13.15 |

**Average of three replications*

### *4.6.3. Effect of different pH on the mycelial growth and biomass production of Lentinula edodes*

Results of growth of strains of *L. edodes* in both solid and liquid (malt extract peptone dextrose) medium adjusted to six different H+ ion concentrations are presented below.

*On solid medium*:

All the six strains of *L. edodes* showed significantly superior radial growth (9.00 cm) at pH 6 which was followed by pH 7 (Plate 22). Radial growth of LE- 2, 3, 4 and 5 at pH 7 was statistically on par with the radial growth of all the strains at pH 6. At pH 8 (8.59 cm to 8.77 cm) and pH 9 (8.40 cm to 8.63 cm) there was a decrease in trend in the mycelial growth of the strains. pH 5 (4.20 cm to 5.50 cm) and 4 (3.13 cm to 3.73 cm) were found to be least significant (Table 18).

*In liquid medium* :

pH 6 was found to be significantly superior in biomass production of all the six strains (50.66 mg/50 ml to 154.00 mg/ 50 ml) (Plate 23). At pH 7, maximum biomass (114.86 mg/ 50ml) was produced by LE-5 strain followed by LE-3 (78.03 mg/ 50 ml), LE-2 ( 61.10 mg/ 50 ml), LE-1 (56.53 mg/ 50 ml) and LE-6 (50.26 mg/ 50 ml broth). Comparatively less biomass production was noticed at pH 8 and pH 9. Maximum biomass production at pH 5 was obtained in LE-5 (50.93 mg/ 50 ml) followed by LE-2 (50.90 mg/ 50 ml), LE-6 (50.86 mg/ 50 ml), LE-1 (50.76 mg/ 50 ml) . Least significant mycelial biomass was recorded in LE-4 (35.63 mg/ 50 ml broth) (Table 19).

## 4.7. SPAWN PRODUCTION TECHNOLOGY OF LENTINULA EDODES

Evaluation of various substrates (Plate 24) for spawn production using the best strain LE-6 of *Lentinula edodes* screened in 4.1.1. are presented below. The best

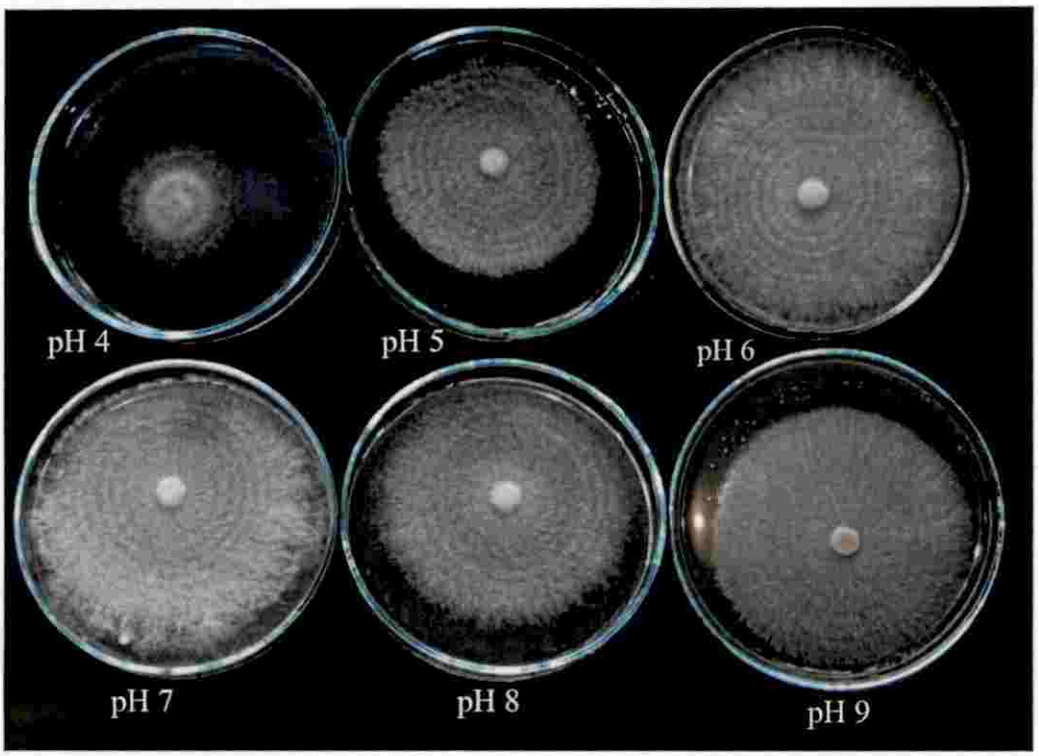



**Plate 22: Growth of *L.edodes* in different pH on solid media**

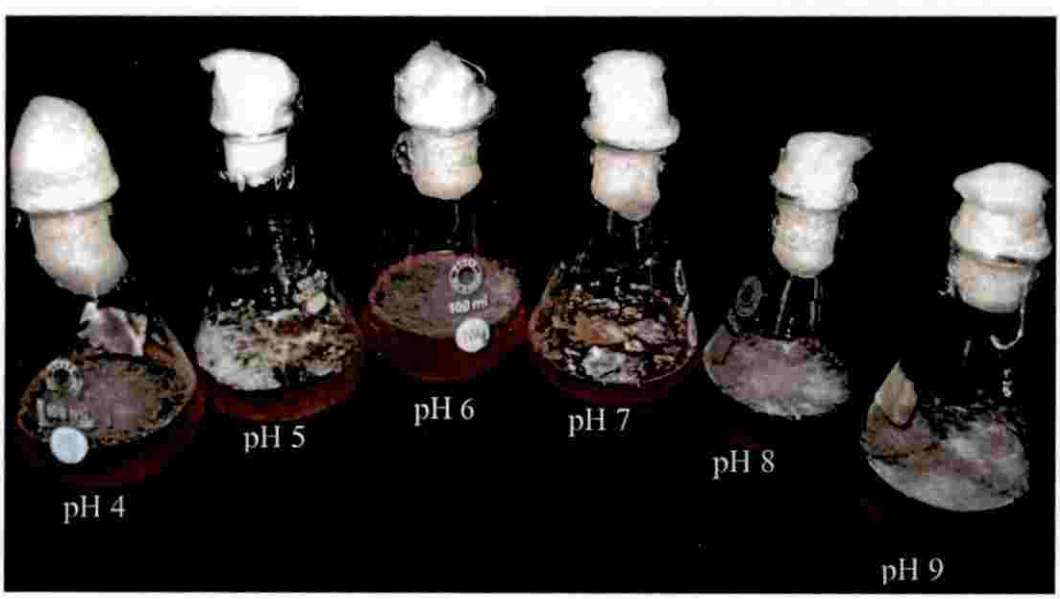



**Plate 23 : Growth of *L.edodes* in different pH in liquid media**

**Table 18. Growth of *L. edodes* strains in different pH on solid media**

| pH | Radial growth 12 DAI (cm) * | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| 4 | 3.37 [d] | 3.57 [d] | 3.70 [e] | 3.73 [e] | 3.54 [d] | 3.13 [d] |
| 5 | 4.93 [c] | 5.50 [c] | 5.17 [d] | 5.33 [d] | 4.20 [c] | 5.50 [c] |
| 6 | 9.00 [a] | 9.00 [a] | 9.00 [a] | 9.00 [a] | 9.00 [a] | 9.00 [a] |
| 7 | 8.68 [b] | 8.73 [ab] | 8.90 [ab] | 8.80 [ab] | 8.68 [b] | 8.82 [ab] |
| 8 | 8.59 [b] | 8.67 [b] | 8.63 [bc] | 8.63 [c] | 8.60 [b] | 8.77 [ab] |
| 9 | 8.50 [b] | 8.50 [b] | 8.53 [c] | 8.40 [bc] | 8.50 [b] | 8.63 [b] |
| CD (0.05 ) | 0.27 | 0.29 | 0.27 | 0.33 | 0.38 | 0.36 |

**Average of three replications*

**Table 19 . Growth of *L. edodes* strains in different pH in liquid media**

| pH | Mycelial dry weight 25 DAI (mg/ 50 ml) * | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| 4 | 45.00 $^{c}$ | 44.70 $^{c}$ | 44.90 $^{c}$ | 35.63 $^{c}$ | 37.46 $^{c}$ | 42.76 $^{b}$ |
| 5 | 50.76 $^{bc}$ | 50.90 $^{c}$ | 45.73 $^{c}$ | 35.63 $^{c}$ | 50.93 $^{c}$ | 50.86 $^{b}$ |
| 6 | 66.50 $^{a}$ | 103.90 $^{a}$ | 109.43 $^{a}$ | 50.66 $^{a}$ | 154.00 $^{a}$ | 60.63 $^{a}$ |
| 7 | 56.53 $^{b}$ | 61.10 $^{b}$ | 78.03 $^{b}$ | 47.96 $^{a}$ | 114.86 $^{b}$ | 50.26 $^{b}$ |
| 8 | 48.40 $^{bc}$ | 43.20 $^{c}$ | 52.23 $^{b}$ | 38.30 $^{e}$ | 36.63 $^{c}$ | 41.40 $^{b}$ |
| 9 | 47.00 $^{a}$ | 50.10 $^{e}$ | 51.63 $^{b}$ | 40.90 $^{bc}$ | 30.13 $^{c}$ | 45.10 $^{b}$ |
| CD(0.05) | 9.66 | 9.03 | 14.13 | 8.55 | 21.83 | 9.63 |

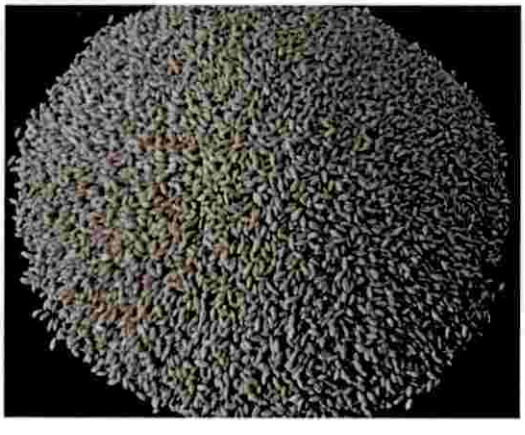
**Average of three replications*

Maize grain

Paddy grain

Ragi grain

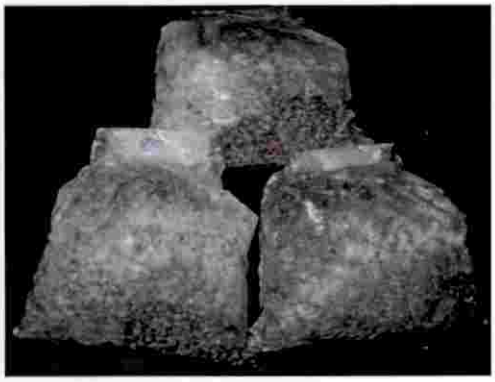
Sorghum grain



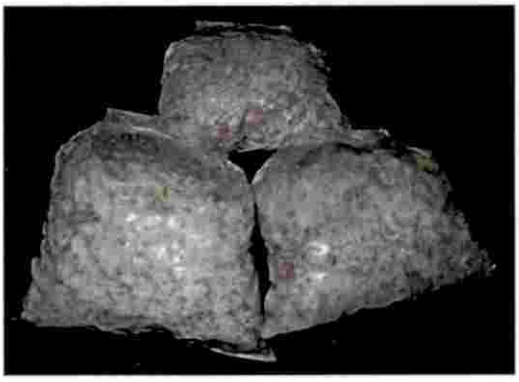
Wheat grain

Sawdust

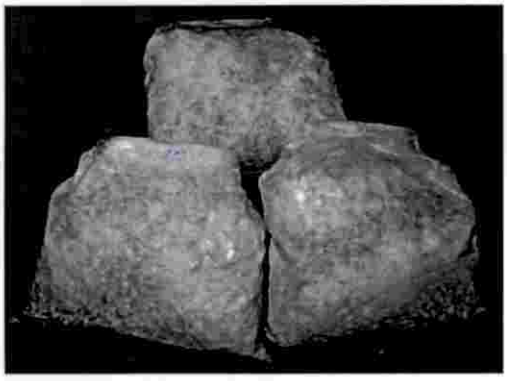
**Plate 24 : Different spawn substrates used for spawn production of *L. edodes***



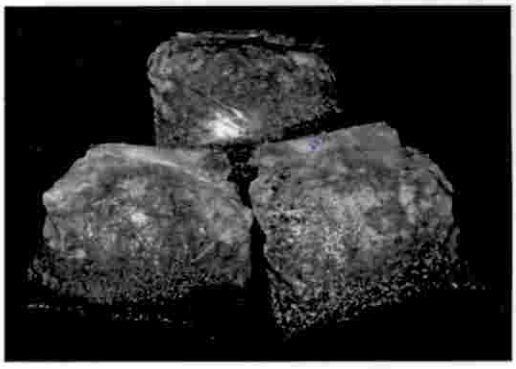
Bajra



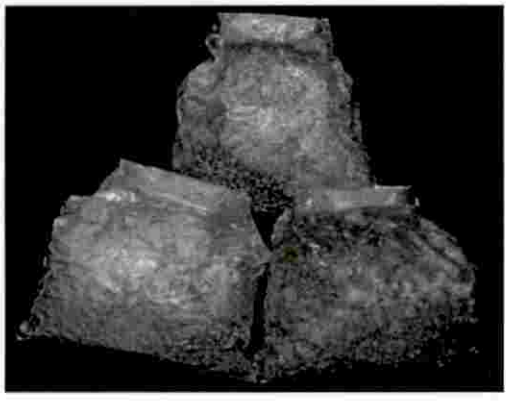
Maize



Paddy



Ragi



Wheat

Sawdust

**Plate 25: Growth of *L. edodes* on different substrates**

substrate was selected based on the minimum time taken for the spawn run and the nature and colour of the mycelial growth .

The grain substrate, maize was found to be significant superior with thick ,white and fluffy mycelium which entirely covered the grain substrate within a minimum period of 16.33 days (Plate 25) (Table 20). Paddy grains were colonized by *L. edodes* in 18.33 days. The growth in paddy grains was thick and fluffy making spawn highly compact.

Colonisation of *Lentinula edodes* on wheat grain was completed within a period of 20.66 days. Mycelial growth was thick and white in this substrate. The thick white but not fluffy growth was observed on sorghum grain substrate within a period of 21.33 days.

Ragi grains was colonized by the culture in 25.67 days producing thick, white and fluffy growth. *L. edodes* culture produced thin, white and root like mycelial growth in the non grain substrate sawdust and proved to be least significant which took 31.67 days to colonise the substrate completely.

## 4.8. CULTIVATION TECHNOLOGY OF LENTINULA EDODES

Results of the experimental trials conducted to standardize the cultivation technology of *L. edodes* in poly bag method (Plate 26) are presented below.

### *4.8.1. Evaluation of substrates*

Experiments on evaluation of combinations of three substrates and nine amendments of all the six strains (LE-1 to LE-6) were conducted by poly bag method (Plate 27 to 32). Results of the observations on growth parameters ( time taken for complete mycelial colonization, time taken for browning of mycelium of mushroom beds and time taken for initiation of sporocarp of mushroom beds ) and yield

**Table 20. Days taken by *L. edodes* for full colonisation on spawn substrates**

| Spawn substrates | Nature of mycelial growth | Colour of mycelial growth | Days taken for spawn run* |
|---|---|---|---|
| Maize | ++++ | White | 16.33 [a] |
| Paddy | ++++ | White | 18.33 [a] |
| Ragi | ++++ | White | 25.67 [c] |
| Wheat | +++ | White | 20.66 [b] |
| Sorghum | +++ | White | 21.33 [b] |
| Sawdust | ++ | White | 31.67 [d] |
| CD (0.05) | | | 3.16 |

**Average of five replications*

++++ - Thick and fluffy growth

+++ - Thick growth

++ - Poor/ thin growth

Whitening phase

Bump formation phase

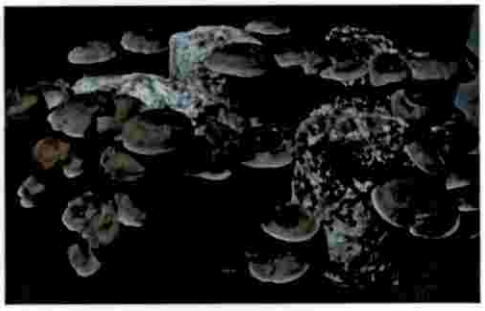
Brown exude formation phase

Browning phase

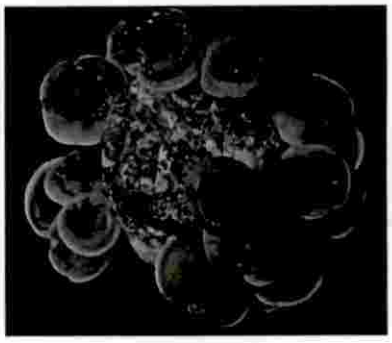
Star like crack formation phase

Pin head initiation phase

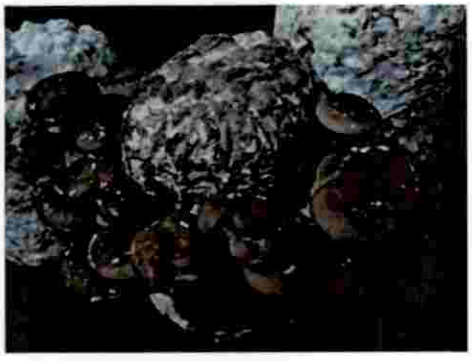
Basidiocarp formation



SD + 20% WB



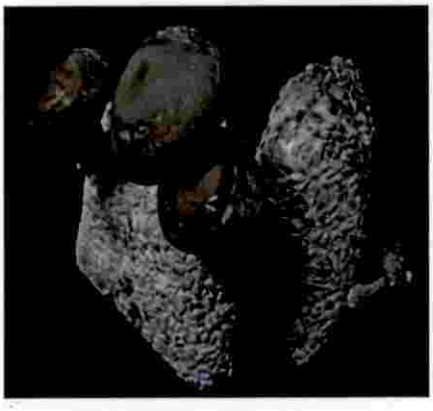
SD + 20 % RB



SD + 10 % WB

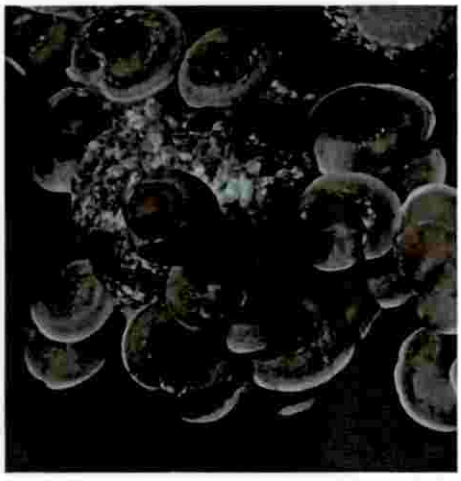
SD + 10% RB

Sawdust



PS + SD + 10 % RB

**Plate 27 : Cultivation of LE- 1 on different substrates**



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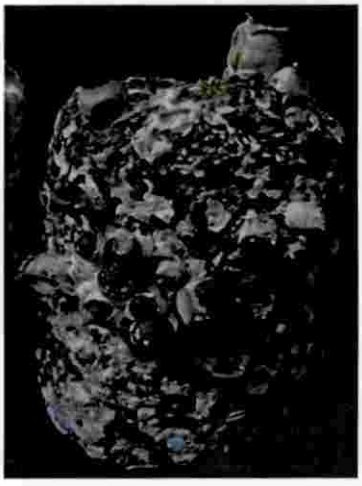

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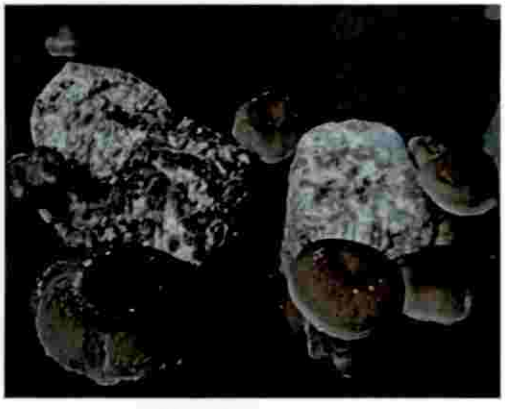


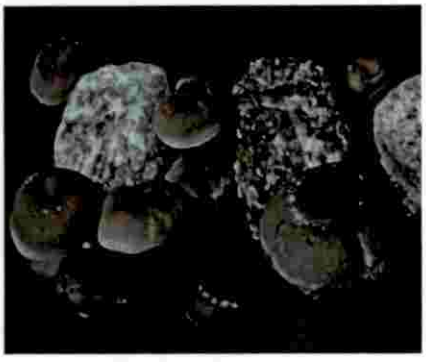
Sawdust

SD + 20 % WB

PS + SD + 20 % RB

SD + 10 % WB

**Plate 28 : Cultivation of LE- 2 on different substrates**



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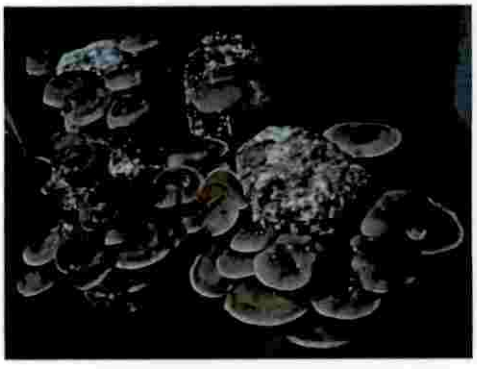
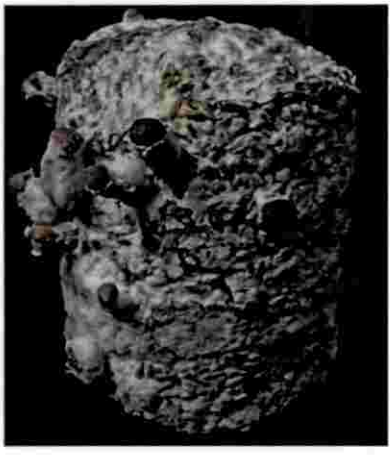
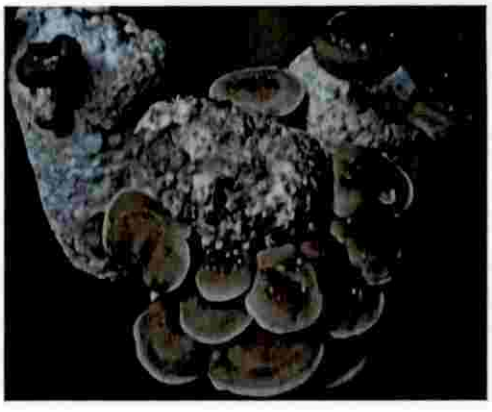


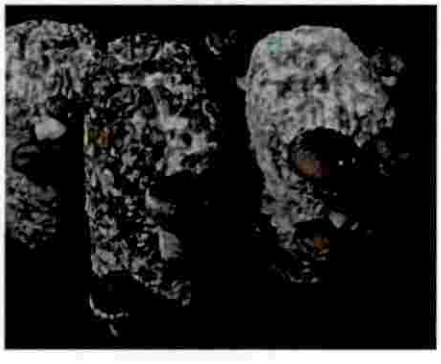
SD + 20 % RB



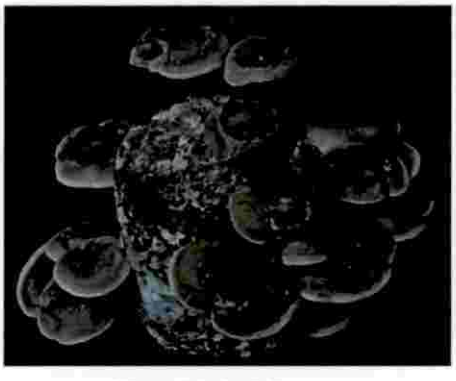
PS + SD + 20 % RB



SD + 20 % WB

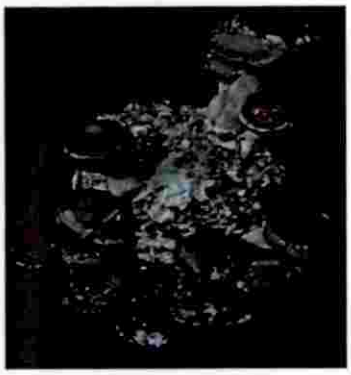


Sawdust



SD + 10 % RB

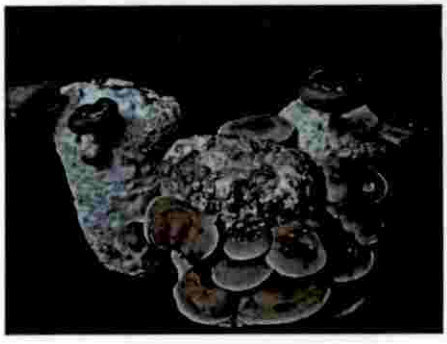
**Plate 29 : Cultivation of LE- 3 on different substrates**



SD + 10 % RB

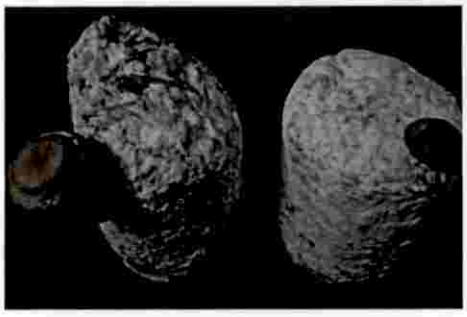
SD + 20 % RB

Sawdust

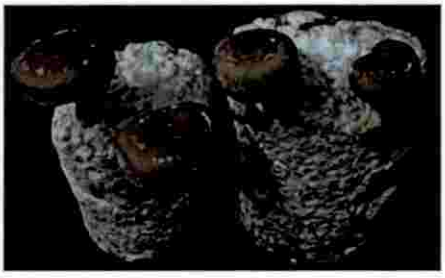


SD + 10% WB

SD + 20 % WB



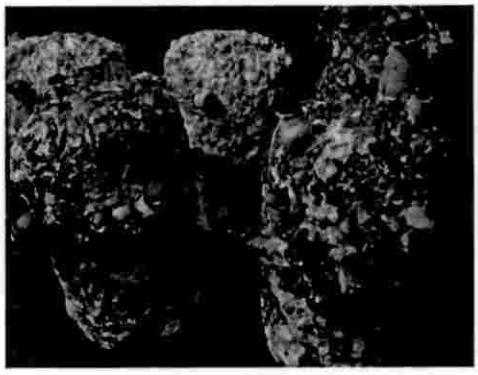
PS + SD + 20 % RB



PS + SD + 10 % RB

**Plate 30 : Cultivation of LE- 4 on different substrates**

Sawdust

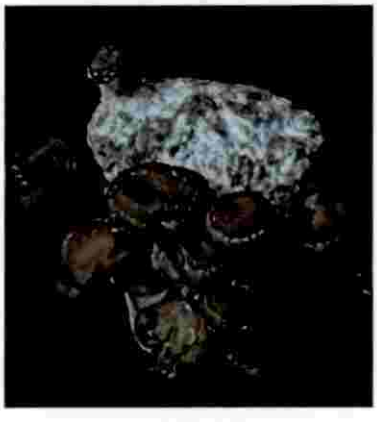


SD + 10 % WB

PS + SD + 10 %

SD + 10 % RB

SD + 20 % RB



SD + 20 % WB

**Plate 31 : Cultivation of LE- 5 on different substrates**

PS + SD + 20 % RB

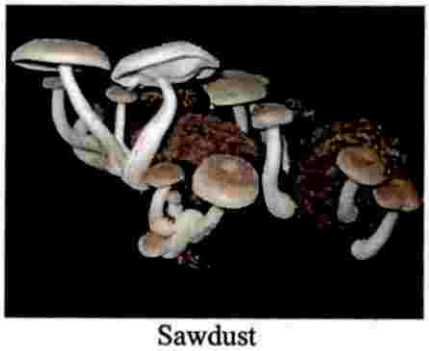
PS + SD + 10 % RB

SD + 10 % RB

SD + 20 % WB

SD + 10 % WB

SD + 20 % RB



Sawdust

**Plate 32 : Cultivation of LE- 6 on different substrates**

parameters (total number of fruiting bodies in different substrates, average yield on different substrates, biological efficiency and total crop growth period) on all the six strains of *L. edodes* are presented below.

**Growth parameters**

***4.8.1.1. Time taken for complete mycelial colonization of the bed substrates***

The inoculated beds which were incubated at room temperature (28 + 2 $^{O}$C) under prevalent humid conditions for 30 days were observed for the time taken by each *L. edodes* strains in colonizing the bed. Results of the observations are presented below

Sawdust amended with 20 per cent wheat bran was significantly superior which took minimum period for mycelial colonization *ie*., LE-2 strain (51.00 days), LE-3 (53.33 days), LE-4 (54.00 days), LE-1 (57.66 days), LE-5 and LE-6 (59.00 days) (Table 21). LE-1 strain took minimum of 57.66 days for complete mycelial colonization which was statistically on par with sawdust + 10 per cent wheat bran (58.33 days), sawdust + 10 per cent rice bran (59.00 days) and sawdust without any amendments (59.66 days). Combination of paddy straw and sawdust amended with 10 per cent and 20 per cent rice bran took 62.30 days and 63.00 days respectively for mycelial colonization. Paddy straw and banana pseudo stem proved to be least significant which took 79.63 days and 83.67 days respectively in completing mycelial colonization.

Days taken for colonization by LE-2 strain varied from 51.00 days (sawdust + 20 per cent wheat bran) to 85.00 days (banana pseudo stem). Sawdust + 10 per cent rice bran substrate took 58.60 days for complete mycelial colonization. Mycelial colonization of *L. edodes* on combination of paddy straw and sawdust amended with 10 per cent rice bran (59.66 days) and 20 per cent rice bran (66.00 days) were on par. LE-3 strain took 59.33 days for completing mycelial run in sawdust which was

**Table 21. Days taken by *L. edodes* strains for complete mycelial colonisation of mushroom beds on different substrates**

| Treatments | Days taken for complete mycelial colonisation | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD + 10 % RB | 62.30 $^{bc}$ | 59.66 $^{c}$ | 71.00 $^{c}$ | 68.33 $^{c}$ | 71.00 $^{c}$ | 68.00 $^{cd}$ |
| PS + SD + 20 % RB | 63.00 $^{c}$ | 66.00 $^{c}$ | 74.00 $^{cd}$ | 71.00 $^{d}$ | 75.00 $^{d}$ | 66.66 $^{c}$ |
| SD +10 % RB | 59.00 $^{a}$ | 54.33 $^{b}$ | 61.33 $^{b}$ | 63.33 $^{b}$ | 75.66 $^{d}$ | 61.00 $^{ab}$ |
| SD + 20 % RB | 59.66 $^{ab}$ | 58.60 $^{c}$ | 60.00 $^{b}$ | 62.00 $^{b}$ | 69.00 $^{b}$ | 63.33 $^{bc}$ |
| SD + 10 % WB | 58.33 $^{a}$ | 55.00 $^{b}$ | 61.66 $^{b}$ | 66.33 $^{bc}$ | 69.66 $^{bc}$ | 62.00 $^{ab}$ |
| SD + 20 % WB | 57.66 $^{a}$ | 51.00 $^{a}$ | 53.33 $^{a}$ | 54.00 $^{a}$ | 59.00 $^{a}$ | 59.00 $^{a}$ |
| Sawdust | 59.66 $^{ab}$ | 60.00 $^{c}$ | 59.33 $^{b}$ | 62.33 $^{b}$ | 67.00 $^{b}$ | 62.33 $^{ab}$ |
| Paddy straw | 79.63 $^{d}$ | 83.66 $^{d}$ | 81.00 $^{de}$ | 83.00 $^{e}$ | 84.00 $^{e}$ | 72.33 $^{e}$ |
| Banana pseudo stem | 83.67 $^{e}$ | 85.00 $^{e}$ | 76.66 $^{d}$ | 81.00 $^{e}$ | 81.00 $^{e}$ | 72.66 $^{e}$ |
| CD (0.05) | 2.93 | 2.93 | 4.77 | 4.53 | 2.33 | 3.94 |

* *Average of five replications*

PS-Paddy straw, SD-Sawdust, RB-Rice bran, WB-Wheat bran

statistically on par with three substrate combinations *viz.*, sawdust + 20 per cent rice bran (60 days) ,sawdust + 10 per cent rice bran (61.33 days) and sawdust + 10 per cent wheat bran( 61.66 days). Combination of the substrates, paddy straw and sawdust amended with 10 per cent and 20 per cent rice bran took 71.00 days and 74.00 days respectively for the mycelial colonization. Longest period for mycelial colonization was recorded in the substrates banana pseudo stem (76.66 days) and paddy straw (81.00 days) without any amendments.

Minimum period for mycelial colonization of LE-4 strain was 62.00 days in sawdust + 20 per cent rice bran followed by 62.33 days (sawdust without amendments) and 63.33 days (sawdust + 10 per cent rice bran). In the combination of the substrates paddy straw and sawdust amended with rice bran @ 10 per cent and 20 per cent respectively, the strain took 68.33 days and 71.00 days for complete mycelial colonization. Period of colonization in banana pseudo stem and paddy straw were 83.00 days and 81.00 days respectively.

Minimum period for mycelial colonization of LE-5 and LE-6 strains was 59.00 days in sawdust + 20 per cent wheat bran. This was followed by 67.00 days (sawdust), 69.00 days (sawdust + 20 per cent rice bran) and 69.66 days (sawdust + 10 per cent wheat bran). Period of colonization in banana pseudo stem and paddy straw were 81.00 days and 84.00 days respectively.

Colonization of LE-6 strain in sawdust + 10 per cent rice bran (61.00 days) closely follows the mycelial colonization in sawdust + 20 per cent wheat bran substrate (59.00 days) which show on par results with sawdust + 10 per cent wheat bran (62.00 days) and sawdust (62.33 days). Maximum days for mycelial colonization was observed in , banana pseudo stem (72.33 days) and paddy straw (72.66 days).

Bump formation and initiation of brown exudates were uniform in the beds colonized by all the six strains.

***4.8.1.2. Time taken for browning of mycelium of mushroom beds in different substrates***

After the bump formation and initiation of brown exudates, the experimental beds were transferred to low temperature conditions for completion of the browning stage. The time taken for browning of mycelium in each strain of *L. edodes* was observed and the results recorded as follows:

LE-1 strain took significantly shorter time (62.66 days) for complete browning in sawdust which was statistically on par with all the treatments except paddy straw + sawdust + 20 per cent rice bran (69.33 days). All the strains failed to enter this stage in paddy straw and banana pseudo stem (Table 22).

In LE-2 strain, browning of the mycelium was completed in 63.33 days in sawdust + 10 per cent rice bran which was statistically on par with 65.33 days ( combination of paddy straw and sawdust amended with 10 per cent rice bran). This was followed by paddy straw + sawdust + 20 per cent rice bran (73.00 days) and sawdust + 10 per cent wheat bran (73.00 days). Longest time (78.00 days) for browning was in sawdust. LE-3 strain took 65.00 days for browning in sawdust + 20 per cent wheat bran which was on par with sawdust + 20 per cent rice bran (67.33 days). Longest period for mycelial browning was recorded in sawdust without any amendments (78.00 days).

In paddy straw and sawdust amended with 10 per cent rice bran, complete mycelial browning in LE-4 strain was recorded in 73.33 days which was statistically on par with sawdust + 10 per cent wheat bran (76.00 days ) and sawdust + 20 per cent wheat bran (77.00 days). The strain took 77.33 days in sawdust, 81.33 days in sawdust + 10 per cent rice bran and 86.30 days in paddy straw + sawdust + 20 per cent rice bran for mycelial browning. Sawdust + 20 per cent rice bran took maximum of 87.00 days for mycelial browning .

**Table 22. Days taken by *L. edodes* strains for browning of mycelium of mushroom beds on different substrates**

| Treatments | Days taken for browning of mycelium | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD + 10 % RB | 64.33 [a] | 65.33 [a] | 76.66 [d] | 73.33 [a] | 83.66 [bc] | 79.00 [d] |
| PS + SD + 20 % RB | 69.33 [b] | 73.00 [b] | 74.00 [cd] | 86.33 [d] | 86.66 [cd] | 76.33 [bc] |
| SD +10 % RB | 64.00 [a] | 63.33 [a] | 79.66 [e] | 81.33 [cd] | 83.66 [bc] | 73.66 [ab] |
| SD + 20 % RB | 64.66 [a] | 74.33 [bc] | 67.33 [ab] | 87.00 [d] | 77.66 [a] | 76.33 [bc] |
| SD + 10 % WB | 65.66 [a] | 73.00 [b] | 74.33 [cd] | 76.00 [ab] | 85.33 [cd] | 75.33 [b] |
| SD + 20 % WB | 63.00 [a] | 77.00 [bc] | 65.00 [a] | 77.00 [ab] | 88.00 [e] | 74.33 [ab] |
| Sawdust | 62.66 [a] | 78.00 [d] | 71.66 [bc] | 77.33 [c] | 81.00 [ab] | 72.36 [a] |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | -- | -- | -- | -- | -- | -- |
| CD (0.05) | 3.23 | 4.89 | 4.88 | 3.89 | 3.77 | 3.70 |

* *Average of five replications*

LE-5 strain completed browning of the mycelium in sawdust amended with 20 per cent rice bran (77.66 days) which was on par with sawdust (81.00 days). Combination of paddy straw and sawdust amended with 10 per cent rice bran (83.66 days) was statistically on par with the same treatment in which paddy straw was combined. Longest time for mycelial browning was recorded in paddy straw + sawdust + 20 per cent rice bran (86.66 days) followed by sawdust + 20 per cent wheat bran (88.00 days).

LE-6 strain took minimum of 72.36 days for browning in sawdust which was followed by sawdust + 10 per cent rice bran (73.66 days), sawdust + 20 per cent wheat bran (74.33 days) and sawdust + 10 per cent wheat bran (75.00 days). Mycelial browning in sawdust + 20 per cent rice bran (76.33 days) was statistically on par with the same treatment in which paddy straw was combined . A maximum of 79.00 days was taken by the strain in paddy straw + sawdust + 10 per cent rice bran for mycelial browning.

### *4.8.1.3. Time taken for initiation of sporocarp of mushroom beds in different substrates*

After the completion of browning stage, physical shock treatment was induced by immersing the beds in cold water for the initiation of sporocarp which appeared as star like cracks. Time taken from the browning to the appearance of the star like cracks was observed and recorded for each *L. edodes* strains as follows:

Among all the strains evaluated, LE-1, 3,4, 6 strains took significantly shorter time (71 days to 83.00 days ) for the sporocarp initiation in sawdust + 20 per cent wheat bran substrate. Period of initiation of sporocarp in LE-2 strain was 84.00 days followed by LE-5 (95.00 days). For all the strains, paddy straw and banana pseudo stem failed for sporocarp initiation (Table 23).

**Table 23. Days taken by *L. edodes* strains for initiation of sporocarp on mushroom beds on different substrates**

| Treatments | Days taken for initiation of sporocarp | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD + 10 % RB | 73.33 $^{ab}$ | 76.33 $^{a}$ | 86.00 $^{d}$ | 95.00 $^{d}$ | 93.66 $^{bc}$ | 86.00 $^{c}$ |
| PS + SD + 20 % RB | 74.00 $^{c}$ | 80.33 $^{b}$ | 84.00 $^{d}$ | 92.66 $^{d}$ | 95.00 $^{c}$ | 84.33 $^{ab}$ |
| SD +10 % RB | 72.66 $^{ab}$ | 75.33 $^{a}$ | 86.33 $^{d}$ | 85.00 $^{c}$ | 85.00 $^{a}$ | 91.33 $^{d}$ |
| SD + 20 % RB | 73.66 $^{c}$ | 82.33 $^{bcd}$ | 75.00 $^{ab}$ | 94.00 $^{d}$ | 86.33 $^{a}$ | 83.33 $^{ab}$ |
| SD + 10 % WB | 74.00 $^{c}$ | 81.00 $^{bc}$ | 77.33 $^{ab}$ | 84.00 $^{ab}$ | 93.33 $^{bc}$ | 84.33 $^{ab}$ |
| SD + 20 % WB | 71.00 $^{a}$ | 84.00 $^{e}$ | 73.66 $^{a}$ | 83.00 $^{a}$ | 95.00 $^{c}$ | 82.33 $^{a}$ |
| Sawdust | 72.00 $^{ab}$ | 85.00 $^{e}$ | 78.66 $^{c}$ | 84.33 $^{ab}$ | 88.00 $^{ab}$ | 86.00 $^{c}$ |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | -- | -- | -- | -- | -- | -- |
| CD (0.05) | 2.42 | 3.49 | 4.36 | 4.08 | 2.87 | 3.00 |

* *Average of five replications*

LE-1 strain took 71.00 days in sawdust + 20 per cent wheat bran for sporocarp initiation which was statistically on par with sawdust (72.00 days), sawdust + 10 per cent rice bran (72.66 days) and paddy straw sawdust + 10 per cent rice bran (73.33 days) . Sawdust amended with 20 per cent rice bran took 73.66 days which was on par with paddy straw + sawdust + 20 per cent rice bran and sawdust + 10 per cent wheat bran (74.00 days).

In LE-2 strain, sporocarp initiation was recorded in sawdust + 10 per cent rice bran (76.33 days) which was statistically on par with the same treatment in which paddy straw was combined. Paddy straw + sawdust + 20 per cent rice bran substrate took 80.33 days for pinhead initiation and was on par with sawdust + 10 per cent wheat bran(81.00 days) and sawdust + 20 per cent rice bran (82.33 days). Longest time for sporocarp initiation was recorded in sawdust + 20 per cent wheat bran (84.00 days) and sawdust (85.00 days).

LE-3 strain, took a minimum of 73.66 days for sporocarp initiation in sawdust + 20 per cent wheat bran which was statistically on par with sawdust + 20 per cent rice bran (75.00 days) and sawdust + 10 per cent wheat bran (77.33 days). Paddy straw + sawdust amended with 20 per cent rice bran took 84.00 days for sporocarp initiation. Paddy straw sawdust combination amended with 10 per cent rice bran took longest time (86.00 to 86.33 days ) for sporocarp initiation.

LE-4 strain showed statistically on par results with sawdust + 10 per cent wheat bran (84.00 days) and sawdust (84.33 days). This was followed by 85.00 days ( sawdust + 10 per cent rice bran). All the three substrates , paddy straw + sawdust + 20 per cent rice bran (92.66 days), sawdust + 20 per cent rice bran (94.00 days) and paddy straw + sawdust + 10 per cent rice bran (95.00 days) were statistically on par.

For sporocarp initiation, LE-5 strain took 85.00 days ( sawdust + 10 per cent rice bran ) which was statistically on par with 86.33 days ( sawdust + 20 per cent rice bran) and 88.00 days (sawdust without any amendments). Sawdust + 10 per cent

wheat bran took ( 93.33 days) which statistically on par with paddy straw + sawdust + 10 per cent rice bran (93.66 days) in initiating the sporocarps. Sawdust + 20 per cent wheat bran and paddy straw sawdust combination amended with 20 per cent rice bran took longest time (95.00 days) for sporocarp initiation.

LE-6 strain took minimum of 82.23 days in sporocarp initiation which was on par with sawdust + 20 per cent rice bran ( 83.33 days), sawdust + 10 per cent wheat bran (84.33 days and combination of paddy straw and sawdust amended with 20 per cent rice bran (84.33 days). Sawdust and combination of paddy straw and sawdust amended with 10 per cent rice bran took 86.00 days for sporocarp initiation.

**Yield parameters**

***4.8.1.4. Total number of fruiting bodies in different substrates***

All the strains produced highest number of fruiting bodies in the sawdust substrate amended with 20 per cent wheat bran and was followed by sawdust amended with 20 per cent rice bran (6.00 to 11.33 ), sawdust + 10 per cent wheat bran ( 3.33 to 9.66 ), sawdust without any amendments ( 3.66 to 8.33 ) and sawdust + 10 per cent rice bran (3.33 to 8.66 ). Lesser number of fruiting bodies were obtained from combinations of paddy straw and sawdust amended with 10 per cent and 20 per cent rice bran( 1.33 to 5.00) (Table 24).

***4.8.1.5. Average yield on different substrates (g/ 500 g)***

Maximum yield of strains LE-1 strain (223.33 g), LE-2 (176.00 g), LE-5( 91.66 g) and LE-6 (290.66 g) were recorded in sawdust amended with 20 per cent wheat bran. LE-3 strain recorded highest yield (136.20 g) in sawdust amended with 10 per cent rice bran and was on par with sawdust amended with 20 per cent rice bran (110.00 g). In LE-4 strain, maximum yield (151.00 g) was obtained in sawdust + 10 per cent rice bran. No yield of sporocarps were recorded in paddy straw and banana pseudo stem without any amendments.

**Table 24. Total number of fruiting bodies of *L. edodes* strains on different substrates**

| Treatments | Number of fruiting bodies | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD +10 % RB | 1.33 $^{c}$ | 2.33 $^{c}$ | 1.66 $^{cd}$ | 1.33 $^{bc}$ | 1.33 $^{cd}$ | 1.66 $^{cd}$ |
| PS + SD + 20 % RB | 2.00 $^{bc}$ | 3.33 $^{bc}$ | 5.00 $^{bc}$ | 2.66 $^{bc}$ | -- | 3.33 $^{bcd}$ |
| SD +10 % RB | 8.66 $^{a}$ | 3.33 $^{bc}$ | 6.33 $^{ab}$ | 5.00 $^{abc}$ | 3.66 $^{abc}$ | 7.00 $^{abc}$ |
| SD + 20 % RB | 11.33 $^{a}$ | 9.66 $^{a}$ | 10.33 $^{a}$ | 6.66 $^{ab}$ | 6.00 $^{a}$ | 9.33 $^{a}$ |
| SD + 10 %WB | 9.66 $^{a}$ | 6.33 $^{ab}$ | 7.66 $^{ab}$ | 5.66 $^{abc}$ | 3.33 $^{abcd}$ | 7.33 $^{ab}$ |
| SD + 20 % WB | 9.66 $^{a}$ | 7.33 $^{a}$ | 9.33 $^{ab}$ | 10.33 $^{a}$ | 5.00 $^{ab}$ | 9.66 $^{a}$ |
| Sawdust | 8.33 $^{ab}$ | 6.66 $^{ab}$ | 8.33 $^{ab}$ | 6.66 $^{ab}$ | 3.66 $^{abc}$ | 7.33 $^{ab}$ |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | --- | -- | -- | -- | -- | -- |
| CD (0.05) | 6.64 | 3.35 | 4.64 | 5.88 | 3.55 | 5.45 |

**Average of five replications*

**Table 25. Average yield of *L. edodes* strains on different substrates (g/ 500 g)**

| Treatments | Average yield | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD +10 % RB | 40.16 $^{bcd}$ | 37.33 $^{cd}$ | 29.00 $^{c}$ | 24.66 $^{cd}$ | 15.00 $^{de}$ | 45.00 $^{de}$ |
| PS + SD + 20 % RB | 27.76 $^{cd}$ | 66.33 $^{bcd}$ | 41.93 $^{bc}$ | 28.16 $^{cd}$ | -- | 38.66 $^{de}$ |
| SD +10 % RB | 133.33 $^{ab}$ | 143.33 $^{ab}$ | 116.10 $^{a}$ | 151.00 $^{a}$ | 36.00 $^{cd}$ | 81.33 $^{d}$ |
| SD + 20 % RB | 100.00 $^{bcd}$ | 106.00 $^{abc}$ | 110.00 $^{ab}$ | 111.66 $^{ab}$ | 66.00 $^{b}$ | 95.66 $^{cd}$ |
| SD + 10 %WB | 130.00 $^{ab}$ | 95.33 $^{bc}$ | 136.20 $^{a}$ | 66.66 $^{bc}$ | 52.00 $^{bc}$ | 150.66 $^{bc}$ |
| SD + 20 % WB | 223.33 $^{a}$ | 176.00 $^{a}$ | 128.66 $^{a}$ | 101.66 $^{b}$ | 91.66 $^{a}$ | 290.66 $^{a}$ |
| Sawdust | 110.00 $^{bc}$ | 108.33 $^{abc}$ | 171.33 $^{a}$ | 55.33 $^{c}$ | 29.33 $^{d}$ | 168.66 $^{b}$ |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | -- | -- | -- | -- | -- | -- |
| CD (0.05) | 101.02 | 78.68 | 73.87 | 45.38 | 21.65 | 64.21 |

**Average of five replications*

Average yield of LE-1 strain varied from minimum of 27.76 g to a maximum of 223.33 g/ 500 g beds. Sawdust + 10 per cent rice bran and wheat bran yielded 133.33 g and 130.00 g respectively which was followed by sawdust ( 110 g) and sawdust + 20 per cent rice bran (100.00 g). Lowest yield was obtained in paddy straw + sawdust + 10 per cent rice bran (40.16 g) and paddy straw + sawdust + 20 per cent rice bran (27.76 g) (Table 25).

LE-2 yielded 143.33 g (sawdust + 10 per cent rice bran substrate), which was on par with 108.33 (sawdust), 106.00 g ( sawdust + 20 per cent rice bran). Paddy straw and sawdust combination supplemented with 20 per cent and 10 per cent rice bran yielded a minimum of 66.33 g and 37.33 g / 500 g substrate respectively.

Maximum yield of 171.33 g/ 500 g bed was obtained in sawdust ( LE-3 strain). This was on par with sawdust + 10 per cent and 20 per cent wheat bran and sawdust + 10 per cent and 20 per cent rice bran which yielded an average of 136.20 g 128.66 g, 116.10 g and 110.00 g respectively. Minimum yield of 41.93 g and 29 g/ 500 g substrates were recorded in paddy straw and sawdust combinations.

Average yield of LE-4 strain varied from 24.66 g to 151.00 g/ 500 g beds. Maximum yield of 151.00 g/ 500 g beds was obtained in sawdust + 10 per cent rice bran which was on par with 111.66 g in sawdust + 20 per cent rice bran which was followed by 101.66 g in sawdust + 20 per cent wheat bran, 66.66 g in sawdust + 10 per cent wheat bran and 55.33 g in the substrate containing only sawdust. Minimum yields of 28.16 g and 24.66 g were obtained in paddy straw - sawdust combinations with 10 per cent and 20 per cent rice bran supplementation.

Among all the six strains, LE-5 strain yielded a minimum of 91.66 g in sawdust + 20 per cent wheat bran followed by 66.00 g in sawdust + 20 per cent rice bran, 52.00 g in sawdust + 10 per cent wheat bran . Yield of 36.00 g was recorded in sawdust + 10 per cent rice bran followed by 15.00 g of sporocarps in paddy straw +

sawdust + 10 per cent rice bran. Sporocarps were not obtained on the substrate combination of paddy straw and sawdust amended with 20 per cent rice bran.

Among all the six strains, maximum yield of 290.66 g was obtained from LE- 6 strain in sawdust + 20 per cent wheat bran followed by sawdust (168.66 g), and sawdust + 10 per cent wheat bran (150.66 g). Sawdust + 20 per cent and 10 per cent rice bran yielded an average of 95.66 g and 81.33 g respectively. Low yields were obtained on combination of paddy straw and sawdust amended with 10 per cent rice bran (45.00 g) which was on par with lowest yield of 38.66 g obtained from the same substrate amended with 20 per cent rice bran.

***4.8.1.6. Biological efficiency (per cent)***

LE-1 strain showed highest biological efficiency of 45.00 per cent in sawdust supplemented with 20 per cent wheat bran which was on par with 30.22 per cent, 27.00 per cent, 26.00 per cent in sawdust, sawdust + 10 per cent rice bran and sawdust + 10 per cent wheat bran respectively. From sawdust + 20 per cent rice bran, biological efficiency of 20.11 per cent was obtained. Minimum biological efficiency of 8.00 per cent and 5.55 per cent was obtained in paddy straw sawdust combinations (Table 26).

Maximum of 35.20 per cent biological efficiency was obtained in sawdust + 20 per cent wheat bran which was statistically on par with 28.66 per cent in sawdust + 10per cent rice bran, 22.11 per cent in sawdust and 21.21 per cent in sawdust + 20 per cent rice bran in the case of LE-2 strain. Biological efficiency of 19.06 per cent was recorded in sawdust + 10 per cent wheat bran which was followed by 13.33 per cent in paddystraw +sawdust + 20 per cent rice bran. Minimum biological efficiency of 7.46 per cent was obtained in paddy straw + sawdust + 10per cent rice bran.

Highest biological efficiency of 35.88 per cent in LE-3 strain was obtained from sawdust which was statistically on par with 27.22 per cent, 27.00 per cent ,

**Table 26. Biological efficiency of *L. edodes* strains (%)**

| Treatments | Strains | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD + 10 % RB | 8.00 $^{c}$ | 7.46 $^{cd}$ | 5.88 $^{cd}$ | 4.93 $^{cd}$ | 3.00 $^{de}$ | 9.00 $^{cd}$ |
| PS + SD + 20 % RB | 5.55 $^{c}$ | 13.33 $^{bcd}$ | 8.44 $^{bcd}$ | 5.66 $^{cd}$ | -- | 7.73 $^{cd}$ |
| SD +10 % RB | 27.00 $^{ab}$ | 28.66 $^{ab}$ | 27.00 $^{a}$ | 30.22 $^{a}$ | 7.20 $^{cd}$ | 16.26 $^{c}$ |
| SD + 20 % RB | 20.11 $^{bc}$ | 21.21 $^{abc}$ | 22.00 $^{abc}$ | 22.33 $^{ab}$ | 13.20 $^{b}$ | 19.13 $^{b}$ |
| SD + 10 %WB | 26.00 $^{ab}$ | 19.06 $^{bc}$ | 27.22 $^{a}$ | 13.33 $^{bc}$ | 10.40 $^{bc}$ | 30.13 $^{b}$ |
| SD + 20 % WB | 45.00 $^{a}$ | 35.20 $^{a}$ | 26.88 $^{a}$ | 20.33 $^{b}$ | 18.33 $^{a}$ | 58.13 $^{a}$ |
| Sawdust | 30.22 $^{a}$ | 22.11 $^{abc}$ | 35.88 $^{a}$ | 11.00 $^{c}$ | 5.86 $^{d}$ | 31.73 $^{b}$ |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | -- | -- | -- | -- | -- | -- |
| CD (0.05) | 20.60 | 15.33 | 17.27 | 9.07 | 4.33 | 13.19 |

***Average of five replications***

26.88 per cent and 22.00 per cent in sawdust + 10 per cent wheat bran, sawdust + 10 per cent rice bran, sawdust + 20 per cent wheat bran and sawdust + 20 per cent rice bran respectively. Paddy straw + sawdust combinations yielded significantly less with biological efficiency of 8.44 per cent and 5.88 per cent.

In LE-4 strain, maximum of 30.22 per cent biological efficiency was obtained in sawdust amended with 10 per cent rice bran which was statistically on par with 22.33 per cent of sawdust + 20 per cent rice bran . Sawdust amended with 20 per cent wheat bran recorded 20.33 per cent biological efficiency which was followed by 13.33 per cent in sawdust + 10 per cent wheat bran and 11 per cent in sawdust. Minimum of 5.66 per cent and 4.93 per cent biological efficiency was recorded in paddy straw and sawdust combinations.

18.33 per cent biological efficiency was obtained from sawdust + 20 per cent wheat bran in the case of LE-5 strain which was followed by 13.20 per cent in sawdust + 20 per cent rice bran, 10.40 per cent in sawdust + 10 per cent wheat bran and 7.20 per cent in sawdust + 10 per cent rice bran.

Maximum biological efficiency among all the six strains were obtained in LE-6 strain with a value of 58.13 per cent in sawdust + 20 per cent wheat bran. This was followed by sawdust (31.73 per cent), sawdust + 10 per cent wheat bran (30.13 per cent), sawdust + 20 per cent rice bran (19.13 per cent) and sawdust + 10 per cent rice bran (16.26 per cent). Minimum of 9.00 per cent and 7.73 per cent biological efficiency was obtained in paddy straw sawdust combinations.

***4.8.1.7. Total crop growth period***

Crop period of LE-1 strain of *L. edodes* varied from 100.00 days to 119.66 days. Significantly high crop period of LE-1 strain recorded was 119.66 days (sawdust + 20 per cent rice bran) which was statistically on par with 117.66 days ( sawdust + 10 per cent rice bran). This was followed by combination of paddy straw

**Table 27. Total crop growth period of *L. edodes* strains (days)**

| Treatments | Strains | | | | | |
|---|---|---|---|---|---|---|
| | LE-1 | LE-2 | LE-3 | LE-4 | LE-5 | LE-6 |
| PS + SD +10 % RB | 109.00 $^{d}$ | 104.33 $^{d}$ | 114.00 $^{a}$ | 123.00 $^{a}$ | 126.33 $^{a}$ | 114.00 $^{ab}$ |
| PS + SD + 20 % RB | 111.33 $^{b}$ | 108.33 $^{c}$ | 112.00 $^{a}$ | 122.00 $^{a}$ | 124.33 $^{ab}$ | 114.33 $^{ab}$ |
| SD +10 % RB | 117.66 $^{a}$ | 103.33 $^{d}$ | 114.33 $^{a}$ | 113.66 $^{b}$ | 119.00 $^{cd}$ | 113.66 $^{ab}$ |
| SD + 20 % RB | 119.66 $^{a}$ | 110.33 $^{abc}$ | 102.66 $^{c}$ | 122.66 $^{a}$ | 115.00 $^{e}$ | 112.66 $^{ab}$ |
| SD + 10 % WB | 110.00 $^{b}$ | 109.00 $^{bc}$ | 103.66 $^{bc}$ | 112.00 $^{b}$ | 121.33 $^{bc}$ | 110.66 $^{b}$ |
| SD + 20 % WB | 105.33 $^{bc}$ | 112.00 $^{ab}$ | 101.66 $^{c}$ | 114.33 $^{b}$ | 122.00 $^{bc}$ | 116.00 $^{a}$ |
| Sawdust | 100.00 $^{c}$ | 113.00 $^{a}$ | 106.66 $^{b}$ | 114.33 $^{b}$ | 116.00 $^{de}$ | 114.00 $^{ab}$ |
| Paddy straw | -- | -- | -- | -- | -- | -- |
| Banana pseudo stem | -- | -- | -- | -- | -- | -- |
| CD (0.05) | 6.24 | 3.49 | 3.26 | 4.79 | 3.75 | 5.13 |

***Average of five replications***

and sawdust amended with 20 per cent rice bran (111.33 days), sawdust + 10 per cent wheat bran (110.00 days) and sawdust + 20 per cent wheat bran (105.33 days). The crop period of the strain was 100.00 days in sawdust substrate without any amendments (Table 27).

The crop growth period of LE-2 strain was maximum in sawdust (113.00 days) which was statistically on par with sawdust + 20 per cent wheat bran (112.00 days) and sawdust + 20 per cent rice bran (110.33 days). This was followed by sawdust + 10 per cent wheat bran (109.00 days) and combination of paddy straw and sawdust substrate amended with 20 per cent rice bran (108.33 days).

Maximum crop growth period of LE-3 strain was recorded in sawdust + 10 per cent rice bran (114.33 days), which was statistically on par with combination of paddy straw and sawdust amended with 10 per cent rice bran (114.00 days) and 20 per cent rice bran (112.00 days). This was followed by sawdust (106.66 days), sawdust + 10 per cent wheat bran (103.66 days) sawdust + 20 per cent rice bran (102.66 days) and sawdust + 20 per cent wheat bran (101.66 days).

Crop growth period was maximum in combination of paddy straw and sawdust amended with 10 per cent rice bran (123.00 days) which was on par with sawdust + 20 per cent rice bran (122.66 days). This was followed by sawdust + 20 per cent wheat bran and sawdust (114.33 days) and sawdust + 10 per cent rice bran (113.66 days).

LE-5 and LE-6 strain took maximum of 126.33 days (paddy straw + sawdust + 10 per cent rice bran) and 116.00 days (sawdust + 20 per cent wheat bran) which was on par with 124.33 days (paddy straw + sawdust + 20 per cent rice bran).

### *4.8.2. Lentinus sp.*

Cultivation trials of *Lentinus* sp. *viz., Lentinus connatus* (Plate 33) and *Lentinus tuber-regium* (Plate 34) were standardized using the two substrates screened *ie.,* paddy straw and sawdust + 20 per cent wheat bran substrate.

#### *4.8.2.1. Time taken for complete mycelial colonization of the bed substrates*

There was significant difference between species and substrates in days taken for complete mycelial colonization. *L. tuber-regium* took minimum of 10.66 days in sawdust supplemented with wheat bran whereas *L. connatus* took 12.33 days for complete mycelial colonization. In paddy straw substrate, *L. tuber-regium* took 18.66 days followed by 19.00 days by *L. connatus* (Table 28) .

#### *4.8.2.2. Time taken for browning of mycelium of mushroom beds in different substrates*

The native isolates were devoid of the browning phase.

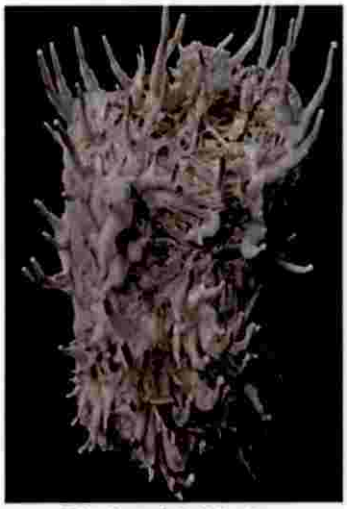
#### *4.8.2.3. Time taken for initiation of sporocarp of mushroom beds in different substrates*

*L. tuber-regium* took 21.33 days ( sawdust) for sporocarp initiation while that of *L. connatus* (18.33 days). In paddy straw substrate, *L. tuber-regium* took maximum of 27.33 days which was followed by 23.66 days by *L. connatus* (Table 29) for complete mycelial colonisation.

#### *4.8.2.4. Total number of fruiting bodies in different substrates*

Maximum number of fruiting bodies were obtained from *L. tuber-regium* (45.33 ) followed by *L. connatus* (35.66) in sawdust amended with 20 per cent wheat bran substrate. Lesser number of sporocarp was recorded in paddy straw substrate by *L. tuber-regium* (24.66) when compared with *L. connatus* (30.33) (Table 30).

Whitening stage



Pin head initiation

Sporocarp formation

Plate 33:Stages in cultivation of *L.connatus*

Whitening stage

Pinhead formation

Sporocarp formation

Plate 34 : Stages in cultivation of *L.tuber-regium*

**Table 28: Days taken by local isolates of *Lentinus* sp. for complete mycelial colonisation on different substrates**

| Treatments | Days taken for complete mycelial colonisation * | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus conatus* |
| SD + 20 % WB | 10.66 [a] | 12.33 [a] |
| Paddy straw | 18.66 [b] | 19.00 [b] |
| CD (0.05) | 1.30 | 1.85 |

**Average of five replications*

**Table 29 : Days taken by local isolates of *Lentinus* sp for initiation of sporocarp on mushroom beds on different substrates**

| Treatments | Number of days for sporocarp initiation * | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus connatus* |
| SD + 20 % WB | 21.33 [a] | 18.33 [a] |
| Paddy straw | 27.33 [b] | 23.66 [b] |
| CD (0.05) | 3.46 | 2.61 |

**Average of five replications*

**Table 30: Total number of fruiting bodies of native isolates of *Lentinus* sp. on different substrates**

| Treatments | Total number of fruiting bodies* | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus connatus* |
| SD + 20 % WB | 45.33 [a] | 35.66 [a] |
| Paddy straw | 24.66 [b] | 30.33 [b] |
| CD (0.05) | 11.29 | 4.71 |

**Average of five replications*

**Table 31: Average yield of native isolates of *Lentinus* sp. on different substrates (g/ 500 g)**

| Treatments | Average yield* | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus connatus* |
| SD + 20 % WB | 293.33 [a] | 233.00 [a] |
| Paddy straw | 183.33 [b] | 153.33 [b] |
| CD (0.05) | 56.21 | 77.97 |

**Average of five replications*

**Table 32: Biological efficiency of native isolates of *Lentinus* sp (%)**

| Treatments | Biological efficiency (%)* | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus connatus* |
| SD + 20 % WB | 58.00 [a] | 36.60 [a] |
| Paddy straw | 46.60 [b] | 30.60 [b] |
| CD (0.05) | 5.60 | 4.26 |

**Average of five replications*

**Table 33: Crop growth period of native isolates of *Lentinus* sp.(days)**

| Treatments | Crop growth period* | |
|---|---|---|
| | *Lentinus tuber-regium* | *Lentinus connatus* |
| SD + 20 % WB | 60.00 [a] | 56.00 [a] |
| Paddy straw | 35.00 [b] | 27.33 [b] |
| CD (0.05) | 3.26 | 2.48 |

**Average of five replications*

#### *4.8.2.5. Average yield on different substrates*

*L. tuber-regium* obtained maximum yield in sawdust based substrate (293.33 gm/ 500 g) followed by 233.00 gm by *L. connatus* in the same substrate. 183.33 gm/500 g yield was obtained in paddy straw substrate by *L.tuber-regium* whereas *L. connatus* yielded 153.33 g/ 500 g bed substrate (Table 31).

#### *4.8.2.6. Biological efficiency (per cent)*

Maximum biological efficiency was recorded in *L. tuber-regium* (58.00) followed by *L. connatus* (36.60) in sawdust amended with 20 per cent wheat bran. Less biological efficiency was recorded in paddy straw by *L. tuber-regium* (24.66) when compared with *L. connatus* (30.33) (Table 32).

#### *4.8.2.7. Total crop growth period*

When compared with *L. connatus* (56.00 days ), total crop growth period was maximum (60.00 days ) for *L. tuber-regium* in sawdust + 20 per cent wheat bran. In paddy straw, the crop period was 35.00 (*L. tuber-regium*) and 27.33 *( L. connatus*) ( Table 33).

### 4.9. NUTRIENT ANALYSIS

The proximate constituents of all the six strains of *L. edodes* were analysed using standard technique and the results are presented (Table 34 ).

The moisture content of *Lentinula edodes* was found significantly higher in LE -3 strain (88.90 per cent ), followed by LE-1 (86.60 per cent), LE- 5 (85.80 per cent) and LE- 4 (85.60 per cent ). LE-6 strain contained moisture content of 81.60 per cent. Minimum moisture content of 79.60 per cent was recorded in LE-2 strain.

LE-6 strain contained significantly high value of carbohydrates (47.66 per cent) which was statistically on par with 47.23 per cent of LE-1 strain. LE-4 showed

**Table 34. Proximate nutrient analysis of strains of *L. edodes***

| Strains | Constituents | | | | | | |
|---|---|---|---|---|---|---|---|
| | Moisture # (%) | Carbohydrates* (g %) | Protein* (g %) | Fibre* (g %) | Vitamin C* (g %) | Ash* (g %) | Lipid* (g %) |
| LE-1 | 86.66 [a] | 40.23 [a] | 20.33 | 27.33 | 3.30 [ab] | 4.36 [a] | 2.90 [bc] |
| LE-2 | 79.66 [c] | 37.83 [bc] | 20.00 | 25.50 | 2.80 [bc] | 4.00 [a] | 3.60 [a] |
| LE-3 | 88.91 [a] | 35.29 [c] | 18.33 | 23.03 | 3.50 [a] | 2.70 [b] | 3.20 [ab] |
| LE-4 | 85.65 [b] | 38.93 [b] | 20.00 | 23.66 | 2.90 [bc] | 4.23 [a] | 3.23 [ab] |
| LE-5 | 85.85 [b] | 38.00 [b] | 21.00 | 22.33 | 3.26 [ab] | 4.20 [a] | 2.46 [c] |
| LE-6 | 81.62 [bc] | 40.66 [a] | 21.66 | 27.00 | 2.53 [c] | 4.40 [a] | 2.90 [bc] |
| CD (0.05) | 4.21 | 4.03 | NS | NS | 0.60 | 0.48 | 0.46 |

* Presented in dry weight basis

a carbohydrate content of 42.93 per cent which was significantly on par with 42.33 per cent of LE-5 strain and 41.83 per cent of LE-2 strain. LE-3 strain was least significant with 37.86 per cent carbohydrate content.

LE-6 strain contained significantly higher protein content of 21.66 per cent which was on par with 21.00 per cent of LE-5 and 20.33 per cent and 20.00 per cent each of LE-1 and LE-2 and LE-3 strains respectively. Least significant value of protein (18.33 per cent) was observed in LE-3 strain.

Fibre content of *L. edodes* varied from 22.33 per cent to 27.33 per cent. Fibre content was significantly high in LE-1 strain (27.33 per cent) which was followed by 27.00 per cent (LE-6), 25.50 per cent (LE-1), 23.66 per cent (LE-5) and 23.03 per cent (LE-3) . LE-5 strain had a minimum fibre content of 22.33 per cent.

Maximum Vitamin C (3.50 per cent) was recorded in LE-3 strain whereas LE-1 and LE-5 showed significantly lower vitamin content of 3.30 per cent and 3.26 per cent respectively. LE-4 and LE-2 strains showed vitamin content of 2.90 per cent and 2.80 per cent respectively. Minimum of 2.53 per cent was recorded in LE-6 strain.

Significantly higher ash content were recorded in LE-6 strain (4.40 per cent) and LE-1 strain (4.36 per cent) which were on par with the values of LE-4 (4.23), LE- 5 (4.20) and LE-2 (4.00). Minimum of 2.70 per cent of ash content was recorded in LE-3 strain. Lipid content of the mushroom varied from 2.46 per cent (LE-5) to a maximum value of 3.60 per cent (LE-2) strain.

Mineral content of the mushroom varied with strains (Table 35). Calcium content ranged from 11.00 mg /100 g (LE-2) to 19.00 mg/ 100 g (LE-6) strain. Maximum magnesium content of 1.10 mg/ 100 g was recorded in LE-4 strain which was followed by 0.95 mg/ 100 g (LE-3), 0.67 mg (LE-5), 0.60 mg (LE-6), 0.54 mg (LE-2) and 0.46 mg (LE-1). There was no significant difference between the strains in iron (1.36 mg to 1.80 mg) and manganese (1.53 to 2.63) content. Maximum

**Table 35. Mineral content of strains of *L. edodes***

| Strains | Minerals (mg/ 100 g)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Calcium | Magnesium | Iron | Manganese | Phosphorous | Potassium | Sodium | Zinc |
| LE-1 | 12.00 $^{b}$ | 0.46 $^{c}$ | 1.80 | 1.80 | 2.82 $^{a}$ | 16.33 $^{b}$ | 16.33 $^{bc}$ | 28.00 $^{ab}$ |
| LE-2 | 11.00 $^{b}$ | 0.54 $^{c}$ | 1.36 | 1.76 | 2.87 $^{a}$ | 25.20 $^{a}$ | 18.00 $^{abc}$ | 28.66 $^{a}$ |
| LE-3 | 11.76 $^{b}$ | 0.95 $^{ab}$ | 1.50 | 1.53 | 2.72 $^{a}$ | 18.00 $^{b}$ | 23.00 $^{ab}$ | 27.00 $^{ab}$ |
| LE-4 | 11.67 $^{b}$ | 1.10 $^{a}$ | 1.36 | 2.63 | 1.65 $^{c}$ | 17.33 $^{b}$ | 23.66 $^{a}$ | 26.33 $^{b}$ |
| LE-5 | 13.66 $^{b}$ | 0.67 $^{bc}$ | 1.40 | 1.70 | 2.80 $^{a}$ | 16.50 $^{b}$ | 15.33 $^{c}$ | 19.66 $^{c}$ |
| LE-6 | 19.00 $^{a}$ | 0.60 $^{c}$ | 1.40 | 1.96 | 2.33 $^{b}$ | 16.90 $^{b}$ | 13.00 $^{c}$ | 28.33 $^{a}$ |
| CD (0.05) | 4.68 | 28.19 | NS | NS | 28.59 | 3.36 | 6.96 | 16.42 |

* Presented in dry weight basis

phosphorous content (2.87 mg) was recorded in LE-2 strain which was on par with 2.82 mg / 100 g (LE-1) and 2.80 mg/ 100 g (LE-5) and 2.72 mg/ 100 g(LE-3). Maximum (25.20 mg) potassium content was recorded in LE-2 strain which was followed by 18.00 mg (LE-3 ), 17.33 mg (LE-4), 16.90 mg (LE-6), 16.50 mg (LE-5) and 16.33 mg/ 100 g (LE-1) strain. LE-2 strain recorded significantly high zinc content ( 28.66 mg/ 100 g) which was statistically on par with 28.33 mg (LE-6), 28.00 mg (LE-1) and 27.00 mg (LE-3). This was followed by 26.33 mg/ 100 g (LE-4) and 19.66 mg/ 100 g(LE-5).

## 4.10. ENZYME ACTIVITY

The enzyme activity of all the six strains of *L. edodes* were analyzed using standard technique and the results are presented below

### *4.10.1 Phenyl alanine lyase (PAL)*

The studies on the changes in PAL activity was carried out to elucidate effects on the six strains of *L. edodes* mushroom. The PAL activity of the strain was expressed μ moles of cinnamic acid equivalent / g of fresh sample / minute as shown in Table 36. The activity of PAL ranged between 2.48-2.62 for the six *L. edodes* strains.

### *4.10.2. Peroxidase (PO)*

The studies of the peroxidase activity were measured as change in absorbance per minute per g of mushroom sample. The activity of peroxidase ranged between 0.15 to 0.56 for *L. edodes* strains (Table 36). Activity was higher in LE-4 strain (0.56) which was followed by LE-1 and LE-2 strains (0.26 each), LE-3 (0.20) and LE-6 (0.15).

**Table 36. Enzyme activity of strains of *L. edodes***

| Strains | Enzyme activity * | | |
|---|---|---|---|
| | PAL (µg of cinnamic acid/ min/g) | Peroxidase (change in absorbance/ min/g) | Poly phenol oxidase( change in absorbance/ min/g) |
| LE-1 | 2.50 | 0.26 | 46.53 |
| LE-2 | 2.60 | 0.26 | 54.58 |
| LE-3 | 2.48 | 0.20 | 53.80 |
| LE-4 | 2.51 | 0.56 | 47.63 |
| LE-5 | 2.62 | 0.20 | 52.13 |
| LE-6 | 2.60 | 0.15 | 57.30 |
| CD (0.05) | NS | 4.13 | 0.168 |

**Average of three replications*

### *4.10.3. Poly phenol oxidase (PPO)*

The polyphenol oxidase activity was measured as change in absorbance per minute per gram of the mushroom sample. There was not much significant difference between the strains of *L. edodes* (Table 36). The activity of poly phenol oxidase was higher in the case of LE-6 strain (57.30) which showed on par results with LE-2 (54.58), LE-3(53.80) and LE-5(52.13). Least value of poly phenol oxidase was recorded in LE-1 strain (46.53).

## 4.11. SHELF LIFE

The study conducted to determine the shelf life of *L. edodes* indicated that mushrooms when stored in polypropylene covers and paper box with perforations had better keeping quality than those kept in polypropylene covers and box with perforations.

Significantly high shelf life of 20.66 days was recorded when mushroom packed in paper box with perforations and kept at refrigerated condition while that of 14.66 days at room temperature. Mushrooms stored paper box without perforation under refrigerator condition had 15.33 days of shelf life. (Table 36).

Mushrooms had a shelf life of 14.66 days when kept in polypropylene cover with perforation at refrigerated condition while that at room temperature (4.33 days). When kept in polypropylene covers without perforation at refrigerator condition, shelf life of 13.00 days where as in open condition, 7.00 days shelf life was recorded.

## 4.12. ORGANOLEPTIC STUDIES

Organoleptic studies were conducted by preparing recipes of both fresh and dried *L. edodes* mushrooms and subjected to sensory evaluation. Six different recipes *viz.*, mushroom cutlet, mushroom masala, soup, scramble, baji from fresh mushrooms and mushroom biscuit from dried mushroom powder were prepared

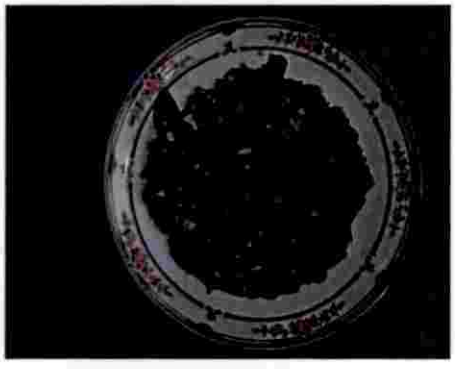
**Table 37. Shelf life of *L. edodes***

| Conditions | Keeping quality * | | Signs of spoilage | |
|---|---|---|---|---|
| | Room temperature | Refrigerator | Room temperature | Refrigerator |
| Polypropylene cover without perforation | 7.00 [b] | 13.00 [c] | Slimy coating, bad odour | Wrinkling |
| Polypropylene cover with perforation | 4.33 [c] | 14.66 [bc] | Bad odour, decay | Brown patches, drying |
| Paper box without perforation | 7.00 [b] | 15.33 [b] | Bad odour, decay | Browning |
| Paper box with perforation | 14.66 [a] | 20.66 [a] | Bad odour | Wrinkling |
| CD (0.05) | 1.53 | 2.10 | | |

**Average of three replications*

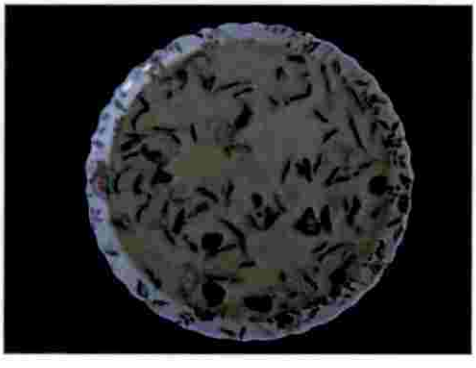
Baji

Scramble



Masala

Biscuit



Soup

Cutlet

**Plate 35: Various recipes tested for cooking quality**

**Table 38. Organoleptic studies of *L. edodes***

| Products | Colour and appearance * | Texture * | Taste* | Flavour* | Overall acceptability* |
|---|---|---|---|---|---|
| Cutlet | 4.60 | 4.20 | 4.60 | 4.20 | 4.35 |
| Soup | 4.50 | 4.00 | 4.80 | 3.90 | 4.20 |
| Masala | 4.30 | 4.50 | 4.50 | 4.60 | 4.80 |
| Scramble | 4.20 | 4.30 | 4.66 | 4.00 | 4.65 |
| Baji | 3.80 | 4.10 | 4.40 | 4.00 | 4.05 |
| Biscuit | 3.50 | 3.80 | 4.00 | 4.20 | 4.00 |

** Average of ten replications*

(Plate 36). The mushroom recipes for their characters like colour and appearance, texture, taste, flavor and overall acceptability were evaluated using a five point score card (Table 37).

Among the different products, cutlets had maximum ranking for colour and appearance and flavor. This was followed by soup which scored 4.50, masala (4.30), scramble (4.20). Baji and biscuit ranked least (3.80 and 3.50 respectively) for colour and appearance.

Mushroom masala ranked first (4.50) for texture which also had excellent flavor (4.60). This was followed by scramble (4.30), cutlet (4.20), baji (4.10), soup (4.00 ) and biscuit (3.80).

Mushroom soup ranked first for taste (4.80 ) which was followed by scramble (4.66), cutlet (4.60), masala (4.50), baji (4.40) and biscuit (4.00).

The overall acceptability was also high for masala, followed by scramble, cutlet, soup, baji. The least preferred product was biscuit with overall acceptability 4.00.

The ranking of the recipes based on the colour and appearance ranged from 3.50 to 4.60, whereas for texture it was 3.80 to 4.50. The ranking of recipes based on the taste and flavor were 4.00 to 4.80 and 3.90 to 4.60 .

## 4.13. CROP MANAGEMENT

### 4.13.1. Pests

The insects during the study comprised of spring tails (Plate 37). Their incidence was found on mushroom beds (Plate 38) during the resting period. They are minute to medium sized collembolans which have stout antenna and silvery white in colour. They feed on mycelium and cause disappearance of mycelium from the substrate.

**Plate 36 : Spring tail**

**Plate 37 : Bed infested with spring tail**

**Plate 38 : Bed infected with *Trichoderma* sp.**

4.13.2. Diseases

The diseases which were found during the study comprised of *Trichoderma* spp. They were found during spawn run period, second harvest and also when relative humidity was high (Plate 37). Infested area turned dark green once the fungus started sporulating. This leads to faster decay of the bed log substrate.

# *Discussion*

## 5. DISCUSSION

*Lentinula edodes* is a highly prized mushroom which is commonly known as Shiitake mushroom. This mushroom prefers a medium temperature for its sporocarp initiation and therefore it has not been recorded so far from the state of Kerala where the average temperature is high throughout the year. The present research was mainly focused to standardize the cultivation technology of *L. edodes* either using a native isolate of the mushroom collected during the survey that was encompassed in the study or if not obtained, using reference strains procured from recognized institutes. A thorough investigation was therefore undertaken throughout Kerala to trace the occurrence of *L. edodes* or any of its related species.

Previously both *Lentinula* sp. and the commonly affiliated mushroom *Lentinus* sp. were accommodated in the Shiitake group of mushrooms. Later, however Pegler (1975) reported many morphological demarcations between the two species indicating that *Lentinus* sp. does not belong to the Shiitake group of mushroom. In the light of observations of Pegler, as well as the other earlier reports on the occurrence of *Lentinus* spp. in Kerala, the native isolates of *Lentinus* sp. were also traced and collected during this survey, in order to undertake a comparative study on the morphological and phylogenetic characters of *Lentinula* sp and *Lentinus* sp.

### 5.1. SURVEY AND COLLECTION

Surveys were conducted during 2012-2014 in different locations of Kerala mainly to collect the mushroom flora of *L. edodes*. Specimens closely resembling *Lentinus* sp. that were spotted during the survey were also collected for assessing their relationship to *L. edodes*. Surveys were conducted in areas comprising of dense vegetations located in different districts of Kerala (Table1) *viz*., Thiruvananthapuram (Vellayani, Balaramapuram, Neyyattinkara, Mangalathukonam, Palode), Kollam (Kottiyam), Idukki, Pathanamthitta, Wayanad (Kalpetta), Malappuram (Aralam), Kannur (Ezhon) and Kasaragod (Cheemany).

As *Lentinula edodes* were not obtained throughout the period of survey conducted, reference strains of Shiitake were procured from recognized institutes *viz.*, LE-1 to LE-5 strains from GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand and LE-6 strain from Maharana Pratap University of Agriculture and Technology, Udaipur. Subsequent studies related to morphology, molecular , cultivation and nutrition aspects were done using the strains procured from these recognized institutes. Boruah *et al.* (1996) reported the presence of *Lentinus edodes* from East Khasi Hills of Meghalaya . Guzman *et al.* (1997) opined that *Lentinula edodes* is confined mainly to temperate, subtropical and tropical regions. According to Birkumar *et al.* (2008), Shiitake cultivation was a potential agro industry for hilly areas of north eastern India including Sikkim, Meghalaya regions where the mushroom grows mainly on dead wood of hard wood trees like oak, betula etc. Thirubhuvanamala *et al.*, 2013 reported that during their survey they had collected *Lentinula edodes* from Nilgiris regions of Western Ghats (Tamil Nadu).

However during each survey conducted from 2012-2014, specimens having typical characters of *Lentinus* had been collected from different locations. These mushrooms were gregarious and lignicolous in habit . Tree stumps of mango, coconut and cashew were found to be the usual spots for the occurrence of these *Lentinus* species. These wild isolates of mushrooms resembling *Lentinus* sp. were classified (VLYN 1 to VLYN 13) based on variations in external features. They include *Lentinus tuber-regium, L. squarrosulus, L. connatus* and *L. giganteum* while two isolates of *Lentinus* were not identified at species level. Survey, collection and distribution of native isolates of *Lentinus* sp. had also been reported earlier (Bose, 1920; Sharma *et al.,* 1985; Manimohan *et al.* 2004; Pires *et al.* 2014).

After each survey, mushroom flora collected were preserved by standard technique and transferred to Department of Plant Pathology, College of Agriculture, Vellayani for conducting morphological studies. Strains of *L. edodes* were also characterized as and when they were procured from the institutes.

## 5.2. MORPHOLOGICAL STUDIES OF LENTINULA SP. AND LENTINUS SP.

Morphological studies of procured reference strains of *L. edodes* and native isolates of *Lentinus* sp. obtained during the survey were conducted to characterize the two mushroom species. A comparative analysis of the results of the study was also made to determine the affiliations between the reference cultures of *L. edodes* and native isolates of *Lentinus* sp. obtained during the survey.

The results showed that all the strains of *L. edodes* had convex shaped pileus and fleshy texture. LE-1 to LE-5 strains were chocolate brown in colour whereas LE-6 strain was golden yellow. Pileus diameter was ranging from 6.50 cm to 11.22 cm. Stipe was cylindrical with central attachment to the pileus. Spores were ellipsoidal in shape with size ranging 3.25 - 4.41 µm x 1.96 -2.35 µm and spore print was pure white in colour. Hyphae were branched with clamp connections at constricted septa and produced club shaped basidium which bore basidiospores on it.

These was in accordance with observations of Pegler (1975) who described *Lentinula edodes* as mushroom with convex shaped with dark vinaceous brown pileus with centrally attached stipe. Spores were ellipsoidal in shape with 5.0-6.5 µm x 3.0-3.7 µm size and pure white spore print. Loosely woven hyphae had 3.0 µm -8.0 µm diameter, inflating to 23.0 µm diameter with a slightly thickened, frequently branching with prominent clamp connections at the constricted septa.

Purkayastha and Chandra (1985) also recorded the diameter of mushroom as 11.00 cm with stipe size of 3.0-4.0 cm x 0.8 -1.3 cm. Pegler (2003) reported the diameter of pileus of *L. edodes* as 5.0 cm to 15.0 cm whereas Gaitan and Mata (2004) reported that diameter of the mushroom was ranging from 5.0 cm to 20.0 cm. Morphological characters of the six procured reference strains were similar to the typical descriptions of *L. edodes* mentioned in these earlier reports.

Therefore the morphological studies of the procured strains of *L. edodes* indicated that these were comparable to the typical *L. edodes* strains described by

Pegler (1975). Further phylogenetic studies were conducted to confirm the identity as presented in the following discussion.

With regard to the native isolates of mushrooms obtained during the survey had typical characters of *Lentinus* sp. described by (Pegler 1977) as having characteristic xeromorphic tough carpophores bearing gills with serrated margins. These sporocarps were classified further into six groups based on slight phenotypic variations.

The isolates (VLYN-1 to VLYN-6) belonging to Class I (Table 4) were concave shaped, white to creamy white in colour and fibrous in texture. Stipe was cylindrical, fibrous and centrally attached to the pileus with crowded gills and smooth margin. The spores were cylindrically shaped with spore size 4.9-8.0 µm x 2.0-3.1µm. The descriptions of the collected isolates belonging to Class I were comparable to *L. squarrosulus* obtained by Manimohan *et al.* (2005) and Leon *et al.* (2013). They observed that the pileus of their isolate *L. squarrosulus* was convex to funnel shaped with wavy margin. Stipe was central to eccentric, gills were decurrent, and the spores were cylindric and nonamyloid.

The characters of VLYN-7 isolate placed in Class II, comprised of convex shaped pileus which was 75.00 cm in diameter and brown in colour and of fleshy texture. The stipe was cylindrical with central attachment. They had cylindric spores which showed cyanophilic spore reaction. Gills were free, soft textured and had 21 number of gills/ cm. Morphological characters observed in VLYN-7 were exactly similar to external features of *L. giganteum* decribed by Pegler (1983) and Manimohan *et al.* (2004).

Isolates (VLYN-8 to VLYN-10) grouped in Class III, were funnel shaped and cream yellow to light yellow in colour with eccentric attachment to the pileus. They had crowded gills which were hardy with smooth margin. Spores were oblong cylindric with spore size varying 6.2-8.0 µm x 2.1 -4.9 µm. Manjunathan, (2011) reported *Lentinus tuber-regium*, as an edible mushroom which was consumed by Kaani tribes of Peechi parai forest of Western Ghats. Manjunathan *et al.* (2011);

Kumar and Kaviyarasan (2012) described *Lentinus tuber-regium* as mushroom having tough leathery rigid and robust basidiocarp with ochraceous cream colour. They observed that its stipe was central to eccentric and was tapering downwards.

The pileus of the isolate VLYN-11 which belonged to Class IV, was funnel shaped, light yellow in colour and of fibrous texture. Stipe was tapering and had eccentric attachment. The isolate had crowded gills and are hardy with smooth margin . Spores were cylindric with size 5.8-8.1 x 2.0 -2.8 µm and white spore print. These characters were similar as described by Lakshmanan *et al.,* 1997 as having white pileus that was depressed in the centre and finally appeared as obliquely infundibuliform to fang shaped with eccentric stipe. Spores were 5.63 – 8.00 × 1.6 - 2.4 µm, ovoid to cylindrical with curved tapered apiculus. Based on these morphological characters observed in this isolate, this mushroom was identified as *L. connatus*.

Isolate VLYN-12 was funnel shaped with dark yellow pileus and had central attachment whereas isolate VLYN-13 was convex shaped with yellow pileus. Stipe was cylindrical with fibrous texture. Both isolates had cylindric spores . The isolates, VLYN-12 and VLYN-13 could not be identified at species level as their morphology characters did not match any descriptions cited in mycokeys.

Morphological studies with native isolates resembling *Lentinus* that were collected during the survey indicated that they possessed the typical characters of *Lentinus* sp. However in order to confirm the identity , molecular characterization of the isolates that resembled two renowned edible species *viz*., *L. tuber-regium* and *L. connatus* were conducted.

## 5.3. MOLECULAR CHARACTERISATION OF PROCURED STRAINS OF LENTINULA EDODES AND LOCAL ISOLATES OF LENTINUS SP.

Molecular characterization of procured strains of *Lentinula edodes* (LE-1 to LE-6) were done by ITS sequencing and RAPD analysis. ITS sequencing of six strains of *L. edodes*, using universal primers of ITS 1 F and ITS 4R yielded a PCR

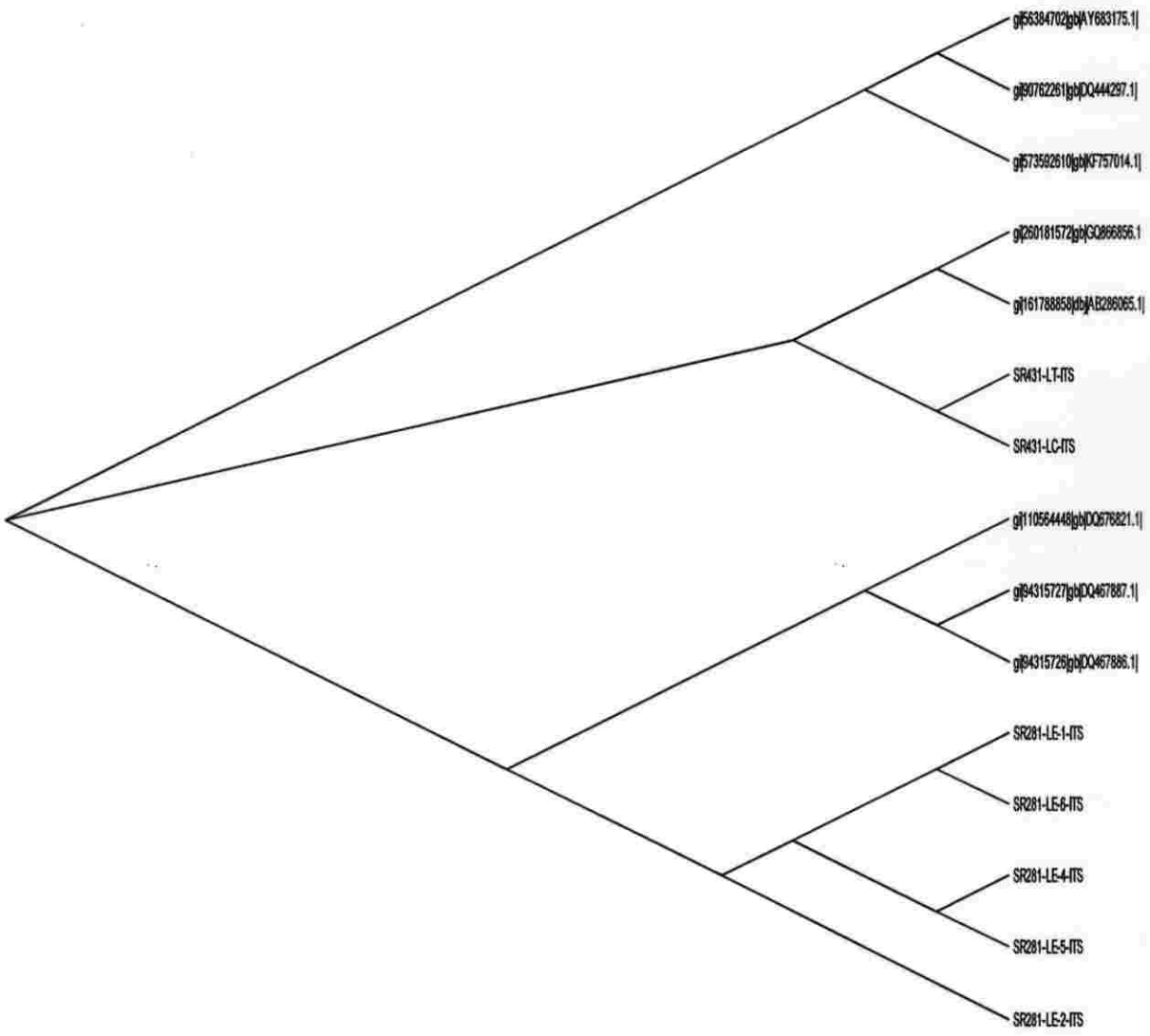



**Fig 3 : Dendrogram showing phylogenetic relationship of *L. edodes* strains and native isolates of *Lentinus* sp. with already known sequences in NCBI database**

product of 700 bp to 800 bp size. But Sharma *et al.* (2014) reported that the size of polymorphic bands of the strains of *L. edodes* ranged from 100 bp -1000 bp and size of ITS 1-2 was (278 bp to 575 bp) and ITS 1- 4 (410 bp to 616 bp) and the results confirmed high degree of divergence between the strains.

The BLAST results showed 99-100 per cent identity of the reference strains (LE-1 to LE-6) with sequences of *L. edodes* available in NCBI database. This confirms that the strains procured from different Institutes, irrespective of the locality, were similar. In the present study of ITS sequencing of *L. edodes* strains, dendogram showed that LE-1,3,4,5 and 6 strains were clustered in one group and LE-2 in another cluster group. Similar results were obtained by Shivani *et al.* (2013) on ITS sequencing of *L. edodes* strains (Le-C and Le-I) which indicated that Le C strain was highly divergent from all the other strains in ITS-1, 5.8 S and ITS-2 region while Le I strain showed high degree of divergence in ITS-1 region only. Likewise, ITS sequencing of strains of *L. edodes* done by Sharma *et al.* (2013) revealed that the strains OE-22, OE-28 and OE-388 were closely related to each other and fall in one group and OE-23 in another group. Strain OE-38 showed 92 per cent similarity to both the groups.

Two native isolates collected during the survey and classified into Class III and Class IV, were selected for molecular analysis based on the account of smoothness, similar to *L. edodes* were sequenced for their confirmation at species level and to check their relationship with the procured strains of *L. edodes*. BLAST results showed that the two native isolates were 99-100 per cent similar to *Lentinus tuber-regium* and *Lentinus connatus* at the same time it was confirmed that they had no phylogenetic relationship with the procured reference strains of *Lentinula edodes* (LE-1 to LE-6).

Affiliation of strains of *L. edodes* with the two identified native isolates *viz.*, *L. tuber-regium* and *L. connatus* were analysed by comparing them with the known sequences in NCBI database. The dendrogram generated indicated that *L. edodes* strains and the native isolates of *Lentinus* sp. were grouped in two entirely different

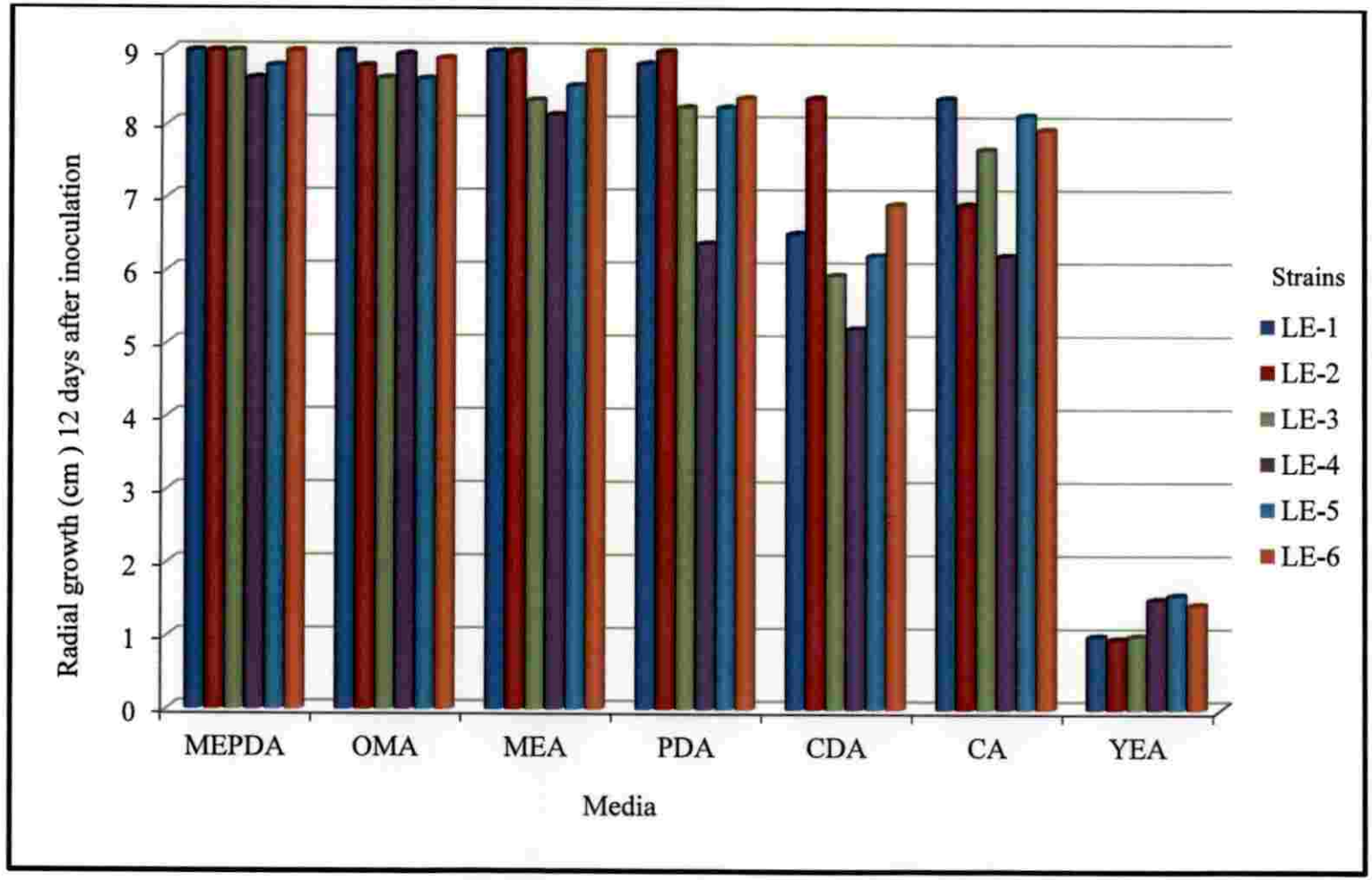



**Fig 4: Growth of *L. edodes* strains on different solid media**

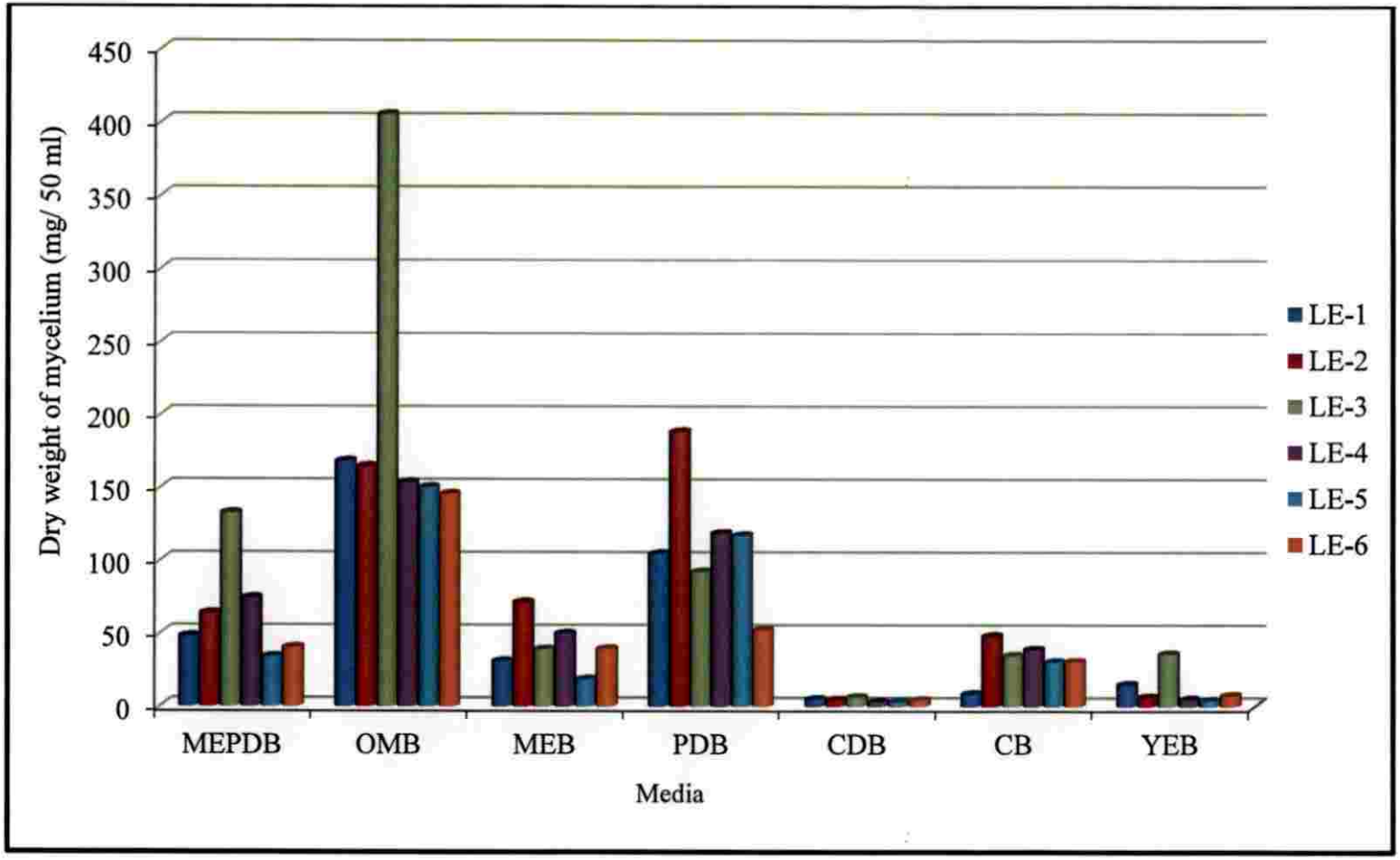



**Fig 5 : Growth of *L. edodes* strains in different liquid media**

clusters which confirmed the divergence between *L.edodes* strains and native isolates of *Lentinus* sp. ( Fig 3). The present study is in accordance to the observations of Molina *et al.* (1992) who confirmed the divergence between the *Lentinula edodes* and *Lentinus* sp.

In the present investigation of RAPD analysis, all the six strains of *L. edodes* were subjected to PCR using 15 random primers (OPA-01 to OPA -15). Of these, five primers (OPA-02, OPA-03, OPA-04, OPA-07, and OPA-09) showed good polymorphism with an average of 77 per cent. The random primers distinguished the strains into four phylogenetic groups. Phylogenetic tree generated using RAPD primers showed that among the six strains, LE-2 and LE-6 strains showed good polymorphism indicating close affiliations between two strains, though they were obtained from two locations (Pantnagar and Udaipur). LE-1 also showed similar to two strains though not up to the same level of polymorphism. All the six strains showed 45 per cent similarity coefficient.

Cristina *et al.* (2001) obtained similar results in the phylogenetic analysis of thirty four *L. edodes* strains using twenty OPA primers where OPA 01 to OPA 05, OPA 07 to OPA 14, OPA 17 to OPA 20 exhibited good polymorphisms. OPA 06, OPA 15 and OPA 16 primers did not amplified *L.edodes* DNA in RAPD amplification. Ravash *et al.,* 2009; Stajic *et al.,* 2005; Staniaszek *et al.*, 2002 also revealed that genetic diversity of mushrooms can be determined using molecular markers like RAPD.

## 5.4. CULTURAL STUDIES OF LENTINULA EDODES

Mycelial growth of six strains of *L. edodes* (LE-1 to LE-6) on seven different culture media (solid and liquid media ) (Fig 4 and Fig 5 ) *viz.*, malt extract peptone dextrose, malt extract, oat meal, potato dextrose, carrot, Czapek Dox and yeast extract were evaluated. The results showed that, radial growth of all the strains were significantly superior in malt extract peptone dextrose agar (MEPDA) which were statistically on par with oat meal agar and malt extract agar. Yeast extract agar

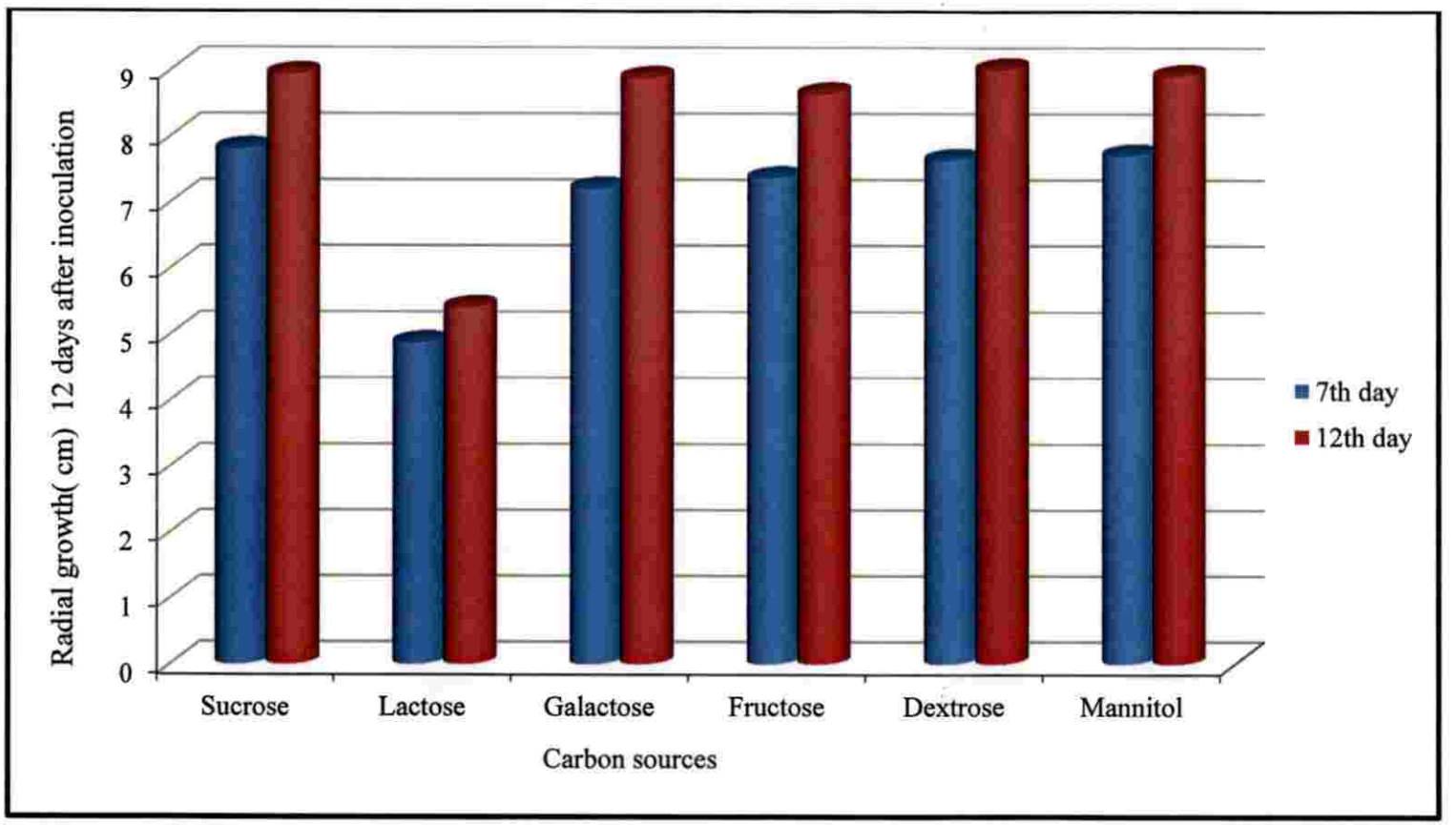



**Fig 6: Growth of *L. edodes* on solid media amended with different carbon sources**

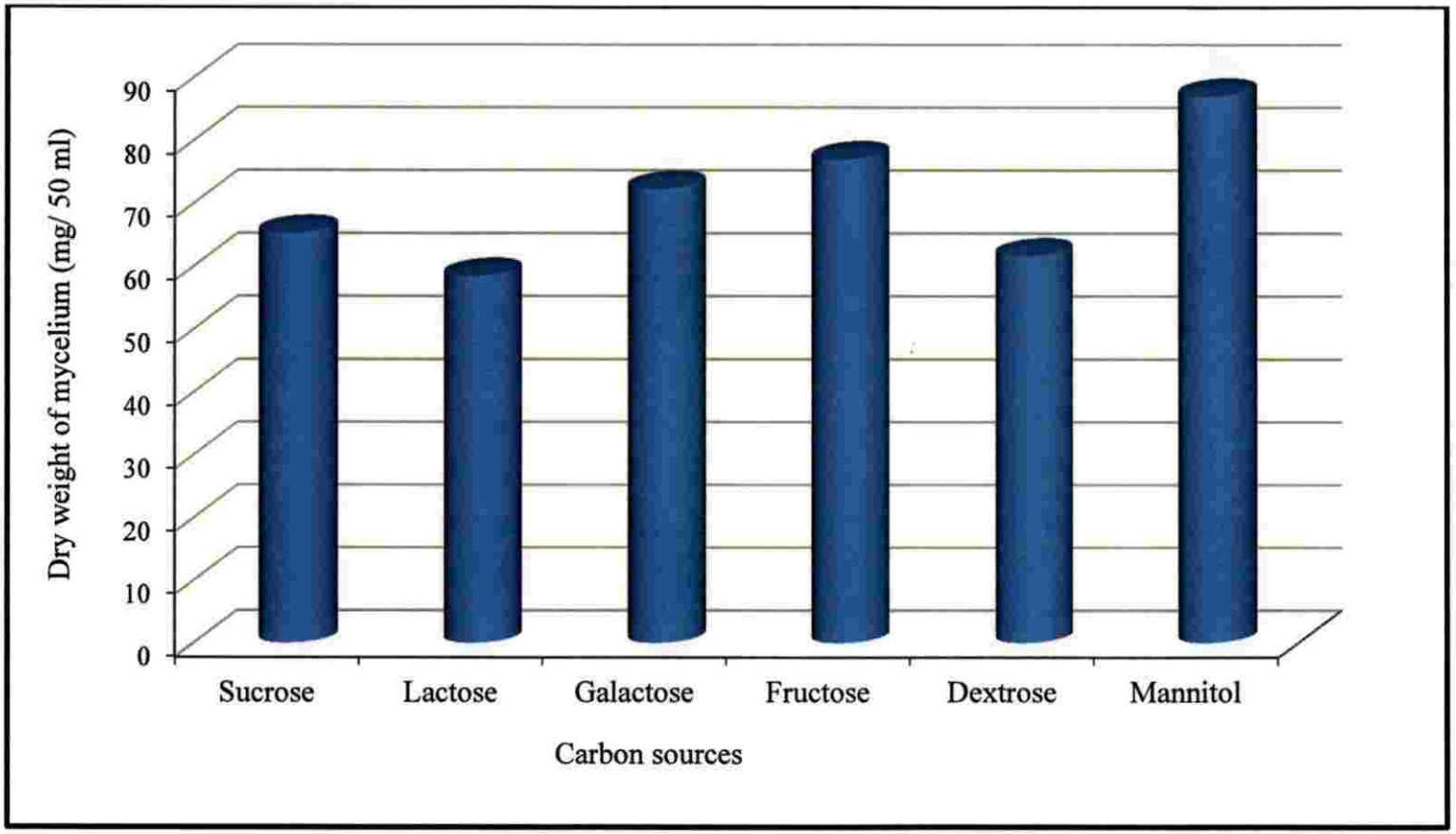



**Fig 7: Growth of *L. edodes* in liquid media amended with different carbon sources**

showed least significant growth in all the strains. LE-6 was the fastest growing in almost all the media when compared to other strains.

Lata and Sharma, (2012) also recorded higher radial growth of *L. edodes* on malt extract peptone dextrose agar . Kannan and Eswaran, (2010 a) recorded maximum mycelial growth in oat meal agar followed by potato dextrose agar medium. In experiments conducted by Puri (2012), highest growth rate of Shiitake culture was obtained on potato dextrose agar medium where least mycelial growth was observed in yeast extract agar.

The results of the study indicated that, oat meal broth produced maximum biomass of the mushroom which was followed by potato dextrose broth (PDB) and malt extract peptone dextrose broth (MEPDB). In the above mentioned experiment, conducted by Lata and Sharma, (2012) among different broth cultures tested, maximum mycelial dry weight was recorded in Asthana and Hawker's medium, followed by malt extract peptone dextrose broth and Walksman's broth. Thus in the present investigation, malt extract peptone dextrose along with oat meal have been screened as the best media for producing maximum mycelial growth of *L. edodes*.

## 5.5. NUTRTIONAL REQUIREMENTS OF LENTINULA EDODES

Different carbon sources namely sucrose, lactose, galactose, fructose, dextrose and mannitol were tested in solid (Fig 6) and liquid (Fig 7) medium (MEPDA) for their efficacy in radial mycelial growth and biomass production of *L. edodes*.

The study indicated that dextrose was the best carbon source followed by mannitol, sucrose, galactose and fructose. Mycelial growth of *L. edodes* was least when lactose was used as carbon source. Among the liquid media tested, significantly high biomass production was obtained when mannitol was used as carbon source which was followed by fructose. Jung *et al.* (2001) recorded sucrose as the better carbon source followed by D-galactose and D-fructose when provided as a sole source of carbon. Song *et al.* (1987) observed that glucose was the best carbon source for submerged mycelial cultures of *Lentinula edodes*. Kaur and Lakhanpal

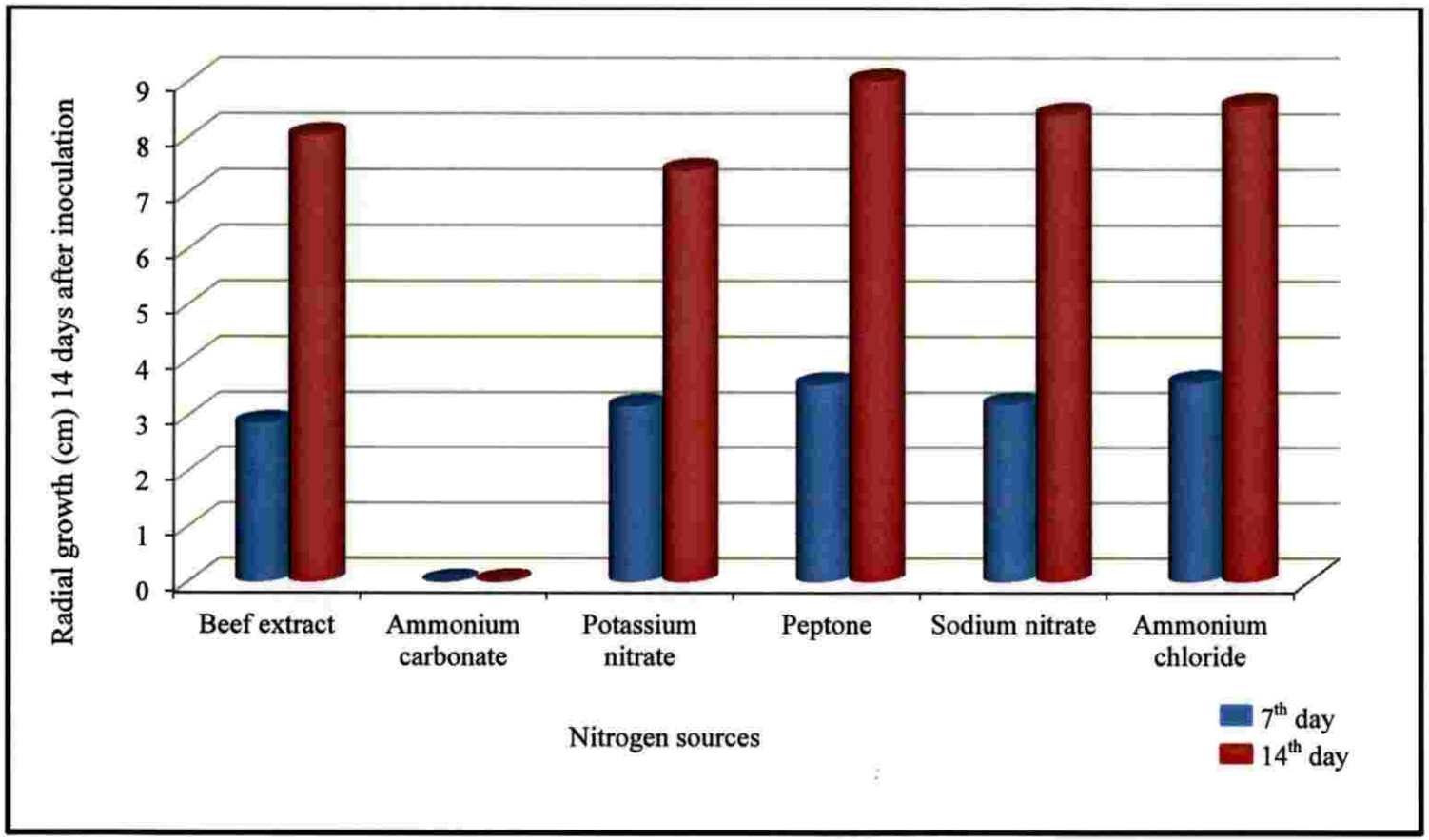



**Fig 8: Growth of *L. edodes* on solid media amended with different nitrogen sources**

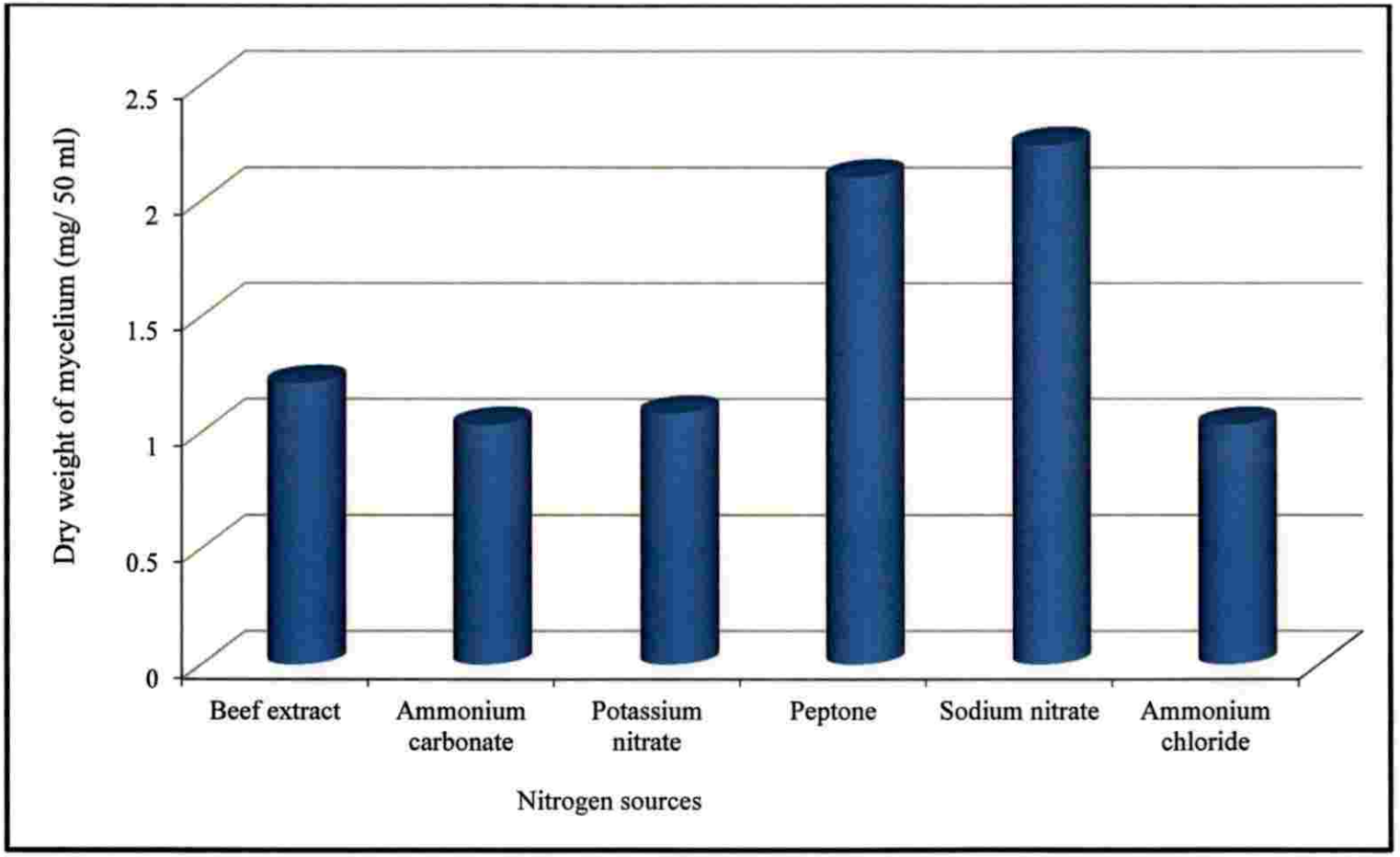



**Fig 9 : Growth of *L. edodes* in liquid media amended with different nitrogen sources**

(1995), in their studies on the effect of nutrients on the vegetative growth of *Lentinus edodes* observed maximum mycelial growth of the mushroom in glucose followed by fructose and sucrose. In the present study also, dextrose was observed as the best carbon source for the growth of *L. edodes*.

Peptone was found to be the best source of organic nitrogen in the experiment conducted to evaluate the effects of both organic and inorganic nitrogen sources on the growth of *L. edodes* in both solid (Fig 8) and liquid (Fig 9) media (Czapek Dox). Inorganic nitrogen sources like ammonium chloride , sodium nitrate, beef extract and potassium nitrate were also suitable in the solid media. There was no growth *of L. edodes* when ammonium carbonate was used as nitrogen source in both solid and liquid media.

In liquid media, sodium nitrate and peptone were equally suitable for the biomass production of *L. edodes*. The growth of *L. edodes* was sparse when inorganic nitrogen sources like ammonium carbonate, potassium nitrate and ammonium chloride were substituted in liquid media. According to the present investigation, organic compound peptone, was the best nitrogen source in both solid and liquid media whereas biomass production of *L. edodes* was highest when inorganic compound sodium nitrate was substituted as nitrogen source. According to Kaur and Lakhanpal, 1995 and Balazs *et al.* (1998), peptone @ 0.30 per cent increased the mycelial growth of *L.edodes* .

## 5.6. ENVIRONMENTAL REQUIREMENTS OF LENTINULA EDODES

Mycelial development of *L. edodes* was assessed at different temperatures, varying light conditions and H + ion concentrations.

Inorder to determine the suitable temperature for optimum growth of all the six strains of *Lentinula edodes* , they were grown in different temperature ranges like 5,10,15,20,25 and room temperature (28 ± 2 $^{O}$C) in both solid (Fig 10) and liquid media (Fig 11) (MEPD). All the strains of *L. edodes* showed significantly high

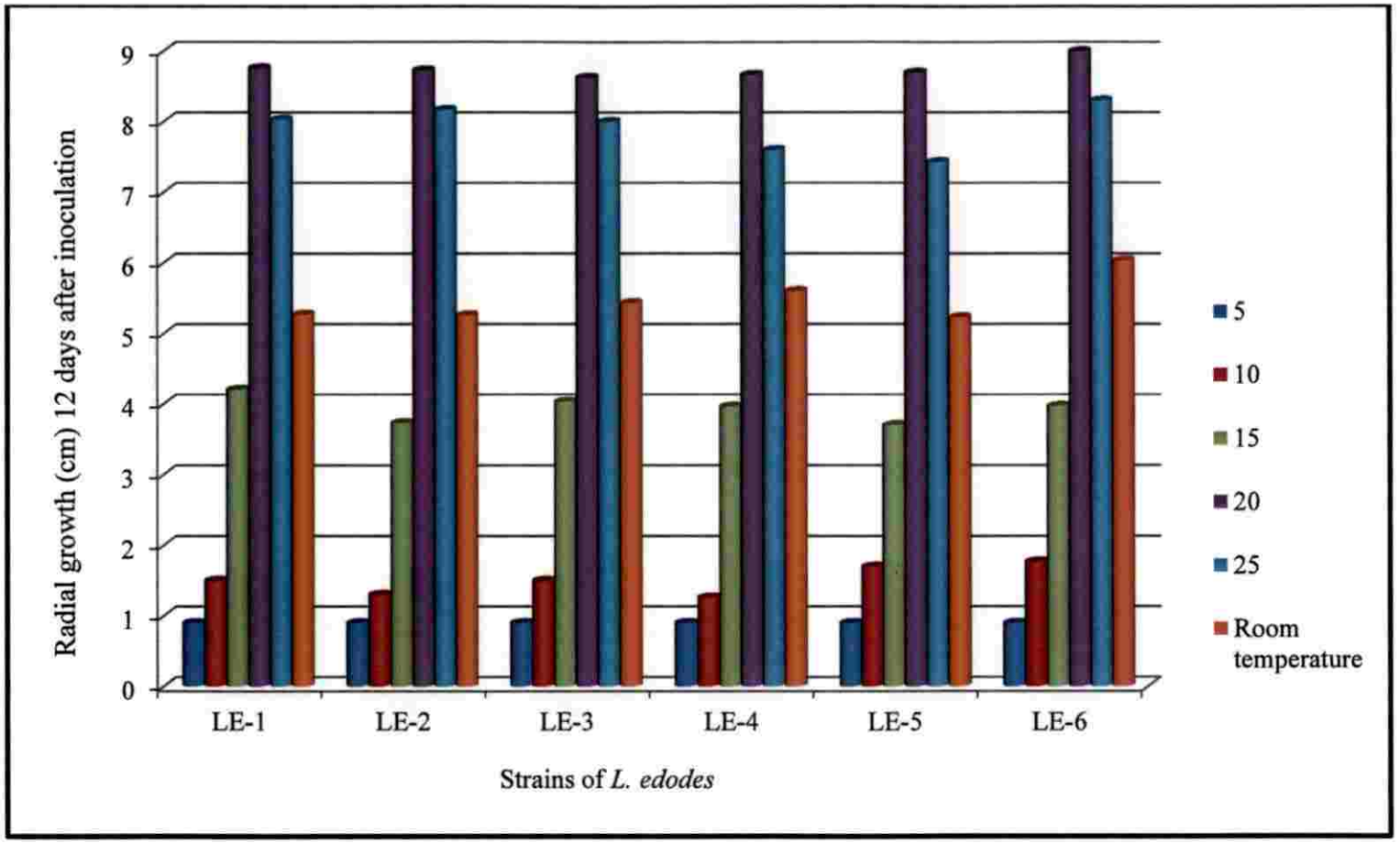



**Fig 10: Growth of *L. edodes* strains at different temperature on solid media**

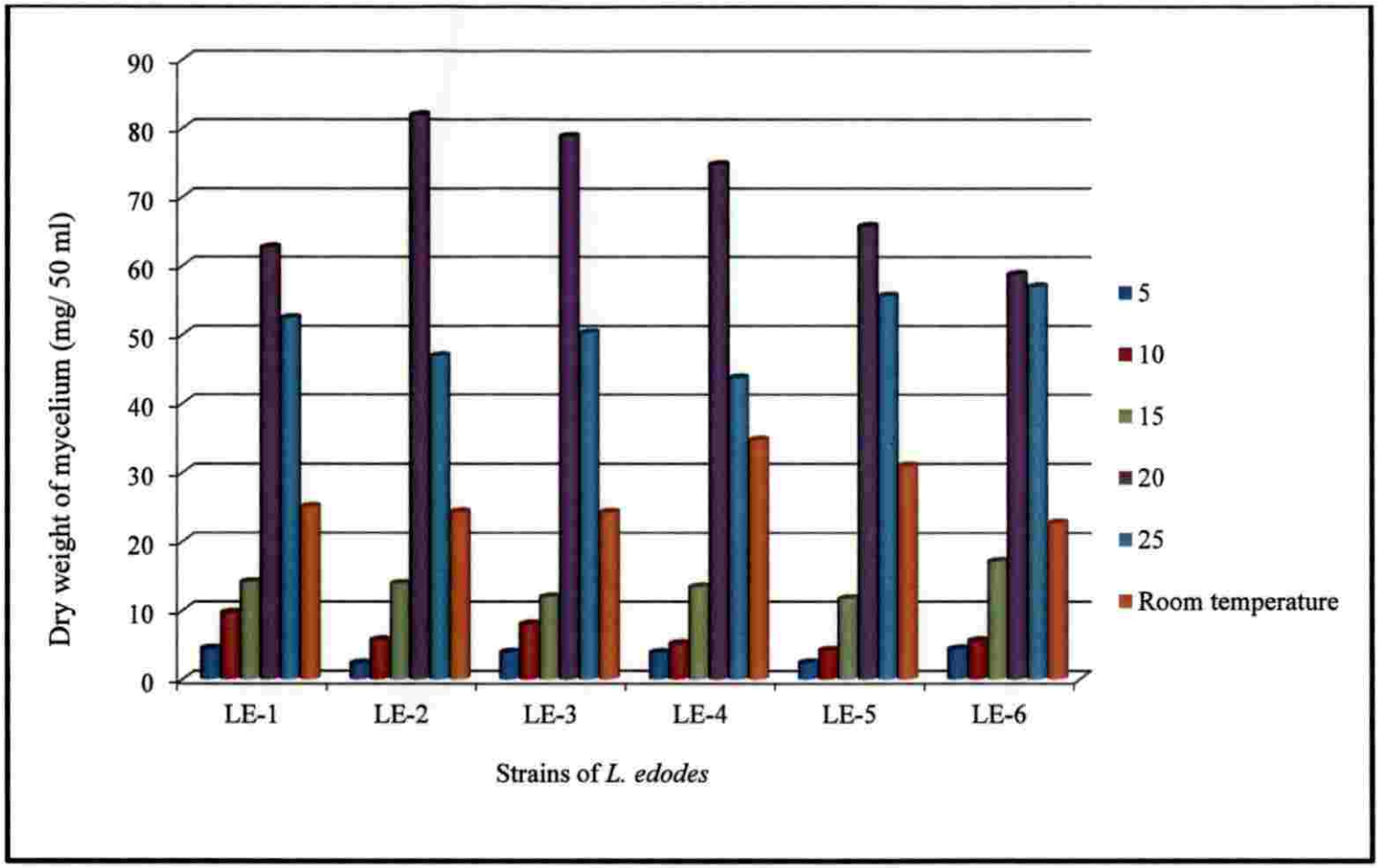



**Fig 11: Growth of *L. edodes* strains at different temperature in liquid media**

radial growth at 20 $^{O}$C. The radial growth of mycelium was lower at 25 $^{O}$C whereas the least growth was recorded at 10 $^{O}$C and 5 $^{O}$C. The optimum temperature for the mycelial growth of *Lentinula edodes* was 20-25 $^{O}$C as observed by Song *et al.* (1987) ; Lata and Sharma, 2012 and Sharma *et al.* (2013). Puri ( 2012 ) observed that there was decrease in average mycelial growth rate of *L. edodes* , when the temperature goes below 15 $^{O}$C and also when the temperature increases upto 35 $^{O}$C. Lata and Sharma, (2012) also observed that with further increase or decrease in temperature, there was a corresponding decrease in the mycelial growth of *L. edodes* which is in accordance with the results obtained in the present study.

Three different ranges of light conditions like ambient light, fluorescent light and darkness were tested for the mycelial growth and biomass production of *L. edodes* in both solid (Fig 12) and liquid media (Fig 13) (MEPD). Maximum radial growth and biomass production of *L. edodes* was recorded in darkness. Mycelia grew better in darkness than under direct strong light which inhibited its growth. Diffused light was required for fruiting body formation in Shiitake (Chen, 2002). Medda *et al.* (2011) reported that diffused light and alternate dark and light conditions showed good result in terms of average yield and biological efficiency *of L. edodes*. Results of the present study indicated that the mycelial and biomass production of *L. edodes* is positively influenced by the decrease in the brightness of light.

In the study conducted to determine the influence of different H+ ion concentration (4,5,6,7,8,9) in both solid (Fig 14) and liquid media (Fig 15) (MEPD) on the growth of different strains of *L. edodes*, pH 6 was observed to be the best for the growth of almost all the strains of *L. edodes*. Growth began to decline at lower and higher concentrations (5 to 4 and 7 to 9 respectively). Campbell (1932) revealed that the affinity of *L. edodes* towards the acidic media was due to the close association of acid production and enzymatic action. Acids might be formed by the action of oxidase on lignin and pentosans to bring about cellulose decomposition making acidic environmental as ideal for better mycelial growth of Shiitake. Khan et al. (1991) and Balazs *et al.* (1996) reported acidic pH of 5.0 as the most suitable for the growth of *L. edodes* . Lata and Sharma (2012) stated that 5.00- 7.50 was the most

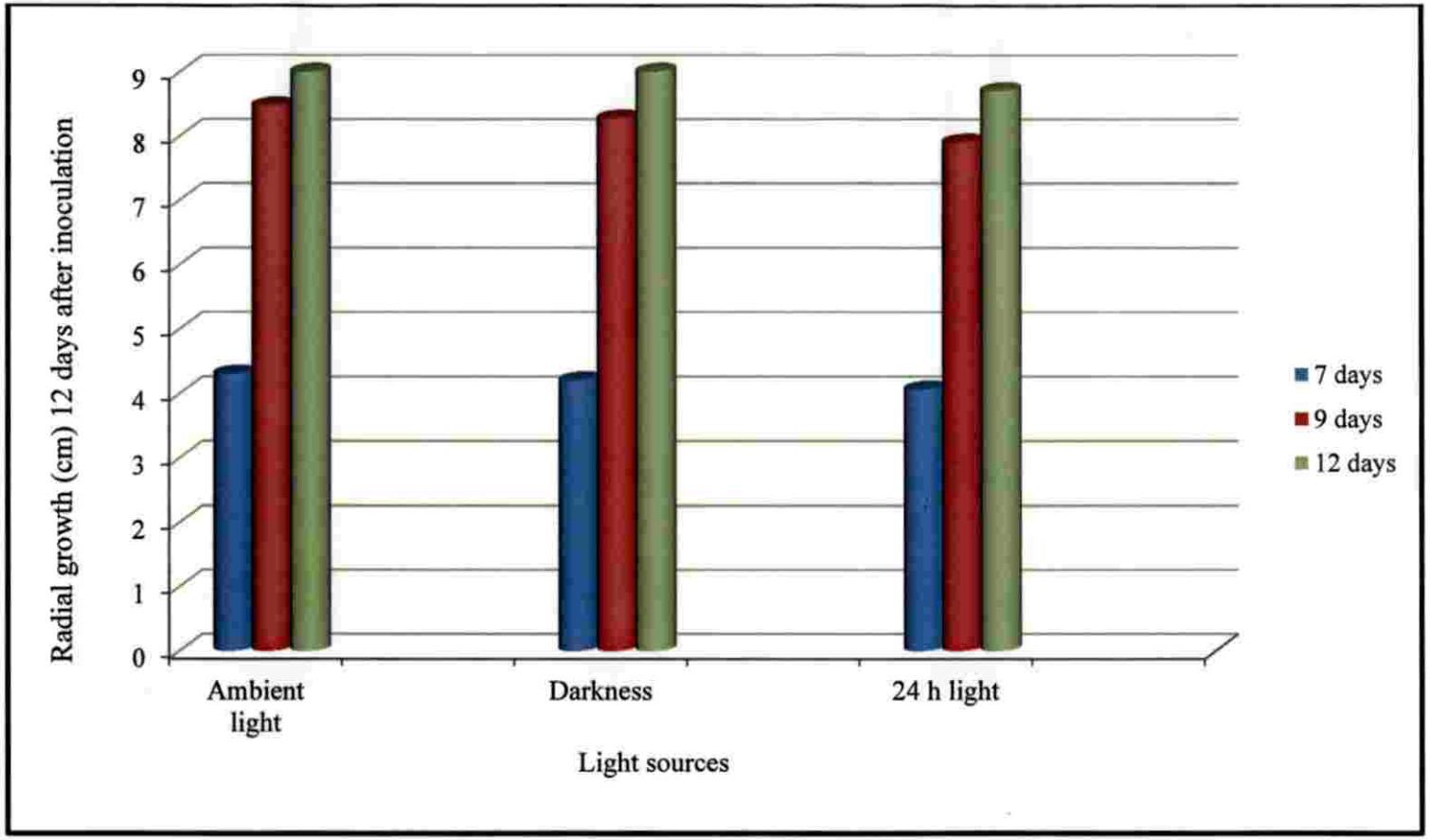



**Fig 12: Growth of *L. edodes* in different light sources on solid media**

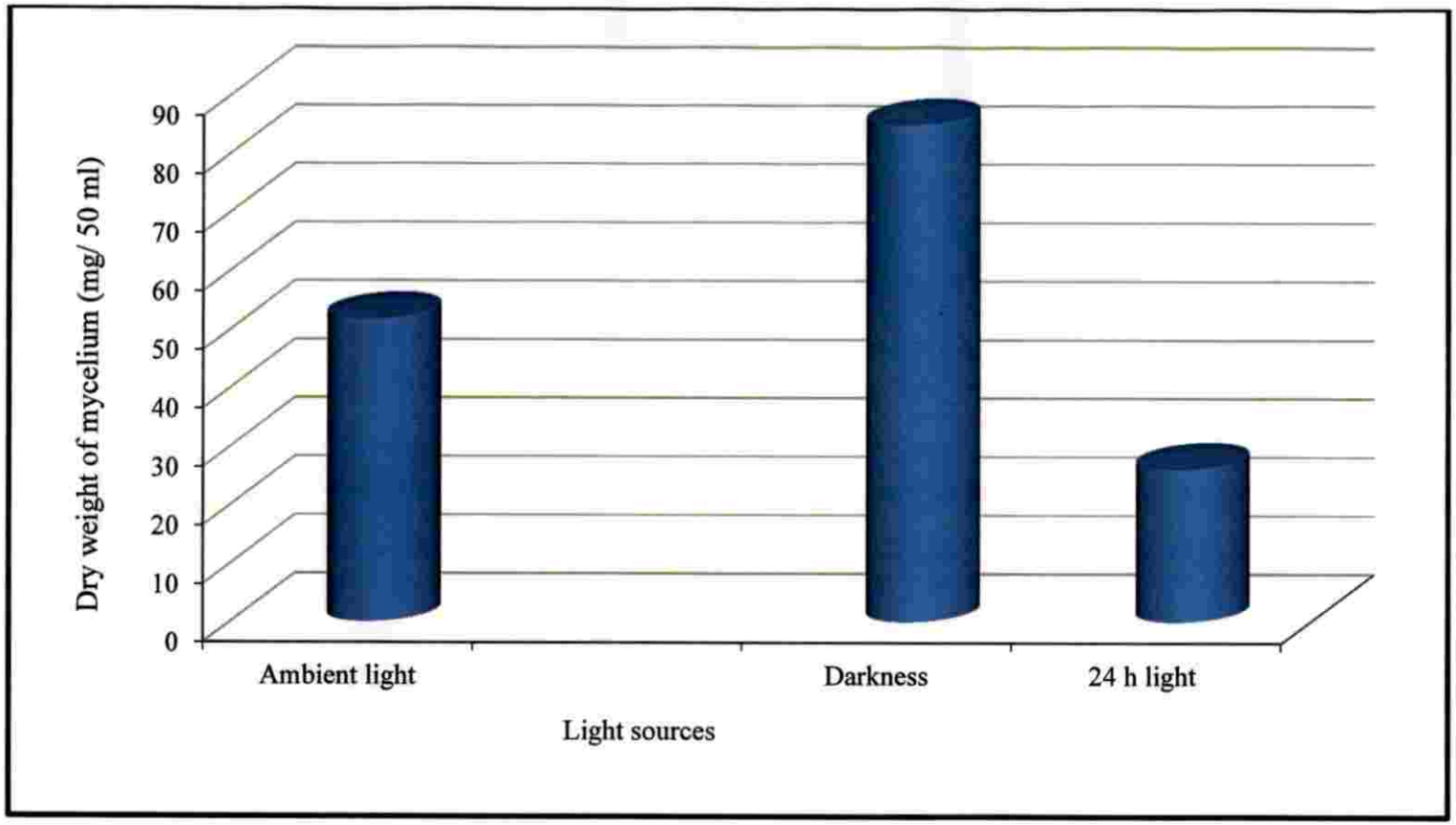



**Fig 13: Growth of *L. edodes* in different light sources in liquid media**

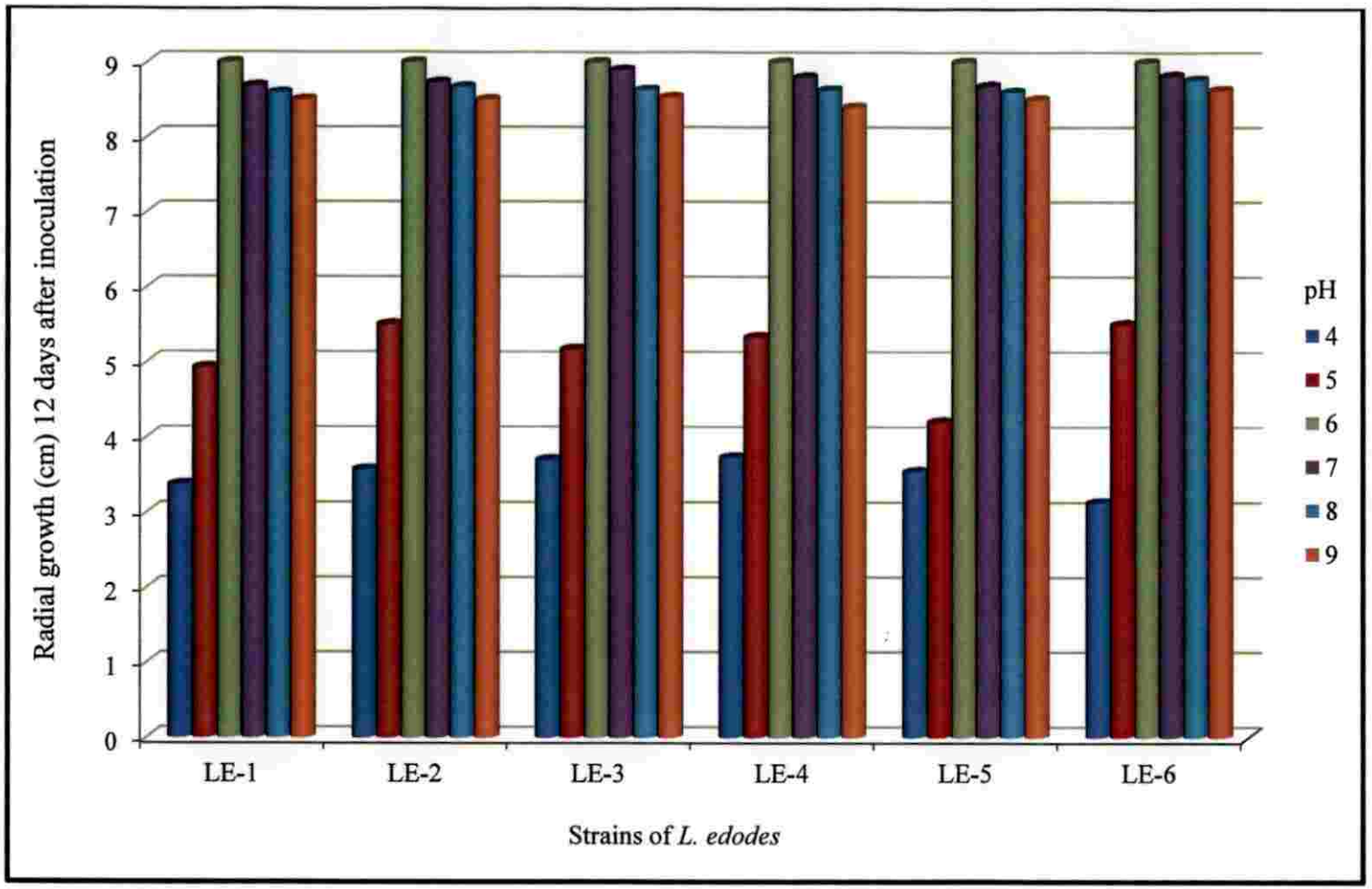



**Fig 14: Growth of *L. edodes* strains in different pH on solid media**

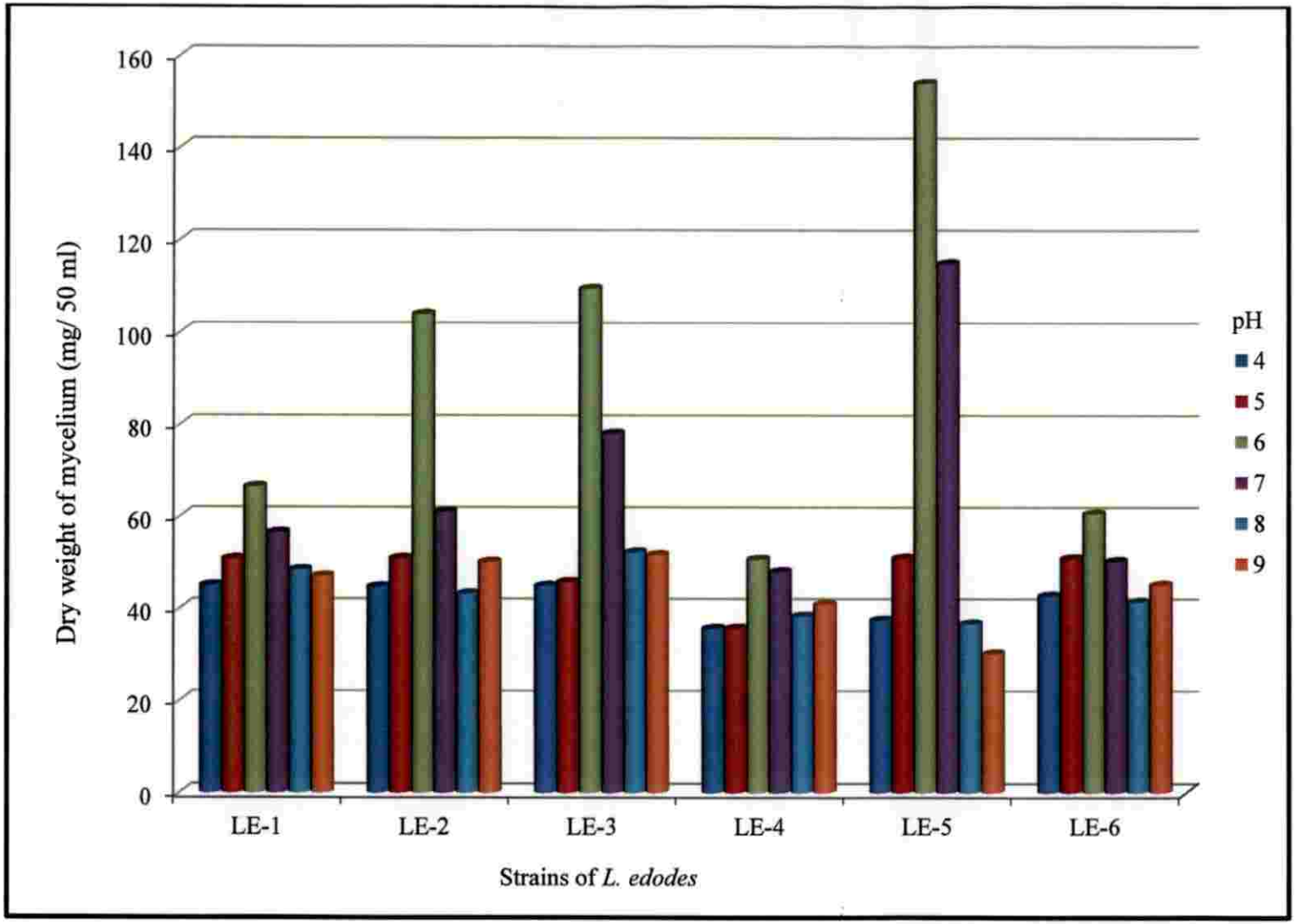



**Fig 15: Growth of *L. edodes* strains in different pH in liquid media**

suitable pH for the growth of *Lentinula edodes*. Sharma *et al.* (2013 a) conducted study to optimize pH for *Lentinula edodes* and the results showed that maximum mycelial growth was obtained at a pH of 7.00. The optimal pH for mycelial growth of *L. edodes* in potato dextrose broth was pH 6.00 ( Aminuddin *et al.*, 2013). In the present study it is evident that the optimum pH for the growth of *L. edodes* is 6, as it declined at lower and higher pH concentrations.

## 5.7. SPAWN PRODUCTION TECHNOLOGY OF LENTINULA EDODES

It was Shozaburo Mimura (1904-1915) discovered the pure culture technique for spawn production of Shiitake mushroom ( Stamets , 2000 a)

In the present study, five different grain substrates *viz.*, paddy, wheat, maize, sorghum , ragi and the non- grain substrate, sawdust were evaluated separately for their effects on the growth of *L. edodes* based on the minimum number of days taken for complete spawn run and the colour and nature of mycelial growth. The best spawn substrate selected based on minimum time taken for the spawn run was maize (16.33 days) followed by paddy (18.33 days) and wheat (20.66 days). Least significant mycelial growth was in sawdust (31.67 days) with the production of thin, root like mycelial growth. Paddy grain was selected for further studies based on the cost effectiveness and less contamination rate.

Kannan and Eswaran (2010 b) tested various grain substrates for their influence on the mycelial growth of *Lentinula edodes*, significantly less period (11.30 days) was taken for completing the spawn run on paddy grains , followed by ill filled paddy (11.50 days) and par boiled wheat (12.10 days). When Puri (2011) conducted a study using locally available poplar and teak sawdust, sorghum and wheat grains for the production of Shiitake spawn, fastest mycelial colonization was observed in sorghum grains which resulted in maximum biological efficiency (6.0 per cent to 8.0 per cent) proving sorghum as ideal substrate. She further observed all types of sawdust amended with cotton waste gave optimum spawn run. Sawdust substrate

supplemented with rice bran provided three kinds of nutrients (starch, nitrogen and minerals) which resulted in fast mycelial growth and also prevented contamination by other microorganisms (Lee, 1991). The present study therefore indicated the suitability of grain substrates like maize and paddy for spawn production while sawdust was not ideal for the same.

## 5.8. CULTIVATION TECHNOLOGY OF LENTINULA EDODES

The use of sawdust substrate for Shiitake cultivation by cylinder method was first evolved and improved by Peng in China in 1983 ( Chang and Miles, 2004). The use of sawdust substrate blocks in sealed polypropylene bags with microporous filters was developed in U.S which is a faster method in giving greater productivity in much shorter growing cycles.

Results of this study showed that all the six strains (LE1 to LE-6) took minimum days for mycelial colonization in sawdust amended with 20 per cent wheat bran. LE-2 strain took shortest time of 51.00 days to entirely colonize the substrate which was followed by LE-3 strain (53.33 days). All the strains took longest period for mycelial colonization in paddy straw and banana pseudo stem. This was in accordance with findings of Ashrafuzzaman *et al.* (2009) where, maximum time for the completion of mycelium run of *L. edodes* was observed in paddy straw. So sawdust combined with wheat bran was served as the best substrate for *L. edodes* cultivation in Kerala.

After completion of spawn run, the days taken by the six strains of *L. edodes* for the browning of the beds were evaluated. Browning of the beds is an indication of maturation of mycelium and the shortest time of 62.66 days for browning phase was taken by LE-1 strain in sawdust without any amendments which was on par with 63.00 days in sawdust + 20 per cent wheat bran. All the six strains failed to enter the browning phase in paddy straw and banana pseudo stem due to the early decomposition of the substrates. Ramkumar *et al.* (2010) reported that when ill filled paddy was amended with calcium carbonate, it enhanced the browning of mycelia (16.40 days), basidiocarp formation (51.80 days), yield (360.70 g/ kg) and biological

efficiency (36.07 per cent) of *Lentinula edodes* when compared to paddy straw, sugarcane bagasse, silver oak sawdust.

Among the six strains tested, LE-1 strain took significantly shorter period for sporocarp initiation in sawdust amended with 20 per cent wheat bran which was statistically on par with LE-3 strain which took 73.66 days in the same substrate. The two substrates, paddy straw and banana pseudo stem did not develop any pinheads, even after incubation of two and half months. This could be attributed to the early decomposition of the substrate by the growth of fungal mycelium.

Wonchull *et al.* (1996) described breeding of *Lentinula edodes* strains for sawdust based cultivation and effect of various methods to stimulate primordia formation. First flush was stimulated by a cold water (12 $^{O}$C) spray, followed by dipping for 24 h for second and third flushes which yielded about 283 g while continuous spray method yielded 245 g. According to Ashrafuzzaman *et al.* (2009), Shiitake mushroom when cultivated on sawdust of hard wood trees like babala, champa, ipil ipil, jack, mango, shisoo and rain tree, pinhead initiation occurred significantly earlier on jack sawdust when compared to other substrates. No primordia was produced on paddy straw substrate which gave no yield and took maximum time in completion of mycelium running.

LE-6 strain recorded maximum yield among all the substrates evaluated. The highest yield of 290.66 g/ 500 g bed substrate was obtained in sawdust amended with 20 per cent wheat bran by LE-6 strain which was on par with 223.33 g by LE-1 strain. This was followed by LE-2 strain (176.00 g) and LE-4 strain (171.33 g) in sawdust substrate without any amendments. Anastazia *et al.* (1982) observed that higher yield was obtained when the different agricultural waste substrates were supplemented with 10 per cent wheat bran.

Maximum biological efficiency of 58.13 per cent was recorded in LE-6 strain which was followed by LE-1 strain in sawdust + 20 per cent wheat bran substrate. Among the different *L. edodes* strains tested on the various substrates, LE-6 strain recorded the highest biological efficiency in sawdust amended with 20 per cent wheat

bran. Lowest biological efficiency of these strains were obtained in combination of paddy straw and sawdust amended with 10 per cent and 20 per cent rice bran.

Lizuka and Takeuchi (1978) formulated a substrate formula of 80 per cent sawdust and 20 per cent bran for the cultivation of *L.edodes*. Royse (1996) reported sawdust as the most popular basal ingredient along with starch based supplements like rice bran, wheat bran, millet, rye and maize for producing Shiitake. Ikegaya (1997) and Kawai *et al.* (1997) reported sawdust as a synthetic medium for the cultivation of Shiitake mushrooms. Fomina *et al.* (1999) proved oak sawdust with 20 per cent rye bran as the favorable substrate for the cultivation of *L. edodes*. Highest yield was obtained in oak and spruce bark combinations when supplemented with varying proportions of wheat grain, wheat bran and limestone, whereas straw based substrates showed a negative response with supplementation (Kilpatrick *et al.*, 2000). Regina and Leonardo (2009) evaluated the use of coconut husk sawdust supplemented with wheat bran or rice bran .Growth and vigor of Shiitake strains increased when supplemented with different proportions of wheat bran or rice bran. Similar results were obtained by Kumar and Sharma (2010) who recorded highest biological efficiency of 115 per cent in sawdust + 20 per cent wheat bran followed by 92 per cent biological efficiency in wheat straw + sawdust.

Puri *et al.* (2011) reported biological efficiency of 3.80 per cent to 24.20 per cent for all sawdust substrates than compared to other agricultural wastes (9.20 per cent to 45.90 per cent). Supplementation of wheat bran @ 10 per cent gave higher yield (80.40 g per 500 g dry substrate) than other supplements in Shiitake cultivation. *L. edodes* when cultivated on sawdust supplemented with different concentrations of wheat bran, rice bran and maize powder, highest number of fruiting bodies (34.8 / 500 gm packet), highest biological yield (153.3/ 500 g powder) and biological efficiency (76.6 per cent) were obtained from sawdust substrate supplemented with 25 per cent wheat bran (Moonmoon *et al.* 2011). Results of the present study are in accordance to the observations of the earlier reports as sawdust amended with rice bran or wheat bran was the suitable substrates for the cultivation of *L. edodes*.

Cultivation trials of two *Lentinus* sp. (*Lentinus tuber-regium and Lentinus connatus*) was standardized using the two substrates screened *ie.*, paddy straw and sawdust + 20 per cent wheat bran. Irrespective of *L. edodes* having various growth phases, native *Lentinus* sp. had only while colonization, sporocarp initiation and harvest stage. Among the two species, *L. tuber-regium* took minimum days for mycelial colonization when compared to *L. connatus*. Likewise, number of days for sporocarp initiation was also less in *L. tuber-regium*. Average yield and biological efficiency was higher in *L. tuber-regium* in sawdust + 20 per cent wheat bran.

## 5.9. NUTRIENT ANALYSIS

The proximate constituents present in all the six strains of *L. edodes* were analysed . The moisture content of *L. edodes* ranged from 79.66 per cent to 88.91 per cent, carbohydrates ( 35.29 per cent to 40.66 per cent), protein (18.33 per cent to 21.66 per cent), crude fibre ( 22.33 per cent to 27.33 per cent), Vitamin C ( 2.53 per cent to 3.50 percent), ash (2.70 per cent to 4.40 per cent) and lipid (2.46 per cent to 3.60 per cent). These results were in accordance with Singh *et al.* (2003) who tested *L. edodes* for various nutrients *viz.*, moisture content (40.07 per cent to 92.92 per cent), crude protein (8.89 per cent -27.29 per cent), fat (1.50 per cent - 8.50 per cent), ash (4.75 per cent -21.00 per cent) and crude fibre (3.25 per cent - 13.00 per cent ). Silva *et al.* (2007) reported that the mushroom mycelium contained high content of proteins, carbohydrates, fibre and vitamins.

Various minerals *viz.*, calcium content was significantly high in LE-6 strain ( 19.00 mg/ 100 g), magnesium content (1.10 mg/ 100 g) in LE- 4 strain. There was no significant difference between the strains in iron and manganese content. Maximum phosphorous content (2.87 mg/ 100 g) was recorded in LE-2 strain which was statistically on par with 2.82 mg/100 g (LE-1) and 2.80 mg/ 100 g (LE-5) and 2.72 mg/ 100 g (LE-3).

Potassium content was maximum (25.20 mg/ 100 g) in LE-2 strain which was followed by 18.00 mg/ 100 g (LE-3). LE-2 strain recorded maximum zinc (28.66 mg / 100 g) which was statistically on par with 28.33 mg/ 100 g (LE-6), 28.00 mg/ 100 g

(LE-1) and 27.00 mg/100 g (LE-3). The results obtained in the experiments done by George *et al.* (2014) were comparable with the results obtained in the present study which revealed that the fruiting bodies of Shiitake contained potassium (6.2 g - 13.2 g/ kg), phosphorous (7.8 g - 54.5 g / kg), magnesium (3.4 g- 6.5 g/ kg), calcium (179.8 mg - 1698.0 mg/ kg), sodium (191.3 mg- 3448 mg/ kg ), aluminium (15.3 mg - 79.5 mg/ kg). Heavy metals included iron (44.4 mg -125.1 mg/ kg), zinc (59.3 mg - 283.9 mg/kg) and copper (13.7 mg -182.4 mg/ kg).

## 5.10. ENZYME ACTIVITY

The strains of *L. edodes* were subjected to enzyme analysis *viz.*, Phenyl alanine lyase, peroxidase and polyphenol oxidase. Phenyl alanine lyase activity of the strains ranged from 2.48 µg to 2.60 µg of cinnamic acid/ min/g. whereas the activity of peroxidase ranged between 0.15 to 0.56 of absorbance /min/ g . The activity of poly phenol oxidase was higher in LE-6 strain (57.30 of absorbance/ min/ g) which was followed by LE-2 (54.58), LE-3(53.80) and LE-5(52.13). According to Kurt and Buyukalaca, (2010); Hernandez *et al.* (2011), increase in enzyme activities during vegetative growth and mycelial regenerative stage was associated with the energetic requirements for initiating fructification whereby enzymes are secreted to digest the substrate to provide carbon and other nutrients.

## 5.11. SHELF LIFE

Shelf life of *L. edodes* was determined by keeping mushrooms in polypropylene cover and paper box with and without perforations at room temperature and also at refrigerated condition. The study showed that mushrooms packed in paper box with perforation in refrigerated condition had better shelf life of 20.66 days when compared with paper box without perforation. According to Santana *et al.* (2008) the shelf life of minimally processed Shiitake mushroom was 10 days when kept at 7 $^{O}$C but less than 5 days at 10 $^{O}$C and 3 days at 15 $^{O}$C. Ramkumar *et al.* (2010) reported that Shiitake mushroom can be stored upto 10 days in perforated polybags under natural condition and up to 30 days in refrigerated condition.

## 5.12. ORGANOLEPTIC STUDIES

Organoleptic studies were conducted by preparing recipes of both fresh and dried *L. edodes* mushroom and subjected to sensory evaluation. The different recipes prepared and evaluated for their characters like colour and appearance, texture, flavor, taste and overall acceptability using a five point score card. Among the various products, cutlets had maximum ranking for colour and appearance and flavor. Mushroom masala ranked first (4.50) for texture which also had excellent flavor (4.60). The overall acceptability was also high for masala, followed by scramble, cutlet, soup, baji. The least preferred product was biscuit with overall acceptability 4.00. Ibrahium and Hegazy (2014) evaluated the effect of partial replacement of wheat flour by different levels (10 per cent , 20 per cent and 30 per cent) of mixture of mushroom powder and sweet potato flour at equal rates in biscuit making and the results showed that increase in corporation of mushroom powder in biscuits increased the protein, fibre, ash, iron, calcium, potassium, phosphorous and amino acid content. Prodhan *et al.* (2015) reported that biscuits can be fortified with protein rich mushroom powder in order to supplement protein in the diet and nutrition.

## 5.13. PESTS AND DISEASES

The incidence of spring tails were found on mushroom beds during the resting period which feed on mycelium and caused disappearance of mycelium from the substrate. *Trichoderma* infection were found during spawn run period, second harvest and also when relative humidity was high.

For a long time, Shiitake was known as *Lentinus edodes* (Berk.) Singer, among mushroom growers. In 1975, Pegler proposed this species to be transferred to *Lentinula* based on microscopic and macroscopic characters. Recent DNA studies conducted by Molina *et al.* (1992), also supported its placement in the *Lentinula* genus. But, mushroom growers continued to use the old name *Lentinus edodes* even during the later period. As there are many reports of *Lentinus* sp. from Kerala, an

attempt was also made in this study to make a comparative evaluation of morphological characters and phylogenetic relationship between the two mushrooms which is a subject of controversy till date. However from this study it is concluded that *Lentinula edodes* is completely divergent from *Lentinus* sp.

This study was mainly conducted to explore the occurrence of Shiitake mushroom (*L. edodes* ) in Kerala, as so far there is no record of the mushroom in this state. Further the study also envisaged the standardization of the cultivation technology of Shiitake mushroom under semitropical conditions of Kerala using native or reference strains of the mushroom. The strain LE-6 of Udaipur which performed best in the artificial cultivation under environmental conditions prevalent in Kerala. Suitable nutrients, substrates and environment parameters required for cultivation of the selected strain was standardized to obtain a satisfactory biological efficiency. This study which is a prime attempt for the cultivation of Shiitake mushroom in Kerala marks the beginning of the artificial cultivation of prized mushrooms which are otherwise recalcitrant to grow in the natural habitat of the state.

# *Summary*

## 6. SUMMARY

Surveys were conducted in different locations of Thiruvananthapuram, Kollam, Idukki, Wayanad, Pathanamthitta, Kannur and Kasargode districts of Kerala. Strains resembling *Lentinula edodes* could not be located in any of the surveyed locations. However, mushroom specimens of *Lentinus* were spotted and collected for making comparative morphological and phylogenetic analysis between *Lentinus* sp. and *Lentinula edodes.*

As natural isolates of *L. edodes* were not collected during the survey, six strains (LE-1 to LE-6) were procured from institutes *viz.*, LE-1 to LE-5 from GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand and LE-6 from Maharana Pratap University of Agriculture and Technology, Udaipur.

Morphological studies of *L. edodes* strains were conducted and their characters were recorded and documented. All the strains were morphologically similar except LE-6 from Udaipur which was larger and had a yellowish tinge unlike all other strains were chocolate brown in colour.

From the morphological characterization of *Lentinus* sp. collected during the survey they could be grouped to six species *viz.*, VLYN- 1 to VLYN-6 *(Lentinus squarrosulus*), VLYN-7 (*Lentinus giganteum*), VLYN-8 to VLYN-10 (*Lentinus tuber-regium*), VLYN-11 (*Lentinus connatus*) ,VLYN-12 and VLYN-13 (unidentified *Lentinus* sp.)

Phylogenetic analysis of all six strains of *L. edodes* using RAPD markers confirmed the variability between the strains. Maximum similarity coefficient of 74.10 per cent was observed between LE-2 and LE-6 strains while LE-2 and LE-4 strains showed a minimum similarity coefficient of 35.70 per cent. Further studies by ITS sequencing showed that all the *L. edodes* strains showed 99-100 per cent similarity with the sequences of *L. edodes* available in NCBI database.

Molecular characterization of *L. edodes* and local isolates of *Lentinus* sp. done by ITS sequencing and RAPD analysis confirmed that there is no phylogenetic relationship between the two species.

Growth of strains of *L. edodes* were tried on different solid media namely potato dextrose agar, malt extract agar, oat meal agar, malt extract peptone dextrose agar, carrot agar, Czapek Dox agar and yeast extract agar for testing their efficiency in supporting the radial growth of *L. edodes*. The radial growth after 12 days indicated that malt extract peptone dextrose agar was superior with a maximum growth of 9.00 cm by almost all the strains. This was followed by malt extract , oat meal and potato dextrose agar. Yeast extract agar was the least preferred media.

The biomass production of *L. edodes* strains in liquid media 25 days after inoculation indicated that oat meal broth was the most promising treatment with 405 mg/ 50 ml of biomass production.

Studies on the effect of carbon sources on radial growth and biomass production of *L. edodes* in solid and liquid media revealed that dextrose and mannitol were the best with a radial growth of 9.00 cm twelve days after inoculation and biomass production of 87 mg/ 50 ml respectively. Least growth was observed in the media amended with lactose as carbon source.

Peptone (9.00 cm) was found to be significantly superior nitrogen source , which was followed by ammonium chloride and sodium nitrate. Sodium nitrate on par with peptone with 2.24 mg/ 50 ml and 2.10 mg/ 50 ml respectively of biomass production was observed in liquid medium.

Six different temperature conditions of 5 $^{O}$C , 10 $^{O}$C , 15 $^{O}$C, 20 $^{O}$C, 25 $^{O}$C and room temperature (28 +2 $^{O}$C) were tested for the production of maximum radial growth and biomass. *L. edodes* attained full growth (9.00 cm) on petriplate 12 days after inoculation at a temperature of 20 $^{O}$C by all the *L. edodes* strains. In liquid

media also, temperature of 20 $^{O}$C supported maximum biomass production of 81.90 mg/ 50 ml.

The influence of different light conditions on the radial growth and biomass production of *L. edodes* was evaluated and it was found that darkness was more efficient in enhancing the radial growth (9.00 cm) and biomass production (84.93 mg/ 50 ml).

In the study conducted to determine the influence of different H+ ion concentration (4,5,6,7,8,9) on the radial growth and biomass production of *L. edodes* strains , pH 6 was observed to be the best for the growth of almost all the strains of *L. edodes*. There was a decrease in radial growth and biomass production at lower and higher H +concentrations.

Five grain substrates of paddy, wheat, maize, sorghum, ragi and non-grain substrate sawdust were evaluated for the time taken for mycelial colonization and nature of mycelial growth. Though maize grain took minimum of 16.33 days for complete mycelial colonization with thick, white, mycelial growth, due to the less contamination rate and cost effectiveness, paddy grains was selected as the best substrate.

Different substrates were evaluated for the development of a cultivation package for shiitake mushroom. LE-1 strain took minimum of 71.00 days for initiation of sporocarp in sawdust + 20 per cent wheat bran substrate. Hard wood sawdust especially of teakwood was used for the study. The substrate based on paddy straw and banana pseudo stem were not found effective for pinhead initiation and thus failed to produce sporocarps. LE-1 produced maximum sporocarp (11.33) in sawdust + 20 per cent wheat bran substrate which was followed by LE-3 (10.63) in sawdust + 20 per cent rice bran substrate. Maximum yield of 290.66 g/ 500 g substrate was obtained in sawdust + 20 per cent wheat bran substrate by LE-6.

Maximum biological efficiency of 58.13 per cent was recorded in LE-6 in sawdust + 20 per cent wheat bran substrate.

Substrates like paddy straw and sawdust amended with 20 per cent wheat bran were evaluated for the development of native isolates of *Lentinus tuber-regium* and *Lentinus connatus* . Minimum days for mycelial colonization and sporocarp initiation of the isolates were observed in sawdust amended with 20 per cent wheat bran. The same substrate yielded with a maximum biological efficiency of 58.00 per cent was obtained in *Lentinus tuber-regium* whereas 36.60 per cent biological efficiency in *Lentinus connatus.*

Nutrient analysis of all the six strains of *L. edodes* showed carbohydrate content ( 35.29 per cent to 40.23 per cent), protein (18.33 per cent to 21.66 per cent), crude fibre (22.33 per cent to 27.33 per cent), Vitamin- C (2.53 per cent to 3.50 per cent), ash (2.70 per cent to 4.40 per cent) and lipid (2.46 per cent to 3.60 per cent). Mineral content of *L. edodes* included calcium (11.00 mg to 19.00 mg/ 100 g), magnesium (0.46 mg to 1.10 mg/ 100 g). There was no significant difference between the strains in iron and manganese. The phosphorous content (1.65 mg to 2.87 mg/100 g ), potassium (16.33m g to 25.20 mg/ 100 g), sodium (13.00 mg to 23.66 mg/ 100 g) and zinc (19.66 mg to 28.33 mg/ 100 g).

The enzyme activity of phenyl alanine lyase of *L. edodes* strains ranged from 2.48 µg to 2.60 µg of cinnamic acid/ min/g whereas the activity of peroxidase ranged between 0.15 to 0.56 of absorbance /min/ g . The activity of poly phenol oxidase was higher in LE-6 strain (57.30 of absorbance/ min/ g) which was followed by LE-2 (54.58), LE-3(53.80) and LE-5(52.13).

Mushrooms packed in paper box with perforation in refrigerated condition had better shelf life of 20.66 days when compared with paper box without perforation and polypropylene cover with and without perforation in the same condition.

Sensory evaluation of mushroom products made from *L. edodes* was carried out by a panel of judges for characters like colour and appearance, texture, flavor, taste and overall acceptability using a five point score card. Mushroom masala scored maximum for texture, taste, flavor and overall acceptability when compared to other recipes like mushroom cutlet, scramble, soup, baji and biscuit.

The incidence of spring tails and *Trichoderma* infection were found on mushroom beds throughout the growing period of *L. edodes*.

As part of the present study, six strains of *Lentinula edodes* were procured, LE-6 was found to be the best *Lentinula* culture based on the cultural studies, malt extract peptone dextrose agar was the most suitable medium, an acidic pH and temperature of 20 $^{O}$C favoured the growth. Paddy was the most suitable spawning material, sawdust amended with 20 per wheat bran was the most efficient substrate and LE-6 strain was superior in terms of biological efficiency.

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# *Appendices*

## APPENDIX – I

## DATA-SHEET

Date of collection .....

Collected by..... Locality .....

### GENERAL

Substrate :

Habitat : Terrestrial / Lignicolous / Epixylose / Coprophilous

Habit : Solitary / Scattered /Gregarious

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**Pileus**

Shape : Convex/infundibuliform/Umbonate/Petaloid/Flabelliform/Depressed

Colour :

Size : Diameter

Thickness

Texture : Soft/Brittle/Fleshy/Fragile/Coriaceous/Membraneous

---

**Stipe**

Shape : Clavate/Cylindrical/Solid/Hollow/Slender

Size : Length :

Diameter :

Attachment to pileus : Lateral/Eccentric/Central/Resupinate

Surface : Glabrous/Scaly/Smooth/Pubescent/Fibrillose

Basal part : Globular/Bulbous/Fusoid/Cylindrical

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## APPENDIX – I (Continued)

**Gills**

| | |
|---|---|
| Arrangement | : Remote/Free/Decurrent/Adnate/Adnexed/Sinuate |
| Texture | : Soft/Brittle/Waxy/Thick/Papery/Opaque |
| Margin | : Smooth/Wavy/Serrate/Fimbriate/Dentate |
| Size | : Number per cm |

**Veil**

| | |
|---|---|
| Type | : Present/Absent |

**Annulus**

| | |
|---|---|
| Type | : Present/Absent |

**Volva**

| | |
|---|---|
| Type | : Present/Absent |

**Spore print**

| | |
|---|---|
| Colour | : |

**Spores**

| | |
|---|---|
| Colour | : |
| Shape | : Ovate/Elliptical/Globose/Epiculate/Cylindrical/Fusiform/ Angular/Echinulate/Recticulate/Ovoid/Pyriform |
| Reaction with | |
| Cotton blue | : Cyanophilic/Acyanophilic |
| Melzer's reagent | : Amyloid/Dextrinoid/Nonamyloid |

## APPENDIX-II

## COMPOSITION OF STAIN USED

1. **Lactophenol –Cotton blue**

| | |
|---|---|
| Anhydrous lactophenol | - 67.0 ml |
| Distilled water | - 20.0 ml |
| Cotton blue | - 0.1 g |

Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid in 3 ml glycerol.

## APPENDIX – III

### Composition of different media

**a) Potato dextrose agar (PDA)**

| | | |
|---|---|---|
| Potato | : | 200 g |
| Dextrose | : | 20 g |
| Agar-agar | : | 20 g |
| Distilled water | : | 1 l |

**b) Malt extract agar (MEA)**

| | | |
|---|---|---|
| Malt extract | : | 25 g |
| Agar-agar | : | 20 g |
| Distilled water | : | 1 l |

**c) Oat meal agar (OMA)**

| | | |
|---|---|---|
| Oats | : | 40 g |
| Agar-agar | : | 20 g |
| Distilled water | : | 1 l |

**d) Malt extract peptone dextrose agar (MEPDA)**

| | | |
|---|---|---|
| Malt extract | : | 20 g |
| Peptone | : | 1 g |
| Dextrose | : | 10 g |
| Agar-agar | : | 20 g |
| Distilled water | : | 1 l |

**e) Carrot agar (CA)**

| | | |
|---|---|---|
| Carrot | : | 200 g |
| Dextrose | : | 20 g |
| Agar-agar | : | 20 g |
| Distilled water | : | 1 l |

**f) Czapek– Dox Agar**

| | | |
|---|---|---|
| $NaNO_3$ | : | 2 g |
| $K_2HPO_4$ | : | 1 g |
| $MgSO_4.7H_2O$ | : | 0.5 g |
| KCl | : | 0.5 g |
| $FeSO_4$ | : | 0.01 g |
| Sucrose | : | 30 g |
| Agar | : | 20 g |
| Distilled water | : | 1 l |

**g) Yeast extract agar (YEA)**

| | | |
|---|---|---|
| Yeast extract | : | 20 g |
| Agar | : | 20 g |
| Distilled water | : | 1 l |

# APPENDIX – IV

## Score card

| | |
|---|---|
| **Colour and appearance** | |
| Excellent | 5 |
| Good | 4 |
| Fair | 3 |
| Poor | 2 |
| Very poor | 1 |
| **Texture** | |
| Very good | 5 |
| Good | 4 |
| Fair | 3 |
| Poor | 2 |
| Very poor | 1 |
| **Flavour** | |
| Very soft | 5 |
| Soft | 4 |
| Slightly fibrous | 3 |
| Fibrous | 2 |
| Very fibrous | 1 |
| **Taste** | |
| Highly acceptable | 5 |
| More acceptable | 4 |
| Acceptable to certain extent | 3 |
| Less acceptable | 2 |
| Not acceptable | 1 |
| **Overall acceptability** | |
| Highly acceptable | 5 |
| More acceptable | 4 |
| Acceptable to certain extent | 3 |
| Less acceptable | 2 |
| Not acceptable | 1 |

# STRAIN EVALUATION AND PRODUCTION TECHNOLOGY OF SHIITAKE MUSHROOM (*Lentinula edodes* (Berk.)Pegler)

**DEEPA RANI C.V**

**Abstract of the**
**thesis submitted in partial fulfilment of the requirement**
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**DOCTOR OF PHILISOPHY IN AGRICULTURE**

**Faculty of Agriculture**
**Kerala Agricultural University**

**DEPARTMENT OF PLANT PATHOLOGY**
**COLLEGE OF AGRICULTURE**
**VELLAYANI, THIRUVANANTHAPURAM 695522**
**KERALA, INDIA**

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# ABSTRACT

The present investigation on 'Strain evaluation and production technology of Shiitake mushroom (*Lentinula edodes* (Berk.) Pegler' was conducted at Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram during the period 2012-2015. The aim of the experiment was to exploit various strains of *Lentinula* spp. for novel production technology and their phylogeny analysis through physiological and molecular studies.

Surveys were collected during pre and post monsoon periods of May to December from different parts of Thiruvananthapuram, Kollam, Wayanad, Idukki, Pathanamthitta, Kannur and Kasargode districts. Six isolates of *Lentinus* sp. (VLYN-1 to VLYN-13) obtained during the survey were identified and compared with procured reference strains of *Lentinula edodes* (LE-1 to LE-5 from GB Pant University of Agricultural and Technology, Pantnagar, Uttarakhand) and LE-6 strain (Maharana Pratap University of Agriculture and Technology, Udaipur) . Morphologically the native isolates of *Lentinus* spp. had concave, funnel and convex pileus with varying colors and were leathery in nature. *L. edodes* strains in contrast had convex pileus with chocolate brown and golden yellow sporocarps which were fleshy and edible.

Phylogenetic analysis of all six strains of *L. edodes* using RAPD markers confirmed the variability between the strains. Maximum similarity coefficient of 74.10 per cent was observed between LE-2 and LE-6 strains while LE-2 and LE-4 strains showed a minimum similarity.coefficient of 35.70 per cent. Further studies by ITS sequencing showed that all the *L. edodes* strains tested in the study showed 99-100 per cent similarity with the known sequences of *L. edodes* available in NCBI database while that of native isolates showed 99- 100 per cent similarity to *Lentinus*

*tuber-regium* and *Lentinus connatus* thus confirming the variability between *Lentinus* and *Lentinula* sp.

All the six strains of *L. edodes*, showed maximum mycelial growth in malt extract peptone dextrose agar in solid and oat meal broth in liquid medium. *L. edodes* strains preferred temperature of 20 °C with an acidic pH of 6. Dark and ambient light conditions favored maximum mycelial growth and biomass production for *L. edodes* culture.

Although a minimum period of 16.33 days was required for full mycelial run in maize grains but due to comparatively less contamination rate in paddy grains which took 18.33 days for completion of mycelial run were selected as best substrate for further studies.

Different substrates were evaluated for the development of a cultivation package for shiitake mushroom. Results showed that LE-1 strain took minimum of 71.00 days for initiation of sporocarp in sawdust supplemented with 20 per cent wheat bran. Hard wood sawdust especially of teakwood was used in the study. The substrate based on paddy straw and banana pseudo stem were not found effective for pinhead initiation and thus failed to produce sporocarps. LE-1 produced maximum sporocarp (11.33) in sawdust + 20 per cent wheat bran which was followed by LE-3 (10.63) in sawdust + 20 per cent rice bran. Maximum yield of 290.66 g/ 500 g substrate was obtained in sawdust + 20 per cent wheat bran by LE-6 strain. Maximum biological efficiency of 58.13 per cent was also recorded in LE-6 in sawdust supplemented with 20 per cent wheat bran substrate.

Substrates like paddy straw and sawdust amended with 20 per cent wheat bran substrates were evaluated for the development of native isolates of *Lentinus tuberregium* and *Lentinus connatus* . Results showed that maximum biological efficiency of 58.00 per cent was obtained by *Lentinus tuber-regium* whereas 36.60

per cent biological efficiency by *Lentinus connatus* in sawdust amended with 20 per cent wheat bran substrate.

Nutrient analysis of all the six strains showed that carbohydrate content ranged between 35.29 per cent to 40.23 per cent, protein 18.33 per cent to 21.66 per cent, crude fibre 22.33 per cent to 27.33 per cent, Vitamin- C 2.53 per cent to 3.50 per cent, ash 2.70 per cent to 4.40 per cent and lipid 2.46 per cent to 3.60 per cent. Mineral content of *L. edodes* included Ca (11.00 mg to 19.00 mg/ 100 g), Mg (0.46 to 1.10 mg/ 100 g), Fe (1.36 mg to 1.80 mg/ 100 g), Mn (1.53 mg to 2.63 mg), P (1.65 mg to 2.87 mg), K (16.33 mg to 25.20 mg), Na (13.00 mg to 23.66 mg) and Zn (19.66 mg to 28.33 mg/ 100 g).

Sensory evaluation of mushroom products made from *L. edodes* was carried out by a panel of judges for various characters of which mushroom masala scored maximum for texture, taste, flavor and overall acceptability when compared to other recipes like mushroom cutlet, scramble, soup, baji and biscuit.

As part of the study, paddy grain was found to be the most suitable substrate for spawn production of *L. edodes* and teakwood sawdust amended with 20 per cent wheat bran was the most efficient bed substrate. LE-6 strain was superior in terms of yield and biological efficiency. Therefore findings of the above investigation recommends the adoption of a suitable cultivation package for shiitake mushroom by using low cost substrates (hardwood sawdust) available in Kerala in plains and hilly regions.