CHAPTER 3
MATERIALS AND METHODS

3.1. Fungal Culture

The pure culture of *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Pleurotus djamor* and *Pleurotus florida* was obtained from the Directorate of Mushroom Research, ICAR Solan, Himachal Pradesh, India. The cultures were maintained on Potato Dextrose Agar (PDA) and 2% Malt Extract Agar (MEA) slants and stored at 4^0 C (Fritsch 1978 and Jong 1978). Sub-culturing was done after every 15 days intervals.

A. Potato Dextrose Agar

Sr. No	Material	Quantity
1.	Potato	400gm
2.	Dextrose anhydrous	20gm
3.	Agar agar Type I	20gm
4.	Distilled Water	1000ml

B. Malt Extract Agar

Sr. No	Materials	Quantity
1.	Malt extract	30gm
3	Peptone	5gm
4	Agar agar Type I	20gm
5	Distilled water	1000ml

C. Water Agar

Sr. No	Materials	Quantity
4	Agar agar Type I	20gm
5	Distilled water	1ltr.

3.2. Histopathological characterization of *Pleurotus* sp.

Transverse sections of the mushroom gills were washed with sterile distilled water and stained with lacto phenol - cotton blue (1:1) and incubated for 5-10 min for staining. After staining mounted with cover slip and observed in the Leica DM3200 microscope using 20X and 40 X magnifications. Photographs were taken using Leica Application Suit (LAS Version 4.4.0) software.

3.3. Preparation of Spawn

Wheat grains were used to prepare the spawn for cultivation. Wheat grains were boiled for 20 min and then water drained off. Then the grains were allowed to dry for 4-5 hours in a clean and dry place. After drying, 0.5% (W/W) $CaCO_3$ and 2% (W/W) $CaSO_4$ was added and mixed well. The grains were then filled in the polypropylene bags (250 gm/bag) and autoclaved at 121^0 C for 1 hour. The grains were then inoculated with actively growing mycelia of *Pleurotus ostreatus*, *P. sajor-caju*, *P. djamor* and *P. florida* and incubated at $25\text{-}28^0$ C for 12-14 days until the mycelia fully cover the grains (Sanchez, 2010) (Figure 2).

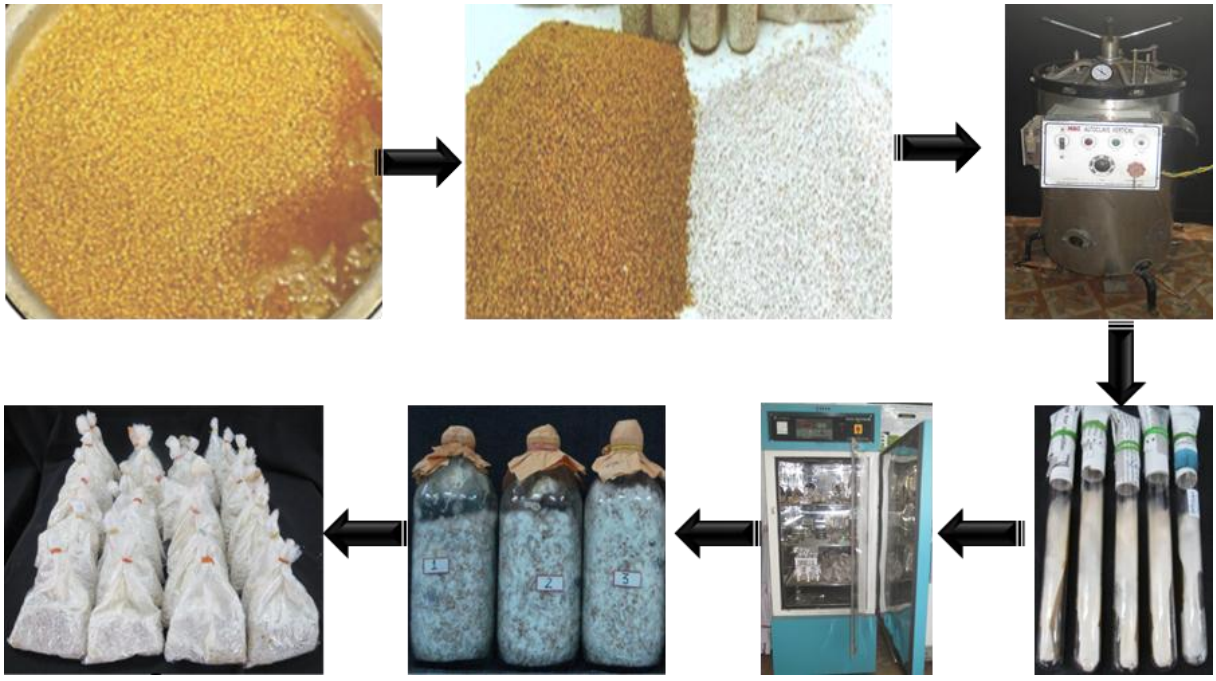

Figure 2: Preparation of grain spawn for the cultivation of *Pleurotus* sp.

3.4. Cultivation of Oyster mushroom

3.4.1. Preparation of substrates

Different agro wastes were used for the cultivation of different species of oyster mushroom. Paddy straw, wheat straw, saw dusts were used either singly or in combination with other substrates. Substrates were collected from the local markets. Straw were dried and chopped in 2-5cm long pieces and washed 3-4 times with tap water. Then the substrates were allowed to soak in water for overnight. Again it was again cleaned 2-3 times until the brown water drained off fully. After cleaning, the substrates were pasteurized at 55-65^0 C for 20-30 min and allowed to cool at room temperature.

3.4.2. Spawning

Spawning was done using different types of containers such as polypropylene bags (12 inch X 18 inch), plastic waste bottles (12 inch X 3 inch dia.), broken laboratory glass goods and paper boxes (30 inch X 12 inch X 12 inch) for the cultivation. Cooled pasteurized substrates were used singly or mixed and layer spawning was done using the fully grown spawn at 100gm/kg substrates following the methodology Sarkar *et al* (2007). The containers were closed tightly and small pores were made for aeration. The containers were then incubated at room temperature in the Mushroom Production Unit, Immuno-Phytopathology Laboratory, NBU (Figure 3) for 10-12 days. Mycelia cover the substrate and the polypropylene bags or the containers were removed and water sprayed over the fully grown mycelia to maintain the 80-90% humidity. After 3-4 days of removal of the container, the pinhead initiation was observed.

3.4.3. Initiation of fruiting body

The pinhead appeared after 4-5 days of opening the bags, lids of bottles or the box cover. Fruiting body was developed at room temperature and 80-90% relative humidity. The fruiting body was harvested from the base carefully so that there will be no damage on the fully grown mycelia. Humidity was again maintained by spraying water after the harvest of the mushrooms for more flushes (Figure 3).

Figure 3: Mushroom production unit for large scale production of *Pleurotus* sp established at Immuno phytopathology laboratory, Department of Botany, NBU

3.5. Biochemical analysis

3.5.1. Determination of moisture content

100 gram of fresh mushroom was weighed and dried in hot air oven at 100-105^{0}C and then cooled in room temperature. Moisture content of mushroom were estimated using the methodology described by Raghuramulu *et al.,* (2003). The moisture content was calculated as following equation

Moisture content (%) = (initial weight − final weight) × 100/weight of sample.

3.5.2. Extraction and estimation of Carbohydrates

Extraction of carbohydrate form the mushroom was done following the methodology of Harbone (1973). 1gm of sample was homogenated using 95% ethanol and then it was kept on boiling water bath for evaporation of the alcoholic fraction. The sample was then resuspended in distilled water. Then it was centrifuged at 10000 rpm for 15 min and the supernatant collected for further analysis.

3.5.2.1. Estimation of total sugar

Total sugar was estimated following the methodology described by Plummer (1973). 1ml of the supernatant was taken and to it 4ml of anthrone reagent (0.2gm anthrone in 100 ml H_2SO_4) was added and then it was kept in boiling water bath for 10 min. the sample was then cooled in tap water and observed at 620 nm weave length.

3.5.2.2. Estimation of reducing sugar

Reducing sugar of mushroom was estimated using the Nelson-Somogy's methodology. 1 ml of supernatant was taken and to it, 1 ml of alkaline copper tartarate added and warmed in water bath for 20 mins. Then it was cooled in tap water and 2 ml of arseno molybdate was added and then observed at 510nm in colorimeter.

3.5.3. Extraction and estimation of soluble protein

3.5.3.1. Mycelia

Mushroom protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). For extraction of protein, different stages of fruiting body were harvested and incubated at -20^0C for 2 hours. The fruiting body was crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05 M Sodium phosphate buffer (PH-7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite, PVPP (polyvinyl pyrollidine phosphate) and 0.5mM magnesium chloride in ice bath. The homogenated mixture was kept for 2h or overnight at 4ºC and then centrifuged at10,000 rpm for 30 min at 4ºC to eliminate cell debris. The supernatant was equilibrated to100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000 rpm) for 30 min at 4º C; the precipitate was dissolved in the same buffer (pH 7.2).

3.5.3.2. Leaf

Soluble protein was extracted from leaves following the method of Chakraborty *et al.*, (1994). Leaf tissues were frozen in liquid nitrogen and ground in 0.05mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$ and 2mM PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm.The supernatant was used as crude protein extract.

3.5.3.3. Estimation of soluble protein content

Soluble proteins were estimated following the method as described by Lowry *et al*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% $CuSO_4$ and 1ml of 2% sodium potassium tartarate, added to 100 ml of 2% Na_2CO_3 in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin-ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin.

3.5.3.4. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detailed analysis of protein profile following the method of (Laemmli, 1970). For the preparation of gel the following stock solution were prepared.

3.5.3.4.1. Preparation of stock solution

Following stock solutions were prepared

A. Acrylamide and N'N' – methylene bis acrylamide

Stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4ºC and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris Buffer

i) 1.5M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4ºC for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4ºC for use.

D. Ammonium persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris- glycine electrophoresis buffer

Tris running buffer consists of 25mM Tris base, 250mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and10 ml of 10% SDS in 1000 ml distilled water.

F. SDS gel loading buffer

This buffer contains 50 mM Tris –HCl (pH 6.8), 10 mM ß- marcaptoethanol, 2%SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M ß- mercaptoethanol, 2 ml of 10%SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

3.5.3.4.2. Preparation of gel

Mini slab gel (plate size 8cm x10cm) was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8 cmx10 cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit (table 6). Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb +1cm). After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Table 6: Composition of resolving and stacking gel for SDS-PAGE

Sl No	Chemical	Resolving Gel (ml)	Stacking gel (ml)
1	Distilled water	2.85	2.10
2	30% acrylamide	2.55	0.5
3	Tris*	1.95	0.38
4	10%SDS	0.075	0.030
5	10%APS	0.075	0.030
6	TEMED**	0.003	0.003

*For 1.5 M tris pH 8.8 in resolving gel and for 1M Tris pH 6.8 in stacking gel

** N,N,N‘,N‘ –Tetramethyl ethylene diamine.

3.5.3.4.3. Sample preparation

Sample (50µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 3 min to denature the proteins samples. After boiling, the sample was loaded in predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97,4000: Biovine Serum Albumin-68,000; Albumin-43,000; Carbolic Anhydrase-29.000; Soybean Trypsin inhibitor-20,000; Lysozyme -14,300) was treated as the other samples and loaded in separate well.

3.5.3.4.4. Electrophoresis

Electrophoresis was performed at 18mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.5.3.4.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved,45 ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper. The gel was removed from the fixer and stained in this stain solution for 4 h at 37ºC with constant shaking at low speed. After staining the gel was finally destained with

destaining solution containing methanol, water and acetic acid (4.5: 4.5:1) at 40ºC with constant shaking until the background become clear.

3.5.4. Determination of total lipid content

Total lipid was determined following the methodology described by Folch *et al.* (1957). Five gram of grinded mushroom was suspended in 50 ml of chloroform**:** methanol (2:1 v/v) mixture then mixed thoroughly and kept for 72 hours. The solution was filtrated and centrifuged at 10000 rpm for 15 min. The upper layer of methanol was removed by pasteur pipette and chloroform was evaporated by heating. The remaining was the crude lipid.

3.5.5. Determination of dietary fibre

Dietary fibre content was estimated from the Arbro Pharmaceuticals, New Delhi following the methodology AOAC (1995). 10 gm mushroom dried powder was taken in a beaker and 200 ml of boiling 0.255 N H_2SO_4 was added. The mixture was boiled for 30 minutes keeping the volume constant by the addition of water at frequent intervals. The mixture was then filtered and washed with hot water until the total acid removed from the residue. Then 200 ml of boiling 0.313 N NaOH was added and boiled for 30 minutes and again the mixture was filtered and washed with hot water till free from alkali. It was then dried overnight at 80-100^{0}C and weighed (We) in an electric balance. Then the sample was heated in a muffle furnace at 600^{0}C for 5-6 hours, cooled and weighed again (Wa). The difference in the weights (We- Wa) represents the weight of crude fibre.

Crude fiber (g/100 g sample) = [100 − (moisture + fat)] × (We-Wa)/Wt of sample

3.6. Application of spent mushroom substrate in crop plants

3.6.1. Experimental Design

Spent mushroom substrate was applied in pot as a soil conditioner for improvement of plant health status. To experiments were performed using the spent mushroom in two species of chili plant. The experimental design is as follows

A. The oyster mushroom (*Pleurotus ostreatus*) was cultivated using four different substrates separately and the spent substrates were dried completely under sunlight and mixed with the soil for experiment. In this experiment, soil was

prepared using different spent mushroom substrates as per the cultivation design of oyster mushroom. The design was like T1=paddy straw, T2= wheat straw, T3= paddy straw+ wheat straw, T4= paddy straw + sawdust and a control (untreated) pot without any treatments. No other organic or inorganic fertilizers were used in the growing media. Seedlings of *Capsicum chinense* were collected from the local market and seedlings were then transferred to the pre-treated pots.

B. In this experiment, seven different treatments were done using the spent mushroom substrate of oyster mushroom and button mushroom. The experiment was performed to compare the effect of spent substrate of different mushroom. Spent mushroom compost of button mushroom was collected from the immuno-phytopathology laboratory, Department of Botany, NBU. The treatments were such as Control (only soil), T1=Soil + Oyster mushroom leachate (100ml/kg soil), T2=Soil + oyster mushroom substrate fresh (250gm/kg soil), T3= Soil + Button mushroom leachate (100ml/kg soil), T4= Soil + Button mushroom spent compost fresh (250gm/kg soil), T5= Soil + Weathered Spent oyster mushroom substrate (250gm/kg soil), T6= Soil + Weathered Button mushroom compost (250gm/kg soil), T7= Soil + Fresh oyster mushroom substrate + Fresh button mushroom spent compost (250gm/kg soil). The experiment was designed in a complete randomized design which was set up in 3 replicates. Pots were placed in open field condition and no other organic or inorganic fertilizer used in the growing media.

C. Application of spent mushroom substrate of *Pleurotus* sp was also performed in *Solanum lycopersicum* and *Amaranthus* sp. Treatment of spent mushroom substrates were done in field condition in two plots (4ftX 4ft) using the freshly harvested spent mushroom substrate. Spent mushroom substrate (1kg) was amended in each plot and kept for 7days. 10day old seedling of *S. lycopersicum* was sowed and seed of *Amaranthus* sp were spread in field. Another two plots were also prepared and no treatment was done which was considered as untreated control plots.

3.6.2. Evaluation of growth promotion of plant

Effect of spent mushroom substrate was evaluate primarily on the morphological growth like plant height, number of leaves, and number of branches were recorded in regular intervals after the transfer of plants. Data was also collected during the flowering and fruiting period.

3.6.3. Extraction and quantification of phosphate from soil, root and leaf

Soil samples (1g, air dried) or plant materials (1g, oven dried) were suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) and activated charcoal (0.01 g) was also added. The extracted samples were shaken for 30 min in a rotary shaker and filtered through Whatman No. 2 filter paper. Quantitative estimation was carried out following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

3.6.4. Extraction and estimation of chlorophyll content

Total chlorophyll of plant leaf was estimated following the methodology as described by Harborne (1973). 1 gm of leaf sample was grinded using 10 ml of 80% acetone and then it was filtrated using the Whatman No 1 filter paper. The absorbance was taken spectrophotometrically at 663 nm and 645 nm and calculated using the following formula

$Chlorophyll_a = [12.7X\ A_{663}] – [2.67\ X\ A_{645}]$ µg/gm tissue

$Chlorophyll_b = [22.9\ X\ A_{645}] – [4.68\ X\ A_{663}]$ µg/gm tissue

Total Chlorophyll = $[20.2\ X\ A_{645}] + [8.02\ X\ A_{663}]$ µg/gm tissue

3.6.5. Extraction and estimation of carotenoid

Carotenoid content was also estimated from the fruit and leaves of the plant treated with spent mushroom substrate. 1gm sample was grinded in dark using 10 ml methanol and then it was filtrated by Whatman No1 paper and used as crude sample for estimation. The absorbance was taken at 480 nm, 645 nm and 663 nm against as described by Jenson (1978). Carotenoid content was estimated by the following formula

Total carotenoid = [A480 - (0.114 X A664) – (0.638 X A645)] µg/gm tissue

3.7. Molecular identification of *Pleurotus* species

3.7.1. Isolation of genomic DNA from fungi

Total genomic DNA from the fungi was isolated by N-cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) method.

3.7.1.1. DNA isolation technique

0.5 g fungal mycelium was taken and grinded with 25 mg PVPP using mini grinder and then it was centrifuged at 10000 rpm 2 min. at 40^0C. The pellet was washed with sterile distilled water and again centrifuged at 10000 rpm 20 min. at 40^0C. Then 675 µl of extraction buffer was added and incubated at 37^0C for 30 min. After that 75µl of SDS (20%) was added and incubated at 65^0C for 2 hours and again centrifuged at 10000 rpm for 10 min at 4^0C.The supernatant was collected in a sterile micro centrifuge tube and equal volume of Phenol: chloroform: isoamyl alcohol (25:24:1) was added and shaken well. Then it was centrifuged at 10000 rpm for 10 min. at 4^0C and equal volume of chloroform: isoamyl alcohol (24:1) was added. It was again centrifuged at 10000 rpm for 10 min. at 4^0C. The aqueous phase was removed and taken in a sterile micro centrifuge tube. 0.6 volumes of isopropyl alcohol was added and incubated at room temperature for 1hour. Then it was centrifuged at 10000 rpm for 10 min. The pellet was washed using 500µl of 70% ethanol and centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 µl sterile distilled water.

3.7.1.2. Purification of genomic DNA

Total genomic DNA from different isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was again suspended in 100 µl 1X TE buffer and incubated at 37^0C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamyl alcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating the DNA quality.

3.7.2. Quantification of isolated DNA

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Bio photometer, Germany). From the stock 1µl DNA was mixed with 49-µl

sterile distilled water to get 50 times dilution. For quantification, DNA absorbance at wave lengths of 260 nm and 280 nm were taken. Quantification was done as follows:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

Pure preparations of DNA have OD_{260}/OD_{280} value 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid will not be possible

3.7.3. Agarose gel electrophoresis

Gel electrophoresis is one of the most important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.7.3.1. Preparation of agarose gel

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gels solidify in 15-20 min.

3.7.3.2. Gel electrophoresis of DNA sample

15µl of sample and 5µl of DNA loading dye mixed properly was loaded in each well of agarose gel (1%). The electrical head of the gel tank was attached firmly and electric supply was applied at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 min. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV trans illuminator and photographed for analysis.

3.7.4. PCR Amplification

3.7.4.1. Reagents and the optimal PCR reaction mixture

PCR amplification of ITS region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl2, 3 mM; dNTP mix, 0.25 mM; Taq DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease free water is used as negative control.

3.7.4.2. ITS PCR analysis

Different species of oyster mushrooms were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^0C for 5 min. followed by 30 cycles of denaturation at 94^0C for 30 sec, annealing at 59^0C for 30 sec and extension at 70^0C for 2 min and the final extension at 72^0C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

Table 7: PCR primers of ITS 4 and ITS6 used for sequencing

	Oligonucleotide Sequences (5'- 3')	GC %	Tm Value	Length	Product Size
ITS 4	TCCTCCGCTTATTGATATG	50	51.0^0C	19	700 bp
ITS 6	GAAGGTGAAGTCGTAACAAGG	60	56.0^0C	21	

3.7.4.3. Amplification conditions

Temperature profile, 94^0C for 2 min. followed by 30 cycles of denaturation at 94^0C for 50 sec, annealing at 48^0C for 30 sec and extension at 72^0C for 1min 30 sec and the final extension at 72^0C for 6 min in a Primus 96 advanced gradient Thermocycler.

3.7.4.4. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the ITS primer pairs by Credora Life Sciences, Bangalore.

3.7.5. Sequence analysis

DNA sequence information was analysed using bioinformatics algorithms tool MEGA 4, as well as the few online software.

3.7.5.1. Chromatogram of sequence

The chromatogram of the DNA sequence was analysed by the software Chromus Lite.

3.7.5.2. Multiple sequence alignment of sequence data

DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

3.7.5.3. BLAST analysis of the sequences

The DNA sequences were analysed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*., 1997) for the different characteristic of DNA sequence for the identification of microorganism. Identification of microorganism was done on the basis of homology of sequence.

3.7.5.4. Submission of rDNA gene to NCBI Genbank

The DNA sequences were deposited to NCBI Genbank through BankIit procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.8. Antioxidant activity of oyster mushroom

3.8.1. Preparation of ethanolic extract of *Pleurotus* sp.

Antioxidant compound is very important for human health. For the estimation of different antioxidant activity, ethanolic extract was prepared of *Pleurotus* species.1gm dried powder o *Pleurotus* sp was suspended in 100 ml 95% ethanol and stirred for 24 h at room temperature. The suspension was then filtered with whatman no 1. Then the filtrate was evaporated by rotary evaporator at 40^0C. The extraction was then resuspended in 95% ethanol and used for further use.

3.8.2. DPPH Scavenging activity

Free radical scavenging activity of mushroom extracts were estimated by DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. 100 µl ethanolic extract of four different species of oyster mushroom (*Pleurotus ostreatus, P. sajor-caju, P. djamor* and *P. florida*) was taken and 5µl DPPH solution was added to it and incubated for 30 min in dark. After incubation absorbance was taken at 517nm against a control. DPPH scavenging activity was measured using the following formula

$$\text{Inhibition \%} = (A_{control} - A_{sample})/A_{control} \text{ X } 100$$

3.8.3. Ferric Reducing Antioxidant Power Assay

2.5ml of (0.2 M) phosphate buffer (pH6.6) was taken in a test tube and to it 2.5 ml of 1% potassium ferricyanide, 1 ml of distilled water and 1 ml of ethanolic extract of the test sample was taken and incubated at 50^0C for 20 min in water bath. Then 2.5ml of 10% trichloro acetic acid was added and mixed well. Then it was centrifuged at 10000 rpm for 15 min. the upper layer of the mixture was collected. 2.5ml of reaction mixture was taken and to it 2.5 ml of distilled water and 300µl of 1% ferric chloride was added and mixed well. Then the absorbance was taken at 700nm in spectrophotometer.

3.8.4. Estimation of flavonoid content

Flavonoid content of the mushroom extracts was done following the methodology of Barros *et al* (2008). 100µl of mushroom extract was taken and 500µl distilled water was added and to which 30µl of 5% sodium nitrate was added and incubated for 5min. Then 60µl of 10% aluminium chloride added and incubated at room temperature for 6 min. After the incubation, 200µl of 1M NaOH and 110 µl distilled water was mixed well and absorbance was taken at 510nm.

3.9. Antidiabetic activity of oyster mushroom

3.9.1. Induction of diabetes

Male swiss albino rats (120-150g) were used for the experimental purpose. The animals were kept into polypropylene cages and maintained in Antisera Reserves of Plant Pathogen, Immuno phytopathology laboratory, Department of Botany, NBU under laboratory conditions as suggested by Niyonzima and Vlientinck (1993). Animals were maintained by feeding bread and pea at 50 gm twice per day before starting the treatment.

Animals were allowed to fast for 15-18 hrs and freshly prepared solution of Streptozotocin (65mg/kg body weight) in 0.1M Sodium citrate buffer pH4.5 was injected intra-peritoneally in a volume of 1ml/kg body weight. After 48 hrs of injection, blood sugar level was observed and rats with more than 200 mg/dl glucose level was considered as diabetic rat and used for further treatment.

3.9.1.1. Chemical used

Streptozotocin (N-Methyl nitrosocarbamoyl-α-D-glucosamine) was used for induction of blood sugar in swiss albino rats. The chemical procured from the HiMedia Laboratory Mumbai (Product code- CMS-1758-250MG).

3.9.2. Experimental Design

In this experiment, 35 rats were used and these were divided into four groups of 5 each.

- **Group-I** untreated rats receiving distilled water designated as Normal control
- **Group-II** rats were induced with Streptozotocin (65mg/kg body weight) was considered as Diabetic control or Negative control
- **Group-III** was induced with Streptozotocin and treated with a commercially available standard drug Metformin tablets and was designated as positive control
- **Group-IV** was Streptozotocin induced rats treated with aqueous suspension of *P. ostreatus* powder administered orally was designated as Sample Treated. Streptozotocin treated rats were fed with aqueous suspension (250mg/kg body weight) of *P. ostreatus* powder for 15 days.
- **Group-V** was Streptozotocin induced rats treated with aqueous suspension of *P. sajor-caju* powder administered orally was designated as Sample Treated. Streptozotocin treated rats were fed with aqueous suspension (250mg/kg body weight) of *P. sajor-caju* powder for 15 days.
- **Group-VI** was Streptozotocin induced rats treated with aqueous suspension of *P. djamor* powder administered orally was designated as Sample Treated. Streptozotocin treated rats were fed with aqueous suspension (250mg/kg body weight) of *P. djamor* powder for 15 days.
- **Group-VII** was Streptozotocin induced rats treated with aqueous suspension of *P. florida* powder administered orally was designated as Sample Treated. Streptozotocin treated rats were fed with aqueous suspension (250mg/kg body weight) of *P. florida* powder for 15 days.

3.9.3. Determination of body weight of experimental rats

The body weight of rats was measured in regular intervals of all rats of each group using the pan balance.

3.9.4. Determination of blood glucose level

Blood sample was collected from the tail vein of two hours fasted rats and glucose level was estimated using the Accucheck Blood Sugar testing kit.

3.9.5. Estimation of blood creatinine

Blood creatinine level was determined using the ERBA-CRE creatinine kit (Product code 120246). 100 µl sample or standard solution was added to 1000µl of working reagent and mixed well. Absorbance was taken after 20th second and 80th second at 505 nm as described by Young *et al* (1975).

Blood creatinine = ΔA of test sample X Conc. of Standard/ ΔA of standard (mg/dl)

3.9.6. Estimation of blood urea

Blood urea was determined using the ERBA-UREA (BUN) kit (Product code 120214). 20µl standard or the taste sample added to 1000µl working reagent and mixed well. Then the absorbance was taken at 340 nm and urea content calculated by following formula

Blood urea = ΔA of test sample X Conc. of Standard/ ΔA of standard (mg/dl)

3.9.7. Estimation of triglyceride

Lipid profile of blood includes Triglyceride which is an important parameter effected by blood sugar. Triglyceride was determined following the methodology of Fossati, (1969) using the ERBA-Triglyceride DES kit (Product code 120211). 1000µl of working reagent was taken and to it 10 µl dist. water, 10µl standard solution and 10µl of test sample was added and incubated at 37^0C for 10 min. after that, absorbance was taken at 505nm and calculated using the following formula

Triglyceride (mg/dL) = Abs. of test X Conc. of standard/Abs of Standard

3.9.8. Estimation of cholesterol

Cholesterol is an important compound that affects the human health. Blood cholesterol level was estimated according the methodology proposed by Roeschlau's *et al*. (1974). This test was performed using the ERBA Cholesterol DES kit (Product code 120194). 1000µl of working reagent was taken and to it 20 µl of test sample (serum) or standard

sample was added. Then it was mixed well and incubated for 10 min at 37^0C. Then the absorbance was taken at 505nm and calculated the concentration according to the following formula

Cholesterol (mg/dL) = Abs. of test X Conc. of standard/ Abs. of standard

3.9.9. Estimation of serum glutathione pyruvate transaminase (SGPT) activity

Serum glutamic pyruvate transaminase is an important enzyme secreted due to abnormalities in blood. SGPT was analysed following the methodology proposed by International Federation of Clinical Chemistry (IFCC) using the ERBA-SGPT kit (Product code120206). 1000µl of working reagent was taken and to it 100µl of test sample were added and aspirated for 10-15 min. Then the absorbance was taken at 340nm. Mean absorbance per min was calculated for each sample and the SGPT activity was calculated using the following formula

International unit (IU/L) =(ΔAbs./min)XTotal vol. X 10^3 /sample vol.XAbsorptivity X P

(Here P= Cuvette light path)

3.9.10. Estimation of serum glutathione oxaloacetate transaminase (SGOT) activity

Serum glutathione oxaloacetate transaminase (SGOT) is one of the major enzyme associated with the liver chronic diseases. SGOT activity was estimated following the methodology proposed by the International federation of clinical chemistry (IFCC; 1980) using the ERBA-SGOT testing kit (Product code- 120204). 1000µl of working reagent was taken in a test tube and to it 100µl of test sample was added, mixed well and aspirated at 37^0 C for 1-2 min. and then the absorbance was taken at 340nm in spectrophotometer. The activity was calculated according to the following formula

International unit (IU/L)=(ΔAbs./min)X Total Vol.X10^3/ sample vol. X AbsorptivityXP
(Here P= Cuvette light path)

3.10. Post-harvest processing of oyster mushroom

3.10.1. Long term processing

3.10.1.1. Drying process

Drying is perhaps the oldest technique known to the mankind for preservation of food commodities for long duration. It is the process of removal of moisture from the product to such a low level that microbial and biochemical activities are checked due to reduced water activity, which makes the products suitable for safe storage and protection against the attack by microorganisms during the storage. Mushroom contains about 90% moisture at the time of harvesting dried to a moisture level down below 10-12%. At a drying temperature of 55-60°C, the insects and microbes on the mushrooms will be killed in few hours, which give us the dehydrated final product of lower moisture content with longer shelf-life. The temperature, moisture of the mushroom and humidity of the air affect the colour of the dried product. Dehydrated mushrooms are used as an important ingredient in several food formulations including instant soup, pasta, snack seasonings, casseroles, and meat and rice dishes. Mushroom dried at higher temperature loose texture, flavour, and colour along with reduced rehydrability. Recently with advances in drying technologies, various drying methods such as solar drying, fluidized bed drying, dehumidified air- cabinet drying, osmo-air drying, freeze-drying, cabinet drying and microwave drying are efficiently used for almost all types of mushrooms. Fruiting body of *Pleurotus* sp were separated according to their size and then dried. Sundry, oven dry and freeze drying process was adopted for drying. In case of sundry, fruiting body of *Pleurotus* sp were kept in a tray covered by a thin cloth for 4 hrs and it took 3-4 days for complete drying. Oven drying was practiced using the hot air oven in which mushroom fruiting body were kept for 1-2 hrs at $35\text{-}40^0$ C which took 3-4days. In case of freeze drying, mushroom fruiting body were stored at 4^0 C for 5-6 days for complete drying.

3.10.1.2. Preparation of mushroom powder

Preparation of mushroom powder is also an effective step for long term storage of mushroom. Mushroom fruit body was dried in following different drying methods such as direct and indirect sun dry, oven dry and freeze dry. After drying, the dried fruitbody were grinded using the mixed grinder and then the powder was sieved. Sieving was done to get the fine grinded powder. Then the powder was kept in airtight container for further use.

3.10.1.3. Preparation of Mushroom biscuits

Delicious and crunchy mushroom biscuits were prepared by using the oyster mushroom powder and various ingredients viz., maida, sugar, butter, mushroom powder, crushed cashew nut, backing soda, and milk powder. Dough was prepared by mixing all the ingredients for 15-20 minutes. After that dough was kept for 10 minutes. Thereafter, thin sheets of dough was made and cut into different shapes of biscuits. These raw cut biscuits were kept in the trays in systematic manner and then these trays were shifted to micro weave oven (600^0C) for baking purpose for 10-15 minutes. After baking, trays were removed from the micro weave oven and cooled; the biscuits were ready for packaging and or for serving. The ingredient like sugar gives desired sweetness; butter gives smoothness to the biscuits. The various ingredients required for preparation of mushroom biscuits are as follows

Ingredients	Amounts
Maida	1000gm
Mushroom powder	150gm
Butter	250gm
Crushed Sugar	To taste
Milk powder	250ml
Cashew nut (crushed)	200gm
Baking Powder	5gm
Water	As required

3.10.2. Short term processing

3.10.2.1. Mushroom soup

Different mushroom dishes were prepared as one of the method of short term processing of oyster mushroom. Oyster mushroom soup was prepared by using the freshly harvested healthy fruiting body of different species of oyster mushroom. Mushroom fruiting body were chopped into small pieces and then dipped into boiling water and kept for 10 min for the dispersal of spores and dirt particles. Then the mushroom was washed and fried with the grinded onion, ginger, salt, and garlic cloves for 15-20 mins. Then the fried paste mixture was boiled for 10-15 min. Then black peeper was added to the soup and served with soup-sticks. The following ingredients were used for the preparation

Ingredients	Amount
Fresh Mushroom	500gm
Onion	100gm
Ginger	50gm
Garlic	10-15cloves
Salt	To taste
Black peeper	To taste
Butter	200gm
Water	1.5ltr.

3.9.2.2. Mushroom pakora

Mushroom pakora was also prepared using the freshly harvested oyster mushroom. The fruit body was washed with warm water and chopped into suitable pieces. Onion, chilli and capsicum were chopped into small pieces. Gram flour paste was prepared and to it onion, chillies, salt, turmeric powder was added and mixed thoroughly. The chopped mushroom was the dipped in the paste and fried in hot edible oil. Then the fried pakora was served with the sauce. Following ratio of ingredients were used to prepare mushroom pakora

Ingredients	Amount
Mushroom	1 kg
Onion	300gm
Capsicum	2pcs
Chili	50gm
Salt	To taste
Turmeric	½ teaspoon
Gram flour	350gm
Oil	As required

3.9.2.3. Mushroom curry

Mushroom curry is also one of the tasty and healthy dish and it was also prepared. Mushroom, tomato, pea and capsicum was washed and chopped, separately. Chopped

onion, grind garlic, and ginger was fried with the cumin seeds until it turns to brown and then the mushroom, tomato and capsicum was added and again fried well. All spices and salt added and again boiled for five minutes.

Ingredients	Amount
Mushroom	500gm
Tomato	200gm
Onion	100gm
Capsicum	2pcs
Garlic	30gm
Ginger	50gm
Oil	As required
Salt	To taste
Turmeric	½ teaspoon
Black peeper	To taste
Chili	20gm

3.11. Promotion and marketing of oyster mushroom

Oyster mushroom cultivation is one of the major earning sources of unemployed youth, women self-help groups. Hands on training of oyster mushroom cultivation, preparation of mushroom spawn management of contaminants as well as post-harvest processing of oyster mushroom was conducted for the easy and efficient training to the economically weaker people. In house training Immuno-phytopathology Laboratory, department of Botany was conducted throughout the year. Some workshop regarding the mushroom technology was also organized by the West Bengal Voluntary Health Association. A stall for the promotion of mushroom technology was set up during the Krishi Mela organized by the Department of Agriculture, Govt. of West Bengal which was very helpful for the rural people even the mushroom growers for better cultivation of oyster mushroom.

3.12. Determination of cost benefit ratio

Pleurotus species is very commonly cultivated in North Bengal region and a large number of mushroom growers cultivating different species of *Pleurotus*. Production

cost and marketing rate is very important factor for economic upliftment. A number of people trained and they are now growing mushrooms successfully. Cost benefit ratio was estimated depending upon the market rates of *Pleurotus* species, total production cost as well as total return. Different markets of Darjeeling and Jalpaiguri districts such as champasari bazar, naxalbari hut, batasi hut, bagdogra hut, shibmandir bazar, fulbari hut, Jalpaiguri hut, barivasa bazar and gate bazar was taken into consideration for market survey data. Different farm visited of the growers who have trained by the Immuno-phytopathology laboratory, department of Botany, NBU for the production cost as well as total cost return survey. Depending upon these field survey data, total cost benefit ratio was calculated using the following formula

Benefit cost ratio= Gross return/ total cost of production