CHAPTER -3
MATERIALS AND METHODS

3.1. Fungal Culture

The pure culture of *Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus djamor, Pleurotus florida, Calocybe indica* and *Agaricus bisporus* 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 (Jong, 1978).

A. Potato Dextrose Agar

Material	Quantity
Potato	400gm
Dextrose anhydrous	20gm
Agar Agar Type I	20gm
Distilled Water	1ltr

B. Malt Extract Agar

Materials	Quantity
Malt extract	30gm
Peptone	2gm
Agar Agar Type I	15gm
Distilled water	1 ltr

C. Richard's synthetic media

Ingredients	**Quantity**
Potassium nitrate	10gm
Monopotassium dihydroge phosphate	5gm
Magnesium sulphate	2.5gm
Ferric Chloride	0.020gm
Sucrose	15gm
Agar agar Type I	20gm
Distilled water	1ltr.

3.2. Maintenance of culture

The culture of *Calocybe indica* was stored in 15˚C and *Agricus bisporus, Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus djamor and Pleurotus florida, P. flabellatus, P. eryngii,* kept in 4˚C using refrigerator. To maintain the pure line of culture sub-culturing was done after every 35 days intervals.

3.3. Molecular identification of *C. indica, A. bisporus* and *Pleurotus sp*

3.3.1. Isolation of genomic DNA from fungal mycelia

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. Liquid nitrogen was used for crushing the cell mass for both cases.

3.1.1.1. Preparation of genomic DNA extraction buffer

The following buffer for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

DNA Extraction buffer composition

1M Tris-HCl pH 8.0

5M Nacl

0.5 mM EDTA, pH 8.0

10% SDS

3.3.1.2. Genomic DNA isolation

The fungal mycelia was grown in PDB for 6-7 days and then harvested. Total genomic DNA was extracted as described by Kuramae-Izioka (1997). The mycelium was ground into the fine powder under liquid nitrogen and suspended in 700 µL extraction buffer. Upon homogenization, the tubes were incubated for 30 minutes at 65ºC. DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitaded with isopropanol. The tubes were centrifuged at 15,400 g for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in TE buffer (pH 8.0) and stored at 4° till further use.

3.3.1.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37^{o} C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol (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 good quality of DNA.

3.3.2. Quanitification of isolated DNA

The pure sample was (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), used to quantify DNA. For quantating DNA,

absorbance was taken at wave-length of 260nm and 280 nm. Quantification was done as follows:

1 OD at 260 nm for double stranded DNA =50ng/µl of dsDNA

1 OD at 280 nm for single stranded DNA=20-33ng/µl 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 quantification of the amount of nucleic acid will not be possible.

3.3.3. Agarose gel Electrophoresis

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

3.3.3.1. 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.

3.3.3.2. Gel Electrophoresis for DNA fraction

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.

3.3.4. PCR amplification

3.3.4.1. Reagents and the optimal PCR reaction mixture

PCR amplification of ITS region was done in 20 µl of the reaction mixture containing PCR buffer, 1X (kappa, SA); Mgcl2, 3mM; dNTP mix, 0.25mM; Taq DNA polymerase, 0.05U; primer, 1 picomol and template DNA, 50 ng, sterile nuclease free water was used as negative control.

3.3.4.2. ITS- PCR analysis

All mushroom fungus was taken up for ITS-PCR amplification.

Genomic DNA was amplified by mixing the temple DNA (50ng), with the polymerase rection buffer, dNTP mixture, primers and Taq polymerase. Polymerase chain reaction

was performed in total volume of 100µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50ng template DNA.

PCR was programed with an initial denaturing at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec. annealing at 59 ^{o}C for 30 sec and extension at 70 ^{o}C for 2 min and the final extension at 72 ^{o}C for 7 min in a primus 96 advanced gradient thermocycler. PCR product (20µl) was mixed with loading buffer (8 µl) 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 electrophoresis.

ITS- PCR primers: The following primers were used to amplify ITS regions

Primer Sequence (5'-3')		GC %	Length	TM Value	Product Size
ITS-1	TCTGTAGGTGAACCTGCGG	57	19	63 ^{0}C	700 bp
ITS-4	TCCTCCGCTTATTGATATGC	45	20	51 ^{0}C	700bp
ITS-6	GAAGGTGAAGTCGTAACAAGG	90	21	56 ^{0}C	700bp

3.3.4.3. Amplification condition

Temperature profile, 94^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.3.4.4. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bidirectionally using the ITS primer pairs by SciGenom, Kerala. DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares. The chromatogram of the DNA sequence was analysed by the software Chromus. All the DNA sequences was edited by using the software BioEdit and aligned with Clustal algorithms.

3.3.5. Sequence analysis

DNA sequence information was analyzed using bio-informatics algorithms tools e.g. MEGA4 software.

3.3.5.1. Chromatogram of sequence

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

3.3.5.2. Alignment of sequence data and Phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Genbank and established fungal taxonomy for identification. Sequences were aligned following the Clustal Walgorithm (Thompson *et al.*, 1994), included in the Megalign

module (DNASTAR Inc.). Multiple alignment parameters used were gap penalty =10 and gap length penalty =10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pair wise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were done manually in order to artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic interference was performed by the UPGMA method (Sneath and Sokal, 1973). Bootstrap tests with 19 replications were conducted to examine the reliability of the interior branches and the validity of these trees obtained. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.*, (2007).

3.3.5.3. BLAST analysis of sequence

The DNA sequences were analyzed 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.

3.3.5.4. Submission of rDNA gene to NCBI Gene bank

The DNA sequences were deposited to NCBI GenBank through Bank-Iit procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.4. Cultivation techniques developed for *Agaricus bisporus, Calocybe indica* and *Pleurotus sp*

3.4.1. Preparation of spawn

3.4.1.1. Spawn preparation using Solid culture

The preparation of mycelial inoculum in Petri dishes and its conservation in test tube slants was performed according to Singh (Singh *et al.*, 2009). The spawn was prepared according to the standard procedure given by Tandon *et al.*, (2004). Spawn was prepared by boiling wheat grains for 15 minutes followed by draining and cooling and calcium carbonate (5g/kg) and gypsum (25g/kg) were added, mixed well and then transferred double polypropylene bags, with a mean thickness of 0.6 mm, and its upper portion was plugged with non-absorbent cotton. The grains were filled up to 2/3rd of 500 ml capacity wide mouthed glass bottles. The bottles were plugged with non-absorbent cotton. These bottles were then sterilized at 121^{o} C (15 lbs pressure) for 1.30

hour and allowed to cool at room temperature. The mycelial inoculum discs (5 mm) of 10 days old were aseptically inoculated in the spawn bottles and incubated at 25± 2^{o} C in dark chamber for mycelial growth and shaking was done after 3 days (Figure.1). The fine mycelial growth on grains is species specific. *Calocybe indica* and four species of *Pleurotus* takes 13-18 days for full mycelial growth but *Agaricus bisporus* takes 25-30 days to cover the Entire grains packet.

3.4.1.2. Spawn preparation using liquid culture

Mother culture was prepared in PDB and kept for growth for 12 days. Then the young and soft mycelial mat was fragmented by shaking the culture flask and used as inoculum for spawn preparation. For preparation of spawn 5 ml of liquid culture was used for 250 gm of grain. This type of culture inoculum technique generally takes less time for spawn growth.

Figure 1: Steps for spawn preparation

3.4.2. Compost preparation for button mushroom (*Agaricus bisporus*)

Composting was carried out in out-door composting where raw materials were mixed and periodically turned. Rice straw was wetted to the maximum capacity, on concrete

floor. Preliminary stack was prepared and wheat bran was added @ 8kg/250 kg straw, urea (3kg/250kg of straw) or poultry manure (10kg/250kg straw) was added and mixed uniformly. The whole mixed material was kept in heap (4ftx5ft) form for five days untouched (Figure. 2G). First turning was done after 5 days. Compost heap was cut from each end and put equally in the central portion. Stack aeration was done by breaking and mixing the material uniformly and kept for next 3 days (Figure.2H). Free turning was done every three days interval upto 11 days and at11th and 17th day addition of gypsum 3kg/250 kg of straw was done. The free turning was continued until the excess ammonia was released from the composting materials. The inner compost temperature was recorded.

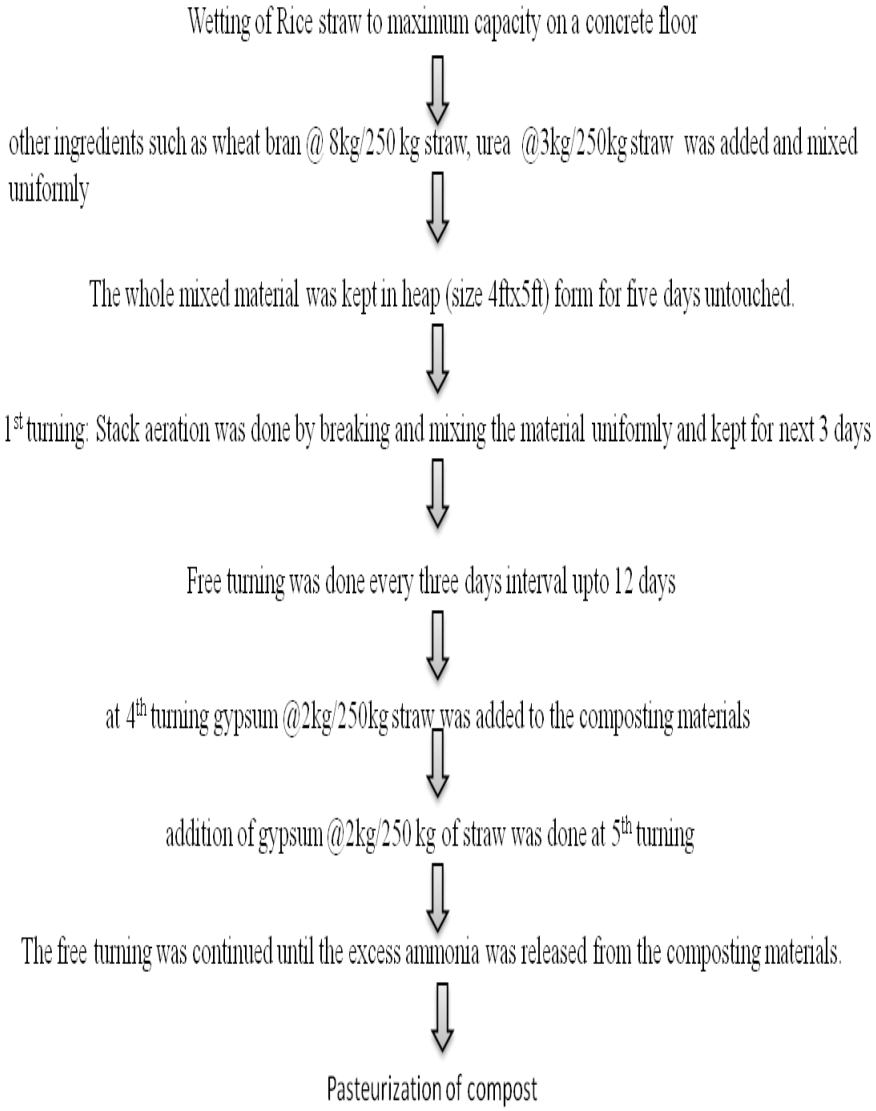

Figure 2: Raw materials and Successive events for preparation of Compost, **A.** paddy straw**, B.** poultry manure. **C.** Gypsum, **D.** wheat bran **E.** washing and mixing of raw material, **F.** piling of the materials, **G.** pile of the composting materials, **H.** turning of the piled materials during which temperature raised upto 85^0C. **I.** Appearance of thermophilic fungi on the compost materials, **J.** mixing of gypsum to reduces the NH_3, **K.** final ready compost.

3.4.3. Substrate preparation for *C. indica* and *Pleurotus sp*

The substrate preparation is same for both the mushroom cultivations. Different agro wastes mainly paddy straw, wheat straw were used for the cultivation of different species of *Pleurotus* and *Calocybe indica*. For cultivation of *C. indica* coconut fiber, sugar can, dried tea leaf were also used. Dried chopped (2-5cm) straw was washed 3- 4 times with tap water. Then the substrates were allowed to soak in water for overnight and washed for 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.

3.4.4. Spawning

For *Pleurotus sp* and *Calocybe indica* cultivation, spawning was done using in layer method in polypropylene bags (12 inch X 18 inch). Cooled pasteurized substrates were used and layer spawning was done using the fully grown spawn @ 100gm/kg substrates

following the methodology Sarkar *et al.*, (2007). The cylinders were closed tightly and small pores were made for aeration. The cylinders were then incubated at room temperature 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. But for button mushroom spawning was done @ 5% 50gm spawn in 1kg compost. Spawning room was maintained as 25°C temperature with 80% relative humidity without ventilation.

3.4.5. Casing placement

Casing soil layer is very important for initiation of primordial for button and milky mushroom. When the substrate was completely colonized by the vegetative mycelium of mushroom fungus, the upper surface of each bag was opened and the surface was applied with casing soil such as garden soil and sand, tea waste, coconut fiber (Figure. 3A-D), hard paper, vermicompost, spent mushroom compost crud and vermicompost form, saw dust to a thickness of 2-3 cm over the spawn run substrate. Water was uniformly and regularly sprayed to keep the surface of substrate moist. Watering was done after and before casing layer placement. When garden soil was used it was pasteurized with 5% formaldehyde. During pasteurization it was kept closed with plastic sheet for 3days till smell of formaldehyde was totally removed and was mixed twice a day. For cultivation of oyster mushrooms (*Pleurotus sp)* no such casing layer was required.

Figure 3 (A-D): Casing materials used for cultivation of *Calocybe indica*, A. young coconut fiber**, B.** tea waste**, C.** vermicompost**, D.** spent mushroom substrate

3.4.6. Fruiting

The fruit body was harvested from the base carefully so that there will be no damage on the grown mycelia. Humidity was maintained by spraying water after the harvest of the mushrooms.

3.5. Histological study of sporocarp

Transverse sections of the mushroom gills were washed with sterile distilled water and stained with lacto phenol-cotton blue (1:1) and incubated for 2-3 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.

3.6. Biochemical analysis of sporocarp

3.6.1. Protein estimation

3.6.1.1. Extraction and estimation of soluble protein

Protein contents in mushrooms fruit body grown on different substrate were determined followed by protocol proposed by Lowry *et al.*, (1951).

3.6.1.2. SDS-PAGE analysis of soluble protein

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.

3.6.1.2.1. Preparation of stock solution

I. 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.

II. Sodium Dodecyl Sulphate (SDS)

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

III. 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.

IV. Ammonium Persulphate (APS)

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

V. 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 and 10 ml of 10% SDS in 1L distilled water.

VI. SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM ß- mercaptoethanol, 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.

3.6.1.2.2. Gel casting

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. 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.

Name of the compound	10% Resolving Gel (ml)	5% Stacking Gel (ml)
Distilled Water	2.85	2.10
30% Acrylamide	2.55	0.50
Tris*	1.95	0.38
10%SDS	0.075	0.030
10%APS	0.075	0.030
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

3.6.1.2.3. 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 30 minutes to denature the proteins samples. After boiling, the sample was loaded in a 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: Bovine 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.

3.6.1.2.4. 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.

3.6.1.2.5. 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, 45ml 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 37 ºC 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 40 ºC with constant shaking until the background become clear.

3.6.1.2.6. Scanning and band analysis

The SDS-PAGE was subjected to scan by BIO-RAD scanner and was analyzed the band number and band intensity of the gel.

3.6.2. Extraction and estimation of carbohydrate

Extraction of carbohydrate from mushroom sample was done following the methodology of Harbone (1973). 1gm sample was homogenated in 95% ethanol and kept on water bath for evaporation of the alcoholic fraction. The residue was then resuspended in distilled water. The sample mixture was centrifuged at 10000 rpm for 15 min and the supernatant was collected for the estimation as crude sample extract.

3.6.2.1. Estimation of total sugar

Carbohydrate content of different parts of fruit body grown in different substrates was measured. Ethanol (95%) was used for extract preparation. Total sugar was estimated at 630nm using Anthrone's reagent. The mushroom tissue was hydrolysed by dilute HCL and then neutralised by NaOH.

3.6.2.2. Estimation of reducing sugar

Determination of reducing sugar content using arseno molybdate was carried out at 620 nm following Nelson-Somogyi Method as described by Plummer (1978). Reducing sugar estimation was done using 2 ml of alkaline copper tartrate in 2ml of ethanolic extract of sample.

3.6.3. Determination of lipid content

Total lipid of the mushrooms were determined following the method of Folch *et al.*, (1957), in which five gm sample mushroom powder was suspended in 50ml of chloroform: methanol (V/V) solution and the mixture will be kept for three days and after incubation the solution was filtered and centrifuged at 10000 rpm for 15 min. the upper layer of the methanol was removed by pipette and chloroform was evaporated by heating. The remaining part was measured as the crude lipid content in the sample.

3.6.4. Determination of moisture content

Moisture content of fresh mushroom sample was measured following the methodology proposed by Raghuramulu *et al.*, (2003). 100 g of fresh mushroom was dried in hot air oven at 100-110 C and cooled in room temperature. The moisture content was calculated using the following formula,

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

3.6.5. Amino acid separation and identification by TLC

Thin-layer chromatography was performed on plates of 15 cm-6cm silica gel Polygram Sil G (Marck). In each case 1.5 cm was measured from the base of the TLC plate, marked with a pencil and labeled. Before applying the sample the TLC plate was charged by heating at 80^{o}C for 1hours. Thin layer chromatography was performed on the ethanolic crude extracts of sample. Capillary tube was used to spot the plates with the sample extract. The spot plates were then placed in a vertical chamber saturated with butanol: acetic acid: water (80:20:20) and covered and ensuring that the solvent was just below the spot. The plate was removed after about two hours when the solvent had risen close to the top edge, marking the distance travelled by solvent with a pencil. It was then dried at room temperature. The dried plate was then placed in a container and ninhydrin solution was sprayed over the plate. Different colour spots developed according the separation of amino acids. The samples were run along with standard amino acid to identify the presence of amino acid in the samples.

3.6.6. Profiling of Bio-constituents in mushroom extract by GCMS

3.6.6.1. Sample and extract preparation

Fresh mushroom *Calocybe indica* was obtained from the cultivation unit. The sample was cleaned, washed under tap water several times to remove the dirt, dried and powdered using a mixer. The powdered mushroom sample was stored in clean bottles until when required. The crude extracts from the mushroom sample was obtained by means of cold extraction method. About 10g of the powdered mushroom sample was added to 100ml of methanol and ethanol in a conical flask, covered with aluminium foil and kept on a rotary shaker for 24 hours at room temperature. The solution was filtered with the help of Whatman No.1 filter paper and the filtrate obtained was evaporated. The dried extracts were then dissolved in methanol and in ethanol and utilized for GC-MS analysis.

3.6.6.2. Gas chromatography-mass spectrometry (GC-MS) programming

GC-MS-QP2010 ultra gas chromatograph was equipped with direct injector with linear velocity. A split injection was used for sample introduction and the split ratio was set to 10:0. The oven temperature program was programmed to start at 50˚c, hold for 2 minutes then ramp at 20˚c per minute to 280˚c and hold for 20 minutes. The helium carrier gas was set to 1.21ml/minute flow rate. ACQ top double focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-3333 software was used for all analyses. High resolution mass spectra were acquired at a

resolving power of 5000 (20% height definition) and scanning the magnet from m/z 40 to m/z 650 at 1 second per scan.

3.6.7. Phenolic profiling by Thin-layer chromatography

Thin-layer chromatography was performed on plates of 15 cm-6cm silica gel Polygram Sil G (Marck). In each case 1.5 cm was measured from the base of the TLC plate, marked with a pencil and labeled. Before applying the sample the TLC plate was charged by heating at 80^0C for 1hours. Thin layer chromatography was performed on the crude extracts of ethanol. Capillary tube was used to spot the plates with the sample extract. The spot plates were placed in a vertical chamber saturated with butanol: acetic acid: water (80:20:20) and covered and ensuring that the solvent was just below the spot. The plate was removed after about two hours when the solvent had risen close to the top edge. It was then dried at room temperature and Folin-ciocalteu phenol reagent solution was sprayed over the plate. Dark blue violet colour spot developed according the separation of phenolic. The samples were run along with standard phenolic.

3.7. Antioxidant activity

3.7.1. Preparation of mushroom extract

All of the edible mushrooms verities were cleaned to remove any residual compost/ soil and subsequently air-dried in the oven at 50 °C for about 3 h. All of the dried mushrooms were ground to fine powder and stored in airtight plastic bags in desiccators at room temperature for further analysis. 10 grams of each of dried mushroom sample was mixed with 100 ml of different solvent such as ethanol, methanol (HPLC grade) and distilled water. Samples were stirred for 24hrs for effective extraction and centrifuged at 2000*g* for 15 min and filtered with filter paper (watchman no. 1). Filtrates were evaporated in rotary evaporator at 40°C and redissolved the residues with their respective solvent at the 20mg/ml wet basis. The solution referred to as ethanolic extract (EE), and methanolic extract (ME), water extract (WE) respectively and stored at 4 °C until the completion of the analysis.

3.7.2. Reducing Power Ability (RPA)

The reducing power of ethanolic extract (EE) of *A. bisporus* was determined according to the method of Oyaizu (1986). Amounts of ml of mushroom extracts sample was mixed with an equal volume of distilled water and 2.5 ml of 0.2 M phosphate buffer, pH 6.6, and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. 2.5 ml of 10% trichloro-acetic acid was added to the mixture and centrifuged at 10000*g* for 15 min. The upper layer of the solution was mixed with 2.5

ml of distilled water and 0.3 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Gallic acid (5-40mg/ml) was used as control.

3.7.3. Free radical DPPH scavenging activity

The effect of EE of the mushroom species *Agaricus bisporus* on DPPH radical was estimated according to the method of Bloise (1958). 1000 µl of mushroom extracts were mixed with 50 µl of DPPH solution that was made by addition of 2ml ethanol in 5mg of DPPH powder. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Inhibition of DPPH free radical (\%)} = \frac{\text{A control} - \text{A sample}}{\text{Absorbance of control}} \times 100$$

An antioxidant value of 100% indicates the strongest antioxidant activity and 95% ethanol as blank and the DPPH and ethanol mixture solution as control was used for absorbance.

3.7.4. Analysis of total Phenolics

3.7.4.1. Sample preparation for quantitative estimation

A fine dried mushroom powder sample (3gm) was extracted by stirring with 100ml of methanol at 25^0C at 150 rpm for 24 hrs and filtered through Whatman no. 4 paper. The residue was then extracted with two additional 100ml of methanol, as described earlier. The combined methanolic extract were evaporated at 40 C to dryness and redissolved in a known concentration of methanol.

3.7.4.2. Folin-Ciocalteu's assay

1 ml of the methanolic extract was mixed with equal volume of Folin-Ciocalteu reagent. After 3 min saturated sodium carbonate solution (1ml) was added to the mixture and adjusted to 10ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used to prepare the standard curve and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

3.7.5. Flavonoid content

The flavonoid contents EE in the mushroom species *Agaricus bisporus* was estimated according to the method of Barros *et al.*, (2008). 100 µl of mushroom extracts was mixed with 500 µl of distilled water and 30 µl of 5% sodium nitrite incubated for 5 min at room temperature in the dark. 60 µl of 10% aluminium chloride ($Alcl_3$) was added to

the mixture solution and incubate for 6 min at room temperature. After incubation 200 µl of sodium hydroxide (NaOH) (1M) and 110 µl distilled water was added. The absorbance of the resulting solution was taken spectrophotometrically at 510 nm. Catechine used as standard curve calibration.

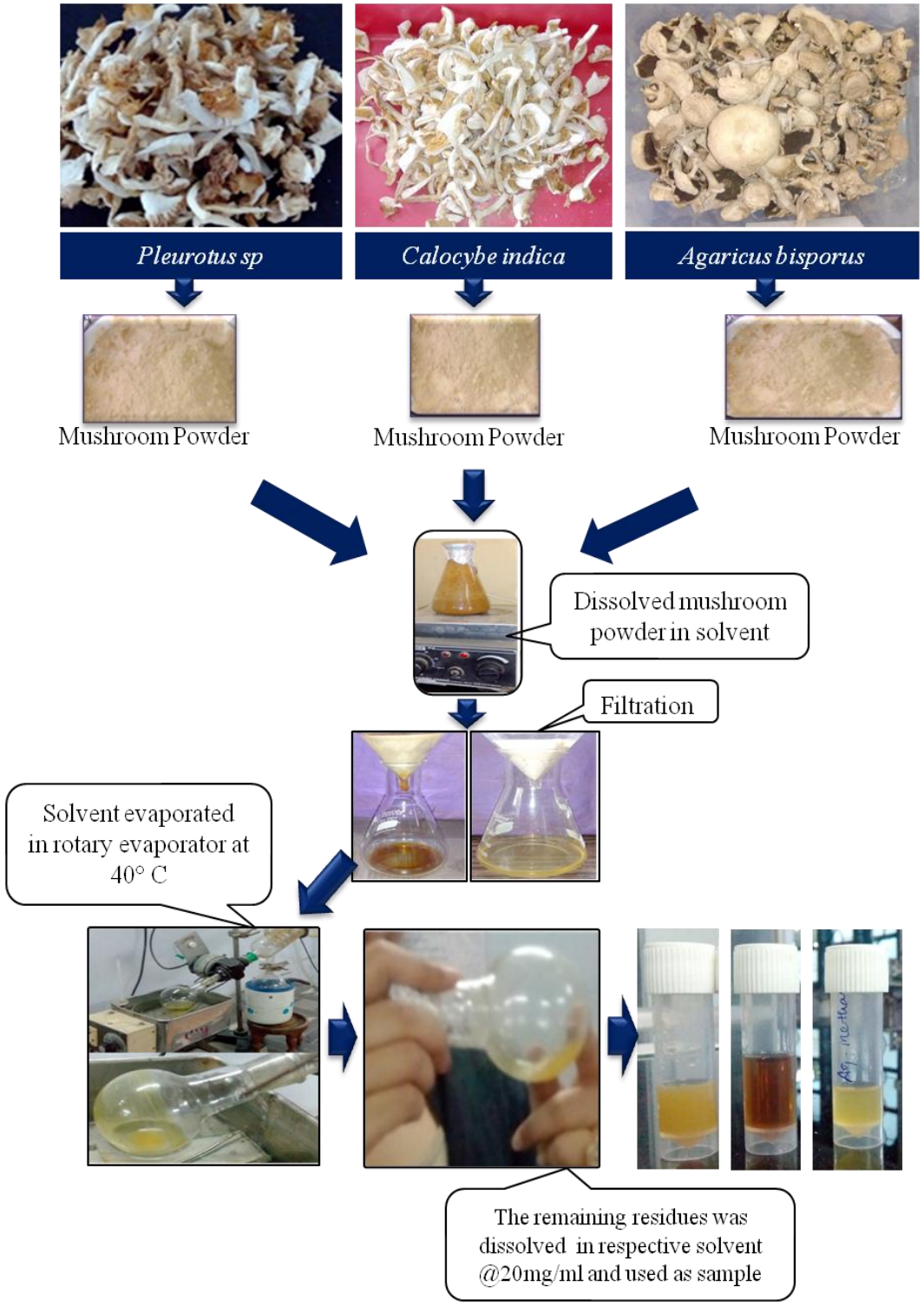

Figure 4: Schematic representation showing extraction process with dry mushroom (fruit body) powder for antioxidant assays

3.7.6. Free amino acid content

Amino acid assay was carried out by ninhydrin colorimetric method proposed by Hwang *et al.,* (1975) with some modification in which 1 ml of sample extract was mixed with 3 ml of water and then 1 ml of ninhydrin was added and kept of boiling water bath for 15 mints. After cooling 1 ml of 50% ethanol added and a ping colour was appeared and absorbance was taken at 540 nm. Increasing absorbance values indicate the increasing concentration of total free amino acid in the sample. Tryptophan was use as standard amino acid. Three solvent such as water extract, ethanolic and methanolic extract used for the determination of total free amino acid concentration in the sample grown in different substrates.

3.7.7. Rapid screening of antioxidants by dot-blot Analysis

Mushroom extract in different solvent was carefully loaded onto a 20cm X 10cm TLC plate (silica gel, Mark) in order to increase concentration (100-300 µl/ml) and allowed to dry for 10 min. along samples the glutathione was loaded as standard. The TLC plate bearing the dry sample spots was treated with 0.05 % 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution by spraying method on the surface of the plate and the plate was allowed to air dry. The stained silica layer of the plate revealed a purple background with yellow spots at the location (samples spots) where radical scavenging capacity presented. The intensity of the yellow color depends upon the amount and nature of the radical scavenge present in the sample (Hatano *et al.,* 1988).

3.7.8. Thin layer chromatography analysis for antioxidant

About 60µl of extract was loaded on TLC plates. The plates were developed in butane: acetic acid: water (8:2:2) to separate various constituents of extracts. The developed plates were air dried. The methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at 0.05% was sprayed on the surface of developed TLC plates and incubated for 10 mints at room temperature. The active antioxidant constituents of the mushroom extracts were detected as yellow spots appeared via reduction of DPPH by resolved spots against purple back ground on the TLC plates.

3.8. Antidiabetic activity

3.8.1. Experimental design

Male albino rates of 5 week-old purchased from Ghosh Enterprise, Kolkata were housed individually in stainless steel cages in a room with control temperature 25 -27^{o} C, a relative humidity of 60 % and 24 hours light. Twenty five Wister albino male rats were used for this study between the months of May to June, 2016. The rats were divided into five groups of five rats each.

Group I- Rats were maintained with normal conventional diet, (normal control),

Group II- Rats were induced with streptozotocin (diabetic control),

Group III- diabetes induced Rats were treated with commercial anti-diabetic medicine Metformin,

Group IV -Diabetes induced rates were fed daily with *ABP* powder water suspension (100mg/kg BW) and

Group V -Diabetes induced rats were fed daily with *C. indica* powder water suspension (100 mg/kg BW).

Group VI- Diabetes induced rats were fed daily with *Pleurotus djamor* powder water suspension (100 mg/kg BW).

Group VII- Diabetes induced rats were fed daily with *Pleurotus sajor-caju* powder water suspension (100 mg/kg BW).

Group VIII- Diabetes induced rats were fed daily with *Pleurotus ostreatus* powder water suspension (100 mg/kg BW).

Group IX- Diabetes induced rats were fed daily with *Pleurotus florida* powder water suspension (100 mg/kg BW).

The administration of sample suspension to rats was given orally once a day for 15 days. Blood glucose level measured in each 24 hours interval **(**Figure5). After the treatment the final blood samples obtained from all the groups and were subjected to different analysis such as estimation the blood WBC content (%), Haemoglobin content (gm/dl), serum urea, serum Creatinine (mg/dl), serum amylase content (U/L), plasma levels of triglyceride, total cholesterol (TC), high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (HDL). Food and water intakes and body weight were recorded daily. Body weight of rats was measured daily. The measurement of food intake was carried out on individual rats. Briefly each cage was supplied a known amount of the specific diet and the water. Each day, the remaining diet and water were measured. Fresh drinking water was also supplied daily. The above processes were followed throughout the experiment. After acclimatization for 1 week, the rats were induced by injection of streptozotocin (Sigma-Aldrich, 65mg/kg body weight) dissolved in citrate buffer at pH 4.5. Two days after STZ treatment, the rats were considered as diabetic as determined blood glucose levels more than 250 mg/dl. After induction of diabetes the rats were maintained on a conventional diet for the duration of the experiment.

Figure 5 (A-B): Maintenance of rats **(A)** and diabetes induction and observation of glucose level in blood **(B).**

3.8.2. Sample preparation

After freeze drying, the dehydrated fruit bodies were milted to a powder approximately less than 1 mm in particular size using grinder. For oral dosing, the mushroom powder was reconstituted in normal mineral water. A dose of mushroom powder suspension of 100 mg/kg BW of rats was used for oral feeding.

3.8.3. Induction of diabetes

Streptozotocin was dissolved in citrus buffer (pH-4.5) and induced in overnight fasted rats by a single intraperitoneal administration of 65 mg/kg BW. Hyperglycemia was

confirmed by the elevated glucose levels in blood after 48hrs. The rats with blood glucose levels higher than 250 mg/dl after 48 hrs of STZ injection were selected for the study.

3.8.4. Measurement of body weight of experimental rats

Every 24 hours interval body weight of each group of rat was measured by a electric balance.

3.8.5. Measurement of blood glucose level

Every 24 hours interval blood glucose level of each group of rat was measured by one touch device.

3.8.6. Measurement of blood serum parameters

Two milliliters of fasting blood samples was obtained from all groups via cardiac puncture after each of the animals had been anaesthetized with chloroform. Each blood sample was collected and blood serum was separated after centrifugation at 4° C at 1100 rpm for 10 minutes. The serum was collected into a vial tube and stored at 20°C until the analysis. Plasma glucose levels, total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) cholesterol and LDL cholesterol were measured spectrophotometrically at 505 nm. Commercial Laboratory kits reagents (Erba Mannheim laboratory analysis kits) were used for all biochemical analysis and their absorbances were read using a UV-Vis spectrophotometer. In this study, lipid parameters in induced diabetic rats treated with two mushroom suspensions was evaluated and compared result with the result (group III) of the commercial anti-diabetes drugs effect in the induced diabetic rats.

3.8.6.1. Creatinine (CRE)

Measure of serum Creatinine (CRE) was done following the Jaffe's Method (Bowers 1980) in which the reaction mixture was prepared with the addition of 1000 µl of working reagent with 100 µl of serum sample. 1000 µl of working reagent with 100 µl of standard solution was used as standard. The initial absorbance (A_1) was taken at 20 sec. after the solution preparation and the final absorbance (A_2) was taken at 80 second after mixing. The absorbance was taken at 505 nm. Distilled water was used as blank. The serum cholesterol was measured following the formula

$$\text{Creatinine content (mg/dl)} = \left[\frac{A_2\text{-}A_1 \text{ of sample}}{A_2\text{-}A_1 \text{ of standard solution}}\right] \times \text{concentration of standard}$$

3.8.6.2. Urea

Serum urea was determined using the ERBA-UREA (bun) kit (product code-120214). Measure of serum urea was done following the GLDH-Urease method proposed by Young (1990) in which the reaction mixture was prepared with the addition of 1000 µl of working reagent with 20 µl of serum sample. 1000 µl of working reagent with 20 µl of urea standard solution was used as standard. The initial absorbance (A_1) was taken at 20 sec. after the solution preparation and the final absorbance (A_2) was taken at 80 second after mixing. The absorbance was taken at 340 nm. Distilled water was used as blank. The serum urea was measured following the formula

$$\text{Urea content mg/dl} = \frac{A_2\text{-}A_1 \text{ of sample}}{A_2\text{-}A_1 \text{ of standard solution}} \times \text{concentration of standard}$$

3.8.6.3. Triglyceride (DES)

Measurement of Serum Triglyceride level following the GPO-Trinder method proposed by McGowan *et al.,* (1983) with slide modification where the reaction mixtures were made by using 1000µl working reagent mixed with 10 µl sample (serum). 1000µl of working reagent with 10 µl of standard solution was used as standard. 10µl of distilled water with 1000 µl of working reagent was used as blank during taking the absorbance. The mixtures ware mixed well and incubated at 37^{o}C for 10 mints. Then the absorbance of the standard and each test was taken at 505 nm against reagent blank. The serum triglycerides was measured using the following the formula**;**

$$\text{Serum Triglyceride content (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times (\text{concentration of standard})$$

3.8.6.4. Total Cholesterol (TC) profiling

Measurement of Serum Cholesterol level following the Chod-Pap method proposed by Roeschlau *et al.,* (1974) with slide modification where the reaction mixtures were made by using 1000µl working reagent mixed with 20 µl sample (serum). 1000 µl of reaction mixture of and 20 µl of standard solution was used as standard. Distilled water with 1000 µl working reagent was used as blank during taking the absorbance. The mixture ware mixed well and incubated at 37^{o}C for 10 mints. Aspirate blank followed by standard and sample. The absorbance of standard and sample was taken against blank in UV-Vis spectrophotometer at 505 nm. The serum cholesterol was measured following the formula**.**

$$\text{Cholesterol content (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times (\text{concentration of standard})$$

3.8.6.5. HDL–Cholesterol

Measurement of Serum High Density lipoprotein Cholesterol level following the Phosphotungstic acid Method proposed by Burstein *et al.,* (1970) where the reaction mixtures were made by using 1000µl working reagent mixed with 50 µl samples. The solution of reaction mixture (1000 µl) and of HDL standard (50 µl) was used as standard. Distilled water (50 µl) with 1000 µl working reagent was used as blank during taking the absorbance. The mixture ware mixed well and incubated at 37^{o}C for 10 mints. Aspirate blank followed by standard and sample. The absorbances of standard and sample was taken against blank in UV-Vis spectrophotometer at 505 nm. The serum cholesterol was measured following the formula**.**

$$\text{HDL-Cholesterol content (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{dilution factor} \times \text{concentration of standard}$$

3.8.6.6. Serum glutathione pyruvate transaminase (SGPT) activity

It is an important liver enzyme secreted due to abnormal level of glucose in blood. Measurement of SGPT level was analyzed following the methodology proposed by International Federation of Clinical Chemistry (IFCC) (Bradley *et al.,* 1972) using the ERBA-SGPT kit (product code 120206). The reaction mixtures were prepared by using 1000µl working reagent mixed with 100 µl of test sample and aspirated for 10-15 min. then the absorbance was taken at 340nm. Mean absorbance per mints was calculated for each sample and the SGPT activity was calculated using the following formula;

$$\text{International unit (IU/L)} = \frac{(\text{Absorbance change/min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}}$$

3.8.6.7. Serum glutathione oxaloacetate transaminase (SGOT) activity

Serum glutathione oxaloacetate transaminase an important liver enzyme associated with the liver chronic diseases. The increased levels are associated with myocardial infarction, liver damage, and muscular dystrophy. Measurement of SGOT activity was analysed following the methodology proposed by International Federation of Clinical Chemistry (IFCC) (Tietz 1986) using the ERBA-SGPT kit (product code 120204). The reaction mixtures were prepared by using 1000µl working reagent mixed with 100 µl of test sample and aspirated at 37° C for 60 sec. then the absorbance was taken at 340nm. Mean absorbance per mints was calculated for each sample and the SGOT activity was calculated using the following formula;

$$\text{International unit (IU/L)} = \frac{(\text{Absorbance change/min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}}$$

[T.V.= Total reaction volume in µl, S.V.= Sample volume in µl
P= Cuvette lightpath=1cm, Absorptivity= Milimolar absorptivity of NADH at 340nm]

3.9. Post harvest practices

3.9.1. Short term processing

3.9.1.1. Mushroom soup

Different mushroom dishes were prepared as one of the method of short term processing. Mushroom soup was prepared by using the freshly harvested mushroom which was 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 onion pieces, ginger, salt, and garlic cloves for 15-20 min. The fried paste mixture was boiled for 10-15 min. Finally black peeper was added to the soup and served with soup-sticks. The following ingredients were used for the preparation

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

3.9.1.2. Mushroom pakora

Mushroom pakora was prepared using the freshly harvested mushroom. The fruit body was washed with warm water and chopped into suitable pieces. Onion, chilli and capsicum were also 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 then dipped in the paste and fried in hot edible oil until brown. Following ratio of ingredients were used to prepare mushroom pakora.

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

3.9.1.3. Mushroom curry

Mushroom, tomato, pea and capsicum were washed and chopped, separately. Chopped onion, grand 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	Quantity
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

3.9.1.4. Mushroom egg scramble

Mushroom, green pea and green chili tomato were washed and chopped, separately. Egg, green pea and tomato were fried until it turns to light brown and then the mushroom and chili was added and again fried well. All spices and salt added and again boiled for five minutes.

3.9.2. Long term processing

3.9.2.1. Drying process

Dehydrated mushrooms are used as an importantingredient in several food formulations including instant soup, pasta, snack seasonings, casseroles, and meat and rice dishes. Fruiting bodies were separated according to their size and then dried. Sun dry, oven dry and freeze drying processes were adopted for drying. In case of sundry, fruiting bodies were kept in a tray covered by a thin cloth for 4 hrs for 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 35-40^0 C for 3-4days. In case of freeze drying, mushroom fruiting body were stored at 4^0 C for 5-6 days for complete drying.

3.9.2.2. Preparation of Mushroom powder

Preparation of mushroom powder is also an effective step for long term storage of mushroom. Mushroom fruit body was dried following different drying methods after which the dried fruitbodies were ground using the mixer grinder and then the powder

was sieved. Sieving was done to get the finely ground powder. Then the powder was kept in airtight container for further use.

3.9.2.3. Mushroom biscuits

Delicious and crunchy mushroom biscuits were prepared by using 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 wave oven (60^0C) for baking purpose for 10-15 minutes. After baking, trays were removed from the micro wave 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	Quantity
Maida	1000gm
Mushroom powder	150gm
Butter	250gm
Crushed Sugar	To taste
Milk	250ml
Cashew nut (crushed)	200gm
Baking Powder	5gm
Water	As required

3.10. Application of spent mushroom substrate for crop improvement

After the cultivated mushroom fruit body has exhausted the nutrients within the substrates and there is no more fruit body to grow, the substrate so called remains is known as spent mushroom substrate. Spent compost or substrate is rich in organic matter and constitute an important source of macro and micronutrients for plants and micro-organisms thereby increase the soil micro-flora, soil biological activity and enhance soil enzyme activity. This spent compost can be used as soil conditioner or organic fertilizer to improve the plant health status. There are many ways to use the spent mushroom substrate. SMS is excellent to spread on top of newly seeded lawns. The SMS provides cover against birds eating the seeds and will hold the water in the soil while the seed germinate. Since some plant and vegetables are sensitive to high salt content in soil, avoid using fresh substrate around these plants. In these case 6 months

or more weathered spent substrate can be used. As a soil amendment, SMS adds organic matter and structure to the soil. Total four field trials have been conducted to evaluate the effect of spent mushroom substrate for improvement of plant health status.

3.10.1. Use of Spent mushroom substrate of *Calocybe indica* in leafy vegetables

Spent mushroom substrate of *C. indica* was used as amendment for leafy vegetables like *Coriandrum sativum*, *Spincia olerecea* and *Amarathus sp*. The spent mushroom substrates were covered with polythene and further fermented for 40 days. Thereafter, the fermented spent substrate was sun dried for 6 hours and used in the experiment field. The experimental was conducted in a randomized complete block design. The seeds of three green leafy vegetables local variety were collected and sowing was done in the sandy land. The experimental field area of 6x7sq ft was supplemented with 5kg of SMS (wet weight) of *C. indica.* Spent substrate was applied in open field condition. These treatments were applied at the depth of 6 inch and mixing properly. SMS was applied and incorporated into the soil manually 2 days before of sowing the seed.

3.10.2. Application of SMC of *Agaricus bisporus* for organic cropping of *Solanum lycopercicum* L.

The experimental design was a randomized complete block design with 15 replicates plants. Each plot (6x7ft) was amended with 5kg of fresh SMC. The SMC was applied and incorporated into the soil at the depth of 10-15cm manually 3 days before of planting the seedlings. Direct planting method was employed. After 3 days of amendment tomato seedlings of 10cm height were transplanted in the untreated soil and the treated soil. The seedlings were planted maintaining the distance of 30 cm from each other's. This experiment was carried out in open field condition. Vegetative growth parameters such as plant height, number of leaves and number of branches was recorded. Flowering time, number of fruits, quality of fruits, fruiting time duration (self-life of plant) was measured. Mycorrhizal root association was also studied. The data generated from these investigations were subjected to test of significance.

3.10.3. Application of SMC and SMS as organic fertilizer over inorganic fertilizer in Bell Pepper (*Capsicum* sp.)

The experiment was conducted at Department of Botany, University of North Bengal, India during the month of May-July, 2015, growing season. The experimental setup was designed as open field potted condition in five replications and the treatments were applied in ten fertilizers combinations. Organic inoculums such as of cow manure, Tricho-compost, PGPR-powder, SMC of *Agaricus bisporus*, SMS of *Calocybe indica* and vermicompost. In this experiment the nine differenttreatments were applied soil

(2kg) + SMC (500gm), soil (2kg) + crude trico-compost (500gm), soil (2kg)+ vermicompost (500gm), soil+ SMC (250g) + vermi-compost (250g), soil+ PGPR, soil + cow dung, soil + urea 200gm, soil+ urea100gm, vermi + PGPR. Control was maintained with only soil and ash. *Capsicum annuum* seeds were procured from local market and Seedlings were grown in mother bed. The seeds were placed in small plastic pots (5 cm diam.) tray in 24◦C ± 2 temperature conditions and were grown for two weeks. They were irrigated thrice weekly. When the seedlings reached the 4-leaf stage, the uniform-height seedlings were transplanted to earthen pots (32 cm diameter) containing different soil mixtures and watered every alternate day.

3.10.3.1. Preparation of plant growth promoting rhizobacteria (PGPR) formulation

The PGPR bacterial isolate *Bacillus altitudinis* (NAIMCC-B01484) was collected from Immuno-phytopathology laboratory, Department of Botany, NBU, West Bengal, India. The isolate was cultured in nutrient Broth. After 48 hours of inoculation the culture medium was centrifuged at 5,000 rpm for 15 mints. The pellet was collected and dissolved in 200 ml distilled water. A pinch of twenty 20 was added to it as adhesive and the suspension was mixed with talcum powder (200ml/kg) using a properly cleaned and fumigated mass mixture machine in a closed chamber. The inoculated talcum powder was packed with proper sealing in aluminum coated packet and stored in low temperature for use.

3.10.3.2. Preparation of tricho-compost

The bio-inoculum tricho-compost was prepared using spent substrate of oyster mushroom (*Pleurotus ostreatus*) as a substrate collected from 'Mushroom Production Unit', Department of Botany, University of North Bengal. The spent mushroom substrate was inoculated with *Trichoderma harzianum* (NAIMCC-F-01966) and kept them in shaded condition (Figure 7). Water sprinkled in regular 2 days interval to maintain moisture. After 55 days of inoculation the brownish tricho-compost was collected and used for experiment.

3.10.3.3. Preparation of vermicompost

A layer about 30-45 cm of organic waste such as aquatic plants i.e. *Eichornia sp*, vegetables waste or spent mushroom substrate was spread into the vermibed (4x4x3ft). The top of the waste layer in the vermibed was sealed with the dry cow dung and earth worm was added to the vermibed and water sprinkled. The vermibed was kept in shade to avoid rain (Figure 7). In regular 3 days interval water sprinkle was applied to avoid the dryness of the composting materials and to maintain the proper moisture in it that

will make favorable environment to the earth worm. The vermi-product was ready in about 40-45 days. The processed vermi-product was brownish black, light in weight with no bad smell. The vermi product was collected by separating earth worm from it and dried and sieved for use.

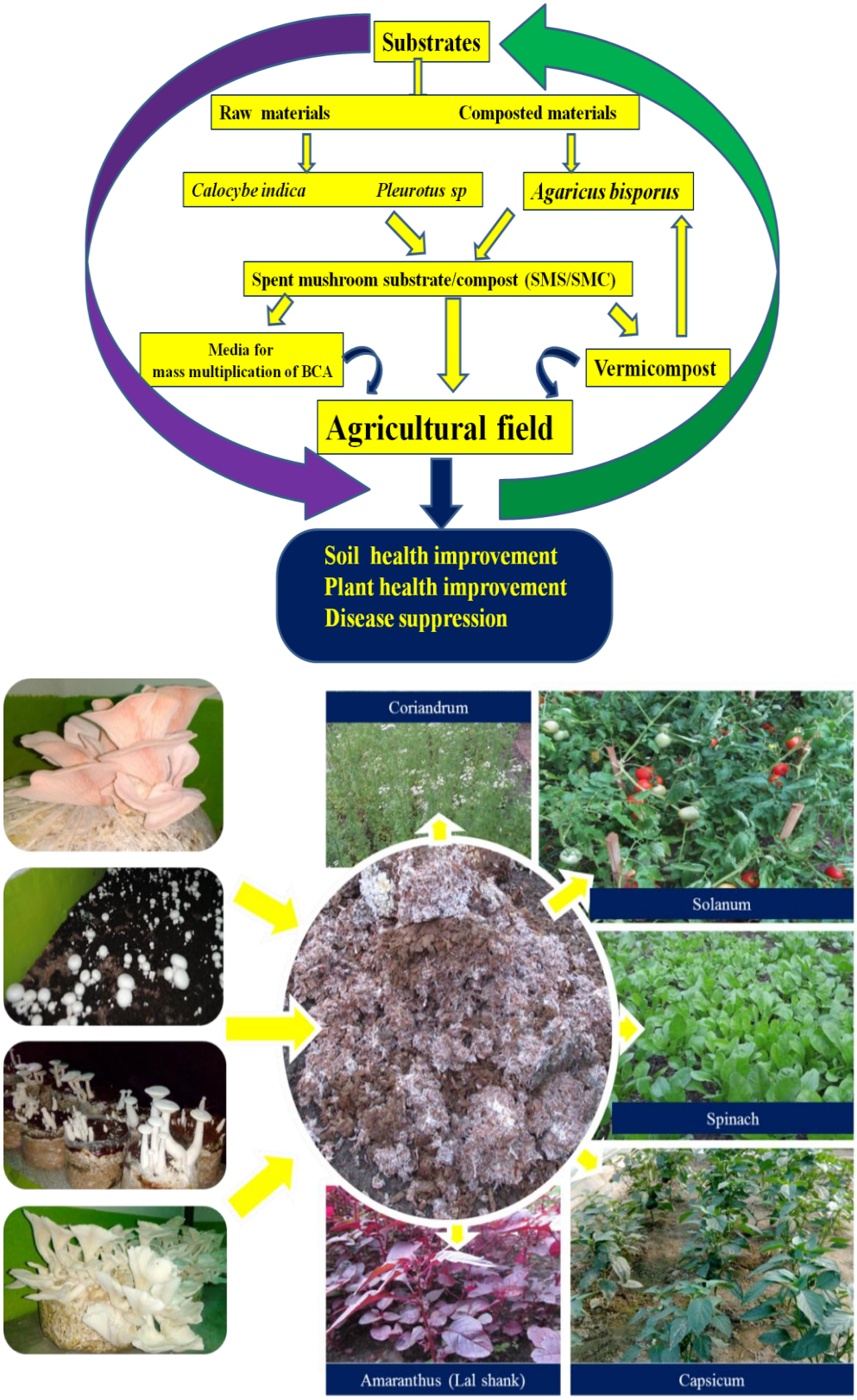

Figure 6: A model scheme of solid waste management through mushroom cultivation

Figure 7: Conversion of spent mushroom substrate into vermicompost and Tricho-compost

3.10.4. Evaluation the effect of spent mushroom substrate in growth parameter of crop plant

Growth parameters in terms of on germination period and plant height, number of leaves, number of branch and leaf size was recorded regularly in an interval basis.

3.10.5. Evaluation the effect of spent mushroom substrate in biochemical levels of plants

3.10.5.1. Extraction and quantification of phosphate from soil, roots and leaves

One gram of oven dried Soil samples and plant samples from each plot was used for extraction and quantitative estimation of phosphate following the ammonium-molybdate-ascorbic acid method as described by Knudsen and Beegle (1988), where dried samples were suspended in 25 ml of the extracting solution made up of 0.025 (N) H_2SO_4 and 0.05 (N) HCL to which activated charcoal .01gm was added, shaken well for 30 mints on a shaker and filtered through Whatman no. 1 filter paper (Mehlich, 1984).

3.10.5.2. Chlorophyll content

Extraction of chlorophyll was done following the methodology proposed by Harborne 1973, by homogenizing one gram plant sample in 80% acetone using mortar and pestle.

The homogenate was filtered through Whatman no. 1 filter paper and the volume made up to 10 ml and chlorophyll was measured from the absorbance taken at 645nm and 663 nm using Arno's formula: Total chlorophyll= [(20.2 A_{645}) + (8.02 A_{663})]µg/ml.

3.10.5.3. Carotenoid content

Carotenoid were extracted and estimated according to the method described by Lichtenthalar HK (1987). Extraction of Carotenoid was done by homogenizing 1gm of plant sample in 80% methanol. The homogenate was filtered using Whatman no. filter paper and the final volume made up accordingly. Carotenoid content was determined by taking the absorbance of filtrate at 480 nm, 645 nm and 663 nm using UV-VIS spectrophotometer.

3.10.5.4. Lycopene content

Lycopene was extracted from the fruit using acetone by making it pulp with the help of mortar and pestle and then the 5gm of the pulp extract was transferred to a separating funnel containing about 20 ml of petroleum ether and mixed gently. 20ml of 5% sodium sulphate solution was added and shaken gently followed by the addition of same volume of petroleum ether. The two phases were separated and the lower aqueous phase was re-extracted until the aqueous phase become colorless. The petroleum ether extract were pooled out and washed once with little distilled water. The washed petroleum extract containing Carotenoid poured into brown bottle and kept aside for 30mints. Then the petroleum ether extract was decanted into a 100ml volumetric funnel containing cotton wool. The sodium sulphate slurry was washed with petroleum ether until it was colorless and washing were also transfer to the volumetric flask. The volume was made up and absorbance was taken in spectrophotometer at 503nm using petroleum ether as blank. Quantification was done on the basis of this absorbance using the formula, Absorbance (1 unit) = 3.1206µg/ml.

3.10.5.5. Ascorbic acid

Plant material was homogenized in cold mortar pestle with 10 ml of 6% of TCA. The filtrate was than extracted at 0^{o} C and 2 ml of DNPH and 1 drop of 10% thiourea added to the 4ml of extract. Mixture kept in water bath for 15 mints and cooled down using ice and after cooling 5ml of 80% H_2SO_4 added to it at 0^{o} C. Quantitative estimation of ascorbic acid was done by following the method as described by Lichtenthalar HK (1987), using ascorbic acid as standard.

3.10.5.6. Determination of total soluble protein content

Protein content (mg gt^{-1}) in plant sample was extracted and estimated following the method described by Lowry *et al.*,(1951) in which one gram of plant sample was crushed in chill condition and protein was extracted using phosphate buffer (pH-7.2). The protein content was determined using BSA as standard.

3.10.5.7. Extraction and estimation of Carbohydrate content of fruits

Extraction of carbohydrate from fruit vegetables was done following the methodology of Harbone (1973). 1gm sample was homogenized in 95% ethanol and kept on water bath for evaporation of the alcoholic fraction. The residue was then re-suspended in distilled water. The sample mixture was centrifuged at 10000 rpm for 15 min and the supernatant was collected for the estimation as crude sample extract.

3.10.5.7.1. Estimation of total sugar

Carbohydrate content of different parts of vegetables was measured. Ethanol (95%) was used for extract preparation. Total sugar was estimated at 630 nm using Anthrone's reagent. The sample tissue was hydrolyzed by dilute HCL and then neutralized by NaOH.

3.10.5.7.2. Estimation of reducing sugar

Determination of reducing sugar content using arseno-molybdate was carried out at 620 nm following Nelson-Somogyi Method as described by Plummer (1978). Reducing sugar estimation was done using 2 ml of alkaline copper tartrate in 2ml of ethanolic extract of sample.

3.10.6. Evaluation of the effect of SMS on Mycorhhizal association and population in the rhizospheric soil of treated crop plant

3.10.6.1. Study of mycorrhizal association in root system

3.10.6.1.1. Root sample Collection and treatment

From each study plot 5 healthy plants were selected. The roots of two variety samples were collected at 12-16 cm soil depth. The root samples were washed thoroughly free of attached soil particles and cut into small (cm) pieces and washed with tap water and boiled in 2% NaOH in water bath for 1 hour. NaOH was then decanted and washed with tap water thrice. The samples were then treated with 1% HCL for 30mints in room temperature. After that the HCL decanted again and the sample was washed thrice with tap water. It was stained with lacto-phenol cotton blue solution (lactic acid: cotton blue: glycerol=1:1:1) and observed under microscopy. Root segments were mounted on glass slide with lacto-phenol and observed compound microscope at 10X magnification. A

minimum 10 segments for each sample were observed for the assessment of percentage root colonization by mycorrhiza. The mycorrhizal colonization percentage was calculated by using following formula.

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{Number of root segments colonized}}{\text{Total number of root segments examined}} \times 100$$

3.10.6.2. Study of Mycorrhizal spore population in the rhizospheric soil

3.10.6.2.1. Isolation and Quantification of AM fungal spores

The AM fungal spores were separated from the soil by wet sieving and decanting technique (Gerdman *et al.*,1963). 100 gram of rhizospheric soil sample was mixed in 1000 ml of distilled water in a large beaker. After 1 hr. the contents of the beaker were decanted through the sieves which were arranged in a descending order from 200 µm to 60 µm size. The process was repeated for thrice. The procedure was repeated until the upper layer of soil suspension is transparent. The retained material on the sieve was decanted into a beaker with a stream of water and estimation of spores was carried out followed by method of Gaur and Adholeya (1994) with modification. A circular filter paper was taken and folded into four equal quadrants. The paper was reopened; two lines were drawn along the two folds to divide the filter paper into four equal quadrants. The suspension was then decanted through the stack of sieves 180 and 60 µm (arranged in decreasing order of mesh size from top to bottom). Same process was repeated 2-3times and the residue from each sieve was collected into Petri plates with little distilled water. Intact AM fungal spores were examined and counted under microscope.

3.11. Application of SMC in disease suppression

3.11.1. Evaluation the effect of SMC extract on radial growth of *Fusarium oxysporum in vitro*

To prepare the water extract 10gm of SMC were suspended in 100ml of sterile distilled water. The suspension was stirred for 2 hours at 40rpm to obtain even particle distribution and then filtered with sterile muslin cloth. Two portions of the suspension were prepared. One portion was sterilized by autoclaving at 121˚ C for 15mins and other was not. One ml of the portion of SMC suspension was poured into sterile petri dishes. 10 ml of cooled molten PDA was aseptically poured into petri-plates and rotated gently to ensure uniform distribution of the extract. Control plates maintained with 1ml sterilized distilled water and cooled molten PDA. The plates were left to solidify. After

solidification of the media, inoculation was done by placing a 5mm diameter mycelia disc taken from a fresh culture (12 days old). All plates were incubated at 25 ±2˚C. Data was collected on mycelia growth and recorded from 3 days after inoculation till when control plates were fully covered with mycelia growth. Percent inhibition in mycelia growth was determined using the formula I=(C-T)/Cx100.

3.11.2. Evaluation of SMC in management *the of Fusarium oxysporum* mediated wilting in *Solanum sp*

3.11.2.1. Inoculum preparation

Conidial suspension was prepared by culturing the fungus on PDA media at 28±2 ˚C for 15 days. 100ml of sterile distilled water was dispensed into the pure culture and the culture was scrapped slightly with a sterile scalpel to dislodge the conidia from the mycelia. The suspension was filtered through a double layer muslin cloth to remove the mycelial fragments. The filtrate was re-suspended in 250ml of sterile distilled water and mixed well. The conidia suspension was used for inoculation.

3.11.2.3. Experimental set up

25 day old egg-plant seedlings were inoculated by standard root dip inoculation method. Seedlings were removed from the pot, shaken to remove adhering particles and washed carefully under running water. The roots were trimmed with a sterile scissor and were submerged in the micro-conidial suspension for 30 mins. The inoculated seedlings were transplanted to six flasks containing sterilized distilled water. In three of these flasks 25 ml of SMC crude extract was added. The set was named as treated inoculated and the other three named as untreated inoculated. Two control set were made, one control set was treated with only normal distilled water and other control with PDB. There are three replica of each set. All sets of replica plants were placed in same growth conditions. The percentage incidence of the wilt was estimated using the formula proposed by Michel *et al.,* (1996). The severity of the wilt disease was assessed every 24 hours starting from 24hours after inoculation up to 15 days.

3.11.3. Evaluation of SMC in management the of *F. oxysporum* mediated wilting in *Citrus reticulata*

The experiment was conducted in potted condition with three replicate. 16” earthen pot was fulfilled by 1.5 kg dry soil which was amended with 500gm fresh spent compost of *A. bisporus*. Seedling of 2years old was transplanted in the pot and observation was

carried out for next eight (8) months. After that again 500gm of SMC was added to the treated potted plant and kept them for 25 days and finally *Fusarium oxysporum* mycelial water suspension was mixed with the soil of all potted plants. The effect and severity of the disease was observed. The presence of pathogen in root and leaf was confirmed by FITC labeling analysis.

3.11.3.1. Extraction and Assay of defense enzyme activity

3.11.3.1.1. ß-1, 3- glucanase (E.C. 3.2.3.39)

Extraction of ß-1, 3- glucanase was done following the methoddescribed by Pan *et al*., (1991). One g of root and leaf samples were crushed inliquid nitrogen and extracted using 5ml of chilled 0.05 (M) sodium acetate buffer (pH-5)by grinding at 4 ºC using mortar and pestle. The extract was then centrifuged at 10000rpm for 15 min at 4 ºC and the supernatant was used as crude enzyme extract.Estimation of the ß-1, 3-glucanase was done by following theLaminarindinitrosalicylate method (Pan *et al*., 1991). The crude enzyme extract of 62.5µl was added to 62.5 µl of laminarin (4%) and then incubated at 40ºC for 10 minutes. Thereaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min inboiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was taken at 500nm. The blank was the crude enzymepreparation mixed with laminarin with zero time incubation. The enzyme activity wasexpressed as µg glucose released min-1 g-1 fresh tissues.

3.11.3.1.2. Chitinase (E.C. 3.2.1.14)

Extraction of chitinase was done by following the methoddescribed by Boller and Mauch (1988) with modifications. 1g root and leaf sample frommandarin plants were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1MSodium Citrate buffer (pH5). The homogenate was centrifuged for 10minutes at 12,000 rpm and the supernatant was used as enzyme source.Chitinase activity was measured according to the method described by (Boller *et al*., 1988). The assay mixture consisted of 10µl Na-acetate buffer (1M) pH 4, 0.4mlof enzyme solution, 0.1ml of colloidal chitin (1mg). Colloidal chitin was prepared as perthe method of (Roberts *et al.*, 1988). After 2h of incubation at 37 ºC thereaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant (0.3ml) was pipetted into a glass reagent tube containing 30µl of potassium phosphatebuffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1hour. After 1h, the pH of the reaction mixture

was brought to 8.9by addition of 70µl of sodium borate buffer (1M) pH9.8. The mixture was incubated in aboiling water bath for 3minutes and then rapidly cooled in an ice water bath.Afteraddition of 2ml of DMAB (ρ-dimethyl-aminobenzal-dehyde) reagent the mixture wasincubated for 20 min at 37 ºC. Therefore absorbance value at 585nm was taken UV-VIS spectrophotometrically. N-acetyl glucosamine (GlcNAc) was used as standard. The enzymeactivity was expressed as µg GLcNAc min $^{-1}$ mg $^{-1}$ fresh tissues.

3.11.3.1.3. Phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5)

Extraction of PAL was done by following the method described by Chakraborty *et al*., (1993) with modifications. 1gm root and leaf sample was crushed in0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß-mercaptoethanol in ice coldtemperature. The slurry was centrifuged in 15,000 rpm for 20 minutes at 4ºC. Supernatantwas collected and after recording its volume, was immediately used for assay or stored at-20ºC. Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 30µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Followingincubation for 1 h at 40 ºC the absorbance at 290nm was read against a blank without theenzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced min^{-1} g $^{-1}$ fresh weight of tissues.

3.11.3.2. Indirect Immune florescence

Indirect immune florescence of root and stemand leaf section was conducted with homologous polyclonal antibody. The fungal mycelium and the samples treated with PAbs raised against *F. oxysporum* and labeled with FITC showed apple green fluorescence.

3.11.3.3. Immuno-localization of Chitinase and Glucanase enzymes by indirect immune-fluorescence staining of leaf, stem and root tissue

Root rot incidence in mandarin plants was successfully reduced after application of SMC in the rhizosphere of mandarin saplings prior to pathogen challenge. Disease reduction was found to be brought about by enhanced activities of key defenseenzymes like chitinase, glucanase, phenylalanine ammonia lyase and peroxidase whichincreased significantly after pathogen challenge.Cellular localization of chitinase in leaves and roots of citrus plants was determined following indirect immunofluorescence test using

FITC binding and treatment with PAbs raised against chitinase and glucanase. Leaf and root sections from untreated control plants and inoculated and treated inoculated plants were taken. Immune-localization of chitinase and glucanase in treated leaf and root sections of citrus plants were observed using FITC after treatment with PAbs raised against chitinase and glucanase.