Chapter III: Research Methodology
3.1 Solan

Solan is a city in the Indian state of Himachal Pradesh (Figure 3.1). The city is situated between Chandigarh and Shimla (state capital), on the Kalka-Shimla National Highway-22. It is known as the "Mushroom city of India" because of the vast mushroom farming in the area. Solan is located at 30.92°N and 77.12°E. It is situated at an altitude of 1600 meters (5249.34 feet) on an average. Solan can be called as a cool station. During winters, Solan experiences light snowfall. Temperatures typically range from -4°C (25°F) to 32°C (90°F) during the year with an average annual temperature 18°C (64°F).

Solan area contains scrub forests on lower elevations and Pinus and Cedrus at higher elevations, mainly pine trees in the city. Oak forests also exist at higher elevations around moist locations. Among conifer species, Pinus roxburghii is the most predominant one. Besides this natural or indigenous vegetation, there is an ornamental plantation too which contains Callistemon, Euphorbia, Grevillea, Jacaranda, Thuja etc.

3.1.1 Sampling Areas for Environmental Isolates:

Environmental samples were collected from decayed wood, bark, soil, debris and sites contaminated with pigeon excreta from the following selected locations of Solan, Himachal Pradesh (H.P.).

1. Bajhol
2. Chambaghat
3. Children Park, Mall Road
4. Degree College
5. Dist Tourism Division Office
6. Jatoli
7. Mohan Park
8. New Bus Stand
9. Old Bus Stand
10. Saproon Parking
11. Shilly
12. Solan Military Station
Figure 3.1 Map of Himachal Pradesh

Study Area-Solan City
13. State Library, Mall Road  
14. Tehsil Office, Kotla Nala

All these locations are covered with different types of vegetation (tree species) but only dominant tree species which were in vast majority were selected for the isolation in the selected locations listed in Table 3.1.1. The following tree species were selected for the fungal isolates:

1. *Callistemon lanceolatus* (Myrtaceae)  
2. *Cedrus deodara* (Pinaceae)  
3. *Eucalyptus* spp (Myrtaceae)  
4. *Euphorbia royleana* (Cactaceae)  
5. *Ficus auriculata* and *Ficus palmata* (Moraceae)  
6. *Grevillea robusta* (Proteaceae)  
7. *Jacaranda mimosifolia* (Bignoniaceae)  
8. *Juglans regia* (Juglandaceae)  
9. *Pinus roxburghii* (Pinaceae)  
10. *Populus deltoides* (Salicaceae)  
11. *Prunus persica* (Rosaceae)  
12. *Quercus leucotrichophora* (Fagaceae)  
13. *Syzygium cumini* (Myrtaceae)  
14. *Thuja occidentalis* (Cupressaceae)

### 3.1.2 Environmental Sample Collection and Screening:

Collection and screening of the environmental samples had been done by the following methods:

A. Sampling of debris, soil and pigeon droppings  
B. Tree swab sampling  
C. Lactophenol cotton blue (LCB) staining for identification  

- During the sampling, timing and weather conditions (maximum and minimum temperature, humidity and rainfall) were taken into consideration. Texture of debris and pigeon dropping samples was noted before processing.
Table 3.1.1 List of Selected Locations or Places for the Isolation of *Cryptococcus* according to the Distribution of Tree species and Pigeon Droppings

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Location</th>
<th>Distribution of Tree species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bajhol</td>
<td><em>Pinus roxburghii, Quercus leucotrichophora,</em> Syzygium cumini, Ficus palmata &amp; Ficus auriculata</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Grevillea robusta, Callistemon lanceolatus, Thuja occidentalis, Jacaranda mimosifolia, Populus deltoides, Eucalyptus spp, Euphorbia royleana</em></td>
</tr>
<tr>
<td>2.</td>
<td>Chambaghat</td>
<td><em>Thuja occidentalis</em></td>
</tr>
<tr>
<td>3.</td>
<td>Children Park</td>
<td><em>Thuja occidentalis</em></td>
</tr>
<tr>
<td>4.</td>
<td>Degree College</td>
<td><em>Eucalyptus spp, Populus deltoides</em></td>
</tr>
<tr>
<td>5.</td>
<td>Dist Tourism Division Office</td>
<td><em>Columba livia</em> (pigeon) Droppings</td>
</tr>
<tr>
<td>6.</td>
<td>Jatoli</td>
<td><em>Callistemon lanceolatus, Jacaranda mimosifolia,</em> Eucalyptus spp, Thuja occidentalis</td>
</tr>
<tr>
<td>7.</td>
<td>Mohan Park</td>
<td><em>Jacaranda mimosifolia</em></td>
</tr>
<tr>
<td>8.</td>
<td>New Bus Stand</td>
<td><em>Columba livia</em> (pigeon) Droppings</td>
</tr>
<tr>
<td>9.</td>
<td>Old Bus Stand</td>
<td><em>Columba livia</em> (pigeon) Droppings</td>
</tr>
<tr>
<td>10.</td>
<td>Saproon</td>
<td><em>Eucalyptus spp</em></td>
</tr>
<tr>
<td>11.</td>
<td>Shilly</td>
<td><em>Juglans regia, Cedrus deodara</em></td>
</tr>
<tr>
<td>12.</td>
<td>Solan Military Station</td>
<td><em>Grevillea robusta</em></td>
</tr>
<tr>
<td>13.</td>
<td>State Library</td>
<td><em>Columba livia</em> (pigeon) Droppings</td>
</tr>
<tr>
<td>14.</td>
<td>Tehsil Office</td>
<td><em>Populus deltoides, Eucalyptus spp,</em> Thuja occidentalis</td>
</tr>
</tbody>
</table>
A. **Sampling of Debris, Soil and Pigeon Droppings**

Environment sampling of debris, soil and pigeon droppings was done by the method proposed by Kidd *et al* (2004).

1. 2 g soil or debris or dry dropping sample was taken.
2. Suspended in 10 ml of sterile distilled water.
3. Vortexed for 5 minutes.
4. Took 0.5 ml aliquots of the soil or debris suspension.
5. Transferred to Sabouraud dextrose agar (SDA) medium.
6. Incubated at 30°C for 10 days.
7. Yeast like creamish colonies of suspected *Cryptococcus* was detected on Sabouraud dextrose agar.
8. Sub cultured to obtain single colony on Sabouraud dextrose agar plates.

B. **Sampling of Trees**

Environment sampling of trees was done by swabbing method of Kidd *et al* (2004).

1. Swabs from test tubes containing saline water were taken.
2. Tree parts like trunk hollows, niche, scars, holes, under bark, shallow cavities and areas of woody debris were swabbed.
3. Then the swabs were transferred to Sabouraud dextrose agar medium.
4. Incubated at 30°C for 10 days.
5. Presence of mucoid and creamish colonies indicated the positive results.

C. **Lactophenol Cotton Blue Staining**

The Lactophenol cotton blue (LCB) wet mount preparation is the most widely used method of staining and observing fungi (Leck, 1999).

1. A drop of 70% alcohol on a microscope slide was placed.
2. Immersed the specimen/material in the drop of alcohol.
3. Added one or at most two drops of the lactophenol cotton blue mountant stain before the alcohol dries out.
4. Held the cover slip between forefinger and thumb, touched one edge of the drop of mountant with the cover slip edge, and lowered gently, avoiding air bubbles. The preparation was then ready for examination.

3.2 Identification and Characterization of Environmental and Clinical Isolates of *Cryptococcus* species complex

**Clinical Strains**

1. Sixteen clinical strains of *Cryptococcus* (15 *C. neoformans* and 1 *C. gattii* isolate) used in this study were procured from National Culture Collection of Pathogenic Fungi (NCCPF), Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (Table 3.2.1).

2. Two clinical strains of *C. neoformans* were procured from Indira Gandhi Medical College (IGMC), Shimla (Himachal Pradesh), India (Table 3.2.1).

**Standard Strains**

- Seven standard ATCC (American Type Culture Collection) strains of *Cryptococcus* were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune and Institute of Microbial Technology (IMTECH), Chandigarh, India (Table 3.2.2).

**Environmental Isolates**

- Fifty isolates collected from the different environmental sources were used for their biochemical identification in the present section.

All the strains were maintained on Sabouraud"s dextrose agar (HiMedia, Mumbai) slants and preserved in 10% glycerol at -20°C. Sub culturing was done regularly to maintain fresh cultures for the experiments. Various morphological, physiological and biochemical methods were used in the identification and characterization of environmental isolates and clinical strains which were following:
Table 3.2.1 List of Clinical Strains of *Cryptococcus* procured from National Culture Collection of Pathogenic Fungi (NCCPF), PGIMER, Chandigarh and IGMC, Shimla, Himachal Pradesh, (India)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Code (<em>Cryptococcus neoformans</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25:49</td>
</tr>
<tr>
<td>2.</td>
<td>25:50</td>
</tr>
<tr>
<td>3.</td>
<td>25:52</td>
</tr>
<tr>
<td>4.</td>
<td>25:61</td>
</tr>
<tr>
<td>5.</td>
<td>25:84</td>
</tr>
<tr>
<td>6.</td>
<td>25:86</td>
</tr>
<tr>
<td>7.</td>
<td>25:102</td>
</tr>
<tr>
<td>8.</td>
<td>25:104</td>
</tr>
<tr>
<td>9.</td>
<td>25:110</td>
</tr>
<tr>
<td>10.</td>
<td>25:141</td>
</tr>
<tr>
<td>11.</td>
<td>25:144</td>
</tr>
<tr>
<td>12.</td>
<td>25:391</td>
</tr>
<tr>
<td>13.</td>
<td>25:404</td>
</tr>
<tr>
<td>14.</td>
<td>25:408</td>
</tr>
<tr>
<td>15.</td>
<td>25:909</td>
</tr>
<tr>
<td>16.</td>
<td>250412 (<em>C. gattii</em>)</td>
</tr>
<tr>
<td>17.</td>
<td>IG-1</td>
</tr>
<tr>
<td>18.</td>
<td>IG-2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>
### Table 3.2.2 Information of Standard Strains of *Cryptococcus*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Organism</th>
<th>ATCC® Number</th>
<th>Source of Isolation</th>
<th>Procured from Culture Collections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Cryptococcus albidus</em></td>
<td>26902</td>
<td>Glacier</td>
<td>IMTECH</td>
</tr>
<tr>
<td>2.</td>
<td><em>Cryptococcus curvatus</em></td>
<td>10567</td>
<td>Sputum</td>
<td>IMTECH</td>
</tr>
<tr>
<td>3.</td>
<td><em>Cryptococcus terreus</em></td>
<td>11799</td>
<td>Soil</td>
<td>IMTECH</td>
</tr>
<tr>
<td>4.</td>
<td><em>Cryptococcus laurentii</em></td>
<td>18803</td>
<td>Palm wine</td>
<td>IMTECH</td>
</tr>
<tr>
<td>5.</td>
<td><em>Cryptococcus luteolus</em></td>
<td>32044</td>
<td>Air</td>
<td>IMTECH</td>
</tr>
<tr>
<td>6.</td>
<td><em>Cryptococcus neoformans</em></td>
<td>34664</td>
<td>Liquor patient of NCL</td>
<td>NCL</td>
</tr>
<tr>
<td>7.</td>
<td><em>Cryptococcus neoformans</em></td>
<td>32045</td>
<td>Fermenting fruit juice</td>
<td>NCL</td>
</tr>
</tbody>
</table>

- IMTECH – Institute of Microbial Technology, Chandigarh, India
- NCL – National Chemical Laboratory, Pune, India
- ATCC – American Type Culture Collection, USA
A. **Morphological Identification**

1. Gram’s staining
2. Lactophenol Cotton Blue (LCB) staining
3. Indian ink staining
4. Wan’s staining

B. **Physiological Examination**

1. Nitrate reduction test
2. Temperature study
3. Carbohydrate assimilation test
4. Carbohydrate fermentation test

C. **Biochemical Characterization**

1. Urease test
2. Bird Seed Agar (BSA) medium test
3. Canavanine Glycine Bromothymol Blue (CGB) agar medium test

**Identification:**

1. **Gram’s staining**

Gram staining is an extremely important technique based on which all bacteria can be differentiated into two distinct groups: Gram-positive and Gram-negative bacteria. Christian Gram (1884) developed this technique (*Bergey et al.*, 1994).

**Method**

1. A freshly grown yeast culture was smeared on a glass slide and heat fixed.
2. The heat fixed smear was flooded with crystal violet for 1 minute.
3. Washed with running tap water for 10 seconds.
4. The slides were then stained with iodine solution for 30 seconds.
5. Washed again with tap water for 5 second and decolorized with acetone for 2 seconds.
6. The cells were then washed with water for 30 seconds and counter stained with safranin.
7. The smear was then observed under a microscope using an oil immersion objective.

2. **Lactophenol Cotton Blue Staining**

The Lactophenol cotton blue (LCB) wet mount preparation is the most widely used method of staining and observing fungi (Leck, 1999). *(See Part C of Section 3.1.2)*

3. **Indian ink Staining**

The mucoid capsule appears as a clear halo that surrounds the yeast cell. The yeast cells may be round, oval or elongate. Buds may be absent, single or rarely multiple and may be detached from the mother cell but enclosed in a common capsule attached (Murray, 1999).

**Method**

1. For suspected Cryptococcal cultures, a wet preparation was made by using saline on a clean glass slide and then a small drop of Indian ink was added and mixed.
2. A cover slip was applied over the mixture and pressed it gently to obtain a thin mount.
3. If Indian ink is too thick (dark), dilute it by 50% with saline.
4. The preparation was allowed to stand for few minutes to settle.
5. Scanned under low power in reduced light.

4. **Wan’s Staining**

Negative staining with Indian ink has been used to identify Cryptococcus. Another staining method utilizes Alcian blue, a dye that reacts selectively with *C. neoformans*, but not other *Cryptococcus* species. To overcome these limitations, a new staining reagent was developed by Wan *et al* for rapid identification of *Cryptococcus* for clinical laboratory diagnosis. The capsulated cells appear clearly visible with Wan’s stain reagent as compared to the Indian ink (Wan *et al.*, 2011).

**Method**

For suspected Cryptococcal cultures, a wet preparation was made by using saline on a clean glass slide, and then a small drop of Wan’s staining reagent was added and mixed.
1. A cover slip was applied over the mixture and pressed it gently to obtain a thin mount.
2. The preparation was allowed to stand for few minutes to settle.
3. Scanned under low power in reduced light.

B. Physiological Examination:

1. **Nitrate Reduction Test**

   Yeast species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. Reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate. The nitrate reduction test is based on the detection of nitrite and its ability to form a red compound when it reacts with sulfanilic acid (reagent A) to form a complex (nitrite-sulfanilic acid) which then reacts with α-naphthylamine (Reagent B) to give a red precipitate (Rhodes et al., 1975).

   **Method**

   1. A special nitrate broth medium was prepared.
   2. Two millilitres of this broth was dispensed per tube, and the tubes were autoclaved for 15 minute at 121°C.
   3. The tubes were heavily inoculated and incubated at 30°C for 48 h.
   4. Six drops each of the sulfanilic acid and α-naphthylamine reagents described by Edwards and Ewing (1972) were added to each tube; each tube was shaken and observed for the presence of a red color that indicated nitrate reduction. A small amount of zinc dust was added to each negative test for confirmation.

2. **Temperature Study**

   Another important step in the identification of yeast is by determining its ability to grow at different temperatures (25°C, 30°C, 35°C and 37°C). It can be used to distinguish C. neoformans from other species (Chakrabarti et al., 1998).

   **Method**

   1. Four Petri plates of malt extract agar were inoculated with the isolate.
2. Incubated all the four Petri plates at four different temperatures (25°C, 30°C, 35°C and 37°C).
3. Examined the Petri plates everyday up to 4-7 days for the presence of growth.
4. Growth must be present in all the Petri plates concluding that the yeast has the ability to grow at different temperatures.

3. Carbohydrate Assimilation Test

Various sugars were used for carbohydrate assimilation study; galactose, sucrose, lactose, mellibiose, xylose etc. A growth around the sugar discs was considered positive for assimilation or utilization of sugars by the organism (Chakrabarti et al., 1998).

Method

1. A yeast suspension from a 24-48 h old culture was prepared in 2 ml of Yeast Nitrogen Base (YNB) by adding heavy inoculums.
2. This suspension was added to the molten agar and mixed well.
3. Entire volume of medium was poured into Petri plate.
4. Petri plates were allowed to settle at room temperature until the agar surface hardened.
5. Various carbohydrate-impregnated discs were placed onto the surface of the agar plate.
6. Incubated plates at 37°C for 3-4 days.
7. The presence of growth around the disc was considered as positive for that particular carbohydrate.

➤ 6mm-diameter disc was punched from Whatman filter paper. Sterilized the disc by placing them in hot air oven for 1 h. Added a drop of 10% filter sterilized sugar solution to each disc. Dried the disc at 37°C and stored at 4°C in airtight container

4. Carbohydrate Fermentation Test

An ability of an organism to produce gas from the sugars is known as fermentation. Production of gas in the Durham’s tube is taken as fermentation test positive. Only acid production may simply indicate that carbohydrate is assimilated (Chakrabarti et al., 1998).
Method

1. Liquid fermentation medium was prepared. Poured into the test tubes (approx. 5ml) and Durham’s tube was placed into each tube. Sterilized by autoclaving at 121°C for 15 minute at 15 pounds pressure. Sterilized sugar discs were added to the medium.

2. The tubes were plugged with cotton plugs.

3. Inoculum preparation was done by suspending heavy inoculum of yeast.

4. Each carbohydrate broth was inoculated with approximately 0.1 ml of inoculum.

5. Incubated the tubes at 25°C up to one week. Examined the tubes every 48-72 h interval for production of acid (pink color) and gas (Durham’s tube).

C. Biochemical Identification:

1. Urease Test

The ability of the yeast to produce urease was tested by inoculating the yeast culture on Christensen’s urea agar supplemented with 2% urea. The ammonia produced by cleavage of urea by urease would increase the pH of the medium and change the indicator color to pink (Klein et al., 2009).

Method

1. Prepared Christensen’s urea agar.

2. A small amount of yeast colony on the agar surface was inoculated.

3. Appropriate controls (C. albicans—negative control, C. neoformans—positive control) were inoculated.

4. Incubated slants at 30°C for 48 h.

5. A deep pink (magenta) color indicated a positive result.
2. **Bird Seed Agar (BSA)**

This test was used for checking the brown pigmented colonies of *Cryptococcus neoformans*. Only *C. neoformans* when grown on the medium containing bird seed agar produced dark brown colonies but other species produced creamish, peach or light brownish colonies (Chakrabarti *et al.*, 1998; Laboratory methods-University of British Columbia, Canada).

**Method**

1. Bird seed agar (BSA) was prepared.
2. The cultures of yeast were streaked on the BSA medium plates.
3. Incubated the plates at 25-30°C and observed every day up to 7 days.
4. *C. neoformans* produced dark brown or black colonies within 3-5 days of inoculation.

3. **Canavanine Glycine Bromothymol Blue (CGB) agar medium**

This test was used to differentiate species *C. neoformans* from *C. gattii*. The later hydrolyses glycine to form ammonia to alter the pH of the medium towards alkalinity thus change its color yellow to blue while the former did not and remain yellow (Kwon-Chung *et al.*, 1982; Chakrabarti *et al.*, 1998; Laboratory methods-University of British Columbia, Canada).

**Method**

1. CGB agar slants or petri plates were prepared.
2. Surface of the medium was inoculated using culture of yeast with inoculating loop.
3. Put a positive control (*C. gattii*) and negative control (*C. neoformans*) simultaneously.
4. Incubated at 25°C for 1-5 days.
5. At the end of 5 days positive result was shown by color change of the medium from greenish yellow (pH 5.8) to cobalt blue (pH 7.0).
3.3 Molecular Characterization of the Environmental Isolates of *Cryptococcus*

Molecular characterization was done by the following steps:

3.3.1 DNA Extraction

3.3.2 Quality Assessment of DNA

3.3.3 Polymerase Chain Reaction (PCR) Amplification of ITS region

3.3.4 PCR Amplification of LSU region

3.3.5 Visualization of PCR Products

3.3.6 Sequencing

3.3.7 *In silico* analysis

3.3.1 DNA Extraction

Genomic DNA was extracted from the 10 selected environmental *Cryptococcus* isolates by using the EPICENTRE Master Pure™ Yeast DNA Purification Kit, according to the manufacturer’s recommendations.

3.3.2 Quality Assessment of DNA

The DNA stock samples were quantified using spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 values was considered to be of good purity. Concentration of DNA was estimated using the formula:

\[
\text{Concentration of DNA (mg/ml)} = \frac{\text{OD } 260}{50} \times \text{Dilution factor}
\]

Quality and purity of DNA were checked by Agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer (Sambrook *et al.*, 2001) was used for submarine gel electrophoresis. Ethidium bromide (1%) was added @ 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30 minute at room temperature. DNA was visualized under UV using UV transilluminator. The DNA was further used for PCR.
3.3.3 Polymerase Chain Reaction (PCR) Amplification of Internal Transcribed Spacer (ITS)

ITS region fragment was amplified by PCR from genomic DNA using internal transcribed spacer (ITS) specific universal primers: IF and IR (Vilgalys et al., 1990; White et al., 1990). Details of Primer Sequence

IF 5’TCCGTAGGTGAACCTGCGG3’
IR 5’TCCTCCGCTTATTGATATGC3’

PCR was carried out in a final reaction volume of 25 µl in 500 µl capacity thin wall PCR tubes in Labnet Thermal Cycler. Composition of reaction mixture for PCR is given in Table 3.3.1. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in Table 3.3.2.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-RNase free water</td>
<td>7.50 µl</td>
<td>--</td>
</tr>
<tr>
<td>2X PCR master mix (MBI Fermentas)</td>
<td>12.50 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Forward Primer (10 pmole/µl)</td>
<td>1.00 µl</td>
<td>10 pmole</td>
</tr>
<tr>
<td>Reverse Primer (10 pmole/µl)</td>
<td>1.00 µl</td>
<td>10 pmole</td>
</tr>
<tr>
<td>Diluted DNA (30ng/µl)</td>
<td>3.0 µl</td>
<td>---</td>
</tr>
<tr>
<td><strong>GRAND TOTAL</strong></td>
<td><strong>25.00 µl</strong></td>
<td><strong>--</strong></td>
</tr>
</tbody>
</table>
Table 3.3.2 Steps and conditions of thermal cycling for PCR (ITS)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2 minute</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Final Denaturation</td>
<td>94°C</td>
<td>30 Second</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Annealing</td>
<td>48°C</td>
<td>30 Second</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td>Extension</td>
<td>72°C</td>
<td>90 Second</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Final Extension</td>
<td>72°C</td>
<td>10 minute</td>
<td>1</td>
</tr>
</tbody>
</table>

3.3.4 Polymerase Chain Reaction of Large Subunit (LSU) Region (28S rDNA)

28S gene fragment was amplified by PCR from genomic DNA using 28S or large subunit (LSU) gene universal primers: DF and DR (Vilgalys et al., 1990; White et al., 1990).

Details of 28S Universal Primer Sequence

| DF       | ACCCGCTGAACCTTAAGC |
| DR       | GGTCGCTGGTTTCAAGACGG |

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tubes in Labnet Thermal Cycler. Composition of reaction mixture for PCR is given in Table 3.3.3. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in Table 3.3.4.
Table 3.3.3 Composition of reaction mixture for PCR (LSU)

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-RNase free water</td>
<td>7.50 µl</td>
<td>--</td>
</tr>
<tr>
<td>2X PCR master mix (MBI Fermentas)</td>
<td>12.50 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Forward Primer (10 pmole/µl)</td>
<td>1.00 µl</td>
<td>10 pmole</td>
</tr>
<tr>
<td>Reverse Primer (10 pmole/µl)</td>
<td>1.00 µl</td>
<td>10 pmole</td>
</tr>
<tr>
<td>Diluted DNA (30ng/µl)</td>
<td>3.0 µl</td>
<td>---</td>
</tr>
<tr>
<td><strong>GRAND TOTAL</strong></td>
<td>25.00 µl</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 3.3.4 Steps and conditions of thermal cycling for PCR (LSU)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>2 minute</td>
<td>1</td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>94°C</td>
<td>30 Second</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>48°C</td>
<td>30 Second</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 Second</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 minute</td>
<td>1</td>
</tr>
</tbody>
</table>
3.3.5 Visualization of PCR Products

To confirm the targeted PCR amplification, PCR products from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % Agarose gel containing Ethidium bromide (1 per cent solution @10 µl/100 ml) at constant 5V/cm for 30 minute in 0.5 X TAE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

3.3.6 Sequencing

Amplified DNA samples were sent to the Xcelris Labs, Ahmadabad (Gujarat), India for sequencing.

3.3.7 In silico Analysis

1. Both ends of each the sequence were verified with the chromatogram file and edited if required. The sequence was converted into FASTA format and saved in notepad.

2. The amplified gene sequences of internal transcribed spacer (ITS) and large subunit (LSU) were used to carry out BLAST (Basic local Alignment Search tool) with nr database of NCBI (National Centre for Biotechnology Information) GenBank using MEGA BLAST algorithm.

3. Sequences of type strains of different Cryptococcus species were selected and aligned using multiple alignment software program ClustalW (MEGA5 Tool).

4. Phylogenetic analysis was done by Neighbor-Joining method. All the phylogenetic analysis was conducted in MEGA5 (Saitou et al., 1987; Tamura et al., 2004; Tamura et al., 2011).

5. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985; Tamura et al., 2004).
3.4 Phenotypic Comparison of Clinical and Environmental Cryptococcus Strains

A total of 41 strains belonged to different sources i.e. hospitals, culture collections and environmental sources were studied for phenotypic characters and possible virulence factors which are listed below:

Clinical Strains

- Sixteen clinical strains of Cryptococcus (15 C. neoformans and 1 C. gattii isolate) used in this study were procured from National Culture Collection of Pathogenic Fungi (NCCPF), Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (3.2.1).

- Two clinical strains of C. neoformans were procured from Indira Gandhi Medical College (IGMC), Shimla (Himachal Pradesh), India (3.2.1).

Standard Strains

- Seven ATCC (American Type Culture Collection) strains of Cryptococcus were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune and Institute of Microbial Technology (IMTECH), Chandigarh, India (3.2.2).

Environmental Isolates

- After molecular characterization, sixteen environmental isolates of C. albidosimilis, C. diffluens, C. flavescens and C. terrestris were used for phenotypic characterization.

All the strains and isolates were maintained on Sabouraud’s dextrose agar (HiMedia, Mumbai) slants and preserved in 10 % glycerol at -20°C. Sub culturing was done regularly to maintain fresh cultures for the experiments. Different types of methods were used in the phenotypic characterization of environmental isolates and clinical strains which are following:
1. **Morphological Characterization**

   Colony phenotypes were analyzed by plating cells from a single colony of each strain onto fresh Sabouraud agar plates. The plates were incubated at 30°C for 7 days; colonies were examined in size, shape and texture (Franzot *et al.*, 1998; Chakrabarti *et al.*, 1998).

**A.) Growth:**
1. Rapid growers - which grew within 2-5 days
2. Intermediate growers - which took 6-10 days for growth
3. Slow growers – which grew in 2-3 weeks

**B.) Surface:**
1. Flat
2. Centre Raised (Dome shaped)

**C.) Texture:**
1. Yeast like
2. Powdery
3. Cottony
4. Velvety
5. Granular
6. Glabrous
7. Mucoid

**D.) Pigmentation**
1. On the surface of colony
2. On the reverse of colony
2. **Enzymatic Activity Profile**

Presence of the four different enzymes in different *Cryptococcus* species were checked by following methods:

A. DNase Activity Test  
B. Hemolysis Activity Test  
C. Phospholipase Activity Test  
D. Protease Activity Test

### A. DNase Activity Test

The method used for detecting extracellular DNase production by yeasts was similar to the procedure used for detecting DNase production by Staphylococci (Cazin *et al*., 1969).

**Method**

1. Plates of DNase test agar supplemented with methyl green were prepared.  
2. By use of straight wire inoculating loop, the organism to be tested was inoculated on the medium.  
3. Incubated the plates at 30°C for 7 days.  
4. After that time, clear or yellowish zones were observed around the colonies.  
5. Assays were repeated at least twice for each strain.  
6. Strains that depolymerized DNA contained in the medium exhibited clear zones surrounding the colonies.

### B. Hemolysis Activity Test

This test is useful in detection of α (alpha) and β (beta) hemolysis activity of an organism. On blood agar, colonies surrounded by greenish zone indicates the alpha hemolysis and clear zone around the colonies indicates beta hemolysis (Linares *et al*., 2007).

**Method**

1. The strains were cultured on Sabouraud dextrose agar for 48 h at 30°C.
2. By use of straight wire inoculating loop, the organism to be tested was spotted onto Sabouraud dextrose agar supplemented with 3% glucose and 7% fresh sheep blood.

3. Plates were incubated at 30°C for 48 h. The presence of a distinctive translucent halo around the inoculum site indicated positive result. 4. The experiments were performed in the duplicate.

C. **Phospholipase Activity Test**

Phospholipase is an extracellular enzyme. In this method, egg yolk digested by phospholipase produces precipitation around colonies (Vidotto *et al.*, 2005). The phospholipase activity was expressed as Pz value (a/b) as described by Price *et al* (1982).

**Method**

1. Sabouraud dextrose agar medium supplemented with calcium chloride, sodium chloride and egg yolk emulsion was inoculated with yeast culture.

2. Incubated at 30°C.

3. Read the results after 96 h.

4. Precipitation zone around the colonies indicated positive result.

5. The ratio of the colony diameter (a) to the total diameter of colony plus precipitation zone (b) was measured as phospholipase activity.

6. The phospholipase activity was expressed as Pz value (a/b) as described by Price *et al* (1982).

- According to the Price *et al* (1982), low Pz values mean high phospholipase production and, inversely, high Pz values indicate low production.

- High Pz group between 1 and 0.700 (+)

- Moderate Pz group between 0.699 and 0.400 (++)

- Low Pz group between 0.399 and 0.100 (+++)

D. **Protease Activity Test**

Determination of protease production was performed by using agar plates containing bovine serum albumin (BSA) according to Aoki *et al* (1994).
**Method**

1. The protein solution was sterilized by filtration and mixed with melted agar.
2. Medium was poured into each Petri dish.
3. 10 μl of cells suspended in 2.5 ml of sterile physiological saline solution were inoculated in each Petri dish (four inoculum were placed in each Petri dish).
4. Incubated at 30°C for 7 days.
5. The precipitation zone around the colonies was considered as positive protease activity.

**3. Pigment Production**

Different tests for pigment production were performed which are the following:

A. Creatinine Dextrose Bromothymol Blue Thymine (CDBT) agar test
B. Littman oxgall agar test
C. Caffeic acid-ferric citrate agar test
D. *Cryptococcus* Differential Agar (CDA) test
E. Rose Bengal agar test
F. Melanization assay
G. L-DOPA drop test

**A. CDBT Test**

It is very useful test in variety differentiation of *Cryptococcus*. It differentiates *C neoformans* var. *neoformans* from *C. neoformans* var. *grubii* and *C. gattii*. Variety *neoformans* produced yellow colored colonies on medium but var. *grubii* was unable to grow on the medium. *C. gattii* was able to change the CDBT medium color into blue (Irokanulo et al., 1994).

**Method**

1. The CDBT medium was prepared.
2. After sterilization, the medium was poured into Petri plates.
3. The *Cryptococcus* cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for 5-7 days.
5. Observed the growth and color of the colonies.

**B. Littman Oxgall Agar Test**

This medium was developed by Littman. The superiority of Littman medium was attributed to better suppression of bacterial and saprophytic fungal growth and to a distinct blue-grey color of *C. neoformans* which facilitated its colonial identification (Littman, 1947).

**Method**

1. Littman oxgall agar medium was prepared.
2. After sterilization, the medium was poured into Petri plates.
3. The *Cryptococcus* cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for 5-7 days.
5. Observed the growth and color of the colonies.

**C. Caffeic Acid-Ferric Citrate Agar Test**

Identification is based on the development of dark brown pigmented colonies by *C. neoformans* on the caffeic agar medium. Other *Cryptococcus* species and common clinically isolated yeasts do not develop the brown color. Only *C. neoformans* is able to produce dark brown colonies (Hopfer et al., 1975a).

**Method**

1. The Caffeic acid-ferric citrate agar medium was prepared.
2. After sterilization, the medium was poured into Petri plates.
3. The *Cryptococcus* cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for 7 days.
5. Observed the growth and color of the colonies.
D. CDA Test

*Cryptococcus* differential agar (CDA) is recommended for the differentiation of *Cryptococcus* species. Colonies of different species developed a variety of blue color. Few species were able to produce brown diffusible pigments on this medium (Chaskes et al., 2008).

**Method**

1. *Cryptococcus* differential agar medium was prepared.
2. The medium was poured into Petri plates.
3. The *Cryptococcus* cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for 7 days.
5. Observed the growth and color of the colonies.

E. Rose Bengal Agar Test

Rose Bengal agar is a selective and commonly used medium for the enumeration of fungi. Rose Bengal is included as a selective agent to inhibit bacterial growth. It is incorporated in the cells of yeasts and molds, turning these colonies pink. In the present study, it was used for checking the pigmentation of colonies of different species (Findley et al., 2009).

**Method**

1. Rose Bengal agar medium was prepared.
2. The medium was poured into Petri plates.
3. The *Cryptococcus* cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for 5-7 days.
5. Observed the growth and color of the colonies.

F. Melanization Assay

*Cryptococcus neoformans* produces melanin in the presence of various substrates, including the L enantiomer of 3, 4-dihydroxyphenylalanine (DOPA). The enzyme laccase catalyses the formation of melanin by oxidizing L-DOPA to brown or black pigment in the yeast cell wall.
Different Cryptococcus species were checked to produce pigment from L-DOPA (Eisenman et al., 2007).

**Method**

1. Chemically defined minimal medium with 1 mM L-DOPA was prepared.
2. The medium was poured into Petri plates.
3. The Cryptococcus cultures were inoculated with the help of inoculating loop.
4. Incubated the plates at 30°C for several days.
5. Examined the plates daily to monitor growth and pigment production.

**G. L-DOPA Drop Test**

This method was developed by Chaskes et al. (1981) to check the rapid melanization of Cryptococcus species.

**Method**

1. Each strain was streaked onto Sabouraud dextrose agar medium.
2. Incubated at 30°C for several days.
3. A toothpick was used to transfer a thick patch of cells to starvation medium.
4. Incubated at 30°C for 2 days.
5. Two drops of a solution of L-DOPA (0.3 %, w/v) were pipetted directly onto the colonies. Pigment formation was monitored visually over a time of 5–60 minute.

**4. Antifungal Susceptibility Testing**

The main purpose of antifungal testing was to know the antifungal susceptibility pattern of different Cryptococcus species (CLSI document M44-A2, 2009).

**Preparation of Inoculum:**

1. Inoculum was prepared by picking five distinct colonies of approximately 1 mm from 24 hours old culture grown on Sabouraud dextrose agar. Colonies were suspended in 5 ml of sterile 0.85% saline.
2. Vortexed the resulting suspension and adjusted the turbidity to yield $1 \times 10^6 - 5 \times 10^6$ cells/ml (i.e. 0.5 McFarland standard).

**Test Procedure:**

1. Plates with Muller Hinton Agar w/ Methylene Blue Dye were prepared for carrying out antifungal susceptibility testing.

2. A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum (turbidity so adjusted, as to obtained semi confluent growth on the Petri plate) and the soaked swab firmly was rotated against the upper inside wall of the tube to remove excess fluid. The entire agar surface of the plate was streaked with the swab three times. The inoculum was allowed to dry for 5-15 minutes with lid in place.

3. The discs were applied using aseptic technique.

4. The plates were inverted and incubated at 30°C within 15 minutes after the discs were applied.

5. Examined each plate after 24-48 h of incubation. Read at 72 h only when insufficient growth was observed after 24-48 h incubation.

6. Recorded the diameters of the zones to the nearest millimetre using a calibrated zone scale.

**5. Inhibition of Urease Activity**

In this method, the differential effect of EDTA (Ethylene Diamine Tetra Acetic Acid) was checked on different *Cryptococcus* species (Roberts, 1985; Kwon-Chung *et al.*, 1987).

**Method**

1. The strains were grown on Sabouraud dextrose agar slants for 48 h at 30°C.
2. A loopful of cells obtained from the 48 h slant cultures was streaked on yeast extract-peptone-glucose agar (YEPG) with 100 µM EDTA (Ethylene Diamine Tetra Acetic Acid) (YEPGE).

3. The cultures were then incubated for 48 h at 30°C and tested for urease test.

4. The rapid urea broth (RUB) was prepared in 2x concentration with urea.

5. Then filter sterilized the broth, the pH of this RUB was 6.8.

6. To test the urease activity, a loopful of cultures grown for 48 h at 30°C on YEPGE agar was suspended in 2 ml of sterile distilled water.

7. The cell suspension was vortexed.

8. 1 ml of the suspension was added to 1 ml of 2 x RUB.

9. The RUB and the cell suspension were kept in an ice bath until they were combined.

10. The RUB culture was then placed in a 30°C shaking water bath.

11. The tube was read every hour for 4 h.

12. Magenta or pink color was considered as positive reaction and orange yellow color was considered as negative reaction.