Studies on Interaction of Human Lung Epithelial Cells with Conidia of *Aspergillus terreus*

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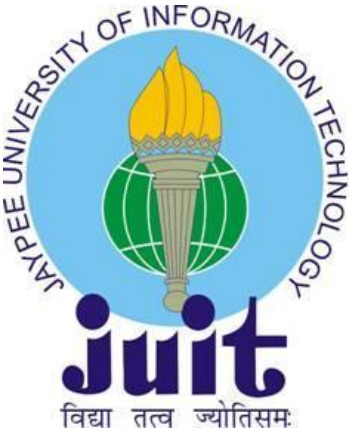

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WAKNAGHAT

CERTIFICATE OF ORIGINALITY

This is to certify that the work titled **"Studies on Interaction of Human Lung Epithelial Cells with Conidia of *Aspergillus terreus*"** submitted by **"Akshay Malhotra"** in partial fulfilment for the award of degree of 2 year Master's Degree program of Biotechnology of Jaypee University of Information Technology, Wakhnaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or Institute for the award of this or any other degree or diploma.

Signature of Supervisor ……

Name of Supervisor Dr. Jata Shankar

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Date …….

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Akshay Malhotra

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SUMMARY

Aspergillus can cause a wide range of human associated diseases depending on the immune status of the host. In patients with an impaired lung function such as asthmatic and cystic fibrosis, aspergillus can allow an onset of allergic bronchopulmonary aspergillosis, which has been recognized as an allergic hypersensitive response to fungal components. The infection associated with *Aspergillus terreus* is not so common as compared to other *Aspergillus* species to cause opportunistic infections in animals and humans. However, there is a sudden increase in the no of cases suffering from *A. terreus* infection and for this reason it is has been classified as a source of fungal infection. As an opportunistic pathogen, it is able to cause both superficial and systemic infections. Inhaling fungal spores, allow them to reach respiratory tract, causing the typical respiratory infection.

It is an important pathogen because of relative amphotericin B resistance and the potential to have rapid and progressive invasive infections in immunocompromised patients. And so, it is important to understand the host (human)-pathogen (*A. terreus)* which is being performed in this particular research. The ex-vivo studies defines the understanding of dual transcriptomics that how the interaction happens between the host-pathogen system and also the pathways and mechanisms are elucidated, that contributes to a better understanding.

The host-pathogen interactions, cell viability, physical and chemical changes in the course of infection with respect to *Aspergillus terreus* (pathogen) and human epithelial cell lines (host), is important to understand and develop a research associated treatment for such sort of infections to avoid the invasive mode of such organisms which can be responsible developing different sets of diseases and disorders in human and animal host, that may not be curable.

Akshay Malhotra Dr. Jata Shankar

Date: Date:

Chapter 1 – Introduction

Fungi is a well-known organism that was once classified under plants, but after knowing the biological role of fungi and different species identified, it has now being classified as kingdom fungi (Bruns 2006). Still fungi having a complex genome, capable of undergoing post transcriptional modification and having a core system of performing protein-protein interactions, it has also considered as a Eukaryotic organism. The fungi with unicellular system are called as yeast and molds whereas the multicellular fungi are capable of producing fruiting forms such as mushrooms. A fungus is differentiated from other micro-organisms on the basis of their cell wall structure. Unlike the cell wall of plants, bacteria and some protists the fungal cell wall contains chitin. But they are very much similar to animals and are generally considered as heterotrophs as they acquire their food by absorbing certain dissolved biomolecules in the environment and by secreting certain digestive enzymes they make their food accessible to them (Shoji, Arioka et al. 2006). Fungi is mobile, except for their spores that any means or source of transportation either air, water or dust, only Some spores possess flagella. Most fungi grow as hyphae, that is a cylindrical, thread-like structure with a diameter of 2–10 µm and even can grow up to a certain height (centimetres). Hyphae are capable of growing at apices (tips); new hyphae are typically formed by emergence of new tips in the presence of pre-existing hyphae as these are the hyphae that give rise to new hyphae and name the process as branching. The combination of apical growth and branching/forking leads to the formation of a mycelium, which has been known as an interconnected network of hyphae. Fungal mycelia can become visible to the naked eye, generally seen on different surfaces and distinct substrates, such as damp walls and spoiled food, where they are commonly called mold. Mycelium is capable of growing on a solid agar media in petri dishes under controlled laboratory conditions. Such cells grown on

Fig 1: Different forms of Fungi

petri-plate are known as colonies. These colonies contains growth shapes and colors (due to spores or pigmentation) that can be utilized as diagnostic characteristic in order to identify certain species or groups (Harris 2008).

The complexity in fungal reproduction mechanism is, reflects the differences in lifestyles and genetic makeup within the distinct kingdom of organisms. It is estimated that a third of all fungi reproduce using more than one method; for example, a particular fungi may reproduce in two well-differentiated stages within the life cycle of a species, one known as anamorph and second is teleomorph. Epigenetic conditions like Environmental factors trigger genetically determined developmental states that results in the formation of certain specialized structures involved in either sexual or asexual reproduction. These structures aid reproduction is capable of transporting by spores or spore-containing propagules. Vegetative spores (conidia) commence asexual reproduction. Mycelial fragmentation happens to be, after a separation of mycelium in such a way that it is fragmented into pieces. Mycelial fragmentation and vegetative spores maintain clonal populations that modify itself and stay associated with a specific niche, and allows more rapid dispersal than sexual reproduction (Heitman 2006). It varies with respect to many parameters associated with sexual reproduction in animals as well as in plants. Different forms of fungal groups exists and can be utilized to differentiate species by morphological variations in sexual physiology and reproductive mechanisms (Guarro, GeneJ et al. 1999), (Taylor, Jacobson et al. 2000). Mating experiments amongst fungal samples may contribute in identifying species on the basis of biological species concepts. The essential fungal groups have initially had rejected based on the morphology of their sexual structures and spores; like, spores containing structures, basidia and asci can be used in the identification of basidiomycetes and ascomyetes. There are certain fungal species that may facilitate a reproductive access between individuals of opposite mating type, whereas others can interact

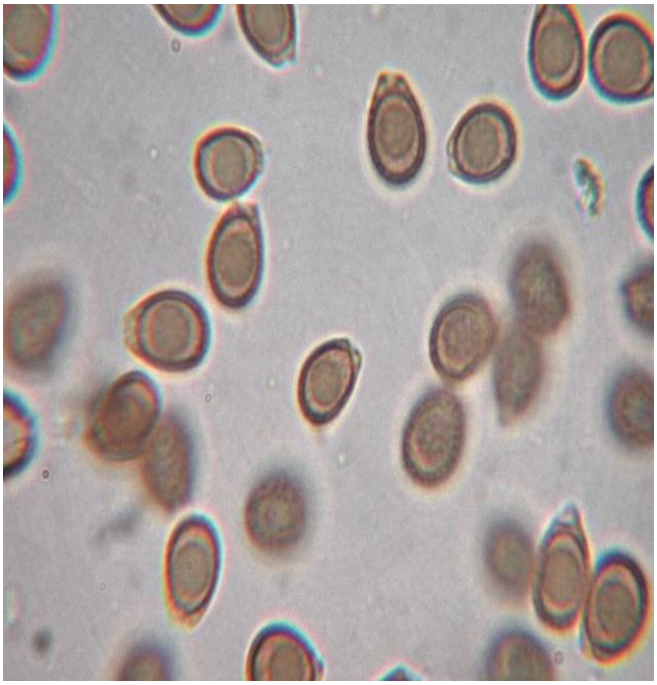


Fig 2: Fungal spores

and sexually reproduce with another individual or may be with itself (Metzenberg and Glass 1990). In ascomycetes, dikaryotic hyphae (the spore-bearing tissue layer) form a characteristic *hook* at the hyphal septum. During cell division, formation of the hook ensures proper distribution of the newly divided nuclei into the apical and basal hyphal compartments. An ascus is then formed, in which karyogamy (nuclear fusion) occurs. Asci are embedded in an ascocarp, or fruiting body. Karyogamy in the asci is followed immediately by meiosis and the production of ascospores. After dispersal, the ascospores may germinate and form a new haploid mycelium. Both asexual and sexual spores and sporangiospores are often actively dispersed by forcible ejection from their reproductive structures. This ejection ensures exit of the spores from the reproductive structures as well as traveling through the air over long distances. Specialized mechanical and physiological mechanisms, as well as spore surface structures (such as hydrophobins), enable efficient

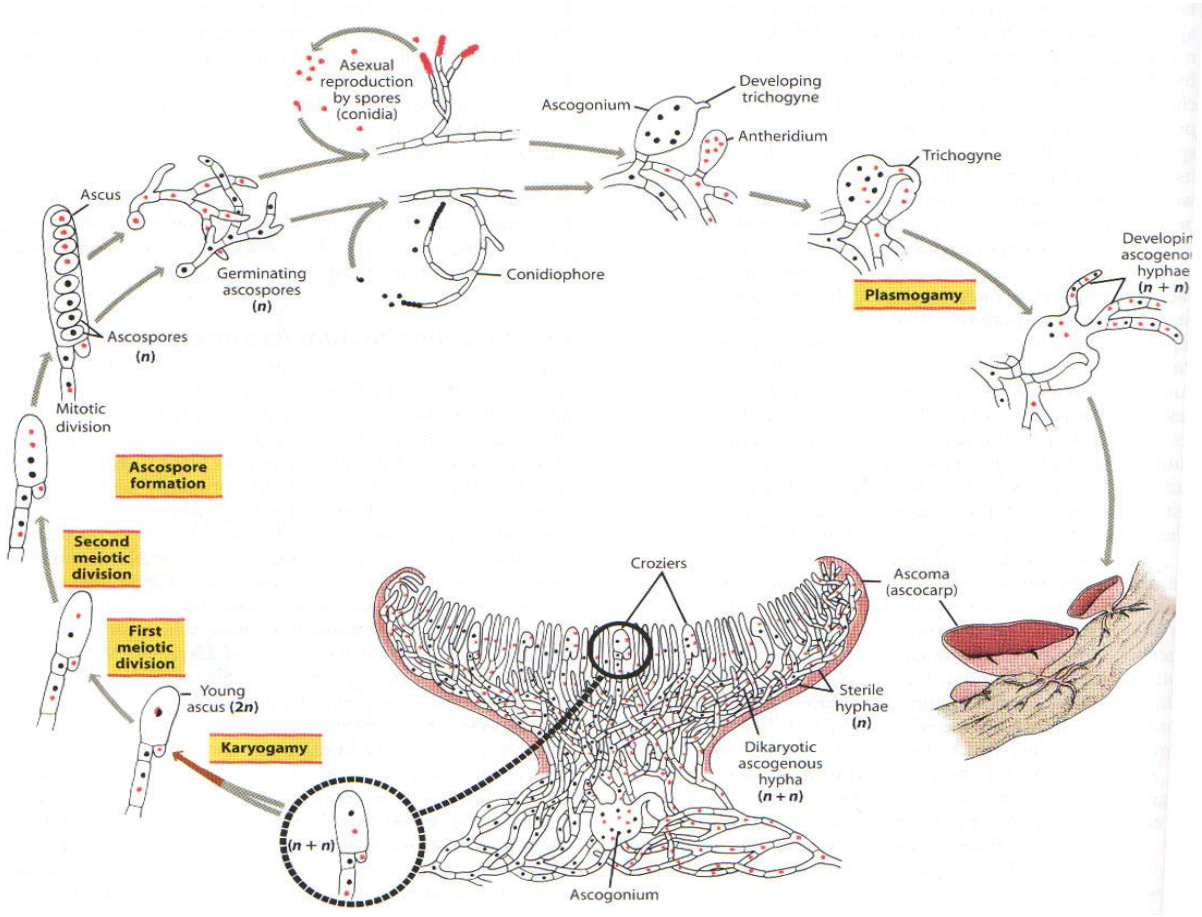


Fig 3: Life cycle Ascomycota

spore ejection (Linder, Szilvay et al. 2005). The forcible discharge of single spores termed ballistospores involves formation of a small drop of water (Buller's drop), which upon

contact with the spore leads to its projectile release with an initial acceleration of more than 10,000g (Pringle, Patek et al. 2005) the net result is that the spore is ejected 0.01–0.02 cm, sufficient distance for it to fall through the gills or pores into the air below. In contrast to plants and animals, the early fossil record of the fungi is meager. Factors that likely contribute to the under-representation of fungal species among fossils include the nature of fungal fruiting bodies, which are soft, fleshy, and easily degradable tissues and the microscopic dimensions of most fungal structures, which therefore are not readily evident. Fungal fossils are difficult to distinguish from those of other microbes, and are most easily identified when they resemble extant fungi. The Ascomycota, commonly known as sac fungi or ascomycetes, constitute the largest taxonomic group within the Eumycota. These fungi form meiotic spores called ascospores, which are enclosed in a special sac-like structure called an ascus. This phylum includesmorels, a few mushrooms and truffles, unicellular yeasts (e.g., of the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Candida*), and many filamentous fungi living as saprotrophs, parasites, and mutualistic symbionts. Prominent and important genera of filamentous ascomycetes include *Aspergillus*, *Penicillium*, *Fusarium*, and *Claviceps*. Many ascomycete species have only been observed undergoing asexual reproduction (called anamorphic species), but analysis of molecular data has often been able to identify their closest teleomorphsin the Ascomycota (Samuels 2006). Because the products of meiosis are retained within the sac-like ascus, ascomycetes have been used for elucidating principles of genetics and heredity (e.g., *Neurospora crassa*) (Radford and Parish 1997).

Aspergillus terreus, also known as *Aspergillus terrestris*, is a fungus (mold) found worldwide in soil. Although thought to be strictly asexual until recently, *A. terreus* is now known to be capable of sexual reproduction (Arabatzis and Velegraki 2013). This saprotrophic fungus is prevalent in warmer climates such as tropical and subtropical regions. Aside from being located in soil, *A. terreus* has also been found in habitats such as decomposing vegetation and dust. *A. terreus* can cause opportunistic infection in people with deficient immune systems. It is relatively resistant to amphotericin B, a common antifungal drug. *A. terreus* is brownish in colour and gets darker as it ages on culture media. On Czapek or malt extract agar (MEA) medium at 25 °C, colonies have the conditions to grow rapidly and have smooth-like walls. In some cases, they are able to become floccose, achieving hair-like soft tufts. Colonies on malt extract agar grow faster and sporulate more densely than on many other media.

A. terreus has conidial heads that are compact, biseriate, and densely columnar, reaching 500 × 30–50 µm in diameter. Conidiophores of *A. terreus* are smooth and hyaline up to 100–250 × 4–6 µm in diameter. The conidia of *A. terreus* are small, about 2 µm in diameter, globose-shaped, smooth-walled, and can vary from light yellow to hyaline. Unique to this species is the production of aleurioconidia, asexual spores produced directly on the hyphae that are larger than the phialoconidia (e.g. 6–7 µm in diameter). This structure might be influential in the way *A. terreus* presents itself clinically as it can induce elevated inflammatory responses. This fungus is readily distinguished from the other species of *Aspergillus* by its cinnamon-brown colony colouration and its production of aleurioconidia. *A. terreus* is a thermotolerant species since it has optimal growth in temperatures between 35–40 °C (95–104 °F), and maximum growth within 45–48 °C (113–118 °F).

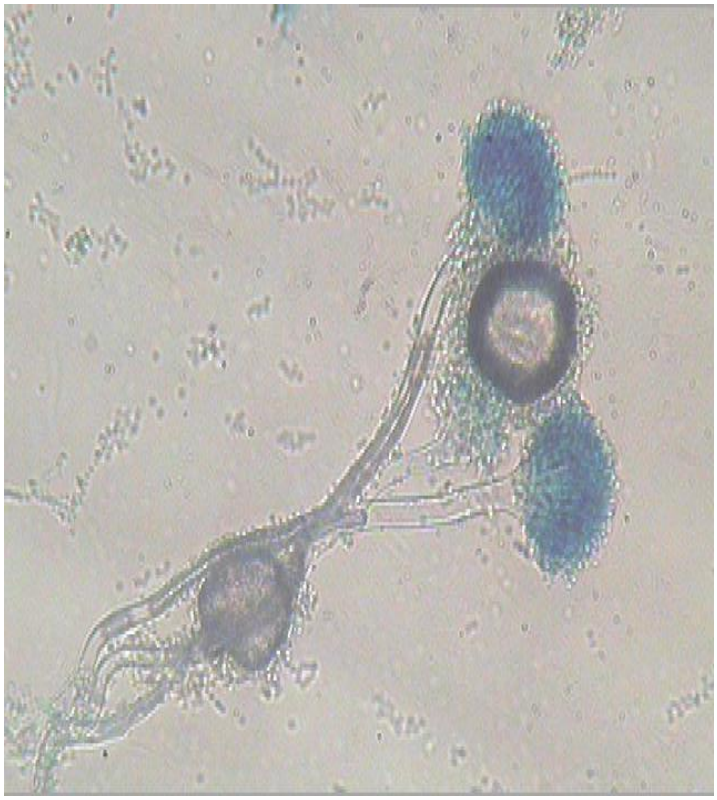


Fig 4: *Aspergillus terreus*

A. terreus, like other species of *Aspergillus*, produces spores that disperse efficiently in the air over a range of distances (Hedayati, Pasqualotto et al. 2007). The morphology of this fungus provides an accessible way for spores to disperse globally in air current. Elevation of the sporulating head atop a long stalk above the growing surface may facilitate spore dispersal through the air. Normally, spores in fungi are discharged into still air, but in *A. terreus*, it resolves this problem with a long stalk and it allows the spores to discharge into air currents like wind. In turn, *A. terreus* has a better chance to disperse its spores amongst a vast geography which subsequently explains for the worldwide prevalence of the fungus. Despite *A. terreus* being found worldwide in warm, arable soil, it has been located in many different habitats such as compost and dust. Eventually, the dispersed fungal spores come into contact with either liquid or solid material and settle onto it, but only when the conditions are right do the spores germinate. One of the conditions important to the fungus is the level of moisture present in

the material. The lowest water activity (A_w) capable of supporting growth of the fungus has been reported as 0.78. Tolerance of relatively low A_w conditions may explain, in part, the ubiquitous nature of this species given its ability to grow is a wide array of places. The soil of potted plants is one common habitat supporting the growth of *A. terreus*, and colonized soils may be important reservoirs of nosocomial infection. Other habitats include cotton, grains, and decomposing vegetation. *A. terreus* contains 30-35 Mbp and roughly 10,000 protein-coding genes.

Genome Assembly	
Genome Assembly size (Mbp}	29.33
Sequencing read coverage depth	1
# of contigs	267
# of scaffolds	26
# of scaffolds >= 2Kbp	26
Scaffold N50	7
Scaffold L50 (Mbp)	1.91
# of gaps	241
% of scaffold length in gaps	0.5%
Three largest Scaffolds (Mbp)	2.75, 2.56, 2.49

Table 1: *Aspergillus terreus* genome

A. terreus produces a number of secondary metabolites and mycotoxins, including territrem A, citreoviridin, citrinin, gliotoxin, patulin, terrein, terreic acid, and terretonin. The fungus also produces a secondary metabolite called lovastatin, a potent drug for lowering blood cholesterol levels in humans and animals. It is an inhibitor to one of the enzymes responsible in the catalyzing steps in cholesterol biosynthesis. Lovastatin is typically produced within fermentation conditions of the fungus. Fast growth of filamentous hyphae in the species *A. terreus* can result in low lovastatin production. To increase the production of this metabolite *A. terreus* requires important nutrients during fermentation. In this case, carbon and nitrogen are very important in fermentation productivity which in turn also increases the biomass of the metabolite lovastatin (Mukhtar, Ijaz et al. 2014). *A. terreus* strains use glycerol and glucose as their best carbon sources for lovastatin production (Patil, Krishnan et al. 2011).

A. terreus is not as common as other *Aspergillus* species to cause opportunistic infections in animals and humans. However, the incidence of *A. terreus* infection is increasing more rapidly than any other *Aspergillus* and for this reason it is considered an emerging agent of infection.

As an opportunistic pathogen, it is able to cause both systemic and superficial infections. Inhalation of fungal spores, which travel down along the respiratory tract, causes the typical respiratory infection. Other infections could also occur, such as onychomycosis and otomycosis (Fernandez, Rojas et al. 2013). *A. terreus* has the ability to cause serious effects in immuno-compromised patients who lack specific immune cells. Specifically, prolonged neutropenia predisposes humans and animals to this fungal disease (Lass-Florl, Griff et al. 2005). *A. terreus* has no adaptation in terms of changing its physical structure when infecting a human or animal host. The fungus continues to grow as the characteristic hyphae filaments. Other pathogenic fungi usually switch over to a different growth stage, mycelia-to-yeast conversion, to best suit their new environment. This process does not occur in *A.terreus* (Lass-Florl, Griff et al. 2005*)*.

Treatment of *A. terreus* is clinically challenging due to its nearly complete resistance to amphotericin B, the fallback drug for serious fungal infections. However, some newer drugs, such as voriconazole, posaconazole, and caspofungin, have shown promise in treating this agent.The laboratory identification of *A. terreus* from clinical specimens can also be difficult. Currently, no rapid immunological tests are availablefor this species, and its correct identification remains dependent on culture. *A. terreus* strains have a tendency to mutate while in the animal host, resulting in a substantial reduction or loss of characteristic spore heads in primary culture. Such strains continue to produce small aleuroconidia similar in appearance to the aleurioconidia of *Blastomyces dermatitidis*. In one study, nearly a third of *A. terreus* infections in hospitals were found to be associated with the presence of potted plants. Elimination of potted plants in the rooms of immunodeficient patients may have a role in prevention of illness. *A. terreus* has also been described in many studies as common to the hospital setting because of outside hospital construction and renovations. The amount of soil and debris reintroduced into the air is capable of travelling through the air and infecting immunosuppressed patients. A simple way to take preventive action is to provide good air filtration and ventilation throughout the hospital rooms. Elimination of inoculum is the key to the prevention of nosocomial infection by *A. terreus* (Flynn, Williams et al. 1993*)*.

Aspergillus is a mould which may lead to a variety of infectious, allergic diseases depending on the host's immune status or pulmonary structure. Invasive pulmonary aspergillosis occurs primarily in patients with severe immunodeficiency. The significance of this infection has dramatically increased with growing numbers of patients with impaired immune state associated with the management of malignancy, organ transplantation, autoimmune and inflammatory conditions; critically ill patients and those with chronic obstructive pulmonary disease appear to be at an increased risk. The introduction of new non-invasive tests, combined with more effective and better-tolerated antifungal agents, has resulted in lower mortality rates associated with this infection. Chronic necrotising aspergillosis is a locally invasive disease described in patients with chronic lung disease or mild immunodeficiency. Aspergilloma is usually found in patients with previously formed cavities in the lung, whereas allergic bronchopulmonary aspergillosis, a hypersensitivity reaction to *Aspergillus* antigens, is generally seen in patients with atopy, asthma or cystic fibrosis (Shah and Panjabi 2016).

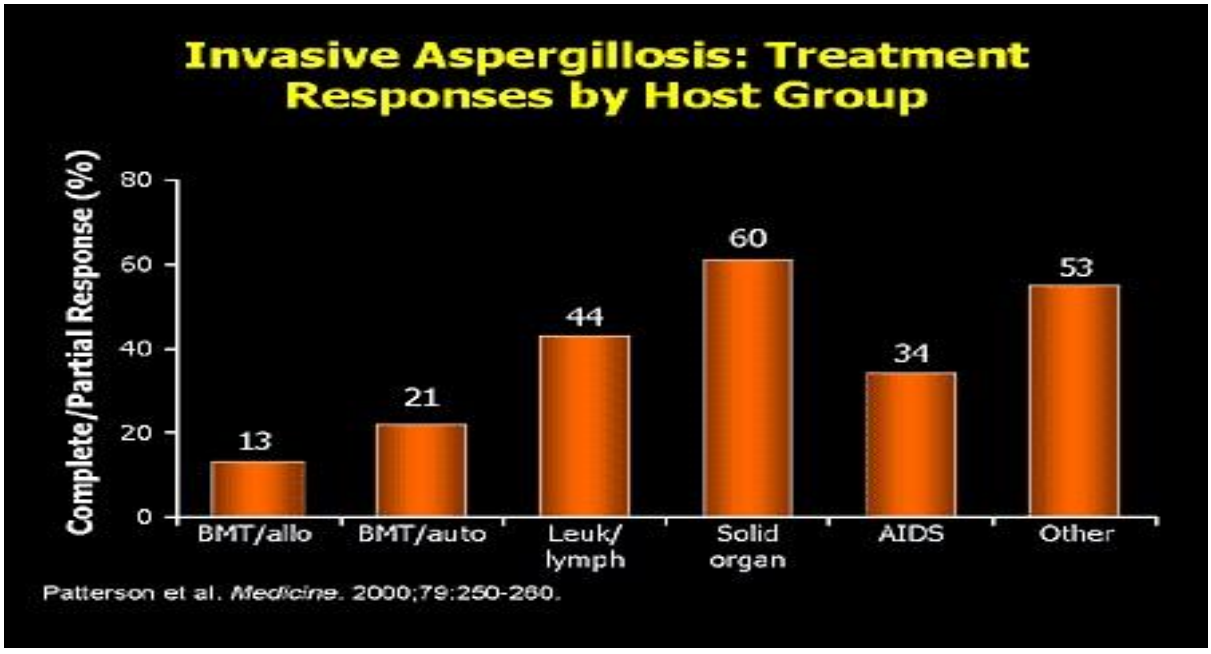
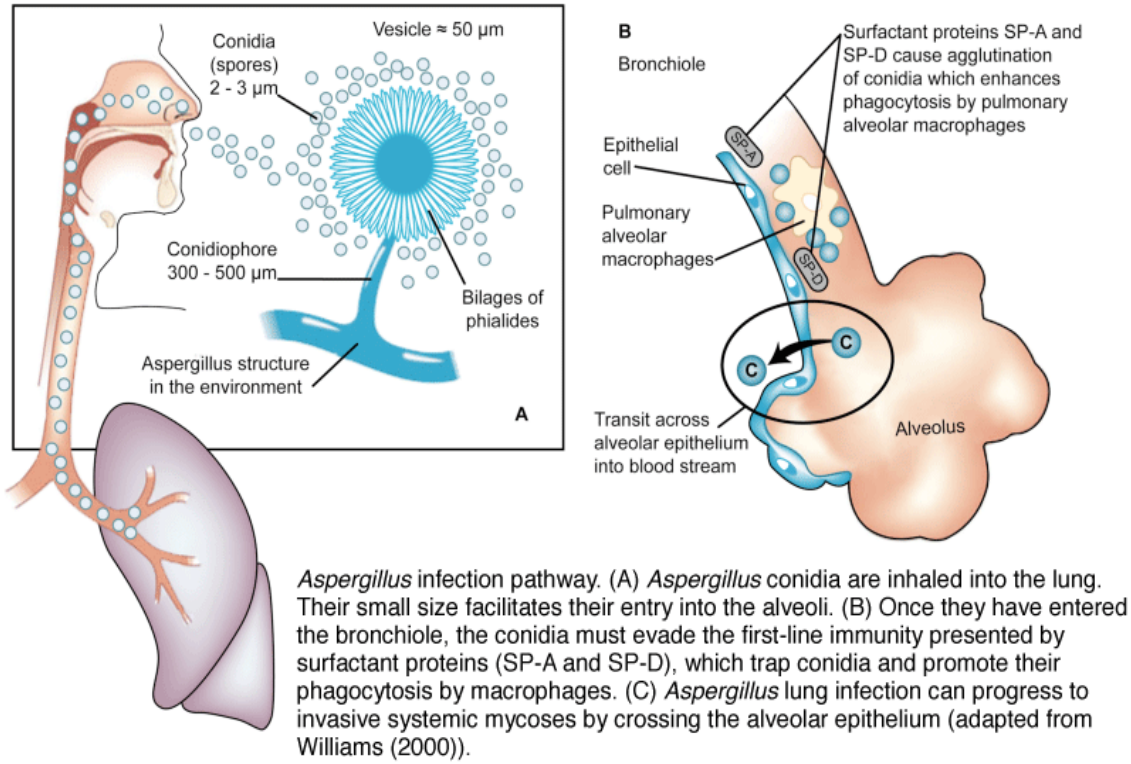


Fig 5: Invasive Aspergillosis: Treatment Responses by Host group

Invasive aspergillosis caused by Aspergillus species (Aspergillus fumigatus, A. flavus, and A. terreus) is life-threatening infections in immunocompromised patients. Understanding the innate and adaptive immune response particularly T-helper cells (TH-cells) against these Aspergillus species and how the different sub-set of TH-cells are regulated by differentiating

cytokines at primary target organ site like lung, kidney and brain is of great significance to human health. This review focuses on presentation of Aspergillus through Antigen presenting cells (APCs) to the naive CD4+ T-cells in the host. The production of differentiating/effector cytokines that activate following TH-cells, e.g., TH1, TH2, TH9, and TH17 has been reported in association or alone in allergic or invasive aspergillosis. Chemokines (CXCL1, CXCL2, CCL1, and CCL20) and their receptors associated to these TH-cells have also been observed in invasive aspergillosis. Cytokines are important in the development of CD4+ TH-cells. Understanding of trafficking of CD4+ TH-cells and their regulation through differentiating/effector cytokines during invasive aspergillosis will be crucial for the targeted immunotherapy. Overall, cytokines and chemokines may serve as prognostic biomarkers that could be followed to assess the effectiveness of treatment response during invasive aspergillosis. Measurement of selected cytokines in the blood samples of aspergillosis patients may be a promising tool for the monitoring of treatment responses. Also, manipulation of cytokine response e. g, IFN-γ or IFN-γ in combination with antifungal drug, IL-37, may be a future avenue for the development of better therapeutic against invasive.



Aspergillus infection pathway. (A) *Aspergillus* conidia are inhaled into the lung. Their small size facilitates their entry into the alveoli. (B) Once they have entered the bronchiole, the conidia must evade the first-line immunity presented by surfactant proteins (SP-A and SP-D), which trap conidia and promote their phagocytosis by macrophages. (C) *Aspergillus* lung infection can progress to invasive systemic mycoses by crossing the alveolar epithelium (adapted from Williams (2000)).

Fig 6: Invasive Aspergillosis: Diagrammatic representation of Infection

Chapter 2 – Review of Literature

Arabitazis *et al.* stated that *Aspergillus terreus* has been recognized as the third most common and important strain of *aspergillus* that is responsible for Aspergillosis. The organism is capable of growing in soil, exists in dust and majorly prevalent in tropical and sub-tropical regions, temperate regions. *Aspergillus terreus* is reproducing asexually and produces conidia with a range of 11-13°C to 45-48°C. The organism can with stand extreme salt concentration and so it is not astonishing to find this growing in coastal areas and salt marshes. It also contributes to society by having certain industrial importance, by producing certain by products like lovastatin, an antihypercholesterolemic agent, as well as it produces different types of toxins. An estimated frequency of 3-12.5% of the patients that have been reported suffering from aspergillosis due to the invasive activity of *Aspergillus terreus*, which has a high mortality rate in oncogenic and haematology patients. It also contains the property of MDR (**M**ulti **D**rug **R**esistance) that makes it different and unique from other strain as it has a developed resistance against known antibiotics, which allows it to be more prone to stay inside the host system without responding to the treatment provided to them. *A. terreus* shows a comparatively high in vitro resistance to the major antifungal drug amphotericin B (minimum inhibitory concentration < 2 mg/L), that confirms its **M**ulti **D**rug **R**esistance property and so it has been a major reason for the research after *A.fumigatus* and *A.flavus* (Arabatzis and Velegraki 2013).

Deak *et al.* studied the morphology of *Aspergillus terreus* and analysed different aspects associated with this organism whose physical structure explains its invasive behaviour with respect to the host system. *Aspergillus terreus* is capable of asexual reproduction and so produces conidia, which grow on conidiophores. Such conidia are considered as phialidic conidia (PC). But there is another type of conidia produced by *Aspergillus terreus* and that too asexually is known as accessory conidia (AC). These accessory conidia grow and reside on the hyphae and are capable of a direct interaction with the host resulting and contributing in an invasive form of infection. It has been hypothesized that the AMB resistance that has been adapted by *A.terreus* is due to these accessory conidia. SEM and TEM were used to study the structure and morphology of these accessory conidia and it was found that these were larger in size as compared to the phialidic conidia of *A.fumigatus* and *A.terreus*, the PC

size for other two aspergilli was around 2-4 µm, the AC for A.terreus were of around 4-7 µm in diameter, with a smooth surface, no rod shaped structure or protuberances. It was also observed a difference in the metabolic activity, germination properties and adherence difference amongst these two distinct conidia germinating in A.terreus. The AC are significantly more adherent as compared to the PC, same was with metabolic activity as well as germination time, AC germinates rapidly and possess better metabolism than PC (Deak, Wilson et al. 2009).

Steinbach *et al.* analysed that *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger*, and *Aspergillus terreus* are the most common elements that allow an onset of Aspergillosis in humans. *Aspergillus terreus* have been considered a new player in activating such disorder but a few in numbers have been registered as actual cases that have found *A.terreus* as the responsible organism. Moreover, it has also been observed that there is a sudden increase in the no of cases at present. The immunocompromised state is the most suitable stage of *A.terreus* to invade the host system and so it has been categorized as an opportunistic pathogen. Amphotericin B resistance results in a low survival rate of the humans and voriconazole has been considered as the primary therapy for Invasive Aspergillosis. The primary therapy in form of voriconazole which was given to the patients suffering from Invasive Aspergillosis were healthier as it was observed that there was an increase in the mortality and so they were able to have the secondary form of therapy which was receiving an oral intake of itraconazole (Renshaw, Vargas-Muniz et al. 2016).

Taylor *et al.* that among the human pathogenic species of *Aspergillus*, *A. fumigatus* is the primary causative agent of human infections, followed by *A. flavus*, *A. terreus*, *A. niger*, and the model organism, *A. nidulans.* In individuals with altered lung function such as asthma and cystic fibrosis patients, aspergilli can cause allergic bronchopulmonary aspergillosis, a hypersensitive response to fungal components. Invasive aspergillosis (IA) is perhaps the most devastating of *Aspergillus*-related diseases, targeting severely immunocompromised patients. Those most at risk for this life-threatening disease are individuals with hematological malignancies such as leukemia; solid-organ and hematopoietic stem cell transplant patients; patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients; individuals with genetic

immunodeficiency such as chronic granulomatous disease (CGD); and individuals infected with human immunodeficiency virus (Dagenais and Keller 2009).

Baddley *et al.* discussed about *Aspergillus terreus* appears to be an emerging pathogen and constitutes a growing proportion of *Aspergillus* isolates in a large no. of patients. Surveillance of *A. terreus* at other institutions may be helpful to better define epidemiology and determine whether this organism is becoming more frequent in relation to other *Aspergillus* species. Clinical data were collected from 41 patients with positive *A. terreus* cultures. Isolates from 34 (83%) of 41 patients were cultured from the respiratory tract, those from three patients (7.3%) were from skin and soft tissues, and isolates from one patient each were from blood, a toenail, the ear canal, and peritoneal fluid. The mean age of patients was 54 years, and 66% were male. Twenty (48.7%) of 41 patients were immunocompromised, with 11 (26.8%) having received solid organ or hematopoietic stem cell transplants. Twenty-four (58.5%) of 41 patients were colonized with *A. terreus*, and in three patients (7.3%), isolates were considered to be contaminants. Of 14 patients (34%) with infection, 11 had invasive aspergillosis, 1 had aspergilloma, 1 had external otitis, and 1 had onychomycosis. Among the 11 patients with invasive aspergillosis, 5 (45.4%) had disseminated disease and the deaths of 8 patients (72.7%) were attributed to *A. terreus* infection (Baddley, Pappas et al. 2003).

Raman *et al.* analysed that Fungi are the most common microorganisms and have clinical importance. Few of them are pathogenic or opportunistic pathogen and results in morbidity and mortality to human beings. There is a rise in opportunistic fungal infections in recent years due to increased incidence of immunocompromised host. After Candida albicans, the leading causes of fungal infections in immunocompromised individuals are from Aspergillus species. Aspergillus is one of the most ubiquitous medically important opportunistic fungi. A. fumigatus, A. flavus, and A. terreus are the leading cause of invasive Aspergillosis in immunocompromised individuals. These species produce conidia at a concentration of around 1–100 conidia per m^3. After the inhalation of Aspergillus conidia, they are entrapped by the lung alveoli and if they are not efficiently cleared from lung, they germinate and establish lung infection termed invasive pulmonary aspergillosis and it may also disseminates to other organs if not treated. The recognition of A. fumigatus conidia and hyphae occurs by PRRs those include soluble receptors and cell-bound receptors. Conidial germination starts with hydrophobic layer degradation and exposure of inner cell wall components mainly

polysaccharides, which includes chitin, β-glucan, mannan, and galactomannan. These are termed as pathogen associated molecular patterns (PAMP), are recognized by PRRs. PRRs soluble receptor such as pulmonary collectins, family of C-type lectins, pentraxin-3, pulmonary surfactant proteinsA and D have been reported in aspergillosis. Further, the cellbound receptors in association with aspergillosis include Toll like receptor-2 (TLR), TLR-4 and TLR-9, which potentially induce the production of pro-inflammatory cytokines and reactive oxygen species through MyD88 signaling pathway (Thakur, Anand et al. 2015).

Jean e*t al.* attempts to investigate the complex nature of the responses that can occur in host-pathogen interactions, dual transcriptomics offers a powerful method of elucidating these interactions during infection. The gene expression patterns of Aspergillus fumigatus conidia or host cells have been reported in a number of previous studies, but each focused on only one of the interacting organisms. In the present study, we profiled simultaneously the transcriptional response of both A. fumigatus and human airway epithelial cells (AECs). 16HBE14o- transformed bronchial epithelial cells were incubated with A. fumigatus conidia at 37°C for 6 hours, followed by genome-wide transcriptome analysis using human and fungal microarrays. Differentially expressed gene lists were generated from the microarrays, from which biologically relevant themes were identified. Human and fungal candidate genes were selected for validation, using RT-qPCR, in both 16HBE14o- cells and primary AECs co-cultured with conidia. It was reported that ontologies related to the innate immune response are activated by co-incubation with A. fumigatus condia, and interleukin-6 (IL-6) was confirmed to be up-regulated in primary AECs via RT-qPCR. Concomitantly, A. fumigatus was found to up-regulate fungal pathways involved in iron acquisition, vacuolar acidification, and formate dehydrogenase activity. this is the first study to apply a dual organism transcriptomics approach to interactions of A. fumigatus conidia and human airway epithelial cells. The up-regulation of IL-6 by epithelia and simultaneous activation of several pathways by fungal conidia warrants further investigation as to better understand this interaction in both health and disease. The cellular response of the airway epithelium to A. fumigatus is important to understand if it is required to improve host-pathogen outcomes (Bessa 1987).

Brock *et al.* studied that alveolar macrophages represent one of the first phagocytes facing inhaled conidia; they compared the interaction of *A. terreus* and *A. fumigatus* conidia with alveolar macrophages. *A. terreus* conidia were phagocytosed more rapidly than *A. fumigatus* conidia, possibly due to higher exposure of β-1,3-glucan and galactomannan on the surface. In agreement, blocking of dectin-1 and mannose receptors significantly reduced phagocytosis of *A. terreus*, but had only a moderate effect on phagocytosis of *A. fumigatus*. Once phagocytosed, and in contrast to *A. fumigatus*, *A. terreus* did not inhibit acidification of phagolysosomes, but remained viable without signs of germination both *in vitro* and in immunocompetent mice. The inability of *A. terreus* to germinate and pierce macrophages resulted in significantly lower cytotoxicity compared to *A. fumigatus*. Blocking phagolysosome acidification by the v-ATPase inhibitor bafilomycin increased *A. terreus* germination rates and cytotoxicity. Although *A. terreus* is frequently found in the environment, IBPA caused by this fungus is less common than infections caused by *A. fumigatus*. However, the outcome of *A. terreus* infections is even more often fatal than *A. fumigatus* infections. The results suggests that *A. terreus* is phagocytosed more rapidly than *A. fumigatus*, β-1,3-glucan and galactomannan significantly contribute to pathogen recognition in the phagocytosis of *A. terreus*, Viable *A. terreus* conidia persist in macrophages after phagocytosis, Phagolysosomes containing *A. terreus* conidia mature and acidify, *A. terreus* is trapped in the non-germinated stage but resistant to acidic pH, Phagocytosed *A. terreus* conidia survive in the lungs of immunocompetent mice, Recombinant expression of the *A. nidulans wA* gene allows *A. terreus*to prevent phagolysosome acidification. (Slesiona, Gressler et al. 2012)

Pugliese *et al.* analysed the in vitro effect of different doses of alpha, beta and gamma human interferons and their interaction on the proliferation rate of human erythroleukemic cell line (K-562) and other cell lines has been evaluated. The results show that the stronger activity is exerted by beta HIF in comparison with alpha HIF. The action is selective and species-specific. beta HIF never elicited an "enhancing" effect on cell proliferation; on the contrary this phenomenon has been observed to occur with alpha and gamma HIF. In our cell system, the interaction between beta and gamma HIF gave a synergistic effect; on the contrary, no interaction has been observed to take place when beta HIF and directly cytotoxic mycotoxins extracted from A. terreus are associated (Pugliese, Vidotto et al. 1986).

Chapter 3 – Methodology

3.1 Overview

- Pathogenic fungal sample culturing
- Staining and morphological studies of pathogenic *Aspergillus terreus*
- Preparation of suspension cultures at different time intervals
- Conidial count under hemocytometer
- Plating, colony formation and CFU count from the suspension cultures, where samples obtained at the conidial stage
- Preservation of the fungal cultures and plates
- RNA isolation from the conidial and hyphal stages
- cDNA formation (PCR) and gel run for the conidial samples
- Protein isolation at conidial; swollen conidia and at the germinating conidial level
- SDS page for protein profiling of conidia; swollen conidia and germinating conidia
- Cell line L132 revival
- Cell lines provided with fungal infection
- MTT assay to analyse the cell viability

3.2 Pathogenic *Aspergillus terreus* sub-cultured and stock prepared

- *Aspergillus terreus* classified as Biosafety level 1 (BSL1) organism was chosen for this project
- *Aspergillus terreus* isolated from patient's induced sputum was cultured by National culture collection of pathogenic fungi, PGI Chandigarh.
- Inorder to understand the functional aspect of host-pathogen interaction, nccpf provided us with this pathogenic strain after having an acceptance from the ethical committee of PGI, Chandigarh and nccpf
- NCCPF no. 860035, *Aspergillus terreus*, was sub-cultured in potato-dextrose agar (1.5%) and also glycerol stocks were prepared to preserve the obtained cultures for further research.

3.3) PROTOCOL 1: Culturing of Pathogenic *Aspergillus terreus* (NCCPF no. 860035)

1) The fungal strain has been obtained from National culture collection for pathogenic fungi, PGIMER (Chandigarh)
2) The fungal strain has been isolated from induced sputum of the patient that is a pure infectious isolate
3) The pathogenic fungal strain that that has been isolated from an infectious patient as it will provide a better understanding between the host epithelial and fungal cells.

Principle: Reviving of the cultures obtained from NCCPF, the fungal strains are required to be cultured in a different medium containing flask. The culturing allow the cells to be always remain alive and sub-culturing is associated with the principle of transferring of the cells from a medium that has been at the verge of consumption of nutrients as well as accumulation of certain toxic by-products by the organism and also to avoid over-population and providing a space for growth.

Materials required:

- Potato Dextrose Broth: 500ml
  10gms of PDB
  2% of AGAR-AGAR
- Distilled water: Making volume upto 500ml
- 70% Ethanol
- Cotton

Equipment Required:

- Beaker
- Measuring cylinder
- Microwave
- Inoculating loop
- Bunsen burner
- Test tubes

PROCEDURE:

1) Firstly 500ml of PDB media is prepared and is kept for autoclaving.
2) After that the media is place
3) Media is poured in the test tubes and slant is formed keeping a thick bud at the base of the test tube
4) Then after the media gets solidified it is kept in room temperature or 37°C overnight for analyzing any contamination in the media
5) After that the colony has been picked up from the stock with the help of inoculating loop and in zig-zag pattern is streaked on the slant
6) The test tubes are kept in incubator at 37°C
7) Growth is observed after every 24 hours.

3.4) PROTOCOL 2: Study the Morphology of *Aspergillus terreus* under Microscope.

1) The structure of the organism is required to be studied as to analyse the stage of growth and life cycle of the organism
2) The identification of formation spores that will later allow the calculation of the conidia which will be the source of infection for the Airway Epithelial Cells (AECs).

Principle: The morphological study of *Aspergillus terreus* was performed by staining the organism with Lactophenol cotton blue stain. It contains certain components which are: Phenol that kills any live organism other than fungi; Lactic acid: Preserves fungal structures and Cotton blue: Stains the chitin and cellulose of the fungal cell wall intensely blue.

Materials Required:

- Culture of Pathogenic *Aspergillus terreus*
- Lactophenol cotton blue
- 70% Ethanol
- Cotton

Equipment Required:

- Optical or light Microscope
- Inoculating loop
- Laminar air flow
- Glass Slides
- Cover slips
- Bunsen Burner

PROCEDURE:

1) Firstly glass slide is placed in a laminar and 1-2 drop of Lactophenol cotton blue is added
2) After that with the help of inoculating loop a small amount of fungal culture is obtained from the culture tube and dipped in the drop of stain placed at the glass slide.
3) After adding the culture to the stain, it is allowed to be getting mixed at that drop of stain and after that cover slip is placed on the top.
4) The slide is then observed under the optical microscope at 4X, 10X and 40X inorder to analyse the morphology of *Aspergillus terreus.*

3.5) PROTOCOL 3: Calculating the Conidia of *Aspergillus terreus.*

1) Conidia define the spores of *A.terreus* and each spore represents on viable cell of the organism that has been formed due to stress conditions.
2) The amount of conidia is required to be analysed and counted inorder to decide that how much amount of conidia should be provided to the Airways Epithelial Cells (AECs) so as to establish an identified and confirmatory infection
3) There are certain dilutions that are being formed as fungal culture contains a large no. of cells and so it is required to dilute the culture inorder to obtain cells in a countable form as well as to acquire the no of cells per ml (CFU)

Materials Required:

- *Aspergillus terreus* culture
- 1X PBS
- Cotton
- 70% Ethanol

Equipment Required:

- Laminar air flow
- 1ml and 100ul pipette
- Microtips- 1ml and 200ul
- Eppendorfs
- Hemocytometer

PROCEDURE:

1) Firstly the culture tubes are obtained and it is allowed to be opened in laminar air flow in presence of Burner
2) After that 1ml of PBS is added to the culture in such a way that it flows on to the culture and then mixing of PBS is done inorder to have a good amount of conidia when dilutions are obtained

3) 1ml of PBS added to the culture is obtained in an Eppendorf and then 2 dilutions are prepared.
4) From 1ml of stock solution 100ul of culture containing PBS son is obtained and transferred to a fresh tube where it is mixed with 900ul of PBS, so this will be considered as dilution 1.
5) From Dilution 1 100ul of solution will be added to 900ul of PBS in a fresh Eppendorf and will be mixed properly. Hence, this will be the dilution 2.
6) After this from Dilution 2 100ul of solution will be placed on Hemocytometer and so no of conidia will be counted

3.6) PROTOCOL 4: Preparation of suspension cultures of *Aspergillus terreus* at different time points

1) Suspension cultures are grown for obtaining *A.terreus* conidia at different stages at different time points.

2) At a duration and time interval of every 3-4 hours different stages of conidia were observed and isolated

3) Conidia, swollen conidia and germinating conidia were obtained by growing them in the suspension cultures of potato dextrose broth, in order to obtain protein or RNA from each of the stage of conidia from the developing *A.terreus.*

Principle: The suspension culture with shaking provides conidia a suitable environment where the conidia are accessible to the nutrients from all around as well as agitation provides them uniformity in utilising the media and constantly taking the nutrient intake. The suspension cultures are also useful in order to obtain a desired product and even any loss of the by product that are mainly secreted by the cells.

Materials Required:

- Potato Dextrose Broth
- Conical flasks
- Stock conidia
- 70% ethanol
- Cotton plugs
- Distilled water
- 50 ml vials
- Glass slides
- Cover slips

Equipment Required:

- Laminar air flow
- 1ml and 100ul pipette
- Microtips- 1ml and 200ul
- Eppendorfs
- Incubator

- Shaker
- Centrifuge
- Optical microscope

PROCEDURE:

1) Take 3 conical flasks and prepare 50 ml of Potato dextrose agar in each of the flask
2) Flask 1 will be for the conidia; Flask 2 will be for swollen conidia and Flask 3 will be for germinating conidia.
3) All the three flasks after inoculation are kept for incubation, at 37°C at 150 rpm.
4) After every time gap of 2 hours the fungal growth is observed and at what stage does the conidia have achieved is visualized under the optical microscope at 10X and 40X.
5) Once the particular stage of fungal growth is achieved the flask representing that particular stage is marked with the no of hours that have been taken to reach to that stage
6) After that the suspension culture is taken in a 50 ml vial and allowed to centrifuge.
7) The centrifugation takes place at 7000 rpm for 25 mins at room temperature (25°C).
8) After centrifugation the supernatant is discarded and the pellet is stored which contains the fungal cell biomass in form of conidia; swollen conidia and germinating conidia.
9) The pellet is stored at -80°C and later is used for either RNA or protein isolation.

3.7) PROTOCOL 5: RNA isolation of the *Aspergillus terreus*

1) The study of *A.terreus* at the genetic level which helps more to understand the functional genomic difference between the wild and pathogenic strain and also amongst the two different pathogenic strains from two different Aspergilli.

2) The RNA isolation later develops a milestone to identify and analyse the presence of a desired gene that may either helps in developing an understanding towards the pathogenic role of an organism.

Principle:

- guanidinium isothiocyanate (powerful protein denaturant) -> inactivation of RNases
- acidic phenol/chloroform -> partitioning of RNA into aqueous supernatant for separation

Materials Required:

- Trizol
- Chloroform
- Isopropanol
- 75% ethanol
- RNase-free water or 0.5% SDS
- Polypropylene microcentrifuge tubes (Eppendorfs)
- Pipettes
- Tips- 200µl; 1000µl

Equipment Required:

- Incubator
- Shaker
- Centrifuge

Procedure:

- **Phase separation:**
    1) Incubate the homogenized sample (see Homogenizing samples) for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.

2) Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.
3) Shake tube vigorously by hand for 15 seconds.
4) Incubate for 2–3 minutes at room temperature.
5) Centrifuge the sample at 12,000 × g for 15 minutes at 4°C. Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.
6) Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
7) Place the aqueous phase into a new tube and proceed to the RNA Isolation Procedure. 8. Save the interphase and organic phenol-chloroform phase if isolation of DNA or protein is desired. See DNA Isolation Procedure and Protein Isolation Procedure for details. The organic phase can be stored at 4°C overnight.

- **RNA Precipitation:**
  1) (Optional) When precipitating RNA from small sample quantities (<106 cells or <10 mg tissue), add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase. Note: Glycogen is co-precipitated with the RNA, but does not inhibit first-strand synthesis at concentrations ≤4 mg/mL, and does not inhibit PCR.
  2) Add 0.5 mL of 100% isopropanol to the aqueous phase, per 1 mL of TRIzol Reagent used for homogenization.
  3) Incubate at room temperature for 10 minutes.
  4) Centrifuge at 12,000 × g for 10 minutes at 4°C. Note: The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
  5) Proceed to RNA wash.

- **RNA wash:**
    1) Remove the supernatant from the tube, leaving only the RNA pellet.
    2) Wash the pellet, with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization. Note: The RNA can be stored in 75% ethanol at least 1 year at –20°C, or at least 1 week at 4°C.
    3) Vortex the sample briefly, then centrifuge the tube at 7500 × g for 5 minutes at 4°C. Discard the wash.
    4) Vacuum or air dries the RNA pellet for 5–10 minutes. Do not dry the pellet by vacuum centrifuge. Note: Do not allow the RNA to dry completely, because the pellet can lose solubility. Partially dissolved RNA samples have an A260/280 ratio

- **RNA suspension:**
    1) Resuspend the RNA pellet in RNase-free water or 0.5% SDS solution (20–50 μL) by passing the solution up and down several times through a pipette tip. Note: Do not dissolve the RNA in 0.5% SDS if it is to be used in subsequent enzymatic reactions.
    2) Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.
    3) Proceed to downstream application, or store at –70°C.

3.8) PROTOCOL 6: cDNA formation and PCR amplification of the product

1) cDNA is formed inorder to check for the presence of a desired gene as in eukaryotic system cDNA is considered as the completely coding region, as post transcriptional modification removes the intronic regions and only exons representing genes are left.

2) As the amount of cDNA synthesised is very less and cannot be visible on the gel due to very low amount, hence it is required to be amplified to increase the amount of sample.

Principle:

Mature mRNA is used as template for preparing cDNA. In fact, cDNA can be produced from any RNA molecule. This conversion is brought about by reverse transcriptase. cDNA can be obtained both from prokaryotes and eukaryotes. Reverse transcriptase is a RNA-dependent DNA polymerase. It acts on a single strand of mRNA. Using mRNA as a template, reverse transcriptase produces its complementary DNA based on the pairing of RNA base pairs. This enzyme executes reactions in the same way as DNA polymerase. It also requires a primer with a free 3'-hydroxy group. For transcribing RNA secondary structures, a reverse transcriptase with high temperature performance is recommended.

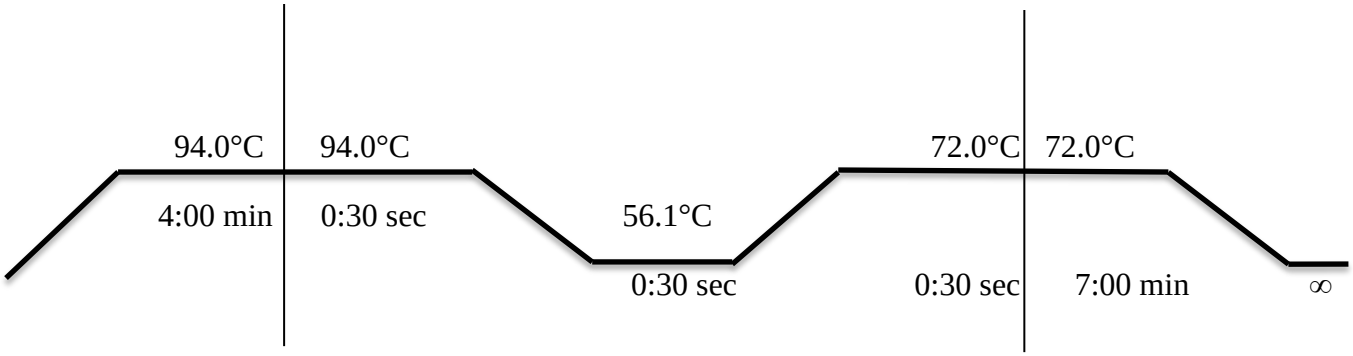
Materials Required:

Protocol

Example of reaction mix preparation.
The volume of each component is for a 20 µL final reaction.

	Volume	Final Concentration
5X cDNA synthesis buffer	4 µL	1X
dNTP Mix	2 µL	500 µM each
RNA Primer*	1 µL	
RT Enhancer	1 µL	
Verso Enzyme Mix	1 µL	
Template (RNA)	1-5 µL	1 ng
Water, nuclease-free (#R0581)	To 20 µL	
Total volume	20 µL	

Procedure:



1) Firstly the reaction mixture obtained from cDNA synthesis Thermo Scientific verso kit and about 1 µl of cDNA template into the mixture.
2) Run the PCR for 35 cycles
3) Once the PCR product is obtained run it on Agarose gel electrophoresis set up.

3.9) PROTOCOL 7: Agarose gel electrophoresis for thr PCR product

1) To confirm that the cDNA has been correctly formed and amplified

2) The PCR product obtained is required to be run on gel so as to confirm that the particular and desired gene has been amplified

Principle:

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode.

Materials Required:

- TAE buffer 1X
- Agarose (1.5%)
- Loading Dye 5X
- DNA ladder (100bps)
- Eppendorfs
- 70 % ethanol
- Pippettes
- Microtips

Equipments Required:

- Microwave
- Agarose gel run set up
- Power source
- Waterbath

Procedure:

1) Weigh out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask.
2) Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA).
3) Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
4) Add ethidium bromide (EtBr) to a concentration of 0.5 μg/ml. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/ml EtBr for 15-30 min, followed by destaining in running buffer for an equal length of time. Note: EtBr is a suspected carcinogen and must be properly disposed of per institution regulations. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however EtBr remains the most popular one due to its sensitivity and cost.
5) Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath Failure to do so will warp the gel tray.
6) Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.
7) Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use
8) Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.
9) Program the power supply to desired voltage (1-5V/cm between electrodes).

10) Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.
11) Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.
12) Remove the lid. Slowly and carefully load the DNA sample(s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples.
13) Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are plugged into the correct slots in the power supply.
14) Turn on the power. Run the gel until the dye has migrated to an appropriate distance.
15) When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
16) Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
17) Remove the gel from the gel tray and expose the gel to uv light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel
18) Properly dispose of the gel and running buffer

3.10) PROTOCOL 8: SDS-Page for the protein product obtained from swollen and germinating conidia

1) In order to check the protein profiling of the two different stages of conidia

2) To analyse the difference amongst the two stages of conidia in association with different sets of protein expressed in them

Principle: Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

Materials Required:

- Protein Marker
- Acrylamide
- Bisacrylamide
- 10% SDS
- Tris-Cl (pH 6.8)
- Tris-Cl (pH 8.8)
- APS
- TEMED
- Loading Dye
- 1X Loading Buffer
- Coomassie blue (Staining solution)
- Destaining solution

Equpments required:

- Transillumiator
- Gel doc-scanner

- SDS-Page assembly
- Power source
- Water bath
- Centrifuge

- **For a 5 ml stacking gel:**

H_2O	**2.975 ml**
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

- **For a 10ml separating gel:**

Acylamide percentage	**6%**	**8%**	**10%**	**12%**	**15%**
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

- **5X Sample buffer (loading buffer):**

10% w/v	**SDS**
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

Procedure:

1) Make the separating gel
2) Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow untill the buffer surface reaches the required level in the outer chamber.
3) Prepare the samples: Mix your samples with sample buffer (loading buffer). Heat them in boiling water for 5-10 min.
4) Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.
5) Set an appropriate volt and run the electrophoresis when everything's done.
6) As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel posessing higher percentage of acylamide, the time will be longer.

3.11) PROTOCOL 9: Bradford assay for the protein estimation of Swollen and germinating conidia

1) The protein content in each of the different conidial stage

Principle: The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250.During the formation of this complex, two types of bond interaction take place: the red form of Coomassie dye first donates its free electron to the ionisable groups on the protein, which causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. These pockets in the protein's tertiary structure bind non-covalently to the non-polar region of the dye via van der Waals forces, positioning the positive amine groups in proximity with the negative charge of the dye. The bond is further strengthened by the ionic interaction between the two. The binding of the protein stabilizes the blue form of the Coomassie dye; thus the amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading.

Materials Required:

- Bradford Reagent
- Distilled water
- Bovine serum albumin
- Rehydration Buffer
- Normal milk
- Soya milk

Equipment Required:

- Spectrophotometer
- 96 well titer plate
- Auto pipettes
- Glass Pipettes
- Beaker

Procedure:

1. Firstly prepare the Bradford reagent and store it in a dark bottle to avoid direct contact with light in order to prevent any catalytic reaction and store it in 4°C.

2. After that prepare BSA concentrations as standards, with a titration of five different concentrations: 100µg/ml, 200µg/ml, 400µg/ml, 600µg/ml, and 800µg/ml. These BSA concentrations provided standard readings in order to analyze and estimate the protein content in the samples.
3. After adding BSA concentrations to the 96 well plates we added only Bradford reagent in two wells which was considered as blanks.
4. Triplicates for soya milk and normal milk were added to the 96 well plate, inorder to avoid error.
5. Under the spectrophotometry at 595 nm the absorbance was obtained corresponding to the protein concentration available in BSA, normal milk and soya milk.
6. The blank readings were subtracted from the actual readings obtained from the three different types of protein samples.
7. BSA concentration readings were obtained and a standard graph was constructed using MS-excel.
8. With the standard graph the sample readings were analyzed and the exact protein content available in normal and soya milk sample was obtained.

3.12) PROTOCOL 10: MTT assay to estimate the Host (L132 epithelial cell lines)-pathogen *(Aspergillus terreus)* interactions

1) In order to analyse the effect of pathogen on the host biological system

2) To study the mode of action, that how the invasion is occurring and which stage of the pathogen is commencing the infection and invading the host.

Principle:

Tetrazolium dye reduction is dependent on NAD (P)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. Therefore, reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD (P)H flux. Cells with a low metabolism such as thymocytes and splenocytes reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction. It is important to keep in mind that assay conditions can alter metabolic activity and thus tetrazolium dye reduction without affecting cell viability. In addition, the mechanism of reduction of tetrazolium dyes, *i.e.* intracellular (MTT, MTS) *vs.* extracellular (WST-1), will also determine the amount of product.

Materials Required:

- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- 96 well Microtiter plate
- Isopropanol
- RPMI media
- Fungal conidia (*Aspergillus terreus)*
- L132 lung-epithelial cell lines
- Pipettes
- Micro tips

Equipments required:

- Laminar Air flow
- Incubator

Procedure:

1) After the interaction of conidia with epithelial cells, there is a 3 times washing of the wells by RPMI media before the monolayer was incubated with MTT at 1mg/ml (300µl/well).
2) After incubation for 2 hrs at 37°C in 5% $CO_{2,}$ media containing MTT was removed and 400 µl of isopropanol was added per well, for a 5 min incubation
3) Then, a 200 µl of ample was transferred to a microtiter plate and the absorbance was determined at 595 nm using a micro reader
4) The experiment was performed in duplicates for each condition.

Chapter 4 – Results

4.1 RESULT 1: Subculturing of NCCPF: 860035 pathogenic fungal strains isolated from Induced sputum.

Fig 7: Stock culture NCCPF no: 860035(*Aspergillus terreus)*

Fig 8: Sub-culture of NCCPF no: 860035(*Aspergillus terreus)*

Observations: *A. terreus* is brownish in colour and gets darker as it ages on culture media. On Potato Dextrose Agar (1.5%) at 37°C colonies have the conditions to grow rapidly and have smooth-like walls. They are able to become floccose, achieving hair-like soft tufts. This fungus is readily distinguished from the other species of *Aspergillus* by its cinnamon-brown colony colouration and its production of aleurioconidia. *A. terreus* is a thermotolerant species since it has optimal growth in temperatures between 35–40 °C, and maximum growth within 45–48 °C

4.2 RESULT 2: Morphological study of *Aspergillus terreus*

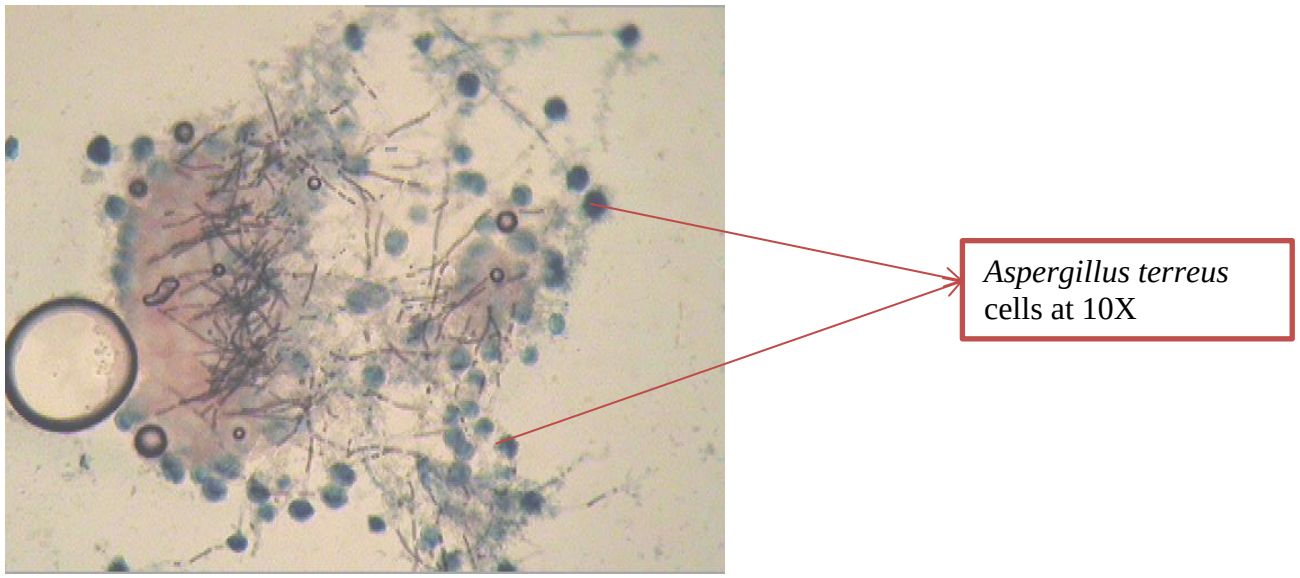


Fig 9: NCCPF no: 860035(*Aspergillus terreus)* at 10X

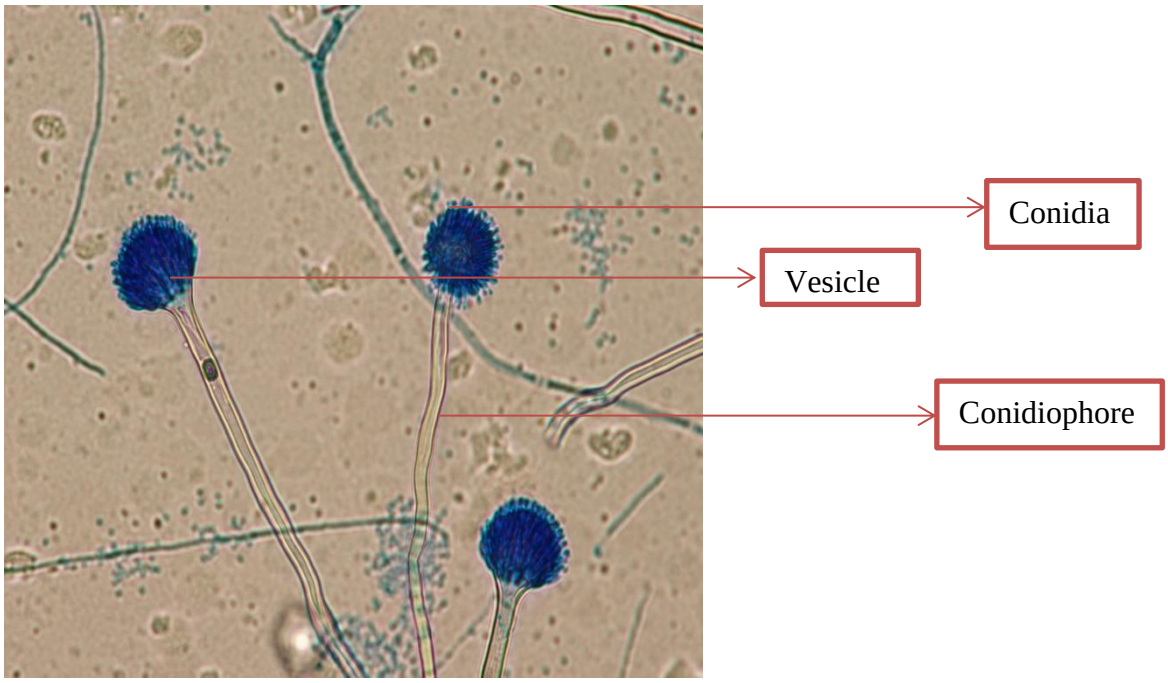


Fig 10: NCCPF no: 860035 (*Aspergillus terreus)* at 40X

4.3 RESULT 3: Counting of the conidia:

Fig 11: Hemocytometer used for counting *A. terreus* conidia

Fig 12: Hemocytometer used for counting *A. terreus* conidia under optical microscope

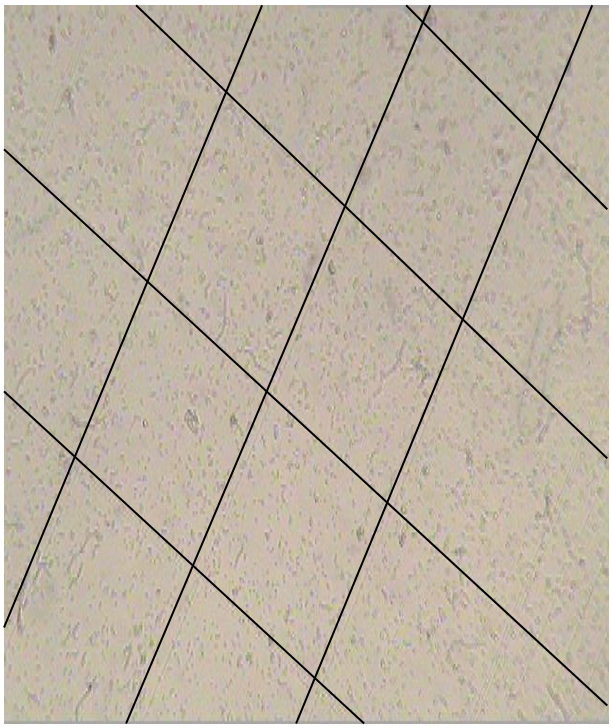


Fig 13: Hemocytometer used for counting *A. terreus* conidia under optical microscope at 10X

Total number of conidia per ml:

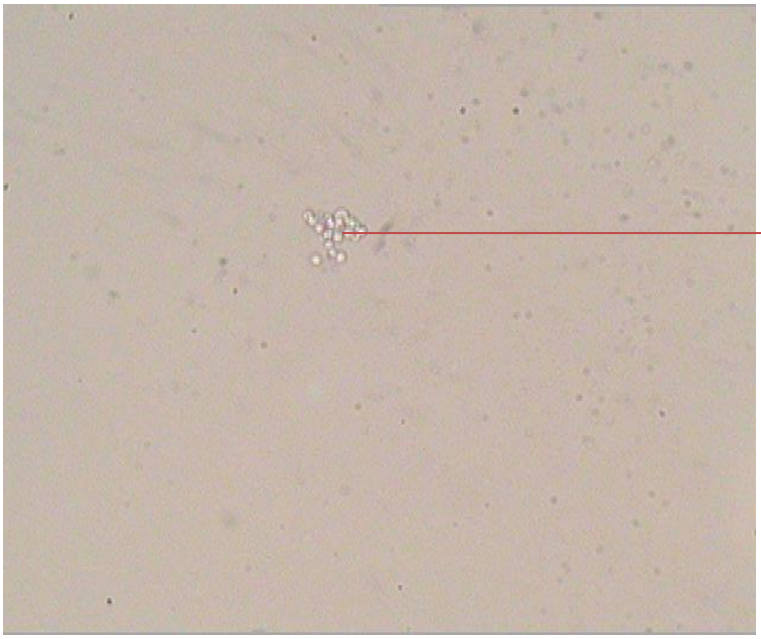
$$\frac{\text{Total no of counted particles X Dilution factor}}{\text{Area of squares counted (mm}^2\text{) X Chamber depth (mm)}}$$

$$1418 \times 10^2$$

$$(0.0025 \times 64) \times 0.1$$

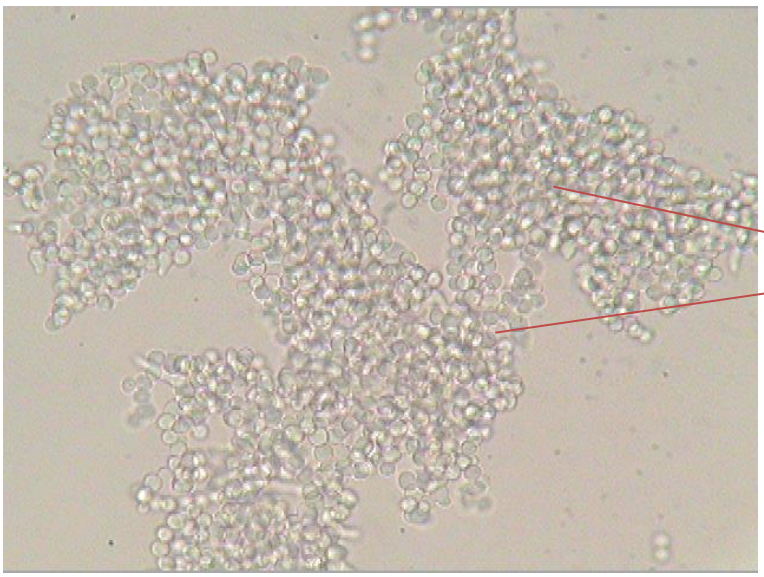
$$= 8.8 \times 10^7 \text{ per ml conidia}$$

4.4 RESULT 4: Suspension cultures containing conidia at different stages – also studying their morphology



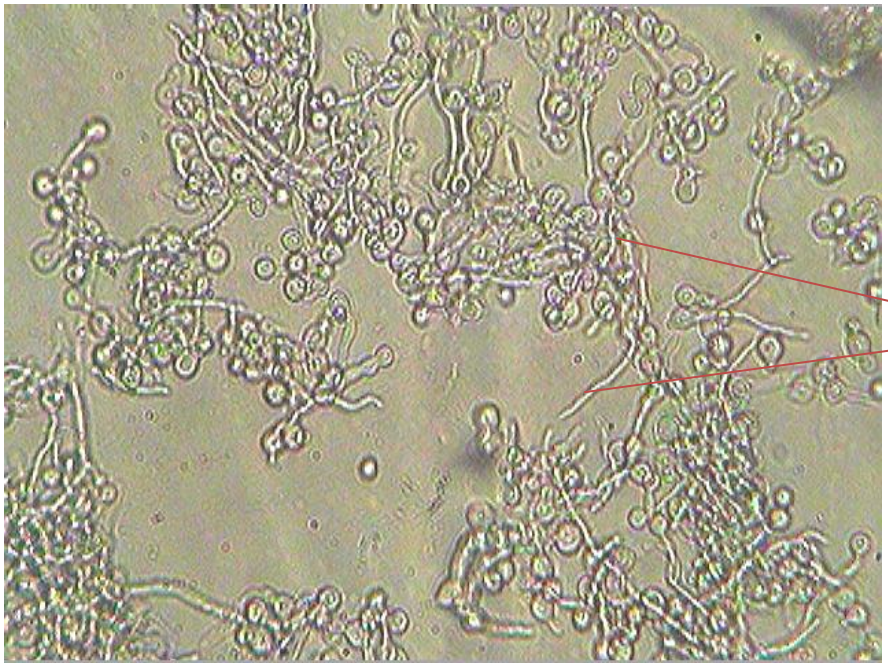
STAGE 1:
Conidia after 2 hours of incubation at 37°C in PDB suspension culture at 150 RPM

Fig 14: *A. terreus* conidia under optical microscope at 40X



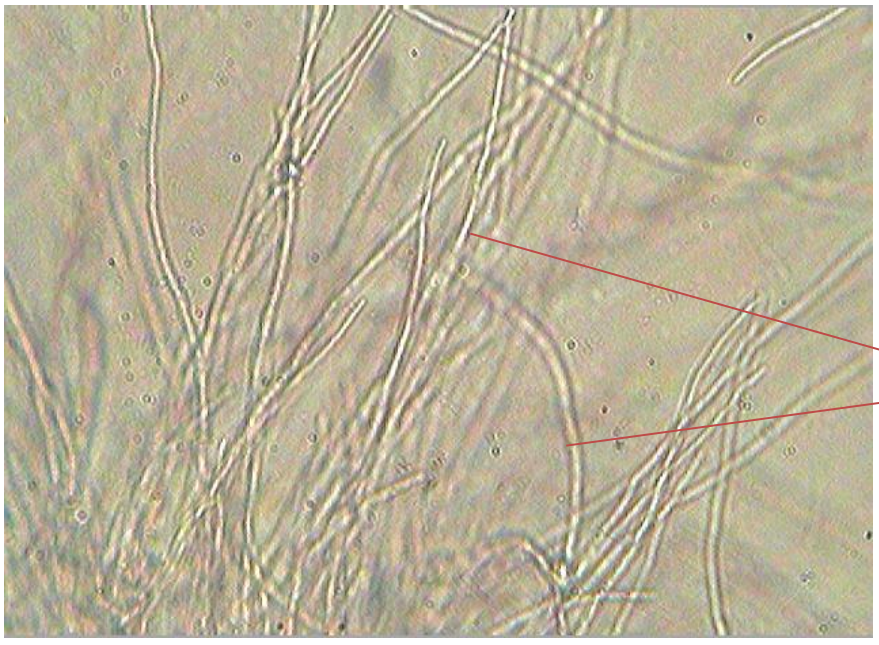
STAGE2:
Swollen Conidia after 6 hours of incubation at 37°C in PDB suspension culture at 150 RPM

Fig 15: *A. terreus* swollen conidia under optical microscope at 40X



STAGE 3:
Conidia after 9 hours of incubation at 37°C in PDB suspension culture at 150 RPM

Fig 16: *A. terreus* germinating conidia under optical microscope at 40X



STAGE 4:
Conidia after 12 hours of incubation at 37°C in PDB suspension culture at 150 RPM

Fig 17: *A. terreus* hyphal stage under optical microscope at 40X

4.5 RESULT 5: RNA isolation from *Aspergillus terreus* swollen conidia and germinating conidia

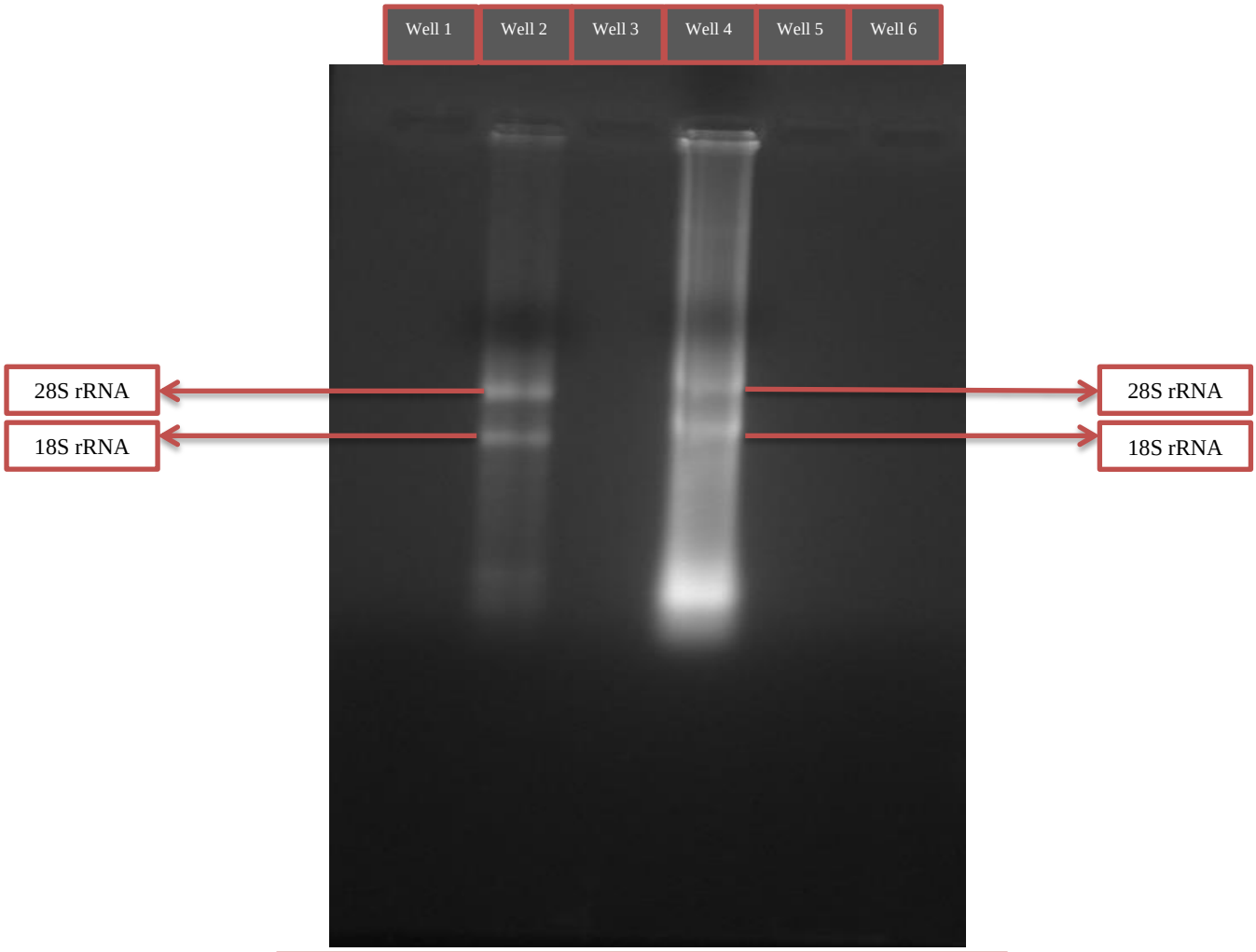


Fig 18: *Aspergillus terreus*- Swollen conidia and Germinating conidia RNA gel image representing RNA bands

Well 1	Empty
Well 2	RNA-Swollen conidia
Well 3	Empty
Well 4	RNA-Germinating conidia
Well 5	Empty
Well 6	Empty

Table 2: Representing RNA gel-wells containing samples

Observation: RNA isolation was done for swollen conidia (at 6hrs) and germinating conidia (8hrs) that was obtained after the *Aspergillus terreus* conidia was cultured in suspension in Potato Dextrose Broth (PDB). The two bands represent for 28S rRNA and 18S rRNA, and so the presence of both the band confirms the RNA has been isolated. These two bands also represents that the organism is fungus rather than any other bacterial contamination as the bands confirms the organism is a eukaryotic system.

4.6 RESULT 6: cDNA formation from RNA isolated from swollen conidia and germinating conidia

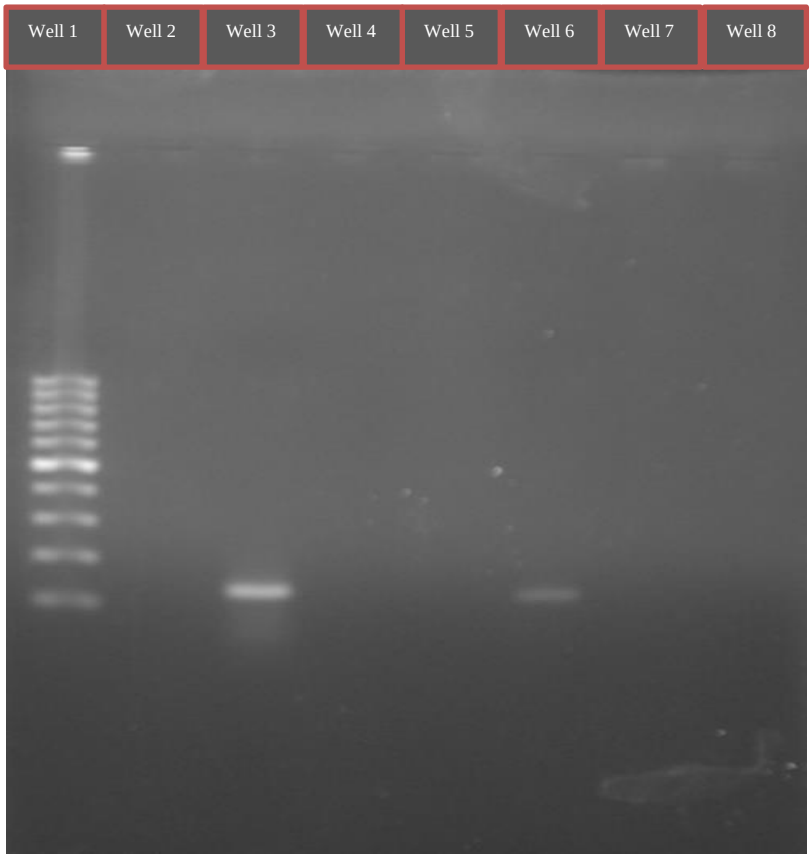


Fig 19: *Aspergillus terreus*- Swollen conidia and Germinating conidia cDNA gel image representing cDNA bands confirming formation of cDNA

Well 1	**DNA ladder (100 bps)**
Well 2	Empty
Well 3	cDNA PCR Product-swollen conidia for Tubulin gene
Well 4	Empty
Well 5	Empty
Well 6	cDNA PCR Product-germinating conidia for Tubulin gene
Well 7	Empty
Well 8	Control

Table 3: Representing cDNA gel-wells containing samples

OBSERVATION: Tubulin gene was tried to identified and checked whether the gene is expressed at the swollen and germinating conidial stage and so primers were designed to confirm the presence and in both the samples that is, swollen and germinating conidia. The bands appeared on the gel corresponding to the DNA ladder confirms the formation of cDNA that contain tubulin gene sequence, and hence it has expressed

Forward 5'GGAATGGATCTGACGGCAAG3'-20 bases
Reverse 3'GGTCAGGAGTTGCAAAGCG5'-19 bases

Primers for Tubulin gene

4.7 RESULT 7: Protein estimation in swollen and germinating conidial stage by Bradford assay

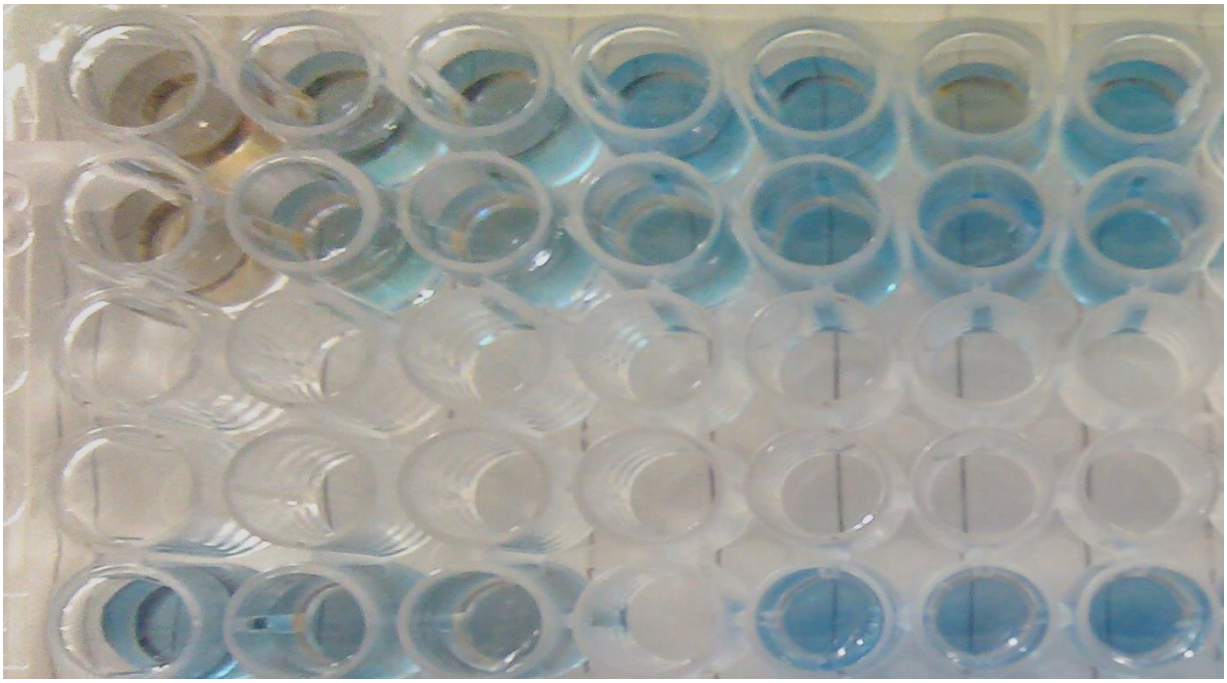


Fig 20: *Aspergillus terreus*- Swollen conidia and germinating conidia protein estimation by Bradford Assay-96 well titer plate

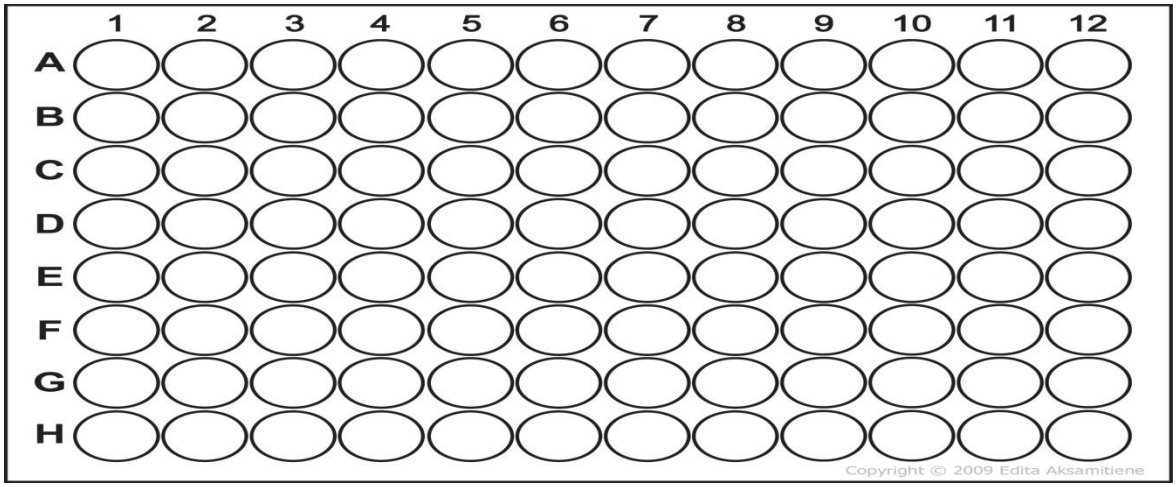


Fig 21: *96*-well titer plate for Bradford estimation

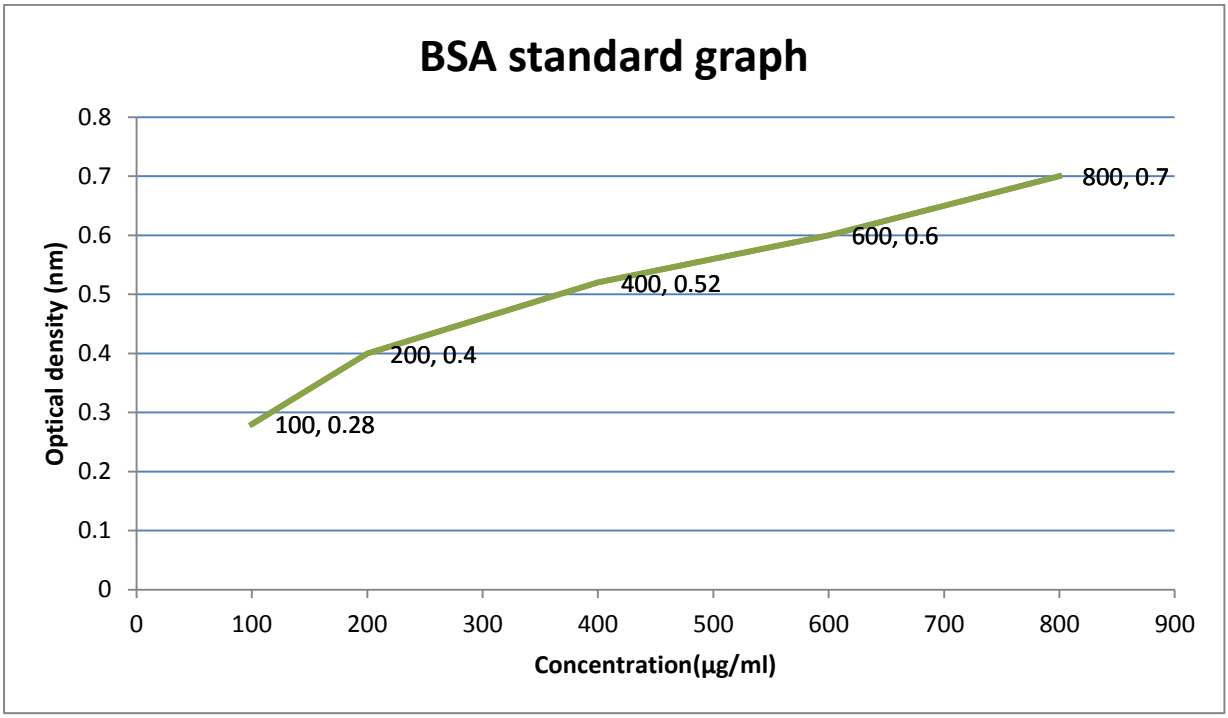


Fig 22: BSA standard graph for protein estimation

Well no	Concentration (µg/ml)	Optical Density (nm)
A1+B1	Blank	0.0006
A3+B3	100 µg/ml	0.28
A4+B4	200 µg/ml	0.4
A5+B5	400 µg/ml	0.52
A6+B6	600 µg/ml	0.6
A7+B7	800 µg/ml	0.7

Table 4: Representing 96-wells titre plate containing Standard BSA concentrations

Well no	Mean Optical Density (nm)	Estimated Protein Concentration (µg/ml)
E1+E2+E3	0.309 (Swollen conidia)	144.539 µg/ml
E5+E6+E7	0.422 (Germinating conidia)	320.026 µg/ml

Table 5: Representing 96-wells titre plate containing Protein samples for conidia; swollen conidia and germinating conidia of *A.terreus*

4.8 RESULT 8: SDS-Page analysis and protein profiling of swollen and germinating conidia

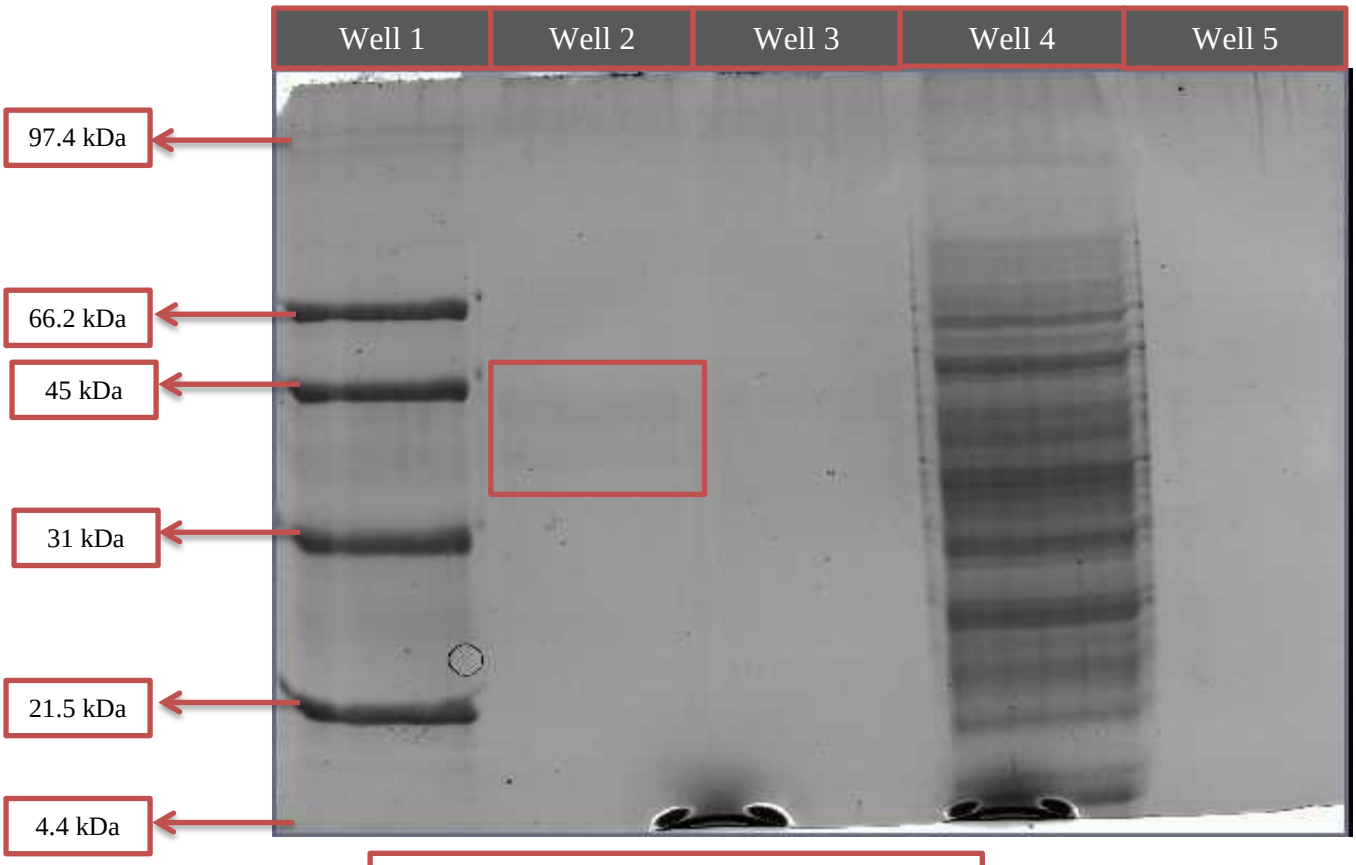


Fig 23: SDS-PAGE gel-wells containing protein samples

Well 1	**Protein Marker (kDa) Low 14.4-97.4 kDa**
Well 2	Protein-swollen conidia
Well 3	Empty
Well 4	Protein-germinating conidia
Well 5	Empty

Table 5: Representing SDS-PAGE gel-wells containing protein samples

OBSERVATION:

Well 1: It represents Low protein marker with a range of 14.4-97.4 kDa proteins.

Well 2: It represents the protein sample isolated from swollen conidia, where it can be seen that there are only 2 band s that have been obtained at the gel. The bands present in a range of 31-21.5 kDa range and there is no other band observed in any other range hence the proteins of this has expressed. The faint bands also explain a low content of protein isolated from swollen conidia at the time of protein extraction.

Well 4: This well represents the protein sample isolated from germinating conidia, where it can be observed that there is a series of bands obtained, and it matches with the corresponding bands of the protein marker. The following bands are 1) between 97.4 and 45 kDa 2) band corresponding to 45 kDa band 3) 2 bands between 45-31 kDa range 4) one more band at 31 kDa 5) 2 bands between 31-21.5 kDa range.

4.9 RESULT 9: *Aspergllus terreus* plating on Potato dextrose Agar, forming colonies and CFU counting

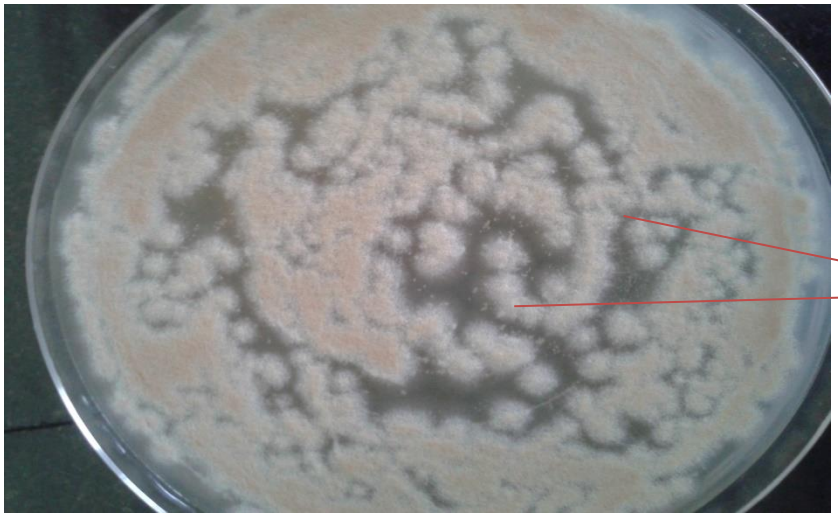


Fig 24: *Aspergillus terreus* colonies on Potato dextrose Agar plate

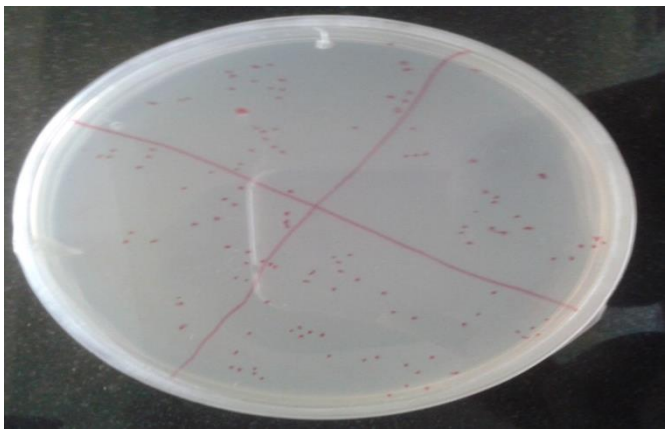


Fig 25: *Aspergillus terreus* on Potato dextrose Agar plate showing total no of colonies grown after keeping culture plates overnight

$$\frac{\text{Total no of CFU}}{\text{Volume plated X total dilution used}}$$

$127/0.1 \text{ X } 10^{-6} = 127 \text{ x } 10^{6} \text{ x } 10 =$ **$1.27 \text{ x } 10^{9}$ conidia per ml**

Observations: On Potato Dextrose Agar (1.5%) at 37°C colonies have the conditions to grow rapidly and have smooth-like walls. They are able to become floccose, achieving hair-like soft tufts. This fungus is readily distinguished from the other species of *Aspergillus* by its cinnamon-brown colony colouration and its production of aleurioconidia. *A. terreus* is a thermotolerant species since it has optimal growth in temperatures between 35–40 °C. The culture plate was kept for overnight at a temperature of 37°C. This allowed the organism to grow as shown in Fig 25. And after an incubation of 48 hours the plate produced a large no of colonies that were merged together as shown in Fig. 24

4.10 RESULT 10: Microscopic analysis host (L132 epithelial cell lines) pathogen (*A.terreus* conidia) interaction

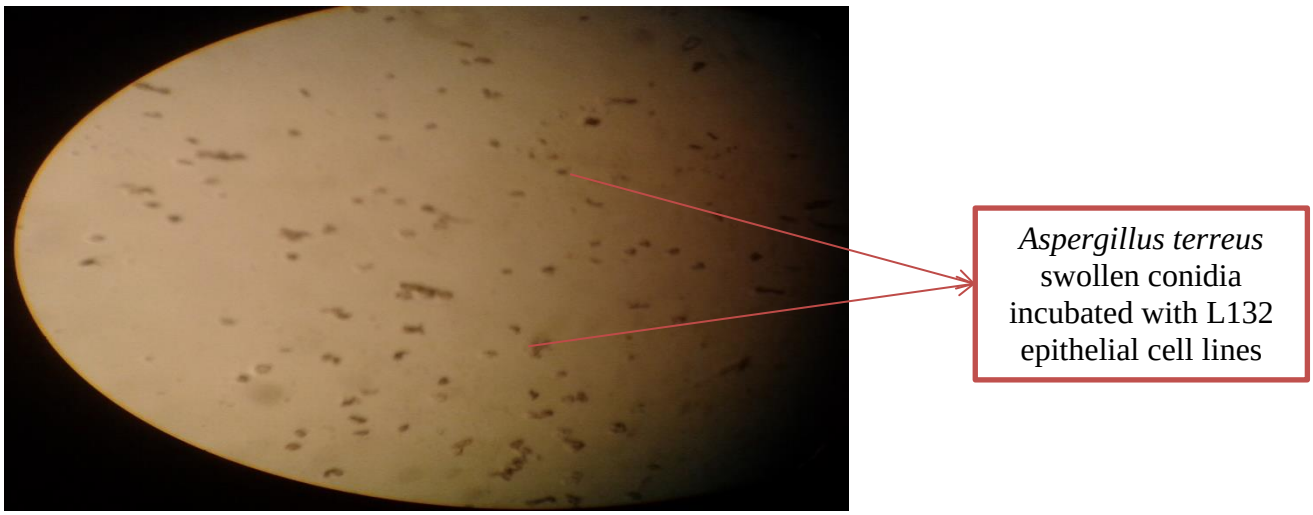


Fig 26: *Aspergillus terreus* swollen conidia

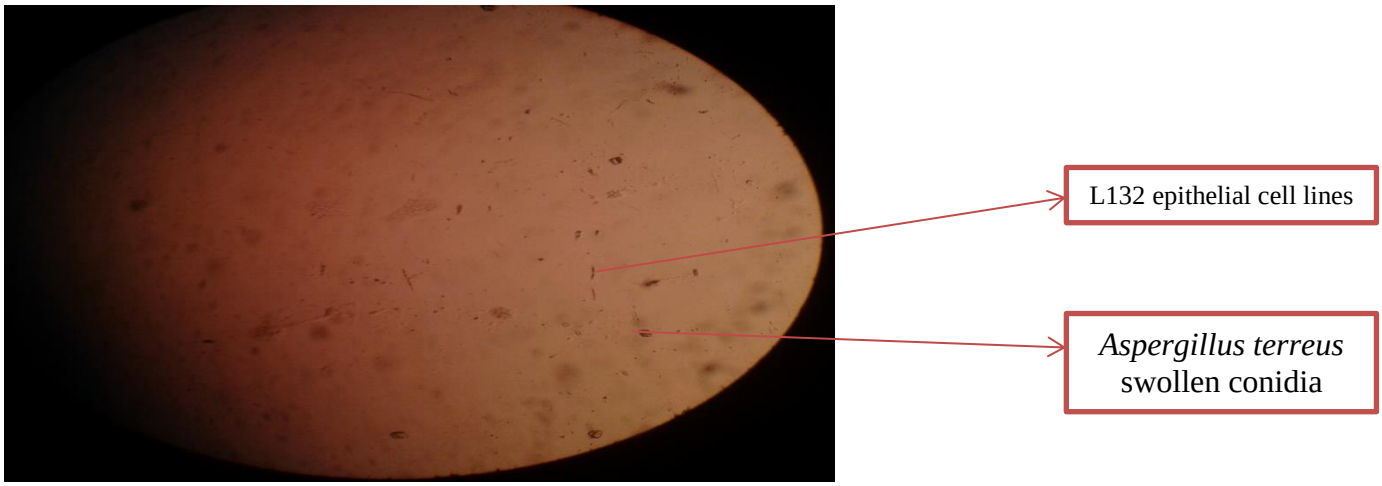


Fig 27: *Aspergillus terreus* swollen conidia and L132 epithelial cell lines

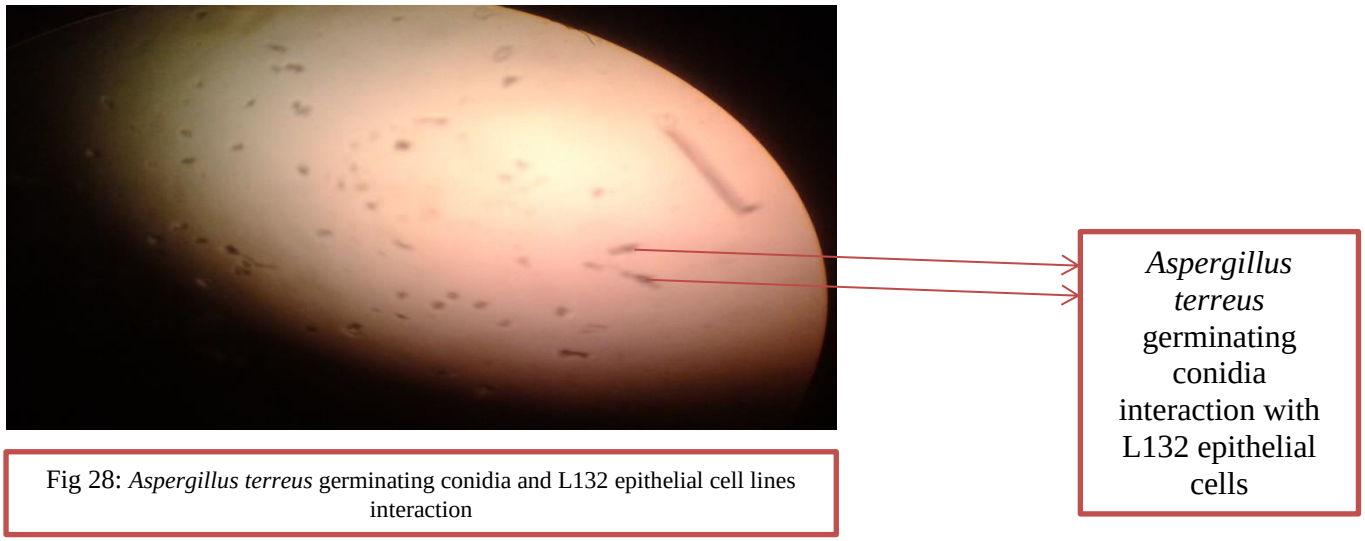


Fig 28: *Aspergillus terreus* germinating conidia and L132 epithelial cell lines interaction

4.11 RESULT 11: MTT assay to check the host (L132 epithelial cell lines) pathogen (*A.terreus* conidia) interaction and cell viability

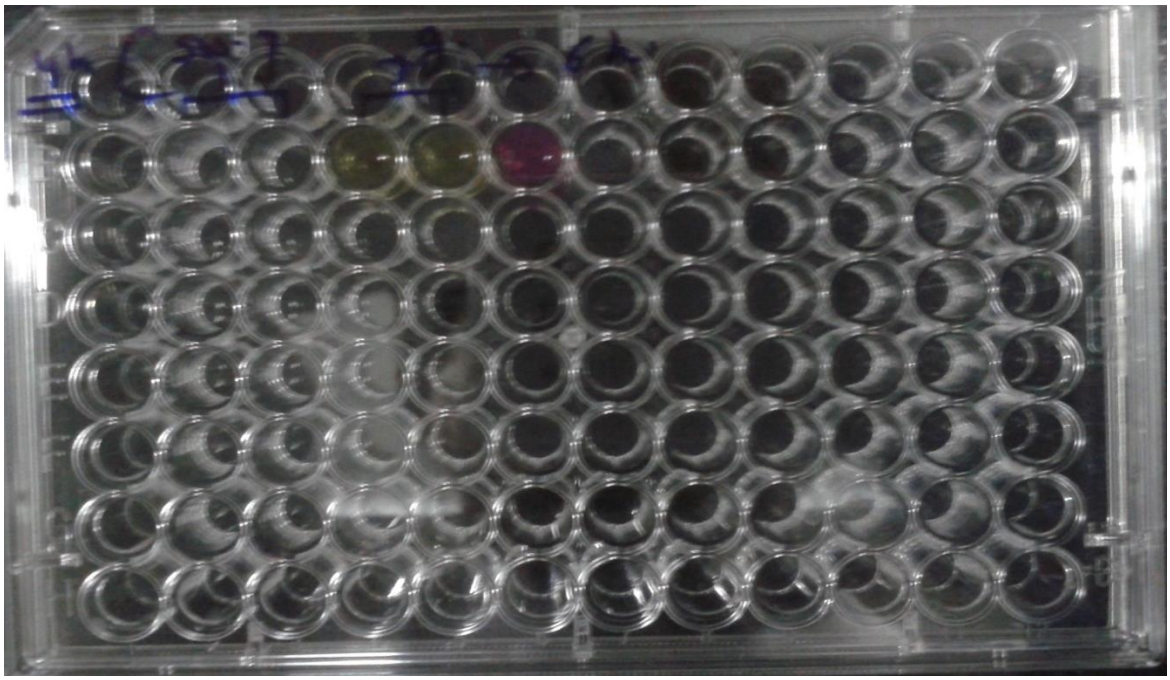


Fig 29: *96*-well titer plate for MTT assay

Wells	Sample	Optical Density (nm)
B3	Blank-isopropanol	0.044
B4	Swollen conidia	0.080
B5	Germinating conidia	0.076
B6	RPMI media	No value

Table 7: Representing 96 wells titre plate containing L132 cells-conidia interaction checked by MTT assay

Cell viability % = (Mean OD/Control OD) x 100%

1) Swollen conidia: Cell viability % = (0.08/0.044) x 100% = 81%
2) Germinating conidia: Cell viability % = (0.076/0.044) x 100% = 72%

Observation: The result explains that the cell viability% for swollen conidia > Germinating conidia which clearly signifies that the invasive property of *Aspergillus terreus* is high in germinating conidia. Though there not much difference in the cell viability % between swollen and germinating conidia but it is very clear by the above result that germinating and swollen both are responsible for Invasive Aspergillosis (IA)

Chapter 5-Conclusion

The project entitled "**Studies on Human Lung Epithelial Cells with conidia of *Aspergillus terreus*"** had involved different techniques and biotechnology tools to identify the entire mechanism of infection caused by *Aspergillus terreus* while invading the lung epithelial cells of Animal system. The study has been done at different levels, firstly, at the genetic level where RNA isolation was performed and nucleic acid was obtained from both swollen conidia and germinating conidia, also cDNA was produced to identify certain common characteristics at the genetic level between different pathogenic strains of level. The morphological studies also set up a bench mark that defines different conidial stages and which stage is actually participating and contributing in Invasive Aspergillosis. The in-silico studies performed in this project involves a host (L132 lung epithelial cell lines)-pathogen (*Aspergillus terreus*) interaction that has led to develop an understanding amongst us that how and at what stage does the pathogen invades the host system. The results via performing MTT assay has been analyzed and the project concludes that the invasion of *Aspergillus terreus* starts at their conidial level and the effect increases as the organism starts growing from conidial to hyphal to mycelium stage. The MTT assay confirmed a decrease in the cell viability when the conidia proceeded from swollen to germinating form. The swollen conidia suggested an L132 epithelial cell viability of 81% whereas germinating conidia showed a cell viability of 72%. Therefore, it confirms that *Aspergillus terreus* is involved and contributes to Invasive Aspergillosis, where during an immune-compromised state the conidia inhaled with a number of at least 1×10^6 conidia inside the lungs may lead to an invasion leading to fungal infection.

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Career Objective:

To enhance my working abilities, professional skills and business efficiencies and to serve the organization in the best possible way with sheer determination and commitment.

Academic Record:

Year	Degree/Examination	Institute	Performance
2014-2016	Master of Technology (Biotechnology)	Jaypee University of Information Technology	7.5 (Upto sem 3)
2010-2014	Bachelor of Technology (Biotechnology)	Jaypee Institute of Information Technology	7.0
2008-2009	Indian Certificate of Secondary Education	Mercy Memorial School	71.67%
2006-2007	Indian Certificate of Secondary Education	Mercy Memorial School	75.43%

Internships:

- Summer internship at DRDO (Defense research & Development Organisation) – Institute of Nuclear Medicine & Allied Sciences (INMAS), New-Delhi, worked on the project entitled – "Analyzing the effect of Phenylthiourea in the formation of blood using zebrafish as a model organism" from 27th May 2013 to 13th July 2013.
- Summer internship at Biotech Park, Lucknow. Worked in the Molecular Biology Department and learnt all possible techniques from 15th Dec 2012 to 14th Jan 2013.
- Summer internship at Dr. Saraswat Pathology, Kanpur. Worked in the Microbiology Department from 13th Dec 2011 to 3rd Jan 2012.

Projects:

- Major Project: **"Evaluation of interaction of Human Epithelial Cells with Conidia of *Aspergillus terreus*"**
- Major Project: "**Amperometric Biosensor for Triiodothyronine (T3)"**
- Internship Project: **"Analyzing the effect of Phenylthiourea in the formation of blood using zebrafish as a model organism"**
- Minor Project: **"Chitosan and its Nutraceutical Benefits"**
- Minor Project: **"Oxidative stress and its effect on ageing"**

Conferences/workshop:

- National Symposium on Computational Systems Biology (2016) [Jaypee University of Information Technology, waknaghat, solan, Himachal Pradesh].

- Malhotra A., Sundari K.* "Role of Natural Sources of Antioxidants in Reducing ROS-Related Ageing". *Indo Global Journal of Pharmaceutical Sciences* (2014). Proceedings of Inernational Conference on Life Sciences, Informatics, Food and Environment (2014). [JIIT Sector-62, Noida-201307: August 29-30, 2014].

- Tiwari A., **Malhotra A.**, Singh, Gaur S.* **"**Role of Chitosan as Nutraceutical and its potential applications*". International journal of pharma and biosciences* (2013). Proceedings of International Conference on Bioproducts and the OMICS Revolution (2013). [JIIT Sector-62, Noida-201307: March 16-17, 2013].
- Workshop based on Nano-particles and their development (2014) [Integral University, Lucknow-226026]

Extra - Curricular Activities/Achievements:

- Won 1st Prize for the poster presentation on the topic "Role of Natural Sources of Antioxidants in Reducing ROS-Related Ageing at Jaypee Institute of Information Technology during the "International Conference on Life Sciences, Informatics and, Food and Environment" in 2014
- Certificate and medallion by Novozymes for participating inter-college public speaking competition-"Voice for BT" at Jaypee University of Information Technology, Waknaghat (H.P) in 2013.
- Awarded certificate for successfully completing the Entrepreneurship course from 1st Jan 2013 to 31st March 2013 offered by Gbiofin Biotechnology.
- Won the 1st prize in the biotechnology event- Troubleshoot in impressions-2013.
- Won 2nd prize in the biotechnology event- in impressions-2013
- Won 3rd prize in *Bioneer-Pioneer*, a Bioventure competition in Impressions- 2012, for Idea of developing a Bifidobacterium-coconut based probiotic drink to combat Dehydration
- Won the 2nd prize in "fashion mantra"-Expressions (painting Hub) event, organized at Impressions 2012.
- Won Certificate during the conference of American society for Microbiology for best Interjection.
- Head Co-ordinator of "ICREATE'' sketching Hub
- Organized "Kalpana"- Sketching competition, at Impression 2012.
- Volunteer "Poste Esitlus"–Ribose (biotechnology hub) event, organized at Impression 2012.
- Coordinated "Event Codon"–Ribose (biotechnology hub) event, organized at Impressions 2012.

Personal Details:

- **Father's Name:** Late Mr. Rajeev Malhotra
- **Mothers's Name:** Mrs. Suchitra Malhotra
- **Date of birth:** 09/12/1991
- **Nationality:** Indian
- **Sex:** Male