ISOLATION AND SCREENING OF HMG-COA REDUCTASE INHIBITOR PRODUCING MICROORGANISMS FROM SOIL SAMPLE

Thesis submitted to Sikkim University as in partial fulfillment of the requirements for the degree

of

MASTER OF PHILOSOPHY (M. Phil.)

in

MICROBIOLOGY

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DECLARATION

I declare that the thesis entitled **"Isolation and Screening of HMG-CoA reductase inhibitor producing microorganisms from soil sample"** submitted by me for the award of the Degree of **Master of Philosophy (M.Phil.) Degree Microbiology** of Sikkim University is my original work. The content of this thesis is based on experiments I have performed myself. The thesis has not been submitted for any other degree to any other University/Institute.

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CERTIFICATE

This is to certify that the thesis entitled **"Isolation and Screening of HMG-CoA reductase inhibitor producing microorganisms from soil sample"** submitted to the **Sikkim University** for the award of **Master of Philosophy (M.Phil.) Degree in Microbiology** embodies the results of bona fide research work carried out by **Ms. ANU ANUPMA** under my guidance and supervision. No part of the thesis has been submitted for any other degree elsewhere.

All the assistance and help received during the course of the investigation have been acknowledged by him.

Place: Gangtok, Sikkim

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Dedicated to my family

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List of abbreviation and symbols

BSA	Bovine serum albumin
CVD	Cardiovascular disease
°C	Degree Celsius
Cm	Centimetre
$CuSo_4$	Cuprous chloride
etc.	*et cetera*
EDTA	Ethylene Diaminetetra Acetate
H_3BO_3	Boric acid
HMG-CoA reductase	3-hydroxy 3-methylglutrayl coenzyme A Reductase
HMGRI	3-hydroxy 3-methylglutrayl coenzyme Areductase inhibitor
KCl	Potassium chloride
K_m	Michaelis constant
K_i	Inhibition constant
L	Liter
LDL	Low Density Lipoprotein
M	Molar
Mg	milligram
ml	milliliter
mm	Millimeter

mM	Millimolar
mol	Mole
nm	Nanometer
NaCl	Sodium Chloride
No.	Number
OD	Optical density
pH	Power of hydronium ion
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RPM	Revolution/rotation per minute
S	Seconds
sp.	Species
T	Temperature
UV	Ultraviolet
VLDL	Very low density lipoprotein
γ	Gamma
μ	Micro
%	Percentage

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Introduction

CHAPTER 1

Fungi are among the most important organisms in the world and are highly versatile in adapting to all kind of environment (Cannon, 1997). Fungi plays a vital role in the global ecological processes, (Bridge and Spooner, 2001) which is dependent on many environmental factors i.e. types of nutrient available, humidity, moisture, degree of aeration, pH, temperature and salinity. These ubiquitous organisms found on earth's biosphere are generally saprophytes (Anderson and Cariney, 2004). Fungi are the major contributors to the soil biomass (Anisworth and Bisby, 1995). They form the major group of organotrophs responsible for the decomposition of organic compounds. Some of the fungal species helps in the biodeterioration and biodegradation of toxic substances in the soil (Wahegaonkar *et al.*, 2011) and also contributes to the nutrient cycle and maintenance of ecosystem (Sharma, 2012).

Fungi play an important role in the various geochemical formation of soil, maintenance of the soil fertility, its structure and improvement. They are also present in plants and animals as parasites or symbionts and play an essential part in the economic importance having both positive and negative effects on human activities (Molitoris, 1995). Strain improved varieties of fungal species are used in brewing, baking, industrial fermentation, biotechnical and pharmaceutical industries and other species can be consumed as food (Kumar and Nagendra, 2010). At the same time pathogenic fungi can cause huge fiscal loss each year through food spoilage, diseases of plants and animals (including humans) (Mueller *et al.*, 2004).

Fungi were used as a medicine from very ancient time. First antibiotic penicillin was developed by Alexender Fleming in 1928 from blue mould belonging to the genus *Penicillium* (Wilson and Talbot, 2009; Endo, 2010). Then the series of antibiotic are developed by using fungus. Today numbers of antibiotics derived from fungi, other than penicillin, are cephalosporin from *Cephalosporium sp.*, griseoflavin from *Penicillium griseofulvum*, Lentinan from *Lentinus sp.*, and Schizophyllan from *Schizophyllum commune* (Kumar and Nagendra, 2010).

During the late 1960's, there was higher reports and cases of CVDs (Cardiovascular diseases) due to hypercholesterolemia and this lead to a vast number of researches leading to the development of cholesterol lowering drugs and inhibitors (Endo, 2008). In the year 1976, Akira Endo discovered a specific competitive inhibitor of the 3-

hydroxy-methylglutaryl (HMG-CoA) reductase enzyme which has the property to reduce the level of cholesterol in the blood. This compound was first isolated from the fungus *Penicillinium citrinum* and was commercially called as Mevastatin. In 1988 Alberts described a more potent inhibitor isolated from *Aspergillus terreus* which was called Lovastatin (Lefera *et al.*, 2001).

3-hydroxy-3-methylglutaryl-CoenzymeA reductase inhibitors (HMGRI) are also known as statins and these are blocking enzymes, which inhibits the HMG-CoA reductase activity. A statin blocks the conversion of HMG-CoA to Melvonic acid and thus controls the biosynthesis of cholesterol. Statins mainly emphasizes on the HMG-CoA reductase activity, which plays a central role in the production of cholesterol in the liver. Increased cholesterol levels have been associated with cardiovascular diseases (CVD), and statins are therefore used in the prevention of these diseases (Pichandi *et al.*, 2011).

HMGRI are the secondary metabolites of the soil dwelling filamentous fungus. It acts as bacteriostatic and bacteriocidal against the bacterial communities residing in its niche because these microbes require sterols and/or other melvonate-derived isoprenoids for their growth (Endo, 2008).

In this study, we looked for the fungal biodiversity which had the potential for HMG-CoA reductase inhibitory activity and has antibacterial and antifungal properties. The agriculture fields of Gangtok, Sikkim were selected as the sampling area for the isolation of these fungal species. Because of its elevation and sheltered environment, Gangtok enjoys a mild, temperate climate all year. Also Sikkim is an organic farming state, hence it is rich in soil fertility, and the chance to find these specific types of HMGRI fungal samples will be maximum so we have chosen its agricultural fields as our sampling source. Hence, our primary aim was to investigate the fungal diversity of the soil and screen the fungal isolates for the HMG-CoA reductase inhibitory activity. Till date, there are no reports of this type of work which has been carried out in this region.

Aims and objectives:

1. Isolation of fungus from the soil samples of the agricultural fields.
2. Characterization of the fungal isolates through their colony morphology.
3. Identification of the fungal isolates by the slide culture method.
4. Effect of the various ranges of temperature on the fungal growth.
5. Effect of the various ranges of pH on the fungal growth.
6. Screening of the fungal isolates for antibacterial activity assay.
7. Screening of HMG-CoA reductase inhibitor production by submerged fermentation of the fungal isolates.
8. Extraction of HMG-CoA reductase inhibitor from the culture filtrate.
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Chapter - 2

Review of literature

CHAPTER 2

Fungi are eukaryotic, spore bearing, heterotrophic organisms, that may reproduce sexually and asexually and includes both single-celled yeasts and multi-cellular filamentous fungi (McLaughlin *et al.*, 2009). Fungi are known to occur ubiquitously in the environment. It is a cosmopolitan, occurring in all aerobic ecosystems. They colonize a wide range of substrates and perform a diverse function. Most common niches harbouring numerous fungal taxa are air, water and soil. Unexplored and unusual habitats might be considered as detrimental to fungal growth (Hawksworth, 1997). But, surprisingly, numerous fungal species are found to grow and survive in such habitats (Haksworth and Rossman, 1997).

According to Hawksworth fungi are a major component of biodiversity, it is estimated that number of fungi present on earth was conservatively estimated at 1.5 million, 72,036 species, but only 5-10% have been describe formally (Hawksworth *et al.*, 1997; Hawksworth, 2001). Schmit and Muller estimates there are a minimum of 7, 12,000 fungal species worldwide (Swer *et al.*, 2011). Actual number of fungi is still unknown; however only 5-13% of the total estimate global species have been describe (Wang, *et al.*, 2008), its main reasons for this is probably the difficulty of growing many fungi in pure culture (Vaiud *et al.*, 2000). A fungus plays an essential role for the survival of other organisms and is crucial in global ecological processes (Poornima *et al.*, 2014). Fungi are very diverse type of organisms, growing in all type of habitats on earth, in water, in soil, in compost or in decaying organic matter as saprophytes (Anderson and Cariney, 2004). But it has been found that more number of microfungi species of fungi exist in soil than in any other environment (Christensen, 1989) because soil is most precious natural resource and also development of fungus is especially favoured by soils having an acidic reaction and where the aerobic condition is likely to be present near the surface (Sharma *et al.*, 2010). They can decompose organic matter, form humus, release nutrients, assimilate soil carbon, and fix inorganic nutrition (Pan *et al.*, 2008). Soil fungi occur in many other interactions and associations, for example as plant, arthropod, nematode or fungal pathogens. Conversely they may be used as a food source by arthropods and other invertebrates, or attacked by antagonistic bacteria. It should however be considered that fungi are present in the soil as both actively growing organisms and as dormant propagules (Bridge and Spooner, 2001).

Extensive researches have been done on the biology of soil fungi in India. Manoharachary *et al.*, 2005 suggested that fungi occupy prime place in the biological world and India has been the cradle for such fungi. Only a fraction of total fungal wealth has been subjected to scientific scrutiny and mycologists have to unravel the unexplored and hidden wealth. One third of fungal diversity of the globe exists in India (Acharya *et al.*, 2010). Out of 1.5 million of fungi, only 50% are characterized until now. Unfortunately, only around 5 –10% of fungi can be cultured artificially.

Table.1: Diversity of fungi (Manoharachary *et al.*, 2005; Hasan and Gupta, 2012)

Phyla	**World**	**India**
Myxomycyina	405	380
Mastigomycotina	308	205
Zygomycotina	55	50
Ascomycotina	2000	745
Basdiomycotina	357	232
Deuteromycotina	4100	468
Total	7270	2080

Sharma *et al.*, 2012 made a detailed study of the soil mycoflora from Katao near Gangtok, India and reported 21 fungal species belonging to the genera of *Aspergillus, Mucorsp*and few species of *Cladosporium sp.* Dutta *et al.*, 2012 four members of Phallaceae were collected from different corners of West Bengal and among them three are reported to be new to India.

Gopal and Kurien, 2013 was conducted detailed study on the fungal diversity in the home gardens of three important district of Kerala and reported that Kerala is one of the hot spot of microbial diversity. 199 fungal isolates were obtained from rhizosphere soil of major crop of home gardens namely coconut, black pepper, coffee, banana, cardamom, arecanut, rubber, cocoa, clove, nutmeg and vanilla. In the present studies they found that black pepper, coconut and banana contain greater diversity of fungi in the homesteads of the three districts. *Penicillium sp.* and *Aspergillus sp.* are most predominant compared to other fungi.

Maheswari and Komalavalli, 2013 was conducted a study in diversity of soil fungi from Thiruvarur district, Tamil Nadu, India deals with diversity and distribution of fungal population in an around soil. The physico-chemical parameters of such soils were identified different Taluk of Thiruvarur District. Totally 35 different species of soil fungi were observed from the soil samples, they were collected from Thiruvarur, Needamangalam, Mannargudi, Valangaiman, Nannilam, Kudavasal and Thiruthuraipoondi Taluk. Among that the identified fungal species like *Aspergillus sp. Pencillium sp. Trichoderma sp. Rhizopus sp.* were predominant in all the soil samples. According to Shiny, *et al.*, 2013 soil mycoflora of different crop fields at Narasannapeta Mandal, Srikakulam district, during the investigation period 232 fungal colonies were observed. The maximum fungal species belongs to Deuteromycotina (200 colonies) and Zygomycotina (11 colonies) and 21 colonies of unknown were observed different culture media namely, Potato Dextrose Agar (PDA) Czapek,s Dox Agar (CZA) and Sabouraud's Dextrose Agar (SA) supplemented with 1% Streptomycin was used as nutrient media for the growth and sporulation of soil fungi. The colonies of *Aspergillus and Penicillium* were predominant in all soil samples of crop fields. Among the isolates, *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus terreus, Penicillium chrysogenum, Penicillium frequentans, Penicillium funiculosum, Alternaria alternata, Curvulari alunata, Trichoderma viride.*

Impact of physical factor on fungal growth:

Fungus growth is depended on various environmental factors (Arnachalam *et al.*, 1997). These environmental factors includes amount and type of nutrient available, available moisture, degree of aeration, pH, temperature, light and physical or chemical soil elements etc. (Shiney *et al.*, 2013). Different type of environmental factor determines the early colonization by producing fungistatic substances which help on secondary colonization.

Nutrient requirements for moulds may vary from mould to mould. Some moulds may thrive well on substrates with high sugar or salt content (Ainsworth and Bisby, 1995. Some may prefer simple sugars while others have the ability to utilize complex sugars. Temperature is one of the prime environmental factor affecting microbial activity, especially fungi growth and reproduction in soil (Moreno, *et al.*, 2009). The

importance of the temperature dependence of soil organisms has been further emphasize recent year due to global warming issue (Pietikainen, *et al.*, 2005). Majority of fungus are mesophilic, i.e., they can grow at temperatures within the range of 10-35°C (Thiyam and Sharma, 2014). Optimum temperatures for growth may range between 15°C and 30°C. However, some moulds such as *Chaetomium thermophilum* and *Penicillium dupontii* are thermophilic, i.e., they can grow at 45°C or higher and fail to grow below 20°C. A few moulds are psychrophilic and are unable to grow above 20°C. A significant number are psychrotolerant and are able to grow both at freezing point and at room temperature. Many moulds species grow well in the dark, but some prefer daylight or alternate light and darkness for them to produce spores. Nearly all moulds require air to grow. One of the most influential factors affecting the fungal community in soil is pH (Penalva *et al.*, 2008). pH strongly influences abiotic factors, such as carbon availability, nutrient availability and solubility of metals. Addition, soil pH may control biotic factors, such as the biomass composition of fungi, in both forest and agricultural soils (Rousk, *et al.*, 2009). All moulds require moisture for growth but the amount required varies widely (Leong *et al.*, 2014). Moulds that are capable of growing at very low water activity are referred to as xerophiles, for examples *Eurotium* species and *Wallemi asebi* (Dantigny *et al.*, 2005). Those that are capable of growing at very high water activity are referred to as hydrophilic, e.g., *Stachybotrys*, *Chaetomium* and *Ulocladium* (Molitoris *et al.*, 2000).

Fungi as source of HMG-COA reductase inhibitor

In 1971 Japanese Biochemist Akira Endo started a project to search for microbial metabolites that would inhibit 3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the synthesis of cholesterol (Gaw *et al.*, 2005).

HMG CoA reducase catalyses the committed step in cholesterol biosynthesis pathway by which HMG CoA is convert to malovonate (Willey, 2010). This step is the four electron reductive deacylation of HMG-CoA to CoA and mevalonate. It is catalysed by HMGR in reactions that proceed as follows:

$$\text{(S)-HMG-CoA} + 2\text{NADPH} + 2\text{H}^+ \longrightarrow \text{(R)-mevalonate} + 2\text{NADP}^+ + \text{CoASH}$$

Where NADP is the oxidized form of nicotinamide adenine dinucelotide, NADPH is the reduced form of NADP, and CoASH is the reduced form of CoA (Istvan, and Deisenhofer, 2001). 3-Hydroxy-3-methylglutaryl–coenzyme a reductase inhibitor (HMGRI) also known as statins are blocking enzyme, which inhibit the HMG-CoA reductase activity.

HMGRI reduce LDL-cholesterol, total cholesterol and slightly increase the high-density lipoprotein and also HMGRI have inflammatory effect. HMGRI are very effective in lowering serum cholesterol levels (Lefera *et al.*, 2001). HMGRI occupy a portion of the binding site of HMG-CoA, thus blocking access of this substrate to the active site (Boyd, 2008). Near the carboxyl terminus of HMGR, several catalytically relevant residues are disordered in the enzyme-HMGRI complexes. If these residues were not flexible, they would sterically hinder statin binding. HMGRI are competitive inhibitors of HMGR with respect to binding of the substrate HMG-CoA but not with respect to binding of NADPH. The K_i (inhibition constant) values for the statin-enzyme complexes range between 0.1 to 2.3 nM, whereas the Michaelis constant, K_m, for HMG-CoA is 4 Mm (Liao and Laufs, 2005).

Certain fungus may produce inhibitors of the enzyme to defend themselves against other microbial agent (Endo, 1992). These inhibitors inhibited the enzyme that resulted in the synthesis of mevalonate which is also required by microorganisms for the maintenance of their cell wall (ergosterol) or cytoskeleton (isoprenoids)

(Ginterand Simko, 2009). Intermediate product of mevalonate pathway includes heme, farnesyl-pyrophosphate, and greanylpyrophosphate (Istvan and Deisenhofer, 2001). These intermediates are important in lipid attachments for the γ subunit of heterotrimeric G-proteins, guanosine triphosphate-binding protein Ras and Ras-like proteins (Rho, Rab, Rac, Ral or Rap) (Liao and Laufs, 2005). Hence, statins act as inhibitors of some G-protein actions and Ras or Ras-like signalling (Galoczy, *et al.*, 2007; Cordle, *et al.*, 2005). Also melvonate is a precursor in the pathway of cholesterol synthesis they presumed that this inhibitor synthesized by the fungi could also suppress the cholesterol synthesis in humans. Further they discovered that this inhibitor inhibited the enzyme HMG-CoA reductase which would reduce the plasma cholesterol levels in humans. These HMG-CoA reductase inhibitors (HMGRI) were called as statins. The first potent reductase inhibitor isolated from *Penicillinium citrinum* was mevastatin (Lefera *et al.*, 2001).

Table 2: Fungus strain used for HMGRI production

Fungal Strains	HMGRI	Reference
Aspergillus terreus	Lovastatin	(Sreedevi *et al.*, 2011)
Aspergillus flavus,	Lovastatin	(Siamak *et.al*, 2003)
Aspergillus parasiticus	Lovastain	(Siamak *et.al*, 2003)
Beauveria bassiana	lovastatin	(Qiao *et al.*, 2012)
Doratomyces	Mevinolin	(Endo *et al.*, 1986)
Monascus ruber	Mevinolin	(Juzlove, *et al.*, 1996)
Monascuspu rpureus	Mevinolin	(Miyake, *et al.*, 2006)
Monascus pubigeru	Lovastatin	(Negishi *et al.*, 1986)
Mucor hiemalis	Lovastin	(Manzoni and Rollini, 2002)
Pleurotus ostrearus	Mevinolin	(Alarcon, *et al.*, 2003)
Penicillium brevicompactum	Compactin	(Brown *et al.*,1978)
Penicillium citrinum	Mevastatin	(Endo et al., 1976)
Pleurotus ostreatus	Lovastatin	(Pushp *et al.*, 2013)
Trichoderma longibrachiatum	Lovastatin	(Samiee, *et al.*,, 2003)
Trichoderma viridae	lovastatin	(Samiee *et al.*,2003)

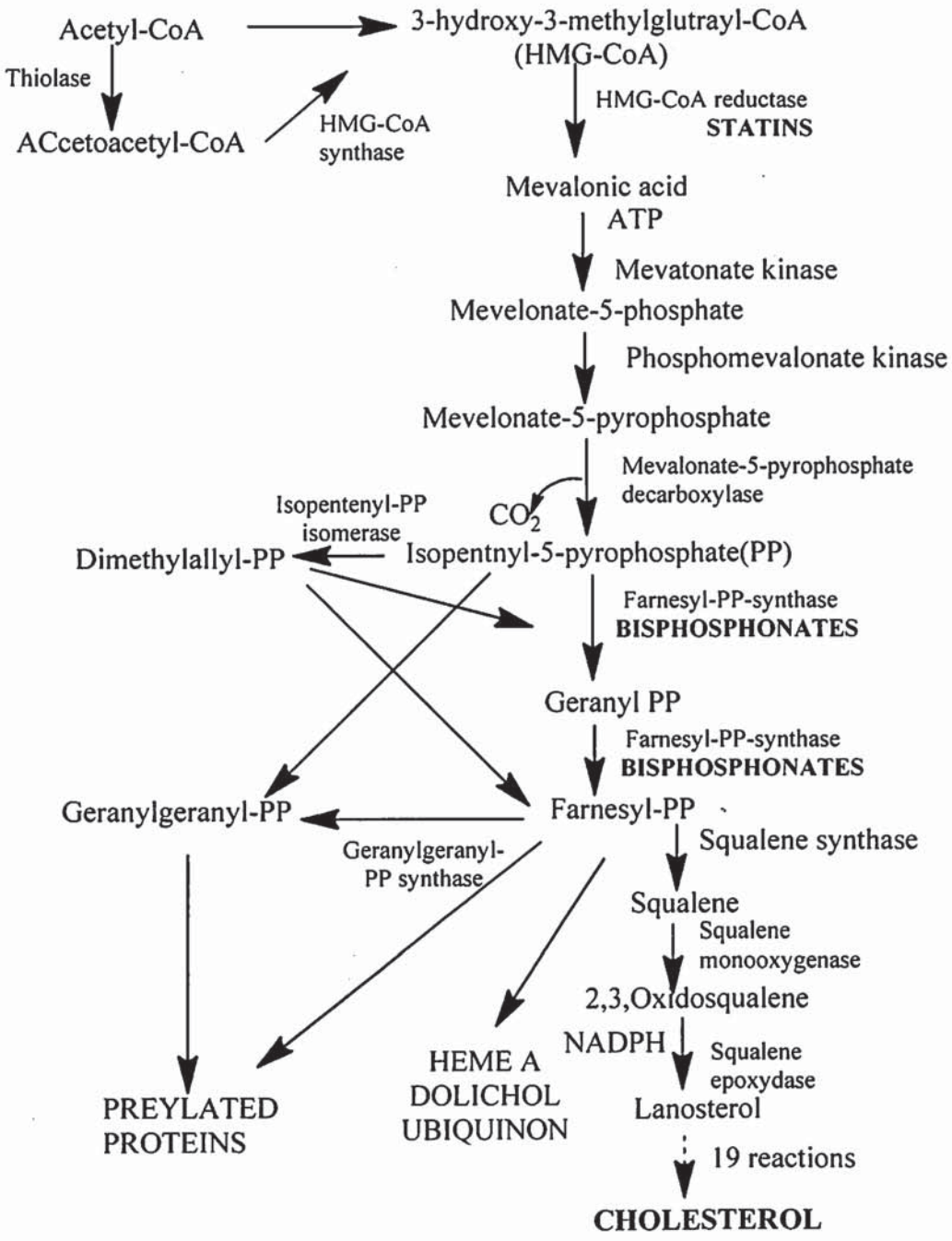


Figure1: The HMG-CoA reductase pathway, which is blocked by statins via inhibiting the rate-limiting enzyme HMG-CoA reductase (Tobert, 2003).

Mechanism of action HMGRI

HMGRI act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the melvonate pathway (Buck, 2002) (figure: 1). HMG-CoA reductase inhibitors are a group of prescription drugs used to lower cholesterol, a white waxy substance that can stick to the inside of blood vessels, resulting in clogged arteries, heart disease, and strokes (Pichandi *et al.*,2011). HMGRI are similar to HMG-CoA on a molecular level (figure: 2) they take place of HMG-CoA in the enzyme and reduce the rate by which it is able to produce melvonate and another molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds (Istvan and Deisenhofer, 2001).

Inhibiting HMG-CoA reductase

By inhibiting HMG-CoA reductase, statins block the pathway for synthesizing cholesterol in the liver (Stancu and Sima, 2001). This is significant because most circulating cholesterol comes from internal manufacture rather than the diet. When the liver can no longer produce cholesterol, levels of cholesterol in the blood will fall. Cholesterol synthesis appears to occur mostly at night, so statins with short half-lives are usually taken at night to maximize their effect (Miettinen, 1982). Studies have shown greater LDL and total cholesterol reductions in the short-acting simvastatin taken at night rather than the morning, but have shown no difference in the long-acting atorvastatin (Pichandi, *et al.*, 2011). Inhibition of HMG-COA reductase enzyme divided into two categories involving directly lipids, or intracellular signalling pathways (Stancu and Sima, 2001).

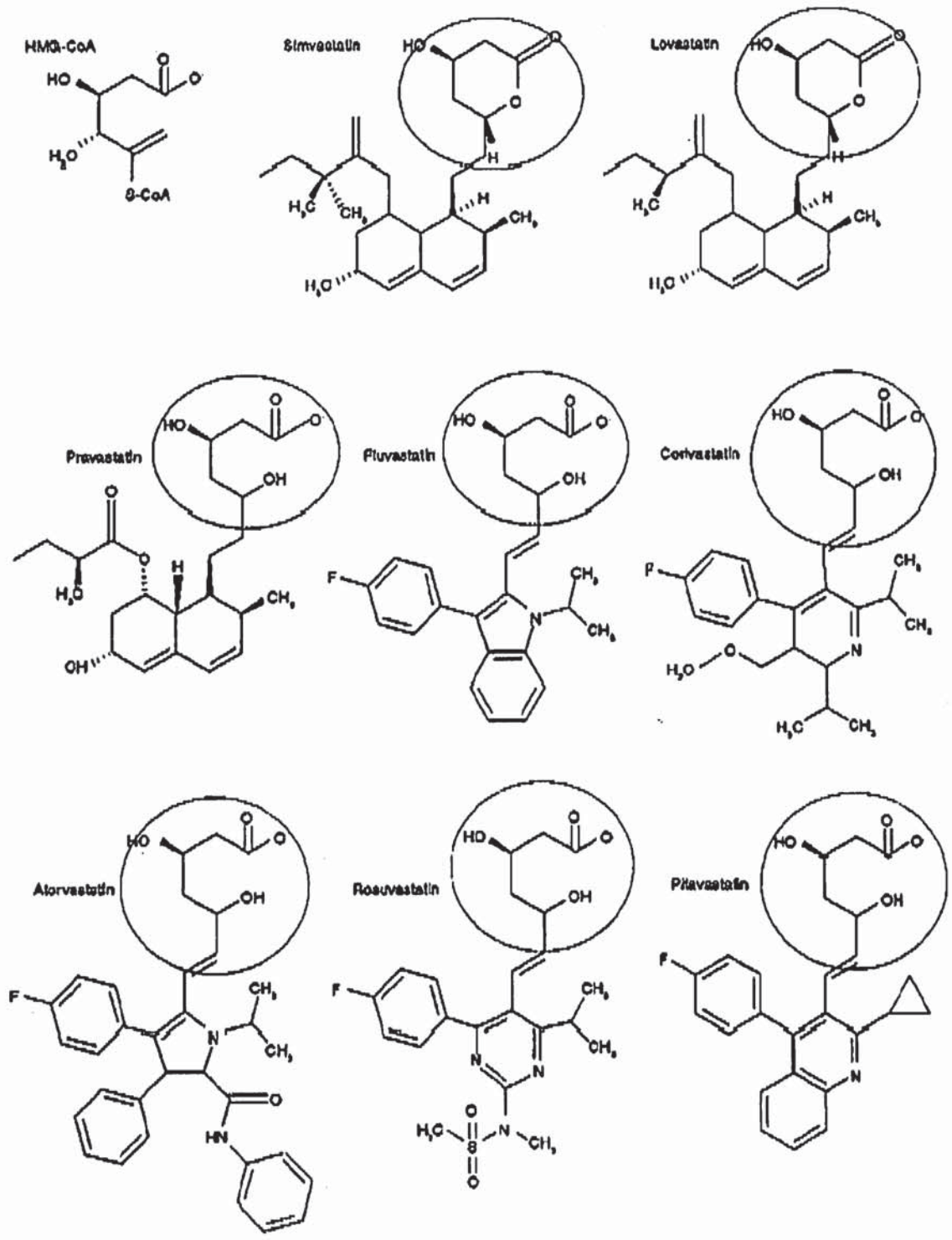


Figure 2: Different types of HMGRI are similar to HMG-CoA on a molecular level (Ose, 2010).

Increasing LDL uptake

Liver hepatocytes cells sense the reduced levels of liver cholesterol and seek to compensate by synthesizing LDL receptors to draw cholesterol out of the circulation (Ma *et al.,* 1986). This is accomplished via protease enzymes that cleave a protein called "membrane-bound sterol regulatory element binding protein ", which migrates to the nucleus and causes increased production of various other proteins and enzymes, including the LDL receptor (Sehayek *et al.,* 1994). The LDL receptor then relocates to the liver cell membrane and binds to passing LDL and VLDL particles (the "bad cholesterol" linked to disease). LDL and VLDL are drawn out of circulation into the liver where the cholesterol is reprocessed into bile salts. These are excreted, and subsequently recycled mostly by an internal bile salt circulation (Goldstein and Brown, 1987).

HMGRI have antibacterial properties. HMG-CoA reductase, the target of HMGRI, is essential in prokaryotes but it is required for biosynthesis of isoprene, not sterols as in eukaryotes. Bacterial HMG-CoA reductase is of a different structural class with an affinity for statins that is 1 10 000 times weaker than the enzyme found in eukaryotes. Thus, it is highly unlikely that the antimicrobial effect can be attributed to a known mechanism of action of HMGRI (Jerwood and Cohen, 2008).

HMGRI have been shown to exert antifungal against the pathogenic yeast yeasts *Candida spp.* and *Cryptococcus neoformans* and the non-pathogenic *Saccharomyces cerevisiae* (Galgocze, *et al.,* 2007). Also antifungal activity observed some filamentous fungi like *Penicillium chrysogenum, Penicillium nalgiovense, Aspergillus giganteus and Aspergillus* (Galgcoz, *et al.,* 2011). HMGRI have pleiotropic activity including of isoprenylation reactions and reduction of signals driving cell proliferation and survival responses. This isoprenoid intermediates that provide lipid attachment sites for activated Ras, Rac, and Rho family members. Many of these downstream products play important role in cellular and subcellular pathways critical for cancer formation and its progression (Campbell, *et al.,* 2006). HMGRI have effect on neurologic diseases, including ischemic and hemorrhagic stroke, Alzheimer disease, Parkinson disease, and multiple sclerosis (Willey, 2010).

Chapter - 3

Materials and methods

CHAPTER 3

3.1 Materials

3.1.1 Culture media used

1. Potato dextrose agar (PDA)

1.	Agar	20.0 g
2.	Dextrose	20.0 g
3.	Potato infusion	200.0 ml
4.	Distilled water	1000 ml
	pH	5.6 ± 0.2 at 25°C

Potato Infusion:

Composition per 10.0mL:

Potatoes, unpeeled and sliced 200.0 g

Preparation of Potato Infusion: Required amount of potato slices were added to 1.0 L of distilled water and gently heated and bring to boiling. After continue boiling for 30 minutes, the infusion is filtered through cheesecloth and the filtrate was used. Agar and dextrose added into filtrate.

2 Potato dextrose broth (PDB)

Composition per liter:

1.	Potatoes, infusion from	200.0 g
2.	Dextrose	20.0 g
	pH	5.1 ± 0.2 at 25°C

Potatoes, Infusion:

Composition per 500.0mL:

Potatoes 300.0 g

Preparation of Potatoes, Infusion: Required amount of potato slices were added to1.0L of distilled water and gently heated and bring to boiling. After continue boiling for 30 minutes, the infusion is filtered through cheesecloth and the filtrate was used. In the filtrate dextrose was added.

3. Nutrient broth

Composition per liter:

1. Peptone	5.0 g
2. NaCl	5.0 g
3. Yeast extract	2.0 g
4. Beef extract	1.0 g
pH	7.4 ± 0.2 at 25°C

Preparation of Medium: Add components to distilled water and bring volume to 1.0L. Mix thoroughly.

4. Seed culture medium

Composition per litre:

1. Corn steep liquor	5 g
2. Tomato paste	40 g
3. Oat meal	10 g
4. Glucose	10 g
5. Trace element stock solution	10 ml
pH	6.8

5. Production media

Composition per litre:

1. Glucose	50 g
2. Yeast extract	20 g
3. Tomato paste	30g
4. Oat meal	20 g
5. Sodium acetate	10 g
6. Ammonium sulphate	5 g
7. Potassium dihydrogen phosphate	2 g
8. Trace element stock solution	10 ml
9. Distilled water	1000ml
pH	7.0

6. Trace element stock solution

Composition per litre:

1.	$FeSO_4.7H_2O$	1 g
2.	$CuCl_2.2H_2O$	25 mg
3.	$CaCl_2.2H_2O$	100 mg
4.	H_3BO_3	56 mg
5.	$(NH_4)_6Mo_7O_{24}.2H_2O$	19 mg
6.	$ZnSO_4.7H_2O$	200 mg
7.	Distilled water	1000 ml

7. Homogenization medium (buffer I)

1. 50 mM potassium phosphate buffer (pH 7.0)
2. 0.2 M sucrose
3. 2 mMdithiothreitol

3.1.2 Reagent

1. Lactophenol cotton blue:

1.	Cotton blue	0.05 g
2.	Phenol crystal	20 g
3.	Glycerol	40 ml
4.	Lactic acid	20 ml
5.	Distilled water	20 ml

Dissolve cotton blue in distilled water. Leave overnight to eliminate insoluble dye and filter it. Then add the phenol crystal to lactic acid in a glass beaker place on magnetic stirrer until the phenol is dissolve. Glycerol and filtrate of cotton blue and distilled water add into the phenol/glycerol/lactic acid solution mix and store at room temperature.

2. Streptomycin sulphate (Hi Media, Mumbai)

To prepared 100 µg/ml streptomycin sulphate solution 10^{-6} gm streptomycin sulphate was added into 100ml of distilled water.

3. Solubilisation buffer

1. 50 mM potassium phosphate (pH 7.0)
2. 0.1(M) sucrose
3. 2 mMdithiothreitol
4. 50 mM KCl
5. 30 mM EDTA

4. Reagent used for Assay of HMG-CoA reductase inhibitory activity by UV/VIS Spectrophotometer

1. 300 µM HMG-CoA
2. 500 µM NADPH,
3. 100 mMNaCl,
4. 1.0 mM EDTA,
5. 2 mMdithiothreitol,
6. 0.5 mM Potassium phosphate buffer (pH 7.0)

5. Reagent used for protein estimation

1. Bovine serum albumin
2. Alkaline $CuSo_4$
3. 1N Folinciocallecure

3.2 Methods

3.2.1 Collection of samples.

The soil samples were aseptically collected from different region of Gangtok, Sikkim, India. Vertical samples were taken from surface, 10 and 20 cm depth in a sterilized sample bags using a sterilized spatula and brought to the laboratory for analysis. The sample was kept at 4°C for storage and preservation for future use.

3.2.2 Isolation fungus from the soil samples of the agricultural fields.

The fungal species were isolated by soil dilution plate method (Waksman, 1922; Johnson and Curl, 1972; Suhail *et al.*, 2006) 1g of soil sample was suspended in 9ml distilled water to make microbial suspension. This suspension was used to make microbial concentration (10^{-1}-10^{-5}). 1ml of microbial suspension of each concentration were added to sterile Petri dishes containing 15ml of sterile potato dextrose agar medium with supplement of streptomycin sulphate (100 µg/ml) and with the help of micropipette and L shaped glass rod spread the diluted sample on the plate. The Petri dishes were then incubated for 4-6 days at 27±1 ^{0}C. Based on colony morphological properties, pure colonies were selected and transfer on another plate then pure culture was preserved in PDA slant and also in distilled water (Makut and Owolewa., 2011: Upendra *et al.*, 2013).

3.2.3 Characterization of the fungal isolates through their colony morphology.

Pure culture of fungal isolates were inoculated into PDA plates and incubated at 28°C for 4 to 5 day for observation of colony morphology (Colony colour, Shape, size, margins elevation and growth rate)and microscopic properties (conidial head, conidiophores, vesicle and conidia) (Upendra *et al.*, 2013; Aneja, 2001).

3.2.4 Identification of the fungal isolates by the slide culture method.

Identification was achieved by taxonomic processes such as direct comparison of specimens and by the use of keys (Tsuneo, 2002). The microscopic examinations of cultures were done by preparing the slide culture. Sterile PDA was poured into sterile petri dishes and allowed to solidify and cut with sterile stainless steel spatula into blocks approximately 5 to 8 mm^2 and placed aseptically sterile microscopic slide. Inoculation of agar block on one or more sides with fungal hyphae or conidia was followed by placement of cover slip on top of it. Then it was incubated at 28^0C until adequate growth and conidiogenesis had occurred. Each cover slip was used to prepare a semi-permanent mount on a standard microscope slide. The top cover glass was lifted off with forceps and wetted on the specimen side with a drop of ethanol (70 to 90%). One drop of fungus mounting medium (e.g., lactophenol cotton blue) was applied to the specimen (Harris, 1986). The slides were observed under Phase Contrast Microscope (Leica DM2700P) and identified with the help of keys given by Tsuneo Watanabe, 2002.

3.2.5 Effect of various ranges of temperature on the fungal growth.

All the fungi were inoculated into Potato Dextrose broth (PDB) and the tubes were incubated at different temperature range (20, 30, 40, 50 and 60°C). Growth was observed till 8^{th} days of incubation.

3.2.6 Effect of various ranges of pH on the fungal growth.

All the fungi were inoculated into Potato Dextrose broth (PDB) containing different pH (5.0, 6.0, 7.0, 8.0 and 9.0) and incubated at room temperature. After incubation for 8^{th} days, growth was observed.

3.2.7 Screening the fungal isolates for antibacterial activity assay.

Antibacterial activity assay (Agar well diffusion method)

The antibacterial activities of the fungal isolates were performed by Agar well diffusion method (Smania *et al.*, 1999). The bacterial strains used were *Escherichia coli* (Laboratory culture), *Bacillus cereus* (Food Microbiology lab SGCC) *Pseudomonas aeruginosa* (MTCC 1034) and *Staphylococcus aureus* (MTCC 7443) (Table 4). About 20ml of sterilized Muller Hilton medium was poured into each sterile Petriplates and allowed to solidify. The test bacterial cultures were evenly spread over the appropriate media by using a sterile cotton swab. Then a well of 0.8 mm was made in the medium, 150µl of each fungal supernatant and crude were transferred into separate well (Samuel *et al.*, 2011).

All the plates were incubated at 37°C for 24 hours. After incubation the plates were observed for formation of clear inhibition zone around the well. The zone of inhibition was measured by taking diameter of the clear zone around the well.

Table 4: List of human pathogens used in present study

S. No	List of human pathogens	Type	Source
1.	*Escherichia coli*	Gram negative	Laboratory culture
2.	*Bacillus cereus*	Gram positive	Laboratory culture
3.	*Pseudomonas aeruginosa*	Gram negative	MTCC 1034
4.	*Staphylococcus aureus*	Gram positive	MTCC 7443

3.2.8 Screening of HMG-CoA reductase inhibitor production by submerged fermentation of the fungal isolates.

The spores were collected and added to 50 ml of seed culture medium. The seed cultures were incubated in a rotary shaker-incubator at 180 *rpm* at 28°C for 24 hours. 10% of the seed broth was inoculated to 50 ml of production medium and incubated in a rotary shaker-incubator at 180 *rpm* at 28°C for 10 days.

3.2.9 Extraction of HMG-CoA reductase inhibitor from culture filtrate.

At the end of 10 days of fermentation, the fermentation broth was acidified to pH 3.0 with 10% 1 N HCl. Then the acidified broth was extracted with equal volume of ethyl acetate under shaking condition (180 *rpm*) at 70°C for 2 hrs. The fungal biomass was separated by filtration using pre-weighed Whatman No.40 filter paper. The filtrates were subsequently centrifuged at 3000 *g* for 10 min and the organic phase was collected. To the 1mL of organic phase 1% Trifluoroacetic acid (10 ml) was added for lactonization process. Then the extract was concentrated at 80°C in a rotary evaporator, diluted to 1 ml with acetonitrile and filtered through a 0.45 µm filter for qualitative and quantitative estimation by UV spectrometry (Upendra *et al.*, 2013).

3.2.10 Extraction of HMG-CoA reductase enzymes.

HMG-CoA reductase was extracted according to the method of Kleinsek *et al.*1997, by slight modification. Fresh liver of chicken was collected and immediately placed in ice-cold homogenization medium which contained 50 mM potassium phosphate buffer (pH 7.0)/0.2 M sucrose/2 mM dithiothreitol (buffer I). Liver was homogenized in homogenization medium (2 ml/g of liver) in a Waring blendor for 15 s, followed by three strokes with a motor-driven Teflon pestle in a Potter-Elvehjem type glass homogenizer (Remi motor RQT 127A,). The homogenate was centrifuged (Sorvall legend XTR centrifuge,) for 10 min at 15,000 × *g* and the supernatant solution was centrifuged at 100,000 × *g* for 75 min in ultracentrifuge (Sorvall MTX-150 Micro ultracentrifuge, Japan). The microsomal pellet was resuspended in buffer I containing 50 mM EDTA and recentrifuged at 100,000 × *g* for 60 min. This pellet was used for isolation of the enzyme. All of the above operations were carried out at 4° C (Jung *et al.*, 2005). Microsomal pellets were frozen at deep freezer for overnight. After

thawing at room temperature the microsomes were homogenized in solubilisation buffer that contained 50 mM potassium phosphate (pH 7.0)/0.1 (M) sucrose/2 mM dithiothreitol/50 mM KCI/30 mM EDTA. A Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle was used. After standing for 15 min at room temperature, the suspension was centrifuged at 100,000 × *g* for 60 min at 20° C. The supernatant solution was collected and used for the assay of HMG-CoA reductase inhibitor activity by UV/ Vis spectrophotometer (L600B00) (Ness *et al.*, 1986).

3.2.11 Assay of HMG CoA reductase inhibitory activity by UV/VIS Spectrophotometer.

The HMG-CoA reductase-inhibitory activity was assayed in UV/VIS Spectrophotometer (Perkin Elmer,) according to Yu *et al.*, 2007 with slight modification. The rate of decrease in absorbance at 340 nm due to the oxidation of NADPH was measured. The spectrophotometer was equipped with a cell holder and maintained at 37°C with Peltier system (Perkin Elmer). The standard assay mixture contained 300 µM HMG-CoA, 500 µM NADPH, 100 mM NaCl, 1.0 mM EDTA, 2 mM dithiothreitol, and 0.5 mM Potassium phosphate buffer (pH 7.0) in a final volume of 500 µl. The reaction mixture containing the enzyme (50 µg/) and all components except HMG-CoA was first monitored to detect any HMG-CoA independent oxidation of NAD(H)P. The reaction was initiated by adding HMG-CoA at 37° C. The initial velocity of the reaction mixture was measured, and net rate of NADPH oxidation was determined by subtracting the rate of its oxidation in the absence of HMG-CoA from the rate observed with both substrates present. HMG-CoA reductases inhibitory activity was calculated by using following formula (Gholamhoseinian *et al.*, 2010):

$$\text{Inhibition\%} = \frac{\Delta\text{Absorbance control} - \Delta\text{Absorbance test}}{\Delta\text{Absorbance control}}$$

3.2.12 Estimation of protein by Lowry method of enzyme.

Estimation of protein was done by according to Lowry *et al.,* 1951 by using bovine serum albumin as standard. To a sample 5 ml in a 10 ml test tube 2 .0 ml of alkaline CuSo4 was added. Mix well and allow to stand for 10 minutes at room temperature. 0.2 ml folin reagent was added very rapidly and mixed within a second, After 30 minutes, the sample absorbance was measurement at 660nm by spectrophotometer.

Chapter - 4

Results

CHAPTER 4

4.1 Isolation fungus from soil samples from agricultural fields

A total of 45 fungal cultures were isolated from the soil samples collected from agriculture fields from surrounding places of Gangtok, Sikkim, India. PDA medium was used for the isolation of fungal isolates (Table: 5).

Table 5: Soil samples obtained from sampling stations

Sample station	Depth in cm	No of isolates	Sample code
Tadong, Gangtok (Sikkim)	10	2	TF10
	20	1	TF20
Bozogari, Gangtok (Sikkim)			
Site A	10	5	BGFA10
	20	6	BGFA20
Site B	10	3	BGFB10
	20	11	BGFB20
Site C	10	11	BGFC10
	20	6	BGFC20

4.2 Characterization of fungal culture by colony morphology

Isolated fungal isolates were characterized by using standard microbiological methods such as morphological properties (Colony colour, shape, size, and margins elevation and growth rate) and microscopic properties (hyphae, conidial head, conidiophores, vesicle and conidia) (Table: 6).

Table 6: Characterization of fungal culture by colony morphology

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony surface	pigment	Hyphae	Spores
TF10-1	White colour colony with yellow spores and yellow brown colour on the reverse	Entire	Velvety	Absent	Septate	Conidiospores
TF10-2	Dark Greenish colour and reverse yellow green colour	Filiform	Cottony	Green yellow pigment	Septate	Conidiospores
TF20-1	White brown, produce brown colour pigment	Filiform	Velvety	Brown pigment	Septate	Conidiospores
BGFA10-1	Cottony growth with white to yellow	Filiform	Cottony	Absent	Nonseptate	Sporangiospores
BGFA10-2	Pink colour	Filiform	Cottony	Absent	Septate	Not observed
BGFA10-3	White colour with green colour rings present	Filiform	Velvety	Absent	Septate	Conidiospores
BGFA10-4	White to off white colony Reverse pale yellow colour	Filiform	Velvety	Absent	Septate	Not observed

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony margin/pattern	Colony surface	pigment	Hyphae
BGFA10-5	Dark green colour and reverse yellow colour	Undulated	Powdery	Absent	Septate	Conidiospores
BGFA20-1	White with dark green colour reverse off white colour	Entire	Velvety	Absent	Septate	Conidiospores
BGFA20-2	White colour with violet colour and reverse dark violet colour	Undulated	Cottony	Absent	Septate	Not observed
BGFA20-3	White colour with green colour reverse pale yellow colour	Entire	Velvety	Absent	Septate	Conidiospores
BGFA20-4	Yellow colour filamentous growth with black spores	Filiform	Cottony	No	Septate	Conidiospores
BGFA20-5	White with dark green colour reverse off white colour	Filiformn, radiated	Cottony	Absent	Septate	Not observed
BGFA20-6	White colour	Filiform	Cottony	Absent	Septate	Sporangiospores

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony surface	pigment	Hyphae	Spores
BGFB10-1	White with green colour and reverse dark brown colour	Filiform	Velvety	Absent	Septate	Conidiospores
BGFB10-2	White colour with violet colour and reverse dark violet colour	Lobated	Cottony	Absent	Septate	Conidiospores
BGFB10-3	White with dark grey colour	Undulated	Velvety	Absent	Septate	Conidiospores
BGFB20-1	White colour with dark grey colour spores and reverse cream colour	Entire, radiated	Velvety	Absent	Septate	Conidiospores
BGFB20-2	Dark green colour and reverse off white colour	Undulated	Powdery	Absent	Septate	Conidiospores
BGFB20-4	Dark green colour and reverse off white colour	Entire	Velvety	Absent	Septate	Conidiospores
BGFB20-5	White colour and reverse yellow colour	Filiform	Cottony	Absent	Aseptate	Sporangiospores

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony surface	pigment	Hyphae	Spores
BGFB20-6	Pink colour with white colour cottony growth and reverse cream colour	Undulated	Cottony	Absent	Septate	Conidiospores
BGFB20-7	Dark green colour and reverse yellow colour	Undulated	Powdery	Pale yellow pigment	Septate	Conidiospores
BGFB20-8	Dark grey colour and reverse cream colour	Entire	Cottony	Absent	Septate	Conidiospores
BGFB20-9	Light greenish yellow to olive and may have a white border	Undulated	Cottony	Absent	Septate	Conidiospores
BGFB20-10	White colour	Filiform	Cottony	Absent	Septate	Not observed
BGFB20-11	Dark green spores surrounded by white colour and reverse white colour	Undulated	Powdery	Absent	Septate	Conidiospores

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	**Colony margin/pattern**	**Colony surface**	**pigment**	**Hyphae**	**Spores**
BGFC10-1	White colour with dark grey colour spores reverse dark brown colour	Lobated	Shrunken with water	Absent	Septate	Conidiospores
BGFC10-2	White colour with green colour spores And reverse dark yellow colour	Undulated	Powdery	Absent	Septate	Conidiospores
BGFC10-3	White colour with dark green rings and reverse cream colour	Undulated	Velvety	Absent	Septate	Conidiospores
BGFC10-4	Light brown colour cottony growth and reverse dark brown colour	Lobated	Cottony	Dark red brown	Septate	Conidiospores
BGFC10-5	White radiated and reverse pale yellow colour	Filiform	Crustaceous	Yellow pigment	Septate	Conidiospores

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony surface	pigment	Hyphae	Spores
BGFC10-6	Dark brown colour and reverse black brown colour	Filiform	Cottony	Dark brown colour pigment	Septate	Conidiospores
BGFC10-7	White colour	Filiform, flowery	Cottony	Absent	Septate	Conidiospores
BGFC10-8	White colour radiated growth and reverse yellow colour	Filiform, radiated	Cottony	Absent	Septate	Not observed
BGFC10-10	Orange colour with dark green colour spores and reverse white colour with light orange colour	Filiform	Powdery	Absent	Septate	Conidiospores
BGFC20-1	White colour with dark green colour spores and reverse pink colour	Entire	Velvety	Absent	Septate	Conidiospores
BGFC20-2	Yellow colour and reverse dark brown colour	Undulated	Shrunken	Dark brown colour	Septate	Conidiospores

Table 6: (Continued)

Isolate code	Colony characteristics				Microscopic characteristics	
	Colour	Colony margin/pattern	Colony surface	pigment	Hyphae	Spores
BGFC20-3	White colour with grey colour and reverse cream colour	Entire	Cottony	Absent	Septate	Conidiospores
BGFC20-4	Dark black colour colony	Entire	Embedded	Yellow pigment	Septate	Conidiospores
BGFC20-5	Dark grey green colour with white colour surrounded and reverse off white colour	Undulated	Embedded	Absent	Septate	Conidiospores
BGFC20-6	Dark green colour with white colour ring around colony and reverse brown colour	Undulated	Embedded	Dark brown colour pigment	Septate	Conidiospores

Following morphological and microscopic characteristics were observed.

1. Isolate Code: TF10 1

Colony morphology:

Colour: white colour colony with yellow spores and yellow brown colour on the reverse.

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidia: formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: with inflated apical cells bearing numerous phialides.....*Aspergillus*

Key to species:

1. Spore masses not green.

Yellow spore mass.....*Aspergillus terreus*

2. Isolate code: TF10-2

Colony morphology:

Colour: dark greenish colour and reverse yellow green colour.

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed ..... Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type..... Phialosporae

Phialosporae

1. Conidia: 1 celled
2. Conidiophores: without inflated apical cells
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: boat-shaped or lunate, with or without appendage not so
6. Conidia: clavate not so
7. Conidiophores well developed
8. Conidia: wet
9. Spore: formed at apical parts of conidiophores
10. Conidiophores: hyaline
11. Conidiophores: irregularly branched ..... *Trichoderma*

(Figure: 3)

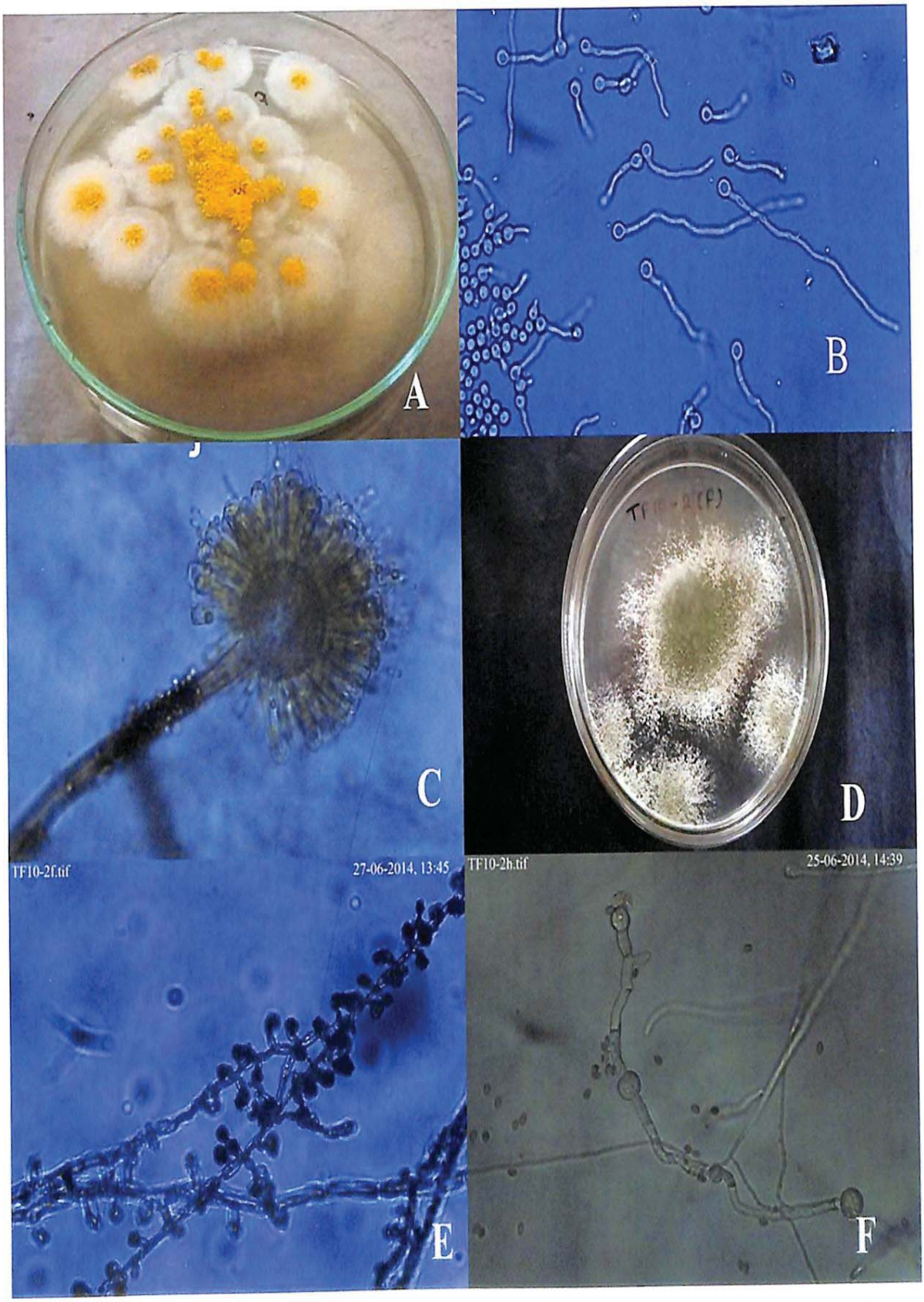


Figure 3: A, D: Colony morphology of isolates TF10-1 and TF10-2 on PDA. B: Spore germination of TF10-1 200X, C: Conidiospores of TF10-1 500X, E: Conidiospores of TF10-2 500X, F: Chlamydospores of TF10-2 500X.

3. Isolated code: TF20-1

Colony morphology:

Colour: white brown grey produce brown colour pigment

Colony margin/pattern: filiform

Colony surface: velvety

Pigment: brown pigment

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed .....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: aleuriosporae type.....Aleuriosporae

Aleuriosporae:

1. Conidia: over 2-celled
2. Conidia: pigmented
3. Conidia: longitudinally and transversely septate (muriform)
4. Conidia: borne directly on hyphae.....*Fumago sp.*

4. Isolated code:BGFA10-1

Colony morphology:

Colour: cottony growth with white to yellow

Colony margin/pattern: filiform

Colony surface: cotton

Pigment: no

Microscopic morphology:

Key to class:

1. Hyphae: non Septate
2. Sporangiospore formed .....Zygomycetes

Key to zygomycetes:

1. Vesicles: not formed between sporangiophore and sporangia
2. Sporangia: globose
3. Sporangia: without apophysis
4. Sporangia: collumellate
5. Columella: not so twisted or coiled
6. Rhizoid: not formed formed just below the sporangiophore
7. Sporagiophore:not partially twisted .....*Mucor*

Key to species:

1. Zygospore not formed
2. Colmellae not so protuberant
3. Sporangiospore: hyaline, ellipsoidal
4. Sporangiospore 5-6.3 µm long.....*M. hiemalis*

(Figure: 4)

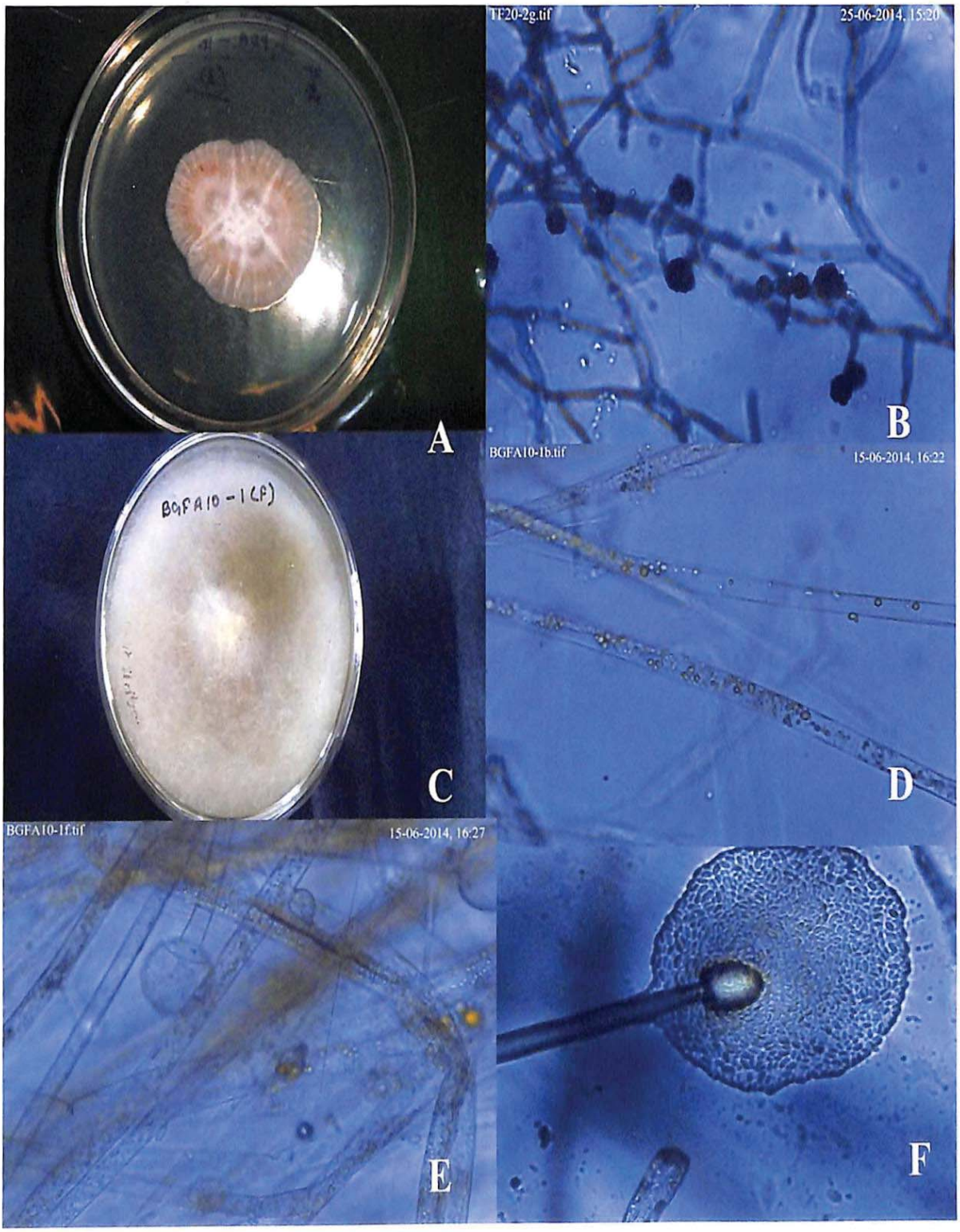


Figure 4: A, C: Colony morphology of isolatesTF20-1and BGFA10-1 in PDA. B: Conidiospores of TF20-1 500X, D: Nonseptate hyphae of BFA10-1 500X, E: Sporangiospores of BGFA10-1 200X, E: Sporangiospores of BGFA10-1 500X.

5. **Isolated code: BGFA10-2**

Colony morphology:

Colour: pink colour

Colony margin/pattern: filiform

Colony surface: velvety

Pigment: no

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed.....Deuteromycetes

6. **Isolated code: BGFA10-3**

Colony morphology:

Colour: white colour with green colour rings present

Colony margin/pattern: filiform

Colony surface: velvety

Pigment: no

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: without inflated apical cells
3. Conidia: hyaline

4. Conidia not globose
5. Conidia boat-shaped or lunate, with or without appendage not so
6. Conidia clavate not so
7. Conidiophores well developed
8. Conidia: wet
9. Spore formed at apical parts of conidiophores
10. Conidiophores hyaline
11. Conidiophores irregularly branched.....*Trichoderma*

Key to species:

1. Setae like hyphae: lacking conidia ovate or others
2. Conidia: globose.....*Trichoderma harzianum*

7. Isolated code: BGFA10-4

Colony morphology:

Colour: white to off white colony reverse pale yellow colour

Colony margin/pattern: lobated

Colony surface: velvety

Pigment: no

Microscopic morphology:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidiospores; formed.....Deuteromycetes

8. Isolated colony: BGFA10-5

Colony morphology:

Colour: dark green colour and reverse yellow colour

Colony margin/pattern: undulated

Colony surface: powdery

Pigment: no

Microscopic morphology:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidiospores; formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: with inflated apical cells bearing numerous phialides.....*Aspergillus*

Key to species

1. Spore masses: green
2. Spore masses: radiate, yellowish green.....*Aspergillus parasiticus*

(Figure: 5)

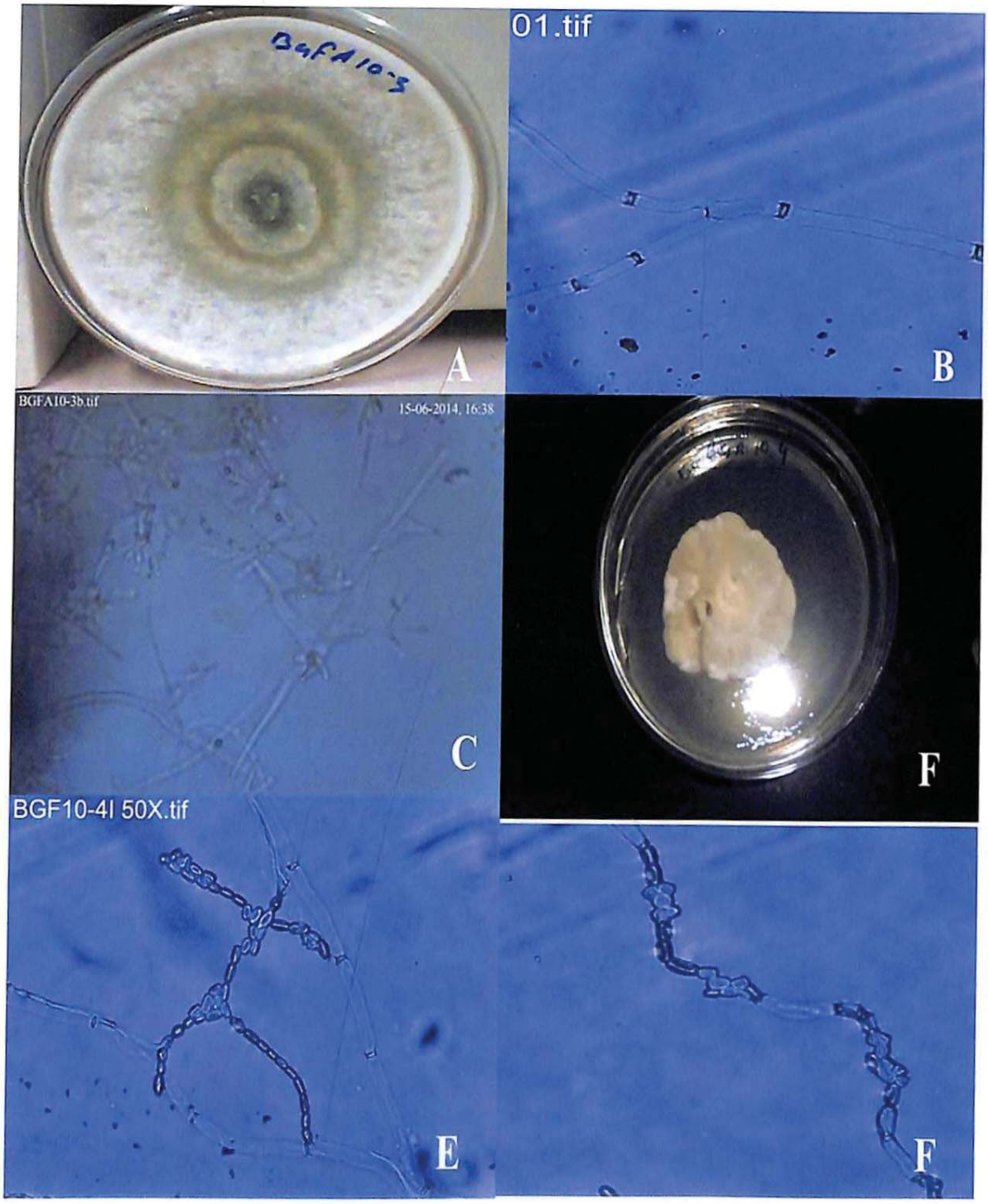


Figure 5: A, F: Colony morphology of isolates BGFA10-3and BGFA10-4 in PDA. B: Septate hyphae BGFA10-3 500X, C: Conidiospores of BGFA10-3 500X, E, F: Conidiospores of BGFA10-4 500X.

9. Isolated colony: BGFA20-1

Colony morphology:

Colour: white with dark green colour reverse off white colour.

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidiospores; formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: with inflated apical cell bearing numerous phialide.....*Aspergillus*

Key to species:

1. Spore masses: green
2. Spore masses: cylindrical, dark green
3. Conidia: under 3µ m in diameter
4. Spore masses: 175-244 µm long.....*Aspergillus fumigants*

10. Isolated code: BGFA20-2

Colony morphology:

Colour: white colour with violet colour and reverse dark violet colour.

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia.....Deuteromycetes

11. Isolated code: BGFA20-3

Colony morphology:

Colour: white colour with green colour reverse pale yellow colour

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia..... Deuteromycets

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type.....Phialosporae

Phialosporae:

1. Conidia:1 celled
2. Conidiophores: with inflated apical cells bearing numerous phialides.....*Aspergillus*

Key to species:

1. Spore mass: not green
2. Spore mass: yellow green colour.....*Aspergillus sp.*

12. Isolated code: BGFA20-4

Colony morphology:

Colour: yellow colour with black colour

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2 Hyphae: without clamp connection
3 Spores: formed
4 Spores: conidia ..... Deuteromycets

Key to deuteromycetes:

1 Conidiomata: not formed
2 Conidia: formed
3 Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1 Conidia:1 celled
2 Conidiophores: with inflated apical cells bearing numerous phialides ..... *Aspergillus*

Key to species:

1. Spore mass: not green
2. Spore mass: black ..... *Aspergillus niger*

(Figure: 6)

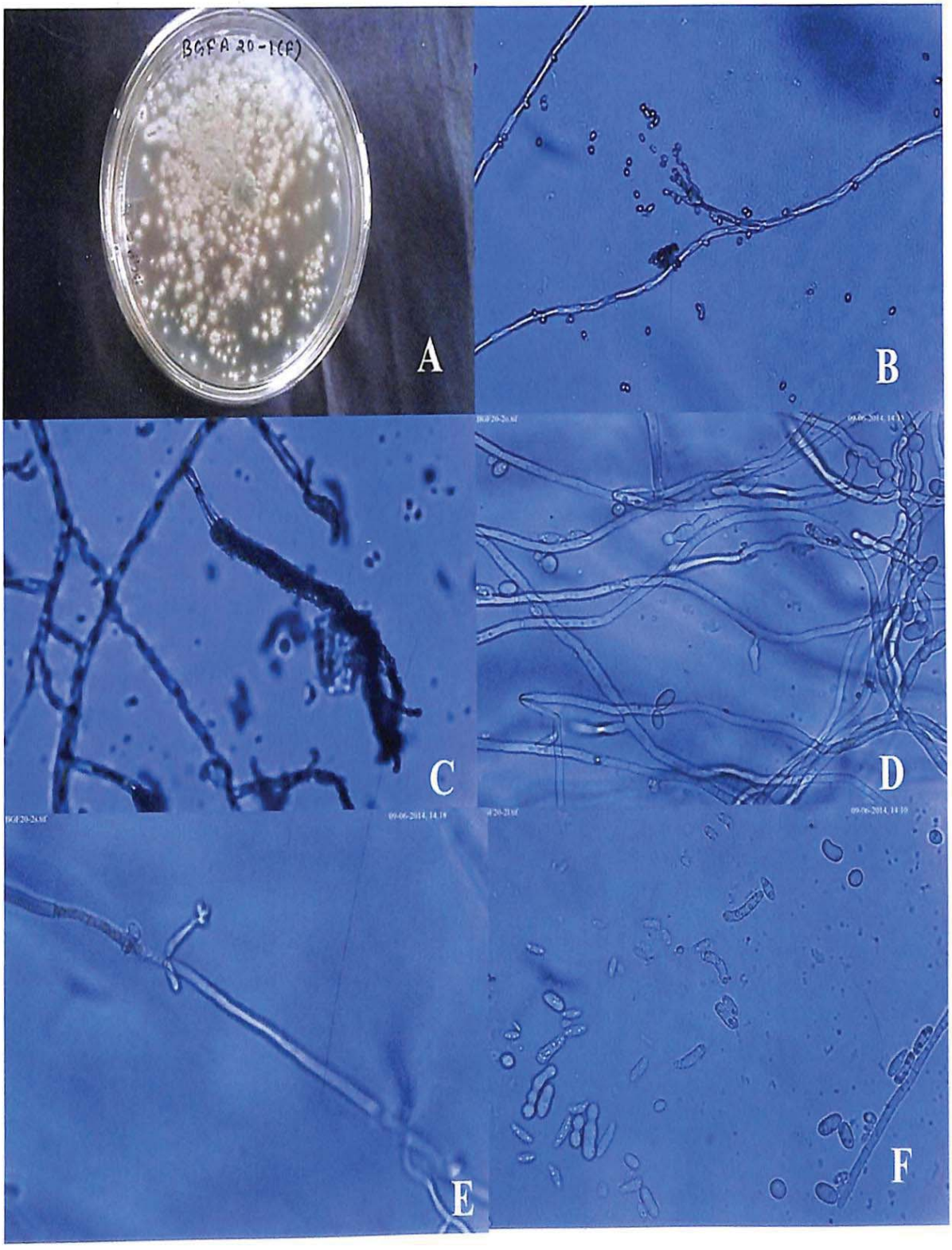


Figure 6: A: Colony morphology of isolates BGFA20-1 on PDA. B: Conidiospores of BGFA20-1 200X. C: Conidiospores and spore mass of BGFA20-1 500X. D-F: Conidiospores of BGFA 20-2 500X.

13 Isolated code: BGFA20-5

Colony morphology:

Colour: white with dark green colour reverse off white colour.

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology:

1. Hyphae: septate
2. Hyphae: with clamp connection ..... Basidiomycetes

14 Isolated code: BGFA20-6

Colony morphology:

Colour: white colour colony with yellow spores

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: aseptate
2. Sporangiospores: formed ..... Zygomycetes

Key to zygomycets:

1. Vesicles: not formed
2. Sporangia: globose
3. Sporangia: without apophysis
4. Sporangia: without collumellate ..... *Mortierella*

16 Isolated code: BGFB10-2

Colony morphology:

Colour: white colour with violet colour and reverse dark violet colour

Colony margin/pattern: lobated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia.....Deuteromycetes

Key to deuteromycetes:

1. conidiomata: not formed
2. Conidia: formed
3. Conidia:phialospore-type .....Phialosporae

Phialosporae

1. Conidia: more than 2 celled
2. Conidiophores: not penicillate with stipe and terminal vesicles
3. Conidia: not formed in long chains, dark
4. Conidia: lunate with a foot cell.....*Fuasrium*

Key to species:

1. Microcondia: formed
2. Microconidia: not catenulate, chlamydospores formed
3. Chlamydospores: solitary or twins
4. Conidiophores: shorter than

macroconidium width.....*F. oxyporum*

17 Isolated code: BGB10-3

Colony morphology:

Colour: white with dark grey colour

Colony margin/pattern: undulated

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidiospores; formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: with inflated apical cell bearing numerous phialide.....*Aspergillus*

18 Isolated code: BGFB20-1

Colony morphology:

Colour: white colour with dark grey colour spores and reverse cream colour

Colony margin/pattern: entire, radiated

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore;conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: formed
2. Conidia: formed
3. Conidia: phialosporae type.....Phialosporae

Phialospore:

1. Conidia: 1celled
2. Conidiophores: without inflated apical cells
3. Conidia: hyaline
4. Conidia: not so globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: limoniform, conidiophores poorly penicillate.....*Paecilomyces*

Key to species:

1. Phialides: verticillate .....*P. puntonii*

19 Isolated code: BGFB20-2

Colony morphology:

Colour: dark green colour and reverse off white colour

Colony margin/pattern: undulated

Colony surface: powdery

Microscopic morphology:

1. Hyphae: septate
2. hyphae:

Key to class

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore:conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type.....Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate..... *Penicillium*

(Figure: 7)

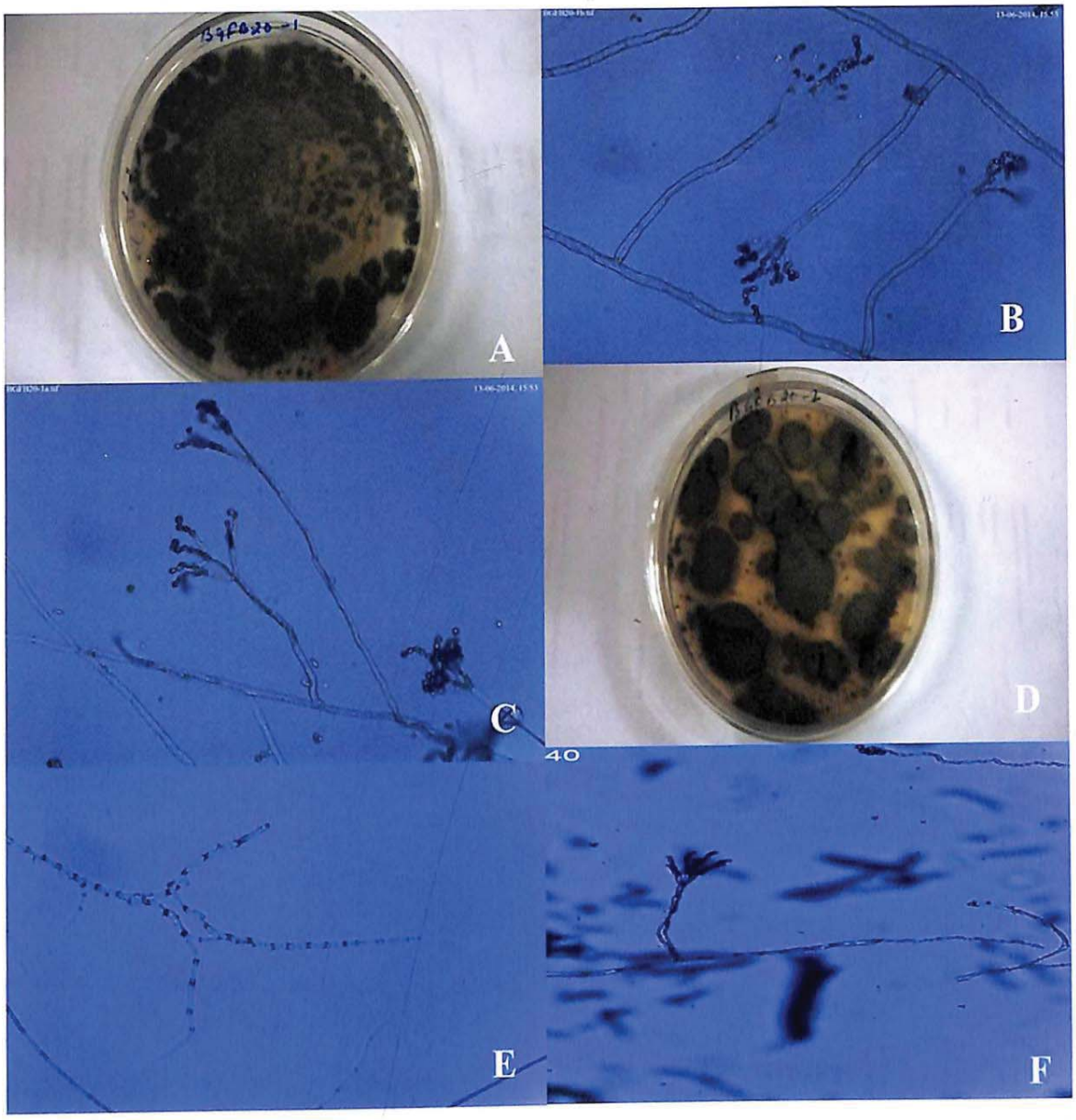


Figure 7: A, D: Colony morphology of isolates BGFA20-1 and BGFA20-2 on PDA. B, C: Conidiospores and conidia of BGF20-1 500X, E: Septate hyphae of BGFA20-2 500X, F: Conidiospores of conidia with BGFA20-2 500X.

20 Isolated code: BGFB20-3

Colony morphology:

Colour: white colour cottony

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: none
4. Sclerotia and other organs: formed
5. Sclerotia: well differentiated
   with rind and medulla.....*Sclerotium sp.*

21 Isolated code: BGFB20-4

Colony morphology:

Colour: dark green colour and reverse off white colour

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore:conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type.....Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

(Figure: 8)

22 Isolated code: BGFB20-5

Colony morphology:

Colour: white colour and reverse yellow colour

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: aseptate
2. Sporangiospores: formed.....Zygomycetes

Key to zygomycetes:

1. Vesicles: not formed
2. Sporangis: globose
3. Sporangia: with apophysis
4. Apophysis: globose .....*Gongronella*

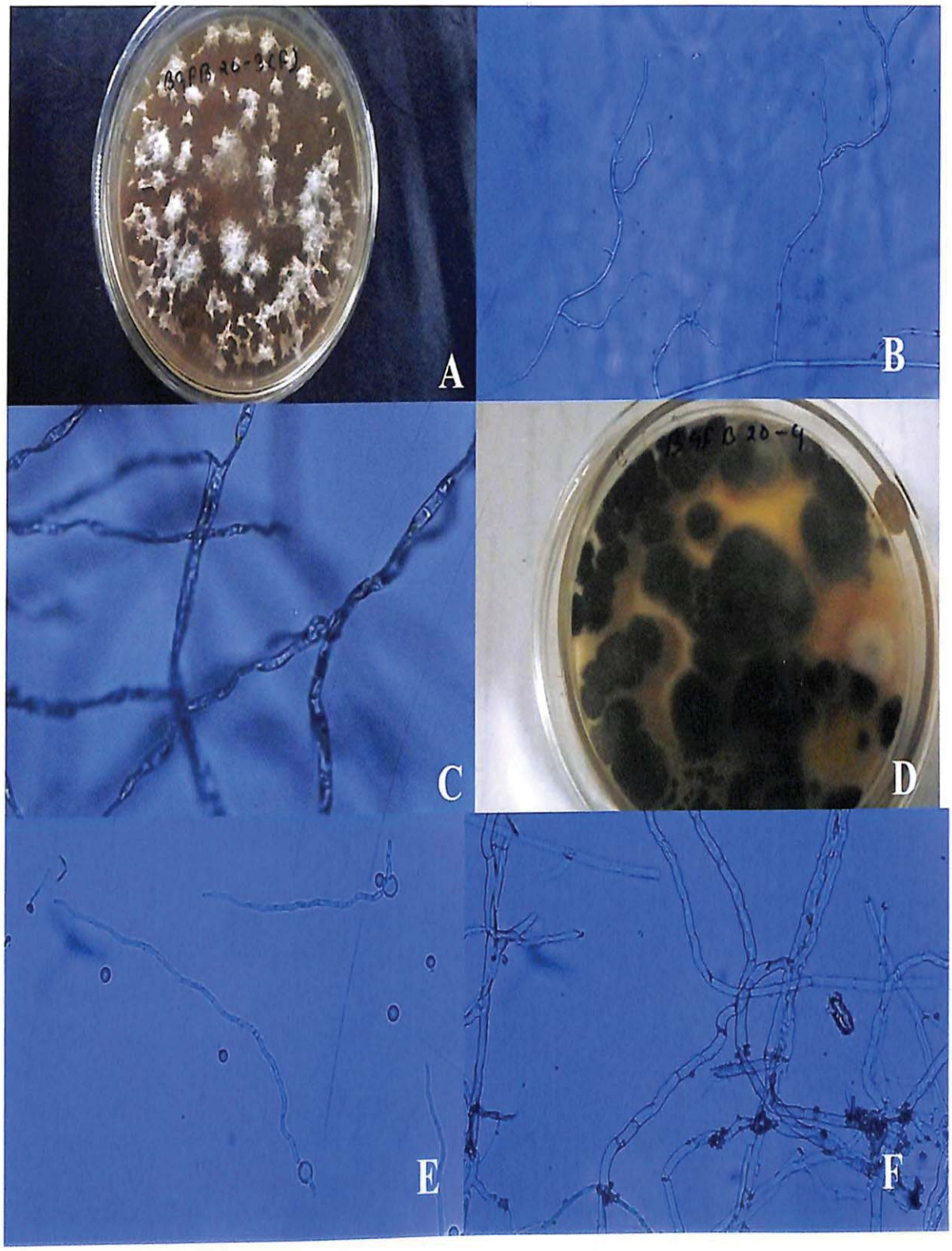


Figure 8: A, D: Colony morphology of isolates BGFB20-3 and BGFB20-4 on PDA. B, C: Septate hyphae of BGFB20-3 200 X. E: Germination of spores of BGFB20-4 200X, F: Septate hyphae of BGFB20-4 500X.

23 Isolated code: BGFB20-6

Colony morphology:

Colour: pink colour with white colour cottony growth and reverse cream colour

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology:

Key to class

1 Hyphae: septate

2 Hyphae: without clamp connection

3 Spores: formed

4 Spore: conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

Key to species:

1. Colonies on PDA: Pinkish tinted
2. Conidia ellipsoidal, 2.8–3.5 µm in diameter . . . . . *P. corylophilum*

(Figure: 9)

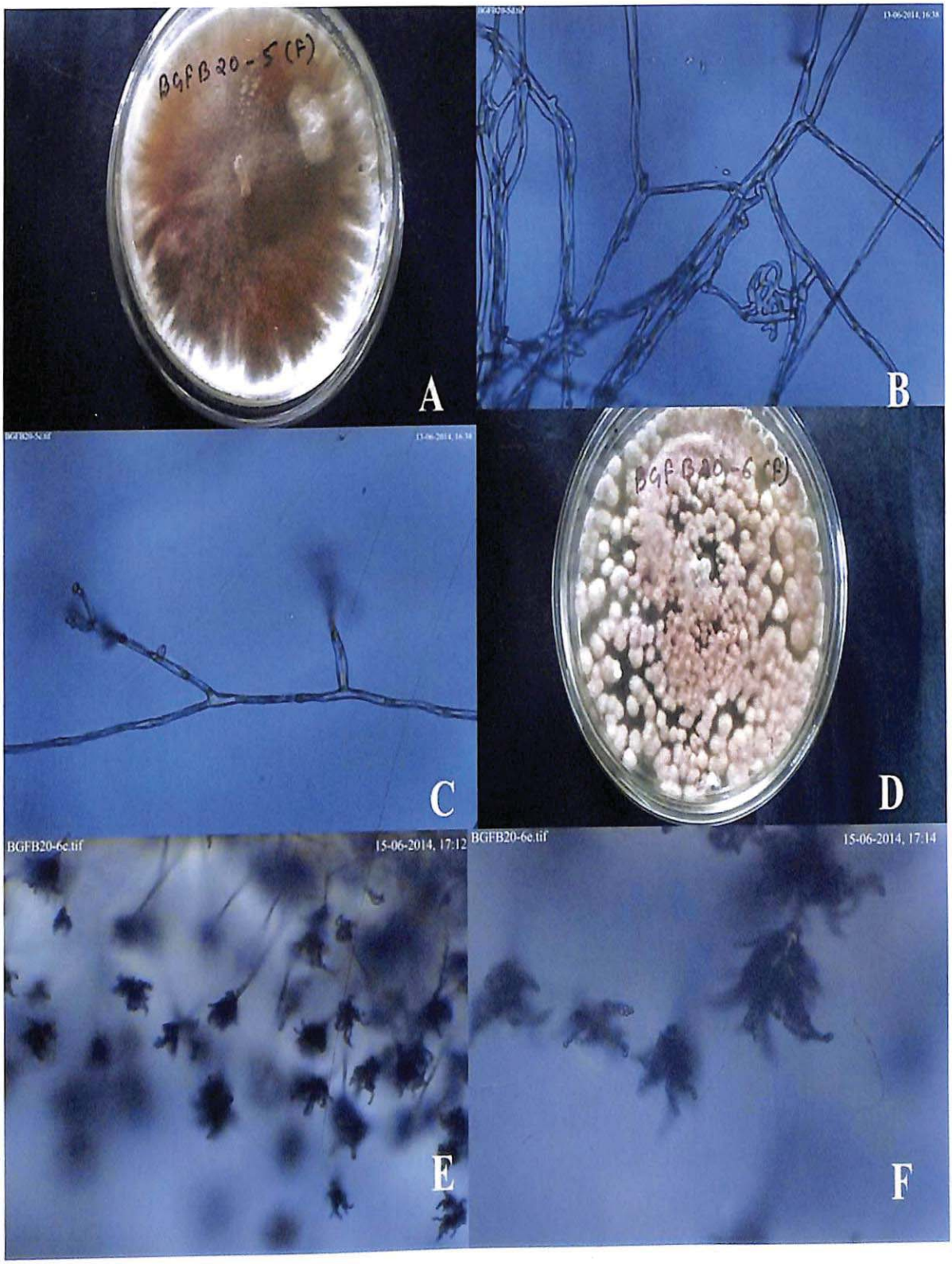


Figure 9: A, D: Colony morphology of isolates BGFB20-5 and BGFB20-6 on PDA. B: Septate hyphae of BGFB20-5 500X. C: Conidiospores with conidia of spores of BGFB20-5 500X, E: Conidiospores with conidia of BGFB20-6 200X, E: Conidiospores with conidia of BGFB20-6 500X.

24 **Isolated code: BGFB20-7**

Colony morphology:

Colour: Dark green colour and reverse yellow colour

Colony margin/pattern: undulated

Colony surface: powdery

Microscopic morphology:

Key to class

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore;conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type .....Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

Key to Species:

1. Colonies on Czapex agar reddish tinted not so
2. Conidia: globose
3. Conidiophores: under 100 µm,
   with pen-shaped phialides ..... *P. lanosum*

25 Isolated code: BGFB20-8

Colony morphology:

Colour: dark grey colour and reverse cream colour

Colony margin/pattern: entire

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore:conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type .....Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

(Figure: 10)

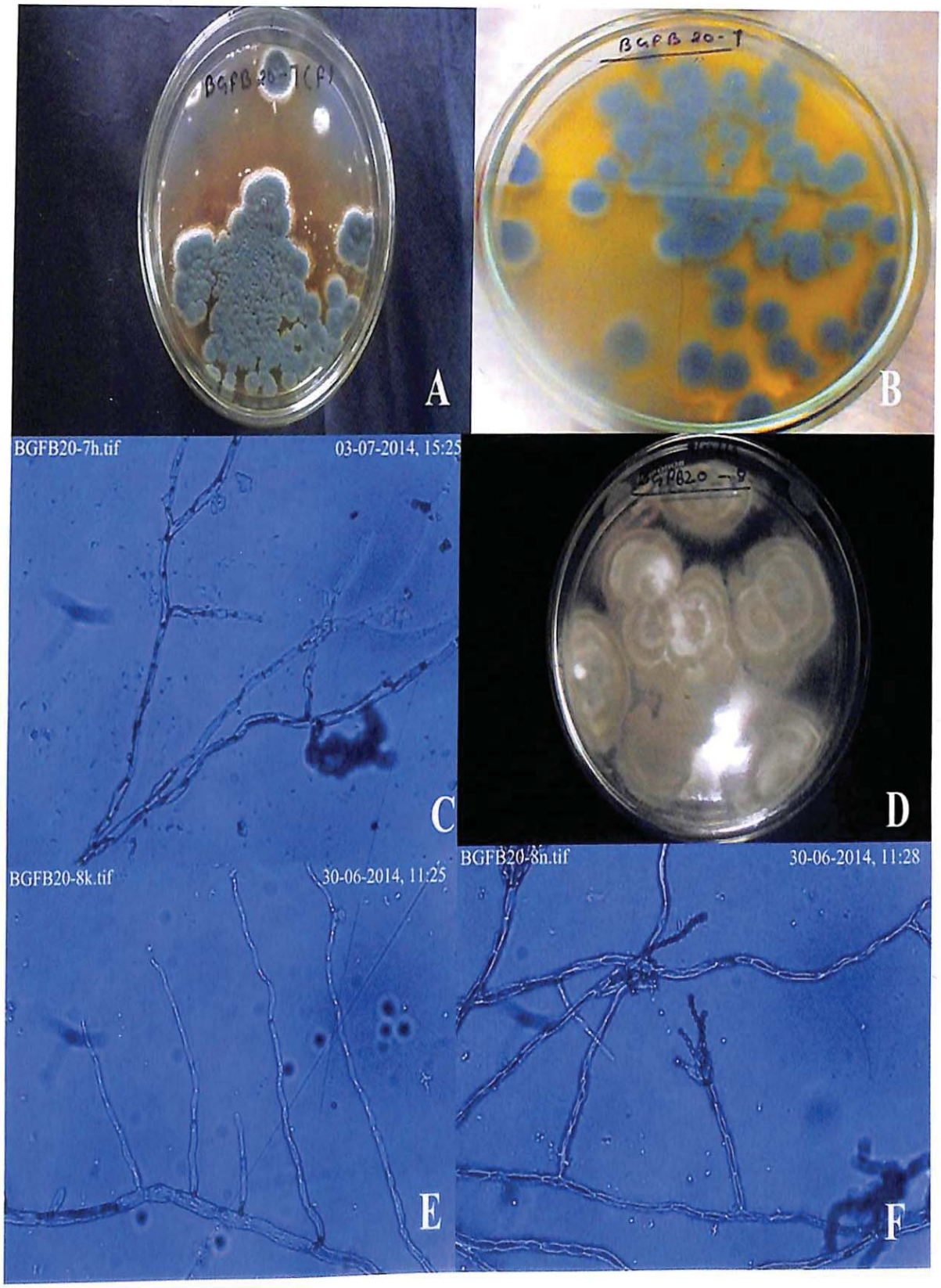


Figure 10: A, B: Colony morphology of isolates BGFB20-7 and BGFB20-7 on PDA. B: Yellow pigment production of BGFB20-7. C, E: Septate hypahe of BGF2B20-7 200X, BGFB20-8 500X. F: Conidiospores with conidia of BGFB20-8 500X.

26 Isolated code: BGFB20-9

Colony morphology:

Colour: Light greenish yellow to olive and may have a white border

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology:

Key to class:

1 Hyphae: septate

2 Hyphae: without clamp connection

3 Spores: formed

4 Spores: conidia ..... Deuteromycets

Key to deuteromycetes:

1 Conidiomata: not formed

2 Conidia: formed

3 Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1 Conidia:1 celled

2 Conidiophores: with inflated apical cells bearing numerous phialides..... *Aspergillus*

27 Isolated code: BGFB20-10

Colony morphology:

Colour: white colour cottony

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: none
4. Sclerotia and other organs: formed
5. Sclerotia: not well differentiated with rind and medulla
6. Hyphae: constricted near branching area..... *Rhizoctonia*

(Figure: 11)

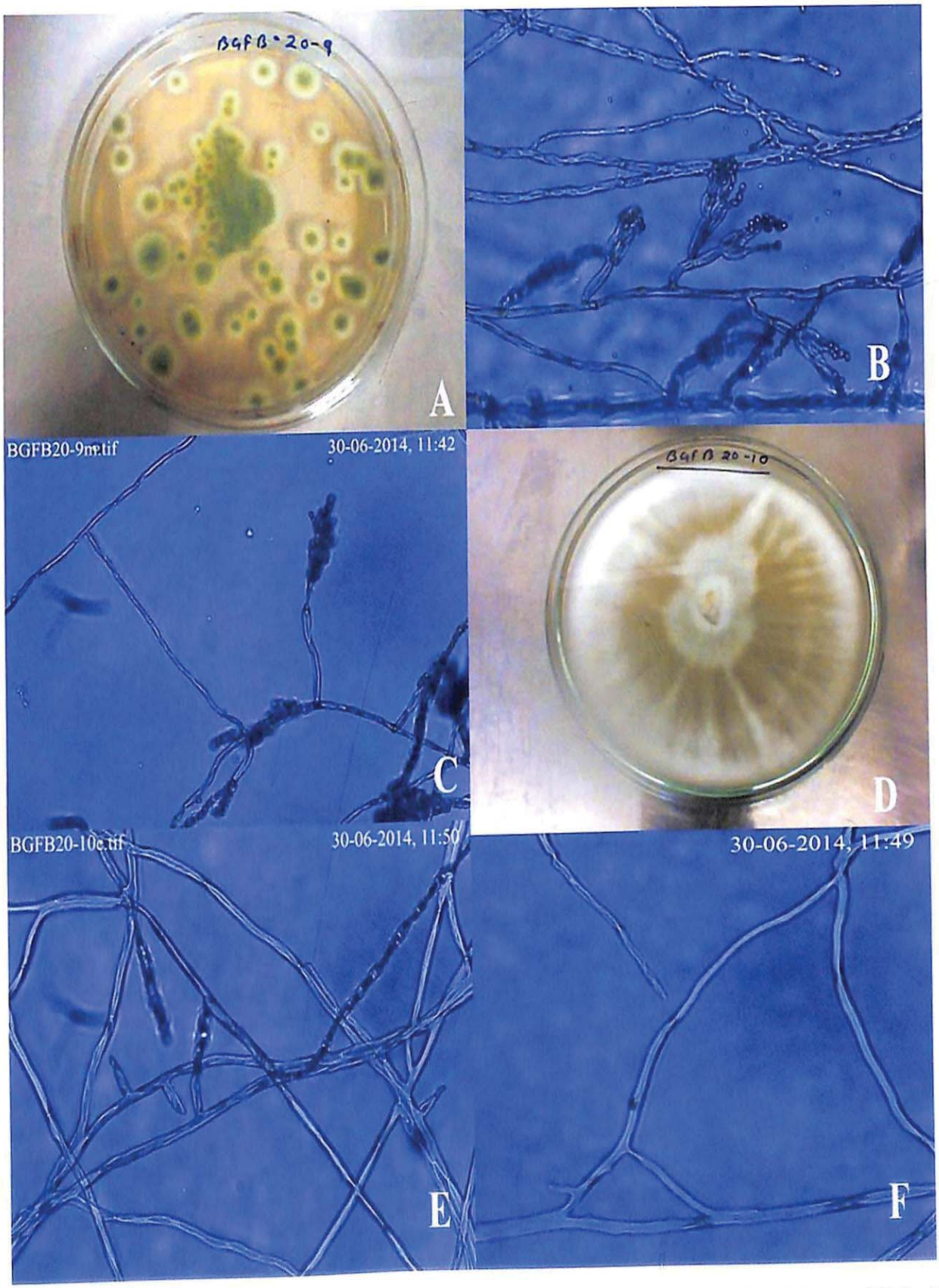


Figure 11: A, D: Colony morphology of isolates BGFB20-9 and BGFB20-10 on PDA. B: Phialide and conidia oh BGFB20-9 500X, C: Conidiophores with spore mass of BGFB20-9 500X, E, F: Septate hypahe of, BGFB20-10 500X.

28 Isolated code: BGFB20-11

Colony morphology:

Colour: Dark green grey spores surrounded by white colour and reverse white colour

Colony margin/pattern: Undulated

Colony surface: powdery

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore; conidia.....Deuteromycete

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

29 Isolated code: BGFC10-1

Colony morphology:

Colour: White colour with dark light yellow grey colour spores reverse dark brown colour.

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophore
12. densely penicillate..... *Penicillium*

Key to species:

1. Colonies on PDA: not reddish
2. Conidia: ellipsoidal, apiculate at one end..... *Penicillium janthinellum*

(Figure: 12)

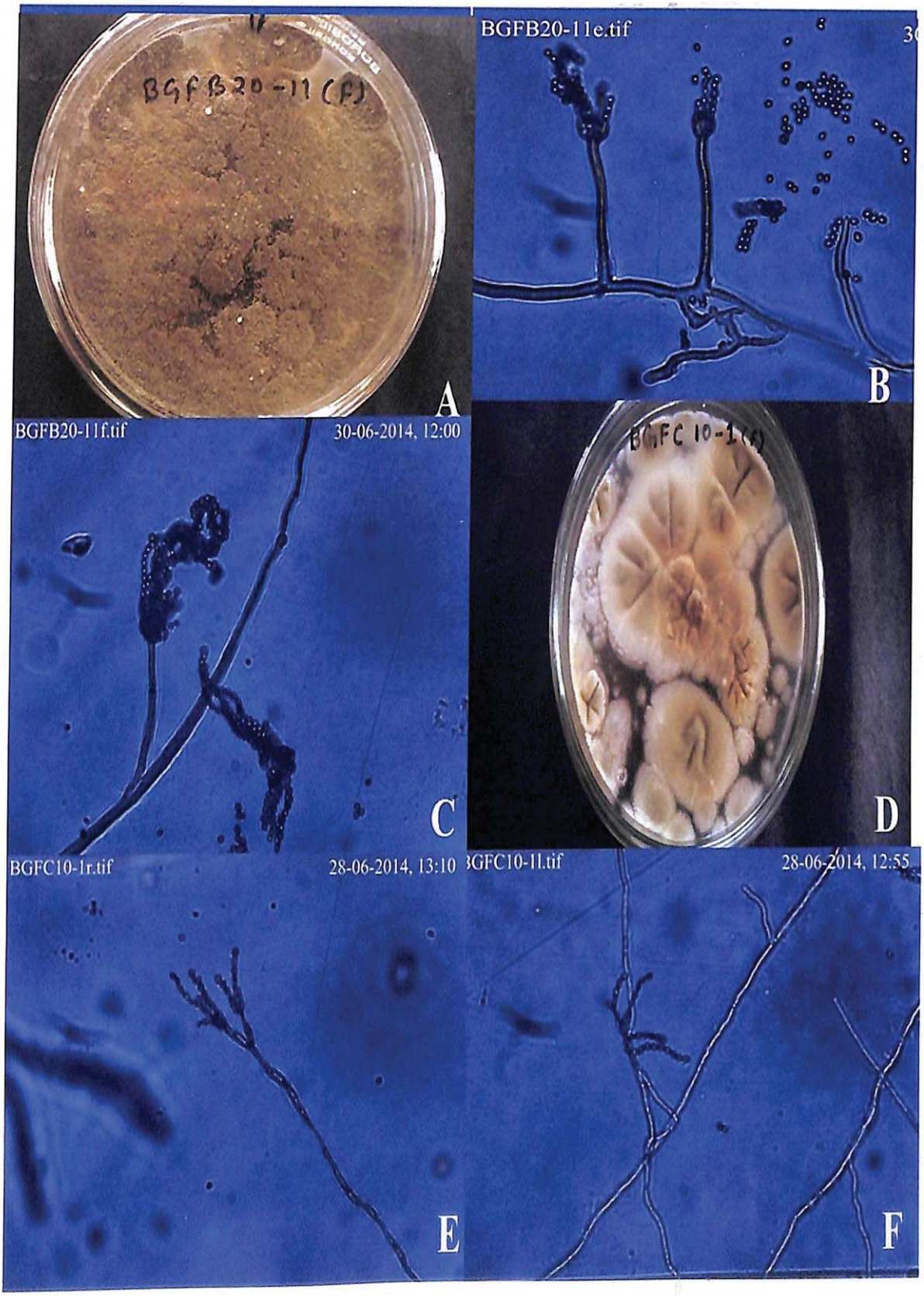


Figure 12: A, D: Colony morphology of isolates BGFB20-11 and BGFC10-1 on PDA. B: Phialide and conidia of BGFB20-11 500X, C: Conidiophores with spore mass of BGFB20-11 500X, E, F: Conidiophores with conidia of BGFC10-1 500X.

30 Isolated code: BGFC10-2

Colony morphology:

Colour: White colour with green colour spores and reverse dark yellow colour

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia .... Deuteromycetes

Key to deuteromycetes

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type Phialosporae

Phialosporae:

1. Conidia: 1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate..... ..... *Penicillium*

Key to species:

1. Colonies on PDA: not reddish
2. Conidia: globose
3. Conidiophores over 100 µm long, with ampulliform phialides..... *P. nigricans*

31 Isolated code: BGFC10-3

Colony morphology:

Colour: White colour with dark green rings and reverse cream colour

Colony margin/pattern: undulated

Colony surface: Velvety

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore: conidia.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia:1-celled
2. Conidiophores: without inflated apical cell
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: globose, conidiophores densely penicillate.....*Penicillium*

Key to species:

1. Colonies on PDA: not reddish
2. Conidia: globose
3. Conidiophores over 100 µm long, with ampulliform phialides .....*P. nigricans* (Figure: 13)

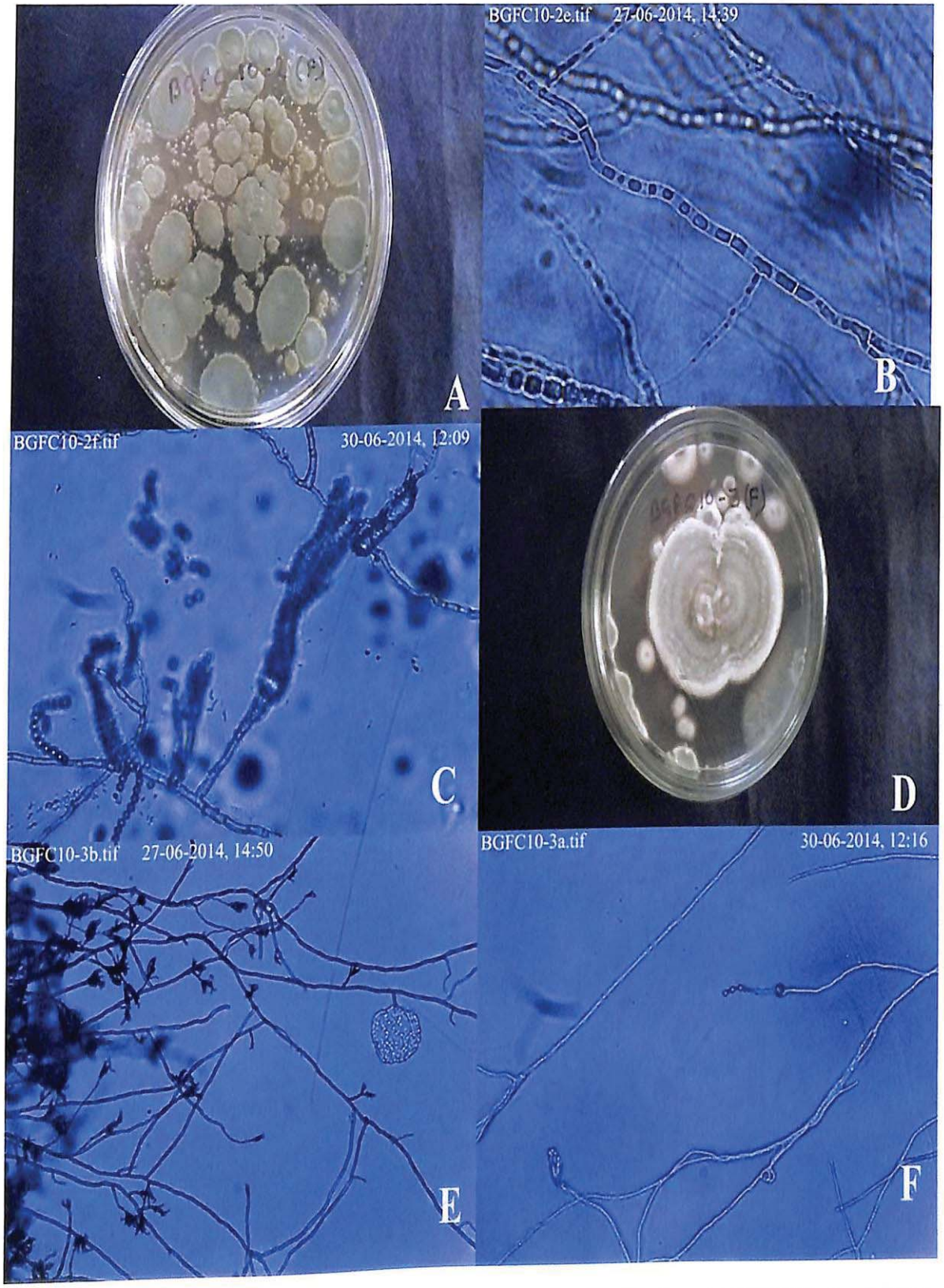


Figure 13: A, D: Colony morphology of isolates BGFC10-2 and BGFC10-3 on PDA. B: Septate hyphae of BGFC10-2 500X, C: Conidiophore with spore mass of BGFC10-2 500X, E: Hyphae with spore of BGFC10-3 100X, F: Conidiophores with conidia of BGFC10-3 200X.

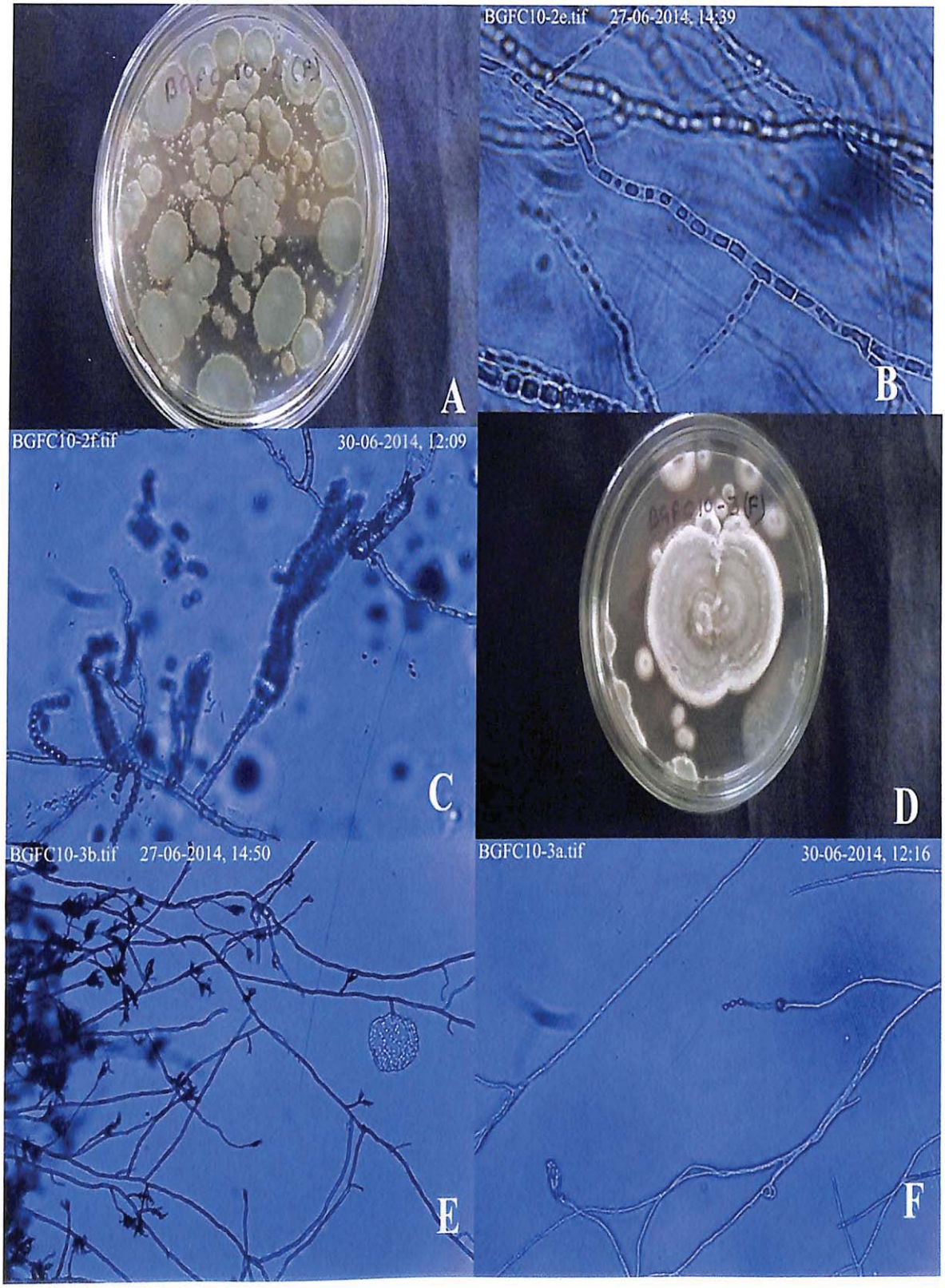


Figure 13: A, D: Colony morphology of isolates BGFC10-2 and BGFC10-3 on PDA. B: Septate hyphae of BGFC10-2 500X, C: Conidiophore with spore mass of BGFC10-2 500X, E: Hyphae with spore of BGFC10-3 100X, F: Conidiophores with conidia of BGFC10-3 200X.

32 Isolated code: BGFC10-4

Colony morphology:

Colour: light brown colour cottony growth and reverse dark brown colour

Colony margin/pattern: lobated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes:

1. conidiomata: formed
2. Conidiomata: sporodochia
   (Or acervuli) formed ..... Sporodochium-former

Sporodochium-Forming Fungi

1. Setae: not formed
2. Conidia: simple
3. Conidia: not with filiform appendages
4. Conidia: 2 celled
5. Conidia: lunar shaped with foot cell ..... *Fusarium*

Key to species:

1. Microcondia: formed
2. Microconidia: not catenulate, chlamydospores formed
3. Chlamydospores: solitary or twins
4. Conidiophores: longer than macroconidium
   length by a few times.
5. Conidiophores mostly branched,
   taller than 100 µm. ..... *F. ventricosum*

33 Isolated code: BGFC10-5

Colony morphology:

Colour: Light brown colour cottony growth and reverse dark brown colour

Colony margin/pattern: lobated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: Phialospore type..... Phialosporae

Phialosporae:

1. Conidia: 1 celled
2. Conidiophores: without inflated apical cells
3. Conidia: hyaline
4. Conidia: not globose
5. Conidia: boat-shaped or lunate, with or without appendage not so
6. Conidia: clavate not so
7. Conidiophores: well developed
8. Conidia: wet
9. Spore mass: formed at apical parts of conidiophores
10. Conidiophores: hyaline
11. Conidiophores: verticillate..... *Verticillium*

(Figure: 14)

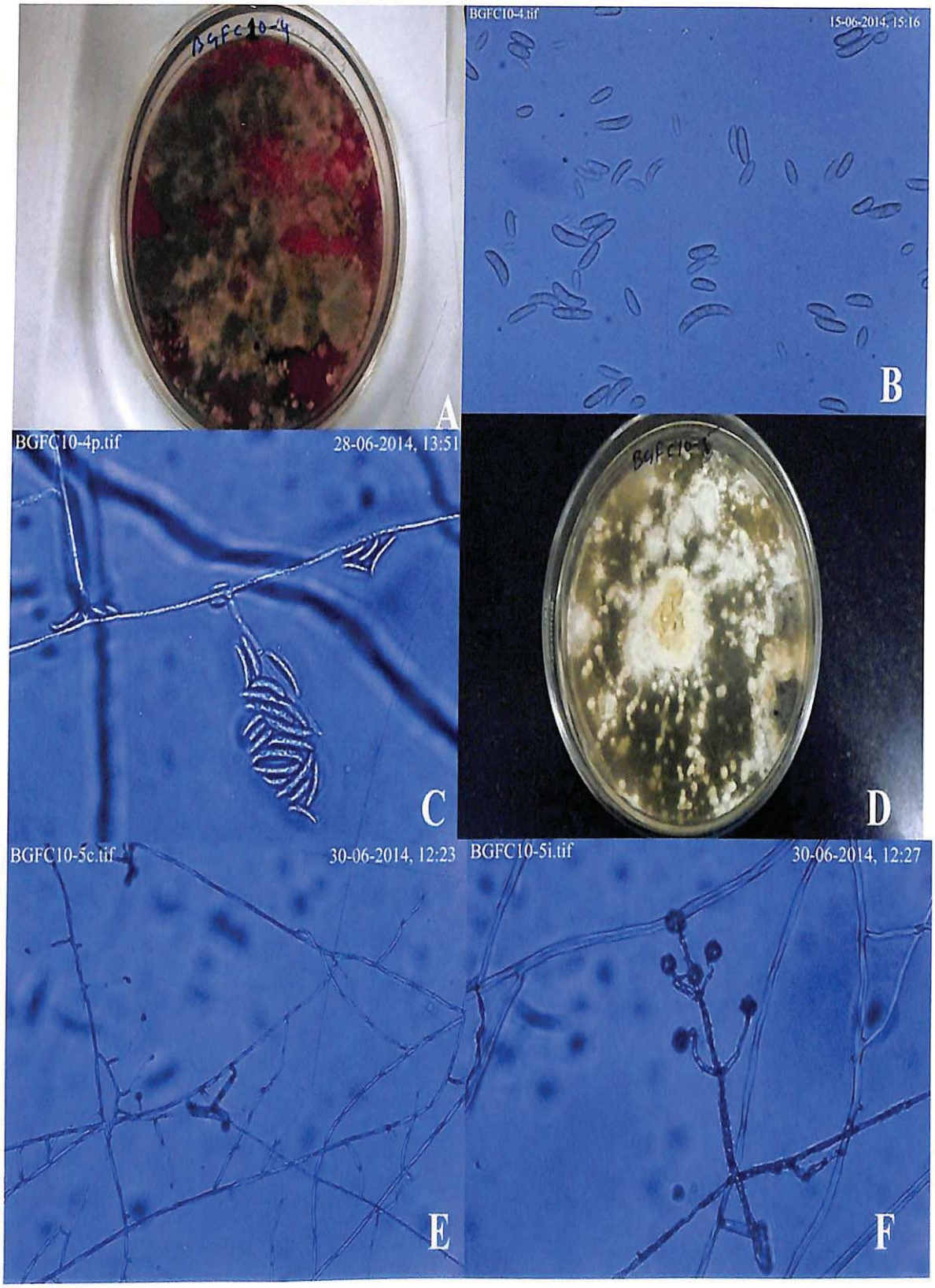


Figure 14: A, D: Colony morphology of isolates BGFC10-4 and BGFC10-5 on PDA. B: spores of BGFC10-4 500X, C: Conidiophore with conidia of BGFC10-4 500X, E: Hyphae with spore of BGFC10-5 100X, F: Conidiophores with conidia of BGFC10-5 500X.

34 Isolated code: BGFC10-6

Colony morphology:

Colour: dark brown colour and reverse black brown colour

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spore:conidia .....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: formed
2. Conidia: formed
3. Conidia: phialosporae .....Phialosporae

Phialospore:

1. Conidia: 1celled
2. Conidiophores: without inflated apical cells
3. Conidia: hyaline
4. Conidia: not so globose
5. Conidia: not boat shaped
6. Conidia: not clavate
7. Conidiophores: well developed
8. Conidia: dry
9. Conidiophores: hyaline, spore aggregate in a row
10. Conidia: not cylindrical
11. Conidia: limoniform, conidiophores poorly penicillate.....*Paecilomyces*

35 Isolated code: BGFC10-7

Colony morphology:

Colour: white colour

Colony margin/pattern: filiform, flowery

Colony surface: cottony

Microscopic morphology:

Key to class:

1 Hyphae: septate

2 Hyphae: without clamp connection

3 Spores: formed

4 Spores: conidia ..... Deuteromycets

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type..... Phialosporae

Phialosporae:

1. Conidia:1 celled
2. Conidiophores: with inflated apical cells bearing numerous phialides..... *Aspergillus*

36 Isolated code: BGFC10-8

Colony morphology:

Colour: White colour cottony

Colony margin/pattern: filiform

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: none
4. Sclerotia and other organs:formed
5. Sclerotia: well differentiated with rind and medulla..... *Sclerotium sp.*

(Figure: 15)

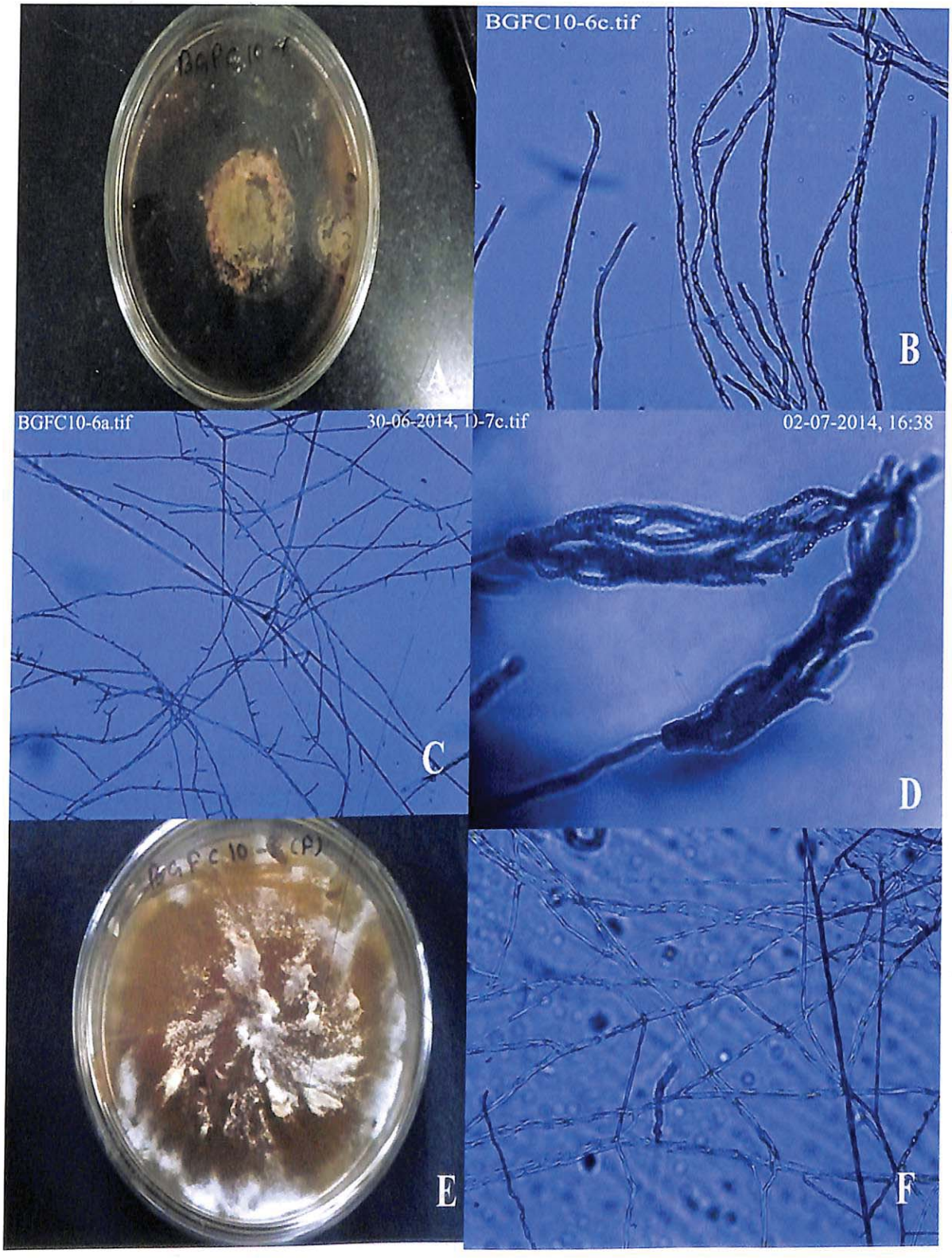


Figure 15: A, E: Colony morphology of isolates BGFC10-6 and BGFC10-8 on PDA. B: Septate hyphae of BGFC10-6 500X. C: Hyphae with spores of BGFC10-6 100X D: Conidiospores with conidia of spores of BGFC10-7 500X, F: Septate hyphae of BGFC10-8 200X.

37 Isolated code: BGFC10-9

Colony morphology:

Colour: White colour with green colour spores

Colony margin/pattern: Filiform, flowery

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia.....Deuteromycetes

Key to deuteromycetes:

1. conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type.....Phialosporae

Phialosporae

1. Conidia: More than 2 celled
2. Conidiophores: not penicillate with stipe and yerminal vesicles
3. Conidia: not formed in long chains, dark
4. Conidia: lunate with a foot cell .....*Fuasrium*

Key to species:

1. Microcondia: formed
2. Microconidia: not catenulate, chlamydospores formed
3. Chlamydospores: solitary or twins
4. Conidiophores: shorter than macroconidium width.....*F. oxyporum*

38 Isolated code: BGFC10-10

Colony morphology:

Colour: Orange colour with dark green colour spores and reverse white colour with light orange colour

Colony margin/pattern: filiform

Colony surface: powdery

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia.....Deuteromycets

Key to deuteromycetes:

1 Conidiomata: not formed

2 Conidia: formed

3 Conidia: phialospore-type.....Phialosporae

Phialosporae:

1. Conidia:1 celled
2. Conidiophores: with inflated apical cells bearing numerous phialides.....*Aspergillus*

(Figure: 16)

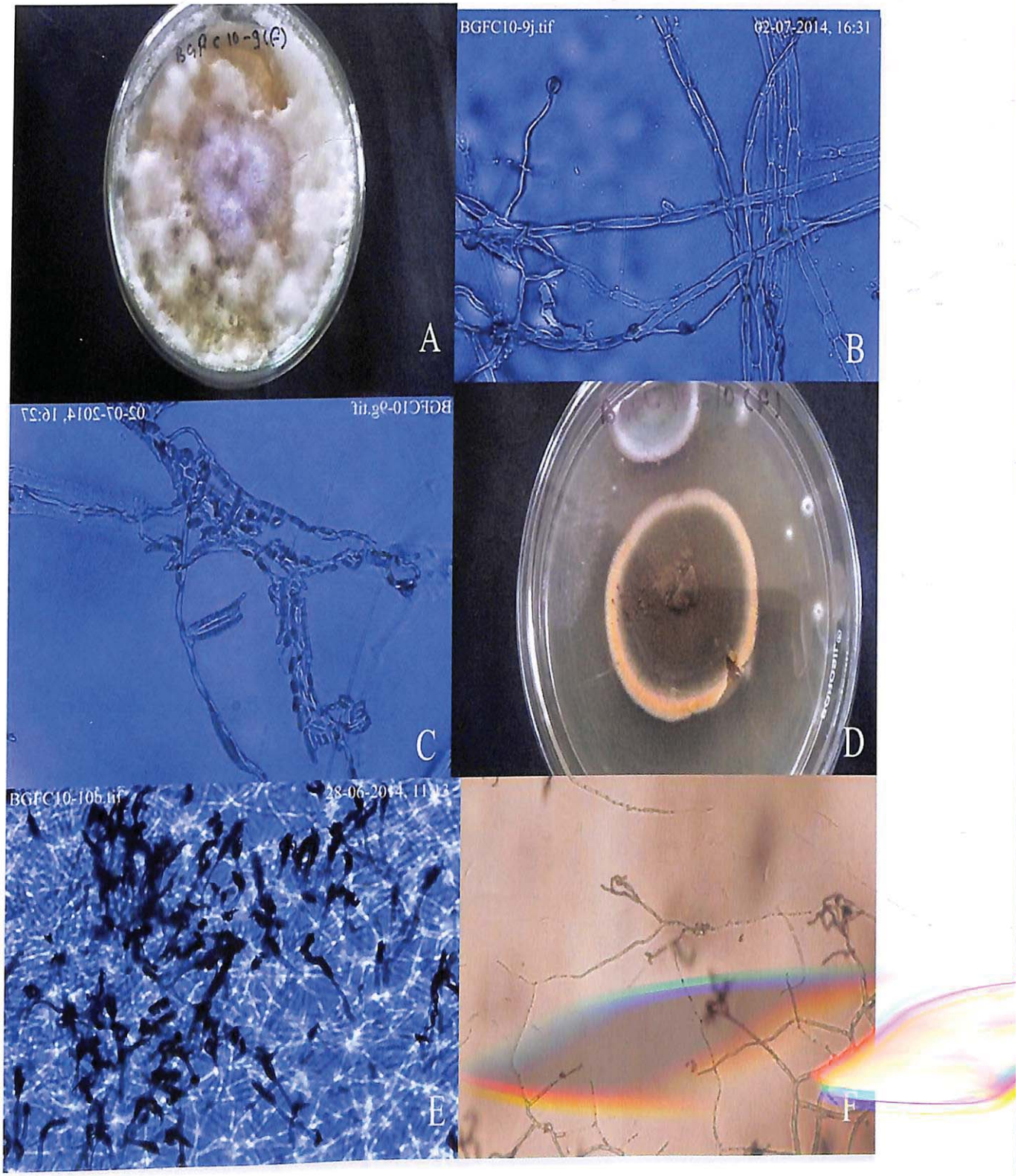


Figure 16: A, D: Colony morphology of isolates BGFC10-9 and BGFC10-10 on PDA. B: Septate hyphae of BGFC10-9 500X. C: Conidiospores with conidia of spores of BGFC10-9 500X, E: Conidiospores with conidia of BGFC10-10, E: Conidiospores with conidia of BGFC10-10 400X.

39 Isolated code: BGFC20-1

Colony morphology:

Colour: white colour with dark green colour spores and reverse pink colour.

Colony margin/pattern: entire

Colony surface: velvety

Microscopic morphology

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed.....Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: formed
2. Conidiomata: synnemata formed.....Synnema former

Synnema forming fungi:

1. Synnema: formed in vitro
2. Seta: formed among synnema, conidia 1-celled .....*Trichurus*

40 Isolated code: BGFC20-2

Colony morphology:

Colour: yellow colour and reverse dark brown colour.

Colony margin/pattern: undulated

Colony surface: shrunken

Microscopic morphology

Key to class:

1. Hyphae: septate
2. Hyphae: without clam connection
3. Spores: formed
4. Conidia: formed .....Deuteromycetes

41 Isolated code BGFC20-3

Colony morphology:

Colour: white colour with grey colour and reverse

Colony margin/pattern: lobated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: arthrospore –type ..... Arthrosporae

Arthrosporae:

1. Conidiophores: poorly developed
2. Conidia: globose
3. Conidia: mainly catenulate ..... *Basipetospora*

(Figure: 17)

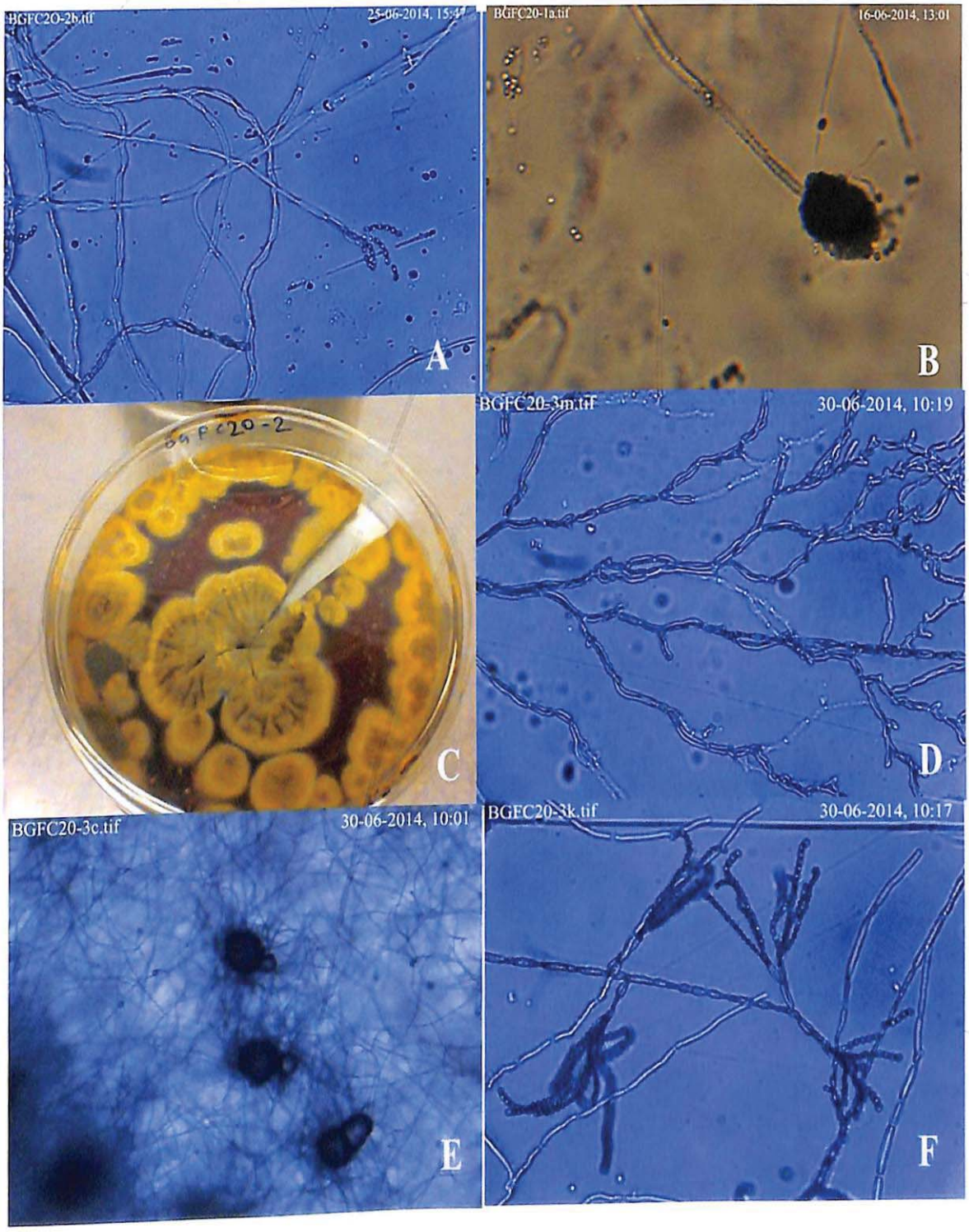


Figure 17: A, B Hyphae with condiospores of BGFC 20-1 500X. C: Colony morphology of isolates BGFC20-2 on PDA. D: Septae hyphae with BGFC20-2 E: Spores with conidia of BGFB20-3 200X, F: Conidiospores with conidia of BGFC20-2 500X.

42 Isolated code: BGFC20-4

Colony morphology:

Colour: Dark black colour colony

Colony margin/pattern: entire

Colony surface: embedded

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes:

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: blastospore-type..... Blastosporae

Blastosporae:

1. Conidiophores: well developed
2. Conidiogenous cells: undifferentiated
3. Conidia: 1-celled
4. Conidia: hyaline
5. Conidia: heterogeneous in size, catenulate in a long chain..... *Monilia*

43 Isolated code: BGFC20-5

Colony morphology:

Colour: White with dark green colour reverse off white colour.

Colony margin/pattern: Entire

Colony surface: Velvety

Microscopic morphology

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Conidiospores; formed.....Deuteromycetes

Key to deuteromycetes:

1 Conidiomata: not formed

2 Conidia: formed

3 Conidia: phialospore type.....Phialosporae

Phialosporae:

1. Conidia: 1 celled
2 Conidiophores: with inflated apical cell bearing numerous phialide.....*Aspergillus*

Key to species:

1 Spore masses: green

2 Spore masses: cylindrical, dark green

3 Conidia: under 3μ m in diameter

4 Spore masses: 175-244 μm long.....*Aspergillus fumigants*

44 Isolated code: BGFC20-6

Colony morphology:

Colour: dark green colour and reverse brown colour

Colony margin/pattern: undulated

Colony surface: cottony

Microscopic morphology:

Key to class:

1. Hyphae: septate
2. Hyphae: without clamp connection
3. Spores: formed
4. Spores: conidia ..... Deuteromycetes

Key to deuteromycetes

1. Conidiomata: not formed
2. Conidia: formed
3. Conidia: phialospore-type ..... Phialosporae

Phialosporae:

1. Conidia: 1-celled
2. Conidiophores: with inflated apical cells bearing numerous phialides ..... *Aspergillus* (Figure: 18)

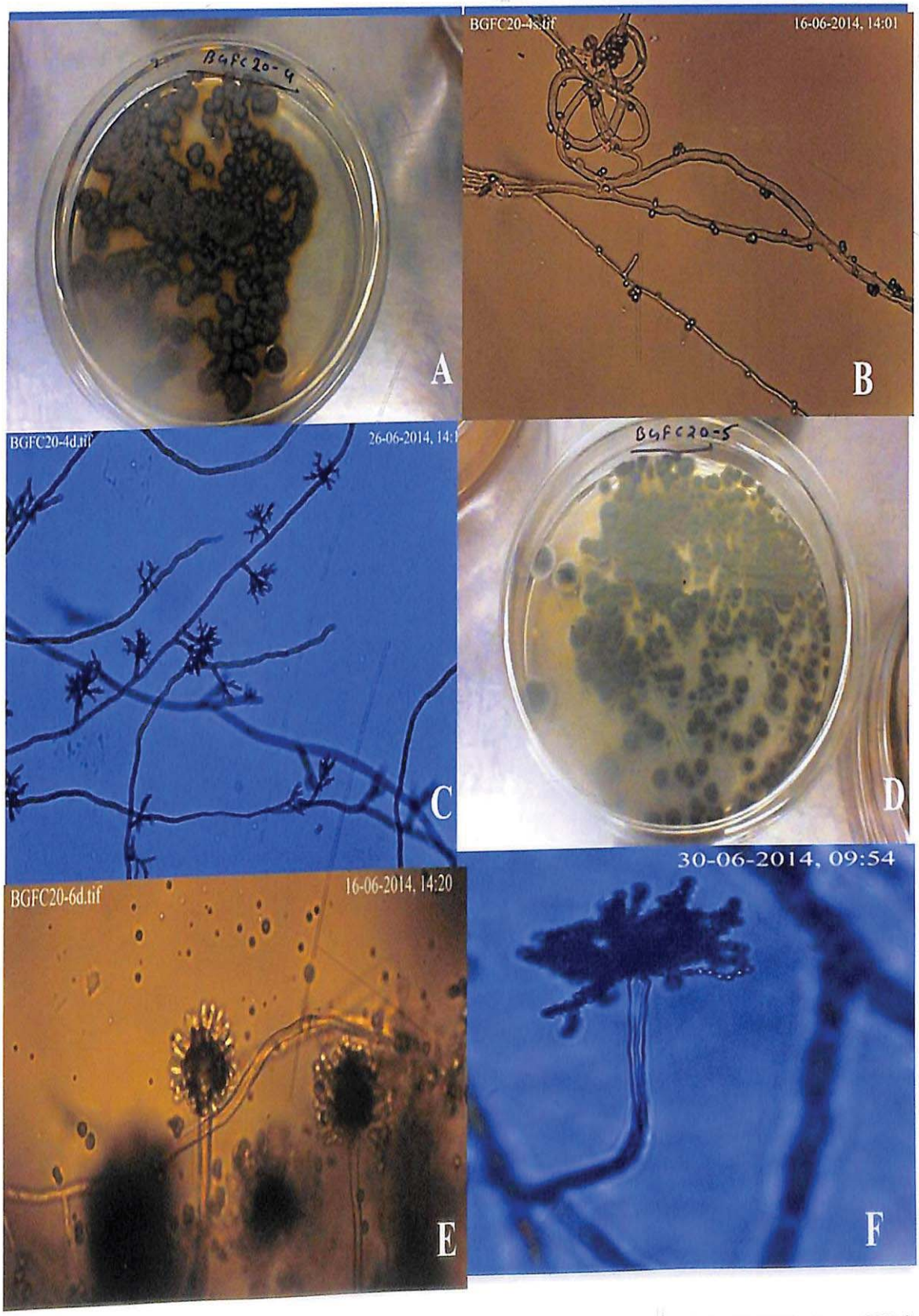


Figure 18: A, D: Colony morphology of isolates BGFC20-4 and BGFC20-6 on PDA. B: Hyphae with spores of BGFC20-4 200X. C: Conidiospores with conidia of spores of BGFC20-4 500X, E, F: Conidiospores with conidia of BGFC20-6 200X & 500X.

Table 7: Presumptive identification of fungal isolates

Sl. No	Strain code	Class	Presumptive identification
1	TF10-1	Deuteromycetes	*Aspergillus terreus*
2	TF10-2	Deuteromycetes	*Trichoderma*
3	TF20-1	Deuteromycetes	*Fumago* sp.
4	BGFA10-1	Deuteromycetes	*Mucor hiemalis*
5	BGFA10-2	Deuteromycetes	Not identified
6	BGFA10-3	Deuteromycetes	*Trichoderma harzianum*
7	BGFA10-4	Deuteromycetes	Not identified
8	BGFA10-5	Deuteromycetes	*Aspergillus parasiticus*
9	BGFA20-1	Deuteromycetes	*Aspergillus fumigants*
10	BGFA20-2	Deuteromycetes	Not identified
11	BGFA20-3	Deuteromycetes	*Aspergillus sp.*
12	BGFA20-4	Deuteromycetes	*Aspergillus niger*
13	BGFA20-5	Basidiomycetes	Not identified
14	BGFA20-6	Zygomycetes	*Mortierella*
15	BGFB10-1	Deuteromycetes	*Penicillium sp.*
16	BGFB10-2	Deuteromycetes	*F. oxyporum*
17	BGFB10-3	Deuteromycetes	*Aspergillus*
18	BGFB20-1	Deuteromycetes	*Paecilomyce puntonii*
19	BGFB20-2	Deuteromycetes	*Penicillium*
20	BGFB20-3	-	*Sclerotium sp.*
21	BGFB20-4	Deuteromycetes	*Penicillium*
22	BGFB20-5	Zygomycetes	*Gongronella*
23	BGFB20-6	Deuteromycetes	*Penicillium corylophilum*
24	BGFB20-7	Deuteromycetes	*Penicillium lanosum*
25	BGFB20-8	Deuteromycetes	*Penicillium sp.*
26	BGFB20-9	Deuteromycetes	*Aspergillus*
27	BGFB20-10	-	*Rhizoctonia*
28	BGFB20-11	Deuteromycetes	*Penicillium sp.*
29	BGFC10-1	Deuteromycetes	*Penicillium janthinellum*
30	BGFC10-2	Deuteromycetes	*Penicillium sp.*

Table 7: Continue

Sl. No	Strain code	Class	Presumptive identification
31	BGFC10-3	Deuteromycetes	*Penicillium nigricans*
32	BGFC10-4	Deuteromycetes	*Fusarium ventricosum*
33	BGFC10-5	Deuteromycetes	*Verticillium*
34	BGFC10-6	Deuteromycetes	*Paecilomyces*
35	BGFC10-7	Deuteromycetes	*Aspergillus*
36	BGFC10-8	Deuteromycetes	*Sclerotium sp*
37	BGFC10-9	Deuteromycetes	*Fusarium. oxyporum*
38	BGFC10-10	Deuteromycetes	*Aspergillus*
39	BGFC20-1	Deuteromycetes	*Trichurus*
40	BGFC20-2	Deuteromycetes	Not identified
41	BGFC20-3	Deuteromycetes	*Basipetospora*
42	BGFC20-4	Deuteromycetes	*Monilia*
43	BGFC20-5	Deuteromycetes	*Aspergillus fumigants*
44	BGFC20-6	Deuteromycetes	*Aspergillus*

4.3 Effect of various ranges of temperature on the fungal growth

Temperature is most important physical environmental factor for regulating the growth and reproduction of which all fungi grow well. The fungal isolate were incubated at 20°C, 30°C, 40°C, 50°C and 60°C at different temperature. They were kept for 8 days incubation. After incubation the growth were observed. All the data has been noted in table: 8 According to data observe that all the fungal isolates grow 20°C to 30°C.

4.4 Effect of the various ranges of pH on the fungal growth

pH conditions are most important physical environmental factor for regulating the fungal growth. The fungal isolate were incubate at different pH 5.0, 6.0, 7.0, 8.0 and 9.0 they were kept for 8 days incubation. After incubation the growth were observed. All the data has been noted in table: 9.

4.5 Screening of the fungal isolates for antibacterial activity assay

The present investigation leads to the antibacterial activities of the isolated fungal species were tested distinctly against human bacterial pathogens in table: 10.

Table 8: Effect of the various ranges of temperature on the fungal growth.

Sl. No	Strain code	20°C	30°C	40°C	50°C	60°C
1	TF10-1	+ve	+ve	-ve	-ve	-ve
2	TF10-2	+ve	+ve	-ve	-ve	-ve
3	TF20-1	+ve	+ve	-ve	-ve	-ve
4	BGFA10-1	+ve	+ve	-ve	-ve	-ve
5	BGFA10-2	+ve	+ve	-ve	-ve	-ve
6	BGFA10-3	+ve	+ve	-ve	-ve	-ve
7	BGFA10-4	+ve	+ve	-ve	-ve	-ve
8	BGFA10-5	+ve	+ve	-ve	-ve	-ve
9	BGFA20-1	+ve	+ve	-ve	-ve	-ve
10	BGFA20-2	+ve	+ve	-ve	-ve	-ve
11	BGFA20-3	+ve	+ve	-ve	-ve	-ve
12	BGFA20-4	+ve	+ve	-ve	-ve	-ve
13	BGFA20-5	+ve	+ve	-ve	-ve	-ve
14	BGFA20-6	+ve	+ve	-ve	-ve	-ve
15	BGFB10-1	+ve	+ve	-ve	-ve	-ve
16	BGFB10-2	+ve	+ve	-ve	-ve	-ve
17	BGFB10-3	+ve	+ve	-ve	-ve	-ve
18	BGFB20-1	+ve	+ve	-ve	-ve	-ve
19	BGFB20-2	+ve	+ve	-ve	-ve	-ve
20	BGFB20-3	+ve	+ve	-ve	-ve	-ve
21	BGFB20-4	+ve	+ve	-ve	-ve	-ve
22	BGFB20-5	+ve	+ve	-ve	-ve	-ve
23	BGFB20-6	+ve	+ve	-ve	-ve	-ve
24	BGFB20-7	+ve	+ve	-ve	-ve	-ve

(+, Present; -, Negative)

Table 8:Continue

Sl. No	Strain code	20°C	30°C	40°C	50°C	60°C
25	BGFB20-8	+ve	+ve	-ve	-ve	-ve
26	BGFB20-9	+ve	+ve	-ve	-ve	-ve
27	BGFB20-10	+ve	+ve	-ve	-ve	-ve
28	BGFB20-11	+ve	+ve	-ve	-ve	-ve
29	BGFC10-1	+ve	+ve	-ve	-ve	-ve
30	BGFC10-2	+ve	+ve	-ve	-ve	-ve
31	BGFC10-3	+ve	+ve	-ve	-ve	-ve
32	BGFC10-4	+ve	+ve	-ve	-ve	-ve
33	BGFC10-5	+ve	+ve	-ve	-ve	-ve
34	BGFC10-6	+ve	+ve	-ve	-ve	-ve
35	BGFC10-7	+ve	+ve	-ve	-ve	-ve
36	BGFC10-8	+ve	+ve	-ve	-ve	-ve
37	BGFC10-9	+ve	+ve	-ve	-ve	-ve
38	BGFC10-10	+ve	+ve	-ve	-ve	-ve
39	BGFC20-1	+ve	+ve	-ve	-ve	-ve
40	BGFC20-2	+ve	+ve	-ve	-ve	-ve
41	BGFC20-3	+ve	+ve	-ve	-ve	-ve
42	BGFC20-4	+ve	+ve	-ve	-ve	-ve
43	BGFC20-5	+ve	+ve	-ve	-ve	-ve
44	BGFC20-6	+ve	+ve	-ve	-ve	-ve

(+, Present; -, Negative)

Table 9: Effect of various ranges of pH on the fungal growth.

Sl. No	Strain code	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
1	TF10-1	+++++	+++++	+++++	+++++	+++++
2	TF10-2	+++++	+++++	++	++	-
3	TF20-1	+++++	+++++	-	-	-
4	BGFA10-1	+++++	+++++	+++++	+++++	+++++
5	BGFA10-2	+++++	+++++	-	-	-
6	BGFA10-3	+++++	+++++	-	-	-
7	BGFA10-4	-	+++++	+++++	+++++	-
8	BGFA10-5	+++++	+++++	+++++	+++++	+++++
9	BGFA20-1	+++++	+++++	+++++	+++++	-
10	BGFA20-2	+++++	+++++	+++++	+++++	-
11	BGFA20-3	+++++	+++++	+++++	+++++	+++++
12	BGFA20-4	+++++	+++++	+++++	-	-
13	BGFA20-5	+++++	+++++	-	-	-
14	BGFA20-6	+++++	+++++	+++++	-	-
15	BGFB10-1	+++++	+++++			
16	BGFB10-2	-	+++++	-	-	-
17	BGFB10-3	+++++	+++++	+++++	+++++	+++++
18	BGFB20-1	+++++	+++++	+++++	+++++	+++++
19	BGFB20-2	+++++	+++++	++++	+++++	+++++
20	BGFB20-3	+++++	+++++	+++++	+++++	-
21	BGFB20-4	+++++	+++++	+++++	+++++	+++
22	BGFB20-5	++	+++++	++	+++++	+++++
23	BGFB20-6	+++++	+++++	-	-	-
24	BGFB20-7	+++++	+++++	+++++	+++++	+++++

(+++++)- Very high growth; (++++)-High growth; (+++) - Intermediate growth; (++) Low growth; (+) Very low growth.

Table 9: Continue

Sl. No	Strain code	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
25	BGFB20-8	+++++	+++++	+++++	+++++	+++++
26	BGFB20-9	+++++	+++++	+++++	+++	-
27	BGFB20-10	+++	+++++	-	-	-
28	BGFB20-11	+++++	+++++	+++++	+++++	-
29	BGFC10-1	+++++	+++++	+++++	+++++	-
30	BGFC10-2	+++++	+++++	+++++	+++++	+++++
31	BGFC10-3	+++++	+++++	+++	++	-
32	BGFC10-4	+++++	+++++	+++++	+++++	-
33	BGFC10-5	+++++	+++++	+++++	+++++	+++++
34	BGFC10-6	+++	+++++	-	-	-
35	BGFC10-7	-	+++++	+++++	-	-
36	BGFC10-8	-	+++++	-	-	-
37	BGFC10-9	-	+++++	-	-	-
38	BGFC10-10	+++++	+++++	+++++	+++++	+++++
39	BGFC20-1	+++++	+++++	+++	-	-
40	BGFC20-2	-	+++++	-	-	-
41	BGFC20-3	+++++	+++++	+++++	+++++	-
42	BGFC20-4	-	+++++	-	-	-
43	BGFC20-5	-	+++++	-	-	-
44	BGFC20-6	-	+++++	-	-	-

(+++++)- Very high growth; (++++)-High growth; (+++) - Intermediate growth; (++) Low growth; (+) Very low growth.

Table 10: Antibacterial activity of isolated fungus strain

Strain code	*Escherchia coli*		*Bacillus cereus*		*Staphylococcus aureus*		*Pseudomonas aeurogenosa*	
	Crude	Supernatant	Crude	Supernatant	Crude	Supernatant	Crude	Supernatant
TF10-1	-ve	-ve	10mm	10mm	12mm	11mm	-ve	-ve
TF10-2	10mm	-ve	12mm	12mm	10mm	-ve	-ve	-ve
TF20-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA10-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA10-2	-ve	-ve	13mm	12mm	11mm	10mm	-ve	-ve
BGFA10-3	-ve	-ve	11mm	10mm	-ve	-ve	-ve	-ve
BGFA10-4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA10-5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA20-1	10mm	-ve	12mm	11mm	10mm	10mm	-ve	-ve
BGFA20-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA20-3	-ve	-ve	11mm	10mm	-ve	-ve	-ve	-ve
BGFA20-4	-ve	-ve	12mm	10mm	-ve	-ve	-ve	-ve
BGFA20-5	10mm	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFA20-6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve; Antibacterial effect not observed

Table 10: Continue

Strain code	*Escherchia coli*		*Bacillus cereus*		*Staphylococcus aureus*		*Pseudomonas aeurogenosa*	
	Crude	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**
BGFB10-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB10-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB10-3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-2	-ve	-ve	15mm	10mm	-ve	-ve	-ve	-ve
BGFB20-3	11mm	10mm	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-5	-ve	-ve	15mm	16mm	13mm	-ve	-ve	-ve
BGFB20-6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFB20-9	-ve	-ve	-ve	12mm	-ve	15mm	-ve	-ve
BGFB20-10	-ve	-ve	13mm	10mm	-ve	-ve	-ve	-ve
BGFB20-11	-ve	-ve	13mm	12mm	14mm	11mm	-ve	-ve

-ve; Antibacterial effect not observed

Table 10: Continue

Strain code	*Escherchia coli*		*Bacillus cereus*		*Staphylococcus aureus*		*Pseudomonas aeurogenosa*	
	Crude	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**
BGFC10-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-4	-ve	-ve	14mm	11mm	-ve	-ve	-ve	-ve
BGFC10-5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-6	-ve	11mm	10mm	-ve	-ve	-ve	-ve	-ve
BGFC10-7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve; Antibacterial effect not observed

Table 10: Continue

Strain code	*Escherchia coli*		*Bacillus cereus*		*Staphylococcus aureus*		*Pseudomonas aeurogenosa*	
	Crude	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**
BGFC10-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-4	-ve	-ve	14mm	11mm	-ve	-ve	-ve	-ve
BGFC10-5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-6	-ve	11mm	10mm	-ve	-ve	-ve	-ve	-ve
BGFC10-7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC10-10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve; Antibacterial effect not observed

Table 10: Continue

Strain code	*Escherchia coli*		*Bacillus cereus*		*Staphylococcus aureus*		*Pseudomonas aeurogenosa*	
	Crude	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**	**Crude**	**Supernatant**
BGFC20-4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
BGFC20-5	16mm	11mm	16mm	11mm	16mm	11mm	-ve	-ve
BGFC20-6	16mm	11mm	16mm	11mm	11mm	10mm	-ve	-ve

Note: -ve; Antibacterial effect not observed

4.6 Screening of HMG-CoA reductase inhibitor production by submerged fermentation and extraction of HMG-CoA reductase inhibitor from the fungal isolates

The fungal cultures were grown under submerged fermentation conditions to screen their potential for lovastatin production. At the end of 10 days of fermentation process organic phase contain lovastatin was extracted through downstream processing. HMG-CoA reductase has been homogeneity from of solubilises extract of chicken liver microsomes.

4.7 Assay of HMG-CoA reductase inhibitory activity by UV spectrophotometer

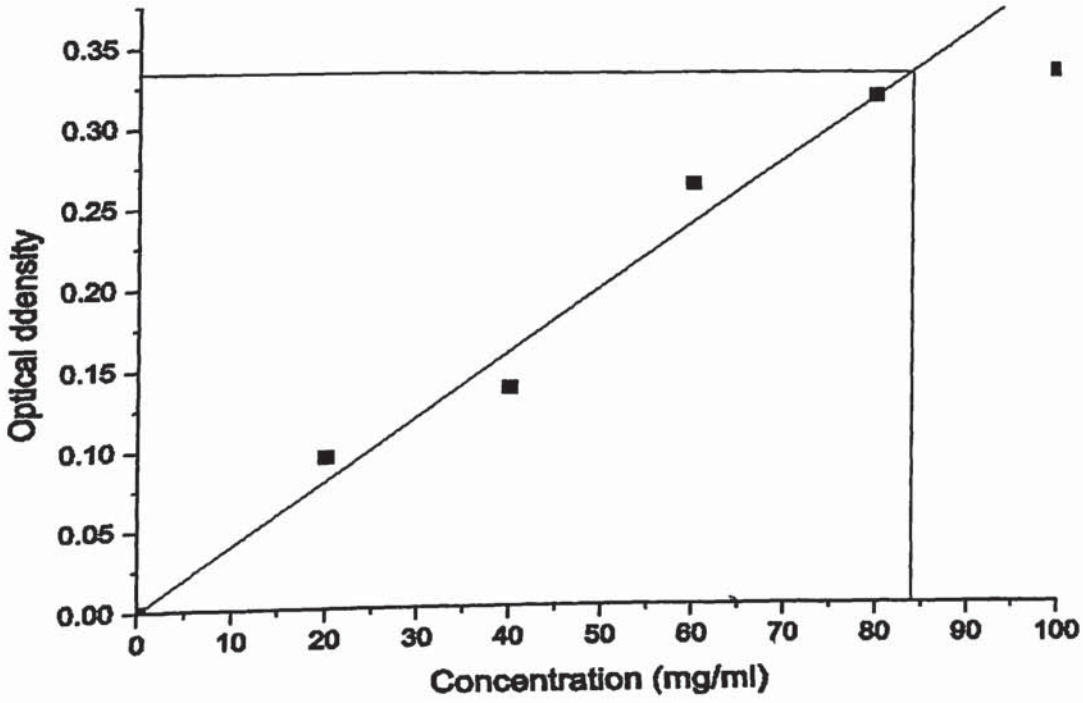
Ten fungal isolates were screen for inhibitory effect on HMG-CoA reductase activity respectively. All ten fungal isolates showed an inhibitory activity 30-96% on HMG-CoA reductase (Table 11).

Table 11: HMGRI activity of the fungal isolates

Sl. No.	Strain code	HMGRI activity%
1	TF10-1	30.8
2	TF10-2	31.0
3	BGFA10-1	95.6
4	BGFA10-3	48.1
5	BGFA20-1	96.2
6	BGFB20-5	84.2
7	BGFB20-6	59.9
8	BGFB20-7	29.5
9	BGFB20-10	42.2
10	BGFB20-11	58.1
11	Lovastatin standard	39.8

4.8 Protein estimation by Lowry method

Protein concentration has been measured by Lowry method 85mg/ml protein was present chicken liver.



Graph 1: Protein estimation optical density vs concentration

Chapter - 5

Discussion

CHAPTER 5

5.1 Isolation fungus from soil samples from agricultural fields

The specialization in mycopopulation of some peculiar types of soils has been differently study by many researchers (Brown, 1958). The fungal diversity of the soil is appreciably affected by physiochemical characters of the soils and environmental complex of the locality Chen and Griffin, 1966; Mishra, 1966; Saksena, 1955). During this study, 45 fungal cultures were recovered from soil samples collected from agriculture fields of Gangtok Sikkim, India. A total of 40 isolates of fungi belonging to Deuteromycetes, Zygomycetes and Basidiomycetes were isolated. Among them Deuteromycetes fungi dominated over the number of Zygomycotes and Basdiomycetes. Some *Aspergillus* species were isolated from soil of forest and cave ecosystems of Taiwan by Hsu, *et al.* (2001). Sharma (2009) studied soil mycoflora of Yumthang valley (Sikkim) and observed that maximum species was belonging to the class Ascomycotina. Microbial analysis of different soil samples of selected site in Obafemi Awolowo University, Nigeria was investigated by Ogunmwonyi *et al.* (2008) they found *Aspergillus niger* as a dominated fungi among all. In the present investigation *Aspergillus* sp found dominated in different fungal species. Baxter, *et al.* (1980) found some fungal species such as *Alternaria alternate, Chrysosporlum pannorum, Cladosporium cladosporioides, Fusarium sp., Mucor hiemalis, M. racemosus* etc. from low temperature region of New Zealand. In the present work we have also found similar fungal genera such as *Mucor, Rhizoctonia* etc. from the low temperature region of Gangtok. Fungi belonging to genera *Acremonium, Aspergillus, Cladosporium, Fusarium* and *Trichoderma* were isolates from Antarctic soils by Singh *et al.* (2006). Species of *Penicillium* and *Trichoderma* were found from different tea growing locations in India by Pandey, *et al.*, 2001.

According to the present work *Aspegillus sp.* (26%) was the most frequent fungal species as shown in table 7. The fungal species *Penicillium sp.* (23%) were the second most frequent fungal species recorded. *Fusarium sp.* (7%) was third most frequent fungal sp. *Trichoderma sp and sclerotium sp.* were forth dominant sp. recorded. The fungi *Basipetospora, Paecilomyces, Rhizoctonia, Trichurus, Paecilomyce puntonii, Basipetospora, Monilia, Mortierella, Gongronella Mucor* and *Verticillium. Aspergillus sp.* were more dominant because may be tolerated inherent

physiological characteristics or by adaptation through a temporary alteration in their developmental pattern which is highly evidenced in this study.

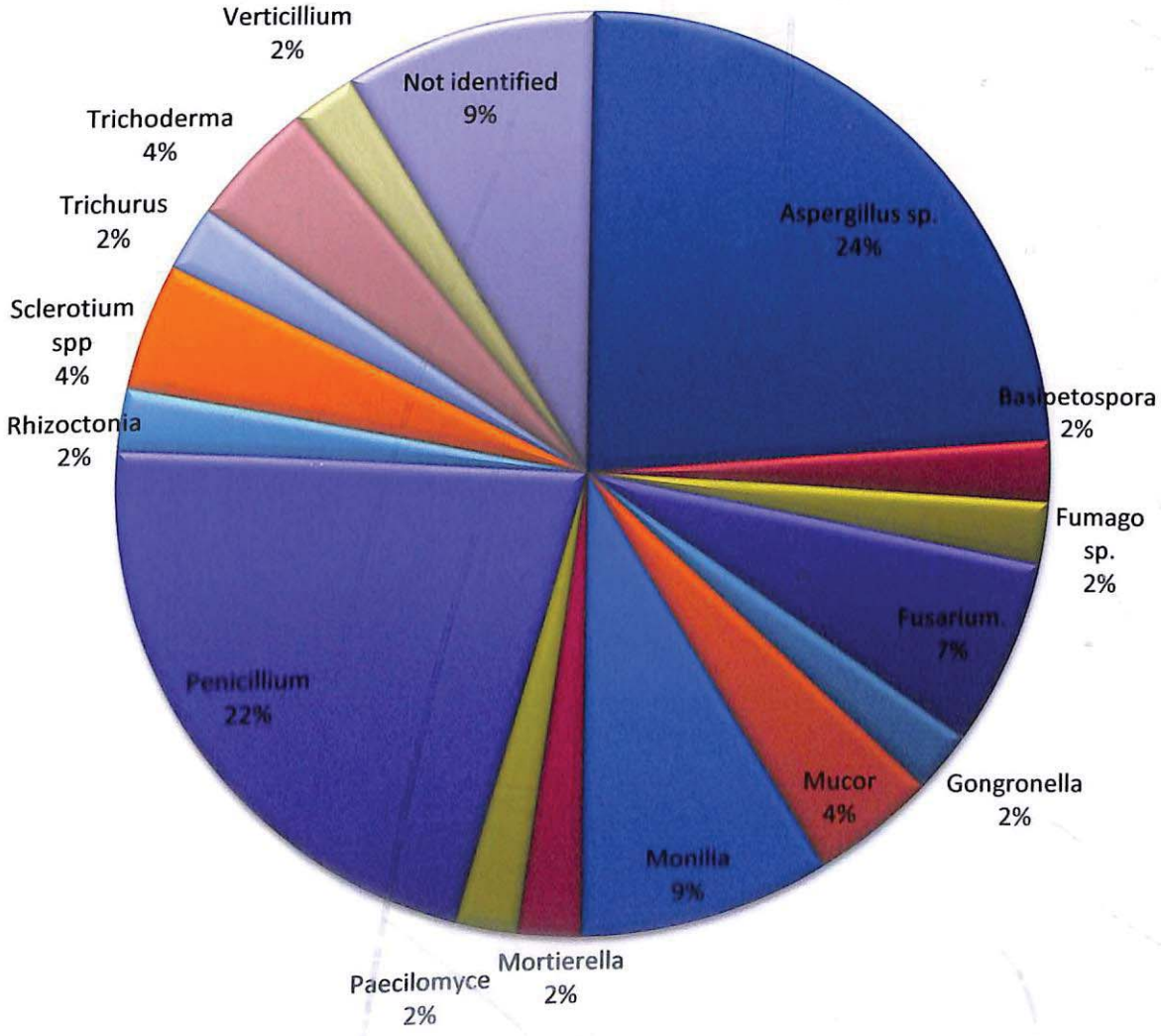


Figure 19: Pie chart showing the percentage of fungal diversity

5.2 Effect of various ranges of temperature on the fungal growth

The temperature is the main factor affecting the fungal population and diversity. The optimum temperature for the growth of all fungal isolates under present study was found to 10-30° C. The growth of these fungal isolates however ceased at 40° C. Similarly Green, (1927) working on working on *Zygorhynchus moelleri* reported that the growth of the fungus increased with rise of temperature up to 26° C but above 26°C it decreased and later ceased at 32° C. The growth of most of the fungi stops at 0° C and only a few fungi are active beyond 40° C (Wolf and Wolf, 1947), whereas

the optimum temperature lies somewhere between the two. *Alternaria tenuis* found best growth and sporulation of the fungus at 24—25° C (Singh and Khanna, 1966). 25-27° C to be the optimum temperature for the growth and sporulation of *Colletotrichum gloeosporioides* and *Chaetostylum* showed best growth at 20° C (Tadon and Verma, 1962). This shows that the fungi are highly sensitive to the temperature of the medium.

5.3 Effect of various ranges of pH on the fungal growth

pH affect the growth of the fungus in two ways. Externally, it can control the degree of dissociation of the inorganic ions in the culture solution. Since dissociation plays a part in the movement of ions in the fungus, degree of dissociation will affect fungus growth (Verma, 1970). Internally it can cause changes in pH in the mycelium. It is clear from Table 9 that all the isolates grew well within a pH range of 6-7. But some fungal grow in pH range 5-9. Maximum growth of fungus was recorded al pH 6. Add general discussion on what pH is optimal for fungus as reported by others (Tandon and Chaturvedi, 1968).

5.4 Screening of the fungal isolates for antibacterial activity assay

Soil fungi have been the major source of the most important antibiotics in human history for decades. In this study, many soil fungal isolates showed antibacterial activity against tested bacteria (Makut and Owolewa, 2011). The results of the zone of inhibition in table 11 shows that all the fungal isolates have antimicrobial activity against at least gram positive bacteria which indicates that these fungi produce some form antimicrobial substance(s) was responsible for inhibiting the test organisms. *Aspergillus terreus* (TF10-1) and *Aspergillus fumigants* (BGFC20-5) was found to inhibit *Staphylococcus aureus and Bacillus cereus* by producing zone of 10mm, 11mm and 12mm but they no effect on *E. coli* and *Pseudomonas aeruginosa* similarly, inhibition zone of 10mm, 12mm, 14mm and 15mm were respectively measured against *Bacillus cereus, staphylococcus aureus* by *Penicillium sp* (BGFB20-11). This shows that these fungal isolates were mostly active against Gram positive bacterial species. It may be due to their nature of the cell wall that only gram positive bacteria were inhibited by these fungi. Gram negative bacteria on the other

hand have lipopolysaccharide layer and hence, the antibacterial substances secreted by these fungi are not effective against them. *Gongronella* (BGFB20-5) was also found to inhibit *Bacillus cereus* and *Staphylococcus aureus.*

5.5 Screening of HMG-CoA reductase inhibitor production by submerged fermentation, extraction of enzyme and activity assayed by UV/vis spectrophotometer

In order to obtain a potent inhibitor of HMG-CoA reductase that would potentially function as a cholesterol lowering agent. Screening process was performed of the fungal extract obtain after extraction process. Ten fungal isolates were only screened for HMG-CoA reductase activity. Out of ten isolates *Mucor hemalis* (BGFA10-1) (95%) and *Aspergillus fumigants* (BGFA20-1) (96%) show the highest activity. *Aspergillus terrus* (TF10-1) (30%), *Trichoderma harzianum* (BGFA10-3) (31%) *(Penicillium corylophilum* (BGFB20-6) (59%) *Penicillium lanosum* (BGFB20-7) (29%).

Changes in HMG-CoA reductase activity related to changes in the overall rate of cholesterol synthesis, suggesting that the inhibition of HMG-CoA reductase would be an effective means of lowering plasma cholesterol in humans (Endo *et al.,* 1985). Various HMG-CoA reductase-inhibitors have been isolated from many microorganisms. Mevastatin, compactin, ML-236A, and ML-236C were isolated from *P. citrinum*, and lovastatin was isolated from *M. ruber* and *A. terreus* (Endo *et al.*, 1976).

Shindia, 1997 investigated 25 fungal species, showed that nearly one-third of the strains were positive for lovastatin production. *Aspergillus terreus* was the best lovastatin producer (84 mg/l) introduced in his article. A number of *Aspergillus terreus* strains were screened for lovastatin production, among them, three produced lovastatin with equivalent or better yield than strain ATCC 20542 originally described for lovastatin production (Sazkacs *et al.*, 1988).

HMG-CoA reductase enzyme has been isolated from fresh chicken liver which contain 84 mg/ml protein.

Chapter - 6

Conclusion

Chapter-6

These findings provide important insights that support our understanding of the diversity of fungi in natural ecosystems since fungi comprise important component of microbial diversity in high altitudes and are considered key organisms in inland ecosystems. Fungi typically constitute more of the soil biomass than bacteria, depending on soil depth and nutrient conditions

Characterizations of the isolates were done through colony and microscopic properties. Only after detail study of the morphology by using slide culture technique and using key for fungal identification all the fungal isolates were identified.

Fungal diversity of any soil depends on a large number of factors of the soil such as temperature, pH, organic contents, and moisture. The fungi obtained from the site of present investigation belong to low temperature region. Now it was concluded that the isolated fungal species were get adopted at the low temperature.

Fungi have been the major source of the most important antibiotics in human history for decades. In the present study the extract of fungal species, were tested for its antibacterial activity Although, this investigation is a primary study, further investigations needs to be embarked upon to determine the type of antimicrobial substances produced or the type of effect they cause on the pathogens, whether static or cidal

HMGRI acts as cholesterol lowering agent by inhibiting HMG-CoA reductase competitively. It decreases LDL level more than other cholesterol lowering drugs. A number of various fungi have ability to produce HMGRI by submerged fermentation process. *Mucor hemalis* (BGFA10-1) (95%) and *Aspergillus fumigants* (BGFA20-1) (96%) show the highest HMGRI activity.

Chapter-6

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