DIFFERENTIATION OF FILAMENTOUS FUNGI BY POLYMERASE CHAIN REACTION (PCR) AND FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

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by
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ABSTRACT

DIFFERENTIATION OF FILAMENTOUS FUNGI BY POLYMERASE CHAIN REACTION (PCR) AND FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

Fourier transform infrared (FTIR) spectroscopy is considered to be a rapid, reliable, sensitive, and a cost-effective technique, which could be used as an efficient tool for microorganism identification. Since bio-molecules, such as lipids, carbohydrates, and nucleic acids, have their own unique 'vibrational' fingerprints and characteristic functional groups, which correspond to specific infrared light frequencies, FTIR spectrum obtained for any compound gives the information on the unique 'fingerprint'. The objective of this study was to investigate the ability of FTIR spectroscopy for differentiating different species of filamentous fungi. In this study, Erkence cultivar olives which were collected from different orchards were used for different fungal strain isolation. The fungi isolates were grown on Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA) at room temperature of 25ºC for 10 days. 15 different genera and 53 species were identified by using Polymerase Chain Reaction (PCR) and characterized in terms of DNA sequencing. FTIR spectroscopy was applied to 71 species as a novel technique to identify fungi. 18 pre-defined species that were collected fom previous studies, were also used for FTIR spectroscopy investigation. Statistical analysis of the data was performed by using a principal component analysis (PCA). FTIR spectroscopy provides a potentially powerful approach to differentiate filamentous fungi.

ÖZET

FİLAMENTÖZ FUNGUSLARIN POLİMERAZ ZİNCİR REAKSİYONU (PCR) VE FOURIER DÖNÜŞÜM KIZILÖTESİ (FTIR) SPEKTROSKOPİSİ İLE AYIRT EDİLMESİ

Fourier Dönüşüm Kızılötesi (FTIR) Spektroskopisi, hedef mikroorganizmanın kimyasal kompozisyonunun belirlenmesi veya fonksiyonel grupların tanımlanmasında kullanılan hızlı, etkili, hassas ve düşük maliyetli bir yöntemdir. Lipidler, karbonhidratlar ve nükleik asitler gibi biyo-moleküllerin özgül kızılötesi ışık frekanslarına karşılık gelen kendine özgü 'titreşimsel' parmak izleri ve karakteristik işlevsel grupları olduğundan, herhangi bir bileşik için elde edilen FTIR spektrumu, o bileşiğin parmak izini oluşturur. Bu çalışmanın amacı, farklı filamentöz fungus türlerini ayırt etmek için FTIR spektroskopisinin yeterliliğini araştırmaktır. Bu çalışmada, farklı bağlardan toplanan Erkence türü zeytinler, farklı küf türü izolasyonu için kullanıldı. Bunun için, fungus izolatları, oda sıcaklığında 25 ° C'de Malt Extract Agar (MEA) ve Czapek Yeast Agar (CYA) üzerinde 10 gün boyunca inkübe edildi. Polimeraz Zincir Reaksiyonu (PCR) kullanılarak 15 farklı cins ve 53 tür belirlendi ve DNA dizilimi açısından karakterize edildi. FTIR spektroskopisi 71 türe yeni bir teknik olarak uygulandı. Önceki çalışmalardan toplanmış 18 tanımlanmış tür de FTIR spektroskopisi için kullanıldı. Sonuçların istatistiksel analizi Temel Bileşen Analizi (PCA) kullanılarak yapıldı. FTIR spektroskopisi filamentöz fungusların ayrılmasında potansiyel olarak güçlü bir yaklaşım sağladığı belirlendi.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

subsp. :	Subspecies
µm :	Micrometer
min :	Minute
g :	Gram
mg :	Miligram
L :	Liter
µl :	Microliter
h :	Hour
rpm :	Round per minute
EPS :	Exopolysaccharide
cfu :	Colony forming unit
UNG:	Uracil N-glycosylase
Mb :	Megabase
DNA :	Deoxyribonucleic Acid
RNA :	Ribonucleic Acid
dNTP :	Deoxynucleotide triphosphate
kbp :	Kilo base pair
PCR :	Polymerase Chain Reaction
MRS :	de Man, Rogosa and Sharpe Medium
EDTA :	Ethylene Diamide Tetra Acetic Acid
TE :	Tris-EDTA
TAE :	Tris Acetate EDTA
SDS :	Sodium Dodecyl Sulfate

CHAPTER 1

INTRODUCTION

Filamentous fungi or molds are fungi that reproduce in the form of multicellular filaments called hyphae (Moore et al., 2011). Molds are a large and taxonomically various number of fungal species and growth of hyphae causes discoloration and a fuzzy appearance, especially on food. The network of these tubular branching hyphae is considered as a single organism and called as a mycelium. The hyphae are usually transparent. Therefore, the mycelium looks like very fine, fluffy white threads on the surface. Cross-walls (septa) could restrict connected compartments throughout the hyphae, each containing one or multiple, genetically identical nuclei. Abundant production of asexual spores (conidia), formed by differentiation at the ends of hyphae, induces the dusty texture of many molds. This forming and shape of these spores are conventionally used for classifying molds. Many of these spores are colored; therefore, most of them can be detected by human eye. Molds are considered to be microbes and can be included in the divisions Zygomycota and Ascomycota (Morgan et al., 2012).

Filamentous fungi have long been used in the production of foods and beverages for centuries. There are many mold species used for producing food additives and processing aids and significant experience is gained for handling those species at the commercial scale. Molds are used in production of foods, enzymes, and smaller metabolites like fatty acids, other organic acids, vitamins and flavor compounds. In some cases, the fungal products are supplied in situ rather than applied exteriorly as in many fermented soy-based foods, and mould-ripened foods that the mold provides flavor (David et al., 2014).

In the recent years there is a significant interest in fungal identification methods based on nucleic acids. These methods have been developed based on the realization that nucleic acid sequences can represent the genetic variations that control morphological and metabolic differences between fungi. Where detailed sequences are available, especially, from the 28S ribosomal RNA subunit phylogenetic schemes have been proposed that can be used as a basis for identification techniques. The methods have been improved rapidly because of developments in sequencing techniques.

Moreover, this enhanced sensitivity of DNA detection techniques and the lots of applications of the PCR (Thomas et al., 2014).

Fourier transform infrared spectroscopy (FTIR) has high regard as a method for differentiation and identification of filamentous fungi. FTIR spectroscopy has been successfully used in differentiation of Aspergillus and Penicillium on genus, species and strain levels (Fischer et al., 2006), airborne filamentous fungi (Santos et al., 2010), various Fusarium species and phytopathogens (Nie et al., 2007), the characterisation of wood-degrading basidiomycetes (Naumann, 2009), and the identification of dermatophytes (Bastert et al., 1999). These studies have shown that there is a potential for FTIR spectroscopy to be used as an adjunct method for the identification and differentiation of fungi.

In this study it was aimed to investigate the potential of employing Polymerase Chain Reaction (PCR) and Fourier transform infrared (FTIR) spectroscopy for identifying and differentiating between different genera of filamentous fungi that were isolated from olives which were belong to Erkence cultivar. The data were analysed by princical component analysis (PCA) to investigate the ability of differentiating these isolates according to their FTIR spectra.

CHAPTER 2

LITERATURE REVIEW

2.1. Molds/Filamentous Fungi

Molds bring about biodegradation of natural materials. It means they are the reason of food spoilage. They need solely a food source – any organic material, such as leaves, wood, paper, or dirt – and moisture (relative humidity > 60%) to grow. Growth occurs when there is adequate amount of moisture. Because molds grow by digesting the organic material andsometimes, new molds grow on old mold colonies. Even though, certain molds can causesome diseases of animals and humans: disease may derive from allergic sensitivity to mold spores, from growth of pathogenic molds within the body, or from the impacts of ingested or inhaled toxic compounds (mycotoxins) produced by molds (Table 2.1.)

Table 2.1. Selected mycotoxins produced by some common indoor molds and other economically important fungi

Genera	Mycotoxins
Acremonium	Citrinin
Alternaria	altenuene, altenusin, alternariol, altertoxin, tenuazonic acid
Arthrinium	nitropropionic acid
Aspergillus	aflatoxin, austin, citrinin, cytochalasin, fumitoxin, nidulotoxin, ochratoxin, patulin, sterigmatocystin, tremorgenicmycotoxins (fumitremorgen, penitrem, territrem, verruculogen), viomellein, vioxanthin, xanthomegnin
Bipolaris	cytochalasin, sporidesmin, sterigmatocystin
Chaetomium	chaetoglobosin, chetomin, chaetochromin, chaetosin, cochliodinol, sterigmatocystin
Cladosporium	cladosporic acid
Claviceps	ergotalkaloids (egrine, ergometrine, ergonovine, ergotamine, ergotoxine, lysergic acid), secalonic acid
Cylindrocarpon	macrocyclictrichothecenes
Diplodia	Diplodiatoxin
Fusarium	fumonisin, fusaric acid, fusarin, fusarochromanone, moniliformin, trichothecenes (deoxynivalinol, T2 toxin), zearlenol, zearalenone
Gliocladium	Gliotoxin
Myrothecium	trichothecenes (roridin, verrucarin)
Paecilomyces	patulin, viriditoxin
Penicillium	citrinin, citreoviridin, citromycetin, erythroskyrin, ochratoxin, griseofulvin, luteoskyrin, oxaline, patulin, penicillic acid, roquefortine, rubratoxin, rugulosin, rugulovasine, tremorgenicmycotoxins (penitrem, territrem, verruculogen), verrucosidin, viomellein, viridicatin, xanthomegnin
Phoma	brefeldin, cytochalasin, secalonic acid, tenuazonic acid
Phomopsis	macrocyclictrichothecenes
Pithomyces	Sporidesmin
Rhizoctonia	Slaframine
Rhizopus	Rhizonin
Sclerotinia	Furanocoumarins
Stachybotrys	griseofulvin, trichothecenes (isosatratoxin, roridin, satratoxin, trichodermol, trichoverrol)
Torula	Cytotoxins
Trichoderma	gliotoxin, koninginin, trichodermin
Trichothecium	roseotoxin, trichothecenes (trichothecin)
Wallemia	Walleminol
Zygosporium	Cytochalasin

(Source: Abbott, S.P. 2002, Mycotoxins and Indoor Molds.Indoor Environment Connections. 3(4):14-24)

The range of native fungal enzymes used in foods is really common and the enzymes have a large range of applications. Molds probably supply 40% of available commercial enzymes (Godfrey et al. 1996). Moreover, molds are promising hosts as cell factories for the production of heterologous enzymes and lots of those enzymes have food applications.

In addition to being added exteriorly during food production, the supply of fungal enzymes is a vital constituentin the production of fermented soy products. Molds play a significant role in biotechnology and food science in the production of several foods, beverages, antibiotics, pharmaceuticals and enzymes (Moore et al. 2011) (Table 2. 2.).

Table 2.2. Uses of Molds

Food application	Useful products	Other process
Baking	Alkaloids	Biobleaching/Biopulping
Brewing	Antibiotics	Biological control agents
Cheese making	Ethanol	Bioremediation of soils
Mushroom cultivation	Enzymes	Coal solubilisation
Oriental food	Gibberellins	Dye intermediates
fermentations	Immunomodulators	Microencapsulation
Quorn micro-protein	Organic acids	Mycorrhizal inoculants
	Polysaccharides	Steroid bioconversion
	Vitamins	Waste treatment

(Source: Fungal Biotechnology by P.H.Hamlyn, North West Fungus Group (NWFG) Newsletter, April 1997)

2.1.2. Foodborne Fungi

Food borne fungi grow as filamentous fungi, which may or may not form mycotoxins. Molds produce conidia (vegetative spores) from conidospores that specialized aerial structures. Certain food borne fungi produce sexual spores, overwhelmingly ascospores, while others might produce thick-walled chlamydospores and other kinds of resting structures. Ascospores of several food borne fungi have enhanced heat resistance compared to vegetative cells. This induce particular problems in the production of heat processed canned fruit, fruit juices, jams and other berry based products (Nolvenn et al., 2014).

Large number of fungal species can possibly damage foods. But, less number of fungal species, the associated fungi (mycobiota) can cause spoilage of a particular food type. Apple rot is mainly induced by *Penicillium expansum*, *P.crustosum, P.solitum,* and *Alternaria alternata*. Wheat and rye grain is generally contaminated in the field by *Fusarium culmorum, F. graminearum, Alternaria alternata* and *Alternaria infectoria*. Cereal grain stored in temperate climates is mostly spoiled by *Penicillum* species such as, *P.verrucusum, P.hordei*, and members of the *P. aurantiogriseum* complex. At lower water activities, *Aspergillus* species such as *A. cadidus, A.versicolor, A. flavus* and *Eurotium* species possess on stored cereals. Spoilage of cheese without preservatives is predominantly caused by *Penicillum commune* and *P. nalgiovense.*In addition causing spoilage, several of the species, mentioned above produce mycotoxins in specific food products (Boysen et al., 2012).

2.1.3. Phytopathogenic Fungi

The relation of fungi and plants is ancient and involves several kinds of fungi. Fungi are significant group of plant pathogens—most plant diseases are caused by fungi—but lower than 10% of all known fungi can colonize living plants (Knogge, 1996). Plant pathogenic fungi represent a relatively small subset of those fungi that are associated with plants. Most fungi are decomposers, taking advantage of the remains of plants and other organisms as their food source. Other types of relations are; the role of fungi as decomposers, as usefulsymbionts, and as cryptic plant colonizers called endophytes.

Thousands of species of plant pathogenic fungi are responsible for 70% of all known plant diseases. All plant pathogenic fungi are considered as parasites, but not all plant parasitic fungi are pathogens. Plant parasitic fungi obtain nutrients from a living plant host, however the plant host doesn't always show any symptoms. Plant pathogenic fungi are parasites and induce disease characterized by symptoms.

We can further divide plant pathogenic fungi by the stage of the plant host that is attacked, for example, seeds, seedlings, or adult plants, and by what part of the plant is affected roots, leaves, shoots, stems, woody tissues, fruits or flowers. A group of fungi including species of *Fusarium*, *Rhizoctonia* and *Sclerotium* cause seed rot and infect

plants at the seedling stage. These pathogens can attack a wide range of plants. Often, seedling pathogens cause damping off symptoms because they occur in wet soils.

Many fungi that kill seedlings can also affect the roots of mature plants and bring about root and crown rot diseases. Infection generally occurs through wounds, and causes in lesions or death of part or all of the root system and crown. Some common root rots of trees are caused by members of phylum *Basidiomycota* in the genera *Armillaria* and *Heterobasidion spp*. produce shoe-string-like bundles of hyphae called rhizomorphs that allow the fungus to grow from one tree to another.

Leaf spot pathogens infect through natural plant spreading such as stomates or by penetrating directly through the host cuticle and epidermal cell wall. To penetrate directly, fungi produce hydrolytic enzymes cutinases, cellulases, pectinases and proteases for destroying the host tissue (Lori et al., 2012). The most important fungal pathogens are; *Magnaportheoryzae, Botrytis cinerea, Puccinia* spp., *Fusarium graminearum, Fusarium oxysporum, Blumeria graminis, Mycosphaerella graminicola, Colletotrichum* spp., *Ustilagomaydis, Melamp soralini* (Ralph et al., 2012).

2.1.4. Cell Components and Cell Wall Structure of Fungi

Unlike plant cells, fungi don't include chloroplasts or chlorophyll. Pigments in fungi are associated with the cell wall. They play a protective role against ultraviolet radiation and can be toxic.

Most fungi are multicellular organisms. They show two main morphological stages: the vegetative and reproductive stages. The vegetative stage consists of a node of slender thread-like structures called hyphae (singular, hypha), whereas the reproductive stage can be more remarkable. The mass of hyphae is a mycelium . It can grow on a surface, in soil or decaying material, in a liquid, or even on living tissue.

The composition of cell wall has lots of different groups of fungi or between the different species of the same group. In the majority of fungi, the wall doesn't involve cellulose but contains a form of chitin (Bowman and Free, 2006).

The suggested formula for fungus chitin is $(C_{22}\,H_{54}\,N_{21})_{n}$. Electron microscope studies show that chitin occurs as lengthened variously oriented microfibrillar units. These are laid down in layers and form the basis of the structural rigidity of fungal cell walls.

The basic structural constituent of the cell wall in the *Zygomycetes* and higher fungi (*Ascomycetes and Basidiomycetes*) is chitin. It is a polysaccharide based on the nitrogen containing sugar (glucosamine). It is probable that more or less closely associated with chitin in the cell wall are pectic materials, protein, lipids, cellulose, callose and minerals.

Ergosterol is the steroid molecule in the cell membranes that replaces the cholesterol found in animal cell membranes (Bernard et al., 2002).

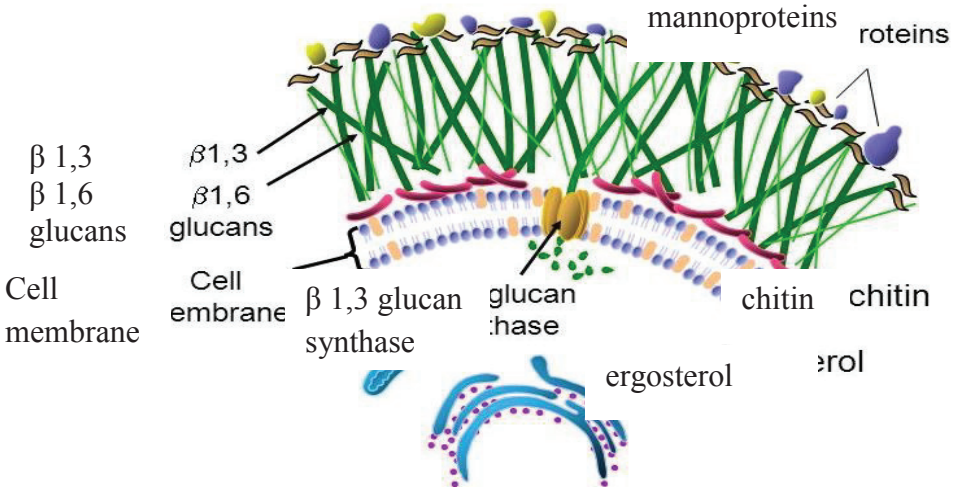

Figure 2.1: Fungal cell membrane and cell wall
(Source: www.nammex.com/redefining-medicinal-mushrooms/)

2.1.5. Genus *Aspergillus*

Aspergillus is defined as a group of conidial fungi. Members of the genus have ability to reproduce where a high osmotic concentration (high sugar, salt, etc.) exists. *Aspergillus* species are considerably aerobic and are found in environments that include large amount of oxygen. They commonly grow as molds on the surface of a substrate, in consequence of the high oxygen amount. Starchy foods (such as bread and potatoes) are generally contaminated by *Aspergillus* spp, and they grow in or on lots of plants and trees.

Aspergillus genus involves a various group of species based on morphological, physiological and phylogenetic features, that considerably affect biotechnology, food production, indoor environments and human health. Moreover, it has high economic and social effects. *Aspergillus* species occur worldwide in diverse habitats. They are

known to spoil food, produce mycotoxins and are generally consider as human and animal pathogens. Furthermore, lots of species are used in biotechnology for the manufacturing of several metabolites likeantibiotics, organic acids, medicines or enzymes, or as agents in various food fermentations. The classification and identification of *Aspergillus* has been based on phenotypic properties. The morphology of the conidiophore, the structure that bears asexual spores, is the most significant taxonomic features used in *Aspergillus* taxonomy. However, in the last decades was profoundly affected by molecular and chemotaxonomic characterization (Joan et al. 2010).

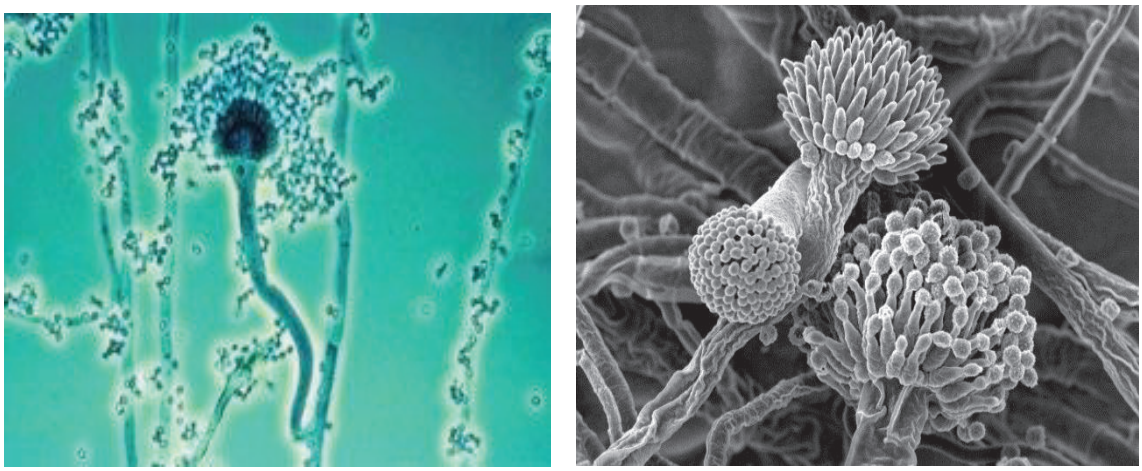

Figure 2.2. *Aspergillus fumigatus*
(Source: www.pfdb.net/photo/nishiyama_y/box20010917/standard/a_fumigatus_e.jpg)

2.1.6. Genus *Penicillium*

Penicillium is a genus of ascomycetous fungi and it is really important for the natural environment. Moreover, they are used in food and drug production. Penicillin, a molecule that is used as an antibiotic, is produced by some *Penicillium* species. The molecule kills or stops the growth of certain kinds of bacteria inside the body and also some species are used in cheese-making. Species of *Penicillium* are ubiquitous soil fungi favoring cool and temperate climates; generally can grow wherever organic material is abundant.

Penicillium is distinguished by their dense brush-like spore-bearing structures called penicilli. The conidiophores are simple or branched. Moreover, they are terminated by clusters of flask-shaped phialides. The spores (conidia) are produced in dry chains from the tips of the phialides, with the youngest spore at the base of the chain,

and are almost always green. Some species are blue color, generally growing on old bread and giving it a blue fuzzy texture.The ability of these *Penicillium* species to grow on seeds and other stored foods depends on their characteristics to grow very well in low humidity and to colonize quickly by aerial dispersion when the seeds have enough moist. Branching is a considerable property for identifying *Penicillium* species. Many species produce highly toxic mycotoxins (Pitt et al. 2000).

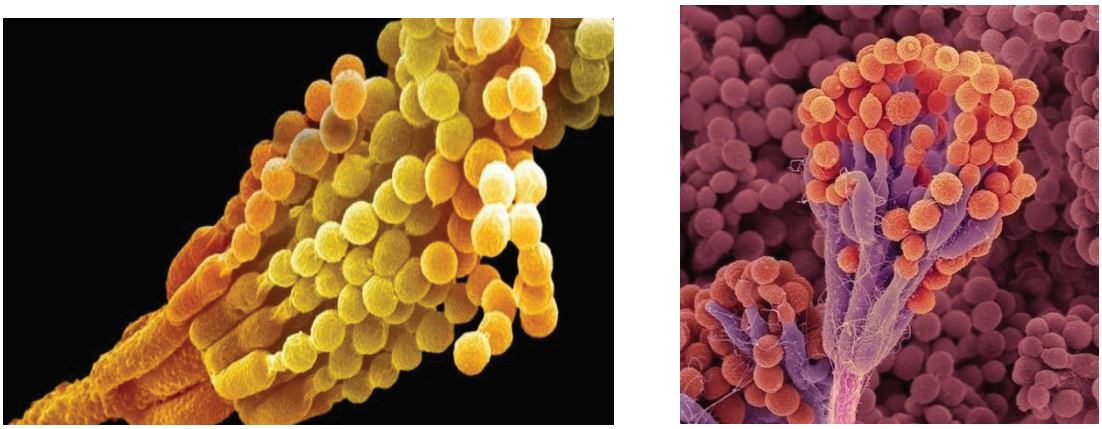

Figure 2.3. *Penicillium notatum*
(Source: cdn.nanxiongnandi.com/bing/Penicillin_EN-GB641807636.jpg)

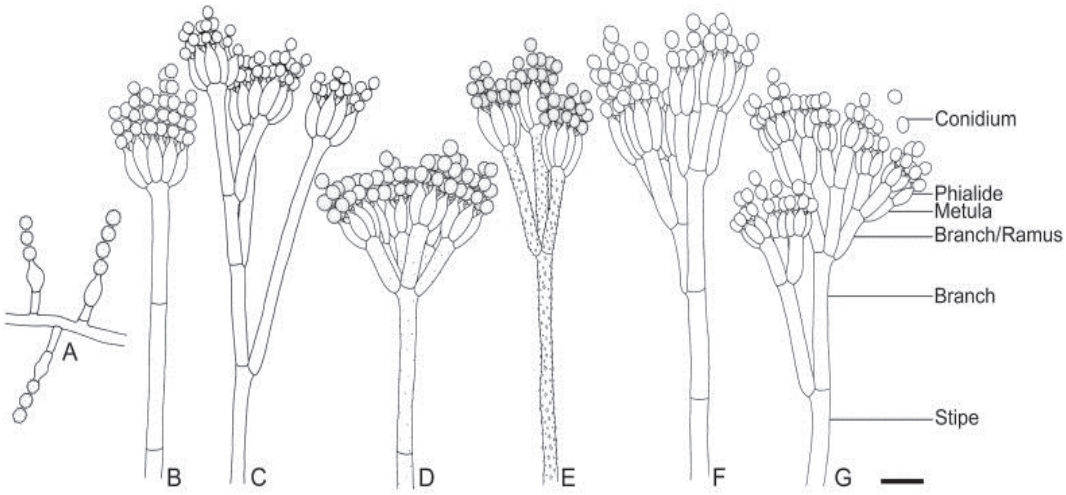

Figure 2.4 Conidiophore branching patterns observed in *Penicillium*. A. Conidiophores with solitary phialides. B. Monoverticillate. C. Divaricate. D, E. Biverticillate. F. Terverticillate. G. Quaterverticillate, terms used for describing parts of conidiophores are given. Scale bar = 10 µm.(Source: Visagie et al., 2014)

2.1.7. Genus *Alternaria*

Alternaria genus is included of ascomycete fungi. *Alternaria* species generally grow as plant pathogen. Theyare also cause of common allergens in humans, growing indoors and causing hay fever or hypersensitivity reactions that sometimes induce asthma. They easily cause opportunistic infections in immune compromised people especially AIDS patients. There are 299 species in *Alternaria* genus; (Kirk et al., 2008) they are ubiquitous in the environment and are a natural part of fungal flora nearly everywhere. Furthermore, they are regular agents of decay and disintegration. The spores are airborne and found in the soil, water, indoors and on objects. The club-shaped spores are single or form long chains. Genus *Alternaria* can reproduce thick colonies that are generally green, black, or gray.

Alternaria species cause of at least 20% of agricultural spoilage; most of losses may reach up to 80% of yield (Nowicki et al., 2012). These fungican induce many human health disorders including on the eyeballs and within the respiratory tract that grow on skin and mucous membranes. Moreover,allergies are commonly seen, but vital infections are uncommon, except in people with compromised immune systems. However, species of this fungal genus are generally vigorous producers of a diversity of toxic compounds. Many species of *Alternaria* modify their secondary metabolites by sulfoconjugation (Kelman et al., 2015). Alternariosis and alternaria toxicosis terms are used for disorders in humans and animals induced by a fungus in this genus.

All *Alternaria* species are not pathogen; some of them are promising as biocontrol agents against invasive plant species. Some species have also been considered as endophytic microorganisms with highly bioactive metabolites**.**

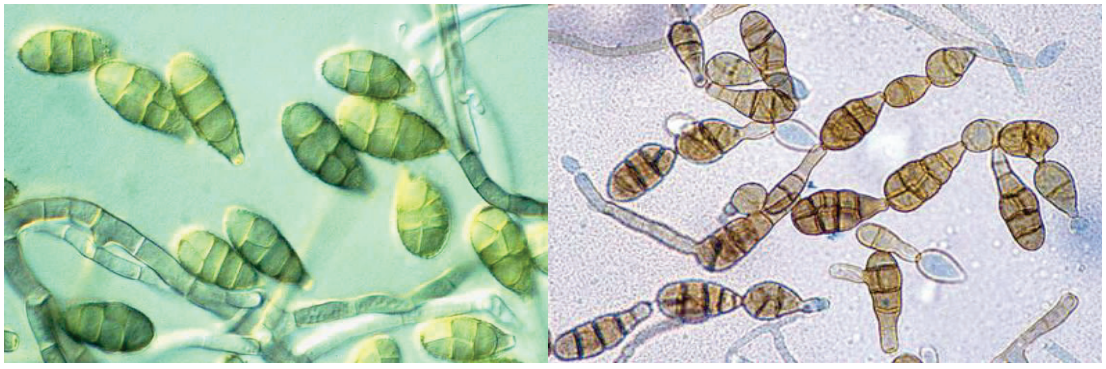

Figure 2.5. *Alternaria alternata*
(Source: http://prgdb.crg.eu/private/organisms/images/5599.gif)

2.1.8. Genus *Cladosporium*

Cladosporium, a genus of fungi, is one of the most common indoor and outdoor molds. Species generate olive-green to brown or black colonies, and have dark-pigmented conidia it is composed in simple or branching chains. Lots of species of *Cladosporium* are frequently live in alive and dead plant material. Some species are plant pathogens; some of them parasitize other fungi. *Cladosporium* spores are wind-dispersed and usually there are large amount of this species in outdoor air. In doors *Cladosporium* species can reproduce on surfaces when humidity is available. *Cladosporium fulvum,* reason of tomato leaf mould, has been ansignificant genetic model, in that the genetics of host resistance are understood (Rivas et al. 2005). *Cladosporium* species are generally highly osmotolerant, growing readily on media containing 10% glucose or 12–17% NaCl (Deshmukh et al., 2005). Most species have very fragile spore chains, due to this characteristic; prepare a mount for microscopic observationextremely tough in which the conidial chains are conserved intact.

Cladosporium species are generally nonpathogenic to humans, but have been reported to bring about infections of the skin and toenails as well as sinuses and lungs. The airborne spores of *Cladosporium* species are substantial allergens, and in big amounts they can virulently influence asthmatics and people with respiratory diseases. *Cladosporium* species does not produce main mycotoxins; however, produce volatile organic compounds such as: hydrocarbons, terpenes, alcohols, carboxylic acids and esters, ketones, aromatic compounds.

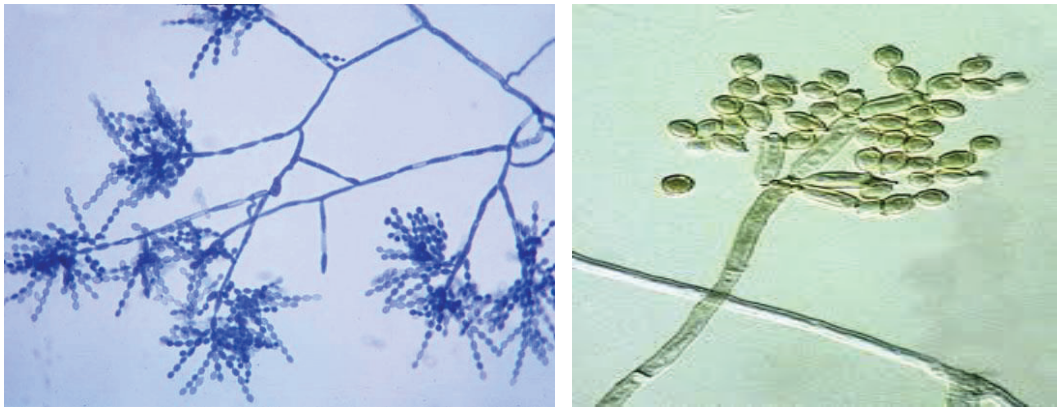

Figure 2.6.*Cladosporium*
(Source: mrnatural.ca/wp-content/uploads/2016/03/cladosporium-mold-species vancouver-mold-lab.jpg)

2.2. Cultural Identification of Fungi

Most of fungi needs simple nutritious. A source of organic nutrients for providing their energy and providing carbon skeletons for cellular synthesis is enough growth of fungi. But, supplied with glucose, lots of fungi can synthesize all their cellular components from inorganic substances, ammonium or nitrate ions, phosphate ions or other minerals such us calcium, potassium, magnesium and iron (Ralph et al., 2012). Fungal growth is influenced by media ingredients, temperature, light, aeration, pH and water activity. Lots of fungi grow very well on Potato Dextrose Agar (PDA). But this media may be so rich, that support the growth of mycelium, with extremely loss of sporulation. Therefore, Potato Carrot Agar (PCA), a starvation media can support sporulation. The majority of filamentous fungi are mesophilic, growing at temperatureswithin the range of between 10-30 °C. However, optimum temperatures for fungi are between 15-30 °C. A lot of fungal species thrive in the dark, but others prefer daylight rather than dark. Moreover, some sporulate better near the ultraviolet light. Filamentous fungi have broad range of pH requirements. Although some of fungi thrive at pH 2 and below, most of fungi thrive over the range pH 3 to 7.

Fungal identification is still fundamentally based on its morphological structures. Therefore, they can be recognized asspecies just by preparing a simple microscopic slide.

The identification of fungi is based on macroscopic and microscopic examination. In addition, macroscopic examination is based on color and nature of the hyphae (Nura et al. 2009).

Different types of fungi will produce different-looking colonies, some colonies may be coloured, some colonies are circular in shape, and others are irregular. A specific terminology is used to describe common colony types. These are form, size, elevation, margin/border, surface, opacity and colour (pigmentation) (Celia et. al., 2012)

In this study the keys to fungi and species of *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* in the followings were used to try for identification of filamentous fungi colonies.

Table 2.3. Key to Fungi

1	Hyphae absent. Colony small, rounded and shiny, commonly white to cream	Go to 2
	Hyphae present, colony cottony, may be coloured	Go to 3
2	Colony with small cells, 0.5 to 2µm diam, single cells cannot be seen under dissecting microscope	Bacteria
	Colony with cells 3 - 10µm diam, single cells can just be seen in smear on slide under dissecting microscope	Yeast
3	Small cells (spores) on stalks can be seen above or in hyphae using the dissecting or compound microscope. Spores may be in a sac-like or round structure.	4
	Spores are invisible, in colony using the dissecting microscope, and on stained mycelia on slide under the compound microscope	Sterile fungus
4	Hyphae lack cr oss walls (examine stained tips of young hyphae on a slide under the compound microscope). Spores in sac held above mycelia (sporangium, diagram over page).	5
	Hyphae have cross walls. Spores commonly held away from hyphae, may be in thick-walled sac (pycnidium)	6
5	Spores held in sporangium, or released from sporangium, hyphae with short darkened "roots" on agar.	*Rhizopus*
	Spores held in sporangium, hyphae lack "roots" into agar	*Mucor*
6	Spores produced in compound pycnidium (diagram over page)	*Phoma*
	Spores formed on free hyphae	7
7	Spores consist of a single cell, not internal walls	8
	Most spores have cross walls, immature spores lack cross walls	14
8	Spores in dry chains when undisturbed	9
	Spores in clumps or clusters, sometimes wet looking	12

(Cont. on next page)

Table 2.3. (cont.)

9	Chains of spores are unbranched	10
	Chains of spores are branched	11
10	Chains of spores held in a brush - like dry cluster, each chain arises from a bottle-like phialide	*Penicillium*
	Chains of spores emerge from phialides which radiate from a swollen vesicle at the top of a specialized coarse hypha	*Aspergillus*
11	Colonies a deep olive to almost black colour, dry spores are generally rounded, lemon shaped or sometimes irregular	*Cladosporium*
	Colonies fawn, spores uniform in shape and size	*Monilia*
12	Colonies flat, creamy, shiny, when young, turning dark with age	*Aureobasidium*
	Colonies fluffy to flat, usually grey to green	13
13	Green masses of spores, white when immature, common in soil	*Trichoderma*
	Grey masses of spores, colony raised and open	*Botrytis*
14	Spores with both vertical and horizontal walls, dark to black	15
	Spores with walls in one direction only, may be pale or dark	17
15	Spores rounded, with walls radiating from centre of spore, held in clusters on short hyphae	*Epicoccum*
	Spores with longitudinal and lateral walls when mature	16
16	Elongate spores formed in branched chains, youngest at tip	*Alternaria*
	Rounded spores formed singly on the sides of short dark hyphae	*Stemphylium*
17	Spores curved, may be dark or pale	18
	Spores cylindrical to rounded, dark, one to many cross walls	*Helminthosporium*
18	Colonies fluffy, white, with curved spores that have one to many cross walls	*Fusarium*
	Colonies dark, spores short, three celled, with central cell larger than the termini	*Curvularia*

(Cont. on next page)

Table 2.4. Key to Species Treated of *Aspergillus*

1	Colonies white, black or in yellow, brown or grey colours	2
2	Colonies in some shade of green	8
	Conidial heads white, often wet	*A. candidus*
2	Conidial heads yellow, some shade of black or brown	3
	Conidial heads dark brown to black	*A.niger*
3	Conidial heads not dark brown to black, but olive, yellow-brown or other shades of brown	4
	Conidial heads columnar, often cinnamon-brown to pinkish-brown	*A.terreus*
4	Conidial heads not columnar, colour yellow or brown	5
	Conidial heads olive to light brown; stripe brown. Hülle cells often produced	*A.ustus*
5	Conidial heads not olive; stripe hyaline or yellowish. Hülle cells absent	6
	Conidial heads pure yellow, conidia smooth to finely roughened	*A.ochraceus*
6	Conidial heads yellow-brown, conidia ornamented	7
7	Conidia conspicuously ornamented with warts and tubercles, outer and inner wall can be distinguished	*A.tamarii*
	Conidiophores typically brown, Hülle cells and *Emericella* teleomorph mostly present	*A.nidulans*
8	Conidiophores not typically brown, *Emericella*teleomorph absent	9
9	Colonies on Czapek or MEA mostly restricted (colony diam. usually less than 1.5 cm within one week)	10
10	Colonies growing faster with a diameter usually larger than 1.5 cm	11
	Colonies variably coloured, conidial heads biseriate, sometimes Hülle cells present	*A. versicolor*

(Cont. on next page)

Table 2.4. (cont.)

	Colonies grey green, conidial heads uniseriate, on MEA or Czapek growing very restricted with poor sporulation, on low water activity media showing better development, Hülle cells not formed	*A.penicillioides*
	Yellow *Eurotium*teleomorph produced in old cultures or on low water activity media, colonies spreading on low water activity media	*A. glaucus*
11	Yellow *Eurotium*teleomorph absent	12
12	Conidial heads yellow-green to dark yellow green	13
14	Conidial heads blue to dark blue green strikingly blue green	15
	Conidial heads predominantly, uniseriate, conidia, dark yellow, green, conspicuously echinulate	*A.parasiticus*
13	Conidial heads uni- and biseriate	14
	Conidia minutely echinulate, yellow green	*A.flavus*
	Conidia irregularly roughened or smooth, greenish olive	*A.oryzae*
15	Conidial heads biseriate, colonies “Delft blue green”	*A.sydowii*
15	Conidial heads uniseriate	16
16	Conidial heads columnar, vesicles broadly clavate, conidia rough to echinulate	*A.fumigatus*
	Conidial heads radiate, splitting into several columns with age, vesicles narrowly clavate, smooth-walled	*A.clavatus*

Table 2.5. Key to Species Treated of *Penicillium*

Colonies white (or very pale greyish-green)	*P.albocoremium*
Colonies in some shade of green, sometimes with yellow aerial mycelium	*P.alii*
Conidiophore with rough stipe	*P.camemberti*
Conidiophore with smooth stipe	*P.nalgiovense*
Colonies on Czapek growing and sporulating poorly (on CYA good growth occur), conidiophores short with distinct large phialides(15-20µm long) and ellipsodial to cylindrical conidia; responsible for citrus rot	*P.digitatum*
Colonies on Czapek and CYA growing and sporulating well, conidiophore with distinct long stripe and usually smaller phialides (5-12µm)	*P.aurantiogriseum*
Conidiophores monoverticillate (simple, unbranched)	*P.glabrum*
Conidiophores branched	*P.aurantiocandidum*
Phialidesacerose (lanceolate), conidiophores, predominantly with terminal whorl of metulae and phialides (biverticillate), occasionally also terverticillate	*P.camemberti*
Phialides flask-shaped, conidiophores biverticillate, terverticillate toquaterverticillate	*P.crustosum*
Colonies growing fast, diameter more than 1.5 cm within one week on MEA	*P.carneum*
Colonies restricted, diameter less than 1.5 cm within one week on MEA	*P.commune*
Colonies funiculose, conidia subglobose-ellipsoidal, 2.5-3.5 µm long	*P.funiculosum*
Colonies velvety, conidia large, ellipsoidal, 3.5-5 (7)	*P.oxalicum*
Conidia rough, ellipsoidal	*P.rugulosum*
Conidia smooth or rough, often fusiform	*P.variabile*

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Table 2.5. (cont.)

Conidiophores predominantly biverticillate	*P.cyclopium*
Conidiophores terverticillate to quaterverticillate	*P.discolor*
Colonies restricted on MEA, mostly growing less than 1.5 cm diam within one week; reverse yellow, metulae 3-5 equal in length	*P.citrinum*
Colonies growing more than 1.5 cm diam on MEA within one week, reverse dark green to blackish after 7 days	*P.corylophilum*
Conidiophore stipe smooth-walled on Czapek agar and MEA, occasionally roughened on MEA	*P.echinulatum*
Conidiophore stipe both on Czapek and MEA finely to distinctly rough or warted	*P.nordicum*
Conidiophores large up to 500-2000 µm tall, compact with 4-6 µm wide stipes	*P.freii*
Conidiophores smaller with stipes of 2.5-4.0 µm wide	*P.hirsutum*
Stipes up to 800 µm, conidia globose to subglobose, colonies restricted	*P.brevicompactum*
Stipes up to 2000 µm, conidia ellipsoidal,colonies more spreading	*P.olsonii*
Colonies velvety, often with yellow exudate and reverse; conidia globose to ellipsoid	*P.chrysogenum*
Colonies with aggregated conidiophores, yellow exudate lacking, conidia subglobose, ellipsoid to cylindrical	*P.hordei*
Phialides short, less than 6.5 µm long, conidiophores often quaterverticillate	*P.griseofulvum*
Phialides mostly longer than 6.5 µm, conidiophores terverticillate	*P.melanocidium*
Colonies 4-5 cm in diam within 14 days; conidia subglobose to ellipsoid; responsible for rot of pomaceous fruit	*P.expansum*
Colonies 2.0-2.5 cm in diam within 14 days;conidia ellipsoid to cylindrical; responsible for citrus rot	*P.italicum*
Conidia echinulate	*P.echinulatum*
Conidia smooth to finely rough	*P.palitans*
Conidiophore stipe conspicuously warted, globose, 4-5 µm in diam, colonies velvety without odour, dark green	*P.roqueforti*

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Table 2.5. (cont.)

Conidiophore stipe rough, but usually not warted, conidia globose to ellipsodial, 3-4.5 µm in diam, colonies fasciculate, often with pronounced odour, reverse not dark green	*P.paneum*
Colonies with yellow mycelium and orange brown exudate	*P.hirsutum*
Colonies without yellow mycelium and orange brown exudate	*P.polonicum*
Colonies on common media Cz and MEA at 25°C in 7 days not exceeding 10 mm in diam, yellow green, conidia (2.5-)2.8-3.2(-3.5) µmdiam, rarely larger	*P.verrucosum*
Colonies on Cz and MEA at 25°C in 7 days usually exceeding 10 mm in diam	*P.requeforti*
Conidia relatively small (2.5-)2.8-3.2(-3.5)µm in diam, when ellipsoidal up to 3.5(-4)µm in length; (weak) growth on CREA, acid production but no base production	*P.solitum*
Conidia relatively large, 3-4(-5)µm in diam, when ellipsoidal up to 4.5-6 µm in length; good growth on CREA, usually both acid and base production	*P.tricolor*
Colonies grey green on MEA, conidia subglobose to ellipsoid, poor growthon CREA	*P.aurantiogriseum*
Conidial blue green on MEA, fast rate & good sporulation on all media, conidia subglose-broadly ellipsoid	*P.polonicum*
Conidial areas dull green to greyish green; in fresh isolates forming crusts mostly after 10 days	*P.crustosum*
Conidial areas in various blue-green or green shades, colonies not forming crusts	*P.verrucosum*
Conidial areas in fresh isolates dark blue-green or dark green; reverse on Czcolourless, yellowish or brownish; conidia (sub)globose	*P.solitum*
Conidial areas in fresh isolates pale blue-green or yellow green or greyish green; reverse on Czcolourless or yellowish conidia subglobose to ellipsoidal	*P.commune*

Table 2.6. Key to Species Treated Of *Alternaria*

1	Conidia produced in unbranched chains on DCMA	*Alternaria tenuissima* (see *A. alternata)*
	Conidia produced in branched clusters on DCMA	2
2	Conidia in unbroken chains, i.e. narrow hyphal elements between sporeslacking; species occurring on wheat and many other substrates	*A. alternata*
	Primary conidia producing secondary conidia, i.e. conidia often separatedby short hyphal lengths; species usually occurring on wheat	*A. infectoria*

Table 2.7.Key To Species Treated Of *Cladosporium*

2	Conidiophores commonly elongating sympodially; conidia usually exceeding 5.0 µm in width, distinctly verrucose	Go to 3
3	Most one-celled conidia (sub) globose, 3-4.5 µm in diameter, finely roughened	*C.sphaerospermum*
	Most one-celled conidia elongate, 3-7(-11) × 2-4(5) µm, smooth-walled or nearly so	*C.clodosporioides*
	One-celled conidia mostly 5.5-13 × 4-6 µm, 2-3 celled conidia also present, somewhat larger	*C.herbarum*
	One-celled conidia mostly 7-17 × 5-8 µm, 2-3 celled conidia common, considerably larger	*C.macrocarpum*

2.4. Molecular Identification of Fungi

Molecular techniques, especially the polymerase chain reaction (PCR) technology, have profoundly affected the molecular biology and the molecular identification of fungi. The combination of molecular techniques and morphology-based identification of fungi helps to distinguish of fungal species and varieties. Databases of genomes and genetic markers are used as sources for molecular barcodes.Moreover, these are being created, and the fungal world is in progression to be unveiled with the help of bioinformatics tools. DNA sequences and other genetic markers supplybig amounts of data that are culture-independent and don't depend on physiological incoherence. Genetic markers permanently reflect the identification information unseen in the genetic code and allow to manage the degree of resolution selecting the proper genes (Youssuf et al., 2008). Molecular approach can overcome lots of limitations of morphological identification technique: (Moore et al., 2011) slow growth of many related fungi with delayed production or lack of property fruiting bodies or macroconidia; (Morgan et al., 2012) the sexual reproduction cycle is not known or lacking with many defective fungi; special nutritional necessities of certain fungi; likeness of macromorphology or micromorphology, or both, at the genus level; (David et al., 2014) antigenic cross-reactivity between significant species and genera; and (Godfrey et al., 1996) possibly dangerous cultures for the health of laboratory personnel.

There are many protocols have been studied for fungal DNA. Most of these protocols are suitable for certain groups or morphological forms of fungi, but may not be efficient for extracting nucleic acids from diverse groups of filamentous fungi (Bolano A. et al., 2001).

Organisms can be identified within mixtures of DNA and without culturing the organisms. This is the major advantage of PCR techniques. Thus, this method is not only specific but also fast.

Probing, in combination with different molecular methods, has been found to be an efficient way for the identification of specific amplicons in a mixture with similar sizes (Sandhu et al., 1995). A large number of probe-based methods have already been developed for the detection and enumeration of various fungi species (Jones 1991). DNA-based diagnostic methods have revolutionized the diagnostic technology in the clinical, forensic science, and in the agricultural sector.

Table 2.8. Molecular Detection Methods of Fungi[*, **]

Detection Method	Sensitivity	Target species	References
Polymerase chain reaction			
18S rRNA gene	50 fg ; 10 fg	*A. fumigatus, A. flavus, A. niger, A. terreus, A. nidulans, C. albicans*	Einsele et al. (1997)
18S rRNA gene	2 spores / reaction	*A. flavus*	Zhou et al. (2000)
18S rRNA gene	10 fg	*A. fumigatus*	Bansod et al. (2008)
Hot start PCR	0.2 GE	*Aspergillus, Candida, Blastomyces, Histoplasma capsulatum, Sporothrix schenckii*	Sandhu et al. (1995)
ver-1 gene	10^2 spores $g^{-}1$	*A. parasiticus*	Shapira et al. (1996)
Calmodulin gene	12.5 pg	*A. carbonicus, A. japonicus*	Perrone et al. (2004)
Calmodulin gene	10 pg	*A. niger, A. tubingensis*	Susca et al. (2007)
Specific primer of aflR	0.5 CFU ≥100 CFU	*A.flavus* *A. parasiticus*	Manonmani et al. (2005)
Real-time PCR of mt tRNA & rRNA	5 copies ml^{-1}	*A. fumigatus, A. flavus, A. niger, A. terreus*	Bolehovska et al. (2006)
RTi-PCR of genomic DNA	SYBR Green I- 5 conidia / reaction TaqMan 50 conidia / reaction	*A. carbonarius*	Selma et al. (2008)
Monochrome light cycler PCR	0.1 pg ; 0.01 pg	*A. flavus, A. fumigatus*	Bu et al. (2005)
Semi-nested PCR	0.1 fg	*A. fumigatus, Rhizopus, Absidia*	Bialek et al. (2005)
Nested PCR of ITS regions	10–100 ag	*A. fumigatus*	Zhao et al. (2001)
Multiplex PCR	1–10 cells	*Aspergillus*	Luo and Mitchell (2002)

(Cont. on next page)

Table 2.8. (cont.)

Combined methods			
LiPA–PCR	ITS–50 pg ITS1–50 fg	*Aspergillus*	Martin et al. (2000)
PCR-EIA	0.5 pg	*Aspergillus*	Hinrikson et al. (2005)
PCR-EIA of 18S rRNA gene	5 pg	*A. fumigatus*	Elie et al. (1998)
Nested-specific PCR-EIA (18S rRNA)	1.7 ng μl^{-1}	*A. fumigatus*	Golbang et al. (1999)
PCR-EIA of mt gene	0.6 fg ml^{-1}	*Aspergillus*	Jones et al. (1998)
DNA fingerprinting method			
RAPD	-	*A. carbonarius*	Fungaro et al. (2004)
DNA microarray			
ITS region of 18S rRNA gene	10 pg	*Aspergillus, Candida*	Hsiao et al. (2005)
Other molecular method			
SPC with immuno-fluorescence labeling	2–10 hyphae / sample	*A. fumigatus*	de Vos and Nelis (2003)
NASBA	10^{7} –10^{11} copies ml^{-1}	*A. fumigatus*	Yoo et al. (2008)

*Different methods used for detecting Aspergillus and other genus.

**CFU Colony-forming unit, GE Genome equivalent, RTi-PCR quantitative Real-time polymerase chain reaction, ITS Intergenic transcribed spacer, LiPA-PCR Line probe assay polymerase chain reaction, PCR-EIA Polymerase chain reaction-enzyme immunoassay, RAPD Random amplified polymorphic DNA, SPC Solid phase cytometry, NASBA Nucleic acid sequence-based amplification.

2.5. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy stands for Fourier Transform Infrared spectroscopy, which is a type of infrared (IR) spectroscopy. In IR spectroscopy, IR radiation passes through a sample, then, the sample absorbs some of the IR radiation while some of them traversed (transmitted). Obtained spectrum represents the molecular absorption and transmission and generates a molecular fingerprint of the sample (Table 2.9.), which means that no two different molecular structures produce the same IR spectrum. FTIR technique could be used to acquire an IR absorption spectrum of a solid, liquid or gas samples. High spectral resolution data over a wide spectral range are concurrently collected by FTIR spectrometer. This offers a big advantage over a separative spectrometer which measures intensity over a narrow range of wavelengths at once (Naumann et al., 1991).

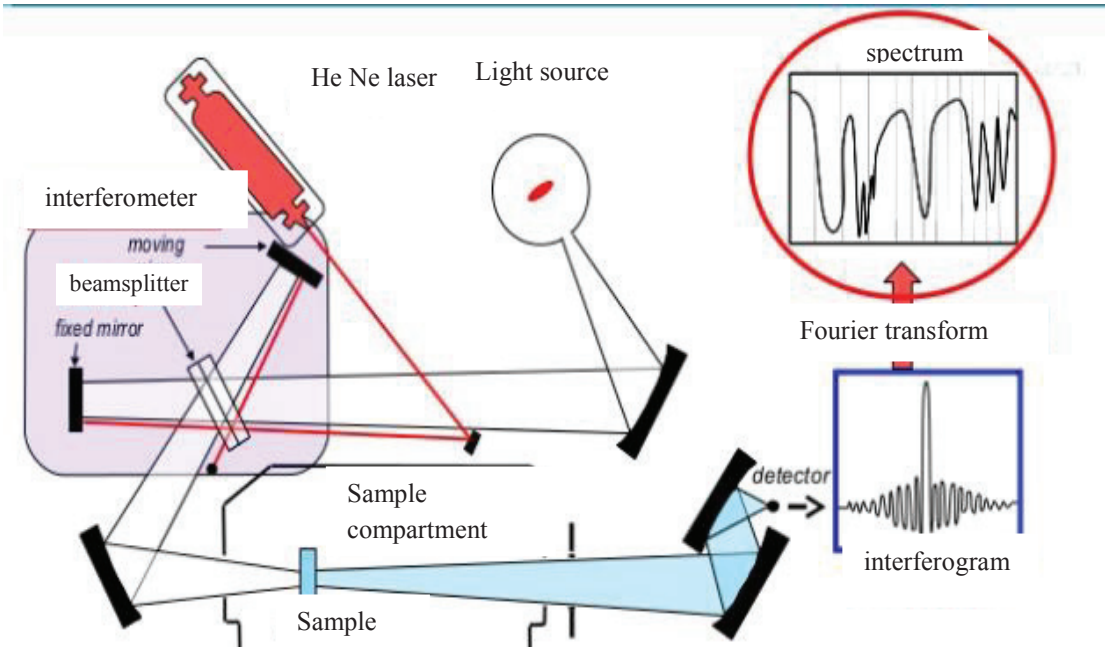

Figure 2.7. Fourier Transform Infrared (FTIR) spectroscopy working principle (Peter et. al., 2006)

Table 2.9. Characteristic IR (infrared) bands

Wavenumber (cm^{-1})	**Assignment**
3640-3610	Hydroxyl (O-H)
~3500	νO-H
3500-3300	Amines (N-H)
~3200	Amide A of proteins
3100-3000	Aromatic rings (C-H)
3080-3020	Alkenes (C-H)
2960-2850	Alkanes (C-H)
2959	$\nu(C\text{-}H_3)as$
2934	$\nu(C\text{-}H_2)as$
2921	ν(C-H2)as (fatty acids)
2898	νC-H (triple bond)
2872	ν(C-H3)s
2500-1900	*Triple bonds*
1900-1500	*Double bonds*
1741–1715	$\nu(C\text{-}H_2)s$ (fatty acids)
~1695	νC=O (carbonic and nucleic acids)
~1685, ~1675	Amide I from antiparallel β-sheets and β-turns of proteins
~1655	Amide I of α-helices of proteins
~1637	Amide I of β-sheets of proteins
1548	Amide II of proteins
1515	"Tyrosine" band
1500	*Deformation/heavy atoms*
1468	$\delta(C\text{-}H_2)$
~1400	ν(C-O)s of COO−
1310–1240	Amide III of proteins
1250–1220	$\nu(P{=}O)as$ of PO_2^-
1200–900	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
1085	$\nu(P{=}O)s$ of PO_2^-
720	C-H rocking of $>CH_2$
900–600	"Fingerprint region"

a ν: stretching; δ: bending; s: symmetric; as: asymmetric. Amide A, I, II and III are typical bands of proteins. Amide A corresponds to νN-H; Amide I, to νC=O of amide groups; Amide II, to νC-N + δN-H coupled out of face, and Amide III, to νC-N + δN-H coupled in face.

(Source: Naumann D. et al. 1991)

FTIR spectroscopic analysis of microorganisms could provide information about the main molecular features of investigated sample rapidly and easily compared to traditional methods. In addition, FTIR spectroscopy is a low cost technique, and small amount of sample is enough for measurement.

Multivariate statistical analysis techniques could be used for the evaluation of spectral data. This spectroscopic technique in combination with multivariate statistical analysis has several applications in microbiology such as determination of the bacterial composition and *Fusarium* cell components, taxonomic classification, determination of the amount of microorganisms, process control, providing information about the microbiological quality control, epidemiological studies and hygiene control. (Mouwen 2005).

Concerning the filamentous fungi, FTIR spectroscopy has already been used for the differentiation and the classification of closely related species like *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus parasiticus* (Garon et al., 2010) *Aspergillus niger*, *Aspergillus ochraceus*, and *Aspergillus westerdijkiae* (Tralamazza et al., 2013). Another recent study investigated the ability of FTIR spectroscopy for the differentiation and classification of clinically related *Trichophyton* species (Ergin et al., 2013). This technique was also used in differentiation and discrimination of *Fusarium* species (Nie et al., 2007). Table 2.10 lists some of the studies related with identification of fungi with FTIR spectroscopy.

Table 2.10. Studies related with identification of fungi by FTIR spectroscopy

Genus	Species	Strain no	Strain source	FT-IR Type	Statistical Method Used	Reference
Aspergillus	*A. flavus*	UBOCC-A-101063 UBOCC-A-106026 UBOCC-A-101060 UBOCC-A-106028	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	A. flavus	UBOOC-A-101061	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	A.flavus	ATCC 16883	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
	A. favus	03121 04759 01652 01656	Culture collection		HCA and PCA	Shapaval et al.
	A. niger	UBOCC-A-112080 UBOCC-A-112082	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	A. niger	01350 04499 01880	Culture collection		HCA and PCA	Shapaval et al.
	A. versicolor	257.97 86.00 278.01	Air		Variance	Guido et al.
	A. versicolor	UBOCC-A-102012 UBOCC-A-101087 CBS 109274 UBOCC-A-101088	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	E.nidulans	29.00	Air		Variance	Guido et al.

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Table 2.10. (cont.)

		30.00 100.00				
	A. ochraceus	87.97 164.01 208.01	Air		Variance	Guido et al.
	A. parasiticus	10.00 104.00 184.00	Air		Variance	Guido et al.
Penicillium	***P. brevicompactum***	192.00 51.01 90.01	Air		Varince	Guido et al.
	P.brevicompactum	UBOCC-A-110065 UBOCC-A-108094	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	P. brevicompactum	UBOCC-A-112048	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	P. brevicompactum	ATCC 9056	Culture collection		PCA	Monika et al.
	P. brevicompactum	04660 04276	Culture collection		HCA and PCA	Shapaval et al.
	P. roqueforti	45.00 92.01 119.01	Air		Variance	Guido et al.
	P. roqueforti	UBOCC-A-109090 CBS 221.30	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	P. roqueforti	UBOCC-A-112026	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	P. roqueforti	04290 01614	Culture collection		HCA and PCA	Shapaval et al.

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Table 2.10. (cont.)

	P. verrucosum	73.01 102.01	Air		Variance	Guido et al.
	P. verrucosum	UBOCC-A-105004 CBS 115508	Culture collection	High-throughput FTIR	PLS-DA	Lecellier et al.
	P. verrucosum	03343 02109 4085 03065	Culture collection		HCA and PCA	Shapaval et al.
	P. expansum	176.00 137.01 216.01	Air		Variance	Guido et al.
	P. chrysogenum	UBOCC-A-110067 UBOCC-A-106023	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	P. chrysogenum	UBOCC-A-112065 UBOCC-A-112108 UBOCC-A-112077	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	P. chrysogeum	ATCC 9179	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
	P. corylophilum	UBOCC-A-112070 UBOCC-A-112081	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	P. paneum	UBOCC-A-111183	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	P. nalgiovense	UBOCC-A-101435 UBOCC-A-102021	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	P. oxalicum	2.00 41.00 12.02	Air		Variance	Guido et al.

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Table 2.10. (cont.)

Alternaria	*A. alternata*	CBS 116329 UBOCC-A-111005 CBS 117143 CBS 916.96	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	A. alternata	ATCC 6663	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Fusarium	*F. oxysporum*	UBOCC-A-108128 UBOCC-A-101157 UBOCC-A-112042	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	F. verticillioides	CBS 119825 CBS 218.76	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	F. equiseti	CBS 414.86 CBS 123566	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	F. langsethiae	01269 03401 03276	Culture collection		HCA and PCA	Shapaval et al.
	F. graminearum	02875 02902 01189 01022	Culture collection		HCA and PCA	Shapaval et al.
Fusarium	-	-	Culture collection	ATR	variance	Salman et al.
Fusarium	-	-	Culture collection	ATR	variance	Erukhimovitch et al.
Mucor	*M. circinelloides*	UBOCC-A-109084 UBOCC-A-101354 UBOCC-A-108126	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	M. circinelloides	04473 01914	Culture collection		HCA and PCA	Shapaval et al.

(Cont. on next page)

Table 2.10. (cont.)

	M. spinosus	UBOCC-A-109053 UBOCC-A-109052	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	M. racemosus	UBOCC-A-109083 UBOCC-A-109051	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	M. plumbeus	02022 03754 02019	Culture collection		HCA and PCA	Shapaval et al.
	M. circinelloides	04473 01914	Culture collection		HCA and PCA	Shapaval et al.
	M. hiemalis	CBS 223.56	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.(2)
	M.mucedo	ATTC 18356	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Eurotium	*E. amstelodami*	CBS 119376 CBS 117323	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	E. amstelodami	03344 02166 02088	Culture collection		HCA and PCA	Shapaval et al.
	E. chevalieri	CBS 522.65 CBS 121704	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	E. chevalieri	02041 02038	Culture collection		HCA and PCA	Shapaval et al.
Rhizopus	*R. oryzae*	02087 02049 02047 02045	Culture collection		HCA and PCA	Shapaval et al.

(Cont. on next page)

Table 2.10. (cont.)

	R. stolonifer	ATCC 14038	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Geotrichum	*G. candidum*	UBOCC-A-103039 UBOCC-A-101170	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
	G. candidum	03865 03033	Culture collection		HCA and PCA	Shapaval et al.
Candida	*C.albican*	ATCC 18804	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Cladosporium	*C. herbarum*	ATCC 28987	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Aureobasidium	*A. pullulans*	UBOCC-A-101092 UBOCC-A-108057 UBOCC-A-101091 UBOCC-A-108047 UBOCC-A-108056	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
Acremonium	*A. strictum*	ATCC 10141	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Lichtheimia	*L. corymbifera*	UBOCC-A-101328 UBOCC-A-103031	Culture collection	High-throughput FT-IR	PLS-DA	Lecellier et al.
Botrytis	*B.cinerea*	ATCC 26806	Culture collection	HATR(Horizontal ATR)	PCA	Monika et al.
Rhizoctonia	-	-	Culture collection	ATR	variance	Salman et al.
Colletotrichum	-	-	Culture collection	ATR	variance	Salman et al.
Verticillium	-	-	Culture collection	ATR	variance	Salman et al.
Pythium	-	-	Culture collection	ATR	variance	Erukhimovitch et al.

2.5.1.Types of Infrared Spectroscopy

The IR region of the spectrum is often divided into three different sub regions:

• Far-IR: 400 – 30 cm^{-1}

• Mid-IR: 4000 – 400 cm^{-1}

• Near-IR: 14000 – 4000 cm^{-1}

The far-IR region is mainly used for evaluating inorganic molecules. The mid-IR region has fundamental absorption bands and supplies a molecular fingerprint. This region is particularly effective for simple structural investigation and raw material ingredient or additive identification through library comparison. Absorption bands in the near-IR region result from combination of the fundamental bands in the mid-IR region. The near-IR region is especially beneficial for food applications, including moisture, fat, and protein content determination.

2.5.1.1. Mid-IR with Attenuated Total Reflectance (ATR) Sampling

ATR is a rapid measuring method that could be used to characterize samples with minimal sample preparation. The benefits of ATR is that sample preparation is less labor-intensive, spectra variation due to sample preparation is minimal, and the effect of sample preparation due to KBr grinding and particle size differences on results is exceedingly reduced (Thompson et al., 2009). ATR is based on the phenomenon of total interior reflection (Bruno, 1999), and measures an inwardly reflected infrared beam that comes into contact with the sample through a zinc selenide (ZnSe) crystal or diamond (Bruno, 1999; Stathopoulou et al., 2008). When a sample is placed in contact with the ATR crystal, the resulting disappearing wave is diminished in the regions of the IR spectrum where the sample absorbs energy (Bruno, 1999) Instead of mixing the bone powder, sample with KBr as in transmission FT-IR, the sample is placed directly on the sampling plate of the equipment over the optic window with the ZnSe crystal.

ATR is optimal for strongly absorbing or thick samples that mostly generate intensive peaks when measured by transmission. ATR works well for these samples because the intensity of the evanescent waves decays exponentially with distance from the surface of the ATR crystal, making the method generally irresponsive to sample thickness.Other solids that are a good fit for ATR include homogeneous solid samples,

the surface layer of a multi-layered solid or the coating on a solid. Even irregular-shaped, hard solids can be analyzed using a hard ATR crystal material such as diamond. Ideal solids including: laminates, coatings, natural powders, solids that can be ground into powder (Peter et. al., 2006).

Moreover, ATR is the mostly the preferred technique for liquid analysis because it only requires small amount of liquid to be placed on the crystal. ATR can be used to analyze free-flowing aqueous solutions, viscous liquids, coatings and biological materials.

Table 2.11. Characteristic infrared absorption frequencies typical of microorganisms and their biomolecular attribution. (Bozza de Almeida et al., 2015)

Frequencies(cm^{-1})	**Molecular bond**	**Vibrational mode**	**Biomolecular attribution**
3200–2800	CH_2, CH_3	Symmetric and asymmetric stretching	Fatty acids Lipids
	N-H	Symmetric stretching	Proteins
1780–1700	C=O	Symmetric stretching	Fatty acids
1695–1625	C=O, C-N	Symmetric stretching	Proteins (amide I)
	N-H	Bending	
1560–1525	C-N	Symmetric stretching	Proteins (amide II)
	N-H	Bending	
1480–1400	CH_3, CH_2	Bending	Lipids
	C=O	Asymmetric stretching	
1300–1200	P=O	Asymmetric stretching	Nucleic acids
1200–900	C-O-C, C-O, P=O, C-C/C-O	Symmetric stretching	Ribose, glycogen, nucleic acids
900–700	C-H	Bending	Aromatic groups

2.6. Principal Component Analysis (PCA)

Principal component analysis is a special technique for identification of a smaller number of uncorrelated variances, called "principal component", from a broad set of data. The aim of principal component analysis is explaining the maximum amount of variable with the fewest number of principal component. PCA is an exploratory multivariate statistical technique for analyzing relationships between various quantitative descriptors or variables (Basilevsky et al., 1994; Everitt and Dunn, 1992). PCA generates a set of expression patterns known as principal component (PCs), and linear combinations of these patterns can be compounded to reflect the features of all of the objects in a given data set.

PCA is usually used as a tool in exploratory data analysis and to generate predictive models. PCA can be done by value decomposition of a data covariance (or correlation) matrix or singular value decomposition of a data matrix, frequently after mean centering (and normalizing or using Z-scores) the data matrix for each attribute (Abdi et al., 2010). The conclusions of a PCA are generally discussed in terms of constituent scores, sometimes called factor scores (the transformed variable values corresponding to a particular data point), and loadings (the weight by which each standardized original factor should be multiplied to obtain the component score (Shaw et al., 2003).

CHAPTER 3

MATERIALS METHODS

3.1. Materials

3.1.1. Fungal Strains

In this study, for the fungal strain isolation olives belong to Erkence cultivar were used. Olives were collected from different orchards. All the samples were collected into sterile jars and kept at -18°C until they were analyzed.

3.1.2. Primers

NL Primer were used for PCR analysis:
Forward Primer: NL1:(5'-GCATATCAATAAGCGGAGGAAAAG-3')
Reverse Primer: NL4: (5'-GGTCCGTGTTTCAAGACGG -3')
Target: 28S DNA, 680 bp.

3.1.3. Culture Media

Potato Carrot Agar with Manganese (PCA-Mn) (Difco 213400), Sabouraud Dextrose Agar (SDA) (Difco 210950) with olive oil, Malt Extract Agar (MEA) (Merck 1.05398), and Czapek Yeast Extract Agar (CYA) (Difco 233810) were used for growth, isolation and identification of the molds. All media were used listed in the Appendix A.

3.2. Methods

3.2.1. Cultural Isolation

Potato Carrot Agar with Manganese (Difco 213400), Sabouraud Dextrose Agar (Difco 210950) with Olive Oil and Malt Extract Agar (Merck 1.05398) were used for isolation of molds from olive samples. All work surfaces were cleaned with a disinfectant solution. It is essential to reduce airflow as much as possible during the transfer of cultures to avoid contamination.To isolate molds, the olive sample was placed in a sterile empty plate. The olive was cut with a sterile knife into 3 pieces. Then it was held the plate with one hand and taken pieces with nippers. The finished plate was had a 3 points from edge to edge (Fig. 3. 1). All plates were incubated at temperatures at 25°C and controlled the growing the colonies 3 times in 10 days.

3.2.2. Pure Culture Isolation

Czapek Yeast Extract Agar (CYA) (Difco 233810) and Malt Extract Agar (Merck 1.05398) were used for each sample. The cooled loop was inserted into the plate and pick up a small quantity of the culture. Then it was gently raised the cover of the plate dish and touched the loop to the top of the dish in 3 points. The cover was lowered and the loop was flamed. All plate dishes were incubated at temperatures at 25°C for 7-10 days.

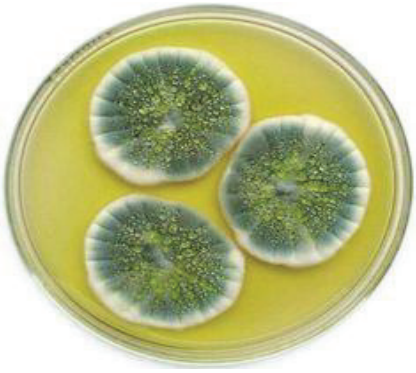


Figure 3.1."Three Point Inoculation Method" in mold cultivation

3.2.3. Culture Examination

3.2.3.1. Measurement of Colony Growth

On the seventh day of incubation, growth and spore germination on the 5°C incubated plate was examined using a stereomicroscope (Zeiss, Germany). Growth at 25°C and 37°C was only visually examined (Pitt, 1991). Diameters of distinct colonies were measured in millimeters on the reverse side of the Petri dish. Each colony that developed from the suspension droplet was measured twice across the widest points. A maximum of six measurements were taken per plate. Colonies where growth was inhibited, or those that developed from stray droplets were disregarded. All measurements were documented.

3.2.3.2. Colony Characteristics

Colony characteristics were assessed visually and stereomicroscopically. Important characteristics included colony texture and colour, conidia production, exudate production and colour there of, pigmentation of mycelium or exudate, diffusion of thepigment into the medium, sclerotia production and buckling of the medium. Other unique characteristics were also noted and all information was documented.

3.2.3.3. Microscopy

A compound microscope (Zeiss) was used for the examination of fruiting structures and conidia. Bright field microscopy of *Penicillium* species requires staining with lactofuschin. Microscopic slide preparations were made from hyphalgrowth on each of the MEA and CYA plates due to certain characteristics being moreapparent on certain media. The colour of the colonies was used for first identification of the *Aspergillus* sections. Other features included presence of sclerotia, production of exudates and soluble pigments in the media. The shapes of conidia head (either globose, radiate, columnar or clavate), seriation (biseriateor uniseriate),vesicle (shape and diameter), the length, width, texture and colour of the conidiophores and conidia (size,

diameter, texture and colour) are the other characteristics used for identification. For *Penicillium* species important characteristics which were observed and noted include mono-, bi-, ter-, or quaterverticillate penicilli nature; stipe size, shapeand texture; conidia shape, size, colour and texture; conidiation and the presence orabsence of conidial chains. For the identification of *Cladosporium*, shape of the conidiophores (geniculate-sinuous, nodulose,location of the conidiogenous loci, dimorphism); ramification of the conidiophores (presence, topology,degree); width of the conidiophores; formation of conidia (solitary or catenate, unbranched orbranched chains);conidial surface ornamentation were the differentiating features in combination with Mycelium (internal, external, both internal and external inbiotrophic species); arrangement of the conidiophores in biotrophic species (solitary, fasciculate); length, septation and thickness of the conidiophore wall; conidiogenous cells (terminal, intercalar, conidiophores reduced to conidiogenous cells; proliferation sympodial,enteroblastic; number and width of conidiogenous loci); conidia (length, width, septation, shape, cell structure).

3.2.4. Molecular Identification

3.2.4.1 DNA Extraction and Polymerase Chain Reaction (PCR)

In order to make molecular identification, firstly, genomic DNA extraction was performed with the mold cultures that were isolated from olives (Bartlett and Stirling, 2003). The mold colony grown in MEA or CYA was mechanically crushed by mortar and beetle in order to crush the fungal cell wall.Then, the genetic material was exposed to the extraction process.The samples were then transferred into 10 mL CTAB extraction buffer and incubated for 30 min at 65°C in a shaking water bath to affect the entire sample surface. Then, an equal volume of chloroform: isoamyl alcohol (24: 1) was added and centrifuged at 2000 g for 10 min at room temperature. Subsequently, supernatant was placed a new tube and an equal volume of isopropanol was added. After this stage, DNA precipitation was expected. However, if DNA could not be observed, samples were washed with 70% ethanol and dried in the open air and 1 ml of 20 μL/ml RNAse containing TE was added to suspend and the samples were store at 4°C.

PCR amplification was performed with 25 µlPCR mixture (5µl genomic DNA, 10 pmol forwardPrimer, 10 pmol of reverse primer, 2.5 µldNTP (2 µM), 2.5µlTaq polymerase buffer (Fermentas), 1.5µl (25 mM) $MgCl_2$ (Fermentas) and 0.24IU Taq polymerase (Fermentas) and 11.26 µl of dH_2O.Genomic DNA amplification was performed with the BIO-RAD C1000 thermal cycler (France) according to the following conditions.The amplified gene region was electrophoresed with a 1% Agarose gel (A4710SIGMA) containing Ethidium bromide (E1510SIGMA) to observe how many base pairs were amplified.100 mV electric current was applied in an electrophoresis tank containing 1% TAE buffer.

1. 94°C for 5 min
2. 94°C for 1 min denaturation
3. 55°C for 2 min binding
4. 72°C for 2 min elongation
5. 72°C for 10 min last elongation

} 40 cycle

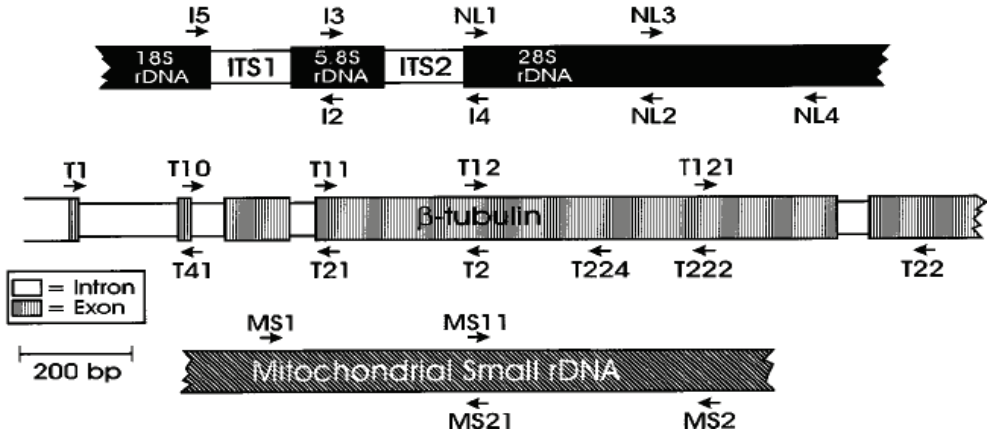


Figure 3.2. Maps of the nuclear ITS and 28S rDNA, b -tubulin gene, and mtSSU rDNA. (Source: https://www.researchgate.net/figure/14201598_fig1_FIG-1-Maps-of-the-nuclear-ITS-and-28S-rDNA-b-tubulin-gene-and-mtSSU-rDNA-Stippled)

3.2.4.2. DNA Sequence Analysis

Amplified PCR products were run on a Sephadex column at 5400 rpm for 2 min in order to remove impurities. Then the cycle sequence is applied according to the following conditions.

1. 96°C for 1 min
2. 96°C for 10 min
3. 53°C for 5 sec
4. 60°C for 4 min
5. 4°C, ∞

} 30 cycle

The most important difference of PCR sequencing from PCR isthe use of primer solely on the single strand to achieve linear amplification product rather than overlapping. Another important difference is the use of DNA sequences in order to interrupt the extension of the DNA single strand DNA merges. DNA sequence analysis was performed by a 3130xL Genetic Analyzer (Applied Biosystems, USA). When the analysiswas completed, the results of the nucleotide sequence were analyzed using software (Finch TV v.1.4.0, Geospiza, Inc., USA); then, cultures were identified by investigating microorganisms with similar sequences in the BLAST server interface at http://www.ncbi.nlm.nih.gov/BLAST/.DNA sequenceanalysis was performed by a 3130xL Genetic Analyzer. After completion of the analysis, the results of the nucleotide sequencing were evaluated by software (Finch TV v.1.4.0,Geospiza, Inc., USA); Cultures were identified by searching for similar sequences in the BLAST server interface at http://www.ncbi.nlm.nih.gov/BLAST/.

Pre-Cycle Sequence Cleaning: Sephadex and Spin columns were used to purify 10-15 µl PCR products before DNA sequencing analysis. (Receiver Columns) Macherey-Nagel

- First, the column was prepared with Sephadex.
- 1gr Sephadex G-50 (Sigma-Aldrich) was dissolved in 15 ml of dH_2O and shaken vigorously (5 min).
- Sephadex650 µl was added to empty spin columns and waited for 20-30 minutes.
- The bottom part of the spin column was removed.
- The column was centrifuge at 5400 rpm for 2 minutes.
- The supernatant was removed.
- The spin column was placed in the sample collection tube.

- 10-15 µl of PCR product was placed in the middle of the spin column that containing Sephadex.
- The column was centrifuge at 5400 rpm for 2 minutes. The PCR product passed to the sample collection tube.

Columns that were cleaned with Sephadex and quantity and quality of PCR products were determined with a NanoDrop-Spectrophotometer (Nano Drop 8000, Thermo Scientific). Then, 260/280 DNA absorbance values (A_{260}/A_{280} index) of the samples were determined to assess the DNA purity**.** If the 260/280 absorbance ratio is between 1.8 and 2, DNA is suitable for sequence analysis. The amount of PCR product should be 3-10 ng/µl for 200-500 base pairs. Then, Cycle Sequence was performed using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). For this,

DNA : 2 µl (3-10ng/µl)

Primer : 1 µl → 3.2 pmol/µl

Buffer : 1 µl →BigDye Terminator v1.1, v3.1 5x Sequencing Buffer

BigDye: 2 µl (3.2pmol/µl)→BigDye Terminator v3.1 Cycle Sequencing Kit

dH_2O: 4 µl

Total: 10 µl

Cleaning After Cycle Sequence: The column was prepared with Sephadex.

- 1gr Sephadex G-50 (Sigma-Aldrich) was dissolved in 15 ml of dH_2O and shaken vigorously (5 min).
- Sephadex 650 µl was added to empty spin columns and waited for 20-30 minutes.
- The bottom part of the spin column was removed.
- The column was centrifuge at 5400 rpm for 2 minutes.
- The supernatant was removed.
- The column was centrifuge at 5400 rpm for 2 minutes. (Column prepared with Sephadex).
- The supernatant was removed.
- The spin column was placed in the sample collection tube.

- 10 µl of the cyclic PCR product was placed 10 µl of the cyclic PCR product in the middle of the spin column containing Sephadex.in the middle of the spin column containing Sephadex.

After Cleaning: Cycle sequenced PCR products passing through the column were transferred to 96 well microplates. If there was less than 10µL of sample in the 96-well microplate, completed with formamide(Hi-Di TM Formamide, Genetic Analysis Grade 25 ml). Septa were placed on 96-well microplate.Microplates were placed on the sequencing device (3130xL Genetic Analyzer, Applied Biosystems) fixed with the microplate bottom and top. In the sequence device, polymer (3130 POP-7 TM Performance Optimized Polymer 7 ml) and buffer for Anode and Cathode poles (Genetic Analyzer 10x Running Buffer with EDTA 25 ml) were used.

For conventional PCR analysis of mold isolates, THERMAL CYCLER (BIO-RAD, C- 1000) was used. Quantities or concentrations (ng/µl) of PCR products were determined using Nanodrop (8 channels, ThermoScientific, USA) before sequence analysis. The DNA integrity of the samples was checked by electrophoresis. The images of the gels obtained by using the Maxi horizontal electrophoresis system (EC340, 20x20 cm, ThermoScientific, USA) were provided by the gel imaging system (VERSADOC, 4000MP, BIO-RAD, USA).

3.2.5. Fourier Transform Infrared (FTIR) Spectroscopy

Studies were performed with a FTIR spectroscopy GX Optica Perkin-Elmer. FTIR spectroscopy analyses were performed according to following procedure:

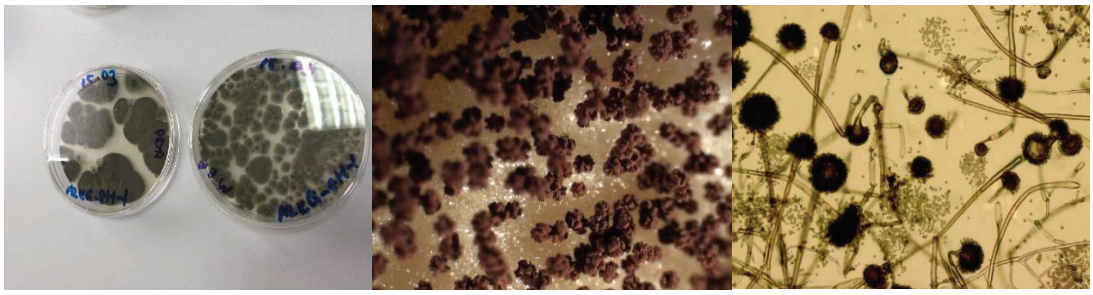
- Each fungal strain which was cultivated on MEA plates, were used for FTIR spectroscopy analyses.
- Spores were collected from MEA plates with clean tool.
- Spores were placed on the ZnSe crystal as a thin layer and placed on the horizontal position.
- Measurements were done at 4 resolutions, 20 replicates at room temperature in the range of 4000-650 cm^{-1}.
- Perkin Elmer Spectrum (version 10.4.3) software was used to display and compare data.

CHAPTER 4

RESULTS

4.1.Cultural Results

The fungi isolates used in the study were grown on Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA) at room temperature of 25ºC for 10 days. Samples were identified according to their morphological (cultural and microscopical) characteristics.

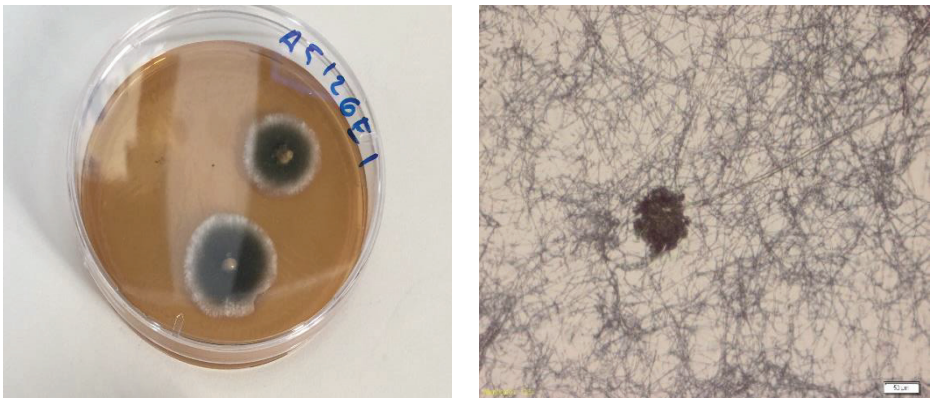


(a) (b) (c)

Figure 4.1. Typical cultural and microscopical features of *Aspergillus fumigatus*. Culture plate (a), stereomicroscope (b) and light microscope (c) images.

(a) (b) (c)

Figure 4.2. Typical cultural and microscopical features of *Aspergillus aculeatus*. Culture plate (a), stereomicroscope (b) and light microscope (c) images.

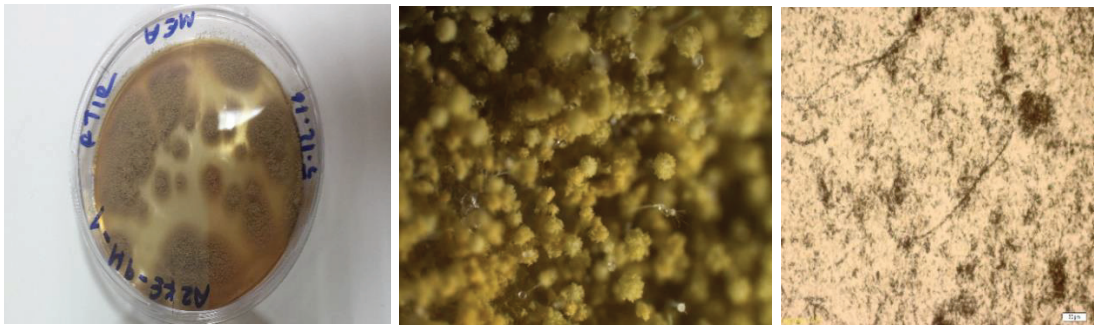


(a) (b)

Figure 4.3. Typical cultural and microscopical features of *Aspergillus sydowii.* Culture plate (a) and light microscope (b) images.

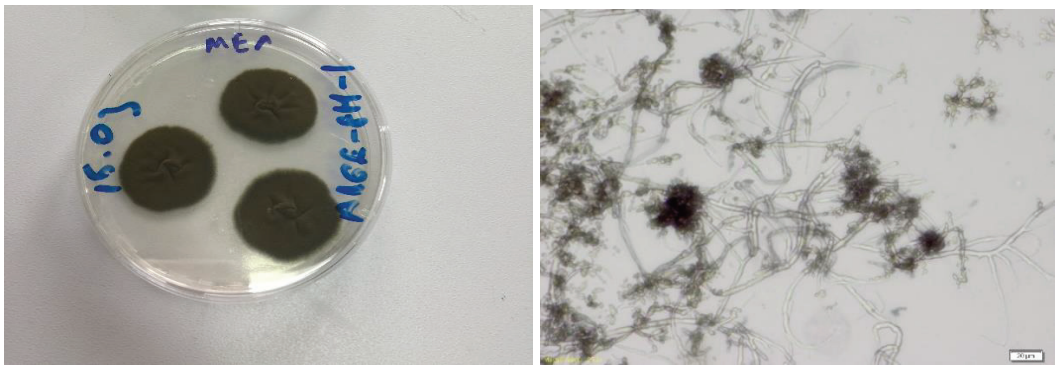
(a) (b) (c)

Figure 4.4. Typical cultural and microscopical features of *Aspergillus japonicus.* Culture plate (a), stereomicroscope (b) and light microscope (c) images.



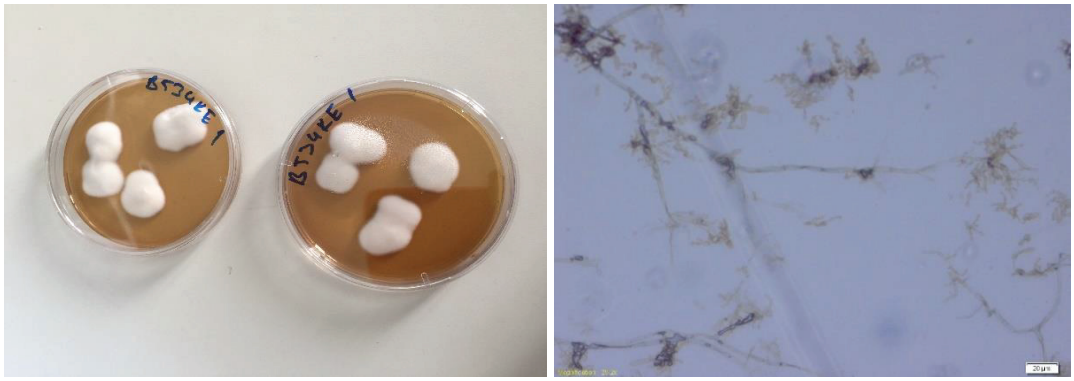
(a) (b) (c)

Figure 4.5. Typical cultural and microscopical features of *Aspergillus ochraceus.* Culture plate (a), stereomicroscope (b) and light microscope (c) images.



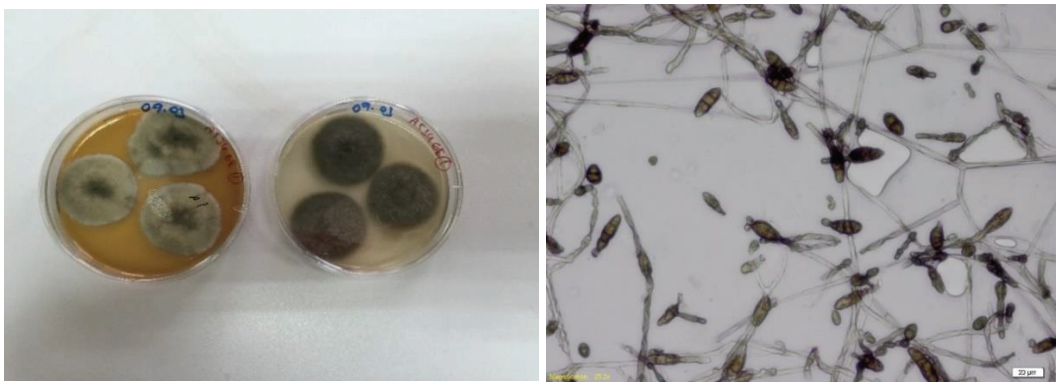
(a) (b)

Figure 4.6. Typical cultural and microscopical features of *Aspergillus ustus.* Culture plate (a) and light microscope (b) images.



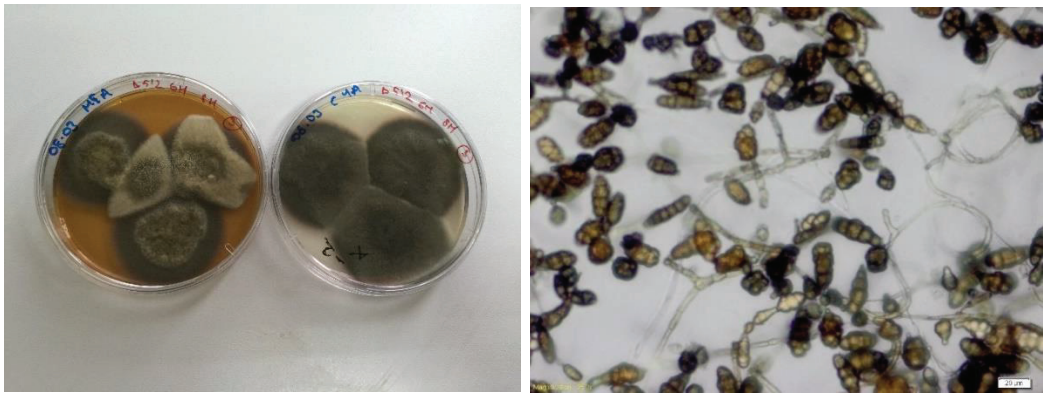
(a) (b)

Figure 4.7. Typical cultural and microscopical features of *Verticillium sp.* Culture plate (a) and light microscope (b) images.



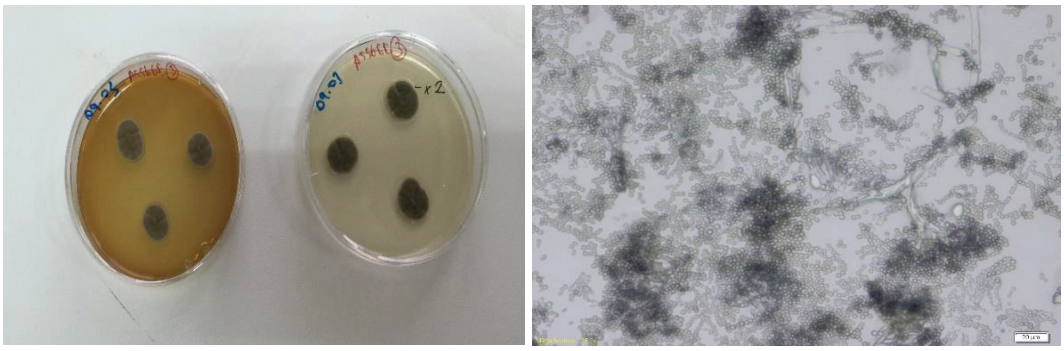
(a) (b)

Figure 4.8. Typical cultural and microscopical features of *Alternaria alternata sp.* Culture plate (a) and light microscope (b) images.



(a) (b)

Figure 4.9. Typical cultural and microscopical features of *Alternaria tenuissima sp.* Culture plate (a) and light microscope (b) images.



(a) (b)

Figure 4.10. Typical cultural and microscopical features of *Penicillium glabrum.* Culture plate (a) and light microscope (b) images.

4.2. PCR Results

4.2.1. Amplification of 28S rDNA Region

25 µl of PCR mixture including 5µl of genomic DNA (gDNA) was amplified by PCR. Two different PCR mixtures were prepared; one of them included primers of NL1- NL4. Amplification of gDNA was visualized by agarose gel electrophoresis under UV light.

The results showed that gDNA from moulds was obtained since the X bp PCR product was generated (Figures 4.11-4.14).

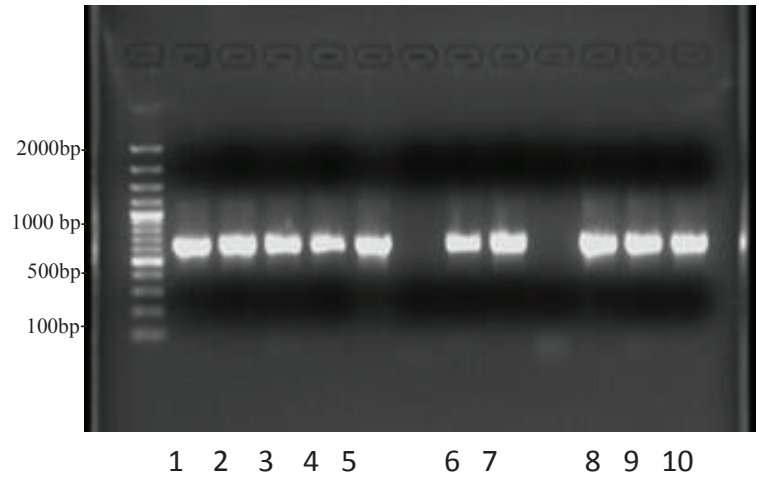


Figure 4.11. First sample group's amplified products of by NLprimers; **Lanes: 1.** 600 bp DNA ladder**,** *Aspergillus japonicus* **2.** *Alternaria alternata*; **3.***Aspergillus sydowii* **4.** *Kalmusia variispora* **5**. *Sarocladium terricola* **6.** *Aspergillus fumigatus* **7.** *Alternaria alternata* **8.***Cladosporium sp.* **9.** *Pleospora tarda* **10.** *Pyronema omphalodes*

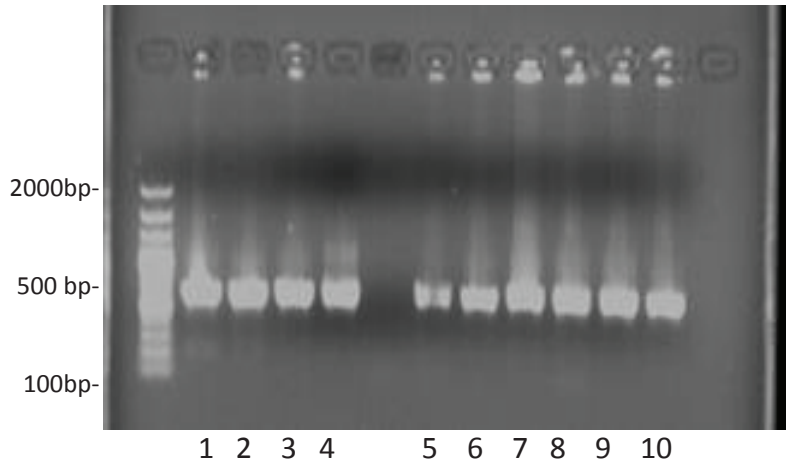


Figure 4.12. Second sample group's amplified products of by NLprimers; **Lanes: 1.** 600 bp DNA ladder**,** *Diaporthe rudis* **2.** *Aspergillus sydowii*; **3.** *Aspergillus fumigatus* **4.** *Alternaria alternata* **5.** *Cladosporium sp.* **6.** *Aspergillus japonicus* **7.** *Alternaria alternata* **8.** *Verticillium sp.* **9.** *Fusarium sp.***10.** *Diaporthe rudis*

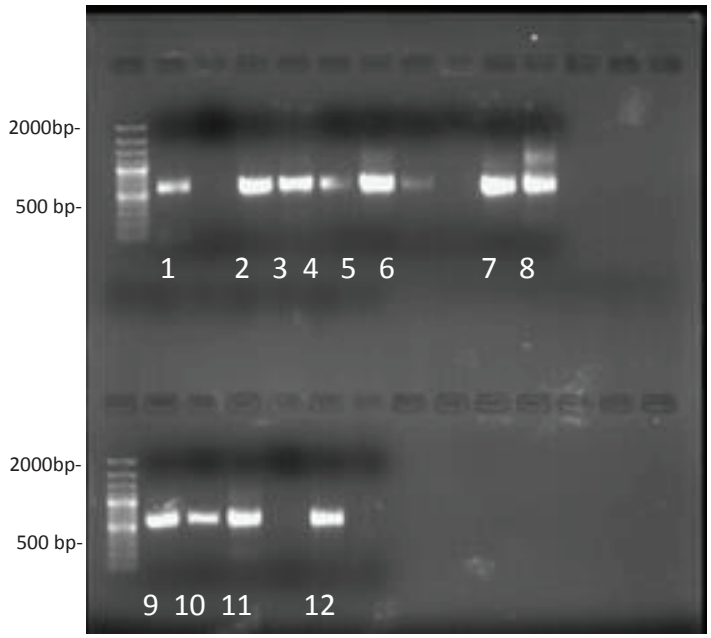


Figure 4.13. Third sample group's amplified products of by NLprimers; **Lanes: 1.** 600 bp DNA ladder**,** *Alternaria alternata* **2.** *Alternaria alternata*; **3.***Aspergillus sydowii* **4.** *Aspergillus fumigatus* **5**. *Cladosporium sp.***6.** *Alternaria leucanthemi* **7.** *Cladosporium sp.* **8.** *Aspergillus ustus* **9.** *Penicillium glabrum* **10.** *Verticillium psalliotae* ***11.*** *Preussia sp.****12****. Paraconiothyrium variabile*

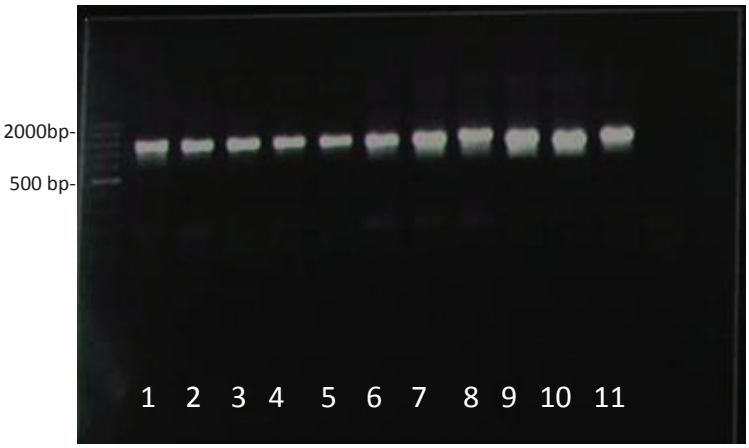


Figure 4.14. Forth sample group's amplified products of by NLprimers; **Lanes: 1.** 600 bp DNA ladder**,** *Aspergillus fumigatus* **2.** *Alternaria alternata*; **3.** *Cladosporium sp.* **4.** *Diaporthe rudis* **5**. *Alternaria alternata* **6.** *Verticillium sp.***7.** *Fusarium sp.***8.** *Aspergillus fumigatus*
9. *Aspergillus sydowii* **10.** *Alternaria alternata* **11.** *Cladosporium cladosporioides*

Isolation of amplifiable DNA from the sample is the most prominent prerequisite for a reliable PCR method. This means also DNA obtained should be intact and free from contaminants such as RNA, DNAases, proteins, enzymes which may interfere the isolation of pure and intact DNA. Moreover, pure mould DNA extraction is quite difficult since the mould cell wall structure affects the ability to extract DNA, efficiently.

On the agarose gels the gDNA extracted from the different moulds gave a single amplicon of 600 bp. Therefore, extracted gDNA was free of RNA contamination and met the standard. Marshall et al. (2003) has stated that the NL primer set had a hidher tolerance for PCR inhibitors as seen in this studyç

To check the quality of the total DNA, the A_{260}/A_{280} ratio was measured in all the filamentous moulds. Ratio obtained for most of the moulds ranged between 1.7 and 2.1 (Table 4.1).

Table 4.1. Purity ratios (A_{260}/A_{280} index) of the DNA extracted from fungal strains*

Code	**Genus/Species**	**A_{260}/A_{280} index**
8HSDA15	*Aspergillus fumigatus*	1.79
8H2DA23	*Aspergillus fumigatus*	2.14
6HSDA39	*Aspergillus sydowii*	1.92
2HPDA33	*Aspergillus fumigatus*	1.98
6H2SD21	*Aspergillus fumigatus*	1.93
8H1DA29	*Aspergillus fumigatus*	1.74
H1PDA8	*Aspergillus japonicus*	1.76
6H3DA31	*Aspergillus japonicus*	1.82
Z10A4	*Aspergillus aculeatus*	1.84
H1S3	*Aspergillus sydowii*	1.92
2H3PDA30	*Aspergillus aculeatus*	2.03
A556KE2	*Aspergillus protuberus*	2.18
B556KE1	*Aspergillus sydowii*	1.95
7H2PDA12	*Aspergillus sydowii*	1.97
8HCZA13	*Aspergillus ustus*	1.84
7HOA71	*Penicillium brevicompactum*	1.71
25HSDA13	*Penicillium glabrum*	1.76
B556GH1	*Penicillium glabrum*	1.83
A512GE3	*Penicillium glabrum*	1.92
B512KE3	*Penicillium glabrum*	1.74

(Cont. on next page)

Table 4.1. (cont.)

B512KH1	*Alternaria alternata*	1.71
A534KE1	*Alternaria leucanthemi*	1.86
B556GH2	*Alternaria alternata*	1.98
B556KE3	*Alternaria alternata*	1.87
B512GH1	*Alternaria alternata*	1.96
A534KE6	*Alternaria alternata*	1.87
B556KH1	*Alternaria alternata*	1.98
B512KH2	*Alternaria alternata*	1.78
B2KE9H1	*Alternaria alternata*	1.98
A2GE9H1	*Alternaria alternata*	1.71
B534KH3	*Cladosporium sp.*	1.82
A534GE2	*Cladosporium sp.*	1.84
A556GE2	*Cladosporium sp.*	1.98
B556GE2	*Cladosporium sp.*	1.92
A534GE6	*Cladosporium cladosporioides*	1.86
B534KE2	*Cladosporium cladosporioides*	1.78
B556KE1	*Cladosporium uredinicola*	1.87
B534GE1	*Cladosporium sp.*	1.92
A2KE9H2	*Fusarium sp.*	1.76
A534GE4	*Verticillium psalliotae*	1.72
A2KE9H2	*Verticillium psalliotae*	1.74
B534KE1	*Verticillium sp.*	1.86
A512GH2	*Verticillium sp.*	1.92
A1GE9H1	*Verticillium sp.*	1.77
B534KH5	*Preussia sp.*	1.75
A3GE9H1	*Kalmusia variispora*	1.82
A3KE9H1	*Dendrothyrium longisporum*	1.74
B2KE9H1	*Paraconiothyrium variabile*	1.86
B534KH4	*Diaporthe rudis*	1.72
A556KE1	*Pleospora tarda*	1.86
B512KE1	*Pyronema omphalodes*	1.88
H10A5	*Aschersonia insperata*	1.71
B2GE9H1	*Sarocladium terricola*	1.87

*** Values were provided as A_{260}/A_{280} index ± SD.**

4.2.2. PCR Blast Results

All isolated samples were analyzed by BLAST after PCR process. Highly similar sequences were selected for BLAST analysis. The data obtained according to the BLAST results are shown in the Table 4.2. 18 isolates were could not be identified by PCR.

% 19 and %81 of the isolates were identified to the genus and species level, respectively. Some of the identified species are rare and in some cases had only a single GenBank deposit (Table 4.2).

With a query cover < 90% and identity around 95% mean suspected new species or genus records in BLAST-N search. In our study only 3 species had query cover 63%, 63% and 96% with identities 99%, 95% and 96%, for *Alternaria alternata*, *Kalmusia variispora* and *Paraconiothyrium variabile*, respectively.

Query coverage is the % of the sequence aligned to a sequence in GenBank. If this effective size of the sequence that have been compared low, it means that overlap with the reference sequence is very low. However, identity around or higher 95% indicates that whatever sequence which overlapped matched very well.

All the mold isolates showed high homology by 28S rDNA sequence of the species or genera with the level of identity 99 and 100%, except for *Preussia* spp. (98%), *Kalmusia variispora* (95%) and *Paraconiothyrium variabile* (96%). Query coverage and alignment identity values ranged from 95-100%, with exceptions of 63% query coverage for *A. alternata* and *K. variispora*.

Table 4.2. Fungal species that were identified by PCR analysis.

	Code	Species/Genus	Max Score	Total Score	Query Cover	Ident	Accession Number
		Aspergillus spp.					
1	8HSDA15	*Aspergillus fumigatus*	1046	1287	100%	100%	**MF352619**
2	8H2DA23	*Aspergillus fumigatus*	1053	1024	100%	99%	**MF352620**
3	6HSDA39	*Aspergillus sydowii*	1026	1026	100%	99%	**MF352621**
4	2HPDA33	*Aspergillus fumigatus*	1038	1038	100%	100%	**MF352622**
5	6H2SD21	*Aspergillus fumigatus*	990	990	100%	100%	**MF352656**
6	8H1DA29	*Aspergillus fumigatus*	992	992	100%	100%	**MF352661**
7	H1PDA8	*Aspergillus japonicus*	1013	1013	100%	100%	**MF352623**
8	6H3DA31	*Aspergillus japonicus*	418	418	97%	99%	**MF352646**
9	Z10A4	*Aspergillus aculeatus*	1029	1029	100%	100%	**MF352624**
10	H1S3	*Aspergillus sydowii*	1024	1024	100%	100%	**MF352625**
11	2H3PDA30	*Aspergillus aculeatus*	1033	1033	100%	100%	**MF352626**

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Table 4.2. (cont.)

12	A556KE2	*Aspergillus protuberus*	1042	1042	100%	99%	**MF352628**
13	B556KE1	*Aspergillus sydowii*	1044	1044	100%	99%	**MF352630**
14	7H2PDA12	*Aspergillus sydowii*	1046	1046	100%	100%	**MF352631**
15	8HCZA13	*Aspergillus ustus*	931	931	100%	100%	**MF352663**
		Penicillium spp.					
1	7HOA71	*Penicillium brevicompactum*	1050	1224	100%	100%	**MF352632**
2	25HSDA13	*Penicillium glabrum*	1038	1185	100%	100%	**MF352627**
3	B556GH1	*Penicillium glabrum*	1042	1221	100%	99%	**MF352629**
4	A512GE3	*Penicillium glabrum*	1033	1033	100%	99%	**MF352633**
5	B512KE3	*Penicillium glabrum*	1038	1038	98%	99%	**MF352671**
		Alternaria spp.					
1	B512KH1	*Alternaria alternata*	1040	1040	100%	99%	**MF352634**
2	A534KE1	*Alternaria leucanthemi*	974	974	100%	100%	**MF352641**
3	B556GH2	*Alternaria alternata*	1002	1002	100%	100%	**MF352642**
4	B556KE3	*Alternaria alternata*	963	963	100%	100%	**MF352645**
5	B512GH1	*Alternaria alternata*	702	702	100%	100%	**MF352647**
6	A534KE6	*Alternaria alternata*	905	905	100%	100%	**MF352648**
7	B556KH1	*Alternaria alternata*	924	924	100%	100%	**MF352651**
8	B512KH2	*Alternaria alternata*	372	372	100%	100%	**MF352658**
9	B2KE9H1	*Alternaria alternata*	974	974	100%	100%	**MF352664**
10	A2GE9H1	*Alternaria alternata*	1029	1029	63%	99%	**MF352669**

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Table 4.2. (cont.)

	Code	Species/Genus	Max Score	Total Score	Query Cover	Ident	Accession Number
		Cladosporium sp.					
1	B534KH3	*Cladosporium sp.*	1035	1357	100%	99%	**MF352635**
2	A534GE2	*Cladosporium sp.*	965	965	100%	100%	**MF352637**
3	A556GE2	*Cladosporium sp.*	970	970	100%	100%	**MF352644**
4	B556GE2	*Cladosporium sp.*	961	961	100%	100%	**MF352654**
5	A534GE6	*Cladosporium cladosporioides*	1038	1350	100%	99%	**MF352636**
6	B534KE2	*Cladosporium cladosporioides*	891	891	100%	100%	**MF352655**
7	B556KE1	*Cladosporium uredinicola*	968	968	100%	100%	**MF352650**
8	B534GE1	*Cladosporium sp.*	1037	1400	100%	99%	**MF352659**
		Others					
1	A2KE9H2	*Fusarium sp.*	929	929	100%	100%	**MF352666**
2	A534GE4	*Verticillium psalliotae*	996	1141	100%	99%	**MF352671**
3	A2KE9H2	*Verticillium psalliotae*	998	1252	95%	99%	**MF352672**
4	B534KE1	*Verticillium sp.*	970	970	100%	100%	**MF352657**
5	A512GH2	*Verticillium sp.*	935	935	100%	100%	**MF352665**
6	A1GE9H1	*Verticillium sp.*	778	778	100%	100%	**MF352668**
7	B534KH5	*Preussia sp.*	976	976	100%	98%	**MF352640**
8	A3GE9H1	*Kalmusia variispora*	841	841	63%	95%	**MF352670**
9	A3KE9H1	*Dendrothyrium longisporum*	368	368	100%	100%	**MF352667**
10	B2KE9H1	*Paraconiothyrium variabile*	929	929	96%	96%	**MF352673**
11	B534KH4	*Diaporthe rudis*	1013	1013	100%	99%	**MF352638**
12	A556KE1	*Pleospora tarda*	994	994	100%	100%	**MF352643**
13	B512KE1	*Pyronema omphalodes*	985	985	100%	100%	**MF352653**
14	H10A5	*Aschersonia insperata*	973	972	100%	99%	**MF352649**
15	B2GE9H1	*Sarocladium terricola*	959	959	100%	100%	**MF352662**

Molecular identification based on small differences in genetic material provides a more accurate and definitive method for distinguishing species. (Fairbanks and Anderson, 1999). Fingerprinting methods generally used for identification include Random Amplified Polymorphic DNA (RAPD) (Fungaro et al., 2004; Hadrys et al., 1992; Lund et al.,2003), Amplified Fragment Length Polymorphic fingerprinting (AFLP) (Vos et al., 1995;Majer et al., 1996; Castella et al., 2002), Polymerase Chain Reaction-RestrictionFragment Length Polymorphism (PCR-RFLP) (LoBuglio and Taylor, 1995; Sequerra et al., 1997; Colombo et al., 2003; Latouche et al., 2003), micro arrays (Hsiao et al., 2005; Schmidt-Heydt and Geisen, 2007), DNA barcoding (Seifert et al., 2007) and DNA sequencing (Vogler et al.,1998; Samson et al., 2004). Variations on PCR identification methods include multiplex PCR assays (Dean et al. 2005; Luo & Mitchell, 2002) and Real-Time PCR (Bolehovska et al., 2006; Bu et al., 2005; Geisen et al. 2004).

Currently, the routine identification of filamentous fungi is based on phenotypic methods that frequently need an expertise in their morphological analysis (Marinach-Patrice et al., 2009). These methods are often time-consuming, laborious, and can lack sensitivity because of the the huge diversity of the fungal species present in food or feed. The molecular methods currently available such as sequencing of the internal transcribed spacer region or other genes of interest are still high cost and difficult to implement in routine laboratory practices (Nilsson et al., 2009).

Traditionally, the identification of fungi has mainly been made through morphological and physiological parameters.Currently, in addition to classical methods, molecular techniques have been used in classification, such as the sequencing of β-tubulin and calmodulin genes, ribosomal RNA genes (rDNA) and their flanking internal transcribed spacers (ITS1–5.8S–ITS2 rDNA region) (Perrone et al., 2004).

Species of *Aspergillus* and *Penicillium*, as well as *Alternaria* spp. are filamentous fungi isolated from olive and olive cake (Roussos et al. 2006). Also, main species isolated from olive and olive cake were *Aspergillus fumigatus* and *Paecilomyces variotii* (Roussos et al. 2006). It should be noted that *P. variotii* was found the best isolate as it degraded 10-40% of the phenolic compounds (Khatab et al., 2013). In this study after *Aspergillus* and *Penicillium* species, *Alternaria* was also the genus which is mostly isolated from olive (Table 4.3).

Alternaria is a genus of asexual or imperfect fungi assigned with the class hyphomycetes and fungi in this genus are anamorphs of ascomycetes, including

members of the *Pleospora*. Both genera are included in the same family (Pleosporaceae) (Table 4.3). *Alternaria* is a dematiaceous mold which includes about 50 species and varieties; including 8 human pathogens (*A. alternata*, *A. brassicicola*, *A. chartarum*, *A. stemphylioides*, *A. dianthicola*, *A. infectoria*, *A. pluriseptata* and *A. tenuissima* and an allergenic *A. iridinis* (Liu 2011). In general saprobic or plant-pathogenic species of *Alternaria* attacks plants under stress especially by those affected by drought, insect infestation or senescence (Liu 2011). From olives besides *A. conjuncta*, *A. breviramosa*, *A. leucanthemi* and *A. tomato*, potential pathogens *A. alternata* and *A. tenuissima* were isolated (Table 4.3). Although the metabolite profiles of *A. alternata* and *A. tenuissima* are very similar, *A. tenuissima* differs from *A. alternata* by the formation of longer conidia (up to 60 μm) in unbranched chains (Simmons 2007). *A. alternata* is the most common saprophytic *Alternaria* species, occuring on a wide variety of sources in contrast to other species. *A. alternata* was also the mostly isolated fungi in this study (9 strains). However, this major pathogen of tomatoes, produces several toxins of which the most important is tenuazonic acid (TA) (Logrieco et al., 2003; Pitt & Hocking, 2009). Less toxic compounds such as alternariol and alternariolmonomethyl ether are usually found in combination and can cause some teratogenic and fetotoxigenic effects in mice (Pitt & Hocking, 2009). The production of one or more of these toxins has been in many sources, also in olives (Logrieco et al., 2003).

Table 4.3. Taxonomical classification of ascomycota (division) fungi (kingdom) identified* and used in this study.

Division	Subdivision	Class	Subclass	Order	Family	Genus	Species
Ascomycota	Pezizomycotina	Eurotiomycetes	Eurotiomycetidae	Eurotiales	Trichocomaceae	*Aspergillus*	*aculeatus*
							fumigatus
							japonicus
							*ochraceus**
							*oxalicum**
							*paxilli**
							protuberus
							sydowii
							ustus
						Penicillium	*brevicompactum*
							glabrum
							*funiculosum**
							*malachiteum**
							*oxalicum**
							*paxialli**
						Emericella	*variecolor**
						Paecilomyces	*variotii**
		Dothideomycetes	Dothideomycetidae	Capnodiales	Cladosporiaceae	*Cladosporium*	*cladosporioides*
							delicatum
							herbarum
							*silenes**
							uredinicola
					Mycosphaerellaceae	*Davidiella**	-
			Pleosporomycetidae	Pleosporales	Didymosphaeriaceae	*Dendrothyrium*	*longisporum*
							*variisporum**
						Kalmusia	*variispora*
						Paraconiothyrium	*variabile*
					Sporormiaceae	*Preussia*	*minipascua*
							-
					Pleosporaceae	*Alternaria*	*alternata*
							*breviramosa**
							*conjuncta**
							leucanthemi

(Cont. on page)

Table 4.3. (cont.)

							tenuissima[*]
							tomato[*]
						Pleospora	*tarda*
Division	**Subdivision**	**Class**	**Subclass**	**Order**	**Family**	**Genus**	**Species**
		Sordariomycetes	Hypocreomycetidae	Hypocreales	Nectriaceae	*Fusarium*	*fujikuroi*[*]
					Clavicipitaceae	*Aschersonia*	*insperata*
							aleyrodis[*]
						Sarocladium	*terricola*
				Glomerellales	Plectosphaerellaceae	*Verticillium*	*psalliotae*
					Glomerellaceae	*Colletotrichum*	*gloeosporioides*[*]
			Sordariomycetidae	Diaporthales	Diaporthaceae	*Diaporthe*	*meduseae*[*]
							rudis
						Phomopsis[*]	-
		Pezizomycetes	Pezizomycetidae	Pezizales	Pyronemataceae	*Pyronema*	*omphalodes*

[*] Mould strains isolated and identified in another study performed by Özcan and Baysal (2013).

Soilborne and strictly asexual reproducing fungus *Verticillium dahliae* is aetiological agent of *Verticillium* wilt of olive (*Olea europea* L.) isolated from olive and olive trees (Bellahcene et al. 2005; Gouveia 2007; Jafary 2011; Jiménez-Díaz et al. 2011; Jiménez-Díaz et al. 2012; López-Escudero & Mercado-Blanco, 2011; Mercado-Blanco et al. 2003; Triki et al., 2011). In this study 5 strains of *Verticillium* were isolated from the olive, however, *V. psalliotae* (2 strains) is the only species identified at species level (Table 4.2.).

Increased incidence of attacks by airborne fungi *Colletotrichum* species (olive anthracnose) have been reported. It is a hemibiotrophic haploid species for which no sexual state has been identified yet (López-Escudero & Mercado-Blanco, 2011).

Paecilomyces species are soil fungi and isect pathogens that 3 species of those are commonly isolated from foods are *Paecilomyces variotii*, *P. lilacinus* and *P. fulvus*. *Byssochlamys spectabilis* and *Byssochlamys fulva* are the teleomorph of the *P. variotii* and *P. fulvus*, respectively. *P. variotii* is a xerophilic (a_w 0.80) fungus and notable for sorbate resistance and has a strong association with edible oils, raw materials containing oil. Therefore, it is also necessary to emphasize the importance of this mould for sorbate preserved and oil containing foods such as olives.

Previous results have shown that the ITS rDNA regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful and reasonably precise in the rapid identification of fungi species (Peterson 2012). Consequently the lack of adapted tools for the food and feed sectors may lead to a misidentification of fungi and generates an uncontrolled risk for these industries. In this context, there is a need for developing simple and rapid approaches adapted to industrial set-ups for the identification of filamentous fungi. FTIR spectroscopy represents a real progress towards efficient mold identification techniques as it is cost-effective and time-saving (2 days instead of 5) over conventional methods. In fact, the filamentous fungi identification based currently on the morphological features analysis is difficult because of the very high phenotypic biodiversity. Morover, the conventional methods require a good knowledge of the fungal strains and are time consuming. The use of molecular approaches based on DNA sequencing for filamentous fungi identification presents some limits like the cost and the application constraints (Alexander, 2002). The most recent promising method is the use of MALDI TOF mass spectrometry for routine fungal identification (Del Chierico et al., 2012; Normand et al., 2013).This method is

rapid, reliable, involves low labor and consumable cost but the equipment is quite expensive as compared to a high-throughput FTIR system (Santos et al., 2010).

4.3. Fourier Transform Infrared (FTIR) Spectroscopy Results

Recent developments concerning FTIR spectroscopy have allowed implementing alternative identification methods adapted to a large range of microbiological samples. Infrared spectroscopy is a vibrational spectroscopic technique which is based on the measurement of the fundamental molecular bond vibrational modes. In this technique, a polychromatic infrared source (400-4000 cm^{-1}) interacts with the sample and the molecules can either absorb or reflect the radiation, whereby vibrational motions are stimulated. Only specific frequencies are absorbed corresponding to their molecular modes of vibration that are characteristic of their chemical bonding, composition, and structure (Bozza de Almeida et al. 2015).

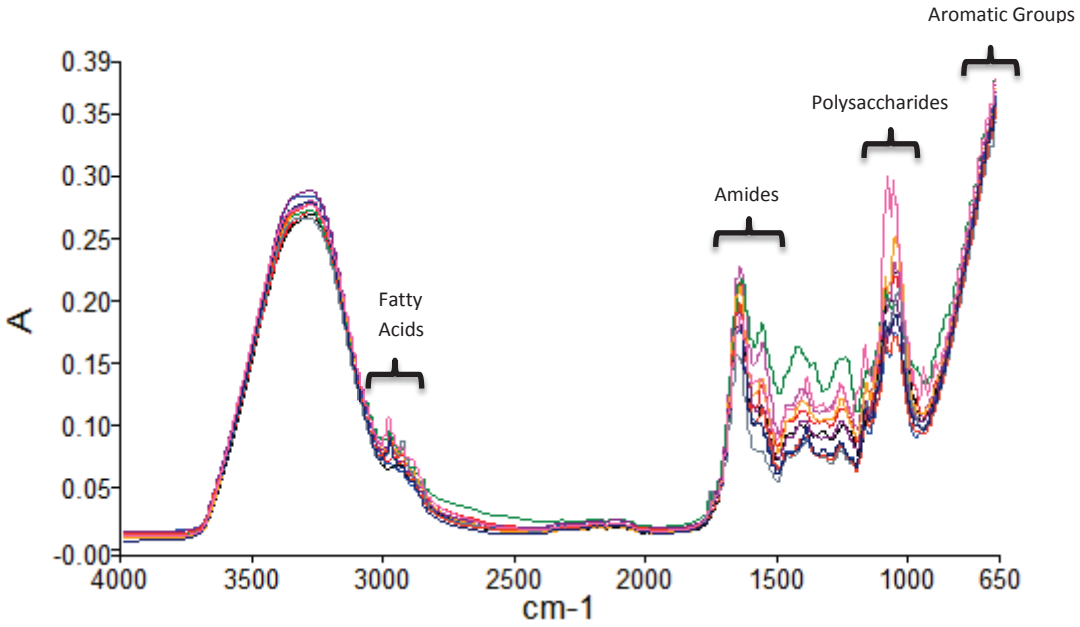
The changes in light absorption at specific frequencies allow determining which molecular groups are present and how they are arranged or interacting. The result is given under the form of an FTIR spectrum. Thus, on this spectrum, each spectral band characterized by its frequency and intensity, reflects "the molecular fingerprint" of the characteristic molecules (Duygu et al., 2009; Naumann, 2000). The spectral profile gives information about important macromolecules like proteins, lipids, nucleic acids and carbohydrates present in cells.

The infrared spectra of fungi show bands, which are specific to certain functional groups of biological origin. According to numerous studies (Lecellier et al., 2015; Salman et al., 2006; Shapaval et al., 1996; Nie et al., 2006 and Monika et al., 2013), the four main regions can be distinguished:

- Fatty acids (3050‑2800 cm^{-1}),
- Amide I and II (1700‑1500 cm^{-1}),
- Polysaccharides (1200‑900 cm^{-1}),
- Aromatic groups (900-500 cm^{-1}).

A total of 71 fungal isolates were tried to be differentiated by FTIR spectroscopy in this study. 53 isolates were identified by PCR before FTIR spectroscopy analysis. 18 pre-defined isolates were collected from previous studies.

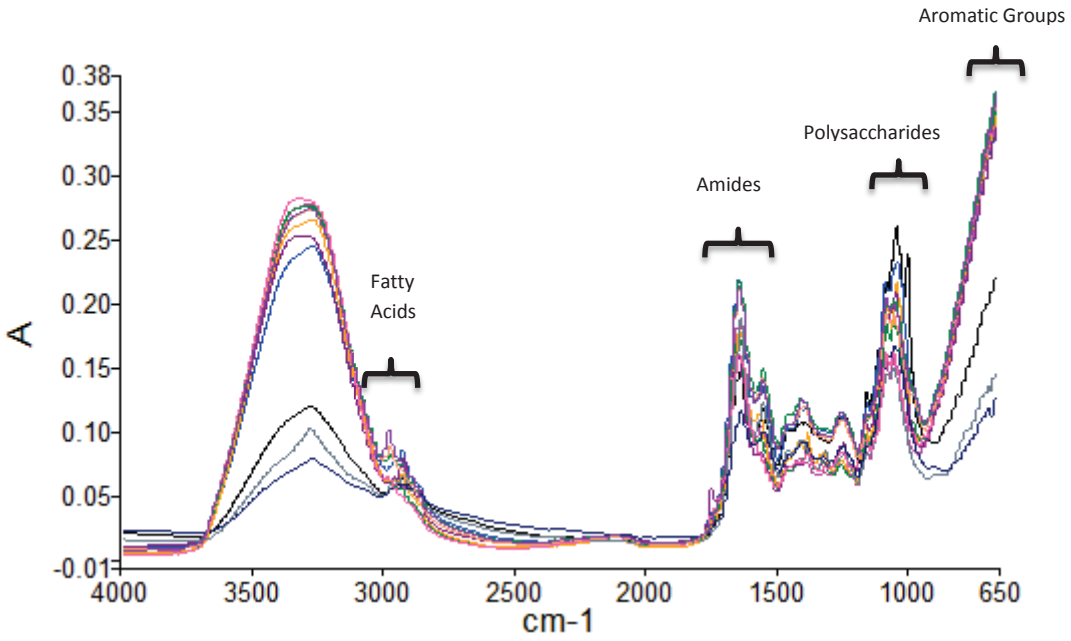
Isolates collected from different fungal samples were compared by using their FTIR spectra in 4000-650-cm^{-1} range. FTIR spectra of each of the *Aspergillus* species are given in the Figure 4.15.



Name	Description
8HSDA15	*Aspergillus fumigatus*
8H2DA23	*Aspergillus fumigatus*
6HSDA39	*Aspergillus sydowii*
2HPDA33	*Aspergillus fumigatus*
H1PDA8	*Aspergillus japonicus*
Z10A4	*Aspergillus aculeatus*
H1S3	*Aspergillus sydowii*
A556KE2	*Aspergillus protuberus*
7H2PDA12	*Aspergillus sydowii*
8HCZA13	*Aspergillus ustus*
8H1DA29	*Aspergillus fumigatus*

Figure 4.15. Comparison of FTIR spectra of different *Aspergillus* species

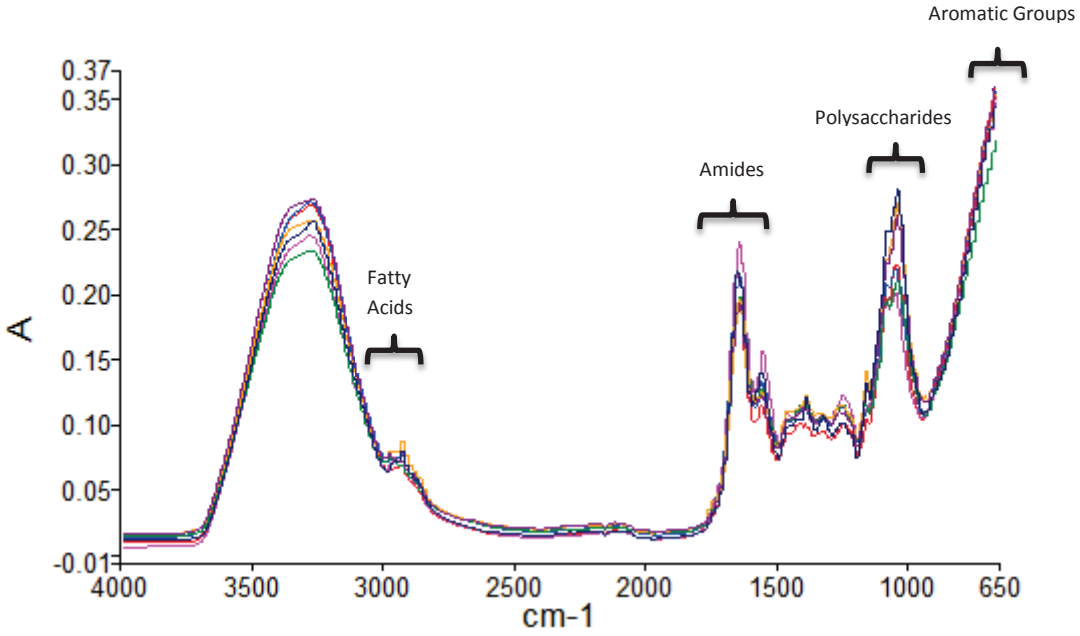
The FTIR spectra were characterised by the amide I and II group around 1650 cm^{-1} and 1550 cm^{-1}, respectively. These bands most likely originate from the amide bond in various proteins and peptides (Maquelin et al., 2002). Around 1200-950 cm^{-1}, C-O-C stretching vibration in polysaccharides, together with the symmetric and asymmetric stretching vibration of PO_2 functional groups from nucleic acids can be observed (Maquelin et al., 2002). FTIR spectrum also shows a series of bands, which are assigned to fatty acids and lipids.



Name	Description
B534KH3	*Cladosporium sp.*
A534GE2	*Cladosporium sp*
A556GE2	*Cladosporium sp.*
A534GE6	*Cladosporium sp.*
B534KE2	*Cladosporium cladosporioides*
B556KE1	*Cladosporium cladosporioides*
B534GE1	*Cladosporium uredinicola*
B556GE2	*Cladosporium sp.*
B556GE2	*Cladosporium sp.*

Figure 4.16. Comparison of FTIR spectra of different *Cladosporium* species

FTIR spectra of each of the *Cladosporium* species are given in the Figure 4.16. Aromatic groups which are in a range of 900-500 cm^{-1} spectra, have been reported that the true "fingerprint region" where most of the bands are unassigned to specific cellular compounds or functional groups (Nie et al., 2006). In the Figure 4.16, it was observed that the spectrum which belong to B534KH3, A534GE6 and B556GE2 coded *Cladosporium sp.* genus at the "fingerprint" region, have different behavior than others. These fungi have 99%, 99% and 100% identification in Blast records, respectively.

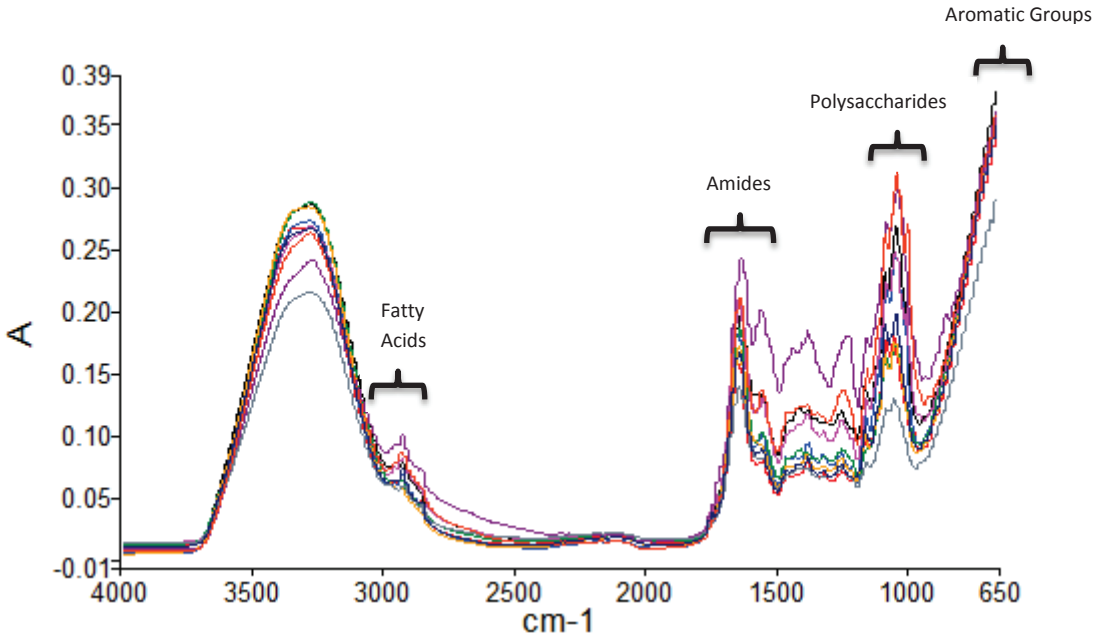


Name	Description
7HOA71	*Penicillium glabrum*
25HSDA13	*Penicillium glabrum*
B556GH1	*Penicillium brevicompactum*
A512GE3	*Penicillium glabrum*
A512KE3	*Penicillium glabrum*
Z10A10	*Penicillium paxaillii*
A3KH3SBRO4	*Penicillium oxalicum*

Figure 4.17. Comparison of FTIR spectra of different *Penicillium* species

In the Figure 4.17., it has observed that the spectrum which belong to B556GH1 coded *Penicillium brevicompactum* strain has extra high picks at amides band or A512GE3 coded *Penicillium glabrum* strain has a different behavior at "fingerprint" region that were refered to 900-700 cm^{-1} wavenumbers. Both fungi has 99% identification in Blast records.

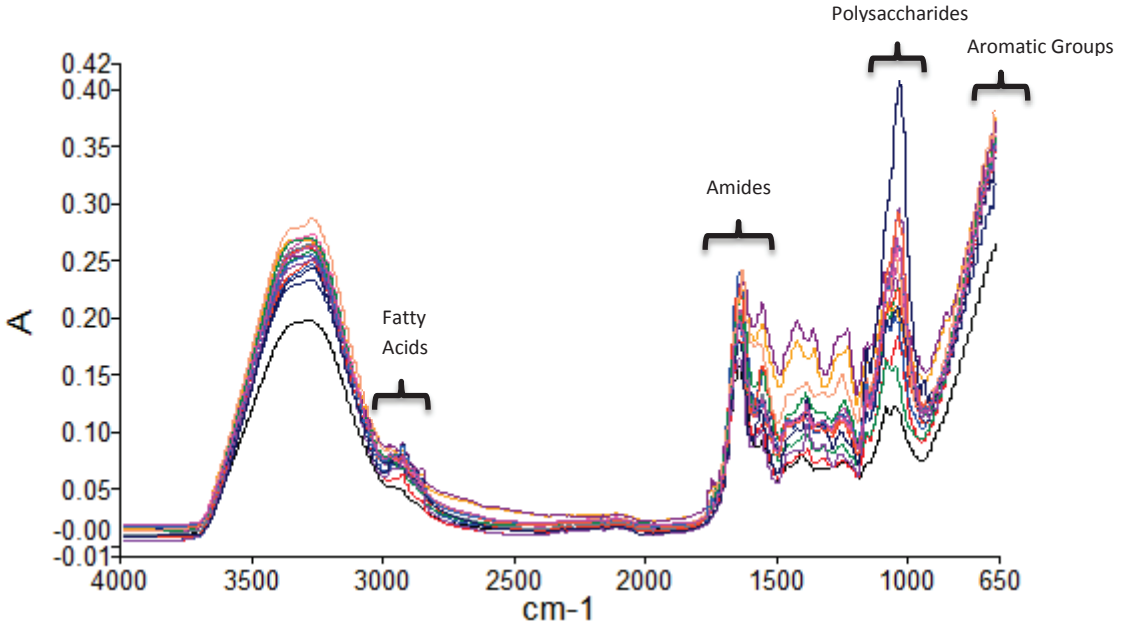
However, it should be noted that absorption intensity in the "fingerprint" region was small for most of microorganisms. For the best classification results, all four bands should be included in analysis. As seen in Figure 4.17., the spectra that has a wavenumber of about 3300 cm^{-1}, an intense, wide band originating from an O-H group was observed. This band was present in spectra of all the species examined. This band could originate from water molecules presented in the cytoplasm or from extracellular water, e.g. from the moisture in the air. Therefore, this area (4000‑3050 cm^{-1}) is not suitable for identification of fungi since it may cause some confusion in interpretation of the results (Szeghalmi et al., 2007).



Name	Description
B512KH1	*Alternaria alternata*
A534KE1	*Alternaria leucanthemi*
B556GH2	*Alternaria alternata*
B556KE3	*Alternaria alternata*
B512GH1	*Alternaria alternata*
A534KE6	*Alternaria alternata*
4HPDA1	*Alternaria tenuissima*
A3GH4PDACRM1	*Alternaria tomato*
A5KH4MEA	*Alternaria brevimosa*
A3GH8PDACRMN	*Alternaria conjucta*
B2KE9H1	*Alternaria alternata*

Figure 4.18. Comparison of FTIR spectra of different *Alternaria* species

Figure 4.18 shows that the spectra of different *Alternaria* species have showed great diversity for each *Alternaria* species, and it was observed not only in the "fingerprint" band (900‑700 cm^{-1}), but also in the remaining fatty acids, amides, polysaccharides bands. For example B2KE9H1 coded *Alternaria alternata* strain has different profile at fatty acids and amides band than the other *Alternaria* strains. On the other hand, A3GH4PDACRM1 coded *Alternaria tomato* strain has a different behavior at "fingerprint" region.



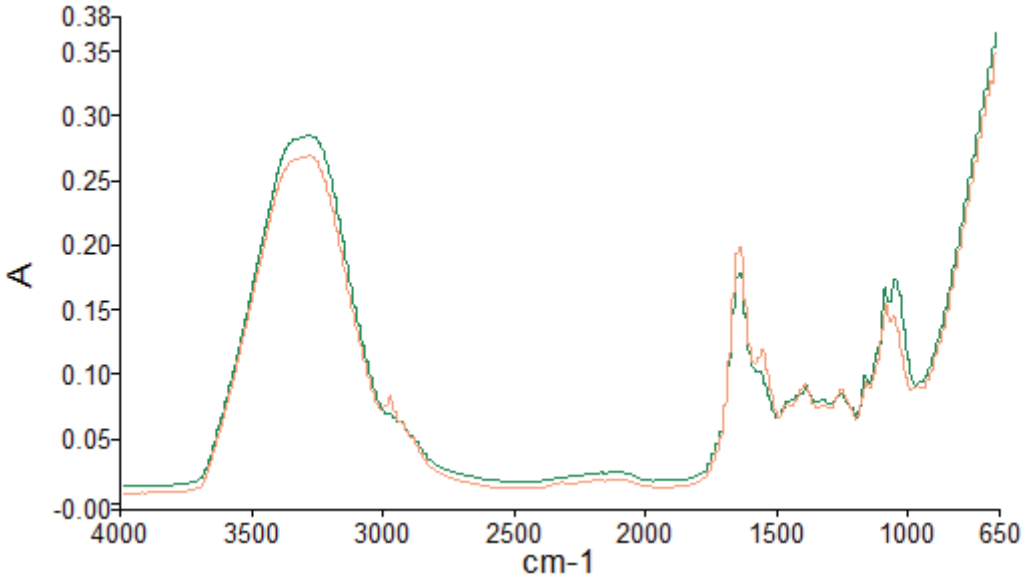
Name	Description
A2KE9H2	*Fusarium sp.*
A534GE4	*Verticillium psalliotae*
A2KE9H2	*Verticillium psalliotae*
B534KE1	*Verticillium sp.*
A512GH2	*Verticillium sp.*
A1GE9H1	*Verticillium sp.*
B534KH5	*Preussia sp.*
A3GE9H1	*Kalmusia variispora*
A3KE9H1	*Dendrothyrium longisporum*
B2KE9H1	*Paraconiothyrium variabile*
B534KH4	*Diaporthe rudis*
A556KE1	*Pleospora tarda*
B512KE1	*Pyronema omphalodes*
H10A5	*Aschersonia insperata*
B2GE9H1	*Sarocladium terricola*
H1OA5	*Colletotrichum gloesprodies*
6HPDA2	*Fusarium fjikori*
8HSDA12	*Paecilomyces variot*

Figure 4.19. Comparison of FTIR spectra of different fungal species

FTIR spectra of various fungal species were compared in Figure 4.19. It can be clearly observed that there are significant differences in the fatty acids, amides, polysaccharides and "fingerprint" regions because of different chemical compositions of different fungal species. But; B2KE9H1 coded *Paraconiothyrium variabile* strain has a very different peak in the polysaccharide range. On the other hand, B2GE9H1 coded *Sarocladium terricola* has a different trend in the fingerprint region. The strains have

96% and 99% identification in Blast records, respectively. These strains have common properties; and they belong to the same tree and they were isolated in the same period.

Comparison of two FTIR spectra of different *Alternaria alternata* strains was performed with spectral analyses by PerkinElmer Spectrum™ (Version 10.4.3) software. The results are given in the Figure 4.20 and 4.21. The correlation of these strains 1 vs. 4 and 2 vs. 4 were found as 0.963 and 0.946, respectively.

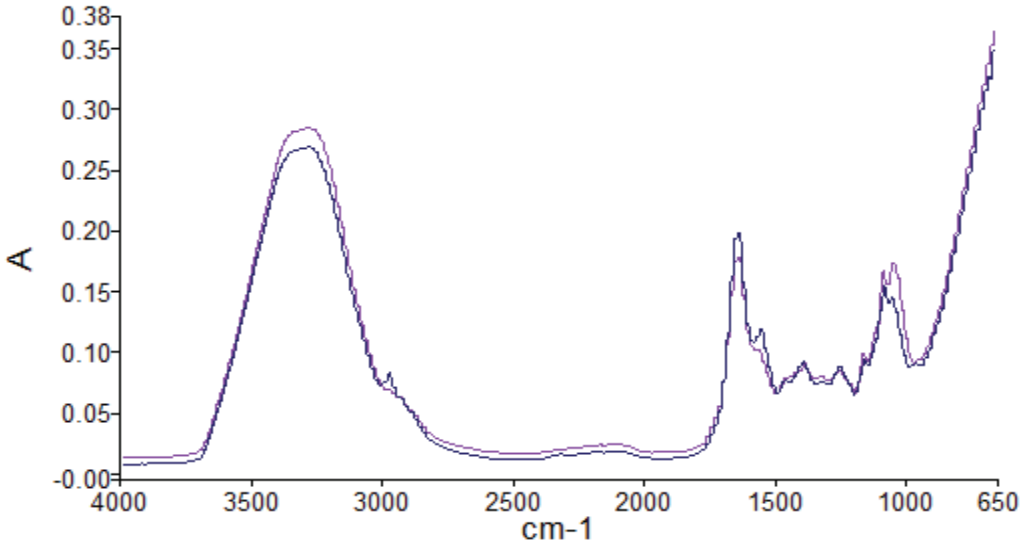


Hit Name	Correlation
Alternaria altenata1& Alternaria alternata4	0.962674

Figure 4.20. FT-IR spectra of *Alternari alternata 1 vs*. 4

Figure 4.20 and Figure 4.21 show that two different *Alternaiia alternata* strains have 2.6% and 5.4% difference which describes the reproducibility of spectral measurements for samples prepared from independent cultures of the different strains, has practical implications for the measurement of FTIR spectra.

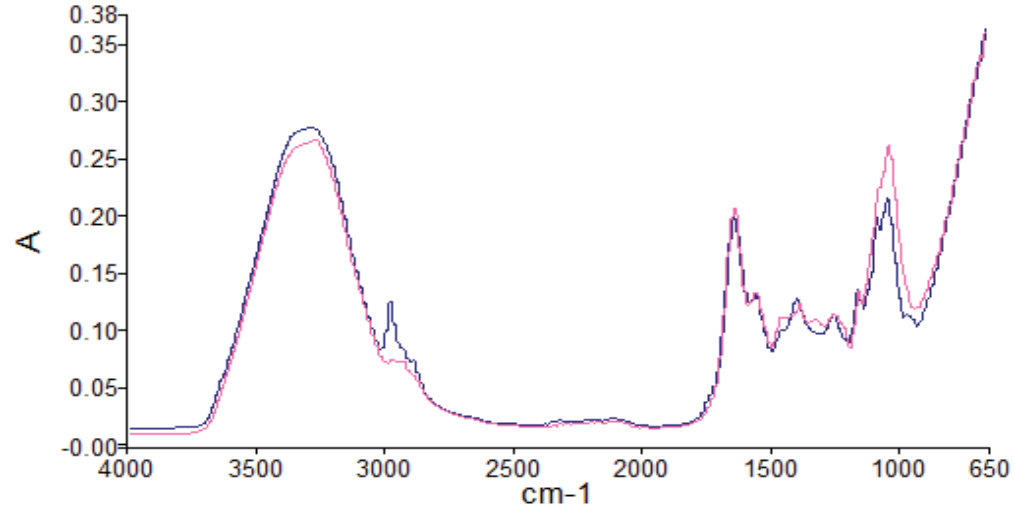
According to Figure 4.20. and Figure 4.21., spectral profiles differ in fatty acid, amides and polysaccharides bands. However, these fungi have similar peaks in the "fingerprint" region. The strains have both 100% Identification in Blast records.



Hit Name	Correlation
Alternaria altenata2 & Alternaria alternata4	0.946492

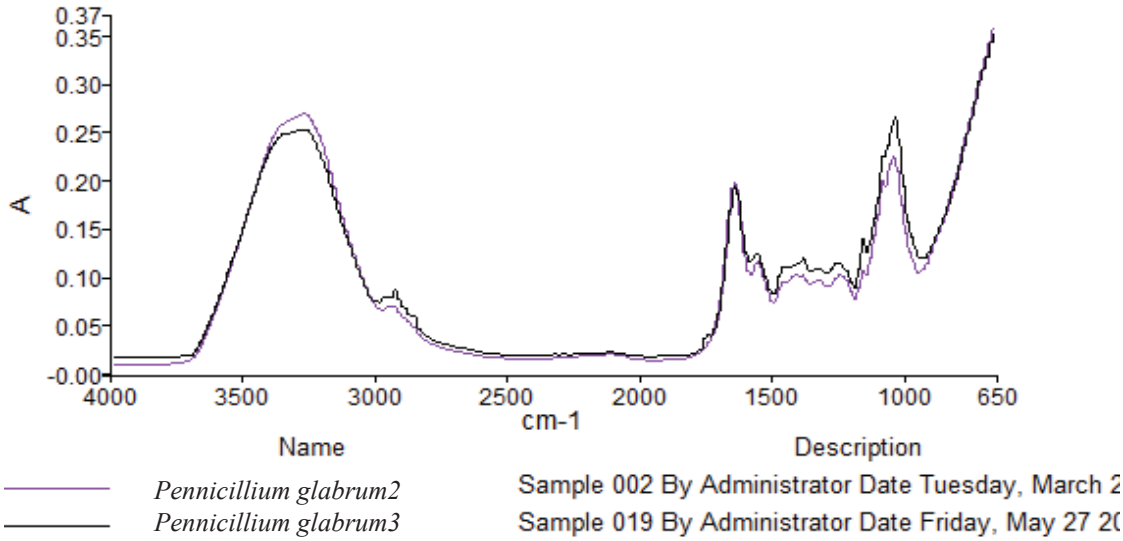
Figure 4.21. FT-IR spectra of*Alternaria alternata* 2 *vs.*4

As could be seen from the Figure 4.22 and 4.23, the correlation of FTIR spectra of two *Aspergillus fumigatus* and *Penicillium glabrum* strains were found as 0.908 and 0.985, respectively.



Hit Name	Correlation
Aspergillus fumigatus4 & Aspergillus fumigatus5	0.907723

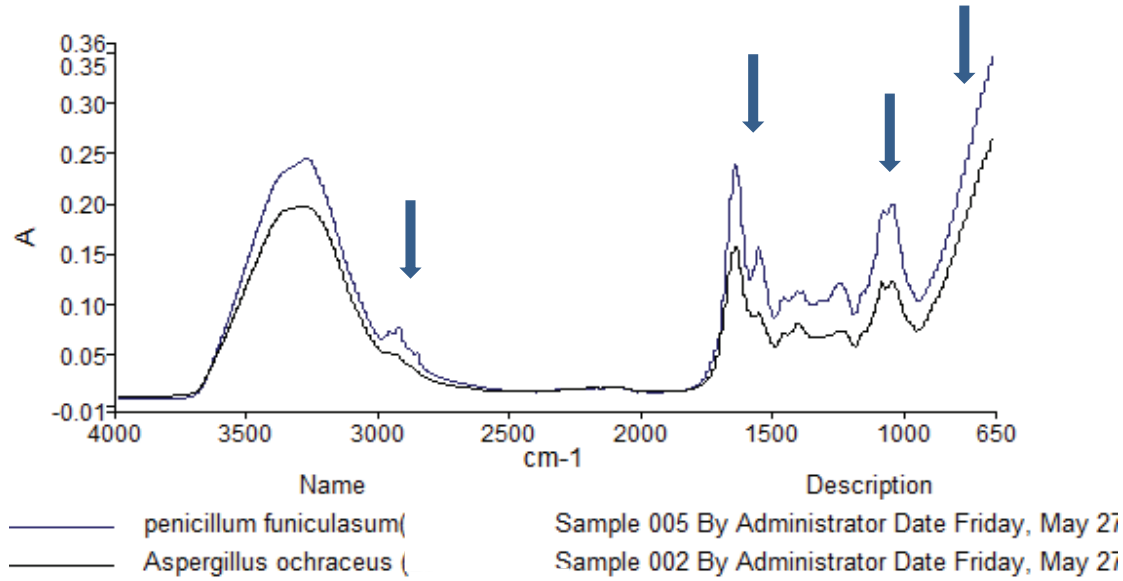
Figure 4.22. FT-IR spectra of *Aspergillus fumigatus* 4 *vs.* 5



Hit Name	Correlation
Pennicillium glabrum2&Penicillum glabrum3	0.98472

Figure 4.23. FT-IR spectra of *Penicillium glabrum* 3 *vs.* 4

It was observed in Figure 4.22 and Figure 4.23, that fungus have differences in fatty acid, amides and polysaccharides bands. But the spectra for both *Aspergillus fumigatus* and *Pennicillium glabrum* strains are largely similar in pattern in their "fingerprint" region.



Hit Name	Correlation
Aspergillus& Penicillum	0.66895

Figure 4.24. FT-IR spectra of *Penicillium funiculosum vs. Aspergillus ochraceus*

Comparison of the FT-IR spectra of *Penicillium funiculosum vs. Aspergillus ochraceus* are given in the Figure 4.24. The correlation of the spectra of these two species were found as 0.669. According to Figure 4.24, these two fungus have different spectroscopic profile in fatty acid, amides, polysaccharides and "fingerprint" regions. FTIR spectroscopy has been successfully applied for the differentiation of *Aspergillus* and *Penicillium* on genus, species and strain levels in the studies reported in the literature (Fischer et al., 2006).

Concerning the filamentous fungi, this method has already been used for the differentiation and the classification of closely related species such as; *Aspergillus fumigatus*, *A. flavus*, and *A. parasiticus* (Garon et al., 2010) and *A. niger*, *A. ochraceus*, and *A. westerdijkiae* (Tralamazza et al., 2013).Another recent study assessed the ability of FTIR spectroscopy for the differentiation and classification of clinically relevant *Trichophyton* species (Ergin et al., 2013). *Fusarium* species were also differentiated and discriminated using this method (Nie et al., 2007).

In most studies related to the identification of filamentous fungi using FTIR spectroscopy, the focus was only made on one genus. Few studies are related to the ability of FTIR spectroscopy for discrimination and identification of several fungal genera and species. FTIR spectroscopy was applied to the identification of airborne fungi belonging to the *Aspergillus*, *Emericella*, and *Penicillium* genera (Fischer et al., 2006). In recent studies, a high throughput protocol for FTIR spectroscopy was developed for the characterization and the identification of 11 strains (5 genera and 7 species) and this protocol was applied to the characterization of 59 food spoilage fungal strains belonging to 10 genera (Shapaval et al., 2010, 2013).

FTIR spectroscopy method was used to differentiate *Aspergillus* and *Penicillium* species on the generic, the species and the strain level. Different strains of one species mostly showed a heterogeneity of below 0.5. Also this method was found to be useful for differentiation of *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporum* at genera and species level. In this study also the FT-IR spectra of the some filamentous fungi (*Verticullum*, *Davidiella*, *Aschersonia*, *Dendrothyrium*, *Sarocladium* and *Dothideomycetes*) was obtained for the first time.

4.4. Principal Component Analysis (PCA) Results

Principal Component Analysis (PCA) was performed in 3000-2800 and 1800-650 cm^{-1} spectral range in this study. These spectrum range provide the ability to determine similarities and differences between particular species of the fungi studied. The second derivative spectra were used for evaluation of the data since it provides better resolution of overlapping peaks.

The spectral region from 1300–900 cm^{-1} is referred as the fingerprint region that confirms the identity of compounds. Within this range, the most important absorptions are the ones stemming from the stretching of the C–O bond of the esters. These absorption ranges of the ester C–O bonds, actually correspond to two asymmetric vibrations that involve the bonds C–C and C–O. In the case of saturated aliphatic esters, the two observed bands appear at 1275–1185 cm^{-1} and at 1160–1050 cm^{-1}. The first band involves the bond stretching between the oxygen and the carbonyl carbon, coupled with C–C stretching. The second one involves the bond stretching between the oxygen and a carbon atom. The band that occurs in the biggest number of waves is usually more intense between two of them (Salman et al., 2010). Fatty acids show characteristic absorbance at the 2950–2845 cm^{-1} wavenumber region.

The spectral ranges selected for PCA analysis correspond more or less with the regions known to be characteristic for certain chemical structures. These are C-H region dominated by fatty acids (3050-2800 cm^{-1}), amide I and amide II region (1600–1700 cm^{-1}) (carbonyl region, C=O ester absorption) and 1500–1600 cm^{-1} (N–H absorption); the spectral region between 1500 and 1200 cm^{-1}; polysaccharides (1200–900 cm^{-1}); and region from 900 to 700 cm^{-1} (many weak unassigned bands which are quite characteristic for the identification of microorganisms called “fingerprint region”) (Monika et. al., 2013).

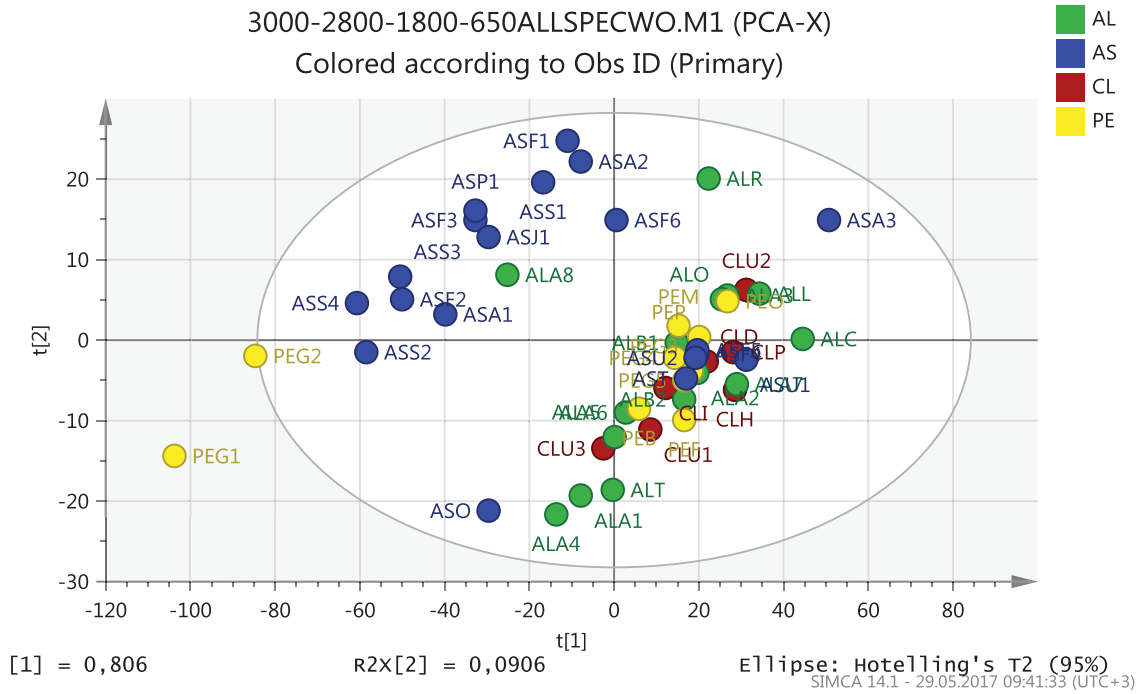


Figure 4.25. PCA analysis score plot of *Aspergillus, Alternaria, Clodosporium* and *Penicillium* species spectral data on 3000-2800, 1800-650 cm^{-1} range.

Figure 4.25. shows the differences between *Aspergillus* and other species as a result of evaluation of their IR spectral data with PCA. It is observed that *Aspergillus* species and *Penicillium* species located in the separate quartiles of the score plot; consequently, this separation might be related to their chemical composition. It is observed in the Figure 4.25. that, 66.6% *Aspergillus* strains are located in the same region.

Aspergillus and *Penicillium* genus are both in *Eurotiomycetes* class. On the other hand, *Alternaria* and *Cladiosporum* genus are both recognized as phylum *Ascomycota*, class *Dothideomycetes* (Frisvad and Jens Christian, 2015). These species, although belonging to different classes, showed biochemical similarities in the IR absorbance bands. According to the literature, *Sordariomycetes and Dothideomycetes* are the most morphologically similar species pairs within *Ascomycota* group (Wang et. al., 2006).

A512GE3 (PEG1) and 25HSDA13 (PEG2) coded *Penicillium glabrum* strains are located at different regions in PCA score plot. However, both fungi has 99% Ident in Blast records, A512GE3 coded *Penicillium glabrum* (PEG1) strain has a different profile in "fingerprint" region which corresponds to 900-700 cm^{-1}.

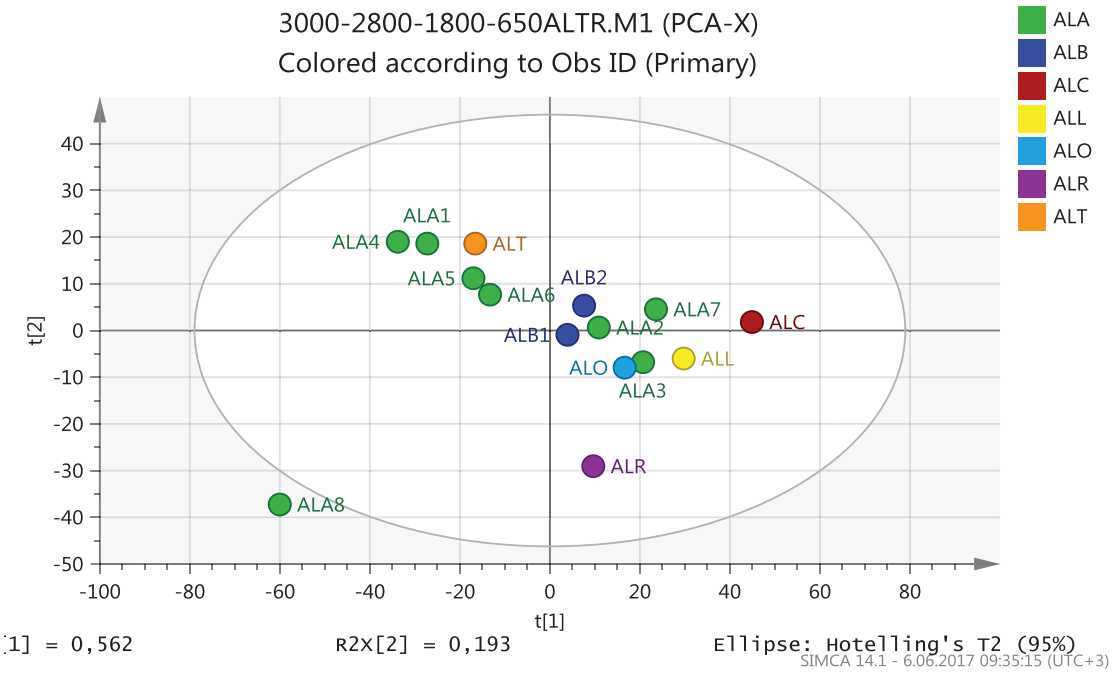


Figure 4.26. PCA analysis score plot of *Alternaria* species spectral data on 3000-2800, 1800-650 cm^{-1} range.

Figure 4.26 shows that 50% of *Alternaria alternata species* are closely located to each other. However, some species are far away from each other. Especially A2GE9H1 coded *Alternaria alternata* strain is located in a very far area in PCA score plot. It is observed that this strain has low (63%) "Query Cover" in BLAST analysis.

B2KE9H1 coded *Alternaria alternata* is located at a different region in PCA score plot. In the Figure 4.18, it has been observed that B2KE9H1 coded *Alternaria alternata* (ALA8) strain has a different spectral profile in fatty acids and amides band than the other *Alternaria* strains. *Alternaria conjucta* (ALC) and *Alternaria brevimosa* (ALR) strains are also located in a different region of the plot. These strains have collected from previous studies.

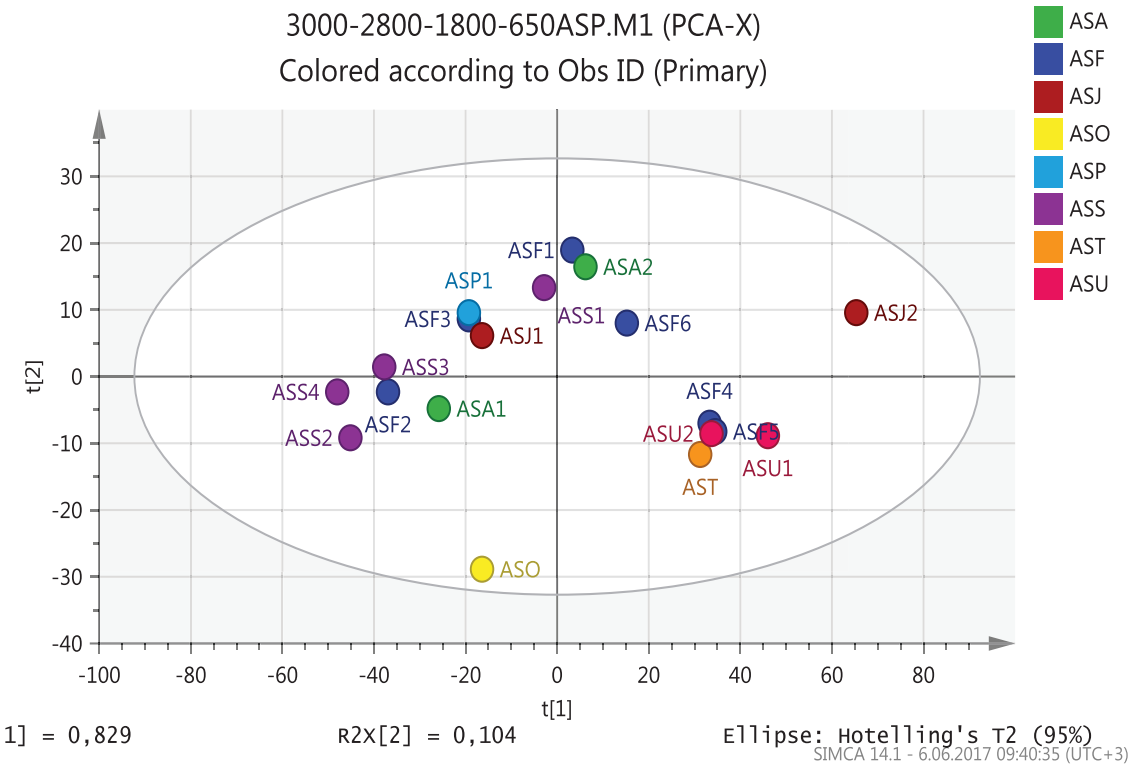


Figure 4.27. PCA analysis score plot of *Aspergillus* species spectral data on 3000-2800, 1800-650 cm^{-1} range.

Figure 4.27 shows that 75% *Aspergillus sydowii* strain is placed in the same group. On the other hand, all of *Aspergillus fumigatus* species are far away from each other. *Aspergillus fumigatus* biomass contain hydroxyl, carboxyl and amine groups on their surface (Ratnasri and Hemalatha, 2015). Observations close to each other in the space of principal components necessarily have similar characteristics.

H1PDA8 and 6H3DA31 coded *Aspergillus japonicus* (ASJ1 and ASJ2) strains are located in a different region. These strains have 99% and 100% Identification in BLAST analysis.

8HCZA13 coded *Aspergillus ochraceus* which is responsible for toxic and carcinogenic fungal secondary metabolite (Ochratoxin A), is collected from previous studies. It is observed that this fungi has located in a very restricted area in PCA score plot.

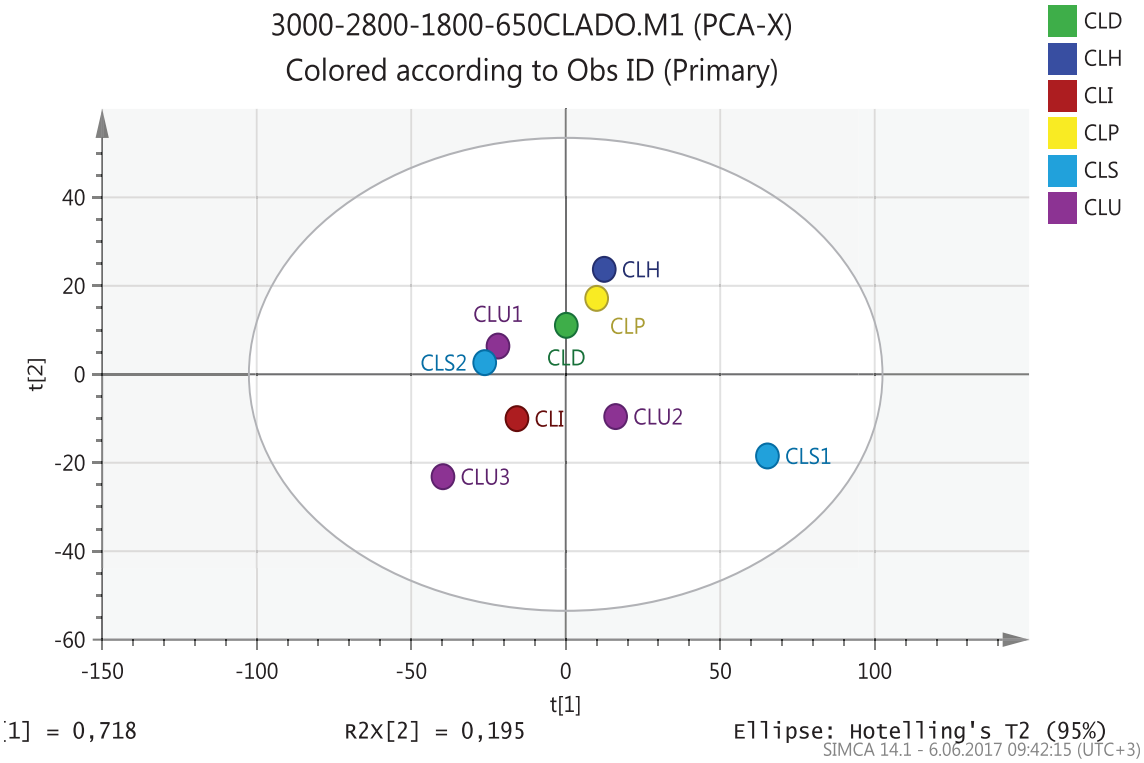


Figure 4.28. PCA analysis score plot of *Cladosporium* species spectral data on 3000-2800-1800, 650cm^{-1} range.

Various *Clostridium* species are compared in Figure 4.28. It is observed that B534KH3 coded *Cladosporium sp.* (CLU2), A534GE6 coded *Cladosporium sp.* (CLU3), B556GE2 coded *Cladosporium sp.* (CLS1) and A556GE2 coded *Cladosporium sp.* (CLS2) are located in different area. These strains have 100% "Identification" in BLAST analysis. But it is observed in Figure 4.16 that the spectra which belong to B534KH3, A534GE6 and B556GE2 coded *Cladosporium sp.* genus have different spectral profile in the "fingerprint" region.

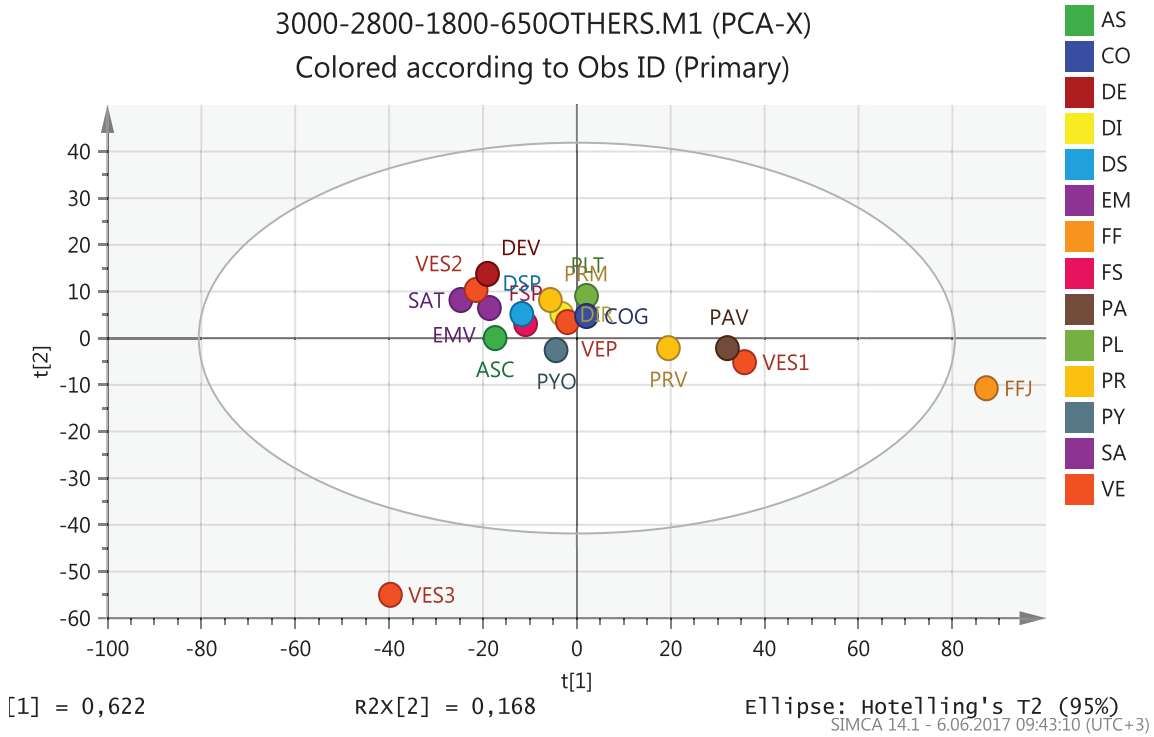


Figure 4.29. PCA analysis score plot of all investigated species spectral data on 3000-2800, 1800-650 cm^{-1} range.

Various fungal strains are compared in Figure 4.29. These strains are recognized as phylum *Ascomycota*, and they belong to different classes. A2KE9H2 coded *Fusarium fujikuroi* (FFJ) *A534GE4* coded *Verticillium psalliotae* (VES3) are located in different parts of the plot. These strains have 99% Identification in BLAST analysis. These strains are both in *Sordariomycetes* class. These strains, although belonging to different classes, showed biochemical similarities in the IR absorbance bands. According to the literature, *Sordariomycetes and Dothideomycetes* are the most morphologically similar species pairs within *Ascomycota* phyla (Wang et. al., 2006).

CHAPTER 5

CONCLUSIONS

In this study, an optimized rapid technique for the detection and identification of fungi isolated from a certain variety of olive (Erkence) is presented. It consists of sensitive and precise method of identification of fungi based on amplification of 28S DNA and molecular typing. Analysis of sequences (28S region) from the database confirmed that this study is effective to differentiate fungi at the species level. NL specific primers have been successfully used for fungal detection and identification in fungi isolates. In this study, filamentous fungi of the most important phyla from the perspective of food spoilage, *Ascomycota*, were analysed.

According to the cultural and molecular results, 4 main fungal genus were identified in the samples: *Aspergillus*, *Penicillum*, *Alternaria*, and *Cladosporium*. In the species level 8 different *Aspergilllus* species; *Aspergillus fumigatus* (6)*, Aspergillus sydowii* (4)*, Aspergillus aculeatus* (3)*, Aspergillus ustus (*2)*, Aspergillus japonicus, Aspergillus protuberus, Aspergillus ochraceus, Aspergillus terreus;* 7 different *Alternaria* species; *Alternaria alternata* (8), *Alternaria tenuissima, Alternaria tomato, Alternaria breviramosa, Alternaria conjuncta, Alternaria leucanthemi,A lternaria burnsii,* 6 different *Penicillium* species; *Penicillium glabrum* (3), *Penicillium brevicompactum, Penicillium malachiteum, Penicillium paxiallii, Penicillium funiculasum, Penicillium oxalicum;* 4 different *Cladosporium* species; *Cladosporium uredinocola* (4), *Cladosporum cladospoides, Cladosporium herbarum, Cladosporium delicatum* were identified. Moreover, *Pyronema omphalodes*, *Colletotrichum gloesprodies, Emercella variecolor, Fusarium fjikori, Fusarium equiseti, Paecilomyces varioti, Diaporthe rudis, Aschersonia aleyrodis, Verticillum psalliote, Preussia minipascua, Dendrothyrium variisporum, Dothideomycetes longisporum, Paraconiothyrium variabile* and *Sarocladium terricola* species were also identified.

FTIR spectroscopy method was reproducibly differentiated some fungi species on the generic, the species, the strain level. This study have shown that FTIR method could be complementary to the methods currently used for fungal identification as it was accurate and allowed a high-throughput analysis using micro-culture.

Principal component analysis of FTIR data was also performed in 3000-2800, 1800-650cm^{-1} range in this study. Among the species examined, main clusters can be distinguished, allowing separation of the fungal species.

In the future, additional reference strains from culture collections will be also included to create a wider infrared database. FTIR spectroscopy could be a supplementary and complementary tool that allows a rapid identification of fungal isolates.

It is predicted that the results obtained in this study might be useful to set up a future reference database to employ FTIR spectroscopy and differentiating between various filamentous fungi present.

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APPENDIX A

RECIPES FOR CULTURE MEDIA

A.1.Potato Carrot Agar + Manganese (PCA-Mn)

10 g carrot and 10 g potato/L medium is half strength of a standard PCA (Simmons, 1992). The basal medium was prepared with 20 g agar (SoBiGel, Bie&Berntsen, Roedovre,Den mark) and autoclaved in a 900 ml volume to which 100 ml autoclaved manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added after cooling to 55 °C. Then media was poured immediately into sterile 90 mmPetri dishes.

•Preparation of Manganese Solution: 15g.$MnCl_2 \cdot 4H_2O$was added to distilled water and brought volume to 100 ml. Blend was mixed thoroughly, filtered and sterilized.Optimum concentration for $MnCl_2 \cdot 4H_2O$solutions (1.5 % w/v)15 g. $MnCl_2 \cdot 4H_2O$– 100 ml deionized water (filtered and sterilized pH= 6.5 at 25°C)

•Preparation of Medium: Potatoes and carrots were washed and peeled. Grate potatoes and carrots were placed in 1 L of tap water. Then they were gently heated and brought to boil. After boiling for 30 minutes, potatoes and carrotswere filtered through cheesecloth. The volume of filtrate was brought to 900 ml. Then 20 g. of agar was added. After gently heating and bring to boiling, they were distributed into flasks and autoclave for 20 minutes.

A.2. Sabouraud Agar + Olive Oil

20 g. Glucose
10 g. Bacteriological peptone
20 ml. Virgin olive oil
0,5 g. Chloramphenicol
0,5 g. Cycloheximide
1 lt. demineralized water

pH was adjusted to 6.0. 12-15 g. agar was added and autoclaved for 15 minutes at 120^{o}C.

A.3. Malt Extract Agar

50 g malt extract agar in 1 liter of distilled water was suspended and brought to the boil to dissolve. Then mixture was sterilized by autoclaving at 115°C for 10 minutes without overheat.

A.4. CYA - Czapek Yeast Extract Agar

1.0 g K_2HPO_4
10.0 ml Czapek Concentrate (see below)
5.0 g Yeast extract
30.0 g Sucrose
15.0 g Agar
1.0 L Distilled water
The mixture was autoclaved at 121°C for 15 minutes.

A.5. Czapek Concentrate:

30.0 g $NaNO_3$
5.0 g KCl
5.0 g $MgSO_4.7H_2O$
0.1 g $FeSO_4.7H_2O$
0.1 g $ZnSO_4.7H_2O$
0.05 g $CuSO_4.5H_2O$
1.0 L Distilled water
The ingredients were dissolved and sterilized at 121°C for 15 minutes.
Final pH must be 6-6.5.

APPENDIX B

FT-IR SPECTRUM OF DIFFERENT FUNGAL SPECIES

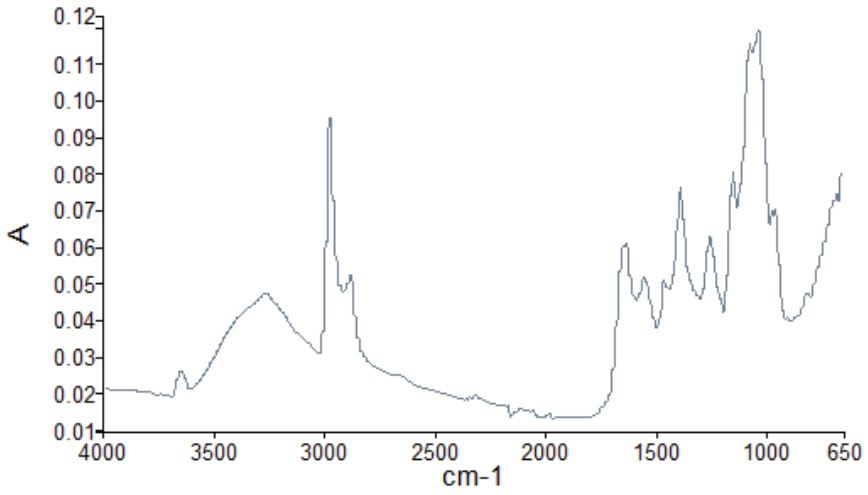

Figure 1. FTIR spectra of*Aspergillus fumigatus1*

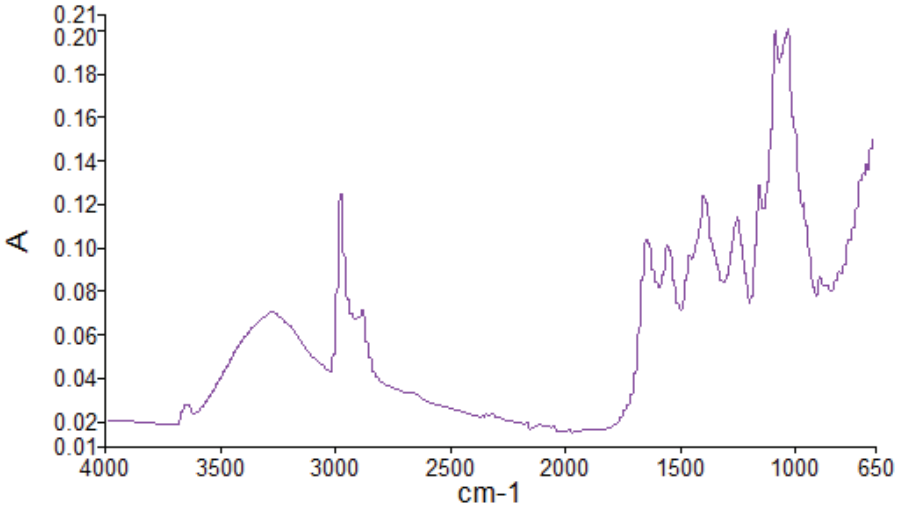

Figure 2. FTIR spectra of *Aspergillus fumigatus2*

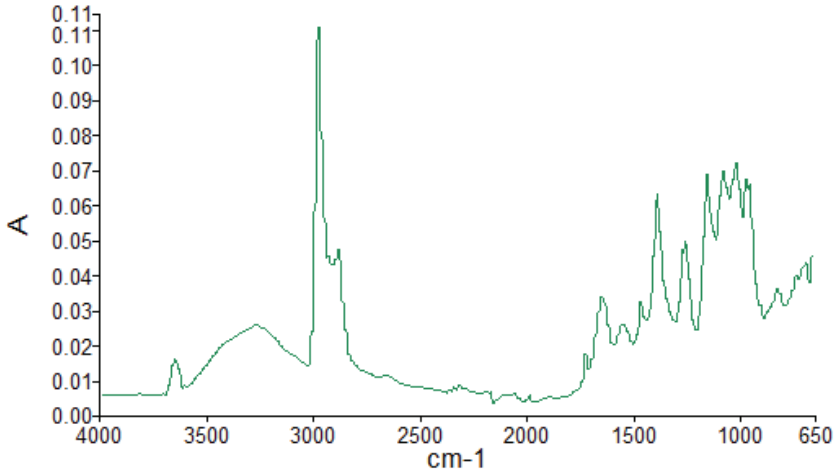

Figure 3. FTIR spectra *of Aspergillus sydowii1*

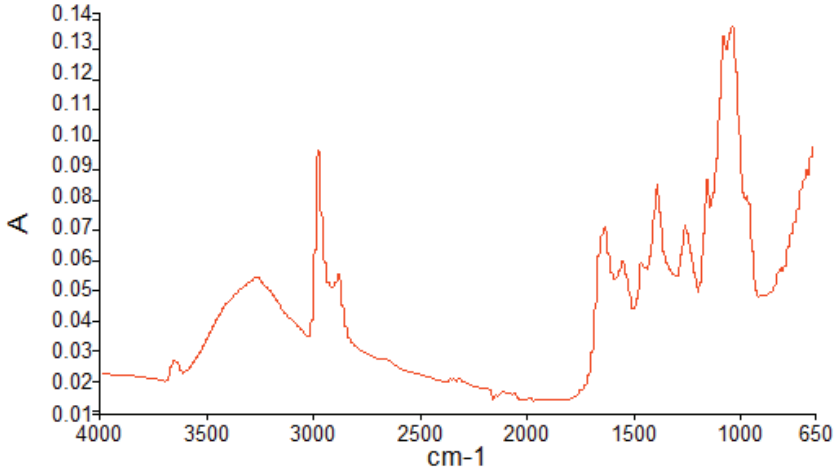

Figure 4. FTIR spectra of *Aspergillus fumigatus3*

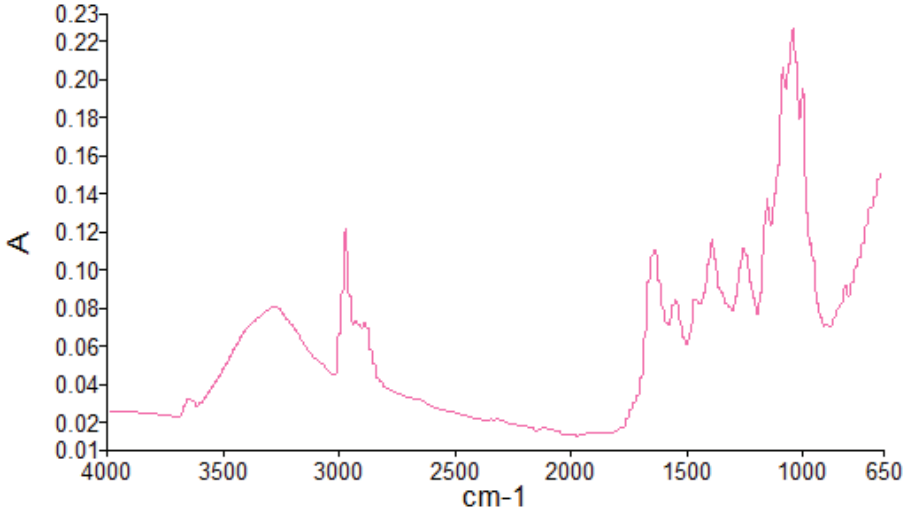

Figure 5. FT-IR spectra of *Aspergillus japonicus*

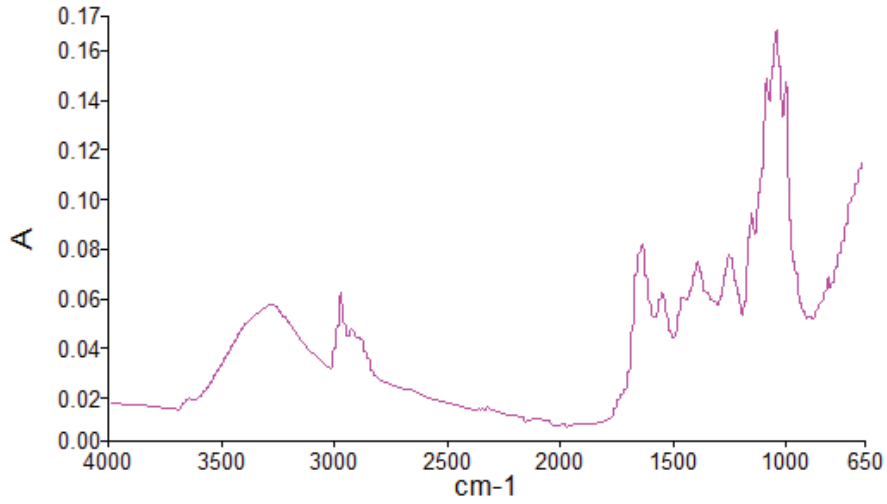

Figure 6. FT-IR spectra of *Aspergillus aculeatus1*

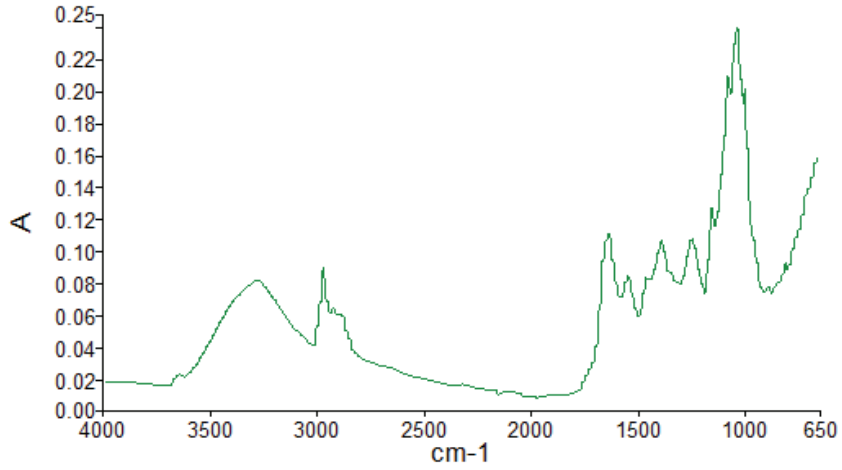

Figure 7. FT-IR spectra of *Aspergillus sydowii2*

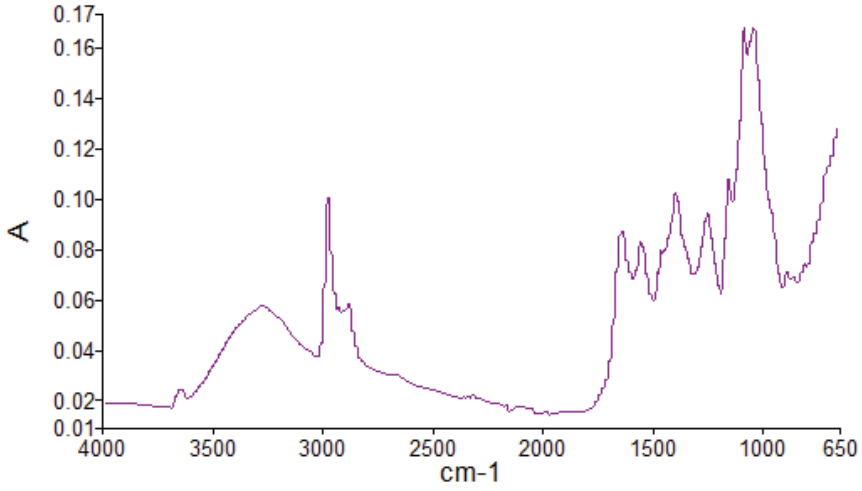

Figure 8. FT-IR spectra of *Aspergillus aculeatus2*

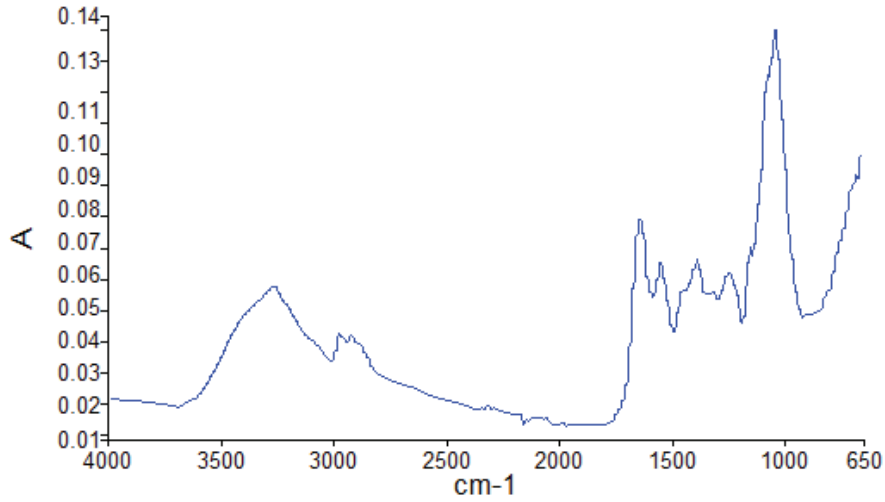

Figure 9. FT-IR spectra of *Penicillim glabrum1*

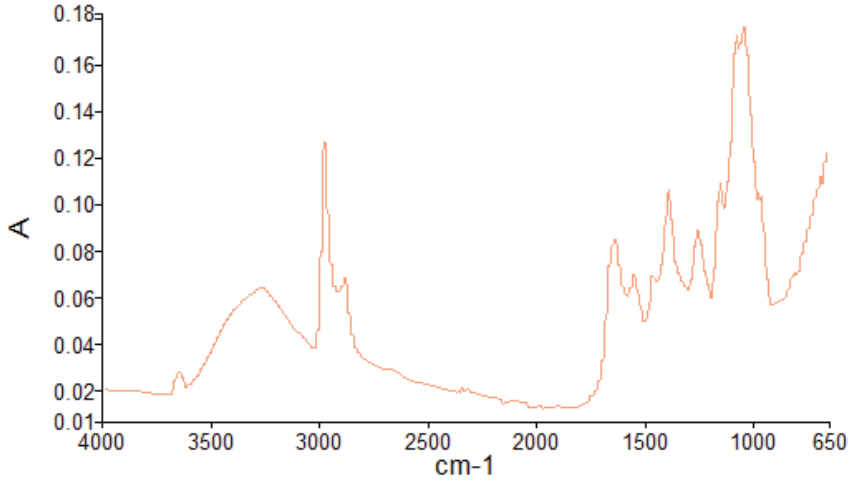

Figure 10. FT-IR spectra of*Aspergillus protuberus1*

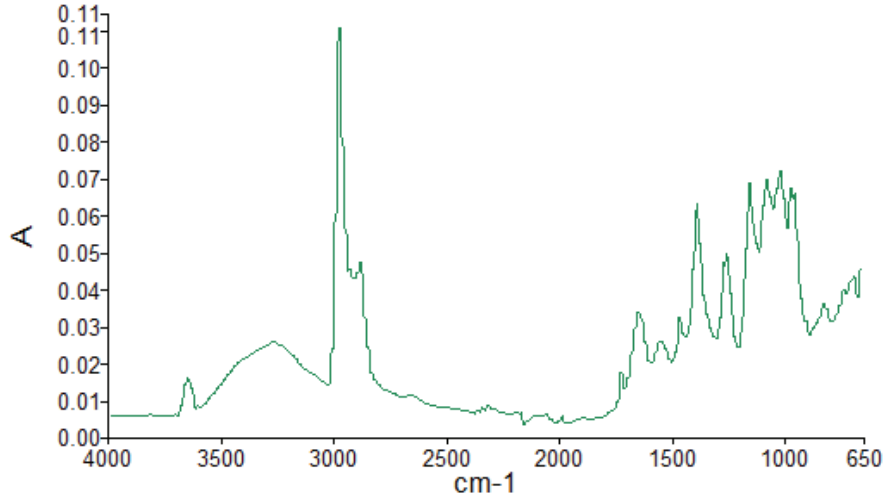

Figure 11. FT-IR spectra of*Penicillium glabrum2*

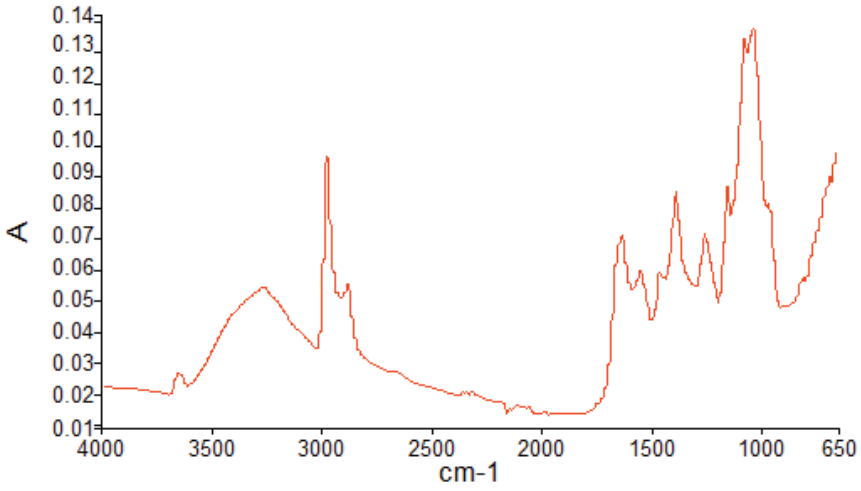

Figure 12. FT-IR spectra of*Aspergillus sydowii3*

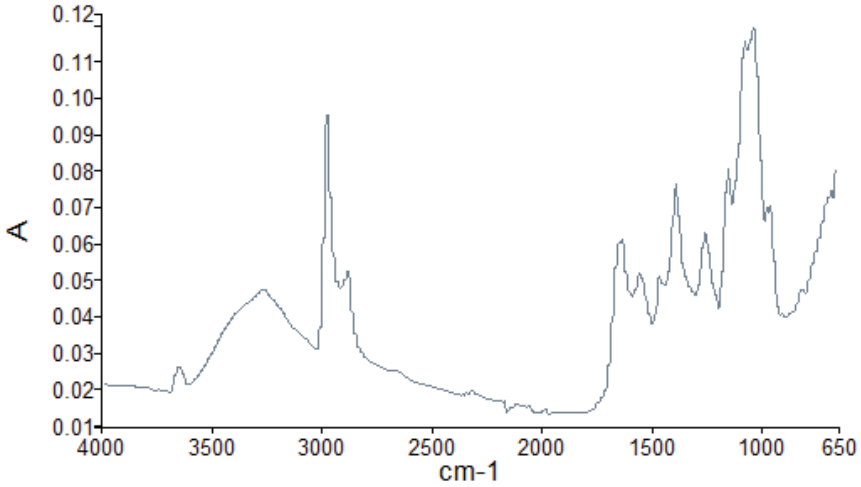

Figure 13. FT-IR spectra of*Aspergillus sydowii4*

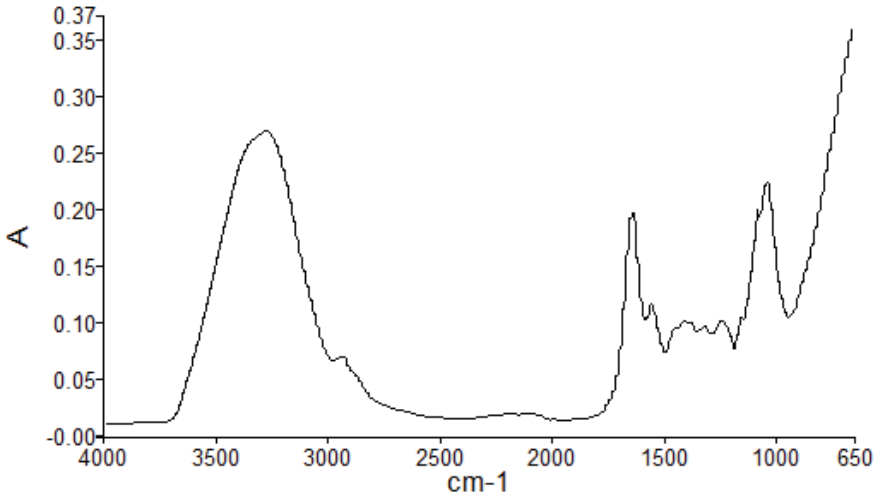

Figure 14. FT-IR spectra of.*Penicillium brevicompactum*

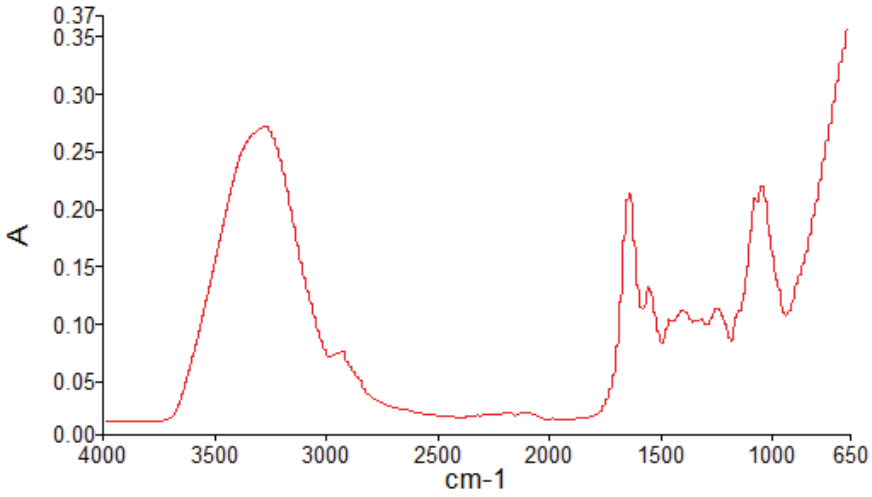

Figure 15. FTIR spectra of*Penicillium glabrum3*

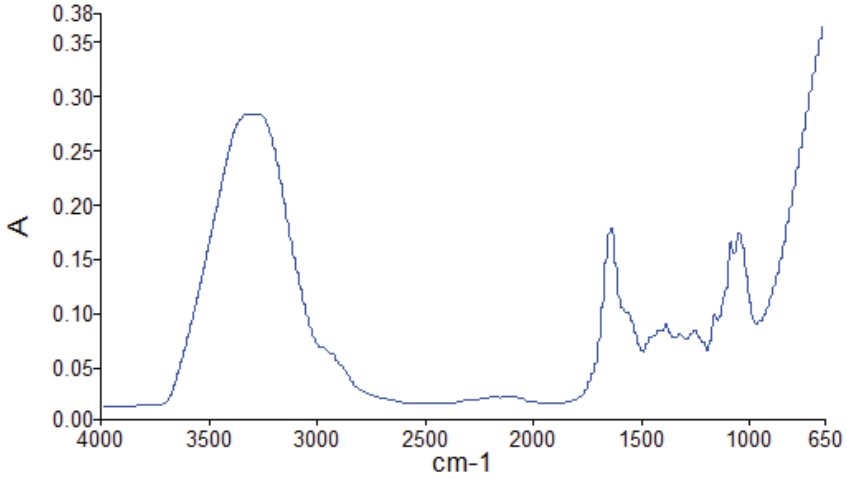

Figure 16. FT-IR spectra of*Alternaria alternata1*

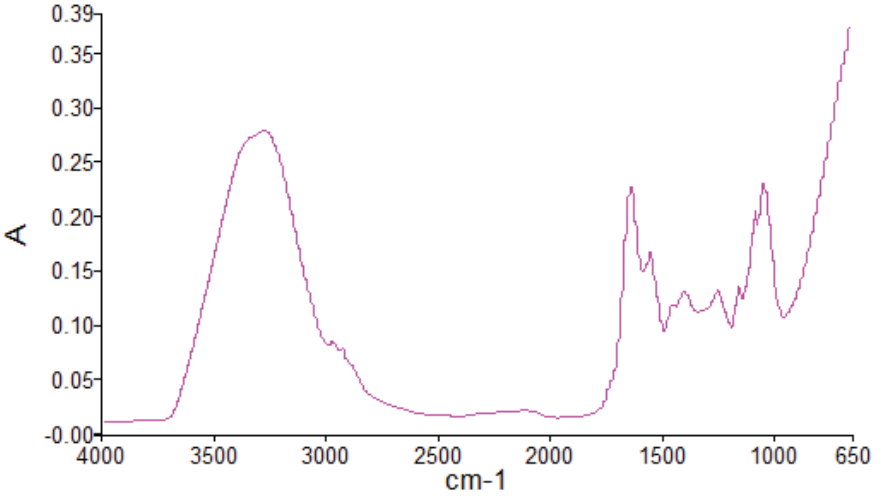

Figure 17. FT-IR spectra of*Cladosporum sp.*

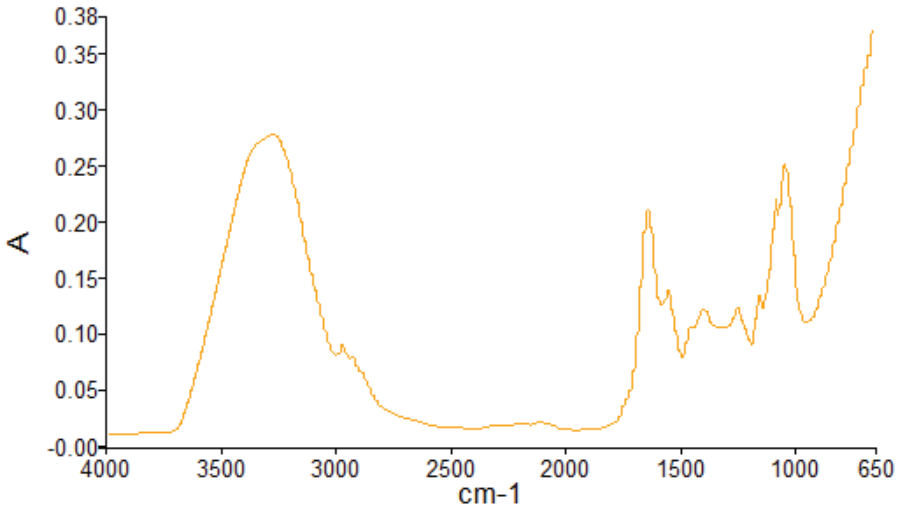

Figure 18. FT-IR spectra of*Cladosporium cladosporioides*

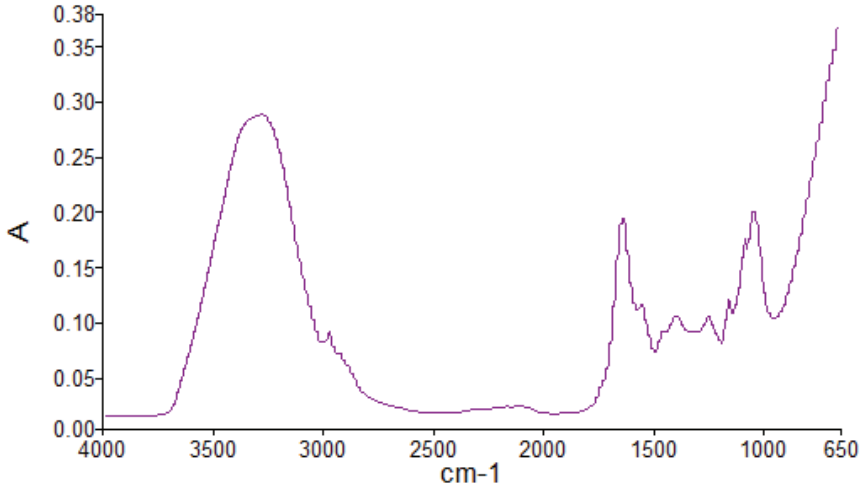

Figure 19. FT-IR spectra of *Cladosporium sp*

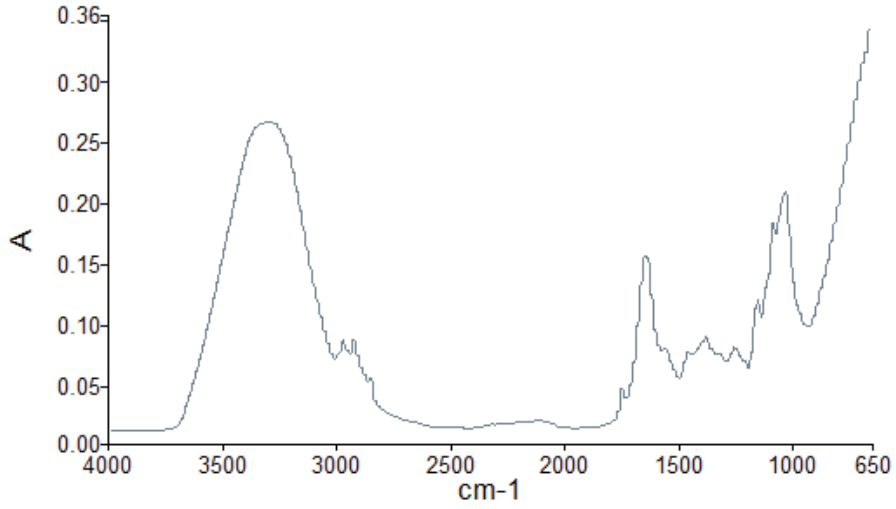

Figure 20. FT-IR spectra of *Diaporthe rudis*

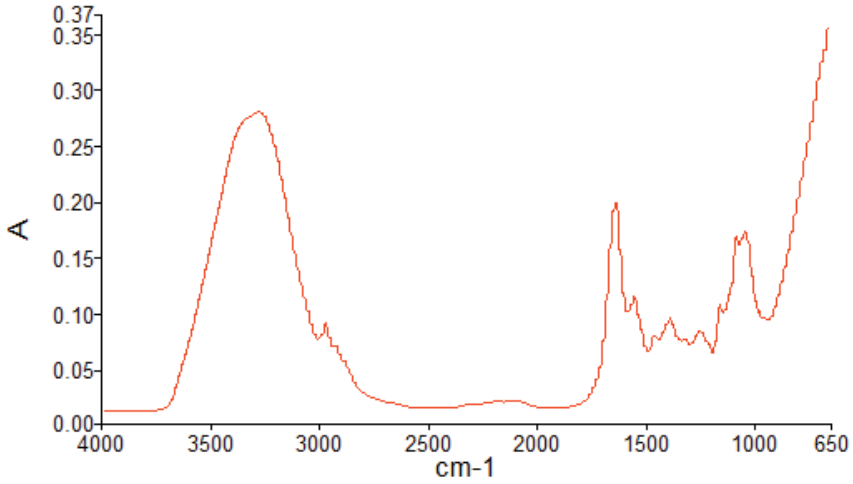

Figure 21. FT-IR spectra of *Verticillium psalliotae*

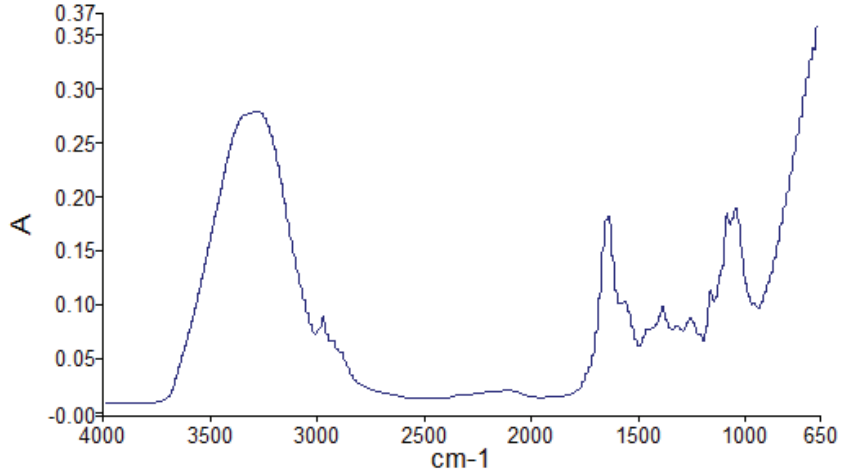

Figure 22. FT-IR spectra of *Preussia sp*

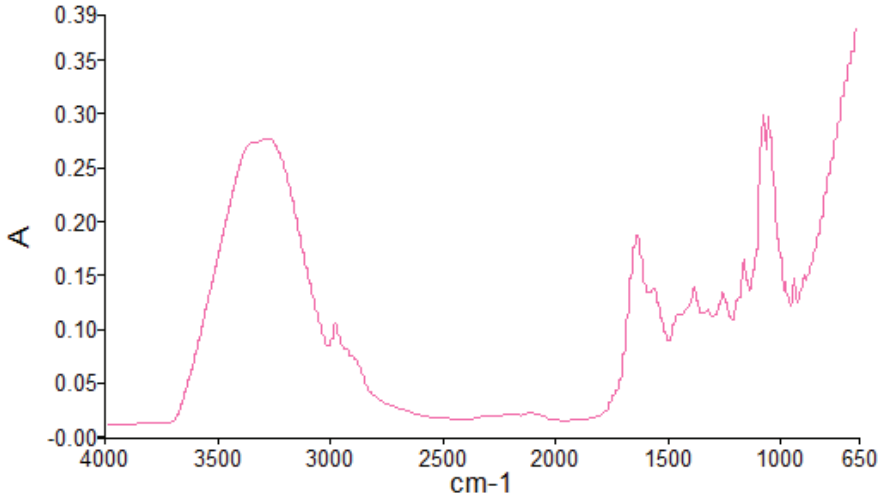

Figure 23. FT-IR spectra of *Alternaria leucanthemi*

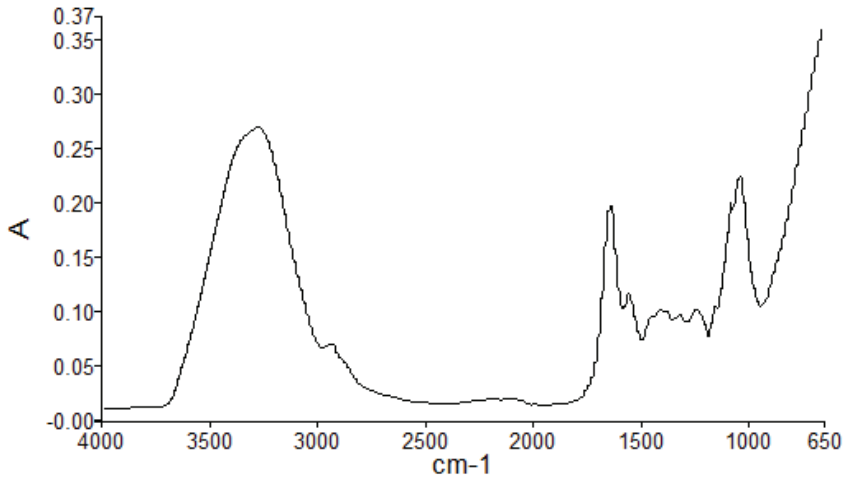

Figure 24. FT-IR spectra of *Alternaria alternata 2*

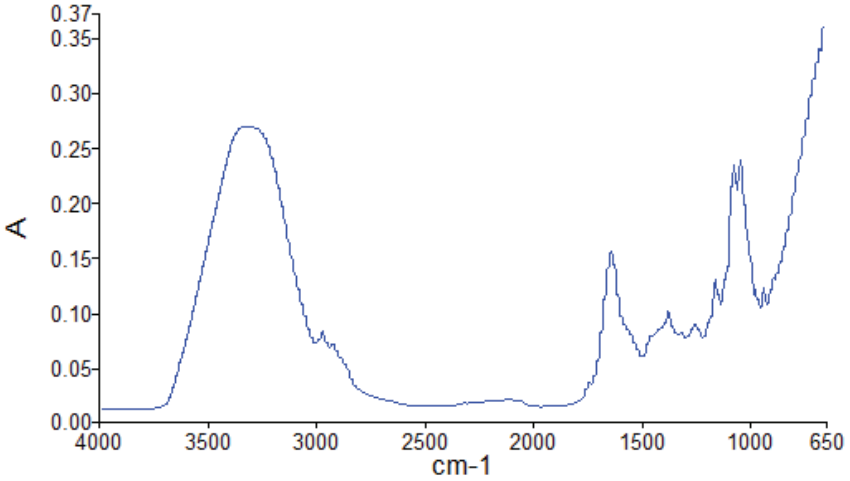

Figure 25. FT-IR spectra of *Pleospara tarda*

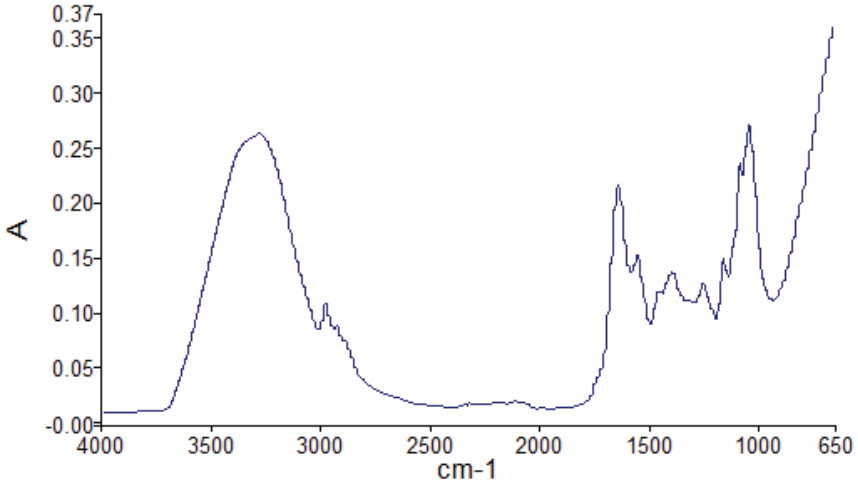

Figure 26. FT-IR spectra of *Cladosporium sp*

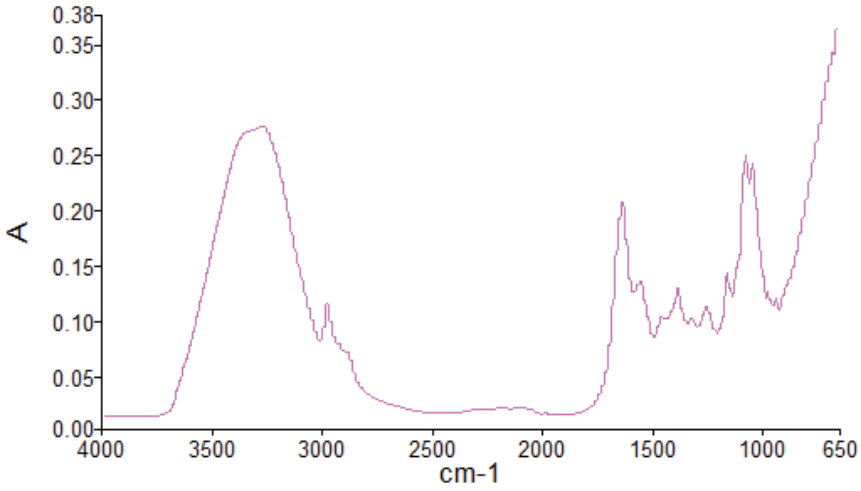

Figure 27. FT-IR spectra of *Alternaria alternata*

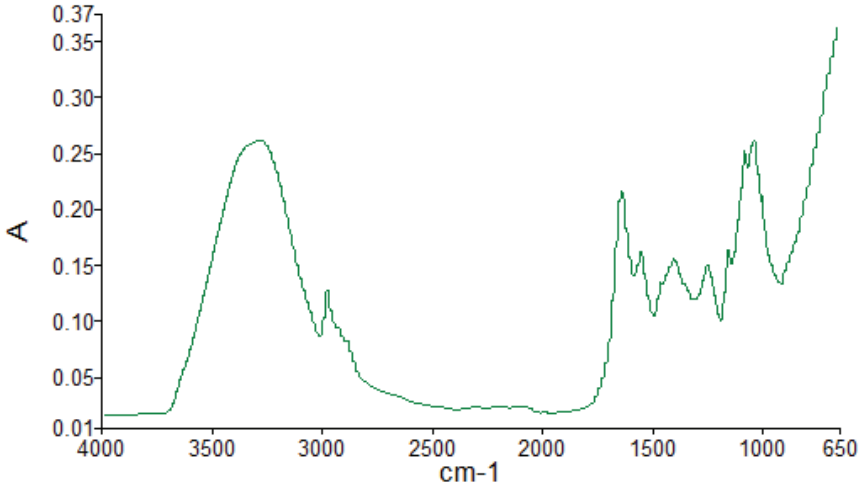

Figure 28. FT-IR spectra of *Aspergillusjaponicus2*

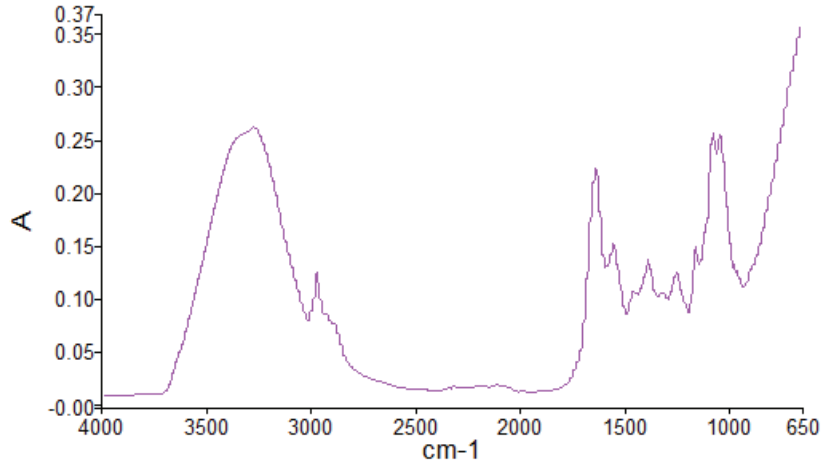

Figure 29. FT-IR spectra of*Alternaria alternata 3*

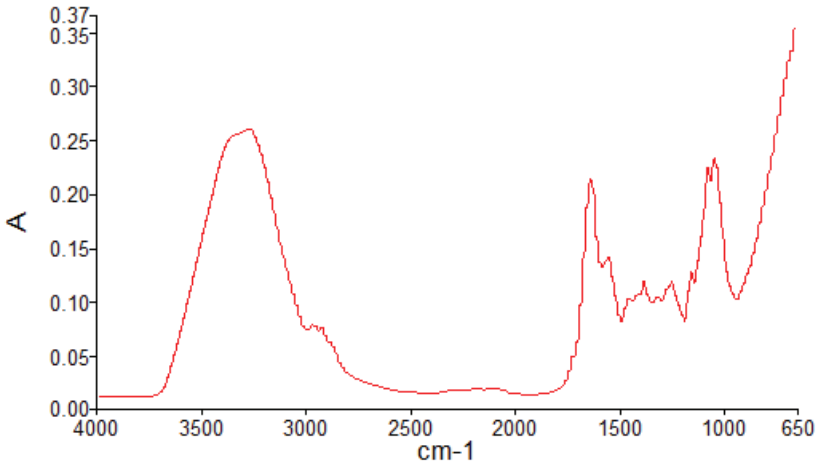

Figure 30. FT-IR spectra of *Alternaria alternata 4*

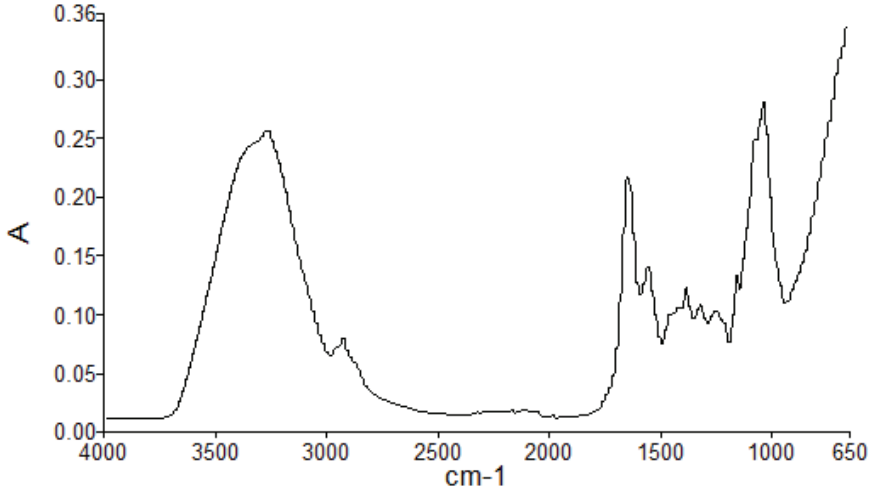

Figure 31. FT-IR spectra of *Aschersonia insperata*

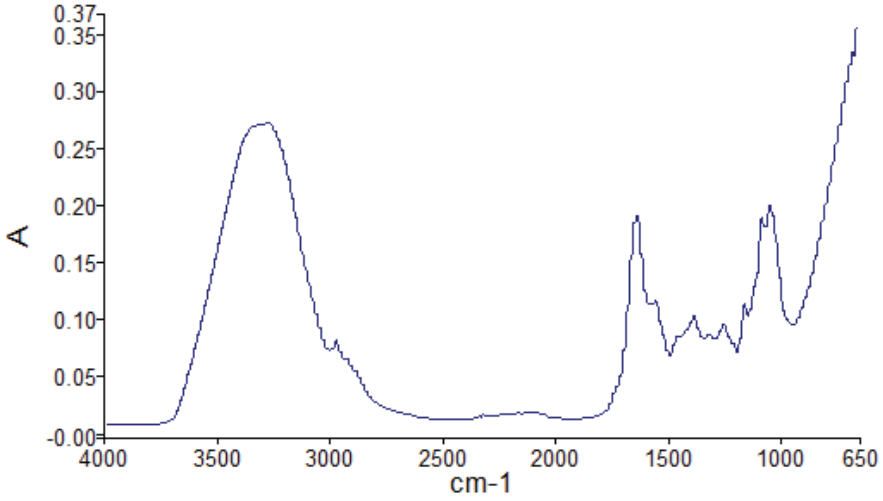

Figure 32. FT-IR spectra of *Cladosporium uredinocola3*

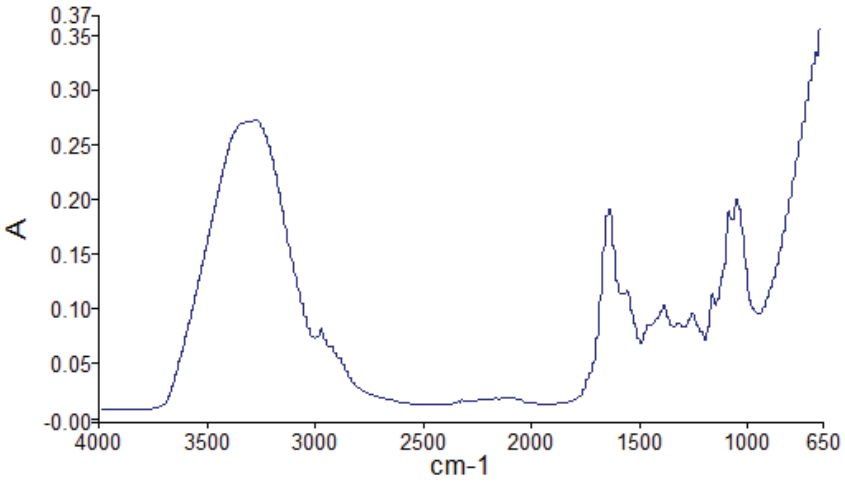

Figure 33. FT-IR spectra of *Alternaria alternata 5*

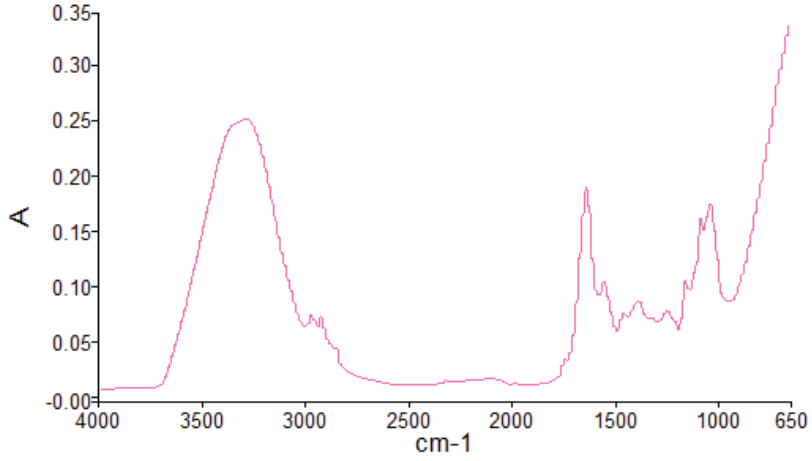

Figure 34. FT-IR spectra of *Verticillium sp*.

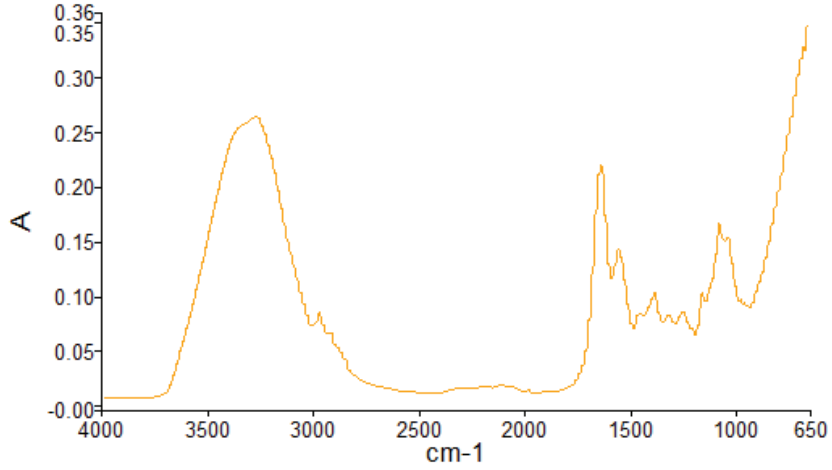

Figure 35. FT-IR spectra of *Pyronema omphalodes*

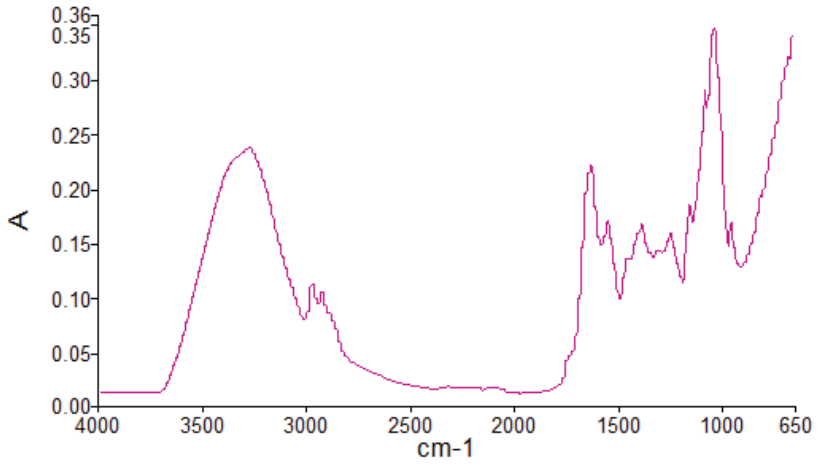

Figure 36. FT-IR spectra of *Cladosporium sp*.

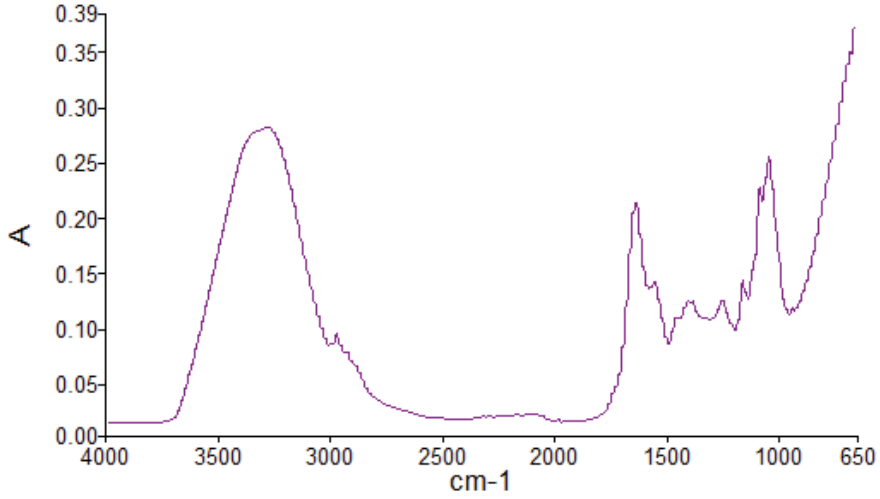

Figure 37. FT-IR spectra of *Cladosporium cladosporioides*

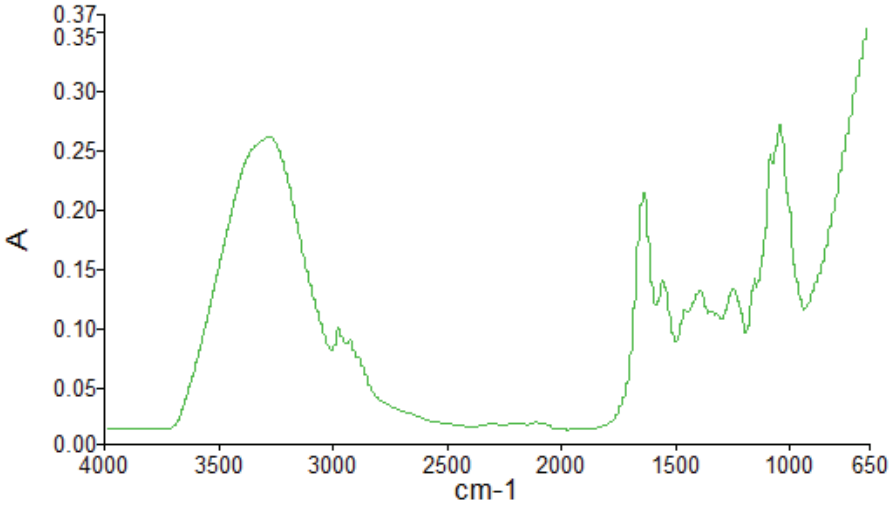

Figure 38. FT-IR spectra of *Aspergillus fumigatus4*

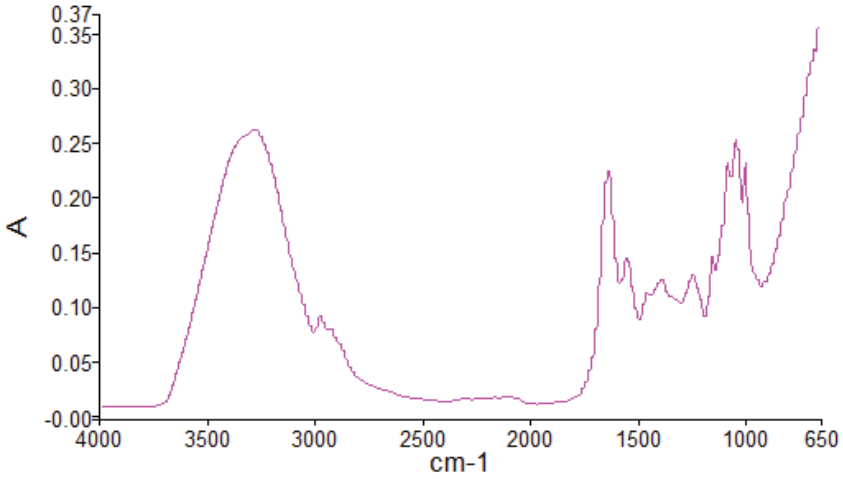

Figure 39. FT-IR spectra of V*verticullum sp1*

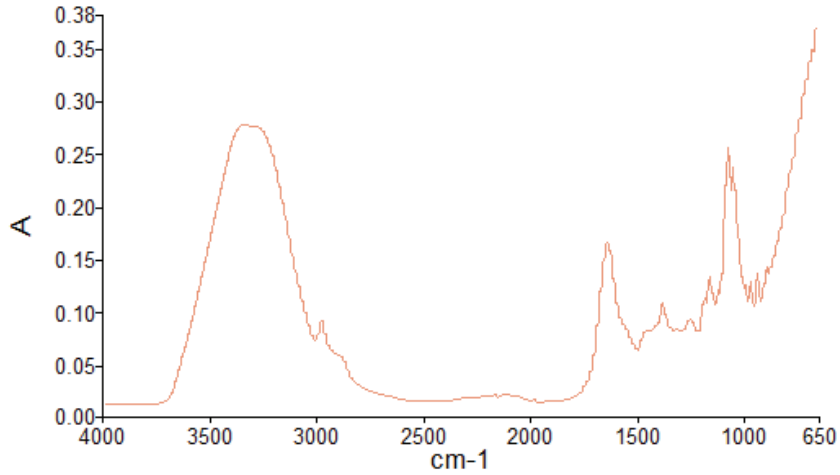

Figure 40. FT-IR spectra of *Alternaria alternata6*

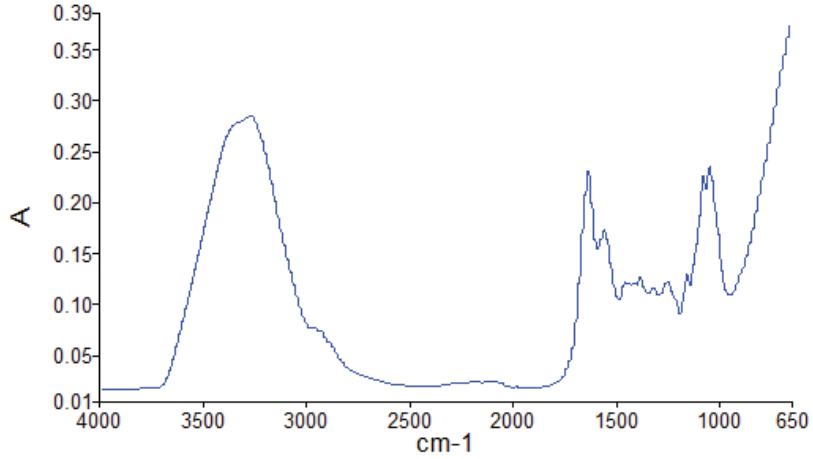

Figure 41. FT-IR spectra of *Alternaria alternata7*

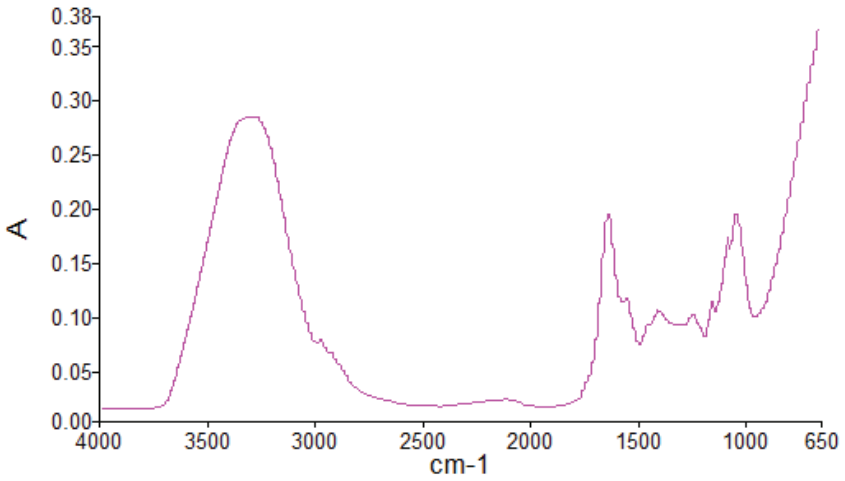

Figure 42. FT-IR spectra of *Cladosporium sp*

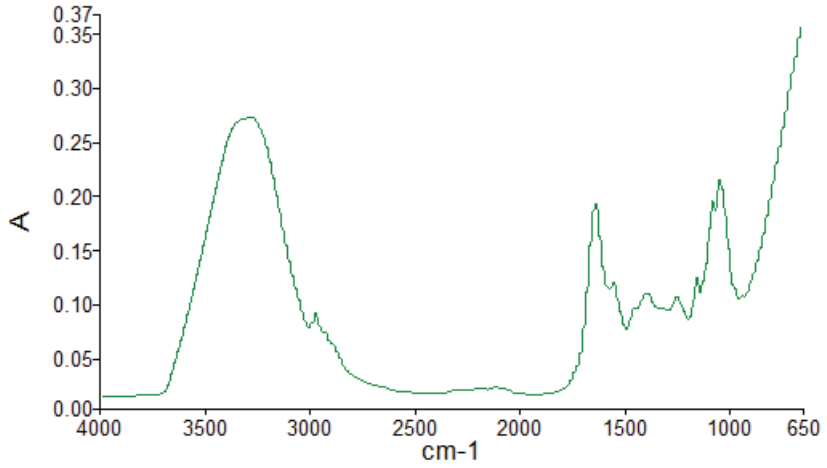

Figure 43. FT-IR spectra of *Cladosporium sp*

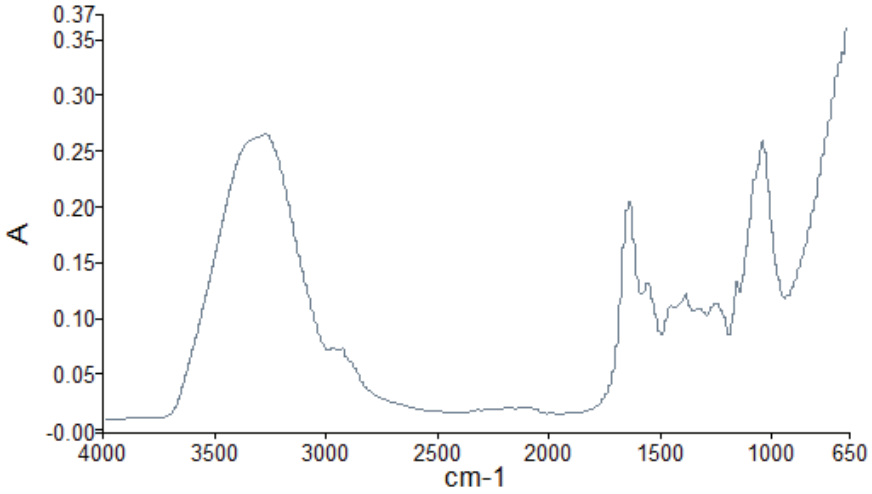

Figure 44. FT-IR spectra of *Aspergillus fumigatus 5*

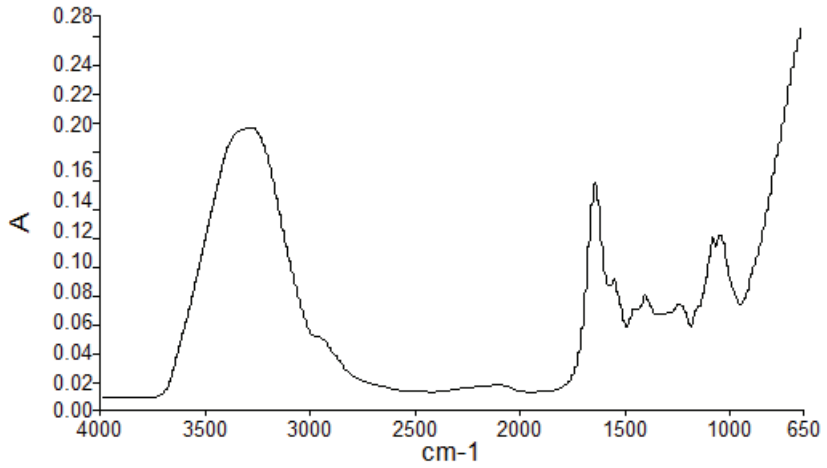

Figure 45. FT-IR spectra of *Aspergillus ochraceus*

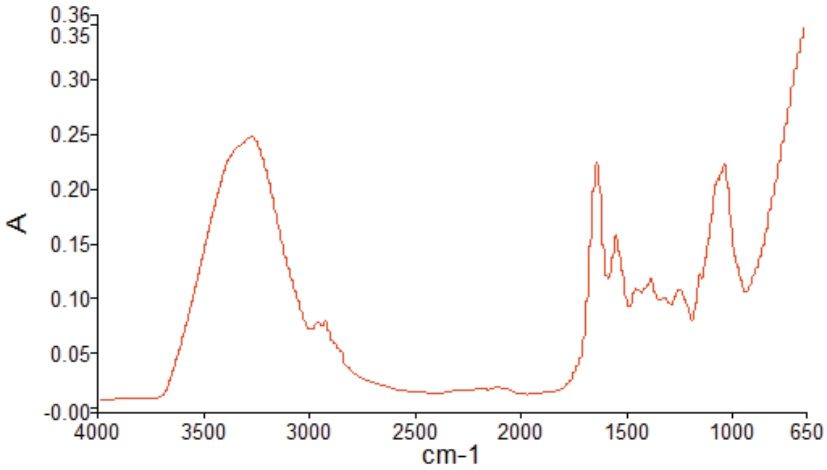

Figure 46. FT-IR spectra of *Aspergillus terrus*

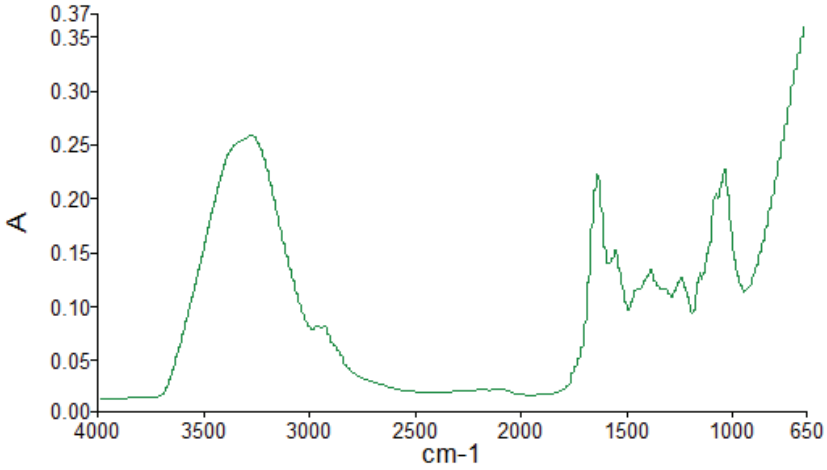

Figure 47. FT-IR spectra of *Aspergillus ustus1*

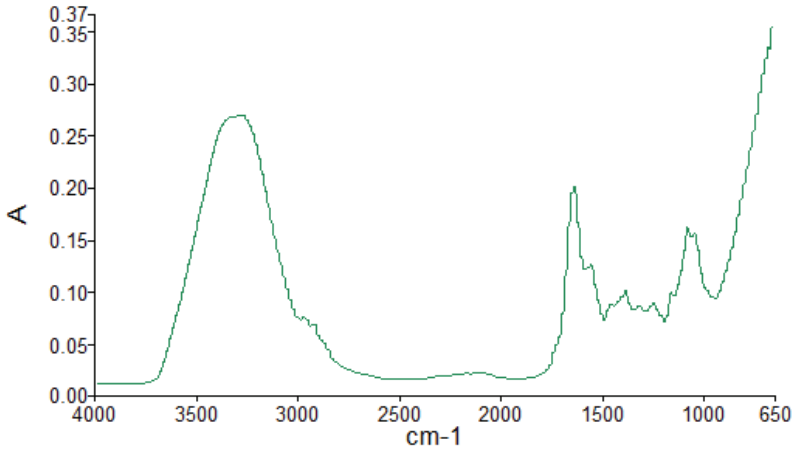

Figure 48. FT-IR spectra of *Alternaria tenuissima*

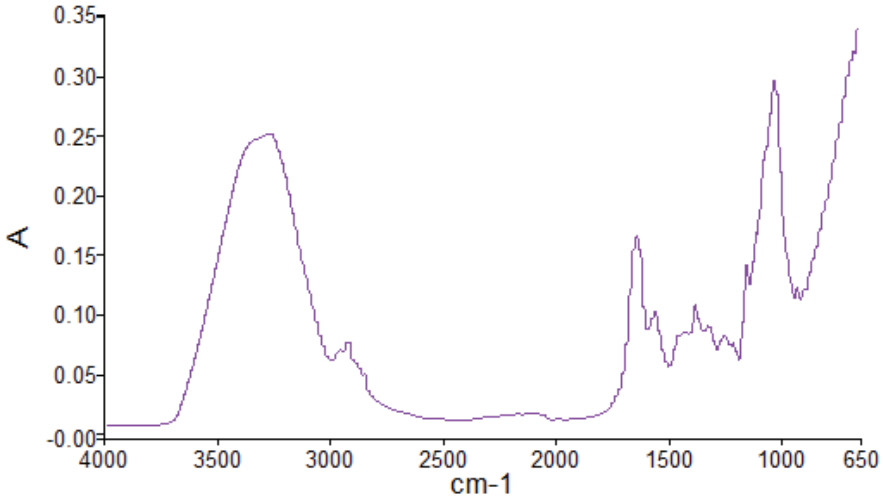

Figure 49. FT-IR spectra of *Colletotrichum gloesprodies*

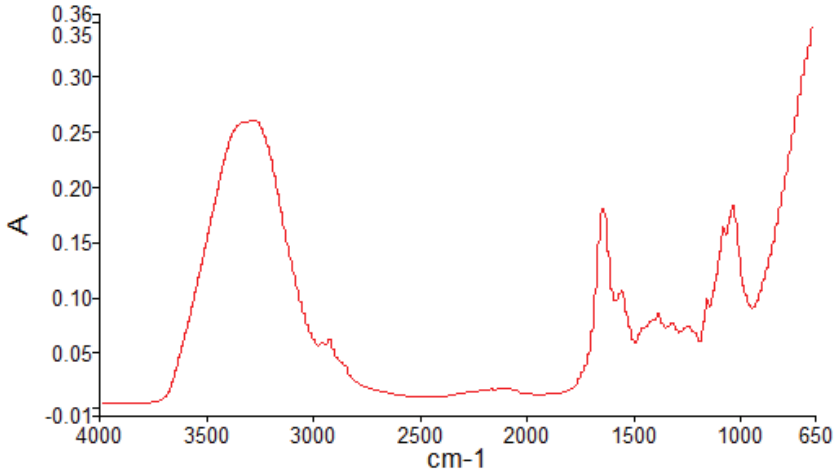

Figure 50. FT-IR spectra of *Emercella variecolor*

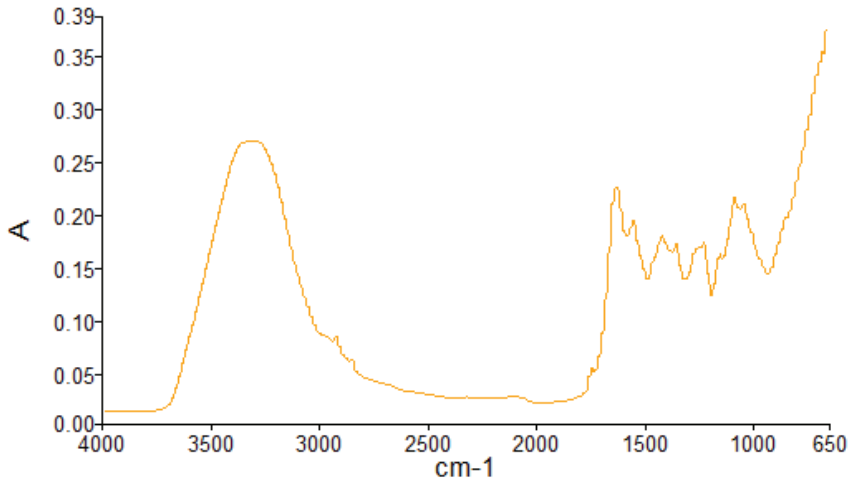

Figure 51. FT-IR spectra of *Fusarium fjikori*

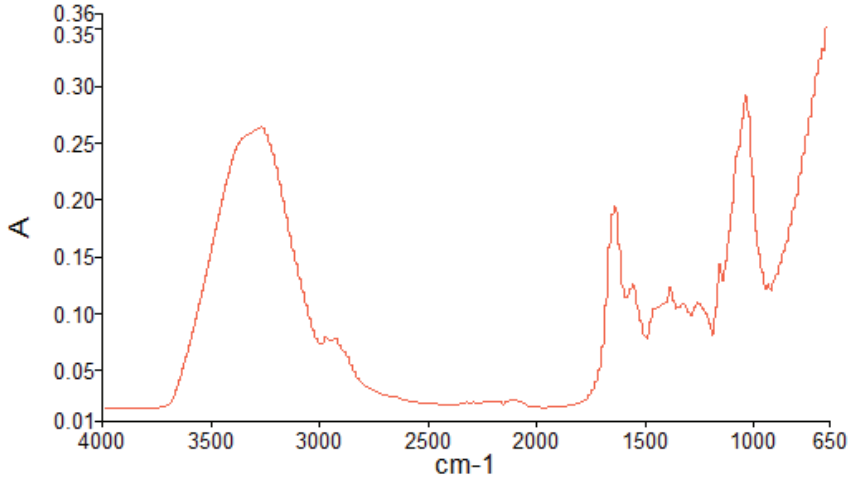

Figure 52. FT-IR spectra of *Paecilomyces varioti*

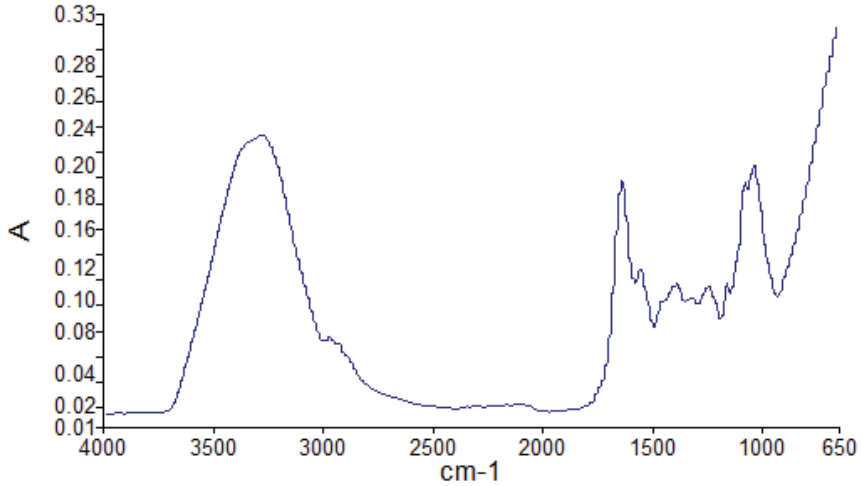

Figure 53. FT-IR spectra of *Penicillum glabrum 4*

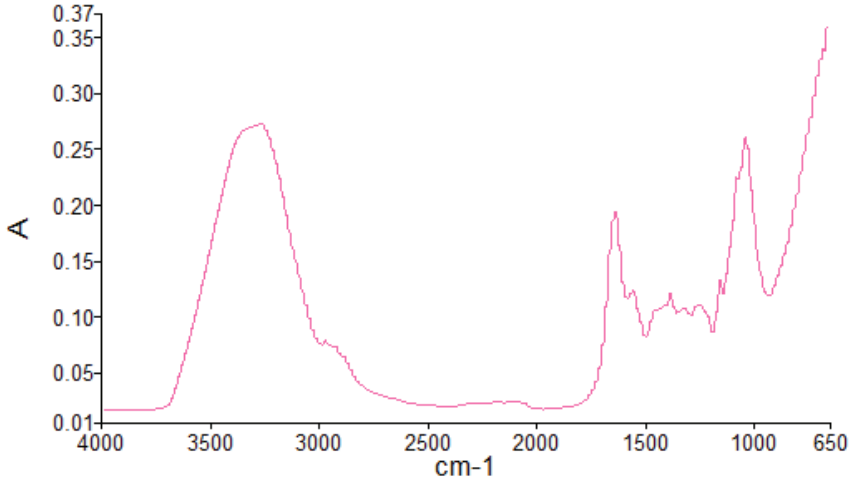

Figure 54. FT-IR spectra of *Penicillum paxiallii*

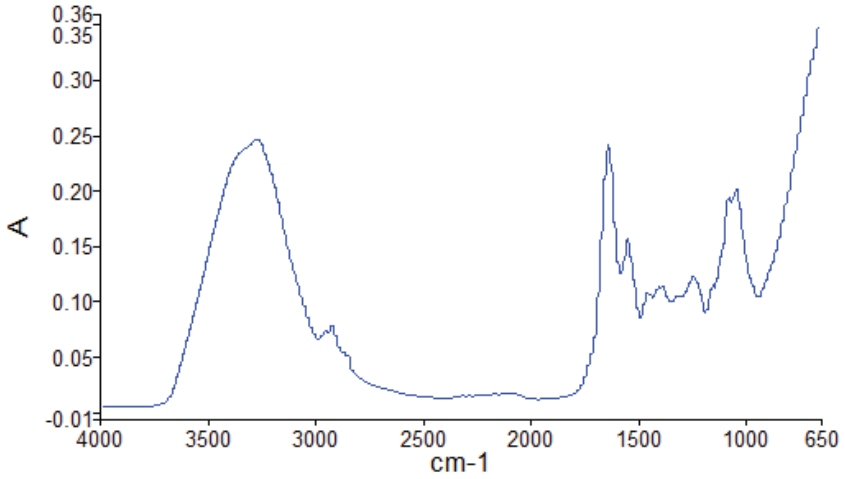

Figure 55. FT-IR spectra of *Penicillum funiculasum*

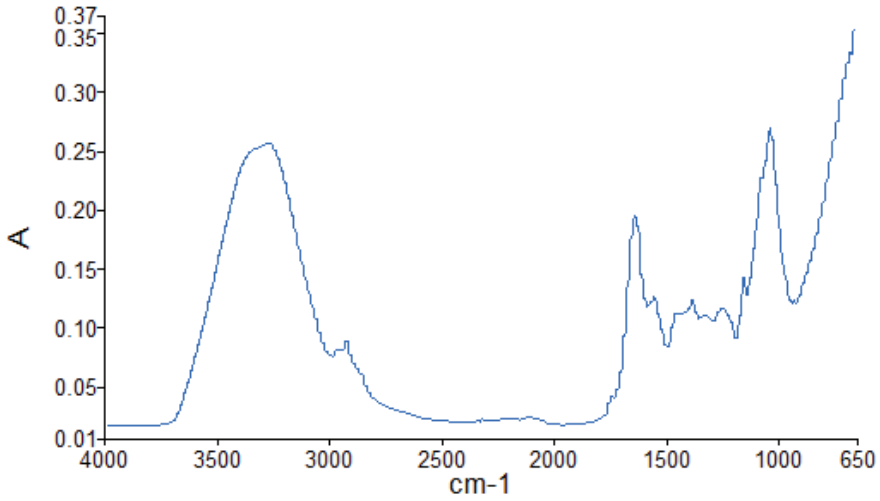

Figure 56. FT-IR spectra of *Penicillum oxalicum*

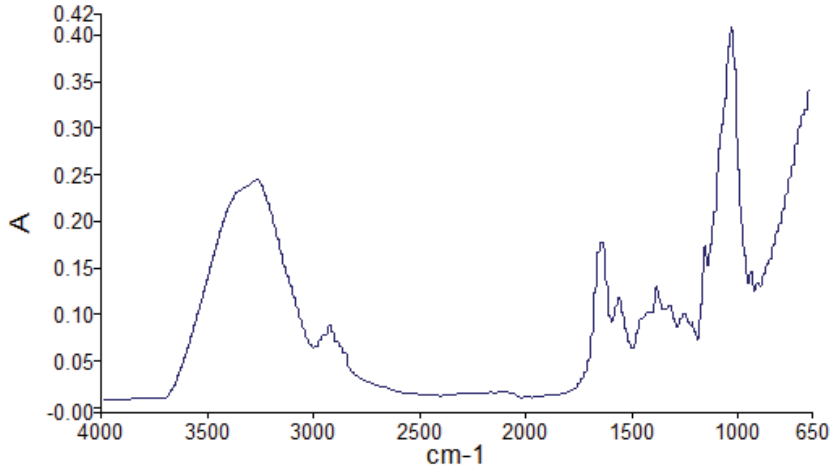

Figure 57. FT-IR spectra of *Alternaria brevimosa*

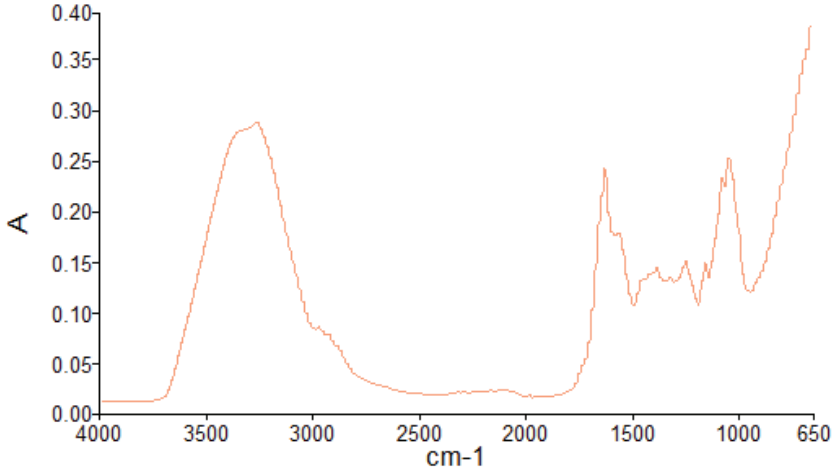

Figure 58. FT-IR spectra of*Alternaria conjucta*

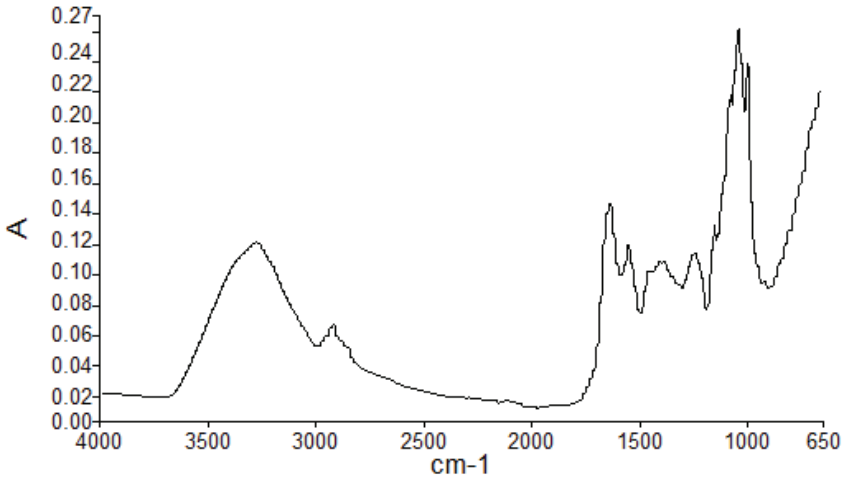

Figure 59. FT-IR spectra of *Aspergillus fumigatus6*

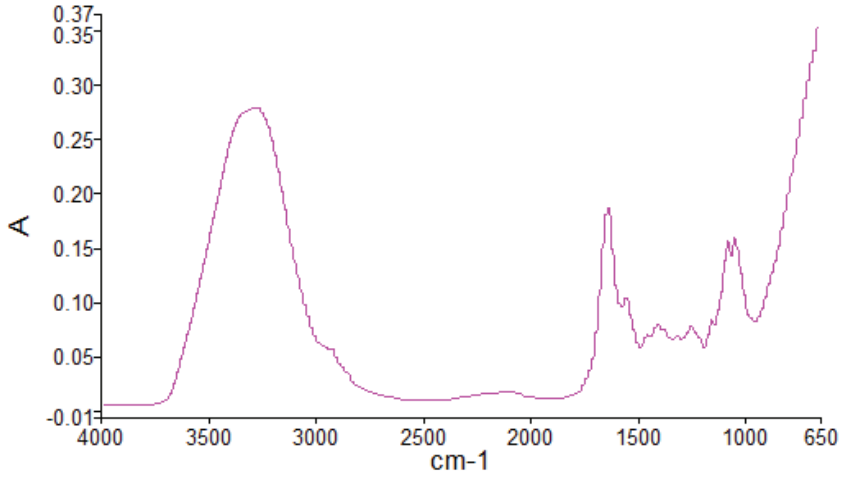

Figure 60. FT-IR spectra of *Sarocladium terricola*

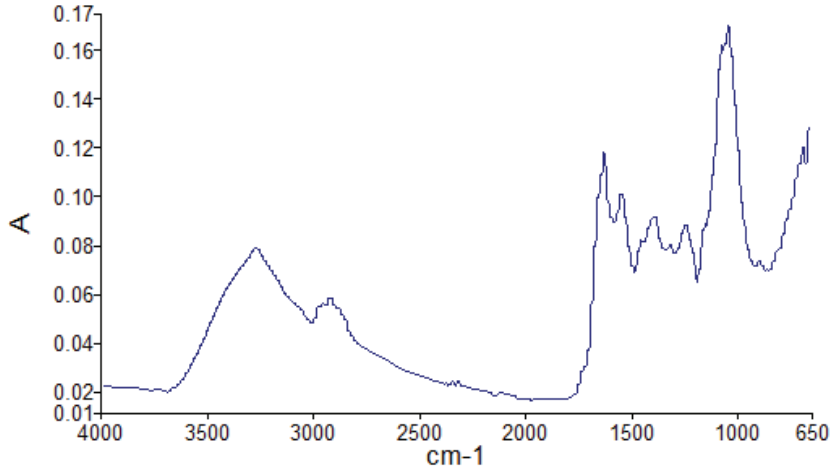

Figure 61. FT-IR spectra of *Aspergillus ustus2*

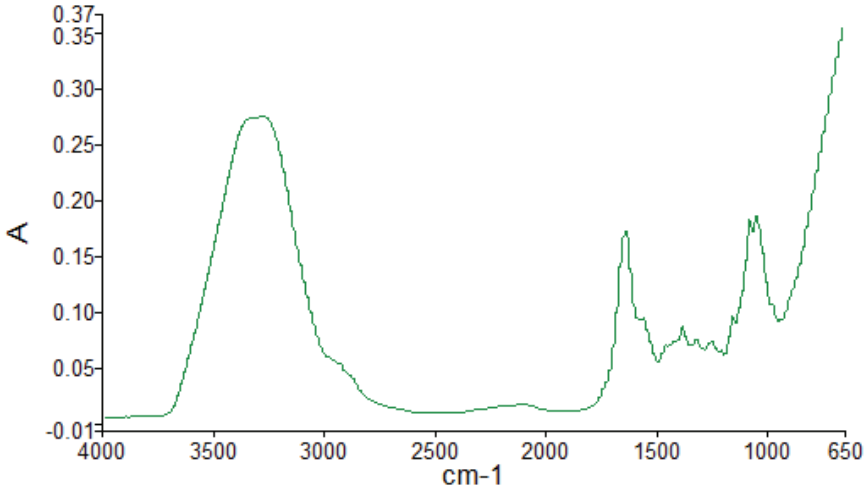

Figure 62. FT-IR spectra of *Verticullum sp2*

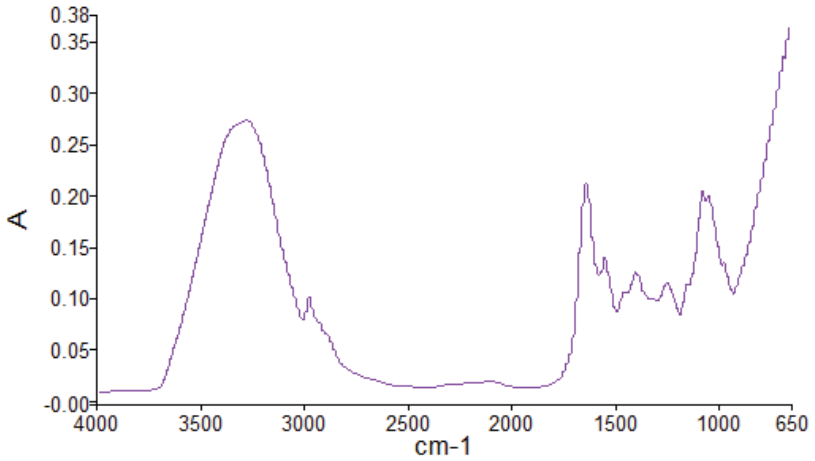

Figure 63. FT-IR spectra of *Penicillium glabrum5*

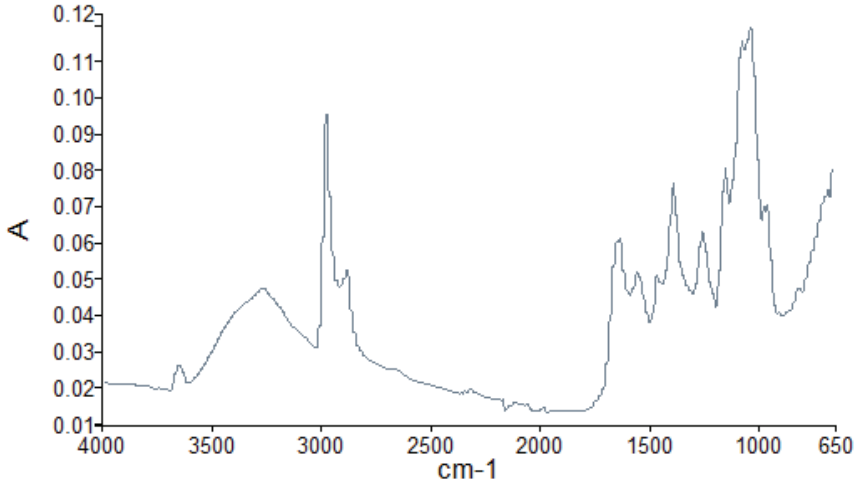

Figure 64. FT-IR spectra of *Aspergillus sydowii4*

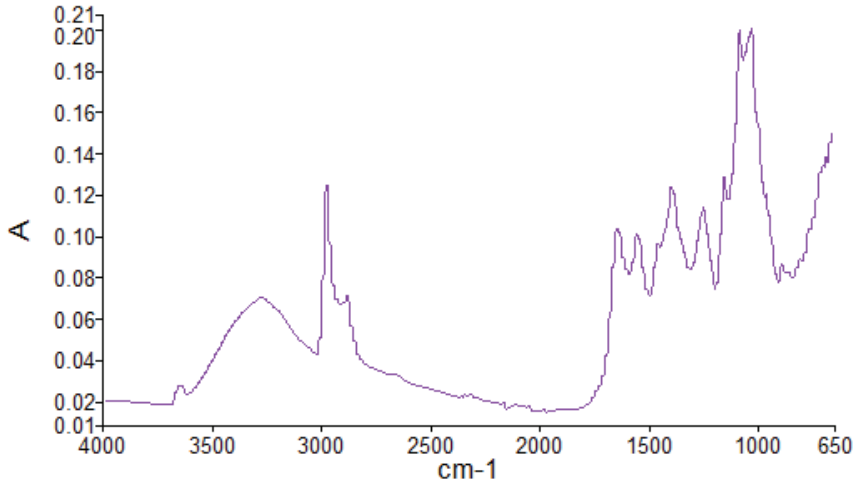

Figure 65. FT-IR spectra of *Aspergillus aculeatus*

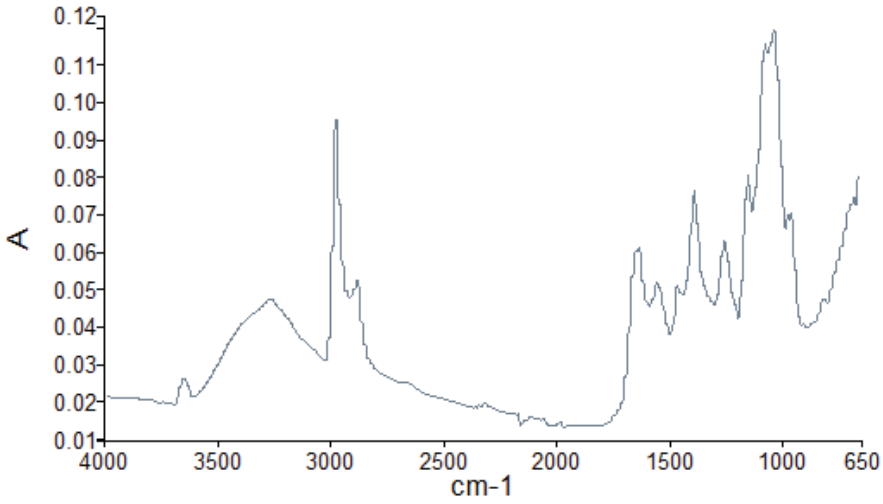

Figure 66.FT-IR spectra of *Aspergillus fumigatus1*

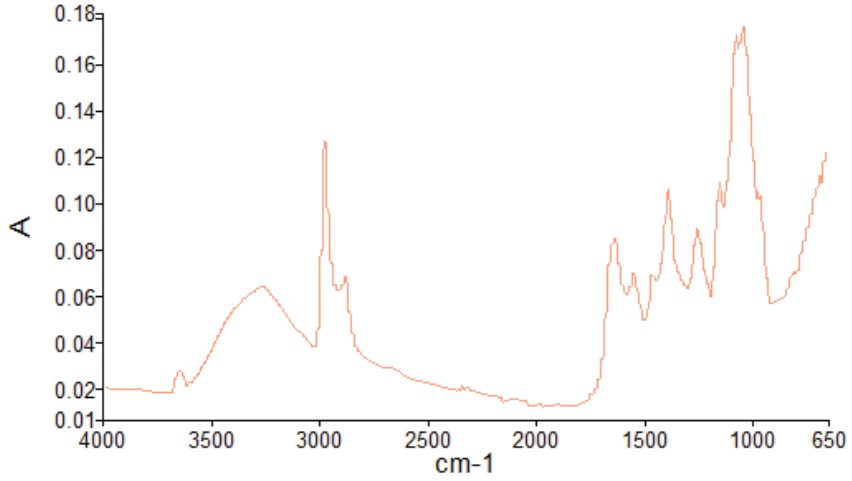

Figure 67.FT-IR spectra of *Aspergillus protuberus1*

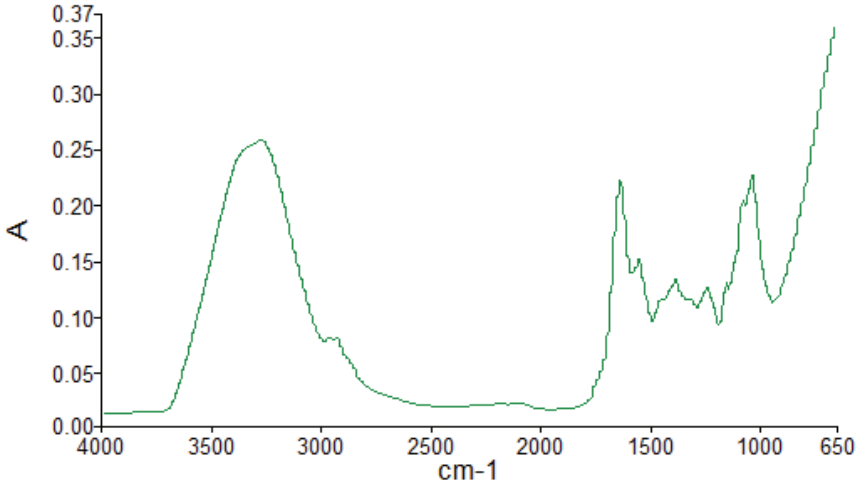

Figure 68. FT-IR spectra of *Aspergillus ustus*

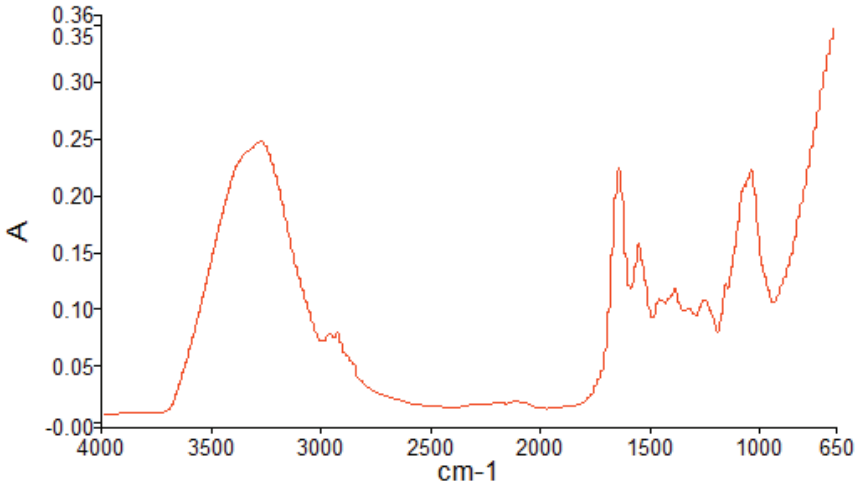

Figure 69. FT-IR spectra of *Aspergillus terreus*

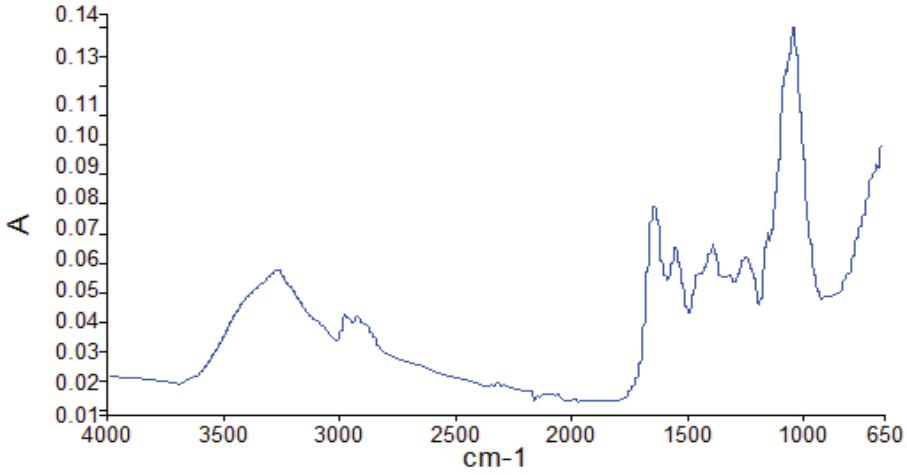

Figure 70. FT-IR spectra of *Penicillim glabrum1*

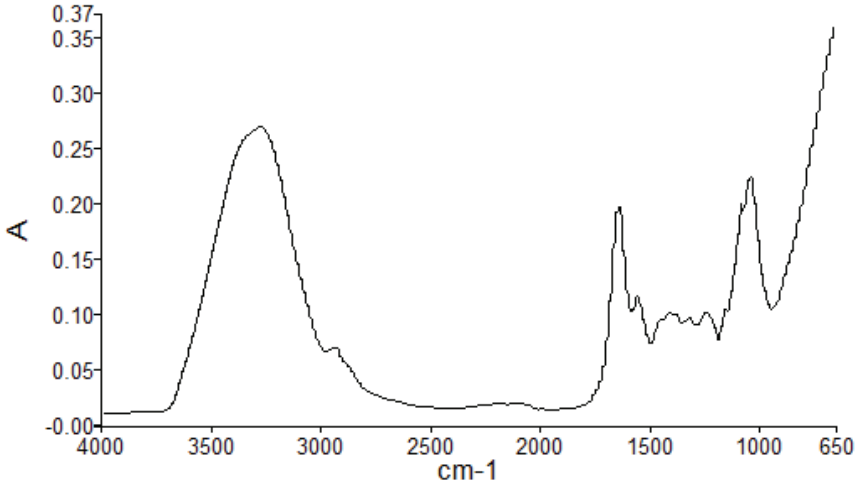

Figure 71. FT-IR spectra of *Penicillium brevicompactum*