Production and Evaluation of Bioactive Metabolites from Newly Isolated Endophytic Fungal Isolates

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بسم الله الرحمن الرحيم

DEDICATED TO MY LOVING

PARENTS AND GRANDPARENTS

ESPECIALLY TO MY

GRANDFATHER (LATE)

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Abdul Haleem

Contents

List of Tables

List of Figures

List of Appendices

List of Acronyms

%	Percentage
°C	Degree Centigrade
µg	Microgram
µl	Micro Liter
ATCC	American Type Culture Collection
Bu	Butanol
CFU	Colony Forming Unit
CH_3Cl	Chloroform
cm	Centimeter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPHH	2,2-diphenyl-1-picrylhydrazyl
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
FCBP	First Fungal Bank of Pakistan
GYP	Glucose Yeast Peptone Agar
IC	Inhibitory concentration
i.e.	That is
LD	Lethal Dose
M	Molar
MeoH	Methanol
mg	Milligram

ml	Milliliter
mm	Millimeter
MHA	Muller Hinton Agar
MRL	Microbiology Research Lab
NA	Nutrient Agar
Nacl	Sodium Chloride
nm	Nanometer
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
QAU	Quaid-i-Azam University
ROS	Reactive Oxygen Species
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
Sp.	Species
TLC	Thin Layer Chromatography
UV	Ultraviolet Radiation

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ABDUL HALEEM

ABSTRACT

The aim of this study is the production and evaluation of bioactive metabolites produced by the endophytic fungi of medicinal plant *Taxus fuana* inhabited in the Himalayan region of Pakistan. A total of 07 endophytic fungal isolates encoded (NFW1, NFW3, NFW6, NFW7, NFW9, NFW11, and NF3L2) obtained as pure culture from MRL QAU, were screened for the production of antimicrobial metabolites and also qualitatively screened for the production of extracellular enzymes (amylase, protease, lipase, and cellulase). All the fungal isolates were positive against at least one test organism. Two out of 07 were negative against all the test bacterial strains (*E. coli, B. spizizenii, S. aureus, S. typhi and S. epidermidis)*. Three isolates shows activity against both Gram positive and Gram negative bacteria. Five isolates exhibit antibacterial effect against Gram positive while 04 isolates possess antibacterial effect against Gram negative bacteria. All the fungal isolates showed antifungal activity against at least one test fungal strain (*A. niger, A.fumigatus* and *Mucor* sp.). Three fungal isolates produce at least one extracellular enzyme. Two fungal isolates NFW9 and NFW3 producing relatively better antimicrobial metabolites were studied for the optimization of operational parameters such as (Incubation time, media, pH value and temperature). SDB medium, pH range 6.5 to 7, incubation time of 14 days and 15°C temperature was optimum for both of the isolates. On the basis of initial screening and optimization studies NFW9 showing better results was further studied for organic extraction of both extracellular and intracellular bioactive metabolites and their preliminary study by TLC, FTIR, protein precipitation and bioautography. TLC analysis showed the production of a wide range of organic metabolites and staining of TLC plates indicate the presence of terpenes and sesquiterpenes which showed inhibitory effect against test bacterial strains in bioautography. The FTIR analysis of organic extracts indicates the presence of functional groups like phenols, amines, Alkenes, alkanes and ketones which may attribute to the antimicrobial activity against test microbial strains.

INTRODUCTION

The emergence of novel pathogenic strains and the development of drug resistant among the present pathogenic organisms increased the need to discover novel and potent bioactive agents to combat with these situations. Natural sources are always remained best solution for these problems because they can directly act as drugs or provide leads for the synthesis of drugs. Among these natural bioactive agents microbial metabolites enjoyed a prime importance.

Living organisms are capable of producing different types of metabolites through metabolic processes. Metabolites are small molecules produced during a metabolic reaction. They may be the intermediates or products of metabolism. Metabolites produced are broadly classified as primary and secondary metabolites. Primary metabolites (required for cell growth, its development and reproduction), while secondary metabolites are not involved in growth but involve in an important ecological function i.e. confer protection against variety of biotic and abiotic stresses.

Any compound, substance or molecule is bioactive if it possesses any activity on living tissue or living organisms. The effect of activity can be beneficial or harmful depending upon the nature of compound, its dose and also the nature of the living organisms or tissues upon which it acts.

Over the years researchers have sought different type of natural sources for the production of bioactive compounds. Among these sources; microorganisms get prior attention over the recent few decades. Since the discovery by Sir Alexander Fleming, he first time discovered penicillin (antibiotic) from fungal specie *Aspergillus notatum* in 1929. The success of various drugs of microbial origin like different antibiotic from fungi, bacteria and *actinomycetes*, i.e. cyclosporine having immunosuppressant properties produced by *Tolypocladium inflatum*, griseofulvin having antifungal activity produced by a fungal sp. *Penicillium griseofulvum*, and the inhibitor of cholesterol biosynthesis lovastatin was produced by a fungal sp. *Aspergillus terreus* has diverted the trend of searching natural bioactive compounds from plants towards microbes (Selim *et al.,* 2012). More than half of the Antimicrobial (almost 60%) drugs approved from FDA consist of natural products. Up till 2006 the 47% of total anticancer drugs present in market while 52% of newly introduced anticancer

chemicals were of natural origin (Newman and Cragg, 2007; Chin *et al.,* 2006). In USA 50% of the drugs prescribed are of natural origin or semi-synthetics which were derived from natural products, and also majority of the chemicals used for crops protection are also of natural origin (Schneider *et al*., 2008). Bioactive Metabolites of microbial origin exceed up to 20000 in number (Berdy, 2005). Among these microbes the most important eukaryotic group is fungi which produce a number of novel bioactive metabolites which are being used directly as drugs or can serve as lead structures for synthetic modification (Mitchell *et al.,* 2008; *Chin et al.,* 2006).

However; recent studies indicate decline in such discoveries. In the context of emerging new diseases and life threatening infections, there is a need to explore novel natural sources for the discovery of novel bioactive leads. Endophytes present one such untapped natural reservoir.

Endophytes are the microbes present inside the tissues of healthy plants without causing any infection. Fungi present in the healthy tissues of plants without causing an apparent infection is known as endophytic fungi (Hyde and Soytong, 2008). Endophytic fungi produce a number of bioactive metabolites which possess anticancer, antimicrobial and other biological activities. Endophytic fungi produce a wide diversity of bioactive metabolites with distinctive structures classified as, quinones, phenolic acids, chinones, flavonoids, benzopyranones, steroids, terpenoids, alkaloids, xanthones and tetralones (Pimentel *et al.,* 2010).

The endophytes inhabited in such an ecological niche which is less explored. These endophytes produce a plethora of bioactive metabolites depending on their ecological niche and the factors that effecting their production. So under lab condition it is extremely necessary to produce such conditions under which they produce bioactive metabolites more sufficiently. The diterpenoid "Taxol" ($C_{47}H_{51}NO_{14}$) also renowned as paclitaxel an excellent anticancer compound initially obtained from yew tree (*Taxus brevifolia)* but isolation of a strain of *Taxomyces andreanae* by Stierle *et al*., (1993) from phloem of *Taxus brevifolia* first time, produces taxol. Food and Drug Administration (FDA) approved this compound for the treatment of various types of cancers. The antineoplastic agent "Camptothecin" ($C_{20}H_{16}N_2O_4$) is an alkaloidal compound can also be successfully produced by endophytic fungi (Wall *et al*., 1996). A multi potent compound "Phenylpropanoids" is produced by endophytic fungi

having antimicrobial, anticancer, antioxidant immunosuppressive and anti-inflammatory properties (L.G. Korkin, 2007).

The discovery of novel and unique bioactive metabolites from endophytic fungi is an important and alternative source to overcome the drug resistance of human, animals and plant pathogens and also to achieve the sufficient amount of new antibiotics against a diverse range of microbial species (Yu *et al*., 2010). The antimicrobial compounds from endophytic fungi can be significantly use not only as drugs but also as food preservatives to avoid food spoilage and food borne diseases (Liu *et al*., 2008). An endophytic fungi *Armillaria mellea* produces antimicrobial agents' sesquiterpene and aryl esters which possess significant antimicrobial activities (Gao *et al.,* (2009). Another antimicrobial metabolite 7-amino-4-methylocoumarin is produced by *Xylaria* sp. this bioactive metabolite is active against a wide range pathogenic microbes including, *E. coli, S. enteritidis, S. typhimurium, S. aureus, A.hydrophila, Shigella* sp., *V. parahaemolyticus, Yersinia* sp., *V.anguillarum, A. niger, C. albicans* especially to *A. hydrophila,* and was suggested to be used as natural preservative in food (Liu *et al*., 2008). *Xylaria* sp. residing in different host plants produces several bioactive metabolites like "sordaricin" with antifungal properties, (Pongcharoen *et al*., 2008)., "multiplolides A and B" possessing activity against *C. albicans* (Boonphong *et al*., 2001)., "1,8-dihydroxynaphthol 1-O-a-glucopyranoside" and "mellisol" having antiviral activities against herpes simplex virus-type 1 (Pittayakhajonwut *et al*., 2005). "Griseofulvin" ($C_{17}H_{17}ClO_6$), produced by Xylaria sp. was potent antimycotic agent used for the treatment of several veterinary and human mycotic diseases (Park *et al., 2005).* Chaetomugilin "A" and "D" is also produced by endophytic fungi, these are strong antifungal agents (Huang *et al*., 2008). "Emodin" ($C_{15}H_{10}O_5$) and "Hypericin" ($C_{30}H_{16}O_8$), produced by endophytic fungi shows antimicrobial activity against a number of Gram positive and Gram negative bacteria and also against various fungal pathogens (Kusari *et al.,* 2008). An endophytic fungi present in *Grevillea pteridifolia* produces novel antibiotics "echinomycin" and "kakadumycin A". "kakadumycin A" is more active against Gram positive bacteria and possess a significant activity against malarial parasite *Plasmodium falciparum.* "Pestalotheol C" shows antiviral activity against HIV virus is produced by an endophytic fungus *Pestalotiopsis theaeof* (Li *et al*., 2008).

Another exciting aspect of endophytic fungi is the production of a variety of enzymes which was (not too much studied) of unique characteristics because of their ecological niches they may produce commercially important and stable enzymes as compared to other microbes. The most abundantly produced endophytic fungal enzymes which have been relatively more studied until now include cellulase, amylases, protease, lipases, laccases, pectinases and xylanases. Further studies are required regarding endophytic fungal enzymes as they have a great potential to produce novel enzymes (Sunitha *et al*., 2013). These enzymes can be used in different industries to overcome the problems associated with present enzymes which are being used for different biotechnological purposes.

In the context of biotechnological importance of endophytic fungi and associated bioactive metabolites, the present study was proposed to explore this exciting potential. For this purpose; endophytic fungi isolated from *Taxus fauna* of Himalayan region of Pakistan was selected. The isolates were analyzed for the production of bioactive metabolites under different experimental conditions giving insight about the optimum conditions which will help to produce the bioactive metabolites on large scale for industrial applications.

AIMS AND OBJECTIVES

The aim of present study is the production and evaluation of bioactive metabolites from newly isolated endophytic fungal isolates of *Taxus fauna* of Himalayan region of Pakistan. The specific objectives of the study were:

- To screen the endophytic fungi for antimicrobial activity by agar well diffusion assay and disk diffusion assay.
- To screen the fungal isolates for the production of commercially important enzymes by plate based assay. (Qualitative Screening).
- To optimize the operational parameters (time, media, temperature and pH) for lab scale cultivation and production of bioactive metabolites of the isolates by shake flask experiments.
- To analyze the nature and preliminary study of bioactive metabolites produced by FTIR, TLC and bioautography.

REVIEW OF LITERATURE

History shows that as far as human beings have been living on earth they used different types of remedies to combat with diseases and to improve their health. Documentations of such remedies can be found 6000 years back. These remedies were obtained from some medicinal plants and it is extremely fascinating that some of those plants are still being used in traditional medicine and also their bioactive ingredients are isolated and are being used in modern medicine. WHO reported that almost 80% population of the world is still using the traditional type of remedies from medicinal plants to cure the diseases. Medicinal products until the beginning of 20^{th} century are being obtained from extracts or powder of medicinal plants tissues. The progress in modern medicine based on pure compound isolation from these plants. These medicinal plants produce a wide range of natural products that can be used for medicinal purposes. Due to the prevalence of diseases and increasing human population the medicinal plants are directly affected as they are being used commercially on large scale to get bioactive natural compounds for medicinal uses. Therefore some of these plants undergoes, almost in endangered zone. Another issue with medicinal plants as a producer of bioactive natural compounds is their slow growth rate and the proper ecological niche where they grow better. Efforts made by chemists to overcome these issues so these compounds are mimicked or synthesized chemically to overcome the situations but they are not too much efficient, ecofriendly and safe as compared to natural products (Suryanarayanan *et al.,* 2009). But due to emergence of drug resistant microbes and endemics and pandemics of certain new diseases, the search for novel natural products with pharmaceutical applications from such sources which are safe, ecofriendly and renewable are of high interest.

The presence of fungi inside the plant tissue was discovered almost 77 years ago by Sampson in 1935. He reported endofungal species from *Lolium* grass however the research on endophytes began in 1977 when the occurrence of endophyte in the plant *Pseudotsuga menziesii* needles was reported by carroll and Bernstein (Rajagopal *et al*., 2012).

The relationship of endophytes with specific plant species or more than one host plant species is described as host specificity, host selectivity, host recurrence or host

preference (Zhou and Hyde, 2001). Host specificity means that the particular endophyte will be inhabited inside a single host species or a group of related host plants but does not found in other unrelated host species in that particular habitat (Holliday, 1998). Endophytes occupy a novel niche and therefore they are still less studied (Nithya and Muthumary, 2011).

Endophytes are the chemical synthesizers inside the plants (Owen and Hundley, 2004). Many of the endophytes produce bioactive metabolites that are used by the plants for their defense against infectious microbes and also increase the tolerance of plants against drought conditions environmental stresses and nutrient deficiencies. Among these bioactive compounds some are proven as drugs and some act as lead structure for novel drug discovery (Guo *et al*., 2008).

After the discovery of a potent anticancer compound producing endophytic fungus *Taxomyces andreanae* the research on endophytes enhanced significantly. Taxol was the world first billion dollars anticancer compound initially obtained from *Taxus brevifolia* (Yew tree). The endophytic fungi in last couple of decades were studied for their potential to produce different bioactive metabolites possessing antibacterial, antifungal, antiviral, insecticidal, and anticancer activities and these endofungal strains shows significant results in all prospects. The bioactive compounds produced by endophytic fungi can be majorly classified as terpenoids, alkaloids, streriods, quinones, phenols, ligans and lactones (Zhang *et al*., 2006; Xu *et al*., 2008). For example, Phodophyllotoxin (PDT) is a potent aryltetralin lignin possessing antioxidant, antiviral, antibacterial, immunostimulant, antireheumatic, and anticancereous properties is produced by two endophytic fungal specie *Alternaria* which was isolated from *Sinpodophyllum* and *Fusarium oxysporum* isolated from *Sabina recurva* (Gao *et al.*, 2007; Kour *et al*., 2008).

Plants are the reservoirs for an unknown number of endophytic microorganisms (Bacon and White, 2000). Endophytic association provides a great potential of biocontroll programs because they are integrated into the host plant system (Cao *et al*., 2005; Clay, 1989). Most common endophytes are fungi, bacteria and actinomycetes, but the diversity of these endophytes, their host range and geographical distributions are unknown (Arnold and Engelbrecht, 2007). According

to Staniek et al., (2008) the most frequently encounter endophytes are fungi. There may be more than a million species of endophytic fungi (Dreyfuss and Chapella, 1994).

In general, the endophytic fungi were previously classified into two major groups, i.e. Clavicipitaceous endophytes (C-endophytes), which reside inside different grasses and non-Clavicipitaceous endophytes (NC-endophytes) inhabit in the asymptomatic internal tissue of ferns, conifers, allies and angiosperms (Rodriguez *et al.*, 2009). Endophytes can be transmitted both vertically and horizontally. Vertical transmission occurs by infecting seeds or vegetative propagation while horizontal occurs via spores (Carroll, 1988). Endophytic microorganisms can enter to the plants tissue usually by roots of the plants however some other aerial parts like flowers, stomata and cotyledons can also be the route of transmission. Primary transmission of C-endophytes takes place vertically, where that they can pass from maternal plants to the offspring by infecting the seeds (Saikkonen *et al.*, 2002).

Interaction between Endophytic Fungus and Host Plant

Earlier in 1970's endophytes were considered as neutral as they have no effect on plants, but later on, with the passage of time many studies revealed that they are involved in the protection of host plants (Azevedo *et al.*, 2000). In turn the endophytes get benefit from their host in the form of organic nutrients, shelter for their better survival, growth, and reproduction and also the successful transmission to the next generation of their host (Muller and Kraus, 2005).

Impact of Endophytes on Host Plants

Endophytes affect their host plants in numbers of way but the exact role is still unclear. Usually the presence of endophytes is beneficial to the host plants. Endophytes are involve directly or indirectly in plant growth by various mechanisms, and also endophtyic metabolites attributed to the fitness of plant and also increase the resistance of their host plants towards biotic and abiotic factors and also towards growth. Some endophytes are reported that they are able to fix nitrogen, increased uptake of phosphorus and production of some important plant growth related hormones like auxin, gibberelins ethylene, abscisins and indole acetic acid (IAA)

(Zou *et al*., 1999; Firakova *et al.,* 2007). Endophytes increases biomass of their host plants by the production of growth hormones directly or induces the production of hormones in the host (Petrini, 1991; Schulz and Boyle, 2005).

Role of Endophytes in Hosts Tolerance to Stress

endophytes help their host plant in combating abiotic or environmental stresses like salt concentrations, drought conditions and elevated temperatures (Malinowski and Belesky, 2000). Dichanthelium lanuginosum a Herbal plant grows in such regions where soil temperature reaches to 57°C, its fitness increases in the presense of an endophytic fungus *Curvularia* specie while endophyte free plant are more sensitive to high soil temperature and water stress conditions (Redman *et al*., 2002). The symbiotic relation of endophytes and plants play major role in the adaption of global climate changes and hence enhance their survival (Rodriguez *et al*., 2004).

Role of Endophytes in Photosynthetic Capacity of Hosts

Endophytes effect on host's photosynthesis has been studied but their effect is not always positive. Pinto *et al.,* (2000) reported that an endophyte *Colletotrichum musa* which is present in banana plant reduces the photosynthetic capacity of their host as compared to endophyte free host.

Role of Endophytes in Resistance against Pathogens and Herbivores (Biological Control)

The plants harboring endophytes are safer from plants pathogen, pests (Akello *et al*., 2007). Endophytes may reduce the herbivory by the production of alkaloids which are toxic for vertebrates and pests (Schardl, 2001). Endophytic fungi can induce resistance to disease in host plants and several mechanisms are proposed. This resistance is related with the genetics of host and its nutritional status (Saikkonen *et al.,* 2002).

Mechanisms of Resistance against Pathogens

Primarily three mechanisms are involved by which the endophytes can decrease the host susceptibility towards pathogens (Mandyam and Jumpponen, 2005).

First mechanism is competition. Pathogenic organisms compete with endophytes for similar resources (Lockwood, 1992). Example is *Fusarium oxysporum* Fo47 is a nonpathogenic fungus inhibits the pathogenic *Fusarium oxysporum* resulting in reduced root rot symptoms in tomato plant (Bolwerk *et al.,* 2005).

Second mechanism that protects host from pathogens is possibly the ability of endophytes to induce the plant so that plant produces phytoalexins or other biocidal compounds or the endophytes produce such compounds their own. *Piriformospora indica* an endophyte induced the production of antifungal compounds from its host plant which are active against plants pathogenic fungi (Rai *et al.,* 2002).

The example of third possible mechanism is root associated unidentified endophyte named as LtVB3 that prohibited the spread of *Verticillium longissima* in *Brasica campestris* plant by the formation mechanical barriers, and cell wall congealing which will leads to 80% reduced symptoms (Narisawa *et al.,* 2004).

Mechanisms of Resistance to Herbivores, and Pests

Mandyam and Jumpponen (2005) proposed the probable mechanisms with the help of which endophytes enhance the resistance of a plant against pests and herbivores.

The first possible mechanism is the improvement of the host plant performance by endophytes that help the plants to overcome the damages of herbivory and pest damage without major noticeable effects on productivity (Gehring and Whitham, 2002).

Second probable mechanism is to change the nutritional chemistry of plant by shifting the carbohydrate and nitrogen ratio (C: N), and phytosterol composition (Schulz and Boyle, 2005). Due to this alteration in nutrients level and their contents the carbohydrate metabolism of the host plants altered results in less susceptibility toward herbivores (Selim *et al*., 2012).

Third mechanism which is possible is the production of toxic compounds by endophytes themselves. For example toxic alkaloid production by foliar endophytes of grasses (Clay and Holah; 1999). In *L. esculentum* the common root endophyte *F.*

oxysporum is responsible for the production of soluble metabolites that are toxic to the roots nematode *Meloidogyne incognita*. This fungus produces same type of metabolites in vitro as well (Hallman and Sikora, 1996). Mandyam and Jumpponen (2005) explained that the extensive colonization of endophytes can prvent root grazing becauce many of the endophytes produces melanized structures and melanin redeuces microbial grazing (Bell and Wheeler, 1986; Griffith, 1994). *Periconia macrospinosa* an endophyte extensively colonizes in grasses produces chlorine containing compounds that may possess antibiotic properties (Mandyam and Jumpponen, 2005).

Environmental Role of Endophytes

Endophytes play a crucial role in ecological community, by reducing environmental degradation, biodiversity loss and the pollution of land and water caused by industrial effluents, toxic insecticides, sewages and poisonous gasses. Biological control with the use of appropriate endophytes is an efficient method and being used in certain environmental remediation and in killing of various insects and pathogens (Guo *et al.,* 2008). It is reported in several reviews that endophytes are associated with a novel application in the field of phytoremediation of heavy metals and xenobiotics. Endophytes play a key role in phytoremediation by improving the growth of plants capable of remediation and increase the uptake of pollutants present in soil by plants or directly involved to accumulate or degrade the pollutant from soil by endophytes themselves (Ma *et al.,* 2011). Endophytes have a mysterious existence and in ecosystem they play a key role in decomposition because they are previously present in dead tissues of plants (Kumaresan and Suryanarayanan, 2002).

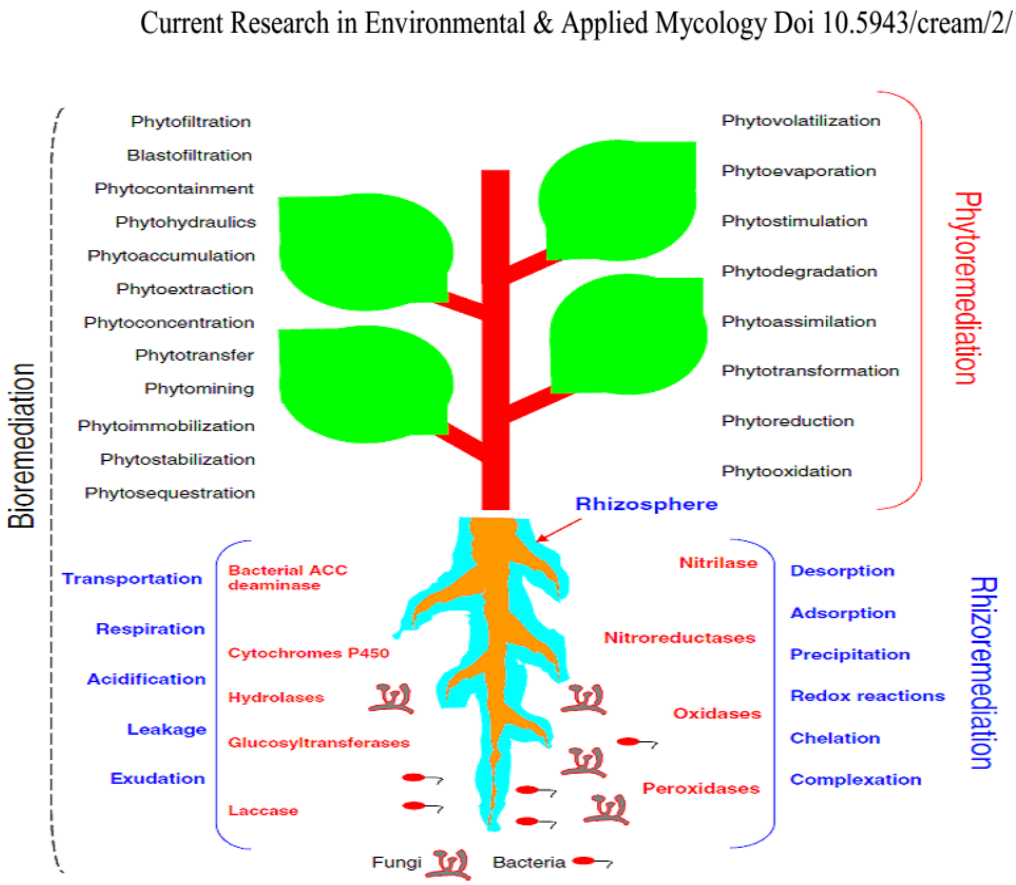


Fig. 12 – Importance of soil–plant–microbial interactions in bioremediation for the cleanup of metals and organics (pesticides, solvents, explosives, crude oil, polyaromatic hydrocarbons), Adapted from (Ma et al. 2011).

Role of Endophytes in Bio-Transformations

Biotransformation is a process in which a compound is chemically changed with the help of living organisms (Borges *et al.*, 2007). Microorganisms have a huge potential to adapt new conditions and are able to metabolize various foreign substances as carbon and nitrogen source when these components are not given to them in the medium where they grow (Doble *et al.*, 2004).

Any compound or molecule can be altered by transforming their moieties or functional groups, with the degradation of their carbon backbone or without the degradation of their carbon backbone. These types of modifications lead to the formation of novel and valuable products which cannot be easily synthesized by chemical methods. Therefore the biotransformation using microorganisms is being popular and received a significant attention for bio-transforming steroids, lipids,

monoterpenes, diterpenes, triterpenes, lignans, alkaloids and various synthetic chemicals carrying out stereospecific and stereoselective reactions which will lead to the production of potential and unique bioactive molecules bearing important pharmaceutical and industrial applications (Borges *et al.,* 2009). Endophytes are responsible for the production of many enzymes (Firakova *et al.,* 2007) so they have the potential to be used in biotransformational processes as they have the better ability of biotransformation due to the production of unique and novel enzymes.

Endophytic fungus *Phomopsis* specie isolated from *Viguiera* is responsible for the biotransformation of tetrahydrofuran lignin grandisin which results in the formation of a new compound dimethoxyphenyl-5-methoxy-tetrahydrofuran. This compound possesses trypanocidal activity against *Trypanasoma cruzi*, a parasite and etiological agent of Chagas disease (Verza et al., 2009).

Bio-Technological Role of Endophytes

Endophytes are capable of producing several novel and known enzymes which can be used in different biotechnological applications such as environmental applications, industrial applications, biotransformation of organic compounds having various advantages over other methods and medical applications (Pimentel *et al.*, 2010).

Enzymes Production by Endophytes

The endophytes primarily produce the enzymes which are necessary to them for colonization in host plant tissues. Many endophytes investigated utilize pectin and xylan display lipolytic activity and are responsible for the production of nonspecific peroxidases, glucanases, chitinases and laccases (Li *et al.*, 2004).

Fungal enzymes are being used in different industries including food, beverages, leather industries, confectionaries and textiles industries to make the process of raw material more simple and easy. The fungal enzymes are usually more stable for commercial sources as compared to enzymes obtained from other sources (Sunitha *et al*., 2013).

Novel xylanases and extracellular cellulases may be obtained from Endophytes from selected host plants or from specifc plant tissues (Leuchtmann *et al*., 1992; Suto *et al*., 2002). Amylase is an industrially important enzyme used in starch degradation a thermostable glucoamylase enzyme is produced from an endophyte isolated from the leaves of maize crop (Stamford *et al.,* 2002).

Cellulose present in all plants is extremely abundant polysaccharide which is non-fossil renewable source of carbon on earth (Coughlan, 1990). The cellulose degradation leads to the production of simple sugars like monosaccharides has got attention as its use in food and fuels (Clarke, 1997; Duff and Murray, 1996). Microbes which are degrading cellulose produces an array of cellulases, these enzymes act synergistically for cellulose degradation (Lynd *et al.,* 2002). The cellulase enzymes which are used for the commercial purposes are usually obtained from *Trichoderma reesei* a filamenteous fungus and also from spore forming fungus *Aspergillus niger*. However, still the waste cellulose cannot be converted into glucose commercially due to low specific activity and inhibition of these enzymes by negative feedback mechanism due to end products. Significant studies on the production of stable cellulases from endophytes are still lacking. Therefore studies for a selective search of endophytic fungal strains producing cellulases and other enzymes should be encouraged. In past studies the emphasis on the production of antimicrobial and antioxidant metabolites from endophytic fungi is much more than biotechnologically important enzymes. Therefore the enzymology of endophytic fungi is relatively new and less explored field and might be a potential source of novel and stable enzymes with huge potentialities (Sunitha *et al*., 2013).

The rate of production of different enzymes by endophytic fungi can be properly monitored when these organisms are grown in large batch culture under proper conditions of enzyme production for purification (Pavithra *et al*., 2012).

Some common enzymes produced by endofungal species are of great interst and also have various applications. Among these enzymes the most commonly produced enzymes reported are Cellulases, amylases, proteases, lipases, pectinases, laccases, chitinases and xylanases. These enzymes play several vital activities for humankind

and thus more studies regarding endophytic fungal enzymes are necessary to get sufficient benefit from the endophytic fungal enzymes.

Endophytic Fungi as Bio Factories for the Production of Bioactive Metabolites

Fungal endophytes are known to produce a wide variety of secondary bioactive metabolites of novel and unique structures majorly classified as terpenoids, xanthanes, steroids, alkaloids phenolisc acids, quinones, chinones, flavonoids, tetralones and benzopyramones. These classes contain a variety of unique and novel compounds of different application especially having anticancer compounds, novel antimicrobials, immunosuppressants, and many other compounds of pharmacological and agrochemical applications (Pimentel *et al.*, 2010; Ding *et al.*, 2010; Strobel *et al*., 2004).

Endophytes are capable of producing novel bioactive secondary metabolites with unique structures which soil isolates can't produce (Schulz *et al.*, 2002). The endophyte produces a variety of secondry metabolites by different pathways that include polyketide pathway, isopernoid pathway and aminoacid derivation pathway (Tan and Zou, 2001). Sauer et al explained that the metabolic pathway for production of secondary metabolites in endophytic fungi mainly occurred by polyketide and nonribosomal peptide pathways by polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) respectively.

However three recognized pathway for the production of secondary metabolites in fungus is estabilished (Fatima N, 2013).

1) Polyketide pathway
2) Mevalonic acid pathway
3) Shikimic acid pathway

Polyketide Pathway

Majority of the secondary metabolites from fungi are produced by polyketide pathway). Polyketides are synthesized by the condensation of one molecule of Acetyl-CoA and at least three molecules of malonyl-CoA. After synthesis these can be

modified by series of reactions and thus they generate a huge number of secondary metabolites (Zhang *et al.*, 2004). Intermediates and Products of the pathway include some color pigments, variety of enzymes and pharmaceutically important compounds including griseofulvin.

Mevalonic acid pathway

Acetyl-CoA is the starting molecule of this pathway in most of the cases (Garraway and Evan, 1984). Important intermediate products and end product of the pathway include diterpenes, sterols, fucosterols,cholesterols, ergosterols tetrapenoids called carotenoids.

Shikimic acid pathway

This pathway is common among fungi, bacteria and plants and produces a number of aromatic compounds (Garraway and Evan, 1984). Important products of this pathway are amino acids Phenyl alaninetyrosine and tryptophane.the antibiotics penicillin and cephalosporin are produced by the same pathway.

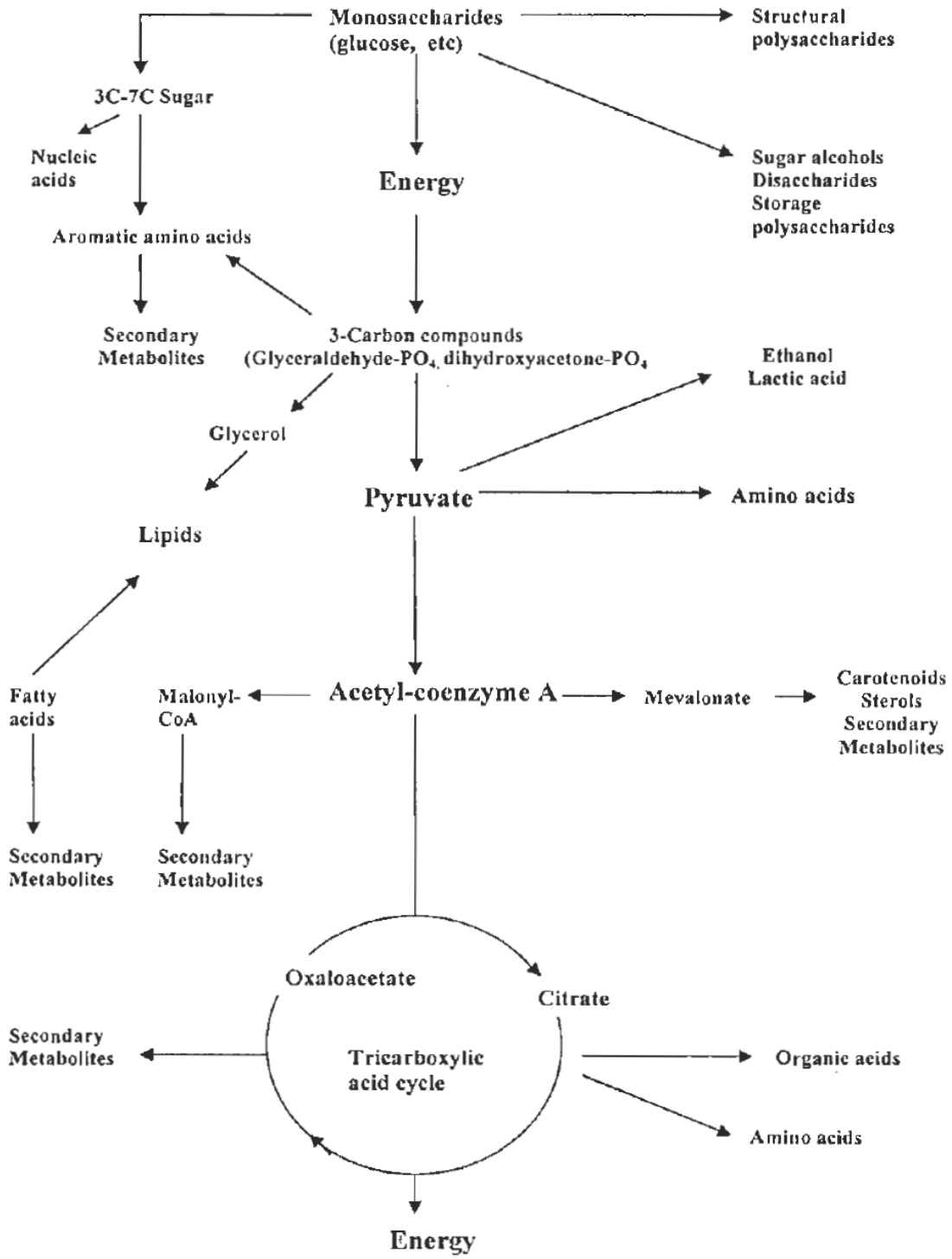


Figure. 1: Metabolic pathways of fungi (Adapted from Fatima N, 2013)

Endophytes produces a plethora of bioactive metabolite in host and studies also reveal that these metabolites are continuously produced by endophytes if they are cultured in fungal media and even after the passage of several generations (Pavithra *et al., 2012*).

Pharmaceuticals applications of Endophytic Fungi

The emergence of multidrug resistant and novel pathogenic microbes is extremely challenging for current treatment protocols therefore novel and efficient drugs are required to combat with such organisms. The bioactive secondary metabolites from microbes are still an attractive and potential source for novel drug discovery. The bioactive secondary metabolites produced by endophytic fungi can directly be used as drug or may act as lead structures for novel drug discovery (Strobel and Daisy, 2003).

Antimicrobial Metabolites Produced by Endophytic Fungi

Microbial metabolites possessing antibiotic activity can be defined as "low molecular weight organic natural product produced by microbes that are antagonistic against other microbes at very low concentrations (Guo *et al.,* 2008).

Liu Dong et al., (2008) reported a bioactive metabolite 7-amino-4 methyl coumarin produced by endofungal *Xylaria* specie YX-28 isolated from *Ginkgo biloba L*. The compound exhibit antimicrobial activity against several micro-organisms including *E.coli, S. typhimarium, S.aureous, Yersinia* sp. *Shigella* sp. *C. albicans* and *A. niger* and was suggest to use as a natural preservative of food. Hypercin ($C_3H_{16}O_8$) and Emodin (C_{15} H_{10} O_5) were produced by an endophytic fungus that was isolated from a medicinal plant found abundantly in sub-continent which shows antimicrobial activity against several species including *E.coli, S.aureus, Klebsiella pneumonia Pseudomonas auroginosa* and fungal species including *C.slbicans and Aspergillus niger* (Kusari *et al.,* 2008). Jianglin Zhao et al., (2010) reported 03 novel steroids that are ergosta-7,22-dien-3β,5α,6β-triol, ergosta-5,7,22-trienol, 5α,8α-epidioxyergosta-6,22-dien-3β-ol, and one nordammarane triterpenoid helvolic acid from an endophytic fungus *Pichia guilliemondii* Ppf9 isolated from a medicnal plant exhibit strong antimicrobial activity.

Antifungal Metabolites Produced by Endophytic Fungi

A novel antifungal compound Oocydin is produced by *Serratia marcescens* sp. was Isolated from *R. penicillata* being considered for use in agricultural purpose because it controls the presence of *Oomyceteous* fungi (Strobel et al., 1999). An endophytic fungus *Pestalotiopsis adusta* contributes significant antifungal activity against plant pathogenic fungi *Fusarium culmorum*, *Verticilliumalb-artrum* and *Gibberella zeae* by producing the antifungal compound Pestalachlorides "A" ($C_{21}H_{21}C_{l2}NO_5$) and B ($C_{20}H_{18}C_{l2}O_5$) (Li *et al.*, 2008).

Atmosukaro et al., (2005) reported an endophytic fungal strain of *Muscodar albus* that produces a volatile organic compound which exhibit both antifungal and antibacterial effect. Qin et al., (2009) studied an endophytic fungus *Chaetomium globosums* obtained from *Ginkgo biloba* produces antifungal compounds classified as chaetomugilin A and Li et al., (2001) isolated a highly efficient antifungal compound ambuic acid from *Monochaetia* specie and *Pastalotiopsis* specie.

Endophytic *Phomopsis* specie isolated from *Mangrove* produces an antifungal compound cytosporone B and C which inhibits the activity of *F.oxysporum* and *C.albicans*. Two novel antifungal metabolites namely phomopsilactone and ethyl 2,4-dihydroxy – 5,6 – dimethyl benzoate were produced by endophytic fungal strains *Phomopsis cassiaspectbilis* shows strong activity against plant pathogenic fungi *cladosporium C.sphaerospermum* and *cladosporide*s (Silva et al., 2005).

Antiviral Metabolites Produced by Endophytic Fungi

Novel and resistant infectious strains of viruses are always remaining threat for not only human health but also for animals, crops and plants. Endophytic fungi produce novel and important antiviral compounds which can be used against these viruses. Many studies revealed the importance of endophytic fungi regarding antiviral agents like the novel cytonic acids A and B that are HCMV protease inhibitors isolated from *Cytonaema* species (Guo *et al.,* 2000). Hinnuliquinone is efficient inhibitor of HIV-1 protease can be produced by an endophytic fungi isolated from the leaves of *Quercus coccifera* (Singh *et al.,* 2004). Pittayakhajonwut *et al*., (2005) reported Mellisol 1-O-

a-glucopyranoside and 1, 8-dihydroxynaphthol were produced by endophytic fungi *Xylaria mellisii* possess antiviral activity against herpes simplex virus type 1.

Antitubercular Compounds

World Health Organization (WHO) estimated that Almost one third population on earth are infected with tuberculosis and 1500 people die each hour from tuberculosis worldwide. After the emergence of multidrug resistant strains of *Mycobacterium tuberculosis* the need of novel drugs increased significantly to combat this alarming situation. Endophytic fungi from medicinal plants can serve as source for the production of novel anti-mycobacterial compounds.

An endophytic Fungus *Phomopsis* sp. isolated from *Garcinia* a medicinal plant produces two anti-mycobacterial metabolites Phomoxanthone "A" and "B" that showed significant results against *Mycobacterium tuberculosis* (Isaka *et al*., 2001). *Phomopsis* sp. inhabiting *Garcinia dulcis* involved in the production of novel metabolites named as Phomonitroester and Phomoenamide which can efficiently inhibit the growth of *Mycobacterium tuberculosis* (Rukachaisirikul. *et al*., 2008). Bungihan *et al*., (2011) isolated two novel metabolites benzopyranones diaportheone "A" and "B" from an endofungal sp., these metabolites causes the growth inhibition of virulent strain of MTB.

Antiparasitic Metabolites Produced by Endophytic Fungi

Parasitic disease is an infectious disease caused by a parasite. The parasitic infections are usually caused by two kinds of organisms, *helminths* and *protozoa.* Malaria is the most common parasitic disease of concern since thousands of years caused by *Protozoan* parasites of the genus *Plasmodium*. Now it is extended to more than 40% population (Robert *et al.,* 2001). Antiparasitc drugs available are costly, with some side effects on health and having limited useful life. Therefore it is required to discover such endophytes that are capable of producing novel and potent antiparasitc compounds.

Hemtasin et al., (2011) studied an endophytic fungus isolated from *Vanilla albindia* that are responsible for the production of aromatic sesquiterpenes phomoarcherins

"A"–"C" which shows antimalarial activity against *P. falciparum* (Hemtasin *et al.*, 2011). Endophytic fungal *phomopsis* sp. produces two novel dimers of xanthone classified as Phomoxanthones "A" and "B" ($C_{38}H_{38}O_{16}$) possesses significant antimalarial effect (Isaka *et al*., 2001). An endophytic *Penicillium janthinellium* isolated from *Melia azedarach* produced a polyketide citrinin ($C_{13}H_{14}O_5$) which shows strong antileishmanial activity (Marinho *et al*., 2005). Endopytic fungal *Mycosphaerella* sp. associated with *Psychotria horizontalis* produced cercosporin which on acetylation converted into a new analogue of cercosporin. Both of these compounds showed strong activity against a number of parasites namely *Trypanosoma cruziand, P.falciparum and L. donovani* (Moreno *et al.,* 2011).

Antioxidant Metabolites Produced by Endophytic Fungi

Reactive oxygen species (ROSs) formed inside the body due to various reasons and are highly toxic for health, produces several pathological conditions like cellular degeneration, DNA damages and carcinogenesis (Huang *et al.,* 2007; Seifried *et al.,* 2007)**.** The metabolites bearing antioxidant activities are important against ROSs because they neutralize these oxygen containing free radicals thus having the ability to be used as therapeutic agent for ROS associated diseases like cancer, hypertension, diabetes mellitus, cardiovascular disease, ischemia/reperfusion injury, atherosclerosis, rheumatoid arthritis, neurodegenerative diseases (Alzheimer and Parkinson diseases), and ageing (Valko *et al.,* 2007).

Fungal enophytes are valuble sources of producing antioxidant compounds. Several novel and efficient antioxidant compounds are produced by them. Pestacin and isopestacin are potent antioxidant compounds produced by *Pestalotiopsis microspora* recovered from *Terminalia morobensis* plant. Amazingly highly active antioxidant phenolic metabolite graphislactone A was obtained from two endofungal species *Microsphaeropsis olivacea* and Cephalosporium IFB-E001 present in *Pilgerodendron uviferum* and *Trachelospermum jasminoides* respectively. This metabolite shows antioxidant activity stronger than ascorbic acid and butylated hydroxytoulene (BHT) which are used as positive standard in the assay (Hormazabal *et al.,* 2005; Song *et al.,* 2005). *Xylaria* sp. an endophytic fungus isolated from *Ginkgo biloba* a medicnal plant produces metabolites of phenolic and flavonoids nature which exhibit strong

antioxidant activities (Liu *et al.,* 2007). Zeng *et al.,* (2011) reported several endophytic fungi from *Scapania verrucosa* a medicnal plant, having tremendous potential of producing novel antioxidant compounds.

Anticancer Metabolites Produced by Endophytic Fungi

Cancer can be defined as a broad group of diseases in which the cells undergoes an uncontrolled and unregulated growth. According to WHO report in the year (2008) 7.6 million people worldwide died due to cancer. Present treatment strategies for cancer are not sufficient enough so novel drugs for cancer therapy should be investigated. Many anticancer compounds exhibit cytostatic activity and they inhibit the assembly of microtubules, eventually causes the programmed cell death (Elisabetta Buommino, *et al.,* 2008). Taxol is wonderful antimitotic agent possessing outstanding activity against a variety of cancers. This compound was first time discovered in a plant named *Taxus brevifolia* (Wani *et al.,* 1971).

Taxol is extremely potent and expensive compound used all over the world against a variety of cancers including breast cancer, lung cancer, and ovarian cancer and also against some tissue proliferating diseases. Taxol appears as white to off white crystalline powder consist of 11 stero centers. Its empirical formula is $C_{47}H_{51}NO_{14}$. Taxol is strong lipophilic and not soluble in water, and having molecular weight of (853.9). Taxol can kill the cancer cells by inhibiting the depolymerization of microtubules (Schiff *et al.,* 1979). In 1993 the isolation of an endophytic fungus *Taxomyces andreanae* from *taxus brevifolia* provides an easy, cheaper and alternative source for the production of taxol (Stierle *et al.,* 1993). Several endophytic fungi belonging to various genera are reported which can produce taxol. These include *Pestalotiopsis microspora,* as *Taxomyces Andreanae, Alternaria alternata, Pithomyces* sp., *Monochaetia* sp., *Bartalinia robillardoidesare Botryodiplodia theobromae* and *Periconia* species. *Pestalotiopsis terminaliae* recoverd from *Terminalia arjuna* produce the greatest amount of taxol (Gangadev and Muthumary, 2009).

Camptothecin is an alkaloid compound with brilliant antineoplastic properties was first time obtained from the wood of *Camptotheca acuminate Decaisne* (*Nyssaceae*)

in China (Wall *et al.,* 1966). Different endophytic fungi produce anticancer compound camptothecin including *Entrophospora infrequens* present in medicnal plant *Nothapodytes foetida* and *Entrophospora infrequens* (Puri *et al.,* 2006). Camptothecin along with its two analogues analogues (9 methoxycamptothecin and 10-hydroxycamptothecin) showing anticancer activity were produced by *Fusarium solani* associated with *Camptotheca acuminate* (S. Kusari *et al.,* 2009).

Immunomodulatory Agents

Several immunomodulatory metabolites are reported from endophytic fungi. The emergence of autoimmune diseases immunosuppressive compound has a considerable value in market. These drugs are also used to avoid graft rejections in organ transplanted patients. Therefore for researcher an alternative source to avail such metabolites, endophytic fungi may be an excellent choice.

Endophytic fungus *Fusarium subglutinans* from *Tripterygium wilfordii* produces two significant immunosuppressive metabolites Subglutinol-A ($C_{27}H_{38}O_4$) and Subglutinol-B that are noncytotoxic diterpene pyrones. These metabolites are very potent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP). An effective immunosuppressive endofungal metabolite Mycophenolic acid ($C_{17}H_{20}O_6$) is obtained from *Penicillium Aspergillus* species, are used for the treatment to avoid graft rejection and in different autoimmune diseases (Larsen *et al.,* 2005).

Insect Repellent Metabolites Produced by Endophytic Fungi

Along with the production of various insecticidal metabolites, some natural bioactive metabolites from endophytes are also reported showing insect repellent properties. Usually parasitic diseases are transmitted by vectors in which majority of them are insects like ticks, flies and mosquitos. The developing countries especially in Pakistan peoples are highly exposed to these vectors especially mosquitos which may transmit malaria and dengue. The mosquito repellent lotions present in market are usually synthetic so need of natural compounds which may be safer than the present ones should be a better option to use in those lotions as well as for other relevant purposes.

Bryn Daisy et al., (2002) reported an insect repellent metabolite Naphthlene from the Endophytic fungus *Muscodor vitigenus* isolated from *Paullinai paullinioides*. Another endophytic fungal species *Periconia*, *Stenella*, and *Drechslera* present in host plant *Azadirachta indica A. Juss*, produces a compound known as Pestasol has excellent insects, repellent properties (Verma *et al.,* 2007).

Many other bioactive metabolites having important bioactivities like antidiabetic, antialgal, anti *H.pylori* and blood regulatory metabolites of endophytic fungi and some metabolites can be served as biofuel are also been reported in different studies. To present an overview on some of the important metabolites, their function, the endophytic fungi responsible for their production and its host plants, studied in the last few years along with their references (Table 2.1).

1

Table 2.1: Bioactive Metabolites of Endophytic Fungi

Bioactive Metabolites	Biological Activities	Enophytic Fungi	Host plants	Reference
Subglutinol A & B	Immunosuppressive	*Fusarium subglutians*	*Tripterygium wilfordii*	Lee et al., 1995
Torreyanic acid	Anticancer	*Pestalotiopsis microspora*	*Orreya taxifolia*	Lee et al.,1996
22-oxa-(12)-cytochalasins	Anticancer	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Wagenaar et al., 2000
Taxol	Anticancer	*Tubercularia* sp. strain TF5	*Taxus mairei*	Jianfeng-Wang et al, 2000
1-butanol ,3-methyl-, acetate	Antimicrobial	*Muscodor albus*	*Cinnamomum zeglanicum*	Strobel, et al., 2001
Naphthalene	Insect repellent	*Muscodor vitigenus*	*Paullinia paullinioides*	Daisy et al., 2002
N-(2-hydroxyphenyl) acetamide, N-(2-hydroxyphenyl) malonamic acid	Bioremediation	*F. tabacinum, F. sambucinum, Plectosporium cibotii,*	*Aphelandra tetragona*	Zikmundov et al., 2002
1,3-dihydro isobenzofurans	Antioxidant	*Pestalotiopsis microspora*	*Terminalia morobensis*	Harper et al., 2003
3-Hydroxypropionic acid	Nematicidal	*Phomopsis phaseoli*	*Betula pendula*	Schwarz et al., 2004
Mevinic acid	Antiinflammatory	*Phomopsisspp.*	*Erythrina cristagalli*	Weber et al., 2004
Graphislactone A	Antioxidant	*Cephalosporium* sp. IFB-E001	*Trachelospmum jasminoides*	Song et al., 2005
Ergosterol, Cerevesterol	Antimicrobial	*2L-5*	*0cimum basilicum*	Md. Aminul Haque et al., 2005
Camptothecin	Anticancer	*Entrophospora infrequens*	*Nothapodytes foetida*	Puri et al., 2005
Radicicol	Cytotoxic	*Chaetomium chiversii* C5-36-62	*Ephedra fasciculata*	Turbyville et al., 2006
Isoflavonoids	Antimicrobial	*Phomopsis* sp.	*Erythrina crista-galli*	Redko et al., 2006
Podophyllotoxin	Anticancer	*Trametes hirsute,*	*Podophyllum Hexandrum*	Puri et al., 2006
Pestasol	Insect repellent	*Periconia, Stenella, Drechslera*	*Azadirachta indica A. Juss*	Verma et al., 2007
Taxol	Anticancer	*Phyllosticta* sp.6	*Ocimum basilicum*	Gangadevi 2007
Caryophyllene, phenylethyl alcohol, 2-phenylethyl ester,bulnesene	Antibiotic	*Muscodor albus* E-6	*Guazuma ulmifolia*	Strobel, 2007; Kluck et al., 2007
Taxol	Anticancer	*Colletotrichum gloeosporioides* (strain	*usticia gendarussa*	Gangadevi & Muthumary, 2008

		JGC-9)		
Cochlioquinone A, Isocochlioquinone A	Anti- parasitic	*Cochliobolus* sp. (UFMGCB-555)	*Piptadenia adiantoides J.F. Macbr*	Campos et al. (2008)
7-amino-4-methylcoumarin	Antimicrobial	*Xylaria* sp.YX-28	*Ginkgo biloba L.*	Liu et al., 2008
Javanicin	Antibacterial	*Chloridium* sp.	*Azadirachta indica A. Juss*	Kharwar et al., 2008
Phomoenamide, Phomonitroester	Anti–tubercular	*Phomopsis* sp. PSU-D15	*G. dulcis*	Rukachaisirikul et al. (2008)
2,6-dimethyl, 3,3,5-trimethyl; cyclohexene, 4-methyl; decane, 3,3,6-trimethyl; and undecane, 4,4-dimethyl (Volatile hydrocarbons)	Biofuel	*Gliocladium roseum* (NRRL 50072)	*Eucryphia cordifolia*	Strobel et al., 2008
Cochliodinol isocochliodinol	Cytotoxic	*Chaetomium* sp.	*Salvia officinalis*	Debbab et al., 2009
Camptothecin, (9-methoxycamptoth ecin, 10-ydroxycamptothecin	Anticancer	*Fusarium solani*	*Camptotheca acuminate*	Kusari, 2009
Taxol	Anticancer	*Fusarium solani*	*Taxus chinensis*	Deng, et al. 2009
Sesquiterpene aryl esters	Antimicrobial	*Armillaria mellea*	*Gastrodia Elata*	Li Wen Gao et al 2009
5-methoxy-7-hydroxyphthalide,(3R,4R)-cis-4-hydroxymellein	Antifungal	*unidentified Ascomycete*	*Meliotus dentatus*	Hussain, et al., 2009
3,4-dimethyl-2-(4´-hydroxy-3´5´ dimethoxyphenyl)-5-methoxy-tetrahydrofuran	Trypanocidal	*Phomopsis* sp.	*Viguiera arenaria*	Verza, et al., 2009
4- hydroxyphthalide,-5-methoxy-7-Hydroxyphthalide (3R,4R)-cis-4-hydroxymellein	Antialgal	*unidentified Ascomycete*	*Meliotus dentatus*	Hussain, et al., 2009
Terpenoid	Antimicrobial	*Phomopsis* sp.	*Plumeria acutifolia*	Nithya, 2010
Propanoic acid, methyl ester, 2-methylbutyl ester, ethanol.	Antibiotic	*Muscodor crispans*	*Ananas ananassoides*	Mitchell, et al., 2010
Phomoarcherins A–C	Antimalarial	*P. archeri*	*V. albindia*	Hemtasin, et al., 2011

2

MATERIALS AND METHODS

The present study was conducted on bioactive metabolites production from endophytic fungal isolates under lab conditions in Microbiology Labs Qauid-i-Azam University Islamabad.

Endophytic Fungal Isolates

A total of 07 Endophytic fungal isolates coded NFW1, NFW3, NFW6, NFW7, NFW9, NFW11 and NF3L2 were obtained from Department of Microbiology, Quaid-i-Azam University Islamabad as pure cultures. These strains were previously isolated by Dr. Nighat Fatima from different tissues of the ever green plant *Taxus fauna* (baccata) from Himalayan region of Pakistan. In the present study, the fungal isolates were screened for their ability to produce bioactive metabolites as well as for the production of commercially important enzymes. All these fungal isolates were preserved as pure cultures on Potato Dextrose Agar (PDA) slants at 4°C, in fungal bank of Microbiology Department Qauid-i-Azam University Islamabad. Pure cultures were maintained on PDA slants and plates. After revival and the confirmation that the strains were grown successfully again in pure form, these strains were screened for the production of bioactive metabolites as well as the production of commercially important extracellular enzymes.

Inoculum Preparation

The inoculum was prepared from 7 days old cultured PDA plates. Mycelia plugs of 3-5mm in size were cut off by sterilized fungal needle and point inoculated on the solid medium at center or two to three different locations of plate while 03 plugs were inoculated per flask in liquid medium.

Qualitative Screening for the Production of Enzymes

Qualitative analysis for the production of four commercially important enzymes including amylase, protease, lipase and cellulase was carried out by adding soluble or suspended substrates in solid medium (plate based assays) (cellulase: Lingappa and Lockwood, 1962; rest of the enzymes: Hankin and Anagnostakis, 1975), (Maria *et al*.,

2005). Two or three mycelia plugs of 3-5mm in size inoculated on plates containing specific medium and incubated in order to determine the production of respective enzyme.

Assay for Amylase Production

The production of amylase was determined by using specified amylase screening medium GYP (Glucose Yeast Peptone Agar medium) by adding 2% soluble starch as reported by (Maria *et al*., 2005). The composition of the medium is given in table 3.1.

Table 3.1: Composition of GYP Agar Medium

Media components	**Concentration**
Glucose	01g/l
Yeast Extract	0.1g/l
peptone	0.5g/l
Agar	1.6 to 2%

After preparation of the required medium pH was adjusted to 6 and then autoclaved. The autoclaved media was poured in sterilized petri plates. These plates were than inoculated with endophytic fungal isolates and incubated for 5 to 7 days at 25°C. After incubation the plates were flooded with the solution of 1% iodine in 2% potassium Iodide for few minutes. The clear zone around the colony shows the activity of amylase (Maria *et al*., 2005).

Screening for Protease Production

Protease production was determined by using GYP (Glucose Yeast Peptone Agar medium) amended with 0.4% gelatin as protein source (Maria *et al*., 2005). The composition of GYP was similar to that used in amylase assay.

Preparation of Gelatin

Gelatin 08g/100ml added in distilled water, its pH was maintained at 6 than separately autoclaved and kept as stock. 0.4% from the stock was added to the autoclaved GYP medium (pH 6) under sterilized conditions. The autoclaved media emended with gelatin was poured in sterilized plates. The plates were than inoculated by the endophytic fungal isolates and incubated for 5 to 7 days at 25°C. After incubation the plates were flooded with saturated aqueous solution of ammonium sulphate for 15 minutes. The clear zone around the colony shows protease activity (Maria *et al*., 2005).

Screening for Lipase Production

The production lipase was determined by using Peptone Agar medium as reported by (Maria *et al*., 2005). The composition of medium is given in table 3.2.

Table 3.2: Composition of Peptone Agar Medium

Media components	**Concentration**
peptone	10g/l
Nacl	5g/l
$Cacl_{2.}2H_2O$	0.1g/l
Agar	1.6 to 2%

Peptone agar medium was prepared, adjusted its pH to 6 and autoclaved. Separately autoclaved tween 80 with pH 6 added 1ml/100ml of peptone agar medium under sterilized conditions. The fungal isolates were inoculated on the plates as described earlier, and incubated for 5 – 7 days at 25°C. Clear zones around the colony show lipase activity (Maria *et al*., 2005).

Screening for Cellulase Production

In this assay the medium used was Peptone Agar medium supplemented with 0.5% sodium carboxymethyl cellulose (Maria *et al*., 2005). The composition of the medium is given in table 3.3.

Table 3.3: Composition of Yeast Extract Peptone Agar Medium

Media components	**Concentration**
Yeast Extract	0.1g/l
Peptone	0.5g/l
Agar	1.6 to 2%

Yeast Extract Peptone Agar medium was prepared, adjusted its pH to 6 and autoclaved. The sterilized medium plates were than inoculated with the fungal isolates according to the procedure described earlier and incubate for 5 to 7 days at 25°C. After incubation the plates were flooded with 1–2 % Congo red for a minute and destained the plates with 1M NaCl for 15 minutes. Clear zones around the colony show cellulase activity (Maria *et al*., 2005).

Evaluation of Fungal Isolates for the Production of Bioactive Metabolites

Primary screening for bioactive metabolites was carried out on the basis of production of extra cellular antibacterial and antifungal metabolites. The starter culture was taken from PDA plates as mentioned earlier and inoculated in SDB medium (250ml flask containing 100ml broth). The culture containing flasks were kept in shaking incubator (150rpm) at 25°C for 21 days. Analysis of fermentation broth for production of extracellular antibacterial and antifungal metabolites was checked at regular interval of 4^{th} day, 7^{th} day, 14^{th} day, and 21^{st} day of incubation against three Gram positive and two Gram negative bacterial and three fungal strains by well diffusion method.

Antibacterial Assay

Antibacterial assay was performed initially by well diffusion method Nithya and Muthumary, (2010) for fermentation broth and by disc diffusion method Baur, (2010) for the samples which were extracted by organic solvents.

Procedure

The test bacterial strains *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidus* (ATCC 12228), *Bacillus spizizenii* (ATCC 6633), *Escherichia coli* (ATCC 10536) and *Salmonella typhimarium* (ATCC 14028) were refreshed overnight at 37°C by inoculating them on nutrient agar plates. The overnight fresh bacterial cultures were used to prepare bacterial suspensions for lawn formation. Bacterial suspension is prepared by adding refreshed test bacterial strains into sterilized normal saline making its turbidity equal to 0.5 McFarland solution in order to get 10^6 CFU per ml. The antibacterial assay was conducted on Muller Hinton Agar (MHA) by pouring 01ml prepared bacterial suspension on sterilized petri plate containing (MHA). A uniform bacterial lawn is prepared by using sterilized cotton swabs. Wells are made at appropriate distance from each other and from the walls of plate by using a sterilized cork borer of 07mm in diameter, and the wells bottom were sealed by pouring a drop of sterilized MHA with the help of a micropipette. Plates were labeled and each well was then loaded with 100µl of sample using micropipettes and allowed two hours without shaking so that sample can diffuse and little shaking can't effect it. After two hours the plates were incubate at 37°C for 24 hours, the antibacterial activity was determined by the formation of clear zones around the wells and the diameter of zones were measured in mm.

Antifungal Assay

Antifungal assay was also performed by well diffusion method for fermentation broth and by disc diffusion method for the samples which were extracted by organic solvents.

Procedure

Spores suspension of fungal strains obtained from first fungal bank of of pakistan (FCBP) *Aspergillus Niger* (0198), *Aspergilus Fumigatus* (66) and *Mucor* Sp (0300) were prepared and 01ml spore suspension was used to make a uniform fungal lawn on PDA plates by using sterilized glass rods. The rest of procedure was same as antibacterial only they are kept at 30°C and the result were noted after 48 hours.

Antioxidant Assay

Free radical scavenging activity of the samples was determined by antioxidant assay using DPPH with slight modification in the method reported by Brand-Williams *et al.*, *(1995).*

Procedure

The DPPH stock solution was prepared by adding 10mg DPPH into 95% ethanol. The working concentration was prepare by diluting the ethanol until its absorbance reaches to 0.980 (±0.02) at 517 nm (control absorbance) using the spectrophotometer. Now 03ml of the working solution is mixed with 100µl of sample dissolved in 95% ethanol at various concentrations in test tubes. The solution was shaken well and incubates for 15 minutes in dark at room temperature. After incubation the absorbance was again taken at 517nm. Ascorbic acid was used as positive reference. The free radical scavenging activity was determined by the following equation.

$$\% \text{ Scavenging} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Cytotoxic Assay

Cytotoxicity of the organic crude extracts was determined by Brine Shrimp Assay as reported by (Maridass, 2008).

Procedure

Artificial sea water was prepared by dissolving 34g of commercial sea salt in 01 liter of distilled water. Brine Shrimp (*Artemia salina*) eggs were hatched in prepared artificial sea water in a shallow rectangular dish containing two separate compartments interlinked with each other by small channels. The eggs 15-20mg was sprinkled in a compartment which was kept in dark, while the other compartment kept under illumination. After 24 hours phototrophic nauplii (Brine Shrimp larvae) were collected by Pasteur pipette from the lightened side.

Transparent glass vials were used in the assay which was half filled with 5ml of artificial sea water along with 10 viable larvae. The organic extracts were dissolved in DMSO and add 100µl into the vial containing larvae and make its total volume up to 10ml by adding artificial sea salt solution. The final concentration of the sample used in assay was 200µg/ml (obtained by adding 02mg sample in 100µl of DMSO). The experiment was performed in triplicate. The vials were kept under light/lamp at temperature 25-28°C and results were noted after 24 and 48 hours by counting the larvae in the vials with magnifying glass. LD_{50} was calculated by using finni software.

Optimization of Different Operational Parameters

Two endophytic fungal isolates coded NFW3 and NFW9 were selected on the basis of screening results for optimization of different operational parameters which are effecting the production of bioactive metabolites. Four different parameters were studied by the strategy one parameter at a time by keeping all the other factors uniform. These operational parameters were

- Incubation time
- Media
- Temperature
- pH

Incubation Time

Effect of Incubation time on the production of bioactive metabolites was studied by growing the fungal strains in 250ml Erlenmeyer flasks containing 150ml SDB for different time duration. Cell free fermentation broth was obtained by centrifugation of a small proportion (2ml) of broth at 10000rpm at 4°C for 10 minutes. The cell free fermentation Broth was checked for the production of antibacterial and antifungal metabolites at regular interval of 4^{th} day, 7^{th} day, 14^{th} day, and 21^{st} day by well diffusion method against test bacterial and fungal strains to determine the optimum incubation time, by keeping all the other conditions same. The fungal isolates were kept at shaking conditions 150 rpm in shaking incubator at 25°C.

Media Optimization

Mineral salt media TM Xu *et al.,* (2008) and SDB were used in this context to check out the effect of media on the production of bioactive metabolites. The cell free fermentation broth after optimum incubation time was analyzed for antibacterial and antifungal assay. All other conditions were at optimum value. The composition of TM media is given in table 3.4.

Table 3.4: Composition of TM Media

Contents	**Composition (g/L)**
sucrose	40g
Ammonium sulphate	3g
Potassium dihydrogen phosphate	2g
Yeast extract	0.8g
Sodium chloride	0.6g
peptone	0.5g
Magnesium sulphate heptahydrate	0.5g
Sodium acetate	0.5g
Sodium benzoate	0.1g
phenylalanine	0.01g

Temperature

The fungal isolates cultured in optimum media (SDB) were kept in shaking incubators set at different temperatures ranging from 15°C to 35°C. These temperatures include 15°C, 20°C, 25°C, 30°C and 35°C. Fungal isolates were kept at different temperatures until for their optimum incubation time. The cell free fermentation broth of fungal isolates incubated at different temperature was checked after their optimum incubation time for its antibacterial and antifungal activity by well diffusion method to determine the optimum temperature for the production of bioactive metabolites.

pH

The appropriate pH for the better production of antibacterial/antifungal metabolites was analyzed by growing the fungal isolates in optimum media at different pH (5, 6, 6.5, 7 and 8) in shaking incubator 150rpm at optimum temperature for optimum incubation time at optimum temperature. All the other conditions were same. The fermentation broth from each pH was then checked for antibacterial and antifungal activity by well diffusion method and the maximum activity shows the optimum pH value.

Protein Precipitation of Cell Free Fermentation Broth of NFW9

100ml of fermentation broth of NFW9 was taken after incubation period at optimized conditions and its protein precipitation was carried out by ammonium sulphate precipitation method.

Ammonium sulphate was grinded in a grinder because powder form is more easily soluble. The protein precipitation was carried out at 4°C to avoid protein degradation on magnetic stirrer plate. The broth was taken in a 250ml flask and placed in a beaker containing ice around the flask to maintain the temperature at 4°C. Than ammonium sulphate was gradually added (1– 3mg at a time) to the fermentation broth after small intervals of time so that the already added ammonium sulphate dissolves completely. Protein precipitation was carried out by using different ammonium sulphate concentrations starts from 20% up to 100% by a regular increase of 10%. After each

concentration the broth was collected and centrifuged at 10000rpm for 15 minutes at 4°C to check either the precipitates are formed or not.

The calculation for ammonium sulphate concentration for precipitation of protein was measured with the help of online ammonium sulphate calculator designed by Encor Technologies.

Organic Extraction

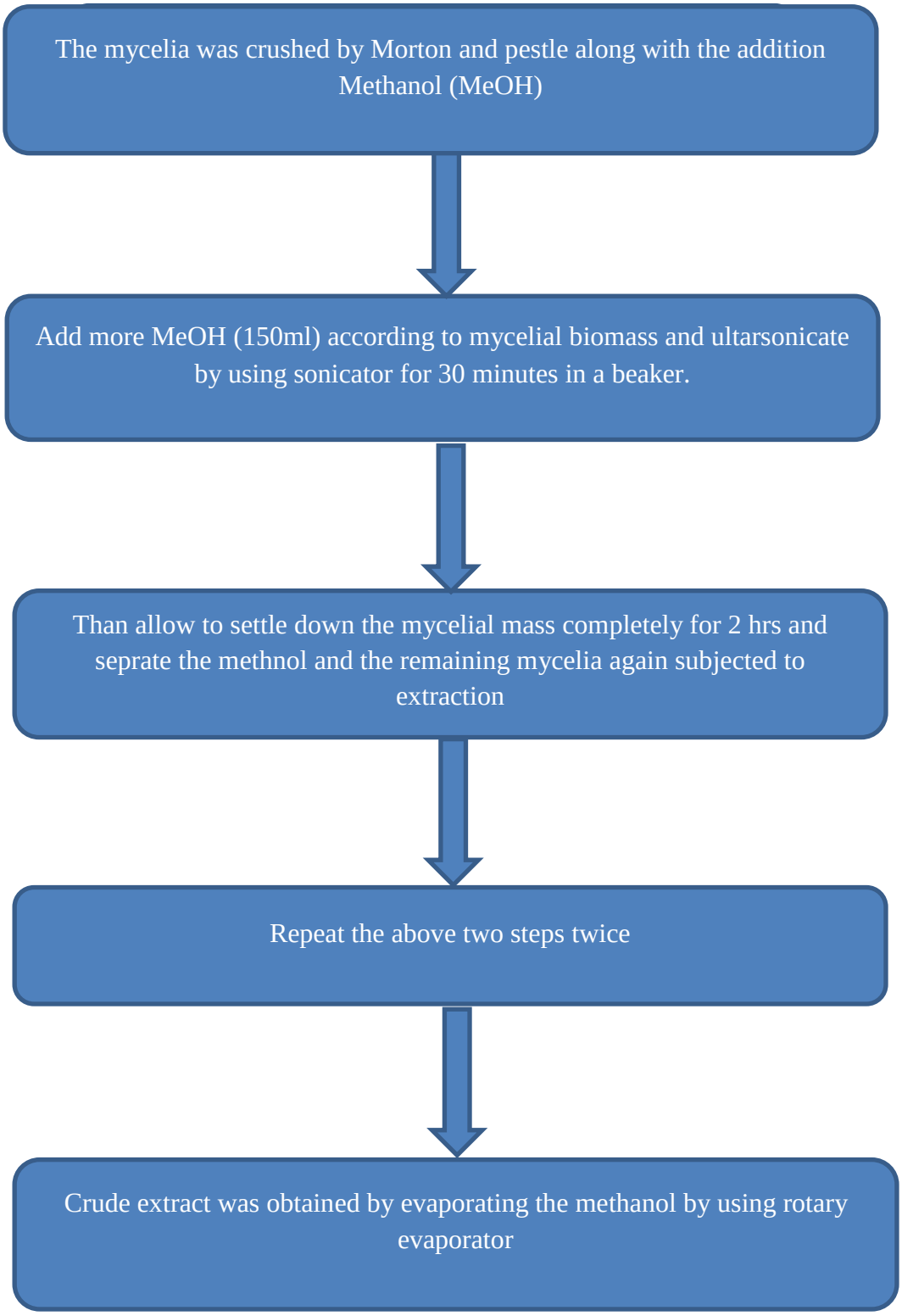
Organic Extract of both the fermentation broth and mycelia was done by using different organic solvents. Mycelial extraction was done by Methanol, while broth extraction was carried out by Ethyl acetate and Butanol. Mycelial biomass was separated from fermentation broth with the help of sterilized filter paper Whattman No.4 and the broth was collected in a sterilized container. The mycelium was dried under room temperature by using sterilized filter paper under sterilized conditions. The fermentation broth was further centrifuge at 14000rpm for 10 minutes to completely remove the biomass and subjected to organic extraction.

Organic Extraction of Broth

Organic extraction of broth was performed as

- 150ml optimized cell free fermentation broth was treated three times with equal amount of ethyl acetate in a separating funnel.
- Every time the mixture was vigorously shaken and then allows forming two separate layers.
- The separated layers were carefully collected in containers and the layer containing organic extract was dried using rotary evaporator in order to evaporate the organic solvent.
- The crude extract was collected in a glass vial and labeled for further analysis.
- The remaining aqueous phase was subjected treat with butanol three times in the same way as already discussed.

Organic Extraction from Mycelia



Thin Layer Chromatography

Thin layer chromatography is a technique used to separate various organic compounds. It is also used to identify a compound by calculating its R_f value and comparing it with the R_f value of a known compound by running both on the same TLC plate. Normal phase TLC plates (Silica coated aluminum plates, silica gel 60 F254, MERCK Germany) were used in this study.

Procedure

TLC plate consist of a stationary phase usually silica gel coated on glass or any inert matter and a mobile phase is usually mixture of miscible solvents. The plate can be divided into different sizes according to need. The sample (organic extract) dissolved in appropriate organic solvent, spotted by glass capillary tube on TLC plate at suitable distance from the bottom to avoid mixing of spot directly with mobile phase in TLC tank. The Plate was labeled with the help of a lead pencil and placed in transparent glass tank with a lid containing a filter paper almost half of the length of glass tank to provide same amount of vapours inside the tank space. This tank should be filled with appropriate solvent so that the spot should not touches the surface of solvent. Allow the TLC plate to develop until its mobile phase reaches almost near to the top (solvent front). Remove the plate from the tank and rapidly mark the top where the mobile phase reached in order to calculate the R_f value (distance travelled by compound divided by the distance travelled by mobile phase). The TLC plate is placed horizontally and allowed to dry and then visualized under UV light of 254nm for fluorescence quenching spots and 365nm wave length for fluorescent spots.

Bioautography

It is technique to detect the antibacterial/antifungal activity of pure or partially pure compounds separated in the form of bands or spots on TLC plate. Bioautography was conducted with slight changes as reported by (Kalinak *et al., 2013*).

Procedure

The TLC plate developed in a suitable mobile phase was placed over MHA and PDA agar plate containing a uniform lawn of test bacterial/fungal strain. Spots visible on TLC plate were marked on another similar TLC plate for comparison so that it can be compared with the TLC plate placed on MHA/PDA agar plate to detect the activity of bioactive compounds on the plate. After half hour remove the TLC plate carefully and the petri plate is allowed to incubate for 5 to 15 hours. After incubation the petri plate was observed for the zone of inhibition (Kalinak *et al., 2013*). The compounds from TLC plate diffuse into the agar medium and if they possess inhibitory activity they will produce a zone of inhibition. The zone of inhibition is noted and corresponding spot on TLC plate was marked.

Fourier Transform Infrared Spectrometers (FTIR)

In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. To analyze the various groups present in the crude extract of different media, FTIR was done by Perkin Elmer spectrum 65, FTIR spectrophotometer equipped with ATR. The sample (10mg) was placed on the sample plate over the crystal plate. Crude extracts of mycelia and broth were analyzed under the range of 600–4000 wave number/cm. the spectra obtained were analyzed with the known compounds spectra by labeling its peaks.

RESULTS

A total of Seven (07) endophytic fungal isolates isolated from *Taxus fuana* used in present study were screened for production of industrially important enzymes as well as production of bioactive metabolites.

Enzyme Assays

Qualitative analysis for the production of extracellular enzymes Amylase, Protease, Lipase, and cellulase was carried out by methods described earlier.

Test fungal isolates incubated for 5 to 7 days at room temperature (25°C) on specific medium until significant growth on plate appeared. The zones observed around the colony to determine the production of respective enzymes.

Although enzymes are not the primary objective of the study but for the sake of convenience they are written earlier so that the rest of results can be written in a sequence.

Table 4.1: Screening of endophytic fungal isolates for the production of different enzymes of commercial importance

Fungal Isolates	Enzymes			
	Amylase	Lipase	Protease	Cellulase
NFW1	-	-	-	-
NFW3	-	-	-	-
NFW6	-	-	-	-
NFW7	++	-	-	-
NFW9	++	-	-	+++
NFW11	-	-	-	-
NF3L2	+++	-	-	-

Results showed that three isolates have potential for enzyme production.

NFW9 isolate showed the production of amylase and cellulase while NFW7 and NF3L2 showed the production of amylase enzyme.

Results indicate that these three isolates could be potential source of commercially important enzymes.

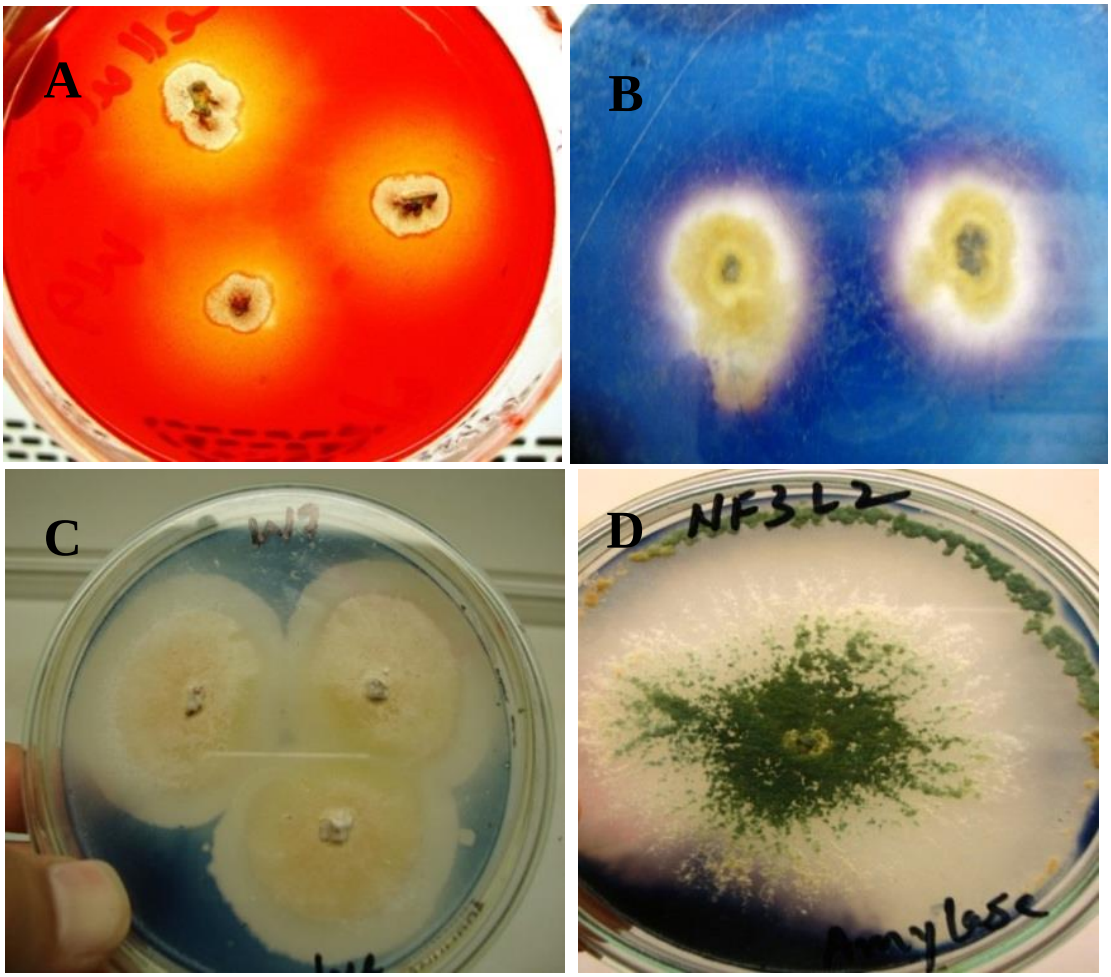


Figure 4.1: Enzyme Assay A: Cellulase activity by NFW9 B: Amylase activity by NFW9 C: Amylase activity by NFW7 D: Amylase activity by NF3L2

Screening of Endophytic Fungal Isolates for the Production of Bioactive Metabolites

All fungal isolates were incubated for liquid state fermentation by using SDB medium. The fermentation broth was tested at regular interval of four days, seven days, fourteen days and after twenty one days of incubation for the production of bioactive metabolites by using well diffusion method. All the isolates were checked against five (05) ATCC test bacterial strains (two Gram negative and three Gram positive) and three fungal strains two *Aspergillus* sp. and one *Mucor* species.

Only NF3L2 fungal isolate showed positive activity both antibacterial and antifungal after 7 days of incubation while rest of the isolates were negative so the table showing results after seven days of incubation was not shown. Majority of the fungal isolates showed optimum activity between 12^{th} to 15^{th} days of inoculation and also at 21^{st} day but the activity was milder.

Results indicate that two wood isolates NFW9 and NFW3 showed positive results in both antibacterial and antifungal assay against all tested strains between 14^{th} –21^{st} day of incubation. These isolates did not exhibit activity after seven days of incubation.

Antibacterial Activity at 14^{th} Day

Fermentation broth of NFW9 and NFW3 showed better results than the other fungal Isolates. The isolate NF3L2 which was active at 7^{th} day showed static activity at 14^{th} day. All other fungal isolates showed significant activity at 14^{th} day.

Table.4.2: Screening for Production of Antibacterial Metabolites by Different Endophytic Fungal Isolates at 14^{th} Day of Incubation

Fungal Isolates	Test Strains				
	E.coli (a)	*B.spizezenii* (b)	*S.aureus* (C)	*S.typhimarium* (d)	*S.epidermidis* (e)
NFW1	-	+	+	-	+
NFW3	+	++	+	++	+
NFW6	-	-	-	-	-
NFW7	-	+	+	+	-
NFW9	++	++	+++	++	+++
NFW11	-	-	-	-	-
NF3L2	+	+	+	+	+

+ (static effect), ++ (positive with a zone of inhibition of less than 8mm

+++ (positive effect with zone of inhibition of almost 8 or above 8mm

Antibacterial activity at 21st Day

Fermentation broth of fungal isolates incubated for 21 days showed milder activity (results shown in table) especially the isolates NFW9 and NFW3 which shows better activity at 14th day**.**

Table.4.3: Screening for Production of Antibacterial Metabolites by Different Endophytic Fungal Isolates at 21st Day of Incubation

Fungal Isolates	Test Strains				
	E.coli (a)	*B.spizezenii* (b)	*S.aureus* (c)	*S.typhimarium* (d)	*S.epidermidis* (e)
NFW1	-	-	+	-	+
NFW3	+	++	+	+	+
NFW6	-	-	-	-	-
NFW7	-	+	+	+	-
NFW9	+	++	++	+	++
NFW11	-	-	-	-	-
NF3L2	-	-	-	-	-

+ (static effect), ++ (positive with a zone of inhibition of less than 8mm

+++ (positive effect with zone of inhibition of almost 8 or above 8mm)

Antifungal activity at 14th Day

Fermentation broth of several Fungal isolates shows fungicidal activity at 14th day shown in table 4.4 among them NFW9 and NFW3 showed better activity than rest of all.

Table.4.4: Screening for Production of Antifungal Metabolites by Different Endophytic Fungal Isolates at 14th Day of Incubation

Fungal Isolates	Test Strains		
	A.niger	*A. fumigatus*	*Mucor spp.*
NFW1	++	+	+
NFW3	++	++	++
NFW6	+	-	-
NFW7	+	-	-
NFW9	+++	+++	++
NFW11	+	+	-
NF3L2	+	+	+

+ (static effect), ++ (positive with a zone of inhibition of less than 8mm)

+++ (positive effect with zone of inhibition of almost 8 or above 8mm)

Antifungal Activity at 21st Day

The antifungal activity at 21st day of many fungal isolates was less than the 14th day (data shown in table) especially the Fungal Isolates coded NFW9 and NFW3 which were more active at 14th day.

Table.4.5: Screening for Production of Antifungal Metabolites by Different Endophytic Fungal Isolates At 21st Day of Incubation

Fungal Isolates	Test Strains		
	A.niger	*A. fumigatus*	*Mucor spp.*
NFW1	+	+	+
NFW3	++	+	+
NFW6	+	-	-
NFW7	+	-	-
NFW9	++	++	++
NFW11	+	+	-
NF3L2	+	+	+

+ (static effect), ++ (positive with a zone of inhibition of less than 8mm)

+++ (positive effect with zone of inhibition of almost 8 or above 8mm)

Selection of Endophytic Fungal Isolates for Optimization Studies

Initial screening results reveal that NFW9 and NFW3 isolates could be potential source of antibacterial and antifungal compounds. Therefore these two isolates were selected for optimization of different operational parameters.

Effect of Media Composition on the Production Bioactive Metabolites

Two types of media Sabouraud Dextrose broth (SDB) and mineral salt medium (TM) were used in the study. Both of the endophytic fungal isolates cultivated on TM medium showed no or static activity against few test strains while on the other hand when these fungal isolates were grown on SDB exhibit antibacterial and antifungal activity under normal conditions results were described in table 4.2 and 4.4 respectively.

Effect of Incubation Time on the Production Bioactive Metabolites

Optimization of incubation time for the fungal isolates NFW9 and NFW3 was carried out on the basis of antibacterial and antifungal activity of their fermentation broth. Fermentation broth was regularly checked for its antibacterial/antifungal activity after 4^{th} day, 7^{th} day, 14^{th} day and 21^{st} day by well diffusion method. Both isolates showed observable activity at 14^{th} day of incubation. Largest zone of inhibition was observed against *S.aureous* among bacteria and *A. niger* among fungi by fermentation broth of NFW9. However the activity of fermentation broth was reduced at 21^{st} day of incubation against test bacterial and fungal strains. Overall results showed that both isolates produced optimum quantity of bioactive metabolites at 14^{th} day of incubation.

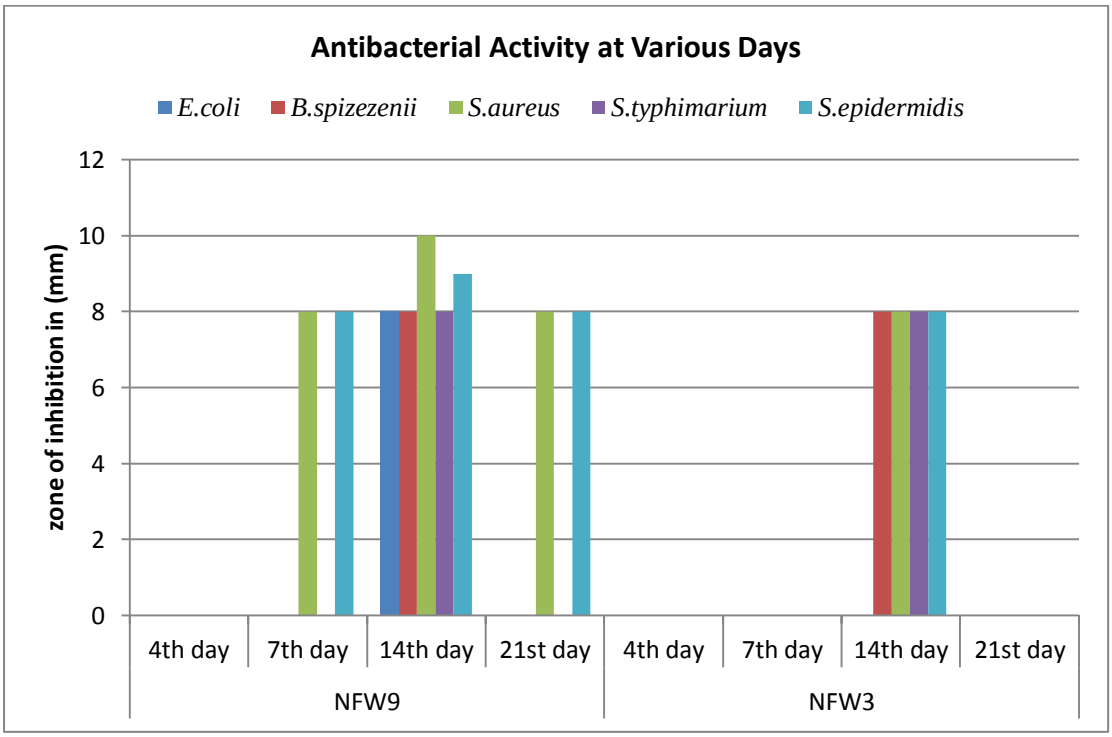


Figure 4.2: Antibacterial activity of fermentation broth of fungal isolates at various days

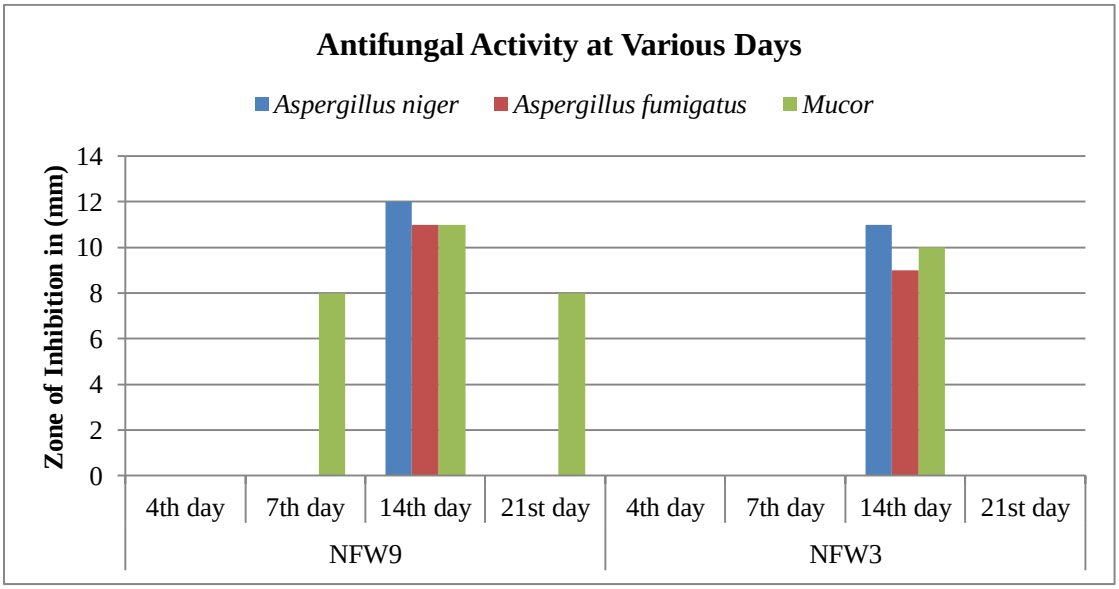


Figure 4.3: Antifungal activity of fermentation broth of fungal isolates at various days

Effect of Temperature on the Production of Bioactive Metabolites

To find out the optimum temperature, the fungal isolates were cultured at different temperature ranges i.e. 15ºC, 20ºC, 25ºC, 30ºC and 35ºC while all the other parameters were kept at optimum value. Minimum temperature used in this study for the production of bioactive metabolite was 15°C ±1°C. The fungal isolates at low temperature below than 15°C were grow slowly and produce less biomass as compared to other temperatures and no growth observed at 35ºC. Results indicate that NFW9 showed maximum zone of inhibition of 19mm against *S. aureus* and 20mm against *A. niger* when cultured at 15°C, this isolate showed significant activity against all the test bacterial and fungal strains at 15°C however the activity gradually reduced at 20ºC, 25ºC. While in case of NFW3 isolate there was no contrast difference of activity at 15°C, 20ºC, 25ºC. NFW3 showed moderate activity against all the test bacterial and fungal strains with zone of inhibition ranging from 09mm to12mm. Both isolates showed total loss of activity at 30ºC.

During temperature optimization one important aspect noted was the growth morphology difference and color change of fermentation broth which can attribute to the production of different metabolites at various temperatures.

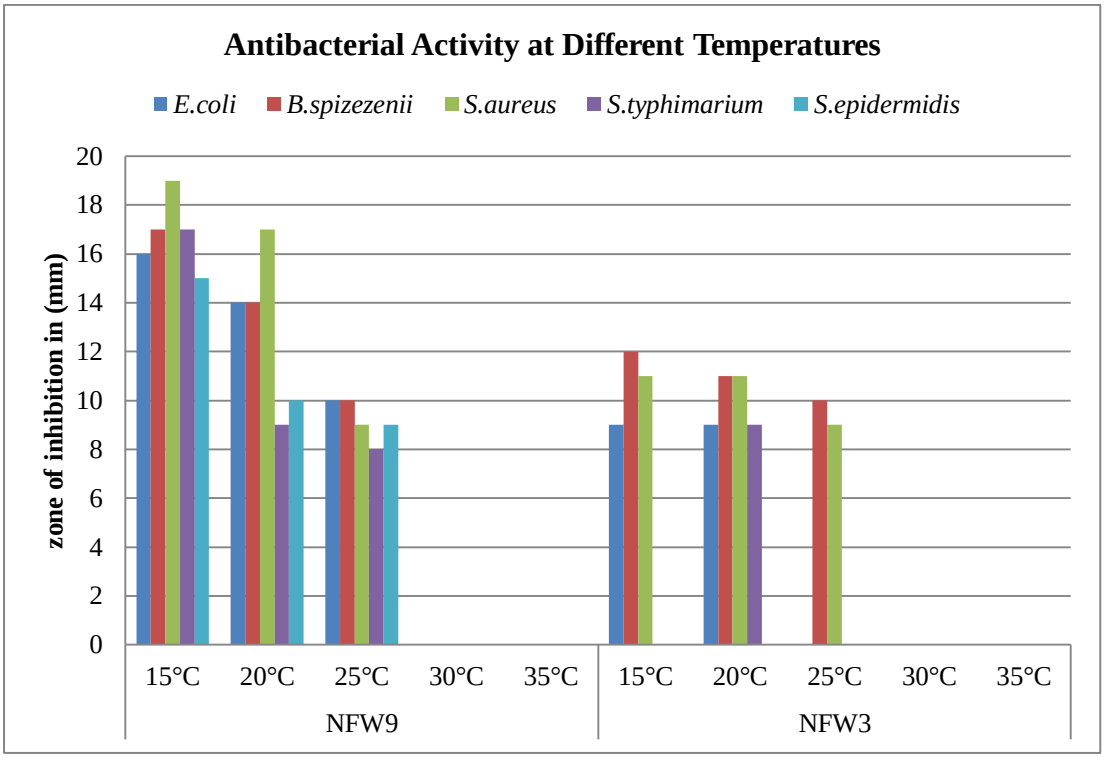


Figure 4.4: Antibacterial activity of fermentation broth of fungal isolates at various temperatures

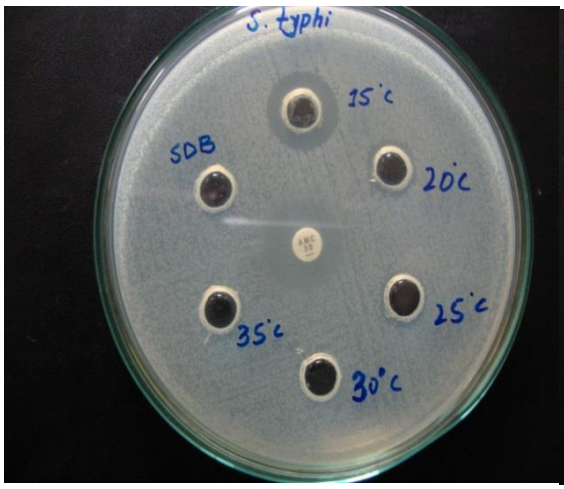


Figure 4.5a: Antibacterial activity at different temperatures

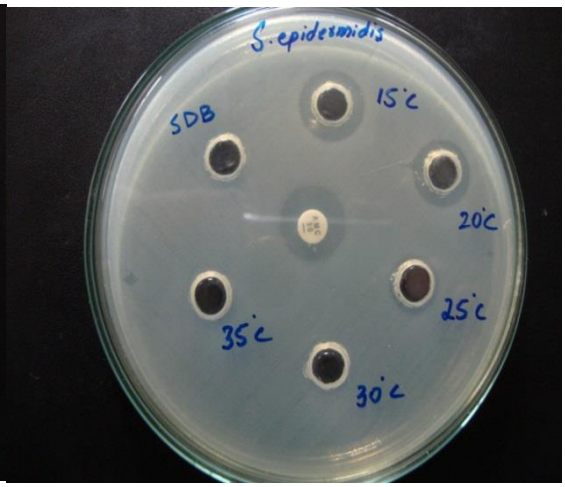


Figure 4.5b: Antibacterial activity at different temperatures

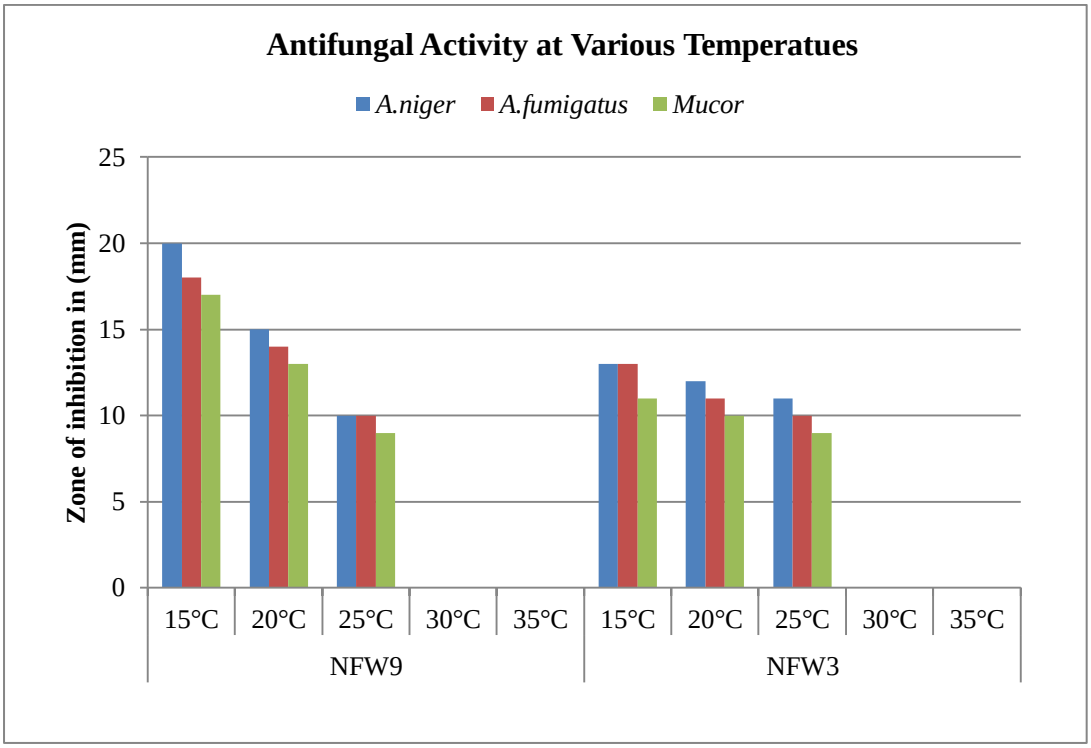


Figure 4.6: Antifungal activity of fermentation broth of fungal isolates at various temperatures

Growth Pattern of NFW3 and NFW9 at Various Temperatures

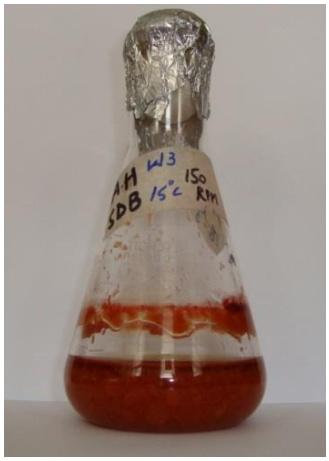


Figure 4.7a: NFW3 producing brown color at 15°C

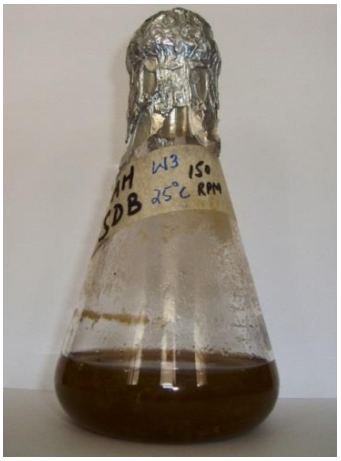


Figure 4.7b: NFW3 producing greenish color at 25°C

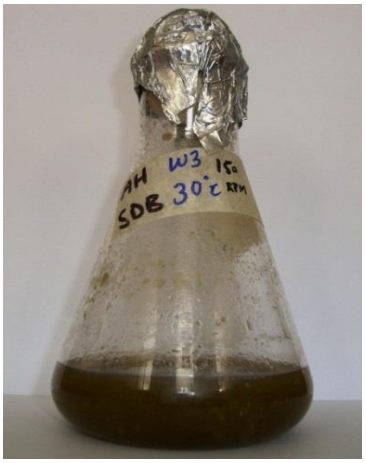


Figure 4.7c: NFW3 producing greenish color at 30°C

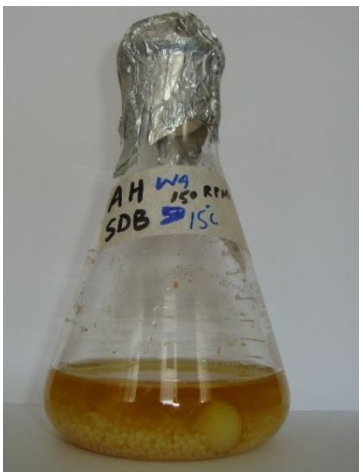


Figure 4.7d: NFW9 producing lemon color at 15°C

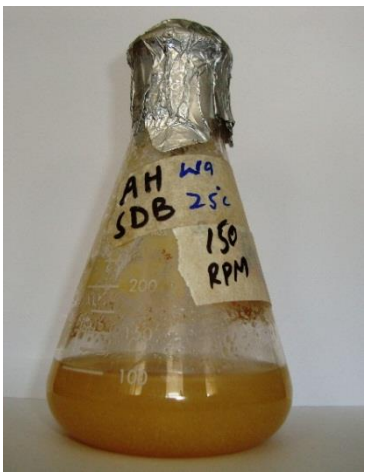


Figure 4.7e: NFW9 producing lemon color at 25°C

Figure 47f: NFW9 producing lemon color at 30°C

pH Optimization

After incubation time and temperature another important factor effecting the production of bioactive metabolites was pH. The fungal isolates were cultured in SDB broth of different pH values (5, 6, 6.5, 7, and 8) to determine the optimum pH value**.**

Results indicates that both isolates showed increase production of bioactive metabolites from pH value 6 to 7 while production reduced at pH value 8. Maximum zone of inhibition 19mm by fermentation broth of NFW9 was against *S. aureus* while NFW3 isolate exhibit maximum zone of inhibition of 13mm against both *S. aureus* and *B. spizezenii* at pH 6.5. However maximum zone of inhibition 21mm against test fungal strain was observed by NFW9 against *A. niger* while NFW3 exhibit 14mm zone of inhibition against *A. niger at* pH 7. These results revealed that both isolates grown at pH value 6.5 to 7 produced optimum concentration of bioactive metabolites.

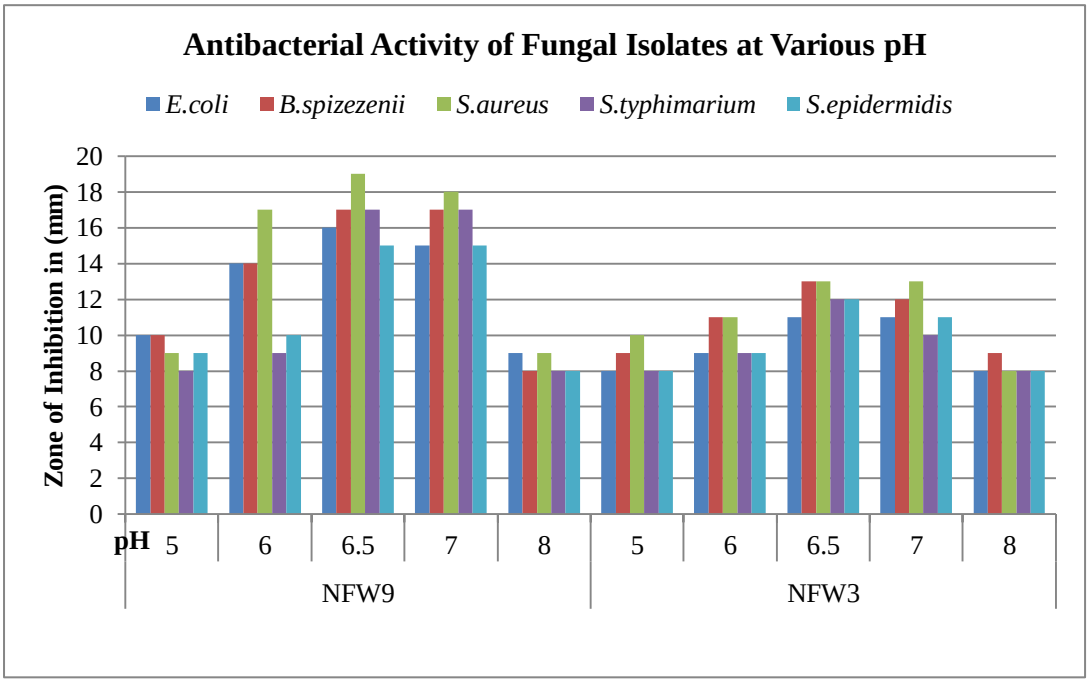


Figure 4.8: Antibacterial activity of fermentation broth of fungal isolates at various pH

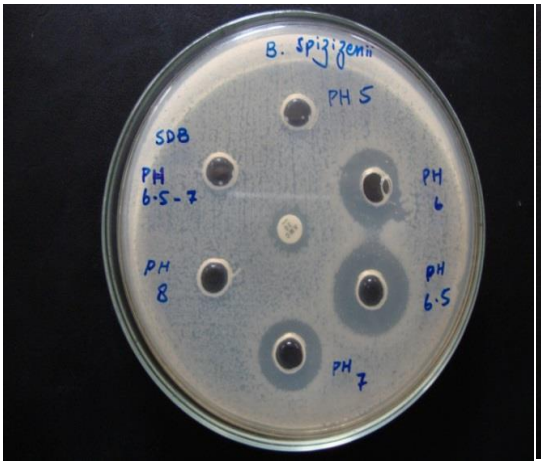


Figure 4.9a: Antibacterial activity at various pH values

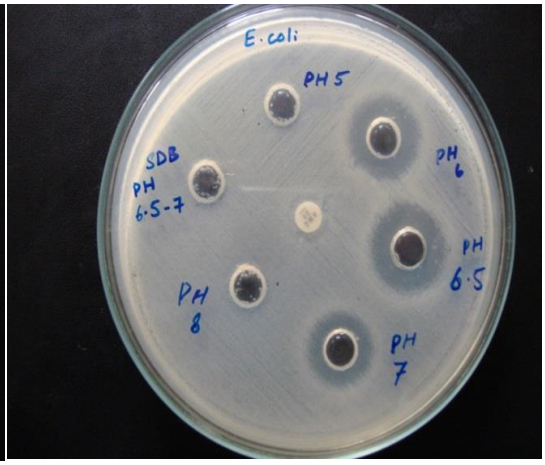


Figure 4.12b: Antibacterial activity at various pH values

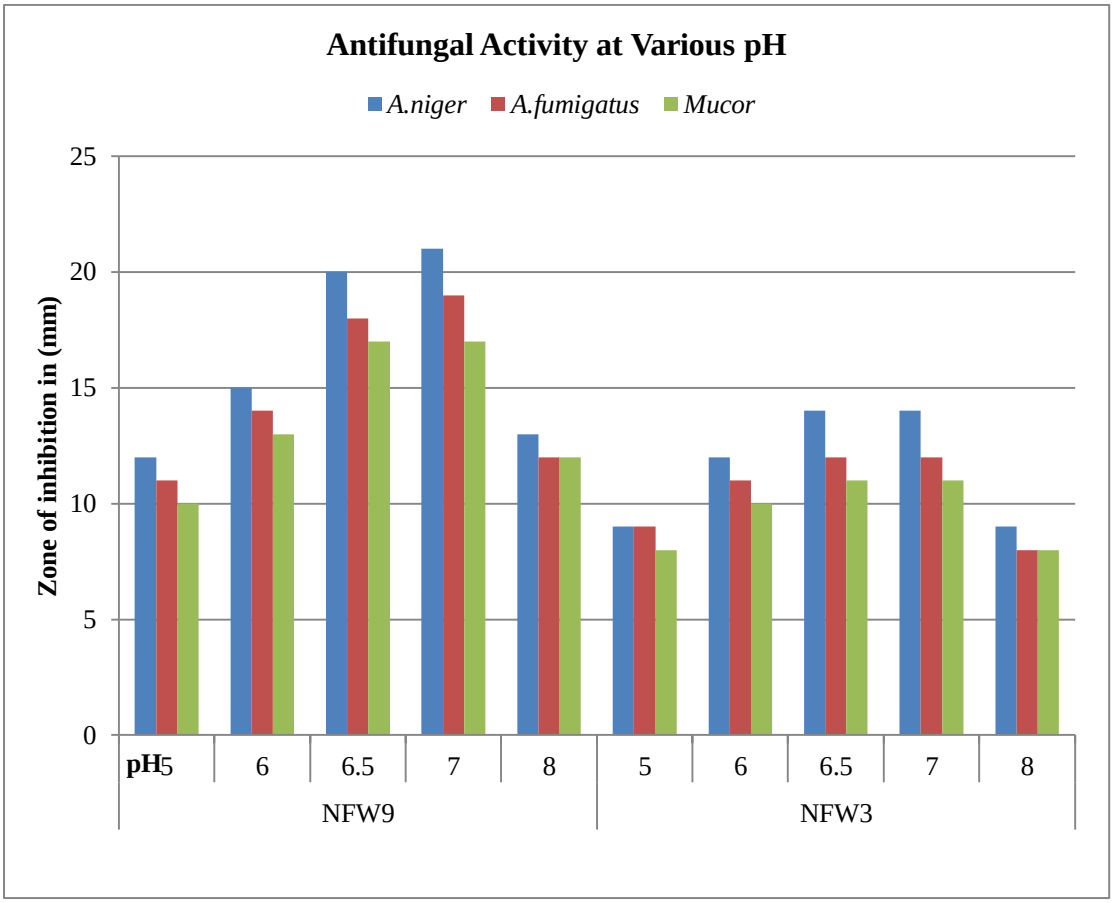


Figure 4.10: Antifungal activity of fermentation broth of fungal isolates at various pH

Fermentation and Organic Extraction

On the basis of better production of bioactive metabolites under optimized conditions NFW9 was further studied for bulk production and extraction of intracellular (extract from mycelia) and extracellular metabolites from fermentation broth. The extraction of fermentation broth was done by two methods i.e. organic extraction (by ethyl acetate and butanol) and protein precipitation (by ammonium sulphate) to obtain the preliminary information about the chemical nature of the bioactive metabolites.

Organic Extraction

After 14 days of incubation of NFW9 isolate in SDB medium under optimum conditions, the fermentation broth was filtered to separate mycelial biomass. The filtered fermentation broth was extracted three times with equal volume of ethyl acetate and dried in rotary to acquire organic crude extract. Crude ethyl acetate extract

10mg obtained from 200ml of fermentation broth. This extract was used against test bacterial and fungal strains by disk diffusion method as well as for TLC, FTIR analysis and also for bioautography. While mycelial mass was freeze dried and extract with methanol after grinding in mortar and pestle and dried by using rotary evaporator. Crude extract obtained was 25mg. This extract was subjected to the same analysis as like the extracts of fermentation broth.

Results of antibacterial and antifungal assay showed that ethyl acetate extract of fermentation broth was less active as compared to butanolic extract with a zone of inhibition ranging from 6.5 to 7mm in diameter at concentration of 1mg per disk (5mm diameter). But butanolic extract of fermentation broth showed significant zone of inhibitions ranging from 7.5 to 9mm at same concentration. While extract of mycelial mass showed lower activity as compared to fermentation broth, its zone of inhibition ranges from 7 to 7.5mm, indicating that potent antimicrobial metabolites were produced extracellular and suitable solvent for extraction is butanol.

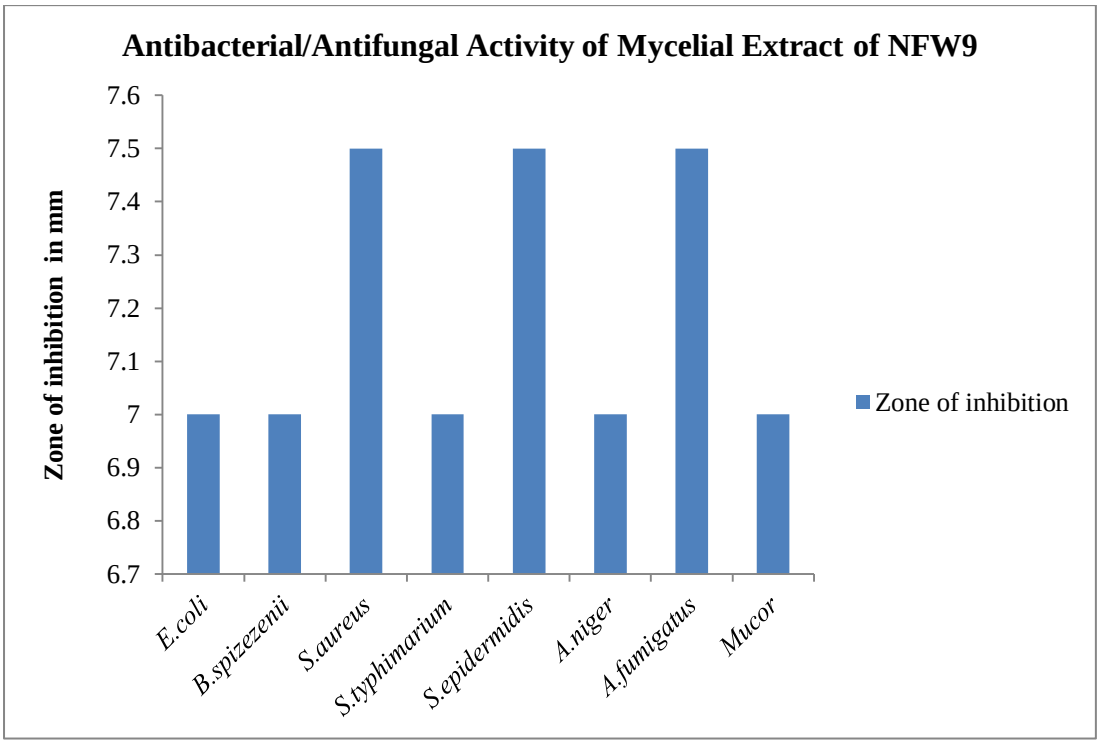


Figure 4.11: Antibacterial/antifungal activity of methanolic extract of mycelia of NFW9

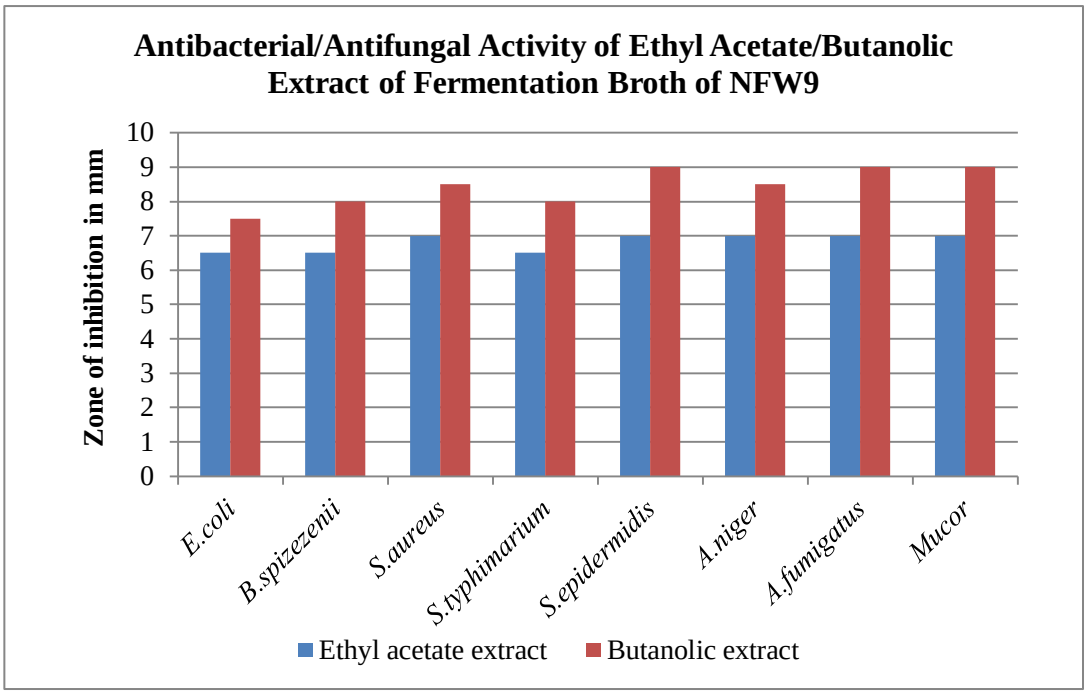


Figure 4.12: Antibacterial/antifungal activity of ethyl acetate/butanolic extract of fermentation broth

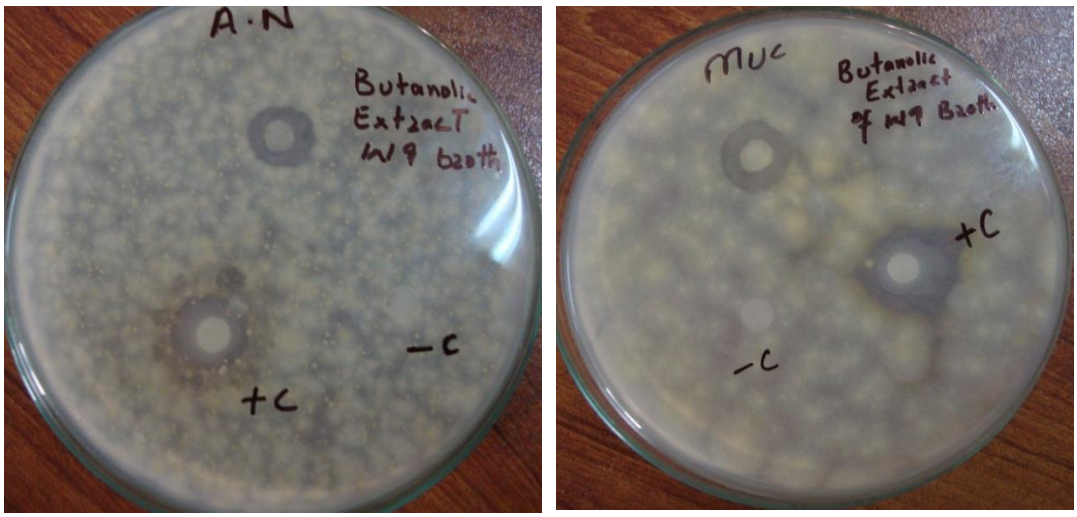


Figure 4.13: Antifungal Activity of butanolic extract of fermentation broth of NFW9

Cytotoxic Activity

The cytotoxic activity of the metabolites produced by NFW9 was studied through Brine Shrimp Assay. The fermentation broth, methanolic extracts of mycelial mass, ethyl acetate and butanolic extracts of fermentation broth all showed lack of cytotoxic activity.

Protein Precipitation

Protein precipitation of optimized fermentation broth was done to find out either the bioactive metabolites are peptidal or not it is observed that protein precipitates formed at two different concentrations of ammonium sulphate (calculated by using online $(NH_4)_2SO_4$ calculator Encor technologies) 60% and 80%. Both the precipitates were dissolved in phosphate buffer (pH7.5) separately and used for antibacterial and antifungal activity it was observed that they have no effect against any of the test bacterial and fungal strain. The combination of both precipitates was also checked to determine the synergistic effect but the results were the same. Results revealed that the protein precipitates were inactive against all the test bacterial and fungal strains. Therefore organic extract of fermentation broth and mycelia were used for further analysis.

Antioxidant Activity of Mycelial and Broth Extracts of NFW9

All the organic extracts were evaluated for their antioxidant potential. Results indicate that the antioxidant activity of butanolic extract was highest among all the extracts i.e. 53% of scavenging activity at 130µg/ml.

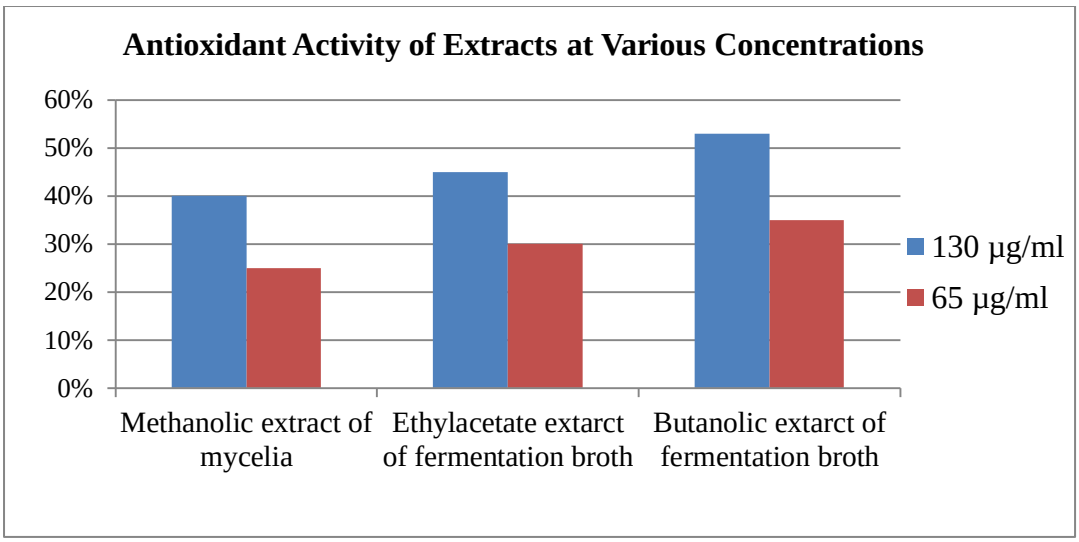


Figure 4.14: Antioxidant Activity of crude organic extracts of NFW9

FTIR of crude extract of fermentation broth and mycelia was performed by using Perkin Elmer spectrum 65, FTIR spectrophotometer equipped with ATR; while TLC was performed on silica coated aluminum foil plates by using different mobile phases.

Thin Layer Chromatography

TLC plates were developed with all three samples (organic crude extracts) by using different mobile phases and maximum separation of metabolites was observed. Spots were viewed under UV light at 366nm for fluorescent spots and at 254nm for fluorescence quenching spots.

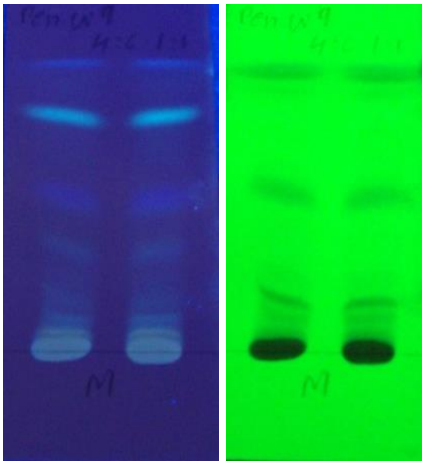


Figure 4.15a: TLC pattern of crude extract of mycelia under UV_{254} & UV_{366}

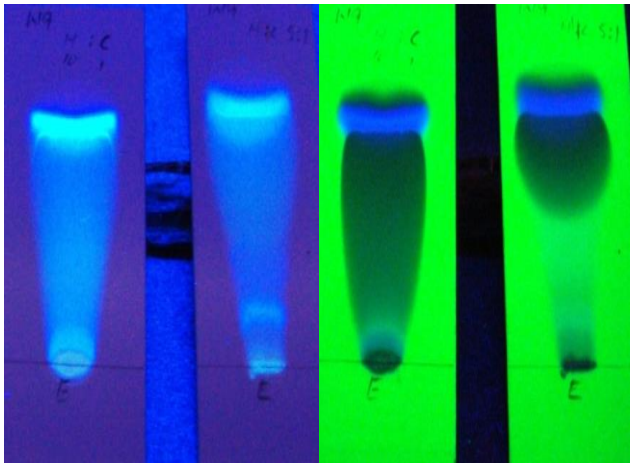
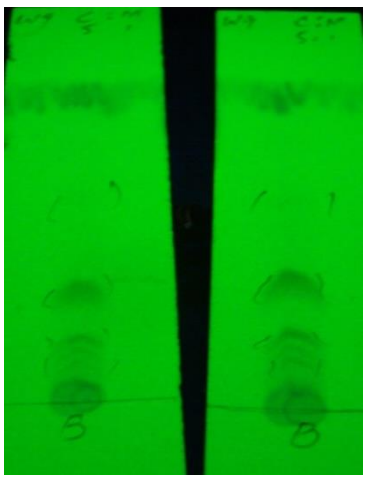


Figure 4.15b: TLC pattern of ethyl acetate extract of fermentation broth under UV_{254} & UV_{366}



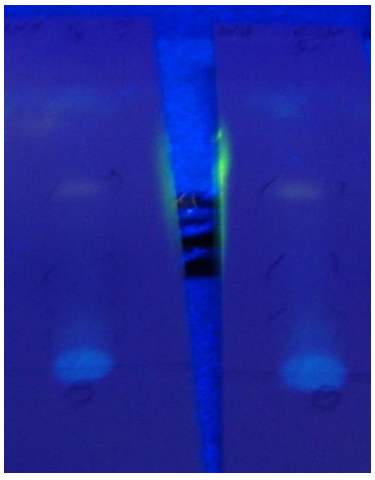


Figure 4.15c: TLC pattern of butanolic extract of fermentation broth under UV_{254} & UV_{366}

Staining of TLC Plates

The TLC plates are stained with Dragon drought reagent and with 15% H_2SO_4. The staining results showed that the TLC plates of all the extracts were Dragon drought negative while methanolic extract of NFW9 gained H_2SO_4 staining.

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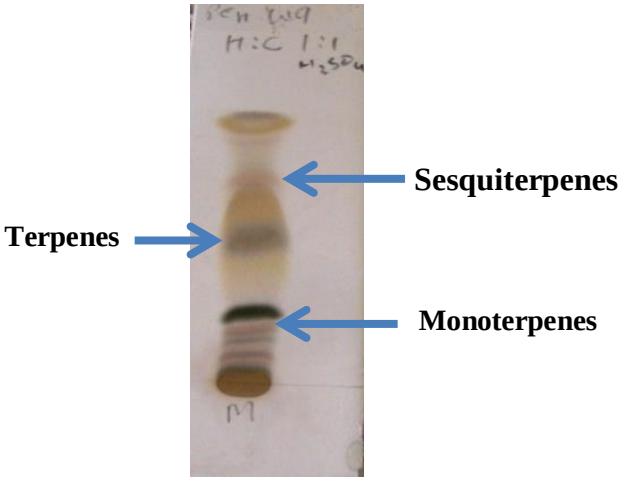

Figure 4.17: sulphuric acid staining

Bioautography

Bioautography of TLC plates revealed that two separate band on ethyl acetate and butanolic extract of fermentation broth showed antibacterial activity against test bacterial strains. The sulphuric acid staining of these TLC plates give information about presence of terpenic compounds. The compounds gain stain initially converted into pinkish colour and on continues heating colur changed into brown, yellow brown, and brownish green. Brown colour indicates the presence of sesquiterpenes while pink give idea about mono terpenes.

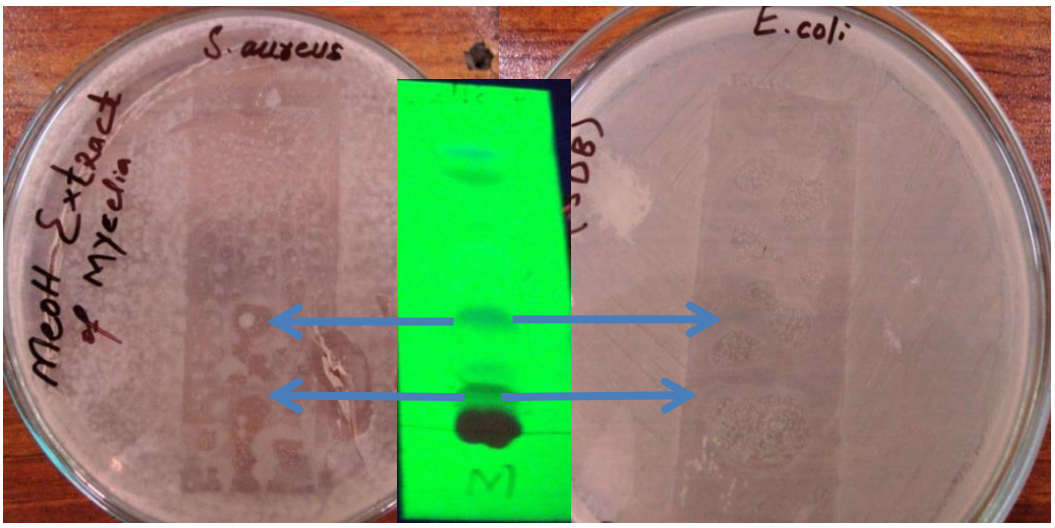

Figure 4.16: Bioautography of TLC plates

FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier Transform Infrared Spectroscopy (FTIR) results revealed the presense of some specific peaks indicating the presense of anthraquinone, azaphilones and terpenes. IR spectra of organic extracts also indicate the presense of functional groups like phenols, alkanes, alkenes, nitro compounds,esters,ketones, chloroalkanes and fluoroalkanes which may attribute to inhibitory activity against test microbial strains.

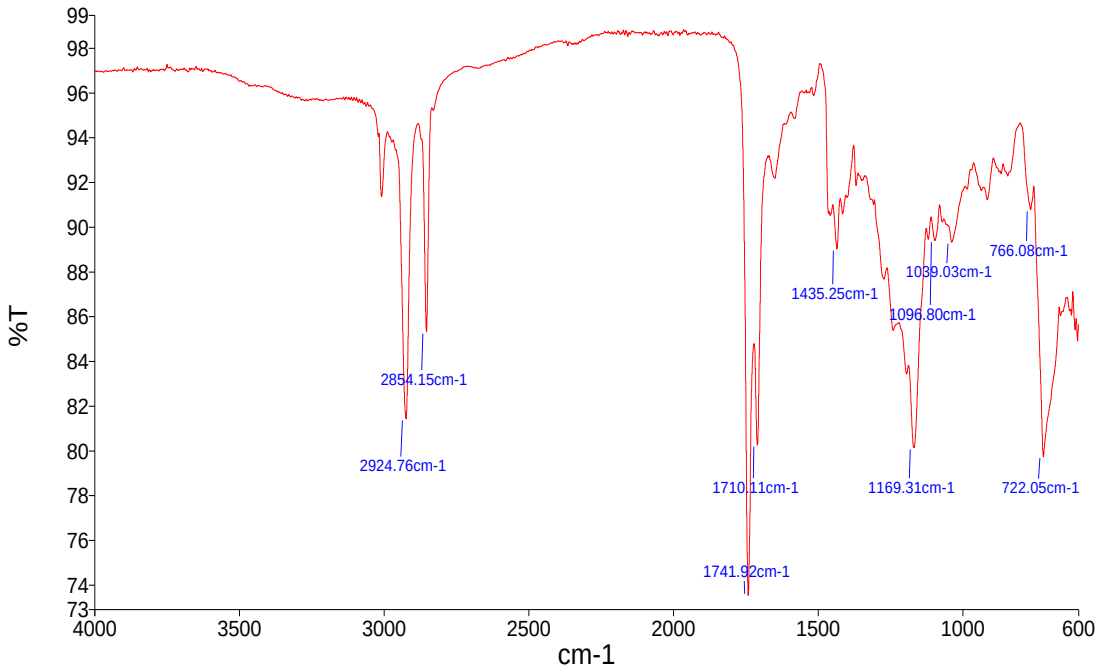

Figure 4.18: IR spectrum of mycelial extract of NFW9

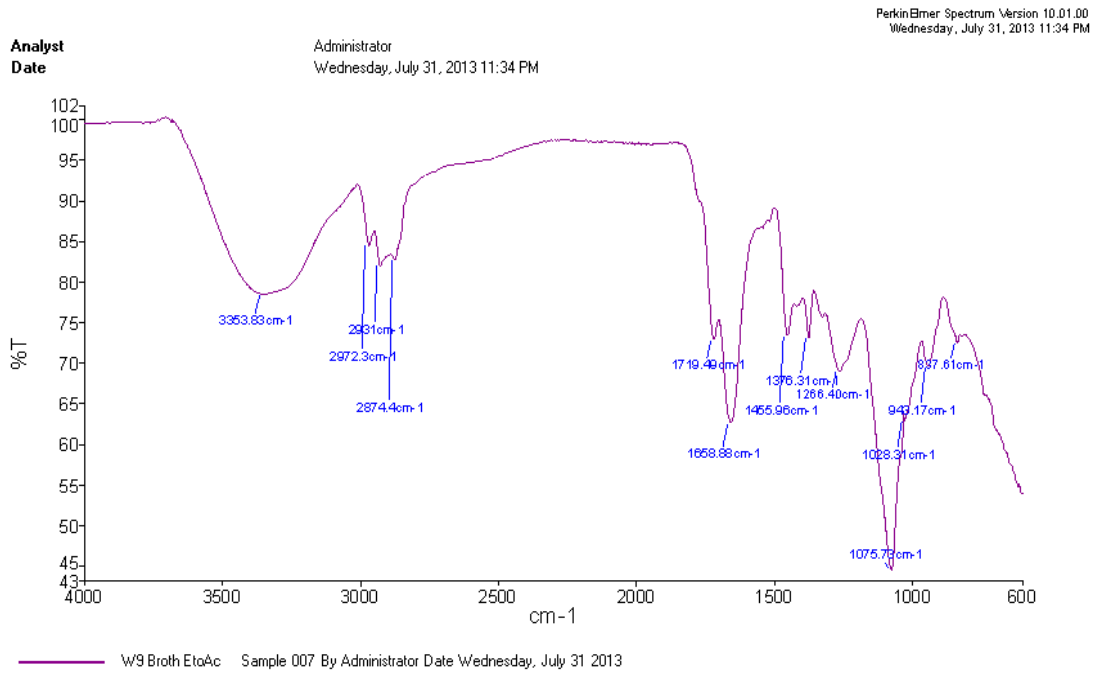

Figure 4.19: IR spectrum of fermentation broth extract of NFW9

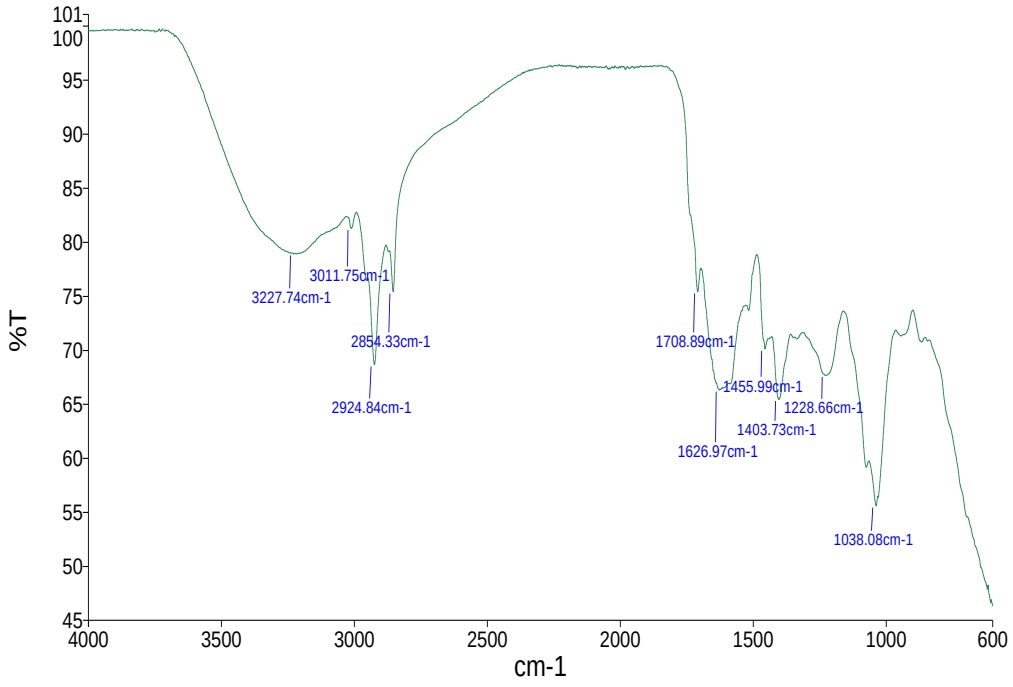

Figure 4.20: IR spectrum of fermentation broth extract of NFW9

DISCUSSION

Endophytic fungi are the organisms residing within the intracellular matrix of almost every plant species on earth without causing any apparent harm to the host. This group of microflora is considered untapped due to their wide range and the hard ecological niches of their hosts. After the discovery of taxol production from endophytic fungi several studies regarding the production of bioactive metabolites had been conducted with promising results. The present study aimed at the production of bioactive metabolites and commercially important extracellular enzymes.

Production of Antimicrobial Metabolites

Endophytic fungi especially those from medicinal plants are excellent source of antimicrobial substances. They produce novel and potent antimicrobial substances which can be leads to produce drugs particularly against resistant microbial strains.

The results of present study showed that all the fungal isolates produced antifungal compounds and five isolates among 07 showed antibacterial activities against test bacterial strains. The antimicrobial metabolites of endophytic fungi have been studied by other different research groups (Gopinath *et al*., 2013; Idris *et al.*, 2013).

k. Tayung and D. K. Jha, (2010) studied 77 endophytic fungal isolates from the inner bark of *Taxus baccata L*. The most frequently found genra includes *Aspergillus, Fusarium and Penicillium.* Ethyl acetate extracts of fermentation culture of 15 isolates shows activity against test bacteria (Gram positive and Gram negative) and Fungi. One of the isolates identified as *Fusarium* sp. show inhibition of all the test bacterial and fungal strains.

The results are in accordance with Gopinath *et al*., (2013) who reported antimicrobial activity of *Phomposis* sp. Isolated from different medicinal plants, after cultivation for 21 days and the culture filtrate was extracted with ethyl acetate to screen their extracellular metabolites for antibacterial activity against three Gram negative and three Gram positive bacteria including, *Escherichia coli, Pseudomonas aeruginosa, Klebsiella Pneumoniae, Micrococcus luteus, Bacillus subtilis,* and *Staphylococcus aureus* respectively. The *Phomopsis* sp.2 isolated from *Guazuma ulmifolia* was positive against all the test bacterial strains.

In another study S.k Jena and K. Tayung, (2013) isolated 458 endophytic fungal isolates from different parts of *Solanum rubrum* and *Morinda pubescence.* The fungal genras frequently found were *Colletotrichum* and *Aspergillus.* The endophytic fungal isolates were grown in PDB at 25°C for 15 days. The cultural filtrate was than screened for antibacterial and antifungal activities against various test strains by well diffusion method (7mm Wells loaded with 100µL sample). Among them 20% of the isolates show activity against Gram positive, 10% was active against Gram negative and 28% inhibit the growth of test fungal strains. Zone of inhibitions ranges from less than 10mm to greater then 15mm. This is coherent with the results of present study where improved antimicrobial activity was found under similar experimental conditions.

Idris *et al.*, (2013) studied 07 endophytic fungal strains to check out the extracellular antibacterial metabolites produced by them. The ethyl acetate extract of all the 07 endophytic fungal isolates was positive against the three test bacterial strains. Similar results were deduced from the proposed study where more than 70% isolates demonstrate antibacterial activity against test bacterial strains. Similarly Ramesha *et al*., (2013) studied 28 fungal isolates from different tissues of *Nerium oleander* and check their antimicrobial activity against bacteria and fungi. Of the isolates 36% shows antimicrobial activity against the test strains. In the present study 71% isolates were positive against test bacterial while 100% positive against test fungal strains. The ability of endophytic fungi to produce bioactive metabolites is highly dependent on the host plant and the conditions where the host inhabited. This might be a reason for the better production of bioactive secondary metabolites.

The results of our study are in agreement with Pavithra *et al., (*2012). This study reports screening of fungal isolates of *Tulsi* plant and screened them for the production of bioactive metabolites (antibacterial and antifungal) and enzymes. Of the isolates 06 showed an inhibitory activity against all the test bacterial and fungal strains with inhibitory zone of 12 mm to 20 mm in diameter. Bharathidasan. R and Panneerselvam. A, (2012) studied endophytic fungal sp. *Penicillium janthinellum, A. conicus and phomosis sp.* for bioactive metabolite production against ten pathogenic bacterial strains. Ethyl acetate extract of *Phomosis* sp. expressed broad spectrum antibacterial activity against all the test strains with zone of inhibitions varies from 6 to 11mm. This complies with the present study where endophytic fungi NFW3 and

NFW9 displayed broad spectrum activity comparable to that of positive control (ciprofloxacin).

You *et al.,* (2012) screened 14 fungal isolates from *R. palmatum* for the production of antibacterial metabolites. Two isolates showed inhibitory effect against all the test strains. The zone of inhibition ranges from 15mm to 32mm. One endophytic fungal isolates shows the maximum zone of inhibition 32mm against *B. subtilus* and *E. coli.* In our study two fungal isolates out of 07 shows inhibitory effect against all the test strains. The maximum zone of inhibitions observed was 23mm in diameter.

Production of Extracellular Enzymes

Enzymes being biological catalysts play crucial roles in biological reactions remains always of great interest. Fungal enzymes Contribute significantly to several industrial processes due to their wide applications. Among the fungi Endophytic fungi are extremely less studied regarding enzymes but due to its unique and distinguish ecological niches they can produce more suitable and stable enzymes as compare to other sources. In the present study endophytic fungal isolates were qualitatively screened for the production of commercially important extracellular enzymes such as amylase, protease, lipase and cellulase. In the present study three endophytic fungal isolates produced amylase while only one isolate produced cellulase.

Sunitha *et al., (*2013) screened 50 fungal isolates of medicinal plants *Alpinia calcarata, Bixa orellana, Calophyllum inophyllum* and *Catharanthus roseus* for the production of extracellular enzymes amylase, cellulase, lipase, protease, laccase and pectinase by plate based assay at pH 6. The results show that 64% isolates were lipase positive, 62% were amylase and pectinase positive, 32% were cellulase positive, 30% were laccase positive and 28% were protease positive. These findings are in closely related to that of the present study however; the relative percentage for different enzymes was comparatively low. Of the isolates, 43% positive for amylase production while only one species (14%) produce cellulase. On the other hands, these results are comparable to that of Pavithra *et al., (*2012). The study showed that. 50% fungal isolates from different parts of *Tulsi* produce amylase and protease while 27.5% shows tyrosinase activity at pH6 after 5 day of incubation.

Maria *et al*., (2005) screened 14 endophytic fungal isolates from *Acenthus ilicifolius* and *Acrostichum aureum* for the production of enzymes including tyrosinase, laccase, chitinase, amylase, protease, lipase and cellulase was carried out by plate based assays. All the fungal isolates were found to be potential producers of lipase and cellulase while few isolates produces amylase and protease and showed lack of production for the rest of enzymes. In our study the amylase was produced by 43% of isolates which is comparable to the study, the cellulase production is less than reported in the study while the rest of two enzymes screened were not produced by the fungal isolates. This may be due to the difference of fungal isolates and their host plants as each fungal strain from different hosts have different tendencies to produce enzymes or some other secondary metabolites.

Production of Antioxidants

Antioxidant compounds can neutralizes the oxygen containing free radicals produced inside the body due to different metabolic reactions and causes ROS linked diseases like DNA damages, cancers, arthritis and many more pathological conditions along with aging (Huang *et al.,* 2008). In the present study the crude extracts, of mycelial mass and cell free supernatant (fermentation broth) was analyzed for antioxidant activity by DPPH radical scavenging activity. In the present study crude organic extracts of mycelia and broth showed moderate antioxidant activity at low concentrations.

Artanti *et al.,* (2011) screened 14 endophytic fungal strains isolated from *Taxus sumatrana.* The mycelia was extracted with methanol while the fermentation broth (PDB) was extracted with ethyl acetate and screened for the bioactive metabolites of antioxidant nature. Antioxidant assay performed using DPPH and the positive control was vitamin C. Only one isolate shows 69.6% scavenging activity while the other isolates shows very low antioxidant activity ranges from 2% to 24%. The results of our study are not too much low they are somehow moderate ranges from 25% to 40% for intracellular (mycelail extract) while 35% to 53% for extracellular (fermentation broth extract) at concentration of 65µg/ml to 130µg/ml respectively.

Optimization of Opertional Parameters for the Production of Antimicrobial Metabolites

It is well known that the media composition, incubation time, temperature and pH has important role not only for endophytic fungi but for all the microbes regarding their potential to produce secondary metabolites.

In the present study, effect of operational parameters such as incubation time, media, temperature and pH on the production of antimicrobial compounds was studied. Incubation time of 14 days, meadia SDB (carbon source is sucrose), and temperature 15℃ and pH value of 6.5 to 7 is optimum for the production of antimicrobial metabolites.

Similar as the present study was conducted by Henrique Pereira Ramos and Suraia Said, (2011) for an endophytic fungal strain *Arthrinium arumdinis* isolated from *Smallanthus arundinis*. The operational parameters relative to the production of antibacterial and antifungal metabolites were studied. Effect of Carbon source in medium, incubation time, temperature and pH values on the production of bioactive metabolites was determined. The optimum temperature was 30°C, Incubation time was two weeks (starts from 6^{th} day until 27^{th} day) optimum pH value was 7 and the carbon source more suitable for the production of bioactive metabolites was sucrose, among glucose galactose and maltose. These results except temperature are very much similar to our study the only difference is the low optimum temperature i.e. 15℃ for our endophytic fungal isolates reflects the ecological niche (Himalayan region) where the climate remains considerably cold.

The results of present study are in agreement with k. Tayung and D. K. Jha, (2008) they reported the optimum culture conditions for the production of antimicrobial metabolites of an endophytic fungal isolate from *Taxus baccata*. The ethyl acetate extract of fermentation broth was screened for antibacterial and antifungal activity at different ranges of temperature, incubation time and pH value. The optimum temperature was 30°C ±1°C, incubation time of 12 days and optimum pH value of 6 was suitable for the production of bioactive metabolites. Again the only contradiction is temperature.

Thin Layer Chromatography

Thin layer chromatography is a technique by which several compounds in a mixture can be separated from each other. In present study a variety of metabolites were observed on TLC plates forming separated bands. On sulphuric acid staining some compounds gained stain and turn to brownish, green brownish and to pinkish color which indicate the presence of monoterpenes and sesquiterpenes.

These results are in agreement with Tanvir *et al*., 2013. They also reported same type of compounds and functional groups in their extracts obtained from endophytic microbes. Also idris *et al.,* reported the similar bands under UV light showing alike fluorescence.

Bioautography

Bioautography is performed to detect the antimicrobial activity of pure or partial pure bands separated on TLC plates. In present study two separated bands of ethyl acetate extract of fermentation broth on TLC plate showed activity against test bacterial strains. sulphuric acid staining of these plates indicate that these compounds are possibly monoterpenes and sesquiterpenes.

These results are similar as reported by Kalinak *et al*., (2013). This study reported that two pure compounds forming separate bands on TLC plate showed antimicrobial activity against some of the test microbial strains.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

FTIR analysis of the crude extracts showed peaks which indicate the presence of different functional groups like phenols, Amine groups, alkyl, alkenes, esters etc. Peaks appeared in the region of 3500-1700 showed the peaks which are reported for anthraquinone compounds by Chen *et al*., (2011). The peaks 1370, 1079 also found in anthraquinone and two similar peaks appeared in the spectra of crude extracts. In another study azaphilones were reported by Hsu *et al*., (2010) and peaks between 3500-1200 reported and few peaks also found in sample tested as 2972, 2931, 1455 and 1376.

The spectrum exhibits an absorption at 3,125 cm^{-1} (C=C), strong carbonyl absorption peaks at 1,745 and 1,750 cm^{-1} (two ester groups, R-COO-R), strong carbonyl

absorption peaks at 1,675 and 1,680 cm^{-1} (two carbonyl groups, -C=0), and two strong absorption peaks at 1,215 and 1,230 cm^{-1} (two ether groups, R-O-R). The spectrum did not exhibit any absorption peaks in areas between 3,200 and 3,600 cm^{-1} Wortmannin IR (neat) 1742, 1685, 1618, 1589, 1575, 1224 cm^{-1}; (Abbas and Mirocha 1988).

CONCLUSIONS

Endophytic fungi are promising source of bioactive metabolites. The present study reveal that majority of the endophytic fungal isolates obtained from *Taxus fauana* of the Himalayan region of Pakistan are active against some pathogenic bacteria and fungi. Among seven screened endophytic fungal isolates the fermentation broth of two isolates NFW3 and NFW9 showed better bactericidal and fungicidal effect against all the test strains. NFW9 is more active than all of the isolates, its fermentation broth inhibit test bacteria and fungi almost equal to the positive control (ciprofloxacin). The organic crude extract of mycelia and fermentation broth of NFW9 also possess moderate antioxidant activity along with the significant antibacterial and antifungal activity. The optimization studies reveal that NFW9 can produce bioactive metabolites under almost normal conditions except low temperature (15°C) and do not require expensive and specific nutrients so it can serve as a potential source for the production of antimicrobial agents on large scale.

Beside the production of bioactive metabolites the endophytic fungal isolates studied were able to produce different enzymes which may be of great interest in different industrial applications.

FUTURE PROSPECTS

Endophytic fungi are a diverse group of microbes and possessing a huge potential to produced novel bioactive metabolites. The present study reflects that the endophytic fungal isolates inhabiting in a medicinal plant *Taxus fuana* of the Himalayan region of Pakistan shows great potential to produce antimicrobial agents and some industrially important enzymes. The present study anticipated that these fungal isolates should be studied in future for

- The isolation of pure antimicrobial agents from the potent fungal isolates such as NFW9 and NFW3.
- To determine the structure of these compounds and their potential use in pharmaceutical applications.
- Molecular screening for some important genes like taxol and others and after confirmation of the presence of desired genes cultivate the specific isolates using different precursors and activators in medium to get desired product (this will reduce labor and save resources and time).
- Recombinant DNA techniques and cloning of desired genes such as taxol in *E.coli* and especially in yeast (because yeast is eukaryote and grow faster than the endophytic fungal isolates studied) and check its expression.
- Metabolic pathways should be studied for potent bioactive metabolites.
- The role and structure of enzymes produced by these isolates their stability along with all other parameters should be studied in detail to get benefit from them.
- Large scale production of these bioactive metabolites and enzymes is necessary to boost up the industry and economy.

REFERENCES

1. Akello, J., Dubois, T., Gold, C.S., Coyne, D., Nakavuma, J. and Paparu, P. (2007) Beauveria bassiana (Balsamo) vuillemin as an endophyte in tissue culture banana (*Musa spp*.). *Journal of Invertebrate Pathology*, 96: 34–42.
2. Amna, T., Puri, S.C., Verma, V., Sharma, J.P., Khajuria, R.K., Musarrat, J., Spiteller, M. and Qazi, G.N. (2006) Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin. *Can. J. Microbiol*, 52: pp 189-196.
3. Arnold, A.E. and Engelbrecht, B.M.J. (2007) Fungal endophytes nearly double minimum leaf conductance in seedlings of a neotropical tree species, *Journal of Tropical Ecology*, 23: pp 369-372.
4. Artanti, N., Tachibana, S., Kardono, L. B., & Sukiman, H. (2011). Screening of endophytic fungi having ability for antioxidative and alpha-glucosidase inhibitor activities isolated from Taxus sumatrana. *Pakistan journal of biological sciences: PJBS*, *14*(22), 1019.
5. Atmosukarto, I. (2005) Isolation and characterization of Muscodor albus I-41.3s, a volatile antibiotic producing fungus. *Plant Science*, 169: pp 854-861.
6. Azevedo, J.L., Maccheroni, J.W., Pereira, J.O. and Araujo, W.L. (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electr. J. Biotechnol*, 3: pp 40-65.
7. Bacon, C.W. and White, J.F.Jr. (2000) An overview of endophytic microbes: endophytism defined In: Bacon CW, White JF Jr (eds) Microbial endophytes. Marcel Dekker, NewYork, USA, pp 3–29.
8. Bell, A.A. and Wheeler, M.H. (1986) Biosynthesis and functions of fungal melanins. *Annual Review of Phytopathology*, 24: pp 4111–4451.
9. Bérdy, J. (2005) Bioactive microbial metabolites: a personal view. *Journal of Antibiotics*, 58: pp 1–26.
10. Bharathidasan, R., & Panneerselvam, A. (2012). Antioxidant Activity of the Endophytic Fungi isolated from Mangrove Environment of Karankadu, Ramanathapuram District. *Int J Pharm Sci Res*, *3*(8), 2866-2869.
11. Bolwerk, A., Lagopodi, A.L., Lugtenberg, B.J.J. and Bloemberg, G.V. (2005) Visulaization of interactions between a pathogenic and a beneficial Fusarium

strain during biocontrol of tomato foot and root rot. *Molecular Plant-Microbe Interactions*, 18: pp 710–721.

12. Boonphong, S., Kittakoop, P., Isaka, M., Pittayakhajonwut, D., Tanticharoen, M. and Thebtaranonth, Y. (2001) Multiplolides A and B, new antifungal 10-membered lactones from *Xylaria multiplex*. *Journal of Natural Products*, 64: pp 965–967.
13. Borges, K.B., Borges, W.D.S., Durán-Patrón, R., Pupo, M.T., Bonato, P.S. and Collado, I.G. (2009) Stereoselective biotransformations using fungi as biocatalysts. *Tetrahedron Asymmetry*, 20: pp 385–397.
14. Brand-Williams M.E *Cuveli*er, and C Berset, 1995 use of a free radical method to evaluate antioxidant activity Food Science and Technology 28; 25 -30.
15. Bryn, H., Daisy., Gary, A., Strobel, U., Castillo, D., Ezra, J.S., David, K., Weaver., Justin, B. and Runyon. (2002) Naphthalene, an insect repellent, is produced by Muscodor vitigenus, a novel endophytic fungus. *Microbiology*, 148: pp 3737–3741.
16. Cao, L.X., Qiu, Z.Q., You, J.L., Tan, H.M. and Zhou, S. (2005) Isolation and characterization of endophytic *Streptomycete* antagonists of *Fusarium wilt* pathogen from surface-sterilized banana roots. *FEMS Microbiol. Lett*, 247 (2): pp 147–152.
17. Carroll, G. (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology*, 69: pp 2-9.
18. Chen, T., Yu, C., & Yang, B. (2011). Structure elucidation and NMR assignments for two new quinones from fructus rhodomyrti of Rhodomyrtus tomentosa. *Chemistry of Natural Compounds*, *47*(4), 524-526.
19. Chin Y.W., Balunas, M.J., Chai, H.B. and Kinghorn, A.D. (2006) Drug discovery from natural sources. *The AAPS Journal*, 8: pp 239–253.
20. Clarke, A.J. (1997) Biodegradation of cellulose: enzymology and biotechnology. Lancaster PA : Technomic Publishing Co. Inc,
21. Clay, K. (1989) Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. *Mycol. Res*, 92 (1): pp 1–12.
22. Clay, K. and Holah, J. (1999) Fungal endophyte symbiosis and plant diversity in successional fields. *Science*, 285: pp 1742–1744.
23. Coughlan, M.P. (1990) Cellulose degradation by fungi In: Microbial enzymes and biotechnology, 2nd ed. Elsevier: New York; pp. 1-36.

24. Ding, L., Munch, J., Goerls, H., Maier, A., Fiebig, H.H., Lin, W. H. and Hertweck, C. (2010) Xiamycin: A pentacyclic indolosesquiterpene with selective anti-HIV activity from a bacterial mangrove endophyte. Bioorg. *Med. Chem. Lett*, 20: pp 6685-6687.
25. Doble, M., Kruthiventi, A.K. and Gaikar, V.G. (2004) Biotransformations and Bioprocesses. Marcel Dekker, New York, USA.
26. Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus*. 12: pp 13-15.
27. Dreyfuss, M. M. and Chapela, I. H. (1994) Potential of fungi in the discovery of novel low-molecular weight pharmaceuticals. In: The discovery of natural products with therapeutic potential (e.d. Gullo V. P.). Butterworth-Heinemann, London, UK: 49-80.
28. Duff, S.J.B. and Murray, W.D. (1996) Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. *Biores. Technol*, 55: pp 1-33.
29. Firakova, S., Sturdíková, M. and Múčková, M. (2007) Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia Bratislava*, 62(3): pp 251-257.
30. Gangadevi, V. and Muthumary, J. (2009) Taxol production by *Pestalotiopsis terminaliae*, an endophytic fungus of Terminalia arjuna (arjun tree). *Biotechnology and Applied Biochemistry*, 52: pp 9–15.
31. Gao, J.M. (2009) Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from Ginkgo biloba. *Bioorganic and Medicinal Chemistry Letters*, 19(6): pp 1572–1574.
32. Gao, L., Huang, J. and Li, J. (2007) Fermentation conditions of *Sinopodophyllum hexandrum* endophytic fungus on production of podophyllotoxin. *Food and Fermentation Industries*, 33: pp 28–32.
33. Gehring, C.A. and Whitman, T.G. (2002) Mycorrhizae-herbivore interactions: population and community consequences. In: Mycorrhizal ecology (Van der Heijden M, Sanders I, eds.): 295–320 Springer, Berlin.
34. Gloer, J.B. (1997) Application of fungal ecology in the search for new bioactive natural products. New York: Springer Verlag, p. 249-68.

35. Griffith, G.W. (1994) Phenol oxidases in *Aspergillus nidulans*: 50 years on. In: Progress in industrial Microbiology, 29: (Martinelli SD, Kinghorn JR eds.): 763–788 Elsevier, Amsterdam.

36. Guo, B., Dai, J., Ng, S., Huang, Y., Leong, C., Ong, W. and Carte, B.K. (2000) Cytonic Acids A and B: Novel Tridepside Inhibitors of hCMV Protease from the Endophytic Fungus *Cytonaema Species. Journal of Natural products*, 63: pp 602-604.

37. Guo, Y., Wang, X., Sun. and Tang, k. (2008) Bioactive natural products from endophytes: a review. *Applied Biochemistry and Microbiology*, 44(2): pp 136–142.

38. Hallmann, J. and Sikora, R.A. (1996) Toxicity of fungal endophyte secondary metabolites to plant parasitic nematodes and soil-borne plant-pathogenic fungi. *European Journal of Plant Pathology*, 102: pp 155–162.

39. Holliday, P. (1998) A Dictionary of Plant Pathology. Cambridge University Press, Cambridge, UK.

40. Hormazabal, E., Schmeda–Hirschmann, G., Astudillo, L., Rodriguez, J. and Theoduloz, C. (2005) Metabolites from *Microsphaeropsis olivacea*, an endophytic fungus of Pilgerodendron uviferum. *Z. Naturforsch*, [C] 60: pp 11–21.

41. Hsu et al., 2010 Monaphilone A (1). Yellowish oil. [R]D 25: þ260.62_ (c 0.32, acetone). IR: νmax (KBr) 3468, 2954, 2926, 2852, 1708, 1644, 1535, 1454, 1405, 1373, 1275, 1194 cm-1. ESIMS: m/z 383 [M þ Na].

42. Huang, W.Y., Cai, W.Z., Hyde, K.D., Corke, H. and Sun, M. (2008) Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity*, 33: pp 61-75.

43. Huang, W.Y., Cai, Y.Z., Xing, J., Corke, H. and Sun, M. (2007) Potential antioxidant resource: endophytic fungi isolated from traditional Chinese medicinal plants. *Economic Botany*, 61: pp 14–30.

44. Hyde, K.D. and Soytong, K. (2008) The fungal endophyte dilemma. *Fungal Diversity*, 33: pp 163–173.

45. Idris, A. M., Al-tahir, I., & Idris, E. Antibacterial activity of endophytic fungi extracts from the medicinal plant Kigelia africana.

46. Isaka, M., Jaturapat, A., Rukseree, K., Danwisetkanjana, K., Tanticharoen, M. and Thebtaranonth, Y. (2001) Phomoxanthones A and B, novel xanthone

dimers from the endophytic fungus *Phomopsis species*. *Journal of Natural Products*, 64: pp 1015–1018.

47. Jena, S. K., & Tayung, K. (2013). Endophytic fungal communities associated with two ethno-medicinal plants of Similipal Biosphere Reserve, India and their antimicrobial prospective. *Journal of Applied Pharmaceutical Science Vol*, *3*(4 Suppl 1), S7-S12
48. Kaliňák, M., Barátová, V., Gallová, E., Ondrušová, Z., & Hudecová, D. (2013). Secondary metabolite production of Epicoccum sp. isolated from lignite. *Acta Chimica Slovaca*, *6*(1), 42-48.
49. Kour, A., Shawl, A.S., Rehman, S., Sultan, P., Qazi, P.H., Suden, P., Khajuria, R.K. and Verma, V. (2008) Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. *World J. Microbiol. Biotechnol*, 24: pp 1115–1121.
50. Kumaresan, V. and Suryanarayanan, T.S. (2002) Endophyte assemblages in young, mature and senescent leaves of Rhizophora apiculata: evidence for the role of endophytes in mangrove litter degradation. *Fungal Diversity*, 9: pp 81–91.
51. Kusari, S., Lamshoft, M., Uhlke, S.Z. and Spiteller, M. (2008) An endophytic fungus from *Hypericum perforatum* that produces hypericin. *Journal of Natural Products*, 71(2): pp 159–162.
52. Kusari, S., Zuhlke, S. and Spiteller, M. (2009) An endophytic fungus from Camptotheca acuminata that produces camptothecin and analogues. *Journal of Natural Products*, 72(1): pp 2–7.
53. Leuchtmann, A., Petrini, O., Petrini, L.E. and Carroll, G.C. (1992) Isozyme polymorphism in six endophytic *Phyllosticta species*. *Mycological Research*, 96: pp 287–294.
54. Li, E., Jiang, L., Guo, L., Zhang, H. and Che, Y. (2008). Pestalachlorides A-C, antifungal metabolites from the plant endophytic fungus *Pestalotiopsis adusta*. *Bioorganic and Medicinal Chemistry*, 16(17): pp 7894–7899.
55. Li, H.M., Sullivan, R., Moy, M., Kobayashi, D.Y. and Belanger, F.C. (2004) Expression of a novel endophytic fungal chitinase in the infected host grass. *Mycologia*, 96: 526–536.

56. Li, J.Y., Tombe, B.O., Bashyal, B., Hess, W.M. and Strobel, G.A. (2001) Ambuic acid, a highly functionalize cyclohexenone with antifungal activity from *Pestalotiopsis sp*., and *Monochaetia sp*. *Phytochemistry*, 56: pp 463-468.

57. Liu, X., Dong, M., Chen, X., Jiang, M., Lu, X. and Yan, G. (2007) Antioxidant activity and phenolics of an endophytic *Xylaria sp*. from Ginkgo biloba. *Food Chemistry*. 105: pp 548–554.

58. Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X. and Zhou, J. (2008) Antimicrobial activity of an endophytic *Xylaria sp*., YX-28 and identification of its antimicrobial compound 7-amino- 4- methylcoumarin. *Applied Microbiology and Biotechnology*, 78(2): pp 241–247.

59. Lockwood, J.L. (1992) Exploitation competition. In: The fungal community– its organization and role in the ecosystem (Carroll GC, Wicklow DT, eds.): 243–263 Dekker, New York.

60. Lynd, L.R., Weimer, P.J., Vanzyl, W.H. and Pretorius, J.S. (2002) Microbial cellulose utilization: fundamentals and bio technology. *Microbiol. Mol. Biol. Rev*, 6: pp 506-77.

61. Ma, Y., Prasad, M.N.V., Rajkumar, M. and Freitas, H. 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnology Advances*, 29: pp 248–258.

62. Malinowski, D.P. and Belesky, D.P. (2000) Adaptations of Endophyte-Infected Cool-Season Grasses to Environmental Stresses: Mechanisms of Drought and Mineral Stress Tolerance. *Crop Science*, 40: pp 923–940.

63. Mandyam, K. and Jumpponen, A. (2005) Seeking the elusive functions of the root colonizing dark septate endophytic fungi. *Studies in Mycology*, 53: pp 173-189.

64. Maria, G. L., Sridhar, K. R., & Raviraja, N. S. (2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *J Agric Technol*, *1*(1), 67-80.

65. Maridass, M. (2008). Evaluation of Brine Shrimp Lethality of< em> Cinnamomum Species. *Ethnobotanical Leaflets*, *2008*(1), 106.

66. Marinho, M.R., Rodrigues-Filho, E., Moitinho, M.D.L.R. and Santos, L.S. (2005) Biologically active polyketides produced by *Penicillium janthinellum* isolated as an endophytic fungus from fruits of *Melia azedarach*. *Journal of the Brazilian Chemical Society*, 16(2): pp 280–283.

67. Mitchell, A.M., Strobel, G.A., Hess, W.M., Vargas, P.N. and Ezra, D. (2008) Muscodor crispans, a novel endophyte from Anans ananassoides in the Bolivian Amazon. *Fungal Diversity*, 31: pp 37–43.

68. Moreno, E., Varughese, T., Spadafora, C., Arnold, E., Coley, P.D., Kursar, T.A., Gerwick, W.H. and CubillaRiosa, L. (2011) Chemical Constituents of the New Endophytic Fungus *Mycosphaerella sp.*, nov. and Their Anti-Parasitic Activity. *Natural product communications*, 6: pp 835-840.

69. Muller, C. B. and Krauss, J. (2005) Symbiosis between grasses and asexual fungal endophytes. *Curr. Opin. Plant Biol*, 8: pp 450-458.

70. Muthumary, J., & Nithya, K. (2010). Secondary metabolite from Phomopsis sp. isolated from Plumeria acutifolia Poiret. *Recent research in Science and Technology*, *2*(4).

71. Narisawa, K., Usuki, F. and Hashiba, T. (2004) Control of verticillium yellows in chinese cabbage by the dark septate endophytic fungus LtVB3. *Phytopathology*, 94: pp 412–418.

72. Newman, D.J. and Cragg, G.M. (2007) Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*, 70: pp 461–477.

73. Nighat Fatima (2013) Isolation and characterization of biologically active secondary metabolites from endophytic fungi of indigenous species of *Taxus* plant PhD thesis Microbiology department Qauid-i-Azam University Islamabad.

74. Nithya, K. and Muthumary, J. (2011) Bioactive metabolite produced by *Phomopsis sp*., an endophytic fungus in Allamanda cathartica linn. *Recent Res. Sci. Technol*, 3 (3): pp 44–48.

75. Owen, N.L. and Hundley, N. (2004) Biodiversity of Marine derived fungi and identification of their metabolites. *Sci. Prog*, 87: pp 79–99.

76. Park, J.H., Choi, G.J., Lee, H.B., Kim, K.M., Jung, H.S, Lee, S.W., Jang, K.S., Cho, K.Y. and Kim, J.C. (2005) Griseofulvin from *Xylaria sp*. strain F0010, an endophytic fungus of Abies holophylla and its antifungal activity against plant pathogenic fungi. *Journal of Microbiology and Biotechnology*, 15: pp 112–117.

77. Patent reference (1988). Wortmannin IR (neat) 1742, 1685, 1618, 1589, 1575, 1224 cm^{-1}; Abbas and Mirocha.

78. Pavithra, N., Sathish, L., & Ananda, K. (2012). Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi. *Journal of Pharmaceutical and Biomedical Sciences (JPBMS), 16*(16).

79. Petrini, O. (1991) Fungal endophytes of tree leaves. In: Andrews JH, Microbial ecology of leaves. Springer, Berlin Heidelberg New York, pp 179–197.

80. Pimentel, M.R., Molina, G., Dion´ısio, A.P., Junior, M.R.M. and Pastore, G.M. (2010) The use of endophytes to obtain bioactive compounds and their application in biotransformation process. *Biotechnol. Res. Int*, 1: pp 1-11.

81. Pinto, R.C.L.S., Azevedo, J.L., Pereira, J.O., Carneiro., Vieira, M.L. and Labate, C.A. (2000) Symptomless infection of banana andmaize by endophytic fungi impairs photosynthetic efficiency. *New Phytologist*, 147: pp 609–615.

82. Pittayakhajonwut, P., Suvannakad, R., Thienhirun, S., Prabpai, S., Kongsaeree, P. and Tanticharoen, M. (2005) An anti-herpes simplex virus-type 1 agent from *Xylaria mellisii* (BCC 1005). *Tetrahedron Letters*, 46: pp 1341–1344.

83. Pongcharoen, W., Rukachaisirikul, V., Phongpaichit, S., Kühn, T., Pelzing, M., Sakayaroj, J., Walter, C. and Taylor, W.C. (2008) Metabolites from the endophytic fungus *Xylaria sp*. PSU-D14. *Phytochemistry*, 69: pp 1900–1902.

84. Qin, J.C., Zhang, Y.M. and Gao, J.M. (2009) Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from Ginkgo biloba. *Bioorganic and Medicinal Chemistry Letters*, 19(6): pp 1572–1574.

85. Rai, M.K., Varma, A. and Pandey, A.K. (2002) Antifungal potential of Spilanthes calva after inoculation of Piriformospora indica. *Mycoses*, 47: pp 479–481.

86. Rajagopal, K., Maheswari, S. and Kathiravan, G. (2012) Diversity of endophytic fungi in some tropical medicinal plants – a report. *Afr. J. Microbiol. Res*, 6 (12): pp 2822–2827.

87. Ramesha. A, Sunitha.V. H, and C. Srinivas (2013) Department of Studies in Microbiology & Biotechnology, Jnanabharathi Campus, Bangalore University, Bangalore – 560 056, Karnataka, India.

88. Ramos, H. P., & Said, S. (2011). Modulation of biological activities produced by an endophytic fungus under different culture conditions. Advances in Bioscience and Biotechnology, 2(6), 443-449.

89. Redman, R.S., Sheehan, K.B., Stout, R.G., Rodriguez, R.J. and Henson, J.M. (2002) Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science*, 298: pp 1581.

90. Rodriguez, R.J., Redman, R.S. and Henson, J.M. (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitigation Adaptation Strategies Global Change*, 9: pp 261–272.

91. Rodriguez, R.J., White, J.F.Jr. and Arnold, A.E. (2009) Fungal endophytes: diversity and functional roles, New Phytologist. doi: 10.1111/j.1469-8137.2009.02773.xx

92. Rosario Nicoletti Elisabetta Buommino Anna De Filippis Maria Pilar LopezGresa Emiliano Manzo Angela Carella Marcella Petrazzuolo Maria Antonietta Tufano. (2008) Bioprospecting for antagonistic Penicillium strains as a resource of new antitumor compounds. *World J Microbiol Biotechnol*, 24: pp 189–195.

93. Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J. and Kirtikara, K. (2008) Metabolites from the endophytic fungus *Phomopsis sp*. PSU-D15. *Phytochemistry*, 69: pp 783–787.

94. Saikkonen, K., Helander, M., Faeth, S.H., Schulthess, F. and Wilson, D. (1999) Endophyte-grassherbivore interactions: the case of Neotyphodiumendophytes in Arizona fescue populations. *Oecologia*, 12: pp 411–420

95. Saikkonen, K., Ion, D. and Gyllenberg, M. (2002) The persistence of vertically transmitted fungi in grass metapopulations. *Proceedings of the Royal Society B; Biological Sciences*, 269: pp 1397–1403.

96. Schardl, C.L. (2001) Epichloe festucae and related mutualistic symbionts of grasses. *Fungal Genetics and Biology*, 33: pp 69–82.

97. Schiff, P.B., Fant, J. and Horowitz, S.B. (1979) Promotion of microtubule assembly in vitro by taxol. *Nature*, 277: pp 665–667.

98. Schneider, P., Misiek, M. and Hoffmeister, D. (2008) In vivo and in vitro production options for fungal secondary metabolites. *Molecular pharmacology*, 5: pp 234–242.

99. Schulz, B., Boyle, C., Draeger, S., Rommert, A. and Krohn, K. (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. *J. Mycological Society*, 106: pp 996-1004.

100. Seifried, H.E., Anderson, D.E., Fisher, E.I. and Milner, J.A. (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. *Journal of Nutritional Biochemistry*, 18: pp 567-579.

101. Selim, K.A., El-Beih, A.A., AbdEl-Rahman, T.M. and El-Diwany, A.I. (2012) Biology of Endophytic Fungi. *Current Research in Environmental & Applied Mycology*, 2(1): pp 31–82.

102. Silva, G.H., Teles, H.L. and Trevisan, H.C. (2005) New bioactive metabolites produced by *Phomopsis cassiae*, an endophytic fungus in *Cassia spectabilis*. *Journal of the Brazilian Chemical Society*, 16(6B): pp 1463–1466.

103. Singh, S.B., Ondeyka, J.G., Tsipouras, N., Ruby, C., Sardana, V., Schulman, M., Sanchez, M., Pelaez, F., Stahlhut, M.W., Munshi, S., Olsen, D.B. and Lingham, R.B. (2004) Hinnuliquinone, a C2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. *Biochemical and Biophysical Research Communications*, 324: pp 108–113.

104. Song, Y.C., Huang, W.Y., Sun, C., Wang, F.W. and Tan, R.X. (2005) Characterization of graphislactone A as the antioxidant and free radical scavenging substance from the culture of *Cephalosporium sp*. IFBE001, an endophytic fungus in Trachelospermum jasminoides. *Biological and Pharmaceutical Bulletin*, 28: pp 506–509.

105. Stamford, T.L., Stamford, N.P., Coelho, L.C. and Araujo, J.M. (2002) Production and characterization of a thermostable glucoamylase from *Streptosporangium sp*. endophyte of maize leaves. *Bioresource Technology*, 83: pp 105–109.

106. Staniek, A., Woerdenbag, H.J. and Kayser, O. (2008) Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. *J. Plant Interact*, 3 (2): pp 75–93.

107. Stierle, G. and Strobel. (1993) Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. *Science*, 260(5105): pp 214–216.

108. Strobel, G. A. and Daisy, B. (2003) Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiology and Molecular Biology Reviews*, 67: pp 491-502.

109. Strobel, G. A., Miller, R.V., Miller, C.M., Condron, D.B., Teplow. and Hess, W.M. (1999) Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina. Microbiology*

110. Strobel, G., Daisy, B., Castillo, U. and Harper, J. (2004) Natural products from endophytic microorganisms. *Journal of Natural Products*, 67: pp 257-268.

111. Sunitha, V. H., Devi, D. N., & Srinivas, C. (2013). Extracellular Enzymatic Activity of Endophytic Fungal Strains Isolated from Medicinal Plants. *World Journal of Agricultural Sciences*, *9*(1), 01-09.

112. Suryanarayanan, T.S., Thirunavukkarasu, N., Govindarajulu, M.B., Sasse, F., Jansen, R. and Murali, T.S. (2009) Fungal endophytes and bioprospecting. *Fungal Biology Reviews*, 23: pp 9– 19.

113. Suto, M., Takebayashi, M., Saito, K., Tanaka, M., Yokota, A. and Tomita, F. (2002) Endophytes as Producers of Xylanase. *Journal of BioScience and Bioengineering*, 9: pp 88–90.

114. Tan, R.X. and Zou, W.X. (2001) Endophytes: a rich source of functional metabolites. *Natural Product Reports,* 18: pp 448–459.

115. Tanvir, R., Sajid, I., & Hasnain, S. (2013). Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens. *Pak. J. Pharm. Sci*, *26*(2), 277-283.

116. Tayung, K., & Jha, D. K. (2010). Antimicrobial endophytic fungal assemblages inhabiting bark of Taxus baccata L. of Indo-Burma mega biodiversity hotspot.*Indian journal of microbiology*, *50*(1), 74-81

117. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, 39: pp 44–84.

118. Verma, V.C., Gond, S.K., Kumar, A., Kharwar, R. N. and Strobel, G. (2007) The Endophytic Mycofloraof Bark, Leaf, and Stem Tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India*). Microbial Ecology*, 54: pp 119-125.

119. Verza, M., Arakawa, N.S., Lopes, N.P., Kato, M.J., Pupo, M.T., Said, S. and Carvalho, I. (2009) Biotransformation of a tetrahydrofuran lignan by the endophytic fungus *Phomopsis sp*. *Journal of the Brazilian Chemical Society*, 20: pp 195–200.

120. Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T. and Sim, G.A. (1966) Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminate*. *Journal of the American Chemical Society*, 88(16): pp 3888–3890.

121. Wani, M.C., Taylor, H.L. and Wall, M.E. (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *Journal of American Chemical Society*, 93: pp 2325-232.

122. Xu, L., Zhou, L., Zhao, J. and Jiang, W. (2008) Recent studies on the antimicrobial compounds produced by plant endophytic fungi. *Natural Product Research and Development*, 20: pp 731-740.

123. You, X., Feng, S., Luo, S., Cong, D., Yu, Z., Yang, Z., & Zhang, J. (2012). Studies on a rhein-producing endophytic fungus isolated from Rheum palmatum *Fitoterapia*.

124. Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., Sun, P. and Qin, L. (2010) Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiological Research*, 165: pp 437–449.

125. Zeng, P.Y., Wu, J.G., Liao, L.M., Chen, T.Q., Wu, J.Z. and Wong, K. H. (2011) In vitro antioxidant activities of endophytic fungi isolated from the liverwort Scapania verrucosa. *Genetics and Molecular Research*, 10: pp 3169–79.

126. Zhang, H.W., Song, Y.C. and Tan, R.X. (2006) Biology and chemistry of endophytes. *Nat. Pro. Rep*, 23: pp 753-771.

127. Zhao, J., Mou, Y., Shan, T., Li, Y., Zhou, L., Wang, M. and Wang, J. (2010) Antimicrobial Metabolites from the Endophytic Fungus *Pichia guilliermondii* Isolated from Paris polyphylla var. yunnanensis. *Molecules*, 15: pp 7961-7970.

128. Zhou, D. and Hyde, K.D. (2001) Host-specificity, host-exclusivity and host-recurrence in saprobic fungi. *Mycological Research*, 105 (12): pp 1449-1457.

129. Zou, W.X. and Tan, R.X. (1999) In: Bioactive Natural Products from Endophytes: A Review. Advances in Plant Sciences, Beijing: China Higher Education Press, 183–190.

Appendix 1: Antibacterial Activity of Fermentation Broth of Fungal Isolates at Various Days

Fungal isolates	Time	Test strains				
		E.coli	*B.spizezenii*	*S.aureus*	*S.typhimarium*	*S.epidermidis*
NFW9	4th day	0	0	0	0	0
	7th day	0	0	8	0	8
	14th day	8	8	10	8	9
	21st day	0	0	8	0	8
NFW3	4th day	0	0	0	0	0
	7th day	0	0	0	0	0
	14th day	0	8	8	8	8
	21st day	0	0	0	0	0

Appendix 2: Antifungal Activity of Fermentation Broth of Fungal Isolates at Various Days

Fungal isolates	Time	Test strains		
		Aspergillus niger	*Aspergillus fumigatus*	*Mucor*
NFW9	4th day	0	0	0
	7th day	0	0	8
	14th day	12	11	11
	21st day	0	0	8
NFW3	4th day	0	0	0
	7th day	0	0	0
	14th day	11	9	10
	21st day	0	0	0

Appendix Iii: Antibacterial Activity of Fermentation Broth of Fungal Isolates at Various Temperatures

Fungal isolates	Temperatures	Test strains				
		E.coli	*B.spizezenii*	*S.aureus*	*S.typhimarium*	*S.epidermidis*
NFW9	15°C	16	17	19	17	15
	20°C	14	14	17	9	10
	25°C	10	10	9	8	9
	30°C	0	0	0	0	0
	35°C	0	0	0	0	0
NFW3	15°C	9	12	11	0	0
	20°C	9	11	11	9	0
	25°C	0	10	9	0	0
	30°C	0	0	0	0	0
	35°C	0	0	0	0	0

Appendix 1V: Antifungal Activity of Fermentation Broth of Fungal Isolates at Various Temperatures

Fungal isolates	Temperatures	Test strains		
		A.niger	*A.fumigatus*	*Mucor*
NFW9	15°C	20	18	17
	20°C	15	14	13
	25°C	10	10	9
	30°C	0	0	0
	35°C	0	0	0
NFW3	15°C	13	13	11
	20°C	12	11	10
	25°C	11	10	9
	30°C	0	0	0
	35°C	0	0	0

Appendix V: Antibacterial Activity of Fermentation Broth of Fungal Isolates at Various pH

	PH	Test strains				
		E.coli	*B.spizezenii*	*S.aureus*	*S.typhimarium*	*S.epidermidis*
NFW9	5	10	10	9	8	9
	6	14	14	17	9	10
	6.5	16	17	19	17	15
	7	15	17	18	17	15
	8	9	8	9	8	8
NFW3	5	8	9	10	8	8
	6	9	11	11	9	9
	6.5	11	13	13	12	12
	7	11	12	13	10	11
	8	8	9	8	8	8

AppendixV1: Antifungal Activity of Fermentation Broth of Fungal Isolates at Various pH

Fungal isolates	PH	Test strains		
		A.niger	*A.fumigatus*	*Mucor*
NFW9	5	12	11	10
	6	15	14	13
	6.5	20	18	17
	7	21	19	17
	8	13	12	12
NFW3	5	9	9	8
	6	12	11	10
	6.5	14	12	11
	7	14	12	11
	8	9	8	8

Appendix VI1: Antibacterial/Antifungal Activity of Organic Extracts of NFW9

	Test Bacterial strains					Test Fungal Strains		
NFW9	*E.coli*	*B.spizezenii*	*S.aureus*	*S.typhimarium*	*S.epidermidis*	*A.niger*	*A.fumigatus*	*Mucor*
Mycelial Extract (methanolic)	7	7	7.5	7	7.5	7	7.5	7
Ethyl acetate Extract (broth)	6.5	6.5	7	6.5	7	7	7	7
Butanolic Extract (broth)	7.5	8	8.5	8	9	8.5	9	9

Appendix VIII: Antioxidant Activity of Organic Extracts of NFW9

	% Scavenging activity of crude extracts at various conc.	
	65µg	130 µg
Methanolic extract of mycelia	40%	25%
Ethylacetate extarct of fermentation broth	45%	30%
Butanolic extarct of fermentation broth	53%	35%