Screening and Identification of Secondary Metabolites Produced by Stress-tolerant *Penicillium* Strains under Extreme Environmental Conditions

THESIS

SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD
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Dedicated

to

My Beloved

Parents

You have to dream before your dreams can come true. – Dr. A. P. J. Abdul Kalam

DECLARATION

I hereby declare that the thesis entitled **"Screening and Identification of Secondary Metabolites Produced by Stress-tolerant *Penicillium* Strains under Extreme Environmental Conditions"** is my own work conducted under the supervision of Prof. D. P. Singh in the Department of Environmental Science, at Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow, and is also approved by Departmental Research Committee (DRC).

I further declare that to the best of my knowledge, the thesis does not contain any part of work, which has been earlier submitted for the award of any other degree either in this university or in any other University/ Deemed University.

Jyoti

(Jyoti)
Department of Environmental Science
Babasaheb Bhimrao Ambedkar University
Lucknow- 226 025

CERTIFICATE

This is to certify that the thesis titled **"Screening and Identification of Secondary Metabolites Produced by Stress-tolerant *Penicillium* Strains under Extreme Environmental Conditions"** submitted by **Ms. Jyoti** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulations -1999 as amended in 2008/2010* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

26/2/18
Supervisor

Dr. D.P. SINGH
Professor
Department of Environmental Sciences
Babasaheb Bhimrao Ambedkar Central University
Lucknow [illegible] (U.P.)

Dwivedi
26.02.18
Head of the Department

Head
Deptt. of Environmental Sciences
B. B. Ambedkar University
RaeBareli Road, Lucknow

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PREFACE

All microorganisms (pro and eukaryotes) have their own adaptation mechanisms against particular environmental conditions and they may survive in different environmental conditions. In a stress condition, organisms may go through some changes in their metabolic processes and develop unique adaptation strategies, especially at the level of their membranes and macromolecules affecting proteins and nucleic acids in particular. These organisms, known as extremophiles, not only tolerate specific extreme condition(s), but usually require these for their growth and survival. Production of extremozymes and secondary metabolites by these microorganisms in extreme conditions is well documented. Fungi are renowned for their ability to synthesize wide variety of small molecules called as natural products or secondary metabolites have both detrimental and beneficial (pharmaceuticals) effects on human endeavors. Because of the bioactive properties of metabolites, many fungal secondary metabolites have been adopted by humans for use in Pharmaceuticals such as antibiotics, cholesterol-lowering agents, tumor inhibitors and immune-suppressants for transplant operations. It has been reported that the production of secondary metabolites by fungus depends upon the nutrient availability, physical and environmental conditions. Thus, the production of fungal secondary metabolites in extreme environmental conditions could be a new approach and may be an area of interest for production of many new natural products which get modified or produced in excess under extreme environmental conditions.

The present study is an attempt to isolate the extremophilic fungi and study their growth, adaptation mechanism and secondary metabolite production under different extreme environmental conditions such as temperature, pH, nutrients, salinity, and heavy metals. The secondary metabolite production by psychrophilic fungal strain was compared with that of mesophilic strains. It was postulated that alterations in membrane structure (protein, nucleic acids, lipid content) of the microorganisms and metabolic conditions under extreme environmental conditions may be regulatory factors for alteration in metabolic production. The changes in functional and structural groups of fungal membrane were analyzed by FTIR study. The identification of produced secondary metabolites (volatile and non-volatile) were also made with the help of UV-Vis absorption spectrometry, Gas Chromatography-Mass spectrometry and High Pressure Liquid Chromatography-photo diode array. The produced metabolites were also used to study their antibacterial and anticancer properties.

Chapter 1 on **'Introduction'** gives the outline of the present work along with the list of objectives of the thesis work to be achieved.

Chapter 2 **'Review of Literature'** is related with the review and citation of the work done by other investigators working in the area of fungal metabolites, their growth and metabolism, production of secondary metabolites under extreme environmental conditions.

Chapter 3 **'Screening, identification and growth characteristics of fungal isolates'** is assigned to isolation of fungal strains, their morphology and biochemical characterization.

Chapter 4 **'Temperature Stress'** deals with the efforts to optimize the ability of fungal isolates to survive under temperature stress. Biochemical and physiological changes including *cellulase* production, lipid analysis and secondary metabolites production of fungal isolates were determined under temperature stress conditions.

Chapter 5 **'pH and Salt Stress'** includes the effect of different pH and salinity on the growth and production of secondary metabolites by fungal isolates.

Chapter 6 **'Nutrient Stress'** includes the evaluation of the effect of nutrients (carbon and nitrogen) on the growth and development of fungal isolates as well as their impact on the production efficiency of secondary metabolites.

Chapter 7 **'Heavy Metal Stress'** deals with the adaptive tolerance of fungal isolates to heavy metals (As III & Cr (VI) in terms of growth, structural changes in membrane and secondary metabolite production.

Chapter 8 **'Identification of Secondary Metabolites'** deals with the identification of volatile and non-volatile compounds of secondary metabolites produced by fungal isolates. The volatile compounds were identified by using Gas Chromatography- Mass spectrometry (GC- MS/MS) and non-volatile compounds by using High Performance Liquid Chromatography- Photo diode Array (HPLC-PDA).

Chapter 9 **'Applications of Secondary Metabolites'** deals with the assessment of applications of secondary metabolites and their potential as antibacterial and anticancer agents.

Chapter 10 **'General Discussion'** with critical analysis of the various parameters followed by **'Summary and Conclusion'.**

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ABBREVIATIONS

μg	Microgram
μl	Microliter
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
18S rRNA	18S ribosomal RNA
^{1}H NMR	Proton Nuclear Magnetic Resonance
A549	Human Lung Cancer cell line
ABA	Abscisic acid
ACP	Acyl carrier
ANOVA	Analysis of variance
	Array
As	Arsenic
As (III)	Arsenite
AT	Acyl transferase
atm	Atmospheric pressure
Aw	Water activity
BM	Basal medium
CDCl$_3$	Deuterated chloroform
CLSI	Clinical and Laboratory Standards Institute
CMC	Carboxy Methyl Cellulose
Conc.	Concentration
Cr(VI)	Hexavalent Chromium
DMSO	Dimethyl sulfoxide

DNS	3, 5- Dinitro salicylic acid
EtBr	Ethidium Bromide
EtOAc	Ethyl acetate
eV	Electronvolt
FIC	Fractional inhibitory concentrations
FICI	Fractional inhibitory concentration index
FPU	Filter Paper Unit
FSC	Forward scatter
FTIR	Fourier Transform Infra-Red
GC-MS	Gas Chromatography- Mass spectrometry
HPLC-DAD	High Pressure Liquid Chromatography-with Diode Array Detection
HPLC-PDA	High Performance Liquid Chromatography-Photodiode
HPLC-UV	High Pressure Liquid Chromatography- with Ultra Violet
HPLC-UV-MS	High Pressure Liquid Chromatography with Ultra Violet Mass Spectrometry
IR	Infra Red
ITS	Internal transcribed spacer region
J&K	Jammu & Kashmir
KBr	Potassium bromide
KR	Ketoreductase
KS	Ketoacyl CoA synthase
LC-MS	Liquid Chromatography- Mass spectrometry
M	Molar
MDR	Multidrug-resistant

mg	Milligram
MHB	Mueller-Hinton broth
MHz	Megahertz
MIC	Minimum Inhibitory Concentration
MPC	Mutation prevention concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT assay	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NIST	National Institute Standard and Technology
nm	Nanometer
OD	Optical Density
OsO_4	Osmium tetroxide
PaβN	Phenylalanine-arginine β-naphthylamide
PBS	Phosphate Buffer Saline
PDA	Potato dextrose agar
PI	Propidium Iodide
ppm	Parts per million
psi	per square inch
PUFA	Poly-unsaturated fatty acids
Rf	Retention factor
RND	Resistance-Nodulation-Division
Rpm	Rotation per minute
RT	Retention Time

SEM	Scanning Electron Microscopy
SSC	Side scatter
TET	Tetracycline
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TNF	Tumor Necrosis Factor
UPLC	Ultra- performance liquid chromatography
UV	Ultra Violet radiations
UV_{max}	Ultra Violet absorbance maxima
UV-Vis	Ultra Violet-Visible
v/v	Volume/volume
viz.	videlicet
w/v	weight/volume
WHO	World Health Organization

CHAPTER-1 GENERAL INTRODUCTION

1.1 Introduction

Innumerable ecosystems had been made during the Earth's evolution and are diverse by large variation in physio, chemical and biological factors creating our environment (Pikuta and Hoover, 2007). Generally, extremes environment referred to pH, salinity and physical extremes known for temperature, pressure and radiation (Burg, 2003). Life exist in all probable places and conditions on the Earth intermingling with the environment and cross species relations. In general, most ecosystems contain the evolutionarily attuned and functionally related functional communities (consortia and populations). The environments with extreme physico-chemical and climatic parameters are inhabited by a wide spectrum of different microorganisms called extremophiles (Pikuta and Hoover, 2007). Extremophiles are organisms which permanently experience environmental conditions which may be considered as extreme in comparison to the physico- chemical characteristics of the normal environment. Microbial extremophiles are the dominant life forms of the extreme environments. They are able to survive in the extreme environments and have developed mechanisms that allow them to cope with a variety of stressors and have evolved several structural and chemical adaptations, which allow them to survive and grow in extreme environments including rocks, geysers, deserts, glaciers, poles and deep sea. These organisms may survive in freezing temperatures and repeated freeze-thaw cycles, desiccation, high or low levels of salinity or pH, and lengthy periods of darkness during winter. The limits of growth and reproduction of microbes varies from –12°C to more than +100°C, pH 0 to 12, hydrostatic pressures up to 1400 atm and salt concentrations of saturated brines. Such extreme factors may vary from one site to another. One of the utmost influencing factors for fungal growth is temperature (Adan, 1994; Carlile, *et al.* 2001) and the understanding of fungal growth affected by temperature of the environment is an important part of fungal physiology (Li, *et al.*, 2008). Some of microorganisms have potential to grow at very low temperature; (-15 to 10°C) these are called as psychrophiles. The environments they inhabit are ubiquitous on earth, as a large fraction of our planetary surface experiences temperature lower than 15°C. Temperature as low as -15 °C are found in pockets of very salty water (brine) surrounded by sea ice. The first discussion of the term "psychrophile" was ended by Schmidt-Nielsen in 1902 by giving the description of bacteria capable of growth at 0°C (Morita, 1975), but Arctic diatoms had already been studied more than one hundred years ago without defining this term (Van Heurck, 1909). Later, the term was also used to refer to a number of species of eukaryotic organisms (diatoms, yeasts, algae, lichens, mosses, insects and also for fishes). The term psychrotroph (also termed psychrotolerant), was retained to denote organisms that have the ability to grow at low temperature, but have their optimal and maximal growth temperature above 15°C and 20°C, respectively. The above identification is a useful one because it has relevance in terms of their respective ecological distributions as psychrophiles are limited to permanently cold environments. A psychrotolerant microorganism may have high metabolism and capable to grow with an extended lag phase at freezing and low temperature, and unlike the psychrophiles they do not die at room temperature and have an optimum growth in the range of mesophilic microrganisms (Pikuta and Hoover, 2007). Usually Fungi are able to live in a relatively large range of temperatures, but their growth rate and metabolism are different at different temperatures even when other conditions, e.g. nutrient and water activity are constant. Normally, the temperature at which a mold has the highest biomass increase rate is accepted as the optimum temperature level of that mold (Carlile, *et al.* 2001). However, it is not well known whether this is also the temperature at which the fungi is growing most efficiently and under least stress.

1.2 Mechanisms of adaptation to temperature stress

According to previously reported reviews described by Pikuta and Hoover (2007), the cold adaptation mechanisms to cold temperatures could be associated with the changes in proteins (more flexible structural and conformation changes), increasing the fluidity of membranes by the changing of the unsaturation degree of fatty acids, alterations in *ante-iso-/iso-* branching patterns, and by shortening in the fatty acid chain length. Likewise, the synthesis of antifreeze glycoproteins and peptides can promote the decrease in freezing point of cellular water. Decline in temperature commonly leads the change in Unsaturation of fatty acid chains, that promotes the increase in the fluidity of the membrane because unsaturated fatty acid groups produce further instabilities to the membrane than saturated chains and it is done by desaturases located in the membrane itself and thus are able to respond faster. Similarly, the average fatty acid chain length may be shortened, which would have the effect of increasing the fluidity of the cell membrane. After a reduction in temperature, an increase in the amount or kind of branched fatty acids also reported. Sometimes, there may be a reduction in the portion of cyclic fatty acids and thus rise in mono-unsaturated straight chain fatty acids may occur. Sometimes, sudden decline in temperature may initiate specific alterations in gene expression of cold shock proteins. Additional adaptive approaches developed by psychrophilic microorganisms comprise the regulation of ion channel permeability, seasonal dormancy and microtubule polymerization (Pikuta and Hoover, 2007). The key adaptive strategy of psychrophiles is the modification of enzyme kinetics, allowing the emergence of metabolic rates compatible to life at low temperatures. The psychrophiles enzymes could be extremely active at low temperatures and could be 10 fold higher than that of their mesophilic homologues.

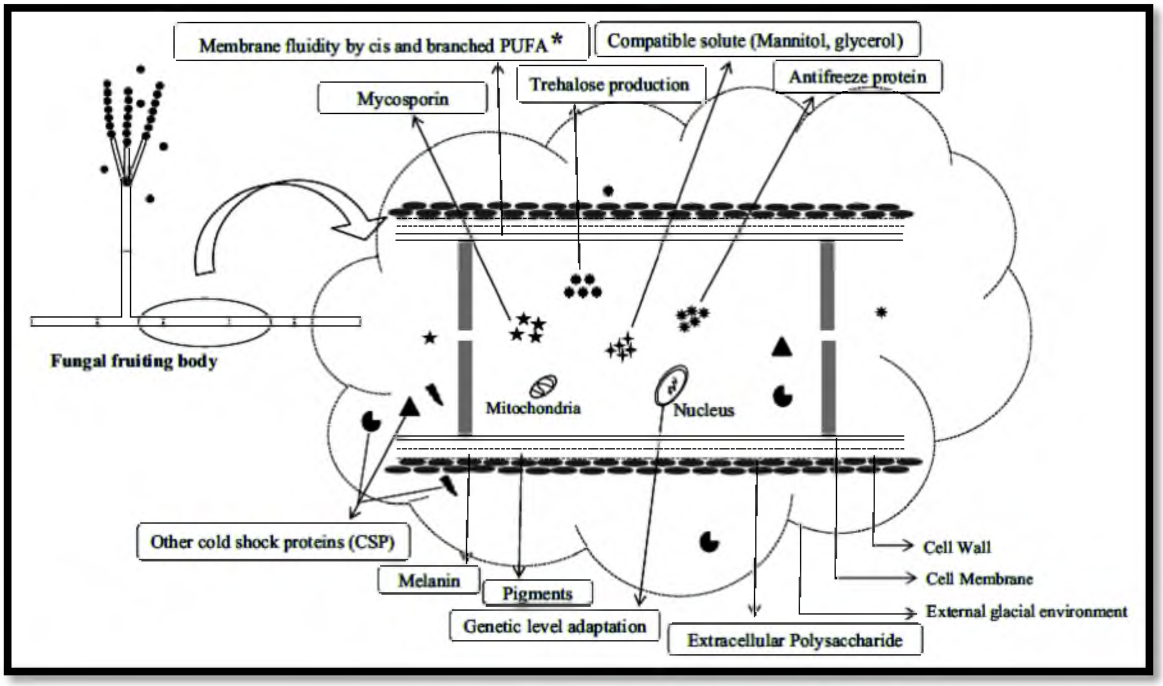


Fig. 1.1 A typical structure of psychrophilic and psychrotrophic fungi and their adaptability mechanisms in low temperature environments, *Poly-unsaturated fatty acids (source: Hassan, *et al.* 2016).

1.3 Secondary metabolites

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. The producer organism can grow in the absence of their synthesis, suggesting that secondary metabolism is not essential, at least for short term survival. A second view proposes that the genes involved in secondary metabolism provide a ‘‘genetic playing field” that allows mutation and natural selection to fix new beneficial traits via evolution. A third view characterizes secondary metabolism as an integral part of cellular metabolism and biology; it relies on primary metabolism to supply the required enzymes, energy, substrates and cellular machinery and contributes to the long term survival of the producer. Fungal secondary metabolites are chemical compounds produced by a limited number of species in a genus, an order, or even phylum, and has a high differentiation power. It consists of compounds produced on one or more media and includes toxins, antibiotics and other outward –directed compounds (Frisvad, *et al.* 2006). A simple classification of secondary metabolites includes three main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates). These compounds are an extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae, and animals. Different classes of these compounds are often associated to a narrow set of species within a phylogenetic group and constitute the bioactive compound in several medicinal, aromatic, colorant, and spice plants or functional foods.

Most of the secondary metabolites may called as ‘stress metabolites’, permitting adaptation to physico-chemical stresses and, in the case of antibiotics, permitting competition against opponent soil microorganisms (Dyson, 2009). The presence of psychrophilic and psychrotrophic fungi in cold environments, including; permafrost, off-shore polar waters glaciers, ice sheets and shelves, freshwater ice, sea ice, icebergs, have been widely studied. To combat harsh conditions, such as very low temperature conditions and others, fungi have adapted special features that are still not fully understood. Although, several cold adaptive mechanisms of psychrophilic fungi have been reported, it is expected that a blend of strategies including production of secondary metabolites, cold-active enzymes, antifreeze proteins, compatible solutes (glycerol), trehalose and polyols (acyclic sugar alcohols) are employed by psychrophiles for their survival. Psychrophilic fungi exist in some of the coldest environments throughout the world because of their great efficiency of adaptation to cold environment. The natural products obtainable from psychrophilic fungi such as proteins, enzymes (cold enzymes), secondary metabolites and compatible solutes are of great interest due to their potential biotechnological applications (eg. Pharmaceuticals). The likely potential has been increasing exponentially with the isolation of new psychrophilic fungal strains, the identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components. There are several examples of extremophilic fungi which show the diversity of microbes in the environment and their unique metabolism towards extreme conditions. A profile of secondary metabolites is reported by the mycologists and is based on fungal extracts. Secondary metabolite production by psychrophilic fungi is also useful in their taxonomical classification and identification. Fungal taxonomy, chemotaxonomy based on secondary metabolites has been reported in large ascomycetes, and basidiomycetes genera. Fungal secondary metabolites are low-molecular weight natural products with restricted taxonomic distribution, often synthesized by non-ribosomal peptide synthetase and the polyketides aflatoxin and sterigmatocystin, which are synthesized through a polyketide pathway, are among the best studied fungal secondary metabolites and their pathways have become paradigms (eg. Penicillin). Because of these bioactive properties, many fungal secondary metabolites have been adopted by humans for use as pharmaceuticals such as immune-suppressants, antibiotics, anticancer, cholesterol-lowering agents and tumor inhibitors. However, the production of secondary metabolites by the fungus depend upon the nutrient availability, physical and environmental conditions. The production of secondary metabolites in extreme environmental conditions could be a part of attention and new approach as the production of natural products may get modified and enhanced under extreme environmental conditions.

1.4 Psychrophilic *Penicillium sp.*

In 1809, the *Penicillium* term (Latin word ''Penicillus'', a structure .similar to pencil) was given by Johann Heinrich Friedrich Link, to a group of fungi which bear brush like conidiophores (asexual fruiting structure) and included the detailed description of three *Penicillium* species of the genus viz., *P. expansum*, *P. glaucum* and *P. candidum* (Raper and Thom, 1949; Houbraken and Samson, 2011). Later, it has been known as a huge genus, comprising 67 % of the total fungal biomass in the soil. Many species of the *Penicillium* genus have been recognized for their ecological and biotechnological applications (Leitão, 2009; Visagie, *et al.* 2009; Khan, *et al.* 2011a, 2011b, 2011c; Gawas-Sakhalkar, *et al.* 2012; Khan, *et al.* 2015 a, 2015 b, 2015 c). John I. Pitt (1973) divided the genus *Penicillium* into four subgenera (*Aspergilloides, Biverticillium, Furcatum* and *Penicillium*) on the basis of their morphological, micro-morphological and growth characteristics (Raper and Thom, 1949). Other example of *Penicillium* species belongs temperate regions and varies in conidial morphology and production of secondary metabolites such as *penicillin* is *Penicillium svalbardense* (Sonjak, *et al.* 2007).

Microfungi of the genus *Penicillium* is one of the most promising sources of physiologically active compounds, including alkaloids, antibiotics, hormones, mycotoxins etc. Production of these compounds is currently intensely searched for among strain of fungi isolated from little studied and practically uninvestigated habitats. Fungi producers, which are representatives of the genus *Penicillium*, differ by growth characteristics and the biosynthesis of secondary metabolites. The transport and excretion of alkaloids metabolites are also confined to certain peculiarities typical of fungi of this genus.

Fungi of the genus *Penicillium* is considered as difficult entity for identifying species using conventional microbiological approaches. The general identification of *Penicillium* fungi by micro and macromorphological features often gives no definite results. The reliability of *Penicillium* fungus classifications to certain species seems possible due to species specific production of various biologically active compounds called secondary metabolites. Fungi of the genus *Penicillium* are known to produce secondary metabolites of various classes of chemical compounds i.e.- ergot, alkaloids, diketopiperazines, quinolines, quinozotrines, azatidine and polyketides.

1.5 Aim of the work

In the present study, the psychrophilic and mesophilic fungi has been isolated from Temperate region of Leh Ladakh (J & K., India) and B. B. A. U. Campus, Lucknow (U. P., India) respectively. The growth pattern and characteristics of *Penicillium* strains under extreme environmental conditions has been studied. The biological activity of produced secondary metabolites by *Penicillium* strains has been examined. An additional objective is to isolate and identify the volatile and non-volatile secondary metabolites produced by *Penicillium* strains has also been done. The information of the category of metabolites produced would help to understand the biological pathways of the fungus and define their role in the growth of the disease symptoms. The differences in the types of chemical structures of the compounds produced by both *Penicillium* strains also tried to investigate. Secondary metabolites extracted from crude extracts of fungal culture on artificial media were screened and identified using high-performance liquid chromatography and biological assays. Other modern current separation and identification techniques, such as HPLC-UV, GC-MS and LC-MS, had been used for the purification of the compounds and their identification in ethyl acetate crude extracts.

Followings are the objectives of the present thesis.

1. **Screening, Identification and Optimization of growth conditions of mesophilic and psychrophilic *Penicillium* strains and their characterization.**
2. **Study on the adaptational features of both the *Penicillium* strains under different environmental conditions.**
3. **Screening of both mesophilic and psychrophilic *Penicillium* strains in terms of secondary metabolite production under different environmental conditions.**
4. **Extraction and characterization of biomolecules present in the secondary metabolite of both the *Penicillium* strains.**
5. **Study on the antimicrobial and anticancer attributes of the biomolecules extracted from the secondary metabolite of each *Penicillium* strain.**

CHAPTER-2 REVIEW OF LITERATURE

MacElory (1974) has proposed the name “extremophile” to specify any organism which can grow well under extremely adverse environmental conditions where most of the organisms are dead. Extremophiles are the good example to understand their functional evolution and stress adaptation in extreme environmental conditions. The exploration of biology of extremophiles has contributed to our understanding about the range of terrestrial life and its biodiversity. It has come as a wonder that not only prokaryotes but also eukaryotes have a great ability to acclimatize in extreme environmental conditions. Extremophilic fungi have aptitude to grow under intense cold, dry, salinity, acidic and deep-sea environments. Nowadays, greater understanding of the survival strategies and mechanisms adapted by the living system under extreme environmental conditions have shown involvement of genetic and biochemical adaptations (Vincent, 2000; Elster, 1999, 2002) such as season induced dormancy, production of exopolysaccharides and pigments, protection from Ultra Violet radiation by forming sheaths, utilization of specialized enzymes and production of various cryo-protectants to prevent cell lysis and slow metabolism during freezing-thaw cycles. With concern to metabolism among the psychrophilic organisms, the phototrophic and organo-heterotrophic types are common.

Morita (1975) defined psychrophiles as organisms that require temperature below 15°C for optimum growth and maximal temperature 20°C for their survival. He also stated that the psychrotolerant organisms may be able to grow at low temperatures with considerable slower rates of metabolism and have their optimum growth in the range of mesophilic organisms (20 - 40°C). It is now very well documented that the psychrotolerant microorganisms are generally the organisms most often found in cold environment, where they have improved nutritional adaptability (Wynn-Williams, 1990) or they may have the horizontal gene transfer from their neighbor mesophiles (Aislabie, *et al*. 2004). However, in the previous decades the knowledge about psychrophilic and psychrotolerant microorganisms and their related habitats have been improved. Mountfort *et al*. (1997) and Reddy, *et al*. (2003) studied about the microbial life present in the polar regions. They discovered and defined the new taxa of psychrophiles. Gounot, (1991) reported about the biotechnological uses of unique cold shock and cold-acclimation proteins and enzymes (proteases, lipases and cellulases) in psychrophilic and psychrotolerant micoorganisms. They suggested that the microbial extremophiles of polar environments are very much important to understand the changes in environment such as global warming, and it is now newly emerging Astrobiology field. By studying more complex coverage of fungi, a more accurate census of gene diversity, regulatory elements and genome organization will be accomplished. Grigoriev, *et al*. (2011) stated that the increased knowledge about Psychrophiles is likely to positively affect the translational science (e.g., biological engineering) in fungal biology and ultimately the successful application of fungi to solve the crucial energy and climate change challenges faced by human civilization. Comparative results of evolutionary studies has helped us to better understand the adaptability of extremophiles. Shared arrays in morphology, characteristics of population and phylogeny have been reported for psychrophilic and halophilic fungi. Since the mid-1990s, studies on fungi growing in different extreme conditions have expanded the domain of investigation extremophilic microbiology, which is mainly focused on prokaryotic microbes. The fungal extremophiles such as halophilic or halotolerant, thermophilic and thermotolerant, osmotolerant, alkalitolerant, oligotrophic, radiation tolerant, psychrotolerant, acid xerotolerant as well as heavy metal tolerant and obligate/facultative anaerobic fungal strains have been investigated in a great variety of environments. The investigations revealed the ample and consistent existence of certain fungal species in extreme environments (Gonclaves, *et al.* 2013). The new and rapid sequencing technologies has facilitated the further interpretation of the microbial diversity present in the ecological units. However further research is necessary for proper documentation of the role of fungi blooming in these rare environments. The survival of microbial cells is determined by their ability to sense and respond to the stressful environment. Habitats with low temperatures such as Arctic and Antarctic, have been investigated to understand the extremophilic fungal diversity. Low temperature environment is responsible for the formation of ice crystals, and also cause low water activity (aw). This is the important factor that stimulates the microbial community in cold environments. Gunde-Cimerman, *et al.* (2003) revealed that the ascomycetous and basidiomycetous yeasts and melanized fungi are the dominant taxa in cold regions and are predominantly symbolized by the genera *Aureobasidium* and *Cladosporium* along with the various species of the genus *Penicillium*. Abyzov, (1993) have reported the *Cladosporium sphaerospermum, C. herbarum* and *Aureobasidium pullulans* from polar-regions and Bergero, *et al.* (1999) have reported about the isolation of microfungi from Acrtic soils including *Acremonium, Geomyces, Mortierella, Phialophora, Phoma, Thelebolus* and other new fungi *Eutypellascoparia, Hyphozymavariabilis* and *Ovadendron sulphureoochraceum*. Gonclaves, *et al.* (2013) isolated several isolates of cold and halotolerant *Penicillium solitum* from Antarctic marine deposits and suggested that this species could be an interesting eukaryotic model to study the structural and functional relationships. A tremendous example of a psychrophilic fungus is the straminipilan fungus *Thraustochytrium antarcticum*, from Ross Sea waters by Bahnweg and Sparrow, (1974). This species has the optimum growth between 2 to 5ºC and had a maximum temperature tolerance upto only 10 ºC. A similar psychrophile, *Schizochytrium aggregatum* was reported by Ulkenn (Raghukumar, 2002).

Fungal secondary metabolites are important source of new compounds for drug and pharmaceutical purposes. *Penicillin* discovered by Fleming in 1928 led to the discovery of potential antibiotics from extracts of microbial broths. Later, continued search for new bioactive chemicals has been carried out (Demain and Fang, 2000). *Penicillium* spp. is difficult to identify by the old and conventional microbiological methods. The common identification methods by using micro and macro morphological features usually gives no final results for *Penicillium* fungi. The consistency in *Penicillium* classification of certain species is likely possible owing to species specific production of different biologically active compounds called "Secondary metabolites" (Sonjak, *et al.*, 2007a). *Penicillium* genus is well-known to produce secondary metabolites of belonging to different classes of chemical compounds such as- ergot, quinolines, quinozotrines, alkaloids, diketopiperazines and polyketides. Kozlovskii, *et al.* (2016) reported the production of secondary metabolites of different groups from *Penicillium* strains isolated from cold environments such as, chrysogines, (chrysogine, 3-acetyl-quinazolin-4(3H)-one, 2-pyruvoylaminobenzamide, 2-(2-hydroxypropionylamino)- benzamide, and questiomycin A), penicillins (penicillin G), roquefortines (3,12 dihydroroquefortine, roquefortine, glandicolines A and B, and meleagrine), xanthocillins (xanthocillin X), and derivatives of tryptophan (*N*-acetyltriptamine and indoleacetic acid).

2.1 Adaptation characteristics of cold tolerant fungi

Adaptation to extreme conditions, fungi have adapted special mechanisms and features to grow well. However, all the mechanisms behind the tolerance of extremely low temperature by psychrophilic fungi is not known (Russell, 1990; Smith, 1993; Weinstein, *et al*. 2000; Snider, *et al*. 2000). Russell, (1990) stated that the growth of psychrophilic fungi at low temperature is possible when all the cell components must be well functional.

2.1.1 Maintenance of fluidity of Plasma membrane

Cell membrane of any microorganism is the first line of defense which protects the cells against unfortunate changes in outer surroundings. Hence, it is necessary to study the membrane function and stability. Cell membrane is comprised of proteins and phospholipid bilayer ordered in several corresponding domains within similar fluidity features (Štrancar, *et al*. 2000; Simons and Toomre, 2000). Therefore, a small alteration in the cell membrane can considerably disturb its metabolism and functions (Hazel and Williams, 1990). It is well-known that the microorganisms existing in Antarctic and other cold environment are capable of inducing conformational changes in their membrane lipids as a major cold tolerance strategy (Russell, 1990). Crowe, *et al*. (1987) stated that the very low temperature, freezing and dehydration can damage the cells by altering the organization of lipids in their cell membrane, which leads to disturbance in the function of a cell membrane. This alteration may be countered by huge augmentation in unsaturation fatty acids. *Cadophora fastigiata*, *Mortierella Antarctica*, *Mortierella alpine* and various other fungal strains from the Antarctic region, are found to produce arachidonic acid (omega-6 polyunsaturated fatty acid) and linoleic acid, when grown at extremely low temperature. The similar reports are available for *Geomyces vinaceus* and *G. pannorum* and other Antarctic strains which bring about changes in fatty acids present in their cell membrane under in cold temperature (Maggi, *et al*. 1991; Finotti, *et al*. 1993). Another similar study, reported about the existence of fatty acids 'stearidonic acids', only in *Mortierella elongate* fungal strains, although the presence of ergosterol were not detected (Weinstein, *et al*. 2000). An increase in the membrane fluidity of psychrotolerant yeast *Rhodosporidium diobovatum* has been documented by Turk, *et al*. (2011), which indicated the role of unsaturated fatty acids in sustaining proper function of plasma membrane at very low temperature. It is observed that low temperature induces the production of several compatible solutes by psychrophilic fungi in order to tolerate the increased osmotic stress and dehydration (Pascual, *et al*. 2002). According to Brown (1978), cellular accumulation of glycerol is an important example of compatible solute. Various sugars including mannitol are reported to provide cryoprotection to the cells during freeze or desiccation (Feofilova, *et al*. 1994; Weinstein, *et al*. 1997; Grant, 2004). These solutes are known to maintain the proper function and stabilization of the cell membrane (Crowe, *et al*. 1984, 1986). According to Jennings (1984), polyols (acyclic sugar alcohols) are known as buffering agents and are the primary soluble carbohydrates found in fungi (Lewis and Smith, 1967). The role of potential polyols as cryoprotectant in fungi has been revealed by relating *Humicola marvinii* with *H. fuscoatra* (Weinstein, *et al*. 1997). Osmoticum and coenzyme regulation are the main functions of polyol in fungi (Jennings, 1984). In addition to this, they protect the cells against freezing stress

(Jennings, 1984). Trehalose is another disaccharide widely found in both reproductive and vegetative stages in fungi (Thevelein, 1984). Trehalose is known as an important compatible solute which helps to enhance the fungal resistance to different environmental stress conditions such as, freezing, desiccation, dehydration and extreme temperature (Lewis, *et al.* 1995; D'Amore, *et al.* 1991). Niederer, *et al* (1992), acknowledged about the increment in concentration of trehalose under extreme low temperature condition. Weinstein, *et al.* (2000) reported an increase in extracellular glycerol and intracellular trehalose in *H. marvinii*, whereas in case of *Mortierella elongate*, only intracellular trehalose was increased. Feofilova, *et al.* (1994), reported increase in cellular content of trehalose and mannitol in case of thermophilic fungus *Myceliophthora thermophila* with optimum growth at 42°C, but inositol content decreased when *M. thermophila* was grown under low temperature stress condition (26°C), signifying enhanced production of trehalose and mannitol as fungal response to low temperature.

2.1.2 Cold-active enzymes

The cold-adapted enzymes supported the proliferation of fungi and other microorganism at extreme cold temperature by providing active sites and more flexibility to the substrate, confirming an increase in the specific activity of enzyme at very low cost of energy (Weinstein, *et al.* 2000; Kuddus, *et al.* 2011). Such kind of enzyme flexibility may be due to related structural features, such as improved surface charge, reduced electrostatic interactions and decrease in the core hydrophobicity (Weinstein, *et al.* 2000). The enzyme flexibility is contributed by replacement of proline residue by glycines on surface loops, decline in the ratio of lysine-arginine, small subunit and reduction in inter-domain and aromatic interaction (Gerday, 2000; Gianese, *et al.* 2001). Fenice, *et al.*

(1998), reported the production of cold active enzymes by some Antarctic fungal species which struggle to survive at very low temperatures. Psychrophilic fungal species, *H. fuscoatra* and *H. marvinii*, isolated from Antarctic soil are well known for production of extracellular protease and inorganic phosphate solubilization enzymes when grown on solid media at low 15°C temperature under laboratory conditions (Weinstein, *et al*. 1997). In the same way, Fenice, *et al*. (1997) reported about the diversified fungal strains isolated from diverse locations of Victoria Land (Antarctic continent) such as mitosporic fungi, ascomycetes, yeast like fungi and sterile mycelial strains produce several extracellular enzymes including protease, DNase, phosphatase, amylase, polygalacturonase glucose oxidase and lipases. Tibbett, *et al*, (1998 a, b) described that the ectomycorrhizal fungal strains of genus *Hebeloma* was able to produce proteolytic and phosphatase enzymes. Extracellular enzymes such as keratinase produced under low temperature conditions also exhibit a virulence factor in animals and plants (Deshmukh, 2002). Some fungal species capable of using Keratin and are termed as keratinophilic, dermatophytes which were isolated from soil sample of glacier banks of Gulmarg, Khilanmarg, Sonamarg and Tangmarg of Kashmir valley include *Chrysosporium keratinophilum*, *Chrysosporium tropicum*, *Ctenomyces serratus*, *G. pannorum*, *Malbranchea sp*., *Microsporum gypseum*, *Microsporum nanum*, *Microsporum van-breuseghemii*, *Trichophyton ajelloi*, *Trichophyton terrestre* and *Uncinocarpus reesii* (Deshmukh, 2002).

2.1.3 Antifreeze proteins

Synthesis of antifreeze proteins is an important strategy of prokaryotic microbes and poikilothermic eukaryotes to continue their life in cold environment (Duman and Olsen, 1993). According to various investigators, these proteins are able to adsorb and attach to the surface of ice as its nucleators (Knight, *et al.* 1993: Sicheri and Yang, 1995). The phenomenon, thermal hysteresis is involved in the binding of antifreeze proteins to ice by lowering the freezing temperature of a solution with no change in its melting point (Urrutia, *et al.* 1992). The thermal hysteresis is different for different types of organism such as in insects it ranges from 2 to 6°C, 1.0 to 1.5°C in fishes, 0.1 to 0.5°C in plants (Urrutia, *et al.* 1992), 0.1 to 0.35°C in bacteria. But in fungi, thermal hysteresis ranges from 0.3 to 0.35°C (Duman and Olsen, 1993; Snider, *et al.* 2000). Antifreeze proteins are able to change pattern of the ice crystal, as it changes the shape of ice crystals from hexagonal to pyramid (Scotter, *et al.* 2006). Antifreeze proteins have been identified in snow molds and are found to be pathogenic to dormant plants when snow covers them (Hoshino, 2005; Hoshino, *et al.* 2003; Snider, *et al.* 2000). Snow molds belong to Basidiomycetes and Ascomycetes taxa and also one pseudo-fungal taxon of oomycetes. Among the above mentioned three taxa, the Antifreeze proteins have only been recognized in *Coprinus psychromorbidus,* which belongs to taxa basidiomycetes (Hoshino, *et al.* 2003). According to Hoshino (2005), various fungi belonging to Ascomycetes taxa isolated from Antarctica, had been found to produce and transform nature of ice crystal, while they are not documented for production of Antifreeze proteins (Hoshino, 2005). Xiao, *et al.* (2010) extracted and purified a new antifreeze protein from Antarctic ascomycetes *Antarctomyces psychrotrophicus*.

2.1.4 Mycosporines

Mycosporines, known as minor secondary metabolites, were first time discovered in spores of several sporulating mycelia of terrestrial fungi (Young and Patterson, 1982;

Bernillon, *et al.* 1984). Mycosporines in non-polar fungi help them to protect themselves from UV radiation. Mycosporine glutaminol, a compound extracted from Deuteromycetes, *Trichothecium roseum* (Favre-Bonvin, *et al.* 1987) and oxo-carbonyl chromophores of these compounds (UVB at 310 nm), are restricted to terrestrial fungi only (Shick and Dunlap, 2002).

2.1.5 Melanin

Melanin is a pigment existing in all forms of biological kingdoms (Eisenman and Casadevall, 2012; Gomez and Nosanchuk, 2003). Melanin is responsible for protection of fungi from many environmental stresses such as UV stress, ionizing radiation, oxidative stress and desiccation (Gorbushina, 2003; Butler and Day, 1998). Melanin is also responsible for fungal pathogenesis and various melanized strains of Antarctic fungal taxa such as *Alternaria alternat*, *Stachybotrys chartarum* and *Ulocladium consortiale* were able to tolerate UV radiation (Domsch, *et al.* 1980). Hughes, *et al.* (2003) examined *P. herbarum*, an isolates from Antarctica, produced a brown pigment melanin, under UV-B radiation. In a similar manner, several other researchers also described about the pigment melanin, a metabolic tool for fungi to resist extreme environmental conditions (Kogej, *et al.* 2004).

2.1.6 Production of secondary metabolites by cold tolerant fungi

Secondary metabolites are biomolecules which are basically not responsible for typical growth and development of an organism, but are commonly produced at advanced levels during a transition from active growth to stationary phase (Agostini-Costa, *et al.* 2012). Frisvad, *et al.*, (2008) suggested that filamentous fungi typically produce species-specific secondary metabolites. Study of species-specific new bioactive compounds is better than isolates specific production of metabolites, this leads us to achieve high chemical diversity of bioactive compounds. Harold Raistrick in 1922, very first time started the systematic study of fungal secondary metabolites and more than 200 mold metabolites have been characterized so far (Raistrick, 1950, Keller, *et al.* 2005). Later, numerous Pharmaceutical companies started various metabolic screening programs at large scale, and by 1950, valuable microbial products with pharmaceutical applications were discovered. The bioactive secondary metabolites exploration has been continued, and several antibacterial/ antibiotics capable of killing bacteria, fungi, viruses, parasites, insects, and protozoa have been discovered which also inhibit the growth of human tumor cells. Various other biomolecules with several biomedical applications including cytotoxic, carcinogenic, mutagenic, teratogenic, enzyme inhibitory, immunosuppressive effects have been documented (Keller, *et al.* 2005).

Fox and Howlett, (2008) suggested that the genes involved in biosynthesis of secondary metabolites, organized into clusters in the control of transcription regulators, with specific effects on the common metabolic pathways. Today, the main interest in present era of research is to identify biosynthetic genes involved in regulation of fungal metabolites. To some extent, fungal secondary metabolism is dependent on the arrangement of chromosomal biosynthetic genes. Whereas eukaryotic genes involved in a single metabolic pathway are mostly scattered all over the genome, desired genes for a fungus to produce a particular secondary metabolite are often assembled and adjacent to one another on the chromosome (Keller and Hohn, 1997). Such group of genes are found in the main stream of filamentous fungi, but their number is not more than 20 genes. Although the main reason behind this grouping of gens is not completely understood. It has been proposed that these gene clusters might have resulted from horizontal gene transfer, with common gene clustering. However, only group of genes for biosynthesis of penicillin has been found (Landan, *et al.* 1990). Besides pathway-specific regulators, production of fungal secondary metabolite is also get affected by common environmental factors; for example variations in temperature, light, pH and nutritional sources such as carbon and nitrogen. It is well known that fungi are able to turn their dynamic high energy in the production of secondary metabolite under certain environmental conditions, or only when they required for their growth. Molecular signals in response to the environmental stresses are naturally transmitted through Cys2His2 zinc-finger proteins, as well as CreA for carbon signaling (Dowzer and Kelly, 1989), AreA for nitrogen signaling (Hynes, 1975), and PacC for pH signaling (Tilburn, *et al.* 1995) these proteins have positive and negative regulatory effects on production of secondary metabolite. The production of penicillin is positively and negatively regulated through CreA and PacC signaling, respectively (Martin, 2000). Mostly aflatoxin production takes place at acidic pH by *A. parasiticus* and *A. flavus*. However, *A. flavus,* an isolate from South Africa, was found to be inhibitory to aflatoxin production at very low pH (Ehrlich, *et al.* 2005). The enzyme Rapamycin (TOR) kinase is responsible for regulating nutrient mediated growth signaling in budding yeast and is found to be associated with the production of gibberellins by *Fusarium fujikuroi* (Teichert, *et al.* 2006). Various researchers suggested that the production of secondary metabolite is also coordinated with the general development of the fungus and also with the environmental conditions. It is beneficial for the fungus to produce certain secondary metabolites only at some appropriate stage of its development.

2.2 Classification of secondary metabolites

According to Keller, *et al.* (2005), there are various different classes of secondary metabolites as mentioned below-

1) Polyketides
2) Non-ribosomal peptides
3) Terpenes
4) Indole alkaloids

2.2.1 Polyketides- They are the most common fungal secondary metabolites and are synthesized by type I polyketide synthases, a multi-domain proteins related to eukaryotic fatty acid synthases. The main difference between polyketides and fatty acids is the complete reduction of the β-carbon present in fatty acids. The ketoacyl CoA synthase (KS), acyl transferase (AT) and acyl carrier (ACP) domains are necessary for polyketide synthesis, whereas the ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains are essential for ketone reduction in the fatty acids, but not found in all the fungal Polyketide enzymes. Polyketides include a collection of compounds such as aurofusarin, a mycotoxin (Shibata, *et al.* 1966), aflatoxin (Bhatnagar, *et al.* 2003), zearalenone (Urry, *et al.* 1966) and pigments of spores (Mayorga and Timberlake, 1992; Watanabe, *et al.* 1998).

2.2.2 Non-ribosomal peptides- These are product of both non proteinogenic and proteinogenic amino acids catalysed by multi-modular, multi-domain enzymes, known as non-ribosomal peptide synthetases (Finking & Marahiel, 2004). Every module of a non-ribosomal peptides is comprised of numerous domains that allow recognition, activation and covalent binding of a module-specific thioester (an amino acid) to the 4′-

phosphopantetheine cofactor, attached to each module through a serine. Later, peptide bonds in between the tethered amino acids. The subsequent release of peptide is done by a thioesterase-like domain i.e. commonly located at the end of the C-terminal of the final module. The domains assigned for these activities are referred as A (adenylation), P (pantothenylation or peptidyl carrier), C (condensation or peptide-bond formation) and TE (thioesterase). Another example of a non-ribosomal peptide synthetase, which produces a linear peptide, is the peptaibol synthetase from *Trichoderma virens* (Wiest, *et al.*, 2002). This enzyme is able to produce an 18-residue linear peptide that includes aminoisobutyric acid, a rarest amino acid.

2.2.3 Terpenes- Camphor and turpentine are the best known examples from the odoriferous plant metabolites. Although, various fungi are reported to synthesize many important terpenes such as carotenoids, gibberellins, aristolochenes, indole-diterpenes and trichothecenes (Keller, *et al.* 2005). They are composed of various units of isoprene, which may be linear or cyclic, unsaturated or saturated, and it is possible to modify them in several other ways. Various other classes of terpenes include monoterpenes, sesquiterpenes, diterpenes and carotenoids. Monoterpenes are produced from geranyl pyro or diphosphate, sesquiterpenes manufactured by farnesyl pyrophosphate, and the last two diterpenes and carotenoids are made up of geranylgeranyl pyro/diphosphate (Keller, *et al.* 2005) (Fig 2.1). Schmidhauser, *et al.* (1990) have investigated about the, Carotenoid biosynthesis in *Neurospora crassa*.

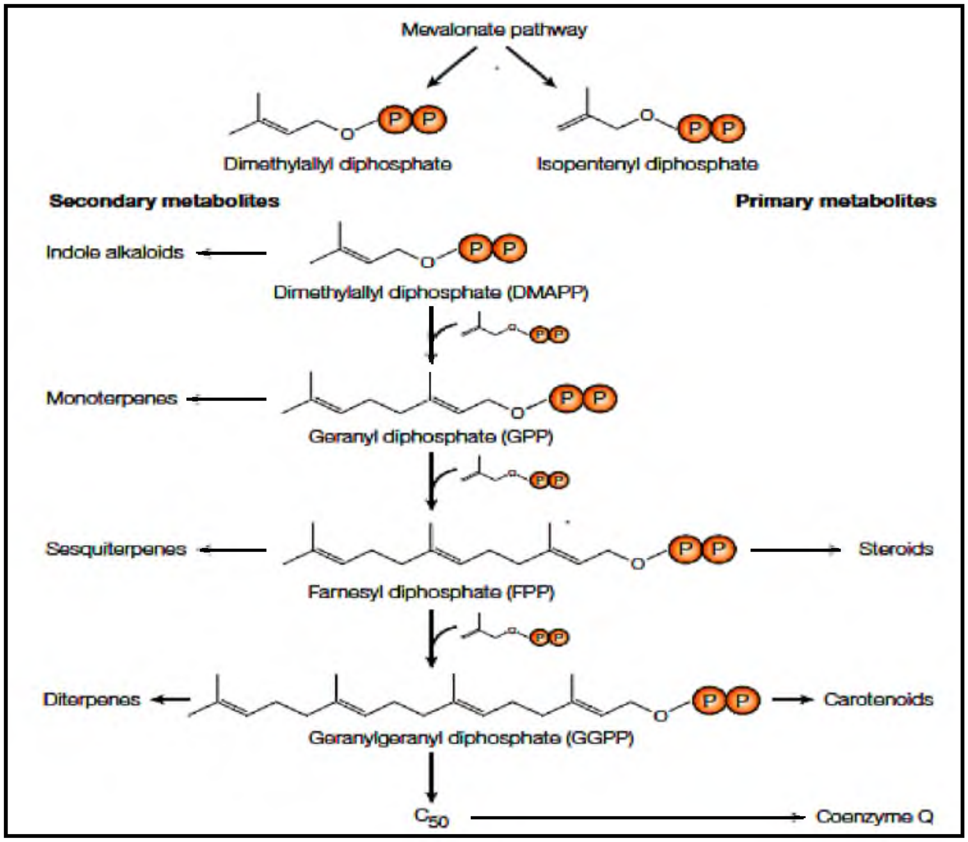


Fig. 2.1 Terpene biosynthetic pathway (source: Keller, *et al.* 2005).

2.2.4 Indole alkaloids- They are generally generated from tryptophan, dimethylallyl pyrophosphate and various amino acids other than the tryptophan, are also used as precursors. The best-agreed pathway is ergotamine synthesis as reported in *Claviceps purpurea* and associated species (Tudzynski, *et al.* 1999). The very first step of pathway includes the prenylation of tryptophan by using dimethylallyl tryptophan synthetase. Consequent to methylation of dimethylallyl tryptophan, a sequence of various steps of oxidation through agro clavine to lysergic acid. Lysergic acid is then stimulated by a single-module of non-ribosomal peptide synthetase, condensed with a tripeptide that is produced by a second non- ribosomal peptide synthetase, and is released as ergotamine. Additional tryptophan derived alkaloids include fumigaclavines and fumitremorgens had studied in *A. fumigatus* and found that there is need of one or more prenylation steps.

Gerhards, *et al.* (2014), reported that *Aspergillus fumigatus* and *Penicillium commune* are able to produce ergot alkaloids whose structural specificity is to share the tetracyclic ergoline nucleus. Clavine ergot alkaloids can be represented by three groups including C-N methyleroyoline derivatives such as festuclavine, epocostaclavine, fungi aclavines and isofumigaclavines (Gerhards, *et al.* 2014). Agroclavine-1 and epoxyagroclavine-1 are allotted to the second group. The 3rd one is comprise of clavine alkaloids with the reformed ring C and D; rugulovasines. A and B aurantioclavine, and cyclopiazonic acid (CPA). Capon, *et al.* (2007) documented that Diketopiperazines is another group of bioactive compounds produced by *Penicillia* comprising cyclic peptides of two amino acids residues and mevalonic acid. It is also known as the precursors for roquefortine and associated alkaloids i.e. 3, 12 dihydroquefortine, glandicolines A and B, meleogrine, oralin are tryptophan, histidine and mevolonic acid.

2.3 Identification of fungal secondary metabolites and Industrial uses

Maximum fungal secondary metabolites are the biomolecules with very difficult structure (Aly, *et al.* 2011; Rateb and Ebel, 2011). Even though they were nearly mapped out in various bio-discovery programs in the era of 1960's and 1980's. The advance molecular tools have provided convincing proof that secondary metabolism in the variety of filamentous fungi unpredictably higher than the originally assumed (Nielsen and Larsen, 2015). This kind of secondary metabolites include the compounds with exceptional ecological roles, predominantly as antibiotics and chemical mediators. As the analysis of diverse and complex fungal natural bio-products is found to be very difficult and the isolation of these compounds is time consuming and resource consuming, also a very labor-intensive assignment. But, the use of high performance liquid chromatography

(HPLC-DAD) coupled to diode-array UV-Vis detection is easier, a very fast and proficient classification of crude extracts of fungal secondary metabolites (Frisvad and Thrane, 1987; Frisvad, *et al* 1989; Horn, *et al.* 1995; Rodrigues, *et al.* 2000; Smedsgaard and Frisvad, 1996; Zhou and hamburger, 1996). It is an extensive technique and now used for several applied applications, such as profiling of mycotoxins produced by molds infected foods (Frisvad, *et al.* 1989), screening of trichotecenes in fungal cultures (Nielsen and Thrane, 2001) and identification of marine mycotoxins produced by *Trichoderma koningii* (Landreau, *et al.* 2002). For isolation and identification of metabolites by several chromatographic steps, including gel-permeation, reversed-phase and normal-phase chromatography can be done. Analysis of fractions of various metabolites by HPLC-UV-MS and the bioassays to achieve the isolation of pure compounds (Iocaa, *et al.* 2016).

In respect of secondary metabolites, both processes of anabolism (biosynthesis of secondary metabolites) and catabolism (periodical uptake of secondary metabolites) are known to occur in *Penicillium* fungi from time to time. *Penicillium* spp. is the unique producers of bioactive compounds, basically produces ergot alkaloids, quinoline, quinazoline alkaloids, benzodiazepine, and polyketides. The psychrophilic fungi are found to be very proficient in production of secondary metabolites that are very unusual to cold environment (Margesin, *et al.* 2008). Psychrophilic microorganisms are key source of cold-adapted enzymes which are economically important, as they can work actively at low temperatures (Georlette, *et al.* 2003). The cold-adapted enzymes or cryoprotectors have wider applications in biotechnological and pharmaceutical fields (Demain and Sanchez, 2009; Petit, *et al.* 2009). These cryoprotectors produced by psychrophilic and psychrotolerant fungi has various applications in cosmetics, medicines, and agriculture field. The fungal isolates from cold environments can be employed as biofertilizers and pigment producer of high medicinal value (Singh, *et al.* 2011; Singh and Palni, 2014). The psychrophilic enzymes are also very cost-effective and provide benefit at low temperatures (Cavicchioli, *et al.* 2002) it works during winter season, offers high yields, increases stereo specificity, decreases unwanted reactions and keeps significant energy in large scale processes, requiring the heating of reactors. The special interests in fungal strains isolated from Arctic and Antarctic climate with toxicogenic potential includes their uses for human welfare and animal health protection including cold-water detergents, food additives and flavor modifying agents, biosensors, and environmental bioremediation (Banerjee, *et al.* 2016). Cold-adaptive enzymes such as poly-galacturonases and alkaline proteases from deep-sea yeast and fungi, respectively are taken with positive consideration (Abe, *et al.* 2006; Damare, *et al.* 2006a, b). Production of alkaline and cold-tolerant proteases has been reported from fungal strains isolated from deep-sea of Central Indian Basin. Various fungal strains are able to grow and produce alkaline proteases under very low 5°C to high temperature (30°C). The *Aspergillus ustus* (NIOCC #20) is reported to produce cold-tolerant protease enzyme at optimum level when grown at 30 °C and 1 atmospheric pressure. The same enzyme was found to be active at range of 5, 30 and 50°C and 300 atmospheric pressure (Damare, *et al.* 2006a, b). The psychrophilic yeasts collected from sediments of supra- and sub-glacials, ice and melting water of two glaciers of the Italian Alps (Forni and Sforzellina-Ortles-Cevedale group) has been studied for production of various enzymes (starch-degrading, lipolytic, esterolytic, proteolytic and pectinolytic activity) at 4°C (Turchetti, *et* *al.* 2008). Zucconi, *et al.* (1996) studied the production of *lipase, urease, chitinase* activity from psychrotolerant *G. pannorum* at temperature less than 25°C. Fenice, *et al.* (1997) reported the enzyme production such as polygalacturonase, pectinase, amylase, cellulose, chitinase, cellulases, phosphatase, glucose oxidase, urease, protease, lipase, RNase and DNase from numerous cold adapted fungal strains, from various cold localities of Victoria (continental Antarctica). A psychrophilic fungi *Sclerotinia borealis* is reported to produce Polygalacturonase (Takasawa, *et al.* 1997). Marshall (1998) reported about the other fungal strain *G. pannorum* isolated from cold regions Antarctica that was able to produce keratinases. Likewise, the fungal spp. of *Cadophora*, *Penicillium*, *Geomyces* and *Cladosporium* were acknowledged for the production of extracellular enzyme endo-1, 4-β-glucanases at 4°C and 15°C (Duncan, *et al.* 2006). Hassan (2015) discovered 77 fungal strains from different places of Pakistan including Batura, Passu and Siachen glaciers and found capable to produce six extracellular enzymes including *protease, amylase*, *cellulase*, *lipase, deoxy-ribonuclease* and *phosphatase*. *Sporobolomyces ruberrimus* was found able to produce 5 extracellular enzymes except phosphatase. As glucose oxidase has a significant application in food industry and bioanalysis, produced by various spp. of *Aspergillus*, *Penicillium, Pleurotus, Alaromyces and Phanerochaete.* However, 03 fungal strains like *Aspergillus niger*, *Penicillium chrysogenum* and *Penicillium amagasakiense* were found to be responsible for industrial scale production (Crueger and Crueger, 1990). To clearing up and reduce viscosity of fruit juices at cold temperature, cold active *pectinase* now been used by various food industries. Mukherjee and Singh (2011) reported about the maximum activity of α-amylase activity at 20°C, suggesting use in food, fabric, baking industry, pulp & bleach, de-sizing of denim jeans. Moreover, they could be used in industries like 'peeling' of leather, dough fermentation, ripening of cheese, wine industry, animal feed and molecular biology (Mayordomo, *et al.* 2000).

2.3.1 Secondary metabolites in Pharmaceutical products

Schulz, *et al.* (2002) had been acknowledged to fungi from temperate and tropical habitats, for production of pharmaceutical products, though the isolation of secondary metabolites from psychrophilic and psychrotrophic fungi are relatively uncommon. But, many researchers has been investigated about the isolation of secondary metabolite production by various psychrophilic fungi. They have found that the fungi thrived in low availability of nutrient in more than 100 million years old sediments of Pacific Ocean floor. This finding indicated the presence of life everywhere, at uncommon places and also creates an opportunity for pharmaceutical companies which are looking for new and more efficient antibiotics to counter the increasing problem of emergence of drug resistant bacteria. From pharmaceutical perspective, the *Penicillium* sp. were found to have great interest. The various *Penicillium* spp. such as *Penicillium lanosum* and *Penicillium soppii* were isolated from very cold soils, capable to produce some economically important secondary metabolites e.g. griseofulvin and cycloaspeptide A (with compounds having antibiotic and antimicrobial activity) (Frisvad, *et al.* 2006). *Penicillium antarcticum*, a halotolerant and psychrotolerant to mesophilic species (McRae and Seppelt, 1999), producing patulin and asperentins. Several other species such as *Penicillium ribium, Penicillium rivulorum* and *Penicillium algidum* reported to produce other medically important secondary metabolites, such as, a cyclic nitro peptide psychrophilin A cycloaspeptide A & D and produced by *P. ribium* (Dalsgaard, *et al.* 2004 a, b; Frisvad, *et al*. 2006), whereas communesin G & H, and psychrophilin B & C were efficiently produced by *P. rivulorum* (Dalsgaard, *et al*. 2004b, 2005a). Similarly, *P. algidum* was able to produce psychrophilin D and cycloaspeptide A & D (Dalsgaard, *et al*. 2005b). These cyclic peptides were found to have varied biological activities including antibacterial, immunosuppressive, and anti-tumor activity, which are typically isolated by fungi from cold habitat (Joo, 2012). Brunati, *et al*. (2009) has documented the antimicrobial activity of fungi belongs to fifteen different genera. Amongst 160 fungal strains, 47 fungi had showed their antibiotic activity against human pathogens such as *E. coli, S. aureus*, *Cryptococcus neoformans* and *C. albicans*. The metabolites named as skyrin and rugulosin sequestered from the Antarctic fungal strain *P. chrysogenum* were successfully explored for insecticidal and medical applications (Sumarah, *et al*. 2005). *Aspergillus fumigatus* R9, isolated from the sediment sample of South Atlantic, has found to produce an antifungal protein, AfAFPR9, that control the antifungal activity against plant pathogens *Fusarium* oxysporum, *Colletotrichum gloeosporioides*, *Alternaria longipes*, *Trichodermaviride* and *Paecilomyces variotii* (Rao, *et al*. 2015). In a previous study, a novel antifungal protein (Pc-Arctin) was successfully purified from *P. chrysogenum* A096 (an Arctic sediment strain) that showed antifungal activity against *P. variotii, A. longipes* and *T. viride* (Chen, *et al*. 2013). Previous studies by Kozlovskii, *et al*., (2003, 2016) documented that fungal species of the genus *Penicillium* isolated from permafrost areas able to produce various secondary metabolites. Recently discovered quinolone compounds and quinocitrinines, have antibiotics and antitumor activitiy.

2.3.2 Secondary metabolites in Bioremediation

Researchers believed that psychrophilic microorganisms are potent to remediate the waste water and contaminated soils in cold weather, where the endogenous microbes were failed to survive and reduced by low temperature. Although, not an adequate amount of work has been done on the bioremediation potentials of the psychrophilic fungi but, in the present era, it is very necessary that this characteristic of cold adapted fungi should be consider for future betterment. However, the Antarctic fungal genera including *Penicillium Trichoderma, Phoma*, *Trichoderma, Mortierella* and *Mollisia* had been studied for the degradation of fuel and hydrocarbons (Hughes, *et al.* 2007). Observation were also made for *Mortierella spp.* that it may be able to use dodecane as their sole source of carbon. This work indicates the impending use of fungal spp. from Antarctic region for hydrocarbon degradation. According to Adams, *et al.* (2006) fungi are important as decomposers in Antarctic environment. Yergeau, *et al.* (2007) have suggested the possible use of fungi as decomposers in Antarctica and explained about the responsible genes for decomposition through microarray survey of the Antarctic Peninsula.

2.3.3 Fungal pigment or lipid production and Commercial uses

Production of Pigment or lipids by cold-adapted fungi, is generally a response to the harsh temperature. A lot of investigators have documented the increase in amount of lipids such as triglycerides, polyunsaturated and fatty acids from cold- adapted fungi (psychrotolerant and psychrophilic) (Weinstein, *et al.* 2000; Weete and Gandhi, 1999: Istokovics, *et al.* 1998). Singh, *et al.* (2014) well informed a fungal strain, *Thelebolus microsporus*, as a cold-adapted (psychrotolerant) fungus, which is able to produce pigments (such as carotenoid) and fatty acids at very low temperature. The various commercial applications of such pigments or lipids is very diverse, such as the linolenic acid may be used for enrichment of food for those suffering from diabetic neuropathy, cardiovascular diseases and eczema (Singh, *et al.* 2014). Likewise, linoleic acid an important aromatic compound reported as a precursor of 1-octen-3-ol, which have been found in most of the fungal strains including *T. microsporus* (Singh, *et al.* 2011, 2014).

2.3.4 Fungal Exopolysaccharide and their uses

The Antarctic strain *P. herbarum* Westend CCFEE 5080 was successfully investigated for exopolysaccharide (EPS) production (Selbmann, *et al.* 2002). Generally, harsh environmental conditions may leads to the production of EPS by fungi i.e. that embedded mycelium grow faster than the unembedded one, on exposure of repeated freeze and thaw cycles (Selbmann, *et al.* 2002). Selbmann, *et al.* (2005) has reported about the production of EPS externally to the hyphae or contiguous to conidia by meristematic black fungi isolated from Antarctica, similar to *Friedmanniomyces endolithicus*. Fungal EPS with bioactive role (Cheung, 1996) and rheological activities (Sutherland, 1994), have pronounced applications in various cosmetic & pharmaceutical industries, food technology & oil recovery (Hisamatsu, *et al.* 1997; Bleicher and Mackin, 1995).

2.3.5 Bio-fertilization capabilities of fungal secondary metabolites

Phosphorus is very important for development of crop plant and their yields. Both organic and inorganic forms of Phosphorus is found in nature. The insoluble inorganic form of Phosphorus (phosphate) present in soil, is insignificant to plants until it converted into soluble form. The process solubilization converted the inorganic phosphorus into organic form, which is a useful form for plants and they may use it through mineralization. Several microorganisms are found to be responsible for the transformation of insoluble phosphates to soluble one through the processes chelation, exchange of reaction and acidification (Reyes, *et al*. 2002; Narsian and Patel, 2000). A number of mesophilic fungi, actinomycetes and bacteria from High Arctic glacier, Kanchanaburi (Thailand) which are responsible for phosphate solubilization have been successfully acknowledged (Nenwani, *et al*. 2010; Stibal, *et al*. 2009; Nopparat, *et al*. 2007). Fungi was found to produce organic acid in response to solubilization of phosphorus and more efficienct than bacteria (Nenwani, *et al*. 2010). Researchers have made attempt to encapsulate fungi that are responsible for phosphate solubilization which have an importance in agricultural and industrial field (Vassileva, *et al*. 1998). Several microfungi such as *Penicillium*, *Aspergillus,* and *Fusarium* were known to produce enzyme phosphatase (Yoshida and Tamiya, 1971; Nozawa, *et al*. 1998; Vassileva, *et al*. 1998; Haas, *et al*. 1991). In the same way, Ectomycorrhizal cold tolerant fungal spp. *Hebeloma* has also been examined for the production of cold active acid phosphatases (Tibbett, *et al*. 1998). Singh, *et al*. (2011) revealed that MacElory, R.D. in 1974 first time reported the cold tolerant phosphate solubilizing fungi present in Arctic soils.

CHAPTER-3 SCREENING, IDENTIFICATION AND GROWTH CHARACTERISTICS OF FUNGAL ISOLATES

3.1 Introduction

Environments with extreme physicochemical parameters were considered as inimical to living being until the microbiologists discovered that they are actually inhabited by a variety of microorganisms (extremophiles). Extremophilic fungi are promising models to understand the functional evolution of stress adaptation. Physiology of these microorganisms not only magnifies our views on the diversity of terrestrial life, but it came as a surprise that eukaryotes have a great capacity to adapt to extreme conditions in a manner similar to prokaryotes. Comparative results of evolutionary studies and shared patterns of morphology, phylogeny and population characteristics of fungi have helped us to better understand the adaptability of extremophilic fungi. In particular, the importance of fungal populations of different geographical or ecological regions also shelter unique adaptations (Alleaume-Benharira, *et al.* 2006; Johannesson and Andre, 2006). Many successful examples of extremophilic fungi have been discovered in different extreme environments such as cold, dry, salty, acidic and deep-sea habitats (Gostinčar, *et al.* 2009a). Several extremophilic fungal species have been isolated and are well documented in considerable numbers from polythermal glaciers (Gunde-Cimerman, *et al.* 2003). *Penicillium* species is well known for their potential role in decomposition, particularly cellulose, in soil ecosystem (Panda, 2011) and saprophytic characteristics under extremophilic conditions, such as high temperature, low water availability, and salinity (Houbraken and Samson, 2011). There are numerous study has been reported on extremophilic *Penicillium* strains and their potential to survive in extremophilic environmental stress conditions (Pitt, 1979; Weinstein *et al.* 1997; Frisvad *et al.*2006, 2015; Sonjak *et al.* 2006, 2007b; Gonclaves *et al.* 2012, 2013; Dhakar *et al.* 2014; Kozlovskii *et al.* 2016).

Dhakar *et al*. (2014) examined 25 fungal cultures of *Penicillium* spp. from Indian Himalayan region, and characterized by following ployphasic approach. They resulted that Potato dextrose agar was the best amongst five different media for the vegetative growth and sporulations of the *Penicillium* strains, microscopic observations revealed biverticillate and monoverticillate are the main branching pattern of sporulation in most of the *Penicillium* isolates. They also reported that exposure to low temperature leads to increased sporulation in *Penicillium* strains as well as all strains were found to be halotolerant as they are able to grown in salt concentration from 10- 20 % and able to grown in wide range of pH from 2 to 14. Hence, they concluded that characteristics like, low temperature tolerance, growth in wide range of pH, high salt tolerance, enhanced sporulation and secondary metabolite production (watery exudates) at low temperature possibly attributed to the ecological resilience of fungi for their existence under low temperature environment. Gonclaves, *et al*. (2013) also reported the low temperature tolerance by *Penicillium* spp. isolated from marine sediments of Antarctic. They also reported about the conidial germination of *Penicillium solitum* at very low temperature and high salt concentration. Also, *P. solitum* showed extracellular amylasic and esterasic characteristics. Frisvad, *et al*., 2010, reported two new species *Penicillium buchwaldii* have terverticillate conidiophores and able to produce asperphenamate (having anticancer property), citreoisocoumarin, communesin A & B, asperetin and 5’-hydroxy-asperentin while *Penicillium spathulatum* have terverticillate and ter-ramulate conidiophores and gradually produces the extrolites including Benzomalvin A & D and anticancer compound asperphenamate. Kozlovskii, *et al*. (2016) concluded that the composite study of culture-morphological properties and profiles of produced exo-metabolites had made them possible for the determination of taxonomical diversity of fungal groups under natural cryopreservation for a very long time period. Similarly, understanding the adaptive response of extremophilic *Penicillium* strains to different growth conditions, physiological and biochemical requirements revealed how this organism survived in its endemic locales. Here, in the present chapter, *Penicillium oxalicum* strain isolated from Temperate region of Leh Ladakh (J&K, India) and mesophilic *Penicillium citrinum* from B.B.A.U. Campus, Lucknow (U.P., India),were studied for their morphological and biochemical characteristics (production of extracellular enzymes). The molecular identification of both fungal strains also been done by amplification of the ITS region of 18s rDNA sequences.

3.2 Materials and Methods

Reagents and Chemicals

Isolation and culture preparation was done by using Potato Dextrose Broth & Agar, Czapek Dox media (Himedia). Basal medium containing KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, KNO_3, and Glucose were obtained from LOBA-CHEMIE. Morphological studies were done by using Olympus Light microscope. Scanning Electron Microscopy (SEM) study was done by JSM-6490LV (JEOL Japan), at Babasaheb Bhimrao Ambedkar University, Lucknow (India). Spectrophotometric analysis was done by using UV-1601-UV-Visible double beam spectrophotometer (Shimadzu, Japan).

3.2.1 Collection of Soil samples

The soil samples were collected from two cities of two states of India namely Leh Laddakh (Jammu & Kashmir) and Lucknow (Uttar Pradesh) India. Soil samples were collected in the same month (June, 2014). The soil samples were taken from a depth of 5 cm and were placed in a sterile plastic bags. The soil samples were then dried under shade and grounded with a pestle and mortar and then sieved through a two mm sieve and stored in refrigerator for further analysis.

3.2.2 Isolation of fungal strains

The fungal strain from soil sample (01 g) was isolated on potato dextrose media (PDA) containing petriplates. To isolate the fungal strains the standard method, serial dilution and spread plate technique were performed. The isolated fungal strains were then incubated at various temperature ranging from 0 to 45°C for 7 days.

After purification of isolated psychrophilic and mesophilic fungal strains, their pure fungal cultures were maintained on PDA petriplates under their respective optimum temperature conditions for further study.

3.2.3 Morphological characterization of fungal isolates

After pure culturing of both *Penicillium* strains on PDA plates, morphological study of 3-7day old culture were carried out. Growth and morphological changes was observed by measuring radial growth (diameter in cm) on PDA containing petriplates, changes in sporulation, color and texture of mycelium and pigment production on both sides of the petriplates were examined. Microscopic examination such as size and length of mycelium, spore shape and size, arrangements of conidia and conidiophores, division of hyphae was examined by using a light microscope, later confirmed by Scanning Electron Microscopy (SEM). For SEM study, the PDA plates were inoculated with a 4-mm mycelial disc from fresh fungal cultures. The cultures were incubated at their respective optimum temperature, and after 7 days, fungi were examined by SEM. Specimens were fixed in 2 % glutaraldehyde in 0.1 M $NaPO_4$ buffer and washed in buffered 1 % OsO_4 for 2 h. The specimen was dehydrated using an ethanol series (10, 25, 40, 60, 75, 85, 95 and 100 %) for 15 min per concentration. The material was dried in a critical point drying apparatus, sputtercoated with gold and viewed with a field emission SEM. For all experiments, the fungus was sub-cultured on PDA containing petriplates. The stock cultures were kept at ±4°C in the refrigerator.

3.2.4 Molecular identification of isolated fungal strains

The **ITS region of 18s rDNA** was successfully amplified using universal primers ITS4 & ITS5 by National Fungal Culture Collection of India (NFCCI-ARI) Pune, India for Molecular identification of both the fungal cultures commenced with the isolation of genomic DNA from pure culture. The sequencing PCR was set up with ABI-BigDye® Terminator 3.1 Cycle Sequencing Kit. The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences & analyzed to reach identity.

3.2.5 Screening of enzyme production using plate test method

A. Casein hydrolysis for protease enzyme

Skimmed milk agar plates were inoculated with 4mm agar plugs of fungal culture under aseptic condition and plates were incubated for 3-7 days under their respective optimum temperature condition. Presence of clear zone around the fungal colonies was indicative of casein hydrolysis (Larsen, *et al.* 1998).

B. *Cellulase* production

Both fungal strains were initially grown on PDA plates. The medium contained (g L^{-1}): potatoes, 300; glucose, 20; agar, 15. The modified basal medium (BM), originally defined by Jonathan and Fasidi, (2001) contained yeast extract, 2.5g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.01 g;KNO_3, 1.55 g and 1000 cm^3 of distilled water, pH value of the medium was adjusted to 5.0. For cellulolytic activity of fungus, 1% Carboxy Methyl Cellulose (CMC), 1.5% agar and 1 ml of Triton-X-100 were also added to the modified basal medium. Fungal agar plugs of 4 mm, size were placed in the center of Petri dishes containing 1 % CMC agar medium and incubated under their respective optimum temperature condition. After incubation at optimum temperature for 3 to 7 d, Petri dishes containing 1% CMC agar media were flooded with an aqueous solution of Congo red (1% Congo red in distilled water) and were shaken at 50 rev/min for 15 min on a shaker. The Congo red solution was then poured off, the plates and then the plates were further flooded with 1 N NaCl and shaken again at 50 rev/min for 15 min. To inhibit the enzymatic activity, 1 N NaOH solution was added, which slightly changed the dye color to brownish-red and inhibited the enzymatic activity (Miller, 1959). The plates with pink to reddish color were considered to be cellulase positive.

C. Starch hydrolysis

Starch agar is the differential medium to test the ability of an organism to produce certain starch hydrolyzing exoenzymes, including α-amylase and oligo-1, 6-glucosidase. Starch molecules are too large to enter the microbial cell, so some fungi secretes exoenzymes to degrade starch into subunits that can then be utilized by the microorganism. Both fungal strains grown on PDA plates were tested for starch hydrolysis test by using modified basal media containing, 0.2% starch, 1.5% agar. Fungal agar plugs of 4 mm size were placed in the center of Petri dishes containing 0.2 % starch agar medium and incubated under their respective optimum temperature conditions. After incubation at optimum temperature for 3 to 7 d, Petri dishes containing 0.2 % starch agar media were flooded with an aqueous solution of Iodine solution (02g Potassium Iodide in 300 ml distilled water) and shaken at 50 rev/min for 15 min on a rotary shaker. Iodine turns blue, purple, or black (depending on the concentration of iodine) in the presence of starch. A clearing zone around the fungal growth indicated that the microorganism has ability to hydrolyze the starch.

D. *Laccase* production

The ability of the fungal strains to secrete extracellular laccase was visualized according to the method of Kiiskinen, *et al*. (2004). The assay plates containing 15 ml of 4% potato dextrose agar amended with 0.01% of guaiacol were incubated at 30°C for 1–3 days. The presence of brick red color around the fungal colony was considered as laccase positive.

E. *Lipase* production

Screening of Lipase production by fungal strains were carried out using basal (mineral) medium (Costa and Peralta, 1999) with 0.5 and 1 % olive oil emulsion as carbon source. The fungal colony showing a halo on this medium were considered lipase positive.

3.3 Results and Discussion

3.3.1 Isolation of fungal strains

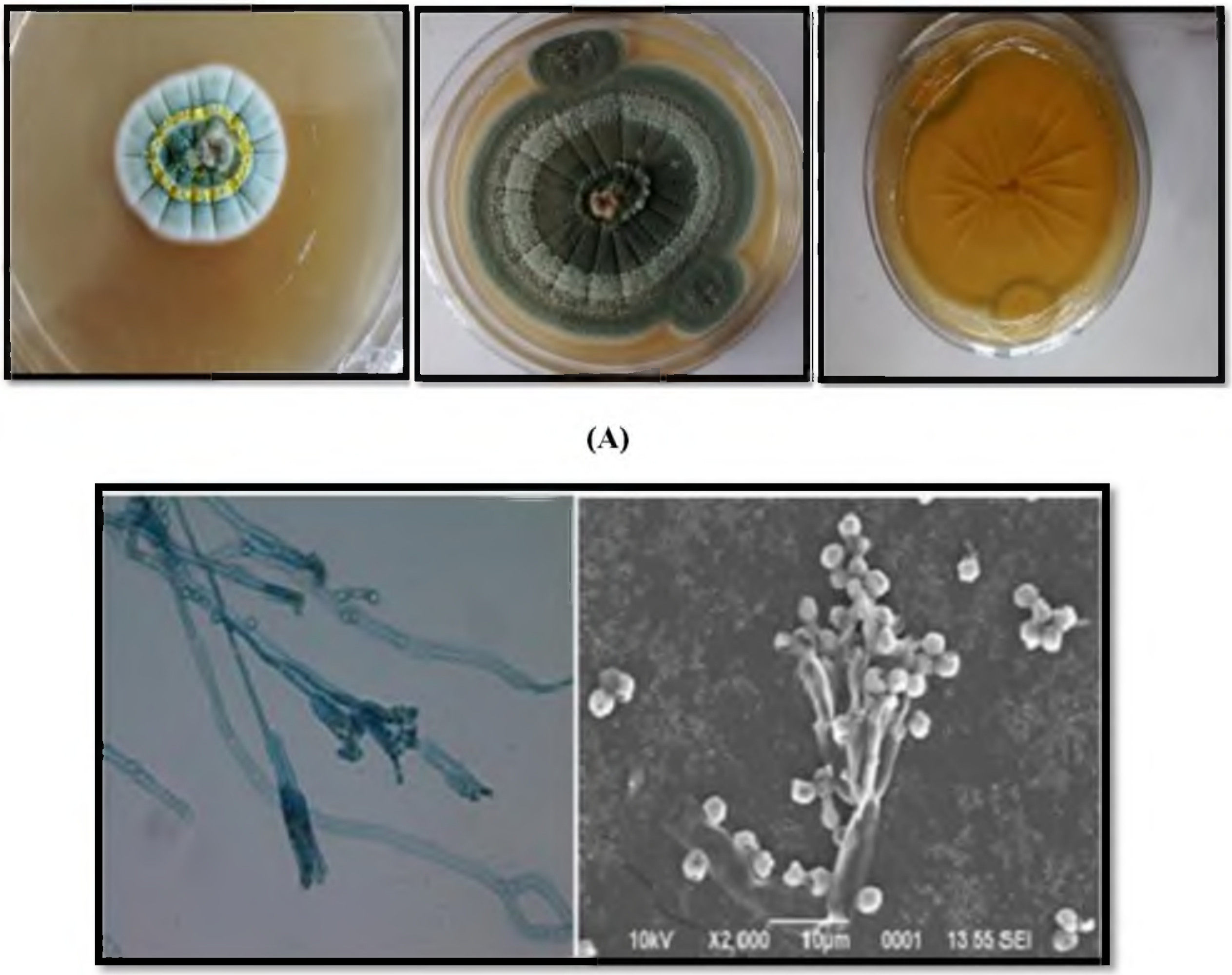
Two fungal strains *P. oxalicum* **(accession no. KR150256)** and *P. citrinum* **(accession no. KR150257)** were isolated on PDA plates from agricultural soil of dry temperate region of Leh Ladakh (J & K), India and garden soil of tropical region of Lucknow (U.P.), India respectively.

Leh is a town in the Leh district of the state Jammu and Kashmir (India) situated with longitude 34°08′43.43′N and latitude 77°34′03.41′E and Lucknow is the capital of the state Uttar Pradesh (India) situated with 26.8°N 80.9°E.

3.3.2 Morphological characterization of fungal isolates

Morphological characteristics and differences among fungal strains have been used to define the fungal diversity as the primary criterion for identifying the fungal strains (Tiwari, *et al*. 2011). In the present study, examination of macroscopic features of psychrophilic fungal strain on petridish containing PDA media showed moderate growth, a green color colony with white periphery and velvet appearance. The back side of the colony on plate was creamish yellow in color (Fig.3.1). Whereas on Czapek Dox media, this psychrophilic fungal strain exhibited slow growth, white matte like appearance with back side white in color. The pigment produced by the psychrophilic fungus on PDA was yellow in color. The mesophilic fungal strain showed rapid growth, dark green color granular appearance and the back side of the colony on PDA plate was reddish in color, whereas on Czapek Dox media this species showed moderate growth, green color with velvet appearance (Fig.3.2). The pigment produced by the mesophilic fungal strain on PDA plates was orange in color. Similar macroscopic results have been reported by Tiwari, *et al.* (2011) for the *Penicillium* strains. In a study reported by Velmurugan *et al.*, (2009) *Penicillium* spp. is capable to produce yellow pigment. Many other fungi are reported as potent pigment producing microorganisms (Babitha, and Pandey, 2007). Hamlyn (1995) reported that anthraquinone, anthraquinone carboxylic acids, pre-anthraquinones are the known pigments produced by filamentous fungi. The present findings showed that both the isolates belong to genera *Penicillium* and are capable to produce pigment under their optimum growth conditions.

Microscopic study using light microscope and Scanning Electron Microscope revealed that the hyphae of psychrophilic *Penicillium* are septate, spore size approx. 2.70 µm and conidiophores are terverticillate. On the other hand, hyphae of mesophilic *Penicillium* were smooth, spore size approx. 2.35 µm and conidiophores are terverticillate. Morphological characters such as elliptical conidia and smooth-walled conidiophores were used for the identification of the fungal strains. The mesophilic *Penicillium* produced globose conidia and visibly rough-walled conidiophores. Based on morphological and microscopic examinations, it was concluded that both *Penicillium* strains isolated from different habitats belong to same genera, but with different characteristics.

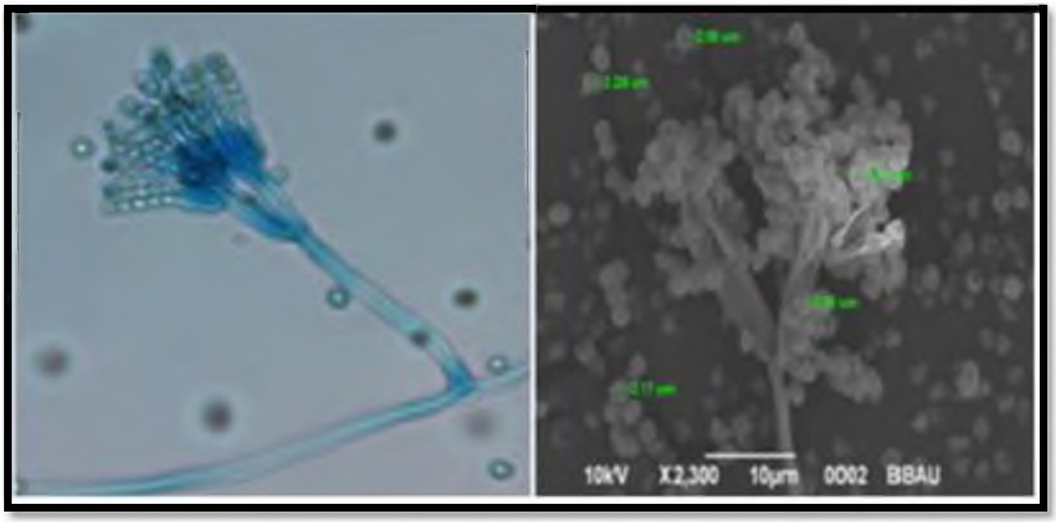


(A)

(B) (C)

Fig. 3.1 Macro and microscopic morphological studies of *Penicillium oxalicum* using photo of petriplates (A), Light microscope (B) and SEM (C) images.

(A)



(B) (C)

Fig. 3.2 Macro and microscopic morphological studies of *Penicillium citrinum* using using photo of petriplates (A), Light microscope (B) and SEM (C) images.

3.3.3 Molecular identification of isolated fungal strains

For 18S rRNA molecular analysis of isolated fungal strains of genera *Penicillium,* The ITS4 & ITS5 universal primers were used for amplification. Based on sequencing of the ITS regions and BLASTn analysis, the psychrophilic *Pencillium* (KR150256) exhibited 99% similarity *Penicillium oxalicum* and nearest similar strain was KX090323.1 *Penicillium oxalicum* strain S1111 and HG798733.1 *Penicillium oxalicum*. Mesophilic *Penicillium* (KR150257) was similar with KP329672.1 *Penicillium citrinum* strain DTO: 133-B6. Based on NCBI database, the relationship of both the *Penicillium* strains with other different *Penicillium* species is given in the form of phylogenetic tree as shown in Fig. (3.3 & 3.4).

A. Gene sequence of *P. oxalicum*

> *P. oxalicum* ITS-rDNA
GCGGCTTCCTCCTGATCGATGCACCTGGTTAAGATTGATGGTGTTCTCCGGCGGGCGCCGGC
CGGGCCTACAGAGCGGGTGACGAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGC
TGCCTTTCGGGCCCGCCCCCCGGAAGCGGGGGGCGAGAGCCCAACACACAAGCCGTGCTTG
AGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGT
TCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTC
TTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTTAGTCAAGTACT
CAGACTGCAATCTTCAGACAAGAGTTCGTTTGTGTGTCTTCGGCGGGCGCGGGCCCGGGGGC
GGATGCCCCCCGGCGGCCGTGAGGCGGGCCCGCCGAAGCAACAAGGTACGATAAACACGGG
TGGGAGGTTGGACCCAGAGGGCCCTCACTCGGT

B. Gene sequence of *P. citrinum*

>*P. citrinum* ITS-rDNA
TCCTCCTGATCGAGGTCCCCTGAGATAATTAAAGGTTGGGGGTCGGCTGGCGCCGGCCGGGC
CTACTAGAGCGGGTGACGAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCC
TTTCGGGCCCGTCCCCCCGGCGGGGGGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGC
AGCAATGACGCTCGGACAGGCATGCCCTCCGGAATACCAGAGGGCGCAATGTGCGTTCAAA
GACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCAT
CGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTAATTTCGTTATAGGTCTCAGA
CTGCAACTTCAGACAGCGTTCAGGGGGGCCGTCGGCGGGCGCGGGGCCCGCCGAGGCAACA
TAGGTTCGGGCAACACGGGTGGGAGGTTGGG

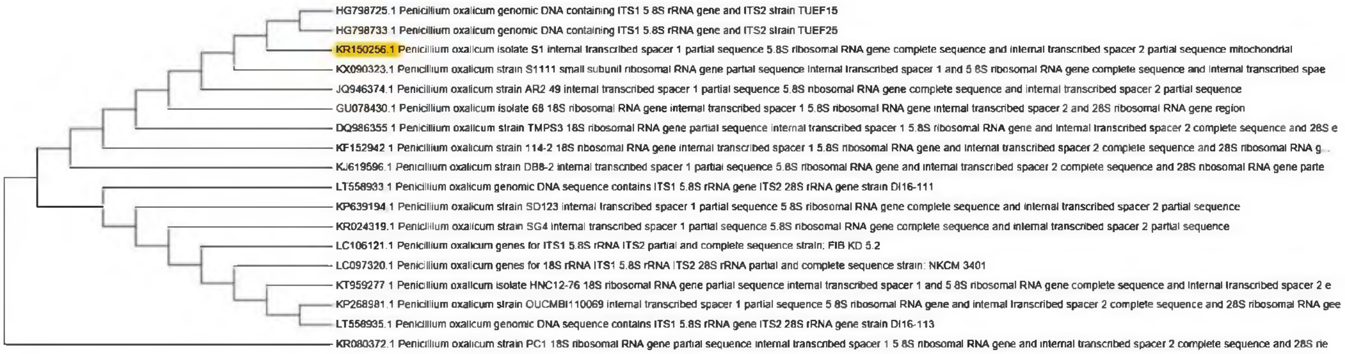


Fig 3.3 Phylogenetic analysis of psychrophilic *Penicillium oxalicum* and reference (R) sequences of its nearest relatives based on the ITS rRNA gene.

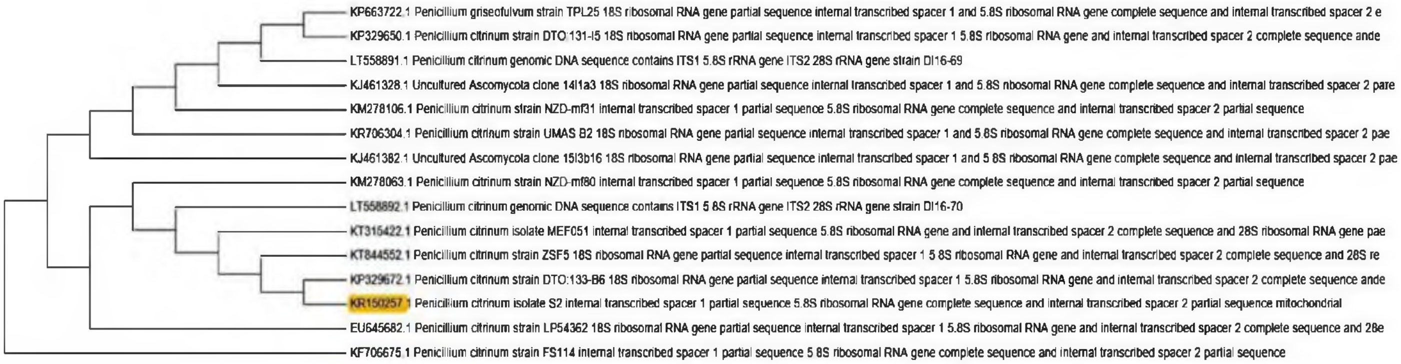


Fig 3.4 Phylogenetic analysis of psychrophilic *Penicillium citrinum* and reference (R) sequences of its nearest relatives based on the ITS rRNA gene.

3.3.4 Screening of enzyme production using plate test methods

A. Casein hydrolysis for protease enzyme

No zone was recorded around both the *Penicillium* fungal colonies grown on Skimmed milk agar petriplates (incubation of 3-7 days) under their respective optimum temperature. Hence, it is clear that the production of protease enzyme was absent in *Penicillium* strains.

B. *Cellulase* production

Earlier, Teather and Wood (1982), showed strong interaction of congo-red with polysaccahrides with connecting β-(1-4)-bound-D-glucopyranosyl units which provided the basis for a sensitive assay for detecting colonies of *cellulase*- producing bacteria. Later Sazci *et al.* (1986) reported the use of Congo red to detect *cellulase*- producing fungi. In the present study, both *Penicillium* strains showed a 2.5 cm clear zone around *P. oxalicum* strain grown on 01 % Carboxy Methyl Cellulose (CMC) containing petriplates. However, less zone (0.25 cm) around *P. citrinum* which was smaller than *P. oxalicum*. Hence, psychrophilic *P. oxalicum* when compared was found to be better celulase producing strain than *P. citrinum* Fig. 3.13 (A&B). Previous studies have reported about the production of cellulolytic enzymes by several species of *Penicillium*. Earlier, Jorgensen *et al.*, (2005), have demonstrated that *Penicillium* strains are producers of cellulolytic complexes with improved synergy due to their high production of *β*-glucosidase and endoglucanase. Especially, *P. funiculosum* ATCC 11797 has been known as an outstanding source of well-balanced cellulolytic complexes (Castro, *et al.*, 2010). Picart, *et al.* (2007) reported high level secretion of *cellulase* by *Penicillium* sp. CR-316 and *Penicillium* sp. CR-313 (isolated from subtropical soil), when grown on mineral media supplemented with rice straw. However, cessation of *cellulase* activity in glucose-supplemented media is a limiting factor. Recently, Maeda, *et al.*, (2013) have been reported the *cellulase* produced by *Penicillium funiculosum* can be used as enzymatic blend in solid-state fermentation process for second generation ethanol production.

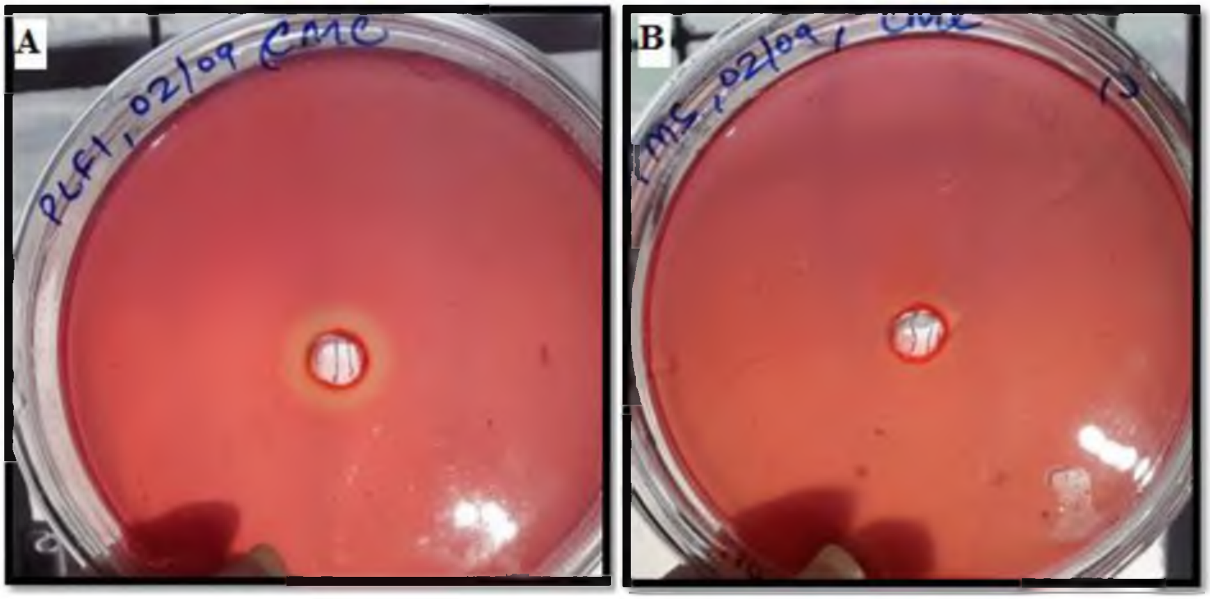


Fig. 3.5 (A & B). Production of *cellulase* enzyme in psychrophilic *P. oxalicum* (A) and mesophilic *P. citrinum* (B) fungal strain.

C. Starch hydrolysis

The *amylase*s hydrolyzing the glycoside bonds at α-1, 4 and α-1,6 bond of starch can be divided into α-*amylase*s; the one which disrupts the inner bonds of the substrate (*endoamylases*); α-*amylase*s. Another hydrolyzing enzyme which breaks the non-reducing ends of the substrate (*exoamylases*) and the *glucoamylases* (*amyloglucosidases*), which releases glucose units from the non-reducing end of starch molecules (Gupta, *et al*., 2003; Norouzian, *et al*., 2006). In the starch hydrolysis test, both *Penicillium* strains grown on 0.2% starch containing petriplates showed a clear zone of 1.5 cm around *P. oxalicum* colony and 0.5 cm zone was recorded around *P. citrinum* colony. Hence, it was concluded that psychrophilic *P. oxalicum* was more efficient in hydrolyzing the starch and production of *amylase* enzyme than *P. citrinum*. Similar results for *amylase* production by 7 fungal strains have been reported by Singh, *et al*. (2014), as indicated by the clear zone (zone of hydrolysis) formed around the fungal colonies. The fungal strains exhibiting a zone of hydrolysis of more than 0.5 cm are considered for amylase production studies (Farias, *et al*.

2010). *Amylases* have a great significance in field of biotechnology with extensive range of applications, for example in textile industry, paper and cellulose, leather, detergents, beer, liquor, bread, and children cereals, liquification and conversion of starch to sugar, animal chow, fermentation industry (vitamins, amino acids, antibiotics), chemical and pharmaceutical industries (Pandey, *et al*. 1999; Butzen and Haefele, 2008). Since, the microbial *amylase* meet industrial demands; they are now being replaced chemical hydrolysis of starch in starch processing industry (Pandey, *et al*. 2000).

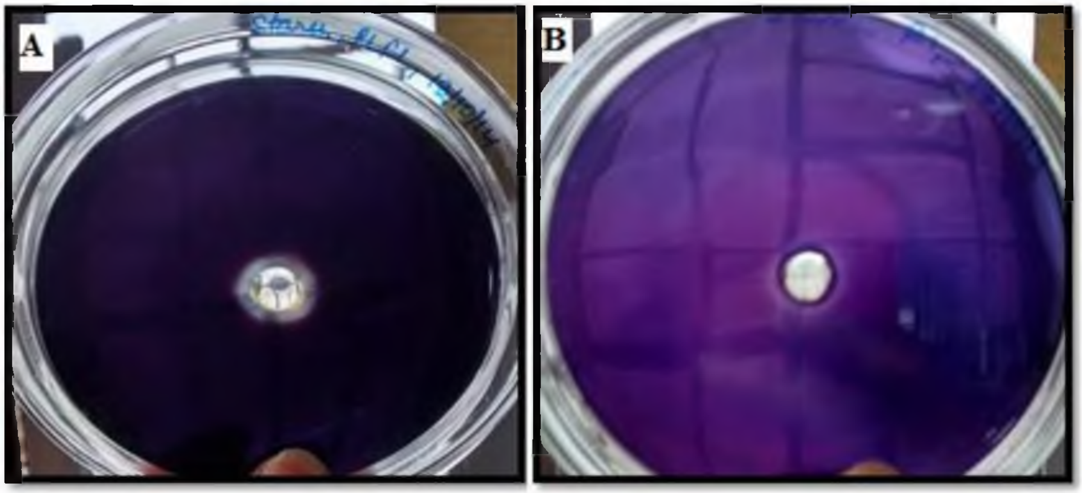


Fig. 3.6 (A & B). Production of *amylase* enzyme in psychrophilic *P. oxalicum* (A) and mesophilic *P. citrinum* (B) fungal strain.

D. *Laccase* production

Since no brick red color zone was recorded in case of both *Penicillium* strains. Hence, it was proved that there was lack of *Laccase* enzyme production in both the *Penicillium* Strains.

E. *Lipase* Production

There was total lack of *lipase* production in both *Penicillium* strains as they failed to grow on olive oil containing petriplates.

Table 3.1: Enzyme activity of both *Penicllium* strains.

Enzyme	*P. oxalicum*	*P. citrinum*
Protease	-	-
Amylase	++	+
Cellulase	++	+
Laccase	-	-
Lipase	-	-

(+) symbolizing production of enzymes, (-) symbolizing no production.

3.4 Conclusion

- Two fungal strains were isolated from agricultural soil sample of temperate region of Leh Ladakh (J & K), India and tropical garden soil of Lucknow (U.P.), India.
- Macroscopic and microscopic study revealed that the fungal isolates belong to genera *Penicillium* with different morphological characteristics.
- Based on sequencing of the ITS regions and comparison with NCBI GenBank database, strains were identified as psychrophilic *P. oxalicum* **(accession no. KR150256)** and mesophilic *P. citrinum* **(accession no. KR150257).**
- Biochemical characteristics of *Penicillium* strains revealed that psychrophilic *P. oxalicum* could efficiently produce extracellular amylase and cellulolytic enzymes when compared with the mesophilic *P. citrinum*. This indicated that *P. oxalicum* can efficiently utilize the starch and carboxy methyl cellulose as the sole source of carbon.

CHAPTER-4 TEMPERATURE STRESS

4.1 Introduction

Temperature is an important parameter affecting the growth and survival of microorganisms (Deegenaars and Watson, 1998). Natural cold environments (low temperature) comprises polar and high alpine soils, waters (glaciers, fresh and marine waters). About 71% of earth's surface is covered by oceans and 90% of its volume has temperature at 5°C or less. At altitudes >3000 m, temperature of the atmosphere is consistently less than 5°C. With the increased in altitude, the temperature decreases gradually and may reach to less than – 40°C. High mountains have snow and ice remains whole year–round due to low temperatures. Thus, cold environment dominates the biosphere. According to Morita, cold environments can be divided into two categories: psychrophilic (permanently cold) and psychrotrophic (seasonally cold or where temperature fluxes into mesophilic range). Owing to precedence, Morita (1975) defined Psychrophiles as organisms capable of growing at about 15 °C or lower and exhibit growth at 0°C or lower. The term psychrotroph (also termed psychro – tolerant), denotes organisms that have the ability to grow at low temperature, but have their optimal and maximal growth temperature at or above 15 °C and 20 °C. Most of the known cultivable microorganisms are mesophiles and occupy temperature niches that are not regarded as extreme. Others that are especially adapted to low-temperature habitats have been defined to be either psychrophilic and psychrotrophic (Russell, 2000). The Leh, Ladakh in J & K has all the characteristics of a cold desert and is accredited with very low temperature, high Ultra-Violet-B radiation, low water & nutrient availability, and repeated freeze and thaw cycles. Psychrophilic fungi are known to survive at extremely low temperature and are commonly found in polar and non-polar habitats (Hassan, *et al.*, 2016). Microorganisms that live in the extreme cold environment usually modify their physiology to ensure their survival and growth under very low cold temperature conditions, show sluggish growth, poor nutrient uptake, disorders in cell membrane and reduction in enzyme activity (Antipova, *et al.* 2011). Many psychrophilic organisms developed adaptation mechanisms to maintain the fluidity of membrane lipids irrespective to ambient temperature. These adaptation mechanisms include alterations in the proportions of various types of lipids and changes in the ratio of lipid/protein (Klein, *et al.* 1999). The most commonly change in the cell membranes is saturation of lipid acyl chains at cold temperatures (Sakamoto, *et al.* 1997; Szalontai, *et al.* 2000). The psychrophilic microorganisms capable to survive at cold temperature owing improved nutritional adaptability (Archer, *et al.*, 2008) and also have unmatched cold shock and cold acclimation proteins and enzymes (Cole and Cox, 1981). Sometimes low temperature condition influence fungal cells by increasing their water viscosity, denaturation of proteins, slowing down of their enzymatic reactions, membrane stability (Russell, 1990; Crowe, *et al.*, 1992). Psychrophilic metabolic changes in psychrotrophic fungi make them more valuable in biotechnological and pharmaceutical fields due to diverse metabolic and morphological characteristics evolved during adaptation to such extreme environments. Such extremophiles are exploited for production of cold-active enzymes, bioactive metabolites and exo-polysaccharides, potential application for biofertilizer and bioremediation (Hassan, *et al.*, 2016). Due to their unique features of producing abundance and varied bioactive sec. metabolites which are therapeutically important such as Penicillin and Lovastatin (Deegenaars and Watson, 1998), fingolimod (Delpiccolo, *et al.*, 2003) and caspofungin (Frisvad, and Samson, 2004), fungi are known as most important organism in medicinal area. The exploration of new bioactive compounds from extremophilic fungi is still going on. The psychrophilic fungi might be a good tool to explore the medically important new bioactive metabolites due to uniqueness of their habitat and changes in their metabolic systems which is responsible for their adaptation under extreme environment conditions (Frisvad, *et al.* 2006). Commonly, *Penicillium* is a genus of ascomycetous fungi and better known for their survival and growth as mesophiles/thermophiles, but very rare to grow at extreme low temperature conditions and have great economic importance in agriculture field and pharmaceuticals such as antibacterial (Gloer, 2007; Gounot, 1991), antifungal biomolecules (Holden, 1982), cholesterol lowering agents (Jonathan and Fasidi, 2001), immune suppressants, and potent mycotoxins (Keating and Figgitt, 2003, Kozlovsky , 1990). Globally, *Penicillium* is known to produce different classes of secondary metabolites for example polyketides, ergot alkaloids, diketopiperazines, quinolones and quinazolines, (Kozlovskii, *et al.* 2013), camazulene and azetidine (Kwon, *et al.* 2002). *Penicillium* is also recognized for the production of essential fatty acids and their therapeutical applications (Lucas, *et al.* 2007) by combating various human diseases (Liu, *et al.* 2001). Earlier studies have revealed about the importance of temperature which affects the growth and survival of the microorganisms (Marella, *et al.*, 2013). In the present study, there is successfully isolation of cold tolerant *Penicillium oxalicum* from the cold deserts Leh, Ladakh (J&K). Efforts have been made to optimize its ability to survive in very low temperature condition. Biochemical and physiological changes in *P. oxalicum* under temperature stress was compared with the mesophilic strain of *P. citrinum* isolated from sub-tropical region i.e., Lucknow (Uttar Pradesh), India.

4.2 Materials and Methods

4.2.1 Temperature-dependent growth of *Penicillium* strains

Isolated fungal strains were grown aseptically under varying temperatures regimes ranging from 0,4,15,25,35 & 45°C for 28 days incubation, in basal medium (50ml) containing yeast extract, 2.5g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.01 g;KNO_3, 1.55 g and 1000 ml of distilled water

(Jonathan and Fasidi, 2001). The liquid medium was supplemented separately with 1% glucose as carbon source.

After incubation time of 28 days, growth was measured in terms of colony diameter (cm) on PDA petriplates and fresh weight (g L^{-1}) of fungal mycelia in basal medium. Harvesting of fungal biomass was done through filtration using Whatman filter paper and fresh weight was taken as follows-

Weight of filter paper before filtration=X_1

Weight of filter paper after filtration=X_2

Fresh weight of biomass = $X_2 - X_1$

4.2.2 Temperature dependent Filter paper assay for *Cellulase* production (FPU Assay)

Temperature dependent Cellulase enzyme production in both the *Penicillium* strains were carried out by using Filter paper assay (Mandels, *et al.*, 1976). Before performance of FPU assay, both *Penicillium* strains were grown in 100 ml Erlenmeyer flask containing 50 ml basal medium with 1 % olive oil as sole source of carbon and incubated for 15 days at different temperature (4-35°C). Separation of enzyme filtrate from fungal biomass was carried out by filtration using Whatmann filter paper and enzyme filtrate was then used to perform FPU assay. Addition of 1.0 ml 0.05 M Na-citrate, pH 4.8, to a test tube containing 0.5 ml enzyme filtrate, diluted in citrate buffer. The test tube was heated up to 50°C and then one filter paper strip (60 mm) was placed in test tube. It was followed by addition of 3.0 ml of 3, 5- Dinitro salicylic acid (DNS) in the above solution. After thorough mixing of solution, the test tube was boiled for exactly 5.0 min. in a vigorously boiling water bath. The same procedure was performed for all samples, enzyme blanks, and glucose standards. Thereafter, test tubes were transferred to a cold water bath. Then, 20 ml deionized or distilled water was added to the mixture and were mixed thoroughly until the 'pulp' has settled well. The resulting colour or the reaction mixture was measured at 540 nm. If the paper pulp does not settle, is done so after stirring with a glass rod (Mandels, *et al.*, 1976).

The cellulase production was calculated by as follows-

FPU/ml = 0.37/enzyme concentration to release 2.0 mg glucose units ml^{-1} (Ghose, 1987)

Derivation of the FPU unit--

The unit of FPU is based on the International Unit (IU)

1.0 IU = 1 μmol min^{-1} of substrate converted

= 1 μmol min^{-1} of "glucose" (reducing sugars as glucose) formed during the hydrolysis reaction

= 0.18 mg min-1 when product is glucose

The absolute amount of glucose released in the FPU assay at the critical dilution is 2.0 mg:

2 mg glucose= 2/0.18 μmol

This amount of glucose was produced by 0.5 ml enzyme in 60 min. i.e. in the FPU reaction:

2.0 mg glucose = 2/0.18 × 0.5 × 60 μmol min^{-1} ml^{-1}

= 0.37 μmol min^{-1} ml^{-1}(IU ml^{-1})

Therefore the estimated amount of enzyme (= critical enzyme concentration = ml ml-1) which releases 2.0 mg glucose in the FPU reaction contains 0.37 units, and:

FPU= 0.37/ enzyme concentration to release 2.0 mg glucose units ml^{-1}

4.2.3 Lipid analysis in *Penicillium* strains

Reagents and Chemicals

For culture preparation, basal media was used containing KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, KNO_3, Glucose, Potato Dextrose Broth and Agar purchased from Qualigens and Himedia (India) were used. For Lipid extraction, chloroform and methanol of mass grade were purchased from Thermofisher Scientific (India). Other reagents and solvents for LC-MS/MS like methanol, acetonitrile and formic acid were purchased from Fluka (St. Louis, MO, USA). Ultra-pure MilliQ water filtered with 0.22 µm were obtained from the Millipore water purification system (Milli-Q synthesis Elix-10, Millipore Corp., Mass., U.S.A.). Standard fatty acids and triglycerides used in this study were as follows: hexaenoic acid (C6:0), octanoic acid (C8:0), decanoic acid (C10:0), dodecanoic acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), octadecanoic acid (C18:0), eicosanoic acid (C20:0), docosanoic acid (C22:0), tetracosanoic acid (C24:0), docosahexaenoic acid (C22:6), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), erucic acid (C22:1), cis-5,8,11,14-eicosatetraenoic acid (C20:4), elaidic acid (C18:1), nervonic acid (C24:1) and petroselinic acid (C18:1) cis-6, 1-monopalmitoyl-rac glycerol , 1-monomyristoyl-rac-glycerol, 1-monolauryl-rac-glycerol, 1-monostearoyl-rac-glycerol, dilaurin, dimyristin, dipalmitin, distearin, trimyristin, tripalmitin, tristaerin, trilaurin. All these chemicals were procured from Supelco (Bellefonte, USA).

A. Extraction of fatty acids

For extraction of fatty acids, the fungal biomass was harvested by filtration using whatman filter paper. The harvested biomass was rinsed with double distilled H_2O and excess moisture was removed by using soft tissue paper. One-gram (fresh weight) of fungal tissue was then placed into 4.0 ml of saponification reagent (15% (w/v) solution NaOH in 50 ml methanol+50 ml water) and homogenized by using homogenizer. The homogenate was then divided into four equal samples and were saponified at 100°C in a water bath for 30 min and cooled at room-temperature. To resulting methylated fatty acids, 2.0 ml of 54% (v/v) 6 N HCl in methanol was added to each tube and samples were then placed at 80°C in a water bath for 10 minutes and thereafter, immediately cooled to room temperature. Next, the aqueous phase (bottom of tube) containing fungal debris was removed with a micropipette, and 3.0 ml of 1.2% NaOH solution was added to each tube; the tube was then rotated end-over-end for 5 min. Finally, the organic phase (top of tube) containing the fatty acid methyl esters was removed from the tubes and placed in a crimp-top gas chromatography vial. The pure form of lipid extracts obtained from the fungal cultures were concentrated in a rotary evaporator and mixed with methanol and then filtered through 0.2 µm filter paper and kept for sonication for 30 min to get a clear solution. Method given by Stahl and Klug, 1996 is little modified and standardized for lipid extraction for LC-MS/MS analysis.

B. LC-MS/MS condition

LC-MS/MS analyses were performed using liquid chromatography (Acquity UPLC, Waters,) connected to API 4000 mass spectrometer (ABSCIEX, Framingham, MA, U.S.A). UPLC was equipped with ACQUITY UPLC®BEH C18 column (1.7µm x 2.1 x 50mm) (Waters, Milliford, NA, U.S.A.) consisting of ethylene bridged hybrid technology and stationary phase of carbon and silicon. Gradient elution was performed in LC to achieve the analysis within 3.5 min run time at 35°C temperature. All the fatty acids were analysed in multiple reactions monitoring scan (MRM) using electrospray ionization in positive polarity mode for the determination of mono-, di-, tri-glycerides. A negative mode was used for fatty acid analysis. Source parameters: CUR- 10.0 psi, GS1- 10.0 psi, GS2- 5.0 psi, CAD- 9.0 psi, temperature- 0°C and ion spray voltage- 4500 eV was optimized and nitrogen gas generated by peak scientific generator were applied throughout the experiment. Compound parameters Parent ion, product ion, declustering potential, entrance potential, collision energy and cell exit potential were optimized for all lipid species.

4.2.4 Extraction of secondary metabolites of *Penicillium* strains grown under different temperature stress conditions

After 28 days, of incubation of both *Penicillium* strains under different temperature regimes (4-35°C), the growth medium of both the *Penicillium* strains was subjected to a liquid-liquid extraction with ethyl acetate (EtOAc) thrice at different time of interval i.e., 3rd, 7th, 15th, and 21st and 28 days. The crude extracts of secondary metabolites were quantified by taking absorption spectra (250-400 nm) of the extract. Solvent ethyl acetate (99.5% purity) of LC grade were used and purchased from Qualigens (Thermofisher Pvt Ltd, India).

4.2.5 FTIR analysis of *Penicillium* biomass

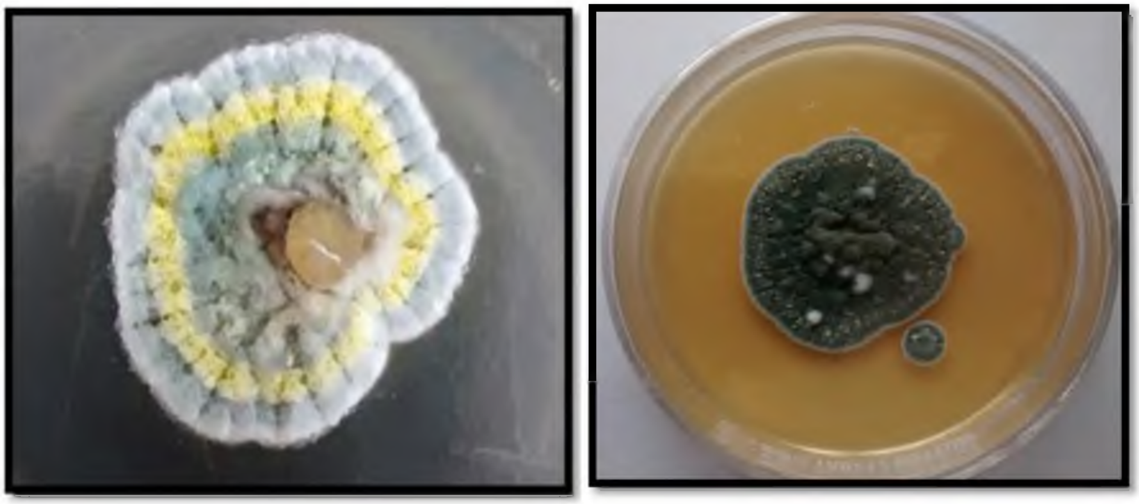
Fungal biomass of both *Penicillium* strains grown at temperature stress condition was harvested, washed with distilled water, dried and grounded. The powdered biomass was supplemented with IR grade potassium bromide (KBr) (1:10), pressed into discs under vacuum using spectra lab pelletizer. The IR spectrum was recorded in the region 4000-400 cm^{-1} using NICOLET 6700 FT-IR (Thermo-Scientific).

4.3 Results and Discussion

4.3.1 Temperature-dependent growth of *Penicillium* strains

Growth was first monitored in terms of colony diameter (cm) on PDA containing petriplates. The mycelial fresh weight (g L^{-1}) of fungal biomass of both *Penicillium* strains was also recorded when the fungal strains were grown in the basal medium incubated at different temperatures (0, 4, 15, 25, 35 and 45°C). Results revealed that 15°C is the optimum growth temperature for *P. oxalicum* with average diameter 8.6 cm on PDA petriplates and fresh weight 63.2 g L^{-1} after 28 day of incubation. However, it could grow well at low temperature at 4°C with av. fresh wt. 59.4 g L^{-1}. The psychrophilic *P. oxalicum* showed sluggish growth at 25°C, 35°C with avg. fresh wt. 20.4 g L^{-1} & 9.2 g L^{-1}, respectively, but it failed to grow at 0°C and 45°C temperature (Fig. 4.2 A & B). On the other side, the optimum growth temperature for *P. citrinum* was 30-35°C with average diameter of 9 cm, and average mycelial fresh wt. 53.8 g L^{-1}, but it grew well at 25°C with mycelial fresh wt. 48.8 g L^{-1} and failed to grow at 0°C and 45°C temperature. Hence, these results suggested that the psychrophilic fungal strain *P. oxalicum* from cold desert of Ladakh was truly psychrophilic fungal strain and *P. citrinum* required moderately high temperature as mesophilic strain (Fig 4.2

A & B). In a similar study by Pandey *et al.*, (2016), *Penicillium* spp. isolated from Indian Himalayan region was reported to have temperature tolerance range from 4-35°C and thus the isolates was psychrotolerants not psychrophilic. It showed unique characteristics to produce extracellular enzymes. Excitingly, in the present study, the both *Penicillium* strain displayed a different colonial morphology under the four tested temperatures. At 4°C and 15°C the *P. oxalicum* showed mycelial green colony with white margins and white to creamish reverse side, was observed. Whereas at 25°C, the same strain showed dusty green conidia with light-brown colour on reverse. An unusual morphology of *P. oxalicum* was observed at 15°C with production of yellow coloured exudates and creamish periphery, the colonies grew vertically and stayed smaller than 5 cm (Fig. 4.1). These morphological variations were noticed following the incubation of the isolate under various temperature regimes, which might be linked to stress response as in other filamentous fungi (Verant, *et al.* 2012).



(A) **(B)**

Fig. 4.1 Petriplates showing growth of psychrophilic *P. oxalicum* (A) and *P. citrinum* with production of yellow coloured and colourless exudates, respectively.

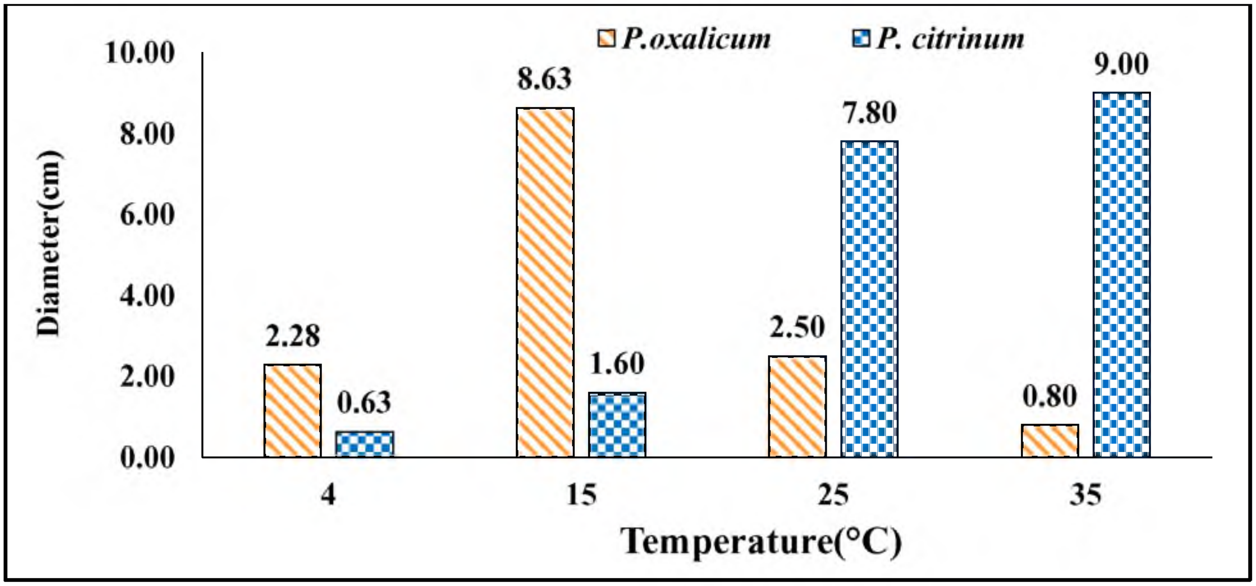


Fig. 4.2 (A) Temperature dependent growth of both *Penicillium* strains on PDA petriplates. Growth was measured in terms of colony diameter (cm).

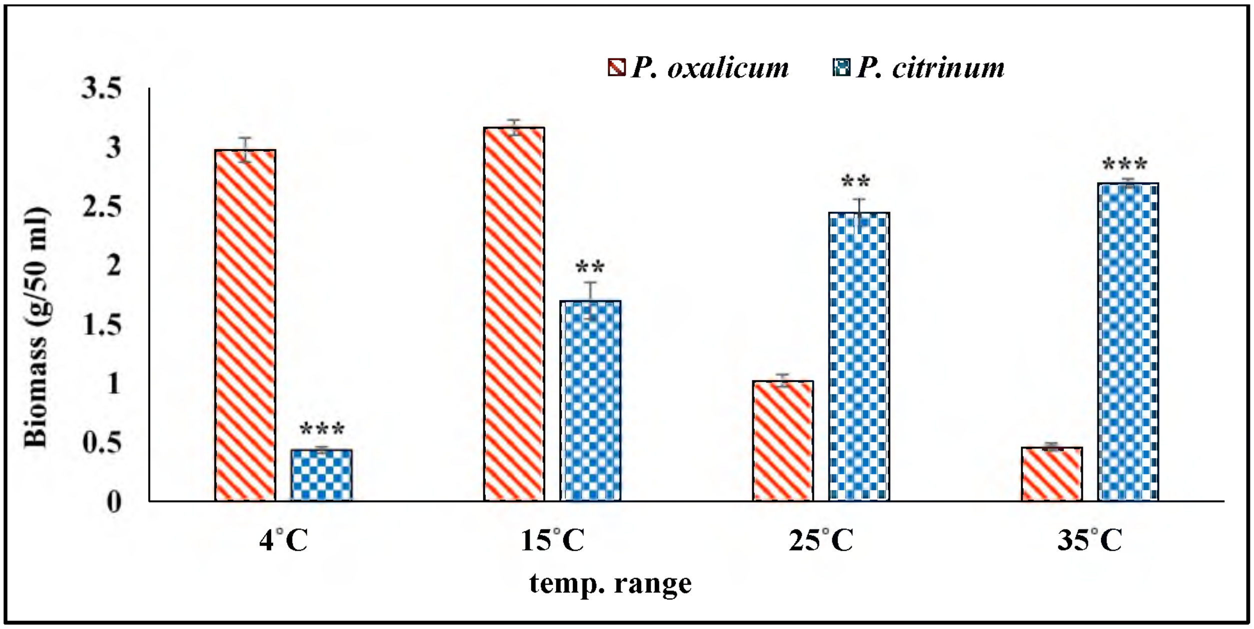


Fig. 4.2 (B) Temperature dependent change in Biomass (g/ 50ml) of both *Penicillium* strains grown in basal medium. Student's paired sample't-test' showing significant difference between both *Penicillium* strains, *p<.0.05, **p<0.01, ***p<0.001. Error bar showing the mean±SD.

4.3.2 Temperature dependent Filter paper assay for production of *Cellulase* enzyme (FPU Assay)

Temperature dependent FPU enzyme assay was performed for production of cellulase enzyme in both *Penicillium* strains. The results revealed higher production of cellulase enzyme (0.76 ± 0.5 units ml^{-1}) by the psychrophilic *P. oxalicum* at its optimum growth temperature (15°C) when compared with that in the mesophilic *P. citrinum* (0.66 ± 0.5 units ml^{-1}) at high temperature 35°C. The pattern of temperature dependent enzyme production in psychrophilic *P. oxalicum* and mesophilic *P. citrinum* was in the order of; 15> 25 > 35> 4°C and 35>25>15>4 °C respectively (Fig. 4.3 (A & B). The results suggested that level of cellulase production in both the strains was dependent on their respective optimum growth supporting temperature. However, overall production of cellulase enzyme was higher in *P. oxalicum* than *P. citrinum*. It has been reported that organisms adopt to the stresses by production of extracellular enzymes, antifreeze proteins, accumulation of sugars and polyols, change in lipid/fatty acids and production of secondary metabolites as a part of their survival strategy (Robinson, 2001). *Aspergillus* and *Penicillium* species dominate the low temperature and saline habitats (Cantrell, *et al.*, 2011) by adapting to peculiar physiological condition. Diversity and applications of various species of *Penicillium* from low temperature environments including glaciers, such as ice from Kongsfjorden (Gunde-Cimerman, *et al.* 2003), Arctic glaciers (Sonjak, *et al.* 2006), Indian Himalayas (Pandey, *et al.*, 2016), cold environments (Margesin and Miteva, 2011), and Antarctica (Konstadinova, *et al.* 2009) has been reported. Most of the cold adaptive fungal species are likely to be inclined towards low temperature for the production of various enzymes and have potential to produce enzymes that are different from the mesophilic species in their catalytic nature and are promising to have advantage in several industrial and biotechnological applications (Feller and Gerday 1997).

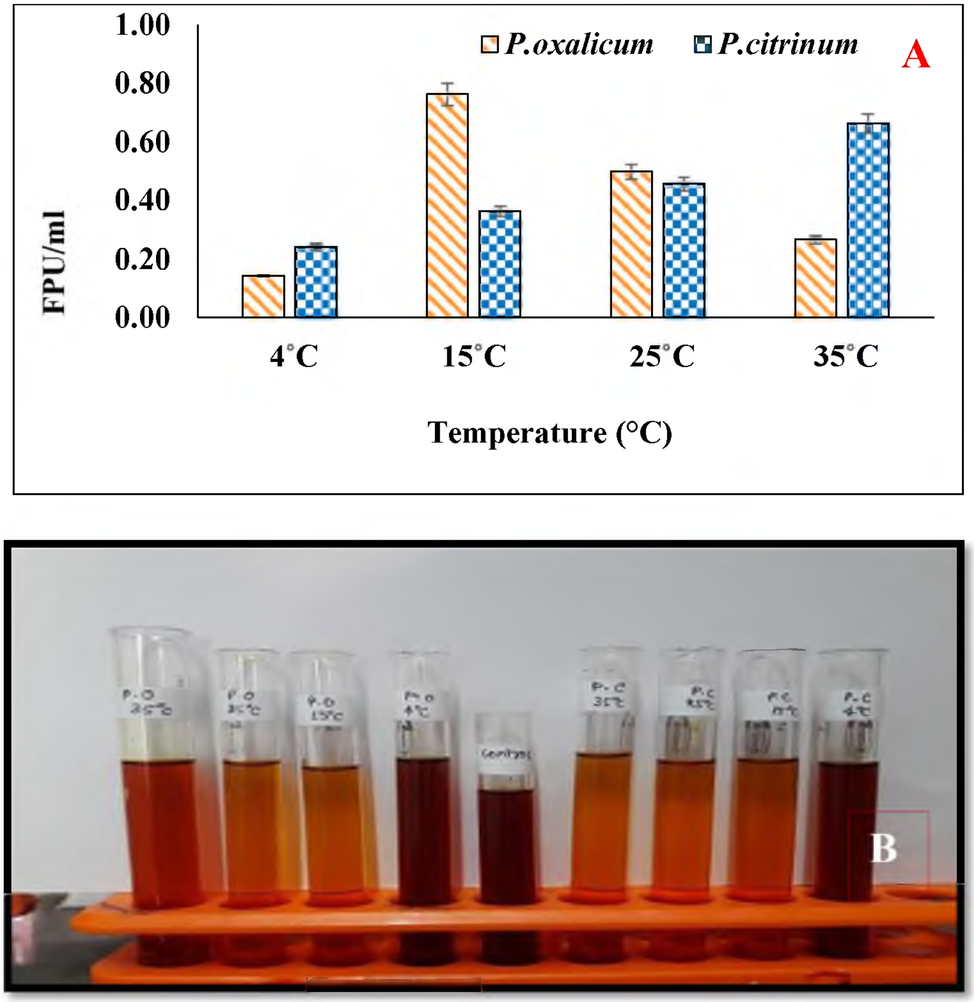


Fig. 4.3 (A&B). Temperature dependent (4-35°C) *Cellulase* activity (FPU/ml) in both the *Penicillium* strains.

4.3.3 Temperature dependent changes in Lipid profile of *Penicillium* strains

The amount and types of lipids in individual fungus may vary from organism to organism as they are known to change with the age, stages of development, nutrition, and environmental conditions (Weete, 1981, Losel, 1989). However, fungi contain abundant oleic acid, palmitic acid, and linoleic acid as major fatty acids, while stearic acid, linolenic acid, palmitoleic acid are present in smaller amounts (Shaw, 1966). In the present study, quantitative analysis of lipid extracts of both *Penicillium* strains grown under different temperature condition (4, 15, 25°C and 35°C) was carried out. A comparison of lipid profile of both strains grown under temperature stress conditions (4 &

35°C) showed presence of major unsaturated fatty acids like linoleic acid and oleic acids. Saturated fatty acid Palmitic acid, which constituted about 80% of the total fatty acid, varied in its concentration with changing growth temperature (Lomascolo, *et al.*, 1994). Major saturated fatty acids obtained in *P. oxalicum* grown at 4°C were Palmitic, Eicosanoic acid and Docosanoic acid, which significantly declined in the cells when grown at 35°C (Fig.4.23). On the contrary, saturated fatty acids in *P. citrinum* grown at 4°C were Decanoic acid, Octadecanoic acid, and Docosanoic acid. A shift in growth temperature of *P. citrinum* from 4°C to 35°C resulted in marginal changes in these fatty acids except Palmitic acid which registered about a four-fold increase with the rise in growth temperature from 4°C to 35°C. (Fig. 4.23). The major unsaturated fatty acids in the *P. oxalicum* grown at 4°C were found to be Linoleic acid, Oleic acid, and Erucic acid, which showed a significant decrease in the unsaturated fatty acid contents when grown at 35°C. However, Nervonic acid registered several fold increase in its content with rise in the growth temperature from 4°C to 35°C. The major unsaturated fatty acid in the mesophilic *P.citrinum* grown at 4°C were Linolenic acid and Linoleic acid. These fatty acids showed marginal increase in the concentration with shift in growth temperature from 4°C to 35°C. However, saturated fatty acids like Elaidic acid, Oleic Acid and Erucic acid contents registered significant rise with shift in growth temperature from 4°C to 35°C. The presence of cis-5,8,11,14 Eicosatetraenoic acid (Arachidonic acid) in the psychrophilic *P. oxalicum,* was exceptionally higher in the cells grown at 4°C, but was found to be absent in *P. oxalicum* strain grown at 35°C. On the other side, presence of this economically important ω-6 polyunsaturated fatty acids (PUFA) in *P. citrinum* cells grown under both high and low temperature growth conditions was negligible. On the contrary, Docosahexaenoic acid was found to be very high in *P. citrinum* cells grown at high temperature (35°C) as compared to that grown at low temperature. Among the triglycerides, 1-monomyristoyl-rac-glycerol and 1-

monostearoyl-rac-glycerol were found to be higher in the psychrophilic *P. oxalicum* strain than *P. citrinum* grown at 4°C (Fig. 4.22.). But the overall triglyceride contents in the *P. oxalicum* declined at 35°C. On the contrary, 1-monomyristoyl-rac-glycerol, 1-monopalmitoyl-rac-glycerol and 1-monostearoyl-rac-glycerol, in the mesophilic *P. citrinum* showed significant increase when the growth temperature was raised from 4°C to 35°C. Overall results demonstrated the triglycerides content was much higher in *P. citrinum* grown at 35°C, while triglyceride content in *P. oxalicum* was higher in cells grown at 4°C. The ratio of unsaturated to saturated fatty acids in *P. oxalicum* was higher at 4°C as compared to that in the cells grown at 35°C. The ratio of unsaturated to saturated fatty acids in *P. citrinum* was little altered due to rise in the temperature from 4°C to 35°C.

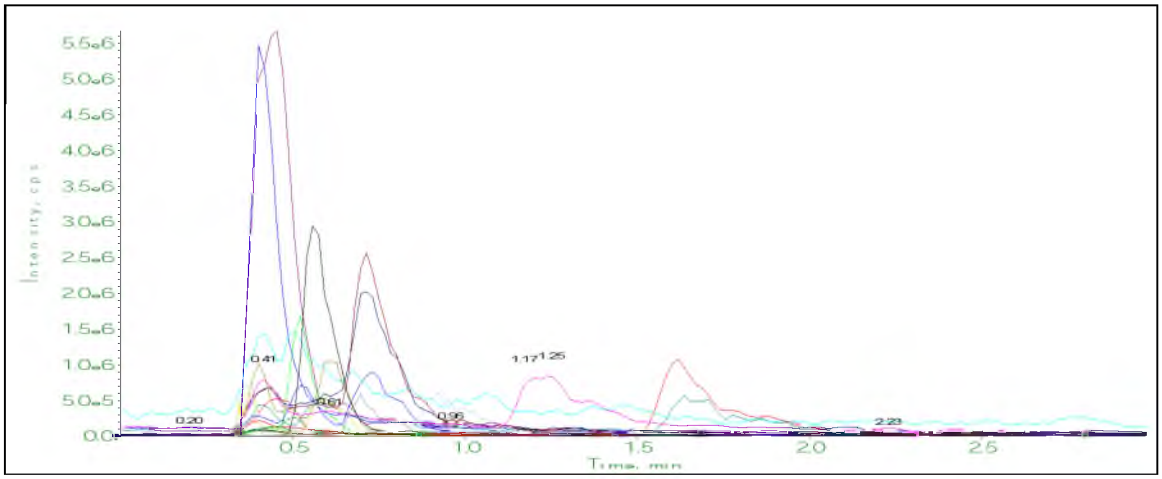


Fig. 4.4 LC-MS/MS spectra of standard fatty acids.

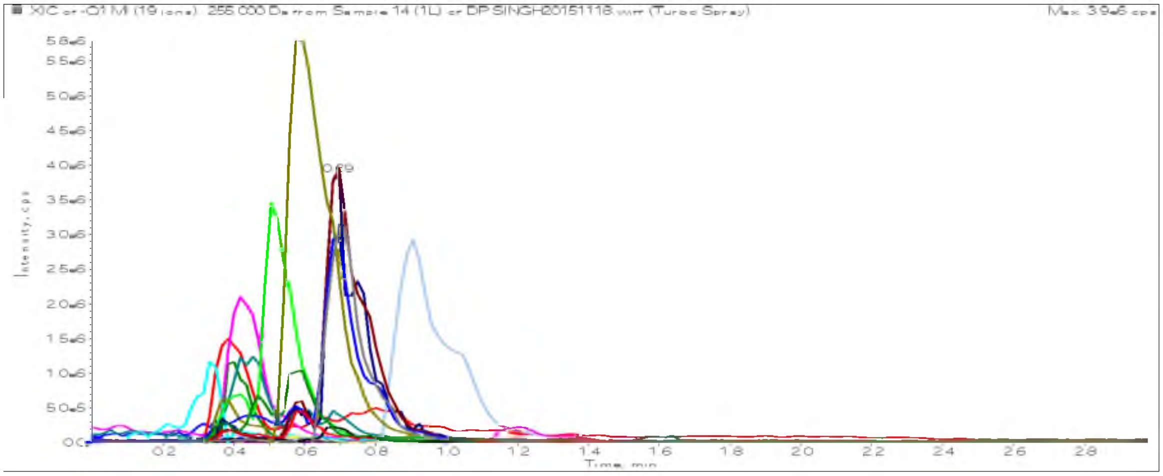


Fig. 4.5 LC-MS/MS spectra of fatty acids of psychrophilic *P. oxalicum* grown at 4°C.

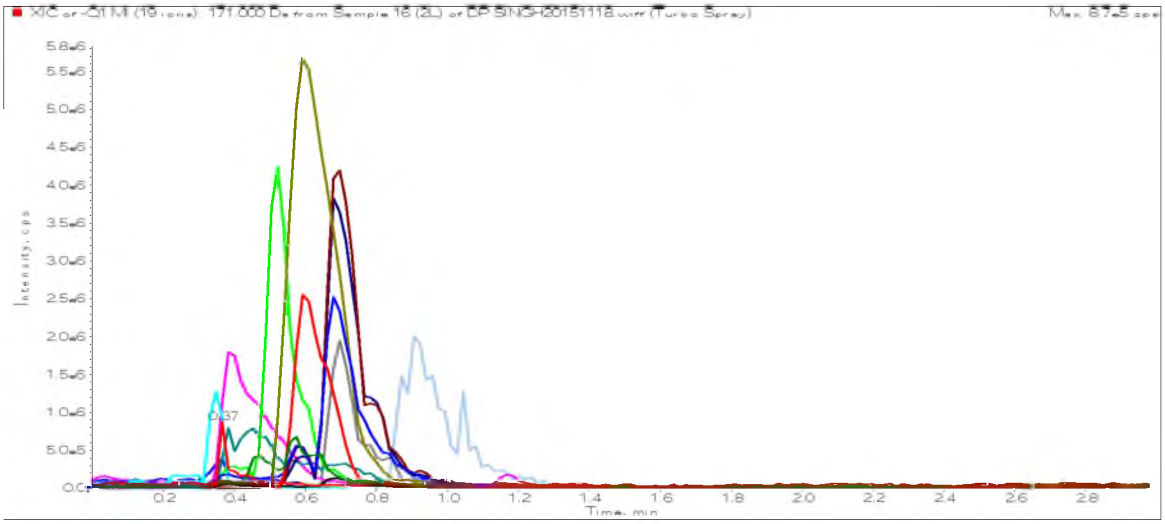


Fig. 4.6 LC-MS/MS spectra of fatty acids of psychrophilic *P. oxalicum* grown at 15°C.

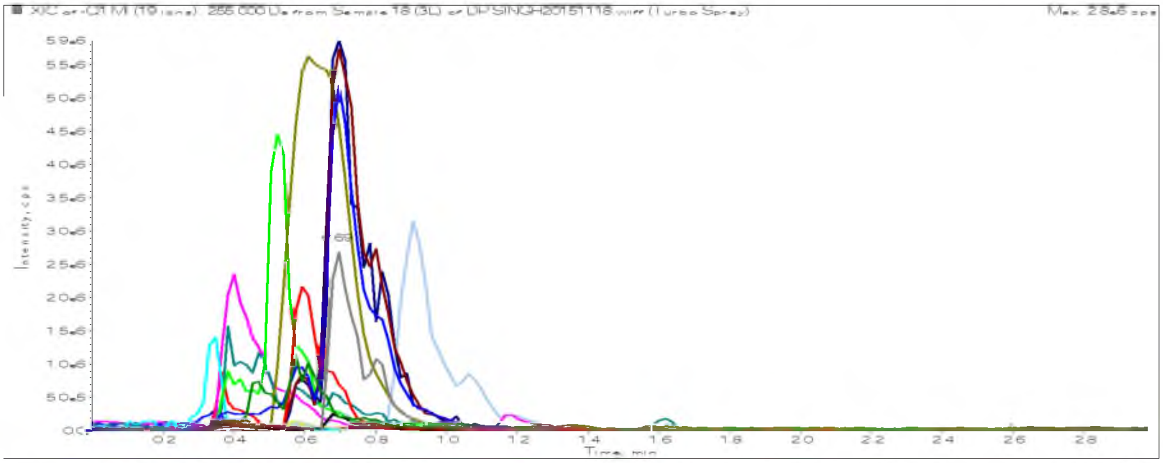


Fig. 4.7 LC-MS/MS spectra of fatty acids of psychrophilic *P. oxalicum* grown at 25°C.

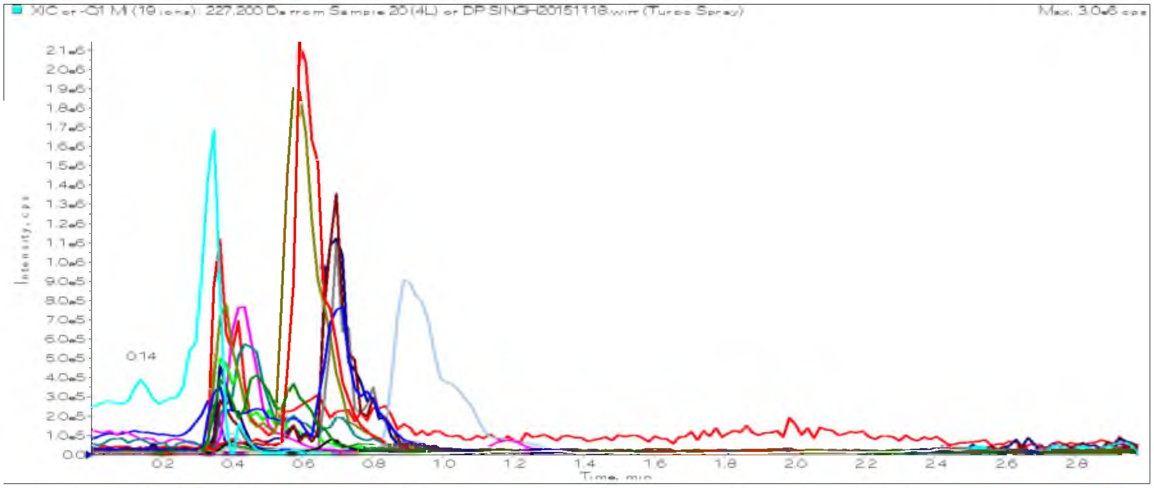


Fig. 4.8 LC-MS/MS spectra of fatty acids of psychrophilic *P. oxalicum* grown at 35°C.

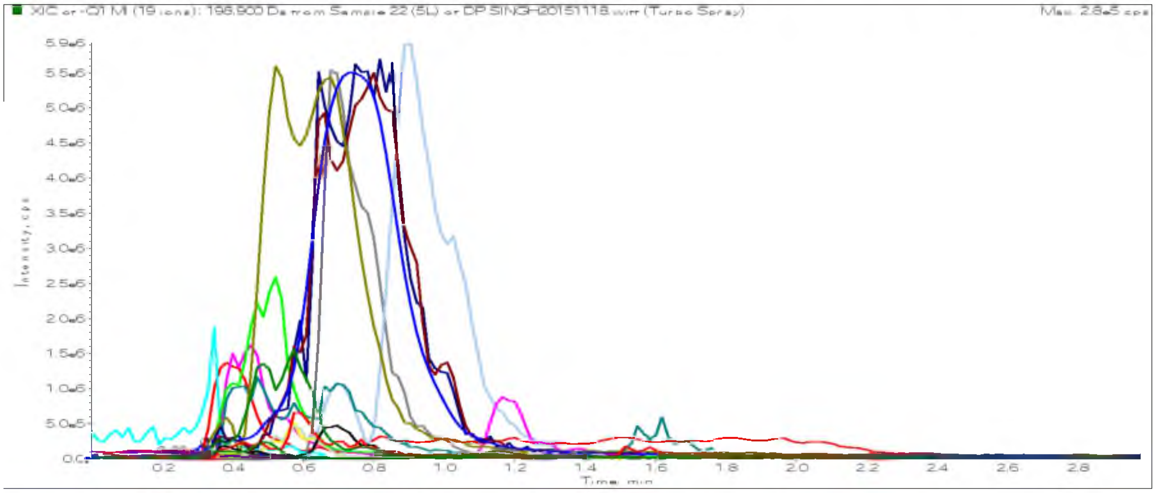


Fig. 4.9 LC-MS/MS spectra of fatty acids of mesophilic *P. citrinum* grown at 4°C.

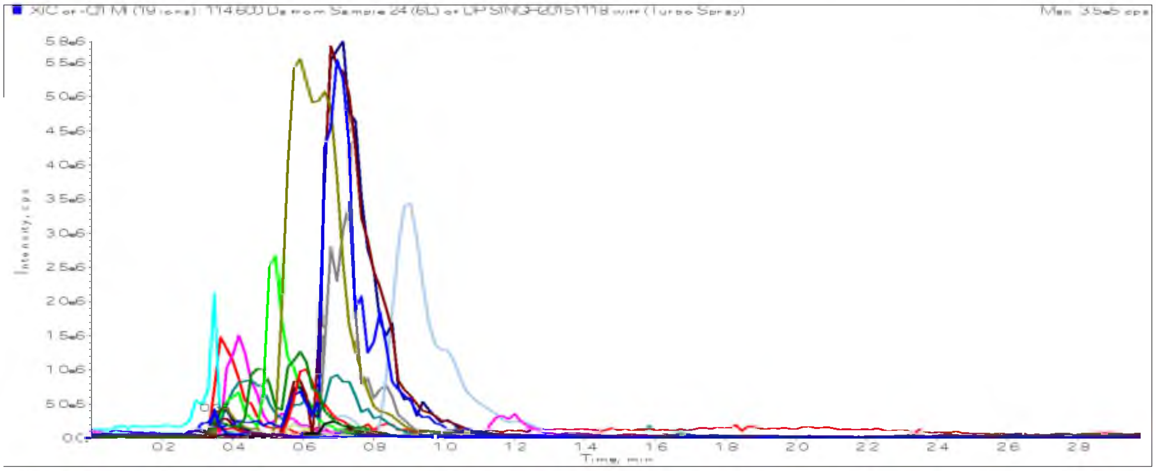


Fig. 4.10 LC-MS/MS spectra of fatty acids of mesophilic *P. citrinum* grown at 15°C.

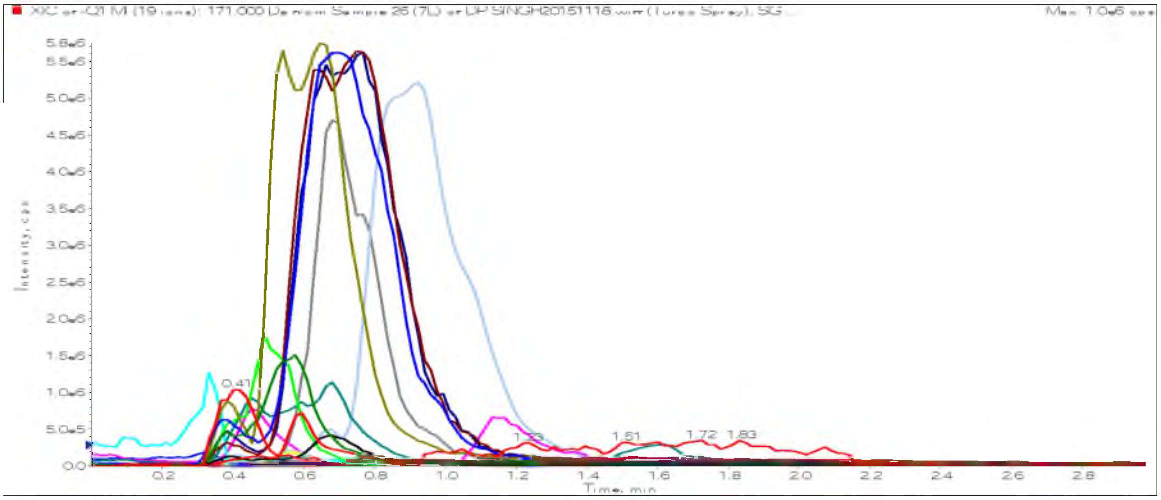


Fig. 4.11 LC-MS/MS spectra of fatty acids of mesophilic *P. citrinum* grown at 25°C.

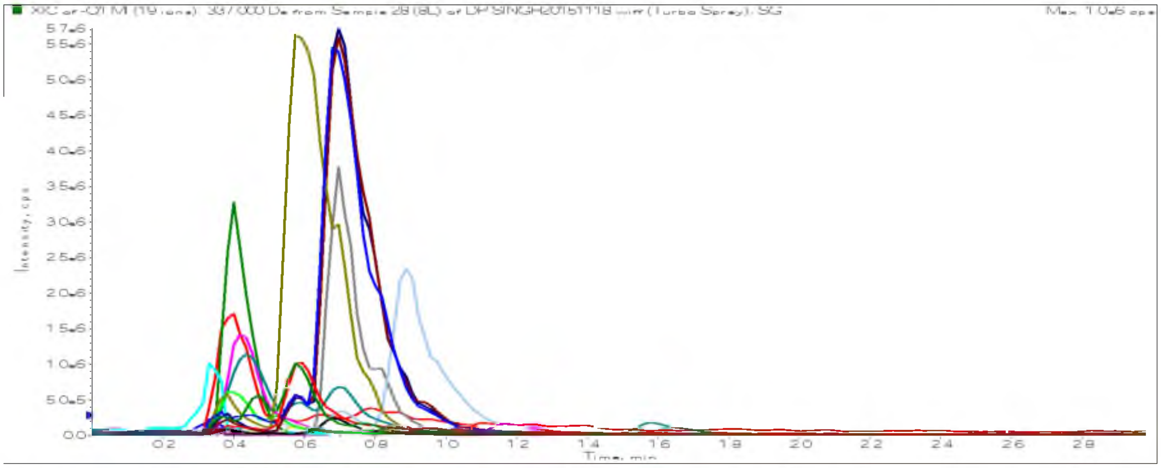


Fig. 4.12 LC-MS/MS spectra of fatty acids of mesophilic *P. citrinum* grown at 35°C.

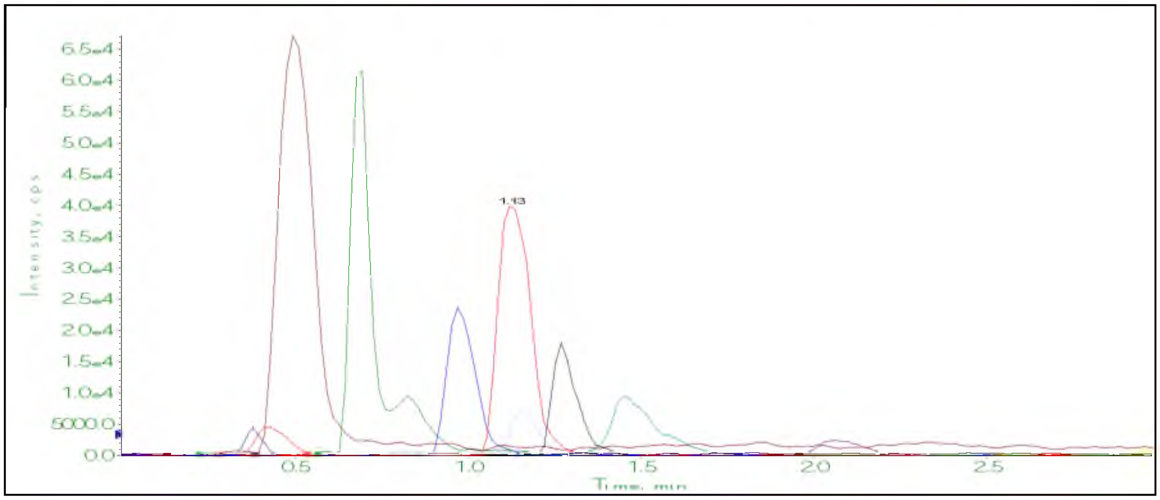


Fig. 4.13 LC-MS/MS spectra of standard Triglycerides.

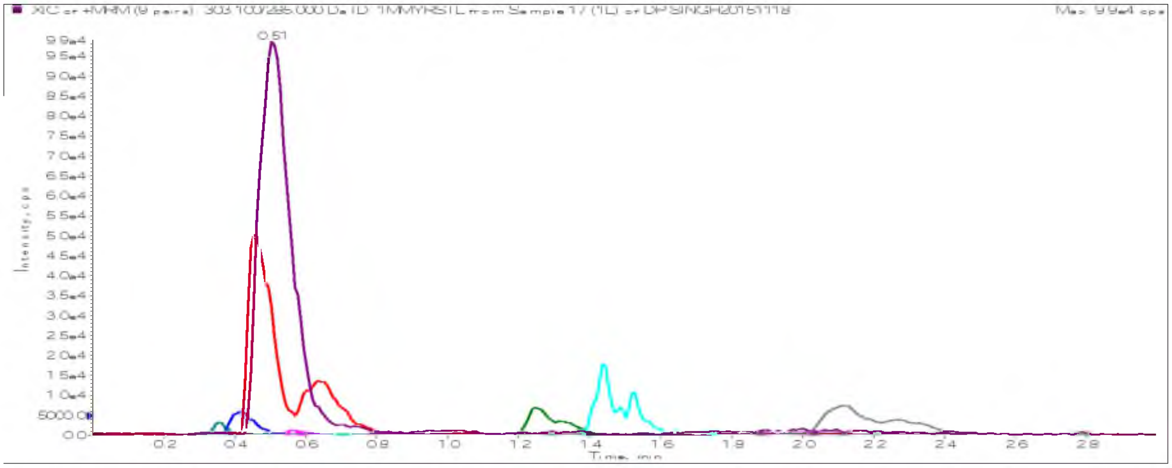


Fig. 4.14 LC-MS/MS spectra of Triglycerides of psychrophilic *P. oxalicum* grown at 4°C.

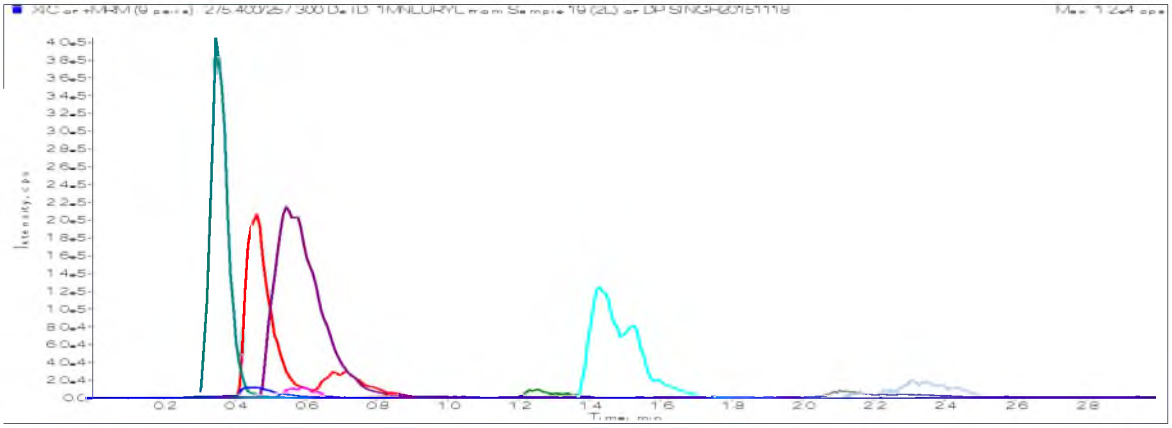


Fig. 4.15 LC-MS/MS spectra of Triglycerides of psychrophilic *P. oxalicum* grown at 15°C.

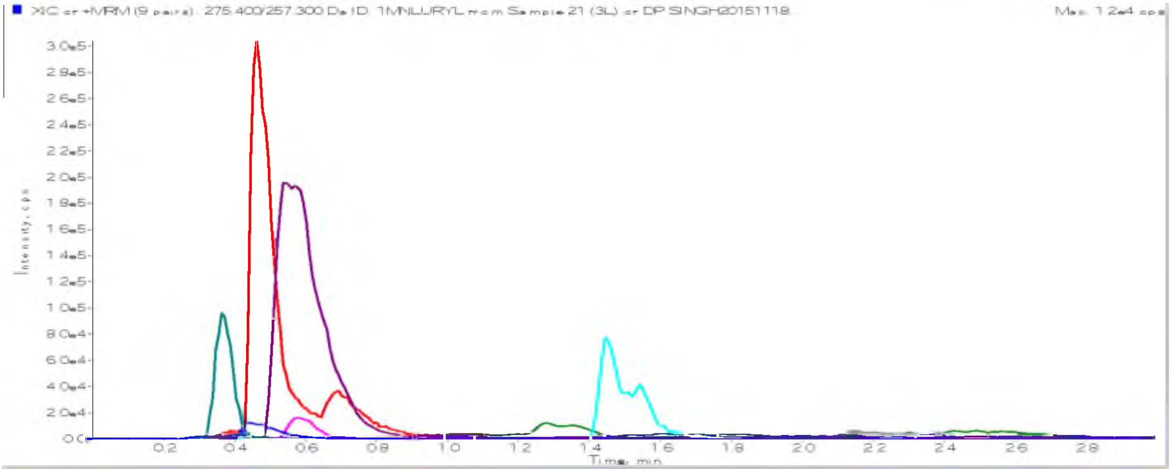


Fig. 4.16 LC-MS/MS spectra of Triglycerides of psychrophilic *P. oxalicum* grown at 25°C.

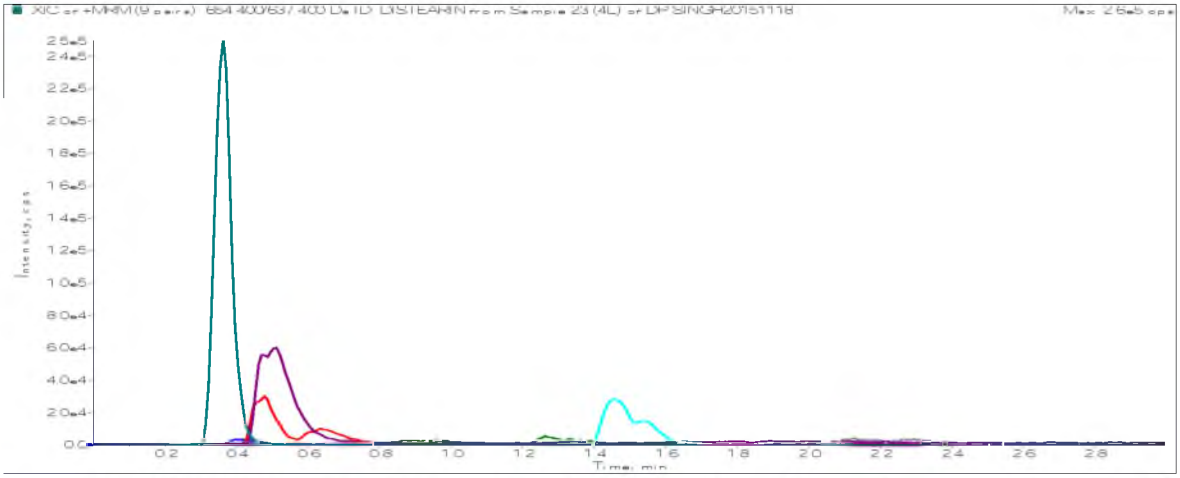


Fig. 4.17 LC-MS/MS spectra of Triglycerides of psychrophilic *P. oxalicum* grown at 35°C.

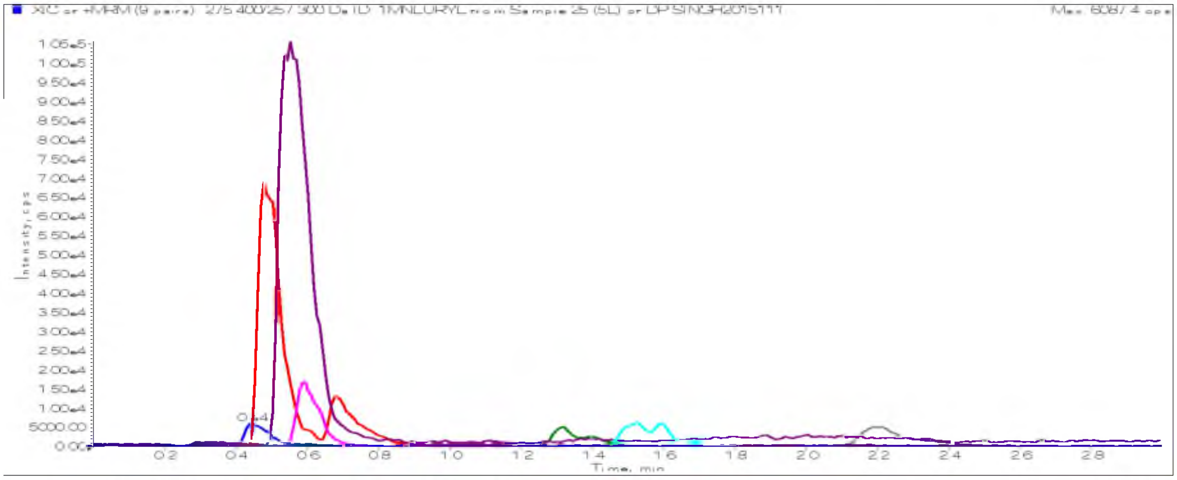


Fig. 4.18 LC-MS/MS spectra of Triglycerides of mesophilic *P. citrinum* grown at 4°C.

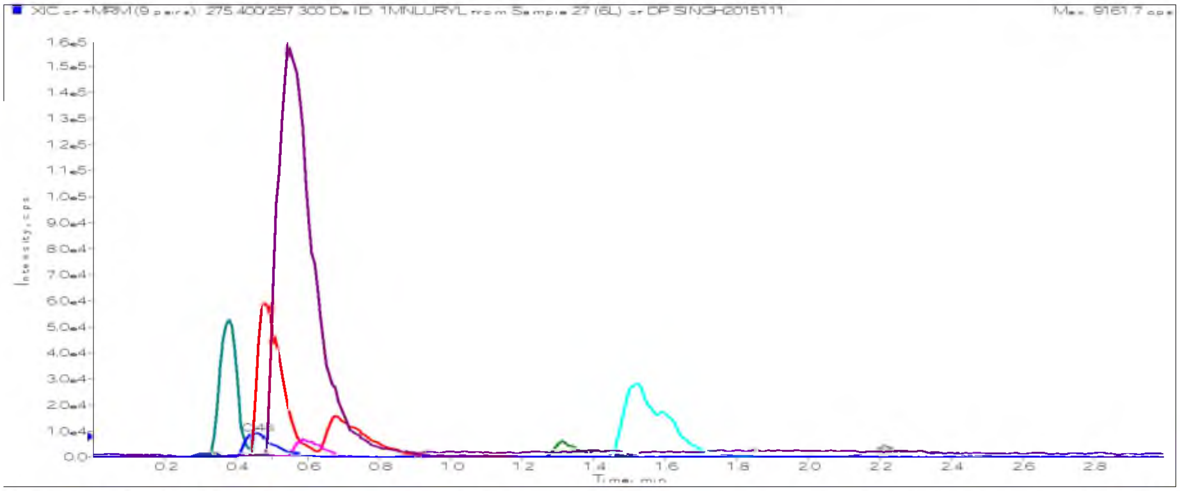


Fig. 4.19 LC-MS/MS spectra of Triglycerides of mesophilic *P. citrinum* grown at 15°C.

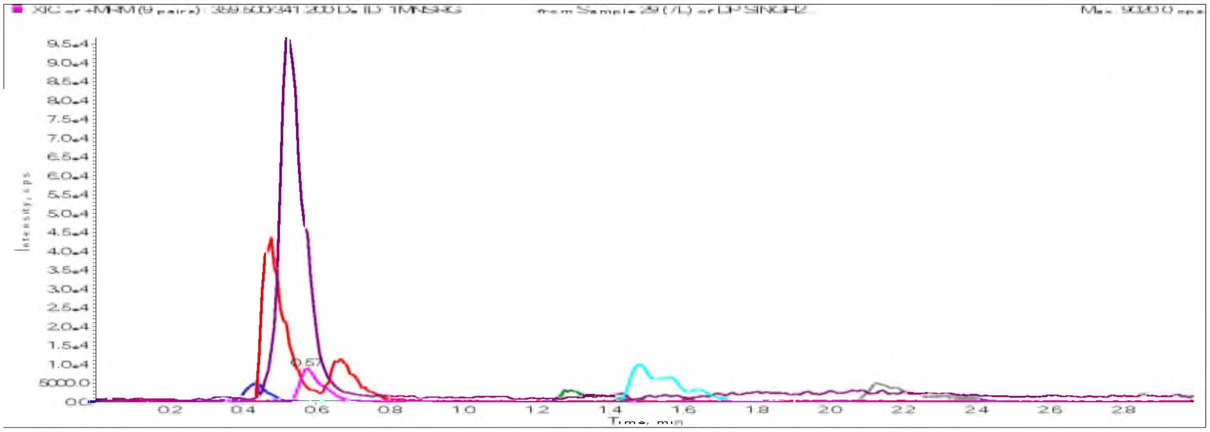


Fig. 4.20 LC-MS/MS spectra of Triglycerides of mesophilic *P. citrinum* grown at 25°C.

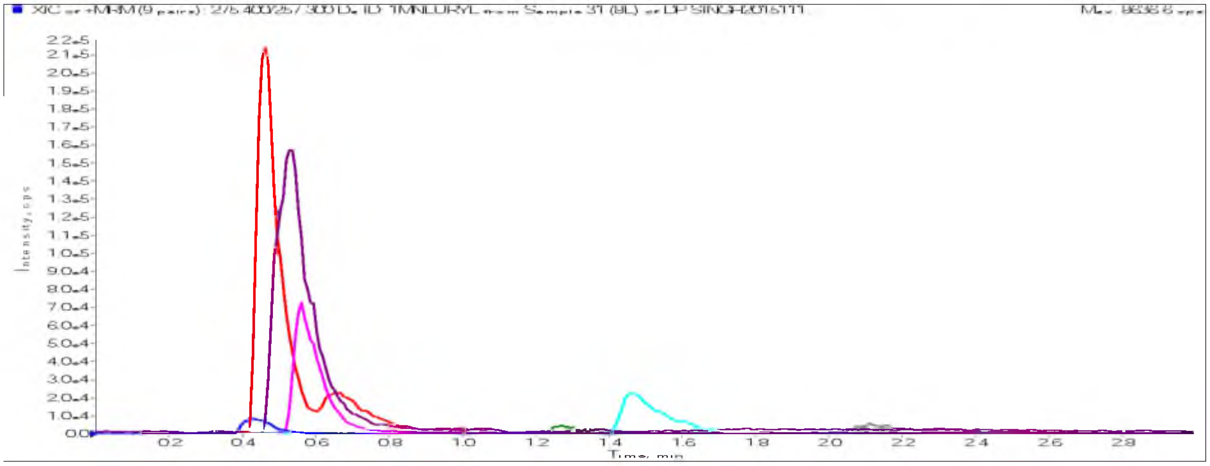
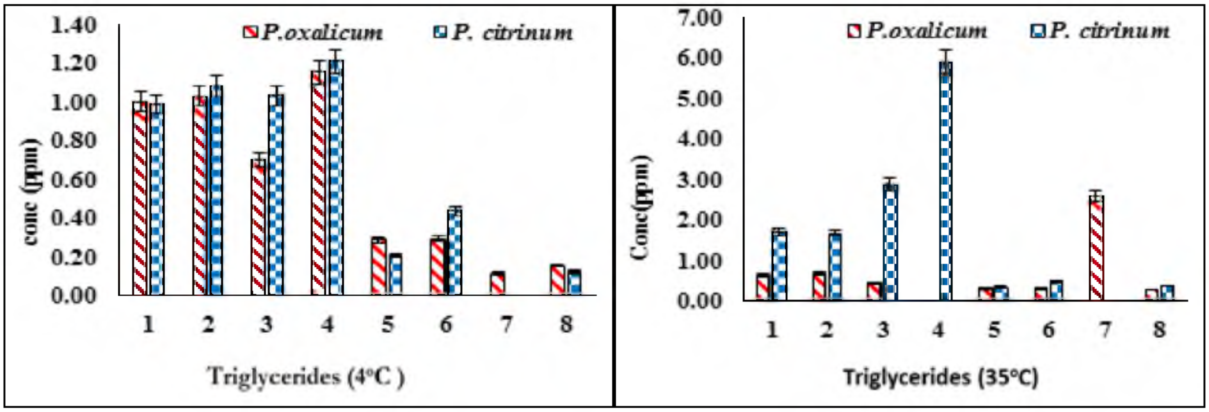


Fig. 4.21 LC-MS/MS spectra of Triglycerides of mesophilic *P. citrinum* grown at 35°C.



1. 1 -monolauroyl-rac-glycerol **2**. 1-monomyristoyl-rac-glycerol **3**. 1-monopalmitoyl-rac glycerol (C16: O) **4**. 1-monostearoyl-rac-glycerol **5**. Dilaurin **6**. Dimyristin (C14: O) **7**. Distearin (C18: O) **8**. Trilaurin.

Fig. 4.22 LC MS/MS analysis of Triglycerides of both *Penicillium* strains grown at 4°C and 35°C temperature.

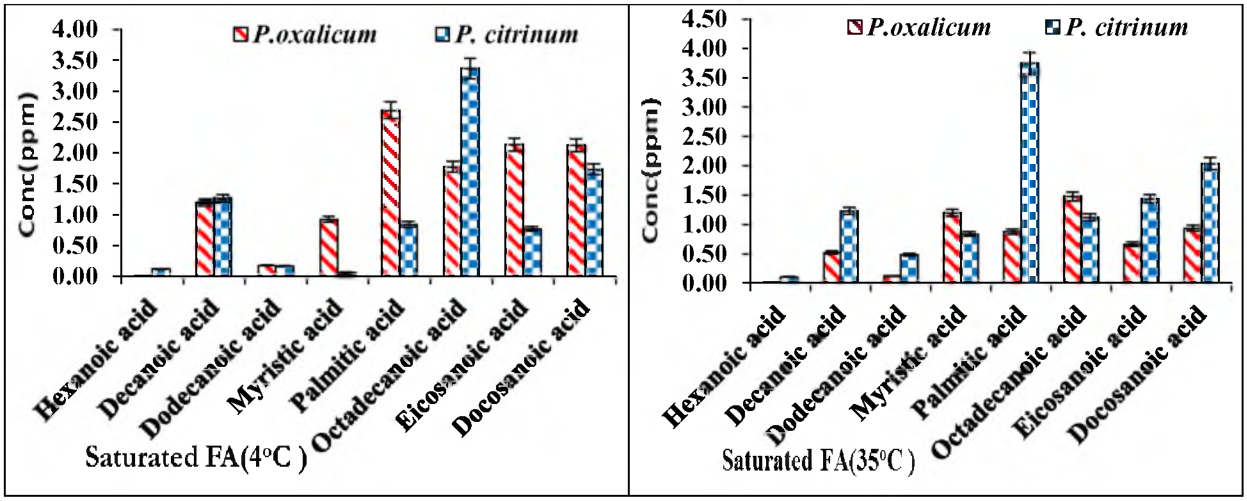


Fig. 4.23 LC MS/MS analysis of Saturated Fatty acids of both *Penicillium* strains grown at 4°C and 35°C temperature.

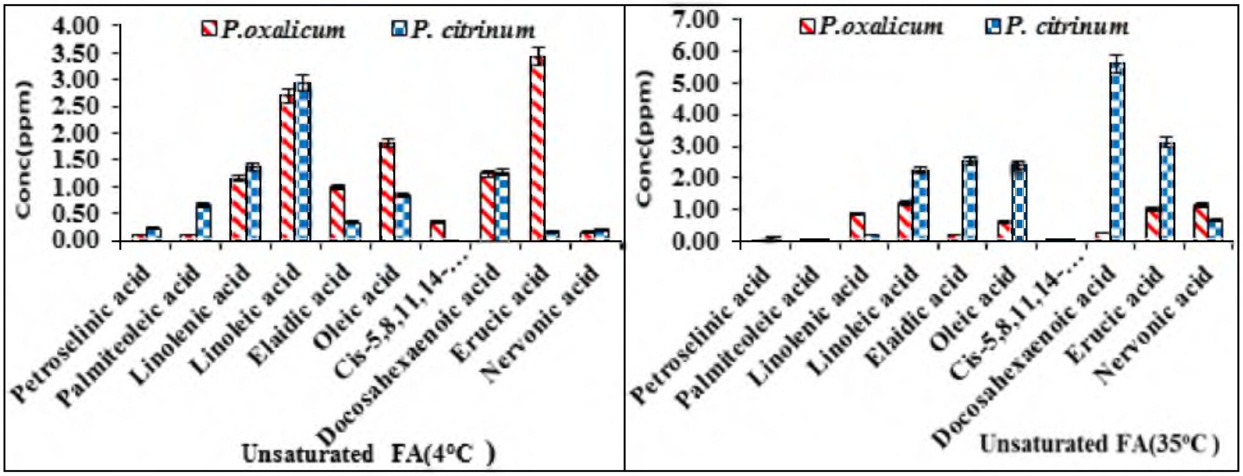


Fig. 4.24 LC MS/MS analysis of Unsaturated Fatty acids of both *Penicillium* strains grown at 4°C and 35°C temperature.

4.3.4 Screening of secondary metabolites produced by *Penicillium* strains under Temperature stress

A fungal secondary metabolites are biochemical compounds produced by a limited number of microorganism as a part of their survived strategy (Keller, *et al.* 2005). A profile of secondary metabolites consists of toxins, antibiotics, and other extracellular compounds. The fungal compounds produced in the secondary metabolites can be used for chemotaxonomic and classification purposes. They are of great physiological importance to the fungus because all the secondary metabolites have power of differentiation. Various studies, have reported the secondary metabolite production by *Penicillium* strains isolated from Permafrost deposits and with their absorption under UV λ_{max} (Zhelifonova *et al.*, 2009; Antipova *et al.*, 2010; Kozlovskii *et al.*, 2013). So, here in the present study, the screening and extraction of secondary metabolites of both *Penicillium* strains grown at different temperature were performed. The fungal culture grown under different temperature stress conditions (4-35°C) in a basal medium was withdrawn at different time interval (3rd, 7th, 15th, 21st, and 28th) days and they were subjected to a liquid-liquid extraction with ethyl acetate (EtOAc). The results showed that the absorption spectra (λ 250-400 nm standardized) of EtOAC extracts from *P. oxalicum,* grown at different temperatures (4, 15, 25, and 35°C), showed distinct increase in absorption wavelength of 275 nm with rise in growth temperature. The absorption spectra of secondary metabolites obtained from the culture broth incubated at 4, 15°C showed reduced absorbance with spectral shift in the absorption peak at λ 260 nm (Fig. 4.25 A). On the contrary, extracted secondary metabolites of *P. citrinum* incubated at different temperature showed increase in absorption at λ 328 nm incubated at 15, 25 and 35°C, while the metabolite extract obtained at temperature 4°C showed negligible absorption. The secondary metabolite extract from mesophilic *P. citrnium* grown at 25°C showed higher absorption peak at λ 258 nm which was subdued under other temperature conditions (Fig. 4.25 B.). Results revealed that the production of secondary metabolites by both *Penicillium* strains, was found to be higher at higher growth temperature (35°C) when compared with obtained from low temperature grown cells. These results revealed that production of secondary metabolites in the cold tolerant *P. oxalicum* strain was also enhanced with increase in the temperature condition. While imposition of low temperature stress on mesophilic *P. citrinum* could not elicit the similar response and exhibited maximum production of metabolites only under optimum growth conditions.

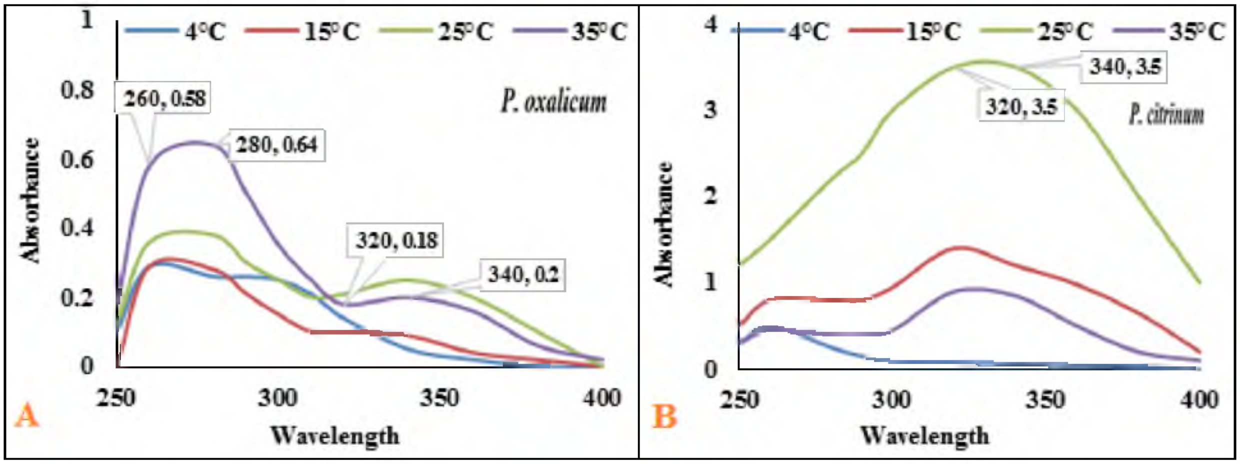


Fig. 4.25 (A&B). Absorbance spectra of EtAOc extract of secondary metabolites from psychrophilic *P. oxalicum* (A) and *P. citrinum* (B) grown under varying temperature conditions.

4.3.5 FTIR analysis of *Penicillium* strains

It is well known that FTIR spectroscopy is a promising, physico chemical analytical method based on the light-matter interaction for the characterization of the energy levels of the atomic bond vibrations and the signals to the main macromolecular constituents including lipids, polysaccharides, nucleic acids, proteins etc. Moreover, the spectral information attained allows attribution, in a qualitative and quantitative mode, thus the FTIR spectrum obtained represents a global "molecular fingerprint" which can be used for characterization, differentiation and identification of microorganisms (Lecellier, *et al.* 2014). Shapaval, *et al.* (2010, 2013) reported the applications of FTIR spectroscopy in the field of identification of fungi at the level of species and genera. In order to achieve the temperature dependent changes in macromolecular constituents of fungi, the FTIR spectra (4000 to 500 cm^{-1}) of biomass of both *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum*) grown under different temperature conditions were compared (Figure 4.28-31). The results exhibited the temperature dependent changes in several functional groups associated with the fungal cell membrane (Klein, *et al.* 1999; Szalontai, *et al.* 2000). In case of psychrophilic *P. oxalicum*, the changes in IR absorption peaks appearing at 3437-420, 2930-27, 1740 cm^{-1} were assigned to asymmetrical stretching of O-H, C-H and C=O of lipids/ester and phospholipids respectively (Fabian, *et al.*, 1995). The temperature dependent changes in IR absorption bands suggested for alteration in functional groups associated with membrane function under different temperature conditions, were recorded at IR peaks at 1078, 1650, 1545 and 1040 cm^{-1} which were assigned for Amide I (predominantly the C=O stretching vibration of the protein) (Sukuta and Bruch,1999;Andrus, 2006), Amide II (Yano et al, 2000; Paluszkiewicz and Kwiatek, 2001), and symmetrical PO_2- stretching in RNA and DNA respectively (Fabian, *et al.* 1995). The result on IR absorption spectrum of psychrophilic *P. oxalicum* grown in the different temperature (4°C-35°C) showed increased absorption as well as shift in their IR peaks at wavenumbers 3377.9 to 3422, 2931 to 2927, 1652 to 1632, 1551 to 1543.9, 1078.2 to 1074.3, 1151.4 to 1150 and 1032.5 to 1042.0 cm^{-1} with the rise in growth temperature (Fig. 4.26-27). The result on IR absorption spectrum of mesophilic *P. citrinum* grown in the different temperature (4°C-35°C) showed increased absorption as well as shift in the IR peaks with the rise in growth temperature at wavenumbers 3434.5 to 3375.5, 2937 to 2928.3, 1652.4 to 1648.2, 1524.3 to 1542.7, 1156.2 to 1154, 1036.1 to 1074.3 cm^{-1} (Fig. 4.28-29). The Two additional peaks emerged at 2884 and 1743.4 cm^{-1} wavelength in the biomass of low temperature grown mesophilic *P. citrinum,* assigned to stretching of C-H (Schulz and Baranska, 2007) and C=O functional groups of lipids respectively (Sukuta and Bruch, 1999).

Further, a comparison of IR spectra of biomass of psychrophilic *P. oxalicum*, grown at 4°C and 35°C, showed emergence of unique IR peak (1456 cm^{-1}) at 35°C, mainly derived from CH_2 group bending in the lipids without contribution from proteins (Cakmak, *et al.*, 2006). This absorbance peak was suppressed in IR spectrum of *P. oxalicum* grown at 4°C, indicating reduced content of saturated lipids (1456 cm^{-1}). On the other hand, the IR absorbance at 1456 cm^{-1} wavenumber in *P. citrinum* was present in the cells grown under both high and low temperature conditions with minor peak shift in the absorption from 1456 to 1452 cm^{-1}. A precise protein-to-lipid ratio (A_{1650}/A_{1740} cm^{-1}) was calculated from the relative absorbance intensity at 1650 cm^{-1}, denoting carbonyl (C=O) group stretching in the proteins, and the IR absorbance at 1740 cm^{-1}, derived from bonding of fatty acyl group with glycerol backbone of the lipids (Shapaval, *et al.*, 2014). A higher ratio of protein-to-lipid in both the strains at high temperature (35°C) might be due to relatively enhanced synthesis of proteins to cope increased protein-lipid interaction (Klein *et al.* 1999). The ratio of Amide I / Amide II (A_{1650}/A_{1560} cm^{-1}) in the low temperature (4°C) grown biomass of *P. oxalicum* was higher when compared with the corresponding values of Amide I / Amide II ratio in the high temp. (35°C) grown biomass. On the other hand, the Amide I/ Amide II ratio in *P. citrinum* was little influenced due to rise in the growth temperature. These results suggested for conformational alteration in protein structure (Ishida and Griffith, 1993) of *P. oxalicum* in response to growth temperature, which was not detected in *P. citrinum*. The lipid/carbohydrate (A_{1740}/A_{1040} cm^{-1}) ratio calculated from IR absorbance spectra of fungal biomass grown under low and high temperature conditions (4° and 35°C, respectively). The results showed increase in the lipid/carbohydrate ratio (A_{1740}/A_{1040} cm^{-1}) in both the *Pencillium* strains grown at high temperature (35°C). This temperature dependent change in lipid/carbohydrate ratio could be due to high rate of respiration and carbohydrate consumption at high temperature. The overall results on IR spectra of biomass grown in different temperature conditions of *P. oxalicum* and *P. citrinum* exhibited temperature dependent alterations in functional groups present in cell membrane which might be contributing to survival strategy of individual strains during stress conditions.

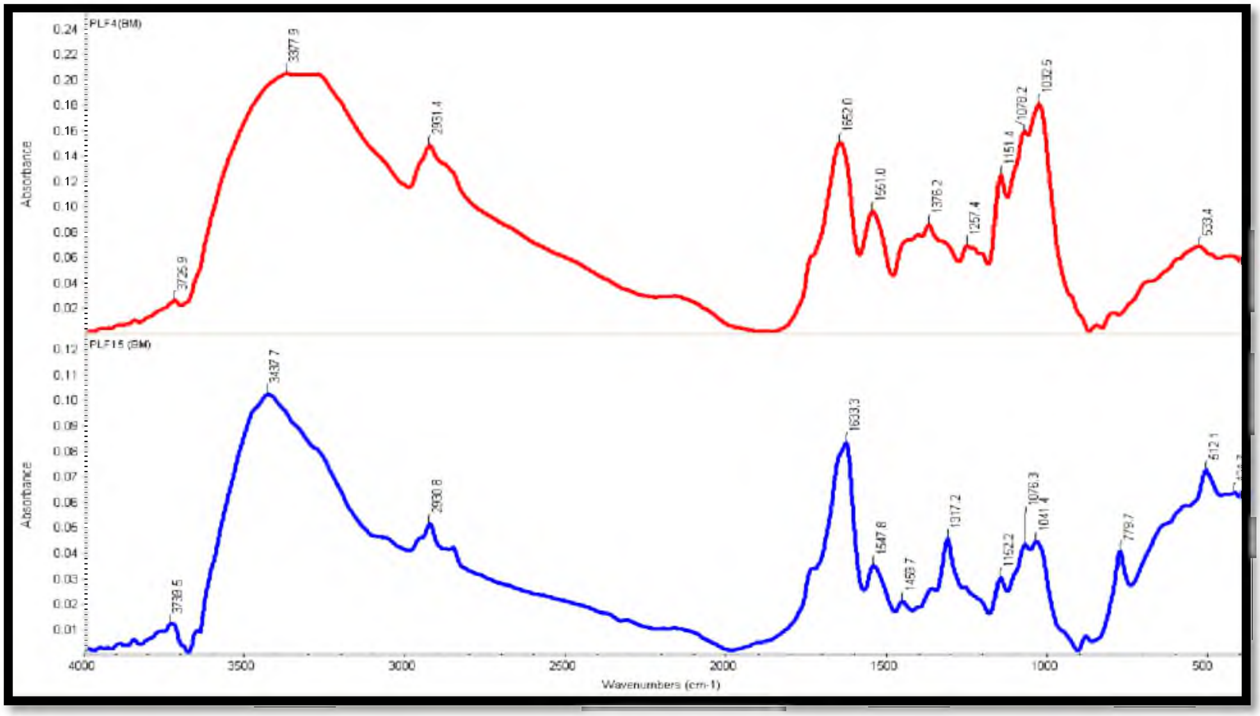


Fig. 4.26 FTIR absorbance spectra of *P. oxalicum* biomass grown at 4°C and 15°C.

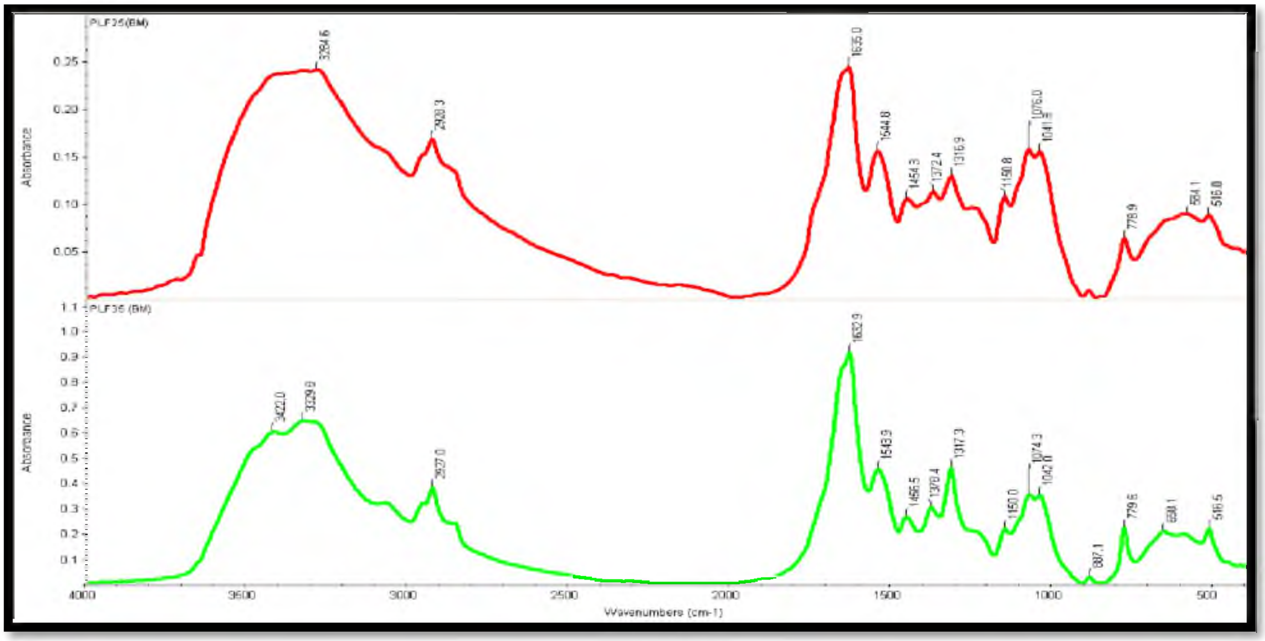


Fig. 4.27 FTIR absorbance spectra of *P. oxalicum* biomass grown at 25°C and 35°C.

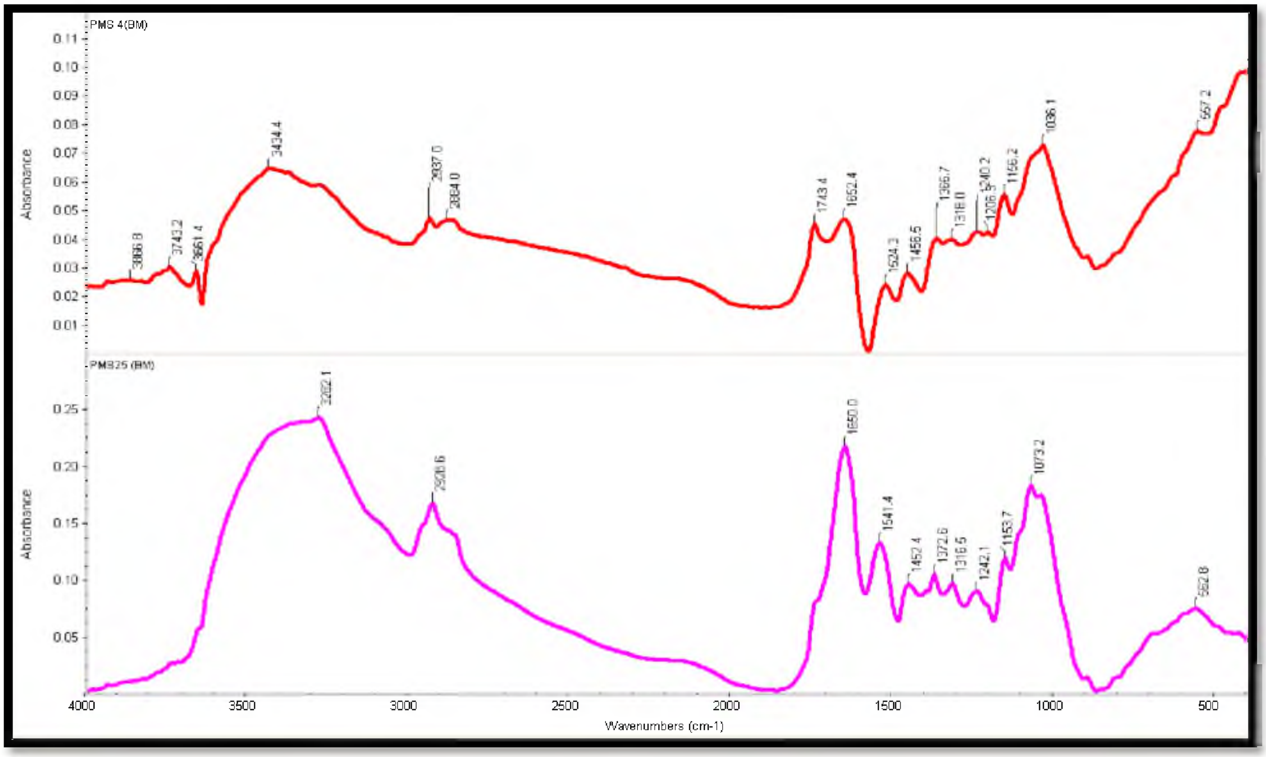


Fig. 4.28 FTIR absorbance spectra of *P. citrinum* biomass grown at 4°C and 15°C.

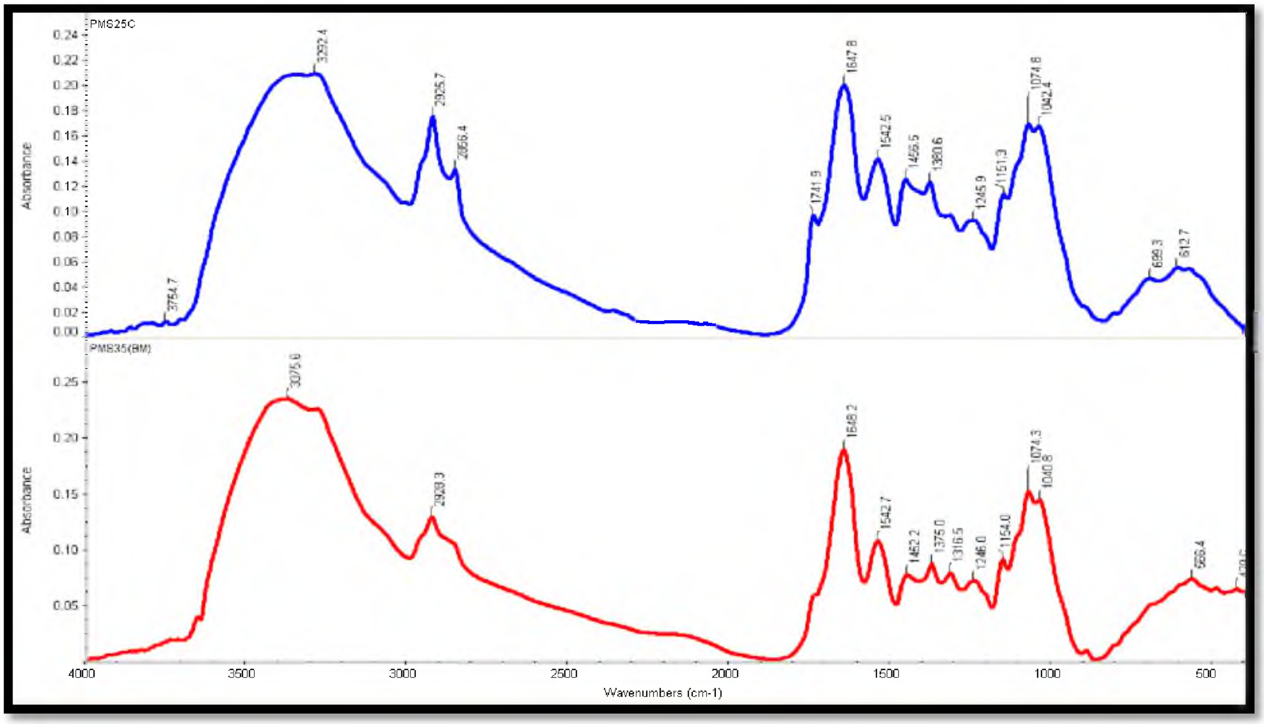


Fig. 4.29 FTIR absorbance spectra of *P. citrinum* biomass grown at 25°C and 35°C.

4.4 Conclusion

- The present work revealed that the psychrophilic *P. oxalicum* has potential to grow at extremely low temperature (4°C) with growth optima at 15°C. The mesophilic *P. citrinum* showed zero tolerance to low temperature and showed optimum growth between 30°C-35°C.
- The results on the production of *Cellulase* enzyme revealed higher production of cellulase enzyme (0.76 ± 0.5 units ml^{-1}) by the psychrophilic *P. oxalicum* at its optimum growth temperature (15°C), when compared with the mesophilic *P. citrinum* at its respective optimum temperature (35°C).
- Lipid profiling of psychrophilic *P. oxalicum* and mesophilic *P. citrinum* revealed abundance of mono fatty glycerols when both *Penicillium* strains were grown at 4°C. Di-fatty acid derivative of glycerolipids were also present. These were scarcely available when both the *Penicillium* strains were grown at higher temperature (35°C). Among the fatty acid, long chain (C_{16}-C_{22}) saturated fatty acids were abundantly present in both the *Penicillium* strains grown under two extreme temperature conditions. However, concentration of Palmitic acid was relatively higher in the mesophilic *P. citrinum* grown at high temperature. Among the unsaturated fatty acids, both *Penicillium* strains grown at low temperature (4°C) showed preferential synthesis of Linolenic acid, Linoleic, Oleic acid and Erucic acid. But at high temperature (35°C), the *P. citrinum*, unlike *P. oxalicum* strain continued to synthesize most of these unsaturated fatty acids. The LC/MS data showed that Docosahexanoic acid and Erucic acid were exclusively synthesized by the *P. citrinum* only

at high temperature, while *P. oxalicum* showed reduced percentage of these unsaturated fatty acid under the same temperature conditions (35°C).

- The present work on the production of secondary metabolites in both *Penicillium* strains showed temperature dependent increase in the quantity of secondary metabolites with rising temperature. These results further revealed that both *Penicillium* strains exhibit quantitative as well as qualitative changes in the contents of secondary metabolites due to changes in the temperature regime.
- FTIR spectra of biomass of both *Penicillium* strains also indicated stress induced species specific and stress dependent compositional alterations in the macromolecules such as lipid, carbohydrate and proteins, perhaps it was a part of their intracellular defense strategy.

CHAPTER-5 pH AND SALT STRESS

5.1 Introduction

Extremophiles are known to survive in extreme environments mainly in terms of temperature, pH, salt, pressure, etc. India is having prodigious diversity in the soil characteristics, specifically with respect to pH of the soil which help in sustenance of the growth and reproduction of microorganisms, especially for fungi. The environmental conditions such as pH, temperature, availability of nutrient, relative humidity, water activity and aeration, play substantial roles in improving production of fungal biomass and have critical influence on spore germination (Mcquilken, *et al.* 1997; Estrada, *et al.* 2000; Sautour, *et al.* 2001; Pardo, *et al.* 2005; Kope, *et al.* 2008). Ryan, *et al.* (2007) reported about the effect of pH on mycelial growth, which again confirmed the regulatory role of ambient pH in biomass production in many fungal species. Penalva and Arst, (2002) reported that many microorganisms, capable of growing over a wide pH range, express a particular gene to control the pH of their growth environment. For example, PacC encodes a transcription factor in *Aspergillus nidulans,* which works as an activator of genes expressed during growth under alkaline conditions and repressor genes those expressed under acidic conditions. The ambient pH may be involved in inhibition of microbial growth by affecting enzyme activity, nutrient availability and the proton gradient through the plasma membrane, as well as cell wall remodeling (Bilgrami and Verma,, 1981, Schmidt, *et al.* 2008). Generally *Penicillium* spp. as saprophytic can grow under different physiological conditions, such as low to high temperature, low water availability, high salt concentrations etc (Houbraken and Samson, 2011). The nutritional requirements and water relations have also been suggested parameters for their identification (Pitt, 1973). A combination of different physicochemical parameters and the usage of new methodologies have been very helpful in controlling the fungal growth and the biosynthesis of mycotoxins, such as Patulin production by *P. expansum* as a function of temperature, pH, and fruit varieties (Morales, *et al.* 2008; Salomao, *et al.* 2009). However, influence of pH and salinity on growth and dimorphism of extremophilic fungal strains are in their infancy. Thus, the efforts are required to optimize the ability of both *Penicillium* strains to survive under different pH conditions (acidic to alkaline), and salinity stress. A major emphasis is required to study the stress induced production of different metabolites. Hence, in order to study the effect of pH and different concentration of salt (NaCl) on growth and production of secondary metabolites by *Penicillium* strains, the fungal strains were grown in the presence of different pH ranges (4.0-9.0) and different salt concentrations (0-15% w/v) in basal medium.

5.2 Materials and Methods

5.2.1 pH dependent growth of *Penicillium* strains

Isolated *Penicillium* strains were aseptically inoculated in autoclaved potato dextrose broth (50 ml) adjusted to varying pH (pH4.0-9.0) by using HCl and NaOH (1 N solution). The fungal inoculated broth was incubated for 21 days. Harvesting of fungal biomass was done through filteration, using whatman filter paper and growth was measured in terms of fresh weight (g L^{-1}) after full incubation time of 21 days.

5.2.2 Effect of Salt (NaCl) concentration on growth of *Penicillium* strains

Isolated fungal strains (*P. oxalicum* and *P. citrinum*) were grown aseptically in the presence of varying concentrations of Sodium chloride (NaCl) [0, 2.0, 5.0, 10 and 15 % (w/v)] supplemented in the basal medium (50ml), which was supplemented separately with 1% (w/v) glucose as carbon source.

5.2.3 Extraction and screening of secondary metabolites under salt (NaCl) stress

Extraction of secondary metabolites from the aqueous growth media was performed by using ethyl acetate as given in chapter 4 **(section 4.2.4).**

5.2.4 FTIR analysis of *Penicillium* biomass grown under pH and salt (NaCl) stress conditions

Fungal biomass of both *Penicillium* strains grown under different pH 4.0-9.0 and NaCl (0-15%) conditions. The mycelia of both the strains were harvested, washed with distilled water. The dried biomass of mycelia was obtained by drying them in incubator at 60°C.The powdered biomass was supplemented with IR grade potassium bromide (KBr) (1:10), pressed into discs under vacuum using spectra lab pelletizer. The IR spectrum was recorded in the region 4000-400 cm^{-1} using NICOLET 6700 FT-IR (Thermo-Scientific).

5.3 Results and Discussion

5.3.1 pH dependent growth of *Penicillium* strains

The *Penicillium* strains were grown in the pH range of 4.0 -9.0.The mycelia were filtered and weighed in terms of gram per litre for both the *Penicillium* strains. The results revealed that the maximum mycelial growth of psychrophilic *P. oxalicum* was recorded at pH 4.0 with avg. fresh weight 152 g L^{-1} , while decline in fresh weight of *P. oxalicum* biomass has been recorded with increase in pH range 5.0-9.0 (acidic to alkaline) i.e. 6.68, 5.82, 4.5, 4.1, 2.98 g in 50 ml basal medium respectively. On the other hand, the maximum mycelial fresh weight of mesophilic *P. citrinum* was recorded at pH 5.0 with average fresh weight 199.4 g L^{-1}. A gradual decline in fresh weight was recorded in both the strains with increase in range of pH 4.0-9.0 (Fig.5.1. & 5.2.). This study indicated that the *Penicillium* strains grew well in the acidic range of pH 4.0-6.0, whereas alkaline pH 7.0-9.0 conditions restricted their growth. Li, *et al.* (2010) reported that the ambient pH has an important effect on the *Penicillium expansum* spores and plays important role in the maintenance of normal cell function by affecting activity of metabolic enzymes and synthesis of DNA, RNA, and proteins. Cogliati, *et al.* (1997) investigated the growth of yeasts *of Penicillium marneffei* at four different pH range (4, 5, 6, & 7.2) and observed enhanced growth at acidic pH compared to neutral pH. The present study also revealed that pH influences the growth and metabolism of *Penicillium* strains. Singh *et al.* (2014) reported the significant difference in the biomass production of *Trichoderma* species at tested pH levels i.e. 4.0 to 8.0 and found that the pH ranging between 5.5 and 7.5 was the most favorable pH range for its growth. Previous several studies reported that numerous fungal isolates including *Fusarium solani*, *F. oxysporum*, *Trichoderma viride* (Verdin *et al*., 2004) and *Aspergillus niger* (Srivastava and Thakur, 2006) cultured in MSM medium could grow at pH 5.5. Hashem *et al.* (2015) also reported that the pH value in the culture medium was an important factor for growth and activity of fungi in agricultural industry and storage of food. As increase in the mycelial growth and extracellular production of secondary metabolite (Ochratoxin) by *Aspergillus carbonarius* was found to be directly proportional to increase in the pH upto 4.5 and 4.0, respectively.

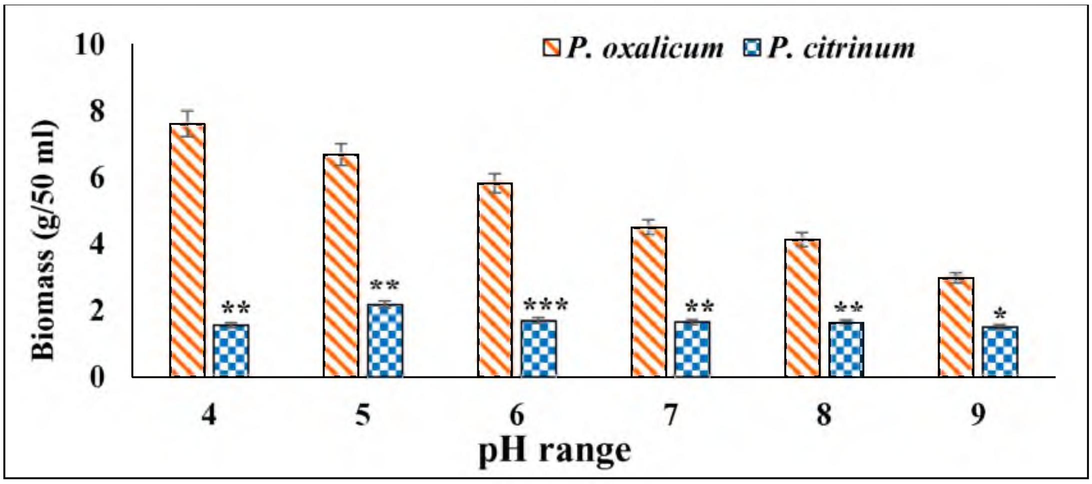


Fig. 5.1 pH dependent (pH 4.0-9.0) growth of *Penicillium* strains grown in basal medium for 21 days. Student's paired sample't-test' showing significant difference between both *Penicillium* strains, *p<.0.05, **p<0.01, ***p<0.001. Error bar showing the mean±SD.

5.3.2 Effect of Salt (NaCl) concentration on growth of *Penicillium* strains

The both *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum)* were grown in basal medium supplemented with different salt (NaCl) concentrations (0-15% w/v). The best growth was recorded at 2% (w/v) salt concentration with average fresh weight 104 g L^{-1} & 48 g L^{-1} for *P. oxalicum* and *P. citrinum,* respectively. Beyond 2 % NaCl concentration, the decline in the growth of both the *Penicillium* strains was recorded with increasing salt concentration. The results (Fig. 5.2) revealed that psychrophilic *P. oxalicum* was relatively more tolerant to higher salt concentration as compared to the mesophilic *P. citrinum*. Several species of *Aspergillus* and *Penicillium* have been documented to dominate the low temperature and saline habitats (Cantrell *et al.* 2011). A great diversity of genera *Penicillium* from cold temperate regions such as glaciers including, Arctic glaciers (Sonjak, *et al.* 2006), Indian Himalayas (Pandey *et al.* 2008), Antartica (Kostadinova, *et al.* 2009), ice from Kongsfjorden (Gunde-Cimerman, *et al.* 2003) and cold environments (Margesin and Miteva, 2011) has been well documented. In a study by Dhakar, *et al.* (2013), the cold tolerant *Penicillium* strains isolated from Himalaya, India, were found to be halotolerant as they were able to tolerate high salt concentration. About 06 species of *Penicillium* were able to tolerate more than 20% salinity (03 species upto 20 % and 03 species upto 15% NaCl concentration). They suggested that the ability of psychrotolerants to grow on high concentration of salt may be attributed to the defense mechanisms involved in coping the stressful to environment. The cold tolerance mechanisms such as antifreeze proteins, lipid/fatty acids, polyols etc. have been attributed to the phenomenon of cold adaptation (Robinson, 2001). Tresner and Hayes, (1971) reported an assessment of NaCl tolerant 975 species of terrestrial fungi from major taxonomic classes. Among them, *Penicillia* and *Aspergilli* were notably found to be the most salt tolerant as they were able to grow in the presence of 20% w/v or more salinity. They also reported that the basidiomycetes, were the least halotolerant as over half the species were unable to survive beyond 2% w/v (NaCl). Equal salt tolerance by several strains of various species may provide a useful taxonomic basis of Classification. In a study by Attaby (2001), it has been reported that the growth of *P. chrysogenum* occurred at 10% NaCl and tolerated NaCl concentration upto 18%. Turk, *et al.* (2007) reported that the increase in optimum range of salinity was responsible for the increased plasma-membrane fluidity of stress-tolerant species and decreased in dominant extremophiles (*Hortaea werneckii*, *Cryptococcus. liquefaciens*). Whereas, plasma membranes of the fungi (*Aureobasidium pullulans* and *Rhodosporium diobovatum*) with a narrow ecological amplitude exhibited different responses. Fungi such as *Hortaea werneckii* survives in the hypersaline waters of salterns and *Cryptococcus liquefaciens* survive in subglacial environments, showed similar profiles of plasma-membrane fluidity in response to raised salinity (Turk, *et al.* 2007). Boumaaza, *et al.* (2015), compared the effect of sodium and calcium salts against growth and sporulation of *Botrytis cinerea* and suggested that the sodium chloride stimulates the growth of fungal mycelium upto 150 ppm and low concentration of calcium salts support the growth. Whereas, higher concentrations of calcium chloride reduced mycelial growth of *B. cinerea* on PDA medium.

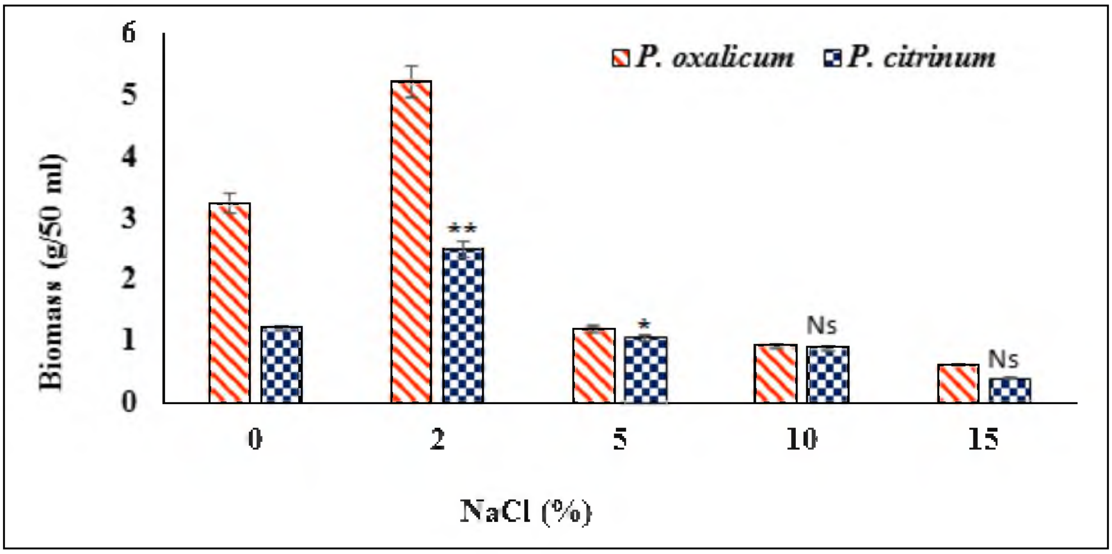


Fig. 5.2 Growth of *Penicillium* strains in response to different concentration of (NaCl, 0-15% w/v). Student's paired sample't-test' showing significant difference between both *Penicillium* strains, *p<.0.05, **p<0.01, Ns=Not significant. Error bar showing the mean±SD.

5.3.3 Extraction and screening of secondary metabolites under Salt (NaCl) Stress condition

Secondary metabolite production of both fungal strains in the presence of different concentration of NaCl concentrations (0-15% w/v) was studied. The samples were withdrawn at different time interval (3rd, 7th, 15th, 21st, and 28th) during the incubation and ethyl acetate (EtOAc) extraction of secondary metabolites was carried out. The absorption spectra (250-400 nm) of EtOAc extracts from *P. oxalicum,* grown under different salt concentrations (0, 2,5,10, and 15% (w/v) showed maximum absorption (0.65, 0.55, 0.17, 0.07 and 0.12) at λ =320, whereas absorption maxima observed at 265 nm was 0.58, 0.5, 0.27, 0.1 and 0.32 (Figure 5.3). On the other side, extracted secondary metabolites of *P. citrinum* showed maximum absorption (0.24, 3.6, 3.6, 1.87 and 0.34) at 328 nm under respective salt concentration 0, 2, 5,10 and 15% (w/v). The other absorption peak (2.6) of secondary metabolite extract of mesophilic *P. citrnium* at 258 nm was recorded in the presence of 5% NaCl. While, the same peak got appeared and showed negligible absorbance at other salt concentrations (Fig. 5.4).These results revealed that the 2% (w/v) NaCl concentration supported the highest production of secondary metabolites in psychrophilic *P. oxalicum,* whereas maximum secondary metabolite production in mesophilic *P. citrinum* was supported by 2 and 5 % NaCl concentrations. It has been reported that high salinity condition promoted the production of bioactive metabolite with antibacterial activity in the fungus (Miao *et al.* 2006). In the present study, excellent growth and production of bioactive metabolite in psychrophilic *P. oxalicum* and mesophilic *P. citrinum* were observed upto 10 % (w/v) and 5% (w/v) salinity respectively. It is evident from the foregoing investigation that the mycelial growth and production of bioactive metabolite in both *Penicillium* strains was greatly enhanced by NaCl concentration. Mathan, *et al.* (2013) investigated the influence of NaCl on the biomass and production of bioactive compounds in *Aspergillus* strain and reported that the NaCl concentration of 5 g L^{-1} was optimal for maximum mycelial growth and 7 g L^{-1} of NaCl in basal medium was optimal concentration for secondary metabolite production. Bhattacharyya and Jha. (2011) also reported the high concentration of NaCl was required for maximum mycelial growth and production of bioactive metabolite in the *Aspergillus* strain.

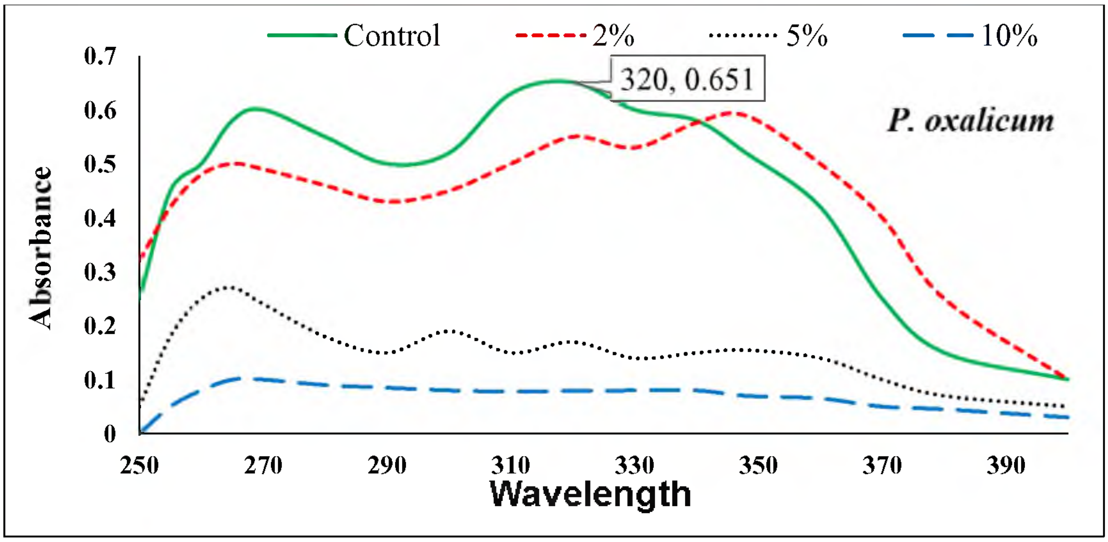


Fig. 5.3 Absorbance spectra (250-400 nm) of EtOAc extract of secondary metabolites of psychrophilic *P. oxalicum* grown under different salinity regimes (0-10% w/v of NaCl).

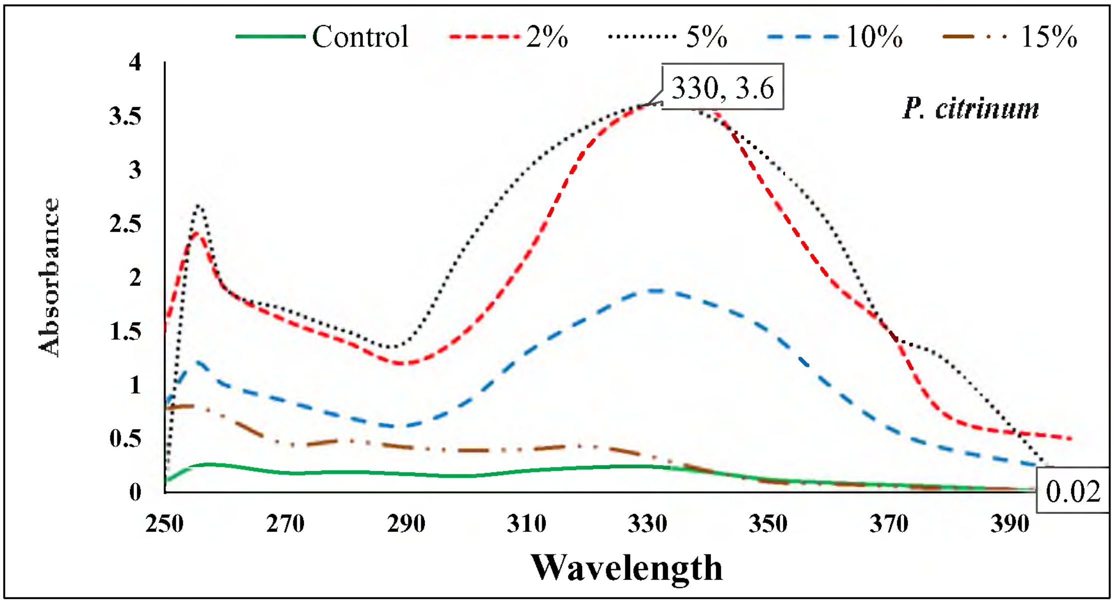


Fig. 5.4 Absorbance spectra of EtOAc extract of secondary metabolites of mesophilic *P. citrinum* grown under different salinity regimes (0-10% w/v of NaCl).

5.3.4 FTIR analysis of *Penicillium* biomass grown under different pH conditions.

The FTIR spectra (4000 to 500 cm^{-1}) of biomass of *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum*) grown under different pH (pH 4.0-9.0) conditions were compared. The significant changes were recorded at wavenumbers 3437-420, 2930-27, 1740, 1078, 1650, 1545 and 1040 cm^{-1}. Appearance and disappearance of new IR absorption peaks were observed in case of *Penicillium* strains grown under different pH stress conditions. The significant changes were recorded at wavenumbers 1650 and 1545 cm^{-1}, representing changes in the Amide I and Amide II functional groups associated and no changes were observed at wavenumber 1740 cm^{-1}, corresponding to C=O stretching of lipids (Andrus, 2006; Paluszkiewicz and Kwiatek, 2001; Shapaval, *et al.*, 2014) . In case of psychrophilic *P. oxalicum* grown (4°C) under different pH conditions exhibited, significant changes at wavenumbers 1651.8 to 1647.8 cm^{-1} and 1539.9 to 1547.1 cm^{-1} assigned to Amide I & amide II groups of Protein, respectively, whereas little changes were observed at wavenumbers 1650.2 to 1648.0 cm^{-1} and 1542 to 1545.1 cm^{-1}, in case of mesophilic *P. citrinum*. The new IR peaks emerged at wavenumbers 2853.0 and 1744.9 cm^{-1} in case of mesophilic *P. citrinum* grown under acidic condition (pH=4.0) assigned to asymmetrical CH_2 stretching of the methylene chains in membrane lipids/ ν_s CH_2 of lipids (Fung et. al., 1996) and C=O stretching mode of lipids/ Ester (C=O) and phospholipids (Fabian et. al., 1995). A Significant shift at wavenumber 1450 cm^{-1} assigned to polyethylene methylene deformation modes (Chiriboga, *et al.*, 1998) was recorded in case of psychrophilic *P. oxalicum* grown under acidic pH(4.0 and 5.0). But the same peak disappeared at pH (6.0 and 8.0). On the other hand, the IR absorption peak at wavenumber 1450 cm^{-1} was absent in the biomass grown at pH=5.0 (best pH for *P. citrinum* growth) and a peak shifted from 1456.6 to 1459.1 cm^{-1} (pH4.0-9.0), assigned to asymmetrical CH_3 bending of the methyl groups of proteins (Fujioka, *et al.*, 2004), was observed at pH 9.0. Overall changes in IR absorption and peak shifts due to change in pH conditions revealed deformation in proteins with structural changes, in both the *Penicillium* strains.

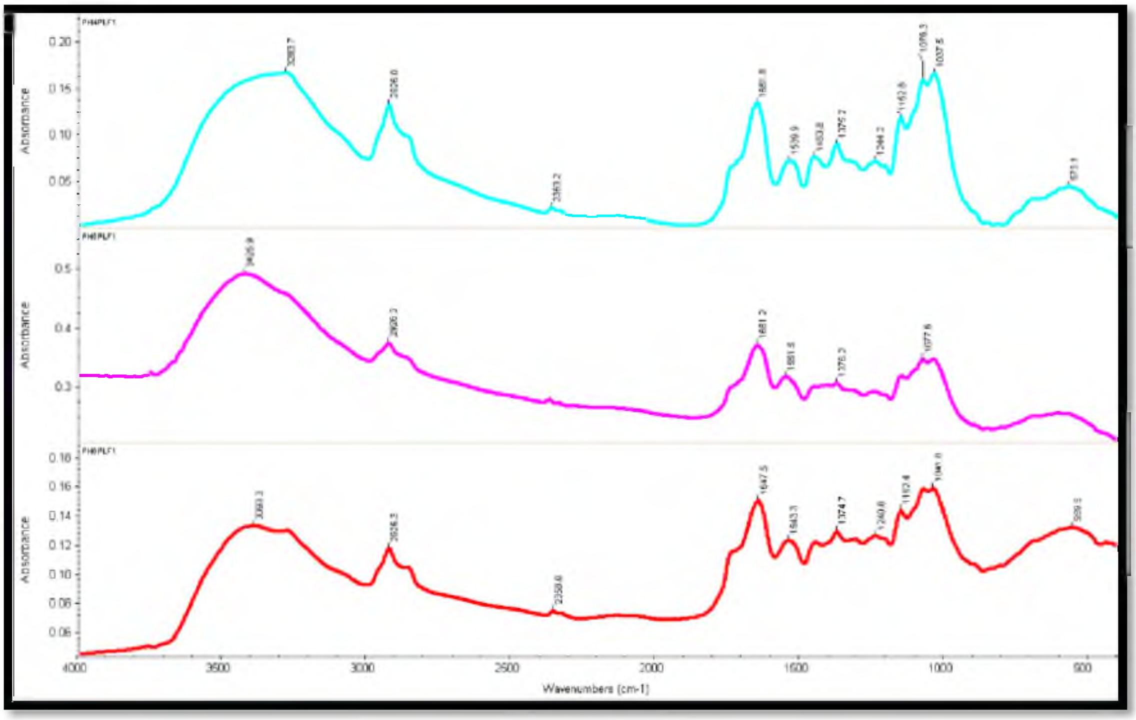


Fig. 5.5 FTIR absorbance spectra (4000-500 cm^{-1}) of *P. oxalicum* biomass grown at pH 4.0-6.0.

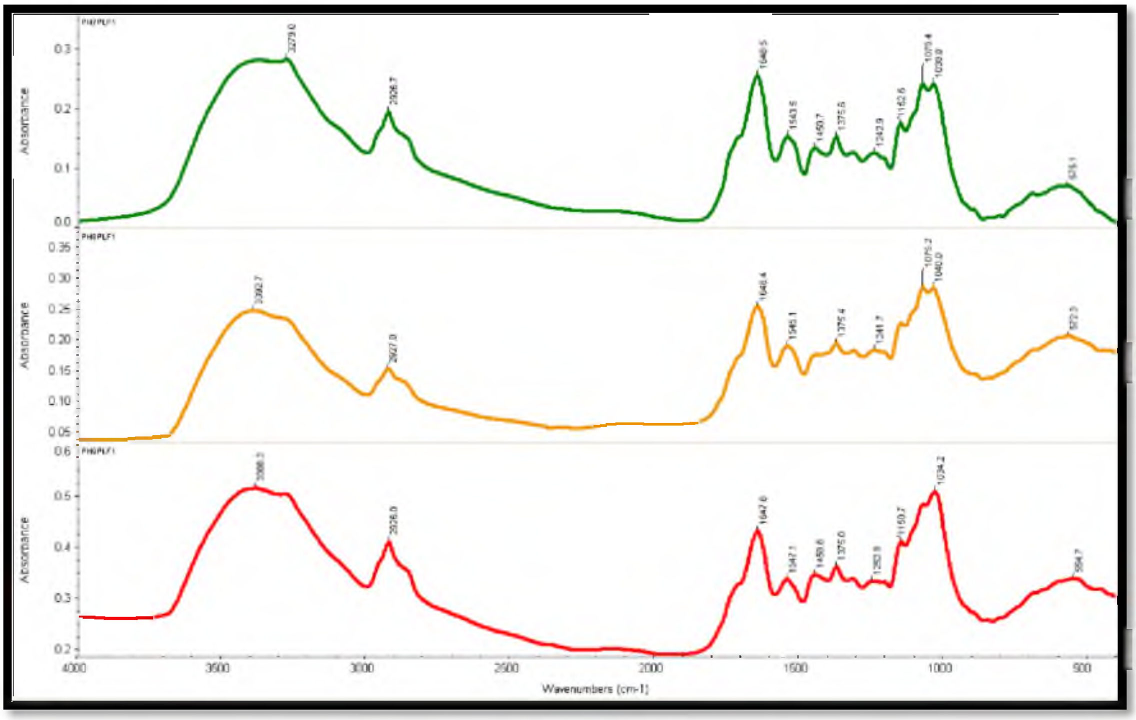


Fig. 5.6 FTIR absorbance spectra (4000-500 cm^{-1}) of *P. oxalicum* biomass grown at pH 7.0-9.0.

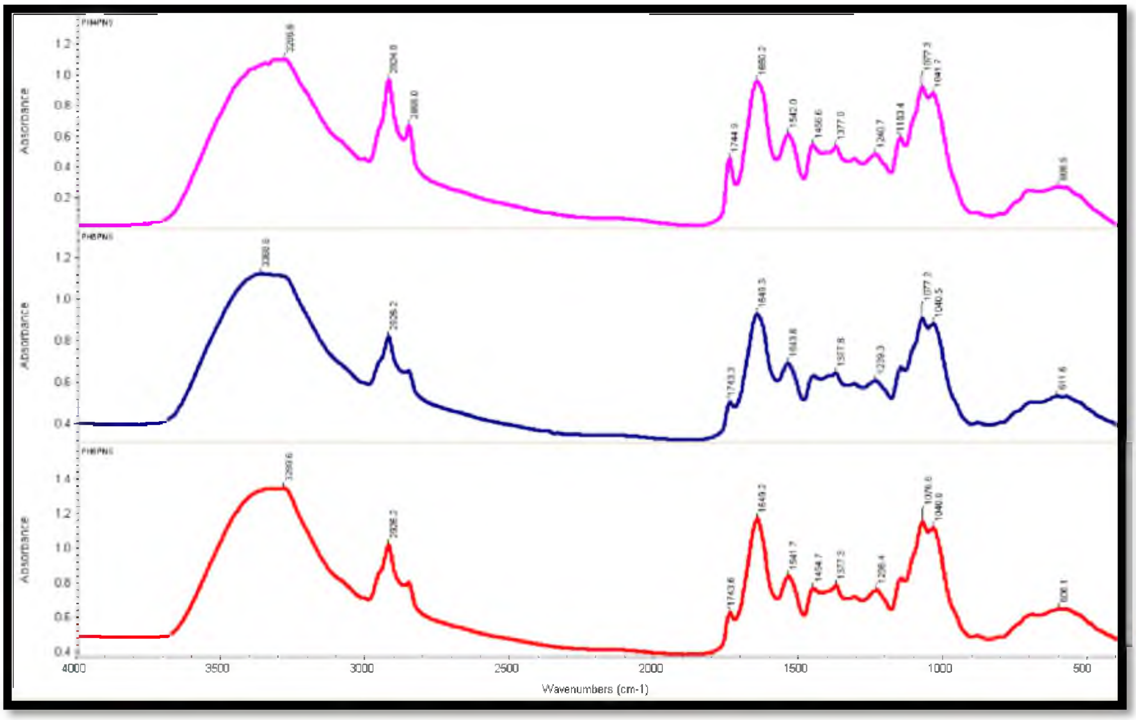


Fig. 5.7 FTIR absorbance spectra of (4000-500 cm^{-1}) *P. citrinum* biomass grown at pH 4.0-6.0.

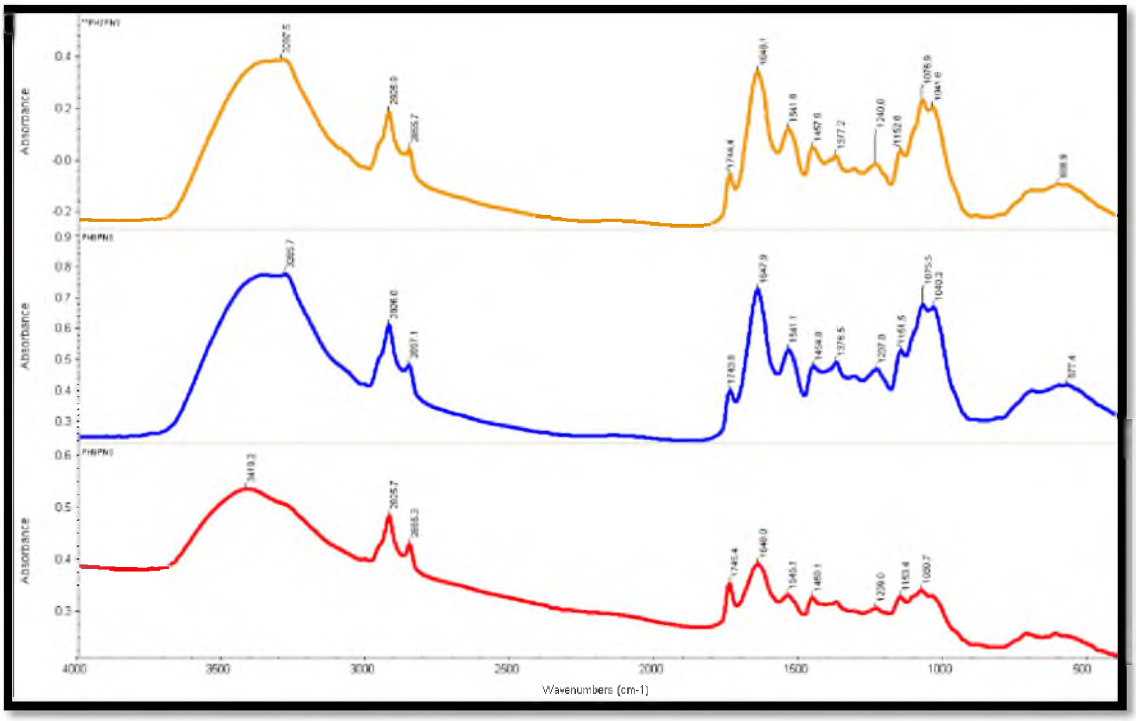


Fig. 5.8 FTIR absorbance spectra (4000-500 cm^{-1}) of *P. citrinum* biomass grown at pH 7.0-9.0.

5.3.5 FTIR analysis of *Penicillium* strains grown under different Salt (NaCl) concentrations

The FTIR spectra (4000 to 500 cm^{-1}) of biomass of both *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum*) grown under different NaCl concentrations (0, 2, 5, 10, 15% w/v) were compared. Emergence of many new peaks and significant peak shift were recorded in IR spectrum of both the *Penicillum* strains due to presence of high salt concentrations, particularly between wavenumber (1220 to 1350 cm^{-1}), assigned to amide III region (Fabian, *et al*. 1995). The C-N stretching, N-H in plane bending, often with significant contributions from CH_2 wagging vibrations and asymmetric PO_2^- stretching are observed in wavenumber region 1220-1240 cm^{-1} (Fabian, *et al*. 1995). In case of psychrophilic *P. oxalicum,* grown in high concentration of salt (5% and 10% w/v) emergence of new IR peak at wavenumbers 1400 and 1403 cm^{-1}, assigned to the symmetric stretching vibration of $-COO^-$ group of fatty acids, $-CH_2$ group of amino acids/ - CH_2 skeletal proteins and symmetric CH_3 bending modes of the methyl groups of proteins showed influence of high salinity on lipid–proteins interaction (Fung, *et al*. 1996; Fujioka, *et al*. 2004). On the other hand, the IR peak at wavenumber 1452-58 cm^{-1}, assigned to methylene (CH_3) and methyl (CH_2) group of lipids (Lecellier, *et al*. 2014), were recorded in case of biomass of mesophilic *P. citrinum* grown under high salinity. A significant peak shift was observed at wavenumber 2925/7 cm^{-1} in case of both *Penicillium* strains grown under high concentration of salt (10 and 15%), was assigned to asymmetrical stretching of CH_2 group of lipids (Fung, *et al*., 1996), when compared with the control (0% NaCl). The result suggested that salt induced changes in membrane lipids occurred in both strains in order to enable them to tolerate high salt concentrations. Whereas, a peak shift at wavenumber 2860 cm^{-1}, assigned to C-H stretching in lipids (Dovbeshko, *et al*. 1997), in case of mesophilic *P. citrinum* suggested for change in lipids, when compared with the control.

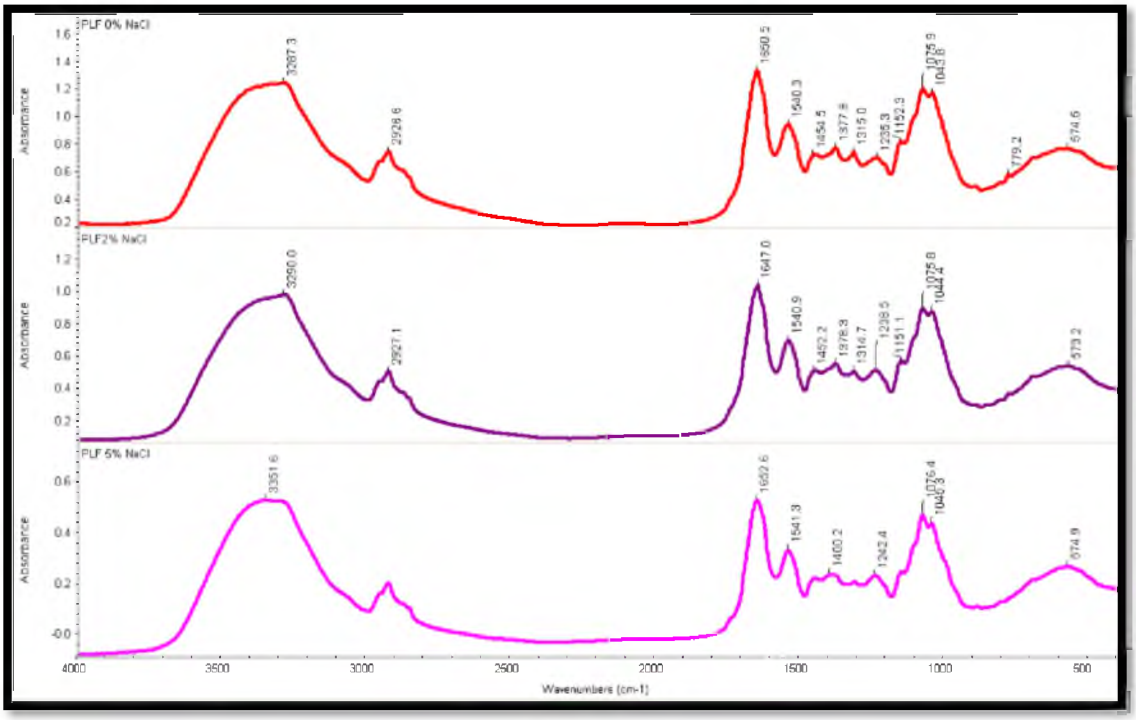


Fig. 5.9 FTIR absorbance spectra of biomass of *P. oxalicum* grown under salinity (0-5% NaCl) stress.

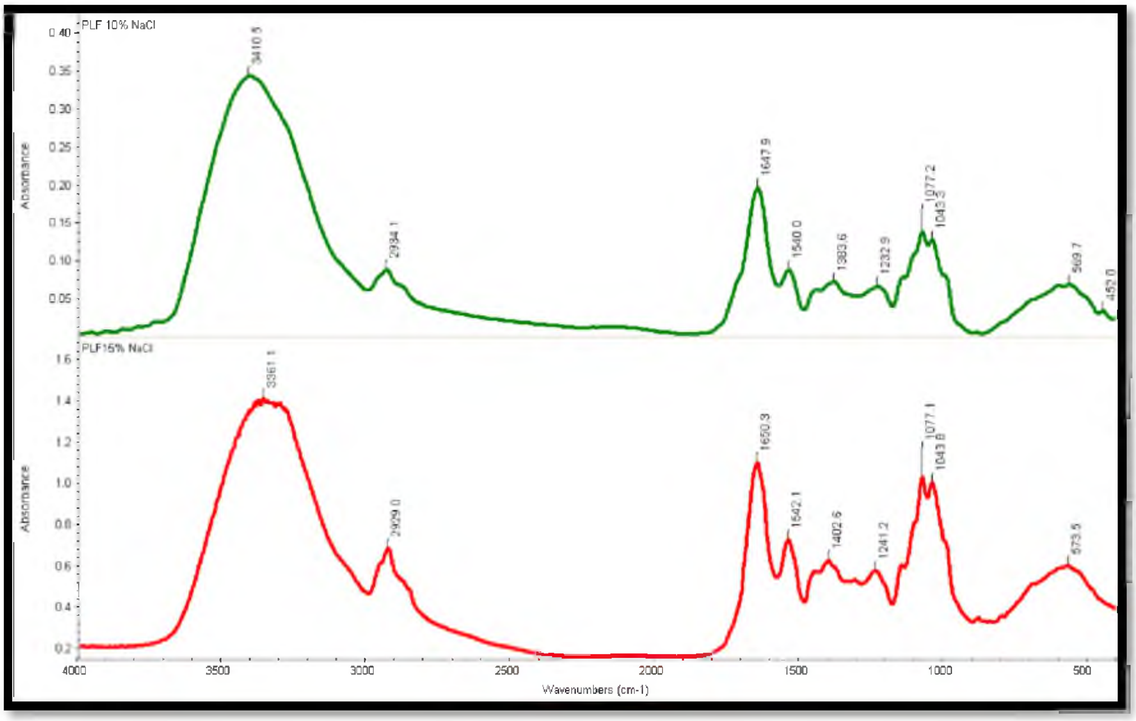


Fig. 5.10 FTIR absorbance spectra of biomass of *P. oxalicum* grown under salinity (10-15% NaCl) stress.

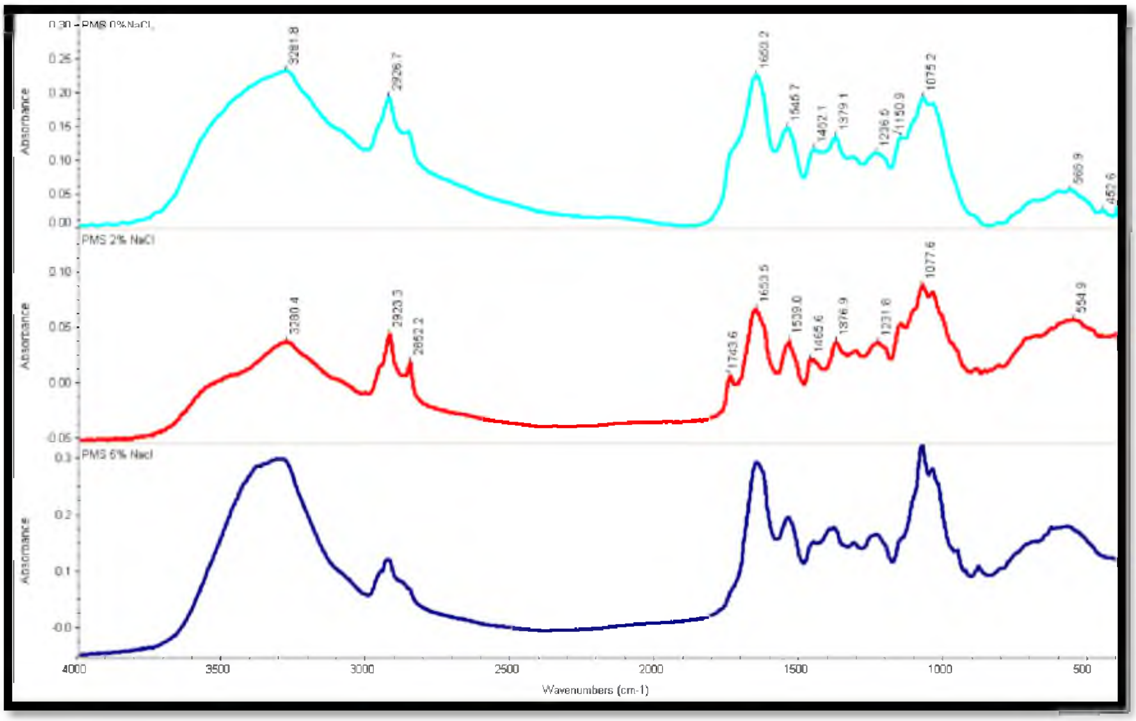


Fig. 5.11 FTIR absorbance spectra of biomass of *P. citrinum* grown under salinity (0-5% NaCl) stress.

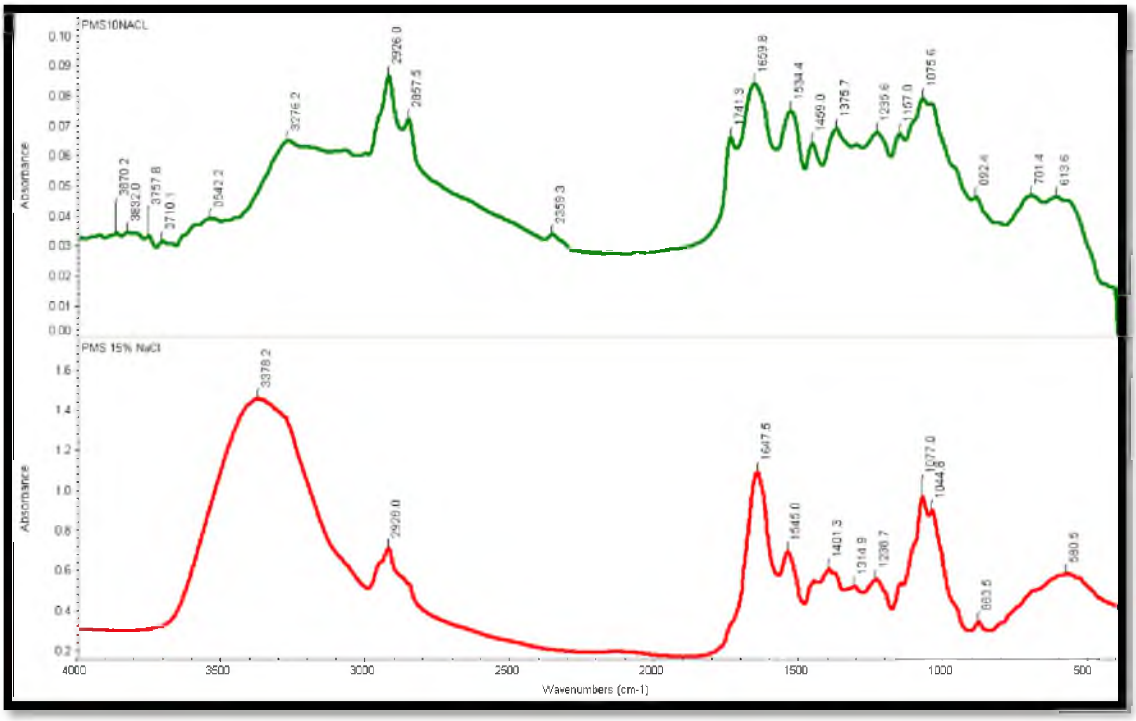


Fig. 5.12 FTIR absorbance spectra of biomass of *P. citrinum* grown under salinity (10-15% NaCl) stress.

5.4 Conclusion

- Both *Penicillium* strains are able to grow on a wide range of pH conditions (pH 4.0-6.0). Optimum pH for the growth of psychrophilic *P. oxalicum* was pH 4.0, while mesophilic *P. citrinum* grew well at pH 5.0. Hence, it is clear that both the *Penicillium* strains are of acidophilic in nature.
- Growth of both *Penicillium* strains recorded under different salinity regimes (0-15% w/v NaCl) revealed that both *Penicillium* strains preferred 2% (w/v) NaCl for their optimum growth. Further, it was observed that psychrophilic *P. oxalicum* was able to tolerate NaCl concentrations (upto 15%, w/v) than *P. citrinum* (10 %, w/v NaCl). Hence, psychrophilic *P. oxalicum* was found to be halotolerant in nature.
- The production of secondary metabolite were reached to its optimum level in *P. oxalicum* at 2% w/v NaCl concentration. Whereas, production of secondary metabolite in *P. citrinum* continued upto 5% (w/v) salt concentrations.
- Analysis of FTIR spectra of biomass of both the *Penicillium* strains showed species specific as well as stress dependent structural and compositional alterations in the lipid and protein component of fungal mycelium.

CHAPTER-6 NUTRIENT STRESS

6.1 Introduction

Several environmental factors influence the fungal growth and metabolite productions. Many of the microbes including fungi live in extreme environments where they are repetitively subjected to extreme pH, temperature, salinity, UV exposure, and lack of nutrients. Availability of nutrients such as carbon and nitrogen play an important role in fungal growth (Zhao, *et al.* 1990). A wide variety of carbon and nitrogen sources enable the fungi to colonize different environmental niches. Production of secondary metabolites also gets influenced by the availability of carbon and nitrogen sources. Sometimes composition of growth media and incubation conditions have a very strong influence on the production of secondary metabolites. It has been well documented that the secondary metabolism depends upon the level of carbon and nitrogen sources, trace elements, phosphate, induction of enzymes of secondary metabolism, catabolic repression and inhibition. The feedback repression and inhibition of secondary metabolism can be controlled by auto-regulators (Betina, 1995). A report by Breitling, *et al.* (2013) revealed that the secondary metabolites are not essential for viability of microorganism, but support the survivability of microbes under varying environmental conditions, for example production of siderophores (chelating agents) protect them against various environmental stresses (such as, metal stress and UV irradiation) and also helps to improve nutrient exchanges with other microorganisms present in ecological niches and also ensure reduction in the hosts population, e.g., plants, animals, or humans. The marine fungus *Arthrinium c.f. saccharicola* studied by Miao, *et al.* (2006) showed that the culture medium had an immense effect on mycelial growth and profile of metabolites. In the pre-genomics era, culture supernatants were screened to identify new bioactive metabolites with activities of interest. These classical methods have been used for activating genes involved in the synthesis of secondary metabolites, by manipulating the culture conditions as represented by the OSMAC (one strain, many compounds) approach (Bode, *et al.* 2002; Craney, *et al.* 2013). This simple and inexpensive method led to the discovery of many novel metabolites and gave first insight into the complex regulatory network. Also by limiting the rate of availability of a single nutrient, for e.g., in continuous fermentations, silent gene clusters could be activated. The present work aimed at evaluating the influence of nutrients such as carbon and nitrogen sources on growth and development of *Penicillium* strains as well as their impact on the production efficiency of secondary metabolites.

6.2 Materials and Methods

6.2.1 Effect of carbon and nitrogen on the growth of *Penicillium* strains

Isolated fungal strains were inoculated on Agar plates containing basal medium supplemented with varying concentrations of carbon and nitrogen sources. The plates were incubated for 15 days at 28± 2°C.

A. Carbon sources

Effect of the different concentrations of carbon sources (glucose, cellulose, and sucrose) on mycelial growth of both *Penicillium* strains was evaluated at their respective optimum temperature and pH conditions for 21 days. The glucose in the basal medium was substituted by each of the carbon compounds so as to provide 1% (w/v) of carbon - a substituent of glucose (10 g L^{-1}) in the basal medium.

B. Inorganic and organic nitrogen sources

Effect of different concentrations of inorganic and organic nitrogen sources (sodium nitrate, ammonium sulphate, yeast extract, glycine, L-tryptophan) on the mycelial growth of *Penicillium* strains was evaluated, replacing inorganic nitrogen compound in glucose supplemented basal medium. A control set without addition of nitrogen source was run parallel. 100 ml Erlenmeyer flasks containing 50 ml of growth medium, were inoculated with a 10 mm surface agar plug from 7 day old culture grown on PDA plates for all the experiments. Three replicates were maintained for each nitrogen source for 21 days. In broth culture, mycelia biomass were filtered through Whatman filter paper No. 1 and moisture content was removed by using tissue paper before taking fresh weight of the biomass.

6.2.2 Extraction of secondary metabolites in both *Penicillium* strains grown under varying C and N conditions

Details of extraction of secondary metabolites was performed by the method mentioned in chapter 4 **(4.2.4).**

6.3 Results and Discussion

6.3.1 Effect of Carbon source on growth of both *Penicillium* strains

The best carbon source for the growth of psychrophilic *P. oxalicum* was found to be 2 % (w/v) glucose with av. fresh wt. 140 g L^{-1}(7 g in 50 ml basal medium) whereas the fungal growth was reduced in the presence of 1 % w/v sucrose and cellulose (Fig. 6.1). The optimum production of biomass (average mycelial fresh weight) in *P. oxalicum* in relation to the carbon source consumed was in the order of glucose> sucrose> cellulose. Whereas, the optimum growth of mesophilic *P. citrinum* was found to be the best in the presence of 2% (w/v) concentration of sucrose with avg. fresh wt. 380 g L^{-1}, followed by glucose and cellulose. The results on psychrophilic *P. oxalicum* exhibiting maximum biomass production in the presence of 2 % glucose, was similar to earlier reports (Chandra, *et al.*, 1977; Oso, 1977a; Fasidi and Jonathan, 1994; Fasidi and Olorunmaiye, 1994). The preferred carbon source for *P. oxalicum* over the other carbon sources was perhaps due to reason that glucose was directly metabolized to produce the cellular energy (Jandaik and Kapoor, 1976; Garraway and Evans, 1984). The carbon sources are also known to influence the sporulation and pigments of spores produced by the fungi. In a study by Basu and Bhattacharyya, (1962) on the evaluation of role of different carbohydrates as carbon source, growth and sporulation of various strains of *Penicillium vermiculatum*, *P. wortmanni* and reported about the importance of carbohydrates and nitrogen compounds for the growth and sporulation of fungi. Prasher, *et al.* (2014) observed the wide range of a low glucose concentration in the liquid medium as a sole source of carbon, favoured the fruiting and sporulation in several fungi. But for many other fungi on agar media, fruiting was always much more rapid and vigorous than in the liquid media containing same glucose concentration. Similar results were recorded in the present study, where a rapid growth and fast sporulation was favoured by the 2% (w/v) concentration of glucose in case of psychrophilic *P. oxalicum*. On the contrary, addition of sucrose and cellulose resulted into extremely slow growth, no sporulation and pigmentation. In case of mesophilic *P. citrinum*, 2 % (w/v) concentration of sucrose favoured the rapid growth, fast sporulation and change in pigmentation.

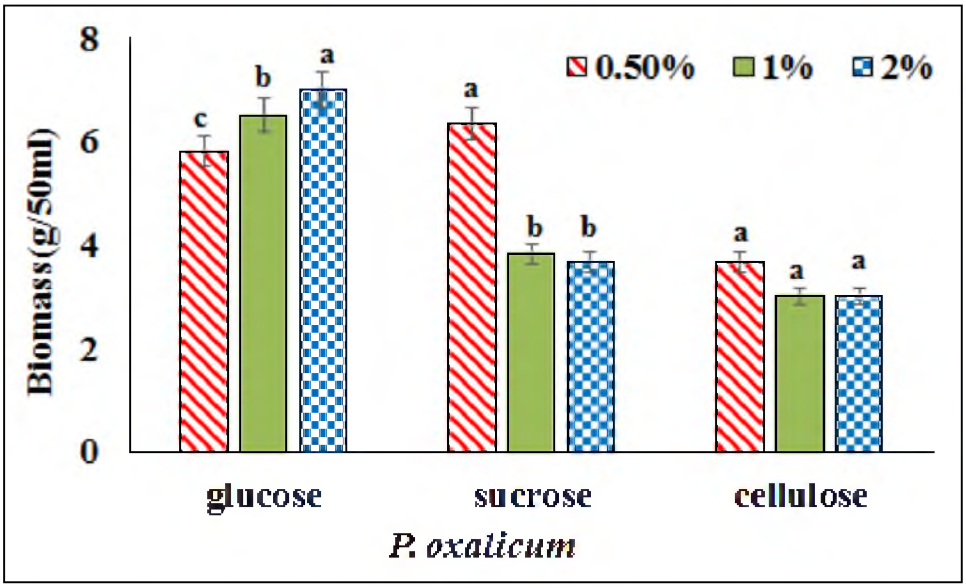


Fig. 6.1 Effect of Carbon sources on the growth of *Penicillium oxalicum* grown in basal medium for 15 days. Different letters within the same group indicate statistically significant difference at $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$[one way analysis of variance (ANOVA)], Error bar showing the mean±SD.

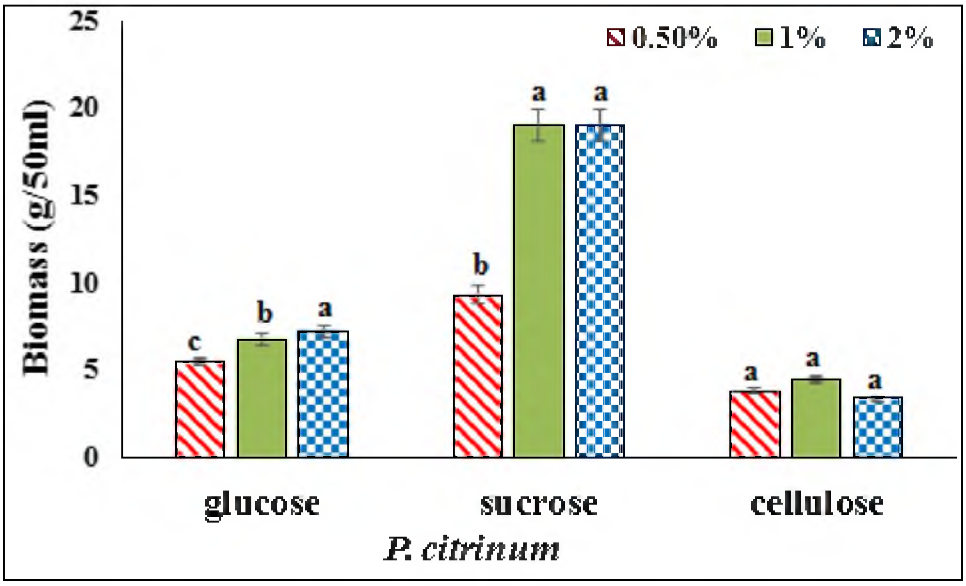


Fig. 6.2 Effect of carbon sources on the growth of *Penicillium citrinum* grown in basal medium for 15 days. Different letters within the same group indicate statistically significant difference at $p<0.05$ [one way analysis of variance (ANOVA)], Error bar showing the mean±SD.

6.3.2 Effect of different nitrogen sources on the growth of *Penicillium* strains

Among the inorganic nitrogen sources, growth of psychrophilic *P. oxalicum* was found to be the best on sodium nitrate (2% w/v) (Fig. 3.10). The least mycelial growth of this fungus was observed in the presence of ammonium sulphate. The optimum level of biomass production (average mycelial fresh weight) of mesohphilic *P. citrinum* relation to the nitrogen consumed was also in the order sodium nitrate> ammonium sulphate (Fig. 3.11). Thus, the best organic nitrogen source for the growth of psychrophilic *P. oxalicum* was 1% (w/v) glycine with highest average mycelial fresh weight 290 g L^{-1}, whereas the *P. citrinum* showed optimum growth in the presence of 2% (w/v) of Glycine with average fresh weight of mycelium 154 g L^{-1}. The growth of both the *Penicillium* strain was reduced in the presence of 2% (w/v) of L-tryptophan. The sporulation and pigmentation in case of psychrophilic *P. oxalicum* was enhanced at 1 % (w/v) glycine as nitrogen source, whereas, 2 % (w/v) L-tryptophan favored the sporulation and deep pigmentation in case of mesophilic *P. citrinum*. Krasniewski, *et al.* (2006) studied the effect of nitrogen sources, the medium was supplemented with glucose as sole source of carbon, suggested that not the just concentration, but the type of nitrogen sources also influenced conidiogenesis in *Penicillium camemberti*; KNO_3 stimulated conidia production while $(NH_4)_2SO_4$ was inhibitory. Similar study by Gutierrez-Rojas, *et al.* (2015) supported the previous study about the effect of different carbon (glucose, sucrose, cassava starch, wheat bran, and rice flour) and nitrogen sources (tryptose, yeast extract, $(NH_4)_2HPO_4$, and KNO_3) on conidiophore and conidia formation in *Penicillium* spp., and revealed that the influence of both carbon and nitrogen sources on conidiophore and conidia morphology and the amount of conidia produced.

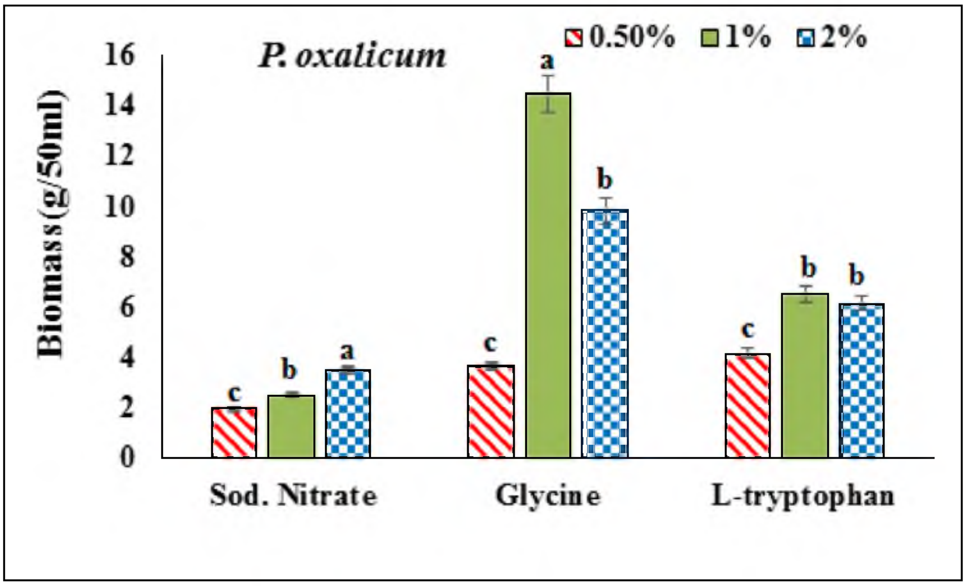


Fig. 6.3 Effect of nitrogen sources on the growth of *Penicillium oxalicum* grown in basal medium for 15 days. Different letters within the same group indicate statistically significant difference at $p<0.05$ [one way analysis of variance (ANOVA)], Error bar showing the mean±SD.

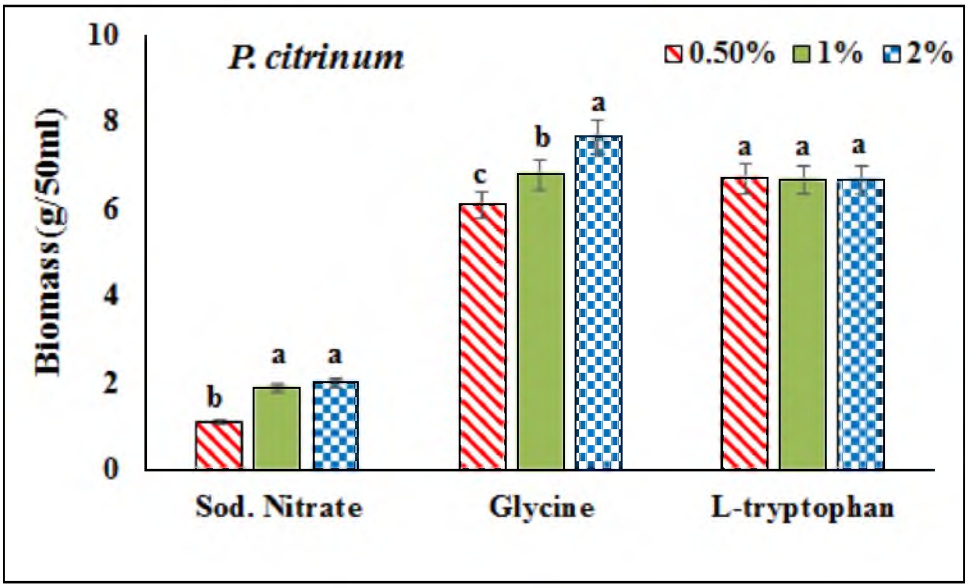


Fig. 6.4 Effect of nitrogen sources on the growth of *Penicillium citrinum* grown in basal medium for 15 days. Different letters within the same group indicate statistically significant difference at $p<0.05$ [one way analysis of variance (ANOVA)], Error bar showing the mean±SD.

6.3.3 Extraction and screening of secondary metabolites under nutritional (C, N) Stress condition

A. Carbon sources

The fungal broth culture was grown under different carbon concentrations [0.5-2.0% (w/v)] was withdrawn at different time interval (3rd, 7th, 15th, 21st, and 28th) during the incubation and was used for extraction of secondary metabolites by using ethyl acetate (EtOAC) thrice. The absorption spectra (250-400 nm) of EtOAC extracts of biomass of *P. oxalicum,* grown under different carbon sources (glucose, sucrose and cellulose) was analysed. The results showed maximum absorption (0.92 and 0.75) of secondary metabolite extract at 320 nm was different in case *P. oxalicum* supplemented with 1% (w/v) glucose and 1% (w/v) sucrose, respectively. Whereas negligible absorbance (0.02) was recorded for secondary metabolites in case of cellulose supplemented *P. oxalicum* (Fig. 6.5). On the opposite side, secondary metabolites of *P. citrinum* showed higher absorption (2.85, 3.10 & 0.52) at 328 nm when grown in the presence of 1% (w/v) glucose, sucrose and cellulose, respectively (Fig.6.6). The secondary metabolite extract of psychrophilic *P. oxalicum* showed maximum absorption (0.82) at 280 nm and mesophilic *P. citrinum* exhibited maximum absorption (1.75) at 290 nm supplemented with glucose 1%(w/v) (Fig. 6.6). But the secondary metabolite extract showed decline in the absorption peaks when the cultures were grown with sucrose and cellulose as carbon source. Further, 1% (w/v) glucose or sucrose concentrations supported the maximum production of secondary metabolites in the *P. oxalicum* and *P. citrinum* strains, respectively. Since the same concentration of these carbon sources supported the optimum growth in both the respective strains. It might be concluded that secondary metabolite production was proportional to mycelial biomass in each strain. An investigation on growth and secondary metabolite production by marine derived fungus *Arthrinium c.f.*

sachharicola by Miao, *et al.* (2006), suggested that the culture medium had a major effect on mycelial growth and metabolite profile. Zain, *et al.* (2009) also supported the present investigation, as they stated that the growth and secondary metabolites production of *Aspergillus terreus, Penicillium janthinellum* and *Penicillium duclauxii* were significantly and simultaneously affected by the type of the growth medium. Whereas in the present study the basal medium supplemented with glucose showed the best mycelial growth and secondary metabolite production in psychrophilic *P. oxalicum*. A study on metabolite production by *A. terreus* in the presence of various carbon sources tested, showed that sucrose was the best carbon source for both biomass and secondary metabolite production (Mathan, *et al.* 2013). Several reports were in support of the present study as they suggests that simple sugar like glucose, fructose, glycerol and sucrose plays an important role to enhance the fungal growth and secondary metabolite production rather than complex carbon sources (Buchanan and Stahl, 1984; Calvo, *et al.* 2002).

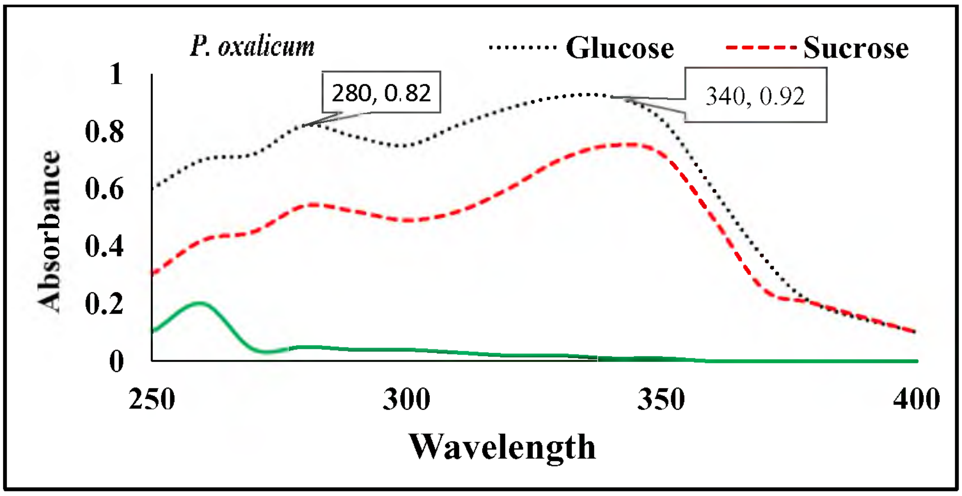


Fig. 6.5 Absorbance spectra (250-400 nm) EtOAc extract of secondary metabolites of psychrophilic *P. oxalicum* grown under different Carbon sources.

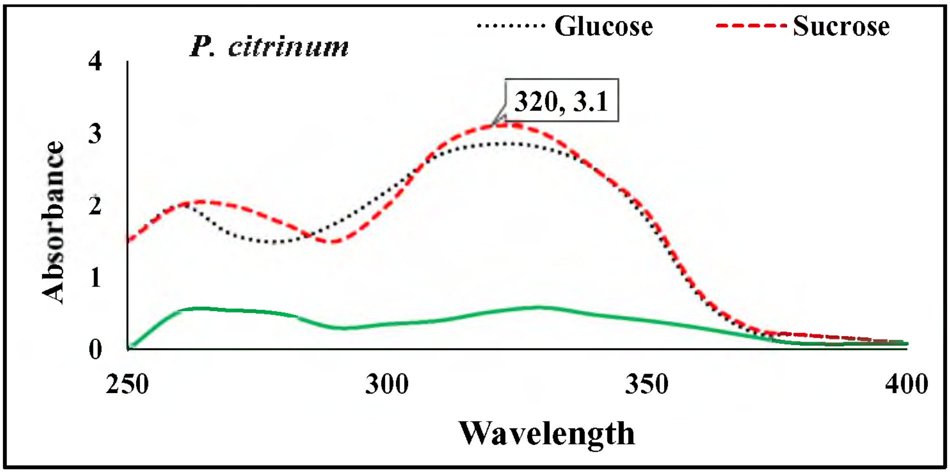


Fig. 6.6 Absorbance spectra (250-400 nm) of EtOAc extract of secondary metabolites of mesophilic *P. citrinum* grown under different Carbon sources.

B. Nitrogen sources

The secondary metabolites of *P. oxalicum,* grown under 1% (w/v) L- Glycine and L-Tryptophan showed maximum absorption values 1.9 and 0.85 at 265 nm, respectively. Whereas negligible absorbance (0.25) was recorded in case of sodium nitrate as nitrogen source (Fig. 6.7). On the opposite side, extract of secondary metabolites of *P. citrinum* grown in the presence of 1% (w/v) glycine, L-tryptophan and sodium nitrate showed maximum absorption 1.87, 0.34 and 0.32, respectively at 328 nm. Some additional peaks of secondary metabolite extract of psychrophilic *P. oxalicum* at 300 nm with maximum absorption 1.2, at 320 nm showed and a small hump at 340 nm (0.78) was recorded in case of glycine, L-tryptophan and sodium nitrate respectively (Fig. 6.7). Whereas, absorbance peaks at 340 nm in case of mesophilic *P. citrnium* with maximum absorption 1.76 was observed at 1% (w/v) glycine concentration (Figure 6.8). This absorbance peak was negligible when *P. citrinum* was grown with L-tryptophan and sodium nitrate as a nitrogen sources. Results revealed that the 1% (w/v) glycine concentration supported the maximum production of secondary metabolite in case of both the *Penicillium* strains (see section 6.3.1). Peighany-Ashnaei, *et al*. (2007) have reported that various nitrogen sources play an important role in maximizing the growth rate as well as production of bioactive compounds. Mathan, *et al.* (2013) reported that the medium supplemented with yeast extract showed optimum growth and secondary metabolite production in *A. terreus*. Findings of Jain, *et al.* (2012), supported the present results and suggested that different carbon and nitrogen sources play key role in fungal growth and metabolite production. They suggested that the fungal growth and biosynthesis of bioactive secondary metabolites by *Penicillium* species were strongly influenced by different sources of carbon and nitrogen. They also concluded that besides the carbon source, the source of nitrogen (sodium nitrate) was important for production of antibiotics in *Penicillium* strain. As many researchers reported that simple sugar like glucose, sucrose, fructose and glycerol help in improvement of growth and secondary metabolite production in microorganisms rather than complex form of carbon sources; such as starch, galactose, mannitol and xylose etc. Simple carbohydrates through their metabolic pathway influence the production of intermediates, leading to primary as well as secondary metabolites (Turner, 1971; Buchanan and Stahl, 1984; Calvo, *et al.* 2002).

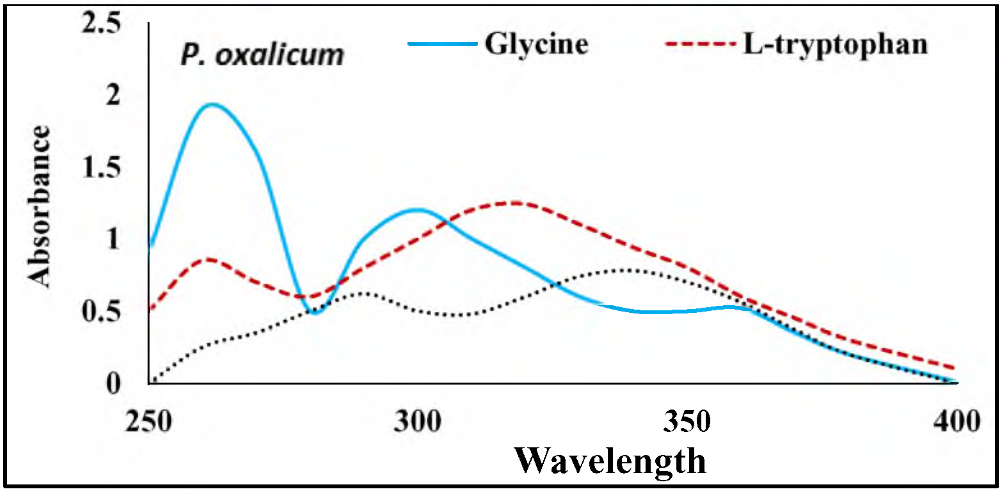


Fig. 6.7 Absorbance spectra (250-400 nm) of EtOAc extract of secondary metabolites of psychrophilic *P. oxalicum* under different nitrogen sources.

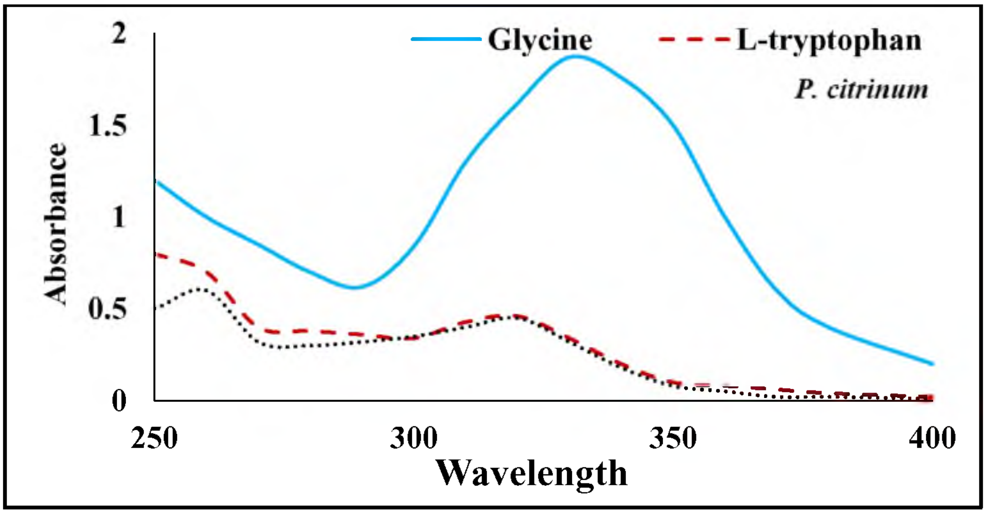


Fig. 6.8 Absorbance spectra (250-400 nm) of ethyl acetate extract of secondary metabolites of mesophilic *P. citrinum* under different Nitrogen sources.

6.4 Conclusion

- The present finding revealed that Glucose 2 % (w/v) is the preferred source of carbon, followed by sucrose for the growth of psychrophilic *P. oxalicum.* In case of *P. citrinum*, sucrose 2 % (w/v) was found to be the best carbon source, followed by glucose. Among the nitrogen sources, glycine (2 % w/v) was found to be relatively better nitrogen source than L- tryptophan for *P. oxalicum,* whereas sodium nitrate was the best nitrogen source in case of mesophilic *P. citrinum* respectively.
- Results of production of secondary metabolite by *P. oxalicum* and *P. citrinum,* showed that the glucose and sucrose were relatively better carbon sources, for respective strain. Among organic nitrogen sources, L-glycine was better nitrogen source for production of secondary metabolite in both the *Penicillium* strains.
- Further results revealed that unlike the carbon source variation, the individual nitrogen source was responsible for preferential synthesis of a particular metabolite as evident from variation in characteristic absorption spectra of secondary metabolites.

CHAPTER-7 HEAVY METAL STRESS

7.1 Introduction

Heavy metals are naturally present in trace amount in soil and as environmental contaminants, they are mainly associated with anthropogenic sources such as industrial activities, agricultural practices, automobile exhaust, coal fired thermal power generation, and municipal incinerators (Rattan, *et al.* 2002; Marshall, *et al.* 2003). The quantum of heavy metal contamination is increasing day by day (Bonaventura, Johnson, 1997) which is a major threat to the environment, public and soil health (Zafar, *et al.* 2006). Heavy metals do not decay naturally and their remediation has become a great challenge nowadays (Bai and Abraham, 2003). Microorganisms can survive in all environments because of their adaptable nature towards the contaminants. Reports on heavy metal tolerance in several fungi are well documented (Hashem and Bahkali, 1994). Cuero, *et al.* (2003) have documented the important role of metal ion concentration in stimulation of fungal growth and metabolite production. Fungi can tolerate and detoxify heavy metals by various mechanisms such as ion exchange, active uptake, complexation, adsorption, extra and intracellular precipitation, valence transformation and many more (Mala, *et al.* 2006; Volesky, 2007). The most important strategy adapted by fungal species is to produce abundant organic acids and secondary metabolites under such environmental stress conditions (Auckloo, *et al.* 2017). Different species of *Aspergillus* and *Penicillium* are well documented for their efficiency to remove heavy metals and exhibit profound capability to produce novel secondary metabolites in stress conditions (Congeevaram, *et al.* 2006; Burgstaller and Schinner, 1993; Ren, *et al.* 2009; Auckloo, *et al.* 2017). The cell wall of fungi is typically composed of chitin (a long linear homopolymer of beta- 1,4-linked Nacetylglucosamine), glucan, mannan, proteins and other polymers that possess carboxyl, phosphoryl, hydroxyl, amino, amine and imidazole functional groups at the surface, and these functional groups contributing surface charges of the fungal biomass to play significant role in heavy metal adsorption. (Bowman and Free, 2006). This chapter introduces the concept of psychrophilic and mesophilic *Penicillium* strains adapted to otherwise different stress condition exhibit adaptive tolerance behaviour to different heavy metals [As III & Cr (VI)]. Heavy metal tolerance in these fungal strains in terms of growth, structural changes in membrane and secondary metabolite production.

7.2 Materials and Methods

Reagents and solvents used

For culture preparation, Potato Dextrose broth/agar and the basal medium containing Yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4$, KNO_3, Glucose was purchased from Qualigens and Himedia (India). The pure culture was maintained on PDA plates and 3 to7th old day culture was used for further experiments.

7.2.1 Plate test for (MIC determination for Arsenic)

Isolated fungal strains (*P. oxalicum* and *P. citrinum*) were grown aseptically under varying concentrations of Sod. Arsenite (As III) ranging from 0, 50, 100, 250, 500 and 1000 mg l^{-1}. The petriplates containing basal medium with constituents: yeast extract, 2.5g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.01 g; KNO_3, 1.55 g and 1000 cm^3 of distilled water and 20 g agar (Jonathan and Fasidi, 2001) were supplemented with As(III) before the inoculation. The petriplates incubated at their respective growth temperatures were routinely observed (03 day time interval) for growth throughout the incubation period (28 days).

7.2.2 Plate test for (MIC determination for Cr VI)

Isolated fungal strains (*P. oxalicum* and *P. citrinum*) were grown aseptically under varying concentrations of di-Potassium dichromate (CrVI) from 0, 01, 2.5, 5.0, 7.5 and 10 ppm. The petriplates containing basal medium with constituents: yeast extract, 2.5g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.01 g; KNO_3, 1.55 g and 1000 cm^3 of distilled water and 20 g agar (Jonathan, 2001) were supplemented with Cr(VI) before the inoculation. The petriplates incubated at their respective growth temperatures and were routinely observed (03 day time interval) for growth throughout incubation period (28 days).

7.2.3 Batch Culture of both *Penicillium* strains in the presence of heavy metals

Pure culture of isolated *Penicillium* strains were inoculated aseptically in the basal medium containing varying metal concentration of As (III) & Cr (VI). The flasks were incubated for 28 days at their respective growth temperatures (as mentioned in chapter 3) under shaking conditions (150 rpm).

7.2.4 Extraction and screening of secondary metabolites of *Penicillium* strains grown under heavy metal stress

Extraction of secondary metabolites from broth culture of both the strains grown under different concentration of Arsenic (AsIII) and Chromium (CrVI) was carried out as discussed in Materials and Methods mentioned in chapter 4 **(section 4.2.4).**

7.2.5 FTIR analysis of *Penicillium* biomass grown under heavy metal stress conditions

Fungal biomass of both *Penicillium* strains grown under heavy metal supplemented conditions [AsIII & Cr (VI)] was harvested. The biomass was washed with distilled water, dried and grounded. The powdered biomass was supplemented with IR grade potassium bromide (KBr) (1:10), pressed into discs under vacuum using spectra lab pelletizer. The IR spectrum was recorded in the region 4000-400 cm^{-1} using NICOLET 6700 FT-IR (Thermo-Scientific).

7.3 Results and Discussion

7.3.1 Plate test for (MIC determination for Arsenic)

The effect of heavy metals on growth of both *Penicillium* strains was assessed on the basis of colony diameter. Results of Arsenic (AsIII) tolerance revealed that the psychrophilic fungal isolate *P. oxalicum* was more tolerant to Arsenic than the mesophilic *P. citrinum*. Psychrolphilic *P. oxalicum* was able to tolerate arsenic upto 1000 mg L^{-1} concentration and grew well at 100 mg L^{-1} with higher 7.75 cm colony diameter on arsenic containing petriplates after 28 days incubation. The mesophilic *P. citrinum* tolerate the arsenic concentration upto 500 mg L^{-1} but showed the best growth at 50 mg L^{-1} with 5.6 cm diameter on arsenic containing petriplates after 28 days of incubation.

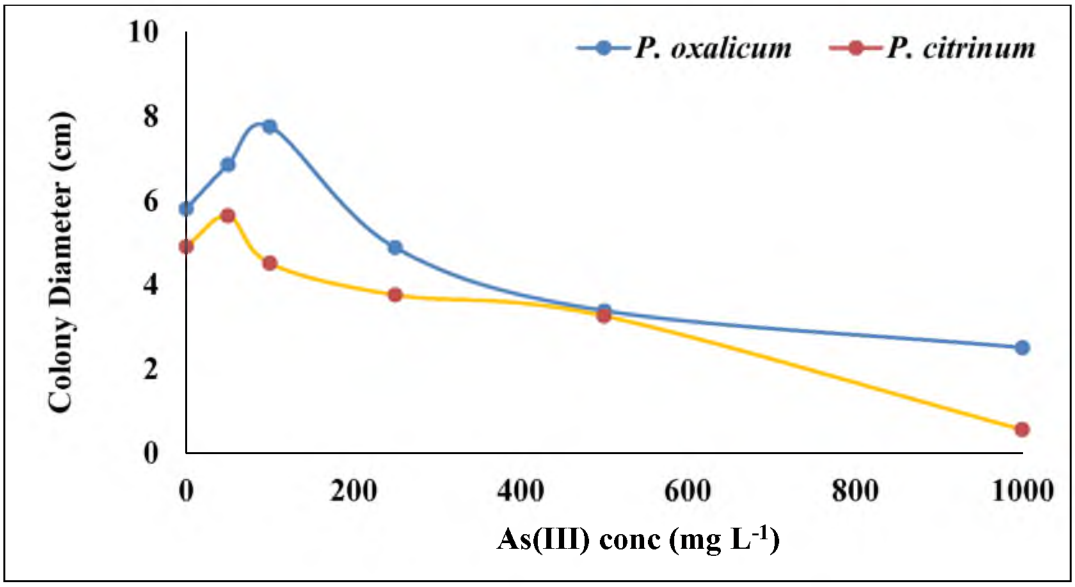


Fig. 7.1 Growth of *Penicillium* strains grown on medium containing As (III) on petriplates.

7.3.2 Plate test for (MIC determination for Cr VI)

Results for Chromium tolerance revealed that the psychrophilic fungal isolate *P. oxalicum* was more tolerant to chromium than the mesophilic *P. citrinum*. Psychrolphilic *P. oxalicum* was able to tolerate Chromium upto 10 mg L^{-1} concentration with growth diameter 2.5 cm, whereas the mesophilic *P. citrinum* was able to tolerate the Chromium concentration upto 01 mg L^{-1} with growth diameter beyond their respective optimum level of 1.9 cm. Further increase in Chromium concentration resulted into decline in growth of both *Penicllium* strains.

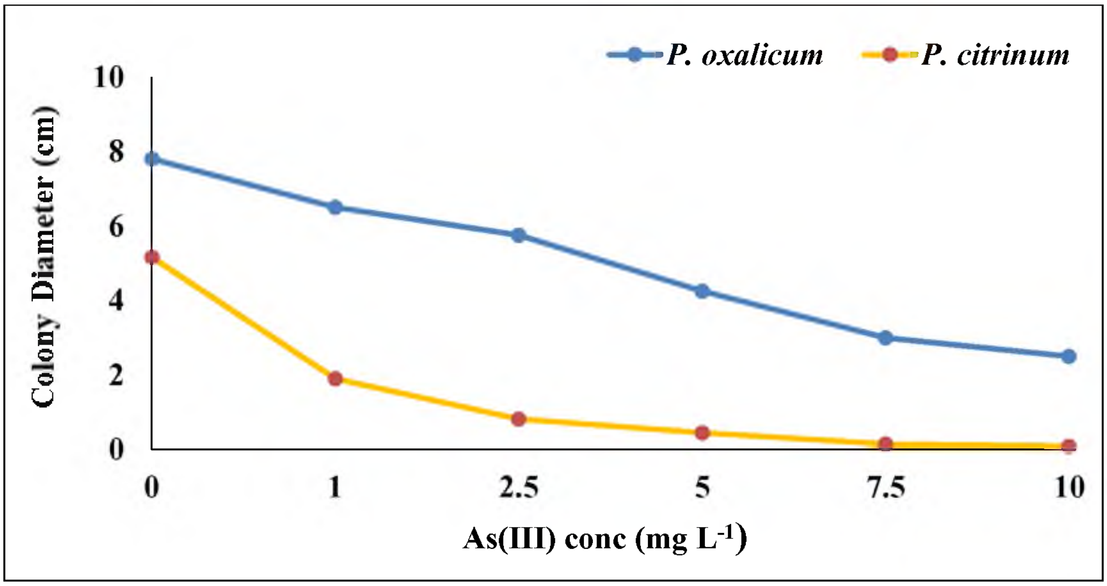


Fig.7.2 Growth of *Penicillium* strains grown on medium containing Cr (VI) on petriplates.

7.3.3 Arsenic (As III) stress (Batch Culture)

Psychrophilic *P. oxalicum* strain was able to tolerate arsenite (As III) concentration up to 1000 mg L^{-1} with average fresh weight 50 g L^{-1} and its best growth was recorded at 100 mg L^{-1} concentration of arsenic with average fresh weight 155 g L^{-1}(Fig. 7.3). On the other hand, the mesophilic *P. citrinum* strain was able to tolerate upto 50 mg L^{-1} concentration of arsenic with average fresh wt. 112.4 g L^{-1}. A further increase in concentration resulted into decline in growth [11 g L^{-1} fresh weight at 1000 mg $L^{-1}$of As (III)] (Fig. 7.3). Hence, the results revealed a significant difference in the level arsenic tolerance in both the *Penicillium* strains which could be attributed to Arsenic adsorption and accumulation efficiency of both the fungal strains. Adeyemi, (2009), also reported that use of fungal strains for bioremediation purposes depends upon their potential to adsorb and accumulate toxic metals. In order to verify the possible bioremediation potential of *Aspergillus niger*, *Serpula himantioides* and *Trametes versicolor*, they observed that different fungal strains have different potential of arsenic accumulation which use as generic character. Srivastava, *et al*. (2011), reported that 15 fungal strains including species of *Penicillium* and *Aspergillus* were able to tolerate very high concentration of sodium arsenate, and thereby, these are considered as potential agents to remove arsenic contamination. Oladipo, *et al*. (2017) reported that three fungal strains (*Fomitopsis meliae, Trichoderma ghanense and Rhizopus microspores*) tolerant to Cu, Pb, and Fe (400-1000 mg kg^{-1}) and could be used as effective agents for bioremediative clean-up of heavy metal contaminated environments. However, little is known about psychrophilic fungal strains and their applications in heavy metal bioremediation in cold environment. So, psychrophilic *P. oxalicum* strain could be a role model with great potential to be used in bioremediation of arsenic in cold environment.

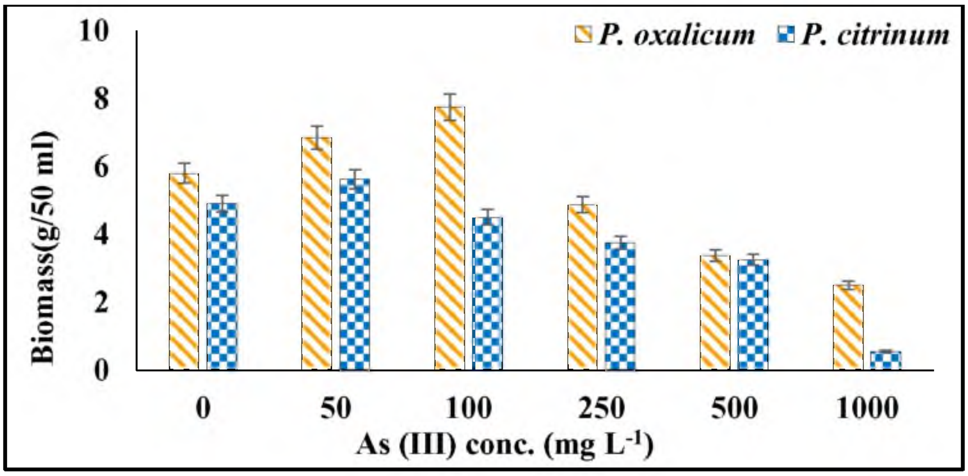


Fig. 7.3 Growth of *Penicillium* strains grown in the presence of As (III) in basal medium.

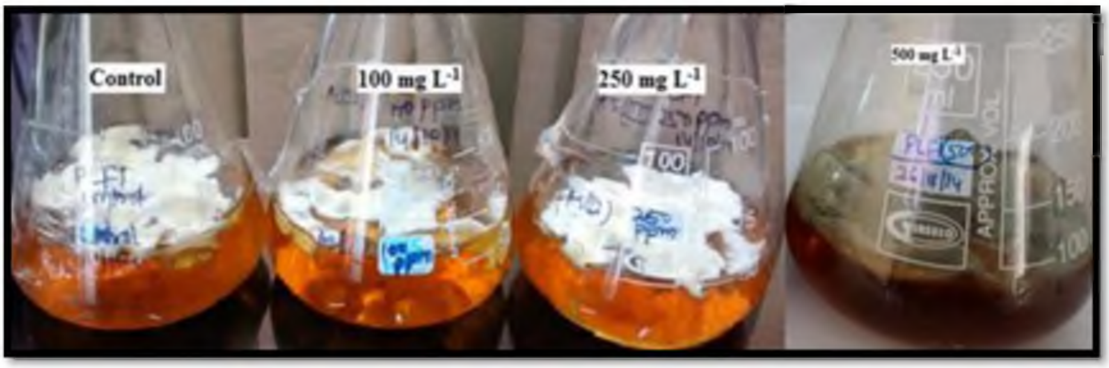


Fig. 7.4 Effect of As (III) concentrations on the growth of *P. oxalicum* at optimum temperature (15°C).

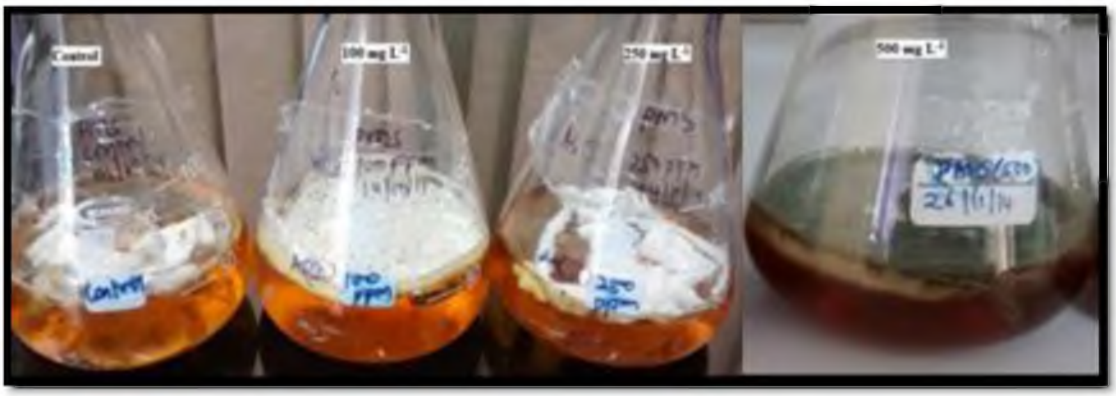


Fig. 7.5 Effect of As (III) concentrations on the growth of *P. citrinum* at optimum temperature (30°C).

7.3.4 Chromium [Cr (VI)] stress (Batch Culture)

The psychrophilic *P. oxalicum* exhibited optimum growth at 01 mg L^{-1} concentration of chromium when grown at varying concentrations (2.5 to 10 mg L^{-1} w/v). A further increase in the metal concentration resulted into decline in the growth of *P. oxalicum*. The average fresh weight of biomass of *P. oxalicum* 19.4 g L^{-1} and *P. citrinum* 07 g L^{-1} at 01 mg L^{-1} chromium concentration was significantly different (Fig 7.6). The chromium tolerance level in *P. oxalicum* was upto 10 mg L^{-1} concentration of chromium with average fresh weight 4.4 g L^{-1}. The av. fresh weight 0.174 g L^{-1} of biomass were recorded for 10 mg L^{-1} chromium concentration indicated negligible growth in *P. citrinum*. A complete disappearance of mesophilic *P. citrinum* was observed at 2.5 mg L^{-1} or higher concentration of Cr (VI) (Fig. 7.6). Hence, these results revealed that the psychrophilic *P. oxalicum* was significantly more tolerant to chromium concentration than the mesophilic *P. citrinum* strains. The occurrence of several other fungi such as *Penicillium, Aspergillus, Rhizopus, Fusarium*, *Geomyces, Paecilomyces* and *Chaetomium* species in the soil polluted by heavy metals (Cu, Cd, Pd, As and Zn) has been reported by other researcher (Babich and Stotzky, 1985; Gadd, 1993). Say, *et al*. (2003) reported the pH-dependent potential of *Penicillium canescens* for cadmium, lead, mercury, and arsenic ions removal from aqueous solution as a biosorbent. In a study by Akhtar, *et al.* (2013), showed that the metal tolerance in filamentous fungi (*Aspergillus>Pithyum>Curvularia*) was common phenomenon, but they respond differently to different metals. There were morphological and physiological differences between fungal genera, and therefore, their response could not be the same to heavy metal ions (Al- Garni, *et al.,* 2009). Volesky, (1990) suggested that the variation in the metal tolerance by fungi, may be due to one or more resistance mechanisms. Generally, the metal tolerance is based on the ionic species associated with the cell surface of fungi and production of extracellular proteins, chitins, and polysaccharides. Hence, the mentioned results suggested that the Cr (VI) tolerant psychrophilic *P. oxalicum* fungi can successfully be used for bioremediation. The use of metal tolerance fungi could be an efficient strategy due to its low cost, high efficiency and eco-friendly nature.

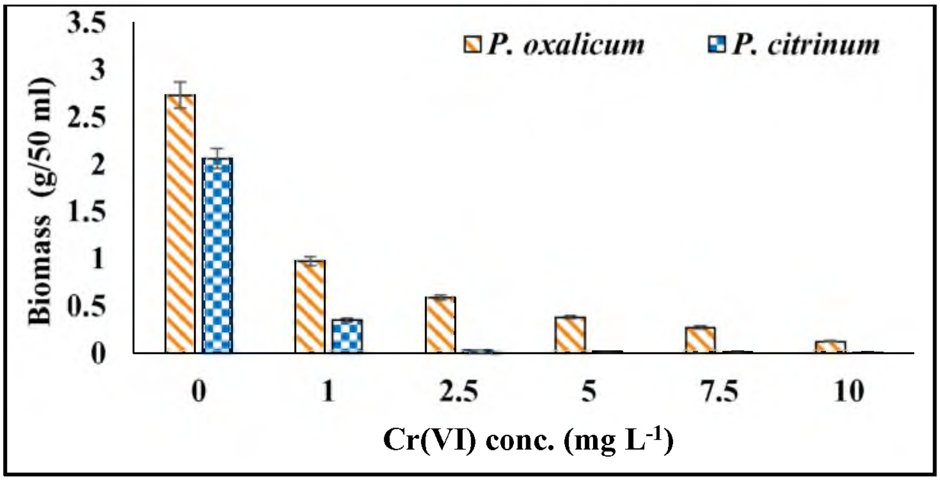


Fig. 7.6 Growth of *Penicillium* strains grown in the presence of Cr (VI) in basal medium.

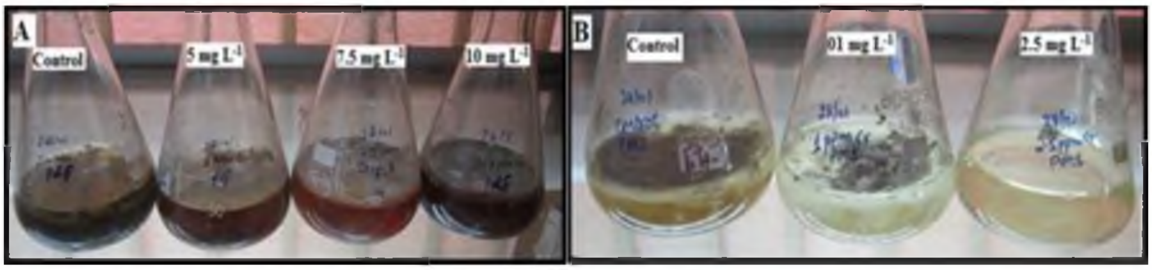


Fig. 7.7 Effect of Cr (VI) on the growth of *P. oxalicum* (A) and *P. citrinum* (B).

7.3.5 Extraction and screening of secondary metabolites of *Penicillium* strains under heavy metal stress

A. Arsenic stress

The absorption spectra (250-400 nm) of EtOAC extracts of secondary metabolites extracted from *Penicillium* strains grown in different concentration of As (III) was analysed. Ethyl acetate (EtOAC) extracts of secondary metabolites of *P. oxalicum,* grown under high conc. of As (III) i.e. 500 mg L^{-1} (w/v), showed high absorption with peaks at 340 nm (0.92), at 328 nm (0.88), at 290 nm (0.82) and at 255 nm (0.76) when compared with absorption spectra of control (without As III) or with secondary metabolites obtained at low conc. of As (III). At 250 mg L^{-1} concentration of As (III) with absorbance with peaks at 350 nm (0.75), at 290 nm (0.54), at 255 nm (0.45) were reduced (Fig. 7.8). On the opposite side, EtOAC extracts of secondary metabolites of

P. citrinum at 250 mg L^{-1} (w/v) conc. of As (III) showed absorption maxima with peaks at 320 nm (3.1), at 310 nm(2.8), at 340 nm (2.5), at 265 nm (2.0), and at 280 nm (1.75). However, a decline in the absorbance of same peaks was observed at high concentration of As (500 mg L^{-1}w/v) with absorbance at 320 nm (2.85), at 265 nm (2.7) and at 255 nm (2.5) (Figure 7.9). The results revealed that the higher conc. of As (III) (500 mg L^{-1}) promoted the production of secondary metabolite in both *Penicillium* strains when compared with the control irrespective of growth.

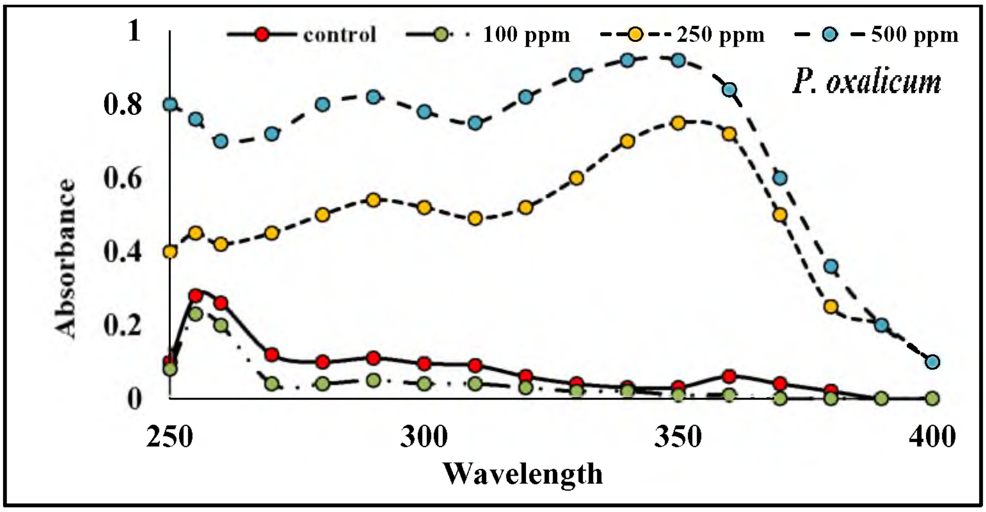


Fig. 7.8 Absorbance spectra of EtOAc extract of secondary metabolites of psychrophilic *P. oxalicum* under As (III) stress.

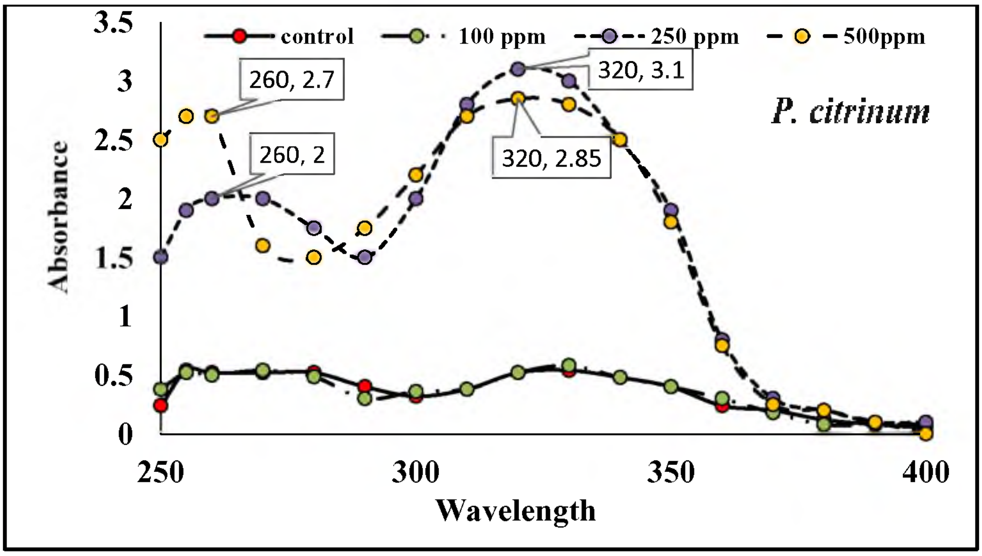


Fig. 7.9 Absorbance spectra of EtOAc extract of secondary metabolites of mesophilic *P. citrinum* under As (III) stress.

B. Chromium stress

The absorption spectra (250-400 nm standardized) of EtOAC extracts of secondary metabolites extracted from different concentration of Cr (VI) was analysed at different interval during incubation time. Ethyl acetate (EtOAC) extracts of secondary metabolites of *P. oxalicum,* grown under the 01 mg L^{-1} (w/v) concentration of Cr (VI) showed overall higher absorption maxima with peaks at 260 nm (0.28), at 270 nm (0.26), at 280 nm (0.24) and at 340 nm (0.21) and emergence of some minor peaks were recorded with absorbance 0.20 at λ 328 nm and 255 nm, at 350 nm(0.19), when compared with the control [without Cr (VI)] (Fig. 7.10). On the opposite side, EtOAC extracts of secondary metabolites of *P. citrinum* under 01 mg L^{-1} (w/v) concentration of Cr (VI) showed absorption maxima with peaks at 328 nm (1.6), at 320 nm (1.55), 1.2 at 260 and 350 nm when compared with the control [without Cr(VI)] (Fig. 7.11). These results revealed that the psychrophilic strain *P. oxalicum* was able to grow and produce more secondary metabolite at higher concentration of Cr (VI) (10 mg L^{-1}). Whereas mesophilic *P. citrinum* showed little growth at higher concentration of Cr (VI), but it was still able to produce high concentration of secondary metabolite at Cr (VI) 01 mg L^{-1}, when compared with psychrophilic counterpart. It is well known that Cr influence the morphology and physiology of soil microorganisms (Endo and Silver, 1995; Cifuentes, *et al.* 1996). For instance, many microorganisms exhibit resistance to deleterious effects of Chrromium. Additionally, chromate gets reduced to Cr (III) by the microorganisms. However, there is a lack of understanding on how the chromium influence the microbial activity especially fungal activity on Cr reduction, adsorption, biodegradation and secondary metabolite production. Heavy metals can influence the soil fungi and diminish their population or weaken the diversity and change the fungal morphology and physiology with overall effect on the growth rate, reproduction processes and enzyme production etc. (Martino, *et al.* 2000). In order to develop a cost-effective and more environmental friendly technique to remove Cr (VI) ions from industrial wastewater and contaminated sites, biological processes, such as bioreduction, bioaccumulation or biosorption using microbial cells, have been examined and are found to be effective in for their Cr (VI) removal (Cheung and Gu, 2003; Volesky, 2003).

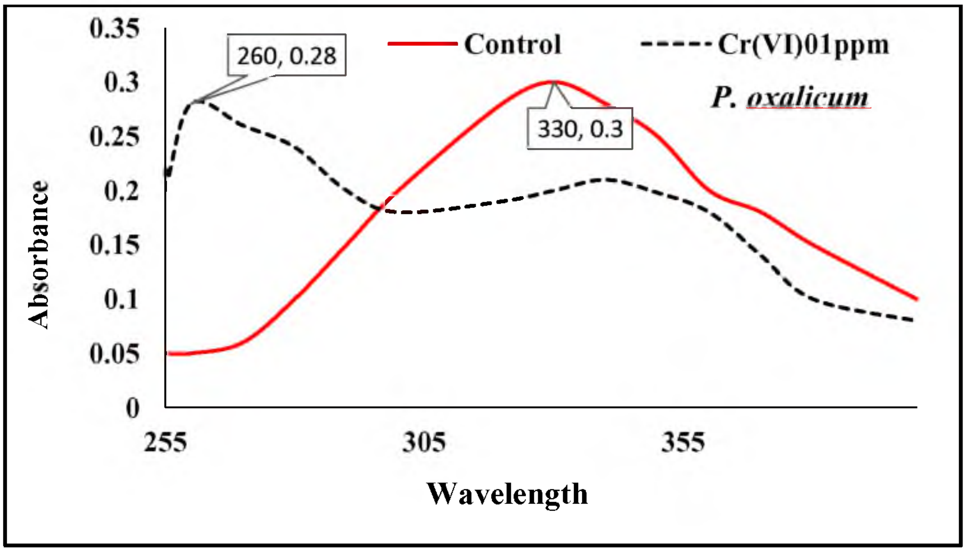


Fig. 7.10 Absorbance spectra of secondary metabolites of psychrophilic *P. oxalicum* under Cr (VI) stress.

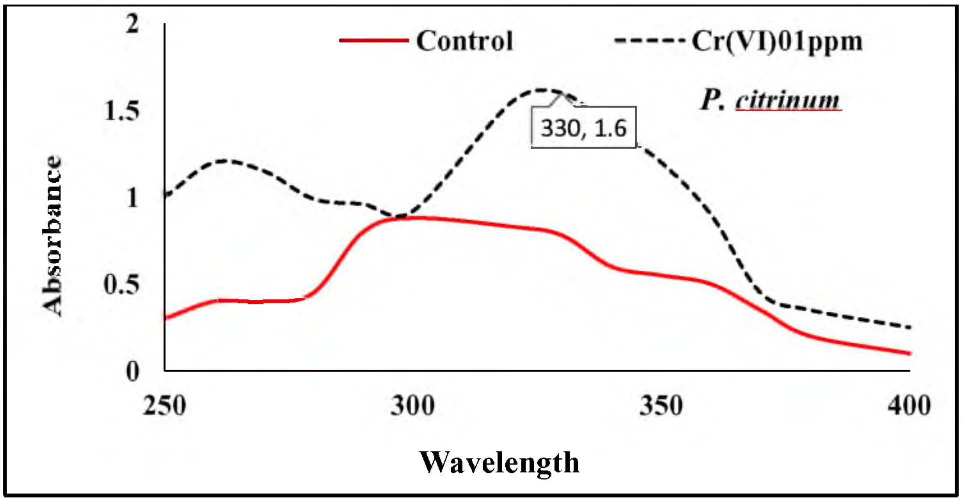


Fig. 7.11 Absorbance spectra of secondary metabolites of mesophilic *P. citrinum* under Cr (VI) stress.

7.3.6 FTIR analysis of *Penicillium* biomass grown in the presence of heavy metals

C. Arsenic (As III) stress

The FTIR spectra (4000 to 500 cm^{-1}) of biomass of both psychrophilic and mesophilic *Penicillium* strains in the presence of varying As (III) concentrations were compared. The result showed overall declined in IR absorption in case of both the *Penicillium* strains treated with 100 mg L^{-1} of As (III) with IR peaks at wavenumber 3288 cm^{-1} assigned to symmetrical O-H stretching and 2929.2 cm^{-1} assigned to stretching C-H (Dovbeshko, *et al.*, 1997), 1649.8 cm^{-1} for unordered random coils and turns of amide I and 1539.8 cm^{-1} for Protein amide II absorption- predominately β-sheet of amide II (Eckel, *et al.* 2001), 1449.1 cm^{-1} for asymmetrical CH_3 bending of the methyl groups of proteins (Wang, *et al.*, 1997), 1380.9 cm^{-1} for Stretching C-O, deformation C-H, deformation N-H (Dovbeshko, *et al.*, 1997), 1242.1 cm^{-1} for Amide III (Chiriboga *et al.* 1998, Andrus and Strickland, 1998), 1152.5 cm^{-1} assigned to glycogen absorption due to C-O and C-C stretching and C-O-H deformation motions (Chiriboga, 1998), 1075.5 cm^{-1} for ν(PO2) symmetric stretching of phosphodiesters (Fujioka, *et al.*, 2004) and 1043.8 cm^{-1} assigned to symmetric stretching of phosphate groups of phosphodiester linkages (Fabian, *et al.* 1995). The results on the FTIR spectrum of psychrophilic *P. oxalicum* treated with As (III) 100 mg L^{-1}showed increase in absorption peak at 1649 cm^{-1} assigned to random coils and turns of C=O, C=N, N-H groups of Amide I (Eckel, *et al.*, 2001; Dovebeshko, *et al.*, 2002) and was found absent in control (without As III). A new IR peak at 1075 cm^{-1} assigned to phosphodiesters (Fujioka, *et al.*, 2004) which was found in case of arsenic treated [As (III)] psychrophilic *P. oxalicum*, but was absent in case of control (without As III). Aside from this, there are no any major changes were found in IR spectrum of biomass of mesophilic *P. citrinum* treated with As (III) 100 mg L^{-1} instead little shift was recorded at wavenumbers 1647.7, 1538.2, 1452.5 cm-

^{1} assigned to amide I & amide II region as compared with control (without As III, Fig 7. 12). The overall results suggested the functional groups present in fungal cell membrane do not play an important role in arsenic removal but the protein deformation, changes in structure of proteins might be caused due to arsenic stress conditions.

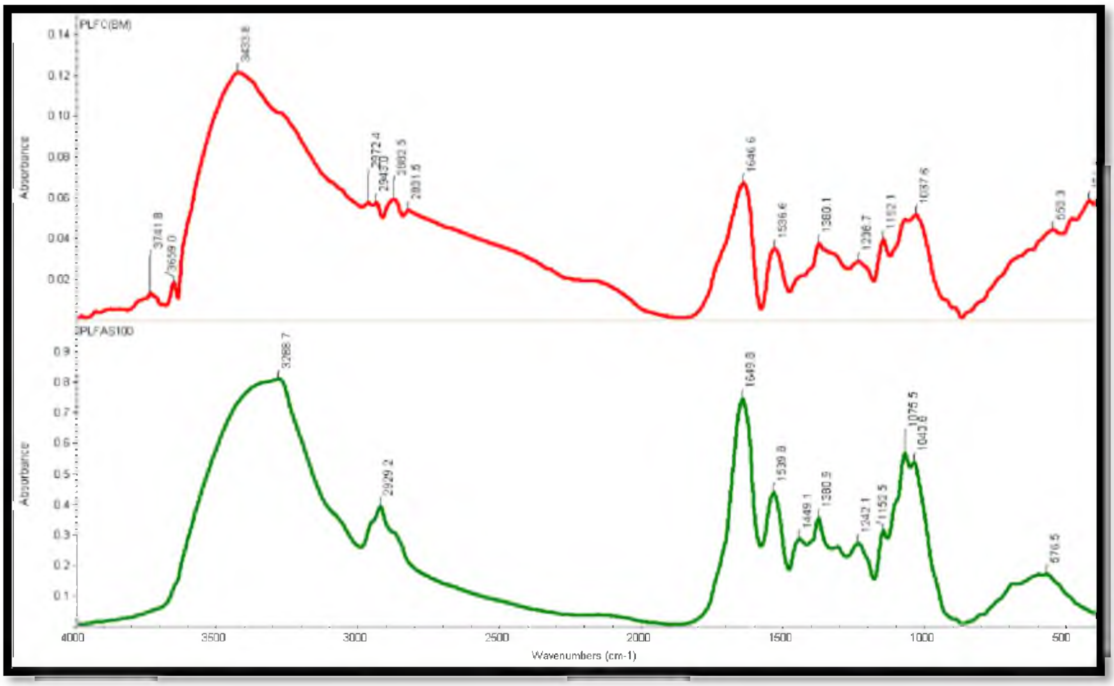


Fig 7.12 FTIR absorbance spectra of biomass of *P. oxalicum* grown with and without As (III) (Control and 100 mg L^{-1} concentration of As III).

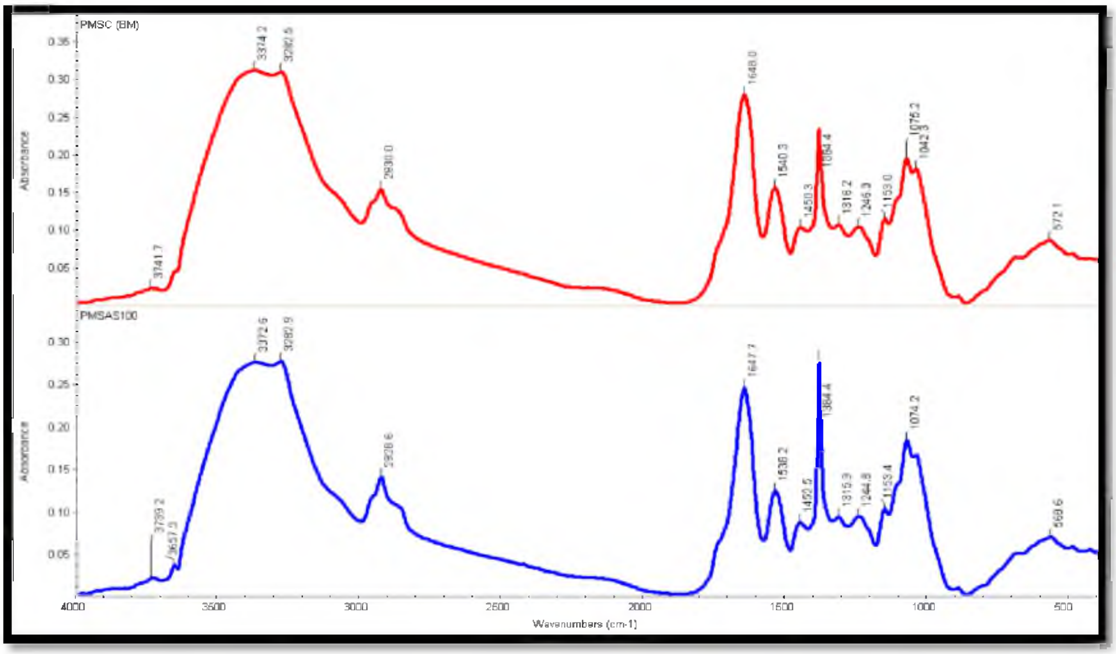


Fig 7.13 FTIR absorbance spectra of biomass of *P. citrinum* grown with and without As (III) (Control and 100 mg L^{-1} concentration of As III).

D. Chromium [Cr (VI)] stress

The FTIR spectra (4000 to 500 cm^{-1}) of biomass of both psychrophilic and mesophilic *Penicillium* strains grown in the presence of Cr (VI) (0- 10 mg L^{-1}) showed overall decline in IR absorption peaks at different wavenumbers. The emergence of IR peak at wavenumber 3072 cm^{-1} assigned to C-H ring (Dovbeshko, *et al.*, 1997), was found in case of psychrophilic *P. oxalicum* treated with 01 mg L^{-1} concentration of Cr(VI) when compared with the control (without chromium, Control). The same peak at wavenumber 3072 cm^{-1} has shifted to 3067.4 cm^{-1} in case of psychrophilic *P. oxalicum* when treated with 2.5 mg L^{-1} concentration of Cr (VI) but it disappeared with the rise in Cr (VI) concentrations. The peak assigned to Amide I (1630 cm^{-1}) altered by different concentration of Cr (VI) and shifted to wavenumbers 1634.3, 1631.2, 1637.9, 1623.9, 1625.0 cm^{-1}. It indicated significant role of proteins in Chromium binding by *P. oxalicum*. On the other hand, there is little shift in peak at wavenumber 1653.2 cm^{-1} was recorded in case of mesophilic *P. citrinum* treated with 01 mg L^{-1} of Cr(VI). There was peak shift in absorption at wavenumber 1222 cm^{-1} to 1227.5 & 1238.7 cm^{-1}, assigned for PO_2^- asymmetric (phosphate I) (Dovbeshko, *et al.*, 1997) and 1500-60 cm^{-1} assigned for Amide II (N-H bending vibration coupled to C-N stretching) was recorded in chromium grown (01,2.5 & 5 mg l^{-1} respectively) biomass. A previous report by Wong, *et al.* (1995) supported the present study as they suggested the α-helix conformational changes of proteins in microbial cell, altered with the increase in Cr (VI) concentration. In a study by Ramrakhiani *et al.*, (2011), FTIR analysis confirmed the involvement of amino, carboxylic, phosphate, sulfonyl and carbonyl groups in Cr (VI) biosorption by heat inactivated biomass of *Termitomyces clypeatus* (an agaric fungus). They also revealed that FTIR study indicated that the biosorption of Cr(VI) followed two subsequent steps, biosorption of $Cr_2O_7^{2-}$ by electrostatic force at the protonated active sites (amino, carboxyl and phosphate groups) and reduction of Cr(VI) to Cr(III) by reductive groups (hydroxyl and carbonyl groups) on the surface of the biomass. Thus the, overall study revealed that the changes in appearance and disappearance in transmittance of peaks and changes on functional groups occurred after Cr binding by methylated amino group, esterified carboxyl and phosphate group biomass(Fig. 7.14-7.16). The clear appearance of peaks ranging from 1500 to 1200 cm^{-1} and 1200 to 1000 cm^{-1}, assigned to C =O stretching in carbonyl or amide I band, N–H bending in amide II and C–N stretching in –CO–NH–, and C–OH, showed involvement of proteins in Cr(VI) binding. The increase of these bands indicated the improvement in biosorption and involvement of N–H of amines, C=O of amides, carboxyl and phosphate groups.

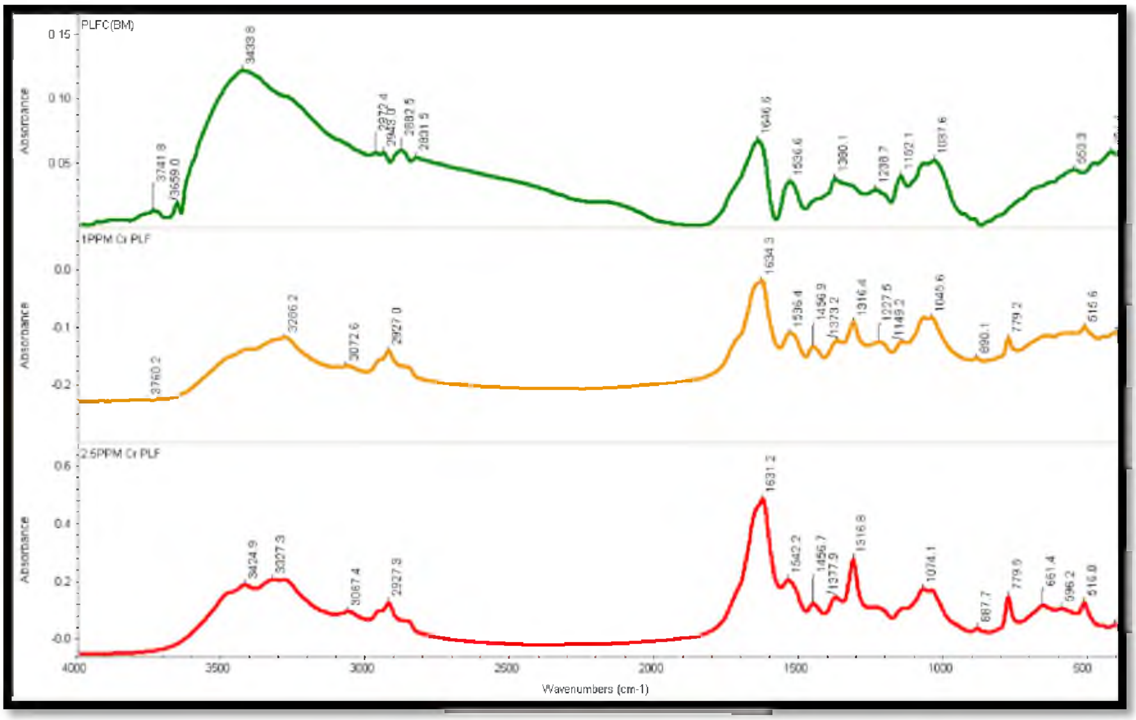


Fig 7.14 FTIR absorbance spectra of biomass of *P. oxalicum* grown with and without Cr (VI) (0-2.5 mg L^{-1} ^{1} concentration of CrVI).

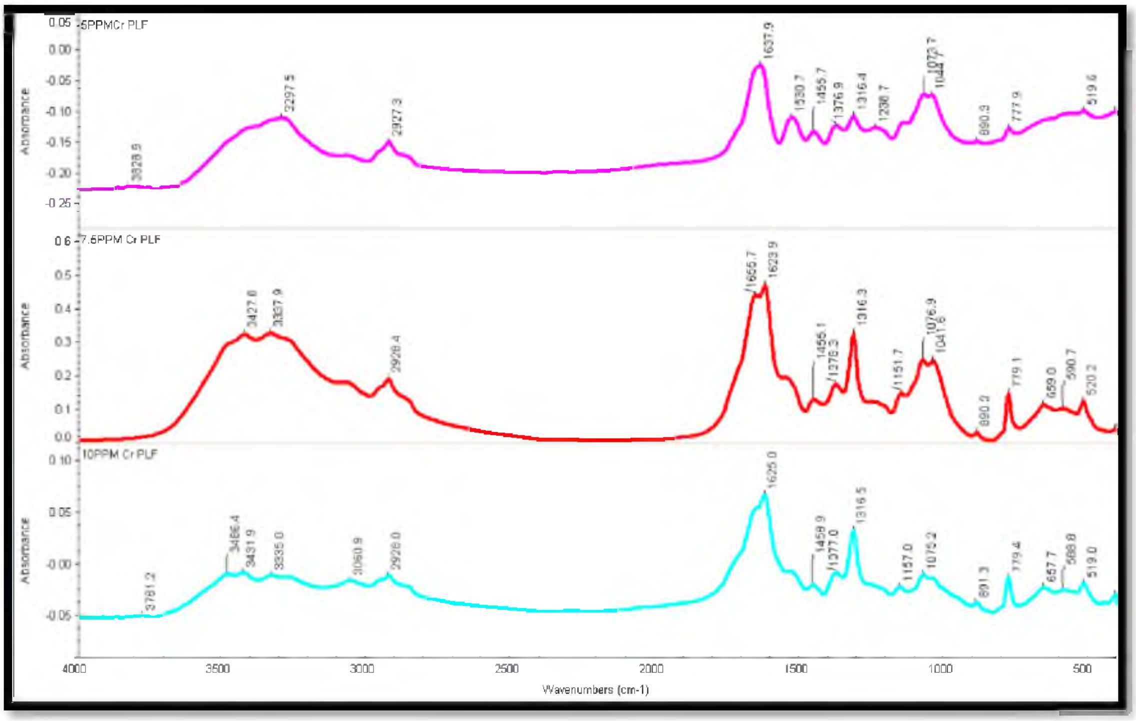


Fig 7.15 FTIR absorbance spectra of biomass of *P. oxalicum* grown in the presence of 05-10 mg L^{-1} concentration of Cr (VI).

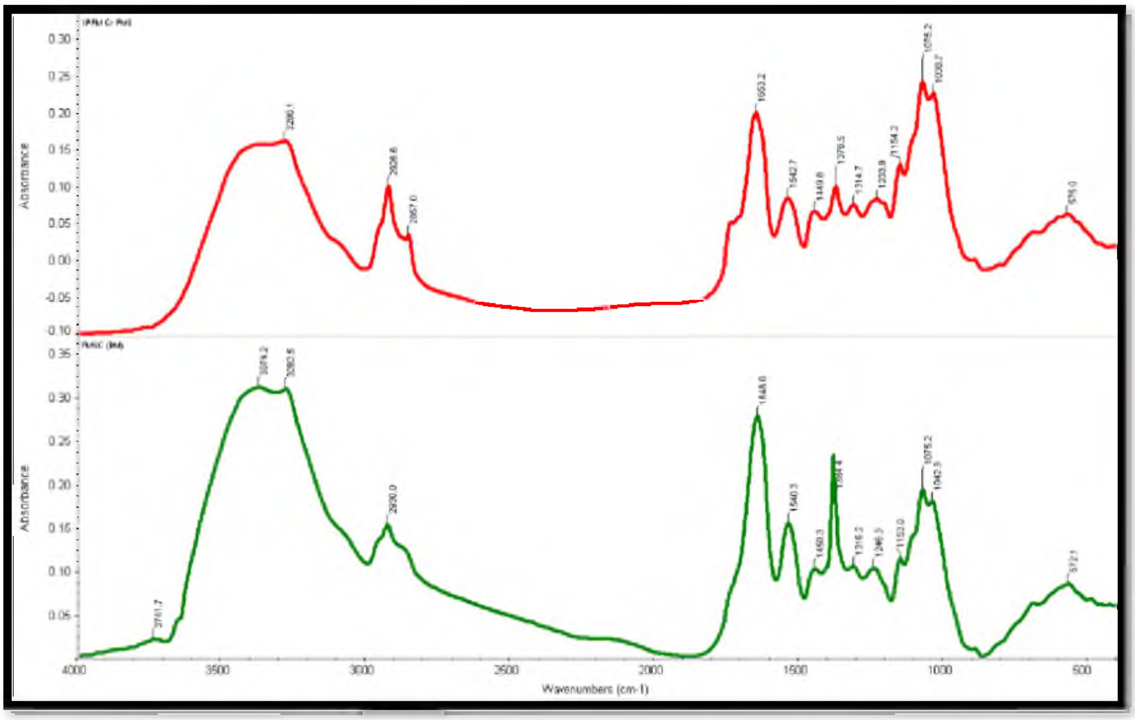


Fig 7.16 FTIR absorbance spectra of biomass of *P. citrinum* grown with and without Cr (VI) (0-1.0 mg l^{-1}).

7.4 Conclusion

- The growth of *Penicillium* strains in the presence of heavy metals, As (III) and Cr (VI) revealed that the psychrophilic *P. oxalicum* was more tolerant to heavy metals than mesophilic strain *P. citrinum*. The psychrophilic *P. oxalicum* was able to grow upto 1000 mg L^{-1} concentration of As (III) and 10 mg L^{-1} concentration of Cr (VI). On the other hand, mesophilic strain *P. citrinum* could grow upto 500 mg L^{-1} of As (III) and only 01 mg L^{-1} concentration of Cr (VI).
- The results on secondary metabolite production revealed that the higher conc. of As (III) (500 mg L^{-1}) and Cr (VI) (10 mg L^{-1}) promoted the production of secondary metabolites in both *Penicillium* strains, when compared with the control (without metal), irrespective of growth.
- The FTIR analysis of *Penicillium* biomass grown in the presence of As (III) indicated protein deformation in both *Penicillium* strains. But the results of FTIR analysis of both *Penicillium* biomass grown in the presence of Cr (VI) revealed disappearance of certain IR absorption peaks and appearance of new peaks related to methylated amino group, carboxyl ester and phosphate group.

CHAPTER-8 IDENTIFICATION OF SECONDARY METABOLITES

8.1 Introduction

The search for novel organic metabolites with their interesting biomedical properties is getting an increasing attention in the present scenario. Production of secondary metabolites by microrganisms under extreme environmental conditions has always been a topic of interest for researchers as the extreme habitat is known to induce some new kind of useful metabolites or harbour new type of microorganisms to produce distinctive metabolites (Magan, 2007). Secondary metabolites are usually bioactive molecules with low molecular weight, and are produced by microorganisms during limited period of their life cycle (Keller, *et al.* 2005). Secondary metabolites share the unusual properties of cellular dispensability. Microorganisms are able to grow without producing these metabolites with restricted taxonomic distribution (only a small group of organisms produce metabolite) (Bennett and Bentley, 1989; Keller, *et al*., 2005).The systematic study of fungal secondary metabolites began in long ago in 1922 under the leadership of Harold Raistrick, who finally characterized more than 200 mold metabolites (Raistrick, 1950). However, it was not until the discovery of *Penicillin* that widespread attention was given to the study of fungal metabolites. By the mid of 20 th century, collection of microbial products with pharmaceutical applications were discovered by pharmaceutical industries. The search for bioactive secondary metabolites has persistently continued, and thousands of compounds which could inhibit the growth of bacteria, fungi, protozoa, parasites, insects, viruses and even human tumor cells have been discovered. Many other molecules with cytotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive, enzyme inhibitory and allelopathic effects have been discovered (Keller, *et al*., 2005). Filamentous fungi are well known producers of unique metabolites which can be used as antibiotics and therapeutic agents etc. (Calvo, *et al*. 2002, Stierle, *et al*. 2006; Hujslovia, *et al*. 2010). A recent literature survey on fungal metabolites has shown about 1,500 compounds that were isolated and characterized in between 1993 to 2001 and more than half of these molecules show antibacterial, antifungal or antitumour activity (Pelaez, 2005). Extremophilic, especially cold tolerant species of fungi are of great importance in biotechnological and pharmaceutical fields due to their adaptation adaptability and survival under extreme environments conditions as well as unique characteristics of product bioactive secondary metabolites and cold-active enzymes (Han, *et al*. 2007; Lopes, *et al*. 2012; Subramani, *et al*. 2013; Wang, *et al*. 2013).The identification of secondary metabolites produced by fungi and the fungal taxonomy for ascomycete genera has been well documented such as species of *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*. Many species of *Penicillium* and *Aspergillus* are reported to be dominant genera under extreme environmental conditions (low temperature and saline habitats) (Cantrell, *et al*., 2011). Recently, Shi, *et al*. (2015) reported that the generation of secondary metabolites or natural products can be intensified by extremotolerant microorganisms inhabiting the stressful environment due to their chelation ability. There are various classes of secondary metabolites and genes associated with fungi which includes polyketides, non-ribosomal peptides terpenes, indole alkaloids and many others (Keller, *et al*. 2005; Hoffmeister and Keller, 2007; Christensen and Kolomiets, 2010). In respect to identification of secondary metabolites, Larsen and Frisvad, (1995) and Springfield, *et al*. (2005) stated that the Gas Chromatography is the greatest technique for best mode of separation of volatile secondary metabolites. However the most bioactive secondary metabolites are not volatile, so different separation methods are required. HPLC or ultra- performance liquid chromatography (UPLC) could be the methods of choice for general separation and simultaneous detection of secondary metabolites. There are two major methods reported for the identification of different peaks eluted from the HPLC, a hyphenated diode array detector (UV-visible spectra) or a hyphenated mass spectrometric detector (Frisvad, 2010). It is also reported that the some secondary metabolites made up of conjugated double bonds, and thus they have more or less specific UV spectra and these UV spectra are often similar to other biosynthetically associated secondary metabolites. (Nielsen, *et al*. 1999; Hansen, *et al*., 2003; Andersen, *et al*. 2005; Larsen, *et al*. 2005). The data for fungal metabolites can be referenced or compared with the Database of 474 mycotoxins and fungal metabolites reported by Frisvad and Thrane (1993) and Nielsen and Smedsgaard (2003). In this chapter, the experiments related with the identification of secondary metabolites produced by both the *Penicillium* strains were performed. The main objective of this work was to identify the volatile and non-volatile compounds produced by *Penicillium* strains. The volatile compounds were identified by using Gas Chromatography- Mass spectrometry (GC- MS/MS) and non-volatile compounds by using High Performance Liquid Chromatography- Photo diode Array (HPLC-PDA). Later the characterization of EtOAc extract containing non-volatile secondary metabolites produced by both *Penicillium* strains was done by UV_{max} absorption by HPLC-PDA. ^{1}H NMR study also done for the purpose of identification of secondary metabolites and the results were matched with the Spectral Database System for Organic compounds (AIST, Japan) and various other research reports.

8.2 Materials and Methods

8.2.1 Culturing of *Penicillium* strains, extraction of metabolites and their UV spectrum analysis

Spores of both the *Penicillium* strains were inoculated in a basal medium (as mentioned in chapter 3& 4). The fungal culture (100 ml) was incubated without agitation for 2 weeks at their respective optimum temperatures. Once the incubation period was completed, the mycelia were filtered off, soaked with ethylacetate (EtOAc) for 24 hrs and organic layer was separated. The aqueous medium was subjected to liquid-liquid extraction with EtOAc. Both EtOAc extracts were combined and concentrated by evaporation under reduced pressure. The EtOAc crude extracts of secondary metabolites was quantified by taking absorption spectra (250-400 nm) by using UV-Visible Spectrophotometer (UV-1601 Shimadzu).

8.2.2 Thin Layer Chromatography analysis

The compounds present in the EtOAc crude extracts of the secondary metabolites were separated by Thin layer Chromatography (TLC). The crude extracts were applied on thin-layer silica gel plates and solvent systems CAP (chloroform/acetone/2-propanol 85:15:20 v/v/v) and chloroform/ methanol/25% NH4OH (90: 10: 0.1 v/v/v) was run to separate the compounds. The TLC plates were sprayed with 6 N sulfuric acid /methanol (1:1 v/v) for detection of compounds (Maiti, *et al.* 2006). The nitrogen containing metabolites and indole alkaloids were detected by Dragendorf reagent and Ehrlich reagent, respectively (Nicoletti, *et al.* 2007). The chromatogram was observed under short and long UV light.

The values of Retention factor (Rf) also calculated as follows-

Rf value = Distance travelled by the solute/Distance travelled by the solvent

8.2.3 GC-MS/MS analysis

Gas chromatography- mass spectroscopy analysis of secondary metabolites was performed in EtOAc crude extracts of both *Penicillium* strains using GC-MS/MS, Triple Quadrupole Mass analyzer (Model Name GC1310 /TSQ8000 Evo system) with liquid auto sampler-Triplus RSH, by Thermofischer Scientific Pvt Ltd. Mumbai (India). The Column selected was –TG- 5MS (30 m × 0.25 mm × 0.25 um) with column conditions- 40°C/5 min, Ramp rate 10°C/min - 260°C/10 min, Injector -260°C, Ion source- 200°C, Interface-260°C, Mass range: 35-550, injection volume: 1.0 µL. The mass spectrum of the unknown compounds was compared with the spectrum of the known compounds by the use of database of (NIST, National Institute Standard and Technology).

8.2.4 HPLC Fingerprinting

The qualitative analysis of secondary metabolites produced by both the *Penicillium* strains was done by using HPLC-PDA, stationary phase, C-18 Symmetry Waters® (4.6×250mm, 5µm), Running conditions included: injection volume, 20µl; mobile phase, Gradient (Water: Acetonitrile); flow rate, 1 ml/min; and detection at PDA (λ=254), 220, 254 & 280 nm.

8.2.5 ^{1}H NMR analysis of secondary metabolites

The 1H NMR spectra of EtOAc extracts of secondary metabolites from *Penicillium* strains were acquired at 300 K on Bruker Biospin Avance-III 800 MHz NMR (Bruker GmBH, Germany) spectrometer equipped with a triple resonance cryoprobe. The ^{1}H NMR spectra of EtOAc extracts of secondary metabolites were manually phased and automated baseline was corrected using TOPSPIN 2.1 (Bruker Analytik, Rheinstetten, Germany). Analysis of purified compounds was acquired in (Deuteratd chloroform: Trifluoroacetic acid), $CDCl_3$: TFA (4:1). NMR analyses were taken in 5 mm NMR tubes.

8.3 Results and Discussion

8.3.1 Thin Layer Chromatography analysis

A single spot on TLC plate with Rf value of 0.5 was observed after sprayed with 6 N sulfuric acid /methanol (1:1 v/v), which carefully scrapped in case of psychrophilic *P. oxalicum* and eluted with EtOAc. The absorption peak at 274.8 nm of separated spot was observed. On the other hand, no spot was observed on TLC plate by spraying with Ehrlich reagent in case of *P. oxalicum*. Though brownish black spot was detected on TLC plate in case of extract of mesophilic *P. citrinum* under short wavelength UV-light. However the same plate stained with Ehrlich reagent showed no spot, and an orange spot was observed when same plate sprayed with Dragendorf reagent with Rf value around 0.3. The spot then carefully scrapped and eluted with EtOAc, which showed absorption peaks at 255.2 and 326.4 nm. The results revealed the presence of some nitrogenous compounds in secondary metabolites of *P. oxalicum*, whereas the presence of alkaloids compounds in sec. metabolites of *P. citrinum* was observed.

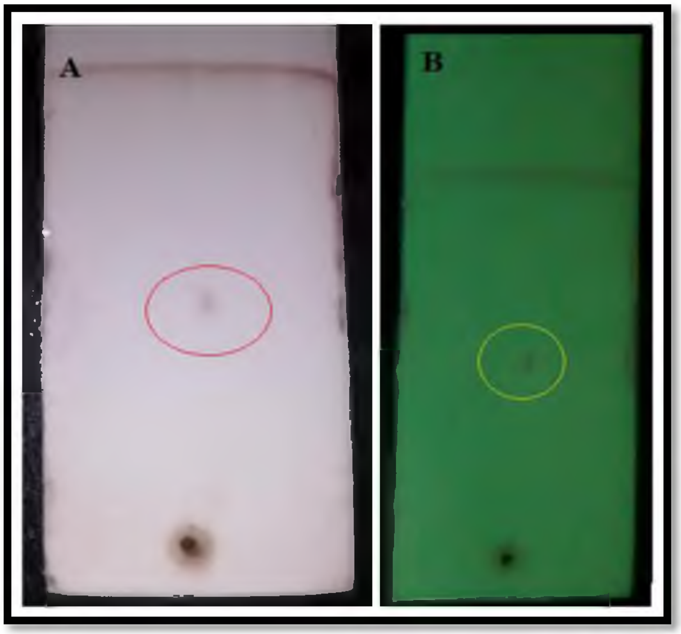


Fig 8.1 (A& B). TLC plate showing spot sprayed with 6 N sulfuric acid/methanol in case of metabolite extract of psychrophilic *P. oxalicum* (A) and black/brown spot under short UV wavelength in case metabolite extract of mesophilic *P. citrinum* (B).

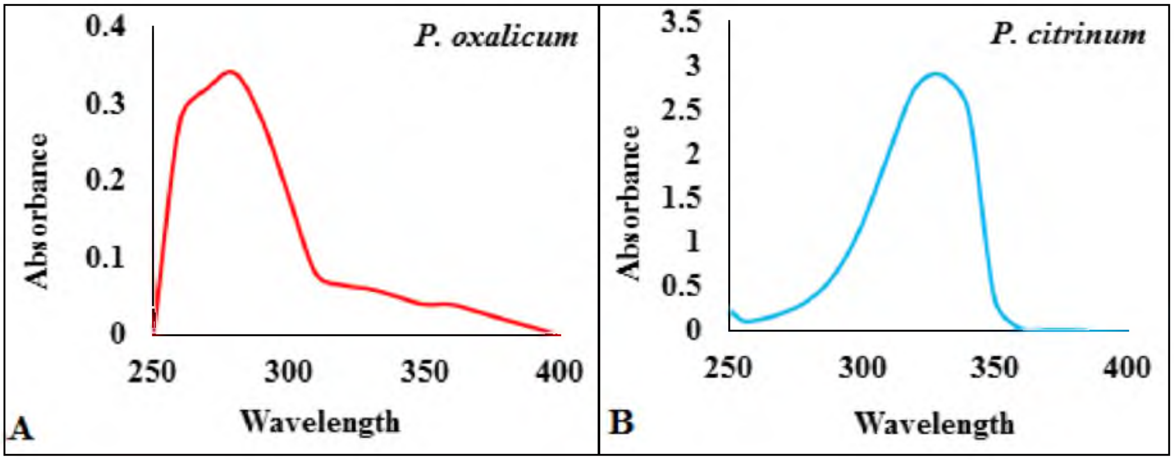


Fig. 8.2 (A&B). Absorbance spectra of compounds scrapped from TLC plate of *Penicillium oxalicum* (A) *and Penicillium citrinum* (B).

8.3.2. GC-MS/MS analysis of the secondary metabolites of *Penicillium* strains

The results of GC-MS chromatogram of the EtOAc extract of volatile sec. metabolites of psychrophilic *P. oxalicum* grown under two different extreme temperatures (4°C and 35°C) revealed the presence of 11 and 16 major peaks at respective temperature (Table 8.1). On the contrary, EtOAc extract of volatile sec. metabolite of mesophilic *P. citrinum* grown at 4°C and 35°C revealed 14 and 12 major peaks at respective temperature (Table 8.2). The common sec. metabolites produced by both *Penicillium* strains under different temperature regimes (4°C and 35°C) comprises 2-dodecanol, 3- dodecene, 1-hexadecanol at low temperature (4°C) and Eicosane, 9-Hexacosene, Dibutyl phthalate, Propanoic acid,2-(aminooxy) at high temperature (35°C). The three unmatched sec. metabolites produced by psychrophilic *P. oxalicum* when grown at low temperature (4°C) comprises 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent[f] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The five unique bioactive compounds produced by psychrophilic *P. oxalicum* grown at high temperature (35°C) includes 2-Methyl-2-propyl methyl phosphonofluoridate, 4(1H) Quinazolinone, 4(1H) Pyrimidinone, Pyridine, 2[(1,1dimethylethyl) thio, 4(3H) Quinolinone, 6-amino-2-methyl-5-nitroso and Phthalic acid, di (2-propylpentyl). Likewise seven unmatched compounds of sec. metabolites produced by mesophilic *P. citrinum* grown at low temperature stress (4°C) comprises Cyclohexanone,4-ethyl-4-methyl-3-(1-methylethyl)-, trans-, 3-Methyl-1,4diazabicyclo [4.3.0] nonan-2,5-dione, N-acetyl, Glycyl-L-proline, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and 11,14-Eicosadienoic acid or its methyl esters. The results of sec. metabolites of mesophilic *P. citrinum* grown under high temperature (35°C) revealed the presence of exceptional derivative of β-lactam antibiotic i.e., 2,4-Azetidinedione, 3,3-diethyl-1-methyl. The additional sec. metabolites produced by this strain at high temperature (35°C) include Methylamine, 2,4-bis(1,1-dimethylethy)-, 2,3-Dimethylhydroquinone and Phenol. These results showed the presence of three unmatched sec. metabolites produced by psychrophilic *P. oxalicum* only when grown at low temperature (4°C), which included 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent [f] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The other five inimitable biochemicals produced by *P. oxalicum* at high temperature (35°C) were 2-Methyl-2-propyl methyl phosphonofluoridate, Pyridine, 2[(1,1dimethylethyl) thio, 4(1H) Quinazolinone, 4(1H) Pyrimidinone, 6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di (2-propylpentyl). These matchless compounds as mentioned above were produced by the psychrophilic *P. oxalicum* only, whereas they were absent in case of mesophilic strain *P. citrinum*. However, few common metabolites produced by *P. oxalicum* respond to change in the growth temperatures in terms of percent increase or decrease in the production of compounds such as Dodecanol at 4°C (9.44%) declined to the level of 1.95% at 35°C. Similarly, percent abundance of 4(1H) Quinazolinone at 4°C (0.44%) inclined to 1.7% at 35°C and abundance of 10-Heneicosene at 4°C (5.95%) inclined to 9.37% at 35°C. Another view of secondary metabolites production proposes that the genes involved in secondary metabolism provide a ‘‘genetic playing field” that allows mutation and natural selection to fix new positive characters via evolution and secondary metabolism to make them an integral part of cellular metabolism (Osorio, *et al.*, 2008). The secondary metabolite quinolone produced by *P. oxalicum* has been reported to have antiprotozoal activity (Pohl, *et al.* 2011), antimalarial, anti-bacterial, antifungal, antihelmintic, cardiotonic, anticonvulsant, anti-inflammatory, anticancer and analgesic properties (Rancic, *et al.*, 2006, Roze, *et al.*, 2011). It is also said to be an alkaloid mycotoxin (Ruisi, *et al.*, 2007). The other important bioactive compound Quinazolinone produced by psychrophilic *P. oxalicum* fungus constitutes a class of drugs that contain a 4-quinazolinone core and functioned as sedatives (Chen, *et al.* 2006). Quinazolinone and quinazoline derivatives, recently been reported as potent antimicrobial and have cytotoxic activities (Jafari, *et al.* 2016). The 2, 4-Azetidinedione, 3,3-diethyl-1-methyl produced by *P. citrinum* is derivative of 2-azetidinone (beta-lactam) ring system is precursor for number of broad spectrum β-lactam antibiotics (Thomas, 1990; Wynn-Williams,1990;Thomas and Cavicchioli, 2000; Singh, 2004; Osorio, *et al.*, 2008; Strader, *et al.*, 2011). Other similar bioactive compound Dodecanol produced by both the *Penicillium* strains, is a saturated 12-carbon alcohol, used in detergents, as lubricating oil, and in pharmaceutical industries. Some unique sec. metabolites produced by *P. oxalicum* under extreme temperature conditions are the biomolecules which need to be explored for their structural details and for application part. Hence, the results revealed unique features of the psychrophilic *P. oxalicum* and its temperature stress dependent production of new secondary metabolites with potential industrial applications, predominantly in pharmaceutical and therapeutical fields.

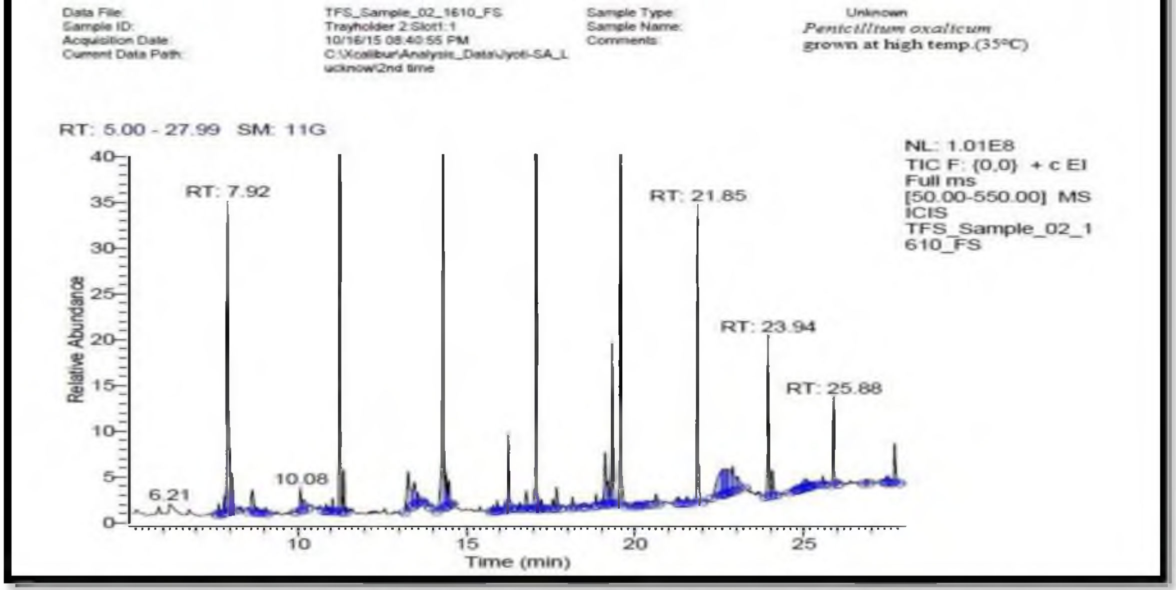


Fig 8.4 Chromatogram of GC-MS/MS analysis of ethyl acetate extract of secondary metabolites produced by *P. oxalicum* grown at temperatures 35°C.

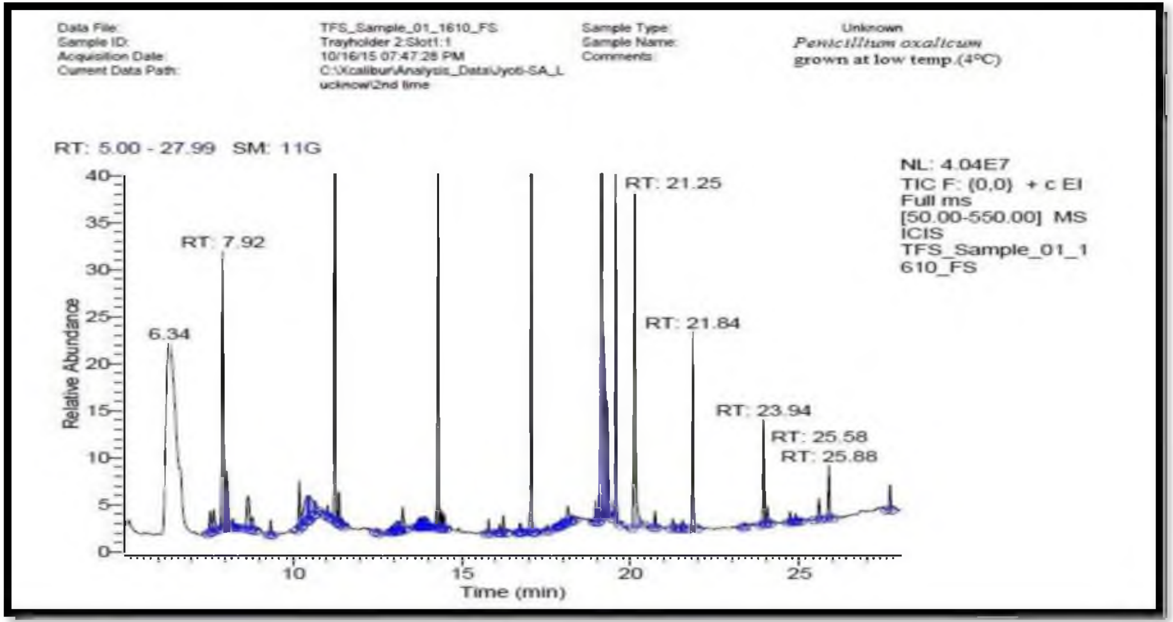


Fig. 8.3 Chromatogram of GC-MS/MS analysis of ethyl acetate extract of secondary metabolites produced by *P. oxalicum* grown at temperatures 4°C.

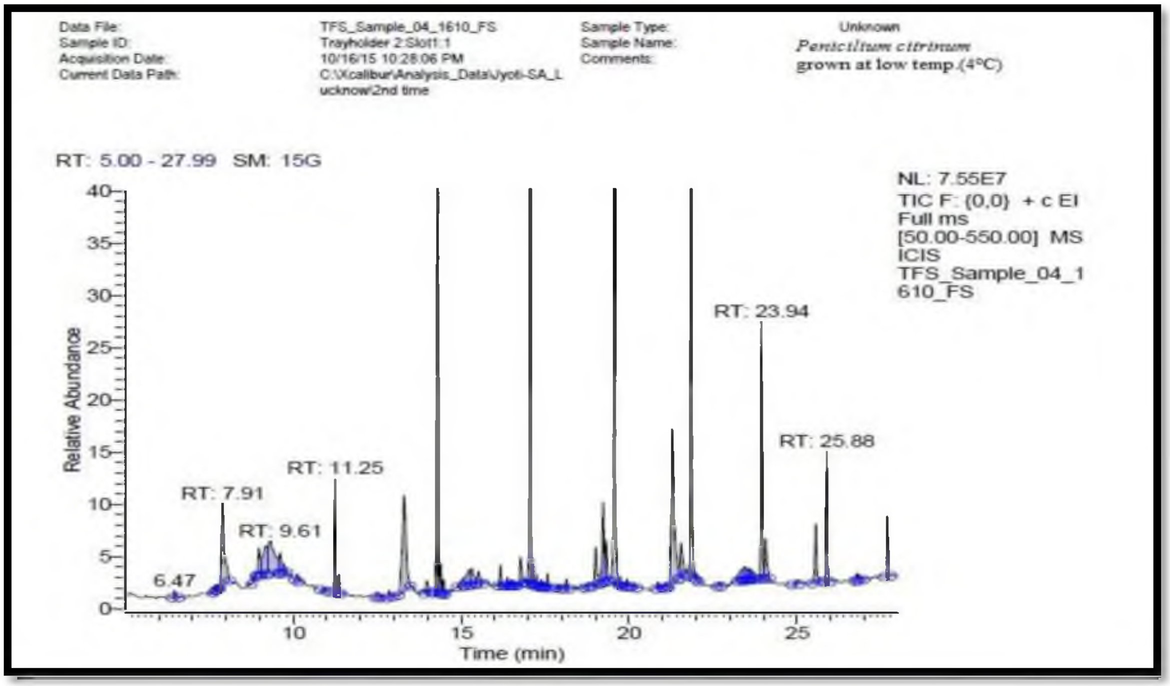


Fig. 8.5 Chromatogram of GC-MS/MS analysis of ethyl acetate extract of secondary metabolites produced by *P. citrinum* grown at temperatures 4°C.

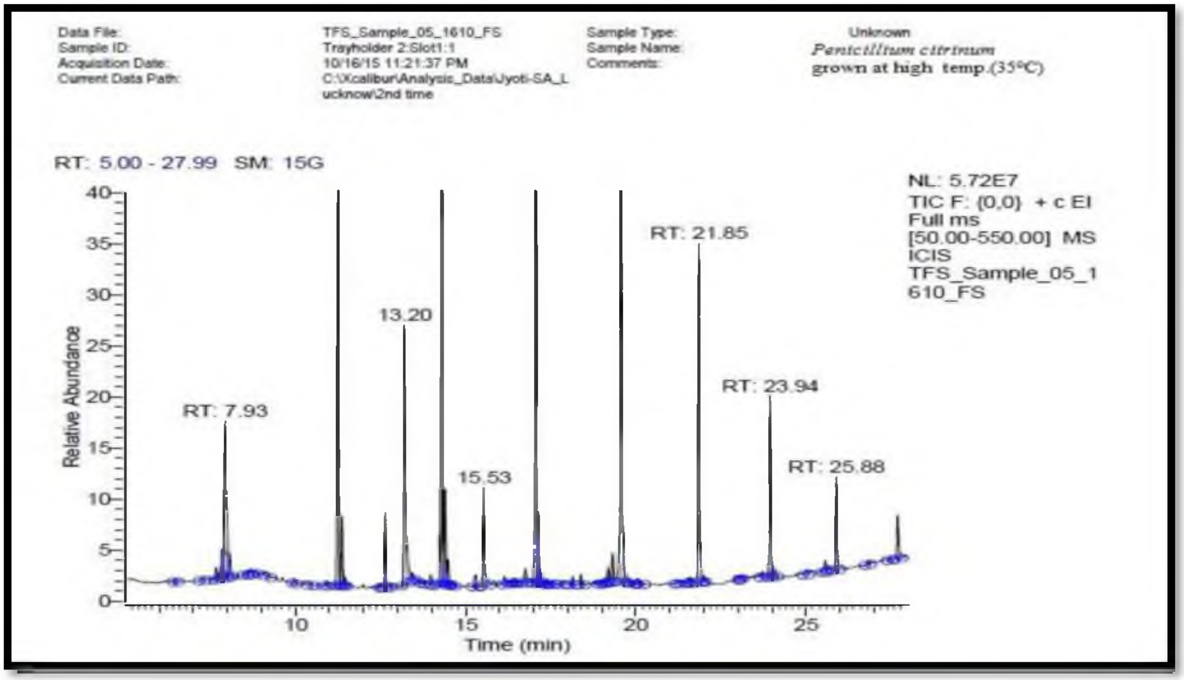


Fig. 8.6 Chromatogram of GC-MS/MS analysis of ethyl acetate extract of secondary metabolites produced by *P. citrinum* grown at temperatures 35°C.

Table. 8.1: Identification of the compounds resulting after GC-MS/MS analysis of ethyl acetate extracted secondary metabolites produced by *P. oxalicum* grown at temperatures 4°C and 35°C.

***P. oxalicum* (4°C)**				
S. No.	**Compounds**	**R_T, min**	**Chemical Formula**	**Abundance (%)**
1	1-Dodecene	7.92	$C_{12}H_{24}$	6.87
2	Cyclotetradecane	11.25	$C_{14}H_{28}$	9.44
3	2-Propyn-1-ol,acetate	14.02	$C_5H_6O_2$	0.13
4	4(1H)Quinazolinone	16.24	$C_8H_6N_2O$	0.45
5	2-Hexadecanol	17.06	$C_{16}H_{34}0$	8.45
6	Propanoic acid,2-(aminooxy)-	19.2	$C_3H_7NO_3$	4.79
7	Dibutyl phthalate	19.32	$C_{16}H_{22}O_4$	2.02
8	2-Hexadecanol	19.56	$C_{16}H_{34}O$	5.15
9	6-Quinazolinol	20.13	$C_8H_6N_2O$	7.77
10	9-Hexacosene	21.84	$C_{26}H_{52}$	3.34

***P. oxalicum* (35°C)**				
S.No.	**Compounds**	**R_T, min**	**Chemical Formula**	**Abundance (%)**
1	3-Dodecene	7.92	$C_{12}H_{24}$	8.15
2	6,7-Dodecanedione	10.08	$C_{12}H_{22}O_2$	0.61
3	2-Dodecanol	11.25	$C_{12}H_{26}O$	12.47
4	2-Methyl-2-propylmethylphosphonofluoridate	13.25	$C_5H_{12}FO_2P$	1.96
5	Pyridine,2[(1,1dimethylethyl)thio	13.81	$C_9H_{13}NS$	0.21
6	1-Hexadecanol	14.3	$C_{16}H_{34}O$	10.27
7	4(1H)Quinazolinone	16.24	$C_8H_6N_2O$	1.7
8	Eicosane	17.1	$C_{20}H_{42}$	0.19
9	4(1H)Pyrimidinone,6-amino-2-methyl-5-nitroso	19.11	$C_5H_6N_4O_2$	1.51
10	Dibutyl phthalate	19.32	$C_{16}H_{22}O_4$	2.82
11	2-Hexadecanol	19.57	$C_{16}H_{34}O$	9.37
12	9-Hexacosene	21.85	$C_{26}H_{52}$	5.55
13	4(3H)Quinolinone	25.27	$C_9H_7NO_2$	0.15

14	Phthalic acid, di-(hex-3-yl)ester	25.5	$C_{20}H_{30}O_4$	0.18

Table 8.2: Identification of the compounds resulting after GC-MS/MS analysis of ethyl acetate extracted secondary metabolites produced by *P. citrinum* grown at temperatures 4°C and 35°C.

***P. citrinum* (4°C)**				
S. No.	**Compounds**	**R$_T$, min**	**Chemical Formula**	**Abundance (%)**
1	Cyclobutanone, 2,2dimethyl	7.91	$C_6H_{10}O$	3.3
2	Cyclotetradecane	11.25	$C_{14}H_{28}$	1.95
3	Cyclohexanone,4-ethyl-4-methyl-3-(1-methylethyl)-,trans-	13.3	$C_{12}H_{22}O$	4.45
4	1-Hexadecanol	14.3	$C_{16}H_{34}0$	11.5
5	3-Methyl-1,4diazabicyclo[4.3.0]nonan-2,5-dione , N-acetyl	16.17	$C_{10}H_{14}N_2O_3$	0.41
6	Glycyl-L-proline	16.77	$C_7H_{12}N_2O_3$	0.74
7	Hexadecane	17.14	$C_{16}H_{34}$	0.39
8	Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	19.01	$C_{11}H_{18}N_2O_2$	1.07
9	2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone	19.23	$C_{10}H_{22}O_3S_2$	1.85
10	9-Hexacosene	19.57	$C_{26}H_{52}$	14.25
11	11,14-Eicosadienoic acid, methyl ester	21.29	$C_{21}H_{38}O_2$	5.96
12	Propanoic acid, 2-(aminooxy)-	21.55	$C_3H_7NO_3$	1.1
13	9-Hexacosene	21.8	$C_{26}H_{52}$	8.88
14	Phthalic acid, di(2propylpentyl)	25.6	$C_{24}H_{38}O_4$	1.37
***P. citrinum* (35°C)**				
S.No.	**Compounds**	**R$_T$, min**	**Chemical Formula**	**Abundance (%)**
1	3-dodecene	7.93	$C_{12}H_{24}$	4.99
2	2-Dodecanol	11.25	$C_{12}H_{26}O$	10.42
3	Methenamine	12.6	$C_6H_{12}N_4$	1.37
4	Phenol,2,4-bis(1,1-dimethylethy)-	13.2	$C_{14}H_{22}O$	6.36

5	2-Hexadecanol	14.3	$C_{16}H_{34}O$	17.32
6	Hexadecane	14.4	$C_{16}H_{34}$	1.64
7	2,3-Dimethylhydroquinone	15.53	$C_8H_{10}O_2$	2.24
8	2,4-Azetidinedione,3,3-diethyl1methyl	16.14	$C_8H_{13}NO_2$	0.29
9	Phthalic acid, hex-3y-l isobutyl ester	18.15	$C_{18}H_{26}O_4$	0.2
10	Propanoic acid,2-(aminooxy)-	19.19	$C_3H_7NO_3$	0.36
11	Dibutyl phthalate	19.32	$C_{16}H_{22}O_4$	0.68
12	9-Hexacosene	21.8	$C_{26}H_{52}$	6.82

8.3.3 HPLC fingerprinting

A study reported by Frisvad *et al.* (1989) discussed about the study of mycotoxins and other secondary metabolites by using HPLC method. The value of this method was enhanced by the use of an alkylphenone retention time index system and diode array detection (Frisvad and Thrane, 1987). Malmstrom, *et al.* (2000), reported the use of HPLC analysis with photo-diode array for detection of metabolites in *Penicillium steckii*. Ioca, *et al.* (2016) also reported the UV spectra, low-resolution mass spectrometry and ^{1}H NMR data are the low cost spectroscopic identification techniques for secondary metabolites. The two sample solutions of EtOAc crude extracts of secondary metabolites of both *Penicillium* strains were prepared and chromatograms were recorded in 70 minutes as shown in figure (8.7-8.14). According to the results of HPLC fingerprinting total 17 and 15 peaks were recorded in case of psychrophilic *P. oxalicum* and mesophilic *P. citrinum,* respectively. Among the peaks, 06 major peaks were identified along with their characteristic UV-Vis spectra, but none of them were identifiable as known compounds as per available literature for characterization of UV absorption spectra of fungal secondary metabolites (Frisvad, 1987; Paterson and Kemmelmeier 1990; Frisvad and Thrane, 1993;

Nielsen and Smedsgaard, 2003; Antipova, *et al.* 2011; Iócaa, *et al.* 2016). The secondary metabolites produced by *P. citrinum* were identified as Quinolactacin C, Abscisic acid, Anofinic acid, Conocenol B, and Aurofusarin (Paterson and Kemmelmeier, 1990; Takahashi, *et al.* 2000; Iócaa, et. al. 2016). Characterization and identification of UV_{max} spectra of secondary metabolites produced by both the *Penicillium* strains are shown in table (8.3). Takahashi, *et al.* (2000) identified Quinolactacins produced by *Penicillium* sp. EPF-6 using UV λ_{max} by HPLC fingerprinting and suggested that it have a unique quinolone skeleton conjugated with a γ-lactam ring. Sasaki, *et al.* (2006) investigated the biosynthesis of Quinolactacin A by a *Penicillium* strain and reported as a Tumor Necrosis Factor (TNF) production inhibitor. The production of Abscisic acid (ABA) in fungi and endophytic fungi as fungal metabolite was reported by several researchers (Assante, *et al.* 1977; Marumo, *et al.* 1982; Oritani and Yamashita, 1985; Tudzynski and Sharon, 2002). Koornneef, *et al.* (1998) and suggested that the ABA have wide applications such as plant growth and their development, including stomatal closure, embryo development, seed germination and seed dormancy, and most importantly they support the plant to survive under environmental stresses, that is why ABA is also called as stress hormone (Tudzynski and Sharon 2002). Gaffoor, *et al.* (2005) reported the mutagenic and cytotoxic properties of aurofusarin. Aurofusarin also been reported to cause chromosomal aberrations in mammalian cell lines (Gelderblom *et al.* 1984). Qin, *et al.* (2011) reported the strong antimicrobial (antibacterial, antifungal and antialgal) activities of anofinic acid against *Microbotryum violaceum*, *Escherichia coli*, *Bacillus megaterium* and *Chlorella fusca*. Liu, *et al.* (2007) and Ying, *et al.* (2013) isolated and identified sesquiterpenes (conocenol B) from cultures of the Basidiomycete *Conocybe siliginea* and *Ceriporia lacerate*, *Huperzia* *serrata*, respectively. Frisvad, *et al*. (1989) stated that the many secondary metabolites could be characterized by the HPLC method and the production of PR-toxin, isofumigaclavine, mycophenolic acid and roquefortine could be confirmed. Thus, use of HPLC can be recommended for good quantitation of the various metabolites and their identification. Sonjak, *et al*. (2005) also reported the differences in secondary metabolites profiles among of *P. crustosum*, isolated from different location and habitats by using the HPLC analysis.

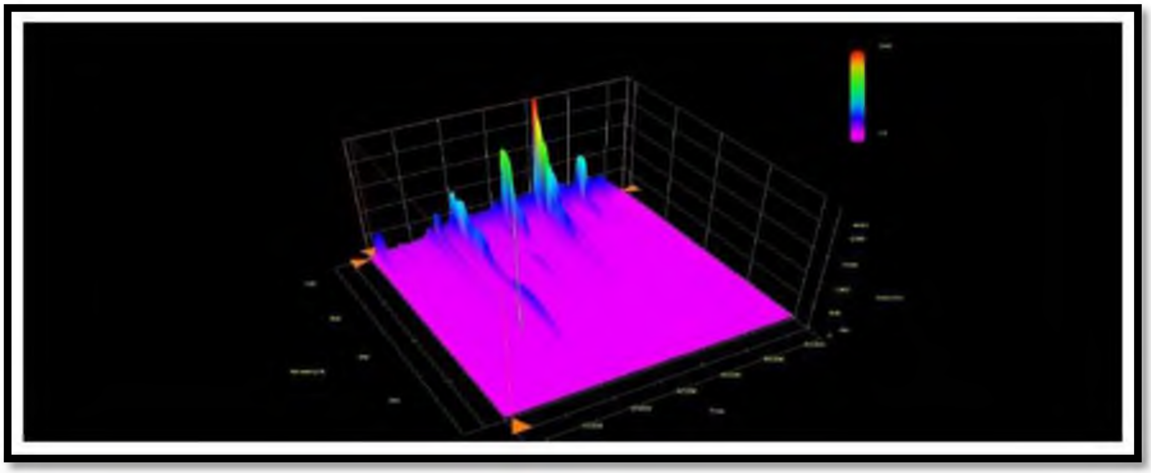


Fig. 8.7 Three dimensional HPLC fingerprint of sec. metabolite of *P. oxalicum*.

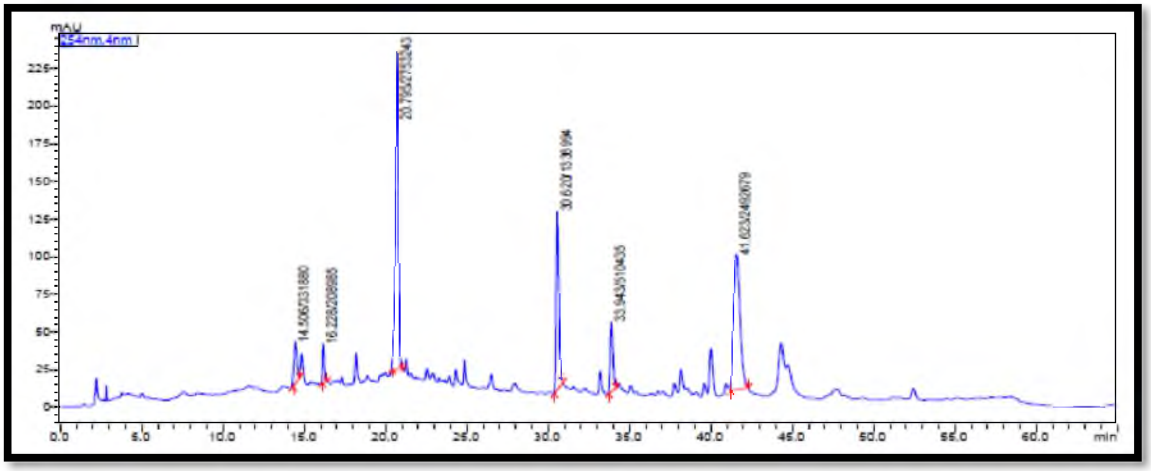


Fig. 8.8 HPLC chromatogram at 254nm of non-volatile secondary metabolite of *P. oxalicum*.

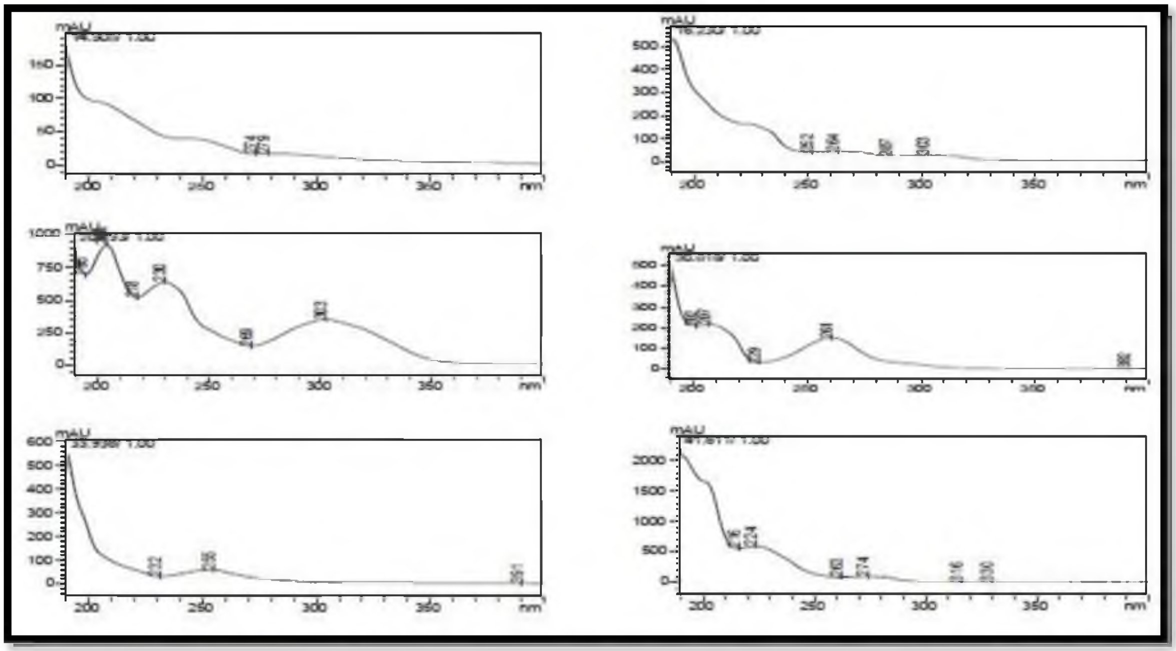


Fig. 8.9 The characteristic UV-Vis spectra of major peak of *P. oxalicum*.

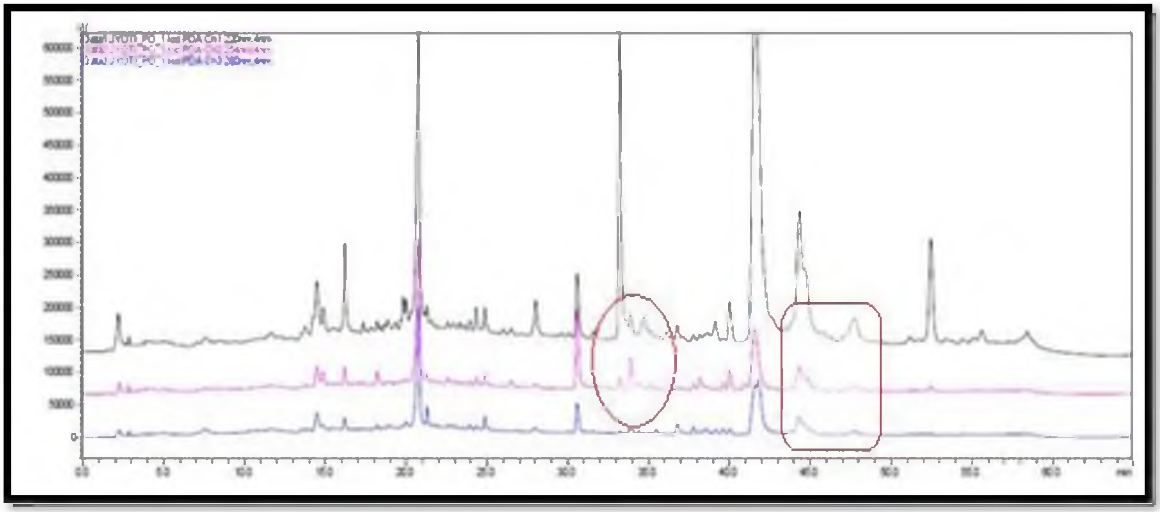


Fig. 8.10 Comparative HPLC chromatogram of sec. metabolite of *P. oxalicum* at 220, 254, 280 nm.

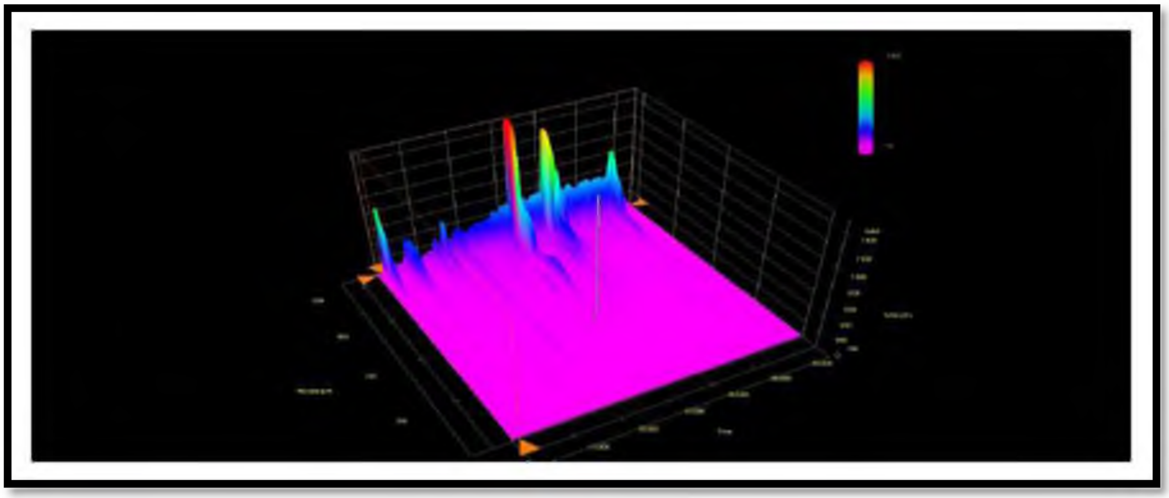


Fig. 8.11 Three dimensional HPLC fingerprint of sec. metabolite of *P. citrinum*.

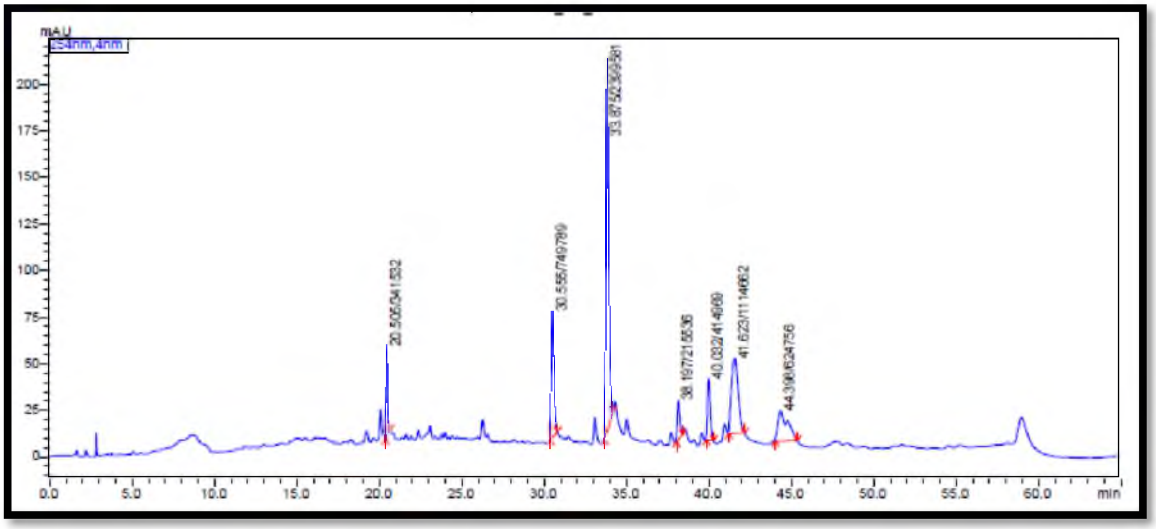


Fig. 8.12 HPLC chromatogram at 254nm of non-volatile secondary metabolite of *P. citrinum*.

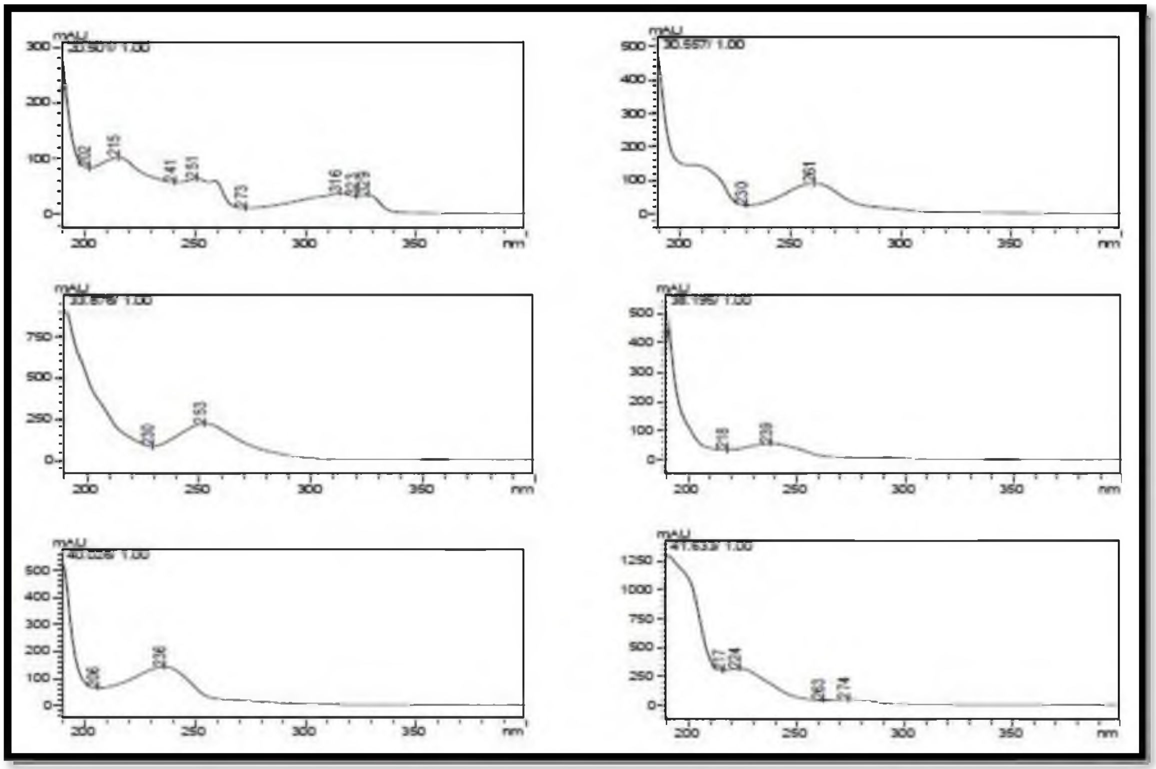


Fig. 8.13 The characteristic UV-Vis spectra of major peak of *P. citrinum*.

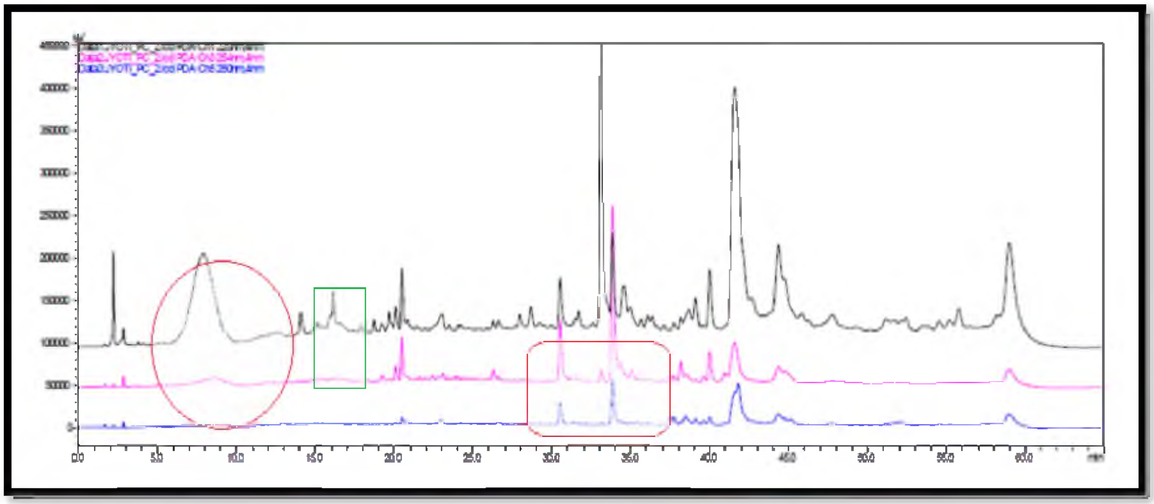


Fig. 8.14 Comparative HPLC chromatogram of sec. metabolite of *P. citrinum* at 220, 254, 280 nm.

Table. 8.3 Identification of non-volatile secondary metabolites produced by *Penicillium* strains.

<table>
<tr><th colspan="5"><i>P. oxalicum</i></th></tr>
<tr><th>RT</th><th>UV$_{max}$ absorbance</th><th colspan="3" rowspan="7">Unidentified</th></tr>
<tr><td>14.905/1.00</td><td>274,279</td></tr>
<tr><td>16.230/1.00</td><td>252,264,303</td></tr>
<tr><td>20.793/1.00</td><td>195,207,261</td></tr>
<tr><td>30.619/1.00</td><td>202,207,261</td></tr>
<tr><td>33.938/1.00</td><td>255</td></tr>
<tr><td>41.611/1.00</td><td>224,263,274</td></tr>
<tr><th colspan="5"><i>P. citrinum</i></th></tr>
<tr><th>RT</th><th>UV$_{max}$ absorbance</th><th>Compound to be</th><th>Potential applications</th><th>References</th></tr>
<tr><td>20.501/1.00</td><td>215,251,316,329</td><td>Quinolactacin B/C</td><td>TNF production inhibitor</td><td>Takahashi, <i>et al.</i> 2000; Sasaki, <i>et al.</i> 2006; Ióca, et. al., 2016</td></tr>
<tr><td>30.557/1.00</td><td>261</td><td>Abscisic acid</td><td>Growth and development of plant and stress hormone</td><td>Ióca, et. al., 2016;
Koornneef, <i>et al.</i> 1998</td></tr>
<tr><td>33.876/1.00</td><td>253</td><td>Aurofusarin</td><td>mutagenic and cytotoxic properties</td><td>Paterson and Kemmelmeier, 1990</td></tr>
<tr><td>38.195/1.00</td><td>239</td><td>Anofinic acid</td><td>antimicrobial (antibacerial, antifungal and antialgal) properties</td><td>Qin <i>et al.</i> 2011; Iócaa, <i>et al.</i>, 2016</td></tr>
<tr><td>40.026/1.00</td><td>236</td><td>Conocenol B</td><td>–</td><td>Iócaa, <i>et. al.</i>, 2016</td></tr>
<tr><td>41.633/1.00</td><td>224,263,274</td><td>–</td><td>–</td><td>–</td></tr>
</table>

8.3.4 ^{1}H NMR analysis of secondary metabolites

Various reports on identification of secondary metabolites produced by different *Penicillium* strains using NMR analysis are documented (Malmstrom, *et al.* 2000; Yang, *et al.* 2016; Iocaa, *et al.* 2016). Proton NMR analysis of secondary metabolites present in ethyl acetate extract of *P. oxalicum* grown at 4°C temperature showed the presence of compound **2-Dodecanol** using Spectral Database System for Organic compounds (AIST, Japan) with **SDBS no-51174** (Fig.8.15). Rest results did not match with any known compounds given in the database (Fig. 8.16-8.18). Hence other proton NMR peaks could not be identified. However active compound present in the crude extract of *P. oxalicum* needs to be further purified and screened, to identify all the unknown compounds using advanced NMR technique such as ^{13}C-NMR, one and two dimension of NMR techniques (DEPT, 1H-1H COSY, HSQC and HMBC) as reviewed in previous literature.

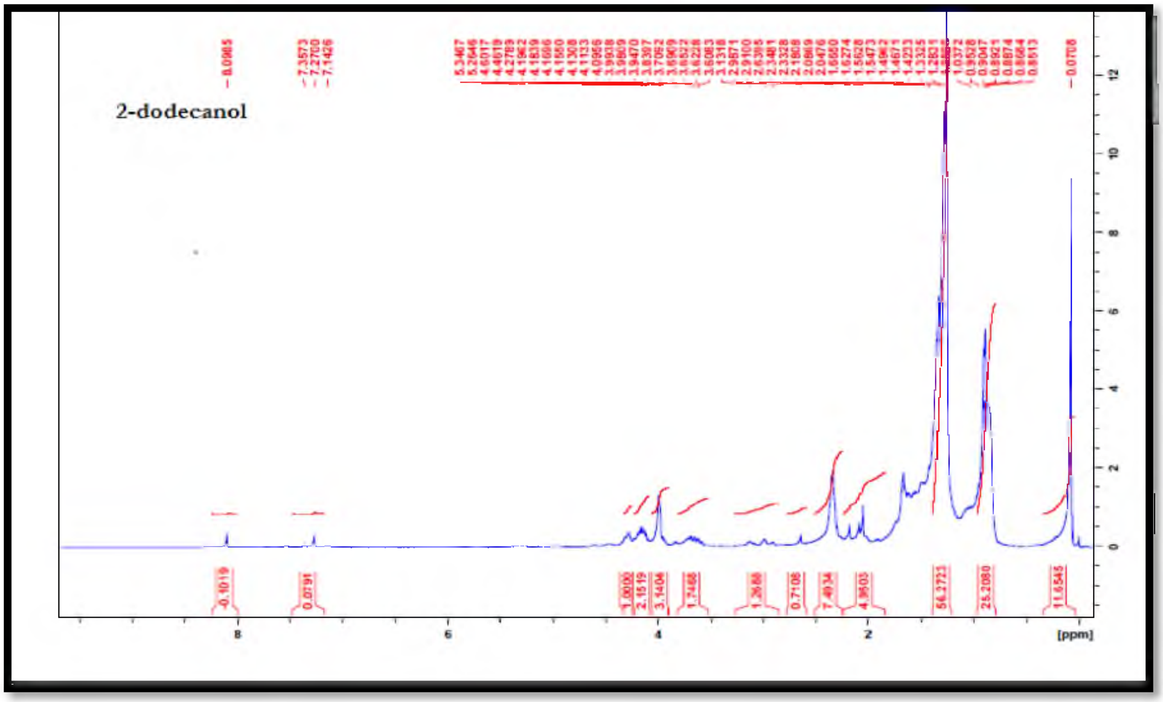


Fig. 8.15 ^{1}H NMR analysis of crude extract of secondary metabolite of *Penicillium oxalicum* at temp. 4°C.

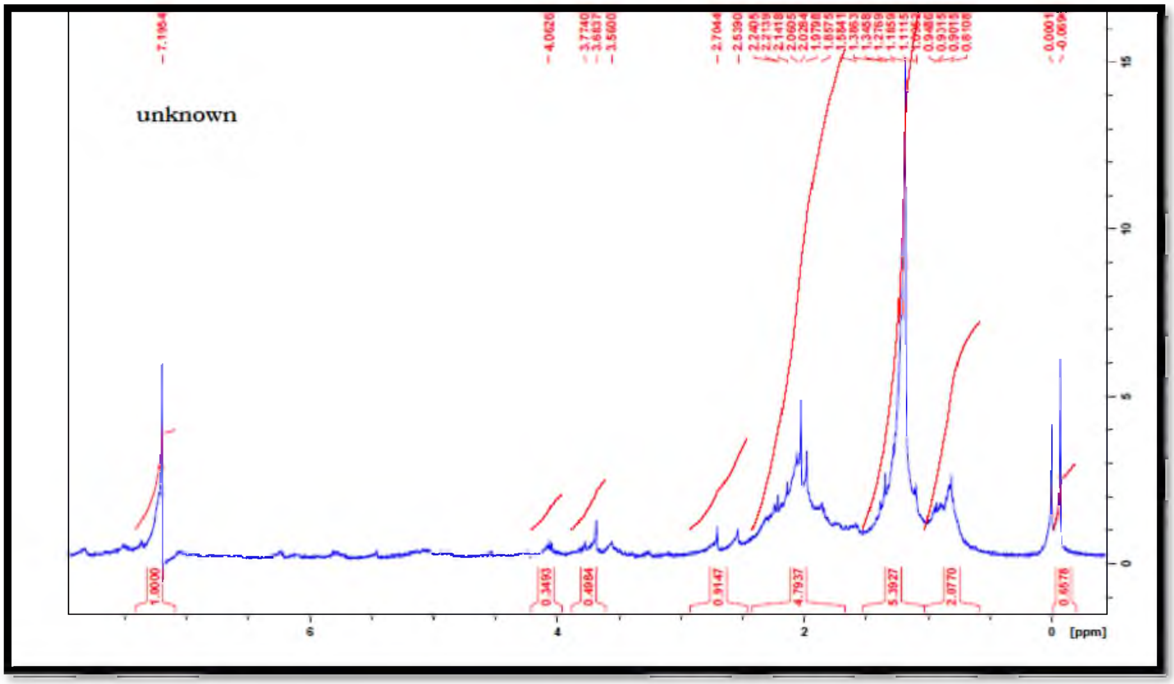


Fig. 8.16 ^{1}HNMR analysis of crude extract of secondary metabolite of *Penicillium oxalicum* at temp. 35°C.

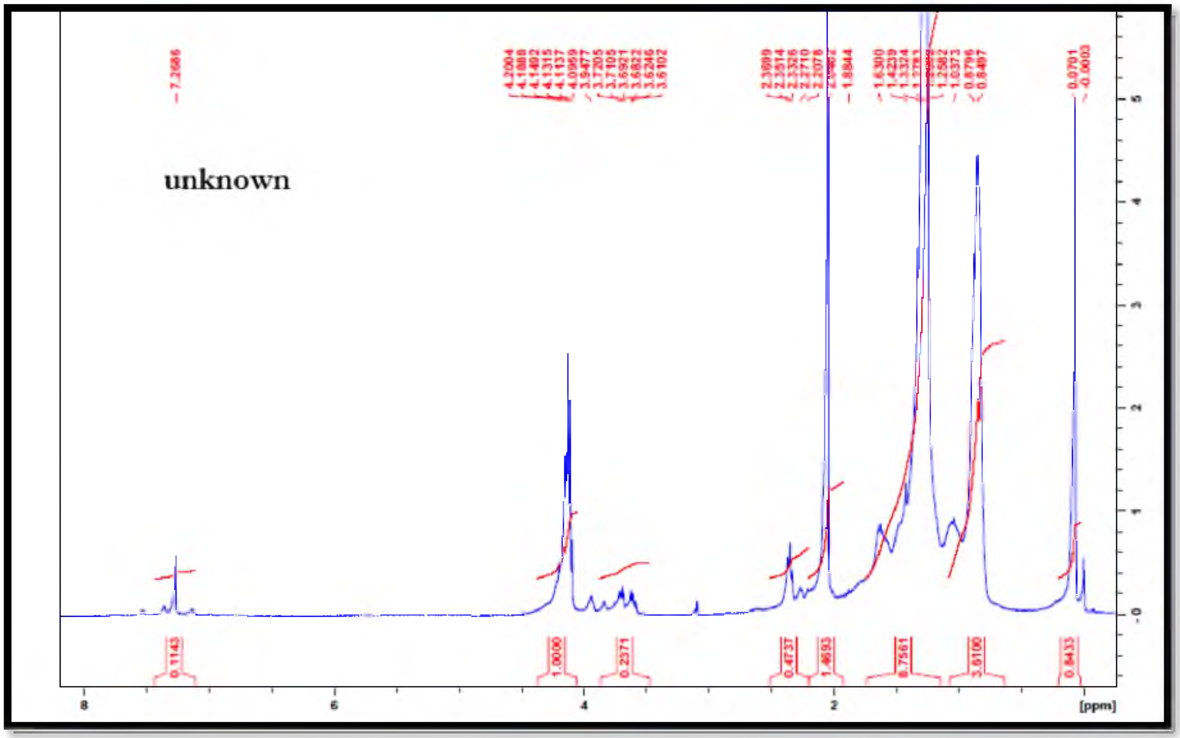


Fig. 8.17 ^{1}HNMR analysis of crude extract of secondary metabolite of *Penicillium citrinum* at temp. 4°C.

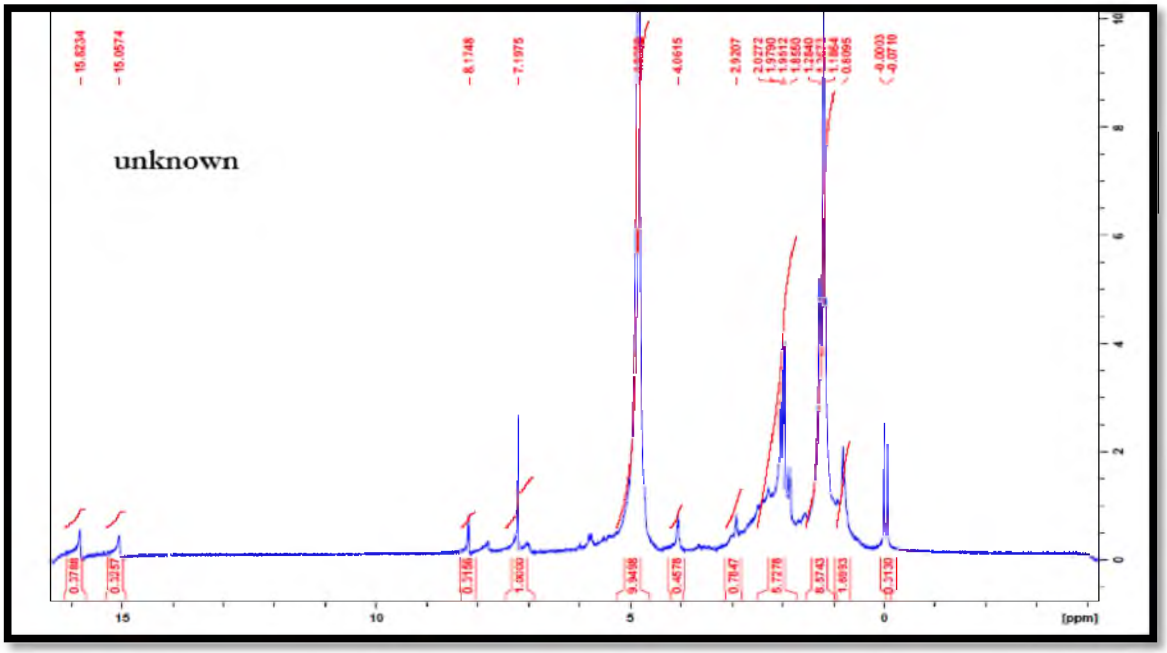


Fig. 8.18 ^{1}HNMR analysis of crude extract of secondary metabolite of *Penicillium citrinum* at temp. 35°C.

8.4 Conclusion

- TLC analysis of EtOAc crude extract of secondary metabolites of *Penicillium* strains suggested that secondary metabolites of *P. oxalicum* include nitrogenous compounds not present in *P. citrinum*, whereas extracts of *P. citrinum* exhibited presence of alkaloids.
- GC-MS/MS analysis of some volatile sec. metabolites produced by both *Penicillium* strains showed the presence of quinoline, quinolinone, azulene, and azetidinedione with known applications in biomedical field.
- HPLC fingerprinting of non-volatile compounds of secondary metabolites indicated the presence of 06 major peaks with unknown and known compounds in psychrophilic *P. oxalicum* and mesophilic *P. citrinum* respectively.
- The ^{1}H NMR study also confirmed about temperature dependent changes in the composition of secondary metabolites produced by psychrophilic *P. oxalicum*, while composition of secondary metabolite produced by *P. citrinum* remained the same except quantitative changes.

CHAPTER-9 APPLICATIONS OF SECONDARY METABOLITES

9.1 Introduction

Microorganisms are highly diverse and are barely explored natural sources of bioactive products as compared to other sources such as plants. The countless bioactive natural products of the microorganisms have the potential of discovering in new molecules for use in drug industry, agricultural and industrial purposes (Keller, *et al*. 2005; Strobel, 2006; Porras-Alfaro and Bayman, 2011; Kodzius and Gojobori, 2015). Various studies based on assessment of microbial populations have shown that only approx. 1% of bacteria and 5% of fungi have been explored for biomolecules and others remain unexplored for their potential role in human prosperity (Heywood, 1995). According to a study on fungal biodiversity, about 1.5 million fungal species exist on the earth, out of these only 5% are now well identified (Hawksworth, 2001). Among these, soil fungi are known to be significant sources of true bioactive secondary metabolites (Bok and Keller, 2004). So far, several antibiotics have been discovered from the secondary metabolites produced by fungi and actinomycetes (Hugo, *et al*. 1987; Anke, 1989). Fungi have provided several bioactive compounds and chemical molecules currently being used as pharmaceutical. Nevertheless, during the last decade, research on the bioactive compounds has increased due to an increasing trend of microbial resistance to the available drugs. Hence, more fungi needs to be examined for new antimicrobial agents. The large number of world population is affected by Cancer (Ali, *et al*. 2011) and thus, there is need to search and screen of novel anticancer drugs for cancer chemotherapy. Moreover, the high toxicity of drugs associated with cancer chemotherapy and their adverse side effects have led to the rise in the demand for novel anticancer and antitumor drugs that are active against untreatable tumor or cancer cells, with less side effects and great therapeutic efficiency (Demain and Sanchez, 2009). The *Penicillium* is well known for production of secondary metabolites and their therapeutic attributes such as antimicrobial/antibacterial, antifungal, anticancer, antitumor, immunosuppressant, cholesterol-lowering agents, anti-arthritis agents, mycotoxins (Li, *et al*., 2001; Yang, 2002; Tan, *et al*., 2006; Han, *et al*. 2007; Petit, *et al*. 2009) and related enzyme inhibitory activities (Zhang, *et al*. 2002; You, *et al*. 2005; Zhou, *et al*. 2007). Many studies reported about the production of numerous anti-tumour compounds from the genera *Penicillium* such as terpenoids, alkaloids, ketones, quinones and their derivatives (Liu, *et al*. 2005) and ergot alkaloids, diketopiperazine, and quinoline alkaloids from *Penicillium* sp. (Antipova, *et al*., 2011). In addition, more than 60% of the anticancer and 70% of the antimicrobial drugs currently in clinical use are natural products or their derivatives (Mc Alpine, *et al*. 2005). A study report by World Health Organization (WHO) revealed that 90% of the bacterial strains are resistant to drugs of first choice. Examples of the bacterial resistance problems on a global scale against methicillin resistance in *Staphylococcus aureus* (MRSA), vancomycin resistance *Enterococci* and *Enterobacteriaceae* producing beta lactamases are common thing (Liang, *et al*., 2012; Calfee, 2012). Drug resistance in bacteria has become a global concern and the search for new antibacterial agents is urgent and ongoing (Liang, *et al*. 2012).The increasing resistance of *P. aeruginosa* to numerous antibiotics, as a result of excessive use of antibiotics and appearance of multidrug-resistant (MDR) in *P. aeruginosa* (Aloush, *et al*. 2006) is becoming more of a challenge to the scientists. In the view of above problems of multidrug resistance in bacteria and lack of novel antibacterial agents against these bacteria, the present study was carried out to assess the drug resistance reversal potential of secondary metabolites of *Penicillium* strains against bacterial strain of *P. aeruginosa* (MTCC-741/ATCC-25668) and it was compared with the EtOAc crude extracts to demonstrate the antibacterial and anticancer property by following MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The cell cytotoxicity was assessed by flow cytometry analysis of Propidium Iodide (P I) uptake by Human Lung Cancer cell line (A549).

9.2 Materials and Methods

9.2.1 Culturing of *Penicillium* strains, extraction of metabolites

Refer to section **8.2.1.** in chapter 8.

9.2.2 Antibacterial Activity

The bacterial inoculum was uniformly spread using sterile cotton swab on a sterile Petri dish containing Nutrient agar in replicates. 100 µL of crude EtOAc extract from both the *Penicillium* strains were added to each of the 2 wells (5 mm diameter holes cut in the agar gel, 5 cm (approx.) apart from each other). The petriplates were incubated for 24 h at 37°C ± 1°C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in terms of colony diameter (mm). For reference, 30 mg streptomycin was used. Tests were performed in duplicate.

9.2.3 Cell Culture

A Human Lung Cancer cell line (A549) procured from National Centre for Cell Sciences, Pune, (India), was maintained at Developmental Toxicology Laboratory, CSIR-Indian Institute of Toxicology Research, Lucknow (India), as per the standard protocols. In brief, the cells were cultured in DMEM/F-12, supplemented with 10% Fetal Bovine

Serum (FBS), 0.2% Sodium Bicarbonate, 100 units/ml Penicillin G sodium, 100 µg/ml Streptomycin Sulfate and 0.25 µg/ml Amphotericin B. Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in an incubator. The medium was changed twice in a week and cultures were passaged at a ratio of 1:6 once in a week. Prior to use of cell line in the experiments, cell viability was ascertained by Trypan blue dye exclusion assay (Strober, 2001). The culture showing viability more than 95% were used in all the experiments. All the experiments were carried out on the cells with passage 18–25 only.

9.2.4 Cell viability by MTT assay

The EtOAc crude extract of secondary metabolite was tested using in-vitro MTT assay against Human Lung cancer cell line (A549) by adapting the method described by Kumar *et al.* (2016). In brief,cells (1×10^4cells ml^{-1}) were seeded in 96-well plates for 24 h under ahigh humid environment containing 5% CO_2 +95% atmospheric air at 37°C. The cells were treated with an EtOAc extract of *Penicillium* strains, using various concentrations of secondary metabolites. Both treated and untreated A549 cell lines were incubated for 24 h.At the end of the incubation, 5 mg ml^{-1}solution of MTT [(3-(4,5- Dimethylthiazol-2-yl)-2,5 Diphenyl tetrazolium Bromide] were added to each well and the cells were incubated for 4 h at 37°C. Viable cells react with MTT to form purple formazan crystal. MTT solution was removed by washing and the cells were lysed using a culture grade DMSO by pipetting up and down several times until the content was homogenized. After 10 min. of incubation, the color was read at 550 nm using multi-well microplate reader (Synergy HT, Bio-Tek, USA). The sets unexposed to MTT were run simultaneously under identical conditions, which served as control.

The MTT assay is only a cell viability assay often used to determine cytotoxicity following exposure to the toxic substance. MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the viable cells. The resulting formazan product,being impermeable to the cell membranes and gets accumulated only in the healthy cells.

9.2.5 Cell Cytotoxicity assay

Briefly, cells of Human Lung Cancer cell line (A549) (5×10^4 /well) were seeded in poly-L-lysine-pre-coated tissue culture slides flasks were allowed to adhere and then they were exposed to different concentrations of EtOAc crude extract of secondary metabolites of *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum*) at concentration of 05 µg/ml, 10 µg/ml and 20 µg/ml and a common control (without extract) for 24 hrs. The crude extract of metabolites of both *Penicillium* strains was dissolved in dimethyl sulfoxide (DMSO).On completion of 24 hour incubation, the cells were collected, washed three times with Phosphate Buffered PBS Saline soln. The crude extract treated cells lines were further mixed with 20 µg/ml of Propidium Iodide (PI) for 15 minutes. Fluorescence of the PI was measured by flow cytometer using BD FACS influx flow cytometer (BD, USA) through an FL-2 filter (580 nm). For the measurement, 10,000 events were acquired. The data were analyzed by BD FACS ™ Software 1.2.0.87. Cell debris, characterized by a low forward scatter/side scatter (FSC/SSC), was excluded from the analysis.

Apoptotic cells were detected by Flow Cytometry using PI staining of DNA fragments to determine sample cytotoxicity. PI binds to DNA by intercalating between the bases with little or no sequence preference. Since PI also binds with RNA, it necessitates treatment of cell lines with ribonucleases to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold. The fluorescence excitation maxima shows red shift by ~30–40 nm and the fluorescence emission maxima shows blue shift ~15 nm. PI is impermeable across the membrane only and generally excluded by the viable cells. Thus, PI is commonly used for identifying dead cells in a population.

9.2.6 Susceptibility tests

Two different fractions, Fraction 1 (*P. oxalicum*) and Fraction 2 (*P. citrinum*) and 2-azetidinone were used for biological activities. The MICs were determined in Mueller-Hinton broth (MHB) using 96-well micro titer plates following the CLSI guidelines for broth micro-dilution (CLSI, 2015). Tested fractions (1&2) and 2-azetidinone were diluted into final concentrations of 1600 µg/mL to 3.12 µg/mL and tested against the most resistant isolate of *P. aeruginosa* (KG-P2). The MIC values were determined by 2-fold serial dilution of broth assay with starting inoculums of 5×10^5cfu/mL. Inoculated plates were incubated at 37°C for 24h and observations were recorded as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Tetracycline was used as positive control, while media (MHB) without culture was used as negative control.

9.2.7 In-vitro combination studies

Combination study was performed by broth checkerboard method as described earlier (Eliopoulos and Wennersten, 2002). Cation-adjusted Mueller-Hinton broth (150µL) was added to each well of the 96-well plate. The last four columns of wells served as controls for *P. aeruginosa* growth and plate sterility. The final concentrations ranged from 12.5 to

1600 μg/mL for tetracycline and from 0.78 to 100μg/mL for tested molecules. Thus, each of the 64 wells had unique combinations of antibiotics and test extracts. The final bacterial inoculum in each well was $5x10^5$cfu/mL except the negative controls. The plates were incubated at 37°C for 24h. The MIC was recorded as the last dilution without any turbidity as per CLSI guidelines 2015. Results were recorded in terms of fold reduction and fractional inhibitory index. To characterize the interaction of tested combinations, fractional inhibitory concentrations (FIC) and fractional inhibitory concentration index (FICI) were determined as given below.

FIC Index = FIC1+ FIC2

FIC1=MIC of drug 1 in combination/MIC of drug 1 alone

FIC2=MIC of drug 2 in combination/ MIC of drug 2 alone

As classically defined, FICI<0.5 represented synergism, whereas FICI (0.5-4.0) represented no interaction, while FICI (>4.0) represented antagonism (Odds, 2003).

9.2.8 Ethidium bromide efflux studies

The fluorometric determination of ethidium bromide efflux was performed as described previously (Viveiros, *et al*. 2010). *P. aeruginosa* KGPG-2 culture was grown to obtain optical density (OD) of 0.6 at 600 nm. The cells were collected by centrifugation and washed with PBS saline buffer. Ethidium bromide (50 mg/L) was added in the bacterial suspension and incubated for 60 min at 25°C in the absence/presence of extracts at 25 mg/L. The EB-loaded bacterial suspension was centrifuged at 16,060 × g for 3 min, the supernatant discarded and the pellets were re-suspended in cold PBS. The tubes were placed on ice. Aliquot of 0.095 mL of the bacterial suspension was distributed to 0.3 mL 96 well plates. Loss of fluorescence was recorded for 30min at a regular interval of 1 min at the excitation and emission wavelength of 530nm and 585nm respectively using spectrofluorometer (FLUOstar Oomega-BMG Labtech, Ortenberg, Germany).

9.2.9 Mutation prevention concentration (MPC)

The MPC of tetracycline (TET) against *P. aeruginosa* MTCC 741 was determined as described previously (Heisig & Tschorny, 1994).Bacterial suspension of 0.1 mL (10^{10} cfu) was plated onto MHA containing TET at 2×MIC, 4×MIC, 8×MIC and 16×MIC. The same concentration of tetracycline was also tested in the presence of these extracts. The mutation frequency was obtained by counting the total number of colonies after 48 h of incubation at 37°C on the drug-containing plate and then dividing by the total number of cfu plated, the mutation frequency was obtained.

9.3 Results and Discussion

9.3.1 Antibacterial activity

Antibacterial activity of crude extracts of secondary metabolites produced by both the *Penicillium* strains are present in Fig. (9.1-9.2). The zone of inhibition indicated that the bacterial strain *Pseudomonas* was more susceptible to the EtOAc crude extract of secondary metabolite produced by *P. oxalicum* than *P. citrinum*. The results showed that there was percent increase 65 % (3.5 fold increase) and 26 % (2 fold increase) in susceptibility of bacterial strain *Pseudomonas* against crude extract of metabolites of *P. oxalicum* and *P. citrinum* strains, respectively when compared with control conditions. There was no antimicrobial activity against reference or control petriplate treated with streptomycin. *Pseudomonas* is known for its multi drug resistance property, as a result of excessive antibiotic administration and it is now known for acquiring antibiotic resistance and cross-resistance between antibiotics and the appearance of multidrug-resistant

(MDRS). These findings demonstrated the antimicrobial attribute of crude extract of secondary metabolite produced by psychrophilic *P. oxalicum* with accurate information regarding the sensitivity patterns of antibiotics. Similar results were reported for antibacterial activity of crude extracts of *P. oxalicum* strain against human pathogens, *B. subtilis, E. coli and S. aureus* (Bisht, *et al*. 2016). Sharma, *et al*. (2016) screened the ethyl acetate and methanolic extracts of fungal culture, *Pestalotiopsis neglecta* using agar diffusion method against gram–positive and gram negative bacteria and were found the extract positively inhibited the growth of the four human pathogens; *B. subtilis, S. aureus, E. coli* and *S. typhimurium*.

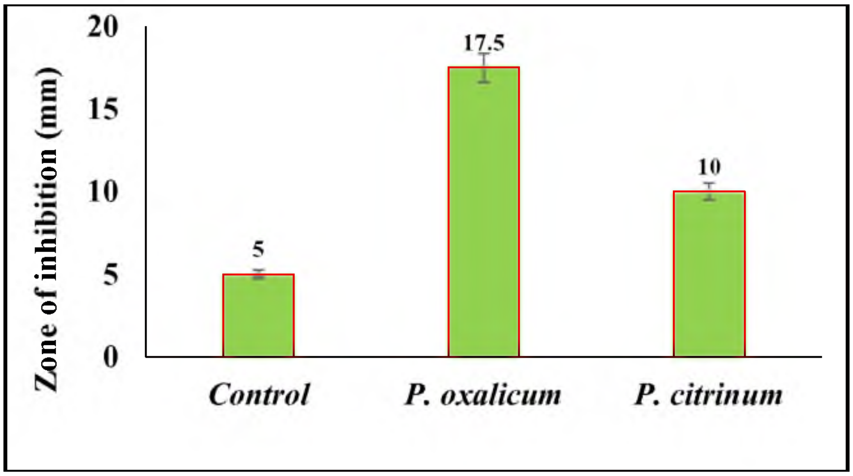


Fig. 9.1 Zone of inhibition of bacterial growth by EtOAc crude extract (100 µL) of secondary metabolites by both the *Penicillium* strain (*P. oxalicum* and *P. citrinum*).

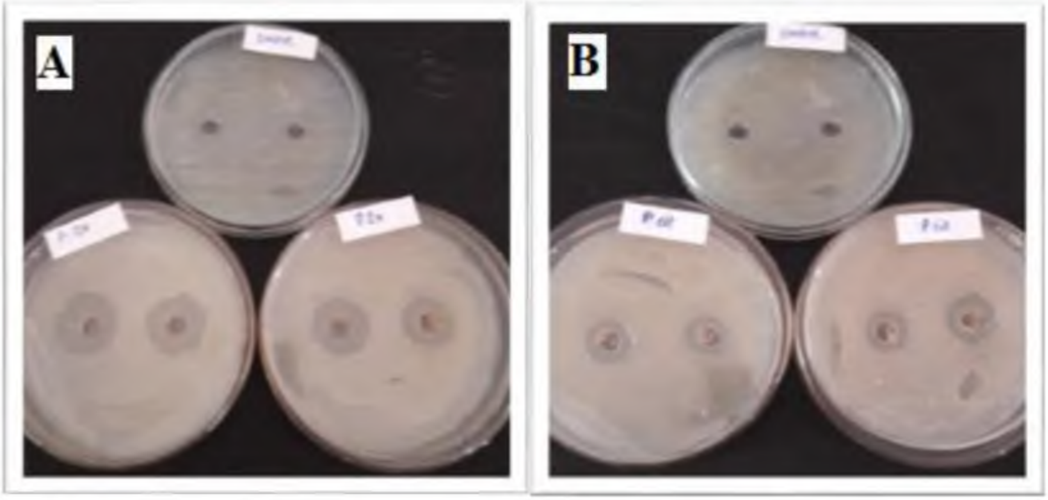


Fig. 9.2 (A&B). Showing zone of inhibition of bacterial growth by EtOAc crude extract of secondary metabolite produced by *P. oxalicum* (A) and *P. citrinum* (B).

9.3.2 Cell viability by MTT assay

Anticancer activity of the crude extract of secondary metabolites of psychrophilic *P. oxalicum* and mesophilic *P. citrinum* on Human Lung cancer cell line (A549) was determined by using different concentrations of crude extract. The MTT assay revealed a dose-dependent decline in percent cell viability as a function of the fungal secondary metabolite. The different concentrations 01,02,04,06 µg/ml and 08µg/ml of crude fungal secondary metabolite of psychrophilic *P. oxalicum* resulted into gradual decline in the percent cell viability (74, 59, 57, 48 and 47 %) respect of A549 cancer cell line. But 10 µg/ml concentration of EtOAc extract of secondary metabolite, there was almost complete cessation of cell viability (13%). The different concentrations (01, 02, 04 and 06µg/ml) of crude extract of secondary metabolite of mesophilic *P. citrinum* also resulted into decline in the percent cell viability by 92, 86, 82 and 80% respectively. At concentrations of 8 and 10 µg/ml, the percent cell viability was reduced to 24 and 22%, respectively. These results suggested that the secondary metabolite of *P. oxalicum* was more cytotoxic to A549 cancer cell lines than the metabolites of *P. citrinum* (Fig. 9.3). A significantly greater cytotoxicity of the crude extract of secondary metabolite from psychrophilic *P. oxalicum* suggested that *P. oxalicum* might be synthesizing some new secondary metabolites with greater anticancer potential. The similar results of antitumor and cytotoxic activities of the *Penicillium* sp. extract against five cancer cell line shave been reported by Devi and Prabakaran (2014). They have demonstrated a promising effect of EtOAc extract (1000µg/ml) against HeLa, A431 and human breast cancer (MCF7) (95, 125 and 275µg/mL respectively), except for HepG2 and A549 the IC50. Shen *et al*. (2013) evaluated the cytotoxic and anti-phytoviral activity of compounds isolated from *P. oxalicum* 0312F_1, which demonstrated moderate inhibitory effect on the proliferation of human gastric cancer cells SGC-7901 and higher inhibitory activity against of the replication of TMV, Sharma, *et al*. (2016) reported the cytotoxic activity of methanol and ethyl acetate extracts of various endophytic fungi on Human Embryonic Kidney(HEK) cell line to check their bioactivity and demonstrated significant effect against the cancer cells, and decrease in cell viability. Li, *et al*. (2012) have studied the cytotoxicity of fungal secondary metabolites against a panel of five human tumor cell lines (HCT-8, Bel-7402, BGC-823, A-549, and A-2780) and reported that the ethyl extract of the whole culture of fungal strain GW-13 showed significant level of in vitro cytotoxicity against P-388 lymphocytic leukemia cells. With more similar findings on anticancer property of secondary metabolite of *Penicillium* sp. have cytotoxic activities against the cancer cell lines such as, the berkelic acid produced by an extremophilic *Penicillium* strain isolated from Berkeley Pit Lake, Butte, Montana, (USA) was investigated in the National Cancer Institute (NCI) for antitumor activity against 60 human cell lines, it showed selective effect against ovarian cancer OVCAR-3 (Stierle, *et* *al*., 2006). The present study clearly demonstrated the cytotoxic potential of crude extract of secondary metabolites from psychrophilic *P. oxalicum* strain and compared with mesophilic *P. citrinum*. However, the secondary metabolite from *P. oxalicum* was more effective against cancer than the *P. citrinum*.

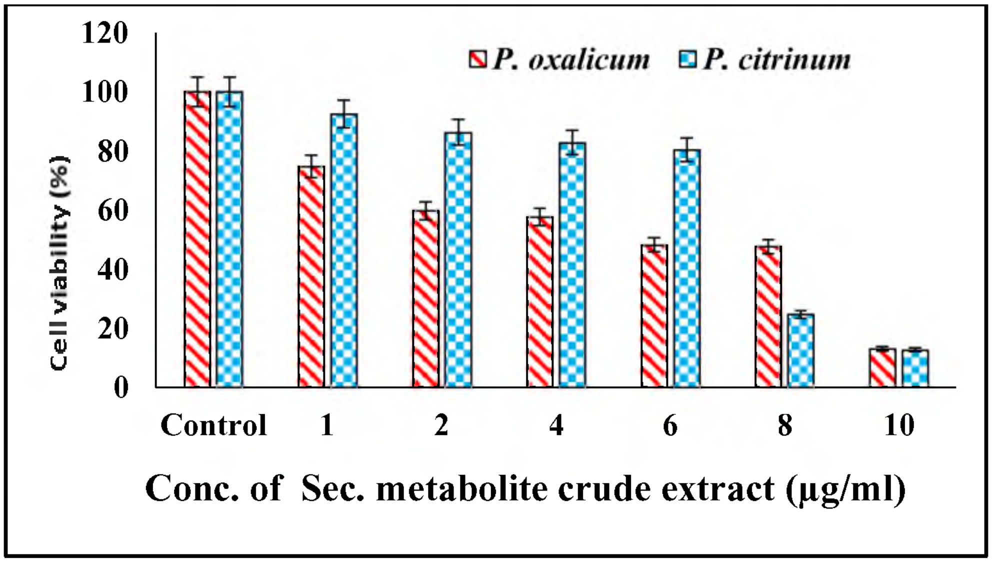


Fig. 9.3 Effect of the different concentrations (0-10 µg/ml) of secondary metabolite extract of both the *Penicillium* strains on cell viability of human lung cancer cell line (A549) using MTT assay.

9.3.3 Cell Cytotoxicity assay

Inhibition of cell proliferation or induction of apoptosis in cancer cells is the most important characteristic of many anti-cancer agents. In the present study, nuclear DNA cleavage (sub-diploid peak) by Propidium Iodide (PI) stain was studied with or without secondary metabolite and it was found that the crude extract of secondary metabolites from *P. oxalicum* was involved in the induction of apoptosis treatment when compared with the metabolites of mesophilic *P. citrinum*. The results showed an increment in percent uptake of PI which directly reflected the death/apoptosis of cancer cells with the increase in the concentration of crude extract of secondary metabolites (5-20µg/ml) of both the *Penicillium* strains. The presence of Secondary metabolites of psychrophilic

P. oxalicum showed higher percentage of PI uptake (8.84, 15.24, 24.22%) by the cancer cells than the metabolites of mesophilic *P. citrinum* (12, 12.69, 15.16% respectively) in a dose dependent manner (Fig 9.4 -9.6.). Hence, results demonstrated that the crude extract of secondary metabolites produced by psychrophilic *P. oxalicum* was more cytotoxic and inhibited cell proliferation in Human Lung cancer cell line (A549) as compared to the mesophilic *P. citrinum*.

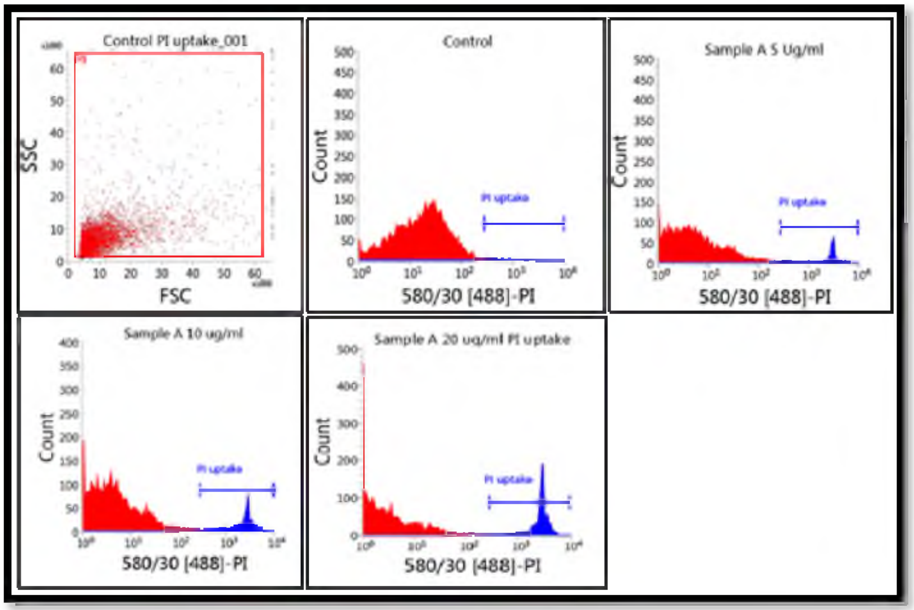


Fig. 9.4 Flow cytometric analysis of PI uptake by cancer cell line (A549) as a function of different doses (5-20 µg/ mL) of EtOAc extracts of secondary metabolite of *P. oxalicum* after 24 hr treatment.

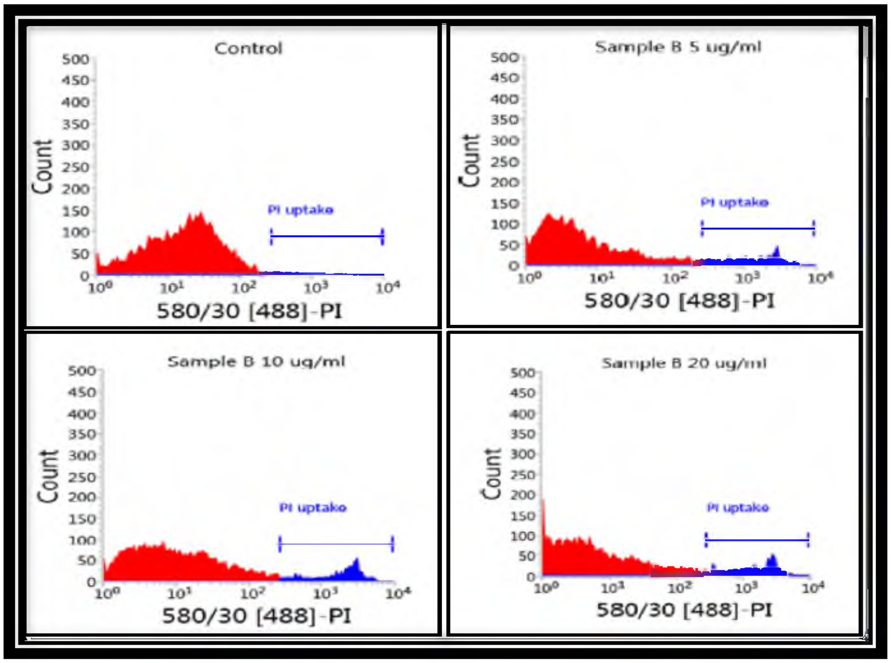


Fig. 9.5 Flow cytometric analysis of PI uptake by cancer cell line (A549) as a function of different doses (5-20 µg/ mL) of EtOAc extracts of secondary metabolite of *P. citrinum* after 24 hr treatment.

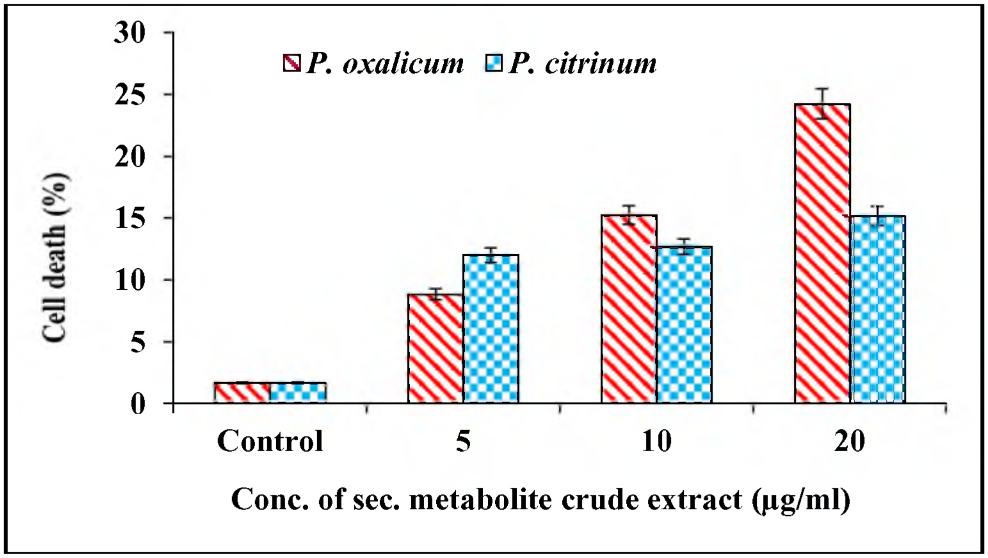


Fig. 9.6 Effect of different doses (5-20 µg/ml) of EtOAc extracts of secondary metabolite of both the *Penicillium* strains on percent cell death in Human Lung Cancer cell line (A549) using PI staining.

9.3.4 Susceptibility tests

Multidrug resistance is governed by the respective gene(s)/protein(s). In the post genomic era these muti-drug resistant gene(s)/protein(s) in the bacteria are promising targets of antibacterial drugs. For developing more effective anti-infective drugs, the structural and functional studies of the bacterial membrane proteins become crucial (Livermore, 2009). In the present study, antibiotic tetracycline was used because of its broad spectrum antibacterial activities, cost efficient, relatively low toxicity and also being the good substrate for efflux pumps (Roberts, 2003). The efflux pump inhibitor phenylalanine-arginine beta-naphthylamide (PaβN) was used due to its Resistance-Nodulation-Division (RND) efflux pump inhibitory potential. Fractions 1 & 2 (*P. oxalicum* & *P. citrinum*) along with the PAβN were evaluated for their antibacterial activity against KG-P2, the most resistant clinical isolate &MTCC-741/ATCC-25668 strain of *P. aeruginosa*. The MICs of these fractions varied from 400-800 mg/L (Table 9.1). The results showed that these fractions (1&2) of crude extract of secondary metabolites (*P. oxalicum* and *P. citrinum*) and 2-azetidinone used in the assay did not possess antibacterial activity of their own, but when they were tested in combination with antibiotic tetracycline, crude extract reduced the MIC of antibiotics by 8 folds (Table 9.1). This 8 fold reduction in the MIC of antibiotics in presence of metabolite fractions, indicated that secondary metabolite of *Penicillium* strains have equivalent drug resistance reversal potential. Further fractionation and isolation of active molecule (s) of secondary metabolites can make them better efflux pump inhibitors. However molecule 2-azetidinone present in the secondary metabolites of *P. citrinum* is known to have wide applications in chemotherapeutic fields such as antimicrobial (Sutariya, *et al.* 2007), antibacterial

(Mistry and Desai, 2005), antimalarial (Nivsarkar, *et al.* 2005), anticancer (Banik, *et al.* 2004) etc. From the foregoing results, it can be predicted that the activity of the fraction is not due to direct action of metabolite molecule, but due to the synergy of other molecules/compounds. Tetracycline at 800mg/L (8 MIC) inhibited the growth of *P. aeruginosa* MTCC-741 indicating its mutation prevention concentration (MPC). However, when MPC of tetracycline was reduced to 400mg/L in combination with metabolite fractions, the results showed that molecules in the metabolites were able to reduce the mutation prevention concentration of TET and suggested the suitability of these biomolecules as a potential therapeutic agent (s) (Table 9.2). The present findings were in accordance to earlier reports wherein the efficacy of antibiotics was enhanced by natural molecules when tested in combinations (Dwivedi, *et al.* 2014; 2015; 2016; Khan, *et al.* 2006). To understand the possible mechanisms of action, of metabolites of *P. oxalicum* and *P. citrinum* with PaβN, their efflux pump inhibitory potential was assessed using ethidium bromide efflux assay. The significant decrease in relative fluorescence units of Ethidium Bromide (EtBr) were observed in non-treated control cells, whereas this decrease in fluorescence was restricted in the set up containing *Penicillium* metabolites (Fig. 9.7). The loss of EtBr fluorescence was inhibited significantly by PAβN, followed by metabolic molecules of *P. citrinum* and *P. oxalicum,* respectively. Accumulation and efflux of ethidium bromide indicated involvement of efflux pumps in the resistance mechanism, particularly in Gram-negative bacteria (Viveiros, *et al.* 2005).

Table 9.1 Interaction study of metabolite fractions of both *Penicillium* strains with tetracycline against KG-P2 of *P. aeruginosa*.

Agent	**MIC (mg/L)**		**FICI**	**Interaction**	**Fold reduction in the MIC of TET**
	Alone	Combination (Compound/TET)			
TET	1600	-	-	-	-
Fraction 1	800	100/400	0.250	Synergy	4
Fraction 2	400	50/200	0.125	Synergy	8
2-azetidinone	800	100/800	0.625	No interaction	2
PAβN*	800	50/100	0.062	Synergy	16

*phenyl arginine β naphthalmide

Table 9.2 Mutation prevention frequency assay in the presence of TET and metabolite fraction of both *Penicillium* strains.

Agents	**Mutation frequency of *P. aeruginosa* with T*Et* *al*one and in combination with compounds**				
	MIC	**2 MIC**	**4 MIC**	**8 MIC**	**16 MIC**
TET(Alone)	4.1x10^{10}	1.5x10^{10}	0.6x10^{10}	<10^{10}	<10^{10}
TET+ *P. oxalicum* (100mg/L)	2.0x10^{10}	0.8x10^{10}	<10^{10}	<10^{10}	<10^{10}
TET+ *P. citrinum* (50mg/L)	1.0x10^{10}	0.2x10^{10}	<10^{10}	<10^{10}	<10^{10}
TET+PAβN (50mg/L)	0.4x10^{10}	<10^{10}	<10^{10}	<10^{10}	<10^{10}

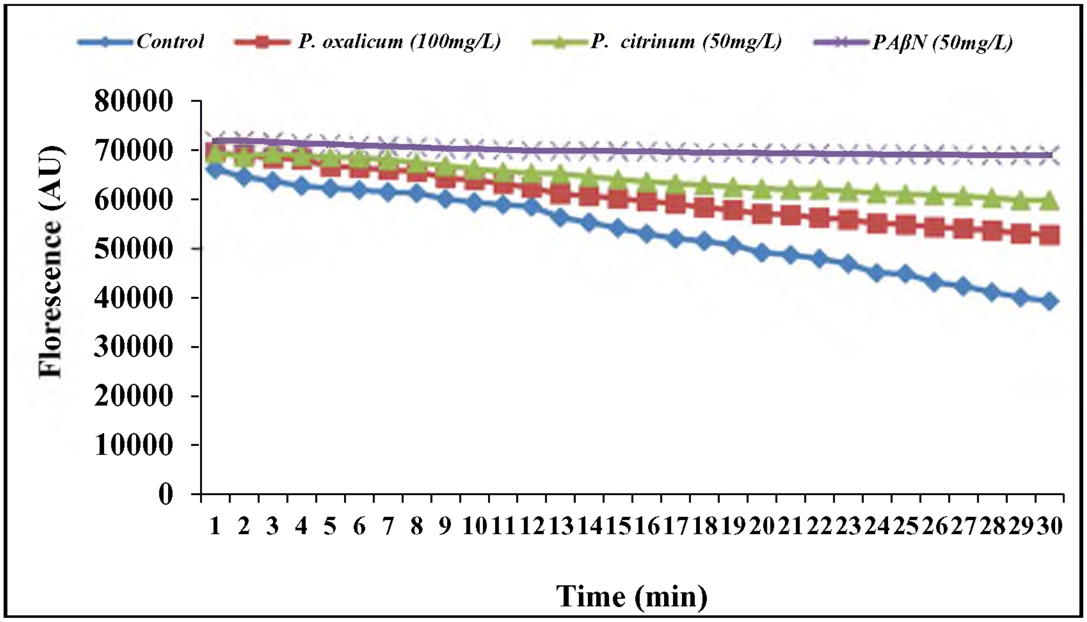


Fig. 9.7 Efflux pump inhibitory potential of metabolites of both *Penicillium* strains and comparison with PAβN (50 mg L^{-1}).

9.4 Conclusion

- The results of antibacterial activity in secondary metabolites of psychrophilic *P. oxalicum* and mesophilic *P. citrinum* showed that there was 3.5 fold and 2 fold increase in susceptibility of bacterial strain *Pseudomonas* against crude extract of metabolites of *P. oxalicum* and *P. citrinum* strains, respectively, when compared with the control.
- These results of Cell viability assay suggested that the secondary metabolite of *P. oxalicum* was more cytotoxic to A549 cancer cell lines than the metabolites of *P. citrinum*. The MTT assay revealed a dose-dependent decline in the percent cell viability as a function of the fungal secondary metabolite of *Penicillium* strains.
- The results of cell cytotoxicity assay showed an increment in percent uptake of PI, which directly reflected the death/apoptosis of cancer cells with increase in the

concentration of crude extract of secondary metabolites of psychrophilic *P. oxalicum* and mesophilic *P. citrinum*.

- The results on antibiotic susceptibility test showed about 8 fold reduction in the MIC of tetracycline that revealed the secondary metabolites of *Penicillium* strains have equivalent drug resistance reversal potential against bacterial strain tetracycline tolerant *P. aeruginosa*.
- Antibacterial and Anticancer activity of secondary metabolites of psychrophilic *P. oxalicum* may lead to a new biotechnological approach to combat MDR (multidrug resistance) and cancer problem. However, it requires a detailed investigation to know the mechanism and mode of action of new biomolecules produced by the stress tolerant *Penicillium* strains.

CHAPTER-10 GENERAL DISCUSSION

The search for novel organic molecules with interesting biomedical properties is drawing increasing attention of scientists world over. Production of secondary metabolites by microrganisms under extreme environmental conditions has held a unique position in search of new kind of useful biomolecules. Secondary metabolites are usually bioactive, low molecular weight, molecules produced by microorganisms during their life cycle and they are usually associated with a specific stage of morphological differentiation (Keller, *et al.* 2005). Psychrophilic fungi have served as novel tool to explore the production of various bioactive secondary metabolites with several applications in pharmaceuticals and biotechnological industries (Leitão, 2009; Visagie, *et al.* 2009; Gawas-Sakhalkar, *et al.* 2012). The present study is an attempt to explore the potential source of new metabolites. The *Penicillium* strains isolated from two different climatic zones were screened, for the production of secondary metabolites under harsh environmental conditions such as, temperature, pH, salinity, heavy metal and nutritional stress. The potential applications of biomolecules of secondary metabolites from isolated *Penicillium* strains such as antibacterial and anticancer properties were also studied.

The evolutionary characteristics of fungi and their shared patterns of morphology, phylogeny and population help us to better understand the adaptability of extremophilic fungi (Alleaume-Benharira, *et al.*, 2006; Johannesson and Andre, 2006). The two different *Penicillium* fungal strains were isolated from two different climatic zones of India; one from agricultural soil of dry temperate region of Leh, Ladakh (J & K), India and another from garden soil of tropical region of Lucknow (U.P.), India. They also exhibited characteristic features of psychrophilic and mesophilic nature when grown under different temperature regime. The macroscopic features of psychrophilic fungal strain grown on petridish containing Potato Dextrose Agar (PDA) media showed moderate growth, a green color colony with white periphery and velvet appearance. Whereas, on Czapek Dox media, this psychrophilic fungal strain exhibited slow growth, white matte like appearance with back side white in color. The pigment produced by the psychrophilic fungus on PDA was yellow in color. On the other side, the mesophilic fungal strain showed rapid growth, dark green color granular appearance and the back side of the colony on PDA plate was reddish in color, whereas on Czapek Dox media this species showed moderate growth, green color with velvet appearance. Similar macroscopic observations were reported by Velmurugan, *et al.*, (2009) and Tiwari, *et al.* (2011), for the *Penicillium* strains. The microscopic morphological study showed that the hyphae of psychrophilic *Penicillium* are septate with spore size approx. 2.70 µm and conidiophores are terverticillate. On the other hand, hyphae of mesophilic *Penicillium* were smooth with spore size approx. 2.35 µm and globose condia, and visibly rough-walled terverticillate conidiophores. Based on morphological and microscopic examinations, it was concluded that both *Penicillium* strains isolated from different habitats belong to same genera, but with different morphological characteristics.

Molecular Identification of isolated *Penicillium* strains, using 18S molecular analysis with ITS4 & ITS5 universal primers showed that *Penicillium* strains were psychrophilic *P. oxalicum* **(accession no. KR150256)** isolated from Leh, Ladakh and *P. citrinum* **(accession no. KR150257)** isolated from tropical region of Lucknow.

It is well known that the fungi are also good producers of extracellular and useful secondary metabolites (Calvo, *et al*., 2002, Keller, *et al*. 2005; Stierle, *et al*., 2006). Krishnan, *et al*. (2016) suggested that the extracellular enzyme production in harsh environmental conditions may be an important component of their survival strategy. In the present study, biochemical characteristics of *Penicillium* strains revealed that psychrophilic *P. oxalicum* could efficiently produce extracellular amylase and cellulolytic enzymes, when compared with the mesophilic counterpart *P. citrinum*. Previous studies have also demonstrated the production of cellulolytic enzymes by several species of *Penicillium* (Jorgensen, *et al*. 2005; Picart, *et al*. 2007). In recent times, Maeda, *et al*. (2013) and Carvalho, *et al*. (2014) have documented that the *cellulase* production by *Penicillium funiculosum* could be used for ethanol production. Various industries have started paying attention on the production of fungal enzymes and secondary metabolites (Varalakshmi, *et al*. 2009). Fungal *amylases* have found important industrial applications such as textile, leather industry, liquification and conversion of starch to sugar, fermentation industries, chemical and pharmaceutical industries (Pandey, *et al*. 1999; Butzen and Haefele, 2008) and the most important advantage of use of fungal amylase, its economical bulk production and ease of manipulation (Saleem and Ebrahim, 2014). Many reports on fungal species as a source of enzyme *amylase* had been documented (Pandey, *et al*. 2005; Gupta, *et al*. 2008; Khan and Yadav, 2011; Irfan, *et al*. 2012). Hence, production of *cellulase* and amylase enzyme in a good amount by psychrophilic *P. oxalicum* for its own survival under adverse conditions can be exploited for commercial purpose. Cold-active enzymes like *amylases, cellulases, lipases, pectinases,* and *proteases* from psychrophilic fungal strains find vast applications in the food, medicine, and detergent industries (Feller and Gerday, 2003; Leary, 2008).

The temperature is one of the most important parameters which directly affects the growth and survival of microorganisms (Deegenaars and Watson, 1998). Psychrophilic fungi, known to survive at extremely low temperature, are commonly found in polar and non-polar habitats (Hassan, *et al.*, 2016). The survival of psychrophilic microorganisms at low temperature might be due to their better nutritional adaptability (Archer, *et al.*, 2008) and unique cold shock and cold acclimation proteins or enzymes (Cole &and Cox, 1981). Thus, the psychrophiles exhibiting unique features of producing cold active proteins, enzymes, and diverse bioactive metabolites with medicinal importance include Penicillin, Lovastatin (Deegenaars and Watson, 1998), fingolimod (Delpiccolo, *et al.*, 2003) and caspofungin (Frisvad, and Samson, 2004). In the present study, the cold tolerant isolate, *P. oxalicum* (psychrophilic) was examined for production of biochemical and physiological changes under low temperature stress (4°C) and results were compared with the mesophilic *P. citrinum* strain under high temperature stress (35°C). The present study showed that the psychrophilic *P. oxalicum* has potential to grow at extremely low temperature (4°C) with growth optima at 15°C but the mesophilic *P. citrinum* showed zero tolerance to low temperature and showed optimum growth at 30°C.

Ryan, *et al.*, (2007) reported about the effect of pH on mycelial growth, which confirmed the regulatory role of ambient pH in biomass production in many fungal species. The ambient pH determines the nutrient availability and the proton gradient across the plasma membrane, as well as cell wall remodeling (Bilgrami and Verma, 1981, Schmidt, *et al*, 2008). Both the *Penicillium* strains grown at different pH (4.0-9.0) under respective optimum growth temperature, showed that the maximum mycelial growth of psychrophilic *P. oxalicum* and mesophilic *P. citrinum* at pH 4.0, whereas decline in fresh weight of both *Penicillium* strains was recorded with increase in pH range (6.0-9.0). Results of the present study were supported by Cogliati, *et al*. (1997) as they reported the best growth of *Penicillium marneffei* at acidic pH, compared to the neutral pH. Singh, *et al*. (2014) reported the significant difference in the biomass production of *Trichoderma* fungal strain at tested at pH 4.0, 4.5, to 8.0 and observed that the pH ranging between 5.5 and 7.5 was the most favorable pH range for its growth. Results on salt dependent growth of *Penicillium* strains revealed that the best growth at 2% (w/v) salt concentration. Beyond 2 % NaCl concentration, growth of both the *Penicillium* strains declined. However, overall results revealed that psychrophilic *P. oxalicum* was relatively more tolerant to higher salt concentration as compared to the mesophilic *P. citrinum*. Cantrell, *et al*. (2011), documented that the several species of *Aspergillus* and *Penicillium* have tendency to dominate the low temperature and saline habitats. Particularly, the *Penicillium* spp. is capable to survive and grow under different physiological conditions, such as high salt concentrations, low to high temperature and low water availability (Houbraken and Samson, 2011). Various other studies supported the present results as they stated the *Pencillium* strains have ability to grow on high concentration of salt which may be attributed to the defense mechanisms involved in coping the stressful environment (Robinson, 2001; Dhakar, *et al*. 2014).

Zhao, *et al*. (1990) suggested that the availability of nutrients such as carbon and nitrogen play an important role in fungal growth and sporulation. Production of secondary metabolites also gets influenced by the availability of nutrients such as carbon and nitrogen sources (Demain, 2000; Prasher, *et al.* 2014). The present study on the influence of nutrients such as carbon and nitrogen sources on growth and development of *Penicillium* strains showed that glucose was the best carbon source for the growth of *P. oxalicum*, as it holds maximum biomass production, whereas optimum growth of mesophilic *P. citrinum* was found to be the best in the presence of 2% (w/v) concentration of sucrose with avg. fresh wt. 380 g L^{-1}. Psychrophilic *P. oxalicum* exhibiting maximum biomass production in the presence of 2 % (w/v) glucose was similar to earlier reports (Chandra, *et al.*, 1977; Oso, 1977a; Fasidi and Jonathan, 1994; Fasidi and Olorunmaiye, 1994). The glucose is preferred carbon source for *P. oxalicum* perhaps due to reason that it was directly metabolized to produce the cellular energy (Jandaik and Kapoor, 1976; Garraway and Evans, 1984). On the other hand, among the different inorganic and organic nitrogen sources (sodium nitrate, ammonium sulphate, glycine, and L-Tryptophan), 2 % (w/v) sodium nitrate and 1% (w/v) glycine was found the best organic nitrogen source for the growth of psychrophilic *P. oxalicum*. Whereas the *P. citrinum* showed optimum growth in the presence of 2% (w/v) of sodium nitrate and glycine.

Fungi are amazing organisms that produce a great diversity of natural products often called secondary metabolites (Calvo, *et al.* 2002) with wider applications in the medical, industrial and agricultural fields (Demain and Fang, 2000). The screening and extraction of secondary metabolites of both *Penicillium* strains grown at different temperatures revealed that the production of secondary metabolites in both the *Penicillium* strains, were higher at higher growth temperature (35°C), when compared with that obtained in low temperature grown cells. The temperature dependent increase in the quantity of secondary metabolites of psychrophilic *P. oxalicum* with rising temperature, might be a survival strategy of the fungus under stress conditions. Whereas, imposition of low temperature stress on the mesophilic *P. citrinum* could not elicit similar response and exhibited maximum production of metabolites only under optimum growth conditions. The biosynthesis of secondary metabolite is directly related to culture conditions (Demain, 1999). The present study on secondary metabolite production in the presence of different carbon and nitrogen sources revealed that 1% (w/v) glucose or sucrose concentrations supported the maximum production of secondary metabolites in the *P. oxalicum* and *P. citrinum* strains, respectively. Several reports suggested that simple sugar like glucose, fructose, glycerol and sucrose play an important role to enhance the fungal growth and secondary metabolite production rather than complex carbon sources (Buchanan and Stahl, 1984; Calvo, *et al.* 2002; Jain, *et al.* 2012). On the other hand, nitrogen results revealed that the 1% (w/v) glycine concentration supported the maximum production of secondary metabolite in case of both the *Penicillium* strains. Peighany- Ashnaei, *et al.* (2007) have demonstrated that various nitrogen sources play important role in maximizing the growth well as production of bioactive compounds. Further, it was observed that both *Penicillium* strains in the presence of different salt (NaCl) concentrations, showed an outstanding quantity of bioactive metabolites in psychrophilic *P. oxalicum* and mesophilic *P. citrinum* upto 10 % (w/v) and 5% (w/v) salinity, respectively. It is evident from the foregoing results that production of bioactive metabolite in both *Penicillium* strains was greatly induced by high NaCl concentration, irrespective of its effect on growth. In a similar study by Mathan, *et al.* (2013), who investigated the influence of NaCl on the biomass and production of bioactive compounds in *Aspergillus* strain, and showed that the NaCl concentration of 5 g L^{-1} was optimal for maximum mycelial growth and 7 g L^{-1} of NaCl in basal medium was optimal concentration for secondary metabolite production. Bhattacharyya and Jha, (2011) also demonstrated that the high concentration of NaCl was required for maximum mycelial growth and production of bioactive metabolite in the fungal strain.

It has been reported that types of lipids in individual fungus may vary from organism to organism as they are known to change with the age, stages of development, nutrition, and environmental conditions (Weete, 1981, Losel, 1989). Shapaval, *et al.*, (2014) documented that the temperature dependent changes in the physical state of the lipids lead to changes in the various metabolic functions of the fungal membrane. In the present study, quantitative analysis of lipid extracts of both *Penicillium* strains grown under different temperature conditions (4, 15, 25°C and 35°C) has been done. The results revealed there was abundance of mono fatty glycerols and di-fatty acid derivative of glycerolipids in both the *Penicillium* strains grown at 4°C. They were scarcely available when both the *Penicillium* strains were grown at 35°C. Among the fatty acid, long chain (C_{16}-C_{22}) saturated fatty acids were abundantly present in both the *Penicillium* strains grown under two extreme temperature conditions. However, concentration of Palmitic acid was relatively higher in mesophilic *P. citrinum* grown at high temperature. Among the unsaturated fatty acids, both *Penicillium* strains grown at low temperature (4°C) showed preferential synthesis of Linolenic acid, Linoleic, Oleic acid and Erucic acid. However, LC/MS data showed that Docosahexanoic acid (omega-3) and Erucic acid (omega-9 unsaturated fatty acid) were exclusively synthesized by the *P. citrinum* at high temperature, while *P. oxalicum* showed reduced percentage of these unsaturated fatty acid under the same temperature conditions (35°C). Hence it is proved that the types of lipids in individual fungus may vary from organism to organism as they are known to change with the age, stages of development, nutrition, and environmental conditions.

The FTIR spectrum of cells represents a global "molecular fingerprint" which can be used for characterization, differentiation and identification of molecules (Lecellier, *et al.*, 2014). Shapaval, *et al.* (2010, 2013) reported the applications of FTIR spectroscopy in the field of identification of fungi at the level of species and genera. Pitt and Hocking (2009), also reported the identification of more than 50 *Penicillium* strains by using FTIR spectra. The temperature dependent changes in both the biomass of *Penicillium* strains, grown under different temperature regimes, showed the changes in IR absorption bands of Amide I, Amide II, symmetrical PO_2- stretching in RNA & DNA, and C=O functional groups of lipids. In case of psychrophilic *P. oxalicum*, the changes in IR absorption peaks appearing at 3437-420, 2930-27, 1740 cm^{-1} were assigned to changes in functional groups of lipids/ester and phospholipids, respectively (Fabian, *et al.* 1995). The temperature dependent changes in IR absorption bands suggested for alteration in functional groups associated with membrane function under different temperature conditions, were recorded at IR peaks at 1078, 1650, 1545 and 1040 cm^{-1} which were assigned for Amide I (Sukuta and Bruch, 1999; Andrus, 2006), Amide II (Yano, *et al.* 2000; Paluszkiewicz and Kwiatek, 2001), and symmetrical PO_2- stretching in RNA and DNA respectively (Fabian *et al.*, 1995). Further, a comparison of IR spectra of biomass of psychrophilic *P. oxalicum*, grown at 4°C and 35°C, showed emergence of unique IR peak (1456 cm^{-1}) at 35°C, mainly derived from CH_2 group bending in the lipids without contribution from proteins (Cakmak, *et al.* 2006). This absorbance peak was suppressed in IR spectrum of *P. oxalicum* grown at 4°C, indicating reduced content of saturated lipids (1456 cm^{-1}).). On the other hand, the IR absorbance at 1456 cm^{-1} wavenumber in *P. citrinum* was present in the cells grown under both high and low temperature conditions with minor peak shift in the absorption from 1456 to 1452 cm^{-1}. A precise protein-to-lipid ratio (A_{1650}/A_{1740} cm^{-1}) was calculated from the relative absorbance intensity at 1650 cm^{-1}, denoting carbonyl (C=O) group stretching in the proteins, and the IR absorbance at 1740 cm^{-1}, derived from bonding of fatty acyl group with glycerol backbone of the lipids (Shapaval, *et al.*, 2014). A higher ratio of protein-to-lipid in both the strains at high temperature (35°C) might be due to relatively enhanced synthesis of proteins to cope increased protein-lipid interaction (Klein, *et al.* 1999). The ratio of Amide I / Amide II (A_{1650}/A_{1560} cm^{-1}) in the low temperature (4°C) grown biomass of *P. oxalicum* was higher when compared with the corresponding values of Amide I / Amide II ratio in the high temp. (35°C) grown biomass. On the other hand, the Amide I/ Amide II ratio in *P. citrinum* was little influenced due to rise in the growth temperature. These results suggested for conformational alteration in protein structure (Ishida and Griffith, 1993) of *P. oxalicum* in response to growth temperature, which was not detected in *P. citrinum*. The overall results on IR spectra of biomass grown in different temperature conditions of *P. oxalicum* and *P. citrinum* exhibited temperature dependent alterations in functional groups present in cell membrane which might be contributing to survival strategy of individual strains during stress conditions.

The results of FTIR spectra (4000 to 500 cm^{-1}) of biomass of *Penicillium* strains (psychrophilic *P. oxalicum* and mesophilic *P. citrinum*) grown under different pH (pH

4.0 to 9.0) conditions revealed that there is significant major changes in IR absorption with peak shifts at wavenumbers 1740, 1078, 1650, and 1545 cm^{-1} assigned to changes in membrane lipids/ ν_s CH_2 of lipids (Fung, *et al.*, 1996), lipids/ Ester (C=O) and phospholipids (Fabian, *et al.*, 1995), deformation in proteins (Amide I and Amide II) (Andrus, 2006; Paluszkiewicz and Kwiatek, 2001; Shapaval, *et al.*, 2014), respectively in both the *Penicillium* strains. On the other hand, emergence of many new peaks and significant peak shift were recorded in IR spectrum of both the *Penicillum* strains due to presence of high salt (NaCl) concentrations (5 & 10 % w/v), particularly at wavenumber 1220 to 1350 cm^{-1} assigned to amide III, C-N stretching and N-H in plane bending (Fabian, *et al*;. 1995) and 1400 and 1403 cm^{-1}, assigned to the symmetric changes in fatty acids, -CH_2 group of amino acids/skeletal proteins and symmetric CH_3 bending modes of the methyl groups of proteins showed influence of high salinity on lipid–proteins interaction (Fung *et al.*, 1996; Fujioka, *et al.*, 2004). The results suggested that salt induced changes in membrane structure in both the strains was perhaps to enable them to tolerate high salt concentrations. Whereas, a peak shift at wavenumber 2860 cm^{-1}, assigned to C-H stretching in lipids (Dovbeshko, *et al.*, 1997), in case of mesophilic *P. citrinum*, suggested for change in lipids, when compared with the control. Hence it can be concluded that the change in IR spectra of biomass in both the *Penicillium* strains showed species specific as well as stress dependent structural and compositional alterations in the lipid and protein component of fungal membrane.

Cuero, *et al.* (2003) documented the important role of metal ions in stimulation of fungal growth and metabolite production. Fungi can tolerate and detoxify certain heavy metals by various mechanisms such as ion exchange, active uptake, complexation, adsorption, extra and intracellular precipitation, valence transformation (Mala, *et al.*, 2006; Volesky, 2007). The most important strategy adapted by fungal species against metal stress is to produce abundant organic acids and secondary metabolites (Auckloo, *et al.*, 2017). Here in the present study, heavy metals [As (III) and Cr (VI)] were selected to study their impact on growth and secondary metabolite production by both *Penicillium* strains. The results revealed that the psychrophilic *P. oxalicum* was more tolerant to high concentration of Arsenic and Chromium than the mesophilic *P. citrinum*. Psychrophilic *P. oxalicum* was able to tolerate arsenic upto 1000 mg L^{-1} concentration and grew well at 100 mg L^{-1} on petriplates supplemented with arsenic as well as in broth culture. Whereas, mesophilic *P. citrinum* strain could tolerate arsenic concentration upto 50 mg L^{-1}. In case of chromium, psychrophilic *P. oxalicum* exhibited optimum growth at 01 mg L^{-1} chromium concentration and showed chromium tolerance upto 10 mg L^{-1} concentration. Whereas mesophilic *P. citrinum* was only able to grow upto 01 mg L^{-1} Chromium concentration, 2.5 mg L^{-1} or higher concentration of Cr (VI) eliminated the growth. Results of production of secondary metabolites in the presence of heavy metal (As III and Cr VI) showed that higher conc. of As (III) and Cr (VI) promoted the production of secondary metabolites in both *Penicillium* strains when compared with their respective control (without metals). However, mesophilic *P. citrinum* showed little growth at higher concentration of Cr (VI), but it was still able to produce high concentration of secondary metabolite at 01 mg L^{-1} of Cr (VI) when compared with the psychrophilic counterpart. Hence, the results revealed a significant difference in the level of metal tolerance and secondary metabolite production in both the *Penicillium* strains which could be attributed to metal adsorption and accumulation efficiency of these fungal strains. Martino, *et al.*

(2000) described that the heavy metals are able to influence the growth of soil fungi and weaken their diversity or change their morphology and physiology with overall effect on the growth, reproduction processes and enzyme and metabolite production etc. A study by Akhtar, *et al.* (2013), suggested that the metal tolerance in filamentous fungi (*Aspergillus>Pithyum>Curvularia*) was common phenomenon, but they respond that differently to different metals as in the present study. Results of FTIR analysis suggested the functional groups present in the cell membrane of both *Penicillium* strains showed changes in the structure of proteins due to arsenic stress conditions, as major changes in IR peaks including shifting and stretching of the peaks were recorded at wavenumber 1650, 1540 and 1242 cm^{-1} assigned to amide I, II and III, respectively (Andrus, 2006; Fabian, *et al.* 1995). A previous report by Wong, *et al.* (1995) suggested that α-helix conformational changes of proteins due to heavy metals. Some changes were also observed in the peaks at wavenumber 1150, 1075 and 1043 cm^{-1} assigned to glycogen absorption, C-O-H deformation motions and stretching of phosphodiesters. Results of the FTIR study of both *Penicillium* strains grown in the presence of Cr (VI) revealed the changes in appearance and disappearance of IR peaks indicating changes in functional groups due to Cr binding by methylated amino group, esterified carboxyl and phosphate group biomass, which were commensuration with FTIR results on both *Penicillium* strains grown in the presence of arsenic. A study by Ramrakhiani, *et al.*, (2011) on FTIR analysis confirmed the involvement of amino, carboxylic, phosphate, sulfonyl and carbonyl groups in Cr (VI) biosorption by heat inactivated biomass of *Termitomyces clypeatus* (an agaric fungus). Thus, we conclude that both *Penicillium* strains can be a good tool for studying the heavy metal stress as well as for stress induced production of secondary metabolites.

In search of novel organic metabolites with interesting biomedical properties, fungi are considered remarkable organisms which continuously produce an extensive range of natural products known as secondary metabolites (Calvo, *et al*. 2002). These bioactive natural products produced by fungi are beneficial to human welfare due to their unique properties such as antibiotic, therapeutic, anticancer, cytotoxic, enzyme inhibitory and many more (Calvo, *et al*., 2002, Stierle, *et al*., 2006; Keller, *et al*. 2005). Extremophilic, especially cold tolerant species of fungi are of immense importance as they survive under extreme environments with evolved characteristics of production of bioactive secondary metabolites and cold-active enzymes (Han, *et al*., 2007; Lopes, *et al*., 2012; Subramani, *et al*., 2013; Wang, *et al*., 2013). Hence, the present study is an attempt to search for novel organic metabolites with unique biomedical characteristics produced by both *Penicillium* strains. The TLC analysis revealed that secondary metabolites of *P. oxalicum* include some mycotoxins, whereas extracts of *P. citrinum* exhibited presence of some alkaloids as evident from their absorbance and color of the chromatogram on the TLC plates. Chromatogram of GC-MS analysis of the EtOAc extract of psychrophilic *P. oxalicum* grown at two different temperatures (4°C and 35°C) showed the presence of 11 and 16 major peaks at respective temperature. On the other hand, EtOAc extract of mesophilic *P. citrinum* grown at 4°C and 35°C exhibited 14 and 13 major peaks at respective temperature. The common metabolites produced by both *Penicillium* strains grown under different temperature regimes (4°C and 35°C) were identified as 3-dodecene, 2-dodecanol, 1-hexadecanol at 4°C and Eicosane, Dibutyl phthalate, 9-

Hexacosene, Propanoic acid, 2- (aminooxy) at high temperature (35°C). The unique metabolites produced only by psychrophilic *P. oxalicum* were 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent[f] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro, 6-Quinazolinol, 2-Methyl-2-propyl methyl phosphonofluoridate, Pyridine, 2[(1,1dimethylethyl) thio, 4(1H) Quinazolinone, 4(1H) Pyrimidinone, 6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di(2-propylpentyl). Whereas, unique bioactive compounds produced by mesophilic *P. citrinum* were Cyclohexanone,4-ethyl-4-methyl-3-(1-methylethyl)-, trans-, 3-Methyl-1,4diazabicyclo [4.3.0] nonan-2,5-dione, N-acetyl, Glycyl-L-proline, Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, 11,14-Eicosadienoic acid or its methyl esters and a derivative of beta-lactam (2,4-Azetidinedione, 3,3-diethyl-1-methyl). The GC- MS results identified some volatile sec. metabolites of *Penicillium* strains which belongs to quinoline, quinolinone, azulene, and azetidinedione class of compounds with their widely known applications in biomedical field. As suggested by various researchers, the secondary metabolite quinolone produced by *P.* oxalicum has been reported to have an antiprotozoal activity (Pohl, *et al.*, 2011), antimalarial, anti-bacterial, antifungal, antihelmintic, cardiotonic, anticonvulsant, anti-inflammatory, anticancer and analgesic activity (Rancic, *et al.*, 2006, Roze, *et al.*, 2011). The other important bioactive compound Quinazolinone produced by psychrophilic *P. oxalicum* fungus constitutes a class of drugs that works as sedatives and contains a 4-quinazolinone core (Chen, *et al.* 2006). Quinazolinone and quinazoline derivatives, recently been reported as potent antimicrobial and have cytotoxic activities (Jafari, *et al.* 2016). The 2,4-Azetidinedione, 3,3-diethyl-1-methyl produced by *P. citrinum* is derivative of 2-azetidinone (β-lactam) ring system a precursor for number of broad spectrum β-lactam antibiotics (Thomas, 1990; Wynn-Williams,1990; Thomas and Cavicchioli, 2000; Singh, 2004;Osorio, *et al.*, 2008; Strader, *et al.*, 2011). Hence, the foregoing results revealed that the unique characteristics of *Penicillium* strains which respond to temperature stress and produce number of new secondary metabolites with potential industrial application, particularly in pharmaceutical and therapeutical fields.

As per the results of HPLC fingerprinting of metabolites, 17 and 15 peaks were detected in case of both psychrophilic *P. oxalicum* and mesophilic *P. citrinum*, respectively. Among these peaks, 06 major peaks were identified with their characteristic UV-Vis absorption spectra in case of psychrophilic *P. oxalicum*, but none of them were found to be known compounds as per the literature reported for characteristic absorption maxima of fungal secondary metabolites (Frisvad, 1981; Paterson and Kemmelmeier, 1990; Frisvad and Thrane, 1993; Antipova, *et al.*, 2011; Iócaa, *et al.*, 2016). On the other hand, metabolites produced by *P. citrinum* were identified as Quinolactacin C, Abscisic acid, Anofinic acid, Conocenol B, and Aurofusarin (Takahashi, *et al.* 2000; Iócaa, *et al.,* 2016). Takahashi, *et al.* (2000) identified Quinolactacins produced by *Penicillium* sp. EPF-6 using UV λ_{max} by HPLC fingerprinting and suggested that it have a unique quinolone skeleton conjugated with a γ-lactam ring. Sasaki, *et al.* (2006) investigated the biosynthesis of Quinolactacin A by a *Penicillium* strain and reported as a Tumor Necrosis Factor (TNF) production inhibitor. Other identified metabolites were reported for their wide applications in different fields such as Abscisic acid (stress hormone) for plant growth and development (Koornneef, *et al.* 1998; Assante, *et al.* 1977), aurofusarin for mutagenic and cytotoxic properties (Gaffoor, *et al.* 2005); and anofinic acid as strong antimicrobial agent (Qin, *et al.* 2011). Various reports on identification of secondary metabolites produced by different *Penicillium* strains using NMR analysis are documented (Malmstrom, *et al.* 2000; Liu, *et al.* 2007; Yang, *et al.* 2016; Iocaa *et al.* 2016). Proton NMR analysis of secondary metabolites present in ethyl acetate extract of *P. oxalicum* grown at 4°C temperature showed the presence of compound **2-Dodecanol** using Spectral Database System for Organic compounds (AIST, Japan) with **SDBS no-51174**. Rest results did not match with any known compounds given in the database. However active compound present in the crude extract of *P. oxalicum* needs to be further purified and identified using advanced NMR technique such as ^{13}C-NMR.

Undoubtedly, the *Penicillium* is known for production of secondary metabolites with antimicrobial/antibacterial, antifungal, anticancer, antitumor, immunosuppressant, cholesterol-lowering agents, anti-arthritis properties (Li, *et al.* 2001; Yang, 2002; Tan, *et al.*, 2006; Han, *et al.* 2007; Petit, *et al.* 2009). Many investigators have reported about the production of numerous anti-tumour compounds such as terpenoids, alkaloids, ketones quinones and their derivatives from marine fungi and *Penicillium* sp. (Liu, *et al.* 2005; Antipova, *et al.*, 2011). In the present investigation antibacterial and anticancer properties of EtOAc extract of secondary metabolites of *Penicillium* strains were estimated again bacterial strain *P. aeruginosa* and Human Lung Cancer cell line (A549), respectively. Preliminary study on the anticancer property by following MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay and cell cytotoxicity by flow cytometry analysis of Propidium Iodide (PI) uptake by Human Lung Cancer cell line (A549) demonstrated that metabolites of psychrophilic *P. oxalicum* was very efficient against cancer cells. The results of antibacterial activity revealed that *Pseudomonas spp.* was more susceptible to crude extract of secondary metabolite of *P. oxalicum* than *P. citrinum* with the zone of inhibition 17.5 and 10 mm, respectively. Similar results were reported for antibacterial activity of crude extracts of *P. oxalicum* strain against human pathogens, *B. subtilis, E. coli and S. aureus* (Bisht, *et al*., 2016). PI stained cancer cells indicated the induction of apoptosis in the cells treated with secondary metabolites of both the *Penicillium* strains commensuration with the result of MTT assay. Hence, these results demonstrated that the crude extract of secondary metabolites produced by psychrophilic *P. oxalicum* was found to be more cytotoxic and inhibited cell proliferation in A549 cancer cells as compared to the mesophilic *P. citrinum*.

The fractions of sec. metabolites of psychrophilic *P. oxalicum* and mesophilic *P. citrinum* along with the PaβN (efflux pump inhibitor) were evaluated for their antibacterial activity against KG-P2 bacterial strain of *P. aeruginosa* (most resistant clinical isolate MTCC-741/ATCC-25668). The results revealed that the metabolite fractions alone did not possess antibacterial activity of their own, but when tested in combination with antibiotic tetracycline these inhibitors reduced the MIC of antibiotics by 8 folds. Several fold reduction in MIC of antibiotics in presence of metabolite fractions (sec. metabolites of both *Penicillium* strains), indicated that these have equivalent drug resistance reverse potential. The results given by Khan, *et al.* 2006; Dwivedi, *et al*. 2014, 2015, 2016 were in accordance with present results.

SUMMARY

Production of secondary metabolites by microorganisms in extreme conditions is well documented. Fungi are well-known for their ability to synthesize wide variety of small bioactive molecules called as secondary metabolites; detrimental (toxins) and beneficial (pharmaceuticals) effects on human welfare (Keller, *et al.* 2005; Gawas-Sakhalkar, *et al.* 2012). Due to these bioactive properties, many fungal secondary metabolites have been adopted by humans for their use as Pharmaceuticals such as antibiotics, cholesterol-lowering agents, tumor inhibitors and immune-suppressants for transplant operations (Keller, *et al.* 2005). The production of secondary metabolites by fungi may depend upon the nutrient availability, physical and environmental conditions. However, the production of secondary metabolites in extreme environmental conditions could be a new approach and may be an area of interest as the production of bioactive molecules may get modified and enhanced.

The present study was an attempt to explore the potential aptitude of two *Penicillium* strains isolated from two different climatic zones, for the production of secondary metabolites under harsh environmental conditions such as, temperature, pH, salinity, heavy metal and nutrient stress. The identification of secondary metabolites produced by *Penicillium* strains were also studied. It is postulated that alterations in microbial membrane (protein, nucleic acids, lipid content) and production of secondary metabolites may occur under different environmental stress conditions, which has been analyzed in the present study by using FTIR, LC-MS, GC-MS, UV-Vis absorption and

HPLC-MS techniques. However, several studies have documented about the production of different secondary metabolites under extreme environmental conditions, but their potential applications in biomedical field is a topic of our interest.

The results are summarized as under:

- The two fungal strains were isolated from two different climatic zones of India, one from agricultural soil of dry temperate region of Leh, Ladakh (J & K), India and another from garden soil of tropical region of Lucknow (U.P.), India.
- The microscopic morphological characters were used for the identification of fungal strains and it was concluded that both fungal isolates belong to *Penicillium* genera.
- Based on sequencing of the ITS regions and comparison with NCBI GenBank database, strains were identified as psychrophilic *P. oxalicum* **(accession no. KR150256)** and mesophilic *P. citrinum* **(accession no. KR150257).**
- The present work revealed that the psychrophilic *P. oxalicum* has potential to grow at extremely low temperature (4°C) with growth optima at 15°C and acidic pH 4.0. The mesophilic *P. citrinum* showed zero tolerance to low temperature and showed optimum growth between 30°C- 35°C and acidic pH 5.0.
- Both *Penicillium* strains preferred 2% (w/v) NaCl for their optimum growth. Further, it was observed that psychrophilic *P. oxalicum* was able to tolerate NaCl concentrations (upto 15%, w/v) than mesophilic *P. citrinum* (10 %, w/v NaCl). Hence, psychrophilic *P. oxalicum* was found to be halotolerant in nature.
- The preferred source of carbon for *P. oxalicum* was glucose, followed by sucrose. For *P. citrinum*, sucrose was the best carbon source. Among the nitrogen sources,

glycine was found to be relatively better nitrogen source than Glycine and Sodium Nitrate in case of both the *Penicillium* strains.

- Biochemical characteristics of *Penicillium* strains revealed that psychrophilic *P. oxalicum* could efficiently produce extracellular *amylase* and *cellulase* enzymes, when compared with the counterpart mesophilic *P. citrinum*.
- Lipid Profiling revealed abundance of mono fatty glycerols and di-fatty acid derivative of glycerolipids in both *Penicillium* strains grown at 4°C. They were scarcely available when the *Penicillium* were grown at 35°C.
- Among the fatty acid, long chain (C_{16}-C_{22}) saturated fatty acids were abundantly present in both the *Penicillium* strains grown under two extreme temperature conditions. However, concentration of Palmitic acid was increased in *P. citrinum* due to rise in growth temperature (35°C).
- Among the unsaturated fatty acids, growth of both the *Penicillium* strains at low temperature (4°C) preferentially synthesized Linolenic acid, Linoleic, oleic acid and Erucic acid. High temperature (35°C) grown *P. citrinum* strain continued synthesis of most of the unsaturated fatty acid such as Docosahexanoic acid (omega-3) and Erucic acid (omega-9) while *P. oxalicum* showed reduced percentage of unsaturated fatty acid. Hence it is proved that the types of lipids in individual fungus may vary from organism to organism as they are known to change with the age, stages of development, nutrition, and environmental conditions.
- The high production of secondary metabolites in both *Penicillium* strains, were at higher growth temperature (35°C), when compared with that obtained in low

temperature grown cells. The temperature dependent increase in the quantity of secondary metabolites of psychrophilic *P. oxalicum* with rising temperature, might be a survival strategy of this fungus under stress conditions.

- The production of secondary metabolite were reached to its optimum level in *P. oxalicum* at 2% w/v NaCl concentration. Whereas, production of secondary metabolite in *P. citrinum* continued upto 5% (w/v) salt concentrations.
- Results of production of secondary metabolite by *P. oxalicum* and *P. citrinum,* showed that glucose and sucrose were relatively better carbon sources, for respective strain. Among organic nitrogen sources, L-glycine was better nitrogen source for production of secondary metabolite in both the *Penicillium* strains.
- The psychrophilic *P. oxalicum* was more tolerant to high concentration of Arsenic and Chromium than the mesophilic *P. citrinum*. Psychrophilic *P. oxalicum* was able to tolerate arsenic upto 1000 mg L^{-1} and grew well at 100 mg L^{-1} medium supplemented with arsenic. Whereas, mesophilic *P. citrinum* strain was able to tolerate arsenic concentration upto 50 mg L^{-1}.
- Psychrophilic *P. oxalicum* exhibited optimum growth at 01 mg L^{-1} chromium concentration and showed chromium tolerance upto 10 mg L^{-1} concentration. Whereas mesophilic *P. citrinum* was able to grow upto 01 mg L^{-1} Chromium concentration, 2.5 mg L^{-1} or higher concentration of Cr (VI) eliminated the growth.
- The production of secondary metabolites in the presence of heavy metal (As III and Cr VI) showed that higher conc. of As (III) 500 mg L^{-1} and 1.0 mg L^{-1}Cr (VI)

promoted the production of secondary metabolites in both *Penicillium* strains when compared with the control (without metals), irrespective of growth.

- FTIR spectra of biomass of both *Penicillium* strains showed species specific as well as stress dependent compositional alterations in functional groups present in fungal membrane or cell such as lipids (especially in saturated fatty acids) and deformation in proteins, perhaps it was a part of their intracellular defense strategy.
- TLC analysis of EtOAc crude extract of secondary metabolites of *Penicillium* strains suggested that secondary metabolites of *P. oxalicum* include some unique metabolites not present in *P. citrinum*, whereas extracts of *P. citrinum* exhibited presence of alkaloids based on their absorbance and color of the chromatogram on TLC plates.
- The results of identification of temperature dependent production of volatile compounds of secondary metabolites using GC-MS/MS, revealed the presence of 53 major peaks by both *Penicillium* strains. The compounds were identified as derivatives of quinoline, quinolinone, azulene, and azetidinedione class of compounds with widely known applications in biomedical and therapeutical fields such as antiprotozoal activity, anti-inflammatory, antimicrobial, cytotoxic and anticancer properties.
- As per the results of HPLC fingerprinting of metabolites, 17 and 15 peaks were detected in case of both psychrophilic *P. oxalicum* and mesophilic *P. citrinum*, respectively. Among these peaks, 06 major peaks were identified with their characteristic UV-Vis absorption spectra in case of psychrophilic *P. oxalicum*, but

none of them were found to be known compounds as per the literature reported. On the other hand, metabolites produced by mesophilic *P. citrinum* were identified as Quinolactacin C, Abscisic acid, Anofinic acid, Conocenol B, and Aurofusarin with several extensive applications.

- The ^{1}H NMR study also confirmed about temperature dependent changes in the composition of secondary metabolites produced by psychrophilic *P. oxalicum*, while composition of secondary metabolite produced by *P. citrinum* remained the same except quantitative changes.
- These results of Cell viability and cell toxicity assay suggested that the secondary metabolite of *P. oxalicum* was more cytotoxic to A549 cancer cell lines than the metabolites of *P. citrinum*.
- The results on antibiotic susceptibility test showed about 8 fold reduction in the MIC of tetracycline that revealed the secondary metabolites of *Penicillium* strains have equivalent drug resistance reversal potential against bacterial strain tetracycline tolerant *P. aeruginosa*.
- Hence, foregoing results revealed that the unique characteristics of the psychrophilic *P. oxalicum* and its temperature stress dependent production of number of new secondary metabolites with potential industrial applications, particularly in pharmaceutical and therapeutical fields.

CONCLUSION

The aim of the present study was to assess the production and identification of secondary metabolites produced by two different *Penicillium* strains under different environmental stress conditions and their potential applications in pharmaceutical and therapeutical fields. The fungal isolates were identified as psychrophilic *Penicillium oxalicum* **(accession no. KR150256)** isolated from agricultural soil of dry temperate region of Leh, Ladakh (J & K), India and mesophilic *Penicillium citrinum* **(accession no. KR150257)** isolated from garden soil of tropical region of Lucknow (U.P.), India. Based on 18S molecular analysis with ITS4 & ITS5 universal primers and they also exhibited characteristic features of psychrophilic and mesophilic nature when grown under different temperature regime. They grew well and produce secondary metabolites under different environmental stress conditions (temperature, pH, salinity, nutrients and heavy metals). The crude extract of secondary metabolites produced by psychrophilic *P. oxalicum* showed better antibacterial and anticancer properties than the extract taken from mesophilic *P. citrinum*.

Results on optimization of environmental conditions for better growth and production of secondary metabolites by both *Penicillium* strains offers an opportunity for the production of secondary metabolites at industrial level. Results revealed that optimum growth of psychrophilic *P. oxalicum* was achieved at low temperature (15°C), acidic pH (4.0) and 2% (w/v) salt concentration. Whereas, mesophilic *P. citrinum* showed optimum growth at temperature (30-35°C), acidic pH (5.0) and 2% (w/v) salt concentration. The glucose and sucrose was the preferred source of carbon for psychrophilic *P. oxalicum* and mesophilic *P. citrinum* fungal strains, respectively. The high concentration of sodium nitrate and glycine, best supported the growth of both *Penicillium* strains. The maximum production of secondary metabolites by both *Penicillium* strains has been achieved at high stress conditions such as high temperature, high salinity, high concentrations of carbon, nitrogen sources and high concentrations of heavy metals (AsIII and CrVI). Hence, it is proved that the environmental conditions play an important role to enhance the fungal growth and secondary metabolite production stated by other researchers.

It is well known that the FTIR spectrum of cells represents a global "molecular fingerprint" which can be used for characterization, differentiation and identification of microbial cell/biomolecules. In the present study, FTIR analysis of biomass of both *Penicillium* strains grown under different harsh environmental conditions has been performed. The results of the study suggested that there was involvement of various components of fungal cell/membrane such as deformations of proteins (amines, amino, amide groups), changes in membrane lipids/phospholoipids, saturated lipids. Such compositional alterations in lipid and protein component of fungal cell/membrane might be contributing to survival strategy of individual strains during different stress conditions.

It is well documented that types of lipids in individual fungus may vary from organism to organism as they are known to change with the age, stages of development, nutrition, and environmental conditions. The results of Lipid Profiling revealed abundance of mono fatty glycerols and di-fatty acid derivative of glycerolipids, long chain saturated fatty acids, in both the *Penicillium* strains grown at low temperature (4°C). Omega 3, 6 unsaturated fatty acids such as Docosahexanoic acid and Erucic acid were abundantly available when the *Penicillium* were grown at high temperature (35°C). Hence it is proved that the types of lipids in individual fungus may be species specific or varies from organism to organism as they are known to change with the age, and environmental conditions.

In search of novel organic metabolites with remarkable biomedical properties, fungi are considered significant organisms which continuously produce an extensive range of natural products known as secondary metabolites. The results of GC-MS/MS revealed the identification of volatile compounds belongs to derivatives of quinoline, quinolinone, azulene, and azetidinedione class of compounds with widely known applications in biomedical and therapeutical fields such as antiprotozoal activity, anti-inflammatory, antimicrobial, cytotoxic and anticancer properties. HPLC fingerprinting of non-volatile compounds of secondary metabolites indicated the presence of unidentified 17 and identified 15 peaks in case of both psychrophilic *P. oxalicum* and mesophilic *P. citrinum*, respectively. The known compounds identified as Quinolactacin C, Abscisic acid, Anofinic acid, Conocenol B, and Aurofusarin in case of mesophilic *P. citrinum*. These compounds are reported to have wide applications in pharmaceutical field (antimicrobial, anticancer, cytotoxic agent) and promoted the plant growth. ^{1}NMR analysis showed the presence of compound 2-Dodecanol in case of *P. oxalicum*, rest compounds are still unidentified.

In the present investigation antibacterial and anticancer properties of EtOAc extract of secondary metabolites of *Penicillium* strains were estimated again bacterial strain *P. aeruginosa* and Human Lung Cancer cell line (A549), respectively. The results of antibacterial activity revealed that *Pseudomonas sp.* was more susceptible to crude extract of secondary metabolite of psychrophilic *P. oxalicum* than mesophilic *P. citrinum*. Preliminary study on the anticancer property of produced secondary metabolites using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay and cell cytotoxicity using flow cytometry analysis on Human Lung Cancer cell line (A549) demonstrated that sec. metabolites of psychrophilic *P. oxalicum* were more efficient against cancer cells than *P. citrinum*. The results on antibiotic susceptibility test showed about 8 fold reduction in the MIC of tetracycline that revealed the secondary metabolites of *Penicillium* strains have equivalent drug resistance reversal potential against bacterial strain tetracycline tolerant *P. aeruginosa*.

Thus, whole study may be concluded as follows-

1. *P. oxalicum* strain was a psychrophilic, acidophile and halophilic in nature.
2. Psychrophilic *P. oxalicum* was a good producer of *cellulase* and *amylase* enzyme than mesophilic *P. citrinum*.
3. At low temperature, content of total lipids, unsaturated fatty acids get declined in case of psychrophilic *P. oxalicum*. However, the content of omega-3 and 9 unsaturated fatty acids rises with rise of temperature in case of mesophilic *P. citrinum*.
4. The psychrophilic *P. oxalicum* was more tolerant to high concentration of Arsenic and Chromium than the mesophilic *P. citrinum*.
5. The environmental stress conditions (temperature, salinity, nutrients and heavy metals) promoted the production of secondary metabolites by both *Penicillium* strains.

6. The psychrophilic *P. oxalicum* showed better antibacterial and anticancer properties than mesophilic *P. citrinum*.
7. Hence, it can be concluded that psychrophilic *P. oxalicum* has inimitable characteristics and able to produce temperature stress dependent various unidentified/novel secondary metabolites with potential applications in pharmaceutical and therapeutical fields.

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Research Article **OMICS International**

Production of Secondary Metabolites from Two *Penicillium* Strains Adapted to Different Temperature Conditions: A Study on Differential Response of Fungal Strains to Temperature Stress

Jyoti S and Singh DP*

Department of Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, India

Abstract

In the present investigation, temperature dependent production of secondary metabolites of two *Penicillium* strains i.e., cold tolerant *Penicillium oxalicum* originally isolated from a low temperature environment of Leh (Ladakh), India and the other one is mesophilic *Penicillium citrinum* (KR150257) isolated from Lucknow (Uttar Pradesh), India. The psychrotolerant *P. oxalicum* can grow at low temperature (4°C) and shows optimum growth at 15°C, while the mesophilic *P. citrinum* exhibits optimum growth temperature at 35°C. The study of secondary metabolites produced by both *Penicillium* strains, studied by UV-Visible Spectroscopy, GC-MS, confirmed the presence of alkaloids, mycotoxins, antibiotics, hydrocarbons and fatty acids. The maximum production of alkaloids by cold tolerant *Penicillium oxalicum* is detected under temperature stress (35°C). On the other hand, mesophilic *Penicillium citrinum* produced maximum alkaloids with different absorption characteristics at 35°C. The GC-MS analysis of secondary metabolites revealed the presence of number of unique biochemical compounds in both the *P. oxalicum* and *P. citrinum* strains grown under temperature stress conditions (35°C and 4°C, respectively). The common biochemical in the secondary metabolites produced by both the *Penicillium* strains grown under temperature stress condition are 3-dodecene, 2-dodecanol and 1-hexadecanol, eicosane, dibutyl, phthalate, 9-hexacosene, propanoic acid, 2-(aminooxy). The three-unique biochemical produced by *P. oxalicum* grown at low temperature (4°C) are 4(1H) Quinazolinone, 1,4,8-Metheno-1H-cyclopent [f] azulene, 3a, 4, 4a, 7, 7a, 8, 9, 9a-octahydro and 6-Quinazolinol. The five-unique biochemical produced by *P. oxalicum* at high temperature (35°C) are 2-Methyl-2-propylmethylphosphonofluoridate, Pyridine, 2[(1,1dimethylethyl) thio, 4(1H) Pyrimidinone,6-amino-2-methyl-5-nitroso, 4(3H) Quinolinone and Phthalic acid, di(2-propylpentyl). The seven unique biochemical produced by *P. citrinum* at low temperature (4°C) are Cyclohexanone, 4-ethyl-4-methyl-3-(1-methylethyl)-,trans-, 3-Methyl-1,4diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl, Glycyl-L-proline, Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, 2,2-Dimethyl-propyl 2,2-dimethyl-propanesulfinyl sulfone, 11,14-Eicosadienoic acid, methyl ester. The unique derivative of β-lactam antibiotic produced by the *P. citrinum* at 35°C is 2,4-Azetidinedione,3,3-diethyl-1-methyl.

Keywords: Penicillium; Secondary metabolites; GC-MS; Temperature stress; Alkaloids

Introduction

Psychrotolerant microorganisms are mostly present in the extremely cold environment [1,2] but exhibit slower growth rates, as they automatically encounter number of growth limiting conditions such as reduced efficiency of nutrient uptake, membrane disorders and decrease in the enzyme activity [3]. The psychrotolerants survive at low temperature due to their better nutritional adaptability [4] and have unique cold shock and cold acclimation proteins and enzymes [5]. Fungi often provide plentiful and diverse bioactive metabolites which are medicinally important such as Penicillin, Lovastatin [6], fingolimod [7] and caspofungin [8]. The search for new and bioactive secondary metabolites is still going on, particularly from the extremophilic microorganisms. The psychrotolerant fungi can be a good tool to explore the new bioactive metabolites of pharmaceutical importance due to uniqueness of their habitat and changes in the metabolic systems, amenable for their adaptation to extreme cold environmental conditions.

Penicillium generally, is a genus of ascomycetous fungi, known for its growth and survival as mesophiles or thermophiles, but rarely in a very low temperature conditions. *Penicillium* have great major economic importance in the field of agriculture and pharmaceuticals and many of its species known to produce a highly diversified spectrum of bioactive secondary metabolites, including antibacterial [9,10], antifungal substances [11], immune suppressants, cholesterol-lowering agents [12], and potent mycotoxins [13,14]. Worldwide, *Penicillium* is also known to produce secondary metabolites such as ergot alkaloids, diketopiperazines, quinolines, quinazolines, polyketides [15], camazulene and azetidine [16]. *Penicillium* is also known for production of essential fatty acids and hydrocarbons and their therapeutical applications [17] by combating a number of human diseases [18]. Production of these biomolecules from *Penicillium* strains is intensely being examined, particularly from the strains of unexplored habitats. Earlier studies have revealed that the temperature is one of the most important factors which affects the growth and survival of these microorganisms [19]. In the present study, there is successfully isolation of cold tolerant *Penicillium oxalicum* from the cold deserts Leh, Ladakh (J&K), India has been done and efforts have been made to screen its secondary metabolite production under temperature stress and that was compared with mesophilic strain of *Penicillium citrinum* isolated from sub-tropical region i.e., Lucknow (Uttar Pradesh), India. Efforts were

***Corresponding author:** Singh DP, Department of Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow-226025, India, Tel: +91-9415575735; E-mail: dpsingh_lko@yahoo.com

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Morphophysiological variations in two *Penicillium* strains isolated from different climatic zones

Jyoti and D. P. Singh[*]

Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.)-226025, INDIA

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ABSTRACT

The present investigation is a comparative study of the morphological and physiological characteristics of two *Penicillium* strains isolated from different climatic regions. A psychrophilic strain *Penicillium oxalicum* isolated from Leh (Ladakh) - a cold desert in J & K (India) was able to grow upto 4°C and other one was a mesophilic *Penicillium citrinum*, isolated from Lucknow (U.P.), India, was able to grow upto 35°C. The Fungal Taxonomical classification of both the strains was primarily based on the morphology of hyphae, spores, and spore-bearing (conidial) structures of isolates. The **ITS region of 18s rDNA** was successfully amplified using universal primers ITS4 & ITS5 for molecular identification fungal isolates. The Psychrophilic strain was identified as *Penicillium oxalicum* (accession no. KR150256) and mesophilic strain as *Penicillium citrinum* (accession no. KR150257). Physiological studies pertaining to preference of growth temperature and nutritional (C, N) conditions on the growth of both the *Penicillium* strains was studied to understand their physiology response. The study revealed interesting results regarding the growth and reproductive behaviour of both *Penicillium* strains adapted to different climatic zones. The temperature range of 4-25°C was found to be optimum range for growth of Psychrophilic *Penicillium oxalicum*. However, maximum growth of the psychrophilic strain was achieved at 15°C at acidic pH 4.0. The mycelial growth of mesophilic *P. citrinum* occurred between 15-35°C at acidic pH 5.0; but its optimum growth was obtained between 25-30°C. The best carbon source for the growth of *P. oxalicum* was glucose, followed by sucrose. On the other hand, the best carbon source for the growth of *P. citrinum* was found to be sucrose, followed by glucose. The best nitrogen source for growth of *P. oxalicum* was found to be sodium nitrate, followed by organic nitrogen glycine, and L-tryptophan. On the contrary, *P. citrinum* could grow well in the presence of both glycine and L-tryptophan. Thus, an opposite morphophysiological characteristics of both the fungal strains could be associated with their adaptation to their respective climatic conditions and might be helpful in their taxonomic classification.

1) INTRODUCTION

Microorganisms that live in the extreme environment usually modify their physiology to ensure their survival and growth under harsh conditions. Psychrophilic fungi are known to survive at extremely low temperature and are commonly found in polar and non-polar habitats [1]. Therefore, optimal temperature for the growth of psychrophiles is $\leq$ 15°C, maximum temperature is $\leq$ 20°C [2]. The Leh, Ladakh in J & K has all the characteristics of a cold desert and is accredited with very low temperature, high Ultra-Violet-B radiation, low water & nutrient availability, and repeated freeze and thaw cycles. Most of the psychrophilic fungi are acclimated to such harsh conditions by adapting various morphological and physiological strategies. Sometimes low temperature condition influence fungal cells by increasing their water viscosity, denaturation of proteins, slowing down of their enzymatic reactions, and membrane stability [3, 4]. Psychrophilic metabolic changes in psychrotrophic fungi make them more valuable in biotechnological and pharmaceutical fields due to diverse metabolic and morphological characteristics evolved during adaptation to such extreme environments. Such extremophiles are exploited for production of cold-active enzymes, bioactive metabolites and exo-polysaccharides, potential application for biofertilizer and bioremediation [1].

Microfungi of the genus *Penicillium* are one of the most promising sources of physiologically active compounds such as alkaloids, antibiotics, hormones, mycotoxins etc. *Penicillium* (a mold) is a very large and omnipresent genus which currently contains approx. 354 accepted species [5]. *Penicillium* spp. are typically fast growing, mostly in green coloured shades, often white, tailing of a dense felt of conidiophores and are recognized by their dense brush-like spore bearing structures. Spore bearing systems in most *Penicillium* spp. are biverticillate, sparsely monoverticillate or terverticillate [6]. Some fungi are tolerant to the external extreme environmental factors such as dryness and low or high temperature [7]. *Penicillium* spp. is one of the fungi that has the ability to survive in the extreme environmental conditions [8, 9]. According to Brandt and Warnock [10], the taxonomical classification of fungi is mainly centred on morphology of hyphae, spores, and spore-bearing (conidial)

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* Corresponding Author: **Dr. D.P. Singh**
Email address: dpsingh_lko@yahoo.com

PUBLICATIONS

RESEARCH PAPERS

1. Jyoti and Singh, D. P. (2016). Production of Secondary Metabolites from Two *Penicillium* Strains Adapted to Different Temperature Conditions: A Study on Differential Response of Fungal Strains to Temperature Stress. Cell. And Mol. Biology, 62-3.

2. Jyoti and Singh, D. P. (2017). Morphophysiological variations in two *Penicillium* strains isolated from different climatic zones. G- J. Environ. Sc. And Tech., 5(1): 125-130.

CHAPTER

3. Jyoti and Singh, D. P. (2016). "Fungi as Biocontrol Agents in Sustainable Agriculture". In: Microbes and Environmental Management, (eds.) Singh, J. S. and Singh D.P. Studium Press (India) Pvt. Ltd.

CONFERENCES

4. Presented a paper entitled "Biodetoxification of Cr (VI) and Membrane surface binding by the Halotolerant fungal strain *Penicillium oxalicum*", Jyoti, Prem Chandra, and D. P. Singh, in International Conference on Environmental Technology and Sustainable Development: Challenges & Remedies, organized by Deptt. of Environmental Science, B. B. Ambedkar University, Lucknow (U.P.), during 21-23rd Feb., 2014.

5. Attended a workshop on Mainstreaming Climate Change Adaptation & Disaster Risk Reduction, organized by organized by Deptt. of Environmental Science, B. B. Ambedkar University, Lucknow (U.P.), on 7th March 2014.

6. Participated a National Seminar on Current Trends In Biological Sciences: Advances and Challenges, organized by Deptt. of Zoology, Janta College, Bakewar, Etawah (U.P.), during 13-14th Dec., 2014.

7. Prsented a paper entitled "Temperature dependent membrane alterations and lipid profile of two *Penicillium* strains", Jyoti, Ankita Asati and D. P. Singh, held at 56th Annual Conference of Association of Microbiologists of India (AMI-2015) & International Symposium on "Emerging Discoveries in Microbiology", held during 7-10th Dec., 2015.

8. Participated a National Conference "BIOKUMBH-2016" on Recent Trends and Advances in Biotechnology, organized by Centre of Biotechnology, University of Allahabad, Allahabad (U.P.), during 20-21st Feb., 2016.

9. Participated an International Conference on Strategies for Environmental Protection and Management (ICSEPM-2016) mini symposium on Environmental Biotechnology, Biorefinery and Solid Waste Management (BRSI), organized by Jawaharlal Nehru University, New Delhi, during 11-13 Dec., 2016, with a paper entitled "Metal tolerance by Psychrotolerant *Penicillium oxalicum* fungal strain isolated from low temperature environment, Leh Ladakh, India. Jyoti and D. P. Singh.

10. Presented a paper entitled "Arsenic stress mediated alterations in the growth and Secondary Metabolites production by two *Penicillium* strains", Jyoti and D. P. Singh. in 58th Annual Conference of Association of Microbiologists of India (AMI-2017) and International Symposium on Microbes for Sustainable Development: Scope & Applications (MSDSA-2017) organized by B. B. Ambedkar University, Lucknow (U.P.), during 16-19th Nov., 2017.

11. Presented a paper entitled "Evaluation of growth and secondary metabolite production by two *Penicillium* strains under heavy metal stress conditions", Jyoti and D. P. Singh, in National Symposium on Biodiversity and Natural Resources For Sustainable Develoment & 37th Annual Session of Academy of Environmental Biology, Lucknow , organized by Deptt. of Zoology, C.C. S. University, Meerut (U.P.), during 24-26th Nov., 2017.

OTHERS

12. Attended a national Seminar on "Application of G.I.S. for Resource Mapping and Planning" sponsored by U.G.C. and organized by Bareilly College Bareilly (U.P.) during 27-28 Nov., 2010.

13. Presented a paper entitled "Electronic waste- An Ecological Menace" in National seminar on Global Environmental Issues: Problems & Prospects, sponsored by U.G.C and organized by Deptt. of Geography, National Post Graduate College, Lucknow, during 19-20th Feb., 2011.

14. Presented a paper entitled "Role of Soil chemistry with reference to Arsenic contamination" in 1st Lucknow Science Congress (LUSCON-2013) organized by B. B. Ambedkar University, Lucknow (U.P.), during 20-21st March, 2013.

15. Participated a seminar on Environment, Education & Society organized by Deptt. of Environmental Science, B. B. Ambedkar University, Lucknow (U.P.).

16. Presented a paper "Application of Plant growth promoting microbes to boon agriculture in India", Jyoti, P. K. Srivasatava, N. Singh and D. P. Singh, in National Conference on Women power in Cutting Edge Biotechnology, organized by Amity University, Lucknow, during 17-18 October, 2013.

17. Presented a paper “Fungal mediated degradation of Polycyclic Aromatic Hydrocarbons”, Jyoti and D. P. Singh, 2nd Lucknow Science Congress (LUSCON-2013) organized by B. B. Ambedkar University, Lucknow (U.P.).

Thank you

“End is not the end, if fact E.N.D. means “Effort Never Dies”

- Dr. A. P. J. Kalam