ASSESSMENT OF *IN VITRO* ANTIMICROBIALS AND ENZYME INHIBITORS FROM INDIAN *MUSCODOR* SPECIES

Dissertation

Submitted in the partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

In

MICROBIOLOGY

Submitted By

JASPREET KAUR BOPARAI

(Reg. No. 301205003)

Under the supervision of

Dr. SANJAI SAXENA

Associate Professor

Department of Biotechnology

Thapar University, Patiala-147004, Punjab

JULY 2014

CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled "Assessment of *in vitro* antimicrobials and enzyme inhibitors from *Muscodor* species" in the partial fulfillment of requirements for the award of degree of Master of Science in Microbiology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period from Jaunary 2014 to June 2014, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Jaspreet Boparai

Patiala

Date: 17 July 2014

Jaspreet Kaur Boparai

(Roll No. 301205003)

This is to certify that the above statement made by candidate is correct and true to the best of my knowledge.

Sanjai Saxena

Dr. Sanjai Saxena

Associate Professor/Supervisor

Dr. Dinesh Goyal

Head of the Department

Department of Biotechnology

Thapar University, Patiala, 147004

CERTIFICATE

This is to certify that the thesis entitled "Assessment of *in vitro* antimicrobials and enzyme inhibitors from *Muscodor* species" being submitted by Ms. Jaspreet Kaur Boparai (Roll no. 301205003) in the partial fulfillment for the requirements for the award of degree of Master of Science in Microbiology, Thapar University, Patiala is a bonafide work carried out under the supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

Dr. Sanjai Saxena
Associate Professor,
Department of Biotechnology
Thapar University

Dr. Dinesh Goyal
Professor & Head
Dept. of Biotechnology
Thapar University

Dr. S.K. Mohapatra
Dean, Academic Affairs
Thapar University, Patiala, Punjab

ACKNOWLEDGEMENT

I owe my profound gratitude and sincere regards to my supervisor Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology, Thapar University, who have been a source of inspiration and guiding light for me throughout my dissertation. His careful corrections during regular meetings and discussions always ensured a more thoughtful approach towards problem solving. I am very grateful to him for teaching me things that I would have never found in any textbooks.

I thank Prof Dr. Dinesh Goyal, Head of the Department, for providing the necessary infrastructure and facilities to carry out my work.

I would like to thank all my lab research scholars Mr. Vineet Meshram, Ms. Neha Kapoor and Ms. Mahiti Gupta for their constant support and guidance and their valuable suggestions during my course work.

I would like to thank my classmates with whom I share numerous memorable moments which made my M.Sc wonderful and really enjoyable. My heartfelt thanks to Shilpi, Preet, Kajal, Manmeet, Ambuj, Pallak, Tanya without whom I could not have imagined life at Thapar University. I would also like to thank my best friends Paramdeep sohi and Jason for their constant support and encouragement, who never failed to give me a boost, and made my life better in every possible way.

My parents and my brother deserve special mention for their inseparable support, love and care. I cannot thank them enough for being the source of strength and inspiration all these years.

Lastly, I thank, almighty for giving me the patience and perservence to move in the right direction.

Date: 17 July 2014 **Jaspreet Kaur Boparai**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

ABBREVIATIONS

Abbreviation	Full form
AWD	Agar Well Diffusion
CDB	Czapek Dox Broth
CZD	Czapek Dox Media
CMC	Carboxymethylcellulose
DMSO	Dimethylsulfoxide
DW	Distill water
G	Grams
Hrs	Hours
IC_{50}	50% Maximal Inhibitory Concentration
M	Molar
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
Min	Minutes
Ml	Milliliters
Mm	Millimeters
MW	Molecular Weight
Nm	Nanometers

OD	Optical Density
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SD	Standard Deviation
TTC	Triphenyl Tetrazolium Chloride
µg	Micrograms
Ml	Microlitre

EXECUTIVE SUMMARY

The current study explores endophytic Indian *Muscodor* isolates for their antimicrobial, enzyme production and α-amylase inhibitory potential. Initially the eight *Muscodor* sp. were subjected for production of cell free culture broths via shake flask method. These culture filtrates were further tested for their antimicrobial potential against a battery of gram positive and gram negative bacteria. Out of all the eight screened *Muscodor* isolates, ethyl acetate fraction of #130 TMDSTYEL exhibited maximum antimicrobial activity whose zone of inhibition ranges from 8-17 mm in agar well diffusion assay followed by #6(B) CCSTITD whose zone diameter ranges from 9-14 mm. Minimal Inhibitory Concentration (MIC) of #130 TMDSTYEL ranges from 62.5-250 μg/ml where as its IC_{50} was found to be ranging between 0.125-2 mg/ml against the tested spectrum of microorganisms. These *Muscodor* isolates were then screened for production of industrially important enzymes like cellulases and amylases via plate assays. #130 TMDSYEL and #6(B) CCSTITD were found to produce α-amylase whereas #1 CCSTITD, #6610 CZSTITD and #2 CCSTITD produced cellulases respectively. The *Muscodor* isolates were also screened for their α-amylase inhibitory potential where #6(B) CCSTITD, #130 TMDSYEL and #16AMLWLS induces inhibition of α-amylase enzyme by 99.19%, 98.59% and 73.46% respectively. The current study is the first attempt to harness the secondary metabolites (apart from volatile organic compounds) produced by *Muscodor* species for biological activities like antimicrobial and enzyme inhibitors. Thus from the above obtained results it can be concluded that Indian *Muscodor* species are prolific producers of biologically active molecules which can be further exploited in pharmaceutical and agro industries.

1.0 Introduction

Emergence of resistance towards antimicrobial agents by the pathogenic microorganisms is becoming a global health problem (Roberta *et al.,* 2013). Infections caused by drug resistant microrganisms results into cost intensive treatments, morbidity and mortality. Both gram negative and gram positive bacteria are getting refractory to the current armamentarium of antimicrobial drugs. *S.aureus* is a gram positive bacterium which is considered as the third most dreaded pathogen which is responsible for an array of chronic infection due to its refractory behaviour as a result of genomic plasticity (Saxena and Gomber, 2010). *Pseudomonas aeruginosa* is another problematic nosocomial pathogen which increases the rate of mortality and morbidity than other bacterial species (Hilmar *et al.,* 2003). The emergence of antimicrobial drug resistance (ADR) is being attributed to a series of societal, technological, environmental and microbial changes. These include increasing populations of susceptible hosts, microbial adaptation and indiscriminate use of antibiotics in therapeutic as well as non therapeutic settings (Kapil, 2005). Although the effect of ADR are documented in developed and developing nations alike, there is a greater potential for harm in developing world where many of the second and third line therapies for drug resistance infections are unavailable, and many of the narrow spectrum antimicrobials available in the developed world expensive for a common man (Fasehun, 1999).

To combat with multidrug resistant microorganism, naturally derived compounds have become popular alternative for anti-microbial agents (Tiwari *et al.,* 2009). Antibiotics sometimes possess adverse effect on host including hypersensitivity, immunosuppressant and allergic reactions wheareas antimicrobials of natural origin treat

infections while simultaneously checking many of the side effects that are associated with the synthetic antimicrobials. Due to the pharmaceutical potential of fungi, secondary metabolites of fungi have been studied for more than 70 years (Rodriguez, 2009). The search for new antimicrobial drugs from fungi started with the discovery of Penicillin, a potent antibiotic against Gram-positive bacteria produced by *Penicillium notatum.* Another milestone in the history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine, which is produced by *Tolypocladium inflatum and Cylindrocarpon lucidum*. It was first discovered as an antifungal metabolite and later found to be immunosuppressive which made cyclosporine useful for the treatment following organ transplantation (Dreyfuss and Chapela, 1994).

Diabetes mellitus is a metabolic disorder characterized by increased blood glucose levels resulting from lack of insulin secretion. It's a global threat and the numbers of cases are expected to rise by 300 million by 2025. India is expected to have large numbers of diabetic cases. The most commonly practised therapies for diabetes include stimulation of endogenous insulin. secretion, enhancement of the action of insulin at the target tissues, oral hypoglycemic agents, such as biguanids and sulfonylureas and inhibition of degradation of dietary starch by glycosidases such as a-amylase and α-glucosidase. Pancreatic α-amylase is a primary enzyme involved in hydrolysis of starch into smaller oligosaccharides comprising of maltose, maltotriose and several oligoglucans. They are further hydrolysed by α-glucosidases and degraded to glucose which on absorption enters the blood-stream. Degradation of dietary starch leads to elevated post-prandial hyperglycemia. The increased level post-prandial glucose correlates to human pancreatic α-amylase in small intestine hence it is an important target

for treatment of type 2-diabetes. Hence, slowing the starch digestion by inhibition of alpha amylase plays a key role in control of diabetes. The current arsenal of inhibitors engaged in clinical use are acarbose and miglitol which act upon glycosidases such as α-glucosidase and α-amylase while others such as and voglibose inhibit α-glucosidase. These drugs are synthetic in their origin and suffer imitations like non specificity, fail to remove other diabetic complications. The main side effects of these inhibitors are gastrointestinal viz., bloating, abdominal discomfort, diarrhea and flatulenc hence herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents (Romila *et al.,* 2010).

Endophytes are micro-organisms residing in a unique and specialized biological niche in the intracellular space between the cells of higher plants without causing any overt symptoms onto the plant they are residing in (Bacon & White, 2002). Endophytes are most abundant in tropical rainforests. Novel endophytes usually have associated with them many novel secondary natural products and various new processes (Strobel, 2006). The list of natural products obtained from endophytes is quite diverse and has potential in pharmaceutical and agrochemical arenas- a) microbial products act as antibiotics (Example-Cryptocandin A- antifungal peptide obtained from *Cryptosporiopsis quercina*), b) endophytic fungal products possess potential as anticancer agents (Paclitaxel, world's first billion-dollar anticancer drug is produced by many endophytic fungus), c) Endophytes are reported to produce products that can be used as antioxidants, anti-diabetic agents, immunosuppressive compounds and a few with insecticidal properties (Nigam *et al.,* 1995).

Muscodor is a sterile whitish endophytic fungi possessing ropy or coiled hyphae and right angle branching. It is non-pathogenic fungus belonging to the Xylariaceae family. The possess the remarkable potential to produce blend of volatile organic compounds, including alcohols, esters, ketones, lipids and acids, which can kill pathogens like molds, bacteria such as *Listeria, Salmonella*. *M. vitigenus* produces napthelene which induces nematicidal activity against certain insects. Potential applications of *M. albus* are currently being investigated and include uses for treating various plant parts and human wastes. Another promising option includes controlling soil borne plant diseases. Volatiles produced by *Muscodor* are non toxic to human and animals. VOCs produced by *Muscodor* sp. also kills some soil borne pathogens, such as *Rhizoctonia solani, Pythium ultimum, Verticillium dahliae, Fusarium aveneaceum, Sclerotinia minor* etc (Mercier *et al.,* 2009). Volatiles produced by Muscodor species also kills many post harvest pathogens such as *Botrytis cinerea, Geotrichum candidum, Monilinia fructicola* and *Penicillium digitatum.* Thus *Muscodor* species holds emormous potential of myco/ biofumigation.

Vast emphasis has been given to harness the volatile organic compounds for mycofumigation potential whereas the other secreted metabolites produced by the same *Muscodor* species have not been exploited at all. There exist only a single report where the organic residues of *Muscodor yucantansis* (Gonzalez *et al.,* 2009) been exploited for biological activities such as antimicrobial activity. Thus the current study is focused on exploring the lead molecules (except volatile organic compounds) produced by endophytic Indian *Muscodor* isolates for their antimicrobials and enzyme inhibition

potential. This is a nascent filed with very scanty work and humongous opportunities taking in account the volatile activites reported by previous *Muscodor* species.

2.0 Review of literature

2.1 Discovery of *Muscodor*

Muscodor albus is an extraordinary endophytic fungus that is renowned for producing volatile antimicrobial. *M albus* was for the first time isolated as an endophytic fungus from the stems of *Cinnamomum zeylanicum* by Prof. Gary Strobel, Emeritus Professor, Department of Plant Sciences and Plant Pathology, Montana State University, USA during his forest forays in Honduras, Central America in 2001. There is an interesting story related to the discovery of *M. albus*. Petri-plates containing plant tissues were placed in large plastic boxes with firmly fitting lids to eliminate the invasion of mites and other fastidious microbes. Endophytic fungal growth was observed in most plant parts after few days of incubation, plates were removed and the emerging fungus were transferred as individually from the hyphal tips onto fresh Potato Dextrose Agar (PDA) plates, after two days incubation only one of the transferred endophytes was observed to grow. Obvious answer to the question of limited oxygen supply in the plastic box on the contrary was the production of volatile antibiotics (volatile organic compounds- VOC's) by the endophytic fungus that remained alive (designated as isolate 620), it was these VOC's that killed or inhibited the growth of other endophytes, thus out came the hypothesis, that an endophyte can produce or generate volatile antibiotic substances with a wide range of biological activities (Strobel & Daisy, 2003).

2.2 Muscodor- endophyte with biological promise

Muscodor is a genus of endophytic fungus that are known from certain tropical tree and vine species of Central/South America & South-Eastern Asia & Australia (Atmosukarto *et al*., 2005, Daisy *et al*., 2002b, Erza *et al*., 2004, Gonzalez *et al*., 2009, Mitchell *et al*.,

2008, Sopalun *et al*., 2003, Strobel *et al*., 2007, Worapong *et al*., 2001, 2002, Meshram *et al*., 2013, Saxena *et al* 2014 a and b). *Muscodor sp*. characteristically produce a mixture of volatile organic compounds (VOC's) that consist primarily of various alcohols, esters, acids, ketones, and lipophilic substances which are lethal to a wide variety of plant & human pathogenic fungi, and bacteria as well as to nematodes and certain insects and act synergistically (Daisy *et al*., 2002a & Strobel *et al*., 2001). *Muscodor sp*. are therefore of high value and promise for bio-control (Strobel 2006) and the discovery of new isolates and taxa of this genus is of major interest to mycologists. Till date fifteen species have been isolated using from different corners of the world. (Table No 2.1)

Name of species	**Host Plant**	**Reference**
Muscodor albus	*Cinnamomum zeylanicum*	Worapong *et al.* ,2001
Muscodor roseus	*Grivillea pteridofolia*	Worapong *et al.* ,2002
Muscodor vitigenus	*Paullinia paullinoides*	Daisy *et al.*, 2002
Muscodor crispans	*Ananas ananassoides*	Mitchell *et al.*, 2008
Muscodor yucatenensis	*Bursera simaruba*	Gonzalez *et al*., 2009
Muscodor fengyangensis	*Actinidia chinensis*	Zhang *et al*., 2010
Muscodor sutura	*Prestonia trifidi*	Kudalkar *et* al., 2011
Muscodor cinnamomi	*Cinnamomum bejolghota*	Suwannarach *et al*., 2010
Muscodor oryzae	*Oryza rufipogon*	Suwannarach *et al*., 2012
Muscodor suthepensis	*Cinnamomum bejolghota*	Suwannarach *et al.*, 2012

Muscodor musae	*Musa acuminate*	Suwannarach *et al*., 2012
Muscodor equiseti	*Equisetum debile*	Suwannarach *et al*., 2012
Muscodor kashayum	*Aegle marmelos*	Meshram *et al.*, 2013
Muscodor tigerii	*Cinnamomum camphora*	Saxena *et al.*, 2014
Muscodor darjeelingensis	*Cinnamomum camphora*	Saxena *et al.*, 2014

Table No 2.1 Showing *Muscodor sp.* reported till date

These have been described on the basis of morphological, physiological, biological and genetic feature (Kudalkar *et al*., 2012, Suwannarach *et al*., 2012, Meshram *et al.*, 2013). These fungi are classified in the family xylariaceae and have unique molecular identity as compared to other genera in the family (Ezra *et al*., 2004, Zhang *et al*., 2010). These are sterile mycelia, non-sporulating, slow-growing, flat, undulating, hyaline, septate mycelia possessing characteristic hyphal coiling, ropyness, right-angle branching.

Mycofumigation is the term applied to practical aspects of *Muscodor* sp. (Strobel *et al*., 2001). The first practical demonstration of its effects against a pathogen was the myco-fumigation of covered smut infected barley seeds for a few days. The seeds were eventually planted and the resulting plants, in contrast to the untreated control group, produced no infected heads (Strobel *et al*., 2001). Mycofumigation is also important for the treatment of fruits in storage and transit (Mercier and Jimenez 2004). Soil treatments have also been effectively used in both field and greenhouse situations (Mercier and Manker 2005; Stinson *et al*., 2003; Jacobsen *et al*., 2004). In these cases, soils are pre-treated with a *M. albus* formulation in order to preclude the development of infected

seedlings. *M. albus* is now being produced, by solid state fermentation, by the ton in order for its use in many practical applications. Besides the above mentioned uses of *Muscodor sp.*, the genus has been exploited for many other purposes which are briefly mentioned below-

The fact that volatiles from *M. albus* kills most storage pathogens exposed *in vitro* opens up new possibilities to develop bio-fumigation as a post-harvest treatment for a range of commodities (Mercier *et al*., 2007). Bio-fumigation with *M. albus* controlled blue mould & gray mould of apple, brown rot of peaches (Mercier *et al*., 2004), green mould & sour rot of lemons (Mercier *et al*., 2005), gray mould of grapes (Mlikota-Gabler *et al.*, 2006), bacterial soft rot (Corcuff *et al.*, 2006). The potential of *Muscodor albus* as a microbial control agent of potato tuber moth in stored potatoes was also explored (Lacey *et al.*, 2006), efficacy of *Muscodor albus* for control of phytophthora blight of sweet pepper & butternut squash was examined in greenhouse study (Camp *et al*., 2008), pupal mortality & adult emergence of western cherry fruit fly exposed to *M. albus* has also been evaluated (Yee *et al*., 2009), seedling diseases on sugar beet & root-knot nematode on tomato have been controlled during in vitro experiments using *M. albus* (Grimme *et al*., 2007), Mycofumigation has been demonstrated as a new alternative to methyl bromide fumigation for control of soil-borne pathogens, many more applications of this genus are being explored each passing day.

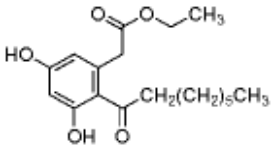
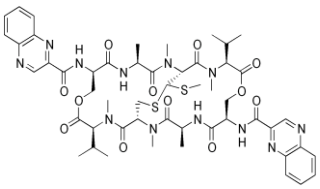
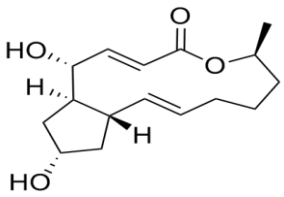
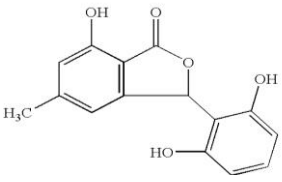
2.3 Endophytic Fungi: Nature hidden treasure

Endophytes comprise of an extremely diverse group of microorganisms that are ubiquitous in plants and maintain a symptomless and unobtrusive union with their hosts for at least a period of their life cycle (Stone *et al.*, 2000; Kusari *et al*., 2012). Endophytes

play an important role in plant symbiosis, rescuing their host from microbial infiltration and stressful conditions (Shipunov *et al*., 2008). The genetic recombination of the endophytes with the host plant enables them to mimic the biological properties of their host and produce analogous bioactive metabolites (Zhao *et al*., 2011; Kusari *et al*., 2012). Thus, endophytic microorganisms are considered as a lucrative source of bioactive metabolites with promising applications in the agrochemical and pharmaceutical industries (Strobel and Daisy 2003; Kudalkar *et al*., 2012).

A wide range of compounds produced by endophytes have been shown to combat pathogens and even cancers. *Pestalotiopsis microspora* an endophytic fungus of *Taxus wallachiana* produces billion dollar anticancer drug Paclitaxal similar to its host which is effective in breast and prostate cancer therapy (Gordon *et al.,* 2005). Fungal endophytes like *Phomopsis* spp., *Fusarium* spp. (Firakova1 *et. al*, 2007), *Pestalotiopsis microspora* (Strobel, 2003), *Gliocladium* spp. (Strobel *et al*., 2008), *Cryptosporiopsis quercina* etc are serving as prolific sources of anticancer, antifungal and antimicrobials (Strobel, 2003). *Streptomyces guanacastepene*, an endophyte from branch of *Daphnosis americana* produces a novel diterpenoid, Munumbicins possessing activity against methicillin-resistant *Staphylococus aureus* and Vancomycin resistant *Enterococcus faecium*. Chaetominine, an alkaloid produced by *Chaetomium sp*. an endophytic fungus from *Adenophora axiliflora* shows cytotoxicity against the human leukemia K562 and colon cancer SW1116 cell lines higher than the drug 5-fluorouracil (Jiao *et al*., 2006) (Table 2.2). Endophytic fungi such as *Gliocladium rosenum, Hypoxylon* sp and *Nodulisporum* sp. produces of mycodiseal. Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the

need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price (Strobel *et al.*, 2003).

S.No.	Secondary Metabolite	Endophytic Fungi	Host Plant	Activiy
1	 **CYTOSPORONE**	*Cytospora sp.*	Rain forest Of Costa Rica	Antibacterial
2	 **KAKADUMYCIN**	*Streptomyces NRRl 30566*	*Grevillea Pteridifolia*	Antimicrobial against gram negative bacteria
3	 **BREFELDINE A**	*Phoma medicaginis*	*Medicago sativa*	Antibacterial
4	 **ISOPESTACIN**	*Pestalotiopsis Microspora*	*Terminalia Morobensis*	Antimicrobial and antioxidant effect

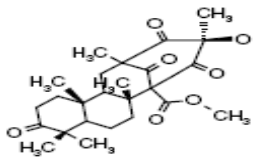
5	 **PREAUSTINOID**	*Penicillium sp.*	*Melia azedarach*	Bacteriostatic effect on *S. aureus, P.aeruginosa, E. Coli*

Table 2.2: Bioactive compounds produced by endophytic fungi

2.4Antimicrobial drug resistance and need for new antimicrobial agents

The evolution of antimicrobial resistant bacteria species stems from a multitude of factors that includes the non-therapeutic use of antimicrobials, the extensive use of these agents as growth enhancers in animal feed (Tomasz, 1994; Cohen, 1992). The emergence of resistance to multiple antibiotics has been reported in pathogens like *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis.*

The past few decades have seen an alarming increase in the prevalence of resistant antimicrobial pathogens in serious infections. In the USA, for instance, 50–60% of >2 million nosocomial infections are caused by antibiotic-resistant pathogens. The prevalence of antibiotic-resistant pathogens isolated from the intensive care unit setting may be significantly higher than in other hospital wards. Infections caused by resistant pathogens represent an important source of morbidity, mortality and increased costs. The possible mechanisms of drug resistance are:

- Changes in membrane permeases (Kurtz, 1998),
- Change in cellular efflux mechanism.

- Changes to a particular fungal "activase" whose action is required before that agent becomes metabolically active (Kurtz, 1998) and
- Mutations that render the target enzyme less sensitive or insensitive to the antibacterial agent.

S. aureus is conceivably the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life threatening infections and its capacity to adapt to different environmental conditions (Lowy, 1998; Waldvogel, 2000). Mechanism of resistance in bacteria occurs due to horizontal transfer of gene (table 1- Lyon and Skurray, 1987). The mortality of *S. aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials (Mylotte *et. al.*, 1987). As rapidly as new antibiotics are introduced, staphylococci have developed efficient mechanisms to neutralize them, exhibiting par excellent darwinism. 70 to 80% of *Staphylococus aureus* isolates are resistant to penicillin. Methicillin and other semi synthetic penicillins were successful in treating penicillin-resistant *Staphylococus aureus* infections until the 1980s, when methicillin resistant *Staphylococus aureus* became endemic in many hospitals. Increasing numbers of *Staphylococus aureus* infections are caused by methicillin- resistant strains (MRSA), including many infections acquired in the community. None of the β-lactam antibiotics (including methicillin derivatives and cephalosporins) are effective in these infections (Franklin, 2003). Vancomycin was once reliable against all gram-positive organisms, including MRSA and *enterococci*, increasing numbers of *Staphylococus aureus* strains now also respond poorly to this antibiotic (Fridkin, 2003). These Vancomycin-intermediate *Staphylococus aureus* strains (VISA) have been found in many countries. 27% population in a healthy community is

the carrier of *Staphylococus aureus* of which 18% are MRSA carrier whereas the prevalence of *Staphylococus aureus* and MRSA in the nasal swabs of health workers is 44.5% and 25% respectively (Saxena *et al.*, 2002). The need for new classes of antibiotic continues to grow as drug resistance erodes the efficacy of current therapies. The antibacterial drugs which appeared to grow almost endlessly in the early decades of antibiotic discovery, has been depleted by the emergence of drug resistant bacterial strains. Ultimately, to conquer drug resistant infections, a new arsenal of antibacterial agents that function by binding to new targets is a necessisity.

2.5 Fermentation

Fermentation is a process very much similar to anaerobic respiration and is carried out in a mixture of nutrients and metabolites essential for the growth and reproduction of the microbe. It is a metabolic process in many microorganisms and involves oxido-reduction reactions resulting in the breakdown of complex organic molecules into various end products with the release of energy. Fermentation is mostly extracellular and is brought about with the help of enzymes released by the microorganisms. The end products or the various intermediate products (primary and secondary metabolites) of the fermentation activities of many microorganisms are highly useful. Hence, microorganisms have been commercially exploited by the fermentation industry. Thus, with reference to industrial microbiology, fermentation may be defined as "a process for the production of useful products through mass culture of micro-organisms." The various intermediate compounds produced during fermentation activity are classified as primary and secondary metabolites. Some of the commercially important metabolites are Secondary metabolites such as antibiotics, toxins, alkaloids, gibberellins, etc. (Samiee *et al*., 2003; Vicente *et al*.,

2001; Rodrigues *et al.,* 2001). Culture broth extracts produced by fermentation of *Guignardia* speceis and *Phomopsis* speceis in Potato Dextose Broth (PDB) which were isolated from leaves of *Aspidosperma tomentosum* and *Spondias mombin,* were evaluated for antimicrobial activity by using agar well diffusion assay (Corrado and Rodrigues, 2004). Spent broth of *Oudemansiella mucida, Oudemansiella radicata* produced by fermentation in PDB also exhibited antimicrobial activity (Anke *et al*., 1990). The qualilty and quantity of metabolites produced also depends on the duration of fermentation. It is difficult to measure the real activities of the mixture even with modern *in vitro* or cell-based assays, as there is lot of interference by other intermediates or byproducts of the fermentation process. The three worst interfering substances are: known compounds or non-specific inhibitors (67%), fatty acids (52%), high molecular substances (48%). In order to decrease or eliminate these interfering substances, extraction with organic solvents is widely applied (85%) whereas others (15%) use fractionated samples.

2.6 *Analytical methods*

2.6.1 Extraction

The precise mode of extraction depends upon the nature of different type of secondary metabolites produced by fungi. Initial screening of different type of fungi for the assessment of bioactivity (prescreen) and purification of the bioactive compounds produced by them is done by using a variety of methods like Soxhlet Extraction, Cold extraction (solvent extraction), Distillation and Acid & Alkaline extraction for obtaining a crude extract (Verma *et al.,* 2007). Distillation is a preferred method for extraction of aromatic oils as well as for solid aromatic samples. Generally solvent extraction (liquid-

liquid extraction) is method of choice since it has an advantage of being carried out at room temperature thereby preventing the loss of heat labile components, which are lost at high temperature.

Liquid - Liquid Extraction is a separation process that takes advantage of the relative solubilities of solutes in immiscible solvents. The solute dissolves more readily and becomes more concentrated in the solvent in which it has a higher solubility. A partial separation occurs when a number of solutes have different relative solubilities in the two solvents used. These are separated on the basis of relative solubility. The distribution coefficient determines the ratio of the concentration of the solute in each solvent. Two immiscible solvents flow in opposite directions. The lighter solvent flows upward while the heavier solvent flows downward. The substance to be separated is in contact with both solvents and is dissolved in each stream according to a ratio determined by the distribution coefficient (Smith *et al.,* 1993). A variety of compounds like the Aureobasidin A, Corynecandin, Arthrichitin, Cispentacin, Lovastatin and Khafrefungin having potential antimicrobial activity have been obtained by solvent extraction (Rosa *et al.,* 2003, Gupte *et al.,* 2002, and Samiee *et al.,* 2003)

2.6.2 Bioassay

Bioassays play an important role in evaluation of a particular bioactivity. A bioassay which is applied to large numbers of initial samples to determine whether or not they have any bioactivity of the desired type is referred to as a prescreen assay. A bioassay used to select materials for detailed individual study is referred to as screen assay. Bioassays are also used to guide fractionation of a crude material towards isolation of the

pure bioactive compounds (monitor), which is referred to as bioassay guided fractionation (Hostettmann *et al.,* 1994; Pieckova *et al.,* 1999)

For these purposes, bioassay tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive. The *in vitro* assessment of antimicrobial susceptibility is done by two methods: Diffusion Assay and Broth Assay.

2.6.2.1 Kirby-Bauer (KB) Disk Assay

Antibiogram is the resistance pattern of a microorganism against a battery of antimicrobial agents. The Kirby Bauer disk assay was used for testing 38 antibacterial drugs against the test microorganisms for profiling their resistance pattern according to Clinical Laboratory Standards Institute (CLSI, USA-formerly National Committee for Clinical Laboratory Standards -NCCLS) guidelines. All the experiments were repeated three times. Based on the diameter of inhibition zone the cultures were classified as sensitive, intermediate or resistant (Mohanty *et al.,* 2010)

2.6.2.2 Pre-Screen Assay- Agar Well Diffusion Assay

The agar diffusion assay was devised by Heatley (1944) as a means of monitoring the extraction and purification of penicillin. Graded doses of solutions of reference standard and 'unknown' were applied to reservoirs [holes cut by a sterile cork-borer] in a layer of agar seeded with an organism sensitive to penicillin. On incubation, a circular clear zone of inhibition surrounded the reservoir in contrast to the turbidity where the organism had multiplied. Heatley showed that the diameter of the circular zone of inhibition was directly proportional to the logarithm of concentration of the penicillin. The zone width was defined as the distance between the edge of the reservoir and the outer edge of the inhibition zone. This technique is popularly referred as Agar Well Diffusion technique,

Radial Diffusion Assay and Cylinder plate Assay (Lehrer, 1991). Agar well diffusion assay is a popular prescreen employed by clinical microbiologists working on the antimicrobial drug development from plant pathogenic fungi (Bonjar, 2004)

2.6.2.3 MIC by microbroth dilution method

Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate, for example when determining the susceptibility of coagulasenegative staphylococci to teicoplanin.

Andrews *et al.,* (2006) observed that the range of antibiotic concentrations used for determining MICs is universally accepted to be in doubling dilution steps up and down from 1 mg/l as required. The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation (this period is extended for organisms such as anaerobes, which require prolonged incubation for growth).

The *in vitro* microbroth dilution assay using 96 well microtitre plate and 3-(4, 5-dimethyl-2-thiazolyl - 5-Diphenyl-2H)-tetrazolium bromide (MTT) assay was performed by Goomber and Saxena (2007) to establish to MIC and MIC_{50} of the alkaloid bioactive fraction. Since visual MIC of each organism varies in the test panel of microorganisms being tested, MIC_{50} is the term used for the minimum MIC which is inhibiting 50% of the microbes in the test panel. The alkaloid bioactive fraction (ABF) was evaluated between

640-10 μg/mL. 50 μl of the bacterial suspension in saline was added to 125 μl of MH broth to achieve a final bacterial cell concentration of 10^5 cells in the test and control wells on the titre plate. Subsequently the plates were incubated for 2.5 hours at 37°C after which 25 μl of the test extract at different concentrations was added. These were then incubated for 24 hours at 37°C. After 24 hours 20 μl of 0.02% MTT was added to each well. The MIC value was taken as the lowest concentration of the ABF where no colour change occurred. The assay was performed in triplicates. Cefixime served as a positive control.

2.7 Enzyme assays

2.7.1 Cellulose degradation

Cellulose is the most abundant biopolymer on earth and holds an important biological role in maintaining the structural rigidity of plant cell walls. The extensive hydrogen bonding network within and between chains means that cellulose is an insoluble, heterogeneous substrate and hence cellulase active enzymes are very different than enzymes that catalyze reactions on soluble substrates. To add to this complexity most fungi contain dozens of enzymes that are active on cellulose and the reason for such redundancy is largely a mystery (Wilson, 2011).

Carboxymethylcellulose (CMC) is soluble cellulose that is an excellent substrate for endocellulases (Zhang *et al.,* 1999). There are several different mechanisms that are used by cellulolytic microorganisms to degrade cellulose, although cellulases are used in all of them. Almost all enzymes that degrade insoluble substrates contain a substrate binding domain, which is usually joined to the catalytic domain by a flexible linker peptide (Shoseyov *et al.,* 2006). Some aerobic fungi like *Trichoderma reesei* degrade cellulose

which is the most common source of commercial cellulase (Martinez *et al.*, 2005). *Acremonium thermophilum, Chaetomium thermophilum, Humicola grisea, Melanocarpus albomyces, Talaromyces emersonii, Thermoascus aurantiacus* produced thermophilic cellulases

2.7.2 Starch degradation

Alpha-amylase has found its application in a range of industries including food, brewing, distilling industry, textile and bioconversion of solid waste etc. Amylases have been reported to be produced by plant, animal and microbial sources, although the microbial amylase production of amylases have been a bit costlier and that's why is a matter of concern (Smith, 2005). There are mainly two types of assays that are used to determine the activity of α-amylase. One is based on measuring the amount of reducing sugars by the dinitrosalicylic acid (DNS) assay or the Nelson-Somogyi method, whereas the other one is based on the decreased staining value of blue starch-iodine complexes (Zeeman, 2007).

2.8 α-amylase inhibition

α- amylase is a prominent enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules (Afifi *et al.,* 2001) whereas α-glucosidase in the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet (Manohar *et al.,* 2002). Inhibitors of α-amylase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kwon *et al.,* 2007).

An effective means of lowering of levels of postprandial hyperglycemia have been offered by α-amylase and α-glucosidase inhibitors (Matsui *et al.,* 2007). Several

inhibitors of α-amylase has been isolated from serve as an alternative drug with increased potency and lesser adverse effects than existing synthetic drugs (Matsuda *et al.,* 2009). Sudha et al 2011 found that the plant extract of the 17 Indian medicinal plants (*Aloe vera, Adansonia digitata, Allium, sativum, Casia fistula, Catharanthus roseus, Cinnamomum verum, Coccinia grandis, Linum usitatisumum, Mangifera indica, Morus alba, Nerium oleander, Ocimum tenuiflorum, Piper nigrum, Terminalia chebula, Tinospora cordifolia, Trigonella foenum-graceum, Zingiber officinale*) inhibited the pancreatic alpha amylases evaluated via starch iodine plate assays and DNS method. The bioactive metabolites belonged to the chemical class of alkaloids, tannins, cardiac glycosides, flavonoids, saponins and steroids (Sudha *et al*., 2011).

2.9 Allelochemical effects of *Muscodor*

Volatiles produced by *M. yucantensis* were lethal to the endophytes *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangiferae*, and to the phytopathogens *Phythophthora capsici, P. parasitica, Rhizoctonia* sp., *Alternaria solani. Fusarium oxysporum* were resistant to the volatiles recovered 86% of its growth after exposure. *Muscodor yucatanensis*, like other *Muscodor* species produces a mixture of volatile compounds when cultured under *in vitro* conditions. These VOCs were found toxic to other endophytic and phytopathogenic fungi, and to plant roots. Apart from this organic extracts of *Muscodor yucantensis* was also evaluated for its inhibitory activity against certain endophytes, plant pathogens and plants. The organic extracts inhibited plants more than endophytic or phytopathogens fungi. *G. mangifera* was the only organism that was significantly facilitated by both extracts despite of concentration. The bioactive compounds in both organic extracts were identified using gas chromatography/mass

spectrometry. The extracts contain twelve allelochemicals including benzene derivatives, phenolic compounds, cyclopentadienes, esters, lactones, alkanes, aldehydes, and carboxylic acids (Martha *et al*., 2010).

3.0 Aim of Study

The current study aims at "Assessment of *in vitro* antimicrobials and enzyme inhibitors from *Muscodor* species".

The objectives of the current study are:

1. Screening endophytic *Muscodor* isolates for *in vitro* broad spectrum antimicrobial activity.
2. Evaluation of in *vitro* α-amylase inhibitory activity of *Muscodor* species.
3. Screening endophytic *Muscodor* isolates for production of industrially important enzymes.

4.0 Materials and methods

4.1 Re-culturing and maintenance endophytic *Muscodor* isolates

This involves preparation of Potato Dextrose Agar (PDA) Plates, sub culturing *Muscodor* species onto PDA plates and then maintaining it over PDA slants.

4.1.1. Preparation of Potato Dextrose Agar (PDA) Plates

39.0 g of PDA (Hi Media) was dispensed in liter lukewarm single distilled water and stirred thoroughly. This was then dispensed in 250 ml Erlenmeyer Flasks and autoclaved at 121°C, 15 psi for 15 minutes. Glass Petri plates were sterilized at 121°C, 15 psi for 20 minutes. Then under sterile conditions 25 ml of the autoclaved PDA was dispensed in sterile 90 mm Petri plates and allowed to solidify at room temperature. The plates were stored in incubator at the temperature 26 ± 2°C until further use.

4.1.2. Sub culturing of *Muscodor* isolates

Eight *Muscodor* isolates were subcultured onto PDA plates and incubated at 26 °C for 7 days. Morphological characters (colony color, size, texture etc) of all the eight cultures were noted after every 24 hours.

4.1.3. Maintenance of Pure culture

A mycelial plug of 5 mm was then inoculated aseptically in PDA slants supplemented and incubated at 26 °± 2°C till the fungal growth was observed.

4.2. Production of culture filtrate

Mycelial plug of 5 mm diameter of 7-days old fungal culture was inoculated in 100 ml pre sterilized Potato Dextrose Broth (PDB) and Czapek Dox Broth (CDB) (Hi Media) respectively in Erlenmeyer flasks (Scott Duran). The flasks were incubated at 120 rpm

and 26 ± 2°C for 15 days for production of secondary metabolites (Rodrigues, 2000; Santamari *et .al,*. 2002; Rosa *et .al.* 2003). After 15 days broth was separated from mycelia by filtration. Filtration is carried out aseptically through muslin cloth and subsequently through Whatman paper 4 (Rodrigues *et al.*, 2000).

4.3. Solvent Extraction

Liquid-liquid extraction procedure was adopted to extract the spent broth of endophytic *Muscodor* isolates. The aqueous layer was lyophilized and then extracted using different solvents like, ethyl acetate, chloroform and hexane (Merck GR). Aqueous layer was extracted three times with each solvent and the solvent layer was pooled. Then organic layer containing compounds of interest was dehydrated with anhydrous sodium sulphate. The organic layer is then collected in a pre-weighed crucible and the solvent is removed. After removal of solvent, stock solutions of extracts (1mg/ml) were prepared in 10% DMSO (Merck GR) and stored at –20ºC till use (Vicente and Cabello, 2001).

4.4. Procurement of test microorganisms

A spectrum of both Gram negative and Gram positive bacterial isolated were procured from the national microbial repository IMTECH, MTCC Chandigarh and National Collection of Type Cultures, United Kingdom (Table 4.1)

S.no	Bacterial strain	Code
1.	*Bacillus subtilis*	MTCC 121
2.	*Escherichia coli*	MTCC 1302
3.	*Pseudomonas aeruginosa*	MTCC 3541
4.	*Pseudomonas putida*	MTCC 102
5.	*Staphylococcus aureus*	MTCC 96

6.	*Staphylococcus aureus*	MTCC 737
7.	*Staphylococcus aureus*	NCTC 6571
8.	*Staphylococcus epidermidis*	MTCC 2639

Table 4.1: Microorganisms used in the antimicrobial testing

4.5. Antimicrobial susceptibility testing

4.5.1. Maintenance of test microorganisms

The bacterial cultures were maintained Muller Hinton broth (MHB) with 2% glycerol and stored at 4 ºC. Activation of the bacterial cultures was carried out by streaking culture from the slants on to a MH agar (MHA) plate (Hi Media) and then incubating them over night at 37 ºC. A single colony was picked from this plate and transferred to Muller Hinton broth (MHB) and incubated for 16-18 hours at 37 ºC prior to the test.

4.5.2. Preparation of 0.5 Mcfarland Standard

0.5 ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2$ .2H20) was added to 99.5 ml of 0.18M H_2So_4 (1% w/v) with constant stirring. Recorded the O.D. of the solution; it should be in the range of 0.08-0.1 at 625 nm (1 $x10^8$ cells/ml). Store the prepared standard solution in brown colored bottle to prevent it from light at room temperature. Vigorously vortex the standard on a vortex mixer prior to use. (NCCLS, 1997).

4.5.3. Agar Well Diffusion Assay (AWD Assay)

Wells of 5 mm were punched with the help of pre sterilized cork borer in 24 hrs old MH agar (Hi Media) plates to provide a depth of 4 to 5 mm. 30µl of the test extract in 10% DMSO was dispensed in the wells and incubated for 15mins. The wells were sealed with molten MH agar. After 15 min the plate was swabbed with 18-24 hrs old, 0.5 McFarland adjusted culture of the test isolate in three different directions. 10% DMSO served as

control. Antibacterial activity was determined by measuring the zone of inhibition (diameter of inhibition zone). All the tests were performed in triplicates. (Das, 2010; Heatley, 1944)

4.5.4. Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by *in vitro* broth dilution assay. 125 µl of MHB was dispensed in the wells of the 96 well micro-titer plate and then 50 µl of 18 hrs old 0.5 McFarland adjusted culture was dispensed to the wells. The plate was incubated for 3 hrs at 37°C/ 120 rpm. Two fold dilutions (Stock 2mg/ml) were prepared for each extract. Then according to the template (Fig 4.1) 25 µl of extract was dispensed to each well. Again the plate was incubated at 37°C overnight. After incubation 30 µl of 2, 3, 5 triphenyl tetrazolium chloride (TTC) was added and 1-2 hr incubation was given. The appearance of pink color due to formation of formazan indicates growth and the minimum concentration at which growth was inhibited (no pink color) represents the MIC (Gomber and Saxena 2007).

Stock	Diluents	Concentration (mg/ml)	Effective Conc (in µg) / 200 µl	Effective Conc (in µg) /ml	Concentration
S1 (1ml)	1 ml	2	50	250	C1
S2 (1ml)	1 ml	1	25	125	C2
S3 (1ml)	1 ml	0.5	12.5	62.5	C3
S4 (1ml)	1 ml	0. 25	6.25	31.25	C4
S5 (1ml)	1 ml	0.12	3.12	15.62	C5
S6 (1ml)	1 ml	0.062	1.56	7.81	C6
S7 (1ml)	1 ml	0.031	0.78	3.9	C7
S8 (1ml)	1 ml	0.015	0.39	1.95	C8

Table 4.2 Twofold dilutions of bioactive extract for MIC estimation

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1S1	C3S1	C6S1	Ctrl								
B	C1S1	C4S1	C6S1	Ctrl								
C	C1S1	C4S1	C7S1	Ctrl								
D	C2S1	C4S1	C7S1	B								
E	C2S1	C5S1	C7S1	B								
F	C2S1	C5S1	C8S1	B								
G	C3S1	C5S1	C8S1	B								
H	C3S1	C6S1	C8S1	B								

Fig. 4.1 Template for MIC estimation

*C1-C8: Different concentrations used, S1: Test microorganism, B: Blank, Ctrl: Control.

4.5.5. Determination of IC_{50}

After formation of formazan (pink color) by addition of TTC, OD was measured at 650 nm in a using a Bioteck Powerwave 340 plate reader, IC_{50} was calculated using the formula:

$$IC_{50} = \frac{\text{Control (C)} - \text{Test (T)}}{\text{Control (C)}} \times 100$$

Where, Control: Values of control wells

Test: Values in test wells

4.6. Enzyme assays

Production of extracellular enzymes by the fungal endophytes was assessed by digestion of suspended or dissolved substrate in agar plates after inoculation with 5 mm mycelial plugs and incubation for 5 days at 26 ± 2ºC. The zone clearance or formation surrounding the fungal colony was measured.

4.6.1. Amylase activity

The fungi were grown on modified Czapek Dox Agar (CDA) medium (Sucrose-30g, Sodium Nitrate ($NaNO_3$)-2.0g, Ferrous Sulphate ($FeSO_4$)-0.01g, Magnesium Sulphate ($MgSO_4$)-0.5g, Kcl-0.5g, Dipotasium Phosphate (K_2HPO_4)-1.0g, Agar-1.5% and distilled water 1000 mL; pH 6) with 1% soluble starch. After incubation at 26 °C for 5 days the plates were flooded with iodine solution. The clear zone formed surrounding the colony was considered positive for amylase activity (Zeeman *et at*., 2007).

4.6.2. Cellulase assay

The fungi were cultured CDA medium (Sucrose-30g, Sodium Nitrate ($NaNO_3$)-2.0g, Ferrous Sulphate ($FeSO_4$)-0.01g, Magnesium Sulphate ($MgSO_4$)-0.5g, Kcl-0.5g, Dipotasium Phosphate (K_2HPO_4)-1.0g, Agar-1.5% and distilled water 1000 mL; pH 6) supplemented with 1% Na-carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2 aqueous Congo red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the cellulase activity (Zhang *et al.*, 1999)

4.6.3. Laccase activity

The fungi were grown on CD agar medium amended with 0.005% α- naphthol, (pH, 6) and incubated at 26 °C for 5-7 days. On oxidation of α-naphthol by laccase, the medium changes from clear to blue (Hankin and Anagnostakis, 1975).

For production of extracellular enzymes all the *Muscodor* isolates were subjected for production of spent broth in CDB medium. A 5 mm mycelial plug was inoculated and the Erlenmeyer flask was incubated at 26 °C over an orbital shaker at 120 rpm for 10 days. After incubation the broth was filtered through Whatmann filter paper 4 and then through 0.22 µm nitro cellulose membrane. The broth was then evaluated via agar well diffusion assay. 30 µl of the broth was dispensed in the plates containing substrates either CMC, starch or α- napthol and incubated at 37°C overnight. The next day starch plates were stained with iodine solution whereas CMC plates were first flooded with congo red dye and then through 1M NaCl. The zone of clearance around the well was then observed. Plates with α- napthol were checked for the formation of blue color around the well for laccase activity. The zone size around the well was diagonally measured in two

directions, their mean and SD was calculated. All the tests were at least repeated three times (Ominyi *et al.,* 2013, Vijayaraghavan *et al.,* 2013).

4.7 α- amylase inhibitory activity of *Muscodor* isolates

4.7.1 Preparation of reagents

0.02 M amylase was prepared by dissolving 90 mg of amylase in 10 ml distilled H_2O. Then 1% starch solution was prepared by adding 0.3 g of starch in 30 ml of distilled H_2O. For the preparation of 1M HCl, 0.1 ml HCl was added in 10 ml of distilled water. Preparation of Iodine solution had been done by dissolving 0.1 g of I_2 and 0.2 g of KI in 10 ml of distilled water.

4.7.2 Standard curve for α-amylase inhibition

Different dilutions of standard α-amylase were prepared by mixing stock amylase solution (1mg/ml) and water in the test tube. The final volume in each of the test tube was 1 ml. Then, 1 ml of 1% starch solution and 200 µl of different concentrations of amylase were added in different test tubes. After this, these test tubes were incubated for 15 minutes at 37°c. Then 100 µl of 1M HCl was added. The reaction had been stopped by the addition of HCl. 500 µl of iodine was added to the above reaction mixture and OD was measured at 620 nm (Ali *et al.,* 2006).

S. no	Conc. (mg/ml)	Amylase stock(ml) (1mg/ml)	Distilled water (ml)	Total vol. (ml)
1.	0	0	2.0	2
2.	0.1	0.2	1.8	2
3.	0.3	0.6	1.4	2
4.	0.5	1.0	1.0	2
5.	0.7	1.4	0.6	2
6.	0.9	1.8	0.2	2
7.	1.0	2.0	0	2

Table 4.3: Showing the preparation of different conc. from α-amylase stock solution

4.7.3 α-Amylase inhibitory assay

40 µl of 0.02M α-amylase was incubated with culture filtrate (40 µl) of *Muscodor* species in a 96 well micro-titre plate. The plate was incubated at 37 °C for 10 minutes. Subsequently, 500 µl of 1% starch solution was added in each well. The plate was again incubated for 15 minutes at 37° C. 20 µl of 1M HCl was added to stop the reaction and left at room temperature for 10 minutes incubation. Then 100 µl of iodine solution (0.1 g of I_2 and 0.2 g of KI in 10 ml distilled water) was added and OD was measured at 620 nm using a Bioteck Powerwave 340 plate reader. Inhibition of α- amylase was measured against a calibration curve of α- amylase with R^2 value of 0.99 and the regression equation was y = - 0.2907x + 3.7124 (Sudha *et al*., 2011)

5.0 Results and discussion

5.1 Sub culturing and Maintenance of *Muscodor* species

All the eight *Muscodor* species produced sterile, whitish colonies over PDA (Fig. 5.1). Pure colonies were transferred to PDA slants supplemented with glycerol. All the *Muscodor* special remained viable for six months over PDA slants. The eight *Muscodor* species under study are listed in Table 5.1.

Culture Code	Host Plant	Sampling	Taxonomic identification
#16 AMLWLS	*A. marmelos*	Wayand, Kerala	*M. kashayum* sp. nov
#6610 CZSTITBRT	*C. zeylanicum*	BRT wildlife sanctuary, Karnataka	*M. strobelii* sp. nov.
#130 TMDSTYEL	*T. divaricata*	Yelandur, Karnataka	*Muscodor yelandurii* sp. nov.
#1 CCSTITD	*C. camphora*	Darjeeling, West Bengal	*M. darjeelingensis* sp. nov
#2 CCSTITD	*C. camphora*	Darjeeling, West Bengal	*M. tigerii* sp. nov
#6 CCSTITD	*C. camphora*	Darjeeling, West Bengal	*M. ghoomensis* sp. nov
#6 (B) CCSTITD	*C. camphora*	Darjeeling, West Bengal	*M. indica* sp. nov
#1639 CCSTITD	*C. camphora*	Darjeeling, West Bengal	*M. camphora* sp. nov

Table 5.1: Eight endophytic *Muscodor* species under study

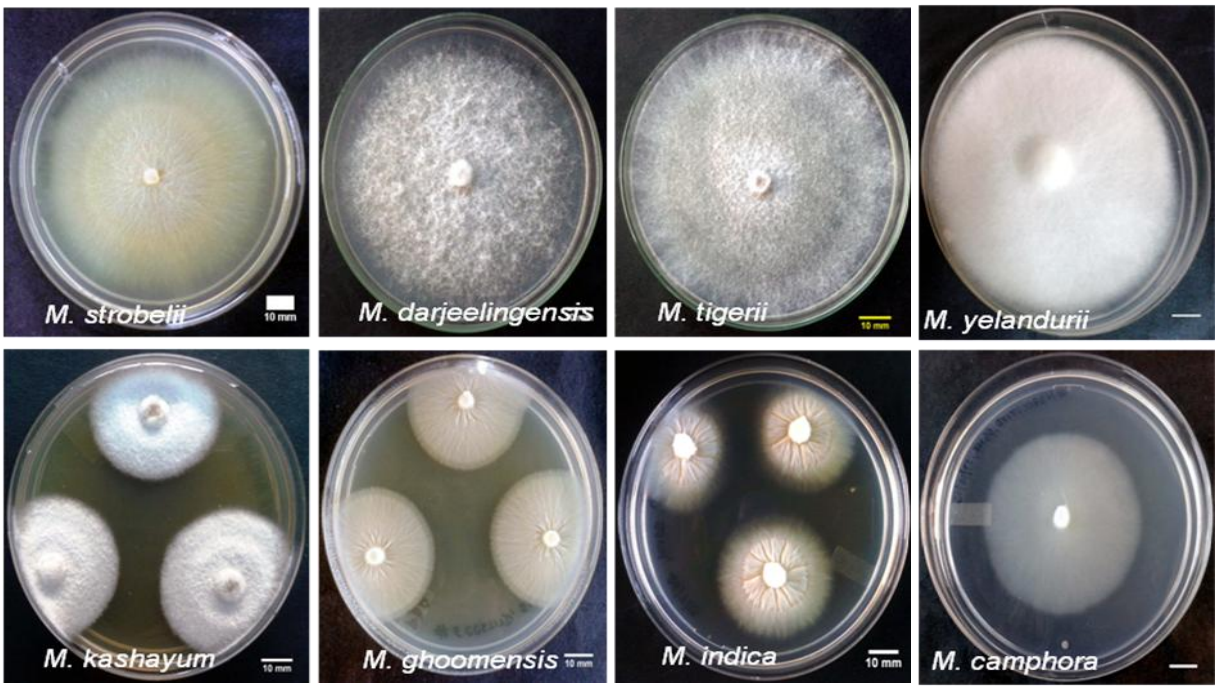


Fig 5.1: Morphological features of Indian *Muscodor* species over PDA medium

5.2 Culture filtrate preparation and Extraction

Potato Dextrose Broth (PDB) and Czapek Dox Broth (CDB) were used as fermentation media for the production of functional secondary metabolites. All the 8 isolates were fermented giving the appropriate conditions. It was found that the final volume of the fermented spent broth falls down after incubation period along with the pH. Initially, 25 ml of the Potato Dextrose broth (PDB) when extracted with 100 ml of three different solvents (EA, $CHCl_3$ and Hexane). Maximum yield was observed in ethyl acetate fraction of #6(B) CCSTITD with 11.8 mg followed by 16 ALMWLS (7.9 mg) and #1639 CCSTITD (6.5 mg) where as minimum yield was yielded by #6610 CZSTITBRT. Similarly in chloroform and hexane fraction maximum yield was yielded by #16 AMLWLS. Whereas in case if CDB the yield was less as compared to PDB. Maximum

yield was obtained in chloroform fraction of #6 CCSTITD followed by #2 CCSTITD (Table 4.2). Stock solutions of all the fractions (1 mg/ml) were prepared in 10% DMSO and further evaluated for their antimicrobial potential using various pre-screen antimicrobial susceptibility assays.

	Yield in PDB (mg)			Yield in CDB (mg)		
Culture Code	EA	$CHCl_3$	Hexane	EA	$CHCl_3$	Hexane
#16 AMLWLS	7.9	8.5	7.8	0.7	1.8	1.1
#6610 CZSTITBRT	2.0	8.3	6.8	0.9	0.8	1.8
#130 TMDSTYEL	5.1	4.7	4.6	1.7	0.9	3.3
#1 CCSTITD	1.3	5.7	6.3	0.8	0.9	1.9
#2 CCSTITD	2.3	3.0	7.8	1.5	5.9	3.7
#6 CCSTITD	3.2	6.4	1.7	0.4	9.1	1.3
#6 (B) CCSTITD	11.8	8.1	4.2	1.6	1.8	1.7
#1639 CCSTITD	6.5	4.2	1.3	2.1	0.6	0.8

Table 5.2: Representing the fermentative product with their yield after extraction with ethyl acetate (EA), Chloroform ($CHCl_3$) and Hexane

5.3 Antimicrobial susceptibility testing:

5.3.1 Agar Well Diffusion Assay

In agar well diffusion assay, antimicrobial activity was only observed in EA fractions of CDB medium whereas no activity was observed in any other fractions ($CHCl_3$ and Hexane). Similarly no activity was observed in any of the three fraction of PDB.

Muscodor sp. #130 TMDSTYEL exhibited maximum broad spectrum antimicrobial potential against the tested battery of organisms with a zone size ranging from 9-17 mm followed by #6 (B) CCSTITD and #1639 CCSTITD whose zone of inhibition ranges between 8-14 mm. None of the fraction was able to inhibit the growth of *B. subtilis* (MTCC 121) and *E. coli* (MTCC 1302) (Table 5.3) (Fig 5.2). Based on the zone diameter, EA fraction can be inferred as intermediate for *S. aureus* and susceptible for *P. aeruginosa* and *P. putida*. Similarly, the other isolates also fall under this category only. The bioactive metabolite might be a non polar compound as it is ethyl acetate residue.

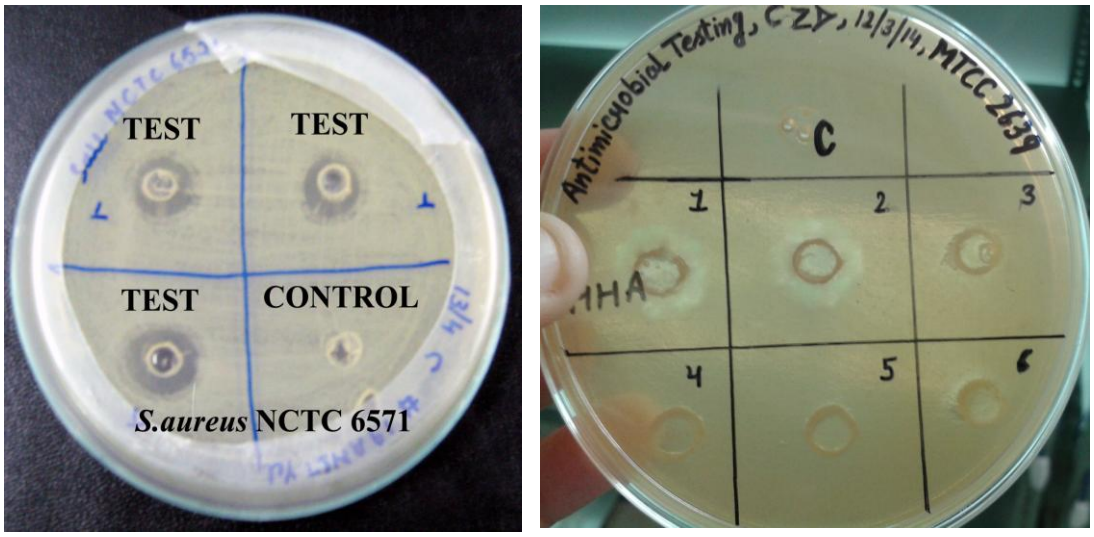


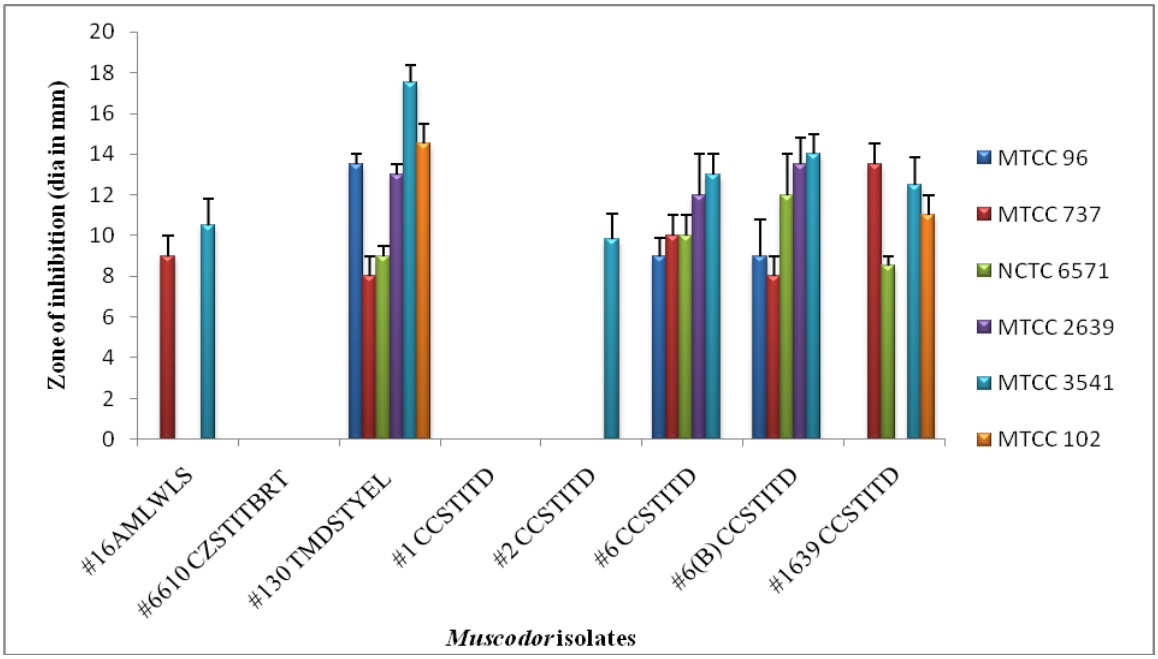
Fig 5.2: Antimicrobial activity of *Muscodor* isolate against *S. aureus* and *S. epidermidis*

Culture Code	Mean Zone of Inhibition (in mm)							
	MTCC 96	MTCC 737	NCTC 6571	MTCC 2639	MTCC 3541	MTCC 102	MTCC 121	MTCC 1302
#16 AMLWLS	-	9 ± 0.5	-	-	10.5 ± 1.32	-	-	-
#6610 CZSTITBRT	-	-	-	-	-	-	-	-
#130 TMDSTYEL	13.5 ± 0.5	8 ± 0.86	9 ± 0.5	13 ± 0.5	17.5 ± 0.86	14.5 ± 0.5	-	-
#1 CCSTITD	-	-	-	-	-	-	-	-
#2 CCSTITD	-	-	-	-	9.8 ± 1.25	-	-	-
#6 CCSTITD	9 ± 0.86	10 ± 1.54	10 ± 1	12 ± 2	13 ± 1	-	-	-
#6(B) CCSTITD	9 ± 1.80	8 ± 0.86	12 ± 2	13.5 ± 1.32	14 ± 1	-	-	-
#1639 CCSTITD	-	13.5 ± 0.86	8.5 ± 0.5	-	12.5 ± 1.32	11 ± 2	-	-
Control	-	-	-	-	-	-	-	-

Table 5.3: Antimicrobial activity of EA extract of Indian *Muscodo*r species using Agar well diffusion assay

*All the tests were performed in triplicate. Their mean± SD was calculated

Test Culture	Code	MIC (µg/ml)	IC_{50} (µg/ml)



Graph 1: Antimicrobial activity of EA extract of Indian *Muscodor* species using Agar well diffusion assay

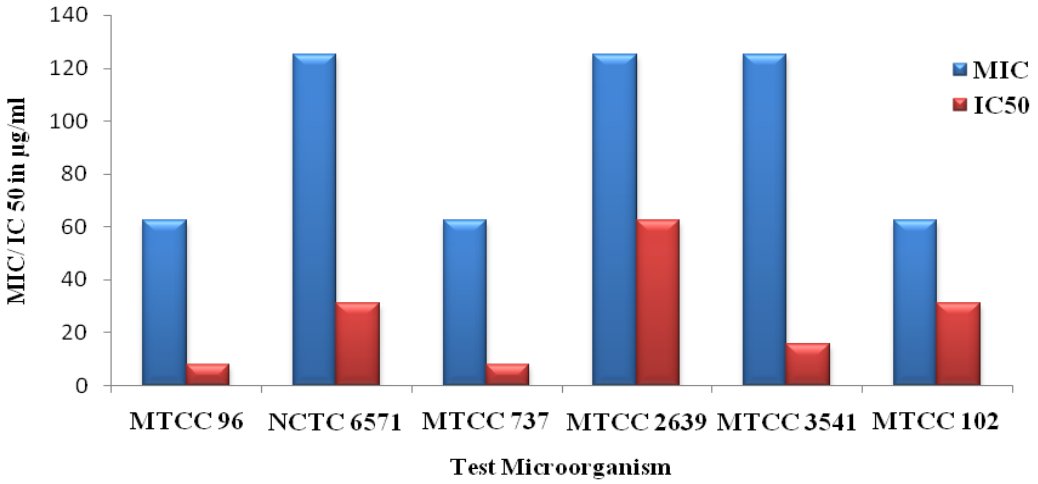
5.3.2 Determination of Minimal Inhibitory Concentration (MIC) and IC_{50}

Ethyl acetate fraction of #130 TMDSTYEL was further subjected for estimation of MIC and IC_{50} against the spectrum of test microorganisms. MIC ranges between 62.5 – 250 µg/ml. EA fraction of #130 TMDSTYEL exhibited lowest MIC against *S. aureus* MTCC 96 whereas highest MIC was observed against *Pseudomonas aeruginosa* MTCC 3541.

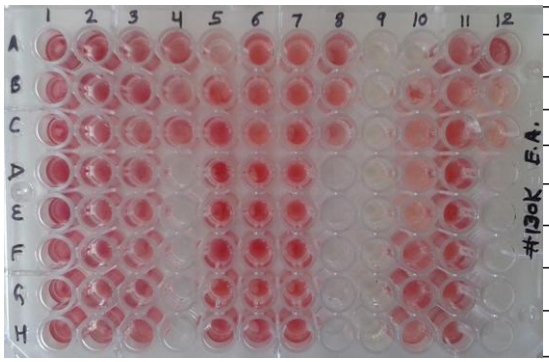
It also exhibited IC_{50} values between 7.8 µg/ml to 62.5 µg/ml. *Staphylococcus aureus* MTCC 96 and MTCC 737 were the most vulnerable bacterial pathogen. *S. epidermidis* exhibited highest IC_{50} whereas for *Pseudomonas* it ranges between 15.62 – 31.25 µg/ml (Table 5.4) (Fig 5.3- 5.4)

S. aureus	MTCC 96	62.5	7.8
S. aureus	NCTC 6571	125	31.25
S. aureus	MTCC 737	62.5	7.8.
S. epidermidis	MTCC 2639	125	62.5
P. aeruginosa	MTCC 3541	125	15.62
P. putida	MTCC 102	62.5	31.25

Table 5.4: MIC and IC_{50} of EA fraction of #130 TMDSTYEL



Graph 2: MIC and IC_{50} of #130 TMDSTYEL against tested microorganisms.



	1	2	3	4	5	6	7	8	9	10	11	12
A	G	G	G	G	G	G	G	G	NG	NG	G	G
B	G	G	G	G	G	G	G	G	NG	G	G	G
C	G	G	G	B	G	G	G	G	NG	G	G	G
D	G	G	G	B	G	G	G		NG	G	G	B
E	G	G	G	B	G	G	G		NG	G	G	B
F	G	G	G	B	G	G	G		NG	G	G	B
G	G	G	G	B	G	G	G		NG	G	G	B
H	G	G	G	B	G	G	G		NG	G	G	B

Fig. 5.3: *In vitro* broth dilution assay for MIC and IC_{50} evaluation

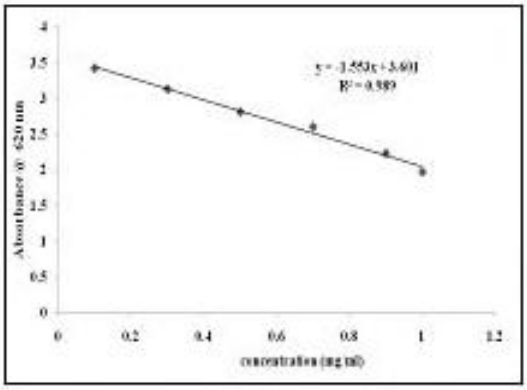
* G: growth; NG: No growth; B: Blank

5.4 α- Amylase Inhibition:

All the eight *Muscodor* isolates exhibited potent α- Amylase inhibitory activity. #6 (B) CCSTITD showed 99.19% inhibition of α- Amylase followed by #130 TMDSTYEL which depicted inhibition of 98.59%. Other *Muscodor* isolates also showed α-Amylase inhibitory potential in the range 54 – 70% (Table 5.6, Fig 5.4).

Culture Code	OD1	OD2	Mean	Control	% inhibition = $\frac{C-T}{C} \times 100$
#1CCSTITD	0.292	0.306	0.299 ± 0.009		69.90%
#2 CCSTITD	0.416	0.42	0.418 ± 0.002		57.90%
#6 CCSTITD	0.311	0.331	0.321 ± 0.014		67.70%
#6(B) CCSTITD	0.005	0.011	0.008 ± 0.004	0.995	99.19%
#16AMLWLS	0.261	0.267	0.264 ± 0.004		73.46%
#1639 CCSTITD	0.321	0.335	0.328 ± 0.009		67.03%
#130TMDSTYEL	0.013	0.015	0.014 ± 0.001		98.59%
#6610 CCSITTD	0.445	0.451	0.448 ± 0.004		54.97%

Table 5.5: α- Amylase inhibitory activity of Indian *Muscodor* species



Graph 3: Standard curve of α-amylase

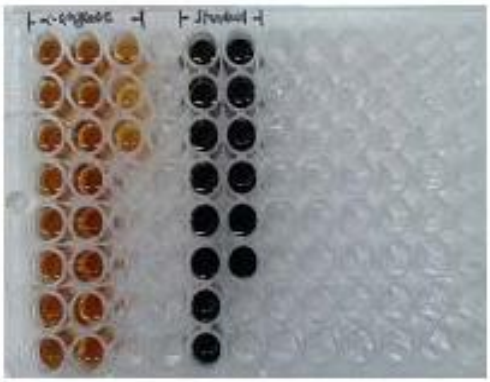


Fig. 5.4 α- amylase inhibitory activity of Indian *Muscodor* species

5.5 Enzyme assays

Muscodor cultures were tested for the efficacy of enzyme production. Following standard protocols, production of cellulose, amylase and laccase were screened (Table 5.6).

In cellulase assay four *Muscodor* isolates showed positive results. #6610 CZSTITBRT, #2 CCSTITD and #1 CCSTITD showed formation of clear zone around the colony after 5 days of incubation when stained with Congo red dye indicating the production of amylase enzyme via CMC degradation. The spent broths also exhibited formation of clear zone around the well depicting extracellular enzyme activity. The zone size varies between 11-14.5 mm. Maximum cellulase production was shown by #6610 CZSTITBRT (Fig 5.5 – Fig 5.6).

Culture Code	Zone of clearance /formation		
	Cellulase activity	Amylase activity	Laccase activity
#16 AMLWLS	13.67 ±1	-	-
#6610 CZSTITBRT	11.5 ± 1.32	-	-
#130 TMDSTYEL	-	12.8 ± 1.44	-
#1 CCSTITD	14.5 ± 1	-	-
#2 CCSTITD	12.1 ± 0.57	-	-
#6 CCSTITD	-	-	-
#6(B) CCSTITD	-	10.6 ± 1.52	-
#1639 CCSTITD	-	-	-

Table 5.6: Production of various enzymes by *Muscodor* species

In amylase assay three isolates showed positive results interpreted by formation of clear zone around colony after dying with iodine solution. # 130 TMDSTYEL and #6(B) CCSTITD. The spent broth of both the isolates also showed degradation of starch thereby conforming the potential of the two *Muscodor* isolates to produce extracellular amylase enzyme. The zone size varies between 10-13 mm (Fig 5.7). None of the *Muscodor* isolate produced laccase enzyme.

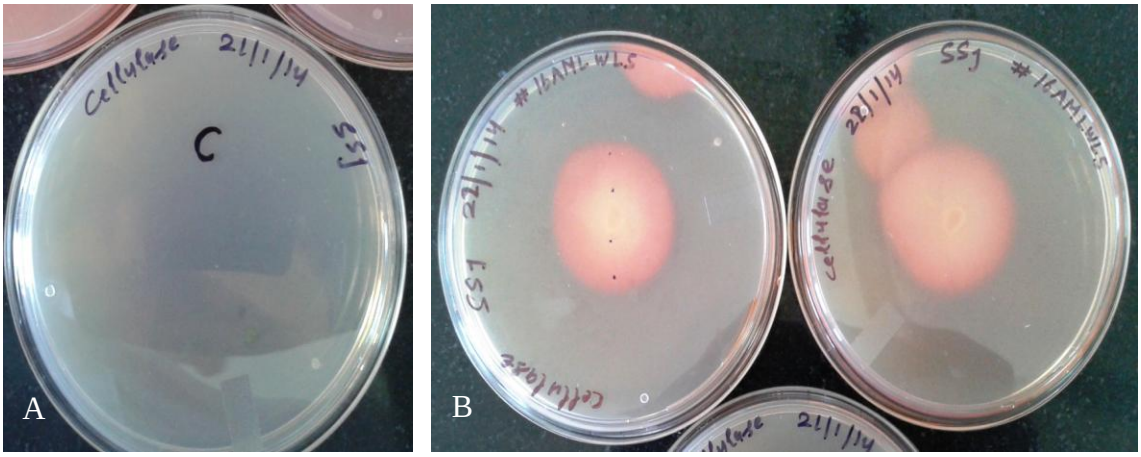


Fig 5.5: Cellulase production by *Muscodor* isolates

A) Control, B) Cellulase production by #16 AMLWLS

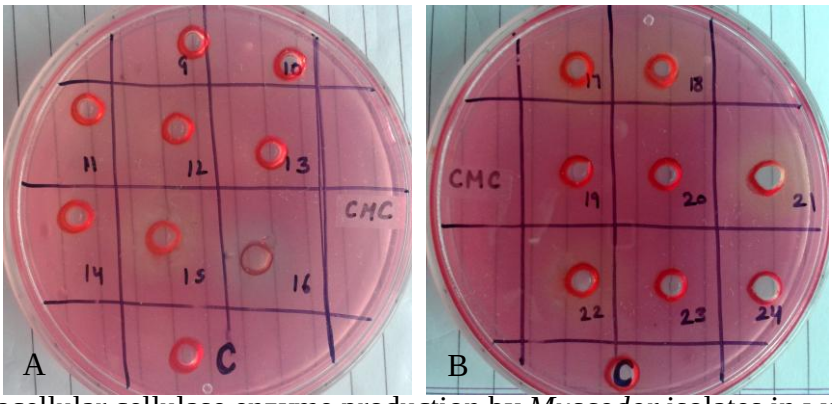


Fig. 5.6: Extracellular cellulase enzyme production by *Muscodor* isolates in well assay

A) Well 15 – 16: #6610CZSTITBRT; B) Well 17–18: #2CCSTITD, Well 21 –22: #1CCSTITD

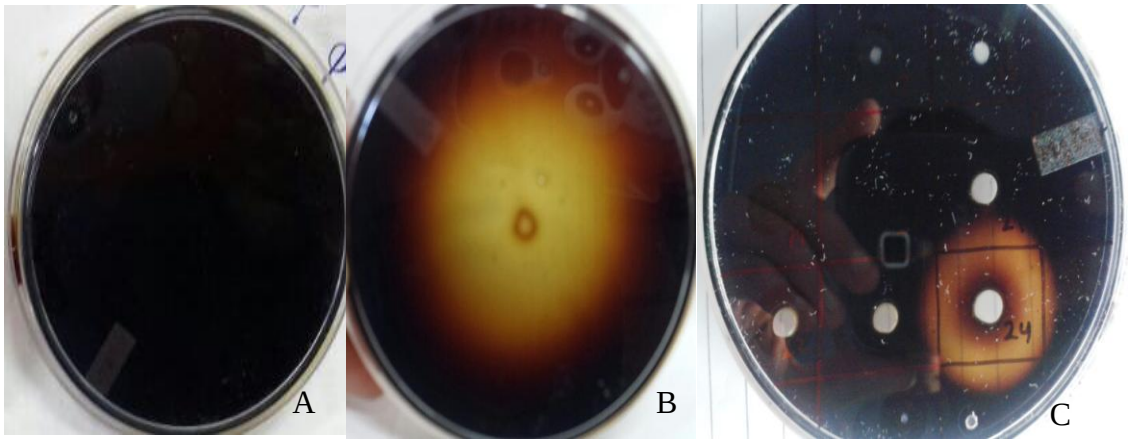


Fig. 5.7: Amylase production by *Muscodor* isolates

A) Control, B) #130 TMDSTYEL, C) Well 24: #130 TMDSTYEL

Organic extracts of eight *Muscodor* cultures were tested for their antimicrobial potential. In well diffusion assay, the maximum zone of inhibition was observed in *Muscodor* culture #130 TMDSTYEL against the Gram positive bacteria (MTCC 96, MTCC 737, MTCC 3541, PP-1). Zone of inhibition is directly proportional to the antimicrobial potential of the compound against the test bacteria. In other words, more the resistant of the micro-organism, smaller will be the zone of size. Methanolic extract of the endophytic fungi obtained from *Orthosiphon stamineus* posses strong antimicrobial activity whose zone sizes varies between 9-26 mm (Tong *et al*., 2011). Similarly fungal endophytes like *Phyllosticta* spp. *Nodulisporium* spp. and *Xylaria* sp.isolated from Dipterocapous trees of Thailand exhibited potent antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aerogenosa and Escherichia coli. Nodulisporium* spp also inhibits Candida albicans (Sutjaritvorakul *et al.,* 2011). The secondary metabolites of an endophytic fungus *O. Japonicus* are also potential antimicrobial agents (Hanqiao *et al.,* 2012). MIC of #130TMDSTYEL against test

micro-organism was between 62.5-125 µg/ml. It is observed that lower the MIC, more susceptible the micro-organism is to the antimicrobial agent. In other words, more resistant the micro-organism, higher the MIC. MIC of the cloxacillin for MRSA strains were in range between 32 – 128 µg/ml as tested via broth dilution method (Islam *et al*., 2008). Similarly, MIC of *Senna alata* for MRSA was found to be 125 and 250 µg/ml (Marzalina *et al.,* 2006). When *Muscodor* cultures were also subjected for the production of industrially important enzymes. It was observed that CMC degradation appeared in three *Muscodor* cultures as yellow-opaque area around the colonies against red colour for undegraded CMC. Result showed that four *Muscodor* cultures (#6610CCSTITD, #16 AMLWLS, #2CCSTITD, #1CCSTITD) exhibit the tendency to degrade cellulose. Other than *Muscodor* species, other reported cellulolytic fungi belonged to *Trichoderma sp., Fusarium sp., Aspergillus sp., Alternaria sp., Penicillium sp. and Rhizopus sp.* (Sadaf *et al.,* 2005). In case of amylase production, starch degradation observed in two *Muscodor* cultures (#130TMDSyel and #6(b) CCSTITD) as clear zone around the colony, against the dark reddish brown undegraded starch. Most efficient starch degradation was observed by *Muscodor* culture #130TMDSYEL. These fungal culture filtrates with a reputation of usefulness in treating diabetes and inhibiting the growth of many other pathogenic fungi and bacteria were examined for α-amylase inhibition using an *in vitro* model. The results of this test showed that *Muscodor* filtrates had α-amylase inhibitory properties. All the *Muscodor* isolates were tested in the α-amylase inhibitory assay and results revealed that #6(b)CCSTITD is a potent α-amylase inhibitor with IC_{50} 99.19%, followed by other two *Muscodor* cultures #130TMDSYEL and #16AMLWLS which induces inhibition of α-amylase enzyme by 98.59 and 73.46% respectively. These

Muscodor cultures thus have the potential to be used as α-amylase inhibitors along with applications in various fields including agro and pharmaceutical industries.

Conclusion

The Indian *Muscodor* species produces bioactive secondary metabolites in their organic extract that possess broad spectrum antimicrobial and enzyme inhibition potential. *Muscodor* species also exhibited cellulase and alpha-amylase production. Thus based on the above study it can be concluded that Indian *Muscodor* species are the prolific source of bioactive compounds which could be further exploited for their possible use in agro and pharmaceutical industries. Mass production, purification, characterization and QSAR studies of the bioactive compounds are warranted. Through studies on host parasite-relationship needs to be done to unveil the mystery of symbiosis between plant and microbe. The life cycle of *Muscodor* is still remains an untold story.

References

1. Ali H., Houghton P.J., Soumyanath A., (2006), α-amylase inhibitory activity of some Malaysian plants used to treat diabetes with particular reference to *Phyllanthus amarus*, Journal of Ethnopharmacology, 107:449-455
2. Andrews J.M., (2001), Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.,* 48 (S1): 5-16
3. Atmosukarto I., Castillo J., Strobel G., (2005), Isolation and characterization of *Muscodor albus*: A volatile antibiotic producing fungus, Plant Sci, 169:854-861
4. Bacon C.W., White J.F.J., (2002), Physiological adaptations in the evaluation of endophytisim in the *Clavicipitaceae,* Marcal Dekker Inc., 23:237-263
5. Caballo A., Gorrochategui J., Vicente F., (2001), Screening of antimicrobial activities in red, green and brown macroalgae from Gran canaria (Canary Islands, Spain), Int. Microbiol., 4:35-40
6. Cohen M.L., (1992), Epidemiology of drug resistance: Implications for a post antimicrobial Era, Science, 257:1050-1055
7. Corcuff J.B., Krim E., Tison F., Foubert-Sanier A., Guehl D., Burbaud P., (2006), Subthalamic nucleus stimulation in patients with Parkinson's disease does not increase serum ghrelin levels, Br J Nutr, 95:1028–9
8. Das K., Tiwari R.K.S., Shrivastava D.K., (2010), Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends, Journal of Medicinal Plants., 4(2):104-111

9. Daisy, B., Strobel, G., Ezra, D., Castillo, U., Baird, G. & Hess, W. M., (2002a). *Muscodor vitigenus* sp. nov., an endophyte from *Paullinia paullinoides*, Mycotaxon 84:39–50
10. Daisy, B. H., Strobel, G. A., Castillo, U., Ezra, D., Sears, J., Weaver, D. K. & Runyon, J. B., **(**2002b**).** Naphthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus, Microbiology 148: 3737–3741
11. De la Rosa L.A., Alfonso A, Vilariño N, Vieytes M.R., Botana L.M., (2003), Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin, Biochem. Pharmacol., 61:827–833
12. Dreyfuss, M.M., Chapela I.H., (1994), potential of fungi in discovery of novel low molecular weight pharmaceuticals, Butterworth-Hainemann, London, UK, 2:49-80
13. Ezra D., Hess W.H., Strobel G., (2004), New endophytic isolates of *Muscodor albus*, Microbio, 150:4023-4031
14. Fasehun, (1999), The antibacterial paradox: essential drugs, effectiveness, and cost, the International Journal of Public Health, 77 (3):211-216
15. Firakova S., Sturdikova M., Muckova M., (2007), Bioactive secondary metabolites produced by microorganisms associated with plants, Biologia. 62:251–257
16. Fuwa H., (1954), A new method of microdetermination of amylase activity by the use of amylase as the substrate, J. Biochem. 41**:**583–603
17. Gomber C., Saxena S., (2007), Anti-staphylococcal potential of Callistemon rigidus, Central European Journal of Medicine, 2 (1):79-88
18. González, María C., Anaya, Luisa A., Glenn, Anthony E., Rubalcava M., Martha L., Bautista H., Blanca E., Hanlin, Richard T., (2009), *Muscodor yucatanensis*, a new

endophytic ascomycete from Mexican chakah, *Bursera simaruba,* Mycotaxon, 110:363-372

19. Gordon M., Cragg, David J. Newman, (2005), Plant as a source of anticancer agents, Journal of Ethnopharmacology, 100:72-79
20. Grimme E., Zidack, N.K., Sikora, R.A., Strobel G.A., (2007), Comparison of *Muscodor albus* volatiles with a biorational mixture for control of seedling diseases of sugar beet and root knot nematode on patato, Plant disease, 91:220-225
21. Hankin L., Anagnostakis S. L. Mycologia, (1975), The use of solid media for the detection of enzyme production by fungi, Mycologia., 67:597-608
22. Hanqiao L., Xing Y., Juan C., (2012), Antimicrobial activities of endophytic fungi isolated from Ophiopogon japonicus, Biomed Central, 1472-6882
23. Hilmar I, Daniel B, Victor K, Birjit S, (2003), The Arabidopsis STICHEL gene is a regulator of trichome branch number and encodes a novel protein, Plant physiology, 131(2):643-55
24. Heatley N. G., (1944), Estimation of vitamin C by Agar Diffusion, Biochem. J., 3:38:61
25. Jacobson S.J., Laurenson P.M., Pillus L., (2004), Functional analyses of chromatin modifications in yeast, Methods enzymol, 377:3-55
26. Jiao Z., Zhang Z.G., Hornyak T.J., Hozeska A., Zhang R.L., Wang Y. *et al*., (2006), Dopachrome tautomerase (Dct) regulates neural progenitor cell proliferation, Dev Biol. 296:396–408
27. Kudalkar P., Strobel G., Hasan S.R.U., Geary G., Sears J., (2012), *Muscodor sutura* a novel endophytic fungus with volatile antibiotic activities. Mycoscience, 53:319–325

28. Lacey L.A., Granatstein D., Arthurs S.P., Headrick H., Fritts R., (2006), Use of entomopathogenic nematodes (Steinernematidae) in conjunction with mulches for control of overwintering codling moth, Entomological Science, (2006), 41:107–119
29. Liang H., Xing Y., Chen J., Zhang D., Guo S., Wang C., (2012), Antimicrobial activities of endophytic fungi isolated from Ophiopogon japonicus (Liliaceae), Biomed. Central Ltd., 12:238
30. Martinez A.T., Speranza M, Ruez-Duenas F.J., Ferreira P., (2005), Biodegradation of lignocellulosics: microbial, chemical and enzymatic aspects of the fungal attack of lignin, Int. Microbiol., 8:195-204
31. Mercier, J. & Jimenez, J. I. (*2004*), Control of decay of apples and peaches by the biofumigant fungus *Muscodor albus*, Postharvest Biol Technol, 31:1–8
32. Mercier J., Manker D.C., (2005), Biocontrol of soil-borne diseases and plant growth enhancement in greenhouse soilless mix by the volatile producing fungus *Muscodor albus*, Crop protection, 24:355-362
33. Mercier J., Santamaria J.I.J, Guerra P.T., (2007), Development of the volatile producing fungus *Muscodor albus* Worapong, Strobel, and Hess as a novel antimicrobial bio-fumigant, Rev Mex Fitopatol., 25:173–179.
34. Mertens, J.A., Skory, C.D. (2006) Isolation and characterization of a second glucoamylase gene without a starch binding domain from *Rhizopus orzae*, *Enzyme Microbial Technology*, 40: 874-880.

35. Meshram V., Saxena S., Neha K., (2013), *Muscodor tigerii* sp. nov.- Volatile antibiotic producing endophytic fungus from the Northeastern Himalayas, Annals of Microbilogy, 4(4):196-204
36. Mitchell A.M., Strobel G.A., Hess W.M., Vargas P.N., Ezra D., (2008), Muscodor crispans, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon, Fung Divers, 31: 37–43
37. Mitchell A.M., Strobel G.A., Moore E, Robison R, Sears J., (2010), Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus, Microbiology, 156: 270–277.
38. Mlikota Gabler F., Fassel R., Mercier J., Smilanick J.K., (2006), Influence of temperature inoculation interval on biofumigation with *Muscodor albus* to control postharvest gray mould on grapes, plant dis., 90:1019-1025
39. Mohanty A., (2010), Physiochemical and antimicrobial study of polyherbal, Pharmaceiglobal, 4(04):1-3
40. Nigam, P. and Singh, D. (1995) Enzymes and microbial system involved in starch processing. *Enzyme Microbial Technology*. 17: 770-778.
41. Ominyi , M.C., Ogbonna, J.C., Nwoba, E.G., Nwagu, K.E., Ukachi , R. (2013), Isolation and screening of α-amylase and glucoamylase producing fungi and their application in bioethanol production, I.J.S.N., 4 (1):44-50
42. Pederson, N. and Nielsen, J., (2000), The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus oryzae* in continuous cultures, Applied Microbial Biotechnology, 53:278-281.

43. Roberta J. Worthington, Melander C., (2013), Combination Approaches to Combat Multi-Drug Resistant Bacteria, Trends Biotechnol, 31 (3):177-184
44. Rodríguez E., Pickard M. A., R. Vázquez-Duhalt, (2000), Industrial dye decolorization by laccases from ligninolytic fungi, Current Microbiol., 38:27–32
45. Rodriguez E., Regina S, Douglas A. Lauffenburger, Steffen K and Peter K., (2009), Discrete logic modeling as a means to link protein signaling networks with functional analysis of mammalian signal transduction, Molecular Systems Biology, 5:331
46. Romila Y., P.B. Mazumder, M. Dutta Choudhury, (2010), A review on antidiabetic plants used by the people of Manipur characterized by Hypoglycemic activity, Journal of Sci. and Tech., 6(1):167-175
47. Saxena S., Gomber C., (2010), Superoxide Dismutase and extracellular Protease expression in clinical isolates of Staphylococcus aureus: A tool for antimicrobial drug discovery, Molecular Cellular Biochemistry, 341(1-2): 217-223
48. Saxena S., (2014), Microbial metabolites for development of ecofriendly agrochemicals, Allelopathy Journal, 33(1)
49. Saxena S., Pandey A., (2002), Evaluation of an indigenous isolate of Alternaria alternata (LC#508) for use as a mycoherbicide for Lantana camara L., Crop protection, 21(1):71-73
50. Sopalun K., Strobel G.A., Hess W.M., (2003), A record of *Muscodor albus*: An endophyte from *Myristica fragrans*, Mycotaxon, 88:239-247
51. Stinson A.M, Strobel G., (2003), Effect of mycofumigation with Muscodor albus and Muscodor roseus on seedling disease of sugarbeet and verticellium wilt of egg plant, Plant Dis., 87:1339-1354

52. Stone, J.K., Bacon, C.W., White, J.F., (2000). An overview of endophytic microbes: endophytism defined. In *Microbial Endophytes*, pp. 3-29. Edited by C. W. Bacon & J. F. White. New York: Marcel Dekker.
53. Strobel G., Daisy B., (2003), Bioprospecting for microbial endophytes and their natural products, Microbiol, Mol., Biol. Rev., 67:491-502
54. Strobel, G. A., Dirkse, E., Sears, J. & Markworth, C. (*2001*)**.** Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus, Microbiology 147:2943–2950.
55. Strobel G.A., Katreena K., Hess W. M., Sears J., Ezra D., Vargas P. N. (2007), *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia* making volatile antibiotics: isolation, characterization and experimental establishment in host plant, Microbiology, 153:2613-2620
56. Strobel G.A., (2006), *Muscodor albus* and its biological promise, J Ind. Microbiol Biotechnol., 33:514-522
57. Suwannarach N., Kumla J., Bussaban B., Hyde K.D., Matsui K., Lumyong S. (2013), Molecular and morphological evidence support four new species in the genus *Muscodor* from northern Thailand, Ann Microbiol., 13:212-223
58. Tiwari, B.K. (2009), Application of natural antimicrobials for food preservation, Journal of Agricultural and Food Chemistry, 57 (14):5987-6000.
59. Tomasz A., (1994), Multiple antibiotic resistant pathogenic bacteria, New England Journal of Medicine, 330:1229-1230
60. Tong, C., Ohyama, T., Tien, A.C., Rajan, A., Haueter, C.M., Bellen, H.J., (2011), Rich Regulates Target Specificity of Photoreceptor Cells and N-Cadherin Trafficking in the Drosophila Visual System via Rab6. Neuron 71(3): 447-459

61. Toye, E., (2009), Laboratory production and Assay of Amylase by fungi and bacteria. *Buscar Manuales*, 2:1-30.

62. Vijayaraghavan P., Prakash S.G., Vincent (2013), A simple method for the detection of protease activity on agar plates using bromocresolgreen dye, J Biochem. Tech., 4 (3):628-630

63. Wilson D.B., (2011), Microbial diversity of cellulose hydrolysis, Ecology and Industrial Microbiology, 14:1-5

64. Wisplinghoff H., Bischoff T., Sandra M. Tallent, Seifert H., Richard P. Wenzel, Michael B. Edmond (2003), Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study, Clin. Infect Dis., 39 (3): 309-317

65. Worapong, J., Strobel, G.A., Daisy, B.H., Baird, G., Hess, W.M. (2002), *Muscodor roseu*s sp. nov., an endophyte from *Grevillea pteridifolia*. *Mycotaxon*, 81: 463-475.

66. Worapong, J., Strobel, G. A., Ford, E. J., Li, J. Y., Baird, G. & Hess, W. M. (*2001*)**,** *Muscodor albus* anam. nov., an endophyte from Cinnamomum zeylanicum, Mycotaxon 79: 67–79

67. Zhang C.L., Wang G.P., Mao L.J., Komon-Zelazowska M., Yuan Z.L., Lin F.C., Druzhinina I.S., Kubicek C.P. (2010), *Muscodor fengyangensis* sp. nov. from south east China: morphology, physiology and production of volatile compounds, Fungal Biol., 114:797–808

68. Yee L.W., Lawrence A.L., Belinda J.B, (2009), Pupal mortality and adult emergence of western cherry fruit fly (Diptera: Tephritidae) exposed to the fungus *Muscodor albus*, Journal of Economic Entomology, 102(6):2041