Effect of pH on Copper-tolerant *Penicillium chrysogenum*

A Dissertation Report for

Course code: MMD 412 Dissertation

Credits : 8

Submitted in partial fulfilment of Masters of Science in Marine Microbiology

By **KUMARI**

RIYA

21P039012

Under the Supervision of Dr. Nikita P. Lotlikar

School of Earth, Ocean and Atmospheric Sciences

MASTER OF SCIENCE
In
MARINE MICROBIOLOGY

Goa University, Taleigao Plateau

Goa, India

403206

April 2023

Examined by: Seal of the School

DECLARATION BY STUDENT

I hereby declare that the present thesis entitled "**Effect of pH on Copper-tolerant *Penicillium chrysogenum***" submitted to the Goa University, for the award of the degree in **Master of Science**, **Marine Microbiology** is a consolidation record of original and independent work carried out by me during the month of **November 2022 – April 2023** , in the School Of Earth, Ocean and Atmospheric Sciences ; Marine Microbiology , Goa University under the supervision and guidance of Dr. Nikita P. Lotlikar , Assistant Professor, Marine Microbiology program, Goa University and that the same has not been submitted to any other University or Institution to form the basis for the award of any Degree, Diploma, Associate-ship or Fellowship.

The record of literature related to the research problem investigated has been duly cited, and the facilities and the research fellowships availed of have been duly acknowledged.

Signature of the Student
Kumari Riya
21P039012
Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences

Date:
Place: Goa University

COMPLETION CERTIFICATE

This is to certify that **Kumari Riya** has satisfactorily completed the thesis entitled "**Effect of pH on Copper-tolerant *Penicillium chrysogenum***" submitted to **Goa University** for the award of the degree in Master **of Science, Marine Microbiology** is a consolidation record of original and independent work carried out by herself, a student of MSc. Marine Microbiology under the supervision and guidance of Dr. **Nikita P. Lotlikar** at the School of Earth, Ocean and Atmospheric Sciences, Goa University, during the period of **15th November 2022 – 15th April 2023**, in partial fulfillment of the requirement of MSc. Marine Microbiology Degree of the University.

It has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

Lotlikar

Signature of Guide
Dr. Nikita P. Lotlikar (Assistant Professor, Marine Microbiology)

Date: 02/05/2023

SCHOOL OF EARTH, OCEAN & ATMOSPHERIC SCIENCES
GOA UNIVERSITY

Signature of the Dean of the School
Dr. Chandrashekher U. Rivonker
Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences
Date:
Place: Goa University

School Stamp

Dr. C. U. Rivonker,
Senior Professor and Dean,
School of Earth, Ocean
& Atmospheric Sciences,
Goa University,
Goa - 403 206.

COMPLETION CERTIFICATE

This is to certify that **Kumari Riya** has satisfactorily completed the thesis entitled "**Effect of pH on Copper-tolerant *Penicillium chrysogenum***" submitted to **Goa University** for the award of the degree in Master **of Science, Marine Microbiology** is a consolidation record of original and independent work carried out by herself, a student of MSc. Marine Microbiology under the supervision and guidance of Dr. **Nikita P. Lotlikar** at the School of Earth, Ocean and Atmospheric Sciences, Goa University, during the period of **15th November 2022 – 15th April 2023**, in partial fulfillment of the requirement of MSc. Marine Microbiology Degree of the University. It has not previously formed the basis for the award of any Degree, Diploma, Associate- ship or Fellowship or any other similar title to any candidate of this or any other University.

Signature of Guide
Dr. Nikita P. Lotlikar (Assistant Professor, Marine Microbiology)

Date:

Signature of the Dean of the School **School Stamp**
Dr. Chandrashekher U. Rivonker
Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences Date:
Place: Goa University

Dedicated to

My Mummy and Papa

And caring Elder Brother……

ACKNOWLEDGEMENT

Good work is a sign of combined efforts of all the ones contributing towards successful completion of it. The success of any project is never limited to the individual who is undertaking the work. Hence my work would be incomplete without thanking all the people who have helped and supported me throughout my period of research and their names deserve to be mentioned with gratitude. I take this opportunity to express my gratitude and thank some of the personalities involved with me during my studies and the completion of this thesis. It was a learning experience to work on the topic, write papers, work responsibly and stay focused. When I look back I realize many people have contributed to this thesis in innumerable ways. It is quite difficult to find the right words to acknowledge everyone. So, if you do not find yourself mentioned in the following pages kindly feel free to drop by, to complain to me about that and help me remember the good of all times…

Above all, I express my deep sincere gratitude to my God for the blessings and good health he had bestowed upon me during my entire research work. First and foremost, my most sincere and deep gratitude to my guide Dr. Nikita P. Lotlikar, Assistant Professor of M.Sc. Marine Microbiology, School of Earth, Ocean and Atmospheric Sciences, Goa University, for the valuable and perpetual guidance, fruitful advice and never ending patience during the research work which has made this thesis a success. Thank you so much Ma'am, for the support and encouragement and making complex situations simple. Ma'am i cannot be grateful and thankful enough for all that you have done for me. I have not only earned a degree, but also learnt to be a better person after knowing you. Thank you once again for being there for me through all the highs and lows of my Master's degree.

I also wish to thank all the Heads of the Department of Marine Microbiology, ever since I joined here, a special thanks to the Program Director, Dr. Priya M. D'Costa (Assistant professor, M.Sc. Marine Microbiology, School of Earth Ocean and Atmospheric Sciences, Goa university) for providing the required materials to complete my entire experiment. Thank you so much Ma'am for providing me glassware.

A special "Thank you" goes to Dr. Varada for helping me in Phase contrast Microscopy, to teach and make me understand about differences in fungal and bacterial growth. Thank you so much Ma'am for answering all my questions and supporting me.

I am thankful to Dr. Samir R. Damare Senior Scientist, CSIR- National Institute of Oceanography and his Student Vruti for getting my samples lyophilized at NIO.

I am grateful for the FTIR analysis carried out at School of Chemical Sciences, Block-E, Goa University, Dr. V. M. S. Verenkar, Dean & Professor of Inorganic Chemistry; and Sir Prajot Chari for the graphical representation of the spectrum.

I am also grateful for the SEM analysis carried by M.G. Lanjewar (Technical Officer-I) at University Science Instrumentation Centre (USIC), Goa University.

I am thankful to Ma'am Sarika Rohidas Naik (Librarian), for providing all the help that was needed in terms of research articles, books and other reference materials.

Not to forget, I am also thankful to the Laboratory assistants and office staff, Ms Vaishali, of School of Earth Ocean and Atmospheric Sciences, Goa university for helping me with everything that was required whenever I needed. My gratitude to my few friends from this university who have all played a role in helping me with my work or by just being there.

I am also deeply indebted to people who indirectly enable me to complete this thesis and made a difference in my life by their out of the way sacrificial help, support and encouragement, providing for what I need. This goes to my Mummy and Papa who struggled and faithfully took care of Me; also, my gratitude to my Elder brother who kept taking care of me during my hard times. Thank you so much for being there with me.

At last but not the least I also want to thank my other family members, cousin relatives, friends and many more well-wishers whose names are not mentioned, but are still in my heart who appreciated me for my work and motivated me. I believe I am lucky to be surrounded by people who have always tried to keep me positive and happy. By saying this, I wish and pray and I am thankful to Almighty God for his blessings and grace who made all the things possible for me till the end.

Kumari Riya

TABLE OF CONTENTS

Chapter One : Diving into Heavy Metal Tolerance: Investigating Fungal Adaptations to Metal Exposure.

1.1 INTRODUCTION

Marine fungi are an ecologically rather than physiologically or taxonomically defined group of organisms. According to the "classic" definition that appears to be universally accepted in the scientific community, marine fungi are divided into two groups, obligate and facultative marine fungi – *obligate* marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, while *facultative* marine fungi are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment (Raghukumar 2008). It has been suggested that indigenous marine species (obligate and facultative) should be separated from nonindigenous species (sometimes referred to as "contaminants" or "transients", *i.e.* terrestrial or

freshwater species that are dormant in marine habitats) based on their germination ability (Kohlmeyer and Kohlmeyer 1979), but in practical terms this is difficult to achieve. Moreover, there is an increasing tendency to identify marine fungi by molecular biological methods that do not require sexually reproducing life stages, for example sequencing of rDNA, instead of traditional approaches based on morphological characteristics.

Marine fungi have an estimated diversity of 1112 marine fungal species in 472 genera (Balabanova et al. 2018). They are frequently present in intertidal zones, and mangroves, but also can be found in extreme environments such as deep-sea sediment, ice and hypersaline waters (Raghukumar C 2008). They act as pathogens and symbionts of other marine organisms, such as algae, corals and sponges and are ecologically relevant due to their performance in biochemical processes such as nutrient regeneration as decomposers of organic matter (Hyde et al. 1998). Species belonging to the genera *Aspergillus*, *Chaetomium*, *Cladosporium, Penicillium* and *Trichoderma* are commonly found in marine environments as facultative marine fungi that originate from terrestrial environments and developed morphological and physiological characteristics that allow them to adapt to marine conditions (Cantrell et al. 2006). *Penicillium* species are among the most common fungi isolated from various outdoor and indoor environments, including marine substrates such as sponges, corals, algae and sand (Hyde et al. 1998; Visagie et al. 2014). In particular, marinederived *Penicillium* species are potential sources of unique bioactive compounds that are produced because of the natural conditions of marine environments (Edrada et al. 2002; Komatsu et al. 2000).

Penicillium chrysogenum the most studied member of a family of more than 350 *Penicillium* species that constitutes the genus (Nielsen et al., 2017), renamed as (*P. rubens).* Like many other species of the genus *P. chrysogenum,* it reproduces by forming dry chains of spores from brush shaped conidiophores. The conidia are typically carried by air current to sites of colonization, the

conidia are from blue to blue-green and molds sometimes exude to yellow pigment. However, based on color itself is not identified, observation by its morphology and microscopic features are needed to confirm its identity. Fungi are ubiquitous members of subaerial and subsoil and in marine environments, often becoming a dominant grouping metal-rich or metal-polluted habitats. Microorganisms have been shown to possess the ability to thrive under extreme pH, temperature and nutrient variability conditions, as well as tolerance to high metal concentrations. Fungi strains, such as *Penicillium*, have shown potential for metal bioleaching. Metals and their compounds can interact with fungi in various ways depending on the type of metal, organism and environment.

They exert toxic effects in many ways, for example by blocking the functional groups of enzymes. The adaptation of fungi exposed to heavy metal ions has been examined to increase the tolerance of fungi for the bioleaching process (Gadd et al. 2010). Biosorption may be simply defined as the removal of substances from solution by biological material (Gadd et al. 2008). Such substances can be organic or inorganic, and in soluble or insoluble forms. Biosorption is the removal of materials (compounds, metal ions, etc.) by inactive, non-living biomass (materials of biological origin) due to "high attractive forces" present between the two.

Biosorption is a physico-chemical process and includes such mechanisms as absorption, adsorption, ion exchange, surface complexation and precipitation. It is a property of living and dead biomass (as well as excreted and derived products) metabolic processes in living organisms may affect physico-chemical biosorption mechanisms, as well as pollutant bioavailability, chemical speciation and accumulation or transformation by metabolism dependent properties. Biosorption are passive, metabolism independent physico-chemical interactions between heavy metal ions and microbial surfaces (Macek and Mackova, 2011).

It could be interpreted that biosorption process consists of two phases: One phase is a solid phase (biomass / sorbent / biosorbent / biological material) and another is a liquid phase (solvent, usually water) containing a dissolved species to be sorbet (sorbate/ metal ion) principally, process, which

is metabolism- independent accumulation of metals, is often rapid (Volesky 2007). Generally, biosorption is a property of certain types of inactive, dead, microbial biomaterials to bind and concentrate heavy metals from even very dilute aqueous solutions (Gupta et al. 2015). Biomass exhibits this property, acting just as a chemical substance, as an ion exchanger of biological origin. It is particularly the cell wall structure of certain algae, fungi and bacteria, which was found responsible for this phenomenon.

However, the feasibility of the application of other fungal species, such as *Penicillium chrysogenum*, in heavy metal stabilization was seldom studied. In addition, the fungal remediation mechanisms involved are complex and vary with fungal species and metals (Hartley et al., 1997). In the mining area, the microbial resources are abundant, and some microorganisms have been resistant to heavy metals for a long time in the polluted environment. Therefore, the isolation and screening of tolerant strains in the mining area is more conducive to the treatment of heavy metal pollution and the prevention of ecological risks. However, the survival and repair effect of microorganisms will be restricted by environmental conditions such as temperature, pH and heavy metal concentration. In general, the higher the tolerance of the fungus, the greater its potential for application for environmental remediation. Currently, *Penicillium* has been shown to be tolerant to a variety of heavy metals. In the presence of high concentrations of heavy metals, some microorganisms with a certain tolerance to heavy metals survive, and some reduce toxicity through biotransformation or metabolic activities.

Fungi are known to be a robust group of microorganisms that can adapt to changing environments efficiently, produce high yield of biomass, and can easily be genetically and morphologically manipulated. Fungi appear to have high resistance to the large amount of heavy metals and simultaneously can accumulate micronutrients (Cu, Zn, Ni, Co and Mn) and non-nutrient metals (Cd, Pb, Hg and Ag) (Gaur and Adholeya, 2004). Cell wall of fungi is composed of chitin, lipids, mineral ions, polysaccharides, polyphosphates, and proteins. Several mechanisms were included in heavy metal bioremediation by fungi as they could be complexed and oxidation states can be

changed, metal ions by extracellular and intracellular precipitation, energetic uptake or by converting the valency of the metal ions, many fungi also can accumulate metals into their spores and mycelium (Gadd et al. 2010).

Metal tolerance/resistance has been defined as "*the ability of an organism to survive metal toxicity by means of one or more mechanisms devised in direct response to the metals concerned*" (Zafar et al. 2013). Metal tolerance by filamentous fungi has been associated with their sites of isolation, toxicity of the metal tested, its concentrations in medium, and on the isolate's competence. However, there is a dearth of knowledge of the growth response and heavy metal tolerance of filamentous fungal species isolated from marine environments. This study was therefore designed to assess the growth response and tolerance/resistance of select filamentous fungus to varied concentration of select heavy metal (Adebusoye et al. 2015).

1.1.1 Metals

Metals: toxicity and pollution

Metals are ubiquitous in the environment, existing as normal constituents of the earth's crust, and are also present in trace amounts in soil, water and plants. They are also introduced into the surroundings by man-made activities. Since they are non-degradable, they persist and accumulate in the environment, giving rise to pollution. "Heavy metals" are chemical elements with a specific gravity that is at least 5 times the specific gravity of water (Tchounwou et al. 2012). Some wellknown toxic heavy-metallic elements are manganese (7.35), iron (7.9), copper (8.93), cadmium (8.65), lead (11.34), arsenic (5.7), and mercury (13.546) (Lide 1992). Metals such as potassium, sodium, magnesium, calcium, cobalt, copper, manganese, iron, molybdenum, nickel and zinc, are nutritionally essential for microbial growth at low concentrations and are therefore referred to as trace elements (Kabata- Pendias 2010, Glass and Orphan, 2012); these elements, or

their derivatives, are commonly found naturally in food, in fruits and vegetables, and also in commercially available multivitamin products. These same metals can be detrimental in excessive levels. Metals, such as Fe, Zn, Cu and Co scanty concentrations are essential elements for metabolism and growth of fungi (Gadd 1986). Fungi and yeasts can also store innutritious elements such as cadmium, mercury, uranium, silver and gold in themselves up to a constant level. In contrast, metals such as mercury, cadmium, lead and tin, are non-essential for biological functions and are therefore grouped as toxic metals (Lide 1992; Duffus 2002); their accumulation over time can cause serious ailment. Essential metal ions are important as they form part of vital enzymes and are used for metabolic processes, such as copper which is an essential cofactor in various redox enzymes systems like cytochrome c oxidase, lysyl oxidase and more (Agranoff and Krishna, 1998). Metals in the aquatic econiches may exist in soluble or as particulate form, and as free hydrated ions or as complex ions, chelated with inorganic ligands, or they may be complexed with organic ligands such as amines, humic or fulvic acids and proteins (Naja et al. 2008). Several adverse health effects of metals are known and exposure to heavy metals in the aquatic ecosystem particularly that of dissolved metals that accumulate in the living tissues throughout the food chain culminating with humans, has become a serious threat today. These natural systems exposed to metal contamination require serious attention for the survival of the biota (Gupta et al. 2000). The toxic levels of metals varies greatly, with virtually all metals, whether essential or nonessential, exhibiting toxicity above a certain threshold concentration, which may be extremely low for a poisonous metal like mercury, and can be comparatively high for an essential element such as sodium in its chloride form.

Furthermore, different forms of the same metal have different biological and toxicological properties. This can be exemplified with sodium chloride, which is essential in our diet; however, sodium metal if swallowed produces serious, even fatal, injury. Likewise, chromium alloys have

been used safely in medical and dental prostheses, but the chromate salt is identified as a carcinogen (Roberts 1999).

Metals play a surprisingly central role in infection processes, as they serve as cofactors in a multitude of enzymes-including many with direct and indirect roles in virulence, such as metaldependent superoxide dismutases (SODs), metalloproteases or melanin-producing laccases (Gerwien et al. 2017). Especially the first-row transition metals- manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), and copper (Cu)-provide the necessary redox and catalytic activity for many important biological processes.

Copper performs a myriad of functions in biological systems, making it an element that is essential for the existence of all currently known life forms. In humans, for example, copper is third in abundance amongst the essential heavy metals, after iron and zinc (Barceloux 1999). All living organisms require copper as a catalytic cofactor for basic biological processes such as respiration (Puig and Thiele 2002). The physiological oxidation states of copper are Cu1+ and Cu2+ (Shleev et al. 2005). In enzymology, copper is somewhat similar to iron in that it functions in a series of oxidases (Whittaker 1999), oxygenases (Blain et al. 2002), and low molecular weight electron transfer proteins that are reminiscent of ferredoxins (Mogi et al. 1994).

Metals which do not have any essential functions are classified under toxic metals. An important aspect of Tolerance may be defined as "*the relative ability of an organism to grow or thrive when subjected to an unfavorable environment factor or toxin*" (Green and Clausen 2003). In general, two mechanisms have been proposed for heavy metal tolerance in fungi; one is extracellular sequestrations and other one is intracellular physical sequestration of metal by binding to protein or other ligands to prevent it from damaging the metal sensitive cellular targets (Igiri et al. 2018).

Metals may enter the human body through food, water, air, or absorption through the skin in agriculture, in manufacturing in pharmaceuticals and industry, or residential settings. Industrial exposure accounts for a common route of exposure for adults while that of; Ingestion is the most common route of exposure in children

(Roberts 1999). Small children may develop toxic levels from the normal hand-to-mouth activity of those who come in contact with contaminated soil or by consuming non-food related objects (Dupler 2001). The environment has been under increasing pressure due to anthropogenic activities and fast growing industrialization including mining, ore and metal refining, metal fabrication, metal plating, manufacture of alloys, steel, batteries, electrical equipment, as well as other industries of petroleum, fuel transportation, pesticides, paints, textile dyes, sludge, waste treatment, and chemical industries. This generates large quantities of toxic aqueous effluents containing toxic metals, metalloids, radionuclides as well as various organic pollutants in the form of particulate matter, aerosols, wastewater, solid waste and sludge. Development of the negative effects on the ecosystems and the health hazards associated with metal pollutants that accumulate throughout the food chain makes it mandatory for industries to follow the standards of pollutant detection and its proper treatment. Thus, the industrial treatment methods aim at preventing or limiting toxic discharges through conventional measures that demand a very high expenditure. As a result, several concepts of biological metal purification and also biological treatment of environmental contamination have been proposed, some of which having been employed in pilot or industrial scale (Bargar et al. 2008; Macek et al. 2008). A more suitable alternative has been found in bioremediation, in which fungi have been used as effective agents (Gadd 1993; Iqbal et al. 2006; Zafar et al. 2007; Osaizua et al. 2014).

In the microbial community, bacteria have been studied to a wide extent, however due to their size-to-mass ratio and limitations of handling, fungi are preferred organisms. Compared to bacteria, yeast or fungi prove to be better models to study heavy metal tolerance as its eukaryotic make up show more similarities at mammalian cell and organelle levels (Borut et al. 2010) besides being tolerant to high concentrations of pollutants (Gadd, 2001). Fungi are known to tolerate and detoxify metals by several mechanisms, including valence transformation of metals, biosorption to cell wall and pigments, decreased transport and sequestration. The sorption capacity of microorganisms such as fungi, algae and plants for removal of heavy metal ions, radio–nuclides and, in some cases, lowering the toxicity of such substances have interested microbiologists, biotechnologists and

environmental engineers (Mishra et al. 2011). Compared to bacterial leaching, fungal leaching has the following advantages: (1) ability to grow under higher ph and thus is more suitable in bioleaching of alkaline solid wastes; (2) ability of excreted metabolites i.e. organic acids to form complexes with metal ions, thus reducing its toxicity to the biomass (Burgstaller and Schinner, 1993; Castro *et al.,* 2000).

The toxicity of HMs toward fungi and fungal-like microorganisms manifests mainly at morphological level (Baldrian 2003; Vashistha and Chaunhary 2019). In turn, the HM-dependent response of filamentous pathogens at cellular level is similar to that in animals and plants since these toxic elements provoke oxidative damage and induce antioxidant machinery.

1.1.2 Metal-tolerant fungi

Metal-contaminated environments, which may be lethal to most microorganisms, leads to the development of metal tolerance mechanisms in some microorganisms present (Wood and Wang 1983). A wide range of fungi from all major taxonomic groups are reported to occur in metal polluted habitats and are capable of surviving as well as growing in the presence of toxic concentrations of heavy metals.

Fungi can adapt and grow under high metal concentrations (Anand et al. 2006), developing tolerance mechanisms (Bellion et al. 2006), such as metal binding by cell-wall and/or melanin pigment, decreased transport or cell wall impermeability, valence transformation, extracellular precipitation of metal ions by production of organic acids or by formation of metal sulfides (Gadd 1993; Gadd 1994; Nies 1999; Baldrian 2003; Sastry et al. 2003; Malik 2004). Functional groups of fungal cell walls involved in binding of metal ions have been documented (Akhtar et al. 1996; Akar et al. 2007; Bairagi et al. 2011; Gazem and Nazareth 2012; Yuan et al. 2012). Intracellular

chelation of metals in the cytosol by a range of functional groups, glutathione and metallothioneins, and increased efflux from the cytosol out of the cell, or into sequestering compartments, are also key mechanisms conferring tolerance (Nies 1999; Bellion et al. 2006; Hemambika et al. 2011; Shoaib et al. 2015). Metal toxicity due to increasing concentrations might occur through the displacement of essential metals from their native binding sites and also results in delay or inhibition of conidiogenesis (Levinskaitė 2001). The nature of metal salts – sulfate, nitrate and chloride, may also add to the toxicity of the metal ion on the growth of fungi, in varying proportions (Shoaib et al. 2015).

The cell wall composition is the characteristic feature of the fungal species implicated in metalbinding (Sağ 2001). The fungal cell wall comprises mainly carbohydrates: chitin (3-39%) and chitosan (5-33%), and to a lesser degree, polyuronide and polyphosphates (2-12%), lipids (2-7%) and proteins (0.5-2.5%), with significant variations between diverse taxonomic groups (Fourest and Roux 1992), giving each group its specific features, which play a role in bestowing on an individual species its distinct metal-binding characteristics. Moreover, metals in excess are known to induce stress in fungi by generating detoxifying enzymes and morphological changes (Lloyd

2002; Taboski et al. 2005; Nazareth and Marbaniang 2008; Gadd 2010; Xie et al. 2010; Gazem and Nazareth 2012; Fomina and Gadd 2014). As a result, several concepts of biological metal purification and also biological treatment of environmental contamination have been proposed, some of which having been employed in pilot or industrial scale (Bargar et al. 2008; Macek et al. 2008). Metals, such as Fe, Zn, Cu and Co scanty concentrations are essential elements for metabolism and growth of fungi (Gadd 1986). Fungi and yeasts can also store innutritious elements such as cadmium, mercury, uranium, silver and gold in themselves up to a constant level.

1.1.3 Metal removal from solution

Various waste biomaterials, micro-organisms: bacteria, fungi, yeast and algae have been reported for the removal of metal ions from aqueous solutions, using live, or treated dead cells of fungi (Kapoor et al. 1999). Biological treatments of waste, offer a means of reduction of toxic metal levels to environmentally acceptable limits in a cost-effective and eco-friendly manner, with minimization of the volume of chemicals, and high efficiency in detoxifying very dilute effluents (Gadd 1994; Volesky and Holan 1995; Magyarosy et al. 2002; Naja et al. 2008; Bairagi et al. 2011). The greatest demand for metal sequestration at present comes from the need to immobilize the metals released to the environment by anthropogenic technological activities (Volesky 2007). The capacity of a given organism for metal tolerance, its removal and/or uptake from solution must be initially determined, in order to evaluate the efficiency of the organism as a potential biosorbent. Types of biosorbents utilized for removal of metals from aqueous solution are agricultural wastes, or biomass of organisms such as bacteria, fungi and algae. The efficiency and capacity of a given biosorbent is influenced by various aspects such as the pH, time of contact, initial metal concentration and type of metal ions. pH of solution: pH influences the solubility of metals and the ionization state of the functional groups of hydroxyl, carboxyl and phosphate of the fungal cell wall that are negatively charged and participate in sequestering the cationic metal ions (Say et al. 2001). At low pH, the cell wall ligands may become protonated by interaction with H3O+ ions of the aqueous medium that competes with the metal cations for the binding sites, thus causing inhibition in binding of the metal ions. As pH decreases, desorption of the metal will also occur. As pH increases, the ligands develop a negative charge, which correspondingly increases sorption capacity for metal ions. The pH of the solution also affects speciation of the metal in solution; at alkaline pH they form hydroxides and get precipitated from solution (Dursun 2006; Naja et al. 2008; Gazem and Nazareth 2012).

Time of contact: Interaction between the fungal cell and metal ions, initially proceeds speedily till equilibrium is attained, since functional groups of the cell wall are amply available for binding of the metal ions, followed by a steady rate seen as a plateau in the sorption curve; with time, the functional ligands get saturated, resulting in a fall in the rate of sorption (Volesky 2004).

Metal removal from solution by microorganisms may occur by the process of biosorption, bioaccumulation and bioprecipitation. Bioaccumulation is an active, metabolically-mediated active transport system process, occurring specifically in living organisms (Tobin et al. 1994; Kapoor and Viraraghavan 1995; Singh 2006; Naja et al. 2008). Bioprecipitation is reported to occur via detoxification of metals by reduction or formation of metal sulfides (Sastry et al. 2003); the precipitation of insoluble metal complexes occurs through the activities of membraneassociated sulfate reductases.

Binding to the cell is called biosorption. The cell surface of microorganisms is negatively charged owing to the presence of various anionic structures, such as glucan and chitin. This gives microorganisms the ability to bind metal cations. Metal tolerance by filamentous fungi has been associated with their sites of isolation, toxicity of the metal tested, its concentration in medium, and on the isolates competence (Maghsoodi et al. 2007).

Biosorption is a physico-chemical process, that occurs by a rapid, passive immobilization of metals by the biomass, independent of metabolic energy, and can therefore be accomplished not only by live cells, but also by dead biomass as well as by cellular components (Fourest and Roux 1992; Zucconi et al. 2003); it forms the main mechanism in fungi, the process occurring mainly through an interaction of metal with functional groups on the cell-wall (Gadd, 1994; Volesky 2007; Naja et al. 2008; Gazem and Nazareth 2012; Yuan et al. 2012). The use of dead biomass is also advantageous as it is unaffected by the toxicity of metals, limited nutrient availability or any other such adverse circumstance (Gadd 1990). Both live and dead cells of fungi can be manipulated to increase their capacity for the sorption of toxic elements and valuable metals (Arıca et al. 2001).

The metal sequestration of fungal cell could be as a result of:

1. Van der Waals forces wherein uncharged atoms and molecules are loosely bound in the matrix by electrostatic attraction;

2. Ionic bonds between a metal cation and an ionic reactive/functional group of the binding matrix of the cell wall: polysaccharides, proteins and pigments involving

3. Chemical groups such as hydroxyl, carboxyl, carbonyl, sulfhydryl, thioether, sulfonate, amino, amido, amine, imine, amide, imidazole, phosphate and phosphodiester groups;

4. Extracellular precipitation, complexation and crystallization;

5. Transformation of metal species by oxidation, reduction, methylation, dealkylation;
6. Intracellular accumulation/ compartmentation energy-dependent, active uptake by live cells and passive removal by sorption; Metal-binding proteins and peptides – metallothioneins, phytochelatins, siderophores;

7. Chromosomal or plasmid mediated sequestration.

These factors may be operative individually or in combination to sequester metals.

1.1.4 Response of fungi to metal stress

Metal stress causes overall reduction in growth of the fungal colony and changes in morphogenesis, such as slow and/or delayed conidial germination and growth, compact mycelial formation. This serves to protect the fungus against the strain of metal toxicity, increased or decreased pigment formation, and delay or inhibition of conidiogenesis (Gadd 1982; Levinskaitė 2001; Ezzouhri et al. 2009; Nazareth et al. 2012). As well as micromorphological anomalies such as irregular mycelia, swollen apical tips, and thickened cell wall which may be due to augmented chitin deposition, as chitin is responsible for giving physical protection, structural form, rigidity and

strength (Cooke and Whipps 1993; Ram et al. 2004; Akar and Tunali 2006; Nazareth and Marbaniang 2008; Farrag 2009). The dematiaceous fungi have an additional line of defense during growth in presence of metals by way of enhanced production of melanin (Henson et al. 1999; Ramsay et al. 1999; Sun and Shao 2007; Belozerskaya et al. 2010; Eisenman and Casadevall 2012). Melanins are also known to aid survival of the fungi under varied stress conditions (Gadd and de Rome 1988; Taboski et al. 2005; Griffith et al. 2007). The variations in cell-wall structure and the functional groups involved, as a growth response to presence of metals, has been determined by scanning electron microscopy and Fourier transform infrared spectroscopy analysis (Césarini 1996; Henson et al. 1999; Pethkar and Paknikar 2001; Bairagi et al. 2011; Iqbal et al. 2011, Gazem and Nazareth 2012; Yuan et al. 2012). Irregularities in morphology produced, are also seen as a part of stress response by fungi to other adverse conditions of extreme environments such as that of pressure (Damare et al. 2006) or salt (Nazareth and Gonsalves 2014).

1.1.5 Application potential of metal-tolerant fungi

Metal-tolerant fungi can be used as economical biological agents in various aspects of industrial biotechnology. Metal nanotechnology is a rapidly growing field of research with vast application potential. Bio-based synthesis of metal nanoparticles has gained great importance as a green technology, without use of harmful chemicals. Biosynthesis of nanoparticles can yield varied sizes and shapes, with controlled monodispersity (Ahmad et al. 2002). Nanoparticles have also attracted attention in the medical field such as that of silver nanoparticles in drugs and in targeted drug delivery systems, magnetic nanoparticles for magnetic resonance imaging and heating mediators for cancer therapy (Ito et al. 2005; Syed et al 2013; Moghaddam et al. 2015). The use of microorganisms in the synthesis of nanoparticles is a relatively new and exciting area of research with considerable potential for development.

1.1.6 Bioremediation

Due to the immense drawbacks of physical and chemical methods to remove metals from solutions. Industries seek alternative bioremediation measures, which are economical and ecofriendly, for pollution control. This may be accomplished through processes such as sorption and accumulation (Fomina and Gadd 2002; Adeyemi 2009), augmentation (Lebeau et al. 2008), solubilization, reduction, precipitation, mineralization and methylation (Gadd 2010). The uptake of metals by fungal biomass appears to involve a combination of two processes; bioaccumulation (i.e active metabolism dependant processes, which may include both transport into the cell and partitioning into intracellular components) and biosorption (i.e the binding of metals to the biomass by processes that do not require metabolic energy. Rather than searching thousands of microbial species for particular metal sequestering features, it is beneficial to look for biomasses that are readily available in large quantities to support potential demand. While choosing the biomaterial for metal sorption, its origin is a major factor to be taken into account. Metal-tolerant fungi can therefore offer potential for application in metal removal in bioremediation measures.

1.1.7 Fungal-Metal Interactions

The increasing applications of fungal-metal interactions have led to the need for research on their contributions in the field of mycoremediation. In nature, metals serve as micronutrients required for fungal growth, however, in excess they can influence homeostatic systems (Ahmad et al. 2013). For example, in the presence of water, copper sulfate (CuSO4) hydrates to copper (2) sulfate pentahydrate (CuSO4 5H2O) and then dissociates into Cu2+ +SO4. Upon dissociation, Cu2+ can then be reduced by fungal proteins for uptake (Bundschuh 2018; Kittler 2010; Mussin et al. 2019).

Some fungal pathogens heavily rely on copper exporters to prevent host-enacted copper toxicity or import machinery to maintain virulence (Festa and Thiele 2011).

Mycoremediation has much significance due to its capacity for a passive, rapid means of metal removal from solution, and the high surface – volume ratio of fungal mass. Mycofiltration, a variation of mycoremediation, uses mycelial mats as biological filters. Mycoremediation can also be used for metal contaminated soils as fungal hyphae can penetrate the soil, having an advantage over bacteria. Fungal tolerance towards multi-metals is of high importance both for fungal survival in contaminated environments and for their application in bioremediation of industrial effluent (Levinskaitė 2001). The success of a mycoremediation process is to obtain the right fungal species for a specific pollutant (Singh 2006). Although studies have been carried out on the use of fungal biomass for metal removal from solution, a further understanding of the varied responses of diverse genera and species, with regard to tolerance levels to different metals, and their metal sorption capacities, would serve towards selection of a more effective and efficient biosorbent. High salt concentrations may also form part of many industrial processes and effluents. The capacity for metal tolerance and sorption by marine-derived fungi with high levels of halotolerance make these isolates valuable for use in bioremediation. *Penicillium* species have been shown to be able to grow at high concentrations of salt as well as in its absence, while also possessing high resistance to heavy metals and hydrocarbon degradation efficiency, and could be used as agents for abatement of these pollutants in hypersaline conditions, as well as in non-saline environment (Marbaniang and Nazareth 2007; Leitão 2009).

1.2 LITERATURE REVIEW

Heavy metals damage cell membranes, alter functioning of enzymes, inhibit protein synthesis, denature protein and damage the structure of DNA. Toxicity is mainly created by the dislocation of essential metals from their real binding sites or ligand interactions (Olaniran et al. 2014). Microbes such as *Penicillium, Aspergillus, Pseudomonas, Sporophyticus, Bacillus* and *Phanerochaete* have been reported for their ability to efficiently remove heavy metals such as chromium, nickel, uranium ions, cadmium and copper ions from polluted environments with heavy metals (Kapoor et al., 1999; Congeevaram et al., 2007). During the last few decades, biosorption of a number of metals such as Al, Au, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Th, U and Zn by a variety of biomass, including bacteria, fungi and algae have been studied (Volesky and Holan 1995; Davis et al. 2003; Mehta and Gaur 2005; Romera et al. 2006). The biosorption of copper and cobalt by filamentous fungi has received a great deal of attention in recent years, as an emerging technology for minimizing the distribution of these heavy metals from mining wastes, e-wastes, and industrial wastewaters (Hussein et al., 2004; Dhankhar and Hooda, 2011; Ahemad and Kibret, 2013). The presence and persistence of elevated levels of heavy metals in the environment causes harmful effects on humans and other organisms mainly due to moderate accumulation over time (Leenu and Sheela 2016). Biosorption is a term which involves the use of microbes to detoxify and control environmental contaminants. Recently, it has received increasing attention to clean up polluted sites (Farhadian et al., 2008). By using non-viable microbial biomass including algae, fungi and bacteria, this alternative method has been considered the preferred approach for removing heavy metals and radionuclides from the aqueous solution (Sar and D'Souza, 2001). Propositions have been made to use filamentous fungi in combination with other microorganism matrices, such as bacteria, to increase biosorption efficiency. The resistance mechanisms developed by these microorganisms mainly for their survival shows that they are highly efficient for detoxifying metal ions in aqueous solutions (Xie et al., 2010; Mohammadian et al., 2017).

1.2.1 Study of Copper metal

Copper contributes to several physiological processes in plants, namely, photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production, including disease resistance (Kabata-Pendias and Pendias 2001). Although copper is an essential element, free copper ions are highly reactive and catalyze formation of toxic reactive oxygen species inside cells (Ercal et al. 2001).

Copper contamination comes from industrial effluents and seepage, pesticides added to soil, from old copper water pipes and corrosive water that comes in contact with pipe fittings or joints. In addition, copper is known as a widespread heavy metal contaminant in industrial wastewater, and removal of copper from wastewater is necessary to minimize ecological impacts (Rehman et al. 2008). Copper resistance has been demonstrated in a number of microorganisms including *Aspergillus niger, Aspergillus oryzae, Penicillium chrysogenum, Rhizopus stolonifer and Saccharomyces crevisiae* (Mattuschka et al. 1993, Huang and Huang 1996; Hashem 1989; Gilotra and Srivastava 1997).

1.2.2 Study of the different pH of the solution

The pH of the solution greatly affects the uptake of ions by fungi. An increase in pH values results in increase in cation uptake which was found with fungi biomass (Kuyucak and Volesky, Paknikar, et al 1993; Mattuschka et al., 1993; Fourest, et al., 1994; Sag and Kutsal, 1995; Modak and Natarajan, 1995). A general trend, which was observed, is that the metal uptake is negligible at very low pH values of pH 1 to 2 due to competition between positive ions for binding sites of the biosorbent; the metal uptake increases with the increase in pH from pH 3 to 5, and an optimum of pH 5 to 7 was reached when the uptake is maximum, beyond which a reduction in the uptake was observed, attributed to reduced solubility and precipitation

of base metals. However, the uptake of precious metals and radionuclides are reported at alkaline pH of 8 to 10 (Modak and Natarajan, 1995). According to (Mahadevan and Tatum 1995), the increase in biosorption when raising the pH would indicate the involvement of negatively charged groups.

At highly acidic conditions, Fr and H30+ ions may compete with metal cations for binding sites on the biosorbent. As the pH levels increase, more ligands with negative charge would be exposed, thereby increasing the attraction of positively charged metal ions, while at neutral to alkaline pH, most metals precipitate, thus making them unavailable for biosorption (Paknikar et al., 1993; Modak and Natarajan, 1995); The receptive sorbent groups can also be metal - specific (Muraleedharan et al., 1991) for example, amongst all the tested metals, such as copper, cobalt, chromium, cadmium, nickel, zinc, gold and silver, the fungus *C.cladosporioides* preferentially adsorbs gold and silver (Pethkar and Paknikar, 1998). A positive influence on sorption at pH controlled conditions by *Rhizopus arrhizus, Penicillium chrysogenum* and Mucor miehei were observed (Fourest, et al., 1994). The biosorption of cadmium and zinc using *P. chrysogenum* was maximum between pH 4 and 6.0, while in case of copper, chromium and lead was pH 2 (*Paknikar et al., 1993*), the maximal sorption at low pH is a possible indication that not all binding sites are electrostatic in nature (Modal and Natarajan, 1995), and could be attributed to coordinate bond formation which is not pH dependent (Paknikar et al., 1993).

Chapter Two : Copper tolerance in *Penicillium chrysogenum*

2.1.1 Research Aims and objectives

Fungal identification can be carried out either using morphological characteristics or molecular biology techniques, i.e., sequencing. To check for different morphology in fungi, one could observe the physical characteristics of the fungal cells and structures, such as the shape and size of the spores, hyphae, and fruiting bodies.

AIM: The aim of this experiment is to assess effect of pH on the copper tolerance of *Penicillium chrysogenum*,

OBJECTIVE ONE: To determine the ability of *Penicillium chrysogenum* to tolerate different levels of metal concentrations.

OBJECTIVE TWO: To check the effect of pH on copper tolerance in *Penicillium chrysogenum*.

2.1.2 Materials and Methods:

The culture used in this study was pre-isolated from Divar mangroves sediment (15°29'N, 74°12' E). Culture was pre-identified as *Penicillium chrysogenum*, NIOSN-M29 (Accession no.) using molecular techniques (Lotlikar 2018). The culture is multi-metal tolerant, hence metal tolerance index (MTI) in percent, was calculated for the selected isolates using the following formula (Akhtar et al. 2013):

MTI = Colony diameter of isolate in presence of metal/Colony diameter of isolates in absence of metal (control) ✕ 100

Based on the MTI results, it was found that chromium was the most toxic metal amongst the three toxic heavy metals (chromium, cadmium and lead) while copper was found to be toxic amongst the essential heavy metals (copper and nickel). All the initial screenings were carried out on media prepared in SW as the isolates were marine-derived, and impact of pH on the bioavailability of metals was not considered. The metal screenings were carried out to shortlist a few isolates and carry out further analysis. Based on screening, six isolates were chosen to study Cr and Cu toxicity on fungi. Since metals are more bioavailable in acidic pH, further experiments were carried out in DW.

- **Subculturing of the spores of NIOSN-M29:** A pre isolated pure culture of NIOSN-M29 was previously stored in sealed glass vials in the form of lyophilised spores. The spores suspended in PDB medium and incubated at room temperature till the spores germinated. Matt growth was observed at the end of 4 days. Further, the matt was subsequently subcultured in 100 ml PDB medium.
- **Metal Tolerance Assay:**

Preparation of metal stock: A stock of 5000 ppm of copper using distilled water was prepared. The metal solution was sterilized by 0.22 µm filter paper (pre- autoclaved) using a syringe filtration technique. Three different concentrations of metal solution were prepared with PDB and CDB media.

Preparation of media with different Cu metal concentrations: The experimental flask with the matt growth was crushed using glass beads with added broth, aseptically in order to break the mycelia uniformly. Three concentrations (50 ppm, 150 ppm, 300 ppm) of Cu were prepared in triplicates both for static and shaker conditions. Negative controls were also maintained for both the conditions in both media. Chloramphenicol (concentration 20 mg of antibiotic in 200µl ethanol was added to the experimental flask (it is an antibiotic which inhibits any bacterial growth) and

then one ml for the culture was dispensed into each flask (control; 50 ppm, 150 ppm, 300 ppm in static and shaker flask).

Incubation period: Both static and Shaker conditions were incubated at room temperature for 1 to 7 days (Figure 1, 2, 3 and 4).

Chapter Three: Results and Discussion

3.1.1 To determine the ability of NIOSN-M29 to tolerate different levels of metal concentrations.

Figure 1: Effect of different concentrations of copper metal in PDB on NIOSN-M29 in Static condition.

Day 1: Static condition

Day 7: Static condition

In static condition, maximum growth was in control flask which had started to sporulate and eluted yellow color, 50 ppm had more growth as compared to 150, 300 ppm concentrations.

Figure 2: Effect of different concentrations of copper metal in PDB on NIOSN-M29 in Shaker condition.

Day 1: Shaker condition

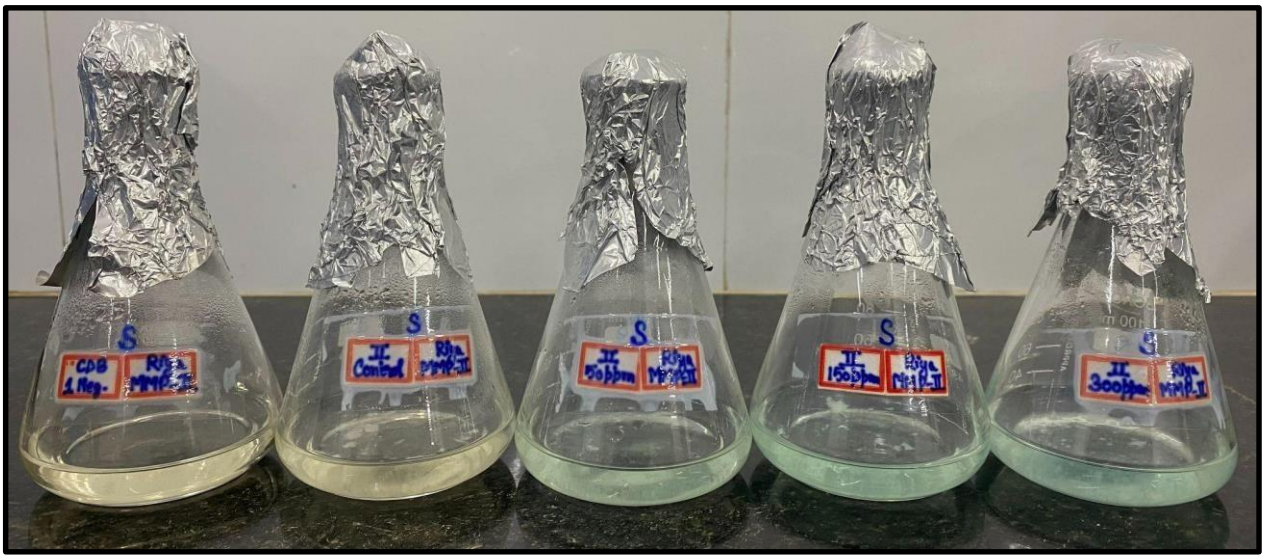
Day 7: Shaker condition

In Shaker condition, maximum growth was seen in control with slimy clumps and round in shape.

50 ppm had more growth as compared to 150 and 300 ppm concentrations.

Figure 3: Effect of different concentrations of copper metal in CDB on NIOSN-M29 in Static condition.

Day 1: Static condition



Day 7: Static condition

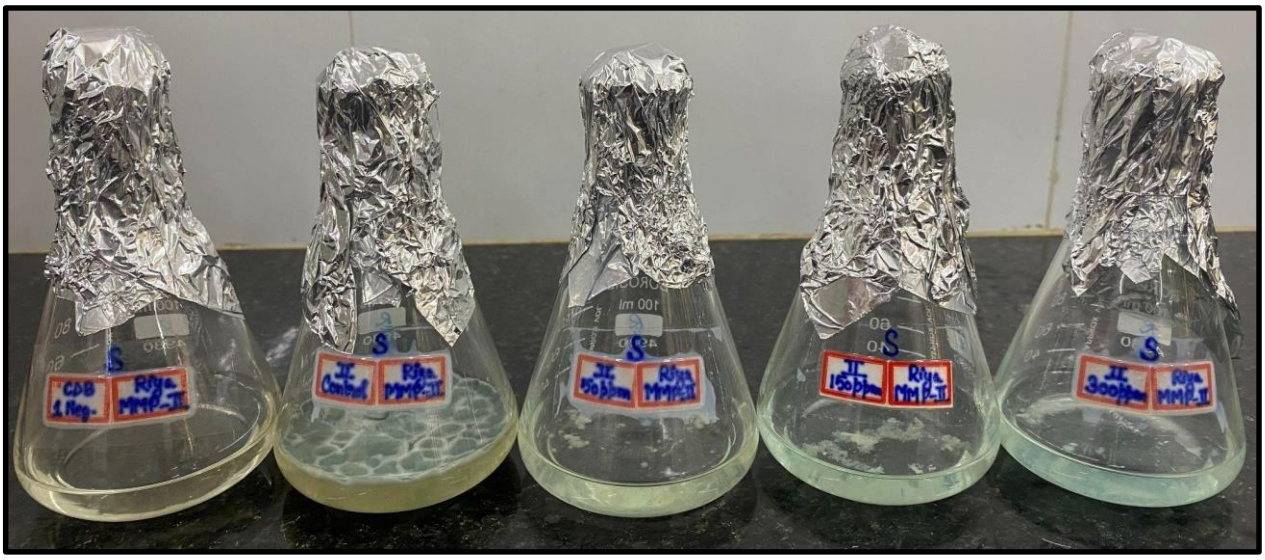


Figure 4: Effect of different concentrations of copper metal in CDB on NIOSN-M29 in Shaker condition.

Day 1: Shaker condition

Day 7: Shaker condition

In Static condition, maximum growth was in the control flask which had sporulated earlier as compared to PDB media, 50 ppm had maximum growth. In Shaker condition, there were larger clumps as compared to PDB media.

(Figure no. 1) On 7th day incubation it shows that the control flask elutes yellow pigment, the yellow pigment produced by NIOSN-M29 in static conditions is likely due to the production of a secondary metabolite called chrysogine or chrysogenin. Chrysogine is a yellowish-orange pigment that is produced by several species of *Penicillium*, including *P. chrysogenum*. The sporulation of NIOSN-M29 on the 7th day of incubation may be a result of the fungal growth and development reaching a certain stage of maturation, where sporulation becomes a natural and necessary part of the fungal life cycle (Figure no. 1, 3). NIOSN-M29 is a filamentous fungus that undergoes a complex life cycle that includes vegetative growth, asexual reproduction through spore formation, and sexual reproduction. The process of sporulation in NIOSN-M29 is triggered by environmental and physiological signals, such as nutrient depletion, pH changes, or the accumulation of specific metabolites.

During the early stages of fungal growth, NIOSN-M29 undergoes vegetative growth, where the fungal mycelium elongates and spreads across the growth medium, absorbing nutrients and building biomass. As the fungal biomass accumulates, the cells begin to differentiate into specialized structures, including conidiophores, which are the structures that produce and release spores.

The clumps formation in a shaker flask during the 7th day of incubation period of NIOSN-M29 may be due to several factors (Figure no. 4). One possible reason is that the fungal mycelia have grown to a certain size and density where they begin to aggregate together to form clumps. This can happen when the nutrient levels in the flask become limiting or when the pH of the media changes.

Another possible reason is that the NIOSN-M29 is producing spores, which can also clump together. Spores are produced as a mechanism for the fungus to spread and propagate, and they can form clumps

when conditions are favorable for their growth. It is also possible that the clumps formation is due to the mechanical agitation of the shaker flask. The shaking can cause the mycelia and spores to collide with each other, leading to the formation of clumps. In any case, the clumps formation is a natural part of the fungal growth process and can be an indication that the culture is healthy and actively growing.

After 7 days of incubation period:

Selection of one set of the replicates were studied for cultural and morphological characteristics of *NIOSN-M29* (Figure no. 7, 8, 9, 10) nature of growth, colour of spores, pigment production, the colony on the reverse and their morphological characteristics, particularly the branching pattern of the conidiophore (Larone et al., 1995; St-Germain and Summerbell, 1996; Sutton et al., 1998; Christensen et al., 1999; de Hoog et al., 2000), as shown in the figures below.

Morphological variations in response to metal: Using phase contrast microscopy.

Lactophenol cotton blue is used to stain the fungal specimens and was observed under 40x and 100x magnifications (Figure no. 7-10), the same was done for different pH levels. As a result, under phase contrast microscopy at 40x and 100x magnification, the hyphal morphology of NIOSN-M29 in static PDB media appeared as follows under different concentrations of copper stress:

Control (no copper stress): The hyphae of NIOSN-M29 appear long, thin, and translucent, with a regular branching pattern. At 40x magnification, the individual hyphae are visible, and at 100x magnification, the branches and septa (cellular walls) become more distinct.

50 ppm of copper stress: The hyphae of NIOSN-M29 may appear slightly shorter and thicker than the control, with some irregular branching patterns. At 40x magnification, the hyphae appear similar to the control, but at 100x magnification, there may be some visible irregularities in the septa.

150 ppm of copper stress: The hyphae of NIOSN-M29 may appear significantly shorter and thicker than the control, with more irregular branching patterns and some areas of constriction. At 40x magnification, the hyphae appear noticeably shorter and thicker than the control, and at 100x magnification, there may be visible constrictions in the hyphae and irregular septa.

300 ppm of copper stress: The hyphae of NIOSN-M29 may appear severely stunted and irregular in shape, with many areas of constriction and some hyphae appearing almost completely collapsed. At 40x magnification, the hyphae appear significantly shorter and thicker than the control and may have a visibly distorted shape. At 100x magnification, there may be visible areas where the hyphae have collapsed or irregular septa.

When NIOSN-M29 is grown in a shaker PDB media and subjected to different concentrations of copper stress, the hyphal morphology appeared as follows under phase contrast microscopy at 40x and 100x magnification:

Control (no copper stress): The hyphae of NIOSN-M29 appear long, thin, and translucent, with a regular branching pattern. At 40x magnification, the individual hyphae are visible, and at 100x magnification, the branches and septa (cellular walls) become more distinct.

50 ppm of copper stress: The hyphae of NIOSN-M29 may appear slightly shorter and thicker than the control, with some irregular branching patterns. At 40x magnification, the hyphae appear similar to the control, but at 100x magnification, there may be some visible irregularities in the septa and slightly more visible branching.

150 ppm of copper stress: The hyphae of NIOSN-M29 may appear significantly shorter and thicker than the control, with more irregular branching patterns and some areas of constriction. At 40x

magnification, the hyphae appear noticeably shorter and thicker than the control, and at 100x magnification, there may be visible constrictions in the hyphae and irregular septa.

300 ppm of copper stress: The hyphae of NIOSN-M29 may appear severely stunted and irregular in shape, with many areas of constriction and some hyphae appearing almost completely collapsed. At 40x magnification, the hyphae appear significantly shorter and thicker than the control and may have a visibly distorted shape. At 100x magnification, there may be visible areas where the hyphae have collapsed or irregular septa, and the branching pattern may be difficult to distinguish due to the severe deformation of the hyphae.

In general, the appearance of NIOSN-M29 under copper stress in a shaker PDB media is similar to that in static PDB media. However, the shaking motion may cause the hyphae to appear more fragmented and distorted under high levels of copper stress.

Under phase contrast microscopy at 40x and 100x magnification, the hyphal morphology of NIOSN-M29 in static CDB media with copper stress are described as follows:

Control (no copper stress): The hyphae of NIOSN-M29 appear as long, thin, and highly branched structures with clear septa. At 40x magnification, individual hyphae are visible, and at 100x magnification, the hyphae appear highly segmented due to the septa present at regular intervals.

50 ppm of copper stress: The hyphae of NIOSN-M29 appear shorter and thicker than the control, with some branches showing irregularities in their shape and size. However, the overall morphology of the fungus is still similar to the control. At 40x magnification, the hyphae appear slightly shorter and thicker than the control, and at 100x magnification, the septa may appear slightly distorted.

150 ppm of copper stress: The hyphae of NIOSN-M29 appear significantly shorter and thicker than the control, with many branches showing irregularities in their shape and size, and some areas of constriction can be observed. At 40x magnification, the hyphae appear noticeably shorter and thicker than the control, and at 100x magnification, the septa appear distorted and the branching pattern becomes less regular.

In both 50 ppm and 150 ppm of copper stress, complete NIOSN-M29 can still be observed, indicating that the fungus is still able to grow and maintain its morphology under these conditions. However, the hyphal morphology is altered, with shorter and thicker hyphae and irregularities in the septa and branching pattern becoming more pronounced at higher concentrations of copper. Overall, in shaker conditions, the hyphal morphology of NIOSN-M29 appears less dense compared to static conditions, with individual hyphae appearing more loosely arranged. As the concentration of copper stress increases, the hyphae appear shorter and thicker, with irregularities in the branching pattern and septa becoming more pronounced. At higher concentrations of copper, significant disruption of the hyphal morphology is observed, with the formation of large clumps of mycelium and distorted hyphae.

At the end of the incubation period, all three flasks were filtered using 0.45µ filter paper and biomass production was estimated. The results were plotted in the form of a graph (Figure no. 5, 6).

Figure 5: The effect of NIOSN-M29 to tolerate different levels of metal concentrations on Static Condition.

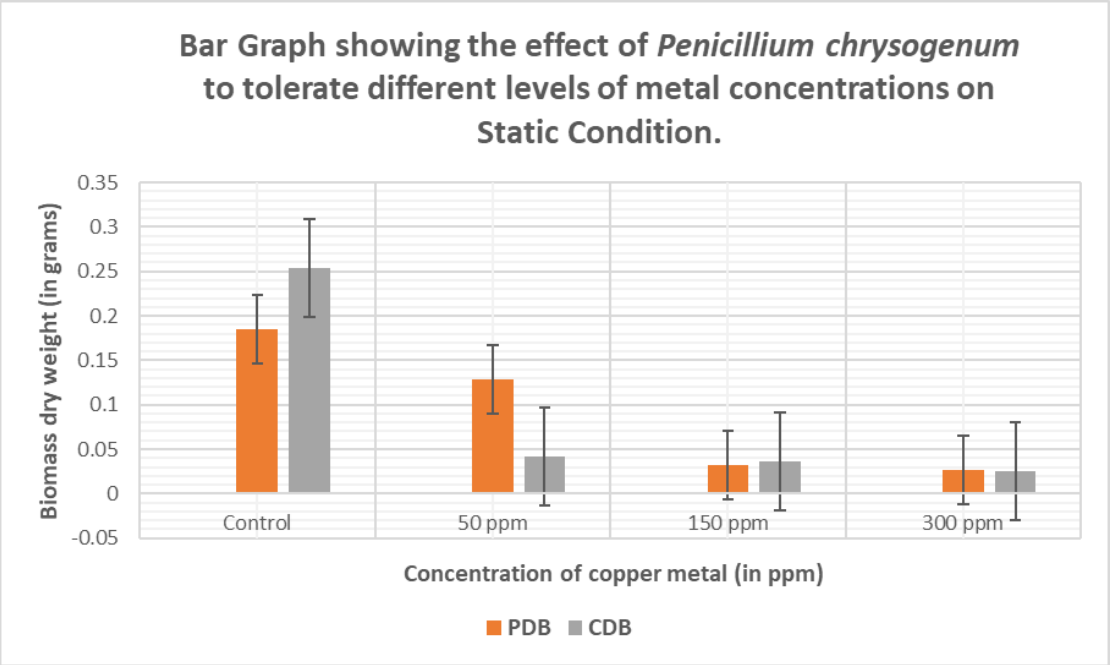
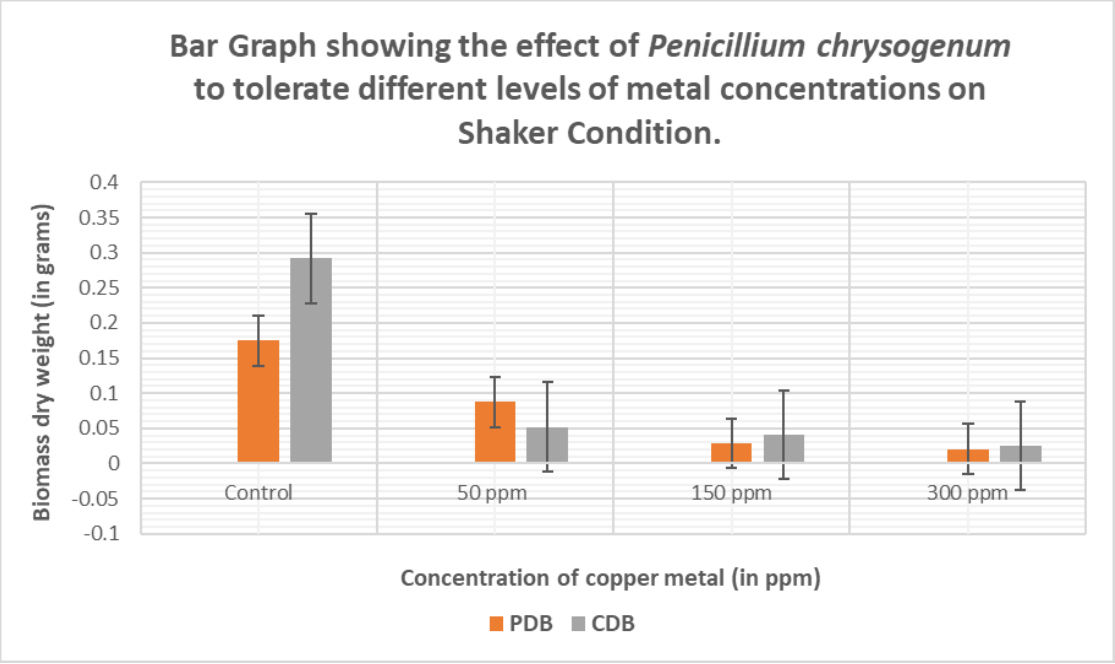


Figure 6: The effect of NIOSN-M29 to tolerate different levels of metal concentrations on Shaker Condition.



As a result, Czapek-Dox broth (CDB) and Potato Dextrose Broth (PDB) are two commonly used media for the growth of NIOSN-M29. Both media have different compositions, which can affect the growth and biomass production of the fungus (Figure no. 5, 6).

CDB is a nutrient-rich medium that contains a balanced mixture of carbon, nitrogen, and mineral sources, which can provide the fungus with all the essential nutrients required for its growth. In contrast, PDB is a medium that contains a high concentration of carbohydrates, especially glucose, which provides a good source of energy for the fungus. However, it lacks some of the essential minerals and trace elements required for optimal growth.

Several studies have compared the growth and biomass production of NIOSN-M29 in CDB and PDB media. These studies have consistently shown that the fungus produces a higher biomass in CDB medium compared to PDB medium. The reasons for this are likely to be multifactorial and may include:

Nutrient availability: CDB medium contains a balanced mixture of nutrients that provide the fungus with all the essential elements required for optimal growth, while PDB medium has a relatively lower concentration of some of these nutrients.

pH: NIOSN-M29 prefers a slightly acidic environment for optimal growth. CDB medium has a pH range of 7.3, while PDB medium has a pH range of 5.1. This difference in pH may be enough to affect the growth and biomass production of the fungus.

Carbon source: PDB medium has a high concentration of carbohydrates, especially glucose, which provides a good source of energy for the fungus. However, the high concentration of glucose in PDB medium can lead to a rapid depletion of nutrients, which may limit the growth and biomass production of the fungus.

In conclusion, Czapek-Dox broth (CDB) has been shown to produce a higher biomass of NIOSNM29 compared to Potato Dextrose Broth (PDB). This may be due to a combination of factors such as nutrient availability, pH, and carbon source.

NIOSN-M29 produced a higher biomass in PDB medium than in CDB medium under 50 ppm copper stress conditions (Figure no. 5, 6).. This suggests that PDB medium may be more suitable for the growth of NIOSN-M29 than CDB medium. The reason for this difference may be due to the different compositions of the two media. PDB medium contains a higher amount of glucose and yeast extract compared to CDB medium, which may provide more nutrients for the fungus to grow and produce biomass. However, it is important to note that the effect of media composition on biomass production may depend on several factors such as the strain of the fungus, the experimental conditions, and the specific nutrients in the media.

High concentrations of copper, such as 150 ppm and 300 ppm, can significantly reduce the biomass production of NIOSN-M29, regardless of the type of medium used (Figure no. 5, 6). At high concentrations, copper can become toxic and damage the cell membrane, proteins, and DNA of the organism, which can lead to growth inhibition and reduced biomass production.

Figure 7: Hyphal Morphology of NIOSN-M29 in PDB Medium Observed under Phase Contrast Microscope.

Static Condition	**40x** Magnification	**100x** Magnification

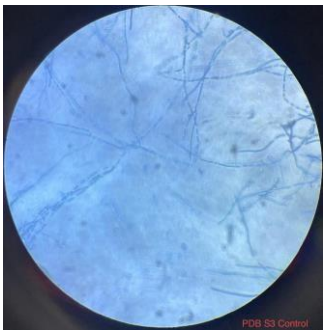
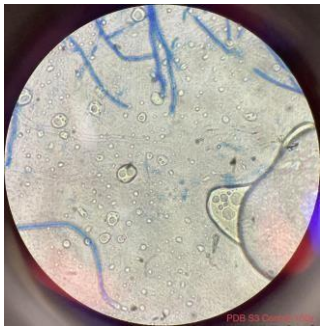
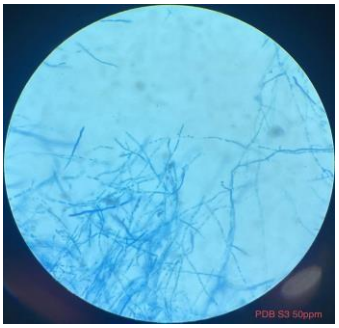
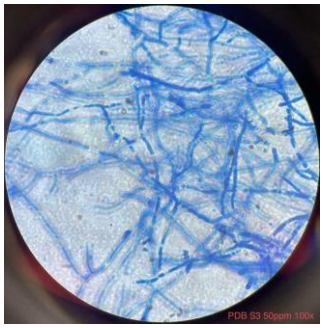
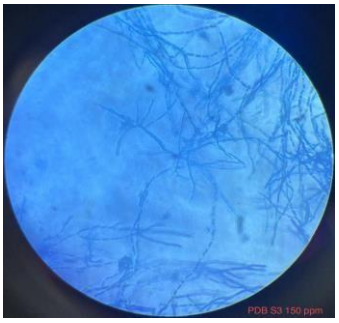
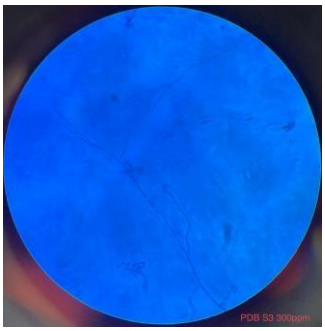
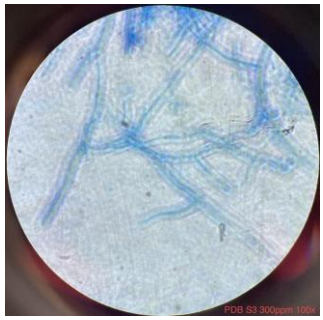
Control PDB		
50 ppm Cu		
150 ppm Cu		
300 ppm Cu		

Figure 8: Hyphal Morphology of NIOSN-M29 in PDB Medium Observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification

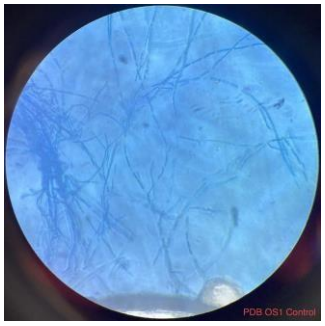
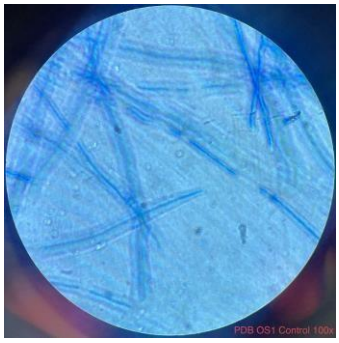
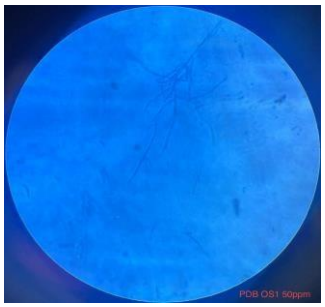
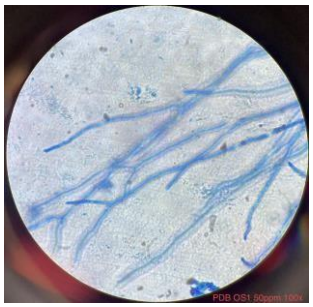
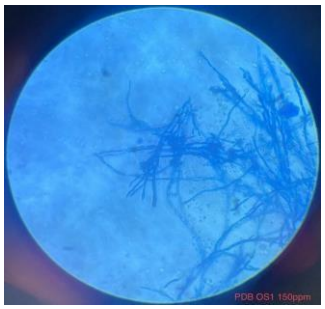
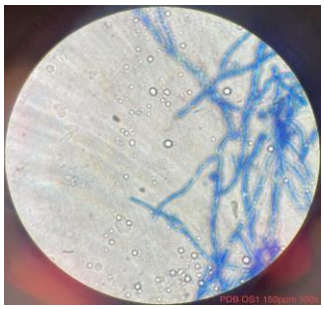
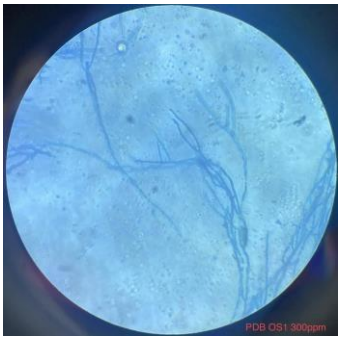
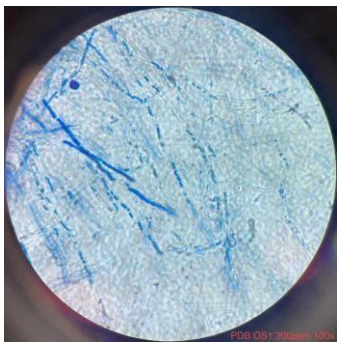
Control PDB		
50 ppm Cu		
150 ppm Cu		
300 ppm Cu		

Figure 9: Hyphal Morphology of NIOSN-M29 in CDB Medium Observed under Phase Contrast Microscope.

Static Condition	**40x** Magnification	**100x** Magnification

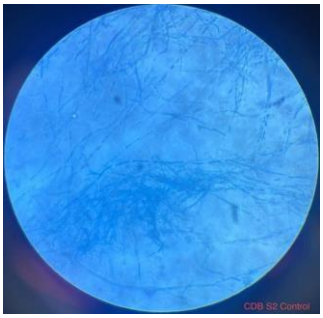
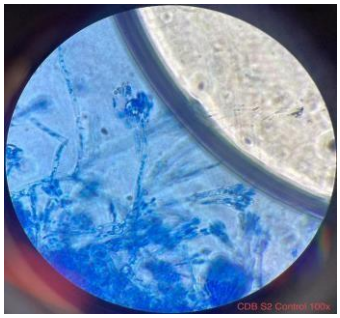
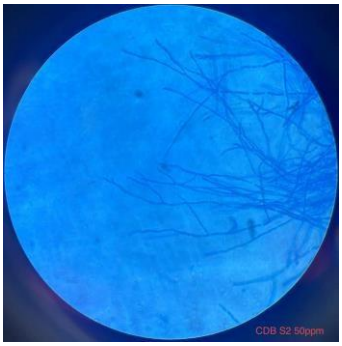
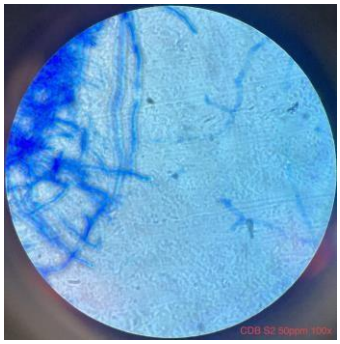
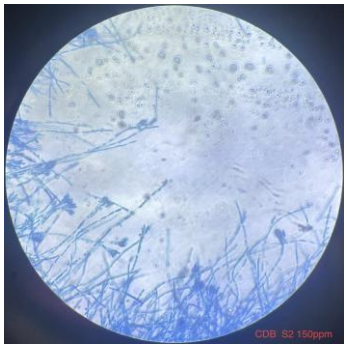
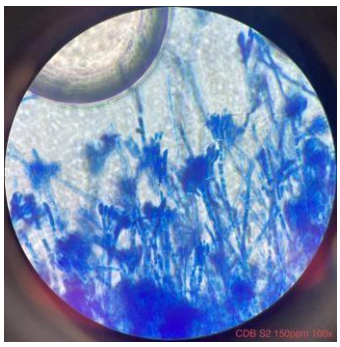
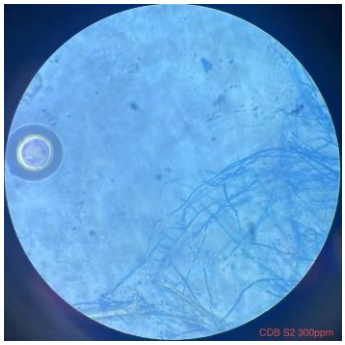
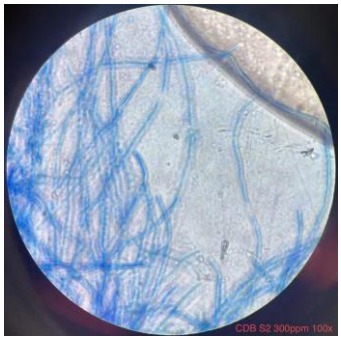
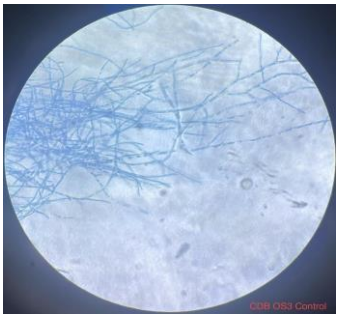
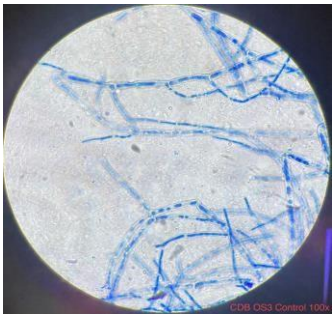
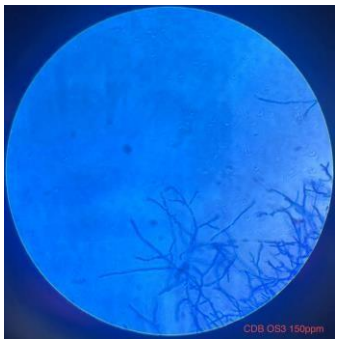
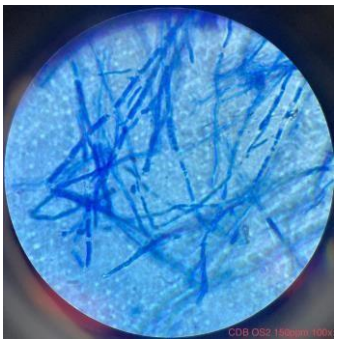
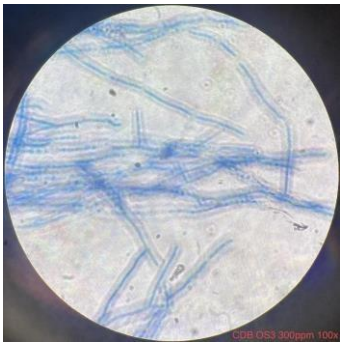
Control CDB		
50 ppm Cu		
150 ppm Cu		
300 ppm Cu		

Figure 10: Hyphal Morphology of NIOSN-M29 in CDB Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification

Control CDB		
50 ppm Cu		
150 ppm Cu		
300 ppm Cu		

3.1.2 To check the effect of pH on copper tolerance in *Penicillium chrysogenum*.

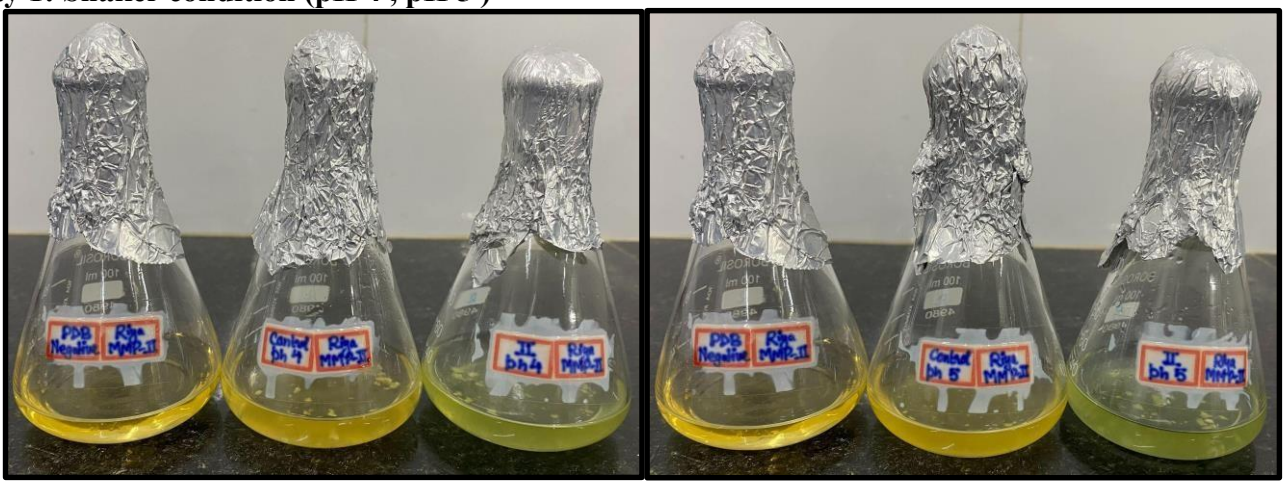
To make different pH parameters, one can use either acidic or basic substances to change the pH of a solution. For an acidic solution, add an acid such as hydrochloric acid, sulfuric acid, or acetic acid to the solution. These acids will donate hydrogen ions (H+) to the solution, thereby decreasing the pH. On the other hand, for a basic solution, add a base such as sodium hydroxide, potassium hydroxide, or ammonia to the solution. These bases will accept hydrogen ions (H+) from the solution, thereby increasing the pH. It is important to note that pH is measured on a logarithmic scale, which means that each unit change in pH represents a tenfold change in acidity or basicity. For example, a solution with a pH of 5 is ten times more acidic than a solution with a pH of 6, and a solution with a pH of 9 is ten times more basic than a solution with a pH of 8. To measure the pH of a solution accurately, use a pH meter or pH paper. pH paper changes color based on the pH of the solution, allowing to determine its approximate pH. A pH meter, on the other hand, gives a more precise measurement of the pH.

Preparation of media with 50 ppm metal concentrations adjusting the pH to 4, 5, 6, 7,8,9:

From 5000 ppm of Cu stock solution, 1.174 ml of Cu solution is added to 18.826 ml of broth (PDB/CDB) and then the pH is adjusted by adding an acid such as hydrochloric acid, sulfuric acid, or acetic acid to the solution. For a basic solution, add a base such as sodium hydroxide, potassium hydroxide, or ammonia to the solution.

Figure 11: The effect of pH 4 and pH 5 on copper tolerance in on PDB media.

Day 1: Shaker condition (pH 4 , pH 5)



Day 7: Shaker condition (pH 4, pH 5)

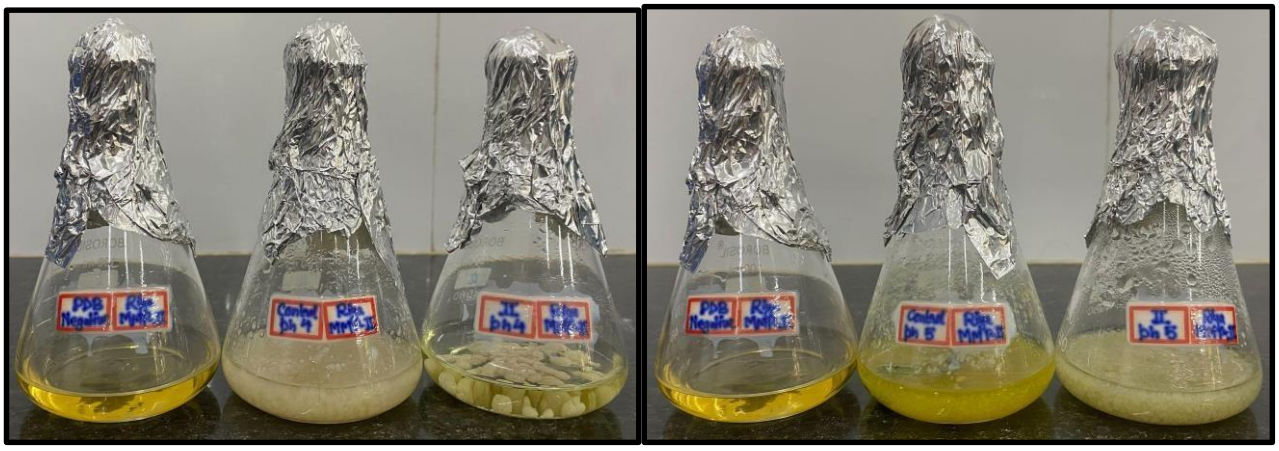
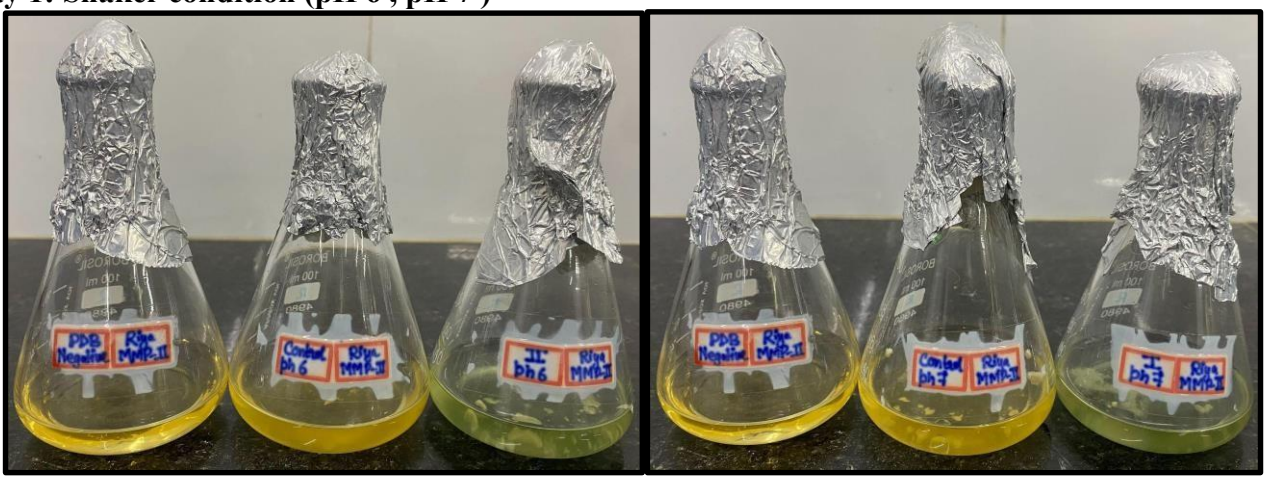


Figure 12: The effect of pH 6 and pH 7 on copper tolerance in NIOSN-M29 on PDB media.

Day 1: Shaker condition (pH 6 , pH 7)



Day 7: Shaker condition (pH 6 , pH 7)

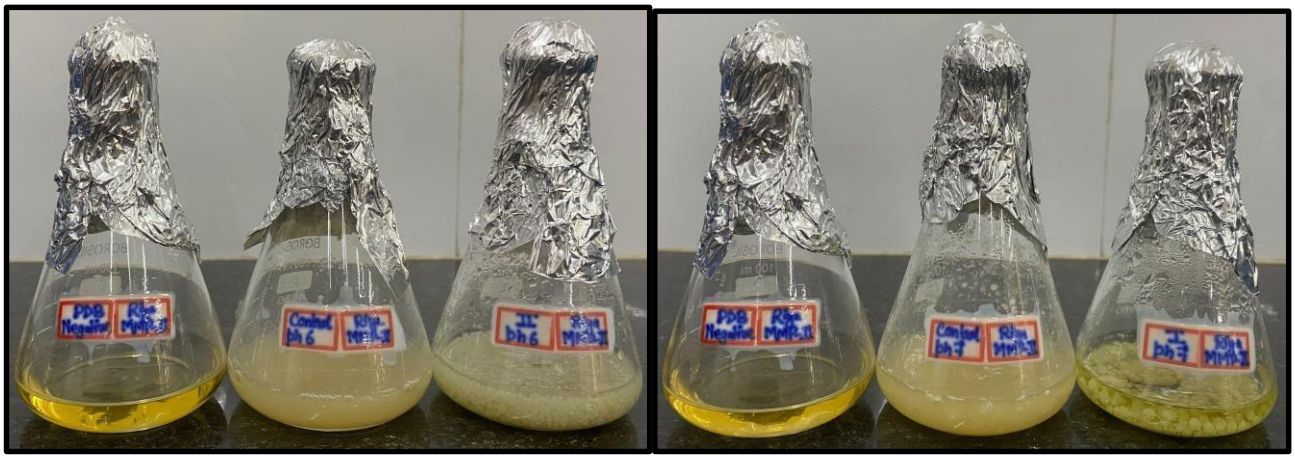
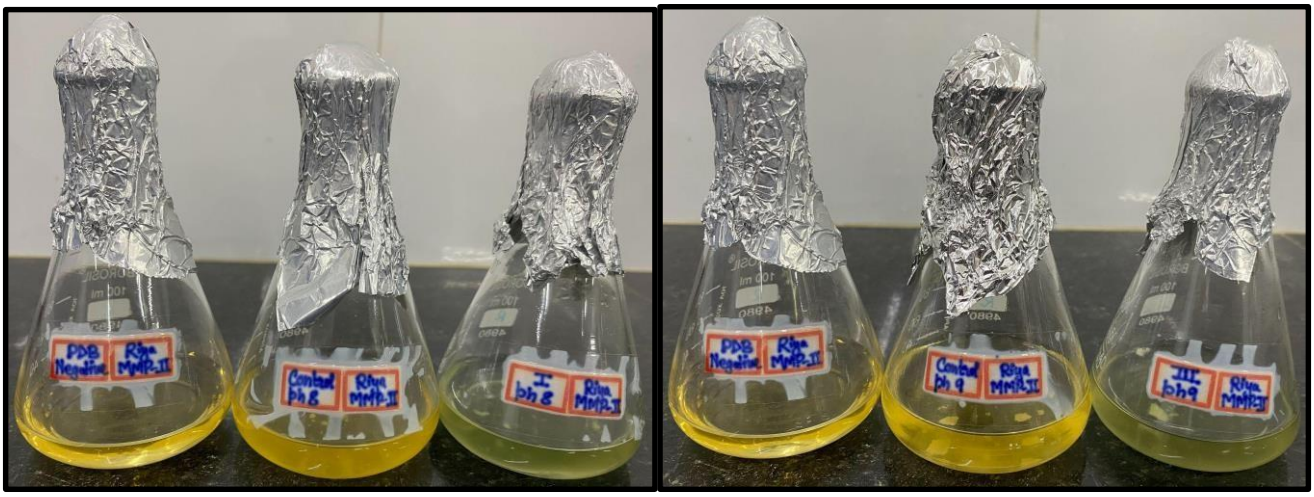


Figure 13: The effect of pH 8 and pH 9 on copper tolerance in on PDB media.

Day 1: Shaker condition (pH 8 , pH 9)



Day 7: Shaker condition (pH 8 , pH 9)

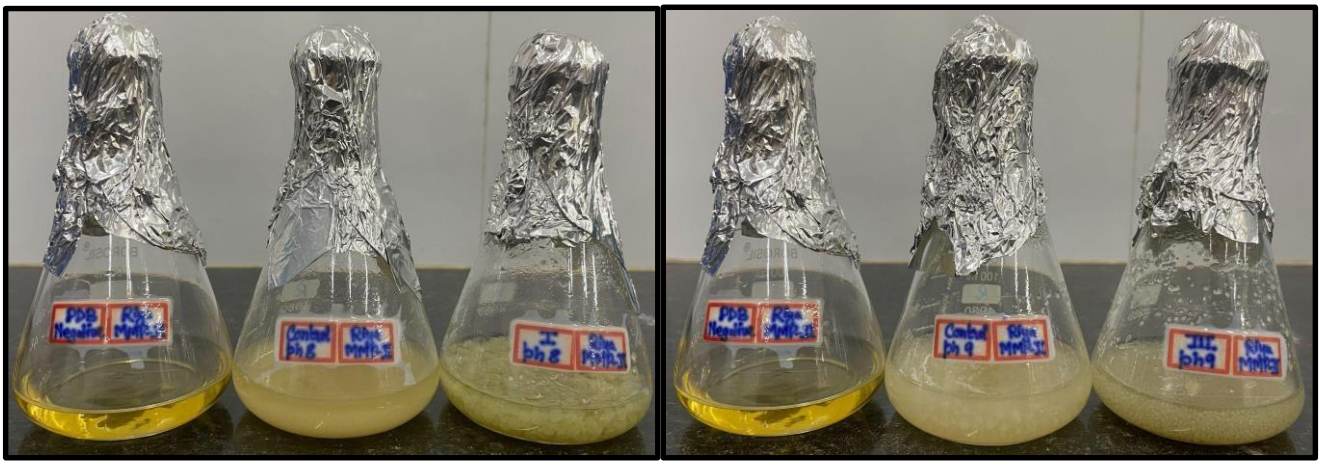


Figure 14: The effect of pH 4 and pH 5 on copper tolerance in NIOSN-M29 on CDB media.

Day 1: Shaker condition (pH 4 , pH 5)

Day 7: Shaker condition (pH 4 , pH 5)

Figure 15: The effect of pH 6 and pH 7 on copper tolerance in on CDB media.

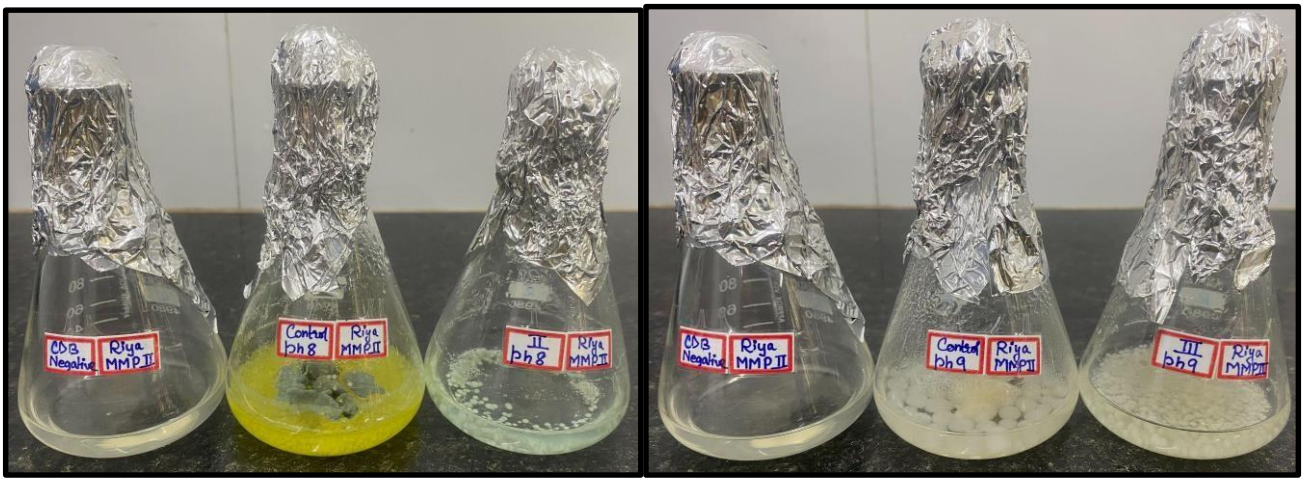
Day 1: Shaker condition (pH 6 , pH 7)

Day 7: Shaker condition (pH 6, pH 7)

Figure 16: The effect of pH 8 and pH 9 on copper tolerance in NIOSN-M29 on CDB media.

Day 1: Shaker condition (pH 8 , pH 9)

Day 7: Shaker condition (pH 8, pH 9)



As a result, 50 ppm metal concentrations were selected as it had higher biomass production compared to other concentrations. Both the media PDB and CDB in triplicates were incubated in shaker condition for 1-7 days (Figure no. 11, 12, 13 PDB) ; (Figure no. 14, 15, 16 CDB). Each pH range was compared with its control, (CDB) pH 4 had clear broth with big clumps (Figure no.

14), pH 5 and 6 had small clumps with slimy appearance, pH 7 had bigger clumps compared to pH 4, pH 8 and pH 9 had similar growth.

(PDB) Control of pH 4, 5, 6 and 8 elutes yellow colour after sporulating as discussed under copper tolerance of NIOSN-M29 (Figure no. 1, 2, 3, 4) control pH 7 which is neutral appears to be slimy and slowly sporulating with big clumps, similar to control of pH 9 ; pH 4, 5 had slow growth whereas pH 6, 7, 8, had similar growth with clear broth and small clumps , pH 9 among all shows a large amount of growth with big clumps (Figure no. 13).

After 7 days of incubation period:

Selection of one set of the replicates were studied for cultural and morphological characteristics under the effect of pH on copper tolerance NIOSN-M29 by using phase contrast microscopy. (Figure no. 18 -23).

As a result, the hyphal morphology of NIOSN-M29 in PDB media at different pH values, as well as controls at each pH value, were observed as:

Control at pH 4: The hyphae are long, thin, and highly branched with clear septa. They appear slightly less dense compared to higher pH values.

Control at pH 5: The hyphae are long, thin, and highly branched with clear septa. They appear slightly denser compared to pH 4 and may appear slightly more segmented at 100x magnification.

Control at pH 6: The hyphae are long, thin, and highly branched with clear septa. They appear slightly shorter and denser compared to pH 4 and pH 5, and may have some irregularities in their shape and size.

Control at pH 7: The hyphae are long, thin, and highly branched with clear septa. They appear similar to the control at pH 6, but may have a slightly higher density.

Control at pH 8: The hyphae are long, thin, and highly branched with clear septa. They appear slightly denser compared to pH 7, and may have a slightly higher density.

Control at pH 9: The hyphae are long, thin, and highly branched with clear septa. They appear similar to the control at pH 8.

pH 4: The hyphae appear longer and thinner than the controls, and may be less dense. They may also appear less segmented at 100x magnification.

pH 5: The hyphae appear similar to pH 4, but slightly denser with slightly more segmentation at 100x magnification. pH 6: The hyphae appear slightly shorter and thicker than the controls, with irregularities in their shape and size. They may also appear slightly denser. pH 7: The hyphae appear similar to pH 6, but may have a slightly higher density. pH 8: The hyphae appear slightly shorter and thicker than the controls, with a slightly higher density.

The septa may appear slightly distorted at 100x magnification.

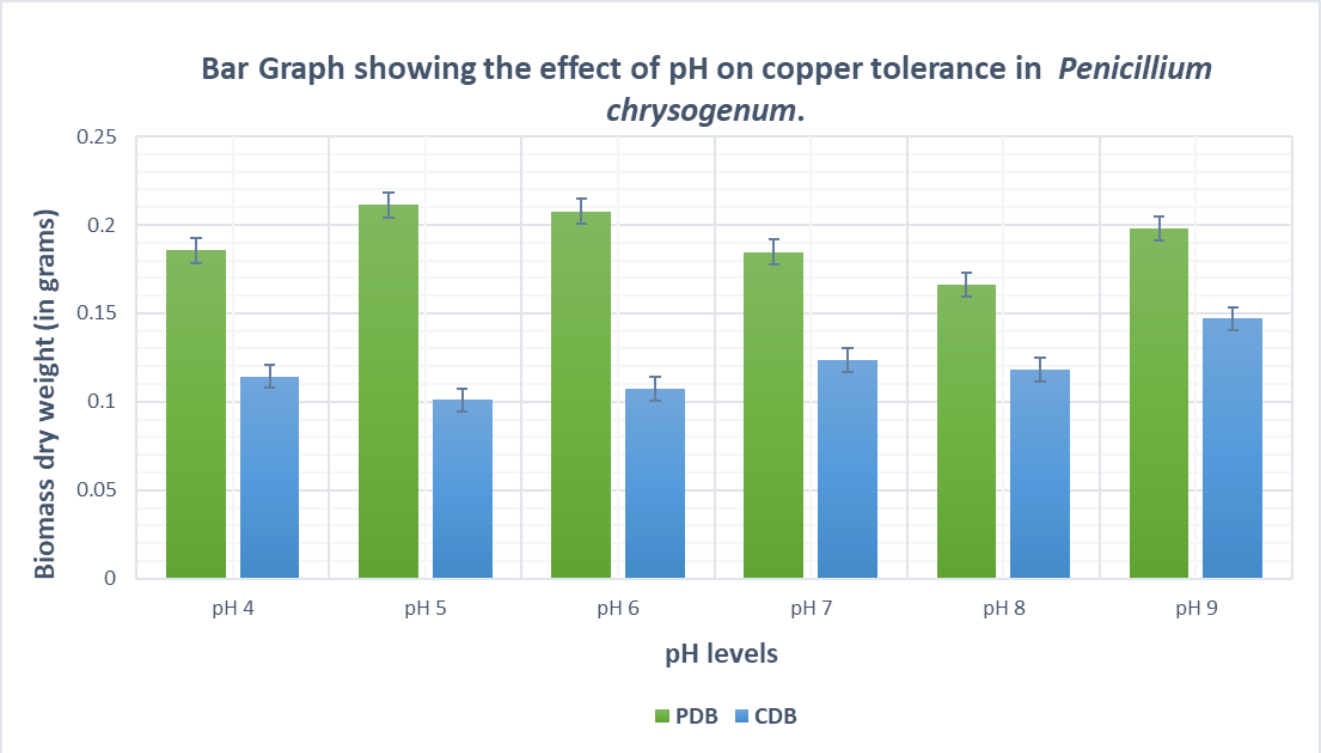
pH 9: The hyphae appear slightly shorter and thicker than the controls, with a slightly higher density. The septa may appear slightly distorted at 100x magnification.

Overall, the hyphal morphology of NIOSN-M29 at different pH values appears to be relatively consistent, with some differences in hyphal length, thickness, density, and segmentation at higher pH values. The hyphae generally maintain their highly branched structure and clear septa at all pH values.

In CDB medium, the hyphal morphology at pH 4 and pH 5, the hyphae appeared to be shorter and more branched compared to the control. At pH 6 and pH 7, there was not much visible change in the hyphal morphology compared to the control. However, at pH 8 and pH 9, the hyphae appeared to be longer and less branched compared to the control. Overall, the hyphal morphology of NIOSN-M29 in CDB medium appeared to be pH-dependent.

At the end of the incubation period, all six flasks of different pH were filtered using 0.45µ filter paper and biomass production was estimated. The results were plotted in the form of a graph (Figure no. 17)

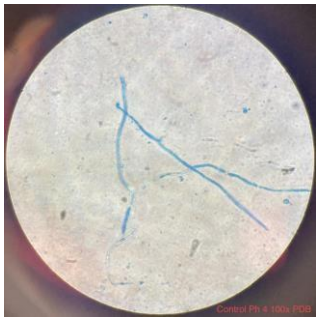
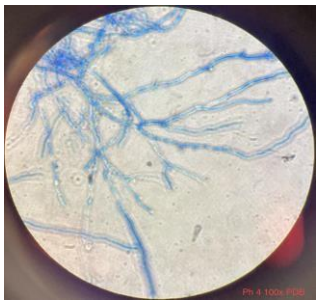
Figure 17: The effect of pH on copper-tolerance in NIOSN-M29 biomass (Comparison of two media PDB and CDB media).



As mentioned earlier, the pH level of PDB and CDB media may differ depending on their specific composition and preparation method. In general, PDB medium has a pH range of 5.6 to 7.0, while CDB medium is usually slightly more acidic with a pH range of 4.8 to 5.5.

Overall, PDB medium is often considered a richer and more nutrient-dense medium than CDB medium, which may explain its ability to support higher biomass production in NIOSN-M29 across a range of pH levels (Figure no. 17). As a result, the effect of pH on copper-tolerance in NIOSN-M29 showed higher biomass in PDB media compared to CDB media (Figure no. 17)

Figure 18: The effect of pH 4 and pH 5 on hyphal morphology of in PDB Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 4		
pH 4		
Control pH 5		

NIOSN-M29

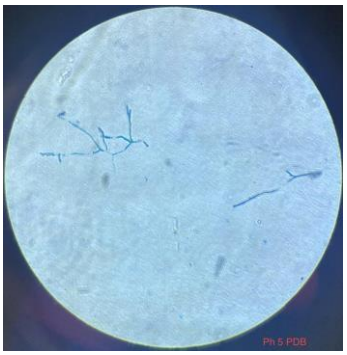
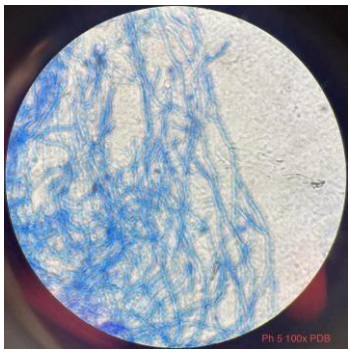
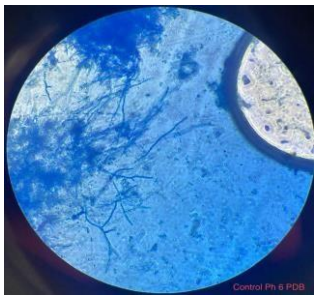
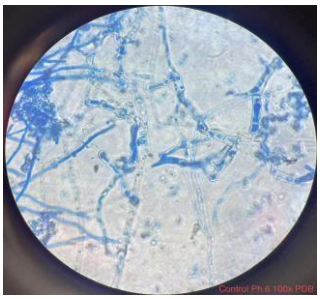
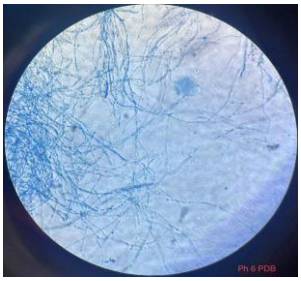
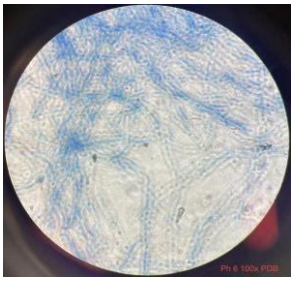
pH 5		

Figure 19: The effect of pH 6 and pH 7 on hyphal morphology of in PDB Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 6		
pH 6		

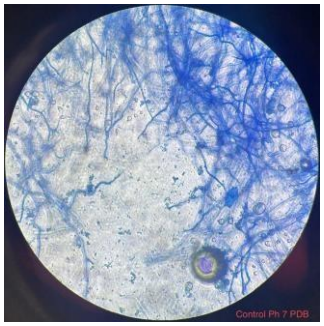
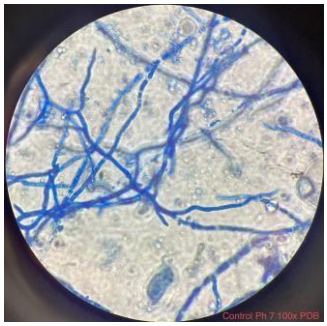
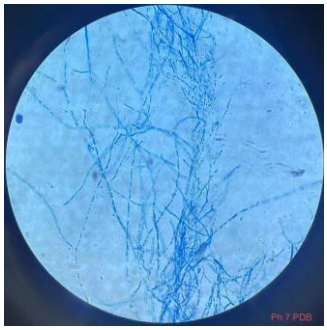
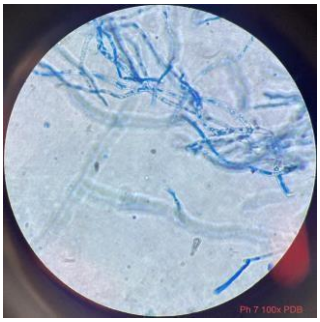
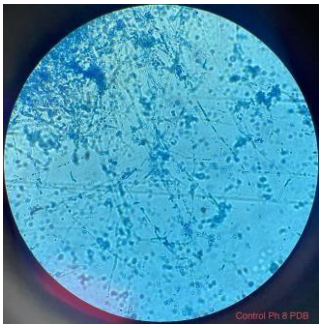
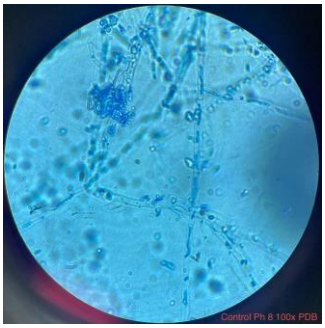
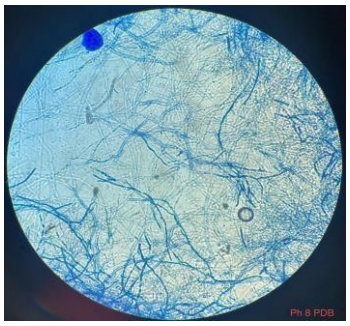
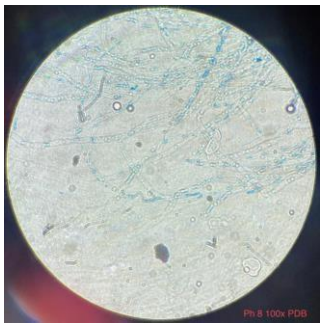
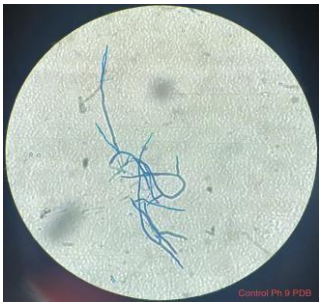
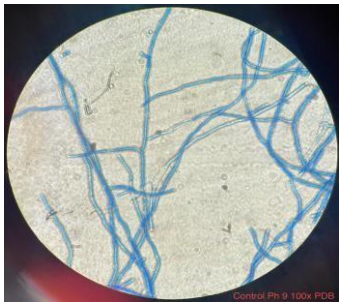
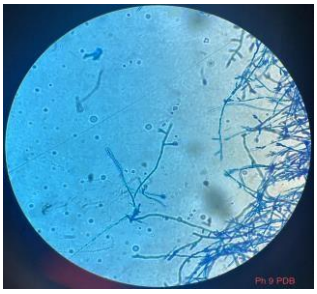
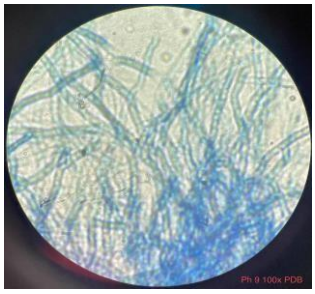
Control pH 7		
pH 7		

Figure 20: The effect of pH 8 and pH 9 on hyphal morphology of in PDB Medium observed under Phase Contrast Microscope.

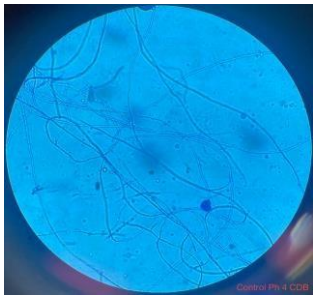
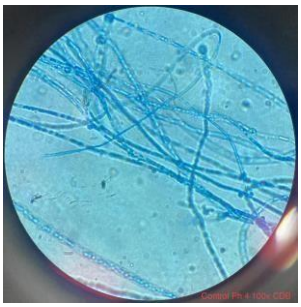
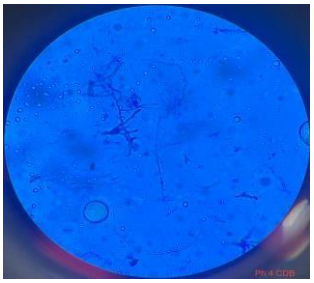
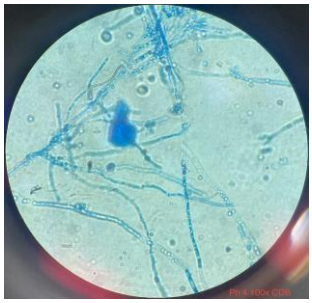
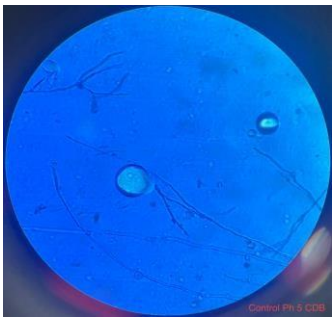
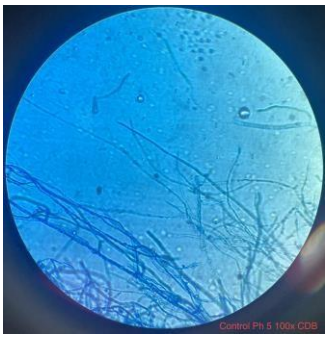
Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 8		

NIOSN-M29

pH 8		
Control pH 9		
pH 9		

NIOSN-M29 in CDB

Figure 21: The effect of pH 4 and pH 5 on hyphal morphology of Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 4		
pH 4		
Control pH 5		

NIOSN-M29 in CDB

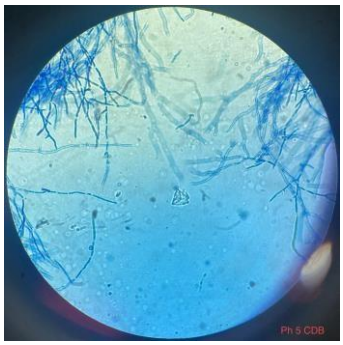
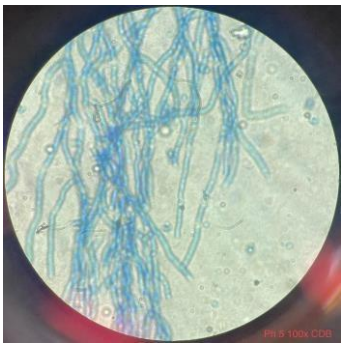
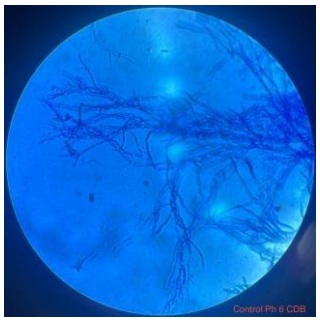
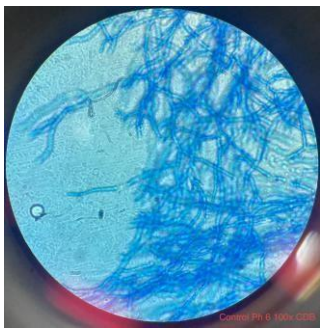
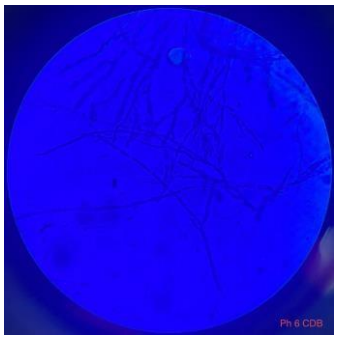
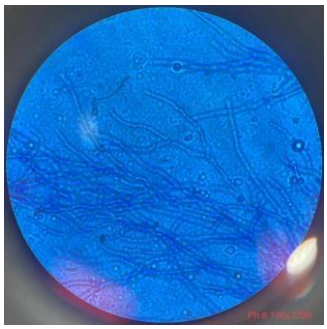
pH 5		

Figure 22: The effect of pH 6 and pH 7 on hyphal morphology of Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 6		
pH 6		

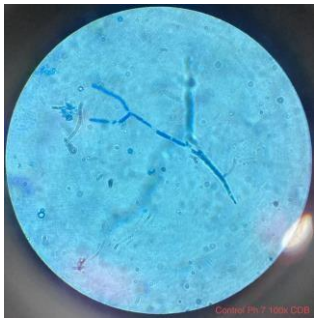
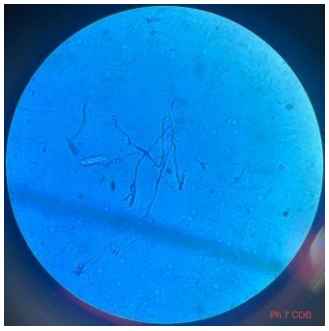
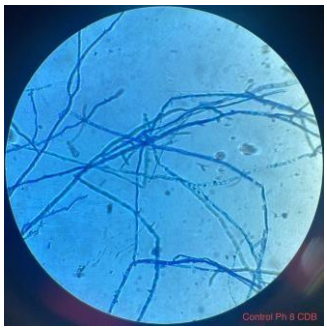
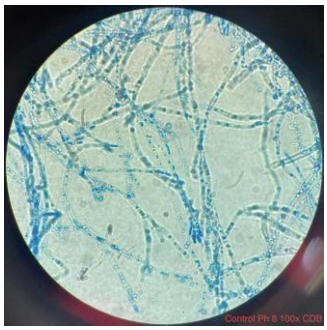
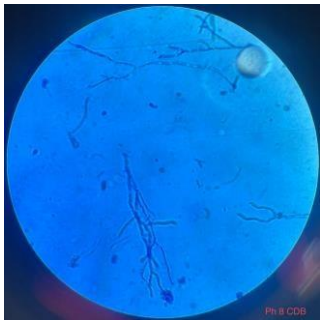
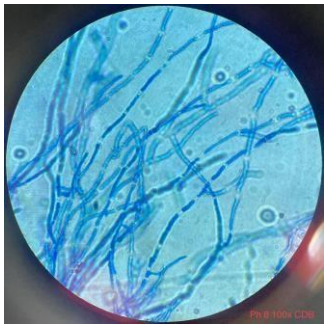
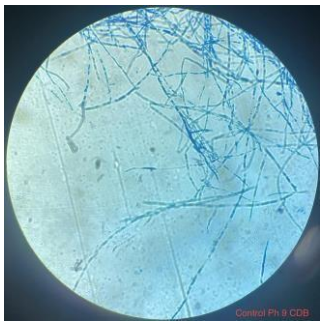
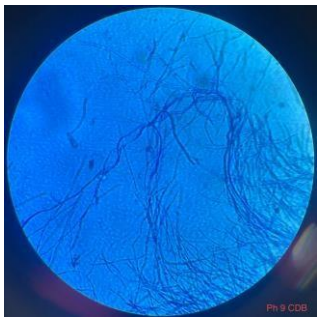
Control pH 7		
pH 7		

Figure 23: The effect of pH 8 and pH 9 on hyphal morphology of Medium observed under Phase Contrast Microscope.

Shaker Condition	**40x** Magnification	**100x** Magnification
Control pH 8		

NIOSN-M29 in CDB

pH 8		
Control pH 9		
pH 9		

3.1.3 Effect of Cu on hyphal morphology of NIOSN-M29 using SEM:

Morphological responses of *NIOSN-M29* to interactions with metal were studied by scanning electron microscopy (SEM) for confirmation of the presence of the metal ion bound to the cell surface. Culture was grown on Czapek Dox broth and Potato dextrose broth as a control and 50 ppm metal concentration to check the tolerance in NIOSN-M29 (Figure no. 24).

The culture sample preparation was carried out using biological fixation and with 2.5% glutaraldehyde solution dehydration method and incubated in room temperature for 1 hour. Samples were then dehydrated with a series of increasing acetone concentrations of 10% ,30%, 50%, 70%, 90% and 100%, each for 10 min. Samples were then air dried (Karnovsky et al. 1965, Lotlikar 2018). Prior to SEM analysis, samples were then placed onto a stub using double-sided sticky tape and mounted in the sputter coater for coating the sample surface. Samples were then placed in the SEM unit to observe surface morphology of the mycelia, conidiophore, phialides and conidial surfaces. The dried biomass samples of control and test cultures obtained as given in (Figure no. 24).

Figure 24: Effects of Cu on hyphal morphology of NIOSN-M29 in PDB media using SEM.

Effects of Copper on hyphal morphology of NIOSN-M29 (in PDB media) using SEM.	
Control	**50 ppm copper**

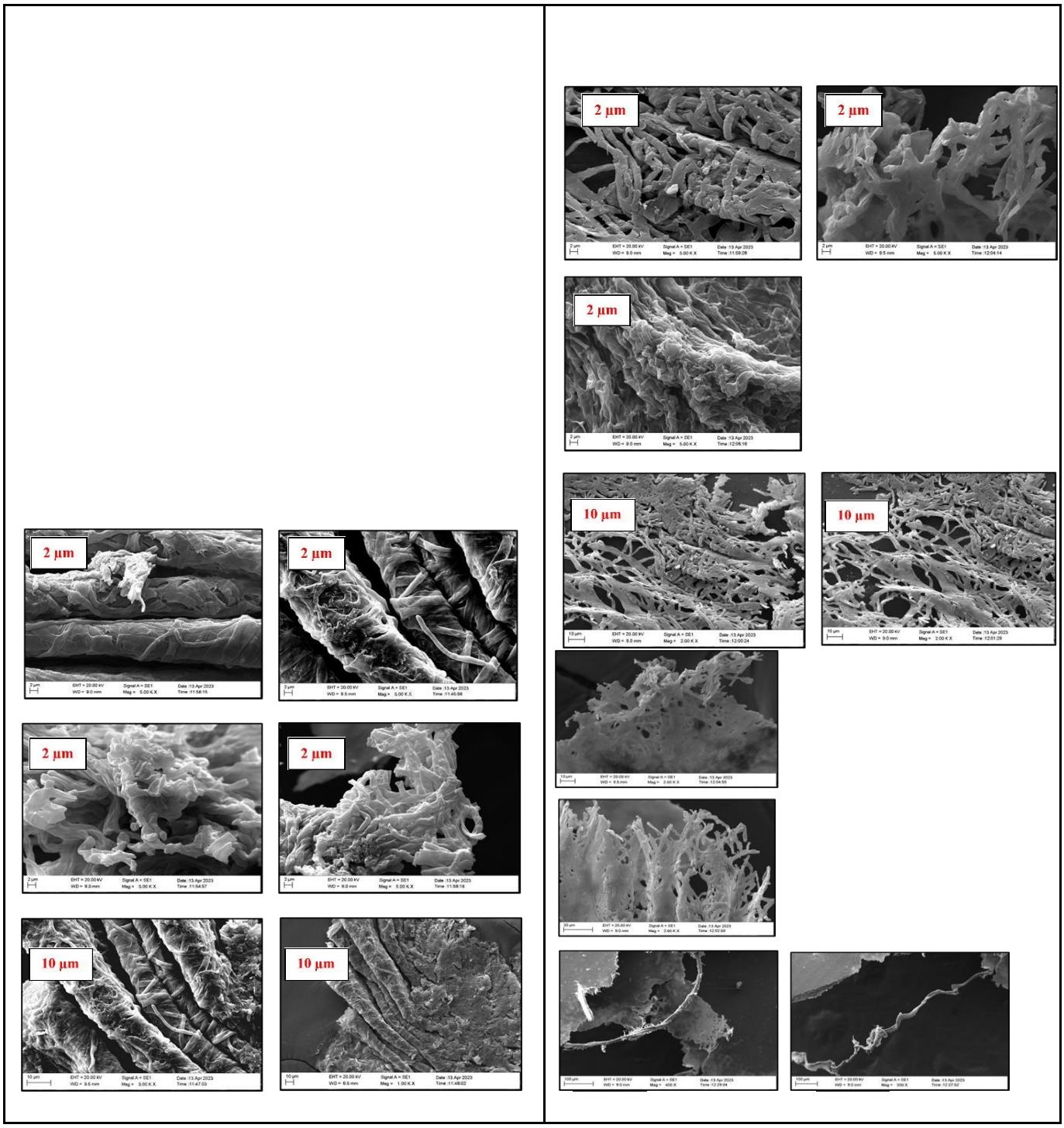
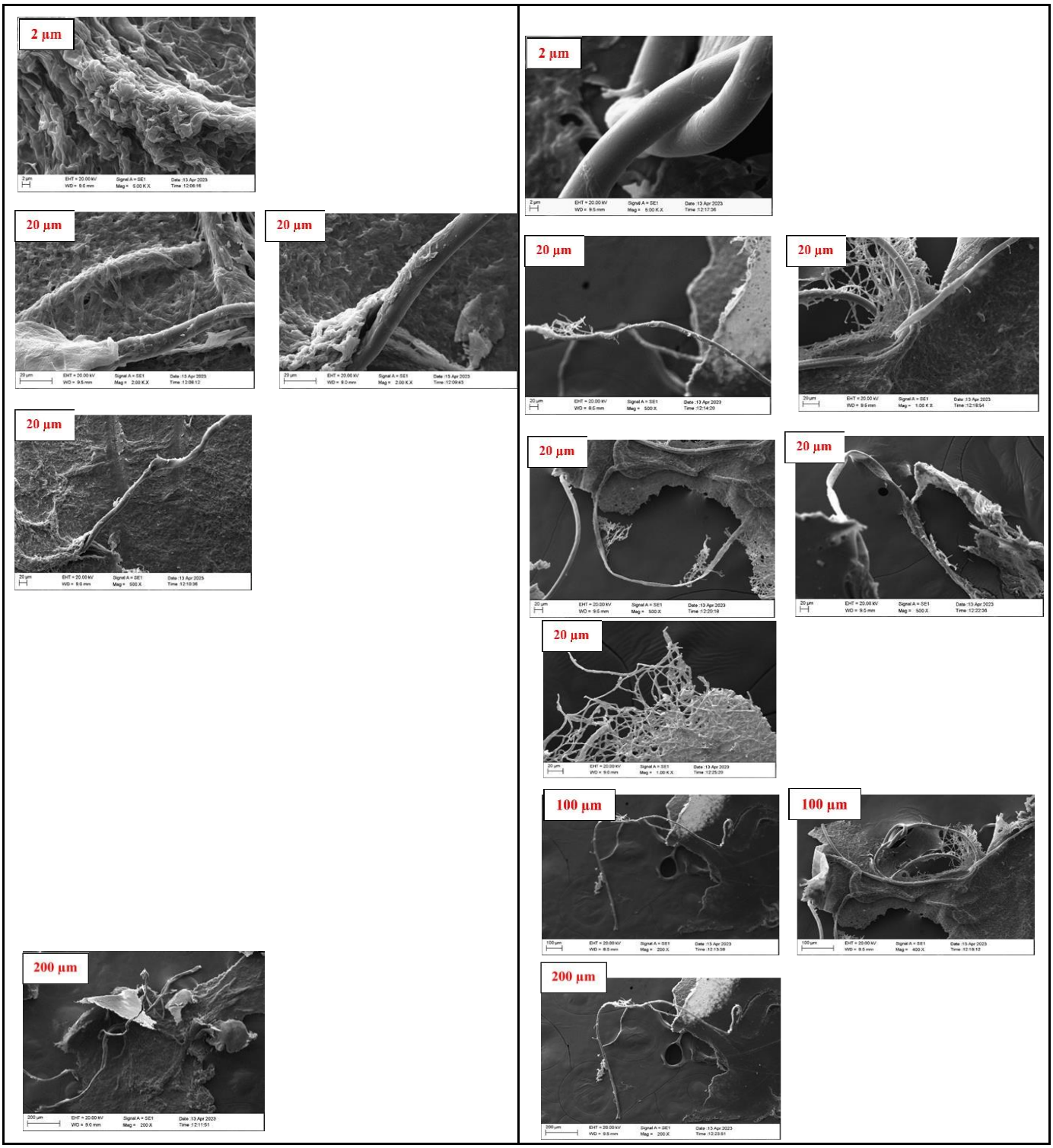


Figure 25: Effects of Cu on hyphal morphology of NIOSN-M29 in CDB media using SEM.

Effects of Copper on hyphal morphology of NIOSN-M29 (of CDB media)using SEM.	
Control	**50 ppm copper**



The response of marine-derived fungi NIOSN-M29 to metal stress was studied based on the morphological appearance of hyphae using SEM. It was observed that the isolate produced

extracellular thread-like substances (Figure no. 24) under Cu stress, which were otherwise absent under the control condition (absence of metal). Similarly, extracellular aggregation that appeared like fine mesh in between the mycelial network was seen at 10µm and 20µm of 50 ppm Cu stress (Figure no. 24). The production of thread-like substances and extracellular aggregates by isolate NIOSN-M29, with response to Cu, led to the inference that biosorption of metals on cell walls, which is a generally occurring phenomenon, might not be the only mechanism for tolerance. The extracellular material produced by these cultures might be responsible for trapping of toxic metal ions so that less/no harm is further caused to the organism (Lotlikar 2018). SEM analysis of NIOSNM29 revealed a highly branched and filamentous structure. Interestingly, it was observed the production of extracellular thread-like substances in response to copper stress. These substances appeared to surround the hyphae and may play a role in metal detoxification. In contrast, these extracellular substances were absent in the control condition. Furthermore, extracellular aggregation that appeared like fine mesh in between the mycelial network was seen 10 µm and 20 µm under 50 ppm Cu stress.

It was also observed significant changes in hyphal morphology compared to the control condition. The hyphae exposed to 50 ppm appeared to be more compact and dense, with more branches and irregularly shaped. These changes suggest that Cu may have a significant impact on the growth and development of NIOSN-M29.

Additionally, the hyphae exhibited signs of cellular damage, including ruptured cell walls and leakage of intracellular contents (Figure no. 24). At 100 µm it provides a detailed view of the surface of the fungal cells. At this magnification, it is possible to observe the shape, size, and surface characteristics of the individual cells, including their structures such as cell walls, vacuoles, cytoplasmic membranes, and organelles.

Scanning Electron Microscopy (SEM) analysis was performed to investigate the effects of copper stress on the morphology of NIOSN-M29 grown in CDB media. The control cells exhibited a smooth surface with regular shapes and sizes, indicating normal growth and development (Figure no. 25). Surprisingly, cells exposed to 50 ppm copper also showed a similar morphology to the control, with no significant alterations in size or surface features (Figure no. 25). The mycelia appeared to be undamaged by copper stress, maintaining a healthy and uniform appearance.

Furthermore, the number of cells in the copper-stressed sample was comparable to that of the control, indicating that copper stress had no significant effect on cell proliferation.

These results suggest that exposure to 50 ppm copper in CDB media did not have a noticeable impact on the morphology and viability of NIOSN-M29, and that the mycelia remained undamaged. However, further studies are required to determine if the organism's metabolism, gene expression, or other physiological processes were affected by copper stress, despite the lack of visible morphological changes.

There are some differences between CDB and PDB media that could affect the SEM analysis of control and 50 ppm copper-stressed NIOSN-M29. Here are a few examples:

- Nutrient composition: CDB (Czapek-Dox broth) and PDB (Potato-Dextrose broth) have different nutrient compositions. CDB is a synthetic medium that contains various salts, amino acids, and carbon sources, while PDB is an undefined medium that contains potato extract, dextrose, and other complex components. These differences in nutrient composition could affect the growth and morphology of NIOSN-M29 and, consequently, the results of SEM analysis.

- pH: CDB and PDB have different pH levels. CDB typically has a pH of 7.3, while PDB has a pH of 5.1. This difference in pH could affect the growth and morphology of NIOSN-M29 and, in turn, affect the results of SEM analysis.

- Copper availability: Copper availability could also differ between CDB and PDB. Copper ions in CDB may be sequestered by various components of the medium, such as amino acids, while copper ions in PDB may be more freely available to the fungus. This could affect the level of copper stress experienced by NIOSN-M29 and, consequently, the results of SEM analysis.

Therefore, the results of SEM analysis comparing control and 50 ppm copper-stressed NIOSN-M29 in CDB and PDB media could potentially differ, and it is important to take into account the specific characteristics of each medium when interpreting the results.

3.1.4 Analysis of FTIR Peaks and Functional Groups in NIOSN-M29 Biomass:

FTIR stands for Fourier Transform Infrared Spectroscopy, which is a type of analytical technique used to identify and analyze chemical compounds based on their absorption of infrared radiation. In FTIR spectroscopy, a sample is exposed to a beam of infrared light, which causes the chemical bonds

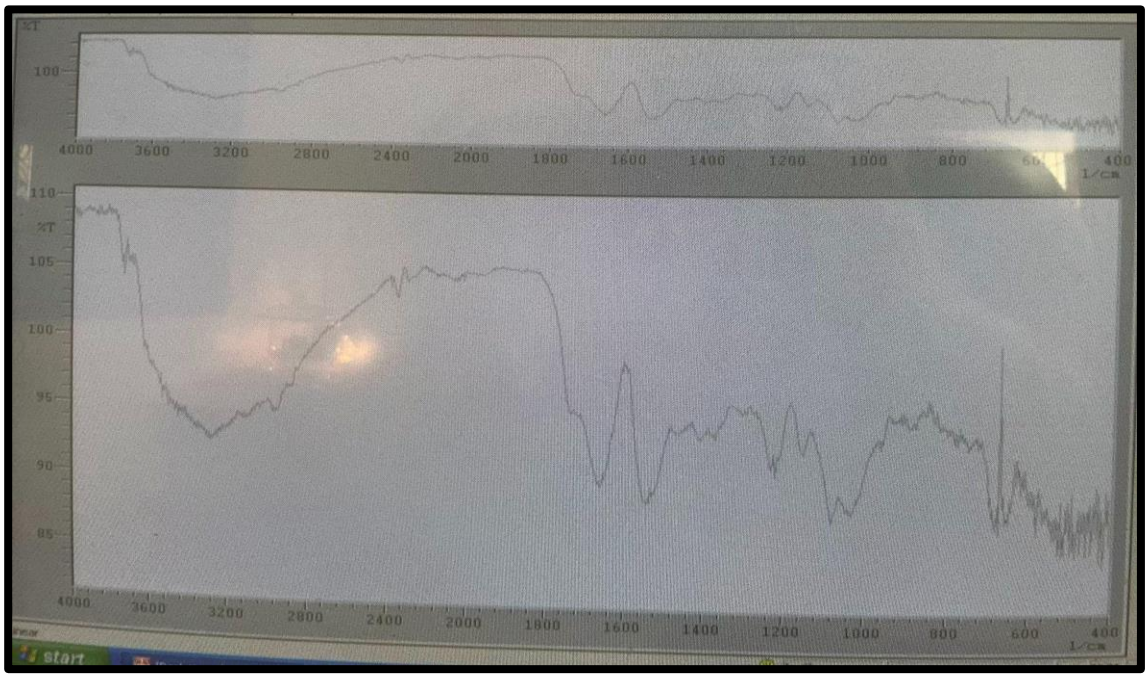
in the sample to vibrate. These vibrations produce a unique spectrum of frequencies that can be measured and analyzed to identify the functional groups and chemical composition of the sample. In order to determine the functional groups involved, the biomass was analyzed using FTIR (Figure no. 26). Functional groups are specific groups of atoms within a molecule that determine its chemical and physical properties, including its reactivity, solubility, and intermolecular interactions. In FTIR spectroscopy, functional groups can be identified by the characteristic peaks in the infrared spectrum. Here are some of the common functional groups that can be detected in FTIR spectra:

- Carbonyl group (-C=O): This group is found in ketones, aldehydes, carboxylic acids, esters, and amides. The characteristic peak for carbonyl group stretching vibration occurs around 1700-1750 cm^-1.
- Hydroxyl group (-OH): This group is found in alcohols, phenols, and carboxylic acids. The characteristic peak for hydroxyl group stretching vibration occurs around 3200-3600 cm^-1.
- Amine group (-NH2): This group is found in primary, secondary, and tertiary amines. The characteristic peak for amine group stretching vibration occurs around 3300-3500 cm^-1.
- Alkene group (-C=C-): This group is found in alkenes and aromatic compounds. The characteristic peak for alkene group stretching vibration occurs around 1600-1680 cm^-1.
- Alkane group (-C-H): This group is found in alkanes and alkyl groups. The characteristic peak for alkane group stretching vibration occurs around 2800-3000 cm^-1.
- Nitrile group (-C≡N): This group is found in nitriles and imines. The characteristic peak for nitrile group stretching vibration occurs around 2200-2300 cm^-1.

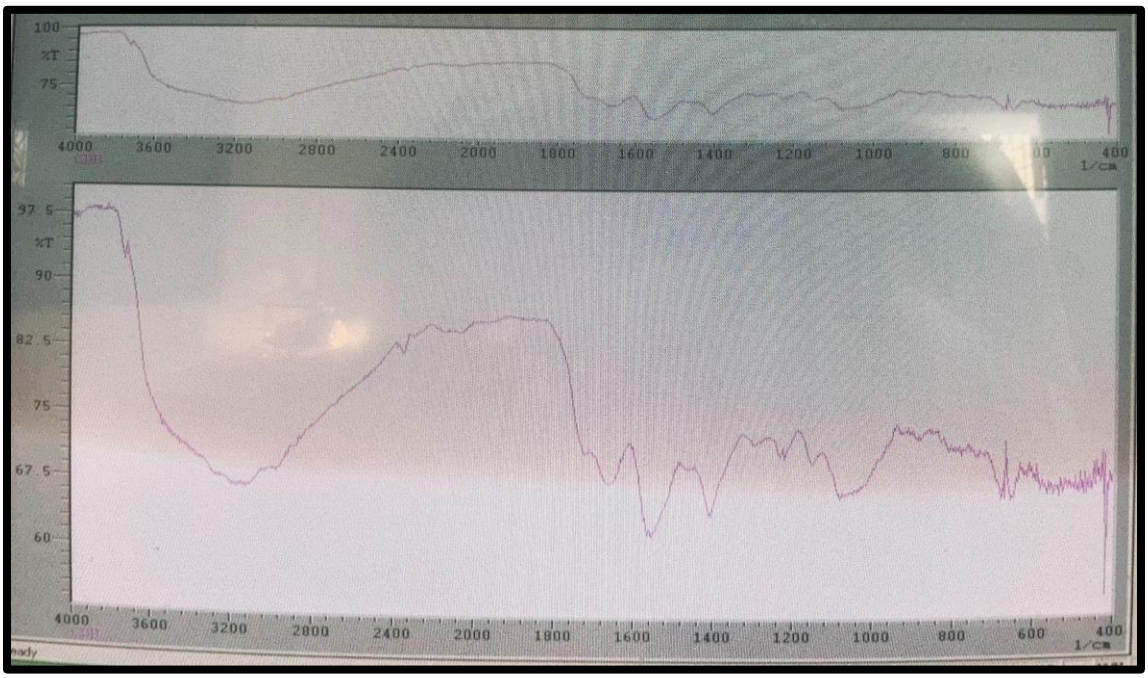
These are just a few examples of the many functional groups that can be detected in FTIR spectra. The exact position and intensity of the peaks may vary depending on the specific molecular structure and other factors.

Figure 26: FTIR Spectroscopic Analysis of NIOSN-M29 biomass.

PDB



CDB



To identify and interpret the peaks in the FTIR spectrum of NIOSN-M29:

- Carbohydrate peaks: NIOSN-M29 is a fungus that produces complex carbohydrates. The presence of carbohydrates in the sample can be identified by the peaks around 1000-1200

cm^-1. The intensity and position of these peaks can provide information about the type and structure of carbohydrates in the sample.

- Protein peaks: Proteins are one of the major components of NIOSN-M29. The amide bands in the spectrum are related to protein and peptide bonds, and they appear in the region between 1600-1700 cm^-1. The intensity and position of these peaks can provide information about the type and structure of proteins in the sample.
- Lipid peaks: The presence of lipids in the sample can be identified by the peaks around 28003000 cm^-1. The intensity and position of these peaks can provide information about the type and structure of lipids in the sample.
- Secondary metabolite peaks: NIOSN-M29 is known for producing a variety of secondary metabolites, including penicillin. The presence of these metabolites can be identified by the peaks around 1000-1800 cm^-1. The intensity and position of these peaks can provide information about the type and structure of the metabolites in the sample.

It is important to note that the significance of each peak in the FTIR spectrum of NIOSN-M29 may depend on various factors, such as the growth conditions of the fungus, the sample preparation method, and the specific analytical technique used.

The FTIR spectra of biomass samples within the range 400–4000 cm are depicted in figure no. 26, As shown in (Figure no. 26) for PDB, the peak at 3600 cm was due to bounded hydroxyl (–OH) stretching vibration and –NH stretching of the protein, as well as the acetamido groups of the chitin fraction. The strong adsorption band at 1800 cm represents C=O stretching vibration and NH deformation.

Similarly, for CDB, the peak at 3900 cm was due to bounded hydroxyl (–OH) stretching vibration and –NH stretching of the protein, as well as the acetamido groups of the chitin fraction. The strong adsorption band at 1700 cm represents C=O stretching vibration and NH deformation.

CONCLUSION

Investigating survival strategy in copper tolerance fungi refers to the study of how fungi that can tolerate high levels of copper in their environment are able to survive and thrive. Copper is a heavy metal that can be toxic to living organisms in high concentrations, including fungi. However, some fungi have developed the ability to tolerate copper by employing different survival strategies. These strategies may include the production of enzymes that can detoxify copper, changes in cellular metabolism to reduce copper uptake, or the formation of specialized structures such as biofilms that can protect the fungi from copper toxicity. Studying the survival strategies of coppertolerant fungi can provide insights into how these organisms are able to adapt to their environment and can help in the development of strategies for bioremediation of copper-contaminated environments. However, the mechanisms by which pH affects copper tolerance in fungi are not well understood. It is thought that pH may affect the availability and solubility of copper ions in the environment, which can in turn affect the uptake and toxicity of copper in fungi. Additionally, pH can affect the activity of enzymes involved in copper detoxification and the production of metallothioneins, which are important factors in copper tolerance.

Overall, the copper tolerance of fungi in different pH environments is a complex and multifaceted phenomenon that requires further research to fully understand. Understanding the mechanisms by which fungi are able to tolerate high levels of copper in different pH environments can have important implications for the development of bioremediation strategies, as well as for the use of fungi in biotechnology and agriculture.

SUMMARY

Penicillium chrysogenum is a filamentous fungus, and its hyphae can display different morphologies depending on various factors such as the growth stage, nutrient availability, and environmental conditions (Andersen et al. 2013). During early growth stages, the hyphae of *P. chrysogenum* are typically thin and elongated, forming a dense network of mycelium. As the fungus continues to grow and spread, the hyphae can branch and form more complex structures, such as a radial pattern of hyphal strands (Frisvad et al. 2004). Another feature of *P. chrysogenum* hyphae is the presence of septa, which are partitions that divide the hyphae into compartments. These septa can be simple or complex, and they play a role in regulating the transport of nutrients and organelles within the hyphae (Brakhage et al. 2011). Overall, the hyphae of *P. chrysogenum* can display various morphologies depending on different factors, and the diversity of hyphal structures can contribute to the adaptability and growth of this fungus in different environments (Krijgsheld et al. 2013).

Research has shown that *P. chrysogenum* has the ability to tolerate and even remove heavy metals from contaminated environments. Heavy metals such as cadmium, copper, lead, and zinc can be toxic to living organisms, including fungi, but *P. chrysogenum* has been found to have mechanisms that allow it to survive in metal-contaminated environments (Kumar et al. 2019). Studies have shown that *P. chrysogenum* can produce extracellular compounds such as organic acids, chelators, and siderophores, which can bind to heavy metals and facilitate their removal from the environment (Ojuederie et al. 2017). This ability makes *P.* chrysogenum a potential candidate for bioremediation, which is the use of living organisms to clean up contaminated environments. In addition to its metaltolerance abilities, *P.* chrysogenum has also been shown to produce bioactive compounds with potential applications in medicine, agriculture, and industry (Das et al. 2011). The combination of its metal-tolerance and bioactive compound production make it an interesting organism for further

research and potential applications. Research has also shown that *P. chrysogenum* can grow and survive in a wide range of pH levels (Al-Gheethi et al. 2017). However, its optimal growth pH varies depending on the specific strain and the type of substrate being used. In general, *P. chrysogenum* prefers a slightly acidic to neutral pH range of 5.0 to 7.0. However, some strains have been shown to tolerate more acidic or alkaline conditions, with growth reported in pH levels ranging from 2.5 to 9.0 (Iqbal et al. 2013). The ability of *P. chrysogenum* to grow in different pH levels is due in part to its ability to produce and secrete enzymes that can modify the surrounding environment. For example, it can produce acid phosphatase, which can hydrolyze phosphate groups from organic molecules and release protons, thereby lowering the pH. *P. chrysogenum* is an important organism for the production of the antibiotic penicillin, and its ability to grow and survive in a wide range of pH levels makes it a useful organism for industrial applications in which pH conditions may vary (Guillemette et al. 2017). However, further research is needed to better understand the mechanisms that allow *P. chrysogenum* to grow and survive in different pH levels. Studies have shown that the biomass of *P. chrysogenum* can vary depending on the concentration of metals in the growth medium (Li et al. 2019). In general, at low to moderate metal concentrations, the biomass of *P. chrysogenum* is not significantly affected. However, at high metal concentrations, the growth of *P. chrysogenum* can be inhibited and lead to reduced biomass production (Olaifa et al. 2020). The specific effect of metal concentration on the biomass of *P. chrysogenum* can vary depending on the type of metal, the strain of the fungus, and the conditions of the growth medium. For example, some studies have reported that copper can have a greater inhibitory effect on the growth and biomass production of *P. chrysogenum* compared to other metals such as cadmium or zinc (Sharma et al.
2013).

Furthermore, some strains of *P. chrysogenum* have been shown to be more tolerant to metal concentrations compared to others, which can result in differences in biomass production (Rajkumar et al. 2008).

Overall, the biomass of *P. chrysogenum* can be affected by metal concentrations in the growth medium, but the specific effect can vary depending on the type of metal, the strain of the fungus, and the conditions of the growth medium. Different types of media can contain different nutrients and environmental conditions that can affect the growth and morphology of *P. chrysogenum* (Liu et al. 2017).

For example, on potato dextrose agar (PDA) media, *P. chrysogenum* typically produces a blue-green spore mass with a characteristic odor, while on Czapek agar media, it produces a powdery bluegreen colony. On malt extract agar (MEA) media, *P. chrysogenum* typically produces a dense, bluegreen mycelium with small, white spores. Therefore, choosing the appropriate growth medium is important for studying the growth and behavior of *P. chrysogenum*, as well as for optimizing the production of specific compounds or products (Al-Gheethi, et al. 2016).

LIST OF FIGURES CONTENTS

APPENDIX

MEDIA:

1. **Potato Dextrose Broth, Granulated**

Ingredients	Gms/litre
• Potatoes, infusion from	200.00
• Dextrose (Glucose)	20.00

Final pH (at 25°C) 5.1 ± 0.2

PREPARATION:

Suspend 24.0 grams in 1000 ml of distilled water.

Mix well and Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

2. **Czapek Dox Broth, Granulated**

Ingredients	Gms/litre
● Sucrose	30.000
● Sodium nitrate	3.000
● Dipotassium hydrogen phosphate	1.000
● Magnesium sulphate	0.500
● Potassium chloride	0.500
● Ferrous sulphate	0.010

Final pH (at 25°C) 7.3 ± 0.2

PREPARATION:

Suspend 35.01 grams in 1000 ml of distilled water.

Mix well and Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

ANTIBIOTIC:

1. **Chloramphenicol**

Mol. Wt.: 323.13

Store it below 30°C, away from light.

PREPARATION:

100 mg of chloramphenicol in 1 ml of ethanol

So,

1mg/10µl concentration

Therefore, dissolve 10 mg of antibiotic in 100µl ethanol

- 20 mg of antibiotic in 200µl ethanol was prepared for this experiment and dispensed into an experimental flask (to inhibit bacterial growth).

METAL:

1. **Copper (II) chloride dihydrate**

   Mol. Wt. 170.48

PREPARATION:

For 5000 ppm

500 mg (0.5g) dissolve in 100 ml distilled water is 5000 ppm.

Filter Sterilize with 0.22µm filter paper (pre- autoclave)

STAINING:

1. **Lactophenol Cotton Blue** was used as a staining solution for fungi.

FIXATION:

1. **Glutaraldehyde solution**
2. **Ethanol** (10%, 30%, 50%, 70%, 90%, 100%) each for 10 mins of incubation.

PREPARATION:

Stock solution of 50% Glutaraldehyde solution.

Take 100µl of 50% and add 1900µl of distilled water that gives 2 ml or 2.5% of the stock solution required for fixation of fungal culture.

REFERENCES

- Puranik PR, Paknikar KM (1999) Biosorption of lead, cadmium, and zinc by Citrobacter strain MCMB-181: characterization studies. Biotechnol Prog. 15: 228- 237.
- Fourest E, Roux J (1992) Heavy metals biosorption by fungal mycelial by-product: mechanisms and influence of pH. Appl Microbiol Biotechnol 37: 399- 403.
- Kuyucak N, Volesky B (1989) The mechanism of cobalt biosorption. Biotechnol Lett. 33: 823- 831.
- Aksu Z, Donmez G (2001) Comparison of copper(Il) biosorptive properties of live and treated *Candida sp*. J Environ Sci Health. 36:367- 381.
- Rehman A, Shakoori FR, Shakoori RA (2008) Uptake of heavy metals *by Stylonychia mytilus* and its possible use in decontamination of industrial wastewater. World .1 Microbiol Biotechnol 24:47- 53.
- Ercal N, Gurer-Orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part 1: mechanisms involved in metal induced oxidative damage. Curr Top Med Chem. 1 (6): 529- 539.
- Kabata-Pendias A, Pendias H (2001) Trace elements in soils and plants. CRC Press (ed.). Florida. EISA. 413 p.
- Whittaker JW (1999) Oxygen reactions of the copper oxidases. Essays Biochem. 34: 155- 172. WHO (2006) Guidelines for drinking-water quality. Vol. 1, Recommendations, - 3rd ed.
- Shleev S. Tkac J, Christenson A, Ruzgas T. Yaropolov Al. Whittaker JW, Corton L (2005)

  Direct electron transfer between copper-containing proteins and electrodes. Biosens

Bioelectron. 20: 2517- 2554.

- Puig S, Thiele DJ (2002) Molecular mechanisms of copper uptake and distribution. Cun-Opinion Chem Biol. 6: 171- 180.
- Barceioux DG (1999) Chromium. Clinic Toxicol. 37:173- 194.
- Barton H. Zachwieja Z, Folta M (2002) Predicted intake of trace elements and minerals via household drinking water by 6-year-old children from Krakow (Poland). Food Addit Contam. 19: 906- 915.
- Baldrian P. Gabriel J (2005) Lignocellulose degradation by *Pleurotus ostreatus* in the presence of cadmium. FEMS Microbiol Lett. 220 (2): 235- 240. Baldrian P (2003) Interactions of heavy metals with white-rot fungi. Enzyme Microb Technol. 32: 78-91.
- Blain I, Slama P, Giorgi M, Tron T, Reglier M (2002) Copper-containing monooxygenases: enzymatic and biomimetic studies of the O-atom transfer catalysis. J Biotechnol. 90 (2): 95-112.
- Hashem AR. (1989) Effect of copper on the growth of *Aspergillus niger. Penicillium chrysogemim* and *Rhizopus stolonifer*. Trans Mycol Soc Japan. 30: 111- 119.
- Gadd GM (1999) Fungal Production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. Adv Microb Physiol. 41: 47- 92. Mehra RK, Winge DR (1991) Metal ions resistance in fungi: molecular mechanisms and their regulated expression. J Cell Biochem 45:30- 40.
- Gadd GM (1993a) Interactions of fungi with toxic metals. New Phytologist 124: 25- 60. Gadd GM (1993b) Microbial formation and transformation of organometallic and organometalloid compounds. FEMS Microbiol Rev. 11: 297- 316.

- Wang Jing-S, Hu Xin-J, Liu Yun-G, Xie Shui-B, Bao Zheng-L (2010) Biosorption of uranium (VI) by immobilized *Aspergillus fumigatus* beads. J Environ Radioact. 101: 504- 508.
- V'ang JL. Chen C. (2006) Biosorption of heavy metals by *Saccharomyces cerevisiae*: a review. Biotechnol Adv. 24: 427- 451.
- Wang JL. (2002) Biosorption of copper(II) by chemically modified biomass of *Saccharomyces cerevisiae*. Process Biochem. 37:847- 850.
- Hussein H, Farag S, Kandil K. Moawad H (2005) Tolerance and uptake of heavy metals by Pseudomonads. Process Biochem. 40: 955- 961.
- Ahmad I, Ansari MI, Aqil F (2006) Biosoiption of Ni, Cr and Cd by metal tolerant *Aspergillus niger* and *Penicillium sp.* using single and multi-metal solution. Indian J Exp Biol. 44: 73- 76.
- White C. Saver JA, Gadd GM (1997) Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. FEMS Microbiol Rev. 20: 503- 516.
- Kapoor A. Viraraghavan T, Cullimore DR (1999) Removal of heavy metals using the fungus *Aspergillus niger.* Bioreso Technol. 70: 95- 104.
- Kapoor A, Viraraghavan T (1998) Removal of heavy metals from aqueous solution using immobilized fungal biomass in continuous mode. Water Res. 32: 1968- 1977.
- Kapoor A, Viraraghavan T (1997) Heavy metal biosorption sites in *Aspergillus niger*. Bioreso Technol. 61: 221 - 227.
- Kapoor A. Viraraghavan T (1995) Fungal biosorption- an alternative treatment option for heavy metal bearing waste water: a review. Bioresourse Technol. 53: 195- 206.

- Congeevaram Sh, Dhanarani S, Park J, Dexilin M, Thamaraiselvi K. (2007) Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. .1 Hazard Mater. 146: 270- 277.
- Zafar S, Aqil F, Ahmad I (2007) Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour Technol 98:2257- 2261.
- Damare SR, Nagarajan M and Raghukumar C (2008) Spore germination of fungi belonging to *Aspergillus* species under deep-sea conditions. Deep-Sea Research I 55: 670 - 678.
- Raghukumar C (2008) Marine fungal biotechnology: an ecological perspective. Fungal Diversity 31: 19-35.
- D'souza, J (1995). Effect of mining on the microbial ecology and the environment in the Bicholim Taluka of Goa, and the study of the possible restoration process in the region. Project report submitted to the Department of Environment, Government of India, New Delhi.
- Fourest E and Roux JC (1992). Heavy metal biosorption by fungal mycelial by-products: mechanisms and influence of pH. Appl Microbiol and Biotechnol, 37: 399-403.
- Fourest E, Canal C and Roux JC (1994). Improvement of heavy metal biosorption by mycelial dead biomass (*Rhizopus arrhizus, Mucor meihei and P. chrysogenum)*: pH control and cationic activation. FEMS Microbiol Reviews, 14: 325-332.
- Fourest E, Serre A, Roux JC (1996). Contributions of carboxyl group to heavy metal binding sites in fungal walls. Toxicol Environ Chem, 54: 1-10.
- Gadd G M and White C (1998). Removal of thorium from simulated acid process streams by fungal biomass. Biotechnol Bioeng, 33: 592-597.

- Gadd GM (1990). Fungi and yeasts for metal accumulation, In: HL Ehrlich and CL Briefly (eds) Microbial. Mineral Recovery, McGraw-Hill, New York, pp 249 - 276.
- Levinskaite L (2001). Fungi of the genus *Penicillium* under the influence of heavy metals. Metal-tolerance of various isolates. Botanica Lithuanica, 7: 79-91.
- Paknikar KM, Palnitkar US and Puranik PR (1993). Biosorption of metals from solution by mycelial waste of *Penicillium chrysogenum.* In: Torma AC, Apel ML, Brierley CL (eds) Biohydrometallurgical Technologies. The Minerals, Metals and Materials Society, Warrendale, Vol II, pp-229-235.
- Pethkar AV and Paknikar KM (1998). Recovery of gold from solutions using *Cladosporium cladosporioides* biomass beads. J Biotechnol, 63: 121-136.
- Rao M, Parvate AV and Bhole AG (2002). Process development for the removal of copper and lead from aqueous solutions using low-cost materials. I J Environ Protection, 22: 17-24.
- Xie, X.-Q., Wang, J., Huang, B.-F., Ying, S.-H., Feng, M.-G. (2010) A new manganese superoxide dismutase identified from Beauveria bassiana enhances virulence and stress tolerance when overexpressed in the fungal pathogen. Applied Microbiology and Biotechnology, 86:1543-1553.
- Volesky, B. (1990) Removal and Recovery of Heavy Metals by Biosorption, Biosorption of Heavy Metals. CRC Press. Boca Raton. FL: 3-43.
- Singh, H. (2006) Mycoremediation: Fungal bioremediation. Wiley-Interscience, Hoboken, New Jersey, 484-532.
- Levinskaitė, L., Smirnov, A., Lukšiene, B., Druteikiene, R., Remeikis, V., Baltrunas, D. (2009) Pu (IV) and Fe (III) accumulation ability of heavy metal-tolerant soil fungi.

- Nukleonika, 54:285-290.
  Levinskaitė L. (2001) Stimulation effect of nickel, cadmium and chromium (VI) on soil micromycetes. Biologia, 4:13-15. Levinskaitė L. (2002) Response of soil fungi to chromium (VI). Ekologija, 1:10-13.
- Gadd, G.M. (1988) Accumulation of metals by microorganisms and algae. Biotechnology - A Comprehensive Treatise, 6:401-433.
- Gadd, G.M. (1990) Heavy metal accumulation by bacteria and other microorganisms. Review Experientia, 46:834-840.
- Gadd, G.M. (1993) Interaction of fungi with toxic metals. New Phytologist, 124:25-60.
- Gadd, G.M. (1994) Interactions of fungi with toxic metals. In: The Genus *Aspergillus*: From Taxonomy and Genetics to Industrial Application (Eds.: Powell, K.A., Renwick, A. and Peberdy, J.F.). Plenum Press, New York, USA, 361–374.

Document Information

INTRODUCTION

Analyzed document	Riya Plagiarism .docx (D164796494)
Submitted	2023-04-23 16:43:00
Submitted by	Priya
Submitter email	priyadcosta@unigoa.ac.in
Similarity	0%
Analysis address	priyadcosta.unigoa@analysis.urkund.com

Sources included in the report

Entire Document

https://secure.urkund.com/view/157632741-612921-171089#/