Plant growth promoting and biocontrol potential of Putative *TRAMETES HIRSUTA* fungal isolate for organic farming and CuO nanoparticle synthesis as antifungal agent.

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By

Binita Khadka

Registration No.:5-2-3-396-2013

Roll no: BT 606/075

Supervisors

Asst. Prof. Alina Shri Sapkota

Dr. Pramod Aryal

Date: ……

RECOMMENDATION

This is to certify that the research work entitled "**Plant growth promoting and biocontrol potential of Putative TRAMETES HIRSUTA fungal isolate for organic farming and CuO nanoparticle synthesis as antifungal agent**" has been carried out by Miss. Binita Khadka under our supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation

…….

Alina Shri Sapkota
(Supervisor)
Assistant Professor
Central Department of Biotechnology
Kitripur, Nepal

……

Dr. Pramod Aryal
(Supervisor)
Visiting senior scientist
Central Department of Biotechnology
Kirtipur, Nepal

Date: ……

CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled "**Plant growth promoting and biocontrol potential of Putative TRAMETES HIRSUTA fungal isolate for organic farming and CuO nanoparticle synthesis as antifungal agent**" presented to the evaluation committee by Miss Binita Khadka is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……..

Prof. Dr. Krishna Das Manandhar
Head of Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Nepal

……..

External Examiner
Dr. Parash Mani Timilsina
Associate professor
Department of Biotechnology
Kathmandu University

……..

Internal Examiner
Dr. Pramod Poudel
Associate professor
Central department of Biotechnology
Tribhuvan university
Kirtipur, Nepal

……

Supervisor
Alina shri sapkota
Assistant professor
Central Department of Biotechnology
Kirtipur, Nepal

……

Supervisor
Dr. Pramod Aryal
Visiting senior scientist
Central Department of Biotechnology
Kirtipur, Nepal

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Glossary Acronyms

CuO	Copper Oxide
FTIR	Fourier Transform Infrared Spectroscopy
NPK	Nitrogen Phosphorus and Potassium
HCN	Hydrogen Cyanide
IAA	Indole Acetic Acid
GA	Gibberellic Acid
CK	Cytokinin
SA	salicylic Acid
BR	Brassinosteroids
CWDEs	Cell Wall Degrading Enzymes
BCA	Biological Control Agent
IPM	Integrated Pest Managment
MIC	Minimal inhibitory Concentration
VOC	Volatile Organic Compound
CSA	Competitive Saprophytic Ability
MCO	Multi copper Oxidase
SE	Solubilization Efficiency
SI	Solubilization Index
AMF	Arbuscular Mycorrhizal Fungi
PSM	Potassium solubilization microorganism
PVK	Pikovskaya
ATP	Adenosine Tri Phosphate
SAR	Systemic Acquired resistance
ISR	Induced systemic resistance
MAMPs	Microbe associated molecular patterns

PR	Pathogenesis Related
GRAS	Generally Recognized As Safe
PDA	Potato Dextrose Agar
F.O	Fusarium Oxysporum
CMC	Carboxy Methyl Cellulose
DNS	3-5 Dinitro Salicylic Acid
HPLC	High Performance Liquid Chromatography
PIRG	Percentage Inhibition Growth Rate
LPCB	Lactophenol Cotton Blue
BSA	Bovine Serum Albumin
EDTA	Ethylene Diamine Tetra Acetic Acid
PCR	Polymerase Chain Reaction
CTAB	Cetyltrimethylammonium bromide
RECAST	Research Center of Applied Science and Technology
CCR	Carbon catabolite repression
NMR	Nitrogen metabolite repression
HD	Halozone Diameter
KSM	Potassium Solubilizing Microorganism
NJ	Neighbor Joining
MUSCLE	Multiple Sequence Comparision by log- Expectation

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ABSTRACT

Fungal plant pathogens are prevalent, with more than19,000 strains causing disease in crop plant globally. Concerns over chemical residues and fungicide resistance have led to a decline in synthetic fungicide use. Therefore, it is vital to explore alternatives against the pathogens that are not only ecologically sound but also preserve soil biodiversity and promote the growth of plants. Our primary objective was to identify potent fungal isolates with cell wall degrading enzymes targeting diverse plant pathogens while exhibiting traits that promote plant growth. Additionally, our research explores the application of Nanotechnology for combating pathogens.

Soil sample was collected from Himalayan region which is noted for its rich biodiversity in beneficial microbes. Slow growing saprophytic fungal isolates was screened following heat shock on soil sample, antibiotics use and CCR mechanism. A total of 11 fungal strain was screened among which 6 isolates were selected for the inhibition test against various plant pathogen. Various cell walls degrading enzymes such as chitinase, cellulase, protease, amylase, pectinase and PGP traits was tested by qualitative as well as quantitative method. The highest cell wall degrading enzyme activity was shown by fungal strain BK1 as well as BK102 whereas the isolate BK102 has more PGP feature. Furthermore, partial purification of the protein was done by ammonium sulfate precipitation method of isolate BK102 strain followed by the dialysis. Partial purification revealed that the protein concentration increases after dialysis. CuO nanoparticle on other hand was synthesized and characterized by FTIR, synthesis was done by the precipitation method followed by the calcination at 500°C of copper sulphate as substrate. The antifungal activity of the formed CuO nanoparticle (1%) has shown the greatly reduce mass of fungal mycelia on the plate with PDA as substrate

Dual culture analysis had shown BK102 (64.38%) as the greatest inhibitor of *A.solani*, whereas for *R.solani* and *Fusarium oxysporum* BK1(60.56%, 62.44%) followed by BK102(58.52%, 56.33%) has been the greatest inhibitor. Isolate BK102 has shown the promising PGP feature such as, NPK, zinc solubilization, organic acid production. Upon ITS sequencing and phylogenetic analysis through neighbor joining method it was found that BK102 isolate has the homology with the fungal strain *Trametes hirsuta*.

Therefore, we would advise using consortia of effective isolates that exhibit a higher proportion of inhibition with PGP properties as a significant plant pathogen antagonist.

Keywords: Bio-control, Plant growth promotion, Dual culture, Antagonist, Fungus, CuO nanoparticle.

Contents

CHAPTER 1

1. Introduction

1.1. Background

The quality and quantity of agricultural products have been significantly impacted by plant pathogens, which include fungi, bacteria, viruses, and nematodes. This poses a major threat to global food production every year and results in significant losses or damage to crops around the world (Dean et al., 2012).chemical pesticides have long been used in agricultural sector against the pest and to enhance crop productivity. Chemical agricultural inputs, such as fertilizers and insecticides, may be used to meet the growing demand for agricultural products brought on by the world's population growth however it has a detrimental effect on soil health and production(Garcia-Rubio et al., 2020) and degrades the environment by producing greenhouse gas emissions, which will greatly contribute to climate change and global warming (Aloo et al., 2022). Such pesticides often contaminate abiotic components of the environment leading to the adverse effect on biodiversity and ecosystem. Pesticides runoff can contaminate the water resources and pose a serious threat to aquatic ecosystem as well (Damalas & Eleftherohorinos, 2011). Fungicide overuse and irrationality harm the ecosystem and have unintended consequences on plants and animals as it cause residual issues, the emergence of disease resistance (P. Choudhary et al., 2018) and various health risks to humans such as neurological disorders ,reproductive problems and certain types of cancer (Damalas & Eleftherohorinos, 2011). Furthermore, the overuse of chemical pesticides can lead to the development of pesticide resistance strain which necessitate the application of even stronger chemical alternative control method (Pretty et al., 2018).

Consequently, it is highly preferred to use conventional farming techniques, which include a variety of strategies and approaches for managing plant diseases, such as genetically engineered plants, heat treatments, UV irradiation, cold storage, and applying elicitors to develop resistant varieties; application of biological control agents; and good horticultural and agronomic practice(Droby, 2006) (Singh & Chawla, 2012).Integrated pest management techniques offer a sustainable and environment friendly strategies for pest control, with minimizing reliance on chemical pesticides. One of the major aspect of IPM is the utilization of Biological control agent such as natural enemies of pests, to manage pest population. BCA includes predators, parasitoids and pathogens that target specific pests while posing minimal risk to non-target organisms and the environment (Gurr et al., 2017).

The term "antagonistic action" in microbiology was first used by (Roberts & Roscoe, 1997)who demonstrated the antagonistic behaviour of bacteria and *Penicillium glaucum* in liquid cultures. In (Hartley, 1921) work, microorganisms with antagonistic potential were introduced into soil, marking the first direct use of biological control against plant diseases. Beneficial microorganisms

(biocontrol and/or plant bio stimulant microorganisms) seem to be the most promising natural products for guaranteeing plant health and the safety and quality of vegetative products. A variety of studies (thousands of published scientific articles) executed over the past few decades have evaluated the effectiveness of multiple BCAs against a range of dangerous fungal diseases; as a result, BCA-based bio formulas are currently marketed (Hobley et al., 2013). Although research on this issue is still underway with the goal of discovering/elucidating better novel mechanisms, optimizing biocontrol activity, and formulating microbial antagonists, the primary mechanisms of action of BCAs have also been clarified (Farzand et al., 2020).

Beneficial microorganisms, mainly fungus, and bacteria, are a rich source of multiple naturally occurring compounds that can control plant diseases on several levels. Fungal antagonists are used as Biocontrol Agents (BCAs) throughout the world as a result of their great efficacy in managing infections and plant diseases. Fungus produces large quantities of bioactive compounds against the plant pathogen and can be used as an agrochemical for crops. Fungus of genera aspergillus, trichoderma and penicillium could be used as the potential antagonist against various fungal as well as bacterial plant disease (Ayaz et al., 2023). Along with this microorganism's aid plant growth by several means such as phosphate solubilization, siderophore creation, nitrogen fixation, and HCN production (Rivera-Méndez et al., 2020).

On the other hand prospective uses of copper oxide nanoparticles (CuO NPs) in agriculture, especially as antifungal agents against plant infections, have drawn interest due to their large surface area-to-volume ratio, which improves their contact with fungal cells and causes growth inhibition and cell death, these nanoparticles have strong antifungal characteristics (El-Abeid et al., 2024).Copper oxide nanoparticle being a multi targeting product can achieve a great result in plant disease management and growth promotion as suggested by (Ashraf et al., 2021). Reduction in the hyphal growth and spore germination with no toxicity for plant growth and production has been noted (Ashraf et al., 2021). Investigation has shown 68 and 65.5% reduction in Fusarium wilt incidence and severity respectively on use of copper oxide nanoparticle. Thus it is suggested that copper oxide nanoparticle can be used in case of nutritional copper deficiency in soil as copper is important micronutrient required for the plant growth promotion as well as antifungal agent (Lopez-Lima et al., 2021) .

1.2. Rational of the study

Use of chemical fertilizer and pesticide has been increasing for the enhanced productivity and control of pests in the agricultural sector, while along with the beneficial impact at shorter period of time it has been resistant to the environment, reduce the soil fertility and number of soil microorganism, ground water pollution. In order to mitigate such problems, sustainable agricultural technique which ensures the food security and maintain the soil microbial diversity should be adopted (Umesha et al., 2018). Use of the biocontrol approach could be the cost effective and environment friendly method to enhance the fertility of soil as well as antagonist activity against the various soil borne disease. Thus our study focuses on the isolation, screening of the antagonistic fungal species from the soil sample. The selected isolates can further be used as antifungal agent against various plant pathogen and also as a bio fertilizer.

1.3. Objectives

1.3.1. General Objective

- To isolate fungal strain producing multiple cell wall degrading enzymes and plant growth promoting traits along with test of antagonist character to various fungal plant pathogen.

1.3.2. Specific objectives

- To isolate and screen the slow growing saprophytic fungal strain from the collected soil sample.
- To access the different cell wall degrading enzyme (laccase, chitinase, cellulase, protease, amylase, pectinase) produced by the screened isolates.
- To study different plant growth promoting features (such as, zinc solubilization, IAA production, NPK, HCN) of the isolated strains.
- To evaluate antifungal activity of the isolate against the fungal pathogen by dual culture technique.
- To perform microscopic and molecular characterization (DNA extraction, PCR, Phylogenetic tree analysis) of the most effective isolate.
- To partially purify the different enzymes.
- To prepare CuO nanoparticle and characterize through FTIR.
- To perform antifungal activity test of CuO nanoparticle on the fungal pathogen.

1.4. HYPOTHESIS

1.4.1. Null hypothesis

Fungal strain antagonist to various fungal pathogen, having multiple cell wall degrading enzymes and plant growth promoting traits will not be isolated.

1.4.2. Alternative hypothesis

Fungal strain antagonist to various fungal pathogen, having multiple cell wall degrading enzymes and plant growth promoting traits will be isolated.

CHAPTER 2

2. literature review

2.1. Biocontrol: An approach of integrated pest management technique

The application of microbes, such as bacteria, yeast, fungi, insects including viruses and bacteriophages (Mendes et al., 2011) that are antagonistic to pathogens and possess features that promote plant growth is known as "biocontrol," and it is one of the most prevalent agricultural techniques. (Lahlali et al., 2022a)When no alternative pathogen remedy has emerged, biocontrol agents, which are highly target-specific and effective against resistant pathogens, can be used. Additionally, biocontrol is an environmentally friendly, and sustainable method that has the potential to replace the conventional chemical and pesticide method to control disease.

Effectiveness of the BCA depends on the screening and selection of antagonistic microbe. Depending on the pathogen's site and nature, antagonistic isolates must be screened(Ghozlan et al., 2020) When introduced to a targeted ecosystem, the quality and quantity of microbes have an impact as BCAs.Utilizing a combination of BCAs, applying an antagonist at the right time, integrating biological and chemical controls, and improving the environment are some of the strategies to enhance the effectiveness. According to (Harman et al., 2004a)the main benefits of using beneficial microbes for plants include the establishment of an antagonistic microbial community in the rhizosphere, suppression of pathogens, a general enhancement of plant health, growth promotion, increased nutrient availability and uptake, and enhanced host resistance to both biotic and abiotic stresses.(Lahlali et al., 2022a) technique employed by BCAs include solubilization and sequestration of inorganic nutrients, induced resistance and inactivation of enzymes produced by pathogens responsible for biocontrol and plant growth promotion (Monte, 2001); (Kubicek et al., 2011).

2.2. Mode of Action of BCA

Microbes interact with pathogens through a wide range of mechanisms to cope with disease. Understanding the BCA's mechanism provides useful insights that can be used to select an effective strain against a phytopathogen (Mishra et al., 2018). Interaction of BCA can be categorized to direct and indirect mechanism, to combat disease and production of factors for various stress-tolerant capacities (Köhl et al., 2019) .

2.2.1. Direct mechanism of BCA

Biocontrol of diseases by BCAs through direct pathogen antagonism is mainly based on four mechanisms, which includes hyper parasitism, competition for space and nutrition, antibiosis, production of secondary metabolites (Köhl et al., 2019).

2.2.1.1. Antibiosis

Antibiosis is outlined as the process that suppress or reduce the growth and/or proliferation of the phytopathogens by the production of antimicrobial compounds, antibiotics, and secondary metabolites. It entails the production of various cell-wall-degrading enzymes like cellulose, xylanase, chitinase, glucanase, lipase, amylase, arabinose, protease, and volatile metabolites such as 6-n-pentyl-2H-pyran-2-one (6-PAP) or microbial volatile chemicals that interfere with phytopathogen metabolism and prevent the pathogen(Howell, 2003) (Alfiky & Weisskopf, 2021). (Kovács et al., 2021) demonstrated that bacteria are involved in the production of various extracellular hydrolytic enzymes, secondary metabolites including trichomycin, gelatinomycin, chlorotrichomycin, and antibacterial peptides produce by trichoderma exhibit antibacterial effect as well act as plant growth promoter as shown by (Yao et al., 2023).(Glandorf et al., 2001)showed genetically engineered pseudomonas putida WCS358r strain produce phenazine, and 2,4-diacetylphloroglucinol (DAPG) had greater capacity to control plant diseases in field grown wheat. Moreover, Transgenic construct containing gene of lytic enzyme of *T. atroviride* have shown inhibition of *R.solani .*

(Toghueo et al., 2016) shows that trichoderma species inhibit the spore germination of *F. solani* with a minimal inhibitory concentration (MIC) of 0.66 mg/ml. Among filamentous fungi, different *Trichoderma* species are known to produce antibiotic compounds as well as volatile organic compound (Harman et al., 2004b). However, for yeasts and bacterial BCAs, VOC production includes several classes of chemicals with antimicrobial activities. VOCs that are involved in the biocontrol activity are alcohols, esters, aldehydes, ketones, terpenes and lactones (Palmieri et al., 2022).

2.2.1.2. Hyperparasitism

Hyperparasitism" a complex physiological process that should be viewed in the broad perspective of microbial competition where the targeted phytopathogen is parasitized through direct contact where in one benefits at the expense of the other. (Witzell et al.,2014).Mycoparasitic relationships can be necrotrophic or biotrophic, and depending on the i ntimate nature of the relationship, they can be further classified as contact necrotrophs, invasiv e necrotrophs, haustorial biotrophs, intracellular biotrophs, or fusion biotrophs. In biotrophic mycoparasitism, the hyperparasite gains the nutrients from host without killing it, where host and mycoparasitic fungus interact in a stable and balanced way (Barea & Jeffries, 1995). The parasite's sporulation, growth, and metabolism are unaffected by biotrophic mycoparasitism during the initial stage. Compared to necrotrophs, biotrophic mycoparasites often have more constrained host ranges (Ram et al., 2018).

Necrotrophic or destructive mycoparasites kill their hosts as a result of their parasitic activity. Necrotrophic hyperparasites, in contrast to biotrophic hyperparasites, are far more suited for c ommercial application since they can be produced in large quantities on artificial media and rec eive their nutrients not only from dead host cells but also from other readily available organic d ebris. Direct hyphal cell wall contact between the host and mycoparasite has the potential to result in host cytoplasmic death, but various hyphal cell wall-degrading lytic enzymes, including chitinase, protease, cellulose, glycans, and exotoxins, are implicated in the host's cytoplasmic death before the hyphal contact (Barnett & Binder, 1973) .

Mycoparasitism suppresses the disease effectively than antibiosis by the eventual Disruption/deletion of the pathogens hyphae takes place (Latz et al., 2018). In case of fungus as pathogen, mycoparasitism involves the use of fungal cell wall degrading lytic enzymes. Considering that fungal cell walls are composed of glucans, chitin and proteins, thus glucanases, chitinases and proteases degrade these biopolymers in mycoparasitism (Gow et al., 2017).

Chitinase, β-glucanases, and proteases were established as key enzymes involved in mycoparasitism (Vázquez-Garcidueñas et al., 1998);(Cortés et al., 1998);(Carsolio et al., 1994). The entophytic microbes feed on the plant behinds its mycoparasitism activity by penetrating hyphae and coil around it (Mukherjee et al., 2022).Mycoparasitism, is a tightly regulated process which involves activation of several signal transduction pathways leading to transcriptional activation/repression of several genes between the microbes (Karlsson et al., 2017), biotrophs and hemibiotrophs obtain nutrients by manipulating the expression of plant genes regulating cell membrane permeability or the genes encoding transporters. (Palmieri et al., 2022),.

Gliotoxin was the first Trichodermaderived molecule shown to have antifungal action and a fun ction in the antagonistic activity against T. virens (Weindling, 1943).
According to Lorito et al. (1994), gliotxin and endochitinase have a synergistic action that preve nts B. cinera spore germination.The pathogenic fungus's cells are lysed by the combined action of hydrolytic enzymes and secondary compounds generated by trichoderma (Druzhinina et al., 2011).Combination of antimicrobial characteristics such as mycoparasitism, antibiosis, IDR and competitive exclusion is involved in the disease suppression (Sharma et al., 2017). *P. guilliermondii* was shown to strongly adhere to hyphae of the plant pathogen *B. cinerea* and to cause hyphal collapse, presumably due to the secretion of hydrolytic enzymes such as glucanase (Wisniewski et al., 1991).

2.2.1.3. Competition for space and nutrition

It is the phenomenon in which the pathogen and the BCAs compete with each other for space and nutrition. Competition among the microbes for nutrients and space is consider as primary mode of biocontrol. (Schaible & Kaufmann, 2005);(Spadaro & Droby, 2016). Competition of nutrient specially (carbon, nitrogen, iron) and space is often suggested as a potential mechanism of action in biological control systems. Inhibition of pathogen due to competition for space and nutrition is seen in (Janisiewicz et al., 2000)which determine the competition between the yeast

like biocontrol agent, Aureobasidium pullulans, and Penicillium expansum for limited nutrients in apple juice.

(Köhl et al., 2019) suggested that Competition between nutrients binds restricted nutrients through the actions of enzymes and other processes. Since these processes occur wherever that saprophytic microbes colonize in a competitive manner and are widespread in the environment. BCAs compete with pathogens mainly for carbohydrate and nitrogen sources such as amino acids (Spadaro & Droby, 2016)along with this micronutrient competition for iron ,phosphorus, sulfur, hydrogen, calcium, and other metals(Ghoul & Mitri, 2016). for microbial growth which is crucial element as shown by the study between BCAs (bacteria, yeasts and fungi) versus pathogens (Filonow, 1998) ,(Palmieri et al., 2022).Iron is an essential micronutrient required in trace amount for the growth of microbes and are key element for the formation of several enzymes catalase and cytochromes), Iron Sulphur cluster (Fe/S) containing electron carriers proteins, and di-iron and mononuclear enzymes, along with this expression of genes need to be in oxidative stress with state is obtained by interacting with the iron, to carry out the essential metabolic activities by the microbes. Strategies exhibited by yeast to respond to iron depletion consists of: (i) activation of systems of iron uptake, (ii) mobilization of intracellular stores of iron, and (iii) metabolic adaptations to iron limitation (Philpott & Protchenko, 2008).

Trichoderma species are regarded as ruthless and effective competitors for nutrients and space. Competition for soluble ferric iron is based on production and/or utilisation of high-affinity chelators termed siderophores (Lemanceau et al., 1992);(Neilands, 1995).Ferric ions from the rizosphere are trapped by a siderophore that Trichoderma secrete, making them inaccessible by competitors' bacteria. Trichoderma against soil-borne and post-harvest pathogens like Botrytis cinerea (Chet & Inbar, 1994);(Harman et al., 2004a), T. asperellum against F. oxysporum f.sp. lycopersici (Segarra et al., 2010), are seen and siderophores may also be advantageous for plant growth and development due to their iron solubilizing activity(Zeilinger et al., 2016). An intrinsic characteristic of a fungus to colonize dead organic substrates by competition is characterized as CSA competitive saprophytic ability by (Ahmad, 1987) .QT5-19 produce the volatile organic compounds (VOCs) and shown to inhibit disease in strawberry caused by pathogen B. cinerea and S. sclerotiorum, whereas the VOCs from the virulent isolate RoseBc-3 of B. cinerea had no detectable antifungal activity thus showing highest competitive saprophytic ability of QT5-19 through extensive mycelial growth and production of antifungal volatiles (Ahmad, 1987).

Microbes with higher CSA should produce antimicrobial substances which are inhibitory to the competitors , tolerate the mycoparasitic effects from the competitors, grow rapidly in response to stress and the transient nutritional stimulus which includes utilizable carbon and nitrogen sources, under which an appropriate extracellular enzymes are secreted for degradation of the complex constituents in the substrates (Kamaruzzaman et al., 2020). Amount of enzyme produced by the organism is directly proportional to the competitive saprophytic ability and

rhizosphere competence, as shown by comparative analysis of different species of trichoderma isolates.(Ahmad, 1987).

Apart from enzyme production for nutrient competition, biofilm formation can be considered as strategy to compete for space among the microbes. Biofilms are the virulence factor of the microbial communities that live and grow on surfaces and can be comprised of a single species or represent multi-species consortia (Costa-Orlandi et al., 2017).

2.2.1.4. Production of various enzymes and toxins

The synthesis of lytic enzymes such as chitinase, glycan's, proteases, and toxins is demonstrated to be increased when there is a nutritional deficiency or when complex biomolecules are present in the media. Necrotrophic bacteria during necrosis aid saprophytic endophytes in obtaining nutrients from decomposing materials; microbes with higher lignin degradation enzyme laccase production would have higher CSA.

Chitin, glucans, and glycosylated proteins make up the majority of the fungal cell wall (Free, 2013). Chitin serves as the major backbone, and glucan, which makes up about 50% to 60% of the dry weight of the cell wall, serves as the filling of the backbone to which components of cell walls are covalently attached, maintaining integrity and mechanical strength of the fungal cell wall.

Chitinase

Chitin linear, insoluble homopolymer composed of beta-1,4-linked subunits of the acetylated amino sugar N-acetyl glucosamine and second most abundant polysaccharide after cellulose is the major structural component in the exoskeletons of the crustaceans, crabs and shrimps, as well as the cell walls of fungi (Elieh-Ali-Komi & Hamblin, 2016),(Abo Elsoud & El Kady, 2019). The cell walls of filamentous fungi consist of up to 20% or more chitin (Seidl, 2008). Chitinases are glycosyl hydrolases present in a wide range of organisms including bacteria, fungi, insects, plants and animals have been reported in(Hoster et al., 2005). Chitinase enzyme is capable of degrading chitin, and this enzyme can be used as a biological fungicide against phytopathogenic fungi, as well as an insecticide against insect pests(Senol et al., 2014). *Trichoderma* species produce a range of enzymes which can degrade the cell walls of fungi among which chitinase produced by *Trichoderma* spp. parasitize *R. solani* by activating the expression of chitinase genes in the absence of accessible nutrients like glucose and cellulose(mature bark), but when easily accessible nutrient is available (fresh bark), *Trichoderma* Spp. seems to be less pathogenic towards *R.solani* (Benhamou & Chet, 1997). Inhibition of up to 35% and 62% of germ tube elongation and mycelial growth of *(Mycosphaerella fijiensis)* causative agent of the Black Sigatoka Disease (BSD) of banana has been demonstrated by the chitinase produced by *Streptomyces galilaeus* CFFSUR-B12(Nunes & Philipps-Wiemann, 2018).

Glucanase (cellulase and amylase)

β-glucans are a heterogeneous group of (sugars) non-starch polysaccharides, consisting of D-glucose monomers linked by β-glycosidic bonds (El Khoury et al., 2012) which are found in the cell walls of the bacteria, fungi, yeasts, algae, lichens, and plants, such as oats and barley (Rahar et al., 2011).In fungal species it is a branched polymer with branches attached to the core polymer β-(1,3)-Glucan by β-(1,6)-linkages which allow the proper binding with the other cell wall components to maintain the proper assembly of cell wall and constitutes around 30 to 80 % of the dry mass of fungal cell wall (Aimanianda et al., 2017).

Glucans are the most abundant polysaccharides in the cell walls of fungi, and their structures are highly variable. They are complex polysaccharides consisting of repeated units of D-glucose linked by glycosidic bonds.(De Felice et al., 2020). Accordingly, their glucose moieties may be joined through either or both alpha (α) or beta (β) mixed α,β-glucans linkages(Synytsya & Novak, 2014), they are either linear or branched, and amorphous or microfibrillar (Ruiz-Herrera & Ortiz-Castellanos, 2019).The most common and linear examples of α- glucan are starch, glycogen, and pullulan, (Preiss, 2009). β-d-glucans are amylose and cellulose, which are the simple polymers of α- and β-d-Glcp, respectively (Caipang & Lazado, 2015). The major component of fungal cell wall β-(1,3)-Glucan are bound together by β-(1,6)-glycosidic linkages which maintain the cell wall assembly (Aimanianda et al., 2017). Glucanase are the enzymes which are usually glycoside hydrolases that cleave the glycan chains of the peptidoglycan(Santin & Cascales, 2017).

Protease

Proteases (also known as peptidases or proteolytic enzymes) are enzymes that breakdown peptide bonds in proteins to release their individual free amino acids. Based on the pH range where they exhibit the greatest activity, these enzymes are classified as acid, neutral, and alkaline proteases: acidic (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0), and alkaline (pH 8.0 to 13.0) (Gupta et al., 2002);(Sabotič & Kos, 2012).

Peptides also regulate the various enzymatic cascade, post translation modification and processing of various enzymes(Rao et al., 1998). Along with the activation of various proenzyme zymogens, which eventually releases other active enzymes from longer, yet inactive, peptide sequences (*Comprehensive Biotechnology*, n.d.) .

protease enzyme can be obtained from the various sources which includes plants, animals, and microorganisms among which microbial protease are most important extensively studied protein accounts for approximately 60% of the total enzyme sales in the world (Zambare et al., 2007)because of their biotechnology potential in various industrial processes such as detergent, textile, leather, dairy and pharmaceutical preparations .(Saran et al., 2007).Among the microbes fungus protease are more preferred for industrial application as they can grow on low cost substrate ,produces more enzyme into culture medium making ease on downstream processing. (Anitha & Palanivelu, 2013). Drug design targeting protease can be done to treat diseases since it is necessary for the pathogenic microbial strain to carry out numerous metabolic processes, including replication, and it can also be employed as a virulence factor(de Souza et al., 2015).

Laccase

The enzyme laccase, often referred to as a lignin-modifying enzyme, is a member of the superfamily of blue multicopper oxidases (MCOs), which are found in many different types of microbes, plants, and fungi (Shraddha et al., 2011).

In industrial host organisms, it is relatively simple to heterologously manufacture laccases, and both ascomycete- and basidiomycete-derived laccase preparations are available commercially. The two groups of fungus that produce a significant amount of laccase are classified as saprophytic and basidiomycetes. The basidiomycetes, in particular Agaricus bisporus, Pleurotus ostreatus, Trametes versicolor, Phanerochaete chrysosporium, and Coprinus cinereus, produce different laccase isoforms among fungal species(Strong & Claus, 2011).In comparison to Ascomycetes and Deuteromycetes the white-rot Basidiomycetes fungi efficiently degrade the lignin which oxidize phenolic compounds to give phenoxy radicals and quinines (Eggert et al., 1996).This enzyme takes part in the oxidation of diverse phenolic and non-phenolic compounds, cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds while reducing oxygen to water without forming toxic byproducts(Shraddha et al., 2011).

Laccase have different industrial applications such as pollution control and bioremediation (Robinson et al., 2001) textile processing like different processes such as bio-bleaching, dyeing, rove scouring, dyeing, finishing, printing, wash-off treatment, dye synthesis &effluent treatment.., food industry processes such as beverage processing, baking, stabilization of wine and beer, and sugar beet pectin gelation (Robinson & Nigam, 2008).The laccase activity in U/ml is calculated by this formula: E.A = A x V/t x e x v Where, E.A = Enzyme activity, A =Absorbance, V =Total mixture volume (ml), v = enzyme volume (ml) t =incubation time, e = extinction coefficient for guaiacol (0.6740 µM/ cm) (D & C, 2019).

Most fungal laccases are active at acidic pH values and lose activity under alkaline condition. According to different research various organism have different optimum temperature and pH between 3.0 and 5.5 for the maximum production of laccase enzyme(Yin et al., 2019).

Several factors such as type of cultivation (submerged or solid state), carbon limitation, and nitrogen source influence the production of laccase(Shraddha et al., 2011). The optimum temperature range for laccase production is between 25°C and 30°C (Thurston, 1994). (Mohorcic et al., 2004)found that mycelia are damaged when fungus is grown in the stirred tank reactor and laccase production by *Trametes multicolour* is considerably decreased. Laccases are inducible enzyme easy to produce heterologous in industrial host organisms. Laccases from WRF usually have masses of 60–80 KDa, acidic p, and are glycosylated (Tuomela & Hatakka, 2011).laccase has an essential role in lignification's of plants whereas it has been shown that it is involved in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis in case of fungal strain (Thurston, 1994).

2.2.1.5. NPK essential macronutrient for plant growth and development

According to several studies, around 17 chemical components play a vital role in plant growth. These elements are categorized as micro and macronutrients based on the amount required by the plant. Micronutrients are chemical elements that plants require in trace amounts, whereas macronutrients are required in greater quantities and play an important role in plant growth and maintenance. Nitrogen (N), Phosphorus (P), Potash (K), Calcium, Sulphur (S), and Magnesium are macronutrients (required in greater quantities), while Iron (Fe), Zinc (Zn), Copper, Boron, Manganese Molybdenum, Chloride, and others are micronutrients (required in smaller quantities) for crop plant growth and development. Uptake of the balance nutrient is required to carry out the plant metabolic and physiological activities for enhancing plant productivity. NPK are the primary macronutrient required about 50–150 lbs./acre. Micronutrient such as (Fe, Mn, Zn, Cu, B, Mo, and Cl) are generally necessary in quantities less than 1 lb./acre(S. Kumar et al., 2021),

Importance of Nitrogen in plant growth promotion

In fungal species, nitrogen is a crucial component for the synthesis of numerous macromolecules, including as proteins, nucleic acids, and chitin, as well as for spore germination and a variety of metabolic processes(Hayer et al., 2014). Nitrogen exists in many different chemical forms, including L-amino acid, ammonium ions, nitrate and nitrite ions, and nitrate ions, which are particularly prevalent in soil. To the contrary, fungi mostly use ammonium and glutamate or glutamine forms of the nitrogen supply (Marzluf, 1993) . In order to address environmental niches and nutritional limitations, fungi species must have the ability to metabolize a variety of nitrogen sources (Tudzynski, 2014).

Utilization of the secondary nitrogen sources involves a group of genes that regulate the uptake and utilization of nitrate, which are activated through the actions of AreA and NirA, transcriptional nitrogen-responsive activators that convert nitrite ions to more preferable ammonium ions, which are otherwise subjected to nitrogen catabolite repression(Meti et al., 2011) (Marzluf, 1997).

When preferred nitrogen sources are available, the extensive transcriptional control system known as nitrogen catabolite repression (NCR) enables the downregulation of genes involved in the utilization of complex nitrogen sources(Fayyad-Kazan et al., 2016). Along with being fungi essential component, nitrogen is a crucial amino acid component that contributes to the formation of a variety of vital proteins and enzymes necessary for normal functioning, biochemical reaction, and building blocks of plant cells. Nitrogen is also a component of the chlorophyll molecule, which enables the plant to capture sunlight energy by photosynthesis, driving plant growth and grain yield (*Https*, n.d.).

Essential role of Phosphate(p) in plant growth

Phosphorus (P) is a major growth-limiting macronutrients required for the proper functioning of plants, deficiencies of which can reduce plant growth and development. It is second most major

element required after nitrogen. However, phosphorus being reactive element exists as insoluble inorganic phosphorus and insoluble organic phosphorus which substantially remains inactive in the soil and thus unavailable to plants. Most soil possess considerable amounts of P, but a large proportion is bound to soil constituents because of its fixation as insoluble phosphates of iron, aluminum, and calcium in the soil. An alternate method (phosphate solubilizing microorganism) has been developed in which helpful microorganisms hydrolyze insoluble phosphorus molecules, both organic and inorganic, into soluble P forms that can be easily assimilated by plants(Kalayu, 2019).Phosphorus is the vital component in every aspect of plant growth and development, from the molecular level to many physiological and biochemical plant activities including photosynthesis development of roots, strengthening the stalks and stems, formation of flowers and seeds, crop maturity and quality of crop, energy production, storage and transfer reactions, root growth, cell division and enlargement, N fixation of the genetic traits in plants and contained within nucleic acids, enzymes, coenzymes, nucleotides, and phospholipids of plant cell. (*Functions of Phosphorus in Plants*, n.d.)

Isolation and characterization of PSM (phosphate solubilizing microorganism) can be done using various growth medium, but a reliable approach first known for initial screening of potential PSM was described by Pikovskaya(PVK) where Tricalcium phosphate was used as insoluble phosphorus source(Ri, 1948). PSM was accessed in terms of the solubilization index (SI), the ratio of total diameter, i.e., clearance zone and the colony diameter (Alam et al., 2002)Microbes apply various approaches to make phosphorus accessible for plants to absorb. These include lowering soil PH, chelation, and mineralization. One of study done by (Yousefi et al., 2011)shows that application of consortia of PSM bacteria (PSB) and arbuscular mycorrhizae fungi (AMF) led to an increase shoot dry matter yield (both SDW and RDW), grain spike number, grain yield of wheat and immediate plant growth by providing easily absorbable P form and production of plant growth hormones such as IAA and GA. Microbes showing PSMs have also shown the biocontrol activity via production of antibiotics ,antifungal metabolites, HCN making it an suitable substitute of inorganic phosphate (Kalayu, 2019).

Role of Potassium (k) on Plant growth regulation

Potassium is the second most essential element after nitrogen for the growth and maintenance of plants. Potassium plays major role in the enhancement of plant product quality, (such as fruit size, appearance, color, soluble solids, acidity, vitamin content, taste, and shelf life), stress tolerance such as drought, excess water, wind, high and low temperature,

Potassium, together with N and P, plays an important role in plant development, influenced by photosynthesis, translocation of photosynthesis, protein synthesis, regulation of stomata, activation of enzymes, and many other processes. It has the major function in plant nutrition, activation of numerous enzymes which affect starch, protein ATP production which regulate the rate of photosynthesis process (*Potassium for Crop Production*, n.d.).

protection of electrical potential gradients in cell membranes, anion-cation balance, turgor regulation, and osmotic regulation in cell as well balance transport of water nutrient and carbohydrate to the plant tissue (Çalişkan et al., 2017).

2.2.1.6. Plant Growth Hormones

Plant hormones (also known as phytohormones) are low molecular weight signaling molecules that influence a variety of physiological processes and are classified into different groups based on their chemical structure which include auxins, cytokinins (CKs), abscisic acid (ABA), gibberellins (GAs), ethylene, jasmonic acid (JA), salicylic acid (SA), brassinosteroids (BRs), and strigolactones (SLs) (Chu et al., 2017) (Bari & Jones, 2009) .Hormone plays vital roles in plant physiological and cellular process such as seed dormancy, growth, metabolism, organ formation, reproduction, and stress responses(Nemhauser et al., 2006) (Umehara et al., 2008). Modulation in the level of different hormones, IAA, GA, CK, JA during the several developmental stages of plant such as fruit ripening, fruit maturation has been observed (Bari & Jones, 2009)

Arabidopsis thaliana has been used as the model plant mostly for the study of innate immunity caused due to the plant hormones(Çalişkan et al., 2017). Plant hormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses(Yang et al., 2013)

Auxin

Auxin are the first group of plant growth hormones discovered. These hormones are derived from indole and involved in plant developmental processes, such as cell division, differentiation and organ formation as well as controls biotic and abiotic stress in plants(Chanclud & Morel, 2016). Based on the location they get produced auxin have variable functions, when produced by apical buds, they promote root growth and development but inhibit lateral bud growth thereby maintaining apical dominance, promotes cell division and differentiation(Dilworth et al., 2017).Besides plants the fungal as well as bacterial strain has the endogenous role of auxin which is involved in symbiotic interactions between plants and bacteria or fungi. They has role in initiation of nodule formation in the nitrogen-fixative bacterial symbiosis (Hirsch et al., n.d.)

Most occurring auxin forms IAA is produced in bacteria from tryptophan through enzymatic steps where tryptophan is converted into indole-3-acetamide by tryptophan-2-monooxygenase enzymes(Zhao, 2010)and finally Indole-3-acetamide is hydrolyzed to form indole-3-acetic acid (IAA). Beside tryptophan, indole-3 pyruvate and tryptophan independent synthesis of auxin in fungal strain has been observed in fungal genera (Reineke et al., 2008).

2.2.1.7. Mycotoxins as a potential biocontrol agent

The use of mycotoxins as biocontrol agents aligns with the growing interest in sustainable and eco-friendly approaches to manage fungal diseases in agriculture. Research in this field is ongoing, and further exploration of the biocontrol potential of mycotoxins holds promise for environmentally friendly strategies in crop protection(Geiser et al., 1998).

Mycotoxins produced by certain fungi have gained attention as potential biocontrol agents against fungal pathogens. These secondary metabolites exhibit antimicrobial properties and can be harnessed for their ability to inhibit the growth and development of other fungi, thereby

serving as bio pesticides. For instance, the mycotoxin gliotoxin, produced by various fungi including *Aspergillus fumigatus*, has been identified for its antifungal activity and potential as a biocontrol agent against plant pathogenic fungi (Scharf et al., 2014).Additionally, aflatoxin, a mycotoxin produced by *Aspergillus flavus*, has demonstrated antifungal properties and is being explored for its biocontrol potential in agricultural settings (Bhatnagar et al., 2006).Furthermore, trichothecenes, mycotoxins produced by *Fusarium* species, have shown promise in controlling fungal pathogens of crops (Desjardins, 2006).These studies highlight the diverse applications of fungal mycotoxins in biocontrol strategies, providing valuable insights for sustainable and eco-friendly approaches to manage fungal diseases. Several metabolites produced by *Metarhizium anisopliae, Beauveria bassiana, Trichoderma* spp., *Fusarium* spp., *Alternaria alternata, F. oxysporum* and *Aspergillus* spp., respectively include destruxins, oxalic salts, trichothecenes, zearalenone, fumonisins, fusaric acid and aflatoxin (Vey et al., 2001).

2.2.2. Indirect mechanism of BCA

Indirect method of plant defense mechanism involves the induction of resistance by stimulating plant defense reactions and stimulating plant growth and soil fertilization. which can be induced by signaling molecules such as salicylic acid, jamonic acid and ethylene leading to the pathway for formation of phytohormones, phytoalexins, and defense enzymes such as phenylalanine ammonia-lyase, chitinase, PR-proteins, and phenolic compounds (Lahlali et al., 2022a).

Plant defences can be induced by pathogenic and non-pathogenic microorganisms as pathogen-associated or microbe-associated molecular patterns (PAMPs or MAMPs), or with certain natural or synthetic chemical compounds(S. Zhang et al., 2022).Based on the nature of the signaling molecule and the regulatory pathways, induced systemic resistance are of 2 types, systemic acquired resistance (SAR), and induced systemic resistance (ISR). Induced systemic resistance and systemic acquired mechanism are both plant defence mechanism against phytopathogen with slight different mechanism where ISR is naturally present in plant and triggered by the action of PGPR or nonpathogenic rizobacteria ,SAR is induced on infection (*Induced Systemic and Systemic Acquired Resistance*, n.d.)

2.2.2.1. Factors involved in induced systemic resistance

Induced systemic resistance(ISR) is the resistance which is naturally present in plant but induced or enhanced by plant associated non-pathogenic rhizobacteria or plant growth promoting rhizobacteria. Various type of nonpathogenic rizobacteria and Fungal species trichoderma, have been shown to produce elicitors like ethylene, jasmonic acid and plant hormones which eventually induce localized or systemic resistance in in plants (Harman et al., 2004).Production of the ethylene is stimulated by wounding or pathogen attack as onset of plant defense(Palmieri et al., 2022).Several studies where MAMP inducing the ISR has been reviewed which shows various type of antibiotics, siderophore (iron chelating compound), VOC acts as elicitiors for ISR in plants (Pieterse et al., 2014). 6-PP which is auxin like compound acts like antifungal agent, PGPR as well as inducer of systemic defense has been demonstrated to reduce the mycelial

growth of phytopathogens such as B. cinerea, R. solani and F. oxysporum(Vinale et al., 2008). Bacterial proteins flagellin and elongation factor EF-Tu acts as the MAMPs. BCAs can initiate plant systemic resistance which involve the production reactive oxygen species, phytoalexins, phenolic compounds, or pathogenesis-related proteins or the formation of physical barriers like modifications of cell walls and cuticles by the induced plant (Wiesel et al., 2014). MAMPs are the receptors of PR proteins glucan, chitin, xylan, e.g., produced by phytophthora megasperma and *Trichoderma viride* which acts as resistance factor of cell wall (Boller & Felix, 2009).

2.2.2.2. Factors involved in systemic acquired mechanism

SAR is the systemic resistance present in the plant body itself, which get activated on exposure to pathogen similar to immune activation after vaccination in human being. Induction of SAR required pathogen attack after which defense system will get activated in the plant for longer period of time for broad range of pathogen. In the infection site SAR develops, with the production of salicylic acid which on triggers hypersensitive response as well as production of the PR proteins, inhibition of virulence factors and control another plant hormone, ethylene.PR proteins act as antibacterial, antifungal and antiviral which include lytic chitinase, lysozyme and peroxidase enzyme which are functional against different pathogens. They also act as chemical messenger for signaling pathogen attack which then activates lignin formation and deposition creating efficient barrier to infecting agents (D. K. Choudhary et al., 2007);(*Induced Systemic and Systemic Acquired Resistance*, n.d.).

plant defence responses may include thickening of cell walls by lignification, deposition of callose, accumulation of low-molecular-weight antimicrobial substances (e.g., phytoalexins), synthesis of various proteins (e.g., pathogenesis-related (PR) such as chitinases, glucanases, and peroxidases) and other bacterial and fungal elicitors (lipopolysaccharides, siderophores, etc.)(Conrath et al., 2015).Several rhizobacteria trigger the salicylic acid (SA)-dependent SAR pathway by producing SA at the root surface whereas other rhizobacteria trigger different signaling pathway independent of SA. The existence of SA-independent ISR pathway has been studied in *Arabidopsis thaliana*, which is dependent on jasmonic acid (JA) and ethylene signaling. Specific *Pseudomonas* strains induce systemic resistance in viz., carnation, cucumber, radish, tobacco, and *Arabidopsis*, as evidenced by an enhanced defensive capacity upon challenge inoculation. Combination of ISR and SAR can increase protection against pathogens that are resisted through both pathways besides extended protection to a broader spectrum of pathogens than ISR/SAR alone(D. K. Choudhary et al., 2007);

Some MBCAs interact with plants by inducing resistance or priming plants without any direct interaction with the targeted pathogen. Antagonists acting through hyper parasitism and antibiosis are directly interfering with the pathogen. Such interactions are highly regulated cascades of metabolic events, often combining different modes of action (Köhl et al., 2019) (Zeilinger et al., 2016).

2.3. Types of pathogen on the basis of location of pathogenicity

2.3.1. Foliar pathogen

Foliar fungal pathogens are a diverse group of microorganisms that specifically target the leaves of plants, causing a range of diseases with detrimental effects on plant health and crop productivity. Various fungal (rust, powdery mildew) bacterial along with viral pathogen cause foliar disease in plants majority of the time, the causative agent is a fungal or fungal-type organism. Foliar pathogen originates in the leaf surfaces and shows symptoms of disease when the pathogen invades the vascular and parenchymal tissues(Beattie & Lindow, n.d.).Infection of the foliar pathogen initially begins, when pathogens invade the leaf and pass through the stomatal pores or break the cuticle after which spore-bearing structures are produced which either pass through the stomatal pores or erupt through the epidermis damaging the cuticle. Infection may also occur within sub stomatal cavity affecting stomatal function and finally results in an alteration in guard cell regulation of stomatal aperture(Grimmer et al., 2012).

foliar disease late blight is caused in celery by the fungus *Septoria apiicola*. It is favored by cool, moist weather (Quiros, 1993)The most significant foliar disease of wheat grows in moderate regions (15–20°C) .Foliar diseases reduce grain yield, particularly associated with reduced photosynthetic area, duration and function. various type of fungal pathogen and the disease caused by them are mentioned in (Mahmood et al., 2017).One prominent example is the fungus Blumeria graminis, causing powdery mildew in a variety of crops. This foliar pathogen thrives in temperate climates and exhibits a broad host range, affecting economically important plants such as wheat, barley, and various grasses. The characteristic powdery white mycelium on the leaf surface is a visual manifestation of the infection. The impact of Blumeria graminis on plant health is multifaceted. The fungus extracts nutrients from host cells, leading to a reduction in photosynthetic efficiency and ultimately affecting the overall growth and yield of the plant(Dean et al., 2012).

2.3.2. Endophytic pathogen

Endophytic fungi, typically regarded as symbiotic organisms residing within plant tissues without causing apparent harm to the host, can occasionally turn pathogenic under specific circumstances.the route of colonization of the pathogenic as well as the endophyte strain varies (Brader et al., 2017) microorganisms may systemically colonize plants from roots to shoots, shoots to flowers or fruits and/or from flowers to fruits and seeds, and they may also cause localized colonization inside/outside plant organs.

One notable example is Colletotrichum species, known for their dual role as endophytes and pathogens. Colletotrichum fungi are implicated in a range of plant diseases, causing anthracnose in various crops. As endophytes, they may establish benign associations within plant tissues; however, environmental factors or host susceptibility can trigger a shift to a pathogenic lifestyle, leading to disease development. The intricate balance between endophytic and pathogenic phases highlights the complex nature of these interactions. (Damm et al., 2009)explores the

taxonomy and diversity of Colletotrichum species, shedding light on their pathogenic potential and ecological roles. Another study by (Rodriguez et al., 2009)delves into the broader aspects of endophytic fungi, emphasizing their ecological significance and potential for plant protection. Understanding the factors influencing the transition from endophyte to pathogen is crucial for developing strategies to manage diseases caused by endophytic fungi. The work by (Ganley et al., 2004)provides insights into the genetic factors underlying pathogenicity in endophytic fungi, contributing to our understanding of the mechanisms driving these complex interactions.

2.3.3. Rizospheric pathogen

The rhizosphere, the soil region influenced by plant roots, is a complex ecosystem where various microorganisms such as (bacteria, fungi, nematodes, protozoa, algae and micro arthropods) (Lynch & Whipps, 1990);(Raaijmakers & Weller, 2001) have the pivotal roles. Among which fungal microbes are of significance due to their potential to cause plant diseases .These fungi establish intimate associations with plant roots, leading to diverse interactions ranging from mutualistic to pathogenic (Raaijmakers et al., 2009).

As pathogens, these fungi can compromise plant health by causing root diseases, affecting nutrient uptake, and ultimately impacting crop yield. Understanding the dynamics of rhizospheric fungal pathogens is crucial for effective disease management strategies in agriculture. Research in this field has highlighted the importance of unraveling the molecular mechanisms underlying the interactions between these fungi and their plant hosts. By elucidating the complex network of signals and responses involved, scientists aim to develop targeted approaches for disease control that minimize environmental impact. This area of study contributes significantly to sustainable agriculture practices by promoting a nuanced understanding of plant-microbe interactions in the rhizosphere. (Gerlin et al., 2021) Only a few groups of bacteria are considered to be soil borne, probably because non-spore forming bacteria cannot survive well in soil for long periods. Bacteria also require a wound or natural opening to penetrate into the plant and cause infection. Examples are *Ralstonia solanacearum*, cause of bacterial wilt of tomato, and *Agrobacterium tumefaciens*, the well-studied causal agent of crown gall (Lipa & Lipa, 2005).Some filamentous bacteria (*Streptomyces*) can also infect plants and are better adapted to survive in the soil. Only a few viruses can infect roots like bacteria, they require a wound to infect the plant and are mostly transmitted by vectors(Campbell, 1996).Whereas the fungal species Fusarium oxysporum penetrates the root initially in the rhizosphere and finally colonize vascular tissue and triggers massive wilting, necrosis, and chlorosis of aerial plant parts(Husaini et al., 2018).

2.4. Fungus versus bacteria as BCA.

Fungi are placed in eukaryotic group of organism, which also includes yeast, mushrooms, and molds. Fungi are non-chlorophytic, spore-forming organism; the true fungi are mostly found in filamentous and branched form are distributed into four divisions: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota(Lutzoni et al., 2004). (S & Singh, 2016) state most of the fungal

species are saprophyte, with approximately 20,000 fungal species being parasites, which cause disease in crop and plants. All plants are attacked by at least one species of fungi or another of phytopathogenic fungi. One or different kinds of plants can be attacked by individual species of fungi (Aloo et al., 2022).

Fungal biocontrol agents, such as species of Trichoderma and Beauveria, are renowned for their ability to colonize the rhizosphere and exert antagonistic effects against soil-borne pathogens. They often employ mechanisms such as competition for nutrients, production of antimicrobial compounds, and induction of systemic resistance in plants. On the other hand, certain bacterial species, like Pseudomonas and Bacillus, are recognized for their biocontrol efficacy through mechanisms such as the production of antibiotics, siderophores, and the induction of systemic resistance. The choice between fungal and bacterial biocontrol agents depends on factors such as the specific pathogen targeted, environmental conditions, and the desired mode of action. Combining both fungi and bacteria in integrated biocontrol strategies holds promise for enhanced efficacy. This dynamic interplay between fungi and bacteria in biocontrol underscores the importance of a holistic approach in sustainable agriculture (Aloo et al., 2022);(Lugtenberg & Kamilova, 2009).

Fungi and bacteria are both potential biocontrol agents for managing fungal plant pathogens, each with its own set of advantages and disadvantages. Fungi, such as Trichoderma and Beauveria, have been extensively studied for their biocontrol capabilities. Fungi has the higher potential to be used as biological control agent, due to its high reproductive rate (sexually as well as asexually), a short generation time, target specific host as well they can survive without host by shifting their mode to saprophytic nature(Thambugala et al., 2020). As they have target specific host, thereby minimizing the impact on non-target organisms, do not leave toxic residues in soil or water sources, which reduces the risk of ecological damage and reduces the chemical load in agricultural system(Zeilinger, 2023). One of the significant advantages of fungal biocontrol agents is their ability to produce a diverse array of secondary metabolites, including antibiotics and enzymes, which can inhibit the growth of pathogenic fungi. These metabolites can disrupt the pathogen's life cycle, providing an effective means of disease control. Additionally, some fungal biocontrol agents establish symbiotic relationships with plant roots, enhancing plant growth and resilience(Harman et al., 2004a);(Whipps, 2001).

Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production, and the ease with which the enzymes can be modified. Further, the enzymes, being normally extracellular, are easily recoverable from the media (Vishwanatha et al., 2010)Proteases production of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since they are normally recognized as GRAS (generally regarded as safe) (Germano et al., 2003).

CHAPTER 3

3. Methods and Methodology

3.1. Collection of test plant pathogen

Different test pathogen which include *Aspergillus solani, Rizoctonia solani, fusarium sp., fusarium oxysporum* were collected from NHRC and was provided by alpha agro Pvt. Ltd. Birgunj (A reputed agricultural industry). Samples were provided in glycerol stock. Collected fungal sample were revived and pure colony was isolated for further re confirmation in PDA plate whereas Molecular level of confirmation was not done as the sample was taken from the authentic source.

3.2. Identification and reconfirmation of test pathogen

Fungal pathogen in glycerol stock was revived using PDA. *Fusarium oxysporum* was grown on oxysporum specific media, whereas the rest of the pathogen did not revive in the selective media *F. oxysporum.* colony morphology of the fungus was observed. Lacto phenol cotton blue staining of the spore was done and the microscopic observation of each fungal spore was done for the determination of the pathogenic strain. Thus, the fungal strain was determined on the basis of specification on media, colony morphology (color, hyphae) and nature of spore.

3.3. Materials required for isolation of antagonistic fungus against plant pathogen

Soil sample, rose Bengal, potato dextrose agar, tannic acid, whey, guaiacol, chloramphenicol, modified media, autoclaved distilled water, Petri plates, test tube, laminar hood, pipette, incubator.

3.4. Area of sample collection

Soil sample was collected from the Infected areas of Nepal, where there is a significant possibility of isolating a resistant microbial population, as well as sample was collected from diverse regions of Nepal with an abundance of beneficial microorganisms. From the eastern part of the hilly area of Goruwale bhanjhyang ,Ilam, Mechi, Nepal. 9 distinct samples, labelled BG01 to BG09, were gathered from various sites. The location detail is mentioned here (BG01 27°6'4''N 87°55'28''E 2840.6m 9211ft, BG02 27°6'1''N 87°55'33''E 2837.5m 9201ft, BG03 27°5'56''N 87°55'49''E 2836.4m 9197ft, BG04 27°5'59''N 87°55'57''E 2791.4m 8818ft, BG05 27°5'59''N 87°55'57''E 2730.7m 8854ft, BG06 27°5'58''N 87°55'57''E 2735.2m 8869ft, BG07 27°3'39''N 87°56'26''E 2735.7m 8871ft, BG08 27°5'59''N 87°55'59''E 2739.3m 8882ft, BG09 27°5'59''N 87°55'59''E 2739.1m 8882ft). The area for sample collection was pristine, microbe-rich terrain. The second location for collecting soil samples was Chitwan, where the plant pathogen *Fusarium* had wreaked devastation on a banana farm. The soil sample was

properly labelled and tightly packed in disinfected plastic bags, which were then kept at CDBT's 4°C storage facility.

3.5. Isolation and screening of beneficial fungal isolate

The antagonistic fungal isolate was screened using various technique which include use of antibacterial agent, heat shock, carbon catabolite repression whose final aim was selection of slow growing antagonist saprophytic fungal isolate. The collected soil sample was initially weighed and 1gm of the sample was mixed with water in a culture tube and incubated for 37 for 30 min to allow the growth of microbial population. Heat shock was given at 80 for 10 min which kills the vegetative cells present in the soil sample. later on incubation of the soil sample at 28℃ for 30min was done to allow the growth of the microbial population. Again heat shock was given for 10min at 80℃ which was supposed to kill fast growing spore former microbes. Then it was incubated at 28℃ for 3hrs. Heat shock was given again at the condition as mentioned above assuming that this step could kill slow grower non spore former.

The soil sample was then prepared for serial dilution, where 1 gm of the soil sample was suspended in 10 ml of distilled water. Tenfold serial dilution was carried out to a dilution of 10^{-5}. Aliquots of 0.1 ml of the dilution of 10^{-2}, 10^{-3} and 10^{-4} was spread into the modified media containing tannic acid and antibacterial agent chloramphenicol by spread plate technique. while at the same time the dilution was spread in to the plate containing whey. This approach was based on the CCR for the selection of the slow growing saprophytic strain that can feed on lignin degradation product or have the glyosidic activity in order to take nutrition for survival. The plates were than incubated at 28 for more than 5days.

With the ultimate goal of selecting a slow-growing opponent saprophytic fungal isolate, the antagonistic fungal isolate was screened utilizing a variety of techniques, including the application of antibacterial agents, heat shock, and carbon catabolite suppression. Initially weighed, 1gm of the acquired soil sample was combined with water in a culture tube and subjected to a 10-minute heat shock at 80°C to destroy any vegetative cells that could still be present. To facilitate the growth of the microbial community, the soil sample was afterwards incubated at 28°C for 30min. Once more, a 10minute heat shock at 80 degrees Celsius was administered to kill fast-growing spore-forming bacteria. Then it was incubated for 3 hours at 28°C.

According to the literature review the saprophytic strain exhibit brownish stain in tannic acid and reddish on the guaiacol, such isolates were labelled and then picked with the help of toothpick and pure colony of the strain was isolated in the PDA plate.

3.6. Criteria for isolate selection

Inclusion criteria

Only the isolate which exhibit brownish and reddish color in the modified media containing TA and guaiacol respectively was selected.

Exclusion criteria

Rest of the isolate which didn't not exhibit color pigmentation in modified media was excluded.

3.7. Microscopic identification of the beneficial fungal isolate

Lacto phenol cotton blue was used for the microscopic identification of fungal isolate.

3.8. Test of different cell wall degrading enzyme activity

3.8.1. Screeening of slow growing saprophytic isolates followed by laccase activity assay.

lignin degrading products, tannic acid and guaiacol was taken as substrate for initial screening of the fungal isolate having laccase activity. In my study, the concentration less than (5mM) of lignin degradation product was calculated after performing toxicity test of that compound. The media composition is mentioned in (APPENDIX II) with ph. (5.5, 6). The isolates were picked with a toothpick and streaked in the plate containing tannic acid, guaiacol, and whey to reconfirm the laccase and glycosidase producing activity of the fungal isolates. Antibacterial agent chloramphenicol was used to inhibit the bacteria present in the soil sample. The Petri plate was incubated at 28 degrees for 5 days. Color change of the media was observed. Diameter of halo zone and colony growth was determined for determination of laccase activity.

Isolates positive for laccase production was subjected for the quantitative estimation of laccase activity in the same media composition at ph. 6 as highest halo zone was observed at that ph. The cultures were inoculated in 30 ml culture tube containing 15 ml of media broth and incubated at 30 degrees without agitation. Fungal growth and enzyme activity were assayed periodically. Autoclaved media broth serves as positive control. the enzyme assay was done by following the procedure as stated (Kalra et al., 2013). Medium was centrifuged after incubation at 10 000 r min−1 for 10 min, and the supernatant was used for estimation of laccase activity. 1ml of each supernatant was taken in a test tube and mixed with 1ml guaiacol and 3ml of sodium acetate buffer (ph. 5.0). For blank mixture of 1ml D/W ,1ml guaiacol and 3ml sodium acetate buffer was used. Both the blank and the enzyme were incubated at 30 degrees for 30 min and absorbance was taken at 450nm using UV vis spectrophotometer.

This activity was determined by monitoring the absorbance for 3–5 min and expressed in U/L by this formula: $E.A = A \times V/t \times e \times v$ (Kalra et al., 2013).

where E.A = Enzyme activity

A = Absorbance
V = Total mixture volume (ml)
v = enzyme volume (ml)

t = incubation time

e = extinction coefficient for guaiacol (0.6740 lM/ cm)

3.8.2. Chitinase activity test

Qualitative test of the chitinase activity was done by streaking the selected isolates in the media plate containing colloidal chitin (1%) and 1.5 % agar for the initial selection at ph. 6 and incubated at 28 degrees Celsius for 2 days. After that 0.5% Congo red was poured into the plate stained for 30 min and de stained using 1M NaCl, after that the formation of halo zone was observed for the chitinase positive isolate. Then the diameter of the zone was measured with the help of scale to determine the chitinase activity of the respective fungi.

For the quantitative analysis, Chitinase enzyme activity was determined using colloidal chitin substrate. Media composition is mentioned in (Appendix I). Isolate which was positive for chitinase was inoculated in the sterilized media broth in a culture tube and incubated at 30 degree Celsius for 4 days without agitation.0.5 ml of 1:10 dilution enzyme solution (obtained after centrifugation of enzyme extract at 12,000 rpm for 5 min) was taken from each isolate separately in a falcon tube and mixed with colloidal chitin (1%) and allowed for incubation at 45 degree for 1 hr. Following incubation, the enzymatic reaction was stopped by using 3ml DNS. The reaction mixture was than heated for 5 min at boiling water bath to completely stop the reaction followed by the centrifugation at 1000 rpm for 15 min. Thus the obtained supernatant was subjected for the measurement of absorbance at 530 nm. Enzyme broth of different time interval (4,8 ,12and 16 days) were taken for study.

For the standard N-acetyl D glucosamine of 1-10 Mm concentration was prepared and absorbance was taken following DNS method as done for enzyme.

3.8.3. Screeninig of cellulase producing isolates and activity asssay.

Cellulose positive isolates were identified by inoculation of equal size of mycelia with the help of 6mm borer in the media containing 1% CMC and 1.5% agar at ph. 6.5 and incubated at temperature of 28 degrees for 5 days. After that 0.5 % Congo red was poured into the petri plates, the isolate having cellulose activity form halo zone whose diameter was noted.

Cellulase positive isolates were subjected for enzyme production in the culture tube containing media composition with Carboxymethyl cellulose 1% as substrate, at pH 6.0, and incubated at temperature of 28°C for several days with constant agitation at (150rpm). The extract was taken at different time interval for the estimation of enzyme activity.

Different concentration of glucose was taken as standard.

3.8.4. Screening of starch hydrolyzing isolates and assay of amylase activity.

Starch hydrolysis test was done by the inoculation of fungal mycelia in 0.2% soluble starch and nutrient agar media in a sterilized condition with ph. 6.0 incubated at the temperature of 30

degrees Celsius for 3 days. Then the media was flooded with grams' iodine solution (1% iodine and 2%potassium iodide). The halo zone formed was measured.

Quantitative analysis of the enzyme amylase was done by inoculation of mycelia of each isolate in culture tube containing autoclaved media with composition as mentioned in (Appendix I) with pH 6.0 at 30°C for several days with constant agitation. The enzyme extract was than centrifuged at 2000 rpm for 5 min to remove the fungal mycelia and strain the supernatant. Taking 0.5 ml of obtained extract was mixed with (1%soluble starch dissolve in citrate phosphate buffer). The mixture of substrate and enzyme extract was then allowed for incubation at water bath 40degree for 30 min. reaction was than terminated using 1ml DNS reagent followed by immersing the test tubes in the boiling water for 5min. After cooling the test tube in the ice bath, absorbance was measured at 540nm.

For blank 1ml of 1% soluble starch in citrate phosphate buffer PH 6.4 was taken.

For standard different concentration of glucose was taken.

3.8.5. Screening of gelatin hydrolyzing isolates and Protease activity test

Gelatin hydrolysis test was done by the inoculation of fungal mycelia in 0.4% gelatin and nutrient agar media in a sterilized condition with ph. 6.0 incubated at the temperature of 30 degrees Celsius for 3 days. Then the media was flooded with grams' iodine solution (1% iodine and 2%potassium iodide). The halo zone formed after the addition of grams' iodine was measured to express the gelatin hydrolysis activity of fungus.

Isolates positive for gelatin hydrolysis was inoculated in the autoclaved Media with composition as mentioned in (Appendix I). maintaining ph. 6.0, and incubated at temperature 30 degree with constant agitation at 150 rpm. The culture medium was centrifuged at 13000 rpm for 5 min. 0.65% of 5ml casein solution was taken in a test tube and incubated at water bath (37 degrees) for 5 min. After that enzyme was added to the solution to make a dilution of 1:10 then 5ml of TCA reagent was added to stop the reaction. The final volume of the enzyme was made 1ml in the solution and incubated at 37degrees for 30min, the solution was then filtered using the syringe filter (0.45microm). In a test tube 2 ml of that solution was taken in which 5ml of sodium bicarbonate and 1 ml of Folins' reagent was added followed by the incubation at 37 degrees for 30min. 2ml of that solution was again filtered by syringe filtered and absorbance of the filtrate was taken at 660nm.

3.8.6. Evalualtion of Pectinanse producig isolates

Pectin degrading fungus was identified by growing the isolates in the Petriplate with the composition of main substrate as pectin 1% and other materials KH_2PO_4 0.2 %, $K_2HPO_4$0.3%, $MgSO_4$ 0.01%, Di ammonium orthophosphate 0.3% maintaining ph. 6.0 and incubated at 30

degrees for 48 hrs. The plates were then poured with grams' iodine solution, where the fungus producing the pectinase exhibit clear halo zone. The diameter of the zone formed was measured. Quantitative analysis of the pectinase activity was not done.

3.8.7. NPK Test

3.8.7.1. Screening of Complex Nitrogen Source utilizing isolates

Peptone was used as the complex nitrogen source, to screen the complex nitrogen using fungus among the selected isolates. The isolates were grown on modified peptone water broth media having composition of 1%peptone, sodium chloride 5g/l, MgCl2 0.1 g/l, CaCl2 0.1g/l for 7 days at 28 degrees. Then 3ml of the extract from each isolate was taken in the test tube and 1ml of Nessler's reagent was added to each test tube. Isolate which can utilize the complex nitrogen would change the color of the extract to orange upon addition of Nessler's reagent. Modified peptone water was set as blank.

3.8.7.2. Phosphate solubilizing test

The solubilization of phosphate through inoculation of selected isolates was detected by using modified single solution reagent method by(Murphy & Riley, 1962). Quantitative estimation of phosphate solubilization was determined by using Pikovskaya broth medium. Available phosphate contents in broth culture were determined by using method as described by(Murphy & Riley, 1962).The pH drop of inoculated broth culture was checked after 2 weeks of incubation. As described by murphy et al the required chemical includes H_2SO_4 (5N), Ammonium molybdate 0.04g/ml, Ascorbic acid (0.0176 g/ml), potassium antimony tartrate (0.00274g/ml) was used to prepare the regent mix. For 50ml of reagent mix ,25ml 5N H_2SO_4, 7.5 ml Ammonium molybdate, 15 ml ascorbic acid and 2.5 ml potassium antimony tartrate was mixed.

The enzyme broth was centrifuged at 13000 rpm for 5 min to remove the mycelial debris.5 ml of the filtrate with 1:10 dilution was taken and mixed with 1ml of reagent mix and kept it for 10 minute. After that absorbance of that reaction mixture was taken at 882nm.

3.8.7.3. Potassium Solubilizing Test

Potassium solubilizing fungus was identified by using potassium aluminum silicate as the complex potassium source. Fungal strain was inoculated in the Petri plate of Alexandro media and incubated at 30 degrees for 5 days. the Petri plates were then flooded with dye Bromothymol blue (Rajawat et al., 2016). The protocol was slightly modified , instead of incorporating dye in the media ,it was flooded after incubation. 5g/l of Bromothymol blue stock solution was prepared in 70% ethanol. Of which 1 ml was taken and mixed with 100 ml water. which was then flooded in the Petri plates and kept for 25 minutes. Fungal strain which can solubilize complex potassium source shows yellow colored halo zone around the colony. The halo zone size and colony diameter were measured after 5 days. The halo zone size was calculated by subtracting the diameter of colony from the total diameter.

3.8.8. Organic acid production test

The isolates were inoculated in the PVK broth with ph. 7.2 for 7 days and incubated at 28 degrees. The broth was then centrifuged at 10000 rpm for 5 min to separate the mycelial debris, the obtained supernatant was then syringe filtered using 0.298micro meter syringe filter. The filtrate was then subjected for HPLC to identify the types of organic acid produced by the fungus.

3.8.9. Zinc solubilizing activity

Saprophytic isolates were screened for zinc solubilization by using media containing D-glucose– 10 g, KCl 4.68 g, NH_4SO_4-1.0 g, $MgSO_4$.0.2g, and Agar 15 g dissolved in deionized water to make 1000 mL volume. Two different complex source ZnO and $ZnCO_3$(0.1%) was used as sole source of zinc to check the ability of isolates to solubilize zinc oxide. The fungal mycelia isolates were inoculated in the respective media and incubated at 28 °C for 5 days. Upon storage of the Petri plates in the fridge at 4°C for 5 days shows clear halo zone around the colony. The halo zone size was calculated by subtracting the diameter of colony from the total diameter.

3.8.10. Auxin production test:

IAA production by the fungal isolates was tested by growing the isolates in the media Sabouraud dextrose broth with L- tryptophan of concentration 2.5 mg/ml at 28 degrees for 7 days. The extract was then taken in a test tube and mixed with salkowskis reagent (2% 0.5M $FeCl_3$ in 35% $HClO_3$ solution). Result obtained was then noted.

3.9. Dual culture Assay for Screening of Biocontol Activities of Isolates against selected phytopathogens.

An agar disc (6 mm) was taken from 4-day old PDA culture plates of each isolate and placed at the periphery of the PDA plates (9 mm). Another agar disc of the same size of each pathogen was also placed at the periphery but on the opposing end of the same Petri dish. An agar disc (6-mm) of the antagonist, isolate was placed 2 cm away from the periphery of the Petri dish, and a same sized agar disc of the test fungus, was similarly placed 2 cm away from the edge of the Petri plate but on the end opposite of isolate. As a control, each test pathogen was placed in a similar manner on a fresh PDA plate.

All pairings were carried out in quadruplicate and incubated at 28° C. Antagonistic activities was tested 7 days after incubation by measuring the radius of the isolated beneficial colony in the direction of the antagonist colony (R2) and the radius of the isolate colony in the control plate (R1). The two readings were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by (Skidmore & Dickinson, 1976).

$$PORG = \frac{R1 - R2}{R1} \times 100\%$$

Observations were continued on the dual culture plates after 7 days of incubation and PIRG was calculated. The number of days required for the antagonist to overgrow the whole colony of pathogen was recorded (Bunbury-Blanchette & Walker, 2019).The degree of antagonisms between each bio agent and test pathogen in dual culture was scored on scale of 1-5 as proposed by (Bell, 1982).

Macroscopic and morphological observations indicating an interaction between species were recorded: formation of a hyphal barrier (thickened mycelial growth between cultures), development of an inhibition zone, abnormal color or exudate production in comparison to axenic culture, and overlapping growth.

3.10. Microscopic identification of the fungal isolates

Lacto phenol cotton blue spore staining of the selected fungal isolates were done and observed under the microscope. A strip of transparent tape was cut and places at the ends between thumb and index finger with the sticky side out which was then pressed against the colony to be identified. After that a drop of lacto phenol cotton blue was placed in a clean glass slide. The tape was then pressed against the LPCB in the slide. Another drop of LPCB was placed on the top of the tape, which was then covered with a clean glass slide and viewed under the microscope.
Spore size, shape, fruiting body, hyphae was observed under the microscope.

3.11. Partial purification of different enzymes using ammonium sulphate

Protein gets precipitated on addition of the salt, thus salting in and salting out process is used for the precipitation of the proteins. The amount of ammonium sulphate to be used to obtain the desired concentration of salt was calculated based on the table given in(Wingfield, 2001).Based on the type of enzyme and the molecular weight desired concentration of ammonium sulphate to be used for the precipitation was calculated. Enzyme production was done by inoculating mycelia of only one isolate BK102 isolates in the respective media for the production of laccase , protease, cellulase, chitinase and phosphatase maintaining ph., temperature and incubation in a shaker having 150rpm for 7 days. After that the broth was centrifuged at 13000 rpm for 5 minutes to separate the debris of mycelia. The supernatant was then treated with different concentration of ammonium sulphate according to the molecular weight of the respective enzyme. For the separation of enzymes, the calculator was used to calculate the amount of ammonium sulphate to make the respective concentration. The supernatant was poured in a falcon tube and the appropriate amount of ammonium sulphate was added. After that the falcon tube was paced in bucket having ice cube and shaked in a shaker at 180 rpm for whole night. The precipitate was collected next day by centrifuging the solution at 2000 rpm for 30 min. The supernatant was mixed again with the ammonium sulphate to make another concentration. The protein concentration extracted was re suspended with 50mm Tris cl. Concentration of the protein was then calculated after the precipitation following Lowry's method. The precipitated

protein was dialyzed for the removal of the salts and other impurities. The concentration of the protein was calculated following the similar method after dialysis and compared.

3.12. Lowry's method of protein precipitation before dialysis

Lowry's method for the estimation of protein was followed for the quantitative analysis of the protein before and after dialysis. BSA was taken as standard at a concentration of (5-100 µg /ml).

Reagent used

A=2%NA_2CO_3 in 0.1N NaOH

B=1%NAK tartarate in H_2O

C= 0.5%$CuSO_4.5H_2O$

Reagent 1= 48ml of A ,1 ml B 1ml c

Reagent 2= 1:1 ratio of Folins phenol(2N) and water

0.5 ml of the protein of dilution of 1:10 is taken in a test tube in which reagent 1 was mixed and stand for 10 min. after that the reagent B was mixed and kept for 30 min at room temperature and the absorbance of both test and standard was taken at 680 nm.

3.13. Dialysis in the dialysis membrane

The dialysis membrane was prepared prior the process. The tubing was cut into the piece of 10 cm and boiled in a large volume of 2% sodium bicarbonate and 1Mm EDTA for 10 min. after rinsing the tube in DW the tube was subjected for boiling for 10min in a 0.001M EDTA. The tube was then submerged in buffer and stored at 4 degrees. Before using the tubing, it was properly washed with distilled water and checked properly.

The diluted protein was kept in the dialysis membrane and properly tied with thread at both ends. It was submerged in a 50mm Tris cl buffer in a beaker and stirred gently. Dialysis was carried out for overnight. The protein obtained after dialysis was again subjected for quantification by Lowry's method. comparative analysis of the concentration of protein was done before and after dialysis. The fermentation broth of 12 days old culture of Phlebia floridensis on 0.5% malt extract} was filtered through WHATMAN filter paper No. 1 and the filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant thus obtained was subjected to total protein precipitation with ammonium sulfate in the range of 50 – 75% saturation. Dialysis was done against 50 mM sodium acetate buffer (pH 4.5), replaced after every 6 – 8 h. Laccase assay was done after 24 and 48 h of dialysis.(Arora & Rampal, 2002).

3.14. Molecular characterization of the fungus

The screened and the identified fungus based on their morphology and the biochemical characterization were further subjected to the molecular characterization to confirm the specific

isolates. So, the genomic DNA extraction and PCR amplification were performed to confirm the specific fungus.

3.14.1. Genomic DNA (g DNA) extraction

For the extraction of fungal genomic DNA fungus was first cultured in the PDA broth for 3-5 days. Of which 1-3 ml of the culture broth was taken in an Eppendorf tube and centrifuged at 12000 rpm for 1 minute. The supernatant was discarded and the pallet was resuspended with 560/570µl TE buffer. Along with this 30 µl of 10% SDS and 3µl of proteinase K(20mg/ml) and incubated at 37°C for 1 hr. After which 100 µl of 5M NaCl was added along with 80 µl of CTAB/NaCl (10%,0.7M) solution. The mixture was then incubated at 65°C for 10 min. Equal volume of chloroform and isoamyl alcohol mixture at the ratio of 24:1 was added and mixed well. The mixture was then centrifuged at 13000 rpm for 5 minutes. Then equal volume of PCI (phenol: chloroform: Isoamyl alcohol) was added and mixed properly which was then centrifuged at 13000 rpm for 5 minutes. The supernatant was then collected in a fresh epitube in which 0.6 ml of chilled isopropanol was added and stored for 5 minute. The mixture was centrifuged at 13000 rpm for 3 min. The supernatant was discarded and pellets was dried and resuspended with about 50 µl TE. The DNA was then subjected for gel electrophoresis and visualized in UV trans illuminator.

3.14.2. Polymerase Chain Reaction (PCR)

The extracted DNA was undergone PCR at Intrepid Nepal Thapathali, Kathmandu targeting fungal ITS region using the primers:

ITS1 : TCCGTAGGTGAACCTGCGG

ITS4 : TCCTCCGCTTATTGATATGC

The PCR components used to carry out the process are mentioned below:

Components	For 1(µL)
Nuclease Free Water	3
Qiagen 2X Master mix	12.5
Primer Forward (ITS1F)	1
Primer Reverse (ITS4R)	1
Q solution	5
Template DNA	2.5
Total	25

The condition used for PCR is mentioned below:

PCR conditions		for n(µL)
Temperature	Time	Cycles
95°	15min	1

94°	30s	5
47°	30s	
72°	30s	
94°	30s	35
52°	30s	
72°	30min	
72°	5 min	1
4°	Forever	1

The sequencing has been carried out at both directions using forward and reverse primers in ABI310 GeneticAnalyzer.

3.14.3. Phylogenetic analysis

The sequences obtained after the sequencing process were subjected to nucleotide blast at Genbank database Release 241 (December 15 2020) using blastn v2.9.0 locally in Lenovo P70 mobile workstation.

3.15. Nanoparticle synthesis by chemical method

Copper oxide nanoparticle was synthesized following precipitation method using copper sulphate $CuSO_4.5H_2O$. 0.1M copper Sulphate solution was prepared in and dropwise addition of 0.1 m NaOH was done under vigorous stirring until ph. reach 14 and kept stirring for overnight until the brownish black ppt. was observed. The ppt. was washed repeatedly with deionized water and absolute ethanol until the ph. of the filtrate reach 7. The washed ppt. was dried initially at 80°C in an incubator for 16 hrs. The supernatant was tested using phenolphthalein if it contains any ammonia or not. After that the ppt. was calcinated at 500°C for 4-5 hr. In the calcination chamber available in RECAST (research center for applied science and technology). The obtained copper oxide nanoparticle was then characterized by FTIR at Alpha agro Pvt. Ltd (Birgunj) for that process.

CHAPTER 4

4. Result and discussion

4.1. Selection of saprophytic slow growing isolates following CCR technique.

The soil sample was heat treated at different range of temperature for the initial screening of the slow growing spore forming fungal isolates. And antibacterial agent chloramphenicol 30µg/ml as well as Rose Bengal was used for the selective fungal screening. The non-spore forming fungus as well as bacterial strain was excluded through the screening process. Slow growing saprophytic strain having laccase activity was selected through CCR mechanism. The modified media composition having whey and lignin degradation phenolic compound were used.

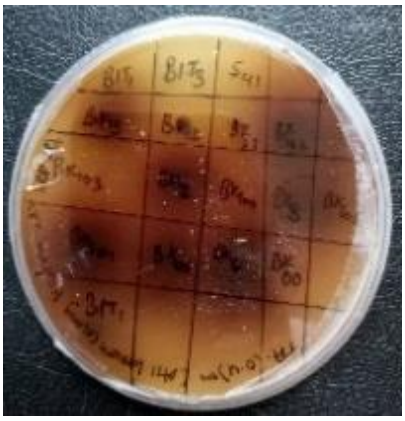

Figure 1: Screening of saprophytic strain in tannic acid media.

By the plate assay 98 isolates were selected based on the halo zone formed on the media containing whey and tannic acid of different concentration. Laccase secretion was monitored by visual color change in the plates. Among 98 isolates ,8 of them were selected based on the reddish brown zone around its colony due to the oxidation–reduction reaction on the media plate by the fungal isolate as shown in the figure. Enhancement in the laccase production has been seen in the media supplemented with aromatic compounds, such as vanillin, guaiacol, catechol, and Gallic acid, Thus such aromatic compound are considered as inducers for fungal enzymes, including laccase according to (Ikehata & El-Din, 2004)

Two different substrates lignin degradation product tannic acid at first was used as the complex carbon source and then guaiacol was used for the initial screening of saprophytic strain producing the laccase enzyme in the media. The reddish brown color developed in the media is due to oxidation of phenolic compound guaiacol by laccase (Kalra et al., 2013). lignin a phenolic compound in plants makes the integrity of cell wall of plants (Ninkuu et al., 2023)is degraded by the enzyme laccase breaking the integrity and making access of the nutrient for fungus showing saprophytic nature of fungi. In competitive niche environment of the rhizosphere, slow growing saprophytic strain which can retain on the lignin degradation product was selected by the reduction in the easily available carbon and nitrogen sources in the media (G. Chandra & Chater, 2014). (Durán-Sequeda et al., 2022) Shows that CCR and NMR mechanism in fungus has been

involved in the regulation of laccase producing gene expression . Taking this into account, the culture media was supplemented with the lignin degradation product tannic acid and guaiacol only for the initial screening of laccase producing strain without nitrogen and carbon sources.

4.1.1. Qualitative analysis of laccase activity

The optimum media pH for laccase secretion varies on different organism and depends on the growth media used. Studied shows that the fungal species has optimum ph. range of 3-7 and temperature of 25–30 °C.in the mesophilic fungus, *Ganoderma lucidum*, have been reported to secrete highest levels of laccase(Ko et al., 2001). Another report shows that the basidiomycetes fungal strain *Trametes hirsuta* has maximum laccase activity at ph. range 5.5-7.5 and temperature 35 °C (Dhakar & Pandey, 2013).
Likewise for the optimization of suitable media condition 2 different ph media was set,5 and 6 .most of the strain showed the optimum activity at ph6 during the plate assay.The strain having the laccase activity showed the wider reddish brown halozone around the fungal colony in guaiacol media ,whereas smaller brownish halozone was observed in media supplemented with tannic acid.

Table 1:laccase enzyme activity at different substrate after 5 day of incubation at 30°C.

ISOLATES	GUAIACOL PH 6	TANNIC ACID PH6
	H.D (In cm)	H.D (In cm)
BK1	3±43	1±83
BK2	3±54	1±92
BK6	3±12	1±01
BK8	2±34	0.5±32
BK7	-	-
BK100	1±63	2±12
BK101	3±82	1±84
BK102	3±35	1±01
BLT1	2±17	1±32
w14	-	-
s41	-	-

The ability of the microbes to utilize much complex carbon source from tannic acid was different from that of guaiacol which is quite simple lignin degradation product.

Plate assay methods shows that the BK101 isolates shows highest enzyme activity at ph. 6 amongst all isolate in guaiacol media. While the isolate BK100 shows highest activity in tannic acid media, on contrary BK8 shows the least activity in that respective media plate. As guaiacol and the tannic acid are the 2 different substrate for laccase, which produces the different color product after the reaction. The laccase isozyme variation could be attributed to the different ecological origins of the species or different culture conditions under which various isozymes

were expressed and/or secreted(You et al., 2014). Single strain of the fungal species can secrete more the one type of isozyme. Production of type of laccase isozyme also depends on the type of inducer present in the media(Ko et al., 2001). Thus the variation in the type of inducer in our media component is the reason for the different color and size of halo zone in the plate due to the differential production of different laccase isozyme.

Laccase is extracellular enzymes produced by different groups of fungi, of which basidiomycetes fungi secrete substantial amount of the enzyme. Earlier studies on production of laccase from Pleurotus sajor caju, Agaricus bisporus, Ganoderma lucidum have been reported. In accordance with this research, the extracellular laccase activity was visually confirmed by using guaiacol in the media. The mycelial showed strong laccase activity with the development of a red zone around the mycelium which was conformity with earlier reports by (Debnath et al., 2018)who also have reported similar red zone around the growth as visual confirmation of extracellular laccase activity.

4.1.2. Quantitative assay of laccase activity at different time intervals (5,10,15 and 20 days)

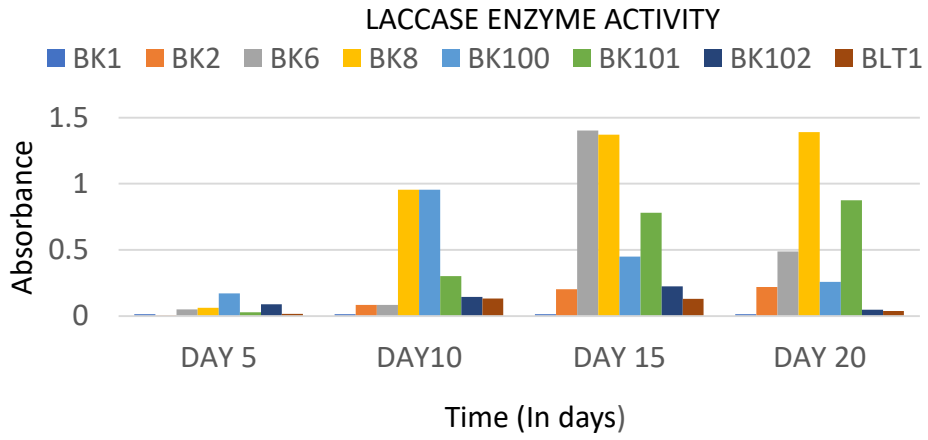

Figure 2: Quantitative assay of laccase activity at different time interval.

All laccase positive fungal isolates were examined for quantification of laccase enzyme activity using guaiacol assay method. The isolate which didn't show any laccase activity (BK7, W14, S41) at guaiacol assay were excluded for the further process. Ph. optimization in the plate assay method shows that the majority of isolates shows the darker and wider halo zone around the colony with ph. 6.0. Which was then maintained for the further enzyme activity test in the liquid media for laccase production. laccase activity reach maximum on the 15th day of incubation and gradually decrease in 20th day for almost all of the isolates. Highest laccase enzyme activity was 0.69 U/ml of isolate BK6 at 15 day of incubation which is less than the laccase production by *T. harzianum* at 6th day (138 h) being 1.286 U/ml as shown by (Abd El Monssef et al., 2016). Reports indicate that the activity of laccase enzyme ranges from 3.5 to 484,000 U L-1(Baltierra-Trejo et al., 2015) . On comparative analysis with this report the range of the enzyme activity at day 15

was between 111U/L to 692U/L for our isolates. The alternation in enzyme activity for quantitative and qualitative assay may be due to the variation in the media component, so for better result the optimization of the media components is required.

4.2. Screening of chitinase producing isolate

4.2.1. Qualitative analysis of chitinase activity

All of the eight screened saprophytic strain has been found positive for chitinase activity. Screening and qualitative estimation of the isolates has been done though plate assay using colloidal chitin (1%) as substrate. After 2 days of incubation the qualitative evaluation of chitinase was done. Isolate BLT1 and BK8 has shown the highest solubilization efficiency (108.33%) and 106.66% respectively.

Table 2: Chitinase enzyme plate assay.

Isolates	H.D(In cm)	C.D(In cm)	S.I	S.E(In %)
BK1	3.4	3.1	2.09	9.67
BK2	3.2	2.1	2.52	52.38
BK6	3.2	2. 2	2.45	45.45
BK8	3.1	1.5	3.06	106.66
BK100	3.2	2.5	2.28	28
BK01	3.1	2.3	2.34	34.78
BK102	3.2	2.3	2.39	39.1
BLT1	2.5	1.2	3.08	108.33

C.D = Colony diameter; H.D = Halo zone diameter;S.I=Solubilization index; S.E = Solubilization efficiency

4.2.2. Quantitative analysis of chitinase activity at different time intervals

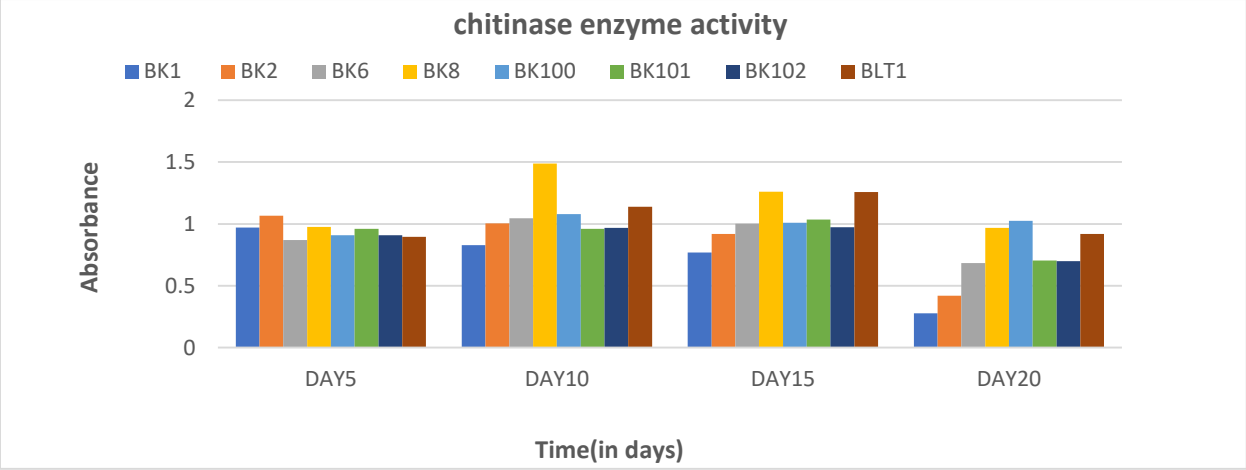

Figure 3: Quantitative evaluation of chitinase enzyme at different time interval.

Majority of the isolate has been seen to produce maximum chitin solubilization at day10 which then eventually decreases with time.BK8 shows the maximum activity at day 10. Calculation of the enzyme concentration was done using the slope value obtained from standard curve. By using the value of enzyme concentration, enzyme activity was calculated which shows that highest enzyme activity (1.813 U/ml/min) produced by isolate BK8 at day 10. While rest of the isolates are also seen to have quite similar range of enzyme activity. Likewise, a fungal isolate *T. longibrachiatum* having chitinase activity 0.55 U/mL was recorded. species such as *T. gamsii, T. virens, T. longibrachiatum and T. asperellum* produced maximum chitinase enzyme of about 0.151 U/mL, 0.107 U/mL, 0.228 U/mL and 0.164 U/mL, respectively as reported by. As demonstrated by (Sandhya et al., 2004).*Trichoderma harzianum* shows the chitinase activity of 14 .7 U/ml at day 5 . similarly, (Asad et al., 2015)shows chitinase activity of *T. harzianum* as 117 U/mL after 96h of incubation.

Table 3: Chitinase enzyme activity of different isolates (in U/ml).

Name of the isolates	DAY5	DAY10	DAY15	DAY20
BK1	1.1	0.909	0.827	0.25
BK2	1.23	1.15	1.034	0.35
BK6	0.96	1.206	1.14	0.75
BK8	1.11	1.813	1.502	0.62
BK100	1.01	1.23	1.15	1.17
BK101	1.089	1.09	1.19	0.74
BK102	1.019	1.10	1.106	0.73
BLT1	1.002	1.33	1.49	1.03

Diverse group of fungus such as *Mucor, Aspergillus, Trichoderma, Penicillium, Trichoderma Lactarius, Pistacia* are involved in the chitinase production(Poria et al., 2021). Chitinase enzyme is involved in defense mechanism as well as the competition for nutrition and space and shows the mycoparasitism property (Homthong et al., 2016) . Thus the isolate producing such bioactive material has been considered to have the control over pathogenic fungi and harmful food pathogen. The research suggests that the optimization of the reaction condition enhances the production of the enzyme. though our work does not involve the optimization process. Chitin a major structural macromolecule and important component of cell wall of bacteria, fungi and higher plants can be hydrolyzed by chitinase, where this enzyme act on the e ß-glycosidic linkages between GlcNAc residues. Similarly, a fungal isolate *T. asperellum* produces the mycolytic enzymes (chitinase and β-1,3, glucanase), which destroy phytopathogen cell walls and shows antimicrobial property(Lahlali et al., 2022b). The accumulation of respective transcripts and enzymatic activity of both chitinase and β-1,3, glucanase seems to be significantly higher as shown by(Win et al., 2021). (Hartl et al., 2012)shows the various fungal strain as chitinase producer and their genetic engineering for enhance chitinase production. (Brzezinska & Jankiewicz, 2012) involves the optimization of the culture media for the fungal strain *A. niger*

which shows enhance production of the chitinase in the media containing colloidal chitin and shrimp shell as substrate, thus we use colloidal chitin as substrate in our work. Highest chitinase producer *Trichoderma harzianum* and *Fusarium chlamydosporum*—are of particular significance, when it comes to plant protection.

4.3. Assessment of amylase activity

4.3.1. Qualitative analysis of amylase activity of fungus

Isolates positive for chitinase activity was subjected for the starch hydrolyzing test.

Table 4: Quantitative assay of starch hydrolysis activity.

Isolates	**Starch hydrolysis activity plate assay at day 5**			
	H.D	C.D	S.E (%)	S.I
BK1	4.4	2.1	109.52	1.477
BK2	3.5	1.7	105.82	1.48
BK6	3.9	1.8	116.66	1.461
BK100	3.6	1.9	89.47	1.527
BK101	3.4	2.0	70	1.588
BK102	3.9	2.0	95	1.512

C.D = Colony diameter; H.D = Halo zone diameter;S.I=Solubilization index; S.E = Solubilization efficiency

Plate assay shows that isolate BK6 having higher starch solubilization efficiency (116.66%) BK1(109.52%). Whereas the isolate BK8 didn't show starch hydrolysis activity along with it isolate BLT1 shows the least hydrolyzing activity. By observing the previous results for Chitinase, Protease and amylase activity BK8 and BLT1 was excluded for the further process.

4.3.2. Quantitative analysis of amylase activity

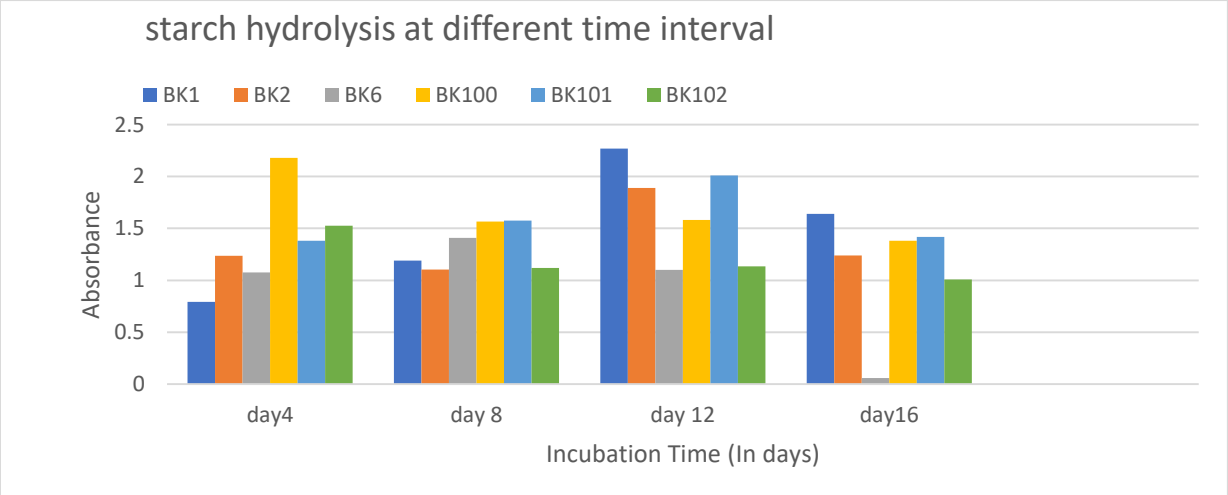

Figure 4: Quantitative analysis of amylase at different time interval.

Quantitative evaluation of the amylase activity was done and the spectrophotometric result was observed at different interval of time 4,8,12,16 days. Almost all screened isolate has been seen to have the optimum amylase activity at day 12. On further incubation of isolate, it has been demonstrated that all isolates amylase activity seems to decline at 16 days. Quantitative data shows that BK1 having higher enzyme activity (90.02U/ml) at day12.And the isolate BK6 having the least activity was discovered. One of the isolate *Aspergillus Terreus* have shown the amylase activity of range 77.53-339 U/ml on different substrates (Ahmed, 2018).(Olguin-Maciel et al., 2019) shows that the fungal strain quantitative assays revealed that the Bm-2 strain produced the amylase enzyme with activity of 193.85 U/mL which is greater comparative to the BK102 (44.33 U/ml at day 12) isolate which is also a *Trametes* species.BK102 species seems to show higher activity at day 4(60.01U/ml) after BK100 isolate in that time thus showing reduced activity with increasing incubation period for that isolate. While a report by (Ali et al., 2017) found shows less α-amylase yield of 33.52 U/ml by *A. Flavus* AUMC 11685 at day 4 of incubation period. . *Bacillus sp*. recorded an optimal amylase activity of about 3800 U/L, whereas Ferreira et al. [45] reported a value of 6500 UI/L on SSF using *R. oryzae* on wheat bran.

Table 5:Quantitative amylase enzyme activity of isolates at different time interval.

Name of isolate	Day4 (U/ml)	Day8 (U/ml)	Day12 (U/ml)	Day16 (U/ml)
BK1	30.47	46.58	90.02	64.66
BK2	48.43	42.98	74.7	50.16
BK6	41.95	54.97	42.9	21.9
BK100	111.78	61.71	62.24	54.18
BK101	49.37	61.99	82.77	55.70
BK102	60.01	43.71	44.33	39.24

4.4. Evaluation of cellulase producing isolates

All of the 6 isolates having significant amylase activity showed cellulase activity during the plate assay. Similarly, the activity was least for 1 isolate BK8 which was then excluded from the further process. After incubation for five days, halo zone formed by the isolates in the media plate containing CMC (1%) has been measured and evaluated for the calculation of cellulase activity of the isolates. Qualitative plate assay shows the clear halo zone for 6 isolate while the isolate BK1, BK2, BK6 shows purple color halo zone in the area of CMC plate hydrolyzed by the fungus. CMC was used as substrate for the production of the endo cellulase in the media (Khoirunnisa et al., 2020).whereas, BK8 has shown the least activity during the plate assay.

Quantitative estimation of cellusase activity was also done which demonstrate that isolate BK102 having higher enzyme concentration 819.819 µg/ml in 7 days of incubation. Glucans a major component of fungal cell wall is hydrolyase by the enzyme cellulase which cleave the β-1,4-glucan bonds in cellulose. Enzyme activity calculation of BK102 was done which was found to be 1.8 U/ml. The result of cellulase enzyme activity appears to be less compared to (Widiana et al., 2019). (Santos et al., 2016)shows the cellulase enzyme activity for *Rhizopus stolonifera* was

12.721 U/mL and for *Aspergillus chevalieri* was 29.377U/ml. (Fitrah Dewi & Muzakhar, 2018)the value of cellulase enzyme activity in *Aspergillus chevalieri* was only 0.7 U/ml which is less in comparison of enzyme activity of our isolate.

4.5. Qualitative analysis of gelatin hydrolysis test and Quantitative protease assay following Lowry's method

Gelatin hydrolysis test has been done, following the incubation of isolate in the media plate containing gelatin as substrate for 72 hr. After which the plate has been poured with the gram's iodine, then the halo zone formed around the hydrolysis zone has been noted. All of the screened 7 isolates has shown the gelatin hydrolysis activity. By measuring the diameter of the colony growth and the solubilization zone the clear zone was calculated. The highest clear zone was found to be 1.9 cm for isolate BK6

Table 6: Growth and halo zone formed by the selected isolates on gelatin agar plate.

Name of isolate	Gelatin hydrolysis isolates	clear zone (total D- colony D) (in cm)
BK1	+	1.7
BK2	+	1.0
BK6	+	1.9
BK100	+	1.7
BK101	+	1.4
BK102	+	1.4

(- no growth, + positive growth)

Calculation of the protease activity by the various isolate was done and tabulated in the table above. The results show that BK1 having higher concentration of protease activity (483 µg/ml) following BK6 (299.6667 µg/ml)and BK102 (248.55µg/ml). on calculation of enzyme activity taking the tyrosine as the standard it was found that BK1 having the highest enzyme activity (2.858 U/ml), BK6 (1.653 U/ml) and BK102 having (1.371 U/ml which is quite less than the enzyme activity shown by *aspergillus niger*(Ahmed, 2018) . Various physical factors like temperature ,pH, incubation time, and innoculam size greatly affect the enzyme activity of microbes(Wajeeha et al., 2020). Which was also favored by (Ellaiah et al., 2002)as, protease production strongly depends on the extracellular Ph. which affects several metabolic and enzymatic processes and transports various components across the cell membranes, thus influencing the cell growth and metabolite production.(Johnvesly et al., 2002) reported that the maximum protease production occurred during the 7th day of incubation for Aspergillus flavus. (Benabda et al., 2019) .Filamentous fungi, such as *Aspergillus, Penicillium* and *Paecylomices* have been identified as great producers of extracellular protease (Suryawanshi & Pandya, 2017).

Table 7: protease enzyme activity assay using Lowry's method.

Isolate	Absorbance	Concentration (µg/ml)
BK1	0.481	483

BK2	0.227	200.7778
BK6	0.316	299.6667
BK100	0.188	157.4444
BK101	0.251	227.4444
BK102	0.27	248.5556

4.6. Qualitatitve test of pectinase activity

Table 8: pectinase activity of isolates.

Isolates	pectin activity plate assay at day 5				
	H.D (In cm)	C.D (In cm)	clearzone (In cm)	E.I	S.E(%)
BK1	3.7	3.0	0.7	2.23	23.5
BK2	3.5	2.8	0.7	2.25	25
BK6	3.0	1.8	1.2	2.66	66
BK100	3.2	2.2	1.0	2.45	45
BK101	3.4	2.2	1.2	2.54	54
BK102	3.1	2.4	0.7	2.29	29

After incubation of the isolate in the plate containing pectin media for 5 days,the halozone formed by the fungal strain have been observed after pouring grams iodine in the plate.Thus the pectin solubilization efficiency and index has been calculated after measuring the diameter of halozone formed by the respective strain following protocol of (Hankin et al., 1971)(EI) = (colony diameter + halo zone diameter) / colony diameter.(P., 2012).Pectinase producer strain are graded based on (Soares et al., 1999).(Kabir & Tasmim, 2019)which demonstrate that 3 isolates BK6, BK100 and BK101 as good pectinase producer while rest are weak producer of pectinase.

4.7. NPK test

4.7.1. Screening of isolates producing ammonia.

Only the six isolates were screened based on the production of cell wall degrading enzyme. Among the six screened isolate it has been seen that only 2 of the fungal isolate was able to utilize the complex nitrogen source like peptone for the production of ammonia. The color change of the culture medium was used to assess the ammonia producing ability, color change of the media from faint yellow to dark orange demonstrate the production of ammonia in the medium. Depending on the quantity of NH4+, ammonium reacts with Nessler's reagent to generate a yellow complex or a yellow-brown complex. further quantification of ammonia production was not done. particularly in nutrient-poor and degraded soils, adding nitrogen-fixing microbes to soil coupled with PGP characteristics can be an effective method to reduce the usage of costly chemical fertilizers (Rahman et al., 2017).

Table 9: isolates producing ammonia from complex nitrogen source from complex nitrogen media (peptone water).

Isolates	Ammonia producing isolates
BK1	-
BK2	-

BK6	-
BK100	-
BK101	-
BK102	+

(- no growth, + positive growth)

4.7.2. Qualitative analysis of potassium solubilizing fungus

Out of the 6 isolates 5 isolates were found to have potassium solubilization activity. After the incubation of the isolates for 5 days in the media plate containing potassium alum as the substrate along with dye, formation of the yellowish halo zone is observed. Which was then measured to find solubilization efficiency of the insoluble potassium. Isolate BK102 shows the higher potassium solubilization efficiency (60%) and SI (2.2). All of the isolates were classified as intermediate (SI ≤ 2.00 ≤ 4.00) potassium solubilizing fungus based on classification of potassium dissolution done by(Setiawati & Mutmainnah, 2016). which also showed that intermediate activity for 33.33% isolates among the 15 isolate. All the KSM were found to be capable of solubilizing K from insoluble K-bearing substrate and the solubilization efficiency ranges from (26-60%).

Table 10: potassium solubilizing activity plate assay.

Isolate	S.I	S.E (%)
BK1	2.29	29.41
BK2	2.35	35.71
BK6	2.4	40
BK100	-	-
BK101	2.26	26.66
BK102	2.22	60

SI=solubilization index, S.E=solubilization efficiency

4.7.3. Quantitative analysis of phosphatase activity

Only the quantitative estimation of phosphatase activity was done in our experiment using triclacium phosphate(TCP)as the complex phoshate source. The finding showed that phosphate solubilization activity increases with incubation time . Screened isolates were chosen for the quantitative estimation of phosphatase activity. The isolates released P levels that ranged from (44.4µg/ml-243.4µg/ml). Amongst 5 screened isolates BK101(243.4 µg/ml) followed by BK102(223 µg/ml) showed the maximum capacity of phosphate solubilization at day 16 of incubation. The phosphatase capacity was observed at 4 days interval for 16 days which shows maximum capacity at day 16 for all of the isolate tested. Similar results were obtained in a study of tricalcium phosphate solubilization by (P. Kumar et al., 2020)They observed P concentration between 20.80 µg/mL159.55 µg/ml. According to (Cao et al., 2018)who isolated PSB from a natural wetland, showed solubilized P up to 125.88 mg/L. In the study of (Aliyat et al., 2020)PSB

isolates showed high solubilization efficiency, with Soluble-P concentrations in the NBRIP medium varying from 101.91 µg/mL to 174.33 µg/ml. Different parameters, such as nitrogen supplies, phosphate sources, TCP concentration, temperature, potassium sources, etc., may have an impact on the PSB's ability to solubilize phosphate (Yadav et al., 2013). It is well acknowledged that microbes solubilize P through processes like acidity, chelation, and exchange reactions (Mahdi et al., 2012).

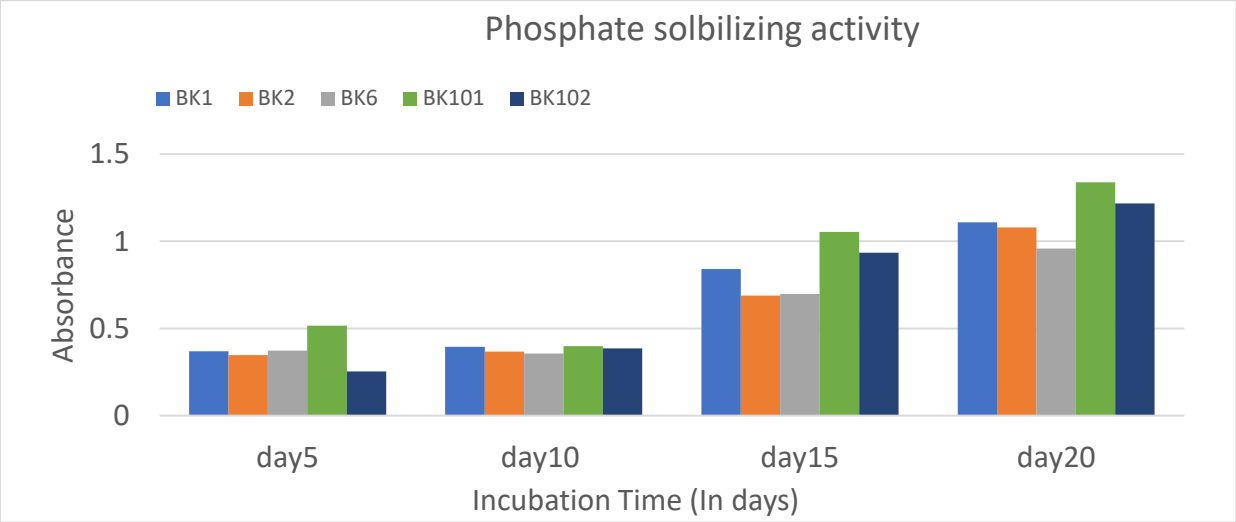

Figure 5: Quantitative test of phosphate solubilizing activity of isolates at different time interval.

4.8. Qualitative test of zinc solubilizing activity

Qualitative evaluation of zinc solubilization efficiency was calculated and tabulated in the chart above. Two different media ZNO and ZNCO3 was used .only the efficiency of zn solubilization of ZNO was evaluated based on the halozone formed in the media. Isolate BK102 (55%) shows the highest Zinc solubilization efficiency.while isoalte BK1 exhibit no activity Solubilization index of zinc is calculated using formula S.I = halo zone diameter- colony diameter /colony diameter as given by (Jagana et al., 2019).Similarly solubilization efficiency was calculated by using formula: S.E = Solubilization Efficiency S.E = halo zone diameter/ colony diameter × 100 following the same article. Among the tested fungus for zinc solubilization, *Phomopsis spp., Aspergillus sp.1, Aspergillus sp.2, and Aspergillus niger* were found to have the highest halozone diameter greater than 40mm which seems to be greater than the halo zone produce by our isolate on doing comparative analysis as shown by (Sutjaritvorakul et al., 2013).Along with this various species of bacteria *Bacillus, Pseudomonas, Azotobacter, and Rhizobium* has shown the ability to solubilize zinc demonstrate by a number of researchers (Hussain et al., n.d.);(Jagana et al., 2019); (Joshi et al., 2013) .Amongst the various substrate used ZNO was solubilized with higher efficiency as demonstrated by (Sutjaritvorakul et al., 2013). which was also observed in our research.

Table 11: Zinc solubilization activity of isolates on plate assay.

Isolates	**zinc producing isolates**		
H.D	C.D	S.I	S.E (in %)

BK1	-	-	-	-
BK2	2.4	2.0	2.2	20
BK6	3.5	2.5	2.4	40
BK100	2.3	1.7	2.35	35.29
BK101	2.2	1.8	2.22	22.22
BK102	3.1	2.0	2.55	55

4.9. Auxin producing fungus

Table 12: IAA producing fungal isolates.

Isolates	auxin production test
BK1	-
BK2	-
BK6	-
BK100	+
BK101	+
BK102	-

(- no growth, + positive growth)

Qualitative evaluation of auxin production by the isolate was done which demonstrate that only the 2 isolates seems to produce auxin among the 6 screened isolates while rest of the isolate didn't exhibit any activity which was evaluated based on their appearance of color change to red during auxin production assay. IAA has important role on plant developmental processes, such as cell division, differentiation and organ formation(Benjamins & Scheres, 2008),senescence (Kim et al., 2011)biotic as well as abiotic stress(Peleg & Blumwald, 2011),. As IAA have a significant impact on plant growth, the ability of rhizobacterial isolates to produce IAA is particularly useful (S. Chandra et al., 2018). IAA promotes root development and growth, which could lead to improved nutrient uptake (Jasim et al., 2014)In the present study, the saprophytic fungal isolate was able to generate IAA. Primary root growth can be significantly impacted by IAA in even tiny doses (Chrouqi et al., 2017).Bacterial and fungal species producing auxin are involved in the symbiotic relationship with plant (Hirsch et al., n.d.)as well as phytopathogenic role. It was suggested that role of auxin depends on the concentration applied, lower concentration favours fungal isolate growth while higher concentration of IAA reduces the spore germination (Sieńczyk et al., 2004).

4.10. Synthesis characterization and antifungal test of copperoxide nanoparticle.

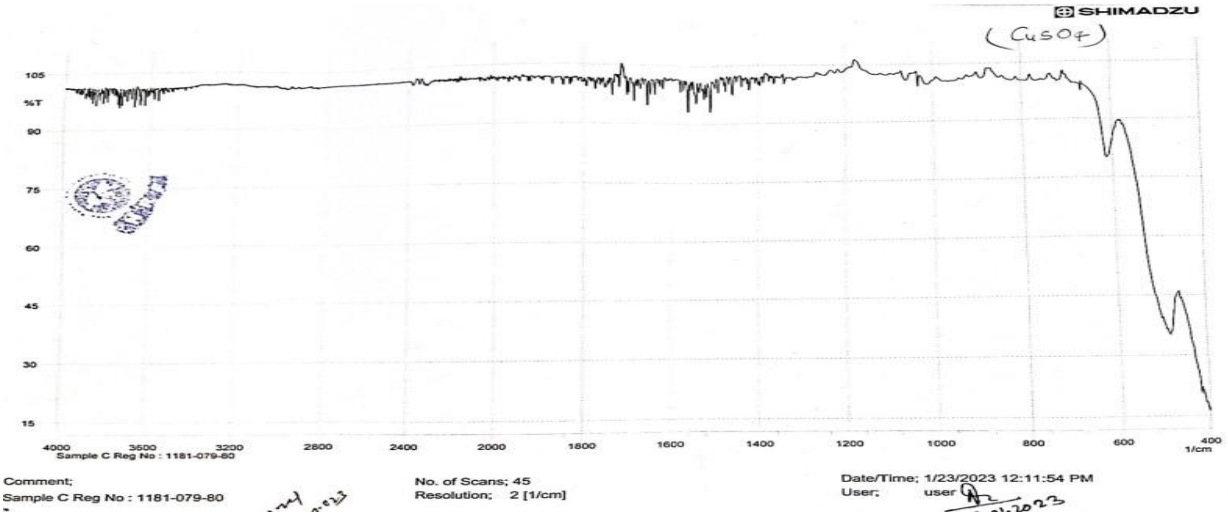

Figure 6:FTIR data of CuO nanoparticle.

Copper oxide (CuO) nanoparticle was formed through the process of precipitation method followed by calcination from the precursor copper sulphate following the procedure of(Phiwdang et al., 2013). Brownish black color ppt. of the copper oxide nanoparticle was formed. which was then characterized through FTIR. Result showed that CuO has characteristic peaks in the FT-IR spectrum at 437.84 cm −1, 516.92 cm −1 and 590.22 cm^-1. Similarly, nanoparticle formed in our process has shown the similar peaks on FTIR results. (Phiwdang et al., 2013)suggested that the calcination process effectively remove residue and lead to the better crystallization of CuO.

The formed CuO was then mixed in the media 1µg/ml and the pathogenic strain was inoculated and allowed for their growth. The growth pattern was observed where the growth of the isolate on PDA served as the control for reference. It shows that the growth of the pathogenic strain was greatly reduced in the media plate containing CuO nanoparticle.

4.11. Microscopic identification and colony morphology of the selected isolate.

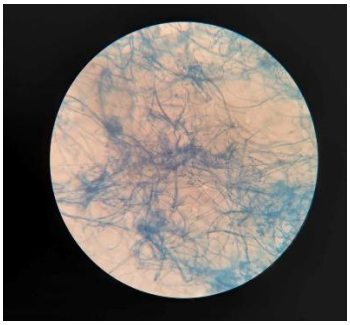

Figure 7: Microscopic observation of BK102 isolate using LPCB staining.

Table 13: Morphological characterstics of fungal isolate BK102.

Colony morphology on PD agar	• White cottony mass with no pigmentation on forward colony. • Light yellow color colony on the reverse.
Microscopic characteristics	• Thread like mycelium with septa .

Similar result was shown by (Dhakar & Pandey, 2013) for the fungal isolate *Trametes hirusta*.

4.12. Molecular characterization of isolate

Isolates positive for most of the enzyme activity and mineral solubilization capacity was chosen as the ideal isolates for further molecular characterization. Genomic DNA of the selected fungus isolate was initially isolated using the CTAB technique and subjected for ITS region based PCR and sequencing. Identification of fungi by PCR technique generally uses the universal primer pair ITS1/ITS4 to amplify the ITS region of ribosomal DNA (rDNA) because of its universal presence, conservative, and abundant presence. The rDNA is the coding region of the genome for the ribosomal DNA component. In addition, the ITS region also has a clear barcode to distinguish species and the presence of inter- and intra-specific nucleotide base variations (Buehler et al., 2017)Obtained DNA sequence is listed in the appendix.

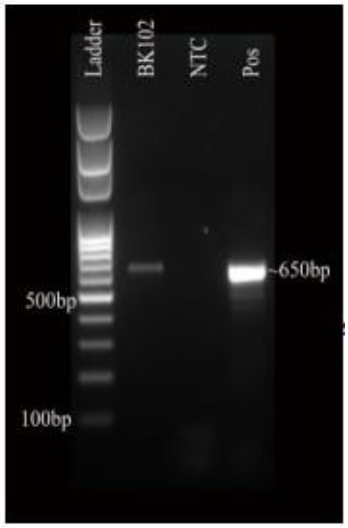

Figure 8: Agarose gel electrophoresis of genomic DNA and ITS PCR products of sample BK102. pos- positive PCR product, NTC- negative control.

4.13. Phylogenetic tree analysis of the isolate

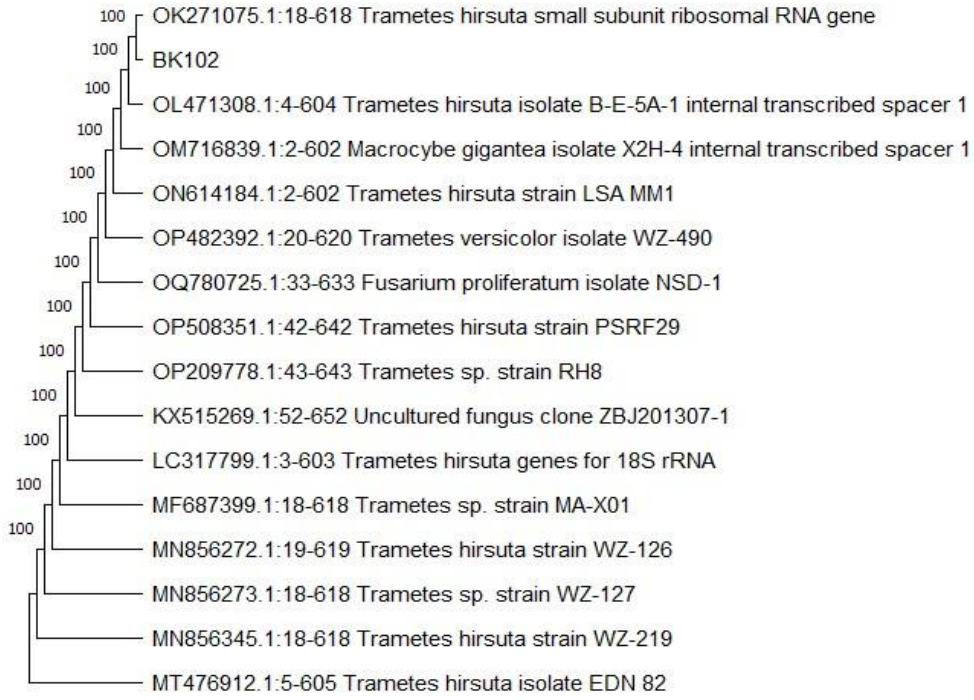

Figure 9: cladogram of isolate BK102 showing Phylogenetic Neighbor joining tree based on ITS rRNA sequences showing Phylogenetic relationship between related species of the genus Trametes.

The selected isolate was demonstrated to be the fungal strain through the sequencing as shown by the fig. 24 the FASTA sequence of the isolate obtained after doing the sanger sequencing was

subjected to nucleotide BLAST's sequence homology analysis at NCBI. Isolate BK102 shows the high percentage identity (99.83%) with Trametes sp.. The sequence that were acquired were added into the MUSCLE algorithm for multiple sequence alignment in MEGA11 software. The data from the batch received were put through a phylogenetic analysis following the neighbour joining method. The phylogenetic analysis was constructed using MEGA.6 software with Neighbor Joining (NJ) (Uncu et al., 2015)and bootstrap test 1000 replications. Figure displays the cladograms obtained by phylogenetic analysis. isolate BK102 was determined to be Trametes.sp. based on the results.

4.14. Partial purification of the protein

Dialysis is a molecular separation procedure based on selective diffusion to change the concentration or composition of solutes. The most common application of dialysis is for the removal of unwanted small molecules such as salts, reducing agents, or dyes from larger macromolecules such as proteins, DNA, or polysaccharides (Wu & Zou, 2017). Chitinase enzyme is currently used in biological control of fungal pathogens because of its ability to degrade chitin in fungal cell walls ((Ulhoa & Peberdy, 1991)protein concentration is listed below in the table which shows enhanced protein concentration after dialysis compared to initial concentration before dialysis. Different amount of ammonium sulphate was used following the literature in order to obtain the higher protein concentration recovery for different protein. (Rabeeth et al., 2011)suggested that percentage saturation range lies in the range of 30-85% as the saturation range varies for different isolates .

Table 14:Comparative analysis of protein concentration before and after dialysis.

Name of enzymes	Cellulase	Chitinase	Laccase	PVK	Protease
Concentration of enzymes before dialysis	248.5556	17.44444	100.7778	125.2222	258.5556
Concentration of enzyme after dialysis	426.3333	355.2222	400.23	379.6667	586.3333

4.15. Dual culture test between screened isolate and the pathogens

Cubes of medium of 6mm was cut from the growing edge of seven day old cultures of each pathogens (*F. oxysporum, A. solani, Fusarium, R. solani*) along with our isolates each of 7 days. A cube of each pathogen and the saprophytic isolates were placed in the Petri plates 2 cm away from the opposite end of edge. Triplicates of each combination was prepared and allowed for incubation at 37 for 5 days.

(i) (ii) (iii) (iv)

Figure 10: Dual culture assay between isolate BK102 and pathogens.(i)BK102 and fusarium sp. (ii) BK102 and A.solani (iii) BK102 and R.solani (iv) BK102 and Fusarium oxysporum.

Observation was made each day after that. For control the isolate and the pathogen were kept alone in the plate. Radial growth of each fungal strain (in control) and dual culture plate was observed for each strain. The percentage inhibition of radial growth of each isolate was calculated using formula when each isolates were inoculated simultaneously. The results are tabulated below. During the dual culture assay along with the radial growth, Macroscopic morphological observations indicating an interaction between species were recorded: inhibition zones, abnormal pigments and/or exudate production formation of a hyphal barrier (thickened mycelial growth between cultures) and overlapping growth can also be interpreted as signs of antagonism FOC (Badalyan et al., 2002); (Hajieghrari et al., 2008).

Table 15: Inhibition percentage of different pathogenic strain by the selected isolates by dual culture method.

Name of isolates	BK1	BK2	BK6	BK100	BK101	BK102
Fusarium	68.88	60	54	57.33	60	56
F.oxysporum	62.44	52.11	52.11	54.92	53.52	56.33
A.solani	52.11	52.05	52.05	54.79	51.21	64.38
R.solani	60.56	56.33	52.11	56.33	57.77	58.52

The inhibition percentage ranges from 50-68% by the different isolate. The PIRG calculated shows that BK1(60.56%) followed by BK102(58.52%) isolate have the greatest inhibition as compared to other for the pathogenic strain *R.Solani* (64.38%). BK1 strain also shows the overlapping property over the mycelia and thick growth over *R. solani*, whereas the isolate BK102 shows quite less overlapping over the pathogen as compared to BK1. One of the report by (Mirsam et al., 2023)shows that antagonistic effect of *R.solani* by *T.asperellum* isolates with a percentage of inhibition ranging from 59.57 to 75.25% which is greater compared to synthetic fungicide treatment/control which was only 56.54%. *R.solani* is a pathogenic soilborne fungus which attack the crops like potato, corn etc. and reduces the production(Rotasouw et al., 2020),(Mirsam et al., 2023) (Lahlali & Hijri, 2010). This isolate causes different symptoms at each plant growth phase, including root, stem, and crown rots, damping-off, as well as foliar and sheath rot (Baker & Martinson, 2020)On getting suitable environmental condition *R.solani* can endure in the soil for long period by either feeding on residual debris or forming sclerotia. Our finding shows that isolated fungal endophyte BK102 which was confirmed to be *Trametes hirusta* having the second

highest percentage inhibition when tested by dual culture method. Other experiment done by (Lahlali & Hijri, 2010)shows the percentage inhibiton of *R.solani* in the range of (10.22-81.81%) by different endophytic isolated where the highest antagonism was shown by *T.atroviridea* .

Similarly, the strain f.oxysporum was inhibited greatly by BK1(62.44%) strain followed by BK102 (56.6%). T. versicolor induced a line of growth between the two fungi and an interaction zone appeared between the fungi as demonstrated by(Ruiz-Dueñas & Martínez, 1996). similarly, BK1 isolate shows dense growth, change in color of mycelia in the interaction zone over the pathogenic strain F.oxysporum. Other pathogenic strain *Fusarium* was inhibited at greater percentage by BK1 strain with percentage inhibition of (68.88%) during dual culture assay. change in the mycelia color from white to yellow was observed at the interaction zone between *Fusarium* and BK1 during the dual culture assay. Along with this overlapping of bk1 isolate was seen over the pathogen.On the other hand the pathogenic strain *A.solani* was greatly inhibited by the endophytic isolate BK102 with inhibition percentage of (64.38%).

(X. Zhang et al., 2020),(Wu & Zou, 2017) Suggested that the the mycoparasitism starts when the hyphae of the pathogen is coiled by the beneficial isolates and secretes a number of cell wall degrading enzymes(CWDEs) containing chitinase, glucanase, and protease causing the abnormalities and lysis of the hyphae of pathogen. The (CWDEs) production was higher for the BK1 isolates, due to which it shows the higher inhibition of the pathogenic isolate.

CHAPTER 5

(summary, conclusion and recommendation)

5.1. Summary

Fungal pathogens can create diverse array of diseases following the various strategies including colonizing the plant cells as necrotrophs or biotrophs. Various types of cell walls degrading enzymes (CWDEs such as xyloglucanases, polygalacturonases, glucanases, cellulases, and pectinases), effectors, toxins of the fungal strain are involved in the phytopathogenic activity. To combat fungal pathogenicity present research was focused on the isolation and screening of fungal isolates having greater enzymatic activities as well as the plant growth promoting factors that might act as gut microbiota in human. The diverse array of the CWDEs (cellulase, chitinase, laccase, amylase) were taken as the parameters to acts as possible antagonist.

Initially the soil sample was collected from the infected area as well the virgin area in Ilam. Following the heat shock and utilizing antibacterial agent in the media spore forming fungal strain was isolated. In order to screen the slow growing potential saprophytic isolate, lignin substrate tannic acid was used in the media. At first 98 isolates were selected based on the halo zone formed in the media plate containing tannic acid. For further process 8 isolates producing reddish halo zone in guaiacol substrate plate was selected. The screened isolates were again tested for their ability to utilize carbon source from the complex lignin degradation product tannic acid. Two different substrate guaiacol and tannic acid were used for the qualitative analysis of laccase production. BK101 shows higher activity at 5day on guaiacol plate, which shows the halo zone diameter (3.82 cm) followed by BK2 (3.54 cm). On the substrate tannic acid, BK100(2.12 cm) followed by BK2 (1.92 cm) and BK101 (1.84 cm). Optimization of the media component, Ph. and temperature was not done for any isolate during the quantitative analysis. variation in the qualitative plate assay and quantitative broth assay in the same media component guaiacol was seen which might be due to elements of media.so optimization of media component is required for the better result.

Chitin an essential component of the fungal strain can be degraded by the enzyme chitinase. Screened 8 saprophytic strain were found to be positive for chitinase. Qualitative analysis of the isolates shows highest solubilization of BLT1 and BK8 strain which was 108.33% and 106.66%. On quantitative analysis BK8 shows the highest activity 1.813U/ml at day 10. Further optimization of the media component and the reaction condition is required. Most of the isolates shows the higher activity at 10 days of incubation and decreases with time.

Other important cell wall component of the fungus is glucanase. Seven isolates were found positive for starch hydrolysis test of which BLT1 shows the least activity where BK8 didn't exhibit any activity. On plate assay of starch hydrolysis test S41(173.33%), BK6(116.66%), BK1(109.52%) has shown the highest solubilization efficiency. Enzyme activity test at different time interval was

done, which shows that BK1 having highest amylase activity (90.02) U/ml at day 12. While on day 8 BK100(118.78U/ml) followed by BK102 (60.01 U/ml) has shown the highest activity. With time the activity decrease afterwards.

All of the screened isolates were found positive for cellulase activity on doing qualitative analysis. However, BK8 have shown the least activity which was then excluded for the further quantification process. CMC was taken as the complex source of cellulose in the media plate. Bk102 (819.81µg/ml) has been found to have highest activity at day 7 on quantitative analysis.

Gelatin hydrolysis test was also done in which BK6 had the highest gelatin hydrolysis activity with clear zone 1.9cm. Protease activity was quantified following Lowry's method. For the quantitative estimation of protease activity casein was used as substrate. BK1, BK101 and BK102 was found to have the highest protease activity which was 483 µg/ml, 299.66 µg/ml, 248.55 µg/ml on day 7 of incubation. The isolate BK8 doesn't exhibit any protease activity which was then excluded for further process.

The isolates were also tested for plant growth promoting traits thus various features such as (NPK, zinc solubilization, auxin production) of the isolates were analyzed. Only 6 isolates (BK1, BK2, BK6, BK100, BK101 and BK102) were tested for PGP traits having significant enzyme activity. While testing for the NPK, it was found that only one isolate (BK102) have ability to produce ammonia when supplemented with complex nitrogen source. Quantitative assay of phosphorus solubilizing activity was done which demonstrate that BK101 and BK102 having the highest amongst all the screened isolates. Among the screened isolates 5 were found to have potassium solubilizing ability however BK100 do not show any activity on plate assay. Where BK102 have shown the highest solubilization efficiency on doing plate assay with solubilization efficiency of 60%. Out of 6 isolates five isolates has shown the ability to solubilize the zinc. Among which BK102(55%) has shown the maximum ability to solubilize zinc. Two isolates BK100, BK101 were able to produce the hormone auxin which acts as a plant growth regulator. However, none of the isolate were positive for HCN production.

On dual culture analysis BK1 (60.56%) and BK102(58.52%) has shown the highest inhibition on the fungal pathogen *R. solani*. For the pathogen *A. solani* the isolates BK102(64.38%) and BK100 (54.79%)has inhibited the isolates by greater percentage. Other pathogen *Fusarium* was greatly inhibited by BK1(68.88%). BK1(62.44%) and BK102(56.33%) isolates were able to inhibit the strain *Fusarium oxysporum* with maximum inhibition percentage.

Beside biocontrol, other approach of controlling the pathogen was also done. For that CuO nanoparticle was synthesized through precipitation followed by calcination method using copper sulphate. On testing in the media plate with PDA and nanoparticle it was found that the mass of the pathogenic strain was greatly reduced by the nanoparticle. The nanoparticle was characterized by the FTIR process.

5.2. Conclusion

In our study 11 fungal strain were isolated denoted as, BK1, BK2, BK6, BK8, BK100, BK101, BK102, BLT1, S41 and W14. The isolates were accessed for different cell wall degrading enzymes as well plant growth promoting traits thus various features such as (NPK, zinc solubilization, auxin production). Dual culture between the pathogen and the antagonist isolate showed BK1 as the most effective strain, while based on the Plant growth promoting traits as well biocontrol trait BK102 strain was quite effective. The isolate having prominent enzyme activity and plant growth promoting feature was BK102 isolate which was then selected for DNA extraction and PCR. Identification of fungi by PCR was done by using universal primer pair ITS1/ITS4 to amplify the ITS region of ribosomal DNA (rDNA) because of its universal presence, conservative, and abundant presence. The obtained sequenced was then processed for phylogenetic analysis through neighbor joining method. The sequence showed 100% sequence similarity with *Trametes hirusta.*

5.3. Recommendation

The following suggestions can be made in light of our study's findings:

- Optimization of ph., temperature, media components for the different enzyme activity.
- Investigating the many environmental elements that either directly or indirectly influence the functions of fungal isolate is important.
- To obtain more effective isolates, more isolates from various ecological zones should be isolated and characterized.
- It is necessary to conduct extensive field experiments so that the significance of these isolate in plant growth promotion may be well understood
- Efficient isolates need to be produced in sufficient quantities, commercialized, and made available to farmers.

CHAPTER 6

6. References

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7. Appendix

APPENDIX I: List of components for media preparations:

Nessler's reagent preparation:

Laccase production media:	**g/L**
Peptone	3g
Glucose	10g
KH_2PO_4	0.6g
K_2HPO_4	0.4g
$ZnSO_4$	0.001g
$FeSO_4$	0.0005g
$MgSO_4$	0.5g
$MnSO_4$	0.05g
Chitinase production media:	**g/L**
KH_2PO_4	0.1g
$MgSO_4$.$7H_2O$	0.001g
NaCl	3.0g
$(NH_4)_2SO_4$	0.7g
Yeast extract	0.05g
Galactose	1%
Colloidal chitin	2g
Cellulase production media:	**g/L**
Sucrose	1.5%
Yeast extract	1%
$(NH_4)2SO_4$	1.4g
KH_2PO_4	2g
$CaCl_2.2H_2O$	0.4g

$MgSO_4.7H_2O$	0.3g
$FeSO_4.7H_2O$	0.005 g
$COCl_2.6H_2O$	0.0037g
$MnSO_4.H_2O$	0.0016g
$ZnSO_4.7H_2O$	0.001g
CMC	10g
Protease production media:	**g/L**
$CaCl_2.7H_2O$	0.4g
KH_2PO_4	7.0g
Na_2HPO_4	2.5g
$MgSO_4.7H_20$	0.5g
$ZnCl_2$	0.1g
NaCl	0.3g
Casein	2.0g
Amylase production media:	**g/l**
KH_2PO_4	1.4g
Ammonium nitrate	10g
KCL	0.5g
$MgSO_4.7H_2O$	0.1g
$FeSO_4.7H_20$	0.01g
Soluble starch	20g
Agar	2.5%
Pectinase production medium	**g/L**
Pectin	10 g
Di-ammonium orthophosphate	3.0 g
K_2HPO_4	2.0 g
$MgSO_4$	0.1 g

Agar	20 g

Final pH 5.5

Media composition for 100 ml of Nessler's reagent

$HgCl_2$	2gm in 50ml dw
NaOH	4g in 20 ml (5M)
KI	5-7 gm in 10 ml

As mentioned composition of respective chemicals were prepared in separate beakers. Potassium iodide solution was mixed to mercuric chloride solution slowly with constant stirring. while doing so, solution will display pinkish yellow ppt. initially, upon addition of more KI the precipitate starts to dissolve slowly displaying light greenish solution. Finally, the NaOH solution was added to maintain the ph. of the reagent and maintained the volume to 100ml using DW. The prepared reagent was then stored in amber bottle in dark place.

Pikovskaya medium (PVK)	**g/L**
Yeast Extract	0.5 g
Dextrose	10.0 g
Calcium Phosphate	5.0 g
Ammonium Sulphate	0.5 g
Potassium Chloride	0.2 g
Magnesium Sulphate	0.1 g
Manganese Sulphate	0.0001 g
Ferrous Sulphate	0.0001 g
Agar	15.0 g

Aleksandrow medium:	**g/l**
$MgSO_4.7H_2O$	0.005g
$FeCl_3$	0.1g
$CaCO_3$	2.0g
Glucose	5.0g
Calcium Phosphate	2.0g
Mica	2.0g

Bromothymol Blue:	(0.05in 10ml solution of 70% ethanol)
Agar	15g
Zinc media:	**g/l**
Dextrose	10g
Ammonium sulphate	1g
KCL	0.2g
K_2HPO_4	0.1g
$MgSO_4$	0.2g
$ZnO/ZNCO_3$	1.0g
Agar	15g
Sabouraud's dextrose agar media:	**g/l**
Dextrose	4%
Peptone	1%
Agar	1.5%
D/W	Maintain the solution to 100 ml

2. **Equipments**

Autoclave Centrifuge

Electrophoresis apparatus

Gel documentation system

High performance liquid chromatography (HPLC)

Hot air oven

Incubator

Laminar airflow cabinet

Microscope Nanodrop

pH meter

Refrigerator

UV spectrophotometer

Water bath

APPENDIX II: List of figures.

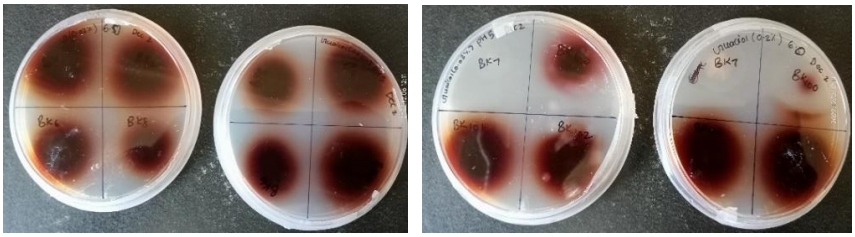


Figure1: Plate assay of laccase enzyme activity in Guaiacol (ph. 5 and 6)

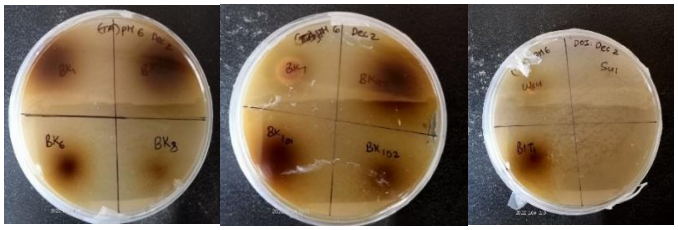


Figure2: Plate assay of laccase enzyme activity in Tannic acid (ph 6)

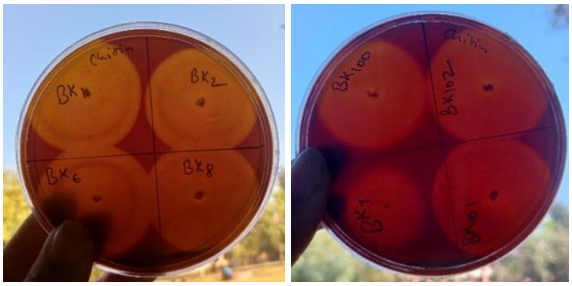


Figure 3: Plate assay of isolates for chitinase activity.

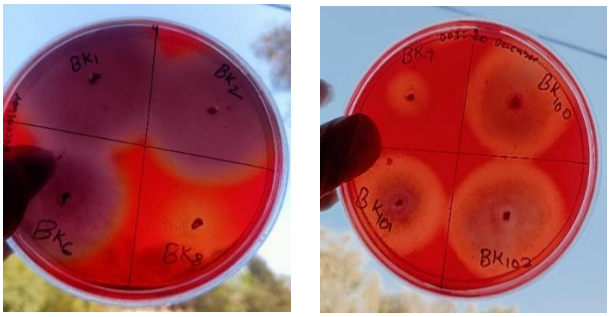


Figure 4: Cellulase plate assay of different isolate on CMC agar plate after 5 day of incubation.

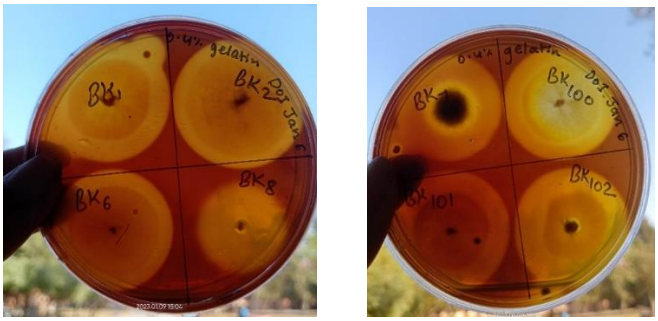


Figure 5: Plate assay of gelatin hydrolysis test after 3 days of incubation at 30°C.

Figure 6: Plate showing the halo zone formed by different isolates on starch agar plate.

Figure 6: Qualitative test of ammonia producing isolates ,using nessler reagent.

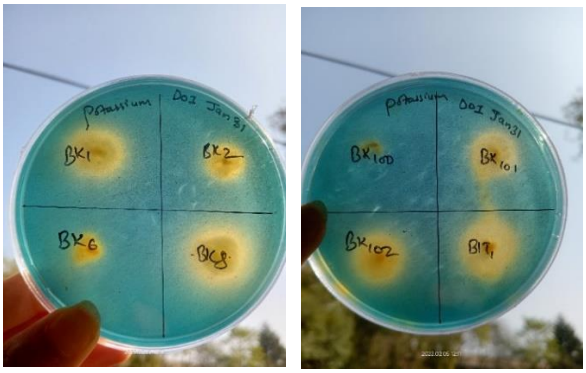


Figure 7: potassium solubilizing activity test by plate assay method.

Figure 8: Plate showing pectin solubilizing isolates in pectin agar plate.

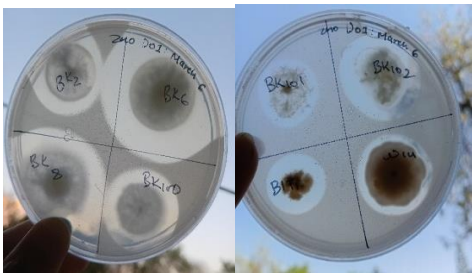
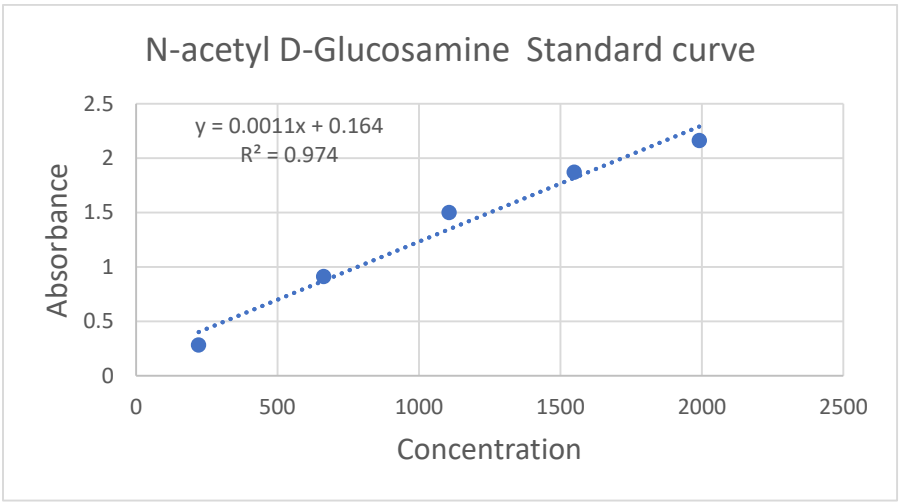
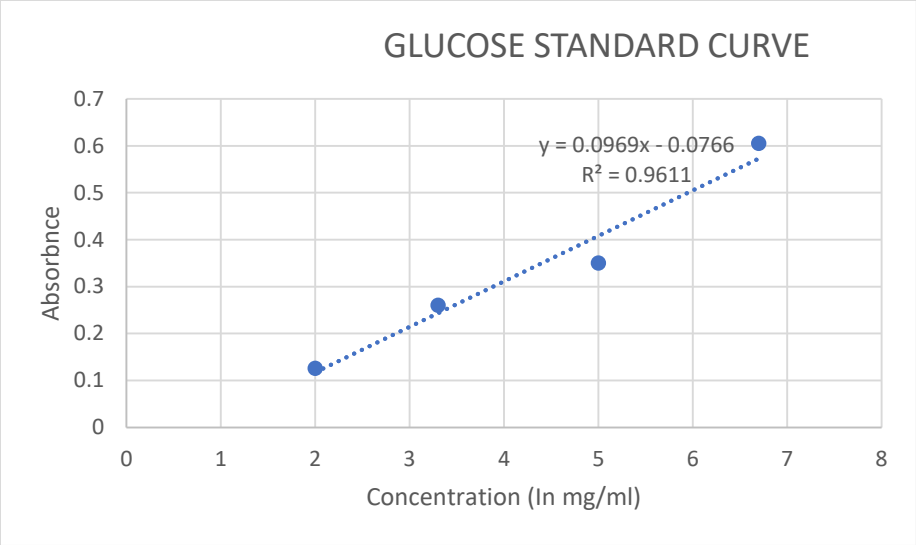


Figure 9: zinc solubilizing test using

Figure 10: screening of auxin producing isolate.

APPENDIX III: Standard calibration curve.




GLUCOSE STANDARD CURVE
y = 0.0969x - 0.0766
R² = 0.9611
Absorbnce
Concentration (In mg/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8