Potential of *Bacillus* spp. Showing Antagonistic Activity against Some Bacterial and Fungal Pathogens

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بسم الله الرحمن الرحيم

CERTIFICATE

This thesis submitted by ***Asad Ali*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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DEDICATION

I dedicated this piece of work to my beloved parents and brothers whose continuous support encourage me to accomplish it with the best of my abilities. I have no words to acknowledge the sacrifices you made and the dreams you had to let go just to give me a shot at achieving mine.

DECLARATION

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

ASAD ALI

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List of Acronyms

GDP	Gross Domestic Product
BCA	Bio Control Agent
OC	Organo-Chlorinated
GRAS	Generally Recognized As Safe
FDA	Food and Drug Administration
PGPB	Plant Growth Promoting Bacteria
AHL	N-acyl-L-homoserine Lactones
NRPS	Non Ribosomal Peptide Synthase
MSM	Mineral Salt Media
SDA	Sabouraud Dextrose Agar
PDA	Potato Dextrose Agar
RPM	Revolution Per Minute
PGPRs	Plant Growth Promoting Rhizospheric Bacteria
FTIR	Fourier Transform Infrared Spectroscopy

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Abstract

The agricultural productivity losses and human health decline due to microbial pathogens have been a serious issue in Pakistan and rest of the world. Present work was designed to isolate soil borne microorganisms having antagonistic ability against notorious phyto and human pathogens. From the initial collection of 08 bacterial isolates, two potent strains of *Bacillus* were screened on the basis of their comparative efficacy against devastating fungal and bacterial pathogens. The strains AAM-1 and AK-47 showed excellent inhibitory indexes against selected pathogens. It was noted that both strains of *Bacillus* showed significant bio-control activity against plant pathogens like *Aspergillus flavus, Fusarium moniliforme, Colletotricum falcatum, Botrytis cinerea, Aspergillus niger, Fusarium oxysporum, Phythopthora capsici* and *Rhizopus oryzae. In addition both isolates showed significant inhibitory indexes against selected human pathogens namely Salmonella typhi, Escherichia coli, Klebsella pneumoniae, Staphylococcus aureus, Acinitobacter baumannii* and *Listeria monocytogenes.* The strain AAM-1 was efficient to suppress *Fusarium* species while AK-47 expressed significant antagonistic activity against *Aspergillus* species, *Fusarium* species, *Botrytis cinerea* and *Colletotricum falcatum*. On the basis of *in vitro* assay, it can postulated that the *Bacillus* strains AAM-1 and AK-47 can be used as bio-protective agent against various plant diseases and can also be used as a source of antibiotic production against aforesaid human pathogens. In addition, their applications as natural pesticides could be very helpful to prevent the adverse effects of chemical pesticides.

INTRODUCTION

Being primary producers in the ecosystem, plants are the principle source of food for all living organisms on earth including humans. The unavoidable environmental changes and natural disasters are continuously posing serious challenges for ecosystem sustainability and supply chain management. In addition microbial pathogens associated with the plants are the major sources of productivity loss at every stage of agricultural processes. In the current situation of food scarcity, plant protection and growth promotion has been the ultimate solution to secure future needs of world (Oerke *et al*., 2012). The environmental and food authorities all over the world has suggested to exploit modern agriculture methods for crop protection and improving agriculture productivity. One of the best method for the plant protection is the use of natural products instead of chemical pesticides for controlling growth and activities of problematic microorganisms. On the other side, application of bio-fertilizers is growing all over the world as an alternative to the synthetic chemicals. Taking together, efforts are being made towards the development of sustainable agriculture (Bhardwaj *et al*., 2014).

According to Pakistan Bureau of Statistics, agriculture is the main pillar of our economy which contributes about 24% of National GDP. This sector provides employment to the half of labor force of the country and serves as largest source of foreign exchange earnings. Our agricultural products mainly comprise of cash and edible crops including tobacco, wheat, sugarcane, rice, cereals, maize, variety of vegetables and fruits. These mentioned products are affected by variety of reasons like low water supply, water logging and salinity and by invading pathogens responsible for major economic losses every year (Coelli and Rao, 2005). Variety of viruses, bacteria and fungi are the serious plant pathogens causing severe damage to the crops. Viral pathogens account for10-15% productivity loss while bacterial and fungal pathogens are responsible for16.5% and 40% loss respectively. Among these, root associated fungal pathogens are the main agents for plant diseases (Berendsen *et al*., 2012).

Strategies for optimized production of crops are adapted worldwide specifically use of hybrid seeds for maximum yield, introduction of genetically modified plants and chemical control of plant pests and pathogens. Of the particular interest, use of chemicals as pesticides has serious and deleterious repercussions. Recently, it has been reported that over dosage of permethrin and D-altrin lead towards cancer progression, skin allergies, asthma etc. when incorporated into the food chain (Carvalho, 2006). In this situation, biological control or biocontrol has emerged an eco-friendly and sustainable approach of restricting pathogen or pathogenic effects on plant. A number of bacterial and fungal strains have been isolated from different mediums to be used as alternative biocontrol agents (BCA). Technically, BCA are those living forms that can suppress the pathogens or their effects. These agents are itself are microorganisms but they have no pathogenic effect on plants as well as on humans and can be used for diverse purposes like in biocontrol strategy against plant and human pathogens, pests control, antibiotic production, fatty acids production which act as antibiotics, enzyme production and also used as a bio fertilizers which promotes plant growth by suppressing pathogens (Raaijmakers et al., 2002).BCA are classified as fungal, bacterial and viral. Reported promising BCAs from fungal domain are *Trichoderma harizanum*, *Ampelomyces quisqualis, Gliocladium catenulatum* etc while from viral domain BCAs are nuclear polyhedrosis virus (NPV) Insect viruses like baculoviruses, bacteriophages etc. while from bacterial domain includes *Pseudomonas aeruginosa*, *Pseudomonas fluorescens,* species from *Bacillus* genus like *Bacillus subtilis, Bacillus pumilis, Bacillus thuringiensis* etc. This study is mainly focused on biocontrol of plant and human pathogens by *Bacillus* species.

The genus *Bacillus* is comprised of hundreds of bacterial species which are ubiquitous and have the potential to be used as auspicious BCAs. Important strains which suppress the growth of pathogens invitro and invivo are screened out and identified as *Bacillus subtilis*. These strains have the ability to suppress the proliferation of fungal pathogens by

showing fungicidal and fungi-static effect. To acquire mentioned effects these antagonists in soil adapt certain strategies to suppress pathogens. Mechanism of suppression in suppressive soils for pathogens is not always clear because it may involve biotic and abiotic factors. General stratagems include production of antibiotics by antagonists, formation of lytic enzymes, through competition for food, or through direct parasitizing of the pathogen which do not allow the pathogen to reach high enough populations to cause severe disease (Compant*et al*., 2005). Disease rate can be controlled by adding suppressive soils to conducive one because former one contains antagonists which proliferate in the later one and consequently halts the production rate of pathogen hence promotes plant health (Janvier*et al*., 2007).

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens *Bacillus* species isolated from rhizosphere possess greater potential to overcome the devastating properties of pathogens whether they are phytopathogens or human ones. They are appealing candidates for biocontrol because of production of endospores which are tolerant to harsh environments like heat and desiccation (Nicholson, 2002). Of greatest interest is *B.subtilis* which was isolated from suppressive soil and is inhibitory in vivo to several phytopathogens.in this study we manipulate selected *Bacillus* species as a biocontrol agents against some important phyto and human pathogens. Variety of soil borne pathogens occupy conducive soil where they proliferate rapidly and cause diseases in growing plants but this flourishing rate is not the same in suppressive soils where the growth is suppressed by some soil antagonists like *Bacillus* species which are promising BCA. *Bacillus* species are also in the list of plant growth promoting rhizospheric bacteria (PGPR) (Kumar et al., 2011). *B. subtilis* has been sold as a treatment for peanut under the name QUANTUM-4000.

Delineating the diverse actions of BCAs mutualism is the first and important association where both species get benefit from each other. Protocooperation is a form of mutualism,

but the organisms involved do not depend exclusively on each other for survival. Many of the microbes isolated and classified as BCAs can be considered facultative mutualists involved in protocooperation, because survival rarely depends on any specific host and disease suppression will vary depending on the prevailing environmental conditions. Further down the spectrum, commensalism is a symbiotic interaction between two living organisms, where one organism benefits and the other is neither harmed nor benefited. Neutralism describes the biological interactions when the population density of one species has absolutely no effect whatsoever on the other. Related to biological control, an inability to associate the population dynamics of pathogen with that of another organism would indicate neutralism. In contrast, antagonism between organisms results in a negative outcome for one or both. Biocontrol can occur when non-pathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control (Weller, 1988).

Overviewing mechanism of action of *Bacillus* spp. as antagonist BCA reveals that they have the potential to be used on large scale against various pathogens to control diseases because it involves adopting of different mechanisms like production of antibiotics including Iturin, bacillomycine ,fengycin production of lytic enzymes that can hydrolyse a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose all of which are found in bacterial or fungal cell wall hence perform suppression of disease by causing alteration in their cell wall (Zhang et al., 2008). Competition for nutrients between pathogens and non-pathogens which is important for limiting disease incidence and severity is also performed by *Bacillus* antagonists.

Reviewing literature suggested that plant root associated fungal pathogens responsible for greater loss both economically and yield wise are *Aspergillus niger* which causes black mold on fruits and vegetables like grapes, onions, apricots and peanuts. *Aspergillus flavus* which is opportunistic human and plant pathogen is responsible for aspergillosis.

Phytophthora capsici that causes blight and fruit rot of green chili, cucumber, watermelon, tomato and snap beans. *Botrytis cinerea* besides causing infection in plants of citrus family like strawberries and grapes is also human opportunistic pathogen causing winegrower's lung. *Colletotricum* a genus of fungi and many of its species are plant-pathogens like *Colletotrichum lindemuthianum* which cause disease of bean pods. *Fusarium oxysporum* root associated fungal plant pathogen which produces three types of spores. Its hosts include potato, sugarcane, garden bean, cowpea and mainly a causative agent of panama disease of banana results in huge loss.

In this study activity of isolated *Bacillus* strains was also checked against selected human pathogens like *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Listeria monocytogenes*. Positive results confirmed that they are promising biocontrol agents and have great potential to be used as a source of antibiotic production in future.

Efficiency of chemical control agents used against pathogens is proficient enough but its damaging effects on both environment and human health is alarming, so biocontrol agents are sustainable and eco-friendly alternatives to overcome the devastating consequences of the former by improving human health conditions and enhancing productivity in agricultural sector.

Aim

To analyze the potential of *Bacillus* species that displayed antagonistic activity against some bacterial and fungal pathogens.

Objectives

- Isolation of *Bacillus* strains from agricultural soils.
- Screening of isolated strains with reference to their biocontrol activity.
- To evaluate the potential of screened *Bacillus* strains against selected fungal phythopathogens.
- To analyze the efficiency of screened *Bacillus* strains against selected human pathogens.
- To isolate and characterize the antifungal and antibacterial metabolites produced by *Bacillus* strains using analytical techniques.
- Designing of pilot scale experiment to evaluate biocontrol efficiency of *Bacillus* strains in vivo.

LITERATURE REVIEW

Human population is tremendously increasing with alarming rate i.e. 80 million per year (United Nations, 1996). This inflated population demands food for survival (e.g. more consumption of meat, milk and agricultural products) which is only possible by increasing agricultural products mainly edible crops because food of all living form is directly or indirectly dependent on plants. However arable land is limited and most of the land which has high potential as regard productivity is already under cultivation. In addition cultivated area is shrinking due to water availability and other resources which are depleting continuously (Barnes *et al*., 1995). Sustainable and increased production in yield is required due to these limitations. Crop protection from animal pests, weeds and pathogens is also a major step for providing food and feed of sufficient quality and quantity. Combined effect of hallmarks of green revolution i.e. use of chemical fertilizers for enhancing soil fertility, irrigation and pest control strategies with the help of synthetic pesticides and genetically modified high yielding seeds usage results in maximizing world food production to double in the past 35 years. Global cropland is almost 40% occupied by the three main staple crops namely wheat, maize and rice (Tilman, 1999) which are the major sources for human nutrition. In order to safeguard productivity to the level necessary to meet the demand, these crops have to be protected from pests. The balance between the growing food demand of the world population and global agricultural output is hard to maintain because demands surpasses the supply rate resulting increase in prices (Smil, 2000)(Lang and Heasman, 2015). Consequently strategies like plant protection in general and crops protection against plant diseases in particular were adopted which play an obvious role in meeting the budding demand for food (Strange and Scott, 2005).

2.1: Major soil borne pathogens

The harvesting of crops is extremely effected by soil related pathogens and it's very problematic to cope with this issue. The ability of the pathogens to persist in soil for

many years has made this problem more serious. Soil related pathogens can be explained as those microorganisms that have the capability to cause different infections to plants. The toxin produced by pathogens are usually infected into the host plant through soil (Larkin, 2008). The most common ailments caused by plant related pathogens includes rots that destroy the ground tissues, decaying of seeds, flaccid vascular bundles and roots and crown rots. The yield and quality of major crops such as rice, sugarcane, tomato, weed and wheat is reduced to a larger extent because of these pathogens. (Fiers*et al*., 2012) (Yin *et al*., 2013). It has been estimated that approximately 1300 species of fungi are responsible for producing different infections in crops. The yearly loss in some significant crops for example peas, scraps, nuts, cassava, coconut, sugar beet, sugar cane, potatoes, vegetables and numerous fruit ranges from 11 to 14%.This loss is equal to the loss of 80 billion dollars per year. Viruses are well- known to effect plant and cause a loss of about 41% as illustrated by International Committee on Taxonomy of viruses. Potatoes, tomatoes, hops, avocado and citrus fruits are known to be damaged by 30 viroids. About 16% of loss in crops yield is because of bacterial infections. 100 species of bacteria are reported to cause different diseases in plants.

2.2: Viral infections in plants

Viruses are the microorganisms that are smaller than bacteria and their genetic components include only little DNA strands. Viruses usually live within their host cells and reproduce to increase their number by using the organelles of host. The color and the structural organization of the pants is altered because of viral infections but in some cases these infections do not have profound effect on the physiology of plants. In most of the cases viral infections are transferred form one plant body to another plant body through asexual reproduction methods including grafting etc.Insects, parasitic plant trembles and nematodes are also used as a source of transmission of various viral infections. Host plant seeds containing virus can also be used as a mode of transmission of viral infections.

Overwhelming is a viral infection caused by more than 700 known viruses which have the ability to survive in wide variety of hosts. It has been estimated that about 60 billion

loss in crop yield had occurred during 2002 to 2003 because of viral infections. About 10% to 15% loss in the productivity of crops is because of viral diseases (Abramovitch and Martin, 2004). Cucumber mosaic virus has the broadest host assortment and can cause infections in 1200 species of dicotyledonous and monocotyledonous angiosperms. Such as bean, banana, beats and tabacco etc. (Gallitelli, 2000). Barely Yellow Dwarf virus is responsible to cause infection in 150 species of the Poaceae family which comprises rice, barley and oats etc.

2.3: Bacterial phythopathogens

Bacteria are unicellular and prokaryotic microbe with no definite nucleus. They are facultative parasites of plants. They have the ability to survive independently in plant host cells. In plants stomata are the main sites of entrance for bacteria. The infections produced by bacteria can be transmitted from one organism to another and they are very difficult to be treated. Bacteria are transmitted simply by flapping water, predominantly wind-propelled rain and overhead irrigation. Some other mode of transmission of bacteria are by means of vectors, and contaminated hands and by various tools. Some disease causing bacteria have the ability to cause infection in one or a few host species, however others, such as *Erwiniacarotovora*subsp. *carotovora*, a soft-rotting bacterium, have a broad host range. Bacteria have the capability to rupture the cell wall of plants and produce different infections.

It has been reported that more than 80 species of bacteria along with its subspecies cause diseases in different plants. Such as, *Xanthomonas* species can be a source of about 350 diverse plant diseases (leyns*et al*., 1984). Bacterial blight is produced by *Xanthomonas oryzae*, *Ralstonia* (*Pseudomonas*) *solanacearum*, which affects the productivity of crops (Ray *et al*., 2011). 20.69 to 74.91 % loss in yield of crop is permitted by bacterial disease i.e. Bacterial canker of pepper (Parshad, 2013). It has been estimated that in 2010 losses due to plant infections were approximately $701.2 million. The worth of the crops used in

this assessment was around $4236.5 million, take about a 16.5 percent total crop damage (zanten *et al*., 2014).

2.4: Fungal phythopathogens

The infections caused by the fungi are considered as polycyclic if species have ability to produce many spores at one time.The infection can be considered as monocyclic if fungi infects a new plant in new season. Likewise, the disease causing fungi might similarly be well-defined by the forms of spores they synthesize and the way by which they pierce the plant. After penetration into the host plant, fungi first produces haustorium and then start growing within pant by consuming the cell contents. This is called as necrotrophic feeding. The infections caused by fungi have broad range of symptoms in host cells e.g. chlorotic and necrotic spots, sifting, cankers, smuts, wet or dry decay, cadavers, guts, hollows, coatings, drowning, wilts and pustules.

It has been reported that more than 10,000 species of fungi cause infections in plants (Agrios, 2005).

There are four main classes of fungi producing wide variety of diseases

- **Plasmodiophoromycetes**

  They are responsible for producing club roots of crucifers, various root diseases and potato scab.

- **Oomycetes**

  The fungi of this class cause damping-off of seeds, blight, downy mildews and rust infections.

- **Zygomycetes**

  Theses fungi causes rot diseases in plants.

- **Ascomycetes and Deuteromycetes**

  They cause blights, leaf spots, cankers and fruit spots.

- **Basidomycetes**

  It causes rust and smut infections (Agrios, 2005).

Rhizoctonia solani is present in the soil matrix and more than 2000 species of plants are targeted by this specie (Parmeter, 1970). *Phytophthora capsici* is responsible for causing root rot disease in black pepper (Suprapta, 2012). Roots, stems, shoots, leaves and spikes are the parts of the black pepper plant affected by this disease. Phytopathogenic *Fusarium* fungi cause numerous diseases of small grain cereals. Among these species, *Fusarium graminearum, F.culmorum, F.poae, F. avenaceum* and *Microdochium nivale* (formerly known as *Fusarium nivale*, teleomorph *Monographella nivalis*) are wide range pathogens of wheat and barley, while *F. graminearum, F. moniliforme*, and *F. subglutinans* on maize, cause important productive losses all over the world and are consequently of countless economic rank.

Additional significance of the disease is the production of poisonous products. Plant pathogens, especially fungi produce toxins as aflatoxins released by *Aspergillus* species in corn and other stored products. About 300 toxins of fungi are well understood.

2.5: Strategies for crops protection

There are many approaches which are being used by mankind to regulate plant infections. Crop variation, which includes the disruption of the life cycle of phytopathogens resulting in their reduced accumulation, was previously quoted in the Roman literature, similarly in great civilizations of Asia and Africa. Three crop rotation schemes are being accomplished in Europe by farmers from 29th century. During the first year wheat and barley crop is cultivated, during second year cultivation of oats occurred (in spring) and during the last year soil is set free. In the agricultural history farmers which are resistant towards disease causing microorganisms are usually selected. In 1905, a Britain researcher R.H.Biffer revealed the scientific experience of assortment and the option of resilient refinement. It was shown single gene is responsible for causing resistance in wheat plant as well as different diseases are also controlled by single gene (Jones *et al.*, 1993). Due to the existence of unidentified strains of pathogens many plant defense approaches including resistance breeding has been distorted. Major drawback of this

approach is that as resistance is not caused by a single gene therefore plant loses its fitness (Kawchuk *et al*., 2001). Alternative technique for regulating plant infections was soil solarization. All above approaches are unpretentious and eco-friendly but these means are not so effective. So farmers practices those procedures that are more competent and accessible, and can fight plant infections easily like chemical and biological techniques.

2.6: Chemical control

The word chemical control refers to such synthesized chemical compounds which are toxic in nature have been used to control pests and pathogens (fungal and bacterial) of varied types from centuries. Depending upon the kind of target pathogen chemical control agents are divided into more than one categories i.e. fungicides, bactericides and nematicides respectively (Ronan Le Cointe *et al*., 2016).

The extreme toxicity of few of such chemical agents makes them able to kill the desired pathogenic organisms and are used routinely as disinfectants, sterilants or fumigants. Rest of the members are not as much toxic and play a vital role in agricultural industry by preventing plant diseases caused by fungal or bacterial pathogens.

2.6.1: Properties of an ideal chemical control agent

Nature of a chemical agent with respect to its toxicity level and mode of action is very important in the following ways:

- It should not affect the plant growth by damaging its structure
- It should be nontoxic to animals and humans who are consuming those pants as a food
- Normal microflora of plant and soil (in which the plant is cultivated) should not get affected permanently by the chemical

- It should not persist in the soil as if the chemicals are of persistent nature they will destroy the fertility of soil turning it into a barren land
- It should be chemically stable after degradation or breakdown into its metabolites
- Composition of the agent should restrict the targeted pathogens to develop resistance against it
- The chemical agent should have enough stable nature that it can easily survive in an environment where some other control agents are present, otherwise severe reactions occurs and toxic metabolites would produce that are extensively damaging for crop
- It should have a longer shelf life
- It should be affordable for all levels of community (Ogle, 1997).

2.6.2: Types of chemical control agents used against pathogens

Depending upon the type of target pathogen, nature of chemical control agent will also change accordingly and hence they are divided into three main categories

- Fungicides
- Bactericides or antibiotics
- Nematicides

Fungicides

As the name indicates they are the chemicals used to target fungal pathogens. They are further divided into two main types:

- Protectant fungicides
- Systemic fungicides

Protective fungicides are those which helps to prevent the infections occur on the surface of the plants. They can act against a broad range of fungi. They form a layer on

the surface of plant but as the plants are continuously growing, new parts are formed and old ones are weathered so during the growth period these chemicals are applied several times at regular intervals to get unthoughtful protection.

Systemic fungicides are the ones who transported inside the plant by absorption and kill the fungi. They are designed in a way that their toxicity doesn't affect the plants metabolism. They not only protect the plant from the fungal infections but also able to diminish the existing fungal infections.

The only problem associated with their use is the development of resistance by the fungal pathogens, to avoid this they are categorized in various groups or classes having small differences in their chemical composition. Alternate use of these groups on a same set of crop prevents the resistance development by fungal agents (Mc Grath *et al*., 2014).

Bactericides and antibiotics

Antibiotics are used to control plant diseases caused by bacterial pathogens. They are special as they are generated by some microorganisms, and have the ability to kill the other ones (bacteria).

On the basis of their mode of action they are divided into two main types:

- Bacteriostatic
- Bactericidal

Antibiotics are divided in different classes, few of them act against the cell wall of bacteria known as **penicillins** and **cephalosporins**. Some of them affect the enzymatic activities of bacteria called **lipiarmycins**, **sulfonamides**, **rifamycin** etc. while few have the ability to destroy the cell membrane of bacteria known as **polymixins**. And few of them like **tetracyclines** target the protein synthesis of bacterial cells (Pankey *et al*., 2004).

Nematicides

They are used to control the parasitic nematodes. They are extensively toxic. Few mainly used nematicides includes **carbamate** and **aldicrab** (Anwar *et al*., 2009).

2.6.3: Advantages and disadvantages of chemical control agents

In order to cover a huge range of chemical compounds we use a term pesticide. It includes fungicides, antibiotics, herbicides, nematicides, rodenticides etc. All other except antibiotics are synthetic compounds.

It is a fact that they brought revolution in the agricultural industry but their use is also associated with a wide range of environmental damages (Akhtar *et al*., 2009).

Advantages

Pesticides or control agents are beneficial in the following ways:

Enhanced crop productivity

In order to understand this lets take an example of grain production in India. During the year 1948-49 it is estimated about 50 million tons and after the use of pesticides the yield increases up to four folds and became 198 million tons till the end of 1996-97 (Employment Information: Indian Labor Statistics, 1994). Like India the productivity rate is also increased in many other countries e.g. corn and wheat yields in USA and United Kingdom.

Decrease in crop losses

While talking about the rice crop when it is grown in dry lands, weeds reduce the production by 37-79% and if the proper irrigation conditions are maintained and rice crop is grown in the presence of water than weed attack can affect the yield rate by 28-48%

respectively. So herbicides are responsible to overcome these losses by increasing the yield (Behera and Singh, 1999).

Good quality food production

Fruits and vegetables are categorized as a major components of healthy and nutritional foods. Their high yields after the use of chemical agents increase their availability and hence provide a better quality food to the consumers. It is reported that fruits and vegetables are the major contributors to decrease the risk of many diseases like heart attack, stroke and many others (Lewis *et al*., 2011).

Control of diseases spread by vectors

Let's take an example of a very common and deadly vector borne disease malaria responsible for about 5000 deaths per day. Control of insects (mosquito in this case) helps to reduce the mortality rate (Cooper *et al*., 2007).

Disadvantages

Besides their usefulness, there are many destructive impacts of chemical control agents both on humans and their environment. Few of them are discussed below

Harmful impact on humans

It is reported that many of the pesticides and proven to have very damaging health effects on humans. According to a study pesticides are responsible for about 1 million deaths per year all around the world.

The people involved in the agricultural and industrial set ups get affected directly. Workers working in the industries which are dealing with the production of such chemical compounds at extreme high risk as they are exposed to many toxic solvents and raw materials used in the manufacturing process. In the same way the farmers are also comes in contact with these chemicals easily. All organo-chlorinated compounds are highly damaging as they are not only toxic but persistent in nature and responsible for the

production of many diseases in humans and other life forms e.g. DDT and its metabolites DDE are responsible to damage the population of eagle by thinning of eggs (McCauley *et al*., 2006).

Few chemical control agents are endocrine disruptors, they resembles like the natural hormones of body and their long term exposure can cause various diseases mainly related to hormonal disorders and reproductive disabilities.

Other than direct exposure to such agents life forms are also affected by the indirect contact from them e.g. they can enter in the body of humans or animals who consume contaminated food (fruits and vegetables) etc. (Akhtar *et al*., 2009).

Environmental damages caused by chemical control agents

Water, air and soil all are get contaminated by the use of pesticides or chemical control agents.

Surface water and ground water contamination

Chemical compounds which are used as fungicides or pesticides etc. enter in the water sources by runoff. Rain water is mainly responsible to take such compounds up to lakes, ponds, rivers and ultimately to the sea waters. From where they get entered into the bodies of all water life forms e.g. fish etc. Along with drinking water humans also consume high concentration of these toxic compounds. A survey reveals that 23 different pesticides were present in waters of Puget Sound Basin, US and it is estimated that concentration of pesticides in urban streams is much more as compare to its agricultural counterpart (Edwards, 2013).

Ground water also get contaminated when the pesticides leech out up to the water table present below the upper layer of soil. According to a survey 58% of water used for drinking purpose and drawn from the earth in Bhopal, India is contaminated with OC pesticides. When such chemicals enter in water table they persist there for many years (Skyes*et al*., 2015).

Contamination of air (atmosphere)

Almost all pesticides are volatile in nature, and most of them are applied on crops in the form of spray. This will lead to a serious hazard as the chemical get entered in the atmosphere and remain there for several years. According to study 80-90% of pesticides escapes into the atmosphere by the process of volatilization. Once it enters the atmosphere it is than found in air, rain, snow and fog of the area (Godish *et al*., 2014).

Such contaminated air is when inhaled by different lifeforms and produce serious respiratory complications as these compounds contain chlorine which is fatal for all life forms (Clifford *et al*., 2016).

Soil contamination or infertility

Chemical control agents are applied on the crops to protect them from pathogens and pests and then they entered in soil on which the crop is cultivated or grown. Soil contamination by pesticides is divided into two main categories.

Persistent pesticides who can bio accumulate in the soil and hydrophobic in nature. Examples of such compounds includes endosulfan, heptachlor, DDT, lindane and their metabolites. Due to their persistent nature use of most of them is prohibited.

Other group contain polar pesticides. They are not persistent in nature and move from one place to another by rain or leeching, hence they are main source of contamination for underground drinking water. They include carbamates, herbicides, fungicides and their metabolites (Yang *et al*., 2014).

These compound are also responsible to decrease the soil fertility by killing the indigenous micro flora of soil which is beneficial in several ways. Microorganisms present in soil are responsible to run all major nutrient cycles e.g. nitrogen cycle etc. so their destruction will also leads to nutrient imbalances of soil and make it non fertile. Chemical control agents are also responsible to affect the symbiotic relationships between plant and microorganism like mycorrhizal relationship (between plant and

fungi). One such example is Triclopyr it destroys the mycorrhizal fungi by its toxicity (Edwards, 2013).

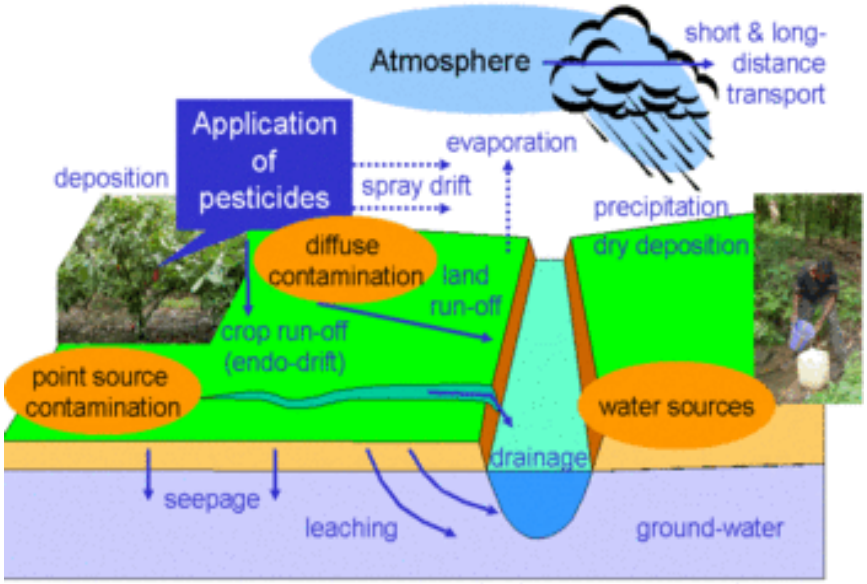


Figure 1: Schematic diagram showing contamination of air, soil and water by chemical control agent

2.7: Biological control

C.F Von was the first who proposed the term biological control in 1914.Biological control or biocontrol both these terms are used widely in different fields of biology most notably in plant pathology where it explains the phenomenon in which microbial antagonists are used to suppress diseases. Bio-control of plant diseases involves the use of an organism or organisms to inhibit the pathogen and reduce disease (Nega, 2014). There are many definitions of bio control however the basic idea involves a sustainable eco-friendly strategy for reducing disease incidence by direct or indirect manipulation of

microbes (Shurtleff and Averre, 1997). This strategy is considered as sustainable, ecofriendly and has efficient potential over chemical control strategy which poses serious hazards to environment and human health (Heydari and Pessarakli, 2010). The active soldiers of biocontrol strategy or the organisms through which suppression of pathogens is made possible are known as Bio Control Agents (BCA). Microbial word possesses a huge diversity that is why it provides endless resource for this particular purpose. This strategy is more reliable because of the complex interactions between organisms, adaptations of bio control agents to different harsh conditions and environments and variety of mechanisms of disease suppression used by antagonists. Bio control of plant pathogens through BCAs has several advantages over chemical control and traditional methods like self-perpetuation (Jong-Chol*et al*., 1987) lack of adverse side effects and lack of resistance in plant pathogens (Mathew *et al*., 2014). Reported bio control agents are from different domains of microorganisms like they may be fungal, viral or bacterial.

2.7.1: BCAs

Microorganisms from different domains are reported as BCAs and they are isolated, identified and used in laboratory and green house for research and experiments. The bio control agents can be insects, fungi, viruses and bacteria (Elad *et al*., 1996).

2.7.2: Fungi as biocontrol agent

Among fungal domain *Trichoderma* specie is considered as the most promising bio control agent against phythopathogenic fungi because of its broad antagonistic spectrum and production of specialized extracellular enzymes. Trichoderma species show high level of genetic and ecological diversity that is why it can be used in variety of commercial products of ecological interest. Several strains of Trichoderma genus have been registered as bio fungicides commercially. Strains like T. harzianum, T. viride, T. atroviride, T. polysporum, T. asperellum, T. gamsii, etc. have been found very efficient and declared as promising bio control agents as well as associated with plant growth promotion. Trichoderma strains show variety of mechanisms to suppress pathogen in

order to eradicate diseases like hyper parasitism, competition for nutrients and production of enzymes. *Penicillium* and *Gliocladium* are also used as BCAs. Due to some properties like their nontoxic nature for environment and mammals, no adverse effects on microflora and vast spectrum with respect to action Trichoderma genus is accepted as efficient and preferable bio control agent.

2.7.3: Bacteria as biocontrol agent

BCA from bacterial domain for laboratory purposes or green house experiments have several advantages over fungal counterpart like their proliferation rate is high, short time is required to show optimized results because of low generation span. Plant diseases whether fungal or bacterial can also be controlled biologically by exploring bacterial BCAs (Gerhardson, 2002).BCAs from this domain mainly includes *Pseudomonas* spp. (Whipps and Lumsden, 2001*), P. putida* (Scher and Baker, 1982). *Pseudomonas fluorescens*, Actinomycetes spp. (Ikotun and Adekunle, 1990), *Agrobacterium radiobacter* (Powell *et al*., 1990), *Bacillus* spp. (Ikotun and Adekunle, 1990). Apart from controlling diseases majority of bacterial BCAs are considered as plant growth promoting bacteria (PGPB). They colonize the rhizosphere consequently antagonize the pathogen by multiple modes of action and causes disease suppression. Competition for substrate and production of inhibitory compounds are the two main recognized mechanisms of actions. Some bacterial strains are responsible for the production of multiple antibiotics which in result suppresses the pathogenic ability of pathogen (Haas and Defago,2005) (Stein, 2005).

Among bacteria *Bacillus* spp. is considered as the most efficient, beneficial and promising BCAs (Stein, 2005). Origin of the first commercially prepared bio pesticides used against pathogens and insects also prepared from genus *Bacillus*. *Bacillus thuringiensis* (Bt) accounts for over 90% of all marketed bio-insecticides and represents a worldwide market of $110 000 000 annually for control of insects. *Bacillus* spp. are considered as safe and convenient to be used as BCAs because of the formation of

endospores and survival in harsh conditions like extreme pH, high temperature and chemical stress (Schisler *et al*., 2004).

2.8: *Bacillus* based biological control of plant pathogens

Any Rod shaped, gram positive, aerobic or facultative anaerobic and endospore forming bacteria falls into genus *Bacillus* which is very diverse group of bacteria including both beneficent and pathogenic bacteria. As according to Bergey's manual 90 different species are reported till 2000 (Logan *et al*., 2009). The genus *Bacillus* is classified into six major groups on the basis of biochemical, physiological and morphological properties. Among *Bacillus* genus *Bacillus subtilis* is studied well and considered as model organism for research in different fields of biology (Earl *et al*., 2007). The genome of *Bacillus subtilis* strain 168 was sequenced for the first time in 1997 which was consist of 4214810 base pairs with protein coding genes numbered as 4100 (Wipat and Harwood, 1999).

Bacillus is ubiquitous i.e. occupy all types of environments including harsh conditions having primary habitat soil where they are found on the surface of the soil, aerial parts of plants and also associated with rhizospheric region of plant roots. They form complex system with other microbial communities where they help in protecting plant. They are also present in water, air and aerosols (Shoda, 2000).

Various characteristics increase the importance of *Bacillus* to be used on large scale in industry like:

- High proliferation rate
- Short generation cycle
- Potential of protein production
- And the most important one is that they are qualified as GRAS (generally recognized as safe) by the FDA

It is used in the production of antibiotics, food supplements, enzymes, fungicides and insecticides (Schallmey*et al*., 2004). 50% of the enzymes are prepared from *Bacillus*

subtilis and related species on large scale. Being a spore former *Bacillus* is used as probiotic like lactobacillus (De Vecchi and Drago 2006).

2.8.1: *Bacillus* as a BCA

Gram negative bacteria like *Xanthomonas, Aerobacter, Flavobacterium, P. flourescens, P.agglomerans* etc. are the main microbiota of plants however the amount of gram positive colonists are less as compared to gram negative (Cook and Baker, 1983). From plant microbiota strains mainly belong to the species *P. agglomerans, P. fluorescens* and *Bacillus* have been reported as BCAs. Several BCAs have potential to control plant pathogens like *P. fluorescens* and *Bacillus subtilis* which are used in control of blight diseases of apple but some of them especially gram negative bacteria have formulation and biosafety issues as they pose health hazards to human beings. For this reason, interest develop in other bacterial groups which are recognized as safe and have the potential to be used as BCAs like species from genus *Bacillus*. *Bacillus* species are selected to be used as BCAs for the following reasons:

- Safety to human beings (Earl *et al*., 2008)
- Wide distribution (Emmert and Handelsman 1999)
- Ability to survive in harsh conditions (Kloeppe*et al*., 2004)
- Recognized as PGPB (Montesinos, 2007)
- Synthesis of compounds which are beneficial for agronomical use (Ongena and Jacques 2008)
- Fast proliferation rate
- Short generation time

Bacillus spp. are of quiet importance in agronomy because they have the potential to be used as biocontrol agents against variety of fungal and bacterial phythopathogens. They are strong antagonists and this property made them different from all other counterparts. They offers a strong contribution to environmental conservation as they are more efficient to suppress plant diseases as compared to agrochemicals (Darko *et al*., 2008). In

last decade several strains of *Bacillus subtilis* have been commercialized against fungal phythopathogens like BS GB03 which was quiet efficient against *Rhizoctonia* and *Fusarium spp.* B. *subtilis* QST713 have the potential to control *Plasmodiophora brassicae* (Lahlali*et al*., 2011). Majority of members of this genus are beneficial and exploited mainly as biopesticides to halt plant diseases. Use of *Bacillus* as insecticide was start in 1938 i.e. *Bacillus thuringiensis* which accounts for more than 70% sale and the activity of which is mediated by the formation of Cry toxins which are highly toxic to insects as they made their intestinal gut perforated and eventually cause death (Cawoy*et al*., 2011). Majority of the commercially available bacterial biocontrol agents are *Bacillus* based (about 50%) (Fravel, 2005). Notable *Bacillus* BCAs are described below

- *Bacillus pumulis* GB34
- *Bacillus licheniformis* strain SB3086
- *Bacillus subtilis* strain GB03
- *Bacillus subtilis* strain QST713
- *Bacillus amyloliquefaciens* strain FZB42

Bacillus has special features due to which they are appealing candidates as BCAs. Some remarkable characteristics are discussed below:

- Production of bioactive compounds which are valuable for agricultural applications
- Production of different metabolites with antimicrobial activity and surface active properties
- Generation of compounds which were implicated in the induction of plant defense mechanism
- Used as plant growth promoting bacteria
- Use in the production of potent antibiotics

2.9: Mechanism of action

Rhizospheric region of plant is occupied by diverse group of microorganisms and they have a complex interaction. *Bacillus* control plant pathogens by variety of mechanisms, for efficient results and to establish it as a successful BCA it is necessary to understand these mechanisms. Following are the main mechanisms:

2.9.1: Hyperparasitism and predation

In hyperparasitism the pathogen is directly attacked by BCA. Hyperparasitism is performed by hyperparasites which is divided into four major classes:

- Predators
- Obligate bacterial parasite
- Facultative parasite
- Hypoviruses

Some BCAs are predators under specific conditions like nutrient deficiency but this behavior is not observed in normal growing conditions like few species of *Trichoderma* have the ability to generate wide range of enzymes which act directly on the fungal cell wall but when fresh bark is used as a substrate they do not attack directly on the phythopathogen like *Rhizoctonia solani* because available cellulose content is enough but as the bark getting decomposed resulting in the deficiency of cellulose then *Trichoderma* spp. switch on their genes to produce chitinases for getting cellulose consequently parasitize R. solani (Benhamou and Chet, 1997). Obligate bacterial parasite includes *Pasteuria penetrans* which is active against root knot nematodes and used as bio control agent. Hypoviruses are also include in the hyperparsites category (Milgroom and Cortesi, 2004).

Some microorganisms secrete and excrete metabolites which interfere with the growth or other activities of the pathogen. Some microbes have the ability to produce specialized lytic enzymes which have the ability to hydrolyze polymeric compounds like cellulose,

hemicellulose, proteins, chitin and DNA. Secretion of these enzymes made possible disease suppression. Potential microbes capable of lysing phythopathogens might be classified as BCAs. For example *Serratia marcescens* used as BCA against *Sclerotium rolfsii* is because of chitin production (Ordentlichet al., 1988).

2.9.2: Competition

Microbe must be efficient enough to compete for nutrients for successful colonization in photosphere. For halting disease severity, competition between pathogen and non-pathogen for nutrient is necessary in which non pathogen is the winner. Damaged plant tissues, exudates and leachates are the host supplied nutrients on plant surfaces. Nutrients can also be obtained from waste products of other organisms. Nonpathogenic plant root associated microbes are in abundance and they protect plant by finishing the nutrients available and rapid colonization resulting in the unfavorable conditions for pathogen proliferation (Kageyama and Nelson, 2003). Competition for micronutrients like iron has also been examined. In the rhizosphere iron concentration is extremely limited and is highly dependent on pH of soil. Iron is present in ferric form in highly aerated soil and is insoluble in water and the concentration is too low to support the growth of microorganisms. For survival in that type of environment iron binding ligands known as siderophores were secreted by microbes which have affinity to confiscate iron from the environment. Commensals microbes have high efficiency of iron uptake and this is the main factor that they colonize aggressively the rhizospheric region and help in the displacement of harmful microorganisms from potential sites of infection (Haas and Defago, 2005).

2.9.3: Pathogen induced resistance

Temperature, light, water, gravity, physical stress and nutrient availability are the environmental stimuli to which plants respond. Plants also respond to different stimuli of chemical nature produced by microbes. These chemical stimuli have the potential to induce plant defense by enhancing resistance to pathogens. Such resistance mainly

depends on the amount, type and source of stimuli. Biochemical pathways used for induced resistant by BCAs are characterized by plant pathologist. Systemic acquired resistance is the first of these pathways which is interceded by salicylic acid. Production of SA is related to pathogen infection i.e. its production is initiated when infection occurs and then expression of PR (pathogens related) proteins is initiated which have different enzymes and some of which are responsible for lysis of invading cells, strengthen boundaries of cell wall to resist infections or initiate death of localized cell (Numbergeret al., 2004).Second pathway is known as induced systemic resistance which is arbitrated by ethylene or jasmonic acid.Trichoderma and Pseudomonas so are the biocontrol agents which induce plant host defense (Haas and Defago, 2005).

2.9.4: Quorum quenching

Quorum quenching is also known as quorum sensing inhibition and it is recently described mechanism to control phythopathogens. In this mechanism bacterial virulence is weakened by the inactivation of quorum sensing molecules. Quorum sensing is actually the cross talk mechanism of microorganisms which is helpful in the adaptation of specific strategy. Pathogenic bacteria also use this mechanism to regulate expression of virulence genes. Quorum sensing is performed by numerous small molecules known as quorum sensing molecules which are very diverse with respect to structure and morphological appearance.N-acyl-L-homoserine lactones (AHLs) are the most common signaling molecules among pathogenic bacteria (Yadeta and Thomma, 2013). Some *Bacillus* species have the abilty to produce AHL lactonase which catalyze hydrolysis of the AHL ring like *B. thringiensis* suppresses plant pathogen *Erwinia carotovora* which is dependent on quorum sensing molecules for proliferation (Dong *et al.,* 2004).

2.9.5: Metabolites production

Majority of BCAs are from bacterial domain and they are able to secrete secondary metabolites which have antibiotic activity. Secondary metabolites vary in size from low molecular weight compounds to high ones and they act at low concentration consequently

effect the growth or metabolic activity of other microbes (Fravel, 1998). BCAs produce variety of antimicrobial compounds as regard to structure, size, nature and performance potential. Antibiotic production is directly proportional to the stress conditions provided to organism and it is also reported that more than one kind of antibiotics are also produced by single microorganism at the same time (Keel *et al.,* 1989). Among all bacterial BCAs *Bacillus* species are considered as the best secondary metabolite producers with efficient antibiotic activity (Ongena and Jacques, 2008).

2.10: *Bacillus* used as a source of antimicrobial peptides

Bacillus species plays a dominant role in the manufacturing of biologically active substances that restrain the growth of plant pathogens. *Bacillus* species have vast genetic diversity for synthesising compounds (like polyketides, non-ribosomal peptides) that have an antibiotic activity e.g. nine gene clusters have been recognized in *B. amyloliquifacians* which are responsible for the synthesis of many beneficial enzymes i.e. non ribosomal peptide synthatase and polyketide synthatases.

Antimicrobial peptides are industrially active compounds. They have tremendous applications in biotechnology and pharmaceutical industries because of their low density, hydrophobicity and rigorousness. *Bacillus* species undergo the manufacturing of antimicrobial compounds by two pathways:

- Ribosomal synthesis of peptides
- Non ribosomal synthesis of peptides

Ribosomal peptide manufacturing

Bacillus species have a potential to undergo the production of biologically active compounds synthesized by peptides i.e. bacteriocins. Bacteriocins are categorized into three divisions according to their structural properties and biological functioning.

Class 1 bacteriocin

They are also known as lantibiotics. They are distinguished by the presence of unusual amino acid residues e.g. lanthionine, methyllanthionine etc. Lanthionine production usually take place by post translational alteration of substances that are ribosomally manufactured including dehydration of serine and threonine deposits.

Class 2 bacteriocin

In contrast to lantibiotics these peptides are non-modified. These peptides have low density and are manufactured ribosomally.

Class 3 bacteriocin

These peptides are of very high density. They are not heat resistant.

More precisely lantibiotics are the molecules with relatively low density. They can be inactivated by the action of proteolytic enzymes. On the basis of structural and functional abilities lantobiotics are divided into two groups A and B.

Type A lantibiotic

They exhibit secondary structure. As they are positively charged they have the capability to act against Gram-positive marked cells. They consist of substilin and ericine. Type A lantibiotics have different protective mechanisms e.g. interference with formation of lantibiotic lipid pore.

Type B lantibiotic

They do not exhibit any charge. They have a globular structure. They usually consist of sublancin, subtilosin and mersacidine.

Subtilin

The most advanced lantibiotic produced by *Bacillus* spp. is subtiline.32 amino acid residues are present in its structure. It has the capability to produce cationic peptide pores

and target the Gram positive cells for antimicrobial activity. Its genetic diversity is responsible for the synthesis of many proteins. spaK and spaR are the two governing components that binds to the motif of DNA and stimulates the expression of genes used for subtilin production.

2.11: Antibiotics from non-ribosomal peptides

Non-ribosomal peptide synthase (NRPSs) is an enzyme used for the manufacturing of non-ribosomal peptides. NRPSs are structured in the form of segments, and they are liable for integration of definite amino acids. NRPSs comprises of three key domains (Fickers, 2012). These three domains catalyse precise reaction for integration of specific units.

- **Adenylation domain (A)**

There are 550 amino acids in this domain. It plays a key role for stimulating the amino acid as amino acyl adenylate (Ongena and Jacques, 2008).

- **Thiolation domain (T)**

There are 80 amino acids in this domain. With the help of phosphopantethienyl group it covalently fixes into the stimulated monomer to the synthetase (Stein, 2005).

- **The Condensation domain (C)**

450 amino acids are present in this domain. It accelerates the formation of peptide bond between the actuated amino acids from two neighboring T segments (Felnagle *et al.*, 2008).

Above three specified domains support NRPSs to manufacture a notable number of specialized structures with wide variety of biological activities that could not be acquired from ribosomal machinery (Stein, 2005).

2.11.1: Surfactins

The composites of surfactin family have fundamental bio surfactant manufacturing ability with lathering and emulsifying possessions. Members of surfactin are amphiphilic in nature. They have capability to embed into the layers of lipids and can hinder with integrity of biological membrane. There are four enzymatic subunits of synthtase complex i.e. *srf*A, *srf*B, srfC and *srf*D. Synthetase complex stimulates the manufacturing of Surfactin molecules e.g. acyltransferase *srf*D helps in the initiation reaction of surfactin production. The initiation reaction comprises the transmission of beta hydroxy fatty acid substrate from coenzyme A to the initial segment of srfA (Steller *et al*. 2004). The production of surfactin is dependent upon the stages of its growth. During stationary phase production of surfactin is persuaded. According to many reports the synthesis of surfactin also proceeds during initial phases of sporulation.

2.11.2: Iturins

Iturin family usually consist of Iturin A, C, D and E, bacillomycin D, F, and L, bacillopeptine and mycosubstilin (Ongena and Jacques, 2008).The chain length of iturins comprises 14 to 17 carbon atoms having beta amino fatty acids (Raaijmakerset*et al*., 2010).The adherents of Iturin family have hemolytic properties. They strongly act in contradiction of a variety of fungi and yeast, but have inadequate antibacterial and antiviral activities (Romero *et al.*,2007).The ability of iturins to act against fungi depends upon the permeability of their membranes. The structures of mycosubstilin and itrucin are almost similar but last two amino acid in mycosbtilin are reversed in itrusin A. Therionin amino acid is present at the terminal end of bacillomycin (Thasana *et al*., 2010).

2.11.3: Fengycins

Fengycin chain length consists of 14 to 18 carbon atoms with a beta hydroxy fatty acid group. Fengycin are cLP with an interior lactone ring in the peptide moiety (Arguelles-Arias *et al*., 2009). The structure of fengycins is either cyclic, branched or contain unusual constituents like existence of ornithine or four D amino acid. As compare to

surfactin and intrucin structure, the existence of fengycin is of broad range but structural variety is quite low. There are two types of fengycin illustrated as fengycin A and fengycin B. These two structures only fluctuate at the amino acid residue of the 6 position. Fengycin A has alanine at 6 position while fengycin B has valine at 6 position respectively. Fengycin has a strong antifungal activity especially against filamentous fungi. NRPSs with five ORF fen A-E are responsible for the manufacturing of fengycins.

2.11.4: Bacilysin

One of the simplest peptide antibiotics well-known includes bacilysin. This antibiotic is a dipeptide comprising L-alanine residues at N terminal end and L-anticapsin at the C terminal end. Bacilysin have antibacterial and antifungal activity. It undergo its activity by anticapsin moiety which lumps the glucosamoine synthase during its uptake in the respective cells.The manufacturing of bacilysin is not related to its sporulation phase (Yazgan *et al.*, 2001). *bac*ABCD gene cluster is tangled in the synthesis of bacilysin.

MATERIALS AND METHODS

The current research was performed at Microbiological Research laboratory (MRL), Department of Microbiology, Quaid-i-Azam University Islamabad.

3.1: Sampling Sites

Soil samples from five different fertile lands were collected. First Sample was taken from agricultural soil of Swabi district where edible and cash crops are grown like wheat, maize, tobacco, watermelons and vegetables. Collection of second sample was done from the bio lawn of Quaid-i-Azam University Islamabad. Third sample was collected from agriculture soil of Sialkot District where rice was grown annually. Collection of fourth sample was done from Abbottabad district where maize crop is cultivated and the final sample was poised from Dera ghazi khan district the soil of which is important for the cultivation of cotton.

Table 3.1: Sampling sites with description of crops grown and sampling duration

S.no	Sampling Sites	Crops Grown	Time
01	Swabi District KPK	Wheat, maize, tobacco, watermelons and vegetables.	June 2016
02	Bio-lawn QAU.	Not used for crop cultivation	April 2016
03	Sialkot district Punjab	Rice paddies	August 2016

04	Abbottabad district KPK	Maize	March 2016
05	Dera ghazi khan district Punjab	Cotton fields	July 2016

3.1.1: Sampling time

All these samples were collected from rhizospheric region of seasonal crops in sterilized zipper bags of 50gm and shifted to MRL Quaid-i-Azam University Islamabad till March 2015.Temprature and moisture was favourable according to crop requirement.

3.2: Sample processing

MSM was prepared for the soil enrichment. One gram of soil was taken from each sample and is poured into autoclaved MSM which was present in 100 ml flasks. These flasks are then incubated in shaking incubators at 37˚C with RPM fixed at 150 for 72 hours. After enrichment serial dilution is performed. Recipe of MSM is given below.

Table 3.2: Components used in MSM enrichment broth

Macronutrients	**Concentration (g/lit)**
K2HPO4.2H2O	10
NaH2PO4.2H2O	5
NaNO3	2
CaCL2.2H2O	0.01
MgSO4.7H2O	0.2
FeSO4.7H2O	0.08
GLUCOSE	20

Table 3.3: Micronutrients used in MSM

Micronutrients	**Concentration (mg/lit)**
MnSO4.4H2O	0.80
ZnSO4.7H2O	1.40
CoCl2.6H2O	1.20
CuSO4.5H2O	1.20

3.3: Serial dilution

36 test tubes were autoclaved i.e. 9 for each sample. Following the proper procedure of serial dilution 10ml normal saline was poured in first test tube and the rest of 8 test tubes were filled with 9ml normal saline. 1ml enriched sample was added in first test tube and is mixed well. Then one ml mixture was taken from 1st test tube and poured into second one. After mixing properly one ml again was taken and shifted to third one and mixed properly. Same method is followed up to 9th test tube of the last sample i.e. 36th one.

3.3.1: Growth medium

For the isolation of only *Bacillus* strains from soil we used commercially available differential media i.e. Hi Chrome *Bacillus* Agar.720 ml *Bacillus* agar was prepared in 1000 ml flask and after autoclaving was poured in 36 autoclaved Petri plates inside safety cabinet such that each plate contains 20 ml media.

Table 3.4: Composition of Hi Chrome *Bacillus* Agar

INGREDIENTS	**g/lit**
Agar	15.0
Peptic digest of animal tissue	10.0

Meat extract	1.0
Sodium chloride	10.0
Chromogenic mixture	3.2
D-Mannitol	10.0
Phenol Red	0.025

3.4: Spreading

When media was solidified 4 stakes of 9 Petri plates was made and named as 1st, 2nd, 3rd and 4th stake for described samples.1st stake was labelled properly from 1 to 9 and on each plate 0.5 ml sample taken from first set of test tubes was spread using spreader in a proper order so that 1st plate was spread by the sample taken from 1st test tube and so on. When spreading of all samples was completed then each stake was wrapped and incubated at 37˚C for 24 hours.

3.5: Isolation from processed samples

After 24 hours' incubation we observe dense growth of *Bacillus* on all 36 plates. Different types of colonies were observed with different morphological appearance, colour and growth pattern. These colonies were sub cultured on freshly prepared autoclaved *Bacillus* agar and incubated for 24 hours to obtain pure colonies. After 24 hours pure strains show vivid growth on *Bacillus* agar plate.

3.6: Initial Screening

All strains were screened out against selected bacterial and fungal pathogens whether they have the potential to control their growth or not. For this purpose, 3 bacterial

pathogens ATCC strains are sub cultured on nutrient agar plates inside safety cabinet and incubated for 24 hours at 37 degree Celsius. Their spore suspension is prepared by taking wire loop half full of culture from each of it in 1ml normal saline taken in autoclaved Eppendorf tubes after 24 hours and compared with McFarland standard solution. Spore suspension of each pathogen was spread on 5 nutrient agar plates by cotton swabs and 3 *Bacillus* strains are point inoculated on each plate with a sufficient space to check and measure zones. The plates are then incubated and results are observed after 24, 48, 72 and 96 hours.

To check antifungal abilities of the strains they are screened against selected fungal phythopathogens provided by plant pathology laboratory NARC.3 fungal plant pathogens are sub cultured on SDA plates by point inoculation method and after 3 days of incubation their spore suspension is prepared by taking wire loop half full of culture from each of it in 1ml normal saline taken in autoclaved Eppendorf tubes and compared with McFarland standard solution. Spore suspension of each fungal pathogen was spread on 5 SDA plates by cotton swabs and 3 *Bacillus* strains are point inoculated on each plate with a sufficient space to check and measure zones. These plates are then incubated and observed after 48, 72, 96, 120 and 144 hours.

3.6.1: McFarland standard preparation

1.17% w/v $BaCl_2.2H_2O$ and 1% w/vH_2SO_4 was used for the preparation of McFarland standard solution. It was prepared by adding 0.5ml of $BaCl_2$ into 99.5mL of H_2SO_4 with proper stirring. This McFarland standard was used for comparison of turbidity of spore suspension.

3.7: Morphological and biochemical identification of selected strains

Microscopic analysis of selected strains was performed by making their smears on slides. Then they are gram stained. For gram staining crystal violet dye was poured over slide and then waits for 60 seconds and washed with distilled water. Gram iodine was than flooded onto the slide and wait for 60 seconds. Then ethanol was poured over slide and

washed quickly with distilled water. Finally safranin was flooded over smear and wait for 60 seconds. Then smear was dried and observed under microscope.

3.7.1: Biochemical identification

Following biochemical tests were performed

Catalase test

Catalase test was performed to check the catalase enzyme production ability of selected strains. For this purposed single colony was picked from one day old culture with wire loop and placed on glass slide. Then one drop of 1% hydrogen peroxide was placed on the culture and observed for bubble formation.

Oxidase test

Cytochrome oxidase enzyme production was identified with this test. To perform this test small size filter paper was taken and then soaked into1% Kovacs reagents. Then filter paper was allowed to dry. Colony from one day old culture was picked and rubbed onto the filter paper. Observe for colour changes.

Citrate test

To check the ability of selected strains for utilization of citrate as a sole carbon and energy source this test was performed. Simmon citrate agar was prepared and slants were made in test tube. With the help of wire loop selected strains were streaked onto the slants and incubated for 24 hours at 37 degree Celsius. After incubation colour change was observed.

Indole test

Indole test was used to determine the ability of selected strains to degrading amino acid tryptophan into indole. Tryptone broth was prepared and strains were inoculated and kept for 24 hours at 37 C. 5 drops of Kovacs reagent was added after incubation. Observe the colour change.

Nitrate reduction test

Nitrate reduction test was performed to check the ability of selected strains to reduce nitrate or not. Strains were inoculated into nitrate broth and incubated at 37 C for 24 hours. After incubation period one drop of sulfanilic acid and one drop of α-naphthylamine was added and observed for color change.

3.8: Enzymes Assays

Following enzymes assays were performed

3.8.1: Endoglucanase Assay

Cellulose producing ability of strains was determined by this assay. Media used was prepared by adding 1% carboxy methyl cellulose into 100 ml nutrient broth. From fresh culture isolated colonies were picked and point inoculated at the center of plate containing media. Plates were incubated at 37 ˚C for 24 hours.

3.8.2: Protease assay (Casein Hydrolysis)

Plates were prepared by pouring 1% casein agar. Strains were inoculated at the centre with the help of wire loop. Plates were incubated for 24 hour at 37 degree Celsius. After incubation plates were flooded with 70% acetic acid and zone of hydrolysis were observed.

Table 3.5: Composition of Casein Containing medium

Agar	15 g
Casein	10 g
Peptone	5 g
Beef extract	3 g
Distilled water	1000 ml

3.8.3: Amylase assay (starch Iodine test)

Amylase assay was performed to check the starch utilizing ability of strain. With the help of wire loop isolated colony was picked and inoculated at the canter of plate. Then plates were incubated at 37 ˚C for 48 hours. After incubation plates were flooded with iodine reagent and observe for zone of hydrolysis.

Table 3.6: Composition of Starch Medium

Agar	15 g
Beef Extract	3 g
Starch	2 g
Peptone	5 g
Distal water	1000 ml

3.9: Preservation of Pure Culture

By sub culturing technique pure culture was obtained. The culture was then maintained on tripton agar slant for short term at 4^{o}C. 30% glycerol was used for long term preservation and stored at -20^{o}C.

3.10: Temperature optimization

Temperature optimization was done to find the optimum temperature at which strains show maximum activity and growth. Strains were grown into nutrient broth and incubated at four different temperatures 10^{o}C, 20^{o}C, 30^{o}C, 40^{o}C at 150 rpm in shaker incubator. After each 24 hour interval OD values were recorded for successive five days**.** Antifungal activity was checked by agar well diffusion method. After incubation zone of inhibition were measured.

3.11: Inoculum optimization

For inoculum optimization loop full of inoculum was inoculated into 50 ml nutrient broth flask and kept for 24 hour at 150 rpm in shaker. 1 ml, 2 ml and 3 ml of inoculum was further transferred to 100 ml nutrient broth in order to obtain 1%, 2% and 3% concentration respectively and incubated for 72 hour at 150 rpm in shaker. The % inoculum was used to determine the antifungal activity against various plant pathogens via agar well diffusion method. The results were recorded by measuring zone of inhibition against pathogens

3.12: Final Tests of the Strains to be used as Biocontrol Agent

Potential of the strains to be used as biocontrol agent against 5 bacterial and 8 fungal phytopathogens was checked by two different methods.

3.12.1: Dual culture method

In this method as the name indicates both cultures are cultured on the same plate. This method was used in two different ways

3.12.2: Central Point Inoculation

Fugal phythopathogens were spread on SDA plates by making their spore suspensions in 1ml normal saline and *Bacillus* strains were inoculated in the center of plate using wire loop.

Bacterial pathogens were spread on nutrient agar plates by making their spore suspensions in 1ml normal saline and *Bacillus* strains were inoculated in the center of plate using wire loop.

3.12.3: Spot inoculation

This method was used for fungal phythopathogens only. In this method fungus and *Bacillus* were inoculated on SDA plate by using wire loop at a distance of 1cm.

3.12.4: Cell Free Culture Test

In this method cell free supernatant was applied to check the efficiency of *Bacillus* strains. For this purpose 2 flask of 100 ml containing 40 ml nutrient broth was autoclaved. After cooling selected strains were inoculated in each flask and incubated in shaking incubator at 37˚C with RPM fixed at 150 for 72 hours. Then 20 ml broth containing culture was taken from each flask in centrifuge cups and centrifuged at 10000 RPM for 20 minutes. After centrifugation pellet settled down and cell free supernatant was applied on plates adapting two methods.

3.12.5: Well methods

In this method wells were made in the center of 5 nutrient agar plates using sterilized borer of 5 mm radius. Spore suspension of 5 bacterial pathogens were prepared in 1 ml

normal saline and spread on plates containing wells which were filled with cell free supernatant with the help of pipette.

This method was also applied for fungal strains in this case wells were made in the center of 8 SDA plates using sterilized borer of 5 mm radius. Spore suspension of 8 fungal phythopathogens were prepared in 1ml normal saline and spread on plates containing wells which were filled with cell free supernatant with the help of pipette.

3.12.6: Disc diffusion method

In this method discs were made from filter paper using paper borer with a diameter of 4mm and autoclaved. These discs were then dissolved in the supernatant for 3 to 5 minutes and placed in the center of 5 nutrient agar plates and 8 SDA plates already spread with the spore suspension of bacterial and fungal pathogens respectively. After positive results of these tests we extracted the metabolites of selected strains.

3.13: Metabolite extraction

After observing efficient results of cell free supernatant we extracted the metabolites produced by both of these strains in order to check their nature. For this purpose, several steps are performed

1) Strains were inoculated in two separate 100 ml autoclaved flasks containing 50 ml nutrient broth. Inoculation was done using wire loop.
2) After 24 hours 1% culture (0.50 ml) was taken from each flask with the help of pipette and inoculated in 1000 ml autoclaved flasks containing 500 ml nutrient broth and incubated in shaker incubator for 72 hours at 37˚C with RPM fixed at 150.
3) After 72 hours' broth was poured in centrifuge cups and centrifuged at 10000 RPM for 20 minutes.
4) After centrifugation supernatant was collected in separate sterilized dry bottle and pellet was collected from all centrifuged cups and stored at 4˚C for further use.

5) Equal amount of organic solvent i.e. ethyl acetate (optimized) was mixed with supernatant and shake properly.
6) The mixture was then poured into separating funnel. After 12 hours two layers are with vivid appearance. Lower layer was separated by opening the knob of separating funnel and discarded.
7) Upper layer was poured in sterilized open mouth vessel like beaker which contains organic solvent in which lipids and other organic materials of strains were dissolved.
8) Beaker was then placed in fume hood and organic solvent used was volatile in nature so it evaporates and at the end we extract small amount of metabolite stick to the walls of beaker which was dissolved in methanol and stored in falcon tube kept at 4˚C.

3.14: Tests for metabolites

Metabolites obtained from strains were also screened whether they had the potential to be used as bio control agent or not. For this purpose, disc diffusion and well methods were used. But in this case discs were dissolved in metabolites and wells were filled with metabolite dissolved in methanol

3.14.1: Well method

In this method wells were made in the center of 5 nutrient agar plates using sterilized borer of 5 mm radius. Spore suspension of 5 bacterial pathogens were prepared in 1ml normal saline and spread on plates containing wells which were filled with metabolite dissolved in methanol with the help of pipette.

This method was also applied for fungal strains in this case wells were made in the center of 8 SDA plates using sterilized borer of 5mm radius. Spore suspension of 8 fungal phythopathogens were prepared in 1ml normal saline and spread on plates containing wells which were filled with metabolite dissolved in methanol.

3.14.2: Disc diffusion method

In this method discs are made from filter paper using paper borer with a diameter of 4mm and autoclaved. These discs are then dissolved in metabolite (dissolved in methanol) for 3 to 5 minutes and placed in the center of 5 nutrient agar plates and 8 SDA plates already spread with the spore suspension of bacterial and fungal pathogens respectively.

3.14.3: Fourier transform infrared spectroscopy (FTIR) of strain AK-47 metabolite

In order to check the nature of metabolite extracted from strain AK-47 FTIR was performed.

3.15: Extraction of Antifungal Protein through Ammonium Sulphate Precipitation

To check the nature of metabolite where they are proteins in nature or not we precipitated proteins present in supernatant. Precipitation of proteins was performed by the following steps

1) Strains were inoculated in 250 ml autoclaved flasks containing 100 ml nutrient broth and then incubated at 37 ˚for 72 hours in shaking incubator with RPM fixed at 150.
2) After 72 hours both cultures are poured in centrifuge cups inside safety cabinet and centrifuged at 10000 RPM for 30 minutes.
3) After centrifugation supernatant was poured in 250 ml sterilized dry flask containing magnet.
4) Flask was kept in plastic bowl surrounded by ice bags. Plastic bowl was placed on magnetic stirrer.
5) Magnetic stirrer was turned on and the RPM was adjusted on 40.

6) Then ammonium sulphate already measured according to online ammonium sulphate calculator was added slowly till 90 minutes.
7) Stirring continues till next 20 minutes and then the flasks were kept at 4c for 16 hours.
8) On next day we centrifuged that supernatant again at 10000 RPM for 30 minutes.
9) Supernatant was separated and the pellet containing precipitated proteins was dissolved in sodium phosphate buffer.

RESULTS

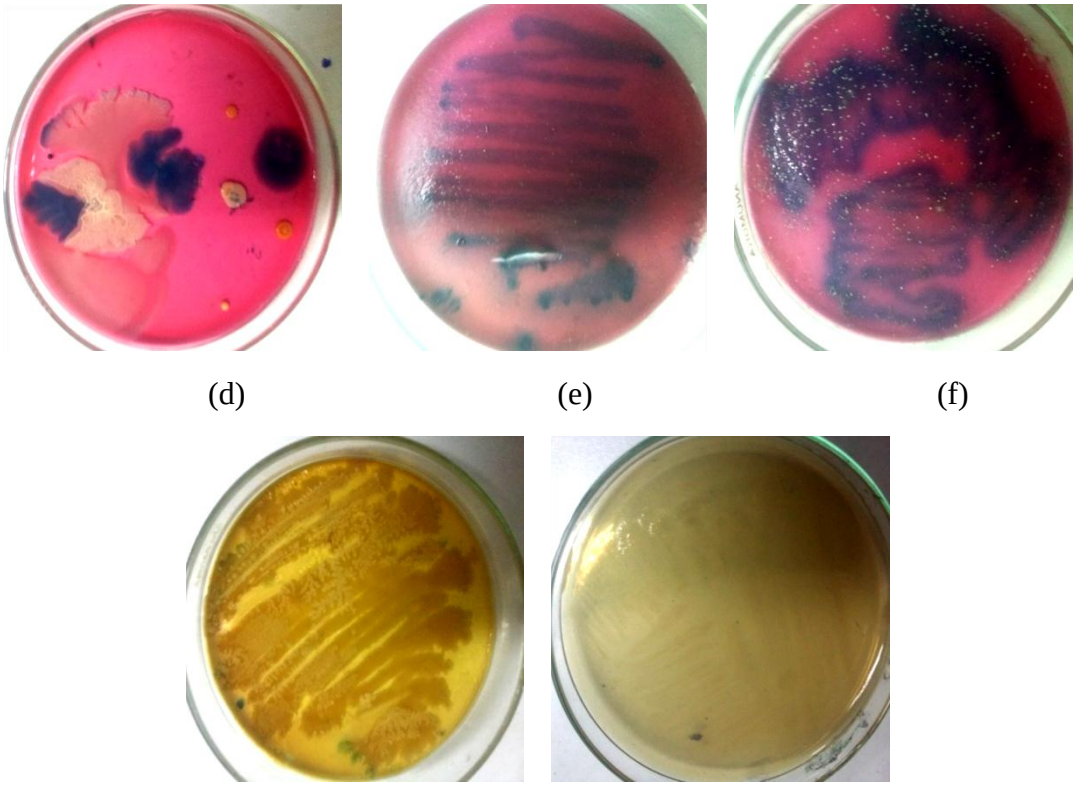
4.1: Isolation and purification

Bacillus strains were isolated initially with different morphological appearance on Petri plates. Some were pink, blue, green, white and their colony patterns differ a lot. Marginal, fluffy, and shiny colonies were observed also. All these colonies were further purified on SDA plates. After 24-hour incubation 8 different colonies were obtained which were coded as, AAM-1, AAM-2, AAM-3, AAM-4, AAM-5, AAM-6, AAM-7 andAK-47. Their colony morphology is described in table 4.1.

Table 4.1: Colony morphology of initial isolates

Colour	**Margins**	**Elevation**
Blue	Slight margins	Low convex
Green	No margins	Low convex
Pink	Wavy margins	Flat
White	Elongated margins	Flat
Yellow	No margins	Flat
Blue	Slight margins	Low convex
Light blue	Slight margins	Low convex
Light green	No margins	Low convex

(a) (b) (c)



(d) (e) (f)

(g) (h)

Figure 4.1: Initial isolates (a-h) showing pigmented growth on Hi Chrome *Bacillus* media with different colony morphology

4.2: Initial screening for antifungal activity

All 8 isolated strains were screened for their antibacterial and antifungal activity on Nutrient agar and SDA plates respectively using dual culture assay. After 24 hour of incubation strains were screened out against bacterial pathogens. Out of 8, 2 strains i-e AK-47 and AAM-1 shows positive results. Antibacterial result was observed after 24 hours while antifungal test were observed after 72 hours. Their results were recorded by measuring zone of inhibition as shown in table 4.2 and 4.3:

Table 4.2: Zone of inhibition of initial isolates against fungal plant pathogens

***Bacillus* strains**	**Zone of Inhibition (mm) against fungal plant pathogens**							
	Fusarium moniliforme	***Fusarium oxysporum***	***Aspergillus flavus***	***Aspergillus niger***	***Colletotricum falcatum***	***Rhizoctonia solani***	***Botrytis cinerea***	***Phytopthora capsici***
AAM-1	35	32	29	27	19	17	20	12
AAM-2	5	5	3	12	11	0	4	4
AAM-3	0	0	4	12	9	0	3	7
AAM-4	5	4	0	7	0	0	8	0
AAM-5	2	2	0	0	5	4	3	7
AAM-6	4	6	3	2	5	2	5	3
AAM-7	5	5	8	6	11	7	8	9
AK-47	42	38	27	30	23	19	18	16

Table 4.3: Antibacterial screening of *Bacillus* strains

***Bacillus* strains**	**Zone of Inhibition (mm) against bacterial human pathogens**					
	Salmonella typhi	***Escherichia coli***	***Klebsiella pneumoniae***	***Staphylococcus aureus***	***Acinetobacter baumannii***	***Listeria monocytogenes***
AAM-1	17.5	25	15	12	22	34
AAM-2	9	7	12	9	11	19
AAM-3	11	11	15	7	9	13
AAM-4	8	12	11	9	5	17
AAM-5	4	6	9	2	7	12
AAM-6	9	8	8	10	9	14
AAM-7	4	5	7	7	6	10
AK-47	26	35	25	14	35	16

4.3: Morphological and biochemical identification of AAM-1 and AK-47

Microscopic analysis of AAM-1 and AK-47 was performed by making their smears on slides. Then they are gram stained. Both of these strains appeared as rod shaped violet in colour hence declared as GPRs. Biochemical analysis show strain was positive for catalase, citrate, and nitrate. Negative results were showed for oxidase and indole. Results are shown in table 4.4:

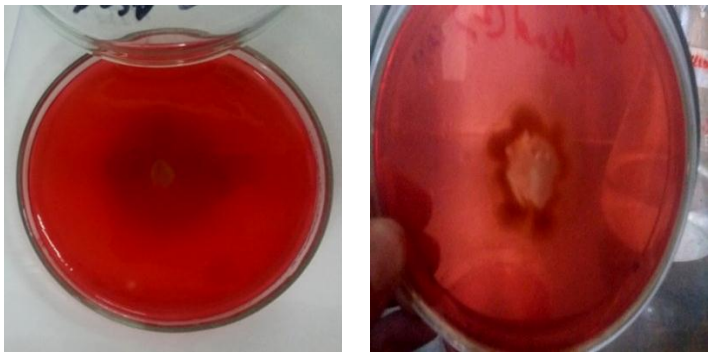
Table 4.4: Biochemical tests analysis

Biochemical test	Results
Catalase	Positive
Citrate	Positive
Oxidase	Negative
Indole	Negative
Nitrate	Positive
Urease	Negative
Methyl red	Negative

4.4: ENZYME ASSAYS

4.4.1: ENDOGLUCANASE ASSAY

After 24 hour incubation period, 0.01% congo red reagent was added and left for 30 minutes. After that plates were washed and flooded with 1 molal sodium chloride solution. Zone of hydrolysis were observed after 5 to 7 minutes, as shown in fig 4.2:

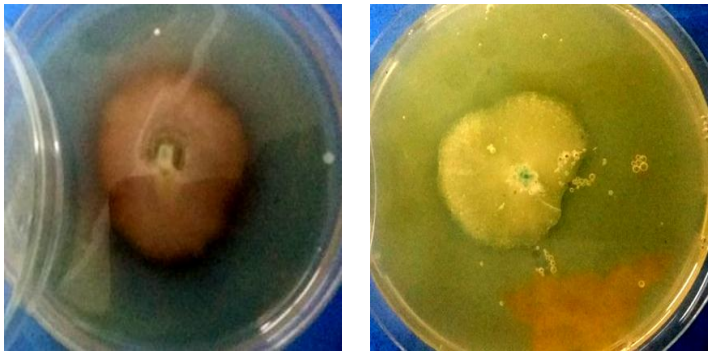


(a) (b)

Figure 4.2: Shows the zone of hydrolysis for endoglucanase (a)AK-47 (b)AAM-1

4.4.2: PROTEASE ASSAY (Casein Hydrolysis)

After 24 hour of incubation plates, 70% acetic acid reagen was poured over plates. After 2 to 3 minutes zone of hydrolysis was observed. Results are shown in fig:4.3:

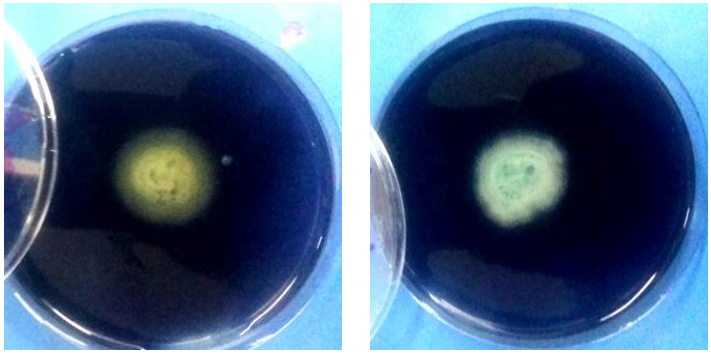


(a) (b)

Figure 4.3: Shows zone of hydrolysis for protease (a) AAM-1 (b) AK-47

4.4.3: AMYLASE ASSAY (Starch Iodine Test)

After 24 hour of incubation time plates were flooded with its reagent i-e iodine solution. Clear zone of hydrolysis was observed after 2to 3 minutes. Results are shown in figure 4.4:



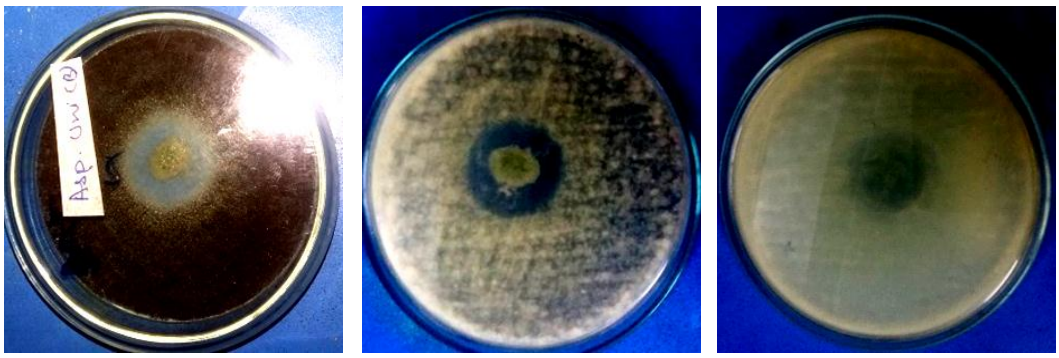
(a) (b)

Figure 4.4: Shows zone of hydrolysis for amylase (a) AAM-1 (b) AK-47

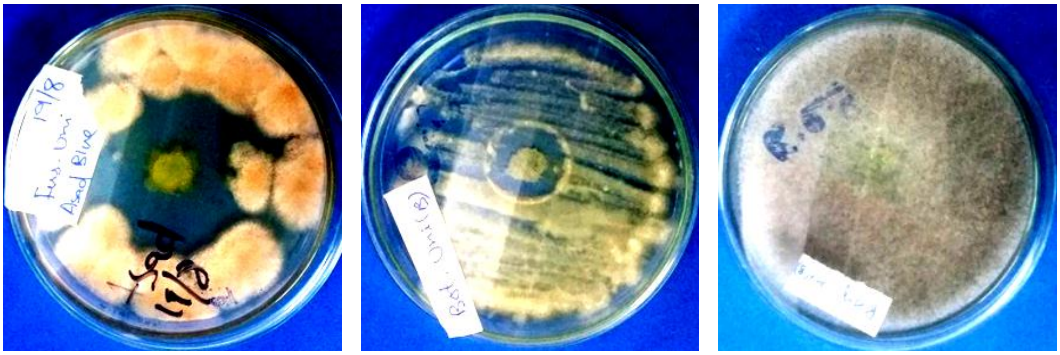
4.5: Final test of AAM-1 and AAM-2

4.5.1: Spreading of fungi and point inoculation of *Bacillus* at center

After 3 days of incubation zone of inhibition against phytopathogens were observed. AAM-1 shows maximum zone against *Fusarium oxysporium* and AK-47 shows maximum zone against *Rhizopus oryzae*. As shown in figures 4.5 and 4.6:

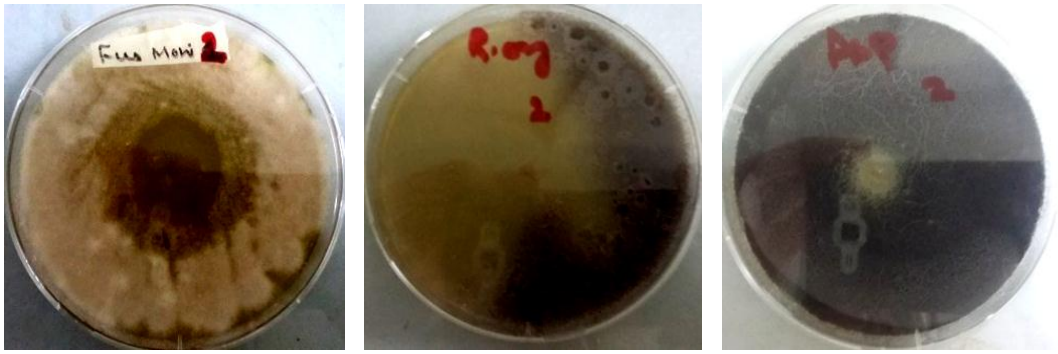


(a) (b) (c)

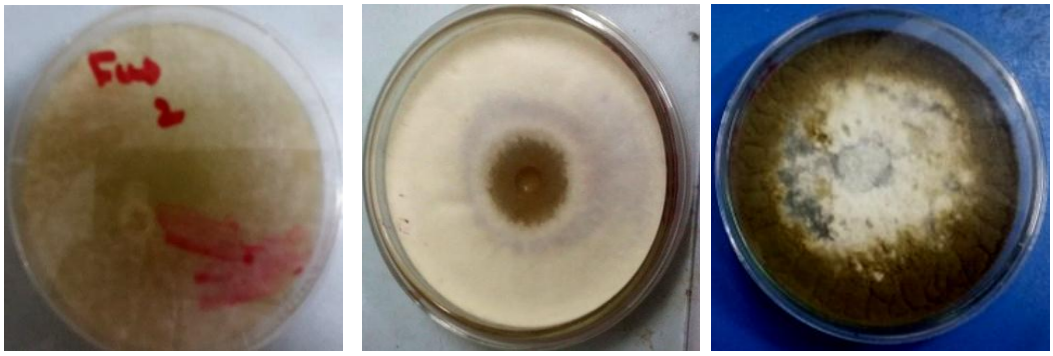


(d) (e) (f)

Figure 4.5: Antifungal activity of strain AAM-1 by spot inoculation of strain at the center and spreading of fungal pathogens (a) *Aspergillus niger* (b) *Phythopthora capsci* (c) *Fusarium moniliform (d) Fusarium oxysporum (e) Botrytis cinerea (f) Rhizopus oryzae*



(a) (b) (c)



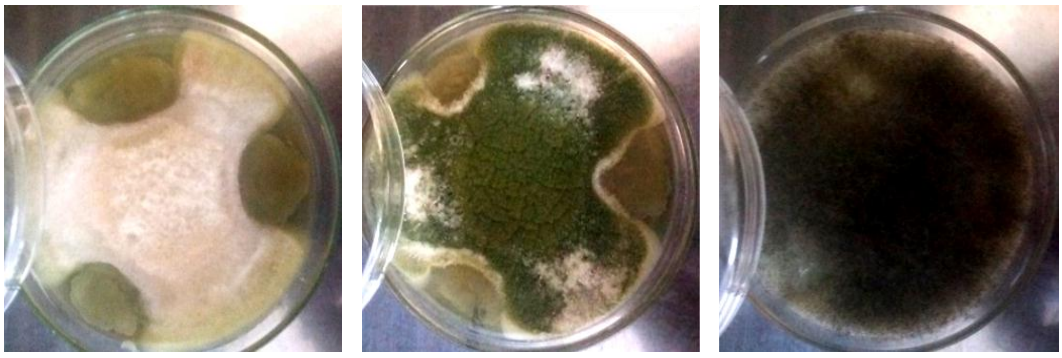
(d) (e) (f)

Figure 4.6: Antifungal activity of *Bacillus* strain AK-47 by spot inoculation of *Bacillus* at the center (a) *Fusarium moniliform* (b) *Rhizopus oryzae* (c) *Aspergillus niger* (d) *Fusarium oxysporum* (e) *Botrytis cinerea* (f) *Aspergillus flavus*

4.5.2: Spreading of fungi and inoculation of *Bacillus* at three positions

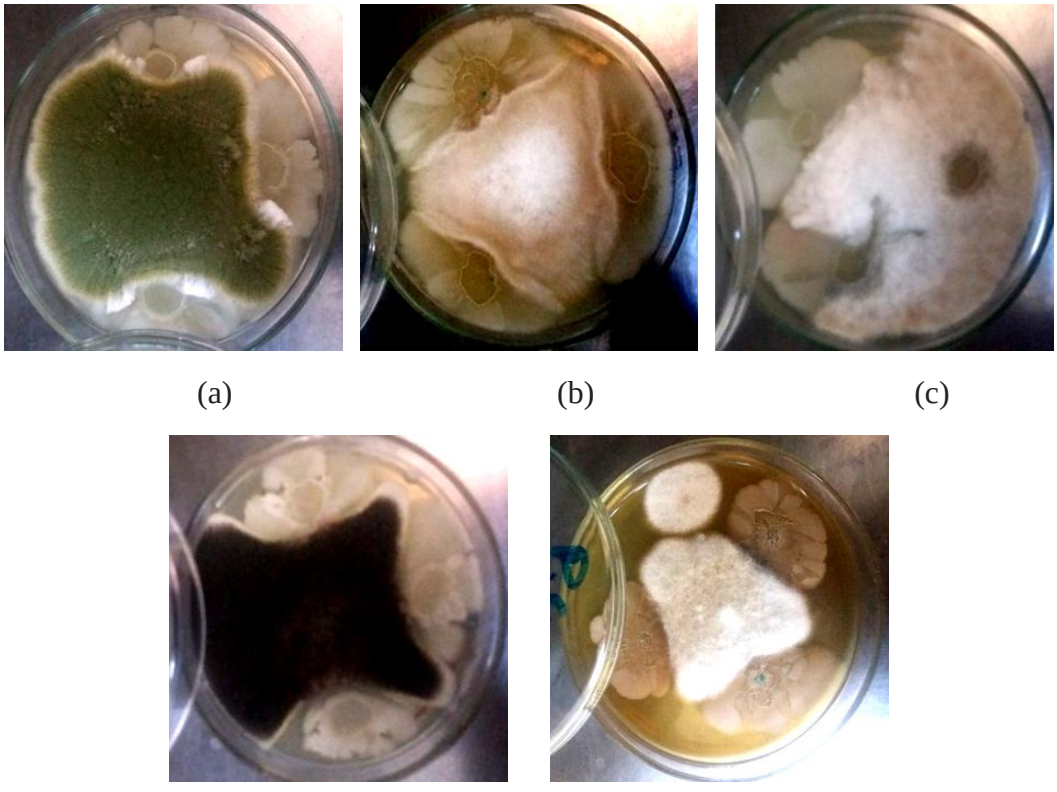
After 72 hours of incubation AK-47 and AAM-1 inhibited the growth of fungal mycelia on SDA plates, as shown in figure 4.7 and 4.8:

(a) (b) (c)



(d) (e) (f)

Figure 4.7: Spot inoculation of *Bacillus* AAM-1 at three different positions inhibit the growth of fungal mycelia (a) *Fusarium oxysporum* (b) *Fusarium moniliform* (c) *Aspergillus niger* (d) *Colletotricum falcatum* (e) *Aspergillus flavus* (f) *Rhizopus oryzae*



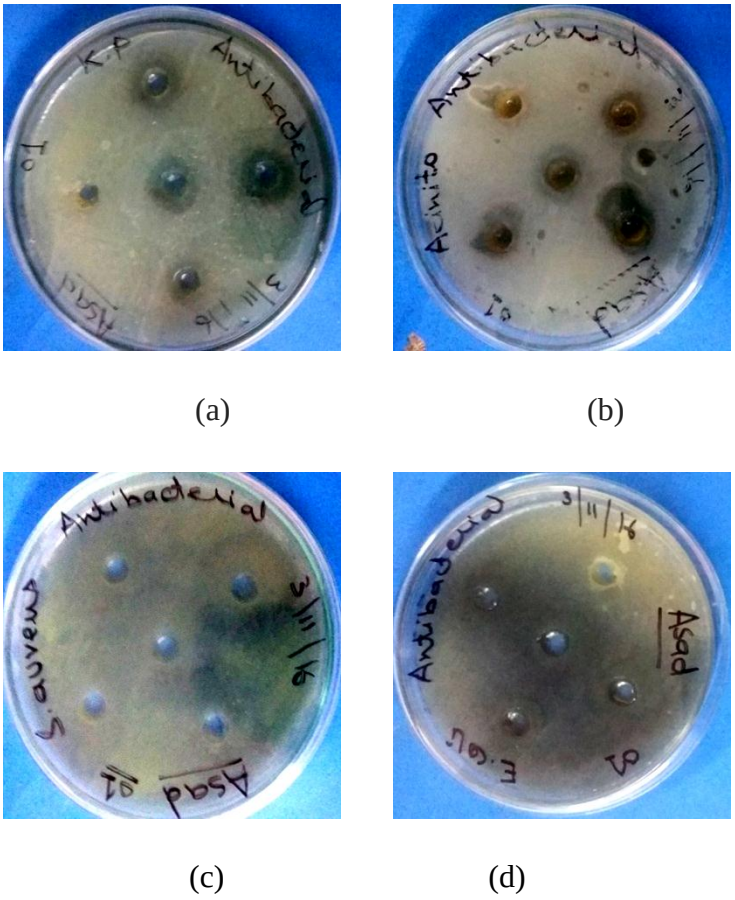
(a) (b) (c)

(d) (e)

Figure 4.8: Spot inoculation of *Bacillus* AK-47 at three different positions inhibit the growth of fungal mycelia (a) *Aspergillus flavus*, (b) *Fusarium moniliform* (d) *Fusarium oxysporum* (d) *Aspergillus niger* (f) *Botrytis cinerea*

4.6: Cell Free Culture Test

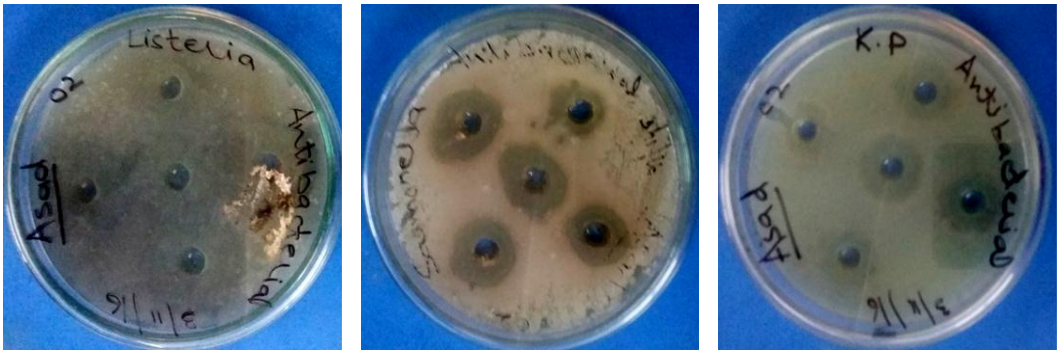
Supernatant of *Bacillus* strains AAM-1 and AK-47 were collected by centrifugation at 10,000 rpm for 10 minutes. Test of supernatant was carried out by using agar well diffusion method for bacterial pathogens. After 24 hours of incubation clear zone of inhibition were observed against selected bacterial pathogen. Zones were measured for analyzing the biocontrol activity. Results are shown in fig 4.9 and 4.10:



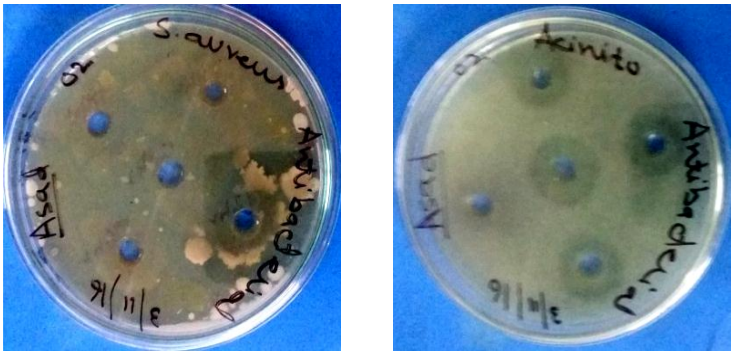
(a) (b)

(c) (d)

Figure 4.9: Antibacterial activity of cellfree culture of AAM-1 via agar well diffusion method againt bacterial pathogens (a) *Klebsiella pneumonia* (b) *Acinetobacter baumannii* (c) *Staphylococcus aureus* (d) *Escherichia coli*



(a) (b) (c)



(d) (e)

Figure 4.10: Antibacterial activity of cellfree culture of AK-47 via agar well diffusion method againt bacterial pathogens (a) *Listeria monocytogenes (b) Salmonella typhi (c) Klebsiella pneumoniae (d) Staphylococcus aureus (e) Acinetobacter baumannii*

4.7: Temperature optimization of strains

Strains AAM-1 and AK-47 were optimized so that their maximum activity and growth can be obtained. For this strain were grown at different temperature i-e 10˚C, 20˚C, 30˚C and 40˚C. OD values at 600 nm were recorded after each 24hrs interval for five

successive days. Maximum growth of AK-47 was recorded at 40˚C for 72 and 96 hours of incubation and for AAM-1 was recorded at 40˚C for 48 hours, while decline in growth was observed by further incubation. Antifungal activity was measured by agar well diffusion method. Antifungal activity was maximum at 40˚C after 72 and 96 hours of incubation for AK-47 and for AAM-1 maximum activity was observed after 48 hour. Results for growth and antifungal activity are depicted in graphical form. As shown in fig 4.11 to 4.20:

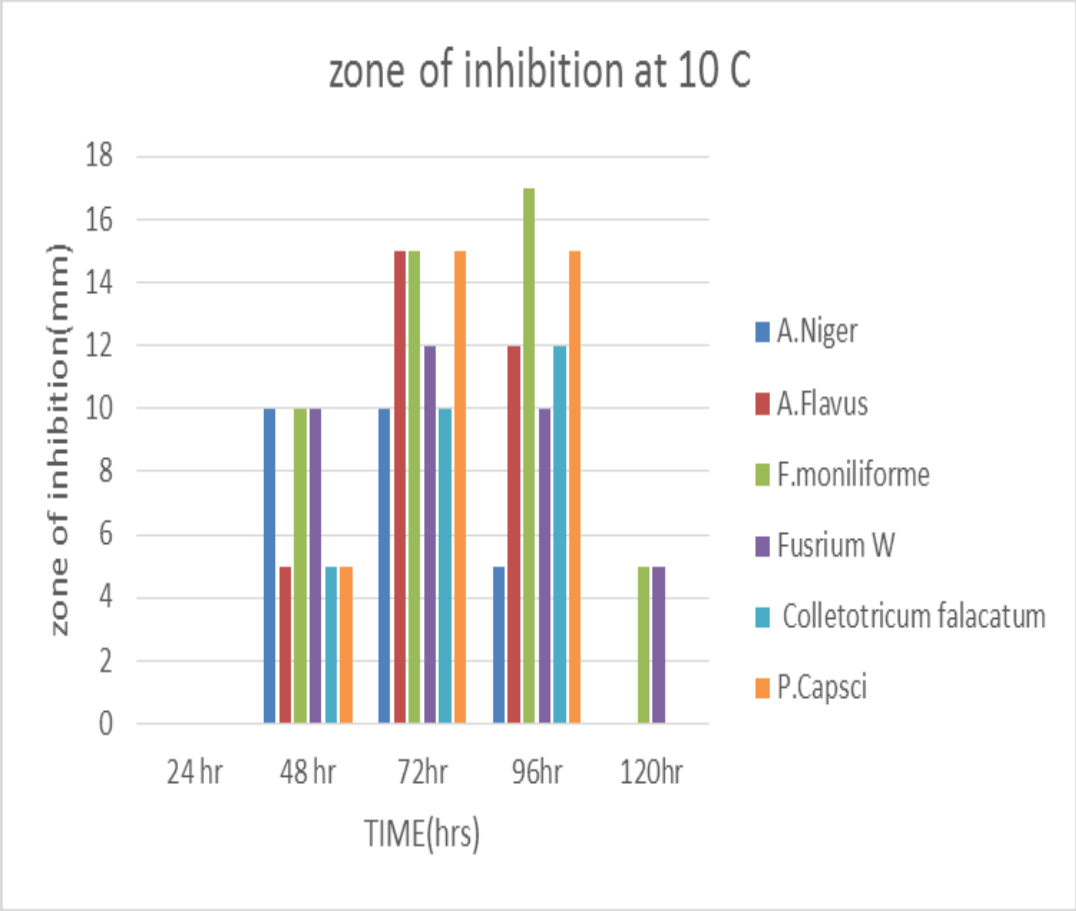


Figure 4.11: AK-47 at 10ºC: Zone of inhibition measured in mm shown on y-axis maximum activity, X-axis shows tested organisms

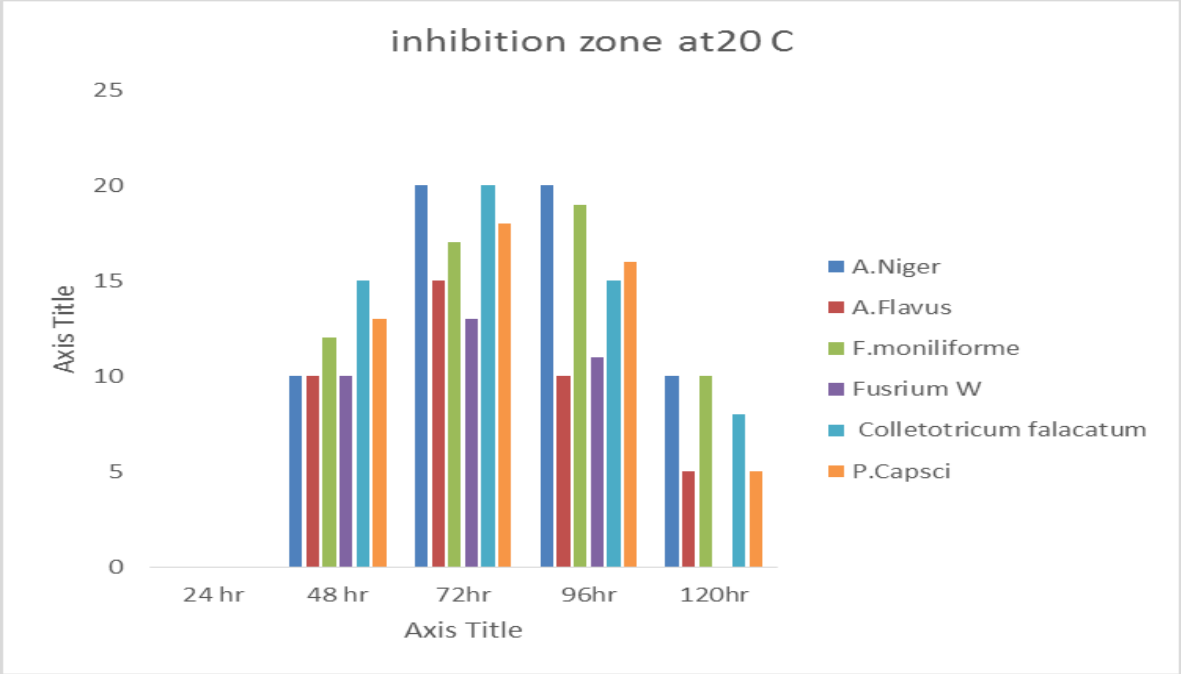


Figure 4.12: AK-47 At 20ºC: Zone of inhibition measured in mm shown on y-axis maximum activity, X-axis shows tested organisms

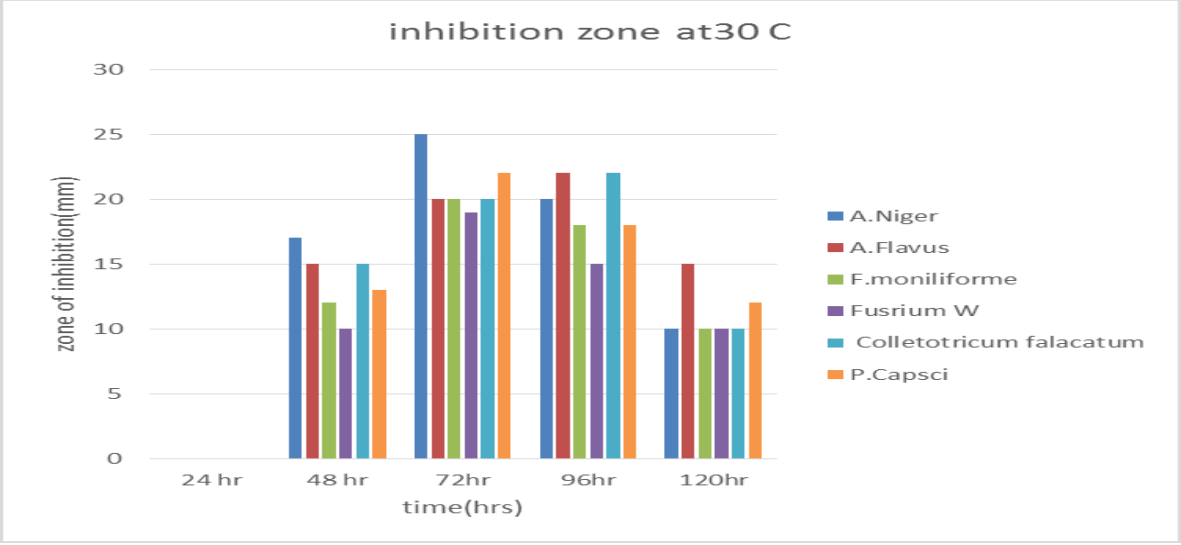


Figure 4.13: AK-47 At 30ºC: Zone of inhibition measured in mm shown on y-axis maximum activity, X-axis shows tested organisms

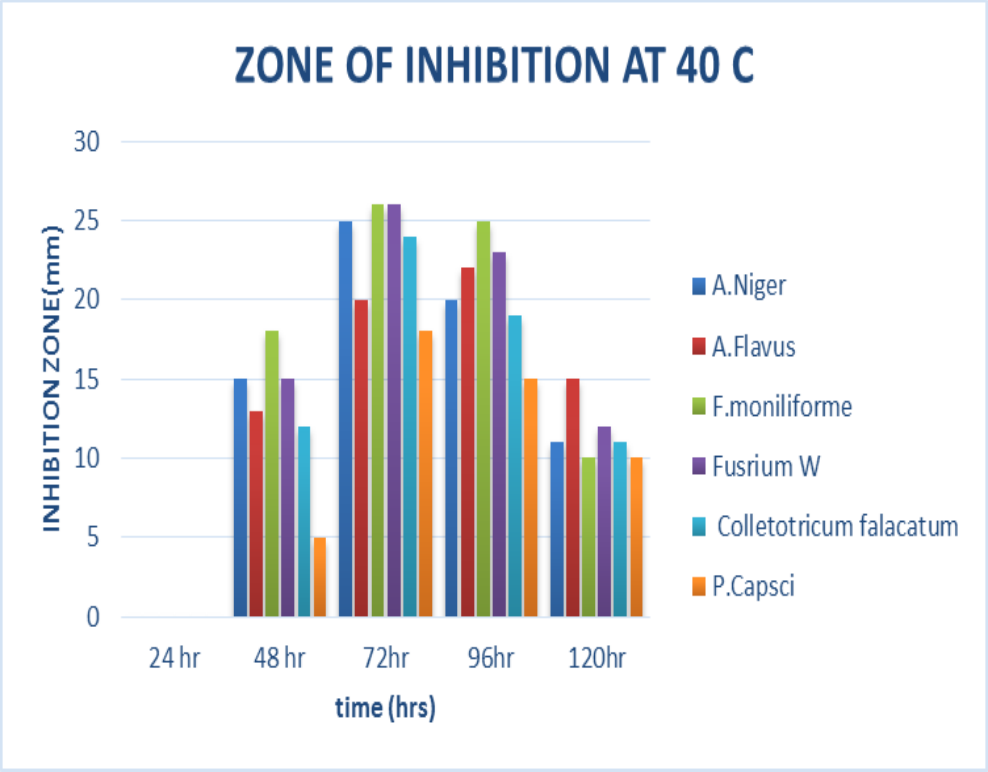


Figure 4.14: AK-47 At 40ºC: Zone of inhibition measured in mm shown on y-axis maximum activity, X-axis shows tested organisms

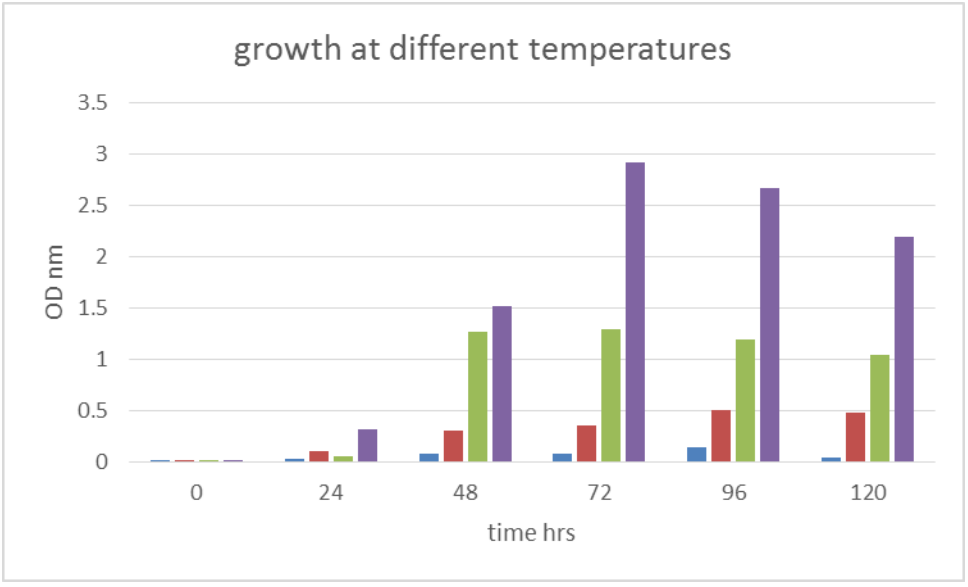


Figure 4.15: Optimum growth analysis of AK-47 at different temperatures

4.8: Inoculum Optimization

Inoculum optimization was performed to obtain maximum activity of strains. After 72 hours of incubation, 1%, 2%, 3% inoculum activity of AK-47 and AAM-1 was checked against fungal pathogen via agar well diffusion method. Zone of inhibition were measured after 72 hours of incubation. 3% inoculum showed maximum zones of inhibition against selected fungal pathogens. % inoculum results are represented in graphical form, as shown in fig 4.16 to 4.18:

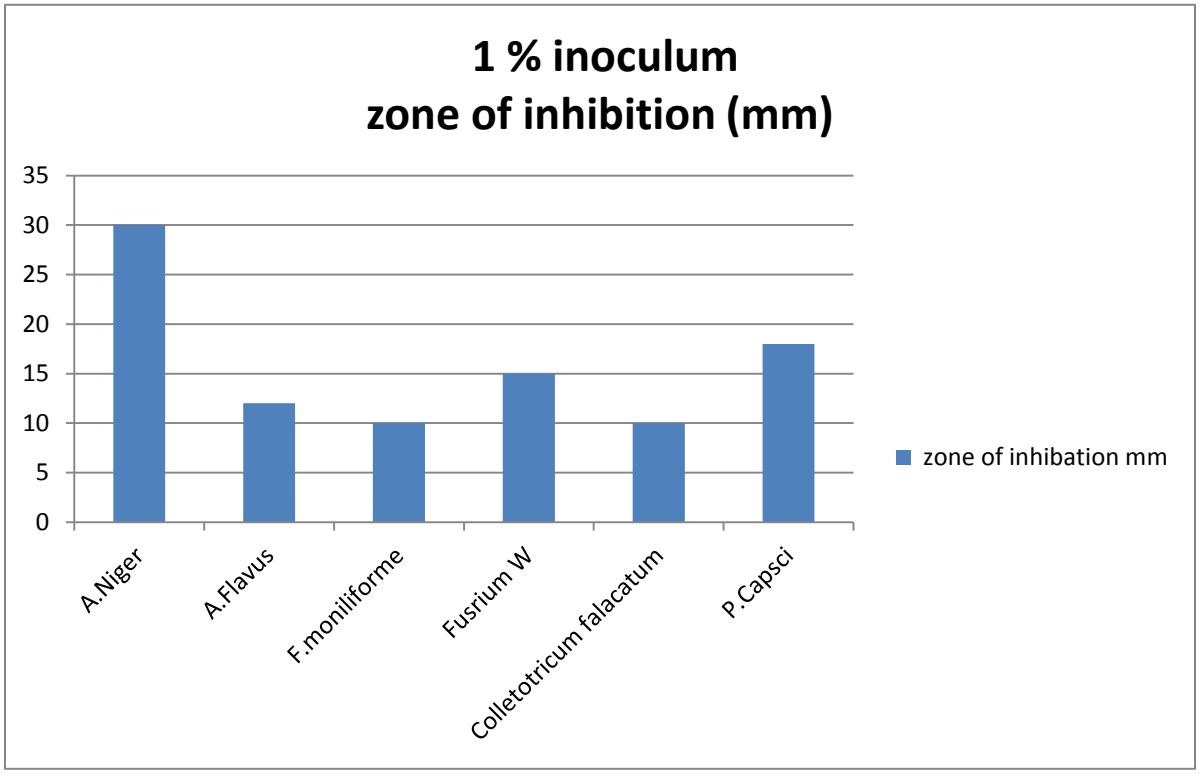


Figure 4.16: For 1% inoculum zone of inhibition measured in mm shown on y-axis maximum activity was observed for *Aspergillus niger*, X-axis shows tested organisms

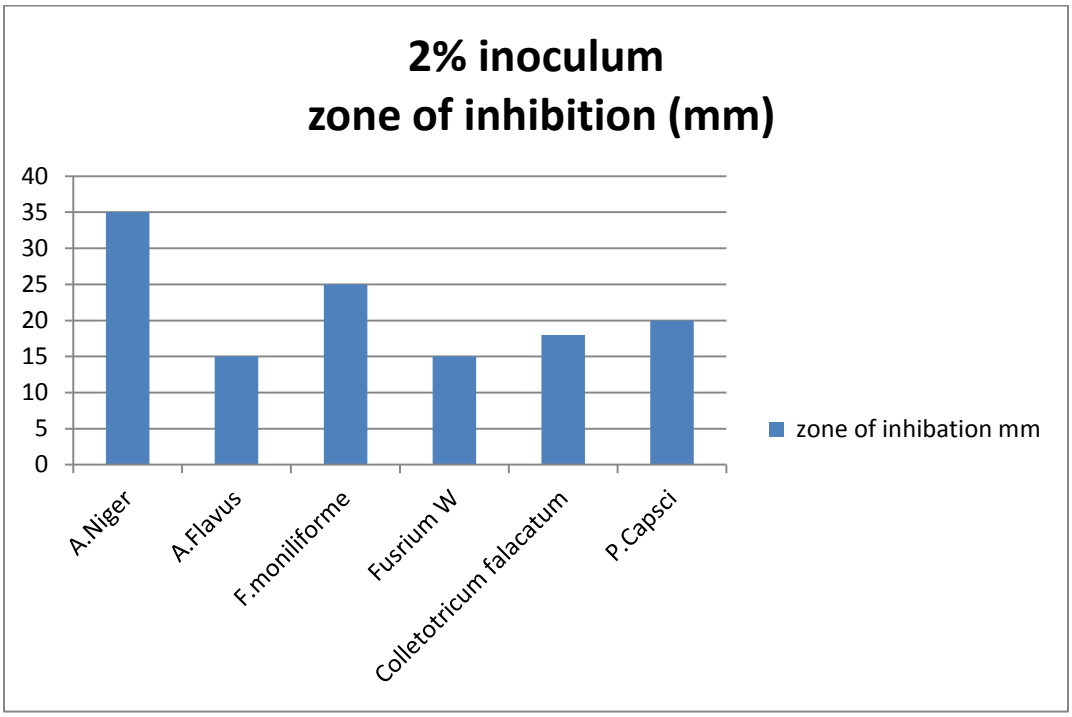


Figure 4.17: For 2% inoculum zone of inhibition measured in mm shown on y-axis maximum activity was observed for *Aspergillus niger*, X-axis shows tested organisms

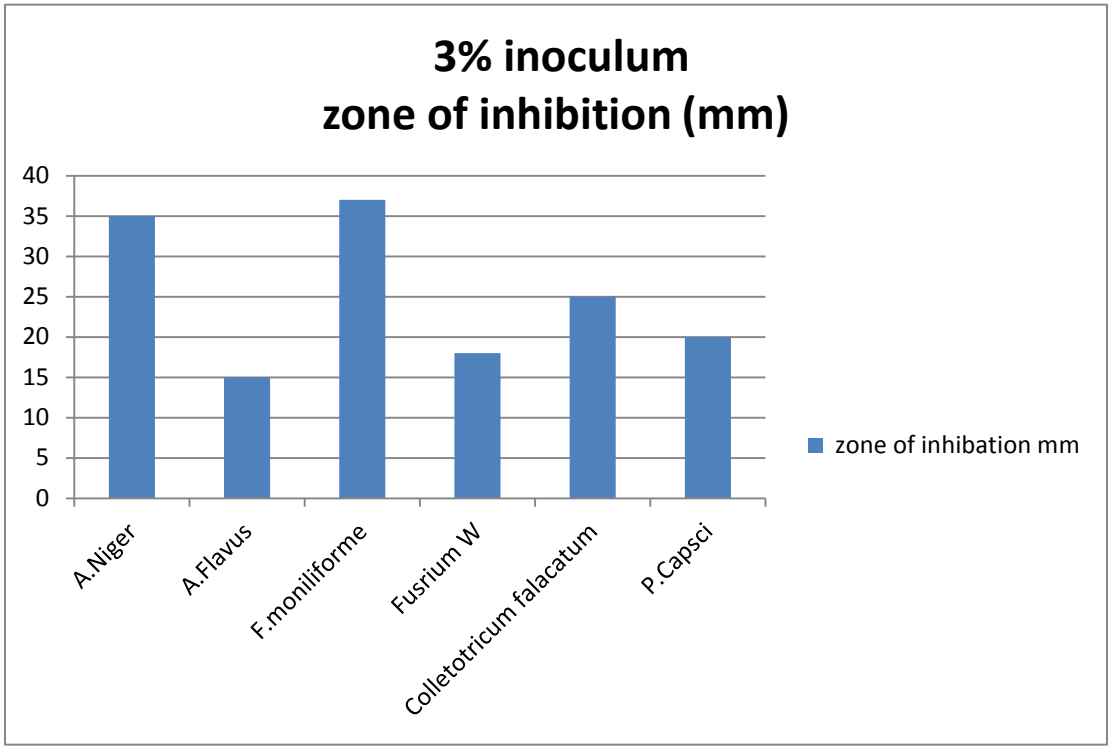


Figure 4.18: For 3% inoculum zone of inhibition measured in mm shown on y-axis maximum activity was observed for *Aspergillus niger*, X-axis shows tested organism

4.9: Extraction of metabolite

Metabolite extraction was done by using ethyl acetate as a solvent. For AK-47 about 0.09g bluish colored crude extract was obtained and for AAM-1 about 0.8g black colored metabolite was extracted.

4.9.1: Tests for metabolites

Crude extract of strains AK-47 and AAM-1 were then dissolved in methanol. Their antifungal and antibacterial activity was checked through disc diffusion method. They shows zone of inhibition against selected fungal pathogens. Results are shown in table 4.4, 4.6, 4.7, 4.8:

Table 4.5: Shows antifungal activity of AK-47 metabolite

Fungal pathogens	**Zone of inhibition (mm)**
Fusarium moniliforme	25
Fusarium oxysporum	20
Aspergillus flavus	21
Aspergillus niger	35
Colletotricumfalcatum	26
Rhizoctoniasolani	11
Botrytis cinerea	18
Phytopthoracapsici	15

Table 4.6: Shows antifungal activity of AAM-1 metabolite

Fungal pathogens	**Zone of inhibition (mm)**

Fusariummoniliforme	19
Fusarium oxysporum	17
Aspergillus flavus	21
Aspergillus niger	22
Colletotricumfalacatum	16
Rhizoctoniasolani	8
Botrytis cinerea	17
Phytopthoracapsici	15

Table 4.7: Shows antibacterial activity of AK-47 metabolite

Bacterial pathogens	**Zone of inhibition (mm)**
Escherichia coli	25
Salmonella typhi	20
Staphylococcus aureus	25
Acinetobacter baumannii	20
Listeria monocytogenes	35

Table 4.8: Shows antibacterial activity of AAM-1 metabolite

Bacterial pathogens	**Zone of inhibition (mm)**
Escherichia coli	24
Salmonella typhi	19
Staphylococcus aureus	24
Acinetobacter baumannii	22
Listeria monocytogenes	29

4.9.2: Fourier transform infrared spectroscopy (FTIR) of strain AK-47 metabolite

Table 4.9: Description of FTIR spectrum of strain AK-47 metabolite

Band assignments	**Functional groups**
3370.99	N-H stretching, aliphatic pri amine
2168.72	S-C≡N stretching, thiocyanate
1641.19	C=N stretching, amine / oxime
1569.50	C-C stretching, cyclic alkene
1406.10	O-H bending, alcohol
1323.57	O-H bending, phenol
1217.38	C-O stretching, vinyl ether
1073.61	C-O stretching, vinyl ether
994.89	C=C bending, alkene
894.89	C=C bending, alkene
838.10	C=C bending, alkene
715.51	C=C bending, alkene
691.25	C=C bending, alkene
623.72	C-Br stretching, halo compound

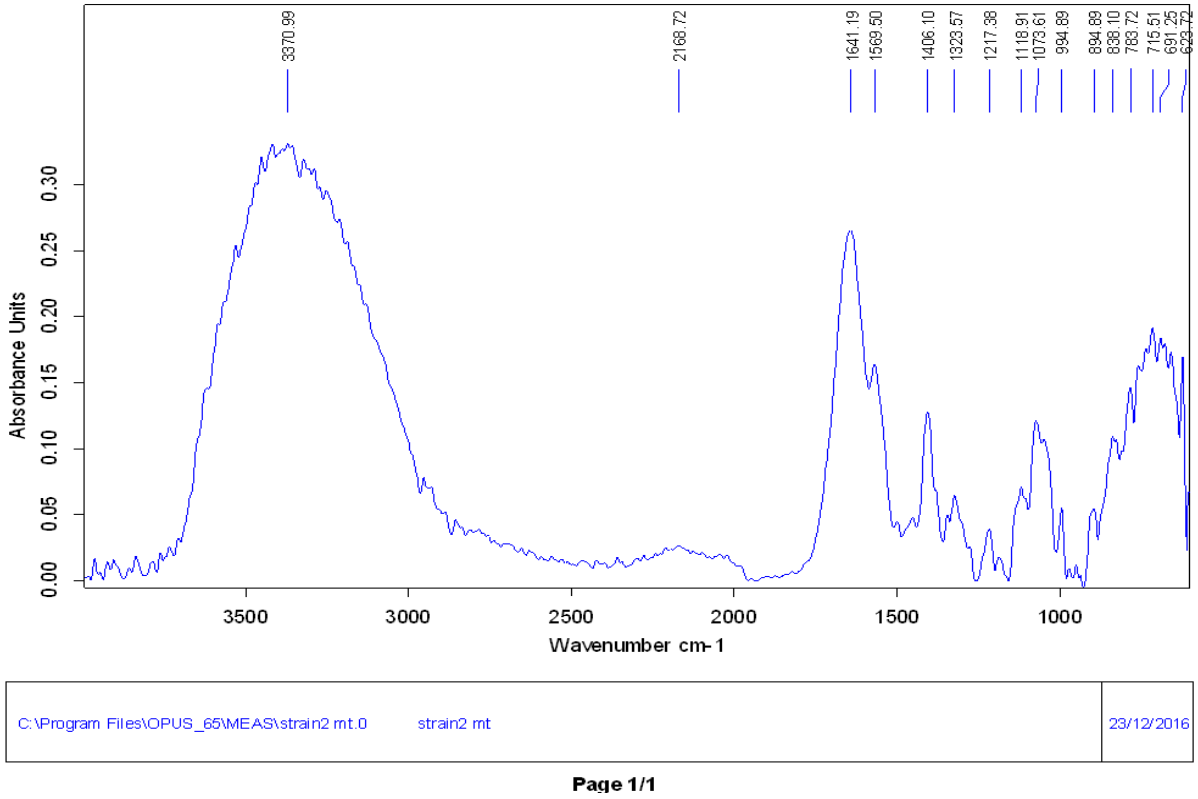


Figure 4.19: FTIR spectrum (absorption) of strain AK-47 metabolite

4.10: EXTRACTION OF ANTIFUNGAL PROTEIN THROUGH AMMONIUM SULPHATE PRECIPITATION

For ammonium sulphate precipitation, three different saturation concentrations i-e 40, 50 and 60% were used. Maximum precipitates were obtained at 60% saturation. Then these precipitates were centrifuged and precipitated protein was separated. Protein then dissolved in sodium phosphate buffer. Antifungal and antibacterial activity of protein was checked by well diffusion method. Only AK-47 shows positive results while AAM-1 do not show results. Zone of inhibition was recorded for AK-47 as shown in table 4.8 and 4.9:

Table 4.10: Shows Ammonium sulphate precipitation results of AK-47 against fungal pathogens

Fungal pathogens	**Zone of inhibition (mm)**
Fusarium moniliforme	20
Fusarium oxysporum	14
Aspergillus flavus	20
Aspergillus niger	30
Colletotrichum falcatum	9

Table 4.11: Shows ammonium sulphate precipitation results of AK-47 against bacterial pathogens

Bacterial pathogens	**Zone of inhibition (mm)**
Escherichia coli	20
Salmonella typhi	15
Staphylococcus aureus	25
Acinetobacter baumannii	19
Listeria monocytogenes	34

4.11: Pilot Scale Experiment of selected strain

The effect of different treatments and growth of seeds were observed after 22 days of experiment. Seeds inoculated with pathogen and biocontrol strain show clear disease suppression. No seed germination or seed with disease symptom was observed for pots containing seed and pathogens. Pots containing seeds inoculated with *Bacillus*, pathogen and PGPR showed not only diseases suppression but also show enhanced growth. The pots containing seed and *Bacillus* strain show increased growth compared with pots containing seed alone. Results are shown table 4.6 and fig 4.19:

Table 4.12: Effect of different inoculum on the growth of seed

SEEDS	AS+S	AS+S+B	AS+S+PGPR	AS+S+PGPR+B	AS+S+B+P	AS+S+PGPR+B+P	NS+S	NS+S+P	NS+S+B+P
RBS	0.5	6	6.8	10	5.6	8.5	2.5	0	4
TS	4	6	7	8	5	7.5	2	0	3
WS	6	19	15	19.5	13	18	0	4	4
MS	7	9	11	15	10	13	4	0	5
OS	1	2	2	3	2	2.5	2	0	2
CS	0	0	0	0	0	0	0	0	0

Table 4.12.a: Key table of 4.10

AS	Autoclaved Soil
S	Seed
RBS	Red Bean Seed
TS	Tomato Seed
WS	Wheat Seed
MS	Maize Seed
OS	Onion Seed
CS	Cucumber Seed
B	*Bacillus* Strain
PGPR	Plant Growth Promoting Rhizospheric Bacteria
P	Pathogen
NS	Normal Soil

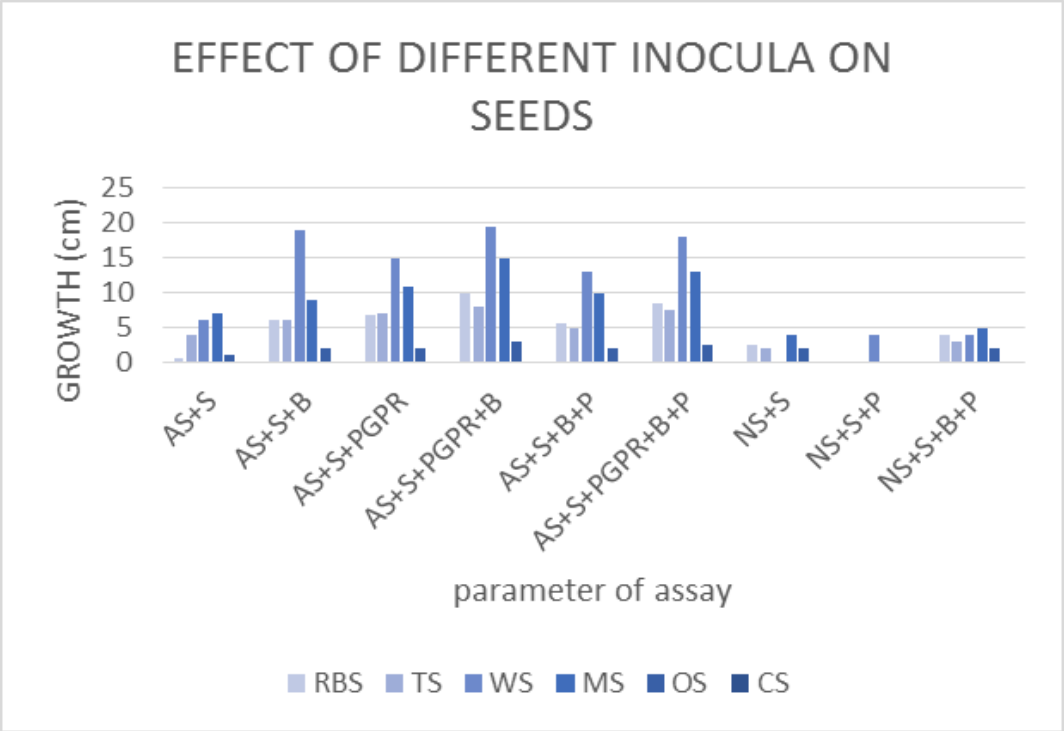


Fig 4.20: Effect of different inoculum on the growth of seeds. Growth measured in cm shown on y axis. X axis shows different parameters (for key check table 4.10.a)

DISCUSSION

Application of the microorganisms as a whole cell or their metabolic products for improving the agricultural yield and inhibiting growth affecting pathogens has been increasing with time. Since many microorganisms in the nature have the ability to limit the growth of other microorganisms, therefore, they are considered as a sustainable alternative to the chemical pesticides. In addition to this these microorganisms stimulates the growth of the plants thus improve the productivity of agricultural products. In view of environmental responsibility, the application of these microorganisms leaves no harmful effects. Keeping in view the promising role of these beneficial microorganisms current research was typically focused to isolate bacterial strains showing excellent potential as biocontrol agents.

Isolation and screening of a potent bacterial strain has been the most important task in any microbiological research. The main theme of isolation is to acquire most productive microbial strain with excellent commercial scope. In present research five soil samples was taken from different agricultural soils for the isolation of microorganisms having antagonistic properties against some serious phythopathogens. Our result indicated that from these soil samples initially eight isolates were obtained on *Bacillus* specific media. Among these strains two *Bacillus* strains AAM-1 and AK-47 showed comparatively better result when tested against fungal and bacterial pathogens. Highest zone of inhibition was calculated against *Fusarium* species, *Aspergillus* species and *Listeria monocytogenes.*

Basically killing other microorganisms is a defense strategy which enables a particular bacterium to survive under a particular environment. For their survival and substrate extrusion these microorganisms produce a wide range of antimicrobial metabolites. It is suggested that the two strains which displayed highest results in initial screening could have develop very effective strategies to antagonize neighboring microorganisms. These strategies may include the extracellular release of antimicrobial chemicals or by competition and antagonism. It is likely that the strains AAM-1 and AK-47 were metabolically more efficient than the other bacterial strains due to their vibrant genetic makeup. Previously Zhang *et al.,* (2008) describe that soil

borne gram positive rods produce antifungal compounds which inhibit the growth of *Aspergillus niger*. Therefore, it is suggested that the strains AAM-1 and AK-47 were biologically evolved with rapid pace than the other, in doing so they have altered their genetic contents and biochemical pathways in such a way that their survival rate is much higher than the others. This could be a possible justification variability in the results obtained in case of present research.

To consolidate the initial findings with higher accuracy various confirmatory assays were performed and their results showed that the strains AAM-1 and AK-47 were more productive than the others however their ability to limit the growth of the agri pathogens or human pathogens was markedly different.

After preliminary screening different confirmatory assays were performed to consolidate the results. Spreading of fungi and spot inoculation of *Bacillus* on PDA plates showed significant zone of inhibition against *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Colletotricum falcatum and Phytophtora capsci*. Maximum zones 35mm were observed against *Listeria monocytogenes* and *Fusarium oxysporum*. Similar study was carried by Ajay Kumar, Pragati Saini and J N Shirivastava (2009), they use *Bacillus,* spot inoculation method against *M.fulvum and Trichophyton* spp. and obtain 16 and 14 mm zone of inhibition respectively. Growths of selected phythopathogens were also inhibited by the strains when they are point inoculated at three different points on the same plate. Similar results was obtained by V.Gouri Shanker *et al.*, (2014), they used *Bacillus* spp. SRB 27 at different position which inhibited the growth of *Fusarium* and *Alternaria* spp. Spreading of bacterial and fungal spore suspension on nutrient agar plates and PDA plates also show inhibitory effect respectively. *Bacillus* inhibits 100% growth of *Aspergillus niger, Fusarium moniliform, and Colletotricum falacatum* and partial inhibition against *Rhizopus oryzae* and *Phytophtora capsci.* Similar result was obtained from the study of Jiang, Shi, Liu, & Zhu, (2014). Spreading of *Bacillus* and point inoculation of fungus also inhibit the growth of pathogenic fungus.

The observed inhibitory activities of AK-47 are likely caused by competition for nutrients. Microbes generally compete with each other for carbon, nitrogen, phosphorus, iron and other

macro and micro nutrients necessary for growth and development (Salvatore and Alessandro, 2008). This mechanism can be correlated with Imran *et al*., (2012) that showed in their study *Bacillus* inhibit the growth of *Fusarium* spp. by competing for the nutrient substances on PDA plate necessary for growth.

Supernatant test of strain AK-47 via agar well diffusion method showed effective result against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium moniliforme* and *Colletotricum falcatum.* Zone of inhibition were measured, maximum zones were obtained against *Aspergillus niger* and *Fusarium moniliform* i-e 35mm and 30mm respectively. Similar studies were conducted by Hammami Imran1, Trabelsi H. Darine and EL Gazzah Mohamed (2012). Crude metabolite extracted from the strains also show antifungal activity via disk diffusion method. It showed zone of inhibition against *Aspergillus niger*, *Aspergillus flavus*, and *Colletotricum falcatum.*

The observed antagonistic activities of the *Bacillus* AK-47 might be due to the production of antimicrobial compounds. It is well reported that *Bacillus* produces different antibacterial and antifungal compounds to control the plant diseases (Zhang *et al*., 2008). Strains of *Bacillus* spp. have been reported to produce different cyclic lipopeptides such as iturin, fengycin, surfactin, bacillomycin D and bacilysin by non-ribosomal synthetases (NRPSs) (Al-Saraireh *et al*., 2015). The study of Kim, Pyoung , Jaewon Ryu, Young Hwan Kim, and Youn-Tae Ch(2010) showed that *Bacillus* spp. produces lipopeptides such as iturin A, surfactin, fengycin to control the fungal pathogen *Colletotrichum gloeosporioides.*

Strain was optimized at different temperatures 10˚C, 20˚C, 30˚C, and 40˚C. Strain show maximum results at 40˚C after 72 and 96 hours of incubation. It is reported that production of secondary metabolites are growth phase dependent and produce during the exponential and stationary phase (Barrios-González & Mejia, 1996). As strain show maximum zone of inhibition after 72 and 96 hours of incubation means that strain produces secondary metabolites in

stationary phase and might be as bacteriocins, iturin A and surfactin. Swain and Ray (2006) report that *Bacillus* spp; produce secondary metabolite subtilin in late phase belongs to the iturin family. So it is possible that the strains show antifungal activity due to the production of subtilin.

Enzyme assay result of strain shows that it produces clear zone of hydrolysis for endoglucanase, amylase and protease. There are many reports on the production of extracellular enzymes by *Bacillus* spp. (Abdel-Aziz *et al*., 2011) (Gupta and Ramnani, 2006). These enzymes are involved in biocontrol activity by cell wall lysis. It is possible that antifungal activity of the strains AAM-1 and AK-47 likely because of these enzymes. This can be correlated with the study of Ashwini and Srividya (2014), who checked the antifungal activity of *Bacillus* spp. against *Colletotrichum gloeosporioides* and shows efficient enzymatic lysis by glucanase and cellulase.

Pilot scale experiment also confirms the potential of AK-47. Different seeds i.e. red beans, wheat, tomato, maize, onion were grown and infected with their respective pathogen. At the same time strain AK-47 was co-inoculated. Strain does not show phytotoxic effect. *Bacillus* strains are considered as plant growth promoting bacteria and this strain promoted growth of the plant in field scale bioassay. This ability of strain is might be due to several reasons most probably by the production of phytoharmones, especially IAA (Ali *et al*. 2009; Kumar *et al*. 2012).Higher auxin level also increases plant growth. Siderophores also have plant promoting affect either by supplying iron to the plant or make it unavailable for pathogens (Rana *et al*., 2012). All these plant growth promoting factors were in accordance with study of Nahid Heidarzadeh & Sareh Baghaee-Ravari (2016). Plant growth significantly increases when *Bacillus* along with PGPR were applied. *Bacillus* strain suppresses the disease completely when applied on infected plants. Different studies shows the antifungal activity of *Bacillus* on experiment field like Xinqi Huang, Nan Zhang, Xiaoyu Yong, Xingming Yang, Qirong Shen (2011) shows antifungal activity of *Bacillus pumilus*. Our findings are in accordance with previous findings regarding the application of *Bacillus* as biocontrol and also plant growth promoter (Babalola 2010; Saha *et al*., 2013; Prashar *et al*., 2013; Zaim *et al*., 2013).

Potential of the Strain AK-47 was also checked against bacterial pathogens and it was surprising that all bacterial pathogens were inhibited by the strain. Bacterial pathogens against which activity of the strain was tested includes *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii* *and Listeria monocytogenes* .It was suggested that the strain has the ability to produce broad spectrum of antibiotics which also inhibits growth of bacterial pathogens.

Our strain shows encouraging results against bacterial and fungal pathogens and can be used as antibiotic and bio-fungiscide respectively. Further investigations are required to reveal the exact mechanism of action in order to improve its efficacy and successful development as biocontrol agent.

CONCLUSION

- Eight *Bacillus* strains were isolated from four different agricultural soils of Punjab and KPK province.
- After initial screening two strains AK-47 and AAM-1 showed efficient results.
- Both these strains inhibit the growth of selected plant and human pathogens.
- Confirming that they have the potential to produce antifungal and antibacterial compounds.
- For this reason metabolite was extracted and analysed using FTIR technique which reveals that variety of functional groups are present and the activity was due the specific functional groups.
- In pilot scale bioassay these strains shows efficient results by suppressing pathogens and also promote plant growth.
- These strains have no phytotoxic effect and considered as PGPR.
- Both of these strains have the potential to be used as a source of antibiotic production to control proliferation of selected human pathogens.

FUTURE PROSPECTS

- By evaluating all the optimum condition of strain its activity can be further enhanced in future
- Through modification at genetic level the antifungal potency of strain can further be enhanced
- A potent biocontrol product is expected if this strain of bacillus is amended with other Biocontrol agents.
- Its plant growth promoting ability should be further evaluated in field experiments.
- Antifungal compounds produce by AAM-1 and AK-47 can be purified, characterized and identified so that it can be used as biopesticide or biofungicide.
- Antibacterial compounds produce by AAM-1 and AK-47 can be purified, characterized and identified so that it can be used as a source of antibiotic production in future.

REFERENCES

1. Oerke, E. C., Dehne, H. W., Schönbeck, F., & Weber, A. (2012). Crop production and crop protection: estimated losses in major food and cash crops. Elsevier.
2. Bhardwaj, D., Ansari, M. W., Sahoo, R. K., &Tuteja, N. (2014). Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microbial cell factories*, *13*(1), 66.
3. Coelli, T. J., & Rao, D. S. (2005). Total factor productivity growth in agriculture: a Malmquist index analysis of 93 countries, 1980–2000. *Agricultural Economics*, *32*(s1), 115-134.
4. Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends in plant science*, *17*(8), 478-486.
5. Carvalho, F. P. (2006). Agriculture, pesticides, food security and food safety. *Environmental science & policy*, *9*(7), 685-692.
6. Raaijmakers, J. M., Vlami, M., & De Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek*, *81*(1), 537-547.
7. Compant, S., Duffy, B., Nowak, J., Clément, C., &Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*, *71*(9), 4951-4959.
8. Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T., & Steinberg, C. (2007). Soil health through soil disease suppression: which strategy from descriptors to indicators?. *Soil Biology and Biochemistry*, *39*(1), 1-23.
9. Nicholson, W. L. (2002). Roles of Bacillus endospores in the environment. *Cellular and Molecular Life Sciences*, *59*(3), 410-416.
10. Kumar, A., Prakash, A., &Johri, B. N. (2011). Bacillus as PGPR in crop ecosystem. In *Bacteria in agrobiology: crop ecosystems* (pp. 37-59). Springer Berlin Heidelberg.
11. Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual review of phytopathology*, *26*(1), 379-407.

12. Zhang, T., Shi, Z. Q., Hu, L. B., Cheng, L. G., & Wang, F. (2008). Antifungal compounds from Bacillus subtilis B-FS06 inhibiting the growth of Aspergillus flavus. *World Journal of Microbiology and Biotechnology*, *24*(6), 783.
13. McMichael, A. J., Haines, A., Slooff, R., &Kovats, S. (1996). Climate change and human health. An assessment by a Task Group on behalf of the World Health Organization the World Meteorological Organization and the United Nations Environment Programme.
14. Barnes, R. F., Miller, D. F., & Nelson, J. C. (1995). *Forages: An introduction to grassland agriculture* (Vol. 1). Ames, IA, USA: Iowa State University Press.
15. Tilman, D. (1999). Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. *Proceedings of the National Academy of Sciences*, *96*(11), 5995-6000.
16. Smil, V. (2000). Feeding the world: A challenge for the 21st century.
17. Lang, T., &Heasman, M. (2015). Food wars: The global battle for mouths, minds and markets. Routledge.
18. Strange, R. N., & Scott, P. R. (2005). Plant disease: a threat to global food security. *Annu. Rev. Phytopathol.*, *43*, 83-116.
19. Larkin, R. P. (2008). Relative effects of biological amendments and crop rotations on soil microbial communities and soilborne diseases of potato. *Soil Biology and Biochemistry*, *40*(6), 1341-1351.
20. Fiers, M., Edel-Hermann, V., Chatot, C., Le Hingrat, Y., Alabouvette, C., & Steinberg, C. (2012). Potato soil-borne diseases. A review. *Agronomy for Sustainable Development*, *32*(1), 93-132.
21. Guo, S., Zhang, J., Sun, H., Salse, J., Lucas, W. J., Zhang, H., ...& Min, J. (2013). The draft genome of watermelon (Citrulluslanatus) and resequencing of 20 diverse accessions. *Nature genetics*, *45*(1), 51-58.
22. Abramovitch, R. B., & Martin, G. B. (2004). Strategies used by bacterial pathogens to suppress plant defenses. *Current opinion in plant biology*, *7*(4), 356-364.
23. Gallitelli, D. (2000). The ecology of Cucumber mosaic virus and sustainable agriculture. *Virus Research*, *71*(1), 9-21.

24. Leyns, F., De Cleene, M., Swings, J. G., & De Ley, J. (1984). The host range of the genusXanthomonas. *The Botanical Review*, *50*(3), 308-356.
25. Ray, S. S., Jain, N., Arora, R. K., Chavan, S., &Panigrahy, S. (2011). Utility of hyperspectral data for potato late blight disease detection. *Journal of the Indian Society of Remote Sensing*, *39*(2), 161.
26. PRASHAD, D. (2013). *MYCOLOGY AND PLANT PATHOLOGY* (Doctoral dissertation, DrYashwant Singh Parmar University of Horticulture and Forestry).
27. Van Zanten, B. T., Verburg, P. H., Espinosa, M., Gomez-y-Paloma, S., Galimberti, G., Kantelhardt, J., ...&Raggi, M. (2014). European agricultural landscapes, common agricultural policy and ecosystem services: a review. *Agronomy for sustainable development*, *34*(2), 309-325.
28. Agrios, G. N. (2005). Plant diseases caused by fungi. *Plant pathology*, *4*.
29. Parmeter, J. R. (1970). *Rhizoctoniasolani, biology and pathology*. Univ of California Press.
30. Suprapta, D. N. (2012). Potential of microbial antagonists as biocontrol agents against plant fungal pathogens. *J ISSAAS*, *18*(2), 1-8.
31. Jones, D. A., Dickinson, M. J., Balint-Kurti, P. J., Dixon, M. S., & Jones, J. D. G. (1993). Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2, Cf-4, Cf-5, and Cf-9 genes for resistance to Cladosporiumfulvum. *Molecular Plant Microbe Interactions*, *6*, 348-348.
32. Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., Van Rooijen, G., Waterer, D. R., ...& Fischer, R. (2001). Tomato Ve disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Sciences*, *98*(11), 6511-6515.
33. Le Cointe, R., Simon, T. E., Delarue, P., Hervé, M., Leclerc, M., & Poggi, S. (2016). Reducing the Use of Pesticides with Site-Specific Application: The Chemical Control of Rhizoctoniasolani as a Case of Study for the Management of Soil-Borne Diseases. *PloS one*, *11*(9), e0163221.
34. Ogle, H. J. (1997). Disease Management: Chemical. Plant Pathogens and Plant Diseases. Berringer, Australia. Rockvale Publications.

35. McGrath, C., Mueller, D. S., & Robertson, A. E. (2014). Fungicide Thoughts for 2014 Season.
36. Pankey, G. A., &Sabath, L. D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clinical infectious diseases*, *38*(6), 864-870.
37. Anwar, A., Groom, M., & Sadler-Bridge, D. (2009). Garlic: from nature's ancient food to nematicide. *Pesticide news*, *84*, 18-20.
38. Aktar, W., Sengupta, D., & Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology*, *2*(1), 1-12.
39. Srinivasan, T. N. (1994). Data base for development analysis Data base for development analysis: An overview. *Journal of development Economics*, *44*(1), 3-27.
40. Behera, T. K., Singh, N., Kalda, T. S., & Gupta, S. S. (1999). Screening for shoot and fruit borer incidence in eggplant genotypes under Delhi condition. *Indian Journal of Entomology*, *61*(4), 372-375.
41. Lewis, L. B., Sloane, D. C., Nascimento, L. M., Diamant, A. L., Guinyard, J. J., Yancey, A. K., & Flynn, G. (2011). African Americans' access to healthy food options in South Los Angeles restaurants. *American journal of public health*.
42. Cooper, J., & Dobson, H. (2007). The benefits of pesticides to mankind and the environment. *Crop Protection*, *26*(9), 1337-1348.
43. McCauley, L. A., Anger, W. K., Keifer, M., Langley, R., Robson, M. G., &Rohlman, D. (2006). Studying health outcomes in farmworker populations exposed to pesticides. *Environmental health perspectives*, 953-960.
44. Edwards, C. A. (Ed.). (2013). *Environmental pollution by pesticides* (Vol. 3). Springer Science & Business Media.
45. Sykes, G., & Skinner, F. A. (Eds.). (2015). *Microbial aspects of pollution*. Elsevier.
46. Godish, T., Davis, W. T., & Fu, J. S. (2014). *Air quality*. CRC Press.
47. Clifford, A., Lang, L., Chen, R., Anstey, K. J., & Seaton, A. (2016). Exposure to air pollution and cognitive functioning across the life course–A systematic literature review. *Environmental research*, *147*, 383-398.

48. Yang, H., Huang, X., Thompson, J. R., & Flower, R. J. (2014). Soil pollution: Urban brownfields. *Science*, *344*(6185), 691-692.
49. Nega, A. (2014). Review on concepts in biological control of plant pathogens. *Journal of Biology, Agriculture and Healthcare*, *4*(27), 33-54.
50. Shurtleff, M. C., &Averre III, C. W. (1997). *Glossary of plant-pathological terms*. American Phytopathological Society (APS Press).
51. Heydari, A., &Pessarakli, M. (2010). A review on biological control of fungal plant pathogens using microbial antagonists. *Journal of biological sciences*, *10*(4), 273-290.
52. Jong-Chol, C., Tsukasa, M., Kazuo, A., Hiroaki, K., Haruki, Y., &Yasuo, O. (1987). Anti-Bacteroidesfragilis substance from rhubarb. *Journal of ethnopharmacology*, *19*(3), 279-283.
53. Mathew, I. L., Singh, D., Singh, R. P., &Tripathi, C. P. M. (2014). Bacillus thuringiensis: The biocontrol agent in a food web perspective.
54. Elad, Y. (1996). Mechanisms involved in the biological control ofBotrytiscinerea incited diseases. *European Journal of Plant Pathology*, *102*(8), 719-732.
55. Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends in biotechnology*, *20*(8), 338-343.
56. Whipps, J. M., &Lumsden, R. D. (2001). Commercial use of fungi as plant disease biological control agents: status and prospects. *Fungal biocontrol agents: progress, problems and potential*, 9-22.
57. Scher, F. M., & Baker, R. (1982). Effect of Pseudomonas putida and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. *Phytopathology*, *72*(12), 1567-1573.
58. Ikotun, T., &Adekunle, F. (1990). Inhibition of growth of some plant pathogenic fungi by some antagonistic microorganisms isolated from soil. *Journal of basic microbiology*, *30*(2), 95-98.
59. Rogowsky, P. M., Powell, B. S., Shirasu, K., Lin, T. S., Morel, P., Zyprian, E. M., ...&Kado, C. I. (1990). Molecular characterization of the vir regulon of Agrobacterium

tumefaciens: complete nucleotide sequence and gene organization of the 28.63-kbp regulon cloned as a single unit. *Plasmid*, *23*(2), 85-106.

60. Haas, D., &Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology*, *3*(4), 307-319.
61. Stein, T. (2005). Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Molecular microbiology*, *56*(4), 845-857.
62. Schisler, D. A., Slininger, P. J., Behle, R. W., & Jackson, M. A. (2004). Formulation of Bacillus spp. for biological control of plant diseases. *Phytopathology*, *94*(11), 1267-1271.
63. Logan, N. A., Berge, O., Bishop, A. H., Busse, H. J., De Vos, P., Fritze, D., ... &Seldin, L. (2009). Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *International journal of systematic and evolutionary microbiology*, *59*(8), 2114-2121.
64. Earl, J. S. (2007, December). The Blackwell companion to social movements. In *Blackwell Publishing Ltd*.
65. Wipat, A., & Harwood, C. R. (1999). The Bacillus subtilis genome sequence: the molecular blueprint of a soil bacterium. *FEMS microbiology ecology*, *28*(1), 1-9.
66. Shoda, M. (2000). Bacterial control of plant diseases. *Journal of bioscience and bioengineering*, *89*(6), 515-521.
67. Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of Bacillus species for industrial production. *Canadian journal of microbiology*, *50*(1), 1-17.
68. Vecchi, E., & Drago, L. (2006). Lactobacillus sporogenes or Bacillus coagulans: misidentification or mislabelling?. *International Journal of Probiotics and Prebiotics*, *1*(1), 3.
69. Cook, R. J., & Baker, K. F. (1983). *The nature and practice of biological control of plant pathogens*. American Phytopathological Society.
70. Sandström, E., Nilsson, C., Hejdeman, B., Bråve, A., Bratt, G., Robb, M., ...&Aboud, S. (2008). Broad immunogenicity of a multigene, multiclade HIV-1 DNA vaccine boosted with heterologous HIV-1 recombinant modified vaccinia virus Ankara. *Journal of Infectious Diseases*, *198*(10), 1482-1490.

71. Emmert, E. A., &Handelsman, J. (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology letters*, *171*(1), 1-9.
72. Kloepper, J. W., Ryu, C. M., & Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by Bacillus spp. *Phytopathology*, *94*(11), 1259-1266.
73. Montesinos, E. (2007). Antimicrobial peptides and plant disease control. *FEMS microbiology letters*, *270*(1), 1-11.
74. Ongena, M., & Jacques, P. (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends in microbiology*, *16*(3), 115-125.
75. Darko, G., Akoto, O., &Oppong, C. (2008). Persistent organochlorine pesticide residues in fish, sediments and water from Lake Bosomtwi, Ghana. *Chemosphere*, *72*(1), 21-24.
76. Peng, G., McGregor, L., Lahlali, R., Gossen, B. D., Hwang, S. F., Adhikari, K. K., ...& McDonald, M. R. (2011). Potential biological control of clubroot on canola and crucifer vegetable crops. *Plant Pathology*, *60*(3), 566-574.
77. Cawoy, H., Bettiol, W., Fickers, P., &Ongena, M. (2011). Bacillus-based biological control of plant diseases. *Pesticides in the modern world—pesticides use and management. InTech, Rijeka*, 273-302.
78. Fravel, D. R. (2005). Commercialization and implementation of biocontrol 1. *Annu. Rev. Phytopathol.*, *43*, 337-359.
79. Benhamou, N., & Chet, I. (1997). Cellular and Molecular Mechanisms Involved in the Interaction between Trichoderma harzianum and Pythium ultimum. *Applied and Environmental Microbiology*, *63*(5), 2095-2099.
80. Milgroom, M. G., &Cortesi, P. (2004). Biological control of chestnut blight with hypovirulence: a critical analysis. *Annu. Rev. Phytopathol.*, *42*, 311-338.
81. Ordentlich, A., Elad, Y., & Chet, I. (1988). The role of chitinase of Serratiamarcescens in biocontrol of Sclerotiumrolfsii. *Phytopathology*, *78*(1), 84-88.
82. Kageyama, K., & Nelson, E. B. (2003). Differential inactivation of seed exudate stimulation of Pythium ultimum sporangium germination by Enterobacter cloacae influences biological control efficacy on different plant species. *Applied and environmental microbiology*, *69*(2), 1114-1120.

83. Haas, D., &Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology*, *3*(4), 307-319.
84. Nürnberger, T., Brunner, F., Kemmerling, B., &Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological reviews*, *198*(1), 249-266.
85. Yadeta, K. A., &Thomma, B. P. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens.
86. Zhang, L. H., & Dong, Y. H. (2004). Quorum sensing and signal interference: diverse implications. *Molecular microbiology*, *53*(6), 1563-1571.
87. Voisard, C., Keel, C., Haas, D., &Dèfago, G. (1989). Cyanide production by Pseudomonas fluorescens helps suppress black root rot of tobacco under gnotobiotic conditions. *The EMBO Journal*, *8*(2), 351.
88. Ongena, M., & Jacques, P. (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends in microbiology*, *16*(3), 115-125.
89. Fickers, P. (2012). Antibiotic compounds from Bacillus: why are they so amazing. *Am J BiochemBiotechnol*, *8*(1), 38-43.
90. Felnagle, E. A., Jackson, E. E., Chan, Y. A., Podevels, A. M., Berti, A. D., McMahon, M. D., & Thomas, M. G. (2008). Nonribosomal peptide synthetases involved in the production of medically relevant natural products. *Molecular pharmaceutics*, *5*(2), 191-211.
91. Ryoo, H. D., Gorenc, T., & Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Developmental cell*, *7*(4), 491-501.
92. D'aes, J., De Maeyer, K., Pauwelyn, E., &Höfte, M. (2010). Biosurfactants in plant–Pseudomonas interactions and their importance to biocontrol. *Environmental microbiology reports*, *2*(3), 359-372.
93. Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., Vicente, A. D., &Bloemberg, G. (2007). Isolation and characterization of antagonistic Bacillus subtilis

strains from the avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology*, *103*(5), 1950-1959.

94. Thasana, N., Prapagdee, B., Rangkadilok, N., Sallabhan, R., Aye, S. L., Ruchirawat, S., &Loprasert, S. (2010). Bacillus subtilis SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated β-amino acid. *FEBS letters*, *584*(14), 3209-3214.
95. Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B., &Fickers, P. (2009). Bacillus amyloliquefaciens GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. *Microbial Cell Factories*, *8*(1), 63.
96. Inaoka, T., Takahashi, K., Ohnishi-Kameyama, M., Yoshida, M., & Ochi, K. (2003). Guanine nucleotides guanosine 5′-diphosphate 3′-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in Bacillus subtilis. *Journal of Biological Chemistry*, *278*(4), 2169-2176.
97. Kumar, A., Saini, P., & Shrivastava, J. N. (2009). Production of peptide antifungal antibiotic and biocontrol activity of Bacillus subtilis.
98. Shanker, S., Pindi, P., & Shankar, G. (2014). Characterization of effective bio-control agent Bacillus sp. SRB 27 with high salt tolerance and thermostability isolated from forest soil sample. *African Journal of Biotechnology*, *13*(25).
99. Jiang, C., Shi, J., Liu, Y., & Zhu, C. (2014). Inhibition of Aspergillus carbonarius and fungal contamination in table grapes using Bacillus subtilis. *Food Control*, *35*(1), 41-48.
100. Imran, H., Darine, T. H., & Mohamed, E. G. (2012). In vitro screening of soil bacteria for inhibiting phytopathogenic fungi. *African Journal of Biotechnology*, *11*(81), 14660-14670.
101. Zhang, T., Shi, Z. Q., Hu, L. B., Cheng, L. G., & Wang, F. (2008). Antifungal compounds from Bacillus subtilis B-FS06 inhibiting the growth of Aspergillus flavus. *World Journal of Microbiology and Biotechnology*, *24*(6), 783..

102. Al-Saraireh, H., Al-Zereini, W. A., & Tarawneh, K. A. (2015). Antimicrobial Activity of Secondary Metabolites from a Soil Bacillus sp. 7B1 Isolated from South Al-Karak, Jordan. *Jordan Journal of Biological Sciences*, *8*(2).

103. Kim, P. I., Ryu, J., Kim, Y. H., & Chi, Y. T. (2010). Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from Bacillus subtilis CMB32 for control of Colletotrichum gloeosporioides. *J Microbiol Biotechnol*, *20*(1), 138-145.

104. Barrios-González, J., & Mejía, A. (1996). Production of secondary metabolites by solid-state fermentation. *Biotechnology annual review*, *2*, 85-121.

105. Swain, M. R., Kar, S., Padmaja, G., & Ray, R. C. (2006). Partial Characterization and Optimization of Production of Extracellular"-amylase from Bacillus subtilis Isolated from Culturable Cow Dung Microflora. *Polish Journal of Microbiology*, *55*(4), 289-296.

106. Gupta, R., & Ramnani, P. (2006). Microbial keratinases and their prospective applications: an overview. *Applied Microbiology and Biotechnology*, *70*(1), 21.

107. Abdel-Aziz, S. M., Hamed, H. A., Mouafi, F. E., & Abdelwahed, N. A. (2011). Extracellular metabolites produced by a novel strain, Bacillus alvei NRC-14: 3. Synthesis of a bioflocculant that has chitosan-like structure. *Life Sci. J*, *8*(4), 883-890.

108. Ashwini, N., & Srividya, S. (2014). Potentiality of Bacillus subtilis as biocontrol agent for management of anthracnose disease of chilli caused by Colletotrichum gloeosporioides OGC1. *3 Biotech*, *4*(2), 127-136.

109. Kumar, P., Dubey, R. C., & Maheshwari, D. K. (2012). Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological research*, *167*(8), 493-499.

110. Rana, A., Saharan, B., Nain, L., Prasanna, R., & Shivay, Y. S. (2012). Enhancing micronutrient uptake and yield of wheat through bacterial PGPR consortia. *Soil science and plant nutrition*, *58*(5), 573-582.

111. Heidarzadeh, N., & Baghaee-Ravari, S. (2015). Application of Bacillus pumilus as a potential biocontrol agent of Fusarium wilt of tomato. *Archives of Phytopathology and Plant Protection*, *48*(13-16), 841-849.

112. Huang, X., Zhang, N., Yong, X., Yang, X., & Shen, Q. (2012). Biocontrol of Rhizoctonia solani damping-off disease in cucumber with Bacillus pumilus SQR-N43. *Microbiological Research*, *167*(3), 135-143.

113. Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology letters*, *32*(11), 1559-1570.

114. Saha, R., Saha, N., Donofrio, R. S., & Bestervelt, L. L. (2013). Microbial siderophores: a mini review. *Journal of basic microbiology*, *53*(4), 303-317.

115. Prashar, P., Kapoor, N., & Sachdeva, S. (2013). Isolation and Characterization of Bacillus sp with In-vitro Antagonistic Activity against Fusarium oxysporum from Rhizosphere of Tomato. *Journal of Agricultural Science and Technology*, *15*, 1501-1512.