ANTAGONISTIC BIOACTIVITIES OF ENDOPHYTIC ACTINOMYCETES

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject: Biochemistry)

By

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(L-2012-BS-286-M)

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Antagonistic bioactivities of endophytic actinomycetes"** for the degree of Master of Science, in the subject of **Microbiology (Minor Subject: Biochemistry)** of the Punjab Agricultural University, Ludhiana is bonafide research work carried out by **Priyanka Kamboj (L-2012-BS-286-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Antagonistic bioactivities of endophytic actinomycetes"** submitted by **Priyanka Kamboj (L-2012-BS-286-M)** to Punjab Agricultural University, Ludhiana, in the partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Microbiology (Minor Subject: Biochemistry)** has been approved by the Student's Advisory Committee along with the Head of the Department after oral examination on the same.

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Abstract

The present study was conducted with a view to evaluate antifungal activity of endophytic actinomycetes isolated from medicinal plants against phytopathogenic fungi. Out of seventy isolates, 11 isolates (6 from *Azadirachta indica* A. Juss, 3 from *Emblica officinalis*, 1 from *Aloe vera* and 1 from *Ocimum sanctum*) were displaying antagonistic activity against *Alternaria alternata, Fusarium oxysporum and Rhizoctonia solani*. None of the isolates exhibited antifungal activity against *Sclerotium rolfsii*. All the isolates were screened for hydrolytic enzyme production, out of which 47 isolates produced amylase enzyme, 25 produced protease enzyme and 16 were having ability to produce chitinase. The extracellular chitinase activity of AR3 an isolate of *Emblica officinalis* was observed to be 0.083 U/ml by using 0.6% colloidal chitin concentration and 0.0639 U/ml with 1% colloidal chitin as substrate concentration. Isolate O9 obtained from *Ocimum sanctum* exhibited 0.080 U/ml chitinase production with 0.6% substrate concentration and 0.0656 U/ml with 1% colloidal chitin concentration. Both the isolates displayed more extracellular chitinase production with 0.6% as compared to 1% colloidal chitin concentration. Scanning electron microscopy (SEM) was conducted to study the effect of potential actinomycete isolates (AR3 and O9) on fungal cell wall. The results revealed the rupture of the *F. oxysporum* mycelial cell wall at the area of interaction between *F. oxysporum* with AR3 and O9. The endophytic actinomycete strain O9 was identified as *Streptomyces rochei* strain KMB 1 by 16S rDNA sequencing. Both the isolates were found to be most promising in terms of seed germination and wilt control under green house conditions. Therefore these isolates may be used as potential biocontrol agents against *Fusarium* wilt caused by *Fusarium oxysporum.*

Keywords: Endophytic actinomycetes, antifungal activity, phytopathogenic fungi, chitinase, biocontrol agent

Signature of Major Advisor **Signature of the Student**

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ਸਾਰਾਂਸ਼

ਹਾਲ ਦੀ ਘੋਖ *ਐਂਡੋਫਿਟਿਕ ਐਕਟੀਨੋਮਾਈਸੀਟੀਜ਼* ਵੱਖ ਔਸਦੀ ਬੂਟਿਆਂ ਦਾ ਫਾਈਟੋਪੈਥੋਜੈਨਿਕ ਉੱਲੀ ਵਿਰੋਧ ਮੁਲਾਂਕਣ ਕਰਨ ਲਈ ਕੀਤੀ ਗਈ । ਸੱਤਰ ਵੱਖਾਂ ਵਿੱਚੋਂ ਗਿਆਰਾਂ ਵੱਖਾਂ (6 ਐਜ਼ਾਡਾਇਰੈਕਟਾ ਇੰਡੀਕਾ ਏ. ਜਸ ਤੋਂ, ਤਿੰਨ ਐਂਬਲੀਕਾ ਔਫੀਸੀਨੈਲਿਸ ਤੋਂ, ਇੱਕ ਐਲੋਵੀਰਾ ਅਤੇ ਇੱਕ ਔਸੀਮਮ ਸੈਂਟਮ ਤੋਂ ਵਿਰੋਧੀ ਗਤੀਵਿਧੀਆਂ *ਅਲਟਰਨੇਰੀਆ ਅਲਟਰਨਾਟਾ*, *ਫੁਜ਼ੇਰੀਅਮ ਔਕਸੀਸਪੋਰਮ* ਅਤੇ *ਰਾਜ਼ੋਕਟੋਨੀਆਂ ਸੋਲੇਨਾਈ* ਦੇ ਵਿਰੁੱਧ ਵਿਰੋਧ ਦਰਸਾਇਆ। ਇਹ ਵੱਖਾਂ ਵਿੱਚੋਂ ਕਿਸੇ ਨੇ ਵੀ ਉੱਲੀ ਵਿਰੋਧੀ ਸਰਗਰਮੀਆਂ *ਸਿਕਲੇਰੋਸ਼ੀਅਮ ਰੋਲਫਸਾਈ* ਦੀ ਵਿਰੋਧਤਾ ਨਹੀਂ ਵਿਖਾਈ । ਸਾਰੀਆਂ ਵੱਖਾਂ ਦੀ ਹਾਈਡ੍ਰੋਲੀਟਿਕ ਇੰਜ਼ਾਇਮ ਉਤਪਾਦਨ ਲਈ ਸਕਰੀਨਿੰਗ ਕੀਤੀ ਗਈ ਜਿਨ੍ਹਾਂ ਵਿੱਚੋਂ 47 ਵੱਖਾਂ ਨੇ ਅਮਾਈਲੇਜ਼ ਇੰਜ਼ਾਇਮ ਦਾ ਉਤਪਾਦਨ ਕੀਤਾ, 25 ਨੇ ਪ੍ਰੋਟੀਏਜ਼ ਇੰਜ਼ਾਇਮ ਅਤੇ 16 ਦੀ ਕਾਈਟੀਨੇਜ਼ ਉਤਪਤੀ ਦੀ ਸਮਰੱਥਾ ਸੀ । AR3 ਇੱਕ ਐਂਬਲੀਕਾ ਔਫੀਸੀਨੈਲਿਸ ਦੀ ਵੱਖ ਦੀ ਐਕਸਟਰਾ ਸੈਲੂਲਰ ਕਾਈਟੀਨੇਜ਼ ਸਰਗਰਮੀ 0.083 ਯੂਨਿਟ/ਮਿਲੀਲੀਟਰ, 0.6% ਕੋਲਾਈਡਲ ਕਾਈਟਿਨ ਘਣਤਾ ਪ੍ਰਯੋਗ ਕਰਕੇ ਵਿਖਾਈ ਗਈ ਅਤੇ 0.0639 ਯੂਨਿਟ/ਮਿਲੀਲੀਟਰ, 1% ਕੋਲਾਈਡਲ ਕਾਈਟਿਨ ਸਬਸਟ੍ਰੇਟ ਘਣਤਾ ਦੇ ਤੌਰ ਤੇ ਵੇਖੀ ਗਈ । O9 ਵੱਖ ਔਸੀਮਮ ਸੈਂਟਮ ਤੋਂ ਲਈ ਗਈ, 0.080 ਯੂਨਿਟ/ਮਿਲੀਲੀਟਰ, ਕਾਈਟੀਨੇਜ਼ ਉਤਪੱਤੀ 0.6% ਸਬਸਟ੍ਰੇਟ ਘਣਤਾ ਦੇ ਨਾਲ ਅਤੇ 0.0656 ਯੂਨਿਟ/ਮਿਲੀਲੀਟਰ, 1% ਕੋਲਾਈਡਲ ਕਾਈਟਿਨ ਘਣਤਾ ਦੇ ਨਾਲ ਦਰਸਾਈ ਗਈ । ਦੋਵੇਂ ਵੱਖਾਂ ਨੇ ਹੋਰ ਜ਼ਿਆਦਾ ਐਕਸਟਰਾਸੈਲੂਲਰ ਕਾਈਟੀਨੇਜ਼ ਉਤਪੱਤੀ 1% ਕੋਲਾਈਡਲ ਕਾਈਟਿਨ ਘਣਤਾ ਦੇ ਮੁਕਾਬਲੇ 0.6% ਕੋਲਾਈਡਲ ਕਾਈਟਿਨ ਘਣਤਾ ਨਾਲ ਦਰਸਾਈ । ਸਕੈਨ ਕੀਤਾ ਇਲੈਕਟ੍ਰੋਨ ਮਾਈਕ੍ਰੋਸਕੋਪੀ (ਸੈਮ) ਫੰਗਲ ਸੈਨ ਕੰਧ ਤੇ ਸੰਭਾਵੀ ਐਕਟੀਨੋਮਾਈਸੀਟੀਜ਼ ਵੱਖਰਾ (AR3 ਅਤੇ O9) ਦੇ ਪ੍ਰਭਾਵ ਨੂੰ ਅਧਿਐਨ ਕਰਨ ਲਈ ਕੀਤਾ ਗਿਆ । ਨਤੀਜੇ ਦੱਸਦੇ ਹਨ ਕਿ AR3 ਅਤੇ O9 ਨਾਲ *ਐਫ. ਔਕਸੀਸਪੋਰਮ* ਵਿਚਕਾਰ ਦਖਲ ਦੇ ਖੇਤਰ ਤੇ *ਐਫ. ਔਕਸੀਸਪੋਰਮ* ਮਾਈਸੀਲੀਅਲ ਸੈੱਲ ਕੰਧ ਦੇ ਹਿੱਸੇ ਤਿੜਕੇ ਹੋਏ ਹਨ । ਐਂਡੋਫਿਟਿਕ ਐਕਟੀਨੋਮਾਈਸੀਟੀਜ਼ ਖਿਚਾਅ O9 ਦੀ 16S rDNA ਕ੍ਰਮਵਾਰ ਨਾਲ *ਸਟਰੈਪਟੋਮਾਈਸਿਜ਼ ਰੋਸਾਈ* ਖਿਚਾਅ KMB-1 ਦੇ ਤੌਰ ਤੇ ਪਛਾਣ ਕੀਤੀ ਗਈ । ਦੋਵੇਂ ਵੱਖਾਂ ਨੂੰ ਬੀਜ ਉੱਤਪਤੀ ਅਤੇ ਮੁਰਝਾਉਣ ਨਿਰਧਾਰਣ ਲਈ ਹਰੀ ਘਰ ਹਾਲਤਾਂ ਵਿੱਚ ਸਭ ਤੋਂ ਜਿਆਦਾ ਠੀਕ ਪਾਇਆ ਗਿਆ । ਇਸ ਲਈ ਇਹਨਾਂ ਵੱਖਾਂ *ਫੁਜ਼ੇਰੀਅਮ* ਵਿਰੁੱਧ ਸੰਭਾਵੀ ਬਾਇਓਕੰਟਰੋਲ ਏਜੰਟ *ਫੁਜ਼ੇਰੀਅਮ ਔਕਸੀਸਪੋਰਮ* ਦੇ ਕਾਰਨ ਕਰਨ ਦੇ ਤੌਰ ਤੇ ਵਰਤਿਆ ਜਾ ਸਕਦਾ ਹੈ।

ਮੁੱਖ ਸ਼ਬਦ: ਐਂਡੋਫਿਟਿਕ ਐਕਟੀਨੋਮਾਈਸੀਟੀਜ਼, ਉੱਲੀ ਵਿਰੁੱਧ ਸਰਗਰਮੀ, ਫਾਈਟੋਪੈਥੋਜੈਨਿਕ ਉੱਲੀ, ਕਾਈਟੀਨੇਜ਼, ਬਾਇਓਕੰਟਰੋਲ ਏਜੰਟ।

ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Currently, there is an increasing public concern regarding the continued use of agrochemicals to control the phytopathogenic fungi. This awareness relies mainly in the noxious effects of the pesticides on the environmental and human health (Cardoso *et al* 2010). Several efforts have been made to find less hazardous options for controlling these plant pathogens among which the biological control using the microorganisms has been demonstrated to be a feasible alternative (Zucchi *et al* 2008) but it is not widely used on commercial scale (Bressan 2003, Medeiros *et al* 2012). Among the biocontrol agents, endophytic microorganisms have raised special attention, mainly due to their crucial role on host-plant development (Firakova *et al* 2007). Since these symbionts are systemically distributed in the plant via metabolic translocation colonizing the same niche of the phytopathogens, they are interesting candidates for the biological control (Rai *et al* 2007). Bacterial endophytes have been demonstrated to inhibit the plant pathogen development as well as to promote the growth of host plants (Hasegawa *et al* 2006). A deeper understanding of the endophyte-host plant interaction can enhance the use of these microorganisms in the agriculture (Araujo *et al* 2000, Lee *et al* 2008).

Actinomycetes have been largely exploited mainly because their capability to produce bioactive compounds, such as antibiotics and lytic enzymes (El-Tarabily *et al* 1997, Berdy 2005 and Clardy *et al* 2006). These compounds have often been related as one of the most important tools to control the soil-borne diseases (Buchenauer 1998) with low environmental impact and toxic effect for humans as well as for animals and well-desired traits for new consumer's requirements (Cardoso *et al* 2010).

The actinomycetes are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil. They play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials. They account for a high proportion of soil microbial biomass and are also important in soil biodegradation and humus formation as they recycle the nutrients associated with recalcitrant polymers, such as chitin, keratin, and lignocelluloses (Goodfellow and Williams 1983, McCarthy and Williams 1992 and Stach and Bull 2005). Actinomycetes are the most economically significant prokaryotes, producing more than half of the bioactive compounds including antibiotics, immunosuppressive agents, antitumor agents and enzymes (Qin *et al* 2009).

Actinomycetes are the Gram positive filamentous bacteria .They represents a large portion of rhizospheric microbial community and are important in the rhizosphere because they can influence plant growth and protect plant roots against invasion by root pathogenic fungi (Crawford *et al* 1993, Tokala *et al* 2002). But in case of aquatic system, they play a

great role in carbon cycle due to their ability to grow at low concentrations of carbonaceous substances and degrade recalcitrant organic matter.

Actinomycetes are also found as endophytes that colonize the plant tissues and posses the potential to produce bioactive compounds similar to their host. Endophytic actinomycetes are those bacteria that reside in the tissue of living plants and do not visibly harm the plants (Stone *et al* 2000). Previously, *Frankia* was recognized as the only endophytic actinomycetes that reside in nodule of non-leguminous plants by fixing nitrogen (Benson and Silvester 1993). Recently, reports of non-*Frankia* endophytic actinomycetes have been significantly increased (Bunyoo *et al* 2009). They were isolated from crop plants such as wheat (Conn and Franco 2004), barley (Coombs *et al* 2004), rice (Tian *et al* 2007) and medicinal plants (Verma *et al* 2009).

Endophytic actinomycetes are particularly, considered as sources of bioactive compounds and various novel compounds (Igarashi *et al* 2007) and secondary metabolites produced by these microbial endophytes could serve as prospective resources of antimicrobial substances, antioxidants, cytotoxic compounds, growth hormones and hydrolytic enzymes of biotechnological applications (Tan and Zou 2001,Yu *et al* 2010). Moreover, most endophytic actinomycetes of medicinal plants can produce important compounds and some of them are new chemical structure (Ezra *et al* 2004, Liu *et al* 2009). The actinomycetes, especially *Streptomyces* species are valuable economical and biotechnological bacteria by providing over two third of antibiotics and bioactive compounds used these days (Baltz 1998). New antibiotics from endophytic *Streptomyces* sp. alnumycin, munumbicins A to D and coronamycins have been reported (Bieber *et al* 1998, Castillo *et al* 2002). Recently, two novel antitumor anthraquinones, lupinacidins A and B were isolated from a new endophytic *Micromonospora* sp. (Igarashi *et al* 2007). Actinomycetes can promote the growth of many field crops by producing plant growth-promoting substances like indole-3-acetic acid (IAA) to help the growth of roots and by fixing nitrogen from the atmosphere and potential sources of novel natural products for exploitation in medicine, agriculture and industry (Kumar *et al* 2011).

Soil actinomycetes have revealed their wide antifungal activity (Tinatin and Nuzrat 2006). They have been shown to protect several different plants to various degrees from soil-borne fungal pathogens (Reddi and Rao 1971). Endophytic actinomycetes which associated with medicinal plants also play important role in protecting their host from phytopathogenic invasions. Previous investigations proved that the endophytic actinomycetes are having high ability to inhibit phytopathogenic fungi is mainly by production of bioactive compounds, such as antibiotics and cell wall degrading enzymes and highlighted their importance as candidates for further investigation in the biocontrol of phytopathogens. *Streptomyces* sp. inhibits the development of a broad range of phytopathogenic fungi and bacteria (Berg *et al* 2001). Actinomycetes produce extracellular enzymes e.g. proteases, chitinases, amylases etc.

Actinomycetes strains are having antagonistic properties can serve as gene donors in developing resistant transgenic plants or use as soil amendments or biofungicide in biological control of the pathogens. In addition, endophytes are known to compete phytopathogens for nutrients (El-Tarabily and Sivasithamparam 2006). They produce siderophore to bind Fe^{3+} from the environment and help to improve nutrient uptake. Endophytic actinomycetes were also reported to hold the ability of triggering plant induced systemic resistance (ISR) (Haas and Defago 2005). *Streptomycetes* are major contributors to the biological buffering of soils and have roles in decomposition of organic matter conducive to crop production (Dastager *et al* 2006). Actinomycetes may also play roles in the colonization and formation of mycorrhiza and certain actinomycetes appear to be hyperparasites of fungi, fungal oospores, or fungal sclerotia. A few actinomycetes have also been shown to produce herbicidal and insecticidal compounds (Crawford *et al* 1993). *Streptomyces* species and a few other *Streptomyces* are among the most producers of biologically active substances such as vitamins, alkaloids, plant growth factors and enzyme inhibitors (Zarandi *et al* 2009).

Keeping in view the extensive utility of endophytic actinomycetes, the present study has been undertaken to fulfill the following objectives:

- To study the antagonistic bioactivities of endophytic actinomycetes isolates against phytopathogenic fungi.
- To evaluate quantitative production of extracellular chitinase by potential isolate**.**
- Molecular characterization of potential isolate through 16S rDNA techniques.

CHAPTER II

REVIEW OF LITERATURE

Endophytes are organisms inhabiting plant tissues whether they are neutral, beneficial or detrimental to hosts (Sturz *et al* 2000). In recent years, many researchers have focused on the bioactivities of endophytes (Strobel and Daisy 2003). In endosphere, endophytes are in protected environment that gives them competitive advantage over organisms of the rhizosphere and phyllosphere. Because of living in a relatively steady environment, endophytes may be much more bioactive (Dowler and Waiver 1974, Andrews 1992). The future use of biological–chemical combinations of endophytes in combination with commercial pesticides applied to seed or seedling could lead to synergistic effects on one or multiple disease causing agents. Moreover, the biological agent could provide continuing effective control into the crops production cycle (Shoda 2000). Now a day, it is well known that excess use of fungicides leads to severe large-scale pollution. With increasing awareness of environmental protection, biological strategies using endophytic actinomycetes are new promising alternatives to solving the contamination problems. Therefore, biological pesticides especially endophytic actinomycetes are an effective and environmental tool in controlling plant diseases instead of using chemical pesticides (Kong and Ding 2001).

Actinomycetes are also found inside plants as endophytes. Actinomycetes are Gram-positive bacteria and are the most widely distributed group of microorganisms in nature. They are also well known as saprophytic soil inhabitants (Takizawa *et al* 1993). They are characterized by having a high G+C content (>55%) in their DNA (Bizuye *et al* 2013) and are known to constitute a large part of the rhizosphere microbiota. Endophytic actinomycetes are particularly considered as potential sources of bioactive compounds and various novel compounds. Most actinomycetes in soil belong to the genus *Streptomyces* (Goodfellow and Simpson 1987) and 75% of the biologically active compounds are produced by this genus. Actinomycetes occur in the plant rhizosphere soil and produce active compounds (Suzuki *et al* 2000). The genus *Frankia* was considered as the only nitrogen fixing bacteria in non-leguminous plants which is extensively studied (Benson and Silvester 1993). Matsumoto *et al* (1998) isolated actinomycetes from fallen leaves and genus *Microbispora* was frequently found. Actinomycetes are the main source of antibiotics and endophytic actinomycetes isolated from medicinal plants have considerable development potential (Mini Priya 2012) and have ability to produce a variety of bioactive metabolites including antibiotics, plant growth promoters, plant growth inhibitors and cell wall-degrading enzymes such as cellulases, hemicellulases and chitinases which can be applied to agricultural packages (Getha and Vikineswary 2002, Igarashi *et al* 2002, Taechowisan *et al* 2003, Hasegawa *et al* 2006 and Sharma 2014). The species belonging to the genus *Streptomyces* constitute 50% of the

total population of soil actinomycetes and 75-80% of the commercially and medicinally useful antibiotics have been derived from this genus. This is true because metabolites of endophytes have the potential to inhibit a numbers of pathogenic microorganisms (Gurney and Mantle 1993). Endophytic actinomycetes live in close association with their host plants and the long co-evolution relationship, there is a real possibility that genes involved in natural products biosynthesis could be exchanged via horizontal gene transfer (HGT) between microbes and plants, resulting in production of plant-derived compounds by a microbe such as the paclitaxel-producing *Kitasatospora* sp. isolated from *Taxus baccata* in Italy (Caruso *et al* 2000, Janso and Carter 2010). This is the first report of production of taxol from endophytic actinomycetes.

A variety of actinomycetes inhabiting in a wide range of plants as symbionts, parasites or saprophytes were reported and most of them belong to the genera, *Streptomyces* and *Microbispora* (Matsumoto *et al* 1998). A total of 55 separate isolates were obtained from 20 plants of *Azadirachta indica* A. Juss. The dominant genus was *Streptomyces,* followed by *Streptosporangium*, *Microbispora*, *Streptoverticillium*, *Sacchromonospora* sp. and *Nocardia* (Verma *et al* 2009).

Ten endophytic actinomycetes strains were isolated from healthy shoots and roots of *Aquilaria crassna.* Analysis of 16S rDNA sequencing shows that they belong to genera *Streptomyces, Nonomuraea, Actinomadura, Pseudonocardia and Nocardia* (Nimnoi *et al* 2010). Moussa *et al* (2011) assigned the isolates to the genus level, which was sufficient to cover a broad spectrum of actinomycete genera in the *in vitro* pilot study, as 17 of the isolates were *Streptomyces*, 12 were *Nocardioides*, 7 were *Kitasatosporia*, 6 were *Pseudonocardia*, 3 were *Actinomadura*, 2 were *Kibdelosporangium*, 2 were *Nocardia* and 2 were *Nocardiopsis.*

Additionally, actinomycetes are important for the production of enzymes, like chitinase (eg. *Streptomyces viridificans*), cellulases (*Thermonospora* sp.), peptidases, proteases (*Nocardia* sp.), Xylanases *(Microbispora* sp.), ligninases (*Nocardia autotrophica*), amylases (*Thermomonospora curvata*), sugar isomerases (*Actinoplanes missouriensis*), pectinase, hemicellulase and keratinase (Solans and Vobis 2003).

Hydrolytic Enzymes

Many microbes particularly bacteria and fungi are currently employed for the production of various industrial enzymes (Souza and Oliveira 2010). Hydrolases cover more than 75% of commercially used enzymes and are often in great demand. These are used in a crude form to make the process economically viable and also to meet the demand of enzyme at a large scale. A wide array of enzymes and their products applied in biotechnological industries and biomedical fields has been reported from various genera of actinomycetes. Actinomycetes have been continuously employed for the production of proteases, cellulases, chitinases, amylases, xylanases, and other enzymes. Several enzymes produced by the

actinomycetes are becoming industrially important for either product manufacture or chemical diagnosis. Kavya *et al* (2012) was carried a study on actinomycetes which have the potential to produce antibiotics as well as enzymes from under-explored mangrove soils collected from Coringa mangrove forest, Andhra Pradesh. Screening tests were performed to determined that *Streptomyces* sp. strain A exhibited amylase (0.59 µmol/ml/min), cellulase (1.388 µmol/ml/min), L-asparaginase (3.67 µol/ml/min), chitinase (1.356 µmol/ml/min) and protease (0.248 µmol/ml/min) activities and specific activities of cellulase, L-asparaginase, chitinase and protease were found to be 10.475 µmol/ml/mg protein, 10.194 µmol/ml/mg protein, 6.027 µmol/ml/mg protein and 0.4332 µmol/ml/mg protein respectively.

Jeffrey *et al* (2011) did the preliminary screening of actinomycetes which showed that 40 isolates produced various bioactivities such as cellulase (45%), galacto-mannanase (5%), xylanase (12.5%), protease (12.5%) and lipase (32.5%). 212 isolates of actinomycetes were isolated from soil samples collected in the area of Serdang, Bangi, Petaling Jaya and Putrajaya. 91 showed the ability to degrade cellulose, 16 for mannan and 90 for xylan (Jeffrey *et al* 2007).

Amylases

Actinomycetes secrete amylases to the outside of the cells to carry out extracellular digestion. Amylases are starch degrading amylolytic enzymes of great significance in biotechnological applications such as food industry, fermentation and textile to paper industries (Pandey *et al* 2000). They hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers composed of glucose units. Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (Rao *et al* 1988). However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey *et al* 2000). Starch hydrolyzing activity was widely distributed in species of *Streptomyces* and some of them can attack and hydrolyze raw starch granules with the release of maltose as the predominant product, such enzymes are used for the industrial conversion of raw starch into sugar for fermentation (Andrews and Ward 1988). Thermophilic and acidophilic amylases have been studied from *Streptomyces erumpens* which are having applications in bakery, brewing, and alcohol industries (Kar and Ray 2008).

They are the important group of enzymes which are employed in the starch processing industry for the conversion of starch to high fructose syrups (Ammar *et al* 2002). One of the focus areas with respect to starch industry is the production of maltooligosaccharides which can be produced with amylases with a very specific mode of action. Thermostable amylases are reported from *Nocardiopsis* sp. which have important applications in bakery and paper industries (Stamford *et al* 2001). The amylase from *Thermobifida* sp. produced maltotriose as the major end product from refined starch and raw sago starch. Such amylases are lucrative catalysts in nutrition and healthcare (Yang and Liu

2004). Besides this, end-product specific amylases can be used for the production of maltooligosaccharides from low cost starch substrates (Godden *et al* 1989).Many actinomycetes have also been reported for the production of cold-active α-amylases and used in textile industries, detergents, bioethanol producing industries.

Amylase enzymes are currently used to accelerate starch degradation in many industrial processes. *Streptomyces* sp., an endophytic actinomycete, is well known as a potential source of hydrolytic enzymes, antimicrobial agents, and many secondary metabolites. Previously, an endophytic *Streptomyces griseoflavus* P4 was isolated from sweet pea root and identified by 16S rRNA sequence analysis was found to be capable of producing amylase enzymes (Tang-um and Niamsup 2012b). Extracellular amylase production by a newly isolated alkali-thermotolerant strain *Streptomyces gulbargensis* DAS 131 was optimized and characterized. The highest amylase production was achieved by growing *S. gulbargensis* DAS 131 in media with 1% starch. Maltose and maltotriose were the main end products of starch hydrolysis, indicating amylase activity. The extracellular amylolytic activity found in *Streptomyces gulbargensis* species. Due to the high alkaline nature of α-amylase produced by *Streptpmyces gulbargensis* DAS 131, it has wide application in detergent and textile industries (Syed *et al* 2009).

Proteases

Proteases are one of most important industrial enzymes accounting for nearly 60% of the total world wide sales. Proteases catalyze the hydrolysis of proteins to peptides and aminoacids. The possibility of using *Streptomyces* for protease production has been investigated because of their capacity to secrete the proteins into extracellular media, which is generally regarded as safe with food and drug administration. *Streptomyces* sp. that produce proteases include *Streptomyces clavuligerus, Streptomyces griseus, Streptomyces rimouses, Streptomyces thermoviolaceus, Streptomyces thermovulgaris* (Mostafa *et al* 2012). The quest for novel proteases and their formulations used for industries like detergents, animal feed and breweries is observed from several decades. Most of the proteases reported from *Streptomyces* sp. are alkali-tolerant, and some of the mare salt tolerant and belong to genera other than the genus *Streptomyces.* Proteases from *Nocardiopsis* sp. are employed as detergents additives (Moreira *et al* 2002) and for the depilation of hides and skins in the leather industry. Dehairing of goat skin by proteases from *Streptomyces* sp. makes the process economically and environmentally feasible (Mitra and Chakrabartty 2005). Microbial alkaline proteases for manufacturing uses are produced mostly from *Streptomyces* sp. and *Bacillus* sp. Actinomycetes, particularly *Streptomycetes* are known to secrete multiple proteases in culture media (Sharmin *et al* 2005).

Proteases from other sources are used in conjunction with enzymes from actinomycetes for recovery of antioxidants from shellfish waste. Protease production was also

carried by growing *Microbispora* sp. on the shellfish waste (Jaouadi *et al* 2010). End products of protein hydrolysis rich in amino acids and peptides serve as a low cost animal feed.

Guravaiah *et al* (2012) isolated 50 actinomycetes. Out of them 40 *Thernoactinomycetes vulgaris,* 10 *Thernoactinomycetes putidus*, 10 *Thermoactinomycetes sacchari,* 10 *Thernoactinomycetes thalpophilus* were screened for their beneficial applications. They studied that the *Streptomyces roseiscieroticus* as an ideal organisam for the industrial production of the extracellular protease enzyme. Several actinomycete isolates were obtained from various biotopes in Manipur. *Nocardiopsis prasina* designated as *Nocardiopsis prasina HA4* was the best producer strain (Ningthoujam *et al* 2009). An alkaline protease producer strain NRC-15 was isolated from Egyptian soil sample and strongly represented a novel species of the genus *Streptomyces*, hence the name *Streptomyces pseudogrisiolus* NRC-15 (Mostafa *et al* 2012).

Chitinase

Chitinases are another class of hydrolases which have gained tremendous importance in the past two decades. Chitin is a major constituent of the shells of crustaceans, exoskeletons of insects, and cell walls of a variety of fungi. Chitinase enzyme is extremely necessary within the biological control of insects (Reguera and Leschine 2001) and plant pathogenic fungi (El-Tarabily *et al* 2000, El-Tarabily 2003). A major mechanism involved in the biological control of plant pathogens is parasitism via degradation of the cell wall. Chitinases produced by various organisms hydrolyzed chitin polymer and have been implicated in biocontrol processes. Chitin is an unbranched homopolymer of N-acetyl glucosamine in α, β-1,4 linkage and is a structural component of cell wall in most of the fungi. The mycolytic activity of antagonists could be mainly due to the lytic enzymes 1,3-β-glucanase and chitinase (Henis and Chet 1975). Chitinases are useful in protoplast preparation from fungi, as biocontrol agents against plant pathogenic fungi, nematodes and are recently used for the extraction of chitin oligomers which are important biomedical products. Chitinases occur in several actinomycetes and possess unique properties in terms of thermostability and activity in wide pH range which makes them suitable for industrial applications (Nawani *et al* 2002). One of their most resourceful applications is the production of chitin oligosaccharides. Chitin oligosaccharides (COS) have anticoagulant, antimicrobial, anticholesteremic, anticancer, wound-healing, antitumor, and antioxidant activities which make them bright candidates for biomedical applications (Bhattacharya *et al* 2007). COS can be recovered from low cost substrates like shrimp, crab, and squid pen waste (Jeon *et al* 2001). Chitinase from *Microbispora* sp. was employed for the recovery of chitobiose, a potential antioxidant which can be used as a food additive and for other biomedical applications (Jaouadi *et al* 2010).This renewable resource can be utilized for the growth of many chitinolytic organisms as well as for the effective recovery of COS at the industrial

level. The disposal of the waste is also carried out effectively by the biological utilization by actinomycetes.

Srividya *et al* (2012) isolated *Streptomyces* sp. 9p that produced 2 most important hydrolytic enzymes- chitinase and β-1, 3 glucanase along with cellulase, lipase and protease. The strain 9p produced relatively high levels of chitinase (696 U/mL) and β -1,3-glucanase (392 U/mL).

An endophytic *Streptomyces* strain P4, isolated from sweet pea root which was effective in restricting the radial growth of *Fusarium oxysporum* f.sp. *lycopersici*, an important phytopathogen of tomato. Scanning electronic microscopic analysis showed that the rupture of the *F. oxysporum* mycelial cell wall occurred at the area of interaction between *F. oxysporum* and *Streptomyces* sp. P4 in a dual culture plate possibly as a result of the chitinolytic activity of chitinase produced by the endophytic actinomycete. Thus, this actinomycete has the potential to be used as a biocontrol agent, thereby reducing the use of chemical fungicides (Tang-um and Niamsup 2012a).

An industrial enzyme chitinase produced by endophytic *Streptomyces* was purified and its antifungal activity was investigated against phytopathogens *i.e. Rhizoctonia solani, Fusarium oxysporum, Alternaria alternate, Aspergillus niger, Aspergillus flavus, Sclerotinia scleotiorum, Phytophthora parasitica* and *Botrytis cinerea*. A chitinase produced by endophytic *Streptomyces hygroscopicus* was found to inhibit the growth of all phytopathogenic fungi (Haggag and Abdallh 2012).

Extracellular chitinase production by a chitinolytic *Streptomyces* sp. PTK19 and crude chitinase efficacy tested against *Fusarium oxysporum* PTK2 and the crude chitinase has been found to dissolve the phytopathogenic fungal cell wall (Thiagarajan *et al* 2011). Shekhar *et al* (2006) purified a bioactive compound from endophytic *Streptomyces violaceusniger* that showed a strong antagonism towards various wood-rotting fungi. In general, the higher chitinase activity was correlated with higher fungal inhibition. An extracellular chitinase from *Streptomyces halstedii* AJ-7, a broad spectrum antifungal biocontrol agent, was characterized and purified by Joo (2005a).

Mane and Deshmukh (2009) obtained 80 actinomycetes from Krishna River in Satara district, India and were screened for their chitinolytic activity on colloidal chitin agar. *Streptomyces canus, Streptomyces pseudogriseolus* and *Micromonospora brevicatiana* were selected on the basis of zone of clearance. *M. brevicatiana* showed maximum activity at 1.2% chitin concentration and *S. pseudogriseolus* showed maximum activity at 1% substrate concentration.

El-Tarabily *et al* (2000) isolated 94 *Streptomycetes* and 35 non-*Streptomycete* actinomycetes from a lettuce growing field, produced high levels of chitinase and were examined in vitro for their ability to suppress the growth of *Sclerotinia minor*, a pathogen

causing basal drop disease of lettuce. The three most suppressive isolates were examined further for their production of β-1,3-glucanase and antifungal activity as well as their ability to colonize the roots and rhizosphere of lettuce *in vitro* and *in planta*. The three isolates, *Serratia marcescens, Streptomyces viridodiasticus* and *Micromonospora carbonacea,* significantly reduced the growth of *S. minor in vitro,* and produced high levels of chitinase and β-1,3-glucanase. *Streptomyces viridodiasticus* also produced antifungal metabolites that significantly reduced the growth of the pathogen *in vitro*.

Actinomycetes have been shown to be a good source for L-asparaginase too. Various actinomycetes, especially *Streptomyces griseus, S. karnatakensis, S. albidoflavus* and *Nocardia* sp. have abilities to produce this enzyme (DeJong 1972, Narayana *et al* 2007 and Mostafa and Salama 1979).

Biocontrol of plant pathogens by endophytic actinomycetes

Endophytic actinomycetes have attracted the attention of researchers as biological control agents of plant pathogens due to their plant colonizing ability and antifungal activities. They have been shown to protect plants against different soil-borne plant pathogens, including *Rhizoctonia solani, Verticillium dahliae, Plectosporium tabacinum, Gaeumannomyces graminis* var. *tritici, F. oxysporum, Pythium aphanidermatum* and *Colletotrichum orbiculare* (El-Tarabily 2003, Coombs *et al* 2004, Cao *et al* 2005, El-Tarabily *et al* 2009 and Shimizu *et al* 2009). Many endophytic actinomycetes, especially those from medicinal plants possess the ability of inhibiting or killing a wide variety of harmful microorganisms like pathogenic bacteria, fungi and viruses. *Streptomyces roseosporus* W9 obtained from wheat plants was displaying maximum antagonistic activity against ten different pathogenic fungi tested (Gangwar *et al* 2012). Thus, there is great application value to develop antimicrobial drugs from endophytic actinomycetes.

Streptomyces hygroscopicus var. *geldanus* grown in sterile soil had shown antagonism against *Rhizoctonia solani,* which causes the pea root rot fungus, via geldanamycin production (Rothrock and Gottlieb 1984). A few previous reports showed that actinomycetes may be useful in protecting plants such as lettuce against damping-off fungi like *Phythium ultimum* (Crawford *et al* 1993). *Paenibacillus* sp.300 and *Streptomyces* sp. 385 which produce chitinases and β-1,3-glucanases, provided excellent control of *Fusarium* wilt of cucumber caused by *F. oxysporum* f. sp. *cucumerinum* in potting medium (Singh *et al* 1999). El-Tarabily *et al* (2000) observed that the combination of *Streptomyces marcescens, Streptomyces viridodiasticus,* and *Micromonospora carbonacea* strains effectively inhibited the growth of *Sclerotinia minor* responsible for vegetable rot.

Ouhdouch *et al* (2001) found isolates of actinomycetes from medicinal plant and tested the antifungal activity against *Candida albicans* and *C. tropicalis* and found that all *Streptomyces* had antifungal activity. Shimizu *et al* (2001) isolated ten actinomycete strains

from field-grown rhododendron plants and the strain MBR-5 was identified as *Streptomyces galbus* which showed significant antagonistic activity against major rhododendron pathogens, *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana*. Getha and Vikineswary (2002) found inhibitory activity of *S. violaceusniger* strain G10 against *F. oxysporum* f.sp. *cubense* the causal pathogen of wilt disease of banana.

More than three hundred isolates of endophytic actinomycetes were screened for their potential for chitinase production. By 16S rDNA analysis, the strain CMUAc130 was phylogenetically closely related to *Streptomyces aureofaciens*. N-acetylglucosamine was a good inducer and expression of the enzyme complex was repressed by several mono- and disaccharides including lactose, mannose, glucose, cellobiose, arabinose, raffinose, sucrose, xylose and fructose. Addition of pectin, starch and carboxymethyl cellulose to the colloidal chitin-containing medium, increased chitinase production. The crude or purified enzyme had potential for cell wall lysis of many phytopathogenic fungi tested (Taechowisan *et al* 2003). In case of soybean, inoculation of selected endophytic actinomycetes, *Streptomyces sp.* isolated from sweet pea which showed antagonistic ability against fungal plant diseases could infect and improve nitrogen uptake of the soybean plant to about 83% compared to uninoculation control treatment and such endophyte could be compatible well with *Bradyrhizobium* (Thapanapongworakul 2003).

Coombs *et al* (2004) examined biocontrol efficacy of endophytic actinomycetes against *G. graminis* var. *tritici* of wheat. Six strains which showed significant biocontrol effects in naturally infested soil tests had varied degrees of antifungal activities. Endophytic actinomycetes with tomato were obtained by Inderiati and Franco (2008) noted that 13 of 15 strains having varying levels of antagonistic activity showed the suppressive effects on damping-off caused by *Rhizoctonia solani* significantly. *Streptomyces thermocarboxydus* showed strong *in vitro* antagonism but resulted in the lowest disease suppression *in vivo*.

Cao *et al* (2004) reported biocontrol activity of an endophytic *Streptomyces* sp. against damping-off disease of tomato seedlings caused by *Rhizoctonia solani*. A typical antagonist among isolated strains was evaluated for its suppressive effect on damping-off disease of cucumber and tomato seedlings. The strain S96 of *Streptomyces* sp. isolated from surface-sterilized banana roots by Cao *et al* (2005) concerned the possible association of siderophore production with biocontrol activity. Culture broth of *Streptomyces halstedii* AJ-7 suppressed the growth of *Phytophthora capcisi* which causes *phytophthora* blight in red peppers (Joo 2005b) leading to abnormal hyphal swelling, degradation, and lysis of mycelia.

Kishore *et al* (2005) described a synergistic effect of *Bacillus circulans* GRS 243 and *Streptomyces marcescens* GPS 5 and their combination inhibited the growth of the mold fungus *Phaeoisariopsis personata* when used as a prophylactic on the leaves. *Streptomyces* sp. strain 3 was found to be a potential biological agent for control of *Fusarium* head blight

(FHB) caused by *F. graminearum* (Nourozian *et al* 2006). Actinomycetes were isolated from soil samples collected from Agriculture Research Center Semongok, Sarawak. All 62 isolates were later purified and subjected to a few enzymatic screening. 48, 46 and 41 isolates showed the ability to secrete cellulase, lipase and protease respectively. The antimicrobial test was observed with *Fusarium palmivora*, *Bacillus subtilis*, *Pantoae dipersa* and *Ralstonia solanacearum* respectively. Six isolates were identified as *Streptomycetes* sp (Jeffrey 2008).

Endophytic strains of *Microbispora rosea* sub sp. *rosea* and *Streptomyces olivochromogenes* were found to be effective in suppressing club root of Chinese cabbage caused by *Plasmodiophora brassicae* (Lee *et al* 2008). These strains were originally isolated from surface-sterilized roots of Chinese cabbage collected from various regions in Korea and reduced the severity of club root by 33-58%. A potent strain of actinomycetes SRA14 was identified as *Streptomyces hygroscopicus*. The strain SRA14 highly produced extracellular chitinase and β-1,3-glucanase indicating that growth suppression was due to extracellular antifungal metabolites present in culture filtrates (Prapagdee *et al* 2008).

Eleven strains of endophytic actinomycetes were isolated from healthy roots of *Acacia auriculiformis* A. Cunn. ex Benth. collected from Bangkok and Nakhonpathom, Thailand. Analysis of 16S rRNA sequencing of those strains revealed that they belong to members of genera *Streptomyces*, *Actinoallomurus*, *Amycolatopsis*, *Kribbella* and *Microbispora*. Three isolates active against test bacteria namely, GMKU 932 (against *Bacillus cereus*), GMKU 940 (against *Bacillus cereus* ATCC 11778 and *Escherichia coli* ATCC 8739) and GMKU 944 (against *Bacillus cereus*, *Staphylococcus aureus* ATCC 25923 and *Ralstonia solanacearum*). Only 4 isolates, GMKU 937, GMKU 938, GMKU 940 and GMKU 944 showed strong activity against *Aspergillus niger*. GMKU 944 showed strongest antibacterial activities against tested bacteria and fungi (Bunyoo *et al* 2009). *Pseudonocardia endophytica* sp. nov., isolated from the pharmaceutical plant *Lobelia clavata* a traditional Chinese medicinal plant, is usually used to treat parotitis and rheumatoid arthritis (Chen *et al* 2009).

El-Tarabily *et al* (2009) studied the potential use of endophytic actinomycetes for controlling *Pythium aphanidermatum* in cucumber. They evaluated the biocontrol potential of three antagonistic isolates of *Actinoplanes campanulatus, Micromonospora chalcea and Streptomyces spiralis,* which produced high levels of cell-wall degrading enzymes (β-1, 3, β-1, 4 and β-1, 6-glucanases). Twenty-three *Streptomyces* isolates showed activity against *Alternaria brassicicola, Collectotrichum gloeosporioides, Fusarium oxysporum, Penicillium digitatum and Sclerotium rolfsii* (Khamna *et al* 2009). *Streptomyces rochei* and *Streptomyces rimosus* from the chickpea were found to be strong antagonists of *Fusarium oxysporum* f. sp. *ciceri.* Actinomycetes of the genera *Promicromonospora* and *Oerskovia*, which are known as rare actinomycetes that were first time found to be endophytic (Qin *et al* 2009).

Streptomycetes from rhizosphere of *Araucaria* were shown to have the ability to inhibit the growth of *Fusarium* and *Armillaria* causing pine rot (Vasconcellos 2009). *Streptomyces alni* isolated from soil of grapevine was found to exhibit antagonistic activity against *F. oxysporum* the causative agent of root rot of grapevine (Ziedan *et al* 2010).

Culture filtrates obtained from cells of *Micromonospora* sp. strain EN43 grown in either minimal or rich medium activated the system acquired resistance and jasmonates/ ethylene pathways, respectively, indicating that two different sets of metabolites are synthesized by endophytic actinomycetes for eliciting the plant defense pathway (Hirsch and Valdes 2010). Recently, a new antimycotic compound saadamycin was isolated from endophytic *Streptomyces* sp. Hedaya48, and it exhibited significant antimycotic activity against dermatophytes and other clinical fungi (El-Gendy and El-Bondkly 2010).

The antifungal activities of endophytic actinomycetes that were recovered from 12 wild medicinal plants in Egypt was determined using the dual culture bioassay against four fungal phytopathogens *Fusarium solani, Phytophthora infestans, Macrophomina phaseolina* and *Botrytis cinerea,* which are the causative agents of tomato's wilt, potato's late blight, soybean's charcoal rot and eggplant fruit rot, respectively as well as *Fusarium oxysporum*. *Streptomyces* sp. 1 and *Kitasatosporia* sp. 5 were prolific producers of fungal inhibitory compounds (Moussa *et al* 2011). One hundred eighty seven actinomycete isolates were obtained from different soil samples of Kalyoubia, Egypt. The most efficient isolate on anticandidial activity was *Streptomyces* isolate *SY*1 which was identified as a strain of *Streptomyces griseolus* (Shahat *et al* 2011).

Endophytic actinomycetes were isolated from surface sterilized leaves of *Catharanthes roseus* (L.) G. Don of family *Apocynaceae*. 20 morphologically different isolates were screened for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris* and for antifungal activity against fungi *Candida albicans, Botrytis cinerea, Curvularia lunata, Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani.* Sixty five percent of the isolates exhibited antimicrobial activity (Kafur and Khan 2011). *Streptomyces olivaceus* strain 115 showed high antifungal activity against *Fusarium oxysporum* f.sp. *melonis,* which causes root rot, vascular wilt and damping off in greenhouse cucurbits. Jeffrey *et al* (2011) observed that antimicrobial activities against various test pathogens; *Ralstonia solanacearum* (10%), *Pantoea stewartii* (20%) and *Bacillus subtilis* (10%). Seven out of eight best producers of bioactivities were identified as *Streptomyces* and hence we can conclude that *Streptomyces* species in the soil is the most significant. These potential actinomycetes can be further exploited for use in various industries such as agriculture, food and paper.

Biological control agents can replace chemical agents to control insect pests and weeds and microbial pathogens. Several bio-fungicides are based on antibiotic metabolites

and hydrolytic enzymes. For example, *Streptomyces griseoviridis* strain K61, a soil borne fungal antagonist which produces aromatic antibiotics with characteristic 7-membered rings in the molecules was commercialized as Mycostop^{R} by Verdera Oy, a Finnish company and *Streptomyces* sp. Di-944 was formulated to suppress *Rhizoctonia* damping-off (Tang-um and Niamsup 2012a).

A potent actinomycete isolate 9p exhibited broad spectrum antifungal property against *Alternaria brassiceae OCA3; Collectotrichum gleosporioides OGC1; Rhizoctonia solani MTCC 4633* and *Phytophthora capsici.* The strain 9p exhibited mixed path antagonism type of mechanisms of biocontrol through the production of mycolytic enzymes. Isolate showed 87.5% germination index when coated onto chilli seeds. This coupled with antifungal properties suggests both the PGPR and biocontrol aspect of the actinomycete *Streptomyces* sp. 9p **(**Srividya *et al* 2012). One-hundred and ninety one isolates of actinomycetes were screened for antifungal activity against anthracnose of long cayenne chilli pepper causing *Colletotrichum gloeosporioides*. The non filtrates of isolates NSP1 and NSP2 were subjected to a 16S rDNA sequencing to reveal its phylogenetic relationship with representative *Streptomyces* and are potentially biological control agent against chilli anthracnose caused by *Colletotrichum gloeosporioides* (Suwan *et al* 2012).

One hundred and forty seven strains of actinomycetes were isolated from soil samples collected from cardamom fields at Western Ghats of South India. The isolate *Streptomyces* sp. Sh7 exhibited promising antibacterial activity against Gram positive and Gram-negative bacteria. Cryomycin a peptide antibiotic is produced by a strain of *Streptomyces* (Ramani and Kumar 2012).

Five hunred and sixty endophytic actinomycetes were isolated from 26 medicinal plant species in Panxi plateau. 60 isolates were selected for 16S rDNA-RFLP analysis and 14 representative strains were chosen for 16S rDNA sequencing. Seven isolates were *Streptomyces* sp. while the remainder belonged to genera *Micromonospora, Oerskovia, Nonomuraea, Promicromonospora* and *Rhodococcus*. Antimicrobial activity analysis combined with the results of amplifying genes coding for polyketide synthetase (PKS-I, PKS-II) and nonribosomal peptide synthetase (NRPS) showed that endophytic actinomycetes isolated from medicinal plants in Panxi plateau had broad-spectrum antimicrobial activity and potential natural product diversity, which further proved that endophytic actinomycetes are valuable reservoirs of novel bioactive compounds (Zhao *et al* 2012).

Mini Priya (2012) isolated actinomycetes from 4 different and unique Indian medicinal plants *Phyllanthus niruri, Withania somnifera, Catharanthus roseus and Hemidesmus indicus*. *Streptomyces sp.5* and *Streptomyces sp.7* isolates showed a wide spectrum of the antifungal activities against *Fusarium solani, Phytophthora infestans, Macrophomina phaseolina, Rhytisma acerinum* and *Botrytis cinerea,* which are the causative

agents of tomato's wilt, potato's late blight, soybean's charcoal rot, tar spot disease and eggplant fruit rot, respectively. Soil actinomycetes isolated in Maseno area (Western Kenya) have antagonistic activity against a wide range of plant pathogens. Actinomycetes were studied for their antifungal effect on *Pyricularia grisea* pathogenic on finger millet (George *et al* 2013).

Manasa *et al* (2013) obtained *Streptomyces* isolates for the protection of the ginger rhizomes from soft rot symptoms. Treatment of tomato seeds with *Streptomyces miharaensis* strain KPE62302H was found to induce a significant reduction in the incidence of *Fusarium* wilt in tomato plants compared with untreated controls. *Streptomyces* sp. C-11 and C-26 isolated from the soil sample showed antagonistic activities against *Fusarium subglutinans* in dual culture test (Sadeghy *et al* 2013). *S. rochei* ACTA 1551 strongly suppressed the growth of *Fusarium oxysporum* f.sp. *lycopersici in vitro*. The strain was able to protect tomato seeds from *Fusarium oxysporum* infection *in vivo.* Kanini *et al* (2013) found inhibitory efficacy of indigenous *Streptomyces* isolates against the soil-borne fungal plant pathogen *Rhizoctonia solani*.

CHAPTER III

MATERIAL AND METHODS

3.1 Endophytic actinomycetes cultures

Actinomycetes isolates used for carrying out antagonistic activity were procured from Department of Microbiology, PAU, Ludhiana (Table. 1). The stock cultures were maintained on Starch Casein Agar slants by regular sub-culturing and stored at 4ºC.

3.2 Fungal cultures

Fungal cultures *Alternaria alternata, Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii* used in the present study were procured from Department of Plant Pathology, PAU, Ludhiana. The fungal cultures were maintained on Potato Dextrose Agar / Glucose Yeast Agar and stored at 4ºC.

Table 1: Occurrence of endophytic actinomycetes fom Medicinal plants

Source	No. of isolates
Azadirachta indica A.Juss	18
Emblica officinalis	13
Ocimum sanctum	16
Aloe vera	6
Mentha arvensis	17
Total	70

3.3 Chemicals

The chemicals used in the investigation were of analytical grade and purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, Qualigens Fine Chemicals, Mumbai.

3.4 Dual culture antagonistic bioassay

The actinomycetes isolates were evaluated for their antagonistic activity against four phyto pathogenic fungi: *Alternaria alternata*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotium rolfsii* by dual-culture *in vitro* assay. Fungal discs (8mm in diameter), 5 days old on PDA at 28ºC were placed at the center of PDA plates. Two actinomycetes discs (8mm) 5 days old, grown on starch casein agar, incubated at 28ºC were placed on opposite sides of the plates, 3 cm away from fungal disc. Plates without the actinomycetes disc served as controls. All the plates were incubated at 28ºC for 14 days and colony growth inhibition (%) was calculated by using the formula: C – T/C x 100, where C is the colony growth of pathogen in control and T is the colony growth of pathogen in dual culture. The zone of inhibition was measured between the pathogen and actinomycetes isolates.

3.5 Screening of isolates for hydrolytic enzyme production

3.5.1 Amylase production

One gram of potato starch suspended in 10 ml of cold distilled water was added to 90 ml of nutrient agar, autoclaved at 121^0C for 20 minutes and poured into sterile Petri plates. After inoculation and incubation of 4-5 days, the plates were flooded with Gram's iodine solution. A clear zone surrounding colony indicates production of amylase.

3.5.2 Protease production

The test for protease production was performed by the procedure described by Gordon and Smith (1955). A 10 % suspension of skimmed milk powder in water and an equal volume of 2% water agar were autoclaved separately and cooled to 55^0C. They were mixed and poured into Petri plates. The actinomycete culture were inoculated in the center and incubated at 28^0C for 48 h. A zone of clearance beneath and around the colony indicated hydrolysis of casein by the production of protease.

3.5.3 Chitinase Production

The test for was performed by the procedure described by Taechowisan *et al* 2003, Tang-um and Niamsup 2012a. The colloidal chitin medium was used. Screening for chitinase production of all the isolates was done by forming 0.6% and 1% colloidal chitin by plate agar assay. Practical grade crab shell chitin powder (Hi- media) was used to prepare colloidal chitin (Berger and Reynolds 1958) as a substrate for growth and enzyme assay. The clear zone around the colonies observed after 7-14 days is an indication for enzyme production. After incubation period, the plates were flooded with 0.1% Congo red solution and observed for the zone of clearance around the colony.

3.5.3.1 Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin (Hi Media) by the modified method of Hsu and Lockwood (1975). Chitin powder (10 g) was slowly added with 150 ml of concentrated HCl and kept for 60 min at 30°C with stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2 l of water at 4–10°C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5 l of distilled water. Washing was repeated 3 times until the pH of the suspension was 3.5. After the above treatment, the loose colloidal chitin was used as a substrate.

3.6 Quantitative production of extracellular chitinase

For the quantitative estimation of chitinase activity 0.6% and 1% colloidal chitin concentration was used. Colloidal chitin broth was used as a production medium. Both the isolates were precultured in colloidal chitin medium. The pH of the media was maintained at 7. Enzyme production was carried out in shake culture (50 ml medium in 250 ml Erlenmeyer flask) incubated at 30ºC in the incubator shaker at 150- 160 rev min^{-1} for 7 days. Spores were inoculated to a concentration of 10^5 ml^{-1}. The supernatant fluid was harvested every day for

7 days by filtration through Whatman no.1 filter paper. Chitinase activity in the supernatant was assayed using 0.6% and 1% colloidal chitin as a substrate and was based on a procedure by Taechowisan *et al* 2003, Tang-um and Niamsup 2012a. The supernatant fluid was added with 2% colloidal chitin in 0.1M acetate buffer at pH 5.0 and the mixture was incubated in a water bath at 50ºC for 1 hr. One mL of Somogyi's reagent was added and the reaction mixture was boiled at 100ºC for 10 min. and cooled to room temperature. Then Nelson's reagent (1mL) was added and the mixture cooled to room temperature for 20 min. After centrifugation of the reaction mixture, the amount of N-acetyl glucosamine (GlcNAc) released in the supernatant was spectrophotometrically measured by the method of Somogyi-Nelson (Green *et al* 1989). The method is based on the 520-nm absorbance given by a coloured complex formed between a copper-oxidised sugar and arsenomolybdate. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 mol of reducing sugar per min. under the conditions of the experiment. All measurements were performed in triplicate.

Standard curve of N-acetylglucosamine

Standard curve was prepared by taking N-acetylglucosamine solution 1mg in 10 ml of distilled water. 0.1 ml of solution was taken and makes the volume 1ml by adding distilled water in the test tube. Then add 1 ml of Nelson reagent. Heat the test tubes vigorously in boiling water bath for 20 minutes. Then cool them and add 1ml of arsenomolybdate reagent. After few minutes take absorbance at 520 nm.

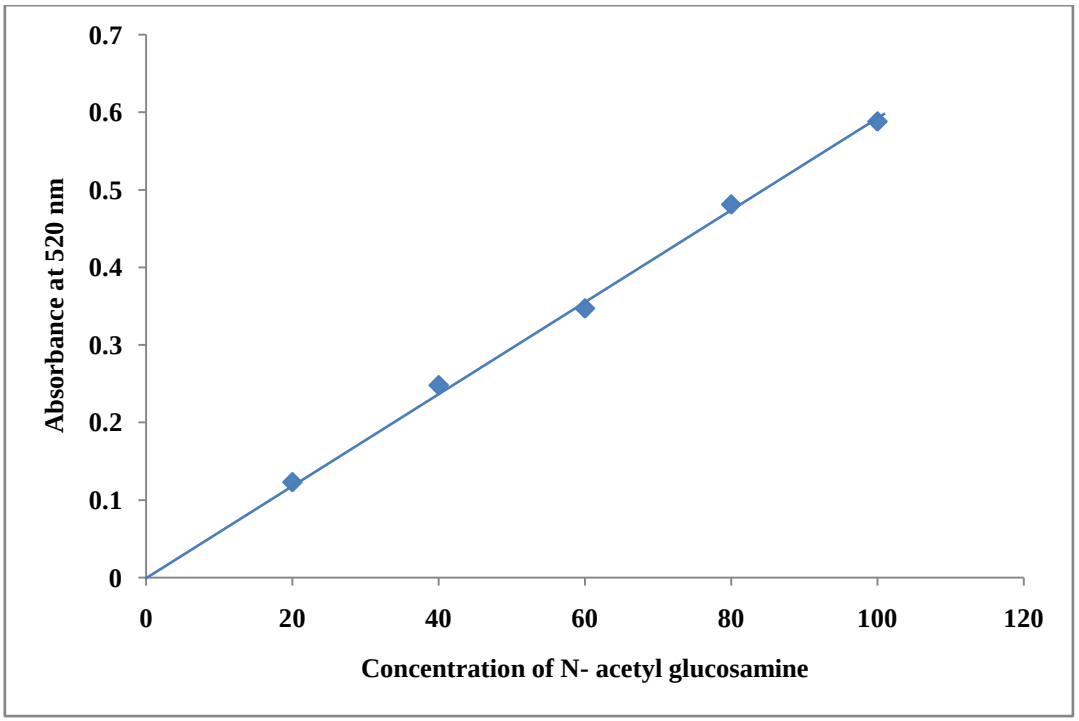

Fig 1: Standard curve of N-acetyl glucosamine

3.7 Scanning electron microscopic (SEM) studies of the antagonistic effect of potential actinomycete isolates on fungal cell wall

Scanning electron microscopy (SEM) of two actinomycetes isolates, AR3 and O9 treated with *Fusarium oxysporum* culture was performed using chemical fixation and liquid osmium fixation technique (Bozzola and Russell 1996). Samples were fixed in 2.5% gluteraldehyde solution (fixative) at 4ºC for 24 hours. After fixation the gluteraldehyde was drained and three washings with 0.1 M sodium cacodylate buffer (pH 7.2) were performed after the interval of 15 minutes at 4ºC. Wash buffer was drained and 1% Osmium tetraoxide (OsO_4) was added for 1-$2_{1/2}$ hours at 4ºC. As OsO_4 is slow penetrating solution the samples were placed in liquid osmium tetraoxide for 2 to 3 hours. Later OsO_4 solution was drained off followed by three washings with the rinsing buffer solution (0.1 M sodium cacodylate buffer) for 15 minutes at 4ºC each. After fixation step dehydration was done by incubating the samples with the ethanol solution of different concentrations starting with 30%, 50% and 70% ethanol solution each for 15 minutes at 4ºC (sample can be stored in 70% ethanol at 4ºC for three to four days). Then further samples were incubated in 80%, 90% and 95% ethanol solution each for 15 minutes. Samples were then incubated three times in the 100% ethanol solution each for 20 minutes at room temperature. The solution was drained off at last and sample was placed in the vacuum dessicator overnight, stubbed and sputter coated with gold in E-1010 Ion sputter coater machine to be viewed under secondary electron imaging mode in Hitachi S-3400N Scanning electron microscope.

3.8 Molecular characterization of potential isolate by 16S rDNA sequencing

DNA was isolated from the endophytic actinomycetes culture. Quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified and further process for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Reverse sequence of 1033bp 16S rDNA gene was used for further analysis. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

3.9 Evaluation of effectiveness of endophytic actinomycetes isolates (AR3 and O9) as potential antagonists against *Fusarium oxysporum* in green house

3.9.1 Inoculum preparation of potential antagonists

The potential isolates were grown in broth medium for 5 days. Healthy seeds of musk melon variety *Punjab sunehri* were surface sterilized with 0.1% $HgCl_2$ for 3 min followed by

treatment with 95% ethanol for 5 minutes and then successive washing with sterilized distilled water. The surface sterilized seeds were immersed overnight in the antagonists suspension containing 10^8 cfu.ml^{-1}.

3.9.2 Fungal inoculum preparation

Inoculum of phytopathogenic fungi *Fusarium oxysporum* was prepared by soaking wheat seeds overnight in water. Sand and soaked wheat seeds were mixed and transferred to 250 ml Erlenmeyer flasks and autoclaved at 121ºC. *Fusarium oxysporum* was grown on potato dextrose agar and discs of fungi were transferred to 250 ml Erlenmeyer flasks containing autoclaved wheat and sand. The flasks were incubated at 25ºC for 7 days. The rate of inoculum applied to the potting mixture was 10 gm of fungi in 9 kg of soil per pot. Inoculum of fungus was added in sterile soil before sowing the seeds.

3.9.3 Soil infestation

Soil was taken from field and sterilized by autoclaving at 121^{0}C for 1 hr for 3 consecutive days. Musk melon seeds variety *Punjab sunehri* were grown in pots, using completely randomized block design (CRD) with and 6 treatments and 3 replications each. Five seeds were sown per pot containing 9 kg of sterile soil. The treatments comprised were: (A) Control without antagonists and *Fusarium oxysporum* (Negative control), (B) *Fusarium oxysporum* inoculation (Positive control), (C) Endophytic actinomycete isolate AR3 (from *Emblica officinalis*) alone, (D) Endophytic actinomycete isolate O9 (from *Ocimum sanctum*) alone, (E) Endophytic actinomycete isolate AR3 + *Fusarium oxysporum*, (F) Endophytic actinomycete isolate O9 + *Fusarium oxysporum*.

3.9.4 Observations to be recorded

3.9.4.1 Percentage of seed germination

Total numbers of seeds germinated were counted and then percent germination was calculated as follows:

$$\text{Germination (\%)} = \frac{\text{Total number of seed germinated}}{\text{Total number of seeds sown}} \times 100$$

3.9.4.2 Plant growth promotion

3.9.4.2.1 Fresh weight of shoot and root

Plants were removed with root system intact and then measured for fresh weight.

3.9.4.2.2 Dry weight of shoot and root

Shoots were randomly selected and uprooted plants from each pot were sun dried and then oven dried at 60^{0}C for 1 day.

3.9.4.2.3 Root and shoot length

Plants were removed with root system intact and then measured for root and shoot length with simple measuring scales separately.

3.9.4.2.4 Incidence of disease (Wilt incidence %)

The plants were counted with wilting symptoms and then wilt incidence was measured as follows:

$$\text{Incidence of disease (\%)} = \frac{\text{Total number of wilted plants}}{\text{Total number of plants}} \times 100$$

3.9.4.2.5 Inhibition of disease development (%)

Inhibition of disease was calculated with comparison of control with other treatments.

$$\text{Inhibition of disease development (\%)} = \frac{\text{Wilt incidence}}{\text{Wilt incidence in control}} \times 100$$

CHAPTER IV

RESULTS AND DISCUSSION

Endophytic actinomycetes have been isolated from a range of plant types including crop plants such as wheat, rice, tomato, carrot and medicinal plants. *Streptomyces* is the predominant species followed by *Micromonospora* and *Nocardia*. Actinomycetes have been used to improve the growth as well as to reduce disease through various mechanisms which are used against pests, diseses and induction of systemic acquired resistance in plants. Actinomycetes have been largely exploited mainly because of their capability to produce bioactive compounds, such as antibiotics and lytic enzymes. Present study was undertaken to screen the antifungal potential of endophytic actinomycetes isolated from medicinal plants and production of extracellular hydrolytic enzymes Furthermore, the potential to control *Fusarium oxysporum using* endophytic actinomycetes isolates was evaluated in muskmelon (variety *Punjab sunehri*) under greenhouse conditions.

4.1 Antagonistic activity of endophytic actinomycete isolates against phytopathogenic fungi

Out of 70 isolates, eleven isolates (6 from *Azadirachta indica* A. Juss, 3 from *Emblica officinalis*, 1 from *Aloe vera* and 1 from *Ocimum sanctum*) were displaying antagonistic activity against one or the other plant pathogenic fungi tested (Table 2). In the present study, AzR3 an isolate of *Azadirachta indica* A. Juss exhibited antifungal activity against *Alternata alternata* with percent inhibition of 33.62%, AzS4, AzR7 (isolates of *Azadirachta indica* A. Juss), A6 (an isolate of *Emblica officinalis*) displayed antagonistic activity only against *Fusarium oxysporum* and AzS13, AzR1, AzS11 (isolates of *Azadirachta indica* A. Juss) exihibited antifungal activity against *Rhizoctonia solani*. AS2 and AS9 isolates from *Emblica officinalis* displayed antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani.* AR3 isolate of *Emblica officinalis* and O9 an isolate of *Ocimum sanctum* exhibited strong antagonistic activity against *Alternata alternata, Fusarium oxysporum* and *Rhizoctonia solani*. AR3 has maximum percent inhibition against *Fusarium oxysporum* (60.69%) (Plate1). None of the isolates were displaying antifungal activity against *Sclerotium rolfsii*. Anitha and Rabeeth (2009) studied *in vitro* tests of interactions between *Streptomyces griseus* strains and some soil borne plant pathogens like *Fusarium oxysporum, Alternaria alternata, Rhizoctonia solani* and *Fusarium solani* of tomato. *Streptomyces griseus* showed maximum percent inhibition of 61.1% against *F.oxysporum* f. sp. *lycopersici.* The inhibition was due to the presence of some inhibitory substance, antibiotics and other enzymes such as glucanases, proteases essential for complete cell-wall lysis.

Out of 146 strains, 10 strains showed antagonistic ability against either *Colletotricum gloeosporioides, Sclerotium rolfsii* or both the fungi. Twenty-one isolates strongly inhibited

the growth of *C. gloeosporioides* whereas two isolates were very active against *S. rolfsii*. Only one isolate SRA14, had a strong antagonistic activity against both the fungi (Prapagdee *et al* 2008).Verma *et al* (2009) observed that endophytic *Streptomyces* isolates AzR031, 008, 047, 030, and AzL025 had very acute activity toward root pathogens. Isolates AzR021 and AzR008 were found to be antagonistic to *Pythium aphanidermatum* while *Nocardia* (AzL025) showed the strongest inhibition against *Pythium oligandrum*.

Kafur and Khan (2011) reported 20 isolates, 11 isolates (55%) exhibited antifungal activity and two of the isolates (Cr 12, Cr 20) inhibited all the filamentous fungi. Out of the five filamentous fungi tested *Curvularia lunata* was inhibited by 8 isolates followed by *Botrytis cinerea* (6 isolates), *Fusarium solani* (5 isolates), *Fusarium oxysporum* (5 isolates) and *Rhizoctonia solani* (4 isolates).

Nine isolates of endophytic actinomycetes obtained from medicinal plants inhibited the growth of at least one or more phytopathogenic fungi. *Saccharopolspora* 0-9 exhibited antagonistic activity against *Aspergillus niger, Aspergillus flavus, Alternaria brassicicola, Botrytis cinerea, Penicillium digitatum, Fusarium oxysporum, Penicillium pinophilum, Phytophthora dresclea* and *Colletotrichum fulcatum* (Gangwar *et al* 2011). Nine *Streptomyces* strains isolated from lentil (*Lens esculentus*), chickpea (*Cicer arietinum* L.), pea (*Pisum sativum*), faba bean (*Vicia faba*) and wheat (*Triticum vulgare*) from Paskerville, South Australia showed inhibiton against *Phytophthora medicaginis* for root rot of chickpea plants (Misk and Franco 2011).

Plant root exudates stimulate growth of rhizosphere actinomycetes that are strongly antagonistic to fungal pathogens while the actinomycetes utilize root exudates for growth and synthesis of antimicrobial substances (Crawford *et al* 1993, Yuan and Crawford 1995). In another study, thirty eight strains of endophytic actinomycetes isolated from surface sterilized wheat and barley roots were tested for their antagonistic activity to wheat root pathogens *Gaeumannomyces graminis, Rhizoctonia solani* and *Pythium* sp. It was observed that out of 38 isolates, 17 isolates displayed significant activity against *Gaeumannomyces garminis* (Coombs *et al* 2004).

Aghighi *et al* (2004) and Moussa *et al* (2011) observed that a small number of endophytic microorganisms had the capability of producing broad-spectrum antifungal compounds and their mechanisms of action of these endophytic actinomycetes are mainly focused on the production of bioactive compounds such as antibiotics, cell wall degrading enzymes and competition for nutrients (El-Tarabily and Sivasithamparam 2006). A number of endophytic actinomycetes suppressed wheat fungal pathogens, including *Rhizoctonia solani, Pythium* sp. and *Gaeumannomyces graminis* var. *tritici*, both *in vitro* and *in planta* indicating their potential use as biocontrol agents (Coombs *et al* 2004).

Table 2: Antifungal activity (% inhibition) of endophytic actinomycetes isolates

Isolate	*Alternaria alternata*	*Fusarium oxysporum*	*Rhizoctonia solani*
AzS4	_	31.01±0.4	_
AzS13	_	_	22.25±0.6
AzR1	_	_	26.67±0.9
AzS11	_	_	29.25±0.9
AzR7	_	46.29±0.5	_
AzR3	33.62±0.5	_	_
AR3	39.68±0.5	60.69±0.1	33.25±0.4
AS2	_	37.03±0.6	24.07±0.6
AS9	_	40.64±0.4	25.77±0.5
A6	_	32.03±0.6	_
O9	35.23±0.1	33.62±0.5	33.62±0.5

*Average ± standard error from three replicates

4.2 Screening of isolates for hydrolytic enzymes production

Starch is an insoluble polymer of glucose which acts as a source of carbon for microorganisms and they have an ability to degrade them. Starch degrading microorganisms transport the degraded form across the cytoplasmic membrane of the cell. Actinomycetes possess the ability to produce amylases that breaks starch into maltose.

All the isolates of endophytic actinomycetes from medicinal plants were screened qualitatively for amylase, protease and chitinase enzymes production. Out of 70 isolates, 32 isolates from *Azadirachta indica* A. Juss, 8 from *Emblica officinalis*, 4 from *Aloe vera*, 2 from *Ocimum sanctum* and 1 from *Mentha arvensis* produced amylase enzyme (Table 3) (Plate 2). Stamford *et al* (2001) reported that the occurrence of amylase in actinomycetes has commonly observed in *Nocardia* and *Streptomyces.* Tang-um and Niamsup **(**2012b) found that an endophytic *Streptomyces griseoflavus* P4 isolated from sweet pea root was found to be capable of producing amylase enzyme using plate agar assay. Marine actinomycetes isolates ACT-A2, ACT-3, ACT-A4, ACT-A5, ACT-A7 and ACT-A15 showed starch hydrolysis by amylase production (Attimarad *et al* 2012).

Proteases produced by actinomycetes are the most important group of secondary metabolites that are widely exploited. *Streptomyces* sp., *Bacillus* sp., *Myceliophthora* sp., *Aspergillus fumigates, Aspergillus awamori and Aspergillus niger* were reported to produce protease enzyme (Balachandran *et al* 2012). Microorganisms are the preferred protease producers as they grow rapidly and require little cultivation area and can easily be subjected to genetic manipulation. The possible use of *Streptomycetes* for enzyme production has been investigated. Several proteases were obtained from *Streptomycetes* and were biochemically

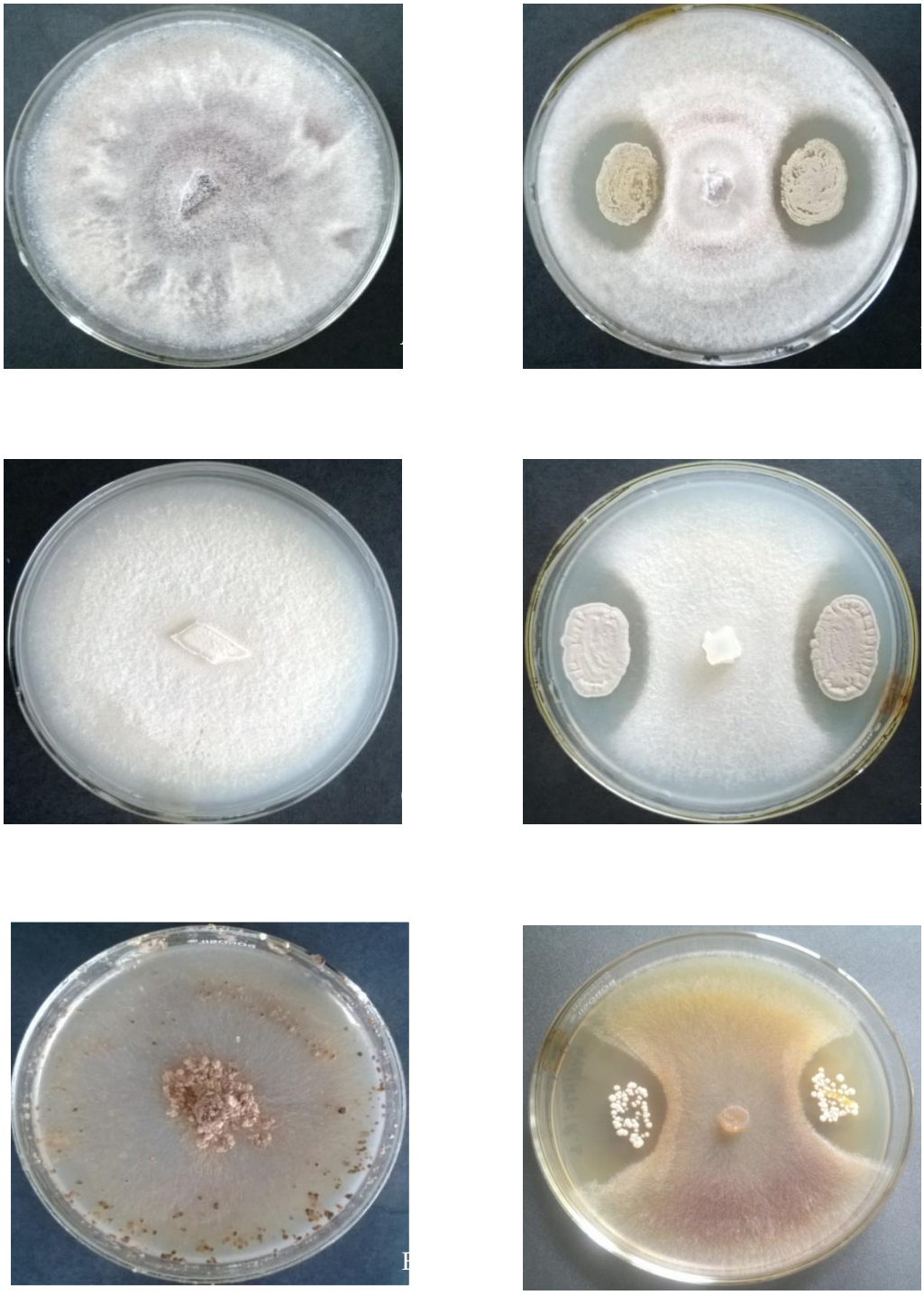

Plate 1: Antifungal activity of endophytic actinomycetes

(A) ***Alternaria alternata*** **(B) AR3 +** ***Alternaria alternata***
(C) ***Fusarium oxysporum*** **(D) O9 +** ***Fusarium oxysporum***
(E) ***Rhizoctonia solani*** **(F) AR3 +** ***Rhizoctonia solani***

characterized as serine protease produced by *Streptomyces pactum*, metallo and serine proteases from *Streptomyces exfoliatus* and aminopeptidase from *Streptomyces rimosus* (Rifaat *et al* 2007).

Fifteen isolates from *Azadirachta indica* A. Juss, 6 from *Emblica officinalis*, 2 from *Ocimum sanctum*, 1 from *Aloe vera* and 1 from *Mentha arvensis* produced protease enzyme (Table 3) (Plate 2). Gurielidze *et al* (2010) reported 48 strains of halophilic actinomycetes and demonstrated the protease activity. It varied from 0.03 to 1.52 unit/ml. Among the studied cultures, protease activity was found in 48% of actinomycetes from the environs of Lake Kumisi, 11% from Krasnogorka, 26% from millary valley and 15% Alazani valley. Highly active protease producers were strains of *Streptomyces rectiviolaceus* 173H isolated from the environs of Lake Kumisi.

Chitin is being the most abundant naturally occurring aminopolysaccharide. Besides being present on most fungal cell walls, it is hydrolysed by three separate enzymes categorized as exochitinase, endochitinase and chitobiase that constitute the chitinase complex. Chitinase-producing bacteria can inhibit fungal growth e.g. plant-pathogenic fungi (Ordentlich *et al* 1988). One possible explanation for its inhibition is the action of chitinases and β-glucanases on chitin or glucan present in these fungal cell walls, acting as protective agents (Inbar and Chet 1991). Actinomycetes particularly the genus *Streptomyces*, which are Gram-positive mycelial bacteria, ubiquitous in soil and are well known producers of many extracellular enzymes with polymer-degrading properties including chitinases (Gupta *et al* 1995).

Sixteen isolates of endophytic actinomycetes, out of which 5 from *Azadirachta indica* A. Juss, 5 from *Emblica officinalis*, 3 from *Aloe vera*, 1 from *Ocimum sanctum* and 1 from *Mentha arvensis* produced chitinase enzyme (Table 3) (Plate 2). Shekhar *et al* (2006) purified a bioactive compound from endophytic *Streptomyces violaceusniger* which showed a strong antagonism towards various wood-rotting fungi and found that chitinase enzymes were associated with this inhibition. Haggag and Abdallh (2012) observed that endophytic *Streptomyces hygroscopicus* produced a high concentration of chitinase which inhibited the phytopathogens and showed larger fungal growth inhibition zones for *Rhizoctonia solani, Sclerotinia scleotiorum, Botrytis cinerea and Fusarium oxysporum* than for *Alternaria alternata, Aspergillus niger, Aspergillus flavus* and *Phytophthora parasitica.*

As the fungal cell wall is rich in chitin (Peberdy 1990) it could be a potential target in biocontrol of fungal phytopathogens. The enzymes could be used directly in biological control of microorganisms (Ordentlich *et al* 1988, Gomes *et al* 2000) or indirectly using purified protein (Ueno *et al* 1990, Gunaratna and Balasubramanian 1994 and Gomes *et al* 2001). Taechowisan *et al* (2003) observed that fourteen isolates produced chitinase and the best strain for chitinase production was strain *Streptomyces aureofaciens* CMUAc130, which produced nearly 2-6 times more enzyme activity than any other isolates.

Table 3: Hydrolytic enzymes production

Isolate	Amylase production	Protease production	Chitinase production
AzS4	+	+	-
AzS6	+	+	-
AzS13	-	+	-
AzL3	+	+	+
AzL5	+	-	-
AzL6	+	+	-
AzR9	-	-	-
AzR14	+	+	-
AzR1	-	+	-
AzS11	+	-	-
AzR7	-	+	-
AzR13	+	+	+
AzR10	+	+	-
AzL2	+	-	+
AzR3	+	+	-
AzR2	-	-	+
AzS10	+	+	-
AzR12	+	+	-
AR12	+	-	+
AR4	+	+	-
AR3	+	+	+
AR16	+	-	+
AL7	+	+	+
AR6	+	-	-
AR14	+	+	-
AS1	+	-	-
AS2	+	+	+
AS9	+	+	+
AR9	+	-	-
AR10	-	-	-
A4	+	-	+
O11	+	+	-
A9	+	-	+
M1	+	+	+
A3	+	-	-
A6	+	+	+
O9	+	+	+
O1	+	-	-

(+) = Growth detected
(-) = Growth not detected

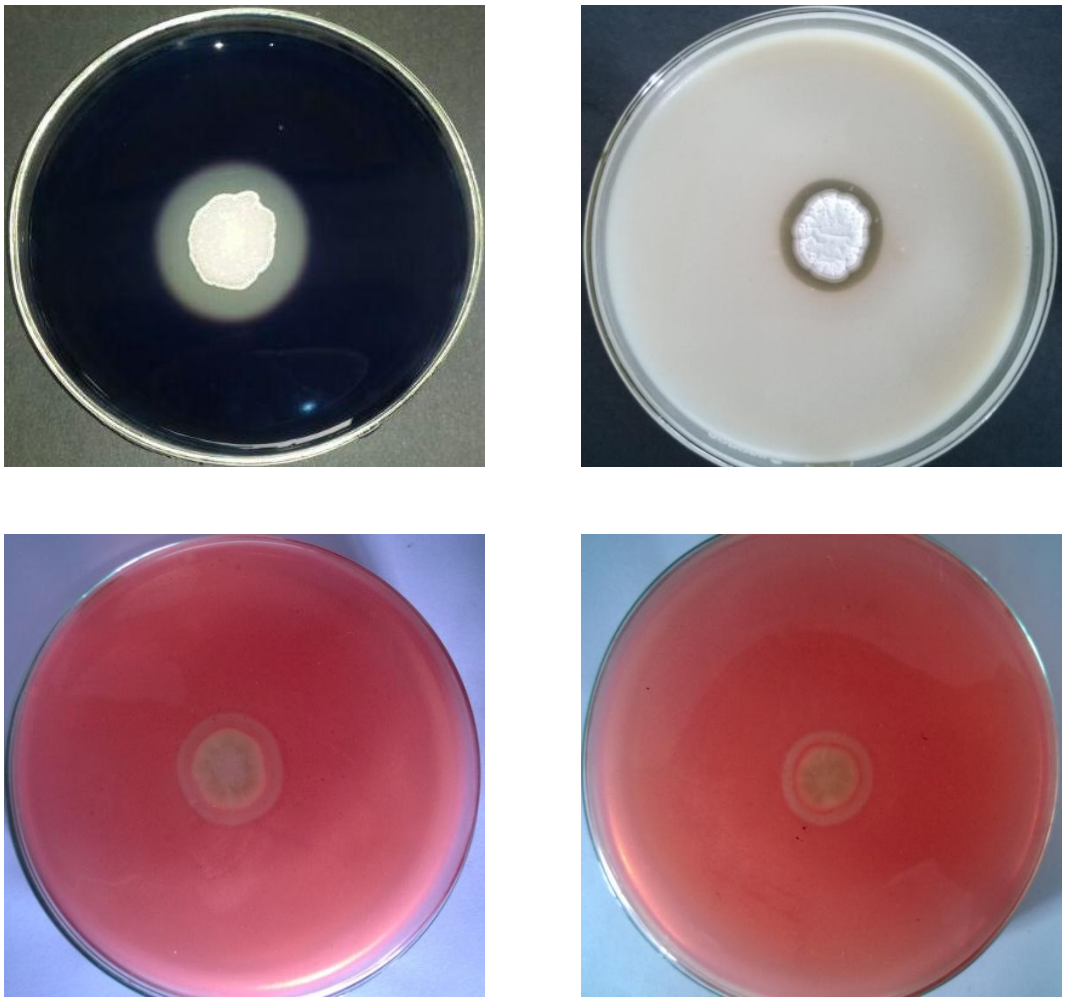

Plate 2: **Hydrolytic enzymes production by endophytic actinomycetes**
(A) Amylase Production
(B) Protease Production
(C) Chitinase Production with 0.6% colloidal chitin
(D) Chitinase production with 1% colloidal chitin

Chitinolytic activity has been implicated in the biocontrol activity of several bacteria, including *Streptomyces* sp. Due to the innocuous nature of these organisms, delivery systems have been developed to introduce endophytic actinomycetes such as *Streptomyces* sp. into plants and the potential for endophytes as biocontrol agents has been explored (Bhattacharya *et al* 2007).

Various hydrolytic enzymes e.g. proteases/peptidases, chitinases/chitosanases, cellulases/endoglucanases, amylases, pectate, lyases were produced by *Streptomyces coelicolor* (Dyson 2009). Microorganisms which secrete a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases. Biological controlling agents can replace chemical agents in controlling pathogenic insects, microbials and weeds. Several biofungicides are based on antibiotic metabolites and hydrolytic enzymes.

Elad *et al* (1982) suggested extracellular cell wall degrading enzymes in the biological control of phytopathogenic fungi by *Trichoderma harzianum*. In addition, actinomycetes especially *Streptomycetes* has been effective in the protection of plants against soilborne fungal pathogens (Crawford *et al* 1993).

4.3 Quantitative production of extracellular chitinase

On the basis of maximum antifungal activity as well as hydrolytic enzymes production, the endophytic actinomycete isolates AR3 and O9 were selected for quantitative production of chitinase enzyme. Screening for chitinase production was done by plate agar assay by using different concentrations of colloidal chitin as a substrate. From a preliminary screening of enzymes by the plate method by using different substrate concentrations, a clear zone surrounding the actinomycetes colonies were observed indicating that AR3 and O9 produced chitinase. But the qualitative analysis showed that the best chitinase production zone was seen by using 0.6% colloidal chitin concentration as compared to 1% colloidal chitin concentration as a substrate. Maximum chitinase activity was observed on 4^{th} day 0.083 U/ml in AR3 an isolate of *Emblica officinalis,* by using 0.6% colloidal chitin concentration. Chitinase activity was maximum during logarithmic or exponential phase. The enzyme activity was increased rapidly in log phase and then slowly decreased in stationary phase of growth. With 1% colloidal chitin substrate concentration, the maximum activity of AR3 was observed 0.0639 U/ml on 5th day (Fig 2). The chitinase activity in AR3 was observed to be maximum with 0.6% colloidal chitin concentration. In case of O9 isolate, chitinase production increased upto 5^{th} day with 0.6% substrate concentration and total production was 0.080 U/ml after 7 days. With 1% colloidal chitin concentration chitinase activity was observed to be highest on 4^{th} day then, slowly reached in stationary phase with the total production of 0.0656 U/ml of extracellular chitinase enzyme (Fig 3). Chitinase activity of O9 isolate was maximum by using 0.6% substrate concentration. Both the isolates were exihibiting maximum extracellular chitinase production with 0.6% colloidal chitin concentration. Similar

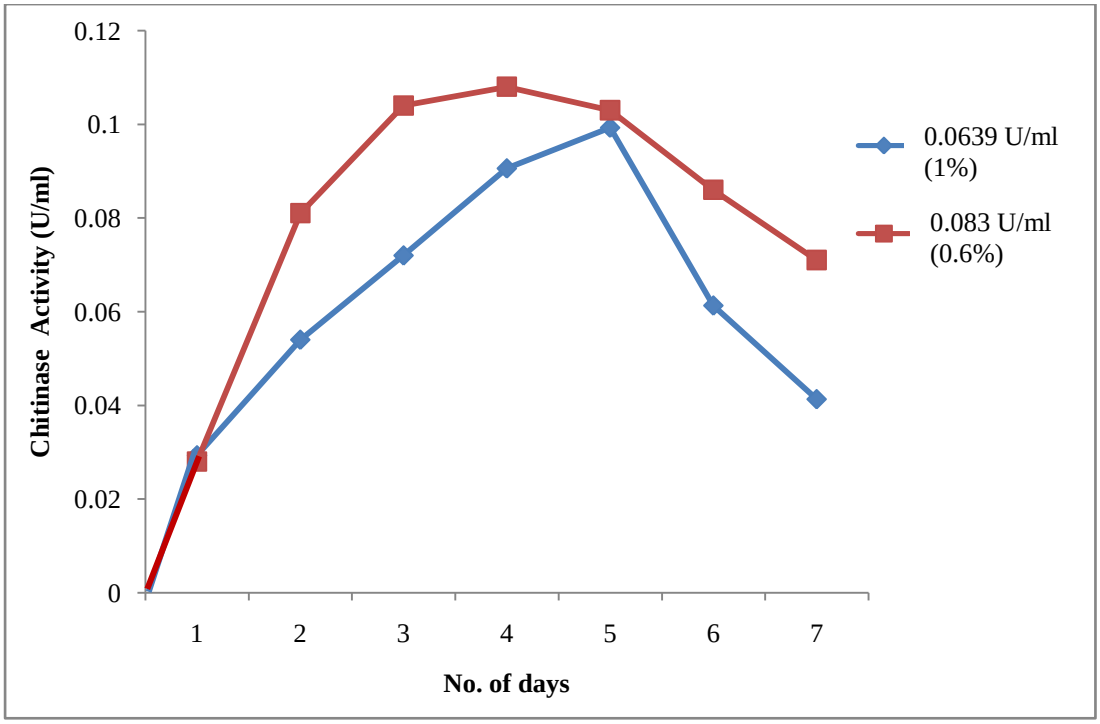

Fig 2: Quantitative production of chitinase by AR3 isolate

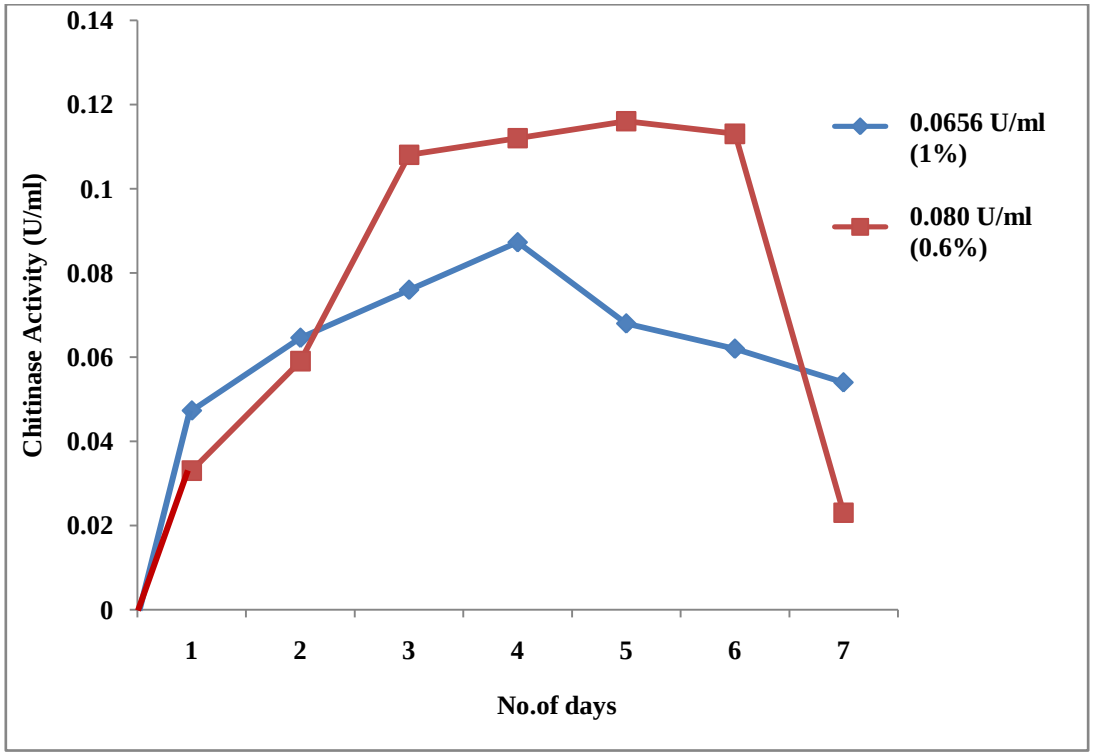

Fig 3: Quantitative production of chitinase by O9 isolate

observations had been made by Young and Bell (1985) and Neugebour *et al* (1991) during production of chitinase from *S. marcescens* and *S. lividans* respectively.The growth of the culture was slow in the beginning and was exponential after 84 h. Enzyme production increased was in exponential phase.

Taechowisan *et al* (2003) reported that strain CMUAc130 more chitinase activity than other isolates. Maximum levels of *Streptomyces aureofaciens* CMUAc130 chitinase production (0.0833 $mUml^{-1}$) was achieved by the addition of 1% colloidal chitin, at 30-40 ºC with 100-150 rev min^{-1} shaking in pH 6.5-7.0 culture after 7 days of incubation. The chitinase activity reported out to be 0.00093 U/mL in *Streptomyces* sp. P4 by using 0.6% colloidal chitin (Tang-um and Niamsup 2012a). Bacteria produce chitinase to digest chitin primarily to utilize it as a carbon and energy source. *Streptomyces* strains are regarded as the major producers of chitinases in soil. *Streptomyces lydicus* WYEC108 (Mahadevan and Crawford 1997) and *Acremonium obclavatum* (Gunaratna and Balasubramanian 1994) also hydrolysed colloidal chitin more rapidly than crude chitin or chitin from fungal cell walls. Beyer and Diekmann (1985) observed cell wall degradation of *Penicillium chrysogenum* by chitinase system of *Streptomyces* sp. ATCC 11238. An increase in chitinase production was observed in *S. aureofaciens* and *S. halstedii* when cultured in a medium containing colloidal chitin supplemented with fungal cell wall preparations (Joo 2005a, Taechowisan *et al* 2003).

Mane and Deshmukh (2009) observed that *Streptomyces canus* showed maximum activity at 1.2% chitin concentration (4.8 unit ml^{-1}) and the activity remained constant up to a concentration of 1.6%. *Micromonospora brevicatiana* showed maximum activity at substrate concentration 1.2% and the activity decreased thereafter. *S. pseudogriseolus* showed activity up to 1% substrate concentration and declined thereafter.

Kavya *et al* (2012) reported that *Streptomyces* strain A has exhibited optimum activity of 5.83µmol/ml/hr when ISP-2 media was used and incubated till day 7 at 37ºC. Similarly, chitinase activity was observed to be 1.356 µmol/ml/hr on day 7 when colloidal chitin media was used.

Priya *et al* (2011) reported *Streptomyces hygroscopicus* - VMCH2 which produced a total chitinase activity of 28.09 units / ml under optimized growth conditions. The maximum chitinase production was observed with 0.2% colloidal chitin at pH 7.0 and 35° C after 8 days of incubation. *S. hygroscopicus* was found to be most active organism when colloidal chitin was added to the medium as sole carbon and nitrogen source. There was no chitinase production until 49 h of incubation, after which the amount increased from 96 to 240 h. The chitinase activity remained until 288 hours during production of chitinase from *Streptomyces marcescens* and *Streptomyces lividans* respectively. The growth of the culture was slow at the beginning and was exponential after 96 h. Enzyme production was increased in exponential phase and more amounts were detected in the stationary growth phase.

The purified chitinase of *Streptomyces halstedii* AJ-7 suppressed the growth of various fungal phytopathogens: *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Stemphylium lycopersici*. The enzyme showed highest antifungal activity against *Fusarium oxysporum* but the purified enzyme had no inhibitory activity against cell wall chitin-containing fungi *Botrytis cinerea* and *Pythium ultimum*, the non-chitin containing fungi *Phytophthora capsici* and *Rhizopus stolonifer* (Joo 2005a).

Haggag and Abdallh (2012) observed that *Streptomyces hygroscopic* produced relatively high levels of chitinase (5.8 U/mg protein) at day 1 of the incubation period. The level of chitinase was sharply increased during the exponential phase and dramatically declined when the cells entered the stationary phase. It was postulated that chitinase produced by the antagonists could be involved in disease control. The production of these enzymes was therefore used as the criteria for selection of potential biocontrol agents against pathogens.

Antagonism could be better explained by polymeric substances such as enzymes, probably acting in synergism in the lysis of the fungal cell-wall. Chitinolytic activity detection, suggest that the antagonism observed could be explained by the enzymatic action on fungal cell-wall or co-operative synergism (Schirmbock *et al* 1994). The absence of their growth in fungal mycelia may be due to the presence of some inhibitory substance or due to the lack of other enzyme systems such as glucanases, essential for complete cell-wall lysis.

4.4 Scanning electron microscopic (SEM) studies of the antagonistic effect of potential actinomycete isolates on fungal cell wall

Scanning electron microscopy was performed by co-culturing *Fusarium oxysporum* and *Emblica officinalis* root endophytic actinomycete isolate AR3 and *Ocimum sanctum* root endophyte O9 that was identified as *Streptomyces rochei* strain KMB-1(GenBank Accession Number KJ020689.1) using 16S rDNA sequencing technique. Scanning electron micrographs showed degradation of *Fusarium oxysporum* cell walls due to secretion of diffusible compounds by AR3 and O9 as compared to control. The control plate of *Fusarium oxysporum* showed the presence of regular vegetative cells and cells having smooth surface with overall intact morphology whereas fungal colony inoculated with AR3 and O9 showed hyphae disrupted and damaged at the edges of the inhibited fungal colonies on the PDA plates. The diameter of the hyphae in *Fusarium oxysporum* culture infected with both the isolates found to be more than the control due to osmosis or due to intake of water into the cells. The cell wall surface of treated fungus appeared to have expanded, indicating that the cytoplasmic structures were flushed out of the cells and spores were partially deformed and reduced in size. Many cells were enlarged and elongated. The isolate O9 i.e. *Streptomyces rochei* strain KMB-1 entered directly into fungal cell wall and caused disruption of hyphae structure. Tang-um and Niamsup (2012a) reported that breakage of the cell walls of *Fusarium oxysporum f.sp.lycopersici* mycelia growing towards *Streptomyces* sp. P4 as compared to control

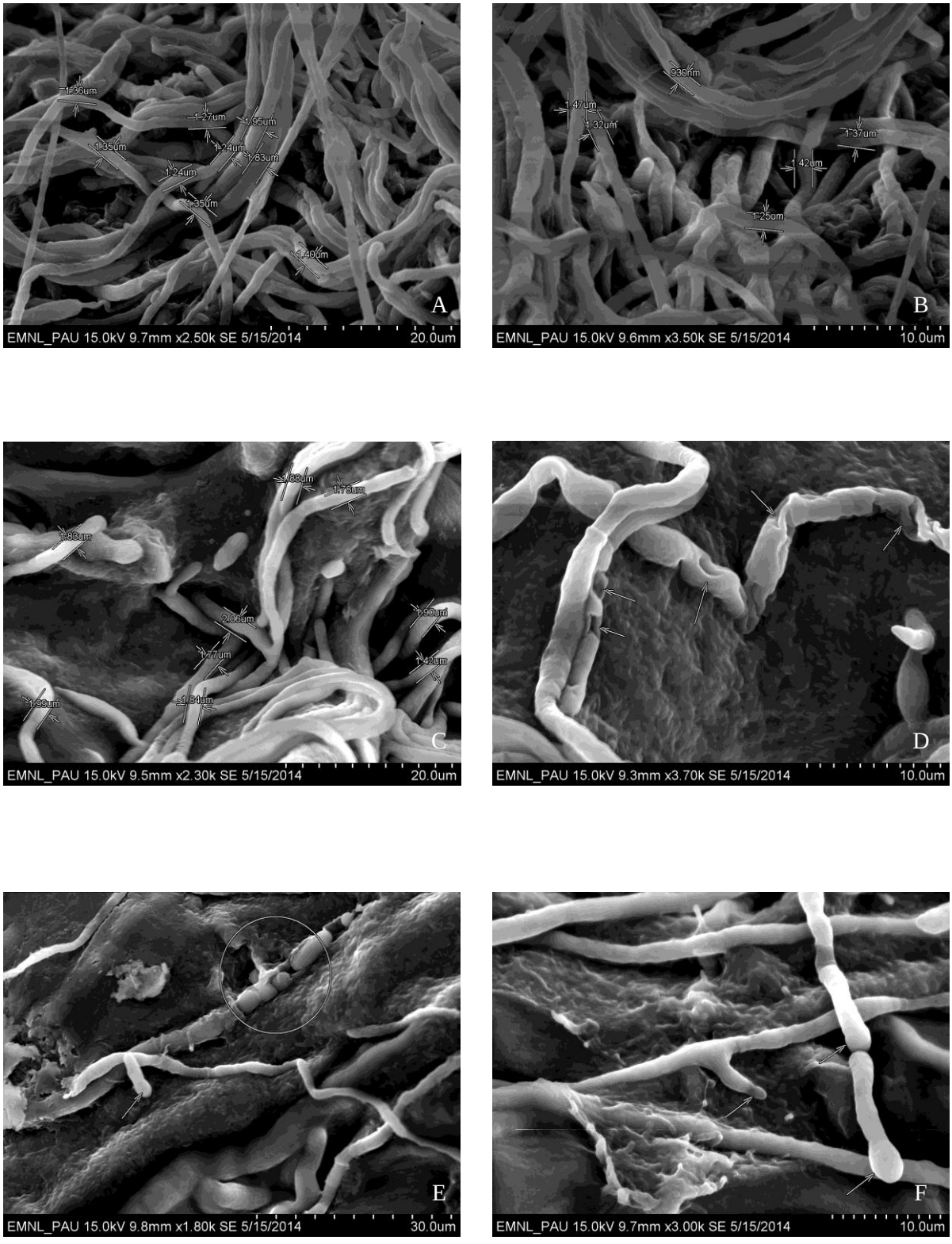

Fig 4: ***Fusarium oxysporum*** **control (A) (B),** ***Fusarium oxysporum*** **infected with AR3 (C) (D),** ***Fusarium oxysporum*** **infected with O9 (E) (F)**

Fusarium oxysporum. The effect was investigated and compared with the control. Prapagdee *et al* (2008) also observed that the antifungal activity of *Streptomyces hygroscopicus* during exponential growth was mainly due to hydrolytic enzymes, while in the stationary phase it was due to secondary thermostable compounds. In addition, there was a report on a positive correlation between chitinolytic and antagonistic activities of *Streptomyces* against the fungi *Collectotrichum sublineolum, Guignardia citricarpa, Rhizoctonia solani* and *Fusarium oxysporum*, but not in the oomycetes *Pythium* sp. and *Phytophthora parasitica*, which contain cellulose as a major cell wall component (Quecine *et al* 2008). He *et al* (2009) reported that endophytic bacteria obtained from *Epimedium brevicornu* degraded hypha of *Sclerotinia sclerotiorum* and the cytoplasm was extravagated outside from the fungal walls.

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces,* are well known as antifungal agents that inhibit several plant pathogenic fungi (Khamna *et al* 2009). The chitinolytic activity of *Streptomyces* sp. obtained from citrus and soybean plants showed high inhibition levels against fungi and the fungal hyphae exhibited a degraded appearance after chitinolytic A8 strain culture treatment. This indicated an inhibitory role of chitinase to plant pathogenic fungi. The *C. sublineolum* hyphae surface-treated with A8 culture filtrate contained many holes, possibly corresponding to lysis zones. However, the hyphal surfaces of both *C. sublineolum* and *Pythium* sp. treated with A8 culture filtrate exhibited a slightly roughened surface, indicating little or no effect of hydrolytic enzymes on these structures (Quecine *et al* 2008).

4.5 Molecular identification by 16S rDNA of potential isolate

The culture, which was labeled as O9 from *Ocimum sanctum* was similar to *Streptomyces rochei* strain KMB 1(GenBank Accession Number KJ020689.1) based on nucleotide homology and phylogenetic analysis (Fig 5) (Table 4).

1. O9-1492R-S011052.ab1: Data obtained with Reverse primer

O9-1492R-S011052 (1033 bp)

GGAACGGGGACACTATGGCGACACTGTGACAACATATAGCGAGACAGGGTCACA
CTCTACGGATACCGTGTCACCATATACAGAGACAGGGTCTAAATATATAGGTTTT
TTGTTACAACATACAGGGCCGGTGTGAAAAAACCCACCGCTTAAGTGAAAACAT
ATTAAAATGGAGGAGAACTTTAAAATCACTTATTGATTTTTGAGATACAATATTG
AGGGTGCTTAAAATTTTGTCAAACGGGCCATTGTAAACAAGCAAGCCCCAAGAC
ATAAGGGGCATGATGACTTGACGGTCGGCCTTACCTTCCTCCGAGTTGACCCCGG
CGGTCTCCCGTGAGTCCCCAGCACCACAAGGGCCTGCTGGCAACACGGGACAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAG
CCATGCACCACCTGTACACCGACCACAAGGGGGACCCTGTCTCCAGGGTTTTCCG
GTGTATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCGTCGAATTAAGCCACATGC
TCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTATCCTTGCGGCCGTA

CTCCCCAGGCGGGGCACTTAATGCGTTAGCTGCGGCACGGACAACGTGGAATGTT
GCCCACACCTAGTGCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTG
TTCGCTCCCCACGCTTTCGCTCTCTCAGCGTCAGTATCGGCCCAGAGATCCGCCTT
CGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCG
ATCTCCCCTACCGAACTCTAGCCTGCCCGTATCGACTGCAGACCCGGGGTTAAGC
CCCGGGCTTTCACAACCGAAAGTGACAAGCCGCCTACGAGCTCTTTACGCCCAAT
ATTCCGGACACGCTTGCGCCCTACATATTACCGCGGCTGCTGGCACGTAGTTAGC
CGGCGCCTTCTTTCTGCAGGTACCGTCACTTTCCTTTCTTCCT

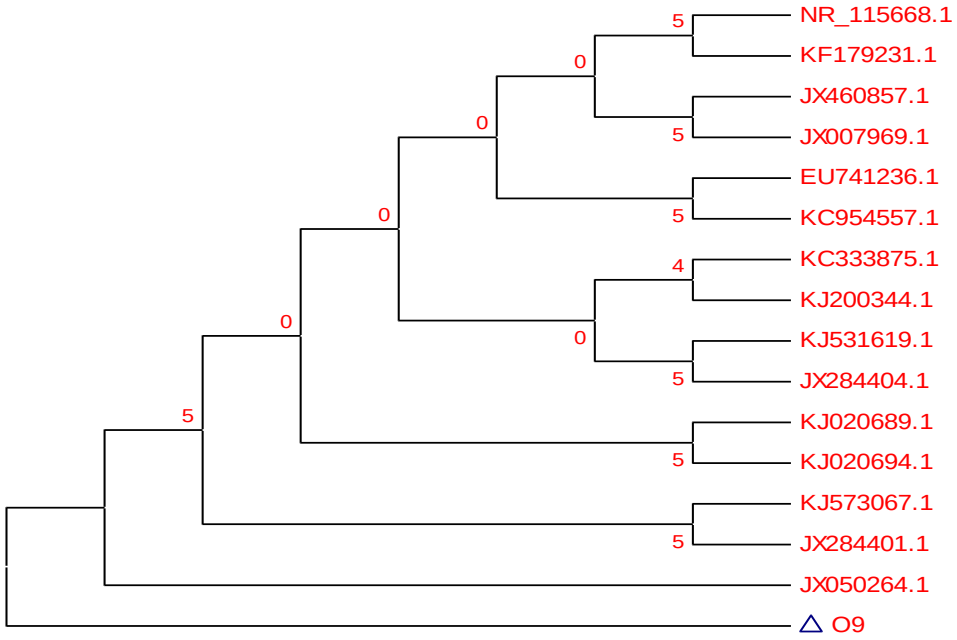

Fig 5: Phylogenetic Tree of evolutionary relationships of 11 taxa

Table 4: Sequence producing significant alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KJ020689.1	*Streptomyces rochei* strain KMB-1	1338	1338	74%	0.0	98%
KC333875.1	*Streptomyces* sp. RK61	1332	1332	74%	0.0	98%
EU741236.1	*Streptomyces mutabilis* strain 13676E	1332	1332	74%	0.0	98%
KJ573067.1	*Streptomyces enissocaesilis* strain EIH4	1330	1330	74%	0.0	98%
NR_115668.1	*Streptomyces enissocaesilis* strain NRRL B-16365	1330	1330	74%	0.0	98%
KF179231.1	*Streptomyces mutabilis* strain ACT147	1330	1330	74%	0.0	98%
KJ531619.1	*Streptomyces rochei* strain CB6J5	1330	1330	74%	0.0	98%
KJ200344.1	*Streptomyces* sp. NEAE-126	1330	1330	74%	0.0	98%
KC954557.1	*Streptomyces mutabilis* strain DSM 40169	1330	1330	74%	0.0	98%
KJ020694.1	*Streptomyces mutabilis* strain BLH-1	1330	1330	74%	0.0	98%
JX460857.1	*Streptomyces djakartensis* strain D5	1330	1330	74%	0.0	98%
JX284404.1	*Streptomyces fungicidicus* strain FMA-204	1330	1330	74%	0.0	98%
JX284401.1	*Streptomyces rochei* strain FMA-91	1330	1330	74%	0.0	98%
JX007969.1	*Streptomyces rochei* strain S41	1330	1330	74%	0.0	98%
JX050264.1	*Streptomyces* sp. DSV15	1330	1330	74%	0.0	98%

4.6 Evaluation of effectiveness of endophytic actinomycetes isolates AR3 and O9 as potential antagonists against *Fusarium oxysporum* in green house

4.6.1 Percentage of seed germination

Maximum seed germination was observed in O9 (100 %) followed by AR3 (93.33 %) and minimum was recorded in uninoculated control (73.33%) followed by treatment with *Fusarium oxysporum* (66.66%) (Fig 6 and 7). Venkatachalam *et al* (2010) observed that the isolate *Streptomyces gibosonni* SAV 01 and *Streptomyces grieseoleutus* SAV 06 induced germination of radish, maize and gram. *S. gibosonni* and *S. grieseoluteus* produced putriscine which induced germination of the seeds. *Streptomyces* produce many extracellular active compounds such as indole acetic acid, phosphate solubilizing substances, chitinase and intracellular siderophores which induce germination of seeds and their growth.

Table 5: Percentage of seed germination

Treatments	Germination % (10 DAS)
AR3	93.33
O9	100
AR3 + *Fusarium oxysporum*	82.00
O9 + *Fusarium oxysporum*	86.66
Control	73.33
Fusarium oxysporum	66.66

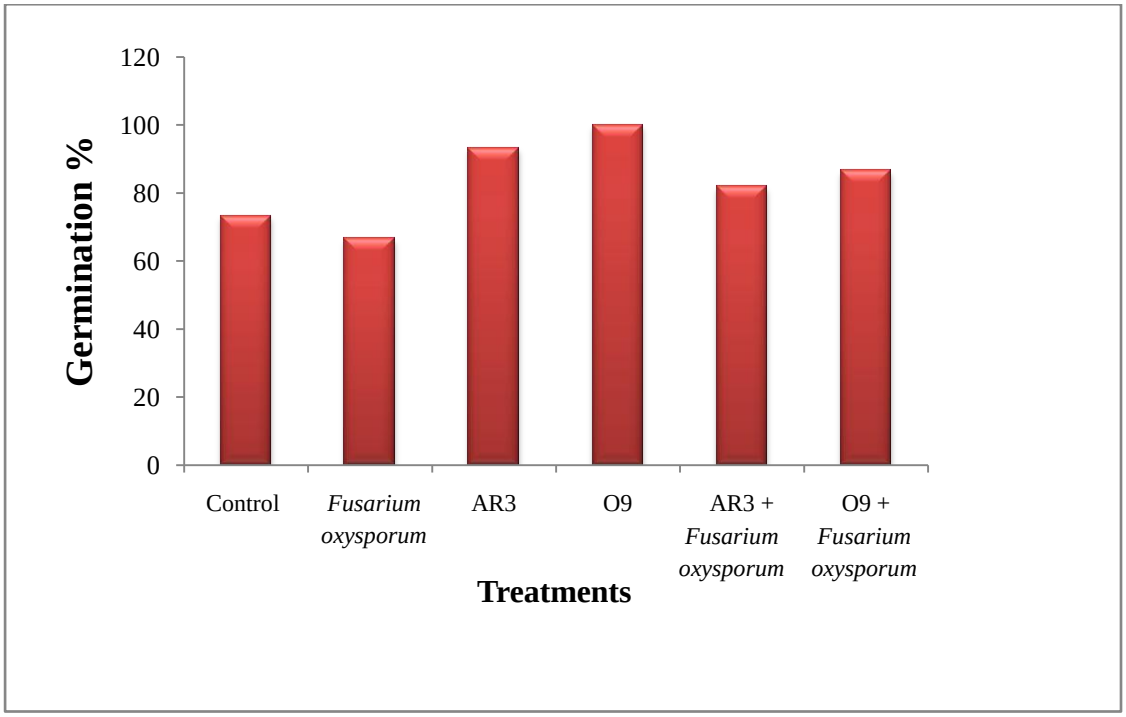

Fig 6: Percent germination under pot condition at 10 DAS

4.6.2 Root length

Data on root length presented in Table 6 showed non-significant difference in all the treatments at 60 days. Maximum root enhancement was recorded in AR3 (11.5 cm plant^{-1} at 60 DAS) followed by O9 (9.50 cm plant^{-1} at 60 DAS) (Fig 8). Root length was measured to be less in treatment with *Fusarium oxysporum* (6.0 cm plant^{-1} at 60 DAS) as compared to AR3 + *Fusarium oxysporum* and O9 + *Fusarium oxysporum*. *Streptomycetes*, along with other bacterial strains belonging to the *Actinomycetales* have the ability to colonize plant root surfaces (Kortemaa *et al* 1994, Tokala *et al* 2002). Under greenhouse conditions in sorghum the *Streptomyces* strains significantly enhanced all the agronomic observations including root length (3-18%) over the un-inoculated control. The *Streptomyces* strains are reported widely in for their PGP potential (Gopalakrishnan *et al* 2013).

Table 6: Effect of isolates on root length of musk melon under pot conditions

Treatments	**Root length (cm plant^{-1})**
AR3	11.5
O9	9.50
AR3 + *Fusarium oxysporum*	7.16
O9 + *Fusarium oxysporum*	8.16
Control	7.0
Fusarium oxysporum	6.0
CD at 5%	NS

4.6.3 Shoot length

The results of shoot length were observed to be significantly different in all the treatments at 15, 30, 45 and 60 DAS as compared to *Fusarium oxysporum* (Table 7). The maximum shoot length was recorded in the treatments with AR3 and O9 isolates alone. But shoot length in AR3+ *Fusarium oxysporum* was recorded to be 50.27 cm plant^{-1} at 60 DAS and 45.25 cm plant^{-1} at 60 DAS in O9 + *Fusarium oxysporum* as compared to *Fusarium oxysporum* (43.11 cm plant^{-1} at 60 DAS). Ara *et al* (2012) reported maximum plant height, weight and minimum disease index (13.9 cm, 3.8 and 0.58 g respectively) in the plants treated with *Streptomyces* strain AS-2(29) after 30 DAS.

Fig 7: Control (A), *Fusarium oxysporum* (B), AR3 (C), O9 (D), AR3 + *Fusarium oxysporum* (E) and O9+ *Fusarium oxysporum* (F)

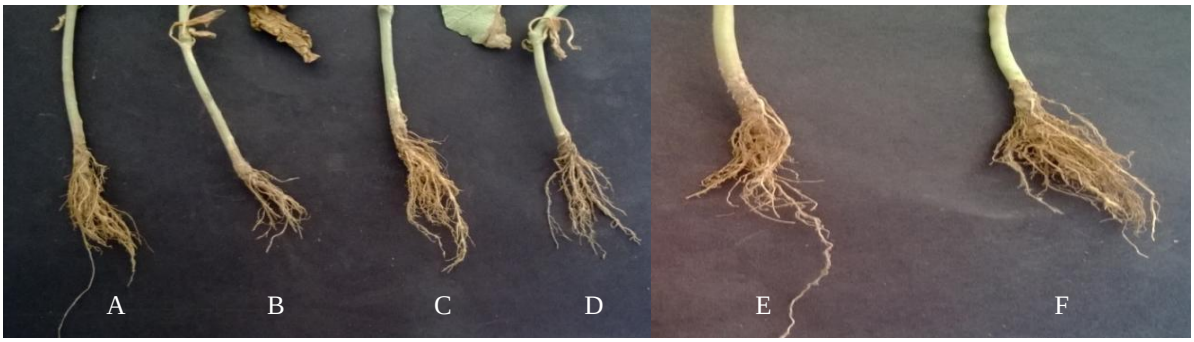

Fig 8: Control (A), *Fusarium oxysporum* (B), AR3 + *Fusarium oxysporum* (C), O9 + *Fusarium oxysporum* (D), AR3 (E) and O9 (F)

Table 7: Effect of isolates on shoot length of musk melon

Treatments	Shoot length (cm plant^{-1})			
	15 DAS	**30 DAS**	**45 DAS**	**60 DAS**
AR3	4.97	12.56	20.40	53.08
O9	4.88	11.98	18.60	47.16
AR3 + *Fusarium oxysporum*	3.02	8.16	15.38	50.27
O9 + *Fusarium oxysporum*	4.04	11.14	17.11	45.25
Control	3.66	8.58	15.15	35.75
Fusarium oxysporum	3.88	11.2	14.41	43.11
CD at 5%	1.20	1.53	1.27	1.29

4.6.4 Root fresh weight

Root fresh weight was observed to be significantly maximum in the treatments with AR3 (0.587 gm plant^{-1}), O9 (0.541 gm plant^{-1}), AR3 with *Fusarium oxysporum* (0.375 gm plant^{-1}) and O9 with *Fusarium oxysporum* (0.404 gm plant^{-1}) as compared with *Fusarium oxysporum* (0.299 gm plant^{-1}) (Table 8). These rhizosphere antagonists significantly increased maize fresh and dry weight of shoot and root, length of root, shoot height as compared to the control *Cephalosporium maydis*, a causal agent of late wilt disease of maize (El-Mehalawyi *et al* 2004).

Table 8: Root fresh weight under green house condition

Treatments	Root fresh weight (gm plant^{-1})
	60 DAS
AR3	0.587
O9	0.541
AR3 + *Fusarium oxysporum*	0.375
O9 + *Fusarium oxysporum*	0.404
Control	0.352
Fusarium oxysporum	0.299
CD at 5%	0.110

4.6.5 Shoot fresh weight

The results of shoot fresh weight were recorded to be significantly different in all the treatments (Table 9). The shoot fresh weight was observed maximum in AR3 (10.09 gm plant^{-1}) and O9 (8.62 gm plant^{-1}) followed by AR3 with *Fusarium oxysporum* (8.40 gm plant^{-1}) and O9

with *Fusarium oxysporum* (7.02 gm plant^{-1}) as compared to treatment with *Fusarium oxysporum* (5.00 gm plant^{-1}).

Table 9: Effect of isolates on shoot fresh weight

Treatments	Shoot fresh weight (gm plant^{-1})
	60 DAS
AR3	10.09
O9	8.62
AR3 + *Fusarium oxysporum*	8.40
O9 + *Fusarium oxysporum*	7.02
Control	6.13
Fusarium oxysporum	5.00
CD at 5%	1.33

4.6.6 Root dry weight under pot condition

The root dry weight was recorded in all the treatments were non significantly different from each other (Table 10). Maximum was observed in AR3 (0.154 gm plant^{-1}) and O9 (0.154 gm plant^{-1}). The root dry weight was more in the treatments with AR3+ *Fusarium oxysporum* (0.130 gm plant^{-1}) and O9+*Fusarium oxysporum* (0.115 gm plant^{-1}) as compared to treatment with *Fusarium oxysporum* (0.111 gm plant^{-1}). Some actinomycetes isolates significantly increased root and shoot dry weight. Such increases have been reported in cauliflower, wheat and potato. This phenomenon may be related to the production of growth regulators by actinomycetes (Kloepper 1993).

Table 10: Effect of actinomycete isolates on root dry weight

Treatments	Root dry weight (gm plant^{-1})
	60 DAS
AR3	0.154
O9	0.154
AR3 + *Fusarium oxysporum*	0.130
O9 + *Fusarium oxysporum*	0.115
Control	0.126
Fusarium oxysporum	0.111
CD at 5%	NS

4.6.7 Effect of isolates on shoot dry weight

Shoot dry weight was observed significantly different in all the treatments (Table 11). The maximum was recorded in O9 (2.63 gm plant^{-1}) and AR3 (2.46 gm plant^{-1}). The shoot dry weight was observed minimum in the treatment with *Fusarium oxysporum* (1.26 gm plant^{-1}) as compared to AR3 with *Fusarium oxysporum* (1.38 gm plant^{-1}) and O9 with *Fusarium oxysporum* (1.47 gm plant^{-1}).

Table 11: Effect of isolates on shoot dry weight

Treatments	**Shoot dry weight (gm plant^{-1})**
	60 DAS
AR3	2.46
O9	2.63
AR3 + *Fusarium oxysporum*	1.38
O9 + *Fusarium oxysporum*	1.47
Control	1.56
Fusarium oxysporum	1.26
CD at 5%	0.652

4.6.8 Incidence and Inhibition of disease development (%) in the plants

Maximum wilt incidence (%) was observed in the treatment with *Fusarium oxysporum* (70%) (Table 12). There was no wilting in the treatments with inoculation of AR3 and O9 isolates alone. Incidence of disease was reported to be maximum in AR3 with *Fusarium oxysporum* (18.18%) and O9 with *Fusarium oxysporum* (16.66%). Similarly, maximum inhibition of disease was observed in AR3 with *Fusarium oxysporum* (25.97%) followed by O9 with *Fusarium oxysporum* (23.8%) (Fig 9). Costa *et al* (2013) reported two *Streptomyces* isolates for the control of *P. aphanidermatum* in cucumber (*Cucumis sativa* L.) under greenhouse conditions. Isolate 16R3B was able to reduce 71% damping-off incidence whereas isolate 14F1D/2 reduced the disease incidence by 36%. Damping off control in cucumber, mainly for the isolate 16R3B suggested for its use in greenhouse cucumber. The culture filtrate of *Streptomyces spectabilis* CMU-PA101 has also been reported to control shallot blotch caused by *Alternaria porri* (Khamna *et al* 2009). Kanini *et al* (2013) reported the ability of *Streptomyces rochei* ACTAI55I to protect tomato seeds from the pathogenic effect of *Fusarium oxysporu*m. The results found under greenhouse conditions with the isolates AR3 and O9 proved their potential as a biocontrol agents to reduce the *Fusarium* wilt caused by *Fusarium oxysporum* in this planting system.

Table 12: Incidence of wilt and inhibition of disease development (%)

Treatments	Incidence of disease (%)	Inhibition of disease development (%)
AR3 + *Fusarium oxysporum*	18.18	25.97
O9 + *Fusarium oxysporum*	16.66	23.8
Fusarium oxysporum	70	-

The mode of action of *Streptomyces* appeared to be the antagonism shown by the production of Tubercidin, produced by *Streptomyces tubercidicus* and *Streptomyces violaceoniger* against *Phytophthora capsici* (Hawang and Kim 1995) and Geldamycin produced by *S. hygroscopicus* against *Rhizoctonia* (Rothrock and Gottlieb 1984).

Five strains of *Streptomyces* sp. (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) isolated from herbal vermi-compost were reported as having potential for biocontrol of *Fusarium* wilt in chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* and the application of *Streptomyces griseoviridis* (Mycostop) reduced the percentage of disease caused by *Fusarium oxysporum* f. sp. *radicis cucumerinum.* (Gopalakrishnan *et al* 2011b). Sreeja and Gopal (2013) observed that the endophytic actinomycete *Streptomyces thermodiastaticus* showed superior performance in plant growth promotion as well as in the management of bacterial wilt in tomato.

Sorghum seedlings inoculated with *Drechslera halodes* which causes leaf spot on sorghum and tomato seedlings inoculated *Alternaria alternata* which causes early blight on tomato when treated with *S. rochei* R92 broth culture extract were found to be free of disease symptoms (zero infection percentage). Seedlings of the positive control have recorded 100% infection. The *n*-butanol extract of *S. rochei* R92 culture was more potent against the phytopathogenic fungi as compared with the tested commercial antifungal agents (Hussein *et al* 2014).

Fig 9: Wilting in different treatments
Control (A), AR3 (B), O9 (C), *Fusarium oxysporum* (D), AR3 + *Fusarium oxysporum* (E) and O9 + *Fusarium oxysporum* (F)

CHAPTER V

SUMMARY

Endophytic actinomycetes are the bacteria that do not visibly harm the plants and can be isolated from the surface of the disinfected plant tissues or extracted from inside of the plants. Endophytes have been demonstrated to improve and promote the growth of the host plants as well as reduce disease symptoms caused by plant pathogens or various environmental stresses. Endophytic actinomycetes are known to produce a plethora of substances e.g. novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds of potential use to modern medicine, agriculture, and industry. They play an important role in decomposition of organic materials, such as cellulose, lignocellulose, starch and chitin in soil. Endophytic actinomycetes have been cited as promising biocontrol agents, either acting directly on fungal cell walls or initiating increased plant responses against disease.

Out of 70 isolates 11 isolates (6 from *Azadirachta indica* A. Juss, 3 from *Emblica officinalis*, 1 from *Aloe vera* and 1 from *Ocimum sanctum*) were displaying antagonistic activity against one or the other plant pathogenic fungi tested. The antifungal activity against all plant pathogenic fungi *Alternaria alternata*, *Fusarium oxysporum*, *Rhizoctonia solani* was observed by AR3 isolate of *Emblica officinalis* and O9 an isolate of *Ocimum sanctum.* AR3 has maximum percent inhibition against *Fusarium oxysporum* (60.69 %). None of the isolates exhibited antagonistic activity against *Sclerotium rolfsii*.

Thirty two isolates from *Azadirachta indica* A. Juss, 8 from *Emblica officinalis*, 4 from *Aloe vera*, 2 from *Ocimum sanctum* and 1 from *Mentha arvensis* produced amylase enzyme. Fifteen isolates from *Azadirachta indica* A. Juss, 6 from *Emblica officinalis*, 2 from *Ocimum sanctum*, 1 from *aloe vera* and 1 from *Mentha arvensis* produced protease enzyme. Sixteen isolates of endophytic actinomycetes, out of which 5 from *Azadirachta indica* A. Juss, 5 from *Emblica officinalis*, 3 from *Aloe vera*, 1from *Ocimum sanctum*, 1 from *Mentha arvensis* were having capability to produce chitinase.

Quantitative production of chitinase was shown by two endophytic actinomycetes isolates. The chitinase activity of AR3 an isolate of *Emblica officinalis*, reported out to be 0.083 U/ml by using 0.6% colloidal chitin concentration and 0.0639 U/ml with 1% colloidal chitin as substrate concentration. In case of O9 isolate of *Ocimum sanctum*, chitinase production was observed to be 0.080 U/ml with 0.6% substrate concentration and 0.0656 U/ml with 1% colloidal chitin concentration. Both the isolates displayed more extracellular chitinase production with 0.6% colloidal chitin as compared to 1% colloidal chitin concentration.

Scanning electron microscopy was done with two co-cultures containing *Fusarium oxysporum* and one of the endophytic actinomycete isolates AR3 and O9. Results showed the

degradation of cell wall of *Fusarium oxysporum* mycelia growing towards the isolates AR3 and O9 as compared to control while the single culture plate of *Fusarium oxysporum* control showed the regular vegetative hypae with intact morphology.

Molecular identification by 16S rDNA sequencing of potential isolate revealed that O9 isolate of *Ocimum sanctum* was similar to *Streptomyces rochei* strain KMB 1 (GenBank Accession Number KJ020689.1) based on nucleotide homology and phylogenetic analysis with a 98% identity score.

The pot experiment on musk melon (variety *Punjab sunehri*) was conducted in green house. The study revealed that:

Maximum percent germination was observed in O9 (100 %) followed by AR3 (93.33%) and minimum was recorded in uninoculated control (73.33%) followed by treatment with *Fusarium oxysporum* (66.66%). Maximum enhancement in root length was recorded in treatment with AR3 (11.5 cm plant^{-1} at 60 DAS) and O9 (9.50 cm plant^{-1} at 60 DAS). Root length was measured to be less in treatment with *Fusarium oxysporum* (6.0 cm plant^{-1} at 60 DAS) as compared to AR3 + *Fusarium oxysporum* (7.16 cm plant^{-1} at 60 DAS) and O9 + *Fusarium oxysporum* (8.16 cm plant^{-1} at 60 DAS). Shoot length recorded maximum in treatments with AR3 and O9 isolates. But AR3+ *Fusarium oxysporum* recorded more shoot length (50.27 cm plant^{-1} at 60 DAS) and in O9 + *Fusarium oxysporum* (45.25 cm plant^{-1} at 60 DAS) as compared to *Fusarium oxysporum* control (43.11 cm plant^{-1} at 60 DAS).

Root fresh weight was observed to be maximum in the treatments with AR3 (0.587 gm plant^{-1}), O9 (0.541 gm plant^{-1}), AR3 with *Fusarium oxysporum* (0.375 gm plant^{-1}) and O9 with *Fusarium oxysporum* (0.404 gm plant^{-1}) as compared to treatment with *Fusarium oxysporum* (0.299 gm plant^{-1}). Shoot fresh weight was observed maximum in AR3 (10.09 gm plant^{-1}) and O9 (8.62 gm plant^{-1}) followed by AR3 with *Fusarium oxysporum* (8.40 gm plant^{-1}) and O9 with *Fusarium oxysporum* (7.02 gm plant^{-1}) as compared to treatment with *Fusarium oxysporum* (5.00 gm plant^{-1}).

Similar root dry weight was observed in AR3 (0.154 gm plant^{-1}) and O9 (0.154 gm plant^{-1}). Root dry weight was more in treatments with AR3 with *Fusarium oxysporum* (0.130 gm plant^{-1}) and O9 with *Fusarium oxysporum* (0.115 gm plant^{-1}) as compared to treatment with *Fusarium oxysporum* (0.111 gm plant^{-1}). Maximum shoot dry weight was recorded in O9 (2.63 gm plant^{-1}) and AR3 (2.46 gm plant^{-1}) and minimum in the treatment having *Fusarium oxysporum* (1.26 gm plant^{-1}) as compared to AR3 with *Fusarium oxysporum* (1.38 gm plant^{-1}) and O9 combined with *Fusarium oxysporum* (1.47 gm plant^{-1}).

Maximum wilt incidence (%) was observed in treatment with *Fusarium oxysporum* (70%). Incidence of disease observed in AR3 with *Fusarium oxysporum* was 18.18% and 16.66% in O9 with *Fusarium oxysporum.* Maximum inhibition of disease was observed in

AR3 with *Fusarium oxysporum* (25.97%) followed by O9 with *Fusarium oxysporum* (23.8%).

Hence, from the present study, it is concluded that the endophytic actinomycetes are effective biocontrol agents against phytopathogenic fungi by inhibiting occurrence of a disease.

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APPENDIX-I

Following media used in the present study

1. Nutrient agar

	g l^{-1}
Beef extract	3.0
Peptone	5.0
NaCl	5.0
Agar	20.0
Distilled water	1000 ml
pH	7.0

2. Glycerol yeast agar

	g l^{-1}
Yeast extract	2.0
Glycerol	5.0 (ml)
Agar	20.0
Distilled water	1000 ml
pH	7.0

3. Glucose yeast extract agar

	g l^{-1}
Glucose	10.0
Yeast extract	5.0
Peptone	5.0
Agar	20.0
Distilled water	1000 ml
pH	6.8-7.2

3. Potato dextrose agar

	g l^{-1}
Potato	250.0
Dextrose	10.0
Agar	20.0
Distilled water	1000 ml
pH	6.5

5. Colliodal chitin agar

	g l^{-1}
Colloidal chitin	10
Yeast extract	0.5
Ammonium sulphate	1.0
Magnesium sulphate hydrated	0.33
Potassium dichromate	1.36
Agar	15
pH	7.0

6. Peptone water

	g l^{-1}
Peptone	10.0
NaCI	5.0
pH	6.8-7.0

7. Starch casein agar

	g l^{-1}
Starch	10
Casein	0.3
NaCl	2.0
K_2HPO_4	2.0
$CaCO_3$	0.02
$MgSO_4.7H_2O$	0.05
$FeSO_4.7H_2O$	0.01
KNO_3	2.0