STUDIES ON SOIL-INHABITING SIDEROPHORE-PRODUCING BACTERIA AND THEIR ROLE IN SUPPRESSION OF PLANT ROOT PATHOGENS

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN SCIENCE (BIOTECHNOLOGY) OF THE
UNIVERSITY OF NORTH BENGAL

Thesis Submitted By

ANASOOYA GHOSH

UNDER THE SUPERVISION OF

Dr. Dipanwita Saha

Dr. Aniruddha Saha

DEPARTMENT OF BIOTECHNOLOGY
UNIVERSITY OF NORTH BENGAL
INDIA
2012

ACKNOWLEDGEMENT

For seeing this thesis to fruition, I would like to thank all the people who have played some role or the other at some point in my life. But mainly I would like to mention those people who played invaluable roles.

Firstly, I would like to thank my supervisor Dr. Dipanwita Saha, Assistant Professor, Department of Biotechnology, University of North Bengal, for training me adequately so as to enable me to carry out this research. Not for a moment did she lose faith in an amateur such as myself, she was always there to offer help even before I could ask.

My heartfelt thanks go to Dr. Aniruddha Saha, Associate Professor, Department of Botany, University of North Bengal, who has always lent a helping hand whenever I pestered him.

I am also indebted to Dr. R Chakraborty, Head of the Department, and Dr. Shilpi Ghosh of Department of Biotechnology, University of North Bengal for their valuable advice and encouragement throughout the course of this work.

Special thanks are due to my lab-mates, Ms. Gargee Dhar Purkayastha, Ms. Sima Mandal and Mr. Ramasish Kumar who were ready to help me whenever I asked for and they made my working place an enjoyable temple of knowledge.

I am highly obliged to Dr. Sourish Dasgupta, Md. Mehbub Isha, Mr. Bikram Saha, Mr. Hrishikesh Mandal for their continuous support throughout the execution of the work.

I express my thanks to all the staffs of the Department of Biotechnology for their assistance, cooperation and support.

I bow my head humbly to thank my parents Sri Subhash Chandra Ghosh and Srimati Uma Mitra Ghosh who made me what I am today. I am also thankful to my sister, Anushreya Ghosh for being with me every moment I needed her. My sincere thanks are also due to my other family members for their help and encouragement.

I would also like to express my gratitude from the core of my heart to Dr. Malay Kanti Karanjai, Principal, Siliguri College, Siliguri for his never-ending cooperative support and encouragement.

Heartfelt gratitude is also due to my friend who showed the way, held my hand in every odds of my life.

Heartiest thanks are given to Dr. Samik Bagchi, Sri Subir Ghosh, Sri Subimal Ghosh, Mr. Kalyan Kumar Datta, Mr. Sanjay Saw, Mrs. Rituparna Sarkar, Mr. Kamal Krishna Singh, Mr. Partho Choudhury, Dr. Milan Haldar and all others who encouraged and stood by me in all the tiring hours.

Anasooya Ghosh

(Anasooya Ghosh) 20/11/2011

PREFACE

The economic stability of India is dependent on the agricultural yield. Great deal of research carried out in the last 50 years by agricultural scientists, has its major thrust on increasing crop productivity. Despite the developments, India has not yet been able to tackle the loss of nutritional value of food items. According to the estimates of the Indian National Commission on Agriculture, fruits and vegetable suffers huge loss of their nutritional value due to lack of proper disease management. In developing countries, agriculture is the driving force for broad-based economic growth. One of the major problems with agriculture now-a-days is the ever increasing demand of enhanced production in order to provide food for the population which is in permanent augmentation. In realizing this, one of the stumbling blocks seems to be the yield losses due to plant pathogen.

Pests, weeds, diseases take a toll on gross production in our country. The country is losing agricultural production worth Rs 1.48 lakh crore annually due to damage from pests, weeds and plant diseases, according to the Crop Care Foundation of India (CCFI) (Sobrinho et al., 2003).

Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market. Additionally, the spread of plant diseases in

natural ecosystems may prevent successful application of chemicals, because of the scale to which such applications might have to be applied. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. These alternatives are those referred to as biological controls (Pal and Gardener, 2006).

Biological control is an effective means of reducing the damage caused by plant pathogens. Biological control of plant diseases involves the use of one non-pathogenic organism to control or eliminate a pathogenic organism. Hence, biological control has attracted a great interest in plant pathology and it becomes important to develop cheaper management practices to control disease and obtain higher yield.

Some soil and water bacteria have been found to naturally promote growth of plants. These bacteria aggressively colonize the roots of plants and prevent the growth and inhibit the virulence of many species of fungi as well as other bacterial species. Production of siderophores by beneficial bacteria that bind iron and make it unavailable to the pathogenic bacteria or fungi is one mechanism by which bacteria can promote the growth of plants. The growth-promoting bacteria produce specific siderophores that cannot be used by the pathogenic organisms because they lack the appropriate siderophore receptor. The pathogens are therefore not able to compete for necessary iron in the environment of the plant root system. The production of siderophores by the biocontrol agents in quantities sufficient to limit Fe^{3+} availability to the pathogen may be used as potent disease control device (Glick and Bashan, 1997).

The work embodied in this thesis was initiated in the year 2007 with broad objectives of controlling crop disease by utilizing the siderophore producing and plant growth promoting rhizobacteria. The status of the work and their results and inferences drawn thereof is presented in seven major chapters and additional supplementary details given as appendix at the end.

Abbreviations

Amp	Ampicillin
A_r	Absorbance of reference
A_s	Absorbance of sample
BCA	biocontrol agent
bp	base pair
BLAST	Basic local alignment search tool
^oC	Degree Celcius
CAS	Chrome Azurol S
CD	Critical difference
CFU	Colony forming unit
cm	centimeter
Cont.	continued
CTAB	cetyl trimethyl ammonium bromide
d	days
DDW	Deionized distilled water
DW	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxy nucleotide triphosphate
dsDNA	Double stranded DNA
EDTA	Ethylene diamino tetraacetic acid
ed.	edition
Eds.	Editors

et al.	*Et alia*
Fig.	Figure
g	gram
h	Hour
HDTMA	hexadecyltrimethyl-ammonium bromide
IAA	Indole-3-acetic acid
IARI	Indian Agricultural Research Institute
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
ITCC	Indian Type Culture Collection
ITS	Internal transcribed spacers
L	Litre
LB	Luria Bertani
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
min	minutes
MR	Methyl Red
VP	Voges–Proskauer
N	normal
NA	Nutrient agar
NB	Nutrient broth

NCBI	National Center for Biotechnology Information
No.	Number
nm	nanometer
ng	nanogram
OD	Optical Density
OD_{260}	Absorbance at 260 nm
OD_{280}	Absorbance at 280 nm
O-F	Oxidation-Fermentation
ONPG	*Ortho*-nitrophenyl-β-D-galactopyranoside
' (prime)	denotes a truncated gene at the indicated side
PCR	polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
psi	Pound per Square Inch
PEDC	percent efficacy of disease control
PGPR	Plant Growth Promoting Rhizobacteria
PIPES	piperazine-1.4-bis(2-ethanesulphonic acid)
PPL	PUSA purple long
rDNA	ribosomal Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
SDW	Sterile Distilled Water
SE	Standard Error
SEM	Scanning electron microscopy
SMA	Skimmed Milk Agar

Soln.	Solution
spp.	Species
SPSS	Statistical Package for the Social Sciences
TAE	Tris acetic acid EDTA
TE	Tris HCl EDTA
TLC	Thin layer chromatography
TRIS	Tris hydroxymethyl amino methane
TSI	Triple sugar iron
TSIA	Triple sugar iron agar
U	unit
UV	Ultra violet
UV-Vis	ultra violet visible
V	Volt
Viz.	Videlicet=namely
v/v	Volume by Volume
w/v	Weight by Volume
X-gal	5- Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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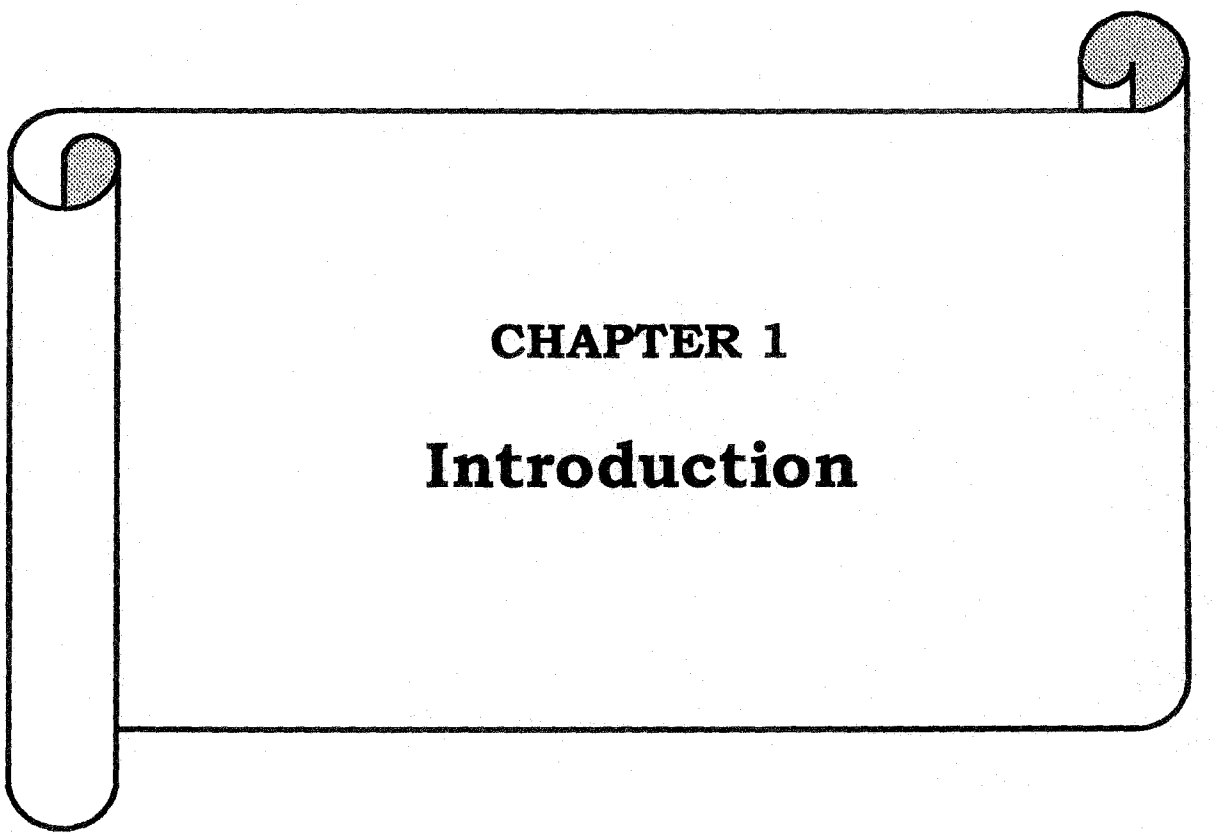


CHAPTER 1

Introduction

INTRODUCTION

1.1 Iron: An essential element

Iron (Fe) is the fourth most abundant element in the earth's crust following oxygen, silicon, and aluminium (Storey, 2005). Iron is a vital element required by almost all living organisms, including bacteria, with the exception of only a few, including *Streptococcus sanguis,* some *Lactobacillus* spp. and *Borrelia burgdorferi* (Archibald 1983; Posey and Gherardini, 2000; Logeshwaran et al., 2009). So it is said that iron is universally required by all living cells. Iron is involved in many important cellular processes such as the electron transport chain and in deoxyribonucleotide biosynthesis and acts as a cofactor for many enzymes, such as ribonucleotide reductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase (Litwin and Calderwood, 1993). It also participates in other significant biological processes, such as photosynthesis, methanogenesis, H_2 production and consumption, respiration, the tricarboxylic acid cycle and gene regulation (Andrews et al., 2003). Iron is also essential in nitrogen fixation, in which the nitrogenase enzyme utilizes iron alone, or molybdenum or vanadium together with iron to reduce atmospheric nitrogen to ammonia. Iron plays a vital role in oxygen transport in both hemoglobin and myoglobin in which oxygen is bound to the Fe (II)-heme (Storey, 2005).

1.2. Siderophore: a carrier

The term siderophore is derived from the Greek word which means "iron carriers". While iron is widespread in the environment, but under aerobic conditions at nearly neutral pH it is present in an extremely insoluble form, such as hematite, goethite, and pyrite or as polymeric oxydehydrates, carbonates, and silicates which rigorously limit the bioavailability of this metal (Matzanke, 2005). It is considered biologically unavailable as it is often found only in the form of highly insoluble Fe(III) ion. In aerobic environment ferric ion (as free molecule) occurs in very low concentration due to the low solubility constant of $Fe(OH)_3$ ($K_{sol}=10^{-38}$). So iron is present as highly insoluble ferric hydroxide complexes which are forms that severely

restrict the bioavailability of iron (Braun et al.1998). The solubility product of $Fe(OH)_3$ is approximately 10^{-38} so by calculation, the concentration of Fe^{3+} at neutral, aerobic conditions is 10^{-17} – 10^{-18} M in the absence of any external Fe(III) chelators. In response to this situation, one of the most common strategies for iron sequestration in an aerobic environment is through the synthesis and excretion of low molecular weight, high affinity chelators, with a very high and specific affinity for Fe(III), known as siderophores. Microbial metabolic products (mainly secondary metabolites) can be classified as siderophores, if

(i) they exhibit ability for iron chelation,

(ii) they participate in active transport across the cell membrane(s) and

(iii) their biosynthesis is regulated by the intracellular iron level.

These siderophores are able to solubilise iron prior to transport into the cell (Winkelmann, 2001). However, they also exhibit affinity to other metals. They are produced by bacteria, fungi and some monocotyledonous plants (Das et al., 2007). Over 500 different siderophores have been identified and are produced by various organisms (Butler and Martin, 2005). The secretion of siderophore in environment and entry of siderophores through cell walls or bacterial membranes is a highly specific process which is regulated by an array of proteins, up to eight in numbers, in most microbes (Matzanke, 2005). The advent of modern molecular biology has enriched us with various methods enabling high-yield production of specific gene products relevant to siderophore-synthesis and -transport, and analyses of structure-function relationships.

1.3 Biocontrol of plant pathogens

Almost all the cultivated crop plants on earth are attacked by plant pathogens which cause different types of diseases which may often lead to considerable damage and loss in yield. Plants provide us not only our food but also it provides us feed, fibre and presently fuel. Therefore, in order to avoid crop-loss and prevent socio-economic disaster especially in the developing countries, more efficient control of pests and diseases is of prime importance. Soil-borne pathogens including fungi, bacteria and nematodes

have deleterious effects on agricultural field and conventional control measures like breeding of resistant varieties and crop rotation fail to reduce disease incidence. The pathogens survive by feeding on root exudates of host plant and reside there.

Chemical pesticides are in use for more than hundred years to combat the plant pathogens that pose a threat to the cultivated crops. It has been used by farmers worldwide as most effective tool in preventing economic losses. However, due to several reasons, use of chemical fungicides for addressing plant disease problems has become unpopular and even unacceptable in some cases. The reasons include negative impact on environment and modified safety regulations, effect on non-target organisms, development of pathogen resistance, increasing cost of pesticides and non-functionality of chemicals in particular cases.

Pathogenic microorganisms affecting plant health are severe threat to crop production and ecosystem sustenance worldwide. As agriculture is advancing and intensifying over past few decades, producers are becoming more and more dependent on chemicals as a relatively reliable method of crop protection. However, increasing use of chemical fungicides causes several deleterious effects, i.e., development of resistance strains of pathogen to that chemical and their non-target environmental impacts (Compant et al., 2005). Furthermore, the increasing cost of such fungicides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of diseases for which chemical solutions are few or sometimes, ineffective.

With more strict regulations on chemical usage along with an increased pressure for minimizing chemical usage, the available number of usable compounds has considerably decreased. In order to meet the growing consumer demand for organic food, a lot of interest has developed on finding an alternative eco-friendly method in plant disease control.

In plant protection studies, the term 'biological control' is used for describing the utilization of living organisms with an aim to restrict the growth and proliferation of pests and pathogens. Biological control in plant pathology pertains to the use of antagonistic microbes for the purpose of disease suppression. These antagonistic microbes affect the growth of pathogens by a variety of mechanisms. These include antibiosis, parasitism, production of cell wall degrading enzymes, degradation of pathogenicity factor and competition for nutrients, space or infection sites (Pal and Gardener, 2006; Whipps, 2001).

Kloepper et al. (1980) were the first to demonstrate the importance of siderophores in the mechanism of biological control. Siderophores mediate the limited amount of iron in the rhizosphere, deprive pathogens of iron and suppress their growth. Many reports are available showing involvement of siderophore in the suppression of plant pathogenic fungi (Bakker et al., 1986; Kloepper et al., 1980; Loper and Buyer, 1991).

The microbial world is enormously rich in its diversity and is an infinite pool of organisms which may be utilized to fight plant pathogens (Emmert and Handelsman, 1999). Study of available literature shows that a wide spectrum of bacteria has been used as inoculums in disease management practices of various crops. They not only control or inhibit plant pathogens, but have often been found to induce resistance and stimulate plant growth (Huang and Wong, 1998; Ross et al., 2000; Berg et al., 2001; Zhang et al., 2002; Sabaratnam and Traquair, 2002; Collins and Jacobsen, 2003; Xue et al., 2009). Soil-borne, non-pathogenic bacteria with the ability to antagonise fungal phytopathogens and thus prevent plant disease represent a realistic alternative to chemical fungicides (Walsh et al., 2001). These bacteria are known by several generic names, including biological control agents (BCAs), plant growth promoting rhizobacteria (PGPR) and biopesticides.

India is the second largest vegetable producer after China with 11% production share in the world based on the information provided by the report of the Working Group on Horticulture, Plantation Crops and Organic Farming for the XI Five Year Plan (2007-12). West Bengal contributes a

significant amount in making India the second largest vegetable producer. There is a wide diversity of horticultural crops grown in West Bengal. The sub-Himalayan region of West Bengal, commonly known as North Bengal, is an agriculturally developed area which cultivates various crops. Major crops include fruits and nuts, vegetables, spices, plantation crops, medicinal and aromatic plants, flowers and ornamentals. Common vegetables are: tomato, brinjal, chilli, cabbage, cauliflower, radish, carrot, pea, lady's finger (okra, bhendi), leafy vegetables, turnip, beet, tapioca etc (Source: National Horticulture Mission Action Plan for West Bengal, September 2005/ http://nhm.nic.in/actionplan/actionplan_wb.pdf). Being largely dependent on agriculture, the economy of North Bengal thrives on the well being of the agricultural system and seeks for more sustainable and eco-friendly way of plant disease control.

1.4. Objectives

A variety of biological control measures are available for application, but further development and effective adoption requires a greater understanding of the intricate interactions among pathogen, biocontrol agents and the environment. The research presented here aims towards a better utilization of soil microbes in limiting fungal diseases of crops. Siderophore production is a beneficial trait of antagonistic microbes as it can not only deprive the plant pathogen of iron but may also provide the plant with an additional iron acquiring pathway that may promote plant growth. But a literature study reveals that siderophore production trait in antagonists have not received due attention; particularly its capacity as a major contributor to biocontrol mechanism has not been emphasized. Hence, it was considered worthwhile to study the soil inhabiting siderophore producing bacteria with antagonistic potential against phytopathogens and characterize the type of siderophore they produce. Additionally a study on how far these bacteria can actually reduce disease occurrence in the plant is also necessary. Therefore the basic objectives of the present study are

1. To isolate siderophore producing bacteria from soil.

2. To study the antifungal activity of the isolated siderophore producing strains in suppressing some plant pathogens *in vitro*.

3. To characterize the selected siderophore-producing and antagonistic strains and their identification.

4. To partially purify and chemically characterize the siderophores produced by the selected strains.

5. To study the efficiency of siderophore producing bacteria in suppressing plant root pathogens *in vivo*.

CHAPTER 2

Literature Review

LITERATURE REVIEW

2.1. Siderophores: An overview

Most organisms require iron as an essential element because of its unique chemical properties, in a variety of metabolic and cellular pathways. Iron-containing cofactors such as iron-sulfur clusters or heme groups are found to be present in more than 100 enzymes acting in primary and secondary metabolism processes. The ability to coordinate and activate oxygen and the possession of ideal redox chemistry (FeII/Fe III) for involvement in electron transport and metabolic processes makes iron most suitable for catalyzing a broad spectrum of redox reactions (Miethke and Marahiel, 2007; Hider and Kong, 2010).

Fe(II) is soluble in aqueous solutions at neutral pH and is hence sufficiently available for living cells if the reductive state is maintained. Generally, Fe(II) can be taken up by ubiquitous divalent metal transporters (Ballouche et al., 2009; Cartron et al., 2006; Miethke and Marahiel 2007). Systems for specific Fe(II) uptake are known in bacteria (Cartron et al., 2006) and fungi (Howard, 1999; Knight et al., 2002; Haas and Keel, 2003). However, in most microbial habitats, Fe(II) is oxidized to Fe(III) either spontaneously by reacting with molecular oxygen or enzymatically during assimilation and circulation in host organisms. In the environment, Fe(III) forms ferric oxide hydrate complexes in the presence of oxygen and water at neutral to basic pH. These complexes are very stable, leading to a free Fe(III) concentration of 10^{-9} to 10^{-18} M (Raymond et al. 2003; Neilands, 1995).

Siderophores coordinated to iron(III) are accumulated by microorganisms by facilitative transport, using a multitude of membrane bound iron–siderophore receptors. Iron is removed from siderophores predominately by a redox-mediated process, the affinity of siderophores for iron(II) being very much less than that of iron(III) (Xiao and Kisaalita, 1998). Some siderophores may be secreted in order to deprive competing organisms of iron (Hamdan et al., 1991; Leong, 1986; Loper and Henkels, 1997), and as such will influence the ecology of the environment occupied by the secreting

colony. For some microorganisms there is a strong correlation between siderophore production and virulence (Fernandez et al., 1998; Koczura and Kaznowski, 2003; Courcol et al., 1997). The genes for the biosynthesis of some siderophores are encoded on plasmids, which facilitates lateral gene transfer (Tolmasky et al., 1988; Salinas et al., 1989).

A common advantage for microbes is the ability to utilize xenosiderophores or heterologous siderophores that is, the siderophores that are produced by other organisms. This indicates that these microbes possess ferric-chelate reductases and/or uptake systems for siderophores not synthesized by themselves (Meyer, 1992; Guan et al., 2001; Llamas et al., 2006). For example, in the plant associated bacteria, *Pseudomonas putida*, utilization of heterologous siderophores enhances levels of iron available to it in the rhizosphere. Apart from using the siderophores produced by themselves, these bacteria have the capacity to utilize siderophores produced by diverse species of bacteria and fungi (Loper and Henkels, 1999; Llamas et al, 2006). Other microorganisms, for example Baker's yeast, refrains completely from siderophore production but is capable of utilizing several exogenous siderophores as iron sources (Lesuisse et al., 2001).

2.1.1. Chemistry of siderophores

Siderophores are divided into three main classes depending on the chemical nature of the moieties that are involved in donating the oxygen ligands for Fe(III) coordination. These are either catecholates (ie, catecholates and phenolates; also termed as "aryl caps") and hydroxamates (Neilands, 1981; Baakza et al., 2004; Neilands, 1995; Holzberg and Artis, 1983; Raymond and Dertz 2004; Wandersman and Delepelaire, 2004; Storey et al., 2006). However, increasing information about new siderophores has led to a more complex classification. Many structures have been elucidated that integrate the chemical features of at least two classes into one molecule, resulting in "mixed-type" siderophores. Some representative structures of various siderophore types are shown in figures 1, 2 and 3.

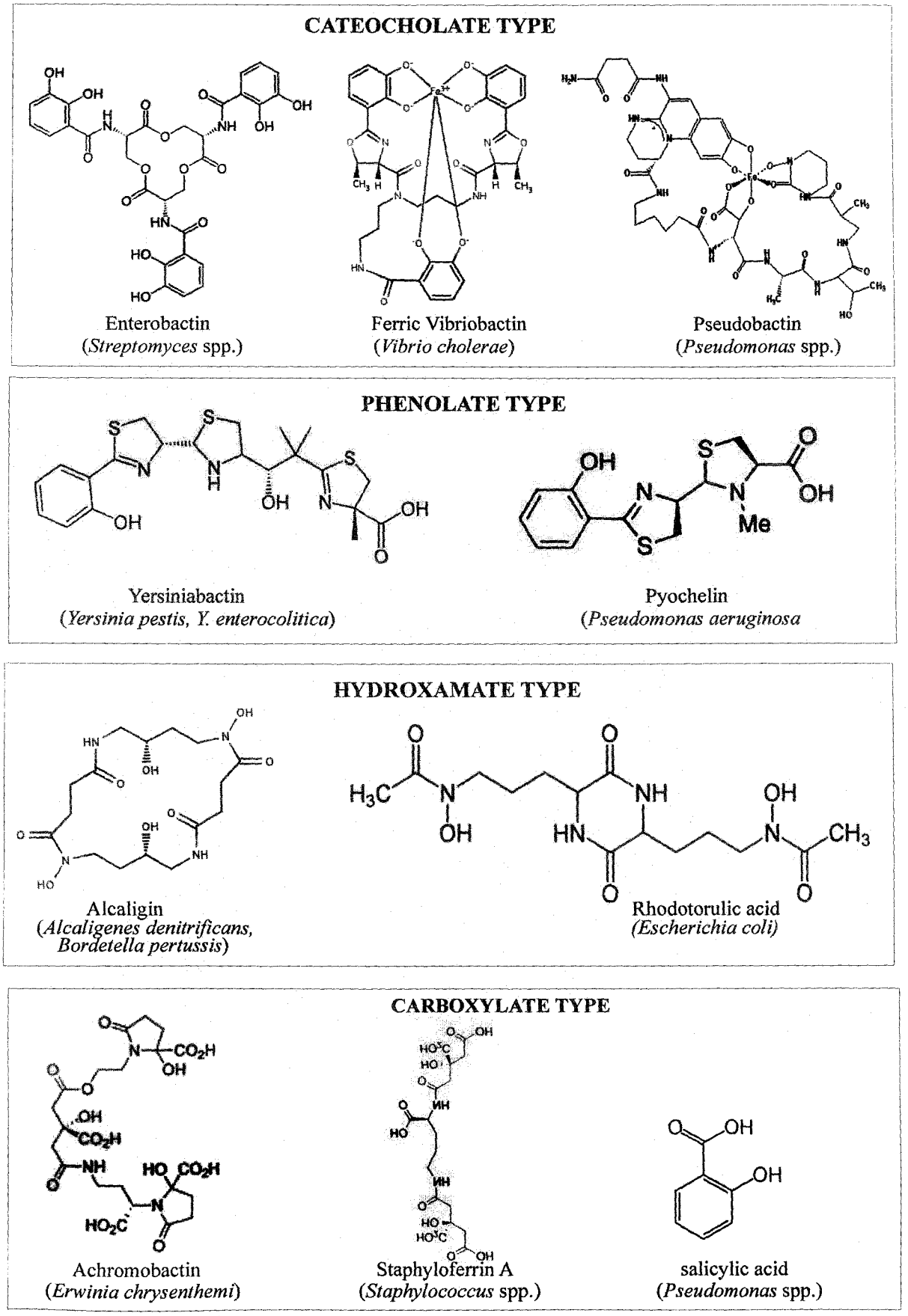
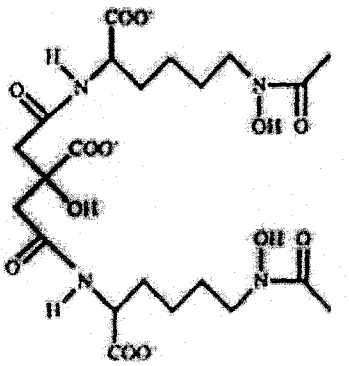


Fig. 1:Representative examples of different types of siderophores and their natural producers.

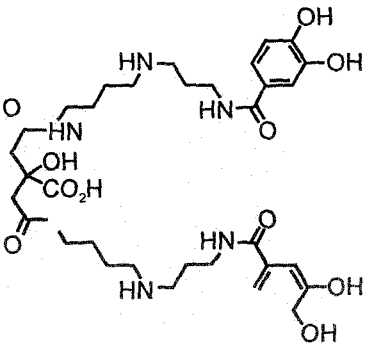
MIXED TYPES

Citrate-hydroxamate



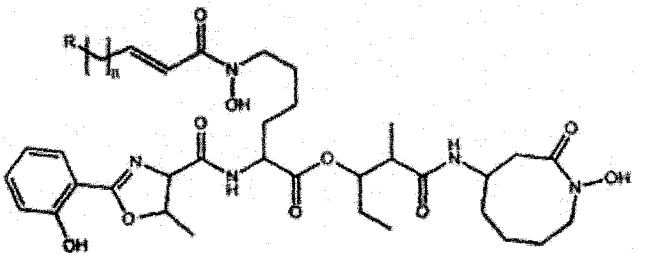
Aerobactin
(*E. coli, Enterobacter* spp.)

Citrate-catecholate



Petrobactin
(*Bacillus anthracis*)

Phenolate-hydroxamate



Mycobactin
(*Mycobacterium tuberculosis*)

Fig. 2: Representative examples of mixed types of siderophores and their natural producers.

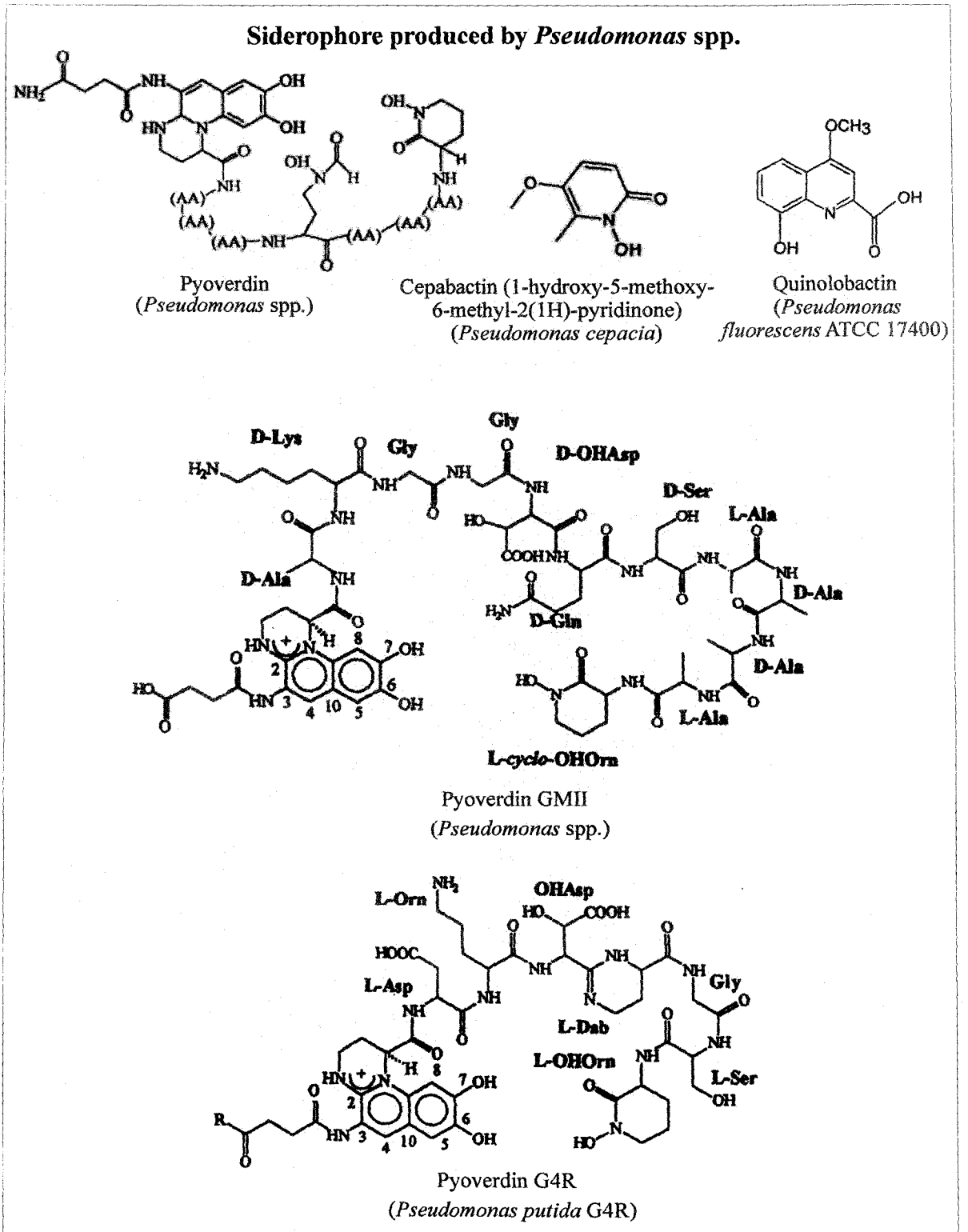


Fig. 3: Representative examples of different siderophores produced by members of the genus *Pseudomonas*.

Siderophores possess a higher affinity for iron(III) than iron(II). Siderophores are designed to form tight and stable complexes with ferric iron. The logic for this selectivity may be that, designing ligands that are selective for iron(II) over the biologically important dipositive cations zinc(II), copper(II), nickel(II) and manganese(II) is difficult, but the problem with iron(III) is more simple as there are not many biologically important tripositive cations. Fe(III) is strongly solvated in aqueous solution by forming an octahedral $Fe(H_2O)_6^{3+}$ complex (Raymond and Dertz, 2004). Because of the gain in entropy, the siderophore donor atoms readily replace the solvent water and surround Fe(III) in a hexacoordinated state which also has an octahedral geometry like the aqueous ion.

The octahedral field is favourable for the formation of the thermodynamically stable high-spin iron(III) species. Depending on the siderophore, the octahedral field may be distorted and sometimes nitrogen or sulfur are included as donor atoms. Such modifications tend to reduce the affinity for iron(III) (Hider and Kong, 2010). But in most cases, if there are less than six donor atoms provided by the ligand, the vacancies are occupied by alternative oxygen donors such as water molecules. Alternatively, siderophores complex with higher stoichiometry as in the cases of rhodoturolic acid that forms Fe_2L_3 complexes (Carrano and Raymond, 1978), pyochelin that forms both FeL and FeL_2 complexes (Tseng et al., 2006), or cepabactin that forms FeL_3 complexes (Klumpp et al., 2005). Mixed complexes were also found to occur for cepabactin and pyochelin, forming 1:1:1 complexes with Fe(III) (Klumpp et al., 2005).

Bidentate ligands can be designed to form proper moleculer patterns of hexadentate structures. There is a range of bidentate ligands that can be incorporated into hexadentate structures; as is the case in enterobactin, the classic example where the three catechol rings are involved (Burnham and Neilands, 1961). Catechol, hydroxamate and α-hydroxycarboxylate are the three main groups that are usually incorporated into siderophore structures, each having a high selectivity for iron(III). Catechol exhibits a

high affinity for iron(III) due to the two ortho-phenolate atoms, as indicated by the associated pKa values.

The hydroxamate moiety has two mesomeric forms, one producing a high charge density on the carbonyl oxygen. This delocalisation of charge can be further increased by additional conjugated side chains leading to an enhanced electron density on the oxygen atom and hence affinity for iron(III) (Hider, 1984). These kinds of modifications are observed in many siderophores, for example mycobactin (Crosa and Walsh, 2002) and ferrichrome A (Eisenhauer et al., 2005). The α-hydroxycarboxylate function also shows a favourable pKa value and thus has a high affinity for iron(III). In comparison to other biologically important cations, iron(III) is able to compete with protons more easily for the alkoxide functional groups which make the α-hydroxycarboxylate function highly selective for iron(III) (Martell and Hancock, 1996). The pKa value of the hydroxyl function can be further decreased by intramolecular hydrogen bonding of the conjugate anion. This is observed in rhizoferrin, where the two hydroxyl pKa values are 10.05 and 11.3 (Silva et al., 2009). In mycobactin, which is a mixed type of siderophore having phenolate-hydroxamate structure (Fig. 2), the hydroxyl-phenyloxazolone coordinates iron(III) by the phenolate anion and the nitrogen atom in the oxazolone ring (Hider, 1984).

The bound Fe(III) is always found in a high-spin d^5 electronic configuration in the siderophore complex. The complex is kinetically stable despite the fact that there is no ligand field stabilization energy provided by this configuration. This is because the oxygen donor atoms that are mainly used in siderophores for iron coordination represent hard Lewis bases thereby allowing additional strong ionic interactions between metal and ligand. Thus, siderophores display an enormous affinity towards Fe(III) (Miethke and Marahiel, 2007). Some hexadentate siderophores possess a higher than expected affinity for iron(III), the effect resulting from the predisposition of the ligands before binding a ferric cation. The presence of the three chiral serine residues in the tri-ester ring of enterobactin (Fig. 1) orientates each of the three catecholate functions to the same side of the ring which causes a

sharp increase in its affinity for Fe(III) (Stack et al., 1993; Raymond et al., 2003).

Proton-independent affinity constants do not reflect the real iron binding capacity of the siderophores under physiological conditions for which complete deprotonation is usually not achieved. A better way of comparing the true relative abilities of different siderophores to bind ferric iron is the pH-analogous pFe value, which gives the negative logarithm (base 10) of the free iron concentration. This refers to a standard convention, the total Fe(III) concentration is 10^{-6} M and the total ligand concentration is 10^{-5} M. Since the pH of the medium strongly influences the chelation efficiency, pFe is a pH-dependent value. Therefore, only at above a pH of 5.0, enterobactin is significantly more efficient as an iron chelator than aerobactin (Raymond and Dertz 2004; Valdebenito et al, 2006).

2.1.2. Siderophore biosynthesis

Siderophore biosynthesis is induced in response to iron limitation. The biosynthesis of siderophores occurs by different mechanisms depending on the chemical nature of the siderophores. In general, the biosynthesis pathways can be distinguished as being either dependent on or independent of non ribosomal peptide synthetase (NRPS) enzymes.

2.1.2.1. Non ribosomal peptide synthetase dependent pathway

NRPSs represent large multienzyme complexes that activate and assemble a broad array of amino, carboxy, and hydroxy acids, leading to a high structural variability of the generally macrocyclic peptidic products (Grunewald and Marahiel, 2006). These multimodular enzymes function as enzymatic assembly lines in which the order of the modules usually determines the order of the amino acids incorporated into the peptide (Marahiel et al., 1997; Fischbach and Walsh, 2006). Each module contains the complete information for an elongation step combining the catalytic functions for the activation of the amino acid by the adenylation domain, the tethering of the corresponding adenylate to the terminal thiol of the enzyme-bound 4´-phosphopantetheinyl (4´-PP) cofactor by the peptidyl

carrier protein domain, and the formation of the peptide bond by the condensation domain (Marahiel et al., 1997; Fischbach and Walsh, 2006). At the end, the product is released by the C-terminal thioesterase domain by hydrolysis or by cyclization via intramolecular condensation. Each adenylation domain recognizes a specific amino acid, and its substrate specificity can be predicted by its sequence (Patzer and Braun, 2009). An NRPS specificity conferring code consisting of 10 nonadjacent amino acid residues in the adenylate domain has been proposed (Stachelhaus et al., 1999). Exceptions to the "colinearity-rule" have been discovered (Fischbach and Walsh, 2006). For example, in the biosynthesis of the siderophores enterobactin and bacillibactin, all the modules in the NRPS are used iteratively, and the thioesterase domain stitches the chains together into a cyclic product (May et al., 2001).

NRPSs are responsible mainly for the synthesis of aryl-capped siderophores. NRPS-dependent siderophore biosynthesis in several human pathogens has been elucidated in detail, e.g., enterobactin synthesis in enteric bacteria such as *E. coli*, *Salmonella enterica*, *Klebsiella* spp., and *Shigella* spp.; yersiniabactin synthesis in *Yersinia* spp.; pyochelin and pyoverdin synthesis in *P. aeruginosa*; vibriobactin synthesis in *V. cholerae*; and mycobactin synthesis in *M. tuberculosis* (Crosa and Walsh, 2002).

The aryl acids 2,3-dihydroxybenzoate (DHB) and salicylate, which are generally used as aryl caps, have to be provided by approaching enzymes before forming the NRPS-catalyzed assembly. In most bacteria, the genes encoding the NRPS and the enzymes for aryl acid synthesis are directly iron regulated via the Fur repressor. The enzymes for DHB and salicylate formation as well as several NRPS domains involved in catecholate siderophore assembly have been extensively characterized, and crystal structures are available in many cases (Kerbarh et al., 2005, 2006; Harrison et al., 2006; Patzer and Braun, 2009).

Salicylate synthesis in *P. aeruginosa* was shown to depend on two distinct enzymes, the isochorismate synthase PchA and the isochorismate-pyruvate lyase PchB (Gaille et al., 2002). The activities of both salicylate and

isochorismate synthases are highly magnesium dependent, which is also the case for the structurally similar chorismate-utilizing enzymes. In the crystal structure of salicylate synthase Irp9 soaked with chorismate, the Mg(II) cofactor was found to be coordinated by two glutamate residues of the active site and the carboxy group salicylate that was found together with pyruvate in the catalytically active crystal, suggesting that this coordination is crucial during catalysis (Miethke and Marahiel, 2007).

2.1.2.2. Non ribosomal peptide synthetase independent pathway

In most cases hydroxamate and carboxylate siderophores are assembled by NRPS-independent mechanisms. The synthesis of siderophores belonging to these two main classes commonly relies on a diverse spectrum of enzymatic activities such as monooxygenases, decarboxylases, aminotransferases, ac(et)yltransferases, amino acid ligases, and aldolases (Challis, 2005). Siderophores synthesized by NRPS-independent pathways are found as virulence factors in several pathogens, e.g., aerobactin in enteric bacteria, alcaligin in *B. pertussis* and *B. bronchiseptica* (Moore et al., 1995; Nishio et al., 1988), staphylobactin in *Staphylococcus aureus* (Dale et al., 2004), and petrobactin in *Bacillus anthracis* (Koppisch et al., 2005).

Hydroxamate moieties are generally built in two steps. The first reaction step is an N-hydroxylation catalyzed by reduced FAD-dependent monooxygenases that use molecular oxygen and a set of amino acids and polyamines as substrates. In most pathways, one oxygen atom is transferred either to the ε-amino group of lysine (aerobactin pathway), else to the δ-amino group of ornithine, or to one amino group of the corresponding decarboxylation products cadaverine and putrescine, respectively (Challis, 2005). In the rhizobactin 1021 pathway, the unusual diamine 1,3-diaminopropane is suggested to be the substrate for N-hydroxylation (Lynch et al, 2001). The hydroxamate functions are introduced to the NRPS-derived mycobactin scaffold in the final synthesis step (Moody et al., 2004; Krithika et al., 2006).

The formylation resulting in free hydroxamic acid moieties or acylation of the hydroxylated amine generally represents the second step yielding the functional hydroxamate and is catalyzed in the case of acylation by acyl coenzyme A transferases. Formylated N5-hydroxy-ornithines are present in pyoverdins and in ornibactin. Acylation of hydroxylated amines is much more frequent. As substrates, coenzyme A derivatives of various carboxy acids such as acetate (aerobactin), succinate, β-hydroxybutyrate (pyoverdins) etc. are used (Crosa and Walsh, 2002; Moody et al., 2004; Miethke and Marahiel, 2007).

2.1.3. Siderophore transport

The intracellular level of iron is carefully monitored in the bacterial cell. A shortage of iron will reduce the growth of bacteria, whereas high concentrations of the metal can be toxic. Therefore, the expression of the iron-acquisition systems is regulated in response to iron, being increased under iron limitation. In addition, iron serves as an important environmental signal for the expression of factors unrelated to iron uptake (Litwin and Calderwood, 1993). The intracellular level of iron is carefully monitored in the bacterial cell. A shortage of iron will reduce the growth of bacteria, whereas high concentrations of the metal can be toxic. Therefore, the expression of the iron-acquisition systems is regulated in response to iron, being increased under iron limitation. In addition, iron serves as an important environmental signal for the expression of factors unrelated to iron uptake (Litwin and Calderwood, 1993).

Siderophore secretion and transport systems have been identified in only a few microorganisms so far. These include mostly gram negative bacteria like *Salmonella typhimurium*, *Escherichia coli* and members of the genera *Pseudomonas*, *Shigella*, *Salmonella*, *Yersinia*, *Vibrio*, *Pseudomonas*, *Bordetella*, *Erwinia* and *Agrobacterium*. Among gram positive bacteria, transport mechanism for siderophores has been identified in *Staphylococcus aureus* and *Bacillus subtilis*. Fungal siderophore transport mechanism has been characterized in *Saccharomyces cerevisiae* and *Aspergillus nidulans* (Haas et al., 2008). The exporters that were found or suggested to be

involved in siderophore release belong to efflux pumps of the major facilitator superfamily (MFS); the resistance, nodulation, and cell division (RND) superfamily; and the ATP-binding cassette (ABC) superfamily (Venturi et al., 1995; Miethke and Marahiel, 2007). In the present review we shall focus only on the siderophore transport mechanism of pseudomonads.

2.1.3.1. Transport of siderophore in *Pseudomonas* sp.

Two strategies are used by Gram-negative bacteria to take up iron under aerobic conditions: via the uptake of heme or via the uptake of iron-siderophore complexes. In both instances, a TonB-dependent outer membrane receptor recognizes the iron-loaded complex. These receptors are large porins with 22 β-strands forming a β-barrel and they are gated, meaning that the pore is constricted by the N-terminal domain of the protein (the "cork"). The N-terminal end of the receptor contains a domain termed the TonB box which interacts with TonB, an inner membrane protein, which, together with ExbB and ExbD transmits the energy of the proton motive force (pmf) to allow the opening of the gate and transport of the ferric complex into the periplasm where a periplasmic binding protein binds and brings the ferrisiderophore to a transporter. In *Pseudomonas aeruginosa*, two heme uptake systems have been described, Phu and Has (Ochsner et al., 2000). In the Phu system, hemoproteins bind directly the receptor and heme is extracted, while in the Has system a hemophore protein, HasAp, is secreted via a type I secretion system and takes heme from hemoproteins, bringing it to the HasR receptor (Létoffé et al. 1998; Ochsner et al., 2000). Once in the cytoplasm of *P. aeruginosa*, heme is degraded by a heme oxygenase encoded by hemO, liberating biliverdin and Fe^{2+} (Wegele et al., 2004; Lansky et al., 2006; Kaur et al., 2009).

2.1.4. Pyoverdines: the high-affinity siderophore from fluorescent pseudomonads

Fluorescent pseudomonads are characterized by the notable phenotypic expression of the production of a green–yellow fluorescent pigment, pyoverdine under conditions of iron limitation (Meyer, 2000; Cornelis,

2010). Pyoverdines contain a peptide moiety, usually between 6–12 amino acids in length, and a dihydroxyquinoline chromophore moiety (Meyer, 2000), which gives pyoverdine its characteristic yellow-green fluorescent appearance. A range of pyoverdines have been identified (Fig. 3), each having a different peptide chain and a generally conserved chromophore conferring the typical colour and fluorescence under UV light (Meyer, 2000; Cornelis and Matthijs, 2002; Ravel and Cornelis, 2003; Visca et al., 2007). Pyoverdines have been shown to be good taxonomic markers at species levels, which led to the development of "siderotyping" based on pyoverdines isoforms separation by isoelectric focusing and cross-uptake of Fe-pyoverdines (Fuchs et al., 2001; Meyer et al., 2007). Sideroptyping is a method recently developed to characterize bacterial strains by the siderophores they produce when grown under iron deficiency. First applied to fluorescent pseudomonads and their main siderophores, the pyoverdins, the method was primarily used for the recognition of new molecules among pyoverdins. Because of the huge diversity of molecules encountered among this siderophore family, the method became useful prerequisite for starting novel structure investigations. Close to 50 structures have been already established and a total of more than 110 structurally different compounds are presently recognized (Meyer et al., 1997, 2007; Bultreys, 2007).

Interest for siderotyping considerably increased when it became evident that all strains belonging to a well defined *Pseudomonas* spp. produce an identical pyoverdin and furthermore, the most species are characterized by specific pyoverdins. Pyoverdins are potent taxonomic makers, opening a new valuable way of bacterial identification and taxonomy within this major genera (Bultreys, 2007). However, sometimes the rule of "one pyoverdine–one species" is not always true since *P. aeruginosa* strains can produce three different pyoverdines (one type per strain) and this is also true for *Pseudomonas putida* (Meyer et al., 1997, 2007). Pyoverdine biosynthesis genes and the gene(s) coding for the receptor are not part of the core genome of fluorescent pseudomonads as revealed in different studies, suggesting that they could have been acquired by horizontal gene transfer (Smith et al., 2005; Bodilis et al., 2009).

Pyoverdines have been shown to be important or even essential for the colonization of host tissues and for virulence in plant and animal pathogens, and, in the case of *P. aeruginosa* shown to be necessary for the establishment of mature biofilms and for competitiveness in soil (Meyer et al., 1996; Handfield et al., 2000; Mirleau et al., 2000; Banin et al., 2005; Yang et al., 2009). Recently, pyoverdine was confirmed to be an important colonization factor for the plant pathogen *P. syringae* and to be necessary for the production of the toxic compound tabtoxin and the quorum sensing molecules N-acyl-homoserine lactones (Cornelis and Aendekerk, 2004; Juhas et al., 2004). An overlap between quorum sensing-dependent and iron-limitation-induced genes has been established in *P. aeruginosa* thereby establishing a link between iron uptake, virulence, and quorum sensing (Taguchi et al., 2010; Zheng et al., 2007; Attila et al., 2008; Oglesby et al., 2008).

Pyoverdine-mediated iron uptake in *P. aeruginosa* has been extensively investigated. It has been observed that the pyoverdine precursor ferribactin is synthesized in the cytoplasm and transported via ABC transporter PvdE to the periplasm, where maturation of the chromophore takes place (Baysse et al., 2002; Imperi et al., 2009). In the uptake process, ferripyoverdine is taken up via the FpvA receptor, and reductive release of iron occurs in the periplasm. This is followed by rapid recycling and excretion of apo-pyoverdine (Imperi et al., 2009).

2.2. Biological control: A concept and case study

"Biological control" and its abbreviated synonym "biocontrol" are common terms which have been used in different fields of biology, but in plant pathology, this term is applied for the use of microbial antagonists, often referred to as biological control agent or BCA to suppress diseases. Biocontrol is considered as a multitrait phenomenon whose success depends on many factors. These include the ability of the microbial inoculant to survive in the rhizosphere and to compete with the resident microbial populations, as well as protecting the plant host against pathogens at both the time and site of infection (Chin-A-Woeng et al., 2000;

Mark et al., 2006). It has long been recognized that there are many naturally occurring bacteria and fungi that are antagonistic to crop pathogens, and consequently have the potential to provide an alternative to chemical fungicides. There has been a large body of literature describing potential uses of plant-associated bacteria as agents stimulating plant growth and managing soil and plant health. Table 1 and 2 enlists several rhizobacteria of the genus *Pseudomonas* that have been reported in literature as potential biocontrol agents. The list is selective rather than comprehensive and is limited to the findings published in the last 10 to 12 years.

Plant growth-promoting bacteria are associated with many plant species and are commonly present in many environments. The most widely studied group is the plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Compant et al., 2005). Bacteria such as those belonging to the *Bacillus* and *Pseudomonas* genera, and fungi in the *Trichoderma* genus have been cited as potential biological control agents (Chet and Inbar, 1994; Chin-A-Woeng et al., 2000; Walsh et al., 2001; Harman et al., 2004). A number of biocontrol products based on these three genera have been have been commercially developed as biocontrol agents. It has been known for many years that they produce a wide range of antibiotic substances and that they parasitize other fungi. They can also compete with other microorganisms; for example, they compete for key exudates from seeds that stimulate the germination of propagules of plant-pathogenic fungi in soil and, more generally, compete with soil microorganisms for nutrients and/or space (Harman et al., 2004).

Table 1: *Pseudomonas* strains reported as biocontrol agents against fungal pathogens of plant

Biocontrol Strains	Tested Plant (Disease)	Target Pathogen	References
Pseudomonas fluorescens strain PfT-8	Tomato (Damping-off)	*Pythium aphanidermatum*	Jayaraj et al., 2007
Pseudomonas fluorescens P5	Wheat (take-all)	*Gaeumannomyces graminis* var. *tritici*	Jing et al., 2004
	Rice (sheath blight)	*Gaeumannomyces graminis* var. *tritici*	
	Cotton (damping off)	*Rhizoctonia solani*	
Pseudomonas putida type A1	Chickpea (wilt)	*Fusarium oxysporum f. sp. ciceri*	Boopathi and Rao, 1999
	Rice (leaf spot)	*Helminthosporium oryzae*	
Pseudomonas fluorescens CHA0	Tomato (crown and root rot)	*Fusarium oxysporum* f. sp. *radicislycopersici*	Duffy and De´Fago 1999
Pseudomonas spp.	Soybean (charcoal rot)	*Macrophomina phaseolina*	Ahmadzadeh et al., 2006
	pistachio (gummosis)	*Phytophthora nicotianae* var. *Parasitica*	
	Bean (damping off)	*Rhizoctonia solani*	
	Pepper (damping off)	*Pythium* sp.	
	cucumber (wilt)	*Fusarium* sp.	
Pseudomonas fluorescens Pf4-99	Chickpea (charcoal rot)	*Macrophomina phaseolina*	Kumar et al., 2007
Pseudomonas fluorescens strain R	Chilli (-)	*Collectotrichum gleosporioides* OGC1	Ramyasmruthi et al., 2012
Pseudomonas fluorescens CHA0	Wheat (take-all)	*Gaeumannomyces graminis* var. *tritici*	Werra et al., 2009
P. fluorescens P3/pME6863	Potato (soft rot)	*Erwinia carotovora*	Molina et al., 2003
	Tomato (crown gall)	*Agrobacterium tumefaciens*	
P. chlororaphis PCL1391 coinoculated with *P. fluorescens* P3/pME6863	Tomato (vascular wilt)	*Fusarium oxysporum f. sp. lycopersici*	
P.fluorescens Pf29 Arp	Wheat (root take all)	*Gaeumannomyces graminis* var. *tritici*(*Ggt*)	Daval et al., 2011
P. reactans B3	Lettuce, sugar beet	*Rhizoctonia solani*	Faltin et al., 2004
P. fluorescens B1	Lettuce, sugar beet	*Rhizoctonia solani*	

Cont.

Cont.

Biocontrol Strains	Tested Plant (Disease)	Target Pathogen	References
Pseudomonas fluorescens (Pf4-92 and PfRsC5)	Chickpea (wilt)	*Fusarium oxysporum f. sp. ciceri (FocRs1)*	Saikia et al., 2005
P. aeruginosa (PaRsG18 and PaRsG27)	Chickpea (wilt)	*Fusarium oxysporum f. sp. ciceri (FocRs1)*	
Pseudomonas fluorescens strains MKB100 and MKB249	Wheat and barley (seedling blight)	*Fusarium culmorum*	Khan et al., 2006
P.frederiksbergens is strain 202	Wheat and barley (seedling blight)	*Fusarium culmorum*	
Pseudomonas sp. strain MKB 158	Wheat and barley (seedling blight)	*Fusarium culmorum*	
P. fluorescens strain WCS374r	Radish (wilt)	*Fusarium* sp.	Bakker et al., 2007
Pseudomonas GRC2	Peanut (charcoal Rot)	*Macrophomina phaseolina*	Gupta et al., 2002
Pseudomonas aeruginosa MR-2, 5, 6, 9, 15 and 18	Tomato (-)	*Sclerotina sclerotiorum*	Deshwal, 2012
Pseudomonas fluorescens CHA0	Cucumber, Maize (-)	*Pythium ultimum*	Maurhofer et al., 2001
Pseudomonas spp. strains RE8,RS13, RS56 and RS158	Radish (wilt)	*Fusarium oxysporum* RS111	De Boer et al., 1999
Pseudomonas fluorescens strains PF1	Rice (sheath blight)	*Rhizoctonia solani*	Nandakumar et al., 2001
Pseudomonas fluorescens PS1	Indian rapeseed (Stem blight)	*Sclerotinia sclerotiorum*	Aeron et al., 2011
Pseudomonas fluorescens EPS62e	Pear (fire blight)	*Erwinia amylovora*	Cabrefiga et al., 2007
Pseudomonas fluorescens CHAO	Pea (-)	*Pythium ultima*	Naseby et al., 2001
Pseudomonas cf. monteilii 9	Groundnut(stem rot)	*Sclerotium rolfsii*	Rakh et al., 2011
P. corrugata strain ICMP 5819	Tomato (grey mildew)	*B. cinerea*	Guo et al., 2007

Cont.

Cont.

Biocontrol Strains	Tested Plant (Disease)	Target Pathogen	References
P. chlororaphis subsp. aureofaciens strain M71	Common cypress (cypress canker)	*Seiridium cardinale*	Raio et al., 2011
P. fluorescens strain BL915	Cucumber (-)	*Rhizoctania solani*	Ligon et al., 2000
Pseudomonas putida strain PCL1760	Tomato (foot and root rot)	*Fusarium oxysporum* f. sp. radicis-lycopersici	Validov et al., 2009
P. fluorescens Pf1,PFV, PFP, PSV	Tea (blister blight)	*Exobasidium vexans*	Saravanakumar et al., 2007
P. aeruginosa SD12	Pyrethrum (root rot and wilt)	*Rhizoctania solani*	Dharni et al., 2012

2.2.1. Common mechanisms of antagonism

Microorganisms as BCAs are widely reported, and in some cases, their modes of action against the plant pathogen have been elucidated. Table 2 lists some selected strains of fluorescent pseudomonads and their different mechanisms of antagonism that has been reported in literature. In hyperparasitism, the pathogen is directly attacked by a specific BCA that kills it or its propagules (Leveau and Preston, 2008). Many microorganisms produce and release lytic enzymes against compounds such as chitin, proteins, cellulose, hemicellulose and DNA sometimes resulting in the direct suppression of plant pathogenic activities (Kobayashi et al., 2002). The best studied mechanism of antagonism is the one mediated by different compounds with antifungal properties (Haas and Keel, 2003). Most microbes produce and secrete one or more compounds with antibiotic activity (Raaijmakers et al., 2002; Gross and Loper 2009), such as the biocontrol strain *Pseudomonas fluorescens* Pf-5, which produces the antibiotics pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol (Loper et al., 2007), or the strain FZB42 of *Bacillus amyloliquefaciens*, which produces various antifungal lipopeptides (Koumoutsi et al., 2004). Other microbial by-products such as hydrogen cyanide (Howell et al., 1988) or

ammonia (Voisard et al., 1989), also may contribute to pathogen suppression. Rhizosphere colonisation is one of the first steps in pathogenesis by soilborne pathogens. For this reason, the trait of some bacteria to colonise the root and to interfere with the biology of the pathogen can be used for biological control of plant diseases (Bloemberg and Lugtenberg, 2001). Other aspect, such as the induction of host resistance, is also an important mode of action to protect against fungal diseases (Ongena et al., 2004; Ryu et al., 2004).

Table 2. Overview of the mechanisms or metabolites involved in the biological control of phytopathogens by *Pseudomonas* strains

Bacterial strains	Suggested Mechanism Involved in Biocontrol Action	References
Pseudomonas putida type A1	Siderophore	Boopathi and Rao 1999
Pseudomonas fluorescens strain PfT-8	Chitinase, b-1,3-glucanase, cellulase, fungitoxic metabolites and siderophores	Jayaraj et al., 2007
Pseudomonas fluorescens CHA0	Antibiotics 2,4-diacetylphloroglucinol (PHL), pyoluteorin (PLT), and pyrrolnitrin and the siderophores salicylic acid and pyochelin	Duffy and De´Fago 1999
Pseudomonas fluorescens Pf4-99	IAA, siderophore	Kumar et al., 2007
Pseudomonas fuscovaginae UPMSP 20	IAA, siderophore, phosphate solubilization	Yasmin et al., 2009
Pseudomonas corrugata UPMSP 2	IAA	
Pseudomonas fluorescens strain R	Siderophore, HCN, phosphate solubilisation and IAA, chitinase	Ramyasmruthi et al., 2012
Pseudomonas spp. strains	Siderophore, HCN and protease	Ahmadzadeh et al., 2006
Pseudomonas fluorescens strains MKB 100 and 249	Fluorescent siderophores, ISR	Khan et al., 2006
Pseudomonas fluorescens CHA0	2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT)	Werra et al., 2009

Cont.

Cont.

Bacterial strains	**Suggested Mechanism Involved in Biocontrol Action**	**References**
Pseudomonas sp. isolate NJ-101	IAA, phosphate solubilization	Bano and Musarrat, 2004
Pseudomonas stutzeri YPL-1	Chitinase, ,B-1,3-glucanase, laminarinase	Lim et al., 1991
Pseudomonas fluorescens WCS374r,WCS417r,Q2-87,2-79 and CHA0r	Siderophore	Tian-hui et al., 2009
Pseudomonas spp.	Siderophore, antibiosis, production of lytic enzymes and ISR	Bakker et al., 2007
Pseudomonas fluorescens NCIM 5164	Siderophore	Bholay et al., 2012
Pseudomonas aeruginosa NCIM 2036	Siderophore	
Pseudomonas fluorescens strain CV6	Siderophore, IAA, HCN, catalase, protease, and phosphatase	Maleki et al., 2010
Pseudomonas aeruginosa GRC1	Chitinase, siderophore, HCN	Gupta et al., 2006
Pseudomonas fluorescens strain Mst 8.2	Siderophore, nitrogen fixation, phosphate solubilization, IAA, hydrolytic enzymes, HCN and antibiotics	Gull and Hafeez, 2012
Pseudomonas spp. M1P3, N1P3 and A1P3	Chitinase, cellulose, β-1,3 glucanase, HCN, siderophore	Saraf et al., 2008
Pseudomonas fluorescens P5	Chitinase	Jing et al., 2004
P. fluorescens P3/pME6863	Lactonase, Phenazine	Molina et al., 2003
Pseudomonas fluorescens Pf29Arp	ISR	Daval et al., 2011
Pseudomonas sp. *PGC2*	Chitinase, β-1,3-glucanase	Arora et al., 2008
Pseudomonas aeruginosa PUPa3	Phenazine-1-carboxamide, IAA	Kumar et al., 2005
Pseudomonas corrugata strain ICMP 5819	HCN, IAA, phosphatase, and protease	Guo et al., 2007

Cont.

Cont.

Bacterial strains	Suggested Mechanism Involved in Biocontrol Action	References
Pseudomonas fluorescens	β -1,3-glucanase , siderophore, Salicylic Acid, HCN	Nagarajkumar et al., 2004
Pseudomonas corrugata	Siderophore, ammonia, lipase and chitinase	Trivedi et al., 2008
P. fluorescens strain Psd	Siderophores, HCN, antibiotics (Phenazine, Pyrrolnitrin)	Upadhyay and Srivastava, 2008
P. aeruginosa SD12	Siderophore, protease, pectinase, cellulase, phosphatase, HCN	Dharni et al., 2012

2.2.1.1. Production of antibiotics

Bacteria that produce antibiotics, which kill pathogens, act via antagonism if their mutants defective in structural genes in the synthesis of this antibiotic are biocontrol negative (Lugtenberg and Kamilova, 2009). For a bacterium to be suitable for biocontrol, it must not only synthesize and release the antibiotic, but also compete successfully with other organisms for nutrients from the root and for niches on the root to deliver the antibiotic along the whole root system (Chin-A-Woeng et al., 2000). Also, the bacterium should escape in sufficient numbers from predators feeding on rhizosphere bacteria, so-called protozoan grazers (Jousset et al., 2006). Furthermore, the bacterium should produce the antibiotic in the right microniche on the root surface (Pliego et al., 2008).

Literature studies have revealed several reports of antifungal metabolites being produced by bio control bacteria *in vitro*, many of which has been shown to be active *in vivo* also. These are mostly produced by fluorescent pseudomonads, which are among the most common bacteria that inhabit the rhizosphere where they interact intimately with the plant roots thereby inhibiting the soil borne pathogens as well as influencing plant disease susceptibility and growth. These includes ammonia, butyrolactones, 2,4-diacetyl phloroglucinol (Phl) (Raaijmakers and Weller, 1998), HCN,

kanosamine (Milner et al., 1996), Oligomycin A (Kim et al., 1999), Oomycin A, Phenazine-1-carboxylic acid (PCA) (Thomashow et al., 1990), pyoluterin (Plt) (Kraus and Loper, 1995), pyrrolnitrin (Pln) (Hammer et al., 1997), viscosinamide (Thrane et al., 1999), xanthobaccin (Nakayama et al., 1999), and zwittermycin A as well as several other uncharacterised moieties. Antibiotics more recently discovered in biocontrol strains are d-gluconic acid (Kaur et al., 2006) and 2-hexyl-5-propyl resorcinol (Cazorla et al., 2006). Volatiles other than hydrogen cyanide, such as 2,3-butanediol, or blends of volatiles (Ryu et al., 2003) can be involved in plant protection. Finally, lipopeptide biosurfactants produced by *B. subtilis* (Ongena et al., 2007) and by pseudomonads (De Bruijn et al., 2007) have been implied in biocontrol. Rhamnolipid and phenazine act synergistically in suppressing soil borne diseases caused by *Pythium* spp. (Perneel et al., 2008).

Despite strong experimental evidence for a positive role of *Pseudomonas* antibiotics in the suppression of a variety of root diseases on many different plants, it is unclear how this happens in situ. An intuitively attractive model of the microbial world could incorporate antibiotic-producing killers, antibiotic-resistant heroes, and antibiotic-sensitive victims. Based on such a view, it may be appealing to construct a theoretical model ecosystem in which "chemical warfare between microbes promotes diversity" (Lenski and Riley, 2002; Haas and Keel, 2003). S´eveno et al. (2001) calculated that on nutrient agar, a biocontrol strain of *P. aureofaciens* produces PCA at 2–3 mg/ml inside the colonies. These concentrations inside microcolonies are one or two orders of magnitude higher than antibiotic concentrations observed in liquid media (S´eveno et al., 2001). Thus, inside microcolonies antibiotic concentrations might be sufficient to inhibit sensitive bacteria and fungi. Such a "stop invasion" strategy is supported by experimental data obtained from antibiotic-producing strains of *Streptomyces* spp. and sensitive *Bacillus subtilis* competitors, showing that sensitive bacteria do not invade the antibiotic producer (unless they have become resistant by mutation). Moreover, in a structured environment, antibiotic production does not improve the ability of the producer to invade a population of sensitive microorganisms (Haas and Keel, 2003). The resident microflora as

well as the plant can strongly influence the expression of antibiotic biosynthetic genes in pseudomonads (Notz et al., 2001, 2002; Kraus and Loper, 1995; Wood et al., 1997; Howell and Stipanovic, 1980). A *phlA0–0lacZ* fusion monitoring DAPG formation in *P. fluorescens* CHA0 is expressed more strongly on maize and wheat roots than on bean and cucumber roots (Notz et al., 2001). This differential regulation is likely caused by differences in exudate composition between monocots and dicots, although the biochemical and genetic details have not been elucidated. Pyoluteorin production by *P. fluorescens* contributes to the biocontrol of *Pythium* damping-off on cress (Maurhofer et al., 1994), but not on cucumber (Kraus and Loper, 1992; Maurhofer et al., 1994). Root exudate-dependent variations in the quantity of pyoluteorin produced were suggested to cause the observed effects (Maurhofer et al., 1994).

2.2.1.2. Signal interference

Many bacteria only express pathogenicity/virulence factors at a high bacterial cell density, sensed when the level of quorum-sensing molecules such as homoserine lactones (AHLs) accumulate in the medium (Bassler, 1999). AHLs are required, for example, for the synthesis of cell-wall-degrading enzymes of the pathogen *Erwinia carotovora*. Signal interference is a biocontrol mechanism based on the degradation of the AHL (Lin et al., 2003), for example, by AHL lactonases of *B. thuringiensis* strains that hydrolyze the lactone ring or by AHL acylases that break the amide link. Recently, it was shown that AHL acylases play a role in the formation of biofilms (Shephard and Lindow, 2008). Lack of biofilm formation by pathogen makes biocontrol easier (Lugtenberg and Kamilova, 2009).

Several antibiotics secreted by the biocontrol *Pseudomonas* strains also require minimal critical bacterial cell density. Biosynthetic genes for the phenazine antibiotics in *P. aureofaciens* 30-84, in *P. chlororaphis* PCL1391 and in *P. aeruginosa* PAO1 are under quorum sensing control (Chin-A-Woeng et al., 2001; Chugani et al., 2001) . The above findings collectively suggest that AHL signaling can have a profound influence on biocontrol efficacy. Both positive and negative influences on AHL-producing biocontrol

strains can be expected from other rhizobacteria. These may stimulate biocontrol when they produce the same AHL signal as does the biocontrol strain. Other rhizobacteria may interfere with biocontrol, either by degrading AHLs or by producing AHL antagonists (Haas and Keel, 2003).

2.2.1.3. Parasitism and production of extracellular enzymes

The ability of bacteria, especially actinomycetes, to parasitize and degrade spores of fungal plant pathogens is well established. Assuming that nutrients pass from the plant pathogen to bacteria, and that fungal growth is inhibited, the spectrum of parasitism could range from simple attachment of cells to hyphae, as with the to complete lysis and degradation of hyphae (Whipps, 2001). If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort has gone into identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. Earlier studies have shown that isolated chitinases are involved in degradation of fungal hyphae and a positive correlation existed between chitinolytic activity and biological control of phytopathgens (Saha et al., 2012b). Chitinolytic enzymes endochitinase (58-kDa) and chitobiase (98-kDa) purified from *Serratia marcescens* B2 showed inhibitory effects on the spore germination of pathogenic fungus *Botrytis cinerea* (Someya et al., 2001). Biological control disappeared equally in two mutants of *S. plymuthica* IC1270 (previously known as *Enterobacter agglomerans*) one of which lost only chitinolytic activity but not antibiotic or proteolytic activity and the other, which lost all the three activities indicating that chitinolytic enzymes contribute significantly to the antagonistic activity of the strain (Chernin et al., 1995). Tn*5* mutants of *E. agglomerans* deficient in chitinolytic activity were unable to protect cotton and expression of the *chiA* gene for endochitinase in *E. coli* allowed the transformed strain to inhibit *R. solani* on cotton seedlings. Similar techniques involving Tn*5* insertion mutants and subsequent complementation demonstrated that biocontrol of

P. ultimum in the rhizosphere of sugar beet by *Stenotrophomonas maltophila* W81 was due to the production of extracellular protease (Dunne et al., 1997). Biocontrol of *Phytophthora cinnamomi* Rands root rot of *Banksia grandis* Willd. was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* Luedemann & Brodsky (El-Tarabily et al., 1996) and control of *Phytophthora fragariae* var. *rubi* Hickman causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of β-1,3-, β-1,4- and β-1,6-glucanases (Valois et al., 1996).

2.2.1.4. Induced systemic resistance

Interaction of some bacteria with the plant roots can result in plants resistant to some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR). ISR was discovered by the findings that resistance can be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* wilt of carnation and by selected rhizobacteria against the fungus *Colletotrichum orbiculare* in cucumber (Van Peer et al., 1991). ISR is dependent on jasmonic acid and ethylene signaling in the plant. Many individual bacterial components induce ISR, such as LPS, flagella, salicylic acid, and siderophores. More recently, cyclic lipopeptides, the antifungal factor Phl, the signal molecule AHLs, and volatile blends produced by *B. subtilis* GB03 and, to a lesser extent, the individual volatiles acetoin and 2,3-butanediol have been added to the list (Ongena et al., 2007; Lugtenberg and Kamilova, 2009). In contrast to many biocontrol mechanisms, extensive colonization of the root system is not required for ISR, as shown by the ISR of *P. fluorescens* WCS365 using root colonization mutants (Dekkers et al., 2000). It is unlikely that a poor colonizer acts through antibiosis, since colonization is the delivery system for antifungal components along the root system. As *Bacillus* strains are not good colonizers, there function as good biocontrol agents was surprising. However, the observation that certain antifungal metabolites can induce ISR explains this phenomenon. Therefore *Bacillus*

strains that can act as biocontrol agents, act through ISR rather than antibiosis (Lugtenberg and Kamilova, 2009).

2.2.1.5. Competition for ferric iron ions

Although competition between bacteria and fungal plant pathogens for space or nutrients has been known to exist as a biocontrol mechanism for many years, the greatest interest recently has involved competition for iron (Whipps, 2001). Under iron-limiting conditions, bacteria produce a range of iron chelating compounds or siderophores which have a very high affinity for ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999). Studies have clearly established that the iron nutrition of the plant influences the rhizosphere microbial community structure (Yang and Crowley, 2000). Iron competition in pseudomonads has been intensively studied and the role of the pyoverdine siderophore produced by many *Pseudomonas* species has been clearly demonstrated in the control of *Pythium* and *Fusarium* species, either by comparing the effects of purified pyoverdine with synthetic iron chelators or through the use of pyoverdine minus mutants (Loper and Buyer, 1991; Duijff et al., 1993). Pseudomonads also produce two other siderophores, pyochelin and its precursor salicylic acid, and pyochelin is thought to contribute to the protection of tomato plants from *Pythium* by *Pseudomonas aeruginosa* (Schroeter) Migula 7NSK2 (Buysens et al., 1996). However, siderophores are not always implicated in disease control by pseudomonads (Schmidli-Sacherer et al., 1997; Ongena et al., 1999).

It has generally been accepted that when antibiosis is carried out on a test plate containing a medium with a low ferric iron concentration, and when the test strain inhibits fungal growth in the absence but not in the presence of added Fe^{3+} ions, the bacterial strain likely produces a siderophore, i.e., a Fe^{3+} ion-chelating molecule. Upon binding the ion, the formed siderophore-Fe^{3+} complex is subsequently bound by iron-limitation-dependent receptors at the bacterial cell surface and the Fe^{3+} ion is subsequently released and

active in the cytoplasm as Fe^{2+}. Bacteria producing high concentrations of high-affinity siderophores in the rhizosphere can inhibit the growth of fungal pathogens when the Fe^{3+} concentration is low, e.g., in acid soils (Lugtenberg and Kamilova, 2009). The dynamics of iron competition in the rhizosphere are often complex. For example, some siderophores can only be used by the bacteria that produce them, whereas others can be used by many different bacteria (Ongena et al., 1999; Loper and Henkels, 1999). Different environmental factors can also influence the quantity of siderophores produced (Duffy and Défago, 1999). There is also the further complication that pyoverdine and salicylate may act as elicitors for inducing systemic resistance against pathogens in some plants (Leeman et al., 1996).

2.2.1.6. Colonization and PGPR traits

Irrespective of mode of action, a key feature of all plant growth promoting rhizobacteria is that they all colonize roots to some extent (Whipps, 2001). This may involve specific attachment as evident in *Pseudomonas fluorescens* 2-79 to the surface of wheat roots (Vesper, 1987); however, such attachment does not seem to be an absolute requirement for colonization (de Weger et al., 1995). Colonization may involve simply root surface development but, endophytic colonization of the root is also known, and the degree of endophytic colonization depends on bacterial strain and plant type. Endophytic growth in roots has been recorded with the PGPR *Bacillus polymyxa* and *Pseudomonas fluorescens* Sm3-RN on spruce (Shishido et al., 1999) and with the biocontrol strains of *Bacillus* sp. L324-92R and *P. fluorescens* 2-79RN on wheat (Kim et al., 1997). Colonization is widely believed to be essential for biocontrol and is found to be significantly correlated with disease control (Weller, 2007).

Plant growth promoting rhizobacteria or PGPR include a diverse assemblage of bacteria representing a broad spectrum of genera (Weller, 2007). PGPR can affect plant growth either directly or indirectly. The direct promotion of plant growth by PGPR for the most part entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment. The direct effects of PGPR

include providing the host plant with i) fixed nitrogen, ii) phosphorus and iron solubilized from the soil and iii) phytohormones such as auxins, cytokinins and gibberelins that are synthesized by the bacterium (Glick and Bashan, 1997). PGPR strains are aggressive colonists of the rhizosphere environment and they can persist for the duration of the growing season (Bahme and Schroth, 1987). PGPR can pre-empt the establishment of other rhizosphere microorganisms through competition for favoured sites on the root and in the rhizosphere (Kloepper and Schroth, 1981).

2.3. Biocontrol of plant diseases by siderophore producing bacteria

Due to requirement of iron for cell growth and metabolism, siderophore mediated acquisition of iron plays a central role in determining the ability of different microorganisms to colonize plant roots and contributes to microbial interactions in the plant rhizosphere. Kloepper et al. (1980) were the first to demonstrate the importance of siderophores in the mechanism of biological control. There are now 500 known siderophores, some of which are widely used by various microorganisms, whereas others are used only by the same microbial species and strains that produce them (Wandersman and Delepelaire, 2004). In this section, the status of research conducted in the last 10 years on utilization of siderophore producing bacteria in controlling plant diseases has been reviewed. The review has been divided into two sections depending on the kind of bacteria utilized: Gram positive and Gram negative.

2.3.1. Gram negative bacteria

Although a range of different bacterial genera and species have been studied, the overwhelming number of papers have involved the use of *Pseudomonas* species (Whipps, 2001). Gram negative bacteria mostly those belonging to the *Pseudomonas* genera, have been cited as potential biological control agents and a number of *Pseudomonas*-based biocontrol inoculants have now been commercially developed. However, other gram negative bacteria like those belonging to the genera *Serratia* (Kamensky et al., 2003, Purkayastha et al., 2010), *Agrobacterium* (Lopez et al., 1989;

Penyalver et al., 2001) and *Enterobacter* (Utkhede et al., 1992; Duponnois et al., 1999) have also been reported as potential biocontrol agents.

Pseudomonads are characteristically fast growing, easy to culture and manipulate genetically in the laboratory, and are able to utilize a range of easily metabolizable organic compounds, making them amenable to experimentation. But, in addition, they are common rhizosphere organisms and are considered as good colonizers. Having appropriate ecological rhizosphere competence may be a key feature for reproducible biological control activity in the spermosphere and rhizosphere. *Pseudomonas* bacteria are of particular interest because of the intrinsic ability of certain strains to colonize the rhizosphere at a high density, to compete successfully with microorganisms, and to produce secondary metabolites with powerful antifungal activity (Bloemberg and Lugtenberg, 2001; Mark et al., 2006).

Penyalver et al., (2001) reported that the iron-binding siderophores were produced in various amounts in response to iron limitation by the bacterial species *A. tumefaciens, A. rhizogenes*, and *A. vitis*. The strain *A. rhizogenes* K84 which was reported earlier as a well known biocontrol agent of crown gall (Lopez et al., 1989), produced a hydroxamate type siderophore in large amount as well as an antibiotic-like substance called ALS8 when grown in iron-deficient medium. Similarly, sensitivity to ALS84 was expressed only when susceptible cells were tested in low-iron media. Southern blotting analysis revealed that the biosynthetic gene from strain K84 is present only in isolates of *A. rhizogenes* that produce hydroxamate-type compounds under iron restricted conditions. Based on physiological and genetic analyses a distinct correlation between production of a hydroxamate siderophore and ALS84 by strain K84 was evident.

Ran et al. (2005) investigated the influence of siderophore production by biocontrol bacteria in relation to ISR in plants. The ability of selected strains of fluorescent *Pseudomonas* spp. to cause induced systemic resistance (ISR) in *Eucalyptus urophylla* against bacterial wilt caused by *Ralstonia solanacearum* was investigated. Two strains, *P. putida* WCS358r

and *P. fluorescens* WCS374r activated ISR when infiltrated into two lower leaves 3-7 days before test inoculation. A mutant of strain WCS358r defective in the biosynthesis of the fluorescent siderophore pseudobactin, did not cause ISR, while the purified siderophore of WCS358r positively induced ISR, suggesting that pseudobactin 358 is the ISR determinant of WCS358. A siderophore-minus mutant of WCS374r induced the same level of disease resistance as its parental strain, but the purified siderophore induced resistance as well, indicating that both the siderophore and unknown, inducing determinant(s) of WCS374r can trigger ISR in *Eucalyptus urophylla.* In another study, Tn5 transposon mutant of the strain *Pseudomonas putida* WCS358 defective in biosynthesis of the fluorescent siderophore pseudobactin was still found to be effective in inducing disease resistance in bean against *Colletotrichum lindemuthianum* infection but the same mutant strain lost its effectivity in tomato against *Botrytis cinerea* infection. Pseudobactin isolated from the parental strain successfully induced ISR in both bean and tomato and protected the plants against their respective pathogens (Meziane et al., 2005). The authors observed that apart from siderophores, other factors such as lipopolysachharides of the bacteria can cause similar effect in these plants.

Saikia et al. (2005) studied the effect of availability of iron on inducing systemic resistance by *Pseudomonas* spp in chickpea against *Fusarium* wilt. Selected isolates of *Pseudomonas fluorescens* (Pf4-92 and PfRsC5) and *P. aeruginosa* (PaRsG18 and PaRsG27) were observed for PGPR activity and ISR against *Fusarium* wilt in chickpea. It was also found out that the *Pseudomonas* spp. could successfully colonized in root of chickpea and significantly suppressed the disease in greenhouse condition. All isolates of *Pseudomonas* spp. showed better disease control in the induced systemic resistance (ISR) bioassay when iron in the nutrient solution was less available. The result of High performance liquid chromatography (HPLC) analysis indicated that all the bacterial isolates produced more salicylic acid, an inducer of ISR at low iron than high iron availability.

Kapsalis et al. (2008) reported that the entomopathogenic bacterial strains *Pseudomonas (Flavimonas) oryzihabitans* and *Xenorhabdus nematophilus*, can be used for suppression of few soil-borne pathogens. *In vitro* antifungal activity of the bacterial strains was tested by studying the mycelium and spore development of soil-borne pathogens, *Pythium* spp. and *Rhizoctonia solani*, the causal agent of cotton damping-off. The role of the antibiotics phenazine-1-carboxylic acid (PCA), HCN and siderophores in the biocontrol activity of these entomopathogenic strains was studied and the result supported earlier evidence that mechanisms of secondary metabolites may be responsible for reducing damping-off diseases.

Chaiharn et al. (2009) reported the possible role of siderophore producing soil bacteria in inhibiting plant pathogenic fungi. A total of 216 bacterial isolates were obtained from soil samples taken from paddy fields in Northern Thailand and the isolates were checked for siderophore production and effectiveness in inhibiting the growth *in vitro* of 4 important rice pathogenic fungi; *Alternaria sp., Fusarium oxysporum, Pyricularia oryzae* and *Sclerotium* sp., the causal agent of leaf spot, root rot, blast and stem rot in rice repectively. It was found that 23% of the bacteria isolated produced siderophore on solid plating medium and liquid medium. In dual culture technique, the siderophore producing rhizobacteria showed a strong antagonistic effect against the tested phytopathogens. The isolate *Pseudomonas aureofaciens* AR 1 was the best siderophore producer overall producing hydroxamate type siderophore and this strain exhibited an *in vitro* antagonistic effect against *Alternaria sp., F. oxysporum* and *P. oryzae.*

Sharifi et al. (2010) demonstrated the role of pyoverdine production in *Pseudomonas fluorescens* UTPF5 in suppression of common bean damping off caused by *Rhizoctonia solani* (Kuhn). *Pseudomonas fluorescens* UTPF5 was isolated from onion field soil and it was observed that this bacterial isolate effectively controlled several phytopathogenic fungi. Pyoverdine type siderophore of this strain was isolated using XAD amberlite column. The plant growth promotion and antifungal properties of bacteria were demonstrated under greenhouse conditions in combination with Fe-EDTA,

Fe-EDDHA and Zn as modulators of pyoverdine production. Amendment with zinc, Fe-EDTA and Fe-EDDHA suppressed the disease inhibition when partially used with UTPF5. 7NSK2 and its pyoverdine mutant, MPFM1, were used as reference strains, the inhibition percent of which was not affected by soil amendment. Iron chelates, especially Fe-EDDHA, increased growth and chlorophyll production by plants. This effect was improved in the presence of bacterial strains. The siderophore mutant MPFM1 did not exhibit satisfactory disease inhibition and growth promotion activity. *In vitro* experiments showed that purified pyoverdine could decrease the fungal growth to the same extent as pyoverdine-producing strain.

The soybean epiphyte *Pseudomonas syringae* pv. syringae strain 22d/93 which showed great potential for controlling *P. syringae* pv. glycinea, the causal agent of bacterial blight of soybean (Volksch and May, 2001) produced a significantly larger amount of siderophores than the pathogen *P. syringae* pv. glycinea produced (Wensing et al., 2010). While *P. syringae* pv. syringae 22d/93 and *P. syringae* pv. glycinea produce the same siderophores, achromobactin and pyoverdin, the regulation of siderophore biosynthesis in the former organism is very different from that in the latter organism. The epiphytic fitness of *P. syringae* pv. syringae 22d/93 mutants defective in siderophore biosynthesis was determined following spray inoculation of soybean leaves. The population size of the siderophore-negative mutant *P. syringae* pv. syringae strain 22d/93ΔSid was 2 orders of magnitude lower than that of the wild type 10 days after inoculation. The results suggested that siderophore production has an indirect effect on the biocontrol activity of *P. syringae* pv. syringae 22d/93. Although siderophore-defective mutants of *P. syringae* pv. syringae 22d/93 still suppressed development of bacterial blight caused by *P. syringae* pv. glycinea, siderophore production enhanced the epiphytic fitness and thus the competitiveness of the antagonist (Wensing et al., 2010).

Kanimozhi and Perinbam (2011) investigated siderophore mediated antagonistic activity of *Pseudomonas fluorescens* Lp1 isolated from soil against common fungal pathogens of plant. The authors optimized the

siderophore production under varied physiochemical conditions and also partially purified the siderophore. Antagonistic activity of *Pseudomonas fluorescens* Lp1 against plant fungal pathogens such as *Aspergillus flavus*, *A. niger*, *Curvularia* sp. and *Fusarium* sp. was evaluated. Inhibition of mycelial growth of tested pathogens was observed upto 52.5%. In a similar study, fifty-eight *Pseudomonas* strains were obtained from the chickpea and green gram rhizosphere and these were tested for siderophore production and colony growth. Diameter of halo zone in CAS agar medium varied with different strains. The antagonistic activities of the isolates were tested against some phytopathogenic fungi i.e., *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium aphanidermatum*. Coinoculation of *Pseudomonas* strain CP56 with *Bradyrhizobium* strain and *R. solani* showed increase in plant dry weight at 60 days in comparison to control uninoculated plants and entirely suppressed the root rot disease under pot house conditions (Sahu and Sindhu, 2011).

Sayyed and Patel (2011) studied the role of siderophore producing bacteria for *in vitro* phytopathogen suppression. The activity of siderophoregenic preparations of Ni and Mn resistant *Alcaligenes* sp. STC1 and *Pseudomonas aeruginosa* RZS3 SH-94B isolated from soil were found to be promising and more effective than chemical pesticide. Both the broth and supernatant of siderophore rich culture showed antagonistic activity against *Aspergillus niger* NCIM 1025, *Aspergillus flavus* NCIM 650, *Fusarium oxysporum* NCIM 1281, *Alternaria alternata* ARI 715, *Cercospora arachichola*, *Metarhizium anisopliae* NCIM 1311 and *Pseudomonas solanacerum* NCIM 5103.

Villalobos et al., (2012) described the activity of siderophore producing bacterium *Burkholderia cepacia* XXVI in biological control of *Colletotrichum gloeosporioides* ATCC MYA 456, the causal agent of anthracnose in mango. Tests for production of IAA and lytic enzyme activities (cellulase, glucanase and chitinase) by *B. cepacia* XXVI were negative, which was an indicative that these metabolites were not involved in the biocontrol effect. Based on halo formation on CAS-agar, as well as colorimetric tests, it was confirmed that strain XXVI produced a hydroxamate siderophore and it was involved

in the growth inhibition of the pathogen on the diagnostic medium. The minimal inhibitory concentration test showed that 0.64 µg /ml of siderophore (Deferoxamine mesylate salt-equivalent) was sufficient to achieve 91.1 % inhibition of the pathogen growth on PDA. The biocontrol capacity against *C. gloeosporioides* ATCC MYA 456 correlated directly with the siderophore production by *B. cepacia* XXVI. Gull and Hafeez (2012) isolated 14 siderophore positive *Pseudomonas* strains and tested them for their biocontrol potential against the phytopathogen *Rhizoctonia solani* using various dual culture assays. The involvement of siderophores in the inhibition of *R. solani* was checked by $FeCl_3$ experiment. The most potent 8 antagonisitic strains were found positive in nitrogen fixation, phosphate solubilization, IAA, various hydrolytic enzymes, hydrogen cyanide and antibiotics production. Spectrochemical analysis indicated that all the bacterial strains produce catecholate type of siderophores. The authors suggested that siderophore production was the key mechanism involved in the antagonism.

Serratia strains have been shown to secrete siderophores under *in vitro* conditions (Kamensky et al., 2003; Purkayastha et al., 2010) but there is no direct evidence of siderophore being the major contributor in biological control. Müller et al. (2009) observed that siderophore production in *S. plymuthica* strain HRO-C48 is not influenced by quorum sensing mechanism mediated by acyl homoserine lactones. However, siderophores seemed to be involved in induction of resistance in cucumber by *Serratia marcescens* 90-166 since it was affected negatively by high iron concentration (Press et al., 1997). The authors observed that a mutant 90-166 strain that was defective in siderophore production failed to induce systemic resistance in cucumber against *Colletotrichum orbiculariae.* Ovadis et al. (2004) studied the regulatory mechanism of siderophore production by *S. plymuthica* strain IC1270. However, neither GrrA/GrrS nor RpoS affected siderophore production by strain IC1270 indicating that these global regulators probably do not have a role in siderophore production.

2.3.2. Gram positive bacteria

Although biocontrol strains of fluorescent pseudomonades have contributed greatly to the understanding of the mechanisms that are involved in phytostimulation and disease suppression, biological preparations from spore-forming *Bacillus* spp. are often preferred due to their long-term viability which facilitates the development of commercial products (Haas and Defago, 2005; Emmert and Handelsman, 1999; Romero et al., 2007). Compared to plant growth–promoting *Pseudomonas* rhizobacteria, relatively little is known about the lifestyle of plant associated *Bacillus* spp., which were originally considered as typical soil bacteria, despite their well-established advantages for beneficial action on plant growth and biocontrol (Compant et al., 2005; Chen et al., 2007).

Sousa et al. (2008) suggested that Streptomycetes strains may be utilized as biocontrol and PGPR agents. They characterized six isolates of streptomycetes that produced siderophores, different extracellular enzymes and IAA, solubilised phosphate, colonized roots of tomato seedlings and grew under different pH and salinity levels. The isolates AC-147, AC-95, and AC-29 produced siderophores in large amount. All isolates colonized tomato roots *in vitro*, and AC-92 grew under all pH and salinity levels tested. In a similar study, Khamna et al. (2009) observed that *Streptomyces* CMU-PA101 and *Streptomyces* CMU-SK126 produced antifungal compounds, IAA and siderophores in high amount and they suggested that these isolates may serve as alternative means to control the phytopathogens.

Li et al. (2008) isolated a chitinase-secreting strain CH2 along with 353 strains from rhizosphere of eggplant. Based on 16S rDNA sequence alignment and several biochemical and physiological characteristics, the strain was identified to be of *Bacillus cereus*. On chitin–Ayers (CA) medium, the strain secreted chitinase. Evaluation of its activity, combined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), showed it to be a 15.0-KD chitinase. On glass slides, germination of the fungal spores was effectively suppressed by the bacterial suspension,

supernatant from the suspension, and 0.005% solution of chitinase extracted from the strain CH2. The optimum pH for chitinase was 7.1 and optimum temperature was 40°C. At that temperature, high-level chitinolytic activity was retained for 10 days. In greenhouse experiments, suspension of the cells of the CH2 strain reduced the severity of *Verticillium* wilt on eggplant by 69.69%, its supernatant by 54.04%, and the enzyme diluted to 0.01% strength by 53.13% in 14 days. The authors suggested that strain CH2 and its chitinase have good commercial potential in controlling *Verticillium* wilt.

Yu et al. (2011) reported the role of siderophore producing *Bacillus* in controlling plant pathogens. In their study they screened for siderophore positive bacteria from rhizospheric soil of pepper in Hainan, China and *Bacillus subtilis* strain CAS15 was obtained. *In vitro* test showed that CAS15 can strongly limit the growth of 15 plant fungal pathogens, with rates of inhibition ranging from 19.26 to 94.07%. Chemical characterization of the siderophore by the ESI-MS and DHB(G) assays showed that CAS15 produced the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin under iron starvation. A pot culture experiment was used to study the effects of *B. subtilis* CAS15 on pathogen development and plant growth. CAS15 reduced the incidence of *Fusarium* wilt in pepper significantly, by 12.5-56.9%, but iron supplementation reduced this biocontrol effect, which suggested that this strain also participated in induced systemic resistance (ISR) in pepper. Additionally, the strain showed strong growth promoting activity with treated plants growing 27.24-54.53% taller than controls. *B. subtilis* CAS15 also enhanced the yield of pepper by increasing the average crop yield per plant by 49.68%.

Almoneafy et al. (2012) worked with about 200 *Bacillus* strains isolated from tomato and potato rhizosphere and they found four strains, *Bacillus amyloliquefaciens* AM1, *Bacillus amyloliquefaciens* D29, *Bacillus subtilis* D16, and *Bacillus methylotrophicus* H8 which showed strong antagonistic activity against the pathogen *Ralstonia solanacearum* T-91 and resulted in

81.1 to 89.0% reduction of disease incidence of bacterial wilt in treated tomato plants. The four strains showed ability to inhibit growth of the three soil-borne fungi, produce indole-3-acetic acid, siderophores and also with exception of strain D16, the other 3 strains were capable of phosphate solubilization. It was also observed that plant height was significantly increased by 22.7 to 43.7% and dry weight by 47.93 to 91.55% compared with non-treated control. In another study, the siderophore and IAA producing strains *Bacillus subtilis* WR-W2 and *Bacillus amyloliquefaciens* MR-AI have been reported to show significant increase in growth when the strains were used as bioinoculum in rice plants (Pusa sugandha III) (Mishra and Kumar, 2012).

CHAPTER 3

Isolation of Siderophore Producing Antagonistic Bacteria From Soil and their Characterization

ISOLATION OF SIDEROPHORE PRODUCING ANTAGONISTIC BACTERIA FROM SOIL AND THEIR CHARACTERIZATION

3.1. Introduction

The rhizosphere was first described by Hiltner (1904) as the volume of soil surrounding plant roots influenced by the living root. Rhizosphere is a dynamic environment, which harbours diverse groups of microbes. Bacteria respond differently to the compounds released by the plant root, and thus different compositions of root exudates are expected to select different rhizosphere communities. Soil microbial communities are often difficult to fully characterize, mainly because they are immensely diverse in genotypic and phenotypic composition, heterogeneous and often quite obscure. With respect to the latter, bacterial populations in soil top layers can go up to more than 10^9 cells per gram soil, and most of these cells are generally unculturable (Garbeva et al., 2004). Traditionally, methods to analyze soil microorganisms have been based on cultivation and isolation; a wide variety of culture media has therefore been designed to maximize the recovery of diverse microbial groups. Some of these soil-borne, non pathogenic microorganisms have the ability to antagonize fungal phytopathogens and thus prevent plant diseases.

The main *in vitro* screening methods that have been performed in plate assays with only one microorganism (mainly searching for lytic enzymes or siderophores production) or with two different microorganisms (mainly searching for antagonistic or parasitic relationships) (Pliego et al., 2011). Antagonistic bacterial-fungal interactions are typically assessed *in vitro* in terms of an unoccupied "inhibition zone" between a bacterial colony and fungal hyphae cocultured on an agar plate. Two-component screening (e.g. dual cultures of a candidate antagonist and a pathogen on agar) is exclusively related to interaction studies, and potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen expressed by an inhibition zone. The antibiotic producing strains

have been studied for their antagonism in this way, and these antibiotics are known to be active against fungi *in vivo* (Knudsen et al., 1997). The production of these antagonistic substances sometimes correlate very well with the biocontrol ability of these bacteria, and the dual culture method has performed reasonably well for their screening (Pliego et al., 2011).

Some authors have argued that this approach is focused on some limited facet of the mechanism of antagonism and that these screening methods may not be suitable and should be avoided (Campbell, 1986). However, screening for this mode of action is easy and inexpensive and permits massive screening of several strains of microorganisms. If the goal is to select microorganisms with high capabilities of natural metabolite production and to develop these natural products for commercial applications, prescreening for antibiosis may be appropriate (Pliego et al., 2011). DNA-based methods currently employed to characterize soil microbial community composition in large part rely upon use of the polymerase chain reaction for amplification of the small subunit rRNA gene. PCR amplification of rRNA genes or other ecologically significant genes generates relatively less biased information. Once sequenced, the amplicons are analyzed for similarity to other known sequences, and the identity of the organism can be suggested based on phylogenetic relatedness (Mazzola, 2004).

Although a number of authors have stressed the importance of appropriate screening procedures (Merriman and Russell, 1990; Deacon, 1991; Campbell, 1994; Knudsen et al., 1997; Whipps, 1997), but, it should be considered that any screening method is selective and therefore, it is to be expected that only a portion of the antagonistic microbiota will be detected (Knudsen et al., 1997; Pliego et al., 2011). As this study aims to utilize antagonistic microbial inoculants for control of fungal diseases in plants, rhizosphere soil bacteria have been screened for two properties: siderophore production and antagonism. Antagonism has been tested against major plant pathogenic fungi relevant to the region of study. Further the selected isolates have been characterized for certain morphological and biochemical

properties. Phylogenetic characterizations have been done by matching their 16S rRNA gene sequences with other bacteria.

3.2. MATERIALS AND METHODS

3.2.1. Isolation of bacteria from soil

3.2.1.1. Collection of sample

Soil samples were collected from 9 different regions of sub-Himalayan West Bengal which included different parts of Darjeeling and Jalpaiguri districts (Fig. 4 and 5) where biocontrol agents have never been applied.

Samples were collected from the rhizosphere of maize (*Zea mays*), mango (*Mangifera indica*), lemon (*Citrus limon*), brinjal (*Solanum melongena*), jute (*Corchorus capsularis*), Potato (*Solanum tuberosum*), Cassia (*Cassia fistula*), tea (*Camellia sinensis*), radish (*Raphanus sativus*), neem (*Azadirachta indica*), wheat (*Triticum sativum*) and grass (*Eleusine indica*). The local regions of sample collection were Bagracote (MB), Damdim (MD), Chathat (LC), Bagdogra (BB), Lataguri (JL), Siliguri (AS), Kamala Bagan (KT), Ellenbarie (NE) and Nagrakata (NG) (Table 3). Geographic Information System (GIS) Locations of the places of sample collection and their respective codes are presented in table 4.

For isolation of bacteria, soil samples which comprised of plant roots with adherent soil were collected in sterilized polythene packets and transported to the laboratory within five hours. At the same time, approximately 100 g soil was collected from the same area in clean glass bottles which would be necessary for preparation of soil extract.

3.2.1.2. Media

Soil extract agar (Barrow and Feltham, 1993) was used to obtain a first level screening of the culturable bacterial strains, which are predominant in a definite rhizospheric soil. It was achieved by maintaining the pH of the medium similar to that of the soil. To prepare soil extract, 75g of soil of the specific rhizosphere was dried and sieved through fine mesh. Air dried soil

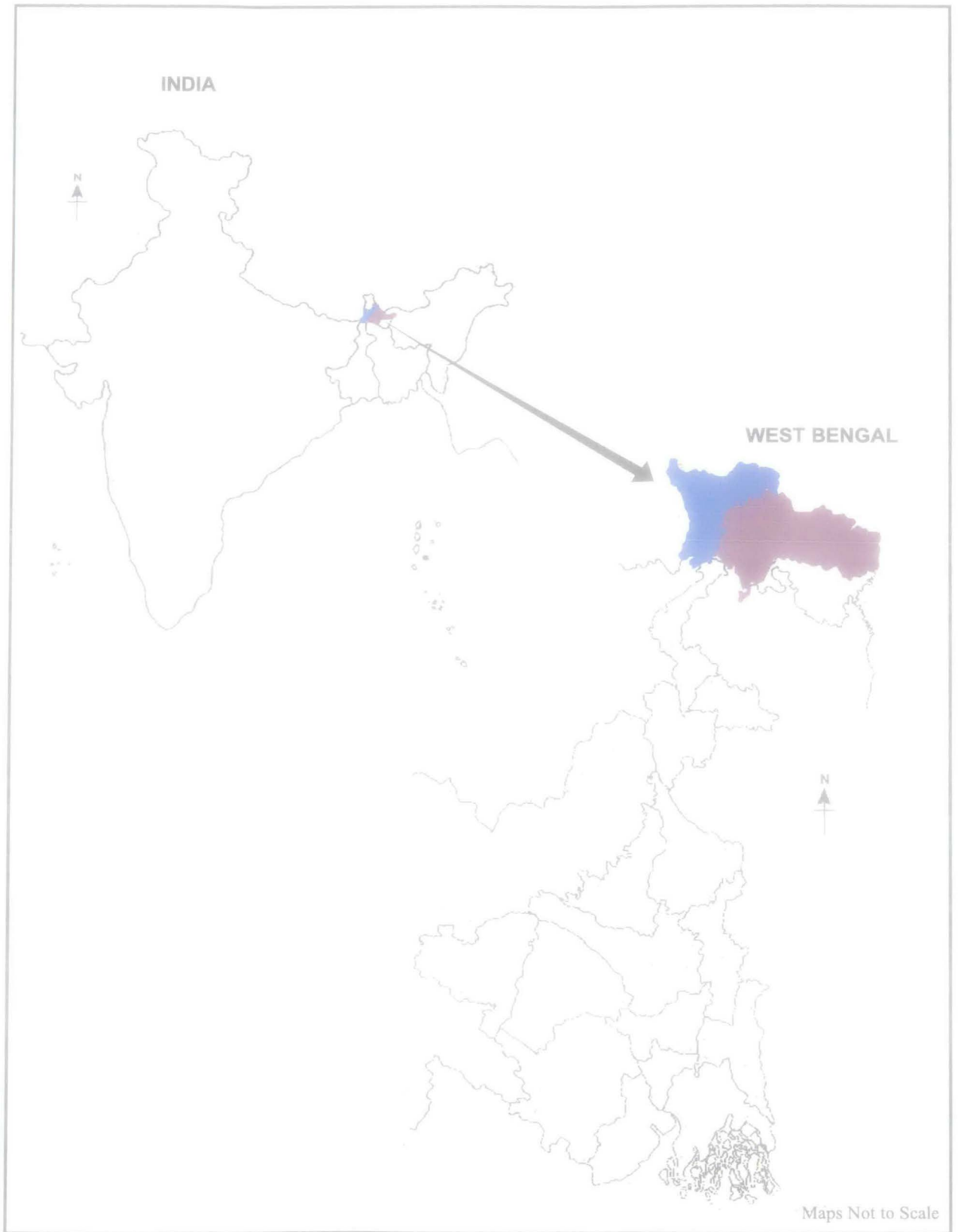


Fig. 4: Map showing present study area in India.

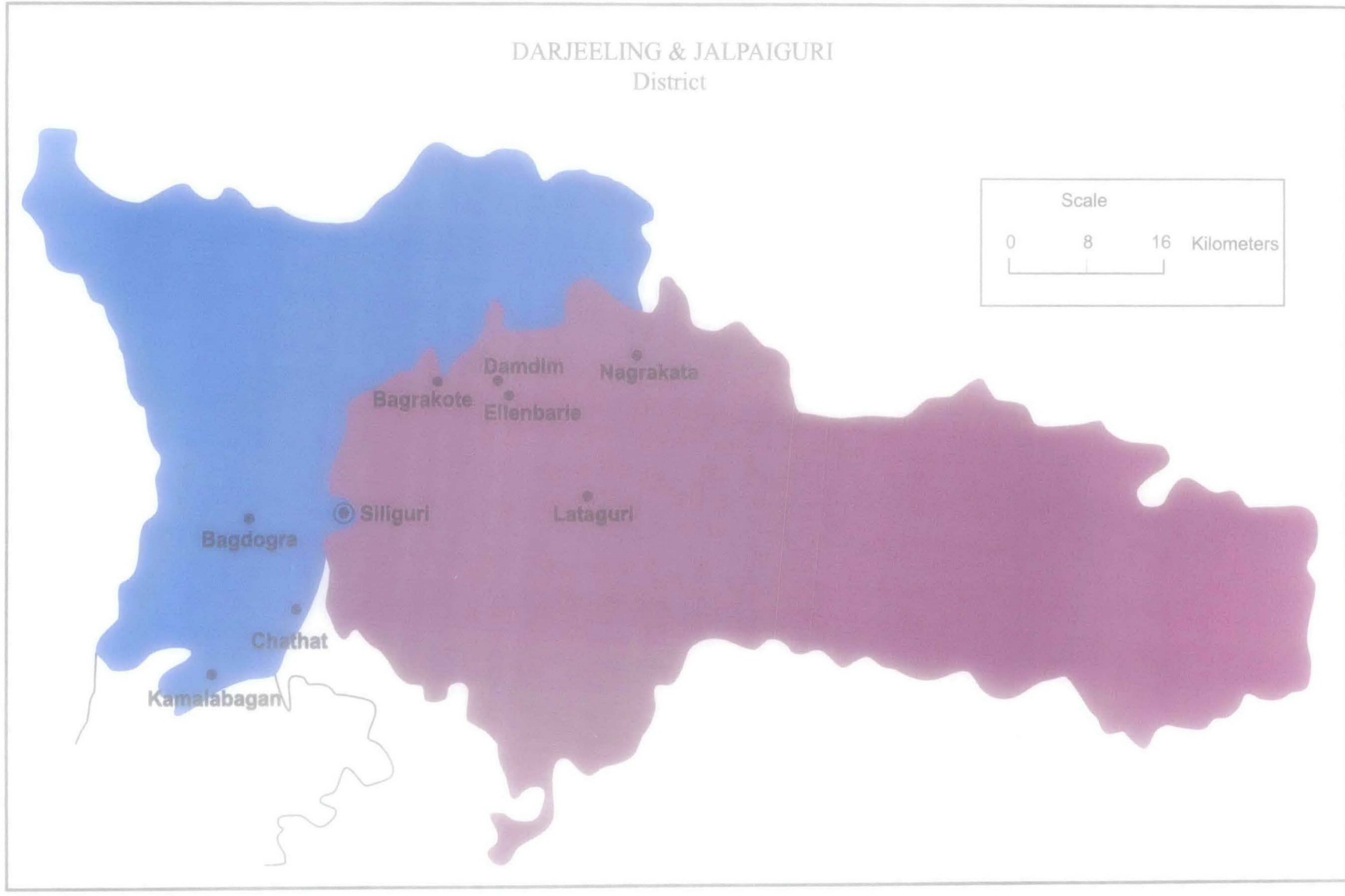


Fig. 5: Map showing locations of collection of rhizosphere soil samples in Darjeeling and Jalpaiguri Districts of West Bengal.

was suspended in 180 ml of distilled water and sterilized by autoclaving at 15 lbs pressure (121°C for 15 min) and allowed to settle down overnight. The top clear layer of the solution was decanted and a pinch of $CaCO_3$ was added and allowed to stand for removal of the turbidity. The resulting clearer solution was filtered through the Whatman filter paper (Grade-II) for removing the traces of fine soil particles. For preparation of soil extract agar medium, peptone, beef extract and agar were added in 5.0 g, 3.0 g and 20.0 g in amount respectively in the 1000 ml clear soil extract solution. Instead of distilled water, soil extract was used, which essentially was enriched with the indigenous minerals and ions of that soil sample.

Table 3: List of local regions of sample collection and the respective source plants

Place of isolation	Source plant (rhizosphere soil)		Code assigned
	Common Name	Botanical Name	
Bagracote	Maize	*Zea mays*	MB
Damdim	Mango	*Mangifera indica*	MD
Chathat	Lemon	*Citrus limon*	LC
Bagdogra	Brinjal	*Solanum melongena*	BB
Lataguri	Jute	*Corchorus capsularis*	JL
Siliguri	Potato	*Solanum tuberosum*	AS
Bagdogra	Cassia	*Cassia fistula*	CB
Kamala Bagan	Tea	*Camellia sinensis*	KT
Ellenbarie	Neem	*Azadirachta indica*	NE
Chathat	Radish	*Raphanus sativus*	CR
Bagracote	Wheat	*Triticum sativum*	WB
Nagrakata	Grass (Indian goosegrass)	*Eleusine indica*	NG

Table 4: Geographic Information System (GIS) Locations of the Places of Sample Collection and Their Respective Codes

Place of Sampling	Code assigned	GIS Location	
		Latitude	Longitude
Bagracote	MB	26.8809° N	88.5734° E
Bagracote	WB	26.8809° N	88.5734° E
Damdim	MD	26.8667° N	88.7500° E
Chathat	LC	26.5800° N	88.3600° E
Chathat	CR	26.5800° N	88.3600° E
Lataguri	JL	26.7060 ° N	88.7662° E
Siliguri	AS	26.7223° N	88.4248° E
Bagdogra	CB	26.6811° N	88.3283° E
Bagdogra	BB	26.6811° N	88.3283° E
Kamala Bagan	KT	26.6110° N	88.33535° E
Ellenbarie	NE	26.7881° N	89.0408° E
Nagrakata	NG	26.9000° N	88.9667° E

3.2.1.3. Isolation process

For isolation of bacteria, the sample packets were opened and soil adhering to roots was collected by gently shaking the roots. Ten gram of soil sample was mixed in 100 ml of sterile Winogradsky solution (Szreniawska and Hattori, 1981) and kept for 2 hours on a shaker (Nielsen et. al., 1998). The resultant solution was serially diluted and bacteria were isolated from each dilution by the spread-plate method. To prepare the dilution series, 1 ml solution was dispensed in another test tube containing 9 ml of sterile distilled water to make a dilution of 10^{-1}. In the similar way, a dilution series from 10^{-1} to 10^{-10} were prepared (Aneja, 2003). Each different dilution was spread onto solidified agar media to obtain bacterial colonies. To do this,

100µl of each dilution was placed on soil extract agar in petriplates of 9 cm diameter and spread by a glass spreader under aseptic condition. In addition to that, each dilution was also spread onto CAS agar media to check the presence of siderophore producing bacteria. The plates were incubated at 30°C for 48 hours. Each single colony was picked from the soil extract agar plates and streaked on sterile nutrient agar plates following quadrant streak method. Pure cultures were maintained on NA slants at 4°C and sub-cultured at regular intervals.

3.2.2. Screening for siderophores producing bacteria using CAS agar medium

All isolated bacterial strains were subjected to screening for siderophores production. Qualitative test for siderophore production was done using Chrome Azurol S agar as described by Husen (2003). The universal Chrome Azurol S-agar medium (Schwyn and Neilands, 1987) was used for selecting the siderophore producing strains from isolated soil bacteria. The medium is a combination of four solutions which were prepared separately and sterilized. Solution 1 (Fe-CAS indicator solution) was prepared by mixing 10ml of 1mM $FeCl_3.6H_2O$ (in 10mM HCl, sterile), 50ml of aqueous solution of CAS (1.21mg/ml) and 40ml aqueous solution of HDTMA (1.82mg/ml). Solution 2 (buffer solution) was prepared by dissolving 30.24 g of PIPES buffer in water. The final pH was adjusted to 6.8 by using 50% KOH solution to obtain a final volume of 800 ml. Next, 15 g agar was added to it and autoclaved. Solution 3 consisted of glucose and mannitol (2g each) in 70 ml distilled water and autoclaved. Solution 4 was prepared by dissolving casamino acid in 30 ml water (10% w/v). This solution was sterilized by filtering through 0.2µ cellulose acetate membrane. All solutions were mixed appropriately to obtain the final medium. It was prepared by first allowing solution 2 to cool after autoclaving and when temperature reached to about 50-55°C, solution 3 and 4 were mixed with it. Finally solution 1 was added to it and mixed generously. The medium appeared blue to dark green in color due to the presence of dye-Fe-CAS complex. Spot inoculation of

bacterial culture onto CAS agar plate was done and incubated at 30°C overnight.

3.2.3. Fungal pathogens used in the study

Seven fungal pathogens were selected as test pathogens considering their agronomic importance in sub Himalayan West Bengal. All pathogens except *Fusarium solani* were isolated as pathogens from various crops of sub Himalayan West Bengal (Saha et al., 2008, 2010; Choudhuri et al., 2008; Mandal et al., 2006).

3.2.3.1. Source of fungal pathogens

Fusarium solani was procured from Indian type culture collection, IARI, Pusa, New Delhi and rest of the pathogens were kindly gifted by Dr. Aniruddha Saha, Department of Botany, University of North Bengal. The details of the source of the strains used in the present study are given in Table 5. All the strains were identified by Dr. A. Saha and the identities of the ITCC strains were confirmed from IARI, New Delhi. Before using the fungi in experiments, they were cultured in PDA and 10 day old fungal cultures were taken in glass slide and observed under microscope by staining with lactophenol-cotton blue.

Table 5: List of fungal cultures used as test pathogen in the current study

Fungal pathogen	Host Plant	Strain identity
Fusarium solani	Brinjal	ITCC-4999
Fusarium equiseti	Brinjal	ITCC- 6566.07
Fusarium graminearum	Brinjal	FSG01
Rhizoctonia solani	Tea	ITCC-5995.05
Colletotrichum gloeosporioides	Brinjal	ITCC-5446.02
Alternaria alternata	Niger	ITCC-6250.05
Lasiodiplodia theobromae	Tea	ITCC-5446.02

3.2.3.2. Maintenance of cultures in PDA

Freshly prepared sterile PDA slants were used for the maintenance of the pathogenic fungal cultures. Pathogens were grown on sterile PDA media and were maintained in two different conditions, *viz.* at low temperature in refrigerator (at 4°C) and at room temperature. At the interval of 2-3 weeks sub culturing was done for maintenance of cultures. Sub culturing was also done for preparation of inoculums for different experiments.

3.2.4. Evaluation of antifungal activity

All siderophore producing bacterial isolates were at first screened by the dual culture technique for *in vitro* antagonism against all fungal pathogens. The isolates which recorded antifungal activity against all pathogens were selected for further evaluation of their antifungal potential.

3.2.4.1. Screening of isolates by dual culture test

In this method, both the fungal pathogen and isolated bacterium was allowed to grow simultaneously in presence of each other. For fungal inoculation, mycelial disc (4 mm diameter) was excised from advancing zones of fungal hyphae in PDA cultures of the pathogens and placed at the centre of a 90 mm diameter petriplate containing PDA. The bacterial isolate was streaked at a distance of 2-3 cm from the centre in a semi-circular fashion. The control plate was prepared by inoculating only the pathogen at the centre of PDA plates. The plates were incubated at 28°C until the fungal growth on the control plate reached the rim of the plate. Inhibition of fungal growth along the bacterial line of streaking indicated antagonistic activity of bacterial isolates.

3.2.4.2. Quantitative test for study of *in vitro* antagonism

For evaluation of antagonistic potential, the dual culture technique as described above was followed. Thus, both the fungal pathogen and selected bacterial strains were allowed to grow simultaneously in a similar way but the bacteria were now streaked circularly at a distance of 20 mm from the central 4 mm fungal inoculum in 90 mm PDA plates. The control plates

were inoculated only with the fungal pathogens. Radial growth of the fungal mycelia was recorded until the fungal growth in the control plates reached the edge of the plates. Percent inhibition of fungal growth was calculated as [(90-d)/90] × 100 where d is the diameter (in mm) of fungal growth in the test plates. The tests were performed in three replications and the data was averaged.

3.2.4.3. Statistical analysis

Statistical analysis was done with the help of Smith's statistical package (version-2.5), developed by Dr. Gray Smith, Pomona College, Claremont-91711, USA and Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois. Standard error was also calculated using this software.

3.2.5. Characterization of selected bacterial isolates

In order to characterize the selected antagonistic bacterial strains, a number of morphological and biochemical tests were conducted (Barrow and Feltham, 1993; Aneja, 2003). Furthermore, Phylogenetic characterization was done by using the partial 16S rDNA sequences which were obtained following PCR amplification. Bergey's Manual of Systematic Bacteriology and Cowan and Steel's Manual for the Identification of Medical Bacteria was studied to determine the identity of bacterial antagonists (Barrow and Feltham, 1993; Brenner et al, 2005; Sneath et al, 1984).

3.2.5.1. Morphological characterization

Morphological characterization included both cell morphology and colony morphology. Studies on cell morphology were conducted under microscope. Colony morphology was studied by observing the colony characteristics in NA plates/slants and in NB.

3.2.5.1.1. Shape and size

To examine the shape and size of the cells, a drop of cell suspension of the bacterium was placed on a clean grease-free slide, heat fixed and stained

with methylene blue and observed under microscope. Diameter was measured after standardizing the stage and ocular micrometer of the microscope.

3.2.5.1.2. Endospore staining

Endospore formation by the isolates was studied by malachite green staining of 3-4 day old culture (Aneja, 2003). A loopful of bacterial colony from 24 h old NA slants was smeared with sterile distilled water on a clean grease-free slide. The smear was heat fixed and the slide was flooded with malachite green. The slide was heated to steaming and the steaming was continued for 10 minutes during which stain was added intermittently. The slide was washed carefully under tap water and was then stained with safranin. Safranin was washed with distilled water after 1 minute. The excess water was blotted off and the slide was air dried and observed under microscope. Presence of green coloured spores inside red vegetative cell indicated endospore formation (Aneja, 2003).

3.2.5.1.3. Colony characteristics

Colony characteristics were examined with young cultures (18-24 h) of all the selected isolates in NA plates or slants or in NB. Different parameters such as elevation, margin of colonies, pigment production, surface were tested following Aneja (2003) and Seeley and Vandemark (1972).

To study growth pattern of bacterial isolates it is mostly seen that the organisms are fast, moderate or slow in growth rate when grown for 24 h at 30°C. The margin of the colonies shows entire, undulate or rough appearance for different strains. The surface of some colony on agar plate shows glistering or shiny form or dull, moist or smooth features. Several colonies appear raised from the agar surface, some may show flat form. Colour of the colony was also observed as it may turn different on pigment production. The cultural characteristics were also studied in NB. Some of the overnight grown culture shows turbidity throughout the broth while some cultures form visible clump or pellicle like growth at the upper layer of medium.

3.2.5.1.4 Motility test

To detect the motility of bacteria, tubes of semi solid motility medium were stab inoculated with a straight needle (Tittsler and Sandholzer, 1936). The tubes were incubated at 30°C and motility was indicated by a diffused zone of growth around the line of inoculation.

3.2.5.2. Biochemical characterization

Biochemical characterization of all the isolated siderophore producing antagonistic bacterial strains was done following standard published methods. Bacteria were subcultured twice from stocks and 24 h old cultures were used for the tests. During the tests, an uninoculated tube or an untreated tube was included in the biochemical experiments as negative control.

3.2.5.2.1. Gram Staining

Each bacterial strain was grown in NA for 24 h. Each bacterium was separately smeared with sterile distilled water at the centre of clean grease free slide. The smear was air dried; heat fixed and was covered with crystal violet for 30 seconds. Each slide was then washed with distilled water for a few seconds and subsequently covered with Gram's iodine solution for 30 seconds. The crystal violet-iodine complex was washed off with 95% ethyl alcohol. Ethyl alcohol was added drop by drop holding the slides in a slanting position against a white background till no colour came from the lower edge of the slide. The slides were washed with distilled water and drained. Safranin was applied to the smears for 1 minute, washed with distilled water and blotted dry with absorbent paper. The stained slides were observed under microscope to study the Gram character and cell morphology of the bacterial isolates. The staining technique was followed after some modification of the method described by Aneja (2003).

3.2.5.2.2. Indole production

Test tubes containing tryptone broth were inoculated with bacterial isolates and one tube was kept as an uninoculated control. Tubes were incubated at

30°C for 48 hours. Next, 1 ml of Kovac's reagent was added to each tube including control. The tubes were gently shaken after intervals of 10-15 min. The test tubes were allowed to stand to permit the reagent to come to the top. A cherry red colour in the reagent layer indicated indole production (Aneja, 2003).

3.2.5.2.3. Methyl Red and Voges–Proskauer Test

MR-VP broths were inoculated with selected bacterial strains and incubated at 30°C for 48 hours. An uninoculated tube was maintained as control. After incubation, culture was divided equally into two tubes for each isolate. To one of the tube marked as MR (for Methyl Red), 5 drops of Methyl red indicator was added and observed for change of colour. The MR indicator remains red in the pH range upto 4 and an appearance of red throughout the broth indicates positive result. If the broth retains the original yellow colour, it shows a negative test.

To the other tube marked as VP, 12 drops of VP reagent I and 2-3 drops of V-P reagent II were added. Tubes were shaken gently for 30 seconds. The reaction was allowed to complete for 15-30 minutes. Development of ruby pink or red color (mostly intense at the top layer of broth culture) indicated a positive test (Aneja, 2003).

3.2.5.2.4. Citrate Utilization

Simmon's Citrate agar slants were inoculated with isolated cultures by streaking and one tube was kept as uninoculated control. All the slants were incubated at 30°C for 48 hours. Bromothymol blue, the indicator in Simmon's citrate agar shows green colour at acidic pH (upto pH 6.8) and turns blue in alkaline medium (pH 7.6 and higher). Observation of colour change from green to blue indicated positive result (Aneja, 2003).

3.2.5.2.5. Nitrate Reduction

Nitrate Broth was inoculated and incubated at 30°C for 48 hrs. One ml of nitrite reagent A followed by 1 ml of reagent B was added. A deep red colour showed the presence of nitrite and thus showed that nitrate had been

reduced and indicated a positive reaction. To tubes, not showing a red colour within 5 min, powdered zinc was added and allowed to stand. Red colour formation confirmed the presence of nitrate in the medium (i.e. not reduced by the organism) (Barrow and Feltham, 1993).

3.2.5.2.6. ONPG Test

Tubes of ONPG broth were inoculated with the selected isolates and incubated at 30°C for 48 hours. An uninoculated tube was maintained as control. β-galactosidase activity was indicated by the appearance of a yellow colour due to the production of o-nitrophenol (Barrow and Feltham, 1993).

3.2.5.2.7. Oxidation or Fermentation of glucose

Tubes containing Hugh and Leifson's O-F medium were inoculated by stabbing with a straight wire. Two uninoculated tubes were used as comparative control. Sterile liquid paraffin was poured over the medium to form a layer of about one cm deep into one of the tubes of each pair. The other tube was left open. The tubes were incubated at 30°C for 5 days. If the blue colour of the medium changed from blue to yellow in the open tube only, it indicated acid production from glucose by oxidation. Fermentative utilization of carbohydrate was indicated by a colour change from blue to yellow in both open and sealed tubes (Barrow and Feltham, 1993).

3.2.5.2.8 Gelatin Liquefaction

Tubes containing gelatin agar media was inoculated and incubated at 30°C for 2 days. The cultures were then allowed to chill by keeping them in ice bath for 15 minutes. The tubes in which the medium did not turn solid even after chilling but remained liquid were considered positive for gelatin liquefaction (Aneja, 2003).

3.2.5.2.9 Phenylalanine Deamination Reaction

Tubes containing phenylalanine agar were inoculated heavily with bacterial isolates and incubated at 30°C for 24 h. Then 0.2 ml of 10% aqueous solution of $FeCl_3$ was run over the growth. A positive reaction was indicated

by appearance of a dark green colour on the slope and in the free liquid accumulated at the base (Barrow and Feltham, 1993).

3.2.5.2.10. DNase activity

Test organisms were inoculated on the agar surface of DNase agar plates by line streaking and incubated at 30±2°C for 36-48 h. The plates appeared greenish blue and a positive result was indicated by formation of a pinkish/clear halo around the bacterial growth. (Kanlayakrit et al., 2001)

3.2.5.2.11. Urease activity

Tubes containing urea broth medium were inoculated and incubated for 48 hours at 30°C. Colour change of the media from yellow to pink indicated positive result (Barrow and Feltham, 1993).

3.2.5.2.12. TSI agar Test

Tubes were inoculated with test organism by first streaking onto the surface of TSI agar slant and then stabbing the medium in the butt region. All tubes were incubated for 24 h at 30°C and observation was recorded. A red coloration in the medium indicated alkaline reaction and yellow was for acid formation. H_2S production was shown by blackening of the medium. The appearance of red coloration in the entire tube (both slant and butt) indicated absence of fermentation (Aneja, 2003).

3.2.5.2.13. Catalase activity

Bacteria were inoculated in NA slants and incubated at 30°C for 24 hrs. After that, 3-4 drops of 10% hydrogen peroxide was allowed to flow over the growth of each slant culture. Effervescence over the surface indicated positive result (Aneja, 2003).

3.2.5.2.14. Oxidase activity

A fresh solution of the reagent was prepared each time of use by adding a loop full of oxidase reagent (tetramethyl-*p*-phenylenediamine dihydrochloride) to about 3 ml of SDW. A filter paper disc was soaked in a

sterile plastic Petri dish with a few drops of the indicator solution and a 24 h culture of the bacteria in NA was smeared across the moist paper with a platinum loop. The appearance of a dark purple colour on the paper within 30 seconds denoted a positive reaction (Barrow and Feltham, 1993).

3.2.5.2.15. Ornithine Decarboxylase Test

Tubes containing decarboxylase agar base media supplemented with ornithine were inoculated with bacterial isolates and incubated at 30°C for 48 h. The tubes were then acidified with 0.1 N HCl drop by drop until the medium was yellow. Then 0.2 ml of a 10% aqueous solution of $FeCl_3$ was added, mixed and observed for colour change. A positive reaction indicates a dark green colour which quickly fades (Barrow and Feltham, 1993).

3.2.5.2.16 Lysine Decarboxylase Test

Tubes containing decarboxylase agar base media supplemented with lysine were inoculated with bacterial isolates and incubated at 30°C for 48 h. The tubes were then acidified with 0.1 N HCl drop by drop until the medium was yellow. Then 0.2 ml of a 10% aqueous solution of $FeCl_3$ was added, mixed and observed for colour change. A positive reaction indicates a dark green colour which quickly fades (Barrow and Feltham, 1993).

3.2.5.2.17. Acid formation from different carbohydrates

All bacterial strains were tested for their ability to utilize different sugars as their sole carbon source and to produce acid. The sugars tested were maltose, glucose, lactose, sorbitol, inositol, mannitol, sucrose, raffinose, rhamnose, trehalose, arabinose, xylose and adonitol. Peptone water broth supplemented with 0.2% phenol red (10ml indicator in 1 L medium) was inoculated with bacterial culture and incubated at 30°C for 24-48 h. The indicator Phenol Red remains red at neutral pH but turns colourless at acidic pH. Thus, the colour change of the broth culture from red to yellow confirms positive result for sugar fermentation and if remains red, is a negative result. The change of colour was recorded by comparing with the uninoculated (control) tube (Aneja, 2003).

3.2.5.2.18. Fluorescence on *Pseudomonas* Agar (For Fluorescein) medium

The ability of isolates to produce fluorescent siderophores was tested by streaking bacteria on *Pseudomonas* Agar (For Fluorescein) (HiMedia) and incubating at 30°C for 48 h. Following incubation, the plates were inspected under UV light source (at 254nm) for emission of fluorescence. The emitted fluorescence was compared visually to that of a standard *P. fluorescens* strain (NRRL B23932) which was also inoculated as a positive control.

3.2.5.3. Phylogenetic characterization

Phylogenetic characterization included partial sequencing of the 16S rRNA gene and comparing the obtained sequences with that of other sequences available in NCBI GenBank. For this, genomic DNA was isolated from each antagonistic bacterial isolate and used as templates for amplification of the target gene. Blast searches were conducted with the amplicon sequences and related species were analysed for final identification of the bacterial strains.

3.2.5.3.1. Isolation of genomic DNA by CTAB method

Genomic DNA was isolated from all the different antagonistic bacterial isolates following the CTAB method (Gomes et al., 2000). At start, a 24 h old NB culture of bacteria was distributed in six 1.5 ml eppendorf tubes and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 20 µl TE buffer. Next, 3 µl Proteinase K (10mg/ml) and 30 µl 10% SDS were added to it, mixed well and incubated at 55°C for 16 hours. Then 80 µl of 1% CTAB in 1M NaCl and 100 µl of 5M NaCl were added to the tubes, mixed and incubated at 65°C for 10 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was transferred to a clean eppendorf tube, and mixed with 0.6 volume chilled 70% ethanol to precipitate the DNA. The eppendorf tubes were centrifuged at 12,000 rpm for 15 min. The DNA pellet obtained after discarding the supernatant was washed twice by adding two volumes of 70% ethanol and centrifuged again at 10,000 rpm for 10 min.

The pellet was allowed to dry for 30-40 min at room temperature and finally re-suspended in 100 µl of TE buffer.

3.2.5.3.2. RNase treatment

For RNase treatment, genomic DNA suspended in 100 µl TE buffer was incubated with 60 µg RNase at 37°C for 30 min. Following incubation, DNA was re-extracted with PCI (Phenol: chloroform: Isoamyl alcohol 25:24:1) and purified DNA was precipitated with chilled ethanol. The DNA was washed twice with ethanol as described above, dried and resuspended in 50 µl of TE buffer (Soni and Kumar, 2009).

3.2.5.3.3. Quantification of DNA

Before quantifying the DNA, the purity was checked by determining the OD_{260}/OD_{280} value. For this, optical density was recorded at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The reading at 280 nm gives the amount of protein in the sample. Pure preparation of DNA has OD_{260}/OD_{280} value in the range of 1.8 to 2.0.

The standard value of 1 O.D. at 260 nm for double-stranded DNA corresponds to 50 ng/µl of dsDNA. For quantification of isolated DNA, the DNA sample (1 µl) was diluted in 50 µl TE buffer (dilution factor = 50) and OD was recorded in a spectrophotometer (Systronics, Visiscan-167).

3.2.5.3.4. Gel electrophoresis

The DNA quality was further checked by agarose gel electrophoresis conducted in a submarine gel electrophoresis system (Bangalore Genei (India) Pvt. Ltd., India). To prepare gel block, 0.8% agarose was suspended in 1X TAE buffer and heated to melt. It was boiled till clear solution was obtained. The solution was allowed to cool to about 50-55°C, following which, ethidium bromide (0.5µg/ml) was added to it, mixed properly and poured into gel casting tray. It was then allowed to solidify. After solidification, the gel was transferred to electrophoresis tank such that the gel block was completely submerged in 1X TAE running buffer. DNA

samples (5µl) were mixed with 1µl gel loading buffer and loaded onto wells. Electrophoresis was run at 50-55 volt for 1 hour and observed under UV transilluminator (Bangalore Genei (India) Pvt. Ltd, Bangalore, India). Presence of sharp and bright fluorescent orange bands confirmed proper DNA isolation.

3.2.5.3.5. PCR amplification of 16S rRNA gene

The 16S rRNA gene of all the antagonistic bacterial isolates was amplified by PCR using their respective genomic DNA as templates. 16S rDNA primers fD1 and 157 rP2 (Weisburg et al., 1991) were used for the amplification (Table 6). PCR was performed in 25µl reaction volume containing 2.5µl of 10X Taq DNA polymerase buffer without $MgCl_2$, 2.5mM $MgCl_2$, 2mM dNTP mix, 2µM primer (each forward and reverse), 50ng template DNA and 3U of Taq polymerase. The reaction mix was amplified using the following program: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min on a thermal cycler (Applied Biosystems GeneAmp PCR 2400).

Table 6: List of primers used in various PCR amplification processes

Name of the Primer	Sequences	References
fD1	5'-AGTTTGATCCTGGCTCA-3'	Weisburg et al., (1991)
157 rP2	5'-ACGGCTACCTTGTTACGACTT-3'	
16S-23S ITS forward	5'AAGTCGTAACAAGGTAG-3'	Kumar et al, (2002)
16S-23S ITS reverse	5'-GACCATATATAACCCCAAG-3'	

3.2.5.3.6. Detection of PCR amplicon in agarose gel

Agarose gel (1%) was prepared as mentioned earlier. PCR products were resolved on 1% agarose gel containing ethidium bromide (0.5µg/ml) at 50V for 1 h in 1X TAE running buffer along with a 500 bp ladder DNA. The resolved amplicons were observed under UV transilluminator and photographed.

3.2.5.3.7. Cloning of PCR amplicons

The successfully amplified 16SrRNA genes of the selected antagonistic strains were cloned before proceeding for sequencing. The pGEM–T Easy Vector System II, purchased from Promega Corporation, Madison, USA, was used following manufacturer's instructions. The PCR products were cloned in the pGEM-T Easy vector without purification of PCR products. For vector ligation, the reaction mixture was as follows: 2X rapid ligation buffer 5µl, pGEM T-Easy vector (50ng) 1.0µl, PCR products 1.5µl, T4 DNA ligase 1.0µl and final volume 10µl made up with deionized water. Ligation reaction was thoroughly mixed by vortexing and stored at 4°C for overnight.

3.2.5.3.7.1. Preparation of competent cells

Escherichia coli JM 109 were grown in 10ml Luria-Bertani (LB) broth in a conical flask and incubated over night at 37°C at 120 rpm. From this flask 100µl culture of *E. coli* cells was transferred to fresh 10 ml LB medium flask and grown for 2-4 h at 37°C until the OD (at 600 nm) attained a value of 0.4-0.6. The culture was taken in microcentrifuge tubes and pelleted at 6,000 rpm for 7 min at 4°C. The supernatant was discarded and the pellet was allowed to dry. To the pellet, 5ml of solution I (80 mM $MgCl_2$ and 20 mM $CaCl_2$) was added and mixed gently and centrifuged at 4000 rpm for 7 min at 4°C. Then the supernatant was discarded and 5ml of Solution II (100 mM $CaCl_2$) was added and mixed gently. Then the micro-centrifuge tubes were incubated in ice for 45 minutes and subsequently centrifuged at 4000 rpm for 7 min at 4°C. The supernatant was discarded and the retained pellet was resuspended in 500 µl Solution II. The resulting cells were competent cells and were further used in the study.

3.2.5.3.7.2. Transformation

To 100µl competent cells taken in fresh microcentrifuge tube, 10µl prepared ligation mixture was added. The eppendorf tube was incubated in ice for 45 min. Following incubation, the tube was placed in a preheated 42°C circulating water bath for 90 sec and then rapidly transferred to an ice bath. Next, the cells were allowed to chill for 10 min and 300 µl LB medium

was added to the tube and incubated at 37°C for 2 h in a rotary shaking incubator.

3.2.5.3.7.3. Blue white screening

Following incubation, the transformed cells (100 µl) were taken out from the eppendorff tube and spreaded on a pre-warmed (at 37°C) LB plates containing ampicillin (100 µg/ml), IPTG (0.5 mM) and X-Gal (80 µg/ml) with the help of plate master (Hi Media Laboratories, India). All plates were kept inside the laminar air flow for 10 min for absorbing the media, sealed with parafilm and incubated in an inverted position at 37°C for overnight. The plates were then observed for formation of blue and/or white colonies. The white coloured recombinants were selected and used for sequencing of the cloned insert.

3.2.5.3.8. Sequencing of cloned PCR products

After obtaining the positive clones, they were sequenced at Bangalore Genei Sequencing Services, India and Xcerlis Labs, Gujarat, India. The sequences of the amplicons are listed in Appendix II. The partial 16S rRNA gene sequences were submitted to NCBI GenBank. The selected bacterial strains were identified by similarity searches of the sequences using the BLAST function of GenBank (Altschul et al. 1990; Tamura, et al. 2007).

3.2.5.3.9. Identification of *Pseudomonas* strains using 16S-23S ITS specific primer

This PCR amplification was performed to identify specifically the fluorescent pseudomonads among the strains identified as *Pseudomonas* sp. PCR amplification was performed on a thermal cycler (Applied Biosystems GeneAmp PCR 2400) with a final reaction volume of 25µl containing 2.5µl of 10X Taq DNA polymerase buffer with 2.5 mM $MgCl_2$, 2mM dNTP mix, 2µM primer (each forward and reverse), 50 ng template DNA and 3U of Taq polymerase. PCR was performed under the following conditions: initial denaturation at 94°C for 5min, followed by 40 cycles at 92°C for 4 min,

28°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. Primers used are tabulated in Table (6).

3.2.5.3.10. Phylogenetic analysis of *Pseudomonas* isolates

The 16S rDNA sequences of the seven *Pseudomonas* isolates were compared with available 16S rDNA sequences of fifty other *Pseudomonas* strains in GenBank databases using the BLAST search facility at the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences of the bacterial strains having similarity range from 96% to 100% with the target sequence were used for sequence alignment. The phylogenetic trees were constructed with the neighbor-joining method by using MEGA 4.0 (Tamura et al., 2007). Confidence in the tree topology was determined by bootstrap analysis using 1000 re-samplings of the sequences (Felsenstein, 1985).

3.3. RESULTS

3.3.1. Isolation of bacteria from rhizosphere soil

Rhizosphere soil samples were collected following random sampling patterns from non-uniformly distributed locations of sub-Himalayan West Bengal including districts of Darjeeling and Jalpaiguri. The source plant was selected depending on the local vegetation and/or cultivation. Serial dilution of the soil samples and spreading onto soil extract agar led to the isolation of altogether 208 bacterial colonies (Fig. 6, 7). The serial dilution plating on CAS agar plate produced orange halo around single colonies (Fig.8a,b). The isolates were coded according to the source soil and place from where it was collected (Table 7).

3.3.2. Screening for siderophore production

All isolates were screened in bluish-green coloured CAS agar medium for selection of siderophores producing strains. Of 208 bacterial isolates, 68 strains (32.7%) were found to produce siderophore. Some isolates failed to grow on CAS agar (Fig. 8c,e). A typical orange halo around colonies indicated siderophore production (Figure 8c,d,e,f). Some strains showed variations in colour of the halo (light yellow to pinkish purple). The intensity

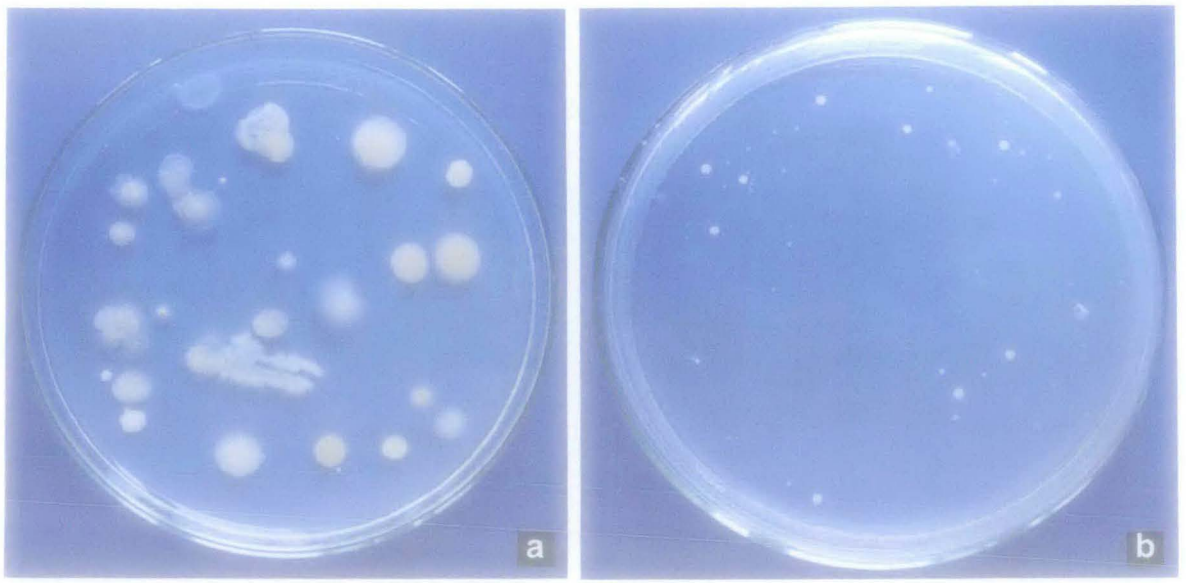


Fig.6: Isolation of bacteria from soil: serially diluted sample spread onto soil extract agar produced isolated bacterial colonies; (a) sample at 10^{-3} dilution and (b) sample at 10^{-5} dilution.

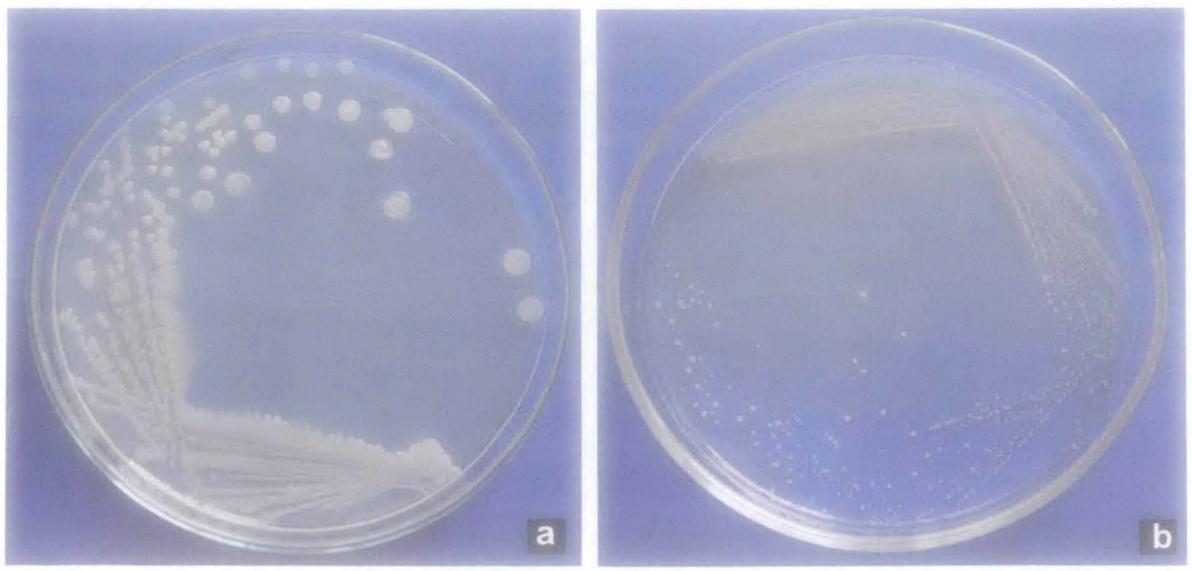


Fig.7: Isolation of pure culture of bacteria by streaking on nutrient agar plates: (a) Strain MB02; (b) Strain BB05.

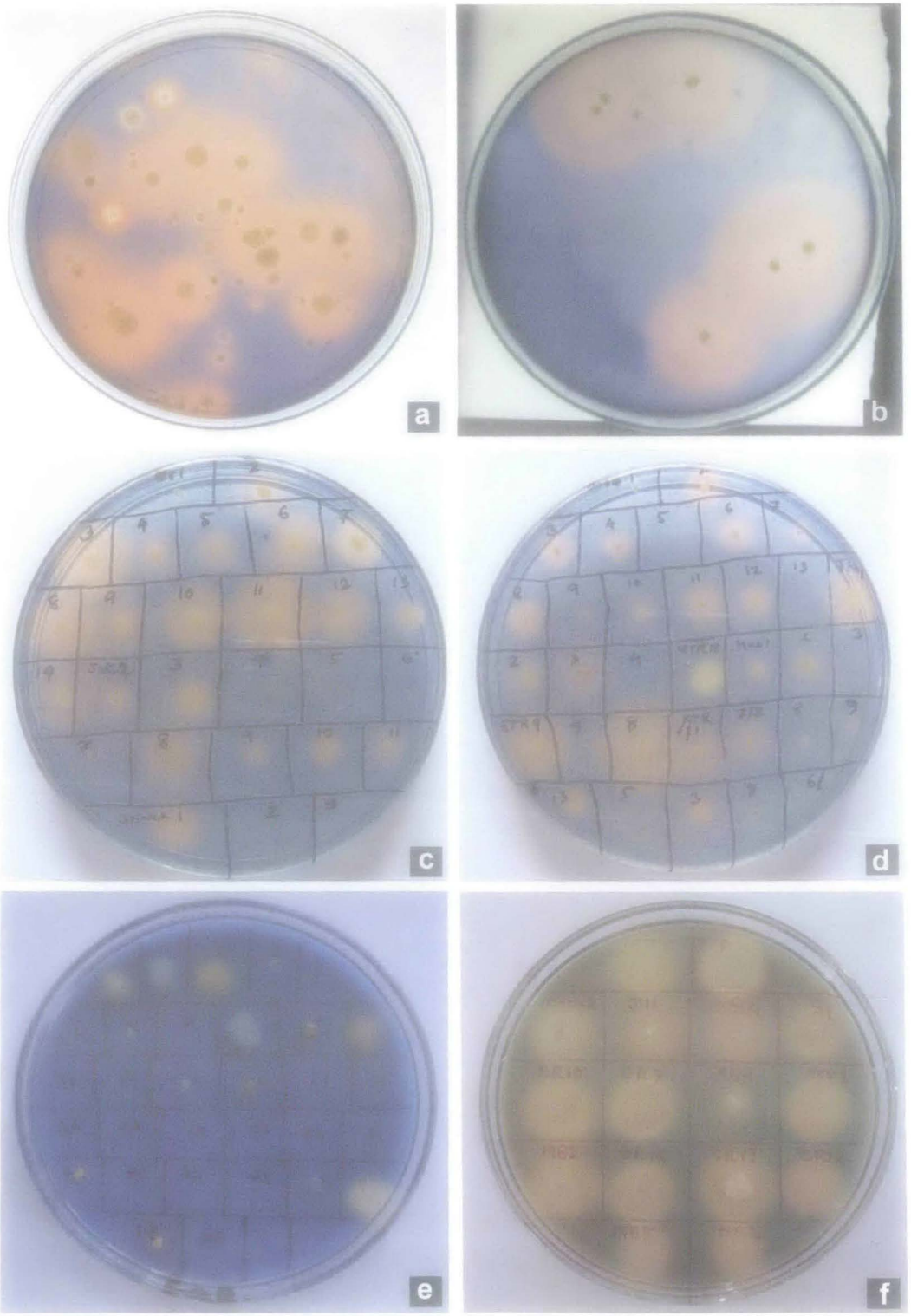


Fig.8: Screening of bacteria in CAS agar plates, the orange halo around bacterial colony indicating siderophore production, (a), (b) serially diluted soil sample spread onto CAS plate, (c),(d),(e),(f) isolated bacteria point inoculated in CAS plates to identify siderophore producing strains.

or size of the halo also differed. The 68 isolates exhibiting robust siderophore production were selected for further studies.

Table 7: List of bacterial isolates from different rhizospheric soil

Source of rhizosphere soil (crop)	Code assigned	Total Number of isolates	Siderophore producing isolates
Maize	MB1-18	18	MB01, MB02, MB03, MB05, MB06, MB07, MB12
Mango	MD1-12	12	MD01, MD02, MD03, MD04, MD05, MD06
Lemon	LC1-9	09	LC02, LC06
Brinjal	BB1-22	22	BB1-BB7
Jute	JL1-8	08	JL04, JL05, JL08, JL10, JL11, JL16, JL17
Potato	AS1-28	28	AS01-AS12
Cassia	CB1-14	14	CB01, CB02, CB06, CB14
Tea	KT1-18	18	KT01-05
Neem	NE1-25	25	NE01, NE06, NE07
Radish	CR1-14	14	CR01, CR02, CR04, CR07-CR14
Wheat	WB1-24	24	WB02
Grass	NG1-16	16	NG04, NG05, NG07
Total number of isolates:		208	68

3.3.3. *In vitro* antagonism of bacterial isolates

All the 68 siderophore producing isolates were screened for the presence of antagonistic activity against seven important phytopathogens. Results showed that 20 bacterial strains among the 68 isolates were capable of inhibiting the mycelial growth of *Fusarium equiseti* (Fig. 9), *Lasiodiplodia theobromae* (Fig. 10), *Rhizoctonia solani* (Fig. 11), *Colletotrichum gloeosporioides* (Fig. 12), *Alternaria alternata* (Fig. 13), *Fusarium solani* (Fig. 14) and *Fusarium graminearum* (Fig. 15) along the bacterial line of streaking. Several isolates besides the selected 20 also showed antagonism

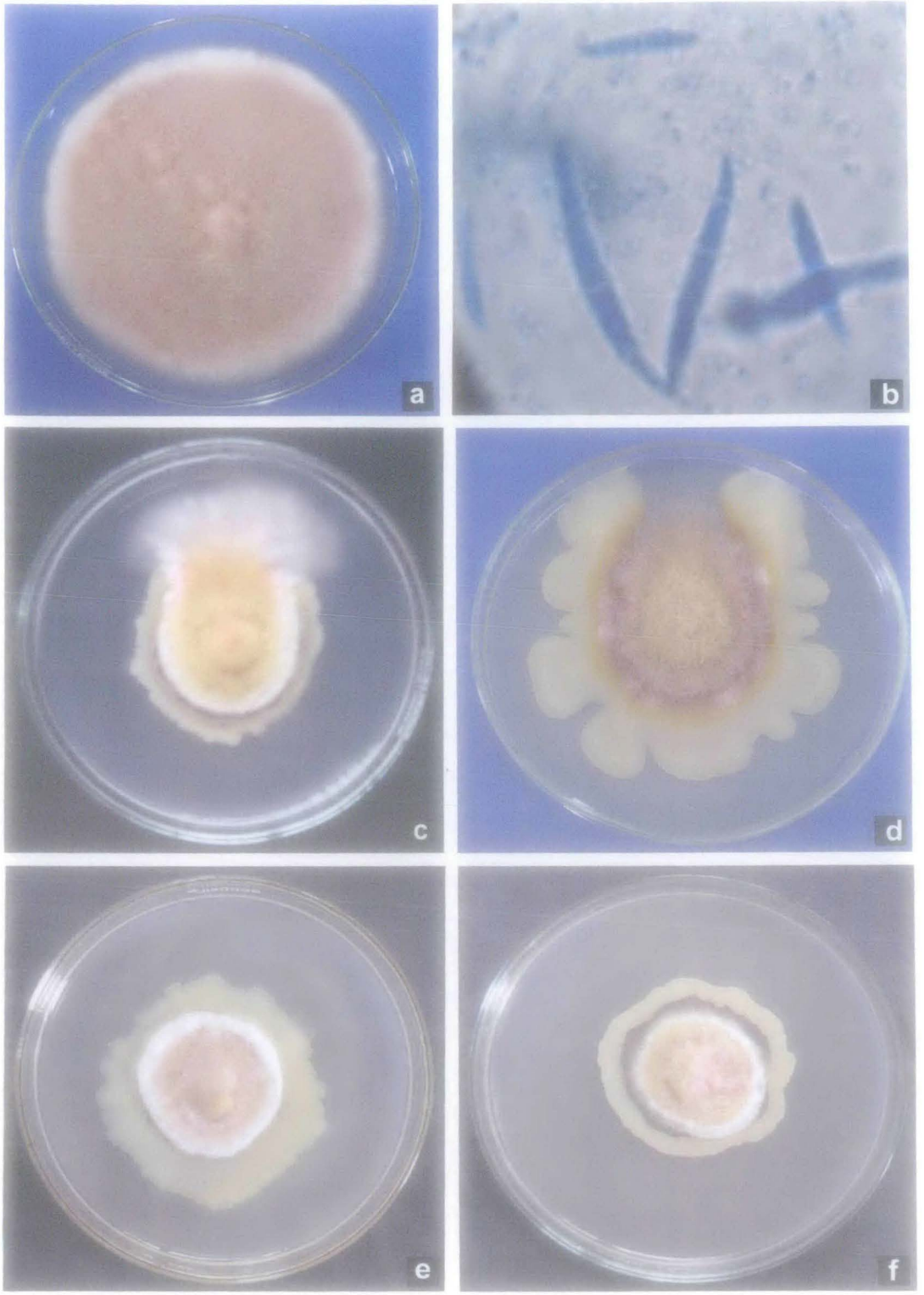


Fig.9: Inhibitory effect of selected bacterial isolate on the growth of *Fusarium equiseti* evident by the dual culture test in PDA plates:

(a) Growth of *F. equiseti* on PDA plate, (b) microscopic observation of spores of *F. equiseti*;

Restricted fungal growth observed during initial screening in presence of strains (c) AS01 and (d) MD01;

Strong antifungal activity exhibited by strains (e) AS01 and (f) AS04.

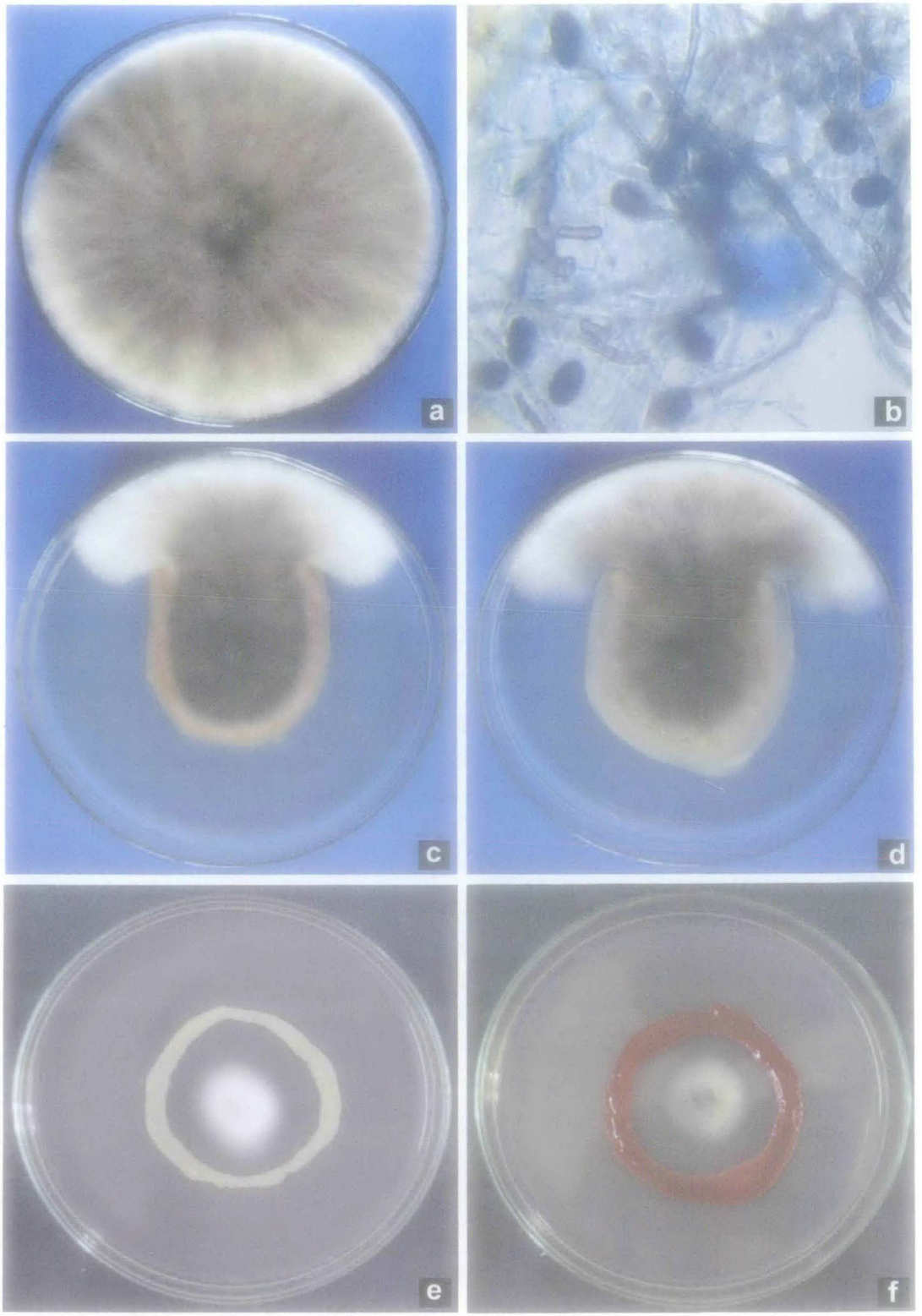


Fig.10: Inhibitory effect of selected bacterial isolate on the growth of *Lasiodiplodia theobromae* evident by the dual culture test in PDA plates:

(a) Growth of *L.theobromae* on PDA plate, (b) microscopic observation of spores of *L.theobromae*;

Restricted fungal growth observed during initial screening in presence of strains (c) MB05 and (d) AS01;

Strong antifungal activity exhibited by strains (e) AS04 and (f) CB02

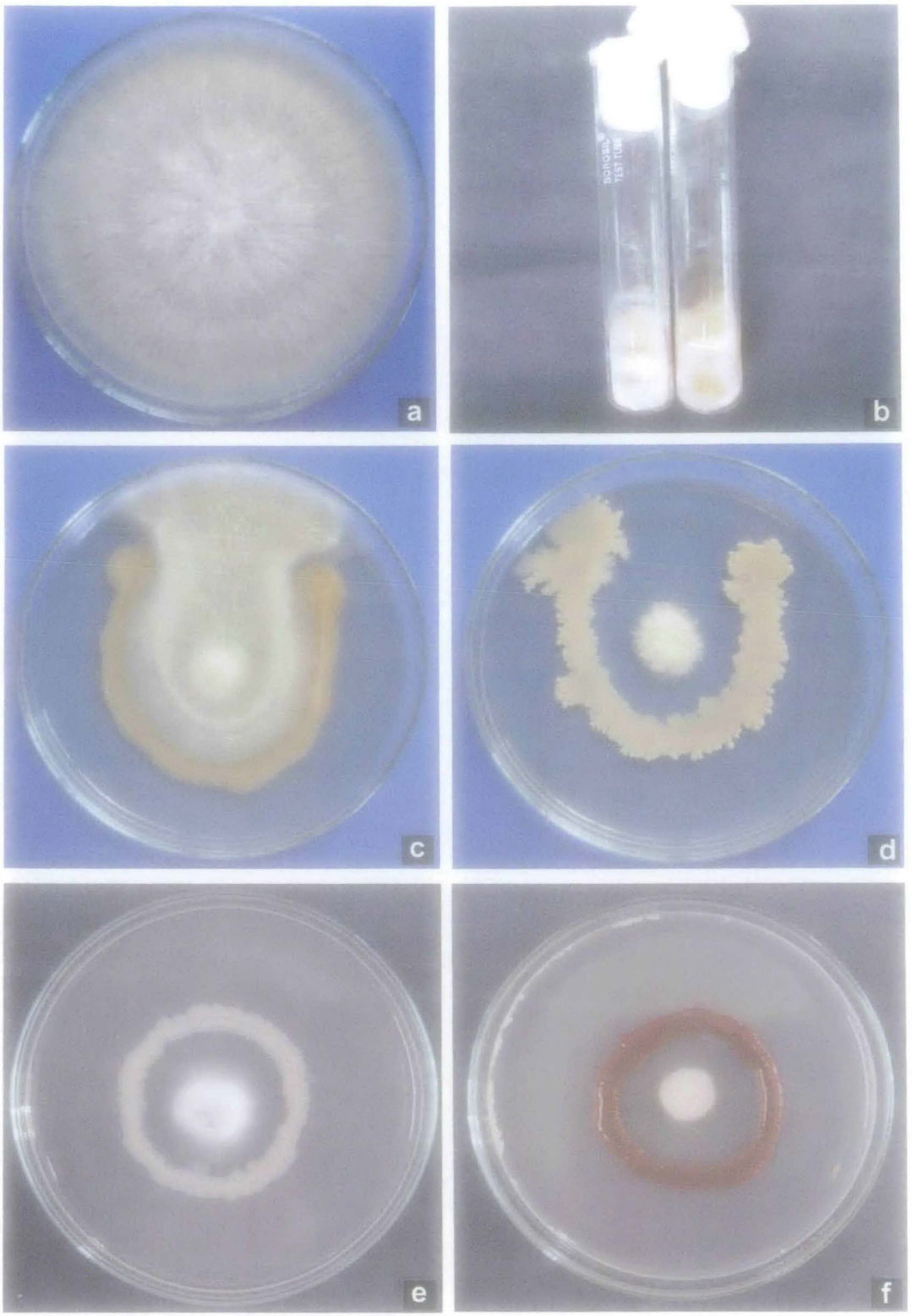


Fig.11: Inhibitory effect of selected bacterial isolate on the growth of *Rhizoctonia solani* evident by the dual culture test in PDA plates:

(a) Growth of *R.solani* on PDA plate, (b) slant culture showing sclerotia of *R.solani*;

Restricted fungal growth observed during initial screening in presence of strains (c) CR07 and (d) KT05;

Strong antifungal activity exhibited by strains (e) AS04 and (f) CB02.

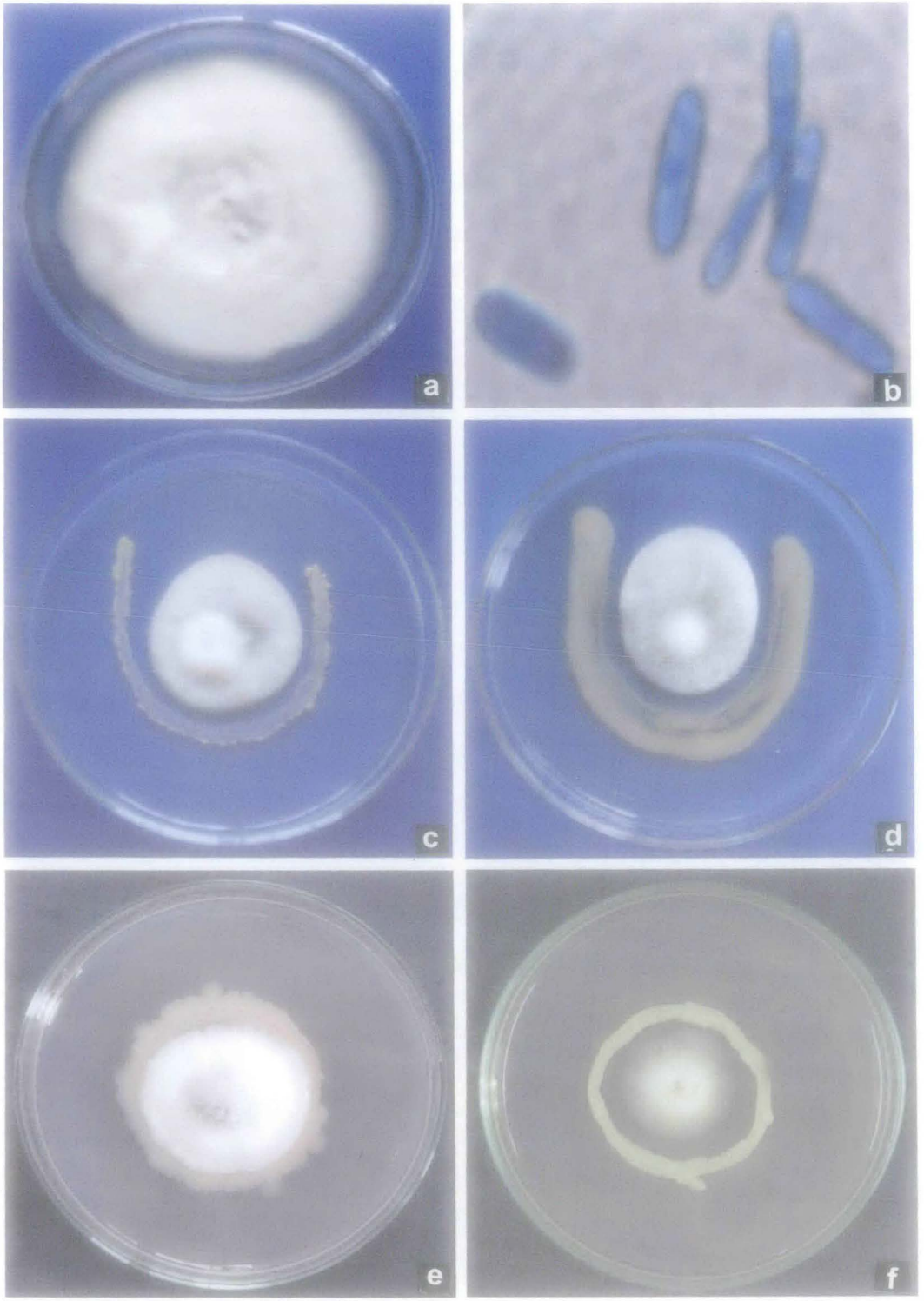


Fig.12: Inhibitory effect of selected bacterial isolate on the growth of *Collectotrichum gloeosporioides* evident by the dual culture test in PDA plates:

(a) Growth of *C. gloeosporioides* on PDA plate, (b) microscopic observation of spores of *C.gloeosporioides;*

Restricted fungal growth observed during initial screening in presence of strains (c) AS01 and (d) CR12;

Strong antifungal activity exhibited by strains (e) AS04 and (f) BB07.

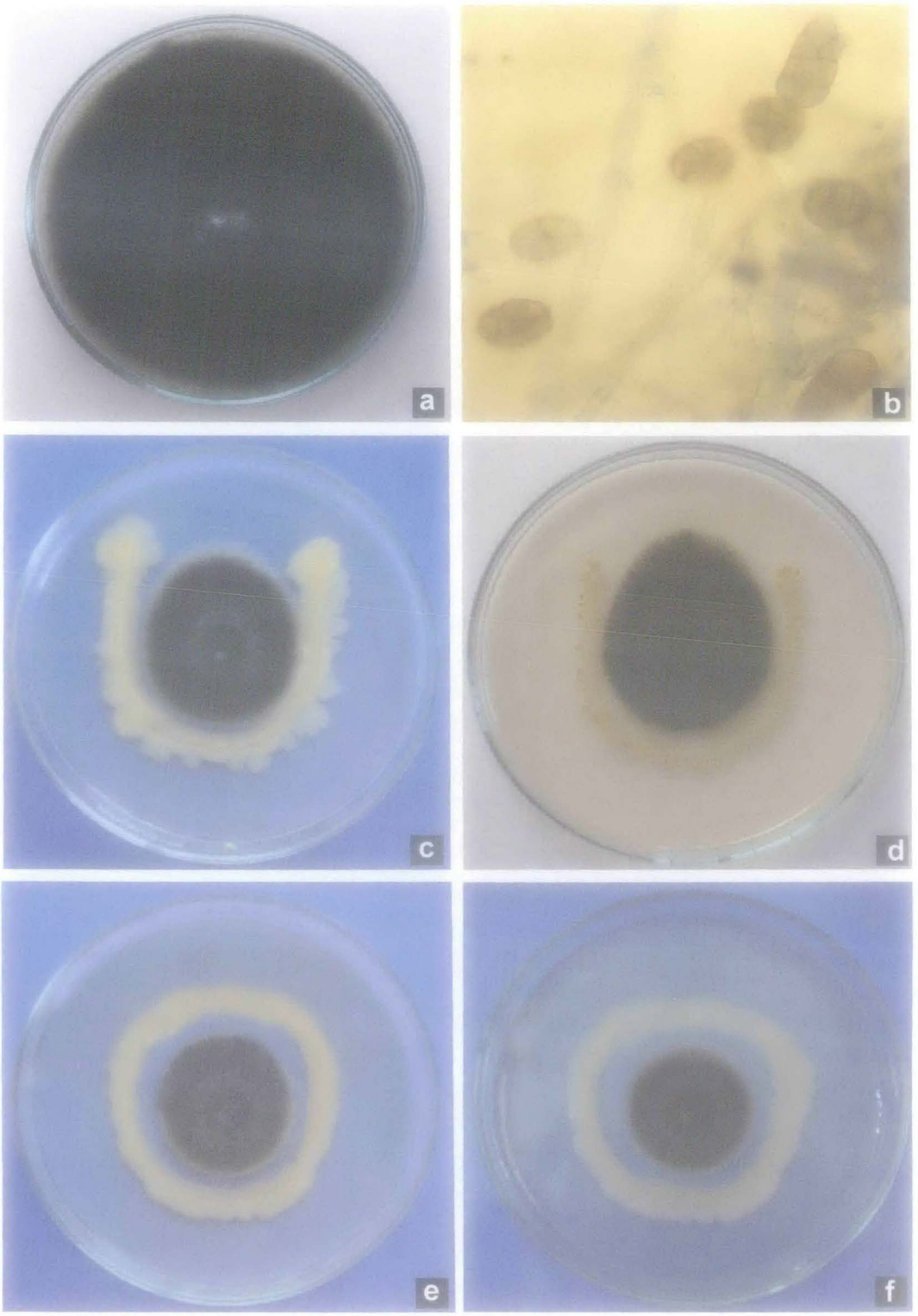


Fig.13: Inhibitory effect of selected bacterial isolate on the growth of *Alternaria alternata* evident by the dual culture test in PDA plates:

(a) Growth of *A.alternata* on PDA plate, (b) microscopic observation of lightly stained spores of *A.alternata*;

Restricted fungal growth observed during initial screening in presence of strains (c) MB02 and (d) MB01;

Strong antifungal activity exhibited by strains (e) CR10 and (f) NG07.

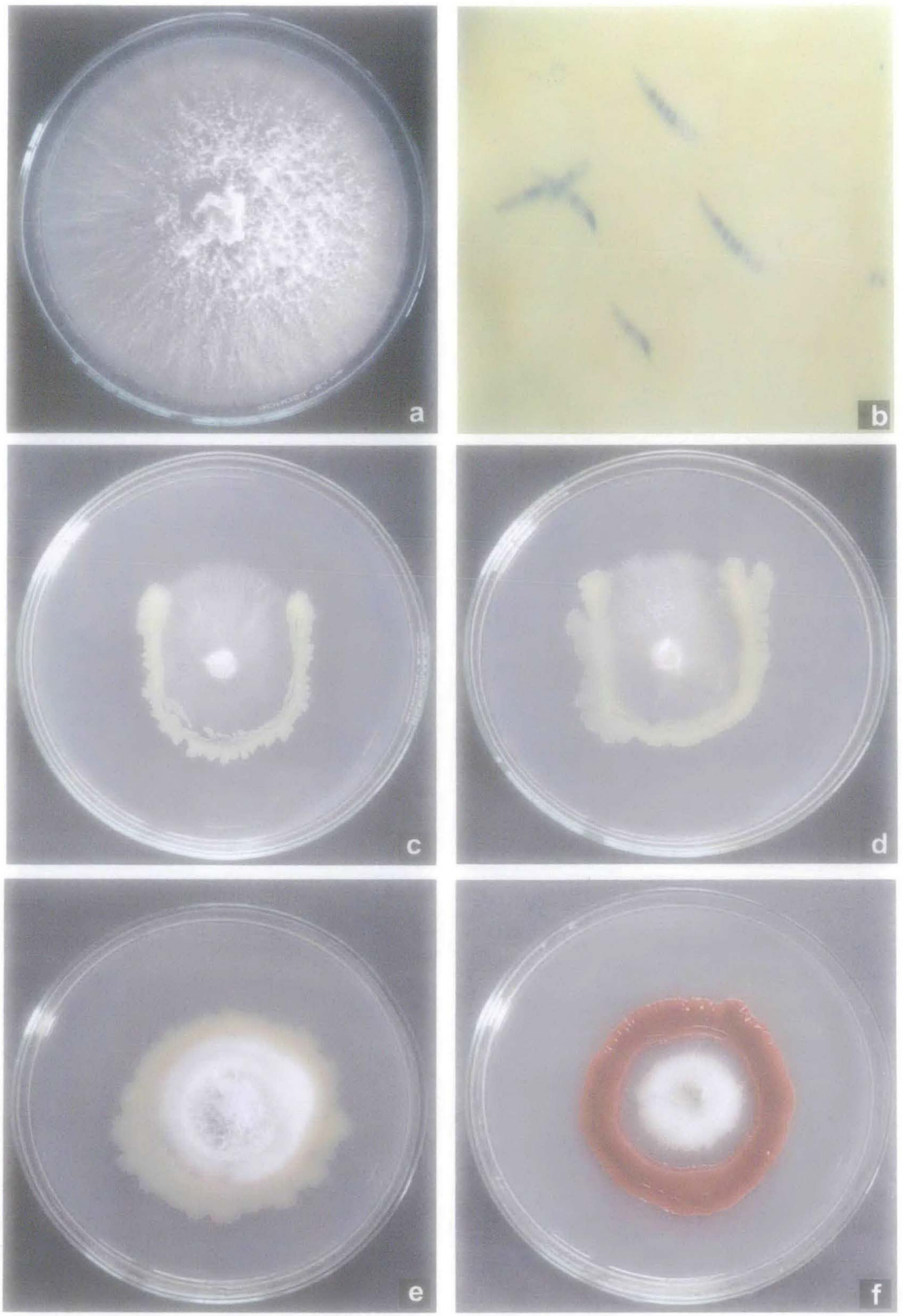


Fig.14: Inhibitory effect of selected bacterial isolate on the growth of *Fusarium solani* evident by the dual culture test in PDA plates:

(a) Growth of *F. solani* on PDA plate, (b) microscopic observation of conidia of *F. solani*;

Restricted fungal growth observed during initial screening in presence of strains (c) AS01 and (d) BB05;

Antifungal activity exhibited by strains (e) AS04 and (f) CB02.

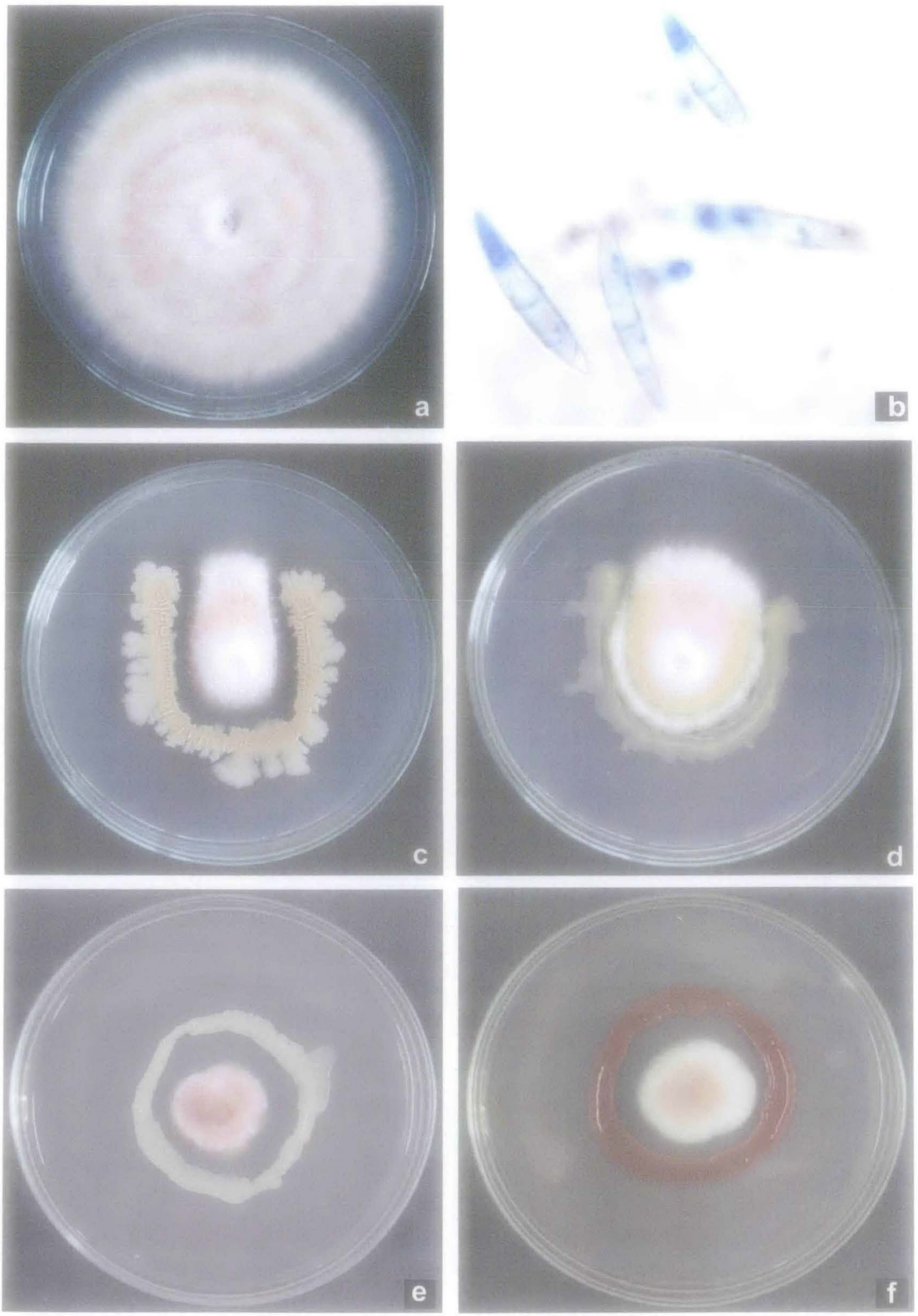


Fig.15: Inhibitory effect of selected bacterial isolate on the growth of *Fusarium graminearum* evident by the dual culture test in PDA plates:

(a) Growth of *F.graminearum* on PDA plate, (b) microscopic observation of conidia of *F.graminearum;*

Restricted fungal growth observed during initial screening in presence of strains (c) NG07 and (d) MD01;

Antifungal activity exhibited by strains (e) AS01 and (f) CB02.

in dual culture tests but not against all the seven pathogens and were therefore not used for further study.

The qualitative study of antagonism was followed by quantitative evaluation of antagonistic potential. The diameter of mycelial growth in dual culture plate was compared with the control plates (plates with only test fungus). Percent inhibition of growth of different pathogens was calculated (Table 8). The results of all dual culture tests showed that isolates AS01 and AS04 were the best antagonists that inhibited the growth of all test pathogens to more than 64%. Other strains showing an overall moderate antifungal activity were BB05, MD01, CR12 and KT05. Highest antagonistic activity was exhibited by BB05 (83%) against *A. alternata*. However, it was not equally effective against all pathogens as it showed only 53% activity against *C. gloeosporioides* and 55% activity against *R. solani*. The antagonistic potential of some strains showed much variation, for example, MD01 showed 82% activity against *R. solani* but only 20% activity against *L. Theobromae*. Similarly, CR07 showed 75% activity against *A. alternata* but only 27% activity against *F. graminearum*. AS01 showed more than 70% activity against four pathogens while AS04, the most promising strain, showed the same against six pathogens out of the seven tested. Considering their overall performance the strains AS01 and AS04 were selected for *in vivo* studies.

3.3.4. Characterization of antagonistic bacterial isolates

Altogether twenty bacterial isolates showing potent antagonistic activity against all selected fungal pathogens were subject to characterization in a polyphasic approach. The approach included morphological, biochemical and phylogenetic studies.

3.3.4.1. Morphological characterization

Morphological studies are summarized in table 9a and 9b. A wide variety of various morphological features were recorded by the strains. All isolates were found to be straight rods but their sizes varied from being long rods (MB02) to coccobacilli (CR12). All strains were motile except JL11.

Table 8: *In vitro* study of antagonistic activity of bacterial isolates against seven fungal pathogens

Bacterial strains	% Inhibition of Growth of Fungal Pathogens*						
	Fusarium equiseti	***Lasiodiplodia theobromae***	***Rhizoctonia solani***	***Colletotrichum gloeosporioides***	***Alternaria alternata***	***Fusarium solani***	***Fusarium graminearum***
BB05	66.6±1.1	61.1±0.7	55.3±0.3	53.2±0.7	83.1±0.4	75.5±0.6	79.8±0.6
BB07	64.4±0.3	63.2±0.6	56.7±0.5	49.8±0.5	64.2±0.3	56.7±0.4	57.6±0.5
JL11	57.2±0.3	24.3±0.3	59.8±0.6	57.5±0.4	50.0±0.8	42.4±0.7	42.3±0.3
MD01	72.2±0.2	19.9±0.5	82.1±0.5	67.7±0.7	63.1±0.5	70.9±0.6	72.2±0.2
CB02	57.8±0.4	62.3±0.4	60.9±0.8	68.7±0.5	57.6±0.4	65.6±0.5	56.7±0.5
AS01	64.5±0.3	70.0±0.4	75.4±0.8	71.0±0.3	65.4±0.5	68.7±0.5	72.3±0.6
AS04	74.5±0.5	73.5±0.5	77.6±0.5	73.2±0.2	64.3±0.2	73.4±0.7	72.1±0.5
CR04	49.0±0.6	34.6±0.3	57.6±0.5	47.7±0.5	55.4±0.6	49.8±0.2	45.6±0.4
CR07	53.5±0.7	57.9±0.5	58.7±0.4	47.5±0.6	75.4±0.4	57.6±0.8	27.8±0.5
CR10	52.1±0.2	22.1±0.6	53.2±0.4	56.4±0.3	31.2±0.2	37.6±0.5	55.7±0.4
CR12	62.3±0.2	59.8±0.2	59.8±0.6	65.3±0.4	70.9±0.3	62.2±0.4	62.1±0.6
CR13	62.3±0.4	24.7±0.8	57.7±0.4	70.9±0.6	50.9±0.3	46.7±0.9	54.4±0.6
CR14	55.6±0.7	45.4±0.6	60.9±0.2	54.7±0.4	63.1±0.7	61.2±0.4	41.2±0.3
MB01	60.9±0.6	58.7±0.1	31.1±0.4	46.5±0.5	57.9±0.8	63.4±0.4	45.5±0.7
MB02	59.8±0.8	57.7±0.7	22.0±0.4	42.1±0.4	64.2±0.7	55.7±0.6	60.1±0.6
MB05	68.9±1.0	61.9±0.4	46.7±0.6	38.3±1.2	63.9±0.5	67.8±0.7	66.8±0.6
NG04	56.6±0.5	50.7±0.6	63.1±0.5	42.1±0.5	55.4±0.8	54.4±0.3	52.1±0.6
NG05	51.0±0.6	32.5±0.7	32.1±0.6	47.6±0.5	61.0±0.6	24.2±0.8	66.6±0.7
NG07	53.1±0.4	42.0±0.6	59.9±0.5	45.4±0.8	54.3±0.6	49.1±0.9	49.8±0.7
KT05	57.8±0.9	53.3±0.5	79.8±0.9	59.7±0.8	63.5±0.5	66.5±0.8	56.8±0.7

'*': Mycelial diameter was measured when the fungal growth in the control plate reached the edge of the 90 mm diameter petriplate; '±': Standard error

Table 9a: Morphological and cultural characteristics of antagonistic bacterial isolates

Bacterial strains	Characteristics									
	Shape	Size(length X breadth) (µm)	Endospore formation[a]	Motility	Colony character					
					Growth	Colour	Surface	elevation	Pigment[b]	In broth
BB05	Rod	1.4X0.7	-	motile	moderate	Off white	Smooth, shiny	Flat	Yellowish green	Turbid with pellicle
BB07	rod	1.6X0.8	-	motile	moderate	Off white	Smooth, shiny	Flat	Yellowish green	Turbid with pellicle
JL11	Long rod	1.5X0.4	-	Non-motile	moderate	white	Smooth, shiny	Low convex	-	Turbid
MD01	rod	1.6X0.7	-	motile	moderate	white	Smooth, shiny	Flat	fluorescent pigment	Turbid with pellicle
CB02	rod	0.9X0.6	-	motile	moderate	red	Opaque, shiny	Low convex	Red pigment	Reddish turbid
AS01	Short rod	1.3X0.8	-	motile	moderate	Off white	Smooth, shiny	Flat	Yellowish green	Turbid with pellicle
AS04	Short rod	1.2X0.7	-	motile	moderate	Off white	Smooth, shiny	Flat	Yellowish green	Turbid with pellicle
CR04	rod	1.3X0.6	-	motile	moderate	white	Smooth, Irregular edge	Flat	-	Turbid
CR07	rod	1.0X0.3	+	motile	moderate	white	entire	Flat	-	Turbid with pellicle
CR10	rod	1.3X0.8	+	motile	Fast growing	white	Dull, opaque	Flat	-	Turbid with pellicle

[a]: '+' indicates endospore formation, '-' indicates no visible endospore; [b]: '-' indicates absence of pigments

Table 9b: Morphological and cultural characteristics of antagonistic bacterial isolates

Bacterial strains	Characteristics									
	Shape	Size(μm)	Endospore formation[a]	Motility	Colony character					
					Growth	Colour	Surface	elevation	Pigment[b]	In broth
CR12	Short rod	0.7X0.6	-	motile	moderate	White	Smooth	low convex	-	Turbid
CR13	rod	1.6X0.6	-	motile	moderate	White	Smooth	Flat	-	Turbid
CR14	rod	3.1X1.0	+	motile	moderate	White	Smooth, translucent	Raised	-	Turbid with pellicle
MB01	Short rod	1.5X0.8	-	motile	moderate	white	smooth	Flat	Yellowish green	Turbid with pellicle
MB02	Long rod	2.1X0.8	+	motile	Fast growing	White	Dull, opaque	Flat	-	Turbid with pellicle
MB05	Rod	3.2X1.1	-	motile	moderate	White, translucent	smooth	Low convex	-	Turbid
NG04	rod	1.6X0.7	-	motile	moderate	brownish	wrinkled	Flat	-	Turbid with pellicle
NG05	Rod	2.9X1.0	-	motile	moderate	White, translucent	smooth	Low convex	-	Turbid
NG07	rod	2.9X0.9	+	motile	moderate	White	Smooth, translucent	Raised	-	Turbid with pellicle
KT05	Long rod	2.1X0.8	+	motile	Fast growing	White	Dull, opaque	Flat	-	Turbid with pellicle

[a]: '+' indicates endospore formation, '-' indicates no visible endospore; [b]: '-' indicates absence of pigments

The isolates CR07, CR10, CR14, MB02, NG07 and KT05 were found to produce spores. The strain CB02 produced a deep red pigment when grown on NA. Several isolates (BB05, BB07, AS01, AS04, MD01 and MB01) produced yellowish green fluorescent pigment in *Pseudomonas* agar plates (for Fluorescin) (Fig. 18c).

3.3.4.2. Biochemical and physiological characterization

The results of biochemical tests performed with twenty bacterial antagonists are tabulated in tables 10a, 10b and 10c. The isolates CR07, CR10, CR14, MB02, NG07 and KT05 were Gram positive strains while rest were Gram negative. Indole production test was positive for two strains, JL11 and NG05 (Fig 16c); other 18 isolates showed negative result for this test. Ten strains were oxidase positive while all strains except CR12 were catalase positive. Eleven strains (CR04, CR07, CR10, CR12, CR13, CB02, JL11, MB02, MB05, NG05, KT05) were found to be positive for ONPG test (Fig. 16a). Isolates CB02, NG05 and JL11 recorded urease production while the rest tested negative (Fig.16b). Fourteen strains were found to be oxidative in OF test while six strains were fermentative (Fig. 18b). Sixteen strains were able to reduce nitrate (Fig. 18a) and three isolates (CR10, MB02 and KT05) were able to produce DNase. Gelatine was found to be liquefied by twelve strains (BB07, CR04, CR07, CR10, CR14, CB02, MD01, JL11, MB01, MB02, NG07 and KT05). Ten strains each were positive for MR and VP test, while six were positive for both. All isolates except CR12 were able to utilize citrate (Fig. 17b). Two strains (MB05 and NG05) were positive for H_2S production. All twenty isolates tested negative for the production of phenyl alanine deaminase. Two strains each were found to be positive for ornithine decarboxylase (CB02 and CR04) and lysine decarboxylase reactions (CB02 and JL11). A wide variation was found in acid production tests with various sugar substrates. All isolates except CR12 were found to produce acid from glucose. CR12 did not produce acid from any of the sugars tested. Acid was produced from maltose by all isolates except CR12 and JL11 (Fig. 17a). On comparison with descriptions depicted in Bergey's Manual, the identities of the isolates upto the genus level was apparent.

Table 10a: Biochemical characters of the antagonistic bacterial isolates

Biochemical Characteristics	Bacterial Antagonists[a]						
	BB05	BB07	AS01	AS04	CR04	CR07	CR10
Gram character	-	-	-	-	-	+	+
Indole production	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	+	+
VP test	-	-	-	-	+	+	+
Citrate utilization	+	+	+	+	+	+	+
Acid in TSIA	+	+	+	+	-	+	+
H_2S in TSIA	-	-	-	-	-	-	-
Gelatin liquefaction	-	+	-	-	+	+	+
Oxidase	+	+	+	+	-	-	-
Catalase	+	+	+	+	+	+	+
O-F	O	O	O	O	F	O	O
ONPG	-	-	-	-	+	+	+
Urease	-	-	-	-	-	-	-
Nitrate reduction	-	+	-	-	+	+	+
DNase production	-	-	-	-	-	-	+
Phenylalanine deaminase	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	+	-	-
Lysine decarboxylase	-	-	-	-	-	-	-
Sugar utilization:							
D-Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	+	+
D-Sorbitol	+	+	+	+	+	-	-
m-Inositol	+	+	-	-	-	+	+
D-Mannitol	-	+	-	+	+	-	+
Sucrose	+	+	+	+	+	+	+
Raffinose	-	-	-	-	+	+	+
L-Rhamnose	-	-	-	-	+	-	+
Trehalose	-	-	-	-	+	-	-
L-Arabinose	+	+	+	+	+	-	+
D-Xylose	-	+	+	+	+	-	+
Adonitol	-	-	-	-	+	-	-

[a] F: Fermentative; O: Oxidative; '+': positive; '-': negative.

Table 10b: Biochemical characters of the antagonistic bacterial isolates

Biochemical Characteristics	Bacterial Antagonists[a]						
	CR12	CR13	CR14	CB02	MD01	JL11	MB01
Gram character	-	-	+	-	-	-	-
Indole production	-	-	-	-	-	+	-
Methyl Red	+	-	+	-	-	-	-
VP test	-	+	+	+	-	+	-
Citrate utilization	-	+	+	+	+	+	+
Acid in TSIA	-	-	+	+	+	+	+
H_2S in TSIA	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	+	+	+	+	+
Oxidase	+	-	-	+	+	-	+
Catalase	-	+	+	+	+	+	+
O-F	O	F	O	F	O	F	O
ONPG	+	+	-	+	-	+	-
Urease	-	-	-	+	-	+	-
Nitrate reduction	-	+	+	+	+	+	+
DNase production	-	-	-	-	-	-	-
Phenylalanine deaminase	-	-	-	-	-	-	-
Ornithine decarboxylase	-	+	-	+	-	-	-
Lysine decarboxylase	-	-	-	+	-	+	-
Sugar utilization:							
D-Glucose	-	+	+	+	+	+	+
Maltose	-	+	+	+	+	-	+
Lactose	-	-	-	-	-	+	-
D-Sorbitol	-	+	-	+	+	+	+
m-Inositol	-	-	+	+	+	-	+
D-Mannitol	-	+	-	+	-	+	-
Sucrose	-	+	-	+	+	+	+
Raffinose	-	-	-	-	-	+	-
L-Rhamnose	-	-	-	-	-	+	-
Trehalose	-	+	-	+	-	-	-
L-Arabinose	-	+	-	-	+	+	+
D-xylose	-	+	-	-	-	-	-
Adonitol	-	-	+	-	+	+	+

[a] F: Fermentative; O: Oxidative; '+': positive; '-': negative.

Table 10c: Biochemical characters of the antagonistic bacterial isolates

Biochemical Characteristics	Bacterial Antagonists[a]					
	MB02	MB05	NG04	NG05	NG07	KT05
Gram character	+	-	-	-	+	+
Indole production	-	-	-	+	-	-
Methyl Red	+	+	-	+	+	+
VP test	+	-	+	-	+	+
Citrate utilization	+	+	+	+	+	+
Acid in TSIA	+	+	+	+	+	+
H_2S in TSIA	-	+	-	+	-	-
Gelatin liquefaction	+	-	-	-	+	+
Oxidase	-	-	+	-	-	-
Catalase	+	+	+	+	+	+
O-F	O	F	O	F	O	O
ONPG	+	+	-	+	-	+
Urease	-	-	-	+	-	-
Nitrate reduction	+	+	+	+	+	+
DNase production	+	-	-	-	-	+
Phenylalanine deaminase	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-
Sugar utilization:						
D-Glucose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Lactose	+	-	-	-	+	+
D-Sorbitol	-	+	-	+	-	-
m-Inositol	+	-	+	-	+	+
D-Mannitol	+	+	-	+	-	+
Sucrose	+	+	+	+	+	+
Raffinose	+	-	-	+	+	+
L-Rhamnose	+	+	-	+	-	+
Trehalose	-	+	-	+	-	-
L-Arabinose	+	+	-	+	-	+
D-Xylose	+	+	-	+	-	+
Adonitol	-	-	-	-	-	-

[a] F: Fermentative; O: Oxidative; '+': Positive; '-': Negative.

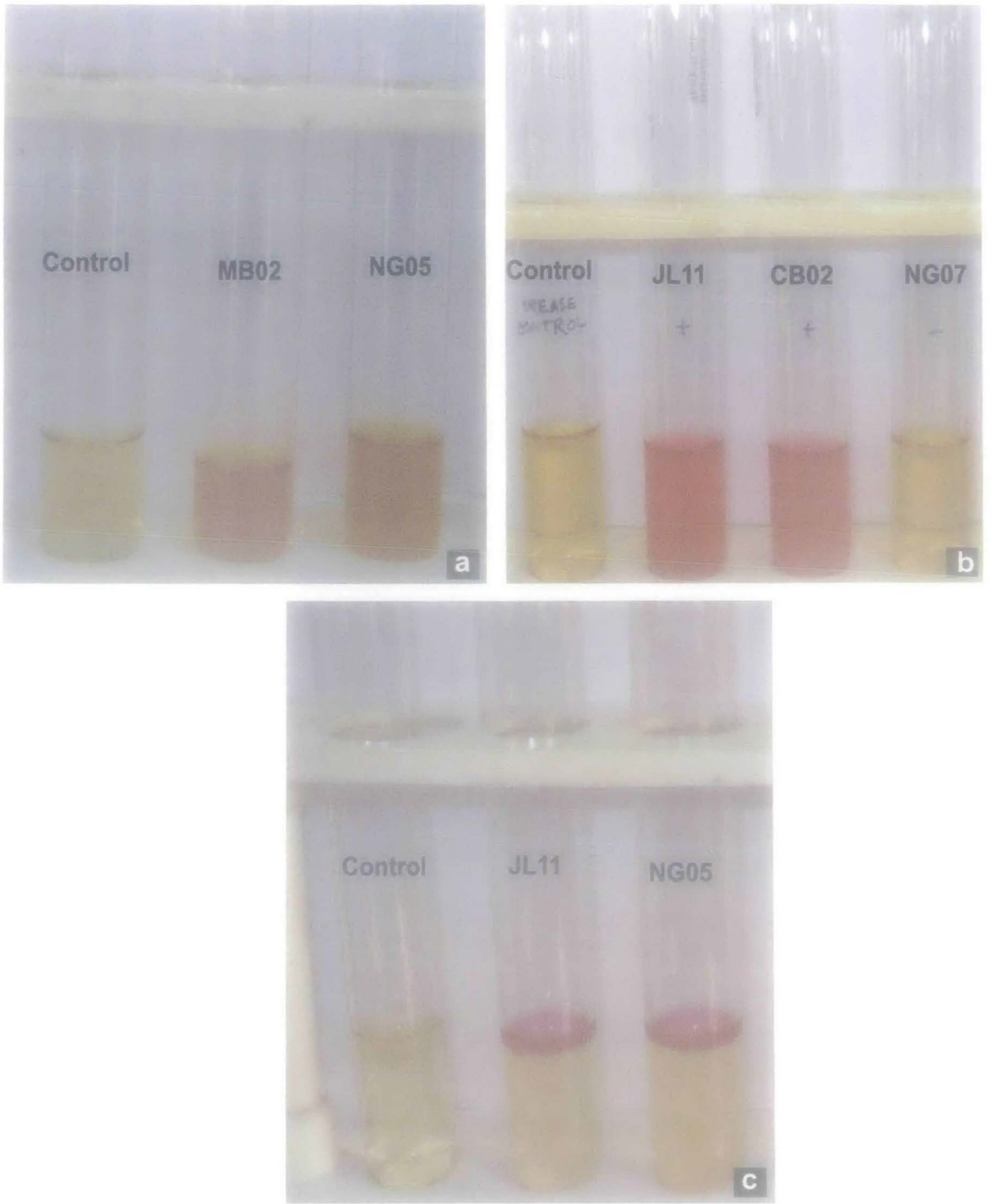


Fig.16: Biochemical characterization of selected bacterial isolates:
(a) ONPG utilization: Appearance of yellow colour shows positive test by strains MB02 and NG05 when compared to control;

(b) Urease production: Appearance of red colour indicates positive test by strains JL11 and CB02. Yellow colour shows negative test by strain NG07 when compared to control;

(c) Indole production: Formation of cherry red ring on top indicates positive test for strains JL11 and NG05 when compared to control.

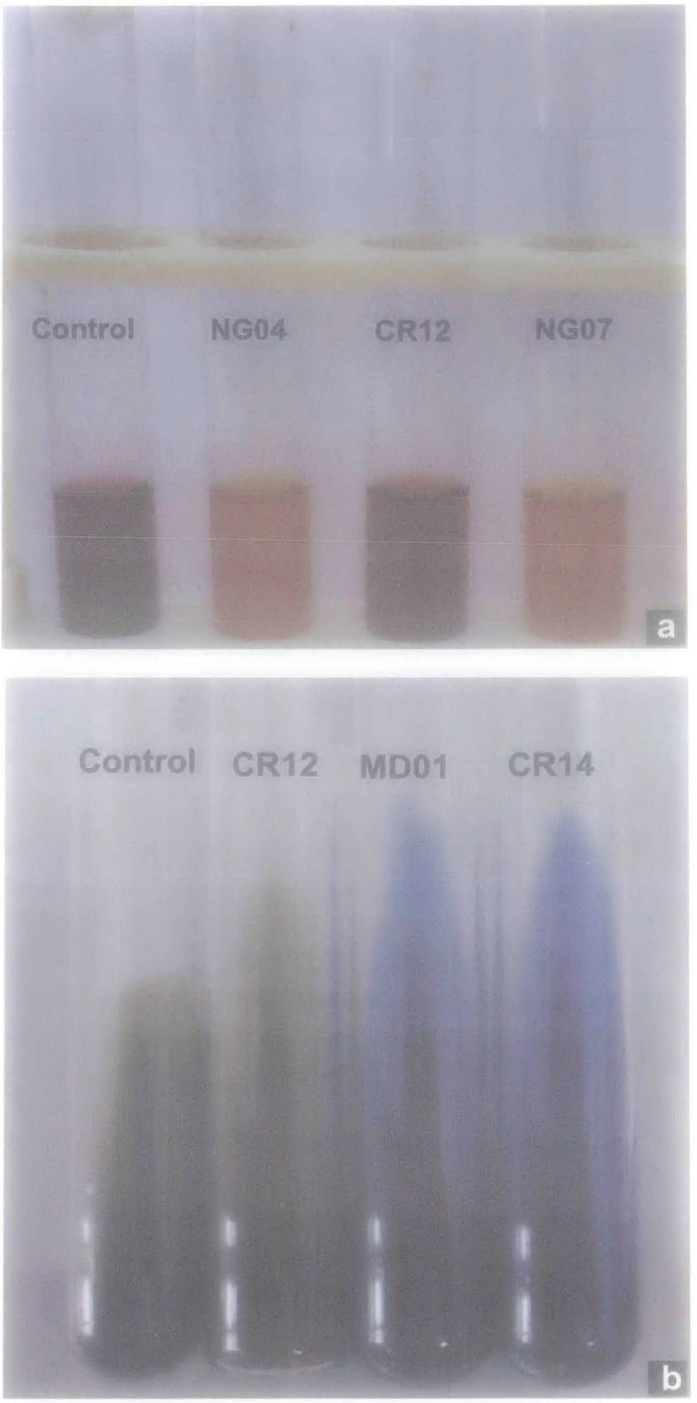


Fig.17: Biochemical characterization of selected bacterial isolates:

(a) Fermentation of maltose: Utilization of maltose is indicated by change in colour from red to yellow by strains NG04 and NG07. Negative test indicated by CR12 due to appearance of red colour when compared to control;

(b) Citrate utilization: Change in colour from green to blue indicates positive test by MD01 and CR14. Green colour showed negative test by CR12 when compared to control.

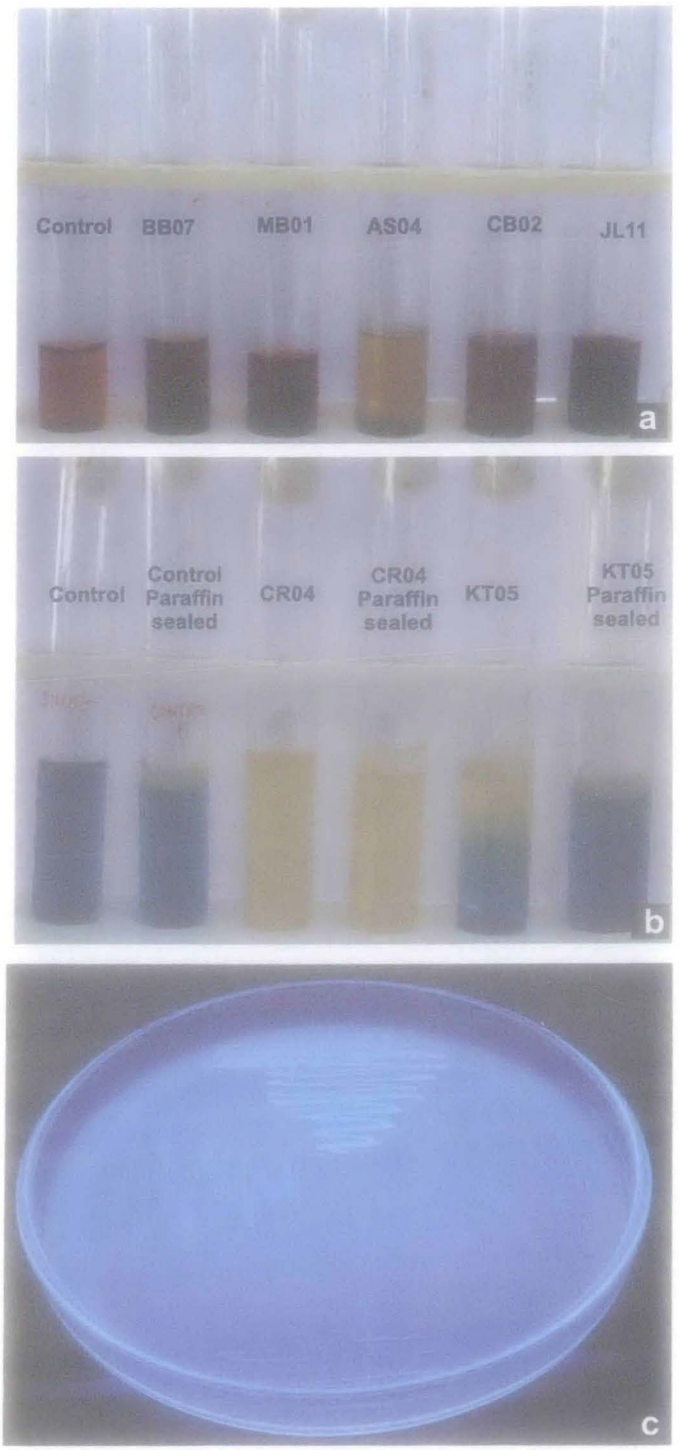


Fig.18: Biochemical characterization of selected bacterial isolates:

(a) Reduction of Nitrate: Deep red colour shows positive nitrate reduction test by strains BB07, MB01, CB02 and JL11 and yellow colour indicates negative result by AS04 when compared to control;

(b) O-F test: Fermentative metabolism shown by yellow colour in both open and paraffin-covered tubes by CR04 and yellow colour in only open tube but not in paraffin covered tube indicates oxidative metabolism by KT05 when compared to blue control tubes;

(c) Fluorescence in *Pseudomonas* agar under UV light by strain AS04.

3.3.4.3. Phylogenetic characterization

The extracted and purified (RNase treated) DNA run in agarose gels produced intense fluorescent bands when viewed in a UV-transilluminator (Fig. 19a). Amplification of the 16SrRNA gene followed by gel electrophoresis of the PCR product resulted in an amplicon of approximate band size 1500bp for each isolate (Fig. 19b). Cloning of the PCR amplification product in pGEM-T easy vector and subsequent transformation of *E. coli* JM109 led to the successful identification of transformed cells via blue white screening (Fig. 19d). All the 16S rRNA gene sequences obtained from the sequencing service provider were deposited in the NCBI GenBank and Accession Numbers were provided (EU661864, EU661866, JX535385, JX960418, KC109315-28, KC117153-4) (Table 11). Alignment of the obtained 16S rDNA sequences with that of the strains from the GenBank database resulted in 98 to 100% similarity matches.

3.3.5. Identification of the antagonistic isolates

The results of molecular analyses were consistent with the biochemical and physiological traits of the isolates as reported in Bergey's Manual of Systematic Bacteriology (1986, 2005). The findings from biochemical characterization studies as well as BLAST searches led to the final identification of individual isolates. The identity of each isolate is listed in Table 11. It was found that out of twenty bacterial isolates, seven belonged to the genus *Pseudomonas*; two strains (AS01, AS04) were *P. putida* and one isolate, BB07, was identified as *P. fluorescens*. The species could not be ascertained for the rest of the pseudomonads (BB05, MD01, NG04 and MB01); hence they were designated as *Pseudomonas* sp. Six isolates were identified as *Bacillus* strains of which one isolate, MB02, was identified as *B. subtilis*. Other five isolates (KT05, CR10, CR07, CR14 and NG07) were identified as *Bacillus* sp. Isolate CR12 was identified as *Alcaligenes faecalis*; and two isolates (MB05 and NG05) were identified as *Citrobacter freundii*. Two isolates were found to belong to the genus *Enterobacter* of which CR04 was identified as *Enterobacter cloacae* while CR13 was designated as

Enterobacter sp. One isolate each were identified *Klebsiella* sp. (JL11) and *Serratia* sp. (CB02).

Table 11: List of identitified antagonistic bacterial isolates and corresponding Accession Numbers

Bacterial strain code	Identified bacteria	GenBank Accession Number
BB05	*Pseudomonas* sp.	KC109321
BB07	*Pseudomonas fluorescens*	JX535385
JL11	*Klebsiella* sp.	KC109327
MD01	*Pseudomonas* sp.	KC109323
CB02	*Serratia* sp.	KC109325
AS01	*Pseudomonas putida*	EU661866
AS04	*Pseudomonas putida*	EU661864
CR04	*Enterobacter cloacae*	KC109315
CR07	*Bacillus* sp.	KC109320
CR10	*Bacillus* sp.	KC117154
CR12	*Alcaligenes faecalis*	KC109316
CR13	*Enterobacter* sp.	KC109317
CR14	*Bacillus* sp.	KC117153
MB01	*Pseudomonas* sp.	KC109322
MB02	*Bacillus subtilis*	JX960418
MB05	*Citrobacter freundii*	KC109318
NG04	*Pseudomonas* sp.	KC109324
NG05	*Citrobacter freundii*	KC109319
NG07	*Bacillus* sp.	KC109326
KT05	*Bacillus* sp.	KC109328

3.3.6. Amplification of fluorescent *Pseudomonas* specific gene

The 16S-23S rRNA intervening sequence (ITS) of the seven *Pseudomonas* isolate was amplified in order to further validate the identity of the *Pseudomonas* strains. The isolates AS01, AS04, BB05, BB07, MB01, MD01

and NG04 and the standard *Pseudomonas fluorescens* strain (NRRL B23932) showed 560bp product (Fig.19c). All these isolates were thus confirmed to be fluorescent pseudomonads. However, isolate NG04 which was also identified as *Pseudomonas* sp. did not produce the requisite band and was therefore not considered to be a fluorescent pseudomonad.

3.3.7. Phylogenetic analysis of the *Pseudomonas* isolates

The phylogenetic relationship among the *Pseudomonas* isolates was established based on their partial 16SrRNA gene sequences. The phylogenetic tree was constructed based on the percent difference in genetic relationships between the allied strains in the NCBI database. Phylogenetic analysis was conducted using MEGA version 4.0. The result of analysis expressing the genetic relations between the seven *Pseudomonas* isolates and 50 other *Pseudomonas* strains has been represented in Fig.20.

3.4. DISCUSSION

Soil-borne pathogens are often difficult to control and conventional methodologies such as crop rotation, breeding for resistant plant varieties and the application of pesticides are insufficient to control root diseases of important crop plants (Weller et al., 2002; Haas and Defago, 2005). After the observation of disease suppressive soils, scientists were attracted to the idea that microorganisms could be used as environment-friendly biocontrol agents for disease management in agriculture (Ownley and Windham, 2004). Considerable evidence has accumulated in the past few decades which indicate that plant associated microorganisms can act as natural antagonists and may be utilized to protect susceptible plants such that they remain almost free of infection despite ample exposure to virulent inoculum of soilborne pathogens (Weller et al., 2002).

Soil represents a highly heterogeneous environment for the microbiota inhabiting it; the different components of the soil provide myriads of different microhabitats. In soil, a wide range of factors affect microbial life such as the plant type, which is a major determinant of the structure of

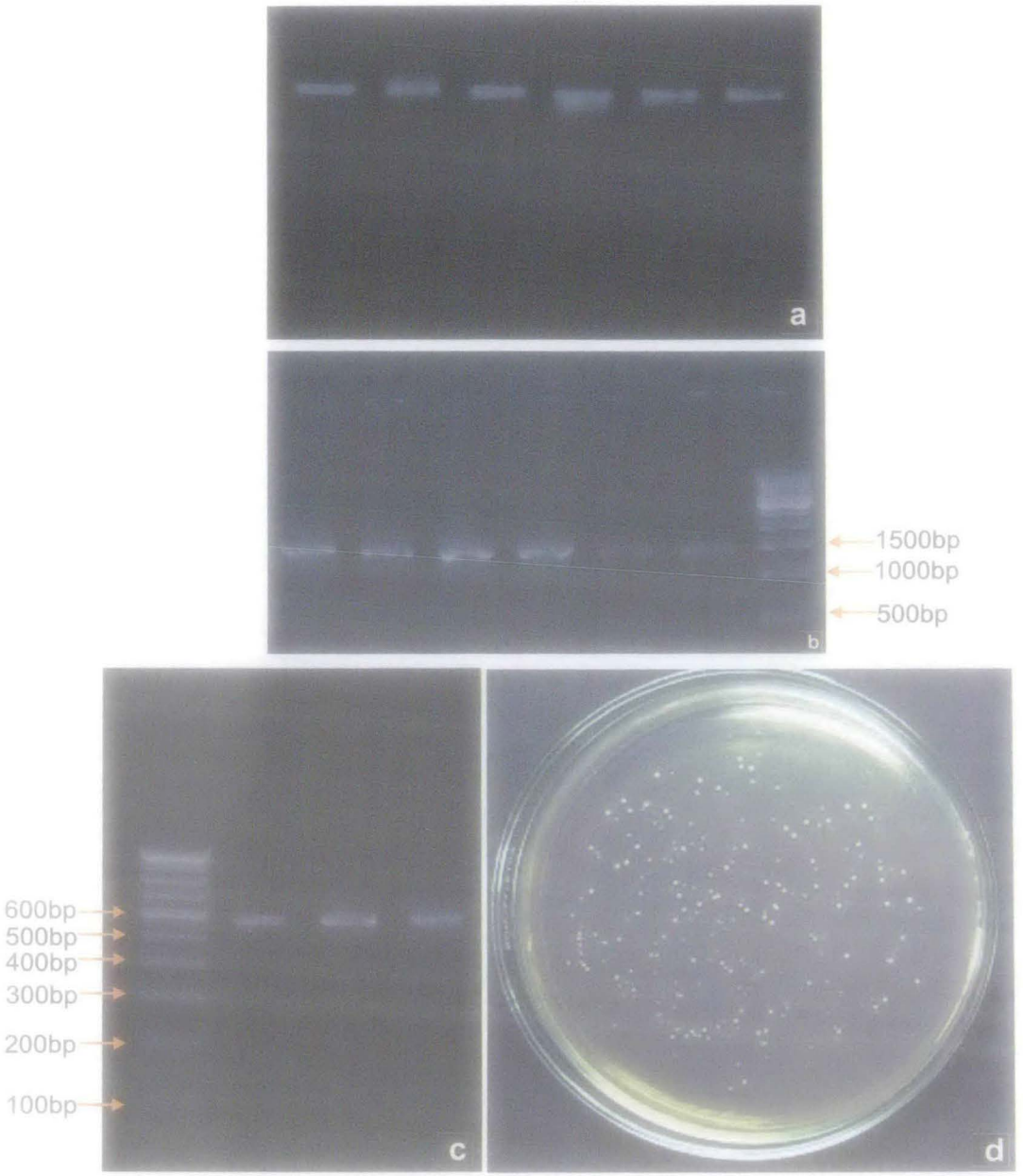


Fig. 19: Agarose gel electrophoresis of (a) extracted genomic DNA of different bacterial strains; (b) 1500 bp long PCR product obtained by amplifying the 16S rRNA gene of different bacterial strains using universal fD1 and 157 rP2 primers, DNA size marker at extreme right (500 bp ladder); (c) 560 bp length PCR product obtained by amplifying using 16S-23S rRNA ITS region gene using primers specific for *Pseudomonas* strains. DNA size marker (100bp ladder) in left; (d) Blue white screening of transformed *E. coli* JM109 cells

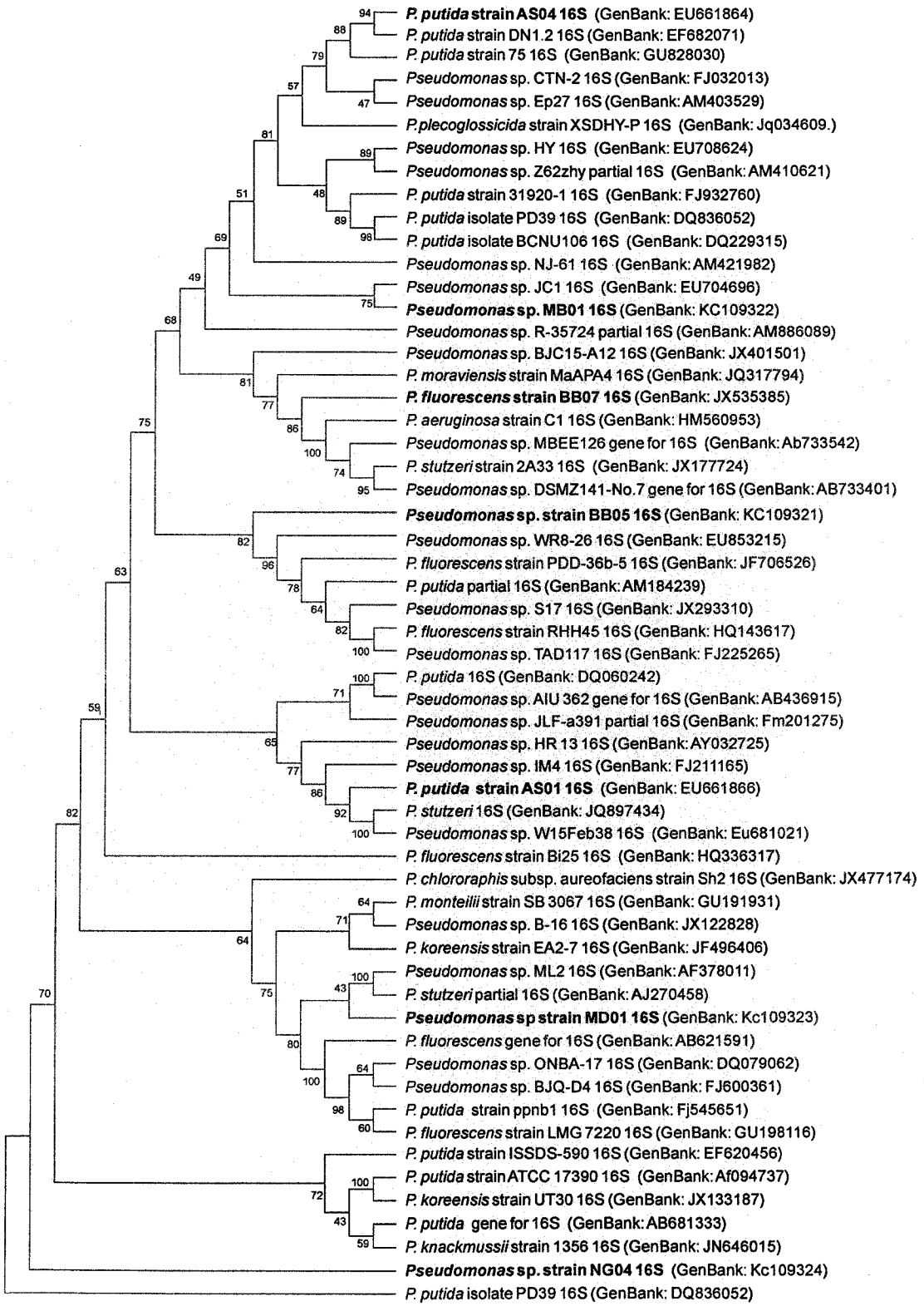


Fig. 20: Phylogenetic tree generated by neighbour-joining method on the basis of partial 16S rDNA sequences showing the position of seven *Pseudomonas* isolates among 50 other member of the genus *Pseudomonas*. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes. The Accession Numbers of the strains are denoted in the parentheses. Emboldened names of the strains indicate those isolated during the present study

microbial communities in soil. Other major factors are soil composition such as pH, presence of organic matter and key nutrients and also the agriculture management regime which has a profound effect on microbial community structure (Garbeva et al., 2004).

In the present study soil sampling sites were selected from a variety of regions and crop fields distributed in the sub-Himalayan plains of the Darjeeling and Jalpaiguri districts. The major collection sites were cultivated fields including tea gardens but natural vegetation was also included; such as grass rhizosphere soil was sampled from Nagrakata. Besides, mango and neem tree rhizosphere soil was also included. Since the microbial activities are 10–1,000-times higher in the vicinity of plant roots than in unplanted soil (Ownley and Windham, 2004) and it harbours both pathogens as well as antagonistic microbes, there is ample opportunity to successfully recover the antagonistic microbes from the rhizosphere region.

Altogether 208 culturable bacterial strains were isolated in this study. Of these, only 68 isolates (32%) were found to be siderophore producing by the CAS agar screening technique. This detection method is sufficiently sensitive in detecting siderophore producing microorganisms especially the gram negative bacteria (Schwyn and Neilands, 1987; Milagres et al., 1999) and is universally accepted (Milagres et al., 1999). Several authors have used it to screen and assay siderophorogenic Gram negative (Majumdar et al., 2007; Chaiharn et al., 2009; Sayyed and Chincholkar, 2010) as well as Gram positive bacteria (Park et al., 2005). The assay has been used for screening fungi (Howard, 1999) and archae (Dave et al., 2006) also. During this study, although in most cases the typical orange halo indicated siderophore production; variations in colour intensity, size of the halo and nature of colour change (purple, pink, yellow) was also noticed. These variations indicated differences in the nature of siderophores produced. The colour intensity can be related to dissimilarities in siderophore concentration (Milagres et al., 1999). Furthermore, several strains failed to grow in the CAS agar medium. Schwyn and Neilands (1987) observed that the detergent hexadecyltrimethyl-ammonium bromide (HDTMA) used in

preparation of the CAS medium may be toxic to some microorganisms. In a study on biocontrol strains of the rice rhizosphere, Chaiharn et al. (2009) observed that only 23.1% of the total bacteria isolated from the rice rhizospheric soil in Northern Thailand produced siderophores when screened by the CAS agar medium. The authors found that apart from exhibiting a wide variation in nature and intensity of colour change of the blue CAS medium, about 14.8% of the strains failed to grow in this medium. Yu et al. (2011) screened the rhizospheric soil surrounding peppers, rubber trees and tomato grown in Hainan, China for siderophore producing bacteria according to the universal CAS agar plate assay. The strain with the largest size of halo, named CAS15 was selected for further biocontrol and plant growth promoting studies. Arora et al. (2001) screened 12 rhizobial isolates for siderophore production via a modified CAS agar method of which only two strains, RMP3 and RMP5 showed orange colour production and yellow-orange coloured halo around the colonies, on CAS reagent overlaid on YEM agar. The authors found variation in size of the halo with RMP5 exhibiting a larger halo in comparison to those of strain RMP3.

The present work was designed to recover the microbes which should bear the inherent ability to outcompete the soil borne pathogens for survival in the natural environment. The scarcity of bioavailable iron in soil habitats and on plant surfaces instigates a furious competition (Loper and Henkels, 1997). Repeated failures or inconsistency in performances of several potential biocontrol agents have been attributed to their poor rhizosphere competence. Rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (Weller et al., 2002; Compant et al., 2005). Given the importance of rhizosphere competence as a prerequisite of effective biological control, competition for nutrients and niches is a fundamental mechanism by which biocontrol agents protect plants from phytopathogens (Compant et al., 2005).

Of the 68 siderophorogenic isolates, 20 strains were found to be antagonistic to all the seven tested pathogens, *F. equiseti*, *L. theobromae* *R. solani*, *C. gloeosporioides*, *A. alternata*, *F. solani* and *F. graminearum*. Several other strains were also found to exhibit antifungal activity against one or more pathogens (data not shown). However, the aim of this study was to isolate the antagonistic strains which were most promising and active against a broad spectrum of pathogens. Thus the strains which failed to restrict the growth of all the test pathogens were not considered for further characterization. Siderophores are secreted in an iron-limiting condition for competitively sequestering the ferric iron from the environment. The antagonistic activity of the siderophore producing strains in the present study was tested in PDA which is a nutrient rich medium; therefore, the limited pathogen growth cannot be attributed to deprivation of iron alone. Thus, additional antagonistic mechanisms are most likely involved such as antibiosis or predation which are causing the restricted growth in dual cultures.

Isolates AS01 and AS04 were the best antagonists as evident by dual culture tests. The inhibition percentage ranged from 64% to 77% against all the test pathogens. However, a maximum of 83% inhibition was exhibited by BB07 against *A. alternata* but the performance was poor against other pathogens such as *R. solani* (55.3%) and *C. gloeosporioides* (53.2%). Similarly MD01 also showed excellent inhibition against *R. solani* (82.1%) but registered only 19.9% inhibition against *L. theobromae*. Given the need for antagonists effective against a wide range of pathogens, the isolates AS01 and AS04 were chosen for *in vivo* studies. Several researchers have used the dual culture assay to identify antagonistic microbes in the environment. Chaiharn et al. (2009) used the dual culture test to detect antagonistic strains against rice pathogens, *Alternaria* sp., *F. oxysporum*, *P. oryzae* and *Sclerotium* sp. In dual culture technique, the siderophore producing rhizobacteria showed a strong antagonistic effect against the *Alternaria* (35.4%), *Fusarium oxysporum* (37.5%), *Pyricularia oryzae* (31.2%) and *Sclerotium* sp. (10.4%) strains tested. *Streptomyces* sp. strain A 130 and *Pseudomonas* sp. strain MW 2.6 in particular showed a significant higher

antagonistic effect against *Alternaria* sp. while *Ochrobactrum anthropi* D 5.2 exhibited a good antagonistic effect against *F. oxysporum*. *Bacillus firmus* D 4.1 inhibited *P. oryzae* and *Kocuria rhizophila* strongly inhibited *Sclerotium* sp. Siddiqui and Shaukat (2002) evaluated the antagonistic activity of the strains CHA0 (*Pseudomonas fluorescens*), IE-6 S+ (*P. aeruginosa*) and 569Smr (*Bradyrhizobium japonicum*) against motile tomato pathogens *Macrophomina phaseolina, F. solani* and *R. solani* by the dual culture test in petriplates containing Czapek Dox agar. Strain CHA0 produced zones of inhibition of 4, 3 and 5 mm, respectively, against *M. phaseolina, F. solani* and *R. solani*. *P. aeruginosa* strain IE-6S+ produced zones of inhibition of 3 and 4 mm, respectively, against *M. phaseolina* and *R. solani* whereas against *F. solani*, the colonies of both organisms met each other and no further growth of either organism was observed. Singh et al. (2010) used the dual culture technique to quantify the antagonistic activity of ten strains of *Pseudomonas aeruginosa* (PN1-PN10) isolated from rhizosphere of chir-pine against *Macrophomina phaseolina*. Of the ten strains, the strain PN1 which recorded maximum siderophore production by the CAS assay was found to exhibit maximum antagonistic activity causing 69% colony growth inhibition. Yu et al. (2011) evaluated the atagonistic potential of siderophore producing strain CAS15 by the dual culture test in PDA plates against 15 fungal pathogens including *F. oxysporum, F. solani* and *C. gloeosporioides*. The rates of inhibition ranged from 19.26 to 94.07%. Arora et al. (2001) screened 12 rhizobial isolates for the ability to inhibit *M. phaseolina* on YEM agar plates. Of all the isolates, only the two siderophore-producing strains, RMP3 and RMP5, showed strong antagonism against *M. phaseolina*. Strains RMP3 and RMP5 showed 72% and 77% inhibition of fungal growth, respectively after 5 days of incubation, compared to control.

All the twenty antagonistic isolates were subjected to characterization studies by a polyphasic approach that included morphological and physiological characterization by general and microscopic observations and biochemical tests; and phylogenetic characterization via 16SrRNA gene sequencing followed by sequence homology searches using software tools (MEGA-4.0). The results of both phenotypic and genotypic tests were

analysed together for appropriate recognition of the antagonistic isolates. In the last decade, because of the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates (Woo et al., 2008). The DNA evidence of the existence of thousands of novel species is clear, but parallel advances in cell culture and phenotyping are needed to actually describe the biology of these bacteria and work with them experimentally. Moreover, it is considered as highly desirable to standardize the phenotypic descriptions of bacteria (Bochner, 2009).

Taxonomical analysis of the twenty antagonistic isolates led to the identification of bacteria belonging to seven different genera, *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia*. Seven out of twenty strains belonged to the genus *Pseudomonas* which was the most commonly isolated genus; and six of these were fluorescent pseudomonads. Within the *Pseudomonas sensu stricto*, which corresponds to the rRNA group I (Palleroni, 1984, 2005, 2008), the fluorescent pseudomonads include all *Pseudomonas* species with the ability to produce fluorescent pyoverdine siderophore(s), noticeably *P. aeruginosa*, *P. syringae*, *P. putida* and *P. fluorescens* (Bossis et al., 2000). Apart from isolate NG04, the other pseudomonads exhibited yellow-green fluorescence in *Pseudomonas* agar plates. This provided an indication that these strains may belong to the fluorescent pseudomonad group. An attempt was made to identify the fluorescent pseudomonads because they are natural siderophore producers and thus should be better colonizers than the non siderophore producers. Therefore, amplification of 16S-23S rRNA ITS region gene using primers specific for fluorescent pseudomonads was performed which led to further corroboration of the results obtained from phenotypic tests. However, the taxonomic studies considering the results of biochemical characterization tests as well as 16S rRNA gene sequences and homology searches could identify only three strains upto the species level. Isolates AS01 and AS04 were identified as *P. putida* while BB07 was identified as *P. fluorescens*. The strains BB05, MB01 and MD01 were maintained as *Pseudomonas* sp. Kumar et al. (2002) used the same 16S-

23S rRNA ITS specific primers to confirm the identity of the fluorescent pseudomonads which were isolated from soil through direct screening in King's B medium. Only the colonies that were fluorescent in the specific medium were used for the taxonomic analysis, therefore all the selected 18 colonies produced the expected amplicon size and were thus confirmed as fluorescent pseudomonads (Kumar et al., 2005).

Fluorescent *Pseudomonas* strains are well known contributors to the disease suppressiveness of soils or protect plants from disease(s) caused by soil-borne fungal pathogens when used as inoculants (Couillerot et al., 2009; Ramette et al., 2011). However, a large number of strains with disease suppression potential are presented as *P. fluorescens* in the literature, but only some of these biocontrol strains actually belong to this species (Sanguin et al., 2008). Many of the other strains correspond in fact to closely-related species from the same '*P. fluorescens*' complex, noticeably *P. kilonensis*, *P. aurantiaca*, *P. thivervalensis* and *P. brassicacearum* (Frapolli et al., 2007), which are often difficult to distinguish from *P. fluorescens*. In addition, a few strains of a different *P. fluorescens* lineage are taxonomically ill-defined and usually referred to as ARDRA-1 based on 16S rRNA gene restriction profiling (Keel et al., 1996; Couillerot et al., 2009). The current state of the *Pseudomonas* taxonomy makes it difficult to assess the phylogenetic distribution of biocontrol agents within *P. fluorescens* and closely-related fluorescent pseudomonads (Bossis et al., 2000). Therefore in this study in order to avoid a probable misrepresentation, some of the present isolates were maintained as *Pseudomonas* sp.

Bacillus was the second most common bacteria among other strains that were isolated in this study. Six isolates were recognized to be *Bacillus* through phenotype characters and phylogenetic considerations (Claus and Berkeley, 1986). MB02 was identified as *Bacillus subtilis* while the rest of the isolates were maintained as *Bacillus* sp. *Bacillus subtilis* isolates are reported to be effective at controlling plant diseases caused either by soil-borne, foliar or post-harvest pathogens (Janisiewicz and Korsten, 2002; Ongena et al., 2005; Arguelles-Arias et al., 2009). Some strains are

incorporated in commercially available biocontrol products (Emmert and Handelsman, 1999; Warrior et al., 2002). *Bacillus* spp. has been reported to suppress a variety of plant diseases including cucurbit powdery mildew (Romero et al., 2007); *Verticillium* wilt in eggplant (Li et al., 2008); take-all in wheat (Liu et al., 2009); *Fusarium* wilt in tomato (Cazorla et al., 2007) and eggplant (Saha et al., 2012a) and damping off in tomato (Leclere et al., 2005). *Bacillus* is highly accepted as biocontrol agent and often preferred to all other types of bacteria due to its spore forming properties which makes it easier to be integrated into formulated biocontrol products with long term viability (Emmert and Handelsman, 1999; Romero et al., 2007).

Among the other bacteria isolated during the present study, *Serratia* spp. are well known bioinoculants and has been reported as strong biocontrol agents against several fungal pathogens (reviewed by Saha et al., 2012b). A siderophore producing strain of *Alcaligenes faecalis* has been reported to suppress the plant pathogens *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum* and *Alternaria alternata* more efficiently than the oraganochlorine fungicide, bavistin (Sayyed and Chincholkar, 2009). However, on the other hand, Siddiqui and Mahmood (1992) reported that *A. faecalis* had an adverse effect on plant growth. *Enterobacter cloacae* EcCT-501, which produces both hydroxamate and catecholate siderophores was found to suppress *Pythium* damping-off of cucumber and other plant hosts. Kavroulakis et al. (2010) reported the inhibitory effects of extracellular products of *Enterobacter* sp. AR1.22, which was found to be the most effective rhizospheric biocontrol agent among six other bacteria selected through *in-vitro* screening against *F. oxysporum*.

In the present study, different types of bacteria with broad spectrum antifungal activity were isolated from the rhizosphere soil of a variety of plants. The antagonistic isolates recovered from the soil appear to have the potential for further analysis of plant protection mechanisms and biocontrol studies. However, in this study, the most potential isolates AS01 and AS04 both of which were identified as *P. putida* were selected for further biocontrol study.

CHAPTER 4

Mechanism of Action of Siderophore Producing Rhizobacteria Showing Antagonistic Activity Against Plant Pathogenic Fungi

MECHANISM OF ACTION OF SIDEROPHORE PRODUCING RHIZOBACTERIA SHOWING ANTAGONISTIC ACTIVITY AGAINST PLANT PATHOGENIC FUNGI

4.1 Introduction

Understanding the mechanism of action of a biological control agent may allow the optimum conditions for implementing biocontrol in a given pathosystem to be determined (Mathre et al., 1999; Cabrefiga et al., 2007). However, assessment of the mechanisms of antagonism is a complex and difficult task, starting with prospective studies to reveal the implications of a given process (Cabrefiga et al., 2007). Plant roots release a wide variety of compounds into the surrounding soil, including ethylene, sugars, amino acids, organic acids, vitamins, polysaccharides, and enzymes. These materials create unique environments for the microorganisms living in association with plant roots, in the rhizosphere. On the other hand, rhizosphere bacteria also have a profound effect on plant health. Rhizosphere colonization is important not only as the first step in pathogenesis of soilborne microorganisms, but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg et al., 2001; Garbeva et al., 2004). Studies on the properties of beneficial bacteria that help them to dominate in the rhizosphere environment and simultaneously exhibit antagonism towards fungal pathogen have attracted a lot of attention of scientists worldwide. Particularly, the mechanism employed by biocontrol organisms in effecting disease control has been the most interesting aspect of biocontrol study (Howell, 2003).

One of the ways in which biocontrol bacteria suppress fungal pathogens is by producing secondary metabolites like antibiotics, siderophore, cell-wall degrading enzymes and hydrogen cyanide (Husen, 2003; Ramirez et al., 2004; Kumar et al., 2005; Ge et al., 2007; Chen et al., 2009). Antibiotics are the most widely studied antifungal metabolites produced by the biocontrol agents to combat the plant pathogens. In several instances, antibiotics have been shown to be particularly effective in suppressing plant pathogens and

the diseases they cause (Ligon et al., 2000; Brodhagen et al., 2005; Kumar et al., 2005; Kumar et al., 2009). Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens (Ge et al., 2007). It has been reported that bacterial and fungal biocontrol agents like *Pseudomonas fluorescens, Bacillus subtilis, Serratia* sp., *Burkholderia cepacia* and *Trichoderma virens* produce a wide range of antibiotics involved in plant disease inhibition (Brodhagen et al., 2005; Shen et al., 2007; Kumar et al., 2009). Expression and secretion of lytic enzymes that can hydrolyze a wide variety of polymeric compounds like cell-wall degrading enzymes, protease and DNase by different microbes can also result in direct suppression of plant pathogen activities (Ramirez et al., 2004; Kumar et al., 2005; Chen et al., 2009).

Unlike microbial phytopathogens, plants are not generally harmed by the localized depletion of iron in the soil caused by PGPR. Most plants can grow at much lower (about 1000-fold) iron concentrations than microorganisms (O'Sullivan and O'Gara, 1992). In addition, a number of plants have mechanisms for binding the bacterial iron-siderophore complex, transporting it through the plant, and then reductively releasing the iron from the bacterial siderophore so that it can be used by the plant (Bar-Ness et al., 1991, 1992; Wang et al., 1993). The ability of siderophores to act as effective "disease-suppressive" agents is affected by the particular crop plant, the specific phytopathogen being suppressed, the soil composition, the bacterium that synthesizes the siderophore, and the affinity of the specific siderophore for iron.

This chapter focuses on the characterization of the selected isolates for secretion of antifungal enzymes *in vitro,* for production of HCN and for presence of the PGPR traits like phosphatase activity and IAA production. Additionally the amount of siderophore released by these bacteria *in vitro* has been quantified.

4.2. MATERIALS AND METHOD

4.2.1. Siderophore production assay

Quantification of siderophore was carried out by the CAS shuttle assay method (Payne, 1994). Bacterial isolates were grown overnight under constant shaking in Fiss glucose minimal media (Vellore, 2001). The medium is an iron restricted medium and thus siderophore production is effectively increased. The overnight grown culture was centrifuged at 10,000 rpm for 15 minutes; the cell-free culture supernatant was mixed with an equal volume of CAS reagent (solution 1 in CAS agar media) and incubated for one hour at 37°C. A control was kept where uninoculated broth was mixed with equal volume of CAS reagent. The absorbance was then measured at 630 nm. This assay method determined the amount of siderophore units present in the antagonistic strain culture. Siderophore content was calculated by using the formula:

$$\% \text{ siderophore units} = [(A_r - A_s)/A_r] \times 100,$$

where, A_r = absorbance of reference (uninoculated medium) at 630 nm and A_s = absorbance of the sample at 630 nm.

4.2.2 Antimicrobial metabolite production

The production of antimicrobial metabolites may be detected *in vitro* by specific tests. All the selected isolates were subjected to the tests listed below. Prior to the experiments, each bacterium was subcultured twice from the stock in NA and finally a 24 h NA culture was used for the test. An uninoculated control was included in all tests for comparison. For preparing culture filtrates, bacterial strains were grown overnight in nutrient broth and cultures were centrifuged at 10,000 rpm for 15 min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius) to obtain the final filtrate which was used in cellulase production and chitinase production tests.

4.2.2.1 Cellulase production

To test for cellulolytic activity, M9 agar medium was amended with 10 g/L cellulose and 1.2 g/L yeast extract. After sterilization of media, it was poured onto petriplates (90 mm diameter) and allowed to solidify. Wells were cut on cellulose agar plates and 100µl bacterial culture filtrates were loaded in the wells. For preparing the culture filtrates, bacterial strains were grown overnight in nutrient broth and cultures were centrifuged at 10,000 rpm for 15 min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius). The plates were incubated at 37°C for 8 days and subsequently stained with 0.1% Congo red solution overnight and destained four times with 1M NaCl at 1 h interval. Plates were checked for the formation of clear halo around the wells that indicated positive result for cellulase production (Cattelan et al., 1999; Kumar et al., 2005).

4.2.2.2. Pectinase activity

For detection of pectinase activity, M9 agar medium was used which was supplemented with 1% pectin (Cattelan et al., 1999; Kumar et al., 2005). Plates were prepared after autoclaving the medium and pouring it onto petriplates (90 mm diameter). Solidified plates were used for streak inoculation of bacterial strains. The inoculated plates were incubated for 48 hours at 30°C and subsequently flooded with 2M HCl. Clear halos around the colonies were considered as positive for pectinase production.

4.2.2.3. Lipase activity

Tween 80 agar plates were used to determine lipase activity. Plates were inoculated by streaking individual bacterial strains on the surface of the agar and incubated over night. An opaque halo of precipitation around the growth indicated hydrolysis of Tween 80 (Barrow and Feltham, 1993).

4.2.2.4. Chitinase activity

Chitinolytic activity was observed by the method of Bargabus et al. (2002). M9 media was supplemented with 0.1% glycol chitosan and 1% agarose.

Bacterial cultures (24 h) in NB were centrifuged at 10,000 rpm for 15 min and the supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius). Wells were made in the plates where 100µl of culture filtrate was added for the detection of chitinase activity. Plates were incubated at 30°C for 48 hours, stained with fluorescent brightener 28 and finally observed under UV light. Positive result was indicated by the presence non-fluorescent lytic zones around wells.

4.2.2.5. Amylase activity

Starch agar plates were used for testing amylase activity (Aneja, 2003). The plates were inoculated with the bacterial antagonists by single streak and incubated at 30°C for 48 hours in an inverted position. The plates were then flooded with iodine solution and after holding for 30 seconds the solution was poured off. Development of clear zone around the bacterial line of streaking in a dark blue or purple background was considered as positive result.

4.2.2.6. Protease activity

Skimmed milk agar plates were used to check for protease activity. Plates were inoculated with bacterial isolates as a single streak and incubated for 24-48 h at 30°C in an inverted position. Formation of a clear zone around the bacterial line of inoculation in the background of a turbid medium indicated positive result (Aneja, 2003).

4.2.3. Evaluation of PGPR traits

All the selected bacterial isolates were tested for two major PGPR traits. Each bacterium was subcultured twice from the stock in NA and finally a 24 h NA culture was used for the test. An uninoculated control was included in all tests for comparison.

4.2.3.1. Indole-3-acetic acid production

Production of IAA was determined according to Patten and Glick (2002). Twenty-four-hour-old bacterial isolates were cultured on Luria-Bertani (LB)

broth supplemented with 5 mM of L-tryptophan and were centrifuged to obtain culture supernatants. The supernatants (1 ml) were mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber, 1951; Ahmad et al., 2005) and the absorbance was measured after 20 min at 535 nm. The concentration of IAA was determined by comparison with the standard curve. IAA standard curve was prepared from a series of known IAA concentrations which were similarly assayed.

4.2.3.2. Phosphatase activity

Phosphatase activity was determined in Pikovskaya's agar medium (Pikovskaya, 1948). The medium was inoculated with bacterial strains and incubated at 30°C for at least 5 days. Development of a clear zone around bacterial growth was considered as positive for phosphatase production.

4.2.4. Detection of HCN Production.

Test for HCN production was carried out by the method of Bakker and Schippers (1987). Bacteria were heavily inoculated in nutrient agar plates supplemented with 4.4 g/L glycine and incubated in an inverted position at 30°C with filter paper strips dipped in picric acid solution (0.5% picric acid in 2% Na_2CO_3 aqueous solution) placed inside the lids. Change of colour of the indicator strip from yellow to brown was considered as a positive result.

4.2.5. Antagonism by crude extracellular products

Crude extracellular products were extracted from the culture of the bacterial isolate *Pseudomonas putida* strain AS04 which showed the highest antifungal activity. The strain was allowed to grow on semi-solid nutrient agar media containing 0.6 % agar at 30°C for 48 h.

4.2.5.1. Preparation of crude extract

The entire semi solid agar containing bacterial culture (300 ml) was crushed and homogenized with 150 ml of 80% aqueous acetone. The mixture was centrifuged and the supernatant containing the antifungal metabolite was collected and condensed to 2 ml under vacuum in a rotary vacuum

evaporator (Eyela A-1000S, Japan) and used for bioassay (Shanahan et al., 1992).

4.2.5.2. *In vitro* antifungal activity of the crude extract

The crude culture extract was tested *in vitro* for antifungal activity against the two fungal pathogens *Fusarium solani* and *F. equiseti*. Fungal inoculum was prepared in sterile distilled water by gently brushing the surface of 10 day old PDA cultures of the fungus with inoculation needle. The suspension of mycelial fragment and spores were collected from each fungus by filtering aseptically through cheese cloth and mixed (1 ml) with 19 ml molten PDA (45°C) and allowed to solidify. For testing the antifungal activity of the crude extract, two wells were cut at a distance of approximately 3cm from each other in the PDA plates seeded with the fungal pathogen and the crude extract (100 µl) was loaded in one of the wells. The other well was loaded with acetone only which served as the control. The plates were incubated at 30°C for 3 days and observed for zone of clearing around the well.

4.2.6. Scanning Electron Microscopy

Scanning electron microscopy was used as a tool to study the interaction between the antagonistic bacteria and the fungal pathogen. Two most potent antagonistic bacterial isolates, *Pseudomonas putida* strains AS01 and AS04 were selected for the study against the pathogens *Fusarium equiseti* and *F. solani*. A dual culture plate was set up as described earlier (Section 3.2.4.1). A mycelial disc (4 mm diameter) of the fungus was placed centrally in sterile PDA plates while the bacteria were streaked circularly at a distance of 20 mm from the centre. One sterile cover glass was placed carefully between the fungus and bacterial line of inoculation (closer to bacterial inoculation line) for obtaining the bacteria-fungus interaction zone over the cover glass. Another plate inoculated by the fungal pathogen was treated as control. Both the plates were incubated at 28°C until the mycelia in the control plate reached the edge of the petriplate. The cover slip was removed after fungal mycelia grew over the cover glass and finally subjected to series of treatment prior to observation under SEM. The treatments were

done following the process of Samaranayake et al. (2005) and Masaphy et al. (1987). Fungal mycelia were fixed with 2.5% glutaraldehyde solution for 1 hour. Glutaraldehyde was removed by slight decanting. This step was followed by dehydration through an ascending series of ethanol: 50%, 70% and 90% (vol/vol) ethanol for 5 min each; two changes with 90% ethanol for 15 min each and with 100% ethanol for 20 min each. All ethanol dilutions were made with distilled, deionized water. After dehydration, the samples were coated with gold (IB2-ion coater, Japan) and observed under scanning electron microscope [Model: Hitachi S-530 (Japan) 1986].

4.3. RESULTS

4.3.1. Siderophore production

In the quantitative assay of siderophore production, it was found that the isolate *Pseudomonas putida* strain AS04 was the highest producer recording 62.33 % units of siderophore followed by MD01 which produced 54.80% units. The lowest amount of siderophore was produced by CR07 with 8.30% (Table 12). BB05, MB01, MB02, NG05 and AS01 showed moderate siderophore production that ranged from 41.7% to 45.8%. A comparative chart is given in fig. 23b, which shows the amount of siderophore produced by different bacterial isolates.

4.3.2. Antimicrobial Metabolite Production

Antagonistic bacterial isolates were found to be capable of producing several hydrolytic enzymes which are considered to contribute towards their antagonistic activity (Table 13). Clear halo under UV light confirmed chitinase production by only three isolates namely AS04 and MD01 and CB02 strains (Fig. 21a) whereas rest showed negative result. Test for cellulase and pectinase production was found to be negative for all twenty isolates. Protease production or casein hydrolysis test was also performed where clear zone around opaque media confirmed positive result; the cultures showing positive result were BB05, BB07, AS01, AS04, CR07, CR10, CR12, CR14, CB02, MD01, MB01, MB02, NG07 and KT05 (Fig. 21b). The strains tested positive for lipid hydrolysis were BB05, AS01, AS04,

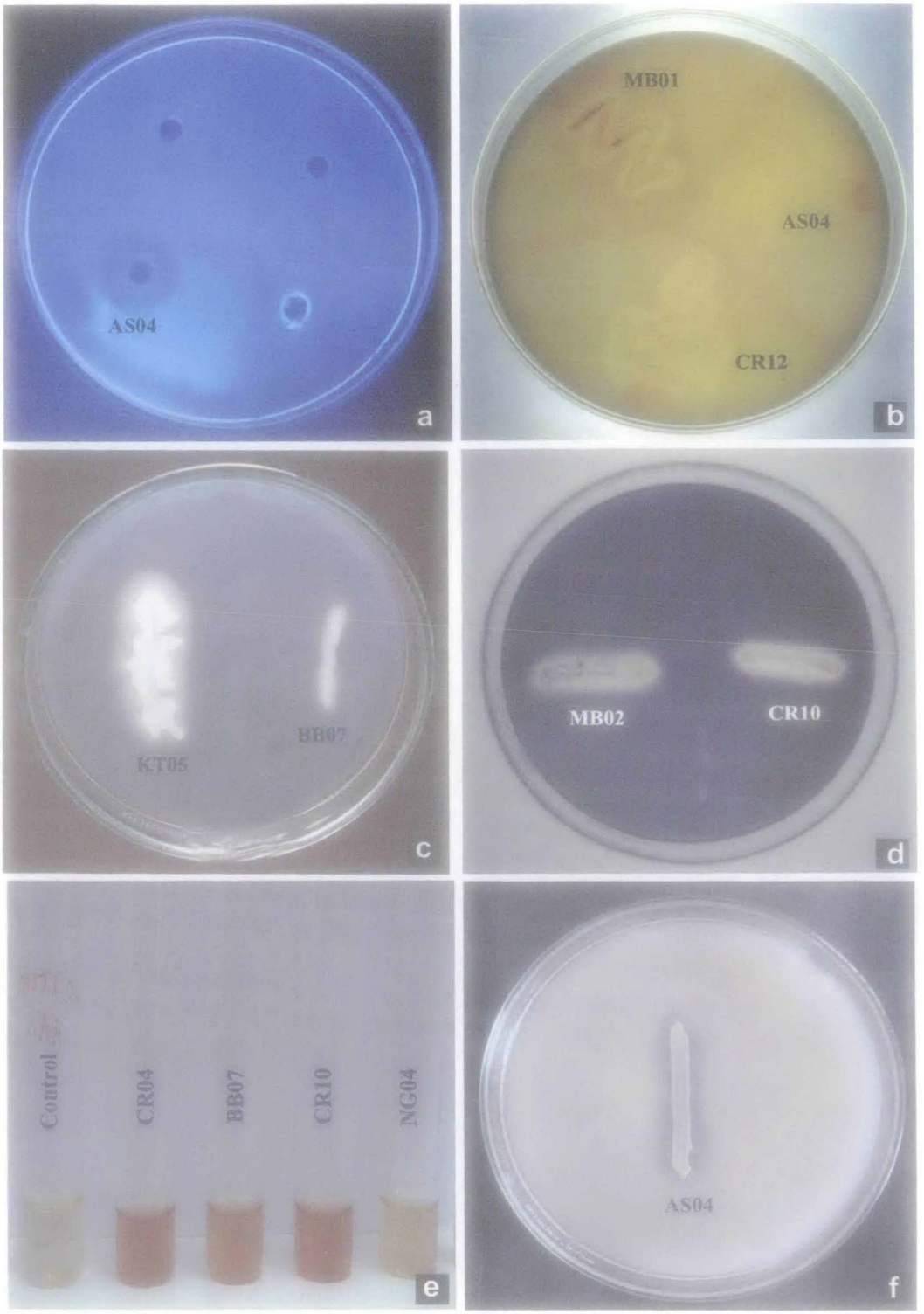


Fig.21: Production of extracellular lytic enzymes by selected antagonistic isolates:

(a) Non-fluorescent zone around the well showing chitinase production by strain AS04; (b) Clearing zone in SMA plate shown by AS04, CR12 and MB01;(c) Turbid zone around bacterial culture showing lipase activity by KT05 and negative result by BB07 showing no turbid zone; (d) Amylase activity evident by clearing zone around purple background by MB02 and CR10; (e) Amount of IAA shown by varying intensity of brown colouration in culture supernatant on addition of Salkowski's reagent by bacterial isolates CR04, BB07, CR10 and NG04. Extreme left tube represents control (f) Phosphatase activity shown by clearing zone in Pikovskaya agar by AS04.

CR07, CR10, CR12, CR13, CR14, CB02, MB02, NG04, NG07 and KT05 (Fig. 21c). The isolates that hydrolyzed starch indicating amylase production were CR04, CR07, CR10, CR14, JL11, MB02, MB05, NG04, NG07 and KT05 (Fig. 21d).

Table12: Amount of siderophore produced by antagonistic bacterial isolates

Bacterial Strains	Absorbance at 630 nm (A_s)	% Siderophore units[a]
BB05	0.14	41.70±0.79
BB07	0.15	37.50±0.29
AS01	0.13	45.80±0.46
AS04	0.06	62.33±0.95
MB01	0.14	41.66±0.34
MB02	0.14	41.66±0.20
MB05	0.17	29.20±0.59
CB02	0.17	29.20±0.49
MD01	0.07	54.80±0.65
CR04	0.16	33.33±0.52
CR07	0.22	08.30±0.40
CR10	0.16	33.33±0.43
CR12	0.20	16.70±0.35
CR13	0.16	33.33±0.46
CR14	0.17	29.20±0.36
JL11	0.19	20.80±0.42
NG04	0.17	29.20±0.53
NG05	0.14	41.70±0.39
NG07	0.16	33.33±0.34
KT05	0.20	16.70±0.35

[a]: % siderophore units = $[(A_r - A_s)/A_r] \times 100$; A_r =0.240 (A_r is the absorbance of uninoculated medium)

Table 13: Production of hydrolytic enzymes related to antagonistic activity by the isolated bacterial strains[a]

Bacterial Isolates	Chitinase	Cellulase	Pectinase	Protease	Lipase	Amylase
BB05	-	-	-	+	+	-
BB07	-	-	-	+	-	-
AS01	-	-	-	+	+	-
AS04	+	-	-	+	+	-
CR04	-	-	-	-	-	+
CR07	-	-	-	+	+	+
CR10	-	-	-	+	+	+
CR12	-	-	-	+	+	-
CR13	-	-	-	-	+	-
CR14	-	-	-	+	+	+
CB02	+	-	-	+	+	-
MD01	+	-	-	+	-	-
JL11	-	-	-	-	-	+
MB01	-	-	-	+	-	-
MB02	-	-	-	+	+	+
MB05	-	-	-	-	-	+
NG04	-	-	-	-	+	-
NG05	-	-	-	-	-	+
NG07	-	-	-	+	+	+
KT05	-	-	-	+	+	+

[a]:'+' tested positive; '-'tested negative.

4.3.3. Detection of HCN Production.

All the twenty anatagonistic isolates showed a negative test for HCN production. This was evident when the colour of the filter paper did not record the expected color shift from yellow to brown.

4.3.4. Antagonism by crude extracellular products

The crude extracellular product of the *Pseudomonas putida* strain AS04 extracted from semi solid culture medium was found to exhibit antifungal activity under *in vitro* condition against *Fusarium solani* (Fig. 22a) and *F. equiseti* (Fig. 22b). This was evident by a clear zone of inhibition around the well containing the culture extracts of the strain AS04. Inhibition zones were not noticeable around the control wells.

4.3.5. Evaluation of PGPR traits

For evaluating PGPR traits, all the antagonistic isolates were tested for IAA production and phosphatase activity. Results showed that all 20 antagonistic isolates produced IAA (Fig. 23a). CR10 produced highest amount of IAA, i.e., 68 µg/ml followed by CR04, BB07 (Table 14). The concentration of IAA was estimated by an IAA standard curve. Streaking of bacterial isolates on the Pikovskaya's agar plate and incubation for 2-5 days at 28°C led to the development of a clear zone indicating positive phosphatase activity. Of the twenty isolates only AS04 produced phosphatase (Fig.17c) on Pikovskaya's agar.

4.3.6. Scanning Electron Microscopy

Scanning electron microscopic studies of the interacting zones of the antagonistic bacteria and plant pathogenic fungi revealed severe mycelial deformities of the pathogens. The results of the study were recorded in photographs (fig. 24a-h). It is clear from the figures that bacterial cells were attached to the hyphal surface of *F. equiseti* (fig 24a). This was accompanied by clearing of the hyphal fluid in some areas (fig 24c). Such events may occur due to secretion of secondary metabolites and diffusible lytic enzymes by this bacterium. Mycelial deformities like hyphal bulging and bursting of the mycelia were also observed in *F. equiseti* in presence of the strain AS04 (Fig. 24b). The strain AS01 also caused degeneration of cell wall, lysis of mycelia and subsequent release of cell contents of *F. solani* (Fig 24e and 24f). Disruption of mycelia of *F. solani* was also observed evidently due to

the action of the strain AS04 (Fig. 24g). The results were compared with respective controls (Fig 24d and 24h).

Table 14: Production of IAA, phosphatase and HCN by antagonistic bacterial isolates[a]

Bacterial strains	Conc. of IAA produced (µg/ml)	Phosphatase production	HCN production
BB05	52.0±0.50	-	-
BB07	60.0±0.87	-	-
JL11	36.0±0.58	-	-
MD01	34.0±0.37	-	-
CB02	47.0±0.34	-	-
AS01	39.0±0.51	-	-
AS04	35.0±0.56	+	-
CR04	60.0±0.68	-	-
CR07	35.0±0.71	-	-
CR10	68.0±0.53	-	-
CR12	26.0±0.73	-	-
CR13	36.0±0.42	-	-
CR14	41.0±0.42	-	-
MB01	36.0±0.55	-	-
MB02	45.0±0.56	-	-
MB05	29.0±0.66	-	-
NG04	19.0±0.68	-	-
NG05	33.0±0.52	-	-
NG07	40.0±0.30	-	-
KT05	55.0±0.68	-	-

[a]:'+' tested positive; '-'tested negative

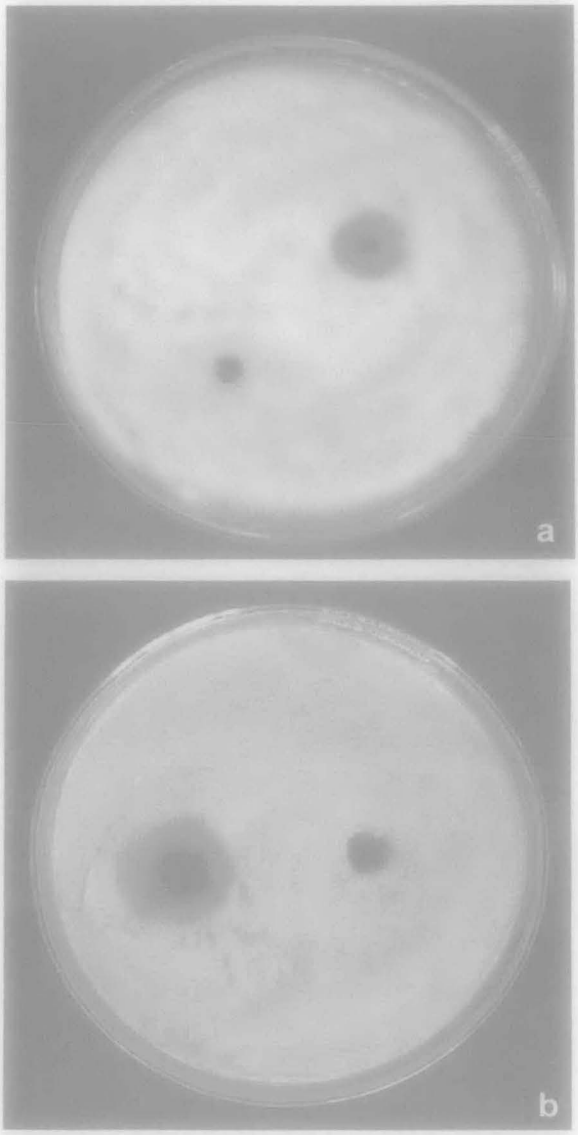
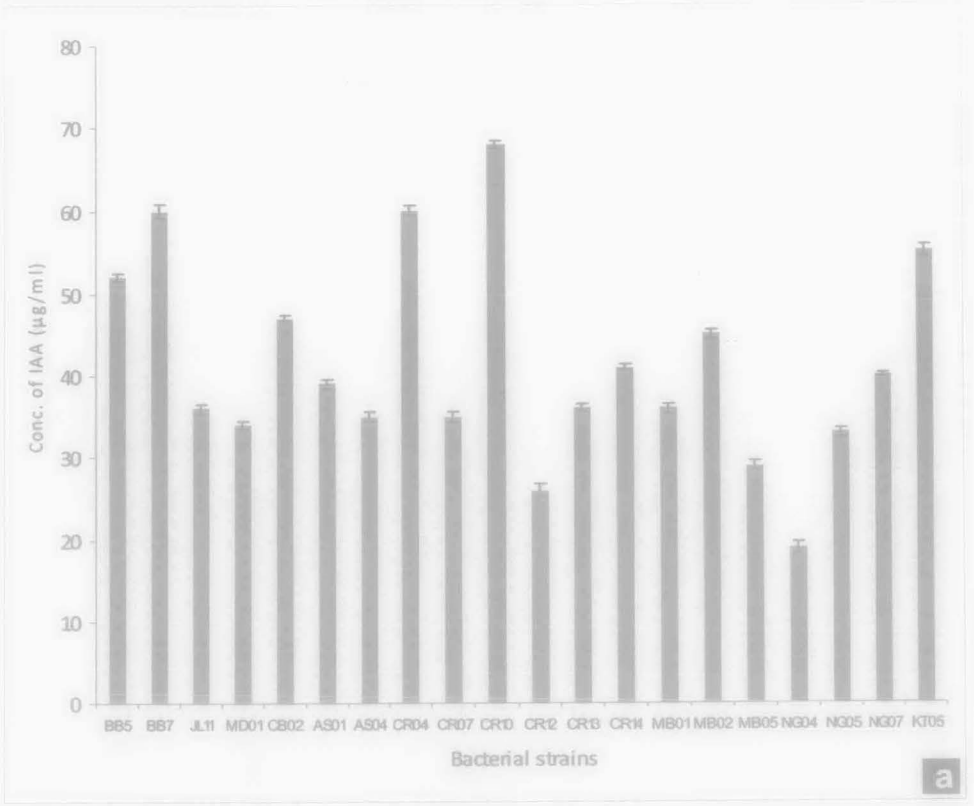


Fig. 22: Inhibition of mycelial growth by crude acetone extract obtained from strain AS04 culture supernatant of; (a) *F. solani* (b) *F. equiseti*



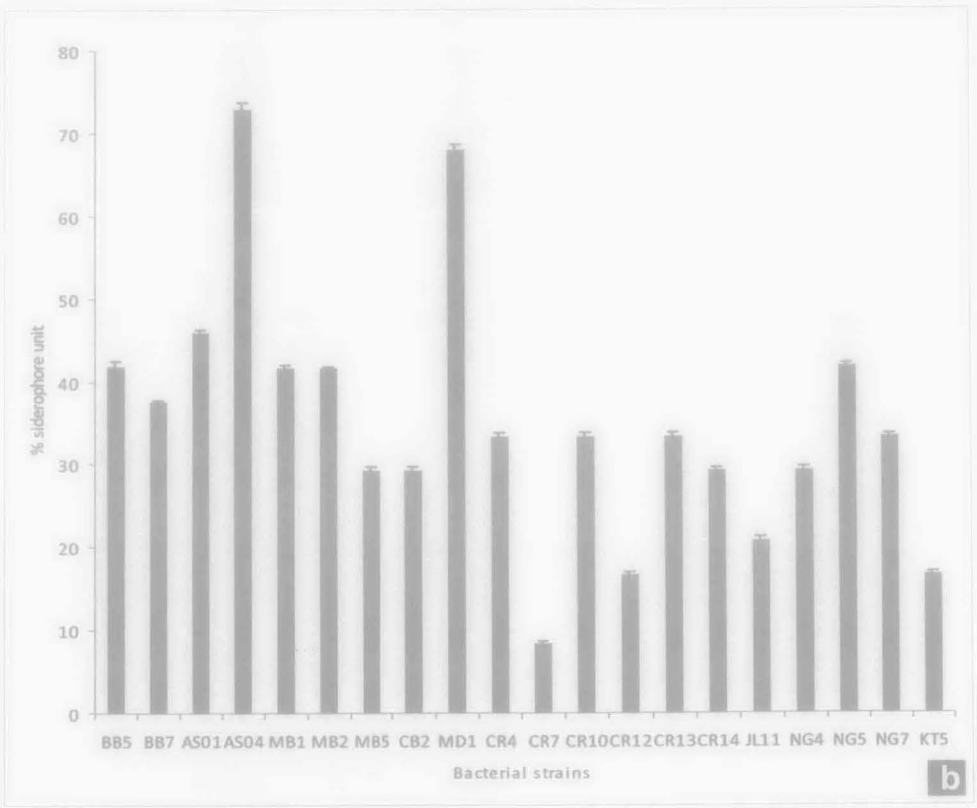


Fig. 23: (a) Amount of IAA produced by the selected antagonistic isolates;

(b)Amount of siderophore produced by selected antagonistic isolates

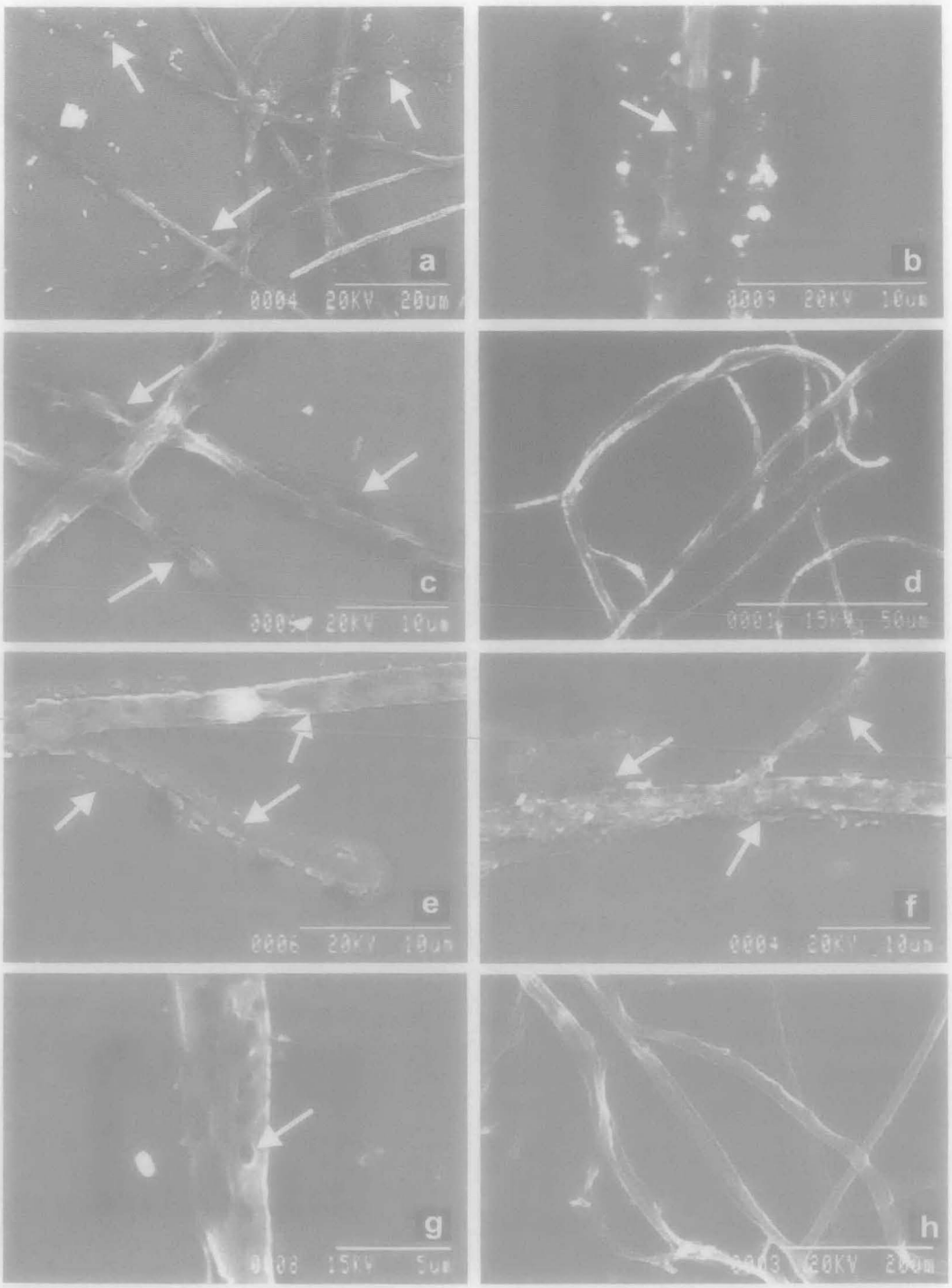


Fig.24: Scanning Electron Microscopic observation of the interaction zone between fungus and antagonistic bacteria grown in PDA plates in dual cultures. The sites of changes are indicated by arrows:

(a) Bacterial cells of *P.putida* strain AS01 attached to the hyphal surface of *F.equiseti* (1500x); (b) Mycelial deformities and lysis of the hyphae of *F.equiseti* by *P.putida* strain AS04 (5000x); (c) Clearing of *F.equiseti* hyphal fluid by *P.putida* strain AS01 (3000x); (d) *F.equiseti* control (1000x); (e) Clearance of mycelia of *F.solani* caused by *P.putida* strain AS01 (4000x);(f)Lysis of mycelia and release of cell contents of *F.solani* by *P.putida* strain AS01 (3000x); (g)Disruption of mycelia of *F.solani* by *P.putida* strain AS04 (6000x); (h) *F. Solani* control (2000x).

4.4. DISCUSSION

Microbes that demonstrate the ability to antagonize plant pathogens are taxonomically diverse. The characteristics that they share that are important determinants of this ability include the aggressive colonization of the plant rhizosphere and the production of antifungal metabolites. Many active biocontrol microbes especially those belonging to the genera *Pseudomonas* and *Bacillus* are known to produce a diverse array of antifungal compounds and in many cases the production of these compounds has been directly correlated with biocontrol activity (Ligon et al., 2000). The biological role of metabolite production by these bacteria appears to be in providing a competitive advantage in the colonization of the rhizosphere, an environment that is rich in plant exuded nutrients. Offensive bacterial colonization by biocontrol strains and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes (Ligon et al., 2000; Compant et al., 2005).

In the present study, the antagonistic isolate, *Pseudomonas putida* strain AS04 recorded maximum siderophore production among all the twenty selected isolates. Several other strains noticeably, MD01, BB05, MB01 and AS01 also produced high amount of siderophores. All these strains were fluorescent pseudomonads. The group comprising of fluorescent pseudomonads derive their name from the yellow green fluorescent pigment pyoverdin which they produce under iron limiting conditions (O'Sullivan and O'Gara, 1992). Several researchers (Gupta et al., 2002; Omidvari et al., 2010) have assayed the amount of siderophore produced by fluorescent pseudomonads. The CAS shuttle assay used in the present study was also used by Sayyed et al. (2005) for determining the amount of siderophore produced by *P. fluorescens* and *P. putida* strains. The authors observed a high amount (87 and 83% units) of siderophore production by these strains. Siderophores have been demonstrated to play a major role in plant disease suppression by some bacterial biocontrol agents (O'Sullivan and O'Gara,

1992). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper and Henkels, 1999). It has been reported that apart from pseudomonads, many gram-positive bacteria including *Bacillus subtilis* synthesize siderophores both of hydroxamate and catecholate type (Temirov et al., 2003).

In the present study, the *P. putida* strain AS04 with maximum antifungal activity was found to exhibit chitinase activity on chitin supplemented plates. Besides, two other strains *Serratia* sp. CB02 and *P. fluorescens* MD01 also produced chitinases. Extracellular lipase activity was evident in most of the tested strains. Extracellular protease can contribute to the ability of bacteria to suppress fungal diseases (Ahmadzadeh et al., 2006) and here, 14 of the 20 displayed proteolytic activity in SMA medium. The extracellular product extracted from the spent culture medium during the present study was found to inhibit the growth of *F. equiseti* and *F. solani* in PDA. Several biocontrol PGPR strains have been found to produce enzymes including chitinase, protease and lipase that can lyse fungal cells (Chet and Inbar, 1994). For example, the role of chitinase produced by antagonistic microorganisms like *Pseudomonas*, *Enterobacter*, *Bacillus*, *Serratia* and *Trichoderma* in inhibition of phytopathogens has been demonstrated by several workers (Nielson et al., 1998; Manjula and Podile, 2005; Jaiganesh et al., 2007). Chitin, an unbranched homopolymer of 1,4-β-linked *N*-acetyl-d-glucosamine, is a major cell-wall component of most phytopathogenic fungi which does not occur in plants or other microbes. Although the physiological function of microbial chitinases has yet to be clarified, there is strong correlative evidence that they are proteins with antifungal activity (Schickler and Chet, 1997). Chitinases, along with proteases and 1,3-β-glucanases, degrade fungal cell walls, inhibit fungal growth at the hyphal tips and have been shown to associate with hyphal walls *in planta* (Schickler and Chet, 1997). Lim et al. (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminarinase, and found that these enzymes could digest and lyse *Fusarium solani* mycelia thereby preventing the fungus from causing crop loss due to

root rot. Similarly, Fridlender et al. (1993) were able to reduce the incidence of plant disease caused by the phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii* and *Pythium ultimum* by using a glucanase-producing strain of *Pseudomonas cepacia* which was able to damage fungal mycelia. Nielsen et al. (1998) reported that in the sugar beet rhizosphere fluorescent pseudomonads inhibit plant pathogenic fungi *Rhizoctonia solani* by production of cell wall-degrading endochitinase. Chernin et al. (1995) observed that three different strains of the biocontrol bacteria *Enterobacter agglomerans* that are antagonistic to fungal pathogens including *Rhizoctonia solani,* possess a complex of four separate enzymes that is responsible for the chitinolytic activity of the bacteria. These bacteria significantly decreased the damage to cotton plants following infection with *Rhizoctonia solani.* Moreover, Tn5 mutants of one of these biocontrol strains that were deficient in chitinase activity were unable to protect the plant against damage caused by the fungal pathogen. Trivedi et al. (2008) observed that the soil bacterium *Pseudomonas corrugata* which showed antagonism against *Alternaria alternata* and *Fusarium oxysporum* recorded lipase and chitinase production in growth medium and they were considered as contributory factors to the antagonistic activity of the strain. Arora et al. (2008) observed that fluorescent *Pseudomonas* isolates PGC1 and PGC2 with antifungal potential against *R. solani* and *P. capsici* produced the enzymes chitinase and beta-1,3-glucanase. Their results indicated the role of chitinase and beta-1,3-glucanase in the inhibition of *R. solani.* Samavat et al. (2011) reported that *Pseudomonas fluorescens* UTPF 68 and UTPF 109 showing antagonism against *R. solani* produced chitinase which was thought to play a role in antagonism. Other authors (Diby et al., 2005; Srividya et al., 2012; Wahyudi et al., 2011; Tabarraei et al., 2011; Viswanathan and Samiyappan, 2001) also reported production of multiple lytic enzymes like protease, lipase and chtinase by *Pseudomonas* strains.

In the present study, all the antagonistic isolates were able to produce the plant growth hormone IAA. Additionally, the most antagonistic *P. putida* strain AS04 was able to produce phosphatase on Pikovskaya's agar plates. Low levels of soluble phosphate can limit the growth of plants. Some plant-

growth promoting bacteria solubilize phosphate from either organic or inorganic bound phosphates, thereby facilitating plant growth (Lugtenberg and Kamilova, 2009). Additionally, it has been documented that IAA production by bacteria associated with plants enhances the development of host plant root system, thereby favouring the growth of crop plants (Patten and Glick, 2002). Plant growth promoting rhizobacteria (PGPR) primarily fluorescent pseudomonads which are aggressive root colonizers and possess ability to solubilise phosphates and produce IAA, play an important role in the biological control of plant diseases caused by soil-borne fungal pathogens (Gupta et al., 2002). Yasmin et al. (2009) isolated 15 rhizobacterial strains from sweet potato rhizoshpere including *Pseudomonas*, *Serratia* and *Klebsiella* which produced phosphatase, IAA and siderophores. These strains were antagonistic against *Rhizoctonia* sp. and *Pythium* sp. Ramyasmruthi et al. (2012) isolated 18 bacterial strains from the rhizophere of brinjal, capsicum and chilli out of which *Pseudomonas fluoresecens* isolate R which controlled *Colletotrichum gloeosporioides* causing anthracnose in chilli was found to produce both phosphatase and IAA. Gupta et al. (2002) isolated 12 fluorescent *Pseudomonas* strains from the rhizosphere which showed a strong antagonistic effect against *Macrophomina phaseolina*, a charcoal rot pathogen of peanut. Of the 12 strains, 11 produced high amount of IAA. Due to its IAA-producing ability, it was found to be effective for promoting the growth of peanut plants. Other microbial by-products like hydrogen cyanide may also contribute to pathogen suppression by blocking the cytochrome oxidase pathway and is toxic to all aerobic microorganisms at picomolar concentrations (Ramette et al., 2003; Kumar et al., 2005; Senthilkumar et al., 2009).

Scanning electron microscopic studies of the interacting zones of the antagonistic bacteria (strains AS01 and AS04) and plant pathogenic fungi (*F. solani* and *F. equiseti*) revealed severe mycelial deformities of the pathogens during the present study. Bacterial cells were found attached to the hyphal surface. Degeneration of cell wall, lysis of mycelia and subsequent release of cell contents was observed. Lim et al. (1991) reported

abnormal hyphal swelling and lysis of hyphae in *F. solani* when co-cultured *in vitro* with the biocontrol strain YPL-1 of *Pseudomonas stutzeri*. Severe mycelial deformations of *Curvularia lunata* caused by *Bacillus* sp. strain BC121 was observed by Basha and Ulaganathan (2002). Senthilkumar et al. (2007) have also reported several structural deformities like hyphal lysis and bulging of the mycelium of *Rhizoctonia bataticola* caused by *Paenibacillus* sp. HKA-15. Arora et al. (2008) observed lysis, distortion, swelling in hyphae of the pathogen *P. capsici* taken from the inhibition zone in microscopic studies on the antagonism of the chitinase producing fluorescent *Pseudomonas* isolates PGC1 and PGC2.

The present siderophore producing antagonistic isolates were found to produce a number of lytic enzymes including chitinase, protease and lipase. Cellulase and pectinase activity was not observed in any of our isolates which can be regarded as a desirable trait because the production of cellulase and pectinase is considered an undesirable characteristic of plant beneficial bacteria (Cattelan et al., 1999). Lack of hydrogen cyanide production is another beneficial trait present in our isolates for HCN is considered to inhibit plant growth and yield due to the interference with cytochrome oxidation (Bakker and Schippers, 1987). The other experimental evidences such as the inhibitory activity of the extracellular crude products as well as the cell wall degradations observed through electron microscope indicate that exoenzymes produced by the present isolated strains can play a crucial role in limiting the growth of the phytopathogens.

CHAPTER 5

Characterization and Purification of Siderophores

CHARACTERIZATION AND PURIFICATION OF SIDEROPHORES

5.1. Introduction

Pyoverdine, the siderophores secreted by fluorescent pseudomonads, is composed of 3 distinct parts, a dihydroxyquinoline chromophore, a variable peptide arm consisting of 6 to 12 amino acids and a dicarboxylic acid (or its cognate monoamide) which is attached to the chromophore. Pyoverdine contains both catechol and hydroxamate functional groups to coordinate iron binding (Meyer, 2000; Cornelis and Matthijs, 2002; Ballouche et al., 2009). Pyoverdines are secreted from the bacterial cell and chelates ferric iron in the environment mainly by the hydroxamate and hydroxyacid groups present within the peptide moiety of the molecule (Loper and Henkels, 1999; Ravel and Cornelis, 2003; Visca et al., 2007).

Detection of siderophores is most readily achieved in iron limited media, which generally means either a synthetic (minimal) recipe or introduction of a complexing agent that will render the iron selectively unavailable (Neilands, 1995). Although most siderophores are either hydroxamates or catecholates, earlier tests based on such functional groups proved unreliable since they are absent from a few siderophores. The chrome azurol sulfonate (CAS) assay has become widely used since it is comprehensive, exceptionally responsive, and convenient (Schwyn and Neilands, 1987). The CAS assay may be applied on agar surfaces or in solution. It is based on the colour change that accompanies transfer of the ferric ion from its intense (extinction coefficient of at least 100,000) blue complex to the siderophore. Since siderophores differ substantially in structure, no uniform procedure is available for their isolation. Most are water-soluble, and it is thus usually expedient to drive the siderophore into an organic solvent, such as benzyl alcohol or phenol-chloroform, in order to eliminate salt (Buyer et al., 1993; Neilands, 1995).

The antimicrobial activity of siderophores can have significant ecological effects. For example, the siderophores of fluorescent pseudomonads are

responsible for antagonism toward various strains of fungi and some *Pseudomonas* spp. that are pathogenic to plants (Buyer and Leong, 1986). In addition, microbial siderophores can serve as iron sources for plants (Bar-Ness et al., 1991; Robin et al., 2007), and the production of a siderophore by *Pseudomonas putida* has been shown to enhance the yield of potato tubers (Bakker et al., 1986). Sometimes bacteria secrete multiple siderophores where each siderophore probably has a specific role in metal acquisition. One molecule may be important for the acquisition of iron, while another may be responsible for transport of some other metal. Pyochelin, one of two siderophores produced by *Pseudomonas aeruginosa* PAO1, has a relatively low affinity for iron. However, pyochelin binds a variety of metals (Cuppels et al., 1987), and regulation of pyochelin synthesis correlates with its relative affinity for Mo(VI), Co(II), and Fe(III) (Visca et al., 1992). Pyoverdine, the other siderophore produced by PAO1, demonstrates a binding affinity and a regulatory response typical of a transport molecule specific for iron (Wendenbaum et al., 1983).

A number of pseudobactins/pyoverdines have been isolated and characterized from several fluorescent *Pseudomonas* species (Budzikiewicz, 1993; Khalil-Rizvi et al., 1997). Purification of the hydroxamate-type siderophore generally includes passing acidified culture supernatant through an amberlite column (XAD-2 or XAD-16), followed by passing the concentrated siderophore through a Sephadex LH-20 hydrophobic column and finally through HPLC on a C18 hydrophobic column (Buyer et al., 1993; Storey, 2005). In the present study, the siderophores produced by all the 20 selected antagonistic isolates were characterized by the standard assays for hydroxamate, catecholate and carboxylate types. Then the hydroxamate siderophore produced by the *Pseudomonas putida* strain AS04 was separated by first passing through amberlite coloumn and then through Sephadex LH-20 hydrophobic column. Before undertaking the purification procedure, the siderophore production by the strain was optimized under different media supplements, temperatures and incubation periods. The most suitable condition for siderophore production was maintained for the

purification process. The partially purified siderophore was scanned in a UV-VIS spectrophotometer.

5.2. MATERIALS AND METHODS

5.2.1. Media for siderophore production

Since siderophores are mainly produced under iron-limiting conditions, the medium used for siderophore production was prepared with restricted amount of iron. Fiss-glucose minimal media (Vellore, 2001) was used as an iron-restricted media. Media was prepared by dissolving 5.04 g KH_2PO_4 and 5.04 g L-asparagine in 960 ml deionized distilled water (DDW) and pH was adjusted to 6.8. After autoclaving, 10 ml of each of the following solutions (each autoclaved separately) was added to the 960 ml sterile media: 50% glucose, 0.005% $ZnCl_2$, 0.001% $MnSO_4$ and 0.4% $MgSO_4.7H_2O$. To minimize the trace amount of iron, all media components were prepared with DDW and all glassware used for media storage and for growth of the culture were treated with concentrated HNO_3 and rinsed with DDW.

5.2.2. Detection of hydroxamate siderophores

The iron-restricted Fiss-glucose minimal media was inoculated with a 24 h culture (NA) of each of the selected 20 bacterial isolates and incubated at 30°C for 30 h. Following incubation, the culture was centrifuged at 10,000 rpm for 15 min to obtain culture supernatant. This supernatant was used for siderophore characterization.

5.2.2.1. Tetrazolium test

Detection of hydroxamate siderophore was carried out following the method of Snow (1954). This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate groups using a strong alkali. The reduction and the release of alkali shows red color. To a pinch of Triphenyl tetrazolium chloride salt, 1–2 drops of 2N NaOH and 1 ml of the test culture supernatant were added. Instant appearance of a deep red colour indicated hydroxamate siderophores.

5.2.2.2. Ferric chloride test

Following the method described by Neilands (1981) hydroxamate siderophore production was confirmed by $FeCl_3$ test. To 1 ml of culture supernatant, 1ml of 2% ferric chloride solution was added. The formation of red or purple colour indicated the presence of siderophore. A peak between 420 and 450 nm of ferrated siderophores indicated its hydroxamate nature.

5.2.3. Detection of catecholate siderophores

For detection of catecholate siderophore, Fiss-glucose minimal media was inoculated with all bacterial isolates and incubated at 30°C for 30 h. Tests were conducted with the culture supernatant obtained from each isolate and used for siderophore characterization.

5.2.3.1. Arnow's test

Arnow's method (Arnow, 1937) was used to determine whether the siderophore produced by the isolated bacterial strains was of catecholate type. This test is performed by mixing the following in order: 1 ml culture supernatant, 0.1 ml 5M HCl, 0.5 ml Nitrite-Molybdate reagent and 0.1 ml 10N NaOH. These were allowed to incubate for 5 minutes for the reaction to occur completely. Catechol gives a yellow color when reacted with nitrous acid and changes to an intense orange-red when made strongly basic (pH 10) (Holzberg and Artis, 1983). Absorbance was measured at 515 nm.

5.2.3.2. Ferric chloride test

The ferric chloride test for detection of catecholate siderophores was performed following the method of Neilands (1981). One ml of culture supernatant was added to 1ml of 2% $FeCl_3$ solution and the absorption maxima at 495 nm indicated the presence of catecholate siderophore.

5.2.4. Detection of Carboxylate siderophores

Spectrophotometric test was conducted following the methodology of Shenker et al., (1995). To 1 ml of culture supernatant, 1 ml of 250 µM $CuSO_4$ and 2 ml of acetate buffer (pH 4) were added. The solution was

scanned in the wavelength range of 190–280 nm and was observed for the peak of absorption of siderophores. An uninoculated medium was kept as blank. The copper complex shows absorption maximum between 190 and 280 nm.

5.2.5. Optimization of siderophore production

Conditions can be optimized to achieve the maximum amount of siderophore production. Hence, before attempting to purify the siderophore compound, optimization is necessary in order to acquire a higher yield of the compound. Of the 20 siderophore producing isolates used in the present study, the *Pseudomonas putida* strain AS04 produced maximum siderophore units in the quantitative test. Therefore, AS04 was selected for optimization and purification experiments.

5.2.5.1. Siderophore production in different media suppliments

Different media combinations were tried in order to optimize siderophore production; Fiss-glucose minimal medium was used as the base which was supplemented with four different carbon and nitrogen sources either individually or in combination. The alternate carbon sources included sucrose (1%) and mannitol (1%). The additional nitrogen sources were $(NH_4)_2SO_4$ and NH_4Cl (1% each). Fiss-glucose medium was supplemented separately with 1% maltose, 1% mannitol, 0.1% $(NH_4)_2SO_4$, 0.1% NH_4Cl, 1% sucrose + 0.1% $(NH_4)_2SO_4$, 1% sucrose + 0.1% NH_4Cl, 1% mannitol + 0.1% $(NH_4)_2SO_4$ and 1% mannitol + 0.1% NH_4Cl. Supplemented media (500 ml) were distributed equally into five 250ml Ehrlenmeyer flasks and sterilized. The flasks were inoculated with a 24 h NA culture of AS04 and incubated for 36 hours at 30°C on a rotary shaker. Following incubation, the cultures were centrifuged at 10,000 rpm for 15 minutes and the culture supernatant was used to measure the percent siderophore units produced (described in section 4.2.1).

5.2.5.2. Siderophore production after different periods of incubation

Fiss-glucose minimal medium (200 ml) taken in two 250 ml flasks (100 ml each) was inoculated with 1.5 ml of a 24 h NB culture of AS04. The culture was grown for a maximum of 40 hours, approximately 5 ml aliquots were removed at each 2-hour interval ranging from 0-40 hours and growth was measured at OD 600 nm. Next, the culture aliquot was centrifunged and culture supernatant was collected for estimation of siderophore.

5.2.5.3. Siderophore production at different incubation temperatures

Siderophore production was assessed after different periods of incubation. Fiss-glucose minimal medium was prepared in six 250 ml flasks holding 50 ml each, sterilized and inoculated with 24 h culture of AS04 in NA. The flasks were incubated at different temperatures (4°, 20°C, 30°C, 37°C, 43°C and 55°C). After 30 hours, growth was measured and the siderophore produced was estimated for each culture and values were recorded.

5.2.6. Purification of siderophore

For partial purification of siderophore, large volumes of culture were grown in the optimized Fiss-glucose medium. Typically it was done by preparing 5 litres of medium and dispensing 150 ml of it in each 500 ml conical flask. A seed culture was grown by inoculating AS04 strain in the same medium and incubated overnight. This fresh culture was used as the seed inoculum to inoculate each flask with 1.5 ml medium. All of the conical flasks were incubated for 30 hours at 30°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 15 minutes. The supernatant was then acidified to pH 2.0 with 6M HCl in order to make the siderophore less soluble in water.

5.2.6.1. Amberlite XAD-2 chromatography

The acidified supernatant of AS04 bacterial culture was passed through a 30X5 cm column packed with Amberlite XAD-2, which binds cyclic compounds. Prior to this, the column was prepared by suspending approximately 60 g of XAD-2 in DDW and the mixture was kept at room

temperature overnight so that the material can completely absorb water. The column was then packed (approximately 20 cm) with the prepared XAD-2 and it was equilibrated with four bed volumes of DDW. Next, the acidified supernatant was passed through the column and the flow-through was collected. When all supernatant had been run, the column was washed with two bed volumes of DDW. This DDW-wash was also collected in a separate container. The column was then eluted with approximately 250 ml of methanol. When the fraction coming out of the column appeared yellow and the flow rate increased, it indicated that only methanol was present in the fraction. This fraction was also collected separately as fraction No. 2. Fractions were collected until the flow-through became colourless. Altogether five 50 ml fractions were collected. The column was then washed with four bed volumes of methanol, followed by four bed volumes of DDW to re-equilibrate the column.

The flow-through, DDW-wash, and all fractions were collected were evaluated for their siderophore content by Tetrazolium salt test. Fractions showing positive result for the siderophore were combined in a 250 ml round-bottom flask and dried on a rotary vacuum evaporator (Eyela, A-1000S, Japan), keeping the temperature at 25°C. The concentrated sample was re-dissolved in 5 ml methanol and stored at -20°C till further purification.

5.2.6.2. Sephadex LH-20 chromatography

Sephadex LH-20 is a material that separates compounds based on their hydrophobicity. It was prepared by suspending 5.0 g LH-20 in methanol and stirring for around 20 minutes. The material was then packed into a 50 x 1.5 cm column (almost upto the top of the column) and was equilibrated with four bed volumes of methanol. The 5ml concentrated sample was loaded on the column and eluted with methanol. Approximately 20 fractions (3ml each) were collected and tested for presence of siderophore by performing thin layer chromatography (TLC). Fractions positive for siderophore were combined in a 100 ml round bottom flask and evaporated to dryness using a rotary vacuum evaporator. The dried sample was then

re-dissolved in 2-3 ml methanol. The sample was stored at –20°C until further studied.

5.2.6.3. Thin layer chromatography

Different fractions of Sephadex LH-20 column were tested for the presence of siderophore by TLC. Concentrated samples of siderophore were spotted on 7X4 cm^2 precoated TLC silica gel 60 F_{254} plates (Merck, India) and were allowed to dry. The plates were developed in n-butanol:acetic acid:DW (12:3:5) until the solvent front reached the top of the plate. Plates were then dried and sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl. The formation of a brown spot indicated a hydroxamate-type siderophore (Storey, 2005).

5.2.6.4. Spectral scan analysis

Spectral scan analysis was done following the method described by (Jalal and van der Helm, 1991). A spectral scan in 300-700 nm was done on the partially purified siderophore sample to characterize the type of hydroxamate. A dihydroxamate shows absorption maxima in the range of 500-520 nm and that of trihydroxamate is in the range of 420-440 nm.

5.3. RESULTS

5.3.1. Detection of siderophores

All the isolates were tested for the type of siderophore produced by each strain. Results, summarized in Table 15, showed that only one type of siderophore was produced by each of the strains.

5.3.1.1. Hydroxamate

Altogether 15 strains out of the 20 tested showed positive result in the tetrazolium test. A red colour appeared immediately indicating the presence of siderophore in the culture supernatants (Fig. 25a). The strains that tested positive were MD01, CB02, AS01, AS04, BB05, BB07, CR07, CR10, CR12, CR13, MB01, MB05, NG04, NG05 and NG07. In the $FeCl_3$ test, a spectral scan showed peaks at 420-440 nm wavelength which confirmed

hydroxamate production by all the 15 strains tested positive in the Tetrazolium test.

5.3.1.2. Catecholate

Arnow's test detected the presence of catecholate siderophore in the culture supernatants of 5 strains out of the 20 tested (Fig. 25b). The strains that tested positive were CR04, CR14, JL11, KT05 and MB02. A spectral scan showed peaks at 495 nm which confirmed catecholate production by all these strains tested positive in the Arnow's test.

Table 15: Types of siderophore produced by bacterial isolates[a]

Bacterial strains	Type of siderophore		
	Catecholate	Hydroxamate	Carboxylate
BB05	-	+	-
BB07	-	+	-
JL11	+	-	-
MD01	-	+	-
CB02	-	+	-
AS01	-	+	-
AS04	-	+	-
CR04	+	-	-
CR07	-	+	-
CR10	-	+	-
CR12	-	+	-
CR13	-	+	-
CR14	+	-	-
MB01	-	+	-
MB02	+	-	-
MB05	-	+	-
NG04	-	+	-
NG05	-	+	-
NG07	-	+	-
KT05	+	-	-

[a] : '+': positive; '-': negative.

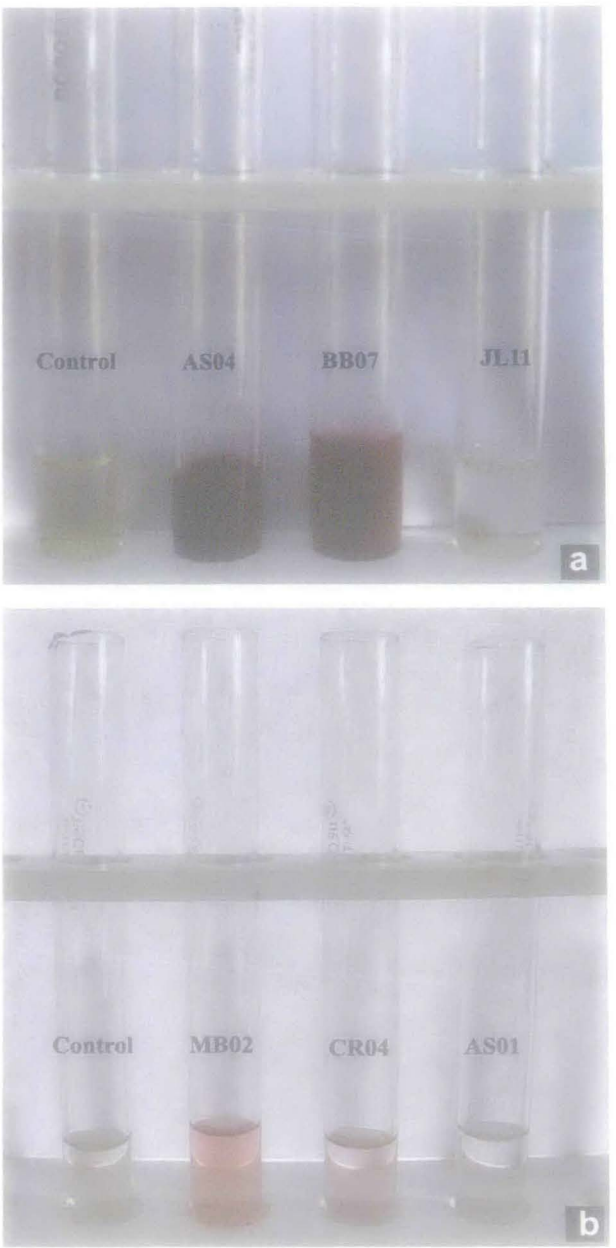


Fig.25: Characterization of siderophore:

(a)Tetrazolium salt test showing immediate red colouration indicating presence of hydroxamate type siderophore produced by bacterial isolates AS04 and BB07. Absence of red colour indicating negative result by isolate JL11 when compared to control;

(b) Arnow's test showing pink colouration indicating the presence of catecholate type of siderophore produced by the isolates MB02 and CR04. Colourless medium indicating negative test by the isolate AS01 when compared to control.

5.3.1.3. Carboxylate

A spectral scan in the 190-280 nm wavelength region did not show characteristic peak indicating that none of the isolated bacterial strains produced carboxylate type of siderophore.

5.3.4. Optimization of siderophores production

5.3.4.1. Media components

In order to achieve maximum siderophore production, the Fiss-glucose minimal medium which was used in the preliminary characterization was required to be optimized. A variety of media combinations were tried to optimize siderophore production. Fig. 26a depicts a graphical representation of the results, which summarizes the effect on siderophore production of each media type tried based on the CAS assay for estimation. The addition of all the media supplements showed some increase in the amount of siderophore production (Table 16). Addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media showed maximum increase in the amount of siderophore produced (from 59.70 to 89.04% units). Next to it, the combination of 1% mannitol and 0.1% NH_4Cl increased the amount of siderophore produced to 81.86% units. Sucrose was better carbon source than mannitol and $(NH_4)_2SO_4$ was better than NH_4Cl as a nitrogen source when tested individually.

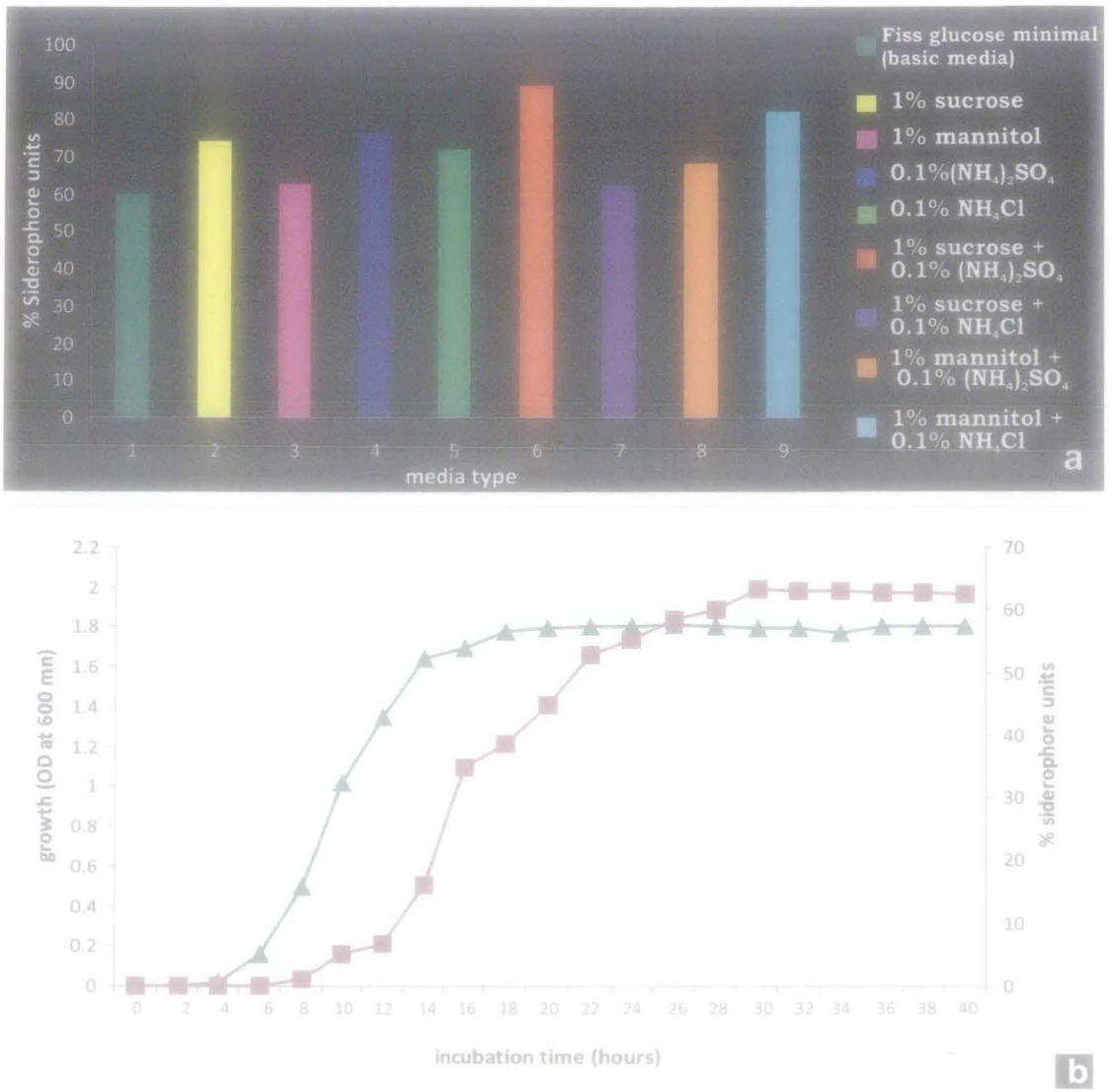


Fig 26: (a) Siderophore production by the bacterial isolate, *Pseudomonas putida* strain AS04 in presence of various medium supplements;

(b) The effects of incubation time on growth (-▲-) and siderophore production (-■-) by *Pseudomonas putida* strain AS04.

Table 16: Siderophore production in various media supplements

Medium Supplements	% Siderophore units
Fiss glucose minimal (basic media)	59.70±0.47
1% sucrose	74.21±0.67
1% mannitol	62.89±0.73
0.1% $(NH_4)_2SO_4$	76.49±0.84
0.1% NH_4Cl	71.92±0.82
1% sucrose + 0.1% $(NH_4)_2SO_4$	89.04±0.62
1% sucrose + 0.1% NH_4Cl	62.00±0.56
1% mannitol + 0.1% $(NH_4)_2SO_4$	68.24±0.67
1% mannitol + 0.1% NH_4Cl	81.86±0.72

5.3.4.2. Incubation time

In order to determine the optimum incubation time for maximum siderophore production by the isolate AS04, the culture was grown for a maximum of 40 hours, and both growth and siderophore production were measured at each 2-hour intervals, starting from zero hour. Fig. 26b shows the growth curve for strain AS04 along with the amount of siderophore produced at the various incubation times measured. Results indicated that AS04 begins producing siderophore after 8 hours post-inoculation, with maximum production occurring at 30 hours (Table 17).

5.3.4.3. Incubation temperature

As described previously, *Pseudomonas putida* strain AS04 was grown at varying temperatures (4°C, 20°C, 30°C, 37°C, 43°C and 55°C) to assess its effects on siderophore production. Figure 27 and Table 18 shows that both growth and siderophore production were highest at 30°C.

Table 17: Siderophore production at different incubation time

Incubation time (h)	Growth (OD at 600 nm)	% Siderophore units
0	0.000	0.00
2	0.002	0.00
4	0.019	0.00
6	0.160	0.00
8	0.498	01.06
10	1.023	05.03
12	1.345	06.78
14	1.664	16.01
16	1.698	34.76
18	1.780	34.64
20	1.782	44.78
22	1.800	52.87
24	1.802	55.34
26	1.811	58.34
28	1.803	60.12
30	1.799	63.23
32	1.797	62.97
34	1.768	62.78
36	1.807	62.79
38	1.806	62.79
40	1.806	62.45

Table 18: Optimization of incubation temperature for highest siderophore production

Incubation temperature (°C)	Growth (OD at 600 nm)	% Siderophore units
4	0.006	0.00
20	1.528	22.62
30	1.842	61.34
37	1.383	37.81
43	0.294	07.43
55	0.011	02.09

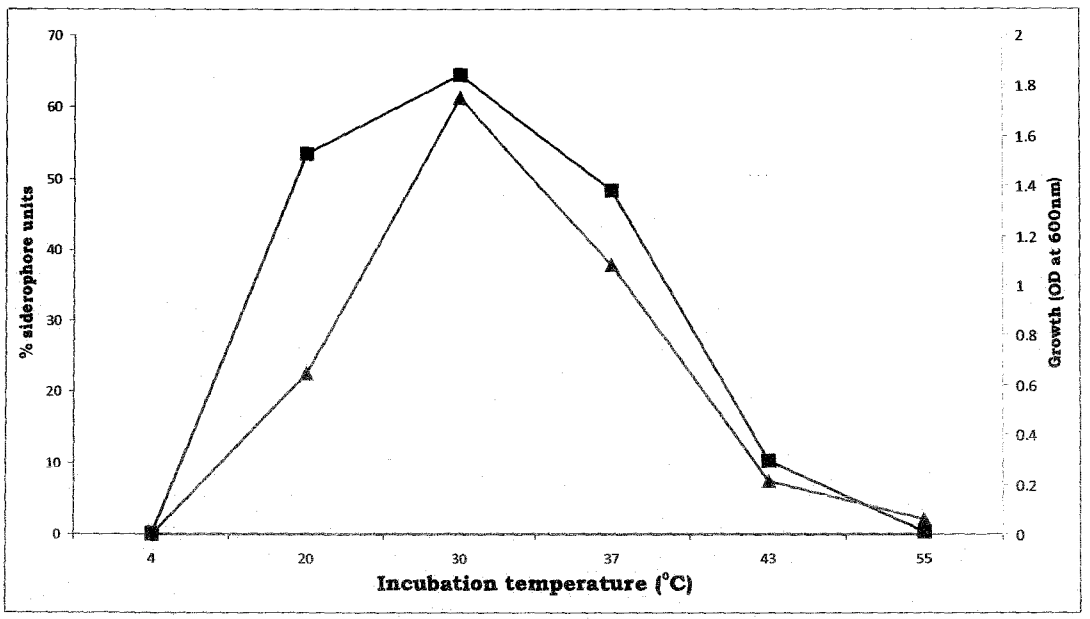


Fig.27: Optimization of siderophore production by *Pseudomonas putida* strain AS04 at various incubation temperatures.

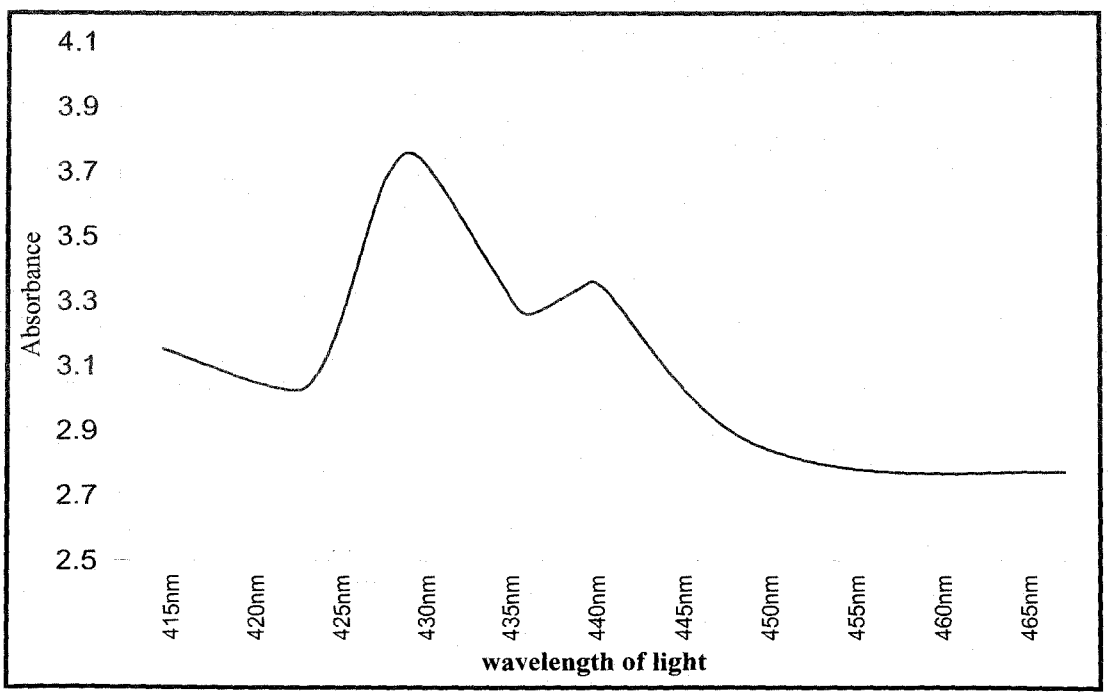


Fig. 28: Spectral scan of partially purified siderophore of *Pseudomonas putida* strain AS04 showing a peak at 430 nm.

5.3.6. Purification of siderophore

5.3.6.1. Amberlite XAD2 chromatography

On Amberlite XAD-2 chromatography several fractions were collected. The first fraction was mostly water and colourless. Fraction 1 was colourless, fractions 2 and 3 were dark yellowish brown in colour and had a putrid odour, and fractions 4 and 5 were light yellow in colour (Fig. 29). The flow-through, DDW wash, and all fractions collected were tested for their siderophore content using Tetrazolium salt test. Fractions 2 and 3 showed the highest amount of siderophore content based on intensity of the colour.

5.3.6.2. Sephadex LH20 chromatography

The fractions tested positive for sideophore content were pooled and concentrated and were further purified by Sepladex LH20 chromatography. Altogether 20 fractions were collected and each fraction was monitored for siderophore content by TLC. Initial fractions were colourless. Fractions 8-11 were pale yellow, the colour intensity increased gradually and the later fractions (fractions 14-19) were brown.

5.3.6.3. TLC

The formation of brown spot by spraying with $FeCl_3$.HCl indicated a hydroxamate-type siderophore. It was observed that fractions 17, 18 and 19 produced intense brown spots (Fig. 30) thus confirming presence of hydroxamate siderophore.

5.3.6.4. Spectral scan analysis

A spectral scan (300-700 nm) with the partially purified siderophore showed a major peak at 430nm (Fig. 28) that confirmed that AS04 produced trihydroxamate type of siderophore.

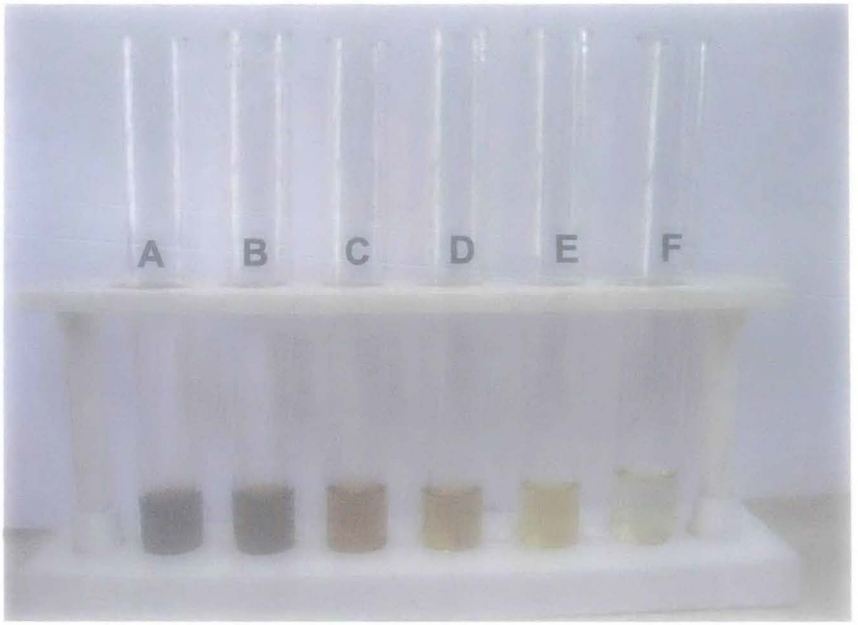


Fig. 29: Tubes with increasing colour intensity, containing Amberlite XAD-2 fractions collected and tested for their siderophore content; A: fraction 2, B: Fraction 3, C: Fraction 4, D: Fraction 5, E: flow-through, F: DDW wash.

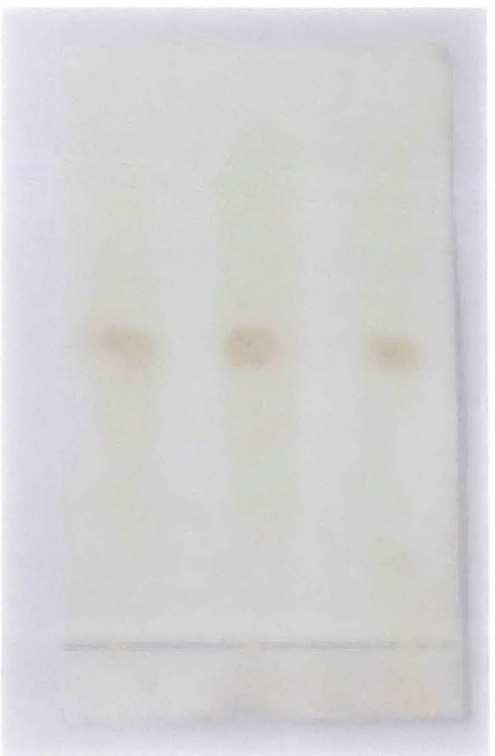


Fig.30: Detection of hydroxamate siderophore as intense brown spots using Thin Layer Chromatography.

5.4. DISCUSSION

The screening of antagonistic bacteria from rhizosphere soil during the present study was based on siderophore production in CAS agar medium. The siderophores produced by each strain was quantified by the CAS shuttle assay and *Pseudomonas putida* strain AS04 was found to be the highest producer of siderophore. In this chapter the siderophore produced by each of the selected antagonistic isolates were chemically characterized and the siderophore produced by strain AS04 was partially purified and analysed by UV spectrophotometry.

Studies on siderophore characterization form a wide array of microorganisms are well documented. The universal chrome azurol S (CAS)-agar plate assay (Schwyn and Neilands, 1987) is the most well-known and widely used method for detection of siderophore production by microorganisms in solid medium (Machuca and Milagres, 2003). This assay is based on a competition for iron between the ferric complex of an indicator dye, chrome azurol Sulphonate (CAS), and a chelator or siderophore produced by microorganisms. The iron is removed from CAS by the siderophore, which apparently has a higher affinity for iron (III). The most positive reaction results in a colour change of CAS-reagent (usually from blue to orange). The blue dye can be applied as a solution test, or alternatively, it can be incorporated in the solid growth medium and used for screening several microorganism siderophore producers by direct plating mode. The authors concluded that the direct plating mode for screening microorganisms is probably applicable to all Gram-negative bacteria. Thus in our study we used siderophore screening which resulted in the isolation of most of the culturable gram negative bacteria. However, Schwyn and Neilands (1987), observed that the detergent hexadecyltrimethyl-ammonium bromide (HDTMA) used in preparation of the CAS medium may be toxic to some microorganisms, especially gram positive organisms and fungi. Thus it was not surprising that in our study, most of the isolates, post-screening were gram negative. We found that only six bacteria out of the twenty selected were gram positive and all of them belonged to the genus *Bacillus*.

Of the twenty antagonistic isolates, 15 strains have been found to produce hydroxamate type of siderophore while the rest 5 produced catecholate type. None of the strains produced carboxylate siderophore. The methodologies followed for the characterization tests; Arnow's test, Neiland's $FeCl_3$ test and the tetrazolium salt test are universally accepted. Several researches have been conducted on characterizing siderophores from fluorescent pseudomonads. Bano and Musarrat (2003) characterized the siderophore produced by *Pseudomonas aeruginosa* NJ-15 and identified it as hydroxamate-type. Chaiharn et al. (2009) isolated siderophore producing antagonistic strains against rice pathogens *Fusarium oxysporum* and *Pyricularia oryzae*. Characterization of siderophores showed that among 18 isolates, all except three were hydroxamate type. *Pseudomonas aureofaciens* AR 1 was the best siderophore producer overall and secreted hydroxamate type siderophore. Gupta et al. (2002) characterized the siderophore produced by antagonistic isolate *Pseudomonas* GRC2. The 48-h-old culture filtrate of the strain showed a major peak at 400 nm when tested by the Neiland's $FeCl_3$ method which revealed that the siderophore was of hydroxamate type. Arora et al. (2001) screened 12 rhizobial isolates for siderophore production of which two strains, RMP3 and RMP5 showed hydroxamate type of siderophore. Santos-Villalobos et al. (2012) isolated *Burkholderia cepacia* XXVI from mango rhizosphere which was found to be effective against the mango pathogen, *C. gloeosporioides*. The bacterium was found to produce siderophore that was involved in the antagonistic activity. The class of siderophore produced by this strain was identified using colorimetric test (Tetrazolium test) and spectrophotometric assays ($FeCl_3$), showing that this metabolite belongs to hydroxamate class, due to the appearance of a deep red colour and the presence of an only peak at 420 nm, respectively.

In the present study, *Pseudomonas putida* strain AS04 was selected for further studies on siderophore characterization and purification. Siderophore production was optimized in order to select the most suitable carbon and nitrogen source that would be best for siderophore production. The amount of siderophore produced increased from 59.70 to 89.04% units

on addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media. When tested individually, sucrose and $(NH_4)_2SO_4$ was better than mannitol and NH_4Cl as carbon and nitrogen sources respectively. The optimum incubation time was evaluated for determining the appropriate time for siderophore extraction and to determine when siderophore production begins in this organism. Results revealed that AS04 begins producing siderophore after 8 hours post-inoculation, with maximum production occurring at 30 hours. Similarly, the optimum incubation temperature recorded for growth and siderophore production were 30°C. Siderophores are secondary metabolites and a culture may begin producing siderophore at the later phases of growth, with production increasing as cultures are grown for longer periods (Storey, 2005). In the stationary phase, the amount of siderophore was increased due to a state of iron starvation attributed to the consumption of this element in the culture medium as a result of bacterial multiplication (Crosa, 1997; Lim et al., 1998; Cowart, 2002; Trivedi et al., 2008).

Siderophore production by the biological control strain *Pseudomonas aeruginosa* was optimised by Villegas et al. (2002). The highest metabolite concentration was obtained in glucose and glutamic medium. Kanimozhi and Perinbam (2011) investigated siderophore mediated antagonistic activity of *Pseudomonas fluorescens* Lp1 isolated from soil against common fungal pathogens of plant. The authors optimized the siderophore production under varied physiochemical conditions and also partially purified the siderophore. The siderophore production was optimum during the incubation time of 24 to 30 h at neutral pH. The yield of 73.0% was obtained in a medium amended with malic acid among organic acids tested, the organic nitrogen source peptone yielded 72.5% and inorganic source urea yielded 75.5%.

Once the growth conditions had been optimized in the present study, it was then possible to produce large amounts of siderophore by growing *P. putida* AS04 in batch cultures. Accordingly, about 5 litres of culture was grown under the optimized conditions and the siderophore was partially purified

by passing acidified supernatant through an XAD-2 column, followed by passing the concentrated siderophore through a Sephadex LH-20 hydrophobic column. At each stage of purification, the tetrazolium salt test was done to track the siderophore. During and after purification through the Sephadex LH-20 column, the concentrated fractions were tested for presence of siderophore in TLC plates. A single brown spot was obtained at each stage indicating the presence of hydroxamate siderophore.

Spectral scan of the purified siderophore showed a peak at 430 nm. Spectral scans (300-700 nm) can indicate whether a dihydroxamate or a trihydroxamate- type siderophore is present (Jalal and van der Helm, 1991). Spectral scans of the purified siderophore isolated from *P. putida* AS04 indicated that it was of trihydroxamate type.

Purification of siderophores from rhizobacteria has been well documented in literature. Kanimozhi and Perinbam (2011) partially purified the siderophore produced by the antagonistic strain *Pseudomonas fluorescens* Lp1 by passing the acidified culture supernatant through an ion exchange chromatographic column containing Amberlite IR120 (Na+) and eluting the siderophore with 50% methanol. Sokol et al. (1992) isolated a novel iron-binding compound named azurechelin from the ethyl acetate extracts of the supernatants from *Pseudornonas cepacia* cultures by Sephadex G-10 column with aqueous methanol 10% v/v as the mobile phase. The structure was determined by UV-VIS spectroscopic analysis. The authors concluded that it was a novel compound with neither the typical characteristics of catechol nor of hydroxamate compounds. Buyer et al. (1993) purified the siderophore of plant-growth-promoting *Pseudomonas* strain B10 by passing the culture supernatant through a column of Amberlite XAD-16 and the red-brown material was eluted with 1:1 water-methanol. The resultant was purified further by gel filtration and identified by a specific ELISA protocol. This methodology was extended to detecting siderophores in the rhizosphere of barley. Fekete et al. (1989) purified hydroxamate siderophore from *Azotobacter chroococcum* B-8 by Sephadex G-10-120 size exclusion column using a 50% methanol-water mobile phase. The orange fractions were

pooled and evaporated under vacuum to about 1 ml before injection of 250-pLI portions onto a reverse-phase high-performance liquid chromatography. Storey (2005) purified hydroxamate siderophore from *R. leguminosarum* IARI 917 by using XAD-2 column followed by Sephadex LH-20 chromatography and HPLC. The siderophore thus purified was analysed by mass spectrometry and the structure was determined as the dihydroxamate siderophore schizokinen.

In the present study characterization of the siderophores from all twenty isolates showed that most were hydroxamate type. The siderophore production from the strain *P. putida* AS04 which recorded highest production during preliminary studies was optimized for three parameters, media composition, temperature and incubation time. The optimized conditions were used for bulk culture of the strain for partial purification of the siderophore. Purification was done by affinity chromatography and LH-20 chromatography that separates compounds based on hydrophobicity, and the resultant compound produced a peak at 430 nm at ferrated state. The compound was identified to be of trihydroxamate type.

CHAPTER 6

In vivo Evaluation of *Pseudomonas putida* Strains AS01 and AS04 as Biocontrol Agents Against Wilt in Brinjal

IN VIVO EVALUATION OF *Pseudomonas putida* STRAINS AS01 AND AS04 AS BIOCONTROL AGENTS AGAINST WILT IN BRINJAL

6.1. Introduction

The eggplant or brinjal (*Solanum melongena* L.), also called aubergine, is one of the most widely grown vegetable crop all over the world. Although brinjal is cultivated in all the continents, it is grown extensively in tropical Asia and Mediterranean countries (Sunseri et al., 2003). Brinjal is a major commercial vegetable crop and is grown all over India except at high altitude (Wesley, 1956). Brinjal is of high nutritive value and has been a staple vegetable in our diet since ancient times. Brinjal fruits are considered especially useful for those who have to maintain low calorie diets. It is very useful as a dietary element of old and sick people due to the unparallel taste as well as the presence of a number of phytochemical compounds in its flesh, which protects against cancer and atheromatosis (Adamicki, 1995; Esteban et al., 1992). As it contains large amount of mineral salts, brinjal fruits are effective in strengthening the heart activity and lowering the level of cholesterol in blood (Adamicki, 1995).

The market demand of brinjal is increasing rapidly leading to an increase in the cultivation of the crop into new areas. West Bengal is the largest producer of brinjal in India sharing 24.13 % of the total yield (http://agriexchange.apeda.gov.in/india%20production/India_Productions.aspxhscode=07093000). It is a major vegetable crop and is cultivated in large areas in sub-Himalayan West Bengal. Brinjal is subjected to attack by many fungi, bacteria, viruses, nematodes and insect pests. Fungal diseases are considered to be a major factor limiting the successful cultivation of the crop. Some common fungal diseases of brinjal are damping-off (caused by *Pythium* sp., *Fusarium* sp., *Rhizoctonia solani* etc.), phomopsis blight (caused by *Phomopsis* vexans), anthracnose (caused by *Colletotrichum gloeosporioides*), fruit rot (caused by *Pythium* spp.), southern blight (caused by *Sclerotium rolfsii*), early blight (caused by *Alternaria solani*) etc. (Pandey,

2010; Najar et al, 2011; National Horticultural Board, Govt. of India, 2007-08).

Wilt and root rot caused by several species of *Fusarium* leads to massive loss of the crop (Chakraborty and Chatterjee, 2007, 2008; Joseph et al., 2008; Akhtar et al., 2010). Because of the increasing restriction in the use of chemical fungicides due to concern for the environment and human health, microbial inoculants have been experimented extensively during the last decade to control wilt and other plant diseases (Siddiqui and Shakeel, 2006; Chakraborty and Chatterjee, 2008; Akhtar et al., 2010).

Fluorescent *Pseudomonas* spp. have emerged as the largest and potentially most promising group of microbes involved in the biocontrol of plant diseases (Weller et al., 2007; Couillerot et al., 2009). This comprises of the species that produce the water-soluble yellow-green siderophore pyoverdine, a feature commonly used to distinguish them from other pseudomonads. The fluorescent pseudomonads include *P. aeruginosa, P. syringae, P. putida* and *P. fluorescens* (Bossis et al, 2000; Palleroni, 2008). Besides the fluorescent pseudomonads, there are some other species that are also reported as biocontrol agents. Table 1 lists some selected strains of pseudomonads which have been used successfully to control diseases in plants. Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents. These include the ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances); (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses (Weller, 2007). In addition, pseudomonads are responsible for the natural suppressiveness of some soils to soilborne pathogens (Weller et al., 2002).

Pseudomonas putida strains are known to be excellent root colonizers and can protect plants by the mechasnism of competition for nutrients and niches (Scher and Baker, 1982; Gotz et al., 2006; Validov et al., 2009). The

aim of this work was to evaluate the *in vivo* biocontrol potential of the most promising antagonistic strains isolated during the present study. As brinjal wilt is a major disease in India, therefore the effect of two *Pseudomonas putida* strains AS01 and AS04 in suppressing wilt incidence in brinjal seedlings caused by *F. solani* were studied under green house conditions.

6.2. MATERIALS AND METHODS

6.2.1. Host plant

The experiment for *in-vivo* evaluation of antagonism showed by the selected bacterial isolates was conducted on brinjal, which is a widely cultivated plant in sub-Himalayan West Bengal.

6.2.1.1. Selection of brinjal varieties

Two varieties of brinjal plants were selected for the present study. Selection was done taking into account, the suitability of growing conditions of each variety and also emphasis was given on the choice of the farmers. All the selected varieties were cultivated at the experimental garden of Department of Botany, University of North Bengal.

6.2.1.2. Collection of selected varieties

Seeds of different brinjal varieties were collected from different places. Seeds of Pusa Purple Long (PPL) variety were collected from 'Indo-Japan Hybrid', Siliguri, District- Darjeeling, India. Seeds of Lalita variety and another locally cultivated variety were collected from local market of Matigara, located in the Darjeeling district of West Bengal.

6.2.1.3. Cultivation of selected varieties

Brinjal cultivation needs a warm sheltered sun-drenched position for optimum growth. It also requires plenty of moisture, well drained soil and the optimum pH value ranges from 5.5 to 6.8. A steady temperature of 25-

30°C is required in the growing season for best yield. All these conditions were maintained during cultivation of the varieties.

6.2.1.4. Raising of seedlings

Seeds of both brinjal varieties were sown six weeks before the plants were set in the garden. Seed sowing was done at 1 cm depth in seedbed prepared in 60cm x 30 cm x 7 cm aluminium trays filled with sterile soil. Soil moisture was maintained by periodic spraying of water and the temperature was maintained in between 25-30°C. Soil was not allowed to dry out during the period of germination. After germination, seedbeds were watered.

6.2.1.5. Transplantation

Brinjal plant requires more care than many other plants during the time of transplantation. Before transplantation, plants need to be hardened off for 10 days. During this period, exposure to sunlight was increased and frequency of watering was reduced for slowing down growth rate. Proper care was taken to avoid wilting. After hardening, the plants were transplanted in the earthen pots (size 20 cm diameter x 15 cm height) filled with sterile soil. For biocontrol experiments, transplantation was done according to experimental requirements.

6.2.2. Pathogenicity test of *Fusarium solani* and verification of Koch's postulates

F. solani was selected as test pathogen because of its agronomic importance. The fungus is a severe pathogen causing wilt and major crop loss in brinjal. The present culture was purchased from IARI, New Delhi and used for biocontrol experiments after verification of Koch's postulates. For this, six-week old potted brinjal plants of three different varieties (PPL, Lalita and a locally cultivated variety) were collected from the experimental garden. Five potted plants of each variety were used for the test and five plants were reserved as uninoculated control. Fifteen day old culture of *F. solani* (grown in twenty-five 250 ml flasks with 100ml PDB in each) was harvested and the mycelial mat along with spores was collected by filtration

through sterile muslin cloth. It was mixed with sterile distilled water (50 g of fresh mycelia in 200 ml water). The mixture was blended in a waring blender to fragmentize the mycelial mat. Soil up to 2 inches deep surrounding the stem of the plants were removed carefully and the whole mixture with spore and fragmented mycelial mat were poured in the pots. Removed soils were replaced and the pots were kept in the experimental screen house under normal conditions of light and temperature. The pots were observed regularly for 14 days and watered with sterile water as necessary. The diseased PPL plants were uprooted and damaged regions of roots were cut into 5-10 mm long pieces. The pieces were first washed with SDW and then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 1-3 min and rewashed with SDW. The pieces were finally transferred aseptically to sterile PDA slants. Isolates were examined after 15 days of inoculation and the identity was confirmed after comparing them with the stock culture.

6.2.3. Disease evaluation process

Evaluation of wilt incidence was done after 8 and 16 days following the method of Chen et al. (1995). The plants were uprooted carefully and symptom severity was graded into five disease classes, as follows: 0 = no disease; 1 = 0-25% of the leaves withered; 2 = 26-50% of the leaves withered; 3 = 61-75% of the leaves withered; 4 = 76-100% of the leaves withered. Based on the classes, the disease index was calculated using the following formula: Disease index = Σ [(P × DC) × 100]/ (T × 4); Where P = plants per class, DC = disease class and T = total number of plants. After calculating disease index, the percent efficacy of disease control (PEDC) was calculated using the formula: PEDC= [(Disease index in untreated control – Disease index in treated plants)/Disease index in untreated control] x100 (Purkayastha et al., 2010).

6.2.4. *In-vitro* studies on growth and antagonism of the antagonistic *Pseudomonas putida* strains AS01 and AS04

The *P. putida* strains AS01 and AS04 were selected for green house experiments as they showed maximum antifungal activity *in vitro*. Before proceeding for plant studies, it was considered worthwhile to study the growth kinetics of these bacteria and assess their *in vitro* antagonistic potential with respect to time in liquid dual cultures.

6.2.4.1. Growth kinetics study

Nutrient broth media (5 ml) in sterilized test tubes were inoculated aseptically with 200µl of 24 hour old culture of the bacterial isolates. The tubes were then incubated at 30°C on an orbital shaker and bacterial growth was recorded at a regular interval of one hour by measuring the absorbance at 600nm till stationary phase was reached.

6.2.4.2. Study of *in vitro* antagonism in liquid culture

A study of *in vitro* antagonism was carried out against the fungal pathogen *F. solani* in liquid cultures. For this, PDB media was prepared, dispensed in 250ml conical flasks and sterilized at 121°C for 15 min. A fungal mycelial disc (4mm diameter) was co-inoculated separately with each of the bacterial isolates, AS01 and AS04 to the PDB medium and incubated at 30°C. A control flask was inoculated with fungal pathogen only. Mycelial dry weight was taken at 24 h intervals up to a period of 6 days by straining the media through muslin cloth and then removing the excess media by blotting dry. The mycelia were then dried in hot air oven and dry weight was measured.

6.2.5. *In vivo* studies for management of *Fusarium* wilt in brinjal

6.2.5.1. Preparation of bacterial inoculum

The bacterial isolates AS01 and AS04 were grown separately in nutrient broth at 30°C for 30 h at 120 rpm in a rotary shaking incubator. The culture thus obtained was centrifuged at 7,000 rpm for 10 min. Cell pellets of bacterial culture were suspended in 0.1 M phosphate buffer (pH 7.0) to

obtain a final concentration of 10^8 CFU per ml and 1% carboxy methyl cellulose was added to it which acted as a binder (Nandakumar et al., 2001).

6.2.5.2. Preparation of fungal inoculum

Fungal inoculum for biocontrol experiments was prepared on wheat seeds. The seeds were rinsed and soaked in distilled water for 10-12 h in 500 ml Erlenmeyer flasks. Subsequently, excess water was drained off. The flasks were filled with the imbibed wheat seeds in one-third of its volume, were autoclaved twice in two successive days. Each sterilized flask was inoculated with five mycelial disks taken from 7-day-old PDA cultures of *F. solani* (Leslie et al., 2006). The inoculated flasks were incubated at 28°C for 20 days and shaken every three days to avoid lump formation and allow uniform growth of the fungus. For soil inoculation, 10 g of the infected seeds were mixed per kg soil in the experimental pots.

6.2.5.3. *In vivo* studies for management of *Fusarium* wilt in brinjal

Six-week-old brinjal seedlings were transplanted to earthen pots (20 cm diameter) containing sterilized garden soil and organic manure (1:1), which was pre-treated with fungal inoculum. The bacterial culture (25 ml) was poured carefully at the root (Nandakumar et al., 2001). Severity of disease was recorded by visual observation with reference to the untreated control plants, where no biocontrol bacterium but only the pathogen was applied. For each treatment 10 healthy plants were selected. All experimental plants were kept in the experimental screenhouse under normal light and temperature conditions and watered with sterile water at regular intervals. The other set of treatment was performed by replacing sterilized garden soil with unsterilized garden soil. In this case, unsterile tap water was used for watering the seedlings. All other experimental conditions were kept similar to that of the sterilized set.

6.2.5.4. Statistical analysis

The experiment was replicated thrice and all data from three independent experiments were pooled and average was calculated. Standard error was calculated using the statistical software SPSS version 11.0.

6.3. RESULTS

6.3.1. Verification of Koch's postulates

All inoculated plants of PPL variety showed typical symptoms of wilt after 7 to 12 days which grew severe by 14th day. The locally cultivated variety showed moderate wilt after 14 days but Lalita was almost resistant. The isolated fungal culture was identified as *F. solani*. This reisolation of *F. solani* from infected plants confirmed it as a pathogen of brinjal and thereby the Koch's postulations were verified.

6.3.2. *In vitro* studies

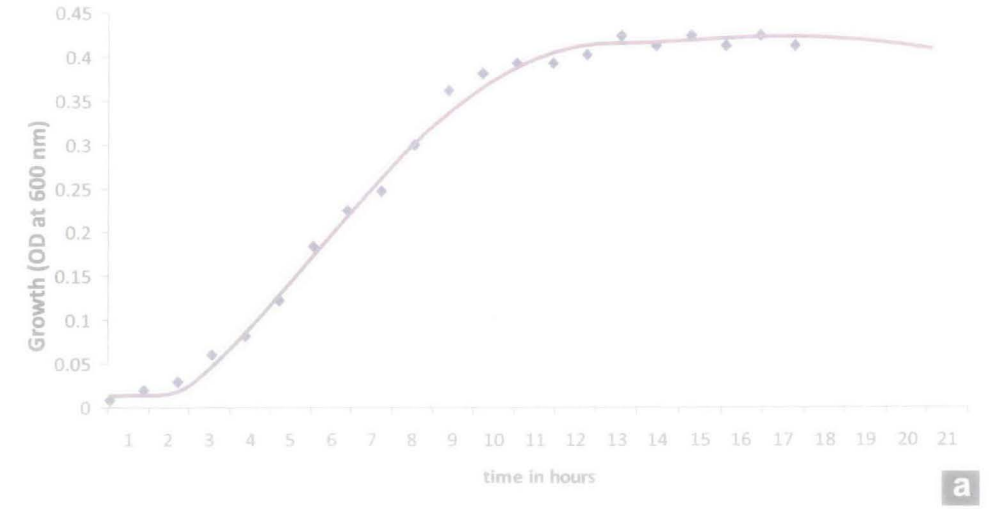
The growth curve of the bacteria is depicted in Fig 31a,b. Both bacteria showed steady increase in OD values upto 15 h and reached a plateau by 16-17 h. There was no significant difference in the growth time and pattern among the two tested strains. In dual cultures, severe restriction of growth of the fungus was noted in flasks inoculated by both the bacteria (Table 19a,b, Fig 31c). The percentage reduction of biomass of *F. solani* after 5 days was found to be 75.72% in AS01 co-inoculated cultures and 71.67% in cultures co-inoculated with AS04. The control flask on the other hand recorded progressive fungal growth upto 5 days after which the growth slowed down. The extent of inhibition in mycelial growth inflicted by the two strains AS01 and AS04 showed little variation when compared to each other.

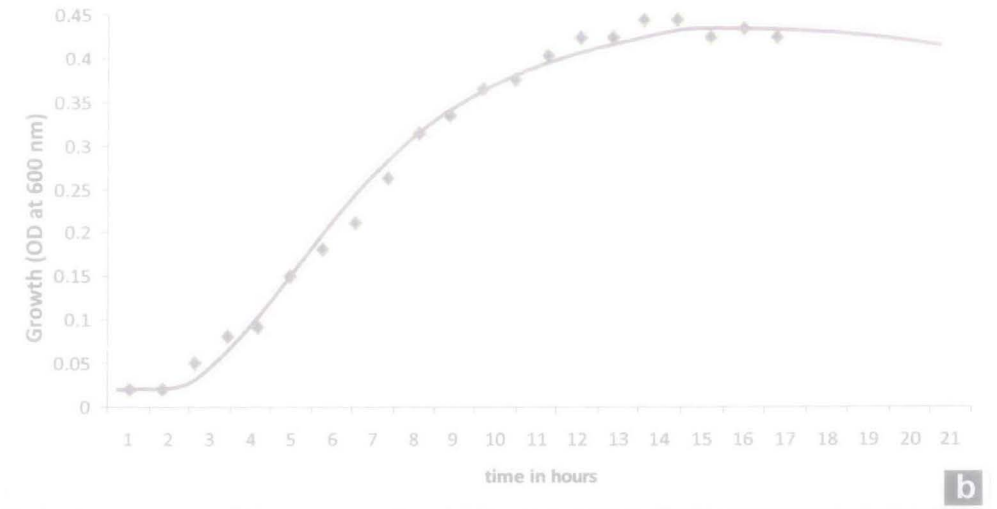
Table 19a: Study of *in vitro* antagonism by *P. putida* strains AS01 against *F. solani* in PDB dual culture assay

Days of incubation	Mycelial dry weight of *Fusarium solani* (g)		% reduction in biomass of *F. solani*
	Control	Inoculated with AS01	
1	1.09±0.03	0.21±0.04	80.73%
2	1.94±0.04	0.39±0.03	79.89%
3	2.83±0.05	0.59±0.04	77.51%
4	4.22±0.04	1.02±0.06	75.82%
5	5.19±0.07	1.26±0.03	75.72%
6	5.26±0.05	1.64±0.04	68.82%

Table 19b: Study of *in vitro* antagonism by *P. putida* strains AS04 against *F. solani* in PDB dual culture assay

Days of incubation	Mycelial dry weight of *Fusarium solani* (g)		% reduction in biomass of *F. solani*
	Control	Inoculated with AS04	
1	1.09±0.03	0.24±0.03	77.98%
2	1.94±0.04	0.40±0.04	79.38%
3	2.83±0.05	0.63±0.04	77.73%
4	4.22±0.04	1.16±0.06	72.51%
5	5.19±0.07	1.47±0.04	71.67%
6	5.26±0.05	1.78±0.04	66.16%





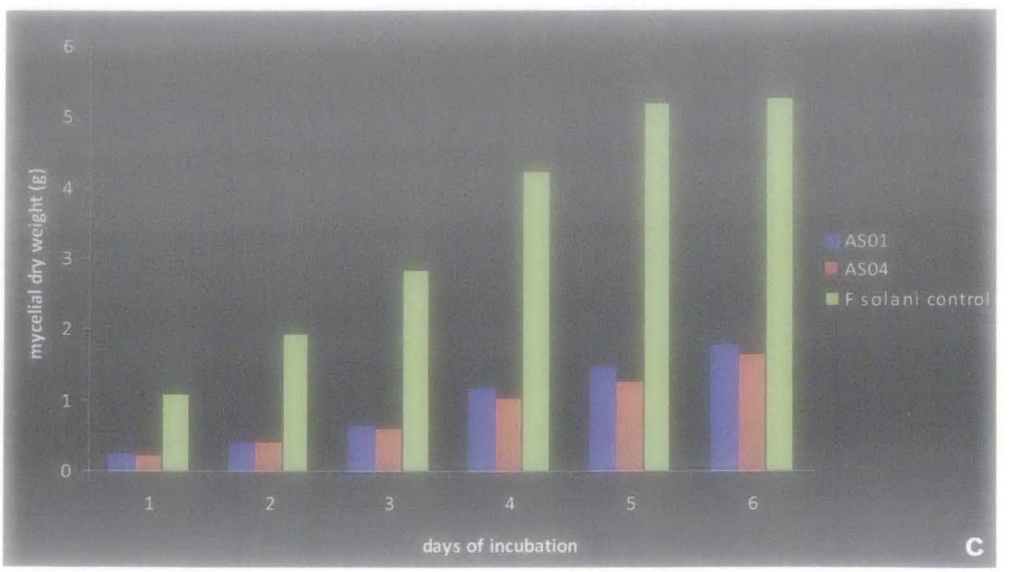


Fig. 31: Study of growth kinetics by turbidometry of *Pseudomonas putida* strains (a) AS01 and (b) AS04;
(c) Study of *in vitro* antagonism by *P. putida* strains AS01 and AS04 against *F. solani* in PDB dual culture assay.

6.3.3. *In vivo* evaluation of biocontrol potential of the antagonistic bacterial isolates AS01 and AS04 against *Fusarium* wilt in brinjal

Table 20 summarizes the results of the *in vivo* evaluation of the strains AS01 and AS04 for their ability to protect brinjal plants against wilt caused by *Fusarium solani*. The strain AS04 showed significant reduction of disease incidence (Fig. 32c) as compared to untreated control (Fig. 32a) within 8 days of treatment, regardless of the soil being sterilized or unsterilized. The disease index in plants treated with bacteria reduced to 25.0 from 82.5, which was recorded in untreated sets. However, in case of sterilized soil, the disease control efficiency was slightly higher (69.6 %) than in unsterilized soil (63.6%) as evident from a lower disease index and higher PEDC value obtained under sterilized soil conditions. There was also a marginal lowering of disease index after 16 days of treatment (Table20, Fig. 32f). The result of treatment with the strain AS01 was also satisfactory as it showed considerable amount of disease inhibition (Fig. 32b,e) as compared to control (Fig. 32a,d). However, the disease control efficacy exhibited by this strain was lower than that showed by AS04. Sterilized soil condition was again found be better in controlling the wilt disease and the disease control efficiency was again recorded to be higher after 16 days of treatment than that of 8 days as evident from the disease index and PEDC values.

Table 20: Inhibition of wilt disease caused by *F. solani* in brinjal seedlings by *Pseudomonas putida* strains AS01 and AS04

Treatments	8 days after inoculation		16 days after inoculation	
	Disease Index	PEDC*	Disease Index	PEDC*
AS01 (sterilized soil)	35.0±0.46	57.5±0.35	30.0±0.58	64.7±0.45
AS01(unsterilized soil)	42.5±0.29	48.5±0.50	37.5±0.26	57.1±0.64
AS04 (sterilized soil)	25.0±0.85	69.6±0.79	22.5±0.36	73.5±0.29
AS04 (unsterilized soil)	30.0±0.66	63.6±0.23	27.5±0.36	68.6±0.30
Untreated control (sterilized soil)	82.5±0.57	0.0±0.0	85.0±0.34	0.0±0.0
Untreated control (unsterilized soil)	82.5±0.29	0.0±0.0	87.5±0.44	0.0±0.0
CD at 5%	0.594	0.725	0.262	0.462

*PEDC: Percent efficacy of disease control. PEDC = [(Disease index in untreated control – Disease index in treated plants)/Disease index in untreated control] x100. Data represent the means ± standard error.

6.4. DISCUSSION

Pseudomonas spp. are ubiquitous in agricultural soils, and are well adapted to growing in the rhizosphere. Favourable characters such as widespread distribution in soil, ability to colonize the rhizospheres of host plants, and ability to produce a range of compounds antagonistic to a number of serious plant pathogens have made these bacteria a subject of intense research as biocontrol agents at the genetic and biochemical level (Anjaiah et al., 1998; De Souza et al., 2003; Pujol et al., 2006). Biocontrol strains have been observed markedly at the root surface, (i.e. the rhizoplane) where they form microcolonies or discontinued biofilms in between the epidermal cells (Couillerot et al., 2009). A large number of these strains have been utilized as inoculums in plant health management practices to control or inhibit plant pathogens and stimulate plant growth (Huang and Wong,

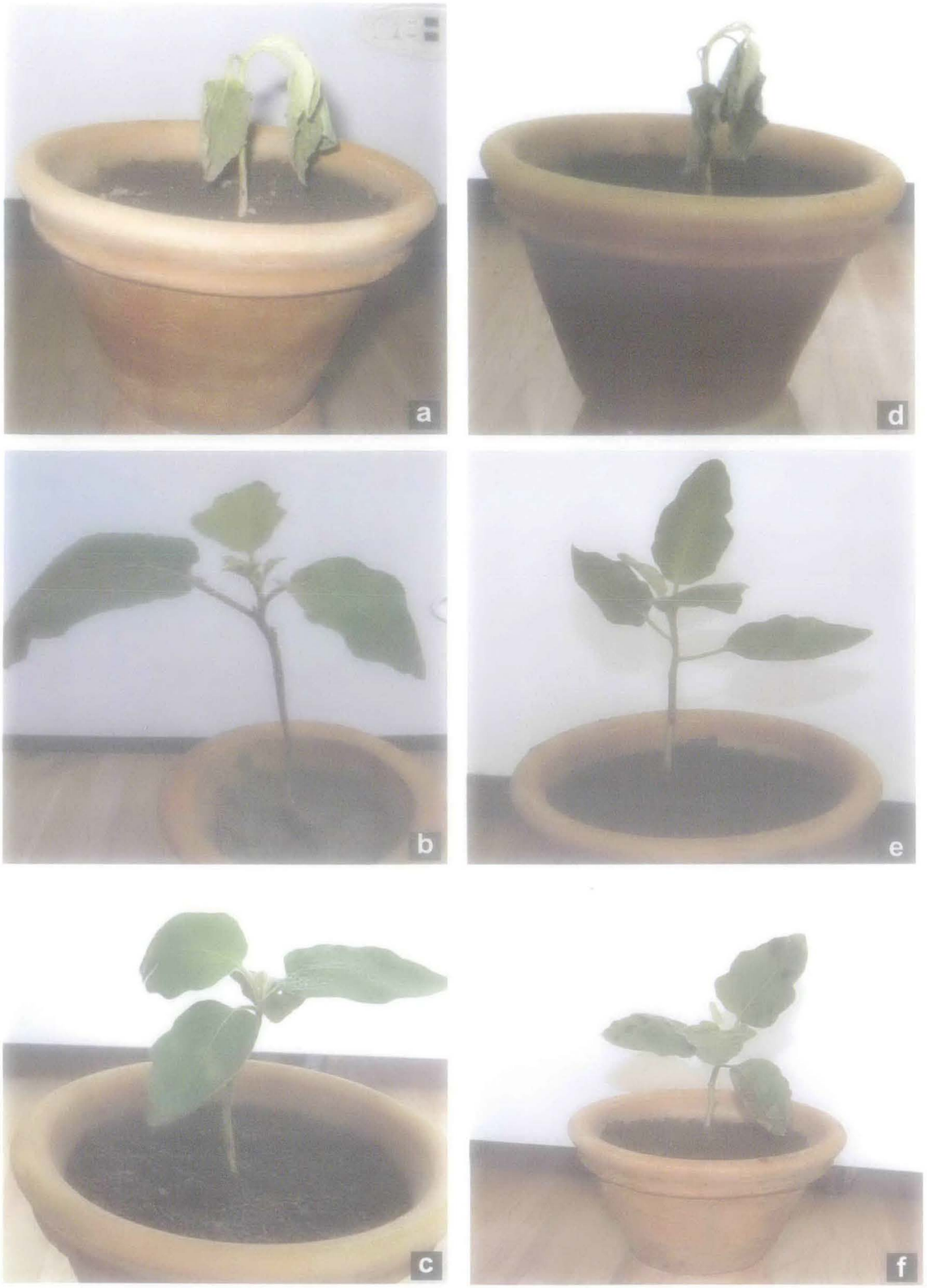


Fig. 32: Suppression of wilt disease in brinjal seedlings by the isolated *Pseudomonas putida* strains under sterile soil conditions:

(a) Considerable wilt in untreated control set after 8 days of inoculation; Disease suppression after 8 days of inoculation by *Pseudomonas putida* strains (b) AS01 and (c) AS04;(d) Severe wilt in untreated control set after 16 days of inoculation; Disease suppression after 16 days of inoculation by *Pseudomonas putida* strains (e) AS01 and (f) AS04.

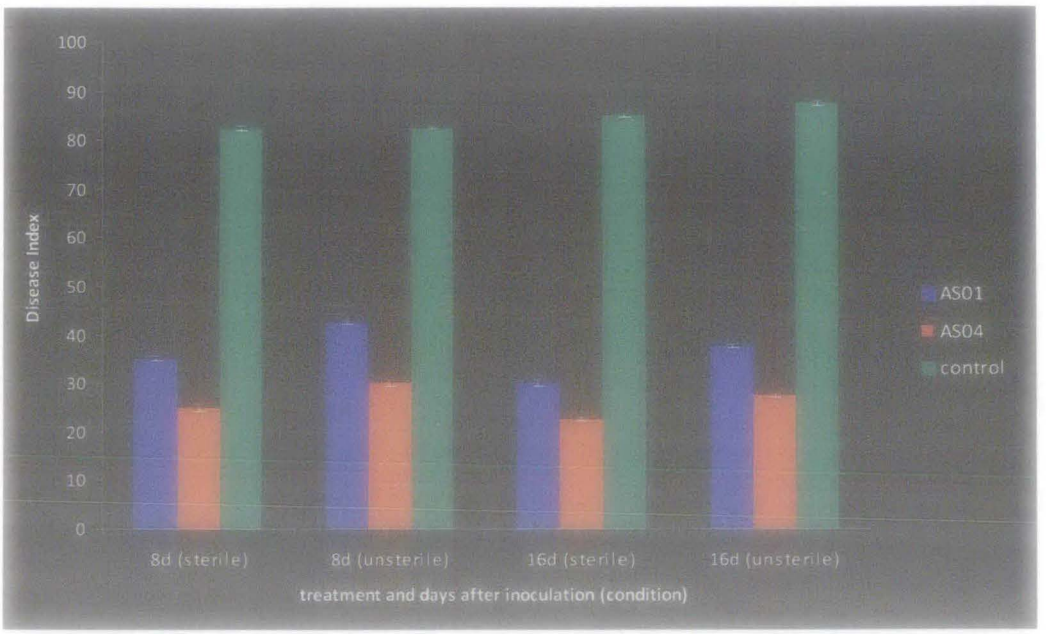


Fig. 33: Disease Index (DI) of wilt in brinjal seedlings inoculated by *F. solani* and treated by isolated antagonistic *Pseudomonas putida* strains AS01 and AS04.

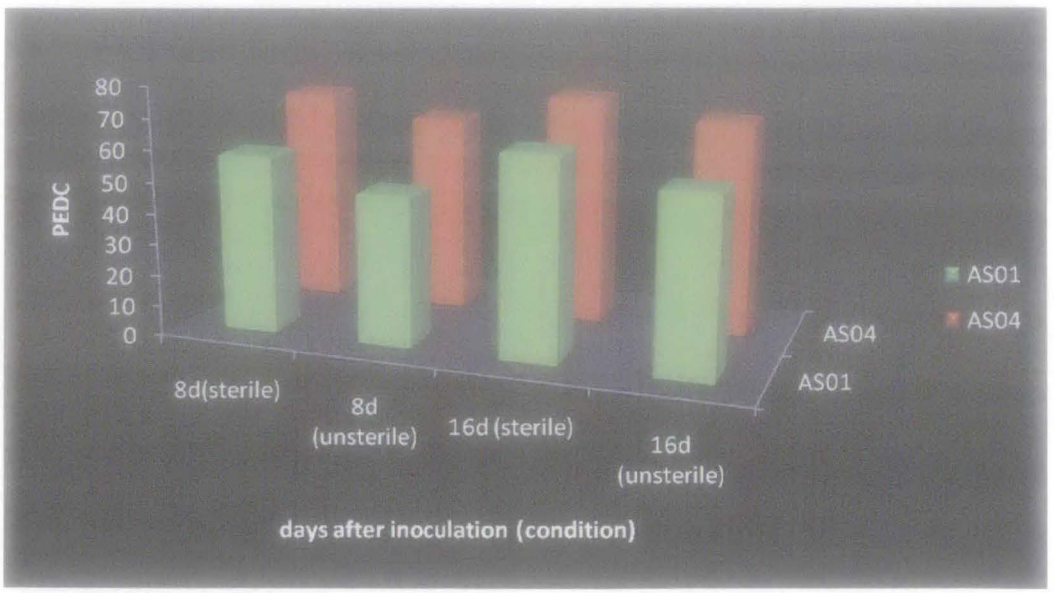


Fig. 34: Percent Efficacy of Disease Control (PEDC) of wilt in brinjal seedlings by the isolated antagonistic *Pseudomonas putida* strains AS01 and AS04 under sterile and unsterile conditions.

1998; Ross et al., 2000; Berg et al., 2001; Zhang et al., 2002; Sabaratnam and Traquair, 2002; Collins and Jacobsen, 2003; Xue et al., 2009).

The present study has shown that *Fusarium* wilt of eggplant can be efficiently controlled by *Pseudomonas putida* strains AS01 and AS04 recovered from the rhizosphere. During the green-house studies, biocontrol bacteria and fungal pathogen were co-inoculated to assess the efficiency of the isolated bacteria in reducing wilt disease in eggplant. Method of co-inoculation was successfully used for controlling phytopathogens *Macrophomina* and *Aspergillus* infecting chickpea by *Pseudomonas* M1P3 (Saraf et al., 2008). *Pseudomonas stutzeri* YPL-1 co-inoculated with the pathogen *F. solani* was also found to suppress the root-rot disease in kidney bean (*Phaseolus vulgaris* L.) to a desirable extent (Lim and Kim, 1995). Method of application is another aspect that contributes significantly towards achieving a good biocontrol efficiency and plant growth promotion (Xue et al., 2009). We have used soil application method of bacterial inoculation as this method has been shown to produce better levels of colonization and biocontrol efficiency than other methods like root dipping (Xue et al., 2009) or seed inoculation (Gotz et al., 2006).

At the onset of this study, the pathogenicity of the *F. solani* strain was confirmed through the verification of Koch's postulates. Next, the growth kinetics of the two *P. putida* isolates was studied. The growth rate was moderate for both strains as they attained stationary phase stage after 16-17 hours. Severe restriction of growth in terms of a low mycelial dry weight was observed in dual cultures in broth. In the *in vivo* study, about 73.5% reduction in disease incidence was demonstrated in the eggplant variety PPL on direct soil application of *P. putida* isolates. The strain AS04 appeared to be more competent than AS01 in controlling pathogen infection. However, the *in vitro* experiments of dual culture in liquid medium did not show any significant difference between these strains in inhibiting the growth of *F. solani.*

A large number of workers previously reported the suppression of root diseases by *Pseudomonas* spp. both in greenhouse and field conditions (Kim

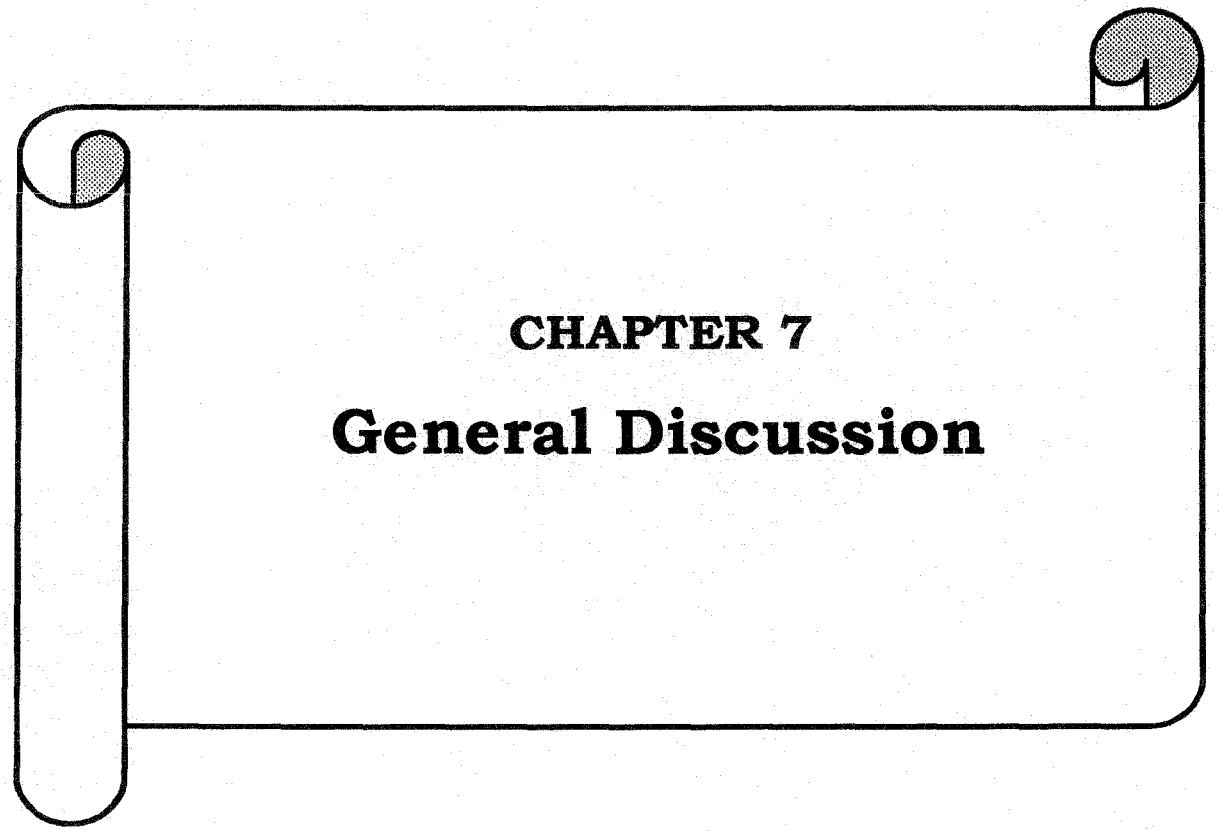
et al., 1994; Remadi et al., 2006; Nihorimbere et al., 2009; Akhtar et al., 2010). Scher and Baker (1982) observed that addition of *P. putida* strain A12 to the soil reduced the incidence of *Fusarium* wilt caused by *Fusarium oxysporum* f. Sp. *lini*. Wilt incidence in flax after 30 days was 42.5% in the control and 10% in the presence of strain A12. Significant disease control was achieved by adding A12 against *Fusarium* wilt in cucumber (lowered to 40% of that in the control) and radish (lowered to 61% of that in the control). Further experimental evidences suggested that competition for iron was responsible for the suppressiveness since iron appeared to be necessary for germ tube elongation of the *F. oxysporum* conidia. During our experiment, although a higher suppression of disease was achieved but the involvement of siderophores could not be ascertained. Several other authors have also suggested that siderophores are major contributors in disease inhibition by fluorescent pseudomonads. For instance, *Pseudomonas fluorescens* EPS62e which was found to be highly efficient in controlling infections by *Erwinia amylovora*, the causal agent of fire blight disease in pear trees did not produce antimicrobial compounds described in *P. fluorescens* species and only developed antagonism in King's B medium, where it produced siderophores (Cabrefiga et al., 2007). The maximum growth rate and affinity for nutrients in immature fruit extract were higher in EPS62e than in *E. amylovora*, but the cell yield was similar. In preventive inoculations of EPS62e, subsequent growth of *E. amylovora* was significantly inhibited. It was concluded that cell-to-cell interference as well as differences in growth potential and the spectrum and efficiency of nutrient use are mechanisms of antagonism of EPS62e against *E. amylovora* (Cabrefiga et al., 2007). Gupta et al. (2002) observed that bacterization of peanut seeds with the siderophore producing fluorescent *Pseudomonas* strain GRC2 resulted in increased seed germination, early seedling growth, fresh nodule weight, grain yield and reduced charcoal rot disease of peanut in *M. phaseolina*-infested soil as compared with control.

Several studies have been conducted to understand the role of siderophores produced by antagonistic bacteria in disease suppression in plants. Experimental evidences accumulated during the last three decades suggest

that competition for iron is responsible for disease suppression by several fluorescent *Pseudomonas* strains. Scher and Baker (1982) studied the effect of *P. putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt. Results revealed that *P. putida* mediated disease suppressiveness in soil may be induced by managing iron availability in the environment. Duijff et al. (1994) found that *P. putida* strain WCS358 alone significantly reduced carnation wilt caused by *F. oxysporum* and showed that siderophore mediated competition for iron was the principal mechanism involved in disease suppression. Siderophore production and inhibition of *F. oxysporum* by the biocontrol strain decreased with increasing iron availability *in vitro* supporting the more effective disease suppression at low iron availability. Biocontrol studies with a Tn5 mutant defective in siderophore biosynthesis showed that the mutant strain did not reduce disease incidence. De Boer et al. (2003) observed that *Fusarium* wilt in radish caused by *F. oxysporum* can be controlled in a more effective way by using multiple *P. putida* strains. The authors used a mixture of the strains *P. putida* WCS358 (defective in siderophore production) and *P. putida* RE8 to study the suppression of wilt in comparison to single strain treatments. They found an enhanced suppression by the combination of the strains than single applications. Leeman et al. (1996) found that iron availability affects induction of resistance to *Fusarium* wilt of radish by the antagonistic strain P. fluorescens WCS374. They suggested that iron chelating salicylic acid and pseudobactin siderophore produced by the strain is involved in the induction of systemic resistance to the disease. Buysens et al. (1996) showed that the plant growth-promoting rhizobacterium *Pseudomonas aeruginosa* 7NSK2 produced multiple siderophores, pyoverdin, the salicylate derivative pyochelin, and salicylic acid and was an efficient antagonist of *Pythium*-induced damping-off. Studies with mutant strains impaired in siderophore production and subsequent complementation tests revealed that siderophore-mediated iron competition could explain the observed antagonism. On the other hand, Ongena et al. (1999) reported that experimental evidences on protection of cucumber by *P. putida* strain BTP1

and its siderophore negative mutant M13 indicate that induction of resistance is the principal mechanism of disease suppression rather than involvement of siderophores.

The present study reveals that the antagonistic isolate *P. putida* AS04 which was recorded as a strong siderophore producer and possess multiple plant growth promoting traits was also capable of reducing incidence of brinjal wilt induced by *Fusarium solani*. Considering all the characters possessed by this strain it may be concluded that *P. putida* AS04 has excellent potential and may be used in experimental trials for managing crop diseases in the field.



CHAPTER 7

General Discussion

GENERAL DISCUSSION

The ability of soil inhabiting microorganisms to inhibit the growth or metabolic activity of plant pathogens has been studied intensively during the last three decades and continues to inspire research in many fields, such as drug discovery and crop protection (Scher and Baker, 1986; Chet et al., 1990; Boer et al., 1999; Cazorla et al., 2006; Ramette et al., 2011). Biological control of deleterious microbes especially fungi by introducing antagonistic microorganisms onto plant surfaces has been the focus of considerable research partly due to the need to minimise the use of hazardous chemical pesticides or fungicides and thereby enhance the sustainability of agriculture and horticulture and also because biocontrol may provide control of plant diseases that cannot, or only partially, be managed by other strategies (Duffy et al., 2003; Compant et al., 2005).

In the present study, twenty different siderophore producing bacterial strains was isolated from the rhizosphere of 12 different plants exhibiting broad spectrum antifungal activity against several fungal pathogens which include the soil borne pathogens, *Fusarium solani*, *F. oxysporum*, *F. graminearum* and *Rhizoctonia solani*. The rhizosphere region is heavily populated by a wide array of microorganisms which include both beneficial and harmful ones. The rhizosphere is the first-line defence for roots against attack by pathogenic fungi (Weller, 1988). Therefore, there is an excellent opportunity to find rhizosphere-competent bacteria in the rhizosphere which are potential biocontrol agents (Chet et al., 1990). Most studies on the biocontrol of plant pathogens focus on a multitude of factors related to the microbial antagonist, that is, recovery of appropriate strains from the rhizosphere, correct identification of the strain, how the isolated antagonists affect pathogens; which mechanisms or metabolites are involved and how far the antagonists can function in the specific environment. Consequently, substantial progress has been made in the identification of microbes involved in suppressing plant pathogens, and in identifying microbial traits that contribute to disease suppression and the competence of introduced strains in biological control of diseases in green house and field conditions.

16s rRNA gene remains an important diagnostic marker for prokaryote identification. However, valid species definitions require phenotypic description. While many recent studies describe molecular characterization of prokaryotes for the purpose of phylogenetic analysis, a concerted effort is underway to use this molecular target for routine identification of pathogens microbiology laboratories and to rapidly characterize those organisms that are recalcitrant to identification because of fastidious growth requirements or unusual biochemical patterns (Kolbert and Persing, 1999). However, just as all bacteria can be described with a powerful common framework of their 16S rRNA gene or genomic DNA sequences, it would also be highly desirable and productive to describe all bacteria by their phenotypes, which reflects their physiology (Bochner, 2009). Growth phenotypes are directly and intimately involved in fundamental aspects of cellular genome and organism evolution and they remain a cornerstone of microbial taxonomy (Bochner, 2009).

For identification of the present strains, a polyphasic approach was adopted that included both phenotypic and genotypic studies. The phenotypic studies included a study of the cell size and morphology under microscope and culture morphology in growth media. Additionally, an array of biochemical tests was conducted to characterize the strains by their phenotypes. In the Phylogenetic approach, 16S rRNA gene sequences of all the isolates were determined. Results of all these studies led to the recognition of the isolates; all were identified to the genus level and nine bacteria were identified to the species level. A wide range of bacteria were recovered from the rhizosphere soil which included *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia*. An attempt was made to phylogenetically analyse the seven *Pseudomonas* isolates; these were *P. fluorescens* (one isolate), *P. putida* (two isolates) and the rest were maintained as *Pseudomonas* sp. Despite the efforts, the species of all the strains could not be ascertained. Further analysis with other phylogenetic markers such as *rpo B*, *rpo D* or *gyr B* may help to determine their identity (Ramette et al., 2011).

A successful biocontrol agent should be efficient in suppressesing the pathogen and reduce disease incidence significantly. Biocontrol agents act against pathogens mainly through the process of antagonism in the form of competition, antibiosis and parasitism (Chet et al., 1990; Yasmin et al., 2009; Werra et al., 2009). The activity is not restricted to only one of these, and, in fact, a combination of mechanisms acts in concert in an efficient biocontrol process (Cazorla et al., 2006; Gupta et al., 2006; Bano and Musarrat, 2002; Garbeva et al., 2004; Duffy and De Fago, 1999). Competition between the biocontrol bacteria and the pathogen can lead to dislodgment of the pathogen (Ligon et al., 2000; Weller et al., 2006; Gupta et al., 2002; Kamilova et al., 2008). Experimental evidences in many studies could find a direct correlation between the *in vitro* activities and the biocontrol action in plants (Maurhofer et al., 1994; Jagdeesh et al., 2001). However, some authors reported that production of lytic enzymes, antibiotics or siderophores or even *in-vitro* antagonism could not be linked to disease suppression (Ongena et al., 1999; Pandey et al., 2000; Ahmadzadeh et al., 2006). Microorganisms compete with each other for food and essential elements in the soil (Schippers et al., 1987; Cazorla et al., 2006; Validov et al., 2009). The availability of iron for assimilation by microorganisms in the rhizosphere environment is extremely limiting (O'Sullivan and O'Gara, 1992). Since almost all living organisms require iron for growth, survival in a heterogeneous environment such as the rhizosphere depends largely on the ability to scavenge sufficient iron from a limiting pool. Siderophores mediate the limited amount of iron in the rhizosphere, deprive pathogens of iron and suppress their growth. Many reports have been published showing siderophore involvement in the suppression of plant pathogenic fungi (Loper and Buyer, 1991; Ongena et al., 1999; Saikia et al., 2005; Sayyed et al., 2005; Sayyed and Patel, 2011; Bholay et al., 2012).

The present antagonistic strains were capable of producing multiple extracellular lytic enzymes such as chitinase, protease and lipase and also exhibited biofertilizer traits such as production of the plant growth hormone IAA and the phosphate solubilising enzyme. Most of them recorded robust

siderophore production by the CAS shuttle assay. The *Pseudomonas putida* strain AS04 in particular was able to produce chitinase, protease and lipase and was also recorded chitinase and phosphatase activity. Besides it showed maximum siderophore production among all isolates. Enzymatic dissolution of cell walls leading to loss of fungal protoplasm is one of the main antagonistic mechanisms involved in the activity of biocontrol agents (Lim et al., 1991; Kim and Chung, 2004). Hence, these observations together with electron microscopic evidences suggest that *P. putida* AS04 has excellent potential to act as biocontrol agent that not only limit pathogen proliferation but also promote plant growth.

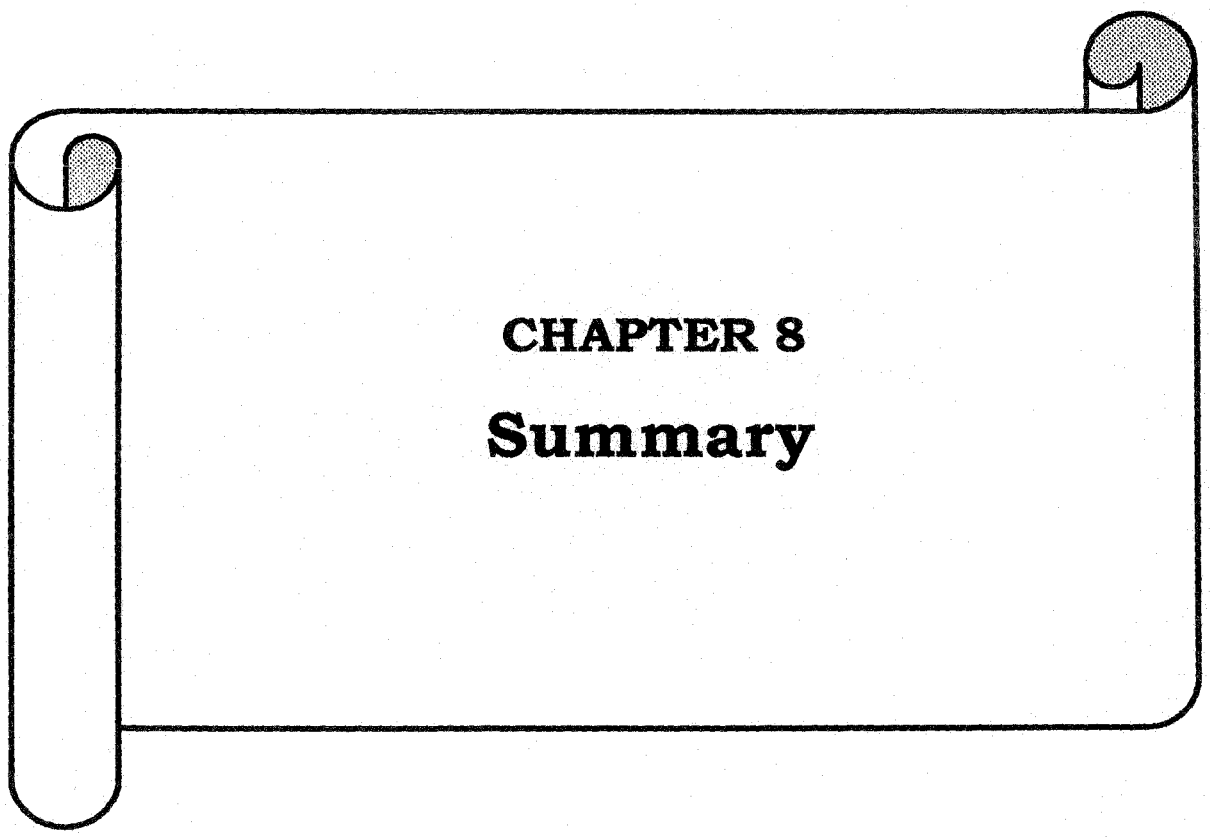
Since siderophore production was the common antagonistic property of all the strains, the siderophores from each strain was chemically characterized using standardized protocols. Of the 20 isolates, 15 strains were found to produce hydroxamate type of siderophore. Siderophores are considered to be one of the major contributory factors towards the biocontrol action of the antagonistic bacteria because apart from depriving the fungal pathogens of iron and thereby limiting their growth; these molecules also supply iron to the plants and aid in plant growth promotion (Shoda, 2000). The conditions required for maximum siderophore production *in vitro* was optimized for further purification of the siderophore from the strain *P. putida* AS04. Results revealed that siderophore production increased from 59.70 to 89.04% units on addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose minimal media. The production of siderophore began after 8-9 hours and reached maximum after 30 hours resembling secondary metabolite production, which is produced during later stages of growth. The optimum temperature was recorded to be 30°C. For siderophore purification, the strain AS04 was cultured in bulk under the optimized conditions and the acidified culture supernatant was passed through XAD-2 column and the bound siderophore was eluted with methanol. The concentrated siderophore was then passed through a Sephadex LH-20 hydrophobic column and the siderophore containing fractions were pooled and concentrated. This partially purified preparation of siderophore was

subjected to spectral scan and a peak at 430nm indicated that the siderophore was trihydroxamate type.

A literature survey on the use of bacterial inoculums in suppression of plant diseases reveal several reports that have warned against associating *in vitro* inhibition with *in vivo* activity (Paulitz and Loper, 1991; Loper and Buyer, 1991; Ongena et al., 1999). Therefore *in vivo* demonstration of disease control is a prerequisite to establish a potential strain as a biocontrol agent. Brinjal is a major crop grown in sub-Himalayan West Bengal and disease problems are many. Wilt and root rot caused by *Fusarium solani* is one of the major factors that limit brinjal production (Chakraborty and Chatterjee, 2007, 2008; Joseph et al., 2008; Akhtar et al., 2010). Two most potential strains isolated during the present study, namely, AS01 and AS04 both of which were identified as *P. putida* was used for the biocontrol experiments. Before the *in vivo* study, the pathogenicity of the *F. solani* strain was confirmed through the verification of Koch's postulates and growth inhibition studies along with growth kinetic studies of AS01 and AS04 were conducted to evaluate the fungal inhibition in liquid culture. Severe retardation of mycelia growth was found in dual cultures in PDB. *In vivo* biocontrol experiments in brinjal seedlings revealed 73.5% reduction in disease incidence in PPL variety on direct soil application of *P. putida* isolates under sterile conditions. The strain AS04 showed higher disease suppression than AS01 in controlling pathogen infection although the *in vitro* experiments of dual culture in liquid medium did not show any significant difference between these strains in inhibiting the growth of *F. solani*. Lack of correlation between *in vitro* inhibition and biocontrol has been documented in literature (De Boer et al., 2007).

It has long been known that the management of iron availability in the rhizosphere environment, through competition for iron can induce suppressiveness to diseases caused by soil borne pathogens (Scher and Baker, 1982). In the present study it was demonstrated that a particular *Pseudomonas putida* strain AS04 with robust siderophore producing ability was able to suppress wilt caused by *Fusarium solani* in brinjal. The bacteria

possessed multiple plant protecting and plant growth promoting traits such as production of chitinase, protease, lipase, phosphatase and plant growth hormone IAA. But how far these properties are responsible in disease suppression in plants remained unknown. Future studies in determining the exact role of these enzymes and siderophore in limiting pathogen proliferation is warranted.



CHAPTER 8

Summary

SUMMARY

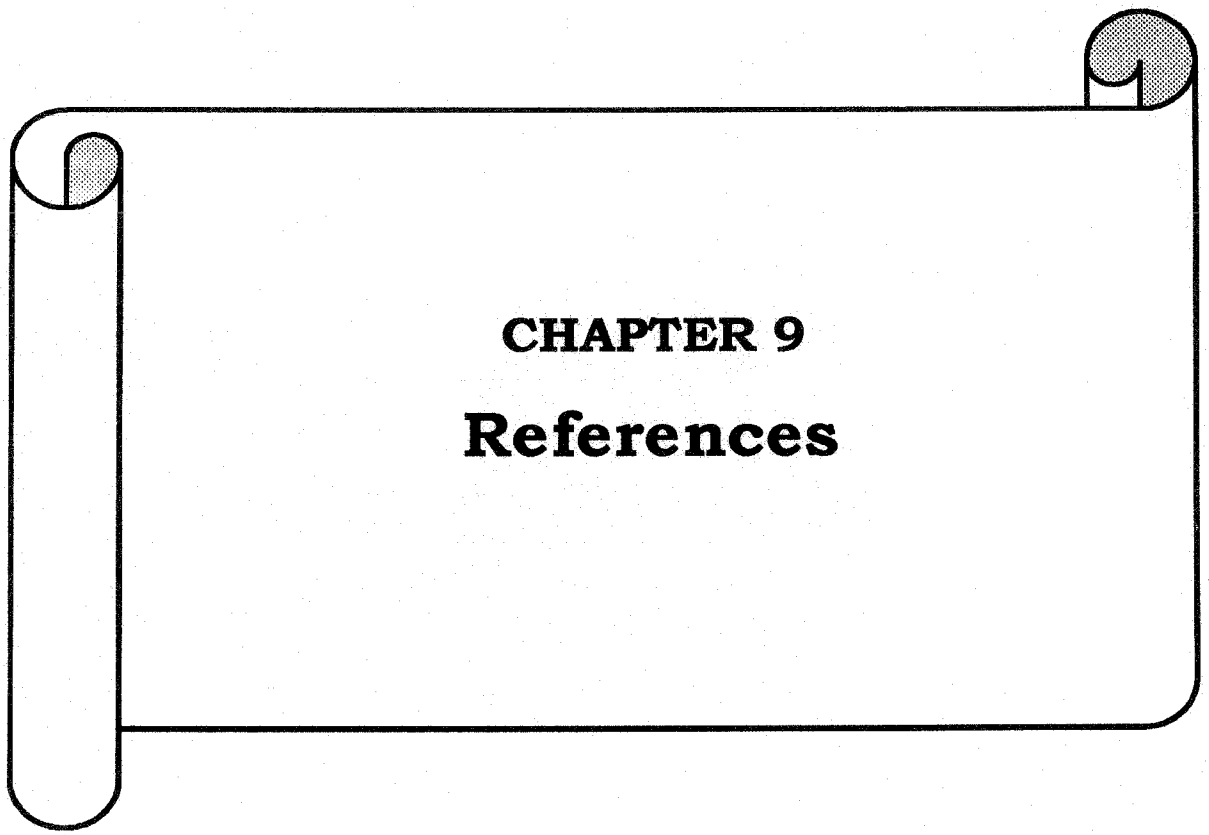
- The present study deals with "Studies on Soil-Inhabiting Siderophore-Producing Bacteria and Their Role in Suppression of Plant Root Pathogens".
- After a short introduction to the work, a brief review of literature on siderophore has been presented. This section deals with the findings of the previous workers with respect to the present line of research. This study includes the chemistry, biosynthesis and transport of siderophore in microorganisms. Extensive study has been done on the siderophores produced by the fluorescent pseudomonads, the pyoverdins. Literature review was also done on the biological control of plant diseases. The common mechanisms of antagonisms are described in brief which included the production of antibiotics; signal interference; parasitism and production of extracellular enzymes; induced systemic resistance; competition for ferric iron ions; root colonization and PGPR traits. The literature review was extended to describe the role of Gram negative and Gram positive rhizosphere bacteria in suppression of plant diseases which are reported as biocontrol agents.
- Basic objectives of the present study were: (i) To isolate siderophore-producing bacteria from soil; (ii) To study the antifungal activity of the isolated siderophore producing strains in suppressing some plant root pathogens *in vitro*; (iii) To characterize the selected siderophore-producing and antagonistic strains and their identification; (iv) To partially purify and chemically characterize the siderophores produced by the selected strains; (v) To study the efficiency of siderophore-producing bacteria in suppressing plant root pathogens *in vivo*.
- The experimental study is depicted in four chapters: (i) Isolation of siderophores producing antagonistic bacteria from soil and their characterization, (ii) Mechanism of action of siderophore producing rhizobacteria showing antagonistic activity against plant pathogenic fungi, (iii) Characterization and purification of siderophores, (iv) *In vivo* evaluation of *Pseudomonas putida* strains AS01 and AS04 as biocontrol agents against wilt in brinjal.

- A detailed description of different experimental procedures and techniques used during the present study are explained in the section of materials and methods of each chapter.
- Chapter 3 deals with collection of rhizosphere soil samples from 9 different locations of Darjeeling and Jalpaiguri districts of West Bengal, India, to isolate different bacterial strains. A total of 208 bacterial isolates were obtained which were then checked for siderophore production on CAS agar. Altogether 68 siderophore producing strains were obtained.
- *In vitro* study of antagonistic activity of 68 siderophore producing bacterial isolates were performed against seven pathogens, namely *Fusarium equiseti, Lasiodiplodia theobromae, Rhizoctonia solani, Colletotrichum gloeosporioides, Alternaria alternata, Fusarium solani,* and *Fusarium graminearum.* Twenty isolates were found to exhibit antagonistic activity towards all the test pathogens.
- The characterization of the antagonistic bacterial isolates was done. Both the morphological and phylogenetic characterizations were performed. For identification of the isolates, 31 biochemical tests were performed followed by the genetic study. The 16S rRNA gene of each bacterium was amplified, cloned and sequenced. Identification was done by similarity searches of the sequences using the BLAST function of GenBank. The results of the phenotypic tests and 16S rRNA gene sequencing revealed that the strains belonged to the genera *Pseudomonas, Klebsiella, Serratia, Enterobacter, Bacillus, Alcaligenes* and *Citrobacter.* The sequences were deposited in NCBI GenBank through BankIt and Accession Numbers were provided for each of the strains (Accession numbers: EU661864, EU661866, JX535385, JX960418, KC109315-28, KC117153-4). Phylogenetic relationship was studied with the seven antagonistic *Pseudomonas* spp. with other 50 species of *Pseudomonas* obtained in GenBank using MEGA 4.0.
- Chapter 4 deals with a study on the mechanism of inhibition of the antagonistic isolates against the plant pathogens. Production of different antimicrobial metabolites by the antagonistic isolates was

observed in specific media. Siderophore production was estimated by the CAS shuttle assay which showed that AS04 was the highest producer. The antimicrobial metabolites produced by different strains included chitinase, amylase, lipase and protease. Moreover, some PGPR traits were also tested which showed that all strains were producers of IAA and one strain, namely AS04 produced phosphatase.

- The interaction of the *Pseudomonas putida* strains AS01 and AS04 with fungal pathogens *Fusarium equiseti* and *F. solani* was studied by Scanning Electron Microscopy. Severe deformities of the fungal mycelia and hyphal lysis were observed.
- The siderophores of each of the 20 isolates were characterized (chapter no. 5) which revealed that 15 strains produced hydroxamate type and 5 produced catecholate type but none of them produced carboxylate type of siderophore.
- The media and growth parameters were optimized for maximum amount of siderophore production by the strain *Pseudomonas putida* AS04. Media supplements, temperature and incubation time were optimized which showed that Fiss glucose minimal media supplemented with 1% sucrose and 0.1% $(NH_4)_2SO_4$ was the best media for siderophore production. Siderophore production began after 8 hours of incubation and reached maximum after 30 hours. The optimum temperature for siderophore production was recorded to be 30°C.
- For partial purification of siderophore culture supernatants were passed through Amberlite XAD-2 column and the siderophore was eluted with methanol. All fractions tested positive for siderophore were combined and concentrated and the concentrate was passed through sephadex LH20 column. Fractions eluted with methanol were monitored for presence of siderophore by TLC using chromogenic spray with $FeCl_3$ in HCl. A brown spot indicated hydroxamate siderophore on TLC plate.
- The partially purified siderophore was studied spectrophotometrically in 300-700 nm visible range and a peak was obtained at 430 nm which confirmed that the sample contained a trihydroxamate type siderophore.

- In the final chapter (chapter no. 6) *in vivo* studies were performed to evaluate the efficacy of *P. putida* strains AS01 and AS04 in suppressing wilt in brinjal caused by *Fusarium solani*. Initially the pathogenicity of *Fusarium solani* was confirmed through verification of Koch's postulates. For this, six-week old potted brinjal seedlings of three different varieties (PPL, Lalita and a locally cultivated variety) were used as host plant and disease index was evaluated.
- The *Pseudomonas putida* strains AS01 and AS04 were grown in liquid culture (PDB) in presence of the pathogen *Fusarium solani* in order to assess the antagonistic activity in liquid media. The percentage reduction of biomass of *F. solani* after 5 days was found to be 75.72% in AS01 co-inoculated cultures and 71.67% in cultures co-inoculated with AS04.
- The isolates AS01 and AS04 were selected for *in vivo* studies for management of *Fusarium* wilt in brinjal seedlings. The brinjal seedlings (var. PPL) were used for this experiment. The disease control efficacy exhibited by strain AS04 was higher than that showed by AS01. AS04 strain exhibited 73.5% disease inhibition while AS01 showed 64.7% inhibition in sterilized soil.
- The findings of the present study have been discussed in detail and compared with the results of other prominent works in each chapter.
- A generalized discussion on the entire work is presented in chapter 7.
- In conclusion, the present study found out some potential soil inhabiting antagonistic bacteria which produced siderophore and several other bioactive principles such as hydrolytic enzymes and IAA. The findings of the study have suggested a possible means to biologically control fungal phytopathogens in an eco-friendly way for a more sustainable agricultural system.



CHAPTER 9

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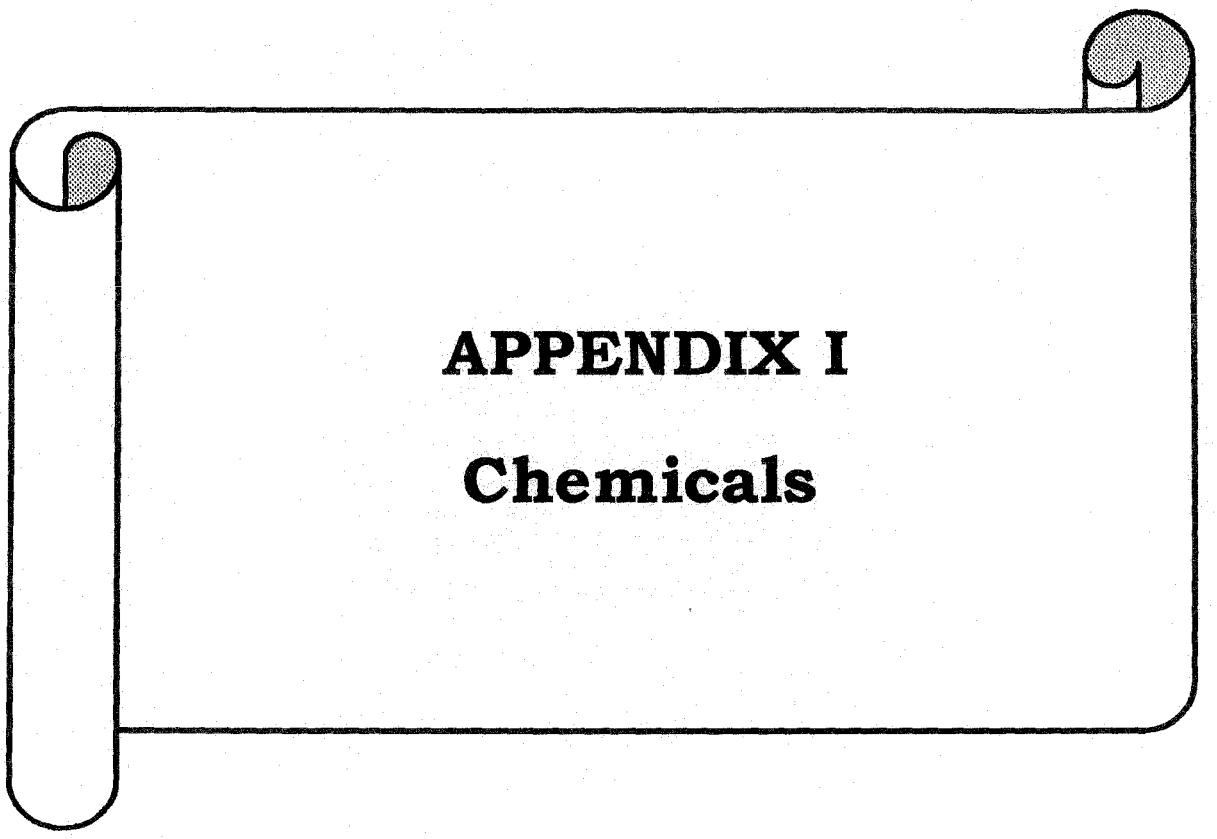


APPENDIX I

Chemicals

CHEMICALS	COMPANY
Acetic acid (glacial)	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Adonitol	HiMedia Laboratories Pvt Ltd, Mumbai, India
Agarose	Lonza, Rockland, ME, USA
Amberlite XAD-2	Supelco Analytical, PA, USA
Ammonium sulphate	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Ampicillin	HiMedia Laboratories Pvt Ltd, Mumbai, India
Amyl alcohol	Sisco Research Laboratories Pvt Ltd, Mumbai, India
D-Arabinose	HiMedia Laboratories Pvt Ltd, Mumbai, India
L-Asparagine	Sisco Research Laboratories Pvt Ltd, Mumbai, India
n-butanol	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Calcium chloride	HiMedia Laboratories Pvt Ltd, Mumbai, India
Carboxy methyl cellulose sodium salt	HiMedia Laboratories Pvt Ltd, Mumbai, India
casamino acid (Casein acid hydrolysate)	HiMedia Laboratories Pvt Ltd, Mumbai, India
chloroform	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Chrome Azurol S	HiMedia Laboratories Pvt Ltd, Mumbai, India
Crystal Violet	Micro Master Laboratories Pvt. Ltd., India
decarboxylase agar base media	HiMedia Laboratories Pvt Ltd, Mumbai, India
p-Dimethyl aminobenzaldehyde	HiMedia Laboratories Pvt Ltd, Mumbai, India
DNA ladder (500 bp)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
DNA ladder (100 bp)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
dNTP mix (2.5 mM each)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Ethidium Bromide	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Ferric chloride (anhydrous)	HiMedia Laboratories Pvt Ltd, Mumbai, India

Ferric chloride hexahydrate	HiMedia Laboratories Pvt Ltd, Mumbai, India
fluorescent brightener 28	Sigma-Aldrich Co., MO, USA
gel loading buffer (6X)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Gram's Iodine solution	Micro Master Laboratories Pvt. Ltd., India
Glucose	HiMedia Laboratories Pvt Ltd, Mumbai, India
Glutaraldehyde (25%)	HiMedia Laboratories Pvt Ltd, Mumbai, India
Glycine	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Glycol Chitosan (chitin)	Sigma Aldrich Co. MO, USA
Cetyl trimethyl ammonium benzaldehyde (CTAB/HDTMA)	Calbiochem, E. Merck (India) Ltd., India
Hydrogen peroxide (30%)	E. Merck (India) Ltd., India
Indole acetic acid	E. Merck (India) Ltd., India
Inositol	HiMedia Laboratories Pvt Ltd, Mumbai, India
IPTG	Promega Corporation, Madison, USA
Iso amyl alcohol	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Lactose	Sisco Research Laboratories Pvt Ltd, Mumbai, India
L-Lysine hydrochloride	HiMedia Laboratories Pvt Ltd, Mumbai, India
Maltose	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Magnesium chloride (25 mM)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Mannitol	HiMedia Laboratories Pvt Ltd, Mumbai, India
Methyl alcohol (Methanol)	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Methyl red	Micro Master Laboratories Pvt. Ltd., India
Methylene Blue	E. Merck (India) Ltd., India
α-Naphthol	Sisco Research Laboratories Pvt Ltd, Mumbai, India
α-Naphthylamine	SD Chemicals, India
ONPG	Sisco Research Laboratories Pvt Ltd, Mumbai, India

L-Ornithine monohydrochloride	HiMedia Laboratories Pvt Ltd, Mumbai, India
pGEM-T Easy Vector System II	Promega Corporation, Madison, USA
Peptone	HiMedia Laboratories Pvt Ltd, Mumbai, India
Pectin	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Perchloric Acid (35%)	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Phenol	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Phenol red	Sisco Research Laboratories Pvt Ltd, Mumbai, India
L-Phenylalanine	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Picric acid	HiMedia Laboratories Pvt Ltd, Mumbai, India
Primers	Sigma Aldrich Co., USA
Proteinase K	Bangalore Genei (India) Pvt Ltd, Bangalore, India
D-Raffinose	HiMedia Laboratories Pvt Ltd, Mumbai, India
L-Rhamnose	HiMedia Laboratories Pvt Ltd, Mumbai, India
Safranin Stain	E. Merck (India) Ltd., India
Sephadex LH-20	Sigma Aldrich Co., USA
D-Sorbitol	HiMedia Laboratories Pvt Ltd, Mumbai, India
Sucrose	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Sodium citrate	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Sodium dodecyl sulphate	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Sodium molybdate dihydrate	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Starch (soluble)	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Sulphanilic acid	E. Merck (India) Ltd., India
10X Taq DNA polymerase buffer F (without $MgCl_2$)	Bangalore Genei (India) Pvt Ltd, Bangalore, India
Taq polymerase (3Unit/µl)	Bangalore Genei (India) Pvt Ltd, Bangalore, India

Tetramethyl-p-phenylenediamine dihydrochloride	HiMedia Laboratories Pvt Ltd, Mumbai, India
Thiamine HCl	Sisco Research Laboratories Pvt Ltd, Mumbai, India
Trehalose	HiMedia Laboratories Pvt Ltd, Mumbai, India
Triphenyl tetrazolium chloride (Tetrazoliun salt)	HiMedia Laboratories Pvt Ltd, Mumbai, India
Tryptone	HiMedia Laboratories Pvt Ltd, Mumbai, India
Urea	HiMedia Laboratories Pvt Ltd, Mumbai, India
X-gal	Promega Corporation, Madison, USA
D-Xylose	HiMedia Laboratories Pvt Ltd, Mumbai, India
Yeast extract	HiMedia Laboratories Pvt Ltd, Mumbai, India

APPENDIX II

Buffers and Reagents

1. Acetate buffer

Stock solution A:

Acetic acid	0.1 M
Distilled water	1000 ml

Stock solution B:

Sodium acetate (tri hydrate)	13.6 g
Distilled water	1000 ml

847 ml Stock solution A and stock solution B 153 ml were mixed to obtain a buffer of pH 4.

2. Phosphate Buffer

Stock solution A:

NaH_2PO_4	23.4 g
Distilled water	1000 ml

Stock solution B:

Na_2HPO_4	21.29 g
Distilled water	1000 ml

28 ml of stock solution A was added to 72 ml of stock solution B to obtain a final solution of pH 7.2.

To obtain a solution of pH 7.0, stock solution A was added to 61 ml of stock solution B.

3. TE buffer

Tris-HCl	10 mM
EDTA	1 mM
Final pH	8.0

4. 1X TAE buffer

50X TAE composition:

Tris base	242 g
Glacial acetic acid	57.1 ml

EDTA (0.5 M)	100 ml
Distilled water (final volume make up to)	1000 ml
Final pH	8.0

To make 1X TAE buffer, 1 ml 50X stock buffer was diluted in 49 ml distilled water to make final volume 50 ml.

5. 0.1% Congo Red Solution

Congo Red	0.1 g
Distilled water	100 ml

6. 1% CTAB in 1M NaCl

CTAB	1.0 g
NaCl	5.8 g
Distilled water	100 ml

5.8 g of NaCl was added to 100 ml distilled water in a conical flask and dissolved completely. The solution was autoclaved at 15 psi for 15 min. After sterilization the solution was allowed to cool down and then 1 g CTAB was added to it and mixed gently.

7. Kovac's reagent

p- dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75 ml
Concentrated HCl	25 ml

The *p*-dimethylaminobenzaldehyde was dissolved in alcohol by gentle mixing and then conc. HCl was added to it with care. The solution was stored at 4°C in dark.

8. Malachite green Stain

Malachite green	5.0 g
Distilled water	100 ml

9. Methylene Blue Stain

Methylene blue (90%)	0.3 g
Distilled water	100 ml

10. Methyl red indicator

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

Methyl red was dissolved in alcohol, distilled water was added to it and the mixture was filtered, stored.

11. Nitrate reagent A

Sulphanilic acid	8.0 g
Acetic acid 5N	1000 ml

12. Nitrate reagent B

α-Naphthylamine	5.0 g
Acetic acid 5N	1000 ml

13. Nitrite-Molybdate Reagent

$NaNO_2$	10.0 g
$Na_2MoO_4.2H_2O$	10.0 g
Distilled water	50 ml

14. Oxidase Reagent (tetramethyl-*p*-phenylenediamine dihydrochloride)

Tetramethyl *p*-phenylene diamine dihydrochloride	1.0 g
Distilled water	100 ml

15. Salkowski's reagent

$FeCl_3$ (0.5 M)	1.0 ml
Perchloric Acid (35%)	50 ml

16. 10% SDS solution

SDS	10 g
Distilled wate	100 ml

To prepare 10% SDS solution, 100 ml distilled water was measured and dispensed in a 250 ml conical flask. It was sterilized by autoclaving at 15 psi for 15 min and allowed to cool down. After the sterilized water was cold

enough (about 40°C) 10 g of SDS was weighed and added to it. It was mixed gently to avoid froth formation.

17. Sodium chloride solution (5M)

NaCl	29.2 g
Distilled water	100 ml

100 ml distilled water was taken in a conical flask and 29.2 g NaCl was added to it. It was mixed thoroughly and sterilized at 15 psi for 15 min.

18. VP reagent I

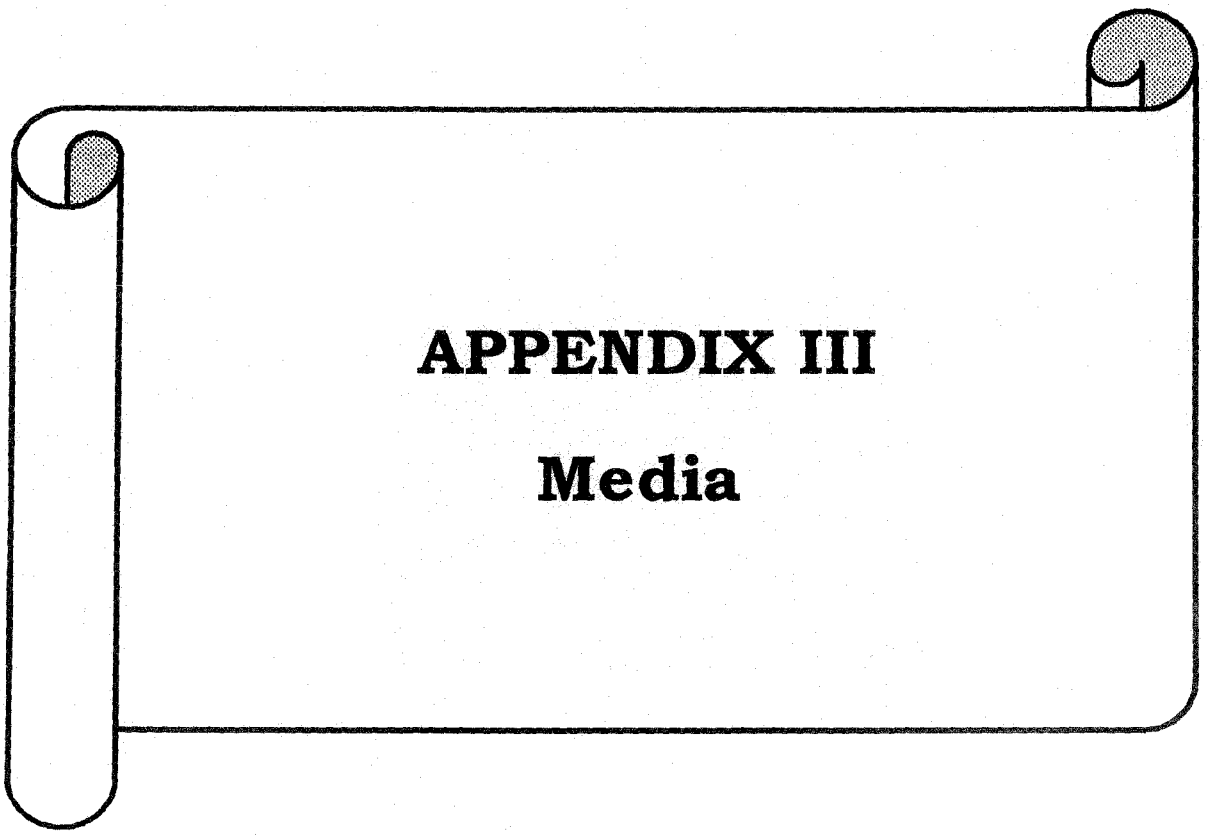
α-naphthol	5.0 g
Ethyl alcohol	95 ml

19. VP reagent II

KOH	40 g
Distilled water	100 ml

20. Winogradsky solution

K_2HPO_4	3.8 g
KH_2PO_4	1.2 g
$MgSO_4.\ 7H_2O$	5.1 g
NaCl	2.5 g
$FeSO_4$	0.05 g
$MnSO_4$	0.05 g
DDW	1000 ml



APPENDIX III

Media

1. Chrome Azurol S (CAS) agar (Schwyn & Neilands, 1987)

CAS agar plates were prepared by mixing all ingredients as described in chapter 3 (section 3.2.2)

Solution 1(Fe-CAS indicator solution):

1mM $FeCl_3.6H_2O$ (in 10mM HCl)	10 ml
CAS solution (1.21mg/ml)	50 ml
HDTMA solution (1.82mg/ml)	40 ml

Solution 2 (Buffer solution):

PIPES buffer	30.24 g
Distilled water	750 ml
Final pH (at 25°C)	6.8

Solution 3:

Glucose	2 g
Mannitol	2 g
Distilled water	70 ml

Solution 4:

10% (w: v) casamino acid (filter sterilized)	30 ml

2. Decarboxylase test medium base (HiMedia, India)

For preparing the decarboxylase medium, decarboxylase test medium base (Falkow base) (HiMedia, India) was used. It was supplemented with L-Ornthine and L-Lysine separately. The composition of the medium is as follows:

Composition:

Peptone	5.0 g
Yeast extract	3.0 g
Dextrose	1.0 g
Bromo cresol purple	0.02 g
Distilled water	1000 ml

9 g of test medium (HiMedia Laboratories, India) was dispensed in 1000 ml distilled water following manufacturer's instruction. Medium was heated to dissolve the components completely. It was divided into 3 equal parts. One part was prepared without adding any amino acid, to the remaining 2 parts L-Ornithine and L-Lysine hydrochloride were added to obtain a final concentration of 0.5%. The medium was dispensed in 3-4 ml quantities in test tubes and sterilized by autoclaving at 15 psi for 15 min.

3. DNase agar (Himedia, India)

Composition:

Tryptose	20.0 g
Deoxyribonucleic acid (DNA)	2.0 g
NaCl	5.0 g
Toluidine blue	0.1 g
Agar	15.0 g
Distilled Water	1000 ml
Final pH (at 25°C)	7.3

The DNase agar (Hi-media Laboratories, India) was weighed 42.1 g and dissolved in 1000 ml distilled water in a conical flask following manufacturer's instruction. It was next heated to melt and sterilized by autoclaving at 15 psi for 15 min. After autoclaving medium was dispensed in sterile petri plates (15 ml each) and allowed to solidify.

4. Fiss Glucose Minimal Media (Vellore, 2001)

Composition:

KH_2PO_4	5.03 g
L-Asparagine	5.03 g
Glucose	5.0 g
$MgSO_4.7H_2O$	40.0 mg
$MnSO_4$	100.0 µg

$ZnCl_2$	500.0 µg
Deionized distilled water	1000 ml

All components were dissolved in distilled water and the resulting medium (150 ml) was dispensed in 250 ml conical flasks. It was autoclaved for 15 min at 15 psi.

5. Gelatin Agar Media (Aneja, 2003)

Composition:	
Tryptone	1.0 g
Yeast extract	5.0 g
Glucose	1.0 g
Gelatin	4.0 g
Potassium monohydrogen phosphate	5.0 g
Distilled water	1000 ml
Final pH (at 25°C)	6.8

All ingredients were weighed and dissolved in distilled water. The medium was heated to melt all ingredients and pH was adjusted to 6.8. The final solution was dispensed in test tubes (5 ml each) and sterilized by autoclaving at 15 psi for 15 min.

6. Hugh and Leifson's O-F media (Barrow and Feltham 1993)

Composition:	
Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Bromothymol Blue (0.2%)	15 ml
Agar	3.0 g
Glucose	10.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.1

All the solids except glucose were dissolved by heating in the water and pH was adjusted to 7.1, filtered, and the indicator was added to it. Then the mixture was sterilized at 121°C for 15 min. Glucose was dissolved in water, sterilized separately and added aseptically to the medium to make a final concentration of 1% and was dispensed in sterile test tubes (5 ml each).

7. Luria-Bertani (LB) broth

Composition:	
Yeast extract	5.0 g
Casein peptone	10 .0 g
Sodium chloride	10.0 g

All media components were dissolved in distilled water and dispensed in test tubes (5 ml each). Test tubes were autoclaved at 15 psi for 15 min.

8. M9 agar basal medium (Miller, 1974)

Composition:	
Na_2HPO_4	6.0 g
K_2HPO_4	4.5 g
NH_4Cl	1.0 g
NaCl	0.50 g
$CaCl_2$	15.0 mg
$MgSO_4.7H_2O$	245.0 mg
Thiamine HCl	10.0 mg
Agar	20 g
Distilled water	1000 ml

M9 agar basal medium was prepared by dissolving all ingredients in distilled water and then it was heated to melt them completely. The basal medium was supplemented with cellulose, pectin or chitin (glycol chitosan) separately to test for different lytic activities. The

supplemented medium was sterilized by autoclaving at 15 psi for 15 min.

9. MR-VP broth (Aneja, 2003)

Composition:	
Peptone	7.0 g
K_2HPO_4	5.0 g
Dextrose	5.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.0

MR-VP broth was prepared by dissolving the components in distilled water and its pH was adjusted to 7.0. The medium was dispensed in test tubes (5 ml each) and sterilized.

10. Nitrate Broth (Barrow and Feltham 1993)

Composition:	
KNO_3	1.0 g
Nutrient Broth	1000 ml

KNO_3 was dissolved in the nutrient broth and mixed properly. The medium was distributed into test tubes (5 ml each) and sterilized at121°c for 15 min.

11. Nutrient Broth (NB) (Aneja, 2003)

Composition:	
Peptone	5.0 g
NaCl	5.0 g
Beef extract	3.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.2

Nutrient broth was prepared by dissolving all ingredients in distilled water and pH was adjusted. The medium was dispensed into test tubes (5 ml each). Test tubes were sterilized by autoclaving at 15 psi for 15 min.

12. Nutrient Agar (NA) (Aneja, 2003)

Composition:	
Peptone	5.0 g
NaCl	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.2

All media components were dissolved in distilled water and heated to melt the agar. It was dispensed in test tubes (5 ml for slants) and autoclaved. To prepare NA plates, the medium was autoclaved and then dispensed in sterile petri plates.

13. ONPG broth (Barrow and Feltham 1993)

Composition:	
ONPG	6.0 g
0.01M Na_2HPO_4	1000 ml

ONPG (O-nitro-phenyl-D-galactopyranoside) was dissolved in the phosphate solution (pH-7.5) at room temperature and sterilized by filtration.

ONPG solution	250 ml
Peptone water	750 ml

ONPG solution was aseptically added to the sterile peptone water, mixed and distributed in 2.5 ml volumes in sterile test tubes.

14. Peptone Water Broth (Barrow and Feltham 1993)

Composition:	
Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.2

The solids were dissolved by heating in water and pH of the solution was adjusted to pH 7.2. The medium was distributed in test tubes (5 ml each) and sterilized at 121°C for 15 min.

15. Phenylalanine agar (Barrow and Feltham 1993)

Composition:	
DL-Phenylalanine	2.0 g
Yeast extract	3.0 g
Na_2HPO_4	1.0 g
NaCl	5.0 g
Agar	20.0 g
Distilled water	1000 ml

All media ingredients were dissolved in distilled water and heated to melt. It was then dispensed in test tubes (5 ml each) and sterilized by autoclaving at 15 psi for 15 min.

16. Pikovskaya's Agar Medium (Pikovskaya, 1948)

Composition:	
Yeast extract	0.5 g
Dextrose	10.0 g
$Ca_3(PO_4)_2$	5.0 g

$(NH_4)_2SO_4$	0.5 g
KCl	0.2 g
$MgCl_2$	0.1 g
$MnSO_4$	0.0001 g
$FeSO_4$	0.0001 g
Agar	15 g
Distilled water	1000 ml

Media components were dissolved in distilled water and then heated to melt. The medium was autoclaved and after that dispensed in sterile perti plates and allowed to solidify.

17. Potato Dextrose Broth (PDB) (Aneja, 2003)

Composition:	
Potato	20.0 g
Dextrose	2.0 g
Distilled water	100 ml

The skin of potatoes were peeled off and cut into 1 cm cubes. The pieces were boiled in 100 ml distilled water till they turned soft. It was then filtered through cheesecloth and the filtrate was collected. Dextrose was added to it and dissolved. The medium was dispensed according to the experimental requirements and autoclaved at 15 lb psi for 15 min.

18. Potato Dextrose Agar (PDA) (Aneja, 2003)

Composition:	
Potato	20.0 g
Dextrose	2.0 g
Agar	2.0 g
Distilled water	100 ml

The PDB was prepared as above and agar was added which was then heated to melt before dispensing in tubes (5 ml for slants). It was

sterilized at 15 psi pressure for 15 min. The medium was dispensed in sterile petri plates and allowed to solidify or in case of slants, the tubes were kept at slanting position till solidified.

19. *Pseudomonas* Agar (For Fluorescein) (HiMedia):

Composition:	
Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue	10.0 g
Anhydrous dibasic potassium phosphate	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15.0 g
Distilled water	1000 ml

37.3 g of the dehydrated medium (Hi-media Laboratories, India) was dissolved in 1000 ml of distilled water containing 10 ml glycerine following manufacturer's instruction. The medium was then heated and sterilized at 121°C for 15 min and dispensed into sterile petri plates.

20. Semi Solid Motility Medium (Tittsler and Sandholzer, 1936)

Composition:	
Beef extract	3.0 g
Peptone	5.0 g
Agar	5.0 g
Distilled water	1000 ml
Final pH (at 25°C)	6.8

The ingredients were mixed and heated to melt. The medium was distributed in test tubes (10 ml each) and autoclaved at 121°C for 15 min. The tubes were then kept upright to solidify for stab inoculation.

21. Simmon's Citrate agar

Composition:	
$(NH_4)_2HPO_4$	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g
Sodium citrate	2.0 g
$MgSO_4$, $7H_2O$	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.0

Medium was prepared by adding all ingredients in distilled water and was heated to melt the agar. The melted medium was dispensed in test tubes (5 ml each) and autoclaved at 15 lb psi for 15 min. The tubes were allowed to stand at an inclined position until solidification.

22. Skim Milk Agar (Aneja, 2003)

Composition:	
Skim milk powder	100.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.2

The ingredients were dissolved in distilled water and its pH was adjusted to 7.2. The medium was autoclaved at 15 psi for 15 min. After that, it was dispensed in sterile petri plates and allowed to solidify.

23. Soil extract agar (Barrow and Feltham, 1993)

Composition:	
Peptone	5.0 g
Beef (Meat) extract	3.0 g
Agar	20.0 g
Soil extract	1000 ml

The ingredients were added to soil extract and sterilized at 15 psi for 15 min. The medium was dispensed in sterile petri plates and allowed to stand for solidification.

24. Starch Agar (Aneja, 2003)

Composition:	
Starch (soluble)	20.0 g
Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1000 ml
Final pH (at 25°C)	7.0

The ingredients except agar were dissolved in distilled water and pH was adjusted. Then agar was added to it and melted by heating. It was next autoclaved at 15 psi for 15 min. The medium was distributed in sterile petri plates and allowed to solidify.

25. Tryptone Broth (Aneja, 2003)

Composition:	
Tryptone	10.0 g
NaCl	5.0 g

$CaCl_2$ (1M)	1.0 g
Distilled water	1000 ml

Tryptone broth was prepared by dissolving all the components in distilled water and distributing them into test tubes (5 ml each). Test tubes were sterilized by autoclaving.

26. TSI agar (Aneja, 2003)

Composition:

Beef (Meat) extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
$FeSO_4.7H_2O$	0.2 g
NaCl	5.0 g
$Na_2S_2O_3.5H_2O$	0.3 g
Agar	20.0 g
Distilled water	1000 ml
Phenol red (0.2% aq. Soln.)	12 ml

The mixture of all ingredients was heated to dissolve the solids in distilled water and the indicator solution (Phenol red) was added, mixed and dispensed into tubes. The media was sterilized at 121°C for 15 min and cooled to form slopes with deep butts, about 3 cm long.

27. Tween 80 Media (Sierra, 1957)

Composition:

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2.2H_2O$	0.1 g

Agar	20.0g
Tween 80	10 ml
Distilled water	1000 ml

The ingredients were dissolved by steaming and pH was adjusted to 7.4. It was sterilized at 121°C for 15 min and cooled to 40-50°C. Tween 80 was filter sterilized and 10 ml of it was added aseptically to flask to give a final concentration of 1% and then dispensed into petriplates.

28. Urea Broth (Barrow and Feltham 1993)

Composition:

Peptone	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Glucose	1.0 g
Phenol red, 0.2% aq. Soln.	6 ml
Urea, 20% aq. soln.	100 ml
Distilled water	1000 ml
Final pH (at 25°C)	6.8

The solids namely peptone, NaCl and KH_2PO_4 were dissolved by heating and adjusted to pH 6.8, filtered and sterilized at 121°C for 15 min. Glucose and phenol red soln. were added to the molten base, steamed for 1h and cooled to 50°C. Urea was sterilized by filtration and added aseptically to the base cooled at 50°C. The medium was aseptically distributed into sterile test tubes.

APPENDIX IV

Nucleotide Sequences

1. Nucleotide sequences of 16S rRNA genes of the siderophore producing bacterial isolates antagonistic towards phytopathogens

1.1 Isolate BB05 (*Pseudomonas putida*)

Accession No. KC109321

Forward Sequence:

GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCG
GATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTT
TGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAA
TACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTG
GTTTGTTAAGTTGGATGTGAAAGCTCAACCCCGGGCCTGGGAACTGCATCCAAAA
CTGGCAAGCTAGAGTACGGTAGAGGATGGGCCTATTAGAATTTCCTGTGTAGCG
GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC
TCCATGGCTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTT
AGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTT
AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGAAGCAA

1.2 Isolate BB07 (*Pseudomonas fluorescens*)

Accession No. JX535385

Forward Sequence:

CTTGCTCCCGGATTCAGCGGCCGACGGGTGAGTAATGCCTAGGAATCTGCCTGG
TAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAG
CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAG
GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT
GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATT
AATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCC
AGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGG
GAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTGGAGTTTCC
TGT

1.3 Isolate JL11 (*Klebsiella oxytoca*)

Accession No. KC109327

Forward Sequence:

CGGAATTGCCGCGGGCCCTAACACATGCAGTCGACGGTAGCACAGAGAGCTTGC
TCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGG
AGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAA
AGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTA
GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT
GACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT

GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA
GAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAA
TAACCTCAGCAATTGACG

1.4 Isolate MD01 (*Pseudomonas fluorescens*)

Accession No. KC109323

Forward Sequence:

TCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGT
CCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCCTATCAGATGAGCCTAGGT
CGGATTAGCTATTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACGGATAGTACTCCTACGGGAG
CAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGT
GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA
ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAATTTCTAGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCGCTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGG
AACTGCATTCAAAACTGTCGATAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTG
TAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC
TGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAATA
CCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTTGAGC
TCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGGTACGGCCGCAAG
GTTAAAA

1.5 Isolate CB02 (*Serratia marcescens*)

Accession No. KC109325

Forward Sequence:

TGGCGGCAGGCTTAACACATGCAAGTCGAGCGGTAGCACAGGGGAGCTTGCTCC
CTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGA
GGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAA
GAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATCCCTAGC
TGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTCTCGGGGTCCGTAAAGCACTTTCAGCGAGAG
AGGAAGCTGGTGAGCTTAATACGCTCATTCAATTGAACGTAACTCGCAGAAGAAG
CACCGGCTAACTCCGGTGCCAGCAGCCGCGGTAATACCGGAGGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGT
GAAATCCCCGGGGCTCAACCCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGT
CTCGTAGAGGGGGGGTAGAATTCCAGGT

1.6 Isolate AS01 (*Pseudomonas putida*)

Accession No. EU661866

Forward Sequence:

AGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGACGGGA
GCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTG
GTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGG

AGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA
GCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGA
GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTG
TGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGC
TAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGC
CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTG
GGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTC
CTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGA
CCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTT
GAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAAC
TTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC
TTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGG
CTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAG
CTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTG
AAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGC
TAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGA

1.7 Isolate AS04 (*Pseudomonas putida*)

Accession No. EU661864

Forward Sequence:

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAG
TCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAAT
GCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATAC
CGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGAT
GAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGAT
CCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGG
GAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACAGACAGAATAAGC
ACCGGCTAACTCTGTGCAAACAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAA
GCCCCGGGCTCAACGTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTA
GAGGGTGGTGGAATTTCCTGTGTGGCGGTGAAATGCGTAGATATAGGAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACGCTGGTAGTCCACGCCGTAAACGATGTCGA
CTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAGCGCATTAAGTTGACC
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGCGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCAAAGCAACGCGAACATCCTTACCAAGG
CCTTGACATGCAGAGAACTTTCCAGAGATGCATTGCAGCCTTCGGGAACTCTGAC
GCAGGTGCTGCATGCCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGATAAGTCC

CGTAACGAGCGCAACTCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTA
AGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCAT
GGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGC
CAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGTGGGAATCGCTAGTAATCGCGAATCAGAATGTC
GCGGTGATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGG
GTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGA
TTCATGACTGGGGTGAAGTCGTAACAAGGTAAAT

1.8 Isolate CR04 (*Enterobacter cloacae*)

Accession No. KC109315

Forward Sequence:

GGAATATTGCACAAGGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTAAGGTTAATAACC
TTGTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGTGCAAGCGTTAAGGATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTAACCTGGGAACTG
CATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGCCTGAATTGGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCG
GCCCTCCCCTGGACAAAGACTGACGCTCATGCGAAAGCGGGGGAGCAAACAGG
ATTAGTACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCC

1.9 Isolate CR07 (*Bacillus thuringiensis*)

Accession No. KC109320

Forward Sequence:

AACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATAATATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC
CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGCTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAA
CTCTGTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTAC
CTAACCAGA

1.10 Isolate CR10 (*Bacillus subtilis*)

Accession No. KC117154

Forward Sequence:

TTGCAAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCT
TCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACC

TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGC
GGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGA
AACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG

1.11 Isolate CR12 (*Alcaligenes faecalis*)

Accession No. KC109316

Forward Sequence:

CGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTGGAGCGG
CCGCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGC
TGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCAT
CCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAC
CTCCAAAGGTATCTCATACGAGATACTGCTGACGGTATCTGCAGAATAAGCACCG
GCTAACTACGTGCCAGCAGCCGCGGTAATAGTAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATAAAGAACTTGAT
TCGATGTGAAATCCCAGGGCTC

1.12 Isolate CR13 (*Alcaligenes faecalis*)

Accession No. KC109317

Forward Sequence:

CGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTGGAGCGG
CCGCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGC
TGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCAT
CCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAC
CTCCAAAGGTATCTCATACGAGATACTGCTGACGGTATCTGCAGAATAAGCACCG
GCTAACTACGTGCCAGCAGCCGCGGTAATAGTAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATAAAGAACTTGAT
TCGATGTGAAATCCCAGGGCTC

1.13 Isolate CR14 (*Bacillus cereus*)

Accession No. KC117153

Forward Sequence:

AAACATTGCGGCGTGCTATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTC
TTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGA
CTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTG
CTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT

ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAA

1.14 Isolate MB01 (*Pseudomonas fluorescens*)

Accession No. KC109322

Forward Sequence:

GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA
AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGCAGTTACCTAATACG
TGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGC
CGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCG
CGTGGTGGTTTGTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
TTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCG
GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC
TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTA
GTGGCGCAGCTACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGA
TAGATTGGTGCCTTCGG

1.15 Isolate MB02 (*Bacillus subtilis*)

Accession No. JX960418

Forward Sequence:

GGGTTCNNAACCCTCGCCTGGNAAGGACTAGGGATAACTCCTGTGAAAAACGGG
GGCTAATACCGGATGGTTGTTTGAACCGCANGGTTCAAACATAAAAGGTGGCTTC
GGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATC
GTANAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAAC
TGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTATAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGATCANGATTAGATACCCTGGTAGTT
CACGCCGTAAACGATTAGTGCTAAGTTGTTAGGGGGTTTCCGCCCCTTATTGCTG
CAGCTTACGAATTAAGNACTNCGCCCTGTGAAGTATGGT

1.16 Isolate MB05 (*Citrobacter freundii*)

Accession No. KC109318

Forward Sequence:

TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGCT
TGTAGGGGGGGTAGAATTCCAGGTTAGCGGTGAAATGCGTAATCTGGAGGCCGG
TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGC
AAACAGGATTAGATCCTGGTAGTCCACGCCGTAACGATGTCGACTTGGAGGTTGC
CCTTGAGGCGTGGCTTCCGGAGCTACGCGTTAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGAAAACTCAAATGAATTGAGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGTGCAACGCGAGAACCTTACCTACTCTTGAATCCAGAGAACTT
AGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGCAGGTGCTGCATGGCTGTC
GTCAGCTCGTGAAATGTTGGGTTAATCCCGCAACGAGCGCAACCCTTATCCTTTGT
TGCCAGCGATCGGCCGGGACTCAAAGGGACTGCCAGTGATAAACTGAGGAAGGT
GGATGACGTCAAGTCATCAGGCCCTTACAGTAGGGCACACACGTGCTACAATGG
CATATACAAAGAAAGCGACCCGCGAGAGCAAG

1.17 Isolate NG04 (*Pseudomonas stutzeri*)

Accession No. KC109324

Forward Sequence:

GACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACATGGGCGAAAGCCTGATC
CCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGA
GGAAGGGCATTAACCTCAATACGTCTAGTGTTTTGACGTTACCGACAGAATAAGC
ACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTAAGTTGAATGTGAAAG
CCCCGGGCTCAACCTGGGAACTGATCCAAAACTGGCAAGCTAGAGTGTTGAATT
CCATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAG
GAAGGAACACCAGTGGCGAAGGCGACCACCTGGGCTAATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAATA
AACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGC
ATTA

1.18 Isolate NG05 (*Citrobacter freundii*)

Accession No. KC109319

Forward Sequence:

CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGCACGAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAA
CTGCATCCGAAACTGGCAGGCAGAGTCTTGTAGAGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGGCCAATGCCACCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCT
TGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCTGGGGAGTACGGC
CGCAAGGTTAAAACCAAATGAATTGACGGGGGCCCGCACAAGCGTGGAGCATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTT
AGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA

TCCTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCAGTGATAACT
GGAGGAAGGATGACGTCAAGTCACATGGCCCTTACGAGTAGGGCTACACACGTG
CTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATA
AAGTATGTCGTAGT

1.19 Isolate NG07 (*Bacillus cereus*)

Accession No. KC109326

Forward Sequence:

GCTAATACCGATAACATTTGAACCGCATGTTCGAAATTGAAAGGCGGCTTCGGCT
GTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTA
AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACG
GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGTTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGGCGCAGGT

1.20 Isolate KT05 (*Bacillus subtilis*)

Accession No. KC109328

Forward Sequence:

ATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGTCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTC
GGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT
AAAGCTCTGTTGTTAGGGAAGAACAAGTACGTTCGAATAGGGCGGTACCTTGACG
GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAG