Bacteriocins for the Control of Phytopathogenic Bacteria

BY

NUSRAT JABEEN

Dissertation Submitted to the Department of crobiology, University of Karachi, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Department of Microbiology
University of Karachi, Karachi-75270
Pakistan

2007

BACTERIOCINS FOR THE CONTROL OF PHYTOPATHOGENIC BACTERIA

Certificate

This is to certify that the research work reported in this dissertation entitled: ***"Bacteriocins for the Control of Phytopathogenic Bacteria"*** *by* ***Nusrat Jabeen****, has been carried out under my supervision and guidance in the Department of Microbiology, University of Karachi. I considered this thesis suitable for submission for the award of degree of Doctor of Philosophy.*

Dr. Sheikh Ajaz Rasool
meritorious Professor and Thesis Supervisor
Department of Microbiology
University of Karachi, Karachi-75270
Pakistan

Dedicated

To My Beloved Parents

List of contents

6. Figures.....91

Acknowledgement

I begin with the name of almighty Allah, the most merciful and benign. Who gave me the opportunity and strength to undertake this research work. All praise to the lord of all, ruler, and divine. The king of the judgment thrown to the alone we bend our heads thy help alone we seek.

I wish to express my perpetual deepest gratitude to my supervisor Prof. Dr. Sheikh Ajaz Rasool, Dean Faculty of Science, University of Karachi, for his excellent guidance, enthusiastic supervision and constant encouragement, without whom I could not work with confidence and satisfaction in the whirlpool of research. I will always be indebt for his affectionate attitude.

My endless regards are due to my research colleague and teacher Mr. Syed Abdus Subhan for his supervision and guidance, keen interest and above all giving his valuable time, suggestions, and attention during my research work. I am also grateful to Ms. Samia Ahmad and Dr. Sadia Saeed, Laboratory of Molecular Genetics, Department of Microbiology, University of Karachi for their cooperation, valuable suggestions, selfless help and encouragement during the study. This study would not have been possible without constant help and insightful suggestions of these people.

My special thanks are due to Mr. Junaid Ahmed Khan, Nuclear Institute of Agriculture Biology, Faislabad, for helping me in carrying out all Field experiments without whom it is not possible for me to do the applied studies.

I am also grateful to Engr. Dr. Khursheed Mehmood, Professor and Mr. Ikram ul Haq TEM operator, Department of Mechanical Engineering, NED University of Engineering and Technology, for their kind cooperation in conducting the experiment on Transmission Electron Microscopy.

I am also obliged to Mr. Mushtaq Hussain and Tanveer Khan (KIBGE) to help me out in computational analysis.

I acknowledge with thanks the generous funding of Pakistan Agriculture Research Council- Agricultural Linkage Programme under research project: F.No.2 23/2003 (ALP) PARC (P)

Profound thanks are extended to all my teachers as well as the non teaching staff Department of Microbiology for their supporting back up.

From the depth of my heart I am thankful to my two special teachers Mr. S. Zubaid Shoeby and Ms. Shazia Tabassum Hakim for their moral support and unconditional affection throughout my life.

I owe my gratitude to my all friends for their support and. encouragement. Specially Ms, Mahwish Baqir (late) who always gave me the confidence.

All words of gratitude seem insufficient to express the indebteness, whole hearted thanks and deepest appreciation to my family, including my gracious parents and my brothers and sisters specially beloved grand mom for their constant love, support and prayers throughout this study and always. Its their believe and faith on me that helped me to overcome the difficulties during the path of research.

May God show his blessings to all of them.

Nusrat Jabeen

SUMMARY

خلاصہ

عوامی حلقوں میں کیمیائی مادوں کے استعمال، خصوصی طور پر ماحولیاتی نکتہ نظر سے اور بیماریوں کی روک تھام کے لیے متبادل کی ضرورت کے احساس میں خاصا اضافہ ہوا ہے۔ نباتی بیماریوں کے خلاف حیاتی قابو کے ایجنٹ کے استعمال سے زیادہ کاشت کا حاصل کرنا اور مویشوں کی محفوظ نسل افزائی کی تائید ہوتی ہے۔ جرثومے بدخلوی کلاں سالمی لحمیہ / پیپٹائیڈ ضد حیاتیے کے طور پر بتائے جا چکے ہیں۔ جو حقیقی جراثیم سے بنتے ہیں اور اپنے مہلک اثر سے اپنے ہی جراثیم اور ان سے منسلک گروہوں پر اثر انداز ہوتے ہیں۔ جرثومے اور دافع خرد جراثیمی پیپٹائیڈ مخفی متبادل کے طور پر توجہ حاصل کر چکے ہیں اور اضافی طور پر جراثیم کش مرکبوں کے طور پر استعمال ہو رہے ہیں۔ غالباً ۹۹ فیصد تمام جراثیم کم از کم ایک جرثومہ بناتے ہیں جبکہ یہ مثبت گرام اور منفی گرام جرثومہ بنانے کے قابل ہوتے ہیں۔ اکثر نباتی امراض زایانہ جراثم معہ *Erwinia*، *Corynebacterium*، *Pseudomonas*، *Xanthomonas* اور *Agrobacterium* کے نمائندوں کے حیاتی سرگرم جوثومے بناتے ہیں۔ یہ جرثومے انتہائی منفرد کارگر لاگت اور یہاں تک کہ معاشی دوست ہوتے ہیں۔

آٹھ انواع جیسے *Xanthomonas oryzae* NAI، *Xanthomonas oryzae* NA2، *Xanthomonas citri* NA3، *Pseudomonas andropogonis* NA4، *Erwinia carotovora* NA5، *Agrobacterium randiobacter* NA6، *Agrobacterium radiobacter* NA7 اور *Erwinia carotovora* NA8 خراب پھلوں، سبزیوں اور کھاد سے علیحدہ کیے گئے۔ تمام الگ کیے گئے نمونوں کی جراثیم نباتی امراض زایانہ کی مخالف حیاتی سرگرمی کے لیے اسکرین کیے گئے جن میں سے صرف تین جو *Erwinia carotovora* NA5، *Agrobacterium radiobacter* NA6 اور *Agrobacterium radiobacter* NA7 جرثومے بنانے کے لیے پائے گئے۔ ان کے جرثومے *Erwiniocin* NA5، *Agrocin* NA6 اور *Agrocin* NA7 بالترتیب مقرر کیے گئے۔ *Agrocin* NA6 اور NA7، *Agrobacterium tumefaciens* B6 کے مخالف سرگرم پائے گئے جبکہ Erwiniocin NA5 ایک میگا جرثومہ کے طور پر سامنے آیا۔ ان کی جراثیم خوروں اور ہائیڈروجن پر آکسائیڈ سے مزاحمتی سرگرمی متعلق نہ ہو سکی۔ Erwiniocin NA5 مثبت گرام اور منفی گرام کے مخالف ایک وسیع مسلسلہ سرگرمی رکھتا ہے (بشمول جراثیم خوروں

کے)۔ اس نے مختلف درجہ حرارتوں کی وسیع ترتیب(60°C, 80°C, 100°C اور 121°C دباؤ) میں مختلف وقتی دورانیوں میں اپنی مخالفت میں استحکام قائم رکھا اور یہ استحکام pH (2-14) کی حد میں بھی قائم رہا۔ مادہ (Erwiniocin NA5) مختلف دھاتی برقوں اور نامیاتی محلولوں' lipase' lysozyme اور catalase کی درستگی میں مزاحم رہا لیکن لحمیہ K اور protease کے لیے حساس رہا۔ Erwiniocin NA5 کا ضد حاتی معائر 160 AU/mL پایا گیا۔اس کی پیداوار ابتدائی لاگرتھم مرحلہ میں شروع اور آخری ساکن مرحلہ تک جاری رہی۔ Erwiniocin NA5 نے جراثیم کش اثر *Xanthomonas oryzae* NA1 پر اس کے ساتھ ہی ساتھ *Ewrinia carotovora* NA8 پر ظاہر کیا۔ Erwiniocin NA5 کی برق پاشی بافتوں کی رق پاشیدگی سے کی گئی۔اس کا سالماتی وزن 12kDa سے کم ناپا گیا(جو 11kDa بذریعہ SPS-PAGE سے تصدیق کرتا ہے)۔ جرثومہ کی امونیم سلفیٹ کے ذریعہ رسوبیت (۸۰ فیصد کی حد تک سیرابی) کی گئی۔ جب صاف جرثومہ کی جزوی عمل تقطیر Sephadex G75 کے کالم استعمال کرتے ہوئے) لحمیہ کی ایک بلند سرگرمی 2.2 تہہ کے ساتھ دیکھی گئی۔ امونیوایسڈ کی بناوٹی اعداد ظاہر کرتے ہیں کہ Erwiniocin NA5 کی 12.44%' تیزابی اور 13.75% بنیادی اور 73.99% تعدلی امینوایسڈ کے اجزا (49.8% پولر اور 50.16% غیر پولر امینوایسڈ کے اجزا) پر مشتمل ہے۔ Erwiniocin NA5 کے برقی خردنگار اس کے فطری منفی دائمی کروی ذروں کو ظاہر کرتے ہیں جس سے قیاس کیا جاتا ہے کہ یہ آبلہ خلیہ پاش مادوں یا ناقص جراثیم خور ذروں (بناسر کے) حبثہ ہیں۔ Erwiniocin NA5 کی پیداوار اضافی جینیاتی کرو موسومل عناصر سے ترتیب دی جاتی ہے۔ Erwiniocin NA5 کا *in vivo* اثر *Xanthomonas oryzae oryzae* (Xoo) کے مخالف دیکھا گیا۔ نتیجتاً جرثومہ اثر انداز پایا گیا(منظم حالات اور ساتھ ہی ساتھ کھلے میدان میں)۔ جرثومے(Erwiniocin NA5 جیسے) کو ضد حیاتی اور مصنوعی محلولوں پر فوقیت دی جاسکتی ہے۔ Erwiniocin NA5 کی پیداوار کا مطالعہ Shake glass' لیب اور Pilot Scale Fermenter جیسی تکنیکوں سے کیا جاسکتا ہے۔

Summary

Interest in biological control has increased recently fuelled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to the use of chemicals for disease control. Use of biocontrol agents against plant diseases has been advocated to get the high yields of crops and safe breeding of livestock. Bacteriocins have been described as extracelluar macromolecular protein/peptide antibiotics produced by certain bacteria which exert their lethal effects on bacteria of the same or the related groups. Bacteriocins and anitimicrobial peptides have attracted attention as potential substitutes or as additions to currently used antimicrobial compounds. Probably 99% of all bacteria generate at least one bacteriocin, while both gram-positive and gram-negative bacteria are capable of producing the bacteriocins. Many phytopathogenic bacteria including members of the *Corynebacterium, Erwinia, Pseudomonas, Xanthomonas* and *Agrobacterium* produce bioactive bacteriocins. These bacteriocins are highly specific, cost effective and are even eco-friendly.

Eight strains namely *Xanthomonas oryzae* NA1, *Xanthomonas oryzae* NA2, *Xanthomonas citri* NA3 *Pseudomonas andropogonis* NA4 *Erwinia carotovora* NA5 *Agrobacterium radiobacter.* NA6 *Agrobacterium radiobacter* NA7 *and Erwinia carotovora* NA8 were isolated from diseased fruits, vegetables and soil. All the isolates were screened for bioactivity against phytopathogenic bacteria whereby only three i.e. *Erwinia carotovora* NA5, *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobacter* NA7 were found to produce bacteriocin. Their

bacteriocins are designated as Erwiniocin NA5, Agrocin NA6 and Agrocin NA7 respectively. Agrocin NA6 and NA7 were found active against *Agrobacterium tumefaciens* B6, however, Erwiniocin NA5 proved to be a mega bacteriocin. The inhibitory activity could not be related to bacteriophages and hydrogen peroxide. Erwiniocin NA5 has a broad-spectrum activity against many gram-positive and gram-negative bacteria (including phytopathogens). It showed stability against a wide range of temperatures (60°C, 80°C, 100°C and pressured 121°C) for varied time periods and remained stable at wide (2-14) pH range. The substance (Erwiniocin NA5) was resistant to treatment with several metal ions and organic solvents, lipase, lysozyme, and catalase but sensitive to proteinase K and protease (suggesting its proteinaceous nature). The antibacterial titre of Erwiniocin NA5 was found to be 160 AU/mL. Its production starts in early logarithmic phase and continues till late stationary phase. Erwiniocin NA5 revealed bactericidal effect on *Xanthomonas oryzae* NA1 as well as on *Ewrinia carotovora* NA8. Erwiniocin NA5 is dialyzable through dialysis membrane; its molecular weight was estimated to be less than 12 kDa, (which was confirmed to be 11 kDa by SDS-PAGE). Bacteriocin was precipitated (up to 80% saturation) by ammonium sulphate. The precipitate was found to contain 290 mg total protein. When partially purified bacteriocin was subjected to gel filtration (using sephadex G75 column) a major active peak of protein (containing 11 mg of total protein as estimated by biuret method) with 2.18 fold purification was detected. The amino acid composition (processed by NCBI genomics server) data revealed that Erwiniocin NA5 contains about 12.44% acidic and 13.57% basic and 73.99% neutral amino acid components (with 49.8% polar and

50.16% non-polar amino acid components). The electron micrograph of native negatively stained Erwiniocin NA5 revealed spherical particles, which may be presumed to be membrane vesicle from lysed cells or defective bacteriophage particles (i.e. empty head) like structure. The production of the Erwiniocin NA5 is regulated by extra chromosomal genetic factor(s). The *in vivo* effect of Erwiniocin NA5 was monitored against *Xanthomonas oryzae* subsp. *oryzae* the causative agent of bacterial leaf blight (BLB) of rice. Accordingly, the bacteriocin was found effective (in controlled conditions as well as in field trials). Bacteriocins (like Erwiniocin NA5) could be preferred over antibiotics and synthetic chemicals. The production of Erwiniocin NA5 may be studied by shake flask, Lab and pilot scale fermenter technologies. (before the question of mass scale production can be addressed).

INTRODUCTION /LITERATURE REVIEW

1. Introduction/Literature Review

1.1. What are Bacteriocins?

Microbes produce an array of microbial defense systems. These include classical antibiotics, metabolic by-products, lytic agents, numerous types of protein exotoxins, and **bacteriocins** (the protein antibiotics). Bacteriocins are the most abundant of a range of antimicrobial compounds facultatively produced by bacteria, and are found in all major bacterial lineages. The abundance and diversity of these potent bioarsenals are clear (Riley and Wertz, 2002a). Bactericidal specificity, restricted to species closely related to their producer, and chemical composition distinguish bacteriocins from other classic antibiotics (Tagg *et al.*, 1976; Vidaver, 1983; Gross and Vidaver, 1990). The widespread resistance of bacterial pathogens to conventional antibiotics has prompted renewed interest in the use of alternative natural microbial inhibitors such as antimicrobial peptides (Chen and Hoover, 2003). These novel substances have been described as extracellular macromolecular protein/peptide antibiotics produced by certain bacteria which exert their lethal effect(s) on bacteria of the same or the related groups (Iqbal, 2001). Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland *et al.*, 2001). The antimicrobial peptides produced by bacteria have been grouped into different classes based upon the producer organisms, molecular size, chemical structure and mode of action, which resulted in different names for putative compounds which turned out to be identical e.g. thiolbiotics,

lantibiotics, microcins, colicins, bacteriocins, to name a few (Kolter and Moreno, 1992). Bacterial species produce one or more antibacterial substances called bacteriocins, which enhance their competitiveness with other related bacterial species (Chuang *et al.*, 1999).

Bacteriocins and anitimicrobial peptides have attracted attention as potential substitutes or as additions to currently used antimicrobial compounds. Probably 99% of all bacteria generate at least one type of bacteriocin. They are proteinaceous compounds of bacterial origin that are generally lethal to bacteria other than the producing strain. Infact, the antagonistic interaction amongst bacteria is due to a wide variety of inhibitory substances produced by a large number of bacteria. The biological control agents compete through the production of antimicrobial substances such as antibiotics/bcteriocins which inhibit the growth of pathogens directly, rather than by preemptive consumption of limiting resources. Bacteriocins are a class of heterogenous inhibitory substances which are the proteins effective against bacteria closely related to the producing strain (Rodriguez-Valera *et al.*, 1982). Bacteriocins have been described in several genera of both gram positive and gram negative bacteria. As such bacteria produce a wide range of inhibitors; it is difficult to decide which of them should be regarded as bacteriocin (Hardy, 1982). These inhibitory products include low molecular weight antitoxins, metabolic products, lytic agents, enzymes, defective bacteriophages and the bacteriocins (Tagg *et al.*, 1976). The bacteriocins are non-replicating bacterial proteins ribosomally synthesized antimicrobial peptides produced by microorganisms belonging to different eubacterial taxonomic branches (Riley and Wertz, 2002a; 2002b). Some observations have

indicated that bacterial protein toxins may also prove to be broad spectrum antagonists (Rasool and Akhtar, 1992; Rasool *et al.,* 1993). Bacteriocins (sometimes) may include defective bacteriophages although they are clearly distinct from the low molecular weight type of

colicins (Lotz, 1976). Thus, bacteriocins are of two main types' viz. true bacteriocins and defective phage particles. It is well known that bacteriocins are small molecular weight proteins produced by some kinds of bacteria which show various biological activities against animals and microorganisms.

A great deal of research into bacteriocins and their uses has been carried out, including characterization of new bacteriocins, modification of bacteriocins by protein engineering (Rollema *et al.*, 1995; Chen *et al.*, 1998), construction of food-grade vectors (Takala and Saris, 2002), regulation and expression of heterologous proteins (De Ruyter *et al.*, 1996), control of flavour and other characterisitcs of fermented food, agricultural, pharmaceutical and veterinary applications of bacteriocin producing bacteria, etc. Bacteriocins produced by lactic acid bacteria (LAB) are highly valued because of their bactericidal activity which is generally recognized as safe (Muriana & Luchansky 1993). Nisin, a typical bacteriocin produced by *Lactococcus lactis*, has already been used in the food industry as an antagonistic additive in more than 50 countries (Cintas, 2001).

Hence, not only do searches continue for new and more effective bacteriocins, but also development is ongoing for optimization of existing bacteriocins to address both biologic and economic concerns (Chen and Hoover 2003). Numerous bacteriocins

have been purified and characterised in great detail, both at biochemical and genetic levels. Still, novel bacteriocins with new properties are reported in an increasing number in recent years (Nes and Diep 2002).

The following criteria are generally applicable to prototype bacteriocins, *i.e.* colicins, which are produced by Gram negative bacteria:

1. A narrow spectrum of activity centered around the homologous species.
2. The presence of an essential, biologically active protein moiety.
3. Bactericidal mode of action.
4. Attachment to the specific cell receptors.
5. Plasmid located determinants of bacteriocin production and host cell bacteriocin immunity.
6. Lethal biosynthesis of bacteriocins i.e. the commitment of the bacteria to produce bacteriocins that will consequently lead to cell lysis.

Bacteriocins of Gram positive bacteria show difference in only two requirements i.e. wide spectrum of activity against organisms of different species and less solid host cell immunity to the homologous bacteriocins (Hamon and Peron, 1963). In particular bacteriocin may give the producing strains a competitive advantage by killing bacteria in the same environment competing for the same resources (Whitford *et al.*, 2001).

1.2. Historical Perspective of Bacteriocins

Gratia (1925) first demonstrated that the filtrates of a particular strain of *Escherichia coli* culture strongly inhibited the growth of another strain of the same species. The inhibitory substance was later named by Gratia and Fredericq (1946) as "Colicins". They also characterized these substances and found them diffusible in agar and cellophane membrane, resistant to heat and chloroform, acetone precipitable and non-antigenic. Later on, analogous substances were found produced by a number of strains belongs to family Enterobacteriaceae including *E. coli, Enterobacter, Salmonella, Shigella* and *Proteus* species (Fredericq, 1946a; 1946b). Colicinogeny is a permanent hereditary character which is not lost by serial transfer from a strain. In some cases, non-colicinogenic mutants were isolated from the strains producing colicins (Amano *et al.*, 1958). Many Col-plasmids also determine the production of sex pili and are able to transfer themselves to other bacteria by conjugation. Colicins have a narrow spectrum of action, killing only strains of Enterobacteriaceae. Some of them act against only a few strains whereas others may have a wide range of action. In general, the strains producing colicins are immune to their own colicins with an exception where the bacteria are sensitive to the same colicins produced by them (Konisky, 1978). The genes for various types of colicins are located on Col-plasmids, which range in size from 0.2% to 3.5% of the length of *E. coli* chromosome (Hardy, 1979). Colicins are classified into 20 types according to their effect on insensitive strains of Enterobacteria (Hardy, 1982).

In recognition to the discovery that non-coliform bacteria may also produce antibiotic substances of the colicin type, the more general term "bacteriocin" was coined by Jacob *et al.* (1953). Bacteriocins were specifically defined as protein antibiotics of the colicin type, *i.e.* molecules characterized for lethal biosynthesis, predominant intra-species killing activity, and adsorption to the specific receptors on the surface of bacteriocin sensitive cells. Antibiotic-like protein substances are also produced by certain strains of Pseudomonas and are called as "Pyocins" (Jacob, 1954). Hamon and Peron (1961) described a substance produce by *Erwinia carotovora*, the causative agent of soft rot in many plant species that is bactericidal towards the same and related species and designated as "Carotovoricin". Yasunaka and Amako (1979) reported some bacterial species which produced one or more bacterial substances called bacteriocins that enhanced their competitiveness with other related bacterial species. Edmondson and Cooke (1979) after analyzing a number of bacteriocin producing strains of *Klebsiella pneumoniae* classified three types of "Klebicins". Plasmid that codes for "Vibriocins" is responsible for killing sensitive *Vibrio cholerae* was reported by Gardner and Snustad (1981). Similarly, members of Halobacteriaceae are reported to produce a wide variety of antagonistic substances called "Halocins" (Rodriguez-Valera *et al.*, 1982). Bacteriocins are produced by members of all major groups of Eubacteria and Archaebacteria (Torreblanca, 1989), they are bactericidal, antibiotic-like substances apparently protein in nature produced by many bacteria and have a killing action on strains of the same or closely related species (Marekova, 2003).

1.3. How do they Work?

Bacteriocins are a heterogeneous group of particles with different morphological and biochemical properties. They range from simple proteins to a high molecular weight complexes; the active moiety of each molecule in all cases seems to be the protein in nature. The majority constitute small cationic and hydrophobic molecules. These bactericidal particles are species specific. They exert their lethal activity through adsorption to specific receptors located on the external surface of sensitive bacteria, followed by metabolic, biological and morphological changes resulting in the killing of such bacteria (Daw and Falkiner, 1996). The Bacteriocin specificity is not well understood, but specific receptors may play a role in their binding to cells. Immunity proteins that sequester the bacteriocins from the cell membrane or expel them to protect the bacteriocin-producing bacteria. Bacteriocin-sensitive bacteria can become resistant, but this characteristic is distinctly different from immunity. Disruption of any of the steps leading to activity (binding, insertion, and poration) could theoretically generate resistant cells, but binding interruption is the most well understood mechanism of resistance. Most bacteriocins are positively charged, and resistant cells alter their lipoteichoic acids, become more positively charged and repel bacteriocins (Russel *et al.,* 2005). Lethal action of a bacteriocin on sensitive bacterial cells seems to occur in two distinct phases, namely, the initial combination of the bacteriocin with specific receptor sites located on the cell wall of the sensitive organism, which is followed by some sequence of events thereby leading to the death of the cell (Bordet and Beumer, 1951). Bacteriocins seem to work by entering the

outer membrane of a susceptible bacterium, congregating in groups, and forming pores that allow the unregulated outflow of essential ions. The target bacterium begins breaking down ATP in a vain attempt to produce enough new protons to recharge the membrane, a futile cycle that quickly results in exhaustion of bacterial ATP. Bacteriocins are most effective in acid while least effective in salty environments. Bacteriocins of gram-positive bacteria such as *Listeria* spp. and *Clostridium botulinum* are ineffective against gram-negative bacteria such as *E. coli* and *Salmonella* spp. which have a protective double walled outer membrane. Several gram-negative bacteriocins are known e.g. the colicins, which are water-soluble cytotoxins secreted by and active against *E. coli*, that form voltage-sensitive ion channels in the bacterial inner membrane that kill the cell by selectively siphoning out key cell nutrients and inhibit protein synthesis (Robert, 1999). Bacteriocins are generally high molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanisms vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16s rRNA, and inhibition of peptidoglycan synthesis (James *et al.*, 1991; de Vuyst and Vandamme 1994; Heu *et al.*, 2001).

1.4. Genetical Aspects of Bacteriocins

The genetic make up of the living organisms consists of nuclear DNA and RNA but in addition, some circular DNA molecules exist in the cytoplasm called 'plasmids'. Most plasmids are dispensable i.e not required for the survival of the cell (Ochiai *et al.*, 1959). Plasmids or episomes are the cytoplasmic (variable accessory

extrachromosomal genetic elements-VAGE) double stranded DNA molecules that harbour specialized genes and have the ability to replicate autonomously in bacterial cells (Lin *et al.,* 1984). The amount of plasmid DNA (in a bacterium) may constitute as much as 1% to 2% of the total cellular DNA. A bacterium may harbour several plasmids (Lim, 1998). Most of the well characterized bacteriocins are coded by plasmid genes and infact, the entire gram positive bacteriocins are regulated by the plasmid genes (Hardy, 1975).

Bacteriocins are agents which are encoded in the genetic material carried by plasmids, with the purpose of killing or inhibiting closely related species or even different strains of the same species. Bacteriocins were evolved out of the need for survival in over populated environment; thus, cells containing the plasmid encoding for a bacteriocin, have the capability of destroying the surrounding cells not having the same bacteriocin plasmid, and the event is compensated by the rate at which the bacteriocin containing cell is able to transpose the plasmid with the required function into other cells, eventually only cells containing a plasmid encoding the bacteriocin will be left in existence. The outcome from the series of these opposing events are governed by the immunity protein, encoded by the *imm* gene, which allows the cell to be resistant to the bacteriocin it is producing, this ensures the cells survival in the circumstance of the bacteriocin being manufactured by the cell itself or the surrounding bacteria. It should also be noted that a naturally occurring plasmid only encodes for one type of bacteriocin, if it encodes for one at all. A much studied bacteriocin is colicin, which is encoded by the Col plasmid(s) and as expected, originates from the bacterial species, *Esherichia coli.* There are several types of

plasmids and many have been identified which do not have any apparent effect on the phenotype of the host cell (cryptic plasmids) while the remaining can be subdivided depending upon their transferability from one host to another. Various criteria are used to classify the plasmids. Thus, "R plasmids" may confer resistance to one or more antibacterial drugs; "Col plasmids" for antimicrobial protein substances (colicins) while "Virulence plasmids" enhance the pathogenicity of bacteria (Hardy, 1987). Plasmids can be either conjugative or non-conjugative. Conjugative plasmids transfer their copies from one bacterium to another and many of them code for conjugating protein tubes (sex pili) through which the DNA passes to the recipient (a process that may be regarded as Horizontal gene transfer). It is well documented that genes for bacteriocin production and immunity are regulated and transcribed simultaneously (Abee, 1995, Nes *et al.*, 1996). Gene-encoded antimicrobial peptides have been observed in virtually every living organism, and the ones produced by bacteria, (i.e. bacteriocins), have attracted widespread scientific attention in recent years as potential chemotherapeutic agents.

1.5. Bacteriocins Vs other Antimicrobial Substances

The discovery of penicillin by Fleming was a historical milestone in the fight against infectious disease. Over the following fifty years or so, pharmaceutical companies discovered and developed over 100 antibiotics effective against a wide range of human pathogens. More recently, the dramatic rise in antibiotic-resistant bacteria has resulted in renewed efforts to find new antimicrobials. Bacteriocins are an attractive focus for drug development because bacteriocins are active against most pathogens,

already exist in nature, are remarkably stable, and are not toxic to human cells. Bacteriocins are produced by bacteria and possess antibiotic properties, but bacteriocins are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can potentially illicit allergic reactions in humans (Cleveland *et al.*, 2001). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous, ribosomaly sunthesised and generally possessing a narrow specificity of action against strains of the same or closely related species (Tagg *et al.*,1976). Low-molecular-weight antibiotics (for example, tetracyclines), lytic agents, toxins, bacteriolytic enzymes, bacteriophage, and metabolic by-products, such as organic acids, hydrogen peroxide, and diacetyl, also function in a somewhat similar capacity, but nonetheless the capability to produce bacteriocins and producer-cell immunity occurs abundantly in prokaryotes, both eubacteria and archaebacteria. Unlike other antimicrobials, the lethal activity of bacteriocins is often (but not always) limited to members of the same species as the producer, suggesting a major role in competition with co specifics (Riley *et al.*, 2002a). The use of bacterial metabolites instead of microbial agents should be recommended for overcoming difficulties that can be found in the application of microorganisms as biocontrol agents (Lavermicocca *et al.* 2002). Several biotechnological companies have developed different therapeutic applications of these compounds ranging from tropical administration to systemic treatment of infections (Saeed *et al.*, 2006).

1.6. Application of Bacteriocins

Among the many different substances known to play a role in bacterial interactions, bacteriocins are the most specific and efficient antagonists. Bacteriocins comprise a large and functionally diverse family of toxins found in most microbial species. They play a critical role in mediating microbial interactions and in maintaining microbial diversity. This diverse group of protein toxins has potential applications in human health, veterinary medicine, crop management, agriculture, poultry, food preservation and bioremediation (Riley and Gillor, 2007). Bacteriocins are an attractive focus for drug development because bacteriocins are active against most pathogens, already exist in nature, are remarkably stable, even are non toxic to human cells, and one could also go for their large scale (industrial) production.

1.6.1. Bacteriocins as Antimicrobials

Microorganisms are engaged in a never-ending arm race. One consequence of this intense competition is the diversity of antimicrobial compounds that most species of bacteria produced (Riley, 1998). The dramatic rise in antibiotic-resistant pathogens has stimulated the efforts to identify, develop or redesign antibiotics; that may be active against multi drug resistant bacteria. One may well divert attention towards the bacteriocins, which hold great promise as the next generation of antimicrobials. Bacteriocins are differ from classical antibiotics in their specific action on closely related bacteria. Accordingly, they can be considered "designer drugs" that target specific bacterial pathogens to solve some of the most challenging problems in infectious disease control scenario. They can also be used as prophylactic agents

against several tropical and systemic diseases in humans and animals. Some of the bacteriocins like "Nisin" has been used in soaps, skin care and personal hygiene products, disinfectants, cleansers, cosmetics and for the treatment of acne (Chen and Hoover, 2003). Antimicrobial peptides have been shown to display both antiviral and antifungal activities *in vitro* and have also been shown to be effective in experimental infections with multidrug resistant *Mycobacterium tuberculosis* (Oakey *et al.*, 2003). Barrett *et al.* (1992) has reported a bacteriocin named as M87–1551 from *Bacillus* spp. which was active against different clinical staphylococcal strains including those isolated from blood and was called mersacidin due to its bactericidal activity against MRSA strains. Staphylococcin Bac 188 has shown significant bioactivity against several clinical isolates of MRSA (Saeed *et al.*, 2006).

1.6.2. Bacteriocins against Cancerous Cells

Bacteriocins have been defined by their bactericidal or bacteriostatic action on closely related bacterial strains, through their specific cytotoxic action. The antitumour activity of colicins E1, E2, E3 and D was examined in several tumor cell lines. Colicins present a new yet unexplored group of natural substances displaying clear cytocidal tumoricidal and oncolytic effects in a manner similar to non colicin bacterial toxins (Davies, 1990). There are some examples according to which bacteriocins have been shown to have cytotoxic effect on animal cells. These bacteriocin/toxin molecules are mostly pore formers. These bacteriocins exert their toxic action on eukaryotic cells through mechanisms that can lead to the induction of necrosis or apoptosis. In the latter case, individual animal cells are eliminated without the

induction of an inflammatory response. Thus, modulation of apoptosis has an immense potential in the treatment of some diseases, particularly cancer (Lagos, 2007).

1.6.3. Replacement Therapy for the Prevention of Dental Caries

Five billion people worldwide suffer from dental caries, making it the most common and chronic infectious disease of humankind caused by *Streptococcus mutans*. A bacteriocin produced by *Streptococcus mutans* strain JH1000 was originally isolated based on its superior ability to colonize teeth, which was due to its production of a bacteriocin capable of killing all other strains of *S. mutans*. Its cariogenic potential was essentially eliminated by genetically modifying its ability to produce lactic acid, which is directly responsible for eroding the teeth mineral. The resulting strain is intended for use in a novel method for prevention of caries called "replacement therapy". A single application of the replacement strain is expected to provide lifelong protection from most of the human tooth decaying organisms. The bacteriocin produced by the replacement strain belongs to the lantibiotic family. Nisin has been used in the dental health care products such as toothpastes and mouthwashes, for inhibition of dental caries and periodontal diseases (James *et al.*, 2007).

1.6.4. Immunological Studies

Antibodies isolated from antisera raised against nisin and Pediocin ACH (conjugated to egg albumin) fostered the development of an enzyme-linked immunosorbent assay (ELISA) and failed to elicit an immune response in mice or rabbit, suggesting their non-toxicity to humans if and when used as food biopreservatives (Brussiere, 2003).

1.6.5. Bacteriocins in Livestock

The utilization of bacteriocin-producing bacteria (BPB) in livestock has been evaluated as a potential alternative to antibiotic usage. Accordingly the use of BPB in farm animals has increased the productivity, improved animal health, and prevented the spread of human pathogens. Colicinogenic bacteria appear to be promising in controlling enterohemorrhagic *E. coli* in cattle and reducing the incidence of neonatal diarrhea caused by enterotoxicogenic *E. coli* in calves. Microcin-producing *E. coli* have been used to reduce *Salmonella* carriage in poultry. Lacticin produced by *Lactococcus lactis* has been reported to be effective to reduce cow's mastitis caused by *Staphylococcus aureus* and *Strepococcus dysgalactia*. These findings indicate that their utilization will be a feasible antimicrobial intervention in livestock for the future (Diez-Gonzalez, 2007).

1.6.6. Bacteriocins in the Food Industry

Many lactic acid bacteria (LAB) used in the food industry produce bacteriocins and thus are a rich source of these natural inhibitors (Cintas *et al.*, 2001). These bacteriocins have widespread potential applications in preservation for improving the safety and quality of foods. Indeed, bacteriocins may be viewed as an innate immunity system which can be inbuilt into food systems to self-protect them against contamination and/or outgrowth with undesirable flora. Vescovo *et al.*, (1996) suggested a bacteriocin produced by *Lactobacillus casei* IMPC LC34 as a biopreservatives for ready-to-use vegetables as it was found effective in reducing total

mesophilic bacteria and the coliform group. Nisin is considered as the star of the bacteriocins. It is the only bacteriocin allowed as a food additive and accepted by the American Food and Drug Administration for use in a variety of products including pasteurized, flavoured and long shelf life milk(s), aged and processed cheese and canned vegetables and soups (O'Connor, 2007). The advantage of these peptides include; their small size, activity directed towards the cell membrane, readily digested in human intestine, non toxic and not inducing any allergy (Cheen and Hoover, 2003).

1.6.7. Bacteriocinogenics as Starter Cultures

Lactic acid bacteria play an essential role in majority of the food fermentations and are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery e.g. *Pediococcus acidilactici* that produces pediocin A1 as lactic starter for the production of dry fermented sausage (Morgan *et al.*, 1996).

1.6.8. Bacteriocins as a Fungicide

Apple scab, caused by the fungus *Venturia inequalis,* is one of the most important apple diseases worldwide. Control of scab has been relied almost exclusively on the use of fungicides. In future the use of fungicides may be prohibited because of human healths, environmental risks and resistance developed by the pathogens. *Pseudomonas syringae* 508 produced antibiotics syringomycin E and G that may kill conidia of *V. inaequalis* (Corral and Burr, 1996). Magnusson *et al.* (2003) reported lactic acid bacteria which were isolated from different environments and screened for antifungal activity and approximately 10% of the isolates showed inhibitory activity and 4% showed strong activity against the indicator mould *Aspergillus fumigatus.*

1.6.9. Bacteriocins as Biocontrol Agents against Phytopathogens

Bacteria occupying plant niches exhibit diverse lifestyles ranging from epiphytic colonization of phyllospheres or rhizospheres to infection of plant tissues as endophytes, symbionts, or pathogens. Bacteriocin production plays a role in the competitive colonization of the plant environment by phytobacteria. A large bacteriocinogenic potential is present in these bacteria, but only a limited number of these allelopathic agents has been characterized biochemically and/or genetically. In a number of cases, proof-of-principle for control of specific bacterial plant diseases, using bacteriocin producers or bacteriocin preparations has been provided e.g. agrocin K84 a bacteriocin produced by *Agrobacterium radiobacter* K84, commercially available which active against crown gall disease (caused by *Agrobacterium tumifaciens*) of many plants (Kerr, 1980)

1.7. Control of Phytopathogenic Bacteria by Bacteriocins

1.7.1. Plant Diseases and Phytopathogenic Bacteria

Bacterial diseases contribute to a world-wide crop loss of roughly 12-15 % annually. Almost any type of plant can be affected by bacteria. Virtually all plant-pathogenic bacteria are bacilli, and almost all occur in their host plants as parasites. Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide (Stéphane *et al.*, 2005). Plant diseases can be defined in many ways but one of the simplest definitions describes disease as any condition in a plant (caused by living and non living agents) that

interferes with its normal growth and development. Diseases or plant health problems can impact plants in many ways since all parts of a plant can be affected [(including flowers, leaves, fruits, seeds, stem branches, growing tips and roots) (Varnam and Evans, 2000; 2002)].

Plant pathogenic bacteria use the living plant tissue as their favoured food source. These bacteria are highly specialized to circumvent plant defenses and to efficiently invade tissues. As a result of infection process, nutrient materials are elaborated which the pathogen is able to utilize. Once invasion of host tissues takes place, secondary responses are initiated in the plant and constitute manifestations of the disease symptoms (Gabriel, 1986; George, 1997).

These symptoms can be classified into four clearly distinct classes:

1 Rapid death of cells of plant cells (tissues) that are invaded by the bacterial pathogen and may result in the appearance and accumulation of dark pigments such as oxidized polyphenols and melanin, often associated with chlorosis in surrounding tissues.

2 Progressive invasion of vascular elements and subsequent necrosis of adjacent tissues may result in wilting of the plant host.

3 Maceration of tissues through the dissolution of cells, the condition characterized by plant pathologists as "soft-rots".

4 Abnormal cell division and cell elongation and expansion, resulting in the appearance of tissue overgrowths such as tumours, nodules, and faciations. (Kado,1992).

1.7.2. Major Genera Comprising Phytopathogenic Bacteria

There are only five genera (or groups) of eubacteria that constitute the majority of the potent plant pathogens. These general groups (genera) include: *Agrobacterium, Coryneforms, Erwinia, Pseudomonas* and *Xanthomonas*.

1.7.2.1. Agrobacterium

The pathogenic members of the family Rhizobiaceae belongs the genus Agrobacterium. The members of this group are gram-negative, aerobic rod bacteria with simple nutritional requirements. Although, previously believed to be ubiquitous in soil, it is now clear that species of this genus are also commonly associated with plant roots (Brisbane and Rovira, 1961). They produce nitrogen-fixing nodules on the roots of legumes, therefore, these species are more appropriately considered as rhizoplane bacteria rather than general soil organisms. Some species of genus Agrobacterium produce crown gall by genetically transforming the normal plant cells into tumour cells (Bishop *et al.*, 1988). One member (***A. radiobacter***) does not cause disease in plants but is considered as "Biopesticide" (*Agrobacterium radiobacter* produces agrocin, which is a bioactive bacteriocin).

Agrobacterium tumefaciens

Agriculture has a long relationship with the bacterium *Agrobacterium tumefaciens*. Plants plagued with a pathogenic strain of this bacterium suffer a debilitating condition called crown gall disease, which is characterized by the out growth of large "gall" tumours around the crowns of the roots that cannot be cured.

Crown gall by *Agrobacterium tumefaciens*

Crown gall disease causes major agricultural losses throughout the world. The disease causes economic losses worldwide, particularly in plant nurseries, stone fruits, orchards and vineyards (Alconero, 1980; Kennedy and Alcorn, 1980). The pathogen attacks 93 plant families, the majority of which are dicotyledonous (De Cleene and De Ley, 1976). The bacterium enters plants through wounds and induces unregulated cell division leading to massive gall formation. Damage caused by galls frequently surpasses that of the initial injury (Young *et al.*, 2001). *Agrobacterium* harbouring a tumor-inducing (Ti) plasmid causes the plant disease crown gall, which is characterized by uncontrolled proliferation of plant tissues (e.g., tumours). In the course of the infection process, a region of the Ti plasmid, the T-DNA, is transferred and integrated into the plant genome (Gelvin, 1992; Zhu *et al.*, 2000). It is not surprising that some of the plant pathogenic bacteria have acquired the ability to

produce certain plant hormones e.g. indole acetic acid (IAA) is a plant growth regulator that effects cell proliferation (Alan, 2001).

As such, some 600 different plant species are susceptible to crown gall disease; the bacterium is no small an enemy to agriculture. This fact makes *Agrobacterium tumefaciens* an agricultural pathogen of the first degree that defines the very word "pathos," the Greek root that means *suffering*. Though, the foreign DNA is quite small, it will hijack the cell and take over the cell's growth, leading to unsightly tumours. Which grow large and sap the energy of the plant and as one tree suffers, so potentially do the others.

1.7.2.2. Pseudomonadaceae

Plant pathogenic members of the family Pseudomonadaceae comprise the largest number in the genera *Pseudomonas* and *Xanthomonas*. The general characteristics associated with these bacteria include: gram-negative rods with polar flagella in mono- or multitrichous arrangement, strictly aerobic and catalase-positive. The diseases caused by this group are economically important and are mostly the ones that cause necrosis of foliar parts, with certain subgroups causing macerative and vascular wilt diseases (George, 1997).

Pseudomonas

The disease causing ability of *Pseudomonas* is vast, *Pseudomonas andropogonis* causes bacterial stripe, characterized by long, narrow, red stripes, first seen on the lower leaves. The stripes range from 1 inch to more than 9 inches in length and tend to be confined between the leaf veins. Bacterial spot, caused by *Pseudomonas*

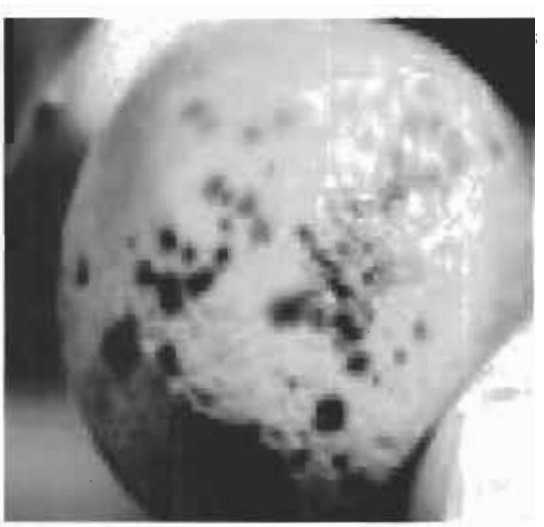
syringae pv. *syringae*, is characterized by small, circular, yellow lesions on the leaves. Old lesions have light-coloured centers surrounded by red borders. The bacteria causing these diseases are believed to survive from one season to another in both the seed and the infected plant debris. They spread from plant to plant by wind-blown, splashing rain and by insects.

Plant pathogenic members of the genus *Pseudomonas* fall into two groups: those elaborating water-soluble pigments, fluorescent to long-wave ultraviolet light, and the ones that are nonfluorescent and accumulate polyhydroxybutyric acid as reserve material. Most fluorescent *Pseudomonas* spp. have at least three ways of carrying out arginine dissimilation. However, the plant pathogenic fluorescent *Pseudomonas* do not use arginine as an anaerobic electron acceptor (via arginine deaminase or ADI pathway) and do not form cytochrome oxidase. An exception is *Pseudomonas syringae* pathovar cichorii, which possesses these properties. When a large number of bacteria are infiltrated into the interveinal panels of leaves of non-hosts, rapid necrosis is usually visible within 24 hours (George, 1997). This "hypersensitive reaction" (HR) serves as a useful additional character for this group.

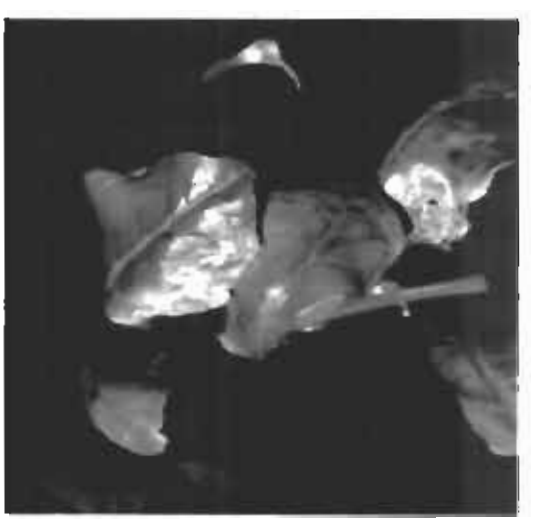
1.7.2.3. Xanthomonas

The general characteristics associated with *Pseudomonas* also apply to *Xanthomonas* spp. and pathovars. However, this group comprises bacteria with straight rods with only polar monotrichous flagellation. The *Xanthomonas* spp. also do not reduce nitrate and are usually devoid of cytochrome oxidase activity. On rich culture medium, they produce yellow-pigmented colonies with copious slime. Although,

there are six species of plant pathogenic *Xanthomonas* (the animal pathogen is known as *X. maltophilia*), the criterion for designating only six plant pathogenic species is based on a few physiological differences, many of which can be affected by a single mutational event, but by modern taxonomic analysis using RFLP (restriction fragment length polymorphisum) have revealed striking dissimilarities among *Xanthomonas compastris* pathovars (Lazo and Gabriel, 1987). The *Xanthomonas* spp. and pathovars infect a wide range of host plants, but each species or pathovar is restricted to certain hosts and therefore, is thought to be the host specific. Basically, the *Xanthomonas* comprise pathogens that cause foliar diseases. Unlike all of the other plant pathogenic bacteria, they infect through natural openings, such as hydathodes at the edges of leaves, as well as through wounds, thus gaining access to the vascular system of the plant. The basis for confinement to the vascular system is unknown, but speculations are that there may be present specific nutritional compounds and chemo-attractants that promote invasiveness (Gabriel *et al.*, 1989).



Citrus canker lesion on fruit



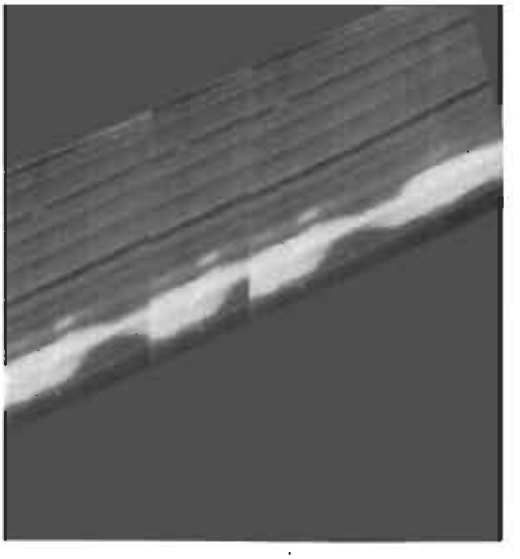
Citrus canker lesions on leaves

Citrus canker is a disease affecting citrus species that is caused by the bacterium *Xanthomonas axonopodis*. Infection causes lesions on the leaves, stems, and fruit of citrus trees, including lime, oranges, and grapefruit.

Bacterial Leaf Blight (BLB) by Xanthomonas campestris pv. oryzae

Blight refers to a specific symptom that can be expressed by plants in response to infection by a plant pathogenic organism. It is simply a rapid and complete chlorosis, browining then death of plant tissues such as leaves, branches, twigs or floral organs. Bacteril blight caused by the pathogens *X. oryzae* subsp. *oryzae*, is destructive disese of rice, which occours throughout the rice-producing areas of the world, and is the most serious disease of rice in Asia and also has been reported from Australia and Latin America, Caribbean region, North America, West Africa, Mali, Nigerian and Senegal (Anonymous, 1987).

Bacterial leaf streak



Bacterial leaf blight

The symptoms of bacterial leaf blight can be divided into leaf blight, wilting (kresek), and pale yellow leaf. Longitudinal lesions developed along leaf edges characterize the leaf blight symptoms. The lesion starts as small water-soaked streaks a few centimeters from the tip, where water pores are distributed. It rapidly enlarges in length and width, forming a yellow lesion with a wavy margin along leaf edges. The lesion turns white to grey later. Lesions may be formed either on one side or both sides of a leaf blade, or sometimes also along the midrib. In young lesions, guttation of white milky ooze is continuously observed early in the morning at the portion of leaf edges where water pores are distributed. The disease occurs on panicle, producing grey to light brown lesions on glumes and resulting in poor fertility and low quality grains (Shaw *et al.*, 1998).

1.7.2.4. Erwinia

The plant pathogenic Enterobacteriaceae are all members of the genus *Erwinia*. Members of this genus are gram-negative, straight, non sporeforming rods with peritrichous flagella. They are facultative anaerobes producing acid from sugars by fermentation. Most members of this family reduce nitrate, produce catalase, and are negative for cytochrome oxidase. All of the *Erwinia* spp are motile and most of them do not show activity for decarboxylases of basic amino acids and urease. Their cellular reserve material is glycogen and most are non fastidious for growth requirements, although growth of *Erwinia amylovora* strains is stimulated by nicotinic acid. The genus *Erwinia* is a member of the family *Enterobacteriaceae* and consists of 18 species that fall into two main groups, the necrogenic or Amylovora g

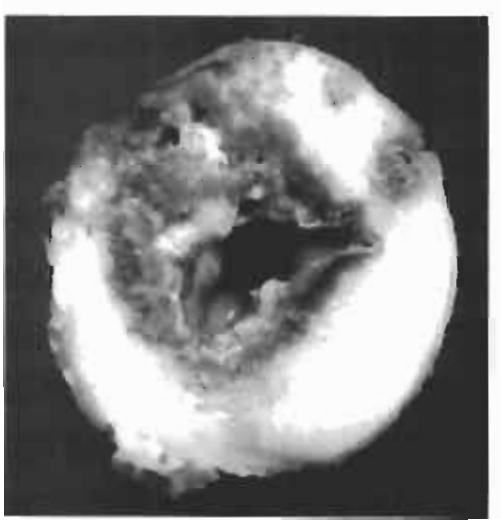
roup and the soft rot or Carotovora group (Kim, 1999; Gallios, 1992). Within the soft rot group, *Erwinia carotovora* and *Erwinia chrysanthemi* are (commercially) the most important soft rotting pathogens. Until 1981, *E. carotovora* contained only two subspecies, *Erwinia carotovora* subsp. *carotovora* (causing soft rot diseases, mainly in storage, on a wide variety of plant species, including potato) and *Erwinia carotovora* subsp. *atroseptica* [causing a vascular disease (blackleg) of potato plants and storage rot of potato tubers]. More recently, a number of new

subspecies have been included, namely, *Erwinia carotovora* subsp. *betavasculorum* that causes soft rot of sugar beet (Thomson, 1981). The *Erwinia* can be separated into three distinct groups on the basis of their pathogenic phenotype: those causing necrotic disease reactions characterized by the formation of cankers (e.g., *E. amylovora* and *E. rubrifaciens*), those causing the maceration of plant tissues (e.g., *E. carotovora* subsp.*carotovora* and *E. chrysanthemi*), and the ones that cause vascular wilts (e.g., *E. stewartii and E. tracheiphila).* Certain pigmented types (usually yellow to orange) may cause leaf galls and overgrowths or may remain epiphytic and nonpathogenic. Thus, the *Erwinia* uniquely comprise bacteria that can cause all four disease phenotypes (Kado, 1999). *Erwinia carotovora subsp.* carotovora is a phytopathogenic bacterium responsible for soft rot disease of many plant species (Kikumoto *et al.,* 1993).

Soft rot Erwinia :

Erwinia carotovora/Pectobacterium carotovorum

The soft rot *Erwinia*, *E. carotovora* spp. *carotovora (Ecc), E. carotovora* spp. *atroseptica (Eca) and E. chrysanthemi* (Ech) are major bacterial pathogens of potato and other crops world wide. They are the members of family Enterobacteriaceae and an alternative genus *Pectobacterium* was proposed (Hauben *et al.,* 1998; Toth *et al.,* 2003). Ech affects crops and other plants in tropical and subtropical regions and Ecc affects crops and plants in subtropical and temperate regions and has widest host range including potatoes. Soft rot *Erwinia* cause general tissue maceration (soft rot disease).



Soft rot/black leg by *Erwinia carotovora* subsp. *carotovora*.

Irregular brownish black soft mushy stem lesion/aerial stem rot by *Erwinia carotovora* subsp. *atroseptica*

This bacterium degrades macromolecules that compose the plant cell and the middle lamella, resulting in maceration of plant tissue. This maceration capacity is derived from the secretion of set of extracellular enzymes that include pectinases, proteases,

cellulases and beta glucosidases. These enzymes occur in multiple enzymatic forms. The bacterial synthesis of isoenzymes might ensure more efficient degradation of polysaccharides present in the plant cell wall (Chang, 2004). The main weapon in the soft rot *Erwinia* arsenal is the coordinated production of high level of multiple exoenzymes including pectinases [(pectate lyase, pectin methyl esterase and polygalacturonase) (McMillan *et al.*, 1994)], cellulases (exhibit endoglucanase activity, break down cellulose in primary and secondary cell walls of the host) and proteases, which break down plant cell walls and release nutrients for bacterial growth (Barass *et al.*, 1994; Pérombelon, 2002).

1.7.3. *Control of Phytopathogenic Bacteria by Bacteriocins*

Interest in biological control has increased recently fuelled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to the use of chemicals for disease control. Infact, all mutagens have some carcinogenic activity. Antibiotic resistant mutants of plant pathogens are undesirable enough, but agricultural use of antibiotics with medical application may result in the selection of antibiotic resistant animal or human pathogen. Examples include different broad host-range R-factors (plasmids) transferred from *E. coli* and *Shigella flexneri* to *Erwinia* species (Chatterjee and Starr, 1972). There are a number of risks in using broad-spectrum chemicals and antibiotics to control plant pathogens. A large variety of commonly used fungicides are mutagenic in both prokaryotic and eukaryotic cells (Bridges, 1975). The prospects for the biological control of bacterial plant diseases with the bacteriocins are immense (Vidavar, 1976). Bacteriocins and antimicrobial

peptides have attracted attention as potential substitutes or as additions to currently used antimicrobial compounds. They are proteinaceous compounds of bacterial origin that are lethal to bacteria other than the producing strain (Joerger, 2003).

An urgent need has been suggested for more selective, less persistent and ecologically more acceptable (desirable) agent for the control of phytopathogens than very broad-spectrum chemicals and antibiotics. Bacteriocins and antimicrobial peptides have attracted attention as potential substitutes or as additives to currently used antimicrobial compounds (Joerger, 2003). Beneficial bacteriocins do have most of the attributes considered desirable for microbial control of insects (Burges and Hussey, 1971) namely appreciable persistence, safety, aesthetic acceptability, ability to control at sub-economical concentrations, predictable control, production with ease, low cost, ease of storage and application. Some non plant origin bacterial isolates may also prove to be the ideal antagonistic bacteriocinogenic strains for the phytopathogenic bacteria (Vernam and Evans, 2000). Bacteriocins with a killing potential specificity against only one or a few strains of a species are useless for control. Usually, the bacteriocins that kill a minimum of 40% of related test strains are considered satisfactory (Vidaver, 1976). The emergence of bacteriocin-resistant mutants should not mitigate against the use of bacteriocins. Such mutants should be checked (tested) for pathogenicity and altered bacteriocin activity because resistance to bacteriocin may be linked to the loss of pathogenicity (Kerr and Htay, 1974).

Many phytopathogenic bacteria including members of the genera *Corynebacterium, Erwinia, Pseudomonas, Xanthomonas* and *Agrobacterium* produce proteinaceous bacteriocins (Heu *et al.*, 2001). These bacteriocins are highly specific; cost effective and safe for the users and the environment alike. The bacterium *Erwinia chrysanthemi* ENA49 was found to produce bacteriocin that is similar to tail fibers of bacteriophages and has activity against *Erwinia, Pseudomonas* and *Xanthomonas* strains. Bacteriocin synthesis is controlled by the determinants located at 68 min of chromosomal (genetic) map (Lysak *et al.*, 1988). A few and much less characterized bacteriocins of phytopathogenic bacteria have been known. Bacteriocins or similar substances (BLIS) have been reported for the following phytopathogens: *Agrobacterium radiobacter* (Kerr and Htay 1974); *A. tumefaciens* (Stonier, 1960; Engler *et al.*, 1975); *Erwinia carotovora, E.chrysanthenii, E. herbicola, E. salicis* (Hamon and Peron, 1961); *E. aroideae* (Kamimiya *et al.*, 1977); *Ps. aptata, Ps. lachrymans* (Hamon *et al., 1961); Ps. Syringae, Ps. glycinea and Ps. phaseolicola* (Vidaver *et al.*, 1972); *Ps. morsprunorum* and *Ps. solanacerum* (Cupples *et al.*, 1975); *Pseudomonas fluorescens* (Gurusiddaiah *et al.*, 1986) *Xanthomonas albilineus, X. beticola, X. juglandis, X. phaseoli* and *X. vesicatoria* (Hamon and Peron, 1962; Rasool, 1992).

1.7.3.1. Bacteriocins of Agrobacterium

Up to 99% decrease in disease was observed by inoculating seeds or roots with non-pathogenic bacteriocin-producing *Agrobacterium radiobacter.* This procedure did control crown gall of peaches in Australia (Kerr 1972; New and Kerr, 1972; Htay and

Kerr, 1974). Comparable results were obtained with inoculation of cherries in the northwestern USA (Moore, 1975; 1979). Bacteriocin- producing non-pathogenic *C. michiganense* was found to control tomato canker (Echandi, 1975). *A. radiobacter*, (producing a nucleotide derivative-agrocin 84) had also been extensively used in Australia and California in order to control *A. tumefaciens* which causes crown gall disease in fruit trees and other dicots (Kerr, 1980). *Agrobacterium radiobacter* strain K1026 is a microbial biopesticide that can be used to treat germinating seeds or roots and stems of certain fruits and nut trees and ornamentals to protect them from crown gall disease. *Agrobacterium radiobacter* K1026, is the result of a simple genetic alteration of a naturally occurring bacterium *Agrobacterium radiobacte* K84 (Ryder, 1994).

1.7.3.2. Bacteriocins of Pseudomonas

Among plant pathogenic Pseudomonds, about 10 species or subspecies are reported to produce bacteriocins (Lacobellis *et al.*, 1995). Syringacin 4-A and W-1 (produced by two strains of *Ps. syringae* pv. *syringae* isolated from corn and beans) are high molecular weight and heat sensitive bcteriocins (Smidt and Vidaver, 1986). Syringacin 4-A was purified and tested against phytopathogenic *Ps. phaseolicola* and *Ps. glycinea.* Purified syringacin 4-A was sprayed on the bean leaves prior to spray inoculation with *Ps. phaseolicola* strain under pressure (water soaking); as little as 3ng per leaf reduced the lesion count from 250 to zero (Vidaver, 1976). *Pseudomonas syringae* pv *ciccaronei* strain NCPPB2355 produced a bacteriocin inhibitory against strains of *Ps. syringae* subsp. *savastanoi*, the causative agent of

olive knot disease. Bacteriocins from *Pseudomonas syringae* pv. *syringae* were isolated and characterized with the aim of using them as biological control agents (Haag and Vidaver 1974; Smidt and Vidaver, 1986). Lavermicocca *et al.*, (1996) reported that *Ps. syringae* pv. *ciccaronei* (isolated from a leaf spot of the carob tree) was specifically able to inhibit the growth of several strains of *Ps. syringae* subsp. *savastanoi*, (causing olive knot disease). Applicative studies on disease control using bacteriocin-producing bacteria were also reported (Chen and Echandi, 1984).

1.7.3.3. Bacteriocins of Xanthomonas

GlycinecinA, a bacteriocin produced by *Xanthomonas axonopodis* pv. glycines 8ra, responsible for the inhibition of several *Xanthomonas* species. GlycinecinA was also produced by culturing *Escherichia coli* DH5 containing biosynthetic genes for glycinecinA, and was tested for its antagonistic effect against *X. vesicatoria* on red pepper and *X. oryzae* pv. *oryzae* on rice. Bacterial leaf spot of red pepper and bacterial leaf blight of rice were significantly reduced by the bacteriocin treatments (Fett *et al.*, 1987). Woo *et al.*, (1998) reported the antimicrobial bioactivity of glycinecinA (produced by *X. campestris* pv. *glycines* 8ra) against several phytopathogenic *Xanthomonas* species, such as *X. axonopodis*, *X. campestris* pv. *campestris* *X. campestris* pv. citri, *X. campestris* pv. *pruni*, *X. campestris* pv. *vesicotoria*., and *X. oryzae* pv. *oryzae*. The control efficacy was as high as or even higher than the chemical treatment of copper hydroxide. These results suggest that the bacteriocin (glycinecin A) is a potential control agent for bacterial plant diseases.

1.7.3.4. Bacteriocins of Erwinia

The genus *Erwinia* belongs to family Enterobacteriaceae. These plant pathogens cause necrotic disease reaction, maceration of plant tissues and vascular wilts (Kado, 1999). Bacteriocin production by the members of the genus *Erwinia* was first reported by Hamon and Peron (1961), who described proteinaceous, narrow spectrum bactericidal substance(s) from *Erwinia carotovora* ssp. *carotovora*, designated as "carotovorocin". Carotovorocin Er was phage-tail like bacteriocin from the partially purified fraction of carotovorocin Er (Kamimiya *et al.*, 1977). Erwinicin, produced by *Erwinia aroideae*, consists of rod-shaped particles (with a size of 14×160 nm). This erwinicin preparation is thermo labile, insensitive to the action of RNase, pronase, DNase and possesses antigenic properties. Molecular weight was found to be 15 megadaltons in succrose gradient (Shukin and Avdienko, 1980). Lysak *et al.* (1978) studied antibacterial activity of 272 strains of *Erwinia*. It was found that 182 (i.e. 66.7 %) of the strains were capable of producing antibacterial substances belonging to the class of 'bacteriocins'. Lysak and Fomichev (1982) reported the induction of bacteriocin synthesis in *Erwinia* cells. Infact, eight bacteriocins produced by *Erwinia* strains were investigated by Lysak (1980). The bacteriocins proved to be protein substances with a molecular weight in the range of 17,000--33,000 and showing differences in their thermal stability and sensitivity to proteolytic enzymes. *Erwinia carotovora* produces two types of bacteriocins i.e. colicin-like and macromolecular carotovorocins (MCTVs). Colicin-like bacteriocin has broad spectrum activity and it primarily lyses *Agrobacterium, Pseudomonas, Klebsiella* spp. and representatives of *E.herbicola* and *E.chrysanthemi*. Macromolecular

carotovorocin primarily lyses *E. carotovora* and *E.coli* strains (Tovkach, 1998). The antibacterial activity of the two types of bacteriocins produced by biocontrol agents (avirulent *E. carotovora* subsp. *carotovora*) may contribute to the suppression of soft-rot disease (Nakatani and Tsuyama, 1973). The bacterium *Erwinia chrysanthemi* ENA49 was found to produce bacteriocin that is similar to tail fibers of bacteriophages and showed bioactivity against *Erwinia*, *Pseudomonas* and *Xanthomonas* strains. Bacteriocin synthesis in *Erwinia* spp. is controlled by the determinants located at 68 min of chromosomal genetic map (Lysak *et al.*, 1988). Evidence has been on record regarding the effectiveness of biocontrol of the soft-rot disease of Chinese cabbage. A biocontrol agent with the trade name 'biokeeper' was developed for the control of this disease in Japan (Takahara, 1994; Kikumoto *et al.*, 1997).

1.8. Genitcs of Bacteriocin from Erwinia

Gram-negative and gram-positive bacteria commonly harbour plasmid-borne genetic determinants for bacteriocin production and of host cell bacteriocin immunity, but according to a subsequent study, these genes are located on chromosomal DNA in *E. carotovora* subsp. carotovora strains (Chuang, 1999). Plasmid genes can easily and stably be inherited without being linked to the chromosome. Plasmids genes may even be exchanged between cells of different species and genera (Hardy, 1987; Rasool *et al.*, 1993a; Peng *et al.*, 1994).

According to the study, out of fifty-two *Erwinia carotovora* strains, sixteen were found to contain extrachromosomal DNA (plasmids) from 2.5kbp to 129 kbp in size (Tovkach, 2001). Some *E. carotovora* strains harboured two to five different plasmids, ranging from 1 Kbp (enough to code for 1 to 2 genes) to over 300kbp (Saunder, 1984). Experiments showed that the cryptic plasmids of *Erwinia* are not responsible for their resistance to antibiotics and are not involved in the synthesis of macromolecular colicin-like carotovoricins (Tovkach, 2001). Curing agents produce a specific effect resulting in the loss of the plasmid from the cell without exerting a mutagenic effect (Marder and Kayser, 1997). Several agents have been reported to accelerate the loss of plasmid(s) from the microorganisms *e.g.* acridine orange, ethidium bromide or acriflavin (Hirota, 1960; Salisbury *et al.*, 1972) or SDS (Aislaibie *et al.*, 1990). Plasmid elimination is also achieved by exposing the host strains to the elevated temperature (Novick, 1990). Biagi and Azevedo (1995) reported the elimination of bacteriocin production in the phytopathogenic bacteria belonging to genera *Erwinia* and *Pseudomonas* when they were treated with ethidium bromide. However, plasmid elimination was not detected after treatment with high temperatures. The curing of bacteriocin plasmid after ethidium bromide treatment suggests the corresponding genes are located on plasmid(s). The presence of plasmid(s) responsible for bacteriocin production was evaluated by the isolation of plasmid by Kado and Leu (1981) and Hardy (1993) and their characterization by horizontal agarose gel electrophoresis (Maniatis *et al.*, 1989).

Research on bacteriocins should be broad-based as they do have most of the desirable attributes of control agents, particularly specificity of action against the target

organism. The greatest potential use of bacteriocins seems to be prophylactic treatment for seeds or tuber-borne diseases, prevention of secondary spread from infected plants, and protection of high value crops (Vidaver, 1976). However, at present, greatest interest resides with the development and application of biocontrol agents for the control of several diseases on seeds, roots, stems and leaves etc (Whipps, 2001).

Pakistan being an agro-economy based country faces challenges to minimize the collosal losses in agricultural produce by encouraging the scientists to conduct extensive and the goal oriented research and to adopt the relevant technologies (Rasool, 1992).

1.9. Bacterial Leaf Blight (BLB) in Pakistan

Rice is a major staple food for thousands of millions of people (for more than half of the world population). Although, Pakistan is not among the top rice producing or consuming countries, it is the fifth largest exporter of rice in the world. It contributed around 5% of the total national export annually and in 2002-03; the value of exported rice was more than US $ 500 million (according to an Economic Survey of Pakistan, 2003-4 by Anonymous, 2004). The yield/ unit area of rice cultivation in Pakistan is far below compared to a number of neighbouring countries. Absence of resistance/ tolerance against diseases in famous basmati varieties cultivated in Pakistan is one of the main reasons for the low yield. Bacterial Leaf Blight (causative agent: *Xanthomonas campestris* pv. *oryzae*) is widespread in Asia (CMI, 1987). BLB is the most serious disease of rice in South East Asia, particularly in Japan, Philippines,

Indonesia and India (Ou, 1985). BLB is increasing in Pakistan in recent years especially in Kaller belt that is famous for producing high quality rice (Khan *et al.*, 2000; Akhtar *et al.*, 2004). In Rice Research Institute, Kala Shah Kaku, Lahore, Pakistan, four fungicides (kasuran, kasumin, copper oxychloride and vitigran blue) and three antibiotics (oxytetracycline, streptomycin and chloramphenicol) were tested as foliar spray, for their effect on bacterial leaf blight, The highest paddy yield among the test treatments was recorded in copper oxychloride followed by vitigran blue, kasumin, streptomycin, oxytetracycline and chloramphenicol. None of the antibiotic treatments in combination with copper oxychloride performed better than the individual applications of either of the treatments (Khan *et al.*, 2005).

MATERIALS & METHODS

2. Materials and Methods

2.1. Materials

2.1.1. Collection of Samples

Different diseased fruits and vegetables were collected from local subzi mandi (Vegetable-Fruit market), local bazaars, Murad Memon Goth (Malir distt. Karachi) and Mitchlle's Farm House (Renala khurd, Punjab, Pakistan) in addition to the soil samples were taken from NIAB Faisalabad agricultural experimental fields.

2.1.2. Procurement of Cultures

Rotten fruits and vegetables and diseased plant materials (stem, leaves etc) were used for the isolation of phytopathogenic bacteria.

2.1.3. Media and Growth Conditions

Yeast peptone glucose agar (YPGA), King's agar, levans agar, nutrient agar (NA), medium 79 (yeast extract manitol agar) and MacConkey agar medium were used for the isolation and identification of phytopathogenic bacteria. All cultures were incubated at 29°C for over night to get maximum growth. Yeast peptone glucose agar and nutrient agar were used to check the bacteriocinogenic potential of the isolates. All the cultures were maintained in vials by growing them in 3mL of nutrient broth and after 24hr incubation overlaid with 0.3mL of 40% glycerol. Vials were stored at -70°C.

2.1.4. *Bacterial strains*

Following organisms were isolated from diseased plants and soils sample(s):

Xanthomonas oryzae **NA1**

Xanthomonas oryzae **NA2**

Xanthomonas citri **NA3**

Pseudomonas andropogonis **NA4**

Erwinia carotovora **NA5**

Agrobacterium radiobacter NA6

Agrobacterium radiobacter NA7

Erwinia carotovora **NA8**

****Agrobacterium tumefaciens B6*** (ATCC culture)

*Source: grape, obtained from Dr. Irfan-ul-Haq, Arid Agriculture University, Rawalpindi, Pakistan.

2.2. *Methods*

2.2.1. *Isolation of Phytopathogenic Bacteria from Diseased Plant Material*

The diseased plant material (tissues of leaves and skin of fruits) were washed with sterilized distilled water and cut into small bits with scalpel; then treated with 10% solution of hypochlorite (bleach) for 1-2 minutes to remove the contaminants, rinsed with distilled water and sections were made. The water-soaked tissue(s) at the lesion of margin was streaked and stabbed across a sterile yeast peptone glucose agar (YPGA) and nutrient agar (NA) medium and incubated at 29°C for over night. Next day isolated colonies were gram-stained and streaked on different media. Different biochemical test (for identification) were then performed.

(Agarwal *et al.*, 1989a; Scott *et al.*, 1992).

Nutrient agar

Peptone	10gm
Sodium chloride	5gm
Yeast extract	3gm
Agar	8gm
Distilled water	1L
pH 7.0	

YPGA (yeast peptone glucose agar)

Yeast	5gm
Peptone	5gm
Glucose	10gm
Agar	8gm
Distilled water	1L
pH 6.5-7.0	

2.2.2. *Isolation of Agrobacterium (for the Bacteriocin Production Potential) from Agriculture Soil Sample(s)*

The soil samples were collected from onion and pepper rhizosphere (region). Dilutions (1:10) of 1 gm of soil were made in sterile distilled water. Then 100 µL from each dilution was spread over medium 79 (yeast peptone mannitol agar) and incubated at 29°C for over night. Next day isolated colonies were gram-stained and streaked on medium 79. Different biochemical tests for identification were then performed (Gabriel *et al.*, 1989).

Yeast Extract Mannitol Agar (Medium 79)

K_2HPO_4	0.5 gm
$MgSO_4.7H_2O$	0.2 gm
NaCl	0.1 gm
$CaCO_3$	3.0 gm
Yeast extract	1.0 gm
Agar agar	15 gm
Distilled water	900 mL
Congo Red*	10.0 mL
Mannitol~	100.0 mL

(pH 6.5-7.0)
*Congo red (4%)
~ Mannitol (10%)
Added into medium after autoclaving

2.2.3. *Identification of bacterial Isolates*

Following characteristics were undertaken for the identification of the isolated organisms (as per Holt *et al.*, 1994).

1. Morpholgy.
2. Colonial characteristics on specific (selective) media
3. Pigment production on (different) media.

4. Utilization of various carbohydrates and synthesis of enzymes (i.e. oxidase and catalase).

In order to identify the isolates API 20E and API 20NE kits (Bio Merieux, France) were used to identify the isolated bacterial cultures.

2.2.4. Preliminary Screening of the Isolates for Bacteriocinogenic Potential of the Phytopathogenic Bacteria

The inhibitory activity of the bacterial isolates was determined by Cross-streak, Stab-overlay and Agar- well diffusion assays.

2.2.4.1. Cross-streak Method

Nutrient agar (NA) plates were inoculated with the producer strain as a streak across the surface of the agar plate and incubated at 29°C for 24h. Next day plates were exposed to chloroform (Merck) vapours for about 20min (to kill the producer). The sensitive/indicator bacterial cultures were then cross-streaked perpendicular to the producer strain and incubated again for overnight at 29°C. Next day plates were observed for inhibition of the growth at each side of the cross streaked producer cultures (as per Pugsley and Oudega, 1987).

2.2.4.2. Stab-overlay Method

Nutrient Agar (NA) plates were stabbed with the producer (plant) pathogens and incubated at 29°C for 24h. Next day plates were exposed to chloroform (Merck) vapours to kill the producing strains (Keeping the plates inverted and 9cm diameter piece of Whatman No.1 filter paper were introduced into the lid and impregnated with

1mL of chloroform and kept closed the lid for 15-20 min). Plates were then overlaid with 3mL nutrient soft agar containing 0.1mL of standardized inoculum (approximately $1x10^8$ CFU/mL) indicator/sensitive organism. Plates were again incubated at 29°C for overnight and observed for clear zones around the producer culture stabs. This method has advantage of testing a number of producer strains against a sensitive strain (Hardy, 1987; Hanlin *et al.*, 1993).

Standardization of Inoculum (of sensitive culture)

1.0mL of overnight indicator/sensitive culture was transferred in to a tube containing 9.0mL of sterile sodium phosphate buffer pH 7.0 to give a dilution of 1:10. A 10-fold dilution of this was made up to 10^{-8}. Absorbance of each dilution was made at 660nm (Spectrophotometer, Spectronic-21, Bausch and Lomb) against uninoculated buffer as blank and number of viable cells were determined by Miles and Misra technique with modifications. Briefly, 10µL of each dilution was spread over the surface of dry nutrient agar plates. The plates were allowed to dry and incubated at 29°C for an overnight. The average number of viable cells per mL was calculated as: number of colonies x 100 x dilution factor. A graph was plotted between absorbance and viable bacterial counts/mL. For standardization of inoculum, different volumes of overnight grown indicator cells (0.1, 0.5, 1.0 and 2.0 mL) were seeded in 3.0mL nutrient soft agar and spreaded over nutrient agar plates. Wells of 5mm diameter were bored and varying volume (20, 40, 60, 80 and 100µL) of the producer supernatant were poured into each well, the plates were incubated at 29°C for 24 hours, zones of inhibition were measured in mm diameter (Iqbal *et.al.*, 1999).

2.2.4.3. Agar- well Diffusion Assay

Nutrient agar plates were overlaid with 3mL nutrient soft agar containing 0.1mL (100µL) of the indicator/sensitive culture (values were calculated through growth curve experiment). Wells were cut into agar plates and 100µL of crude bacteriocin preparation was placed into each well. The plates were incubated and zones of inhibition were measured in mm (Muriana and klaenhammer, 1991; Schillinger *et al.*, 1991).

Preparation of Bacteriocin

Culture (used to screen for bacteriocinogenic potential) was inoculated into nutrient broth (10mL) and incubated at 29°C for over night. Next day centrifuged at 10000g for 30 min and supernatant was collected and filtered through 0.45µm pore size filters in order to eliminate any possibility of remaining cell/contamination (pH of the supernatant was also checked which was already 7.0). The cell free supernatant was referred as crude bacteriocin preparation (Muriana and klaenhammer, 1991).

2.2.5. Detection of Lytic Bacteriophages

(To rule out the lytic activity by the bacteriophages)

Reverse-side technique was applied, which prevents the contact of bacteriophages with the producer and the indicator strains. The producer culture was stabbed on nutrient agar and next day the indicator strain was poured on to the face opposite to the one where the producing strain was grown, in order to avoid the contact between the indicator and the producing strains (Iqbal *et al*, 1999). In another experiment the

agar plugs were picked up (cut) from the area of zones of inhibition and emulsified in Nutrient broth followed by agar well diffusion (for bioactivity check).

2.2.6. *Inhibitory Activity due to Hydrogen Peroxide*

To rule out the inhibitory activity by hydrogen peroxide the method of Muriana and Klaenhammer (1987) and Jimenez-Diaz (1993) was followed. For this the enzyme catalase was added to the bacteriocin preparation at a final concentration of 0.5 mg/mL and processed as per agar well diffusion method. The controls included: bacteriocin preparation (as positive control) and catalase, (as negative control).

2.2.7. *Determination of the Bacteriocin Titer*

Bacteriocin was titrated by two fold serial dilutions up to 1:64 in PBS (50mM Phosphate buffer saline) using agar well diffusion method, and was expressed as activity (arbitrary) units/mL. One arbitrary unit (AU) of bacteriocin is defined as reciprocal of the last serial dilution demonstrating inhibitory activity (Mary-Harting et al., 1972; Muriana and Klaenhammer, 1991).

Calculation

$$\text{AU/mL} = \frac{\text{Reciprocal of the highest dilution x 1000}}{\text{Volume of bacteriocin added}}$$

50mM sodium phosphate:

To obtain 100mL buffer of pH 7.0, 30mL of 0.2M sodium hydroxide (Merck) is added to 50mL of 0.2M disodium hydrogen phosphate (Merck) diluting with distilled water upto 100mL.

2.2.8. *Physical Characterization*

2.2.8.1. *Effect of pH*

a. Stability of Bacteriocin at Different pH (values)

Bacteriocin preparation was adjusted to different pH levels between 1.0 to 14 with 1 M NaOH (Merck) or 1 M HCL (Merck) (pH-meter; Metler, Germany). Samples were maintained for 1 hour at 29°C. All the samples were then re-adjusted to neutral (7.0) pH by using same acid/base and assayed for bioactivity by agar well diffusion (Todorov, *et al.,* 2003).

b. Activity of Bacteriocin at Different pH

Bacteriocin preparation was mixed with 10mM potassium phosphate buffer of different pH range (1-14). Samples were kept (maintained) for 1 hour at 29°C and assayed for bioactivity by agar well diffusion (Bhunia *et al.,* 1988).

2.2.8.2. *Effect of temperature*

The cell free supernatant and partially purified bacteriocins were kept at -20°C, 0°C, and 4°C. The activity was assyed after different time intervals for up to six months. Thermostability of bacteriocin preparation was determined by heating 2.0mL of the preparation at 60°C, 80°C and for 100°C for 30 minutes. Samples were removed, cooled and assayed for remaining (residual) activity. Thermostability was also checked at autoclaving temperature (121°C at 15 lbs/in^2) for varying time intervals (Rasool *et al.*, 1996).

2.2.9. *Biochemical Characterization*

2.2.9.1. *Biomolecular Nature of Activity*

The sensitivity of antibacterial substance to different enzymes was examined to eradicate the biomoleculr nature of its antibacterial activity (by agar well diffusion assay). Enzymes used in this study include: catalase, lipase, lysozyme (dissolved in 50mM sodium phosphate buffer pH 7.0) protease and proteinase K (dissolved in Tris HCL buffer of pH 8.0) (All from Sigma, USA). These enzymes were used at a final concentration of 1mg/mL as per Muriana and Klaenhammer, 1991). The bacteriocin preparations (treated with either of the enzymes) were placed into wells and incubated at 29°C for overnight. Next day, presence or absence of zone of inhibition against sensitive strains was observed [positive control (bacteriocin) and negative control (enzyme) were also run]. In order to stop the reaction treated all the test at 98°C for few minutes.

50mM sodium phosphate: to obtain 100mL buffer of pH 7.0, 30mL of 0.2M sodium hydroxide (Merck) is added to 50mL of 0.2M disodium hydrogen phosphate (Merck) diluting with distilled water upto 100mL

Tris-HCI: to prepare 100mL Tris-HCI buffer of pH 8.0; 0.2M Tris (Merck) was mixed with 0.2M HCI (Merck) to attain pH 8.0.

2.2.9.2. *Effect of Organic Solvents on the Bioactivity of Erwiniocin NA5*

Equal volume of Erwiniocin NA5 was mixed with different concentrations of organic solvents (butane, propane, methanol, acetone, chloroform and formalin) in 1%, 5% and 10% concentration (pre-cooled at 4°C). All the organic solvents were Sigma trade marked except chloroform (of Bio Red). Samples were stirred and incubated at

29°C for 2hours and further processed through agar well diffusion assay (Iqbal *et al.*, 1999; Ahmad *et al.*, 2004). Effect of chloroform was also observed by exposing the 24hours old (stabbed) culture for 60 minutes overlaid with appropriate sensitive cells and zone size was measured next day (Controls included the bacteriocin preparation and the solvents alone).

2.2.9.3. *Effect of Metal Ions on the Bioactivity of Erwiniocin NA5*

Equal volume of Erwiniocin NA5 was mixed with 1mM solutions of different metal ions (including $BaCl_2$, $MnCl_2$, $CdCl_2$, $MgSO_4$, $CsCl_2$, $ZnSO_4$, $FeSO_4$ and $NiSO_4$). All the metal ions were trade Sigma marked except $CdCl_2$, $ZnSO_4$ and $NiSO_4$ (of BioRed). Samples were stirred and incubated at 29°C for 2hours and further processed through agar well diffusion assay with their respective positive (bacteriocin) and negative (1mM solutions of metal ions) controls (Ahmad *et al.*, 2004).

2.2.10. *Effect of Various Media on the Production of Bacteriocins*

The antagonistic activity of the isolate (*Erwinia carotovra* NA5) was determined by stabbing in prepoured Luria agar, nutrient agar and BHI agar (Oxoid), and incubation 29°C for 24 hours. The plates were exposed to chloroform (to kill the producer cells), and 3.0 mL nutrient soft agar containing 0.1mL (of $1x10^8$ CFU/mL) of sensitive culture was poured over the plates and incubated at 29°C. After an overnight incubation, zones of inhibition around the producer colonies were measured and documented (as per Laukova, 1992).

2.2.11.*Effect of Agar Concentration on the Production of Bacreriocins*

The antagonistic activity of the isolates was determined by stabbing in pre-poured nutrient agar (NA) plates containing agar in varied concentrations (0.6, 0.8,1, 1.2, 1.4, 1.6, 1.8 and 2%) and incubating at 29°C for 24 hours (Rasool *et al.*, 1996). Further procedure was same as described in the proceeding reaction.

2.2.12. *Lacuna Counts*

The lacuna assay (Okezi; *et al.*, 1962; Hardy, 1987) was performed to determine the frequency of bacteriocin producing cells from the population of representative Bac^+ bacteria. Producer culture (*Erwinia carotovora* NA5) was grown in nutrient broth under optimum conditions. Next day (10 fold) serial dilutions were made in 1.0mL of sterile 50mM sodium phosphate buffer up to 10^{-8}. Then 100µL from each dilution was spread on to nutrient agar plates to get isolated colonies. Plates were incubated at 29°C for overnight. Next day, producer culture was killed with chloroform (vapours) exposure and 3.0mL of nutrient soft agar containing 0.1mL of sensitive culture ($1x10^8$ CFU/mL) was poured over the plates and incubated at 29°C. Thereafter, the lacunae showing (zones of inhibition) colonies were counted and average was taken. The lacuna frequency was calculated as the ratio of lacuna forming cells to the sum of lacuna forming cells and viable bacterial count.

Calculation

$$\mathbf{LF = \frac{LFC}{LFC+VBC}}$$

Where:

LF = Lacuna frequency

LFC = Lacuna forming cells

VBC = Viable bacterial cells

2.2.13. *Kinetics of Erwiniocin NA5 Production*

Synthesis of Erwiniocin NA5 was monitored during the growth cycle by growing the producer strain *Erwinia carotovora* NA5 overnight in 5mL nutrient broth. Next day OD_{530} was measured and 100µl of the culture was transferred to 350mL of fresh nutrient broth making its O.D. not more than 0.01 and followed by incubation in a shakobator (200 rpm). OD_{530} was recorded after every hour, and 1.0mL samples were centrifuged (10000g for 30 minutes) and supernatants were assayed for bacteriocin bioactivity by agar-well diffusion assay (as per Parrot *et al.*, 1989).

2.2.14. *Effect of Erwiniocin NA5 on Sensitive Cells/Mode of Action*

Stationary phase sensitive cells of *Xanthomonas oryzae* NA1 (grown overnight in nutrient broth at 29°C) were harvested by centrifugation (10000g for 10 min), and resuspended in 50mM sodium phosphate buffer (pH 7). 2mL of sensitive cells suspension was added to 18mL of Erwiniocin NA5. The control constituted 2mL culture and 18mL nutrient broth. The mixtures were incubated and the samples were drawn at 0, 0.5, 1.0, 2.0, and 4.0 hours. Absorbance of each sample was recorded at 620 nm. Same procedure was repeated using logarithemic phase sensitive cells (grown for 2-3 hours in nutrient broth at 29°C) as per Bhunia *et al.* (1991) and Biagi and Azevedo (1992).

2.2.15. *Molecular Mass Estimation (by using Dialysis Membrane)*

The diffusibility of Erwiniocin NA5 through dialyzable membrane (pore size <12kDa cut-off membrane) was checked to estimate its molecular weight. For this, 24h old stabbed culture was covered with sterile piece of dialyzable membrane and then overlaid with 3ml soft agar. After 24h incubation, results were recorded (as per Parrot *et al.*, 1989; Ahmed and Rasool, 2003).

2.2.16. *Partial Purification of ErwiniocinNA5*

For the production of Erwiniocin NA5, *Erwinia carotovora* N5 was grown in nutrient broth at 29°C for 24h. The bacterial cells were separated by centrifugation at 10000g for 45min at 4°C. The supernatant (1 Liter) was filter sterilized by passing it through 0.45µm filter (Millipore MA, USA) and concentrated to (3-5 fold) using a prechilled (at 4°C) rotary evaporator (Buchi, Germany). Ammonium sulfate (Merck, Germany) was added slowly to the concentrated supernatant with constant stirring at 4°C till the level of 80% saturation to attain the optimum precipitation of protein. The system was held overnight and the precipitate was recovered by centrifugation at 14000g for 60min at 4°C. The resulting pellet was solubilized (suspended) in 200mL of 50mM sodium phosphate buffer (pH 7.0), and designated as 'crude preparation'. The antibacterial activity of this sample was assayed using sensitive strain and antagonistic activity was determined by agar well diffusion method. Protein concentration was measured by Biurett method (Harris and Angel, 1989).

Calculation

To calculate the number of grams of ammonium sulfate (g) to be added to one liter to get the desired concentration, following equation (Harris, 1989) was used:

$$g = \frac{533\,(S_2-S_1)}{100-0.3S_2}$$

Where, S_1 = Starting concentration

S_2 = Final concentration, g = No of gram of ammonium sulfate

2.2.17. Ultra- filtration of Crude Ammonium Sulphate Precipitates

Preliminary fractionation of the crude ammonium sulphate preparation was achieved by ultra-filtration using 10kDa cut-off membrane. Both < 10 KDa fractions were concentrated to a minimal volume by rotary evaporator diluted directly in 50mM sodium phosphate buffer (pH 7.0) and designated as "Erwiniocin NA5". The antibacterial activity was further assayed using agar well diffusion assay and antimicrobial titer was determined in terms of arbitrary units/mL. The protein concentration at each step was measured by the method of Biurett (Harris and Angel, 1989), using BSA as the standard protein.

2.2.18. Size Exclusion Chromatography of Erwiniocin NA5

Partially purified bacteriocin (the active fraction of > 10 kDa after ultra-filtration) was further subjected to gel chromatography using Sephadex G-75 column (30 x1.5 cm), of Amersham-Pharmacia Biotech, USA equilibrated and eluted with 50mM sodium phosphate buffer of pH 7.0. The flow rate was maintained at 3mL/15 minutes and

elute was monitored at 280 nm. The active fractions thus obtained were collected and pooled for further bio- activity (Harris and Angel, 1989; DeCourcy, 2004).

2.2.19. *Protein Estimation of Bacteriocin by Biuret Method*

Prepared Biuret reagent by dissolving 1.5gm of copper sulfate ($CuSO_4$. $5H_2O$) and 6gm of sodium potassium tartarate in 500mL of distilled water. Added 300mL of 10% w/v sodium hydroxide and made up the volume to 1 litre with water. 0.5mL of the sample protein (bacteriocin) was added into 2.5 mL of Biuret reagent, kept it for 30 minutes and measured the absorbance at 540nm against a blank containing 0.5mL of the sample buffer plus 2.5 mL of Biuret reagent. The stock solution of 5 mg/mL (in distilled water) of BSA (Bovine serum albumin, Sigma USA) were used as standard. Optical density was read at 660nm. A standard curve of absorbance as function of initial protein concentration was plotted and was used to determine the protein concentration of the bacteriocin sample (Harris and Angel, 1989).

2.2.20. *SDS-PAGE and Assay for Antibacterial Peptide*

The tentative molecular mass and the electrophoretic characterization of the Erwiniocin NA5 active fractions from ammonium sulfate precipitation and gel filtration chromtography were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970; Perbal, 1988). A cook tail of calibration markers were used as molecular weight standard (Bio Labs. UK. Catalog No. P7708S), which include MBP-Beta galactosidase (175kDa), MBP-Paramyocin (83kDa), MBP-C13D (62kDa), Aldolase (47.5kDa), Triosphosphate isomerase (32.5kDa), Beta lactoglobulin A (25kDa), Lysozyme (16.5kDa) and A protinin (6.5kDa).

a. Sample Preparation

Protein samples and molecular weight standards were denatured and reduced completely before electrophoresis. This is usually achieved by mixing the protein solution (preferably in low ionic strength buffer such as 0.0625 M Tris-HCl, pH 6.8 i.e. a 1:7 dilution of the stacking buffer without SDS) with an equal volume of 2X sample buffer (solution7) in an Eppendorf tube, and heating this mixture for 5 minutes at 95°C-100°C. The treated sample was then chilled on ice before loading on to SDS gel.

b. Gel Concentration

1. 1.5mm thick 8% polyacrylamide gel (30.8%T:2.6%C). Acrylamide monomer solution [29.8g acrylamide and 0.8g N,N Bis-methylene-acrylamide] (Sigma)

c. Uniform Gel Preparation

Following solutions were mixed in separate filtration flasks

Solution	**Separating Gel**	**Stacking Gel**
30%Acrylamide	2.7 mL	2.7 mL
Separating gel buffer (pH 8.8)	2.5 mL	-
Stacking gel buffer (pH 6.8)	-	2.5 mL
Water	4.8 mL	4.8 mL
10% SDS	0.1mL	0.1mL
After 5 min with regular agitation, then added		
10% APS	0.05 mL	0.05 mL
99% *TEMED	10 µL	5 µL

(*TEMED: N′,N′,N′,N′.Tetramethyl Ethylenediamine 99%)

Swirled gently to mix well and transferred the separating gel solution to the gel sandwich with a plastic syringe fitted with a blunt needle. Allowed the gel to polymerize (for about 15-30 minutes) at room temperature. After polymerization, inserted a comb into the gel sandwich, and introduced the pre-prepared stacking gel solution (mixture) with a syringe and immediately inserted the comb. After polymerization of the stacking gel, removed the comb carefully from the gel sandwich and placed in electrode chamber (Bio Red USA), 20µL of treated sample and the standard protein markers were loaded in vertical slab gel (Chrambach and Jovin, 1984; Pirzada, 2005).

Reagents for SDS PAGE

1. **Acrylamide Monomer Solution (30.8%T,2.6%C)**

Acrylamide	30g
Bisacrylamide	0.8g
Water up to	100mL

   Filter through Buchner funnel with Whatman filter paper No.1. Store at 4°C in a dark bottle.

2. **Separating Gel Buffer (1.5M Tris-HCl ,pH 8.8)**

Tris	18.15g
1M HCl	~ 48mL
Water up to	100mL

   Adjust pH to 8.8.

3. **Stacking Gel Buffer (0.5 M Tris-HCl ,pH 6.8)**

Tris	6.0 g
Water	40mL

   Titrate with 1 M HCl (~48L) to pH 6.8, make up to 100 mL with water

4. **SDS Solution (10%w/v)**

SDS	10gm
Water up to	100mL

SDS	4mL of solution 4
2- mercaptoethanol	1mL
Sucrose	2g
Bromophenol blue (0.4%)	1mL
Water up to	10mL

Store this soulution frozen in 1 mL aliquots

8. **Staining solution (0.1% Coomassie Blue R-250, 41.7% methanol, 16.7% acetic acid)**

Coomassie blue R-250	1.2 g
Methanol	500mL
Glacial acetic acid	200mL
Water	500mL

Note: Dissolve the dye in methanol first and then add acid and water. It is better to use a freshly prepared solution (every time). Filter it through whatmann filter paper

9. **Destaining solution (30% methanol, 10% acetic acid)**

Methanol	300mL
Glacial acetic acid	100mL
Water up to	1L

d. Gel Overlay Assay for Antimicrobial Bioactivity Directly on SDS-PAGE

Direct detection of the antimicrobial activity by gel overlay method was performed as described by Bhunia *et al.*, (1991). Briefly, after running a 10% gradient gel electrophoresis (SDS-PAGE) the gels were removed and cut into two vertical parts. One half containing the samples and molecular weight standards was stained with Coomassie Brilliant Blue R-250. The second half of the gel was assayed for antimicrobial activity by gel overlay method. This was done by soaking the gel in 20% isopropanol and 10% acetic acid (v/v) for 2hr and then washing it in distilled water for 4hr. The gel was then placed on a nutrient agar plate and overlaid with 5mL top agar (0.6%) containing 100µL of exponential culture of the indicater strain. After overnight incubation (at 29°C), the plate-gel combination was examined for zone of inhibition.

2.2.21. *Bacteriocinogenic Potential of Miscellaneous Bacteria*

Bacteriocin producers from non plant origin were also monitored against classical phytopathogens (causing infections in rice, citrus fruits, sorghum, potato etc.). Staphylococcin Bac 188 [produced by *Staphylococcus aureus* AB188; isolated and characterized in laboratory of molecular genetics (LMG)] was checked for antimicrobial activity against phytopathogens.

2.2.22. *Location of Genetic Determinants by Curing Technique*

In order to determine the location (production) of the bacteriocin production regulation marker(s), the curing (elimination) experiments were performed using

interchelating dyes (acridine orange, ethidium bromide) and sodium dodecyl sulphate (SDS).

2.2.22.1. *Ethidium Bromide and Acridine Orange Mediated Curing*

For this purpose, the producer (culture) was grown overnight in 4mL nutrient broth. Next day, 0.2mL of culture was added in 4mL nutrient broth and incubated (at 29°C) in shakobator for 2- 4 hours. The log phase 200µL culture was then added to 2mL broth containing different concentrations (20µg/mL to 10mg/mL) of ethidium bromide/acridine orange. Positive and negative controls were also run. Positive control contained only cells (without ethidium bromide/acridine orange) while negative control contained only ethidium bromide/acridine orange. All the tubes were incubated (in dark) at 29°C for overnight. Next day, the tubes containing the lowest concentration of ethidium bromide/acridine orange (in which growth was still viable/present) were selected and a loopful was streaked on nutrient agar plate(s) and incubated. Colonies from the plates were subsequently checked for the bacteriocinogenesis by stab-overlay method (Hirota, 1960; Salisbery *et al.*, 1972).

2.2.22.2. *Curing by Sodium Dodecyl Sulphate (SDS)*

Overnight producer broth culture (20µL) was inoculated into 2 mL of broth containing 10% SDS. It was then incubated for overnight at 29°C. Dilutions were made, plated and incubated to obtain isolated colonies. The colonies were analyzed for the presence of plasmid gene mediated activity (bacteriocin production) by stab-overlay method (Hardy, 1993).

2.2.23. *Plasmid Isolation*

1. Filled a microcentrifuge tube (eppendroff) with saturated bacterial culture grown in nutrient broth. Spinned the tube in microcentrifuge for 1 minute. Discarded the supernatant and drained the tube briefly on paper towel.

2. Repeated step 1 in the same tube, filling the tube again with more bacterial culture. The purpose of this step is to increase the starting volume of cells so that more plasmid DNA (preparation) can be procured. Spinned the tube in microcentrifuge for 1 minute. Poured off the supernatant and drained the tube on paper towel.

3. Added 0.2 mL ice-cold solution 1 to cell pellet and resuspend cells as much as possible using disposable transfer pipette.

4. Added 0.4 mL of solution 2, capped tubes and inverted five times gently. Tubes were then kept at room temperature for 5 minutes.

5. Added 0.3 mL ice-cold solution 3, capped tubes and inverted five times gently. Incubated tubes on ice for 10 minutes.

6. Centrifuged tubes for 5 minutes. Transferred supernatant to fresh microcentrifuge tube using clean disposable transfer pipette. Avoided taking any white precipitate during the transfer. It is recommended (to be safe side) to leave a little supernatant behind to avoid accidentally taking the precipitate.

7. Filled remainder of the centrifuge tube with isopropanol and kept at room temperature for 2 minutes.

8. Centrifuged the tubes for 5 minutes. A milky pellet was obtained at the bottom of the tube. Decanted the supernatant without dumping out the pellet. Drained the tube on paper towel.

9. Added 1mL of ice-cooled 70% ethanol. Caped tube and mixed by inverting several times. Spinned the tubes for 1 minute. Poured off supernatant and drained the tube on paper towel.

10. Allowed the tube to dry for ~5 minutes. Added 50μL TE to the tube. Centrifuged the tube briefly to pool TE at bottom of tube.

11. RNAase can be added to TE at final concentration of 20 μg/mL. DNA is ready for use and can be stored indefinitely in the freezer. (Kado and Leu, 1981; Hardy, 1993; Zhou *et al.*, 1990).

Reagents for plasmid isolation

Solution 1 (per 500mL):	
50mM glucose	9mL of 50% glucose
25mM Tris-HCl pH 8.0	12.5mL 1 M Tris-HCl pH 8.0
10mM EDTA pH 8.0	10mL 0.5 M EDTA pH 8.0
Added H_2O to make up 500mL.	
Solution 2 (per 500mL):	
1% SDS	50mL of 10% SDS
0.2 N NaOH	100mL of 1 N NaOH
Added H_2O to make up 500mL.	
Solution 3 (per 500mL):	
3 M K^+	300mL 5 M Potassium acetate
5 M Acetate	57.5mL glacial acetic acid
Added H_2O to make up 500mL	
TE (per 100mL):	
Tris-HCl pH 8.0	1mL 1 M Tris-HCl pH 8.0
1 mM EDTA	0.5mL 0.5 M EDTA pH 8.0
Added H_2O to make up 100mL	

2.2.24. *Agarose Gel Electrophoresis*

Twenty mL of 0.6% agarose was melted in boiling water bath and 1.25µL ethidium bromide (10 mg/mL stock solution) was added when temperature dropped to 45°C. The melted agarose was poured after assembling the gel-casting tray with comb at one end and allowing to set. 1 x TAE was poured in the tank (Bio Red, USA) to sub-merge the gel. 10 µL of the sample was loaded in the well(s) with the equal volume of loading buffer and electrophoresis was carried out at 100 volts for 30-45 min. Thereafter, gel was removed and examined over UV transilluminator (UV Products, UK) for observing the plasmid DNA bands (Maniatis *et al.*, 1989; Ghazala 2000) followed by camera/photo session [Digital camera (Creative, China)].

Running buffer (TAE buffer)

this is prepared by diluting 100mL of 10x gel buffer to 1 litre distilled water and adding 50µL of stock solution of ethidium bromide (5mg/mL).

10X Tris-Acetate-EDTA buffer (pH 8.2)

Tris	48.4g
Sodium acetate	27.2g
Na_2 EDTA	3.72g
Distilled water	1000mL

Adjust the pH 8.2 with glacial acetic acid

Loading buffer

PEG6000	1g
Bromophenol blue	2mg
Distilled water	10mL

2.2.25. *In vivo Gene Transfers (direct mating/conjugation)*

Inoculated the donor and the recipient bacterial strains in 5mL nutrient broth tube separately, incubated at 37°C for 24 hours and transferred 0.2mL of each culture in 15mL of nutrient broth in 50 mL flask. Incubated at 37°C in shaking water bath for 2 hours.

Prepared the following mixture in three tubes.

	Donor Control	**Recipient Control**	**Transconjugant**
Donor	0.2 mL	-	0.2mL
Recipient	-	1.8mL	1.8mL
Plain growth	1.8mL	0.2mL	-
Total Volume	**2.0mL**	**2.0mL**	**2.0mL**

The above mixtures were shaken well; all the tubes were incubated at 37°C for 3-4 hours. 2-3 loopfull of culture from each tube were streaked on respective plates. Incubated the plates at 37°C for 24 hours and selected/searched the transconjugants for the transfer of the Bac+ marker transfer to the recipient (Ghazala, 2000).

2.2.26. *Restriction of Plasmid*

Pipetted the following into a sterile microcentrifuge tube:

a) DNA (upto 10µg)	10µL
b) 10x reaction buffer	2µL
c) Restriction enzyme (10 units)	2µL
(*Hind* III & *Eco*R1 from sigma, USA)	
d) Distilled water	6µL
Total volume	20µL

Mixed the contents by tapping the tube and by centrifugation for 30 sec, kept the tube for 1 hour at 37°C then transferred to a water bath maintain at 37°C and left for 10 minutes. Pipetted 5µL of the mixture into another eppendrof tube and add 5µL loading buffer to it (Old and Primrose, 1985). Further steps were followed as described in 2.2.24 section (agarose gel electrophorasis).

Reagent(s) for restriction

10mM Tris-HCl, pH 7.5

10mM $MgCl_2$

10mM 2-mercaptoethanol

50mM NaCl

2.2.27. Transmission Electron Microscopy (TEM) of Bacteriocin (Macromolecules)

TEM is used to elucidate the structure of the ultramicroscopic macromolecules like bacteriophages and bacteriocins. For this, applied 10µL of the sample (crude & partially purified bacteriocin preparation) on freshly carbon coated (300 mesh sized) copper grid and blotted off the excess fluid after 5 minutes. Washed the grid four times with sterile distilled water. For negative staining 1% uranyl acetate (Merck) is used. After 2-3 minutes, again blotted off the excess fluid, air dried and observed under JOEL JEM-100SX Transmission Electron Microscope (Japan) after an acceleration of 80kV. Areas of interest were imaged at 40,000-80,000 magnification. Exposed the micrographs for 1 second and developed in full strength Agfa developers for 5 minutes (Nguyen *et al.*, 1999; Jabrane *et al.*, 2002).

2.2.28. *Protein Sequence Retrieval*

Erwinia carotovora open reading frames (ORF) were retrieved from TIGR genomics server according to its genomic synteny. The obtained loci were segregated into groups which have homology to putative phage proteins. DNA and the primary protein sequences of the indicated gene loci were retrieved from the NCBI genomics server. Molecular weight and pI were deduced from expasy proteomics server. The amino acid compositions were calculated and analyzed statistically. By comparative spectral, physico-biochemical and protein homology studies tentative amino acid composition of the understudy bacteriocin(s) was estimated (Jeremy, 2001).

2.2.29. *Field Trials*

2.2.29.1. *Isolation and Purification of Xoo Before In Vivo Experiments*

The diseased plants with BLB were recognized with the appearance of yellow to white water soaked stripes at the margin of the infected leaves. They were cut into small pieces and placed on nutrient agar plates and left in incubator at 29°C overnight for the isolation of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Yellow colonies were picked and purified on fresh nutrient agar plates. Identification and characterization of the pathogen were done with the help of the Technical bulletin on seed-borne diseases and seed health testing of rice (Agarawal *et al.*, 1989a); identification was also done by using API 20NE kit (Bio Merieux, France). Confirmation of the pathogen(s) was done by performing experiments according to Koch's postulates.

2.2.29.2. *Screening of Rice Varieties/ lines in Field/in Planta Experiments*

Seven entries/varieties from Rice Research Station, Kala Shah Kaku Lahore, two from rice breeding group, NIAB (Faisalabad) and one from NARC (Islamabad) were grown in rice pathology nursery. Seeds were broadcasted on dry land (0.5ft.x 13 ft., raised beds, 0.5ft. for each entry), patted with bare hands and a thin layer of fine crushed decayed farmyard manure (FYM) was spread over them, and covered with wheat straw, then watered with a hand sprinkler thrice a day. The nursery was flooded for first time after 4th day of sprouting. Thirty-five days old nursery was transplanted with 9 inches row-to-row and plant-to-plant distance.

Inoculum of *Xanthomonas oryzae* pv. *oryzae* was made in nutrient broth by keeping at 29°C for 48hrs in shaker incubator and suspended in distilled water to get 10^8 cells/mL. Plants were inoculated at pre panicle stage. Scissors dipped in the inoculum were used to cut one-fourth part of 10- 12 leaves per plant.

A simple test to differentiate leaf blight consists of immersing the cut end of the basal part of an infected leaf in a dilute solution of basic fuchsin for 1-2 days (Goto, 1965). The area of latent bacterial infection beyond the visible lesion remains unstained and appears as green spots with undulate margins clearly separated from the stained healthy part of the leaf. This reaction only occurs with *X. oryzae* pv. *oryzae* and not with other blight organisms. (Limitation of this technique is that young flag leaves and short old leaves do not stain well).

http://www.eppo.org/QUARANTINE/bacteria/Xanthomonas_oryzae/XANTOR_ds.pdf.

After 24 hours of incubation half of the concentration of Amonium sulphate precipitated) bacteriocin was sprayed on *Xoo* inoculated plants in field, while 1/6 dilution were used in control condition. The Copper oxycholride in 0.3% (concentration i.e. 0.3gm/L) was applied as standard. Data were collected after three weeks of inoculation using the following scale for BLB (Anonymous 1996).

2.2.30. Statistics

Data were analyzed by one way analysis of variance followed by Annova multiple-comparisons test. Where <0.05 was accepted as indicating statistical significance.

2.2.31. Scale for BLB (for field test, lesion area)

Infection %	**Score**	**Host Behaviour**
0-3%	1	Highly Resistant
4-6%	2	Resistant
7-12%	3	Resistant
13-25%	4	Moderately Resistant
26-50%	5	Moderately Resistant
51-75%	6	Susceptible
76-87%	7	Susceptible
88-94%	8	Highly Susceptible
95-100%	9	Highly Susceptible

****Field trials/experiments were conducted in collaboration with/at NIAB (Nuclear institute of Agriculture and Biology) Faislabad.**

RESULTS

3. Results

The present research findings pertain to the isolation of bacterial phytopathogens from different diseased fruits, vegetable and soil (**Figure 1,2,3**). The isolated bacteria were identified on the bases of morphological, cultural and biochemical considerations (**Table 1, 2 and 3**). API 20 E and API 20 NE kits were used for the final identification of the bacterial isolates (**Figure 4a & 4b**). Three methods (stab-overlay, agar-well diffusion and cross-streak) were used to check the bacteriocinogenic potential of the isolates (**Figure 5, 6 & 7**). The isolated phytopathogenic bacterial strains were screened for cross bacteriocinogenic potential (bioactivity). Out of eight isolates only three were found bacteriocin producers i.e. *Erwinia carotovora* NA5, *Agrobacterium radiobacter* NA6 and *Agrobacterium radiobacter* NA7. Among these, *Agrobacterium radiobacter* NA6 (its bacteriocin is designated as Agrocin NA6) and *Agrobacterium radiobacter* NA7 (producing Agrocin NA7) showed sensitivity against each other and *Agrobacterium tumefaciens* B6 (**Figure 5**). However, *Erwinia carotovora* NA5 (producing Erwiniocin NA5) showed activity against *Xanthomonas oryzae* NA1, *Xanthomonas oryzae* NA2, *Agrobacterium radiobacter* NA6, *Agrobacterium radiobacter* NA7 *and Erwinia carotovora* NA8 (and also against miscellaneous clinical isolates showing that Erwiniocin NA5 carries broad based bioactivity (**Table 17**). Results of cross streak method (**Figure 5**) are depicted in **Table 4** which are different from the results obtained by stab-overlay (**Figure 6**) and agar-well diffusion methods (**Figure 7; Table 5**). These shifting results may be due to the difference in the rate of diffusion of

bacteriocin in agar medium in the three methods. Hence, Erwiniocin NA5 showed broad-based bioactivity in cross streak method. The Activity units (AU/mL) of Erwiniocin NA5 were found to be 160 AU/mL (**Figure 8; Table6**); it retained full bioactivity at wide pH range of 2-14, (**Figure 9 and 10; Table 7 and 8**). The heat sensitivity threshold shows that Erwiniocin NA5 is heat stable as bioactivity remained intact after heating at different temperatures and even after autoclave i.e. 121°C at 15 lbs/in^2 for 15min (**Figure 11; Table 9**). Lacuna percentage in *Erwinia carotovora* NA5 against *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1 was 87% and 79% respectively. It was found resistant to lipase, lysozyme and catalase (for excluding the possibility of H_2O_2 action and the presence of any active lipid moiety in Erwiniocin NA5) but complete loss of activity was observed after protease and proteinase K treatment (**Figure 12; Table 10**). Erwiniocin NA5 was also found resistant to different organic solvents (at 1% v/v) including chloroform, butanol, propanol, methanol, ethanol, acetone and to different metal ions (at 1mM conc.) including BaCl2, MnCl2, CdCl2, MgSO4, CsCl2, ZnSO4, FeSO4 and NiSO4 (**Figure 13;Table 11 and 12**). The inhibition zones of Erwiniocin NA5 were still present even after seeding indicator culture in opposite direction, indicating that lytic bacteriophages were not responsible for the bioactivity. The best activity (measured as zone size in mm) was observed at 1.4% of agar when effect of various concentrations of agar on bacteriocin production (**Table 13**) and diffusion were checked. It was also observed that Erwiniocin NA5 was best produced on Nutrient agar compared to YPGA and LB agar (**Table 14**). Production of Erwiniocin NA5 started during early logarithmic phase and continued till late stationary phase (**Graph**

1). However, the maximum production of Erwiniocin NA5 was observed after eight hours of incubation. The mode of action (bacteriostatic, bactericidal or bacteriolytic) of Erwiniocin NA5 was also investigated against *Xanthomonas oryzae* NA1 and *Erwinia carotovora* NA8 (on the growing and the stationary phase cells). Erwiniocin NA5 possesses bacteriocidal mode of action (**Graph 2a and 2b**). Erwiniocin NA5 was dialyzable through dialysis membrane cut-off point <12kDa. Hence molecular mass of Erwiniocin NA5 was roughly estimated to be less than 12 kDa (**Figure 14**). Bacteriocin was precipitated (up to 80% saturation) by ammonium sulphate (**Figure 15**). The crude preparation (cell free supernatant) of Erwiniocin NA5 contained 4800mg total protein, precipitate was found to contain 290 mg, while pooled active elute (fraction) of Erwiniocin NA5 (Sephadex G75 column purified fraction) contained 11mg of total protein (**Table 15**). Partially purified bacteriocins were subjected to gel filteration using sephadex G75 column, giving a major active peak of protein (**Figure 16; Graph 3**) and SDS PAGE analysis followed by commassie blue staining showed a major band of 11 kDa carrying bioactivity [(measured as zone of inhibition against the sensitive strain) (**Figure 17**)]. Transmission electron microscopy of crude and partially purified Erwiniocin NA5 showed spherical particles which may be presumed membrane vesicle or phage related/defective phage particle (**Figure 18**). Statistically analyzed (by bioinformatics) amino acid composition (from NCBI genomics server) retrieved 12.44% acidic and 13.57% basic with 49.84% polar and 50.16% non polar amino acid components (**Table 16**). The bioactivity of Erwiniocin NA5 showed broad range bioactivity against various Gram - and Gram + clinical isolates (**Table 17**).

It was noted that Erwiniocin marker(s) was cured after ethidium bromide treatment suggesting (**Figure 19; table 18**) the possibility of the presence of Bac marker on the extra chromosomal genetic element i.e. possibly on plasmid, which seems to be non conjugative (**Table 20**). Plasmid isolation from uncured and cured *Erwinia carotovora* NA5 strains revealed that the uncured strain retained the plasmid in contrast to the cured strain (**Figure 20**). The molecular weight of the plasmid was determined by horizontal agarose gel electrophoresis with marker (known) DNA i.e. λ (Lambda) DNA *Hind* III digest. Accordingly, the molecular weight (in terms of Kbp) of Bac plasmid was found to be about 23 kb (**Figure 21**).

Effect of Erwiniocin NA5 in control **(Table 21a & 21b; Figure 22, 23)** and field conditions (**Table 22a & 22b; Figure 24, 25, 26**) on different varities of rice (including IR-6, Bas-370, Sup-Bas, IRBB-62, Bas-385, Bas-2000, PK-369943, KSK-203, 202 & 203, EF-1-20-6-10, EF-1-30-4-1) effected with *Xanthomonas oryzae oryzae,* the causative agent of BLB of rice. Significant differences/ reduction in lesion size were found in disease score among different varieties after Erwiniocin NA5 application compared to the control, in field as well as in control conditions.

Bacteriocin producers from non-plant origin were also monitored against phytopathogens. Accordingly Staphylococcin Bac188 showed antimicrobial activity against *Erwinia* NA5, *Xanthomonas citri* NA3, *Agrobacterium sp.* NA7 *Pseudomonas andropogonis* NA4 and *Xanthomonas oryzae* NA2. Hence, Staphylococcin Bac188 could be a choice against the phytopathogenic bacteria (**Table 23**).

TABLES

4. Tables

Table 4.1: Morphological and Cultural Characteristics of the Isolated Strains/Bacteria

Source	Gram reaction	Cultural characteristics	Isolated organisms
Oryza sativa (Rice) from Faislabad Agriculture soil	Gram negative rods	Cream to yellow coloured, round and smooth colonies on nutrient agar.	*Xanthomonas oryzae* NA 1
Oryza sativa (Rice) from Shaikhupora Agriculture soil	Gram negative rods	Yellow coloured, round, smooth and pinpointed colonies on nutrient agar.	*Xanthomonas oryzae* NA2
Citrus ourantium (Orange)	Gram negative rods	White colored round and smooth colonies on nutrient agar.	*Xanthomonas citri* NA3
Sorghum vulgare (Sorghum)	Gram negative rods	White colored round, smooth and pinpointed colonies on nutrient agar.	*Pseudomonas andropogonis* NA4
Solanum tuberosum (Potato)	Gram negative rods	Pink to orange pigmented, round, smooth and pin pointed colonies on nutrient agar.	*Erwinia carotovora* NA5
Soil sample (From pepper rhizosphare)	Gram negative rods	White colored large, round, smooth and dry colonies. Red pigmented colonies on Medium 79.	*Agrobacterium radiobacter* NA6
Soil sample (from onion rhizosphare)	Gram negative rods	White coloured large, round, smooth and dry colonies. Red pigmented colonies on Medium 79.	*Agrobacterium radiobacter* NA7
Solanum tuberosum (Potato)	Gram negative rods	Orange pigmented, round, smooth and pin pointed colonies on nutrient agar	*Erwinia caortovora* NA8

Table 4.2: Biochemical Characteristics of the Isolates

	Catalase	Oxidase	Gelatin liquifaction	Nitrate reduction	Esculin hydrolysis	Urea hydrolysis	Growth at 37°C	Levan production
Xanthomonas oryzae NA1	+	-	+	-	+	-	-	-
Xanthomonas oryzae NA2	+	-	+	-	+	-	-	-
Xanthoomonas citri :NA3	+	+	-	+	+	+	-	-
Pseudomonas andropogonis NA4	-	+	-	+	+	-	-	+
Erwinia carotovora :NA5	+	-	+	+	+	+	-	+
Agrobacterium radiobacter NA6	+	+	+	+	+	-	-	-
Agrobacterium radiobacter NA7	+	+	+	+	+	-	-	-
Erwinia carotovora NA8	+	+	-	+	+	-	-	+

Key: + = Test is positive
- =Test is negative

Table 4.3: Plants, Diseases and the Isolates

Source	Disease caused	Isolated organism
Oryza sativa (Rice)	Bacterial leaf blight (BLB)	*Xanthomonas oryzae* NA1
Oryza sativa (Rice)	Bacterial leaf blight (BLB)	*Xanthomonas oryzae* NA2
Citrus ourantium (Orange)	Citrus canker	*Xanthomonas citri* NA3
Sorghum vulgare (Sorghum)	Bacterial stripe	*Pseudomonas andropogonis* NA4
Solanum tuberosum (Potato)	Soft rot	*Erwinia carotovora* NA5
Soil sample (from pepper agricultural field)	Soil borne	*Agrobacterium radiobacter* NA6
Soil sample (from onion agricultural field)	Soil borne	*Agrobacterium radiobacter* NA7
Solanum tuberosum (Potato)	Soft rot	*Erwinia carotovora* NA8

API 20E and API 20NE kits were used for final identification of the bacterial isolates.

Table 4.4: Cross Bacteriocinogenic Activity of Isolated Phytopathogenic Bacteria (by Cross- streak Method)

Producer strains	Sensitive strains							
	Xanthomonas oryzae NA1	*Xanthomonas oryzae* NA2	*Xanthomonas citri* NA3	*Pseudomonas andropogonis* NA4	*Erwinia carotovor* NA5	*Agrobacterium radiobacter.* NA6	*Agrobacterium radiobacter.* NA7	*Erwinia carotovora* NA8
Xanthomonas oryzae NA1	-	-	-	-	-	-	-	-
Xanthomonas oryzae NA2	-	-	-	-	-	-	-	-
Xanthomonas citri NA3	-	-	-	-	-	-	-	-
Pseudomonas andropogonis NA4	-	-	-	-	-	-	-	-
Erwinia carotovora. NA5	+	+	+	+	-	+	+	+
Agrobacterium radiobacter NA6	-	-	-	-		-	+	-
Agrobacterium radiobacter NA7	-	-	-	-	-	+		-
Erwinia carotovora. NA8	-	-	-	-	-	-	-	-

Key: + = zone of inhibition 20-40 mm
- = no zone of inhibition

Table 4.5: Cross Bacteriocinogenic Activity of Isolated Phytopathogenic Bacteria (by Stab-overlay and Agar-well Diffusion Method)

Producer strains	Sensitive strain								
	Xanthomonas oryzae NA1	*Xanthomonas oryzae* NA2	*Xanthomonas citri* NA3	*Pseudomonas andropogonis* NA4	*Erwinia carotovor* NA5	*Agrobacterium radiobacter* NA6	*Agrobacterium radiobacter* NA7	*Erwinia carotovora* NA8	*Agrobacterium tumefaciens* B6 *(ATCC)*
Xanthomonas oryzae NA1	-	-	-	-	-	-	-	-	-
Xanthomonas oryzae NA2	-	-	-	-	-	-	-	-	-
Xanthomonas citri NA3	-	-	-	-	-	-	-	-	-
Pseudomonas andropogonis NA4	-	-	-	-	-	-	-	-	-
Erwinia carotovora. NA5	+	+	-	-	-	+	+	+	-
Agrobacterium radiobacter NA6	-	-	-	-		-	+	-	+
Agrobacterium radiobacter NA7	-	-	-	-	-	+		-	+
Erwinia carotovora. NA8	-	-	-	-	-	-	-	-	-

Key: + = zone of inhibition 15-30 mm
- = no zone of inhibition
* = was a gift from Dr.Irfan-ul-Haq (Arid Agriculture University, Rawalpindi)

Table 4.6: Titration of Erwiniocin NA5 Against *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1

Sensitive culture	Arbitrary units(AU/mL)
Erwinia carotovora NA8	160
Xanthomonas oryzae NA1	160
Agrobacterium radiobacter NA6	160

Arbitrary units (AU/mL) constitute the highest dilution of the bacteriocin which gives definite zone of inhibition, calculated as

$$\text{AU/mL} = \frac{\text{Reciprocal of the highest dilution} \times 1000}{\text{Volume of bacteriocin added}}$$

Table 4.7: Activity of ErwiniocinNA5 at Extended pH Range

pH	Activity units (AU/mL)
2	160
3	160
4	160
5	160
6	160
7	160
8	160
9	160
10	160
11	160
12	160
13	160
14	160

Note. pH was adjusted by using range of sodium phosphate buffers

Table 4.8: Stability of Erwiniocin NA5 at Extended pH Range

pH	Activity units (AU/mL)
2	80
3	160
4	160
5	160
6	160
7	160
8	160
9	160
10	80
11	80
12	80
13	80
14	40

Note: pH was adjusted by using 1M NaOH & 1M HCl, after 2hr of incubation Erwiniocin NA5 was neutralized by using the same reagents.

Table 4.9: Effect of Temperature Range on the Bioactivity of Erwiniocin NA5

Temperature	Activity units (AU/mL)
Low*	
-20°C	160
0°C	160
+4°C	160
High	
60°C for 30 min	160
80°C for 30 min	160
100°C for 15min	160
121°C for 15min at 15lbs/inch^2	80

* The bacteriocin preparations could be stored at -20°C, 0°C and 4°C without loss of activity for prolonged time

Table 4.10: Effect of Different Enzymes on the Bioactivity of Erwiniocin NA5

Treatment	Activity units (AU/mL)
Control	160
Protease	160
Proteinase K	160
Lipase	0
Catalase	0
Lysozyme	0

Table 4.11: Effect of Organic Solvents on the Bioactivity of Erwiniocin NA5

Solvents	Activity units AU/mL of Erwiniocin in the Presence of Different Concentrations of Organic Solvents		
	1%	5%	10%
Acetone	40	0	0
Butanol	160	160	160
Chloroform	160	160	160
Ethanol	40	0	0
Methanol	40	0	0
Propanol	160	160	160

Table 4.12: Effect of Metal Ions on the Bioactivity of Erwiniocin NA5 (in terms of activity units)

Metal ions (1mM)	Activity units (AU/mL)
$BaCl_2$	160
$MnCl_2$	160
$CdCl_2$	160
$MgSO_4$	160
$CsCl_2$	160
$ZnSO_4$	160
$FeSO_4$	160
$NiSO_4$	160

Table 4.13: Effect of Agar Concentration on the Production of Erwiniocin NA5 against *Xanthomonas oryzae* NA1 and *Erwinia carotovora* NA8

Agar %	Zone of inhibition (in mm)	
	Xanthomonas oryzae NA1	*Erwinia carotovora* NA8
1.0	30	35
1.2	30	35
1.4	30	35
1.6	26	27
1.8	24	27
2.0	20	21
2.2	14	14

Table 4.14: Effect of Different Media on the Production of Erwiniocin NA5

Media	Post production activity units (zone of inhibition in mm)
Nutrient agar	30
Luria basal agar (LB)	23
Yeast peptone mannitol agar (YPGA)	25

Table 4.15. Differential Purification Profile of Erwiniocin NA5

Sample/Step	Volume (mL)	[1]Total activity (AU)	[2]Total protein (mg)	[3]Specific activity (AU/mg)	[4]Recovery (%)	[5]Purification (fold)
Cell free supernatant/ Crude extract	1000	160000	4800	33.3	100	1
Precipitates (after NH_4SO_4 precipitation)	50	16000	290	55.17	10	1.6
Sephadex G75 column. Purified fraction (active elution fraction 9,10,11)	10	800	11	61.5	0.5	2.18

Key:

[1]Activity unit (AU/mL) = Reciprocal of the highest dilution x 1000/volume of bacteriocins added.

[2]Total protein (mg)= (protein estimated by Biuret method)

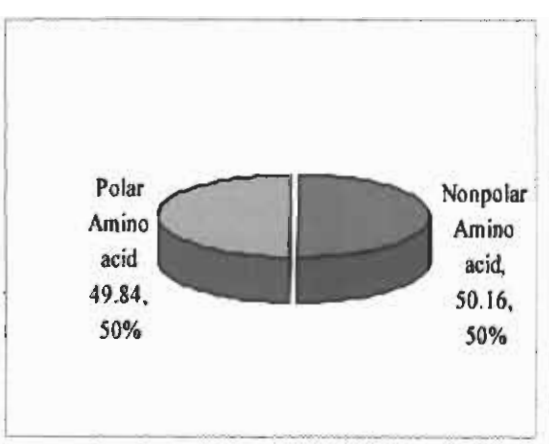
[3]Specific activity (AU/mg)= Total activity of the subsequent purification step/Total protein of the same step

[4]Recovery (%)= Total activity of subsequent step x 100/Total activity of the crude preparation

[5]Fold purified= Specific activity of subsequent step/Specific activity of the crude preparation

Table 14.16. Amino Acid Composition of Erwiniocin NA5 by Computational Analysis

Amino Acid	3 Letter (abbreviation)	1 Letter (abbreviation)	Polarity	Acidity or Basicity	% of Amino Acid in Erwiniocin NA5
alanine	ala	A	nonpolar	neutral	7.9
arginine	arg	R	polar	strongly basic	5.7
asparagine	asn	N	polar	neutral	4.28
aspartic acid	asp	D	polar	acidic	5.28
cysteine	cys	C	nonpolar	neutral	1.37
glutamic acid	glu	E	polar	acidic	7.16
glutamine	gln	Q	polar	neutral	4.27
glycine	gly	G	nonpolar	neutral	6.75
histidine	his	H	polar	weakly basic	2.17
isoleucine	ile	I	nonpolar	neutral	9.7
leucine	leu	L	nonpolar	neutral	7.09
lysine	lys	K	polar	basic	5.7
methionine	met	M	nonpolar	neutral	3.3
phenylalanine	phe	F	nonpolar	neutral	3.2
proline	pro	P	nonpolar	neutral	2.9
serine	ser	S	polar	neutral	7.46
threonine	thr	T	polar	neutral	4.42
tryptophan	trp	W	nonpolar	neutral	1.45
tyrosine	tyr	Y	polar	neutral	3.4
valine	val	V	nonpolar	neutral	6.5



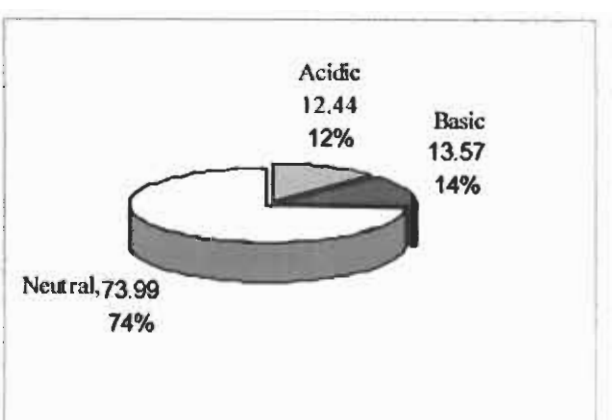


Table 4.17. Bacteriocinogenic Potential of Erwiniocin NA5 on Diverse (Miscellaneous) Bacteria

Sensitive/indicator strains	Erwiniocin NA5	
	A	B
Gram-negative bacteria		
Escherichia coli BU40	1/1	26
Escherichia coli 5014	1/1	21
Escherichia coli WT	3/5	20
Klebsiella pneumoniae	2/2	23
Pseudomonas aeruginosa	0/2	nil
Salmonella typhi	0/2	nil
Salmonella typhi paraA	2/2	15
Salmonella typhi paraB	0/2	nil
Shigella dysenteriae	0/2	nil
Shigella sonnei	0/1	nil
Shigella flexnari	2/2	19
Gram-positive bacteria		
Bacillus subtilus	7/10	20
Bacillus pumilus	1/1	41
Corynebacterium diphtheriae	0/2	nil
Corynebacterium hoffmanii	0/2	nil
Micrococcus luteus	2/2	21
MRSA	2/2	24
Staphylococcus aureus	0/10	nil
Staphylococcus epidermidis	3/3	43
Staphylococcus saprophyticus	0/2	nil
Streptococcus fecalis	0/2	nil
Streptococcus pyogenes	0/2	nil

Key:
*inhibitory activity was determined by stab-overlay method.
A= number of sensitive strains/number of strains tested; **B**=Average zone of inhibition (mm).
Nil = No zone of inhibition.
Note: all strains were obtained from Dept. of Microbiology University of Karachi

Table 4.18: Location of Genetic Determinants by Curing Technique

Curing agents	No.of colonies screened	No. of colonies cured	No. of colonies Uncured	Percentage of curing
Ethidium bromide	300	231	69	77
Acridine orange	300	0	300	0
SDS	300	0	300	0

Table 4.19: Counter Selective Antibiotic Markers for Bacterial Conjugation

Bacterial strains	Ampicillin (µL)			Erythromycin (µL)			Streptomycin (µL)			Tetracycline (µL)			Gentamycin (µL)		
	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100
Erwinia carotovora NA5	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
Xanthomonas oryzae NA1	S	S	S	S	S	S	R	R	R	S	S	S	S	S	S
Pseudomonas andropogonis NA4	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
Klebsiella pneumoniae	R	R	R	S	S	S	R	R	R	S	S	S	R	R	R
Escherichia coli	S	S	S	R	R	R	R	R	R	S	S	S	S	S	S
Pseudomonas aeruginosa	R	R	R	S	S	S	R	R	R	R	R	R	S	S	S

Key: R = Resistant
S = Sensitive

Table 4.20: Recombination via Conjugation (for *in vivo* Bac$^+$ marker transfer)

Donor (Bac$^+$)	Recipient (Bac$^-$)	Transconjugants
Erwinia carotovora NA5	*Xanthomonas oryzae* NA1	-
	Pseudomonas andropogonis NA4	-
	Klebsiella pneumoniae	-
	Escherichia coli	-
	Pseudomonas aeruginosa	-

Note: *K. pneumoniae, E. coli* and *Ps. aeruginosa* were obtained from Dept. of Microbiology University of Karachi

Table 4.21a: Efficacy of Erwiniocin NA5 to Control BLB Disease of Rice in Controlled Conditions (Average disease scores of three replicates)

Entries/ varieties of rice (*Oryza sativa*)	Control (plant infected with *Xoo*)	Test (plant infected with *Xoo* and treated with Erwiniocin NA5)
IR-6	8.5	8.5
Bas-370	8.5	5.5
Sup-Bas	8.5	6
IRBB-62	5	2.5
Bas-385	9	5.5
Bas-2000	9	6.5

Rice varities from Faislabad (NIAB)

Table 4.21b: Efficacy of Erwiniocin NA5 to Control BLB Disease of Rice in Controlled Conditions (Average disease scores of three replicates)

Entries/ varieties of rice (*Oryza sativa*)	Control (plant infected with *Xoo*)	Test (plant infected with *Xoo* and treated with Erwiniocin NA5)
IR-6	9	8.9
Bas-370	9	7.5
Sup-Bas	9	7.5
IRBB-62	6	4.5
Bas-385	9	5.5
Bas-2000	9	7

Rice varieties from Sheikhupura

Table 4.22a: Efficacy of Erwiniocin NA5 to Control BLB Disease of Rice (in field conditions)

Entries/ varieties of rice (*Oryza sativa*)	Control (plant infected with *Xoo*)				Test (plant infected with *Xoo* and treated with Erwiniocin NA5			
	R-1	R-2	R-3	Avg.	R-1	R-2	R-3	Avg.
PK-369943	5.4	6.1	7.0	6.16	6.6	7.6	6.8	7.00
KSK-203	5.0	7.6	5.6	6.08	7.4	6.6	5.5	6.50
KSK-202	7.4	7.0	6.3	6.93	7.4	6.4	6.4	6.73
KSK-201	8.0	6.6	6.2	6.93	8.0	5.9	7.2	7.03
IR-6	6.3	6.6	7.0	6.63	7.8	6.5	4.4	6.23
Bas-370	6.3	8.0	5.0	6.43	6.9	6.9	4.1	5.97
EF-1-20-6-10	4.3	3.9	2.7	3.63	3.4	4.2	2.8	3.47
EF-1-30-4-1	5.9	5.5	6.8	6.07	3.3	4.4	7.2	4.97
Sup-Bas	7.0	6.5	6.9	6.80	7.1	4.5	5.5	5.70
Bas-2000	7.7	7.7	5.3	6.90	6.8	7.1	6.1	6.67

Xoo= *Xanthomonas oryzae* subsp. *oryzae*

Table 4.22b: Efficacy of Erwiniocin NA5 to Control BLB Disease of Rice in Field Conditions (Average disease scores of three replicates)

Entries/ varieties of rice (*Oryza sativa*)	Control (plant infected with *Xoo*)	Test (plant infected with *Xoo* and treated with Erwiniocin NA5)
Pk-369943	6.16 abcd	7.00 ab
KSK-203	6.08 abcd	6.50 abcd
KSK-202	6.93 abcd	6.73 abcd
KSK-201	6.93 abc	7.03 ab
IR-6	6.63 abcd	6.23 abcd
Bas-370	6.43 abcd	5.97 abcd
EF-1-20-6-10	3.63 ef	3.47 f
EF-1-30-4-1	6.07 abcd	4.97 bcdef
Sup-Bas	6.80 abcd	5.70 abc
Bas-2000	6.90 abcd	6.67 abcd

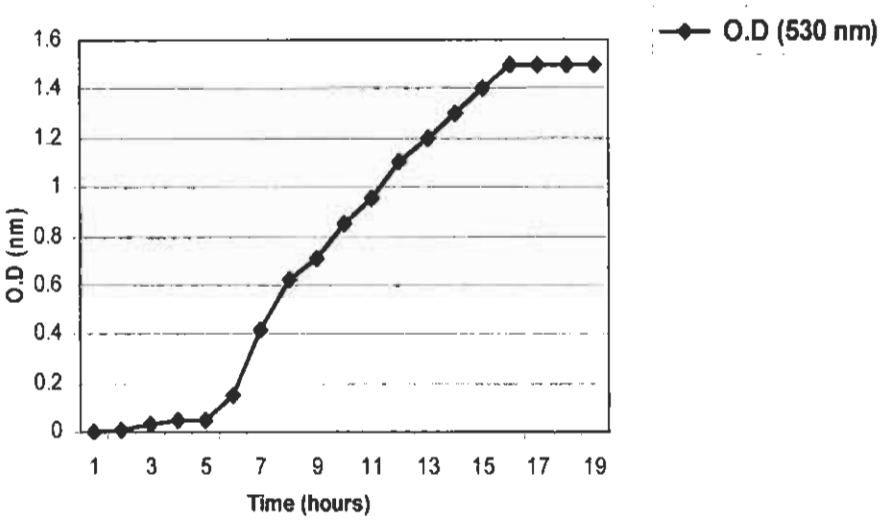
Table 4.23: Bacteriocinogenic Potential of Staphylococcin Bac 188 Against Isolated Phytopathogenic Bacteria by Agar-well Diffusion Method

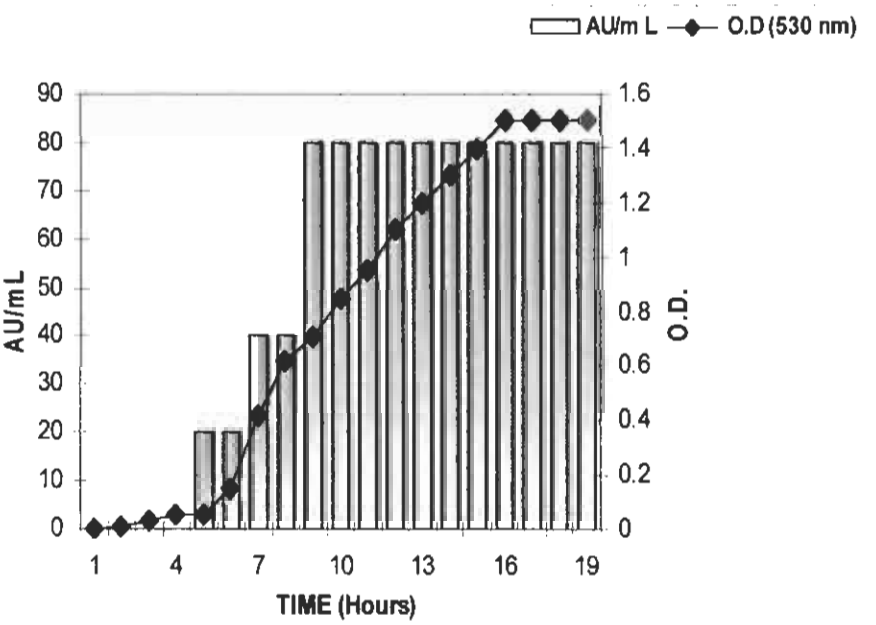
Sensitive phytopathogenic strains	Staphylococcin Bac 188 (zone of inhibition in mm)
Xanthomonas oryzae NA2	+
Xanthoomonas citri NA3	+
Pseudomonas andropogonis NA4	+
Erwinia carotovora NA5	+
Agrobacterium sp NA7	+/-
Erwinia carotovora NA8	+

Key: + = zone of inhibition 15-30 mm
- = no zone of inhibition
+/- = variable

GRAPHS

5. *Graphs*

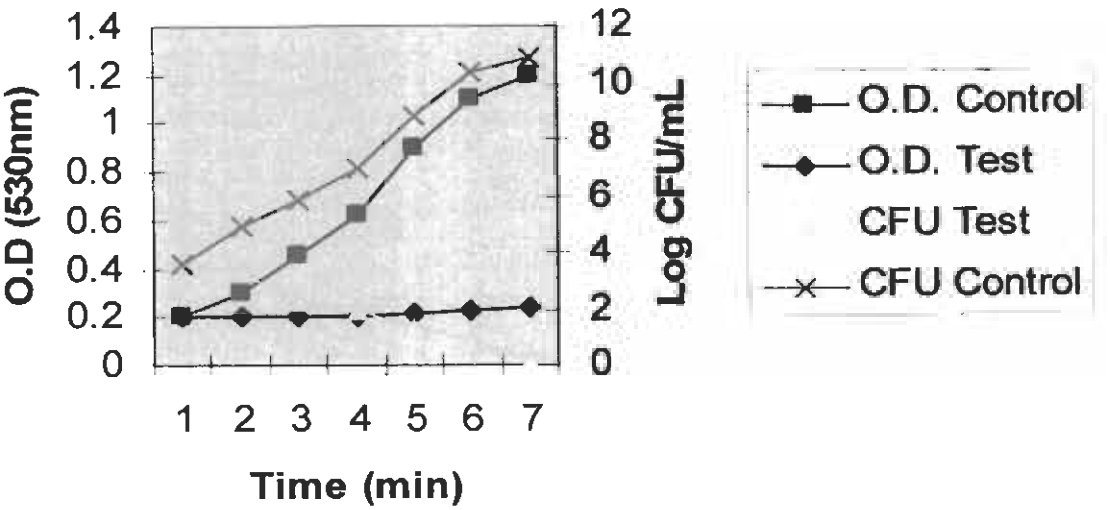




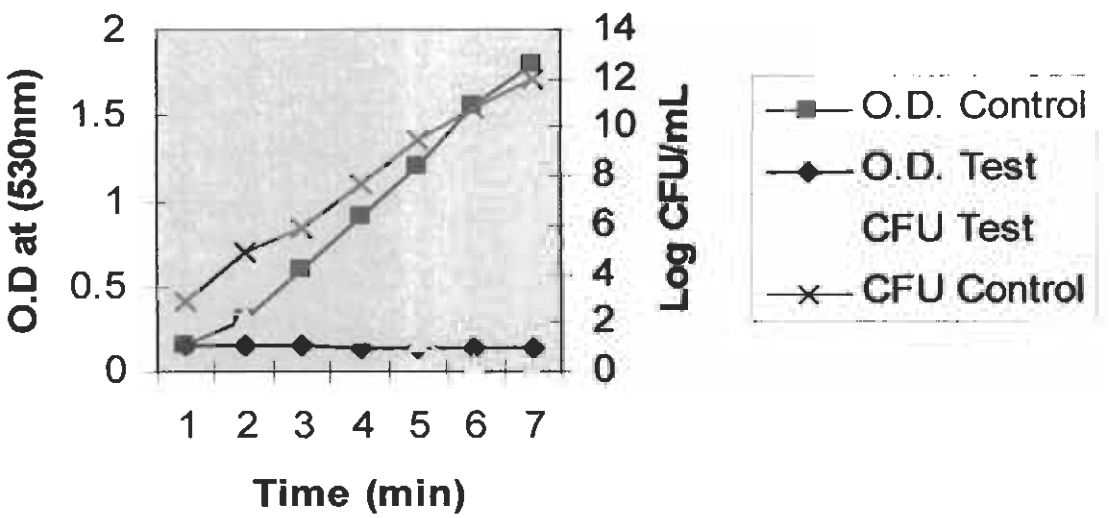
Graph 5.1:

Production of Erwiniocin NA5 during growth cycle of *Erwinia carotovora NA5*. Samples were taken at different time intervals and the absorbance was measured at O.D. 530nm (shown by line) and Erwiniocin NA5 production was determined in terms of AU/mL by agar- well diffusion assay (shown by bars)

2a.

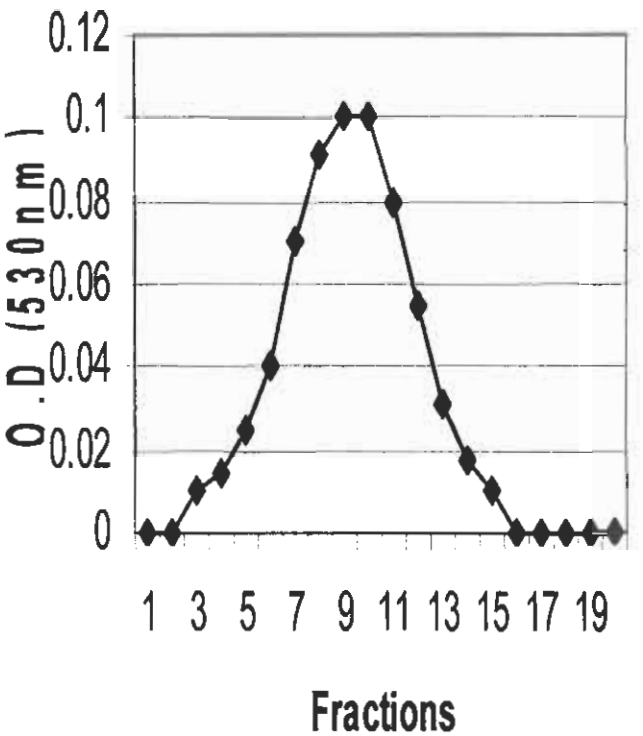


2b.



Graph 5.2:

Bactericidal effect of Erwinioicin NA5 on stationary phase cells (2a) and growing phase cells (2b) of *Xanthomonas oryzae* NA1. Samples were obtained at different time intervals and O.D. 530nm was measured



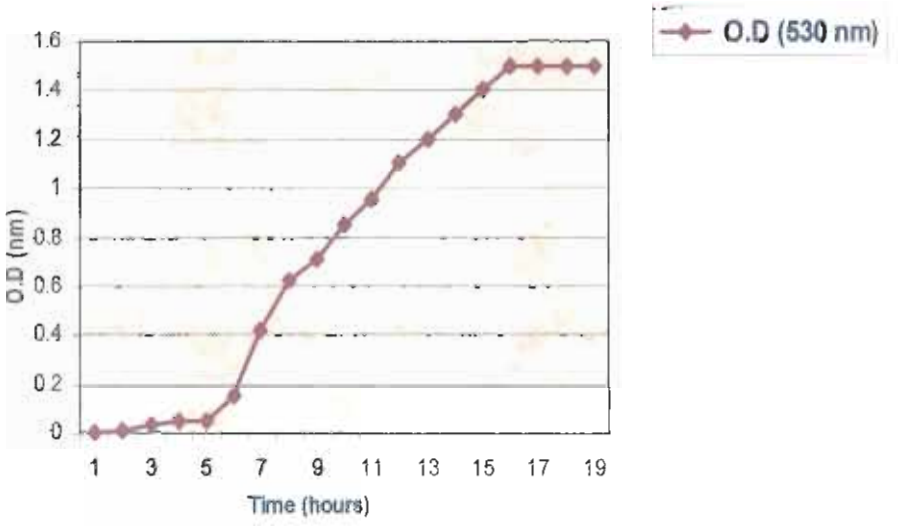
Graph 5.3:

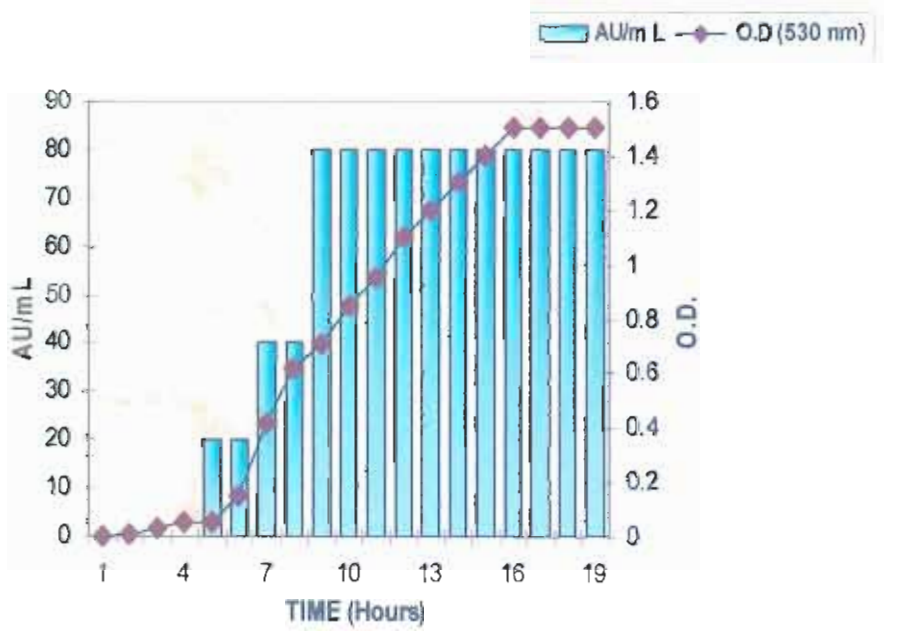
Column chromatography showing single active peak of Erwiniocin NA5

(The value constitute the average of three readings)

GRAPHS

5. Graphs

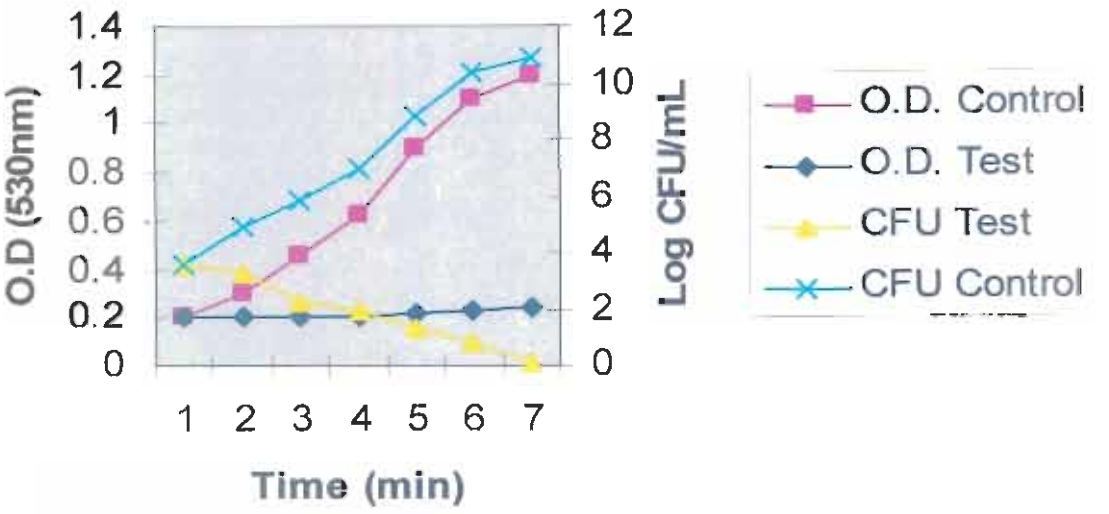




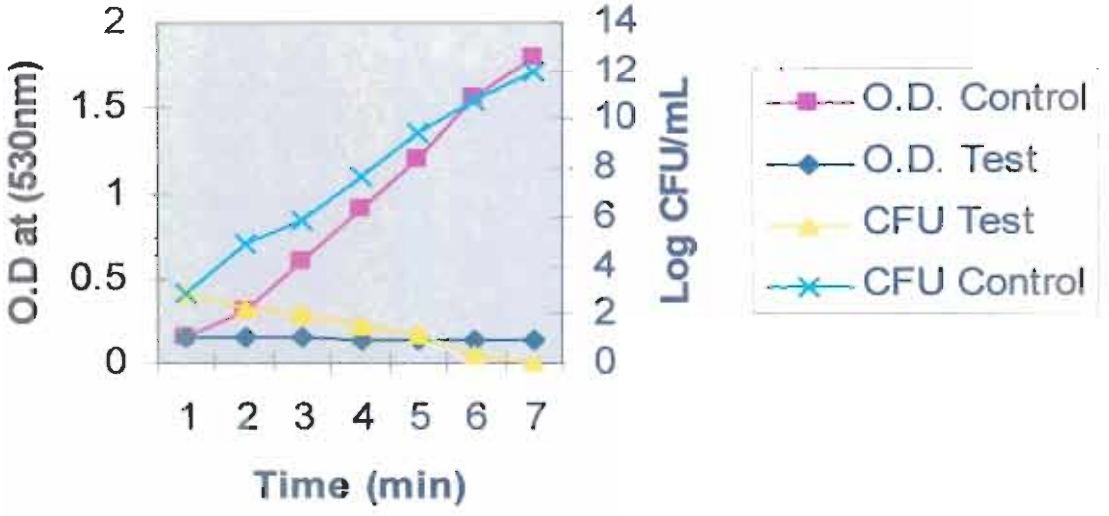
Graph 5.1:

Production of Erwiniocin NA5 during growth cycle of *Erwinia carotovora NA5*. Samples were taken at different time intervals and the absorbance was measured at O.D. 530nm (shown by line) and Erwiniocin NA5 production was determined in terms of AU/mL by agar- well diffusion assay (shown by bars)

2a.

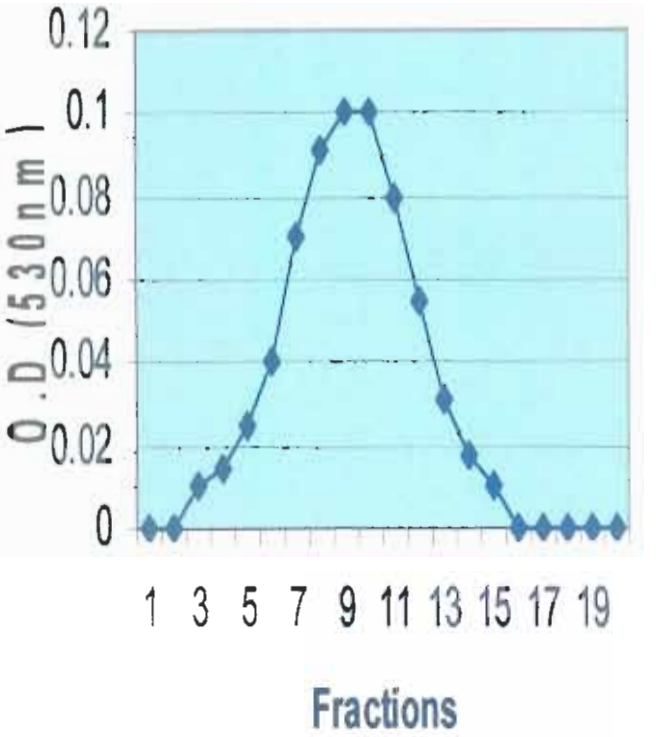


2b.



Graph 5.2:

Bactericidal effect of Erwinioicin NA5 on stationary phase cells (2a) and growing phase cells (2b) of *Xanthomonas oryzae* NA1. Samples were obtained at different time intervals and O.D. 530nm was measured



Graph 5.3:
Column chromatography showing single active peak of Erwiniocin NA5
(The value constitute the average of three readings)

FIGURES

6. Figures

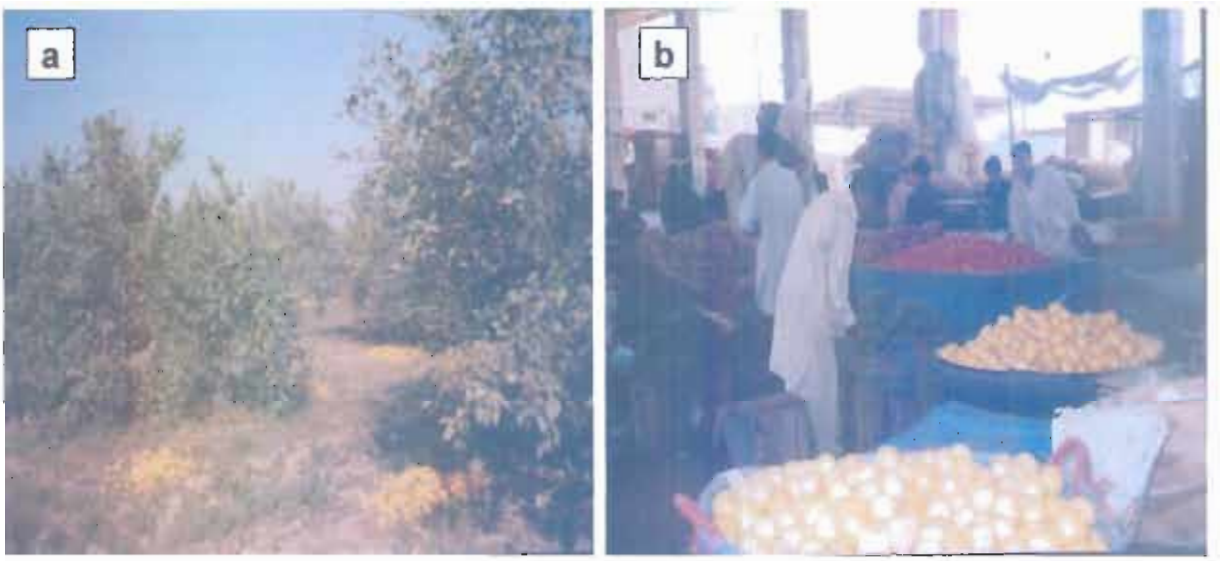


Figure 6.1. Collection Sites

a. Mitchels farm-house, Renala Khurd (Punjab)
b. Vegetable and fruit market, karachi

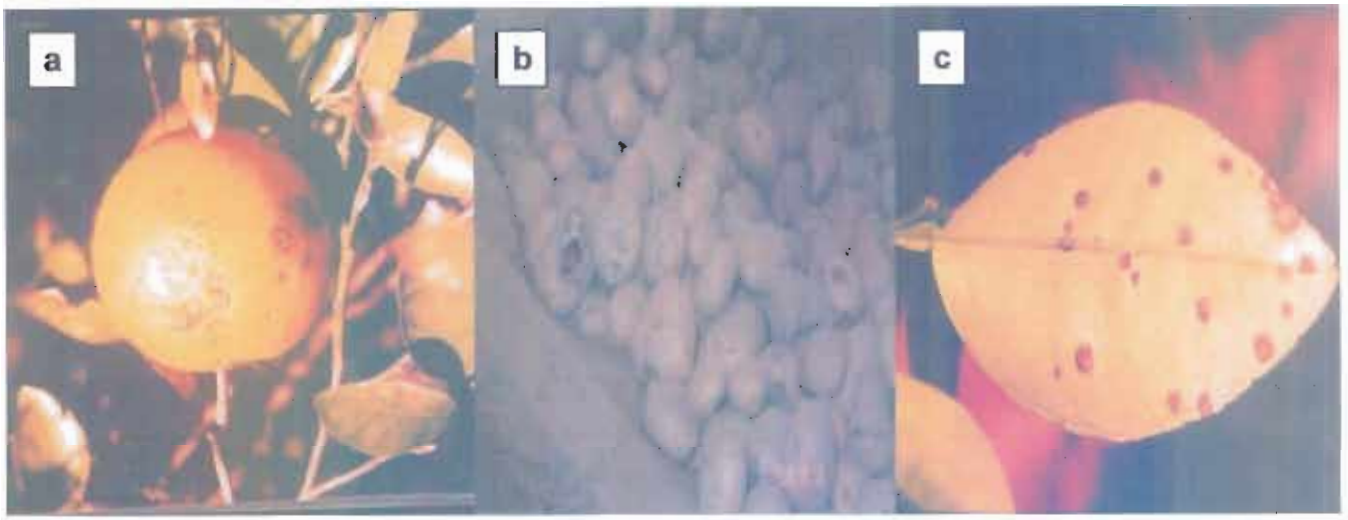


Figure 6.2: Infected Fruits, Leaves and Vegetable

a. Infected oranges (source for the isolation of *Xanthomonas citri* NA2)
b. Infected potatoes (source for the isolation of *Erwinia carotovora* NA5)
c. Infected leaf (source for the isolation of *Pseudomonas andropogonis* NA4).

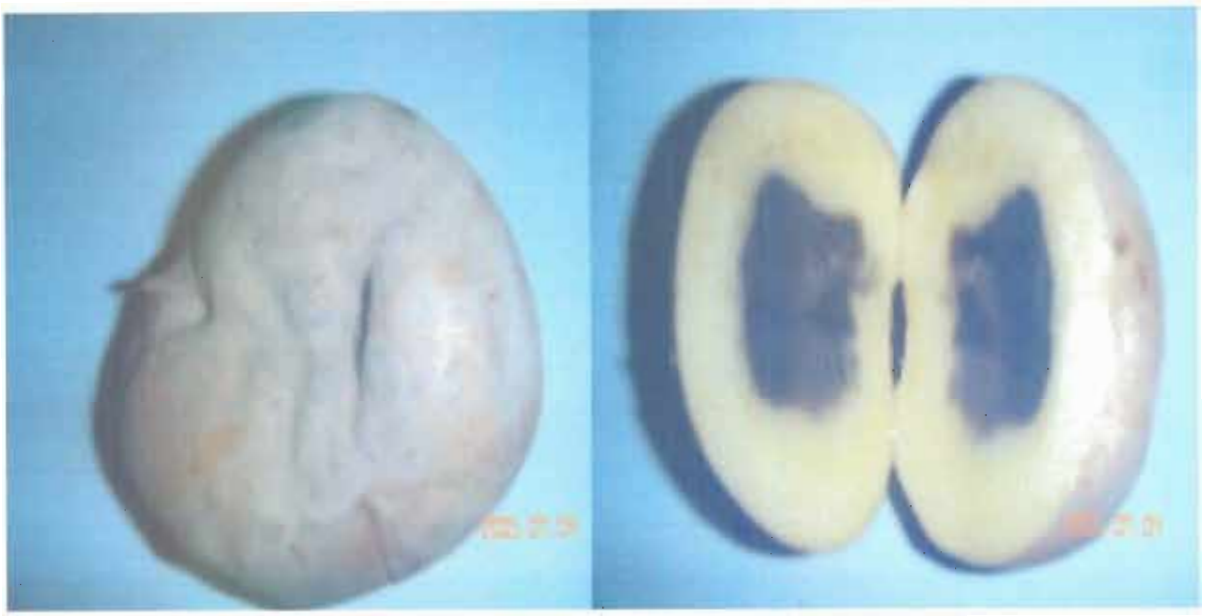


Figure 6.3. Soft Rot of Potato

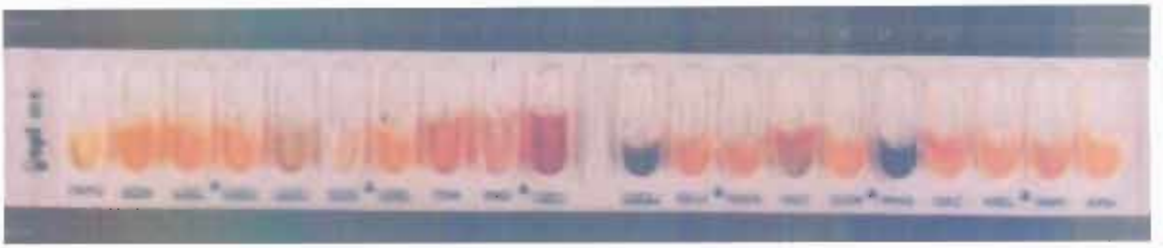


Figure 6.4a. API 20 E Strip Showing the Biochemical Reactions (for *Erwinia carotovora* NA5)

Figure 6.4b. API 20 NE Strip Showing the Biochemical Reactions (for *Agrobacterium radiobacter* NA6)

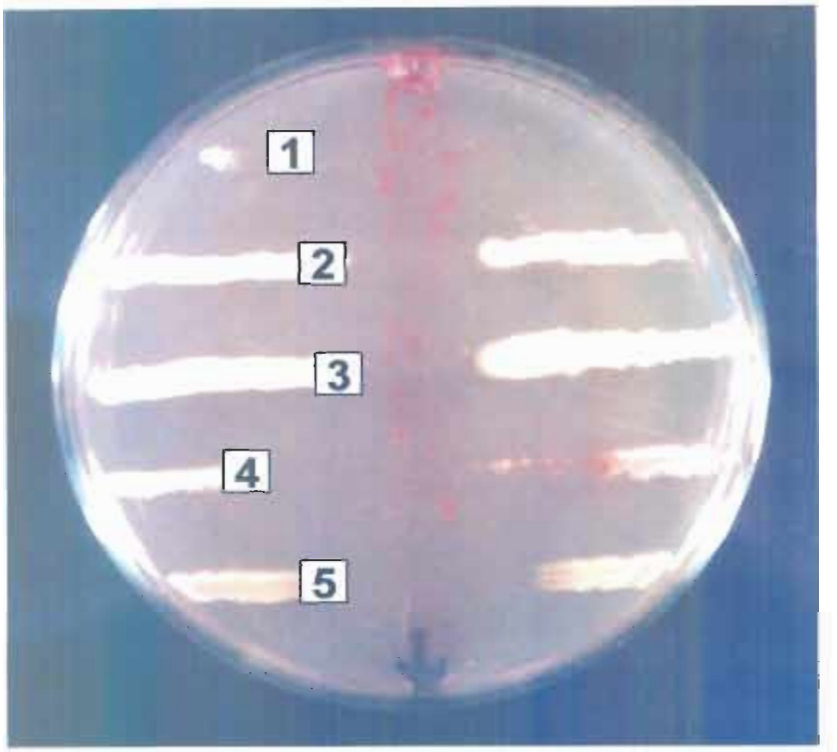


Figure 6.5. Cross-streak Method, Demonstrating Bacteriocinogenesis by *Erwinia carotovora* NA5 against Different Indicator Strains

1) *Xanthomonas oryzae* NA1
2) *Agrobacterium radiobacter* NA6
3) *Pseudomonas andropogonis* NA4
4) *Erwinia carotovora* NA8
5) *Xanthomonas citri* NA3

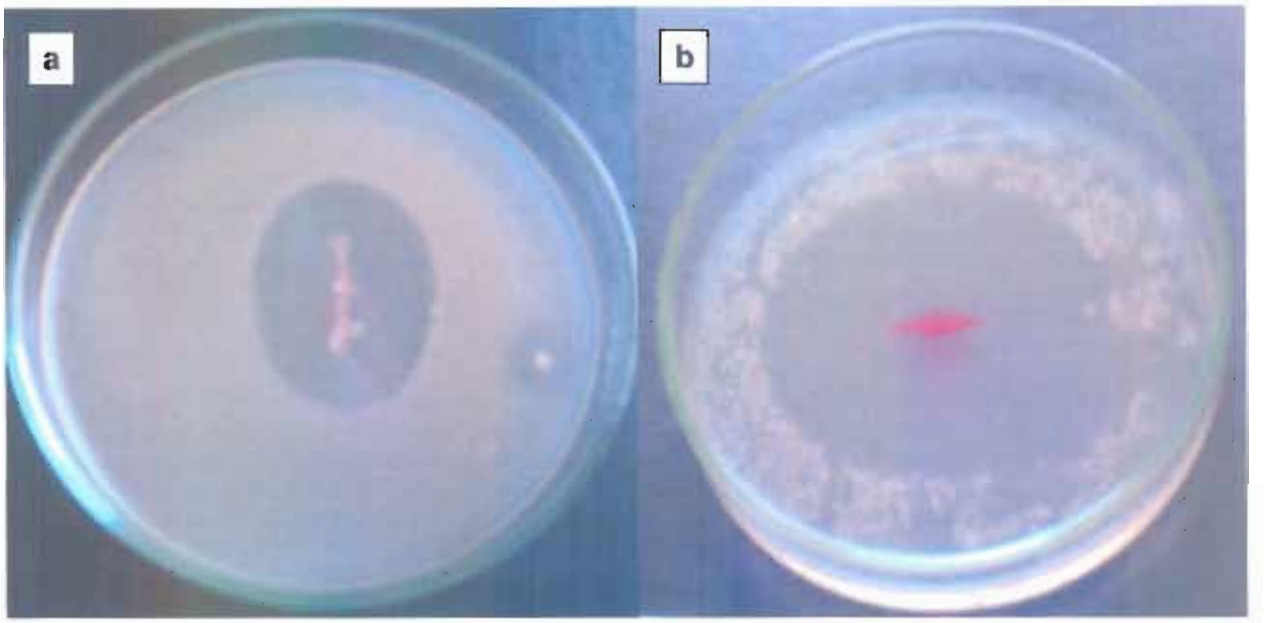


Figure 6.6. Stab-overlay Approach Producer *Erwinia carotovora* NA5 against

a. *Xanthomonas oryzae* NA1
b. *Agrobacterium radiobacter* NA6

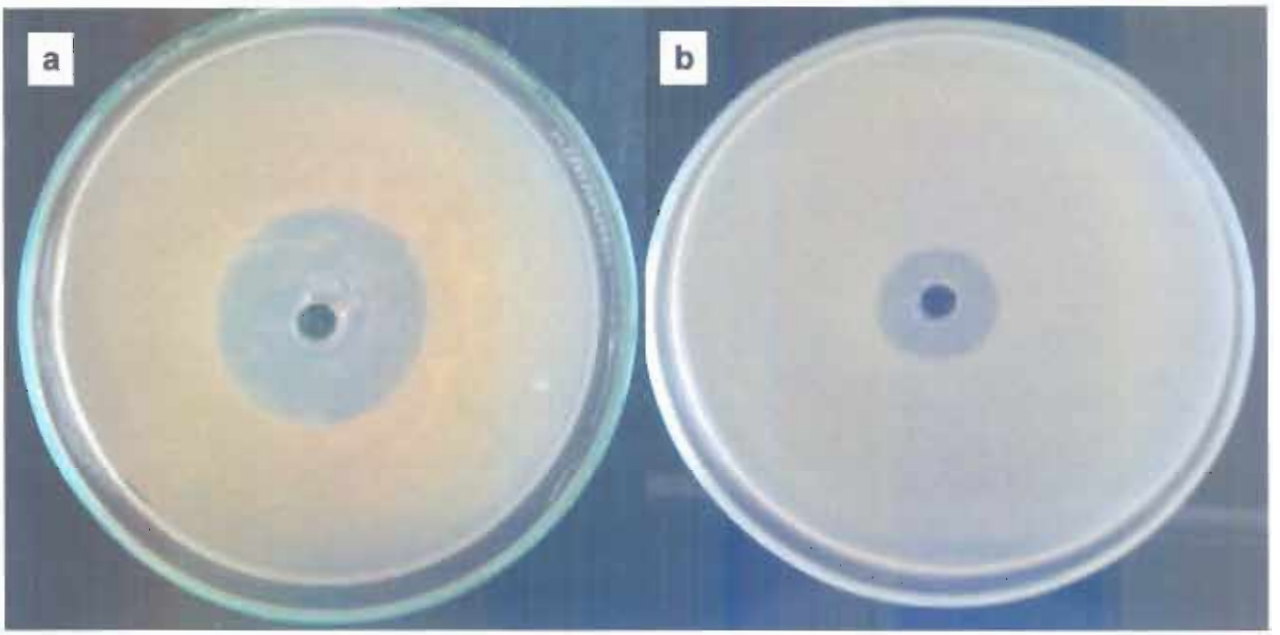


Figure 6.7. Agar-well Diffusion Method

a. Erwiniocin NA5 against *Erwinia carotovora* NA8
b. Agrocin NA6 against *Agrobacterium tumifaciens* B6

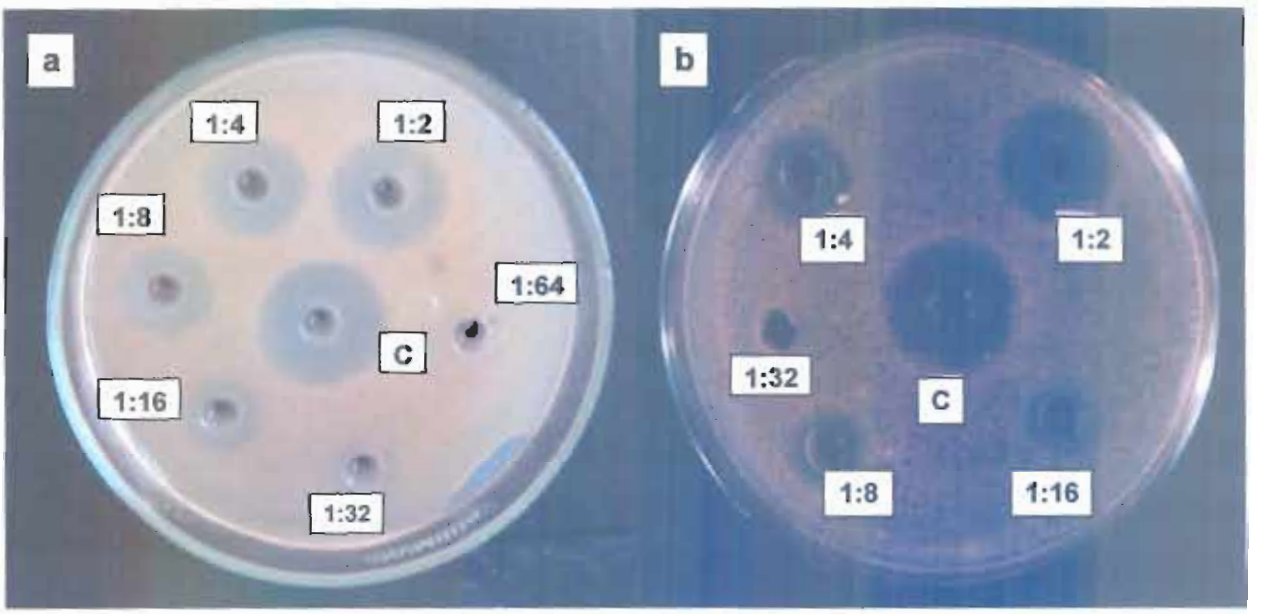


Figure 6.8. Titration of Erwiniocin NA5

a. ***Erwinia carotovora*** **NA8(as indicator strain)**
b. ***Agrobacterium radiobacter*** **NA6 (as indicator strain)**

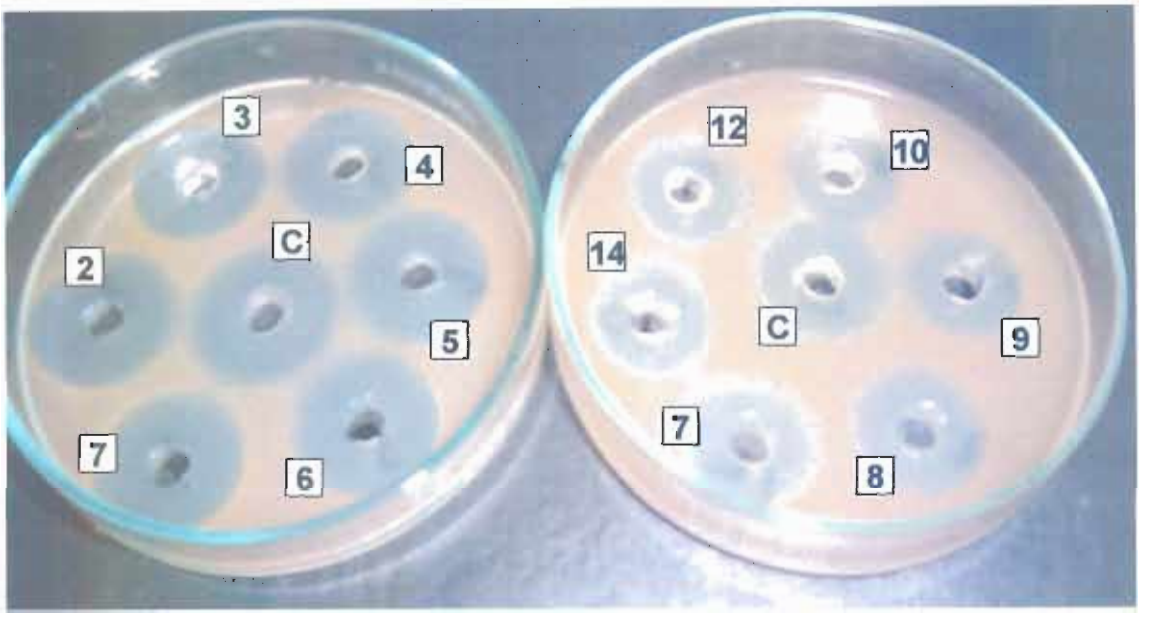


Figure 6.9. Residual Activity of Erwiniocin NA5 at Different pH (without neutralizing after 2-14 pH range treatment)

Note. pH was adjusted by using range of potassium phosphate buffer

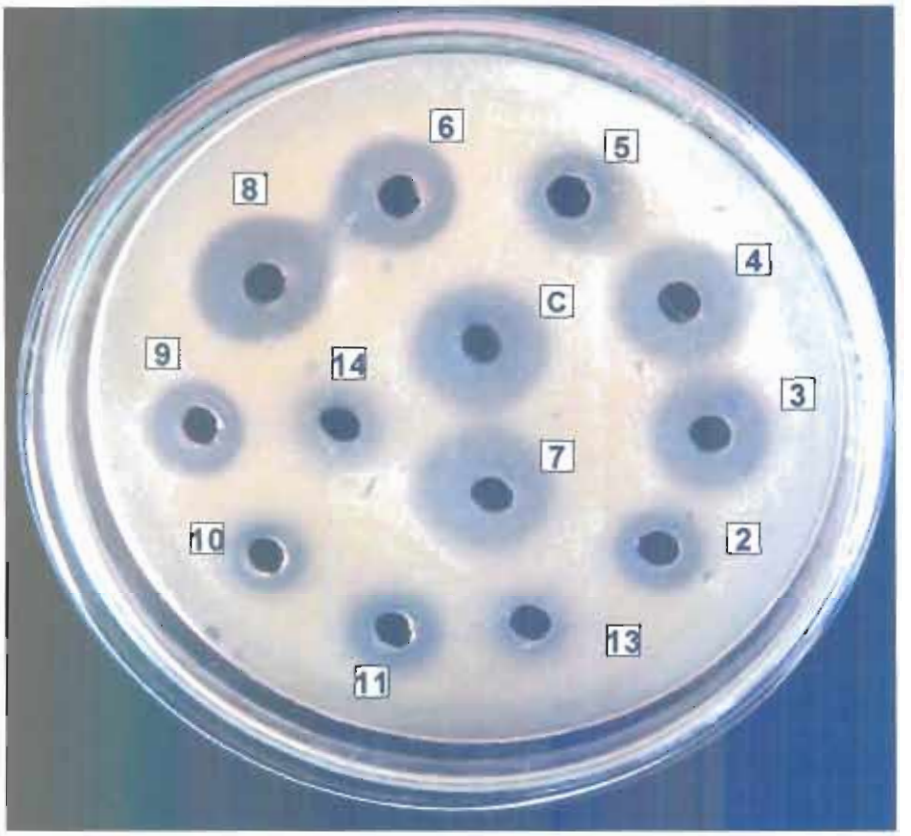


Figure 6.10. Stability of Erwiniocin NA5 against pH Range (2-14) Treatment Followed by Neutralization

Note: pH was adjusted by using 1 M NaOH & 1 M HCl, after 2hr of incubation Erwiniocin NA5 was neutralized by using the same reagents

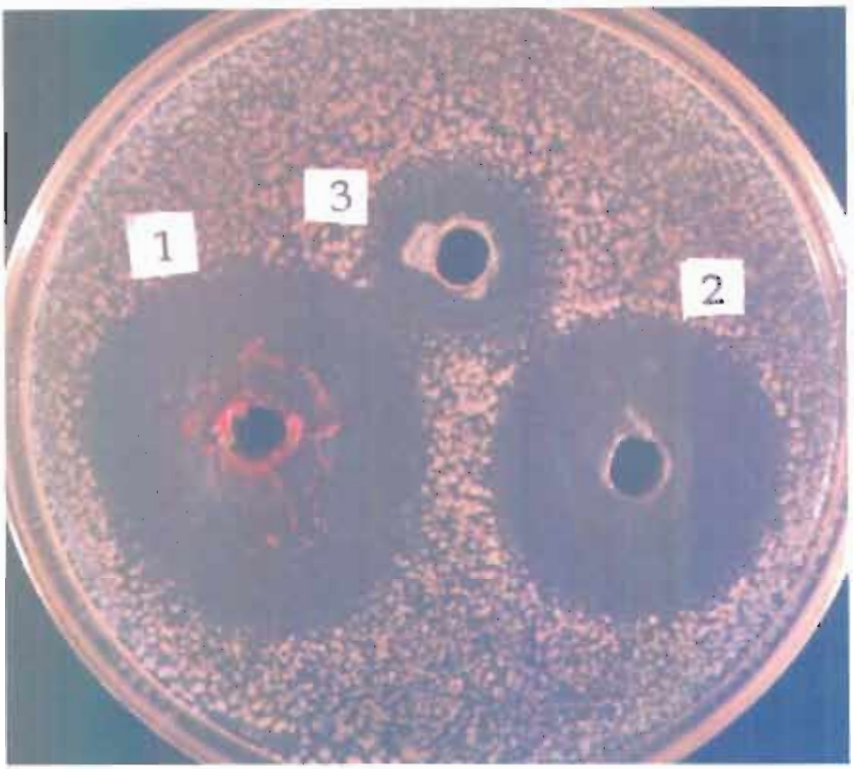


Figure 6.11. Effect of Different Temperatures on the Bioactivity of *Erwinia carotovora* NA5 against *Agrobacterium radiobacter* NA6

1. Un treated Erwiniocin NA5
2. Treated Erwiniocin NA5 (100°C)
3. Treted Erwiniocin NA5 (Autoclaved)

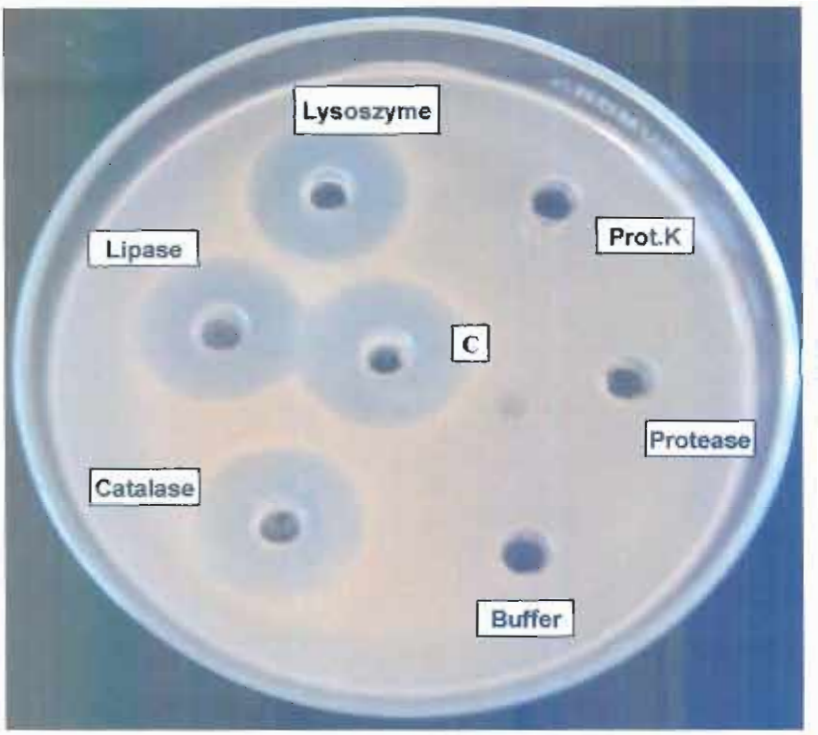


Figure 6.12. Effect of Different Enzymes on Erwiniocin NA5

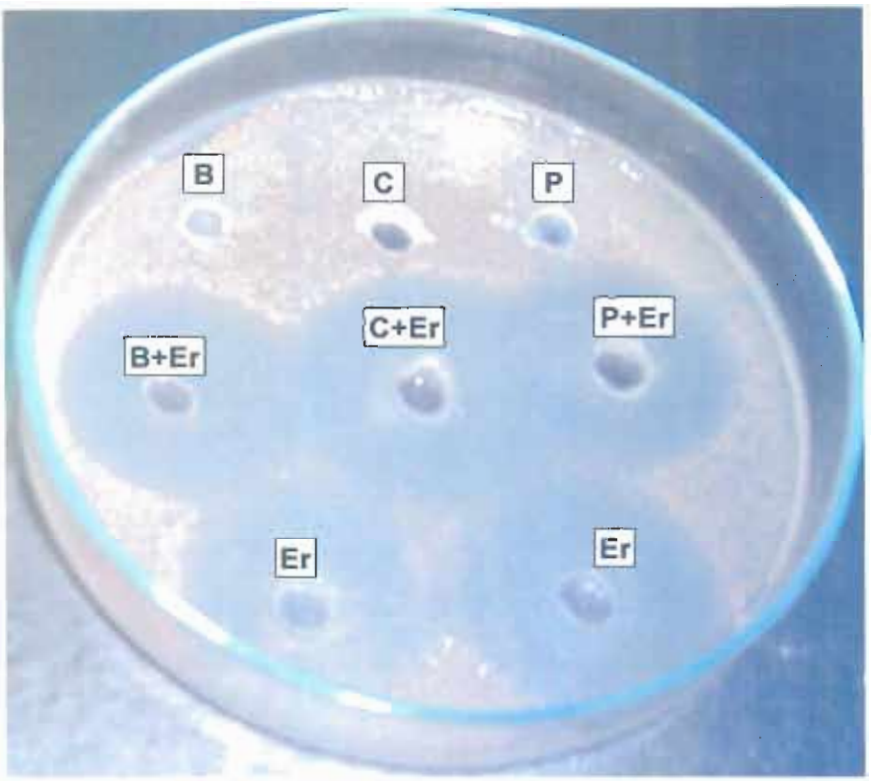


Table 6.13. Effect of Organic Solvent (5%), on the Bioactivity of Erwiniocin NA5, against *Erwinia carotovora* NA8

Er. Erwiniocin NA5 (control)
B. Butanol
C. Chloroform
P. Propanol

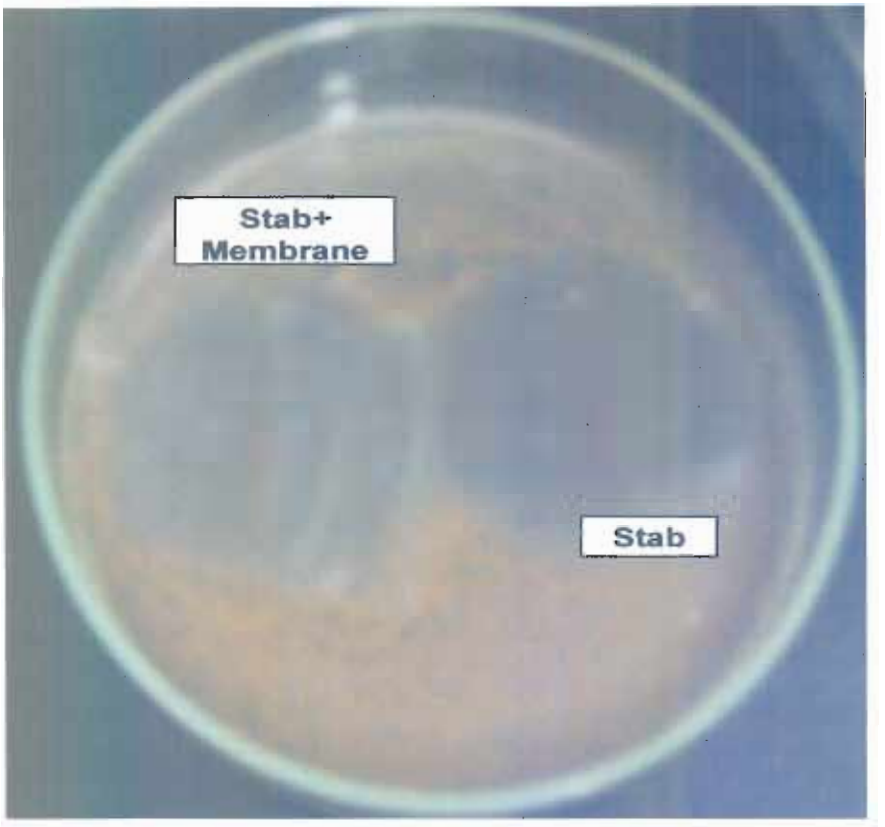


Figure 6.14. Approximated Molecular Mass Estimation of Erwiniocin NA5 (using Dialysis Membrane of <12 kDa cut out size)

a. Stabbed producer effect with overlaid membrane
b. Plain stabbed and overlay (without dialysis membrane)

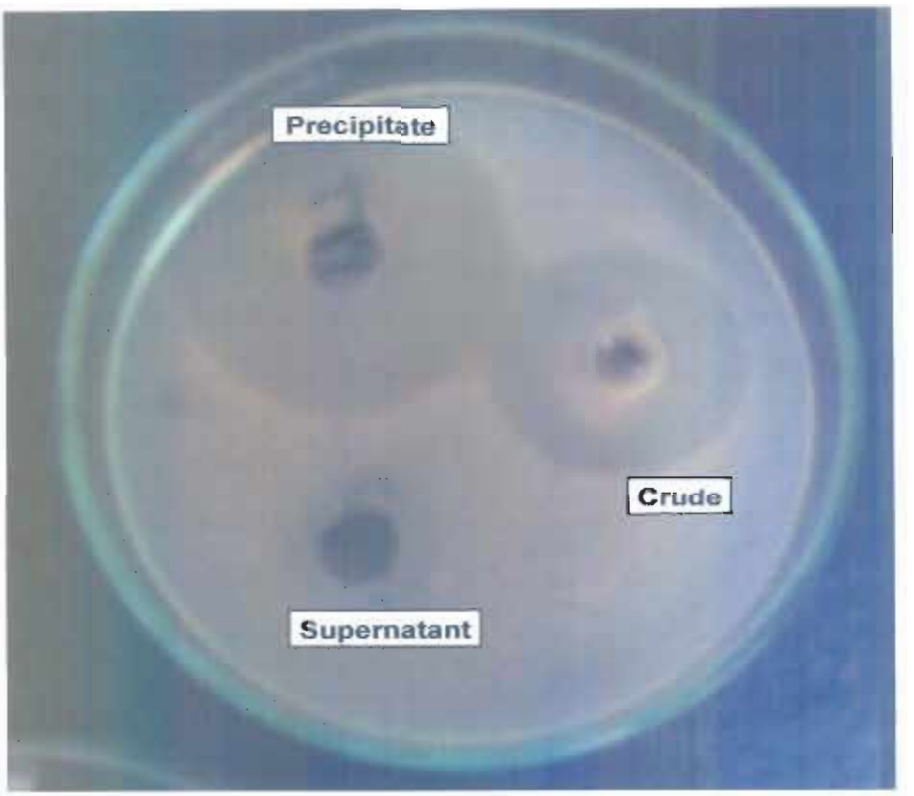


Figure 6.15. Bio-activity of Crude and Amonium Sulphate Precipitated (80%) Erwinioccin NA5 against *Erwinia carotovora* NA8

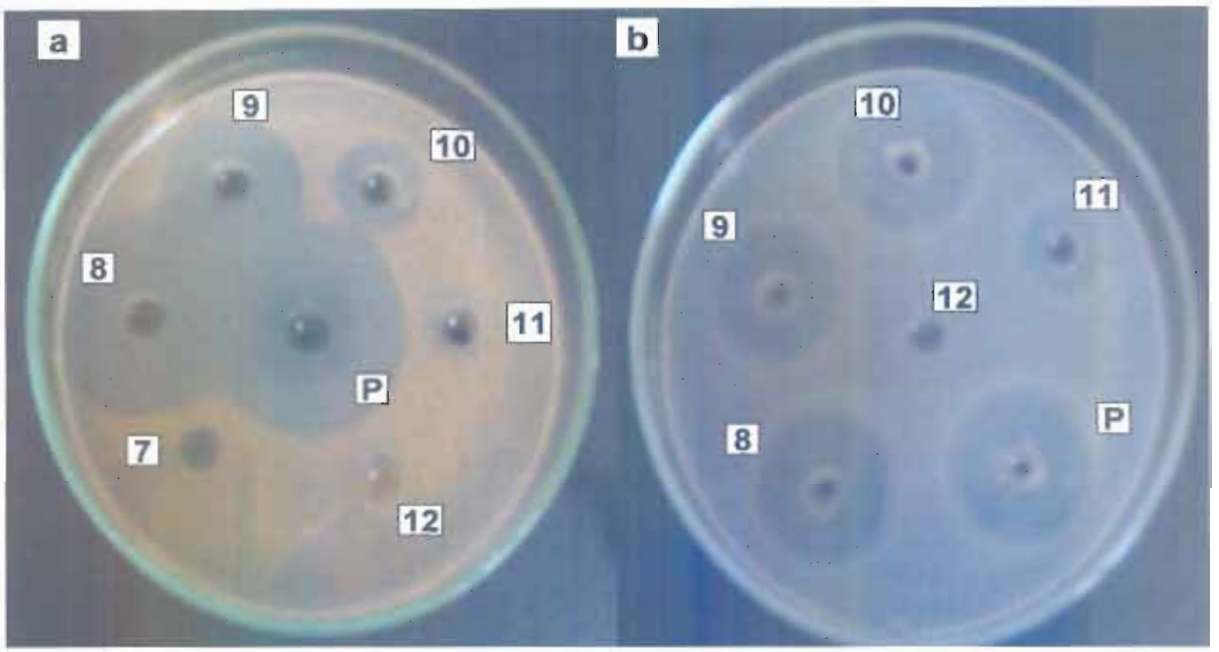


Figure 6.16. Bioactivity of Erwiniocin NA5 Fractions after Size Exclusion Column Chromatography (Sephadex G75)

a. Against *Erwinia carotovora* NA8
b. *Xanthomonas oryzae* NA1 **(the numbers on the zones reflect the fractions of the peak)**
p. Pooled fraction

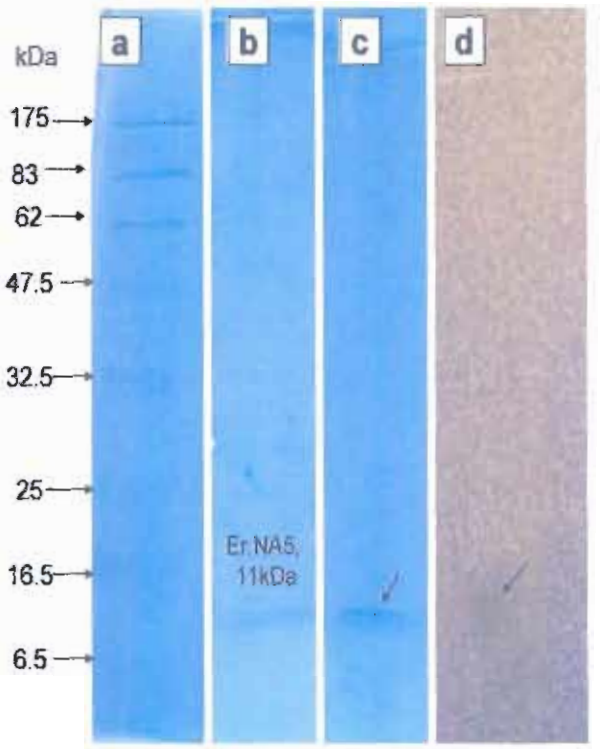


Figure 6.17. 8% Polyacrylamide Gel Electrophoresis of the Erwiniocin NA5 Preparations, Coomasie Brilliant Blue R-250 Stained Gel: Molecular Weight Markers (a); Active Fraction obtained after Size Exclusion Gel Chromatography (b); Ammonium Sulfate Precipitated Erwiniocin NA5 (c); Detection of Erwiniocin NA5 activity on SDS PAGE against *Erwinia carotovora* NA8 (d)

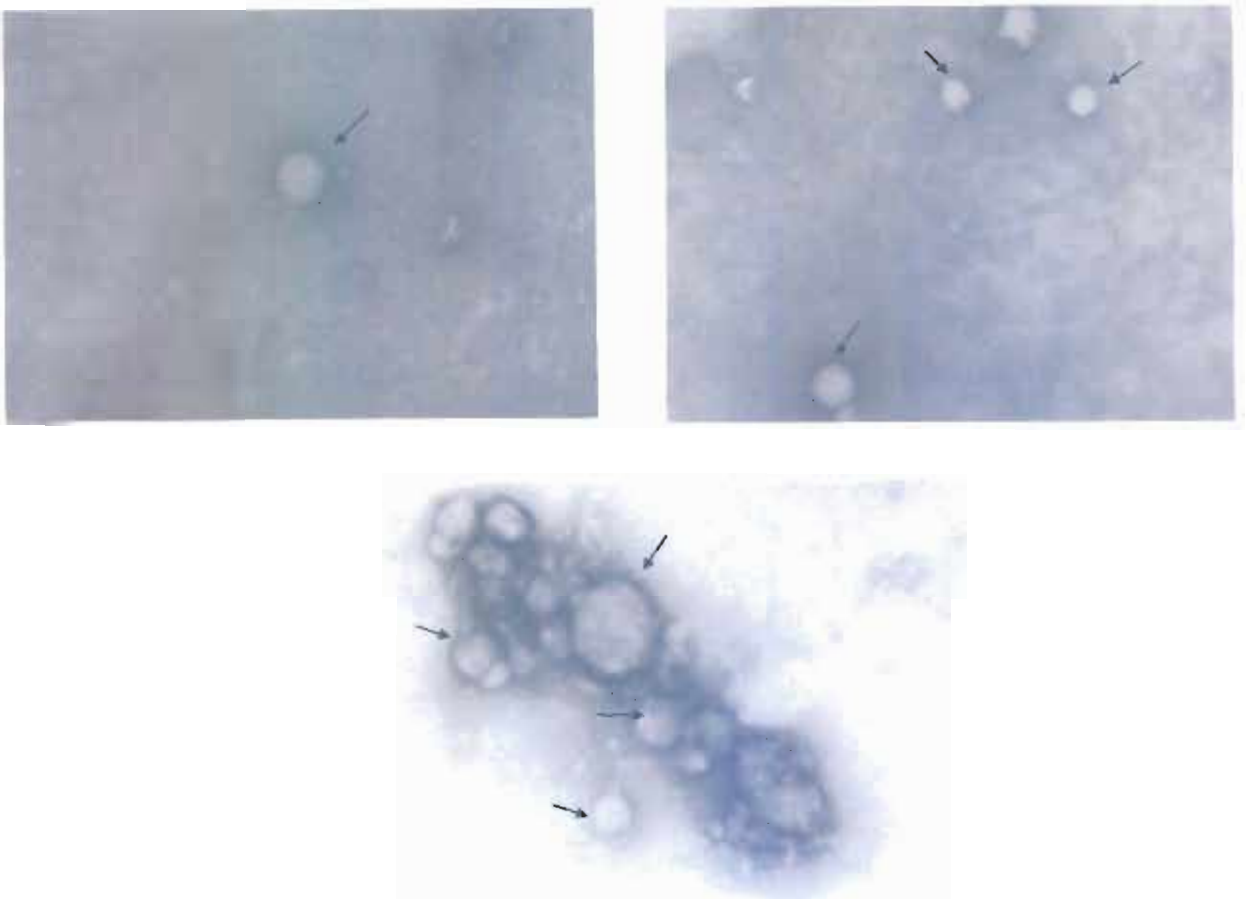


Figure 6.18. TEM of Erwiniocin NA5 (Crude Preparations)

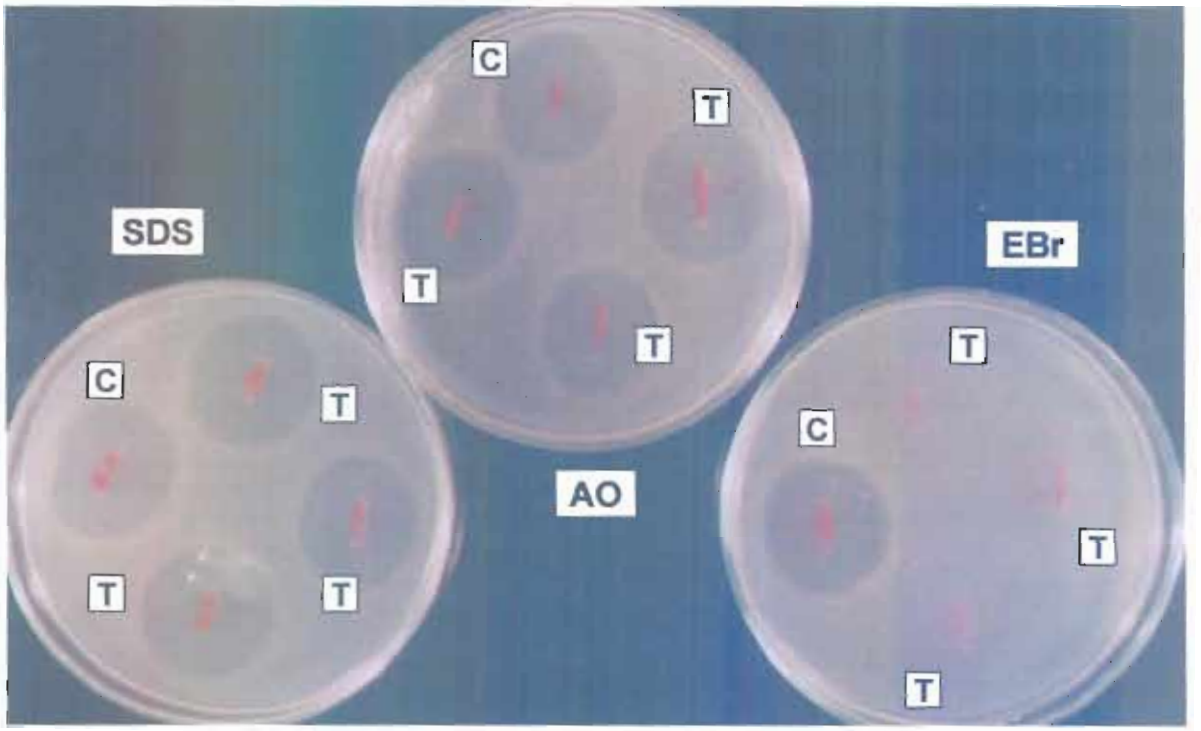


Figure 6.19. Plasmid Curing (For Erwiniocin NA5 bac^{+} Marker)

SDS: Sodium dodecyl sulphate
AO: Acridine orange
EBr: Ethydium bromide
C: untreated(control)
T: Test (treated)

Figure 6.20. Characterization of Cured and Uncured Strains (Plasmid Isolation and Agarose Gel Electrophoresis)

Plasmid DNA (mini prep) from:
C: Uncured cells
B: Uncured cells
A: Cured cells

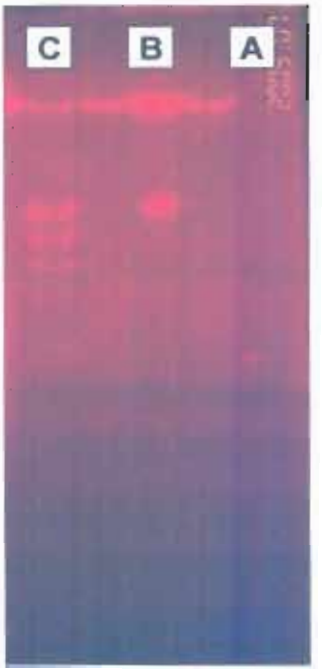


Figure 6.21. Molecular Characterization of Mini Prep Plasmid DNA (molecular weight estimation)

A. Cured
B. bac $^{+}$ plasmid
C. lambda *Hind* III digested

Note: the plasmid was not restricted by using *Hind* III & *Eco*R I Enzyme. That's why the molecular weight of the uncut DNA was estimated by using lambda *Hind* III digested plasmid.

Figure 6.22. General View of the Rice Plant Grown Under Control Conditions

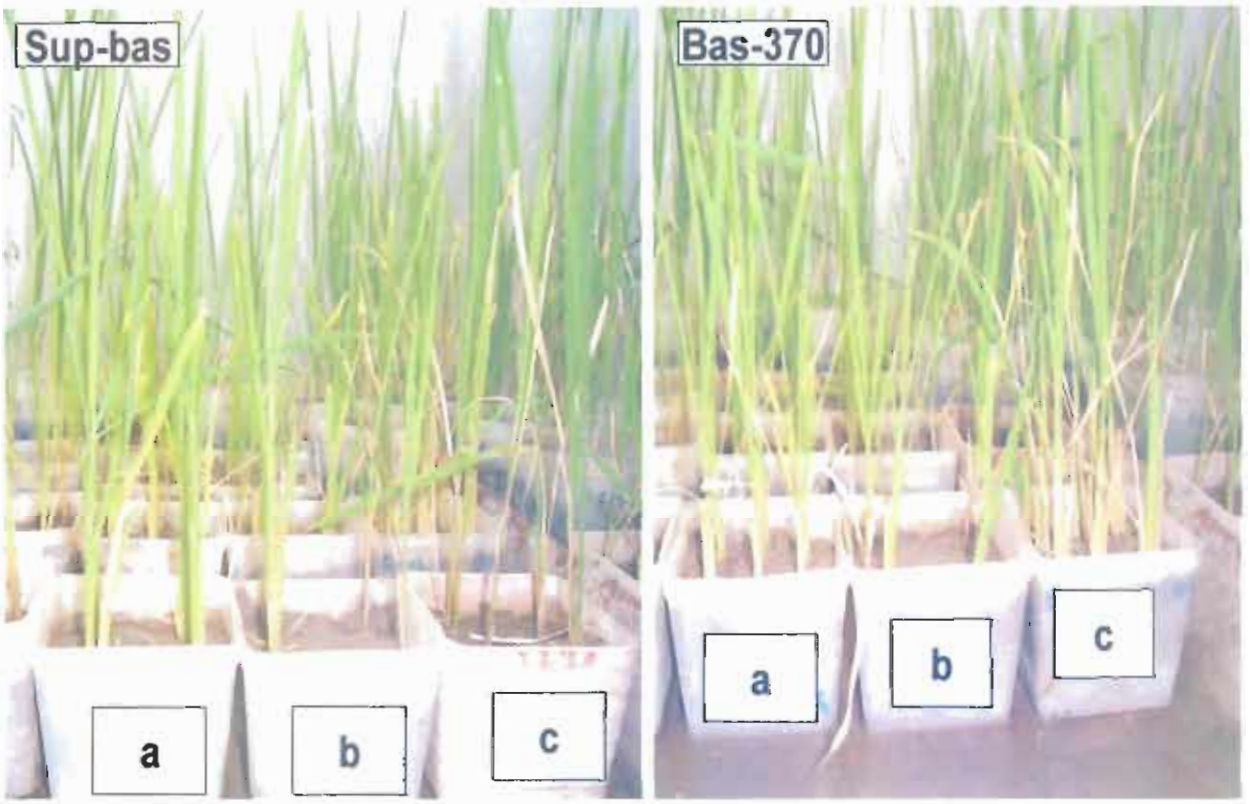


Figure 6.23. Bioactive Potential of Erwiniocin NA5 against BLB affected Rice Varieties

a & b. treated with Erwiniocin NA5
c. control (only infected)

Figure 6.24. Rice Fields (general view) at NIAB Faislabad (Pakistan)

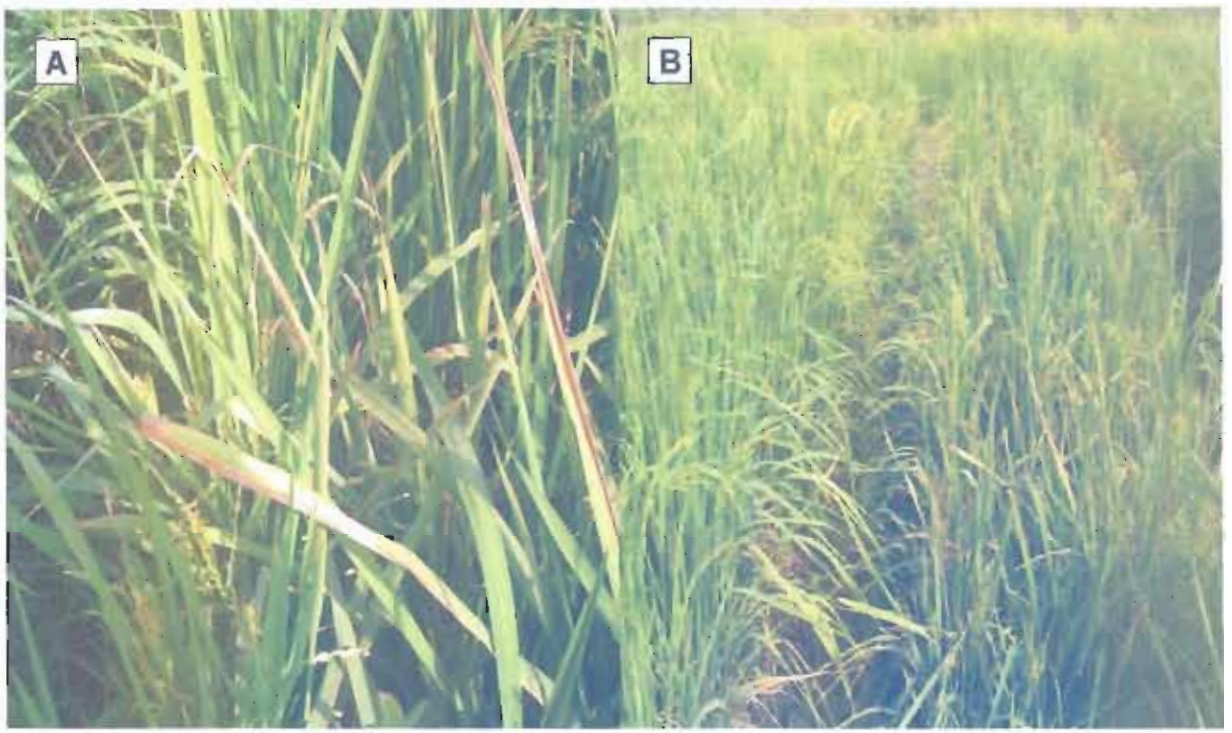


Figure 6.25. Open Field Trials Showing the Efficacy of Erwiniocin NA5 against BLB Infected Rice (bas-370)

a. Untreated
b. Treated

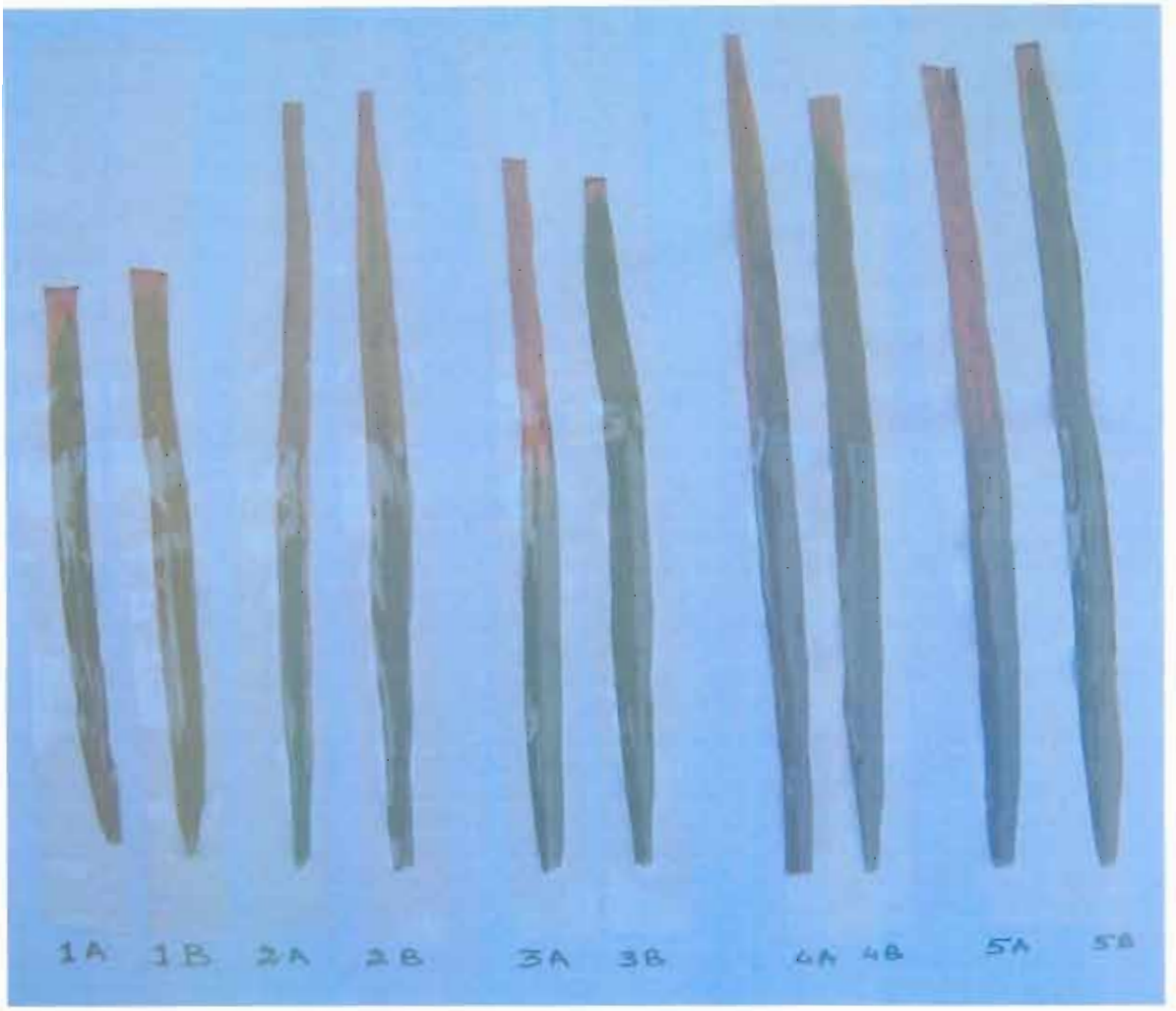


Figure 6.26. Cut and Paste Pictures of the BLB Infected (A. Treated and B.Untreated with Erwiniocin NA5) Rice Leaves from Different Rice Varieties

variety 1 = EF- 1-20-6-10
variety 2= IR-6
variety 3= bas-370
variety 4=Sup-bas
variety 5= 2000

DISCUSSION

7. Discussion

7.1. Isolation and Identification of Phytopathogenic Bacteria

Phytopathogenic bacteria can utilize the living plant cells as favoured food source. These bacteria are highly specialized to circumvent plant defences and efficiently invade tissues and cause diseases (George, 1997). Eight phytopathogenic bacteria isolated from different diseased fruits, vegetables and soil rhizosphere(s) were identified according to Burgey's manual and were finally identified by using API 20E and API 20NE kits.

7.2. Bacteriocins as Biocontrol Agents (Aginst the Emerging Threat to Phytopathogens)

Many bacterial species produce peptide antibiotics, called bacteriocins that often have antimicrobial effect on closely related bacteria (Bizaani *et al.*, 2005). These compounds, because of their potential application in agriculture, food and pharmaceutical industries, are extensively studied as natural bioperspectives. Many phytopathogenic bacteria including members of the *Corynebacterium*, *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Agrobacterium* produce proteinaceous bacteriocins (Heu *et al.*, 2001). These bacteriocins are highly specific, cost effective (and are even eco-friendly) and appear to be excellent candidates for agricultural use in controlling plant pathogens. Agrocin and their derivatives produced by

Agrobacterium radiobacter are extensively used against *A. tumefaciens,* the causative agent of crown gall disease (Kado, 2002) especially in Europe, America and Japan. Although, control of olive knot disease is very difficult, the use of copper compounds is one of the conventional practices to reduce the symptoms. However, the diffused resistance to copper bactericides among pathovars of *Ps. syringae* requires the development of alternative control methods for bacterial pathogens, such as the use of biological control products to lower toxic pesticide residues on fruits and vegetables and to avoid environmental accumulation of chemicals and the consequent development of resistance among pathogens (Wilson and Backman, 1999). Lavermicocca *et al.* (1999; 2002) purified a bacteriocin produced by *Ps. syringae* pv. *ciccaronei,* a bacterium isolated from a leaf spot lesion on a carob tree (the bacteriocin selectively inhibited the growth of *Ps. syringae* subsp. *savastanoi).* Glycinecin A (produced by *Xanthomonas axonopodis* pv. *glycines*) specifically inhibits the growth of bacteria belonging to *Xanthomonas* species. The control efficacy was as high as or even higher than the chemical treatment of copper hydroxide. These results suggest that the bacteriocin is a potential control agent for bacterial plant diseases (Fett *et al.*, 1987). The bacterium *Erwinia chrysanthemi* ENA49 was found to produce bacteriocin that is similar to tail fibers of bacteriophages and showed bioactivity against *Erwinia, Pseudomonas* and *Xanthomonas* strains (Lysak *et al.*, 1988). There are other examples of biological control that involve antibiosis and human dimensions that influence their use. It has been proposed that bacteriocins may play a key role in bacterial population dynamics (Riley, 1998). To date, in Pakistan, there is no evidence to use the bacteriocins, rather the hazardous antibiotics

and chemicals like boric acid, cupric oxychloride and cupric oxide etc. have been in use ever since. Thus, the bacteriocins are of intrest for potential application in food and agriculture industry because of their selective antimicrobial activity, safe for the users and the environment, non hazardous and their technologically favourable properties. The aim of the present study was to develop the biopesticide/bacteriocins for the control of plant bacterial diseases from indigenous phytopathogenic bacteria and understanding the significance with particular reference to agricultural potential of the bacteriocin(s) compared to frequently in practice unsafe chemicals. The present study is based on the isolation and identification of the phytopathogenic bacteria, screening of the best bacteriocin producer, the purification, physico-chemical characterization, genetical aspects, inhibitory spectrum and applied aspects of the bacteriocin (of Erwiniocin NA5 from the best producer i.e. *Erwinia carotovra* NA5).

7.3. Screening for Bacteriocinogenic Strains

The detection of bacteriocin production was monitored using three conventional strategies i.e. agar-well diffusion, stab-overlay and cross-streak methods. In stab-overlay and agar-well diffusion methods, bioactivity was checked using a number of producer strains, against only one indicator culture while in cross-streak method, the inhibitory activity was checked using only one producer strain against a number of sensitive cultures. The objective of this study was to compare some of the most commonly used methods for the detection of bacteriocin activity. Application of these screening procedures has provided useful preliminary information for the

identification of possible bacteriocinogenic organisms. Three isolates (*Erwinia carotovora* NA5, *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobcter* NA7) out of the eight isolates were found bacteriocinogenic. Interestingly, all three isolates did produce the bacteriocin in all the three screening procedures. Previously bacteriocin production by other plant pathogens was reported by Heu *et al.* (2001) and Chuang *et al.* (1997). Out of three bacteriocin producing strains two (i.e. *Agrobacterium radiobacter* NA6 and, *Agrobacterium radiobcter* NA7) were effective against each other thereby showing their commitment to "professional cohabitational antagonism", and these bacteriocinogenic strains were also effective against *Agrobacterium tumefaciens B6* (the causative agent of crown gall in grapes). These results indicate the narrow spectrum of antibacterial activity by the protein metabolites of the three producer strains (i.e. *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobcter* NA7). The biological control of *Agrobacterium tumefaciens* (for the control of crown gall disease) by using *Agrobacterium radiobacter* K84 is well known (Nicholas *et al.*, 1998). However, in case of the third producer strain (*Erwinia carotovora* NA5), cross-streak method was found more effective as Erwiniocin NA5 possessed antibacterial activity against all the isolated phytopathogens (in cross streaks) but was less active in stab-overlay and agar-well diffusion methods. *Erwinia carotovora* var. atroseptica and *Erwinia carotovora* var. carotovora were sensitive to the same bacteriocins but the two serogroups of var atroseptica could be differentiated from each other on the basis of sensitivity to one bacteriocin (Crowley and Debour, 1980). Erwiniocin NA5 was also effective against several clinical isolates. While Nguyen *et al.*, (2001) reported narrow-spectrum

activity of carotovorocin Er, the inhibitory spectrum of Erwiniocin NA5 was found broad spectrum, also showing activity against *Staph epidermidis,* Methicillin resistant *Staphylococcus aureus, Bacillus sublitus, Micrococcus luteus and* Gram-negative bacteria including phytopathogenic strains e.g *Xanthomonas oryzae, Erwinia carotovora* and also against *E. coli, Klebsiella pneumoniae, Shigella flexneri and Salmonella typhi para A.* The results are in agreement with an earlier investigation on the bacteriocin of Erwinia by Tovkach (1998) who reported the bioactivity of bacteriocin carotovoricin against *Agrobacterium, Pseudomonas, Klebsiella and E. coli strains. E. herbicola and E. chrysanthemi* ENA49 also produced bacteriocins with a broad range antibacterial activity against several *Erwinia, Pseudomonas and Xanthomonas* strains (Lysak, 1980). The degree of antagonism (measured in mm of zones of inhibition) by Erwiniocin NA5 varied from organism to organism and it may include factors like the receptors/target sites present on the indicator cells surface (targets for most of the bacteriocins may be modified to some extant), charge distribution on the cell, cell wall composition and diffusion of bacteriocin into medium. There may be other reasons for differential zone sizes viz age of the culture(s) and amount of inocula stabbed on the solid medium, composition of the medium, agar concentration, depth (10mm) of medium and conditions of incubation of bacteriocinogenic strains (these factors may influence the yield of bacteriocin). Laukova and Marekova (1992) reported that bacteriocin production is affected in most cases by environmental milieu, including the characteristics of the culture medium and incubation conditions.

7. 4. Excluding the Possibility of the Inhibitory Effect by Lytic Phage or Hydrogen Peroxide (H_2O_2)

Experiments were performed to rule out of the inhibitory effect by bacteriophages or H_2O_2. Bacteriocins, unlike bacteriophages do not carry the genetic determinants necessary for self replication within the susceptible organism. Presence of zone of inhibition after seeding the indicator culture in opposite direction indicates that lytic bacteriophages are not responsible for the inhibition zones thus, when a block from zone of inhibition was cut and emulsified in sterilized broth, no zone of inhibition was observed, thereby, suggesting that the inhibitory molecules constitute bacteriocins and not the bacteriophages (since only bacteriophages can be propagated on the indicator strains). Stability of the activity of bacteriocin in the presence of catalase enzyme ruled out the possibility of antagonistic activity of bacteriocin due to hydrogen peroxide or organic acids (Kim, 1993). The inhibition due to H_2O_2 could not be observed in Erwiniocin NA5, as earlier reported by Parrot *et al.* (1990) and Ahmed *et al.* (2004).

7.5. Titration of Erwiniocin NA5

The methods for assessing the efficiency of antimicrobial compounds have largely been developed to encounter the needs for further microbiological assays. The most widely used method is the agar well diffusion assay. Infact, various modifications of agar well diffusion assay are widely used and partly standardized for testing the bioactivity of antimicrobial agents (Skytta and Mattila-Sandholm, 1991). A

definition widely accepted for the titration of an inhibitory substance is the reciprocal of the highest dilution of the agent (bacteriocin) exhibiting a definite inhibition zone when the sample volume varies within a range of 5-100μL (Muriana and Klaenhammer, 1991; Van Reenen *et al.*, 1998). Since the size of inhibition zone depends upon the rate of diffusion, misleading results may be obtained if the agent (to be screened) is hydrophobic and does not properly diffuse into the agar. Agar well diffusion assays (AWDA) were used to quantitate the bacteriocin activity (Lambert and Pearson, 2000). Methods for the assay of bacteriocin activity are usually derived from those of the antibiotics. AWDA was routinely used to measure the bacteriocin activity. The titer of Erwiniocin NA5 was 160AU/mL against *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1. Activity units (AU) were found different against different sensitive strains. The results in hand supported by the ones reported by Nguyen *et al.* (2001) who showed the varied bactericidal titers of carotovorocin Er preparation against different strains of Erwinia *carotovora* subsp. *carotovora* strains EC-2,P7 and 645Ar.

7.6. Physicochemical Characterization of Erwiniocin NA5

Crude and partially purified Erwiniocin NA5 preparation (subjected to different heat treatments including autoclave pressure and heat) was found thermostable. Its bioactivity remained intact after several months of refrigeration. Our results could be confirmed by previous report (Tovkach, 1998) regarding the thermostability of colicin-like carotovorocins CCTV. Lysak (1980) studied eight bacteriocins from *Erwinia* strains with different thermal stabilities, sensitivity to proteolytic enzymes,

dialyzability through cellophane membrane(s). Bacteriocins from *Erwinia* showed sensitivity to proteolytic enzymes and were proved to be protein in nature (Shukin, 1980). These properties strongly suggested that the bacteriocin like activity of the strains could be defined as a (typical) bacteriocin. Therefore, it was designated Erwiniocin NA5. In an earlier report, Erwinicin from *Erwinia aroideae* was found resistant to the action of DNase and RNase (Biagi, 1995). The Erwiniocin NA5 remained stable when mixed with phosphate buffer of different (2-14) pH values. The bioactivity of Erwiniocin NA5 (when adjusted the pH with 1M solutions of NaOH and HCl and neutralized by the same reagents after 2hr of incubation) remained intact but the activity units were decreased towards low acidic and low alkaline pH levels. This variation in bioactivity (compared to stability) could be explained as when the pH was adjusted by using 1M NaOH and HCl, the pH remained the same (for 2hr) till its neutralization compared to its stability when range of potassium phosphate buffers was added to Erwiniocin NA5 in 1:2 dilution, where the exact pH value could not be retained. This artifact led to the observed decreased in AU/mL (hence zone size) at lowest acidic and alkaline pH values.

Catalase enzyme also could not eliminate the bioactive potential of Erwiniocin NA5 thereby, ruling out the possibility of hydrogen peroxide mediated antagonism by the producer strain. Similarly, Erwiniocin NA5 was found resistant to various solvents namely, acetone, formaline, ethanol, methanol, propanol, butanol and chloroform at concentration of 1% and propanol, butanol and chloroform at 10% concentration. Further, treatment of Erwiniocin NA5 with 1mM solutions of several metal ions did not lead to the change in its bioactivity.

Bacteriocinogenic activity of *Erwinia carotovora* NA5 was checked by growing the producing strains in nutrient agar medium with variable percentage of agar *i.e.* 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.6. Maximum zone of inhibition (30 mm against *Xanthomonas oryzae* NA1 and 34 mm against *Erwinia carotovora* NA8) was observed in the presence of 1.4% of agar while the marginized zone of inhibition was found in the presence of 2.6% of agar. The difference in zones of inhibition is clearly indicative of the low diffusibility at higher agar concentration(s). Chuang (1999) also reported 1.4% agar as optimum for the production and diffusion of Carotovorocin. Maximum production of Erwiniocin NA5 was observed in nutrient agar compared to LB and YPGA media. Similarly, Erwiniocin NA5 was comfortably dialyzable through dialysis membrane

7.7. Growth Kinetics

Maximum bacteriocin yield in a culture may be observed at different phases of the growth cycle. In the present study, the (extracellular) production of Erwiniocin NA5 starts in early logarithmic phase, its activity reaches maximum after 8 hours of incubation and the activity is retained till late stationary phase. After reaching the maximal bacteriocin activity in the medium during the active growth phase, often a drastic decrease in soluble bacteriocin activity occurs. This disappearance of bacteriocin activity was presumed to be proteolytic inactivation, protein aggregation and adsorption of the bacteriocin molecules to the cell surface of the bacteriocin producing cells (Callewrt and Vust, 2000). It appears Erwiniocin NA5 is not encountered by bacteriocin inactivators and enzymatic digestion.

7.8. Mode of Action of Erwiniocin NA5

Bactetriocins are bacterial products with specific bactericidal activity, generally towards bacterial strains and species closely related to the producer strains (Dykes, 1995). Determination of the effect of antibacterial substance (Erwiniocin NA5) on logarithemic and stationary phase cells showed decrease in the CFU/mL, while optical density (O.D. $_{530}$), after addition of Erwiniocin NA5 remained the same in test and in the positive control (only with sensitive strain). However, prolonged incubation did not result in the increase in optical density thereby, suggesting that Erwiniocin NA5 exerts a bacteriocidal rather than bacteriostatic effect on *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1 strains. Tovkach (1998) reported lysis of various bacterial strains (*E. carotovora, E herbicola, E. coli* and *Ps. syringae*) by bacteriocins of *Erwinia carotovora*. The present findings are in agreement with Hamon and Peron's observations (1961) who described the substance(s) produced by *Erwinia carotovora* subsp. *carotovora* (the causative agent of soft rot disease in many plant species) exerted bactericidal effect towards the same and the related species of bacteria.

7.9. Immunity

In this study the resistance of *Erwinia carotovora* NA5 against its own (produced) bacteriocin by stab and overly and agar-well diffusion methods was also demonstrated, as the producer was not inhibited by its own protein product. Bacteriocin producers are generally immune to their own bacteriocin because of a

self-protection mechanism that is specific for the bacteriocin produced (Sahl and Bierbaum, 1998; Ennahar *et al.*, 2000). Same is true for almost all the bacteriocin-producing bacteria (Nes and Holo, 2000).

7.10. Lacuna Counts

The percentage of cells producing Erwiniocin NA5 out of the whole population was determined by lacuna assay. It is performed to determine the frequency of bacteriocin production by *Erwinia carotovora* NA5. Lacunae are the clearings (halos) produced in the lawn of the indicator bacterial strains by the bacteriocins released from individual cells. Hardy (1987) reported that percentage of lacuna forming cells among *E. coli* was found between 0.01-10%. In our experiments, lacuna percentage in *Erwinia carotovora* NA5 against *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1 was 87% and 79% respectively. Results show that lacuna percentage was higher against Erwinia *carotovora* NA8 compared to (against) *Xanthomonas oryzae* NA1; this difference may be due to the fact that bacteriocins work best against the closely related strains. Bacteriocins are the proteins (or protein complexes) with bactericidal activity directed against species that are usually closely related to the producer bacterium (Tagg, 1976). Not all bacteriocinogenic strains form lacunae, either because individual cells do not form sufficient bacteriocin leading to visible clearings or because the bacteriocins remain largely cell-bound and are not diffused from individual cells (Hardy, 1982).

7.11. Molecular Weight Estimation of Erwiniocin NA5 (by using Dialysis Membrane)

An estimate of the molecular weight of Erwiniocin NA5 was done by placing dialysis membrane (cut off <12000Da) on stabbed producer culture and overlaying with sensitive culture. Presence of zone of inhibition around stabbed colonies with and without dialyzing membrane indicates that the Erwiniocin NA5 was dialyzable through dialysis membrane with a molecular weight less then 12000Da. As per Parrot *et al.* (1989) all the inhibitory substances (CFNS prepration) can pass through dialysis membrane (pore size <10000Da) suggesting its molecular weight to be <10000Da.

7.12. Activity Directed Purification of Erwiniocin NA5

The crude (culture supernatant) Erwiniocin NA5 of *Erwinia carotovora* NA5 was subjected to rotary evaporator for 3-5 fold concentration and then purified by sequential ammonium sulfate precipitation (80% saturation) followed by high speed centrifugation (14000g) the protein pellet obtained after centrifugation was dissolve in 50mM phosphate buffer, protein estimation (by Biuret method) and antibacterial activity in terms of AU/mL were determined. 80% precipitation of culture supernatant of *Erwinia carotovora* NA5 with ammonium sulfate resulted in 10% recovery of the total Erwiniocin NA5 activity. The specific activity in crude culture supernatant was 33.3 AU/mg which was increased to 55.2 (AU/mg). Ogunbanwo *et al.* (2003a) reported that bacteriocins of *L. plantarum* F1 and *L. brevis*

OG1 were recovered following 60% saturation of the culture broths with ammonium sulfate with an increase to specific activity of 9.4 and 5.2 AU/mg proteins respectively. Salting out or ammonium sulfate precipitation is useful for concentrating dilute solutions of proteins (Whitford *et al.*, 2001). It is also useful for fractionating a mixture of proteins. Since large proteins tend to precipitate first, smaller ones will stay in solution. Thus, by analyzing various salt fractions, one can find conditions where the protein (under study) precipitates and many of the other proteins in the mixture stay in solution. The end result is that one is able to increase the purity of the protein of interest while concentrating it using an ammonium sulfate fractionation approach. Precipitation by addition of ammonium sulfate (neutral salt) is probably the most commonly used method for fractionating proteins by precipitation. The precipitated protein is usually not denatured and activity is recovered upon re-dissociating the pellet. In addition, these salts can stabilize proteins against denaturation, proteolysis or bacterial contamination (Harris and Angel, 1989). Usually, an ammonium sulfate cut is taken in order to obtain a higher degree of purification.

Erwiniocin N5 was dialyzable through dialysis membrane with an MW cut-off <120 000, suggesting its molecular weight to be <12kDa. Since the ammonium sulphate precipitated Erwiniocin NA5 was completely ultra-filtrated using a membrane with an MW cut-off of <10,000, further the fraction of purified Erwiniocin NA5 was subjected to conventional gel filtration chromatography on Sepahdex-G75 column equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.0. This group separation resulted in a major (active) peak. The antibacterial

activity was determined by analysis on *Erwinia carotovora* NA8 and *Xanthomonas oryzae* NA1 plates whereby the bacteriocin activity resided in peak fractions (number 9,10,11,12). Jabrane *et al.* (2002) reported the presence of only one peak containing bioactivity against *Erwinia amylovora* B87 from *Serratia plymithicum* J7. Size exclusion gel chromatography resulted in the increase in specific activity (61.5 AU/mg) from the one obtained after ammonium sulfate precipitation (55.17 AU/mg) and a recovery of 0.5% of the total antibacterial activity from 10% (after ammonium sulfate precipitation). Nguyen *et al.* (1999) reported the 26-fold purification of Carotovoricin Er after ultracentrifugation (120,000xg) and sucrose density gradient centrifugation of cell lysate of mitomycin-C treated *E. carotovora* Er with approximately 35% recovery. While in case of Erwiniocin NA5 after column chromatography (Sephadex G75) 2.2 fold purification level was achieved, this difference may be due to the uninduced compared to ultracentrifugted mitomycin-C induced Carotovoricin Er. However the recovery of the activity of Erwiniocin NA5 decreased with the subsequent step in purification, clearly suggesting the protein/peptide nature of the active components. These results were further confirmed by electrophoretic characterization of partially purified and separated fraction of protein by SDS _ PAGE and followed by gel overlay bioactivity assessment.

7.13. Sodium Dodicyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS- PAGE)

It is well known that bacteriocins are small molecular weight proteins produced by certain kinds of bacteria which show various biological activities against animals and microorganisms (Muriana and Luchansky, 1993). While searching the exact molecular weight estimation of the Erwiniocin NA5, the purified protein gave a single band corresponding to a MW of about 11kDa in SDS-PAGE analysis. The detection of bacteriocin activity directly in SDS-PAGE is simple, sensitive, rapid and the most widely used method. The corresponding band giving inhibitory activity can be eluted from the gel for further studies. In the course of detection of Erwiniocin NA5 activity directly in the gel, same methodology (Bhunia *et al.*, 1987) with slight modifications (Crupper and Iandolo, 1996) was followed. The electrophoregram of CBB-stained gel which showed a major band of 11 kDa; the gel used for detection of antibacterial activity, a clear zone of inhibition of the indicator cells (*Erwinia carotovora NA8*) corresponds with 11 kDa protein, indicating that Erwiniocin NA5 could be associated with low molecular mass protein. From these properties, the active component of the strain *Erwinia carotovora* NA5 was placed in the category of bacteriocin and the name Erwiniocin NA5 was proposed. Two types of Bacteriocins (carotovoricins) were reported from *Erwinia carotovora:* those simillar to the colicins (Lysak 1978; Slade 1984) of low molecular weight bacteriocin, and the phage tail type macromolecular carotovoricin (Crowely 1980; Zink 1985, Tovkach, 1984). The low molecular weight bacteriocin/ colicin like protein has been reported in *Erwinia*

carotovora bacteriocins, SDS-PAGE of the purified carotovorocin Er showed that the sheath, core, and tail fiber types consist of single major protein of 50, 19 and 68 kDa, respectively (Nguyen 1999). In addition to these proteins, protein bands corresponding to 78,76,46,44,39 and 35 kDa were detected in the mitomycin-C induced purified carotovoricin Er. These results prompt Nguyen (2001) to study whether or not the carotovoricin Er preparation contains multiple types of carotovoricin Er with different host specificities. The un induced *Erwinia carotovora* NA5 produces the low molecular weight protein (bacteriocin/ Erwiniocin NA5) which may be related to low molecular weight reported colicin like bacteriocin from *Erwinia carotovra* strains.

7.14. Transmission Electron Microscopy

Transmission electron microscopy (TEM) allowed visualizing the Erwiniocin NA5. Accordingly, Erwiniocin NA5 molecules resembled empty bacteriophage /defective phage or it might be membrane vesicles because of their uneven size and shape. These results are in agreement with Nguyen *et al.* (1999) according to whom, the spherical particles obtained from the crude preparation of carotovoricin Er seemed to be membrane vesicles from the lysed cells of *Erwinia carotovora* Er, because they were not homogenous in size and shape. Crowely and Debour (1980) tested several *Erwinia carotovora* serogroups for the bacteriocin activity; among these eight bacteriocin producing strains were found and the bacteriocins partially purified by ammonium sulfate fractionation and high-speed centrifugation followed by electron microscopy; bacteriocins from all eight strains were morphologically similar to the

bacteriophage tail. Our observations are different from the previously reported findings/observations, where these molecules were described as high molecular weight phage related bacteriocins [(resembling phage tails) (Nguyen *et al.*, 1999)]. However these bacteriocins were mitomycin-C induced followed by several chemical treatments with several steps of high speed centrifugation i.e 8000-120,000xg. Whereas the uninduced crude and precipitated Erwiniocin NA5 of low molecular weight was used in the present studies (molecules resembling the defective phage head or might be membrane vesicles of the lysed cells.

7. 15. Computational Analysis for Amino Acid Composition

For amino acid composition of Erwiniocin NA5, statistical analysis was done by applying bioinformatics. Complete protein sequencing (from NCBI genomics server) a low molecular weight hypothetical phage related protein was selected and by taking mean value of each amino acid it was assumed that our low molecular weight bacteriocin Erwiniocin NA5 preparation carries 7.9% alanin, 5.7% arginine, 4.28% asparagine, 5.28% aspartic acid, 1.37% cystein, 4.27% glutamin, 7.16% glutamic acid, 6.75% glycine, 2.17% histidine, 9.7% leucin, 7.09% isoleucine, 5.7% lysine, 3.3% methionine, 3.2% phenylalanine, 2.9% proline, 7.46% serine, 4.42% threonine, 1.45% tryptophane, 3.4% tyrosine and 6.5% valine. Among these the acidic and basic amino acid components are respectively 12.44% and 13.57% with 49.8% polar and 50.16% non-polar amino acid components (Jeremy, 2001).

7.16. Inhibitory Spectrum of Erwiniocin

To elucidate weather the Erwiniocin NA5 was involved in the antagonistic relationships within bacterial communities, this bacteriocin was tested against several bacterial strains by stab-overlay, cross-streak and agar-well diffusion assay. Lysak *et al.*, (1978) studied antibacterial activity of 272 *Erwinia* strains and found that only 66.9% of strains were capable of producing (spontaneously) antibacterial substance belonging to the class of bacteriocins. The inhibitory spectrum of Erwiniocin NA5 was found to be broad spectrum which includes activity against *S. epidermidis, Bacillus subtilus, Micrococcus luteus and* against all Gram-negative phytopathogenic bacteria used in this study and also against *E. coli, Klebsiella pneumoniae, Shigella flexneri and Salmonella typhi para A.* The broad host range antibacterial activity was also reported by Tovkach (1998) who reported the activity against *Agrobacterium, Pseudomonas, Klebsiella* spp., *E. coli strains, E. herbicola and E. chrysanthemi* whereas Hamon and Peron (1961) reported narrow spectrum "Carotovorocin" (a bacteriocin produced by *E. carotovora* subsp. *carotovora).* Among other species of (genus) *Erwinia, Erwinia chrysanthemi* ENA49 was also found to produce bacteriocin with a broad range of antibacterial activity against several *Erwinia, Pseudomonas* and *Xanthomonas* strains (Lysak *et al.*, 1988*).* The degree of antagonism (as per size zones of inhibition) by Erwiniocin NA5 varied from organism to organism. There may be a number of reasons for differential zone size(s) viz age and the amount of inocula stabbed into the solid medium, composition of the medium, agar concentration, depth of the medium, conditions of incubation of the producer and

the (modified) receptors present on the indicator cell surface. Laukova and Marekova (1992) reported that bacteriocin production is affected in most cases by environmental milieu, including the characteristics of the culture medium and the incubation conditions.

7.17. Determination of bac⁺ Marker/ Genetic Determinant(s) of Bacteriocin Production

It is revealed that gene(s) responsible for the bacteriocin production in *Erwinia carotovora* NA5 (i.e. Erwiniocin NA5) are located on plasmid (using curing technique involving interchelating dye ethidium bromide). Biagi and Azevedo (1995) reported the elimination of bacteriocin production phenotype in the phytopathogenic bacteria belonging to *Erwinia and Pseudomonas* genera when they were treated with ethidium bromide. The isolation of plasmids from phytopathogenic bacteria including *E. carotovora* strains, is difficult because of the specific structure of their cell walls and the presence of interfering cellular components (Kado and Liu, 1981). Plasmid DNA was isolated using standard procedures of Hardy (1978) and Kado and Liu (1981) the procedure was found appropriate for the isolation of plasmids not only from *E. coli* and *A. tumefaciens* cells but also from *Erwinia carotovora* cells. It can be seen that about 30% of the *Erwinia carotovora* strains (studied) have plasmids ranging from 2.5 to 129 kbp in size. Plasmid DNA was almost free of chromosomal DNA and other impurities (e.g. RNA and proteins). These preparations of plasmid DNA were used for restriction analysis carried out with the restriction enzyme Hind III. This enzyme restricted plasmid

DNA giving the streaky bands in the agarose gel electrophoresis. It would be of more interest to use (more than one restriction enzymes) for complete and clearly resolvable/bandable restriction of Erwiniocin NA5 plasmid of *Erwinia carotovora* NA5. Tovkach (2001) also used multiple restriction enzymes for characterization of plasmid(s) of *Erwinia carotovora*. The analysis showed that the uncut plasmids of *Earotovora carotovora* NA5 strain was about 23kb in size but appears to be a 'non conjugative'. Bacteriophages and plasmids are the major vehicles for the transfer of the genetic information among bacteria. The conjugative plasmids are able to mediate their own transfer or transfer of the mobilizable plasmid, by cell to cell contact (Hardy, 1993). In case of *Erwinia carotovora* NA5, several strains (including: *Xanthomonas oryzae* NA1, *Pseudomonas andropogonis* NA4, *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*) were tested for conjugal transfer of bac^+ marker of Erwiniocin NA5 (present on plasmid) but seems to be non conjugative, it may be due to the absence of F^+ factor or any enzyme required for this horizontal transfer or incompetent recipient. Col E1 plasmid is a mobilizable but nonconjugative plasmid and has provided the most definite evidence that transfer requires a plasmid-encoded nuclease, and a specific base sequence which contains the cutting site (Frefielder, 1987).

7.18. Field Trials

Mega bacteriocin(s) producer namely *Erwinia carotovora* NA5, the producer of Erwiniocin NA5 has shown bioactivity against different groups of bacteria including phytopathogenic and the clinical (bacterial) isolates. The aim of the study is to use this bacteriocin *in vivo*; benefits of the results would be to reduce the colossal losses of yield of fruits, vegetables and crops by using the effective bacteriocin preparation(s) and the producers showing/synthesizing possibly the ideal bacteriocins (against phytopathogens). Thus, an appreciable economic loss and budget incurred on import of synthetic pesticides (along with their far reaching health hazards) could be safeguarded. The use of bacteriocins has been acclaimed as one of the safest means to control the plant diseases of microbial origin

In general, bacterial diseases of plants are very difficult to control because of the lack of effective chemicals. Antibiotics could be used, but they are expensive and, in any case, the compounds that are valuable for human therapy are not allowed to be used in agriculture. The most effective alternative is the use of copper, which is potentially phytotoxic, polymorphic control with streptomycin has been associated with the emergence of streptomycin resistance in *Erwinia amylovora*, in addition this antibiotic cannot be used in many countries because of its use as human and animal medicine (Sobiczewski, 1997). For the nopaline inducing strains of *A. tumefaciens,* there is a highly effective biocontrol system, discovered and developed by Kerr (1972) in Australia and has been used in Australia since 1973 the first commercial biological control agent for any plant disease. It is now used world-wide, and

marketed by several companies under a range of trade names (e.g. "Galltrol"). Bacteriocin-producing, avirulent *E. carotovora subsp. carotovora* mutants (A-f-39 and B-e-19) were used as biological control agents against the pathogenic strains 2T-2 and TT-4, which cause soft rot of Chinese cabbage. A-f-39 and B-e-19, developed from 2T-2 and TT-4, respectively, inhibited the *invitro* growth of their respective parents. The biological control of these mutant strains was compared with strain M403, a mixture of A-f-39 and M403, and an agrochemical (basic dithianon-copper chloride). A-f-39 was comparable to M403 in terms of biological control efficacy, and was more efficient than the agro-chemical. The mixture of A-f-39 and M403 consistently gave the best results (Kyeremeh *et. al.*, 2000).

A part of the present studies was to develop a bacteriocin (Erwiniocin NA5) from an indigenously isolated phytopath bacterium against other phytopath bacteria which are fatal for our agricultural based economy, like *Xanthomonas oryzae* NA1 (*in vitro* as well as *in vivo*) the causative agent of bacterial leaf blight (BLB) in rice, and also to reduce the increase emergence of antibiotics and chemicals resistance in pathogens.

7.18.1. Experiments/ Trials under Controlled Conditions

For this, Seeds of different varieties/ entries were sown in plastic pots (4"X 4") and were kept in growth incubator room (temp. 30°C, humidity 80%, and light intensity 10000lux for 12 hrs. daily). At three leaves stage plants were inoculated using clip method. Scissors dipped in the inoculum were used to cut tip of the leaves and after 24 hrs of inoculation $1/6^{th}$ dilution of Erwiniocin NA5 of crude preparation (ammonium sulphate precipitated) was used. To compare the efficiency of

Erwiniocin NA5 with the commercially used/available bactericide (Copper oxychloride) was used in concentration of 3mg/mL (0.3%) and also served as positive control. Data were recorded after ten days of inoculation using the scale (presented in materials and methods) for BLB. Significant differences were found in disease scores among different varieties after treatment with Erwiniocin NA5 compared to – ve control (untreated). Some varieties did show reduction in disease score after bacteriocin/Erwiniocin NA5 application; for example reduction was observed in Bas-385, IRBB-62, Bas-370, Bas-super and Bas-2000 on the application of Erwioniocin NA5. While the copper oxychloride treated plants (+ve control) resulted almost incomplete inhibition but far reaching health hazards of such chemical need to be considered or such chemicals need to be replaced with biopesticides.

7.18.2. Field Experiments/ Field Trial Conditions

10 rice varieties were used in field experiments for monitoring the efficiency of Erwiniocin NA5. The first five entries/verities include the coarse type and the last five belonged to basmati type. Significant differences were found in disease score among some varieties after treatment with Erwiniocin NA5; while no significant difference was observed in some varities after the treatments (i.e no reduction in disease incidence and extent). Reduction was observed after treatment with bacteriocin (compared to the -ve control) in KSK-202, EF-1-20-6-10, EF-1-30-4-1, Bas-super, Bas-2000 and IR-6. The performance of Erwiniocin NA5 could be rated "satisfactory" as 70% rice entries showed reduction in disease manifestation reaction. Half of the concentration of partially purified Erwiniocin NA5 (ammonium sulphate

precipitated) was used. A total of 180mL of diluted toxin was used to spray on 60 plants (two replication per plants), 10 entries/verities per replication (with three replications) were used. It means three mL (per plant) diluted toxin was used. 80,000 plants are recommended per acre for optimum yield but usually 50,000 plants are grown by the farmers in our country. 150,000 mL (150 Litres) of half of the concentration i.e 75 Litre of Erwiniocin NA5 were required per acre which is definitely not feasible. But if the results of the growth room where $1/6^{th}$ dilution was found effective on relatively young plants) are analyzed then the requirement of the Erwiniocin is far low i.e only 1.5mL of Erwiniocin ($1/6^{th}$ dilution) is required to spray on one plant and the requirement of the bacteriocin will be 12.5 Litre per acre (which seems still so high). It is thus, recommended that more concentrated form of partially purified Erwiniocin NA5 should be used. After all, the use of the bacteriocin (as biocontrol agent) is better compared to the antibiotics or chemicals like streptomycin, cupric oxide, copper oxychloride and borex which are frequently used for the eradication of plant diseases in our country. Analysis of variance of the % of inhibition are significantly higher ($P<0.05$) in both (in field and in control room).

Sakthivel and Mew (1991) reported that treatment with nonpathogenic bacteriocin-producing strains of *Xanthomonas compestris* pv. *oryzae* reduces the incidence and severity of bacterial leaf streak in rice plants. *Xanthomonas axonopodis pv. glycines* 8ra produces a bacteriocin called glycinecinA, which specifically inhibits the growth of bacteria belonging to *Xanthomonas* species. GlycinecinA was effective against *X. vesicatoria* on red pepper and *X. oryzae pv. oryzae* on rice. The optimum

concentration of glycinecinA for the control in the greenhouse and in the field was 12800 AU/mL. In this study, the activity of glycinecinA on rice cv. Milyang 23 and red pepper cv. Nogkwang leaves continued for 7-8 days, during which the pathogen populations remained at low levels. Bacterial leaf spot of red pepper and bacterial leaf blight of rice were significantly reduced by the bacteriocin (glycinecinA) treatments. The control efficacy was as high as, or even higher than the chemical treatment of copper hydroxide. These results suggest that the bacteriocin (glycinecin A) is a potential control agent for bacterial diseases (Jeon *et al.*, 2001).

7.19. Questions about Possible Antibiotic Resistance and Chemical Toxicity

Reference to the role of antibiotics in animal agriculture and challenge to livestock and human health; there is movement within the livestock industry, as well as at the legislative level, to diminish the threat of antibiotic resistance among human pathogens. We have also learnt that there are many other sources of resistance, including antibiotic use in crops and organic pest control. It has been asked whether antibiotic-resistance genes could inadvertently be transferred from the plant cells to bacteria in the guts of animals and humans, making the bacteria resistant to antibiotics and thus rendering some antibiotics less useful for treating the bacterial diseases. This question has been the subject of extensive scientific and regulatory review around the world. It has been consistently concluded that antibiotic-resistance markers are unlikely to be transferred from the cells of plants to the bacteria. An additional conclusion is that even if the markers were transferred, they would be of

little relevance to human health and safety (U.S. Food and Drug Administration, 1994; European Commission, 1996). The use of antibiotic resistance markers has been internationally recognized and reviewed by the Organization for Economic Cooperation and Development, the United Nations World Health Organization, and the Food and Agricultural Organization; as well as regulatory agencies in various countries including Argentina, Canada, Japan, United States, and European countries. These agencies have repeatedly concluded that these genes have never been shown transferred from crops derived through biotechnology to the bacteria in nature.

Antibiotics are sprayed on high value crops such as apples to prevent bacterial diseases such as fire blight a devastating disease that can quickly burn and run though an orchard caused by the bacterium *Erwinia amylovra.* Antibiotics are one of growers' strongest tools against this disease. The two main antibiotics that are commercially applied are streptomycin and oxy-tetracycline. Through last decade Integrated Pest Management (IPM) is trying to reduce the chemical use for biological control (Reid, 2002). Biological control costitutes the manipulation of one organism to reduce the severity of crop damage caused by another. For plant diseases, this involves microorganisms such as bacteria, fungi and viruses. These antagonistic organisms control disease in a number of ways. Antibiosis, the production of protein antibiotics, is one mode of action. However, as we have seen in animal agriculture the continued exposure to a low dose of an antibiotic can lead to resistance in pathogens. Furthermore, antibiotic producing organisms can spread their genetic material through pathogen communities. Besides, the resistance to antibiotics/agro chemicals in pathogens, the cytotoxic effect of such agrochemicals are also

considerable for human health. Human lymphoid cells of LAZ-007 cell line, incubated with 10^{-4} to 10^{-6} molar of eight different organochlorine pesticides had dose related cytotoxicity, mitotic depression and cell cycle traverse inhibition (Sobti, 1983).

The overall findings reported in this study lead us to conclude that bacteriocin from *Erwinia carotovora* NA5 has the potential to control/ decrease the survival of the causative agent (*Xanthomonas oryzae* pv. *oryzae*) of BLB disease. Further studies on the application of this bacteriocin on plants are required to assess the number of treatments needed to obtain effective disease control and to ascertain the persistence of the bactericide (Bacteriocin- Erwiniocin NA5)

The present findings have served the purpose of prompting new interest in bacteriocins produced by plant pathogens that can be convincingly considered an alternative biocontrol system useful in reducing the hazards associated with the use of synthetic pesticides. Biological control of crown gall is a fascinating example of the intersection of microbiology and societal values. Valid formulations of Erwiniocin NA5 to ensure adequate effectiveness of the bactericide under natural environmental conditions should be pursued.

7.20. Need of Biopesticide use in Pakistan

According to UN Food & Agriculture Organization (FAO, 2005), Pakistan (one of the top paddy rice producers) exported 18 million metric ton of rice. The FAO corporate document repository reports (Country, Regional and International Organization

Reports, Agenda item No. 3, Country reports 4.1.13) that Pakistan has hot and dry climate with around 20 million hectares under field crops, orchards, groves and plantation. Insect pests diseases and weeds are the major constraint causing, leading to an average losses of around 20 percent annually. The major diseases include rusts, foliar spots, root and crown rots, leaf curl, leaf blight and bunchy top viruses, powdery mildew, and malformation etc. resulting in lowering of productivity and hence the economic returns. The economic condition of small and marginal farmers, having limited financial resources is particularly becoming acute. Our agriculture provides a major share of GDP and is a big foreign exchange source for Pakistan. Pesticide and other agro-chemical about 7300 tones (Bajwa, 1999) according to Country, Regional and International Organization Reports, Agenda item No. 3, Country reports 4.1.13 consumption has risen by 40 percent since 1987, yet the rapid increase in pesticide use has not solved the pest related problems and incomes of small cotton growers have fallen as they are spending more and more on pesticides. Thus, the need to formulate the biopestisides to overcome the obstacles to trade/economy is evident.

Bajwa (1999) reported in an agriculture tribune the net deficits in the total food grain requirements (demand over supply) in our country are expected to be about 1.3 million tonnes in 2005 which may swell to about 24 million tonnes by the year 2020. What has gone wrong with our agriculture and technology generation and its adoption that growth in food production is becoming unsustainable to meet our future requirements?

We have to generate and disseminate specific systems of management of crops, inputs and natural resources including bacteriocins as biopesticide for each agro-climatic region to sustain high productivity and ensure profit maximisation while at the same time conserve our rather fragile agro-ecosystem.

7. 21. Control of Phytopathogenic Bacteria by non Plant Associated Microbes

Some non-plant origin bacterial isolates may prove to be the ideal bacteriocinogenic antagonists for the phytopathogenic bacteria (Vernam and Evans, 2000). Keeping this in mind we checked the activity of Staphylococcin Bac 188 (produced by n indigenous clinical *Staphylococcus aureus* AB 188 (Iqbal *et al.*, 2001) against all the isolated phytopathogenic strains. Accordingly, all the isolates were found sensitive. Hence, Staphylococcin Bac 188 may also be regarded a bioactive agent against the phytopathogens for future strategies (Laukova and Marekova, 1993; Saeed *et al.*, 2006).

CONCLUSSION

8. *Conclusions*

- ✓ Eight strains namely *Xanthomonas oryzae* NA1, *Xanthomonas oryzae* NA2, *Xanthomonas citri* NA3, *Pseudomonas andropogonis* NA4, *Erwinia carotovora* NA5, *Agrobacterium radiobacter* NA6, *Agrobacterium radiobacter* NA7 *and Erwinia carotovora* NA8 were isolated from diseased fruits, vegetables and the soil near onion and pepper rhizosphere.

- ✓ The isolates were identified on the base of morphological, cultural and biochemical considerations. The identification was confirmed by using API 20 E and API 20 NE kits.

- ✓ All the isolates were screened for antibacterial activity against phytopathogenic bacteria whereby only three i.e. *Erwinia carotovora* NA5, *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobacter* NA7 were found to produce bacteriocin. Their bacteriocins are designated as Erwiniocin NA5, Agrocin NA6 and Agrocin NA7 respectively.

- ✓ *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobacter* NA7 have shown antagonistic activity against each other. *Agrobacterium radiobacter* NA6 *and Agrobacterium radiobacter* NA7 were also effective against *Agrobacterium tumefaciens* B6 the causative agent of crown gall.

- ✓ Erwiniocin NA5 (a mega bacteriocin) has exhibited broad range bactericidal activity.

- ✓ Bioactive component of Erwiniocin NA5 is sensitive to proteolytic enzyme i.e. proteinase K and protease which is suggestive of its protein nature.

- ✓ Erwiniocin N5 is thermostable and resistant to different pH values (range).

- ✓ The production of Erwiniocin NA5 starts in early logarithmic phase and continues till late stationary phase.

- ✓ Erwiniocin NA5 is dialyzable through dialysis membrane (the molecular mass of Erwiniocin NA5 was roughly estimated to be less than 12 kDa. This was further confirmed by SDS PAGE).

- ✓ The bacteriocin was precipitated (up to 80% saturation) by ammonium sulphate. The precipitate was found to contain 5.8mg/mL of protein. When partially purified bacteriocins was subjected to gel filtration (using sephadex G75 column) a major active peak of protein (having 1.1mg/mL of protein estimated by biuret method) with 2.5 fold purification was obtained.

- ✓ According to the amino acid composition (NCBI genomics server), the protein is estimated to contain 12.44% acidic and 13.57% basic amino acid components.

- ✓ The *Invivo* effect of Erwiniocin NA5 was monitored against *Xanthomonas oryzae oryzae (Xoo)* the causative agent of bacterial leaf blight (BLB) of rice and was found effective. Therefore bacteriocins like Erwiniocin NA5 should be preferred over antibiotics and synthetic chemicals.

- ✓ The Erwiniocin production property (by *Erwinia carotovora NA5*) has been found to be located on a plasmid of high molecular weight. This plasmid borne property seems to be transferable by *in vivo* gene manipulations but *invitro* this plasmid seems to be non conjugable.

- ✓ Some non plant associated bacteriocins have also shown antibacterial activity against all the isolated phytopathogens (responsible for a number of diseases in fruits, vegetables and crops).

- ✓ The over all findings reported in this study lead us to conclude that Erwiniocin NA5 produced by *Erwinia carotovora* NA5, can be a good candidate for the development of the biopesticide against the BLB (bacterial leaf blight) pathogen *Xanthomonas oryzae pv. oryzae* and can be considered as an alternative biocontrol agent and useful replacement to hazard associated use of chemicals, antibiotics and synthetic pesticides. Further, shake flask-based, Lab scale fermenter and pilot scale fermenter research based project(s) need to be developed as the findings out of the present project are significant and carry a tremendous potential and call for futuristic approaches in this direction.

EXPECTED BENEFITS

8. Expected benefits

Most important benefits of the results would be to reduce the colossal losses of yield of fruit, vegetables and crops by using the effective bacteriocin preparation(s) and the producers having the possible ideal bacteriocin (against phytopathogens). Thus, an appreciable economic loss and budget incurred on import of synthetic pesticides (and their far reaching health hazards) could be safeguarded. The use of bacteriocins has been acclaimed as one of the safest means to control the plant diseases of microbial origin. The production of Erwiniocin NA5 should be studied by shake flask, Lab and pilot scale fermenter technologies before mass scale production possibilities are explorable.

BIBLIOGRAPHY

10. Bibliography

Abee T. (1995). Pore-forming bacteriocins of gram positive bacteria and self protection mechanisms of producer organisms. FEMS Microbiol. Lett. 129: 1-9

Agarwal P. C., Mortensen C.N. and Mathur B. (1989a). Seed-born diseases and seed health testing of rice. Technical bulletin No. 3, Phytopathological paper No. 30. CAB Int. Mycological Inst. (CMI) Kew, Surrey, UK. pp. 58-59.

Ahmed S. and Rasool S. A. (2003). Isolation and biochemical characterization of mutacin VSM43 isolated from human oral *Streptococcus mutans* VSM43. Pak. J. Pharm. Sci. 16 (2): 43-50.

Ahmad S., Iqbal A. and Rasool S. A. (2004). Isolation and biochemical characterization of enterocin ESF100 produced by *Enterococcus faecalis* ESF100 isolated from a patient suffering from urinary tract infection. Pak. J. Bot. 36: 145-158.

Aislaibe J., Bej A.K., Hurst H., Rothenberger S. and Atlas R.M. (1990). .Microbial degradation of quinolones and methylquinolones. Appl. Environ. Microbial. 56:345-351.

Akther M.A., Zakria M. and Abbasi F.M. (2004). Trends in occurrence of bacterial leaf blight of rice in Pakistan. Pak. J. Phytopathol. 16(1): 69-71.

Alan V., Murillo J. and Jackson R.W. (2001). The roles of plasmids in phytopathogenic bacteria: mobile arsenals? Microbiology Reviews 147: 763-780.

Alconero, R. (1980). Crown gall of peaches from Maryland, South Carolina and Tennessee and problems with biological control. Plant Dis. 64: 835-838.

Amano T., Goabel W. F. and Smidth M. (1958). Colicin K III. The immunological properties of substances having colicine K activity. J. Exp. Med. 108: 731-752.

Anonymous (1987). CMI Distribution maps of plant diseases. 304(5): 23-25

Anonymous (1996). Standard Evaluation System for Rice. INGER Genetic Resource Center, IRRI, Manila, Philippines. 4th ed: 20-21

Anonymous (2004). Pakistan Economic Survey, 2003-4, Government of Pakistan.pp 101.

Bajwa M.S. and Chahal G.S. (1999). Precision farming — need of next century. Agriculture tribune in India.

Barras F., Gijsegem V. F. and Chatterjee A. K. (1994). Extracellular enzymes and pathogenesis of soft rot Erwinia. Ann. Rev. Phytopathol. 32: 201- 234.

Barrett M. S., Wenzel R. P. and Jones R. N. (1992). *In vitro* activity of mersacidin (M87-1551), an investigational peptide antibiotic tested against Gram-positive bloodstream isolates. Diag. Microbiol. Infect. Dis. 15: 641–644

Bhunia A. K., Johnson M. C. and Ray B. (1987). Direct detection of an antibacterial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate polyacrylamide gel electrophoresis. J. Ind. Microbiol. 02: 319-322.

Bhunia A. K, Johnson M. C. and Ray B. (1988). Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65: 261-268.

Bhunia A. K., Johnoson M.C. and Ray B. (1991). Mode of action of pediocin AcH from *pediococcus acidilatici* H on sensitive bacterial strains. J. Appl. Bacteriol. 65: 261-268.

Biagi-De C. M. R. and Azevedo J. L. (1992). Detection of bacteriocins produced by plant pathogenic bacteria from the genra Erwinia, Pseudomonas and Xanthomonas. Sci Agricol. Piracicaba 49(1): 1-8.

Biagi-De C. M. R. and Azevedo J. L. (1995). Elimination of bacteriocin production in phytopathogenic Erwinia and Pseudomonas. Scientia Agricol. 52(1): 429-435

Bishop A. L., Katz B. H. and Burr T J. (1988). Infection of grapevines by soilborne *Agrobacterium tumefaciens biovar 3* from population dynamics in host and nonhost rhizospheres. Phytopathology 78: 945–948 .

Bizani D., Motta S.A., Morrisy J.A.C., Terra R.M.S, Souto A.A. and Brandelli A. (2005). Antibacterial activity of cerein 8A, a bacteriocin like peptide produced by *Bacillus cereus*. Int. Microbiol. 8: 125-131.

Bordet J. and Beumer J. (1951). Antibiotiques colibacillaires et recepteurs appropries. Rev. Belg. Pathol Med. Exp. 21: 245-250.

Bridges B. A. (1975). Induction of streptomycin resistant mutations in *Escherichia.* Mutation Res. 19: 33-41

Brisbane P. G., and Rovira A.D. (1961). A comparison of methods classifying rhizosphere bacteria. J. Gen. Microbiol. 26: 776-781.

Brussiere J. L. (2003). Animal models as indicators of immunogenicity of therapeutic Proteins in humans. Dev Biol (Basel). 112: 135-139.

Burges H. D. and Hussey N. W. (1971). Microbial control of insect and mites. Academic press. New York. pp. 861.

Callewaert R. and Vust L.D. (2000). Bacteriocin production with *Lactobacillus amylovora* DCE471 is improved and stabilized by Fed-batch fermentation. J. Appl. Environ. Microbiol. 66(2): 606-613.

Chang-Long A.N., Lim W. J., Hong S. Y., Kim E. J., Shin E. C., Kim M K., Lee J.R., Park S. R., Woo J. G., Lim Y. P. and Yun H.D. (2004). Analysis of *bgl* operon structure and characterization of beta-glucosidase from *Pectobacterium carotovorum* subsp. *carotovorum* LY34. Biosci. Biotech. Biochem. 68(11): 2270-2278.

Chatterjee A.K. and Starr M.P. (1972). Genetic transfer of episomic elements among *Erwinia* opecies and other Enterobacteria: F'*lac*$^{+}$. J. Bacteriol. 111(1):169-176.

Chen, W. Y., and Echandi E. (1984). Effects of avirulent bacteriocin-producing strains of *Pseudomonas solanacearum* on the control of bacterial wilt of tobacco. Plant Pathol. (Oxford) 33:245-253.

Chen P., Novak J., Kirk M., Barnes S. Qi F. and Caufield P.W. (1998). Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. Appl Environ. Microbiol. 64: 2335–2340.

Chen H. and Hoover D. G. (2003). Bacteriocins and their food applications. Comprehen. Rev. Food Sci. Food Safety 2: 82-100.

Chrambach A. and Jovin T.M. (1984). Electrophoresis 4: 190-204

Chuang D.Y., (1997). M.A. Thesis Tohoku University, Sendai, Japan

Chuang D.Y., Kyeremeh A.G., Gunji Y., Takahara Y., Ehara Y., and kumoto.T. (1999). Identification cloning of an and *Erwinia carotovora* subsp. carotovora bacteriocin regulator gene by insertional mutagenesis. J. Bacteriol. 181:1953-1957.

Cintas L. M., Herranz C., Hernandez P. E., Casaus M. P and Nes I.F. (2001). Review: Bacteriocins of lactic acid bacteria. Food Science Technol. Internet,. 7(4): 281-305.

Cleveland J., Montville T. J., Nes I. F. and Chikindas M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. Interl. J. Food Microbiol. 71: 1-20.

CMI Distribution Maps of Plant Diseases. (1987). No. 304. ed.5; Fig. 14.1.

Corral G. M. R, Burr T.J., Matteson M.C., Smith C.A. and Takemoto J.Y. (1996). The use of *Pseudomonas syringae* 508 for Biocontrol of Apple Scab *(Venturia*

inaequalis). Poster abstract, Cornel community conference on biological control, April 11-13.

Crowly C.F. and DeBoer S. H. (1980). Sensitivity of some *Erwinia carotovora* serogroups to macromolecular Bacteriocins . Can . J. Microbiol. 26 (9):1023-1028

Crupper S. S. and Iandolo J. J. (1996). Purification and partial characterization of a novel antibacterial agent (Bac1829) produced by *Staphylococcus aureus* KSI1829. Appl. Environ. Microbiol. 62: 3171-3175.

Cupples D., Hanson R. and Kelmen A. (1975). Abstr. Ann. Meet. Am. Phytopathol. Soc. Aug. 10-14 USA, N.O.155.

Davies, J. (1990). What are antibiotics? Archaic functions for modern activities. Mol. Microbiol. 04: 1227-1232.

Daw M.A and Falkiner F.R. (1996). Bcteriocin: Nature, function and structure. Micron. 27(6):467-479.

De-Cleene, M. and De Ley, J. (1976). The host range of crown gall. Bot. Rev. 42:389-466

De-Courcy K. (2004). Column chromatography information manual. Fralin Biotechnology Center. Virginia Technology. pp. 5-17.

De-Ruyter P.G., Kuipers O.P and deVos W.M. (1996). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol.62:3662
–3667.

Diez-Gonzalez F. (2007). Use of Bacteriocins in Livestock. Research and application in bacteriocins. Horizon Biosciences. Chapter 7. ISBN-10: 1-904933-23-8 , ISBN-13: 978-1-904933-23-6.

Dykes G. A., (1995). Bacteriocins: ecological and evolutionary significance. Trends Ecol. Evol. 10: 186-189.

Echandi E. (1975). Biological control of bacterial canker of tomato with bacteriocins from *Corynebacterium michiganens*. Abstr. Ann. Meet. Am. Phytopathol. Soc., Aug. 10-14, p. 154.

Edmondson A. S. and Cooke E. M. (1979). The development and assessment of a bacteriocin typing method for *Klebsiella*. J. Hygn. 82: 207-223.

Engler G., Holsters M., van Montagu M., Schell J., Hernalsteens J.P. and Schilperoort R. (1975). Agrocin 84 sensitivity: A plasmid determined property of *Agrobacterium tumefaciens*. *Molec. Gen. Genet.* 138:345–349.

Ennahar S., Sashihara T., Sonomoto K. and Ishizaki A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Lett. 24: 85–106.

Ercolani G. L. and Caldarola M. (1972). *Pseudomonas ciccaronei* sp.n., agente di una maculatura fogliare del Carrubo in Puglia. *Phytopathologia Mediterranea.* 11:71-73.

European Commission. (1996). Opinion of the Scientific Committee on Plants on the genetically modified cotton line, insect-tolerant. http://europa.eu.int/comm/dg24/health/sc/scp/out18_en.html.

FAO corporate document repository. Report of the twenty-second session of the Asia and Pacific Plant Protection.Country, regional and international organization reports (Agenda item No. 3)

Fett W. F., Dunn M. F., Maher G. T. and Maleeff B. E. (1987). Bacteriocins and temperate phage of *Xanthomonas campestris* pv. glycines. Curr. Microbiol. 16:137-144.

Fredericq P. (1946a). Surla sensiblite et I' activite antibiotique dues Staphylocoques C. R. Scancse. Soc. Biol. Paris 140: 1167-1170.

Fredericq P. (1946b). Surla pluralite des recepteurs d' antibiose de Escherichia coli. Compt. Rend. 140: 1189 -1191.

Freifelder D. (1987). "Microbial Genetics" (Jones and Barlett Publishers, Inc. Boston). Pp. 1-87

Gabriel D. W. (1986). Specificity and gene function in plant-pathogen interactions. ASM News. 52:19–25.

Gabriel D. W., Kingsley M. T., Hunter J. E. and Gottwald T. (1989). Reinstatement of *Xanthomonas citri (ex Hasse) and X. phaseoli (ex Smith) to species and reclassification of all X. campestris pv. citri strains.* Int. J. Systemat. Bacteriol. 39:14–22.

Gallois, A., Samson R., Ageron E. and Grimont P. A. D. (1992). *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (*Cichorium intybus* L.). Int. J. Syst. Bacteriol. 42:582-588

Gardner E. J. and Snustad D. P. (1981). Principles of Genetics, 6th edition, John Wiley and Sons, New York pp. 1-121

Gelvin S.B. (1992). Chemical signaling between *Agrobacterium* and its plant host. *In*: Molecular signals in plant-microbe communications. Verma DPS (ed.). CRC Press, Boca Raton, Fla. pp. 137–167.

George N.A. (1997). Plant Pathology.Fourth Eddition. Academic Press, New York. pp.415-417.

Ghazala S. S. (2000). Plasmids of indigenous *Pseudomonads*: Characterization and gene manipulation. M.Phill thesis University of Karachi pp 1-190.

Goto M. (1965). A technique for detecting the infected area of bacterial leaf blight of rice caused by *X. oryzae* before symptom appearance. *Annals of the Phytopathological Society of Japan* 30:37-41.

Gratia A. (1925). Suran remargiatte example d' antagonisme en tredeux souches de colibacille. Comptes Rendus de la Societe de Biologie 93: 1040-1047.

Gratia A. and Fredericq P. (1946). La technique de la "double couche" de gelose et la distribution des germes producteurs de colicines dans les matieres fecales normales et pathologiques de l'homme et des animaux. Comptes Rendus de la Societe de Biologie 104: 1032-1038.

Gross, D.C. & Vidaver, A.K. (1990). Bacteriocins. In: *Methods* in Phytobacteriology (eds Klement, Z. Rudolph, K. & Sands, D.C), pp. 245 249. Budapest: Akademiai Kiado

Gurusiddaiah, S., Weller, D.M., Sarkar, A., and Cook, R.J. (1986). Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. *Antimicrobial Agents and Chemotherapy* 29:488–495. |

Haag W. L. and Vidaver A. R. (1974). Purification and characterization of syringacin 4-A, a bacteriocin from *Pseudomonas syringae* 4-A. Antimicrob. Agents Chemother. 6 (1): 76-83.

Hamon Y. and Peron Y. (1961). Les propriete antagonistes reciproques parmi les *Erwinia.* Discussion de la position taxonomique de ce genre. C. R. Acad. Sci. 253: 913-915.

Hamon Y. and Peron Y. (1962). Bacteriocin, possible txonimic elements for certain bacteria. C. R Hebd Seances Acad. Sci. 254: 2868-2870..

Hamon Y. and Peron Y. (1963). Quelques re marques sourles bacteriocines produites parles microbes grain positifs. C. R. Acad. Sci. Paris 257: 1191-1193.

Hanlin M. B., Kalchayanand N., Ray P. and Ray B. (1993). Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. J. Food. Prot. 56(03): 252-255.

Hardy K.G. (1975). Colicinogeny and related phenomena.Bacteriol. Rev.39:464- 515.

Hardy K. G. (1979). Bacteriocins. University of Kent at Canterbury, pp. 109-123.

Hardy K. G. (1982). Bacteriocins. In: Burns, R. G. and Slater, J. H. (eds.): Experimental Microbial Ecology, Chapter 21, Black well Scientific Publishers, Edinburgh, pp. 368-379.

Hardy K.G. (1987). Plasmids- A Practical Approach.IRL press.Oxford, England pp.42.

Hardy K.G. (1993). Plasmids: a practical approach. (IRL.Press, New York) pp 11.

Harris E.L.V. and Angal S. (1989). Protein Purification Methods: A Practical Approach. IRL Press, Oxford. pp. 210-290.

Hauben L., Moore E. R., Vauterin L. Steenackers M. Mergaert J., Verdonck L. and Swings J. (1998). Phylogenetic position of phytopathogens within the Enterobacteriaceae. Syst. Appl. Microbiol. 21: 384 -397.

Heu S., Oh J., Kang y., Ryu S., Cho S. K., Cho y. and Cho M. (2001). *gly* gene cloning and expression and purification of Glycinecin A, a bacteriocin produced by *Xanthomonas campestris* pv.glycines 8ra. Appl.Environ.Microbiol.67 (9):4105-4110.

Hirota Y. (1960). The effect of Acridine dyes on mating type factor in *E. coli.* Proc. Natl. Acad. Sci. USA. 46: 57- 64.

Holt J.G. Krieg N.R, Smith P.H., Staley J.T and Williams S.T. (1994). Facultatively anaerobic gram-ve rods. In: Bergey's Manual of Determinative Bacteriology. Group 5. 9th Edition. Williams & Wilkins, Baltimore, Maryland pp. 175-289.

Htay K. and Kerr A. (1974). Biological control of crown gall: Seed and root inoculation. J. Appl Bacteriol. 37:525-530

Iqbal A., Ahmed S., Ali S. A., and Rasool S. A. (1999). Isolation and partial characterization of Bac201: A plasmid-associated bacteriocin-like inhibitory substance from *S. aureus* AB201, J. Basic Microbiol, 39: 325-329.

Iqbal A., Ali S. A., Abbasi A., Volter W. and Rasool S. A. (2001). Production and some properties of Bac201: a bacteriocin-like inhibitory substance from *S. aureus* AB201. J. Basic Microbiol. 3:25-36-41.

Jabrane A., Sabri A. Compere P., Jacques P., Vandenberghe I., Beeumen J.V. and Thonart P. (2002). Chareterization of Serracin P, a phage- tail likebacteriocin, and its activity against Erwinia amylovora, the fire blight pathogen. Appl. Environ . Microbiol . 68(11): 5704-5710.

Jacob F., Lwoff A., Siminovitch A. and Wollman E. (1953). Definition de quelques termes relatifs a la lysagenie. Ann. Inst. Pasteur. 84: 222-224.

Jacob F. (1954). Biosynthese induit et mode d' action d' une pyocine, antibiotique de *Pseudomonas pyocyanea*. Ann. Inst. Pasteur. 86: 149-160.

James R., Lazdunski C. and Pattus F. (1991). Bacteriocins, microcins, and lantibiotics. NewYork : Springer-Verlag. pp. 519.

James L. S., Ravi S. and Hillman J.D. (2007). Lantibiotic Production by *Streptococcus mutans*: Their Uses in Replacement Therapy for the Prevention of Dental Caries and as Antibiotics for the Treatment of Various Infectious Diseases. In: Research and application in bacteriocins. Horizon Biosciences. Chapter 6. ISBN-10: 1-904933-23-8 , ISBN-13: 978-1-904933-23-6.

Jeon Y.H., Cho M. J., Cho Y.S. and Hwang I. (2001). Effect of glycinecinA on the control of bacterial leaf spot of red pepper and bacterial leaf blight of rice. J. Plant Pathology. 17(5): 249-256.

Jeremy D., Lowell A., Umayam., Tanja Dickinson., Erin K., Hickey and Owen w. (2001). The comprehensive microbial resource. Nucleic Acid Res. 29(1): 123-125.

Jimenez-Diaz R., Rios-Sanchez R. M., Desmazeaud M., Ruiz-Barba J. L. and Piard J. C. (1993). Plantacirins S and T, two bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. Appl. Environ. Microbiol. 59(05): 1416-1424.

Joerger R.D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. Poult Sci 82: 640-647.

Kado C.L. and Liu S.T. (1981). Rapid Procedure for Detection and isolation of large and small plasmids, *J. Bacteriol.* 145(3): 1365–1373.

Kado C. I. (1992). Plant Pathogenic Bacteria. In: A. Balows, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer (eds.) The Prokaryotes, Second Edition. Springer-Verlag, New York. Chapter 27, pp. 659-674.

Kado C. I. (1999). Plant pathogenic bacteria. In: Dworkin M., Falkow S., Rosenberg E., Schleifer K. H., and Stackebrandt E. (eds.), The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd ed., release 3.0, 29 May2004.Springer-Verlag,NewYork .http://link.springerm/link/service/books/10125/(2004)

Kado CI. (2002). Horizontal transmission of genes by *Agrobacterium* species .In syvanen M and Kado CI. Eds. Horizontal gene transfer 2nd edition, Academic press, London pp.1- 67

Kamimiya S., Izaki K. and Takashi H. (1977). Bacteriocin in *Erwinia aroideae* with tail like structure of bacteriophages. Agric. Biol. Chem. 41: 911-912

Kennedy, B.W. and Alcorn, S.M. (1980). Estimates of USA crop losses to prokaryotic plant pathogens. Plant Dis. 64:674-676.

Kerr A. (1972). Biological control of crown gall: Seed inoculation. J. Appl. Bacteriol. 35: 493-503.

Kerr A. and Htay K. (1974). Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. 4:37-43.

Kerr, A. (1980). Biological control of crown gall through produc- tion of agrocin 84. Plant Disease. 64: 25-33.

Khan J. A., Jamil F. F. and Gill M A. (2000). Screening of Rice varieties/lines against Bakanae and Bacterial Leaf Blight (BLB). Pak. J. Phytopathol. 12: 6-11.

Khan T.Z., Yasin S., Ayub M., Shah J.A. and Ahmad M.(2005). Effect of different chemicals and antibiotics on bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) of rice. Mycopath 3(1-2):57-59.

Kikumoto T., Ma S., and Takahara Y. (1993). Biological control of the soft rot disease of Chinese cabbage. 3. Interactions of avirulent and virulent strains of *Erwinia carotovora* subsp. *carotovora* on the petiole of Chinese cabbage, abstr. 195, p. 315-316. *In* Abstracts of the papers presented at the Annual Meeting of the Society, 1993. The Phytopathological Society of Japan, Nara, Japan. pp. 315-316.

Kikumoto T., Kyeremeh A. G., Chuang D. Y. and Gunji Y. (1997). Biological control of the soft rot disease of Chinese cabbage with avirulent mutant strains of *Erwinia carotovora* subsp. *carotovora*, p. 118-119. *In* A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino (ed.), Proceedings of the Fourth International Workshop on Plant Growth-Promoting Rhizobacteria. Japan-OECD Joint Workshop

Kim W. J. (1993). Bacteriocins of lactic acid bacteria: Their potential as food biopreservative. Food Rev. Int. 9(02): 299-313.

Kim W.S., Gardan L., Rhim S.L. and Geider K. (1999). *Erwinia pyrifoliae* sp. nov ., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). Int. J. Syst. Bacteriol. 49:899-906.

Kolter R. and Moreno F. (1992). Genetics of ribosomally synthesized peptide antibiotics. Ann. Rev. Microbiol. 46:141-163.

Konisky J. (1978). The Bacteriocins. In: "The Bacteria" (eds. L.N. Ornston and J.R. Sokatch). Academic Press, New York, 6:71-136

Kyeremeh A.G., Kikumoto T., Chuang D.Y., Gunji Y.C., Takahara Y. and Ehara Y. (2000). Biological control of soft rot of Chinese cabbage using single and mixed treatments of bacteriocin-producing avirulent mutants of Erwinia carotovora subsp. carotovora. J. Gen. Plant Pathol. 66(3):264-268.

Lacobellis N. S., Contesini A.M. and Surico G. (1995). Bacteriocin production by *Pseudomonas syringae* subsp. *savastanoi*. Phytopathol. Mediterr. 34:15-22.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227: 680-685.

Lagos R. (2007). Cytotoxic Activity of Bacteriocins Against Eukaryotic Cells. In: Research and application in bacteriocins. Horizon Biosciences. Chapter 5. ISBN-10: 1-904933-23-8 , ISBN-13: 978-1-904933-23-6

Lambert R. J. W. and Pearson, J. (2000). Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J. Appl. Microbiol. 88(5): 784-790.

Laukova A. (1992). The effect of culture medium on bacteriocin production in some bacterial strains. Veter. Med. (Praha). 37(12): 661-666.

Laukova A. and Marekova M. (1993). Antimicrobial spectrum of bacteriocin-like substances produced by staphylococci. Folia. Microbiol. 38: 74-76.

Lavermicocca P., Lonigro S.L., Caputo L., Evidente A. and Marciano C.E. (1996). Metaboliti antibatterici prodotti da *Pseudomonas syringae* pv. *Ciccaronei. Petria*, 6: 245 246.

Lavermicocca P., Lonigro S. L., Evidente A., and Andolfi A. (1999). Bacteriocin production by *Pseudomonas syringae* pv. *ciccaronei* NCPPB2355. Isolation and partial characterization of the antimicrobial compound. J. Appl. Microbiol. 86:257-265.

Lavermicocca P., Lonigro S.L., Valerio F., Evidente A. and Visconti A.(2002) Reduction of Olive Knot Disease by a Bacteriocin from *Pseudomonas syringae* pv.ciccaronei Appl Environ Microbiol, 68(3):1403-1407.

Lazo G.R. and Gabriel D.W. (1987). Conservation of plasmid DNA sequence and pathovar identification of strains *Xanthomonas campestris.* Phytopathol.. 58:1677-1681.

Lim D. (1998). Microbial genetics. In: Microbiology. 2nd edition (Sieveres,E.Ed.).W.C.B/McGraw-Hill, New York pp. 1-189

Lin E.C.C., Goldstein R. and Synanen M. (1984). Bacteria, Plasmids and Phages- An Introduction to Molecular Biology .Harvard University Press, Massachusetts, USA. pp. 1-79

Lotz, W. (1976). Defective bacteriophages, the phage tail-like particles. Progress in Molecular and Subcellular Biology 4: 53 -102.

Lysak V. V., Lobanok T. E. and Fomichev I. K. (1978). Bacteriocinogenic activity of strains of the genus *Erwinia.* Antibiotiki 23(6): 509-513.

Lysak V. V. (1980). Some properties of bacteriocins from Erwinia. Prikl Biokhim. Mikrobiol. 16(3): 372-376.

Lysak V. V. and Fomichev I. K. (1982). Induction of bacteriocin synthesis in Erwinia cells. Antibiotiki 27(3): 165-170.

Lysak V. V., Prokulevvich, V. A. and Fomichev I. K. (1988). Mapping of the locus encoding the bacteriocinogenicity trait in *Erwinia chrysanthemi ENA49.* Mol. Gen. Mikrobiol. Virusol. 5: 24- 27.

Magnusson J., Strom K., Roos S., Sjogren J. and Schnurer J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol. Lett.. 14:219(1):129-135.

Maniatis T., Fritsch E.F. and Sambrook J. (1989). Molecular cloning: A Labortary manual.Cold Spring Harbour,New York. pp. 1-232

Marder H. P. and Kayser F. H. (1977). Transferable plasmids mediating multiple antibiotic resistance in *Streptococcus faecalis. subsp. Liquifaciens.* Antimicrob. Agents Chemother. 12(2): 261-269.

Marekov M., Lukova A., DeVuystL., Skaugen N. and Nes I. F. (2003). Partial characterization of bacteriocins produced by environmental strain *E faecium* EK13. J. Appl. Microbiol. 94: 523-530

Mary-Harting A., Hedges A. J. and Berkely R. C. W. (1972). Methods for studying bacteriocins, In J. R. Norris, and D. W. Ribbons (ed.), Methods in microbiology, Academic Press, New York, N.Y. vol. 7A, pp. 315-422

McMillan G. P., Barrett, A. M. and Pérombelon, M. C. M. (1994). An isoelectric focusing study of the effect of methyl-esterified pectic substances on the production of extra cellular pectin isoenzymes by soft rot Erwinia spp. J. Appl. Bacteriol. 77: 175-185.

Moore L. W. (1975). Biological control of crown gall with an antagonistic Agrobacterium species. Abstr. Ann. Meet. Am. Phytopathol. Soc., Aug. 10-14. p.157.

Moore L.W. and Warren G. (1979). *Agrobacterium radiobacter* strain 84 and biological control of crown gall. *Ann. Rev. Phytopathol.* 17:163–179.

Morgan S., Ross R. P. and Hill C. (1996) Increasing starter cell lysis in cheddar cheese using a bacteriocin-producing adjunct. J. Dairy Sci. 80:1-10.

Muriana P. M. and Klaenhammer T. R. (1987). Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. 53(03): 533-560.

Muriana P.M and Klaenhammer T.R. (1991). Purification and partial characterization of lactacin F,a bacteriocin produced by *Lactobacillus acidophillus* 11088. Appl Environ. Microbiol.57(1): 114-121.

Muriana P.M. and Luchansky J.B. (1993). Biochemical methods for purification of bacteriocins. In Bacteriocins of Lactic Acid Bacteria (ed. Hoover, D.G. & Steenson, L.R) SanDiego Academic, Press pp.41-61.

Nakatani F. and Tsuyama H. (1973). Production of two kinds of antibacterial agents by isolates of *Erwinia carotovora*. J. Fac. Agric. Iwate Univ. 11:245-253.

Nes I. F., Diep D. B., Haverstein L. S., Brurberg M. B., Eijsnik V. and Holo H. (1996). Biosynthesis of bacteriocin in lactic acid bcteria. Antonie van Leeuwenhoek Int. J. Mol. Microbiol. 70:113-128

Nes I. F. and Holo H. (2000). Class II antimicrobial peptides from lactic acid bacteria. Biopolymers. 55: 50-61.

New P.B and Kerr A. (1972). Biological control of crown gall: field measurement and glass house experiments. J. Appl. Bacteriol. 35:279-286.

Nguyen H. A., Tomita T., Hirota M., Sato T. and Kamio Y. (1999). A simple purification method and morphology and component analyses for carotovorocin Er, a phage tail like bacteriocin from the plant pathogen *Erwinia carotovora Er.* Biosci. Biotechnol. Biochem. 63 (8): 1360- 1369.

Nguyen H.A, Tomita T., Hirota M., Kaneko J., Hayashi T. and Kamio Y. (2001). DNA Inversion in the Tail Fiber Gene Alters the Host Range Specificity of Carotovoricin Er, a Phage-Tail-Like Bacteriocin of Phytopathogenic *Erwinia carotovora* subsp. *carotovora* Er J. Bacteriol. 83 (21): 6274-6281.

Nicholas C. M., Ahmadi A. R. and Clare B. G. (1998). Construction of a Range of Derivatives of the Biological Control Strain *Agrobacterium rhizogenes* K84: a Study of Factors Involved in Biological Control of Crown Gall Disease. Applied andEnviron.Microbiol.64(10):3977-3982.

Novick R.P. (1990) Molecular Bilogy of the Staphylococci. VCH. Pub. Inc. New.York. pp.87.

O'Connor E.B., Ross R.P. and Hill C. (2007). Application of Bacteriocins in the Food Industry. Research and application in bacteriocins. Horizon Biosciences. Chapter 9. ISBN-10: 1-904933-23-8 , ISBN-13: 978-1-904933-23-6.

Ochiai K., Yamanaka T., Kimura K., and Sawada O. (1959). Studies on inheritance of drug resistance between *Shigella* and *Escherichia coli* strains. Nipponji Shimpo 18: 61-66.

Ogunbanwo S. T., Sanni A. I. and Onilude A. A. (2003a). Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. Afr. J. Biotechnol. 2(7): 179-184.

Oakey, L., Caroll, K, McClean, S., Kelleher, F., Costello, M. and Behan, J. (2003). Antimicrobial peptides - alternative to antibiotics? The Irish Scientist. 2(11): 85

Okezi H., Stockes B. A. D. and Smith S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. J. Gen. Microbiol. 28:671-676.

Old R.W. and Primrose S.B. (1985). Principles of Gene Manipulation: An Introduction to Genetic Engineering. 3rd Ed. Blackwell scientific publication Oxford. pp 123-125

Ou S. H. (1985). Rice diseases. CAB International Mycological Institute, Kew, Surrey, U.K.

Parrot M., Charest M. and Lavoie M. C. (1989). Production of inhibitory substances by *streptococcus mutans.* Can. J. Microbiol. 35: 366-372.

Parrot M., Caufield P. W. and Lavoie M. C. (1990). Preliminary characterization of four bacteriocins from *Streptococcus mutans*. Can. J. Microbiol. 35: 366-372.

Peng J. B., Yan W. M. and Bao X. Z. (1994). Plasmid and transposon transfer to *Thiobacillus ferrooxidans*. J.Bacteriol.176 (10): 2892-2897.

Perbal B. (1988). Basic methods in Molecular Biology. In: A practical Guide Line to Molecular Cloning. Second Edition. John and Wiley and Sons. Inc. pp. 11-35.

Perombelon M. C. M. (2002). Potato disease caused by soft rot Erwinias. An over view of pathogenesis. Plant pathol. 51:1-12

Pirzada, .Z.A. (2005) Bacteriocins from marine environment: screening, purification and molecular characterization. M.Phil. Thesis. Departement of Microbiology, University of Karachi. pp.1-193.

Pugsley A. P. and Oudega B. (1987). Methods for studying colicins and their plasmids. In: Plasmid: A practical Approch, Hardy, K.J. (eds.): IRL, Press, Oxford, Washington, D.C., pp.105-161.

Rasool S. A. and Akhtar M. (1992). Bacteriocinogenic strains among indigenous clinical *Pseudomonads.* Kar. Univ. J. Sci. 20 (1&2): 155-166.

Rasool S. A. (1992). "Bacteriocins: the protein antibiotics". (Fazleesons (Pvt) limited Karachi). pp. 1-92.

Rasool S.A., Ahmed B. and Iqbal A. (1993 a) Multiple drug resistance among indigenous clinical staphylococci. Proceedings of 13th Pakistan Congress of Zoology; Zoological Society of Pakistan.(A.Muzaffar and A.R.Shakoori Eds.) 13: 439-447.

Rasool S. A., Ahmed S. and Iqbal A. (1996). Streptococcins of indigenous hemolytic streptococci. Nat. Prod. Lett. 8: 67-74.

Rasool S. A. (2004). The science of DNA testing. RADS. 1(2): 8-9.

Reid J. (2002). Antibiotics in crops and biological control. Yates association. Cornell Cooperative Extension. pp.1-33

Riley A. (1998). Molecular mechanisms of bacteriocin evolution. Annu Rev Genet. 32:255-78.

Riley M.A. and Wertz J. E. (2002a). Bacteriocins: evolution, ecology and application. Ann. Rev. Microbiol. 56: 117-137.

Riley M.A. and Wertz J. E. (2002b). Bacteriocins diversity: ecological and evolutionary perspectives. Biochimei. 84: 357-364.

Riley M.A. and Gillor O. (2007). Research and application in bacteriocins. Horizon Biosciences. Chapter abstract. ISBN-10: 1-904933-23-8 ,ISBN-13: 978-1-904933-23-6.

Robert Jr. A. (1999). Nanomedicine, Volume I: Basic Capabilities, Landes Bioscience, Georgetown.pp.1-13

Rodriguez-Valera F., Jeuz G. and Kushne D. J. (1982). Halocins: the salt dependent bacteriocins produced by extremely halophilic rods. Can. J. Microbiol. 28: 151-154.

Rollema H.S, Kuipers O.P., Both P., de Vos W.M. and Siezen R.J. (1995). Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Appl. Environ. Microbiol. 61: 2873–2878

Russell J.B., Houlihan A.J., Rose J.E. and Angert E.R. (2005). Bacteriocins of gram-positive bacteria and their application in biotechnology. In: Recent Research Developments in Applied Biotechnology. Pandalai SG (ed). Kerala, India. Research Signpost. 2: pp. 87-101.

Ryder M. (1994). Key issue in the deliberate release of genetically manipulated bacteria. Bacterial Genetics and Ecology 15:139-146.

Saeed S., Rasool S. A., Ahmed S., Khanum T., Khan B. M., Abbasi A. and Ali S. A. (2006). New insights in staphylococcin research: bacteriocin and/or bacteriocin like inhibitory substance(s) produced by *S. aureus* AB188. World Journ of Microbiol & Biotech. 22: 713-722.

Sahl H.G., Bierbaum G. (1998). Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria. Ann. Rev. Microbiol. 52: 41-79.

Sakthivel N. and Mew T. W. (1991). Efficacy of bacteriocinogenic strains of *Xanthomonas oryzae* pv. *oryzae* on the incidence of bacterial blight disease of rice (*Oryza sativa* L.). Can. J. Microbiol. 37:764-768.

Salisbery V., Hedges R. W. and Datta N. (1972). Two modes of curing transmissible bacterial plasmid. J. Gen. Microbiol. 70: 443-452.

Saunder J.R. (1984). Genetics and evolution of antibiotic resistance. Br. Med. Bull. 40: 54-60.

Schillinger U. and Kaya M. and Lucke F. K. (1991). Behaviour of *Listeria monocytogenes* in meat and its control by bacteriocin producing strain of *lactobacillus sake*. J. Appl. Microbiol. 70: 473-478.

Schillinger U. and Lucke F.K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *J. Appl. Bacteriol.* 70:473-478.

Scott J. C., Sahl H.G., Carne A. and Tagg J.R. (1992). Lantibiotic-mediated anti-lactobacillus activity of a vaginal *Staphylococcus aureus* isolate. FEMS Microbiol Lett. 72:97–102.

Shaw J. J., Settles L. J. and Kado C. I. (1988). *Transposon Tn4431 mutagenesis of Xanthomonas campestris pv. campestris*: characterization of a nonpathogenic mutant and cloning of a locus for pathogenicity. Mol. Plant-Microbe Interact. 1: 39–45

Shukin N. N. and Avdienko I. D. (1980). Properties of bacteriocin of *Erwinia aroideae*. Zh. Mikrobiol. Epidemiol. Immunobiol. 2: 77-81.

Skytta E. and Mattila-Sandholm, T. (1991). A quantitative method for assessing bacteriocins and other food antimicrobials by automated turbidometry. J. Microbiol. Meth. 14: 77-88.

Slade M.B. and Tiffin A.I. (1984). Biochemical and Serological Characterization of *Erwinia*. Methods Microbiol. (15):227-293.

Smidt M. L. and A. K. Vidaver. (1986). Isolation and characterization of syringacin W-1, a bacteriocin produced by *Pseudomonas syringae* pv. syringae. Can. J. Microbiol. 32:231-236.

Sobiczewski P., Deckers T. and Pulawska J. (1997). Fire blight (*Eriwinia amylovora*): some aspects of epidemiology and control. Research Institute of Pomology and Floriculture, Skierniewice, Poland pp. 1-27

Sobti R. C., Krishan A. and Davies J. (1983). Cytokinetic and cytogenetic effect of agricultural chemicals on human lymphoid cells *invitro*. Archives of Toxicology 52(3): 221-231.

Ste´phane C., Brion D., Jerzy N., Christophe C and Essaid A.B. (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl. Environ. Microbiol. 71(9): 4951–4959.

Stonier T. (1960). *Agrobacterium tumifaciens* conn.11. Production of an antibiotic substance. J. Bacteriol. 79:889-895.

Takahara Y. (1994). Development of the microbial pesticide for soft- rot disease. PSJ Biocontrol Rep. 4:1-7. (Japanese).

Takala TM and Saris PE. (2002). A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene nisI. Appl Microbiol Biotechnol. 59: 467–471.

Tagg J. K., Dajani A. S. and Wannamaker L. W. (1976). Bacteriocins of Gram positive bacteria. Bacteriol. Rev. 40: 722-756.

Thomson, S. V., Hildebrand D.C. and Schroth M.N. (1981). Identification and nutritional differentiation of the *Erwinia* sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. Phytopathology 71: 1037-1042

Todorov S., Vaz-Velho M. and Dicks L.M.T. (2003). Isolation and partial characterization of Bacteriocins produced by four lactic acid bacteria isolated from

traditional South African beer. Elect. J. Environ. Agric. and Food Chemistry. http://ejeafche.uvigo.es/2(4)2003/022242003.pdf.ISSN 157-4377.

Torreblanca M., Meseguer I. and Rodriguez-Valera F. (1989). Halocin H6, a bacteriocin from *Haloferax gibbonsii.* J. Gen. Microbiol. 135: 2655-2661.

Toth .I.K., Bell K.S., Mria. C., Holeva C. and Birch P. R.J. (2003). Review: Soft rot Erwiniae: from genes to genomes. Mol. Plant Pathol. 4(1): 17-30.

Tovkach F. I., Grigoryan Y. A., Ruban V.I. and Kishko Y. G. (1984). Polylysogeny in *Erwinia carotovora* 268 R. Microbial. Zh. 46(3): 71-76.

Tovkach F. I. (1998). Biological Properties and Classification of *Erwinia carotovora* Bacteriocins. Mikrobiologiia 67(6): 636-642.

Tovkach F. I. (2001). Isolation and preliminary characterization of cryptic plasmids from *Erwinia carotovora.* Mikrobiologiia 70(6): 629-697.

U.S. Food and Drug Administration. (1994). Secondary Direct Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; Aminoglycoside 3'-phosphotransferase II. Federal Register 59:26700-26711.

U.S. Food and Drug Administration. (1998). Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants pp. 28. http://vm.cfsan.fda.gov/~dms/opa-armg.html.

Van Reenen C. A., Dicks L. M. T. and Chikindas M. L. (1998). Chikindas, M.L. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum.* J. Appl. Microbiol. 84:1131-1137.

Vernam A.H. and Evans M.C (2000): "Environmental Microbiology". Manson publication London. pp. 1-128

Vernam A. H. and Evans M. C. (2002). Environmental Microbiology. Manson publication. London. pp. 29-31.

Vescovo M., Torriani S., Orsi C., Macchiarolo F. and Scolari G. (1996). Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables J. Appl. Bacteriol. 81(2): 113-119.

Vidaver A. K., Mathys M. L., Thomas M. E. and Schuster M. L. (1972). Bacteriocins of phytopathogens *Pseudomonas syringae, Ps. glycinea and Ps. phaseolicola*. Can. J. Microbiol. 18: 705-713.

Vidaver A. K. (1976). Prospects for control of phytopathgenic bacteria by bacteriophages and bacteriocins. Ann. Rev. Phytopathol. 14: 451- 465.

Vidaver A.K. (1983). Bacteriocins: the lure and the reality. Plant Disease 67: 471 475.

Vuyst L. D. and Vandamme E. (1994). Bacteriocins of lactic acid bacteria, (Blackie, London, United Kingdom). pp. 539

Whipps J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487–511.

Whitford W. F., McPherson M. A., Foster R. J. and Teather R. M. (2001). Identification of bacteriocin-like inhibitors from rumen Streptococcus spp. and isolation and characterization of Bovicin 255. Appl. Environ. Microbiol. 67(2): 569-574.

Wilson M. and Backman P. A. (1999). Biological control of plant pathogens. In: J. R. Ruberson (ed.), Handbook of pest management. Marcel Dekker, Inc., New York, N.Y. pp. 309-335.

Woo J., Heu S. and Cho Y.S. (1998). Influence of growth conditions on the production of a bacteriocin, glycinecin, produced by *Xanthomonas campestris* pv. glycines 8ra. Korean J. Plant Pathol. 14:376-381.

Yasunaka K., and. Amako K. (1979). Morphology of bacteriocins. Protein Nucleic Acid Enzyme 24:719-726.

Young J.M, Kuykendall L.D, Martinez-Romero E., Kerr A. and Sawada H. (2001). A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of Agrobacterium Conn 1942 and Allorhizobium undicola de Lajudie et al. 1998 as new combinations*: Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. vitis*. Int. J. Syst. Evol. Microbiol. 51: 89–103.

Zhou C., Yang Y. and Jong A. (1990). Mini-Prep in Ten Minutes. Biotechiniques 08(02): 172-173.

Zhu J., Oger P.M., Schrammeijer B., Hooykaas P.J.J., Farrand S.K. and Winans S.C. (2000). The bases of crown gall tumorigenesis. J. Bacteriol. 182:3885–3895

Zink R.T., Engwall J.K., McEvoy J. L. and Chatterjee A. K. (1985). *rec A* is required in the induction of pectin lyase and carotovoracin in *Erwinia carotovora* subsp. Carotovora. J.Bacteriol. 164:390-396.

http://www.eppo.org/QUARANTINE/bacteria/Xanthomonas_oryzae/XANTORds.pdf.

APPENDICES

Appendices

Appendix A

Equipments

Autoclave	(Qualitron, Pakistan)
Balance (Electrical)	(Sartorius, Germany)
Centrifuge	(Heraceus, Germany)
Centrifuge (Refrigerated)	(Heraceus, Germany)
Colorimeter	(Erma INC, Japan)
Column (30 x1.5 cm)	(Amersham-Pharmacia Biotech, USA)
Filter paper (Whatmann)	(Japan)
Fridge	(Sanyo Japan)
High speed centrifuge	(Hittech, Germany)
Horizontal and, Vertical gel electrophorasis apparatus.	(Bio Red, USA)
Incubator	(Qualitron, UK)
Micropipette variable Justor (02uL to 20uL, 20Ul-200uL, 100uL-1000uL)	(TreffLab, Switzerland)
Micropipette variable Justor (02uL to 5000uL)	(Socorex, Switzerland)
Microscope	(Laboval-4, Germany)
Microwave oven	(Goldstar, Japan)
Millipore filters	(Millipore, USA)
pH meter	(EDT, U.K.)
Rotary	(Buchi,Germany)
Refrigerated incubator	(Uniquip, Japan)
Refrigerator	(Dawlance, Pakistan)
Shaking incubator	(Staurt, Scientific, UK)
Transilluminator	(UV Products, USA)

Shaking incubator	(Staurt, Scientific, UK)
Transilluminator	(UV Products, USA)
Universal indicator pH Strips	(Merck, Germany)
Vortex mixer	(IKA, Germany)
Water bath	(China)

Glassware

Beakers	(Pyrex, Japan)
Flasks	(Pyrex, Japan)
Measuring cylinders	(Pyrex, Japan)
Petri plates	(Germany)
Tubes (5, 10 mL)	(Pyrex, Japan)
Screw-capped tubes	(Pyrex, Japan)
Cuvettes	(Pyrex, Japan)

Media

Agar agar	(Oxoid, Germany)
Nutrient broth	(Oxoid, Germany)
Yeast extract	(Oxoid,Germany)
Peptone	(Oxoid, Germany)
Luria Basal Broth	(Oxoid, Germany)

Chemicals

Acetone	(Sigma, USA)
Acridine orange	(Sigma, USA)
Agarose	(Sigma, USA)
Ammonium sulphate	(Sigma, USA)
Barium chloride	(Merck, Germany)
Bromophenol blue	(Aldrich USA)
Butanol	(Sigma, USA)
Cadmium chloride	(Merck, Germany)
Calcium chloride	(Merck, Germany)

Calcium carbonate	(Merck, Germany)
Catalase	(Sigma, USA)
Cesium chloride	(Merck, Germany)
Copper sulfate	(Merck, Germany)
Chloroform	(Merck, Germany)
Disodium hydrogen phosphate (Na_2HPO_4)	(Merck, Germany)
Dipotassium hydrogen phosphate (K_2HPO_4)	(Merck, Germany)
Ethanol	(Merck, Germany)
Ethidium bromide	(Sigma, USA)
Ethylene diamine tetra acetic acid (EDTA)	(Sigma, USA)
Ferrous sulfate	(Merck, Germany)
Formaline	(Merck, Germany)
Gram staining reagents	(Merck, Germany)
Glucose	(Merck, Germany)
Glycerol	(Merck, Germany)
Hind III λ DNA digest	(Sigma, USA)
Hydrochloric acid	(Merck, Germany)
Lipase	(Sigma, USA)
Lactose	(Merck, Germany)
Magnesium sulfate	(Merck, Germany)
Maltose	(Merck, Germany)
Manganese chloride	(Merck, Germany)
Methanol	(Sigma, USA)
Mercaptoetanol	(BDH)
Nickel sulfate	(Merck, Germany)
Potassium chloride	(Merck, Germany)
potassium iodide	(Merck, Germany)
Propanol	(Sigma, USA)
Proteinase K	(Sigma, USA)
Protease	(Sigma, USA)
RNAse	(Fermantas, Germany)

Sephadex G-75	(superfine grade ICN, USA)
SDS	(Sigma, USA)
Silver nitrate	(Merck, Germany)
Sucrose	(Merck Germany)
Sodium chloride	(Merck, Germany)
Sodium Hydroxide (NaOH)	(Merck, Germany)
Sodium potassium tartarate	(Sigma, USA)
Sodium potassium gluconate	(Sigma, USA)
Zinc sulfate	(Merck, Germany)

Appendix B

Compositions of Media

Brain heart infusion (BHI) Agar

Calf brain infusion solids	12.5g/L
Beef heart infusion solids	5g/L
Protease peptone	10g/L
NaCl	5g/L
Dextrose	2g/L
Disodium phosphate	2.5g/L
Agar	20g/L

pH = 7.4

Nutrient Agar

Peptone	10.0 g/L
Sodium chloride	5.0 g/L
Yeast extract	3.0 g/L
Agar	08.0 g/L

pH = 7.0

Nutrient Broth

Peptone	10.0 g/L
Sodium chloride	5.0 g/L
Yeast extract	3.0 g/L

pH = 7.0

YPGA (yeast peptone glucose agar)

Yeast	5gm
Peptone	5gm
Glucose	10gm
Agar	8gm
Distilled water	1L

pH 6.5-7.0

Kings B Medium

Proteose peptne No3	20gm
Glycerol	10mL
K_2HPO_4	1gm
$MgSo_4$ 7 H_2O	1.5gm
Agar	15gm
Distilled water	1L

Levans Medium

Yeast extract	2gm
Peptone	5gm
NaCl	5gm
Sucrose	50gm
Agar	20gm
Distilled water	1L

Luria Basal Broth

Peptone	10.0 gm
Yeast extract	1.0gm
NaCl	8gms
Distilled water	1L
pH 7.0	

Luria Basal Agar

Peptone	10.0 gm
Yeast extract	1.0gm
NaCl	8gms
Distilled water	1L
Agar	
pH 7.0	

Yeast Extract Mannitol Agar (Medium 79)

Distilled water	900 mL
K_2HPO_4	0.5 gm
$MgSO_4.7H_2O$	0.2 gm
NaCl	0.1 gm
$CaCO_3$	3.0 gm
Yeast extract	1.0 gm
Agar agar	15 gm
Congo Red*	10.0 mL
Mannitol~	100.0 mL

pH 6.5-7.0

*Congo red (4%)

~ Mannitol (10%)

Added into medium after autoclaving

Composition of Buffers

Sodium Phosphate Buffer (50mM)

a) 0.2 M Sodium hydroxide.

b) 0.2 M Disodium hydrogen phosphate.

a) 0.2 M Sodium hydroxide

Molecular weight of NaOH	40
0.2 M NaOH	8.0 g/l

b) 0.2 M Disodium hydrogen phosphate

	141.96
Molecular weight of Na_2HPO_4	28.39 g/l

To obtain the buffer of pH 7.0, 60 ml of 0.2M NaOH is added to 100 mL of 0.2 M of Na_2HPO_4 and make up the volume upto 100ml using distilled water.