STUDIES ON PHOSPHOLIPIDS FROM ALAMETHICIN SENSITIVE AND RESISTANT MUTANTS OF *BACILLUS CEREUS*

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

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IN
DAIRYING
(ANIMAL BIOCHEMISTRY)

BY

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KARNAL - 132001 (HARYANA), INDIA

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To My Loving
Father & Grandmother

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This is to certify that the thesis entitled, **"STUDIES ON PHOSPHOLIPIDS FROM ALAMETHICIN SENSITIVE AND RESISTANT MUTANTS OF *BACILLUS CEREUS*"** submitted by **Mr. RONDA VENKATESWARLU** towards the partial fulfilment of the award of the degree of **Master of Veterinary Science in Dairying (Animal Biochemistry)** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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ABSTRACT

The present project entitled "Studies on Phospholipids from Alamethicin Sensitive and Resistant mutants of *Bacillus cereus*" was proposed to understand the mechanism of resistance by *Bacillus cereus* with the following objectives; selection of alamethicin sensitive strain and resistant mutant of *Bacillus cereus*, phospholipids profile and fatty acyl side chain analysis of alamethicin sensitive strain and resistant mutant and confirmation of phospholipids as target molecules using *in vitro* assay. IC_{50} for sensitive strain was 2.0 µg/ml. Selected resistant mutant was phenotypically stable after 10 successive subculturings showing IC_{50} of 9.75 µg/ml. Total cellular lipids were extracted and resolved by TLC and visualized with iodine, molybdate and ninhydrin. Phospholipids with ethanolamine as head group decreased in the resistant mutant by 8.3 % compared to sensitive strain. The amount of amino containing phospholipids in resistant mutant increased by 4.86 % compared with sensitive strain. Fatty acid chain analysis of total cellular lipid extract revealed the increase in the ratio of unsaturated to saturated fatty acids. The increase was from undetectable quantity in sensitive to 12.38 % in resistant mutant. There was increase in long chain fatty acids in resistant mutants and decrease in short chain fatty acids of sensitive strain of *B. cereus*. Decrease in colorimetric response in biomimetic biosensor assay using polydiacetylene-lipid extract vesicles of resistant mutant in comparison to sensitive strain confirmed phospholipid molecules as target for alamethicin action. This study provide first example of existence of alamethicin resistant mutants among sensitive population of *Bacillus cereus*.

सारांश

वर्तमान अध्ययन, बैसिलिस सिरियस के एलामेथीसीन संवेदी तथा रोधक की भिन्न उत्परिवर्तियों को चुनने के उदे्श्य से किया गया तथा बैसिलिस सिरियस के एलामेथीसीन संवेदी तथा रोधक भिन्न किस्मों के फास्फोलिपीड परिच्छेदिका तथा वसीय एसाइल चेन का आकलन तथा बाहय परीक्षणों द्वारा फासफोलिपीड को लक्ष्य की तरह पुष्टि की गई। एलामेथीसीन को झिल्ली छेद की उत्पति की प्रक्रिया के लिए केटाइनिक ए एम पी माडल के रूप में अध्ययन किया गया। संवेदी किस्म के लिए आई सी 50 2.0 माइक्रो ग्राम/मिलीलिटर तथा रोधक प्रजाति के लिए जो 10 उपसंर्वधन प्रक्रियाओं को दौरान स्थिर था, 9.75 माइक्रो ग्राम/मिलीलिटर पाया गया। कुल कोशिकीय वसा निकाली गई एवं टी एल सी के द्वारा वियोजित कर तथा आयोडिन, मालीबिडेट तथा नीनहाइड्रीन का प्रयोग कर परिणाम देखे गऐ। टी एल सी के परिणामों में रोधक में, संवेदी किस्मों के मुकाबले ईथानोल आमाईन फास्फोलीपिड में 8.3 प्रतिशत गिरावट दिखाई। एमीनो फास्फोलीपीड़ की मात्रा में रोधक किस्मों में 4.86 प्रतिशत की बढोतरी हुई। वसीय अम्ल श्रृखला आंकलन में कुल कोशिकीय वसा के निष्कर्ष में दर्शाया कि सवेंदी तथा रोधी के बीच असतृप्त एवं सतृप्त के बीच अनुपात में नग्नय से 12.38 प्रतिशत की वृद्धि हुई। रोधक वेरिंयट के पॉलीडाईएसीटाइलीन वसीय निचोड में बायामाइमिट्रीक सैंसर का प्रयोग कर, संवेदी वीरेयंट के मुकाबले कैलोरीमिट्रीक प्रतिक्रिया में गिरावट थी, जो एलामिथीसलन के फास्फोलीपीड के लक्ष्य की पुष्टि करती है। यह अध्ययन बैसिलिस सिरियस के संवेदी किस्मों को बीच एलामेथीसीन रोधक किस्मों की उपस्थिति का उदाहरण है तथा रोधक क्षमता को फास्फोलिपीड के अग्र समूह तथा फैटी एसाइलसाइड चैन को जोड़ता है।

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LIST OF ABBREVIATIONS

%	percentage
µg	microgram
µg/ml	microgram per milliliter
µM	micromolar
^{0}C	degrees Celsius
^{0}F	degrees Fahrenheit
ACP	amino containing phospholipid
AMP	antimicrobial peptide
AR	analytical reagent
CAMD	computer aided molecular design
cfu/ml	colony forming unit per milliliter
Cm	centimeter
CM	cytoplasmic membrane
CR	colorimetric response
DMPG	dimyristoyl phosphatidylglycerol
DOPG	dioleoyl phosphatidylglycerol
DPPG	dipalmitoyl phosphatidylglycerol
DSPE	distearoyl phosphatidylglycerol
FAMEs	fatty acid methyl esters
FDA	food and drug administration
GA	genetic algorithm
GLC	gas liquid chromatography
GRAS	generally regarded as safe
hr	hour
IC_{50}	Inhibitory concentration at fifty percent of growth
LPS	lipopolysaccharide
mg	milligram
min	minute
ml	milliliter

mm	millimeter
N	normality
ND	not detectable
NCDC	national collection of dairy cultures
OD	optical density
OM	outer membrane
PC	phosphatidylcholine
PDA	polydiacetylene
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PL	phospholipid
PMF	proton motive force
PS	periplasmic space
QSAR	quantitative structure activity relationship
R_f	retardation factor
SAR	structure activity relationship
TLC	thin layer chromatography
UV	ultra violet
V/V	volume by volume
WHO	world health organization

1. INTRODUCTION

Bacillus cereus is a gram-positive, motile, facultative anaerobic spore former that produces heat stable and heat labile toxins. These toxins produce two types of syndromes. A rapid-onset emetic syndrome characterized by nausea and vomiting associated with boiled rice kept at ambient temperature for prolonged period and a slow-onset diarrhoeal syndrome characterized by abdominal cramps and diarrhoea associated with meat, vegetable and dairy products (Hauge *et al*., 1955; Goepfert *et al.,* 1972). Emetic toxins are extremely resistant to heat (can survive 90 min at 126 °C) and diarrhoeal toxins are inactivated at 56 °C in 5 min. It is ubiquitously distributed in nature especially in soil, on vegetables, cooked and processed foods. It is undoubtedly the most important of the aerobic spore forming species found in milk (Ahmed *et al.,* 1983; Coghill and Juffs, 1979). *B. cereus* is one of the organisms responsible for the spoilage of pasteurized milk and cream referred to as "sweet curdling" (Overcast and Atmaram, 1974) and "bitty cream" (Billing and Cuthbert, 1958). Spore forming and psychrotrophic properties enable *B. cereus* to survive pasteurization as well as to grow in milk at refrigerated storage conditions (Coghill and Juffs, 1979). Christiansson *et al.* (1989) have shown that the incidence of toxigenic *B. cereus* is high in milk and cream and milk is a suitable medium for *B. cereus* to produce toxins even at 8 °C. There is a wide range of natural heat resistance of *B. cereus* spores. *Bacillus cereus* has been identified as the cause of 42% of food-borne disease outbreaks in canteens serving the German military (Kleer *et al*., 2000). *Bacillus cereus* was reported as the cause of 14 outbreaks and 691 cases of food borne illness in the USA during 1993–1997.

Food safety programmes are increasingly focusing on a farm-to-table approach including preservation of foods as an effective means of reducing food borne hazards. Now-a-days consumers are concerned about the synthetic chemicals used as preservatives in foods, and there is resulting trend towards less processed food. A solution to this dilemma may be the use of natural antimicrobial peptides (AMPs) produced by living organisms. The AMPs produced by bacteria are called as bacteriocins. By definition, bacteriocins are

proteins (proteinaceous complexes), which exhibit bactericidal effect against sensitive bacteria (Klaenhammer, 1993). There has been a resurgence of interest for research on bacteriocins in the last decade for application as natural food biopreservatives. Bacteriocins being AMPs are non-toxic because of their inactivation by human digestive tract proteases. In addition, no flavour or textural changes are associated with their use as biopreservatives in different food systems. Affirmation of GRAS (Generally Regarded as Safe) status by US Food and Drug Administration to “Nisin” (a bacteriocin produced from *Lactococcus lactis*) as direct human food ingredient supported the rise of interest in the study of bacteriocins and other AMPs for food biopreservation.

Many of the pathogenic bacteria are becoming resistant to existing antibiotics and antibiotic resistance issue has become a major public health concern. Vadyvaloo *et al.* (2002) reported *Listeria monocytogenes* resistant to a class IIa bacteriocin. Bernhard *et al.* (1978) reported *B. cereus* strains resistant to high levels (up to 1 mg/ml) of ampicillin, colistin, and polymyxin and demonstrated pBC16 plasmid that conferred tetracycline resistance.

The emergence and spread of spontaneous resistant mutants to antimicrobial peptides and bacteriocins threaten the safety of their use as food biopreservatives. Hence there is need to tackle this resistance to enable biopreservatives’ continuous use. This forces the application of antimicrobial peptides in combination (Hanlin *et al*., 1993) resulting in search of effective newer AMPs against resistant varieties. Isolation of antimicrobial peptide producing cultures against the resistant strains through screening of AMPs is time consuming and not necessarily sure of finding an appropriate antimicrobial peptide. Even an effective AMP producing strain is isolated; there are chances that the resistance may develop against effective AMPs over a period of time. On the other hand designing of AMPs using rationale approach such as computer aided molecular design (CAMD) is preferred these days. However designing of antimicrobial peptides needs structure activity relationship (SAR) information between target molecule and antimicrobial peptide.

Alamethicin, an AMP produced by fungus *Trichoderma viride*, is a cationic AMP which forms pore in the membrane of sensitive bacteria (Chen *et al*.,

2002) and is used as model antimicrobial peptide to understand various phenomenon related pore formation.

Hence AMPs as ‘food biopreservatives’ are new alternative to chemical preservatives with promising future as biotechnological tools. Slight variations in the structure of pre-existing antimicrobial peptides and bacteriocins using structure activity relationship (SAR) information, beyond doubt will broaden their potential as antimicrobial peptides to tackle the resistance issue. Therefore the project entitled “Studies on Phospholipids from Alamethicin Sensitive and Resistant mutants of *Bacillus cereus*” was proposed to understand the resistance mechanism by *Bacillus cereus* with the following objectives:

1. Selection of Alamethicin sensitive strain and resistant mutant of *Bacillus cereus*.
2. Phospholipids profile and fatty acyl side chain analysis of Alamethicin sensitive strain and resistant mutant of *Bacillus cereus.*
3. Confirmation of phospholipids as target molecules using *in vitro* assay.

Bacillus cereus is a gram-positive, spore forming, motile, facultative anaerobe that produces heat stable and heat labile toxins. These toxins produce two types of syndromes. A rapid-onset emetic syndrome characterized by nausea and vomiting associated with boiled rice kept at ambient temperature for prolonged period and a slow-onset diarrhoeal syndrome characterized by abdominal cramps and diarrhoea associated with meat, vegetable and dairy products (Hauge *et al.*, 1955; Goepfert *et al.,* 1972). Emetic toxins are extremely resistant to heat (can survive 90 min at 126 °C) and diarrhoeal toxins are inactivated at 56 °C in 5 min. It is ubiquitously distributed in nature especially in soil, on vegetables, cooked and processed foods. It is undoubtedly the most important of the aerobic spore forming species found in milk (Ahmed *et al.,* 1983; Coghill and Juffs, 1979). *B. cereus* is one of the organisms responsible for the spoilage of pasteurized milk and cream referred to as “sweet curdling” (Overcast and Atmaram, 1974) and “bitty cream” (Billing and Cuthbert, 1958). Spore forming and psychrotrophic properties enable *B. cereus* to survive pasteurization as well as to grow in milk at refrigerated storage conditions (Coghill and Juffs, 1979). Christiansson *et al.* (1989) have shown that the incidence of toxigenic *B. cereus* is high in milk and cream and milk is a suitable medium for *B. cereus* to produce toxins even at 8 °C. There is a wide range of natural heat resistance of *B. cereus* spores. *Bacillus cereus* has been identified as the cause of 42% of food borne disease outbreaks in canteens serving the German military (Kleer *et al.*, 2001). *Bacillus cereus* was reported as the cause of 14 outbreaks and 691 cases of food borne illness in the USA during 1993–1997.

Despite remarkable advances in food science and technology, food borne illness is a rising cause of morbidity in all countries. Furthermore, food borne illness is a major cause of preventable death and economic burden in most countries. Unfortunately, most countries have limited data on food borne diseases and its impact on public health. It is only recently that the burden of food contamination and food borne disease have been systematically assessed and quantified. The economic cost associated with food borne diseases caused by microorganisms has only recently been estimated. In the US, the costs of

human illness due to seven specific pathogens have been estimated to range between US $6.5 billion to $34.9 billion (Buzby *et al.,* 1997). The medical costs and value of lives lost from five food borne infections in England and Wales were estimated at UK £300-700 million annually (Robert, 1996). The cost of an estimated 11,500 cases of food poisoning per day in Australia was calculated at AUD $2.6 billion annually (Australia New Zealand Food Authority, 1999).

Apart from causing food borne illness, *B. cereus* is now recognized as an infrequent cause of non-gastrointestinal infections in drug addicts, immunosuppressed, neonates and postsurgical patients. Ocular infections are the commonest type of severe infections (David *et al.*, 1994) including endophthalmitis, panophthalmitis and keratitis. It also causes septicemia, meningitis (Gaur *et al.*, 2001) and endocarditis (Steen *et al.,* 1992). Recently Latsios *et al*. (2003) reported a case of liver abscess caused by *B. cereus* that resulted in abscess rupture and acute peritonitis.

Many of the pathogenic bacteria are becoming resistant to existing antibiotics and antibiotic resistance issue has become a major public health concern. Nisin a bacteriocin produced by *Lactococcus lactis* was approved by FDA for biopreservation of foods. Vadyvaloo *et al.* (2002) reported *Listeria monocytogenes* resistant to a class IIa bacteriocin. Bernhard *et al.* (1978) reported *B. cereus* strains resistant to high levels (up to 1 mg/ml) of ampicillin, colistin, and polymyxin and demonstrated pBC16 plasmid that conferred tetracycline resistance.

2.1 RESISTANCE TO CHEMICAL ANTIBIOTICS: AN UNSOLVED AND GROWING PROBLEM

During 70 years of the antibiotic era in the treatment of human infectious diseases, pathogenic bacteria have developed relentlessly with clinically significant resistances to one class of antibiotic after the other (Levy, 1997). The major antibiotics used till date had four main targets namely 1) cell wall biosynthesis, 2) protein biosynthesis, 3) DNA replication and repair and 4) folate biosynthesis. The mechanisms by which resistance has developed to all these classes are mainly through inactivation of the antibiotic, efflux of the antibiotic and modification of the susceptible molecular target. These properties are achieved through mutation in the gene or acquiring the gene of resistance (Walsh, 2003).

It is widely accepted among the clinicians, medical researchers, microbiologists, and pharmacologists, that antibiotic resistance will, in the very near future, leave healthcare professionals without effective therapies for bacterial infections. Apart from the bacterial infections, food poisoning has also become a core issue due to increased incidence. Many of the food poisoning bacterial isolates of *Bacillus cereus* are also showing resistance to antibiotics (Bernhard *et al.*, 1978).

2.2 AMPs: A NATURL ALTERNATIVE TO CHEMICAL ANTIBIOTICS AND POTENTIAL TO COMBAT RESISTANTCE

It is the fate of all antibacterials to be fought off by the pathogens they target. The continuous and indiscriminate use of antibiotics has resulted in multi-resistant bacterial strains all over the world. The fact that process is accelerating has been alarming the public health officials (Powledge, 2004). The alarming emergence of resistance among bacteria has led the World Health Organization (WHO) to announce antimicrobial resistance as a main concern (WHO, 1995). For the food microbiologist, there is no doubt that it is necessary to avoid the distribution of bacteria with mobilizable antibiotic resistance. There is a lack of efficient and safe preservatives in the food industry. Over the years, massive use of common food safety barriers led to the development of resistance by various food microorganisms (Lin *et al.*, 1991). Enteric bacteria are especially tolerant to adverse environmental conditions such as low pH and high salt concentrations, which limits efficiency of some preservation methods. Consumers demand for natural, preservative-free and minimally processed foods and worldwide concern regarding disease outbreaks caused by food-related pathogens have created a need for development of new classes of antimicrobial agents. The priority for the next decades should be focused in the development of alternative drugs and/or the recovery of natural molecules that would allow the consistent and proper control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens and easy to produce. The discovery of two classes of antimicrobial peptides, non-ribosomally synthesized (Hancock and Chapple, 1999) and ribosomally synthesized peptides, of wider distribution (Boman, 1995; Broekaert *et al.*, 1997; Ezekowitz and Hoffmann, 2003; Hancock and Lehrer, 1998; Hoffmann *et al.*, 1999; Zasloff, 2002) present

in bacteria, insects, amphibians, crustaceans and other eukaryotes and plants - provided a new therapeutic strategy to fight micro organisms. These are termed 'natural antibiotics', because they are active against a wide spectrum of microorganisms, including bacteria, filamentous fungi, protozoan and metazoan parasites (Liu *et al*., 2000; Vizioli and Salzet, 2003). AMPs are often produced as closely related multi-member families that may vary only in a few amino acid residues. Despite the enormous variety of sequences and structures, AMPs possess certain common features (Andreu and Rivas, 1998; Boman, 1995; Hancock and Lehrer, 1998). They are usually made of less than 50 amino acids, bear a net positive charge due to an excess of basic (often lysine and/or arginine) over acidic residues, and contain about 50% hydrophobic amino acids. They often fold into 3-dimensional amphipathic structures stabilized by cysteine disulphide bridges. Linear peptides lacking cysteines tend to fold, only upon contact with membranes, into a variety of amphipathic helices, pleated-sheets, loops, or less defined extended structures in which positively charged hydrophilic domains are well delineated from hydrophobic domains. These features seem to be the main factors affecting their known and diverse biological activities.

Alamethicin, a peptide antibiotic produced by the fungus *Trichoderma viride* is a small, 20-amino acid peptide that forms a voltage dependent ion channel in lipid bilayers (North *et al*., 1994). Because of its strong voltage dependence and elaborate channel behavior (Hall *et al*., 1984), it has been of interest as a model for voltage-dependent gating, and it has served as a model for peptide-membrane interactions (Cafiso, 1994). Figure 2.1 is the crystal structure of Alamethicin. The peptide is a-helical in its N-terminal domain but has some 3_{10} character toward its C-terminus. This helix is laterally amphipathic and is believed to form a conductive aggregate. A number of models have been proposed for the gating of alamethicin, and these are discussed in detail in recent reviews (Cafiso, 1994; Sansom, 1993; Wooley and Wallace, 1992). In the absence of a gating voltage, some models place the alamethicin helix at the membrane interface, but most models place the peptide across the lipid bilayer.

Table 2.1 Different antimicrobial peptides produced from plants, insects, amphibians, animals and humans (Zasloff *et al.*, 2002).

AMPs	ORIGIN
Nisin, Leucocin A, Mundticin KS, Curvacin, Bavaricin A, Mesenterocin, Pediocin PA - 1	Bacteria
Alamethicin	Fungus
Magainin, Pexiganan, Ranalexin	Amphibians
Cercopin A, Thanatin, Tachyplasin, Drosomycin, Apidaecin, Pyrrhocoricin	Insects
Dermaseptin -1, Bactenecin, Protegrin – 1, Defensins, Hepicidin, Indolicidin, Histatin 5	Mammals
Thionin, Crambin	Plants

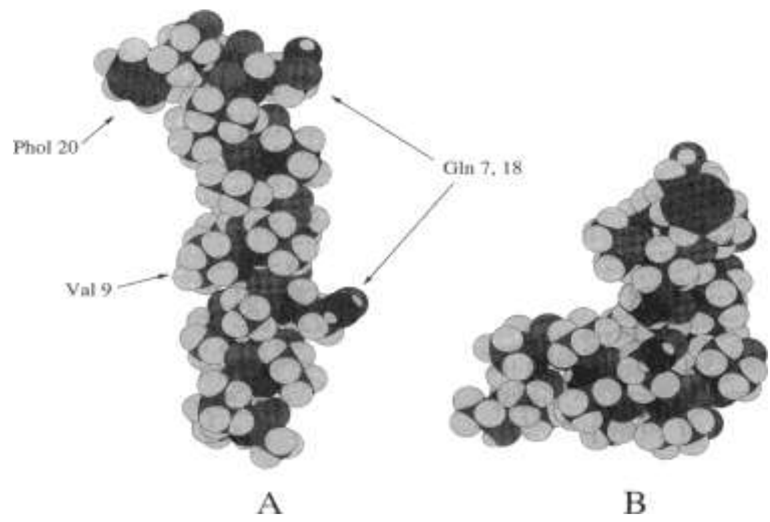


Fig 2.1 The crystal (Fox *et al.*, 1982) (A) and time-averaged solution (North *et al.*, 1994) (B) structures for Alamethicin. The sequence of Alamethicin is *Ac*-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phl.

AMPs produced by bacteria are known as bacteriocins. Tagg *et al.* (1976) defined bacteriocins as bactericidal proteinaceous compounds that kill closely related bacteria. Klaenhammer (1993) observed that bacteriocins might take many forms and elicit bactericidal activity beyond species that are closely related to or confined within the same ecological niche. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins that are quite different from the classical peptide antibiotics, which are made through

enzymatic condensation of free amino acids. Because of their proteinaceous nature, bacteriocins are synthesized via common ribosomal biosynthesis mechanism involving transcription and translation. Bacteriocins kill their target by causing dissipation of Proton Motive Force (PMF) and leakage of small intracellular substances through pore formation in the cell membrane of sensitive bacteria. There has been a resurgence of interest for research on bacteriocins in the last decade for application as natural biopreservatives. Bacteriocins are non-toxic because of their inactivation by human digestive tract proteases. In addition, no flavour or textural changes are associated with their use as biopreservatives in different food systems. Affirmation of GRAS (Generally Regarded as Safe) status by US Food and Drug Administration to “Nisin” (a bacteriocin produced from *Lactococcus lactis*) as direct human food ingredient supported the rise of interest in the study of bacteriocins for food biopreservation. Recently Hidalgo (2007) showed Enterocin AS-48, a bacteriocin from *Enterococcus feacalis* AS-48 is very stable with respect to pH and temperature and has a broad spectrum of action against numerous bacteria, including pathogenic bacteria transmitted by food, "which makes it a suitable molecule to use as a biopreservative."

2.3 MECHANISM OF ACTION OF ANTIMICROBIAL PEPTIDES

The mechanism of action of AMPs seems to involve multiple targets. The most cited target is the plasma membrane while more recent studies suggest intracellular targets at least for some peptides (Brogden, 2005; Zasloff, 2002).

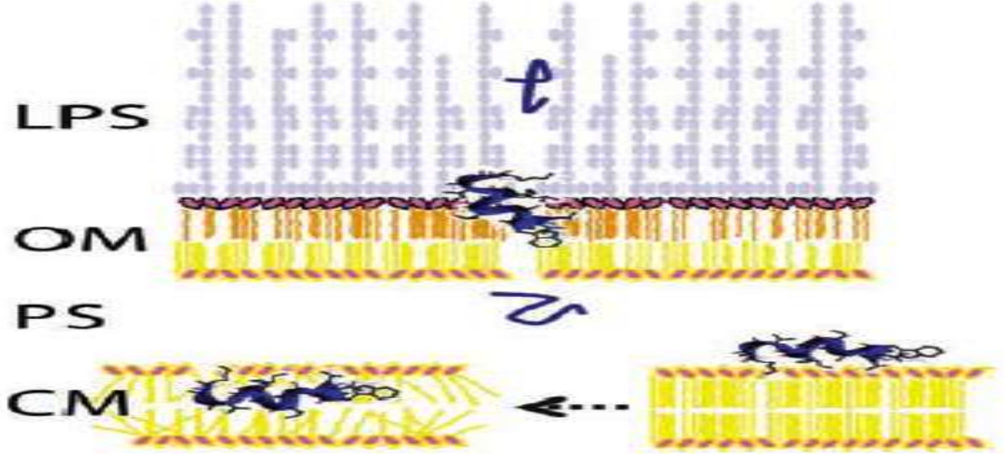


Fig 2.2 Proposed antibacterial mechanism of action of linear AMPs

Whether representing a final or intermediate step in the mechanism of action, it is clear that the interaction of AMPs with the plasma membrane plays an important role in their biological activity.

Unfolded cationic peptides associate with the negatively charged surface of the outer membrane (OM) and neutralize the charge over a patch of the membrane or competitively displace divalent cations from their binding sites on lipoplysacharides (LPS) creating cracks through which peptides can cross the outer membrane. In the periplasmic space (PS), the peptides adhere to negatively charged phospholipids in the cytoplasmic membrane (CM), which induces their amphipathic fold. Insertion of multiple monomers within the membrane lipid core ultimately leads to disruption of the membrane structure and function.

Several models were proposed to understand the action of cationic AMPs and bacteriocins, which forms the pore in cell membrane of the target organism. AMPs act in a multi-step process of binding, insertion, and formation of pore, leading to dissipation of proton motive force (PMF) and leakage of intracellular substances resulting in cell death. Those models include barrel-stave model, carpet and toroidal models.

2.3.1 Barrel – Stave model

Nisin and class IIa bacteriocins were shown to exhibit barrel stave model for their action (Ojcius and Young, 1991; Sahl, 1991). Alamethicin form barrel stave aggregates with broad probability of distribution of size (number of peptides in aggregate). This distribution has been shown to depend on characteristics of lipid bilayer (Cantor, 2002).

According to the barrel stave model (Fig 2.3), peptides bound to membrane recognize each other and oligomerize, and the oligomer inserts into the hydrophobic core of the membrane, forming a transmembrane pore. Upon oligomerization, antimicrobial peptides orient themselves, allowing the hydrophobic surface to interact with the hydrophobic core of the membrane and the hydrophilic surface to point inward to create a hydrophilic pore.

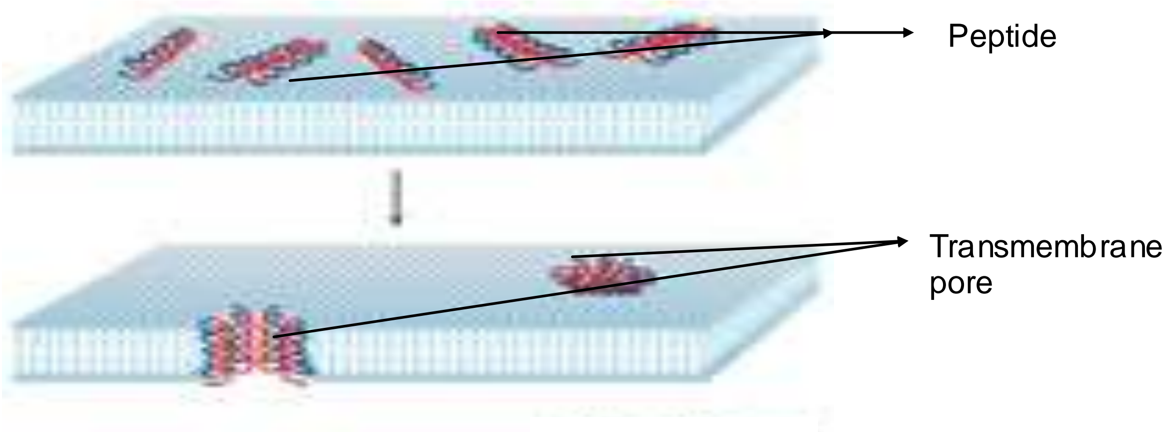


Fig 2.3 Barrel stave model for AMPs action

2.3.2 Toroidal model

In the toroidal model (Fig 2.4), peptides similarly bind and interact with lipid head groups, imposing a positive curvature strain on membranes (e.g., magainin 2) and producing channels where the polar head group region expands to form ‘‘toroidal’’ pores (Matsuzaki *et al.*, 1996)

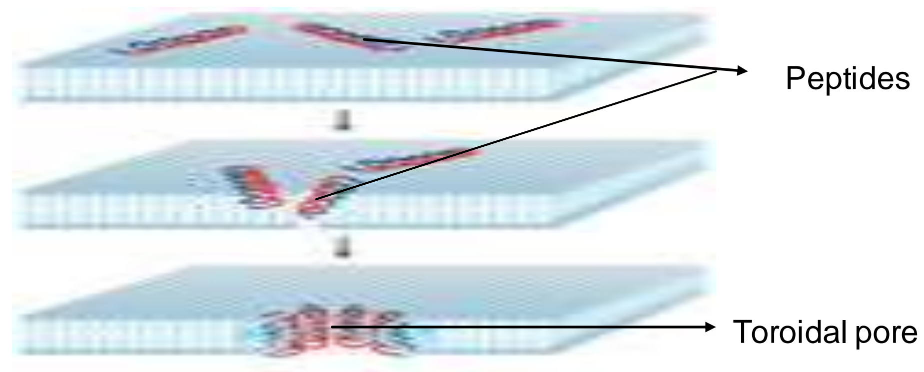


Fig 2.4 Toroidal model for AMPs action

2.3.3 Carpet model

The carpet model (Fig 2.5), suggests that antimicrobial peptides initially bind to and cover the surface of the target membrane. The electrostatic interaction between the peptide and the lipid head group imposes strain in the

membrane, and membrane permeation is induced only at sites where local peptide concentration is higher than certain threshold values (Shai, 2002).

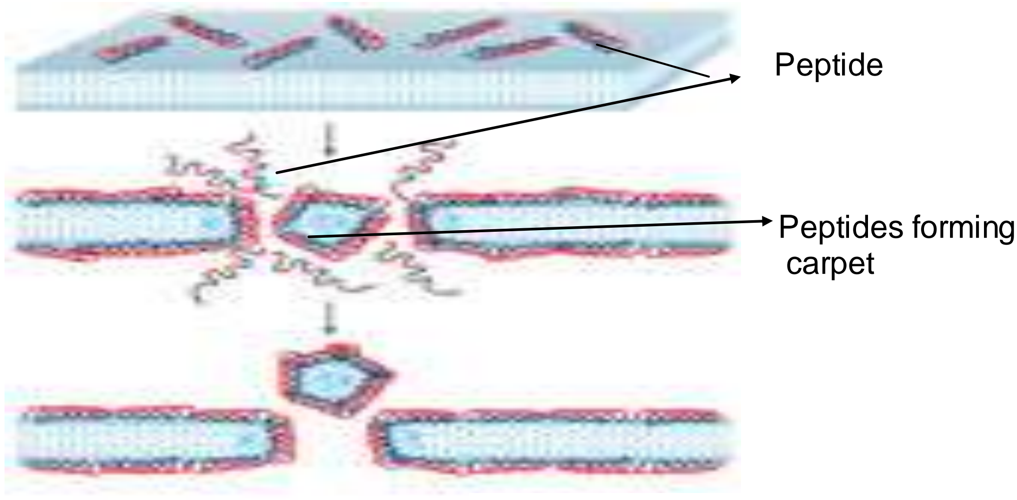


Fig 2.5 Carpet model for AMPs action

2.4 RESISTANCE TO AMPs INCLUDING BACTERIOCINS

Most AMPs exert their bactericidal activity through pore formation in the bacterial membrane, which occurs through four-step process of binding, insertion, aggregation, and formation of pore. The sensitivity of cells to AMPs is therefore thought to be influenced by the membrane lipid composition, which might act on any of the four steps (Mazzotta *et al.*, 1997). Earlier a model of pore formation by AMPs containing YGNGV motif explicitly stated that fatty acyl side chains of the membrane phospholipids govern the specificity of inhibition (Shiv Kumar, 2000; Sood and Sinha, 2003).

Indeed it is the fate of all antimicrobials to be eventually fought off by the organisms they target, as shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997). It has been reported that food poisoning organisms like *Staphylococcus aureus* and *Listeria monocytogenes* are developing resistance to antimicrobial peptide, Nisin (Ming and Daeschel, 1995; Gravesen *et al.*, 2001). It also got resistant to platelet microbicidal protein, a small cationic

peptide that possesses potent microbicidal activities against common blood stream pathogens (Bayer *et al*., 2000). Hanlin et *al.* (1993) and Harris *et al*. (1992) reported the existence of spontaneous sub-populations of variants of *Listeria monocytogenes* resistant to Nisin and Pediocin AcH. Development of spontaneous *Listeria monocytogenes* resistant mutants to class IIa bacteriocins such as Mesenterocin has been reported (Rekhif *et al*., 1994). *Listeria monocytogenes* resistant mutants to bacteriocin Leucocin A has been reported by Vadyvaloo *et al*. (2002). Sakayori *et al*. (2003) reported development of resistance to mundticin KS by *Enterococcus faecium*.

The investigation of the changes in the membrane phospholipids in the bacteria during the process of gaining resistance to AMPs is supported by various reports. Li *et al.* (2002) reported that temperature and surfactant induced membrane modification in *Listeria monocytogenes* by changing its membrane lipid composition and thereby affected its sensitivity to Nisin. Using GLC for the analysis of fatty acids, they showed that the cold adapted cells had increased amount of shorter branched chain fatty acids than normal cells and were more sensitive to Nisin. Shorter chains increased membrane fluidity thereby making the membrane more sensitive to Nisin. The amount of straight chain fatty acids of different chain lengths increased in presence of Tween 20, and such cells were also found to be more sensitive to Nisin. Limonet *et al.* (2002) reported variations in fatty acid composition of mutants of *Leuconostoc* and *Weissela* strains resistant to the class IIA bacteriocin mesenterocin 52A. They showed that resistant strains contained more saturated fatty acids when cultured with Mesenterocin 52A. Sakayori *et al.* (2003) separated and identified individual lipid classes of Mundticin KS resistant mutants of *Enterococcus faecium* on TLC and used GLC for fatty acid estimation. The amount of unsaturated fatty acids was found to be lower, possibly saturated fatty acids change the physical properties of the membrane making it more rigid. Also the putative zwitterionic amino-containing phospholipids (ACP) in such mutants increased whereas amounts of phosphatidylglycerol and cardiolipin decreased as compared to wild type strains. An increased level of ACP might inhibit binding of bacteriocin by positively charging the membrane. In contrast to these findings Vadyvaloo *et al.* (2002) reported that *Listeria monocytogenes* strains

resistant to class IIa bacteriocins showed increased levels of desaturated phosphatidyl glycerols. Chen *et al,* (1996) also reported that the saturated state of the phosphatidylglycerol acyl chains in vesicles had little effect on the binding affinity of pediocin PA-1 for the vesicles. However, their fluorescence results showed that the penetration of the bacteriocin into a bilayer of the saturated dimyristoyl-phosphatidylglycerol was deeper than into a bilayer of unsaturated dioleyl-phosphatidylglycerol (DOPG). The DOPG is therefore thought to be less favorable for efficient membrane permeabilization due to its higher fluidity. Bayer *et al.* (2000) reported elevated levels of longer chain, unsaturated membrane lipids in *Staphylococcus aureus* resistant mutants in presence of thrombin induced platelet microbicidal protein (t-PMP1), a small cationic innate immunity peptide. Younsi *et al*. (2000) reported increased unsaturated to saturated fatty acids ratio in Amphotericin B resistant mutants of *Kluyveromyces lactis*. Mazzotta *et al*. (1997) also reported more rigid nature of the membrane in Nisin resistant *Listeria monocytogenes* strains.

2.5 STRATEGIES TO COMBAT AMP RESISTANCE

The world of antibiotic drug discovery and development is riddled with and driven by the necessity to overcome antibiotic resistance in pathogenic microbes. Despite the success to date in antimicrobial development, the inexorable, ongoing emergence of resistance worldwide continues to spur the search for novel antimicrobial agents to replace and/or supplement conventional antibiotics. Some methods, which effectively help to tackle the resistance issues, are combinatorial use of AMPs. Hanlin *et al*. (1993) tested antibacterial efficiency of two bacteriocins from lactic acid bacteria, pediocin AcH, and nisin individually and in combination against several gram-positive bacterial strains including some involved in food spoilage and food borne diseases. They showed that pediocin AcH, and nisin in combination were more antibacterial than whey they were used alone. The antilisterial efficiency of three bacteriocins from lactic acid bacteria i.e. lactocin 705, enterocin CRL35 and nisin was tested in broth, individually and in combination against *Listeria monocytogenes* and *Listeria innocua*. Greater antilisterial activity was observed when bacteriocins were used in combination (Vignolo *et al*., 1996). However,

further enrichment of subpopulation resistance to antimicrobial peptides threatens the safety of bacteriocins use even in combination, which necessitates addition of another effective AMP in biopreservation panel. This demands search for additional effective AMPs. This could be achieved through brute force isolation of AMP producing bacterial strains, which is a time consuming, and laborious method. On the other hand computer aided molecular designing (CAMD) can be used as more effective method.

2.5.1 Isolation of AMP producing bacterial strains

Historically, the majority of antibiotics were discovered by screening natural product collections for antibacterial properties and subsequently optimizing lead compounds. It involves laborious screening of immense libraries of compounds for the desired properties and activity. A conventional 'synthesize and test' methodology starts with a hypothesis, generates a number of candidate peptides to test, conducts experiments on a selection of these peptides, and uses the results to confirm or falsify the hypothesis. These methods are time consuming and tedious processes with low efficiency and once the desired molecule has been obtained and come into widespread use, resistance may develop and again the whole process has to be repeated because shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997).

2.5.2 Computer Aided Molecular Design of AMPs

Recently significant progress has been made in the use of Computer-Aided Molecular Design (CAMD) techniques for the design of novel molecules possessing desired properties. The approach is best used in parallel with more traditional 'synthesize and test' methods and typically relies on two stages:

(1) Forward Modeling: The use of non-linear modeling methods such as Neural Networks (NN) to predict molecular properties, often called Quantitative Structure Activity Relationships (QSARs). These methods build predictive models based on experimental data. They may use molecular parameters

derived from the 3-D structure of the peptide, or a structural description of the molecule (Venkatasubramaniam *et al*., 1994).

(2) Model Inversion / Optimization: it is the use of optimization algorithms to invert the QSAR models to find new molecules of high activity. The inversion is generally one-to-many, e.g. there are many molecules that have the same activity, and so the inversion is more reasonably treated as an optimization problem in the space of all molecules of the particular class. Genetic Algorithm (GA) has been found to be a successful way of designing molecules in this way (Holland, 1975; Goldberg, 1989).

Method	**Efficiency**
GA	> 90/1250 = 7.2%
Monte Carlo	1/200 = 0.5%
Random	4 / 52000 = 0.008%

Table 2.2 Efficiency comparison of the genetic algorithm against other standard optimization techniques. The GA is an order of magnitude more efficient (Patel *et al*., 1998)

The GA is a search technique used in computing to find true or approximate solutions to optimization and search problems and Monte Carlo is a computational algorithm for simulating the behavior of various physical and mathematical systems, and for other computations.

AMPs, mostly cationic and amphipathic amino acid sequences, found in living species and produced in large quantities at sites of infection and/or inflammation (Zasloff, 2002) seem to be the ideal candidates for such manipulations. But for these applications knowledge of the antimicrobial peptide and its interaction with the target is important to provide information for forward modeling. Once the structure of the peptide with more efficacies is known, simple fine-tuning of the AMPs would lead to the drug of choice.

Certain indubitable advantages of peptides pertaining to simplicity, activity spectra, and bacterial resistance over known preservative agents advocate their potential for food preservation. Nisin, an antimicrobial peptide originating from bacteria, is so far the only FDA-approved peptide for food preservation. However, growing number of reports describe the potential of animal-derived antimicrobial peptides as food preservatives. Classically, these peptides are encoded by small genes, with conserved sequences and patterns that make their cloning easy, and should allow easy expression and both small and large-scale purification. There are definite peptide composition and activity relationships that could be relevant to the design and synthesis of future AMPs (Li, et *al.,* 2002). The problem of designing new peptides that possess specific properties, such as bactericidal activity, is of wide interest.

Slight variations in the structure of pre-existing peptides might broaden their potential as AMPs (Marshall and Arenas, 2003). Structure-activity relationship (SAR) studies of various AMPs demonstrated that the molecular size of native peptides can be significantly reduced while maintaining antimicrobial properties and sometimes improving those (Chen *et al*., 2003). Similar optimization attempts were reported also using non-native model peptide sequences. SAR studies of synthetic model AMPs aiming at understanding their mechanism of action resulted in a 14-residue, long peptide 8K6L, composed of 8 lysine and 6 leucine residue. The peptide showed bactericidal effect against *E. coli* O157, reducing its population by 6 log units after 1 hour of incubation at the concentration of 50 µg/ml.

There are growing numbers of reports using biotechnology techniques either for SAR studies or for mass production of AMPs. Future challenges lie in our ability to adapt these extraordinary compounds to perform tasks specifically related to food safety. Better understanding of the mode(s) by which AMPs rapidly eliminate microorganisms should provide solid grounds for engineering new and upgraded derivatives with optimized potency and stability under the range of incubation conditions typical to food.

2.6 IN-VITRO ESTIMATION OF INHIBITION

Cabo *et al*. (1999) used bioassay method for determining the bacteriocins, which was based on quantification of growth inhibition produced in a sensitive microorganism. However, bioassay method has a number of inconveniences. One of the problems of an assay method based on the diffusion of bacteriocins in agar is that bacteriocins do not diffuse very well in gels. Factors like diffusion time for the extract and the density of the agar used also have influence in the inhibition pattern. Finally, quantification is semi-quantitative and conditioned by subjective interpretations. Chen *et al.* (1996) reported physiological interaction of an AMP, Pediocin PA-1 with target membranes prepared by lipid extract of *Listeria monocytogenes* and carboxyfluorescein. Pediocin PA-1 caused the time and concentration dependent release of entrapped carboxyfluorescein from the vesicles. This in-vitro assay using liposomes containing carboxyfluorescein requires complicated spectrofluroscence procedure. These limitations of bioassay method and carboxyfluorescein efflux assay can be overcome by simple colorimetric biosensor assay.

Development of in-vitro colorimetric assays for high throughput screening of compound libraries is becoming increasingly important for drug discovery and identification of molecules having therapeutic promise. Assays providing rapid and easy evaluation of interactions between pore forming antimicrobial peptides and lipid bilayers could significantly improve screening for substances with effective antimicrobial properties, as well as contribute to the elucidation of structural and functional properties of antimicrobial peptides. In this direction the polydiacetylene vesicles appear to be a promising tool. The polymerization reaction between diyne monomers resulting in a PDA (polydiacetylene) chain is very sensitive to the surrounding environment and packing of terminal substituents. The reaction proceeds as a 1, 4-addition reaction among diyne monomers. UV light initiates the reaction by forming a diradical species, which is then able to attack other diacetylene containing monomers. Depending upon the substituents on the polymer backbone, resulting PDA crystals may have anywhere from a purple-blue to a yellow-

orange appearance. These colors can change in response to a variety of environmental conditions such as temperature and pH (Cheng and Stevens, 1998). PDA vesicles are readily synthesized by sonicating an aqueous solution containing the lipids, which gets incorporated into the vesicles (Okada *et al.*, 1999). The association is driven by the same hydrophobic forces, which lead to self-assembly of cell membranes. Following association of the diacetylene monomers and other lipids into vesicles, polymerization is initiated with UV-light. Reaction progress is monitored easily by the appearance of a blue color. Diacetylene monomers absorb at wavelengths less than 300 nm, rendering them colorless, while lipid substituted PDAs absorb at wavelengths around 600 nm, rendering them blue.

Kolusheva *et al.* (2000) demonstrated that particles composed of phospholipids and polymerized polydiacetylene (PDA) lipids exhibit striking color changes upon interactions with AMPs. The color changes in the system occur because of the structural perturbation of the lipids following their interaction with AMPs. Sonicated and UV-polymerized assemblies of lipid molecules and PDA form vesicular particles that exhibit blue color due to the electronic delocalization within the conjugated PDA backbone (Carpick *et al.*, 2000). Furthermore, such systems undergo specific blue to red transitions induced by diverse biological and chemical processes (Cheng and Stevens, 1998; Jonas *et al.*, 1999; Charyck *et al,* 1993; Reichert *et al.,* 1995). The lipid molecules in the vesicles essentially form 'microdomains' within the PDA matrix which do not affect the polymerization and blue color of the vesicle solution. It has been shown that the lipid/PDA assemblies closely mimic lipid bilayer environments within cellular membranes (Jonas *et al.*, 1999). This colorimetric lipid platform has been previously used for the study of diverse membrane-associated processes, including phospholipase cleavage (Jelinek *et al.*, 1998), membrane disruption by antimicrobial peptides (Kolusheva *et al.*, 2000), ion sensing (Kolusheva *et al.*, 2000), antibody–epitope recognition (Kolusheva *et al.*, 2001), and the activities of membrane penetration enhancers (Evrard *et al.*, 2001). The application of this colorimetric assay for analysis and screening of membrane interactions of lipophilic enzymes, peptides, and ions and for study of the effects of lipid composition upon membrane properties was shown by

Rozner *et al.* (2003) who also demonstrated that the lipid/polymer platform facilitates screening of peptide–membrane interactions in multi-component mixtures. The colorimetric vesicles can incorporate lipid species from different cellular sources facilitating analysis of the contribution of molecular components to membrane properties and lipid interactions.

Wyrsta *et al*. (2001) recently utilized PDA vesicle constructs to examine membrane interaction activity in a library of amphiphilic polypeptides. The authors were interested in determining structure-activity relationships (SAR), which may suggest important factors for designing new antimicrobial peptides. A library of peptides, which varied in length, amino acid composition, and percent hydrophobicity, were synthesized and screened with PDA vesicles. Wyrsta *et al*. utilized one of five α-amino acids as their hydrophobic residue: Ala, Phe, Leu, Ile, or Val. Amino acids Ala, Phe, and Leu are α-helix favoring while Ile and Val are β-sheet favoring amino acids. Lys was the cationic residue used in all peptides. Vesicles containing a 10, 12-tricosadiynoic acid and a cellular membrane lipid (PC, PG or PE) were exposed to each of the members of the library. Generally, peptides with high hydrophobic content (20-50%) exhibited the greatest colorimetric responses. Interestingly, only peptides that contained α-helix favoring amino acids produced any colorimetric response. However colorimetric assay using lipid/PDA assemblies are simple and economically feasible method and can be used as best alternative to carboxyfluorescein efflux assay. If the colorimetric response can be correlated to antimicrobial activity, it may provide some useful information about SAR for antimicrobial peptides**.**

3. MATERIALS AND METHODS

In the present investigation on "Studies on Phospholipids from Alamethicin Sensitive and Resistant Mutants of *Bacillus cereus*", selection of an alamethicin sensitive strain of *Bacillus cereus* and its resistant mutant was carried out to ascertain changes in both polar head groups and hydrophobic fatty acyl chains of phospholipids upon acquisition of resistance against alamethicin and confirm these changes using *in vitro* assay.

3.1 SELECTION OF SENSITIVE STRAIN AND ITS RESISTANT MUTANT

Selection of an alamethicin sensitive strain of *Bacillus cereus* and its resistant mutant was carried out using standard microbial culturing and spot on the lawn method with *Bacillus cereus* NCDC66 grown without and with 5 times of IC_{50} Alamethicin concentration.

3.1.1 Microbial Culturing

3.1.1.1 Bacterial cultures

Bacterial cultures were procured from National Collection of Dairy Cultures (NCDC) in freeze-dried form. Cultures were transferred to nutrient broth aseptically and subcultured twice before use. Strain morphology was studied by Gram staining and colony characters on 1.2 % nutrient agar plates.

3.1.1.2 Growth Media

Nutrient broth (Table 3.1) was used for the culture of *Bacillus cereus* strains. Hard and soft nutrient agar was prepared by adding 1.5 % and 0.75 % agar powder to nutrient broth. Both of the media were sterilized at 121 °C for 20 minutes.

3.1.1.3 Preservation of Bacterial Cultures

For short-term preservation, streaks of the culture were made on nutrient agar slants, incubated at 37 °C overnight and stored at 2 – 8 °C. For current use, preservation was achieved after overnight growth of the culture in nutrient broth tubes and stored at 5 °C.

Table 3.1 Composition of nutrient broth

Component	g/l
Peptone	15
Yeast extract	3
Dextrose	1
Sodium chloride	6
pH	7.0±0.2

3.1.1.4 Preparation of Alamethicin Solution

Five mg of alamethicin (Sigma-Aldrich) was weighed and dissolved in 200 µl of 95 % ethanol (freshly distilled). This solution was made up to 1 ml with sterile 1% sodium bicarbonate solution to give a stock solution of 5 mg/ml. The stock solution was dispensed as 100 µl aliquots in sterile 0.5 ml eppendorfs and stored at -20 °C till use.

3.1.1.5 Setting Inoculum Size

Portions containing 1–5 × 10^5 cfu were calculated by plate count method using McFarland 0.5 turbidity standard.

3.1.1.5.1 Preparation of McFarland 0.5 Turbidity Standard

One percent sulphuric acid and 1.175% barium chloride solutions were prepared and stored in dark. Fifty µl of 1.175 % barium chloride was added to 9.95 ml of 1% sulphuric acid in a test tube comparable to that of culture tube, to give a turbidity of 0.5 McFarland standard.

3.1.1.5.2 Inoculum Preparation

Ten ml of overnight grown culture was centrifuged at 3000 rpm for 5 min and suspended in sterile saline. It was diluted further with sterile saline till its turbidity is visually comparable to McFarland 0.5 turbidity standard using a comparison grid containing black lines. The suspended culture in saline was serially diluted and plated on to nutrient agar to determine the cfu/ml.

3.1.2 Spot on the lawn assay

Nutrient hard agar plates were overlaid with 6 ml of nutrient soft agar inoculated with 5 µl of overnight-activated test culture. Plates were allowed to set for 15 – 30 min. Plate was divided into 1.5 cm × 1.5 cm squares. Ten µl of

alamethicin stock solution (5 µg/µl) was taken in a sterile eppendorf. Five µl of this solution was kept as first spot. The remaining 5 µl was double diluted serially with 5 µl of diluent (95 % ethanol: 1% sterile sodium bicarbonate in the ratio of 1: 4) and spotted on to agar plates. A negative control spot was also kept with diluent alone. Antibiotic solutions were allowed to diffuse for 1 hr at room temperature and kept in incubator at 37 °C for overnight. Inhibitory zones were observed and the last spot showing inhibition was recorded.

3.1.3 Determination of IC_{50}

IC_{50} was determined using the broth assay method described by Cabo *et al.*, (1999). Briefly, nine hundred and ninety µl of sterile nutrient broth was taken in sterile eppendorfs. To these 5 µl of alamethicin (5 mg/ml) solution was added in double dilutions after diluting with diluent (95 % ethanol: 1 % sterile sodium bicarbonate in the ratio of 1: 4). This gave a final concentration of 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, …0.04 µg/ml of alamethicin in broth. To these tubes 5 µl of test culture containing 1 – 5 × 10^5 cfu /ml was added. A positive control was setup with broth and test culture and a negative control with broth alone. All the tubes were mixed well and incubated at 37 °C overnight. Optical density (OD) of the culture was taken at 600 nm with negative control as blank. From OD data, dose response of inhibition was calculated using the formula for inhibition, $I = 1-(A_m/A_o)$ where, A_m is OD of the culture at different concentrations of alamethicin and A_o is OD of the positive control. IC_{50} was determined as the concentration of the alamethicin resulting in 50% inhibition of the test culture.

3.1.4 Determination of Growth Curve

To 13 tubes of 5 ml sterile nutrient broth, 5 µl of overnight grown culture was added and incubated at 37 °C till the end of experiment. OD of the culture was recorded at 600 nm from 0 to12 hr with 1 hr interval using fresh media as blank.

3.1.5 Selection of Resistant Mutants

Resistant mutants were selected using the method described by Rekhif *et al.*, (1994). Sensitive culture containing 1–5 × 10^5 cfu was added to nutrient broth containing alamethicin at 5 and 10 times of IC_{50}/ml and incubated at 37

°C for 36 hr or until visible growth was seen. After 36 hr, the broth culture was serially diluted and plated or streaked on to nutrient agar without alamethicin. The plates were incubated at 37 °C for 24 hr. Colonies were randomly selected and propagated in nutrient broth for 10 successive subcultures. IC_{50} was determined to assess resistance development.

3.2 DETERMINATION OF CHANGES IN PHOSPHOLIPIDS COMPOSITION

Total cellular lipids were extracted from sensitive strain and resistant mutant. Changes in head group were ascertained using TLC. Quantification of phospholipid content for each resolved spot was carried out through phosphorous estimation. In addition, changes in fatty acyl side chains were achieved using GLC.

3.2.1 Extraction of total cellular lipids

Total cellular lipids were extracted using following modified Bligh and Dyer (1959) method.

3.2.1.1 Materials

1. Culture of *Bacillus cereus* NCDC66 sensitive strain and resistant mutant
2. Chloroform (AR grade)
3. Methanol (AR grade)
4. Hydrochloric acid - 2 N (AR grade)
5. Physiological saline (0.9 %) – 9 g of sodium chloride dissolved in distilled water and solution made to 1000 ml.
6. Acidified Physiological Saline (pH – 2.0) – pH of physiological saline adjusted with hydrochloric acid.

3.2.1.2 Procedure

One liter of sterile nutrient broth was inoculated at 1 % level with overnight-activated cultures and incubated at 37 °C overnight. Cells were harvested by centrifugation at 5000 × g for 20 min at room temperature in 500 ml polypropylene bottles. Supernatant was discarded and cell pellet resuspended in 50 ml of physiological saline. This was centrifuged at 5000 × g for 20 min at room temperature. Supernatant was discarded and cell pellet

resuspended in 5 ml (one sample volume) of physiological saline. To this 20 sample volumes of choloroform: methanol (2:1 v/v) and 0.13 sample volumes (0.65 ml) of 2 N hydrochloric acid were added in the order. The suspension was vortexed for 30 sec to get a single phase and incubated at 37 °C for 90 min with occasional agitation. To this 8.3 sample volumes of acidified physiological saline (pH – 2.0) i.e. 41.5 ml was added and vortexed for 30 sec. It was then centrifuged at 190 × g for 5 min at room temperature to facilitate separation of two phases.

The upper aqueous phase was removed and to the lower chloroform phase, 6.6 sample volumes (33 ml) methanol and 8.3 sample volumes (41.5 ml) of acidified saline (pH – 2.0) were added. This was vortexed well for 30 sec and centrifuged at 190 × g for 5 min at room temperature. The upper aqueous layer was discarded and lower chloroform phase was allowed to evaporate in a dry glass beaker to get total lipid extract. The lipid extract was stored at -20 °C.

3.2.2 Thin layer chromatography (TLC)

Different classes of lipids from total lipid extracts obtained from sensitive strain and resistant mutant were resolved using TLC for identification and quantification (Katz *et al*., 1999).

3.2.2.1 Preparation of TLC Plates

Ninety ml of distilled water was poured into 40 g of silica gel G in a mortar and mixed well using pestle to get uniform slurry without air bubbles. Four dry grease free, glass plates (20 cm × 20 cm) were placed on an ebonite board in a row to provide a smooth surface. The Desaga applicator was filled with silica gel slurry and rolled over plates with uniform slow motion to completely cover the plates with 0.5 mm thickness. The plates were allowed to dry for 15 min and activated at 110 °C for 1 hr in an oven. Activated plates were not kept in exposed air for more than half an hour before spotting.

3.2.2.2 Solvent Mixture and Equilibration

One hundred ml of mobile phase solvent mixture was prepared by mixing chloroform: methanol: distilled water: glacial acetic acid (65: 25: 4: 1 v/v). It was poured in to TLC developing chamber (25 cm × 25 cm × 10 cm)

lined inside with filter paper on three sides. The tank was covered with glass lid and kept undisturbed for 2 hr for equilibration to take place.

3.2.2.3 Spotting TLC Plates

Known quantities of phospholipid standards and lipid extracts from sensitive strain and resistant mutant were dissolved in chloroform: methanol (2:1 v/v) to give a final concentration of 5 – 10 µg/µl. With a micropipette, 4 µl of each standard was spotted on the plate in each lane. Ten µl of a mixture of standard phospholipids was also spotted in a separate lane. Similarly, extracted lipids from sensitive strain and resistant mutant were spotted in separate lanes. All the spots were allowed to dry before developing the TLC plates.

3.2.2.4 Development of TLC Plates

Spotted plate was gently lowered in to TLC tank and lid replaced immediately. The level of the mobile phase in tank was not allowed to touch the spots directly. The solvent front started moving upward and reached less than 1 cm from the top in approximately 55 min. Subsequently, the plate was taken out and the solvent front was marked with a marker. The mobile phase was allowed to evaporate to dry the plate before staining.

3.2.2.5 Identification of lipids

Different classes of resolved lipids were visualized using specific stains viz. iodine vapors for general lipids, ammonium molybdate for phospholipids and ninhydrin for amino group containing phospholipids (ACPs). The various classes of lipids were identified in comparison with standard lipids.

3.2.2.5.1 General lipids

Developed plate was kept in iodine chamber (a TLC tank with few iodine crystals) for visualization of general lipids. Plate was removed and spots marked with a marker on back side of the plate. The distance of spots from the spotting line was measured. Retardation factor (R_f) value of the spots was calculated using the formula:

$$\text{Retardation factor } (R_f) = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$$

3.2.2.5.2 Phospholipids

Phospholipids were visualized using ammonium molybdate reagent, which was prepared by dissolving 0.25 g ammonium molybdate in 1 ml of warm distilled water. Eighty mg of metallic copper was placed in the solution. This was chilled over ice and 1 ml concentrated sulphuric acid was added which resulted in a deep blue colored solution. It was kept at room temperature for 2 hr with occasional shaking. 40 ml-distilled water was then added and shaken to give a light brown solution. Copper metal was then removed and 3.2 ml of concentrated sulphuric acid added. Resulting solution remained light brown and was stored at 2 – 8 °C. The solution was freshly prepared every week.

Spots on TLC plates were initially identified with iodine vapors and position of spots marked. Iodine was allowed to evaporate and then using a nebulizer, ammonium molybdate spray reagent was uniformly sprayed. Plates were then kept in an oven set at 90 °C for 5 min or till the blue colored spots appeared.

3.2.2.5.3 Amino Group Containing Phospholipids

Two hundred mg ninhydrin was dissolved in 100 ml of 95 % ethanol to give ninhydrin spray reagent. This solution was stored in dark and is stable for months. Spots on TLC plates were initially identified with iodine vapors and position of spots marked. Iodine was allowed to evaporate and then using a nebulizer, ninhydrin spray reagent was uniformly sprayed. The plate was kept in an oven at 110 °C for 10 min or until dark pink spots appeared.

3.2.3 Quantification of phospholipids

The phospholipids were quantified by estimating the phosphorus content in the resolved spots of thin layer chromatograms (Kahovcova and Odavic, 1969). Charring chromatographic plates with sulphuric acid releases phosphorous; which on reaction with Hahn and Luckhaus' reagent gives blue color. Color developed can be estimated by taking absorbance at 700 nm and amount of phosphorous determined from standard curve. Multiplying the amount of phosphorous with a factor 25 gives the amount of phospholipids in each spot.

3.2.3.1 Materials

1. Sulphuric acid - 50 %

2. Sulphuric acid - 10 N

3. Standard phosphorous solution (stock) - 35.1 mg of potassium dihydrogen phosphate was dissolved in distilled water. One ml of 10N sulphuric acid was added and volume made up to 100 ml with distilled water to give a solution of 80 µg/ml phosphorous. Working standard phosphorous solution was prepared by diluting the stock solution 5 times to give a solution of 16 µg/ml phosphorous.

4. Hahn and Luckhaus' reagent – 6.85 g sodium molybdate dihydrate and 400 mg hydrazine sulphate was dissolved in 100 ml of distilled water. To this, 100 ml concentrated sulphuric acid and 500 ml distilled water were added. After cooling the solution, volume was adjusted to1000 ml, with distilled water.

3.2.3.2 Standard curve of phosphorous

To 5 numbers of test tubes 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml of standard phosphorous solution (working) was added and volume made to 4 ml with distilled water. One tube with 4 ml distilled water alone was set as blank. To these tubes 1 ml of Hahn and Luckhaus' reagent was added and heated in a boiling water bath for 30 min. The tubes were then cooled and absorbance recorded at 700 nm using spectrophotometer against blank. The amount of phosphorous and absorbance were plotted on a graph to get the standard curve.

3.2.3.3 Procedure

Spots on TLC plates were initially identified with iodine vapors and position of spots marked. Iodine was allowed to evaporate. The plate is then sprayed with 50 % sulphuric acid and kept in an oven set at 180 °C for 1 hour. Equal amounts of the charred spots were then scrapped in to test tubes. Silica gel without lipids was also scrapped to set up blank. To these tubes 4 ml of distilled water was added, mixed well and 1 ml Hahn and Luckhaus' reagent was added. All the tubes were then heated in a boiling water bath for 30 min. The tubes were cooled and centrifuged at 2000 rpm for 2 min to sediment silica. The absorbance of the blue color developed was taken at 700 nm. From

the standard curve, the amount of phosphorous in each spot was calculated (Table 3.2 and Fig 3.1).

Table 3.2 Standard curve of phosphorous

Amount of phosphorous (µg)	**OD (700 nm)**
0	0
3.2	0.191
6.4	0.372
9.6	0.577
12.8	0.762
16	0.959

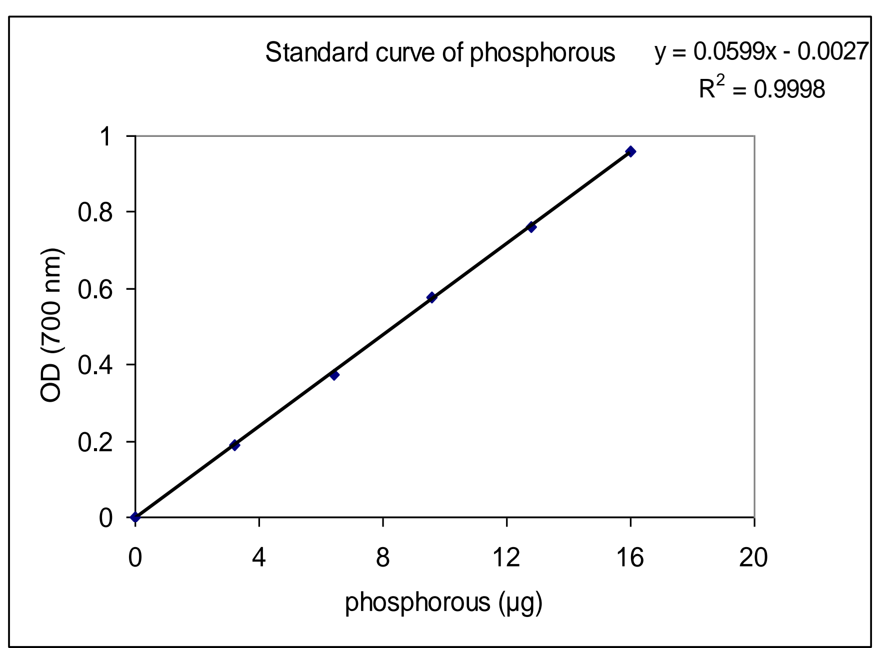


Fig 3.1 Standard curve of phosphorous

3.2.4 Gas liquid chromatography (GLC)

Phospholipids extracted from TLC plates and total lipid extract from alamethicin sensitive strain and resistant mutant were transesterified to get fatty acid methyl esters (FAMEs) for further analysis using GLC (Sakayori *et al.*, 2003).

3.2.4.1 Extraction of Phospholipids from TLC Plates

The resolved phospholipid spots from TLC plates was scrapped and taken in 1.5 ml eppendorf. It was dissolved in 1 ml chloroform and 0.5 ml methanol was added and kept for 1 hr at 4 °C. It was then vortexed and centrifuged at 5000 × g for 10 min. The supernatant was collected. The residue was washed twice with chloroform: methanol (2:1) and supernatant pooled. The pooled supernatant was allowed to dry and residue dissolved in 1 ml chloroform. 5 – 10 mg of total lipid extract was also dissolved in 1 ml of chloroform.

3.2.4.2 Transesterification

Phospholipids from TLC plates and lipid extract from sensitive and resistant mutant were taken in teflon coated screw capped test tubes. To these tubes 3 ml of methanol: hydrochloric acid: chloroform (10: 1: 1 v/v) was added i.e. 2.5 ml methanol, 0.25 ml hydrochloric acid and 0.25 ml chloroform. The tubes were kept in an oven set at 90 °C for 60 min. Then the tubes were taken out and cooled to room temperature.

3.2.4.3 Extraction of Fatty Acid Methyl Esters (FAMEs)

To the transesterified mixture in the tubes, 1 ml distilled water was added followed by 3 ml of hexane: chloroform (4:1 v/v) i.e. 2.4 ml hexane and 0.6 ml chloroform. The upper hexane phase was collected and extraction repeated.. Both the aliquots were pooled and allowed to evaporate. The residue was weighed and then dissolved in known volume of chloroform to give final concentrations of FAMEs in the range of 10 – 100 ug/ul.

3.2.4.4 Operating Conditions

Fatty Acid Methyl Esters (FAMEs) were analyzed on MICHRO 9100 Gas chromatograph using supelcowax – fused silica capillary column of 60 m ×

0.32 mm × 0.25 μm film thickness. Initial oven temperature was kept at 150 °C and an isothermal time of 2 min was allowed. Then a ramp rate of 5 °C /min was used to bring the temperature to 220 °C and an isothermal time of 18 min was allowed. Injector temperature was kept at 230 °C and flame ionization detector (FID) at 240 °C. The whole program took 34 min to complete.

3.2.4.5 Injection of the sample

Once the oven and FID reached their set temperature, FID was ignited using the ignition knob. Ignition was detected by formation of moisture condensate on a glass slide. One μl of standard or sample containing FAME(s) was taken in a chloroform washed micro-syringe and injected into the column through the polysilicone diaphragm. The system was started to run and detector response recorder was started in the computer interfaced GLC, simultaneously. The sample was allowed to run till the completion of the program. The recorder response was saved in a separate file for further analysis.

3.3 MEMBRANE INTERACTIONS BY A BIOMIMETIC COLORIMETRIC LIPID/POLYDIACETYLENE ASSAY

A colorimetric in vitro assay developed by (Kolusheva et al., 2000) was used to ascertain degree of inhibition via colorimetric response obtained upon interaction of alamethicin with biomimetic membranes prepared from total cellular lipid extract from sensitive strain and resistant mutant.

Table 3.3 Preparation of 2mM phospholipid-PDA vesicles

Type of vesicles	10,12-tricosadiynoic acid (mg)	Lipid (mg)	Mole ratio
Pure PDA	1.38	-	-
DMPG – PDA	0.83	1.102	4: 6
DPPG – PDA	0.80	1.192	4: 6
DSPE – PDA	0.83	1.196	4: 6

Phospholipid–PDA vesicles were prepared by taking specific quantities of phospholipids and 10, 12-tricosadiynoic acid in a polypropylene test tube (Table 3.3). To this 2.0 ml of triple distilled water was added and mixed well. This aqueous mixture was probe sonicated using Branson sonicator for 5 – 6 min at 70 °C. The resulting opaque solution was cooled to room temperature and kept at 4 °C overnight. The solution was irradiated using UV light for 20 – 30 min in laminar airflow. The resulting solution exhibited intense blue color.

Thirty-two µl of the alamethicin stock (2.55 mM) was added to 8 µl of 5 mM tris buffer (pH 8.5) to give 2.04 mM working solution. 20 µl of working solution was used for one vesicle solution and the remaining 20 µl was serially double diluted to give various concentrations of alamethicin. Diluent was prepared by mixing distilled water and 5 mM tris buffer (pH 8.5) at 4: 1 ratio.

Twenty µl of double diluted alamethicin solution was added to 0.2 ml phospholipid – PDA vesicle solution at concentration of 1 mM vesicle solution, 2 mM tris buffer (pH-8.5). The pH of all the solutions was maintained at 8.5. Following addition of alamethicin of various concentrations, the resulting solutions were diluted to 1 ml using distilled water. Absorbance of the solutions was taken at 500 nm and 640 nm in a spectrophotometer. Colorimetric response (CR) was calculated using the following formula:

$$\%\text{Colorimetric response (CR)} = (PB_0 - PB_I)/PB_0 \times 100$$

Where, $PB_0 = A_{640}/(A_{640} + A_{500})$ for control (vesicles without alamethicin)

$PB_I = A_{640}/(A_{640} + A_{500})$ for samples (vesicles with alamethicin)

From the colorimetric response of pure PDA vesicles with alamethicin, effective CR for phospholipid-PDA vesicles was calculated as follows:

Effective CR = CR of phospholipid-PDA vesicles - CR of pure PDA vesicles

Table 3.4 Alamethicin interactions with pure PDA vesicles

Alamethicin (μM)	**Log_2Alamethicin (μM)**	**%Colorimetric response (CR)**
6.37	2.671	0.38
12.75	3.672	0.40
25.5	4.672	0.60
51	5.672	1.26
102	6.672	2.37
204	7.672	4.64

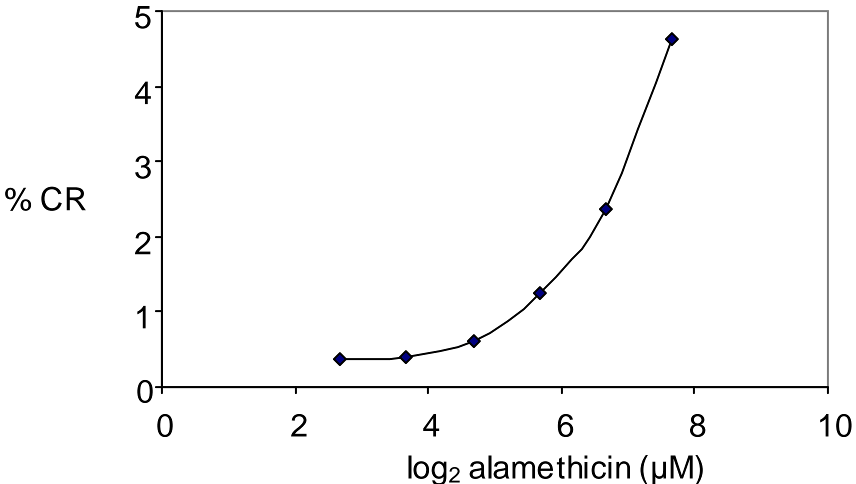


Fig 3.2 Colorimetric response of pure PDA vesicles

Table 3.5 Alamethicin interactions with DMPG-PDA vesicles

Alamethicin (μM)	**Log_2Alamethicin (μM)**	**%Colorimetric response (CR)**	**Effective % CR**
6.37	2.671	5.36	4.98
12.75	3.672	5.85	5.45
25.5	4.672	6.54	5.94
51	5.672	7.07	5.81
102	6.672	12.936	10.59
204	7.672	25.53	18.89

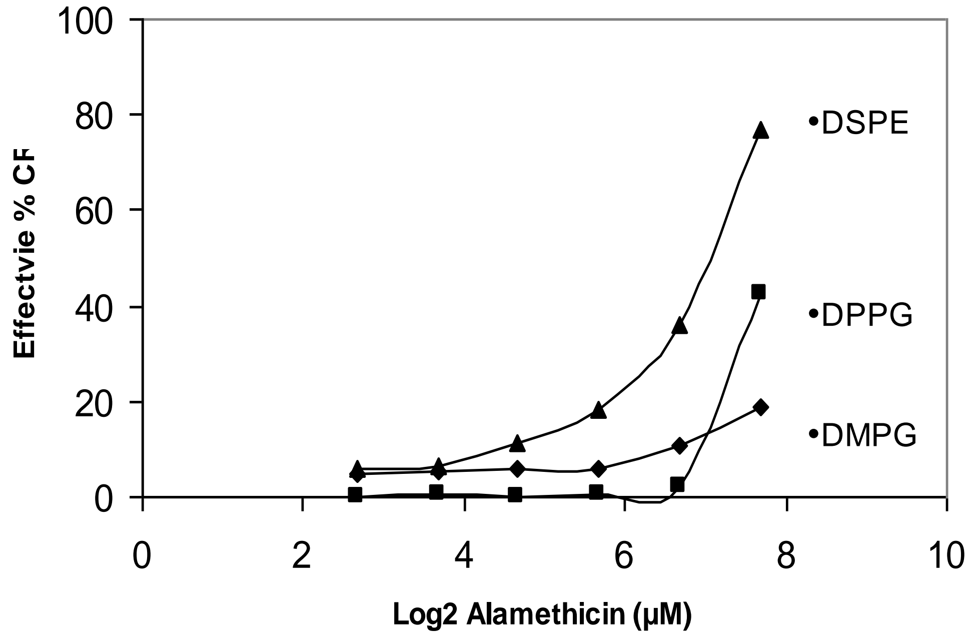
Table 3.6 Alamethicin interactions with DPPG-PDA vesicle

Alamethicin (μM)	**Log_2Alamethicin (μM)**	**%Colorimetric response (CR)**	**Effective % CR**
6.37	2.671	0.58	0.20
12.75	3.672	0.68	0.28
25.5	4.672	0.75	0.15
51	5.672	1.78	0.52
102	6.672	4.42	2.05
204	7.672	46.90	42.26

Table 3.7 Alamethicin interactions with DSPE-PDA vesicles

Alamethicin (μM)	**Log_2Alamethicin (μM)**	**%Colorimetric response (CR)**	**Effective % CR**
6.37	2.671	6.03	5.65
12.75	3.672	7.04	6.64
25.5	4.672	11.98	11.38
51	5.672	19.43	18.17
102	6.672	38.57	36.20
204	7.672	81.64	77.00

Fig 3.3 Colorimetric response of Phospholipid-PDA vesicles to various concentrations of alamethicin



Similarly, vesicle solutions incorporating lipids extracted from sensitive strain and resistant mutant were prepared at 1:1 mass ratio. 4.1 mg of extracted lipids from sensitive and resistant mutant and 4.1 mg of 10,12-tricosadiynoic acid were weighed into a polypropylene tube and vesicle preparation was done as described above.

Titration of these vesicles was done as described for standard phospholipids-PDA vesicles and effective percent colorimetric response was calculated (Table 3.5, 3.6, 3.7; Fig 3.3)

4. RESULTS AND DISCUSSION

The present investigation "studies on phospholipids from alamethicin sensitive and resistant mutants of *Bacillus cereus*" was undertaken to determine the changes in composition of phospholipids and their fatty acyl chains upon acquiring resistance to antimicrobial peptide (AMP) alamethicin and confirmation of these changes using an *in vitro* assay.

4.1 SELECTION OF A SENSITIVE STRAIN OF *BACILLUS CEREUS* AND ITS RESISTANT MUTANT

The sensitivity of three strains of *Bacillus cereus* NCDC240, NCDC250 and NCDC66 was determined by spot on lawn assay. Bacillus cereus NCDC66 was showing more sensitivity (least dilution giving zone of inhibition-1.56 µg/ml). The other two cultures did not show any zone of inhibition. Based on the sensitivity on agar spot assay *B. cereus* NCDC66 was selected (Plate I, II). The growth of the culture showed a lag phase of 2 hr, log phase of 4 hr and finally reached stationary phase at around 6 to 7 hr (Fig 4.1). IC_{50} of alamethicin for Bacillus *cereus* NCDC66 was found to be 2.0 µg/ml (Table 4.1; Fig 4.2).

The sensitive culture did not show any growth for prolonged incubation at alamethicin concentration of 20 µg/ml (10 times) of IC_{50}. Visible growth was seen after 36 hr in 10 µg/ml alamethicin concentration. IC_{50} was determined after 10 successive subcultures. From dose-response inhibition curve, IC_{50} for resistant variant (Fig 4.3) was determined as 9.5 µg/ml. Increase in IC_{50} confirmed the development of resistance.

4.2 COMPARATIVE ANALYSIS OF PHOSPHOLIPIDS BETWEEN ALAMETHICIN SENSITIVE AND RESISTANT MUTANT OF *BACILLUS CEREUS*

4.2.1 Changes in phospholipid head group

Total cellular lipid extract of sensitive strain could be resolved in three spots (Plate III). Spot 1, was detectable with iodine vapors, and molybdate spray reagent but not with ninhydrin. Tentatively it may be identified as amino

group lacking phospholipid. Spot 2 was detectable with iodine, molybdate and ninhydrin. Its R_f was close to that of distearoyl phosphatidylethanolamine (DSPE) hence it may be phosphatidylethanolamine (Table 4.3; Plate IV). Spot 3 was detectable with iodine, molybdate and ninhydrin. Tentatively it was identified as amino group containing phospholipid (Plate V).

Similarly total cellular lipid extract of resistant variant showed three spots whose R_f were similar to the sensitive strain spots. Spots 1, 2, and 3 could be identified as phospholipid lacking amino group, phosphatidylethanolamine and amino group containing phospholipids respectively (Plates VI, VII, VIII). There were significant difference in the density of the spots; its quantitative analysis was carried out.

4.2.2 Phosphorous estimation of resolved spots on TLC

Amount of amino group lacking phospholipid in spot 1 increased in the resistant variant (11.22 %) as compared to sensitive strain (7.73 %). Amount of phosphatidylethanolamine (spot 2) decreased from 77.43 % in sensitive to 69.10 % in resistant variant (8.3 % decrease). Amino group containing phospholipids (spot 3) increased by around 4.86 % from 14.84% in sensitive strain to 19.7% in resistant mutant (Table 4.5; Fig 4.3). Therefore there is an overall slight decrease in amino group containing phospholipids due to slight increase in amino group lacking phospholipids (+3.49 %), decrease in phosphatidylethanolamine (-13.33 %) and slight increase in amino group containing phospholipids (ACP). There is no significant change in the phospholipid head groups.

4.2.3 Fatty acyl chain analysis of sensitive strain and resistant mutant

Fatty acids were identified by comparing their retention times with known standard FAMEs (Table 4.6 and Appendix I-a). Fatty acids C12:0, C14:0, C16:0, C18:0 could be observed in total cellular lipid extract of sensitive strain whereas fatty acids with C18:1 and 18:2 were not detectable (Table 4.7 and Appendix I-b, I-c). In spot 1, fatty acids C12:0, C14:0, C16:0, C18:0 could be observed (Table 4.6 and Appendix I-d, I-e). Spot 2 revealed fatty acids C12:0,

conc. of alamethicin (µg/ml)	**OD (600 nm)**	**Inhibition**
0.04	0.445	0.032
0.09	0.415	0.097
0.19	0.412	0.103
0.39	0.404	0.122
0.78	0.36	0.224
1.56	0.263	0.427
3.12	0.153	0.667
6.25	0.036	0.920
12.5	0.001	0.998
25.0	0.001	0.998
positive control	0.460	0

Table 4.1 Dose-response inhibition of alamethicin sensitive *Bacillus cereus* NCDC66

conc. of alamethicin (µg/ml)	**OD (600 nm)**	**inhibition**
0.24	0.427	0.209
0.49	0.415	0.231
0.98	0.409	0.242
1.87	0.406	0.248
3.75	0.38	0.296
7.5	0.326	0.356
15.0	0	1
30.0	0	1
positive control	0.540	0

Table 4.2 Dose-response inhibition of alamethicin resistant mutant of *B. cereus* NCDC66

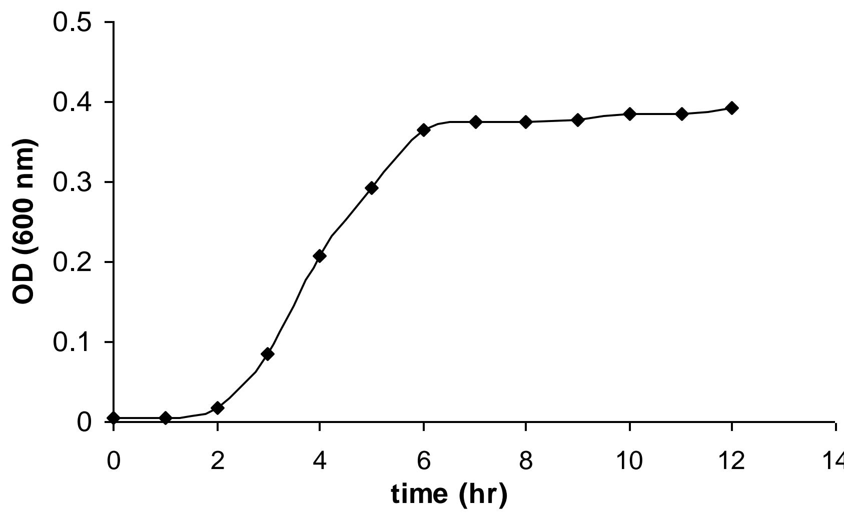


Fig 4.1 Growth curve for *B .cereus* NCDC66

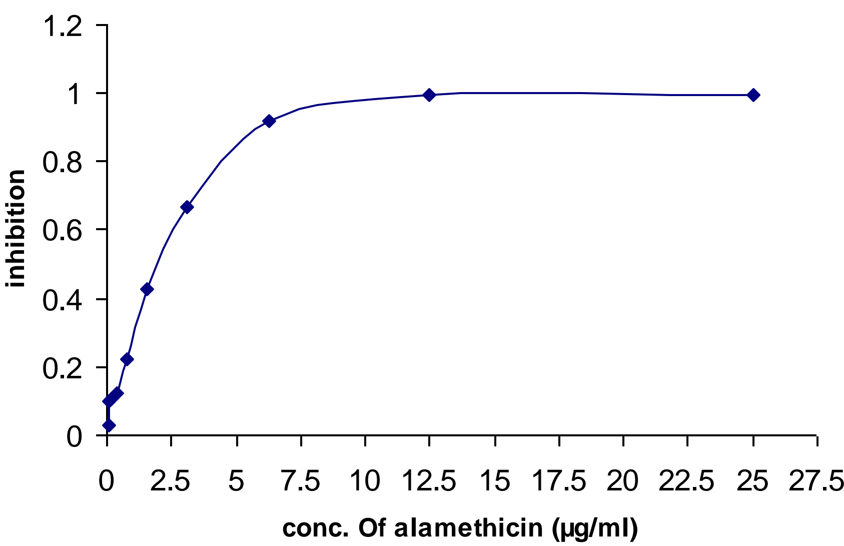


Fig 4.2 Dose-response inhibition curve for alamethicin sensitive *Bacillus cereus* NCDC66

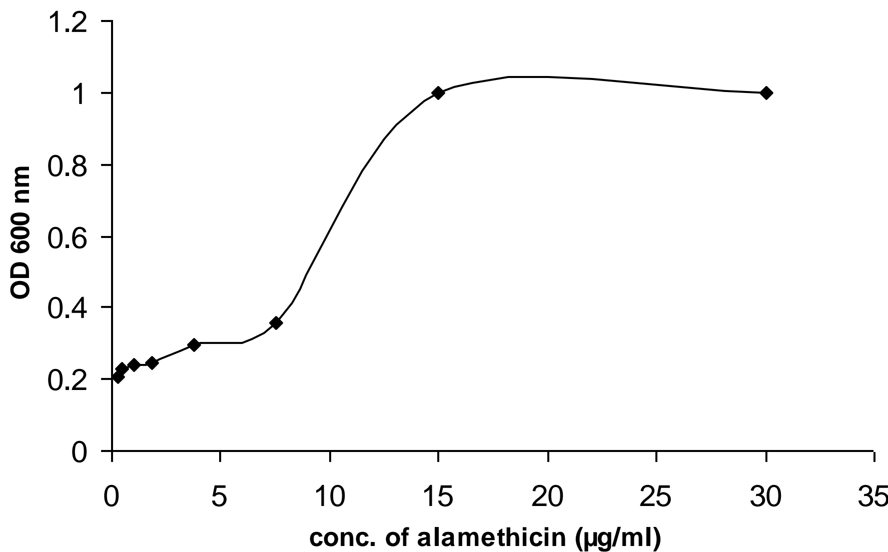


Fig 4.3 Dose-Response inhibition curve for alamethicin resistant mutant of *B. cereus* NCDC66

R_f values	Spot 1	Spot 2	Spot 3	DMPG	DPPG	DSPE
Iodine	0.81	0.66	0.37	0.60	0.60	0.78
Molybdate	0.82	0.72	0.44	0.66	-	0.72
Ninhydrin	ND	0.76	0.38	ND	ND	0.76

Table 4.3 Retention factor (R_f) values of different spots of sensitive *B. cecreus* NCDC66 lipid extract and standard phospholipids resolved by Thin-layer chromatography after visualization with iodine vapors, molybdate reagent and ninhydrin.

R_f values	Spot 1	Spot 2	Spot 3	DMPG	DPPG	DSPE
Iodine	0.86	0.68	0.36	0.60	-	0.70
Molybdate	0.88	0.68	0.36	0.60	-	0.70
Ninhydrin	ND	0.68	0.36	ND	ND	0.68

Table 4.4 Retention factor (R_f) values of different spots of resistant mutant *B. cereus* NCDC66 lipid extract and standard phospholipids resolved by Thin-layer chromatography after visualization with iodine vapors, molybdate reagent and ninhydrin.

C14:0, C18:0 (Table 4.5 and Appendix I-f, I-g), where as fatty acids C14:0, C16:0, C18:0 could be observed in spot 3 (table 4.7 and Appendix I-h, I-i).

In total cellular lipids of resistant mutant fatty acid C12:0 was not detectable. Fatty acid C14:0 decreased from 16.58 % in sensitive to 10.16 % in resistant variant. Similarly, fatty acid C16:0 has decreased from 47.37 % in sensitive to 35.76 % in resistant mutant. However, there is considerable increase in C18:0 from 18.78 % in sensitive strain to 36.03 % in resistant variant. Overall there is an increase in long chain fatty acids and decrease in short chain fatty acids in resistant mutant compared to the sensitive strain. Fatty acid C18:1 was detectable at the level of 11.78 % and C18:2 at the level of 0.6 % in resistant mutant, whereas fatty acids C18:1 and C18:2 could not be detected in sensitive strain (Table 4.7).

4.3 IN VITRO CONFIRMATION OF RESISTANCE ACQUISITION THROUGH CHANGES IN PHOSPHOLIPID COMPOSITION

Figure 4.6 and Table 4.8 revealed a decrease in effective colorimetric response in vesicles prepared with lipid extracts from resistant variant (Table 4.8 and Plate X) as compared to that of the sensitive strain (Table 4.8 and Plate IX). At 204 μM alamethicin concentration the resistant lipid vesicles showed effective % CR of 6.10 whereas, the sensitive lipid vesicles showed a response of 18.89 (Table 4.8).

4.4 DISCUSSION

The present investigation on “Studies on Phospholipids from Alamethicin Sensitive and Resistant Mutants of *Bacillus cereus*” revealed the existence of alamethicin resistant mutants among sensitive population of *B. cereus* NCC66. There were significant differences in the levels of head groups of phospholipids and fatty acyl side chains. Further, evaluation of the *in vitro* alamethicin membrane interactions confirmed the acquisition of resistance to change in phospholipid composition.

In this study successful isolation of resistant mutant confirmed the existence of resistant mutants among sensitive population. This is the first study reporting resistant mutants to alamethicin in the sensitive population of

B. cereus. Previously Bernhard *et al.* (1978) reported *B. cereus* strains resistant to high levels (up to 1 mg/ml) of ampicillin, colistin, and polymyxin and demonstrated pBC16 plasmid that conferred tetracycline resistance. Both polymixin and alamethicin are pore forming peptides. Similarly resistant mutants of *Listeria monocytogenes* has been reported against nisin (Verheul et al., 1997; Davies and Adams, 1994, Gravesen et al., 2001), leucocin A (Vadyvaloo et al., 2002), pediocin (Chen et al., 1996), class IIa bacteriocins (Gravesen et al., 2001), mundticin (Sakayori *et al*., 2003). In addition resistant mutants of *Staphylococcus aureus* against cationic antimicrobial peptide i.e. thrombin induced platelet microbicidal protein (Bayer *et al.,* 2000), Leuconostoc or Weissella against mesenterocin 52a and 52b. Sakayori *et al*. (2003) reported that the frequency of resistant mutants of different classes(10^{-6} – 10^{-7}) was independent of mundticin KS concentration.

Since there was not significant change in the levels of amino group containing phospholipids, it appears that acquisition of resistance by *B. cereus* is not related with the levels of amino group containing phospholipids. There is increase in unsaturated fatty acids and long chain fatty acids in the resistant mutant. This change in the composition of the membrane could be the reason for increase in IC_{50} in resistant mutant. There is decrease in colorimetric response in the biomimetic membrane of the resistant mutant that clearly proves the involvement of membrane change in increase of IC_{50}.

spots	sensitive		resistant	
	µg P	% P	µg P	% P
1	0.85	7.73	2.67	11.22
2	8.51	77.43	16.57	69.1
3	1.63	14.84	4.74	19.7

Table 4.5 Composition of phospholipids in terms of phosphorous (P) content for alamethicin sensitive and resistant *B. cereus* NCDC66.

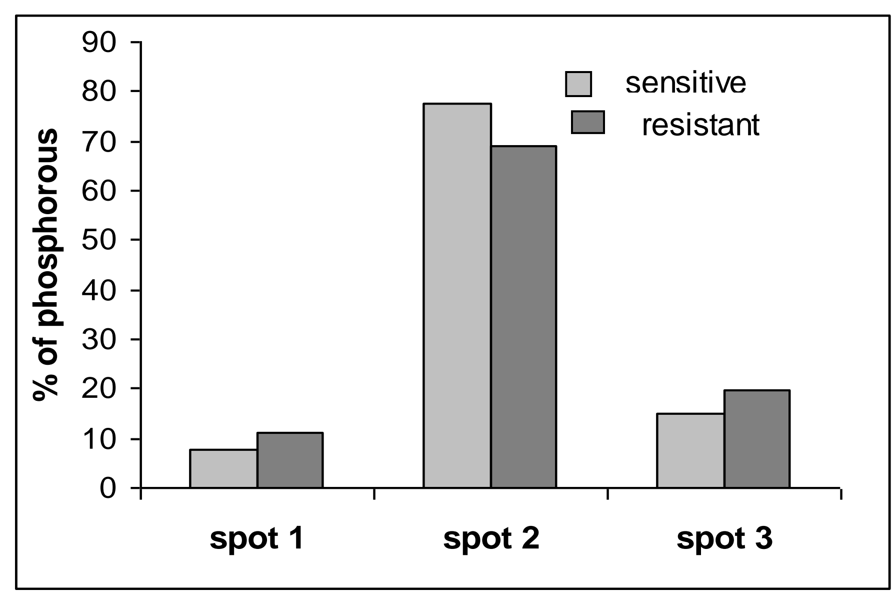


Fig 4.4 Comparison of phospholipid composition in sensitive strain and resistant mutant of *B. cereus* NCDC66

Alamethicin (µM)	Log_2Alamethicin (µM)	% Colorimetric response (CR)		Effective % CR	
		S	R	S	R
6.37	2.671	5.36	0.59	4.98	0.21
12.75	3.672	5.85	0.71	5.45	0.31
25.5	4.672	6.54	0.81	5.94	0.21
51	5.672	7.07	1.83	5.81	0.57
102	6.672	12.936	2.51	10.59	0.14
204	7.672	25.53	10.74	18.89	6.10

Table 4.8 Effective colorimetric response of biomimetic vesicles prepared with lipid extracts of alamethicin sensitive strain and resistant mutant of *Bacillus cereus*

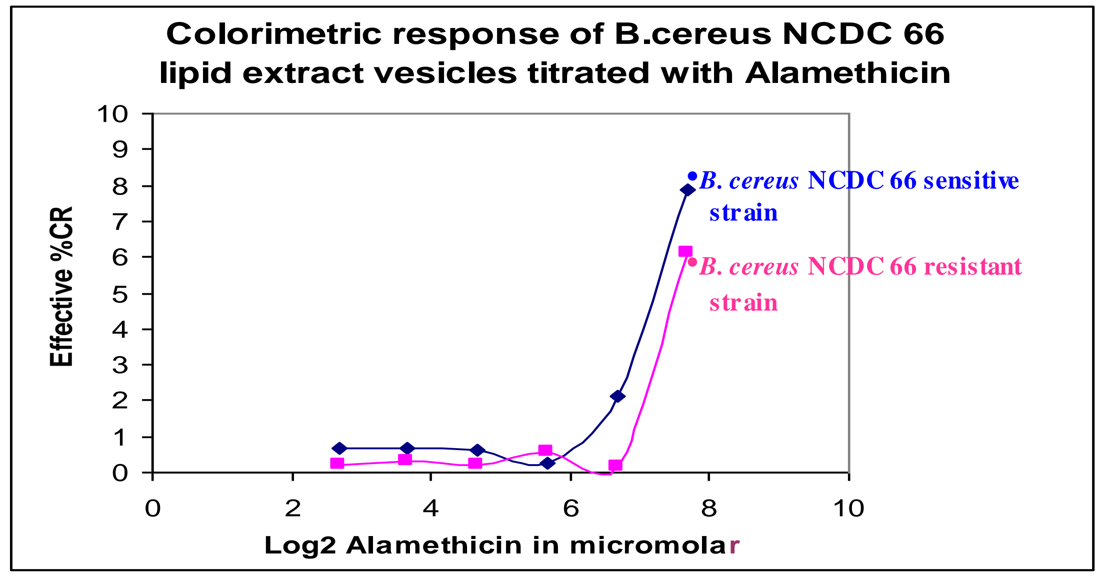


Fig 4.5 Effective colorimetric response of biomimetic vesicles prepared with lipid extracts of alamethicin sensitive strain and resistant mutant of *Bacillus cereus*

FAMEs	C 12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Rt (range)	3.11-3.93	5.53-6.25	8.09-9.75	12.37-13.27	13.08-13.31	13.59-14.99
Rt (avg.)	3.44	5.81	9.07	12.96	13.20	14.27

Table 4.6 Retention times (Rt) of standard fatty acid methyl esters (FAMEs)

FAMEs	C12:0		C14:0		C16:0		C18:0		C18:1		C18:2	
strain	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
cellular lipids	1.94	ND	16.58	10.16	47.37	35.76	18.78	36.03	ND	11.78	ND	0.60
spot 1	1.51	ND	2.39	7.85	40.94	37.16	4.67	6.74	ND	ND	ND	ND
spot 2	3.32	ND	62.45	ND	ND	4.53	7.78	23.18	ND	6.26	ND	0.45
spot 3	ND	ND	6.29	ND	19.41	2.68	24.15	35.31	ND	4.38	ND	ND

Table 4.7 Percentage peak areas of fatty acids in sensitive strain and resistant mutant of *Bacillus cereus* NCDC66. ND- not detectable.

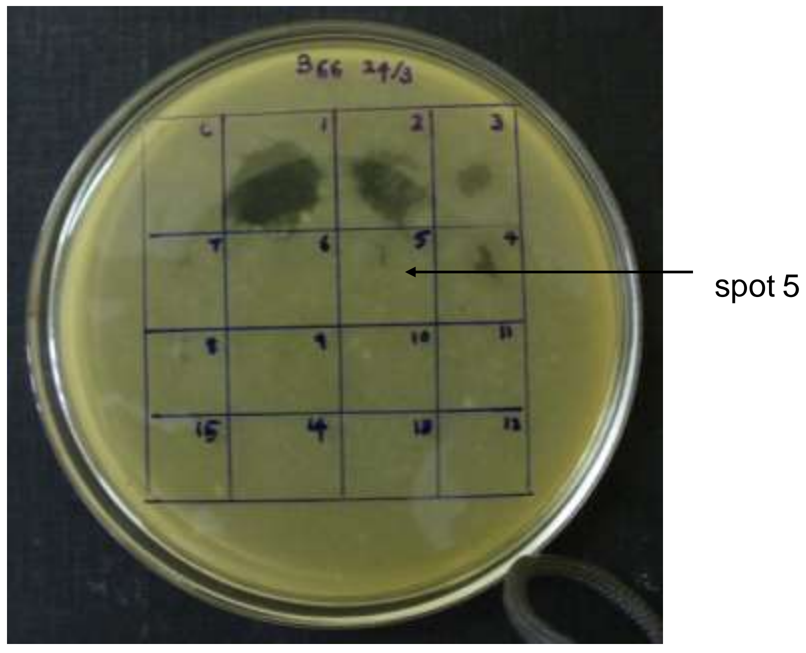


Plate I ***Bacillus cereus*** **NCDC66 showing inhibition zones in spot on lawn assay with alamethicin. The last spot (5) showing inhibition is 1.56 µg.**

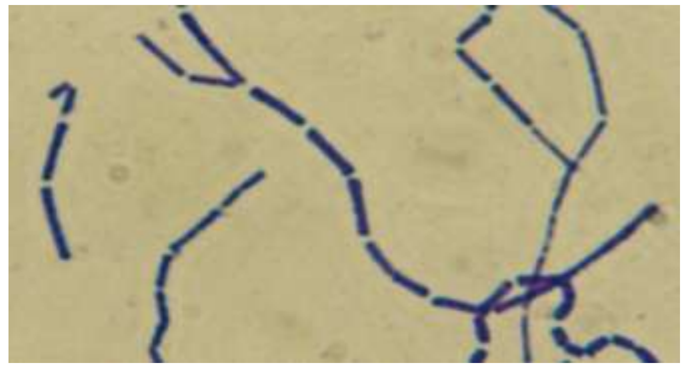


Plate II Photomicrograph of ***Bacillus cereus*** **NCDC66 –Gram staining.**

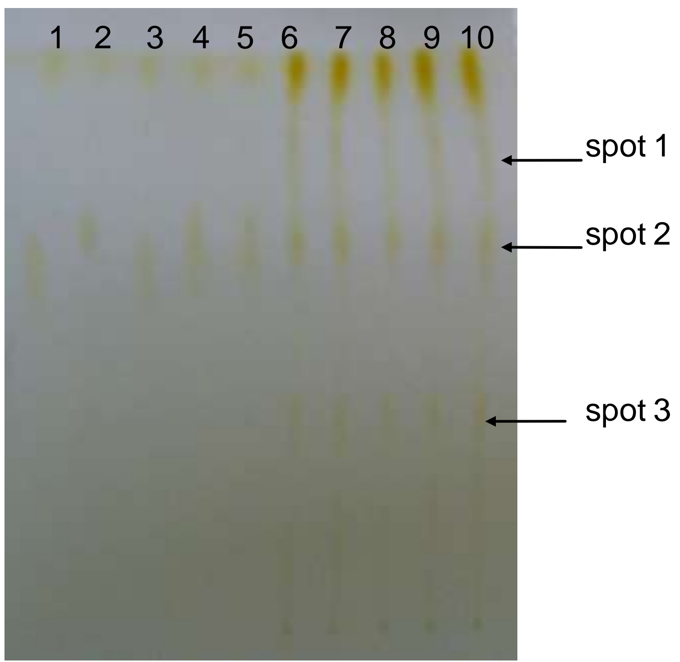


Plate III **TLC analysis of lipids extracted from alamethicin sensitive *B. cereus* NCDC66 visualized with iodine vapors. Lanes: 1, DMPG; 2, DSPE; 3, DPPG; 4,5-DPPG + DSPE; 6-10, lipids extracted from alamethicin sensitive *B. cereus* NCDC66**

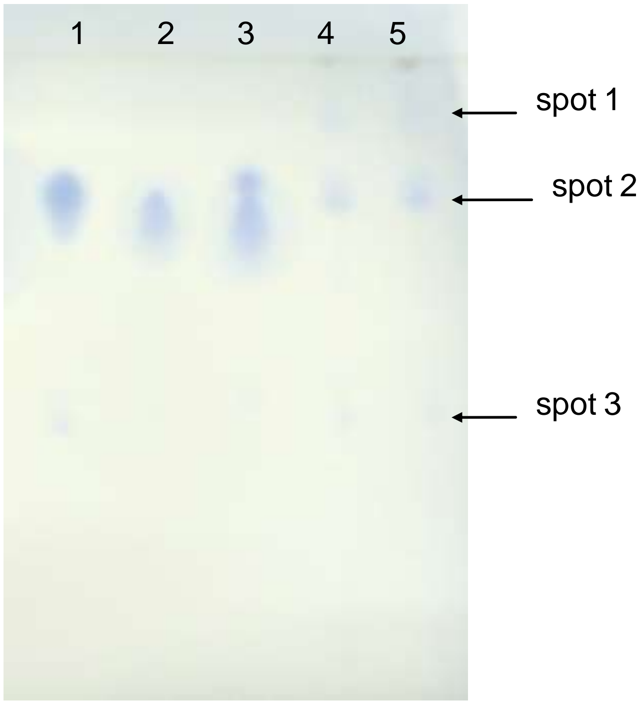


Plate IV **TLC analysis of lipids extracted from alamethicin sensitive *B. cereus* NCDC66 visualized with molybdate reagent. Lanes: 1, DSPE; 2, DPPG; 3, DPPG+DSPE; 4, 5- lipids extracted from alamethicin sensitive *B. cereus* NCDC66**

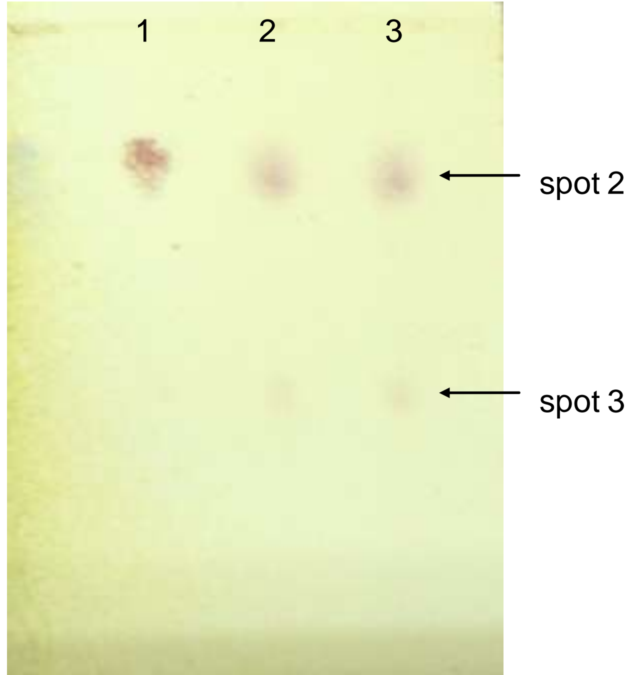


Plate V **TLC analysis of lipids extracted from alamethicin sensitive *B. cereus* NCDC66 visualized with ninhydrin. Lanes: 1, DSPE; 2, 3- lipids extracted from alamethicin sensitive *B. cereus* NCDC66.**

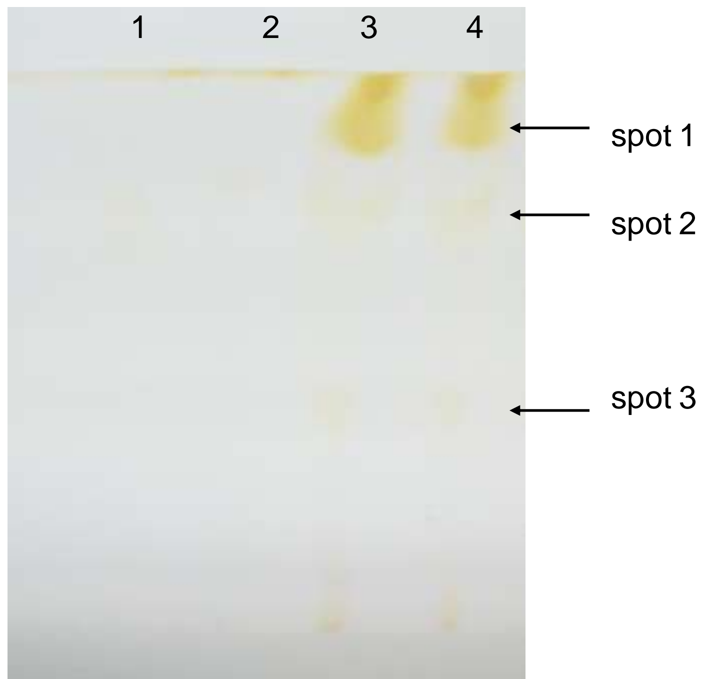


Plate VI **TLC analysis of lipids extracted from alamethicin resistant mutant *B. cereus* NCDC66 visualized with iodine vapors. Lanes: 1, DMPG; 2, DSPE; 3,4- lipids extracted from alamethicin resistant mutant *B. cereus* NCDC66.**

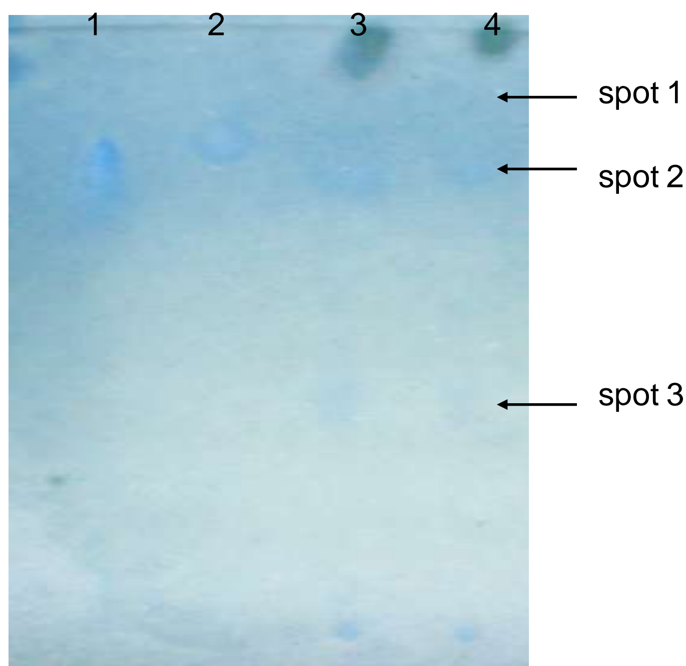


Plate VII **TLC analysis of lipids extracted from alamethicin resistant mutant *B. cereus* NCDC66 visualized with molybdate reagent. Lanes: 1, DMPG; 2, DSPE; 3,4- lipids extracted from alamethicin resistant mutant *B. cereus* NCDC66.**

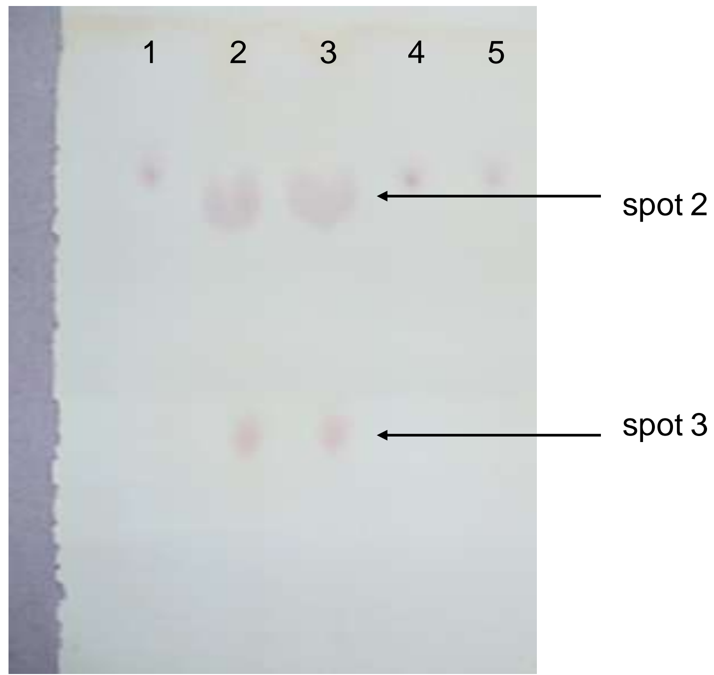


Plate VIII **TLC analysis of lipids extracted from alamethicin sensitive *B. cereus* NCDC66 visualized with ninhydrin. Lanes: 1,4,5- DSPE; 2, 3- lipids extracted from alamethicin sensitive *B. cereus* NCDC66**

Plate IX **Color transition due to interaction of different conc. of alamethicin with vesicles prepared from lipid extracts of alamethicin sensitive *B. cereus* NCDC66 and PDA. 1, control; 2, 204 µM; 3, 102 µM; 3, 51 µM; 4, 25.5 µM; 5, 12.75 µM; 6, 6.37 µM.**

Plate X **Color transition due to interaction of different conc. of alamethicin with vesicles prepared from lipid extracts of alamethicin resistant *B. cereus* NCDC66 and PDA. 1, control; 2, 204 µM; 3, 102 µM; 3, 51 µM; 4, 25.5 µM; 5, 12.75 µM; 6, 6.37 µM.**

5. SUMMARY AND CONCLUSIONS

In the present investigation "Studies on Phospholipids from Alamethicin Sensitive and Resistant Mutants of *Bacillus cereus*", selection of an alamethicin sensitive strain of *Bacillus cereus* and its resistant mutant was carried out to ascertain changes in both polar head groups and hydrophobic fatty acyl chains of phospholipids upon acquisition of resistance against alamethicin and confirm these changes using *in vitro* assay.

Selection of an alamethicin sensitive strain of *Bacillus cereus* and its resistant mutant was carried out using standard microbial culturing and spot on the lawn method with *Bacillus cereus* NCDC66 grown without and with 5 times of IC_{50} Alamethicin concentration. IC_{50} of alamethicin for Bacillus *cereus* NCDC66 was found to be 2.0 µg/ml. IC_{50} for resistant mutant was determined as 9.5 µg/ml. This is the first study reporting resistant mutants to alamethicin in the sensitive population of *B. cereus.*

Total cellular lipids were extracted from sensitive strain and resistant mutant. Changes in head group were ascertained using TLC. Quantification of phospholipid content for each resolved spot was carried out through phosphorous estimation. Total cellular lipid extract of sensitive strain could be resolved in three spots. There was no significant change in the levels of amino group containing phospholipids. In addition, changes in fatty acyl side chains were achieved using GLC.

In total cellular lipids of resistant mutant fatty acid C12:0 was not detectable. Fatty acid C14:0 decreased from 16.58 % in sensitive to 10.16 % in resistant mutant. Similarly, fatty acid C16:0 has decreased from 47.37 % in sensitive to 35.76 % in resistant mutant. However, there is considerable increase in C18:0 from 18.78 % in sensitive strain to 36.03 % in resistant mutant. Overall there is an increase in long chain fatty acids and decrease in short chain fatty acids in resistant mutant compared to the sensitive strain. Fatty acid C18:1 was detectable at the level of 11.78 % and C18:2 at the level of 0.6 % in resistant mutant, whereas fatty acids C18:1 and C18:2 could not be detected in sensitive strain. There is increase in unsaturated fatty acids and

long chain fatty acids in the resistant mutant. This change in the composition of the membrane could be the reason for increase in IC_{50} in resistant mutant.

A colorimetric in vitro assay developed was used to ascertain degree of inhibition via colorimetric response obtained upon interaction of alamethicin with biomimetic membranes prepared from total cellular lipid extract from sensitive strain and resistant mutant. A decrease in effective colorimetric response in vesicles prepared with lipid extracts from resistant mutant as compared to that of the sensitive strain was observed.

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Appendices

REPORT

Sample Name: Standard mix - C12 - C14 - C16 - C18 - C18:2 **Data File:** .... mix\Def24.Dat

Method File: standards.MET

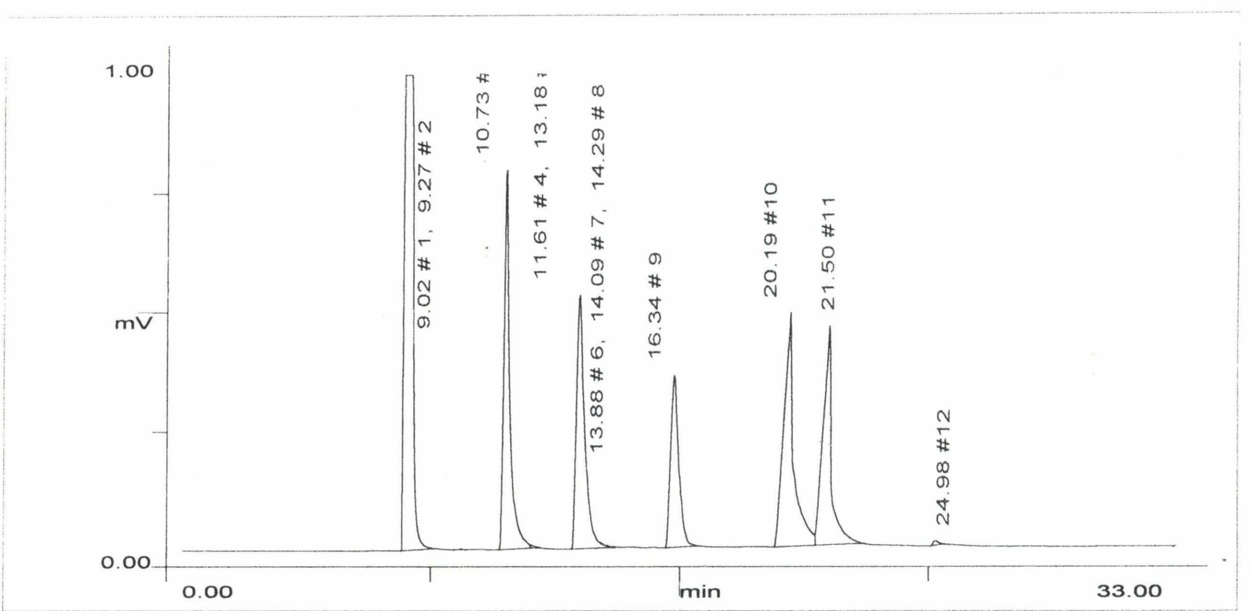
Detector: FID. **System:** GC

Date: 25 May 2007 **Time:** 11:42:6

Run: ch1: 2

Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.38

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	9.02	0	1251	0.0000	0.0024	BB	0.011
2	9.27	89	10494	0.0741	0.0201	BB	0.071
3	10.73	37086	11684315	30.8970	22.3832	BV	0.161
4	11.61	259	69381	0.2158	0.1329	S	0.205
5	13.18	23392	10883933	19.4883	20.8499	BB	0.268
6	13.88	94	11377	0.0783	0.0218	TTT	0.139
7	14.09	66	5985	0.0550	0.0115	TTT	0.106
8	14.29	0	1883	0.0000	0.0036	TTT	0.060
9	16.34	15981	7166258	13.3141	13.7281	BB	0.270

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
10	20.19	21596	12205951	17.9920	23.3825	BV	0.268
11	21.50	21022	10027073	17.5138	19.2085	VB	0.259
12	24.98	446	133413	0.3716	0.2556	BB	0.210
		1e+05	52201314				

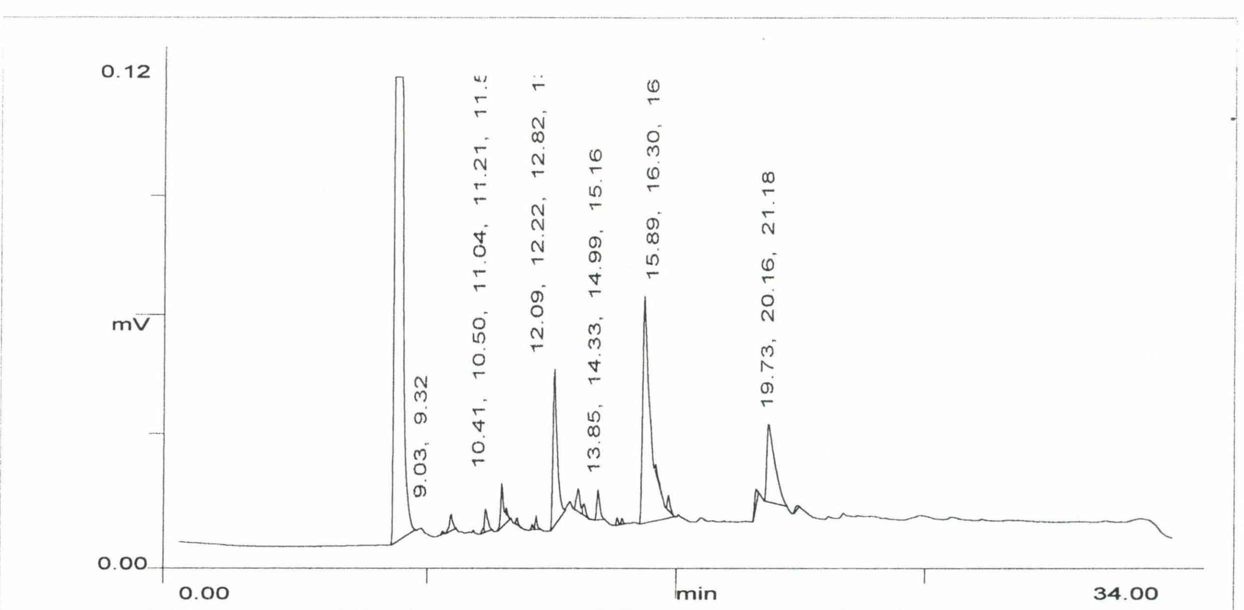
Summary :

TOTAL PEAKS :	12
MUL. FACTOR :	1.0000
SAMPLE AMT.	100.0000
DILUTION :	1.0000
Syssuit(IP) :	

REPORT

Sample Name: B. cereus/Total lipid extract/Sensitive
Data File: ... res\bcres2.Dat
Method File: standards.MET
Detector: FID.
System: GC
Date: 17 May 2007
Time: 11:27:34
Run: ch1: 1
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent Peak - 7.0

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	9.03	0	2296	0.0000	0.0982	BB	0.052
2	9.32	191	33025	2.4690	1.4130	BB	0.093
3	10.41	0	4797	0.0000	0.2052	BV	0.068
4	10.50	274	42859	3.5419	1.8337	VB	0.098
5	11.04	508	84763	6.5667	3.6266	BB	0.082
6	11.21	78	6540	1.0083	0.2798	TTT	0.096
7	11.57	85	8807	1.0988	0.3768	BB	0.060
8	12.09	0	5735	0.0000	0.2454	BV	0.055
9	12.22	155	16312	2.0036	0.6979	VB	0.057

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
10	12.82	1778	394536	22.9835	16.8801	BB	0.139
11	13.67	272	49596	3.5160	2.1219	BV	0.128
12	13.85	127	22915	1.6417	0.9804	VB	0.117
13	14.33	329	56477	4.2528	2.4163	BB	0.106
14	14.99	95	8925	1.2280	0.3818	BP	0.055
15	15.16	80	7160	1.0341	0.3063	PB	0.055
16	15.89	2460	1128099	31.7994	48.2653	BB	0.240
17	16.30	116	18681	1.4995	0.7993	TTT	0.221
18	16.74	174	25217	2.2492	1.0789	TTT	0.120
19	19.73	155	20260	2.0036	0.8668	BB	0.106
20	20.16	859	392891	11.1039	16.8097	BB	0.276
21	21.18	0	7398	0.0000	0.3165	BB	0.115
		8e+03	2337288				

REPORT

Sample Name: B. cereus - tot. lipid extract - Res Resistant Data File: ...ens\efsp1.2.Dat

Method File: standards.MET

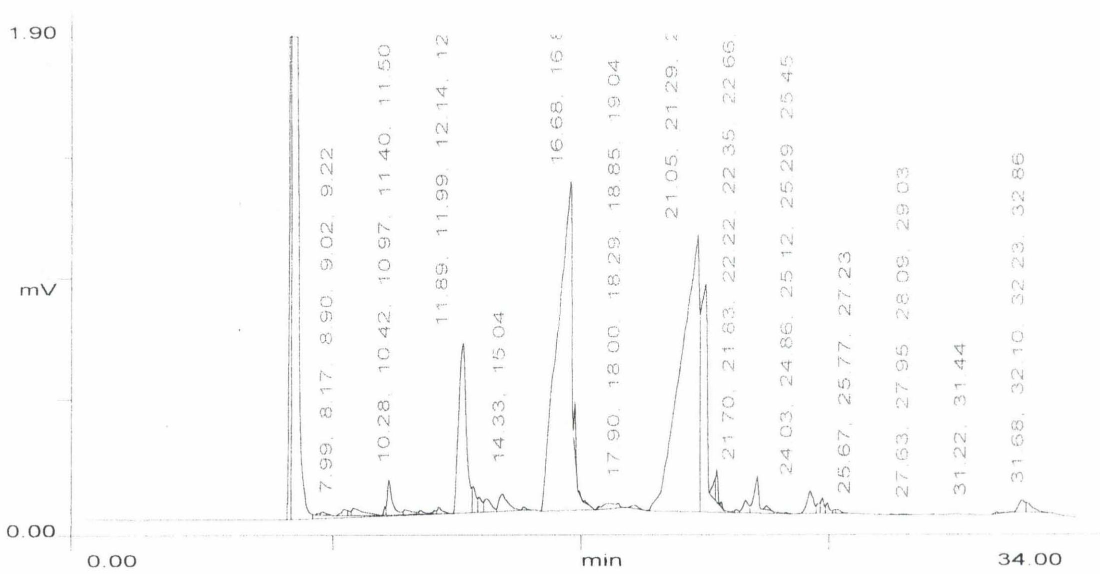
Detector: FID. System: GC

Date: 8 May 2007 Time: 11:11:40

Run: ch1: 1

Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.09

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	7.83	772	1781047	0.2933	1.1777	S	0.082
2	7.99	287	50665	0.1091	0.0335	TTV	0.123
3	8.17	603	213616	0.2291	0.1413	TVP	0.218
4	8.90	1079	335116	0.4100	0.2216	TPV	0.183
5	9.02	881	131228	0.3348	0.0868	TVV	0.117
6	9.22	1263	824879	0.4799	0.5455	TVP	0.382
7	10.28	1614	137467	0.6133	0.0909	TPV	0.063
8	10.42	6232	1706196	2.3680	1.1282	TVV	0.142
9	10.97	935	385212	0.3553	0.2547	TVV	0.311

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
10	11.40	394	44402	0.1497	0.0294	TS	0.079
11	11.50	668	197438	0.2538	0.1306	TVV	0.177
12	11.89	300	29868	0.1140	0.0198	TS	0.076
13	11.99	649	95888	0.2466	0.0634	TVV	0.120
14	12.14	1312	327080	0.4985	0.2163	TVT	0.112
15	12.97	29679	12946471	11.2770	8.5609	PV	0.270
16	13.31	4663	1098003	1.7718	0.7261	VV	0.202
17	13.52	2777	688399	1.0552	0.4552	VV	0.208
18	13.76	2333	981037	0.8865	0.6487	VV	0.298
19	14.33	3051	1592457	1.1593	1.0530	VV	0.328
20	15.04	593	140124	0.2253	0.0927	VB	0.153
21	16.68	58779	49405655	22.3341	32.6697	BV	0.530
22	16.82	11961	1049515	4.5448	0.6940	TTT	0.109
23	16.96	792	67702	0.3009	0.0448	TTT	0.137
24	17.15	536	96366	0.2037	0.0637	TTT	0.213
25	17.90	886	258477	0.3367	0.1709	S	0.068
26	18.00	937	293882	0.3560	0.1943	BV	0.158
27	18.29	967	187759	0.3674	0.1242	VB	0.087
28	18.85	584	223402	0.2219	0.1477	BV	0.298
29	19.04	282	36495	0.1072	0.0241	S	0.071
30	19.30	90	8205	0.0342	0.0054	PV	0.071
31	21.05	48552	50375081	18.4481	33.3107	VV	0.694
32	21.29	39930	16239728	15.1721	10.7386	VV	0.270
33	21.60	3632	550597	1.3800	0.3641	TTV	0.194
34	21.70	6404	771453	2.4333	0.5101	TVV	0.101
35	21.83	1262	127400	0.4795	0.0842	TVT	0.098
36	22.22	273	36796	0.1037	0.0243	S	0.101
37	22.35	578	102038	0.2196	0.0675	VV	0.137
38	22.66	2259	638143	0.8583	0.4220	VV	0.213
39	23.08	6635	2183059	2.5211	1.4436	VB	0.153
40	23.38	834	171527	0.3169	0.1134	TTT	0.257
41	24.03	102	22740	0.0388	0.0150	TTT	0.167
42	24.86	4081	1446740	1.5506	0.9567	BV	0.248
43	25.12	1745	321476	0.6630	0.2126	VV	0.142
44	25.29	2784	542960	1.0578	0.3590	VV	0.150
45	25.45	1888	365741	0.7174	0.2418	VV	0.131

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
46	25.67	548	85383	0.2082	0.0565	S	0.115
47	25.77	666	186753	0.2531	0.1235	BB	0.186
48	27.23	80	12622	0.0304	0.0083	BB	0.096
49	27.63	218	41989	0.0828	0.0278	BV	0.101
50	27.95	193	34905	0.0733	0.0231	VV	0.147
51	28.09	146	23456	0.0555	0.0155	VB	0.109
52	29.03	129	22501	0.0490	0.0149	BB	0.109
53	31.22	358	95935	0.1360	0.0634	BB	0.147
54	31.44	0	3163	0.0000	0.0021	TTT	0.082
55	31.68	0	6807	0.0000	0.0045	BB	0.096
56	32.10	2160	735942	0.8207	0.4866	BV	0.246
57	32.23	1673	707158	0.6357	0.4676	S	0.240
58	32.86	152	41727	0.0578	0.0276	S	0.145
		3e+05	151227869				

Summary :

TOTAL PEAKS : 5
MUL. FACTOR : 1.0000
SAMPLE AMT. 100.0000
DILUTION : 1.0000
Syssuit(IP) :

REPORT

Sample Name: B. cereus - Sensitive - Spot1 Data File: ... sens\bcsp1.Dat

Method File: standards.MET

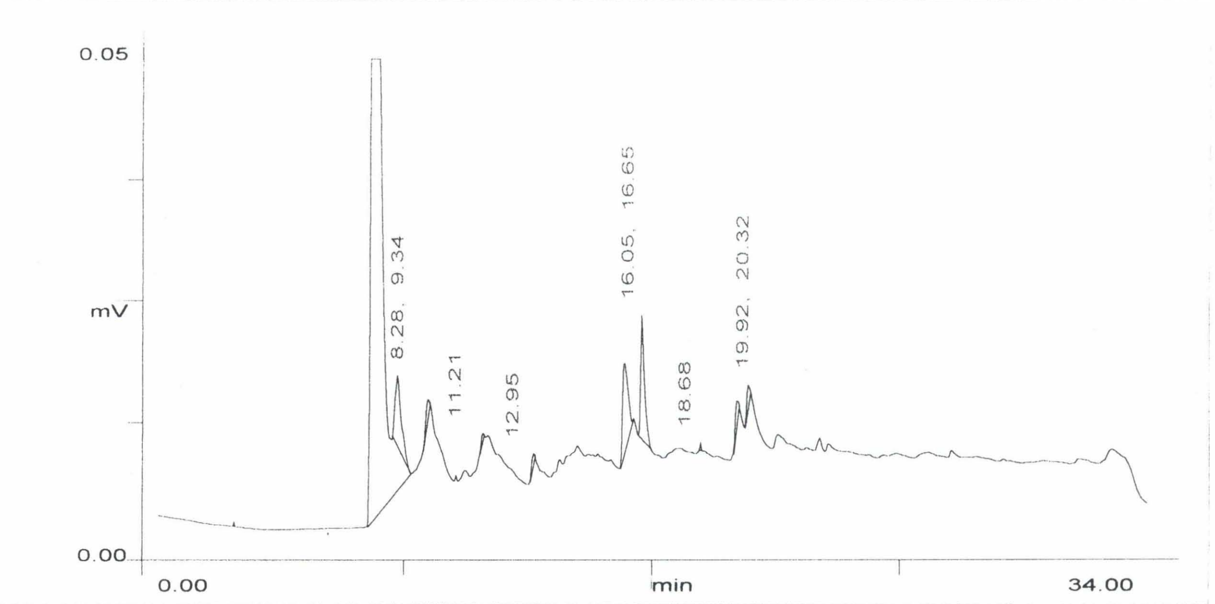
Detector: FID. System: GC

Date: 5 May 2007 Time: 13:13:10

Run: ch1: 3

Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.38

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	8.28	296	0	21.2644	0.0000	TTT	0.333
2	9.34	0	12848	0.0000	4.5885	BB	0.101
3	11.21	0	4204	0.0000	1.5014	BB	0.074
4	12.95	0	6653	0.0000	2.3761	BB	0.082
5	16.05	380	110860	27.2989	39.5926	BB	0.257
6	16.65	526	113763	37.7874	40.6294	BB	0.123
7	18.68	0	2152	0.0000	0.7686	BB	0.030
8	19.92	99	16536	7.1121	5.9057	BB	0.123
9	20.32	91	12986	6.5374	4.6378	BB	0.115

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
		1e+03	280002				

Summary :

TOTAL PEAKS : 9
MUL. FACTOR : 1.0000
SAMPLE AMT. 100.0000
DILUTION : 1.0000
Syssuit(IP) :

REPORT

Sample Name: B. cereus - Resistant - Spot 1 **Data File:** ... res\bcrsp1.Dat

Method File: standards.MET

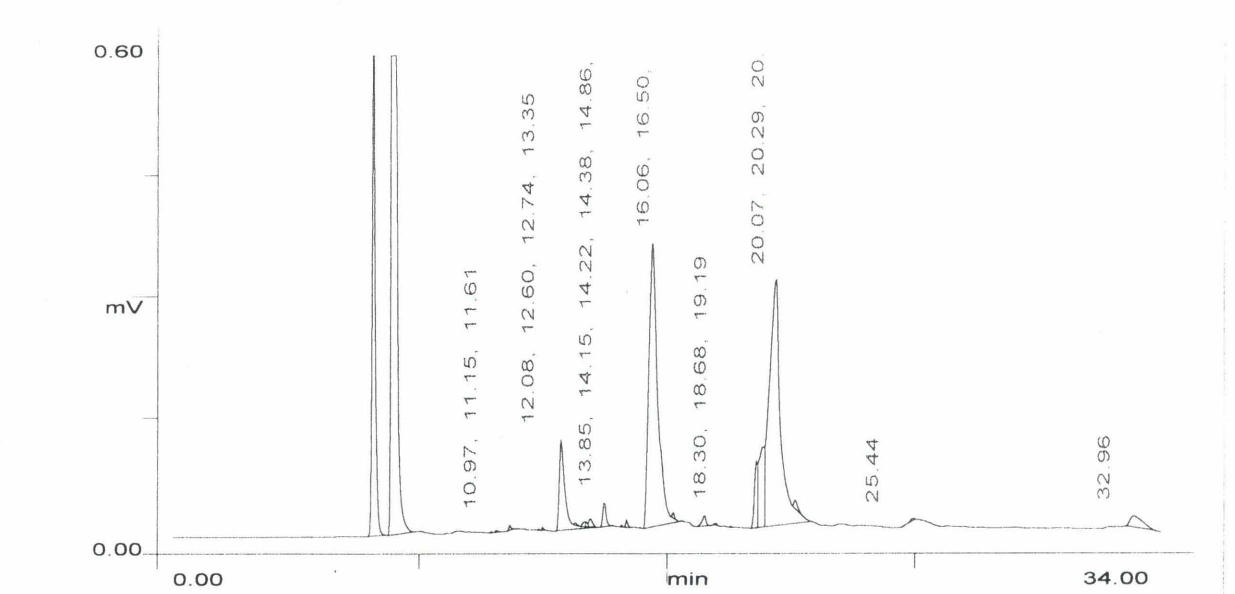
Detector: FID. **System:** GC

Date: 18 May 2007 **Time:** 11:29:32

Run: ch1: 1

Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.85

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	10.97	0	5898	0.0000	0.0272	BV	0.074
2	11.15	107	13065	0.2204	0.0602	VB	0.071
3	11.61	281	31951	0.5787	0.1472	BB	0.063
4	12.08	0	1657	0.0000	0.0076	BB	0.041
5	12.60	0	4143	0.0000	0.0191	BP	0.046
6	12.74	175	15695	0.3604	0.0723	PB	0.049
7	13.35	5018	1652493	10.3349	7.6118	BB	0.183
8	13.85	140	25894	0.2883	0.1193	TTT	0.205
9	14.15	267	39089	0.5499	0.1801	TTV	0.115

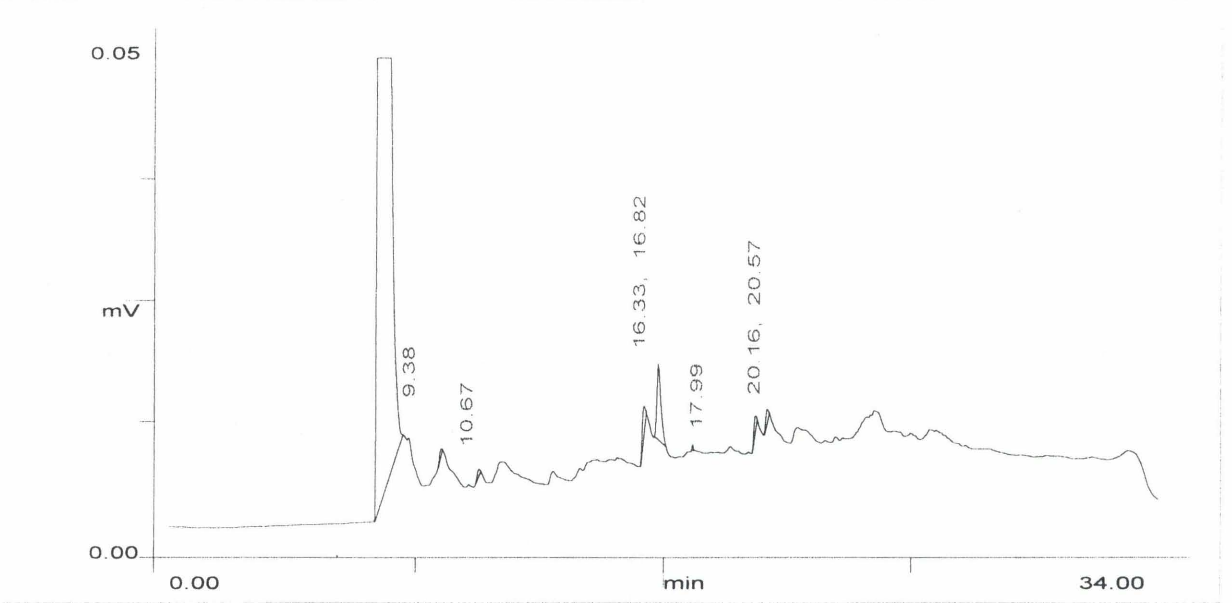
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
10	14.22	316	32320	0.6508	0.1489	TVV	0.093
11	14.38	471	102771	0.9701	0.4734	TVT	0.156
12	14.86	1375	239539	2.8319	1.1034	BB	0.109
13	15.45	128	9722	0.2636	0.0448	BP	0.049
14	15.62	486	46432	1.0009	0.2139	PB	0.052
15	16.06	0	1648	0.0000	0.0076	BB	0.035
16	16.50	15487	7813714	31.8964	35.9922	BB	0.289
17	16.79	563	30259	1.1595	0.1394	TTT	0.076
18	17.22	323	33627	0.6652	0.1549	TTT	0.115
19	17.49	0	1494	0.0000	0.0069	TTT	0.068
20	18.30	618	137187	1.2728	0.6319	BB	0.131
21	18.68	103	13872	0.2121	0.0639	BB	0.109
22	19.19	0	4612	0.0000	0.0212	BB	0.052
23	20.07	3649	598939	7.5153	2.7589	BV	0.112
24	20.29	4381	1417548	9.0229	6.5296	VV	0.243
25	20.75	13440	8843109	27.6805	40.7338	VB	0.371
26	21.42	506	111055	1.0421	0.5116	TTT	0.238
27	25.44	98	20579	0.2018	0.0948	BB	0.117
28	32.96	622	461174	1.2810	2.1243	BB	0.445
		5e+04	21709485				

Summary :

TOTAL PEAKS : 28
MUL. FACTOR : 1.0000
SAMPLE AMT. 100.0000
DILUTION : 1.0000
Syssuit(IP) :

Sample Name: B. cereus-Sensitive-Spot 2 | Data File: ... sens\bcsp2.Dat
Method File: standards.MET
Detector: FID. | System: GC
Date: 5 May 2007 | Time: 12:36:26
Run: ch1: 2
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.18

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	9.38	0	3118	0.0000	2.7029	BB	0.057
2	10.67	0	3831	0.0000	3.3210	BB	0.096
3	16.33	96	18307	22.8029	15.8700	BB	0.120
4	16.82	325	72049	77.1972	62.4580	BB	0.123
5	17.99	0	1377	0.0000	1.1937	BB	0.014
6	20.16	0	8981	0.0000	7.7855	BB	0.096
7	20.57	0	7693	0.0000	6.6689	BB	0.106
		421	115356				

Summary :

TOTAL PEAKS :	7
MUL. FACTOR :	1.0000
SAMPLE AMT.	100.0000
DILUTION :	1.0000
Syssuit(IP) :	

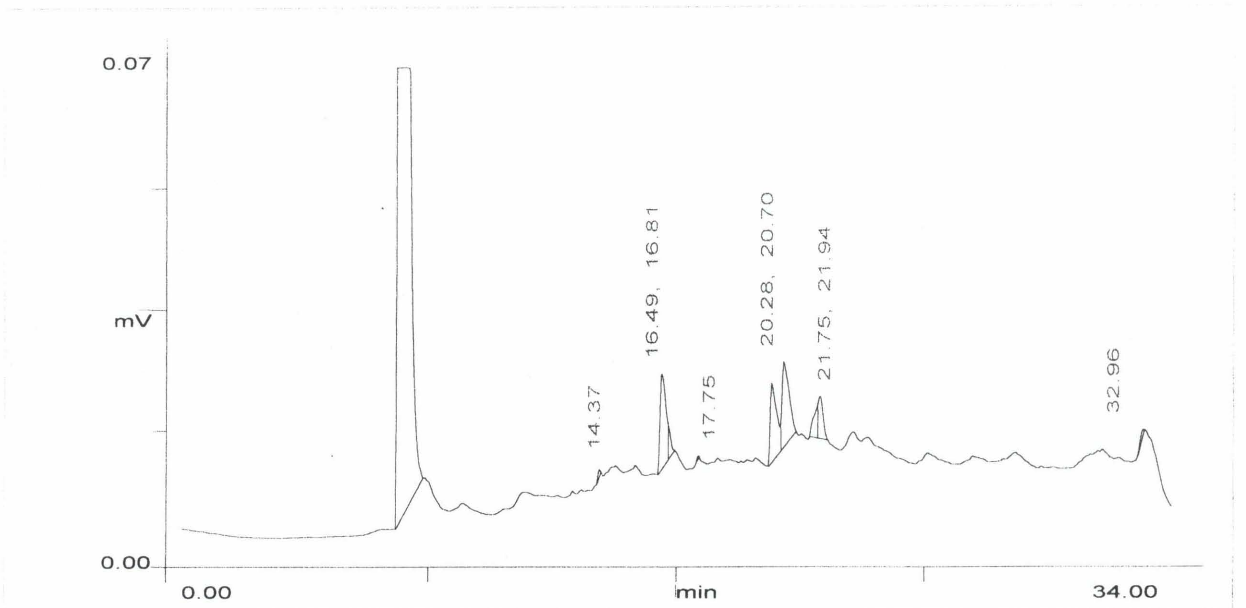
REPORT

Sample Name: Bc/Res/Spot2
Method File: standards.MET
Detector: FID.
Date: 18 May 2007
Run: ch1: 2
Type of Analysis : Percent On Area and Height

Data File: ... res\bcrsp2.Dat

System: GC
Time: 12:7:18

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent Pk - 7.47

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	14.37	0	5165	0.0000	0.7135	BB	0.079
2	16.49	581	183618	27.7990	25.3637	BV	0.265
3	16.81	126	32954	6.0287	4.5521	S	0.134
4	17.75	0	2084	0.0000	0.2879	BB	0.068
5	20.28	479	167837	22.9187	23.1839	BV	0.287
6	20.70	508	202395	24.3062	27.9574	VB	0.298
7	21.75	136	45353	6.5072	6.2648	S	0.221
8	21.94	260	74943	12.4402	10.3521	BB	0.194
9	32.96	0	9590	0.0000	1.3247	BB	0.085

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
		2e+03	723939				

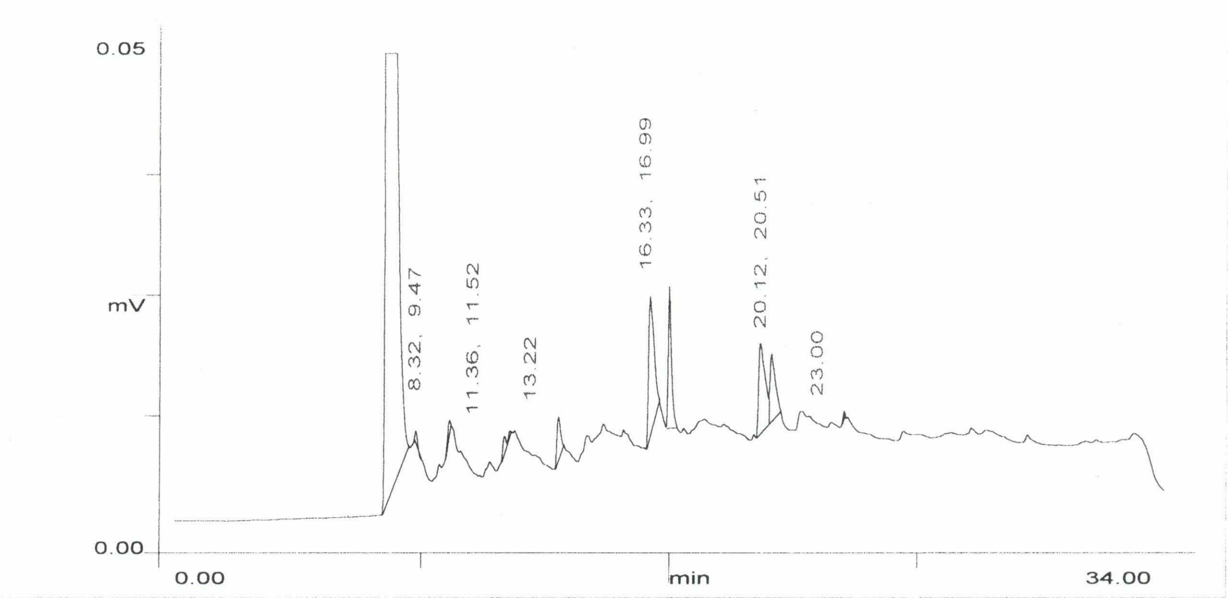
Summary :

TOTAL PEAKS : 9
MUL. FACTOR : 1.0000
SAMPLE AMT. 100.0000
DILUTION : 1.0000
Syssuit(IP) :

REPORT

Sample Name: B. cereus - Sensitive - Spot 3 Data File: ... sens\bcsp3.Dat
Method File: standards.MET
Detector: FID. System: GC
Date: 5 May 2007 Time: 11:55:50
Run: ch1: 1
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.30

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	8.32	0	7891	0.0000	1.3490	BB	0.090
2	9.47	0	9429	0.0000	1.6119	BB	0.090
3	11.36	68	11417	3.2015	1.9517	BP	0.128
4	11.52	0	2501	0.0000	0.4275	PB	0.074
5	13.22	170	36484	8.0038	6.2369	BB	0.147
6	16.33	591	173329	27.8249	29.6304	BB	0.227
7	16.99	624	112573	29.3785	19.2442	BB	0.106
8	20.12	385	140073	18.1262	23.9453	BV	0.289
9	20.51	286	88641	13.4652	15.1530	VB	0.229

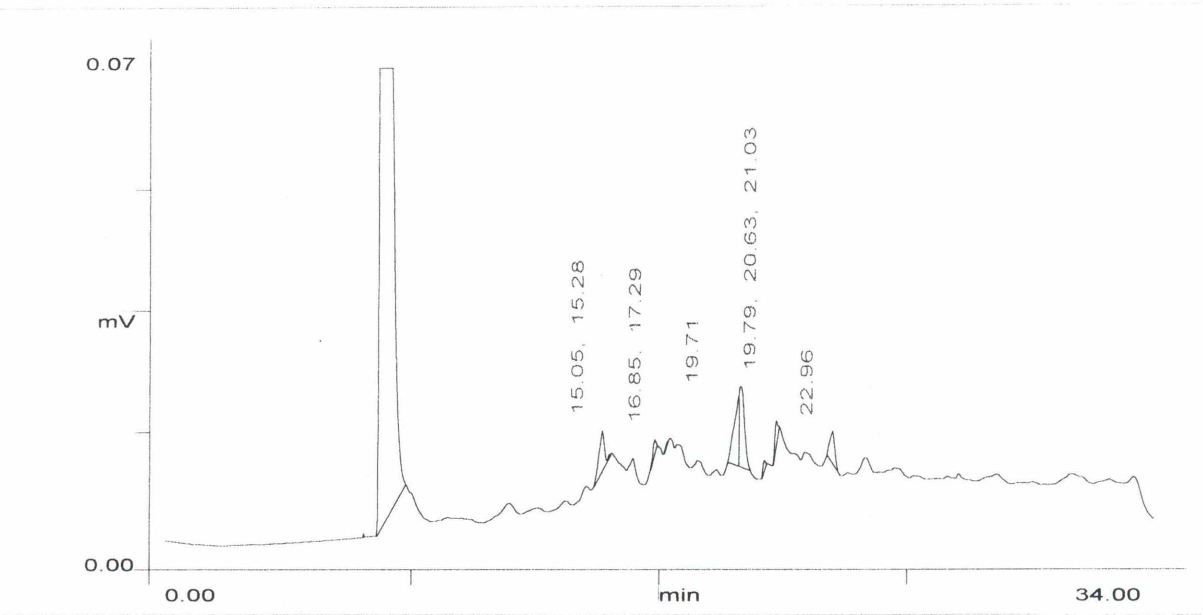
No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
10	23.00	0	2633	0.0000	0.4501	BB	0.057
		2e+03	584971				

Summary :

TOTAL PEAKS : 10
MUL. FACTOR : 1.0000
SAMPLE AMT. 100.0000
DILUTION : 1.0000
Syssuit(IP) :

Sample Name: B. cereus-resistant-Spot3 Data File: ... res\bcrsp3.Dat
Method File: standards.MET
Detector: FID. System: GC
Date: 18 May 2007 Time: 12:46:36
Run: ch1: 3
Type of Analysis : Percent On Area and Height

Pk.Wdth	Peak Thrsh.	Area Rej.	Ht.Rej.	Time Scale
4	30	5	4	20.0



Solvent RT - 7.45

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
1	15.05	241	73878	15.4685	16.7056	BP	0.197
2	15.28	0	1484	0.0000	0.3356	PB	0.060
3	16.85	82	11821	5.2632	2.6730	BB	0.109
4	17.29	0	2716	0.0000	0.6142	BB	0.046
5	19.71	413	111482	26.5083	25.2087	S	0.197
6	19.79	478	155666	30.6804	35.1997	BB	0.205
7	20.63	0	7837	0.0000	1.7721	BB	0.109
8	21.03	137	19306	8.7933	4.3655	BB	0.120
9	22.96	207	58046	13.2863	13.1256	BB	0.216

No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Peak Width
		2e+03	442236				

Summary :

TOTAL PEAKS :	9
MUL. FACTOR :	1.0000
SAMPLE AMT.	100.0000
DILUTION :	1.0000
Syssuit(IP) :	

APPENDIX-II

Sl. No.	Chemical	Company/ catalogue number
1	Silica gel G	S.D.Fine – Chem. Limited
2	Dextrose (AR)	Sisco Research Laboratories Pvt. Ltd.
3	Chloroform (AR)	Sisco Research Laboratories Pvt. Ltd.
4	Methanol (AR)	Sisco Research Laboratories Pvt. Ltd.
5	Alamethicin	Sigma - Aldrich
6	Peptone(bacteriological grade)	S.D.Fine – Chem. Limited
7	Yeast extract powder bacto	S.D.Fine – Chem. Limited
8	Hydrochloric acid (AR)	S.D.Fine – Chem. Limited
9	Hexane (LR)	S.D.Fine – Chem. Limited
10	10,12-tricosadiynoic acid	Sigma - Aldrich
11	Agar powder (bacteriology)	Sisco Research Laboratories Pvt. Ltd.
12	Sodium chloride (AR)	Sisco Research Laboratories Pvt. Ltd.
13	Acetic acid	Sisco Research Laboratories Pvt. Ltd.
14	Sodium bicarbonate	Hi media
15	Ninhydrin	Sisco Research Laboratories Pvt. Ltd.
16	Ammonium molybdate	Sisco Research Laboratories Pvt. Ltd
17	Iodine	Sisco Research Laboratories Pvt. Ltd.
18	Barium chloride	Sisco Research Laboratories Pvt. Ltd

APPENDIX-III

LABORATORY EQUIPMENTS

Sl. No.	Equipment	Brand name
1	Thin Layer Chromatoset	Super fit
2	TLC developing chamber	Perfit
3	TLC plates	Perfit
4	Distillation plant	Scientronic
5	Laminar air flow chamber	Widsons scientific works
6	Microcentrifuge	Remi (RM12C)
7	Gas chromatograph	Michro 9100, Netel chromatographs
8	Microlitre syringe	Hamilton
8	Oven	Akashdeep, Scientronic
9	pH meter	Cyberscan 500
10	Refrigerated high speed micro centrifuge	Sigma K15
11	Electronic weighing balance	Sartorious BP211D
12	Electronic weighing balance	GR – 200, AND
13	Spectrophotometer	Specord 200, Analyticjena
14	Centrifuge	Hitachi
15	Incubator (37 ^{o}C)	Scientronic Instruments