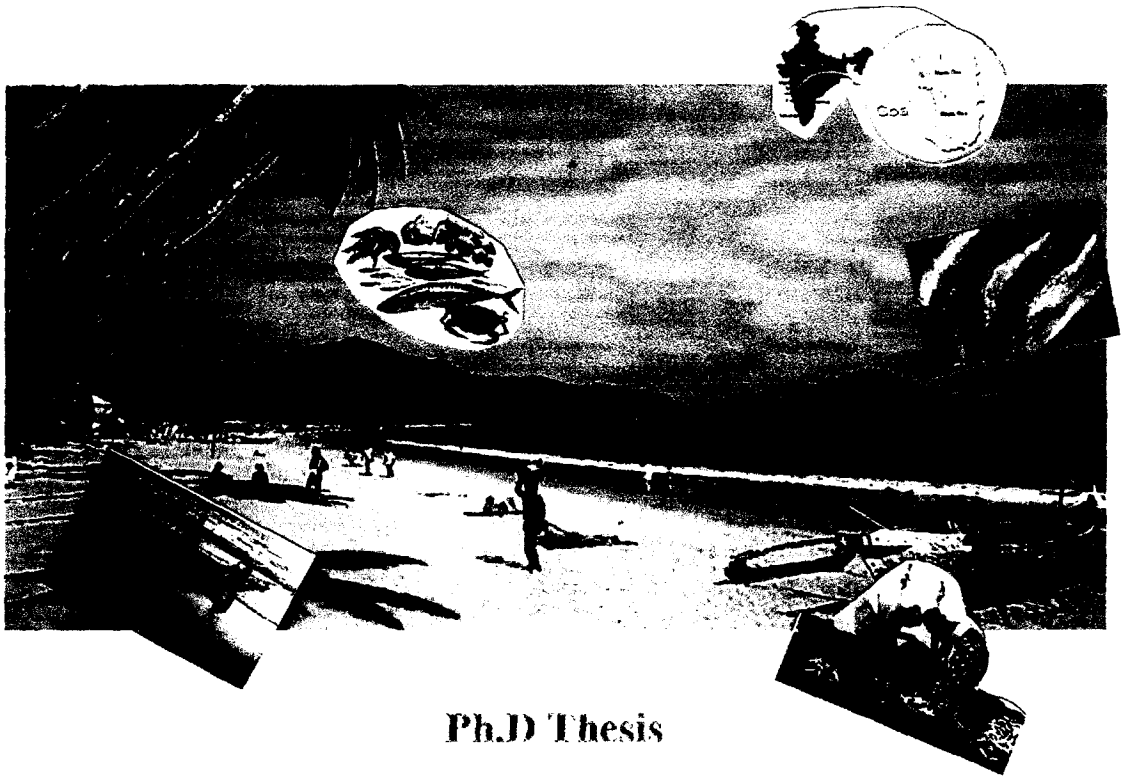
SCREENING, DETECTION AND CHARACTERIZATION OF BACTERIAL FISH PATHOGENS IN COASTAL REGION OF GOA



Ph.D Thesis

By

Anju Pandey

579
PAN/Scr

DEPARTMENT OF MICROBIOLOGY
GOA UNIVERSITY
TALEGAO PLATEAU
GOA- 403206

2011

T-512

"Screening, detection and characterization of bacterial fish pathogens in the coastal region of Goa"

Thesis submitted
to

GOA UNIVERSITY

For the degree
of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

by

Anju Pandey

Under the guidance of

Dr. Santosh Kumar Dubey
Professor
Department of Microbiology
Goa University,
Goa, India.

2011

गोंय विद्यापीठ
ताळगांव पठार
गोंय - ४०३ २०६
फोन : ०८३२-६५१९०४८
फॅक्स : +०९१-८३२-२४५१९८४/२४५२८८९

Goa University
Taleigao Plateau, Goa - 403 20
Tel : 0832-651904
Fax : +091-832 -2451184/245288
Email : registrar@unigoa.ac.in
Website : www.unigoa.ac.in

CERTIFICATE

This is to certify that Anju pandey has worked on the thesis entitled **"Screening, detection and characterization of bacterial fish pathogens in coastal region of Goa"** in my Laboratory of Environmental Microbiology and Biotechnology at Goa University, under my supervision and guidance. This thesis, being submitted to Goa University, Taleigao Plateau, Goa for the award of the degree of Doctorate of Philosophy in Microbiology, is an original record of work carried out by the candidate herself and has not been submitted for the award of any other degree or diploma of this or any other University in India or abroad.

24/10/2011

Date:

Santosh Kumar Dubey
Santosh Kumar Dubey.

Dr. Santosh Kumar Dubey
Research Guide &
Associate Professor
Department of Microbiology

STATEMENT

I hereby state that this thesis for the Ph.D. degree on "Screening, detection and characterization of bacterial fish pathogens in coastal region of Goa" is my original contribution and the thesis or any part of it has not been previously submitted for the award of any degree/diploma of any other University.

Date: ~~24th Dec 2010~~ 24/10/2011

Anju Pandey
Department of Microbiology
Goa University, Goa

ACKNOWLEDGEMENT

The execution as well as accomplishment of this humble endeavor of mine has been possible only due to the valuable guidance and precious support of all the people who, in one way or other, have been benevolent and forthcoming to assist me in their best possible manner.

First and foremost, I owe my deepest gratitude to my Guide, Dr. Santosh Kumar Dubey for his encouragement, his relentless efforts and his patience in guiding me through the entire period of my research work. His vast scientific experience and considerable knowledge of the subject has been immensely advantageous to me and kept me improving ultimately leading to the completion of the research. There is no gainsaying that successful conclusion of this work has been wholly dependent on his precious guidance, support and advice.

I would also like to take this opportunity to extend my earnest thanks to Prof. (Dr.) P.V. Desai, Ex-Dean and Prof. (Dr.) G.N. Naik, Dean, Faculty of life sciences, Dr. S.K. Shyama, Associate Professor, Zoology Department for their critical appraisal of my research work periodically and enriching it with their valuable insights. A special thanks of mine goes to Dr. Sanjeev Ghadi, Associate Professor, Department of Biotechnology, for extending the facilities of the lab in Biotechnology Department for my research work.

I am also extremely grateful to all the faculty members of the Microbiology Department, Prof. (Dr.) Saroj Bhosle, Dr Irene Furtado, Dr. Sarita Nazreth and Dr. Sandeep Garg for their genuine support, advices and patronage during the continuation of my work. In addition, let me also express my heartfelt appreciation for all the assistance extended to me by our office staff members. I feel obliged to Mr Shashi, Mr Budhaji, Mrs. Saraswati, Mr. Ladu and Mr. Domnic for their cooperation and the technical assistance, whenever needed.

Lest I forget, I wish to acknowledge the financial support provided by Goa University in the form of Research Studentship.

It gives me utmost pleasure to thank those who have been part of my life at the precinct of the University. Their cheerful presence has not only enlivened my stay at the Department but has also given me the confidence to turn to them for any help at any time without any glitch. My sincere thanks to Akshaya, Teja, Brenda, Nimali, Celisa, Lakshangy, Mufida, Shweta, Milind, Trelita, Pramod, Marielou, Valerie, Christabel, Subojit, Sanika and Sherryan.

All the words seem to be inadequate to express my gratitude to my dear friend Vidya who had been of immense help and support during my initial stage of research work and kept inspiring me even after migrating from Goa. I would also like to thank Neelam, Bramha, Naveen, Krishnamurthy, Christna, Rahul for the much needed encouragement provided by them.

I would also like to utilise this opportunity to express my reverence to my parents for their blessings and to remember the back up, encouragement and their unending support in every venture of my life. Let me also express my gratefulness for the sacrifices made by my better-half Arun and two adorable sons of mine – Yajush & Tarush, in accommodating and putting up with the strenuous schedule of my research work.

Finally, may be in the last but certainly not in the least, I thank almighty God, for giving me the strength, courage and perseverance to successfully complete this research work.

Anju Pandey

ABBREVIATIONS

ABS	Absorbance
APS	Ammonium per sulphate
Bps	Base pairs
°C	Degree Celsius
cfu	Colony forming units
Conc.	Concentration
EDTA	Ethylene diamine tetra acetic acid
ECP	Extracellular proteins
Fig.	Figure
GC	Gas chromatography
HCl	Hydrochloric acid
Hrs	Hours
dNTPs	Dioxyneucleoside triphoshate
kDa	Kilo Dalton
LB	Luria Bertani Broth
M	Molar
Mg	Milli gram(s)
ml	Milli letre
Min	Minute(s)
NaCl	Sodium Chloride
NA	Nutrient agar
O.D	Optical density
PAGE	Poly arcylamide gel electrophoresis
Rpm	revolution per minute
SDS	Sodium dodecyl sulphate
Sp.	Species
TEMED	Tetra methyl ethylene diamine
UV	Ultra violet
V	Volts
α	Alpha
β	Beta
μ	Micro

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d Pyrrolo(1,2-a) pyrazine-1,4-dione,

hexahydro-3 (-2- methyl propyl)

e Pentafluoro propionic acid heptadecyl ester

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CHAPTER I

INTRODUCTION

Introduction

1.1 General

Goa is a small state situated at the western coast of India with a coast line of 105 kms and livelihood of the coastal inhabitants depends entirely on marine resources including seafoods. It is interesting to note that Goa is well known for its international cuisine prepared using seafood viz. fish, prawn and shell fish blended with local spices, besides their use as staple food for majority of Goan coastal population. The rich fish diversity of the Goan coast includes Mackerel, Seer fish, Pomfrets, Sardines, White sardines, Butter fish, Silver belly, Cuttle fish or Squids ,Catfish and Mullets. Goa has an active fishing area of about 20,000 square kms (105 km long coastline and inland waterways of about 555 kms). India is the fourth largest producer of fish in the world and second largest producer of inland fish. Fish products also play an imperative role in Goan economy, as export of fish through Mormugao harbour during the year 2008-09 was 21,328 tonnes, which realized a foreign exchange worth Rs 96.89 crores (Economic Survey: Directorate of Planning, Statistics & Evaluation, Govt. of Goa, 2009-2010). There is huge demand of ready to eat seafood products in the international market which is exported extensively.

Prevalence of various pathogens in the aquatic environment of susceptible fish, shrimp, shellfish and other sea foods is a matter of serious concern for environmental microbiologists and fishery scientists involved in monitoring sea

food pathogens, affecting their survival and bioproductivity. Occurrence of diseases is a significant setback to successful aquafarming not only in India but worldwide. Bacteria are among the most common pathogens and prevalent cause of morbidity and mortality among cultured and wild fish populations causing major losses to the aquaculture industry.

1.2 Marine fish pathogens

There are two types of diseases that affect fish population, non-infectious (environmental, nutritional or genetic), and infectious (caused by infectious microbes). The latter are contagious and pose a universal threat to the aquaculture and wild fish industry and subsequently to public health. These diseases may be caused by many pathogenic organisms' viz. bacteria, fungi, viruses and protozoa. These pathogenic organisms may be present in the environment or carried by other fish. Infectious disease results from a series of complex interactions of the pathogen, host and surrounding environment. Many characteristics of the pathogen are directly relevant to the disease development which includes whether the microorganism is always associated with infection of the host (obligate) or it can survive in the absence of the host (facultative) (Engelkin et al., 1991, Hedrick, 1998; Karunasagar and Karunasagar 1999; Klinger and Floyed, 2008).

Among various fish pathogens, bacteria constitute the major group posing significant threats to the fishery and aquaculture industry world wide.

Fungal diseases are one of the major problems among fish diseases, second only to the bacterial infections. According to fish pathologists mycoses

deprives fish of its natural strength hindering the internal function of the organ responsible for consequential fish death (Roberts, 1989; Ramaiah, 2006). Mycoses are infections due to fungi or in other words, are the condition in which fungi pass the resistance barriers of the human or animal body and establish infections. Saprolegniasis is among the most common fungal diseases of fish and fish eggs caused by various groups of aquatic fungi; primarily *Saprolegnia*, *Achlya* and *Aphanomyces*. Gross signs are grayish white, cotton-like growths on the skin, gills, eyes, or fins that may invade deeper tissues of the body (Stueland et al., 2005; Mousavi et al., 2009; Yan-Zhi et al., 2001). Branchiomycosis is another fungal disease of gill tissues characterized by respiratory distress and gill necrosis. The causative agents are *Branchiomyces sanguinis* and *B. demigrans* (Rehulka, 1991; Klinger and Fransis, 2008). In ichthyosporidiosis symptoms are small round occasionally ulcerated black granulomas in the fish skin. Internally, numerous granulomas are observed in many visceral organs. Causative agents are *Ichthyophonus hoferi* and *I. gasterophyllim* (Spanggaard, et al. 1994; Kocan,et al. 2004, 2009).

Viruses are important fish pathogens, often species-specific and tissue-specific. It is difficult to isolate viral agents from many fish tissues because there may not be a commercially available cell-line for an individual fish species (Wolf, 1988; Walker and Winton, 2010). Herpes virus is one of the prominent pathogens responsible for infections in salmonids, carps, catfish and ornamental fish koi. Rhabdovirus is responsible for viral hemorrhagic septicemia in salmonids (Meyers and Winton,1995; Mortensen et al.,1998;

Tordo et al., 2005, Kim and Faisal, 2010) and spring viremia of carp (Ahne et al., 2002). Infectious pancreatic necrosis (IPN) in salmonids and other fishes is another significant disease with high mortality rate caused by Birnavirus (Varvarigos and Way, 2002; Pikarsky et al., 2004). Lymphocystis is caused by Iridovirus (Smail and Munro, 2001).

Animal parasites also affect fish health, growth and their survival. Isopods associated with many species of commercially important tropical marine fishes around the world cause significant economic losses to fishery industry by killing, stunting or damaging fish population (Ravichandran et al., 2008, 2009). Some parasites can be considered serious threat for mariculture, but have not been reported in wild populations (Guo and Woo, 2009). Certain parasites are common in both wild and cultured fish (Kırkosek et al., 2007; Vignon *and Sasal*, 2010) but seldom reported in mortality episodes. However, their pathological concerns should not be neglected. Gill Flukes (*Dactylogyrus* sp.), anchor worms (*Lernaea* sp.), fish lice (*Argulus* sp.), mites (*Hydrachnellae* sp.), leeches (*Placobdella montifera*) and skin flukes (*Gyrodactylidiasis* sp. and Trichodina sp.) are few commonly known fish parasites (Munday et al., 2001; Fioravanti et al., 2006; Nowak, 2007).

1.3 Marine bacterial fish pathogens

Bacterial disease outbreaks impose a significant constraint in fish and shellfish production. Bacterial pathogens are smallest pathogenic agents causing serious fish diseases. Pathogenicity of microorganisms is a characteristic feature generally related to species, families and order of fishes as hosts.

The fish pathogenic bacteria cause pathogenicity in three different ways:

(i) fermentative disintegration and destruction of the affected tissues of the host.

(ii) involvement of endotoxins liberated especially during the breakdown of the bacterial cells.

(iii) secretion of water soluble poisonous exotoxins hasten the process of pathogenicity.

Bacterial diseases are responsible for massive mortalities in both culturable and wild fish population worldwide and most of the causative microorganisms are naturally occurring opportunist pathogens which invade the tissue of a fish susceptible to infection. These diseases generally begin as an external infection on the skin, fins, gills, or oral cavity in the form of grayish-white or yellow erosive lesions that can progress to form deep hemorrhagic ulcers in the underlying muscles. External infection is often concurrent with systemic infection and results in sub-acute mortalities (Wang et al., 2009; Dumpala et al., 2010).

Potentially pathogenic bacteria associated with fish, shellfish and other seafoods are *Photobacterium damselae, Photobacterium leiognathi, Photobacterium phosphoreum, Vibrio parahaemolyticus, Vibrio cholerae, Vibrio vulnificus, Vibrio salmonicida, Vibrio anguillarum, Vibrio alginolyticus Escherichia* sp., *Mycobacterium* sp., *Streptococcus* sp., *Aeromonas hydrophila, A. salmonicida, Salmonella* sp., *Listeria monocytogenes, Clostridium botulinum, C. perfringens, Shigella* sp., *Edwardsiella tarda, Flavobacterium* sp. and *Pseudomonas* spp. (Jayasekaran, et al. 1996: Lipp and Rose, 1997; Chattopadhyay, 2000; Karunasagar and Karunasagar, 2000; Novotany et al., 2004; Ringo et al.,

2004; Gopal et al., 2005; Schindler and Shelef, 2006; Vinh et al., 2006; Mohanti and Sahoo, 2007; Noriega-Orozco et al., 2007; Austin and Austin, 2007; Jutfelt et al., 2008; Maiti et al., 2009; Pulkkinen et al., 2009; Aberoumand, 2010).

Due to infection of these bacterial pathogens fish may display many behavioural and physical abnormalities which indicate the nature and degree of infections. It should be emphasized however, that many symptoms are common to a multitude of bacterial diseases (Austin and Austin, 1987, 2007; Inglis et al., 1993).

Some of the important disease symptoms and possible cause of infections are as follows:

Sluggish behaviour

With some infections notably those caused by *Clostridium botulinum, Mycobacterium spp., Nocardia spp., Vibrio anguillaram and* V. *ordalii*, the fish may become very inactive and often ceases feeding. In this state death may quickly follow.

Twirling, spiral or erratic movement

This is indicative of neurological damage. The offending pathogens include *Clostridium botulinum, Edwardsiella ictaluri and* Eubacterium *tarantellus*.

Faded pigment

With this condition attributed to a reduction in melanin content the fish becomes very pale. This may occur due to infections caused by *Edwardsiella tarda, Mycobacterium* spp. and *Nocardia* spp.

Darkened pigment

An enhancement of pigment synthesis results from infection with *Cytophaga psychrophila, Streptococcus* spp. , *V. aliginolyticus* and *Yersinaia ruckeri.*

Exophthalmia ('pop-eye')

This occurs due to infections by *Aeromonas hydrophila, Edwardsiella ictaluri, Mycobacterium* spp., *Nocardia* spp., *Renibacterium salmoninarum, Staphylococcus epidermidis, Streptococcus* spp. and *Yesinia ruckeri.*

Hemorrhage in the eye

Presence of prominent blood spots in the eye is caused by *Streptococcus* spp. and *Yersinia ruckeri* ('salmonid blood spot').

Hemorrhage in the mouth

Essentially blood spots become apparent on the roof of the mouth; a phenomenon attributed to infection by *Pseudomonas anguilliseptica, Vibrio anguillaram, V. ordalii* and *Yersinia ruckeri.*

Erosion of the jaws/mouth

This condition occurs principally due to infections by *Flexibacter maritimus* and *Yersinia ruckeri.*

Hemorrhage in the opercula region

This occurs due to infections by *Pseudomonas anguilliseptica* and *Streptococcus* spp.

Gill damage

This includes the presence of swollen gill lamellae, clubbing and gill rot, which are symptoms of gill disease caused by *Cytophaga aquatilis,*

Flavobacterium branchiophila, *Flexibacter columnaris*, *Vibrio alginolyticus*, *V. anguillaram* and *V. ordalii.*

White nodules on the gills

This is characteristic of infections caused by *Edwardsiella tarda.*

Fin rot

Presence of badly damaged (rotten) fins is indicative of infection by *Aeromonas hydrophila*, *Mycobacterium* spp., *Nocardia* spp. and *Pseudomonas fluorescens.*

Hemorrhage at the base of fins

Presence of clusters of tiny hemorrhagic lesions at the base of the fins is associated with disease caused by *Pseudomonas fluorescens*, *Streptococcus* spp., *Vibrio parahaemolyticus, Vibrio anguillaram, V. ordalii* and *Yersina ruckeri.*

Tail rot

This occurs due to infection by *Flexibacter maritimus*, *Mycobacterium* spp., *Nocardia* spp. and *Pseudomonas fluorescens.*

Saddle-like lesions on the dorsal surface (columnaris, saddle back disease)

This condtition develops with infections by *Cytophaga psychrophila*, *Flexibacter columnaris* and *Sporocytophaga* sp.

This is a common condition with infections by *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella ictaluri*, *Flexibacter columnaris*, *Lactobacillus* spp. , *Mycobacterium* spp., *Nocardia* spp., *Pseudomonas anguilliseptica,*

chlororaphis, P. fluorescens, V. parahaemolyticus, Vibrio cholerae and V. vulnificus.

Ulcers

Ulcers in fish occur due to infections caused by *Acinetobacter sp., Aeromonas salmonicida, Mycobacterium spp., Nocardia spp., Proteus retttgeri, Renibacterium salmoninarum, Staphylococcus epidermidis, Vibrio parahaemolyticus, Vibrio alginolyticus* and V. *damsela.*

External abscess

These may be present due to infections by *Aeromonas hydrophila, Edwardsiella tarda, Lactobacillus spp.* and *Renibacterium salmoninarum.*

Furuncles (boils)

These are characteristic of infections by *Aeromonas salmonicida.*

Blood-filled blisters on the flank

These have so far only been described in infections by *Renibacterium salmoninarum* and *Lactobacillus* spp.

Protruded anus (internal)

This is symptomatic of infection by *Edwardsiella tarda* and *Plesiomonas shigelloides.*

Hemorrhage around the vent

This occurs in vibriosis, caused by *Vibrios.*

Apart from these apparent external symptoms careful internal observation of diseased fish may reveal the presence of following distinct abnormalities:

Distended abdomen

This condition where the abdomen is often filled with ascitic fluid should not be confused with overfeeding. The presence of a distended abdomen is sign of many diseases caused by *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella ictaluri*, *E. tarda*, *Lactobacillus* spp., *Pseudomonas chlororaphis*, *P. fluorescens*, *Renibacterium salmoninarum* and *Streptococcus* spp.

Gas filled hollows in the muscle

These are generally evil smelling and characteristic of *Edwardsiella tarda* infections.

Ascitic fluid in the abdominal cavity

This is responsible for abdominal swelling, a trait common of many bacterial diseases including those caused by *Aeromonas hydrophila, A. Salmonicidia*, *Edwardsiella ictaluri*, *E. tarda, Flavobacterium sp.*, *Lactobacillus spp.*, *Lactobacillus spp.*, *Pseudomonas chlororaphis*, *P. fluorescens*, *Renibacterium salmoninarum*, *Staphylococcus epidermidis*, *Streptococcus* spp. and *Vibrio alginolyticus*.

Petechial (pin-prick) hemorrhage on the muscle wall

These are common to many diseases including those caused by *Acinetobacter* sp. *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella ictaluri*, *Lactobacillus* spp., *Pseudomonas chlororaphis*, *P. fluorescens*, *Renibacterium salmoninarum*, *Vibrio anguillaram*, *V. ordalii* and *Yersinia ruckeri*.

Hemorrhage in the air bladder

This symptom is characteristic of *Acinetobacter* infection.

Liquid in the air bladder

This has been associated with 'vibriosis' caused by *Vibrios*.

White nodules (granulomas) in the internal organs

These have been reported in some diseases, notably those caused by *Edwardsiella tarda*, *Flavobacterium piscicida*, *Mycobacterium* spp., *Nocardia* spp., and *Pasteurella piscicida*.

Swollen kidney

This occurs mainly due to infections caused by *Edwardsiella ictaluri* and *Renibacterium salmoninarum*.

False membrane over the heart and/ or kidney

This has been attributed only to bacterial kidney disease, which is caused by *Renibacterium salmoninarum*.

Haemorrhage in the peritoneum

This is caused by *Flavobacterium* sp., *Pseudomonas anguilliseptica*, *P. fluorescens*, *Vibrio alginolyticus*, *V. anguillaram* and *V. ordalii*.

Swollen intestine containing yellow fluid

This infection is attributed to *Vibrio alginolyticus* and *Yersinia ruckeri*.

Hemorrhage in the internal organs

This is common symptom of most bacterial fish diseases including those caused by *Acinetobacter* sp., *Aeromonas hydrophila*, *A.salmonicida*, *Edwardsiella ictaluri*, *E. tarda*, *Flavobacterium* sp., *Lactobacillus* spp., *Streptococcus* spp., *Vibrio alginolyticus*, *V. anguillaram*, *V. cholerae*, *V. ordalii* and *Yersinia ruckeri*.

1.4 Bacterial fish pathogens - Human infections:

Outbreaks of food-borne human illnesses continue to be a major problem worldwide (Karunasagar, 2008). Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common depending on the season, patient's contact with fish related environment, dietary habits and status of immune system. These are often bacterial species facultatively pathogenic to both fish and human beings and may be isolated from fish with or without apparent symptoms of the disease. The source of infection may be fish stored either for food or recreation (Acha and Szyfres, 2003).

The bacterial diseases transmitted to humans through sea food range from mild gastroenteritis to life threatening syndromes such as bacteremia, septicemia and meningitis etc. *Edwardsiella tarda* is an important cause of hemorrhagic septicemia in fish and also of gastro and extra-intestinal infections in humans. Bacteria related food poisoning (caused by excessive histamine production in the muscles of certain fish as a result of amino acid breakdown by bacteria) is the most common infection among human beings but fewer than 20 out of several thousands of different bacteria actually are the culprits.More than 90 percent of the cases of food poisoning each year are caused by *Clostridium perfringens, Campylobacter* sp., *Vibrio cholerae* (Karunasagar et al., 1990; Dalsgaard et al., 1995), *Vibrio parahaemolyticus* (Okuda et al., 1997; Chowdhury et al., 2000; Lesmana et al., 2001; Rahimi et al., 2010), *Vibrio vulnificus, Bacillus cereus, Aeromonas hydrophila* (Maiti et al., 2009), *Staphylococcus sp., Salmonella* sp., *Listeria monocytogenes* and entero-

pathogenic *Escherichia coli* (Kim et al., 2007; Vestby et al., 2009). Interestingly, all the serious cases of food poisoning were result of consumption of raw or improperly cooked fish. Apart from the consumption of pathogenic bacteria infected fish, other sources of infection could be injuries due to fish bites (Seiberras et al., 2000), fish thorns (Said et al., 1998) or simple contacts with the pathogen while handling fish or processing fish in the food industry(Von Reyn et al., 1996; Bhatty et al., 2000; Lunden et al., 2000; Acha and Szyfres, 2003; Vinh et al., 2006).Severe skin related disorders in humans are results of infections from such bacterial pathogens. Food borne diseases are among the most serious public health concerns and cause of morbidity such as septicemia, soft tissue necrosis, skin rashes, wound infection and nausea (Hoi et al., 1998; Bisharat et al., 1999; Novotany et al., 2004; Vinh et al., 2006).

1.4.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a gram-negative, curved, rod-shaped, motile, enteric bacterium found in marine and estuarine environments worldwide. It is halophilic, oxidase positive, facultatively aerobic and does not form spores. It colonizes filter feeding animals such as oysters, crabs, mussles and fish but can also be found free-living in seawater. Similar to other vibrios, selective medium for *V. parahaemolyticus* is thiosulfate-citrate-bile salts- sucrose (TCBS) agar. On TCBS, *V. parahaemolyticus* isolates appear as distinct green colonies. Virulence can be determined by β hemolysis of red blood cells using

Wagatsuma blood agar, although newer methods use specific DNA/gene probes.

V. parahaemolyticus, first identified by Fujino *et al.* (1953), as a cause of foodborne illness is a human pathogen widely distributed in the marine environment. This microorganism is frequently isolated from a variety of seafoods, particularly shellfish. Consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, and abdominal cramps. In India Chaterjee *et al.* (1970) first isolated *V. parahaemolyticus* from patients of gastroenteritis in Calcutta, India (Venugopal et al., 2001). Subsequently emergence of pandemic O3 : K6 strain of *V. parahaemolyticus* was reported by Okuda *et al.*(1997). Chakraborty and Surendran (2008) also reported the presence of pathogenic, trh positive, *V. parahaemolyticus* strains in the marketed seafood from the areas in and around Cochin, India.

This pathogen is a common cause of food borne human illnesses in many asian countries including China, Japan, Taiwan and Southeast asian countries and is also recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States. Infections caused by V. *parahaemolyticus* have increased globally in the last few years (Wong et al., 1999; Kaufman et al., 2002).

V. parahaemolyticus responsible for grave infections in the form of hemolytic anemia, septicemia, necrotizing fascitis and even reactive arthritis

have been reported (Zide et al.,1974; Tamura et al.,1993; Payinda, 2008; Tena et al., 2010).

1.4.2 *Aeromonas hydrophila*

Aeromonads are gram-negative, facultative anaerobic bacteria found in most of the aquatic ecosystems worldwide. *Aeromonas hydrophila* mainly prefers areas with a warm climate but are also found in fresh, salt, marine, estuarine, chlorinated and un-chlorinated water. *Aeromonas hydrophila* is non-spore former, facultative anaerobic, straight rod with rounded ends (bacilli to cocco-bacilli shape) usually from 0.3 to 1.0 μm in width and 1 to 3 μm in length and motile by a polar flagella. DNase-toluidine blue - ampicillin agar, is best media for isolation of the species.

Aeromonas hydrophila, an opportunistic bacterial pathogen was isolated first time from humans and animals in the 1950s. *Aeromonas hydrophila* is associated with diseases mainly found in fish and amphibians as they live in aquatic environment. For instance red leg disease of frogs which causes internal, sometimes fatal hemorrhage is linked to this pathogen. When infected with Aeromonas *hydrophila* fish develop ulcers, tail rot, fin rot, and hemorrhagic septicaemia. Hemorrhagic septicaemia causes lesions that lead to scale shedding, hemorrhage in the gills and anal area, ulcers, exophthalmia, and abdominal swelling (Austin and Austin, 1987, 2007; Inglis et al., 1993; Karunasagar et al., 1995; El-Barbary, 2010).

Aeromonas hydrophila causes gastroenteritis in humans mostly in young children and immuno-compromised adults (Subhash kumar et al., 2006; Erova

et al., 2007; Mukhopadhyay et al., 2008). A number of water and food-borne outbreaks associated with *Aeromonas hydrophila* have been reported. This organism is typically linked to gastroenteritis, but in majority of cases it has been implicated in skin, soft-tissue and muscle infections, extra-intestinal and deep wound infections, cellulitis, meningitis, bacterimia, soft tissue infections, peritonitis and broncho-pulmonary infections, in humans with compromised immune systems (Janda, 1991; Gold and Salit, 1993; Seetha et al., 2004; Markov et al., 2007; Tulsidas et al., 2008).

The virulence factors of these pathogenic bacteria include toxins with hemolytic, cytotoxic, and enterotoxic activities. Along with many other toxins, Aerolysin is the main pore forming toxin produced by this pathogen (Sha et al., 2002; Yousr, et al., 2007; Zhu et al., 2007).

1.4. 3 *Acinetobacter sp.*

Acinetobacter is Gram-negative, a-sporogenous, non-motile, catalase-positive, oxidase-negative bacillus which was previously placed in *Achromobacter*, (0.9 - 1.6 μm in diameter and 1.5 - 2.5 μm in length), straight, becomes spherical in the stationary phase of growth. Their unusual morphology can be used in diagnosis of infection. In smears prepared from clinical specimens or from one-day old blood-agar plate, cells of *Acinetobacter* appear quite spherical and grouped in pairs (i.e. a coccobacillary diplobacillus). Most strains of *Acinetobacter* grow well on MacConkey agar (without salt). Although officially classified as non-lactose-fermenting, they are often partially lactose-fermenting when grown on MacConkey agar. When grown in the presence of

penicillin or in repeated subcultures, elongated club-shaped cells are seen. Although considered to be Gram-negative, cells are frequently difficult to de-stain, resulting in dark, Gram-positive areas. On Mueller-Hinton agar medium the colonies are smooth, non-pigmented, and generally iridescent. Cells are mucoid when they are encapsulated. Because of their ubiquitous nature and clinical importance members of the genus *Acinetobacter* continue to attract interest. While the majority of strains described have been isolated from clinical sources, some of them include environmental strains. Overall, the ecology of species belonging to the genus *Acinetobacter* is not well elucidated. The genus *Acinetobacter* comprises 17 validly named and 14 unnamed (genomic) species. The knowledge of biology and ecology of Acinetobacter at species level is limited because identification of *Acinetobacter* at species level is difficult (Carr et al., 2003; Gerischer, 2008).

The presence of ulcers and hemorrhage on fish surface, muscles and internal organs are result of infections caused by *Acinetobacter* sp. (Austin and Austin, 1987, 2007). Although not very common fish and food borne pathogen still there are many reports of *Acinetobacter* caused infections in fish (Ramos and Lyon, 2000; Barnes et al., 2010).

Acinetobacter spp. are widely distributed in nature and can survive on various surfaces (both moist and dry). Occasional *Acinetobacter* strains were isolated from food stuffs (Lahiri, 2004). Even after their usual commensal status, they are emerging as important opportunistic pathogens because they are rapidly evolving their multi-drug resistance (MDR) mechanism and are often

involved in various nosocomial infections that can be severe, such as bacteremia, meningitis, or pneumonia, both in highly developed countries and elsewhere (Mondal et al.,1991; Giamarellou et al., 2008; Sharma et al., 2009).Shiga toxins are one of the major virulence factor in Acinetobacter sp. (Doughari et al., 2009).

1.4.4 *Staphylococcus arlettae*

Staphylococcus is Gram-positive bacteria, appear round (cocci), and form grape-like clusters, produce catalase, possess an appropriate cell wall structure (with peptidoglycan and teichoic acid) and G + C content of DNA in a range of 30-40 mol %. *Staphylococcus sp.* is facultative anaerobe, grows in the presence of bile salts and is catalase positive. Growth also occurs in a medium with 6.5% NaCl. On Baird Parker medium, *Staphylococcus spp.* grow fermentatively, except for S. *saprophyticus* which grows oxidatively. One of the most important phenotypic features used in the classification of staphylococci is their ability to produce coagulase, an enzyme that causes blood clot formation.

Staphylococcus can cause a wide variety of diseases in humans and other animals through either toxin production or penetration. Staphylococcal toxins are a common cause of food poisoning, as it can grow in improperly-stored food items.

Staphylococcus arlettae was first described by Schleifer et al. (1985). *Staphylococcus arlettae* is coagulase negative. Other species which are coagulase negative have long been regarded as non- pathogenic but in recent years their important role as pathogens and their increasing role in human

infections have been demonstrated. Virulence factors are not as clearly defined as they are in S. *aureus* but there is a negligible difference in the pathogenicity of these two species (Burriel and scott, 1998; Sampimon et al., 2009).

Most of the virulence factors described have been reported from S. *aureus* but some of these have been found in other species also.These virulence factors could be cell associated components, exoenzymes, exotoxins and endotoxins. One of the prominent characteristics is its capability to survive in changing environment by switching on the selected set of genes (Gyles et al., 2010). Although *Staphylococcus arlettae* has not been widely associated with fish and human diseases but changing scenario of pathogens and pathogenicity has not exempted the species as they have been isolated from marine fish, seafood including clinical isolates from humans (Vilhelmsson et al., 1997; Couto et al., 2001; Kim et al., 2008; Lopez-Cortes et al., 2008).

1.5 Environmental optimas for the growth of bacterial fish pathogens

Microorganisms like any other living organisms require an external environment customized and tailored to their specific metabolic needs and capabilities. The ability of a organism to grow in a specific environment relies on the inherent presence of certain specific genes and encoded proteins/enzymes that facilitate the growth. For instance, salinity tolerant microbes possess genes to produce and orient compatible solutes to offset the increased osmotic pressure of their hypertonic environment. Likewise, growth in an alkaline environment requires production of proteins that can offset the over-abundance of hydroxyl ions and the charge differential of the environment.

Although the aspects of the external physical environment are infinite, the three most important and basic requirements are salinity, temperature and pH which play a very important role in growth of microorganisms. Although, each organism is capable of growth outside their "optimal" environment, it grows best when a certain aspect of the environment is kept at optimal level. When environmental conditions are optimized organisms can grow to their full biotic potential which happens only in highly controlled laboratory situations such as bioreactors.

pH

pH is an important environmental condition to consider, as an excess of either hydrogen ions (H^+), as exists in acidic environment or an excess of hydroxyl ions (OH^-), as exists in alkaline environment, can be equally detrimental to cells, not adapted to those conditions. Extreme acidic and alkaline conditions will denature membrane proteins and quickly cause death of cells. While a vast majority of eukaryotes are categorized as neutrophiles, enjoying an environmental pH close to 7.0, many bacteria require acidic (acidophiles) or alkaline (alkaliphiles) conditions to grow or even survive. Although many bacteria are obligate acidophiles or obligate alkaliphiles, all bacteria maintain an intracellular environment near a neutral pH of 7. pH requirements for growth of microorganisms can be easily determined by inoculating them into media buffered to maintain a certain pH. A spectrophotometer can then be used to quantitatively measure the relative amounts of growth at different pH and a pH optima can be determined.

Temperature

Microbes grow over a broad temperature range that extends from below 0°C to above 100°C. Changes in temperature have a direct effect on the structural integrity of organic molecules viz. nucleic acids and proteins as well as the rate of metabolic reactions of organisms. These organisms are also classified according to their temperature requirements relative to human body temperature (i.e. 37°C). Organisms that prefer this "normal" environment are called mesophiles, while those who thrive in colder environments are termed psychrophiles and those requiring higher temperatures are called thermophiles or even hyperthermophiles. A fourth class of organisms the psychrotrophs prefer mesophilic conditions but can also grow at 4°C. For any of the organisms listed above, below normal ambient temperature slows down metabolic reactions and thickening of the cytoplasm which results not in death but in extremely slow growth. On the contrary, higher than normal temperature causes irreversible enzyme denaturation, cell membrane dissolution and ultimately cell death.

Salinity (NaCl)

The availability of water is a critical factor that affects the growth of all cells. Microorganisms live over a range of water activity (a_w from 1.0 to 0.7).

Salts is one of the major factors affecting A_w in surrounding microbial s (a_w of seawater = 0.98).

The only common solute in nature that occurs over a wide concentration range is NaCl and some microorganisms are named based on their growth

response to NaCl. Microorganisms that require some NaCl for growth are **halophiles**. **Mild halophiles** require 1-6% NaCl, **moderate halophiles** require 6-15% NaCl and **extreme halophiles** requiring 15-30% NaCl for growth belong to the **archaea**. Bacteria that are able to grow in presence of moderate NaCl levels, even though they grow best in the absence of NaCl, are called halotolerant.

1.6 Virulence, Virulence factors and Pathogenesis

Disease can be defined as 'any deviation from or interruption of the normal structure and function of any part of the body' and the pathogen is 'a disease causing organism'.

Pathogenesis is defined as a process of pathogen mediated disease progression and mechanism underlying it. This term can also be used to describe the developmental stage of the disease, such as acute, chronic and recurrent.

Pathogenicity refers to the ability of an organism to cause disease. Virulence, a term often used interchangeably with pathogenicity, refers to the degree of pathogenesis caused by the organism. The extent of the virulence is usually correlated with the ability of the pathogen to multiply within the host and may be affected by different factors and depending upon the conditions may exhibit different levels of virulence.

Bacterial pathogenicity is result of combination of factors, including resistance to environmental threats and to the host's defenses, growth capability,

localization in the host, tissue specificity, resource obtaining mechanisms and the bacterium's own defenses to aggression.

A variety of bacterial products, often harmful and specific to each strain, are involved in the microorganism's survival, adhesion and growth in the host and as a result contribute to the infection and cause of disease.These factors responsible for the pathogenicity of the bacteria are called virulence factors.

The virulence factors of bacteria can be divided into a number of functional types which are as follows:

(i) adherence factors, so that the organisms can attach to cells, fimbrae or other capsular polysaccharides;

(ii) invasion factors, that facilitate cell invasion which could be enzymes, including haemolysin;

(iii) Capsules or other surface components which enhance the survival rate of bacteria in the host by acting as protective mechanism; and

(iv) Bacterial toxins in the form of exotoxins and endotoxin which exert profound biologic effects on the host and may be lethal.

These virulence factors are encoded by specific genes viz. *vhh*, *chiA*, *vhpA*, $toxR_{Vh}$, *loxR, tdh, trh, ure, hlyA, stx, etc.* which represent virulence factors used by the bacteria for attachment to and degradation of eukaryotic cells.

The typical genome of a pathogenic bacterium consists of a core genome shared with related commensals. Often, the virulence encoding genes are contained in extended genetic continuums termed as pathogenicity islands. The PAI's vary in size from a few kilobases to large chromosomal regions, and

their base composition and codon usage often differ from the host genome. It has been noted that many virulence genes are associated with genomic islands (GI's; clusters of genes of probable horizontal origin), and the first GIs identified were infact called pathogenicity islands (Schmidt and Hensel, 2004; Ho Sui et al., 2009). Bacterial protein secretion systems are crucial for pathogenicity as they allow export of virulence factors, enzymes or toxins in order to invade a host cell through the outer membrane. T3SS, T4SS, T5SS and T6SS genes, encoding all protein secretion systems in bacterial pathogens are associated with genomic island (Cornelis, 2006; Pukatzki, et al., 2006; Persson et al., 2009).

Some critical bacterial species display both virulent as well as avirulent strains eg. *Vibrio colerae*. Any virulence factors observed to be present in pathogenic strains from diverse bacterial genera, with no detectable homologs in non-pathogenic strains of the same genera, are considered good candidates for being classified as pathogen associated, for example hemolysins. Such genes could be identified within a diverse bacterial genome data set, and examined for common features of such genes with the hypothesis that they may play more virulence-specific roles in pathogens. Aerolysin is a well-known pore-forming toxin that was first purified from *Aeromonas hydrophila* and later from A. *sobria* (Fujii et al., 1998). Presence of virulence genes viz. *vhh*, *chiA*, *vhpA*, $toxR_{Vh}$, *loxR* or lux R and serine protease, that are found in human pathogenic vibrios such as *Vibrio cholerae*, *Vibrio paraha-emolyticus* and *Vibrio vulnificus* and aquatic pathogenic *Vibrio anguillarum* were detected in

Vibrio harveyi isolates (Ruwandeepika et al., 2010).

A curated data set of virulence factors is available through the Virulence factor database (VFDB) which may be cross-referenced with current bacterial genome data sets (Chen et al., 2005; Yang et al., 2008).

Some times plasmids also play an important role in regulating virulence factors as reported by Majumdar et al., (2007) in an *Aeromonas hydrophila* infection in cat fish which lost its virulence after curing. Plasmids such as pJM1 of *V. anguillarum* are well explored for their role in *Vibrio* pathogenicity (Tolmasky and Crosa, 1991; Di Lorenzo et al., 2003; Hazen et al., 2007). Other examples of plasmid mediated virulence are *Yersinia* sp. *Mycobacterium* sp. and *Salmonella* sp. (Bhaduri et al., 1990; Cornelis et al., 1998; Ranger et al., 2006; Wiesner et al., 2009).

1.6.1 PCR mediated detection of specific bacterial pathogens

PCR (polymerase chain reaction) is a technique widely used in molecular biology, microbiology, genetics, medical and biological research labs and many other branches of life sciences. This technique was developed in 1983 by Kary Mullis (Barlett and Stirling, 2003). It is used to amplify millions of copies of a specific region of a DNA strand (the target DNA).The name, polymerase chain reaction, comes from the DNA polymerase used for amplification. In addition, target DNA template, Two primers (flanking to target DNA), dNTPs, divalent cations (magnesium) and PCR buffers are used FOR the reaction mixture.

Reaction comprises of the repeated cycles of three different temperatures using Thermal cycler (denaturation, annealing and extension).

Traditionally, PCR is performed in a PCR tube and when the reaction is complete the products of the reaction (the amplified DNA fragments) are analysed by agarose gel electrophoresis followed by visualisation using gel documentation system.

Molecular methods offer increased sensitivity and specificity over traditional microbiological techniques for the detection of pathogens as the later is very time consuming. It is imperative to confirm the presence or absence of pathogenic organisms in fish or the surrounding environment and to monitor the changes in abundance of these organisms quickly (Altinok and Kurt, 2003; Lui et al., 2009). PCR mediated detection of pathogens is most efficient, cost effective and faster technique.

Further problems arise due to virulent and avirulent strains such as among *V.cholerae,* only serotypes O1 and O139 cause cholera and environmental isolates of **serotype** O1 non-toxigenic strains also exist. Among *V. parahaemolyticus* only less than 2% of environmental strains are pathogenic. These are characterized by their ability to produce a thermostable direct hemolysin (TDH) or TDH- related hemolysin (TRH)(Koch et al., 2001; Karunasagar et al., 2002). Since these virulence factors are encoded by specific genes specific for each bacteria, these genes have been chosen as PCR targets to be used for easy detection of pathogens in the seafoods.

Some pathogen specific genes are listed below:

Some pathogen specific genes are listed below:

Pathogen	Virulence genes	References
Vibrio cholerae	*ctx*	Karunasagar et al.,1995; Koch et al., 2001
V. parahaemolyticus	*tdh, trh*	Karunasagar et al.,1996; Parvathi et al., 2006; Raghunath et al., 2007.
V. vulnificus	*Vvh*	Gray and Kreger, 1985; Parker and Lewis, 1995.
L. monocytogenes	*iap, hly, actA*	Portnoy et al.,1992.
Salmonella	*inv, hns*	Hoorfar et al., 2000.
Enterohemorrhagic *E.coli*	*stx, eae*	Kumar et al., 2001, 2004; Neilsen and Andersen, 2003.
Enterotoxigenic *E.coli*	*St, Ctx*	Franck et al., 1998
Aeromonas hydrophila	*ahh1, hlyA, aer*	Wang et al., 2003.

1.6.2 Drug (antibiotic) resistance in bacterial pathogens

Antibiotics are natural substances secreted by bacteria and fungi to kill other bacteria that are competing for limited nutrients. The heavy use of antibiotics in rearing of fish could be detrimental to the health of the fish but also to aquatic

animals and humans (Cabello, 2006). This practice encourages bacterial resistance to drugs and could lead to the evolution of antibiotic resistant strains of bacteria in animals, humans and fish. During the past few decades however, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in humans, agriculture and aquaculture (Akinbowale et al., 2006; Interaminense et al., 2010).

These antibiotics exhibit four different major modes of action:

(i) **interference with cell wall synthesis** by β-lactams which inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer. These antibiotics include penicillins, cephalosporins, carbapenems and monobactams.

(ii) **inhibition of protein synthesis** by macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins and oxazolidinones

(iii) **interference with nucleic acid synthesis** due to binding to the specific sites of 30S and 50S subunits of the bacterial ribosomes and subsequent inhibition of bacterial growth.

(iv) **inhibition of a metabolic pathway,** for instance block in the pathway for folic acid synthesis results in the death of the bacterium.

Antimicrobial resistance in bacteria is an accelerating and accumulating problem. Spread of resistance amongst micro-organisms may affect the ability to treat infectious diseases in cultured animals and presents a major threat to public health as well. A more judicious approach to the use of prophylactic (preventive) antibiotics is necessary.

Drug resistance mechanisms of bacteria include presence of genes encoding enzymes, such as β -lactamases, that destroy the antibacterial agent before it can have an effect. Second mechanism is based on efflux pumps that

extrude the antibacterial agent from the cell interior before it can reach its target site and exert its detrimental effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produce altered bacterial cell walls that no longer contain the binding site for the antimicrobial agents (McManus, 1997; Tenover, 2006; Clark et al., 2009).

Some species of bacteria are innately resistant to more than one class of antimicrobial agents. In such cases all strains of that bacterial species are likewise resistant to all the members of those antibacterial classes. Development of acquired drug resistance is of greater concern where initially drug susceptible population of bacteria become resistant to that antibacterial agent and proliferate under the selective pressure of use of that agent which could be as a consequence of **mutations** in the susceptible strains or due to **horizontal gene transfer** from resistant to susceptible strains in the environment.

Traditionally Vibrios *are* considered highly susceptible to virtually all antimicrobials but very recently, studies conducted in South Carolina identified highly resistant strains of *V. para-haemolyticus* and *V. vulnificus* (Han et al., 2007). About 50 multi-drug efflux transporters were reported in *V. parahaemolyticus,* according to their primary structural similarities (Matsuo et al., 2007).

1.6.3 Role of plasmid in multi-drug resistance and pathogenesis

Multi-drug resistance or antimicrobial resistance is the best-known example of rapid adaptation of bacteria to a new enviro-nment. Role of plasmids in

antimicrobial resistance in both gram +ve and gram –ve bacteria, has been studied extensively (Courvalin et al., 1978; Bremner, 1979). Plasmids are well known to encode functions essential to antibiotic resistance, heavy metal resistance, metabolic functions and production of antibiotics, toxins and virulence factors (Synder and Champness, 1997). Plasmid mediated resistance is the transfer of antimicrobial-resistance encoding genes which are carried on plasmids. Resistance genes encoded in plasmids are often located within genetic elements called transposons. These elements include the transposase function that enables the transposon to recombine into the bacterial chromosome or plasmids.

Resistance genes are often located on extrachromosomal genetic elements or in segments inserted within the chromosome that originate from other genomes.The acquisition of a new gene may occur by genetic transformation but when resistance genes are located on plasmids, they can be mobilised by conjugative transfer. In this process mobilizable DNA molecules can be transferred from a donor to a recipient cell via a contact-dependent transmission and energy-driven processes (Waters, 1999).

There are reports of simultaneous presence of a resident virulence plasmid and a resistance plasmid in some bacterial sp. Since most of the resistance genes are located on transferable elements, it is possible that such events could represent occasions for the capture of resistence determinants by virulence plasmids. The linkage of virulence and resistance on the same plasmid could represent a relevant step to bacterial adaptation and such traits transferred to

other bacterial populations could be of high relevance in bacterial pathogenicity (Carrattoli, 2003).

Plasmid-mediated resistance to amoxycillin, tetracycline and chloramphenicol in *Staphylococcus aureus* in the most successful and adaptable human pathogen with ability to mediate the production of drug inactivating enzymes viz. beta-lactamases and other functions was recently reported (Daini and Akano 2009).

In another report by Guglielmetti et al. (2009), the transferability, of a large plasmid that harbors a tetracycline resistance gene '*tet* (S)' to fish and human pathogens was assessed using electrotrans-formation and conjugation. These results demonstrate that the antibiotic resistance genes in the fish intestinal bacteria have the potential to spread both to fish and human pathogens, posing a risk to aquaculture and human health.

1.7 Hemolytic activity

Hemolysis is the breakage of the red blood cell's (RBC's) membrane causing the release of the hemoglobin and other internal components into the surrounding fluid. Hemolysis is visually detected by showing a pink to red tinge in serum or plasma. **Hemolysis of red blood cells in agar plates is categorized into two types of hemolysis**:

(i) **α-hemolysis** also referred as incomplete hemolysis detected by dark and greenish agar under the colonies.This is sometimes called *green hemolysis* because of the color change in the blood agar. The colour change is caused

by hydrogen peroxide produced by the bacterium which oxidizes hemoglobin to green methemoglobin.

(ii) **β-hemolysis** or complete hemolysis of red blood cells. In this case blood agar media around and under the colonies appears light yellow and transparent.

(iii) If an organism does not induce hemolysis, it is said to display **gamma hemolysis**.

Hemolytic activity is displayed by the bacteria due to hemolysins, the exotoxin proteins produced by bacteria. Bacteria may use hemolysins as a means to obtain nutrients from host cells. For instance, iron may be a limiting factor in the growth of various pathogenic bacteria. Red blood cells are rich in iron-containing heme. Lysis of these cells releases heme into the surrounding environment, allowing the bacteria to take up the free iron which is normally maintained at low concentrations within the host body.

Hemolysins are one of the important virulence factors of many bacterial pathogens but their mode of action may vary, for example *Aeromonas hydrophila* produces two types of hemolysins (Ljungh et al., 1987; Castilho et al., 2009). A well-defined, clear hemolysis produced by *Vibrio parahaemolyticus* on specially prepared media has been considered closely related to its entero-pathogenicity and has been termed "the Kanagawa phenomenon" by Japanese investigators studying *V. parahaemolyticus* (Miyamoto et al., 1969; Karunasagar et al., 1984, 1996). Other examples of

pathogenic bacteria exhibiting hemolytic activity are *Edwardsiella tarda* (Hirono et al., 1997; Zheng et al., 2007).

1.8 Exoenzymes and their relevance in virulence

An exoenzyme or extracellular enzyme is an enzyme which is secreted by the cells and works outside of that cell. It is usually used for breaking down large molecules that would not be able to enter the cell otherwise. Bacteria secrete exoenzymes to hydrolize macromolecules into smaller molecules capable of being transported across the cytoplasmic membrane into the cytoplasm which is the site of most bacterial metabolism.

Extracellular enzymes produced by bacteria are one of the major factors for their pathogenesis. Chitinase, lipase, protease and lecithinase are among these enzymes catalizing the process of infection. Some pathogenic bacteria are able to produce and secrete enzymes that compromise cell structure of the host and enable the bacteria to work their way further into the body. Interestingly, virulence factors also are secreted proteins or enzymes such as invasins, lecithinase and other common lipases and proteases (Secades and Guijarro, 1999; Lebrun et al., 2009).

Kinase enzymes convert inactive plasminogen to plasmin which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in spreading bacterial lesions allows more rapid diffusion of the infectious bacteria.These enzymes usually act on the animal cell membrane by insertion into the membrane by forming a pore that results in cell lysis as in case of alpha-toxins or by hydrolyzing phospholipids removing their polar head group

by enzymatic attack on phospholipids, which destabilizes the membrane. These are **phospho-lipases** or they may act as **lecithinases** and destroy lecithin (phosphatidylcholine) in cell membranes. Blood cell lysing enzymes are important exotoxins i.e. hemolysins (Songer, 1997; Rossignol et al., 2008).

Lipase is an exoenzyme that hydrolyzes lipids into fatty acids and glycerol acts as an important extra-cellular virulence factor affecting several immune system functions through free fatty acid generated by lipolytic activity in animals and humans. The amplification of small fragment of *lip H3* gene was used to confirm *A. hydrophila* isolated from aquatic environment (Swaminathan et al., 2004; Bender and flieger, 2010).

Proteases hydrolyze proteins into polypeptides and amino acid subunits. Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms as they have the potential to destroy the structural and functional proteins that constitute host tissues as well as important in host defense. Proteases play a crucial role in numerous pathologic processes as they have been linked to a number of degenerative diseases viz. arthritis, tumor invasion and metastasis.

Damage of host tissues by degrading elastin, collagen and proteoglycan by *Pseudomonas aeruginosa* is partly done due to activity of alkaline protease and elastase (Sakata et al., 1993).

Flavobacterium columnare produces several extracellular proteases that are believed to be important virulence factors contributing to the branchial and cutaneous necrosis (Dumpala et al., 2010). Requirement of catalase (*KatB*) is

reported for H_2O_2 and phagocyte-mediated killing in *Edwardsiella tarda* (Rao et al., 2003). Some other important virulent exoenzymes include hyaluronidase (attacks connective tissues), collagenase (breaks down collagen) and neuraminidase (damages intestinal mucosa) (Harrington, 1996; Schmitt, 1999).

1.9 Major Bacterial toxins

Toxin is a poisonous substance produced by living cells or organisms. Bacterial toxins are toxins produced by bacteria and possess a significant role in bacterial pathogenesis and have been recognized as the primary virulence factor(s) for a variety of pathogenic bacteria. In fact these are the first bacterial virulence factors identified. There are mainly two types of bacterial toxins: (i) cell-associated endotoxins and (ii) extracellular diffusible exotoxins (Masignani et al., 2006; Saha and Raghav, 2007).

Endotoxins are cell-associated substances that are structural components of Gram –ve bacteria. Most endotoxins are located in the cell envelope and refer specifically to the lipopoly-saccharide (LPS) or lipooligosaccharide (LOS) located in the outer membrane of Gram-negative bacteria.Soluble endotoxins may be released from growing bacteria or from cells that are lysed as a result of effective host defense mechanisms or by the activities of certain antibiotics. Endotoxins are heat stable generally act in the vicinity of bacterial growth or presence and are released even after death of bacteria. Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins along with other molecules are predominantly (though not exclusive) responsible for the initiation of

sepsis by the generation of pro- and anti-inflammatory mediators, cytokines, coagulation factors, adhesion molecules, myocardial depressant substances and heat shock proteins (Aepfelbacher and Essler 2001; Peters et al., 2003). Bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, *Aeromonas* and other pathogens release endotoxins which result in septicemia, toxic shock syndrome and sometimes food poisoning (Imani and Akhlaghi, 2004; Nya and Austin, 2010).

Exotoxins, well-characterized family of toxins, are usually secreted by bacteria during the exponential growth phase and act at a site remote from bacterial growth. However, some bacterial exotoxins act at the site of pathogen colonization and may play a role in invasion. In some cases, exotoxins are released by lysis of the bacterial cell e.g. *Clostridium botulinum* neurotoxins (BoNTs, serotypes A-G) and the *Clostridium tetani,* tetanus neurotoxin (Singh et al., 1995; Henderson et al., 1997). Exotoxins are usually proteins minimally polypeptides which act enzymatically or directly on host cells and stimulate a variety of host responses. Exotoxins get denatured by heat, acid, and proteolytic enzymes. Some of these have very specific cytotoxic activity and are highly specific in substrates utilized and mode of action. Site of damage indicates the location of the substrate e.g. enterotoxin, neurotoxin, leukocidin or hemolysin. A specific toxin is generally specific to a particular bacterial species e.g. only *Clostridium tetani* produces tetanus toxin and diphtheria toxin is produced exclusively by *Corynebacterium diphtheria*.

Some protein toxins have fairly broad cytotoxic activity and cause non-specific death of various types of cells or damage to tissues eventually resulting in necrosis. Some exotoxins act locally to degrade tissue matrices or fibrin, allowing bacterial invasion and promoting their growth serve as **invasins**. This includes collagenase, hyaluronidase and streptokinase and pore-forming toxins. These invasins degrade membrane components such as phospholipases and lecithinases.

Many protein toxins consist of two components: **subunit A** responsible for the **enzymatic activity** of the toxin and **subunit B** is concerned with binding to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native (A+B) toxin. Isolated A subunits are enzymatically active but lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native toxin), but they are nontoxic (Karunasagar et al., 1984; Alouf and Popoff, 2006; Todar, 2008).

1.9.1 Extra-cellular proteins as important exotoxins in bacteria

Extra-cellular proteins (ECPs) of bacterial pathogens are the proteins secreted by them in the surrounding environment which are exotoxins and mostly work enzymatically. Bacterial protein toxins are the most powerful human poisons known and retain high activity at very high dilutions. Bacterial toxins have been defined as soluble substances that alter the normal metabolism of host cells with deleterious effects on the host (Schlessinger and Schaechter 1993). Bacterial ECPs in the form of exotoxins such as shigatoxins, hemolysins and

enterotoxins of specific strains play a very significant role in bacterial pathogenesis of fish and humans. (Santos et al., 1988; Doughari et al., 2009; Alperi and Figueras, 2010).

Bacterial protein toxins have different mode of action such as damaging cell membranes, inhibiting protein synthesis, activating second messenger pathways or activating the host immune response.

Exotoxins that mediate the cellular damage of extracellular matrix, do so by either enzymatic hydrolysis as in *Clostridium perfringens* (Egerer and Satchell, 2010) or pore formation by α toxins as in *Staphylococcus aureus* (Lind et al., 1987). Alpha-toxins (Hemolysins) are cytolytic to a variety of cell types including human monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells. Pore-forming toxins, as the name suggests, insert a transmembranous pore into a host cell membrane, thereby disrupting the selective influx and efflux of ions across the membrane (Palmer et al., 1993; Hume et al., 2000).

Exotoxins which target cells by inhibiting protein synthesis act upon elongation factors and ribosomal RNA e.g. shiga toxins, also called verotoxins produced by *E.coli* (STEC) and *Shigella dysenteriae* (Jackson, 1991; Taylor, 2008; Miko et al., 2009). Stx inactivate ribosomal RNA so that the affected ribosome can no longer interact with elongation factors.

Toxin activation or modification of secondary messengers can cause dramatic alterations to signal transduction pathways critical in maintaining a variety of cellular functions. Examples of these exotoxins include CNF1

(exhibiting membrane ruffling; the formation of focal adhesions and actin stress fibers; enlarged multinucleated cells) and diarrheagenic heat-stable toxin (**STs)** responsible for extra-intestinal infections. Distinct STs are produced by a variety of enteropathogenic microorganisms including entero-toxigenic *E. coli* (ETEC), *S. aureus*, *V. cholerae*, *Vibrio mimicus*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Klebsiella* sp.(Santos et al.,1988; Daalsgaard et al.,1995; Qadri et al., 2004; Favre et al., 2006; Ostyn et al., 2010). Enterotoxin serotypes initiate potent stimulation of the immune system, pyrogenicity and enhancement of endotoxin induced shock in the host (the pyrogenic toxin super antigens or PTSAgs).

1.10 Antimicrobials in marine bacteria, characterization and their Significance

The marine environment is a special ecological niche for many unique microorganisms, which produce biologically active compounds to adapt to particular environmental conditions (Fenical, 1993; Bultel - Ponce 1998; Penesysan et al., 2010). There is an increasing demand of therapeutic drugs from diverse natural resources.Earlier studies which focused on terrestrial plants and microbes proved extremely fruitful, yielding many organic biologically useful compounds including anticancer drugs (Davidson, 1995). But the potential contribution of marine organisms to the discovery of new bioactive molecules is also very challenging (Patra et al., 2009).

Several bacteria present in aquatic ecosystems possess the ability to inhibit the growth of other microorganisms by producing antimicrobial substances such as antibiotics and bacteriocins.Their inhibitory mechanisms

include (i)production of antibiotics, bacteriocins, siderophores, lysozymes and proteases and (ii) alteration of pH through production of organic acids (Jorquera et al.,1999). Many bacterial strains have been reported as major pathogens for fish, molluscs and crustaceans causing mass mortalities world wide.These pathogens are equally responsible for human infections as well.Recently, several biologically active substances have been isolated from marine bacteria.Taking these facts into account and in order to explore potential bioactive agents that can control the growth of fish pathogenic bacteria several antagonistic strains were isolated in Kagoshima prefecture, Japan, in 2001. Some of these strains were studied for bacteriolytic activity resulting from the production of proteolytic enzymes. In addition, these bacterial strains were found to produce low molecular weight compounds inhibitory to the growth of *Vibrio* strains (Castillo et al., 2008).

There were many reports about antibacterial activity shown by marine bacteria viz. *Pseudomonas, Yersinia, Aeromonas, Brevi-bacterium, Bacillus* and *Alteromonas* (Burkholder et al., 1966; Gauthier and Breittmayer 1979; Shiozawa et al., 1997; Jorquera et al.,1999 ; Jayanath et al., 2001, 2002a, 2002b: Khalil et al., 2006; Ahmed et al., 2008; Rahman et al., 2010).

OBJECTIVES

of the Research

Objectives of the Research

- **Isolation, screening and enumeration of bacterial seafood pathogens in the aquatic waters of the coastal region of Goa.**
- **Physiological, biochemical and molecular detection of major seafood pathogenic bacteria.**
- **Prevalence and biodiversity studies of bacterial pathogens in the west coast of India.**
- **Pathogenicity studies of the extracellular toxins (Enzyme assay and hemolytic properties of bacterial culture),SDS-polyacrylamide gel electrophoresis analysis of extracellular proteins (ECPs) and partial characterization. Antimicrobial activity assays of pathogenic microbes.**
- **Correlation of plasmid profiles and broad range antibiotic drug resistance with their pathogenic properties. Transformation of resistant genes into *E. coli*.**

CHAPTER II

METHODOLOGY

Materials and Methods

2.1 Sample collection

Economically important fish of different species or varieties viz. Silver Belly, Mullet, Cat Fish, Pearl Spot and Sardines were collected from different coastal sites such as Miramar, Colva, Arossim, Betim and Siridao, of Goa.

These fish samples had visible disease symptoms viz. blackening of tissues, lesions, rots, ulcers and hemorrhage on their fins, mouth parts, gills and also on the surface of other body parts. After collection fish samples were transported to the laboratory for further studies.

2.2 Determination of total viable count and isolation of bacteria from infected portions of fish

Infected portions with visible disease symptoms of the fish samples were washed thoroughly with sterile deionized distilled water under sterile conditions and swabbed with sterile cotton wool. The swab was suspended in saline water (0.9 % NaCl). Ten fold serial dilutions (up to 10^{-7}) were made using saline and 100µl of last three dilutions i.e. 10^{-5}, 10^{-6} and 10^{-7} were spread plated on nutrient agar (Hi media) in triplicates. Agar plates were incubated at room temperature (29 ^{0} C) for 24 hrs. In order to determine total viable count as colony forming units (cfu) / ml at each dilution, average of number of colonies on three plates was calculated.

Thirty isolated and discrete bacterial colonies were selected and streaked on separate nutrient agar plates. Subsequently purified, isolated colonies were subcultured in nutrient broth and preserved for further characterization.

2.3 Identification of bacterial isolates based on morphological, biochemical and molecular characteristics

Morphological characterization of bacterial isolates from diseased fish samples was done following the method of Cruickshank et al. (1972). Isolates were Gram stained and observed under the light microscope at 100 x magnifications to study the cell morphology.The colony characteristics of bacterial isolates viz. size, shape, colour, margin, elevation, consistency and opacity were observed and recorded. Common biochemical tests as per Bergey's manual (Appendix B and C) along with Hi-Media (India) kit based biochemical tests were also performed in order to tentatively identify these bacterial isolates and result were finally interpreted according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Four bacterial isolates, out of these thirty, were selected for further characterization and designated as SA1, SB3, SC3 and SD4. Further confirmation of genus and species of these selected isolates was done by 16S rDNA sequencing and NCBI –BLAST search (Altschul et al., 1990). The genomic DNA was extracted from the bacterial isolates and used as template for PCR amplification of the 16S rDNA fragment using a single set of oligonucleotide primers to the universally conserved sequences at the 5' and 3'

termini of the eubacterial gene encoding 16 S rRNA. Following eubacterial forward and reverse primers (Wilson et al.1990) were used for PCR reaction:

27 f 5'- AGAGTTTGATCCTGGCTCAG - 3'

1492 r 5'- GGTTACCTTGTTACGACTT - 3'

These primers (oligos) were procured from MWG Biotech India Pvt. Ltd., Bangalore, India.

2.2 Determination of environmental optimas for growth (pH, Salinity and Temperature)

Initial inoculum was prepared by growing the selected bacterial isolates for 24 hrs in NB (nutrient broth) (Appendix A.1). Volume of the inoculum to be used was decided as per the absorbance of the inoculum at 600 nm so as to get the absorbance of approximately 0.05 at 0 hr to start the growth experiment. Each experiment was conducted in triplicate in 150 ml flasks with 30 ml NB to confirm the accuracy of the results. Shaker speed of incubator shaker was adjusted to 200 rpm and temperature was set 29 ^{0} C. Absorbance of the culture suspension was recorded at 600 nm every 2 hrs using UV- Vis spectrophotometer (Shimadzu, Japan) till they reach stationary growth phase.

(i) pH

0.5ml of the inoculum was added in 150ml flasks with 30ml NB with medium pH separately adjusted to 5, 7 and 9 respectively.These flasks were incubated in incubator shaker with shaker speed 200 rpm and at room tempreture (i.e. 29°C). Absorbance of the culture suspension was recorded at 600 nm every 2

hrs using UV-Vis spectrophotometer (Shimadzu, Japan) till they reach stationary growth phase. pH 7 can be considered as control.

(ii) Salinity

0.5ml or appropriate volume of the inoculum was added to three separate 150ml flasks containing 30 ml of Nutrient Broth supplemented with three different NaCl levels viz. 1.5%, 3% and 5% respectively. These culture flasks were incubated in incubator shaker at 200 rpm and at room temperature (i.e. 29°C). Culture flask without NaCl and 0.5% NaCl (i.e. basal level of NaCl in NB) served as controls.

(iii) Temperature

0.5 ml of the inoculum was added in four 150 ml flasks containing 30 ml NB and culture flasks were incubated in incubator shaker at 200 rpm and at four different temperatures separately viz. 22°C, 27°C, 37°C and 42°C.

According to the average of three readings for each of the three parameters, line diagram was plotted, in order to correlate the growth pattern/behavior of the selected bacterial isolates with time and also determine their environmental optimas.

2.5 Detection of exoenzymes

All the experments for exoenzyme production and activity were carried out at room temperature (i.e. 29°C) by spot inoculation on appropriate agar plates containing media with respective enzyme substrate. Agar plates were observed after 24 hrs to check the results.

(i) Lipase

The bacterial isolates were spot inoculated on Oil agar plate containing 0.5 % coconut oil and incubated at room temperature for 24 hrs. The plate was flooded with aqueous solution of rhodamine B (0 .002 %) and orange fluorescence was observed using UV- Transilluminator (Photodyne, USA).

(ii) Lecithinase

The selected bacterial isolates were spot inoculated on nutrient agar plate supplemented with 0.5% egg yolk and production of lecithinase and lipase along with proteolytic activity was observed after 24 hrs. Lecithinase degrades the lecithin present in the egg yolk producing an insoluble, opaque precipitate in the medium, surrounding it's growth. Lipase breaks down free fats present in the egg yolk causing an iridescent, and "oil on water" sheen on the surface of the colonies. Proteolysis is seen as clear zones (halos) in the growth medium surrounding the colony.

(iii) Protease

The selected bacterial isolates were spot inoculated on caseine agar plates (nutrient agar with 2 % skimmed milk) and also stab inoculated in 10 % gelatine agar (NB + 10 % gelatine) in the test tube. Proteolytic activity was observed as clearance zone on agar plate and liquefaction in gelatine agar tubes respectively after 24 hrs and 48 hrs incubation respectively.

(iv) Amylase

The bacterial isolates were spot inoculated on nutrient agar plate supplemented with 0.4 % starch. After 24 hrs of incubation, plate was flooded with Gram's iodine solution and observed on white box for clearance zone of hydrolysed starch as starch intact in the medium binds with iodine to give dark purple colour to the media.

(v) **Glucosidase**

Bacterial isolates were spot inoculated on nutrient agar supplemented with 0.01% esculine and 0.05% ferric citrate. After 24 hrs of incubation, plates were observed for hydrolysis of esculin in the form of dark brown complex. Hydrolysis of the aesculin, a glucoside results into aesculetin (6,7-dihydroxycoumarin) and glucose. The aesculetin forms dark brown or black complexes with ferric citrate.

2.6 Protease assay

Pattern of protease production Vs growth for only two selected bacterial isolates, SC3 and SB3 was determined as other two isolates did not show enzyme activity. These isolates were grown overnight in 5 ml LB broth (Apendix A.2). Fresh 50 ml LB broth was inoculated with overnight grown cell suspension to get absorbance (600 nm) of 0.05 at 0 hr. Absorbance of bacterial cells was recorded at 600 nm at 2 hr intervals for growth.Protease production was determined at 280 nm following Kunitz method (Kunitz, 1947). Line diagram was plotted, in order to observe the production pattern of protease enzyme with reference to growth of the selected bacterial isolate.

2.7 Hemolytic activity :

Blood agar was prepared by supplementing Trypticase soy agar (TSA) with blood (Apendix A.3). In order to check whether these bacterial isolates were able to lyse blood cells, selected isolates were streaked on separate blood agar plates and incubated. Plates were observed after 24 hrs for α hemolysis in form of dark green diffusion or β hemolysis in form of clearance zone around bacterial growth. .

2.8 Isolation of extra-cellular proteins (ECPs) and their partial characterization by SDS-PAGE analysis

2.8.1 Isolation of ECPs

The selected bacterial isolates were grown overnight to get initial inoculum.100 µl of the inoculum was added to 50 ml LB broth followed by overnight incubation at 37 ^{0} C and shaker speed of 200 rpm. Overnight grown culture suspensions were harvested at 5000 rpm at 4 ^{0} C for 15 mins. Supernatants were precipitated by addition of 10 % (w/v) Trichloroacetic acid (TCA) with overnight incubation at 4 $^{\circ}$C. Further centrifugation at 11000 rpm for 20 mins resulted in pellet containing extra-cellular proteins which was suspended in 50 µ l 1M Tris-HCl buffer with pH 8 (Apendix D.2) (Yoh et al., 2003).

2.8.2 Preparation of SDS-PAGE Gels

The glass plates after thoroughly washing and drying were wiped with acetone and clamped together with spacers in place. The assembly was sealed using 1% molten agar. All the reagents (Appendix D.1) used for separating gel were taken in a clean flask and mixed well to prepare 12% of acrylamide gel.

Ammonium per sulphate (APS) and TEMED (Tetra methyle ethylene diamine) were added just before pouring the gel. The mixture was added in between the 2 plates using a 10 ml pipette, upto ¾ th of the space. Isobutanol was added above the resolving gel to get a uniform surface. The isobutanol was drained out and the surface of the gel was washed with distilled water to remove traces of un-polymerized resolving gel mix or isobutanol. Similarly, stacking gel solution was cast over the resolving gel. A comb was inserted into the stacking gel and allowed to polymerize. After completion of polymerization, the gel assembly was placed in electrophoresis chamber. Samples were loaded in the wells with the help of a syringe. Electrode buffer was added to upper and lower tanks. Electrodes were connected to power pack and the gel was run at constant voltage of 30 volts until the tracking dye reached the bottom of the gel. Plates were separated carefully after removing the spacers and the gel was placed in staining solution (Laemmli et al., 1970).

2.8.3. Commassie Blue staining of acrylamide gels:

The gel was placed in staining solution for 6-8 hrs. It was then destained in solution I for 1h and in solution II (Appendix D.1) until clear bands appear. Gel was washed with deionised water and stored in the 30 % glycerol.Gel doc system(Alpha Innotech, USA) was used to record theses gels in visible light.

2.9 Detection of antimicrobial activity by selected bacterial isolates

Antibacterial activity of these selected bacterial isolates was tested by

extracting crude cell extract and checking its influence on the growth of E. coli and other isolates by agar well diffusion assay.

2.9.1 Preparation of crude cell extract

Ethyl acetate extraction procedure was followed with a slight modification to extract antimicrobial metabolites from all the four bacterial isolates (Wratten et al., 1977). Bacterial isolates were grown on sea water based yeast extract (SYEP) agar. (Appendix A.4). After 48 hrs, agar along with the bacterial cell mass was cut into pieces and suspended in ethyl acetate to extract the antibacterial metabolites overnight. Overnight suspension was decanted followed by centrifugation to get cell free supernatant. Supernatant was subjected to evaporation at 40°C for final recovery of crude cell extract (Ahmed et al., 2008).

2.9.2 Antimicrobial bioassay (agar well diffusion assay)

In order to check the antibacterial activity of the cell extract, SYEP agar (1.2%) was poured in the plates, small wells of about 6 mm diameter were made in the agar plates and bottom of the wells were sealed by 0.7% molten SYEP agar (Abraham, 2004). 100 µl of crude cell extract of each of the bacterial isolates (SA1, SB3, SC3 and SD4) were separately poured in the wells and allowed to diffuse in the agar media for four hours. Different indicator bacterial strains were spread plated on separate SYEP agar plates. Each plate had three wells with different cell extracts and one well with control; fourth isolate was used as indicator. Ethyl acetate (100 µl) was used as a control to check its inhibitory effect.

2.9.3 Characterization of crude cell extract by GC-MS

Identification of the antibacterial metabolites in the crude cell extract of selected bacterial isolates was done using GC-MS (Gas chromatography - Mass spectro-photometry) analysis by injecting 1ul of sample into a RTX-5 column (7 m x 0.32 mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan and Helium (3ml /min) was used as a carrier gas. The following temperature gradient program was used: 75° C for 2 min followed by an increase from 75^0 to 175° C at a rate of 50° C per min and finally 7 min at 175° C. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds (Seto and Kanamori-Kataoka, 2005).

2.10 Antibiotic susceptibility test

Disc diffusion method was followed to check the response of the selected bacterial isolates to commercially available antibiotics in terms of susceptibility or resistance (Bauer et al., 1966). Overnight grown bacterial suspensions of all the four bacterial isolates were spread plated on Mueller Hinton agar (Hi media, India) plates, octadiscs (OD-007 and 014) from Hi Media, India containing multiple antibiotics were carefully placed in the centre of the agar plates and incubated at room temperature (29^0C) for 24 hrs. Sensitivity/resistance of the individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic

disc susceptibility test approved by National Committee for Clinical Laboratory Standards. (Zone size interpretative chart, Hi Media).

2.11 Study of Plasmid profile of bacterial isolates

2.11.1 Extraction of plasmid DNA

Extraction of plasmid DNA from the four isolates was carried out following Alkaline lysis method (Birnboim and Doly, 1979). For each isolate a single bacterial colony was transferred into 10 ml of Luria Bertani broth and incubated overnight at 29°C at 200 rpm. 1.5 ml of culture was taken in microfuge tube and was centrifuged at 8,000 rpm for 5 mins at 4°C. The supernatant was discarded leaving the bacterial cell pellet as dry as possible. The pellet was suspended in 100µL of ice-cold glucose tris – EDTA buffer, freshly prepared lysis buffer (200µl) was subsequently added and the contents were mixed by inverting the tube gently. The microfuge tube was incubated on ice for 5 mins followed by addition of 150 µL of acetate buffer, the contents were gently mixed by inversion and incubated for 10mins at 4°C. The tubes were centrifuged at 11,000 rpm for 5 mins at 4°C. The supernatant was transferred to a fresh microfuge tube and the plasmid DNA was precipitated with double volume of the ice-cold ethanol. The contents were mixed gently and allowed to stand for 1 hr on ice. The microfuge tube was centrifuged at 12,000 rpm for 5 mins at 4°C. The pellet was resuspended in 70% (v/v) chilled ethanol and centrifuged at 12,000 rpm for 5 mins at 4°C.The supernatant was discarded and the pellet was air dried and resuspended in appropriate volume

of TE buffer(depending on pellet volume) containing RNase (20µg/ml) (Appendix D.2). Plasmid DNA was stored at -20°C.

2.11.2. Agarose gel electrophoresis of plasmid DNA

(i) Preparation of Agarose gel

Agarose gel 0.8% (w/v) was prepared in 1x- TAE buffer (pH 8.0) (Appendix D.2). To the molten agarose (50 ml), 1 µl of Ethidium bromide (10 mg/ml) was added to get final concentration of approximately 4µg/ml and poured in the casting tray with comb fixed appropriately at one end. The agarose gel was allowed to set at room temperature. The casting tray containing agarose gel was placed in the horizontal electrophoresis chamber filled with 1 X TAE buffer and the comb was removed.

(ii) Loading of DNA sample and electrophoresis

DNA sample (10µl), mixed with 3 µl of 6x DNA loading dye was loaded in the well of agarose gel using pipetteman (capacity: 0.5-10ul). The lid of the electrophoresis chamber was closed. The electrodes were connected to the power pack using connecting cords and voltage was set at 80 volts constantly to perform electrophoresis for 2 hrs (Sambrook et al., 1989).

(iii) Visualization of DNA

After gel electrophoresis, the gel was observed in a Gel documentation system and the photograph of the ethidium bromide stained bands in the gel was captured using this Gel documentation system (Alpha Innotech, USA).

2.12 Transformation of plasmid DNA of ampicillin resistant bacterial isolates into *E.coli* HB 101

Isolates SB3 and SC3, resistant to ampicillin were selected for transformation experiment following heat shock method (Hanahan, 1983) in order to confirm that ampicillin resistance was plasmid mediated.

The competent cells (*E.coli* HB 101) were thawed on ice, 100 µl was transferred into pre chilled 15 ml Falcon (2059 polypropylene) tubes. 1.7µl of freshly diluted (1:10) ß- mercaptoethanol (SIGMA) was added to these competent cells, giving a 25 mM final concentration. The tubes were swirled gently and kept on ice for 10 mins, swirling gently every 2 mins. 10 µl of plasmid DNA (50ng) from each isolate was added to the cells and swirled gently. As a control, 1 µl of pUC 18 plasmid DNA was added to another 100 µl aliquot of cells and swirled gently. The tubes were incubated on ice for 30mins and heat pulsed in a 42°C water bath for 45 secs. After the heat shock the tubes were incubated on ice for 2 mins. 0.9 ml of preheated (42°C) SOC (Appendix A. 6) was added and incubated at 37°C for 1 hr in incubator shaker with shaker speed of 225 rpm.

2.13 Screening of ampicillin resistant clones

2.13.1 Plating of transformation mix on LB agar plates with Ampicillin

100 µl of the transformation mix was spread plated on separate LB agar plates containing 50µg/ml ampicillin. Plates were incubated at 37°C for 16 hrs and observed for colonies (transformants). *E. coli* HB101 cells transformed with pUC 18 plasmid DNA served as the positive control while the negative control was set by plating *E.coli* HB101 competant cells on LB agar plate with 50µg/ml ampicillin.

2.13.2 Repilca plating of colonies obtained after initial screening

The transformants obtained were replica plated on LB agar plates with 50 µg/ml ampicillin and were incubated at 37°C for 24 hrs.

2.14 PCR amplification and agarose gel electrophoresis of virulence encoding genes

Genes *tdh* and *hly*, encoding virulence factors in two of the bacterial isolates SB3 and SC3 respectively were PCR amplified using corresponding gene specific primers. Genomic DNA of the isolates was used as DNA template for the PCR reaction. PCR kits were purchased from Bangalore Genei, India. PCR was carried out using 0.5 ml PCR microfuge tubes and PTC-150 MiniCycler (M J research, USA).

2.14.1 Isolation of genomic DNA

Genomic DNA of bacterial cells was isolated using PCI method (Sambrook et al., 1989).For each isolate a single bacterial colony was transferred into 10 ml of Luria Bertani broth and incubated overnight at 29°C at 200 rpm. 1.5 ml of culture suspension was taken in microfuge tube and centrifuged at 8,000 rpm for 5 mins at 29°C. The supernatant was discarded leaving the bacterial cell pellet as dry as possible. The pellet was suspended in 100µl of lysozyme(10mg/ml stock solution) and left at room temperature for 15 mins. 100 µl of isolation solution was added, stir mixed gently and incubated in water bath at 65°C. After 15 mins. 500µl of isolation buffer was added and mixed following addition of 700 µl of tris buffered phenol (1%). Mixture was centrifuged for 5 mins at 11,000 rpm and approximately 600µl of aqueous

phase was transferred into a new eppendorf tube. Equal volume of PCI (Phenol: Chloroform: Isoamyl alcohol) mixture (25:24:1) was added to the aquous phase and mixed. After centrifugation for 5 mins at 11,000 rpm, 500µl of aqueous phase from this solution was taken in a clean microfuge tube and again centrifuged in same conditions after adding and mixing equal volume of C:I (24:1).

The aqueous phase from the last centrifugation step was transferred to a new microfuge tube and the genomic DNA was precipitated with double volume of the ice-cold ethanol for an hour. The microfuge tube was centrifuged at 12,000 rpm for 5 mins at 4°C and the pellet was resuspended in 70% (v/v) chilled ethanol, centrifuged at 12,000 rpm for 5 mins at 4°C.The supernatant was discarded and the pellet containing genomic DNA was air dried and dissolved in appropriate volume of TE buffer containing DNase free RNase (20µg/ML) (AppendixD.2). Genomic DNA was preserved at -20°C.

2.14.2 Molecular detection of *tdh* gene

Presence of virulence gene, *tdh* was demonstrated by PCR amplification using genomic DNA as template and gene specific forward and reverse primers (Tada et al., 1992)

Forward primer: 5'- GGTACTAAATGGCTGACATC -3'

Reverse primer: 5'- CCACTACCACTCTCATATGC -3'

PCR was carried out using 0.5 ml PCR tubes and PTC-150 MiniCycler (M J research, USA).

The reaction mixture (50µl) consists of following components:

Template DNA	5µl (0.5µg),
10x PCR buffer	5µl (with 15 mM $MgCl_2$),
F and R primers	6µl (30 ng each)
dNTP mix	4 µl (250 µM each dNTP),
Taq DNA polymerase	2 µl (1 U/µl)
Milli-Q deionized ultrapure water	28 µl

PCR conditions were as follows:

Pre-denaturation : 94 °C for 5 mins

Denaturation : 94°C for 1 min

Annealing: 55°C for 1min

Extention: 72°C for 1 min

Additional extention: 72° C for 10 mins after completion of 30 cycles.

2.14.2 Molecular detection of *hly* gene

Similarly virulence gene, *tdh* was demonstrated by PCR amplification using genomic DNA as template and gene specific forward and reverse primers: (Wong et al., 1998)

Forward primer: 5'- GGCCGGTGGCCCGAAGATACGGG -3'

Reverse primer: 5'- GGCGGCCGCCGGACGAGACGGG -3'

PCR was carried out using 0.5 ml PCR tubes and PTC-150 Mini cycler (M J research, USA).

The reaction mixture (50µl) contains following:

Template DNA	5µl (0.5µg),
10x PCR buffer	5µl (with 15mM $MgCl_2$),
F and R primers	3µl (75 ng of each)
dNTP mix	4 µl (250 µM each dNTP),
Taq DNA polymerase	2 µl (1 U/µl)
Milli-Q deionized ultrapure water	31 µl

PCR conditions were as follows:

Pre-denaturation:	95 °C for 5 mins,
Denaturation :	95°C for 1 min,
Annealing :	66°C for 1min
Extention :	72°C for 1 min

Additional extention: 72° C for 10 mins after completion of 30 cycles.

2.14.3 Visualization of PCR product

The PCR products (amplicons) along with 100 bps DNA marker (Genei , India) were electrophoresed on 1% agarose gel (prepared as described earlier, in 2.11.2) and documented using Gel documentation system (Alpha Innotech, USA). The approximate size of PCR products (amplicons) was determined by comparing them with 100 bps DNA marker which was run parallel.

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CHAPTER III

RESULTS AND DISCUSSION

Isolation, Screening and Identification of Bacterial Isolates

Isolation, screening and identification of the bacterial isolates

3.1 Total Viable Count

Irrespective to the site of collection of fish samples (Fig.3.1), total viable count of bacteria (cfu/ml) was significantly high from the infected fish samples (Figs 3.2 - 3.9).The high viable count was due to direct swabs from wounded body surface of fish, harboring a significant population of pathogenic as well as non-pathogenic bacteria. The highest viable count of bacteria was observed from two of the Miramar samples whereas lowest viable count was found in the sample from Bogmalo (Table 3.1). High viable count of bacteria in Miramar samples may be attributed to contamination of aquatic environment from domestic waste and sewage from Panjim city.

3.2 Identification of the bacterial isolates by morphological and biochemical Characterization

After determining total viable count (cfu/ml) of the bacteria from various fish samples, thirty isolates were selected for further identification and characterization. Colony morphology (Fig.3.10) and gram characters along with biochemical characteristics of the isolates as per Bergey's Manual of Systematic bacteriology

(Krieg and Holt, 1984) lead to their tentative identification. Most of the isolates were Gram –ve rods, apart from two Gram +ve cocii and seven Gram +ve rods (Table 3.2). On the basis of biochemical test results (Table 3.3), bacterial isolates were identified as *Vibrio* spp., *Aeromonas* spp., *Streptococcus* spp., *Micrococcus* sp., *Bacillus* sp., *Lactobacillus* sp., *Flavobacterium* sp., *Alteromonas* spp., *Proteus* spp., *Salmonella* sp. and *Shigella* sp. Interestingly, all these bacterial isolates are commonly known fish and human pathogens which have been characterized up to species level (Dalsgaard et al.,1995;Goh et al.,1998; Daniels et al.,2000; Karunasagar et al., 2002; Sanath Kumar et al., 2003; Huss et al., 2004; Koh et al.,2009; Pulkkinen et al., 2009; Akoachere et al.,2009; Das et al.,2009; Bhowmick et al., 2010; Popovic et al., 2010; Upadhyay et al., 2010). Since bacterial identification based on biochemical characteristics is not considered very reliable, identification only up to genus level has been documented (Table 3.4). It is interesting to note that *Vibrio* spp. were found more predominant among these fish pathogens which is quite common in the west coast of India. There have been frequent reports of *Vibrios* in seafood and coastal waters from other parts of India, whereas not much work has been done on this aspect from west coast of India, specially Goa (Chatterjee et al., 1970, Lalitha et al., 1983; Venu, 2001; Karunasagar et al., 2002; Quintoil et al., 2007; Chakraborty et al., 2008; Karunasagar, 2008; Shekhar et al., 2008; Raghunath et al., 2009).

3.3 Molecular identification of selected isolates by 16S rDNA sequencing and BLAST search

Out of thirty, four isolates, each from separate samples were selected for further characterization and studies (Fig.3.11- 3.14). These four isolates were subjected to PCR amplification of 16S rDNA followed by DNA sequencing and BLAST search for identification.

Out of selected bacterial isolates, Isolate SA1 has been isolated from a Silver belly fish showing hemorrhage on eye and tiny hemorrhagic spots on the dorsal surface of the body. Based on cell morphology, gram characters and biochemical tests SA1 was identified as *Micrococcus* sp. but 16S rDNA sequencing and BLAST search confirmed it as *Acinetobacter* sp. strain An2 (Fig. 3.15;Table 3.5). This may be attributed to typical characteristics of *Acinetobacter* spp., which display variable gram characters, and some times are mistaken for gram +ve cocci (Lahiri et al., 2004). Even otherwise the knowledge of the biology and ecology of *Acinetobacter* at species level is limited. Bouvet and Grimont (1986) added to its taxonomy six new species, but still identification of *Acinetobacter* at species level is not very well defined (Carr et al., 2003; Gerischer, 2008). Although *Acinetobacter* species are important nosocomial pathogens (Lahiri et al., 2004; Berezin et al., 2010), occasionally isolated from different fish species (Ramos and Lyon, 2000; Okoro et al., 2010), they also retain a very important place as fish

pathogens (Austin and Austin, 2007). Sen (2005) described this species as one of the main constituent of bacterial flora and cause of spoilage of several marine fish of Indian waters. This could result into human infections in turn through contact or other means.For instance bloody diarrhoea due to *Acinetobacter* infection has been reported (Grotiuz et al., 2006).

Isolate SB 3 screened from the lesions on fin and other body parts of a Mullet was identified as *Vibrio parahaemolyticus* according to biochemical test results. This was further confirmed by 16S rDNA sequencing and BLAST search as *Vibrio parahaemolyticus* (Accession No. FJ 386958) and designated as strain An3 (Fig. 3.16; Table 3.5). While comparing the 16S rDNA sequence, this isolate showed 97% homology with *Vibrio parahaemolyticus* RIMD 2210633 (Accession No. A000031.2).

V. parahaemolyticus, has been implicated for food poisoning due to consumption of contaminated fish and its presence in estuarine and coastal waters along with its association with fish and shellfish is well established (Joseph et al., 1982; Su and Liu, 2007).

In India, *V. parahaemolyticus* was first reported from cases of gastroenteritis in Calcutta by Chaterjee et al., (1970) followed by Mazumdar et al., (1977) and Quintoil et al., (2007). Involvement of *V. parahaemolyticus* in gastroenteritis among some staff members at the Christian Medical College and Hospital, Vellore following a social get-together has further emphasized on the public health hazard

caused by this microorganism in India (Lalitha et al., 1983). Another study was conducted by Chakraborty et al., (2008) for detection of *Vibrio parahaemolyticus* in seafood samples collected from the markets of Cochin located at the southwest coast of India. Research group lead by Prof. Karunasagar has documented various reports on isolation and characterization of *Vibrio parahaemolyticus* since they have studied this seafood pathogen extensively (1984, 1986, 1990, 1996, 2001, 2002, 2005, and 2009).

Isolate SC 3 was screened from a cat fish with deep hemorrhage on its dorsal fin and symptoms of blackening of tissues. This isolate was identified as *Aeromonas hydrophila* based on biochemical and molecular (16S rDNA) analysis (Accession No. FJ 386959) and showed 98% homology with *Aeromonas hydrophila* (Accession No. AB368776.1). This strain was designated as An 4 (Fig. 3.17; Table 3.5). *Aeromonas hydrophila* is known to infect cat fish world wide (*Alagappan* et al. 2009).

Aeromonas hydrophila is a major fish pathogen, widely distributed throughout the world responsible for adverse effect to aquaculture systems causing haemorrhagic septicaemia, epizootic ulcerative syndrome (EUS) and abdominal dropsy in fish (Roberts et al., 1986; Ho et al., 1990; Das et al., 2005). *A. hydrophila* has been reported to be associated with epizootic ulcerative syndrome in India and South East Asian countries. Strains of *A. hydrophila* are capable of causing extraintestinal and deep wound infections, gastroenteritis, cellulitis,

meningitis, bacteremia, soft tissue necrosis, peritonitis, broncho-pulmonary infections and other infections in humans with compromised immune systems (Janda, 1991; Chang et al., 1997; Asmat and Gires, 2002). In India their have been frequent reports of *A. hydrophila* infections from eastern as well as western coasts (Seetha et al., 2004; Das et al. 2005; Khushiramani et al. 2007; Majumdar et al. 2007; Maiti et al. 2009; Singh et al.2010).

Fourth bacterial isolate, SD 4 was isolated from a Pearl spot fish, with necrotic ulcurs on its body surface. Based on biochemical tests it was tentatively identified as *Streptococcus sp.* Subsequently, 16S rDNA sequence analysis confirmed its identity as *Staphylococcus arletta* (Accession No. FJ 386956) because this isolate showed 97 % homology with *Staphylococcus arlettae* strain CM18 (Accession No.EU660331.1). This isolate was designated as strain An1 (Fig. 3.18; Table 3.5).

Although *Staphylococcus arlettae* has primarily been associated with goat and poultry microflora, isolation of this strain from fish has opened up new avenues for investigations as the species is not well explored and survival techniques of this genus may help in acquiring virulence in changed environment (Gyles et al., 2010). *Staphylococcus* spp. are known as important human and animal pathogens causing a number of severe infectious diseases. Besides the major pathogen *Staphylococcus aureus*, other species which were considered to be non-pathogenic until recently may also be involved in serious infections (Stephan et al., 2004).

It was interesting to note that screening and identification of all these bacterial isolates showing specific symptoms on the respective fish samples from where they were isolated confirmed that these bacterial pathogens were responsible for the diseased condition of their respective fish hosts.

Chapter III

Tables and Figures

Table 3.1 Total viable count of bacteria from infected fish samples.

Samples	Colony forming units/ ml		
	cfu x 10^5	cfu x10^6	cfu x10^7
mir SA	80.7	77.3	53.3
mir SB	180	126	117.3
bet SC	186	101	57.3
bet SD	85.3	53	37.3
sir SE	78	30	17.3
sir SF	118.7	57.3	18.7
col SV	83.7	63.7	33.3
aro SL	103.3	73.3	46.7
mir SO	146	120	96.7
bog SG	96.7	37.3	17.3

Table 3.2 Colony morphology and gram characters of selected bacteria from infected fish samples

Isolate	Colony morphology and gram characters
mir SA -1	Gram(+ve), cocci,1 mm,white, opaque with raised, glistening colonies with entire edge and viscous consistency.
mir SA -2	G(+ve), rods with 2 mm,white, irregular, dull, opaque, umbonate colonies with rhizoidal edges and butyrous consistency.
mir SA -3	G (+ve), rods with 2 mm,white, irregular, dull, opaque, umbonate colonies with rhizoidal edges and butyrous consistency.
mir SA -4	G(+ve), rods with 2 mm, pale, circular, raised ,translucent, glistening colonies with entire edge and viscous consistency.
mir SB-1	G(–ve), rods with 1-2 mm, pale, circular, raised , opaque, glistening colonies with entire edge and viscous consistency.
mir SB-3	G(-ve), rods, 1 mm pale, circular, translucent, convex, glistening colonies with entire edge and butyrous consistency.
bet SC-1	G(-ve), rods, 1-5 mm ,orange , opaque, raised, glistening colonies with entire edge and butyrous consistency.
bet SC-2	G(+ve), rods with 2 mm, yellow, circular, flat, transparent, glistening colonies with entire edge and butyrous consistency.
bet SC-3	G (-ve) rods, 2 mm, cream , opaque, convex , smooth, colonies with entire margins and butyrous consistency.
bet SD-2	G (-ve) rods , 4 mm,pale, circular ,raised , opaque, glistening colonies with entire edge and butyrous consistency.
bet SD-4	Gram(+ve) cocci ,1 mm,cicular ,white, opaque, convex smooth colonies with entire edge and viscous consistency.
sird SE-1	G (-ve), rods,1-2 mm ,bright yellow, circular ,raised , opaque, glistening colonies with entire edge and viscous consistency
sird SE-2	G (+ve) rods, 2-10 mm, circular, brown, transparent, flat, dull colonies with dentate margins and butyrous consistency.
sird SE-3	G (–ve) rods, 1-2 mm, circular, peach, opaque, convex, smooth colonies with entire edge and butyrous consistency.
sird SE-4	G (–ve) rods , 2-6 mm, circular, creamy, raised, translucent, smooth colonies with entire edge and butyrous consistency.
Sird SE-5	G (–ve) rods, 2-4mm , circular, orange, circular opaque, umbonate , smooth colonies with entire edge and powdery consistency.

Table 3.2 Continues

Isolate	Colony morphology and gram characters
sird SF -1	G (-ve), rods, 1 mm pale ,circular, opaque, flat , dull colonies with lobate margins and powdery consistency.
sird SF-2	G (-ve), rods,2-4 mm , circular, peach , translucent , umbonate, smooth colonies with entire edge and powdery consistency.
sird SF-3	G (-ve) ,rods,5-10 mm, irregular ,brown , transparent,umbonate, dull colonies with lobate margins and powdery consistency.
sird SF-4	G (-ve) ,rods,2-4 mm, circular, peach, translucent , umbonate, smooth colonies with entire edge and powdery consistency.
col SVQ	G (-ve), rods,1-2 mm, pale, circular, raised , opaque, glistening colonies with entire edge and viscous consistency.
col SVS	G(-ve), rods,2-4 mm, pale, circular, raised , opaque, glistening colonies with entire edge and viscous consistency.
col SVO	G(-ve), rods, 2-4 mm, pale, circular, raised, opaque, glistening colonies with entire edge and viscous consistency.
bogSG	G (+ve) ,rods, with 2 mm ,white, irregular , dull , opaque , umbonate colonies with rhizoidal edges and butyrous consistency.
aro SL-1	G (-ve) ,rods,1-2 mm, pale, circular ,raised , transparent, glistening colonies with entire edge and butyrous consistency.
aro SL-2	G (-ve), rods ,1-2 mm,,pale, circular ,raised , opaque, glistening colonies with entire edge and butyrous consistency.
mir SO-5	G (-ve) ,rods,1-2 mm, pale, circular,raised , opaque, glistening colonies with entire edge and butyrous consistency.
mir SO-6	G (-ve), rods ,1-2 mm,pale, circular ,raised , opaque, glistening colonies with entire edge and butyrous consistency.
mir SO-11	G (+ve),rods,1-2 mm,pale, circular,raised, opaque, glistening colonies with entire edge and butyrous consistency.
mir SO-12	G (+ve), rods,1-2 mm, pale, circular, raised, opaque, glistening colonies with entire edge and butyrous consistency.

Table 3.3 Biochemical characteristics of selected bacterial isolates.

Biochemical characteristics of isolates										
TESTS	**Bacterial isolates**									
	SA1	**SA2**	**SA3**	**SA4**	**SB1**	**SB3**	**SC1**	**SC2**	**SC3**	**SD2**
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	-	+	+	+	+
Ammonia From Peptone	-	+	-	-	+	+	-	-	-	-
M.R.	+	-	+	+	+	+	-	-	+	-
V.P.	-	-	-	-	-	-	-	+	-	+
Indole	-	-	+	-	-	-	-	-	+	+
Urease	-	-	-	-	+	-	-	-	-	-
Citrate	+	+	+	-	+	-	-	-	+	+
Gelatinase	+	+	+	+	+	+	-	+	+	+
Phenylalanine Deamination	D	-	-	-	D	-	-	+	-	-
Starch Hydrolysis	-	+	+	+	+	+	-	+	+	-
Esculin Hydrolysis	+	+	+	+	+	+	D	-	+	+
Casein Hydrolysis	-	-	+	+	+	+	-	+	+	+
Lipase Activity	+	+	+	-	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	-	-	+	-
H2s Production	-	-	-	-	-	-	-	-	+	+
T S I	- -	+ -	+ +	+ -	+ -	+ -	+ D	+ +	+ +	+ +
O / F	+/ +	+/ +	+/ +	+/ +	+/ +	+/ +	+/ +	- -	-/+	-/+
Growth On TCBS Agar	+	+	+	+	+	+	+	+	+	+
Growth On MacConkey's Agar	+	-	-	-	-	-	-	+	+	+
Growth On Ss Agar	-	-	-	-	-	-	-	-	+	+
Pigmentation On Nutrient Agar	-	-	-	-	-	-	+	+	+	-

Biochemical characteristics of isolates										
Tests	**Bacterial isolates**									
	SD4	**SE1**	**SE2**	**SE3**	**SE4**	**SE5**	**SF1**	**SF2**	**SF3**	**SF4**
Catalase	+	+	+	+	-	+	+	+	+	+
Oxidase	-	-	-	+	+	-	+	+	+	-
Ammonia From Peptone	-	-	-	-	-	-	-	-	-	-
M.R.	+	-	-	-	+	+	-	-	-	-
V.P.	-	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	+	+	+	+	-	+
Urease	-	-	-	-	-	-	-	-	-	-
Citrate	-	+	-	-	-	-	-	-	+	-
Gelatine	+	+	+	+	+	+	+	-	-	+
Phenylalanine Deamination	-	-	-	-	-	-	-	-	D	-
Starch Hydrolysis	-	-	+	+	+	+	+	+	-	+
Esculin Hydrolysis	+	-	+	+	+	+	+	+	+	+
Casein Hydrolysis	-	D	+	+	-	+	+	+	+	-
Lipase Activity	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	-	-	+	+	+	-	+	-	-	-
H2s Production	-	-	-	-	+	-	-	-	-	-
T S I	- +	- -	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
O / F	+/ +	-	+/ +	- -	-/+	+/ +	- -	+/ +	+/ +	-/+
Growth On TCBS Agar	+	-	-	+	+	-	-	-	+	-
Growth On MacConkey's Agar	+	-	-	-	+	-	-	-	–	-
Growth On Ss Agar	-	-	-	-	+	-	-	-	+	-
Pigmentation On Nutrient Agar	-	+	+	+	-	+	-	-	-	+

Biochemical characteristics of isolates										
Tests	**Bacterial isolates**									
Catalase	**SVO**	**SVS**	**SVQ**	**SL2**	**SL3**	**SO5**	**SO6**	**SO11**	**SO12**	**SG4**
Oxidase	+	+	+	+	+	+	+	+	+	+
Ammonia From Peptone	-	+	+	+	+	-	-	-	+	+
M.R.	+	+	+	-	+	-	-	+	+	-
V.P.	-	-	-	-	-	+	+	+	+	-
Indole	+	+	+	+	+	-	-	-	--	-
Urease	-	-	-	-	--	+	-	+	-	-
Citrate	+	+	+	+	+	+	+	+	+	--
Gelatine	+	+	+	+	+	+	-	+	-	-
Phenylalanine Deamination	+	+	+	+	-	+	D	+	D	D
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+
Esculin Hydrolysis	-	-	-	-	-	+	-	-	-	-
Casein Hydrolysis	-	-	-	-	-	-	-	-	-	-
Lipase Activity	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+
H2s Production	+	+	+	+	+	+	+	+	+	-
T S I	H	H	+ +	H	H	H	+ -	H	H	H
O / F	-/+	-/+	-/+	+/ +	+/ +	+/ +	+/ +	-/+	-/+	+/ +
Growth On TCBS Agar	+	+	+	+	+	+	+	+	+	+
Growth On MacConkey's Agar	-	-	-	-	-	-	-	-	-	-
Growth On Ss Agar	+	+	+	+	+	+	-	+	+	+
Pigmentation On Nutrient Agar	-	-	-	-	-	-	-	-	-	-

Table 3.4 Tentative identification of bacterial isolates based on biochemical tests

Sampling sites	Fish host	Isolate Code	Bacterial isolate
SIRIDAO	SILVER BELLY	SA 1	*Micrococcus sp.*
		SA 2	*Bacillus sp.*
		SA 3	*Lactobacillus sp.*
		SA 4	*Bacillus sp.*
MIRAMAR	MULLET	SB 1	*Vibrio sp.*
		SB 2	*Vibro sp.*
		SB 3	*Vibrio sp.*
BETIM	CAT FISH	SC 1	*Vibrio sp.*
		SC 2	*Aeromonas sp.*
		SC 3	*Aeromonas sp.*
BETIM	PEARL SPOT	SD 2	*Micrococcus sp.*
		SD 4	*Streptococcus sp.*
SIRIDAO	CAT FISH	SE 1	*Flavobacterium sp.*
		SE 2	*Alteromonas sp.*
		SE 3	*Lactobacillus sp.*
SIRIDAO	CAT FISH	SE 4	*Alteromonas sp.*
		SE 5	*Alteromonas sp.*
MIRAMAR	MULLET	SF 1	*Vibrio sp.*
		SF 2	*Vibrio sp.*
		SF 3	*Vibrio sp.*
		SF 4	*Vibrio sp.*
COLVA	SARDINE	SVS	*Proteus sp.*
		SVQ	*Proteus sp.*
AROSSIM	SARDINE	SL 2	*Proteus sp.*
		SL3	*Proteus sp.*
MIRAMAR	CAT FISH	SVO	*Vibrio sp.*
		S0 5	*Vibrio sp.*
		S0 6	*Proteus sp.*
		S0 11	*Bacillus sp.*
		S0 12	*Bacillus sp.*

Table 3.5 Molecular identification of selected bacterial isolates by 16S rDNA sequencing and BLAST search

Isolate	Identified as	Homology with	% Homology
SA 1	*Acinetobacter* sp. strain An2 (Accession No.FJ 386957)	*Acinetobacter* sp. WW21 (Accession No. EF433555.1)	**96**
SB 3	*Vibrio parahaemolyticus* strain An3 (Accession No. FJ 386958)	*Vibrio parahaemolyticus* RIMD 2210633 (Accession No. A000031.2)	**97**
SC 3	*Aeromonas hydrophila* strain An 4 (Accession No. FJ 386959)	*Aeromonas hydrophila* (Accession No.AB368776.1)	**98**
SD 4	*Staphylococcus arlettae* strain An1 (Accession No. FJ 386956)	*Staphylococcus arlettae* strain CM18 (Accession No.EU660331.1)	**97**

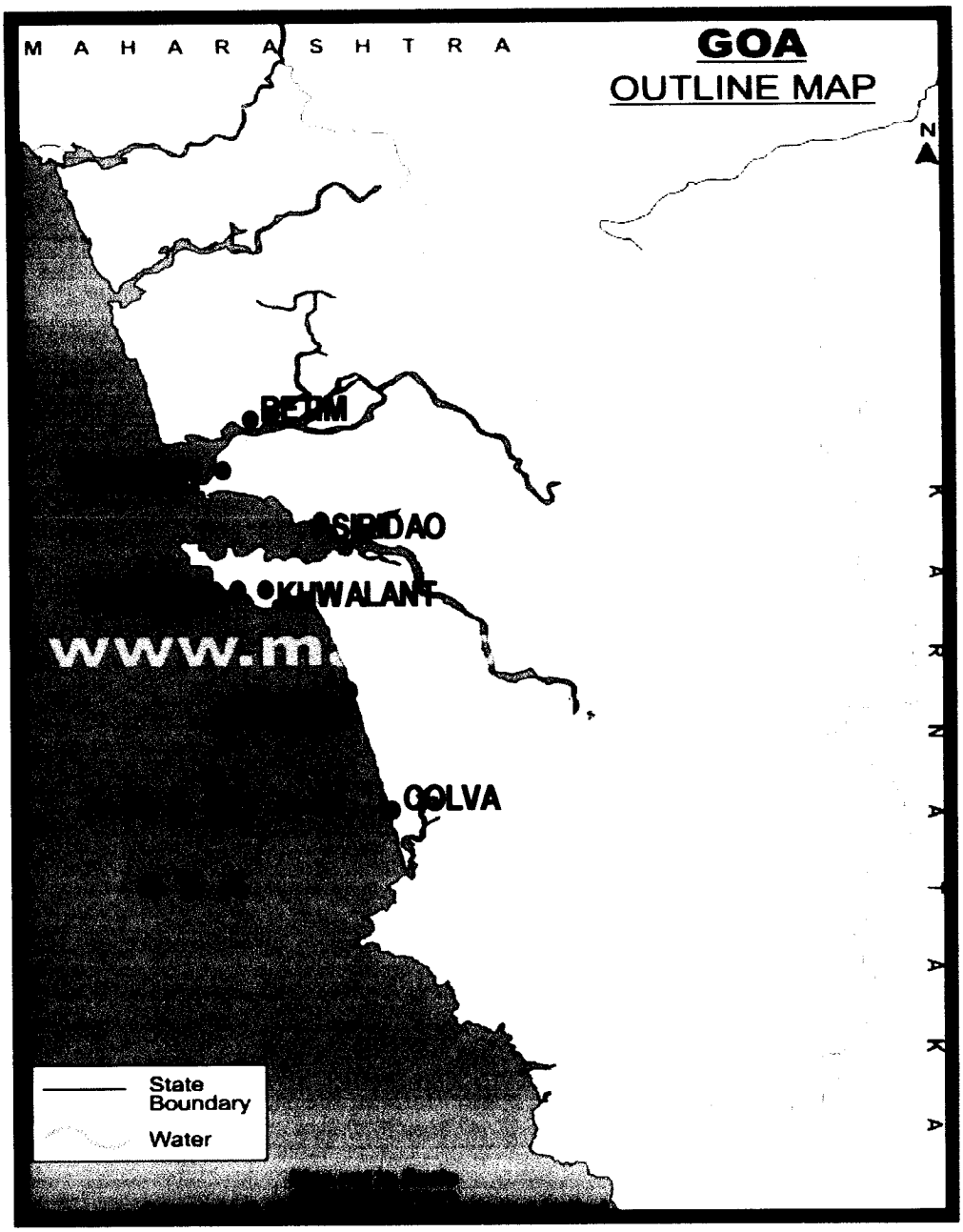


Fig. 3.1 Sample collection sites of Goa.

FISH SHOWING VARIOUS DISEASE SYMPTOMS

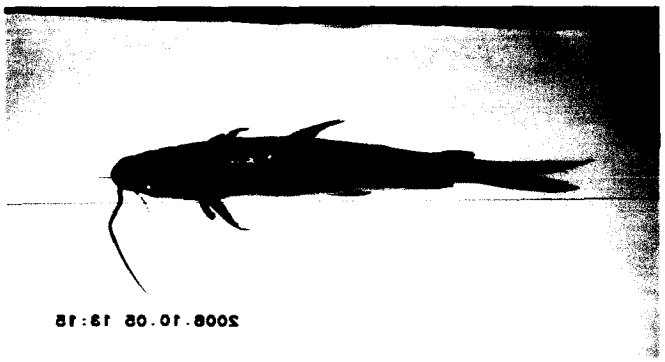


Fig 3.2 Cat fish from Miramar site with necrotic ulcers near the fin

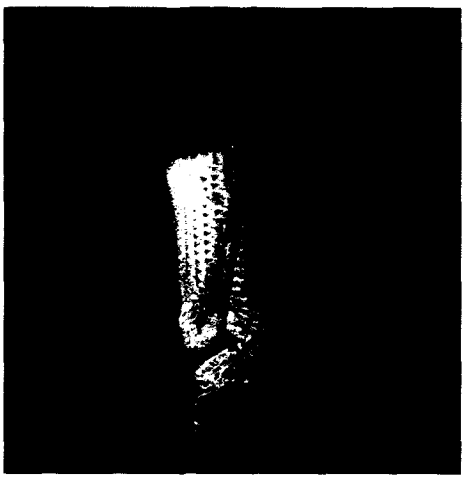


Fig. 3.3 Pearl spot from Betim showing necrotic lesions at tail area

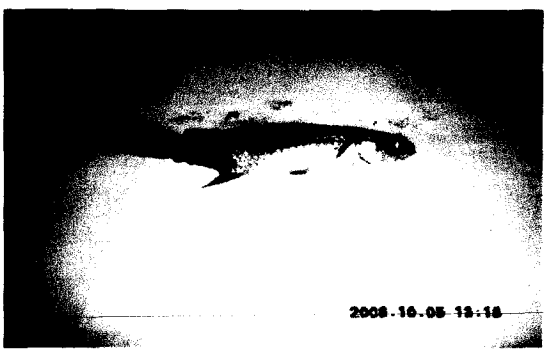


Fig 3.4 Cat fish from Miramar site with lesions at fins and adjacent body parts

Fig. 3. 5 Hemorrhagic ulcers on the mouth part of mullet From Betim

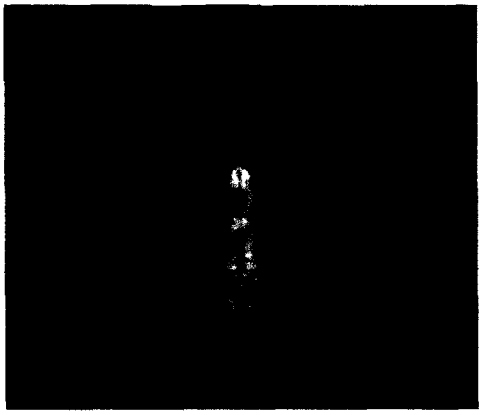


Fig. 3.6 Cat fish from Colva with hemorrhage and blackening of tissue on body parts

Fig. 3.7 Sardin from Arossim with prominent symptoms of fin rot

Fig. 3.8 Necrosis on the body of mullet fom Siridao

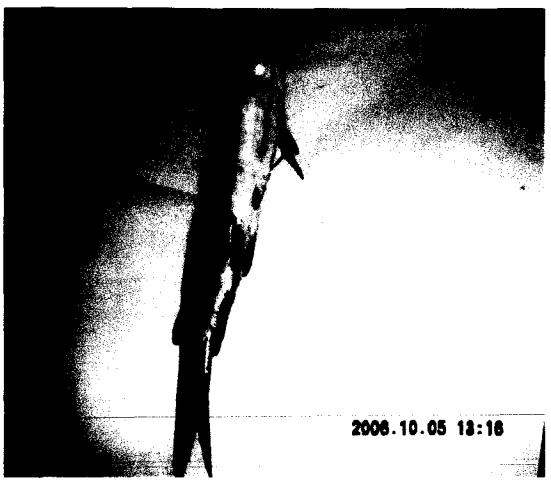
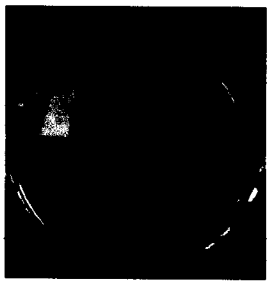
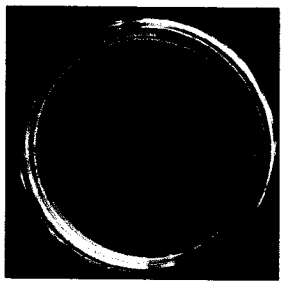


Fig. 3.9 Cat fish from Betim showing blackening of tissue

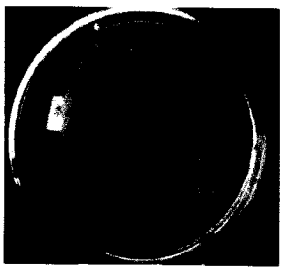
Isolate SE 4



Isolate SB 3

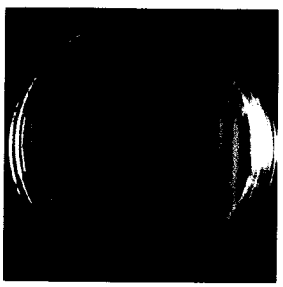


Isolate SE 1

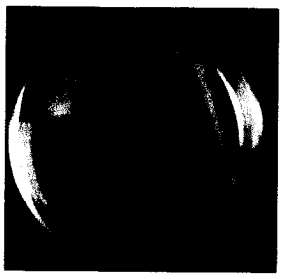


Isolate SE 5

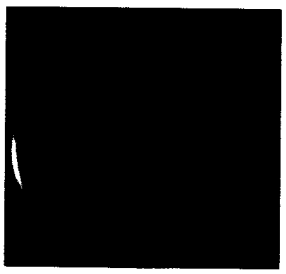
Isolate SD 2



Isolate SF 2



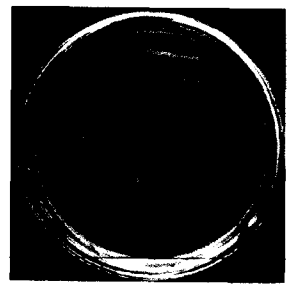
Isolate SA 2



Isolate SA 3

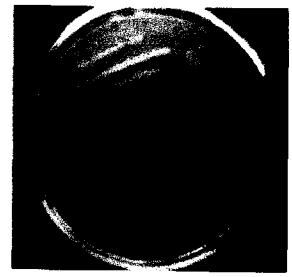
Fig. 3.10 Colony morphology of selected bacterial isolates out of thirty

Isolate SF 3

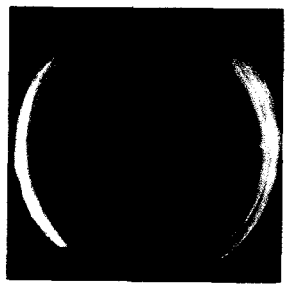


Isolate SC 1

Isolate SF 4

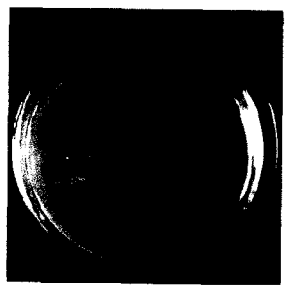


Isolate SE 2



Isolate SE 3

Isolate SC 2



Isolate SF 1

Isolate SO 6

Fig. 3.10 Continues

Fig. 3.11 Colony morphology and Gram character of *Acinetobacter* sp. strain An 2

Fig. 3.12 Colony morphology and Gram character of *Vibrio parahaemolyticus* strain An 3

Fig. 3.13 Colony morphology and Gram character of *Aeromonas hydrophila* strain An 4

Fig. 3.14 Colony morphology and Gram character of *Staphylococcus arlettae* strain An 1

>SA1
AAAAGGCAAGTCGAGCGGAGAGAGGTAGCTTGCTACCGATCTTAGCGGCGGACGGGTGAGT
AATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGAATGCTAATACCGCATA
CGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCGGA
TTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATG
ATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTG
TAAAGCACTTTAAGCGAGGAGGAGGCTACTCTAGTTAATACCTAGAGATAGTGGACGTTACTC
GCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTT
AATCGGATTTACTGGGCGTAAAGCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAAATCCCC
GAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAAT
TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAAGGCAGCCAT
CTGGCCTAACACTGACGCTGAGGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGC
TAACGCGATAAGTAGACCGCCTGGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGGTGGTTTATTCGATGCACGCGAGACCTTACCTG
GCCTTGACATAGTAGACTTCAGAGATGGATTGGTGCCTTCGGGACTACATAACAGTGCTGCAT
GCTGTCGTCAGCTCGTGTCTTGAATGTGGTAGTCCCGGCACGAGCGCACCTTTCTTATTGCAG
CGGTATGTCGGGACTTTATAGATCTGCATGACAAACTGAGAAGGCGGGACGACCTTCAAGCC
CATCATGC

Fig. 3.15 16S rDNA sequence of Acinetobacter sp. strain An 2

>SB3
TGTGACCGGGCCTAAACATGCAAGTCGAGCGGAAACGAGTTATCTGAACCTTCGGGGGACGA
TAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGAT
AACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGG
GCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAA
GGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTG
TAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCA
GGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGG
CAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGA
GGTTGTGGCCTTGAGCCGTGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGGAGTAC
GGTCGCAAGATTAAACTCAATGAATTGACGGGGGCCCGCACAGCGGTGGAGCATGTGTTTAA
TTCGATGCACGCGAGACTTACCTACTCTGACATCCAGAAGAACTTCAGAGATGATTGTGCTTC
GGACTCTGAGAACGTGCTGCATGGCTGTCGTCAGCCTCGTGTTGTGAATGGTGTAGTTCCCG
CACGGGCCACAACTTATCTTGTTGCAGCGGATAGTCGAATCAGGAAACTGCGGTAAAAGGAA
GAAGTGTGGGGG

Fig. 3.16 16S rDNA sequence of Vibrio parahaemolyticus strain An 3

>SC3
GCATGGCGGGCAGGCCTAACAATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTT
GCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACA
GTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCC
TTGCGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGC
CGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAG
CTAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG
CGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCC
AGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGGACAAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAG
GCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAATCACGCATGAGGAGTACGGCC
GCTAGGAT

Fig. 3.17 16S rDNA sequence of Aeromonas hydrophila strain An 4

>SD4
TTTCGGGCCGGCCCCCAATACATGCCCAGTCCGAGCGAACAGATAAGGAGCTTGCTCCTTTG
ACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCC
GGGAAACCGGGGCTAATGCCGGATAACATTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTT
TTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAATGGCTTACCAA
GGCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGT
GTAAGTAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATA
TGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGC
GTGGGGATCAACAAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAAGTGT
AAGGGGGTTTCCGCCCCTTAGTGCTGCGCTAACGCATAAGCACTCCGCCTGGGGAGTACGAA
CGCCAAGGTTGAAACTCAAGGAATGACGGGGACCGCACCAACCGCTGAAGCATGTGGTTTAA
TTTCGAGGCAACGCG

Fig. 3.18 16S rDNA sequence of *Staphylococcus arlettae* strain An 1

CHAPTER IV

RESULTS AND DISCUSSION

Physiological, Biochemical and Molecular Characterization Of Bacterial Isolates

Physiological, biochemical and molecular characterization of selected bacterial isolates

4.1 Environmental optima for the growth of bacterial fish Pathogens

Even after the capability of microorganisms to survive and grow in absence of optimum environmental conditions, environmental optima play a significant role in the growth and biological activites of the organism at its full potential. Keeping in view the importance of this factor the investigation was undertaken to ascertain the environmental optimas for the growth of the selected isolates.

4.1.1 Optimal pH for growth

With a view to decide the optimum pH for the growth of selected bacterial isolates, pH of the growth medium was set at 5, 7 and 9 separately in order to cover acidic, neutral and alkaline range of pH.

Acinetobacter sp. strain An 2 was able to grow at the pH range neutral to alkaline (7 and 9) but failed to grow at acidic pH i.e. 5 (Fig.4.1).

Vibrio parahaemolyticus strain An 3 exhibited good growth at pH 7 but growth at pH 9 was slightly better indicating its preference for alkaline conditions. This strain demonstrated slow growth at pH 5 till 8 hrs but fast growth decline was noticed afterwards (Fig.4.2).

Aeromonas hydrophila strain An 4 showed equally good growth at both neutral and alkaline pH (pH 7 & 9), but took about 6 hrs to get acclimatized to

the acidic pH(pH 5) before entering into log phase of growth. At this pH, the bacterial strain also showed faster decline in growth (Fig.4.3).

Staphylococcus arlettae strain An1 although showed preference for neutral pH for growth than alkaline pH i.e. pH 9, acidic growth condition (pH 5) significantly repressed the growth of this strain (Fig.4.4).

Variable pH is an important limiting factor for survival and growth of bacteria in fish and other hosts, although their response depends upon the type of acids present, but these facts are useful during processessing of fish products. It is interesting to mention that results of our study go hand in hand with earlier reports on growth response of bacterial fish pathogens with reference to environmental variable, pH (Aberoumand, 2010).

4.1.2 Optimal salinity (NaCl) for growth

Keeping in view the vital role played by salt in the form of NaCl for many bacterial cells, different levels of NaCl were amended in the growth media (NB) to determine the optimum salinity for growth of selected pathogenic bacterial isolates.

Acinetobacter sp. strain An 2 showed significant growth at 0 %, 0.5 % and 1.5 % NaCl levels which revealed that NaCl is not essential for its growth. Interestingly, this strain also tolerates upto 3% NaCl but 5% NaCl significantly repressed the growth of this strain (Fig.4.5).

Vibrio parahaemolyticus strain An 3 showed very interesting growth pattern as it exhibited no growth in the absence of NaCl whereas significant growth in the range of 0.5-5 % NaCl levels (Fig.4.6). These studies have

clearly revealed that *Vibrio parahaemolyticus* strain An 3 is a halophile which can grow even in the presence of 5 % NaCl.

Aeromonas hydrophila strain An 4 exhibited best growth at 1.5 % NaCl, moderately good growth at 3 % while it could tolerate upto 5%. This strain could also grow without NaCl but response was poor (Fig.4.7).

Staphylococcus arlettae strain An1 also preferred NaCl as it could barely survive in the medium without NaCl and grew well at even at 5% NaCl with optimum growth at 0.5% and very slight decrease in the growth with the rise in NaCl concentration (Fig.4.8).

4.1.3 Optimal temperature for growth

A wide range of temperature was considered in order to check optimum temperature for growth of selected bacterial strains.

Acinetobacter sp. strain An 2 was able to grow at the variable temperatures such as 22°C, 27°C , 37°C and 42°C, but optimum temperature for growth was 37 °C (Fig.4.9).

Vibrio parahaemolyticus strain An 3 grew very well at 27°C as well as 37 °C, but growth at 22 °C was comparatively very slow, it could grow and survive even at 42°C (Fig.4.10).

Aeromonas hydrophila strain An 4, which could not survive higher temperature i.e. 42°C, also exhibited excellent growth at 27°C and 37 °C and slightly less growth at 22°C (Fig.4.11).

Staphylococcus arlettae strain An1 could also survive both at lower (22°C) and higher (42°C) temperatures but optimum growth was observed at 37 °C and 27°C (Fig.4.12).

It was interesting to note that the results of our study were analogous to the information already documented regarding environmental optimas of the specific bacterial isolates, except *Acinetobacter* spp. Interestingly except *Acinetobacter* sp. strain An2, other pathogens proved the suitability of marine environment for their luxuriant growth. *Acinetobacter* strain An2 might have adopted the marine environment.This strain exhibited different results regarding pH and Salinity preferences but since the strain was isolated from marine fish, it is well established that it has adopted to the marine environment with reference to pH range and salinity for its survival and growth. *Acinetobacter* spp. has been shown to grow at various temperatures. Most of the environmental isolates of *Acinetobacter* prefer incubation temperatures ranging from 20 °C to 30°C but the upper limit differs among different species as some strains of *Acinetobacter* species can grow up to 44°C (Weaver and Actis,1994).

In case of *Vibrio parahaemolyticus,* optimum pH is 7.8-8.6, but it is able to survive a wide range of pH i.e. 4.8-11. NaCl is required for the growth of this bacterial isolate which can grow at 0.5 -10 % NaCl range with 3% NaCl as optimum value. Similarly the optimum temperature reported is 37°C, while it is capable of growth at temperature range 5 - 43°C (Oliver and Kaper, 1997). Growth is very rapid under optimum conditions.Sea foods from tropical region

are known to have high risk of *Vibrio parahaemolyticus* infection due to high temperature of sea water thus the occurence and distribution of this pathogen is all year round (Venugopal et al. 2001; Zulkifli et al., 2009).

Mesophilic *Aeromonads* are halotolerant and are associated with direct discharges to the sea or via rivers and streams. In their study, Hazan et al. (1978) concluded that although *A. hydrophila* was not generally considered to be a marine bacterium, it could be found naturally in marine systems which interface with fresh water. In general, their populations in saline waters were higher than in freshwater. With preference to slightly alkaline pH, moderate growth of A. *hydrophila* at 5 °C is an interesting observation (Ramos and Lyon, 2000; Cho et al., 2003; Vivekanandhan et al., 2003).

Since not much work has been done on *Staphylococcus arlettae*, it can be compared with common *Staphylococcus* sp.This bacterial strain prefers neutral to slightly alkaline pH of the growth medium, but tolerates a wide range of pH (pH 4 - pH 10), temperature (15°C - 45°C) and salinity up to 15 % (Jamshidi et al., 2008).

Temperature and pH are two very important limiting factors for the survival of bacteria in fish and fish products as these important facts are considered during pasteurization and heat treatment, since at room temperature sea foods are likely to deteriorate very fast. Similarly freezing and storage of these foods at low temperature slows down bacterial growth and deterioration of fish which is mediated by some enzymatic and chemical processes (Aberoumand, 2010; Okoro, et al, 2010).

However, the optimal temperature for growth of many pathogenic mesophiles is 37 °C(98 °F),which is normal human body temperature, most microorganisms associated with marine fish of Indian ocean are mesophilic with a fair fraction of thermophiles.Even in tropical sea waters, fish harbour a very small fraction of psychrophilic bacteria (Sen, 2005).

4.2 Detection of exoenzymes produced by the selected bacterial isolates

The selected bacterial strains were found to produce several different exoenzymes viz. amylase, glucosidase, lecithinase, lipase and protease which were clearly demonstrated on suitable media containing specific substrates.

Vibrio parahaemolyticus strain An 3 and *Aeromonas hydrophila* strain An 4 were observed to be more efficient in production of all the above exoenzymes.

Vibrio parahaemolyticus strain An 3 and *Aeromonas hydrophila* strain An 4 exhibited amylase, glucosidase and lecithinase production on respective media. *Acinetobacter* sp. strain An 2 and *Staphylococcus arlettae* strain An1 were able to produce exoenzymes, lecithinase and glucosidase respectively (Figs.4.13 - 4.15).

All the four strains demonstrated lipase production on oil agar medium (Fig. 4.16). On tween 80, *Aeromonas hydrophila* strain An 4 *and Acinetobacter* sp. strain An 2 exhibited better lipase production compared to *Vibrio parahaemolyticus strain* An 3 (Fig. 4.17) which illustrated stronger positive reaction of the enzyme on egg yolk agar compared to these two strains.

All the four strains demonstrated positive results for protease production by liquefaction of gelatine. Protease production by *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4 *on* milk agar was very prominent (Fig. 4.18), while *Acinetobacter* sp. strain An 2 demonstrated protease production on egg yolk agar.

Among all these exoenzymes tested, lipase, protease and lecithinase play a major role in pathogenesis of bacteria as virulence factors while role of amylase and glucosidase in pathogenesis is yet to be established.

Bacteria secrete exoenzymes to hydrolize macromolecules into smaller molecules capable of being transported across the cytoplasmic membrane, and thus help in establishment of infections in case of pathogens. Virulence factors are secreted proteins or enzymes such as invasins, lecithinase, other common lipases and proteases (Leburn et al., 2009). Presence of lecithinase in our pathogenic strains confirms its role as invasin to promote bacterial invasion resulting in severe wound infections in respective fish host. It may also cause invasion of human tissues due to exposure to the pathogen.

Lipids organized in bilayers, constitute the core structure of all cell membranes, such as plasma membrane as well as those of intracellular organelles. In contrast, bacterial protein toxins are water-soluble molecules. However, all bacterial protein toxins interact first with cell membranes by recognizing specific receptors, which are lipid or another molecule but anchored to the lipid bilayer (Geny and Popoff, 2006). Lipolytic enzymes are utilized by a variety of bacterial pathogens to support their establishmant

during infection process (Bender and Flieger, 2010). Lipase is also an important extra-cellular virulence factor, damaging the host tissue lipids and affecting several immune system functions through free fatty acid generated by lipolytic activity as in Acinetobacter spp. (Berezin and Towner, 1996) and Aeromonas *hydrophila* (Cascon et al., 1996; Singh et al., 2010).

Proteases play a crucial role in numerous pathologic processes. Arthritis, tumor invasion and metastasis infections along with a number of degenerative diseases have been linked to one or more proteolytic enzymes (Brown, 1994). Microbial proteases are well established virulence factors in a variety of diseases caused by microorganisms. The virulence of *Pseudomonas aeruginosa* is multi-factorial, but it is partly determined by exo-products such as alkaline protease and elastase, which are responsible for the damage of tissues by degrading elastin, collagen and proteoglycans. These enzymes have been also shown to degrade proteins that function in host defense *in vivo* (Sakata et al., 1993; Vermelho et al., 1996; Lantz, 1996). Leung et al., (1988) suggested that proteases also play an important role in causing massive tissue damage in the host which may aid the establishment of infection (Secades and Guijarro, 1999). Sudheesh and Xu (2001) reported possible role of extracellular proteases in pathogenicity of Vibrio *parahaemolyticus* in tiger prawn.

4.2.1 Protease assay

Protease enzyme assay vs growth was done using two strains viz. *Vibrio parahaemolyticus* strain An 3 and Aeromonas *hydrophila* strain An 4. Both of these bacterial strains interestingly revealed commencement of protease

production during late log phase of growth which was 14 hrs for *Aeromonas hydrophila* strain An 4 and 18 hrs for *Vibrio parahaemolyticus* strain An 3, with maximum production during stationary growth phase i.e. 26 hrs and 22 hrs respectively (Figs. 4.19, 4.20). This phenomenon clearly revealed the dubious nature and capability of these protease producing pathogens causing damage to host cells even after in its stationary growth phase.

4.3 Hemolytic activity

Obvious evidence of hemolytic activity were demonstrated by *Acinetobacter* sp. strain An 2, *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4, in the form of clearance zones around the colonies on the blood agar (β hemolysis), where as *Staphylococcus arlettae* strain An1 exhibited prominent α hemolysis (Figs. 4.21 - 4.24).

Hemolysin, a cytotoxin is one of the very important factors which decides the virulent character of bacterial pathogens although their mode of action may vary. *Vibrio parahaemolyticus* is divided into two groups based on hemolysis in a special blood agar medium (Wagatsuma medium) and the hemolysis observed in this medium has been referred as the "Kanagawa phenomenon". This term was given and described as early as 1969 by Miyamoto. It has been well recognized that Kanagawa phenomenon positivity in *Vibrio parahaemolyticus* is closely associated with human pathogenicity over the period of time (Chun et al., 1975; Nair et al., 1985; Kaysner et al., 1992; Zulkifli et al., 2009; Honda and Iida, 1993; Chawdhury et al., 2000; Kim and Kim, 2001).

In *Aeromonas hydrophila* also hemolysin plays an important role as virulence and lethality factors (Aoki and Hirono, 1991). The formation of a hemolysin from *Aeromonas hydrophila* was studied by Caselitz (1966) who found that most strains produced haemolysin and the activity accumulated extracellularly in the liquid media (Wretlind and Heden, 1973).

4.4 Detection of pathogen-specific toxin encoding virulence genes by PCR amplification

Since all the isolates were producing hemolysins, genes, encoding these virulencs factors from two of the isolates were PCR amplified using *tdh* and *hlyA* specific primers and genomic DNA of *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* respectively as DNA templates.

4.4.1 Amplification of *tdh gene*

Presence of *tdh* gene (amplicon: 263 bps) in *Vibrio parahaemolyticus* strain An 3 was clearly demonstrated by PCR amplification which is known to encode TDH toxin (Fig. 4.25).

tdh is the gene which encodes protein toxin, thermostable direct hemolysin (TDH) responsible for Kanagawa phenomenon in *Vibrio parahaemolyticus.* Although *tdh* gene is the thermostable direct hemolysin found in very low numbers (1-7%) among environmental and sea samples (Depaola et al., *2000;* Lee et al., 2002; Dileep et al., 2003), we have evidently demonstrated the presence of *tdh* gene (263 bps amplicon) in *V. parahaemolyticus* causing infections in *Mugil cephalus* which is a known virulence factor of the pathogen (Osawa et al., 2002; Okura et al., 2003).

Group of researchers lead by Prof. Karunasagar (1996) have also reported detection *tdh* gene in Kanagawa positive strains of *Vibrio parahaemolyticus* by PCR. This assay could detect contamination of seafood homogenate when PCR was performed using lysate prepared directly from fish homogenates.These researchers have been working extensively on detection of *V. parahaemolyticus* virulence encoding genes including *tdh* by PCR amplification and other advanced molecular techniques (Karunasagar et al., 2002; Dileep et al., 2003; Deepanjali et al., 2005; Gopal et al., 2005; Raghunath et al., 2008). A rapid, sensitive and highly reproducible SYBR green dye based real-time PCR assay was also developed by them for detection of *tdh* positive pathogenic *Vibrio parahaemolyticus* (Tyagi et al., 2009).

4.4.2 Amplification of *hly A gene*

PCR amplification of hly *A* gene encoding beta-hemolysin in Aeromonas *hydrophila* strain An 4, evidently revealed presence of an amplicon of 597 bps which encodes hemolysin (Fig. 4.26).

Toxins with hemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas* spp. The hemolytic activity of *Aeromonas hydrophila* is related to both hemolysin (*aerA* and *hlyA*) and cytolytic enterotoxin *(aer)* genes. Several virulence factors have been identified in bacterial strains isolated from a number of sources. It is possible that more than one gene is involved in hemolytic/enterotoxic activity in the same strain (Falcon et al., 2005; Jiravanichpaisal et al., 2009; Castilho et al., 2009). A variety of potential virulence factors and toxins have been characterised in *Aeromonas*

hydrophila, and significant variation is seen even in hemolysin gene hlyA. An hlyA amplicon of 1500 bps was reported by some authors (Kim et al., 2003; Yousr et al., 2007) whereas Wong, et al., (1998) reported hlyA amplicon of 597 bps. It is interesting to mention that in our investigation we have also got *hly A* amplicon of 597 bps while using same primers which has been supported by many other researchers (Heuzenroeder et al., 1999; Gonzalez-Serrano, 2002; Falcon et al., 2005; Seethalakshmi et al., 2008; Bhowmik et al., 2009; Yogananth et al., 2009).

4.5 Characterization of extracellular proteins (ECPs)

Virulence factors are characteristic feature of many pathogens, one of them is extracellular protein secreted by the pathogenic bacteria.

During our investigation, characterization of extracellular proteins (ECPs) of all the four bacterial fish pathogens by SDS-PAGE analysis revealed several significant ECPs ranging in molecular mass from 30 kDa - 97 kDa (Fig. 4.27). Interestingly, prominent protein bands of 23 kDa was noticed in case of *Vibrio parahaemolyticus* strain An 3 and 50 kDa in case of *Aeromonas hydrophila* strain An 4 which may very well be correlated with their characteristic TDH and HLY toxins respectively.

Acinetobacter sp. strain An 2 demonstrated a prominent band of 68 kDa which could be correlared with its Shiga toxin. Shiga toxin is a multi-subunit protein made of one molecule of A subunit responsible for the toxic action of the protein and five molecules of B subunits. These toxins can be classified into two antigenic groups, Stx-1 and Stx-2 that include (especially Stx-2), an

important number of genotypic variants. Since our strain also produced β hemolysin, we may correlate this specific ECP of *Acinetobacter* strain to shiga toxin (Fig.4.21). It is interesting to mention that the presence of shiga toxin in a strain of *Acinetobacter haemolyticus* causing bloody diarrhoea has already been reported (Grotiuz et al., 2006).

Many other bacterial species produce Shiga toxin (Stx) which has been associated with bloody diarrhoea, thrombocytopenic thrombotic purpura, and hemolytic-uremic syndrome (HUS) in humans (Polard et al., 1990; Grotiuz et al., 2006).

Staphylococcus arlettae strain An1 demonstrated the presence of a very prominent protein band of 33 k Da (Fig. 4.27) which could be correlated with *Staphylococcal* α toxin as the strain also demonstrated very prominent α hemolysis (Fig. 4.24).

Alpha toxin, in its monomeric form, is a water-soluble protein that hexamerizes in the target membrane, creating a stable trans-membrane pore. The size of the pore formed by the alpha toxin does not allow intracellular proteins to escape from the cells, whereas low molecular weight molecules are released and can thus be exchanged in permeabilized cell preparations. Molecular weight of *Staphylococcal* α toxin is reported to be 33-34 k Da (Lind et al., 1987; Palmer et al., 1993; Hume et al., 2000; Geny and Popoff, 2006).

Two haemolytic toxins, haemolysin and aerolysin have been reported in *Aeromonas hydrophila*. Hemolytic proteins are commonly isolated from pathogenic bacteria, and β-hemolysins are one of the important bacterial

virulence factors. Hemolysins and related proteins containing cystathionine β synthase (CBS) domains are bacterial toxins that function by assembling identical subunits into a membrane spanning pore (Erova et al., 2007). The molecular mass of *Aeromonas* hemolysins range from 49-60 k Da (Asao et al., 1984; Bloch and Monteil, 1989; Aoki and Hirono, 1991; Buckley, 1992; Erova et al., 2007). A virulent protein of 52 kDa in *Aeromonas hydrophila* was demonstrated by Cipriano (2001), whereas a hemolysin of 50 k Da was reported in *Aeromonas hydrophila* (Asao et al., 1984; Erova et al., 2007) but same hemolysin of 51 k Da was reported by Bloch and Monteil (1989).

We have also demonstrated presence of a prominent protein band of approximately 50 k Da in extracellular protein fraction of this pathogenic strain *Aeromonas hydrophila* strain An 4 encoded by *hlyA* gene and further confirmed the presence of hemolysin encoded by this gene. The extracellular proteins (ECPs) of A. *hydrophila* have been implicated in its pathogenicity (Akoi and Hirono, 1991). Conspicuous protease activity along with hemolytic activity shown by *Aeromonas hydrophila* strain An 4 further strengthens its pathogenic potential as it has already been proved that presence of both the proteins enhances the intensity of virulence in this fish pathogen (Roguluska et al., 1994).

Studies have implicated that the thermostable direct hemolysin (TDH), among several hemolysins produced by *V. parahaemolyticus* are responsible for the "Kanagawa phenomenon". Therefore, presence of the *tdh* gene marked by a β-type hemolysis on Wagatsuma agar serves as markers for pathogenic

strains (Honda et al., 1980; Chawdhury et al., 2000). Thermostable direct hemolysin (TDH) secreted from *V. parahaemolyticus* has been considered a major virulence factor and its connections with pathogenicity has already been established (Honda et al., 1976; Tada *et al.*,1992; Honda and lida, 1993; Zulkifli et al., 2009). Since *V. parahaemolyticus* strain An 3 also possessed TDH encoding gene amplicon of 263 bps and a prominent protein band of 23 kDa on SDS- PAGE we can infer from our experiments that this pathogenic bacterial strain possessed TDH toxin.

Amongst various mechanisms developed by pathogenic bacteria to cause diseases, toxins (including ECPs) play an important role since they are responsible for the majority of host symptoms during infection by pore formation and enzymatic activities towards host cell membranes (Popoff, 2005).

Chapter IV

Figures

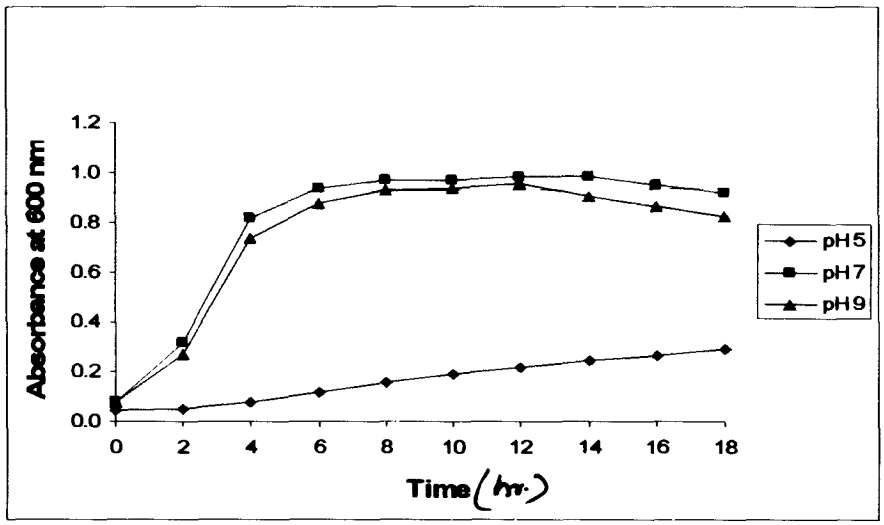


Fig. 4.1 Growth of *Acinetobacter* sp. strain An 2 at different pH

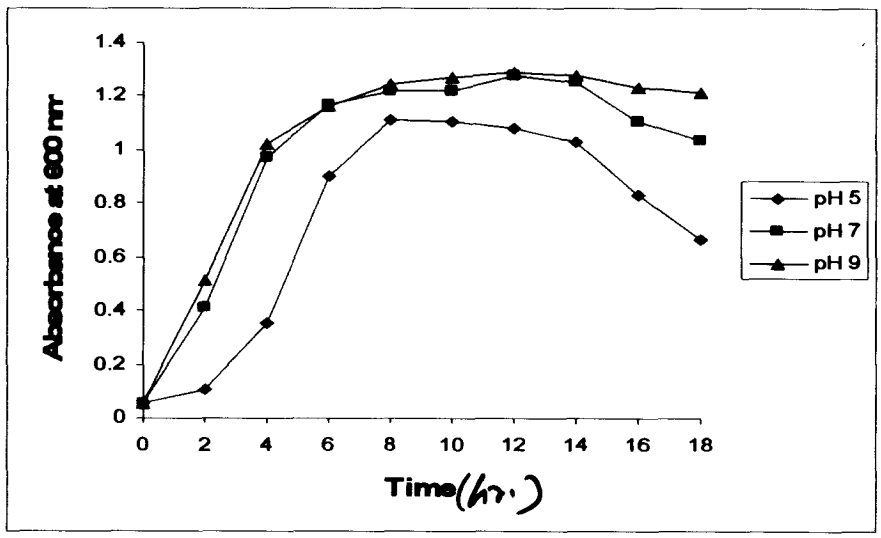


Fig. 4.2 Growth of *Vibrio parahaemolyticus* strain An 3 at different pH

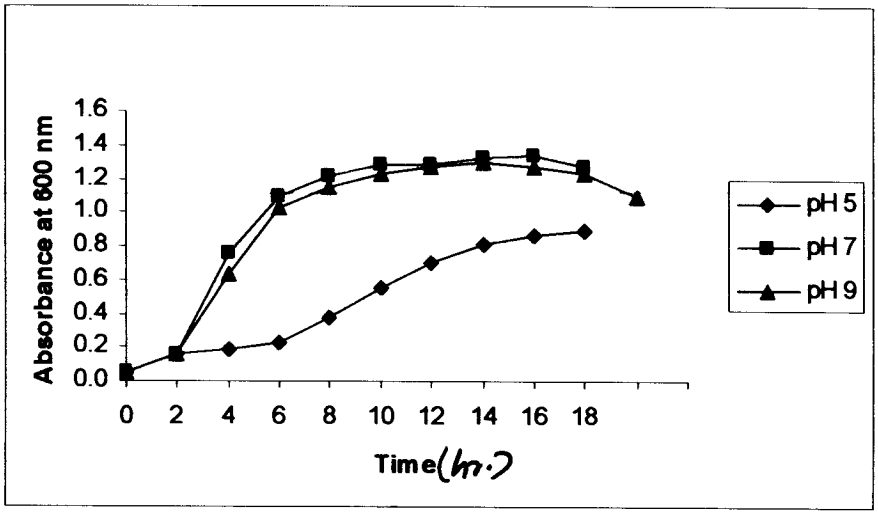


Fig. 4.3 Growth of *Aeromonas hydrophila* strain An 4 at different pH

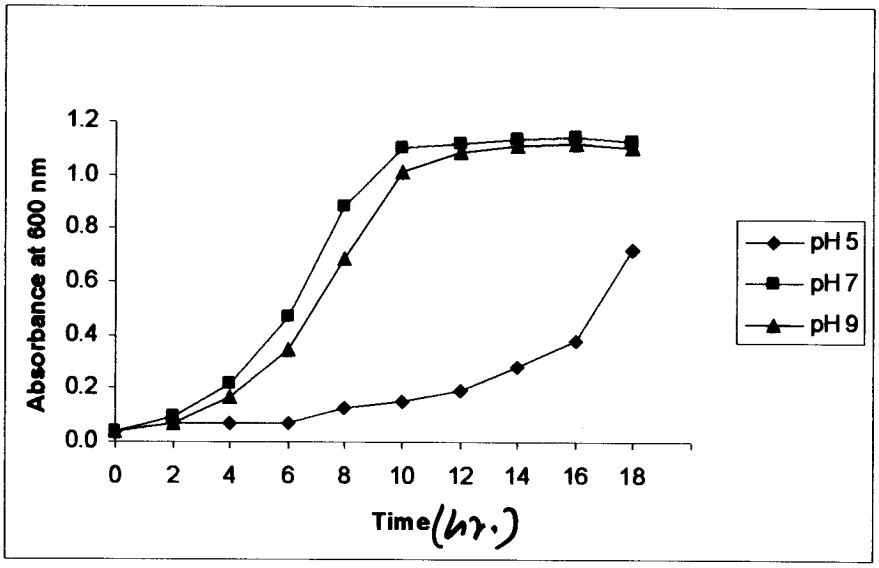


Fig. 4.4 Growth of *Staphylococcus arlettae* strain An1 at different pH

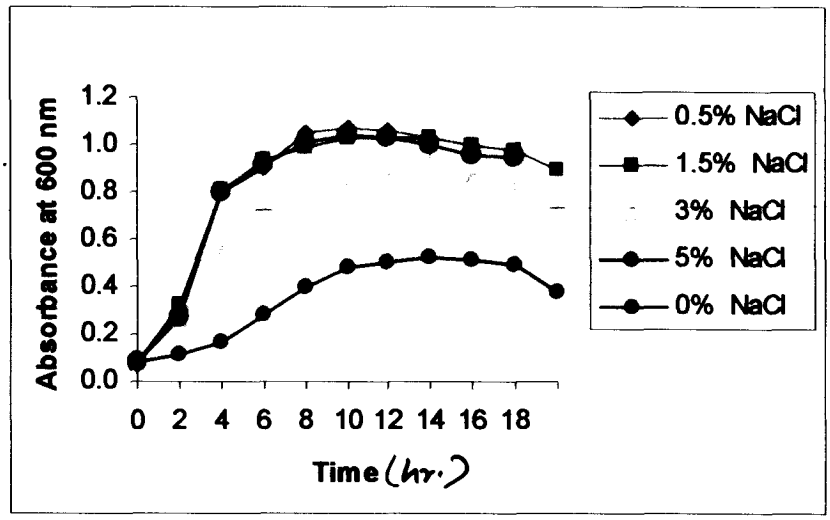


Fig. 4.5 Growth of *Acinetobacter* sp. strain An 2 at different NaCl conc.

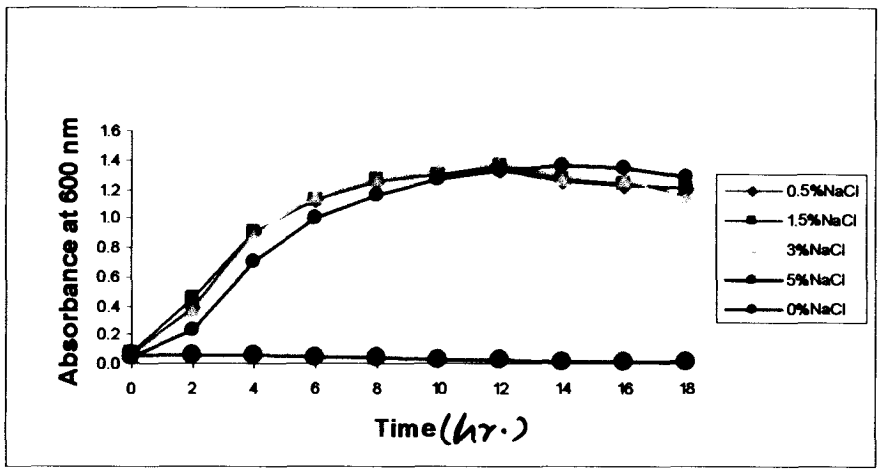


Fig. 4.6 Growth of *Vibrio parahaemolyticus* strain An 3 at different NaCl conc.

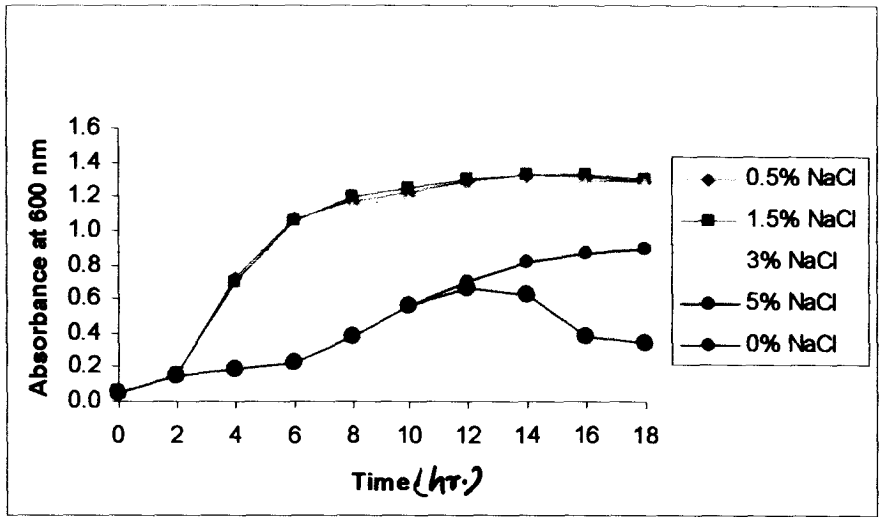


Fig. 4.7 Growth of *Aeromonas hydrophila* strain An 4 at different NaCl conc.

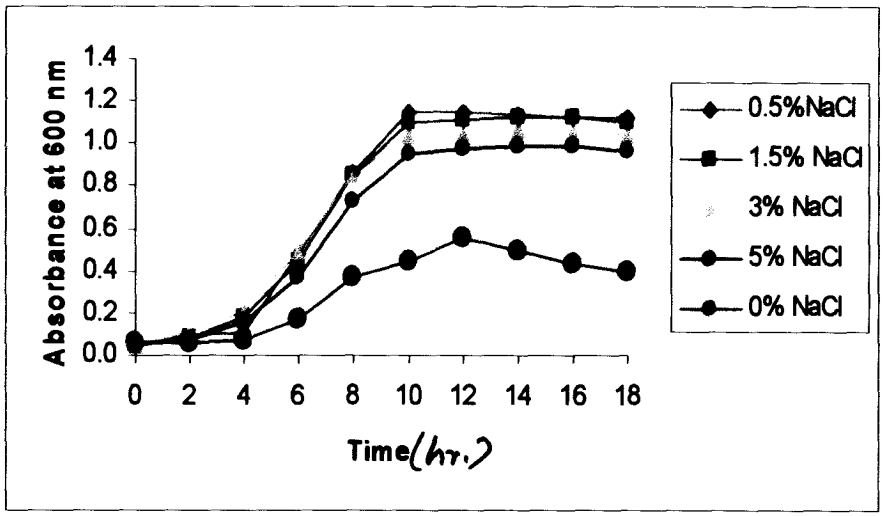


Fig. 4.8 Growth of *Staphylococcus arlettae* strain An1 at different NaCl conc.

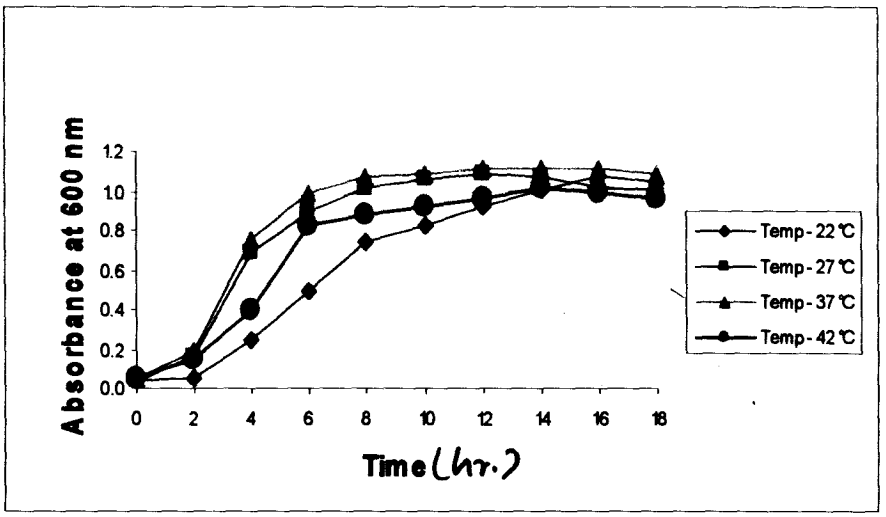


Fig. 4.9 Growth of *Acinetibacter* sp. strain An 2 at different temperatures

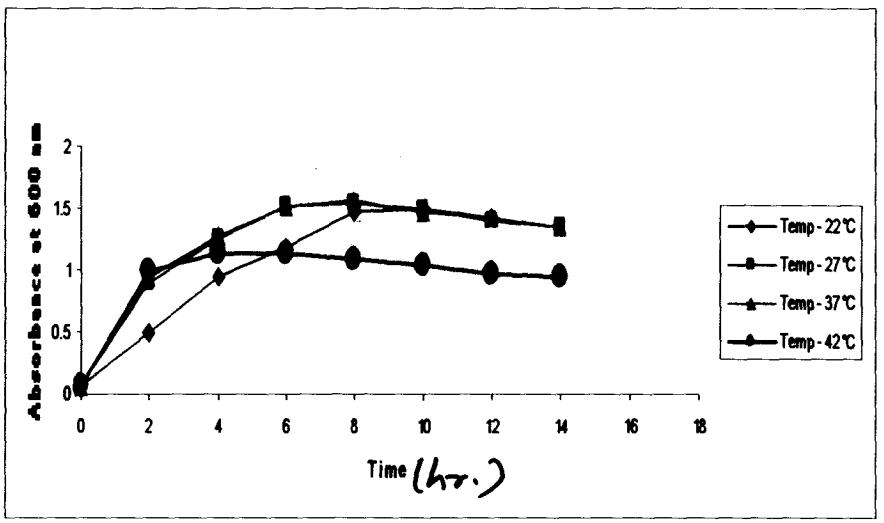


Fig. 4.10 Growth of *Vibrio parahaemolyticus* strain An 3 at different temperatures

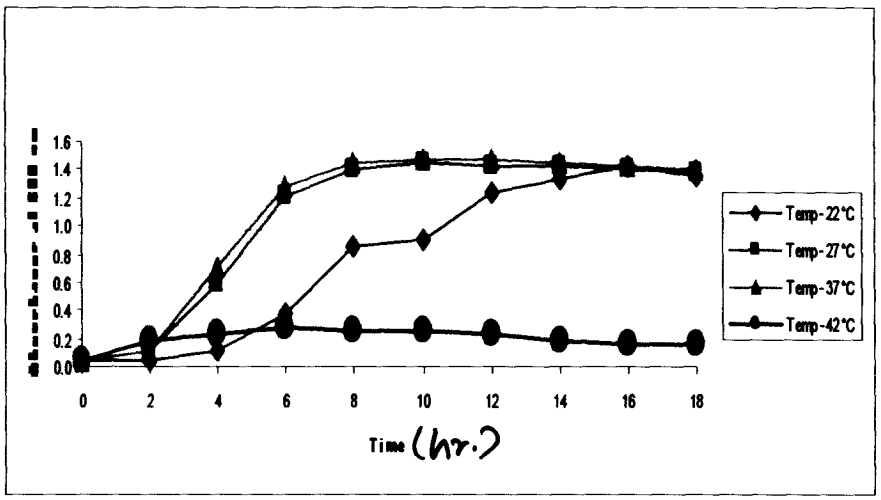


Fig. 4.11 Growth of *Aeromonas hydrophila* strain An 4 at different temperatures

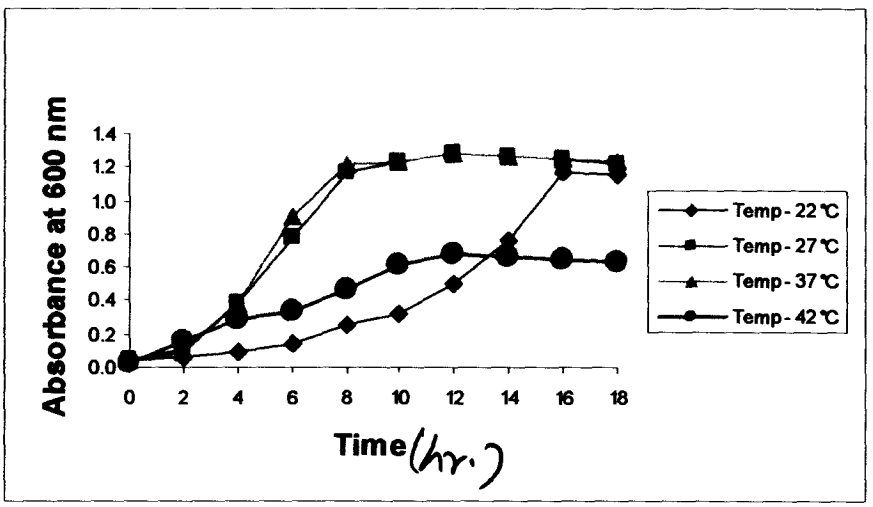


Fig. 4.12 Growth of *Staphylococcus arlettae* strain An 1 at different temperatures

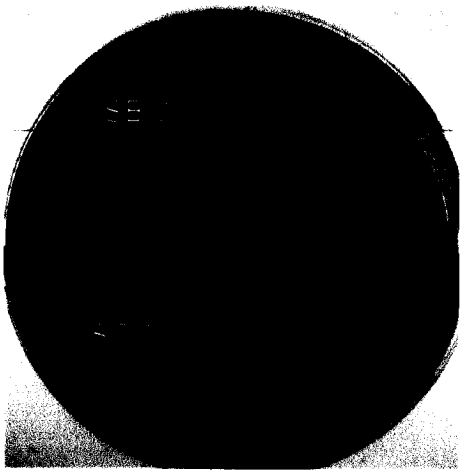


Fig. 4.13 Amylase production by isolates SB 3 and SC3

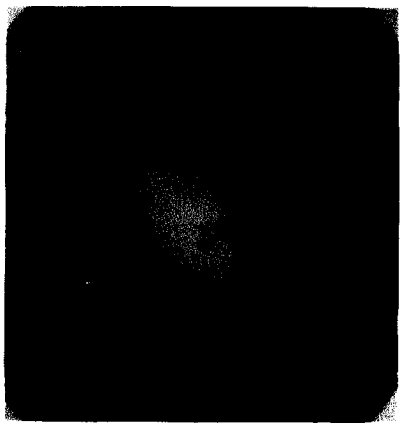


Fig. 4.14 Glucosidase production by isolates SB 3, SC3 and SD 4

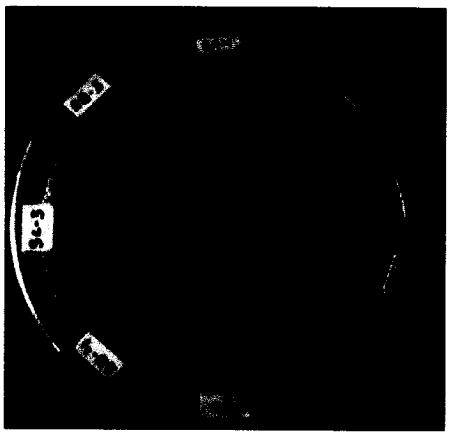


Fig. 4.15 Lecithinase, lipase and protease production by isolates SA 1, SB 3 and SC3 on egg yolk agar

Isolate SA 1 ***Acinetibacter*** **sp. strain An 2**

Isolate SB 3 ***Vibrio parahaemolyticus*** **strain An 3**

Isolates SC3 ***Aeromonas hydrophila*** **strain An 4**

Isolates SD 4 *Staphylococcus arlettae* strain An 1

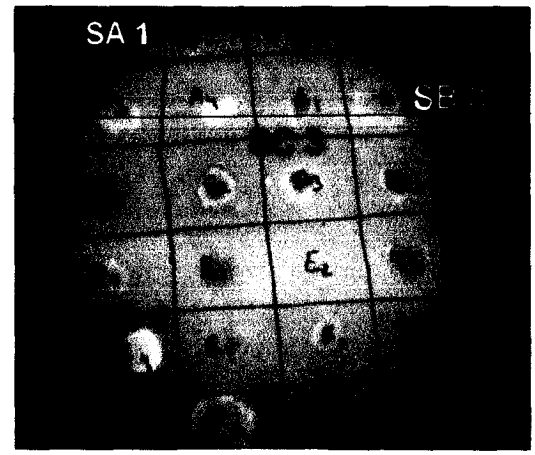


Fig 4.16 Lipase production by the isolates SA1, SB 3, SC 3 and SD4 on oil agar

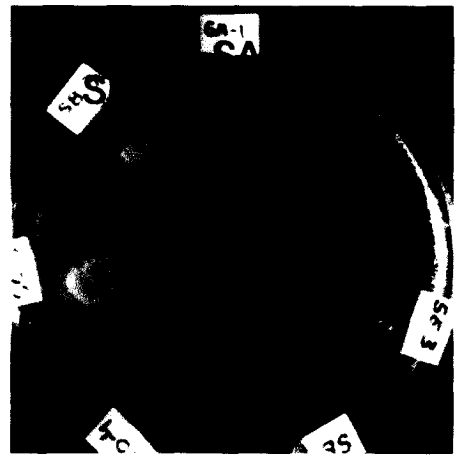


Fig 4.17 Lipase production by the isolates SA 1, SB 3 and AC 3 on tween 80

Fig. 4.18 Protease production by the isolates SB 3 and SC 3 on milk agar

Isolate SA 1 *Acinetibacter* sp. strain An 2

Isolate SB 3 *Vibrio parahaemolyticus* strain An 3

Isolates SC3 *Aeromonas hydrophila* strain An 4

Isolates SD 4 *Staphylococcus arlettae* strain An 1

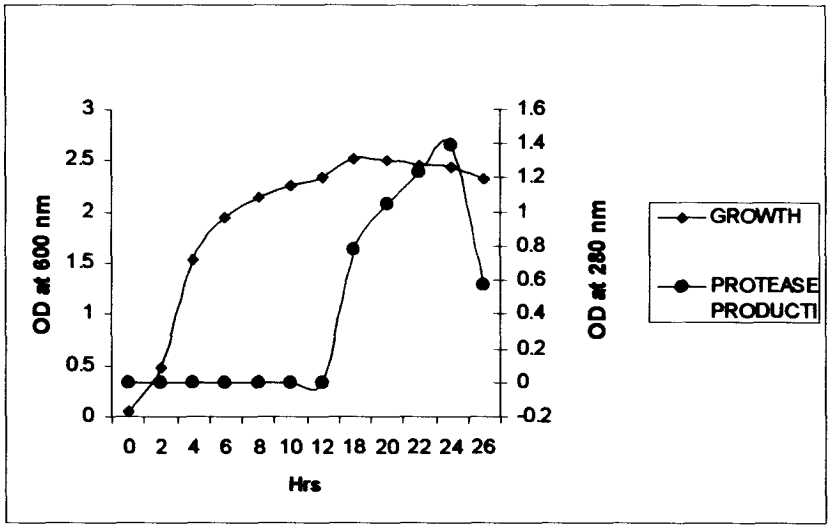


Fig. 4.19 Growth vs. protease production by *Aeromonas hydrophila* strain An 4

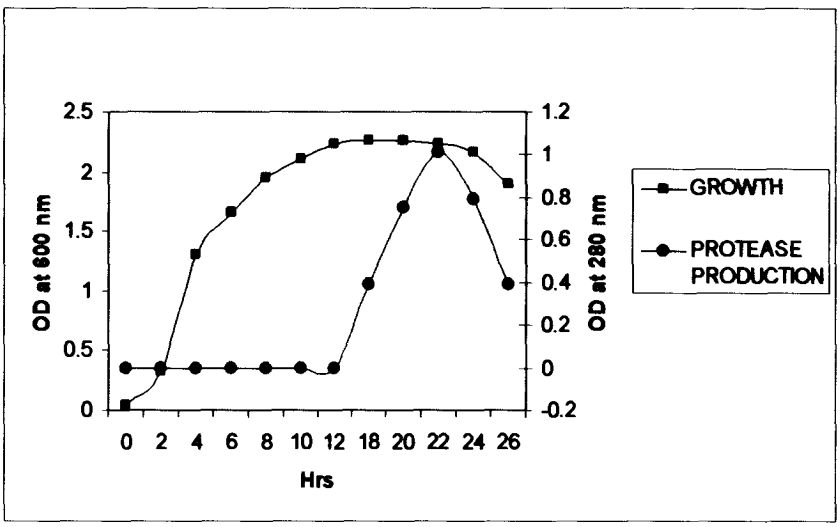


Fig. 4.20 Growth vs. protease production by *Vibrio parahaemolyticus* strain An 4

Hemolytic Activity

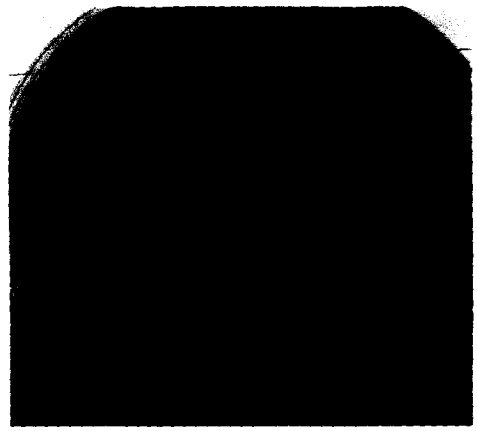


Fig. 4.21 Hemolytic activity by *Acinetobacter* sp. strain An 2

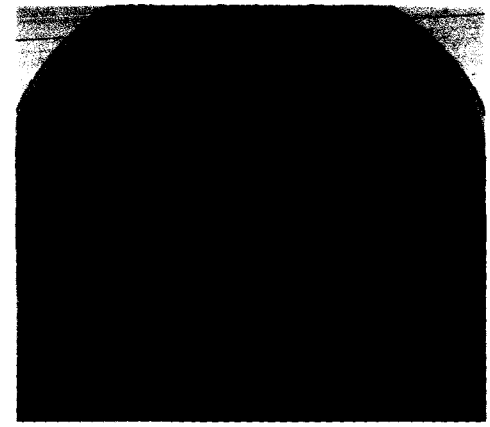


Fig. 4.22 Hemolytic activity by *Vibrio parahaemolyticus* strain An 3

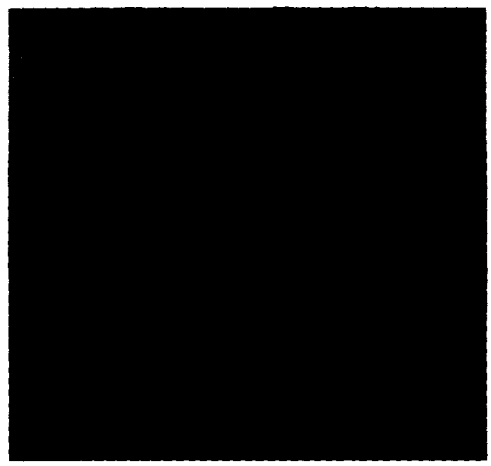


Fig. 4.23 Hemolytic activity by *Aeromonas hydrophila* strain An 4

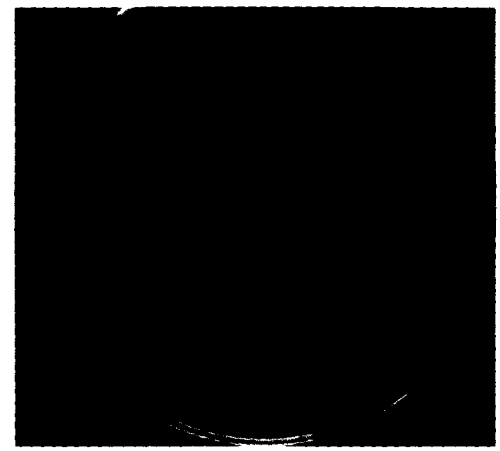


Fig. 4.24 Hemolytic activity by *Staphylococcus arlettae* strain An 1

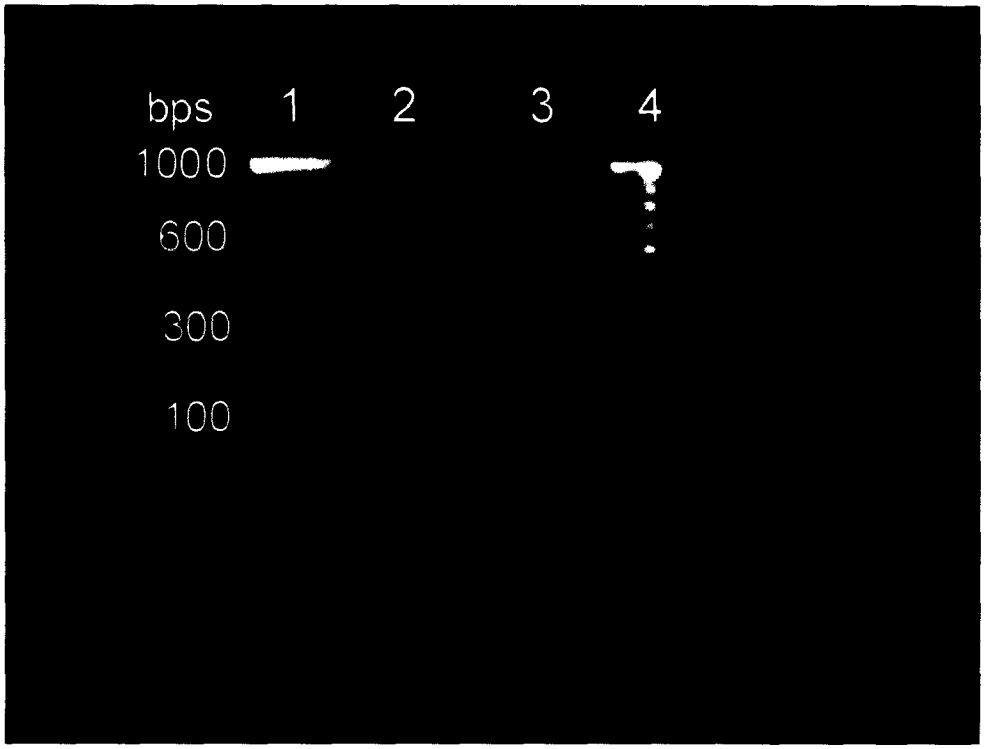


Fig. 4.25 PCR Amplification of *tdh* gene from genomic DNA of *Vibrio parahaemolyticus* strain An 3

Lanel and 4:100 bps DNA marker,

Lane 2: *tdh* amplicon (263 bps)

Lane 3: (-)ve control

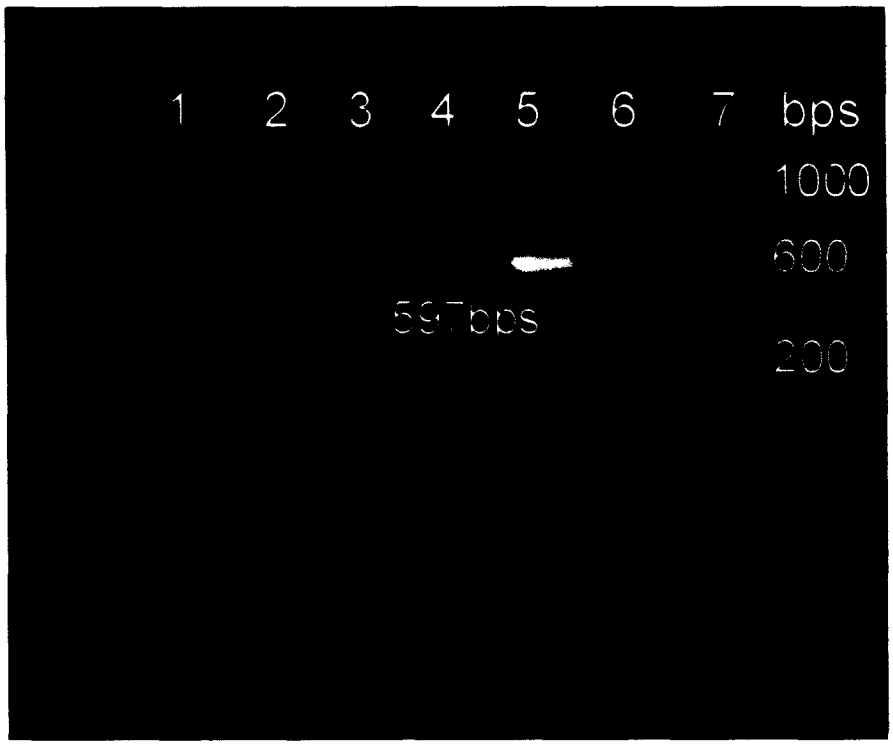


Fig. 4.26 PCR Amplification of *hly* gene from genomic DNA *Aeromonas hydrophila* strain An4

Lane 1and 7: 100 bps DNA ladder

Lane 2:(-) ve control

Lane 4 : *hly* (-ve) Isolate SA1

Lane 5: *hly A* amplicon (597 bps),

Lane 6: *hly* A (-ve) Isolate SD4

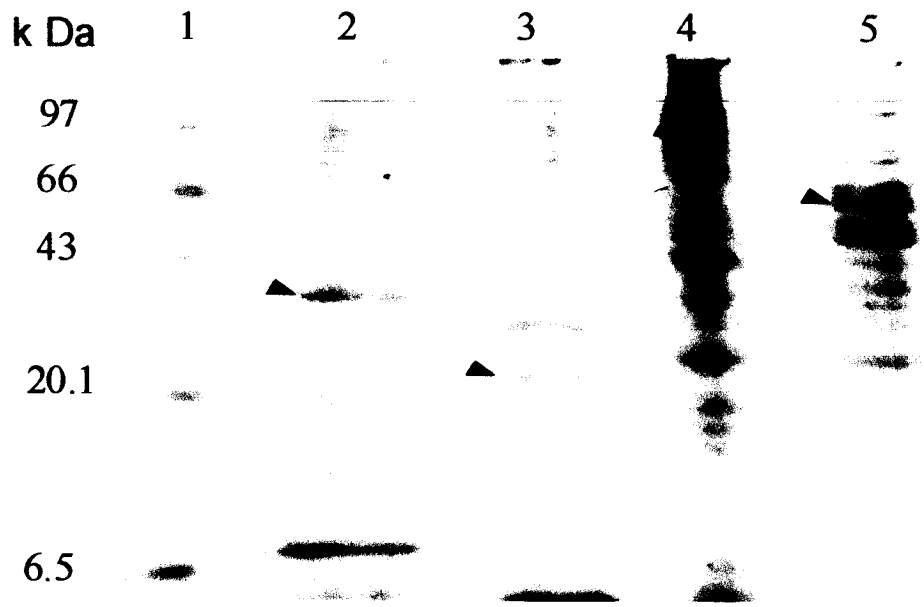


Fig. 4.27 SDS -PAGE analysis of ECPs of bacterial isolates

Lane 1: Protein molecular weight marker ,

Lane 2: Isolate SD 4 (alpha toxin-33 kDa),

Lane 3: Isolate SB 3(TDH toxin-23 kDa)

Lane 4: Isolate SA 1(Shiga toxin-68 kDa)

Lane 5: Isolate SC 3 (Hly toxin-50 kDa)

Isolate SD 4 Staphylococcus arlettae strain An 1
Isolate SB 3 Vibrio parahaemolyticus strain An 3
Isolate SA 1 Acinetobacter sp. strain An 2
Isolate SC 3 Aeromonas hydrophila strain An

CHAPTER V

RESULTS AND DISCUSSION

Biological Characterization of the Selected Isolates With reference to Antibiotic Activity and Responce to Antibiotics

Biological characterization of the selected isolates with reference to antibacterial activity and response to antibiotics.

5.1 Antimicrobial bioassay (agar well diffusion assay)

Antibacterial activity of these bacterial pathogenic isolates by agar well diffusion method clearly revealed antibacterial activity of the ethyl acetate cell extract of all the three gram negative isolates viz. *Acinetobacter* sp. strain An 2, *Aeromonas hydrophila* strain An 4 and *Vibrio parahaemolyticus* strain An3, against *E.coli*, since zone of clearance/inhibition for indicator bacteria was observed around the wells containing the cell extract. Only *Staphylococcus arlettae* did not antagonise the growth of *E.coli* (Fig.5.1). Encoruraged by the results, antagonistic activity of remaining three pathogens was again tested against each other and results obtained were quite interesting (Table 5.1).

Crude cell extract of *Acinetobacter* sp. strain An 2 prepared in ethyl acetate showed its antagonistic characteristic by inhibiting the growth of gram (-ve) pathogenic bacterial indicators viz. *Aeromonas hydrophila* strain An 4 and *Vibrio parahaemolyticus* strain An3, whereas it could not inhibit the growth of gram (+ve) *Staphylococcus arlettae* strain An1 possibly due to different cell wall constituents and less suceptibility of this gram positive indicator bacteria to antimicrobial metabolites produced by the test organism (Figs.5.2 and 5.5).

Antagonistic characteristic of crude cell extract of *Aeromonas*

hydrophila strain An 4 as manifested by growth inhibition of gram (-ve) indicator bacteria viz. *Acinetobacter* sp. strain An 2, *Vibrio parahaemolyticus* strain An3, and gram (+ve) *Staphylococcus arlettae* strain An1(Figs.5.3, 5.4 and 5.5).

Agar well diffusion experiment clearly demonstrated the antagonistic characteristic of crude cell extract of *Vibrio parahaemolyticus* strain An3 as manifested by growth inhibition of gram (-ve) indicator bacteria viz. *Acinetobacter* sp. strain An 2, *Aeromonas hydrophila* strain An 4, and gram (+ve) *Staphylococcus arlettae* strain An1(Figs. 5.2, 5.3 and 5.4).

During the last decades, however, repeated isolation of known metabolites and a reduced hit-rate of novel compounds from marine macroorganisms were observed. Hence, natural product chemists are turning their interest to so far less investigated drug sources, such as marine fungi and bacteria, marine natural products play an increasingly important role in biomedical research and drug development, either directly as drugs or as lead structures for bioinspired chemical drug synthesis (Molinski et al., 2009; Debbab et al., 2010). Recently, several biologically active substances have been isolated from marine bacteria. There were many reports about antibacterial activity shown by marine bacteria viz. *Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus* and *Alteromonas* (Gauthier and Breittmayer, 1979; Shiozawaet al., 1997; Jorquera et al., 1999; Khalil et al., 2006; Ahmed et al., 2008; Rahman et al., 2010).

Keeping in the view the importance of antibacterial activity against pathogenic bacteria, results of our investigations were very significant. There have been

few reports on production of antimicrobial substances by *Aeromonas* (Lategan and Gibson, 2003; Khalil et al., 2006) and *Vibrios* (Sugita et al., 1997; Long and Azam, 2001; Castro et al., 2002; Hjelm et al., 2004; Norhana and Darah, 2005).

5.2 Characterization of crude cell extract by GC-MS

GC-MS analysis of crude ethyl acetate cell extract of *Acinetobacter* sp. strain An2 clearly revealed the presence of several important organic metabolites. Butylated hydroxytoluene (BHT) is one of the major fractions of cell extract i.e. 10 % followed by Pyrrolopyrazines (8.6 %), Benzoquinone (8.5 %), Benzene dicarboxylic acid (6.2 %), and Penta -fluoropropionic acid (6.0 %) in the cell extract of *Acinetobacter* sp. strain An2 (Table 5.2, Figs. 5.6 and 5.7 a - 5.7 f).

GC-MS analysis of crude ethyl acetate cell extract of *Aeromonas hydrophila* strain An 4 also revealed the presence of several important organic metabolites which includes Butylated hydroxytoluene (BHT) as major constituent (7.25%) followed by Pyrrolopyrazines (6.5 %), pyrrolo-pyridine (0.65%) and other phenolic compounds (Table 5.3; Figs.5.8 and 5.9 a- 5.9 e).

Compared with the terrestrial microorganisms, the secondary metabolites produced by marine organisms have novel and unique structures owing to their complex living circumstances and species diversity thus their bioactive potentials are much Stronger (Rinehart, 2000; Schwartsmann et al., 2001). Competition among microbes for space and nutrients in marine environment is a powerful selection pressure which endows marine microorganisms to produce

many natural products of medical and industrial value (Armstrong et al., 2001). Several antimicrobial substances have been reported from these marine microorganisms due to the specialized role they play in their respective hosts (Burgess et al., 1999; Holmstrom et al., 2002).

Presence of Butylated hydroxytoluene (BHT) in the cell extract of this *Acinetobacter* sp. as a major constituent demonstrated that this antiviral metabolite can be purified in large quantities and used against lipid containing human and bacterial viruses viz. Herpes simplex virus and Φ 6 (Snipes et al., 1975; Wanda et al., 1976). Besides Butylated hydroxytoluene, other metabolites such as Pyrrolopyrazine, Benzoquinone, Benzene dicarboxylic acid, Penta-fluoropropionic acid and a phenol derivative were also present in the cell extract of the test organism which are well known potential antimicrobials (Proestos et al., 2008; Mishra et al., 2009).

GC-MS analysis of crude ethyl acetate cell extract of *Vibrio parahaemolyticus* strain An 3 clearly revealed the presence of several important antimicrobial organic metabolites such as Indole, Phenyl acetic acid, N-(3 - methyl - 1, 2, 4 - oxadiazol -5 - yl)- 1- Pyrrolidine, Carboximidamide, Pyrrolopyrazines, Tetramethyl pyrazine and other Phenolic compounds (Table 5.4; Figs. 5.10 and 5.11a- 5.11g).

Novel and unique structures of the secondary metabolites produced by marine organisms also have forced researchers to view the marine environment from different perspective and marine microorganisms as new biomedical sources. Until now more extensive and focussed efforts to discover new

antibiotics have involved the terrestrial environment mainly due to ease of availability, isolation and culture conditions. By the end of 2008, approximately 3000 microbial metabolites were reported from marine environment (Rahman et al., 2010).

GC-MS analysis of crude ethyl acetate cell extract of *V. parahaemolyticus* strain An 3 demonstrated an interesting concoction of compounds with significant antimicrobial activity. A well known antagonistic compound has already been reported from *V. parahaemolyticus* as Vibrindole A, a bis-indole derivative (Bell et al., 1994) along with other indole derivatives (Kobayashi et al., 1994; Velury et al., 2003). In addition other metabolites viz. Phenyl acetic acid, Pyrrolidine carboximidamide, Pyrrolopyrazines, Tetramethyl pyrazine and phenolic compounds are also well known antimicrobials tested against different bacteria, fungi and other microbes (Kim et al., 2004; Somers et al., 2005; Chaudhary et al., 2006; Kumar et al., 2008; Farzaliev et al., 2009; Roy et al., 2010).

It is interesting to note that even pathogenic strains can be used as biocontrol agents against other pathogens due to inherent presence of various antibacterial organic compounds. Thus antibacterial metabolites synthesized by the test organism may serve as valuable drugs to control pathogenic bacterial strains causing fish and human diseases and isolation of novel bacterial strains with antimicrobial activity suggests that marine ecosystem is a valuable source of antimicrobials.

5.3 Antibiotic susceptibility of selected bacterial strains

In 20th century, major developments in antibacterial drug discovery and other means of infection control took place. With respect to bacterial infections, the situation dramatically improved when penicillin became available for use in the early 1940s. However, the euphoria over the potential conquest of infectious diseases was short lived. Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance (Tenover, 2006).

Results of antibiotic susceptibility test, however in our investigations were quite interesting, although susceptible to many of the antibiotics, these pathogens exhibited characteristic resistance to several antibiotics tested (Table 5.5; Fig.5.11 – 5.15). All the four isolates were resistant to ampicillin, Ciprofloxacin and Nitrofurontoin.

Acinetobacter sp. strain An2 was resistant to Carbenicillin, Kanamycin, Cephalothin and Colistin methane sulphonate in addition to above three antibiotics. Tetracycline interferes with protein synthesis at the ribosomal level. Although tetracycline resistance among *Acinetobacter* spp. have been documented (Guardabasi et al., 2000), our strain was found susceptible to it. Emergence and spread of *Acinetobacter* species, resistant to most of the available antimicrobials and their various resistance mechanisms is an area of great concern. It is now being frequently associated with health related infections (Manchanda et al., 2010). Jennifer et al., (2010) have reported many strains of *Acinetobacter* from Asia which are resistant to majority of known antibiotics.

Vibrio parahaemolyticus strain An 3, *Aeromonas hydrophila* strain An 4 and *Staphylococcus arlettae* strain An1 established themselves as excellent examples of bacteria showing multi-antibiotic resistance (MAR) as they were resistant to most of the common antibiotics tested with exception of a few of them (Table 5.5). As described previously, the presence of antimicrobial agents at low concentration through continued usage may lead to the development of drug-resistant bacterial strains and subsequently into multiple antibiotic resistance (MAR) in bacteria. This will ultimately result in transfer of resistance to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases. Multi-drug resistant pathogenic bacteria have become ubiquitous in raw sea food (Roque et al., 2001; Heritier et al., 2005; Manjusha et al., 2005).

Staphylococcal antibiotic resistance has been associated with β-lactamase production and other functions but most of the literature available is on *S. aureus* (Diep et al., 2008; Daini and Akano, 2009; Machon et al., 2010). *Staphylococcus arlettae* strain An1, however remains a rare strain showing multi-antibiotic resistance but not much data is available to compare.

Studies have shown that streptomycin, rifampicin, kanamycin, tetracycline and polymixin B were active against *Vibrio* spp. Hasegawa *et al.* (1986), reported resistance to chloramphenicol, aminoglycoside-3 and beta-lactam antibiotics. Traditionally, *Vibrios* are considered highly susceptible to the majority of antibiotics except ampicillin. During the past few decades, antimicrobial resistance has emerged and evolved in many strains (Han et al., 2007), which

could be attributed to about 50 multi-drug efflux transporters reported in *V. parahaemolyticus* (Matsuo et al., 2007).

Aeromonas species also produce a β-lactamase and are therefore resistant to penicillins and first-generation cephalosporins. *Aeromonas* species are universally resistant to penicillin, ampicillin, carbenicillin, ticarcillin and erythromycin. Some were resistant to gentamycin and amoxicillin too (Saha and Pal, 2002). In Taiwan, *Aeromonas* strains are more resistant to drugs viz. tetracycline, co-trimoxazole trimethoprim, sulfamethoxazole, aminoglycosides and some second- or third-generation cephalosporins than *Aeromonas* strains from the United States and Australia (Tsai, et al., 2005). Mukhopadhyay et al. (2008) reported that tetracycline and co-trimoxazole are routinely included in medicated fish feed could be the reason of developing resistance to these drugs. Our Aeromonas *hydrophila* strain An 4 however was susceptible to these drugs but one of the most important problems involving treatments with antibiotics against *Aeromonas hydrophila* isolated from fish is that antibiotic resistance develops readily. Antimicrobial agents most active against *Aeromonas* are third-generation cephalosporins, carbapenems and quinolones (Tulsidas et al., 2008).

5.4 Plasmid profile of bacterial isolates

Role of plasmids in antimicrobial resistance in both gram +ve and gram –ve bacteria has long been studied. Plasmids encode functions essential to antibiotic resistance, heavy metal resistance, metabolic functions or synthesis of antibiotics, toxins and virulence factors. Plasmids possess genes essential for the initiation and control of their replication. Some plasmids also contain genes

that ensure stable inheritance, such as equipartitioning during cell division or conjugal transfer (Courvalin et al., 1978; Synder and Champness, 1997; Guardabasi et al., 2000; Carattoli, 2003; Guglielmetti et al., 2009; Umamaheshwari and Anbusaravanan, 2010).

In view of the significance of the plasmids in antibiotic resistance, plasmids were isolated from selected bacterial pathogens.All the bacterial strains possessed a small plasmid.

Acinetobacter sp. strain An 2 displayed the presence of smallest plasmid DNA of 1.0 kb, *V. parahaemolyticus* strain An 3 had plasmid DNA of approx. 1.2 kb, plasmid DNA of *Staphylococcus arlettae* strain An1 was approx. 1.5 kb while *Aeromonas hydrophila* strain An 4 demonstrated heaviest plasmid DNA of approx. 2.0 kb on agarose gel (Fig.5.16).Therefore, presence of these plasmids may be correlated with multi-drug resistance displayed by our four selected fish pathogenic bacterial strains.

Plasmid mediated resistance to antibiotics in *Vibrios* (Hasegawa et al., 1986), *Aeromonas* (Chang and Boltont, 1987; Adams et al., 1998; Ye et al., 2010), *Acinetobacter* (Guardabasi et al., 2000) and *Staphylococci* (Daini and Akano, 2009; Machon et al. 2010) have already been reported.

5.5 Transformation of plasmid DNA of ampicillin resistant bacterial isolates into *E.coli* HB 101

In order to confirm the location of antibiotic resistance genes on the plasmid, plasmid DNAs of two isolates viz. *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4, were successfully transformed into

competent cells, *E coli* HB 101. Transformation of *E.coli* HB101 host with this small plasmid resulted in appearance of 23 colonies (transformants) in case of *Aeromonas hydrophila* strain An 4 and 11 colonies (transformants) in case of *Vibrio parahaemolyticus* strain An 3 on LB agar plate containing ampicillin (10μg/ml) (Fig. 5.17). All the transformants gave positive results on replica plating on LB agar plate containing ampicillin (10μg/ml) which confirmed the purity of transformants.

Our results have clearly demonstrated that the fish pathogenic bacteria possessing multi-antibiotic resistance genes have the potential to spread both to fish and human population posing a serious threat to aquaculture as well as consumer safety.It is interesting to note that dissemination of antimicrobial resistance takes place frequently by conjugative DNA exchange in bacteria (Bremner, 1979; Diab et al., 2002; Carattoli, 2003).

Transformation and conjugation studies using ampicillin and tetracycline to check the transferability of plasmid mediated MDR has been of consistent reliability (Noor et al., 2004; Kehrenberg et al., 2006; Lim et al., 2009).

Chapter V

Tables and Figures

Table 5.1 Antibacterial activity of selected isolates

Crude Cell Extract of Isolate	Indicator Bacteria
SA 1	*E.coli,* SC 3, SB 3,
SC 3	*E.coli,* SB 3, SA 1,SD 4
SB 3	*E.coli,* SC 3, SA 1, SD 4

Table 5.2 Major antibacterial metabolites present in ethyl acetate cell extract of *Acinetobacter* sp. Strain An 2

Antibacterial Metabolites	Retention Time (Minutes)	% In Test Volume
Butylated hydroxytoluene (BHT)	12.625	10.0
Benzoquinone, 3, 5- di tert-	19.842	8.5
Pyrrolo-(1,2-a)pyrazine-1,4- dione hexahydro-3(2-methylpropyl)	20. 033	6.6
1,2- Benzene dicarboxylic acid	20.442	6.2
Penta-fluoropropionic acid heptadecyl ester	24. 242	6.0
Pyrrolo-(1,2-a)pyrazine-1,4- dione, hexahydro-3(2-methyl phenyl)	24. 417	2.0
Phenol 4-(1,1,3,3-tetra-methyl butyl)	15.425	1.0

Table 5.3 Major antibacterial metabolites present in ethyl acetate cell extract of *Aeromanas hydrophila* srain An 4

Antibacterial Metabolites	Retention Time (Minutes)	% In Test Volume
Butylated hydroxytoluene (BHT)	12.625	7.25
Pyrrolo-(1,2-a) pyrazine-1,4- dione, hexahydro-3 (2-methylpropyl)	20. 033	4.6
Pyrrolo-(1,2-a)pyrazine-1,4-dione, hexahydro-3(2-methyl phenyl)	24. 417	1.9
Phenol 4 - (1,1,3,3 – tetra-methyl butyl)	15.425	0.65
Pyrrolo-(1,2- a) pyridine	10.042	0.44

Table 5.4 Major antibacterial metabolites present in ethyl acetate cell extract of *Vibrio parahaemolyticus* srain An 3

Antibacterial Metabolites	Retention Time (Minutes)	% In Test Volume
Indole	9.842	3.0
Phenyl acetic acid	9.3	2.0
N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-Pyrrolidine carboximidamide	19.75	1.86
Pyrrolo-(1,2-a) pyrazine-1,4 dione,hexahydro-3 (2-methylpropyl)	20. 033	1.3
Phenol 4 - (1,1,3,3-tetramethyl butyl)	15.425	0.76
Nonyl-Phenol	16.492	0.6
Tetramethyl pyrazine	6.775	0.23

Table 5.5 Response of selected bacterial isolates to different antibiotics

Antibiotics	Zone of clearance (Diameter)			
Concentration (µg/ml)	***Acinetobacter* sp. strain An2**	***Aeromonas hydrophila* strain An4**	***Vibrio parahaemolyticus* strain An3**	***Staphylococci arlettae* strain An1**
Amikacin-(Ak) 10	7 mm	7 mm	7 mm	0 mm **R**
Carbenicillin-(Cb) 100	5mm **R**	5mm **R**	7 mm	3 mm **R**
Ciprofloxacin-(Cf) 10	4 mm **R**	4 mm **R**	3 mm **R**	0 mm **R**
Co-Trimazine-(Cm) 25	9 mm	9 mm	12 mm	6 mm
Kanamycin–(K) 30	6 mm **R**	6 mm **R**	4 mm **R**	11 mm
Nitrofurontoin-(Nf) 300	7 mm **R**	7 mm **R**	2 mm **R**	0 mm **R**
Streptomycin-(S) 10	14 mm	14 mm	8 mm **R**	10 mm **R**
Tetracycline-(T) 30	14 mm	14 mm	6 mm **R**	1 mm **R**
Ampicillin-(A) 10	0 mm **R**	0 mm **R**	0 mm **R**	0 mm **R**
Cephalothin-(Ch) 5	0 mm **R**	0 mm **R**	0 mm **R**	3 mm
Colistin methane - sulphonate-(Cl) 25	1 mm **R**	1 mm **R**	1 mm **R**	9 mm
Gentamycin (G) 10	7 mm	7 mm	4 mm **R**	0 mm **R**
Sulphatriad (SI) 200	7 mm	7 mm	9 mm	3 mm **R**
Tetracycline (T) 25	9 mm	9 mm	8 mm	3 mm **R**
Co-Trimoxazol (Co) 25	12 mm	12 mm	8 mm	7 mm

R. Resistant

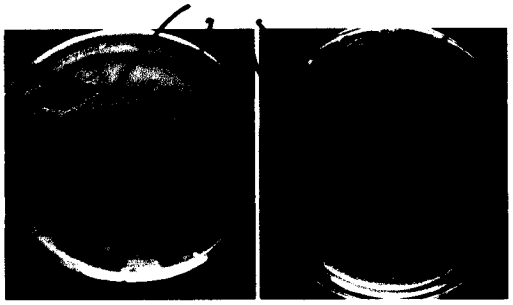


Fig. 5.1 Growth inhibition of *E. coli* by cell extracts of SA1, SB 3 and SC 3.

Fig. 5.2 Growth inhibition of SB 3 by cell extracts of SA1 and SC 3.

Fig. 5.3 Growth inhibition of SD 4 by cell extracts of SB 3 and SC 3.

Fig. 5.4 Growth inhibition of SA 1 by cell extracts of SB 3 and SC 3.

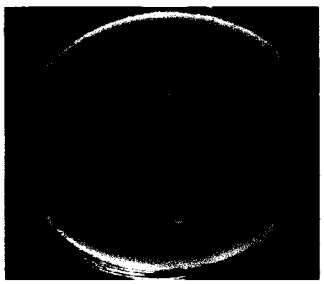


Fig. 5.5 Growth inhibition of SC 3 by cell extracts of SA 1 and SB 3.

C Control
Isolate SA 1 *Acinetibacter* sp. strain An 2
Isolate SB 3 *Vibrio parahaemolyticus* strain An 3
Isolates SC3 *Aeromonas hydrophila* strain An 4
Isolates SD 4 *Staphylococcus arlettae* strain An 1

GC-MS Facility: AIF *Analysis Report*
Jawaharlal Nehru University
New Delhi – 110067
India

Sample Information

Analyzed by	: Ajai Kumar
Analyzed	: 6/15/2009 2:51:49 PM
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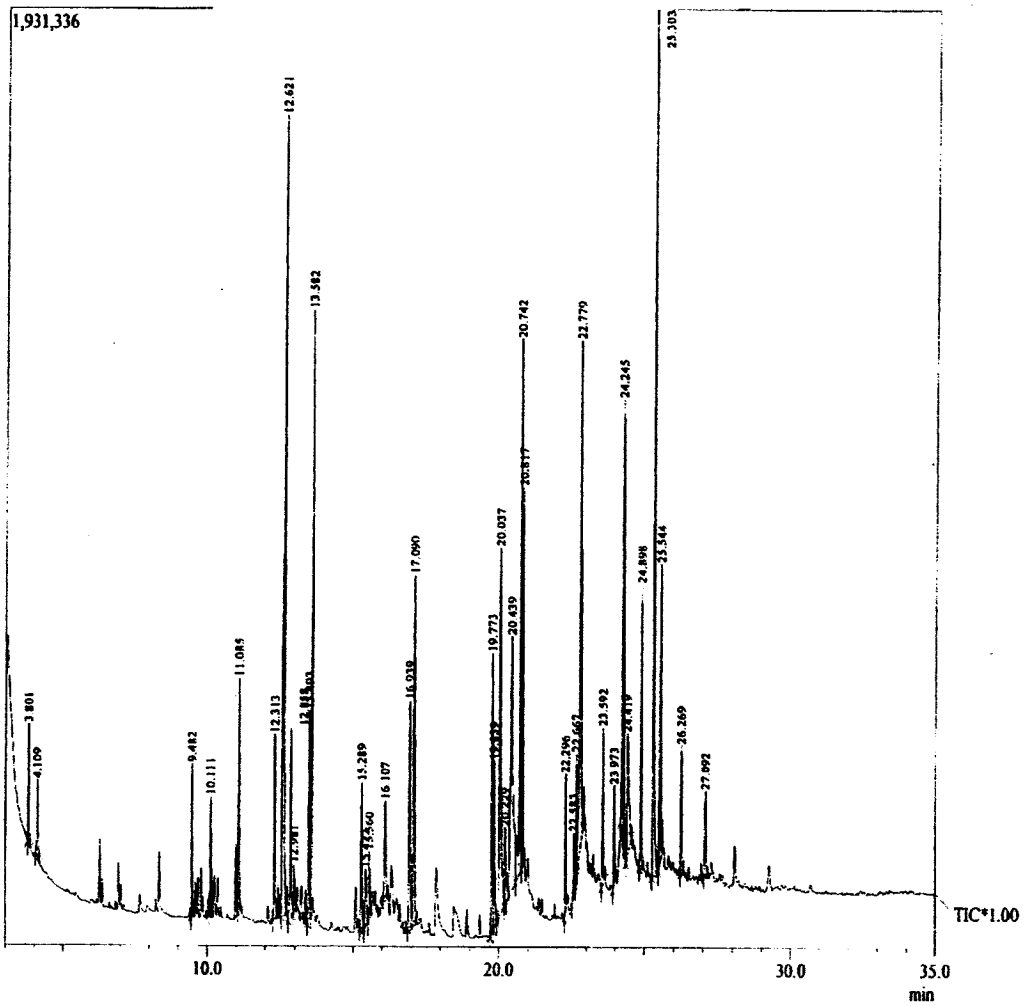

Fig. 5.6 Chromatogram of crude cell extract of *Acinetobacter sp.* strain An 2

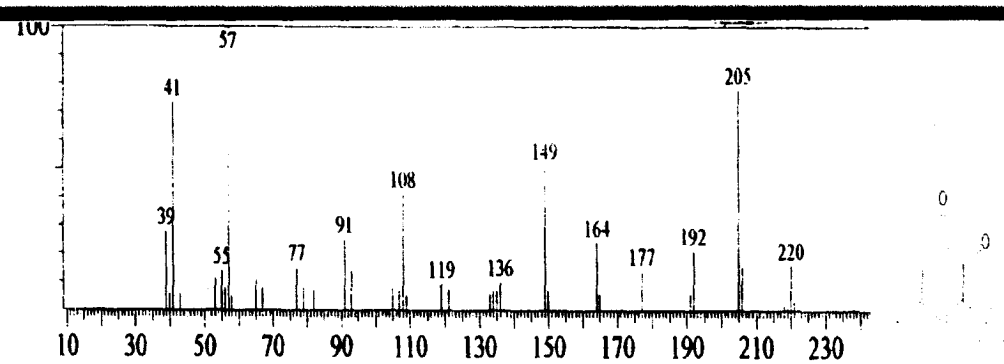

Fig. 5.7a O-Benzoquinone ,3,5 –di tert butyl- [Peak 41]

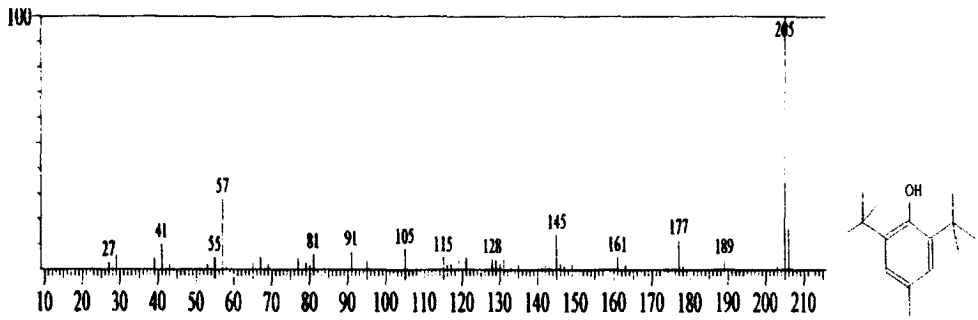

Fig. 5.7 b Butylated Hydroxytoluene (BHT) - [Peak 205]

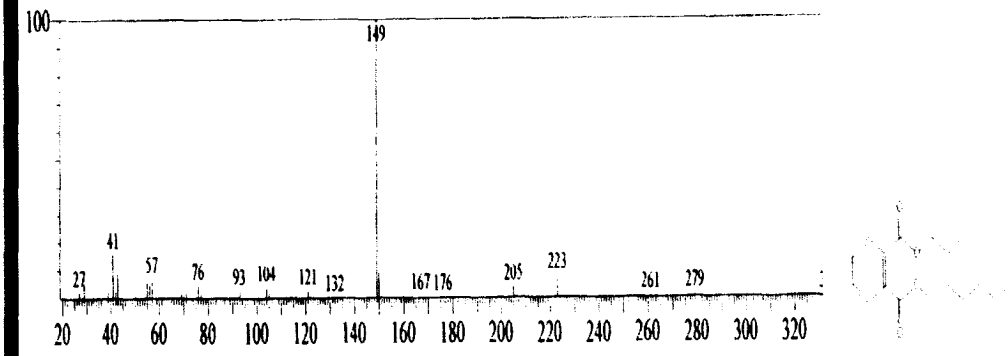

Fig. 5.7 c 1,2- Benzenedicarboxylic acid ,butyloctyl ester -[Peak 149]

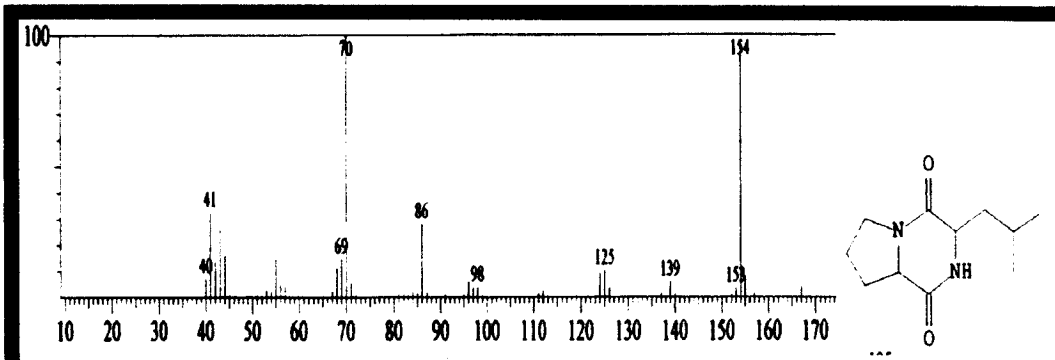

Fig. 5.7 d Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2- methyl propyl)- [Peak 70]

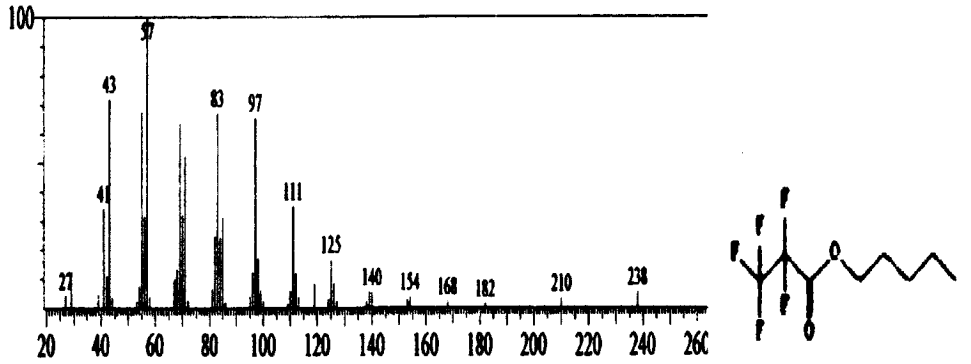

Fig. 5.7 e Pentafluoro propionic acid heptadecyl ester -[Peak 57]

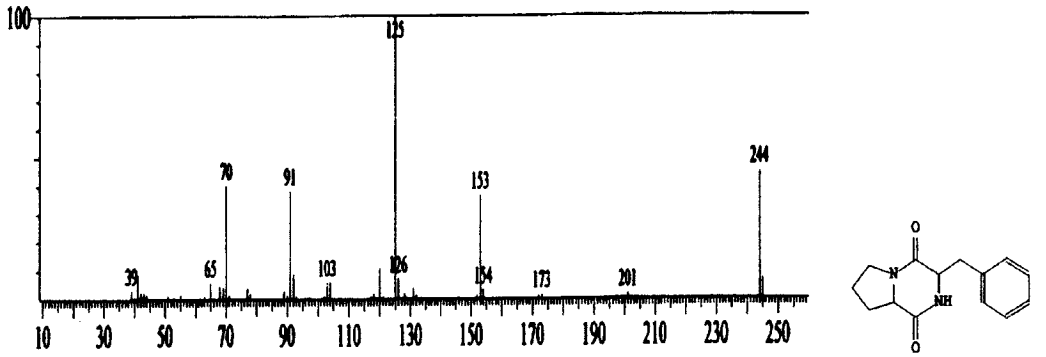

Fig. 5.7 f Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2- phenyl methyl)- [Peak 125]

GC-MS Facility: AIF *Analysis Report*
Jawaharlal Nehru University
New Delhi – 110067
India

Sample Information

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Analyzed : 6/15/2009 3:56:22 PM
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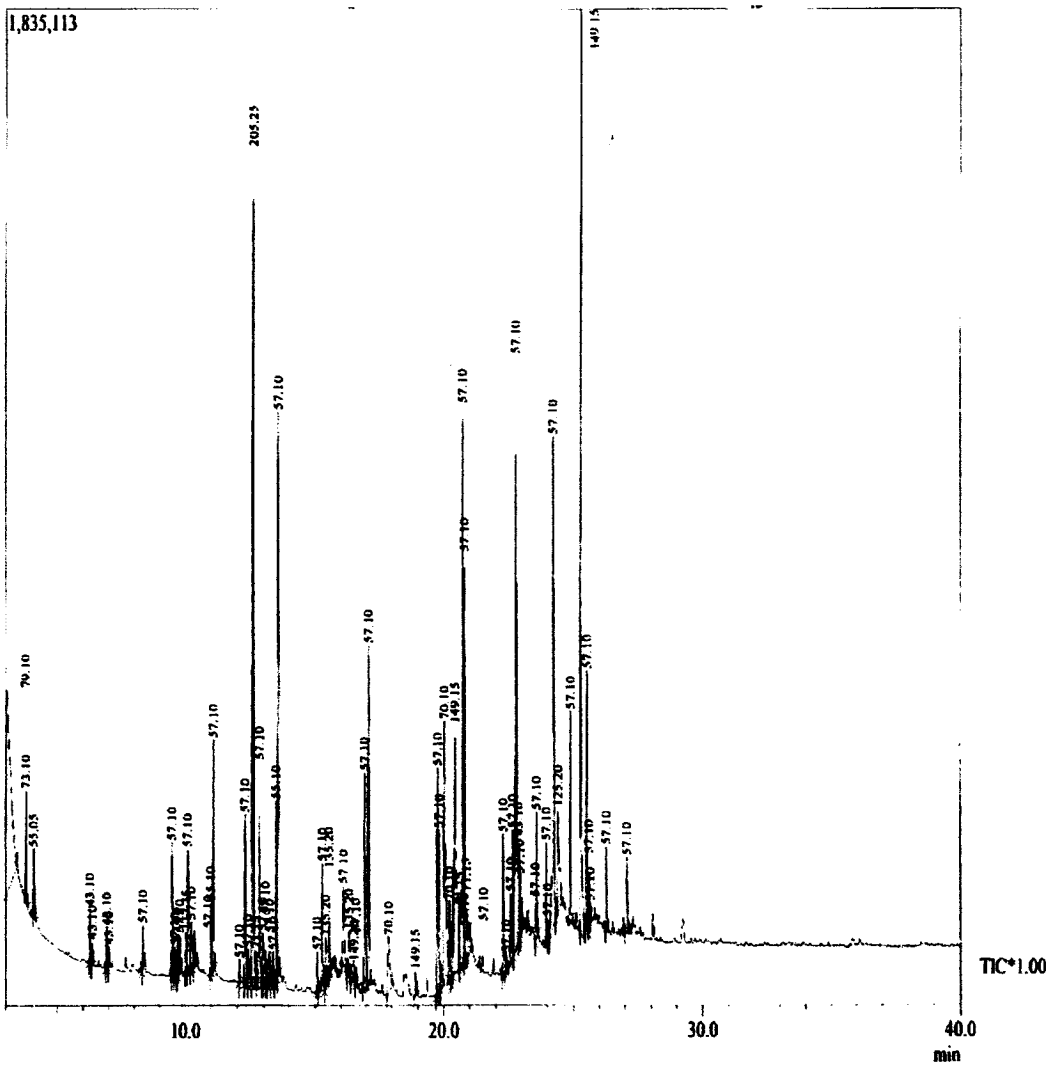


Fig. 5.8 Chromatogram of crude cell extract of *Aeromonas hydrophila* strain An 4

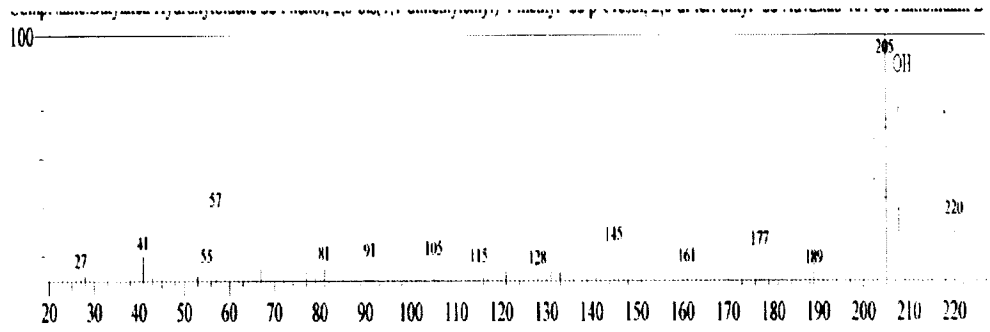


Fig. 5.9 a Butylated Hydroxytoluene(BHT) - [Peak 205]

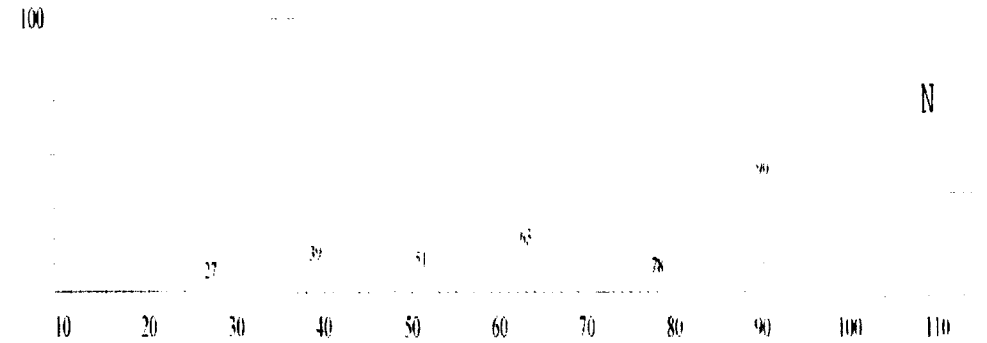


Fig. 5.9 b Pyrrolo[1,2-a] pyridine [peak 117.15]

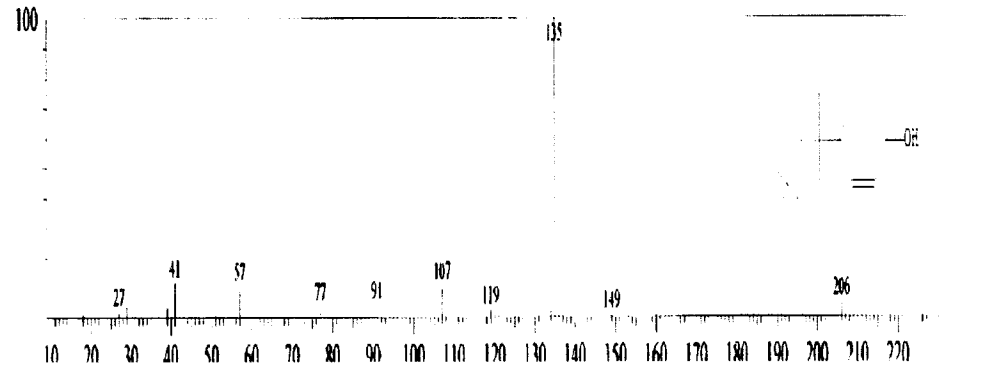


Fig. 5.9 c (1,1,3,3-tetramehyl butyl) –Phenol [Peak 135]

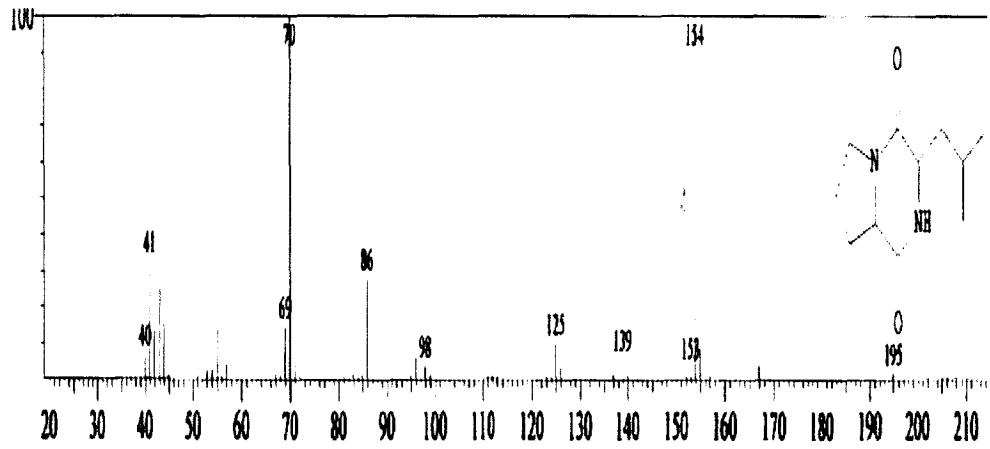


Fig. 5.9 d Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2- methyl propyl)- [Peak 70.1]

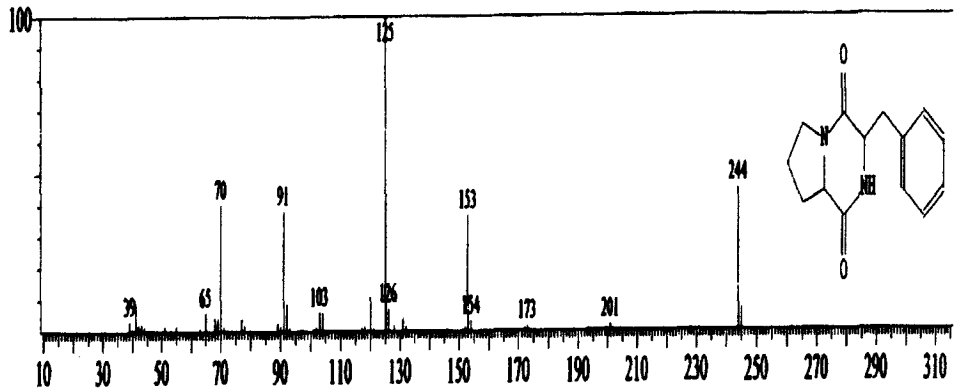


Fig. 5.9 e Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2- phenyl methyl)- [Peak 125]

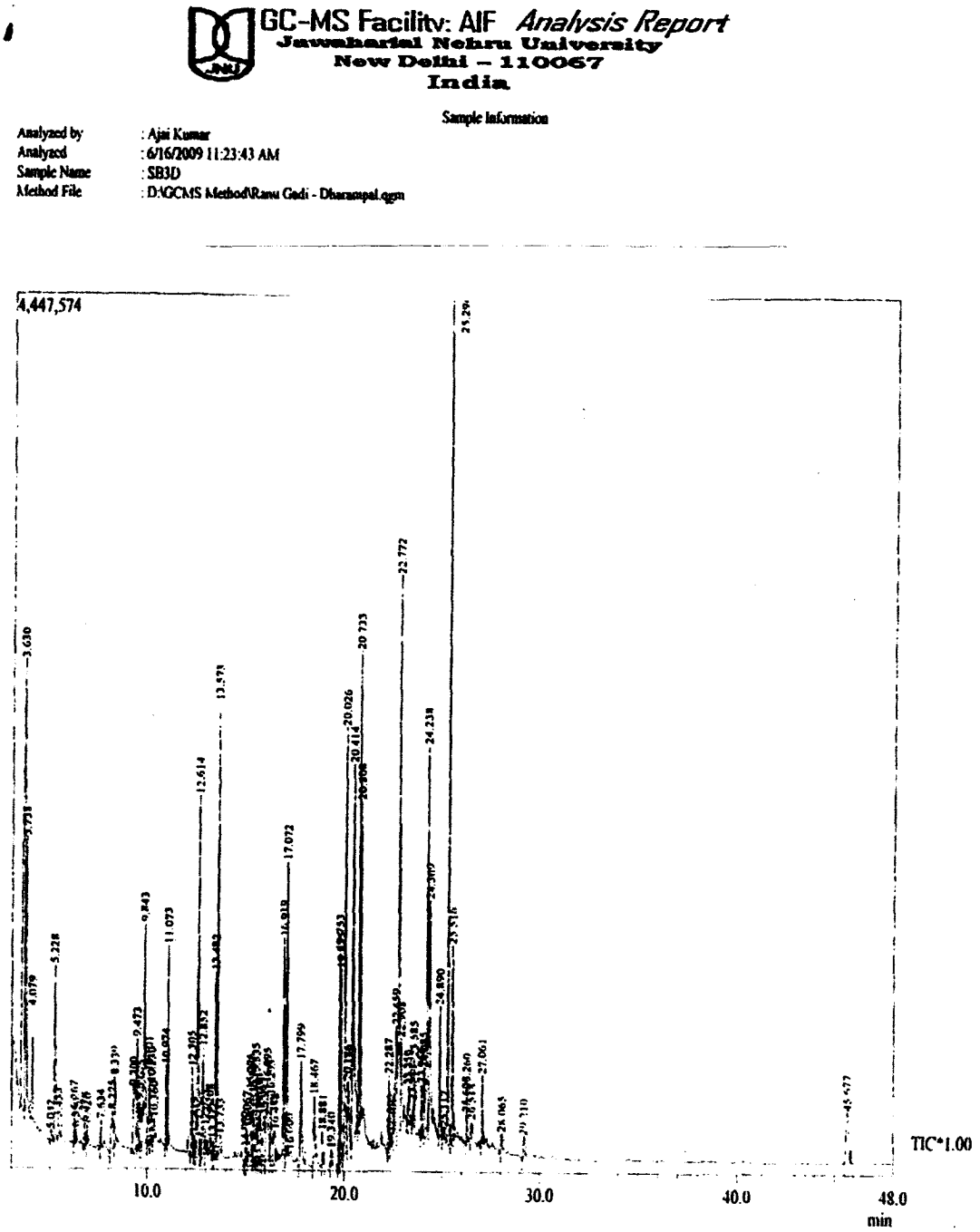


Fig. 5.10 Chromatogram of crude cell extract of *Vibrio parahaemolyticus* strain An 3

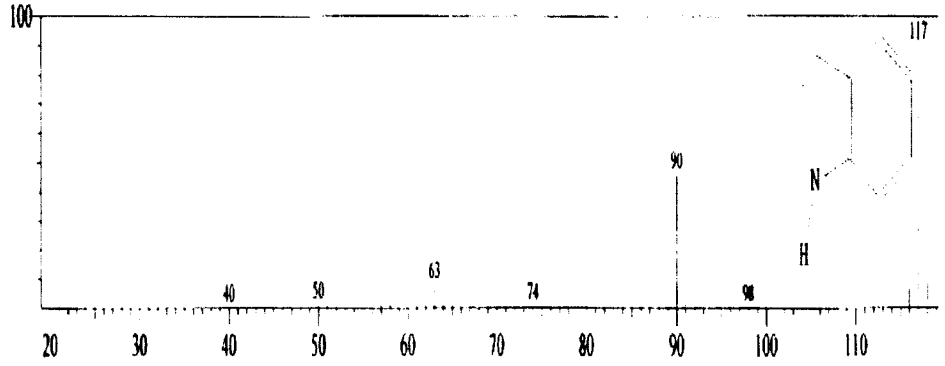


Fig. 5.11a Indole (Peak 117)

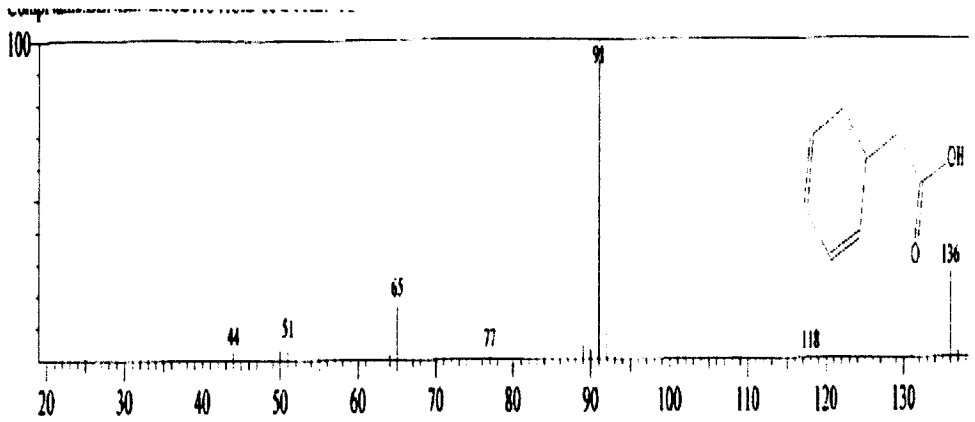


Fig. 5.11 b Phenyl acetic acid (Peak 91)

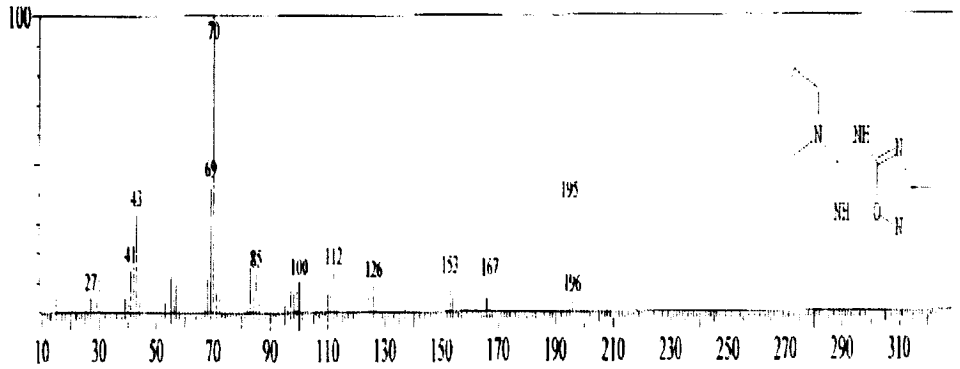


Fig. 5.11c N-(3-methyl-1,2,4-oxadiazol-5-yl)-1 Pyrrolidinecarboximid amide (Peak70)

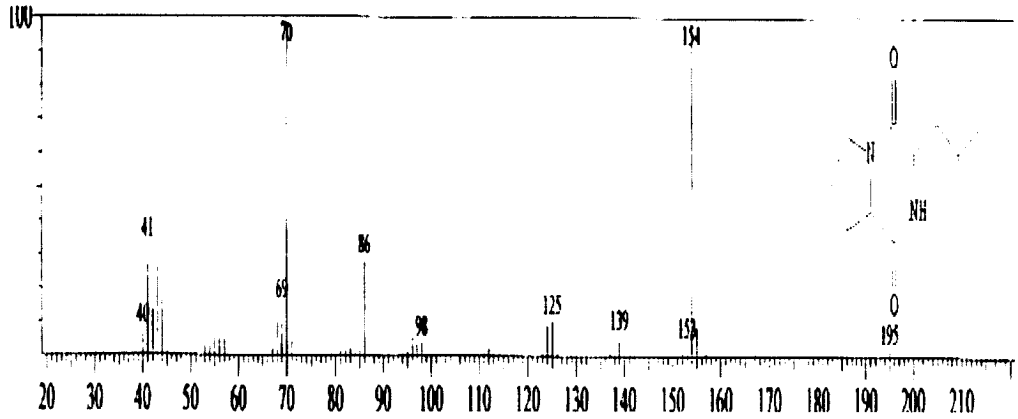


Fig. 5.11d Pyrrolo- (1,2-a)pyrazine-1,4- dione hexahydro-3(2 methylpropyl) (Peak 154)

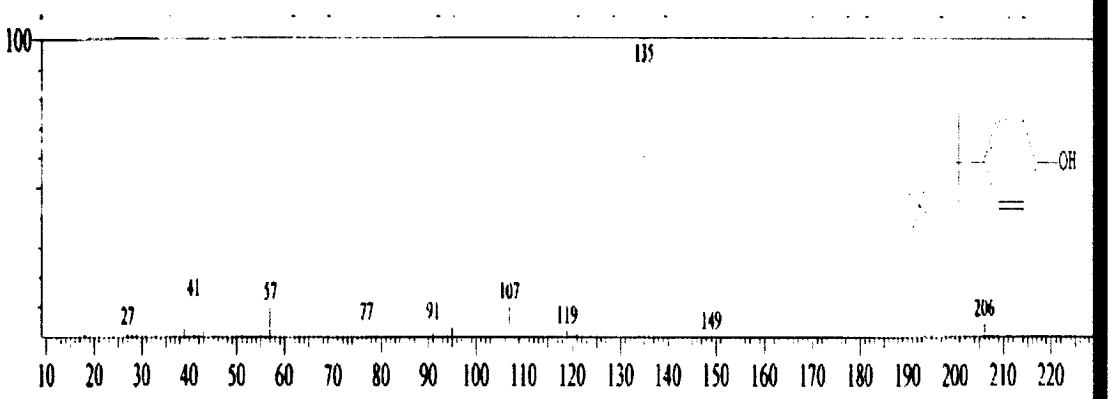


Fig. 5.11e Phenol, 4- (1,1,3,3-tetramethyl butyl) (Peak 135.2)

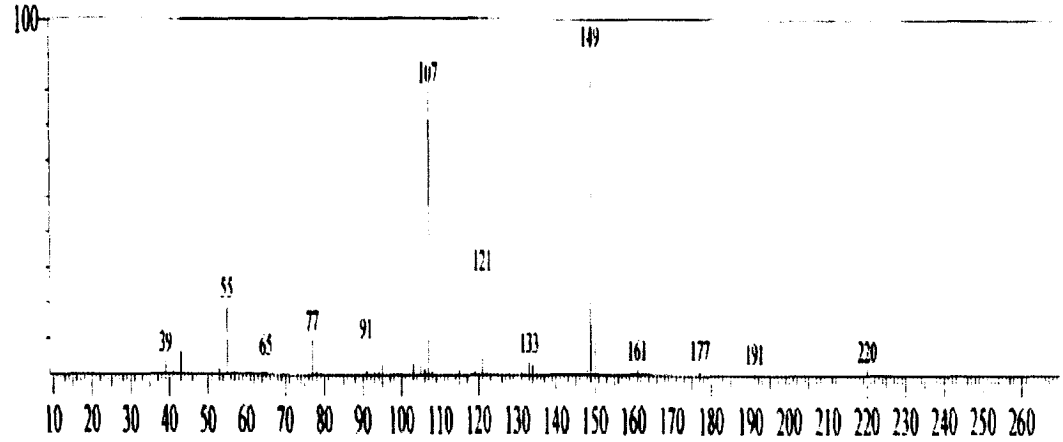


Fig. 5.11f Nonyl-Phenol (Peak 149)

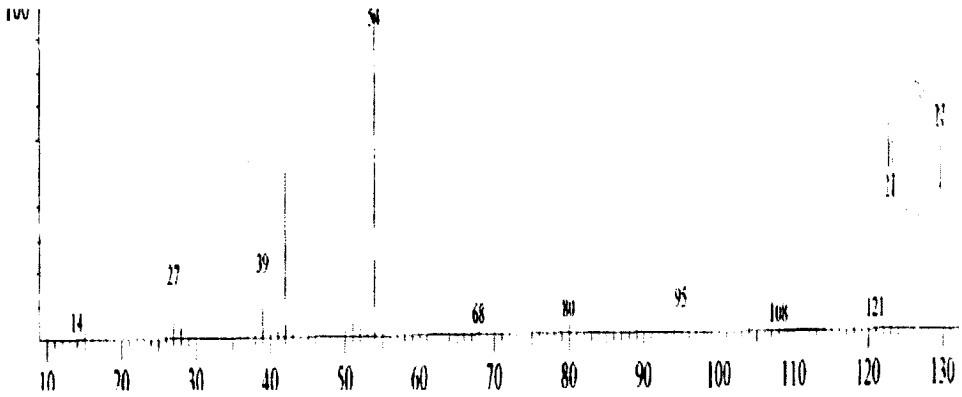


Fig. 5.11g Tetramethyl pyrazine (Peak 54)

RESPONSE OF SELECTED BACTERIAL ISOLATES TO DIFFERENT ANTIBIOTICS

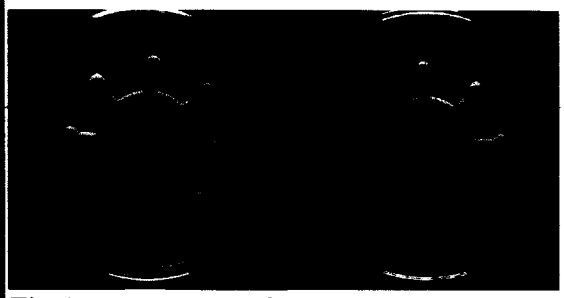


Fig.5.12 Response of *Acinetobacter* sp.strain An 2 to different antibiotics

Fig.5.13 Response of *vibrio parahaemolyticus* Strain An 3 to different antibiotics

Fig.5.14 Response of *Aeromonas hydrophila* strain An 4 to different antibiotics

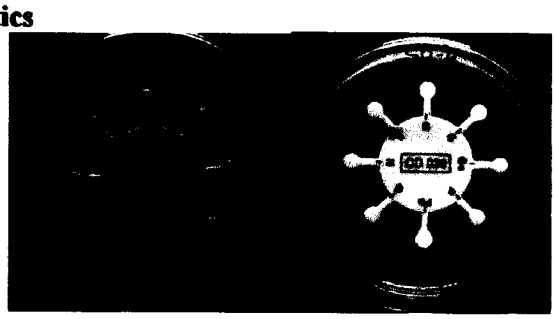


Fig.5.15 Response of *Staphylococcus arlettae* strain An 1 to different antibiotics

PLASMID PROFILE OF SELECTED ISOLATES

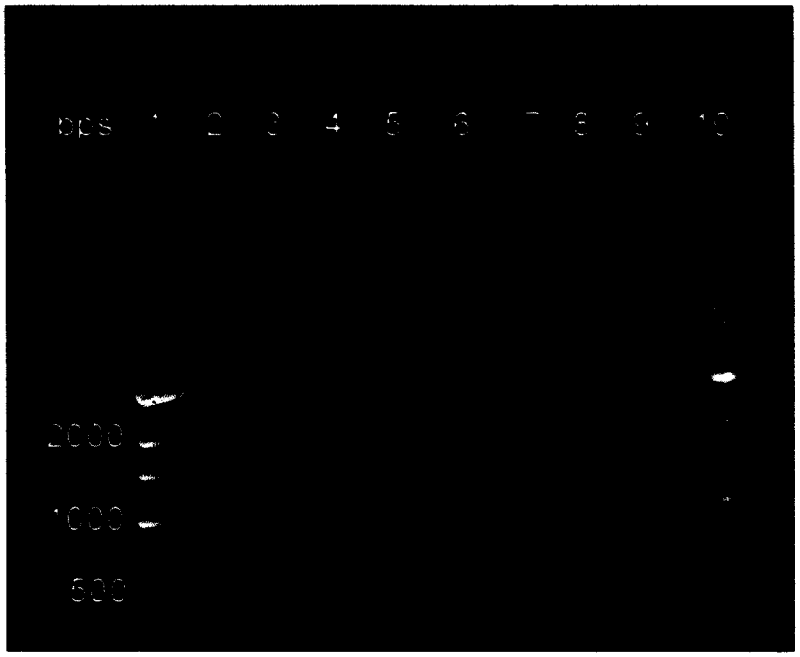


Fig. 5.16 Plasmid profile of selected isolates

Lane 1 : Supermix DNA ladder,

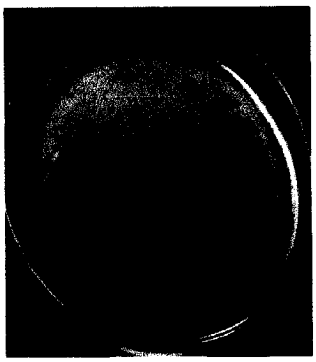
Lane 3: ***Acinetobacter*** **sp. strain An 2(isolate SA1)**

Lane 4 and 5: ***Vibrio parahaemolyticus*** **strain An 3 (isolate SB3)**

Lane 7: ***Aeromonas hydrophila*** **strain An4 (isolate SC3)**

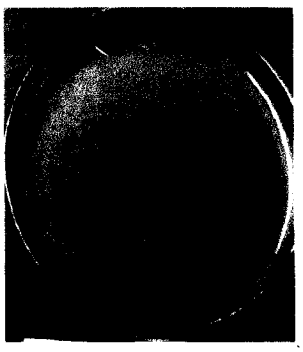
Lane 9: ***Staphylococcus arlettae*** **strain An1(isolate SD4)**

CONFIRMATION OF PLASMID MEDIATED ANTIBIOTIC RESITANCE BY TRANSFORMATION

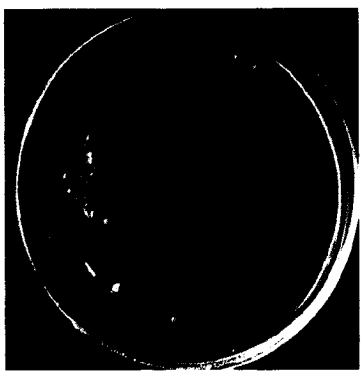


***E. coli* HB101 on LB agar without ampicillin**

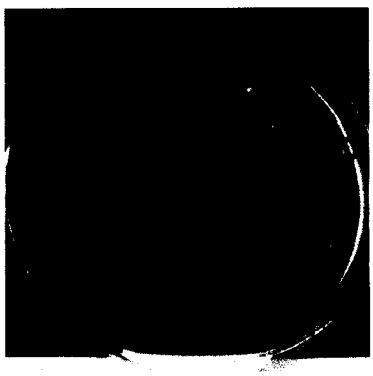
***E.coli* HB101 + pUC19 on LB + Ampicillin (+ ve control)**



***E. coli* HB101 on LB+ Amp. (- ve control)**



***E. coli* HB101+ pSC 3 on LB + Ampicillin (23 +ve transformants)**



***E. coli* HB101 + pSB 3 on LB + Ampicillin (11 +ve transformants)**

Fig. 5.17 Confirmation of plasmid mediated antibiotic resitance by Transformation

Salient Features

- Collection of fish samples from different fishing sites of Goa and isolation of bacterial pathogens from various visibly infected fish with symptoms in the form of lesions and hemorrhage on the body parts was done. Viable count of bacterial fish pathogens was very high as the isolation was done by direct swabs from the wounded areas.
- Morphological and gram characteristics along with biochemical characteristics of thirty selected isolates lead to their tentative identification as *Vibrio* spp., *Aeromonas* spp., *Streptococcus* spp., *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp., *Lactobacillus* sp., *Flavobacterium* sp., *Alteromonas* spp., *Salmonella* and *Shigella* sp. *Among these fish pathogens, Vibrio* spp. were found to be more prevalent.
- Further confirmation the identification of four of the selected isolates by PCR amplification of 16S rDNA and sequencing followed by BLAST search of bacterial isolates further was done. Which confirmed their identity as *Staphylococcus arlettae, Acinetobacter* sp., *Vibrio parahaemolyticus*, and *Aeromonas hydrophila* which were designated as strain A1, A2, A3 and An 4 respectively.

- Growth optimas of all four bacterial pathogenic isolates for environmental conditions viz. pH (. pH 5 pH 7 and pH 9), salinity with reference to NaCl concentrations (0%, 0.5%, 1.5%, 3% and 5%) and temperature (22 °C, 27°C, 39°C and 42°C). It has been revealed that coastal marine environment is optimal for the growth and proliferation of these pathogenic isolates except strain An2.
- Selected bacterial isolates were found to produce several different exoenzymes viz. protease, lipase, amylase, glucosidase and lecithinase which was clearly demonstrated on suitable media. Enzyme assay vs. growth for protease for two bacterial isolates was also done following Kunitz method. The assay study was done with two of the isolates *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4. Both of the bacterial isolates interestingly revealed commencement of protease production during late log phase.

- Hemolytic activity was demonstrated by all the selected bacterial fish pathogens, *Acinetobacter* sp. strain An 2, *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4, in the form of clearance zones around the colonies on the blood agar (β hemolysis), where as *Staphylococcus arlettae* strain An1 exhibited prominent α hemolysis.
- Genes, encoding virulencs factors from two of the isolates were PCR amplified and observed using *tdh* and *hlyA* specific primers and

genomic DNA of *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4 respectively as DNA templates. Presence of *tdh* gene (amplicon : 263 bps) and hlyA (amplicon : 597 bps) encoding beta-hemolysin, were evidently demonstrated on agarose gel.

- Characterization of extracellular proteins (ECPs) of selected bacterial fish pathogens was done by SDS-PAGE analysis, which exhibited several significant ECPs ranging in molecular mass from 30 kDa - 97 kDa. Interestingly , prominent protein bands of 23 kDa was revealed in case of *Vibrio parahaemolyticus* strain An 3 and 50 kDa in case of *Aeromonas hydrophila* strain An 4 which may very well be correlated with their characteristic TDH and HLY toxins respectively. *Acinetobacter* sp. strain An 2 demonstrated a prominent band of 68 kDa and *Staphylococcus arlettae* strain An1 demonstrated the presence of very prominent band of 33 kDa which could be correlared with Shiga toxin and staphylococcal α toxin respectively.

- In order to check the antibacterial activity of the cell extract, of selected strains, the agar well diffusion method had been followed. Experiment clearly revealed antibacterial activity of the ethyl acetate cell extract of three strains, *Vibrio parahaemolyticus* strain An 3, *Aeromonas hydrophila* strain An 4 *Acinetobacter* sp. strain An 2 since zone of clearance/inhibition for indicator bacteria was observed around the wells containing the cell extract.

- In order to identify the compounds responsible for the antibacterial activity GC-MS analysis of the cell extracts of these three selected fish pathogenic bacterial isolates was performed. Analysis revealed presence of significant amount of important antimicrobial compounds viz. phenol, benzene hexa-toluene, Indoles, Nonyl phenol, Pyrrolopyrazine etc. These antibacterial compounds were as effective as any other commercially available antibiotics.
- Response of these bacterial pathogens to various commercially available antibiotics in terms of susceptibility/resistance was checked. Each selected bacterial isolate was exposed to 16 antibiotics. Although susceptible to several of the antibiotics these pathogens exhibited characteristic resistance to many antibiotics tested. All the four isolates were resistant to ampicillin, Ciprofloxacin and Nitrofurontoin.
- In order to confirm the location of antibiotic resistance genes on the plasmid, plasmid DNAs of two strains were successfully transformed into *E coli* HB 101.Two bacterial pathogens, *Vibrio parahaemolyticus* strain An 3 and *Aeromonas hydrophila* strain An 4 confirmed plasmid mediated ampicillin resistance .

IMPORTANCE AND FUTURE PROSPECTS OF THE RESEARCH WORK

- Seafood safety is a global health issue and the food borne diseases create major health crisis. Therefore, detection of microbial pathogens in seafood is the solution to the prevention and recognition of problems related to human health and safety. Fish pathogens including bacteria pose a serious threat to the ornamental fish industry, aquaculture and public health. Keeping in view the importance of sea food including fish, there is urgent need of extreme precautions to ensure the quality of seafood which can only be achieved by regular monitoring and control of bacterial fish pathogens.
- Since there has not been much work done on monitoring and characterization of bacterial fish pathogens from Goa, it is worthwhile and most demanding to study bacterial fish pathogens with reference to screening, identification, morphological, physiological, biochemical and molecular characterization.
- The conventional method of detection and monitoring of the fish pathogenic bacteria is very cumbersome and time consuming, so there is a need to optimize a molecular assay for quick identification of these pathogens based on PCR amplification of pathogen specific virulence genes viz. *tdh, trh, hly* etc. which encode specific toxins. Molecular techniques can be used to solve these problems and increase the

sensitivity and specificity of pathogen detection, since molecular diagnostic techniques are faster and more sensitive.These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, *in situ* hybridization and microarray.

- The acquisition of virulence and antibiotic resistance genes by horizontal transfer might increase the ability of bacterial pathogens to infect aquatic organisms by increasing their virulence to a specific host by broadening their host range. The detection of such genes may forewarn the hatchery and aquaculture operators about a potentially virulent pathogen and thus help to develop management measures to handle the problem of bacterial sea food pathogens.
- These studies will also culminate in possible development of potent and suitable vaccines against important bacterial fish pathogens targeting pathogen specific virulence gene(s).
- Our primary interest should be practical management of pathogens in newly developing aquaculture, along with human infections and according to our investigations, even pathogenic strains can be used as biocontrol agents against other pathogens due to the inherent presence of antimicrobial compounds in the form of secondary metabolites. Although one of the emerging fields of research, very little work has been done on screening and characterization of antibiotics, bacteriocins and other antimicrobials from microbes of marine ecosystem. Therefore, there is an emerging trend to isolate and characterize antimicrobials

from marine fish pathogens which may serve as valuable drugs to control various fish pathogens causing fish and human infections.

Apendices

Appendix-A

A.1 Nutrient Broth

Peptone	10.0 gm
Beef extract	3.0 gm
Sodium-chloride	5.0 gm
Double distilled Water	1000ml

pH adjusted to 7.0 with 0.1 N NaOH

For preparing nutrient agar, 1.5 gm of agar (Bacto-Difco) was added to 100 ml nutrient broth, digested in water-bath and sterilized the medium using autoclave for 20 minutes at 15 psi.

A.2 Luria Bertani (LB) Broth

Tryptone	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	10.0 gm
Double distilled water	1000 ml
Adjusted to pH 7.0 with	0.1 N NaOH

For preparing LB agar, 1.5 gm of agar is added to 100ml LB broth, digested in water-bath and sterilized the medium using autoclave for 20 minutes at 15 psi.

A.3 Trypticase Soy Agar (TSA)

Tryptone	17.0 gm
Soytone - enzymatic digest of soybean meal	3.0 gm
Sodium Chloride	5.0 gm
K_2HPO_4	2.5 gm
Double Distilled water	1000 ml

Agar 15.0 gm

pH adjusted to 7.0 with 0.1 N NaOH

Sterilized the medium using autoclave for 20 minutes at 15 psi.

A.4 Sea Water based Yeast Extract Peptone Agar (SYEP)

Peptone 10.0 gm

Yeast extract 10.0 gm

Aged Sea water 750 ml

Double Distilled water 250 ml

Agar 12.0 gm

pH adjusted to 7.0 with 0.1 N NaOH

Sterilized the medium using autoclave for 20 minutes at 15 psi.

A.5 SOB

Deionized H_2O 950 ml

Tryptone 20.0 gm

Yeast extract 8.0 gm

NaCI 0.5 gm

Shake until the solutes have dissolved. Add 1 ml of $MgCl_2$-Mg SO_4 Solution (12 gm $MgSO_4$ + 9.5 gm $MgCl_2$). Adjust the volume of the solution to 100 ml with deionized distilled H_2O and sterilize by autoclaving for 20 minutes at 15 psi.

A.6 SOC

Deionized H_2O 950 ml

Tryptone 20.0 gm

Yeast extract 8.0 gm

NaCl 0.5 gm

Shake until the solutes have dissolved. Add 1 ml of $MgCl_2$ –$MgSO_4$ solution (12 gm $MgSO_4$ + 9.5 gm $MgCl_2$).Add 1ml of 2M Glucose or 2 ml of 20% Glucose. Adjust the volume of the solution to 100 ml with

deionized distilled H_2O and sterilize by autoclaving for 20 minutes at 15 psi.

Appendix-B

Composition of Gram stain reagents

Crystal violet

Solution A- 2 gm of crystal violet dissolved in 20ml ethanol.

Solution B – 0.8 gm Ammonium oxalate dissolved in 80 ml distilled water.

Mixed solution A and B and filtered through Whatman filter paper No.1.

Gram's Iodine

Dissolved 1 gm Iodine and 2 gm Potassium iodide in 300 ml distilled water and filtered through Whatman filter paper No. 1 (diameter:12.5 cm).

Safranine

2.5 gm Safranine was dissolved in 10 ml ethanol and final volume was brought to 100 ml with distilled water. Solution was filtered through Whatman filter paper No.1.

Gram staining procedure

Prepared smear of the organism on a glass slide and heat fixed it. Flood the smear with crystal violet for a minute. Washed with tap water and flooded with gram's iodine for a minute.Washed with tap water and decolorized with 70% ethanol prepared in distilled water. Counter stained with safranine for 45 seconds, washed with tap water, blot dried with tissue paper and examined under oil immersion microscope (100 x magnification).

Appendix C

C.1 Biochemical media used for identification of bacteria based on Carbohydrate fermentation

Peptone	5.0 gm
Beef extract	3.0 gm
* Sugar	0.5 gm
Distilled water	1000 ml
O-Cresol red	0.01 gm
pH	10.5 adjusted using 10 % Na_2CO_3 solution

* arabinose, glucose, mannitol and xylose

Media with Durhams tube was autoclaved at 15 psi for 20 minutes. Tubes inoculated and incubated at room temperature(29 ^{0} C) for 24-48h. Change in color and presence or absence of gas bubble was observed. Uninoculated tubes serve as the control.

Nitrate reduction test Media

Peptone	5.0 gm
Beef Extract	3 .0 gm
KNO_3	1.0 gm
Distilled water	1000 ml
Na_2CO_3	10.0 gm
pH	10.5

Nitrate broth is inoculated and incubated at room temperature(29 ^{0} C) for 24- 48 hrs. After incubation, 5 drops of sulfanilic acid and 5 drops of napthylamine were added. Red coloration indicated a positive test while in a

negative test, red coloration is observed after addition of 5 mg of zinc. Uninoculated tubes served as the control.

Citrate utilization test

Simmons citrate agar

Ammonium dihydrogen phosphate	1.0 gm
Diammonium phosphate	1.0 gm
Sodium chloride	5.0 gm
Magnesium Sulphate	2.0 gm
Sodium carbonate	10 .0gm
Agar	20 .0gm
Distilled water	1000 ml
pH	10.5

Inoculate Simmon's citrate agar slants by means of stab inoculation and incubate for 24-48 hrs at room temperature(29 ^{0} C). Observe the slants for presence or absence of growth. Citrate utilizers were indicated by the presence of growth and a blue colour on the slant.

Catalase test

Three or four drops of 3% (v/v) hydrogen peroxide were mixed with a loopful of culture in a plate. Evolution of gas bubbles caused by liberation of free oxygen was indicative of Catalase positive organisms.

Oxidase test

A filter paper strip was soaked in Tetra methyl paraphenylene diamine (TMPD) dye. A loopful of fresh bacterial culture was smeared on the moist filter paper. Production of deep purple colour in 5-10 seconds indicated a positive Oxidase test.

Triple Sugar Iron Agar (1000 ml)

Peptone	10 gm
Tryptone	10.0 gm
Yeast extract	3.0 gm
Beef extract	3 .0 gm
Saccharose	10.0 gm
Lactose	10 .0 gm
Dextrose	1.0 gm
Ferric ammonium citrate	0.3 gm
Sodium chloride	5 .0 gm
Sodium Thiosulphate	0.3 gm
Phenol red	0.024 gm
Agar	12.0 gm
pH	7.2

Disolved the media components and sterilized using autoclave for 20 minutes at 15 psi.

TSI Agar contains three sugars (Dextrose, Lactose and Sucrose), phenol red for detecting carbohydrate fermentation and Ferrous ammonium sulfate for detection of Hydrogen sulfide production (indicated by blackening in the butt of the tube).

Carbohydrate fermentation is indicated by a yellow colouration of the medium. If the medium in the butt of the tube becomes yellow (acidic), but the medium in the slant becomes red (alkaline), the organism being tested ferments only Dextrose (Glucose). A yellow (acidic) color in the slant and butt indicates that the organism being tested ferments Dextrose, Lactose and/or

Sucrose. A red (alkaline) color in the slant and butt indicates that the organism being tested is a non-fermenter.Hydrogen sulfide production results in a black precipitate in the butt of the tube. Gas production is indicated by splitting and cracking of the medium.

Gelatin liquefaction

Nutrient Gelatin

Peptone	5.0 gm
Beef extract	3.0 gm
Gelatin	120.0 gm
Distilled water	1000 ml
Sodium carbonate	10.0 gm
pH	10.5

Inoculate tubes and incubate at room temperature (29 ^{0} C)for 24-48 h, the tubes were refrigerated for 30 min and the media were observed. Liquid medium after refrigeration showed a positive test.

Starch hydrolysis

Starch agar medium

Peptone	5.0 gm
Beef extract	3 .0 gm
Soluble starch	2.0 gm
Agar	20 .0 gm
Distilled water	1000 ml
Sodium Carbonate	10 .0 gm
pH	10.5

Inoculated starch agar plates by spot inoculation and incubated at room temperature
(29 ^{0} C) for 24-48 hrs, flooded the plates with Gram's Iodine for 1 min and poured off the excess stain. Clear zone surrounding the colony indicated a positive test.

Casein Hydrolysis

Inoculated milk agar plates and incubated at room temperature (29 ^{0} C) for 24-48 hrs, then examine the plates for the presence or absence of a clear area around the colony. A clear area around the bacterial colony indicates a positive proteolytic activity.

Hugh and Leifson's test

Peptone	2.0 gm
NaCl	5.0 gm
K_2HPO_4	0.3 gm
Glucose	10.0 gm
o-Cresol red	0.01 gm
Sodium carbonate	10 .0 gm
Distilled water	1000 ml
pH	10.5

Heat the media in boiling water bath and cool immediately. Inoculate overnight grown culture in the medium and dispense into two tubes. The medium of one tube was overlayered with sterile liquid paraffin. Growth and color change of the indicator dye
(o-Cresol red) was observed in the two tubes. Strict aerobes grow only in aerobic conditions. Facultative anaerobes grow in both aerobic and anaerobic conditions. The anaerobic organisms grow only in anaerobic conditions.

C.2 Reagents for biochemical tests

Reagents for nitrate reduction

Solution A (Sulfanilic acid)

Sulfanilic acid 8.0 gm

Acetic acid (5N) one part of glacial acetic acid added to 2.5 parts of distilled water

Distilled water to make 1 L

Solution B (Alpha-Napthylamine)

Alpha-Napthylamine 5 gm

Acetic acid (5N) 1 L

Appendix –D

D.1 Stock Solutions for SDS-PAGE

Acrylamide- bis-acrylamide solution (Monomer solution): 29% Acrylamide and 1 % (w/v) N, N-metnyhlene bis-acrylamide was dissolved in warm distilled water. pH adjusted to 7.0 and stored in amber bottles at 4°C and used within 30 days.

Resolving gel buffer (1.5 M Tris, pH 8.8): prepared by dissolving 18.615 gm Tris, in 70 ml Distilled water water and added 100 µl of 10 % Sodium dodecyl sulphate(SDS) in distilled water . The pH of the solution was adjusted to 8.8 using concentrated HCl, final volume was made up to 100 ml with Distilled water and solution was stored at 4°C.

Stacking gel buffer (1.0 M Tris, pH 6.8): Prepared by dissolving 12.11 gm Tris in 70ml distilled water and added 50 µl of 10 % SDS in distilled water.

The pH of the solution was adjusted to 6.8 using concentrated HCl, final volume was made up to 100 ml with distilled water and stored at 4°C.

Ammonium per sulfate (APS, 10 % w/v): Prepared by dissolving 0.1 gm of APS in 1.0 ml distilled water .The solution was prepared afresh each time.

Electrophoresis buffer (Glycine buffer)

Composition of 1X buffer is as follows:

Tris	3.0 gm
Glycine	14.4 gm
SDS (10%)	10 ml
Distilled water	to make 1000ml
pH	8.4

Tracking dye:

50 % Sucrose	10 ml
Bromophenol blue	10 mg

Staining Solution

Coomassie Brilliant Blue G- 250 solution was prepared by dissolving 0.25 gm Coomassie Brilliant Blue G- 250 in 100 ml of 25 % Methanol, 10 % Glacial acetic and
65 % distilled water.

Destaining Solution I

Methanol	40 ml
Acetic acid	10 ml
Distilled water	50 ml

Destaining Solution II

Methanol	5 ml

Acetic acid 7 ml

Distilled water 88 ml

Preparation of gel monomer

The composition of the resolving and stacking gels (10 ml) bfvdcxis as follows:

Solution	Resolving gel (12 %)	Stacking gel (5%)
Monomer	4.0 ml	1.7 ml
1.5M Tris, pH 8.8	2.5 ml	-
1.0 M Tris, pH 6.8	-	1.25 ml
10 % SDS	0.1 ml	0.1 ml
10 % APS	0.1 ml	0.1 ml
Distilled water	3.3 ml	6.8 ml
TEMED	5 µl	10 µl

Staining and destaining procedure:

Coomassie blue staining: The gel was stained in Coomassie Brilliant Blue G 250 solution Staining was carried out overnight: followed by destaining under mild shaking using de staining solution I for 3-4 hours and destaining solution II for several hours till the protein bands became clearly visible with no background colour. The gel were dried and preserved between cellophane sheets.

D.2 Buffers and reagents for plasmid DNA extraction and agarose gel electrophoresis

(1) 1M Tris-HCl (pH 8.0)

Dissolve 15.76 gm of Tris-HCl in 50 ml distilled water and adjust the pH to 8 using NaOH. Make up the volume to 100 ml using distilled water.

(2) 0.5 M Tris-HCl (pH 8.0)

Dissolve 7.88 gm of Tris-HCl in 50 ml distilled water and adjust pH to 8.0 using NaOH. Make up the volume to 100 ml using distilled water.

(3) 0.1 M Tris-HCl (pH 8.0)

Dissolve 1.576 gm in 50 ml distilled water and adjust pH to 8.0 using NaOH. Make up the volume to 100 ml using distilled water.

(4) TE buffer (pH 8.0)

Tris Cl 10 mM

EDTA 1 mM

Diluted with distilled water, adjusted pH to 8.0

and make up the volume to 100 ml using distilled water.

(4) 70% ethanol: 70 ml of ethyl alcohol + 30ml distilled water

(5) Lysozyme (15 mg/ml)

15 mg Lysozyme + 1 ml Tris-HCl (pH 8.0), vortex gently and store at

20^{0} C.

(6) Tris- Acetate EDTA (TAE) buffer (50x)

Tris base 24.2 gm

Glacial acetic acid 5.71 ml

0.5 M EDTA 5 ml

Dissolve in 40 ml distilled water, adjust the pH to 8.

Make the volume to 100 ml using distilled water.

(7) Glucose buffer

Glucose 50 mM (9.0 gm)

EDTA 10 mM (20 ml, 0.5 M EDTA) (pH 8.0)

Tris 25 mM (25ml, 1 M) (pH 8.0)

Adjust the pH and make up the volume to 1000 ml with Distilled water.

(8) Lysis buffer (100 ml)

NaOH 0.8 gm

SDS 1.0 gm

(9) Acetate buffer

Potassium acetate 29.4 gm (or 60ml of 5M)

Glacial Acetic acid 11.5 ml

Adjust the pH to 5.2 and make up the volume to 100 ml with Distilled water.

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Research Publications

- **Anju Pandey**, Milind M. Naik and Santosh K. Dubey (2010). Organic metabolitesproduced by *Vibrio parahaemolyticus* srain An 3 isolated from Goan Mullet, inhibit bacterial fish pathogens. African Journal of Biotechnology. 9(42): 7134-7140 **(Published)**.

- **Anju Pandey**, Milind M. Naik and Santosh K. Dubey (2010). Hemolysin, protease and EPS producing pathogenic *Aeromonas hydrophila* strain An4 shows antibacterial activity against marine bacterial fish pathogens. Journal of Marine Biology. 2010:1-9 **(Published).**

- **Anju Pandey**, Milind M. Naik and Santosh K. Dubey (2010). Biological characterization of marine fish pathogen, Acinetobacter sp. strain An2 producing antibacterial metabolites. Journal of Scientific and Industrial Research. 17: 135-141**(Published).**

- Milind Naik, Kashif Shamim, **Anju Pandey** and S. K. Dubey (2011). Bioremediation of metals mediated by marine bacteria, **In: Role of Microbes in Environmental Management and Industry** (Eds. Johri, B.N., Satyanarayana, T. and Prakash, A.), Springer Press **(In Press)**.

- **Anju Pandey** and Santosh K. Dubey (2011). Biological characterization of *tdh* and *hly* positive, β hemolysin producing pathogenic, strains of

Vibrio parahaemolyticus and *Aeromonas hydrophila* from marine fishes of Goa. Journal of Food Safety **(Communicated).**

- **Anju Pandey**, Gracinda, Maria Mesquita, S. M. Singh, and S. K. Dubey 2010. Isolation and characterization of a psychrotolerant bacteria from Antarctic Ocean. Current Science **(Communicated)**.

- .Milind Mohan Naik, **Anju Pandey** and Santosh Kumar Dubey (2011). P-type ATPase and MdrL efflux pump mediated lead and multi-drug resistance in estuarine bacterial isolates from Goa, India. Letters in Appl. Microbiol.**(Communicated).**

- Milind Mohan Naik, **Anju Pand**ey and Santosh Kumar Dubey (2011). Biological characterization of Lead- enhanced exopolysaccharide from lead resistant *Enterobacter cloacae* strain P2B. Biometals (Communicated).

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Hindawi Publishing Corporation
Journal of Marine Biology
Volume 2010, Article ID 563205, 9 pages
doi:10.1155/2010/563205

Research Article

Hemolysin, Protease, and EPS Producing Pathogenic *Aeromonas hydrophila* Strain An4 Shows Antibacterial Activity against Marine Bacterial Fish Pathogens

Anju Pandey, Milind Naik, and Santosh Kumar Dubey

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Taleigao Plateau, Goa, 403206, India

Correspondence should be addressed to Santosh Kumar Dubey, santoshdubey.gu@gmail.com

Received 9 June 2010; Revised 22 August 2010; Accepted 1 October 2010

Academic Editor: Pei-Yuan Qian

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A pathogenic *Aeromonas hydrophila* strain An4 was isolated from marine catfish and characterized with reference to its proteolytic and hemolytic activity along with SDS-PAGE profile (sodium dodecyl sulphate-Polyacrylamide gel electrophoresis) of ECPs (extracellular proteins) showing hemolysin (approximately 50 kDa). Agar well diffusion assay using crude cell extract of the bacterial isolate clearly demonstrated antibacterial activity against indicator pathogenic bacteria, *Staphylococcus arlettae* strain An1, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3, and *Alteromonas aurentia* SE3 showing inhibitory zone >10 mm well comparable to common antibiotics. Further GC-MS analysis of crude cell extract revealed several metabolites, namely, phenolics, pyrrolo-pyrazines, pyrrolo-pyridine, and butylated hydroxytoluene (well-known antimicrobials). Characterization of EPS using FTIR indicated presence of several protein-related amine and amide groups along with peaks corresponding to carboxylic and phenyl rings which may be attributed to its virulent and antibacterial properties, respectively. Besides hemolysin, EPS, and protease, *Aeromonas hydrophila* strain An4 also produced several antibacterial metabolites.

1. Introduction

Aeromonas infections are one of the most common bacterial diseases diagnosed in marine and cultured freshwater fish. *Aeromonas hydrophila* is found in diverse habitats, including soil, water, and is pathogenic to warm and cold-blooded animals [1]. Aquatic environment along with seafood is thus important potential source for the transmission of *Aeromonas hydrophila* resulting in human infections. *Aeromonas* spp. have been involved in wound infections, sepsis, outbreaks of water, and food-borne gastroenteritis [2]. Virulence in *Aeromonas hydrophila* is multifactorial which consists of aerolysins, hemolysins, enterotoxins, and proteolytic enzymes which play significant role in pathogenesis.

EPS, (exopolysaccharides) also play very important role in the interaction between bacteria and their environment as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances [3]. In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock [4]. Despite their importance, very few studies have been done on chemical characterization of EPS produced by *Aeromonas hydrophila*.

Over 120 of modern drugs have been isolated from terrestrial microorganisms, which includes important antibiotics and other drugs [5]. Although the ocean covers more than 70% of the earth's surface, microbial bioactive compounds of marine origin have been largely unexplored [6]. Marine environment is a special niche for many unique microorganisms, which produce bioactive compounds to adapt to particular environmental conditions [7]. There is an increasing demand of therapeutic drugs from diverse natural resources. Earlier studies focused on terrestrial plants and microbes proved extremely fruitful, yielding many organic

bioactive compounds including anticancer drugs [8]. But the potential contribution of marine organisms to the discovery of new bioactive molecules is very challenging [9]. Several bacteria present in aquatic ecosystems possess the ability to inhibit the growth of other microorganisms by producing antimicrobials and other molecules, namely, antibiotics, bacteriocins, siderophores, lysozymes, and proteases [10]. Many bacterial strains cause major diseases in fish and marine invertebrates resulting in mass mortalities worldwide and are equally responsible for human infections as well. Recently several novel bioactive substances such as Vibrindole, zafrin, nigrospoxydon pyrroles, pyrazines, and phenolic compounds have been isolated from marine bacteria. Several marine antagonistic bacteria including *Pseudoalteromonas* sp.A1-J 11, *Vibrio alginolyticus*, and *Pseudomonas* sp. 55b-11 were isolated from Japan which produced low molecular weight compounds inhibitory to the growth of *Vibrio* strains [11]. There are many reports about antibacterial activity shown by marine bacteria, namely, *Pseudomonas, Yersinia, Brevibacterium, Bacillus*, and *Alteromonas* [9, 12–15]. Recent studies however have targeted bacteria from an increasing assortment of nutrient rich and nutrient poor marine habitats including the surfaces or interior spaces of marine invertebrates in order to fully assess the potential of marine bacteria as a natural drug source.

In the present investigation, we have isolated and characterized *Aeromonas hydrophila* strain An4 from marine catfish with reference to its proteolytic, hemolytic activity and SDS-PAGE profile of ECPs showing hemolysin along with inhibitory activity against three-gram negative pathogenic bacterial isolates, namely, *Acinetobacter sp.* strain An2, *Vibrio parahaemolyticus* strain An3, and *Alteromonas aurentia* strain *SE3* and one-gram positive *Staphylococcus arlettae* strain An1. We have also identified and characterized its several antibacterial organic metabolites by GC-MS and EPS by FTIR.

2. Material and Methods

2.1. Bacterial Strain

2.1.1. Screening and Identification of Marine Bacteria. The test bacterial strain was isolated by serial dilutions of the swab suspension from infected regions of mouth, fins, and gills of a catfish (*Ictalurus punctatus*) with visible symptoms of hemorrhage and lesions. Determination of colony characteristics and gram staining of the test bacterial isolate was followed by biochemical tests using Hi-Media (India) kit to tentatively identify as per Bergey's Manual of Systematic Bacteriology [16]. Further confirmation of genus and species of the test bacterium was done by 16S rDNA sequencing and NCBI-BLAST search [17]. PCR amplification of the 16S rDNA was done using eubacterial forward and reverse primers: 27 f (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492 r (5′-GGTTACCTTGTTACGACTT-3′) (MWG Biotech India Pvt. Ltd., Bangalore, India), according to the standard procedure [18].

2.2. Detection of Proteolytic Activity and Assay. The bacterial isolate was spot inoculated on nutrient agar plate supplemented with 2% skimmed milk and stab inoculated in 10% gelatine agar in test tube, and proteolytic activities were observed in the form of clearance zone and liquefaction, respectively, after 24 hours and 48 hours of incubation. Protease production assay was done in broth, following Kunitz method [19].

2.3. Hemolytic Activity. Hemolytic activity of the pathogenic isolate was detected by streaking the isolate on trypticase soy agar plate supplemented with human blood [20].

2.4. Isolation of Extracellular Proteins (ECPs) and SDS-PAGE Analysis. The bacterial isolate was grown overnight in 5 mL LB broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl) for preculturing.100 μL of this culture suspension (inoculum) was added to 50 mL LB broth and incubated overnight at 37°C at shaker speed of 200 rpm. Culture suspension was harvested at 5000 rpm at 4°C for 15 minutes. Supernatant was precipitated by addition of 10% (w/v) trichloroacetic acid with overnight incubation at 4°C. Further centrifugation at 11000 rpm for 20 minutes resulted in pellet containing extracellular proteins which was suspended in 50 μL of 1 M Tris-HCl buffer (pH 8). Protein sample mixed with sample solubilizing buffer (10 μL) was analyzed by SDS-PAGE using 12% acrylamide followed by Coomassie blue staining [21].

2.5. Extraction and Purification of Exopolysaccharide (EPS). 48-hour grown bacterial culture (100 mL) was centrifuged at 8000 rpm for 20 minutes. Pellet was resuspended in 300 μL EDTA solution (10 mm EDTA + 1.5 mM NaCl) and heated at 50°C for 3 minutes in order to extract cell-bound EPS. Suspension was centrifuged, and supernatant was decanted and mixed with previous supernatant and pressure filtered through cellulose nitrate filters. EPS was precipitated by adding three volumes of chilled ethanol to the filtrate and incubating overnight at 4°C. EPS was recovered by centrifugation, and to remove impurities, it was dialysed (molecular weight cutoff of 8 kDa; Sigma-Aldrich Chemic GmbH, Seelz, Germany) against distilled water at 4°C for 24 hours. EPS was lyophilised and stored.

2.6. Fourier-Transformed Infrared Spectroscopy. Major functional groups of purified EPS were detected by FTIR [22]. Pellets for infrared analysis were obtained by grinding 2 mg of EPS with 200 mg of dry KBr the mixture was pressed into a 16 mm diameter mould. The FTIR spectra was recorded on a SHIMADZU-FTIR 8201PC instrument (Shimadzu, Japan) in the 4000–400 cm^{-1} region and spectra traced with a Hewlett Packard plotter.

2.7. Antibiotic Susceptibility Test. Overnight grown bacterial suspension (0.1 mL) of indicator pathogenic bacteria *Staphylococcus arlettae* strain An1 (accession no. FJ386956), *Acinetobacter sp.* strain An2 (accession no. FJ38695), *Vibrio parahaemolyticus* strain An3 (accession no. FJ386958), and

Alteromonas aurentia strain SE 3 was spread plated on Mueller Hinton agar plates; octadiscs (Hi Media, India) were placed in the center of the agar plates and incubated at room temperature (27°C) for 24 hours. Sensitivity of the individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic disc susceptibility test approved by NCCLS [23].

2.8. Preparation of Crude Cell Extract. Modified ethyl acetate extraction procedure was followed to extract antimicrobial metabolites from the test bacterium [24]. Cells were grown on SYEP agar (seawater-based yeast extract peptone agar). After 48 hours, agar along with the cells was cut into pieces and suspended into ethyl acetate to extract the antibacterial metabolites. Overnight suspension was decanted followed by centrifugation to get cell-free solvent. Solvent was subjected to evaporation at 40°C for final recovery of crude extract [6]. Simultaneously 48-hour old culture suspension of the test bacterium was centrifuged to get cell-free supernatant.

2.9. Antimicrobial Bioassay (Agar Well Diffusion Test). In order to check the antibacterial activity of the cell extract, SYEP agar (1.2%) was poured in the plates, small wells of about 6 mm diameter were made in the agar plates, and bottom of the wells was sealed by 0.7% molten SYEP agar [6]. 100 μL crude cell extract and cell-free supernatant were poured in the wells separately and allowed to diffuse in the agar for 4 hours. Different indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 μL) was used as control.

2.10. GC-MS Analysis of Crude Cell Extract. Identification of the antibacterial metabolites was done by GC-MS analysis, injecting 1ul of sample into an RTX-5 column (7 m × 0.32 mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan, and Helium (3 mL/min) was used as a carrier gas. The following temperature gradient program was used: 75°C for 2 minutes followed by an increase from 75°C to 175°C at a rate of 50°C per min and finally 7 minutes at 175°C. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compound.

3. Results

3.1. Identification of Marine Bacteria. The test bacterium was gram (−ve) rod which formed tiny, circular, white, opaque, raised, glistening colonies with entire edge.This isolate hydrolysed, starch, esculin casein, and gelatin exhibited positive reactions for oxidase, catalase, MR, indole, citrate, and lipase, and negative once for VP and urease.These morphological and biochemical characteristics tentatively established the identity of this isolate as *Aeromonas* sp. which was designated as strain An4. 16S rDNA sequencing and BLAST search further confirmed the identity of this bacterial isolate as *Aeromonas hydrophila* strain An4 (accession no. FJ386959). This strain exhibited 98% homology with *Aeromonas hydrophila* (accession no. AB 368776.1).

FIGURE 1: Protease activity shown by the *Aeromonas hydrophila* strain An4.

FIGURE 2: β Hemolytic activity shown by *Aeromonas hydrophila* strain An4. On blood agar.

3.2. Protease Activity and Assay. Aeromonas hydrophila strain An4 exhibited significant protease production in the form of clearance zone and liquifaction (Figure 1). This pathogenic bacterial isolate interestingly revealed commencement of protease production during late log phase (18 hours) with maximum level of production around 22 hours of incubation which is stationary growth phase of bacterial pathogen (Figure 3).

3.3. β Hemolysis. Aeromonas hydrophila strain An4 interestingly demonstrated a very clear β hemolysis in the form of clearance zone along the streak on blood agar plate within 24 hours of incubation at room temperature (Figure 2).

3.4. SDS-Page Analysis of Extracellular Proteins. The protein profile of the extracellular fraction clearly demonstrated the presence of protein bands ranging between 35 to 97 kDa with a significantly prominent band of 50 kDa corresponding to the virulence protein, hemolysin of *Aeromonas hydrophila.* (Figure 4).

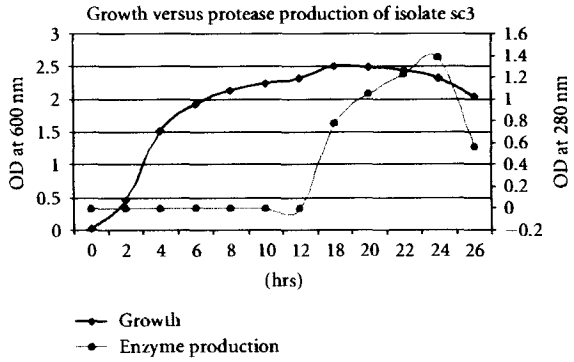

FIGURE 3: Growth versus Protease production in *Aeromonas hydrophila* strain An4.

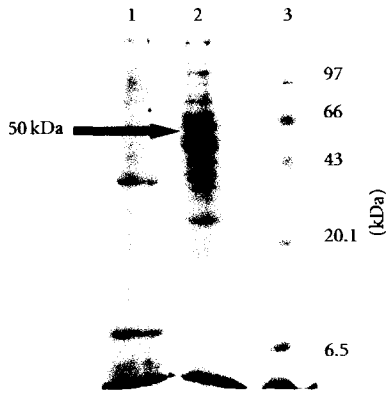

FIGURE 4: SDS-PAGE analysis of extracellular protein fraction. (1) Control. (2) Extracellular protein fraction from *Aeromonas hydrophila* strain An4 showing hemolysin (50 kDa). (3) Protein molecular weight marker.

3.5. Fourier-Transformed Infrared Spectroscopy. The FTIR spectrum of the EPS revealed characteristic functional groups such as C–H stretching peak of methyl group at 2918.30 cm^{-1} and stretching peak of alkene group at 3024.38 cm^{-1}. Absorption peaks ranging between 667.37 cm^{-1}–881.47 cm^{-1} are assigned to phenyl rings. Amide I, amide III (primary amine CN stretch), and amide IV showed absorption peak at 1643.35 cm^{-1}, 1020.34 cm^{-1}, and 466.77 cm^{-1}, respectively. Conspicuous absorption centered between 2524.05 cm^{-1} and 2627.82 cm^{-1} corresponded to carboxylic acids, and aliphatic primary amine showed NH stretching peak at 3302.13 cm^{-1} (Figure 5).

3.6. Antibiotic Susceptibility Test for the Indicator Bacteria. All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested and were also resistant to few antibiotics (Table 1).

3.7. Antibacterial Activity of the Crude Cell Extract of the Test Organism. Agar well diffusion experiment clearly demonstrated the antagonistic characteristic of crude cell extract of *Aeromonas hydrophila* strain An4 as manifested by growth inhibition of gram (−ve) indicator bacteria, namely, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3, *Alteromonas aurentia* strain SE 3, and gram (+ve) *Staphylococcus arlettae* strain An1. Interestingly, ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 caused remarkably wider inhibitory zones as compared to most of the common antibiotics tested. Interestingly, cell-free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on the growth of indicator bacteria. This clearly revealed that intracellular antibacterial metabolites are present inside the cells of *Aeromonas hydrophila* strain An4 without being secreted out (Figure 6 and Table 1).

3.8. GC-MS Analysis of the Crude Cell Extract. GC-MS analysis of crude ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 clearly revealed the presence of several important organic metabolites which includes Butylated hydroxytoluene (BHT) as major constituent (7.25%) followed by pyrrolopyrazines (6.5%), pyrrolo-pyridine (0.65%), and other phenolic compounds (Figure 7; Table 2).

4. Discussion

Two haemolytic toxins, haemolysin and aerolysin, have been reported in *A. hydrophila*. Hemolytic proteins are commonly isolated from pathogenic bacteria, and β-hemolysins are one of the important bacterial virulence factors. Hemolysins and related proteins containing cystathionine β synthase (CBS) domains are bacterial toxins that function by assembling identical subunits into a membrane-spanning pore [25]. The molecular mass of bacterial hemolysins ranges from 49 to 60 kDa [25–29]. We have demonstrated presence of a prominent protein band of approximately 50 kDa in extracellular protein fraction of this pathogenic isolate which indicates the possible presence of the bacterial toxin, hemolysin [26].

It has been suggested that proteolytic enzymes of fish pathogen, *Aeromonas hydrophila*, play an important role in causing massive tissue damage in the host which may facilitate establishment of infection [30]. Conspicuous protease activity along with hemolytic activity shown by *Aeromonas hydrophila* strain An4 further strengthens its pathogenic potential as it has already been proved that presence of both the proteins enhances the intensity of virulence in this fish pathogen [31]. EPSs also play a very important role in virulence of pathogenic bacteria as they mediate the interaction between pathogenic bacteria and their environment through adhesion to the host [32, 33]. FTIR analysis of EPS of *Aeromonas hydrophila* strain An4 clearly revealed presence of many protein-related amine and amide groups indicating the possible presence of bacterial toxins. Therefore we can assume that EPS may also contribute significantly in pathogenesis. EPS could also account for antibacterial activity against indicator organisms due to presence of several phenolic and carboxylic groups which is evident from corresponding stretching peaks in FTIR spectrum of the EPS. Competition among microbes for space and nutrients in

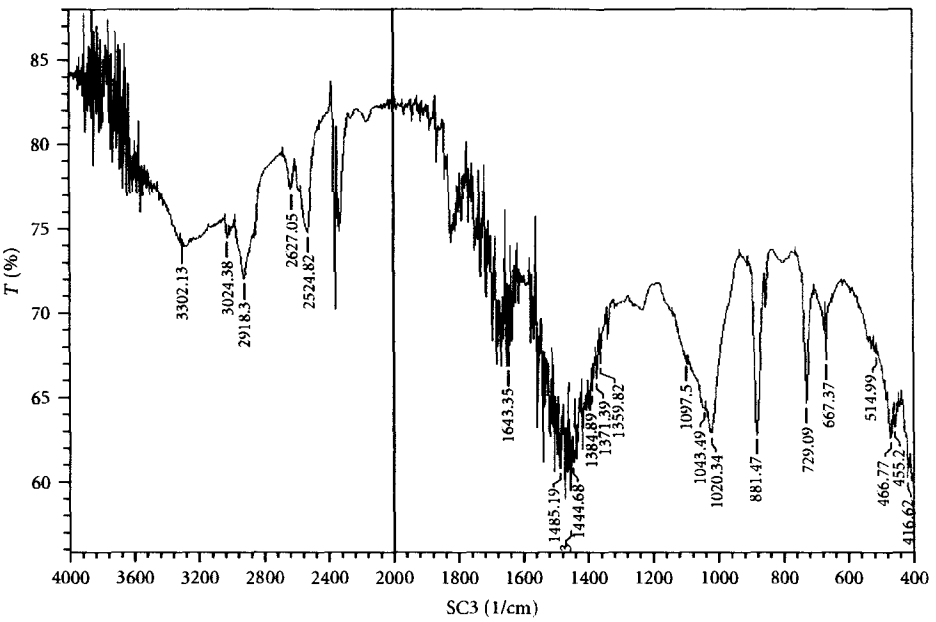

FIGURE 5: FTIR Spectrum of EPS of *Aeromonas hydrophila* strain An4.

TABLE 1: Comparison of antibacterial activity of *Aeromonas hydrophila* strain An4 with commonly used antibiotics on indicator bacterial isolates.

Antibiotics	Concent-ration (μg/mL)	Zone of inhibition (mm) Indicator bacteria			
		Vibrio parahaemolyticus strain An3	*Acinetobacter* sp. strain An2	*Alteromonas aurentia* strain *SE3*	*Staphylococcus arlettae* strain An1
Ciprofloxacin-(Cf)	10	3	4	0	0
Cotrimazine-(Cm)	25	12	9	6	6
Kanamycin-(K)	30	4	6	10	11
Nitrofurantoin-(Nf)	300	2	7	0	0
Streptomycin-(S)	10	8	14	6	10
Tetracycline-(T)	30	6	14	2	1
Ampicillin-(A)	10	0	0	14	0
Cephalothin-(Ch)	5	0	0	13	3
Colistin methane sulphonate-(Cl)	25	1	1	7	9
Gentamycin-(G)	10	4	7	0	0
Streptomycin-(S)	10	3	5	2	3.5
Cotrimoxazol-(Co)	25	8	12	7	7
Cell-free supernatant	100 μL	0	0	0	0
Bacterial crude extract	100 μL	10	11	12	10.5
Ethyl acetate	100 μL	0	0	0	0

marine environment is a powerful selection pressure which endows marine microorganisms to produce many natural products of medical and industrial value. Compared with the terrestrial microorganisms, the secondary metabolites produced by marine organisms have more novel and unique structures, owing to their complex living circumstances and species diversity [34]. Several antimicrobials have been reported from marine microorganisms associated with animals due to their specialized role in their respective hosts [35].

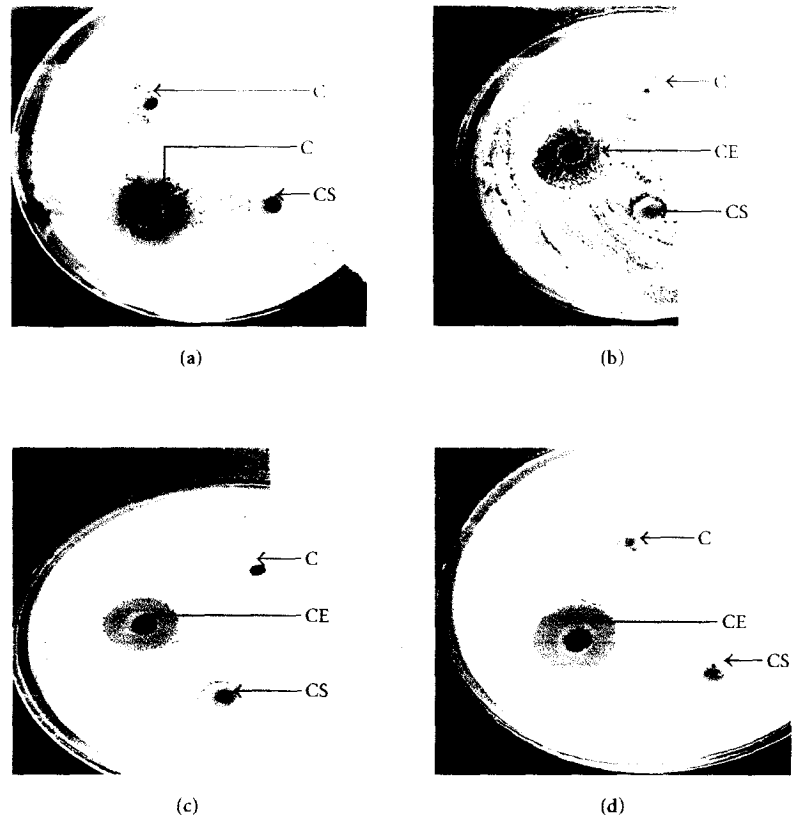

FIGURE 6: Antibacterial activity of the crude cell extract of the *Aeromonas hydrophila* strain An4 on indicator bacteria. (a) *Staphylococcus arlettae* strain An1, (b) *Acinetobacter* sp. strain An2, (c) *Vibrio parahaemolyticus* strain An3, and (d) *Alteromonas aurentia* strain SE 3. C: control (Ethyl acetate); CE: cell extract; CS: culture supernatant.

TABLE 2: Major antibacterial metabolites present in ethyl acetate cell extract *Aeromonas hydrophila* strain An4.

Antibacterial metabolites	Retention time	% in test volume
Butylated hydroxytoluene	12.625	7.25
Pyrrolo-(1,2-a) pyrazine-1,4- dione, hexahydro-3 (2-methylpropyl)	20.033	4.6
Pyrrolo-(1,2-a) pyrazine-1,4- dione, hexahydro-3 (2-methyl phenyl)	24.417	1.9
Phenol, 4-(1,1,3,3-tetramethyl butyl)	15.425	0.65
Pyrrolo(1,2-a) pyridine	10.042	0.44

Presence of Butylated hydroxytoluene (BHT) in the cell extract of this test bacterium as a major constituent demonstrated that this important antiviral compound can be used against Herpes simplex virus and Φ 6 [36, 37]. In addition, other metabolites, namely, pyrrolopyrazines and phenolics, were also present in the cell extract of the test organism which are also well-known antimicrobials [38]. Since cell-free supernatant of the test organism could not inhibit the growth of indicator bacteria, it was assumed that these antimicrobial compounds were present inside the cell as well as in the cell exudates. Treatment of infections is compromised worldwide by the emergence of bacteria resistant to multiple antibiotics. In general bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. One way to prevent antibiotic resistance is by using new compounds which are not based on existing synthetic antimicrobial compounds. GC-MS analysis of crude ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 clearly demonstrated the presence of several important organic metabolites which are known antagonist to bacteria, viruses, and fungi [9, 12, 36, 39, 40]. It is interesting to note that even pathogenic strains can be used as biocontrol agents against other pathogens due to inherent presence of various virulence factors, namely hemolysin, proteases, and EPS along with several antibacterial organic compounds.Thus

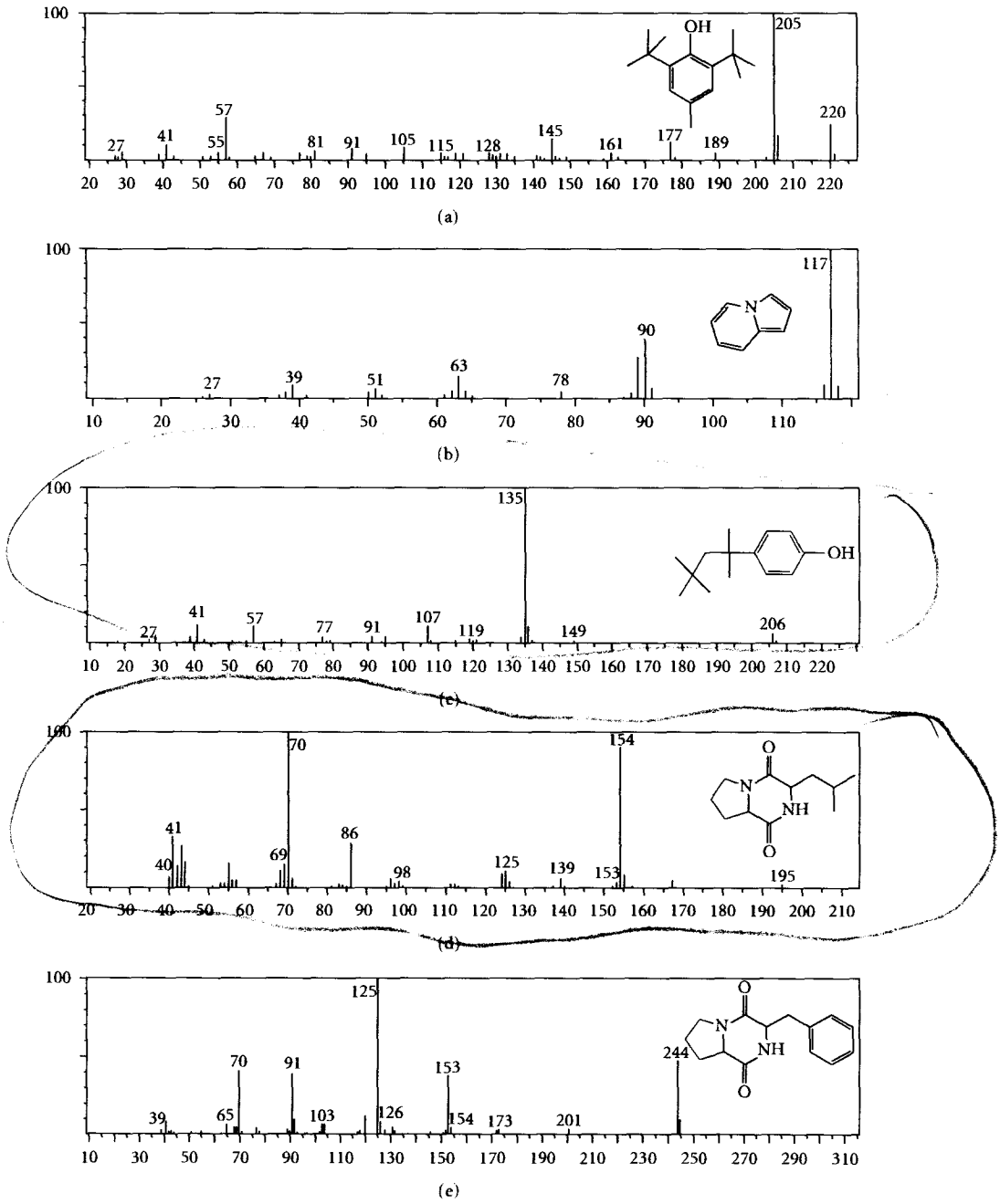

FIGURE 7: Molecular structure and corresponding GC-MS peaks of major antibacterial metabolites from crude cell extract of *Aeromonas hydrophila* strain An4. (a) Butylated Hydroxytoluene(BHT)—[Peak 205], (b) Pyrrolo[1,2-a] pyridine [peak 117.15], (c) (1,1,3,3-tetramehyl butyl)-Phenol [Peak 135], (d) Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2-methyl propyl)—[Peak 70.1], and (e) Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2-phenyl methyl)—[Peak 125].

antibacterial metabolites synthesized by the test organism may serve as valuable drugs to control pathogenic bacterial strains causing fish and human diseases, and isolation of novel bacterial strains with antimicrobial activity suggests that marine ecosystem is a valuable source of antimicrobials.

Acknowledgments

A. Pandey is grateful to Goa University, Goa, India for the financial assistance in the form of Ph.D. studentship during this research work. The authors are also thankful to Mr. Ajay Kumar from Advanced Instrumentation Facility, Jawahar Lal Nehru University, New Delhi for the GC-MS analysis.

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African Journal of Biotechnology Vol. 9(42), pp. 7134-7140, 18 October, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Organic metabolites produced by *Vibrio parahaemolyticus* strain An3 isolated from Goan mullet inhibit bacterial fish pathogens

Anju Pandey, Milind M. Naik and S. K. Dubey*

Department of Microbiology, Laboratory of Bacterial Genetics and Environmental Biotechnology, Goa University, Goa, India.

Accepted 17 September, 2010

Identification and action of several antibacterial metabolites produced by a fish pathogen *Vibrio parahaemolyticus* strain An3 from marine ecosystem of Goa has been demonstrated. Antibacterial activity of the crude cell extract of the test bacterium has been evaluated against indicator pathogenic bacterial strains such as *Acinetobacter* sp. An2, *Aeromonas hydrophila* strain An4, *Staphylococcus arlettae* strain An1 and *Alteromonas aurentia* strain SE3 by agar well diffusion method which clearly demonstrated comparatively more significant inhibitory effect on indicator bacteria as compared to several commonly used antibiotics. Gas chromatography mass spectrometry (GC-MS) analysis of crude cell extract of the test organism interestingly revealed presence of indole, phenyl acetic acid, n-(3-methyl-1, 2, 4-oxadiazol-5-yl) - 1- pyrrolidine carboximidamide, pyrrolopyrazines, tetramethyl pyrazine and other important phenolic compounds which may be responsible for antibacterial activity against indicator microorganisms tested. It has been clearly demonstrated that *V. parahaemolyticus* strain An3 produced several medically important organic metabolites during cultivation suggesting it as a potential candidate for production of several antibacterial metabolites to control pathogenic bacterial strains causing serious fish and human diseases.

Key words: Antibacterial, gas chromatography mass spectrometry, metabolites, pathogenic bacteria, well diffusion.

INTRODUCTION

There is an increasing demand of therapeutic drugs from diverse natural resources. After many years of extensive research, the importance of terrestrial bacteria as source of valuable bioactive compounds has been very well established and exploited. As a result, the ocean and metabolites of marine organisms including associated microorganisms have now become the main focus of drug discovery research (Finical, 1993).These studies are concerned with bacteria and fungi isolated from sea water, sediments, invertebrates and fish (Kelecom, 2002). Bacteria occurring in aquatic ecosystems may have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances such as antibiotics and bacteriocins. Their inhibitory mechanisms include: (i) Production of antibiotics, bacteriocins, siderophores, lysozymes, and proteases and (ii) alteration of pH through production of organic acids (Jorquera et al., 1999).

Vibrio spp. are common inhabitants of aquatic environment and are found free living as well as associated with various marine organisms such as squids, shrimps, corals, fish, molluscs, seagrasses and sponges. Some species are found as symbionts in specialized luminous organs of marine fish and invertebrates, whereas a number of other species are well-known pathogens of humans or

*Corresponding author. E-mail: santoshdubey.gu@gmail.com. Tel: 091-832-6519359, 091-832-2217770, 091-9923568466. Fax: 091-832-2225201.

Abbreviations: GC-MS, Gas chromatography mass spectrometry; **PCR** , polymerase chain reaction; **SYEP,** sea water based yeast extract peptone agar; **TCBS,** thiosulfate citrate bile salts sucrose; **TSI,** triple sugar iron; **ONPG,** o-nitrophenyl-β-d-galactopyranoside; **VP,** Voges-Proskauer; **BLAST,** basic local alignment search tool.

marine animals (Thompson et al., 2004). *Vibrio paraheamolyticus* is one of the leading pathogens causing fish and human diseases. Recently, several biologically active substances have been isolated from marine bacteria. There are many reports about antibacterial activity shown by marine bacteria; *Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus* and *Alteromonas* (Gauthier and Breittmayer, 1979; Shiozawa et al., 1997; Jorquera et al., 1999; Khalil et al., 2006; Ahmed et al., 2008; Rahman et al., 2010). There are few reports on *vibrios* producing antimicrobial substances (Sugita et al., 1997; Long and Azam, 2001; Castro et al., 2002; Hjelm et al., 2004; Norhana and Darah, 2005). In our investigation we have reported organic metabolites of a fish pathogenic strain of *V. parahaemolyticus* which inhibited the growth of other bacterial fish pathogens. We have further characterized these metabolites by gas chromatography mass spectrometry (GC-MS).

MATERIAL AND METHODS

Isolation and screening of marine bacteria

Different marine fishes with visible symptoms of hemorrhage and lesions on their body parts were selected; infected regions such as mouth, fins and gills were washed under sterile conditions with sterile deionized double distilled water and swabbed with sterile cotton wool. Suspension of this swab was prepared in saline and used for isolation of pathogenic bacteria on nutrient agar plates by serial dilutions.

Morphological characterization

The colony characteristics of the selected bacterial isolate; size, shape, colour, margin, elevation, consistency and opacity were observed and recorded. The selected bacterial isolate was gram stained and observed under the light microscope at 100 x magnification to study the cell morphology.

Identification of test bacterium using biochemical tests

Hi-Media (India) kit for biochemical tests was used to tentatively identify this bacterial isolate and results were interpreted according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Molecular identification

Further confirmation of genus and species of the test bacterium was done by 16S rDNA sequencing and NCBI–BLAST search (Altschul et al., 1990). The genomic DNA was extracted from the bacterial isolates and used as template for polymerase chain reaction (PCR) amplification of the 16S rDNA fragment (1400 bps) according to standard procedure (Sambrook et al., 1989). The following eubacterial primers were used for PCR amplification:

27 f (5'- AGAGTTTGATCCTGGCTCAG -3')
1492 r (5'- GGTTACCTTGTTACGACTT -3')

These primers were purchased from MWG Biotech India Pvt. Ltd., Bangalore, India.

Antibiotic susceptibility test

Overnight grown bacterial suspension (0.1 mL) of indicat pathogenic bacteria *Acinetobacter sp.* strain An2 (Accession n FJ38695), *Aeromonas hydrophila* strain An4 (accession n FJ386959), *Staphylococcus arlettae* strain An1 (accession n FJ386956) and *Alteromonas aurentia* strain SE3 was spread plate on Mueller Hinton agar plates; octadiscs (OD-007 and 014 from H Media, India) containing multiple antibiotics were carefully placed i the center of the agar plates and incubated at room temperatur (27°C) for 24 h. Sensitivity of the individual indicator bacteri isolate to a particular antibiotic was determined according to th performance standards of antibiotic disc susceptibility test approve by National Committee for Clinical Laboratory Standards (Bauer e al., 1966).

Preparation of crude cell extract

Ethyl acetate extraction procedure was followed with a slight modif cation to extract antimicrobial metabolites from the test bacteriur (Wratten et al., 1977). Test organism was grown on sea wate based yeast extract peptone agar (SYEP) agar. After 48 h, aga along with the cells was cut into pieces and suspended into ethy acetate to extract the antibacterial metabolites. Overnight suspend sion was decanted followed by centrifugation to get cell free solven Solvent was subjected to evaporation at 40°C for final recovery o crude extract (Ahmed et al., 2008). Simultaneously, 48 h old cultur suspension of the test bacterium was centrifuged to get cell fre supernatant.

Antimicrobial bioassay (agar well diffusion test)

In order to check the antibacterial activity of the cell extract, SYEF agar (1.2%) was poured in the plates, small wells of about 6 mm diameter were made in the agar plates and bottom of the wells were sealed by 0.7% molten SYEP agar (Abraham, 2004). 100 µ crude cell extract and cell free supernatant were poured in the wells separately and allowed to diffuse in the agar media for four hours. Different indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 µl) was used as a control to check its inhibitory effect.

GC-MS analysis of crude cell extract

Identification of the antibacterial metabolites was done by GC-MS analysis; injecting 1 µl of sample into a RTX-5 column (7 m x 0.32 mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan and Helium (3 ml/min) was used as a carrier gas. The following temperature gradient program was used: 75°C for 2 min followed by an increase from 75 to 175°C at a rate of 50°C per min and finally 7 min at 175°C. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds.

RESULTS

Morphological and biochemical characterization of test bacterium

This bacterial isolate appeared as spherical (2 - 3 mm diameter), dark green colonies on thiosulfate citrate bile

Table 1. Comparison of antibacterial activity of *V. parahaemolyticus* strain An3 with commonly used antibiotics on indicator bacterial isolates.

Antibiotic (µg/ml)	Zone of clearance (diameter) of indicator bacteria			
	Acinetobacter sp. strain An2 (mm)	*Aeromonas hydrophila* strain An4 (mm)	*A. aurentia* strain *SE3* (mm)	*S. arlettae* strain An1 (mm)
Amicacin- (Ak) 10	7	7	3	0
Carbenicillin- (Cb) 100	5	5	4.5	3
Ciprofloxacin- (Cf)10	4	4	0	0
Co-Trimazine- (Cm) 25	9	9	6	6
Kanamycine– (K) 30	6	6	10	11
Nitrofurontoin- (Nf) 300	7	7	0	0
Streptomycin- (S) 10	14	14	6	10
Tetracycline- (T) 30	14	14	2	1
Ampicillin- (A) 10	0	0	14	0
Cephalothin- (Ch) 5	0	0	13	3
Colistin methane sulphonate- (Cl) 25	1	1	7	9
Gentamycin- (G) 10	7	7	0	0
Streptomycin- (S) 10	5	5	2	3.5
Sulphatriad- (Sl) 200	7	7	6	3
Tetracycline- (T) 25	9	9	3	3
Co-Trimoxazol- (Co)25	12	12	7	7
Cell free supernatant	0	0	0	0
Bacterial crude extract	11	10	12	10
Ethyl acetate	0	0	0	0

salts sucrose (TCBS) agar. Biochemical tests revealed that this motile bacteria exhibited positive activity for oxidase, gelatinase, arginine dihydrolase and lysine decarboxylase enzymes, whereas negative activity for o-nitrophenyl-β-d-galactopyranoside (ONPG), Voges-Proskauer (VP) and urease enzyme. On triple sugar iron (TSI) slant, it showed alkaline slant and acidic butt. Based on specific biochemical and morphological characters and as per Bergey's manual of systematic bacteriology, this fish pathogenic bacterial strain was tentatively identified as *Vibrio* sp.

PCR amplification of ribosomal gene encoding 16S rDNA followed by DNA sequencing and Basic Local Alignment Search Tool (BLAST) search clearly confirmed the identity of this bacterial pathogen as *V. parahaemolyticus*. Subsequently, we designated this pathogenic isolate as *V. parahaemolyticus* strain AN3 (Accession No. FJ386958). We have also confirmed presence of tdh gene (amplicon size: 264 bp), characteristically encoding thermostable direct hemolysin in *V. parahaemolyticus* (Data not shown).

Antibiotic susceptibility test for the indicator bacteria

All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested and were also resistant to few antibiotics (Table 1).

Antibacterial activity of the crude cell extract of the test organism

Agar well diffusion experiment clearly demonstrated the antagonistic characteristic of crude cell extract of *V. parahaemolyticus* strain AN3 as manifested by growth inhibition of gram (-ve) indicator bacteria; *Acinetobacter* sp. strain An 2, *Aeromonas hydrophila* strain An4, *Alteromonas aurentia* strain SE 3, and gram (+ve) *S. arlettae* strain An1. Interestingly, ethyl acetate cell extract of *V. parahaemolyticus* strain AN3 caused remarkably wider inhibitory zones as compared to most of the common antibiotics tested. Interestingly, cell free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on the growth of indicator bacteria. This clearly revealed that these antibacterial organic metabolites are present inside the cells of *V. parahaemolyticus* strain AN3 without being secreted out (Figures 1a, b, c, d and Table 1).

GC-MS analysis of the crude cell extract

GC-MS analysis of crude ethyl acetate cell extract of *V. parahaemolyticus* strain AN3 clearly revealed the presence of several important antimicrobial organic metabolites which included indole, phenyl acetic acid, N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidine, carboximidamide,

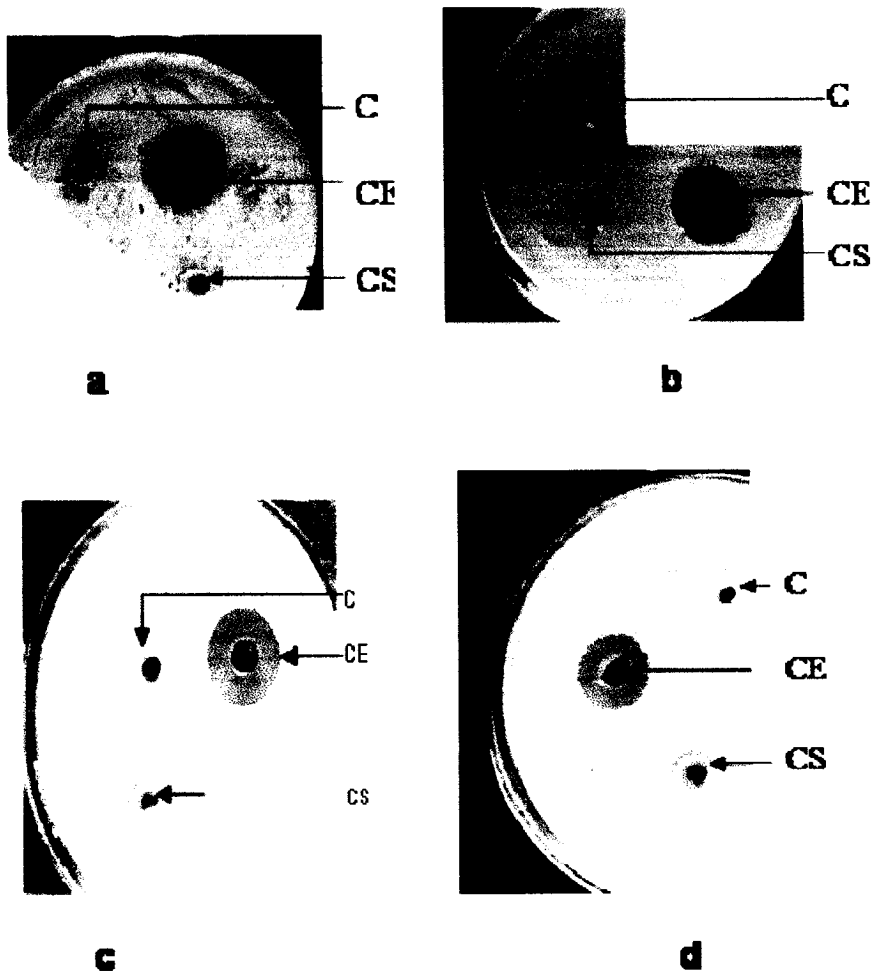

Figure 1. Antibacterial activity of the crude cell extract of the *V. parahaemolyticus* strain An3 on indicator bacteria. a. *S. arlettae* strain An1; b. *Acinetobacter* sp. strain An2, c. *A. hydrophila* strain An 4; d. *A. aurentia* strain SE3; C, Control (ethyl acetate); CE, cell extract; CS- culture supernatant.

pyrrolopyrazines, tetramethyl pyrazine and other phenolic compounds (Figures 2a - g and Table 2).

DISCUSSION

Marine microorganisms including bacteria, fungi and microalgae have received increasing attention during past few years due to increasing re-isolation of previously discovered compounds. Novel and unique structures of the secondary metabolites produced by marine organisms also have forced researchers to view the marine environment from different perspective and marine microorganisms as new biomedical sources. Until now, more extensive and focussed efforts to discover new antibiotics have involved the terrestrial environment mainly due to ease of availability, isolation and culture conditions. By the end of 2008, approximately 3000 microbial metabolites were reported from marine environment (Rahman et al., 2010).

GC-MS analysis of crude ethyl acetate cell extract of *V. parahaemolyticus* strain An3 demonstrated an interesting concoction of compounds with significant antimicrobial activity. A well known antagonistic compound has already been reported from *V. parahaemolyticus* as Vibrindole A, a bis-indole derivative (Bell et al., 1994) along with other indole derivatives (Kobayashi et al., 1994; Velury et al., 2003). In addition, other metabolites such as phenyl acetic acid, pyrrolidine carboximidamide, pyrrolopyrazines tetramethyl pyrazine and phenolic compounds are also well known antimicrobials tested against different bacteria fungi and other microbes (Kim et al., 2004; Somers et al., 2005; Chaudhary et al., 2006; Kumar et al., 2008 Farzaliev et al., 2009; Roy et al., 2010).

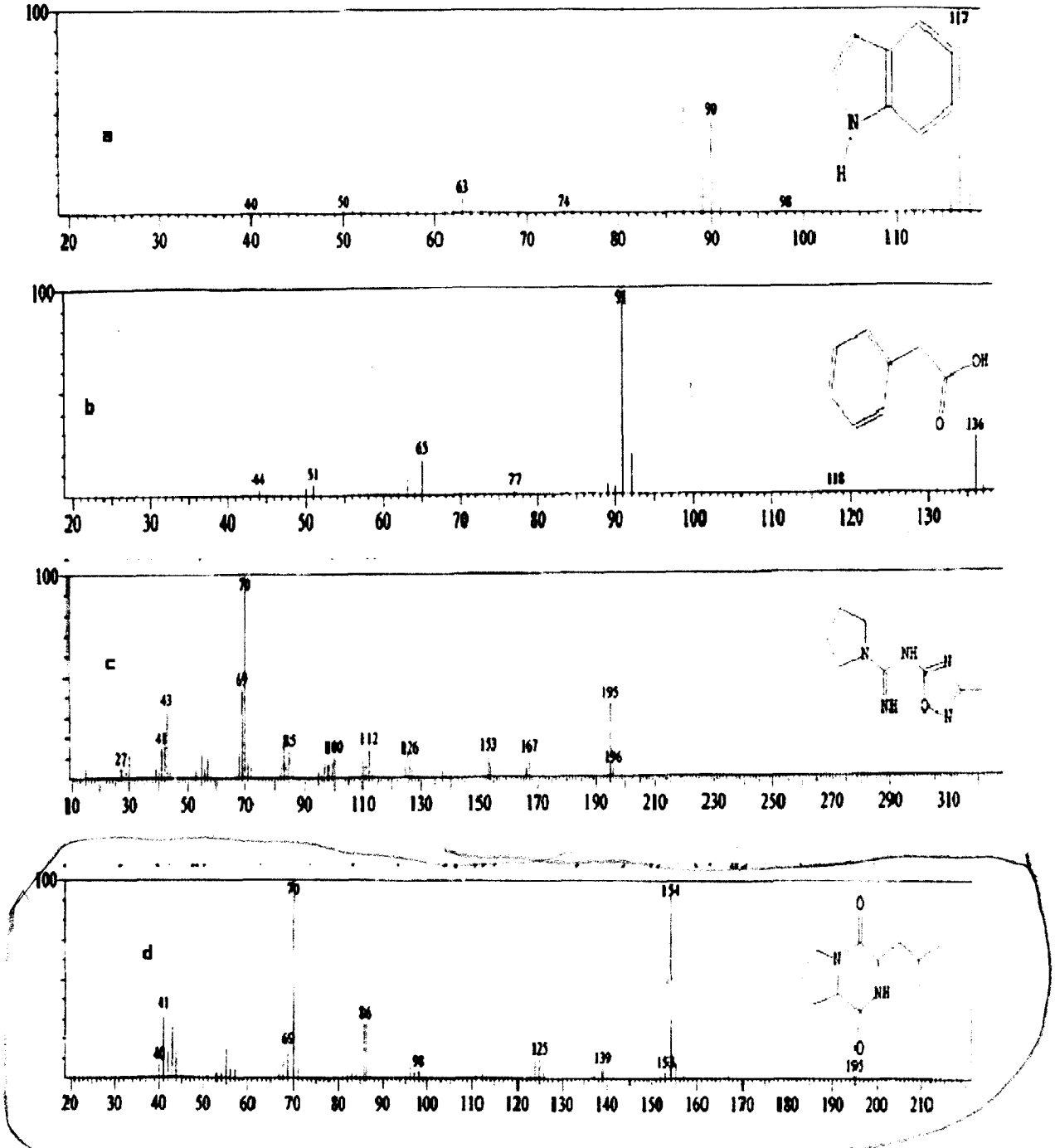

Figure 2. Structure and GC-MS spectrum of major antibacterial metabolites present in crude cell extract of *V. parahaemolyticus* strain An3: **a.** indole (Peak 117); **b.** phenyl acetic acid (Peak 91); **c.** N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidinecarboximidamide (Peak 70); **d.** pyrrolo- (1,2-a)pyrazine-1,4- dione hexahydro-3(2-methylpropyl)(Peak 70); **e.** phenol, 4-(1,1,3,3-tetramethyl butyl) (Peak 135.2); **f.** nonyl-phenol (Peak 149); **g.** tetramethyl pyrazine (Peak 54).

Currently, the treatment of infections is compromised worldwide by the emergence of multi-drug resistant bacteria. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. One way to prevent antibiotic resistance is by using new compounds which are not based on existing

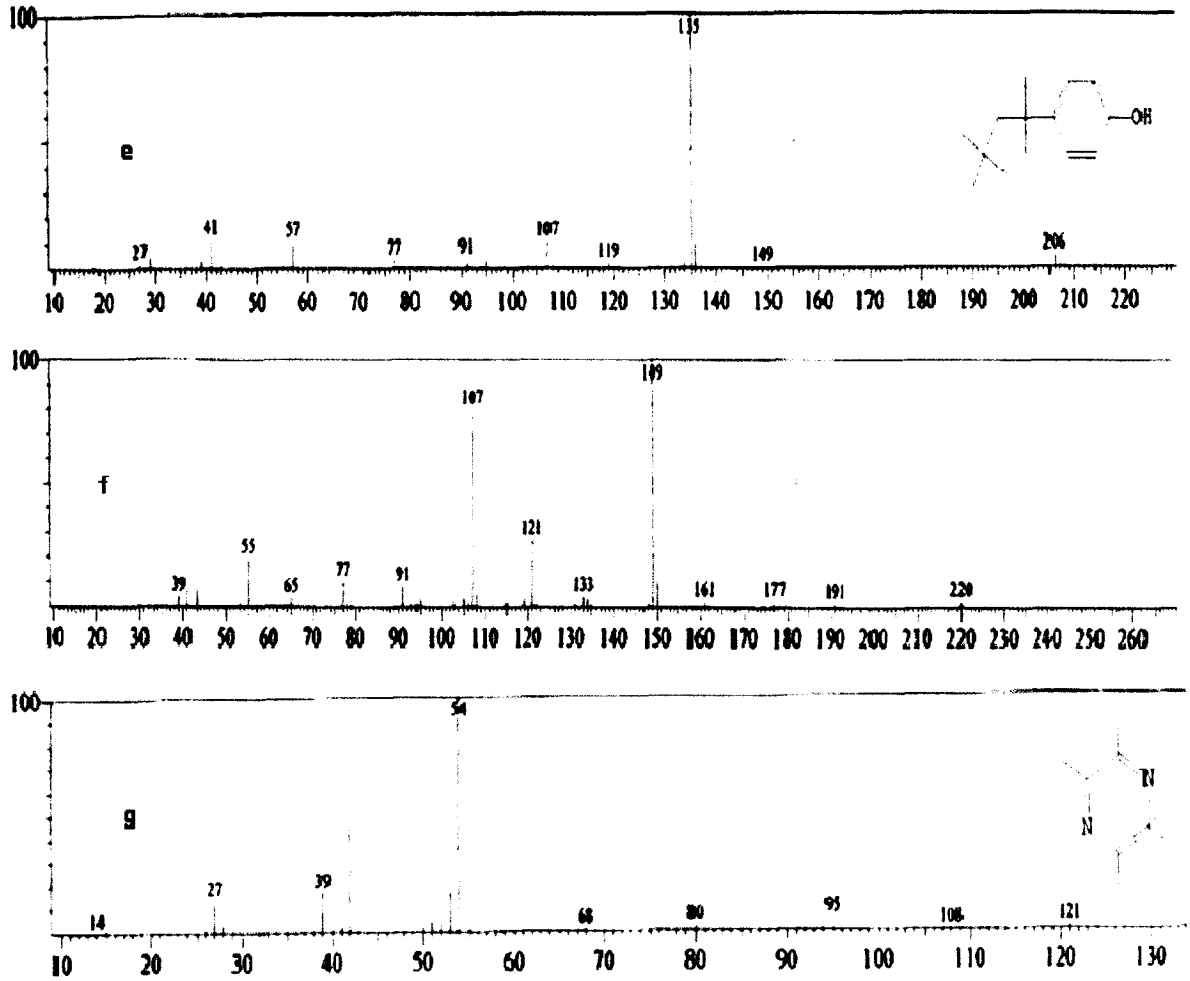

Figure 2. Contd.

Table 2. Major antibacterial metabolites present in ethyl acetate cell extract of *V. parahaemolyticus* strain An3.

Antibacterial metabolite	Retention time (mins)	% in test volume
Indole	9.842	3.0
Phenyl acetic acid	9.3	2.0
N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-Pyrrolidinecarboximidamide	19.75	1.86
Pyrrolo-(1,2-a)pyrazine-1,4- dione, hexahydro-3(2-methylpropyl)	20. 033	1.3
Phenol, 4- (1,1,3,3-tetramethyl butyl)	15.425	0.76
Nonyl-phenol	16.492	0.6
Tetramethyl pyrazine	6.775	0.23

synthetic antimicrobial compounds. In the present investigation, we have tried to explore the production of novel antimicrobials in order to prevent antibiotic resistance in pathogenic bacteria.

Our primary interest should be practical management and control of infectious pathogens in newly developed aquaculture sites and human infections. It is interesting to note that even pathogenic strains can be used as biocontrol agents against other pathogens due to the inherent presence of antimicrobial compounds in the form of secondary metabolites. Thus antibacterial metabolites synthesized by the test organism may serve as source of

valuable antimicrobial drugs to control pathogenic bacterial strains causing fish and human diseases.

ACKNOWLEDGEMENTS

Anju Pandey is grateful to Goa University, Goa, India for financial assistance in the form of Ph.D studentship during this research work. Authors are also thankful to Mr. Ajay Kumar from Advanced Instrumentation Facility, Jawahar Lal Nehru University, New Delhi for GC-MS analysis.

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Journal of Scientific & Industrial Research
Vol. 70, February 2011, pp. 135-141

Biological characterization of marine fish pathogen, *Acinetobacter* sp. strain An 2 producing antibacterial metabolites

Anju Pandey, Milind Mohan Naik and Santosh Kumar Dubey*

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Goa 403 206, India

Received 23 July 2010; revised 31 December 2010; accepted 07 January 2011

This study presents antibacterial activity of several organic metabolites produced by a fish pathogen, *Acinetobacter* sp. strain An 2, from marine ecosystem of Goa. Cell extract demonstrated stronger inhibitory effects on Gram negative bacteria as compared to common antibiotics. GC-MS analysis of crude cell extract revealed presence of potential antimicrobials (butylated hydroxytoluene, phenol, pyrrolo-phenol, benzo-quinone, pyrrolo-pyrazine, phthalic acid butyl octyl ester and penta-fluoro-propionic acid hepta-decyl ester). FTIR analysis of its exopolysaccharide (EPS) revealed presence of amine, amide, carboxylic and phenyl groups. Therefore, this strain can be exploited as a potential candidate for several antibacterial drugs to combat bacterial pathogens causing serious fish and human diseases.

Keywords: *Acinetobacter* sp., Antibacterial activity, Crude cell extract, EPS, Indicator

Introduction

Although ocean covers more than 70% of earth's surface, microbial bioactive compounds of marine origin have been largely unexplored[1]. Marine microorganisms produce biologically active compounds to adapt to particular environmental condition[2-4]. Earlier studies reported[5-7] many organic biologically useful compounds including anticancer drugs and potential contribution of marine organisms including bacteria to the discovery of novel bioactive molecules. Several bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances (antibiotics and bacteriocins). Other inhibitors include siderophores, lysozymes, proteases, and organic acids[8]. Several biologically active substances, isolated from marine bacteria (*Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus*, and *Alteromonas*), have demonstrated antibacterial activity[6,9-13]. Endophytic (*Acinetobacter baumannii*) secrete an antifungal compound, which inhibit growth of some fungal plant pathogens[14]. Microbial exopolysaccharide (EPS) plays an important role in interaction between bacteria and their environment[15] and is reported useful in adhesion, nutrient aquisition, heavy metal sequestration, detoxification of toxic compounds and protection against osmotic shock[16].

This study presents isolation of a fish pathogenic strain of *Acinetobacter*, which inhibits growth of three Gram negative pathogenic indicator bacterial isolates (*Vibrio parahaemolyticus* strain An 3, *Aeromonas hydrophila* strain An 4 and *Alteromonas aurentia* strain SE 3). This study also demonstrated its antibacterial activity by agar well diffusion assay and detected antibacterial organic metabolites in crude cell extract (CCE) by GC-MS and in EPS by FTIR.

Experimental Section

Bacterial Strain

Isolation and Screening of Marine Bacteria

Marine fishes with visible hemorrhagic symptoms and lesions on their body were screened from Goa, and infected body parts (mouth, fins and gills) were washed with sterile deionized double distilled water and swabbed with sterile cotton wool. A suspension of swab was prepared in saline and used for isolation of pathogenic bacteria on nutrient agar plates by serial dilutions. Discrete colonies were picked up for further characterization.

*Author for correspondence
Tel: 91-832-6519359; Fax: 91-832-2225201
E-mail: dubey_santosh1@rediffmail.com;
santoshdubey.gu@gmail.com

Morphological Characterization and Identification of Test Bacterium

Colony characteristics of selected bacterial isolate (size, shape, colour, margin, elevation, consistency and opacity) were observed and recorded. Selected bacterial isolate was Gram stained and observed under light microscope at 100 x magnification to study cell morphology. Hi-Media (India) kit for biochemical tests was used to tentatively identify selected bacterial isolate as per Bergey's Manual of Systematic Bacteriology[17].

Molecular Identification

Genus/species of test bacterium were identified by 16S rDNA sequencing and NCBI–BLAST search[18]. Genomic DNA, extracted from bacterial isolates, was used as template for PCR amplification of 16S rDNA fragment (1400 bps) as per standard procedure[19]. Eubacterial primers [27 f (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492 r (5'- GGTTACCTTGTTACGACTT -3')] from MWG Biotech India Pvt Ltd, Bangalore, India were used for PCR amplification.

Preparation of Crude Cell Extract (CCE)

Ethyl acetate extraction procedure with a slight modification was followed to extract antimicrobial metabolites from test bacterium[20]. *Acinetobacter* sp. was grown on SYEP agar (sea water based yeast extract peptone agar). After 48 h, agar along with cells was cut into pieces and suspended into ethyl acetate to extract antibacterial metabolites. Overnight suspension was decanted followed by centrifugation to get cell free solvent. Solvent was subjected to evaporation at 40°C for final recovery of crude extract[9]. Simultaneously, 48 h old culture suspension of test bacterium was centrifuged to get cell free supernatant.

Antimicrobial Bioassay (Agar Well Diffusion Assay)

To check antibacterial activity of cell extract, SYEP agar (1.2%) was poured in plates, small wells (diam, 6 mm) were made in agar plates and bottom of wells were sealed by 0.7% molten SYEP agar[1]. CCE and cell free supernatant (100 µl) were poured in wells separately and allowed to diffuse in agar media for 4 h. Indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 µl) was used as a control to check inhibitory effect.

Antibiotic Susceptibility Test

Overnight grown bacterial suspension (0.1 ml) of indicator pathogenic bacteria *V. parahaemolyticus* strain An 3 (accession no. FJ386958), *A. hydrophila* strain An 4 (accession no. FJ386959), *S. arlettae* strain An1 (accession no. FJ386956) and *A. aurentia* strain SE 3 was spread plated on Mueller Hinton agar plates, octadiscs (OD-007 and 014 from Hi Media, India) containing multiple antibiotics were carefully placed in the centre of agar plates and incubated at room temperature (27°C) for 24 h. Sensitivity of individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic disc susceptibility test approved by national committee for clinical laboratory standards[21].

GC-MS Analysis of Crude Cell Extract

Antibacterial metabolites were identified by GC-MS analysis by injecting sample (1 µl) into a RTX-5 column (7m x 0.32mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan and Helium (3 ml/min) was used as a carrier gas. Temperature gradient program was used at 75°C for 2 min followed by an increase from 75° to 175°C at a rate of 50°C per min and finally 7 min at 175°C. The m/z peaks (mass to charge ratio) characteristic of antimicrobial fractions were compared with those in mass spectrum library of corresponding organic compounds.

Extraction and Purification of Exopolysaccharides (EPS)

Bacterial culture (100 ml) grown for 48 h was centrifuged at 8000 rpm for 20 min. Pellet was resuspended in 300 µl EDTA solution (10 mM EDTA + 1.5 mM NaCl) and heated at 50°C for 3 min to extract cell bound EPS. Suspension was centrifuged and supernatant was decanted and mixed with previous supernatant and pressure filtered through cellulose nitrate filters. EPS was precipitated by adding three volumes of chilled ethanol to filtrate and incubating overnight at 4°C. EPS was recovered by centrifugation and impurities were removed by dialysis (molecular weight cut-off - 8 kDa; Sigma- Aldrich Chemic GmbH, Seelz, Germany) against distilled water at 4°C for 24 h. EPS was lyophilised and stored until needed for FTIR analysis.

Fourier - Transformed Infrared Spectroscopy (FTIR) of EPS

Major functional groups of purified EPS were detected by FTIR 22. Pellets for IR analysis were obtained by grinding EPS (2 mg) with dry KBr (200 mg), and mixture was pressed into a mould (diam, 16 mm). FTIR spectra was recorded on a SHIMADZU – FTIR 8201 PC instrument (Shimadzu, Japan) in 4000-400 cm^{-1} region and traced by Hewlett Packard plotter.

Results and Discussion

Identification of Marine Bacteria

Test bacterial isolate was identified based on morphological, biochemical and molecular characteristics. Gram (-ve) coccobacillus formed tiny, circular, white, opaque, raised, glistening colonies with entire edge. This nitrate reducing isolate was also positive for oxidase, MR, catalase, citrate and lipase, whereas negative for VP, indole, urease, gelatinase and amylase. These characteristics tentatively established identity of this isolate as *Acinetobacter* sp., which was designated as strain An 2. PCR amplification of ribosomal gene encoding 16S rDNA (1126 bps) followed by DNA sequencing and BLAST search further confirmed identity of this bacterial isolate as *Acinetobacter sp.* strain An 2 (Accession no. FJ38695). Interestingly, 96% DNA sequence homology was found with *Acinetobacter* sp. WW21 (Accession no. EF433555.1).

Antibacterial Activity of Crude Cell Extract of Test Organism

Marine epiphytic bacteria of intertidal seaweeds (17%) showed antibacterial activity[23] against *Staphylococcus aureus.* In present study, CCE of *Acinetobacter* sp. strain An 2 prepared in ethyl acetate showed antagonistic characteristic by inhibiting growth of Gram (-ve) pathogenic bacterial indicators (*A. hydrophila* strain An 4, *V. parahaemolyticus* strain An 3 and *A. aurentia* strain SE 3), whereas it could not inhibit growth of Gram (+ve) *S. arlettae* strain An 1, possibly due to different cell wall constituents and less suceptibility to antimicrobial metabolites produced by test organism (Table 1). Thus ethyl acetate cell extract of *Acinetobacter* sp. caused remarkably bigger inhibitory zones as compared to common antibiotics tested. Antibacterial metabolites synthesized by test organism may serve as valuable drugs to control pathogenic bacterial strains causing fish and human diseases. Cell free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on growth of indicator bacteria, indicating that antibacterial metabolites of *Acinetobacter* sp. are intracellular and not secreted out in growth medium (Fig. 1).

Antibiotic Susceptibility of Indicator Bacteria

All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested. Few indicator bacterial isolates were resistant to common antibiotics tested (Table 1). Treatment of infections is compromised worldwide by emergence of bacteria resistant to multiple antibiotics. Although drug resistance is mainly attributed to chromosomal genes of pathogenic bacteria, it is most commonly associated with extra-

Table 1—Comparison of antibacterial activity of *Acinetobacter* sp. strain An 2 with commonly used antibiotics on indicator bacterial isolates

Antibiotics conc., µg/ml	Indicator bacteria Zone of clearance, mm (diameter)			
	Vibrio parahaemolyticus strain An3	*Aeromanas hydrophila* strain An4	*Alteromonas aurentia* strain SE 3	*Staphylococcu arlettae* strain An 1
Amicacin- (Ak) 10	7	7	3	0
Carbenicillin- (Cb)100	7	5	4.5	3
Ciprofloxacin- (Cf) 10	3	4	0	0
Co-Trimazine-(Cm) 25	12	9	6	6
Kanamycine – (K) 30	4	6	10	11
Nitrofurontoin-(Nf) 300	2	7	0	0
Streptomycin- (S) 10	8	14	6	10
Tetracycline- (T) 30	6	14	2	1
Ampicillin- (A) 10	0	0	14	0
Cephalothin- (Ch) 5	0	0	13	3
Colistin methane -sulphonate-(Cl) 25	1	1	7	9
Gentamycin- (G) 10	4	7	0	0
Streptomycin- (S) 10	3	5	2	3.5
Sulphatriad- (SI) 200	9	7	6	3
Tetracycline- (T) 25	8	9	3	3
Co-Trimoxazol- (Co) 25	8	12	7	7
Cell free supernatant, 100 µl	0	0	0	0
Bacterial crude extract, 100 µl	12	11	14	0
Ethyl- acetate, 100 µl	0	0	0	0

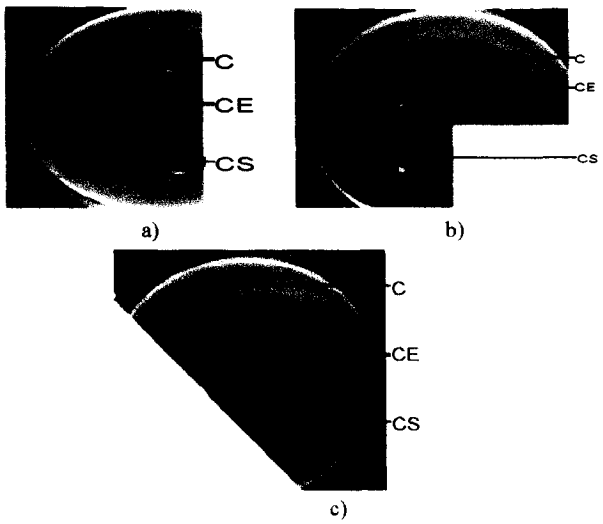

Fig. 1—Antibacterial activity of crude cell extract of *Acinetobacter* sp. An 2 on indicator bacteria: a) *Aeromonas hydrophila* strain An 4; b) *Vibrio parahaemolyticus* strain An3; c) *Alteromonas aurentia* strain SE 3 [C- Control (Ethyl acetate); CE- Cell extract; CS- Culture supernatant]

chromosomal elements acquired from other bacteria in the environment. These include different types of mobile DNA segments (plasmids, transposons and integrons), which genetically govern efflux pumps for multiple drug resistance in environmental bacterial isolates[24]. Thus drug and antibiotic susceptible indicator bacterial isolates may possibly lack these drug efflux pumps.

GC-MS analysis of Crude Cell Extract

GC-MS analysis of crude ethyl acetate cell extract of *Acinetobacter* sp. strain An 2 revealed presence of several important organic metabolites, which exhibit antimicrobial activity against bacteria, viruses and fungi[9,10,25-27]. Butylated hydroxytoluene (BHT) is one of the major fractions of cell extract (10%) followed by Pyrrolopyrazine (8.6%), Benzoquinone (8.5%), Benzene dicarboxylic acid (6.2%), and Penta -fluoropropionic acid (6.0%) (Fig. 2).

Compared with terrestrial microorganisms, secondary metabolites produced by marine organisms have novel and unique structures owing to their complex living circumstances and species diversity, thus their bioactive potentials are stronger[25,28]. Competition among microbes for space and nutrients in marine environment is a powerful selection pressure, which endows marine microorganisms to produce many natural products of medical and industrial value[29]. Several antimicrobial substances have been reported from these marine microorganisms due to their specialized role in respective hosts[30,31]. Presence of (BHT) in cell extract of *Acinetobacter* sp. as a major constituent demonstrated that this antiviral metabolite can be purified in large quantities and used against lipid containing human and bacterial viruses (Herpes simplex virus and Ô 6)[27,32]. Besides BHT, other metabolites (Pyrrolopyrazine, Benzoquinone, Benzene dicarboxylic acid, Penta-fluoropropionic acid) and a phenol derivative were also present in cell extract of test organism, which are well known potential antimicrobials[33,34].

Fourier-Transformed Infrared Spectroscopy (FTIR) of EPS

FTIR spectrum of EPS revealed characteristic functional groups (C-H stretching peak of methylene group at 2924.09 cm^{-1} and stretching peak of alkene group at 3278.99 cm^{-1}). Absorption peaks (667.37 - 881.47 cm^{-1}) are assigned to phenyl rings and one broad OH stretch at 3562.00 cm^{-1}. Amides showed absorptions at: Amide I, 1560.19 cm^{-1} and 1643.35 cm^{-1} [1560.19 (N-H bend)]; amide III (primary amine CN stretch), 1643.35 cm^{-1}; and amide IV, 1020.34 cm^{-1} and 466.77 cm^{-1}. Conspicuous absorption (2542.18 - 2632.83 cm^{-1}) corresponds to carboxylic acids and aromatic primary amine and showed CN stretching peak at 1232.51 cm^{-1} respectively (Fig. 3). Presence of several amine, amide, phenol and carboxylic groups in FTIR spectrum of EPS further confirm antibacterial activity of test bacterium.

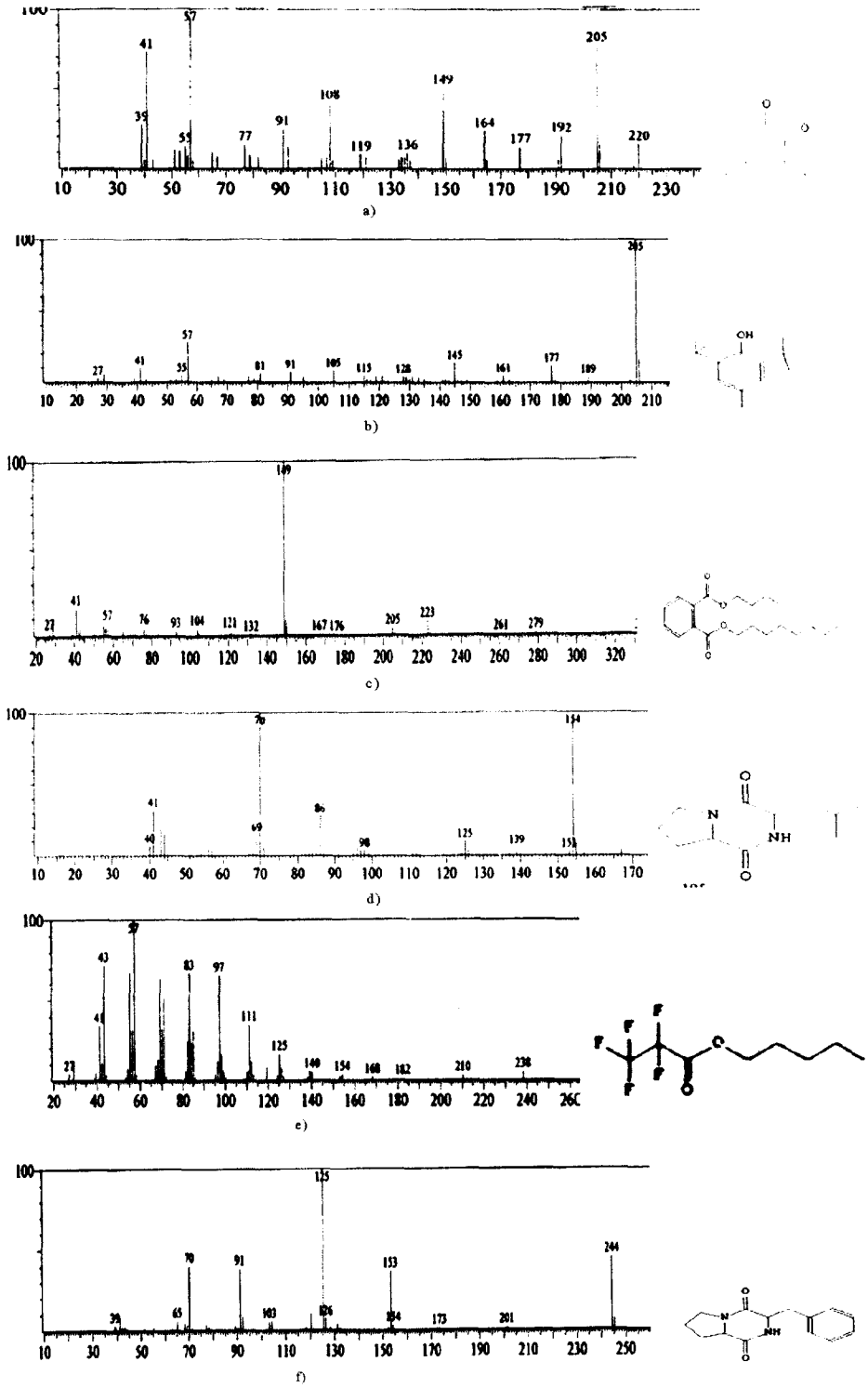

Fig. 2—Structure and GC-MS Spectrum of major antibacterial metabolites present in the crude cell extract of *Acinetobacter* sp. An 2: a) O-Benzoquinone, 3, 5 – di tert butyl- [Peak 41]; b) Butylated Hydroxytoluene (BHT) - [Peak 205]; c) 1, 2- Benzenedicarboxylic acid ,butyloctyl ester -[Peak 149]; d) Pyrrolo(1, 2-a) pyrazine-1, 4-dione, hexahydro-3 (-2- methyl propyl)- [Peak 70]; e) Pentafluoro propionic acid heptadecyl ester -[Peak 57]; and f) Pyrrolo(1, 2-a) pyrazine-1, 4-dione, hexahydro-3 (-2- phenyl methyl)- [Peak 125]

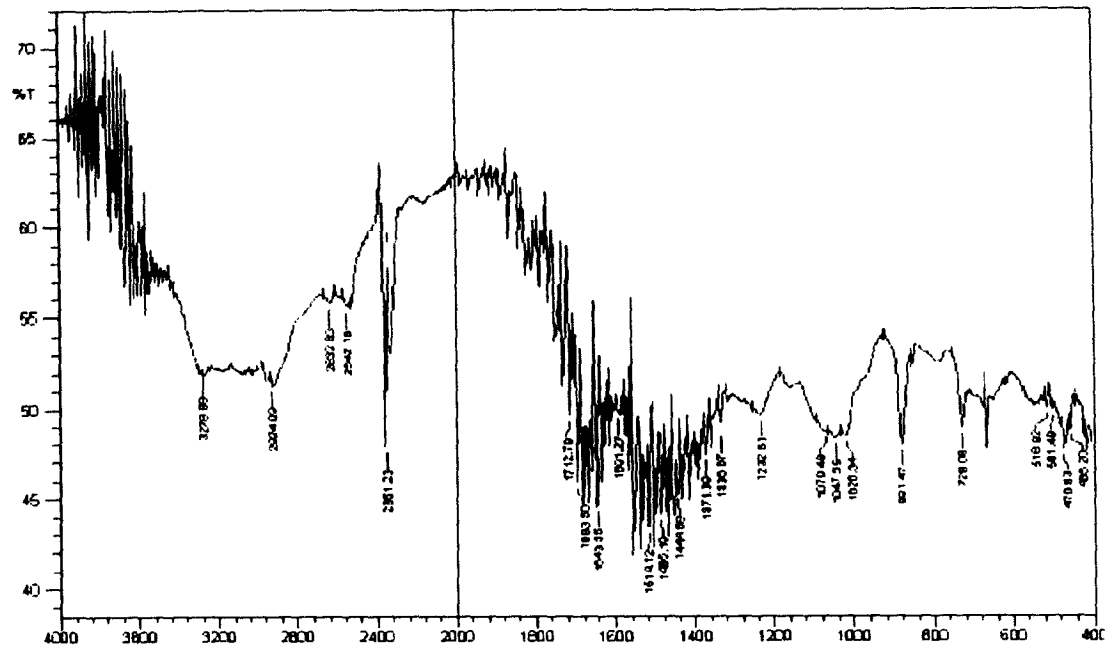

Fig. 3—FTIR spectrum of EPS of *Acinetobacter* sp. An 2

Conclusions

Agar well diffusion assay of CCE of test organism demonstrated stronger inhibitory effects on Gram negative indicator pathogenic bacteria as compared to inhibitory action of many commercially used antibiotics. GC-MS analysis of CCE revealed presence of several potential antimicrobials [BHT (major fraction, 10%), phenol, pyrrolo-phenol, benzo-quinone, pyrrolo-pyrazine, phthalic acid butyl octyl ester and penta-fluoro- propionic acid hepta-decyl ester]. FTIR analysis of EPS revealed several protein related antimicrobial amine and amide groups along with peaks corresponding to carboxylic and phenyl rings. Thus fish pathogen appears to be a potential candidate to produce several antibacterial metabolites along with unique antiviral organic compound to control pathogenic bacterial strains causing serious fish and human diseases.

Acknowledgments

Anju Pandey gratefully acknowledges Goa University, Goa, India for financial assistance as Ph D studentship during this research work. Authors also thank to Mr Ajay Kumar (Advanced Instrumentation Facility, JNU, New Delhi) for GC-MS analysis.

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Chapter 29
Bioremediation of Metals Mediated by Marine Bacteria

Milind M. Naik, Anju Pandey, and Santosh Kumar Dubey

Abstract Metals are an intrinsic part of the earth's crust. With rapid industrialization and urbanization, enormous amounts of industrial wastes including metal wastes are accumulating, which require special treatment. Wastes from mining and metal refining industries, sewage sludge, power plant wastes and waste incineration plants often contain substantial amounts of toxic heavy metals viz. Hg, Cd, Pb, As, Sb, Zn, Cu which pose serious treat to the environment and need to be removed from the source of pollution.

Microorganisms from metal polluted habitats possess a variety of inherent mechanisms to tolerate high levels of toxic metals which include precipitation of metals as phosphate, sulphide, carbonate; volatilization via methylation/ethylation; physical exclusion in membranes and extracellular polymeric substances (EPS); energy driven metal efflux system and intracellular sequestration mediated by metallothionein like proteins. For the last several decades, metal resistant microorganisms including marine bacteria have been considered a potential alternative for heavy metal recovery and bioremediation resulting in the development and refinement of many bioremediation technologies for removal of toxic metals form contaminated soils and aqueous mining and industrial wastes/effluents. Interestingly, these bioremediation technologies are economically viable, environmental friendly and value added processes.

Keywords Bioremediation • Metallothioneins • Heavy metals • Marine environment • Biosurfactants • Biosorption

M.M. Naik (✉) • A. Pandey • S.K. Dubey
Department of Microbiology, Goa University, Taleigao Plateau, Goa 403206, India
e-mail: dubey_santosh1@rediffmail.com

T. Satyanarayana et al. (eds.), *Microorganisms in Environmental Management: Microbes and Environment*, DOI 10.1007/978-94-007-2229-3_29,
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