A study of an antibacterial compound from salt pans of Goa

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

Tonima Kamat

Goa University,
Taleigao Goa
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Research Guide

Dr. Savita Kerkar

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2012

Dedicated to my family……..

DECLARATION

I hereby declare that the thesis entitled **"A study of an antibacterial compound from salt pans of Goa"**, submitted for the Degree of **Doctor of Philosophy** in **Biotechnology** to the Goa University, has been carried out by me at Department of Biotechnology, Goa University under the supervision of **Dr. Savita Kerkar** (Research Guide).

The work is original and has not been submitted in part or full by me for any other degree or diploma to any other university/institute. Materials obtained from other sources have been duly acknowledged in the thesis.

Place: **Tonima Kamat**

Date: (Research Scholar)

Acknowledgements

The work presented in this thesis is a compilation of ideas, challenges, excitement, and also ofcourse the techniques and research carried out during the course of my Ph.D. I take this opportunity to acknowledge all the people who have contributed in some way or the other to help me get where I am today.

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I am falling short of words to appreciate the sacrifice, encouragement and efforts put in by my husband Sameer for the completion of this thesis.

Tonima :)

Contents

Abbreviations

%	Percentage
℃	degree Celsius
µg	microgram
µg/L	Micrograms per liter
µL	micro litre
µM	micro molar
µm	micrometer
amu	Atomic mass unit
bp	Base pairs
CID	Collision induced dissociation
d.f.	Degree of freedom
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic acid disodium salt
ESI-MS	Electrospray ionisation-Mass spectrometry
g /L	grams per litre
g	gram
gDNA	Genomic DNA
h	Hour(s)
Hz	Hertz
Kb	Kilo base pairs
L	Litre
m/z	mass to charge ratio (amu)
mg	milli gram
min	Minute(s)
mL	milli litre
mM	milli molar
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
Psu	percentile salinity units
rDNA	Ribosomal DNA
R_f	Retention factor
rpm	Rotations per minute
RT	Room temperature
SC	*Staphylococcus citreus*
sp.	Species
spp.	Species (plural)
UV	Ultraviolet
V	Volt
v/v	volume / volume
VP	Voges Proscure
w/v	weight / volume
β	Beta

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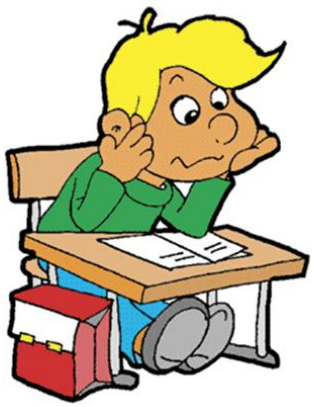
Chapter One

Introduction



An extreme environment is defined relative to human tolerance. These environments include conditions such as high temperature, pH, pressure, salt concentration; high levels of radiations; harmful heavy metals/toxic compounds; low temperature, pH, nutrient concentration and water availability. With reference to the prevailing conditions various extreme environments are known in nature such as hot springs, alkaline lakes, hypersaline lakes, deserts, ocean beds, acidic springs etc. Interestingly, a wide variety of microbes survive and flourish in such harsh habitats. The hypersaline environment refers to habitats with high salt contents and includes salt lakes, salt deserts, soda brines, salt marshes, marine ponds etc. These may be found in inland areas or along the coast. Besides the above mentioned natural hypersaline environments there are also man-made hypersaline environments, like solar salt pans, which are mostly found along the coastal regions and are known to commercially produce natural solar salt.

Fig. 1: Widely studied salt pans of the world

Solar salt pans consist of a series of inter-linked enclosures with a discontinuously increasing salinity gradient, due to the evaporation of seawater. Salinities here vary from 4 to 350 psu. Besides the obvious high salt content in the salt pans, the other stress conditions also include high temperature and heavy solar radiations. Solar salt pans have been studied with respect to the diversity of the inhabiting microorganisms (Ventosa et al., 1998; Bidle et al., 2005; Baati et al., 2008; Baati et al., 2010a; Baati et al., 2010b; Baati et al., 2011). The salinity in these ecological niches is condusive to halotolerant and halophilic microorganisms. Such an environment imposes a selective pressure on the residing organisms. Inspite of such harsh conditions wide spectra of microbes including heterotrophic and methanogenic archaea; photosynthetic lithotrophs and heterotrophic bacteria, as also photosynthetic and heterotrophic eukaryotes are found to exist in these pans. The insight into the mechanisms involved in adaptation of salt pan microbes to extreme conditions such as varying degrees of salt concentrations, high temperatures, solar radiations have also contributed to major research findings. The halophilic and halotolerant microorganisms from salt pans exhibit diversity in terms of mechanisms used to withstand the large osmotic pressure exerted by their highly saline surrounding medium. These stressful conditions in such environments impose selective pressures on the residing flora & fauna and thus organisms may be compelled to produce metabolites to make its surrounding conducive for its survival (Margesin and Schinner, 2001; Oren, 2002; Oren, 2008).

Marine microorganisms have already proven their capacity to yield novel molecules with bioactivity (Fiedler et al., 2005; Lam, 2006). Many free-living and sediment inhabiting marine bacteria have been shown to produce secondary metabolites which exhibit antibacterial properties (Burgess et al., 1991). The first antibiotic from a marine

bacterium was identified and characterized by Burkholder et al. (1966). Being a marine environment, salt pans may contribute microbes having the potential to produce bioactive compounds (Meseguer and Rodgriguez, 1985; Satyanarayana et al., 2005; Mizushina et al., 2009). Ventosa and Nieto (1995) reviewed various biotechnological applications and potentialities of halophilic microbes. According to Oren (2002b) some applications of halophilic microorganisms are centuries old, and have existed since long before microbiological aspects of the processes were understood. Though most of the research on solar salt pans so far has focused on isolating and identifying novel microorganisms, application based research is already fast catching up.

In India, major saltpans are located along the coastal regions of Gujarat, Tamil Nadu, Maharashtra, Karnataka, Orissa, West Bengal and Goa. Major findings have been reported from marine salterns near Bhavnagar, Gujarat (Dave and Desai, 2006); the salt pans located in Vedaranyam, Thondi and Tuticorin, Tamil Nadu in the Palk Strait region of Bay of Bengal (Vijayakumar et al., 2007) and solar salterns in Kelambakkam, Marakanam and Vedaranyam, Tamil Nadu (Manikandan and Kannan, 2009) and mainly have focused on the microbial diversity. Some of the novel species have been reported from salt pans (Kumar et al., 2007; Srinivas et al., 2007; Venkata Ramana et al., 2010; Chanan et al., 2011). Other research on Indian salterns are on antibacterial potential of salt pan actinomycete (Dhanasekaran et al., 2005); organic solvent tolerance of Halobacterium sp. SP1 (1) and its extracellular protease (Akolkar et al., 2008) and production of poly-3-hydroxyalkanoic acids (Biswas et al., 2009). Bioprospecting of microbes from salt pans in India is comparatively still unexplored. Few reports are available from Indian salterns (Vidyasagar et al., 2007; Jayappriyan et al., 2010). Deepika and Kannabiran (2010) have isolated actinomycetes from marine soil samples collected at

the Ennore saltpan and screened them for biosurfactant and heavy metal resistance activity. Few attempts have been made to explore microbes from salt pans as potential producers of antimicrobial agents (Dhanasekaran et al., 2005; Dhanasekaran et al., 2008-09; Suthindhiran and Kannabiran, 2009).

The state of Goa encompasses an area of 3702 km^2 with a coastline of about 101 km. Marine salt pans in Goa are thalassohaline, multipond systems with sequential salinity gradients up to a maximum of 500 during the peak salt-manufacturing season i.e. between November to May. There are about 16 salt pans in Goa which are functional in the production of natural salt. The solar salts, thus produced have been exploited for its special virtues since ancient times and various applications including traditional household uses such as pickling, preservation of fish, plant fertilizer, using the medicinal attributes of natural salt etc. are still prevalent in India. However, studies from Goan saltpans are very limited. Raghavan and Furtado (2005) have studied the effect of aniline on carotenoid pigments of haloarchaeal cultures. Kamat and Kerkar (2011) have highlighted the pharmaceutical potentials of bacteria from saltpans of Goa.

Numerous new antibiotics in the market have shown high efficacy in the treatment of new diseases. However, problems such as multiple drug resistance arising due to irrational drug use, coupled with the unavailability of novel molecules and increased rates of re-isolation of known molecules emphasizes a need to explore novel and more efficient molecules, utilizing the unexploited untapped salt pans resources. If adaptation of microbes to the hypersaline environments is a result of the metabolic changes, then the production of new bioactive compounds as metabolites may be expected (Imada et al., 2007).

Our approach in the present study has been to investigate salt pans of Goa for potential microbes (bacteria and actinomycetes) and to find a candidate culture capable of producing antibacterial compound. The research was carried out with the following objectives:

1. Isolation of bacteria/actinobacteria from water and sediment samples from salt pans.
2. Screening isolates for production of bioactive molecules.
3. Optimization of production and growth of a candidate bacterium.
4. Taxonomy of the candidate bacterium.
5. Purification and characterization of the bioactive compound.

Chapter Two

Review of Literature

2.1. Bioactive compounds from marine sources:

The marine environment is an interesting source for research and development, yet the potential of the domain as the basis for new biotechnologies remains largely unexplored. Vast majority of marine organisms are yet to be identified. Even for many of the known organisms there is insufficient knowledge to permit their intelligent management and application. Marine organisms are of great value for two main reasons. First, they constitute a major share of the earth's biological sources. Second, marine organisms often possess unique metabolic pathways, reproductive systems and sensory and defense mechanisms because they have adapted to extreme environmental conditions ranging from cold polar seas (-2°C) to hot hydrothermal fluids (300°C) at the sea floor ("Hydrothermal Vents") and also to very high hydrostatic pressures (500-1000 atm). Another unique feature of these organisms, including microbes, is their ability to tolerate high salt concentrations (even up to 10-35% NaCl in salt pans). Interest in marine biotechnology has been growing in recent years and developments in molecular biology promise fundamentally new approaches and opportunities for identifying, using and managing biological resources from the seas and other bodies of water. Initial work undertaken, in the field of bioactive compounds from marine sources, in 1960s, 1970s and probably the early 1980s was driven by an interest in the chemistry of new compounds rather than in their biological activities. Later, from research funding perspective it became more and more necessary to link the chemistry of the new compounds with their biological properties (Blunden, 2001).

Numerous bioactive metabolites with antimicrobial, cytotoxic, antiviral, anti-inflammatory, anticoagulant and antiparasitic properties have been reported from marine invertebrates, seaweeds, fish, sea snakes, marine mammals, microbes and also from

microbes associated with marine flora and fauna (Okami, 1986; Jha and Zi-rong, 2004; Zhang et al., 2005; Wiese et al., 2009; Debbab et al., 2010). These compounds have been reported during 2003-04 from 29 countries all over the world including India. Several researchers have described and reviewed the biomedical potential of marine natural products (Burkholder and Sharma, 1969, Jensen and Fenical, 1994; Proksch et al.1993; Faulkner, 2000; Donia and Hamann, 2003; Fiedler et al. 2005;). Since majority of the marine invertebrates have no means of physical defense against predators, it is logical to theorize that symbiotic marine microorganisms are the original producers of these bioactive compounds (Zhang et al., 2005). An excellent example of this are sponges which are host organisms for various symbiotic microorganisms such as archaea, heterogeneous bacteria, cyanobacteria and microalgae with antimicrobial, cytotoxins, antifouling agents, antibiotics, and anti-inflammatory and antiviral compounds (Hentschel et al., 2001). Lee et al. (2001), in their review, have focused on sponge-symbiotic microorganisms as a source of various natural products. It is expected that, if a symbiotic microorganism from which some natural products are derived can be cultured, then the microorganism could be used in a mass production of the bioactive compounds. They also highlighted that microorganisms would be the targets for marine drug discovery in the 21st century. Among the microbes, actinomycetes and bacteria have been studied extensively.

2.1.1. Bioactive compounds from marine actinomycetes:

Actinomycetes are well known sources of structurally diverse natural products with broad ranges of biological activities such as antibiotic, anticancer, immunosuppressant and veterinary agents (Moore et al., 2005). Recently researchers have shown increasing interest in studying marine actinomycetes (Ward and Bora, 2006; Das et al., 2006). Many

known as well as novel compounds started being reported at a high frequency thus indicating that marine actinomycetes have the ability to produce novel metabolites which in turn have the potential for development into useful commercial products (Imada, 2005). Although the exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past few years. Okami et al., in 1976, reported a new antibiotic aplasmomycin from *Streptomyces griseus* isolated from shallow sea mud at Koajiro inlet of Sagami Bay in Japan. Lam (2006) reviewed novel secondary metabolites isolated from marine actinomycetes from 2003 to 2005. These include compounds with diverse biological activities such as anticancer, antibacterial, antifungal, anti-inflammatory etc. Marine Actinomycetes have been also shown to be a new source of compounds against the human malarial parasite (Prudhomme et al., 2008). Magarvey et al. (2004) isolated 102 marine-derived actinomycetes from subtidal marine sediments collected from the Bismarck Sea and the Solomon Sea off the coast of Papua New Guinea. Biological activity testing of fermentation products from these newly isolated marine-derived actinomycetes revealed that more than a few had activities against multidrug-resistant gram positive pathogens, malignant cells, and vaccinia virus replication. *Streptomyces* is the largest antibiotic-producing genus in the microbial world discovered so far. Previously known antibiotics isolated from terrestrial *Streptomyces* strain (such as enterocin) and terrestrial *Micromonospora* strain (such as ikarugamycin) have been also reported from marine *Streptomyces* and *Micromonospora* strains respectively So also, novel antibiotics (such as abyssomicins) have been reported from marine *Verrucosispora* strains (Fiedler et al., 2005). Such findings only confirm the fact that marine actinomycetes represent a new and an important source of bioactive secondary metabolites.

Research on marine actinomycetes in India has been restricted to Maharashtra, Kerala, Tamil Nadu and Andhra Pradesh. Whereas reports covering Gujarat, Goa, Karnataka, Orissa, West Bengal and Andaman & Nicobar group of islands are scarce (Sivakumar et al., 2007). As clearly evident, in India, marine actinobacterial research has been restricted to the coastal ecosystem while the deep sea oceanic floors still remain untapped. Bioprospecting of actinobacteria for bioactive molecules has not been investigated substantially in extremophilic environments in India. Some findings with regards to various compounds such as carotenoids, nanoparticles, bioemulsifiers from actinomycetes have been reported from Indian habitats (Sastry et al., 2003; Kokare et al., 2007; Dharmaraj et al., 2009). Few reports are also available on biosurfactants from terrestrial and marine actinomycetes but their commercial applications and production conditions are yet to be looked into (Ballav et al., 2012). Studies on various enzymes such as proteases, gelatinases, amylases, lecithinases, cellulases and ureases from the actinomycetes strains isolated from the coastal sediments of Konkan coast of Maharashtra have been reported by Gulve et al. (2011). Raja et al. (2010) have described marine actinobacteria producing amylase inhibitors against both prokaryotic and eukaryotic amylases isolated from mangrove rhizosphere of *Rhizophora mucornata* in Vellar estuary, East coast, India. Production of bioemulsifiers from actinomycetes of Alibag, Janjira and Goan coastal marine sediments has been studied by Kokare et al. (2007).

2.1.2. Bioactive compounds from marine bacteria:

Okami (1993) reviewed the potential of marine bacteria for production of diverse new bioactive metabolites and enzymes of both academic and practical interests. Debbab et al., 2010 have reviewed the bioactive compounds from marine bacteria and fungi. Some

of the marine natural products have already undergone clinical trials (Newman and Cragg, 2004). Promising anticancer clinical candidates such as salinosporamide A only indicate the incredible riches of drug leads hidden just beneath the ocean surface (Williams, 2008). Among bacteria, *Bacillus* strains are capable of surviving under adverse conditions and hence are ubiquitous in nature. Few reports are published on the study of the *Bacillus* species isolated from the marine environment. Gugliandolo et al. (2003) isolated a novel thermophilic, halophilic marine *Bacillus aeolius* from Eolian Islands (Italy) utilizing hydrocarbons; having surfactant activity and producing an exopolysaccharide. There are reports on a few bacilli of marine origin which are capable of producing unusual metabolites differing from those isolated from terrestrial bacteria (Jensen and Fenical, 1994) which include an antibiotic, 3-amino-3-deoxy-D-glucose, a new glucanase, and cyclic acylpeptides (Ivanova et al., 1999). Numerous reports are available on antimicrobial potential of marine *Bacillus* spp. Antipathogenic and antibiofilm properties (Musthafa et al., 2011), antibacterial activity (Mondol et al. 2011), anti-biofilm (Nithya et al., 2010) and anti-fungal lipopeptides (Chen et al. 2010) have been reported from various marine *Bacillu*s spp. Mukherjee et al. (2009) reported extracellular synthesis and purification of antimicrobial biosurfactants from marine *Bacillus circulans.* Some important products isolated from marine microbes are given in Table 2.1.

S.No.	Product	Source	Characteristics	Reference
1	Antibiotic against Gram-positive bacteria	Salt-tolerant and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1	Isolated from alkaline soil of Saurashtra University Campus, Rajkot, India	Vasavada et al., 2006
2	Aplasmomycin (antibiotic)	*Streptomyces griseus*	Isolated from shallow sea mud at Koajiro inlet of Sagami Bay in Japan	Okami et al., 1976
3	Istamycins	Marine actinomycete *Streptomyces tenjimariensis* SS-939	*In vitro* activity against both Gram-negative and Gram-positive	Okami et al., 1979

			bacteria, including those with known resistance to the aminoglycoside antibiotics	
4	Antibiotic	Heterotrophic marine bacteria	Isolated from seawater, sediments, phytoplankton, and zooplankton of Suruga, Sagami, and Tokyo Bays and from soft corals and sponges collected from the Taiwan coast	Nair and Simidu, 1987
5	Oncorhyncolide	Bacterium	Isolated from seawater	Needham et al., 1991
6	Antibiotic	Marine photosynthetic bacterium *Chromatium purpuratum* NKPB 031704	First report of antibiotic	Burgess et al., 1991
7	Stylostatin 1 (cell growth inhibitory cycloheptapeptide)	Marine sponge *Stylotella aurantium*	From Papua New Guinea	Pettit et al., 1992
8	Palmitoleic acid	Marine cyanobacteria	Potential for preventing cerebro- and cardiovascular disease, and for enhancing the function of vascular smooth muscle cells	Matsunaga et al., 1995
9	Lychenisin A	Thermo- and halotolerant *Bacillus licheniformis* BAS50	Biosurfactant	Yakimov et al., 1995
10	Novel 190-kDa protein with antibacterial activity	Biofilm forming marine bacterium D2	Isolated from the surface of the tunicate *Ciona intestinalis*	James et al., 1996
11	New anticancer antibiotics Pelagiomicins	New marine bacterium *Pelagiobacter variabilis*	Isolated from a macro alga *Pocockiella variegata* collected at Palau	Imamura et al., 1997
12	PM-94128 a new isocoumarin antitumor agent	Marine *Bacillus* sp. PhM-PHD-090	Isolated from a marine sediment	Canedo et al., 1997
12	Thermo-stablealkaline serine proteases	Halotolerant *B. licheniformis*	Enhanced production in the presence of sea water	Manachini and Fortina, 1998
13	Salinosporamides with cytotoxic activity	*Salinospora tropica*	The Bahamas	Jensen, 2005
14	Marinomycins with cytotoxic	Genus *Marinophilus*	-	Jensen, 2005

	activity			
15	Enzyme inhibitors	Actinomycetes from various samples collected from marine environment	Use in medicine and agriculture	Imada, 2005
16	Antibiotic against Gram-positive bacteria	Actinomycetes from sediments from neritic sea water	Production only in presence of seawater	Imada, 2005
17	Tetrodotoxin	Actinomycetes collected from marine environment	First report of actinomycetes from marine environment that produce tetrodotoxin	Imada, 2005
18	Endo-1, 4-β xylanase	Haloalkaliphilic *Nesterenkonia* sp.	Sua pan evaporator ponds in Botswana	Govender et al., 2009
19	Enzyme	Halotolerant, thermophilic *B. licheniformis* S-86	Organic-solvent-tolerant esterase	Torres et al., 2009
20	Cytotoxic and antimicrobial compound	*Saccharopolyspora salina* VITSDK4	Bay of Bengal, India	Suthindhiran and Kannabiran, 2009
21	Antimicrobial compounds	Sponge-associated *Bacillus licheniformis* SAB1	Active against Gram positive, Gram negative bacteria and fungus *Aspergillus fumigatus*	Devi et al., 2010
22	Exo-polysaccharide	Seaweed associated (endophytic) *B. licheniformis*	Various biotechnological and industrial applications	Singh et al., 2011
23	Antibiotic against selective human pathogenic microorganisms	*Streptomyces rochei* (MTCC 10109)	Isolated from sea water sample of Visakhapatnam coast of Bay of Bengal, India	Reddy et al., 2011

Table 2.1: Products from marine microbes

The potential of marine bacteria and actinomycetes as an exciting resource for the discovery of new classes of therapeutics is already apparent. Bioactive compounds reported from the marine environment are depicted in the Fig. 2.1. It is clearly apparent that least numbers of bioactive products have been isolated from marine salterns as compared to other marine ecosystems and hence salt pans present scope for research in the direction of finding bioactive compounds.

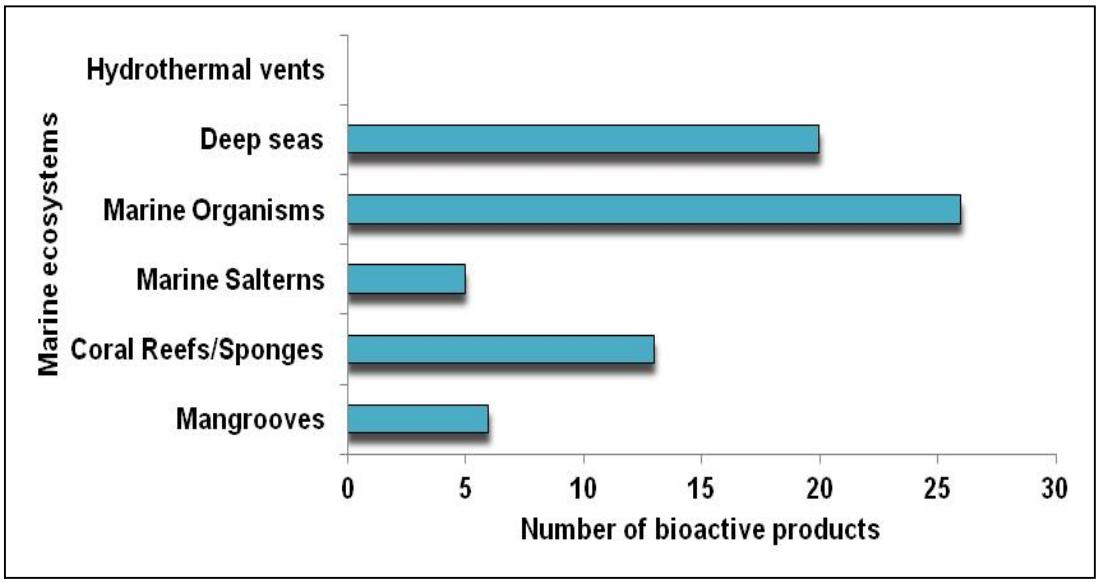


Fig. 2.1: Bioactive compounds reported from the marine ecosystems (Kerkar, 2007)

2.2. Solar salt pans:

Solar salt pans are hypersaline ecosystems and thus extreme environments. The salinity in these ecological niches is conducive to halotolerant and halophillic microorganisms. Solar salt pans have been studied all over the world with respect to the diversity of the inhabiting microbes (Ventosa et al., 1998; Litchfield and Gillevet, 2002; Bidle et al., 2005; Yeon et al., 2005; Oren, 2008; Baati et al., 2010a and b). The most widely studied salt pans from all over the world are presented in the introduction section. The research on salt pans published during 1970-2012 has been depicted in Fig.2.2. As seen from the figure there were maximum publications during 2001 to 2010. Many new species have been identified during 2001-2010 and the overall research on salt pans had got a major boost during this period. The research applications are still in infancy, considering the wealth of the novel species existing in salt pans, as most of these studies are based on culture methods.

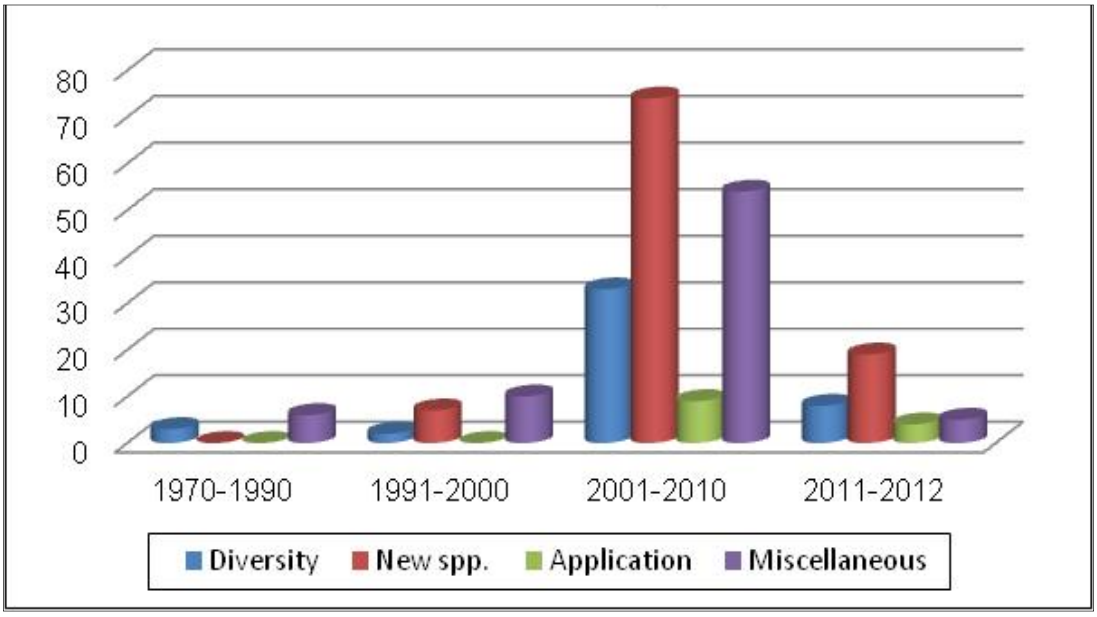


Fig. 2.2: Research published on salt pans during 1970 to 2012 based on data from http://www.ncbi.nlm.nih.gov/pubmed/

2.2.1. Diversity of solar salt pans:

Litchfield et al. (2000) have described the NaCl-dominated salt pans to be inhabited by a rich variety of microorganisms, and each pond with a characteristic flora adapted to the prevailing salt concentration, ranging from seawater to NaCl saturation. They also emphasized that though reports on the biology of solar saltern systems in various parts of the world suggest a high degree of similarity in the microbiology, some differences may be prevalent due to the changes in incident radiation, temperature, nutrient availability, residence time in the ponds etc. A workshop on microbes and biogeochemistry of halophilic microorganisms was held in June 1997, Jerusalem, Israel by A. Oren, M Mevarech and A Kaplan (1999) which gives a comprehensive picture of the state of research on halophilic archaea, bacterium and eukarya and the place of these intriguing microorganisms in nature. The predominant genera in some of the salt pans of the world are tabulated in Table 2.2. Studies on the diversity, source/locations and references of the

microorganisms and novel species from salt pans are tabulated in Table 2.4 and 2.5 and are given at the end of the review.

S. No.	Name of Predominant Genera	Location of saltern	Reference
1	Halotolerant *Vibrio, Flavobacterium, Alcaligenes*, *Alteromonas* and *Chromobacterium*	Alicante (Spain)	Ventosa et al., 1982
2	*Salinivibrio, Acinetobacter, Flavobacterium* and the *Pseudomonas–Alteromonas–Alcaligenes* group	Spanish saltern ponds	Rodriguez-Valera et al., 1985
3	*Vibrionaceae, Pseudoalteromonadaceae, Halomonadaceae, Alteromonadaceae, Idiomarinaceae, Bacillus*, *Halobacillus*, *Jeotgalibacillus* and *Pontibacillus*	Taean-Gun (Korea)	Yeon et al., 2005
4	*Pseudoalteromonas, Flavobacterium*, *Chromohalobacter*, *Halomonas*, *Salegentibacter*, *Halobacillus*, *Salinicoccus*, *Staphylococcus* and *Tetragenococcus*	Alexandria (Egypt)	Ghozlan et al., 2006
5	*Halorubrum*, *Haloarcula*, *Halomonas*, *Halovibrio*, *Salicola* and *Salinibacter* genera and a new archaeal genus	Baja California (Mexico)	Sabet et al., 2009
6	*Halomonadaceae*, the *Vibrionaceae*, the *Alteromonadaceae*, the *Idiomarinaceae*, the *Alcanivoracaceae* and *Firmicutes*	Sfax (Tunisia)	Houda Baati et al., 2010
7	*Dunaliella*	Vedaranyam, Tamil Nadu (India)	Jayappriyan et al., 2010

Table 2.2 Distribution of predominant genera in salt pans around the world

2.2.2. Bioactive potential of salt pans:

If adaptation of microbes to these hyper saline environments is a result of the metabolic changes, then the production of new bioactive compounds as metabolites may be expected (Imada et al., 2007). The ecological significance of these compounds, if any, remains a mystery (Williams and Vickers, 1986). It is intriguing to investigate why microbes produce compounds with biological activities. Several reasons can be responsible for this. Microbes adapt to various environments by producing molecules which contribute in making their environment conducive to their survival. Novel compounds may also be produced to help in competition in a given niche. In case of

symbiotic microbes living with marine invertebrates, the microbes produce and secrete bioactive molecules which act as a chemical defense in protecting the host as well as themselves from predators and in turn the microbes get nutrition and shelter from the host.

2.2.3. Bioactive compounds and applications of microbes from salt pans:

Halocins are bacteriocins produced by halophilic Archaea and a type of archaeocin. Some of the halocins characterized and studied include halocin H4 from *Haloferax mediterranei* R4 (Messeguer & Rodriguez-Valera, 1985); halocin H6 from *Haloferax gibbonsii* (Torreblanca et al., 1989) and halocin S8 from an uncharacterized extremely halophilic rod –strain S8 (Shand et al., 1999). Ventosa and Nieto in 1995 have reviewed present or potential applications of halophiles such as the production of polymers (polyhydroxyalcanoates and polysaccharides), enzymes and compatible solutes as well as use of these microbes in enhanced oil recovery, cancer detection and biodegradation of toxic compounds. Antimicrobial and cytotoxic activities have also been demonstrated by bacteria from salterns in China (Chen et al., 2010). Other products from salt pans such as bacteriorhodopsin; compatible solutes; enzymes (hydrolases & isomerases); and biopolymers (biosurfactants, exopolysaccharides, liposomes, poly (γ-D-glutamic acid), lectins & bioplastics) have also been reported (Margesin & Schinner, 2001; Oren, 2002). Govender et al. (2009) isolated and screened eighty seven bacterial isolates, from Sua pan evaporator ponds in Botswana, for xylanase, mannanase and cellulase activity. Apart from these studies, some researchers have delved on topics such as ants found in salt pans, volatile compounds of salts, planktonic ciliate diversity etc. With this backdrop, salt pans have also been explored for various processes, and applications. Some examples of applications and other research have been listed in Table 2.3.

S. No.	Applications of microbes and other research	Source	Reference
1	Biofilm-associated indole acetic acid producing bacteria	Salterns at Nerul and Curca, Goa, India	Kerkar et al.,2012
2	Survival of extremely and moderately halophilic isolates after UV-B or oxidative stress	Tunisian solar salterns	Trigui et al., 2011
3	Proteomic insight into phenolic adaptation of a moderately halophilic *Halomonas* sp. strain AAD12	Çamaltı Saltern area, located in the Aegean Region of Turkey	Ceylan et al., 2011
4	Volatile compounds from marine salt	Salt pans of Aveiro, Portugal	Silva et al., 2010
5	The metavirome of a hypersaline environment (characterization of the viral assemblage through a metagenomic approach)	Crystallizer pond (CR30) from a multi-pond solar saltern in Santa Pola (SE Spain)	Santos et al., 2010
6	Desert ants, *Cataglyphis fortis*, use olfactory landmarks to pinpoint the nest	Salt-pans of Tunisia	Steck et al., 2009
7	Role of biofilms in the sedimentology of actively forming gypsum deposits	Guerrero Negro, Mexico	Vogel et al., 2009
8	Metagenomic islands of hyperhalophiles: the case of *Salinibacter ruber*	-	Pasić et al., 2009
9	Community structure and seasonal dynamics of planktonic ciliates along salinity gradients	Solar saltern of the Yellow Sea	Lei et al., 2009
10	[FeFe] hydrogenase genetic diversity provides insight into molecular adaptation in a saline microbial mat community	Microbial mats from saltern evaporative ponds in Guerrero Negro, Mexico	Boyd et al., 2009
11	Organic solvent tolerance of *Halobacterium* sp. SP1(1) and its extracellular protease	Kandla salt pans, India	Akolkar et al., 2008
12	Sensitivity of *Haloquadratum* and *Salinibacter* to antibiotics and other inhibitors: implications for the assessment of the contribution of Archaea and Bacteria to heterotrophic activities	Salterns of the Israel Salt Company in Eilat	Elevi and Oren, 2008
13	Rearing of *Fabrea salina* Henneguy (Ciliophora, Heterotrichida) with three unicellular feeds	Multi-pond salterns (Sfax, Tunisia)	Guermazi et al., 2008
14	Dihydroxyacetone metabolism in *Salinibacter ruber* and in *Haloquadratum walsbyi*	Saltern crystallizer ponds in Eilat, Israel	Elevi and Oren, 2008
15	The Raman spectroscopic biosignatures of halotrophic cyanobacterial extremophiles	Sabkha evaporitic salt pans	Edwards et al., 2006
16	Red and transparent brine shrimps (*Artemia parthenogenetica*): a comparative study of their cestode infections	Odiel salt pans in Spain	Sánchez et al., 2006

17	Structure of euhalothece-362, a novel red-shifted mycosporine-like amino acid, from a halophilic cyanobacterium (*Euhalothece* sp.)	Hypersaline saltern pond in Eilat, Israel	Volkmann et al., 2006
18	Buoyancy studies in natural communities of square gas-vacuolate archaea	Saltern crystallizer ponds of Eilat, Israel	Oren et al., 2006
19	Biogeochemistry of an iron-rich hypersaline microbial mat (Camargue, France)	Salin-de-Giraud, France	Wieland et al., 2005
20	Salinity responses of benthic microbial communities	Solar saltern (Eilat, Israel)	Sørensen et al., 2004
21	Osmotic shock stimulates de novo synthesis of two cardiolipins in an extreme halophilic archaeon	Saltern ponds of Margherita di Savoia in southern Italy	Lopalco et al., 2004
22	Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence in situ hybridization	Brine samples of a solar saltern crystallizer pond from Mallorca, Spain	Rosselló-Mora et al., 2003
23	Mercury and lead tolerance in hypersaline sulfate-reducing bacteria	Salt pans of Goa	Harithsa et al., 2002
24	Recommended design for more accurate duplication of natural conditions in salt marsh creation	Texas, USA	Darnell and Smith, 2002
25	First report of mycosporine-2-glycine, the major mycosporine-like amino acid in a unicellular Cyanobacterium (*Euhalothece* sp.)	Gypsum crust on the bottom of a hypersaline saltern pond in Eilat, Israel	Kedar et al., 2002
26	Taxonomic study of extreme halophilic archaea	Salar de Atacama, North Chile	Lizama et al., 2001
27	Characterization of novel methyl-branched chain fatty acids from a halophilic *Bacillus* species	Salt pans of Burgas in Bulgaria	Carballeira et al., 2001
28	The contribution of halophilic bacteria to the red coloration of saltern crystallizer ponds	Solar saltern crystallizer ponds in Santa Pola near Alicante and Balearic island of Mallorca, Spain	Oren and Rodríguez-Valera, 2001
29	Pulsed-field gel electrophoresis analysis of virus assemblages present in a hypersaline environment	Multi-pond solar saltern in Alicante, Spain	Díez et al., 2000
30	Comparisons of the polar lipid and pigment profiles	Solar salterns in Newark, California, USA, and Eilat, Israel	Litchfield et al., 2000
31	Hypersaline waters in salterns - natural ecological niches for halophilic black yeasts	Marine salterns Sečovlje in Slovenia, along the Adriatic coast	Gunde-Cimermana et al., 2000
32	Novel archaeal phylotypes	Alkaline saltern at Lake Magadi, Kenya, East Africa	Grant et al., 1999
33	Growth potential of halophilic bacteria isolated from solar salt environments:	Four different solar salt environments	Javor, 1984

34	Biosorption of halobacterial strains against heavy metals Ni^{2+}, Al^{3+} and Hg^{2+} metal ions can be used as a potential source for the bioremediation of metals	India	Williams et al., 2012
35	*Egretta garzetta* as a bioindicator of trace element pollution	Thyna salt pans (in the gulf of Gabès)	Abdennadher et al., 2011
36	Three new compounds from *Aspergillus terreus* PT06-2	Sediment of the Putian sea saltern, Fujian, China.	Wang et al., 2011
37	The extremely halophilic bacterium *Salicola marasensis* IC10 accumulates the compatible solute betaine	Solar saltern on Isla Cristina (southern Spain)	Moreno et al., 2010
38	Production of poly-3-hydroxyalkanoic acids by a moderately halophilic bacterium, *Halomonas marina* HMA 103	Solar saltern of Orissa, India.	Biswas et al., 2009
39	Novel 3'-phosphoadenosine-5'-phosphatases reveal insight into molecular determinants of salt tolerance of black yeasts	Extremely halotolerant *Hortaea Werneckii*	Vaupotic et al., 2007
40	Alkaline protease production by the marine yeast *Aureobasidium pullulans*	Sediment of saltern in Qingdao, China	Chi et al., 2007
41	Carotenoid pigments of haloarchaeal cultures exposed to aniline	Estuarine salt pans of Goa, India	Raghavan and Furtado, 2005
42	Diversity of haloarchaeal bacteriorhodopsins	Different hypersaline waters from sea salt manufacturing facility near Alicante, Spain	Papke et al., 2003

Table 2.3: Applications of microbes and other research in salt pans

2.3 National scenario:

In India, major salt pans are located in Okha, Dholera, Bhavnagar (Gujarat); Parangipettai, Cuddalore (Tamil Nadu); Ribandar, Batim, Curca, Arpora, Nerul (Goa). The studies on these salt pans mainly focus on the diversity of the microbes prevailing (Dave & Desai, 2006; Vijayakumar et al., 2007; Manikandan and Kannan, 2009). Deshmukh (2004) isolated ten species classified in six genera of keratinophilic fungi and related dermatophytes from the soil samples from twenty salt pans and their vicinity around Mumbai and Thane. Asha et al. (2005) isolated extremely halophilic archaea from saltern crystallizer ponds located in peninsular coast of India. Nowlan et al. (2006) reported *Bacillus okhensis* a novel species from salt pans of Okha, Gujarat. Ashok Kumar

et al. (2011) collected and identified halophilic algae from Kelambakkam salt pan, in the outskirt of Chennai.

2.4 Need for newer antibacterial agents:

Microbial infections can generally be treated with several antibiotics and hence resistance to an antibiotic does not translate directly into therapeutic failure. However, it increasingly limits therapeutic options. In case of some significant pathogens, in which serious resistance problems exist, few effective antibiotics now remain. Genus *Staphylococcus* is one of the most significant representatives of such pathogens. During the last decade, pharma companies have developed novel antimicrobial agents to combat multi drug resistant (MDR) gram-positive bacteria namely, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, resulting in several new compounds with novel mechanisms of action, for example, linezolid and daptomycin (Giske et al., 2008). However, infections caused by MDR gram-negative bacilli such as extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella* spp., MDR *Pseudomonas aeruginosa*, and carbapenem-resistant *Acinetobacter* spp. have become a growing problem. So far no antibiotic from a new class has been developed specifically for MDR gram-negative bacilli though the glycylcycline tigecycline has in vitro activity against many MDR gram-negative bacilli, the drug was not developed specifically for the purpose of treating infections caused by such bacteria (Giske et al., 2008).

Despite the vast number of novel compounds isolated from marine organisms and the biological activities attributed to many of them, very few have either been actually marketed as pharmaceutical products or are being developed. There are probably several reasons why such few compounds isolated from marine sources have been developed as

drugs. Most of the compounds having promising biological properties have complicated chemical structures implying that the synthesis of these would be hard and expensive. A complex compound extracted from a marine organism, probably in very small quantity and often from a relatively inaccessible area, has a remote chance of consideration for development by the pharmaceutical industry. Hence, though the marine organisms are valuable sources of new biologically active chemical structures, unless either the compounds or a derivative of them can be readily synthesized, they are of little commercial interest to the pharmaceutical industry (Blunden, 2001). Genomics, combinatorial chemistry and target-based screening programmes have received a lot of attention in recent years as the methods of choice in the search for new antibiotics (Hughes, 2003). Optimization for screening methods used for the identification of antimicrobials from natural sources is of great significance (Valgas et al., 2007). The surfacing of new infectious diseases, the reappearance of several infections that seemed to have been controlled and the increase in bacterial resistance have created the necessity for studies directed towards the development of new antimicrobials. Hence problems such as multiple drug resistance arising due to irrational drug use coupled with the unavailability of novel molecules and increased rates of re-isolation of known molecules emphasizes a need to explore novel and more efficient molecules, utilizing the unexploited untapped salt pans resources It has been observed that during the last decades there has been a rise in the rates of re-isolation of known metabolites and a reduction in the hit-rate of novel compounds from marine macroorganisms. This has compelled the natural product chemists to concentrate on drug sources, such as marine microbes, which are vast untapped reservoirs of metabolic diversity. Watve et al. (2001) suggested that if the screening efforts are maintained constant, the rate of discovery of new compounds from this genus will not decline for several decades to come. Williams (2008) suggests that if

properly developed, marine bacteria could provide the drugs needed to sustain us for the next 100 years in our battle against drug-resistant infectious diseases. This study is an attempt of exploring actinobacteria and bacteria from the salts pans of Goa for bio-prospecting of antibacterial compounds and characterizes the compound as unless either the compounds or a derivative of them can be readily synthesized, they are of little commercial interest to the pharmaceutical industry.

S. No.	Diversity	Source/Location	Reference
1	Spatial and seasonal prokaryotic community dynamics	Sfax solar saltern in Tunisia	Boujelben et al., 2012
2	Non carotenogenic strains of *Dunaliella bardawil* and *Dunaliella parva*	Salt pans of Tamil Nadu, India	Jayappriyan et al., 2011
3	New abundant microbial groups in aquatic hypersaline environments	Ponds of the Santa Pola salterns near Alicante (Spain) from ponds of Bras del Port salterns, located near Alicante, Spain (38° 12' N, 0° 36' W)	Ghai et al., 2011
4	Molecular community analysis of magnesium-rich bittern brine	Tunisian solar saltern	Baati et al., 2011
5	Isolation and characterization of a Phosphate-Solubilizing halophilic bacterium *Kushneria* sp. YCWA18	Sediment of Daqiao Saltern on the Coast of Yellow Sea of China	Zhu et al., 2011
6	Characterization of heterotrophic prokaryote subgroups by combining flow cytometry cell sorting and phylogenetic analysis	Solar saltern of Sfax in Tunisia	Trigui et al., 2011
7	Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes	Hypersaline sediments of a solar saltern in Mallorca, Spain	Munoz et al., 2011
8	Bacterial communities in the 'petola' microbial mat	Sečovlje salterns (Slovenia)	Tkavc et al., 2011
9	Morphological, toxicological and molecular characterization of a benthic *Nodularia* (first evidence of a non-nodularin-producing *Nodularia* isolate in Atlantic salt pan ecosystems and its potential ecotoxicity against *Artemia* sp.)	Atlantic estuarine salt pans	Lopes et al., 2010
10	Biodiversity of Archaea and floral	Two inland saltern ecosystems in the Alto Vinalopó Valley, Alicante, Spain	Zafrilla et al., 2010
11	Novel prokaryotic diversity in sediments of Tunisian multipond	Solar saltern in Sfax (Tunisia)	Baati et al., 2010b

	solar saltern		
12	Microbial community of salt crystals processed from Mediterranean seawater based on 16S rRNA analysis	Salt crystals retrieved from the bottom of a solar saltern, located in the coastal area of the Mediterranean Sea (Sfax, Tunisia)	Baati et al., 2010a
13	Diversity of *Haloquadratum* and other haloarchaea	Three, geographically distant, Australian saltern crystallizer ponds	Oh et al., 2010
14	Ultrastructure and molecular phylogeny of two heterolobosean amoebae, *Euplaesiobystra hypersalinica* gen. et sp. nov. and *Tulamoeba peronaphora* gen. et sp. nov.	Extremely hypersaline Korean solar saltern	Park et al., 2009
15	Diversity and stratification of archaea in a hypersaline microbial mat	Guerrero Negro, Baja California Sur, Mexico	Robertson et al., 2009
16	Phylogeography and local endemism of the native Mediterranean brine shrimp *Artemia salina*	Mediterranean Basin and South Africa	Muñoz et al., 2008
17	Morphology and molecular phylogeny of *Trimyema koreanum* n. sp., a ciliate	The hypersaline waters of solar saltern in Korea	Cho et al., 2008
18	Prokaryotic community profiles at different operational stages	Greek solar saltern	Tsiamis et al., 2008
19	Identification and phylogenetic analysis of an okenone-containing halophilic purple sulfur bacterium (strain 283-1 is a new isolate of *Marichromatium* genus, named as *Marichromatium* sp. 283-1)	Dongfeng saltern, Qingdao, China	Yang et al., 2008
20	Prokaryotic diversity of a Tunisian multipond solar saltern	Multipond solar saltern of Sfax, Tunisia	Baati et al., 2008
21	Microbial diversity analysis by 16S rRNA-based methods	Former salterns in southern Taiwan	Wang et al., 2007
22	Searching for species in haloarchaea	Spanish saltern and a natural saline lake in Algeria	Papke et al., 2007
23	Ultrastructure and phylogenetic placement within *Heterolobosea* of the previously unclassified, extremely halophilic heterotrophic flagellate *Pleurostomum flabellatum* (Ruinen 1938)	Korean saltern pond	Park et al., 2007
24	Haloarchaeal communities in the crystallizers of two adriatic solar salterns	Adriatic solar salterns in Ston, Croatia and Slovenian Secovlje salterns	Pasić et al., 2007
25	Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats	Sea saltern in Slovenia and a deep-sea salt brine from the Mediterranean	Sorokin et al., 2006
26	Diversity of halophilic archaea	Crystallizers of Adriatic Secovlje salterns	Pasić et al., 2005

27	Diversity of culturable organotrophic bacteria	Solar saltern ponds in Taean-Gun, Chungnam Province, Korea	Yeon et al., 2005
28	Combined use of cultivation-dependent and cultivation-independent methods indicates that members of most haloarchaeal groups are cultivable	Australian saltern crystallizer pond	Burns et al., 2004
29	Isolation of dermatophytes and other keratinophilic fungi	Vicinity of salt pan soils of Mumbai, India	Deshmukh, 2004
30	Characterization of functional bacterial groups in a hypersaline microbial mat community	Mediterranean saltern (Salins-de-Giraud, Camargue, France)	Fourçans et al., 2004
31	Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies	Solar saltern in Spain; an alkaline lake in Nevada, USA; and a small pond from a slag heap of a potassium mine in Germany	Ochsenreiter et al., 2002
32	Microbial diversity and complexity in hypersaline environments: A preliminary assessment	Eilat, Israel saltern; Mono Lake and the lower salinity ponds at the Shark Bay saltern in Western Australia	Litchfield and Gillevet, 2002
33	Diversity and distribution in hypersaline microbial mats of bacteria related to *Chloroflexus* spp.	Saltern in Guerrero Negro, Mexico	Nübel et al., 2001
34	Polyphasic taxonomy of *Nesterenkonia halobia*	Saltern located in Huelva, Spain	Mota et al., 1997
35	Genus *Prochromadora* with a redescription of *P. orleji*	Marine saltern in Qingdao, Shandong, the People's Republic of China	Tarjan et al., 1991
36	Isolation and characterization of moderately halophilic non motile rods	Solar saltern, saline soils and the sea, near Alicante (Spain)	Quesada et al., 1985

Table 2.4: Microbial diversity in salt pans

S. No.	Novel species	Source/Location	Reference
1	*Halomonas smyrnensis* sp. nov., a moderately halophilic, exopolysaccharide producing bacterium	Camalti Saltern Area, wildlife reserve in Sasalı, İzmir province located in Aegean Region of Turkey	Poli et al., 2012
2	*Halomonas beimenensis* sp. nov.	Beimen, an abandoned saltern in Southern Taiwan.	Wang et al., 2012
3	*Halorubellus salinus* gen. nov., sp. nov. and *Halorubellus litoreus* sp. nov., novel halophilic archaea	The Gangxi marine solar saltern near the Weihai city of Shandong Province, China	Cui et al., 2012
4	*Virgibacillus campisalis* sp. nov.	Marine solar saltern in Korea	Lee et al., 2012
5	*Gramella jeungdoensis* sp. nov.	Solar saltern in Korea	Joung et al., 2011
6	*Gracilibacillus bigeumensis* sp. nov., a moderately halophilic bacterium	Solar saltern soil of Bigeum island in south-west Korea	Kim et al., 2011
7	*Salinisphaera orenii* sp. nov.	Solar saltern pond in Gomso Bay, Republic of Korea	Park et al., 2011
8	*Caenispirillum salinarum* sp. nov., a novel bacterium of the family *Rhodospirillaceae*	Solar saltern of Kakinada, Andhra Pradesh, India	Chanan et al., 2011
9	*Halomicrobium zhouii* sp. nov., a halophilic archaeon	Marine solar saltern in Jiangsu, China	Yang and Cui, 2011
10	Characterization of *Halorubrum sfaxense* sp. nov., a new halophilic archaeon	Solar Saltern of Sfax in Tunisia	Trigui et al., 2011
11	*Pontibacter salisaro* sp. nov.	Clay tablet solar saltern in Jeungdo, Republic of Korea (34°59'47″N 126°10'02″E)	Joung et al., 2011
12	*Marinobacter daqiaonensis* sp. nov., a moderate halophile	Sediment of Daqiao saltern (Yellow Sea salt pond) in Qingdao, on the east coast of China	Qu et al., 2011
13	*Halobellus clavatus* gen. nov., sp. nov. and *Halorientalis regularis* gen. nov., sp. nov., two new members of the family *Halobacteriaceae*	Two artificial marine solar salterns (the Tainan and the Taibei) in eastern China	Cui et al., 2011
14	*Halomonas rifensis* sp. nov., an exopolysaccharide-producing, halophilic bacterium	Solar saltern in Chefchaouen, Morocco	Amjres et al., 2011
15	*Echinicola jeungdonensis* sp. nov.	Solar saltern in Jeungdo, Republic of Korea	Kim et al., 2011
16	*Halomonas daqiaonensis* sp. nov., a moderately halophilic, denitrifying bacterium	Sediment of Daqiao saltern, Jimo, Qingdao, on the east coast of China	Qu et al., 2011
17	*Halolamina pelagica* gen. nov., sp. nov., a new member of the family *Halobacteriaceae*	The Taibei marine solar saltern near Lianyungang city, Jiangsu province, China	Cui et al., 2011
18	*Haloplanus aerogenes* sp. nov., an extremely halophilic archaeon	Taibei marine solar saltern Jiangsu province, China	Cui et al., 2011

19	*Halogranum gelatinilyticum* sp. nov. and *Halogranum amylolyticum* sp. nov.	Tainan marine solar saltern near Lianyungang city, Jiangsu province, China	Cui et al., 2011
20	Description of *Ectothiorhodospira salini* sp. nov.	Anoxic sediment of a saltern at Kanyakumari, India	Venkata Ramana et al., 2010
21	*Halorussus rarus* gen. nov., sp. nov., a new member of the family *Halobacteriaceae*	Taibei marine solar saltern in Jiangsu, China	Cui et al., 2010
22	*Halosarcina limi* sp. nov., a halophilic archaeon	Marine solar saltern in eastern China	Cui et al., 2010
23	*Halopelagius inordinatus* gen. nov., sp. nov., a new member of the family *Halobacteriaceae*	Rudong marine solar saltern in Jiangsu, China	Cui et al., 2010
24	*Oceanobacillus locisalsi* sp. nov.	Marine solar saltern in Republic of Korea	Lee et al., 2010
25	*Halogeometricum rufum* sp. nov., a halophilic Archaeon	Marine solar saltern, in Jiangsu, China	Cui et al., 2010
26	*Haloplanus vescus* sp. nov., an extremely halophilic Archaeon	Marine solar saltern in China	Cui et al., 2010
27	*Halogranum rubrum* gen. nov., sp. nov., a halophilic archaeon	Rudong and Haimen solar salterns	Cui et al., 2010
28	*Bacillus halochares* sp. nov., a halophilic bacterium	Solar salterns of Mesolongi, Greece	Pappa et al., 2010
29	*Haladaptatus litoreus* sp. nov., an extremely halophilic Archaeon	Marine solar saltern in Jiangsu, China	Cui et al., 2010
30	*Planococcus salinarum* sp. nov..	Marine solar saltern in Korea	Yoon et al., 2010
31	*Alkalibacillus flavidus* sp. nov.,	Marine solar saltern of the Yellow Sea, Korea	Yoon et al., 2010
32	*Virgibacillus byunsanensis* sp. nov.	Marine solar saltern of the Yellow Sea, Korea	Yoon et al., 2010
33	*Jeotgalibacillus salarius* sp. nov.	Marine saltern located on the coast of the Yellow Sea, Korea	Yoon et al., 2010
34	*Salimicrobium flavidum* sp. nov.	Marine solar saltern of the Yellow Sea,Korea	Yoon et al., 2009
35	*Marinobacter szutsaonensis* sp. nov.	The Szutsao saltern in southern Taiwan	Wang et al., 2009
36	*Salinihabitans flavidu*s gen. nov., sp. nov.	Marine solar saltern in Korea	Yoon et al., 2009
37	*Marinimicrobium locisalis* sp. nov.	Marine solar saltern of the Yellow Sea, Korea	Yoon et al., 2009
38	*Psychroflexus salinarum* sp. nov.	Marine solar saltern of the Yellow Sea, Korea	Yoon et al., 2009
39	*Halomonas ilicicola* sp. nov., a moderately halophilic bacterium	Solar salterns of Santa Pola, located on the Mediterranean coast of Spain	Arenas et al., 2009
40	*Thiohalorhabdus denitrificans* gen. nov., sp. nov., an extremely	Sediments of hypersaline inland lakes in south-eastern Siberia and a	Sorokin et al., 2008

	halophilic, sulfur-oxidizing, deep-lineage gammaproteobacterium	Mediterranean sea solar saltern	
41	*Thiohalospira halophila* gen. nov., sp. nov. and *Thiohalospira alkaliphila* sp. nov., novel obligately chemolithoautotrophic, halophilic, sulfur-oxidizing gammaproteobacteria	Sea saltern of the Adriatic Sea	Sorokin et al., 2008
42	*Halomonas nitroreducens* sp. nov., a novel nitrate- and nitrite-reducing species	Solar saltern in Cahuil, a region next to Pichilemu (Chile)	González-Domenech et al., 2008
43	*Halobacillus seohaensis* sp. nov.	Marine solar saltern in Korea	Yoon et al., 2008
44	*Salegentibacter salinarum* sp. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2008
45	*Virgibacillus chiguensis* sp. nov.,	Chigu saltern in southern Taiwan	Wang et al., 2008
46	*Lentibacillus salinarum* sp. nov.	Marine solar saltern in Korea	Lee et al., 2008
47	*Salegentibacter salarius* sp. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2007
48	*Idiomarina salinarum* sp. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2007
49	Reclassification of *Marinococcus albus* Hao et al. 1985 as *Salimicrobium album* gen. nov., comb. nov. and *Bacillus halophilus* Ventosa et al. 1990 as *Salimicrobium halophilum* comb. nov., and description of *Salimicrobium luteum* sp. nov.	Marine solar saltern in Korea	Yoon et al., 2007
50	*Microbulbifer celer* sp. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2007
51	*Halorubrum litoreum* sp. nov., an extremely halophilic archaeon	Marine solar saltern in Fujian, China	Cui et al., 2007
52	*Halomonas kribbensis* sp. nov., a novel moderately halophilic bacterium	Solar saltern in Korea	Jeon et al., 2007
53	*Halochromatium roseum* sp. nov.	Marine solar saltern at Kakinada, India	Kumar et al., 2007
54	*Marinobacter salicampi* sp. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2007
55	*Halobacillus campisalis* sp. nov., containing meso-diaminopimelic acid in the cell-wall peptidoglycan	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2007
56	*Chromohalobacter salarius* sp. nov., a moderately halophilic bacterium	Solar saltern in Cabo de Gata, Almeria, southern Spain	Aguilera et al., 2007
57	*Rhodobium gokarnense* sp. nov.,	Saltern in Gokarna, India	Srinivas et

	a novel phototrophic alphaproteobacterium		al., 2007
58	*Haloferax larsenii* sp. nov., an extremely halophilic archaeon	Solar saltern in Zhe-Jiang Province, China	Xu et al., 2007
59	*Haloquadratum walsbyi* gen. nov., sp. nov., the square haloarchaeon of Walsby	Saltern crystallizers in Australia and Spain	Burns et al., 2007
60	*Halomonas indalinina* sp. nov., a moderately halophilic bacterium	Solar saltern in Cabo de Gata, Almeria, southern Spain	Cabrera et al., 2007
61	*Maribius salinus* gen. nov., sp. nov., isolated from a solar saltern and *Maribius pelagius* sp. nov., cultured from the Sargasso Sea, belonging to the *Roseobacter* clade	Hypersaline water of a solar saltern in Korea	Choi et al., 2007
62	*Bacillus taeanensis* sp. nov., a halophilic Gram-positive bacterium	Solar-saltern sediment from the Tae-An area of Korea	Lim et al., 2006
63	*Citreimonas salinaria* gen. nov., sp. nov., a member of the *Roseobacter* clade	Solar saltern in Korea	Choi and Cho, 2006
64	*Bacillus seohaeanensis* sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall	Solar saltern at Taean in Korea	Lee et al., 2006
65	*Halocafeteria seosinensis* gen. et sp. nov. (Bicosoecida), a halophilic bacterivorous nanoflagellate	Korean solar saltern	Park et al., 2006
66	*Chromohalobacter nigrandesensis* sp. nov.	Lake Tebenquiche on the Atacama Saltern, Chile	Prado et al., 2006
67	*Jannaschia seosinensis* sp. nov.	Hypersaline water of a solar saltern in Seosin, Korea	Choi et al., 2006
68	*Palleronia marisminoris* gen. nov., sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium belonging to the 'Alphaproteobacteria'	Saline saltern on the Mediterranean seaboard in Murcia (Spain)	Martínez-Checa et al., 2005
69	*Halobacillus yeomjeoni* sp. nov.	Marine solar saltern in Korea	Yoon et al., 2005
70	*Halomonas taeanensis* sp. nov., a novel halophilic bacterium	Solar saltern at Taean in Korea	Lee et al., 2005
71	*Halomonas almeriensis* sp. nov.	Saltern in the Cabo de Gata-Níjar reserve in Almería, south-east Spain	Martínez-Checa et al., 2005
72	*Algoriphagus locisalis* sp. nov.	Sea water of a marine solar saltern of the Yellow Sea, Korea	Yoon et al., 2005
73	*Pontibacillus marinus* sp. nov.	Solar saltern in Korea	Lim et al., 2005
74	Algoriphagus yeomjeoni sp. nov.	Marine solar saltern in the Yellow Sea, Korea	Yoon et al., 2005
75	*Idiomarina seosinensis* sp. nov.	Hypersaline water from a solar	Choi and

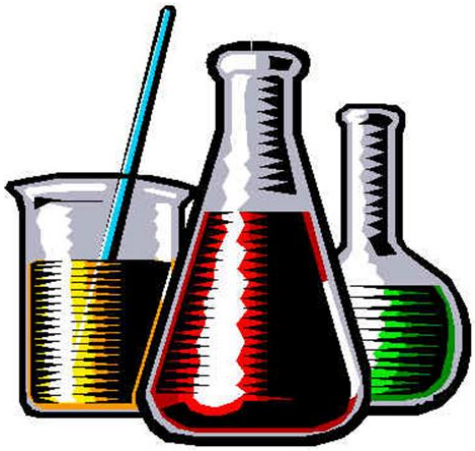
		saltern located in Seosin, Korea	Cho, 2005
76	*Salinimonas chungwhensis* gen. nov	Solar saltern in Korea	Jeon et al., 2005
77	*Pontibacillus chungwhensis* gen. nov., sp. nov.	Solar saltern in Korea	Lim et al., 2005
78	Transfer of *Bacillus halodenitrificans* Denariaz et al. 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov.	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2004
79	*Halomonas koreensis* sp. nov.	Solar saltern of the Dangjin area in Korea	Lim et al., 2004
80	*Marinibacillus campisalis* sp. nov.	Marine solar saltern in Korea	Yoon et al., 2004
81	*Halobacillus locisalis* sp. nov., a halophilic bacterium	Marine solar saltern of the Yellow Sea in Korea	Yoon et al., 2004
82	Isolation and characterization of spirilloid purple phototrophic bacteria forming red layers in microbial mats of Mediterranean salterns: description of *Halorhodospira neutriphila* sp. nov.	Salin-de-Giraud (Rhône delta)	Hirschler-Réa et al., 2003
83	Taxonomic characterization of *Haloferax* sp. (*H. alicantei*) strain Aa 2.2:description of *Haloferax lucentensis* sp. nov.	Spanish saltern located in Alicante	Gutierrez et al., 2002
84	*Haloferax alexandrinus* sp. nov., an extremely halophilic canthaxanthin-producing archaeon	Solar saltern in Alexandria (Egypt)	Asker and Ohta, 2002
85	*Salinibacter ruber* gen. nov., sp. nov.	Saltern crystallizer ponds in Alicante and Mallorca, Spain	Antón et al., 2002
86	*Halorubrum tebenquichense* sp. nov., a novel halophilic archaeon	Atacama Saltern, Chile	Lizama et al., 2002
87	*Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium	Soil samples collected from a saltern at Asilah (Morocco)	Bouchotroch et al., 2001
88	*Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete	Saltern in Kunsan, Republic of Korea	Chun et al., 2000
89	Isolation and characterization of *Desulfovibrio senezii* sp. nov., a halotolerant sulfate reducer	Solar saltern in California	Tsu et al., 1998
90	Ch2, a novel halophilic archaeon	Marine solar saltern in Geelong, Australia	Nuttall and Dyall-Smith, 1993
91	New species of *Pseudodiaptomus* (Copepoda:Calanoida)	Salt pans of the Gulf of Kutch, India	Madhupratap and

			Haridas, 1992
92	*Bacillus persepolensis* sp. nov. and *Piscibacillus halophilus* sp. nov	hypersaline lake Howz-Soltan, Iran	Amoozegar et al. (2009a and b)
93	*Bacillus okhensis* sp. nov.	Okha, Gujarat, India	Nowlan et al. (2006)

Table 2.5: Novel Species isolated from salt pans

Chapter Three

Materials and Methods



3.1. Study Area:

3.1.1. Selection of site:

Seven different salt pans were monitored to select appropriate salt pans having high biomass and diversity. Based on the bacterial diversity, 2 salt pans Ribandar and Batim that lie in the Tropical Mandovi-Zuari estuarine system of Goa, (West coast of India) (Fig.3.1) were selected and sampling was localized to the crystallizer pans. The geographical positions of both the sites were recorded with a Global Positioning System (GPS) (eTrexVista™ personal navigator®, Garmin International, Inc., USA).

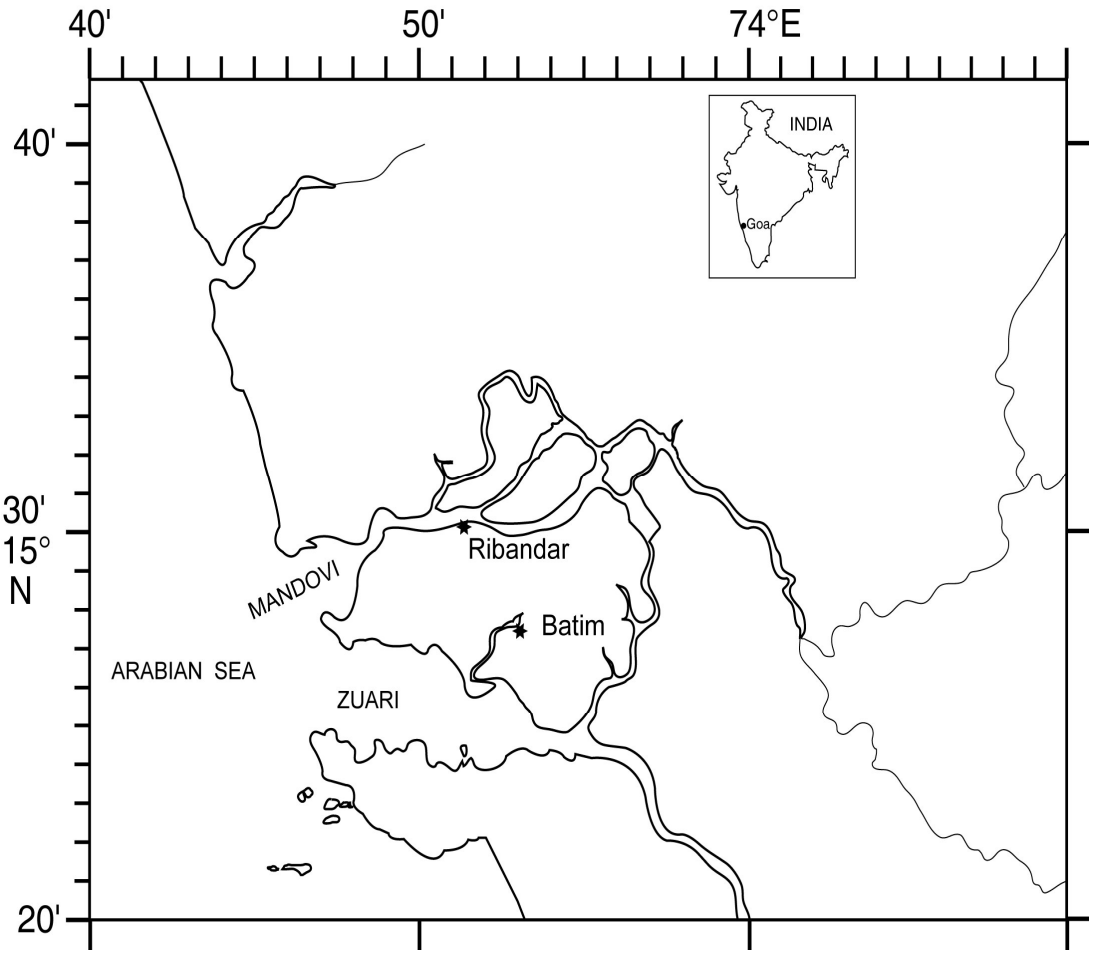


Fig.3.1: Map showing location of the two salt pans.

3.1.2. Description of the Study Area:

Ribandar Saltpan:

The Ribandar salt pan site is situated at 15° 30′ 8.1″ N and 073° 51′ 19.6″ E. The total area of this saltpan is 12,329.12 m^2 and is connected to the Arabian Sea via the Mandovi River (Plate 3.1). It is a part of reclaimed area with natural as well as man-made mangrove plantations in the vicinity and is under the influence of semi-diurnal tides. Ribandar salt pans receive water from the Arabian Sea. The site is also under the influence of human activity since the river is used for transportation of ferry boats, fish trawlers as well as barges with iron ore. Samples were collected from two locations at 15° 29′ 58.1″ N 073° 50′ 49.2″ E (2007) and 15° 30′ 8.1″ N 073° 51′ 19.6″ E (2008).

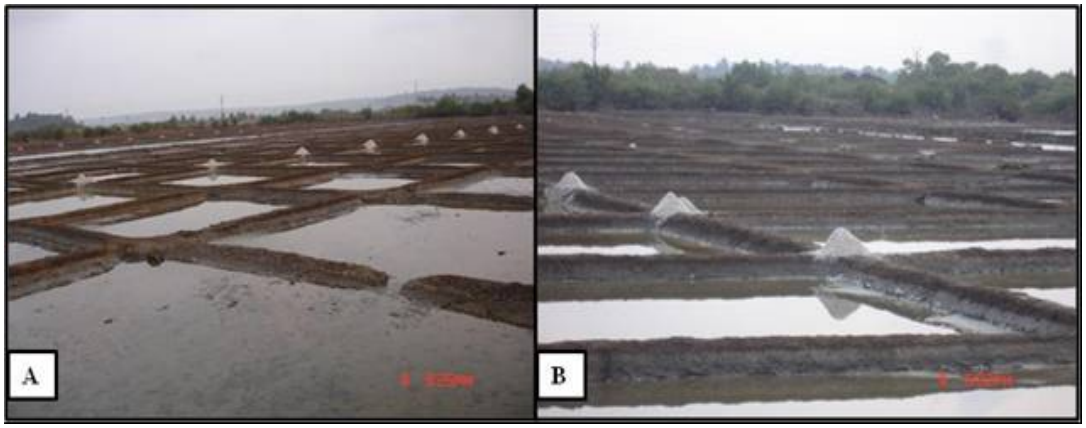


Plate 3.1: A: Ribandar salt pan B: Sampling site

Batim Saltpan:

Batim salt pans are located at 15° 27′ 28.6″ N and 073° 52′ 50.6″ E. These salt pans are relatively pristine and thus free from anthropogenic activity (Plate 3.2). Both the sites receive heavy annual rains (125 cm) during the monsoon season. Mandovi and Zuari are the two major rivers of Goa. A number of small rivulets along the course join the main channels of these rivers that are interconnected by man-made Cumbarjua canal. Samples

were collected from two locations at 15° 27′ 27.6″ N 073° 52′ 58.0″ E (2007) and 15° 27′ 28.6″ N 073° 52′ 50.6″ E (2008)

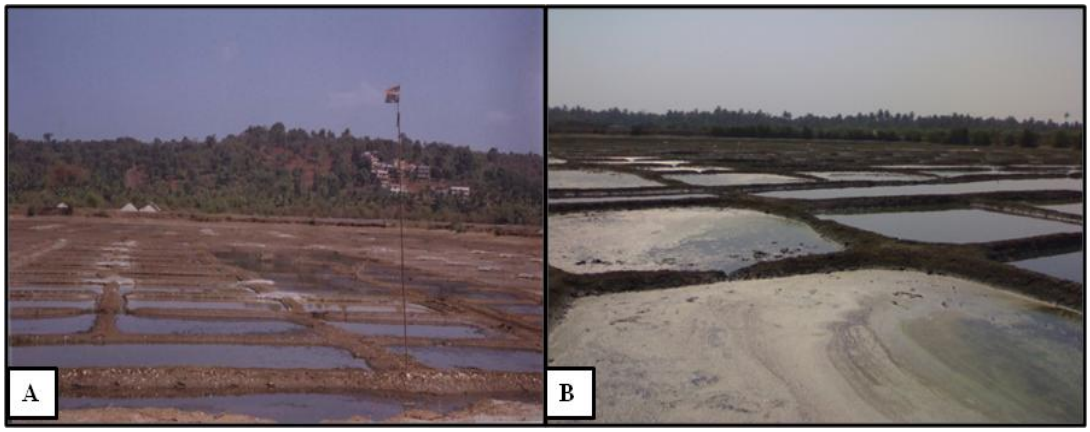


Plate 3.2: A: Batim salt pan B: Sampling site

3.1.3. Frequency of sampling:

Sampling was restricted to the peak salt manufacturing season (pre-monsoons) during the months of May 2007 and May 2008 at Ribandar as well as Batim. Three samples were collected and pooled together, from each sampling site, during the two seasons. Water samples were collected in sterile disposable bottles. Sediment cores (10 cm) were collected using a 1.5 inch diameter graduated PVC pipe. The cores were sealed at both ends with sterile core caps to prevent direct contact with air. The samples were transported to the laboratory in an ice box for further hydrographic parameters (physico-chemical) and microbiological analysis.

3.2. Hydrographic parameters:

The hydrographic parameters recorded at the sampling site were temperature, salinity and pH for the overlying saltpan water. Temperature was measured with a field thermometer

(76 mm immersion, ZEAL, England). Salinity was measured using a hand-held refractometer (S/Mill-E, ATAGO Co. Ltd., Japan) calibrated to zero with distilled water. Whenever salinity was above 100 psu, the sample was diluted with distilled water (1:5) before being recorded. The pH was measured using a digital pH meter (pH Tester 30, Eutech Instruments, Thermo Scientific, USA) after calibrating with standard buffers.

3.3. Microbiology:

3.3.1. Isolation of bacteria/actinobacteria:

Serial dilutions of salt pan water (10 mL) and sediment (10 gm) samples were carried out up to 10^{-5} with sterile salt pan water and 0.1 mL of the serially diluted sample (10^{-3}, 10^{-4} and 10^{-5}) was spread plated, in duplicates, on media M1 to M7 (Appendix 1) and Nutrient Agar (NA)+5% salt. Out of the 8 media used, seven were prepared in filtered sea water (35 psu) and one, NA+5% salt was prepared in distilled water. The plates were incubated at 37°C until the appearance of colonies. Hundred and nineteen strains were isolated, purified, sub cultured and stored at 4°C (Table 3.1). All the media and media components used for the experiments were procured from HiMedia Laboratories Pvt. Limited, India, unless otherwise specified.

Media of isolation	Total number of isolates
M 1	18
M 2	27
M 3	10
M 4	5
M 5	1
M 6	33
M 7	8
NA + 5% salt	17
Total	119

Table 3.1: Total number of isolates

3.3.2. Growth studies of the isolates between media:

The isolates were tested for their ability to grow within the different media used for isolation i.e. in media M1 to M7. The isolates were spot inoculated on to the agar plates and incubated at 37°C for 48 h. The growth was recorded.

3.3.3. Growth in different concentrations of salt (NaCl):

The isolates were streaked on to NA plates with varied salt concentrations of 0%, 5% and 10%. Plates were incubated at 37°C for 48 h and growth was recorded.

3.3.4. Test organisms and growth media:

Twenty clinical pathogens were obtained from Goa Medical College & Hospital, Goa, India and used as test organisms (Table 3.2). The pathogens were maintained on NA and sub cultured at an interval of 30 days. All the necessary precautions were followed during the handling of clinical pathogens.

No.	Code	Name of the culture	Gram character	Disease caused/ Type
1	AB	*Acinetobacter baumanii*	Gram negative	Nosocomial *A. baumannii* bacteremia
2	AH	*Aeromonas hydrophila*	Gram negative	Dysenteric gastroenteritis, cellulitis
3	CD	*Citrobacter diversus*	Gram negative	Neonatal meningitis
4	CF	*Citrobacter freundii*	Gram negative	Enterotoxigenic (produces toxins in the gastrointestinal tract that cause such things as vomiting, diarrhea, and other symptoms of food poisoning)
5	ECATCC	*Escherichia coli* ATCC 25922	Gram negative	ATCC strain
6	KP	*Klebsiella pneumoniae*	Gram negative	Pneumonia, Bacteremia and meningitis
7	MM	*Morganella morganii*	Gram negative	*Proteus morganii* -infection of urinary tract
8	PM	*Proteus mirabilis*	Gram negative	Infection of urinary tract
9	PATCC	*Pseudomonas* ATCC 27855	Gram negative	ATCC strain
10	PP	*Pseudomonas* spp. (Pigmented)	Gram negative	*Pseudomonas* bacteremia, meningitis, folliculitis

11	SPA	*Salmonella paratyphi* A	Gram negative	Gastroenteritis and enteric fever
12	ST	*Salmonella typhi*	Gram negative	Typhoid
13	STM	*Salmonella typhimurium*	Gram negative	Food poisoning
14	SB	*Shigella boydii*	Gram negative	Bacillary dysentery
15	SF	*Shigella flexneri*	Gram negative	
16	VC	*Vibrio cholerae*	Gram negative	Cholera
17	MRSA	Methicillin Resistant *Staphylococcus aureus*	Gram positive	Furunculosis, pyemia, osteomyelitis, suppuration of wounds, and food poisoning
18	MSSA	Methicillin Sensitive *Staphylococcus aureus*	Gram positive	
19	SATCC	*Staphylococcus* ATCC 25923	Gram positive	ATCC strain
20	SC	*Staphylococcus citreus*	Gram positive	Furunculosis etc.

Table 3.2: Clinical pathogens used in the study

3.4. Primary screening of isolates for antibacterial compound:

Preliminary screening was performed on 94 isolates which showed consistent growth by the "cross streak method". The isolates were streaked as a 'ribbon' on NA plates, in triplicates and incubated at 37°C for 48 h. For slow growing cultures, the culture ribbons were incubated for 7 days. At the end of the incubation period, the test pathogens were streaked perpendicular to the ribbon and the plates were incubated again for 16-24 h at 37°C. The observations were recorded as mm of inhibition. A positive control was kept to check the growth of all the pathogens on NA. The pathogens were streaked on to the NA and the plates were incubated for 16-24 h at 37°C.

3.4.1. Selection of candidate culture:

Among the 94 isolates, 5 isolates namely 6, 7, 12, 17 and TSK71 with the most promising antimicrobial potential were tested using Kirby-Bauer disc diffusion method. The isolates were grown in 50 mL NB at 120 rpm, 37°C for 3 days. After every 24 h, 1 mL of each

culture was centrifuged at 8000 rpm. Sterile discs (Whatman filter no. 1) 6 mm each, were impregnated with 10 µL of the culture supernatant and allowed to air dry under aseptic conditions. The test pathogen *Staphylococcus citreus* was suspended in 0.85% sterile saline (6.25 x 10^7 cells per mL), vortexed and spread plated on NA. The impregnated discs were then placed on the culture lawn along with a positive control (streptomycin 10 µg) and incubated at 37ºC for 16-24 h. The diameter of the zones of complete inhibition was measured to the nearest whole mm. Assays were carried out in triplicates. The active cultures were stored in NB as 20% glycerol stock at -80ºC for further studies. The candidate culture TSK 71 was selected for further studies.

3.5. Identification of the candidate culture TSK 71:

The candidate culture TSK 71 was identified using polyphasic approach of biochemical, FAME and 16S rDNA sequence analysis.

3.5.1. Classical Identification:

Identification was based on morphological, physiological, biochemical characteristics as outlined in Bergey's Manual of Systematic Bacteriology (1986).

3.5.1.1. Morphology:

Bacterial morphology was discerned from Gram stained cell preparations and scanning electron microscopy. Glass pieces (1 cm) were cut from a slide and the bacterial suspension of TSK 71 was uniformly spread on these pieces and air dried. The samples were then sequentially dehydrated in 2, 10, 20, 50, 80, 90, 95, 100% concentration of acetone for 15 minutes each, before, finally dehydrating them again in 100% acetone for 30 minutes. The samples were stored in fresh 100% acetone until they were processed for CPD (Critical point drying). The slide pieces were pasted on copper stubs with a double sided tape and sputter coated (with gold) for 40 seconds using SPI-MODULE™ Sputter

Coater (SPI Supplies Division of STRUCTURE PROBE, INC.). The stubs were then stored under vacuum & observed in JEOL JSM-5800LV Scanning Microscope (England) and photographed.

3.5.1.2. Motility:

The culture was checked for motility by hanging drop method (Collins and Lyne, 1985).

3.5.1.3 Presence of spore:

Presence of endospores was confirmed using Schaeffer-Fulton's staining method. The stained slides were observed using LM-52-3001 Lynx microscope.

3.5.1.4 Catalase Test:

Loopful of culture TSK 71 was placed on clean dry slides. A drop of 3% hydrogen peroxide solution (20 volumes 6% w/v H_2O_2 LR, s d fine) was added. The slide was observed after 5 minutes for bubbles either macroscopically or with a low-power microscope.

3.5.1.5. Carbohydrate utilization:

The candidate culture TSK 71 was tested for utilization of carbohydrates using KB009 HiCarbohydrate™ Kit (Part A, B and C) as per the manufacturer's instructions (HiMedia Laboratories Pvt. Limited, India). The tests are based on the principle of pH change and substrate utilization. The test involves change of colour of the medium containing individual carbohydrates (31) from red to yellow due to acid production if the test is positive. Medium remains red in colour if the test is negative. A single colony was inoculated in 10 mL M1 broth and grown for 6-8 h. From this flask, 50 µL was inoculated into each well of the strip. The strip was then incubated overnight at 37°C and results were recorded after 16-24 h as per the chart provided with the strip.

3.5.1.6 Biochemical tests:

The culture was also tested for the presence of ONPG decarboxylase, esculin hydrolysis, citrate utilization and malonate utilization using KB009 HiCarbohydrate™ Kit (Part A, B and C). The protocol followed for inoculation of the kit was same as mentioned in section 3.5.1.5.

ONPG decarboxylase Test: Medium changes from colourless to yellow if the test is positive and remains colourless if the test is negative.

Esculin Hydrolysis: Colour of the medium changes from cream to black if the test is positive and remains cream in colour if the test is negative.

Citrate utilisation: Colour of the medium changes from yellowish green to blue if the test is positive and remains yellowish green in colour if the test is negative.

Malonate utilisation: Colour of the medium changes from light green to blue if the test is positive and remains light green in colour if the test is negative.

Other biochemical tests performed on the culture TSK 71 included VP, gas from glucose, growth in anaerobic agar, growth in 7% NaCl, nitrate reduction, starch and casein hydrolysis and growth at 50°C and 65°C by standard protocols described by Collins and Lyne (1985).

3.5.1.7. Antimicrobial susceptibility test:

The candidate culture TSK 71 was grown on M1 agar for 16-18 h and 0.1 mL saline suspension was spread on Muller Hinton agar plates. The antibiotic discs (Hi-Media, India) were placed on the lawn of the culture and incubated overnight at 37°C. Thirty antibiotics and their concentrations used for the test have been listed in Table 3.3. The test was performed as per the CLSI methodology provided by the HiMedia Laboratories Pvt. Ltd., Mumbai, India (Wikler et al., 2007). The zones of inhibition were recorded in mm

after incubation. The sensitivity and resistance of TSK 71 to a given antibiotic was determined with reference to the chart provided. All assays were carried out in triplicates.

No.	Code	Name of the antibiotic	Concentration (µg)
1	A10	Ampicillin	10
2	Ac30	Amoxyclav (Amoxycillin/Clavulanic acid)	30 (20/10)
3	Ak30	Amikacin	30
4	As10/10	Ampicillin/Sulbactam	10/10
5	C30	Chloramphenicol	30
6	Ca30	Ceftazidime	30
7	Cd2	Clindamycin	2
8	Ce30	Cephotaxime	30
9	Cf5	Ciprofloxacin	5
10	Ch30	Cephalothin	30
11	Co25	Co-Trimoxazole (Sulpha/Trimethoprim)	25 (23.75/1.25)
12	Cp30	Cephalexin	30
13	Cu30	Cefuroxime	30
14	Do30	Doxycyline Hydrochloride	30
15	E15	Erythromycin	15
16	G10	Gentamicin	10
17	K30	Kanamycin	30
18	L10	Lincomycin	10
19	Le5	Levofloxacin	5
20	M5	Methicillin	5
21	N30	Neomycin	30
22	Na30	Nalidixic Acid	30
23	Nf100	Nitrofurantoin	100
24	Of5	Ofloxacin	5
25	Ox1	Oxacillin	1
26	P10	Penicillin-G	10 units
27	S10	Streptomycin	10
28	T30	Tetracycline	30
29	Tb10	Tobramycin	10
30	Va30	Vancomycin	30

Table 3.3: Antibiotic discs used for antimicrobial sensitivity test

3.5.2. Fatty Acid Methyl Ester (FAME) Analysis:

The culture was streaked on TSBA (Trypticase soy broth agar) plates and incubated at (28±1°C) for 24 h and used for extraction.

Harvesting:

Colonies were harvested from the most dilute quadrant exhibiting confluent growth along the streaking axis by gently scraping the surface of the culture medium with a sterile 4 mm inoculating loop. The loop with the cells was inserted into a clean dry 13 mm x 100 mm screw cap culture tube; the cells were wiped off the loop and onto the lower inner surface of the culture tube within 10 mm of the bottom of the culture tube. The loop was removed and sterilized.

Saponification:

Reagent 1, the methanolic base, was pipetted (1.0±0.1 mL) into each of the culture tubes. Each tube was tightly sealed with a clean Teflon lined screw cap and vortexed for 5-10 seconds. The sample tubes were then placed into boiling or circulating water bath at 95-100°C. After five minutes the tubes were removed from the boiling water, cooled slightly and vortexed for 5-10 seconds. They were then returned to the water bath. The tubes were checked for leakage, as evidenced by the presence of bubbles rising in the tube. The caps of leaking tubes were retightened (if bubbling continued, the sample was cooled to RT and then transferred to a new culture tube). The heating of tubes in the water bath was continued for an additional 25 minutes. After a total of 30 minutes of saponification in the water bath, the tubes were removed and placed in a pan of cold tap water to cool.

Methylation:

Each tube was uncapped, and 2.0±0.1 mL of Reagent 2, the methylation reagent, was added to each tube. The tube was then tightly capped and vortexed for 5-10 seconds. Because of an excess of reagents, granular precipitate (salt) may form. In such a case the

tube was heated in an 80±1°C water bath for 10±1 min, removed and quickly cooled to RT by placing it in a tray of cold tap water. The tubes were shaken to speed the cooling process.

Extraction:

Each tube was uncapped and 1.25±0.1 mL of Reagent 3, the extraction solvent, was added to each tube. The tubes were tightly sealed and placed in a laboratory rotator and gently mixed end over end for 10 minutes. Each tube was uncapped and using a clean Pasteur pipette for each sample the aqueous (lower) phase was removed and discarded.

Base wash:

Reagent 4 (3.0±0.1 mL), the base wash, was added to each tube. The tubes were tightly capped and gently rotated end over end for 5 minutes. Brief centrifugation (three minutes at 2000 rpm) was done to clarify the interface between the phases in case an emulsion was present. Alternatively a few drops of a saturated ACS grade NaCl/water solution were added to the tube to aid in breaking the emulsion. The tube was held vertically and rotated rapidly between the palms of the hands, and allowed to settle for a few minutes.

Transfer of extract to sample vial:

The sampler vials were labeled for identification. Each tube was uncapped and using a clean Pasteur pipette for each sample, about 2/3 of the organic (upper) phase from the tube was transferred to a clean GC sample vial. Care was taken not to transfer any of the aqueous (lower) phase into the auto sampler vial. The sampler vial was tightly sealed with a cap.

Analysis in gas chromatography:

2 µL of sample was injected into the column (25 m silica capillary) using the automatic sampler. The oven temperature was increased from 170 to 310°C in each run at 5°C per minute. Each fatty acid present in the extract is sensitive to a particular temperature.

When temperature is increased, the fatty acids get volatilized at a particular retention time and will burn in FID (Flame Ionization Detector). FID detected each of the fatty acids as a signal and transferred to the CHEMSTATION software (software which controls all the GC paramaters). The CHEMSTATION visualizes the raw data as a chromatogram. The raw data was then transferred to software SHERLOCK for matching the fatty acid profile of the unknown organism (Sasser, 1990; Pendergrass and Jensen, 1997). Details of all reagents are given in Appendix 1.

3.5.3. 16S rDNA sequencing:

All the kits and chemicals/enzymes used were from Bangalore Genei, India. Genomic DNA was isolated from TSK 71 using GeneiPure™ Bacterial DNA Purification Kit and was PCR amplified using universal bacterial primers (27F and 1525R) for 16S rDNA gene. PCR reaction was setup as follows:

Genomic DNA (~50ng):	1.0 µl
Taq Buffer A (10X):	2.5 µl
dNTP mix (10 mM each) :	0.5 µl
Forward Primer (10pmole/µl):	1.0 µl
Reverse Primer (10pmole/µl):	1.0 µl
Taq DNA polymerase enzyme:	1U
Sterile distilled water: to make up the volume to	25µl

Reactions were carried out with 5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min; 55°C for 45 sec and 72°C for 1.30 min. A final extension was carried out at 72°C for 10 min. The PCR amplified product was separated electrophoretically on 1% agarose gel along with StepUp™ 500 bp DNA ladder. The details of the reagents used are given in Appendix 1. Approximately 1.5 kb fragment was gel purified and bi-

directionally sequenced using the forward, reverse and an internal primer (Bangalore Genei, India). The analysis of partial 16S rDNA sequence was done by BLAST search at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The sequence has been deposited in GenBank (GenBank Accession Number JF411056).

3.6. Studies on the antibacterial compound of culture TSK71:

3.6.1. Growth and Bioassay of antibacterial compound:

For growth studies, 100 µL of sub cultured TSK 71 (7.6×10^7 cells per mL) was used as inoculum. Growth of TSK 71 was checked by recording the O.D. using UV mini 1240 UV-VIS Spectrophotometer (Shimadzu, Japan) at 600 nm at 8, 16, 24, 32 and 48 h intervals. The production of antibacterial compound (bioactivity) was also monitored, at these intervals, by performing disc diffusion assay. After a definite interval, 1 mL of culture from the flask was centrifuged at 10,000 rpm (Microcentrifuge 5417R, Eppendorf, USA) for 5 minutes. Sterile discs (Whatman filter no. 1) 6 mm each, were impregnated with 10 µL of the culture supernatant and allowed to air dry under aseptic conditions. The test pathogen *Staphylococcus citreus* was suspended in 0.85% sterile saline (6.25×10^7 cells per mL), vortexed and spread plated on NA. The impregnated and dried discs were then placed on the culture lawn and incubated at 37ºC for 16-24 h along with negative controls. The zones of inhibition were recorded in mm. Assays were performed in triplicates.

3.6.2. Optimization of growth and antibacterial compound production:

Temperature, pH, salt concentration, carbon source, nitrogen source, and aeration were tested for optimization of growth and production of antibacterial compound by TSK 71.

Temperature:

Before optimizing the temperature for growth and production of antibacterial compound, tolerance of the culture TSK71 to different temperatures was established. To determine the temperature tolerance TSK71 was streaked on NA plates and incubated at temperatures between 20 to 65°C (i.e. 20, 22, 25, 40, 45, 50, 55, 60 and 65°C). The plates were kept at respective temperatures for 15 min before streaking.

pH:

The effects of pH on growth and bioactivity were determined at different pH between 4.0 and 11.0. Nutrient broth amended with salt was prepared using 100 mM Tris buffer and pH was adjusted to 4, 5, 6, 7, 8, 9, 10 and 11 using 0.1 N HCl or NaOH. The flasks were inoculated and incubated at 37ºC and 120 rpm. The growth and bioactivity were assessed as mentioned in section 3.6.1.

Salt concentration:

Salt requirement for the growth and bioactivity was determined at different salt concentrations between 0 and 20%. Nutrient broth was prepared using 100 mM Tris buffer and pH was adjusted to 6 using HCl. The salt was added to get 0, 2, 3.5, 6, 8, 10, 12, 15 and 20%. The flasks were inoculated and incubated at 37ºC and 120 rpm. Growth and bioactivity were assessed as mentioned in section 3.6.1. Subsequently an experiment was carried out to check the salt requirement between 0 and 2% (i.e. 0, 0.5, 1 and 2%).

Carbon source:

Based on the carbohydrate utilisation tests performed on the candidate culture nine carbon sources viz. Arabinose, Xylose (aldopentoses), Dextrose, Mannose (aldohexose), Glycerol, Sorbitol, Mannitol (sugar alcohols), Fructose (ketohexose), Sucrose (disaccharide) were selected for optimization. Each substrate was added in 0.4% concentration to the Minimal media (Appendix 1). The flasks were inoculated and incubated at 37ºC and 120 rpm. Growth and bioactivity were assessed as mentioned in

section 3.6.1. Subsequently 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1% concentrations of sucrose in Minimal media were used and growth and bioactivity were assessed as mentioned earlier in section 3.6.1.

Nitrogen source:

Eight nitrogen sources, as shown in Table 3.4, were selected for optimization, each added in 0.2% concentration to the Minimal media containing 0.6% sucrose as carbon source. The flasks were inoculated and incubated at 37ºC and 120 rpm. Growth and bioactivity were assessed as mentioned in section 3.6.1. Based on the best nitrogen source, potassium nitrate was added to the flasks containing Minimal media with 0.6% sucrose as carbon source in 0.2, 0.4, 0.6, 0.8 and 1% concentrations. The flasks were inoculated and incubated at 37ºC and 120 rpm. The growth and bioactivity were assessed as mentioned earlier in section 3.6.1.

Sr. no.	Nitrogen source	Code	Type/Source
1	L-Glutamic acid, sodium salt	GA	Organic
2	Urea	U	Organic
3	Ammonia solution about 30%	A	Inorganic
4	Potassium nitrate	PN	Inorganic
5	Yeast extract	YE	Complex, microbial origin
6	Beef extract powder	BE	Complex, animal origin
7	Peptone, bacteriological	P	Complex, microbial origin
8	Soya peptone	SP	Complex, plant origin

Table 3.4: Details of nitrogen sources used for optimization

3.6.3. Optimization of the TSK medium:

Temperature:

For checking the growth and bioactivity in TSK medium (Appendix 1) the culture TSK71 was inoculated and incubated at 120 rpm at 25°C, RT (30±0.2°C), 37°C and 60°C. The flasks were kept at the respective temperatures, 30 minutes before inoculation. The growth and bioactivity were assessed as mentioned in section 3.6.1.

Aeration:

To check the requirement of aeration, the culture TSK71 was inoculated in TSK media and incubated at 37°C under shaker (120 rpm) and static conditions. The growth and bioactivity were assessed as mentioned in section 3.6.1.

Growth curve of TSK71 under optimized conditions:

The culture TSK 71 was sub cultured and 500 µL (O.D.~0.5) was inoculated in 200 mL TSK medium and incubated at 37°C, 120 rpm. The growth was measured at 600 nm, as O.D., at 2 hourly intervals for 24 h followed by 32, 48, 56, 72, 80 and 96 h intervals. Bioactivity was also assessed simultaneously (as mentioned earlier section 3.6.1.), with the same time intervals.

3.6.4. Purification of antibacterial compound:

Bacterial strain TSK 71 was grown in TSK media for 32 h at 37±0.2°C, 120 rpm using 0.25% seed innoculum. The culture was harvested by centrifugation at 10,000 rpm (REMI Cooling Compufuge CPR30, Remi, India) at 10°C for 10 min. Various experiments were carried out to establish the best protocol for extraction of active antibacterial compound from the culture TSK 71. Fresh cell free culture broth or lyophilised cell free culture broth was extracted using organic solvents with increasing polarity starting with petroleum ether followed by chloroform and ethyl acetate. All the organic extracts were dried completely on a rotary vacuum evaporator (Roteva EQUITRON®, India) and tested for activity (as mentioned in section 3.6.1) in their

respective solvents. The results were not consistent and hence other procedures were tried using resins. The detail of the final protocol is as follows. The supernatant was treated with 4g/40mL Diaion HP-20 resin (Supelco, USA) at 120 rpm, RT (30±0.2°C) for 30 minutes. The mixture was then packed as a column in a sterile plastic syringe. The column was washed with sterile MilliQ water and eluted with HPLC grade methanol. A portion of this extract was kept aside for checking antibacterial activity as mentioned in section 3.6.1 as well as for analyses by Thin Layer Chromatography (TLC).The remaining crude extract was dried and dissolved in 20% v/v acetonitrile: water. An aliquot of this extract was kept aside as fraction F1 for testing the bioactivity. The remaining extract was resolved on activated Strata column C-18-E (Phenomenex Inc., USA) and collected as fraction F2. Activation of column was done by washing with 1:1 HPLC grade methanol: MilliQ water. The column was eluted with 50% & 80% acetonitrile and the eluate was collected as fractions F3 & F4 respectively. All the fractions were checked for bioactivity as mentioned in section 3.6.1. The fractions were also monitored on TLC.

3.6.5. Characterisation of the antibacterial compound:

Silica gel 60 sheets (Merck & Co. Inc., NJ, USA) were used for TLC analysis. TLC chamber was saturated with the solvent system (mobile phase) n- butanol: acetic acid: water in the ratio 4:1:5. All the samples for TLC were spotted with the help of glass capillary tubes and allowed to air dry. The samples were resolved on TLC sheet, till the solvent front (mobile phase) reached nearly the end of the sheet. The TLC sheet was then removed carefully from the chamber using a forceps and allowed to air dry and then placed in a hot air oven until the smell of acetic acid disappeared (about 5-10 min). The

spots were then visualized by spraying ninhydrin in methanol followed by heating at 80°C for 3-4 min.

For mass spectrometric analysis electrospray ionization (ESI) Quadropole time of flight (QTOF) QSTARXL MS/MS Applied Biosystem instrument equipped with the MDS Sciex Analyst software (Concord, Ontario, Canada) was used. The active sample was dissolved in methanol and was directly infused at a constant flow rate of 10 µl/ min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: nebulizer gas (N2) 28 (arbitrary units); curtain gas (N2) 18 (arbitrary units); ion spray voltage 5700 V; declustering potential (DP) 120 V; focusing potential (FP) 365 V; declustering potential (DP2) 14 V and collision gas (CAD) 3 (arbitrary units). Full-scan data acquisition was performed, scanning from m/z 100 to m/z 2000 in the profile mode and using a cycle time of 1 s. A collision energy (CE) of 30-40 V was selected to obtain the most intense product ions. The mass analysis was carried out using the TOF analyzer of the instrument. Proton NMR was recorded on Bruker 400 MHz machine.

3.6.6. Minimum inhibitory concentration (MIC) of the purified compound:

The active fraction F3 (100 µL) was lyophilized completely and 200 µL of 50% acetonitrile was added to it. From this stock, various dilutions (1:2, 1:5, 1:10, 1:20, 1:50 and 1:100) were prepared. The bioassay was carried out in triplicates as mentioned in section 3.6.1.

Chapter Four

Results

4.1. Hydrographic parameters:

The temperature of the overlying water of sampling stations of Ribandar and Batim salt pans was ~ 45°C at both the sampling periods (2007 and 2008). At Batim, the temperature dropped to 38°C due to intermittent pre-monsoon showers during 2007 sampling period. Consequently, the salinity also dropped to 220. However, the average salinity of the overlying water at both the stations was > 310 psu. The pH values of the overlying water varied from a minimum of 6.43 in Batim (2008) to 7.09 in Ribandar and Batim (2007). The average pH values at both the locations were not significantly different (Table 4.1).

Parameters	Ribandar		Batim	
	2007	2008	2007	2008
Temperature (°C)	45 (0.12)	45.3 (0.15)	38 (0.25)	45
Salinity (psu)	320	310 (5.77)	220 (5.77)	395
pH	7.09	6.63 (0.02)	7.09	6.43 (0.02)

Table 4.1: Hydrographic parameters recorded during sample collection (The values in parenthesis indicate ±SD)

4.2. Isolation and screening of bacteria/actinobacteria:

A total of hundred and nineteen strains were isolated which comprised of bacteria (94%) and remaining actinobacteria. The number of bacterial and actinobacteria isolates from water and sediments of Ribandar and Batim are shown in Fig.4.1. Actinobacteria were not observed in the overlying waters of Ribandar salt pan during both the sampling periods whereas four isolates were detected in Batim. The number of bacterial isolates from water and sediment were almost equal at Batim.

Maximum number of morphotypes were observed on M6 media (33) followed by M2 (27) whereas only one morphotype isolate was detected on M5 media. At Ribandar 40% isolates were observed on M6 whereas at Batim only 15% were observed (Fig. 4.2 A and B). Maximum isolates (22%) from Batim were observed on NA+5% salt. Only 3% isolates from Ribandar were observed on M3 media as compared to 14% from Batim.

4.2.1. Growth of isolates in different media:

Qualitative analysis of growth on various media indicated that 97% isolates showed dense growth on M2 media followed by M1 (87%) irrespective of the media from which they were isolated (Fig. 4.3).

4.2.2. Growth of isolates in different concentrations of salt:

Salt was not a stringent requirement for growth of most of the isolates (79%). Only 21% isolates were stringent for their salt requirement. Nearly half the isolates could grow in 10 % salt while 65% could tolerate up to only 5% salt (Fig. 4.4). Their preference to grow in varied concentrations of salt reflects their indigenous saltpan habitat and the halotolarant nature of the isolates.

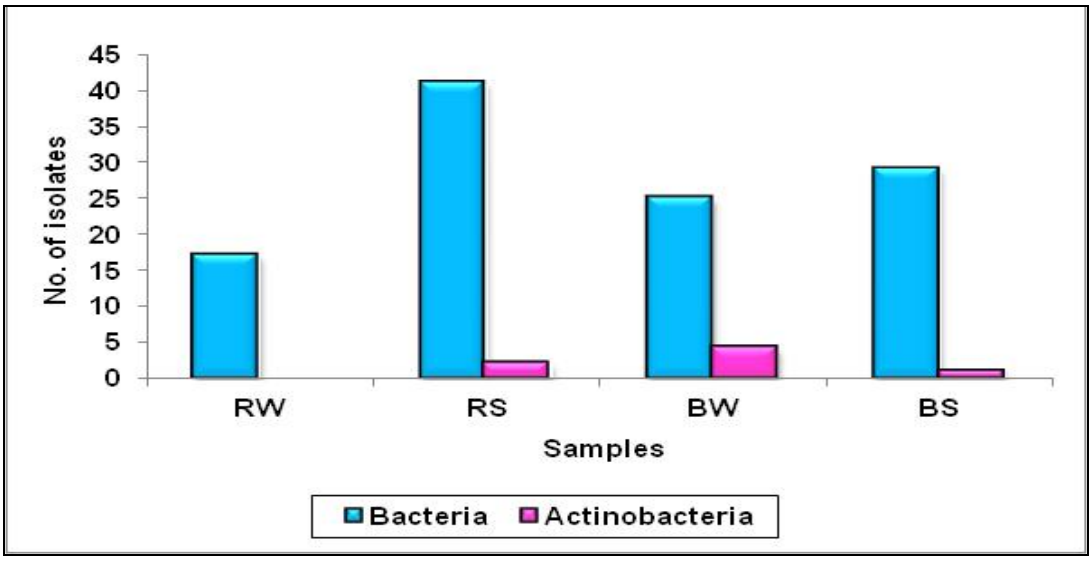


Fig. 4.1: Number of isolates from water (W) and sediment(S) samples of Ribandar (R) and Batim (B)

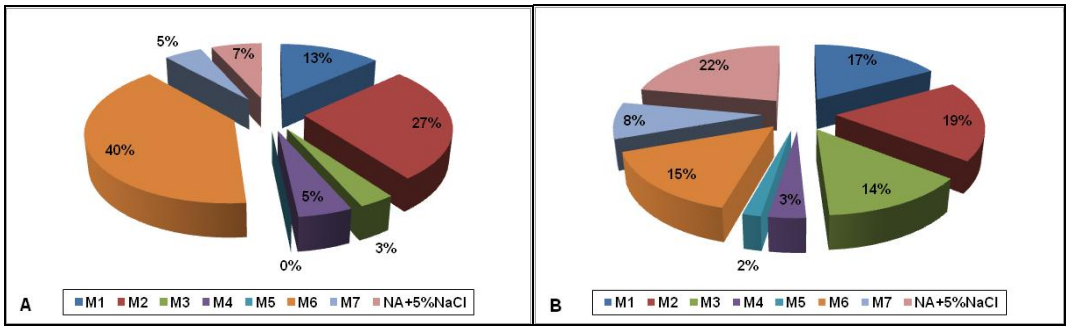


Fig. 4.2: Number of isolates from A: Ribandar and B: Batim obtained on various media

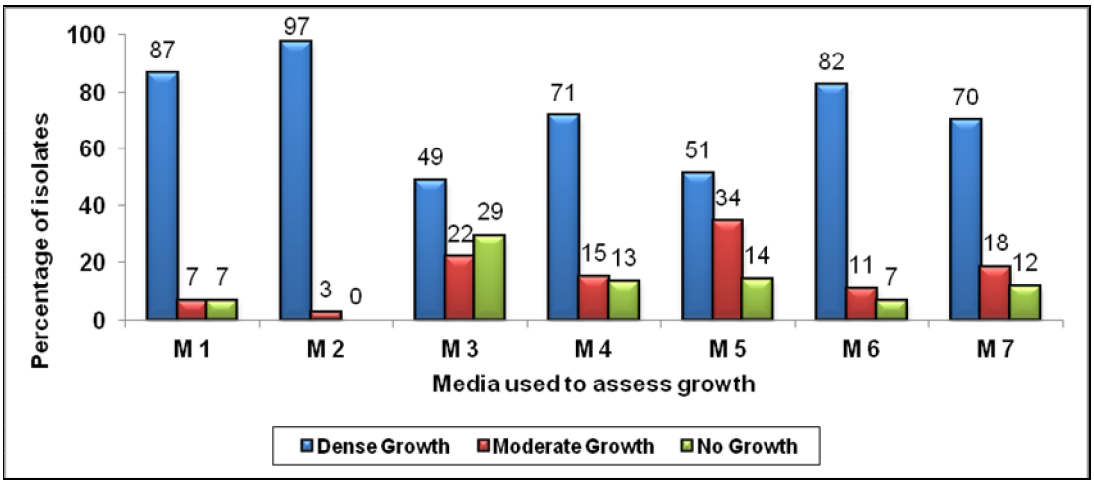


Fig. 4.3: Growth of isolates on various media

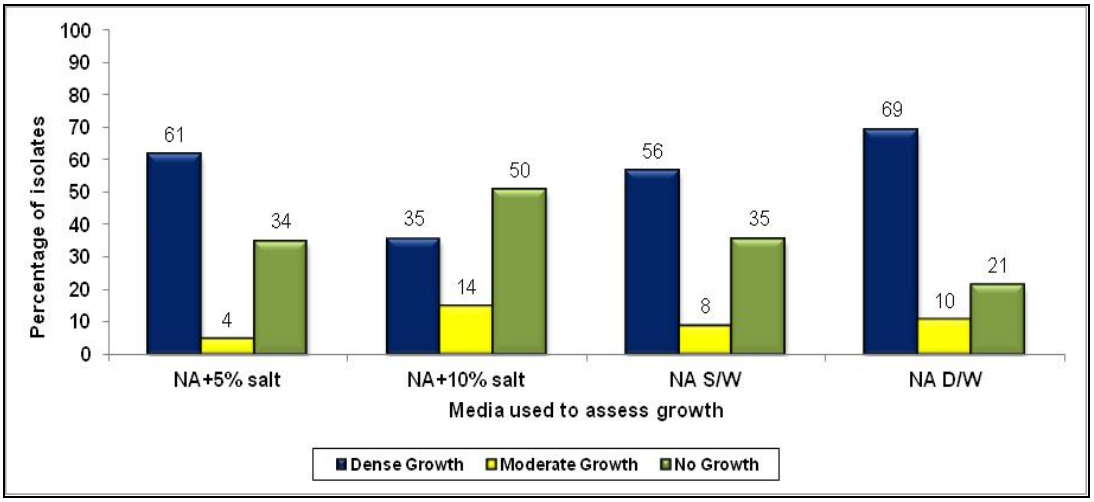


Fig. 4.4: Growth of isolates on various concentrations of salt

4.2.3. Antibacterial activity:

Only 31 isolates out of 94 were found to be potentially active. Less than 2/3rd of these active isolates were bactericidal in action and antagonistic to Gram positive bacteria while majority of the actives were bactericidal towards Gram negative test cultures (Fig. 4.5). Antibacterial activity depends on the test organisms. Not all the Gram negative test cultures were inhibited. One of the active isolates exhibited only bacteriostatic activity while nine were bacteriostatic as well as bactericidal. Two out of 31 active isolates were actinomycetes. From the potential active isolates, 65% were from water samples while the rest were from sediment samples from the salt pans (Fig. 4.6). With respect to bactericidal activity, maximum actives (11) were isolated on NA+5% salt.

Out of the 31 actives, two isolates inhibited Gram positive pathogen *Staphylococcus citreus* (SC); eighteen inhibited Gram negative pathogens viz. AB, CF, PP, SPA, ST, SB, SF & VC whereas eleven (4 from Ribandar and 7 from Batim) were inhibiting both Gram positive and Gram negative test cultures.

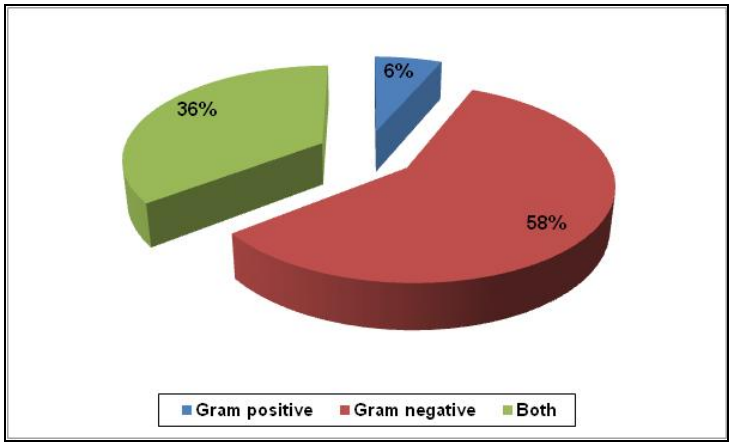


Fig. 4.5: Percentage of isolates showing bactericidal activity against Gram positive and Gram negative test cultures

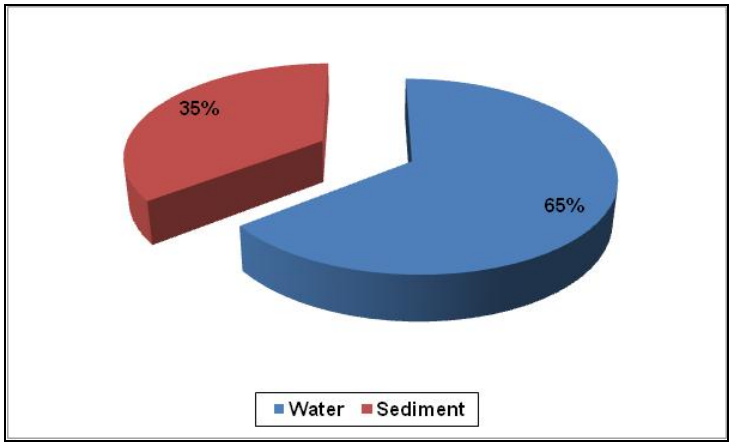


Fig. 4.6: Percentage of isolates showing actives from water and sediment samples

4.2.4. Antibacterial activity of six potential candidates:

Out of the 11 active isolates inhibiting both Gram positive and Gram negative test cultures, only six showed broad spectrum of activity and larger inhibition zones (Fig. 4.7) and isolate TSK 32 and TSK 71 had very similar inhibitory profiles. However, in broth it was found that the isolate TSK 71 exhibited maximum inhibitory activity ($p<0.05$). There were no significant differences ($p>0.05$), over time (three days incubation), in the bactericidal action of TSK 71 on SC (Fig. 4.8). Statistical analyses were carried out using one way ANOVA (Microsoft Office Excel version 2007). Pearson's correlation coefficient was used to establish the significance.

4.3. Characterisation of the TSK 71:

4.3.1. Classical taxonomy:

Microscopic observation of the isolate TSK 71 isolated from overlying water of Batim saltpan revealed a Gram positive character; motile; strongly catalase positive and morphologically appeared as straight, long rods in chains, with endospores (Plate 4.1). The SEM images of TSK 71 were observed at 5000X where in TSK 71 appeared as elongated rods in chains. According to the scale mentioned, the cell size was approximately 1.95-2.73 μm in length and 0.65 μm in width (Plate 4.2).

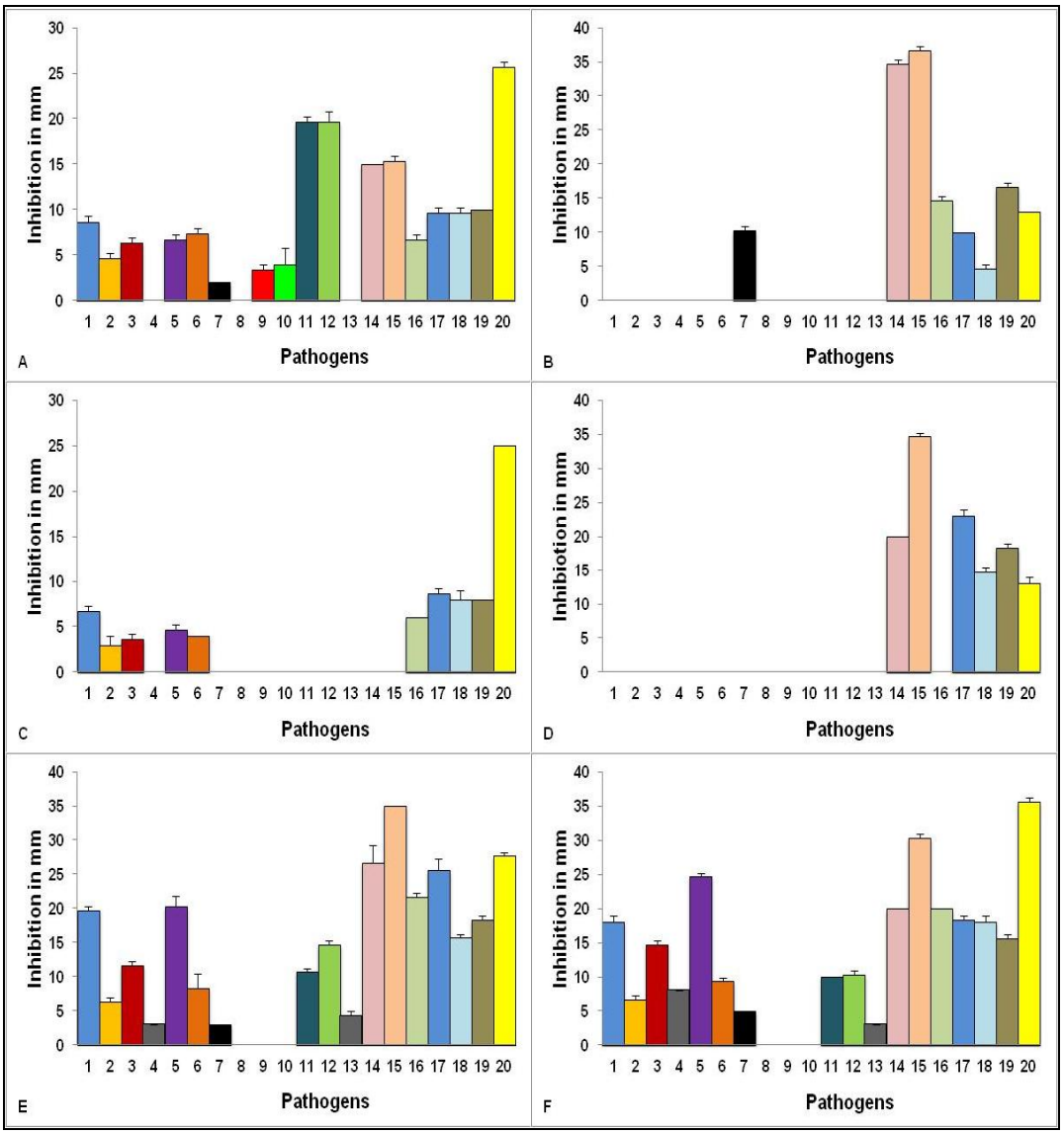


Fig. 4.7: Antibiotic profiles of six active isolates. A: Profile of isolate 6; B: Profile of isolate 7; C: Profile of isolate 12; D: Profile of isolate 17; E: Profile of isolate TSK32; F: Profile of isolate TSK71. Pathogen codes are given in section 3.3.4. of Materials and Methods.

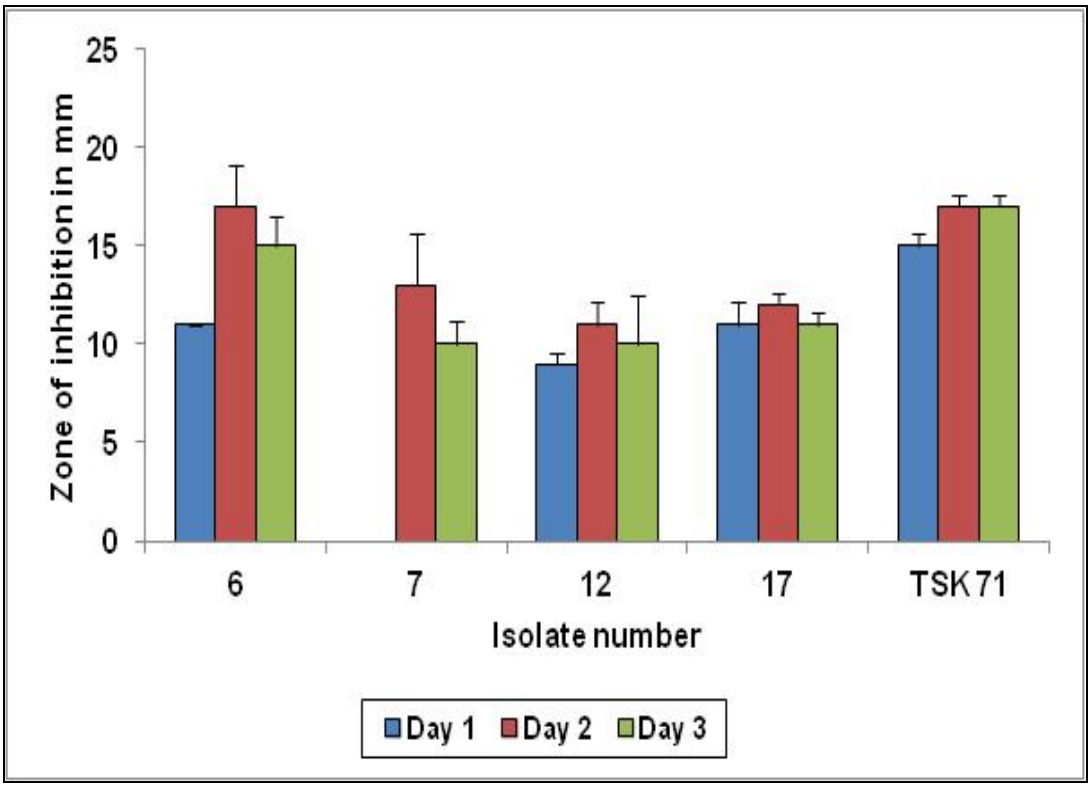


Fig. 4.8: Inhibition profiles of the 6 isolates on SC over a period of three days

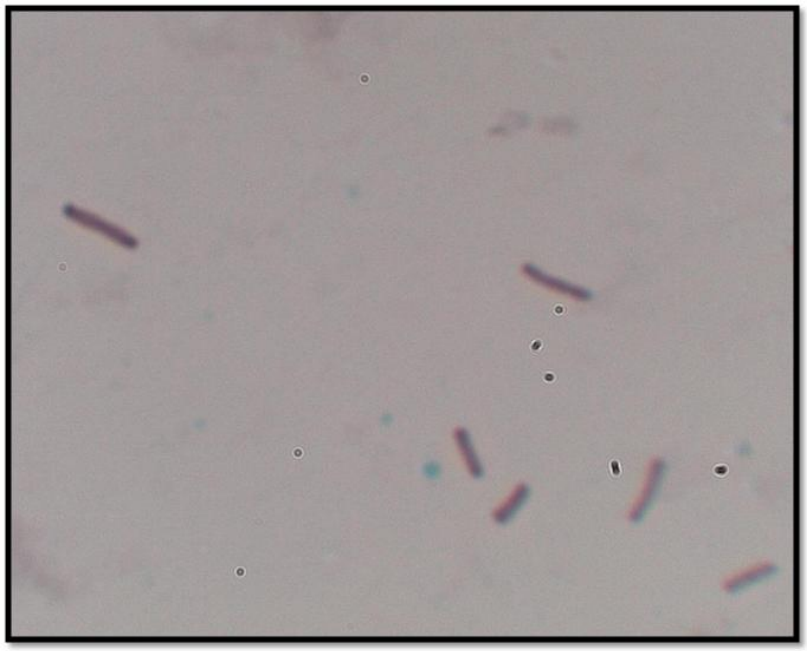


Plate 4.1: Presence of endospores in rods

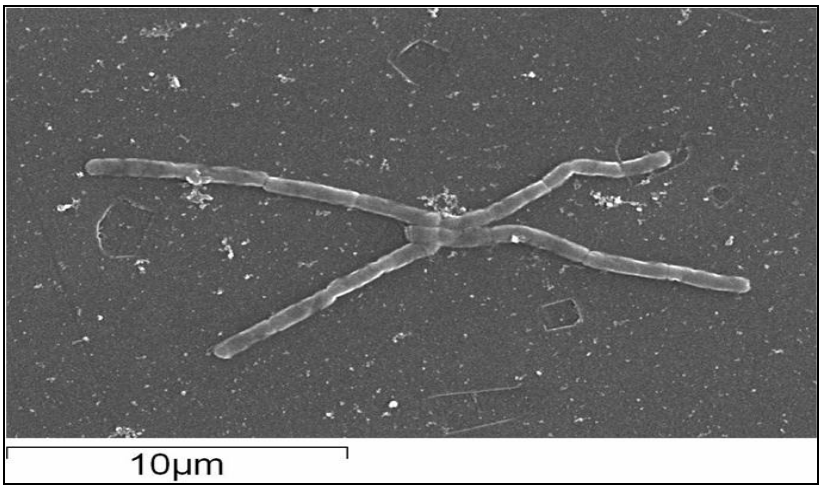


Plate 4.2: Scanning electron micrograph image showing TSK 71 under 5000X magnification

Ability of TSK 71 to utilize carbohydrates and other biochemical tests are given in Table 4.2. As seen from the table, most of the carbohydrates were not utilized by TSK 71 except for dextrose, mannose, glycerol, sucrose, fructose, esculin and citrate. Xylose, arabinose, mannitol and sorbitol did not give 100% positive reaction when tested. TSK 71 was able to hydrolyse polymers such as starch and casein. The results of antimicrobial susceptibility test are given Table 4.3. Out of 30 antibiotics tested TSK 71 was sentitive to most of them except Ceftazidime, Clindamycin, Erythromycin and Lincomycin. Based on the morphological and biochemical characteristics, the isolate is tentatively identified as *Bacillus* sp.

Further Fatty Acid Methyl Ester (FAME) analysis (Plate 4.3 A and B) showed that the culture is *Bacillus licheniformis* (similarity index > 0.5). It was further confirmed by 16S rDNA sequencing. Other promising isolates were identified by the above method, details of which are given in Appendix 2.

Test	TSK 71
Lactose, Maltose, Galactose, Raffinose, Trehalose, Melibiose, Inulin, Sodium gluconate, Salicin, Glucosamine, Dulcitol, Inositol, Adonitol, α Methyl-D-glucoside, Ribose, Rhamnose, Cellobiose, Melezitose, α Methyl-D-mannoside, Xylitol, Malonate and Sorbose	-
Dextrose, Mannose, Glycerol, Sucrose, Fructose, Esculin and Citrate	+
L-Arabinose and D-Arabinose, Xylose, Sorbitol, Mannitol	+/-
Gas from glucose	-
VP and ONPG	+
Growth in anaerobic agar	+
Growth in 7% NaCl	+
Nitrate reduction	+
Starch hydrolysis	+
Casein hydrolysis	+
Growth at 50 ^{0}C	+
Growth at 65 ^{o} C	-
Catalase	+

Table 4.2: Carbohydrate utilization and other biochemical tests for TSK 71

Name of the antibiotic	**S/R/I**
Amikacin, Amoxyclav (Amoxycillin/Clavulanic acid), Ampicillin, Ampicillin/Sulbactam, Cefuroxime, Cephalexin, Cephalothin,Chloramphenicol, Ciprofloxacin, Co-Trimoxazole (Sulpha/Trimethoprim), Doxycyline Hydrochloride, Gentamicin, Kanamycin, Levofloxacin, Methicillin, Nalidixic Acid, Neomycin, Nitrofurantoin, Ofloxacin, Oxacillin, Streptomycin, Tetracycline, Tobramycin, Vancomycin	**S**
Ceftazidime, Clindamycin, Erythromycin, Lincomycin	**R**
Cephotaxime, Penicillin-G	**I**

Table 4.3: Antibiotic sensitivity test of TSK 71 using 30 antibiotics: S: Sensitive; R: Resistant; I: Intermediate

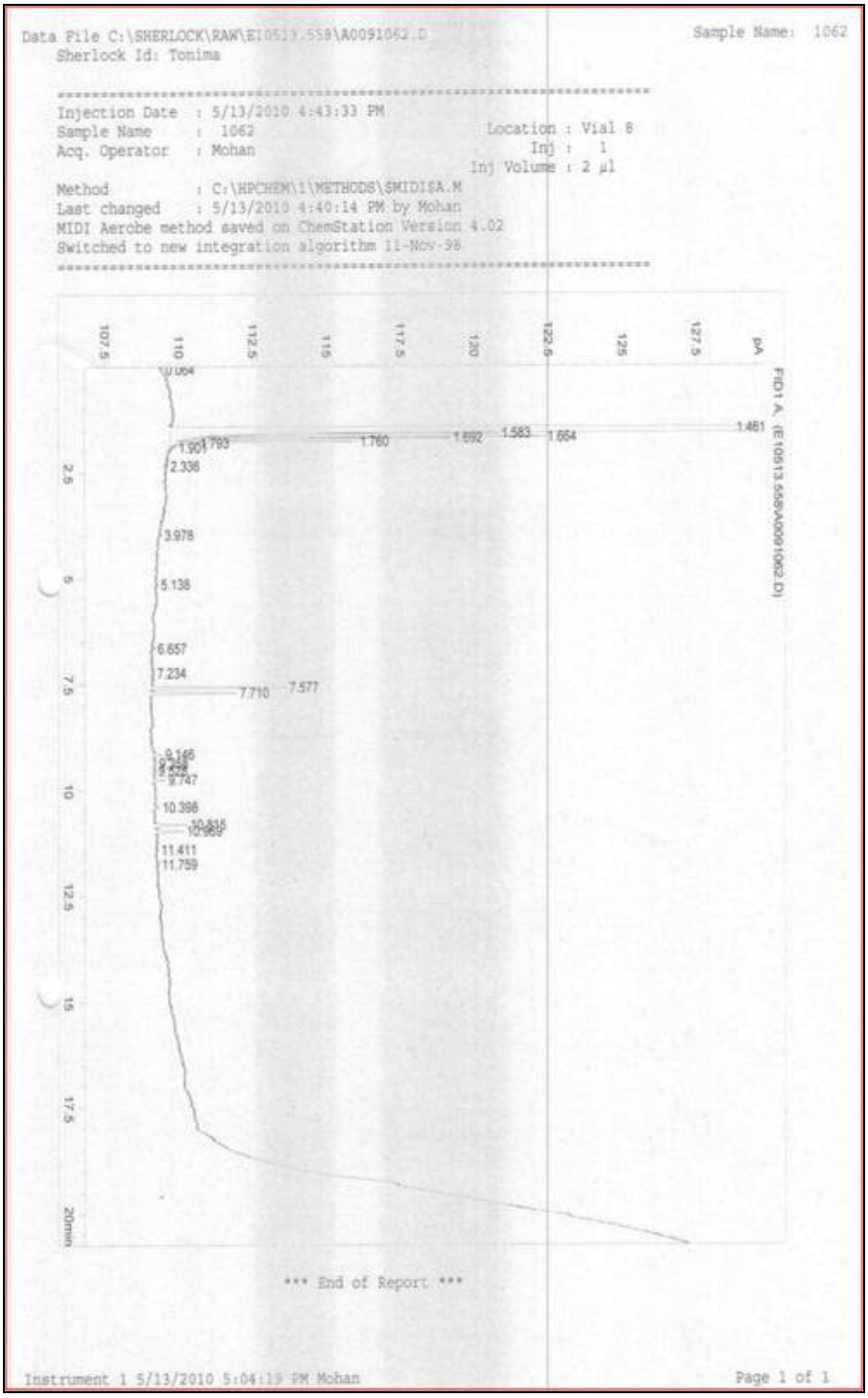
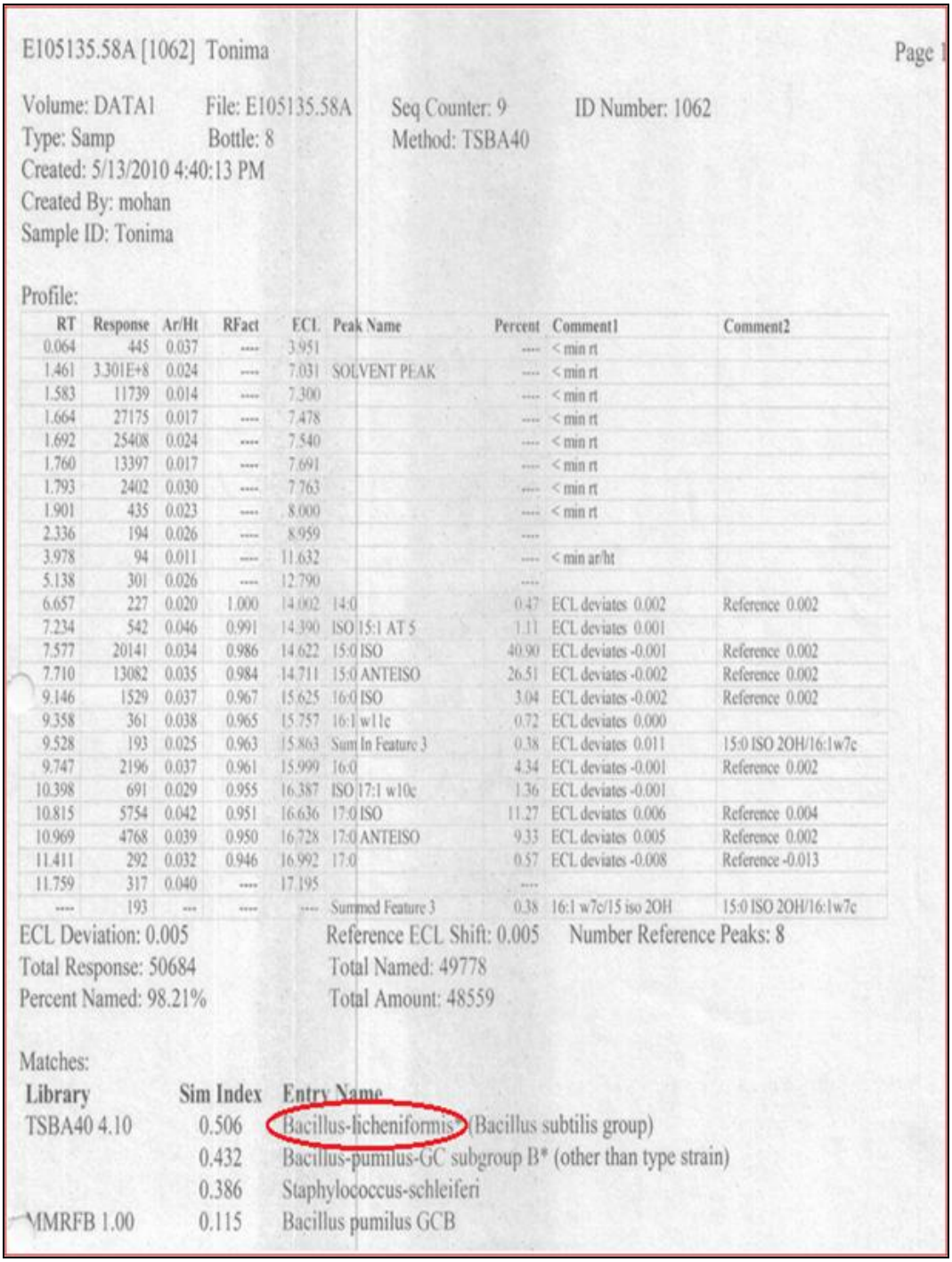


Plate 4.3: A: FAME profile of isolate TSK 71



E105135.58A [1062] Tonima

Page 1

Volume: DATA1 File: E105135.58A Seq Counter: 9 ID Number: 1062
Type: Samp Bottle: 8 Method: TSBA40
Created: 5/13/2010 4:40:13 PM
Created By: mohan
Sample ID: Tonima

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.064	445	0.037	----	3.951		----	< min rt	
1.461	3.301E+8	0.024	----	7.031	SOLVENT PEAK	----	< min rt	
1.583	11739	0.014	----	7.300		----	< min rt	
1.664	27175	0.017	----	7.478		----	< min rt	
1.692	25408	0.024	----	7.540		----	< min rt	
1.760	13397	0.017	----	7.691		----	< min rt	
1.793	2402	0.030	----	7.763		----	< min rt	
1.901	435	0.023	----	8.000		----	< min rt	
2.336	194	0.026	----	8.959		----		
3.978	94	0.011	----	11.632		----	< min ar/ht	
5.138	301	0.026	----	12.790		----		
6.657	227	0.020	1.000	14.002	14:0	0.47	ECL deviates 0.002	Reference 0.002
7.234	542	0.046	0.991	14.390	ISO 15:1 AT 5	1.11	ECL deviates 0.001	
7.577	20141	0.034	0.986	14.622	15:0 ISO	40.90	ECL deviates -0.001	Reference 0.002
7.710	13082	0.035	0.984	14.711	15:0 ANTEISO	26.51	ECL deviates -0.002	Reference 0.002
9.146	1529	0.037	0.967	15.625	16:0 ISO	3.04	ECL deviates -0.002	Reference 0.002
9.358	361	0.038	0.965	15.757	16:1 w11c	0.72	ECL deviates 0.000	
9.528	193	0.025	0.963	15.863	Sum In Feature 3	0.38	ECL deviates 0.011	15:0 ISO 2OH/16:1w7c
9.747	2196	0.037	0.961	15.999	16:0	4.34	ECL deviates -0.001	Reference 0.002
10.398	691	0.029	0.955	16.387	ISO 17:1 w10c	1.36	ECL deviates -0.001	
10.815	5754	0.042	0.951	16.636	17:0 ISO	11.27	ECL deviates 0.006	Reference 0.004
10.969	4768	0.039	0.950	16.728	17:0 ANTEISO	9.33	ECL deviates 0.005	Reference 0.002
11.411	292	0.032	0.946	16.992	17:0	0.57	ECL deviates -0.008	Reference -0.013
11.759	317	0.040	----	17.195		----		
----	193	---	----	----	Summed Feature 3	0.38	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c

ECL Deviation: 0.005 Reference ECL Shift: 0.005 Number Reference Peaks: 8
Total Response: 50684 Total Named: 49778
Percent Named: 98.21% Total Amount: 48559

Matches:

Library	Sim Index	Entry Name
TSBA40 4.10	0.506	Bacillus-licheniformis* (Bacillus subtilis group)
	0.432	Bacillus-pumilus-GC subgroup B* (other than type strain)
	0.386	Staphylococcus-schleiferi
MMRFB 1.00	0.115	Bacillus pumilus GCB

Plate 4.3: B: Table indicating the percentage of major fatty acids in the isolate TSK 71

4.4. Production and characterization of antibacterial compound from *Bacillus licheniformis* (TSK 71):

pH :

The culture TSK 71 could grow at a wide range of pH from 5 to 10 showing its tolerance to an acidic and alkaline range. The variation in growth was significant (F =4.96, P =0.000695 and d.f. =7). However, good growth was observed at pH 5-7. At pH 5 the growth increased from 0.66 to 5.88 O.D. Whereas at pH 9 the growth was 0.13 O.D. which was 5 times lower than at pH 5. Though the growth was observed at alkaline pH 9 to 10, the alkaline pH was not conducive (Fig. 4.9 A-E). TSK 71 exhibited similar trend of growth at pH 6 and 7whereas at pH 8 and 9 the growth slowed down. The optimum pH range for growth was pH 5 to 7. Antibacterial activity was observed at a pH range from 5 to 10 (Fig. 4.9 A-E). Maximum zone of inhibition (21.7 mm) was obtained at pH 6 which remained consistently high up to 48 h. Thus the production of the antibiotic compound varied with pH and was growth related.

Salt concentration :

The culture exhibited growth over wide range of salt concentration from 0 to 20% (Fig. 4.10 A-E). Unlike pH there was no uniform trend in the growth at lower salt concentrations and within 24h. However, at higher concentration (2-10% salt) and longer period of incubation, the growth increased and further increase in concentration and period of incubation brought about a decrease in the growth. The least growth was at 20% salt concentration. In case of antibiotic production, maximum production was observed within 16 h and was almost constant up to 10% salt concentration with time. Though there was a variation in the production, the zone diameter varied from 14 to 19 mm only. The graph (Fig. 4.10 A-E)

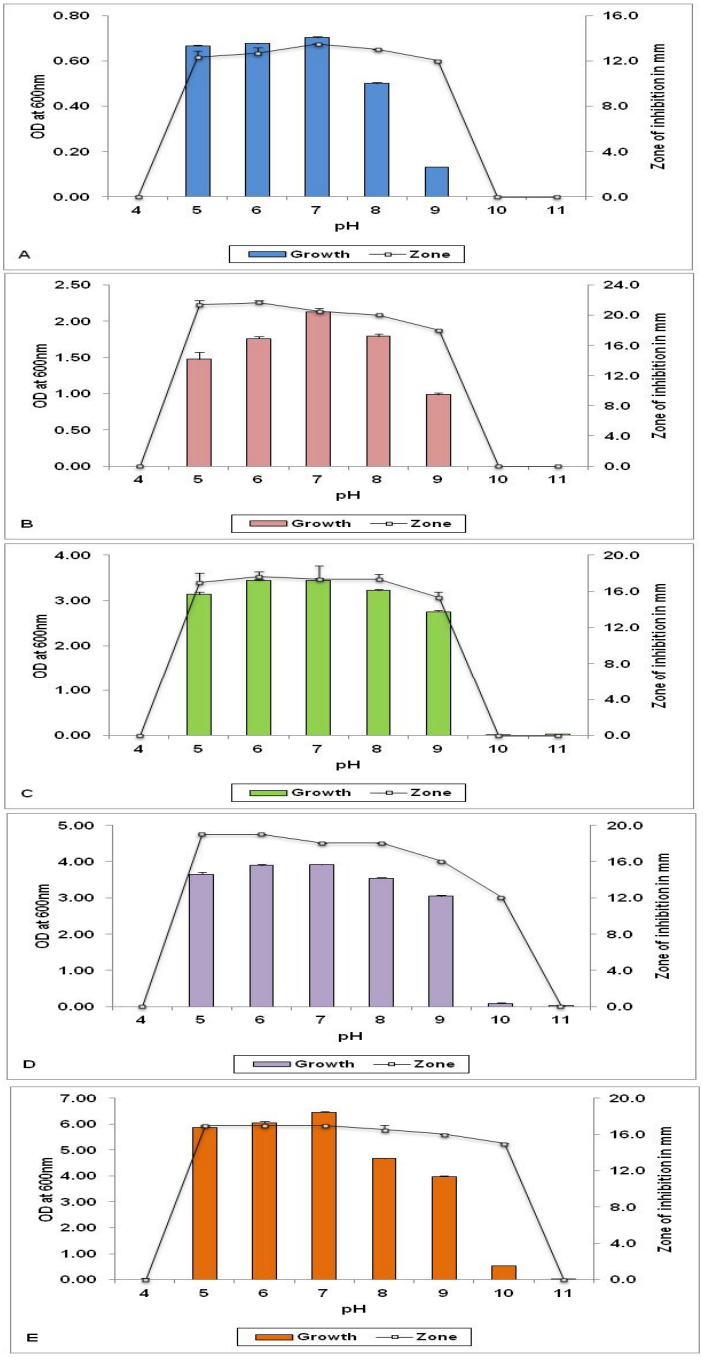


Fig. 4.9: Growth and production of antibacterial compound by TSK71 at various pH: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h (±Standard deviation, *n*=3). For Fig. 4.9 B to E, the actual OD has been equated (OD 2 = 0.2).

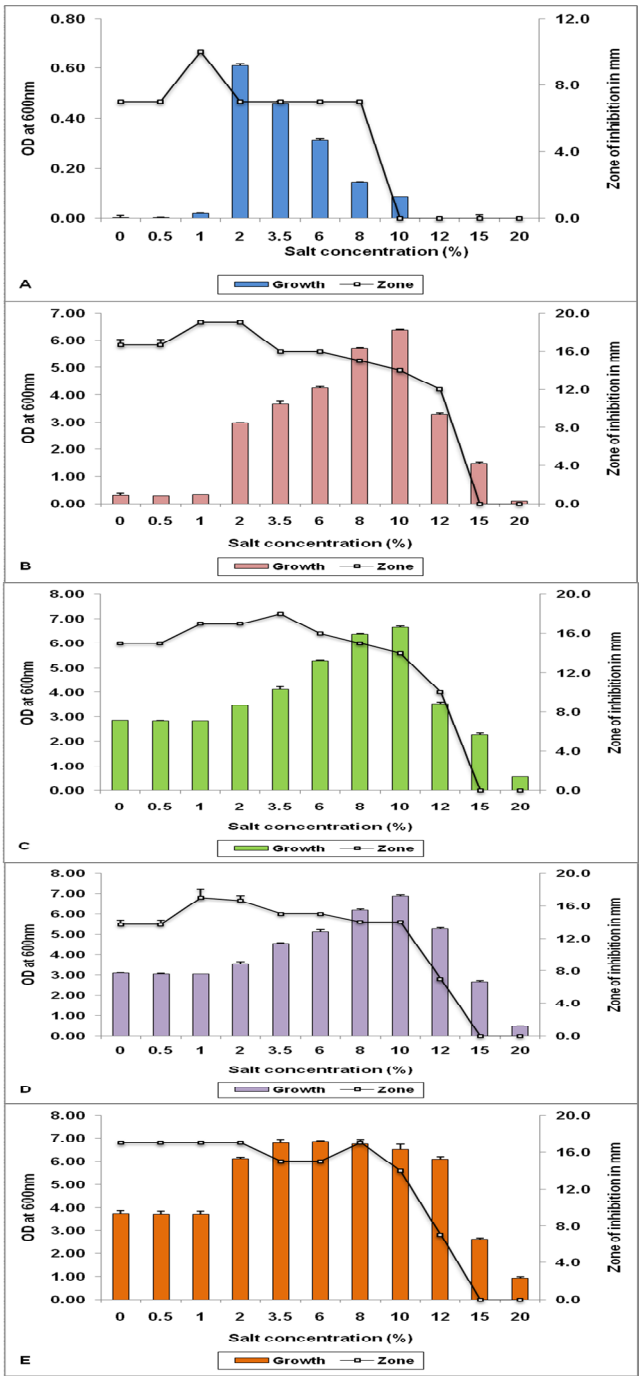


Fig. 4.10: Growth and production of antibacterial compound by TSK71 at various salinity: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h (±Standard deviation, *n*=3). For Fig. 4.10 B to E, the actual OD has been equated (OD 2 = 0.2).

shows variation in both narrow and broad range salinity. Though the production of antibacterial compound was seen at 0% salt concentration, 1% salt concentration seemed to be sufficient.

Carbon source:

The culture TSK 71 showed growth with all the carbon sources tested viz. arabinose, xylose, dextrose, mannose, fructose, sucrose, glycerol, sorbitol, mannitol (Fig. 4.11 A-E). However the growth varied with time. The culture showed similar trends in its growth pattern with dextrose, mannose, fructose, glycerol, and mannitol. Among the sugar alcohols, TSK 71 preferred glycerol and mannitol over sorbitol whereas among the monosaccharides dextrose and fructose were preferred over others indicating a preference for aldo and keto hexoses over pentoses. In general, among monosaccharide good growth was observed with dextrose after 24 h (0.63 OD.). After 48 h most of the carbon sources were utilized with sucrose showing the maximum growth indicating that the culture preferred a disaccharide over the monosaccharides. Antibacterial activity was detected in the presence of all the nine carbon sources tested (Fig. 4.11 A-E). Though growth was observed after 8 h, production of antibacterial compound with individual carbon source was observed only after 16 h. Among all the carbon sources, maximum production was observed in sucrose (0.4%) and it remained almost constant up to 48 h.

Varying concentration of sucrose (0.05 to 1%) showed that the growth increased with increase in time (Fig. 4.12 A-E). For 0.05% concentration the O.D. was 0.01 and reached a max of 0.4 O.D. whereas at higher concentration of 0.8% the growth increased 40 times more.

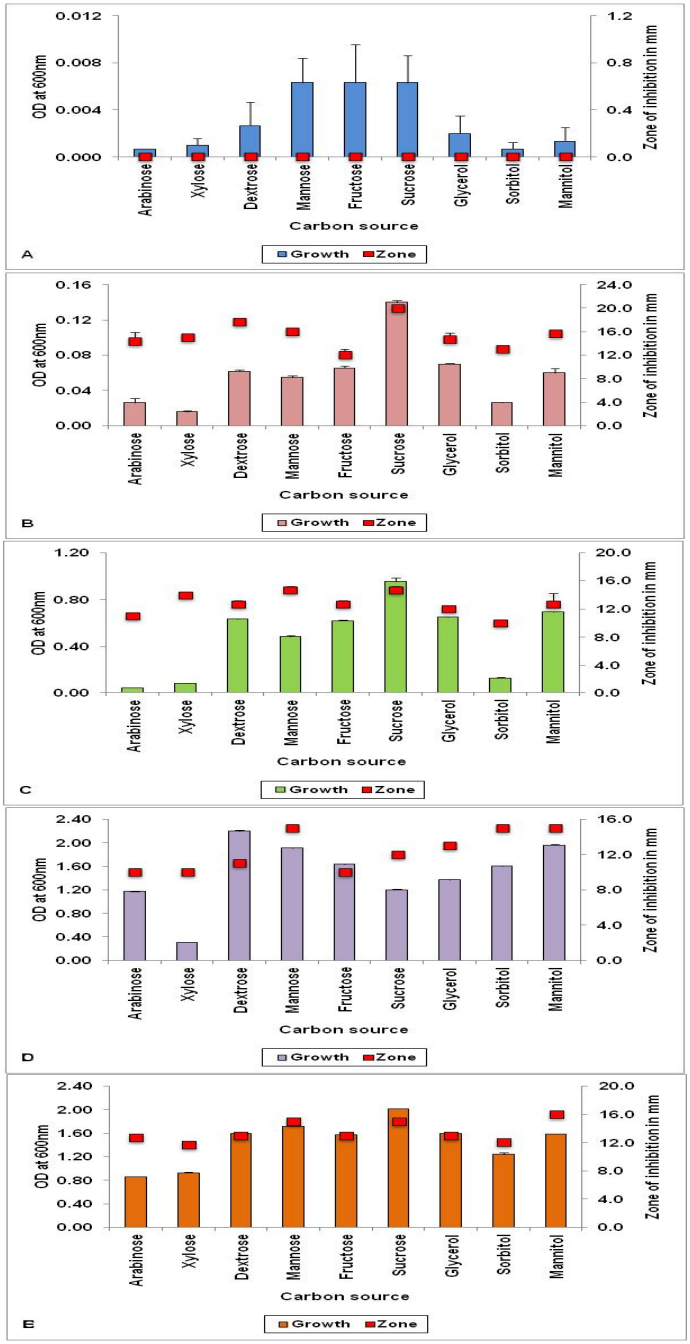


Fig. 4.11: Growth and production of antibacterial compound by TSK71 with different carbon sources: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h. For Fig. 4.11 C to E, the actual OD has been equated (OD 2 = 0.2).

Based on the growth study it was found that though the growth was slow at 0.8% initially with 0.01 O.D., it improved remarkably with time and maximum growth was attained at this concentration (2.14 O.D.). Antibacterial activity of TSK 71 was detected at 0.1 to 1% sucrose (Fig. 4.12 A-E). At 0.05% antibacterial activity was absent. At 16 h, maximum zone of inhibition (12 mm) was observed when 0.6% sucrose was used as carbon source. At 0.6% the zone remained consistently high till 48 h. Sucrose at 0.6% concentration was the best for production of antibacterial compound by TSK 71.

Nitrogen source:

All the eight nitrogen sources used supported growth as well as antibacterial production of TSK 71 (Fig. 4.13 A-E). In case of defined nitrogen sources (GA, U, A and PN) growth was comparatively less than that in case of complex nitrogen source (YE, BE, P, SP). Among defined nitrogen sources maximum growth was seen with GA at the end of 24 h (2.67 O.D.) and among complex nitrogen sources maximum growth was seen with BE within 16 h (2.13 O.D.). In the presence of defined nitrogen source, maximum production of antibacterial compound was seen with PN (0.2%) and in case of complex sources the production was nearly same with YE and BE. Highest zone of inhibition was noted with PN (22 mm).

Varying concentration of PN (0.2 to 1%) showed that the culture could grow at all the concentration and the growth was almost same at 48 h (Fig. 4.14 A-E). Maximum production of antibacterial compound was observed at 0.6% and the peak attained at 48 h.

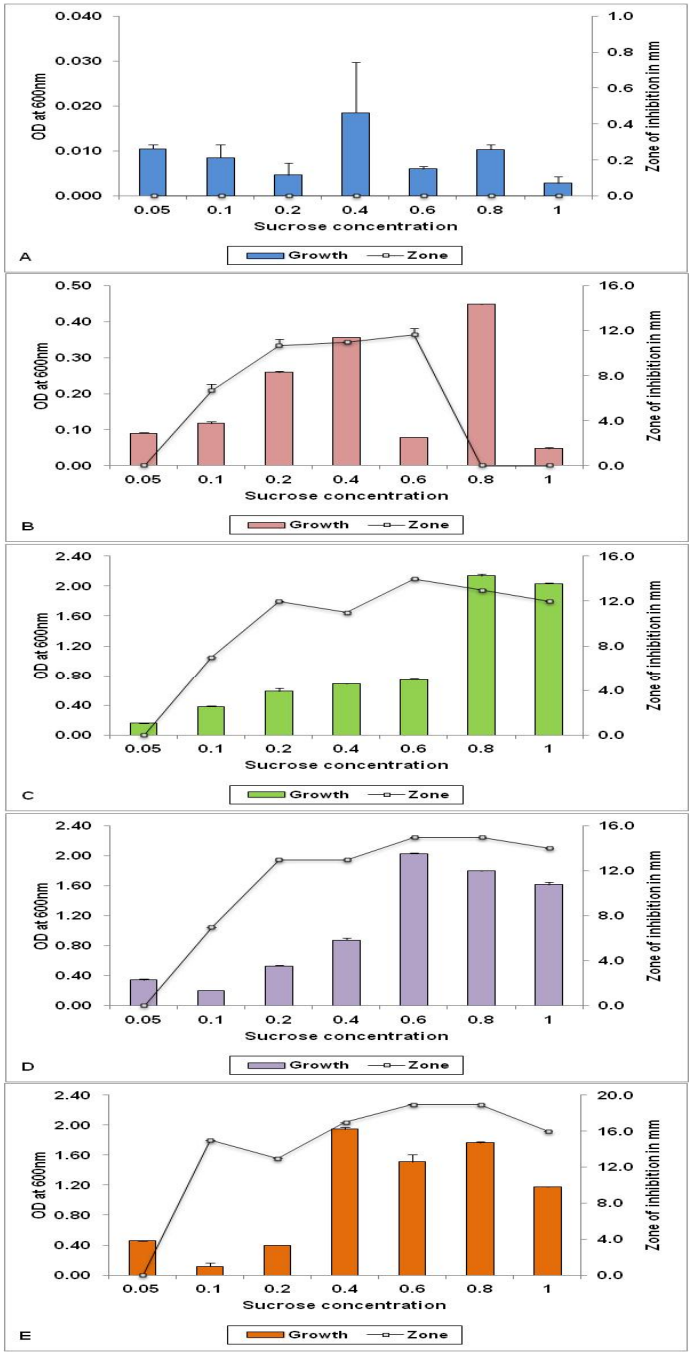


Fig. 4.12: Growth and production of antibacterial compound by TSK71 at various concentrations of sucrose: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h. For Fig. 4.12 C to E, the actual OD has been equated (OD 2 = 0.2).

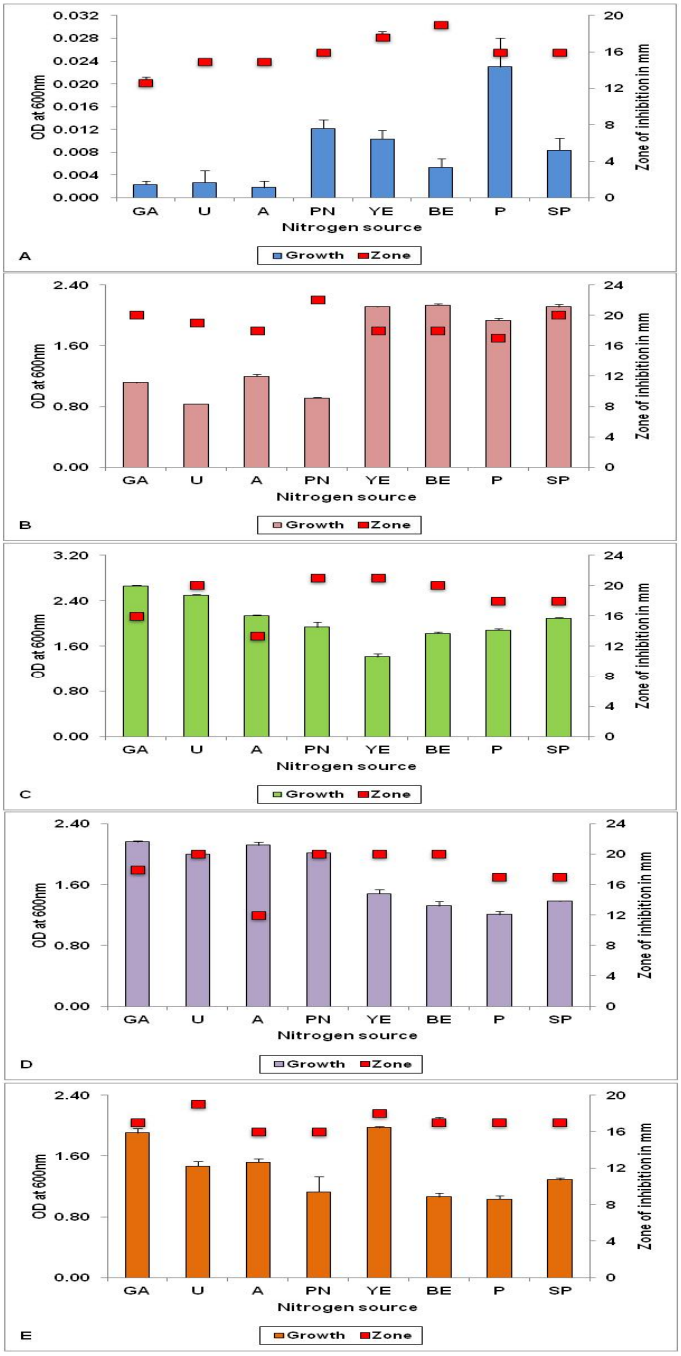


Fig. 4.13: Growth and production of antibacterial compound by TSK71 with different nitrogen sources: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h. For Fig. 4.13 B to E, the actual OD has been equated (OD 2 = 0.2).

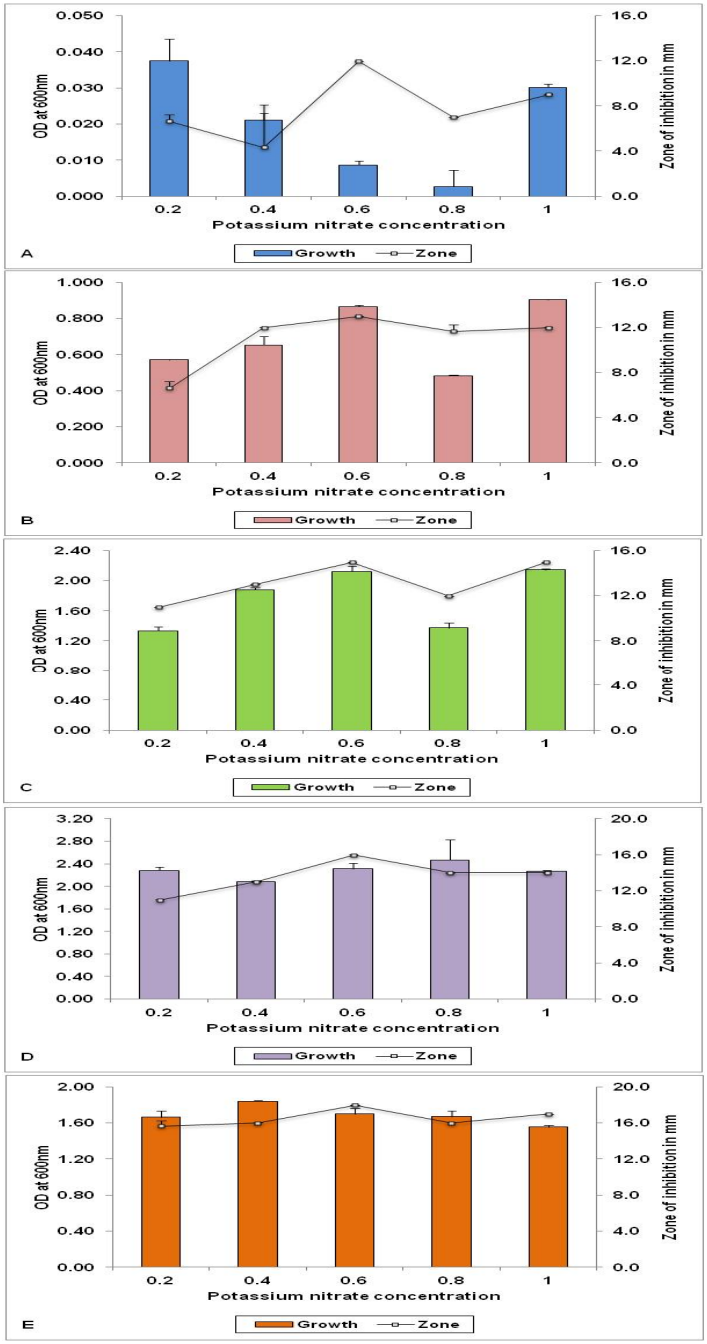


Fig. 4.14: Growth and production of antibacterial compound by TSK71 at various concentrations of potassium nitrate: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h. For Fig. 4.14 B to E, the actual OD has been equated (OD 2 = 0.2).

Temperature:

Preliminary growth conditions for temp showed that TSK 71 could grow from 20 to 55°C. However, good growth was observed between 25 to 45°C. Further studies in liquid medium showed that the maximum growth was at 37°C after 32 h (Fig. 4.15 A-E). Antibacterial activity was observed at RT (30±2°C) and 37°C. There was a significant variation in the production of antibacterial compound (F =18.3, P =0.00023and d.f. =2).

Aeration:

The culture showed enhanced growth as well as antibacterial activity when incubated on a shaker (Fig. 4.16 A-E). However, only growth and no activity was noted under static conditions. For further studies, the culture TSK 71 was incubated under shaker conditions (120 rpm). Significant variation was found between static and shaker conditions (F =14.12, P =0.0056 and d.f. =1) for antibacterial compound production.

Based on this analysis the optimum condition and media composition for maximum production were as shown in Table 4.4

Media composition	Quantity
5X M9 salt solution (Composition g/L: $Na_2HPO_4.7H_2O$ 64, KH_2PO_4 15, salt 2.5 and NH_4Cl 5)	200 mL
Salt	9.5 g
1M $MgSO_4.7H_2O$	2 mL
1M (Fused) $CaCl_2$	100 µL
Sucrose	6 g
Potassium nitrate	6 g
pH (set using 0.1 N HCl)	6.0
Distilled water quantity sufficient to make	1000 mL

Table 4.4: TSK Media composition for optimum production of bioactive compound from TSK 71

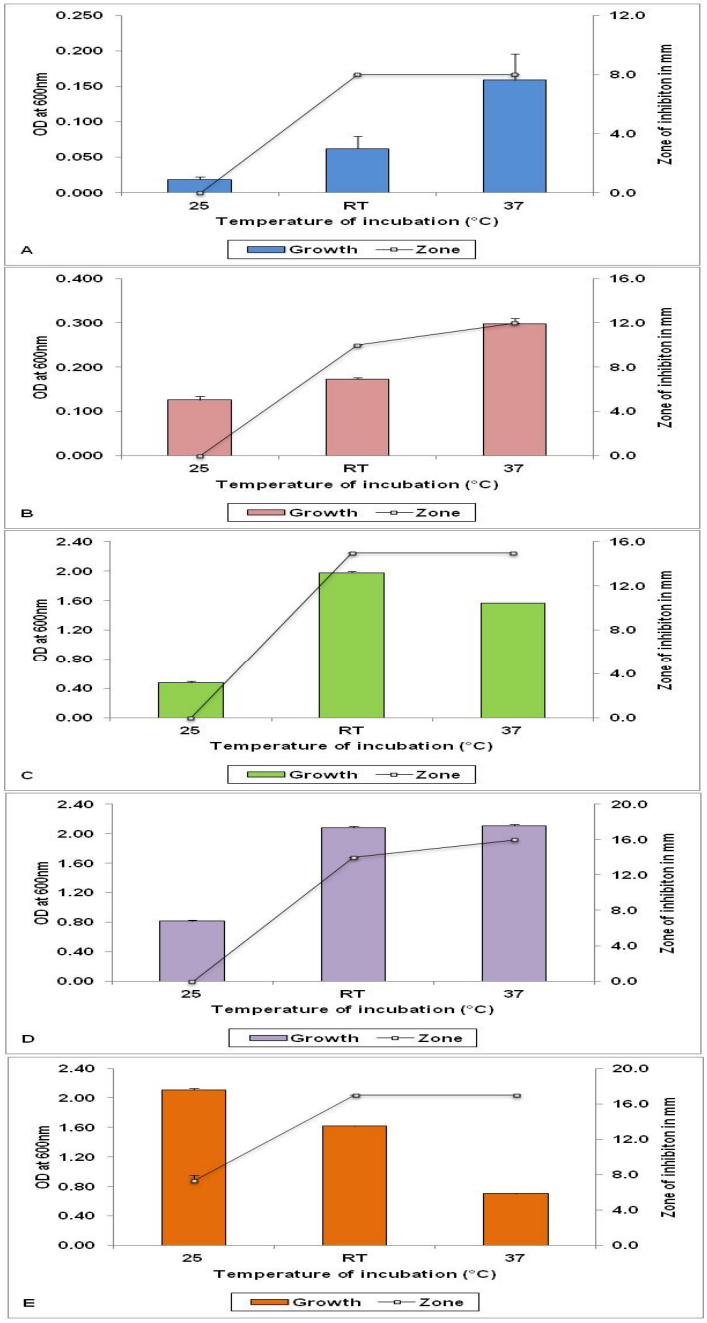


Fig. 4.15: Growth and production of antibacterial compound by TSK71 at various temperatures of incubation: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h (RT=30±2°C). For Fig. 4.15 C to E, the actual OD has been equated (OD 2 = 0.2).

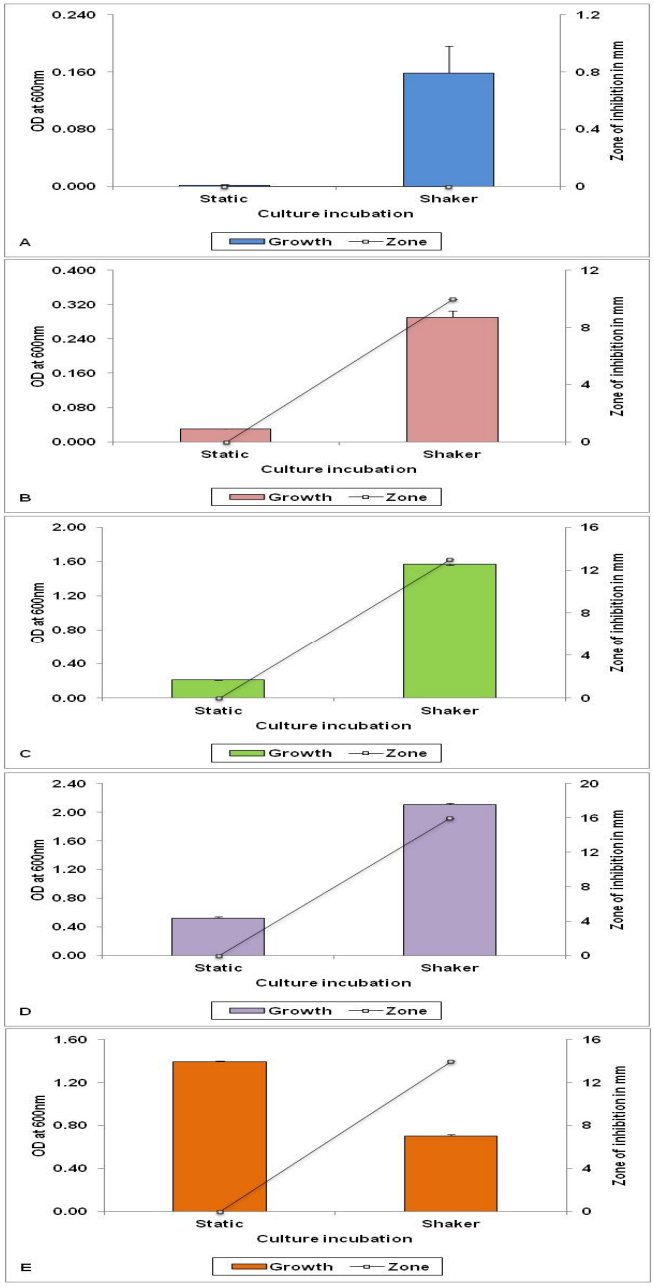


Fig. 4.16: Growth and production of antibacterial compound by TSK71 under static and shaker conditions: A: 8 h; B: 16 h; C: 24 h; D: 32 h; E: 48 h. For Fig. 4.16 C to E, the actual OD has been equated (OD 2 = 0.2).

4.4.1. Growth curve and production of *Bacillus licheniformis* TSK 71 in TSK media:

In TSK media, the culture exhibited a diauxic growth curve (Fig. 4.17) with a second lag phase from 18 to 24 h. The antibacterial compound was detected from 14 h and its production increased as the culture entered the second lag phase at 18 h. The antibacterial activity increased at the end of 24 h. During the second log phase, the highest antibacterial activity at 32 h (zone of inhibition 15.7 mm) was observed. The zone of inhibition remained constant after 48 h (14 mm) suggesting a constant amount of antibiotic being produced.

4.5. Characterisation of the antibacterial compound:

Purification of antibacterial compound:

The methanol extract was active against *Staphylococcus citreus* (SC). Furthermore, three out of the four fractions viz. F1, F3 and F4 were active against SC (Plate 4.4). Highest zone of inhibition was obtained with fraction F1 (25 mm ± 2.08, n = 3), followed by F3 (22 mm ± 0.58, n = 3) and F4 (20 mm ± 4.36, n = 3).

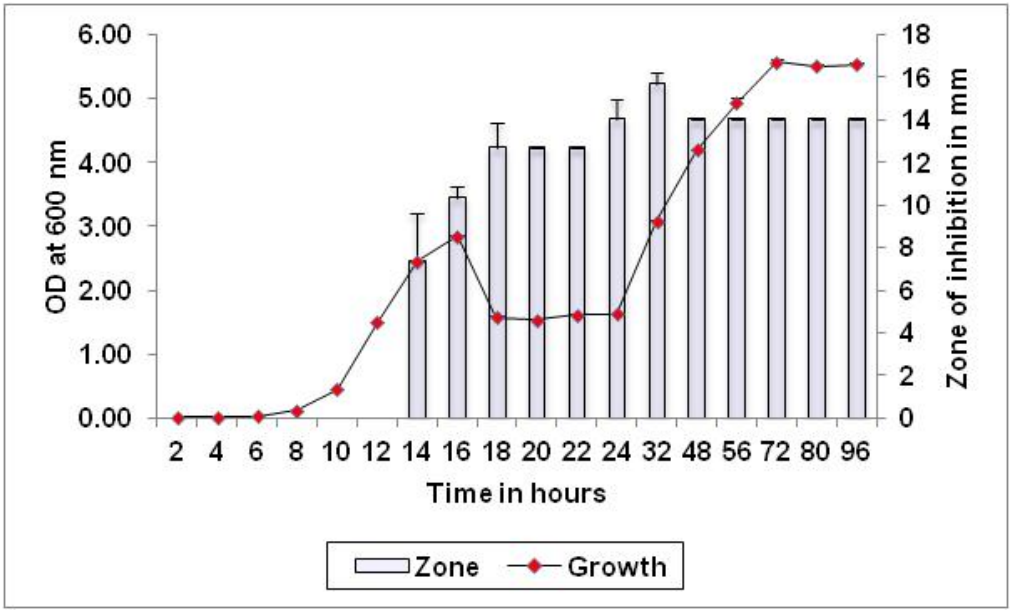


Fig. 4.17: Growth curve and production of antibacterial compound by TSK71 in TSK media

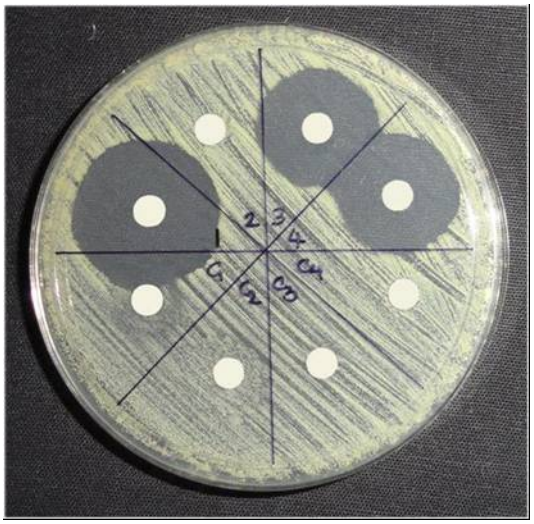


Plate 4.4: Bioassay of fractions showing antibacterial activity against *Staphylococcuscitreus*: 1-4: F1-F4 and C1-C4: respective solvent controls for F1-F4

TLC analysis:

TLC analysis showed pink spots, on detecting with ninhydrin reagent, in methanol extract and fractions F1, F3 and F4. The methanol extract showed mixture of 3-4 compounds. Fraction F1 was impure showing similar spots as in case of methanol extract. Fraction F3 had a similar spot as in F4 but F3 showed a more intense spot at $R_f = 0.57$. It also showed other spots as in methanol extract but with much less intensity. The pink spot with ninhydrin reagent indicates that the major compound in F3 could be with amine groups or peptidic in nature. Plate 4.5 shows the TLC profile showing crude methanol extract and F3 containing antibacterial compound from *Bacillus licheniformis* TSK 71.

Spectral analysis:

The electrospray ionisation mass spectrometer (ESI-MS) of F3 showed several doubly charged molecular ions in the region m/z 700-800 (Fig. 4.18). The m/z 712 is protonated doubly charged molecular ion $[M+H]^{+2}$. The peak at m/z 723 is sodiated adduct $[M+Na]^{+2}$ and m/z 731 is potassiated adduct $[M+K]^{+2}$. Thus, the protonated molecular ion is m/z 1424. The literature reports suggested that it could be bacitracin molecule. Bacitracin is a complex of closely related peptides which are produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* which exhibit activity against Gram-positive organisms (Azevedo *et al*., 1993). Bacitracin A is a peptide with molecular weight 1423 amu, containing a total of 12 amino acid residues: seven in a ring and five in a side chain. In bacitracin A, the N-terminal isoleucine (Ile) residue is condensed with the adjacent cysteine (Cys) residue to form an inter-residue 2-thiazoline ring. To confirm this, doubly charged ions were subjected to collision induced dissocation (CID).

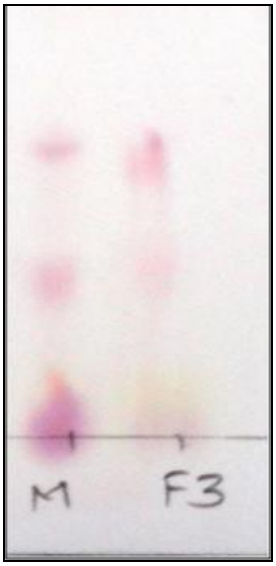


Plate 4.5: TLC profile showing crude methanol extract and fraction F3 containing antibacterial compound from *Bacillus licheniformis* TSK 71: M: methanol extract; F3: purified fraction 3

The fragment ions are designated as b and y ions according to the nomenclature by Roepstorff and Fohlman, 1984. The doubly protonated molecular ion at m/z 712 was subjected CID with a collision energy of 35%. The product ion spectrum is shown in Fig. 4.19. The spectrum contains all the y ions with m/z 869, 983, 1111, 1224 and 1339, generated from the cleavage of the peptide bonds to produce singly charged product ions. Besides this, the b ions are formed at m/z 199, 313, 441and 554. The product ions formed are depicted in the scheme 1. The prominent fragment ion with m/z 869 corresponds to the intact ring part of Bacitracin A. The fragment ions at m/z 227 and 356 corresponds to $[Lys\text{-}Asn]^+$ and $[Orn\text{-}Lys\text{-}Asn]^+$ of the ring part as shown in scheme 2. Accompanying these ions, is another ion formed at m/z 670 by loss of NH_3 ($b–NH_3$) at lysine as shown in scheme 1.

Further, the ESI-MS of the commercially available Bacitracin A was also carried out for the confirmation of the structure (Fig. 4.20). It showed similar doubly charged molecular ions in the regions m/z 700-800. The CID analysis (Fig. 4.21) of the standard Bacitracin A at m/z 711.9 was similar to that observed for F3. And thus, the molecule isolated from *Bacillus licheniformis* is Bacitracin A. Simultaneously, proton NMR of F3 and commercial bacitracin in deuterated methanol were also recorded. Fraction F3 showed similar profile as that of commercial bacitracin confirming that the major compound in F3 is bacitracin (Fig. 4.22 and 4.23).

In Fig. 4.20 and 4.21 please note that during this analysis the mass spectrometer was calibrated with standard Reserpine, hence the m/z values are more close to the true value while during the F3 analysis there is mass shift by 0.5 unit.

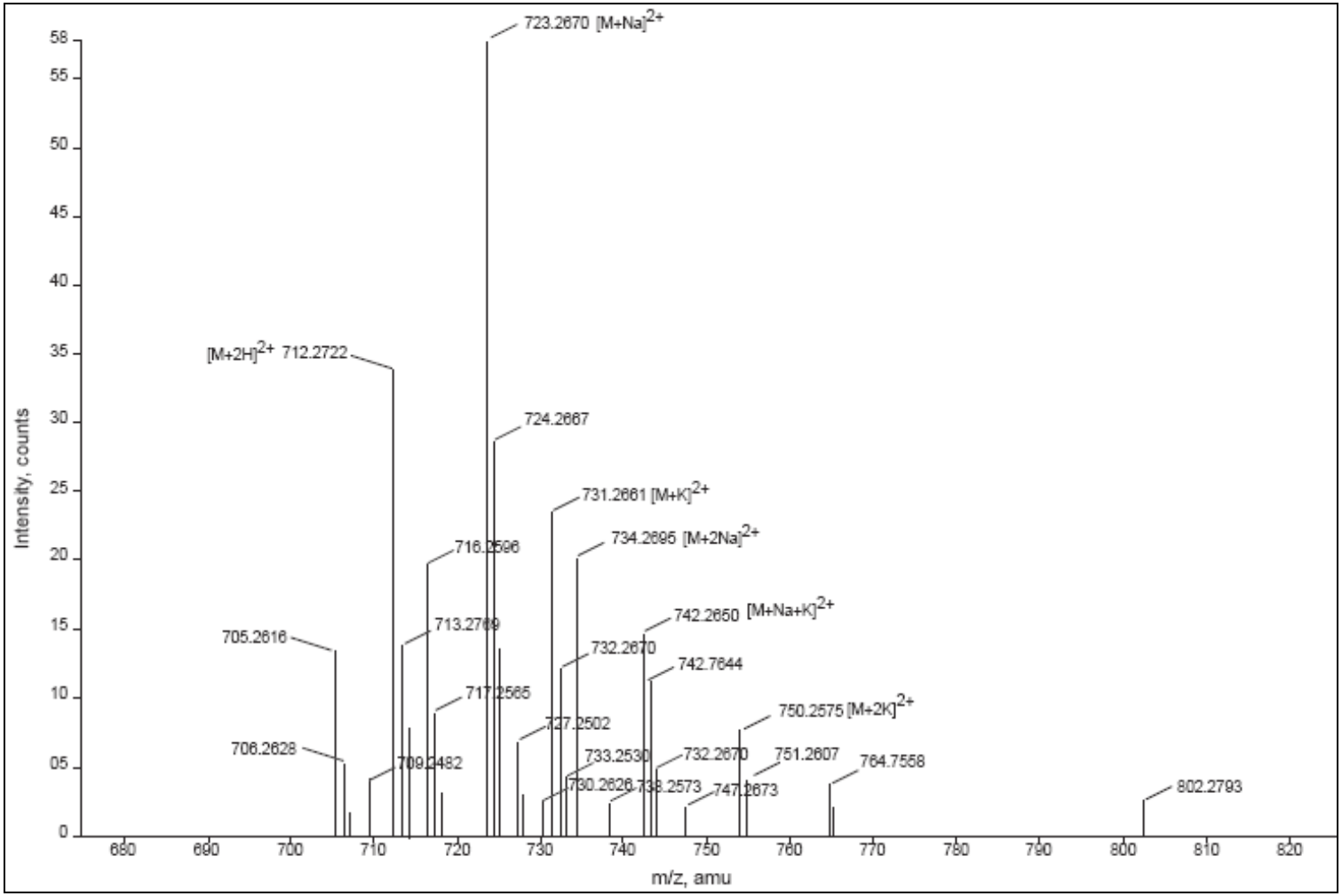


Fig. 4.18: ESI-MS spectrum of fraction F3

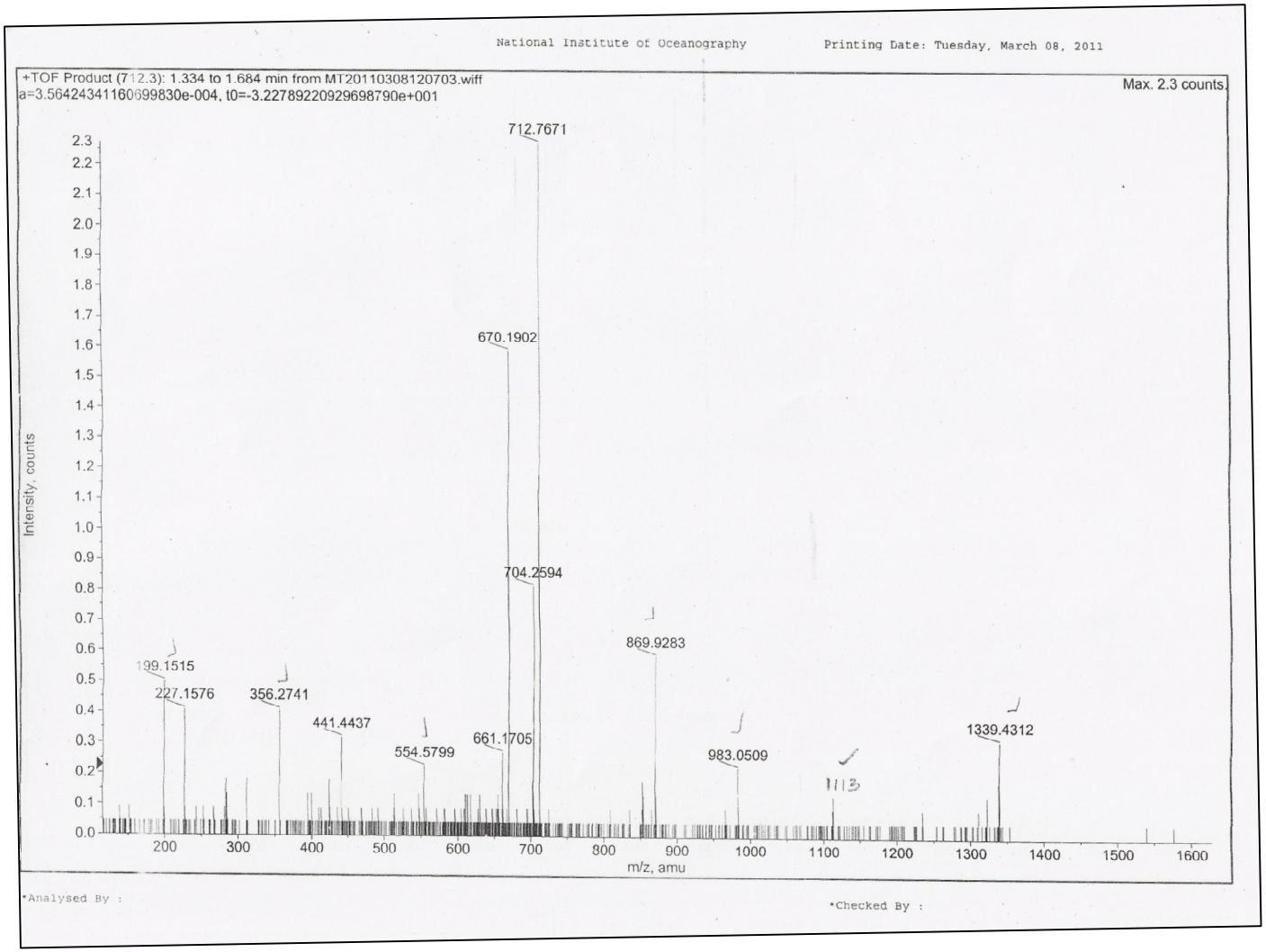
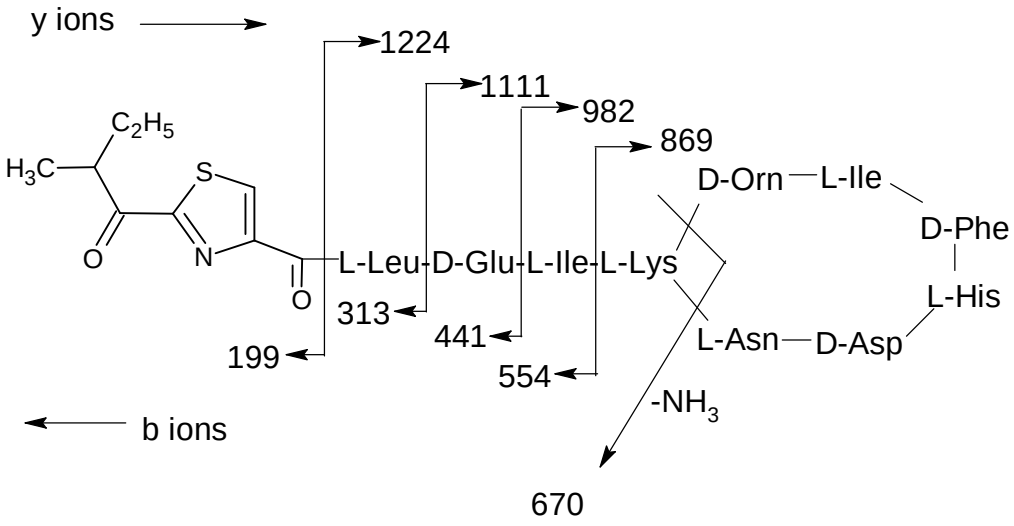
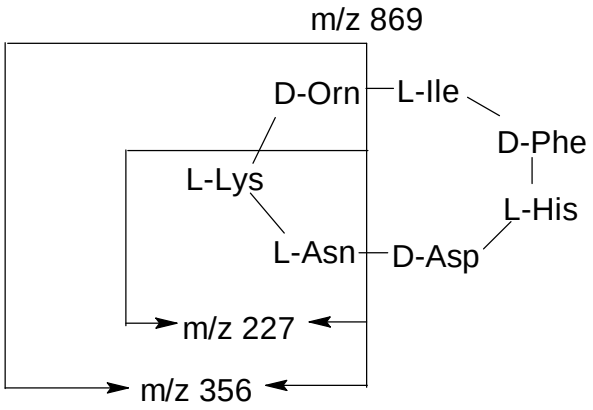


Fig. 4.19: Product ion spectrum at m/z 712.76 of fraction F3



Scheme 1: Mass fragmentation of Bacitracin A



Scheme 2: Mass fragmentation of ring structure of Bacitracin A

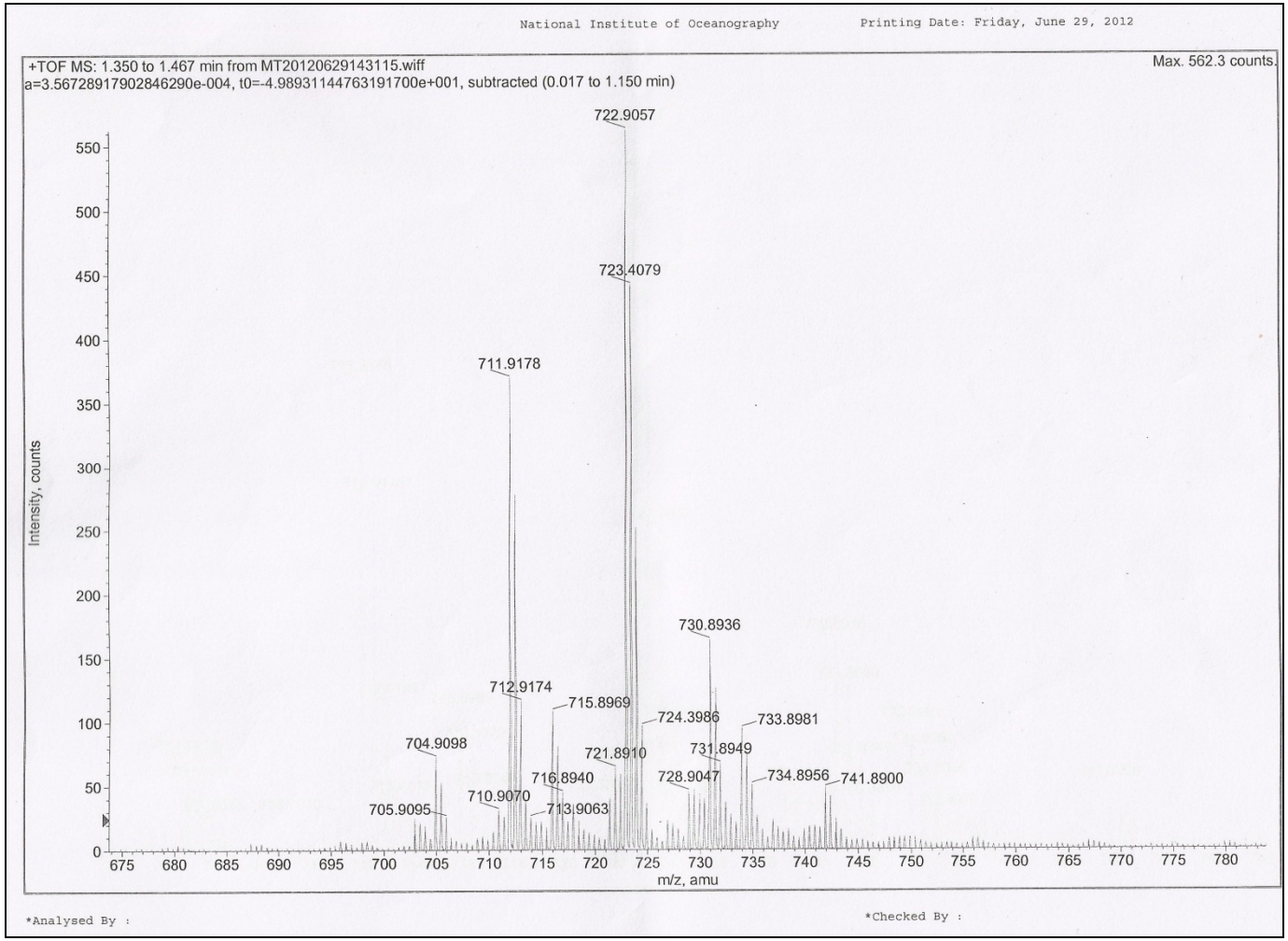


Fig. 4.20: ESI-MS spectrum of the commercial Bacitracin

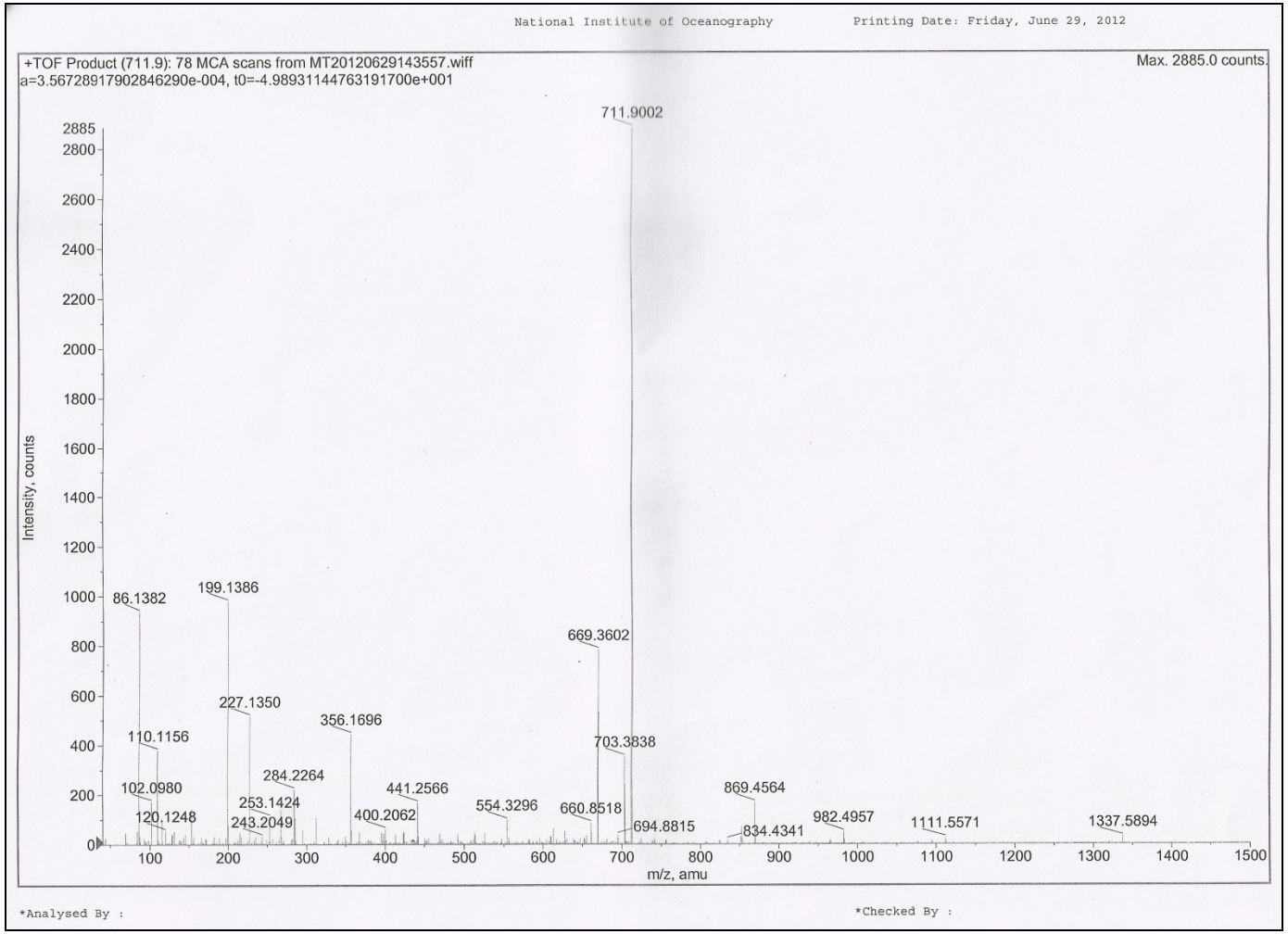


Fig. 4.21: Product ion spectrum at m/z 711.9 of commercial Bacitracin

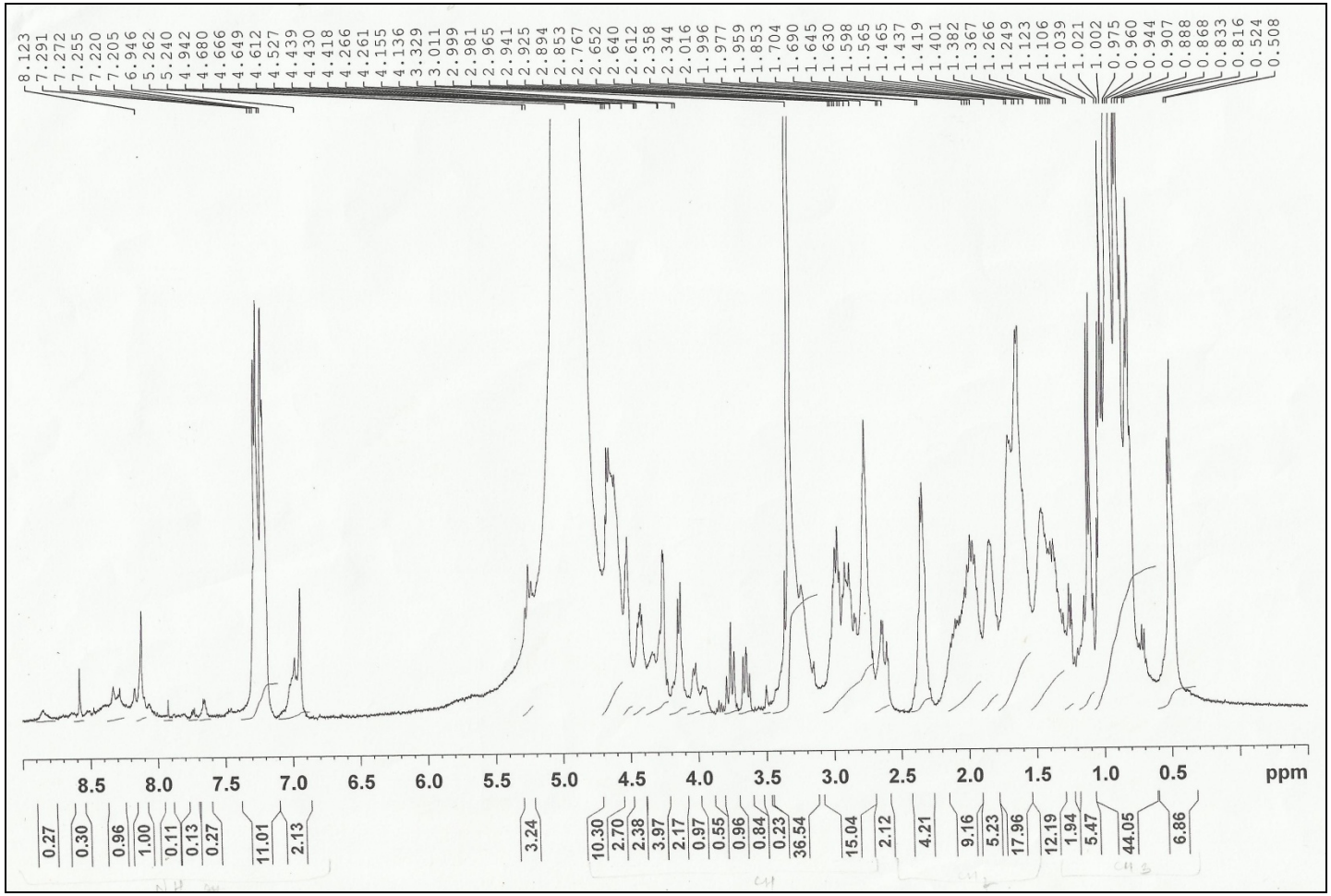


Fig. 4.22: Proton NMR spectrum of fraction F3

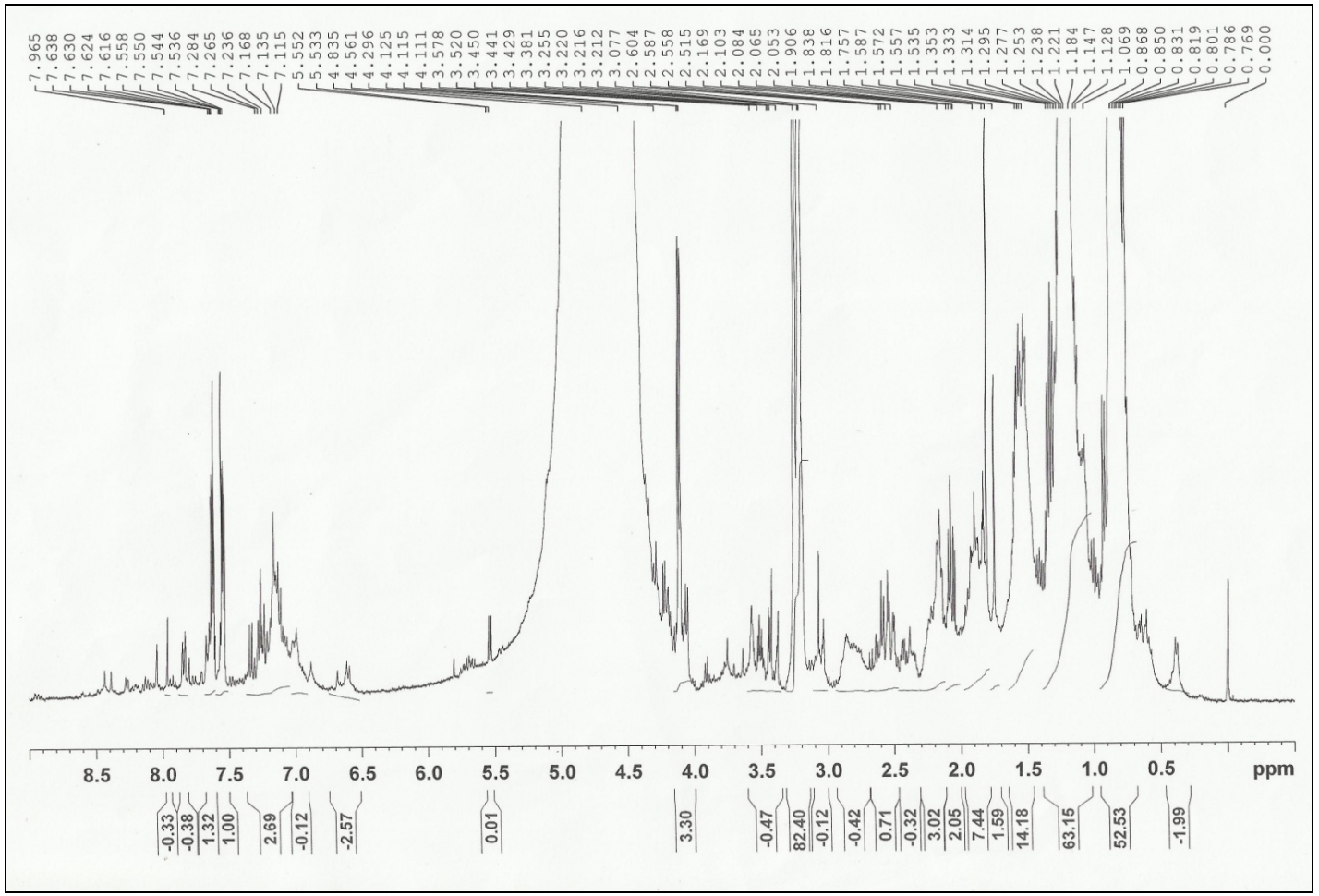


Fig 4.23: Proton NMR spectrum of commercial Bacitracin

TLC profiles of fraction F3 and standard Bacitracin were also compared. A slightly diffused spot at R_f = 0.57 in TLC profile of fraction F3 was observed as compared to the standard Bacitracin used.

4.6. Minimum inhibitory concentration (MIC) of the purified compound:

Table 4.5 shows zones of inhibition with variations in the concentrations of the lyophilized purified fraction 3. The MIC of the purified compound was 590mcg/ml when *Staphylococcus citreus* was the test culture.

Sample	Concentration (µg/ml)	Zone of inhibition (mm)
Undiluted	2950	15.7 (± 0.58)
1:2 dilution	1475	12
1:5 dilution	**590**	7.3 (±0.58)
1:10 dilution	295	0
1:20 dilution	147.5	0
1:50 dilution	59	0
1:100 dilution	29.5	0

Table 4.5: Concentrations used to determine MIC of the antibacterial compound

(±Standard deviation, *n*=3)

Chapter Five

Discussion

Solar salterns are found worldwide and located on or near the seashore. Seawater containing 5 to 20% sodium chloride is evaporated in the salterns and finally approaches 40%. Here the sodium chloride precipitates, leaving an overlying layer of water high in potassium and magnesium and still containing some sodium and calcium. These hypersaline environments originated by evaporation of seawater are called thalassohaline environments. It is classified as extremophilic environment. Microbes sense, adapt and respond to the combined high salt concentrations with extremely high pH values of this environment. In the present study, salinities ranged from 220 – 395 psu in both the salt pans. Such high salinity was recorded earlier from salt pans of Goa by Kerkar and Loka Bharathi (2007). Unlike East Coast salt pans, the pH of both the salt pans was less and ranged from 6.4 to 7.0 during sample caollection. Vijayakumar et al. (2007) reported the pH of salt pans of Vedaranyam, Thondi and Tuticorin in Palk Strait region of Bay of Bengal, India to be in the range 9.1-9.4 which are comparatively more alkaline compared to the Batim salt pan. The microbial life gets adapted to the hypersaline environments which support halophilic and halotolerant microorganisms (Manikandan and Kannan, 2009). Halophiles appear to be highly diverse in their metabolic potentials and have developed mechanisms to cope with the osmotic stress caused by the high salt concentrations. Vijayakumar et al. (2007) report actinomycetes to be omnipresent and that their population dynamics is often influenced by the available nutrients and the physico-chemical conditions of the ecosystem. Goodfellow and Williams (1983) report actinomycetes to represent a small fraction of the bacterial population in marine sediments. However a recent report by Lam (2006) suggests that indigenous actinomycetes not only exist in the oceans, but are also widely distributed in different marine ecosystems. Also culture-independent studies have revealed that marine environments contain a high diversity of actinomycetes that are seldom if at all, recovered

by culture dependent methods. In the present study 119 bacterial cultures, which include bacteria (94%) and actinobacteria (6%) were isolated on 8 different media i.e. media M1 to M7 prepared in sea water and NA prepared in distilled water supplemented with 5% salt. Occurrence of low density of actinobacteria had been reported from other saltpans such as Vedaranyam, Thondi and Tuticorin in Palk Strait region of Bay of Bengal, (Vijayakumar et al.,2007) and from Porto Novo and Thaikkalthurai, Tamil Nadu, India (Dhanasekaran et al., 2008-2009). It has been commonly observed that actinobacteria are inhibited from forming colonies on plates that are crowded with unicellular bacteria, which could be the reason for obtaining fewer colonies of actinobacteria since no pre-treatment or specialized method for exclusive isolation of actinobacteria was carried out.

Litchfield and Gillevet (2002) have studied the metabolic diversity and the phylogenetic complexity of the microbial communities in solar salterns and have asserted the need to develop more versatile media for the cultivation of diverse bacteria in hypersaline environments. Most of the isolation was done on antibiotic amended medium to prevent the bacterial and fungal growth. In present study, antibiotic additions were avoided as the aim was to isolate bacteria as well as actinomycetes on diverse media (8 different media). Manikandan and Kannan (2009) assessed the diversity of cultivable microorganisms in three different media from three solar salterns along the shoreline of Bay of Bengal in Tamil Nadu, India. Interestingly, even such hostile environments periodically support diverse morphotypes. M6 which was peptone yeast extract iron agar was found to be the most preferred media for isolation since it retrieved the highest number (33) of morphotypes. There was variation in the morphotypes depending on the site and source as 97% showed dense growth on M2 media followed by M1 (87%). In the present study, most of the isolates (79%) did not require salt for growth, nearly 50% isolates could

tolerate up to 10% and 61% isolates could tolerate up to only 5% salt. Magarvey et al. (2004) reported that strains of *Micromonospora* could tolerate up to 4% salt but did not require the presence of salt for growth. Numerous reports affirm that in general, actinomycetes isolated from marine sources are of terrestrial origin and reside in the marine ecosystem as spores or resting propagules (Jensen et al., 1991). Halotolerant bacteria are known to survive and even flourish in a relatively low salt environment such as sea water (Grant et. al., 1998). Thus we speculate that some of the salt tolerant bacteria are likely to have a terrestrial origin, invading the salt pans from the surrounding ecosystems. Litchfield et al. (2000) have described the NaCl-dominated salt pans to be inhabited by a rich variety of microorganisms, and each pond with a characteristic flora adapted to the prevailing salt concentration, ranging from seawater to NaCl saturation. They also emphasized that though reports on the biology of solar saltern systems in various parts of the world suggest a high degree of similarity in the microbiology some differences may be prevalent due to the changes in incident radiation, temperature, nutrient availability, residence time in the ponds etc.

The antibacterial compound producing microorganisms, detected using cross streak method, in these salt pans were considerably high and 33% displayed either bacteriostatic or bactericidal activity. Usually 10-25% of the marine bacteria are known to display antibacterial activity; however Chen et al. (2010) reported that more than 50% of the halophilic bacteria tested exhibited some antibacterial activity. Salt pans which are influenced by high human activity and surrounded by mangroves with organic nutrients derived from metabolic processes or death and decay of plant biomass and constant nutritional inputs from external sources alleviate the necessity for bacterial populations to exhibit antibiosis to survive competition. Among the 31 active isolates, 35.5% were from

sediment while 64.5% were isolated from overlying waters of the salt pan. Out of the six most promising isolates one was from the sediments whereas 5 were from the water and 66.7% were from Batim, where four showed antibacterial activity and 33.3% were from Ribandar, which included two isolates showing activity. Most antibacterial studies from salt pans have concentrated on only actinomycetes. These studies have revealed very low % of antibacterial activity (Suthindhiran and Kannabiran, 2009). Though actinomycetes were isolated from salt pans only two cultures produced antibacterial compound and were not very consistent. One hundred and sixteen strains were isolated from the Marakkanam coast of the Bay of Bengal, on screening for bioactive marine actinobacteria, only 7 isolates exhibited broad spectrum activity. *Saccharopolyspora salina* VITSDK4 was profoundly antagonistic with fungal and Gram positive pathogens (Suthindhiran and Kannabiran, 2009). Dhanasekaran et al. (2005) have reported three actinomycetes, from salt pan regions of Cuddalore and Parangipettai, Tamil Nadu, India, showing promising antibacterial activity against eight test organisms. Dhanasekaran et al. (2008-2009) have also reported six broad spectrum antibacterial *Streptomyces* out of nine (66.7%) from salt pan soil. In this study, out of the 31 actives 93.5% were bacterial isolates.

Kamat and Kerkar (2003) have reported a halotolerant *Acinetobacter* sp. from salt pans of Ribandar, Goa, antagonistic to MRSA. In another study, on antimicrobial activity screening, by Chen et al. (2010) it was observed that most of the isolated halophilic bacterial strains from Weihai solar saltern, China inhibited Gram positive bacteria, human pathogenic fungi and plant pathogenic fungi. In the present study, only 6% of isolates inhibited Gram positive test cultures whereas 58% inhibited Gram negative and 36% could inhibit both Gram positive as well as negative test cultures. Gram-positive bacteria are generally more susceptible to antibiotics than Gram-negative bacteria (Peláez et al., 1998).

In the present study, strains producing antibacterial compound belonged to the genus *Bacillus*. The phylogenetic study by Ettoumi et al. (2009) confirms the widespread of the *Bacillales* members in deep-sea environments and highlights their large diversity based on culture-dependent approach. Generally *Bacillus* spp. is abundant in marine sediments and very scarce in marine waters. However, 4 of the 6 most active strains have been isolated from the overlying salt pan waters. Being ubiquitous in the marine environment explains the fact that they could survive in diverse conditions such as overlying sea water and sediments of salt pans. Phylogenetic analysis of moderately halophilic bacteria isolated from the Weihei solar saltern in China has revealed that Gammaproteobacteria and Firmicutes were predominant among the isolates (Chen et al., 2010). Occurrence of *Bacillus* in salt pans is already reported from salt pans over the world (Lim et al., 2006; Nowlan et al. 2006; Pappa et al., 2010).

In the present study, broad spectrum antibiosis was exhibited by isolated *Bacillus* spp., suggesting that this genus was one of the dominant groups producing antibacterial compounds in the salt pans. The present isolate, a *Bacillus licheniformis* producing an antibacterial compound has not yet been isolated from salt pans. Govender et al. (2009) have reported isolates Sua-BAC012, Sua-BAC017 and Sua-BAC019 affiliated with *Bacillus licheniformis*, from Sua pan evaporator ponds in Botswana, capable of producing different degrading enzymes. Sadfi-Zouaoui et. al. (2008) have illustrated that some *Bacillus* strains, including *Bacillus licheniformis* isolated from different hypersaline soils of Tunisian Sebkhas produced an antifungal compound. The Strain TSK 71 which produced maximum antibacterial compound consistently was characterised as a moderately halophilic, Gram positive, facultative anaerobe *Bacillus licheniformis*. Moderately halophilic species belonging to the genera *Bacillus* have been reported from

salt pans (Kerkar, 2004). Facultative anaerobic characteristic are found in diverse niches, commonly found in soil, sediments and microniches of anoxic waters. Ettoumi et al. (2009) have reported 96 phylogenetically diverse bacilli from the marine sediments of 17 distinct stations of 5 oceanographic campaigns which included *Bacillus licheniformis*. Though literature provides valuable information, numerous differences exist between various strains of *B. licheniformis* and thus the characteristics of each strain have to be examined individually. *Bacillus licheniformis* strain TSK 71 has been isolated from the overlying waters of Batim salt pan which may have possibly gained entry in these waters from spores surfacing the plumage of some indigenous or migratory birds and has adapted to the hypersaline waters over time. This strain has been isolated at a salinity of 395 psu. However laboratory experiments showed a good growth of this strain at 10% salt (sodium chloride) and a tolerance range from 0-20% salt. *Bacillus* strains from marine origin are not in reality known to display specific traits (Ettoumi et al., 2009). Even halotolerance, a property thought to be a characteristic of marine bacteria is not exclusive and the majority of marine bacilli do not depend on sea water containing media for their growth. Only *B. marinus* proved to be dependent on sodium and potassium ions for growth and is regarded as true marine species (Ruger and Richter, 1979). More recent reports, Ruger et al. (2000) readdress the question on the existence of obligate marine *Bacillus* sp. This strain luxuriantly grew at 37°C and could tolerate temperatures as high as 55°C. The temperature of overlying waters of the Batim salt pans fluctuated from 28-50°C and this strain was isolated at 45°C. The pH of the salt pan water during isolation was 6.43 which were comparable to the optimum pH for growth in the laboratory. *Bacillus licheniformis* TSK 71 could utilize arabinose, xylose, dextrose, mannose, fructose, sucrose, glycerol, sorbitol and mannitol. Antimicrobial susceptibility test was carried out for TSK 71 and results were recorded as sensitive or resistant to the antibiotic

concentration tested. The candidate culture in the present study *Bacillus licheniformis* TSK 71 was sensitive to most of the antibiotics tested including cefuroxime, cephalexin, gentamicin, methicillin and tetracycline but was resistant to ceftazidime, clindamycin, erythromycin and lincomycin.

It is well known that the production of most of the bioactive compounds depends on the composition of the culture medium. The general idea in designing a culture medium for any microorganism is to provide a balanced mixture of the required nutrients at concentrations that will allow good growth and produce maximum desired compound (Nair and Simidu, 1987). In the present study, the optimum conditions such as pH; salt concentration; carbon source, nitrogen source and their concentrations; temperature and aeration for growth as well as production of antibacterial compound were assessed. Optimal growth of *B. licheniformis* TSK 71 was observed between pH 5 to 7 and 10% salt concentration when incubated at 37°C. However, optimal antibacterial activity was observed at pH 6 and 1% salt concentration when incubated at 37°C under aerobic condition. *B. licheniformis* TSK71 could grow under static as well as shaker conditions indicating that aeration was not an essential parameter for growth. However, aeration was an indispensable parameter for antibiotic production. Variation in the optimum growth and production of bioactive compounds by different strains of *B. licheniformis* from different regions has been reported earlier. For example Williams et al. (1990) have reported a strain PWD-1 which showed optimum rate of growth in nutrient broth at 45 to 50°C and pH 7.5. Martirani et al. (2002) have reported a thermophilic *B. licheniformis* 490/5 with optimal growth rate at 65°C producing a bacteriocin. *B. licheniformis* B3-15, from a marine hot spring, grew aerobically from 25 to 60°C with an optimum temperature of 45°C; pH range 5.5–9 with an optimum at 7 and a range 0–7% (w/v) NaCl with

optimum 2% (w/v) NaCl (Maugeri et al., 2002). Yakimova et al. (1997) have reported four strains of *B. licheniformis* isolated from Northern German oil reservoirs at depths of 866 to 1520 m which could grow on a variety of substrates at temperatures of up to 55°C and at salinities of up to 12% NaCl. The strains also produced extracellular polymer both aerobically and anaerobically over a wide range of temperatures, pressures and salinities, though the production was optimal at temperatures around 50°C and at salinities between 5 and 10% NaCl. While evaluating the best carbon source maximum antibacterial activity was observed when sucrose (0.6%) was used, suggesting the preference of a disaccharide (sucrose). Antibacterial activity was consistently high in sucrose. Sucrose therefore was the growth limiting nutrient which also influenced the rate of spore formation and the activity. Sucrose as preferred substrate was shown for *Bacillus licheniformis* cluster by Pukall et al. (2008) and Ghaly et al. (2007). It could be one of the strategies (osmoadaptation) of all three domains of life from hypersaline environment to exclude salts from the cytoplasm as much as possible, and to accumulate organic solutes to provide osmotic balance (Oren, 2002). Maximum growth and antibiotic production was observed with beef extract (0.2%) and potassium nitrate (0.6%) respectively. Beef extract is a complex nitrogen source of animal origin whereas potassium nitrate is an inorganic source of nitrogen. The study indicates that different sources of nitrogen are required for growth and antibiotic production. Haavik (1981) has also reported the effects of amino acids upon antibacterial compound like bacitracin production by *Bacillus licheniformis* as amino acids are precursors of both bacitracin synthetase and bacitracin itself. It is likely that some of the amino acids participate in control mechanisms for bacitracin formation. Anthony et al. (2009) demonstrated that high concentration of yeast extract, alkaline pH and elevated temperature improved the production of antibacterial peptides by *B. licheniformis* AnBa9 whereas magnesium sulphate, starch and soybean meal enhanced

the production of enzymes in *Bacillus licheniformis* NCIM-2042 (Bhunia et al., 2010). The present study showed that the production of antibacterial compound by *Bacillus licheniformis*, from the saltpan was enhanced with potassium nitrate (0.6%) and sucrose (0.6%). Sharma et al. (2010) have described antifungal activity of hexane extract of culture filtrate of *B. licheniformis* MTCC 7445. The culture when grown in production media with initial pH 6.0-6.2 produced secondary metabolites such as 1-methyl pyrrolidene, 1-methyl cyclohexene, 4,4-dimethyl cyclohexane, ethyl-4-ethoxybenzoate, 2-butoxyethanol, naphthalene, ter butyl benzene and phenoxy acetic acid with antifungal activity. Lichenicidin VK21, Lchα and Lchβ, a novel two-component antibiotic with potential in the development of new drugs was isolated from *Bacillus licheniformis* VK21 at 45°C (Shenkarev et al., 2010).

Bacillus licheniformis is a close relative of *Bacillus subtilis*, with wide applications in biotechnology, used in the manufacture of enzymes, antibiotics, growth hormone (Prashanth and Mathivanan, 2010; Li et al. 2010), biochemicals and consumer products. It is a well studied industrial organism and has proven to be a source of many therapeutically useful molecules such as bacteriocins, lichenicidin etc. *Bacillus* antibiotics share a full range of antimicrobial activity: bacitracin, pumulin, laterosporin, gramicidin and tyrocidin are effective against Gram-positive bacteria; colistin and polymyxin are anti-Gram-negative; difficidin is broad spectrum; and mycobacillin and zwittermicin are anti-fungal. Antibiotics produced by the aerobic spore formers are usually, but not always, polypeptides. *Bacillus licheniformis* strain P40 produces a bacteriocin-like substance (BLS), in TSB broth at 30°C for 48h (Teixeira et al., 2009). Several strains of *B. licheniformis* are known for the production of bacitracin, but only a few have been reported to produce other types of antibiotics, such as the phosphorus-containing triene

proticin (Nesemann et al. 1972), bacilysin (Kluger et al. 1990) and the licheniformins (Callow and Work 1952). Gálvez et al., 1993 have reported the isolation, purification and characterization of the antifungal peptide A12-C produced by *B. licheniformis* A-12. The antibacterial compound of *Bacillus licheniformis* TSK 71 from the Batim salt pan was Bacitracin A and production depended on the nitrogen and carbon sources. Such factors influencing the production of bacitracin by *B. licheniformis* has been reported earlier (Hanlon and Hodges, 1981). Though it is not the first report of bacitracin from *Bacillus licheniformis* but for the first time it is reported from a salt pan isolate.

The bacitracin antibiotics consist of a group of closely related cyclic dodecapeptides. They are peptide antibiotics produced non-ribosomaly by some strains of *Bacillus licheniformis* and *Bacillus subtilis* (Azevedo *et al.*, 1993; Ohki *et al.*, 2003) and function as an inhibitor of cell wall biosynthesis by inhibiting dephosphorylation of lipid pyrophosphate. Different types of bacitracins like A, A1, B, C, D, E, F, F1, F2, F3 and G have been isolated. Of these, Bacitracin A has been considered most potent antibiotic, whereas Bacitracin B & C are less potent and the rest of the bacitracins possess very little antibacterial activity. In the present study, mass spectral analysis revealed a doubly charged protonated molecular ion $[M+H]^{2+}$ at m/z 712.3 for protonated molecular ion $[M+H]^{+}$ at m/z 1424. The literature reports suggested that it was comparable to Bacitracin A. The result was confirmed by product ion spectrum at m/z 712.3 and also by comparison with ESI-MS and product ion analysis of commercially available bacitracin. Bacitracin A is a peptide with molecular weight 1423 amu, containing a total of 12 amino acid residues: seven in a ring and five in a side chain. In bacitracin A, the N-terminal isoleucine (Ile) residue is condensed with the adjacent cysteine (Cys) residue to form an inter-residue 2-thiazoline ring. In this study, it was confirmed by subjecting doubly

charged ions to collision induced dissocation (CID). The prominent fragment ion with m/z 869 was found to correspond to the intact ring part of Bacitracin A. The fragment ions at m/z 227 and 356 corresponded to $[Lys\text{-}Asn]^+$ and $[Orn\text{-}Lys\text{-}Asn]^+$ of the ring part. Another ion was formed at m/z 670 by loss of NH_3 ($b–NH_3$) at lysine. The proton NMR spectra of fraction F3 and commercial Bacitracin were also comparable confirming that the major compound in F3 is Bacitracin A.

During the initial screening *B. licheniformis* TSK 71 showed antibiotic activity against Gram positive as well as Gram negative test pathogens. However, after further purification of the compound from TSK 71 antibiosis was prominently seen only against *Staphylococcus citreus*. Bacitracin antibiotic is known to be most effective against Gram positive and a few Gram negative species of bacteria (Brunner, 1965). Bacitracin may be bactericidal or bacteriostatic in action. It inhibits bacterial cell wall synthesis by preventing the incorporation of amino acids and nucleotides into the cell wall (Stone, 1971). The commonly occurring bacitracins are relatively stable in acidic solutions and unstable above pH 9. The bacitracin isolated in the present study was stable at pH 6 to 9 since during optimization studies pH of medium increased from 6 to 9 and the activity was retained. Perhaps this mechanism facilitated the organism to thrive in salterns which are normally alkaline. It is very soluble in water and methanol. Also the fraction F3 could retain antibacterial activity against *Staphylococcus citreus* for up to 1 year when stored at 4°C.

A MIC value of a compound implies its probability for pharmaceutically useful (Rios et al., 1988). MICs are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. They are used as

a research tool to determine the in vitro activity of new antimicrobials. The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration. For disc diffusion assays, the amount of test compound which diffuses into the agar medium is not uniform, making it difficult to quantify MIC values. However, the definition of MIC value is not always clear. In diffusion assays, MIC is defined as the lowest concentration of test compound producing a visible inhibition effect. While in dilution assays, MIC is defined as the lowest concentration of test compound totally inhibiting growth. The MIC of the purified compound was 590mcg/ml against *Staphylococcus citreus*. Lipoamides showed no antimicrobial activity against *S. aureus* and *P. aeruginosa* with MIC values above 100 µg/ml. Reasons for the relatively high MIC values could be that the extracts tested are still in a partially pure form, or that the active compounds are present in very low concentrations.

Ecological significance:

Antibiotics function to kill or inhibit the growth of other organisms. Different mechanisms have been postulated for the production of antibacterial compounds from the ecological point of view. In nature antibacterial compounds provide a competitive advantage to the producing species. A possibility is that competition exists between strains of antibiotic producing species itself. A further variation of competition involves the excretion of the antibiotic during spore germination in order to eliminate competitors in the immediate environment of germinating spore or is a means of keeping the cellular machinery working when cell growth is not possible due to unfavorable conditions. Another hypothesis currently being debated is that synthesis of an antibiotic is a method of avoiding cell death due to unbalanced growth or it provides a method of detoxification

of the various functions postulated for antibiotics. The one which has received most attention is the view that antibiotics are important compounds in cellular differentiation i.e. transition from vegetative phase to spores. In the salt pan, the exact mechanism cannot be deduced but being a halotolerant and spore former, *B. licheniformis* produces bacitracin which could be a competitive edge against other bacteria or to eliminate competitors in the immediate environment of germinating spore.

Summary and conclusion

Summary:

During the past few decades, a large number of compounds have been isolated from marine organisms and many of these have been reported to have biological activities, some of which are of interest from the point of view of potential drug development. Marine microbes from various marine ecosystems have contributed significantly towards the novel bioactive microbial products. Compared to other marine ecosystems, the salt pans have not been explored adequately for the wealth of products they are capable of providing. Man-made solar salterns are unique hypersaline extreme environments and harbor halophilic and halotolerant flora and fauna. These ecosystems have gained increasing importance in past few decades and lot of research has focused on isolating and identifying novel microorganisms, processes occurring, and products including enzymes from microbes prevailing in salt pans.

The thesis on "**A study of an antibacterial compound from salt pans of Goa**" is an attempt to explore the salt pans of Goa for potential candidate bacterium producing a novel antibacterial compound. Solar salt pans are unique hypersaline environments consisting of increasing salinity gradient, due to the evaporation of seawater. In this study, crystallizer ponds of two topographically different salt pans of Goa viz. Ribandar and Batim have been investigated with an objective to isolate a candidate culture (bacteria/actinobacteria) capable of producing antibacterial compound. The candidate culture has been characterized by polyphasic approach. The antibacterial compound production has been optimized in a defined medium and the compound has been purified and characterized.

The highlights of the results are listed below**:**

Isolation and screening of bacteria/actinobacteria from water and sediment samples from saltpans:

- A total of 119 cultures were isolated from Ribandar and Batim salt pans of Goa, India out of which majority were bacteria (94%).
- Out of the eight media used for isolation, M6 (peptone yeast extract iron agar) was found to be the best media with reference to the maximum numbers and morphotypes obtained.
- Subsequent to isolation, evaluating the growth of the above 119 isolates on different media showed that 97% of the bacterial isolates grew densely on M2 media followed by M1 media (87%) irrespective of the media used for isolation.
- Thirty one isolates were potentially active with a majority inhibiting Gram negative test cultures. About 58% of these were from Batim and most of them (65%) were from salt pan waters. Of the 31 isolates, two inhibited Gram positive pathogen *Staphylococcus citreus*, 18 inhibited Gram negative pathogens whereas 11 inhibited Gram positive and negative test cultures. Six isolates exhibited broad spectrum activity and larger zones of inhibition.
- TSK 71 was selected as the best candidate for study as it showed the maximum inhibition zone against *Staphylococcus citreus* and the production of the active compound was consistent.

Taxonomy and identity of the candidate bacteria:

- The halotolerant *Bacillus licheniformis* TSK 71 strain was isolated from the overlying water of Batim salt pan.

- The culture could grow at wide pH range (5.0 to 10.0), salt concentration (0 to 20%) and temperature range (20-55°C) and was resistant to few antibiotics (ceftazidime, clindamycin, erythromycin and lincomycin). It could grow in the absence of aeration.
- It utilized limited carbon sources (arabinose, xylose, dextrose, mannose, fructose, sucrose, glycerol, sorbitol and mannitol) and various organic, inorganic and complex nitrogen sources of microbial, animal and plant origin.
- It could hydrolyse polymers like starch and casein and showed the presence of amylase, protease, β - galactosidase and catalase enzymes.
- Polyphasic approach including biochemical characterisiation, FAME analysis and 16S rDNA sequencing proved all methods to be congruent and identified the candidate bacteria TSK 71 to be *Bacillus licheniformis*.
- This is the first report of a *Bacillus licheniformis* strain, isolated from a salt pan, which produces an antibiotic.

Optimisation of growth of TSK 71 and antibacterial compound production:

- Though TSK 71 could tolerate wide range of pH, salt concentration and temperature, best growth was observed at pH 5.0 to 7.0, salt concentration 10% and at 37°C.
- The best carbon and nitrogen source was sucrose and beef extract respectively with a distinct diauxic growth curve with sucrose.
- Interestingly, initiation of the antibiotic production commenced at the end of the first phase of growth at the expense of glucose and not at the end second phase of growth at the expense of fructose.

- Optimised conditions for production of antibacterial compound were: TSK media containing 0.6% sucrose, 0.6% potassium nitrate, 1% salt concentration, pH 6.0 and temperature 37°C at 120 rpm on a shaker.

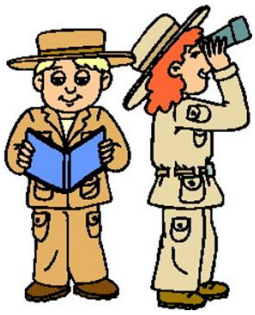
Purification and characterisation of the bioactive compound:

- The antibacterial compound was purified and NMR and mass spectral analysis revealed it to be a peptide, Bacitracin A. NMR and Mass spectra of the compound were comparable to the commercial Bacitracin. A slightly diffused spot at Rf = 0.57 in TLC profile of fraction F3 was observed as compared to the standard Bacitracin used.
- The MIC of the purified compound was 596 µg/ml against *Staphylococcus citreus.*
- During antibiotic production the pH of the media increased from 6 to 9 and the antibiotic was stable. Perhaps this helped the organism thrive in salterns at this high pH.
- The compound retained antibacterial activity against *Staphylococcus citreus* even after 8-12 months of storage at 4°C.

Conclusion:

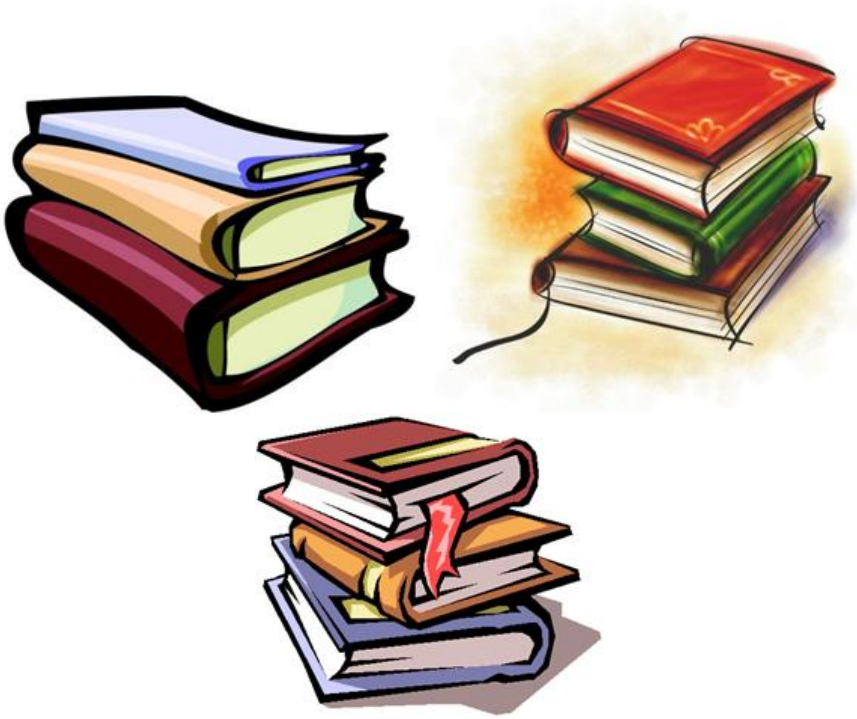
- The antibacterial compound purified and characterized as a peptide Bacitracin A showed maximum inhibitory activity against *Staphylococcus citreus*.
- Other salt pan bacteria from Batim and Ribandar, Goa could have interesting antibiotic producing profiles and hence could be looked upon as a potential resource of antibacterial metabolites.

Future prospects



- Presence of *B. licheniformis* in an extreme environment such as salt pan indicates a possibility of other similar organisms in these ecosystems and also suggests avenues for utilizing these microbes for various applications.
- There is a need to develop methods to isolate less-culturable microbes from these ecosystems.
- Attempts could also be diverted to use culture-independent methods to identify microbes capable of producing bioactive metabolites.
- Understanding the ecological context of some these extremophiles in depth would help pursue other microbes for similar applications using throughput analysis for optimizations.
- Most importantly, it would be pertinent to explore the truly halophilic forms to harness their potential.

Bibliography



Abdennadher A, Ramirez F, Romdhane MS, Ruiz X, Jover L & Sanpera C (2011) Little Egret (Egretta garzetta) as a bioindicator of trace element pollution in Tunisian aquatic ecosystems. *Environ Monit Assess* **175**, 677-684.

Aguilera M, Jimenez-Pranteda ML, Kharroub K, Gonzalez-Paredes A, Durban JJ, Russell NJ, Ramos-Cormenzana A & Monteoliva-Sanchez M (2009) *Marinobacter lacisalsi* sp. nov., a moderately halophilic bacterium isolated from the saline-wetland wildfowl reserve Fuente de Piedra in southern Spain. *Int J Syst Evol Microbiol* **59**, 1691-1695.

Akolkar AV, Deshpande GM, Raval KN, Durai D, Nerurkar AS, Desai AJ (2008) Organic solvent tolerance of *Halobacterium* sp. SP1(1) and its extracellular protease. *Journal of Basic Microbiology* **48**, 421-425.

Amjres H, Bejar V, Quesada E, Abrini J & Llamas I (2011) *Halomonas rifensis* sp. nov., an exopolysaccharide-producing, halophilic bacterium isolated from a solar saltern. *Int J Syst Evol Microbiol* **61**, 2600-2605.

Amoozegar MA, Sanchez-Porro C, Rohban R, Hajighasemi M & Ventosa A (2009a) *Bacillus persepolensis* sp. nov., a moderately halophilic bacterium from a hypersaline lake. *Int J Syst Evol Microbiol* **59**, 2352-2358.

Amoozegar MA, Sanchez-Porro C, Rohban R, Hajighasemi M & Ventosa A (2009b) *Piscibacillus halophilus* sp. nov., a moderately halophilic bacterium from a hypersaline Iranian lake. *Int J Syst Evol Microbiol* **59**, 3095-3099.

Anthony T, Rajesh T, Kayalvizhi N & Gunasekaran P (2009) Influence of medium components and fermentation conditions on the production of bacteriocin(s) by *Bacillus licheniformis* AnBa9. *Bioresour Technol* **100**, 872-877.

Anton J, Oren A, Benlloch S, Rodriguez-Valera F, Amann R & Rossello-Mora R (2002) *Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. *Int J Syst Evol Microbiol* **52**, 485-491.

Arenas M, Banon PI, Copa-Patino JL, Sanchez-Porro C, Ventosa A & Soliveri J (2009) *Halomonas ilicicola* sp. nov., a moderately halophilic bacterium isolated from a saltern. *Int J Syst Evol Microbiol* **59**, 578-582.

Asha KRT, Vinitha DA, Kiran SG, Manjusha WA, Sukumaran N, Selvin J (2005) Isolation and Cultivation of Halophilic Archaea from Solar Salterns Located in Peninsular Coast of India *The Internet Journal of Microbiology* **1**.

Ashok Kumar N, Viji D, Baluswami M (2011) A short term study of algal flora of a salt pan near Chennai. *International Journal of Recent Scientific Research* **2**, 292-296.

Asker D & Ohta Y (2002a) *Haloferax alexandrinus* sp. nov., an extremely halophilic canthaxanthin-producing archaeon from a solar saltern in Alexandria (Egypt). *Int J Syst Evol Microbiol* **52**, 729-738.

Asker D & Ohta Y (2002b) PCR and blot hybridization for rapid identification of *Haloferax* species. *Can J Microbiol* **48**, 468-472.

Azevedo EC, Rios EM, Fukushima K & Campos-Takaki GM (1993) Bacitracin production by a new strain of *Bacillus subtilis*. Extraction, purification, and characterization. *Appl Biochem Biotechnol* **42**, 1-7.

Baati H, Guermazi S, Amdouni R, Gharsallah N, Sghir A & Ammar E (2008) Prokaryotic diversity of a Tunisian multipond solar saltern. *Extremophiles* **12**, 505-518.

Baati H, Guermazi S, Gharsallah N, Sghir A & Ammar E (2010a) Microbial community of salt crystals processed from Mediterranean seawater based on 16S rRNA analysis. *Can J Microbiol* **56**, 44-51.

Baati H, Guermazi S, Gharsallah N, Sghir A & Ammar E (2010b) Novel prokaryotic diversity in sediments of Tunisian multipond solar saltern. *Res Microbiol* **161**, 573-582.

Baati H, Jarboui R, Gharsallah N, Sghir A & Ammar E (2011) Molecular community analysis of magnesium-rich bittern brine recovered from a Tunisian solar saltern. *Can J Microbiol* **57**, 975-981.

Ballav S, Dastagar SG & Kerkar S (2012) Biotechnological significance of Actinobacterial research in India. *Recent Research in Science and Technology* **4**, 31-39.

Bebout BM, Carpenter SP, Des Marais DJ, Discipulo M, Embaye T, Garcia-Pichel F, Hoehler TM, Hogan M, Jahnke LL, Keller RM, Miller SR, Prufert-Bebout LE, Raleigh C, Rothrock M & Turk K (2002) Long-term manipulations of intact microbial mat communities in a greenhouse collaboratory: simulating earth's present and past field environments. *Astrobiology* **2**, 383-402.

Bidle K, Amadio W, Oliveira P, Paulish T, Hicks S & Earnest C (2005) A phylogenetic analysis of haloarchaea found in a solar saltern. *BIOS* **76**, 89-96.

Biswas A, Patra A & Paul AK (2009) Production of poly-3-hydroxyalkanoic acids by a moderately halophilic bacterium, *Halomonas marina* HMA 103 isolated from solar saltern of Orissa, India. *Acta Microbiol Immunol Hung* **56**, 125-143.

Blunden G (2001) Biologically active compounds from marine organisms. *Phytother Res* **15**, 89-94.

Bhunia B & Dey A (2012) Statistical Approach for Optimization of Physiochemical Requirements on Alkaline Protease Production fromBacillus licheniformis NCIM 2042. *Enzyme Research* **2012**, 1-13.

Bouchotroch S, Quesada E, del Moral A, Llamas I & Bejar V (2001) *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **51**, 1625-1632.

Boujelben I, Gomariz M, Martinez-Garcia M, Santos F, Pena A, Lopez C, Anton J & Maalej S (2012) Spatial and seasonal prokaryotic community dynamics in ponds of increasing salinity of Sfax solar saltern in Tunisia. *Antonie Van Leeuwenhoek*.

Boyd ES, Spear JR & Peters JW (2009) [FeFe] hydrogenase genetic diversity provides insight into molecular adaptation in a saline microbial mat community. *Appl Environ Microbiol* **75**, 4620-4623.

Brunner R & Machek G (1965) Polypeptides. *The antibiotics. Volume II. Intermediate antibiotics* **2**.

Burgess GJ, Miyashita H, Sudo H & Matsunaga T (1991) Antibiotic production by the marine photosynthetic bacterium *Chromatium purpuratum* NKPB 031704: localization of activity to the chromatophores. *FEMS Microbiol Lett* **68**, 301-305.

Burkholder PR, Pfister RM & Leitz FH (1966) Production of a pyrrole antibiotic by a marine bacterium. *Appl Microbiol* **14**, 649-653.

Burkholder PR & Sharma GM (1969) Antimicrobial agents from the sea. *Lloydia* **32**, 466-483.

Burns DG, Camakaris HM, Janssen PH & Dyall-Smith ML (2004) Combined use of cultivation-dependent and cultivation-independent methods indicate that members of most haloarchaeal groups in an Australian crystallizer pond are cultivable. *Appl Environ Microbiol* **70**, 5258-5265.

Burns DG, Janssen PH, Itoh T, Kamekura M, Li Z, Jensen G, Rodriguez-Valera F, Bolhuis H & Dyall-Smith ML (2007) *Haloquadratum walsbyi* gen. nov., sp. nov.,

the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. *Int J Syst Evol Microbiol* **57**, 387-392.

Cabrera A, Aguilera M, Fuentes S, Incerti C, Russell NJ, Ramos-Cormenzana A & Monteoliva-Sanchez M (2007) *Halomonas indalinina* sp. nov., a moderately halophilic bacterium isolated from a solar saltern in Cabo de Gata, Almeria, southern Spain. *Int J Syst Evol Microbiol* **57**, 376-380.

Canedo LM, Fernandez Puentes JL, Perez Baz J, Acebal C, de la Calle F, Garcia Gravalos D & Garcia de Quesada T (1997) PM-94128, a new isocoumarin antitumor agent produced by a marine bacterium. *J Antibiot (Tokyo)* **50**, 175-176.

Carballeira NM, Miranda C, Lozano CM, Nechev JT, Ivanova A, Ilieva M, Tzvetkova I & Stefanov K (2001) Characterization of novel methyl-branched chain fatty acids from a halophilic Bacillus species. *J Nat Prod* **64**, 256-259.

Callow RK & Work TS (1952) Antibiotic peptides from *Bacillus licheniformis*; licheniformins A, B and C. *Biochem J* **51**, 558-568.

Ceylan S, Akbulut BS, Denizci AA & Kazan D (2011) Proteomic insight into phenolic adaptation of a moderately halophilic *Halomonas* sp. strain AAD12. *Can J Microbiol* **57**, 295-302.

Chanan R, Korpole S & Pinnaka AK (2011) *Caenispirillum salinarum* sp. nov., a novel bacterium of the family Rhodospirillaceae isolated from a solar saltern. *Int J Syst Evol Microbiol*.

Chen L, Wang N, Wang X, Hu J & Wang S (2010) Characterization of two anti-fungal lipopeptides produced by *Bacillus amyloliquefaciens* SH-B10. *Bioresour Technol* **101**, 8822-8827.

Chi Z, Ma C, Wang P & Li HF (2007) Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Bioresour Technol* **98**, 534-538.

Cho BC, Park JS, Xu K & Choi JK (2008) Morphology and molecular phylogeny of *Trimyema koreanum* n. sp., a ciliate from the hypersaline water of a solar saltern. *J Eukaryot Microbiol* **55**, 417-426.

Choi DH & Cho BC (2005) *Idiomarina seosinensis* sp. nov., isolated from hypersaline water of a solar saltern in Korea. *Int J Syst Evol Microbiol* **55**, 379-383.

Choi DH & Cho BC (2006) *Citreimonas salinaria* gen. nov., sp. nov., a member of the Roseobacter clade isolated from a solar saltern. *Int J Syst Evol Microbiol* **56**, 2799-2803.

Choi DH, Cho JC, Lanoil BD, Giovannoni SJ & Cho BC (2007*) Maribius salinus* gen. nov., sp. nov., isolated from a solar saltern and *Maribius pelagius* sp. nov., cultured from the Sargasso Sea, belonging to the Roseobacter clade. *Int J Syst Evol Microbiol* **57**, 270-275.

Choi DH, Yi H, Chun J & Cho BC (2006) *Jannaschia seosinensis* sp. nov., isolated from hypersaline water of a solar saltern in Korea. *Int J Syst Evol Microbiol* **56**, 45-49.

Chun J, Bae KS, Moon EY, Jung SO, Lee HK & Kim SJ (2000) *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete isolated from a saltern. *Int J Syst Evol Microbiol* **50 Pt 5**, 1909-1913.

Collins CH, Lyne PM, Grange J & Falkinham III J (2004) Microbiological Methods. *Butterworth and Heinemann Publications*.

Cui HL, Gao X, Li XY, Xu XW, Zhou YG, Liu HC & Zhou PJ (2010) *Haloplanus vescus* sp. nov., an extremely halophilic archaeon from a marine solar saltern, and

emended description of the genus Haloplanus. *Int J Syst Evol Microbiol* **60**, 1824-1827.

Cui HL, Gao X, Li XY, Xu XW, Zhou YG, Liu HC & Zhou PJ (2010) *Halosarcina limi* sp. nov., a halophilic archaeon from a marine solar saltern, and emended description of the genus Halosarcina. *Int J Syst Evol Microbiol* **60**, 2462-3466.

Cui HL, Gao X, Sun FF, Dong Y, Xu XW, Zhou YG, Liu HC, Oren A & Zhou PJ (2010) *Halogranum rubrum* gen. nov., sp. nov., a halophilic archaeon isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **60**, 1366-1371.

Cui HL, Gao X, Yang X & Xu XW (2010) *Halorussus rarus* gen. nov., sp. nov., a new member of the family Halobacteriaceae isolated from a marine solar saltern. *Extremophiles* **14**, 493-499.

Cui HL, Gao X, Yang X & Xu XW (2011) *Halolamina pelagica* gen. nov., sp. nov., a new member of the family Halobacteriaceae. *Int J Syst Evol Microbiol* **61**, 1617-1621.

Cui HL, Gao X, Yang X & Xu XW (2011) *Haloplanus aerogenes* sp. nov., an extremely halophilic archaeon from a marine solar saltern. *Int J Syst Evol Microbiol* **61**, 965-968.

Cui HL, Li XY, Gao X, Xu XW, Zhou YG, Liu HC, Oren A & Zhou PJ (2010) *Halopelagius inordinatus* gen. nov., sp. nov., a new member of the family Halobacteriaceae isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **60**, 2089-2093.

Cui HL, Lin ZY, Dong Y, Zhou PJ & Liu SJ (2007) *Halorubrum litoreum* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int J Syst Evol Microbiol* **57**, 2204-2206.

Cui HL, Mou YZ, Yang X, Zhou YG, Liu HC & Zhou PJ (2012) *Halorubellus salinus* gen. nov., sp. nov. and *Halorubellus litoreus* sp. nov., novel halophilic archaea isolated from a marine solar saltern. *Syst Appl Microbiol* **35**, 30-34.

Cui HL, Sun FF, Gao X, Dong Y, Xu XW, Zhou YG, Liu HC, Oren A & Zhou PJ (2010) *Haladaptatus litoreus* sp. nov., an extremely halophilic archaeon from a marine solar saltern, and emended description of the genus Haladaptatus. *Int J Syst Evol Microbiol* **60**, 1085-1089.

Cui HL, Yang X, Gao X, Li XY, Xu XW, Zhou YG, Liu HC & Zhou PJ (2010) *Halogeometricum rufum* sp. nov., a halophilic archaeon from a marine solar saltern, and emended description of the genus Halogeometricum. *Int J Syst Evol Microbiol* **60**, 2613-2617.

Cui HL, Yang X, Gao X & Xu XW (2011) *Halobellus clavatus* gen. nov., sp. nov. and *Halorientalis regularis* gen. nov., sp. nov., two new members of the family Halobacteriaceae. *Int J Syst Evol Microbiol* **61**, 2682-2689.

Cui HL, Yang X, Gao X & Xu XW (2011) *Halogranum gelatinilyticum* sp. nov. and *Halogranum amylolyticum* sp. nov., isolated from a marine solar saltern, and emended description of the genus Halogranum. *Int J Syst Evol Microbiol* **61**, 911-915.

Darnell TM & Smith EH (2002) Recommended design for more accurate duplication of natural conditions in salt marsh creation. *Environ Manage* **29**, 813-823.

Das S, Lyla PS & Khan A. (2006) Marine microbial diversity and ecology: importance and future perspectives. *Current Science* **90**, 1325-1335.

Dave SR & Desai HB (2006) Microbial diversity at marine salterns near Bhavnagar, Gujarat, India. *Current Science* **90**, 497-500.

Debbab A, Aly AH, Lin WH & Proksch P (2010) Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* **3**, 544-563.

Deepika L & Kannabiran K (2010) Biosurfactant and Heavy Metal Resistance Activity of *Streptomyces* spp. Isolated from Saltpan Soil. *British Journal of Pharmacology and Toxicology* **1**, 33-39.

Deshmukh SK (2004) Isolation of dermatophytes and other keratinophilic fungi from the vicinity of salt pan soils of Mumbai, India *Mycopathologia* **157**, 265-267.

Devi P, Wahidullah S, Rodrigues C & Souza LD (2010) The sponge-associated bacterium *Bacillus licheniformis* SAB1: a source of antimicrobial compounds. *Mar Drugs* **8**, 1203-1212.

Dharmaraj S, Ashokkumar B, & Dhevendaran K 2009. Fermentative production of carotenoids from marine actinomycetes. Iranian. J. Microbiol. 1(4):36-41.

Dhanasekaran D, Rajakumar G, Sivamani P, Selvamani S, Panneerselvam A & Thajuddin N (2005) Screening of salt pans actinomycetes for antibacterial agents. . *The Internet J. Microbiol.* **1**.

Dhanasekaran D, Thajuddin N & Panneerselvam A (2008-2009) Distribution and ecobiology of antagonistic streptomycetes from agriculture and coastal soil in TamilNadu, India. . *J. culture collections* **6**, 10-20.

Diez B, Anton J, Guixa-Boixereu N, Pedros-Alio C & Rodriguez-Valera F (2000) Pulsed-field gel electrophoresis analysis of virus assemblages present in a hypersaline environment. *Int Microbiol* **3**, 159-164.

Donia M & Hamann MT (2003) Marine natural products and their potential applications as anti-infective agents. *Lancet Infect Dis* **3**, 338-348.

Edwards HG, Mohsin MA, Sadooni FN, Nik Hassan NF & Munshi T (2006) Life in the sabkha: Raman spectroscopy of halotrophic extremophiles of relevance to planetary exploration. *Anal Bioanal Chem* **385**, 46-56.

Elevi Bardavid R, Khristo P & Oren A (2008) Interrelationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds. *Extremophiles* **12**, 5-14.

Ettoumi B, Raddadi N, Borin S, Daffonchio D, Boudabous A & Cherif A (2009) Diversity and phylogeny of culturable spore-forming Bacilli isolated from marine sediments. *J Basic Microbiol* **49 Suppl 1**, S13-23.

Faulkner DJ (2000) Marine natural products. *Nat Prod Rep* **17**, 7-55.

Fiedler HP, Bruntner C, Bull AT, Ward AC, Goodfellow M, Potterat O, Puder C & Mihm G (2005) Marine actinomycetes as a source of novel secondary metabolites. *Antonie Van Leeuwenhoek* **87**, 37-42.

Fourcans A, de Oteyza TG, Wieland A, Sole A, Diestra E, van Bleijswijk J, Grimalt JO, Kuhl M, Esteve I, Muyzer G, Caumette P & Duran R (2004) Characterization of functional bacterial groups in a hypersaline microbial mat community (Salins-de-Giraud, Camargue, France). *FEMS Microbiol Ecol* **51**, 55-70.

Galvez A, Maqueda M, Martinez-Bueno M, Lebbadi M & Valdivia E (1993) Isolation and physico-chemical characterization of an antifungal and antibacterial peptide produced by Bacillus licheniformis A12. *Appl Microbiol Biotechnol* **39**, 438-442.

Ghai R, Pasic L, Fernandez AB, Martin-Cuadrado AB, Mizuno CM, McMahon KD, Papke RT, Stepanauskas R, Rodriguez-Brito B, Rohwer F, Sanchez-Porro C, Ventosa A & Rodriguez-Valera F (2011) New abundant microbial groups in aquatic hypersaline environments. *Sci Rep* **1**, 135.

Ghaly AE, Arab F, Mahmoud NS & Higgins J (2007) Production of Levan by *Bacillus licheniformis* for use as a soil sealant in earthen manure storage structures. . *Am J Biotechnol Biochem* **3**, 47-54.

Giske CG, Monnet DL, Cars O & Carmeli Y (2008) Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother* **52**, 813-821.

Gonzalez-Domenech CM, Martinez-Checa F, Quesada E & Bejar V (2009) *Halomonas fontilapidosi* sp. nov., a moderately halophilic, denitrifying bacterium. *Int J Syst Evol Microbiol* **59**, 1290-1296.

Goodfellow M & Williams ST (1983) Ecology of actinomycetes. *Annu Rev Microbiol* **37**, 189-216.

Govender L, Naidoo L & Setati ME (2009) Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1, 4-β xylanase from a newly isolated haloalkaliphilic *Nesterenkonia* sp. *African Journal of Biotechnology* **8**, 5458-5466.

Grant S, Grant WD, Jones BE, Kato C & Li L (1999) Novel archaeal phylotypes from an East African alkaline saltern. *Extremophiles* **3**, 139-145.

Grant WD, Gemmell RT & McGenity TJ (1998) Halobacteria: the evidence for longevity. *Extremophiles* **2**, 279-287.

Guermazi W, Elloumi J, Ayadi H, Bouain A & Aleya L (2008) Rearing of Fabrea salina Henneguy (*Ciliophora, Heterotrichida*) with three unicellular feeds. *C R Biol* **331**, 56-63.

Gugliandolo C, Maugeri TL, Caccamo D & Stackebrandt E (2003) *Bacillus aeolius* sp. nov. a novel thermophilic, halophilic marine *Bacillus* species from Eolian Islands (Italy). *Syst Appl Microbiol* **26**, 172-176.

Gulve RM, & Deshmukh AM (2011) Enzymatic Activity of Actinomycetes Isolated From Marine Sediments. *Recent. Res. Sci. Tech.* **3**, 80-83.

Gunde-Cimermana N, Zalarb P, de Hoogc S & Plemenitasd A (2000) Hypersaline waters in salterns - natural ecological niches for halophilic black yeasts. *FEMS Microbiol Ecol* **32**, 235-240.

Gutierrez MC, Kamekura M, Holmes ML, Dyall-Smith ML & Ventosa A (2002) Taxonomic characterization of *Haloferax* sp. *(" H. alicantei*") strain Aa 2.2: description of *Haloferax lucentensis* sp. nov. *Extremophiles* **6**, 479-483.

Haavik H (1981) Effects of amino acids upon bacitracin production by *Bacillus licheniformis*. *FEMS Microbiology Letters* **10**, 111-114.

Hanlon GW & Hodges NA (1981) Bacitracin and protease production in relation to sporulation during exponential growth of *Bacillus licheniformis* on poorly utilized carbon and nitrogen sources. *J Bacteriol* **147**, 427-431.

Harithsa S, Kerkar S & Loka Bharathi PA (2002) Mercury and lead tolerance in hypersaline sulfate-reducing bacteria. *Mar Pollut Bull* **44**, 726-732.

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C & Hacker J (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol Ecol* **35**, 305-312.

Hirschler-Rea A, Matheron R, Riffaud C, Moune S, Eatock C, Herbert RA, Willison JC & Caumette P (2003) Isolation and characterization of spirilloid purple phototrophic bacteria forming red layers in microbial mats of Mediterranean salterns: description of *Halorhodospira neutriphila* sp. nov. and emendation of the genus *Halorhodospira*. *Int J Syst Evol Microbiol* **53**, 153-163.

Imada C (2005) Enzyme inhibitors and other bioactive compounds from marine actinomycetes. *Antonie Van Leeuwenhoek* **87**, 59-63.

Imada C, Koseki N, Kamata M, Kobayashi T & Hamada-Sato N (2007) Isolation and characterization of antibacterial substances produced by marine actinomycetes in the presence of seawater. *Actinomycetologica* **21**, 27-31.

Imamura N, Nishijima M, Takadera T, Adachi K, Sakai M & Sano H (1997) New anticancer antibiotics pelagiomicins, produced by a new marine bacterium *Pelagiobacter variabilis*. *J Antibiot (Tokyo)* **50**, 8-12.

Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova NM, Mikhailov VV, Yumoto N, Shigeri Y, Taguchi T & Yoshikawa S (1999) Characterization of *Bacillus* strains of marine origin. *Int Microbiol* **2**, 267-271.

James SG, Holmstrom C & Kjelleberg S (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. *Appl Environ Microbiol* **62**, 2783-2788.

Javor BJ (1984) Growth potential of halophilic bacteria isolated from solar salt environments: carbon sources and salt requirements. *Appl Environ Microbiol* **48**, 352-360.

Jayappriyan KR, Rajkumar R & Rengasamy R (2011) Unusual occurrence of non carotenogenic strains of *Dunaliella bardawil* and *Dunaliella parva* in India. *J Basic Microbiol* **51**, 473-483.

Jayappriyan KR, Rajkumar R, Sheeja L, Divya S & Rengasamy R (2010) *Recent Research in Science and Technology* **2**, 54-62.

Jensen PR & Fenical W (1994) Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Annu Rev Microbiol* **48**, 559-584.

Jensen PR, Mincer TJ, Williams PG & Fenical W (2005) Marine actinomycete diversity and natural product discovery. *Antonie Van Leeuwenhoek* **87**, 43-48.

Jensen PR, Dwight R & Fenical W (1991) Distribution of Actinomycetes in Near-Shore Tropical Marine Sediments. *Appl. Environ. Microbiol.* **57**, 1102-1108.

Jeon CO, Lim JM, Lee JR, Lee GS, Park DJ, Lee JC, Oh HW & Kim CJ (2007) *Halomonas kribbensis* sp. nov., a novel moderately halophilic bacterium isolated from a solar saltern in Korea. *Int J Syst Evol Microbiol* **57**, 2194-2198.

Jha RK & Zi-rong X (2004) Biomedical Compounds from Marine organisms. *Mar. drugs* 2,123-146

Joung Y, Kim H, Ahn TS & Jon K (2011) *Pontibacter salisaro* sp. nov., isolated from a clay tablet solar saltern in Korea. *J Microbiol* **49**, 290-293.

Joung Y, Kim H, Jang T, Ahn TS & Joh K (2011) *Gramella jeungdoensis* sp. nov., isolated from a solar saltern in Korea. *J Microbiol* **49**, 1022-1026.

Kamat T & Kerkar S (2004) Studies on a bioactive compound produced by a halotolerant saltpan isolate. *Conference on Microbiol of Tropical Seas (COMITS)* **Nat Inst of Oceanography, Goa**, Abstract.

Kerkar S, Raikar L, Tiwari, A. Mayilraj, S. Dastager S. (2012) Biofilm-associated indole acetic acid producing bacteria and their impact in the proliferation of biofilm mats in solar salterns. *Biologia* **67**, 454-460.

Kerkar S (2004) Ecology of Hypersaline Microorganism. Marine Microbiology, Facets and Opportunities. National Institute of Oceanography. Edited by Ramaiah, N.

Kerkar S (2007) Bioactive compounds from the marine environment. *National Seminar on New Trends in Biotechnology* **Nat Inst of Oceanography, Goa**, Abstract.

Kerkar S & Loka Bharathi PA (2007) Stimulation of Sulfate-Reducing Activity at Salt-Saturation in the Salterns of Ribandar, Goa, India. *Geomicrobiology Journal* **24**, 101-110.

Kedar L, Kashman Y & Oren A (2002) Mycosporine-2-glycine is the major mycosporine-like amino acid in a unicellular cyanobacterium (*Euhalothece* sp.) isolated from a gypsum crust in a hypersaline saltern pond. *FEMS Microbiol Lett* **208**, 233-237.

Kim H, Joung Y, Ahn TS & Joh K (2011) *Echinicola jeungdonensis* sp. nov., isolated from a solar saltern. *Int J Syst Evol Microbiol* **61**, 2065-2068.

Kim P, Lee JC, Park DJ, Shin KS, Kim JY & Kim CJ (2011) *Gracilibacillus bigeumensis* sp. nov., a moderately halophilic bacterium from a solar saltern soil. *Int J Syst Evol Microbiol*.

Kokare CR, Kadam SS, Mahadik KR & Chopade BA (2007) Studies on bioemulsifier production from marine *Streptomyces* sp. S1. *Indian. J. Biotechnol.* **6**, 78-84.

Kokare CR, Mahadik KR, Kadam SS & Chopade BA (2004) Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* species AH1 from the west coast of India. *Current Science* **86**, 593-597.

Kumar PA, Srinivas TN, Sasikala C & Ramana Ch V (2007) *Halochromatium roseum* sp. nov., a non-motile phototrophic gammaproteobacterium with gas vesicles, and emended description of the genus Halochromatium. *Int J Syst Evol Microbiol* **57**, 2110-2113.

Kutlu M, Mutlu MB, Aydogan G & Guven K (2008) *Salmonella* mutagenicity analysis of water samples from Camalti Saltern. *Environ Monit Assess* **145**, 237-241.

Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. *Curr Opin Microbiol* **9**, 245-251.

Lee JC, Lim JM, Park DJ, Jeon CO, Li WJ & Kim CJ (2006) *Bacillus seohaeanensis* sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall. *Int J Syst Evol Microbiol* **56**, 1893-1898.

Lee SY, Choi WY, Oh TK & Yoon JH (2008*) Lentibacillus salinarum* sp. nov., isolated from a marine solar saltern in Korea. *Int J Syst Evol Microbiol* **58**, 45-49.

Lee SY, Kang CH, Oh TK & Yoon JH (2012) *Virgibacillus campisalis* sp. nov., from a marine solar saltern. *Int J Syst Evol Microbiol* **62**, 347-351.

Lee SY, Oh TK, Kim W & Yoon JH (2010) *Oceanobacillus locisalsi* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **60**, 2758-2762.

Lee YK, Lee J-H & Lee HK (2001) Microbial Symbiosis in Marine Sponges. . *The Journal of Microbiology, December* **39**, 254-264.

Lei Y, Xu K, Choi JK, Hong HP & Wickham SA (2009) Community structure and seasonal dynamics of planktonic ciliates along salinity gradients. *Eur J Protistol* **45**, 305-319.

Li Y, Yang S & Mu B (2010) Structural characterization of lipopeptide methyl esters produced by Bacillus licheniformis HSN 221. *Chem Biodivers* **7**, 2065-2075.

Lim JM, Jeon CO & Kim CJ (2006) *Bacillus taeanensis* sp. nov., a halophilic Gram-positive bacterium from a solar saltern in Korea. *Int J Syst Evol Microbiol* **56**, 2903-2908.

Lim JM, Jeon CO, Park DJ, Kim HR, Yoon BJ & Kim CJ (2005) *Pontibacillus marinus* sp. nov., a moderately halophilic bacterium from a solar saltern, and emended description of the genus Pontibacillus. *Int J Syst Evol Microbiol* **55**, 1027-1031.

Lim JM, Jeon CO, Song SM & Kim CJ (2005) *Pontibacillus chungwhensis* gen. nov., sp. nov., a moderately halophilic Gram-positive bacterium from a solar saltern in Korea. *Int J Syst Evol Microbiol* **55**, 165-170.

Lim JM, Yoon JH, Lee JC, Jeon CO, Park DJ, Sung C & Kim CJ (2004*) Halomonas koreensis* sp. nov., a novel moderately halophilic bacterium isolated from a solar saltern in Korea. *Int J Syst Evol Microbiol* **54**, 2037-2042.

Litchfield CD & Gillevet PM (2002) Microbial diversity and complexity in hypersaline environments: a preliminary assessment. *J Ind Microbiol Biotechnol* **28**, 48-55.

Litchfield CD, Irby A, Kis-Papo T & Oren A (2000) Comparisons of the polar lipid and pigment profiles of two solar salterns located in Newark, California, USA, and Eilat, Israel. *Extremophiles* **4**, 259-265.

Lizama C, Monteoliva-Sanchez M, Prado B, Ramos-Cormenzana A, Weckesser J & Campos V (2001) Taxonomic study of extreme halophilic archaea isolated from the "Salar de Atacama", Chile. *Syst Appl Microbiol* **24**, 464-474.

Lizama C, Monteoliva-Sanchez M, Suarez-Garcia A, Rosello- *Halorubrum tebenquichense* Mora R, Aguilera M, Campos V & Ramos-Cormenzana A (2002) sp. nov., a novel halophilic archaeon isolated from the Atacama Saltern, Chile. *Int J Syst Evol Microbiol* **52**, 149-155.

Lopalco P, Lobasso S, Babudri F & Corcelli A (2004) Osmotic shock stimulates de novo synthesis of two cardiolipins in an extreme halophilic archaeon. *J Lipid Res* **45**, 194-201.

Lopes VR, Antunes A, Welker M, Martins RF & Vasconcelos VM (2010) Morphological, toxicological and molecular characterization of a benthic *Nodularia* isolated from Atlantic estuarine environments. *Res Microbiol* **161**, 9-17.

Magarvey NA, Keller JM, Bernan V, Dworkin M & Sherman DH (2004) Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Appl Environ Microbiol* **70**, 7520-7529.

Manikandan M, Kannan, V. (2009) Diversity of microorganisms in solar salterns of Tamil Nadu, India. *World J. Microbiol. Biotechnol.* **25**, 1007-1017.

Margesin R & Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* **5**, 73-83.

Martirani L, Varcamonti M, Naclerio G & De Felice M (2002) Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of Bacillus licheniformis. *Microb Cell Fact* **1**, 1.

Martinez-Checa F, Bejar V, Martinez-Canovas MJ, Llamas I & Quesada E (2005) *Halomonas almeriensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium from Cabo de Gata, Almeria, south-east Spain. *Int J Syst Evol Microbiol* **55**, 2007-2011.

Martinez-Checa F, Quesada E, Martinez-Canovas MJ, Llamas I & Bejar V (2005) *Palleronia marisminoris* gen. nov., sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium belonging to the 'Alphaproteobacteria', isolated from a saline soil. *Int J Syst Evol Microbiol* **55**, 2525-2530.

Matsunaga TT, H. Miura, Y. Yamazaki, T. Furuya, H. Sode, H. (1995) Screening of marine cyanobacteria for high palmitoleic acid production *FEMS Microbiology Letters* **133**.

Maugeri TL, Gugliandolo C, Caccamo D & Stackebrandt E (2002) Three novel halotolerant and thermophilic *Geobacillus* strains from shallow marine vents. *Syst Appl Microbiol* **25**, 450-455.

Meseguer I & Rodriguez-V, F. (1985) Production and purification of halocin H4. *FEMS Microbiology Letters* **28**, 177-182.

Mizushina Y, Motoshima H, Yamaguchi Y, Takeuchi T, Hirano K, Sugawara F & Yoshida H (2009) 3-O-methylfunicone, a selective inhibitor of mammalian Y-

family DNA polymerases from an Australian sea salt fungal strain. *Mar Drugs* **7**, 624-639.

Mondol MA, Kim JH, Lee HS, Lee YJ & Shin HJ (2011) Macrolactin W, a new antibacterial macrolide from a marine *Bacillus* sp. *Bioorg Med Chem Lett* **21**, 3832-3835.

Moore BS, Kalaitzis JA & Xiang L (2005) Exploiting marine actinomycete biosynthetic pathways for drug discovery. *Antonie Van Leeuwenhoek* **87**, 49-57.

Moreno Mde L, Garcia MT, Ventosa A, Iglesias-Guerra F & Mellado E (2010) The extremely halophilic bacterium *Salicola marasensis* IC10 accumulates the compatible solute betaine. *Syst Appl Microbiol* **33**, 308-310.

Mota RR, Marquez MC, Arahal DR, Mellado E & Ventosa A (1997) Polyphasic taxonomy of *Nesterenkonia halobia*. *Int J Syst Bacteriol* **47**, 1231-1235.

Mukherjee S, Das P, Sivapathasekaran C & Sen R (2009) Antimicrobial biosurfactants from marine Bacillus circulans: extracellular synthesis and purification. *Lett Appl Microbiol* **48**, 281-288.

Munoz J, Gomez A, Green AJ, Figuerola J, Amat F & Rico C (2008) Phylogeography and local endemism of the native Mediterranean brine shrimp *Artemia salina* (Branchiopoda: Anostraca). *Mol Ecol* **17**, 3160-3177.

Munoz R, Lopez-Lopez A, Urdiain M, Moore ER & Rossello-Mora R (2011) Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. *Syst Appl Microbiol* **34**, 69-75.

Musthafa KS, Saroja V, Pandian SK & Ravi AV (2011) Antipathogenic potential of marine *Bacillus* sp. SS4 on N-acyl-homoserine-lactone-mediated virulence factors production in *Pseudomonas aeruginosa* (PAO1). *J Biosci* **36**, 55-67.

Nair S & Simidu U (1987) Distribution and significance of heterotrophic marine bacteria with antibacterial activity. *Appl Environ Microbiol* **53**, 2957-2962.

Needham GR & Teel PD (1991) Off-host physiological ecology of ixodid ticks. *Annu Rev Entomol* **36**, 659-681.

Nesemann G, Prave P, Sukatsch D & Vertesy L (1972) [A polyene antibiotic from bacteria]. *Naturwissenschaften* **59**, 81-82.

Newman DJ & Cragg GM (2004) Advanced preclinical and clinical trials of natural products and related compounds from marine sources. *Curr Med Chem* **11**, 1693-1713.

Nithya C, Begum MF & Pandian SK (2010) Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. *Appl Microbiol Biotechnol* **88**, 341-358.

Nowlan B, Dodia MS, Singh SP & Patel BK (2006) *Bacillus okhensis* sp. nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan. *Int J Syst Evol Microbiol* **56**, 1073-1077.

Nubel U, Bateson MM, Madigan MT, Kuhl M & Ward DM (2001) Diversity and distribution in hypersaline microbial mats of bacteria related to *Chloroflexus* spp. *Appl Environ Microbiol* **67**, 4365-4371.

Nuttall SD & Dyall-Smith ML (1993) Ch2, a novel halophilic archaeon from an Australian solar saltern. *Int J Syst Bacteriol* **43**, 729-734.

Ochsenreiter T, Pfeifer F & Schleper C (2002) Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. *Extremophiles* **6**, 267-274.

Oh D, Porter K, Russ B, Burns D & Dyall-Smith M (2010) Diversity of Haloquadratum and other haloarchaea in three, geographically distant, Australian saltern crystallizer ponds. *Extremophiles* **14**, 161-169.

Okami Y (1986) Marine microorganisms as a source of bioactive agents. . *Microbial ecology* **12**, 65-78.

Okami Y, Okazaki T, Kitahara T & Umezawa H (1976) Studies on marine microorganisms. V. A new antibiotic, aplasmomycin, produced by a streptomycete isolated from shallow sea mud. *J Antibiot (Tokyo)* **29**, 1019-1025.

Okami Y, Hotta K, Yoshida M, Ikeda D, Kondo S & Umezawa H (1979) New aminoglycoside antibiotics, istamycins A and B. *J Antibiot (Tokyo)* **32**, 964-966.

Okami Y (1993) The search for bioactive metabolites from marine bacteria. *J Marine Biotechnology*, 1: 59-65.

Ohki R, Tateno K, Okada Y, Okajima H, Asai K, Sadaie Y, Murata M & Aiso T (2003) A bacitracin-resistant *Bacillus subtilis* gene encodes a homologue of the membrane-spanning subunit of the Bacillus licheniformis ABC transporter. *J Bacteriol* **185**, 51-59.

Oren A, Mevarech M & Kaplan A (1999) *Microbioloy and biogeochemistry of hypersaline environment, CRC press, Boca Raton, USA.*

Oren A (2002) Molecular ecology of extremely halophilic Archaea and Bacteria. *FEMS Microbiol Ecol* **39**, 1-7.

Oren A (2008) Microbial Life at High Salt Concentrations: Phylogenetic and Metabolic Diversity. *Saline Systems* **4**.

Oren A, Pri-El N, Shapiro O & Siboni N (2006) Buoyancy studies in natural communities of square gas-vacuolate archaea in saltern crystallizer ponds. *Saline Systems* **2**, 4.

Papke RT, Douady CJ, Doolittle WF & Rodriguez-Valera F (2003) Diversity of bacteriorhodopsins in different hypersaline waters from a single Spanish saltern. *Environ Microbiol* **5**, 1039-1045.

Papke RT, Zhaxybayeva O, Feil EJ, Sommerfeld K, Muise D & Doolittle WF (2007) Searching for species in haloarchaea. *Proc Natl Acad Sci U S A* **104**, 14092-14097.

Pappa A, Sanchez-Porro C, Lazoura P, Kallimanis A, Perisynakis A, Ventosa A, Drainas C & Koukkou AI (2010) *Bacillus halochares* sp. nov., a halophilic bacterium isolated from a solar saltern. *Int J Syst Evol Microbiol* **60**, 1432-1436.

Park JS, Cho BC & Simpson AG (2006) *Halocafeteria seosinensis* gen. et sp. nov. (Bicosoecida), a halophilic bacterivorous nanoflagellate isolated from a solar saltern. *Extremophiles* **10**, 493-504.

Park JS, Simpson AG, Brown S & Cho BC (2009) Ultrastructure and molecular phylogeny of two heterolobosean amoebae, *Euplaesiobystra hypersalinica* gen. et sp. nov. and *Tulamoeba peronaphora* gen. et sp. nov., isolated from an extremely hypersaline habitat. *Protist* **160**, 265-283.

Park JS, Simpson AG, Lee WJ & Cho BC (2007) Ultrastructure and phylogenetic placement within Heterolobosea of the previously unclassified, extremely halophilic heterotrophic flagellate *Pleurostomum flabellatum* (Ruinen 1938). *Protist* **158**, 397-413.

Park JS, Cha IT, Kim SJ, Shin KS, Hong Y, Roh DH & Rhee SK (2011) *Salinisphaera orenii* sp. nov., isolated from a solar saltern. *Int J Syst Evol Microbiol*.

Pasic L, Bartual SG, Ulrih NP, Grabnar M & Velikonja BH (2005) Diversity of halophilic archaea in the crystallizers of an Adriatic solar saltern. *FEMS Microbiol Ecol* **54**, 491-498.

Pasic L, Rodriguez-Mueller B, Martin-Cuadrado AB, Mira A, Rohwer F & Rodriguez-Valera F (2009) Metagenomic islands of hyperhalophiles: the case of Salinibacter ruber. *BMC Genomics* **10**, 570.

Pasic L, Ulrih NP, Crnigoj M, Grabnar M & Velikonja BH (2007) Haloarchaeal communities in the crystallizers of two adriatic solar salterns. *Can J Microbiol* **53**, 8-18.

Pela´ez FC, J. Arenal, F. Basilio, A. Cabello, A. Matas, M. T. D. Garcia, J. B. Del Val, A. G. Gonzalez, V. Gorrochategui, J. Hernandez, P. Martin, I. Platas, G. Vicente, F. (1998) Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycol Res* **102**, 755–761.

Pendergrass SM & Jensen P A (1997) Application of the gas chromatography-fatty acid methyl ester (GC-FAME) system for the identification of environmental and clinical isolates of the family Micrococcaceae. . *Appl Occup Environ Hyg* **12**, 543-546.

Pettit GR, Cichacz Z, Barkoczy J, Dorsaz AC, Herald DL, Williams MD, Doubek DL, Schmidt JM, Tackett LP, Brune DC & et al. (1993) Isolation and structure of the marine sponge cell growth inhibitory cyclic peptide phakellistatin 1. *J Nat Prod* **56**, 260-267.

Poli A, Nicolaus B, Denizci AA, Yavuzturk B & Kazan D (2012) *Halomonas smyrnensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium from Camalti Saltern Area, Turkey. *Int J Syst Evol Microbiol*.

Prado B, Lizama C, Aguilera M, Ramos-Cormenzana A, Fuentes S, Campos V & Monteoliva-Sanchez M (2006) *Chromohalobacter nigrandesensis* sp. nov., a moderately halophilic, Gram-negative bacterium isolated from Lake Tebenquiche on the Atacama Saltern, Chile. *Int J Syst Evol Microbiol* **56**, 647-651.

Prashanth S & Mathivanan N (2010) Growth promotion of groundnut by IAA producing rhizobacteria Bacillus licheniformis MML 2501. . *Archives of Phytopathology and Plant Protection* **43**, 191-208.

Proksch E, Holleran WM, Menon GK, Elias PM & Feingold KR (1993) Barrier function regulates epidermal lipid and DNA synthesis. *Br J Dermatol* **128**, 473-482.

Prudhomme J, McDaniel E, Ponts N, Bertani S, Fenical W, Jensen P & Le Roch K (2008) Marine actinomycetes: a new source of compounds against the human malaria parasite. *PLoS One* **3**, e2335.

Pukall R, Schumann P, Clermont D & Bizet C (2008) *Bacillus aeolius* DSM 15084T (=CIP 107628T) is a strain of Bacillus licheniformis. *Int J Syst Evol Microbiol* **58**, 1268-1270.

Qu L, Lai Q, Zhu F, Hong X, Zhang J, Shao Z & Sun X (2011) *Halomonas daqiaonensis* sp. nov., a moderately halophilic, denitrifying bacterium isolated from a littoral saltern. *Int J Syst Evol Microbiol* **61**, 1612-1616.

Qu L, Zhu F, Zhang J, Gao C & Sun X (2011*) Marinobacter daqiaonensis* sp. nov., a moderate halophile isolated from a Yellow Sea salt pond. *Int J Syst Evol Microbiol* **61**, 3003-3008.

Quesada E, Bejar V, Valderrama MJ, Ventosa A & Ramos Cormenzana AR (1985) Isolation and characterization of moderately halophilic nonmotile rods from different saline habitats. *Microbiologia* **1**, 89-96.

Raghavan TM & Furtado I (2005) Expression of carotenoid pigment of halo-archael cultures exposed to aniline. *Environmental Toxicology* **20**, 165-169.

Raja SSG, Sivakumar K & Thangaradjou T (2010) Screening of marine actinobacteria for amylase enzymes inhibitors. *Indian. J. Microbiol.* **50**, 233-237.

Reddy NG, Ramakrishna DPN & Raja Gopal SV (2011) A Morphological, Physiological and Biochemical studies of Marine Streptomyces rochei (MTCC 10109) Showing Antagonistic Activity Against Selective Human Pathogenic Microorganisms. *Asian Journal of Biological Sciences* **4**, 1-14.

Rios JL, Recio MC & Villar A (1988) Screening methods for natural products with antimicrobial activity: a review of the literature. *J Ethnopharmacol* **23**, 127-149.

Robertson CE, Spear JR, Harris JK & Pace NR (2009) Diversity and stratification of archaea in a hypersaline microbial mat. *Appl Environ Microbiol* **75**, 1801-1810.

Rodriguez-Valera F, Ruiz-Berraquero F & Ramos-Cormenzana A (1980) Behaviour of mixed populations of halophilic bacteria in continuous cultures. *Can J Microbiol* **26**, 1259-1263.

Roepstorff P & Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom* **11**, 601.

Rossello-Mora R, Lee N, Anton J & Wagner M (2003) Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence in situ hybridization. *Extremophiles* **7**, 409-413.

Ruger HJ & Richter G (1979) Bacillus globisporus subsp. marinus subsp. nov. *Int. J. Syst. Bacteriol.* **29**, 196-203.

Ruger HJ, Fritze D & Sproer C (2000) New psychrophilic and psychrotolerant Bacillus marinus strains from tropical and polar deep-sea sediments and emended description of the species. *Int. J. Syst. Evol. Microbiol.* **50**, 1305-1313.

Rutten AM (1997) [Supply of medicines for the Dutch whaling and salt trade in de seventeenth century]. *Bull Cercle Benelux Hist Pharm*, 3-10.

Sadfi-Zouaoui NE, B. Hajlaoui, M. R. Fardeau, M. L. Cayaol, J. L. Ollivier, B. Boudabous, A. (2008) Ability of moderately halophilic bacteria to control grey mould disease on tomato fruits. . *J Phytopathol* **156**, 42–52.

Sanchez MI, Georgiev BB, Nikolov PN, Vasileva GP & Green AJ (2006) Red and transparent brine shrimps (*Artemia parthenogenetica*): a comparative study of their cestode infections. *Parasitol Res* **100**, 111-114.

Santos F, Yarza P, Parro V, Briones C & Anton J (2010) The metavirome of a hypersaline environment. *Environ Microbiol* **12**, 2965-2976.

Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids. . (Vorob'ev). *MIDI Technical Note # 101*.

Sastry M, Ahmad A, Khan MI & Kumar R 2003. Biosynthesis of metal nanoparticles using fungi and actinomycete. Curr. Sci. 85(2):162-170.

Satyanarayana T, Raghukumar C & Shivaji S (2005) Extremophilic microbes: Diversity and perspectives. . *Curr Sci* **89**, 78-90.

Sharma PK, Goel M, Dureja P & Uniyal PL (2010) Isolation and identification of secondary metabolites from hexane extract of culture filtrate of *Bacillus licheniformis* MTCC 7445. *Arch Phytopathol Plant Protection* **43**, 1636-1642.

Shenkarev ZO, Finkina EI, Nurmukhamedova EK, Balandin SV, Mineev KS, Nadezhdin KD, Yakimenko ZA, Tagaev AA, Temirov YV, Arseniev AS & Ovchinnikova TV (2010) Isolation, structure elucidation, and synergistic antibacterial activity of a novel two-component lantibiotic lichenicidin from *Bacillus licheniformis* VK21. *Biochemistry* **49**, 6462-6472.

Silva I, Rocha SM, Coimbra MA & Marriott PJ (2010) Headspace solid-phase microextraction combined with comprehensive two-dimensional gas

chromatography time-of-flight mass spectrometry for the determination of volatile compounds from marine salt. *J Chromatogr A* **1217**, 5511-5521.

Sivakumar K, Sahu MK, Thangaradjou T & Kannan L (2007) Research on marine actinobacteria in India. *Indian J. Microbiol.* **47**, 186-196.

Sneath PHA (2005) Endospore forming Gram positive rods and cocci. . *Bergey's Manual of Systematic Bacteriology, Williams & Wilkins, USA.* **2: The Proteobacteria**, 1104-1139.

Sorensen KB, Canfield DE & Oren A (2004) Salinity responses of benthic microbial communities in a solar saltern (Eilat, Israel). *Appl Environ Microbiol* **70**, 1608-1616.

Sorokin DY, Kovaleva OL, Tourova TP & Muyzer G (2010) *Thiohalobacter thiocyanaticus* gen. nov., sp. nov., a moderately halophilic, sulfur-oxidizing gammaproteobacterium from hypersaline lakes, that utilizes thiocyanate. *Int J Syst Evol Microbiol* **60**, 444-450.

Sorokin DY, Tourova TP, Braker G & Muyzer G (2007) *Thiohalomonas denitrificans* gen. nov., sp. nov. and *Thiohalomonas nitratireducens* sp. nov., novel obligately chemolithoautotrophic, moderately halophilic, thiodenitrifying Gammaproteobacteria from hypersaline habitats. *Int J Syst Evol Microbiol* **57**, 1582-1589.

Sorokin DY, Tourova TP, Lysenko AM & Muyzer G (2006) Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats. *Microbiology* **152**, 3013-3023.

Sorokin DY, Tourova TP, Muyzer G & Kuenen GJ (2008) *Thiohalospira halophila* gen. nov., sp. nov. and *Thiohalospira alkaliphila* sp. nov., novel obligately

chemolithoautotrophic, halophilic, sulfur-oxidizing gammaproteobacteria from hypersaline habitats. *Int J Syst Evol Microbiol* **58**, 1685-1692.

Srinivas TN, Kumar PA, Sasikala C, Ramana Ch V & Imhoff JF (2007) *Rhodobium gokarnense* sp. nov., a novel phototrophic alphaproteobacterium from a saltern. *Int J Syst Evol Microbiol* **57**, 932-935.

Steck K, Hansson BS & Knaden M (2009) Smells like home: Desert ants, Cataglyphis fortis, use olfactory landmarks to pinpoint the nest. *Front Zool* **6**, 5.

Stone JK & Strominger JL (1971) Mechanism of Action of Bacitracin: Complexation with Metal Ion and C55-Isoprenyl Pyrophosphate. *PNAS. USA.* **68**, 3223-3227.

Suthindhiran K & Kannabiran K (2009) Cytotoxic and antimicrobial potential of Actinomycete species *Saccharopolyspora saline* VITSDK4 isolated from the Bay of Bengal coast of India. . *Am. J. Infect. Dis.* **5**, 90-98.

Tarjan AC, Davis JS & Nguyen KB (1991) The Genus Prochromadora with a Redescription of P. orleji from a Marine Saltern in the People's Republic of China. *J Nematol* **23**, 491-501.

Teixeira ML, Cladera-Olivera F, Santos JD & Brandelli A (2009) Purification and characterization of a peptide from *Bacillus licheniformis* showing dual antimicrobial and emulsifying activities. . *Food Research International* **42**, 63–68.

Tkavc R, Gostincar C, Turk M, Visscher PT, Oren A & Gunde-Cimerman N (2011) Bacterial communities in the 'petola' microbial mat from the Secovlje salterns (Slovenia). *FEMS Microbiol Ecol* **75**, 48-62.

Trigui H, Masmoudi S, Brochier-Armanet C, Barani A, Gregori G, Denis M, Dukan S & Maalej S (2011) Characterization of heterotrophic prokaryote subgroups in the Sfax coastal solar salterns by combining flow cytometry cell sorting and phylogenetic analysis. *Extremophiles* **15**, 347-358.

Tsiamis G, Katsaveli K, Ntougias S, Kyrpides N, Andersen G, Piceno Y & Bourtzis K (2008) Prokaryotic community profiles at different operational stages of a Greek solar saltern. *Res Microbiol* **159**, 609-627.

Tsu II, Huang C, Garcia J, Patel BKC, Cayol JL, Baresi L & Mah RA (1998) Isolation and characterization of *desulfovibrio senezii* sp. nov., A halotolerant sulfate reducer from a solar saltern and phylogenetic confirmation of *desulfovibrio fructosovorans* as a new species. *Arch Microbiol* **170**, 313-317.

Valgas C, Machado de Souza S, Smania EFA & Smania A Jr. (2007) Screening methods to determine antibacterial activity of natural products *Braz. J. Microbiol.* **38**, 369-380.

Vasavada SH, Thumar JT & Singh RP (2006) Secretion of potent antibiotic by salt-tolerant and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1. *Current Science* **91**, 1393-1397.

Vaupotic T, Gunde-Cimerman N & Plemenitas A (2007) Novel 3'-phosphoadenosine-5'-phosphatases from extremely halotolerant Hortaea werneckii reveal insight into molecular determinants of salt tolerance of black yeasts. *Fungal Genet Biol* **44**, 1109-1122.

Venkata Ramana V, Sasikala C, Veera Venkata Ramaprasad E & Venkata Ramana C (2010) Description of *Ectothiorhodospira salini* sp. nov. *J Gen Appl Microbiol* **56**, 313-319.

Ventosa A, Marquez MC, Garabito MJ & Arahal DR (1998) Moderately halophilic gram-positive bacterial diversity in hypersaline environments. *Extremophiles* **2**, 297-304.

Ventosa A & Neito JJ (1995) Biotechnological applications and potentialities of halophilic microorganisms. . *World J Microbiol Biotechnol* **11**, 85-94.

Vidyasagar MP, S. Jayalakshmi, S. Sreeramula, K. (2007) Optimization of culture condition for the production of halothermophilic proteas from halophilic bacterium *Chromohalobacter* sp. TVSP10. *World Journal of Microbiology and Biotechnology* **23**, 655-662.

Vijayakumar R, Muthukumar C, Thajuddin N, Panneerselvam A & Saravanamuthu R (2007) Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycetologica* **21** 59-65.

Vogel MB, Des Marais DJ, Turk KA, Parenteau MN, Jahnke LL & Kubo MD (2009) The role of biofilms in the sedimentology of actively forming gypsum deposits at Guerrero Negro, Mexico. *Astrobiology* **9**, 875-893.

Volkmann M, Gorbushina AA, Kedar L & Oren A (2006) Structure of euhalothece-362, a novel red-shifted mycosporine-like amino acid, from a halophilic cyanobacterium (Euhalothece sp.). *FEMS Microbiol Lett* **258**, 50-54.

Wang CY, Ng CC, Chen TW, Wu SJ & Shyu YT (2007) Microbial diversity analysis of former salterns in southern Taiwan by 16S rRNA-based methods. *J Basic Microbiol* **47**, 525-533.

Wang CY, Wu SJ, Ng CC, Tzeng WS & Shyu YT (2012) *Halomonas beimenensis* sp. nov., isolated from Beimen, an abandoned saltern in southern Taiwan. *Int J Syst Evol Microbiol*.

Wang X, Xue Y & Ma Y (2009) *Sediminibacillus albus* sp. nov., a moderately halophilic, Gram-positive bacterium isolated from a hypersaline lake, and emended description of the genus *Sediminibacillus Carrasco* et al. 2008. *Int J Syst Evol Microbiol* **59**, 1640-1644.

Wang Y, Wu YH, Wang CS, Xu XW, Oren A, Zhu XF & Wu M (2008) *Halomonas salifodinae* sp. nov., a halophilic bacterium isolated from a salt mine in China. *Int J Syst Evol Microbiol* **58**, 2855-2858.

Wang Y, Zheng J, Liu P, Wang W & Zhu W (2011) Three new compounds from *Aspergillus terreus* PT06-2 grown in a high salt medium. *Mar Drugs* **9**, 1368-1378.

Ward AC & Bora N (2006) Diversity and biogeography of marine actinobacteria. *Curr Opin Microbiol* **9**, 279-286.

Watve MG, Tickoo R, Jog MM & Bhole BD (2001) How many antibiotics are produced by the genus Streptomyces? *Arch Microbiol* **176**, 386-390.

Wiese J, Thiel V, Nagel K, Staufenberger T & Imhoff JF (2009) Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. *Mar Biotechnol (NY)* **11**, 287-300.

Wieland A, Zopfi J, Benthien M & Kuhl M (2005) Biogeochemistry of an iron-rich hypersaline microbial mat (Camargue, France). *Microb Ecol* **49**, 34-49.

Wikler MA, Cockerill FR, Craig WA, Dudley MN, Eliopoulos GM, Hecht DW, Hindler JF, Low DE, Sheehan DJ, Tenover FC, Turnidge JD, Weinstein MP, Zimmer BL, Ferraro MJ & Swenson JM (2007) Performance Standards for Antimicrobial Susceptibility Testing. *Seventeenth Informational Supplement, CLSI 27* **1**, 1-177.

Williams ST & Vickers JC (1986) The Ecology of Antibiotic Production. *Microbial ecology* **12**, 43-52.

Williams DM, Ritcher DS, MacKenzie JMJ & Shih JCH (1990) Isolation, identification and charcterization of a feather degrading bacterium. . *Appl. Environ. Microbiol* **56**.

Williams GP, Gnanadesigan M & Ravikumar S (2012) Biosorption and bio-kinetic studies of halobacterial strains against Ni2+, Al3+ and Hg2+ metal ions. *Bioresour Technol* **107**, 526-529.

Williams PG (2008) Panning for chemical gold: marine bacteria as a source of new therapeutics *Trends in Biotechnology* **27**, 45-52.

Xu XW, Wu YH, Wang CS, Oren A, Zhou PJ & Wu M (2007) *Haloferax larsenii* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int J Syst Evol Microbiol* **57**, 717-720.

Yadav DN & Sarin MM (2009) Ra-Po-Pb isotope systematics in waters of Sambhar Salt Lake, Rajasthan (India): geochemical characterization and particulate reactivity. *J Environ Radioact* **100**, 17-22.

Yakimov MM, Timmis KN, Wray V & Fredrickson HL (1995) Characterization of a New Lipopeptide Surfactant Produced by Thermotolerant and Halotolerant Subsurface Bacillus licheniformis BAS50 *Applied and Environmental Microbiology* **61**, 1706-1713.

Yang S, Lian J, Zhao C, Ma W & Qu Y (2008) [Identification and phylogenetic analysis of an okenone-containing halophilic purple sulfur bacterium]. *Wei Sheng Wu Xue Bao* **48**, 571-576.

Yang X & Cui HL (2012) *Halomicrobium zhouii* sp. nov., a halophilic archaeon from a marine solar saltern. *Int J Syst Evol Microbiol* **62**, 1235-1240.

Yeon SH, Jeong WJ & Park JS (2005) The diversity of culturable organotrophic bacteria from local solar salterns. *J Microbiol* **43**, 1-10.

Yoon JH, Jung SY, Jung YT & Oh TK (2007*) Idiomarina salinarum* sp. nov., isolated from a marine solar saltern in Korea. *Int J Syst Evol Microbiol* **57**, 2503-2506.

Yoon JH, Jung SY, Kang SJ, Jung YT & Oh TK (2007) *Salegentibacter salarius* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **57**, 2738-2742.

Yoon JH, Jung SY, Kang SJ & Oh TK (2007*) Microbulbifer celer* sp. nov., isolated from a marine solar saltern of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **57**, 2365-2369.

Yoon JH, Kang KH, Oh TK & Park YH (2004) *Halobacillus locisalis* sp. nov., a halophilic bacterium isolated from a marine solar saltern of the Yellow Sea in Korea. *Extremophiles* **8**, 23-28.

Yoon JH, Kang SJ, Jung SY, Lee CH & Oh TK (2005) *Algoriphagus yeomjeoni* sp. nov., isolated from a marine solar saltern in the Yellow Sea, Korea. *Int J Syst Evol Microbiol* **55**, 865-870.

Yoon JH, Kang SJ, Jung YT, Lee KC, Oh HW & Oh TK (2010) *Virgibacillus byunsanensis* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **60**, 291-295.

Yoon JH, Kang SJ, Jung YT, Lee MH & Oh TK (2010) *Alkalibacillus flavidus* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **60**, 434-438.

Yoon JH, Kang SJ, Jung YT & Oh TK (2007) *Halobacillus campisalis* sp. nov., containing meso-diaminopimelic acid in the cell-wall peptidoglycan, and emended description of the genus Halobacillus. *Int J Syst Evol Microbiol* **57**, 2021-2025.

Yoon JH, Kang SJ, Jung YT & Oh TK (2009) *Marinimicrobium locisalis* sp. nov., isolated from a marine solar saltern, and emended description of the genus Marinimicrobium. *Int J Syst Evol Microbiol* **59**, 2260-2263.

Yoon JH, Kang SJ, Jung YT & Oh TK (2009) *Psychroflexus salinarum* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **59**, 2404-2407.

Yoon JH, Kang SJ, Lee CH, Oh HW & Oh TK (2005) *Halobacillus yeomjeoni* sp. nov., isolated from a marine solar saltern in Korea. *Int J Syst Evol Microbiol* **55**, 2413-2417.

Yoon JH, Kang SJ, Lee SY, Oh KH & Oh TK (2010*) Planococcus salinarum* sp. nov., isolated from a marine solar saltern, and emended description of the genus Planococcus. *Int J Syst Evol Microbiol* **60**, 754-758.

Yoon JH, Kang SJ, Lee SY & Oh TK (2009) *Salinihabitans flavidus* gen. nov., sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **59**, 2561-2564.

Yoon JH, Kang SJ & Oh TK (2005) *Algoriphagus locisalis* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **55**, 1635-1639.

Yoon JH, Kang SJ & Oh TK (2007) Reclassification of *Marinococcus albus* Hao et al. 1985 as *Salimicrobium album* gen. nov., comb. nov. and *Bacillus halophilus* Ventosa et al. 1990 as *Salimicrobium halophilum* comb. nov., and description of *Salimicrobium luteum* sp. nov. *Int J Syst Evol Microbiol* **57**, 2406-2411.

Yoon JH, Kang SJ & Oh TK (2008) *Halobacillus seohaensis* sp. nov., isolated from a marine solar saltern in Korea. *Int J Syst Evol Microbiol* **58**, 622-627.

Yoon JH, Kang SJ, Schumann P & Oh TK (2010) *Jeotgalibacillus salarius* sp. nov., isolated from a marine saltern, and reclassification of *Marinibacillus marinus* and *Marinibacillus campisalis* as *Jeotgalibacillus marinus* comb. nov. and *Jeotgalibacillus campisalis* comb. nov., respectively. *Int J Syst Evol Microbiol* **60**, 15-20.

Yoon JH, Kim IG, Schumann P, Oh TK & Park YH (2004) *Marinibacillus campisalis* sp. nov., a moderate halophile isolated from a marine solar saltern in Korea, with emended description of the genus *Marinibacillus. Int J Syst Evol Microbiol* **54**, 1317-1321.

Yoon JH, Lee MH, Kang SJ & Oh TK (2007) *Marinobacter salicampi* sp. nov., isolated from a marine solar saltern in Korea. *Int J Syst Evol Microbiol* **57**, 2102-2105.

Yoon JH, Lee MH, Kang SJ & Oh TK (2008) *Salegentibacter salinarum* sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* **58**, 365-369.

Yoon JH, Oh TK & Park YH (2004) Transfer of *Bacillus halodenitrificans* Denariaz et al. 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. *Int J Syst Evol Microbiol* **54**, 2163-2167.

Zafrilla B, Martinez-Espinosa RM, Alonso MA & Bonete MJ (2010) Biodiversity of Archaea and floral of two inland saltern ecosystems in the Alto Vinalopo Valley, Spain. *Saline Systems* **6**, 10.

Zhu F, Qu L, Hong X & Sun X (2011) Isolation and Characterization of a Phosphate-Solubilizing Halophilic Bacterium *Kushneria* sp. YCWA18 from Daqiao Saltern on the Coast of Yellow Sea of China. *Evid Based Complement Alternat Med* **2011**, 615032.

Zhang L, An R, Wang J, Sun N, Zhang S, Hu J & Kuai J (2005) Exploring novel bioactive compounds from marine microbes. *Curr Opin Microbiol* **8**, 276- 281.

Appendix

Appendix 1

Media	Name	Ingredients	Composition g/L in filtered sea water
M1	Tryptone yeast extract agar	Casein enzymic hydrolysate	5.00
		Yeast extract	3.00
		Agar	20.00
		Final pH (at 25°C)	7.0±0.2
M2	Yeast malt agar	Peptic digest of animal tissue	5.00
		Yeast extract	3.00
		Malt extract	3.00
		Dextrose	10.00
		Agar	20.00
		Final pH (at 25°C)	6.2±0.2
M3	Oat meal agar	Oat meal	20.00
		Trace salts solution	1.00 mL
		Agar	18.00
		Final pH (at 25°C)	7.3±0.2
M4	Inorganic salt starch agar	Starch, soluble	10.00
		Dipotassium phosphate	1.00
		Magnesium sulphate	1.00
		Sodium chloride	1.00
		Ammonium sulphate	2.00
		Calcium carbonate	2.00
		Ferrous sulphate	0.001
		Manganous chloride	0.001
		Zinc sulphate	0.001
		Agar	20.00
		Final pH (at 25°C)	7.2±0.2
M5	Glycerol Asparagine agar base	L-Asparagine	1.00
		Dipotassium phosphate	1.00
		Trace salts solution	1.00 mL
		Glycerol	10 mL
		Agar	20.00
		Final pH (at 25°C)	7.4±0.2
M6	Peptone Yeast extract Iron agar	Peptic digest of animal tissue	15.00
		Proteose peptone	5.00
		Yeast extract	1.00
		Ferric ammonium citrate	0.50
		Dipotassium phosphate	1.00
		Sodium thiosulphate	0.08
		Agar	15.00

		Final pH (at 25°C)	6.7±0.2
M7	Tyrosine agar base	L-Asparagine	1.00
		L-Tyrosine	0.50
		Dipotassium phosphate	0.50
		$MgSO_4.7H_2O$	0.50
		Sodium chloride	0.50
		Ferrous sulphate hepta hydrate	0.000011
		Copper chloride dihydrate	0.000027
		Cobalt chloride hexahydrate	0.00004
		Sodium molybdate dihydrate	0.000025
		Zinc chloride	0.00002
		Boric acid	0.00285
		Manganese chloride tetra hydrate	0.0018
		Sodium tartarate	0.00177
		Agar	20.00
		Final pH (at 25°C)	7.3±0.1
Minimal media		Distilled water	700 mL
		5X M9 salt solution (Composition g/L: $Na_2HPO_4.7H_2O$ 64, KH_2PO_4 15, salt 2.5 and NH_4Cl 5)	200 mL
		Salt	9.5g
		1M $MgSO_4.7H_2O$	2 mL
		1M (Fused) $CaCl_2$	100 µL
		Distilled water quantity sufficient to make	1000 mL
		pH	6.0
TSK Media		Minimal media	1000mL
		Sucrose	6g
		Potassium nitrate	6g
Trace Salt Solution			Per 1000 mL
		$FeSO_4.7H_2O$	1 g
		$MnCl_2.4H_2O$	1 g
		$ZnSO_4.7H_2O$	1 g

Primer Type	Primer Sequence (5'-3')	Expected Size of Product
Forward universal bacterial primer (27f)	AGAGTTTGATCMTGGCTCAG	1,500 bp
Reverse universal bacterial primer (1525r)	AAGGAGGTGWTCCARCC	

1% agarose gel details:

50X TAE Buffer for 500mL
121 g Tris base in 250 mL ddH_2O
Stir to dissolve add 28.6 mL acetic acid add 50 mL 0.5M EDTA pH 8.0 measure in graduated cylinder and add ddH_2O to 500 mL.

1X TAE Buffer for 500mL
10 mL 50X TAE buffer 490 mL ddH_2O

1% agarose gel in TAE for 100mL
1g agarose
100 mL 1X TAE

Microwave 1 minute to get into solution, do not allow to boil over if crystals not dissolved, swirl and microwave for additional 20 seconds-1 minute after microwaving, add 5uL ethidium bromide solution (10 mg/ml) per 100 mL pour in gel – takes about 20 minutes to set.

Reagents for FAME analysis:

Reagent 1: The methanolic base (NaOH 45g, Methanol HPLC grade 150 ml, Deionized distilled water 150 ml).

Reagent 2: The methylation reagent (6N HCL-325 ml, Methanol HPLC grade-275 ml).

Reagent 3: The extraction solvent (Methanol HPLC grade-200 ml, methyl tert-butyl ether-200 ml).

Reagent 4: The base wash (NaOH-10.8 g, deionized distilled water-900 ml).

Appendix 2

Details of the sequences submitted in GenBank:

S.no.	Isolate code	GenBank Accession Number	Significantly similar to	Nearest homolog
1	TSK 71	JF411056	*Bacillus licheniformis* (GeneBank Accession No.DQ167473)	*Bacillus subtilis* (GeneBank Accession No. EU221345)
2	6	JF430795	*Bacillus licheniformis* (GeneBank Accession No.GQ280087)	*Bacillus subtilis* (GeneBank Accession No. FJ493055)
3	7	JF411053	*Virgibacillus* sp. (GeneBank Accession No.GQ407221)	*Virgibacillus* sp. (GeneBank Accession No. GQ407220)
4	12	JF411054	*Bacillus licheniformis* (GeneBank Accession No.AY842874)	*Bacillus licheniformis* (GeneBank Accession No. AY842876)
5	17	JF411055	*Bacillus subtilis* subsp. *subtilis* (GeneBank Accession No. GQ375226)	*Bacillus subtilis* (GeneBank Accession No. AY971360)

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Publications

Manuscripts Published:

- **Bacteria from salt pans: a potential resource of antibacterial metabolites. Tonima Kamat** and Savita Kerkar. *Recent Research in Science and Technology*, 2011, 3(9): 46-52.

- **Pharmaceutical potentials of bacteria from saltpans of Goa, India. Tonima Kamat** and Savita Kerkar. *International Journal of Pharmaceutical Applications*, 2011, 2(3):150-154.

- **Antimicrobial potential of *Bacillus marismortui*, a salt pan isolate of Cavellosim, Goa, India. Tonima K. Kamat**, Shashi Kiran and Savita Kerkar. *International Journal of Advanced Biotechnology and Research*, 2011, 2(3): 321-328.

Abstracts published at conferences:

- **Salt pan microbes: Potential factories of diverse metabolites.** Shuvankar Ballav, **Tonima Kamat**, Flory Pereira and Savita Kerkar. Abstracts of 52nd Annual Conference of Association of Microbiologists of India (AMI), International Conference on Microbial Biotechnology for Sustainable Development, Panjab University, Chandigarh, November 3-6, 2011, Page 126.

- **Biotechnological potential of bacteria from saltpans of Goa, India. Tonima Kamat** and Savita Kerkar. Abstracts of North Zone Meet of the Indian Society of Mycology and Plant Pathology (ISMPP) and Symposium on Microbial Diversity and Bioprospecting, University of Jammu, Jammu, 29-30 October, 2010, Page 30.

- **Profiling the bioactive potential of an *Acinetobacter* sp. from a Ribandar saltern in Goa. Tonima Kamat** and Savita Kerkar. Abstracts of National Seminar on New Trends in Biotechnology, Goa, 2007.

Manuscripts submitted:

- **Bacitracin from a halotolerant *Bacillus licheniformis* TSK 71 isolated from salt pan of Goa, India.**

Manuscripts under preparation:

- **Actinobacterial diversity from Ribandar salt pan, Goa, India.**

- **Halotolerant bacteria as potential probiont in shrimp aquaculture.**

Other publications:

- **Screening marine organisms for antimicrobial activity against clinical pathogens.** Prabha Devi, Solimabi Wahidulla, **Tonima Kamat**, Lisette D'Souza. *Indian Journal of Geo-Marine Sciences*, Volume 40(3) June 2011 Pp. 338-346.
- **Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of citrinin.** Prabha Devi, Lisette D'Souza, **Tonima Kamat**, Celina Rodrigues and Chandrakant G. Naik. *Indian Journal of Marine Sciences*, Volume 38(1) March 2009 Pp.38-44.
- **Diffuse reflectance infrared fourier transform spectroscopic (DRIFTS) investigation of *E.coli, Staphylococcus aureus* and *Candida albicans.*** Lisette D'Souza, Prabha Devi, **Tonima Kamat** and Chandrakant G Naik. *Indian Journal of Marine Sciences*, Volume 38(1) March 2009 Pp.45-51.
- **Marine-derived fungi as a source of proteases. Tonima Kamat**, Celina Rodrigues and Chandrakant G Naik. *Indian Journal of Marine Sciences*, Volume 37(3) September 2008 Pp. 326-328.

Other conferences attended:

- **Antimicrobial potential of marine invertebrates from off the south coast of India. Tonima Kamat**, Prabha Devi, Lisette D'Souza and Chandrakant Govind Naik. Abstracts of International Symposium on New Frontiers in Marine Natural Product Research, National Institute of Oceanography, Goa, 2007.
- **Marine Organisms: Their Potential Application as Antibiotic Agents. Tonima Kamat**, Prabha Devi, Lisette D'Souza and Chandrakant Govind Naik. Abstracts of Asia Pacific Marine Biotechnology Conference, National Institute of Oceanography, Kochi, 2006.
- **Production of Citrinin by *Penicillium chrysogenum* and a report on its antibiotic activity.** Prabha Devi, Lisette D'Souza, **Tonima Kamat**, Celina Rodrigues and Chandrakant Govind Naik. Abstracts of Asia Pacific Marine Biotechnology Conference, National Institute of Oceanography, Kochi, 2006.
- **Application of Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) in microbiology.** Lisette D'Souza, Prabha Devi, **Tonima Kamat**, Chandrakant G. Naik. Abstracts of Asia Pacific Marine Biotechnology Conference, National Institute of Oceanography, Kochi, 2006.
- **Antibacterial potential of marine associated fungi.** Celina Rodrigues, **Tonima Kamat**, Rajesh Parvatkar, Ashootosh Tripathi and C. G. Naik. Proceedings of National seminar on prospecting fungal diversity in tropical India, its conservation and applications in biotechnology, Agharkar Research Institute, Pune, Maharashtra, 2006.
- **Microbial symbionts – a source of industrially important enzymes. Tonima Kamat**, Celina Rodrigues and C. G. Naik. Proceedings of 7th National Symposium on Biochemical Engineering and Biotechnology (BIOHORIZON – 2005) IIT, Delhi.
- **Studies on a bioactive compound produced by a halotolerant saltpan isolate. Tonima Kamat**, and Savita Kerkar**.** Abstract volume of Conference On Microbiology of Tropical Seas (COMITS), National Institute of Oceanography, Goa, 2004.

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Bacteria from Salt Pans: A Potential Resource of Antibacterial Metabolites

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Abstract

Marine salt pans are important ecological niches which inhabit halobacteria. These bacteria tolerate and thrive in salt concentrations ranging from 0.5 to more than 5 M in which only very few other organisms are able to survive. Bacteria from marine salt pans of varying salinities of 220 to 395 psu were isolated during the peak salt harvesting season and screened to evaluate their antibiotic producing potential. In this report, a total of 119 bacteria were screened on 12 different solid media supplemented with either natural salt or sea water or distilled water to check their substrate utilization and salinity requirement. Based on their morphological variations, 94 isolates were further screened for their antagonistic properties, against 20 different clinical pathogens. Thirty one isolates were found to produce antibacterial compounds of which, 21 showed bactericidal action and one was bacteriostatic while 9 isolates exhibited both bacteriostatic and bactericidal activity. Eleven isolates were broad spectrum antibiotic producers. This study provides information regarding the applied value of potential halotolerant and halophilic isolates as pharmaceutically important microorganisms.

Key Words: Bioactivity, Hypersaline, Secondary metabolites, Salt pans

Introduction

Marine salt pans in Goa are thalassohaline, multi-pond systems which are interconnected; allowing a discontinuous salinity gradient. These ponds have a continuous inflow of sea water which is evaporated for the commercial production of sodium chloride i.e. natural salt.

Microbes from salt pans are yet to be fully explored as potential producers of antimicrobial agents. However, few reports are available on the antimicrobial potential of microorganisms in Indian salterns. Dhanasekaran *et al.* (1) have reported antibacterial potential of salt pan actinomycetes from Tamil Nadu, India. Kamat and Kerkar (2) have carried out studies on a marine salt pan bacterium, producing a broad spectrum antibiotic, from Goa, India.

In the present study our goal was to focus on antibiotic producing potential of salt pan bacteria from two marine salterns namely Batim and Ribandar from North Goa. The bacterial isolates have been screened against twenty human pathogens to establish their inhibitory activity. Additionally, studies have been carried out on the substrate utilization and salinity requirement of the isolates. Because of the growing interest in the study of secondary metabolites from marine environments these hypersaline ecosystems could be highly promising habitats for the discovery of microorganisms capable of producing novel and useful bioactive compounds for the future development of medicine, agriculture and biotechnology industry.

Materials and Methods

Sample collection and isolation of marine bacteria:

Sampling was restricted to the crystallizer ponds from the salt pans of Batim and Ribandar, Goa, India during the pre-monsoon i.e. the peak salt manufacturing season of 2007 and 2008. Water samples (four) from overlying salt pans, were collected in sterile disposable bottles, chilled immediately on ice and transported to the laboratory for processing within a 24h period. The salt pan water salinity, temperature and pH were measured at the site using S/Mill-E Hand-held refractometer (ATAGO, Japan) and pHTestr 30 (Eutech Instruments, Thermo Scientific, USA) respectively. Sediment samples (four) were collected using a 10 cm corer. Core samples were sealed in sterile plastic bags and transported at 4°C in an ice box. For the isolation of hypersaline bacteria in brief, serial dilutions were carried out with sterile salt pan water and 0.2 ml of the sample was spread plated, in duplicates, on International *Streptomyces* Project (ISP) media 1 to 7 (ISP 1: Tryptone Yeast extract agar, ISP 2: Yeast Malt agar, ISP 3: Oat meal agar, ISP 4: Inorganic salt Starch agar, ISP 5: Glycerol Asparagine agar base, ISP 6: Peptone Yeast extract Iron agar, ISP 7: Tyrosine agar base) (3) and Nutrient Agar (NA) +5% crude NaCl. All the media used were prepared in filtered sea water except NA+5% crude NaCl which was prepared in distilled water. The plates were incubated at 37 °C for 72h. The strains isolated were purified, sub cultured and

stored at 4 °C. All the media and media components used for the experiments were procured from Hi Media, Mumbai, India unless otherwise specified.

Growth and tolerance to salinity:

All the isolates were spotted on 12 different agar media to assess their growth and their tolerance to salinity. The media used for the study included NA (may also be referred as NA D/W i.e. NA prepared using distilled water), NA+10% crude NaCl, NA prepared using sea water (NA S/W) and Kuster's agar prepared in sea water as well as the 8 media used for isolation as mentioned above. The isolates were streaked on respective agar plates and incubated at 37 °C for 24-48h.

Antibacterial activity:

Twenty clinical pathogens namely *Acinetobacter baumanii* (AB), *Aeromonas hydrophila* (AH), *Citrobacter diversus* (CD), *Citrobacter freundii* (CF), *Escherichia coli ATCC 25922* (ECATCC), *Klebsiella pneumoniae* (KP), *Morganella morganii* (MM), *Proteus mirabilis* (PM), *Pseudomonas ATCC 27855* (PATCC), *Pseudomonas spp. (Pigmented)* (PP), *Salmonella paratyphi A* (SPA), *Salmonella typhi* (ST), *Salmonella typhimurium* (STM), *Shigella boydii* (SB), *Shigella flexneri* (SF), *Vibrio cholerae* (VC), *Methicillin Resistant Staphylococcus aureus* (MRSA), *Methicillin Sensitive Staphylococcus aureus* (MSSA), *Staphylococcus ATCC 25923* (SATCC), *Staphylococcus citreus* (SC) were obtained from Goa Medical College & Hospital, Goa, India to be used as test organisms against isolates. Preliminary screening was performed on 94 isolates by the cross streak method. The isolates were streaked as a ribbon on NA plates, in duplicates, and incubated at 37 °C for 48 h. After incubation, the test pathogens were streaked perpendicular to the ribbon and the plates were incubated again for 16-24 h at 37 °C. The observations were recorded as mm of inhibition.

Secondary screening by Kirby-Bauer disc diffusion method:

Six isolates namely 6, 7, 12, 17, TSK 32 and TSK 71 with most promising antimicrobial potential were tested. The isolates were grown in 50 mL Nutrient broth (NB) at 120 rpm, room temperature (28 ± 2 °C) for 3 days. After every 24 h, 1 mL of each culture was centrifuged at 8000 rpm. Sterile discs (Whatman filter no. 1) 6mm each, were impregnated with 10 µL of the filter sterilised culture supernatant and allowed to air dry under aseptic conditions. The test pathogen *Staphylococcus citreus* was suspended in 0.85% sterile saline (6.25 x10^7 cells per mL), vortexed and spread plated on NA. The impregnated discs were then placed on the culture lawn along with positive control (Streptomycin 10µg) and incubated at 37 ºC for 24 hours. The zones of inhibition were recorded in mm. Assays were carried out in triplicates.

Characterization of the active isolates:

The six active isolates were tested for utilization of 35 carbohydrates using KB009 HiCarbohydrate™ Kit (Part A, B and C) from Hi Media, Mumbai, India. The bacterial suspension grown in ISP-1 media was inoculated into KB009 strips and incubated at 37 °C. Results were recorded after 24 h. Presence of endospores was confirmed using Schaeffer-Fulton's staining method.

DNA was isolated from the active isolates and was PCR amplified using universal bacterial primers for 16S rDNA. The PCR amplified product was separated electrophoretically on 1% agarose gel. Approximately 1.5kb fragment was gel purified and sequenced.

Antimicrobial susceptibility test for active isolates:

Active isolates were grown for 16-18 hours and 0.1 mL suspension was spread on Muller Hinton agar plates. The antibiotic discs Cephalexin (30 µg), Tetracycline (30 µg), Cefuroxime (30 µg), Lincomycin (10 µg), Methicillin (5 µg), Gentamicin (10 µg) from Hi Media, India were placed on the lawn of the respective culture and incubated overnight at 37 °C. The test was performed as per the CLSI methodology provided by the HiMedia Laboratories Pvt. Ltd., Mumbai, India (4). The zones of inhibition were recorded.

Results and Discussion

The bioactive molecules currently available in the market have been obtained after decades of intensive screening and research. The past successes make discovering new bioactive metabolites from microbial sources much more difficult, since thousands of compounds are described in literature (5, 6). Newer niches are frantically being explored to screen potential producers of novel bioactive molecules (7).

Marine actinomycetes viz. *Streptomyces* and *Micromonospora* strains have been reported to produce antibiotics like enterocin and ikarugamycin which have previously been isolated from terrestrial strains whereas abyssomicins, a novel family of antibiotics, produced by marine *Verrucosispora* strains (8), marinomycins by *Marinispora* sp., Salinosporamide A (NPI-0052) by *Salinispora tropica* and many other novel metabolites produced by marine actinomycetes have been reported (9). In India, a halophilic *Actinopolyspora* species AH1, with antibacterial activity, was isolated from the sediments of Alibag coast of Maharashtra. The strain exhibited antagonistic activity against Gram positive bacteria and some fungi. (10).

Salt pans are an extreme environment, which inhabit organisms that survive at very high salinities, high temperatures and withstand severe solar radiations. To survive in such extreme environment, these organisms are known to produce secondary metabolites. These metabolites can sometimes be of great importance due to their bioactive potential. Hence organisms isolated from these environments could serve as a source for the discovery of novel secondary metabolites.

Sample collection and isolation of marine bacteria:

We have explored two salt pans in north Goa namely Batim and Ribandar to investigate the antibiotic producing potential of these bacteria. The sampling parameters are recorded in Table 1. Hundred and nineteen bacterial isolates which include bacteria (94%) and actinobacteria (6%) were isolated on eight different media. The number of isolates obtained on various media are shown in Table 2. Maximum number of isolates (33) were obtained on ISP 6 media. Nearly equal number of isolates i.e. 60 from Ribandar and 59 from Batim were obtained from both the sampling sites.

Table 1. Sampling parameters for collections during pre-monsoon season of 2007 and 2008.

Physical parameters during sampling	Sampling site 1 (May 2007)	Sampling site 2 (May 2007)	Sampling site 1 (May 2008)	Sampling site 2 (May 2008)
Place	Ribandar	Batim	Ribandar	Batim
GPS data	N 15° 29' 58.1" E 073° 50' 49.2"	N 15° 27' 27.6" E 073° 52' 58.0"	N 15° 30' 8.1" E 073° 51' 19.6"	N 15° 27' 28.6" E 073° 52' 50.6"
Temperature	45°C	38°C	45.3°C	45°C
Salinity (psu)	320	220	310	395
pH	7.09	7.09	6.63	6.43

Table 2. Isolates obtained on various media.

Media of isolation	No. of isolates May 2007	No. of isolates May 2008	Total no. of isolates
ISP No. 1	0	18	18
ISP No. 2	11	16	27
ISP No. 3	5	5	10
ISP No. 4	2	3	5
ISP No. 5	0	1	1
ISP No. 6	9	24	33
ISP No. 7	5	3	8
NA + 5% NaCl	5	12	17
Total	37	82	119

With salinities at or near sodium chloride saturation hypersaline environment of salt pans supports only limited microbiota. These NaCl saturated salt pans allow the growth of moderately halophilic and halotolerant bacteria (11). Manikandan and Kannan (11) assessed the diversity of cultivable microorganism from three solar salterns along the shoreline of Bay of Bengal in Tamil Nadu, India. They used three different media to isolate halophiles. Vijayakumar *et al.* (12) have reported 19 actinomycetes isolates in the salt pans of Vedaranyam, Thondi and Tuticorin in Palk Strait region of Bay of Bengal, India. Dhanasekaran *et al.* (3) have isolated 9 *Streptomyces* isolates from salt pan soil (1 from Porto Novo and 3 from Thaikkalthurai, Tamil Nadu, India).

Growth and tolerance to salinity:

Qualitative analysis of growth on various media indicated that 97% isolates showed dense growth (matt or confluent growth where colonies are merged and uncountable) on ISP 2 media followed by ISP 1 (87%). Most of the isolates (79%) did not require salt for growth, and only 21% isolates did not show growth in the absence of crude NaCl. Nearly 50% of the isolates could tolerate up to 10 % crude NaCl while 61% could tolerate up to only 5% crude NaCl. The results are shown in Figure 1. There are numerous reports which support that most actinomycetes isolated from marine sources are of terrestrial origin and reside in the marine ecosystem as spores or resting propagules (13). Thus some of the salt tolerant bacteria are likely to have a terrestrial origin, entering the salt pans from the surrounding ecosystems.

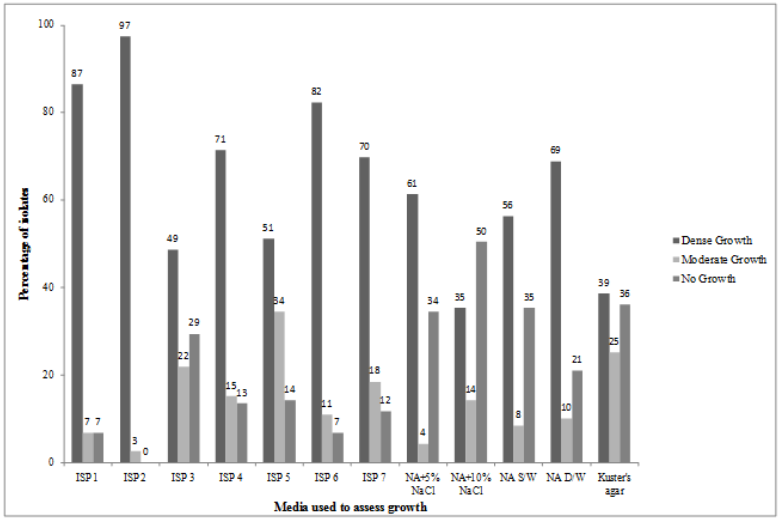

Figure 1. Growth of isolates on various media and salt tolerance study.

Antibacterial activity

Ninety four isolates, based on morphological differences, were screened for antibacterial activity. Numerous screening methods to determine antimicrobial activity of natural products have been discussed in the literature (14, 15). We used simple cross streak method as described above. When no growth of pathogen was seen it was considered to be bactericidal action whereas when few colonies (compared with the control) were seen it was considered to be bacteriostatic activity. Only 31 isolates were found to be potentially active. Of these, 21 showed bactericidal action [Isolate codes: 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 16, 17, 18, 21, 22, TSK 7, TSK 11, TSK 28, TSK 38 and TSK 40] and one was bacteriostatic [Isolate code: TSK 31] while nine were bacteriostatic as well as bacteriocidal [Isolate codes: TSK 10, TSK 19, TSK 24, TSK 32, TSK 33, TSK 43, TSK 44, TSK 45 and TSK 71]. Figure 2 shows preliminary screening by cross streak method for isolate no. 6. Out of the 31 actives, two isolates [Isolate codes: TSK 38 and TSK 40] inhibited Gram positive pathogen SC; eighteen [Isolate codes: 1, 2, 3, 5, 8, 10, 13, 16, 18, 21, 22, TSK 7, TSK 10, TSK 11, TSK 24, TSK 28, TSK 31 and TSK 33] inhibited Gram negative pathogens viz. AB, CF, PP, SPA, ST, SB, SF & VC whereas eleven [Isolate codes: 6, 7, 9, 12, 17, TSK 19, TSK 32, TSK 43, TSK 44, TSK 45 and TSK 71] were broad spectrum antibiotic producers. The profiles of most significant activity are shown in Figure 3.

Secondary screening by Kirby-Bauer disc diffusion method:

Secondary screening was performed on the six most promising isolates, namely 6, 7, 12, 17, TSK 32 and TSK 71. During primary screening a maximum inhibitory zone was observed with SC and hence chosen for secondary screening. Amongst the six cultures tested for activity against SC, TSK 71 had the maximum activity ($p<0.05$). Also there were no significant differences ($p>0.05$), over time (three days tested), in the bactericidal action of TSK 71 on SC. Hence, as shown in Table 3, the maximum zone of inhibition was exhibited by TSK 71 consistently for 3 days.

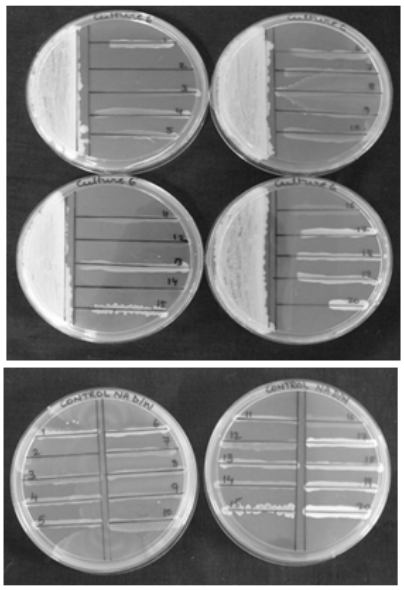

Figure 2. Preliminary screening by cross streak method. Top four plates show preliminary screening by cross streak method for isolate no. 6 and bottom two plates show growth of pathogens on NA (control). 1: *Acinetobacter baumanii*; 2: *Aeromonas hydrophila;* 3: *Citrobacter diversus;* 4: *Citrobacter freundii;* 5: *Escherichia coli ATCC 25922 ;* 6: *Klebsiella pneumoniae;* 7: *Morganella morganii;* 8: *Proteus mirabilis;* 9: *Pseudomonas ATCC 27855;* 10: *Pseudomonas spp. (Pigmented);* 11: *Salmonella paratyphi A;* 12: *Salmonella typhi;* 13: *Salmonella typhimurium;* 14: *Shigella boydii;* 15: *Shigella flexneri;* 16: *Vibrio cholerae;* 17: *Methicillin Resistant Staphylococcus aureus;* 18: *Methicillin Sensitive Staphylococcus aureus;* 19: *Staphylococcus ATCC 25923;* 20: *Staphylococcus citreus*.

Table 3. Zone of inhibition in mm against *Staphylococcus citreus*.

Culture no.	Zone of inhibition in mm		
	Day 1	Day 2	Day 3
6	11	17	15
7	0	13	10
12	9	11	10
17	11	12	11
TSK 32	14	17	16
TSK 71	15	17	17

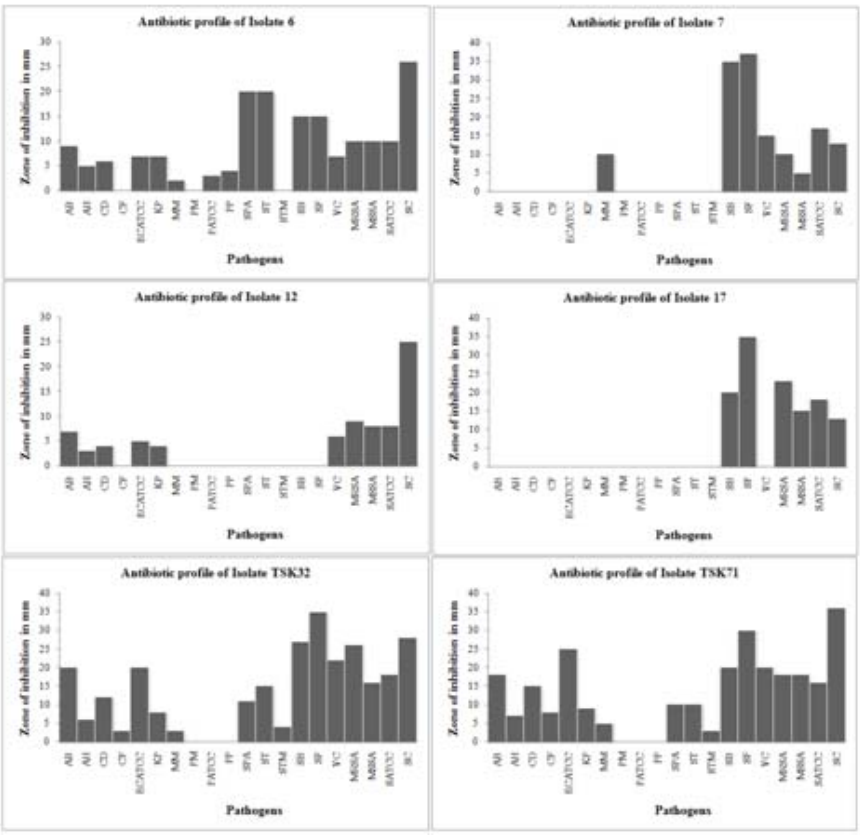

Figure 3. Antibiotic profiles of active isolates showing most significant bactericidal activity. A: Profile of Isolate 6; B: Profile of Isolate 7; C: Profile of Isolate 12; D: Profile of Isolate 17; E: Profile of Isolate TSK32; F: Profile of Isolate TSK71. AB: *Acinetobacter baumanii;* AH: *Aeromonas hydrophila;* CD: *Citrobacter diversus;* CF: *Citrobacter freundii;* ECATCC: *Escherichia coli ATCC 25922;* KP: *Klebsiella pneumonia;* MM: *Morganella morganii;* PM: *Proteus mirabilis;* PATCC: *Pseudomonas ATCC 27855;* PP: *Pseudomonas spp. (Pigmented);* SPA: *Salmonella paratyphi A;* ST*: Salmonella typhi;* STM: *Salmonella typhimurium;* SB: *Shigella boydii;* SF: *Shigella flexneri;* VC: *Vibrio cholerae;* MRSA: *Methicillin Resistant Staphylococcus aureus ;* MSSA: *Methicillin Sensitive Staphylococcus aureus;* SATCC: *Staphylococcus ATCC 25923;* SC*: Staphylococcus citreus*.

Suthindhiran and Kannabiran (16) have reported antimicrobial activity of *Saccharopolyspora salina* VITSDK4 isolated from a salt pan marine soil sample collected at the Marakkanam coast of the Bay of Bengal, India. One hundred and sixteen strains were isolated on screening for bioactive marine actinobacteria, from which only 7 isolates exhibited broad spectrum activity. *Saccharopolyspora salina* VITSDK4 was profoundly antagonistic with fungal and Gram positive pathogens. Gokulkrishnan et al. (17) have reported antimicrobial activity of marine bacteria isolated from the Mangalore coast, along the west coast of India. Out of 38 isolates only 21 showed activity during primary screening against test organisms. Of which 3 were active against Gram negative, 10 against Gram positive and 12 against both Gram positive and Gram negative organisms. Kamat and Kerkar (2, 18) have reported a halotolerant *Acinetobacter* sp. from salt pans of Ribandar, Goa producing antibacterial compound. Halocins have been reported to be produced by extremely halophilic bacteria. Some of the halocins characterized and studied include halocin H4 from *Haloferax mediterranei* R4 (19), halocin H6 from *Haloferax gibbonsii* (20) and halocin S8 from an uncharacterized extremely halophilic rod –strain S8 (21). Dhanasekaran *et al.* (1) have reported three actinomycetes, from salt pan regions of Cuddalore and Parangipettai, Tamil Nadu, India, showing promising antibacterial activity against eight test organisms. Dhanasekaran *et al.* (22) have also reported six broad spectrum antibacterial *Streptomyces* out of nine (66.7%) from salt pan soil. Four out of nine (44.44%) strains also showed extra- and intra cellular antifungal activity.

Characterization of the active isolates:

As shown in Table 4, the isolates TSK 32 and TSK 71 could utilize six carbohydrates and the carbohydrate utilization pattern of TSK 32 resembled that of TSK 71. The colony morphology on various media and salinity tolerance pattern of both the isolates was also similar. Depending on their substrate utilization and morphology TSK 32 and TSK71 were found to be similar. The isolate number 6 and 12 could utilize eighteen carbohydrates while isolate 17 could utilize ten carbohydrates. The carbohydrate utilization pattern of isolate 7 was stringent. The isolate metabolised mannose, glycerol, salicin and inositol. All the isolates showed the presence of endospores.

Table 4. Biochemical characterization of active isolates.

Sr.No.	Test	6	7	12	17	TSK 32	TSK 71
1	Lactose	-	-	-	-	-	-
2	Xylose	-	-	-	-	-	-
3	Maltose	+	-	+	-	-	-
4	Fructose	+	-	+	+	+/-	+/-
5	Dextrose	+	-	+	-	+	+
6	Galactose	+	-	-	-	-	-
7	Raffinose	-	-	-	-	-	-
8	Trehalose	+	-	+	-	-	-
9	Melibiose	-	-	+	-	-	-
10	Sucrose	+	-	+	+	+/-	+/-
11	L-Arabinose	+	-	+	+	+/-	+/-
12	Mannose	+	+	+	+	+	+
13	Inulin	+	-	-	+	-	-
14	Sodium gluconate	-	-	+	-	-	-
15	Glycerol	+	+	+	+	+	+
16	Salicin	+	+	+	-	-	-
17	Glucosamine	+	-	-	-	-	-
18	Dulcitol	-	-	-	-	-	-
19	Inositol	+	+	+	-	-	-
20	Sorbitol	+	-	+	+	-	-
21	Mannitol	+	-	+	+	-	-
22	Adonitol	-	-	-	-	-	-
23	A Methyl-D-glucoside	-	-	-	-	-	-
24	Ribose	+	-	+	+	-	-
25	Rhamnose	-	-	-	-	-	-
26	Cellobiose	+	-	+	-	-	-
27	Melezitose	-	-	-	-	-	-
28	A Methyl-D-mannoside	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-
30	ONPG	-	-	+	-	+	+
31	Esculin	-	-	-	-	+	+
32	D-Arabinose	+	-	+	+	+/-	+/-
33	Citrate	-	-	-	-	+	+
34	Malonate	-	-	-	-	-	-
35	Sorbose	-	-	-	-	-	-
36	Gram character	Positive	Positive	Positive	Positive	Positive	Positive

Based on 16S rDNA sequence and biochemical characterization we assign isolate number 6, 12, 17 and TSK 71 to the genus *Bacillus* (GenBank accession numbers JF430795, JF411054, JF411055 and JF411056 respectively) and isolate number 7 to *Virgibacillus* (GenBank accession number JF411053). *Bacillus* spp. are also marine sediment inhabitants. Nowlan *et. al.* (23) have reported *Bacillus okhensis* sp. nov., a halotolerant and alkalitolerant bacterium from a natural salt pan near Okha, Gujarat, India.

Bacillus okhensis (23) isolated from salt pan in Gujarat utilized dextrose, mannose, L-arabinose, galactose, ribose, xylose and rhamnose but not glycerol, mannitol, and sucrose. Our four *Bacillus* isolates viz. 6, 12, TSK 32 and TSK 71 could utilize dextrose, mannose, glycerol and sucrose but not xylose and rhamnose. Isolate 17 showed a similar pattern but could not utilize dextrose. Only isolate 6 could utilize galactose while isolate number 6, 12 and 17 could utilize ribose and mannitol.

Antimicrobial susceptibility test was carried out for all the active isolates and results were recorded as sensitive or resistant to the antibiotic concentration tested. Our *Bacillus* isolates (6, 12 and TSK 71) were sensitive to methicillin, cefuroxime, cephalexin and also gentamicin and tetracycline but resistant to lincomycin as shown in Table 5. Isolate 17 showed a similar sensitivity pattern but was sensitive to lincomycin as well. Nowlan *et al.* (23) reported that *Bacillus okhensis* was sensitive to lincomycin, methicillin, cefuroxime and cephalexin but resistant to gentamicin and tetracycline.

Table 5. Antibiotic sensitivity test.

Name of the antibiotic	Concentration (µg)	6	7	12	17	TSK 71
Cephalexin	30	S	S	S	S	S
Tetracycline	30	S	S	S	S	S
Cefuroxime	30	S	S	I	S	S
Lincomycin	10	R	S	R	S	R
Methicillin	5	S	S	I	S	S
Gentamicin	10	S	S	S	S	S

I: Intermediate; R: Resistant; S: Sensitive

To conclude, our results indicate that salt pan bacteria from Batim and Ribandar have an interesting antibiotic producing profile and hence can be looked upon as potential resource of antibacterial metabolites. Very few reports are available on the antimicrobial potential of the cultures isolated from Indian salt pans. This study encourages exploration of other salt pans in the discovery of potential halotolerant and halophilic isolates as pharmaceutically important microorganisms with a possibility of being "novel antibiotic" producers.

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References

[1] Dhanasekaran, D., N. Thajuddin and A. Panneerselvam. 2008-2009. Distribution and ecobiology of antagonistic streptomycetes from agriculture and coastal soil in Tamil Nadu, India. J. Culture Collections, 6:10-20.

[2] Kamat, T. and S. Kerkar. 2004. Studies on a bioactive compound produced by a halotolerant salt pan isolate. Conference on Microbiology of the Tropical Seas (COMITS), National Institute of Oceanography, Goa. MB (P) 10.

[3] Shirling, E.B. and D. Gottlieb. 1966. Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol. 16:312-340.

[4] Wikler, M. A., F. R. Cockerill, W. A. Craig, M. N. Dudley, G. M. Eliopoulos, D. W. Hecht, J. F. Hindler, D. E. Low, D. J. Sheehan, F. C. Tenover, J. D. Turnidge, M. P. Weinstein, B. L. Zimmer, M. J. Ferraro and J. M. Swenson. Jan 2007. Performance Standards for Antimicrobial Susceptibility Testing, Seventeenth Informational Supplement, CLSI 27 (1):1-177.

[5] Donadio, S., P. Monciardini, R. Alduina, P. Mazza, C. Chiocchini, L. Cavaletti, M. Sosio and A. M. Puglia. 2002. Microbial technologies for the discovery of novel bioactive metabolites. J. Biotechnol. 99 (3):187-198.

[6] Yarbrough, G.G., D.P. Taylor, R.T. Rowlands, M.S. Crawford and L.L. Lasure. 1993. Screening microbial metabolites for new drugs-Theoretical and practical issues. The J. Antibiotics. 46(4):535-544.

[7] Motta, A.S., F. Cladera-Olivera and A. Brandelli. 2004. Screening for antimicrobial activity among bacteria isolated from the Amazon Basin. Braz. J. Microbiol., 35:307-310.

[8] Fiedler, H-P., C. Bruntner, A.T. Bull, A.C. Ward, M. Goodfellow, O. Potterat, C. Puder and G. Mihm. 2005. Marine actinomycetes as a source of novel secondary metabolites. Antonie van Leeuwenhoek 87:37-42.

[9] Lam, K. S. 2006. Discovery of novel metabolites from marine actinomycetes. Curr. Opin. Microbiol. 9:245-251.

[10] Kokare, C.R., K.R. Mahadik, S.S. Kadam and B.A. Chopade. 2004. Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* species AH1 from the west coast of India. Curr. Sci. 86 (4):593-597.

[11] Manikandan, M. and V. Kannan. 2009. Diversity of microorganisms in solar salterns of Tamil Nadu, India. World J. Microbiol. Biotechnol. 25:1007-1017.

[12] Vijayakumar, R., C. Muthukumar, N. Thajuddin, A. Panneerselvam and R. Saravanamuthu. 2007. Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. Actinomycetologica 21(2):59-65.

[13] Jensen, P.R., R. Dwight and W. Fenical. 1991. Distribution of Actinomycetes in Near-Shore Tropical Marine Sediments. Appl. Environ. Microbiol. 57 (4):1102-1108.

[14] Devienne, K.F. and MS.G. Raddi. 2002. Screening for antimicrobial activity of natural products using a Microplate Photometer. Braz. J. Microbiol., 33:166-168.

[15] Valgas, C., S. Machado de Souza, E.F.A. Smania and A. Smania Jr. 2007. Screening methods to determine antibacterial activity of natural products. Braz. J. Microbiol. 38:369-380.

[16] Suthindhiran, K. and K. Kannabiran. 2009. Cytotoxic and antimicrobial potential of Actinomycete species Saccharopolyspora saline VITSDK4 isolated from the Bay of Bengal coast of India. Am. J. Infect. Dis. 5 (2):90-98.

[17] Gokulkrishnan, K., Kusuma, S. and K. Boopalan. 2011. Antimicrobial activity of marine bacteria isolated from the Mangalore coast, West Coast of India. Rec. Res. Sci. Tech., 3(4):15-17.

[18] Kamat, T. and S. Kerkar. 2007. Profiling the bioactive potential of an *Acinetobacter* sp. from Ribandar saltern in Goa. National Seminar on New Trends in Biotechnology, Goa. BC-07.

[19] Messeguer, I. and F. Rodriguez-Valera. 1985. Production and purification of halocin H4. FEMS Microbiol. Lett. 28:177-182.

[20] Torreblanca, M., I. Messeguer and F. Rodriguez-Valera. 1989. Halocin H6, a bacteriocin from *Haloferax gibbonsii*. J. Gen. Microbiol. 135:2655-2661.

[21] Shand, R. F., L. B. Price and E. M. O'Connor. 1999. Halocins: Protein antibiotics from hypersaline environments. In: Oren, A. (Ed.), Microbiology and biogeochemistry of hypersaline environments. CRC Press, Inc., Boca Raton, FL. 24, pp. 295-305.

[22] Dhanasekaran, D., G. Rajakumar, P. Sivamani, S. Selvamani, A. Panneerselvam and N. Thajuddin. 2005. Screening of salt pans actinomycetes for antibacterial agents. The Internet J. Microbiol., 1 (2).

[23] Nowlan, B., M.S. Dodia, S.P. Singh and B.K.C. Patel. 2006. *Bacillus okhensis* sp. nov., a halotolerant and alkalitolerant bacterium from an Indian salt pan. Int. J. Sys. Evol. Microbiol. 56:1073-1077.

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PHARMACEUTICAL POTENTIALS OF BACTERIA FROM SALTPANS OF GOA, INDIA

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ABSTRACT:

Solar salt pans consist of a series of inter linked pans where gradients of salinity occur due to evaporation of seawater. Salinity in these ponds reaches as high as 400 psu during the peak salt-producing season and as low as 5 psu during the monsoons. Salt pans are extreme environments which inhabit organisms that thrive high salinities, temperatures and withstand severe solar radiations. Such organisms are capable of producing interesting metabolites which may benefit mankind. Salt pan water, salt and sediment samples were collected from nine saltpans from North and South Goa to isolate bacteria. The bacterial extracts have been screened for various biological activities to ascertain their biomedical importance. A total of 63 out of 1178 cultures were found to be active showing antioxidant, anti gastric ulcer, antifungal, memory enhancing activity and activity against neurological disorders, anticancer, amylase, amylase inhibitory, protease and protease inhibitory activity. This study highlights the biomedical potential of bacteria from salt pans and encourages further exploration of such bacteria for therapeutic activities.

Keywords: anticancer, bioactive compounds, salt pans, therapeutic agents.

[I] INTRODUCTION

Marine microbial metabolites have gained tremendous importance in past few decades due to their potential to be 'molecules of the future'. With the increasing problem of drug resistance there is a dire need to isolate, identify and utilize newer molecules of biomedical importance. Newer niches are constantly being sought to identify potential producers of bioactive molecules. Saltpans are an extreme environment, which inhabit organisms that survive very high salinities, high temperatures and withstand severe solar radiations. Hence these organisms could serve sources of novel secondary metabolites. Various halophilic and halotolerant microbes inhabiting the salt pans are yet to be fully explored as potential producers of pharmaceutically significant molecules. Few reports are available on their antimicrobial potential in India [1,2,3].

Goa's traditional salt industry is said to have been a major supplier of salt to the country and an exporter to some foreign countries since the 10th century. But since 2002, only about 16 salt pans are in use for the commercial production of natural salt. Salinity in these ponds ranges from 10 to 400 psu during the peak salt-manufacturing season i.e. between November to May. We have isolated 1178 bacteria from water, salt and sediment samples from nine saltpans and screened the lyophilised aqueous extracts of these bacteria for various biological activities.

[II] MATERIALS AND METHODS

2.1. Sample collection:

Nine saltpans from North and South Goa were selected for sampling. Sampling was restricted to the crystallizer ponds from the salt pans of viz. Ambeli, Arpora, Cavellosim, Curca, Morjim, Nerul, Ribandar, Shiroda and Siridao. Water samples from overlying saltpans, were collected in sterile disposable bottles and chilled on ice. Sediment samples were collected using a 10 -cm corer and salt crystals were collected using a sterile spatula. Core samples and salt crystals

were sealed in sterile plastic bags and transported at 4°C in an ice box and processed within a 24 h period.

2.2. Isolation of bacteria:

In brief, serial dilutions were carried out with sterile salt pan water and 0.2ml of the sample was spread plated, in triplicates, on Nutrient Agar (NA) supplemented with 5% NaCl. The plates were incubated at 37°C until the appearance of colonies. The strains isolated were purified, sub cultured and stored at 4°C. All the media and media components used for the experiments were procured from HiMedia, Mumbai, India unless otherwise specified.

All the isolates growing on these plates were further tested for growth on media C agar [composition in g/L Soluble starch 25, Glucose 10, Yeast Extract 2, Calcium carbonate 3, Trace salt solution 1ml; pH 7.5 (Trace salt solution g/100mL FeSO4.7H2O 0.5, CuSO4.5H2O 0.5, ZnSO4.7H2O 0.5 & MnCl2.4H2O 0.5] and media D agar [composition in g/L Tryptone 15, Soyatone 5, Sodium chloride 5, agar 15; pH 7.3] plates and were selected for further processing.

2.3. Preparation of extracts:

Isolated colonies were inoculated into 40ml of media C or media D broth and kept on shaker for 7 days. The broth was sonicated using an ultra sonicator Vibra cell™ sonicator [Sonics & Materials, Inc. Danbury, CT, USA] with a cycle of 40 seconds with pulse and 40 seconds without pulse, twice. The contents were centrifuged at 10,000 rpm for 20 minutes. The tubes were always kept immersed in ice during the process. The supernatant was decanted and dispensed as 1ml aliquots, frozen and lyophilized using [CoolSafe™ 110 Freeze Dryer, Scanvac, DK]. The freeze dried samples were dispatched to various Council of Scientific and Industrial Research (CSIR) laboratories to evaluate their antioxidant (Indian Institute of Chemical Technology, Hyderabad), anti gastric ulcer against Helicobacter pylori (Indian Institute of Chemical Biology, Calcutta), antifungal (Central Institute of Medicinal and Aromatic Plants, Lucknow), memory enhancing activity & activity against neurological disorders such as anti anxiety, anti dementia, anti depressant & anti parkinsons (Central Drug Research Institute, Lucknow) and anticancer (Indian Institute of Integrative Medicine, Jammu) activity.

The isolated colonies were directly tested for amylase, amylase inhibitory, protease and protease inhibitory activity using plate assay method. MD (Media D) plate was prepared with 1% Starch (soluble starch). Cultures were inoculated on MD Starch agar plates and kept for 48 hours minimum and 3-4 days maximum incubation. Amylase producers were detected with iodine. The colony producing amylases exhibited a clear halo on a blue background which was recorded. Iodine vapours from the plate were allowed to evaporate and subsequently the plate was flooded with commercial amylase Diastase (Alpha amylase) (s d fine-chem) 1% solution (100 micro liter per plate) and incubated for 15 minutes. The amylase was discarded and again the plate was flooded with iodine. Amylase inhibitors exhibited a dark blue halo on a clear plate. The halo size was recorded. To detect protease producers and inhibitors Media D plate was prepared with 1% purified skimmed milk (HiMedia, India), cultures were inoculated and incubated for 48 hours (minimum) and 3-4 days (maximum). The protease producers showed a halo around the colony. The plates were flooded with commercial protease 1% Typsin (HiMedia, India) solution and allowed to react for 15 minutes. The Trypsin was decanted and a white halo was observed around the colonies showing protease inhibitory activity.

[III] RESULTS AND DISCUSSION

Microbes produce secondary metabolites as a response to the effects of the surrounding biotic and abiotic environment. The presently known secondary microbial metabolites exhibit a wide number of varied and versatile bioactivities. The marine microbes, especially those from extreme environments like salt pans and other hypersaline environments represent a massive under developed resource. In this regard, screening and isolation of large number of potential isolates from diverse environments, facilitates the availability of large number of cultures for the depository and in turn would enable us to obtain better isolates for the production of drugs against specific varied diseases. Such potential and novel marine microbes could then be exploited for large scale production of vital drugs. For achieving the above objectives screening for potential bioactive compounds from these hypersaline marine bacteria; water, sediment and salt crystals were explored.

In all 1178 bacteria were isolated from water, sediment and salt crystals from nine different salt pans of Goa. Colonies appeared on their respective solid media within 24 h of incubation. All isolates except BGUM 59 MC showed luxurious growth on Media D whereas BGUM 59 MC showed better growth on Media C. The culture extracts were sent to various laboratories for testing their respective bioactivities. Isolates were tested, in house, for amylase producing & inhibitory activity as well as protease producing & inhibitory activity. Sixty three cultures were found to produce metabolites of pharmaceutical significance (Refer Figure 1). Details of the culture extracts showing various activities are given in Table 1.

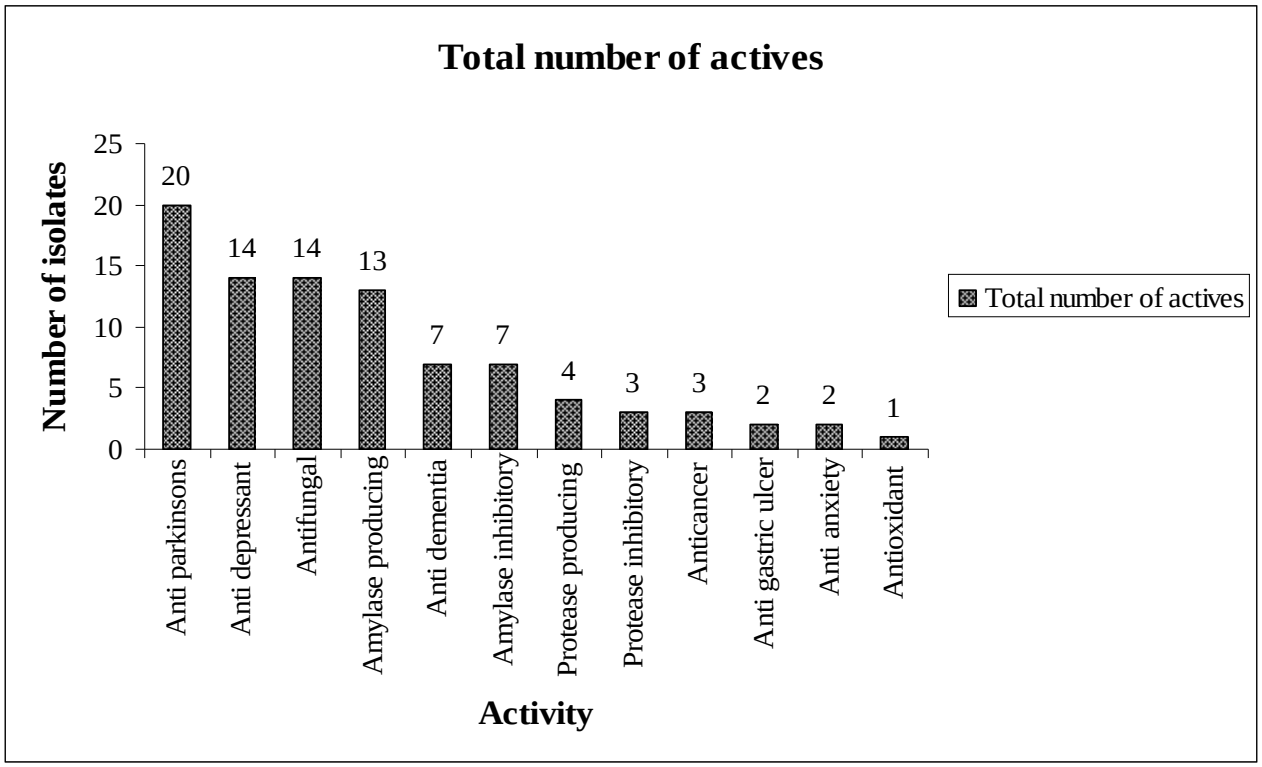

Figure 1: Activity profile of sixty three bacterial isolates.

From the active cultures, a maximum of 32% showed anti Parkinson's activity followed by 22% which showed anti depressant & antifungal

activity, each. Seven cultures (11%) showed anti dementia activity. Three isolates showing anti cancer activity (against colon and uterine cancers) were consistent in their in vitro activity, however in vivo trials are in progress. Two isolates showed anti gastric ulcer activity (against *Helicobacter pylori*) & anti anxiety activity and only 1 culture showed antioxidant activity. When the cultures were tested for enzyme production, 21% i.e. 13 isolates produced amylases while only 6% exhibited protease activity. When tested for enzyme inhibitors 11% produced amylase inhibitors while nearly 5% produced protease inhibitors.

Novel metabolites produced by marine actinomycetes have shown promising antibacterial, anticancer, antifungal, anti inflammatory, anti malarial and neuritogenic activities [4]. Debbab et al. (2010) [5] have reviewed some of the recent new bioactive compounds from marine bacteria and fungi. Bacteria from Weihai solar saltern, China have been screened for antimicrobial and cytotoxic activities [6]. Though there are reports on diversity of microbes from salt pans in India [7,8,9,10] not much is found in literature specifically with respect to bioactive compounds from salt pan isolates. Dhanasekaran et al.(2005) [1] have reported salt pan actinomycetes producing antibacterial activity. *Saccharopolyspora salina* VITSDK4, isolated from a saltpan marine soil sample collected at the Marakkanam coast of the Bay of Bengal, India, was profoundly antagonistic with fungal and Gram positive pathogens [11]. Kamat and Kerkar (2004, 2007) [2,3] have reported a halotolerant *Acinetobacter* sp. from saltpans of Ribandar, Goa producing antibacterial compound.

Activity	Laboratory	Culture No. BGUM_ _ _
Antioxidant	IICT, Hyderabad	009MD.
Anti gastric ulcer	IICB, Calcutta	158MD,159MD.
Antifungal	CIMAP, Lucknow	005MD,006MD,007MD,038MD,047MD,058MD,066MD,072MD,073MD,078MD ,133MD,136MD,165MD,186 MD.
Memory enhancing activity & activity against neurological disorders (i) to (iv)		As detailed below
(i) Anti anxiety	CDRI, Lucknow	837MD,1091MD.
(ii) Anti dementia	CDRI, Lucknow	059MC,262MD,346MD,348MD,370MD,740MD,741MD.
(iii) Anti depressant	CDRI, Lucknow	100MD,107MD,256MD,257MD,264MD,265MD,298MD,327MD,461MD,465MD ,471MD,472MD,473MD,474MD.
(iv) Anti Parkinson's	CDRI, Lucknow	102MD,103MD,109MD,236MD,299MD,305MD,307MD,312MD,313MD,314MD ,315MD,359MD,373MD,375MD,376MD,440MD,457MD,458MD,799MD,806M D.
Anticancer	IIIM, Jammu	014MD,016MD,017MD.
Amylase producing	In house testing	005MD,014MD,016MD,017MD, 059MC,072MD, 078MD,102MD, 236MD,315MD,440MD,465MD,837MD.
Amylase inhibitory	In house testing	109MD,262MD,307MD,314MD,373MD,375MD,799MD.
Protease producing	In house testing	016MD,236MD,264MD,806MD.
Protease inhibitory	In house testing	014MD,465MD,799MD.

Table 1: Details of culture extracts showing various activities

Vidyasagar et al. (2007) [12] have reported *Chromohalobacter* sp. TVSP101 from solar

salterns of Tuticorin, Tamilnadu, India producing halothermophilic protease.

After our preliminary findings, efforts are now in progress to partially purify the active ingredient and confirm the activity. To our knowledge, for the first time in India, such large numbers of halobacteria have been screened for various biological activities. There is a need to continue exploring saltpan ecosystem for more potential cultures, some of which could hold promising solutions to the evolving drug resistance in human pathogens.

[V] CONCLUSION

The present study highlights the biotechnological significance of bacteria from saltpans and emphasizes that these microbes are probably a source for the discovery of novel secondary metabolites.

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REFERENCES

[1] Dhanasekaran D, Rajakumar G, Sivamani P, Selvamani S, Panneerselvam A, Thajuddin N (2005) Screening of salt pans actinomycetes for antibacterial agents. *The Internet J. Microbiol.* 1:2.

[2] Kamat T and Kerkar S (2004) Studies on a bioactive compound produced by a halotolerant saltpan isolate. Conf on Microbiol of Tropical Seas (COMITS), 13-15 Dec 2004. Nat Inst of Oceanography, Goa. MB (P) 10 (Abs).

[3] Kamat T and Kerkar S. (2007) Profiling the bioactive potential of an Acinetobacter sp. from Ribandar saltern in Goa. Natl Sem on New Trends in Biotechnol, 11-12 Jan 2007. Nat Inst of Oceanography, Goa. BC-07 (Abs).

[4] Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. *Curr Opin Microbiol* 9:245-251.

[5] Debbab A, Aly AH, Lin WH and Proksch P (2010) Bioactive compounds from marine bacteria and fungi. *Microbial Biotechnol* 3:544-563.

[6] Chen L, Wang G, Bu T, Zhang Y, Wang Y, Liu M, Lin X (2010) Phylogenetic analysis and screening of antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai solar saltern (China). *World J Microbiol Biotechnol* 26:879-888.

[7] Dave SR and Desai HB (2006) Microbial diversity at marine salterns near Bhavnagar, Gujarat, India. *Curr Sci* 90:497-500.

[8] Vijayakumar R, Muthukumar C, Thajuddin N, Panneerselvam A, Saravanamuthu R (2007) Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycetologica* 21:59-65.

[9] Dhanasekaran D, Thajuddin N, Panneerselvam A (2008-2009) Distribution and ecobiology of antagonistic streptomycetes from agriculture and coastal soil in Tamil Nadu, India. *J culture collections* 6:10-20.

[10] Manikandan M and Kannan V (2009) Diversity of microorganisms in solar salterns of Tamil Nadu, India. *World J Microbiol Biotechnol* 25:1007-1017.

[11] Suthindhiran K and Kannabiran K (2009) Cytotoxic and antimicrobial potential of Actinomycete species Saccharopolyspora salina VITSDK4 isolated from the Bay of Bengal coast of India. *Am J Infect Dis* 5:90-98.

[12] Vidyasagar M, Prakash S, Jayalakshmi S K and Sreeramulu K (2007) Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium Chromohalobacter sp. TVSP101. *World J Microbiol Biotechnol* 23:655-662.

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ANTIMICROBIAL POTENTIAL OF *BACILLUS MARISMORTUI*, A SALT PAN ISOLATE OF CAVELLOSIM, GOA, INDIA

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ABSTRACT:

A halotolerant, Gram positive, motile, rod was isolated from the salt pan sediments of Cavellosim, Salcette, Goa, India and screened for its antimicrobial potential. The strain showed an inhibitory effect specifically to *Staphylococcus citreus.* The isolate showed optimal growth as well as antibiotic production with 4% natural salt and could tolerate up to 6% salt. Dextrose and L-Tryptophan were found to be the best carbon and nitrogen sources for optimum antibiotic production. Based on morphological, biochemical, 16S rDNA sequencing and phylogenetic analysis, the strain was identified as *B. marismortui*.

Keywords: *Bacillus marismortui;* Bioactivity; Hypersaline; Secondary metabolites; Salt pan

[I] INTRODUCTION

The hypersaline environments, with salinities far more than normal sea water, generally originate as a result of evaporation of sea water. Solar salterns are artificial, shallow ponds producing halite (NaCl). They are often built as multi-pond systems, in which sea water is transferred to a series of ponds, with increase in the salinity at each stage up to halite saturation. This makes the multi-pond solar salterns a popular environment for studies on halotolerant and halophilic microorganisms [1].

Compounds such as halocins, glycine, betaine, glutamine, N acetyl-β-lysine and carbohydrates like α-glucosyl glycerate have been reported to be the products of bacteria inhabiting these salterns and possessing bioactivity. It is surmised that marine environments like solar saltern may yield newer strains which may prove to be a rich resource of newer bioactive metabolites.

Our group has been involved in isolation and screening of bacteria and actinomycetes from salt pans of Goa, for more than a decade. Here we report, the antimicrobial potential of culture no. 284 isolated from the sediments of a salt pan of Cavelossim, Goa, India. The optimal conditions for antibiotic production have been elucidated by using variants of carbon and nitrogen sources in the media composition.

[II] MATERIALS AND METHODS

2.1. Sample collection and isolation of bacteria:

All the chemicals and media components used in the study were procured from HiMedia, India unless otherwise specified. The salt pan sediment samples were collected from salt pans of Cavellosim, Salcette, Goa, India; using a sterile 10 cm corer. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were spread plated on Media D containing 1.5% tryptone (Pancreatic digest of casein), 0.5% soyatone (Papain digest of Soyabean meal), natural salt 0.5% and agar 2 %. The pH of the media was adjusted to 7.2 before sterilization at 121°C and 15 psi pressure. The cultures were preserved on Media D supplemented with 2% w/v NaCl and glycerol, adjusted to a pH 7.2 and stored at 4°C.

2.2. Characterisation of culture no. 284:

The strain was characterized by morphological, biochemical and phylogenetic analysis. Micro morphology of culture was examined by photomicrography (Gram's staining method) and Scanning electron microscopy (SEM). Sodium chloride tolerance of the strain was also determined. The utilization of carbon and nitrogen source was carried out by Arahal et. al. [2] method. Various biochemical tests performed for the identification of culture no. 284 are listed in Table 1 and Table 2. The ability of strain 284 to produce enzymes and enzyme inhibitors was also assessed.

2.3. Antimicrobial susceptibility test:

A lawn of the culture no. 284 seeded on Muller Hinton agar plates was checked for sensitivity with 29 different antibiotic discs (HiMedia, India). The plate was incubated for 16-24 h at 37°C. After the incubation, the zones of inhibition were measured using a zone measurement scale. The sensitivity or resistance of culture no. 284 to the antibiotic was interpreted with reference to the table provided.

The data was compared with Bergey's manual of Determinative Bacteriology to identify the isolate.

2.4. Phylogenetic analysis:

Genomic DNA of the strain was isolated [3] and 16S rDNA sequencing was carried out using eubacterial universal primers. The sequence alignment was done using Clustal W software and a phylogenetic tree was prepared using Neighbour Joining plot [4].

2.5. Screening of culture no. 284 for antimicrobial activity:

The screening method consisted of two steps: Primary and secondary screening. Primary screening was carried out by 'microbial inhibition spectrum' [5] on Media D. The test organisms used were *Citrobacter freundii*, *Citrobacter diversus*, *Acinetobacter baumanii*, *Salmonella typhimurium*, *Salmonella paratyphi* A, *Proteus mirabilis*, *Candida albicans*, *Klebsiella pneumoniae*, *Morganella morganii*, *Staphylococcus citreus*, *Escherichia coli* ATCC 25922, *Pseudomonas* sp. (pigmented), Methicillin resistant *Staphylococcus aureus* (MRSA), *Shigella boydii*, *Salmonella typhi*, *Pseudomonas* ATCC 27855, *Staphylococcus* sp. ATCC 25923, *Shigella flexineri*, Methicillin sensitive *Staphylococcus aureus* (MSSA) and *Vibrio cholerae*.

Secondary screening was performed by disc diffusion method using media D broth against the test organism *Staphylococcus citreus*. Sterile filter paper discs (Whatman#1, 5mm diameter) were dipped in Media-D culture broth and allowed to absorb. The dried discs were then placed on actively growing lawn of *S. citreus*. The inhibition zones were measured after an incubation period of 16-24 h at 37°C using Hi media zone scale.

2.6. Evaluation of salt, carbon and nitrogen source for optimal antibiotic production:

Single colony of culture no. 284 was inoculated into media D with 0, 3.5, 5 and 10% natural salt in distilled water at pH 7.2 and incubated at room temperature (28±2°C) on rotary shaker at 150 rpm. Growth was recorded at 600 nm at every 6 h interval.

Optimization of carbon source with 1% Dextrose, Ribose and Malonate as sole carbon source in Minimal media [K2HPO4 0.2 g, KH2PO4 0.2 g, MgSO4 0.06 g, natural salt 2g, Distilled water 100 ml (pH 7.0-7.5)] was carried out.

The best Nitrogen source was discerned from amino acids viz. L-Tyrosine, L-Tryptophan, L-Asparagine, L- Lysine and L- Glutamic acid in Minimal media supplemented with 3.5 % natural salt.

The best carbon and nitrogen source was subsequently used in the antibiotic production media. The pH was maintained between 7.0 to 7.5 and the culture was incubated at room

temperature (28±2°C) on rotary shaker at 150 rpm.

The antibiotic production in the newly designed media incorporating the optimal natural salt concentration, carbon and nitrogen source was assayed by agar disc diffusion method on a lawn of *S. citreus*, with a media control. The inhibition zones were measured after an incubation period of 16-24 h at 37°C using Hi media zone scale.

2.7. Partial purification of bioactive compound:

The bioactive compound produced by culture no. 284 was partially purified by organic solvent extraction method using petroleum ether, chloroform & ethyl acetate [6]. These extracts were individually checked for inhibitory properties using *S. citreus* as test culture and were also scanned from 190 to 1100 nm (UV mini 1240, UV-Vis Spectrophotometer, Shimadzu) and the peaks were recorded.

2.8. Strain improvement study:

Strain improvement was done using Ultraviolet light (UV) mutagenesis to check an increase in the antibiotic production. The culture grown in phosphate buffer saline was exposed to UV light at intervals of 10, 15, 30 and 45 seconds. After exposure the resistant cells were further checked for an increase in antibiotic activity.

[III] RESULTS AND DISCUSSION

The culture no.284 isolated from the salt pan sediments of Cavellosim, Goa, India exhibited good growth on Media D, with a light brown pigmentation. The morphological & physiological characteristics of the isolate are presented in Table 1. Carbohydrate utilization tests are shown in Table 2. Culture no. 284 was a Gram positive, motile rod shaped bacterium (Figure 1). The isolate was quite specific in its carbohydrate utilization pattern. Out of 33 carbohydrates tested only 3 were utilized. It utilized ribose very scantily but could ferment dextrose and malonate. These 3 sugars were used as carbon sources to optimize the antibiotic potential of culture no.284. Dextrose, at a concentration of 0.5%, was found to be the best carbon source as compared to Malonate and Ribose. It also utilized citrate as a sole carbon source. The isolate seemed to be a rich source in producing significant enzymes viz. Protease, Catalase, Oxidase, Urease and Lysine and Ornithine decarboxylase.

The antimicrobial spectrum of culture no. 284 is shown in Table 3. The culture was resistant towards 6 antibiotics and sensitive towards 21 antibiotics. It also showed intermediate sensitivity to Lincomycin and Gentamicin.

Table: 1: Biochemical characterization of culture no. 284

Sr.No.	Characteristics	Results
1	Gram staining	+
2	Pigment	Light brown
3	ONPG	-
4	Lysine decarboxylase	+
5	Ornithine decarboxylase	+
6	Nitrate reduction	-
7	H_2S production	-
8	Casein hydrolysis	+
9	Starch hydrolysis	-
10	Citrate utilization	+
11	Phenylalanine deamination	-
12	Catalase	+
13	Oxidase	+
14	Motility	+
15	Amylase inhibitor	-
16	Protease inhibitor	-
17	Urease	+

+ Positive - Negative

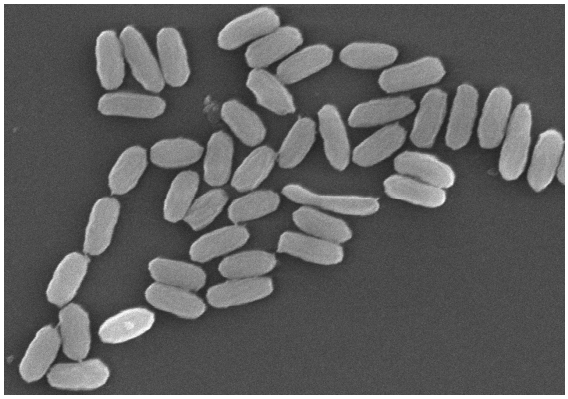

Fig: 1. Scanning Electron micrograph of *Bacillus marismortui*

Table: 2. Carbohydrate utilization of culture no. 284

Sr.No.	Carbohydrate	Results
1	Lactose	-
2	Xylose	-
3	Maltose	-
4	Fructose	-
5	Dextrose	+
6	Galactose	-
7	Raffinose	-
8	Trehalose	-
9	Melibiose	-
10	Sucrose	-
11	L-Arabinose	-
12	Mannose	-
13	Inulin	-
14	Sodium gluconate	-
15	Glycerol	-
16	Salicin	-
17	Glucosamine	-
18	Dulcitol	-
19	Inositol	-
20	Sorbitol	-
21	Mannitol	-
22	Adonitol	-
23	α-methyl-D-glucoside	-
24	Ribose	-/+
25	Rhamnose	-
26	Cellobiose	-
27	Melezitose	-
28	α-methyl-D-mannoside	-
29	Xylitol	-
30	Esculin	-
31	D-Arabinose	-
32	Malonate	+
33	Sorbose	-

\+ Positive - Negative -/+ Scanty utilization

The 16S rDNA sequence of culture no.284 was 1533 bases and showed 99% similarity to *Bacillus marismortui*. The percentage similarity was on the basis of homology studies done using NCBI BLAST database. A phylogenetic tree, as represented in Figure 2, was prepared on the basis of similarity index. Based on its morphological, chemical properties and phylogenetic analysis, culture no.284 was classified as *Bacillus marismortui*. *Bacillus marismortui* was first reported from the Dead Sea [7]. Arahal et. al. [8] proposed the transfer of *Bacillus marismortui* to the genus *Salibacillus* as *Salibacillus marismortui* comb. nov. Later it was proposed by Heyrman et. al. [9] to combine *Virgibacillus* and *Salibacillus* in a single genus, *Virgibacillus* and *Bacillus marismortui* was renamed as *Virgibacillus marismortui* comb. nov.

The antibiotic produced by *B. marismortui*, in the present study, was very specifically active against *Staphylococcus citreus* among the 20 pathogenic cultures tested. The antibiotic did not react with any of the Gram negative organisms tested, nor with *Candida albicans* and the other three species of *Staphylococcus* viz. Methicillin resistant *Staphylococcus aureus*, Methicillin sensitive *Staphylococcus aureus* and *Staphylococcus* sp. ATCC 25923. The species specific antagonistic effect of the antibiotic produced was intriguing. Bacitracin, a cyclic polypeptide, is the most common antibiotic produced by *Bacillus* sp. Few reports are available on marine *Bacillus* sp. producing antimicrobial compounds [10,11,12,13,14]. But to the best of our knowledge, *B. marismortui* has not yet been reported of producing any antibacterial compounds.

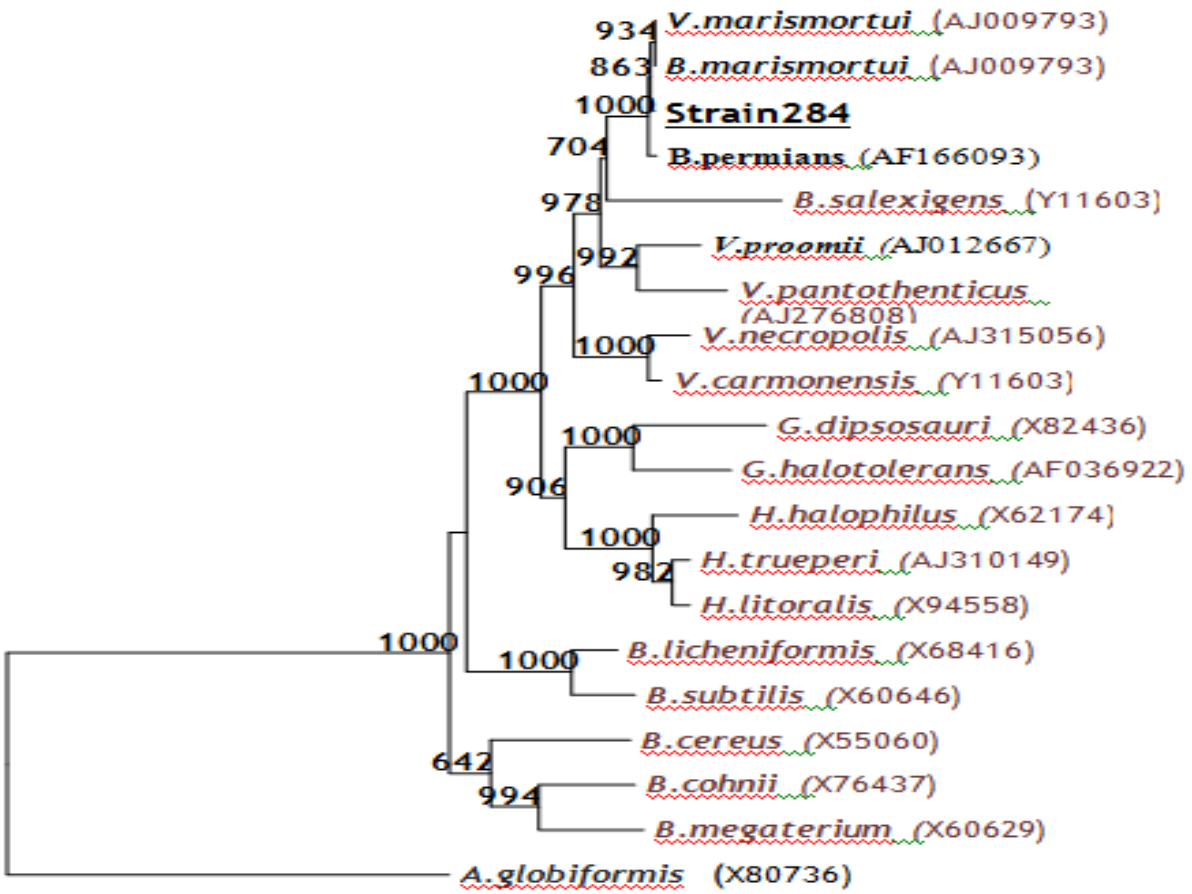

Fig: 2. Phylogenetic neighbour-joining tree based on 16S rDNA sequences showing the relationship between culture no. 284 and related members of the genus. *A.globiformis* was used as an outgroup. Numbers at nodes indicate levels of bootstrap support ≥ 50% based on a neighbour-joining analysis of 1000 re-sampled data sets. The tree is based on neighbour-joining inferences for only complete or nearly complete sequences. GenBank accession numbers are given in parentheses. Bar 1% sequence variation.

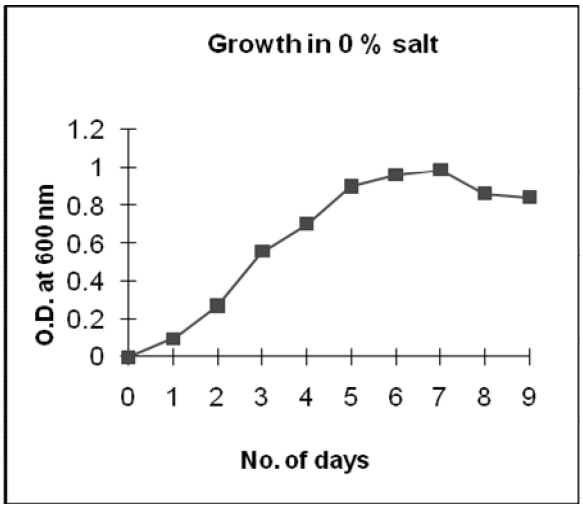

Fig. 3A. Growth curve of *Bacillus marismortui* in 0 % salt.

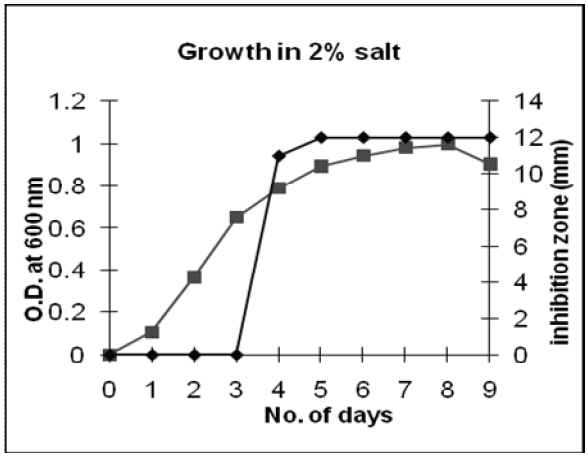

Fig. 3B. Growth and antibiotic production of *Bacillus marismortui* in 2% salt.

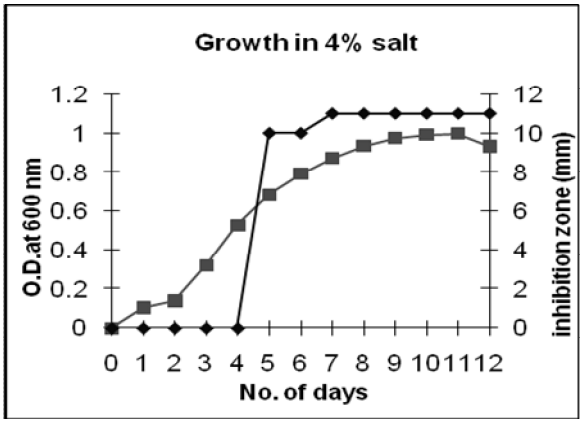

Fig. 3C. Growth and antibiotic production of *Bacillus marismortui* in 4% salt.

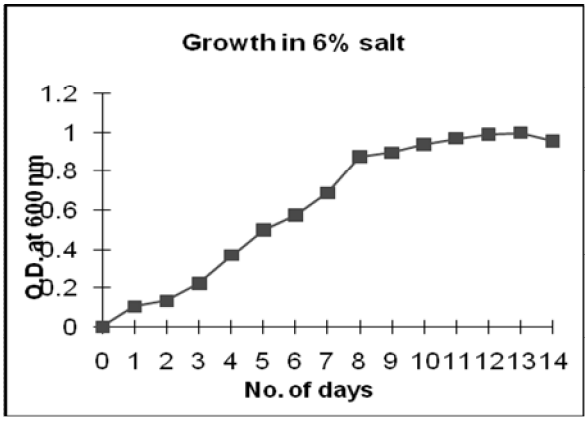

Fig. 3D. Growth curve of *Bacillus marismortui* in 6% salt.

The strain exhibited salt tolerance up to 6% as shown in Figure 3D and may be placed in intermediate salt tolerance group. It exhibited a sigmoid growth pattern with 0 % salt (Figure 3A) with no antibiotic production. However an addition of 2 % (Figure 3B) to 4 % (Figure 3C) natural salt stimulated the antibiotic production indicating the antibiotic production during the stationary phase observed from the 4th till the 9th day at 2 % salt concentration. At 4 % salt concentration, the highest antibiotic production was from the 4th day till the 12th day with uniform antibiotic activity. However, beyond 4% salt, the growth pattern switched back to a sigmoid curve similar to the 0% growth pattern previously observed with no antibiotic activity. The culture, thus, was found to be halotolerant, requiring a minimal 2% salt for the antibiotic production. At 6% NaCl concentration, growth was observed without antibiotic production. Imada et al. [15] have also reported actinomycetes requiring presence of sea water for the production of antibacterial compounds.

Maximum antibiotic production was observed when dextrose and L- tryptophan were used as carbon and nitrogen source, respectively. It is well known that, many factors such as carbon source, nitrogen source, temperature, aeration etc. affect the antibiotic production in different microorganisms [16]. Of these, carbon and nitrogen sources are two of the most critical factors that can affect antibiotic production. A new media was designed which enriched the growth as well as antibiotic production in *B. marismortui* in which the carbon and nitrogen ratio was similar to soyapeptone: tryptone ratio (1:3) in Media D. The media had the following composition in g/100ml: Dextrose 0.5, L-Tryptophan 1.5, K2HPO4 0.2, KH2PO4 0.2

MgSO4 0.06, NaCl 2, Distilled water 100 ml (pH 7.0-7.5).

The partial purification of the antibiotic, showed an activity in the petroleum ether fraction suggesting a hydrophobic nature of the antibacterial compound. The partially purified fraction was scanned from 190 to 1100 nm and a sharp peak, with an absorbance of 1.349, was obtained at 301 nm in 1/100 dilution, as shown in Figure 4.

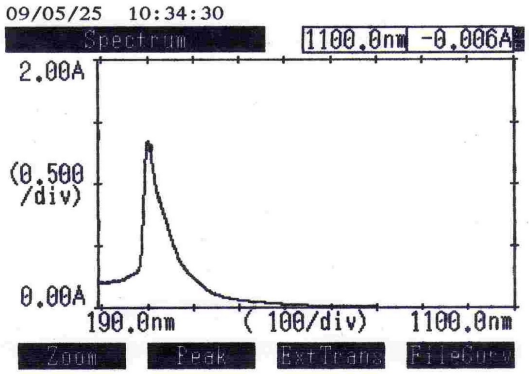

Fig. 4. Spectral analysis of partially purified compound.

The antibacterial compound was produced extracellularly and easily diffused in to the medium, confirmed initially with an antibiogram and later by disc diffusion method as seen in Figure-5.

The isolate showed insignificant change in growth rate as well as amount of antibiotic production subsequent to UV treatment.

Fig. 5. Culture no. 284 showing antibacterial activity against *Staphylococcus citreus.*

[V] CONCLUSION

In conclusion, the results from present study are promising and need further studies with respect to purification, characterisation and identification of the antibacterial compound produced by *Bacillus marismortui.*

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REFERENCES

[1] Kerkar, S. [2004] Ecology of Hypersaline Microorganisms. In *Marine microbiology: Facets & Opportunities*, ed. by N. Ramaiah, pp.53-67.

[2] Arahal, D. R., Dewhirst, F.E., Paster, B.J., Volcani, B.E. & Ventosa, A. [1996] Phylogenetic analysis of some extremely halophilic archaea isolated from the Dead sea water, determined on the basis of their 16s r RNA sequences. *Appl. Environ. Microbiol.*, 62:3779-3786.

[3] Sambrook, J. and Russell, D. W. [2001] Molecular cloning: A laboratory manual, 3rd edition, Volume 3, Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. A8.9-A8.11.

[4] Saitou, N. and Nie, M. [1987] The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4:406-425.

[5] Casida, L. E. [2001] Industrial Microbiology, New Age International (P) Limited, New Delhi, pp.55-75.

[6] Riguera, R. [1997] Isolating bioactive compounds from marine organisms. *J. Mar. Biotechnol.*, 5:187-193.

[7] Arahal, D.R., Marquez, M.C., Volcani, B.E., Schleifer K.H. and Ventosa, A. [1999] *Bacillus marismortui* sp. nov., a new moderately halophilic

species from the Dead sea. *Int. J. Sys. Bacteriol.*, 49:521-530.

[8] Arahal, D. R., Ma!rquez, M. C., Volcani, B. E., Schleifer, K. H. and Ventosa, A. [2000] Reclassification of *Bacillus marismortui* as *Salibacillus marismortui* comb. nov. *Int. J. Syst. Evol. Microbiol.*, 50:1501–1503.

[9] Heyrman, J., Logan, N. A., Busse, H-J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Swings, J. and De Vos, P. [2003] *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. *Int. J. Syst. Evol. Microbiol.*, 53:501–511.

[10] Nithya, C., Gokila Devi, M. and Pandian, S. K. [2011] A novel compound from the marine bacterium *Bacillus pumilus* S6-15 inhibits biofilm formation in Gram-positive and Gram-negative species. *Biofouling*, 27:5, 519–528.

[11] Musthafa, K. S., Saroja, V., Pandian, S. K. and Ravi, A. V. [2011] Antipathogenic potential of marine *Bacillus* sp. SS4 on N-acyl-homoserine-lactone-mediated virulence factors production in *Pseudomonas aeruginosa* (PAO1). *J. Biosci.*, 36(1), 55–67.

[12] Mondol, M. A. M., Kim, J. H., Lee, H-S., Lee, Y-J. and Shin, H. J. [2011] Macrolactin W, a new antibacterial macrolide from a marine *Bacillus* sp. *Bioorg. Med. Chem. Lett.*, 21:3832–3835.

[13] Berrue, F., Ibrahim, A., Boland, P. and Kerr, R. G. [2009] Newly isolated marine *Bacillus pumilus* (SP21): A source of novel lipoamides and other antimicrobial agents. *Pure Appl. Chem.*, 81:6, 1027–1031.

[14] Jaruchoktaweechai, C., Suwanborirux, K., Tanasupawatt, S., Kittakoop, P. and Menasveta, P. [2000] New Macrolactins from a Marine *Bacillus* sp. Sc026. *J. Nat. Prod.*, 63:984-986.

[15] Imada, C., Koseki, N., Kamata, M., Kobayashi, T., and Hamada-Sato, N. [2007] Isolation and characterization of antibacterial substances produced by marine actinomycetes in the presence of sea water. *Actinomycetologica*, 21:27-31.

[16] Gupte, M.D. and Kulkarni, P.L. [2002] A study of antifungal antibiotic production by *Streptomyces chattanoogensis* MTCC 3423 using full factorial design. *Lett. Appl. Microbiol.*, 35:22–26.