STRUCTURE AND FUNCTIONAL CHARACTERIZATION OF THERAPEUTIC PROTEIN ISOLATED FROM MARINE ORGANISM

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DECLARATION

I hereby declare that the thesis entitled ***"STRUCTURE AND FUNCTIONAL CHARACTERIZATION OF THERAPEUTIC PROTEIN ISOLATED FROM MARINE ORGANISM"*** *submitted by me for the Degree of Doctor of Philosophy in Bioinformatics-Zoology interdisciplinary is the result of my original and independent research work carried out under the guidance of* ***Dr. V. Aldous. J. Huxley,*** *Assistant Professor and Research guide, Biotech Research Laboratory, Department of Zoology, Thiru. Vi. Ka Govt. Arts College, Thiruvarur -3 and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.*

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Date: *(Signature of the Candidate)*

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CONTENTS

LIST OF FIGURES	*viii*
LIST OF TABLES	*ix*
LIST OF ABBREVIATIONS	*xii*
Chapter I	*1*
Objectives of the Study	*3*
Review of Literature	*4*
1.2.1 Introduction	*4*
1.2.2 Marine Natural Products	*5*
1.2.3 Marine Sponge as a bioactive substance	*5*
1.2.4 Microbial diversity of marine sponges	*7*
Chapter II	*24*
2.1 Introduction	24
2.2 Materials and Methods	28
2.2.1 Collection and Identification of Sponges	*28*
2.2.2 Isolation of Antibiotic Producing	*29*
2.2.3 Quantitative Analysis of Endosymbionts	30
2.2.4 Antibacterial activity of endosymbionts	*30*
2.2.4.1 Test microorganisms	*30*
2.2.4.2 Antibacterial assay	*31*
2.2.4.3 16S rRNA partial sequencing	32
2.2.4.4 Phylogenetic Tree	33
2.3. Results	34
2.4. Discussion	43
Chapter III	*48*
3.1 Introduction	52
3.2 Materials and Methods	52

3.2.1 Growth curve of potent antimicrobial agent producing bacteria 52
3.2.2 Determination of Antibiotic production time 52
3.2.3. SDS-PAGE 52
3.2.4 Separation of molecular complexes by 2D BN SDS-PAGE 54
3.2.5 Peptide sequencing through LC-MS/MS 55
3.2.5 Peptide sequencing through LC-MS/MS 55
3.3 Results 57
3.4 Discussion 62
Chapter IV 66
4.1 Introduction 66
4.2 Materials and Methods 72
4.2.1 Peptide Sequence Information from MALDI TOF Data 72
4.2.2 MASCOT 75
4.2.3 Peak studio 75
4.2.4 Signal peptide prediction 76
4.2.5 Comparative Modeling 77
4.2.6 Structure and Functional Study 78
4.3 Results 79
4.7 Discussion 117
SUMMARY 126
REFERENCES 130

LIST OF FIGURES

Fig No	Figure name	Page No
2.1	Location of Collection Centers along the Kanyakumari Coast	28
2.2	The identified species of sponges	35
2.3	SrRNA partial sequencing	40
2.4	Phylogenetic Tree	42
3.1	Growth curve of ESB3	59
3.2	BN- PAGE of Axinella donnani symbionts	60
3.3	Mass Analysis in MS	61
4.1	Steps in comparative modeling	78
4.2	MASCOT Analysis	82
4.3	MS spectrum for peptide with Table value	85
4.4	Annotated MS/MS spectrum	86
4.5	Signal IP result of Gram-negative organism	89
4.6	Signal IP result of Gram-positive organism	90
4.7	Signal IP result of Eukaryotic organism	92
4.8	Signal IP SEQUENCE PREDICTION	93
4.9	Template Structure (1BO0 A Chain)	94
4.10	Modeled structures using Modeller9V8	96
4.11	Ramachandran plot of Modeled structure1	97
4.12	Ramachandran plot of Modeled structure2	98
4.13	Ramachandran plot of Modeled structure3	100
4.14	Ramachandran plot of Modeled structure4	101
4.15	Ramachandran plot of Modeled structure 5	103
4.16	Secondary structure of Modeled structure 5	104
4.17	Topology structure of N and C terminul in the 3D structure	105
4.18	Beta sheet regions in the 3D structure.	106
4.19	Beta hairpin regions in the 3D structure	107

4.20	Beta bulge regions in the 3D structure	108
4.21	Strands regions in the 3D structure	109
4.22	Helix and its starting and endingregions of the 3D structure	110
4.23	Turns in the Modeled structure	111
4.24	Turn regions in the Modeled structure	112
4.25	Gamma turns in the Modeled structure	113
4.26	Disulphide regions in the Modeled structure	114
4.26	Cleft residues in the Modeled structure	115
4.27	Binding site regions in the Modeled structure	116
4.28	Binding surfaces in the Modeled structure	116

LIST OF TABLES

Table no	Table name	Page no
1.1	The biopotential of endosymbiotic bacteria of marine products	09
2.1	The collected sponges and their codes with collection spots	28
2.2	Test organisms	31
2.3	Collection and Identification of Sponges	34
2.4	Isolation of Antibiotic Producing Endosymbiotic Bacteria	36
2.5	Quantitative Analysis of endosymbionts	37
2.6	Antibacterial potential of different sponge endosymbionts against common pathogenic bacteriaactivity of endosymbiont	38
2.7	The antibacterial activity of potent endosymbiont (ESB-3)	39
3.1	Characterization of endosymbiotic bacteria	57
3.2	Results of growth curve of ESB3	58
4.1	List of m/z ratios excluded from MS/MS analysis. Contains known contaminants and interference spectra from an unknown origin.	74
4.2	The peptide masses from the sequence	80
4.3	Search parameters of MASCOT	81
4.4	Summary results for proteins producing a significant score from a Mascot search	83
4.5	Matched Peptides	84
4.6	Matched peptides shown in bold red	84
4.7	Annotated Mass Value	87

4.8	Secondary structure elements	106
4.9	Beta sheet details	106
4.10	Beta Hairpin regions	107
4.11	Beta bulges regions	108
4.12	Strands regions	109
4.13	Helix regions	110
4.14	Beta turns,types and its sequences	111
4.15	Gamma turn types and its	114
4.16	Disulphide regions	115

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
Y	Gamma
µl	Micro Litre
3D	Three Dimensional
CAN	Acetonitrile
amo A	Ammonia Monooxygenase Subunit A
ASABF	Ascaris suum antibacterial factor
BHA	*butylated hydroxyanisole*
BHT	*butylated hydroxytoluene*
BN	Blue Native
CN	Clear-native
C-terminal	Corboxyl
DTT	Dithiothreitol
ESB	Endosymbiotic Bacteria
ESI	electrospray ionization
GC	Gel Chromotography
HPLC	High Performance liquid chromatography
HMA	*high-microbial abundance*
IAM	Iodoacetamide

kDa	Kilo Dalton
LC	Liquid Chromotography
LMA	*low-microbial-abundance*
MS	mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MTCC	*Microbial Type culture collection*
Mm	Milli Meter
MD	Molecular Dynamics
NCBI	National Centre for Biotechnology Information
NP	Natural Products
N-terminal	Amine
ORF	Open Reading Frame
rRNA	Ribosomal Ribo Nucleic Acid
PAGE	Poly Acrylamide Gel Electrophoresis
PDB	Protein Databank
SDS	Sodium Dodecyl Sulphate
TOF	time-of-flight
TM	Transmembrane
UV	Ultra violet

CHAPTER I

PREFACE

Natural products have long been used as foods, fragrences, insecticides, medicines etc., The marine environment may contain over 80% of worlds plant and animal species and which covers a wide range of variations produced by their environment. This marine habitate has extreme temperature variation (freezing to 350^{o} C), pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and has extensive photic and non-photic zones. Despite of that the biodiversity in the marine environment far exceeds that of the terrestrial environment.

In recent years, many bioactive compounds have been extracted from various marine organisms like seaweeds, seagrass, tunicates, sponges, softcorals, seahares, nudibranches and bryozoans etc., The search for new metabolites from marine organisms has resulted in the isolation of more than 12000 novel metabolites, many of which are endowed with pharmacodynamic properties. Among the isolated potential metabolites, the sponges are the predominant contributors.

Approximately 15000 sponges have been described in the world and most of them live in marine waters. According to Thomas (1994) India has more than 5000 species of marine sponges. But only 486 species has been described and reported. A range of bioactive metabolites has been found in about 5000 from 500 marine sponges, most of them are bioactive.

However, an increasing role has been played by sponge associated microorganisms (ectosymbionts) in the production of antibiotics and other drugs for the treatment of serious diseases. Nearly 50,000 natural products have been discovered from microorganisms. Over 1410,000 of these are reported to have biological activity and over 100 microbial products are in use today as antibiotics, antitumour and agrochemicals (Joseph and Lipton, 2004). In spite of such success in drug discovery from associated microbes have received very little attention. The difficulty in the search of metabolites from sponge associated or marine bacteria in mainly due to the non-cultivability of the majority over 99% (Rajeev and Xu, 2004).

The fact that sponge associated bacteria in particular are one of the richest source of known and novel bioactive compounds including toxin with pharmaceutical applications is unquestionable. Among the three classes of sponges, the class Demospongiae groups producing more antimicrobial compounds than the other. The main reason for that is having more endosymbionts (38% their biomass is contributed by bacteria. (Jean et al., *2000)*

Over the last decades a diverse group of sponge associated microbial metabolites are chemically elucidated and tested for biological activity. The bioactive metabolites isolated from sponge associated microbes could be divided into steroids, terphenoids, quinines, peptides, neucleosides etc., However the number of compounds which are taken up for the field is scanty.

Reports displayed that the lot of steroids, terphenoids and other compounds structural elucidation and functional characterization. But the protein researches are still in infancy. So the present study intended to elucidate the structure and function of antimicrobial peptide isolated from eight marine sponge collected from south Peninsular coast of India.

1.1 OBJECTIVES OF THE STUDY

- *To isolate and characterize sponge associated bacteria (ectosymbionts) through traditional and molecular approach.*
- *To determine the antimicrobial profile of endosymbiotic bacteria.*
- *To identify the low molecular weight peptides expressed during endosymbionts growth.*
- *To characterize the molecular weight properties and sequencing of novel peptide.*
- *To determine the 3D structure of the novel peptide with their functional properties.*

1.2. REVIEW OF LITERATURE

1.2.1 Introduction

Natural products (NPs) have been the source of most of the active ingredients of medicines. More than 80% of drugs developed so far are from natural products. The comparison of the information presented on sources of new drugs from 1981 to 2007 indicates that almost half of the drugs approved since 1994 are based on natural products (Samuel and Ravikumar, 2011). NPs produce a multitude of organic compounds that have antimicrobial activity. The antimicrobial compounds found in NPs are of interest because antibiotic resistance is becoming a worldwide public health concern especially in terms of food-borne illness and nosocomial infections (Terry et al., *2001; Vasiljeva* et al., *2005; Chia-Lin* et al., *2006; Xie* et al., *2008). Naturally occurring antimicrobials are being sought as replacements for synthetic preservatives such as parabens (ethyl, methyl, butyl and propyl parabens), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are under scrutiny (Darbre* et al., *2004; Darius* et al., *2004). The compounds are found in NPs include alliin/allicins, isothiocyanates, plant pigments (Cutter, 2000), hydrolytic enzymes, proteins, essential oils (Calkins* et al., *1995), and phytoalexins or phenolic compounds (Cutter, 2000). Normally, NPs are grouped into two based on their origin, the terrestrial and marine.*

1.2.2 Marine Natural Products

The world's oceans cover more than 70% of the earth's surface and represent an enormous resource for the discovery of chemotherapeutic agents. Given the diversity of marine organisms and habitats, marine natural products encompass a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures representing biosynthetic schemes of stunning variety (Vasiljeva et al.*, 2007). Over the past 30–40 years marine organisms have been the focus of a worldwide effort for the discovery of novel natural products. A small number of marine plants, animals and microbes have already yielded more than 12,000 novel chemicals with hundreds of new compounds still being discovered every year (Marwa and Mark, 2003). To date majority of these chemicals have been identified from marine invertebrates of which sponges predominate (Lee* et al.*, 2007).*

1.2.3 Marine Sponge as a bioactive substance

Marine sponges are the richest sources of pharmacologically-active chemicals (Laport et al.*, 2009). To date, more than 5300 different products have been isolated from sponges and their associated microorganisms (Laport* et al.*, 2009). Blunt* et al.*, (2010) in a Natural Product Report review, described 287 new compounds from marine sponges isolated in 2009. Remarkable examples of bioactive secondary metabolites isolated from marine sponges are hemiasterlin (E7974) and discodermolide. Hemiasterlin (E7974), is a cytotoxic tripeptide originally isolated from the marine sponge Hemiasterella minor, currently in*

Phase I trial (Talpir et al., *1994). The polyketide natural product discodermolide, isolated from the marine sponge Discodermia dissoluta has potent cytotoxicity to human and murine cell lines (Kingston* et al., *2011). Certain marine sponges have been recognized as potentially rich sources of various bioactive compounds. According to the MarinLit database, around 319 compounds have been reported from the genus* Xestospongia, *244 compounds from the genus* Theonella, *222 compounds from the genus* Halicondria *and 118 metabolites have been reported from the genus* Aplysina *among other sponges. Of special interest to this project are the species* Amphimedon compressa, Aiolochroia crassa *and* Theonella swinhoei *for the protease inhibitory activity of their crude extracts observed in previous studies (Degel, 2006).*

Marine sponges Amphimedon compressa *is abundant in Florida, the Bahamas and the Caribbean (Angermeier, 2011), known to produce useful natural products, such as cytotoxins and antifouling agents (Jeanteur* et al., *2006).* A. compressa *belongs to the family Haliclonidae, whose color varies from brown with green shades to red or purple-brown (Albrizio* et al., *1995). At least seventeen secondary metabolites have been reported from the sponge* A. compressa *such as 2-hydroxydocosanoic acid, 2-hydroxytricosanoic acid (Carballeira and Lopez, 1989) 17-tricosenal, 21-octacosenoic acid, 19-pentacosenal, 19-hexacosenal, 16-tricosenoic acid, 18-tricosenoic acid, 16-pentacosenoic acid, 18-pentacosenoic acid, 19-pentacosenoic acid, 20-hepacosenoic acid (Carballeira* et al., *1992), methyl 2-methoxyhexadecanoate, 8,8'-dienecyclostellettamine , 8,8'-dienecyclostellettamine (Xu* et al., *2007), amphitoxin (Albrizio* et al., *1995) and*

amphiceramides A and B (Costantino et al., *2009). Amphitoxin, for example, showed a broad spectrum of biological activities including antimicrobial and anti-feedant activities (Jeanteur* et al., *2006).* Aiolochroia crassa *is a marine sponge of the order Verongida, which is massive, with lobate or, more rarely, ramose processes, yellow to violet in color (Ciminiello* et al., *1995).* A. crassa *is characterized chemically by a series of secondary brominated metabolites, biogenetically arising from bromotyrosines (Albrizio* et al., *1994). Around twenty compounds have been isolated from the sponge* A. crassa *including N-methyl-aerophobin-2, aerophobin-1, aerophobin-2, purealidin L, isofistularin-3 (Assmann* et al., *1998), araplysillin III, hexadellin C (Hamann* et al., *1999). Fistularin-3 was active in vitro against* Mycobacterium tuberculosis *(Hamann* et al., *1999). A recent study of the structural and physico-chemical properties of three-dimensional skeletal scaffold of the marine sponge* A. crassa, *showed that these fibrous scaffolds have a multilayered design and are made of chitin (Ehrlich* et al., *2010). Natural polymers like chitin are widely used in the biomedical field because of their high biocompatibility and the enriched functionalities being capable of integrating well with a variety of ligands (Rejinold* et al., *2011).*

1.2.4 Microbial diversity of marine sponges

Many marine sponges are associated with dense and phylogenetically diverse microbial consortia including bacteria, archaea and single-celled eukaryotes (fungi and microalgae), that can account for nearly half of the animal's biomass (Hentschel et al., *2006, Michael* et al., *2007). Sponges are filter-*

feeders capable of processing enormous volumes of seawater, providing a rich source of microorganisms. Two different sponge types in respect of their association with bacteria have been called "high-microbial abundance" (HMA) and "low-microbial-abundance" (LMA) sponges (Hentschel et al., *2002).*

Sponge's harbour a rich diversity of microorganisms in their tissues and in some case constitute up to 40% of the biomass, e.g. the Mediterranean sponge Aplysina aerophoba *(Friedrich* et al., *2007). The sponge-associated bacteria are host-specific because each of the tested four sponges from the same geographical location has different predominant bacterial diversity. Sponge* Callyspongia australiensis *has the greatest bacterial diversity, with the four bacteria phyla* Proteobacteria, Bacteroidetes, Firmicutes, *and* Actinobacteria, *followed by the sponge* Dendrilla avara *with the two phyla* Proteobacteria *and* Bacteroidetes, *and the sponge* Halichrondria *with the phylum* Proteobacteria *(Zhi-Yong Li,* et al., *2006). Though similar bacteria with closely related phylogenetic relationships were found among different sponges, the sponge-associated predominant bacterial community structures differ.*

The biopotential of endosymbiotic bacteria of marine products are presented in Table – 1.1

Table 1.1 The biopotential of endosymbiotic bacteria of marine products

Order	***Family***	***Species***	***Symbiont***	***Compound***	***Property***	***Reference***
Dictyoceratida	*Thorectidae*	Dysidea *sp.*	Vibrio *sp. (γ-Proteobacteria)*	*Tetrabromo-diphenyl Ethers*	*Cytotoxic, antibacterial*	*Elyakov* et al., *1991*
	Spongiidae	Hyatella *sp.*	Vibrio *sp. M22-1 (γ- Proteobacteria)*	*Andrimid*	*Antibiotic*	*Oclarit* et al., *1994*
		Spongia *sp. (Hawaii)*	Myrothecium verrucaria *973023 (Deuteromycota (fungus))*	*3-hydroxyroridin E*	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				13'-acetyl-trichoverrin B	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Roridin A	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Roridin L	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Roridin M	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Verrucarin M	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Verrucarin A	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Isororidin A	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Epiroridin E	*Antileukemic, antitumor*	*Amagata* et al., *2003*
				Trichoverrin A	*Antileukemic, antitumor*	*Amagata* et al., *2003*

	Dysideidae	Lamellodysidea herbacea *(Great Barrier Reef, Australia)*	Oscillatoria spongeliae *(Cyanobacteria)*	*Trichoverrin B*	*Therapeutic (unknown action)* *Therapeutic (unknown action)*	*Flowers* et al., *1998* *Flowers* et al., *1998*
		Dysidea avara	*Unidentified bacterium*	*2-methylthio-1,4-Naphthoquinone*	*Antiangiogenic, Antimicrobial*	*Hentschel* et al., *2001*
		Ircinia fasciculate	Penicillium chrysogenum *(Ascomycota (fungus))*	*Sorbicillactone A*	*Antileukemic, anti HIV*	*Bringmann*,et al., *2003*
	Thorectidae	Hyrtios *sp.*	Aspergillus niger	*Asperazine*	*Antileukemic,*	*Cheng* et al*., 1994*
		Hyrtios altum	Vibrio *sp.*	*Trisindoline*	*Antibiotic*	*Kobayashi* et al., *1994*
		Hyrtios proteus *(Dry Tortugas National Park,*	Aspergillus niger *(Ascomycota (fungus))*	*Asperazine* *Malformin C*	*Antileukemic, Cytotoxic* *Antitumor*	*Speitling* et al., *2007*
		Fascaplysinopsis reticulate	Pseudoalteromonas maricaloris *KMM 636T (γ-Proteobacteria)*	*Bromo-alterochromide A*	*cytotoxic*	*Blunt* et al., *2009*
Verongida	*Aplysinidae*	Aplysina aerophoba ***(*** *Banyuls sur Mer)*	Bacillus *sp. SB8 (Firmicutes)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Bacillus *sp. SB8 (Firmicutes)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Micrococcus *sp. SB58* (*Actinobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*

			Enterococcus *sp. SB91 (Firmicutes)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Arthrobacter *sp. SB95 (Actinobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Unidentified bacteria SB122	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Unidentified bacteria SB144	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB6	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB55	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB63	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB89	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB156	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB197	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB202	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB207	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			α-Proteobacteria SB214	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Vibrio halioticoli *SB177 (γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB181(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*

			Pseudo-alteromonas *sp. SB182 (γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB183(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB185(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseud-oalteromonas *sp. SB186 (γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB192(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB194(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB200 (γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB208 (γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*
			Pseudo-alteromonas *sp. SB213(γ-Proteobacteria)*	*Unidentified compound*	*Antibacterial*	*Zheng* et al., *2000*

Astrophorid	*Ancorinidae*	Jaspis aff. johnstoni *(Indo-Pacific)* Stelletta tenuis *(South China Sea)*	*Hyphomycete fungus (Deuteromycota (fungus))* Alcaligenes faecalis *A72 (β-Proteobacteria)*	*Chloriolin B* *Cyclo-(L-Pro-L-Phe)*	*Antitumor* *Antimicrobial*	*Cheng* et al., *1994* *Li, 2009*
Chondrosida	*Chondrillidae*	Chondrosia reniformis *(Elba, Italy)*	Penicillium rugulosum *(Ascomycota (fungus))*	*Prugosene A1*	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene A2	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene A3	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene B1	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene B2	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene C1	*Anti-infective*	*Sufrin* et al., *2009*
				Prugosene C2	*Anti-infective*	*Sufrin* et al., *2009*
Dendroceratida	*Darwinellidae*	Dendrilla nigra *(Vizhinjam, India)*	Streptomyces dendra *sp.* nov.*MSI051 (Actinobacteria)*	*Unidentified compound*	*Antibacterial*	*Selvin and Lipton, 2004*
		Dendrilla nigra *(Kanyakumari, India)*	Streptomyces *sp. BLT7 (Actinobacteria)*	*Unidentified compound*	*Antibacterial*	*Selvin* et al., *2009*
		Dendrilla nigra *(South east coast, India)*	Nocardiopsis dassonvillei *MAD08 (Actinobacteria)*	*Acetic acid,-butyl-ester*	*Antimicrobial*	*Selvin* et al., *2009*

				Ethanol, 2-(octyloxy)	*Antimicrobial*	*Selvin* et al., *2009*
				2-Isopropyl-5-methyl-1-heptanol	*Antimicrobial*	*Selvin* et al., *2009*
				Butylated-hydroxytoluene	*Antimicrobial*	*Selvin* et al., *2009*
				Cyclohexane-carboxylic acid, hexyl ester	*Antimicrobial*	*Selvin* et al., *2009*
				Diethyl- phthalate	*Antimicrobial*	*Selvin* et al., *2009*
				Pentadecanal-	*Antimicrobial*	*Selvin* et al., *2009*
				1-Tridecanol	*Antimicrobial*	*Selvin* et al., *2009*
				9-Octadecenal	*Antimicrobial*	*Selvin* et al., *2009*
				Hexadecanoic acid,	*Antimicrobial*	*Selvin* et al., *2009*
				methyl- ester	*Antimicrobial cholesterolemic, nematicide, antiandrogenic, hemolytic*	*Selvin* et al., *2009*
				n-Hexadecanoic-acid	*Antimicrobial cholesterolemic, nematicide, antiandrogenic, hemolytic*	*Selvin* et al., *2009*
				Hexadecanoic-acid, ethyl ester	*Antimicrobial cholesterolemic, nematicide, antiandrogenic, hemolytic*	*Selvin* et al., *2009*

				9-Octadecenoic-acid-(Z)-, methyl-ester	*Anti-inflammatory, antiandrogenic, cancer-preventive, dermatitigenic,*	*Selvin* et al., *2009*
				9-Octadecenal Hexadecanoic acid, methyl-ester	*Anti-inflammatory, antiandrogenic, cancer-preventive, dermatitigenic, hypo-cholesterolemic, anemiagenic*	*Selvin* et al., *2009*
				Oleic Acid	*Anti-inflammatory, antiandrogenic, cancer-preventive, dermatitigenic, hypo-cholesterolemic, anemiagenic*	*Selvin* et al., *2009*
				(E)-9-Octadecenoic-acid ethyl ester	*Anti-inflammatory, antiandrogenic, cancer-preventive, dermatitigenic, hypo-cholesterolemic, anemiagenic*	*Selvin* et al., *2009*

				9-Octa-decenamide-(Z)	*Anti-inflammatory, antiandrogenic, cancer-preventive, dermatitigenic, hypo-cholesterolemic, anemiagenic*	*Selvin* et al., *2009*
Hadromerida	*Spirastrellidae*	Spirastrella vagabunda *(Indonesia)*	*Unidentified fungus*	*14,15-secocurvularin*	*Antibiotic*	*Abrell* et al., *1996*
	Suberitidae	Suberites domuncula *(Northern Adriatic Sea)*	*α-Proteobacterium MBIC3368 (isolate 2)*	*Unidentified compound*	*Antimicrobial, hemolytic*	*Webster* et al., *2001*
			Idiomarina *sp. (γ-Proteobacteria)*	*Unidentified compound*	*Hemolytic*	*Webster* et al., *2001*
			α-Proteobacterium MBIC3368 (isolate 1)	*Unidentified compound*	*Antiangiogenic, antimicrobial, hemolytic, cytotoxic*	*Thakur* et al., *2003*
Halichondrida	*Axinellidae*	Ptilocaulis trachys *(Enewetak Atoll, Marshall Island, Pacific Ocean)*	Myrothecium *sp. JS9 (Deuteromycota (fungus))*	*Roridin A* *Roridin D*	*Antifungal* *Antifungal*	*Xie* et al., *2008* *Xie* et al., *2008*

		Axinella *sp. (South China Sea)*	Penicillium citrinum *(Ascomycota (fungus))*	*Isocyclocitrinol A*	*Antibacterial*	*Amagata* et al., *2003*
		Axinella *sp. (Papaua New Guinea)*	Penicillium *sp. (Ascomycota (fungus))*	*isocyclocitrinol A 22-acetyl-isocyclocitrinol A*	*Antibacterial*	*Amagata* et al., *2003*
		Axinella verrucosa *(Mediterranean Sea)*	Acremonium *sp. (Ascomycota (fungus))*	*Oxaline* *Griseofulvin* *Communesin B* *Communesin C* *Communesin D*	*Anti-proliferative* *Antifungal* *Antileukemic* *Antileukemic* *Antifungal*	*Koizumi* et al.,*2004* *Koizumi* et al.,*2004* *Koizumi* et al.,*2004* *Koizumi* et al.,*2004* *Koizumi* et al.,*2004*
		Axinella damicornis *(Mediterranean Sea)*	Aspergillus niger *(Ascomycota (fungus))*	*Efrapeptin E* *Efrapeptin F* *Efrapeptin Eα* *Efrapeptin G*	*Cytotoxic, antibacterial* *Cytotoxic, antibacterial* *Cytotoxic, antibacterial* *Cytotoxic,*	*Boot* et al.,*2004* *Boot* et al.,*2004* *Boot* et al.,*2004*

				Efrapeptin H *RHM1*	*antibacterial* *Cytotoxic, antibacterial* *Cytotoxic, antibacterial*	*Boot* et al.,*2006* *Boot* et al.,*2009* *Boot* et al.,*2006*
	Halichondriidae	Axinella damicornis *(Mediterranean Sea)*	Aspergillus niger *(Ascomycota (fungus))*	*Bicoumanigrin* *Aspernigrin B*	*Anticancer, cytotoxic* *Neuroprotective*	*Hiort* et al., *2004* *Hiort* et al., *2004*
		Halichondria okadai Halichondria okadai *(Japan)*	Alteromonas *sp. (γ-Proteobacteria)* Trichoderma harzianum *OUPS-N115 (Ascomycota (fungus))*	*Trichodenone A* *Trichodenone B* *Trichodenone C*	*Antileukemic, cytotoxic* *Antileukemic, cytotoxic* *Antileukemic, cytotoxic*	*Kelecom, 2002* *Usami* et al., *2000* *Usami* et al., *2000*
	Halichondria okadai	Rubritalea *HOact23*^{T} *(Verrucomicrobiae)*	Squalenifasciens *xylosyl esters A* *Dia-polycopenedioic acid xylosyl esters B* *Dia-polycopenedioic acid xylosyl esters C*	*Dia-polycopenedioic acid A* *Dia-polycopenedioic acid A* *Dia-polycopenedioic acid*	*Antioxidant* *Antioxidant* *Antioxidant*	*Shindo* et al., *2008* *Shindo* et al., *2008* *Shindo* et al., *2008*

		Halichondria Panacea	*Unidentified bacterium*	*Unidentified compound*	*Neuroactive*	*Perovic* et al., *1998*
		Halichondria Panacea *(Adriatic coast, Croatia)*	Microbacterium *sp. Actinobacteria)*	*1-O-acyl-3-[R-glucopyranosyl-(1-3)- (6-O-acyl-R-manno-pyranosyl)]-glycerol*	*Antitumor*	*Wicke* et al., *2000*
		Halichondria japonica *(Osaka Bay, Japan)*	Gymnascella dankaliensis *OUPS-N134 (Ascomycota (fungus))*	*Gymnostatin A*	*Antileukemic, cytotoxic*	*Numata* et al., *1997*
				Gymnostatin B	*Antileukemic, cytotoxic*	*Numata* et al., *1997*
				Gymnostatin C	*Antileukemic, cytotoxic*	*Numata* et al., *1997*
				Gymnostatin F	*Antileukemic, cytotoxic*	*Mayer* et al., *1999*
				Gymnostatin G	*Antileukemic, cytotoxic*	*Mayer* et al., *1999*
				Gymnostatin Q	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Gymnostatin R	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Gymnasterone A	*Antileukemic, cytotoxic*	

				Gymnasterone B	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Gymnasterone C	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Gymnasterone D	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Dankastatin A	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Dankastatin B	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*
				Dankasterone A	*Antileukemic, cytotoxic*	*Amagata* et al., *2008*

Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the true source of these metabolites or are intricately involved in their biosynthesis (Proksch et al., *2002). Convincing evidence for the involvement of microorganisms in natural product synthesis has been complied for the tropical sponges* Dysidea herbacea *and* Theonella swinhoei*, in which the producing microbe is a cynobacterium in the former and a bacterium in the latter (Proksch* et al., *2002). Thus an alternative strategy targeting the microorganisms associated with sponges for the screening of bioactive natural products may prove to be an effective approach to circumvent the associated difficulties of dealing with the organism itself. The discoveries of products derived from marine sponges and associated bacteria, which have shown* in vivo *efficacy or potent* in vitro *activity against infectious and parasitic diseases, including bacterial, viral, fungal and protozoan infections (Laport* et al., *2009). Many marine natural products have successfully advanced to the late stages of clinical trials, manzamine A (activity against malaria, tuberculosis, HIV, and others), lasonolides (antifungal activity) and psammaplin A (antibacterial activity).*

Bacterial populations in HMA are in the range of 6.4 x 10^8 *– 1.5 x* 10^9 *bacteria g-1 (Vacelet and Donadey, 1977, Hentschel* et al., *2006). So far, more than 25 bacterial phyla have been reported from sponges (Webster and Taylor, 2011, Bringmann* et al., *2007). The dominant bacterial taxa in marine sponges are Proteobacteria, Actinobacteria, Chloroflexi, Firmicutes, Acidobacteria and Cyanobacteria (Hentschel* et al., *2001, Michael* et al., *2007, Matthias* et al., *2011).*

Marine microorganisms are well-known for being capable of producing bioactive secondary metabolites. Between 1985 and 2008, around 850 compounds were isolated from marine microorganisms including bacteria, fungi and phytoplankton (Hu et al., *2011) A wide range of chemical and functional diversity has been observed among bioactive compounds such as polyketides, alkaloids, fatty acids, peptides and terpenes (Tresa, 2010). Most of the compounds isolated from marine microorganisms have shown biological properties such as antimicrobial, antitumor and anticancer activities. The phylum Actinobacteria dominates in the production of therapeutic compounds followed by Proteobacteria. Among fungi, members of the Ascomycota are predominant producers of bioactive molecules and members of Deuteromycota are also a potential group for exhibiting bioactivity (Tresa, 2010).*

The Indian coastline measures about 8129 km (Rejinold et al., *2011), which is distributed among nine coastal states and four Union Territories. The Gulf of Mannar, in South East coast of India, alone has 295 species of sponges, 180 species of marine algae and seaweeds, 190 species of gastropods, etc. (Rodrigues* et al., *2004). Several reports have appeared on the characterization of the antimicrobial activity of marine macroorganisms collected off the Indian coastline (Schmidt et al., 2000; Salvatore* et al., *2003; Rodrigues* et al., *2004).*

Studies involving the screening, isolation and characterization of bioactive compounds from marine bacteria are yet to be undertaken on a systematic scale. The antimicrobial bacterial species from the Indian coastal waters has been studied and efforts were taken to characterize the bacteria systematically. More over that the study also

concentrated the bioactive protein characterization, structure elucidation and functional sites identification using bioinformatics tools.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF ENDOSYMBIOTIC BACTERIA FROM CHOSEN INDIAN SOUTH PENINSULAR COAST SPONGES

2.1 INTRODUCTION

Sponges are the primitive multicellular, filter feeding and sessile animals associated with most of the microorganisms include bacteria, cyanobacteria and fungi. They harbor these microorganisms in their tissue as extracellular and intracellular nature. According to Vacelet and Donadey (1977), the associated microorganisms may constitute up to 40% of sponge bodymass. Perusal of literature indicated that the relations of sponges with bacteria. The relationship between the sponge and microorganisms are complex and complicated. These complexity of these microbes induces the microorganisms induce to synthesize effective metabolites with vast biodiversity.

The 50% of the isolated bacteria from Haliclona simulans *possessed antimicrobial activity (Kennedy* et al.*, 2009). Until the beginning of 2011, 30 bacterial phyla and 2 archaeal phyla have been detected in sponges (Lee* et al.*, 2007) Crude extracts of sponges of the genus* Petrosia*, (Mirna* et al.*, 2007*) Placospongia, Plakortis, Polymastia, Ptilocaulis, Scopalina, Spongia, Stelleta, Suberites, Terpios, Thethya, Timea, Topsentia, Toxocalina *and* Trachicladus *showed no antibacterial activity or displayed halii of inhibition between 5 and 10 mm against a few bacteria strains* (*Kweyu* et al.*, 2008).* Axinella infundibuliformis *showed strong antibacterial activity against* methicilin *resistant.*

Marine organisms have the specific relationship with numerous microorganisms. The sponge microbial association is a topic of research since a long time. Sponges are host organisms for various symbiotic microorganisms such as archaea (Lee et al.*, 2010), bacteria (Jean* et al.*, 2000), cyanobacteria (Rajeev and Xu Zi, 2004), and microalgae (McCook* et al.*, 2009) and other sponges (Wilcox* et al.*, 2002). In the first report (Fang Liu* et al.*, 2011) of intracellular archaeal symbionts in marine sponges described from* Holoxea *sp. Thus, we have focused on sponge-symbiotic microorganisms as a source of various natural products. In the present study an attempt has been made to find out the antibacterial activity of endosymbionts were determined against 10 pathogenic bacteria of Human and Shrimp.*

The characterization of the antimicrobial activity of marine microorganisms collected off the Indian coastline. Studies involving the screening, isolation and characterization of bioactive compounds from marine bacteria are yet to be undertaken on a systematic scale. In this juncture, the present study projected to take new efforts to isolate and characterize the bioactive potential of marine bacteria associated with selected sponges found in the south peninsular coast of India.

16S rRNA used to reveal the community structure and diversity of the predominant bacteria associated with other organisms. The first investigation of the predominant bacterial community and diversity of the sponges were determined by (Zhi et al.*, 2006) in S.* tenui, Halichrondria, D. avara*, and* C. australiensis. *Recently Jin and Jong (2012) studied the 16S rDNA from* Penares incrustans*, Based on DGGE. In the same year In-Hye Jeong* et al.,*2012 reporeted the same studies in the sponge* Spongia *sp. The sponges* Aplysina aerophoba *and* Aplysina cavernicola *of the strains of 16S rDNA genes has phylogenetically*

different clusters (Ute et al.*, 2001). These findings suggested that the identified strains may contribute to the search for new sources. Therefore, morphological criteria as well as sequence information and the determination of phylogenetic relationships are considered to be necessary for the identification of microorganism.*

Considering the importance of anti microbial activities in the sponge Axinella donani *for the identification of the unknown bacteria has to be devoted in these studies. This chapter of the thesis is tended to detect the 16S rRNA sequence with the evolutionary relationship towards different diversity of the bacterial strains.*

The present chapter was initiated with following objectives:

- *To collect the diverse marine sponges from the southern peninsular coast of India.*
- *To isolate the Antibiotic Producing Endosymbiotic Bacteria through traditional and molecular approach.*
- *To determine the Quantitative Analysis of Endosymbionts.*
- *To assay the Antibacterial activity of endosymbiont against Human pathogen, and Shrimp pathogen.*
- *To detect the partial 16S rRNA for the identification of the unknown bacteria*

- *To determine the phylogenetic tree for the study of evolutionary relationship of the bacterial strains.*

2.2 MATERIALS AND METHODS

2.2.1 Collection and Identification of Sponges

For the commercial, economical and sustainable production of drugs from marine sponges, the cost effective collection methods were carried out. The Arokyapuram, Muttom and vizhinjam coasts of southern peninsular coast of India were chosen for the continuous availability of various types of sponges. Ecofriendly "bycath" method of collection was followed for the collection of sponges.

Fig 2.1. Location of Collection Centers along the Kanyakumari Coast

The collected specimens were identified with the help of Dr. P.A. Thomas, Emeritus Scientist, CMFRI, Vizhinjam. The code and the collected sponges were tabulated in the Table 2.1

Table 2.1 The collected sponges and their codes with collection spots

Sl. No	***Sponge***	***Code***	***Collected places***
1	Sigmadocia carnosa	*MS-1*	*Vizhinjam*
2	Ircinia fasciculate	*MS-2*	*Vizhinjam*
3	Callyspongia diffusa	*MS-3*	*Vizhinjam*
4	Zygomycale angulosa	*MS-4*	*Arokyapuram*
5	Clathria*(thalysias)* vulpine Var dichela	*MS-5*	*Arokyapuram*
6	Clathria gorgonids	*MS-6*	*Muttom*

7	Phloeodictyon sp.	*MS-7*	*Muttom*
8	Axinella donnani	*MS-8*	*Arokyapuram*

2.2.2 Isolation of Antibiotic Producing Endosymbiotic Bacteria (APEB)

For the isolation of endosymbionts from the collected sponges were cultured in three different medias such as Nutrient agar media, Zobell marine agar and Zobell marine agar+sponge extracts were used as a substrate. Initially, the seperated sponge extract were serially diluted with Normal saline (NS) and were streaked and on the appropriate plates.

2.2.3 Quantitative Analysis of Endosymbionts

1 sq cm piece of sponges such as Sigmadocia carnosa *(MS-1),* Ircinia fasciculate *(MS-2),*Callyspongia diffusa *(MS-3),* Zygomycale angulosa *(MS-4),* Clathria *(thalysias)* vulpine var dichela *(MS-5),* Clathria gorgonides (*MS-6)* , Phloeodictyon sps*(MS-7)* and Axinella donnani *(MS-8) were cultured in the broth culture of appropriate media Nutrient agar + sponge extracts and Zobell marine agar + sponge extracts and the number of viable colonies were counted through poure plate method. The No of colonies produced in 1c.m^2 area of sponge is calculated.*

2.2.4 Antibacterial activity of endosymbionts

2.2.4.1 Test microorganisms

The antibacterial activity of endosymbiont was determined against 10 pathogenic bacteria purchased from MTCC (Microbial Type culture cullection) , Candigarch. The test organisms and their source are given in the Table 3.

Table 2.2 Test organisms

Sl.No	Bacteria	Gram stain
1	Micrococcus luteus	GRAM POSITIVE
2	Bacillus cereus	GRAM POSITIVE
3	Bacillus subtilis	GRAM POSITIVE
4	Staphylococcus aureus	GRAM POSITIVE
5	Staphylococcus epidermidis	GRAM POSITIVE
6	Escherichia coli	GRAM NEGATIVE
7	Proteus vulgaris	GRAM NEGATIVE
8	Pseudomonas aeruginosa	GRAM NEGATIVE
9	Vibrio alginolyticus	GRAM NEGATIVE
10	Aliivibrio fischeri	GRAM NEGATIVE

2.2.4.2 Antibacterial assay

An agar-well diffusion method was employed for determination of antibacterial activities (NCCLS, 1999). The Petri plates were prepared with 20 ml of sterile MHA. The plates were allowed to solidify for 5 minutes and the tested cultures were swabbed on top of the solidified media and allowed to dry for 10 min. Wells (4.6 mm in diameter) were cut from the agar with a sterile borer and 40 µl extract solutions were delivered into them. The extracts (well) were placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 35°C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of inhibition zone. The zones of inhibition were measured in mm. Assay was carried out in triplicate and control plates were also maintained.

2.2.4.3 16S rRNA partial sequencing

The DNA was extracted using the method as described by Chen and Kuo (1993) with slight modifications and visualized on 1.0% agarose gel. The 16S ribosomal DNA (rDNA) was amplified by PCR using 16SF: GAATCATATGCTGCGCCGTC *and 16SR:* ATCGGAACGCCATCCACTTC *as primers. Thermal cycling was performed in an Applied BioSystem GeneAmp PCR system 2700 using Taq Polymerase (Fermentas) according to the following program: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 1 min., 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 minutes. The PCR product after visualization was purified from the gel using PCR product purifying kit of Wizard SV Gel and PCR Cleanup System (Promega). The sequencing was performed by the DNA Sequencer ABI 3130 Genetic Analyzer (Applied BioSystems). 16S rDNA sequence was compared to other prokaryotic 16S rDNA sequences by using the similarity rank analysis*

Service of NCBI (BLAST). For the construction of phylogenetic tree and determination of the nearest database neighboring sequences, the sequences of isolates were aligned using CLUSTAL X program version 1.8 (Larkin et al., *1997). Phylogenetic tree was constructed using the neighbor joining algorithm of CLUSTAL X and displayed by using Tree View. The sequences for the closest neighbors (approx. bp 1600) were used for this purpose. A total of 40 sequences were aligned from the genus* Bacillus *sequences available at NCBI GenBank database.*

2.2.4.4 Phylogenetic Tree

Phylogenetic analysis was performed with the MEGA 4.0 program (Molecular Evolutionary Genetics Analysis, Version 4.0). The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method.

2.3. RESULTS

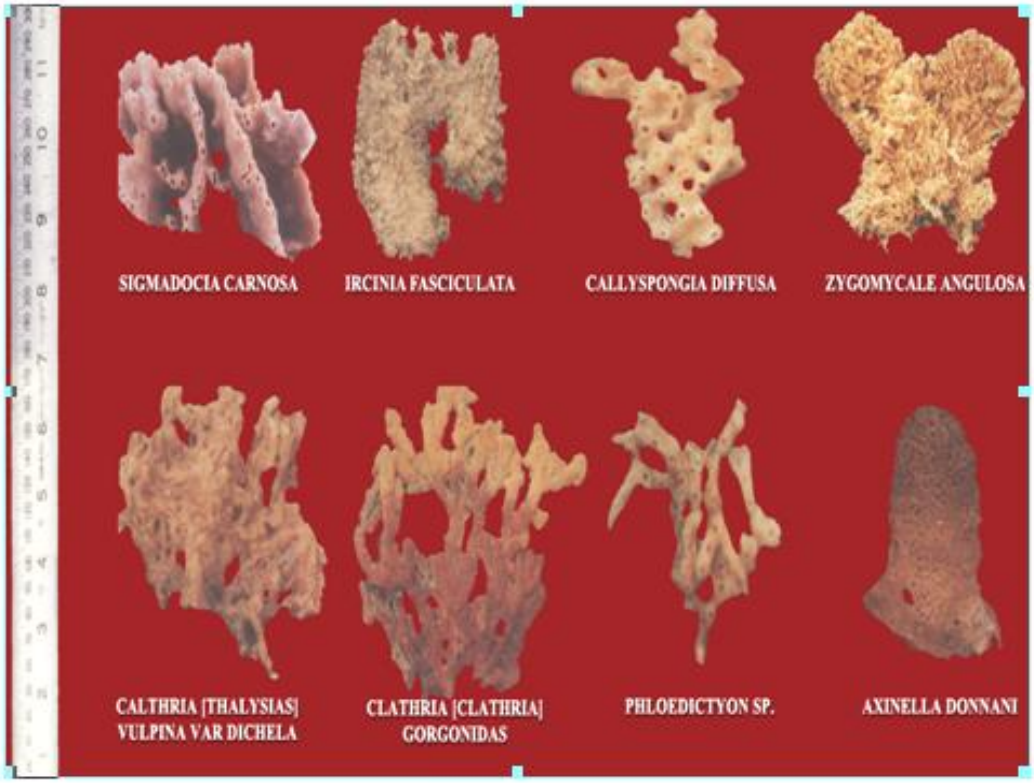
Species of sponges used for the experiments were collected from various region of southern peninsular coast of India. The collected sponges and its colour and collection spots were given in Table 2.3.

Table 2.3 Collection and Identification of Sponges

Sl.No.	***Sp.***	***Colour***	***Collection spot***
1	Sigmadocia carnosa	*Brown*	*Vizhinjam*
2	Ircinia fasciculate	*Brownish yellow*	*Vizhinjam*
3	Callyspongia diffusa	*Yellow*	*Vizhinjam*
4	Zygomycale angulosa	*Yellow*	*Arokyapuram*
5	Clathria*(thalysias)* vulpine Var dichela	*Brownish Yellow*	*Arokyapuram*
6	Clathria gorgonids	*Reddish Yellow*	*Muttom*
7	Phloeodictyon sp.	*Pink*	*Muttom*
8	Axinella donnani	*Black*	*Arokyapuram*

Identified Sponge species

Fig 2.2 The identified species of sponges



Isolation of Antibiotic Producing Endosymbiotic Bacteria

For the isolation of endosymbionts, which is present in the eight species of sponges, were shown in Table 2.4. In this the Sigmadocia carnosa *has three types of endosymbionts in zobell marine agar /broth isolation. But in the case of nutrient agar isolation, only two types of bacteria were obtained. In the case of* Axinella donnani, *three types of endosymbionts were seen in normal nutrient and marine media. But he same sponge produced four different types of endosymbionts in the nutrient media, which is supplemented, with low level of*

sponge extracts. Apparently the same trends were noted in all other sponge types. In Callyspongia *and* Axinella*, were produced different types of fluorescent colour and black colour bacteria were observed respectively. In the specialized media were produced for the effective isolation of bacteria, contain low level of specific sponge extracts.*

Table 2.4 Isolation of Antibiotic Producing Endosymbiotic Bacteria

Sponge sp.	**No of colonies produced in various media**			
	Nutrient agar	***Zobell marine agar***	***Zobell marine agar+sponge extracts***	***Total***
Sigmadocia carnosa	***2***	***3***	***8***	***13***
Ircinia fasciculate	***2***	***3***	***4***	***9***
Callyspongia diffusa	***6***	***6***	***8***	***20***
Zygomycale angulosa	***2***	***2***	***4***	***8***
Clathria *(thalysias)* vulpine var dichela	***2***	***2***	***5***	***9***
Clathria gorgonids	***2***	***2***	***3***	***7***
Phloeodictyon sp.	***2***	***2***	***4***	***8***
Axinella donnani.	***3***	***3***	***4***	***10***

Quantitative Analysis of endosymbionts

The results of quantitative analysis indicated that the sponges contain a considerable amount of bacteria. In Sigmadocia, Callyspongia *and* Axinella *contains very thick bacterial population. The results are shown in Table 6.*

Table 2.5 Quantitative Analysis of endosymbionts

Sponge	***No of colonies produced in 1c.m^2 area of sponge***	
	Nutrient agar + sponge extracts	***Zobell marine agar + sponge extracts***
Sigmadocia carnosa	***7***	***9***
Ircinia fasciculate	***5***	***5***
Callyspongia diffusa	***4***	***6***
Zygomycale angulosa	***9***	***10***
Clathria *(thalysias)* vulpine var dichela,	***5***	***6***
Clathria gorgonides	***6***	***5***
Phloeodictyon sps	***6***	***8***
Axinella donnani	***9***	***13***

Antibacterial potential of different sponge endosymbionts against common pathogenic bacteriaactivity of endosymbiont

Antibacterial activity of endosymbionts against common bacterial pathogens is shown in Table.2.6.

Table 2.6 Antibacterial potential of different sponge endosymbionts against common pathogenic bacteriaactivity of endosymbiont

Bacteria	***ANTIBACTERIAL ACTIVITY***	
	GRAM POSITIVE	***GRAM NEGATIVE***

	Micrococcus luteus	Bacillus cereus	Bacillus subtilis	Staphylococcus aureus	Staphylococcus epidermidis	Escherichia coli	Proteus vulgaris	Pseudomonas aeruginosa	Vibrio alginolyticus	Aliivibrio fischeri
ESB - 1	-	+	++	-	+++	++	+	-	+	++++
ESB – 2	++	++++	-	+++	+	-	++	+	+++	-
*ESB – **3***	**+++**	**++**	**++**	**++**	**++**	**-**	**++++**	**-**	**+**	**++**
ESB – 4	-	++	+	-	+	+++	-	++	-	-
ESB – 5	+	-	+++	++	-	+	-	-	-	+
ESB – 6	++	+	-	-	+++	-	+	++++	-	+
ESB – 7	++	++	++++	++	+	+	-	-	-	+
ESB – 8	++++	-	+	+	-	++	-	+	+++	+
ESB - 9	-	+	++	++	-	-	+	++	-	+
ESB - 10	++	+	-	+	++	+++	-	-	+	-
ESB - 11	-	++	+	-	-	-	+	++	++	+++
ESB - 12	-	-	-	++++	+	+	++	+++	-	-
ESB - 13	++	++++	+	-	-	+	+++	++	-	+

++++=30; +++=20-30mm; ++=10-20mm; +=1-10mm - = No Activity

The antibacterial activity of potent endosymbiont (ESB- 3)

The antibacterial activity of potent endosymbiont (ESB- 3) was determined against 10 pathogenic bacteria of Human and Shrimp. The 10 pathogens used are very commonly seen pathogens. Details are in the Table 2.7.

Table 2.7 The antibacterial activity of potent endosymbiont (ESB- 3)

Pathogens	**Bacterial Species**	***Zone diameter produced by ESB – 3 endosymbiont***

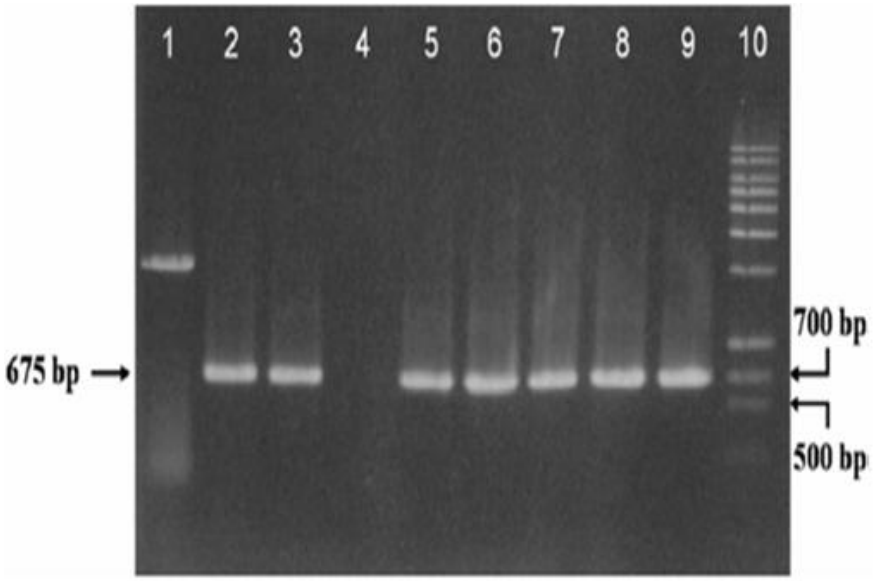
		20°C	***37°C***
Shrimp pathogens	Vibrio esturiances	++	+
	Vibrio alginolyticans	++	-
	V. harvae	+	_
	Aeromonas hydrophila	++++	++
	Pseudomonas aerogenosa	+++	+
Human pathogens	Streptococcus hemolyticus	++	_
	V. fisheri	+	_
	E.coli	+++	+
	Morgenella morgenii	++	_
	Bacillus cereus	+	-

++++=30; +++=20-30mm; ++=10-20mm; +=1-10mm - = No Activity

16 S rRNA

The Fig 2.3 represented the partial sequencing of 16SrRNA. It was reported that the unknown bacteria which was used to identify the both ESB3 and ESB 7 are Bacillus subtilis.

Fig 2.3 16 SrRNA partial sequencing



Lane 1	*: 50 bp Molecular weight marker (promega)*
Lane 2&3	*: Bacillus subtilis strain marker*
Lane 4	*: Blank*
Lane 5 &7	*: ESB 3*
Lane 6,8 &9	*: ESB 7*
Lane 10	*: Molecular weight marker (vivagen)*

FASTA sequence

>ESB 3

GGGTATAAACTGGAGGAGCCAAAAATGAATGAATAAATACCGTGTGCGCCTTTTTTCTGTATTCGTTGTCTGTATGA
TTCTGGTGTTTTGTGTCCTCGGGCTGTTTTTACAGCAGCTTTTTGAAACATCTGATCAAAGGAAAGCAGAGGAACAC
ATTGAAAAAGAAGCAAAATACTTGGCTTCGCTTCTTGATGCCGGCAATCTGAATGATCAAGCAAATGAGAAAATCAT
TAAAGATGCAGGCGGCGCGCTTGATGTGAGCGACGGCAAGGTGCTGTGTCAACAACAGATAACAAGCTTTATTACGG
ACTTTCACTTAGAAGCGAAGGTGAAAAAACAGGATATGTGCTGCTTTCCGCCTCTGAAAAAAGCGACGGCTTAAAAG
GCGAATTGTGGGGGATGCTGACGGCCAGTCTTTGTACCGCATTTATCGTCATTGTTTATTTTTATTCCAGCATGACC
TCACGTTATAAAAGGTCAATTGAGTCAGCCACAAATGTAGCCACAGAACTCTCTAAGGGCAACTACGATGCACGGAC
ATACGGCGGCTATATTAGGCGTTCTGATAAGCTAGGACATGCGATGAACAGCCTTGTATGCATTGAAAACATTGGCT
CCGGGCTGATTATGATCGACGGAAGAGGCTTTATTAATCTCGTAAACAGGTCTTACGCCAAGCAGTTTCATATCAAT
CCGAATCATATGCTGCGCCGTCTTTATCACGATGCATTTGAACATGAAGAAGTGATCCAGCTTGTCGAAGACATTTT
TATGACGGAGACAAAGAAATGCAAGCTGTTAAGACTTCCGATCAAAATAGAACGGCGTTATTTTGAAGTGGATGGCG
TTCCGATTATGGGGCCGGACGATGAATGGAAAGGAATTGTGCTCGTTTTTCATGACATGACGGAAACAAAGAAATTA
GAGCAGATGAGGAAGGATTTTGTGGCCAATGTTTCTCATGAGCTGAAAACGCCGATTACGTCATAAAAGG

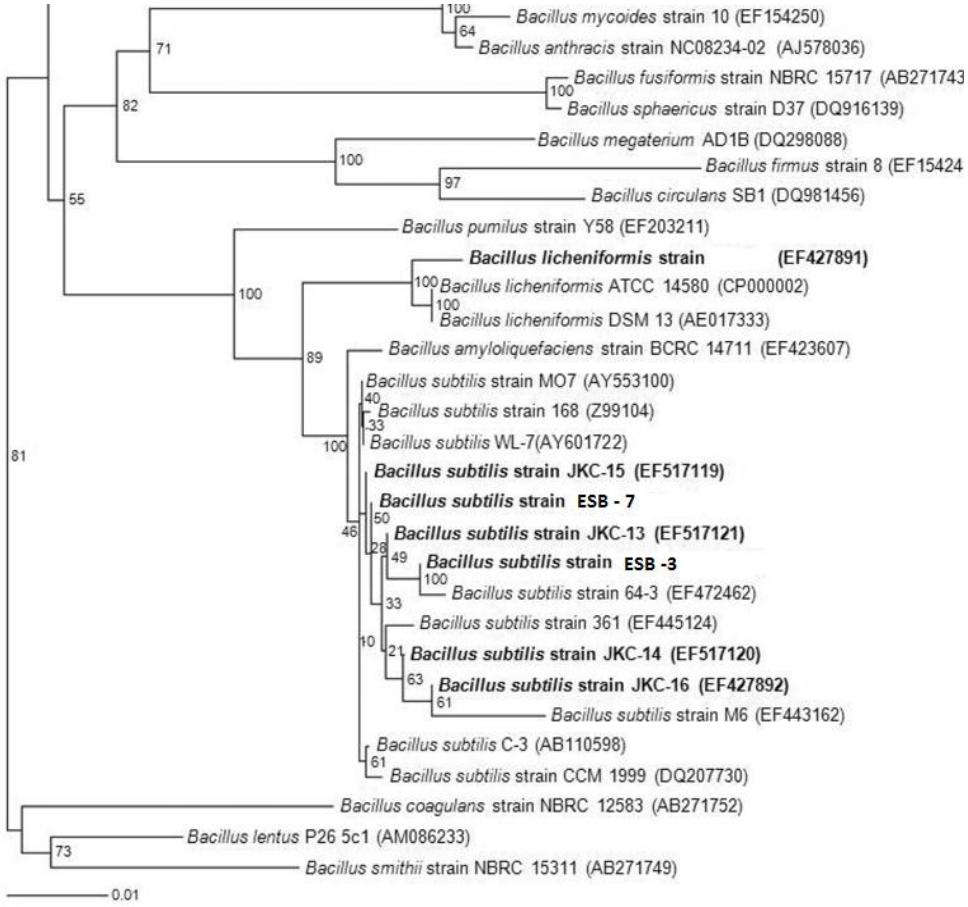
>ESB 7

GGGTATAAATTAGAGGAGCCAAAAATGAATGAATAAATACCGTGTGCGCCTTTTTTCTGTATTCGTTGTCTGTATGA
TTCTGGTGTTTTGTGTCCTCGGGCTGTTTTTACAGCAGCTTTTTGAAACATCTGATCAAAGGAAAGCAGAGGAACAC
ATTGAAAAAGAAGCAAAATACTTGGCTTCGCTTCTTGATGCCGGCAATCTGAATGATCAAGCAAATGAGAAAATCAT
TAAAGATGCAGGCGGCGCGCTTGATGTGAGCGCATCCGTTATCGATACTGTCAACAACAGATAACAAGCTTTATTAC
GGACTTTCACTTAGAAGCGAAGGTGAAAAAACAGGATATGTGCTGCTTTCCGCCTCTGAAAAAAGCGACGGCTTAAA
AGGCGAATTGTGGGGGATGCTGACGGCCAGTCTTTGTACCGCATTTATCGTCATTGTTTATTTTTATTCCAGCATGA
CCTCACGTTATAAAAGGTCAATTGAGTCAGCCACAAATGTAGCCACAGAACTCTCTAAGGGCAACTACGATGCACGG
ACATACGGCGGCTATATTAGGCGTTCTGATAAGCTAGGACATGCGATGAACAGCCTTGAGACGGCAAGGTGCTGTAT
GGGTCAAACGGGAGAGGATCGGCTGCTGACAGTCATTGAAAACATTGGCTCCTTATGATCGACGGAAGAGGCTTTAT
TAATCTCGTAAACAGGTCTTACGCCAAGCAGTTTCATATCAATCCGAATCATATGCTGCGCCGTCTTTATCACGATG
CATTTGAACATGAAGAAGTGATCCAGCTTGTCGAAGACATTTTTATGACGGAGACAAAGAAATGCAAGCTGTTAAGA
CTTCCGATCAAAATAGAACGGCGTTATTTTGAAGTGGATGGCGTTCCGATTATGGGGCCGGACGATGAATGGAAAGG
AATTGTGCTCGTTTTTCATGACATGACGGAAACAAAGAAATTAGAGCAGATGAGGAAGGATTTTGTGGCCAATGTTT
CTCATGAGCTGAAAACGCCGATTACATCAATAAAAGG

Phylogenitic Tree

Numbers on nodes indicate Bayesian posterior probability values. The trees were constructed with the neighbour-joining method. Genetic distances were computed by Kimura's two-parameter model.

Fig.2.4 Phylogenetic tree



2.4 DISCUSSION

The South Peninsular coast was found to be an excellent area for the collection of marine sponges (Joseph Selvin & Lipton, 2004). They are a part of the benthic fauna and live in all areas of the marine world. So, the present study eight sponges were collected from southern peninsular coast of India. The sponges Sigmadocia carnosa, Ircinia fasciculate *and* Callyspongia diffusa *are collected from Vizhinjam,* Zygomycale angulosa, Clathria *(thalysias)* vulpine Var dichela *and* Axinella donnani *from Arokyapuram and* Clathria gorgonids *and* Phloeodictyon sp.collected *from Muttom.*

Symbiotic bacteria are common in sponges. The wider biosynthetic capabilities of marine sponges reported to be associated with the symbiotic microorganisms. It has been estimated that 99% of sponge associated bacterial endosymbionts are uncultivable under laboratory condition using available media. The available (Sunil et al*., 2010) media with sponge endosymbionts isolation isolates only 0.06 and 0.1% of total bacteria in* Candidaspongia flabellate *and* Rhopaloeides odorabile. *The present study clearly indicated that the sponge extract supplemented media produced more bacterial growth in most of the sponge tested. In* Auxinella donnani*, the predominant numbers of bacterial colonies were noted in all culture media than the others. A total of 34 bacterial strains were isolated: 10 on zobell marine agar, 11 on Marine-agar and 13 on Marine/extract supplemented-agar in* A. donnani *All 34 strains were assayed for antimicrobial activity against indicator bacteria. Likewise* Callyspongia diffusa *a total of 20 bacterial strains were isolated: 6 on zobell marine agar, 6 on Marine-agar and 8 on Marine/extract supplemented-agar and* Sigmadocia

carnosa *a total of 13 bacterial strains were isolated: 8 on zobell marine agar, 3 on Marine-agar and 3 on Marine/extract supplemented-agar also gave notable results. The quantitative analysis the sponges* Sigmadocia, Callyspongia *and* Axinella *contains very thick bacterial population as Nutrient agar/ sponge extracts of both contain 9 and Zobell marine agar/sponge extracts contain 10,13nm respectively.*

The antimicrobial potential of actinomycetes *associated marine sponges from the Bay of Bengal coast shows (Gandhimathi,* et al., *2008) high antimicrobial activity against Gram negative bacteria such as* Pseudomonas aeruginosa *and* K. pneumonia. *(Rodrigues, 2004)* H. cribricutis *inhibited* Klebsiella species *considerably and was weakly active against* S. aureus *and* S. flexineri. *Yonghong Fang* et al., *(2011) reported as* Ircinia sp. *exhibited mild antibacterial activity only against* S. aureus. *The* Haliclona *sp. collected form Indonesia yielded a triterpene ketide, Halicotriol B with weak antimicrobial activity against* S. aureus *and* Bacillus subtilis *(Tim and chris, 2004). The extracts of Andaman Sea sponges do not contain broad-spectrum antimicrobial substances (Patchara* et al., *2010). The sponge extracts of* C. longitoxa *and* C. diffusa *were found to be most effective against* C. quiquefasciatus *Larvae (Baby Joseph and Sujatha, 2011) and the extracts evaluated,* Clathria longitoxa *(Henschel),* Callyspongia diffusa *(Ridley)* Sigmadocia carnosa *(Dendy),* Haliclona pigmentifera *(Den.), and* Dendrilla nigra *(Dend.) showed significant insecticidal activity.Touati* et al., *(2007) revealed that* Hymeniacidon sp, Agelas mauritania, Halichondrida sp. *and* Oceanopia sp.*and hexane extract of* Tedania *sp. 1 possess very potent activities against the fungi* Candida albicans *and* C.neoformans Agelas mauritania *showed*

selective activity against C.neoformans *and the antibacterial activities of* Aplysinopsis *sp.,* Halichondrida *sp. and* Oceanopia *sp against* C.neoformans

The sponge Ectyobatsella enigmatica *has effective bactericidal activity against* Streptococcus pyogenes *and it is ineffective against* Pseudomonas aeruginosa *and* Klebsiella *sp. (Prabha* et al*., 2010). Four gram positive symbiotic bacteria (*Pseudomonas aeruginosa, Escherichia coli, Vibrio esturiances *and* V.cholera) *associated with the sponge* Callyspongia diffusa *but no activity against the bacteria (Boobathy* et al., *2009). The sponge* P. soror *is found as an ectosymbiont on* C. novaeguineaei *and it has antimicrobial activity against Shrimp pathogen (Eric and Olliff, 2011). The isolation of the sponge associated bacterium* Oscillatoria spongeliae *grow inhyperosmotic medium (Urban* et al*., 1999). The symbiosis between the Californian sponge* Axinella mexicana *and the psychrophilic crenarchaeote (Michael*, et al*., 2007).*

The sponges Agelas oroides *and* Axinella damicornis *collected from Tunisian Mediterranean and tested against 10 human pathogens, shows antibacterial activity (Ines* et al*., 2007). The Australian marine sponge*, Axinella *sp., have bactericidal activity against* Helicobacter pylori*, a Gram-negative bacterium associated* Myrothecium *sp. (Nechev, 2002). The previous study reported by Joseph Selvin and Lipton (2004) the sponge* D.nigra *exhibited broad spectrum antimicrobial activity,* Axinella donnanin *in narrow spectrum and growth in all the three medium and it has least antimicrobial activity (25.0 %). They have done the antimicrobial activity against shrimp pathogens. The present study also strongly supported to the existing studies that* Axinella donnani *showed broad spectrum antimicrobial activity against 10 pathogenic bacteria of Human and Shrimp. Among the thirteen strains*

ESB-3 showed high antibacterial activity against the pathoges also reported the strains isolated on Marine/extract supplemented-agar inhibited more than 80% of indicator strain.

Phylogenetic analysis based on small 16S rDNA fragment can be useful for understanding the basic relationship among strains (Cebron et al., *2004). The DGGE fingerprints is based on approximate 194 bp 16S rDNA-V3 fragments, with phylogenetic relationship of 5% sequence divergence (Zhi-Yong Li* et al., *2006). The predominant bacterial group found in the* H. simulans *total 16S rRNA gene library was the g-*Proteobacteria*, which constituted 77% of clones obtained (Kennedy* et al., *2008). The remaining sequences were affiliated with the a- (4%), b- (5%) and d-*Proteobacteria *(<1%),* Planctomycetes *(1%),* Verrucomicrobia *(5%),* Lentisphaerae *(4%),* Actinobacteria *(1%),* Spirochaetes *(> 1%) and* Bacteroidetes *(2%) phyla. Recently, Rani Juneius and Selvin isolated two strains from marine sponge and identified by 16S rRNA sequencing as* Rhodobacter spharoides *MSB 57 and* Rhodopseudomonas palustris *MSB 55. It is closely related to our studies but the study interpreted with the ORF analysis. But the present study indicated the functional proteome studies.*

In the present study reported that the sponge Axinella donani *endosymbionts has anti microbial activities. The 16S rRNA demonstrated the identification of the unknown bacteria which was used to identify the both ESB3 and ESB 7 are* Bacillus subtilis.

CHAPTER III

PROTEIN PROFILE ANALYSIS OF ANTIMICROBIAL SUBSTANCE PRODUCING ECTOSYMBIOTIC BACTERIA

3.1 INTRODUCTION

The bacteria associated with marine invertebrates are a rich source of bioactive metabolites. Natural products like sponges have potential bioactive compounds. But most of the compounds are unknown. The microbial inhabitants of sponge species have been characterized with molecular tools (Dettmer et al.*, 2010). Bioactive substances from sponges have shown anticancer, antibacterial, antifungal, antiviral, antiprotozoal, anthelmintic, anti-inflammatory, immunosuppressive, neurosuppressive, and antifouling activities. The research for bioactive compounds in sponges began during the 1950s (Melika* et al.*, 2010). Many of these natural products have interesting biomedical potential. Manoalide is one of the first sesterterpenoid to be isolated from a marine sponge* Luffariella variabilis*, was found to be an antibiotic (Laport* et al.*, 2009). This is the only example of antibiotic seterterpenoid*

discovered so far (Yoo et al*., 2001). Recently Maushmi* et al., *(2012) studied bioactive proteins from* Spongosorites halichondriodes*, collected from Mumbai coastal area. Antimicrobial activity of* Amphilectus fucorum *and Eurypon with* Proteobacteria *and* Pseudovibrio *spp. (Margassery* et al., *2012).*

The protein fractions isolated from Streptomyces *sp. of associated marine sponges namely* Callyspongia diffusa, Mycale mytilorum, Tedania anhelans *and* Dysidea fragilis *collected from Vizhinjam port, situated in the South-West coast of India has huge antimicrobial potential (Hentschel* et al., *2006).*

The vast numbers of marine secondary metabolites represent an extreme challenge for Large-scale protein profiling. To understand physiological processes, insight into protein complexes is very important. Through a combination of blue native gel electrophoresis and LC–MS/MS, were used to isolate protein complexes and identify their potential subunits (Martin, et al*., 2010). Protein complexes play a critical role in many biological processes. Most proteins are, at some time point in the lifespan of the cell, involved in complex formation with multiple protein interaction partners (Matthias, 2011). Identifying the component proteins in a protein complex is an important step towards the understanding of the complex and in elucidating the related biological activities.*

The Blue Native DIGE is a useful method for studying protein separation on the analytical (microgram) scale (Ilka, et al., *2006). It covers a mass range of native proteins and complexes from o100 kDa to B10 MDa. Protein– protein interactions also study using BN-PAGE.(Frank Krause, 2006) membrane protein complexes solubilized with zwitterionic*

detergents like CHAPS and 3[(3- cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) were separated by BN-PAGE.Thus the BN-PAGE promotes the functional proteomic study.

In the 1990's mass spectrometry suddenly became important in proteomic research. Up to that time this analytical technique had faced the problem that there was no satisfying method generating ions from large, volatile analytes such as peptides and proteins without unwanted and significant fragmentation (Aebersold and Goodlett, 2001; Hanjo et al., *2003) Methods to measure relative expression of proteins labeled with stable isotopes have emerged that create* m/z *differences for peptides and proteins that can be measured in the mass spectrometer. Past research on structure elucidation of chemical process by GC/MS has been reported from several marine organisms; however, the GC instrument cannot be used for reliable identification of specific substances. In GC/MS analysis configured mainly for volatile chemicals won't detect very high molecular weight chemicals or very low molecular weight chemicals. LC-MS approah to proteomics generally involves protease digestion and denaturaction followed by LC-MS with peptide mass fingerprinting or LC-MS/MS (tandem MS) to derive sequence of individual peptides.*

The present study used the LC-MS/MS analysis for the identification of peptide masses and its characteristics. The following objectives of the chapter II are

- *To characterize the endosymbiotic Bacteria.*
- *To study the Antibiotic production time of ESB3 strain.*

- *To study the molecular profiling studies for the identification of secondary metabolites using 1D and 2D electrophoresis.*

- *To characterize the protein using BN PAGE.*
- *To determine the antimicrobial peptide sequence of sponge* A. donnani using *LC- MS/MS.*

3.2 MATERIALS AND METHODS

3.2.1 Growth curve of potent antimicrobial agent producing bacteria

From the antimicrobial assay the potent antimicrobial agent producing bacteria was isolated and their growth pattern was studied.

3.2.2 Determination of Antibiotic production time

The peak time of antibiotic activity production metabolism was determined by taking the ECP of broth cultures in different time intervals and they are loaded in Poly Acrylamide Gel Electrophoresis, with molecular weight markers.

3.2.3. SDS-PAGE

The purity,polypeptide subunits and molecular wt of the haemolymph were determined by sodium dodecyl sulphate (SDS)-PAGE according to the method of Laemmli (1970).

Reagents

1. *Acrylamide stock solution. 30% Acrylamide and Bisacrylamide (30:0.08 g) dissolved in 50 ml and made upto 100 using double distilled water.*
2. *Separation gel buffer (1.5 M pH 8.8 Tris) 18.16g Tris dissolved in 50 ml of distilled water and adjusted to pH 8.8 with HCL.*
3. *Sticking gel buffer (Tris-Glycine buffer, pH) 6.056 g Tris dissolved in 25 ml of distilled water, adjusted to pH 6.8 with HCL.*
4. *Running gel buffer (Tris- Glycine buffer, pH 8.3) 30 g of Tris and 14g Glycine dissolved in 500 ml and made upto 1,000 ml with distilled water.*
5. *Staining solution: 250 g of Coomassie brilliant blue R-250 was dissolved in 500 ml of methanol; Acetic acid: H_2O (145:10:45) mixture.*
6. *Destaining solution: Methanol: Acetic acid: H2O in the ratio 145: 10: 45 was used as destainer.*

Sample solubilizing buffer

SDS	-	*1g*
Stacking gel buffer (pH 6.8)-	*2.5ml*	
B-mercaptoethanol blue	-	*1 ml*
Sucrose	-	*3g*
Bromophenol blue	-	*1 pinch*

Made upto 10 ml using distilled water and heated for 30 min at 100^{o} C.

Electrophoresis was carried out in 20cm slab gel of 0.7 mm thickness with 5% and 10% sodium dodecyl sulphate (SDS) - PAGE for stacking and resolving gel, respectively in the presence of 0.5% of SDS dissolved in 1.5 M Tris- Hcl. pH 8.8. Samples and Molecular markers of wide range (97 kDa- 14 kDa- Bangalore Geni) were boiled at 100^{o}C for 3 min with sample solubilizing buffer. The denatured haemolymph and molecular wt standards were loaded in separate wells and electrophoresed at constant current (30 mA) at 30^{o}C in electrophoresis running buffer, pH 8.3. The gel was fixed in 10% TCA for 30 min, stained in 0.25% Coomassie brilliant blue (30 min) and destained. Molecular mass of the protein bands was determined by the method of Weber and Osborn (1969). The presence of a disulphide bond between polypeptide subunit was also investigated based on the reduction with 2-mercaptothanol. The gels were stored in 7% acetic acid.

3.2.4 Separation of molecular complexes by 2D BN SDS-PAGE

Hydrophobic proteins and complexes are first solubilized with a mild nonionic detergent, like Triton X-100 or digitonin. Digitonin, the preferred detergent as it is the mildest, allows the separation of intact super-complexes. Coomassie Blue is added to the sample and cathodal running buffer and remains bound to all hydrophobic proteins and to

many water-soluble proteins by hydrophobic interactions even when an electric field is applied. Coomassie Blue is anionic so all protein-dye complexes become negatively charged in the pH 7.5 buffer used, and the complexes migrate towards the anode. Separation of protein complexes occurs according to size in the range 10 kDa to 10 MDa. These protein-dye-complexes are soluble in the absence of detergent, which minimizes the risk of denaturation. Aggregation of the proteins is also prevented because of their overall negative charge. Detection of the proteins and complexes is straightforward as the attached blue dye makes them visible. Porosity gradient gels from 4 to 16% T are employed: this allows large super complexes to enter the gel, prevents small complexes and single proteins from migrating out of the gel, and applies a band-sharpening effect.

After the first dimension electrophoresis is complete, the lanes containing the separated complexes are cut out with a sharp knife or ruler edge, equilibrated in SDS solution, and embedded into a stacking gel layer of a second dimension discontinuous SDS gel. During this process the complexes fall apart into their components (subunits) to form protein-SDS micelles that separate in the SDS gel according their molecular sizes. A Tris-tricine buffer system is preferred over the conventional Tris-glycine gel, because it offers an improved resolution of low molecular weight proteins. Gels containing non-labeled proteins can be stained after the separation with Coomassie Blue, silver stain, or with a fluorescent stains such as Deep Purple or Sypro Ruby (Frank, 2006).

3.2.5 Peptide sequencing through LC-MS/MS

Ammonium sulphate precipitated peptides were placed in in 2 mL tubes, 50-100uL of 20mM DTT (dithiothreitol) in 100mM Ammonium Bicarbonate was added, and incubated for 1 h at 60°C. DTT solution was removed and 50-100uL of 55mM IAM (iodoacetamide) in 100mM ammonium bicarbonate was added and incubated at room temp for 45 min in the dark.

The solution was removed and the band was rinsed for 10 min with 100mM ammonium bicarbonate, and then for another 10 min with ACN (acetonitrile). The solvent trace of ACN was removed with Vacuum rotavapor. 1 mL of 50 mM ammonium bicarbonate was mixied with a 20 ug vial of Sequencing Grade trypsin. The 50 uL trypsin was added to the peptide mixture and incubated for 45 min on ice. 50mM ammonium bicarbonate was added to cover the expanded gel slice and incubated on a shaker overnight at room temperature.

The solution was removed and 50 uL of 5% acetonitrile/0.1% TFA was added to the peptide mixure and shacked 15 min, room temp. Finally 50 uL of 50% acetonitrile/0.1%TFA was added to the peptide mixure for 15 min with shaking. The eluates were condensed with vacuum dessicator to about 10 uL volumes.

The sample was subjected to removal of cations in the base extension reaction product using SpectroCLEAN resin. 24 µL of water and 12 mg of resin were added to the Peptide extract. The mixture was placed in a rotator for 30 min at room temperature.

The final product was dispensed to the Agilent 1100 series 2D Nano LC MS Instrument. The mass spectrometric data was acquired and recorded.

3.3 RESULTS

Characterization of endosymbiotic bacteria

The Table 3.1 resulted that the morphological, physiological and biochemical characteristics of the two strains ESB3, ESB7. The Gram staining indicated that the strain ESB3 was Gram positive with coccus shape bacteria and ESB7 as Gram positive bacteria, forming Bacilli. Biochemical characterization showed that all strains were catalase and oxidase-positive and non-fomenters for sugars such as glucose, sucrose or lactose.

Table 3.1 Characterization of endosymbiotic bacteria

Characterization	***Results***	
Shape	*coccus*	*Bacilli*
Spore formation	+	+
Motility Test	*Motile*	*Motile*
Indole Test	*-ve*	*-ve*
Citrate utilization	*+ve*	*+ve*
Methyl Red Test	*+ve*	*-ve*
Voges - Proskauer Test	*+ve*	*+ve*
Catalase Test	*+ve*	*+ve*
Oxidase Test	*+ve*	*-ve*

Table 3.2

Carbohydrate Fermentation Test	*+ve*	*-ve*
Triple sugar ion test	*+ve*	*-ve*
Casein hydrolysis	*+ve*	*+ve*
Gas production	*-ve*	*-ve*

Results of growth curve of ESB3

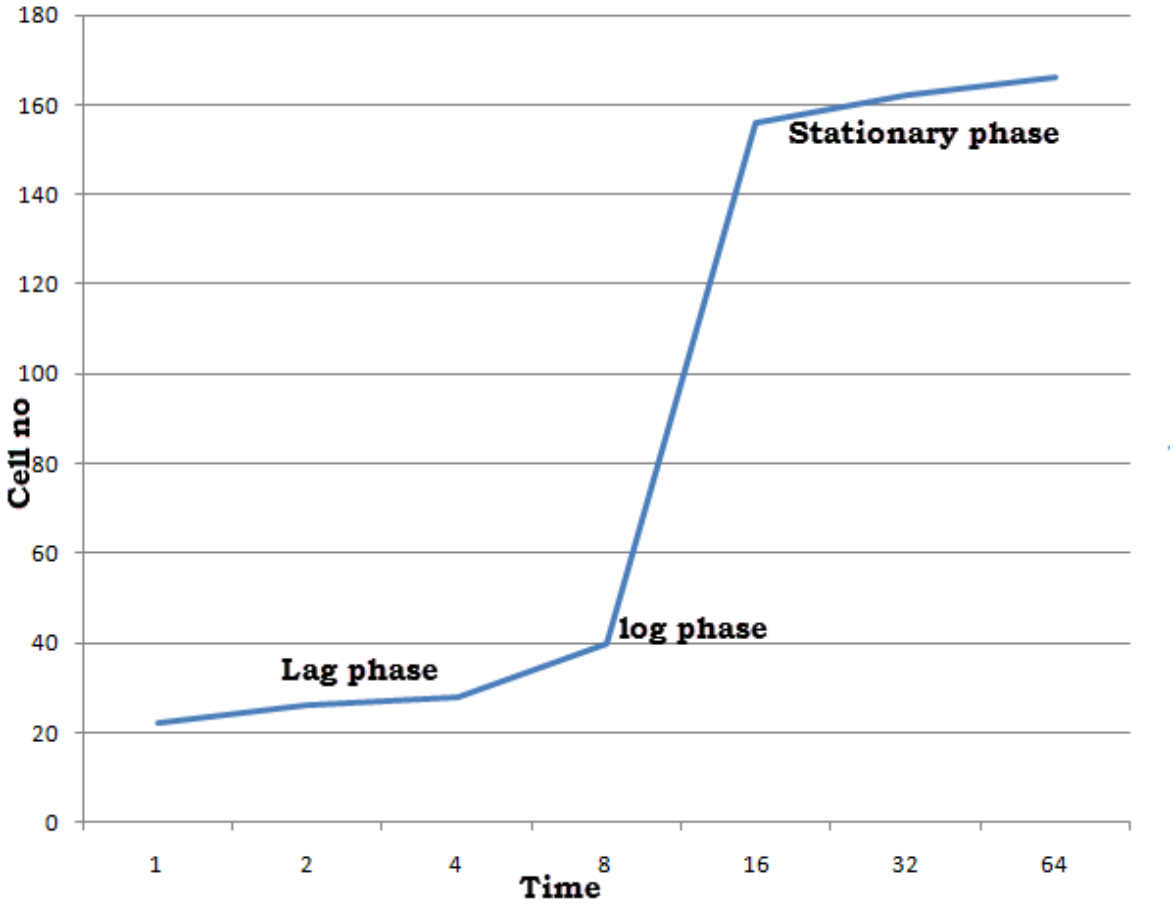
The growth of ESB 3 was determined and plotted on the Table 3.2 and Fig 3.1

Time (hrs)	***CFU/ml***
0 (inoculation)	*20*
1	*22*
2	*26*
4	*28*
8	*40*
16	*156*
32	*162*
64	*166*

Growth curve of ESB3

The growth pattern of the selected endosymbions was shown in Fig 3.1. Based on the result, the log phase starts after 4th hour and it was attain a stable phase at 18th to 24hours. The growth of ESB3 was determined at different intervals of time.

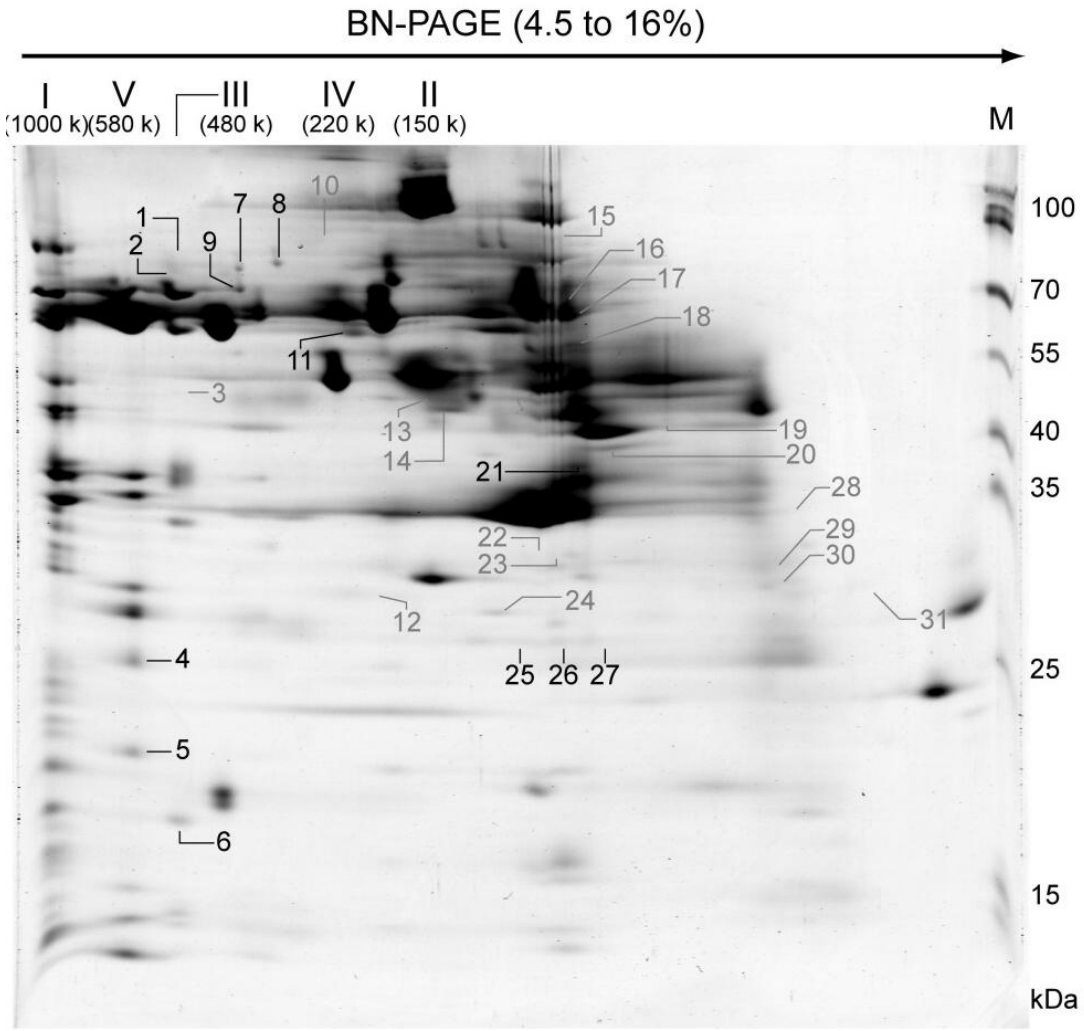
Fig 3.1 Growth curve of ESB3



BN-PAGE

Separation of molecular complexes by 1D BN-PAGE (left panel) and identification of their constituent individual complexes by 2D BN/BN-PAGE (right panel). The 2D BN-PAGE used dodecylmaltoside (DDM) in the cathode buffer. I, V, II1, IV and II, indicate the individual monomeric complexes.

Fig 3.2 BN- PAGE of* Axinella donnani *symbionts



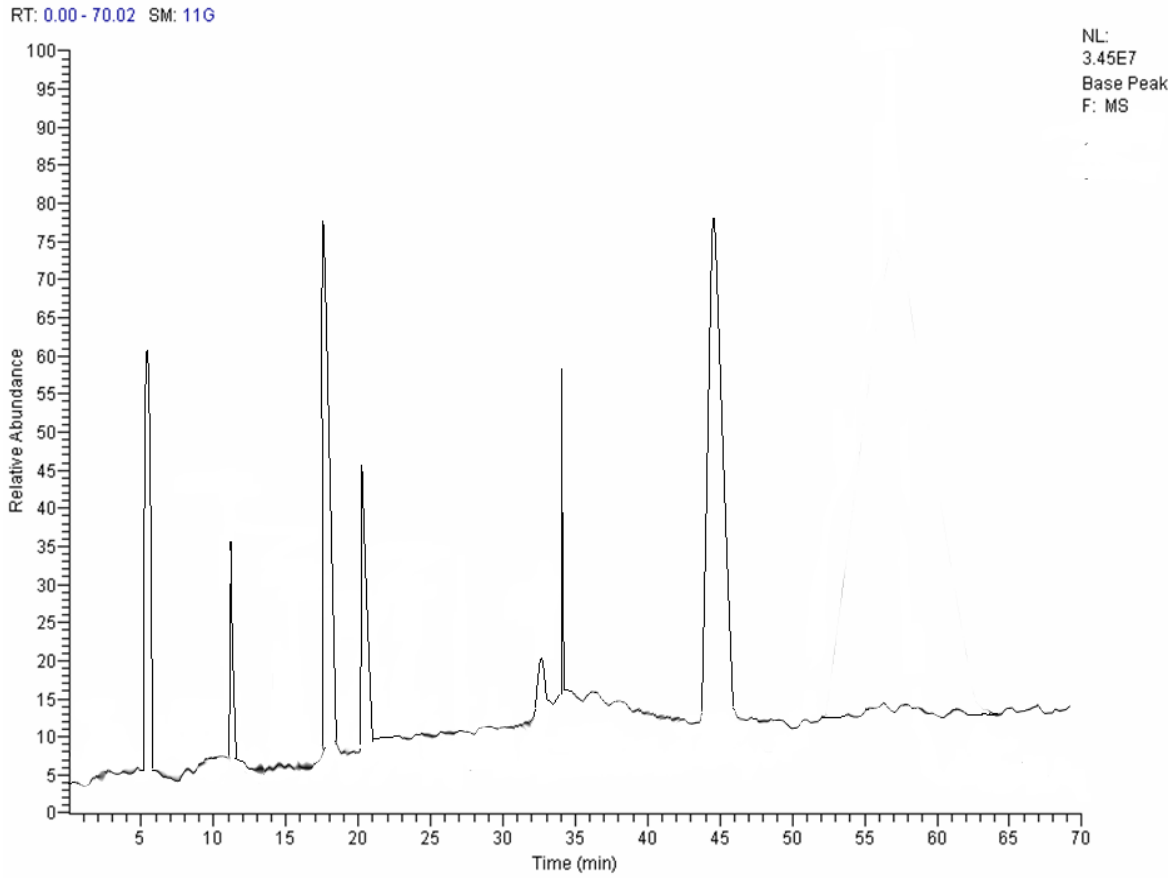
2D BN-PAGE *1D BN-PAGE*

Peptide sequencing through LC-MS/MS

The result shows the LC MS/MS Analysis of Antibacterial peptide purified from sponge associated bacteria. The peaks indicate the mass of the protein. The monoisotopic mass is indicated as high peak the value is 3587.815 and the next lower peak indicates the average mass and the value is 3587.815.The following characteristics are also below:

**** Cys reduced** *** N-term free** *** C-term free***

Fig 3.3 : Mass Analysis in MS



3.4

DISCUSSION

In most studies on microbes associated with sponges the taxonomic classification of the microbes was based exclusively on morphological characteristics and in many cases identification was possible only at the genus level (Jutta, et al.*, 2011). The Brazilian sponges*

associated bacterial strains Pseudomonas fluorescens *H40 and H41 and* Pseudomonas aeruginosa *H51 exhibited antimicrobial activity against both Gram-negative and Gram-positive bacteria, including strains such as vancomycinresistant* Enterococcus faecium *and multiresistant Klebsiella* pneumonia *(Olinda* et al.*, 2010).* Bacillus pumilus *Pc31 and Pc32,* Pseudovibrio ascidiaceicola *Pm31 and Ca31* and Pseudovibrio denitrificans *Mm37 strains were more effective against Gram-positive bacteria (Rong-Bian* et al., *2011). Actinomycete strain AZS 17 was isolated as an endosymbiotic microorganism from a marine sponge* Hymeniacidon *sp. (Petra* et al., *1999). (poly-APS) isolated from the marine sponge* Reniera sarai *act as potent anticholinesterase agents. A symbiotic fungal strain* Myrothecium *sp. JS9 in the marine sponge* Axinella *sp. was found to be an efficient producer of most effective antifungal metabolites roridin A and D (Tresa Remya* et al., *2010). In the present study resulted the morphological, physiological and biochemical characteristics of the two strains ESB3, ESB7. The Gram staining indicated that the strain ESB3 was Gram positive with coccus shape bacteria and ESB7 as Gram positive bacteria, forming Bacilli. Biochemical characterization showed that all strains were catalase and oxidase-positive and non-fomenters for sugars such as glucose, sucrose or lactose. Interestingly, the three strains showed antimicrobial activity only when grown at 20^{o}C, while no antimicrobial activity was detected at 37^{o}C. The growth pattern of the selected endosymbionts also studied. Based on the result, the log phase starts after 4th hour and it was attain a stable phase at 18th to 24hours. These results promote to study the new metabolites.*

BN- PAGE is used for one-step isolation of microgram amount of membrane protein complexes from biological membranes as Solubilized bovine heart mitochondrial complexes

I–V, pyruvate dehydrogenase complex (P), and oxoglutarate dehydrogenase complex (O) were separated according to their indicated masses on a linear 3.5-16% acrylamide gradient gel for BN-PAGE. Subunits of the native complexes were separated by tricine-SDS-PAGE using a 13% T, 3% C gel type (Ilka Wittig, et al., *2000*). *This type of 2-D gel was first employed by Schägger and Pfeiffer (2000) to dissociate digitonin-solubilized bovine heart respiratory supercomplexes I1III2IV0–4 into their individual complexes I, III2 and IV, as well as ATP synthase dimers into monomers by application of 0.02% DDM to the cathode buffer.*

In MS analysis the analytes can be performed by the relative intensity response from the mass spectrometer. Stable isotopes can be incorporated into proteins by metabolic, covalent, or enzymatic labeling and expression ratios are measured by comparing peak areas for the protein or peptide ions measured in the mass spectrometer. According to Meenupriya et al., *2011 the fungal extract of* Aspergillus terreus *was separated using TLC and it characterized by GC-Ms. The chromatogram reveals the presence of different functional groups 15-methyl, tricycle[6.5.2(13,14).0(7,15)]pentadeca-1,3,5,7,9,11,13-heptene with molecular formulaC16H14with molecular weight 206 Da, 3,4-Dihydro-2H-1,5-(3-t-butyl) benzodioxepine with molecular formula C13H18O2 with molecular weight 206 Da, 2,6-dimethyl-N-(2-methyl-α-phenylbenzyl)aniline with molecular formula C22H23N with molecular weight 301 Da and the structural compound of α- Campholene aldehyde with Chemical formula C10 H16 O with molecular weight 152 Da. Valentin Bhimba* et al., *(2011) studied the GC-MS analysis in marine sponge* Dendrilla nigra *for the identification of main phycoconstituents like antibacterial, antioxidant, anti-inflammatory*

*and anticancer activities. In 2007 Christopher John Freeman Reported defensive metabolites from two species of Ircinia (*I. felix *and* I. campana*) are the species of Aplysina (*A.fulva*), Peaks labeled 1K5 (*I. felix*) and 1K3 (*I. campana*) represent peaks with spectra corresponding to FTAs. The volatile compounds were obtained by Salvatore De Rosa,* et al.*, (2003) for steam distillation in sponge* Suberites domuncula *using Lickens-Nickerson apparatus and analysed by GC/MS. Two pure compounds from* Monanchora *that were identified as 4-hydroxybenzaldehyde and indole-3-carboxaldehyde based GC-MS results (Samuel* et al. *2011).*

In this context the results of the present study illustrate the structure elucidation using LC-MS/MS Analysis of Antibacterial peptide purified from sponge associated bacteria, that yields fragment patterns with data files. The peaks indicate the mass of the protein. The monoisotopic mass is indicated as high peak the value is 3587.815 and the next lower peak indicates the average mass and the value is 3587.815.

CHAPTER IV

IDENTIFICATION OF BIOACTIVE PEPTIDES AND STRUCTURAL STUDIES

4.1 INTRODUCTION

During the decade of the 1990s, changes in MS instrumentation and techniques revolutionized protein chemistry and fundamentally changed the analysis of proteins. These changes were catalyzed by two technical breakthroughs in the late 1980s: the development of the two ionization methods electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Cole, 1997). These methods solved the difficult problem of generating ions from large, nonvolatile analytes such as proteins and peptides without significant analyte fragmentation. Because of the lack or minimal extent of analyte fragmentation during the ESI and MALDI processes, they are also referred to as "soft" ionization methods (Ruedi and David, 2001). Several authors also reported that MALDI-TOF MS is the one way to identify the polypeptides (Fenselau, 2001; Bright et al., *2002; Dickinson* et al., *2004; Friedrichs* et al., *2007). The analysis of polypeptides using MALDI-TOF MS may be a relatively cheap, rapid, and reliable method for species identification. The major disadvantage of this method is the lack of guarantee that the active component represents a new structure. This method was applied first time to the tissue of a marine sponge* Stylissa caribica *(Achim* et al., *2009). MS/MS spectra are then searched against these protein candidates to compute an identity score which is dependent on the number and quality of fragment ion assignments (Alberts* et al., *1998). For the evaluation analysis*

Mascot program is used. It is a widely-used database search engine that implements a probability-based scoring algorithm. This search engine identifies proteins by searching both peptide masses and MS/MS spectra against a database (Silas et al.*, 2005). In Mascot, a peaks list containing peptide masses are submitted to either identify proteins by PMF or for selecting a pool of protein candidates for scoring. MS/MS spectra are then searched against these protein candidates to compute an identity score which is dependent on the number and quality of fragment ion assignments (Sudeep* et al.*, 2011).*

PEAKS de novo *sequencing is an automated approach that uses MS/MS spectra to construct* de novo *sequences independently of databases. These* de novo *sequences are then used to infer protein identities using PEAKS DB or SPIDER.* De novo *sequencing begins with a pre-processing step to ensure only good quality spectra are retained (Silva* et al.*, 2010). Thousands of sequences are then constructed, with the highest scoring matches selected for confidence scoring (Zhang* et al*, 2011).*

The extension peptide of amino-terminal is signal. The signal peptide is essential for both targeting the proteins to the membrane and their subsequent translocation across the membrane. The majority of the proteins that are destined for translocation across the bacterial inner membrane are synthesized as pre-proteins with an amino-terminal peptide extension called the signal peptide. A molecular machine called the translocase recognizes the secretary proteins and assists in their travel across the membrane (Mark and Natalie and Natalie, 2011). The first identified A. suum *ASABF-γ peptide possesses the signal peptide regions as aa20 to aa83 (Anne Kuusksalu, 2006). In the same year Werner,* et al.*, revealed the Wnt signaling in siliceous sponges for the identification of immune system and the*

apoptotic system. Perusal literatures (Michaël Manuel et al., *2006) and (Dirk* et al., *2006) reported the phylogenetic signals in siliceous sponges and Demosponges. According to Martin Pfannkuchen, 2010 studied the autofluorescence signals in Porifera. The quorum sensing signals of* Dendrilla nigra *and* Axinella donnani *play an important role in symbiotic relation (Joseph* et al., *2006). The signal peptide is essential for both targeting the proteins to the membrane and their subsequent translocation across the membrane. The importance of signal peptides was shown in 1999 when Günter Blobel received the Nobel Prize in physiology or medicine for his discovery that "proteins have intrinsic signals that govern their transport and localization in the cell" (Blobel, 2000). So, present study used two types of networks in SignalP 4.0 and predicts the regions using PrediSi server.*

The structure of the protein is essential for the study of function. (Fiser et al., *2000) There are hundreds of thousands of protein sequences but only several thousand protein folds available (Eran Eyal, 2007). Refolding experiments show that the protein sequence defines a unique native fold which is, in most cases, the free energy minimum. In theory, this free energy minimum can be computed from quantum mechanics and thus predict the structure from the sequence. In practice,* ab-initio *and molecular dynamics (MD) methods are too slow or too inaccurate. Although there have been significant improvements in the* ab initio *(Bradley* et al., *2003) and threading methods (Kinch,* et al.,*2003), comparative modeling gives the most accurate results if a known protein structure that is sufficiently similar to the modeled sequence is available (Baker* et al., *2001).*

The functional properties of proteins depend upon their three dimensional structures, which result from particular amino acid sequences folding into tightly packed domains

(Raphael and Manuela, 2006). Proteins with little or no sequence identity can have remarkably similar secondary structure patterns and tertiary folds. The motif of peptide has similar size but unrelated functionally and originate from biologically diverse sources (α helix,β sheet, Beta hairpins, strands, beta turns, gamma turn and disulphide (Pallaghy et al., *2008). Richardson in 1981 derived the topology of the beta sheet using the nomenclature of protein. Beta hairpins consist of two beta-strands which are antiparallel and hydrogen bonded together (connected by at least one bridge) (Bancinyane* et al., *1989). A beta bulge is a region of irregularity in a beta sheet, where the normal pattern of hydrogen bonding is disrupted. In 1993 Edith* et al., *provided the beta strands and main-chain hydrogen bonds. The Corresponding hydrogen bonding patterns for parallel classic and wide bulges can also be found. Richardson in 1981 found that the majority of disulphides could be classed as left handed spirals or right handed hooks. In 1994, Hutchinson and Thornton used the nomenclature VIa1 and VIa2 to distinguish between two subclasses of type VIa turns with the phi, psi angles of residue i+1 in the beta and polyproline region of the Ramachandran plot. A Gamma turn is defined for 3 residues i, i+1, i+2 if a hydrogen bond exists between residues i and i+2 and the phi and psi angles of residue i+1 fall within 40 degrees of one of the following 2 classes (Rose* et al., *1985, Milner-White* et al., *1988). Likewise in the present study identified the regions of the functional properties of the newly identified protein. The functional properties like α helix,β sheet, Beta hairpins, Beta bulges, strands, Helixes, beta turns, gamma turn, disulphides, Binding sites and Binding surfaces have been studied.*

In marine environment sponges are the rich source of bioactive elements. The peptide based structural studies are very important possess to lead target identification for drug

designing. No research has yet been performed concerning the peptide sequence with their protein structural motif study in the antimicrobial peptide sequence of sponge Axinella donnani *endosymbionts.*

Considering these aspects and the requirements to detect the peptides from sponge associated bacteria towards different levels of structure has to be evaluated. Hence the present chapter was initiated with the following objectives:

- *To collect the MS and MS/MS spectra from these proteins using MALDI-TOF/TOF MS*
- *To study the Peptide Sequence Information from MALDO TOF analysis.*
- *To determine the antimicrobial peptide sequence of sponge* A. donnani *endosymbionts using LC-MS/MS.*
- *Identify these proteins using a combined MS and MS/MS search against Swissprot protein database using Mascot*
- *Make new identifications and confirm existing identifications using PEAKS DB and SPIDER*
- *To analyze the signal peptide cleavage site with gram positive, gram negative and eukaryotic bacteria.*
- *To predict the signal peptide sequences.*
- *To predict the three dimensional structure using the peptide sequence and evaluate the model.*
- *To study the functional regions which present in the 3D structure.*

4.2 MATERIALS AND METHODS

4.2.1 Peptide Sequence Information from MALDI TOF Data

A matrix was prepared by adding 10 mg of CHCA to 1 ml of ACN and 0.1 % TFA (1:1 v/v), briefly vortexed and centrifuged at 10,000 g for 5 min. The supernatant was then transferred to a clean tube for use. Tryptic peptides were resuspended in 1 µl of a CHCA matrix and spotted onto a 384 Opti-TOF 123 x 81 mm MALDI plate and left to crystallise. The MALDI plate was loaded into an AB SCIEX MALDI-TOF/TOF 5800 mass spectrometer and left for 30 min for pressures to equilibrate. The m/z ratio of precursor ions was acquired in MS mode using a reflector positive ion method and a 355 nm diode pulse laser. TOF/TOF TM Series Explorer TM 4.0 software was used to set up the method using the following settings: mass range of 800-4000 Da and a focus mass of 1500 Da; continuous stage motion with a velocity of 600 µm and 200 shots per spectrum, with the first 10 shots discarded and a laser intensity of 3510 and pulse rate of 400 Hz. A processing method was also used to specify the criteria surrounding the collection of spectra, which required a minimum S/N ratio of 15, local noise window of 50 and a cluster area S/N optimisation of 5.

The MALDI TOF/TOF mass spectrometer was externally calibrated using a TOF/TOF calibration mixture made specifically for TOF/TOF instruments (AB SCIEX,

Framingham, Massachusetts). This calibration mixture contains peptides with a known m/z ratio and ncludes: des-arg1-bradykinin 904.4680 m/z; angiotensin I 1296.6850 m/z; glu1-fibrinopeptidase 1570.6770 m/z; ACTH peptides 2093.0870 m/z, 2465.1990 m/z and 3657.9294 m/z. The criteria set used for calibration was a minimum S/N ratio of 15, with a mass tolerance of +/- 0.1 m/z and a minimum of 3 peaks required to match. An interpretation method was used to specify the criteria for peptides entering MS/MS. Precursor ions required an S/N ratio greater than 20 and needed to be within the mass range of 800-4000 Da. Fifteen of the strongest precursor ions that met these requirements were selected for MS/MS.

An exclusion list (Table 12) was also used to study the common contaminants and interference spectra from entering MS/MS. This was the reference table. Trypsin and matrix fragments are common contaminants, while polyethylene glycol is a product of the materials used. Other contaminating peptides excluded were peptides found in the calibration mix. Interference spectra Masses were observed from a negative control with no protein. Their origin is unknown. Adducts with masses of 21.982 and 37.956 Da were also excluded. MS/MS was carried out using the positive ion 1KV operating mode with CID on. Low power CID was used along with metastable suppressor. Each sub-spectrum which passed acceptance was accumulated, with acceptance criteria requiring an S/N ratio greater than 4. Stop conditions were initiated when 5 sub-spectra passed acceptance. The stage mode used was a continuous stage motion at a velocity of 1200 μm. Laser intensity was set at 4650, with 100 shots per spectrum allowed and a pulse rate of 1000 Hz. The processing method used specified a minimum S/N ratio of 10, local noise window of 250 and a cluster area S/N

optimisation of 10. MS/MS mode was externally calibrated using the angiotensin I precursor ion with a m/z of 1296.6850. Acceptance required a minimum S/N ratio of 1, mass tolerance within +/- 0.1 m/z, with a minimum of 4 peaks to match.

Table 4.1 List of m/z ratios excluded from MS/MS analysis. Contains known contaminants and interference spectra from an unknown origin.

m/z	*Name*	*Tolerance (+/-)*	*m/z*	*Tolerance (+/-)*
659.384	*Trypsin*	*0.03*	*1007.646*	*0.03*
805.417	*Trypsin*	*0.03*	*1017.66*	*0.03*
861.06	*CHCA*	*0.1*	*1019.659*	*0.03*
877	*Polyethylene glycol*	*0.1*	*1033.683*	*0.03*
906.505	*Trypsin*	*0.03*	*1051.684*	*0.03*
1020.503	*Trypsin*	*0.03*	*1131.684*	*0.03*
1153.574	*Trypsin*	*0.03*	*1133.688*	*0.03*
1175.523	*Trypsin*	*0.03*	*1151.681*	*0.03*
1296.68	*Angiotensin 1*	*0.03*	*1165.704*	*0.03*
1433.721	*Trypsin*	*0/03*	*1265.716*	*0.03*
1493.599	*Trypsin*	*0/03*	*1279.725*	*0/03*
1676.777	*Trypsin*	*0/03*	*1300.83*	*0/03*
1774.851	*Trypsin*	*0/03*	*1302.83*	*0/03*
2093.08	*ACTH (clip 1-17)*	*0/03*	*1302.83*	*0/03*
2163.057	*Trypsin*	*0.03*	*1416.853*	*0.03*
2193.003	*Trypsin*	*0.03*	*1434.855*	*0.03*
2193.995	*Trypsin*	*0.03*	*1448.866*	*0.03*
2273.16	*Trypsin*	*0.03*	*1548.884*	*0.03*
2289.155	*Trypsin*	*0.03*	*1562.901*	*0.03*
2305.15	*Trypsin*	*0.03*	*1618.007*	*0.03*
2465.19	*ACTH (clip 18-39)*	*0.03*	*1718.019*	*0.03*
2514.339	*Trypsin*	*0.03*	*1732.034*	*0.03*
2550.233	*Trypsin*	*0.03*	*1901.177*	*0.03*
2612.181	*Trypsin*	*0.03*	*2015.207*	*0.03*
2613.35	*Trypsin*	*0.03*	*2162.99*	*0.03*
3211.475	*Trypsin*	*0.03*	*2289.084*	*0.03*

4.2.2 MASCOT

Peak lists were transferred to ProteinPilot TM 3.0 as DAT files using the Peaks to Mascot functionality of TOF/TOF Series Explorer. The Spot-Based MS/MS functionality of ProteinPilot TM was used for carrying out the Mascot search, which used the following search parameters: trypsin as the enzyme, with a maximum of one missed cleavage; carbamidomethylation of cysteine was as a fixed modification and oxidation of methionine as a variable modification; MALDI-TOF/TOF as the instrument of choice, along with monoisotopic mass values with a +1 charge and mass tolerance values were set at ± 50 ppm for peptide masses and ± 0.05 Da for fragment ions. A search was carried out against Swissprot protein database. The Swissprot database is made up of 537505 protein sequences, while the Swissprot database contains 190795142 residues. Both were downloaded in FASTA format from Swissprot (http://www.expasy.org).

4.2.3 Peak studio

ABI 4700 Data Extractor (Bioinformatics Solutions, Waterloo, Canada) was used to obtain MS/MS peak lists in the form of PKL files from TOF/TOF Series Explorer. PEAKS studio 5.3 (Bioinformatics Solutions) refined these peak lists using the following parameters: correct precursor mass; +1 charge state; recommended quality filter of 0.65; peak centroiding, charge deconvolution and deisotoping. PEAKS de novo sequencing was performed selecting carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. The mass tolerance was set at ± 50 ppm for peptide masses and ± 0.8 Da for fragment ions, while trypsin was also specified. An average local confidence value of 30 or greater was applied to filter de novo sequences. De novo sequences with an average local confidence value of 50 or greater were searched against

both a Swissprot database using PEAKS DB. The search was carried out for each individual protein spot by specifying trypsin as the enzyme used with a maximum of one missed cleavage. Carbamidomethylation of cysteine was selected as a fixed modification and oxidation of methionine as a variable modification. Monoisotopic mass values were also selected along with a peptide mass tolerance of ± 50 ppm and fragment ion tolerance of ± 0.8 Da.

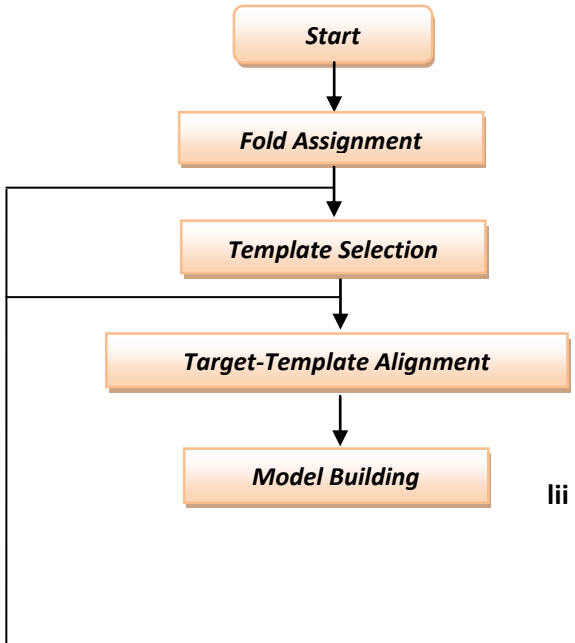
4.2.4 Signal peptide prediction

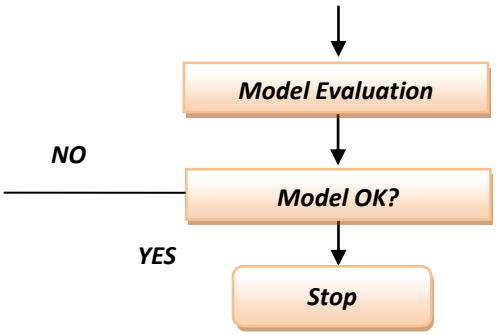
The peptide sequence was submitted to the SignalP 4.0 server to predict the presence and location of signal peptide cleavage sites from different organisms like Gram-positive Bacteria, Gram-negative Bacteria, and eukaryotes (Petersen et al.*, 2011). The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks. (Hiller K*, et al., *2000*), *to identify the signal peptide sequences the peptide sequence was submitted to the PrediSi (Prediction of Signal peptides). It is a tool for predicting signal peptide sequences and their cleavage positions in bacterial and eukaryotic amino acid sequences. PrediSi allows the evaluation of whole proteome datasets, which are currently accumulating as a result of numerous genome projects and proteomics experiments. The method employed is based on a position weight matrix approach improved by a frequency correction which takes in to consideration the amino acid bias present in proteins.*

4.2.5 Comparative Modeling

MODELLER is used for comparative modeling of protein three-dimensional structures. To predict protein structure by comparative modeling, it needs target sequence and known template structure. The template was identified using PDBsum database. To identify the query coverage the target sequence with the template structure are aligned (Marti-Renom et al.*, 2000) the resulted structures produced five different models and the structures are evaluated using PROCHECK analysis with the help of Ramachandran plot. The flow chart represents the steps which followed in comparative modeling shown in Fig.4.1.*

Fig 4.1: Steps in comparative modeling





4.2.6 Structure and Functional Study

ProFunc - prediction of protein function from 3D structure .The ProFunc server is used to identify the biochemical function of a protein from its three-dimensional structure. It uses a series of methods, including fold matching, residue conservation, surface cleft analysis, and functional 3D templates, to identify both the protein's likely active site and possible homologues in the PDB. In this page the Modeled 3D structure was submitted and views the resulted functional sites like N and G terminal, α-helix, β-sheet, disulphide, Beta hairpin, Turns, strands, Binding sites and Binding surfaces.

4.3 RESULTS

Mass Spectrum data file

BEGIN IONS

TITLE=Antibacterial.dta

CHARGE=3+

PEPMASS=538.264850666667

296.9246 1666.0

297.6315 1930.0

298.4435 61272.0

299.8151 58944.0

300.7426 46628.0

314.0497 3681.0

664.3140 1753.0

684.9474 7434.0

733.9299 995.0

775.1658 16399.0

831.1580 4442.0

847.6647 1940.0

863.0175 868.0

END IONS

The above illustration represents the Mass of the spectrum. This is the input file of MASCOT analysis. The format should be of data file.

Peptide Sequence Information from MALDI TOF Data

The peptide masses from thesequence are:

Theoretical pI : 7.82 / Mw
average mass : 7057.19 / Mw
monoisotopic mass : 7052.65/Mw

[Theoretical pI: 7.82 / Mw (average mass): 7057.19 / Mw (monoisotopic mass): 7052.65]

The peptide masses from the sequence

The peptide mass values of the sequence are showed in Table 4.2. Based upon the position, the mass value and its peptides identified. The high mass is in the position of 17-32, the peptide is VLLIASVLLSGNILAR and the resultant mass is 1652.0468.

Table 4.2: The peptide masses from the sequence

Mass	***Position***	***#MC***	***Peptide sequence***
1652.0468	*17-32*	*0*	*VLLIASVLLSGNILAR*
1001.4298	*10-16*	*0*	*EYFHCFR*
868.4015	*1-8*	*0*	*MSASTCLR*
848.4261	*37-43*	*0*	*SDQPFVR*
819.4206	*49-56*	*0*	*TETIGGNK*
685.4130	*57-62*	*0*	*LTPIIE*
620.2886	*44-48*	*0*	*ETDQK*
535.2544	*33-36*	*0*	*QMEK*

Peptide Sequence

MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVR

ETDQKTETIGGNKLTPIIE

M.W.: 7057.29

Query Parameters

Table 4.3: Search parameters of MASCOT

The parameters used in the MASCOT search are showed in Tale 4.3. A good mass range for trypsin is 1000 to 3500 Da.

Type of search	***Peptide Mass Fingerprint***
Enzyme	*Trypsin*

Fixed modifications	*Carbamyl (K)*
Variable modifications	*Carbamidomethyl (C)*
Mass values	*Monoisotopic*
Protein Mass	*35 kDa*
Peptide Mass Tolerance	*± 1.2 Da*
Peptide Charge State	*1+*
Max Missed Cleavages	*1*
Number of queries	*13*

MASCOT Scores

A histogram shows that the Mascot score distribution for the top 50 best matching proteins. Scores in the green shaded area represented random matches, the high score reported as 25. It compares the resultant mass fingerprint with theoretical protein/peptide libraries. Top Score of the search is 28 for YO35_BPHP1 with uncharacterized 58.7 kDa protein in lys 3'region of *Haemophilus phage*.

Fig 4.2 : MASCOT Analysis

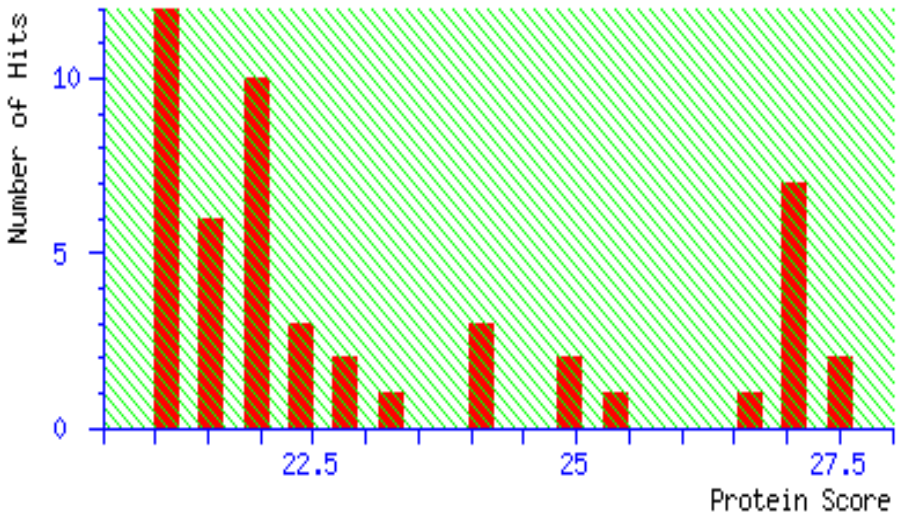


Table 4.4 Summary results for proteins producing a significant score from a Mascot search

AC No	*Sore*	*Expect*	*Calculated pI*	*Nominal mass (M_r)*	*Mass values matched*	*Protein sequence coverage %*	*Taxonomy*
YO35_BPHP1	*28*	*9.6e+02*	*5.45*	*60319*	*5*	*5*	*Haemophilus phage HP1*
PRL_BALBO	*25*	*1.7e+03*	*5.84*	*23185*	*4*	*11*	*Balaenoptera borealis*
TRL21_ARATH	*24*	*2.2e+03*	*24939*	*8.72*	*4*	*10*	*Arabidopsis thaliana*
PRL_CAMDR	*24*	*2.3e+03*	*5.98*	*23344*	*4*	*11*	*Camelus*

							dromedarius
PRL_MUSVI	*23*	*2.6e+03*	*5.98*	*26564*	*4*	*9*	*Neovison vison*
COLB_HORSE	*24*	*2.3e+03*	*5.98*	*23344*	*4*	*11*	*Camelus dromedarius*
PRL_AILME	*22*	*3e+03*	*6.14*	*26649*	*4*	*9*	*Ailuropoda melanoleuca*
PRL_FELCA	*22*	*3e+03*	*6.25*	*26696*	*4*	*9*	*Felis catus*
PRL_PIG	*22*	*3e+03*	*6.50*	*26597*	*4*	*9*	*Sus scrofa*
GPX3_YEAST	*22*	*3.2e+03*	*8.30*	*19533*	*4*	*14*	*Saccharomyces cerevisiae S288c*

Table 4.5 Matched Peptides

Sl No	***Observed***	***Mr(expt)***	***Mr(calc)***	***Delta***	***Start –End***	***Miss***	***Peptide***
1	*664.3140*	*663.3067*	*662.2871*	*1.0196*	*381 – 385*	*0*	*R.ETDQK.I*
2	*684.9474*	*683.9401*	*684.4058*	*-0.4657*	*528 – 533*	*0*	*K.LTPIIE.-*
3	*733.9299*	*732.9226*	*733.3541*	*-0.4315*	*241 – 245*	*1*	*R.RQMEK.T*
4	*847.6647*	*846.6574*	*847.4188*	*-0.7614*	*337 – 343*	*0*	*R.SDQPFVR.T*
5	*863.0175*	*862.0102*	*861.4192*	*0.5910*	*416 – 423*	*0*	*K.TETIGGNK.T*

No match to: 296.9246, 297.6315, 298.4435, 299.8151, 300.7426, 314.0497, 775.1658, 831.1580

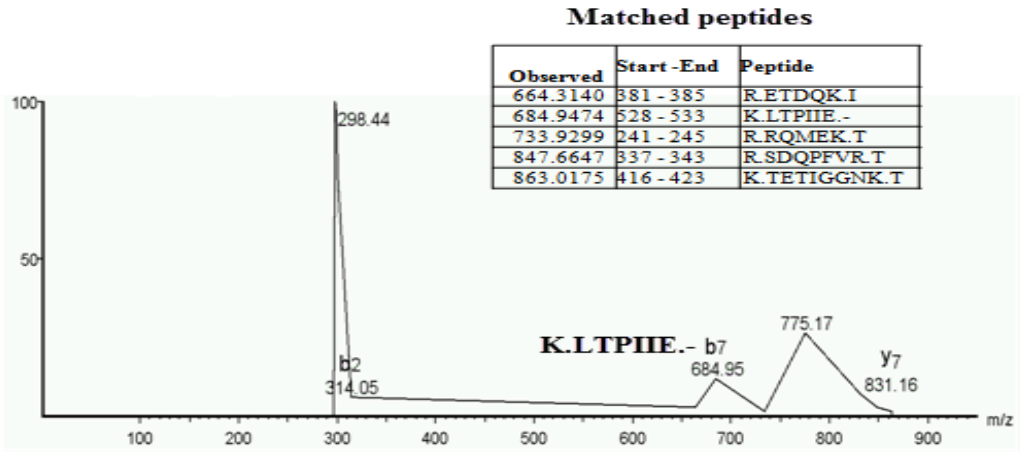
Table 4.6. Matched peptides shown in bold red.

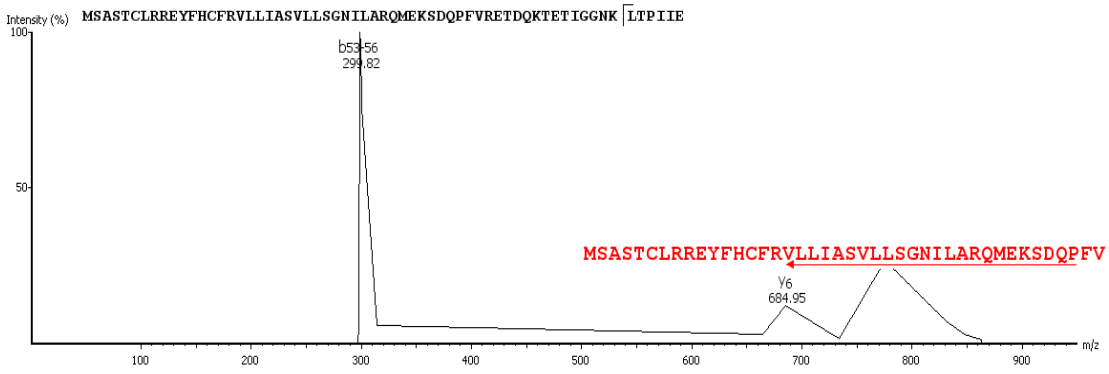
1	MKIIKTCLID	GEELELADEM	IILELNNTGR	GFVTVRTEKD	CIGKSAVFEM
51	GEYDHYYKWF	DGIVEREQSA	ENGYKKLFIR	EKVAVFEKPL	NCSHRHITLR
101	DLCAWITSQT	KIPVKVPQAD	YADTPISLFT	HNGSGYQLLA	NIGRQYQIAD
151	YMWQQSPDGS	LFVGSHKDSR	WAGKNIEFDE	SMTLTSGSND	MTIPITAAIR
201	PGAIINGNKI	QKVELSGDDY	VLSRENLGKD	GKPEQKSPER	**RQMEK**TFPEL
251	AGGYHLPKYA	KVVGIADPSS	GGDISDPFRP	KYAVELQLLD	ENGNEDKTVP
301	VYPAAPLPVT	STGSQGGDFA	FPEVGTMVEV	GFAYGR**SDQP**	**FVR**TMLAQGK
351	TVPSVAPGEQ	LKQQRPEVFE	RTDAAGNKIR	**ETDQK**ITDKS	FERHIETDSE
401	VKQIGTSNVA	IDSDK**TETIG**	**GNK**TVSVLGS	INDMTASNRT	VGTGGTLQEK
451	IVGLAQRVSD	EKNKFVAPLS	YMGTEAQNIF	RLLEDTIQLL	GEVASTLATH
501	THRGSPPPDQ	ASTFNQQANK	AKTIKGK**LTP**	**IIE**	

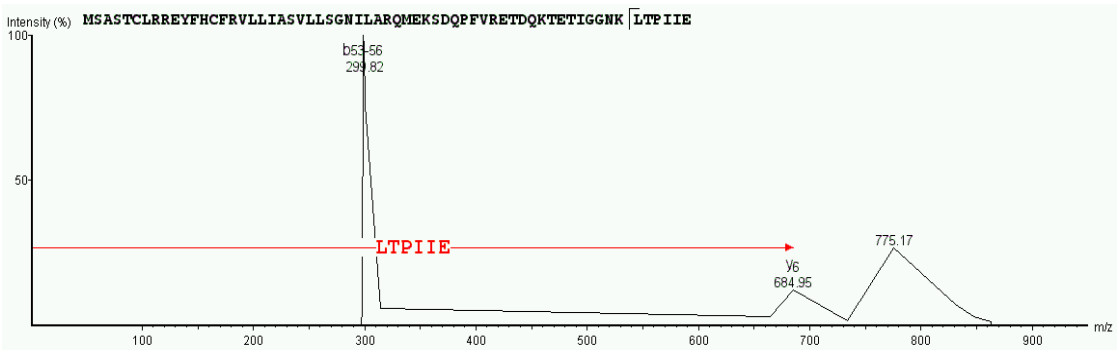
MS spectrum for selected proteins

This is an MS/MS spectrum of the tryptic peptide MSASTCLRREYFH CFRVLLIASVLLSGNILARQMEKSDQPFVRETDQKTETIGGNKLTPIIE. This data was collected on an ion trap mass spectrometer.

Fig 4.3 MS spectrum for peptide with Table value





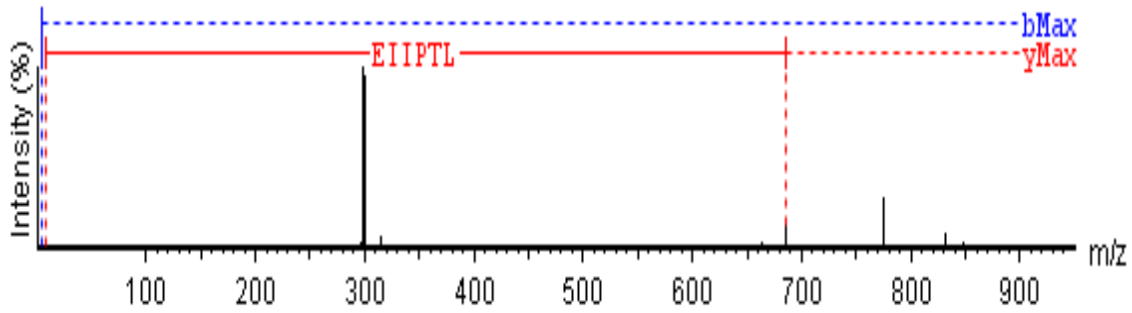

Intensity (%)
MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVRETDQKTETIGGNK|LTPIIE
100
50
b53-56
299.82
LTPIIE
y6
684.95
775.17
100
200
300
400
500
600
700
800
900
m/z

85

Annotated MS/MS spectrum

Annotated MS/MS spectrum accompanied with a spectrum alignment of the peptide MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVRETDQKTETIGGNKLTPIIE for protein spot. Also included is a sequence alignment between the *de novo* and database sequence and peptide identification results obtained from a PEAKS DB search.

Fig 4.4 Annotated MS/MS spectrum



Annotated Mass Value

The b ions appear to extend from the amino terminus, sometimes called the N-terminus, and y ions appear to extend from the carboxyl terminus, or C-terminus. While readily observed and diagnostic for b ions, a ions occur at a lower frequency and abundance in relation to b ions. The a ions are often used as a diagnostic for b ions, such that a-b pairs are often observed in fragment spectra.

Table 4.7 Annotated Mass Value

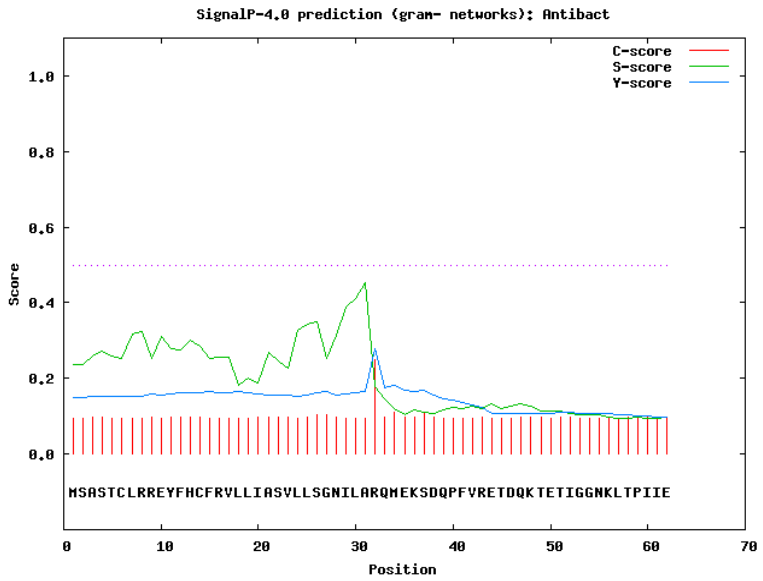
#	B	Sequence	Y	#
1	132.05	M	6922.62	62
2	219.08	S	6835.58	61
3	290.12	A	6764.55	60
4	377.15	S	6677.52	59
5	478.20	T	6576.47	58
6	581.21	C	6473.46	57
7	694.29	L	6360.38	56
8	850.39	R	6204.27	55
9	1006.49	R	6048.17	54
10	1135.54	E	6204.27	53
11	1298.60	Y	6048.19	52
12	1145.67	F	5919.13	51
13	1582.73	H	5756.07	50
14	1685.74	C	5609.00	49
15	1832.80	F	5471.94	48
16	1988.90	R	5368.93	47
17	2087.97	V	5065.76	46
18	2201.06	L	4966.69	45
19	2314.14	L	4853.61	44
20	2427.23	I	4740.52	43
21	2498.26	A	4627.44	42
22	2585.29	S	4556.40	41
23	2684.36	V	4469.37	40
24	2797.45	L	4770.30	39
25	2910.53	L	4257.22	38
26	2997.56	S	4144.13	37
27	3054.58	G	4057.10	36
28	3168.63	N	4000.08	35
29	3281.71	I	3886.04	34
30	3394.80	L	3772.95	33
31	3465.83	R	3659.87	32
32	3621.93	Q	3588.83	31
33	3749.99	Q	3432.73	30
34	3881.03	M	3304.64	29
35	4010.08	E	3173.63	28
36	4138.17	K	3044.59	27
37	4225.20	S	2916.49	26
38	4340.23	D	2829.46	25
39	4468.29	Q	2714.44	24
40	4565.34	P	2586.38	23
41	4712.41	F	2489.32	22

42	*4811.48*	*V*	*2342.26*	*21*
43	*4967.58*	*R*	*2243.19*	*20*
44	*5096.62*	*E*	*2087.09*	*19*
45	*5197.67*	*T*	*1958.04*	*18*
46	*5312.70*	*D*	*1857.00*	*17*
47	*5440.75*	*Q*	*1741.97*	*16*
48	*5568.85*	*K*	*1613.91*	*15*
49	*5669.90*	*T*	*1485.82*	*14*
50	*5798.94*	*E*	*1384.82*	*13*
51	*5899.99*	*T*	*1255.73*	*12*
52	*6013.07*	*T*	*1154.68*	*11*
53	*6070.09*	*G*	*1041.59*	*10*
54	*6127.11*	*G*	*984.57*	*09*
55	*6241.16*	*N*	*927.55*	*08*
56	*6369.25*	*K*	*813.51*	*07*
57	*6482.34*	*L*	*684.95*	*06*
58	*6583.38*	*T*	*572.33*	*05*
59	*6680.44*	*P*	*471.28*	*04*
60	*6793.52*	*I*	*374.23*	*03*
61	*6906.60*	*I*	*261.14*	*02*
62		*E*	*148.06*	*01*

Signal Peptide Prediction

The graphical output of Signal IP result of Gram-negative organism is showed in Fig.4.5 comprises three different scores, C, S *and* Y. *The cleavage site score of the peptide is 0.249 with the position of 32. The* S-score *is the predicted signal peptide value, the position is 31 with 0.454 and 'Y-score' is a combination of C- and S-scores as described in Methods, the score value is 0.280 with the location of 32. The predicted cleavage site is between position 31 and 32. In a summary line below the plot we show the calculated D-score, the associated cutoff value and which networks were used to make the prediction, in this case: "D=0.281 Dcutoff=0.510, Networks=SignalP-noTM"*

Fig 4.5 : Signal IP result of Gram-negative organism



Output

# Measure		Position	Value
max.	C	32	0.249
max.	Y	32	0.280
max.	S	31	0.454
mean	S	1-31	0.283
D	1-31	0.281	0.510 NO

Name=Antibact SP='NO' D=0.281

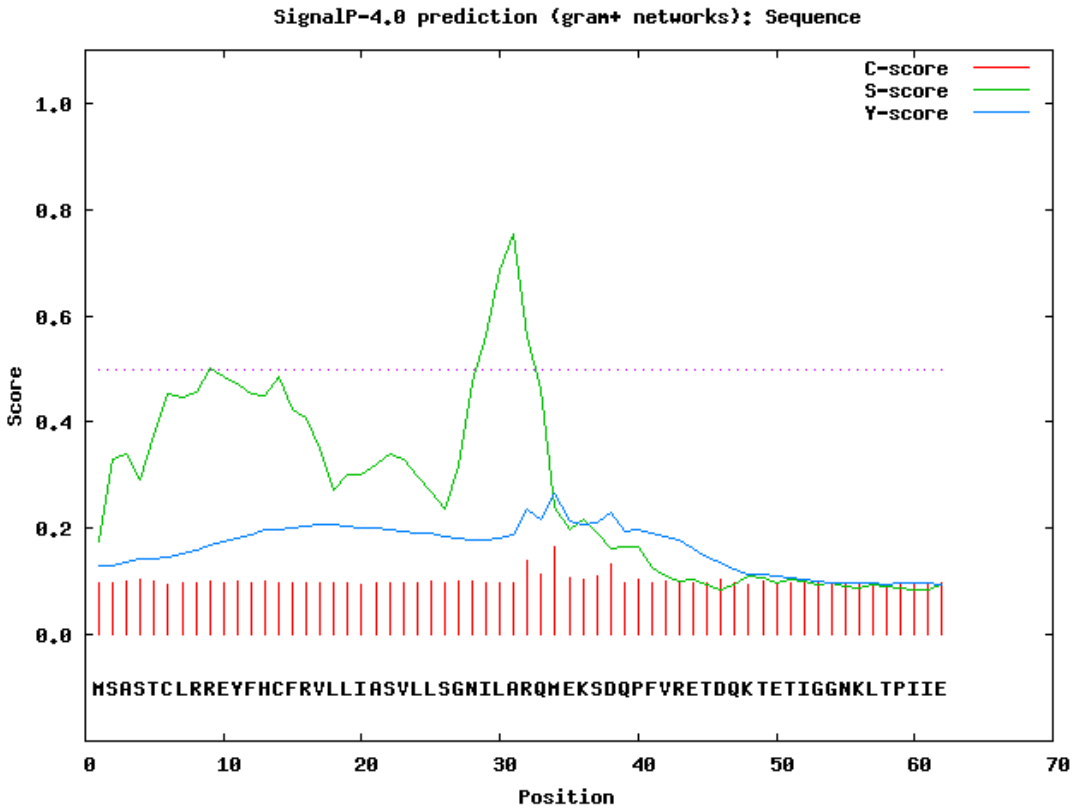
D-cutoff=0.510 Networks=SignalP-TM

Signal IP result of Gram-positive organism

The graphical output of Signal IP result of Gram-positive organism is showed in Fig 4.6. The cleavage site score of the peptide is 0.165 with the position of 34. The S-score *is the predicted signal peptide value, the position is 31 with 0.753 and 'Y-score' is a combination*

of C- and S-scores as described in Methods, the score value is 0.267 with the location of 34. The predicted cleavage site is between position 31 and 34. The calculated D-score,the associated cutoff value and which networks were used to make the prediction, in this case: "D=0.321 D cutoff=0.450, Networks=SignalP-noTM".

Fig 4.6: Signal IP result of Gram-positive organism



# Measure	Position	Value
max. C	34	0.165
max. Y	34	0.267
max. S	31	0.753
mean S	1-33	0.406

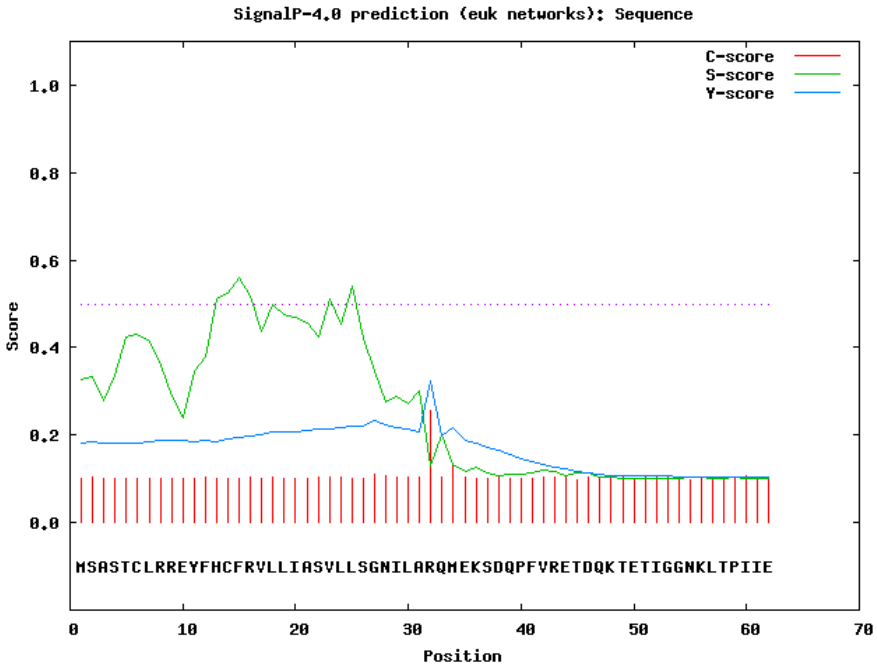
D 1-33 0.321 0.450 NO

Name=Sequence SP='NO' D=0.321 D-cutoff=0.450 Networks=SignalP-TM

Signal IP result of Eukaryotic organism

The graphical output of Signal IP result of eukaryotic organism is showed in Fig 4.7. The cleavage site score of the peptide is 0.255 with the position of 32. The S-score *is the predicted signal peptide value, the position is 15 with 0.561 and 'Y-score' is a combination of C- and S-scores as described in Methods, the score value is 0.323 with the location of 32. The predicted cleavage site is between position 15 and 32. The calculated D-score, the associated cutoff value and which networks were used to make the prediction, in this case: "D=0.354 D cutoff=0.500, Networks=SignalP-noTM"*

Fig 4.7 : Signal IP result of Eukaryotic organism



Output

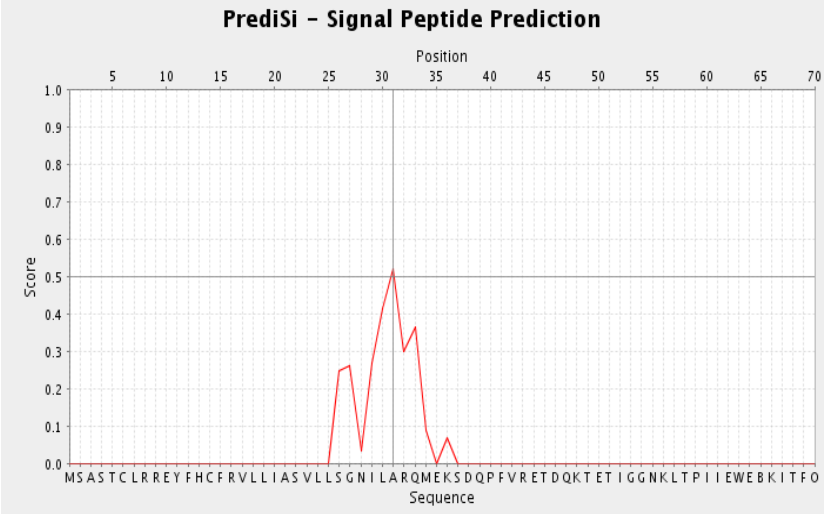
# Measure	Position	Value		
max. C	32	0.255		
max. Y	32	0.323		
max. S	15	0.561		
mean S	1-31	0.401		
D	1-31	0.354	0.500	NO

Name=Sequence SP='NO' D=0.354 D-cutoff=0.500 Networks=SignalP-TM

Signal IP result of Eukaryotic organism

The result of the signal peptide sequence is plotted in the Fig 4.8. The ptedicted sequence regions areindicated as red colour. The cleavage position is 31 and its cleavage sites are LSGNILARQMEKS with the score value of 0.5192.

Fig 4.8 : Signal IP result of Eukaryotic organism



Comparative Modeling – Structural study

Sequence of the target peptide

Matrix **:** *Eukarya*

Truncation **:** *62 residues*

Cleavage position : *31*

Score **:** *0.5192*

Secreted protein **:** ***predicted for secretion***

Peptide Sequence

MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVR

ETDQKTETIGGNKLTPIIE

FASTA Fomat Sequence

>Sponge new

MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVR
ETDQKTETIGGNKLTPIIE

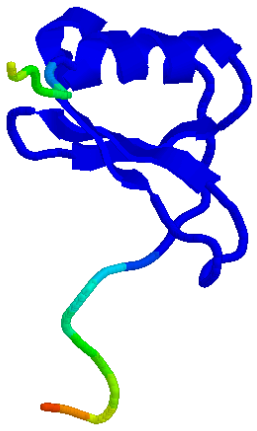
UniProt
PDB seq:
MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVRETDQKTETIGGNKLT
PIIE

---------+---------+---------+---------+---------+---------+--

10 20 30 40 50 60

Template Structure (1BO0 A Chain)

Fig 4.9 : Template structure (1BO0 A chain)



Input file

>P1;TARGET

sequence:TARGET:::::::0.00: 0.00

*MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVRETDQKTETIGGNKLTPIIE**

Alignment file

aln.pos 10 20 30 40 50 60

1BO0A QPVGINTSTTCCYRFINKKIPKQRLESYRRTTSS--
HCPREAVIFKTKLDKEICADPTQKWVQDFMKH

TARGET ----M-SASTC----------
LRREYFHCFRVLLIASVLLSGNILARQMEKSDQPFVRE

*_consrvd ** ** * * * ** * * **

_aln.p 70 80

1BO0A LDKKTQT---PKL-----

TARGET TDQKTETIGGNKLTPIIE
*_consrvd * ** * **

Script File for Alignment

```
from modeller import *
env = environ()
aln = alignment(env)
mdl = model(env, file='1BO0', model_segment=('FIRST:A','LAST:A'))
aln.append_model(mdl, align_codes='1BO0A', atom_files='1BO0.pdb')
aln.append(file=' TARGET.ali', align_codes=' TARGET ')
aln.align2d()
aln.write(file=' TARGET -1BO0A.ali', alignment_format='PIR')
aln.write(file=' TARGET -11BO0A.pap', alignment_format='PAP')
```

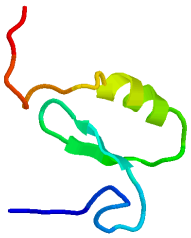
Script File for Model Building

```
from modeller import *
from modeller.automodel import *
```

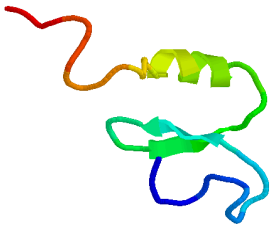
```
env = environ()
a = automodel(env, alnfile='TARGET-1BO0A.ali',
              knowns='1BO0A', sequence='TARGET',
              assess_methods=(assess.DOPE, assess.GA341))
a.starting_model = 1
a.ending_model = 5
a.make()
```

Fig 4.10 : Modeled structures using Modeller9V8

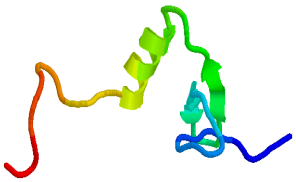
Modeled Structure1



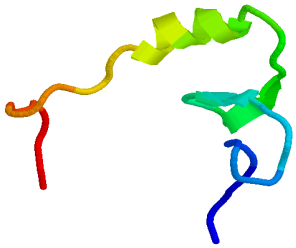
Modeled Structure2



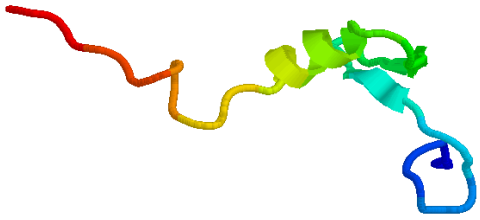
Modeled Structure3



Modeled Structure4

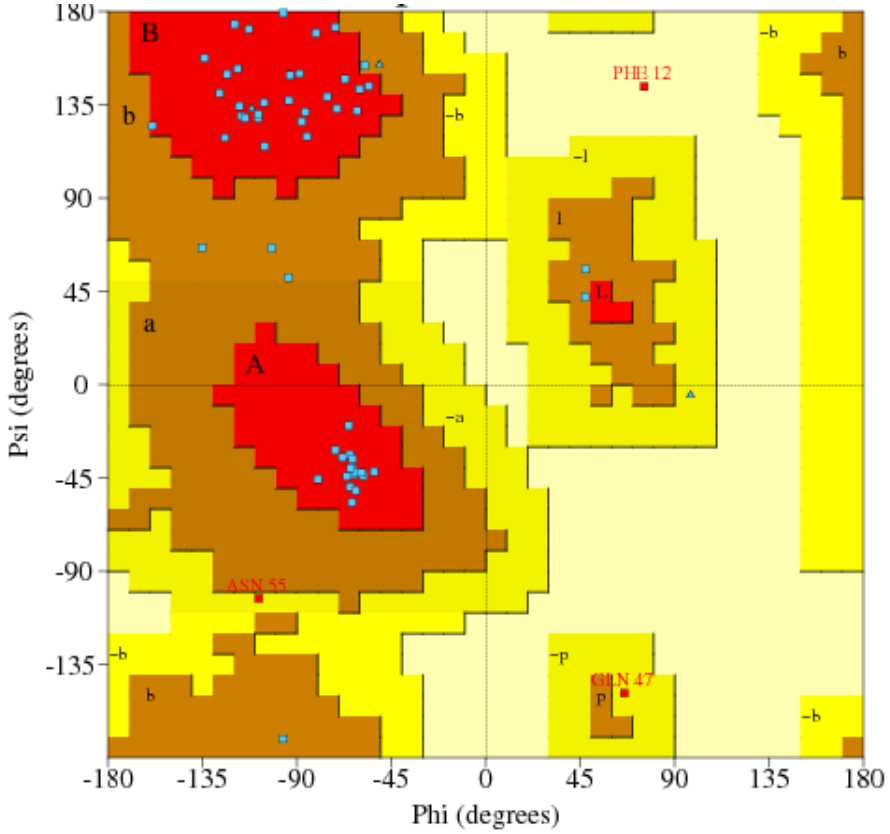


Modeled Structure5



Structure Evaluation

Fig 4.11 : Ramachandran plot of Modeled structure1



Ramachandran Plot statistics

	No. of residues	***%-tage***
	------	------
Most favoured regions [A,B,L]	*45*	*81.8%**
Additional allowed regions [a,b,l,p]	*7*	*12.7%*
Generously allowed regions [~a,~b,~l,~p]	*2*	*3.6%*
Disallowed regions [XX]	*1*	*1.8%**
	----	------
Non-glycine and non-proline residues	*55*	*100.0%*
End-residues (excl. Gly and Pro)	*2*	
Glycine residues	*3*	
Proline residues	*2*	

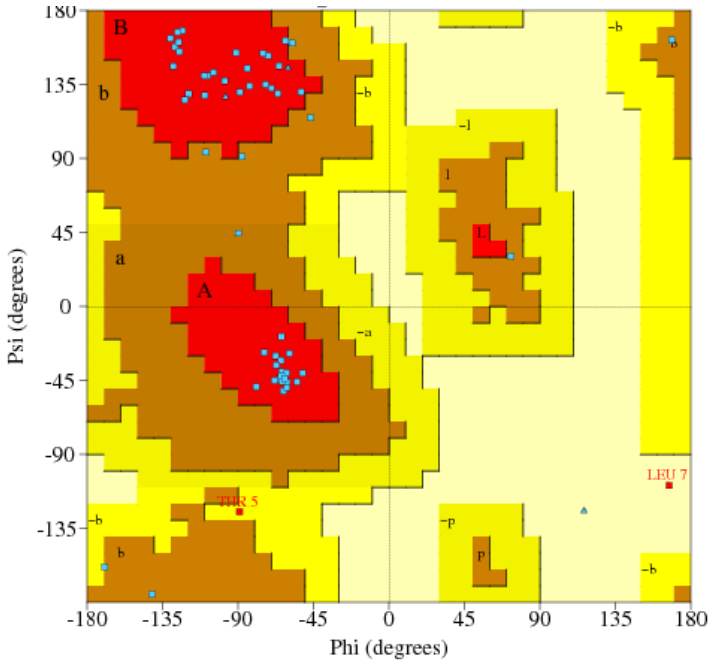
Total number of residues	*62*	

2. G-Factors

Parameter	***Average Score***

Dihedral angles:-	
Phi-psi distribution	*-0.49*
Chi1-chi2 distribution	*- 0.64**
Chi1 only	*0.26*
Chi3 & chi4	*0.21*
Omega	*-0.37*
	-0.34
	=====
Main-chain covalent forces:-	
Main-chain bond lengths	*-0.24*
Main-chain bond angles	*-0.39*
	-0.33
	=====
OVERALL AVERAGE	*-0.33*
	=====

Fig 4.12 : Ramachandran plot of Modeled structure2



PROCHECK statistics

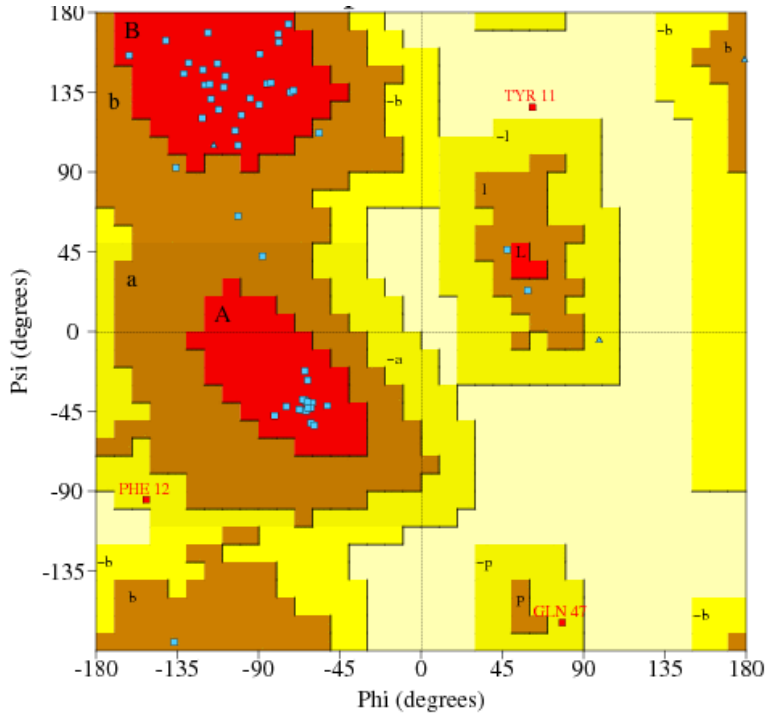
1. Ramachandran Plot statistics

	No. of residues	***%-tage***
Most favoured regions [A,B,L]	*44*	*80.0%***
Additional allowed regions [a,b,l,p]	*9*	*16.4%*
Generously allowed regions [~a,~b,~l,~p]	*1*	*1.8%*
Disallowed regions [XX]	*1*	*1.8%**
Non-glycine and non-proline residues	*55*	*100.0%*
End-residues (excl. Gly and Pro)	*2*	
Glycine residues	*3*	
Proline residues	*2*	
Total number of residues	*62*	

2. G-Factors

Parameter	***Average Score***
Dihedral angles:-	
Phi-psi distribution	*-0.43*
Chi1-chi2 distribution	*-0.11*
Chi1 only	*0.01*
Chi3 & chi4	*0.28*
Omega	*-0.01*
	-0.11
Main-chain covalent forces:-	
Main-chain bond lengths	*-0.23*
Main-chain bond angles	*-0.23*
	-0.23
OVERALL AVERAGE	*-0.16*

Fig 4.13 : Ramachandran plot of Modeled structure3



Ramachandran Plot statistics

1. Ramachandran Plot statistics

	No. of residues	***%-tage***
	------	------
Most favoured regions [A,B,L]	*45*	*81.8%**
Additional allowed regions [a,b,l,p]	*7*	*12.7%*
Generously allowed regions [~a,~b,~l,~p]	*2*	*3.6%*
Disallowed regions [XX]	*1*	*1.8%**
	----	------
Non-glycine and non-proline residues	*55*	*100.0%*
End-residues (excl. Gly and Pro)	*2*	
Glycine residues	*3*	
Proline residues	*2*	

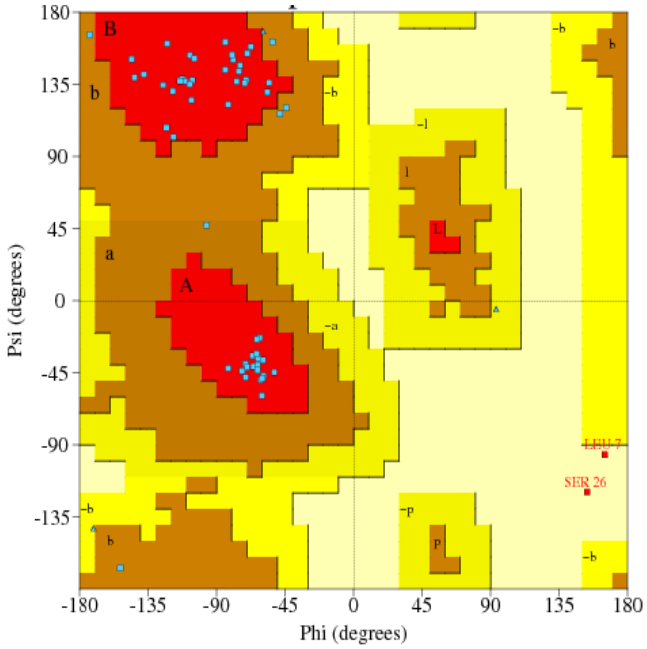
Total number of residues	*62*	

2. G-Factors

Parameter	Average Score

Dihedral angles:-	
Phi-psi distribution	*-0.34*
Chi1-chi2 distribution	*0.05*
Chi1 only	*0.28*
Chi3 & chi4	*0.60*
Omega	*-0.05*
	0.00
	=====
Main-chain covalent forces:-	
Main-chain bond lengths	*-0.26*
Main-chain bond angles	*-0.37*
	-0.33
	=====
OVERALL AVERAGE	*-0.12*
	=====

Fig 4.14 : Ramachandran plot of Modeled structure 4



1. Ramachandran Plot statistics

	No. of residues	%-tage
	------	------

Most favoured regions [A,B,L]	47	85.5%*****
Additional allowed regions [a,b,l,p]	6	10.9%
Generously allowed regions [~a,~b,~l,~p]	0	0.0%
Disallowed regions [XX]	2	3.6%*****
	----	------
Non-glycine and non-proline residues	55	100.0%
End-residues (excl. Gly and Pro)	2	
Glycine residues	3	
Proline residues	2	

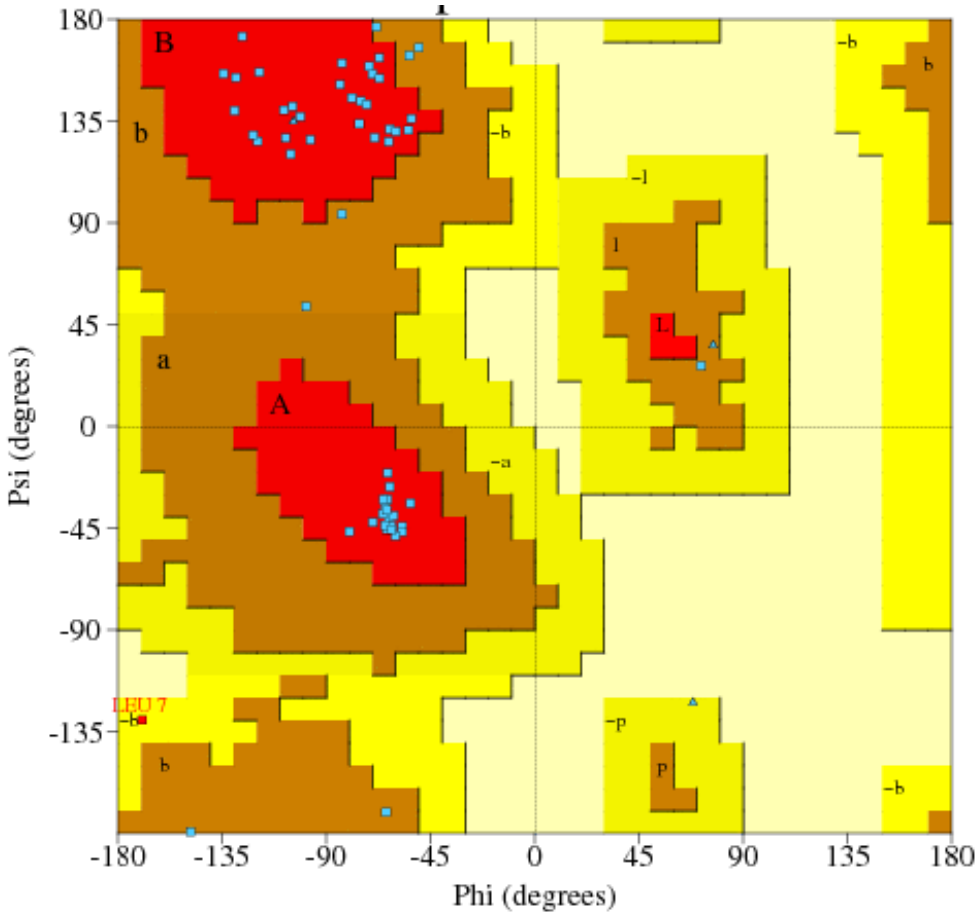
Total number of residues	62	

2. G-Factors

Parameter	**Average Score**

Dihedral angles:-	
Phi-psi distribution	-0.28
Chi1-chi2 distribution	-0.53*
Chi1 only	-0.13
Chi3 & chi4	0.20
Omega	-0.18
	-0.23
	=====
Main-chain covalent forces:-	
Main-chain bond lengths	-0.27
Main-chain bond angles	-0.34
	-0.31
	=====
OVERALL AVERAGE	-0.26
	=====

Fig 4.15 : Ramachandran plot of Modeled structure 5



Ramachandran Plot statistics

	No. of residues	***%-tage***
	------	------
Most favoured regions [A,B,L]	*46*	*83.6%**
Additional allowed regions [a,b,l,p]	*8*	*14.5%*
Generously allowed regions [~a,~b,~l,~p]	*1*	*1.8%*
Disallowed regions [XX]	*0*	*0.0%*
	----	------
Non-glycine and non-proline residues	*55*	*100.0%*
End-residues (excl. Gly and Pro)	*2*	
Glycine residues	*3*	
Proline residues	*2*	

Total number of residues	*62*	

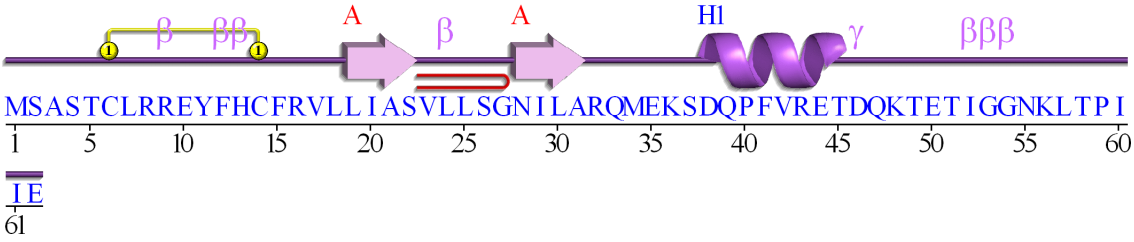
2. G-Factors

Average

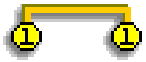
Parameter	*Score*
---	-----
Dihedral angles:-	
Phi-psi distribution	*-0.29*
Chi1-chi2 distribution	*0.00*
Chi1 only	*-0.24*
Chi3 & chi4	*0.37*
Omega	*0.02*
	-0.05
	=====
Main-chain covalent forces:-	
Main-chain bond lengths	*-0.28*
Main-chain bond angles	*-0.35*
	-0.32
	=====
OVERALL AVERAGE	*-0.15*
	=====

Structure and Functional study

Fig 4.16 Secondary structure of Modeled structure



Keys

Secondary structure	***Helix Strand***		*Helices labelled H1, H2, ... and strands by their sheets A, B, ...*
Motifs	*β beta turn Y gamma turn*		*Beta hairpin*
Disulphides			*Disulphide bond*

Topology structure of N and C terminal

Fig 4.17 : Topology structure of N and C terminul in the 3D structure

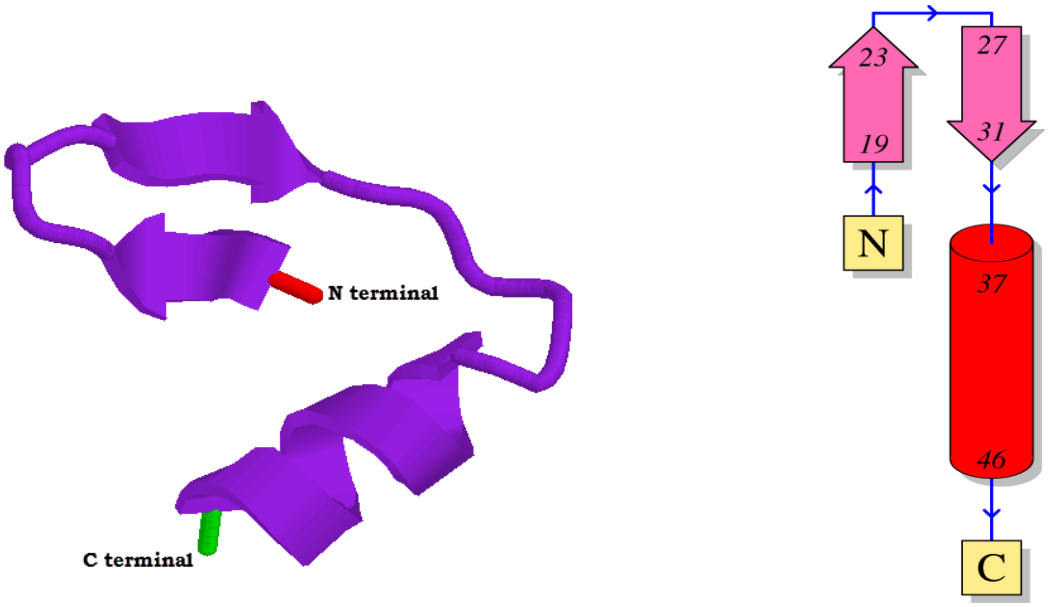


Table 4.8:Secondary structure elements

The summarised secondary structural elements showed in Table 4.8.

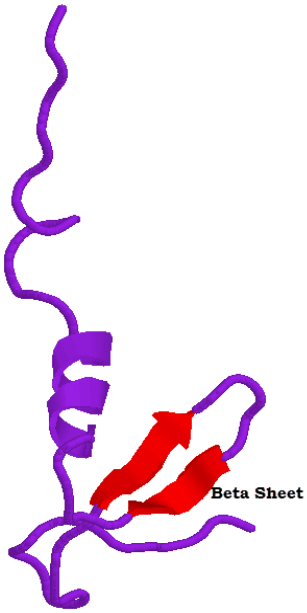
Strand	*Alpha Helix*	*3-10 helix*	*Other*	*Total Residues*
8(12.9%)	*8(12.9%)*	*0(0.0%)*	*46 (74.2%)*	*62*

Table 4.9: Beta sheet details

The resulted 3D structure has 2 strands of bets sheets and it shows red color in the Table 4.9.

Sheet	***No. strands***	***Type***	***Barrel***	***Topology***
A	*2*	*Antiparallel*	*N*	*1*

Fig 4.18 Beta Sheet regions in the Modeled 3D structure



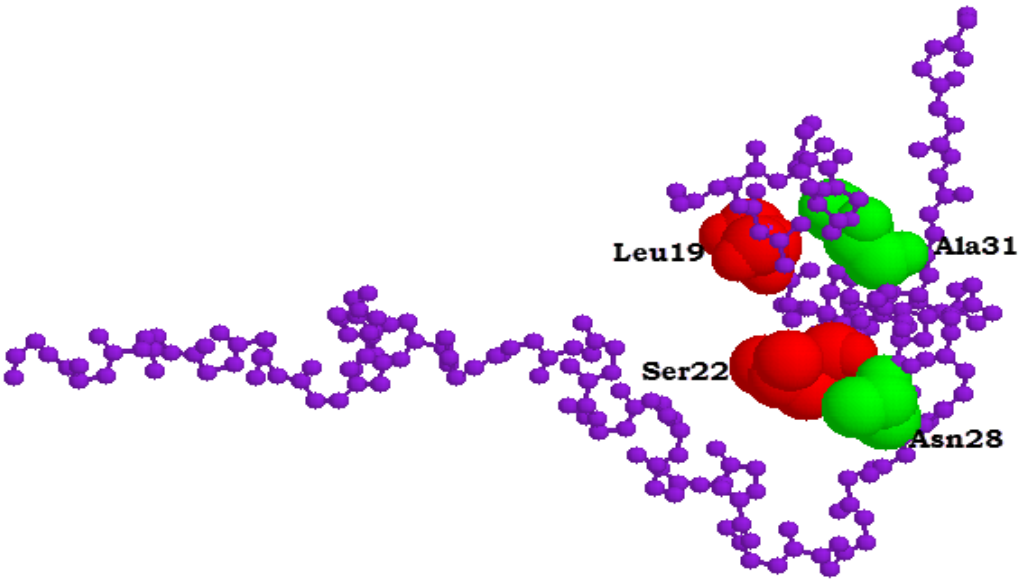
Beta Hairpin details

The Table 4.10 shows the hairpin regions of the protein. It is resulted as four beta hairpin regions like Leu19, Ser22, Asn 28 and Ala 31.These four regions are showed in Fig 4.19. It indicates as Leu and Ser in red colour and Asn and Ala in green colour.

Table 4.10 Beta Hairpin details

Strand 1			*Strand 2*			*Hairpin*
Start	*End*	*Length*	*Start*	*End*	*Length*	*Class*
Leu19	*Ser22*	*4*	*Asn28*	*Ala31*	*4*	*3:5*

Fig 4.19 Beta Hairpin regions in the Modeled 3D structure



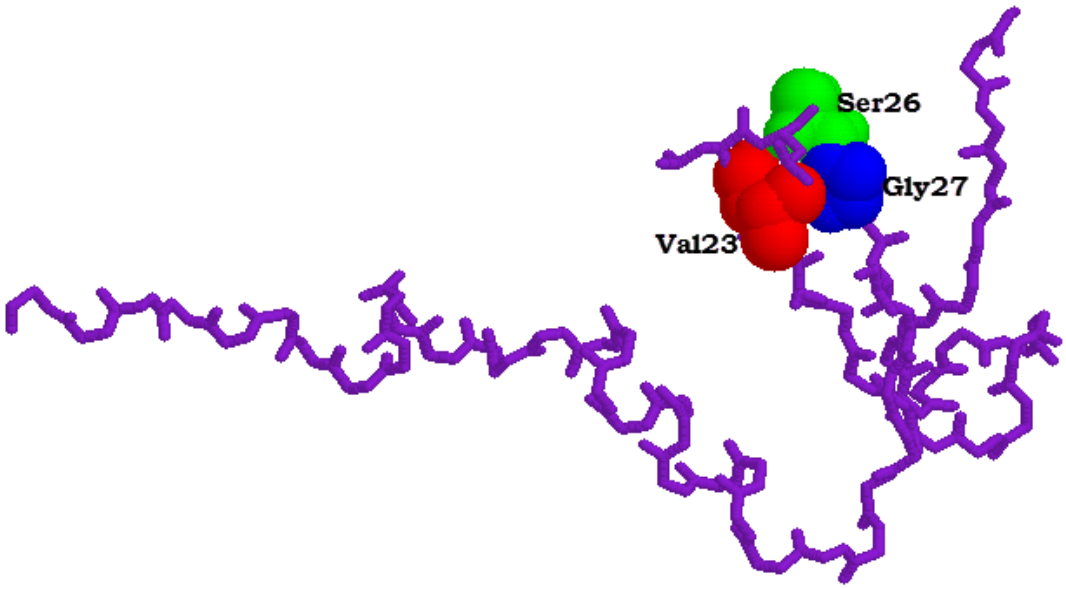
Beta bulge regions

The Table 4.11 noted the beta bulge regions and its type. The regions showed in Fig 4.20. It shows that Res X is Val23 and its colour indication is red, Res 1 is Ser 26 – the colour indication is green and the Res 3 is Gly 27- the colour indication is blue. The type of the bulge is Antiparallel.

Table 4.11 Beta bulge regions

Bulge type	***Res X***	***Res 1***	***Res 2***	***Res 3***	***Res 4***
Antiparallel G 1	*Val23*	*Ser26*	*Gly27*		

Fig 4.20 Beta bulges in the Modeled 3D structure



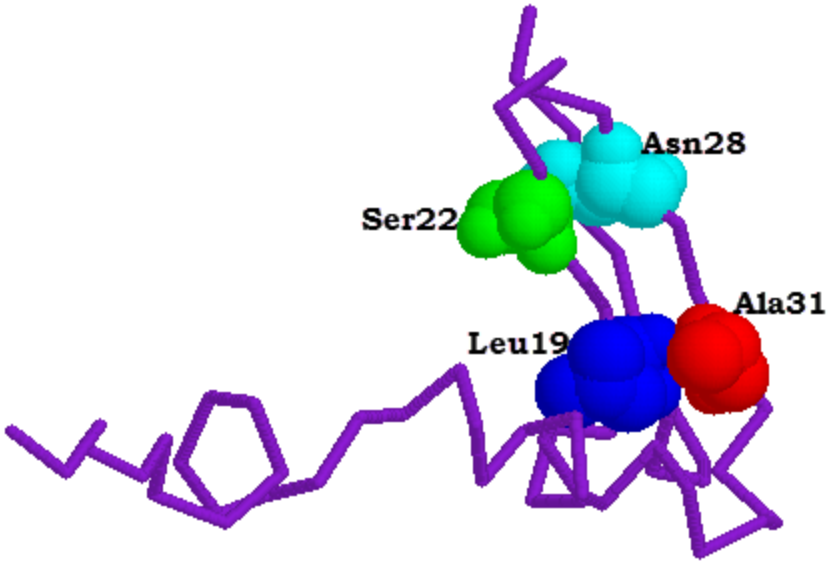
strands regions

The Table 4.12 shows the strand regions and its sheets. The regions indicated in the Fig 21. The starting regions of the strands are Leu19, Asn 28 and its colour indication is Leu-blue, Asn- cyan. The ending regions are Ser22, Ala31 ant itscolour indication Ser-green, Ala- red.

Table 4.12 strands regions

Start	***End***	***Sheet***	***No Residues***
Leu19	*Ser22*	*A*	*4*
Asn28	*Ala31*	*A*	*4*

Fig 4.21 Strand reg... ...he Modeled 3D structure



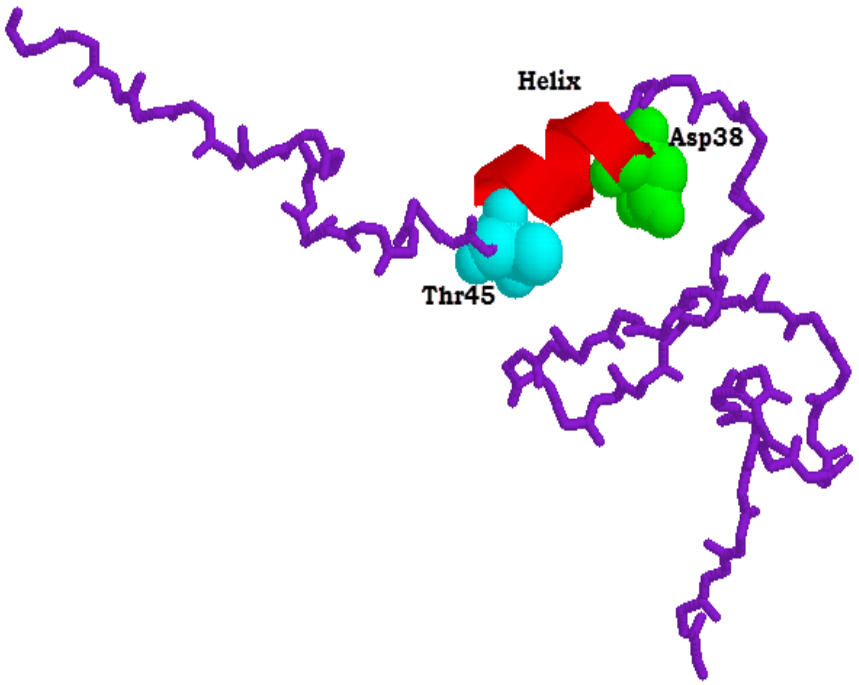
Helix Regions

The Table 4.13 shows the helix regions of the modeled strucyure. The type of the helix is H and the srarting and ending regions are Asp 38 and Thr 45.The Regions showed in the Fig 4.22.The helix is indicated as Red color and the starting and ending regions are showed as cyan and green colour.

Table 4.13 Helix Regions

Start	***End***	***Type***	***No Residues***
Asp38	*Thr45*	*H*	*8*

Fig 4.22 Helix Helix and its starting and endingregions of the Modeled 3D structure



Beta turns

The Table 4.14 resulted the Turn regions, sequences.Totally seven turns noted that is indicated as red colour in Fig 4.23. The turn 5 and turn 7 has H- bond also.

Table 4.14 Beta turns

Sl.No	***Turn***	***Seuence***	***Turn Type***	***H-bond***
1	*Arg8-Tyr11*	*RREY*	*IV*	***No***
2	*Tyr11-Cys14*	*YFHC*	*IV*	*No*
3	*Phe12-Phe15*	*FHCF*	*VIII*	*No*
4	*Val23-Ser26*	*VLLS*	*I*	*Yes*
5	*Thr51-Gly54*	*TIGG*	*II*	*No*
6	*Ile52-Asn55*	*IGGN*	*IV*	*No*
7	*Gly53-Lys56*	*GGNK*	*IV*	*Yes*

Fig 4.23 Turns in the Modeled 3D structure

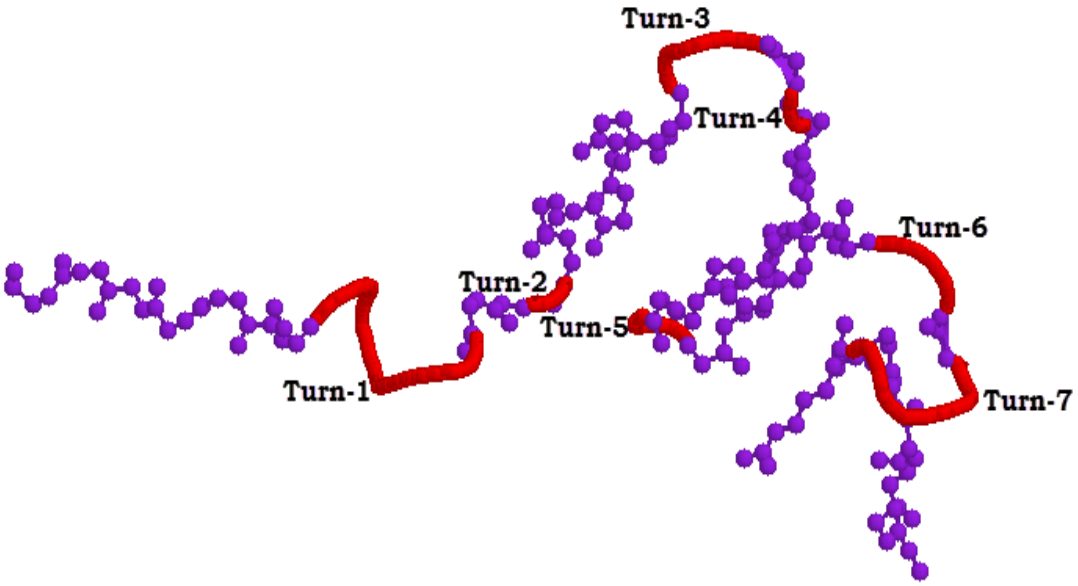
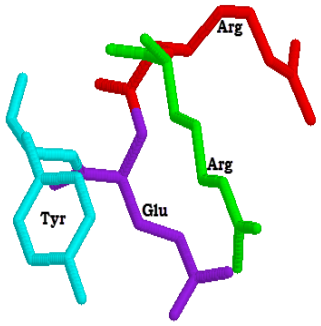
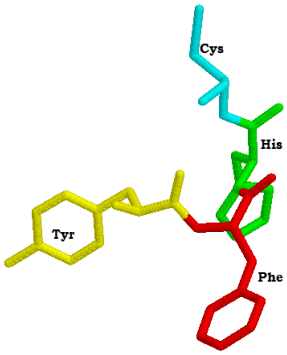


Fig 4.24 Specific Turn regions in the Modeled 3D structure

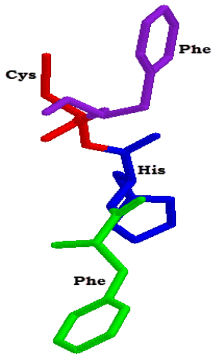
Turn-1



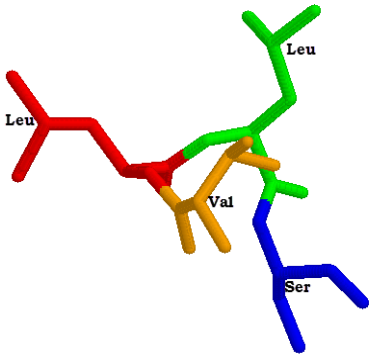
Turn-2



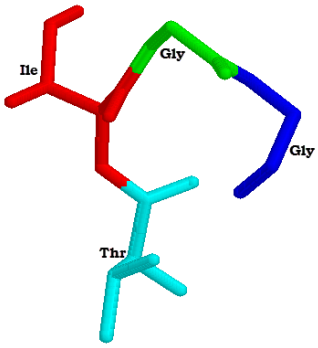
Turn-3



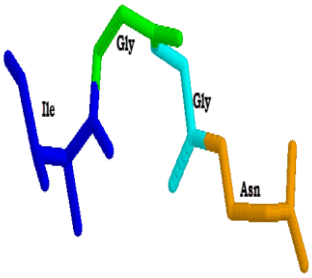
Turn-4



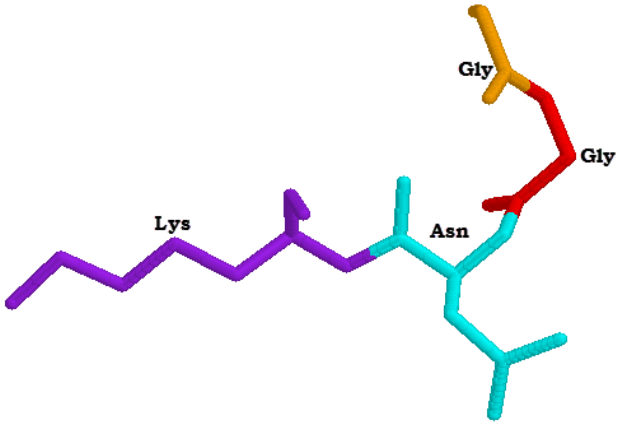
Turn-5



Turn-6



Turn-7



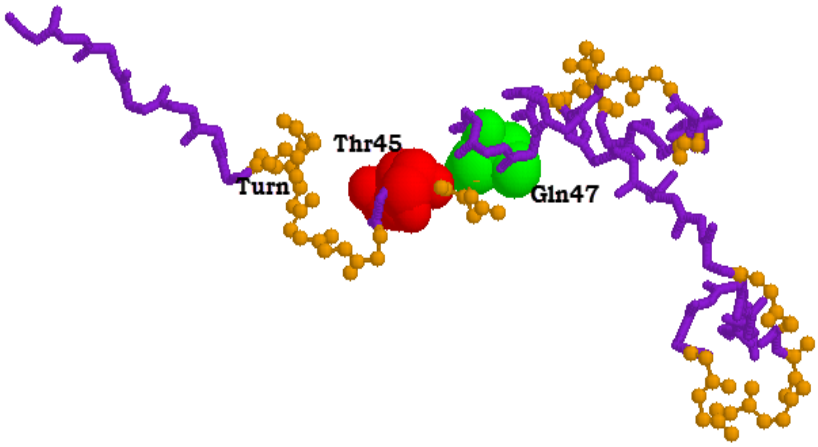
Gamma Turn regions

The Table 4.15 shows the gamma turn regions, sequence and type. The staring region is Thr45 and Ending region is Gln47. The colour indication is Thy45 as red and Gln47 as blue .The sequence regions are TDQ and the type of the gamma turn is inverse, which is indicated as orange colour in the Fig 4.24.

Table 4.15 Gamma Turn regions

Start	***End***	***Sequence***	***Turn Type***
Thr45	*Gln47*	*TDQ*	*INVERSE*

Fig 4.25 Gamma Turns in the Modeled 3D structure



Disulphide Regions

The Table 4.16 indicates the disulphide regions of the 3D structure. 1st

Cysteine and 2nd Cysteine are the disulphide regions. The regions showed in Fig 4.25.

1st Cysteine-red, 2nd Cysteine- blue.

Table 4.16 Disulphide Regions

1st Cysteine	***2nd Cysteine***
6	*14*

Fig 4.26. Disulphide regions in the Modeled 3D structure

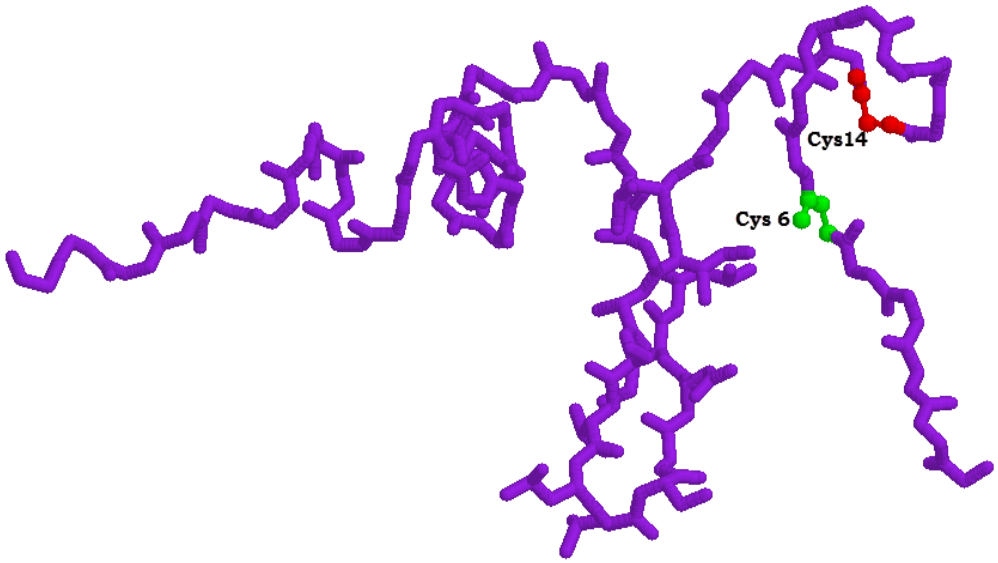


Fig 4.27 Clefts . .n the Modeled structure

Clefts

	Volume	R1 ratio	Accessible vertices		Buried vertices		Average depth		Residue type						Ligands
1	1799.72	1.26	49.35	10	7.57	3	9.55	3	3	3	8	9	0	1	0
2	1430.16	0.00	53.00	6	11.13	1	12.03	1	1	4	4	8	1	0	0
3	881.72	0.00	49.89	8	11.02	2	11.98	2	2	3	4	1	0	3	0
4	372.09	0.00	56.86	4	5.35	5	7.19	4	1	0	3	2	1	0	1
5	165.38	0.00	49.39	9	4.71	7	4.95	7	1	1	0	0	2	0	0
6	127.83	0.00	66.67	1	6.03	4	0.00	8	2	0	1	3	0	0	0
7	121.08	0.00	59.30	3	4.36	9	0.00	9	2	1	0	0	0	0	0
8	110.53	0.00	62.50	2	4.57	8	4.98	6	2	0	0	3	0	0	0
9	94.50	0.00	52.01	7	3.69	10	5.11	5	2	0	0	1	1	0	0
10	103.78	0.00	54.87	5	4.87	6	0.00	10	1	0	1	0	0	1	0

Protein structure

Residue-type colouring

Positive	Negative	Neutral	Aliphatic	Aromatic	Pro & Gly	Cysteine
H,K,R	D,E	S,T,N,Q	A,V,L,I,M	F,Y,W	P,G	C

Fig 4.28 Binding sites regions in the Modeled structure

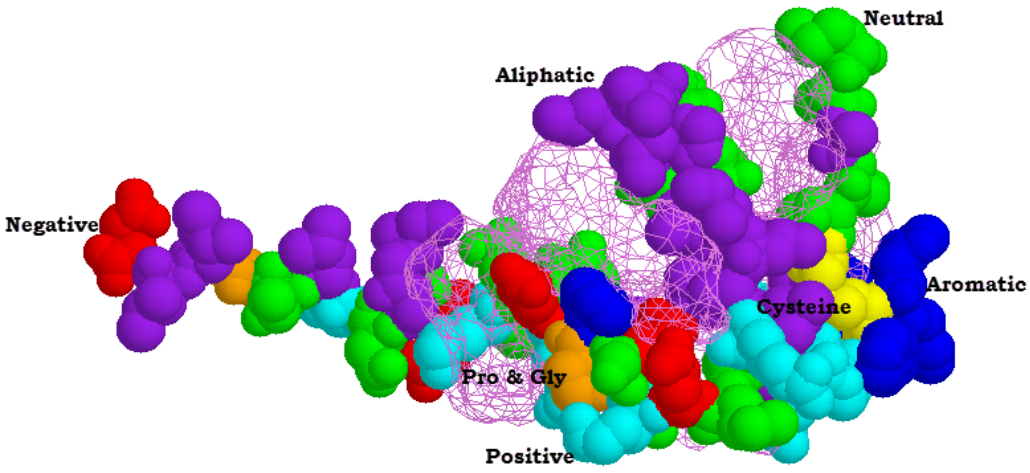
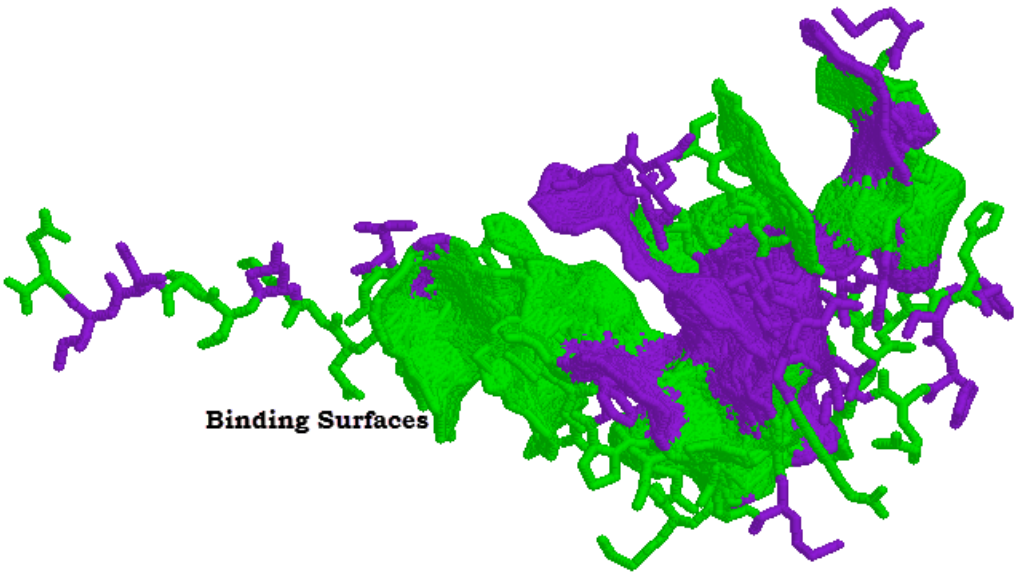


Fig 4.29 Binding surfaces in the Modeled structure



4.4 DISCUSSION

MALDI-TOF is a technique which is used for detection, identification, and characterization of micro-organisms. MALDI-TOF mass spectrometric fingerprinting characterization of toxic cyanobacteria *and the mass range from m/z=2.000 to 20.000 is recorded (Elke Dittmann* et al.*, 1997). The isotopic cluster detected at* m/z *= 828.8 whereas at* m/z *= 781.4 a previously unobserved non-halogenated substance occurred and they consistent with a Br4Cl pattern. Although they are identified the isotopic and theoretical values they are not identified the peptides which presented in the corresponding position. The present study of MALDI-TOF represented the Theoretical pI as7.82 / Mw,average mass as 7057.19 / Mw and monoisotopic mass as 7052.65/Mw. The corresponding positions and its peptide sequences also indicated. The mass of 1652.0468 is in the position of 17-32 and the peptide sequence is VLLIASVLLSGNILAR. Likewise the mass value of EYFHCFR peptide sequence is 1001.4298 and its corresponding position is 10-16. The position of 1-8 has the mass value 868.4015 with MSASTCLR peptide sequences. The mass value of 848.4261 with 37-43 position has the peptide sequence of SDQPFVR. The 49-56 position has the TETIGGNK sequence with 819.4206 mass values. Similarly 685.4130 have the peptide sequence of LTPIIE with the position of 57-62. The 44-48 position has the ETDQK peptide sequences with 620.2886 mass values. Finally the least value of 535.2544 with the position of 33-36 with QMEK peptide sequences.*

The resultant part of MALDI-TOF illustrates the Mass of the spectrum. This is the input file of MASCOT analysis. The format should be of data file. The existing studies showed that (Robert et al.,*2005) the Rigorous analysis of the data generated from these analyses was performed using two different algorithms (MASCOT and Digger) along with manual validation of all peptide matches. The Digger algorithm was used to search the IT data against both databases while MASCOT searches of both IT and oMALDI-Qstar data were performed exclusively using the LudwigNR database. The proteome in the fungal genus* Trichoderma*, and the spectra was submitted in SARAMI package and found the peptides (Sophie* et al.*, 2010). According to the study of (Hanjo Lim* et al.*, 2003) the* Methanococcus jannaschii *and* Pyrococcus furiosus*, using MALDI-TOF peptide mass mapping and calibrated spectra were submitted to database searches using the PROQUEST peptide mass mapping software. Recently the structure elucidation of biomacromolecules has been investigated by MALDI-TOF analysis (Achim Grubea* et al.*, 2009). They applied MALDI-TOF-MS to the sponge* Stylissa caribica *and the spectra referred with LIFT technology. But the present study used the PepMerge algorithm with peak studio 6 for spectra file visualization and* de novo *peptide sequence analysis.*

The metaproteomic study was carried out in the sponge Cymbastela Concentri *(Michael* et al.*, 2006). This work is closely relkated with the present study. But, they are validated the results of MASCOT and peptide view in manually. So, the present study used* de novo *analysis. The MASCOT analysis used the type of search is Peptide Mass Fingerprint, A good mass range for trypsin is 1000 to 3500 Da. So the Enzyme trypsin is used. The fixed modifications should be in the molecule of Carbamyl (K) and the variable modifications in*

Carbamidomethyl (C). The Monoisotopic mass values used in the mass of 35 kDa. The Peptide Mass Tolerance is ± 1.2 Da and its Peptide Charge State is 1+. There are 13 queries and Max Missed Cleavage is 1.The resulted MASCOT analysis, the high score value is 28 with the corresponding highly similar protein is (YO35_BPHP1) lys 3'region of Haemophilus phage. *There are five observed mass values (664.3140, 684.9474, 733.9299, 847.6647 and 863.0175) matched with the peptides R.ETDQK.I, K.LTPIIE.-, R.RQMEK.T, R.SDQPFVR.T and K.TETIGGNK.T. The characterization of proteins of interest used the standard database search engines (MASCOT) can be accomplished, searches should be performed using a 'broad species database' which does not provide optimal confidence in protein annotation. Therefore, it becomes necessary to determine partial or complete amino acid sequences using either manual or automated* de novo *peptide sequence analysis methods. Therefore, PEAKS assisted* de novo *sequencing was performed.*

The generated sequences were used to perform homology searches to characterize the protein identification (Myriam et al., *2009). Since the* Macaca mulatta *has an incomplete protein database, the sequences generated from each spectrum were used for protein identification by sequence homology in the mammalian database using either the PEAKS or SPIDER software (Software Protein Identifier). Therefore, the SPIDER software was useful when the* de novo *sequencing gave partially correct sequence tags and at instances where a segment of amino acids was replaced by another segment with approximately same masses (Han, 2005). The peak interpretation of y and b ion series gave the conformation of the peptide of VFGTLGSDDSGLFGK from* D. saccharalis *(Silva,* et al, *2010). A total of 362* de novo *tags were generated with HCD fragmentation and 139 with ETD for Spider Venom*

Peptides. Homology searches were performed using the identified de novo *tags and a funnel-web spider protein database derived from the Arachnoserver spider toxin database (www.arachnoserver.org). Likewise the present sytudy analyse the peptides against swissprot database with CED and the resultant conformation peptide is LTPIIE.*

The essential cleavage of the signal peptides embedded the pre-proteins during the translocation cycle. The signal peptidase (SPase) from Escherichia coli *is a membrane-bound endopeptidase with two amino-terminal transmembrane segments and a carboxy-terminal catalytic region which resides in the periplasmic space and it exposed hydrophobic surface. (Mark and Natalie, 2011). Most of the researchers reported the phylogenetic signals in Metazoa (Andrea* et al., *2006) in* Flustrellidra hispida, *Demospongiae (Michael* et al., *1998) in* Rhabdocalyptus dawsoni, *Porifera (Erik* et al., *2009) in* Oscarella carmela. *The result of the present study reported the location of signal peptide cleavage sites from different organisms like Gram-positive Bacteria, Gram-negative Bacteria, and eukaryotes used signal 4.0 server. Based on the Gram-Negative bacteria it shows that the graphical output of three different scores,* C, S *and* Y. *The cleavage site of the peptide is in the position of 32. The* S-score *for the signal peptide prediction is reported for every single amino acid position of the sequence, with high scores indicating that the corresponding amino acid is part of a signal peptide ie., 31 and the y score is in the position of 32.The Gram-positive based bacteria that the cleavage site of the peptide is in the position of 34,* S-score *position is 31 and the y score position also 34. The eukaryotic group based signal IP resulted as the cleavage site is in the position of 32 and its Y score position also the same of cleavage site. The S score position is 15. The signal peptide sequence regions plotted in the graph as red*

colour. The cleavage position is 31 and its cleavage sites are LSGNILARQMEKS with the score value of 0.5192.This study noticed the amino acid composition of the peptide sequence of the singal peptide sequence is remarkable.

It is generally useful to know the three dimensional structures of the proteins for the Identification of functional similarities of homologous proteins. In the absence of an experimentally determined structure, comparative or homology modeling can provide a useful model of a proteinThe present work used the template structure of 1BO0 A Chain from PDBsum. The Target sequence with template structure was aligned and the structure modeled using MODELLER9v8 software. There are five models generated. To find out the best model evaluation performed.

The researchers reported that (Gunasekaran et al.,*1996) a data set consisting of 110 high resolution, non-homologous, protein crystal structures from the Brookhaven Protein Data Bank was examined. The data set consisted of a total of 18,708 non-Gly residues, which were characterized on the basis of their backbone dihedral angles (f, c). Residues falling outside the defined ''broad allowed limits'' on the Ramachandran map were chosen and the reported B-factor value of the a-carbon atom was used to further select well defined disallowed conformations. Based upon the reveals the modeled structures are evaluated using PROCHECK analysis. Models were evaluated as a whole as well as in the individual regions It is reported that the Modeled structure1 has 45 residues with 81.8% most favoured regions with 1.8% disallowed regions. The modeled structure 2 has 80.0% favoured regions with 44 residues and 1.8% disallowed regions. The modeled structure 3 has the 81.8% of favoured regions with 45 residues and disallowed regions of 1.8%. The modeled structure 4*

has the 45 residues of 85.5% of allowed regions and 3.6% of disallowed regions. The modeled structure 5 has 83.6% of allowed regions with 46 residues and there is no disallowed region. Among these 5 models the modeled structure 5 is the best model. Because rest of the all models have disallowed regions. It has high percentage of favored regions also. Even though the modeled structure 4 has high favored regions it has 3.6% of unallowed regions. So modeled structure 5 leads to study the functional sites.

The functional sites are used for structural genomics study. So, the present study used computational approaches and identified the functional sites of the modeled 3D structure. It reported that 8(12.9%) strand, 8(12.9%) alpha helix, and the other elements 46 (74.2%) with62 residues. The functional part of the protein is N and C terminus. The N terminus is started in the position of 19 and the C terminus is 46. The topological structure also demonstrated. The 3D structure has 1anti parallel beta sheet sheet with strands. The sheets are indicated in red colour.

There is 1 beta hair pin with four beta hairpin regions like Leu19, Ser22, Asn 28 and Ala 31 was identified. The regions indicated as Leu and Ser in red colour and Asn and Ala in green colour.The 3D structure has 1 antiparallel beta bulge also. The regions Res X, Res1 and Res2. ResX is Val23 and its colour indication is red, Res 1 is Ser 26 the colour indication is green and the Res 3 is Gly 27, the colour indication is blue. The structure also has 2 strands of A sheet with four residues. The starting regions of the strands are Leu19, Asn 28 and its colour indication is labelled in Leu-blue, Asn- cyan. The ending regions are Ser22, Ala31 ant its colour indication Ser-green, Ala- red.

The 3D structure has 1 helix with 8 residues. The type of the helix is H and the srarting and ending regions are Asp 38 and Thr 45. The helix is indicated as Red color and the starting and ending regions are showed as cyan and green colour.There are7 beta turns noted in the 3D structure. The turn 5 and turn 7 has H- bond also. Turns are lebelled in red colour.The turn 1 has the regions from Arg8 to Tyr11, the sequence is RREY and the type of the turn is IV. Turn 2 has the regions of Tyr11-Cys14 and its sequence is YFHC and the type is IV. The third turn has the regions of Phe12-Phe15 the regions of FHCF with VII type. The fourth turn has the sequence of VLLS with Val23-Ser26 regions of I type. The turn 5 has the regions of Thr51-Gly54 with TIGG sequence II type. The sixth turn has the IGGN sequence of Ile52-Asn55 regions of IV type. The turn 7 has the regions from Gly53 to Lys56 in the sequence of GGNK with IV type. The regions of each turn demonstrated. The 3D structure has 1 gamma turn with two regions. The staring region is Thr45 and ending region is Gln47. The colour indication in Thy45 as red and Gln47 as blue .The sequence regions are TDQ and the type of the gamma turn is inverse, that is indicated as orange colour. The final element reported as disulphide. The disulphide regions of the 3D structur is 1st Cysteine and 2nd Cysteine. The color indication is 1st Cysteine-red, 2nd Cysteine- blue.

The protein association is involved in a large array of biological processes like ligand-receptor interactions. It associated with cellular response to its environment, trafficking through export and fusion proteins, and antibiotic resistance mechanisms induced by efflux pumps. The evidence showed (Broutin et al.*, 2005) that OprM is embedded into the surfactant bilayers of the sponge phase. The present study shows the clefts regions of the 3D structure that is ligand binding sites like positive, negative, neutral, aliphatic, aromatic, Pro,*

Gly and cysteine. The regions of positive are H,K,R and the regions are indicated in cyan colour. The negative regions are D,E and it labeled with red colour. The Neutral regions are S,T,N,Q and the colour indication is green. The Aromatic and aliphatic regions are as follows F,Y,W and A,V,L,L,M and the colours are purple and blue. The Cys and Gly regions are P,G and the color displayed is orange. The cysteine regions of C with yellow colour. The binding surfaces also noted in the result and the color indication is green.

SUMMARY

Marine sponges and their associated bacteria have been proven to be a rich source of novel secondary metabolites with therapeutic usefulness. The Indian coastline measures about 8129 km, among which the Gulf of Mannar, in South East coast of India, alone has 295 species of sponges, 180 species of marine algae and seaweeds, 190 species of

gastropods, etc. Several reports have appeared on the characterization of the antimicrobial activity of marine macroorganisms collected off the Indian coastline. Studies involving the screening, isolation and characterization of bioactive compounds from marine bacteria are yet to be undertaken on a systematic scale. In this juncture, the present study projected to take new efforts to isolate and characterize the bioactive potential of marine bacteria associated with selected sponges found in the south peninsular coast of India.

For the isolation of antimicrobial therapeutic protein from the ectosymbionts of sponges were carried out using standard microbiological protocols. Eight different sponges were collected from south peninsular coast of India and their associated bacteria were also isolated using different culture media. Apart from the commercial media, sponge extracts were also supplemented to promote more bacterial growth. The results clearly indicated that the sponge extract supplemented media produced more bacterial growth in most of the sponge tested. In Auxinella donnani*, the predominant numbers of bacterial colonies were noted in all culture media than the others. A total of 34 bacterial strains were isolated: 10 on zobell marine agar, 11 on Marine-agar and 13 on Marine/extract supplemented-agar in A. donnani. The three cultivation conditions showed no significant differences regarding the number of recovered strains, colony morphology or size. All 34 strains were assayed for antimicrobial activity against indicator bacteria. Three strains isolated on Marine/extract supplemented-agar inhibited more than 80% of indicator strain. Interestingly, the three strains showed antimicrobial activity only when grown at 20^{o}C, while no antimicrobial activity was detected at 37^{o}C. This suggests that temperature can also influence the capacity of the bacteria to produce antimicrobial substances.*

The morphological characteristics of the seven strains with antimicrobial activity showed that colonies were pigmented (white, cream, yellow or red) and Gram staining indicated that the strains were Gram-negative bacteria, forming short rods. Biochemical characterization showed that all strains were catalase and oxidase-positive and non-fomenters for sugars such as glucose, sucrose or lactose. The partial 16S rRNA gene sequences obtained were aligned using ABI Prism software (Perkin Elmer Applied Biosystems) and compared to sequences retrieved by the queries generated by BLAST of GenBank Database. Phylogenetic analysis was performed with the MEGA 4.0 program (Molecular Evolutionary Genetics Analysis, Version 4.0). The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method. RAPD-PCR typing was performed with primer 272 for the three strains. Based on that it was confirmed that the strain ESB3, ESB7 and ESB13 were two Bacillus sps *and* Pseudomonas sps *respectively.*

Molecular profiling of this secondary metabolite isolated from ectosymbiont bacteria were carried out using 1D and 2D gel electrophoresis. The protein characterization using SDS PAGE analysis revealed the presence of different peptides with the molecular weight 814 kDa to 3.5 kDa. The amino acid sequences were identified using MALDI-TOF analysis and the results confirmed the presence of enzyme trypsin. The timing of antibiotic synthesis study revealed that the antimicrobial peptide may be synthesized after 18hrs of bacterial culture in low temperatures.

The sequence of target peptides **(MSASTCLRREYFHCFRVLLIASVLLSGNILARQMEK SDQPFVRETDQKTETIGGNKLTPIIE)** was searched using MASCOT analysis. It compares the

resultant mass fingerprint with theoretical protein/peptide libraries. Top Score of the search is the protein in lys 3'region of *Haemophilus phage*. For the conformation analysis the *de novo* sequencing analysis was performed by Peak studio.

Signal peptides are both targeting and translocation proteins across the membrane region. The signal peptide regions are identified by the SignalP 4.0 server to predict the presence and location of signal peptide cleavage sites from different organisms like Gram-positive Bacteria, Gram-negative Bacteria, and eukaryotes. The signal peptide sequences are predicted by a computational tool as PrediSi (Prediction of Signal peptides). The cleavage site is LSGNILARQMEKS with the position of 31 and its score value is 0.5192.

Three dimensional structure of the protein is used to study the function of a protein. The template structure id is (1BO0 A chain), identified by PDBsum. The 3d structure determined by Modeller9v8 in the comparative modeling approaches and the modeled structure was validated using PROCHECK with the reference of Ramachandran plot. The final structure has 83% of allowed with no disallowed regions. The functional properties like helix strand, β turn, gamma turn, Beta hairpins, Beta bulge, disulphide regions, binding site and binding surfaces are determined by ProFunc. In the future investigation, this approach is proposed to study the metabolic pathway construction and de novo *drug designing.*

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