Phylogenetic diversity, significance and future prospects of heterotrophic bacteria associated with marine microalgae

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Certificate

*This is to certify that the thesis entitled "**Phylogenetic diversity, significance and future prospects of heterotrophic bacteria associated with marine microalgae**" is a bonafide record of research work carried out by Mrs. **Sandhya S V (Reg. No 4204)** under my guidance and supervision in the Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Marine Microbiology**, Cochin University of Science and Technology, Cochin. The thesis, as a part or whole has not been presented before, for the award of any degree, diploma, associateship in any university. I further certify that all the relevant corrections and modifications suggested by audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.*

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Declaration

*I hereby do declare that the thesis entitled "**Phylogenetic diversity, significance and future prospects of heterotrophic bacteria associated with marine microalgae",** is a genuine record of research work carried out by me under the supervision of **Dr. K.K. Vijayan**, Director, Central Institute of Brackishwater Aquaculture, Chennai, and that no part of this work, has previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles of any University or Institution.*

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Sandhya S. V.

ABBREVIATIONS

3’	:	Three prime DNA end
5’	:	Five prime DNA end
β	:	Beta
°	:	Degree
°C	:	Degree Celsius
λ	:	Lambda
µ	:	Micro
µl	:	Microlitre(s)
%	:	Percentage
θ	:	Theta
AgNp	:	Silver nanoparticle
ANOVA	:	Analysis of variance
CAS	:	Chrome azurol S
CFU	:	Colony forming unit
cm	:	Centimeter
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-Diphenyl-1-picrylhydrazyl
EDTA	:	Ethylenediaminetetraacetic acid
EOC	:	Extracellular organic carbon
et al.	:	And others
Fig.	:	Figure
FT-IR	:	Fourier Transform Infra Red
g	:	Gram(s)
h	:	Hour(s)
HDTMA	:	Hexadecyltrimethylammonium bromide
IAA	:	Indole-3-acetic acid

L	:	Litre(s)
m	:	Meter(s)
mA	:	Milliampere
mg	:	Milligram(s)
min	:	Minute(s)
ml	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar
mV	:	Millivolt
NCBI	:	National Center for Biotechnology Information
nm	:	Nanometer
OD	:	Optical density
OTU	:	Operational taxonomic unit
PCR	:	Polymerase chain reaction
pH	:	Hydrogen ion concentration
PICRUSt	:	Phylogenetic investigation of communities by reconstruction of unobserved states
ppt	:	Parts per thousand
PUFA	:	Polyunsaturated fattyacid
r DNA	:	Ribosomal DNA
rpm	:	Revolutions per minute
S	:	Second(s)
SEM	:	Scanning electron microscope
sp.	:	Species
TCBS	:	Thiosulfate citrate bile salts sucrose
TEM	:	Transmission electron microscope
UV	:	Ultra violet

v/v	:	Volume per volume
Vis	:	Visible
w/v	:	Weight per volume
XRD	:	X-ray diffraction
ZMA	:	Zobell marine agar
ZMB	:	Zobell marine broth

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Chapter 1

General Introduction

General Introduction

The oceans which cover more than 70 % of earth's surface are thriving with tremendous diversity of living microorganisms and hence represent the largest biome on earth (Penesyan et al. 2010, Amin et al. 2012). Nearly half of the global productivity occurs in ocean which is mediated by ubiquitous photosynthetic organisms referred to as phytoplankton (Geng and Belas 2010, Ramanan et al. 2016). The organic carbon produced by these photoautotrophs are utilised by heterotrophic bacteria and thereby remineralise large portion of organic matter to CO_2. It was assumed that half of the ocean's primary productivity is converted to dissolved organic matter by bacteria (Cho and Azam 1988, Geng and Belas 2010, Amin et al. 2012). Thus, phytoplankton and bacteria affect different trophic levels of aquatic food chain and are considered as structural pillars of the aquatic ecosystem (Natrah et al. 2014, Ramanan et al. 2016). These foremost functional entities together drive oceanic biogeochemical cycles and thereby ensure a balance in the nutrient cycles and energy flow (Natrah et al. 2014). Therefore, microalgal-bacterial interaction and influence of their interaction on each other and on ecosystems has attracted recent research interest (Ramanan et al. 2016).

Microalgae – bacteria interactions

The coexistence of microalgae and bacteria can be traced back to billion years ago. This coevolution which has revolutionized life on earth in

many aspects was a significant step in the evolutionary hierarchy of life (Natrah et al. 2014, Ramanan et al. 2016). Their cooccurrence in common habitat for more than 200 million years, fostering multitude of possible interaction between these two groups over evolutionary time scales (Amin et al. 2012). Further, the term 'Phycosphere' was coined by Bell and Mitchell in 1972 to describe "a zone that may exist extending outward from an algal cell or colony for an undefined distance, in which the bacterial growth is stimulated by the extracellular products of the alga". This algal microhabitat covers a variety of algal bacterial interaction which can be either positive or negative (Grossart 1999, Grossart and Simon 2007, Fuentes et al. 2016). Studies revealed that the bacterial association has a significant impact on algal growth and metabolism and could potentially implied in future algal biotechnology industry (Natrah et al. 2014, Fuentes et al. 2016). Recent studies demonstrate that distinct bacterial phylotypes were associated with different microalgae (Sapp et al. 2007, Amin et al. 2012, Schwenk et al. 2014). The community composition of bacteria in algal phycosphere might be depend on ability of bacteria to assimilate specific algal exudates or cope with antibacterial compounds released by the microalgae (Watanabe et al. 2008, Desbois et al. 2009, Natrah et al. 2014). The bacterial groups such as *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes* and *Bacilli* were among the most prominent bacterial phylotypes found in association with microalgae (Nicolas et al. 2004, Sapp et al. 2007, Amin et al. 2012, Lakaniemi et al. 2012, Natrah et al. 2014). However, knowledge on microalgal-bacterial associations is rather limited due to the tedious task of separating the partners which are naturally bound to each other (Fuentes et al. 2016). The mechanism of interaction has been found to be different in each study. Many phycosphere bacteria secrete several growth

promoters (eg: vitamin B_{12}, indole-3-acetic acid) which can improve the physiological state of phytoplankton host (Cole 1982, Croft et al. 2005, de-Bashan and Bashan 2008, Guo and Tong 2014). It has also been noted that bacterial association provide favourable ambient conditions for microalgae and produce stable microalgal culture with delayed death phase (Natrah et al. 2014). Concurrently, microalgae excrete carbon sources and other products that have a positive effect on bacteria which can be manifested by stimulation of bacterial DNA synthesis, enhancement of bacterial gene transfer and increased bacterial biofilm formation (Murray et al. 1996, Espeland et al. 2001, Matsui et al. 2003, Natrah et al. 2014). The interactions between microalgae and bacteria do not always have beneficial consequences; rather, may have inhibitory effects. Subsets of algicidal bacteria are able to enter the phycosphere and release active molecules that can lyse algal cells (Amin et al. 2012, Natrah et al. 2014). Similarly, many microalgae reported to produce various compounds like different types of fatty acids, glycosides, terpenes, polyunsaturated aldehydes and chlorophyll *a* derivatives that possess antibacterial activity (Bruce et al. 1967, Seraspe et al. 2005, Desbois et al. 2008, Ribalet et al. 2008, Natrah et al. 2014). A summary of interaction that occurs between microalgae and bacteria are shown in Fig. 1.1.

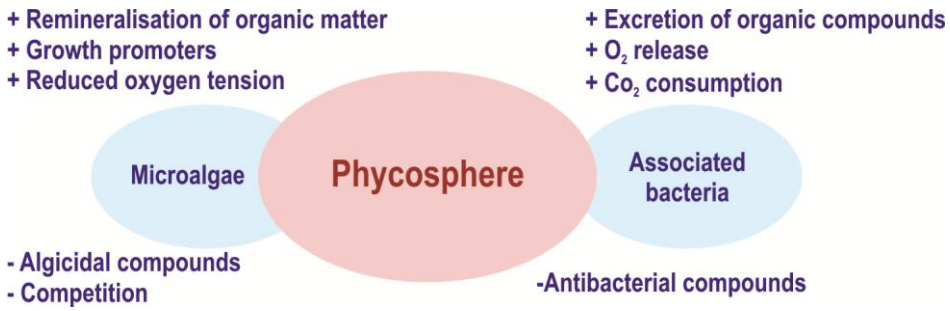

Fig. 1.1. A summary of algal-bacterial interactions

Types of algal – bacterial interactions

The following bacteria – phytoplankton interactions are predominantly studied in phycosphere:

Mutualism

Mutualism is a biological interaction in which both partners are benefitted each other (Atlas and Bartha 2007). Bacteria and microalgae form mutualistic relationship in which algal growth is enhanced by bacterial products such as remineralised nutrients, vitamins and other growth factors whereas bacteria in turn benefit from phytoplankton exudates (Haines and Guillard 1974, Cole 1982, Grossart 1999, Grossart and Simon 2007). A study done by Croft et al. (2005) showed that bacteria belonging to the genus *Halomonas* supplied cobalamin (vitamin B_{12}) to its phytoplankton host in exchange for fixed carbon. Such nutrient exchange plays a significant role in cycling of nitrogen, sulphur, carbon and phosphorus in aquatic ecosystems (Fuentes et al. 2016, Ramanan et al. 2016). Moreover, there are studies highlighting the role of bacteria in algal growth as nitrogen suppliers, especially in oligotrophic environment (Watanabe et al. 2005, Hernandez et al. 2009, Fuentes et al. 2016). Most of the studies pointed out that in order to release these algal stimulatory compounds, bacteria must benefit from improved growth of microalgae. Otherwise, they would not release these metabolically expensive extracellular products (Fukami et al. 1992, Mouget et al. 1995). According to Natrah et al. (2014), the same might be true for microalgal release of organic carbon.

Commensalism

In a commensal relationship, one population benefits while the other remains unaffected (Atlas and Bartha 2007). An example of commensalism is that *Chlamydomonas reinhardtii* uses vitamin B_{12} supplied by bacteria,

although bacterial partner do not make use of organic carbon released by algae (Kazamia et al. 2012, Fuentes et al. 2016). Similarly, commensalic bacteria gain benefit from algal host without causing any negative effect (Grossart 1999). However, there is a transient line that separates mutualism and commensalism, and even parasitism and these interactions may shift from one type to another. In this sense, there are studies that report a shift from commensalism to parasitism when the phytoplankton becomes stressed. The exact mechanisms behind such shifts remain unclear (Fuentes et al. 2016).

Parasitism

In a relationship of parasitism, one population benefits at the expense of other and exerts negative effects on it (Fuentes et al. 2016). Bacteria act as parasites on phytoplankton. They can penetrate and become lodged on the periplasmic space of the host cell and may lead to lysis and death of algal host (Grossart 1999). Many algae produce antibiotic compounds to prevent bacterial parasitism (Sastry and Rao 1994, Grossart 1999). Another form of parasitism is the competition for existing nutrients which results in slower growth rates of algae (Ramanan et al. 2016).

In summary, there exist a variety of interaction between algae and bacteria which have beneficial or detrimental effect to algal growth.

Applications of microalgae – bacteria interactions

The knowledge on algal – bacterial interactions can be explored for various aquaculture, biotechnological and environmental applications. Aquaculture is the fastest growing food producing sector in the world and microalgae forms an important part of diet of many aquaculture organisms, especially in the larval rearing systems (Banerjee et al. 2010, FAO 2014, Natrah et al. 2014). The associated bacteria can enhance the growth as well as the

chemical composition of microalgae (Fuentes et al. 2016). The improved quality of live feed will definitely increase the growth and health of aquatic organisms. In addition, a suitable combination of microalgae and beneficial bacteria might also lead to a better shellfish larval settlement (Natrah et al. 2014, Fuentes et al. 2016). Moreover, the associated bacteria could prevent the entry of pathogens into the larval rearing system by competitive exclusion which can be further explored as a novel strategy to control bacterial disease outbreaks in aquaculture sector (Regunathan and Wesley 2004, Santos and Reis 2014). Hence, it is expected that a well-selected consortium of phycosphere bacteria might significantly improve the productivity, efficiency and sustainability of aquaculture (Natrah et al. 2014).

Another important application of algal – bacterial interaction is the role of bacteria in microalgal aggregation. Nowadays, algal biomass harvesting by biofloc technology gaining more and more acceptance since it can significantly reduce biomass production costs (Natrah et al. 2014, Fuentes et al. 2016). Similarly, algal – bacterial systems have been extensively used in wastewater treatment. Van der Ha et al. (2012) reported the use of methane oxidising bacteria and microalgae for the removal of methane from anaerobically treated wastewater. The effective use of algal – bacterial interactions in metal bioremediation has also been documented. Algae require trace quantities of several metals for their growth and metabolism. At the same time, higher levels of metals are toxic to algae. In this regard, algal- bacterial community can mutualistically detoxify metals from metal rich environment (Ramanan et al. 2016). The application algal – bacterial interaction in degradation of many organic pollutants and toxic pesticides was also documented in previous studies (Subashchandrabose et al. 2011, Subashchandrabose et al. 2013). The algal – bacterial interactions can be further exploited as a platform for biodiesel production, electricity generation, biogas, bioethanol and biohydrogen production (Ramanan et al. 2016). It was reported that *Geobacter*, an electricity producing bacteria can coexist with

algae and can synergistically produce electricity using light microbial cells (Rosenbaum et al. 2005, He et al. 2009).

Overall, algal –bacterial interactions have broad-spectrum applicability in various fields including aquaculture, wastewater treatment, bioprospecting, bioremediation and energy generation.

Objectives of the study

A greater insight on algal – bacterial interactions may allow more effective utilisation of microalgae in commercial systems including aquaculture. Although these interactions may be of significant importance, only limited information on algal microhabitat is still available. A few studies reported the use of phycosphere bacteria for practical purposes (Natrah et al. 2014, Fuentes et al. 2016, Ramanan et al. 2016). Hence, more research is needed to develop a suitable algal – bacterial system for manifold beneficial effects. Thus, the present study was designed to unravel the phylogenetic diversity, significance and applications of heterotrophic bacteria associated with marine microalgal species having relevance in aquaculture.

Major objectives of the present study include:

- To study the **diversity of culturable bacteria** associated with **stock cultures of selected marine microalgae** and **microalgal mass culture system of a finfish hatchery**
- To study the **entire bacterial diversity** in microalgal habitat with special reference to a potential *Isochrysis galbana* isolate using **metagenomic approach**
- To study the **symbiotic association** of culturable heterotrophic bacteria with *Isochrysis galbana*

- To evaluate the efficacy of microalgae associated bacteria on **survival, growth and mysis conversion rate** of shrimp larvae, *Penaeus indicus*, a candidate penaeid shrimp used in shrimp farming
- To screen the heterotrophic bacteria associated with *Isochrysis galbana* for various **biotechnological applications**

The thesis is presented in eight chapters. The first chapter comprises of a general introduction including the importance of the work. The second chapter deals with the isolation, characterisation and phylogenetic diversity of culturable bacteria associated with certain stock cultures of marine microalgae. The biochemical, enzymatic and antibacterial characteristics and tolerance to various abiotic stress factors of isolated bacterial strains are also presented in this chapter. The third chapter comprises of bacterial diversity in microalgal production system of a marine finfish hatchery with special reference to the mass culture of *Chaetoceros gracilis*. Phycosphere of *Isochrysis galbana* was selected as a representative for in-depth studies on algal-bacterial interactions. The fourth chapter gives an account of entire diversity and functional role of bacteria associated with *I. galbana*. The fifth chapter projects the symbiotic association of culturable bacteria in *I. galbana* culture. Effect of bacterial symbionts on algal growth and nutrient profile and heterotrophic growth of bacterial symbionts on algal extra cellular carbon were presented in this chapter. The sixth chapter highlights the efficacy of phycosphere bacteria in shrimp larval rearing system using Indian white shrimp, *Penaeus indicus* as a model. The seventh chapter deals with the emerging applications of two bacterial strains- *Alteromonas* sp. MBTDCMFRI Mab 25 and *Labrenzia* sp. MBTDCMFRI Mab 26 associated with *I. galbana*. The major findings of the work are summarised in eighth chapter and the references given in the chapters are furnished at the end of the thesis.

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Chapter 2

Phylogenetic diversity of culturable bacteria in stock cultures of marine microalgae (The microalgal repository of Marine Biotechnology Division, CMFRI, Cochin)

Contents

2.1 Abstract
2.2 Introduction
2.3 Materials and Methods
2.4 Results and Discussion

2.1 Abstract

First time feeding using cultivated microalgae are an essential source of nutrition to several farmed finfish, shellfish and many other commercially significant aquaculture species, in their larval rearing phase. Knowledge on microalgae associated microhabitat is important for the development of a successful, healthy hatchery rearing system. Therefore, in the present study efforts were made to isolate, characterise and determine the phylogenetic diversity of bacteria associated with cultured microalgae, which are used as live feeds in many finfish and shellfish hatcheries. From ten selected microalgal cultures being maintained at microalgal repository of Marine Biotechnology Division, Central Marine Fisheries Research Institute (Cochin), 34 bacterial isolates were obtained with total bacterial counts of 10^1 to 10^5 CFU ml^{-1}. Most notably, the absence *Vibrio* spp., the major aquaculture pathogen in all tested microalgae suggests the suitability of these microalgae

for use in aquaculture systems. Phylogenetic analysis based on 16S rDNA sequencing revealed that the bacterial phylotypes associated with these microalgae were affiliated to *Gammaproteobacteria*, *Alphaproteobacteria* and *Flavobacteriia* classes. The genus *Marinobacter* (47%) was found to be the most predominant cultivable bacterium followed by *Alteromonas*, *Labrenzia*, *Oceanicaulis*, *Ponticoccus*, *Stappia* and *Rheinheimera*. Bacteria belonging to the genera *Gaetbulibacter* and *Maritalea* were also detected and it is the first report of association of these bacterial groups with microalgae. The biochemical, enzymatic and antibacterial characteristics and tolerance to various abiotic stress factors of these bacterial isolates are also described in the present chapter. Altogether, the present study gives an insight into the phycosphere of cultivated microalgae, which can be further explored for improving the productivity and reliability of indoor and outdoor microalgal culture systems.

2.2 Introduction

Bacteria and microalgae are two numerically dominant groups of microbes in the aquatic ecosystem (Flandez 2011). It has been realised that there is a close association between them under natural as well as in experimental conditions (Sapp et al. 2007, Krohn-Molt et al. 2013). The 'phycosphere' is a region where microalgae release many nutritional exudates; thus it is a favourable microenvironment for diverse subsets of bacteria (Sapp et al. 2007, Natrah et al. 2014). Bacteria in the phycosphere can either be free-living or directly attached to the phytoplankton surfaces (Grossart 1999). Moreover, when microbial partners come closer to the surface of the phytoplankton, various molecular mechanisms that promote bacterial attachment on the surface

of their host are activated (Geng and Belas 2010). In algal habitats, these interactions may be either symbiotic, parasitic, commensal or competitive (Grossart and Simon 2007, Fuentes et al. 2016, Ramanan et al. 2016).

Bacteria are found as close associates of microalgae cultured under laboratory conditions, and may have a direct influence on algal growth and metabolism (Schwenk et al. 2014). Very little is known about the selection of specific types of bacteria by the phytoplankton (Jasti et al. 2005, Giroldo et al. 2007); however, this is probably influenced by the chemical microenvironment created by the host (Penesyan et al. 2010). For example, *Silicibacter* sp. isolated from dimethylsulfoniopropionate (DMSP) producing *Pfiesteria piscicida* dinoflagellate cultures showed chemotactic response to DMSP and other dinoflagellate molecules (Miller et al. 2004). Conceição et al. (2010) reported that microalgal cultures harbour a broad spectrum of bacteria belonging to the groups *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Bacilli*. These bacterial partners normally enhance the algal growth by producing growth stimulating factors such as vitamins, minerals and other essential nutrients and also play a role in the regeneration and remineralisation of organic compounds (Natrah et al. 2014). Thus, the associated bacteria that decompose extracellular products of microalgae participate in biogeochemical cycling and play an important part in the microbial loop (Jasti et al. 2005, Sapp et al. 2007, Natrah et al. 2014). In some cases, the production of algicidal compounds by certain bacteria ensures the environmental balance in nutrient cycle and energy flow.

Marine microalgae are widely used as larval feeds or feed additives in the larval rearing systems of aquatic animals. The productivity of a hatchery

system mainly depends on the quality and quantity of these live feeds (Flandez 2011). Also, maintenance of a proper balance of diverse microflora associated with these live feeds is essential for a successful culture environment in commercial hatcheries (Schulze et al. 2006, Natrah et al. 2014). Sometimes the microalgae might stimulate pathogenic bacteria, especially *Vibrio* spp., which exerts an overall negative effect on the aquaculture rearing system (Natrah et al. 2014). For example, Elston et al. (2008) reported that microalgal stock cultures were contaminated with *Vibrio* spp. with concentrations as high as 2.01×10^6 CFU ml^{-1} in a shellfish hatchery system. Hence, it is crucial to know the phytoplankton and their associated microenvironment in order to attain stable and reliable microalgae cultivation. Against this background, the present work aims to isolate, characterise and determine the phylogenetic diversity of culturable bacteria associated with ten commercially important marine microalgal live feed species grown in laboratory conditions.

2.3 Materials and Methods

2.3.1 Microalgae strain selection and culturing

Ten molecular and biochemically characterised microalgal strains isolated during 2009 to 2015 (Preetha 2017) were selected, based on their significance as aquaculture live feed, from the microalgae culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, south India) (Table 2.1). All isolates were maintained in F/2 seawater medium as monoalgal cultures in the algal germplasm at 22± 1° C under light-dark conditions (16:8 h, 40-50 µmol photons $m^{-2}s^{-1}$) (Anderson 2005, Preetha et al. 2012).

Table 2.1 Details of microalgae used for the study

Strain Code	Nomenclature	Algal types	Class	GenBank Acc No.	Source
S107	*Synechococcus* sp.	Blue-green algae	*Cyanophyceae*	KM087987	Unknown[a]
S002	*Isochrysis galbana*	Golden-brown algae	*Prymnesiophyceae*	JF708124	Unknown[a]
S072	*Chlorella* sp.	Green algae	*Treboxiophyceae*	JF708157	Marine Research Hatchery, CMFRI, Kochi[b]
S082	*Tetraselmis* sp.	Green algae	*Prasinophyceae*	JF708168	Seawater, Poompuhar, Tamil Nadu
S135	*Dunaliella salina*	Green algae	*Chlorophyceae*	JF708161	Seawater, Calicut, Kerala
S078	*Nannochloropsis oceanica*	Heterokont algae	*Eustigmatophyceae*	JF708165	Unknown[a]
S019	*Thalassiosira* sp.	Centric diatom	*Bacillariophyceae*	ND[c]	Mangalavanam mangrove, Kochi, Kerala
S065	*Chaetoceros* sp.	Centric diatom	*Bacillariophyceae*	JF708154	Seawater, Njarakkal, Kerala
S043	*Navicula* sp.	Pennate diatom	*Bacillariophyceae*	JF708144	FortKochi ship channel, Kochi, Kerala
S092	*Nitzschia* sp.	Pennate diatom	*Bacillariophyceae*	ND[c]	Hypersaline lake, Pulikat, Tamil Nadu

[a] Maintained as pure culture in live feed collection of CMFRI, Kochi

[b] Obtained as an invader in *Arthrospira platensis* marine open tank culture

[c] Not Determined

(Strain code for all isolates starts with MBTDCMFRI)

2.3.2 Isolation of bacteria associated with microalgae

For the isolation of associated bacteria, 10 ml of microalgal culture at their early stationary phase (~13 to 15 days old culture) was filtered through a 1.2 µm membrane filter (Pall, USA). The filter cake obtained was rinsed with 0.85% sodium chloride (NaCl) to remove the free living bacteria. The filtered microalgae were suspended in 1 ml of 0.85% NaCl and vortexed. The mixture was serially diluted and plated on Zobell Marine Agar (ZMA) (Himedia, India) and thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India). The plates were incubated at 30°C for 24 to 96 h. After incubation, the total colony counts were taken and morphologically different colonies were selected and purified. The purified isolates obtained were preserved and maintained as glycerol stocks at −80°C for future use.

2.3.3 Identification and molecular phylogeny of bacteria

The total genomic DNA was extracted from all bacterial isolates using a phenol-chloroform enzymatic extraction method (Sambrook and Russell 2001). 16S rDNA amplification was carried out using universal primers NP1F (5'-GAG TTT GAT CCT GGC TCA-3') and NP1R (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Pai et al. 2010) and the PCR conditions described as per Nair et al. (2012). The amplified PCR products were purified (HiPura PCR product purification kit, Himedia) and sequenced by the Sanger sequencing method. The 16S rDNA sequences of the isolates were compared with the sequences available in the EzTaxon database and identified up to generic level (Kim et al. 2012). Multiple alignment was done through the CLUSTALW algorithm (Thompson et al. 1994) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987). Evolutionary analysis was conducted in MEGA6 and tree topologies were evaluated by bootstrap analysis of 1000 data sets (Tamura et al. 2013). The distances were computed using the Kimura 2 parameter method. The 16S rDNA sequence of one isolate Mab 04 (*Alteromonas* sp.) was not included in the analysis: as the sequencing reaction of this isolate repeatedly failed with NP1F, the primer NP1R was used to sequence the products. All the obtained sequences were submitted to the NCBI GenBank (Accession Nos. KR004791 to KR004798 and KR004801 to KR004826).

2.3.4 Characterisation of bacterial isolates

2.3.4.1 Biochemical characterisation

The bacterial isolates (10^6 to 10^8 CFU ml^{-1}) were characterised for physiological and biochemical properties using the potassium hydroxide (3% KOH) string test, oxidase, catalase, citrate utilisation, decarboxylation of arginine, ornithine and lysine and fermentation of different sugars following standard microbiological methods (Krieg and Holt 1984). The media used in this study were prepared with 1% sea salt (Sigma, USA).

2.3.4.2 Enzymatic assay

All bacterial isolates were screened for the production of various hydrolytic exoenzymes such as amylase, casease, lipase, gelatinase, cellulase and urease (Nair et al. 2012). Purified bacterial isolates were spotted in the substrate amended nutrient agar prepared with 1% sea salt and were incubated at 30°C for 48 to 72 h. The activity was measured as the growth or zone around colonies with or without addition of reagents.

2.3.4.3 Antibacterial assay

The isolates were tested for antagonistic activity against five common aquaculture bacterial pathogens using a well diffusion assay (Valgas et al. 2007). The pathogens used were *Vibrio harveyi* 101, *V. anguillarum* A1, *V. alginolyticus* 101 (Central Institute of Brackish-water Aquaculture, Chennai), *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (Microbial Type Culture Collection, Chandigarh). Overnight grown cultures were inoculated into a freshly prepared Zobell marine broth (Himedia, India) and incubated for 48 h. After incubation, 50 µl of cell free suspension was poured into the wells punctured on the pathogen pre-swabbed Mueller Hinton Agar (Himedia, India) plates with 1% NaCl. These were then incubated at 30°C for 24 to 48 h and the results were recorded as a zone of inhibition observed around the wells.

2.3.4.4 Abiotic stress tolerance assay

The bacterial tolerance to fluctuating physiochemical conditions were detected by growth in gradients of salinity, temperature and pH (Nair et al. 2012). To detect salinity tolerance, the isolates were inoculated into medium (0.5% peptone; 0.3% yeast extract; pH 7 ± 0.2) (Himedia, India) supplemented with different concentrations (0, 2, 5 and 10% w/v) of sea salt (Sigma, USA). To study the temperature and pH tolerance, isolates were grown on ZMA plates incubated at 20 to 60°C and pH 5 to 9, respectively.

2.4 Results and Discussion

It is now established that both culture dependent and independent techniques for understanding the diversity of bacteria have their own bias problems and neither technique can simply be substituted for the other (Al-Awadhi et al. 2013). The ultimate goal of this study is to explore the growth stimulating role of bacteria associated with microalgae for final application in aquaculture. For such studies cultivable microorganisms were required. In the present work, 34 morphologically and biochemically different bacterial isolates (Strain Codes Mab 01 to Mab 36 [01–22 and 25–36], see Table 2.2 below) were obtained from ZMA plates inoculated with suspensions of marine microalgal cultures (Fig. 2.1). Their total counts in microalgal cultures were in the range of 10^1 to 10^5 CFU ml^{-1} and all the isolates obtained were Gram-negative. This was in agreement with the studies of Simidu et al. (1971) and Sini (2012) which reported the dominance of Gram negative bacteria in the colonisation and association of microalgae. However, these findings contradict those of one previous study that reported that most of the isolates obtained from microalgal cultures of *Tetraselmis chuii* and *Chlorella minutissima* were Gram-positive (Makridis et al. 2006**)**. There was no bacterial growth on TCBS agar plates, which indicates the absence of the aquaculture pathogens like *Vibrio* spp. in all of the selected microalgal cultures. Similar results are reported in several studies, even though this bacterial genus is ubiquitous in marine environments (Salvesen et al. 2000, Makridis et al. 2006, Conceição et al. 2010). The absence of *Vibrio* may be due to competitive exclusion by phycosphere bacteria, whereby they outcompete the pathogens and prevent their invasion of the niche which they already occupy (Natrah et al. 2014, Santos and Reis 2014). Thus, findings of present investigation indicate that it is safe to use these strains of microalgae as a live feed in aquaculture rearing systems.

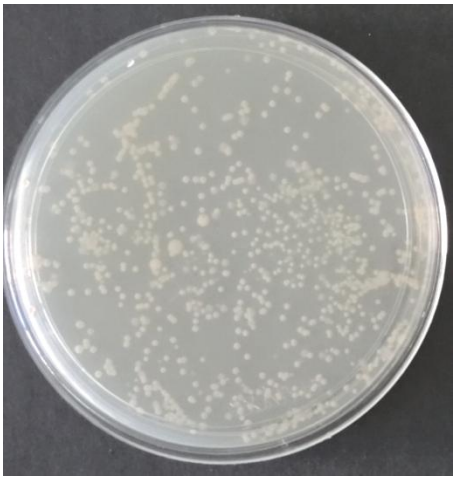

Fig. 2.1. Bacterial colonies on ZMA plates inoculated with microalgal suspension

Phylogenetic analysis of bacterial isolates based on partial 16S rDNA sequences showed they shared 94 to 100% identity with known bacterial genera. The 16S rDNA sequences of the isolates showed maximum similarity to the genera *Marinobacter, Alteromonas, Labrenzia, Ponticoccus, Oceanicaulis, Stappia, Gaetbulibacter, Maritalea* and *Rheinheimera*. Details of the isolates and their accession numbers are shown in Table 2.2. A neighbour-joining tree was constructed with 16S rDNA sequences, separated the isolates into three different clades as *Gammaproteobacteria, Alphaproteobacteria* and *Flavobacteriia* (Fig. 2.2). Out of the 34 culturable bacterial isolates obtained, 22 were from the class *Gammaproteobacteria,* distributed among genera such as *Marinobacter*, *Rheinheimera* and *Alteromonas.* From the class *Alphaproteobacteria*, 11 isolates belonged to 5 different genera *Labrenzia, Ponticoccus, Oceanicaulis, Maritalea* and *Stappia.* From the class *Flavobacteriia,* only one isolate, belonging to the genus *Gaetbulibacter*, was documented. These results confirm earlier reports that microalgae were allied to these bacterial classes (Nicolas et al. 2004, Sapp et al. 2007, Conceição et al. 2010, Amin et al. 2012, Le Chevanton et al. 2013).

Table 2.2 Identification of culturable bacteria associated with microalgae using 16S rDNA sequence data

Isolated bacterial strains					Reference strains in EzTaxon database		
Source	Strain code	GenBank Accession No	Bacterial count (CFUml-1)	Phylogenetic group	Closest relative	Similarity (%)	GenBank Accession No
Chaetoceros sp.	Mab 01	KR004810	4.6×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.93	EF028328
	Mab 02	KR004811	3.8×10^4	*Oceanicaulis* sp.	*Oceanicaulis stylophorae*	98.84	HM035090
	Mab 03	KR004812	2.3×10^4	*Labrenzia* sp.	*Labrenzia suaedae*	99.56	GU322907
Thalassiosira sp.	Mab 04	KR004826	2×10^2	*Alteromonas* sp.	*Alteromonas marina*	99.42	AF529060
	Mab 05	KR004813	2.35×10^5	*Oceanicaulis* sp.	*Oceanicaulis stylophorae*	99.39	HM035090
	Mab 06	KR004814	3×10^2	*Ponticoccus* sp.	*Ponticoccus litoralis*	96.64	EF211829
	Mab 07	KR004824	1×10^2	*Alteromonas* sp.	*Alteromonas macleodii*	99.33	CP003841
	Mab 08	KR004815	4×10^1	*Rheinheimera* sp.	*Rheinheimera aquimaris*	99.00	EF076757
Nitzschia sp.	Mab 09	KR004816	9.8×10^3	*Marinobacter* sp.	*Marinobacter salsuginis*	99.71	EF028328
	Mab 10	KR004817	1.02×10^5	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	100.00	FO203363
	Mab 11	KR004818	3.8×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.71	EF028328
Navicula sp.	Mab 12	KR004819	8×10^3	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	100.00	FO203363
	Mab 13	KR004820	9.6×10^2	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	98.54	FO203363
	Mab 14	KR004821	1×10^3	*Maritalea* sp.	*Maritalea mobilis*	100.00	EU255260
D. salina	Mab 15	KR004791	2×10^3	*Gaetbulibacter* sp.	*Gaetbulibacter jejuensis*	100.00	FJ490367
	Mab 16	KR004792	1.96×10^4	*Marinobacter* sp.	*Marinobacter salsuginis*	99.93	EF028328
	Mab 17	KR004793	2×10^4	*Marinobacter* sp.	*Marinobacter algicola*	98.08	ABCP01000031
	Mab 18	KR004794	1.2×10^4	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	99.71	FO203363
Chlorella sp.	Mab 19	KR004795	5.1×10^3	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.15	DQ235263
	Mab 20	KR004796	6.4×10^3	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.00	DQ235263
Tetraselmis sp.	Mab 21	KR004797	4.47×10^5	*Labrenzia* sp.	*Labrenzia aggregata*	100.00	AAUW01000037
	Mab 22	KR004798	3×10^1	*Alteromonas* sp.	*Alteromonas macleodii*	99.42	CP003841
I. galbana	Mab 25	KR004801	2.5×10^3	*Alteromonas* sp.	*Alteromonas macleodii*	99.50	CP003841
	Mab 26	KR004822	1×10^3	*Labrenzia* sp.	*Labrenzia aggregata*	100.00	AAUW01000037
	Mab 27	KR004823	2×10^3	*Marinobacter* sp.	*Marinobacter algicola*	94.17	ABCP01000031
N. oceanica	Mab 28	KR004802	1×10^3	*Labrenzia* sp.	*Labrenzia aggregate*	100.00	AAUW01000037
	Mab 29	KR004803	1.4×10^4	*Stappia* sp.	*Stappia stellulata*	99.85	AUIM01000013
	Mab 30	KR004804	6×10^3	*Marinobacter* sp.	*Marinobacter lipolyticus*	99.29	ASAD01000031
Synechococcus sp.	Mab 31	KR004825	4×10^3	*Alteromonas* sp.	*Alteromonas macleodii*	99.28	CP003841
	Mab 32	KR004805	5×10^2	*Marinobacter* sp.	*Marinobacter vinifirmus*	97.97	DQ235263
	Mab 33	KR004806	5×10^2	*Stappia*sp.	*Stappia stellulata*	99.85	AUIM01000013
	Mab 34	KR004807	9.8×10^4	*Marinobacter* sp.	*Marinobacter vinifirmus*	98.00	DQ235263
	Mab 35	KR004808	6.4×10^4	*Ponticoccus* sp.	*Ponticoccus litoralis*	98.40	EF211829
	Mab 36	KR004809	2.4×10^4	*Marinobacter* sp.	*Marinobacter hydrocarbonoclasticus*	99.71	FO203363

(Strain code for all isolates starts with MBTDCMFRI)

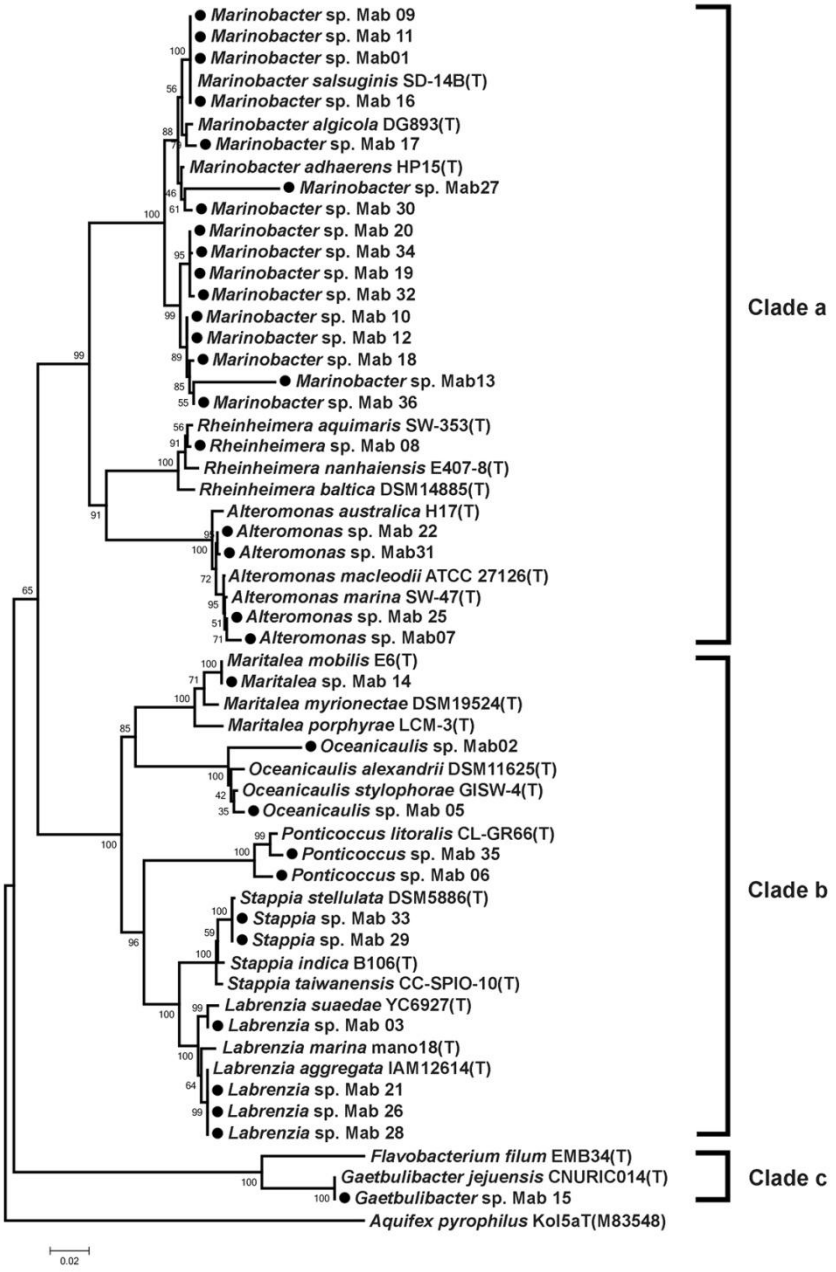

Fig. 2.2. Neighbour-joining phylogenetic tree based on partial 16S rDNA sequence of culturable bacterial strains isolated by this study (strain codes Mab 01 to Mab 36) and reference strains from the EzTaxon database. Strain codes for all bacterial isolates starts with MBTD CMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India)

Clade a: *Gammaproteobacteria*; **Clade b**: *Alphaproteobacteria*; **Clade c**: *Flavobacteriia*

Mircoalgae, such as *Chaetoceros* sp., *Thalassiosira* sp. and *Nannochloropsis oceanica*, harbour diverse culturable bacterial groups belonging to four different genera. At the same time, only one genus, *Marinobacter*, was encountered in the phycosphere of *Nitzschia* sp., *Dunaliella salina* and *Chlorella* sp.. However, Schwenk et al. (2014) isolated *Loktanella* sp. and *Agrobacterium* sp. from *Chlorella pyrenoidosa* and *Nitzschia microcephala* cultures. Also, Guo and Tong (2014) isolated three symbiotic bacterial strains from *Chlorella vulgaris* ATCC 13482 culture, which were shown to be close relatives of *Pseudomonas alcaligenes*, *Elizabethkingia miricola* and *Methylobacterium radiotolerans*. *Rheinheimera* sp. was detected only from the diatom *Thalassiosira* sp. even though there is a report of its isolation from other diatom aggregates (Grossart et al. 2009). Similarly, Schwenk et al. (2014) reported the isolation of *Flexibacter* sp., *Seohaeicola saemankumensis*, *Roseobacter* sp. and *Erythromicrobium* sp. from laboratory maintained cultures of *Isochrysis* sp. However, in the present study, bacterial isolates belonging to the genera *Labrenzia* and *Alteromonas* were obtained from *Isochrysis galbana* culture. At the same time, the study by Sharifah and Eguchi (2011) supports isolation of *Stappia* sp. from *Nannochloropsis oceanica* culture. Concurrently, the bacterial genera *Maritalea* and *Gaetbulibacter* were isolated from the diatom *Navicula* sp. The bacterial genus *Marinobacter* was isolated from most of the microalgal species (*Synechococcus* sp., *Chlorella* sp., *Dunaliella salina*, *Nannochloropsis oceanica*, *Chaetoceros* sp., *Navicula* sp. and *Nitzschia* sp.), and it comprised 47% of total bacterial isolates obtained. Their predominance indicates their close affiliation with the phycosphere of diverse groups of microalgae (Jasti et al. 2005, Amin et al. 2012, Natrah et al. 2014). Likewise, many previous studies report a microalgal association with *Alteromonas*, which supports isolation of similar bacterial genera from *Nannochloropsis oceanica*, *Isochrysis galbana*, *Tetraselmis* sp., *Thalassiosira* sp.

and *Chaetoceros* sp. (Jasti et al. 2005, Sapp et al. 2007, Ali et al. 2010, Amin et al. 2012, Le Chevanton et al. 2013). Two other genera isolated in current study, *Oceanicaulis* and *Labrenzia*, were previously reported to be associated with the toxic dinoflagellates *Alexandrium tamarense* and *A. lusitanium*, respectively (Strömpl et al. 2003, Fiebig et al. 2013). Many culturable bacterial genera identified in this study were also previously documented to be associated with macroalgae; for example, *Marinobacter* and *Labrenzia* were associated with the green alga *Bryopsis* (Hollants et al. 2011, Hollants 2012), *Alteromonas* with seaweeds from the Gulf of Mannar (Janakidevi et al. 2013), *Stappia* with green alga *Ulva intestinalis* (Ali et al. 2010) and *Maritalea* with the red alga *Porphyra yezoensis* (Fukui et al. 2012). However, the bacterial isolate belonging to genus *Gaetbulibacter* is reported here for the first time in algal association.

The physiological and biochemical characteristics of the bacterial isolates are shown in Table 2.3. All the isolates were oxidase positive except Mab 03 (*Labrenzia* sp.), Mab 20 and Mab 34 (*Marinobacter* spp.). Only 6 bacterial isolates Mab 02 (*Oceanicaulis* sp.), Mab 09, Mab 10, Mab 13, Mab 18 and Mab 30 (all *Marinobacter* spp.) were catalase negative. In the decarboxylation assay, most of the isolates were positive for at least one substrate, except the cultures Mab 03 (*Labrenzia* sp.) and Mab17 (*Marinobacter* sp.) which were negative in all the 3 tests. The metabolic utilisation of tested sugars was lacking in most of the isolates, since they were found to be fermentation negative. However, the isolates Mab 30 (*Marinobacter* sp.) and Mab 35 (*Ponticoccus* sp.) were able to ferment many sugars (sucrose, lactose, maltose, mannose, galactose, xylose, glucose and raffinose) as shown in Table 2.3. Also all *Labrenzia* spp. (Mab 03, Mab 21, Mab 26 and Mab 28) obtained in this study were xylose fermenters. Brown (1991) reported differences in the sugar composition of polysaccharides from

microlagae belonging to different species and classes. This variation in the sugar composition might contribute to the difference in the sugar fermentation capability of their bacterial counterparts.

Table 2.3 Physiological and biochemical characteristics of culturable bacterial isolates associated with microalgae

Strain code	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Mab 01	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 02	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 03	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Mab 04	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 05	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 06	+	+	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-
Mab 07	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 08	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 09	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 10	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 11	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 12	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 13	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 14	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 15	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 16	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 17	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Mab 18	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 19	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 20	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 21	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-
Mab 22	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 25	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 26	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-
Mab 27	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 28	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-
Mab 29	+	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-
Mab 30	+	+	-	-	+	-	+	+	-	+	+	+	-	+	+	+	+
Mab 31	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 32	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 33	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Mab 34	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Mab 35	+	+	+	+	+	-	+	-	-	-	+	+	-	-	-	+	-
Mab 36	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-

Biochemical: A - KOH string test; B - Oxidase; C – Catalase;
Decarboxylation: D - Arginine; E - Ornithine; F – Lysine;
Utilisation: G - Citrate;
Fermentation: H - Sucrose ; I - Sorbitol ; J - Lactose; K - Maltose ; L - Mannose ; M - Arabinose; N - Galactose; O - Xylose; P - Glucose ; Q – Raffinose
(Strain code for all isolates starts with MBTDCMFRI)

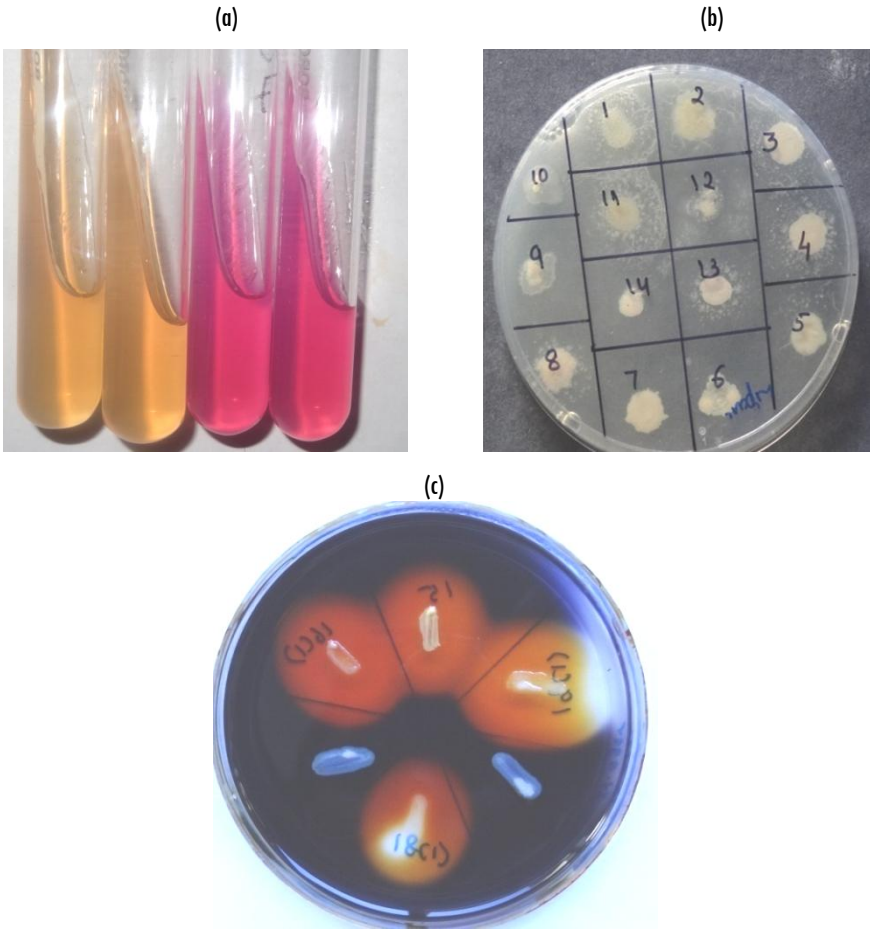

Fig. 2.3. Enzymatic assay of bacterial strains associated with microalgae: urease assay (a); lipase assay (b); amylase assay (c)

Many culturable bacterial isolates associated with microalgal cultures were capable of producing the hydrolytic exoenzymes gelatinase (61.8%) and urease (58.8%). Only 44.1 and 35.3% of the isolates exhibited lipase and amylase activity, respectively. Mab 33 (*Stappia* sp.) associated with *Synechococcus* sp. was the only isolate detected positive for cellulase, and none of the isolates exhibited casease activity (Table 2.4, Fig. 2.3). These results indicate that most of the isolated bacterial strains can hydrolyse algal exudates and act as remineralisers of various organic compounds which lead to the release of nitrogen, phosphorus and carbon compounds to the phycosphere.

This can enhance and sustain the growth of microalgae for a prolonged period of time (Grossart 1999, Sini 2012, Natrah et al. 2014). The extracellular enzyme activity of microalgal cultures can also not be denied (Patil and Mahajan 2016). In non-axenic microalgal cultures, it may be enhanced by the hydrolytic enzyme activity of bacteria associated with them.

Table 2.4 Enzymatic and abiotic stress tolerance assay of culturable bacterial isolates associated with microalgae

Strain Code	Enzymatic Assay						Tolerance Assay		
	R	S	T	U	V	W	X	Y	Z
Mab 01	+	-	-	-	+	-	5-9	20-45	20-100
Mab 02	-	-	-	-	++	+	5-9	20-45	20-100
Mab 03	-	-	-	-	++	-	5-9	20-45	20-50
Mab 04	+	+	-	-	-	++	5-9	20-45	20-100
Mab 05	-	-	-	-	-	+	5-9	20-45	20-100
Mab 06	-	-	-	-	-	+	6-9	20-45	20-50
Mab 07	+	+	-	-	-	++	5-9	20-45	20-100
Mab 08	+	++	-	-	++	++	5-9	20-45	20-100
Mab 09	-	-	-	-	-	+	5-9	20-45	20-100
Mab 10	+	-	-	-	+	+	5-9	20-45	20-100
Mab 11	+	-	-	-	+	-	5-9	20-45	20-100
Mab 12	+	-	-	-	+	+	5-9	20-45	20-100
Mab 13	+	-	-	-	+	+	5-9	20-45	20-100
Mab 14	-	-	-	-	-	+	6-9	20-45	20-100
Mab 15	-	+	-	-	-	+	5-8	20-40	20-100
Mab 16	+	-	-	-	-	-	5-9	20-45	20-100
Mab 17	-	++	-	-	-	-	5-9	20-40	20-100
Mab 18	+	-	-	-	+	+	5-9	20-45	20-100
Mab 19	-	++	-	-	+	+	5-9	20-45	20-100
Mab 20	-	++	-	-	-	+	6-9	20-45	20-100
Mab 21	-	-	-	-	++	-	5-9	20-45	20-100
Mab 22	+	+	-	-	-	++	5-9	20-45	20-100
Mab 25	+	+	-	-	-	++	5-9	20-45	20-100
Mab 26	-	-	-	-	++	-	5-9	20-45	20-100
Mab 27	+	-	-	-	+	-	5-9	20-45	20-100
Mab 28	-	-	-	-	++	-	5-9	20-45	20-100
Mab 29	-	-	-	-	++	+	5-9	20-45	20-100
Mab 30	-	-	-	-	-	-	5-9	20-45	20-100
Mab 31	+	+	-	-	-	++	5-9	20-45	20-100
Mab 32	-	++	-	-	+	+	6-9	20-45	20-100
Mab 33	-	-	+	-	++	-	5-9	20-45	20-100
Mab 34	-	++	-	-	+	+	5-9	20-45	20-100
Mab 35	-	-	-	-	++	-	5-9	20-40	20-50
Mab 36	+	-	-	-	++	-	5-9	20-45	20-100

R - Lipase; S - Amylase; T - Cellulase; U - Casease; V - Urease; W - Gelatinase; X - Hydrogen ion concentration (pH); Y - Temperature (°C); Z - Salinity (ppt); - no enzymatic activity; [+] zone in diameter 10 - 20 mm; [++] zone in diameter 20 - 30 mm

The obtained bacterial isolates were screened for their antibacterial activity against five major aquaculture pathogens belonging to the genus *Vibrio*, but none of the isolates was found to possess antagonistic activity. The bacterial isolates were assessed for their tolerance against different abiotic stress factors like salinity, temperature and pH and the results are given in Table 2.4. In salinity tests, Mab 03 (*Labrenzia* sp.), Mab 06 and Mab 35 (*Ponticoccus* spp.) showed growth only up to 50 ppt. The rest of the isolates showed growth up to 100 ppt indicating that most of them are halotolerant species. All the isolates grew well at 20°C. The maximum temperature tolerance, beyond which no growth was observed, for Mab 15 (*Gaetbulibacter* sp.), Mab 17 (*Marinobacter* sp.) and Mab 35 (*Ponticoccus* sp.) was 40°C. All other isolates showed growth up to 45°C. Mab 06 (*Ponticoccus* sp.), Mab 14 (*Maritalea* sp.), Mab 20 and Mab 32 (*Marinobacter* spp.) showed maximum growth between pH 6 and 9, whereas Mab 15 (*Gaetbulibacter* sp.) showed optimum growth at pH 5 to 8. All other isolates grew well at pH 5 to 9. Thus, these results showed that microalgae cultures are associated with versatile groups of bacteria that can survive under diverse physiological stresses. Open mass culture of marine microalgae takes place in conditions marked by seasonal fluctuations in salinity, temperature etc. (Adenan et al. 2013). Hence, the abiotic stress tolerance would help better adaptation of these bacterial strains towards their phytoplankton host.

The results of the present study clearly indicate the existence of a strong and close association between bacteria and microalgae, including under artificial conditions which makes the phycosphere a hotspot of complex interactions (Sapp et al. 2007, Schwenk et al. 2014). When compared to algae, associated bacterial biomass is low but it can complement the live feed used in aquaculture with many growth factors and improve success rates in larval

rearing (Nicolas et al. 2004). However, in addition to beneficial effects, many associated bacterial groups are reported to display algicidal activity (Natrah et al. 2014). Thus, presence of bacteria plays a pivotal role in energy, nutrient and ecological balance (Cole 1982). Hence, in order to optimally benefit from microalgal−bacterial interaction, it is crucial to increase understanding of the various aspects of interactions which still remain unexplored. Current knowledge on bacterial groups associated with diverse microalgal hosts can be further extended to develop a consortium of suitable bacteria with wide applications in microalgal mass culture. Thus, the present study on microalgal bacterial flora will provide a basis for further research to improve stability, productivity and sustainability of large scale production of microalgae.

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Chapter 3

Phylogenetic diversity of culturable bacteria in microalgal mass culture system of a marine finfish hatchery (West Coast Hatcheries & Research Centre Pvt Ltd., Alappuzha)

Contents

3.1 Abstract
3.2 Introduction
3.3 Materials and Methods
3.4 Results and Discussion

3.1 Abstract

Microalgae, a major live feed in larviculture of finfish and shellfish always coexist with associated bacteria. Hence a better understanding of algal-bacterial interaction is essential for maintaining a stable environment in intensive larval rearing tanks. Therefore, an effort was made in the present study, to determine the phylogenetic diversity of culturable bacteria associated with microalgal production system of a marine finfish hatchery with special reference to the mass culture of *Chaetoceros gracilis*. The sequencing of 16S rDNA of representative from each phylotypes revealed that the associated microflora belong to the classes *Gammaproteobacteria, Alphaproteobacteria* and *Bacilli*. In particular, members of *Marinobacter* genus showed higher degree of association followed by *Leisingera, Alteromonas, Nautella*, *Halomonas* and *Ruegeria.* The association of bacterial groups belonging to the genera

Idiomarina, Albidovulum and *Staphylococcus* were also detected and from the perusal of available literature, it is the first report on their microalgal association. The variation of bacterial diversity in microalgal habitat with changes in environmental conditions was also discussed in the present work. In overall, the present study gives a greater insight to the algal microhabitat which would be vital for improving stability, productivity, sustainability and reliability of large scale microalgal cultivation and their feeding to the target aquaculture species.

3.2 Introduction

When agriculture production is satiated, to feed the increasing population, we need to look towards alternative food production systems such as aquaculture which has registered a continuous growth trajectory across the world. It is estimated that 62 % of the fish consumed by the world's ever growing population will be produced by aquaculture by 2030 (FAO 2014). Microalgae possess high nutritional content and hence they are vital as feed and as life support system in the early life stages of cultured aquatic organisms (Flandez 2011). Other than the nutritional support, these microalgal live feeds may have an impact on bacterial communities of rearing tanks since they always coexist with bacteria in natural aquatic ecosystem (Salvesen et al. 2000, Guo and Tong 2014). The previous chapter clearly confirmed the presence of diverse bacterial groups in microalgal habitat and the concentration of culturable bacteria varied from 10^1 to 10^5 CFU ml^{-1} of algal culture. According to Nicolas et al. (2004) the algal cultures were associated with more number of bacteria than sea water and their impact on larvae may depend on their concentration. These bacterial counterparts might greatly improve the nutritional quality of rearing animal since they can enhance growth and chemical composition of phytoplankton host (Natrah et al. 2014,

Fuentes et al. 2016). For example, Toi et al. (2014) reported the production of healthier *Artemia* cultures through the co-ingestion of algae and bacteria. Thus the interaction between microalgae and bacteria play a key role in productivity and sustainability of aquaculture (Natrah et al. 2011). Moreover, results of the previous chapter suggest the potential of these associated bacteria in preventing the invasion of pathogenic bacteria in algal habitat by competitive exclusion. In addition to these beneficial aspects, inhibitory effects of associated bacteria on algal growth and metabolism were also reported (Cole 1982, Natrah et al. 2014, Fuentes et al. 2016). Thus, in order to determine the impact of these associated bacteria on the microbial environment in aquatic hatcheries, the first step is to study the diversity of microalgal bacterial flora (Nicolas et al. 2004). The chemical composition of microalgae varies with the changes in physical and chemical environment and it may also have an influence on the growth of associated bacterial communities (Salvesen et al. 2000). In this context, the present work aims to study the phylogenetic diversity of culturable bacteria associated with the microalgal production system of a marine finfish hatchery. The samples have been collected in every three month interval for a period of one year to specifically study whether environmental factors have an influence on microflora of microalgal habitat.

3.3 Materials and Methods

3.3.1 Sample collection

The microalgae (*Chaetoceros* sp.) culture samples from various stages of mass culture i.e., from 250 ml flask, 1 L flask, 10 L cylinder, 100 L outdoor tank, 500 L outdoor tank and 2 ton outdoor tank were collected during March 2013 to December 2013 from a marine finfish hatchery at Alappuzha, Kerala, India (West Coast Hatcheries & Research Centre Pvt Ltd.) (Fig. 3.1).

The same microalgal strain was maintained in the microalgae culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) as strain *'Chaetoceros garcilis* MBTD-CMFRI-S172', after morphological and molecular identification (18S rDNA sequence similarity; GenBank Acc No: KM087981).

Fig. 3.1. Sample collection from different stages of microalgal mass culturing

3.3.2 Isolation, identification and molecular phylogeny of associated bacteria

The isolation, identification and molecular phylogeny of bacteria associated with mass culture of *Chaetoceros* sp. were carried out as described in section 2.3.2 and 2.3.3. In brief, 10 ml of microalgal culture from different stage of mass culturing was filtered, vortexed, serially diluted and plated on both Zobell Marine Agar (ZMA) and thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India). The total genomic DNA was extracted from all bacterial isolates and 16S rDNA amplification was carried out using universal primers NP1F (5'-GAG TTT GAT CCT GGC TCA-3') and NP1R (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Sambrook and Russell 2001, Pai et al. 2010). The isolated bacterial strains were identified upto generic level based on their 16S rDNA sequence similarity with sequence available in EzTaxon database and evolutionary history was inferred using neighbour-joining method (Saitou and Nei 1987, Kim et al. 2012). Since the sequencing reaction of six isolates (WC 01, WC 32, WC 36, WC 39, WC 40, WC 46) repeatedly failed with the primer NP1F, only NP1R was used to sequence their 16S rDNA genes. Similarly, for the isolate WC 59 the primer NP1F alone was used. Hence their sequences were not included in the phylogenetic analysis. All 16S rDNA sequences used for phylogenetic analyses were submitted to the NCBI GenBank.

3.3.3 Bacterial diversity analysis

Bacterial diversity was measured by calculating Simpson reciprocal diversity index. It was defined as $1/\sum n(n-1)/N(N-1)$ where n is the number of organisms of a particular genus and N is the number of organisms of all genera (Suchodolski et al. 2008).

3.4 Results and Discussion

In the present study, the culturable bacteria associated with the microalgal production system of a selected marine finfish hatchery were isolated and identified. For bacterial isolation, Zobell marine agar was used which is previously reported as reference medium to study bacterioplankton (Nicolas et al. 2004, Lebaron et al. 2001). After incubation, growth of diverse subsets of bacteria was found on ZMA inoculated with microalgal cultures. The microalgal suspensions were inoculated also on TCBS agar plates in order to determine whether any pathogenic bacterial groups were associated. It was reported that sometimes the microalgae might stimulate the growth of pathogens and it can exert an overall negative effect to the aquaculture production system (Natrah et al. 2014). Also, Gomez-Gil et al. (2002) observed better growth of aquaculture pathogen like *Vibrio alginolyticus* in the presence of *Chaetoceros muelleri*. But in contrary to their observations, no bacterial growth on TCBS plates were observed which indicated the absence of *Vibrio* spp.. As suggested by Santos and Reis (2014) it may be due to the competitive exclusion by phycosphere bacteria and the present results confirm the safety of using this live feed in larval rearing systems.

Totally, 69 bacterial isolates were obtained (Strain code WC 01- 14, 19-73; Table 3.1) and their 16S rDNA sequences shared 88-100 % similarity with known bacterial genera in EzTaxon database. The molecular identification revealed that they showed maximum similarity to the genera *Marinobacter, Leisingera*, *Nautella, Alteromonas, Idiomarina*, *Halomonas, Albidovulum, Ruegeria* and *Staphylococcus*. A neighbour-joining phylogenetic tree constructed with their 16S rDNA sequences separated the obtained bacterial isolates into three different clades as *Gammaproteobacteria* (78.26%), *Alphaproteobacteria* (20.29%) and *Bacilli* (1.45%) (Fig. 3.2). The class *Gammaproteobacteria* comprise 54 isolates belong to four different

genera *Marinobacter, Alteromonas, Idiomarina* and *Halomonas*. 14 bacterial isolates belong to the genera *Nautella*, *Albidovulum*, *Leisingera* and *Ruegeria* were documented from the class *Alphaproteobacteria*. From the class *Bacilli* only one isolate was obtained and it was *Staphylococcus* sp.

Table 3.1 Identification of culturable bacteria associated with microalgal production system using 16S rDNA sequence data. Strain codes for all bacterial isolates starts with MBTD CMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India)

Month	Stage of mass culturing	Strain Code	GenBank accession no.	Phylogenetic group	Similarity (%)
March	250 ml	WC 01	KU572438	*Marinobacter* sp.	99.86
		WC 02	KU554452	*Marinobacter* sp.	97.49
	1 L	WC 03	KU554453	*Marinobacter* sp.	97.45
		WC 04	KU554454	*Marinobacter* sp.	100
		WC 05	KU554455	*Leisingera* sp.	98.03
	10 L	WC 06	KU554456	*Marinobacter* sp.	97.45
		WC 07	KU554457	*Marinobacter* sp.	100
	100 L	WC 08	KU554458	*Marinobacter* sp.	100
	500 L	WC 09	KU554459	*Marinobacter* sp.	97.45
		WC 10	KU554460	*Marinobacter* sp.	100
		WC 11	MF991457	*Alteromonas* sp.	90.50
		WC 12	KU554461	*Nautella* sp.	100
	2 Ton	WC 13	MF991458	*Alteromonas* sp.	90.50
		WC 14	KU554462	*Nautella* sp.	100
June	250 ml	WC 54	KU554496	*Marinobacter* sp.	99.28
		WC 55	KU554497	*Marinobacter* sp.	99.14
		WC 56	KU554498	*Alteromonas* sp.	99.78
		WC 57	KU554499	*Marinobacter* sp.	99.93
	1 L	WC 58	KU554500	*Idiomarina* sp.	98.77
		WC 59	MF991460	*Halomonas* sp.	98.86
	10 L	WC 60	KU554501	*Leisingera* sp.	98.23
		WC 61	KU554502	*Marinobacter* sp.	99.85
		WC 62	KU554503	*Marinobacter* sp.	99.14
		WC 63	KU554504	*Marinobacter* sp.	99.86
	100 L	WC 64	KU554505	*Marinobacter* sp.	100
		WC 65	KU554506	*Marinobacter* sp.	97.59
	500 L	WC 66	KU554507	*Marinobacter* sp.	100
	2 Ton	WC 67	KU554508	*Marinobacter* sp.	97.5

		WC 68	KU554509	*Marinobacter* sp.	97.64
		WC 69	KU554510	*Idiomarina*sp.	100
		WC 70	KU554511	*Marinobacter* sp.	99.86
		WC 71	KU554512	*Marinobacter* sp.	99.64
September	250 ml	WC 19	KU554466	*Alteromonas* sp.	99.71
		WC 20	KU554467	*Nautella*sp.	100
		WC 21	KU554468	*Albidovulum*sp.	99.93
	1 L	WC 22	KU554469	*Marinobacter* sp.	100
		WC 23	KU554470	*Marinobacter* sp.	100
		WC 24	KU554471	*Marinobacter* sp.	100
	10 L	WC 25	KU554472	*Marinobacter* sp.	100
		WC 26	KU554473	*Marinobacter* sp.	99.93
	100 L	WC 27	KU554474	*Marinobacter* sp.	99.93
		WC 28	KU554475	*Marinobacter* sp.	100
		WC 29	KU554486	*Staphylococcus* sp.	99.93
	500 L	WC 30	KU554487	*Marinobacter*sp.	100
		WC 31	KU554488	*Leisingera*sp.	98.25
		WC 32	MF991459	*Marinobacter*sp.	**88.61**
	2 Ton	WC 33	KU554489	*Leisingera* sp.	98.11
		WC 34	KU554490	*Marinobacter*sp.	97.27
		WC 35	KU554491	*Marinobacter*sp.	100
December	250 ml	WC 36	KU572440	*Marinobacter*sp.	99.53
		WC 37	KU554492	*Ruegeria*sp.	99.92
		WC 38	KU554493	*Idiomarina*sp.	97.29
	1 L	WC 39	KU572441	*Leisingera*sp.	99.01
		WC 40	KU572442	*Leisingera*sp.	99.48
	10 L	WC 41	KU554494	*Marinobacter*sp.	99.93
		WC 42	KU554495	*Nautella* sp.	100
		WC 43	KU554476	*Marinobacter*sp.	99.79
		WC 44	KU554477	*Marinobacter*sp.	99.71
	100 L	WC 45	KU554478	*Marinobacter*sp.	99.93
		WC 46	KU572443	*Leisingera*sp.	98.94
		WC 47	KU554479	*Marinobacter*sp.	99.86
		WC 48	KU554480	*Marinobacter*sp.	99.93
	500 L	WC 49	KU554481	*Marinobacter*sp.	100
		WC 50	KU554482	*Marinobacter*sp.	99.77
		WC 51	KU554483	*Nautella* sp.	100
		WC 52	KU554484	*Marinobacter*sp.	99.93
		WC 53	KU554485	*Marinobacter*sp.	98.42
	2 Ton	WC 72	KU554513	*Marinobacter* sp.	97.87
		WC 73	KU554514	*Marinobacter* sp.	97.18

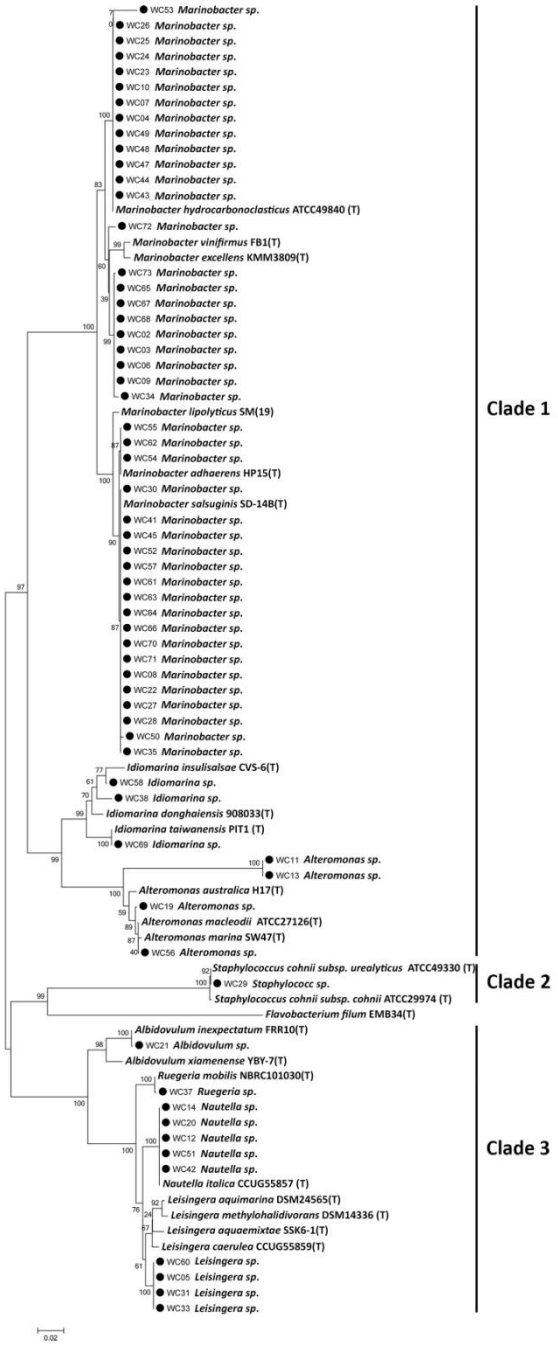

Fig. 3.2. Neighbour-joining phylogenetic tree based on partial 16S rDNA sequence of culturable bacterial strains isolated by this study and reference strains from the EzTaxon database. Strain codes for all bacterial isolates starts with MBTD CMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India). **Clade 1**: *Gammaproteobacteria*; **Clade 2**: *Bacilli*; **Clade 3**: *Alphaproteobacteria*

Previously, bacterial groups belonging to four different genera such as *Marinobacter*, *Oceanicaulis*, *Labrenzia* and *Alteromonas* were obtained from laboratory maintained culture of *Chaetoceros* sp. (MBTDCMFRI S065, GenBank Acc No. JF708154) (Sandhya et al. 2017). In the present study *Marinobacter* spp. were obtained from most of the stages of microalgal production system throughout the year (66.67 %). Similarly the association of *Alteromonas* spp. was also observed except in the month of December. There are many reports which support isolation of these bacterial genera from microalgal culture (Jasti et al. 2005, Sapp et al. 2007, Ali et al. 2010, Amin et al. 2012, Le Chevanton et al. 2013, Natrah et al. 2014). At the same time, neither *Labrenzia* nor *Oceanicaulis* were obtained from any stages of the mass culturing of selected *Chaetoceros* sp.. In addition to *Marinobacter* and *Alteromonas*, seven other bacterial groups (*Leisingera, Nautella , Idiomarina, Halomonas, Albidovulum, Staphylococcus* and *Ruegeria*) were found to be associated with different stages of *Chaetoceros gracilis* production system. The genus *Leisingera* is a member of *Roseobacter* clade within the family *Rhodobacteraceae*. They are reported to be present in various marine habitats including symbiosis with algae (Vandecandelaere et al. 2008, Riedel et al. 2013). Similarly Oh et al. (2011) observed the association of *Nautella* sp. with marine dinoflagellate *Cochlodinium polykrikoides*. Likewise, Porsby et al. (2008) supported isolation of *Ruegeria* sp. from microalgal production system. In addition to that Arora et al. (2012) documented close association of three bacterial strains including *Ruegeria* sp. with marine microalgae *Tetraselmis indica*. Also, *Halomonas* sp. identified from this study was previously documented to be associated with microalgae *Alexandrium minutum* (Palacios et al. 2006). However, it is the first report on microalgal association of bacterial groups belonging to the genera *Idiomarina, Albidovulum* and *Staphylococcus*.

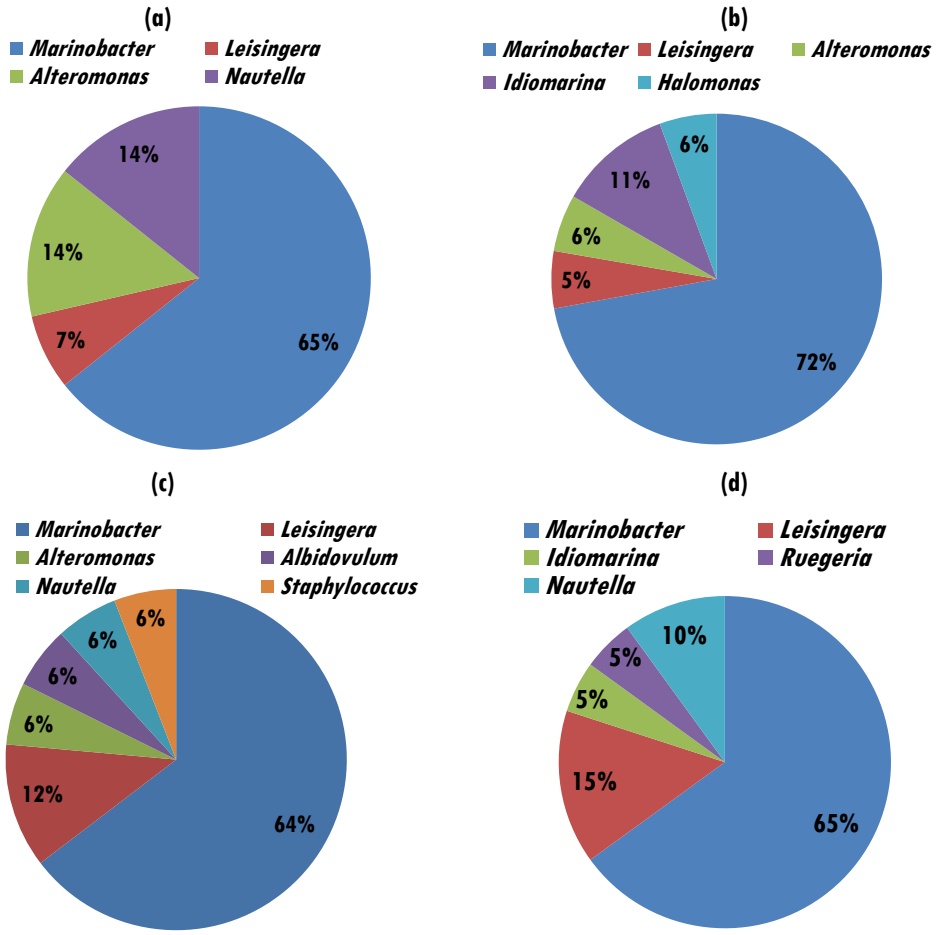

Fig. 3.3. Variation of culturable bacterial diversity in microalgal production system during each sampling: March 2013 (a); June 2013 (b); September 2013 (c); December 2013 (d)

It was observed that *Marinobacter* spp. were predominantly present in the selected microalgal production system which clearly indicated a close association of this bacterial genus with the selected strain of *Chaetoceros gracilis*. However, association of other bacterial groups showed considerable variation in each sampling. Variation of bacterial diversity during each sampling is shown in Fig. 3.3. Bacterial diversity in each sampling was measured by calculating Simpson reciprocal diversity index and is shown in Table 3.2. Simpson reciprocal diversity index yield information about bacterial diversity and high value for the index indicate high bacterial diversity

(Suchodolski et al. 2008). The maximum bacterial diversity was obtained in the month of September followed by March, December and June. The results suggested that there is a variation in bacterial diversity with changes in physical and chemical factors. This was found to be in agreement with one previous study which reported that bacteria – phytoplankton interactions are highly variable with environmental conditions (Grossart 1999). It may be due to the changes in the chemical composition of microalgae with varying environmental conditions (Reitan et al. 1994, Salvesen et al. 2000). Thus the results achieved in the present study indicated that the chemical microenvironment created by phytoplankton host might have an influence on the growth of associated bacterial community. Also, the phycosphere bacteria may be influenced by the algal cell number and growth conditions which could vary considerably between sampling (Salvesen et al. 2000).

Table 3.2 Simpson reciprocal diversity index (1/D) of each sampling

Month	Simpson reciprocal diversity index (1/D)
March	2.39
June	1.95
September	2.43
December	2.32

The present study revealed that microalgal production system of aquatic hatcheries was associated with diverse bacterial groups. Hence microalgae represent a repetitive source of bacterial inoculation into the larval rearing tanks since they are added at regular intervals into the system to maintain specific algal density, as live feed (Salvesen et al. 2000). This repetitive inoculation of bacteria through microalgal addition might have a significant effect on the microflora of water and larvae. Makridis et al. (2006) reported that the bacteria associated with the live feed play an important role in the exponential proliferation of bacteria in the fish gut during the early

development of the larvae. Also, microalgae associated bacteria can outcompete the pathogens and could have a positive impact on aquaculture disease control (Natrah et al. 2014, Fuentes et al. 2016). On the whole, it is clear that enhanced larval growth and development is attributed not only by the high nutritional value of the live feed but also by the algae-bacteria interactions (Skjermo and Vadstein 1993). Present study is an attempt to improve our knowledge on algal-bacterial interaction which could be vital for successful hatchery larval rearing. Future research may consider the effect of these interactions in larval growth and development. Thus the gathered information can be further explored for developing a suitable consortium of bacteria that have wide spectrum applicability in aquaculture.

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Chapter 4

Metagenomic profiling of bacteria associated with marine microalgae with special reference to *Isochrysis galbana* MBTDCMFRI S002

Contents

4.1 Abstract
4.2 Introduction
4.3 Materials and Methods
4.4 Results and Discussion

4.1 Abstract

This study was focused on assessing the entire bacterial diversity, including the uncultivable bacterial assemblage associated with marine microalgae, *Isochrysis galbana* MBTDCMFRI S002 by using metagenomic approach. Next generation sequencing technologies have been applied for sequencing of 16S rDNA V3 region. The data set comprised of 1190 Operational Taxonomic Units based on which phylum, class, order, family, genus and species distribution was determined. A total of 44 different bacterial genera mostly from the classes *Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Flavobacteriia, Acidimicrobiia, Sphingobacteriia* were detected in the study. In addition to these known bacterial groups several unknown groups (the sequences do not have any alignment against taxonomic database) were also present in the studied algal habitat. The bacterial diversity within the samples was analysed by calculating Shannon, Chao1 and observed species metrics. The functional profile

of bacterial communities was predicted using PICRUSt analysis. The results of the present study indicated that these associated bacterial communities are mainly involved in environmental information processing, genetic information processing, membrane transport and nutrient metabolism. These functions may mediate their interaction with phytoplankton host and thus improve bacterial survival in algal habitat. Overall, the present study enhances the understanding of algal microhabitat in terms of diversity and functional role of associated microbial community, including both cultivable and non-cultivable bacterial symbionts.

4.2 Introduction

The brown-golden marine microalga *Isochrysis* could certainly be counted among those phytoplankton that make excellent live food in aquaculture. The genus is comprised of marine, free-living, unicellular flagellate which is classified as: *Prymnesiophyta* (Phylum); *Prymnesiophyceae* (Class); *Isochrysidales* (Order); *Isochrysidaceae* (Family). *Isochrysis* has been considered as one of the most favoured types of live feed for so long since it is rich with very high levels of the polyunsaturated fatty acids such as docosahexaenoic acid (DHA), stearidonic acid and alpha-linolenic acid as well as vitamins and colour-enhancing pigments (Salvesen et al. 2000, Conceição et al. 2010, Preetha 2017). Hence, among different groups of microalgae, *I. galbana* was selected as a representative for further studies on algal-bacterial interactions.

The association of bacteria with microalgae is ubiquitous, playing major roles in the evolution and diversity of microalgae (Nan et al. 2011). For a detailed investigation of algal-bacterial interaction, the first step is to study the bacterial diversity in algal habitat (Nicolas et al. 2004). It was reported that non-axenic cultures of microalgae harbours diverse groups of bacteria

belonging to the classes *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Bacilli* (Conceição et al. 2010). In addition, several uncultivated bacteria are noted in the phycosphere of microalgae (Krohn-Molt et al. 2013). Many times, microbial interaction studies are hampered by difficulties in cultivating these bacterial symbionts (Nan et al. 2011). Consequently, metagenomic technologies have become powerful tools for investigating interactions of microorganisms with their environment and host (Krohn-Molt et al. 2013). These methods rely on direct analysis of bacterial DNA without the need to culture individual species, hence avoiding culture bias (Nocker et al. 2007, Powell et al. 2012). Traditional metagenomic sequencing was carried out using labour intensive techniques which include cloning, colony picking, plasmid extraction and sequencing, and, consequently, most studies analysed fewer than hundred clones per sample (Sanschagrin and Yergeau 2014). Hence, only a small fraction of bacterial diversity was unravelled by these studies (Wemheuer et al. 2014). The advent of next generation sequencing has tremendously simplified these routine metagenomic sequencing procedures (Sanschagrin and Yergeau 2014). These studies provided in-depth investigation of bacterial communities in diverse ecosystems and revealed existence of many taxa not known from previous less sensitive approaches (Wemheuer et al. 2014). However, only very few studies have focused on metagenomes associated with microalgae (Powell et al. 2012, Krohn-Molt et al. 2013, Williams et al. 2013). In this background, the present study was aimed to better define microbial communities inherent to algal habitat with special reference to *I. galbana* MBTDCMFRI S002 by using metagenomic technologies. Additionally, PICRUSt analysis (phylogenetic investigation of communities by reconstruction of unobserved states) was also used to predict functional profile of associated microflora.

4.3 Materials and Methods

4.3.1 *I. galbana* culture

Isochrysis galbana MBTDCMFRIS002 maintained at microalgae culture collection of Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) was used for this study (Preetha 2017).

4.3.2 DNA extraction

The total genomic DNA was extracted from 10 ml liquid culture of *I. galbana* at late growth phase following modified phenol-chloroform enzymatic extraction method (Wu et al. 2000, Preetha et al. 2012).

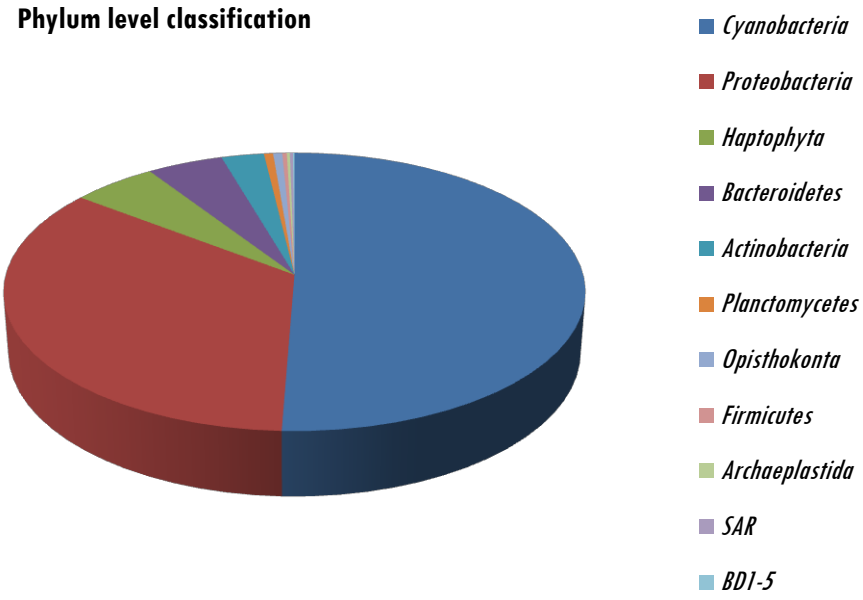
4.3.3 16S rDNA amplicon sequencing and analysis

Next generation sequencing approaches were used for metagenomic analysis V3 region of 16S rDNA. 16S rDNA amplicon sequencing and analysis was performed with a Illumina MiSeq platform at AgriGenome Labs Private Limited, Cochin. Reads having sequence shorter than 150 bp and an average phred score under 30 were excluded from analysis. Fastq quality checking (Base quality, base composition, GC content) was also performed to ensure the quality of the sequence obtained from sequencer. Usually a paired-end sequence from V3 metagenomics contains some portion of conserved region, spacer and V3 region. As a first step, the spacer and conserved region were removed from paired-end reads. After trimming the unwanted sequences from original paired-end data a consensus V3 region sequence is constructed using ClustalO program. Multiple filters such as, conserved region filter, spacer filter and mismatch filter were performed to take further only the high quality V3 region sequences for various downstream analyses. Chimeras were

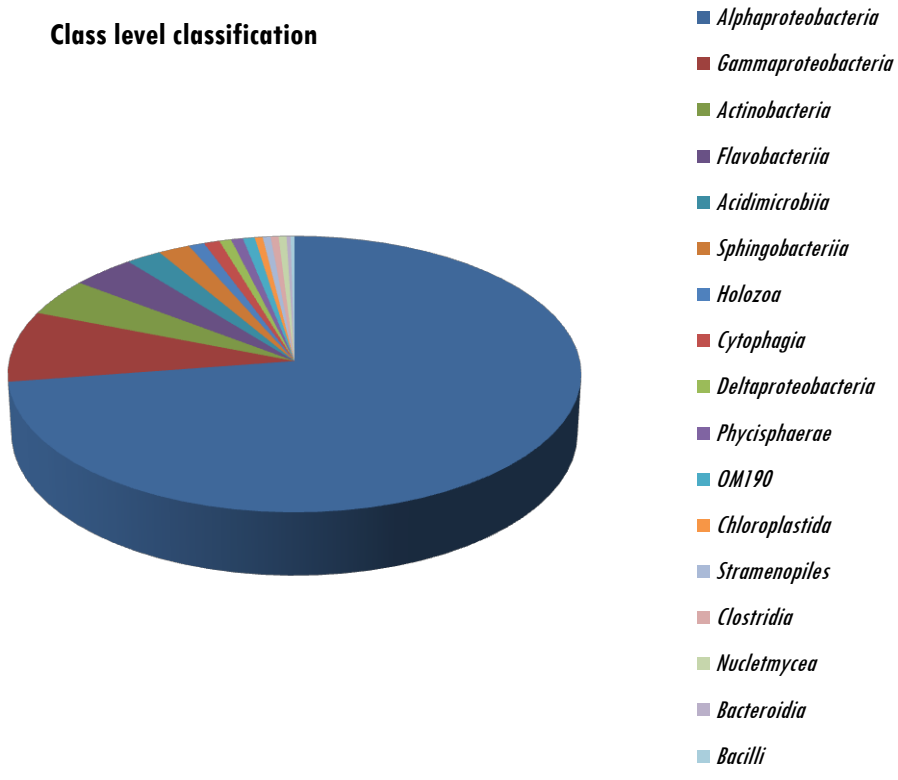
also removed using the de-novo chimera removal method UCHIME implemented in the tool USEARCH. Pre-processed reads from all samples were pooled and clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity using Uclust program (similarity cutoff = 0.97) (Lozupone et al. 2013, D'Argenio et al. 2014). QIIME program was used for the entire downstream analysis (Caporaso et al. 2010). Representative sequence was identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program (DeSantis et al. 2006 a, b). Further, these representative sequences were aligned against reference chimeric data sets. Then, taxonomy classification was performed using RDP classifier against SILVA OTUs database. Sequence data was deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the accession number SRR6740228. Microbial diversity within the sample was further analysed by calculating alpha diversity indices such as Shannon, Chao1 and observed species metrics using QIIME software. Functional profiles of bacterial communities were predicted using PICRUSt analysis.

4.4 Results and Discussion

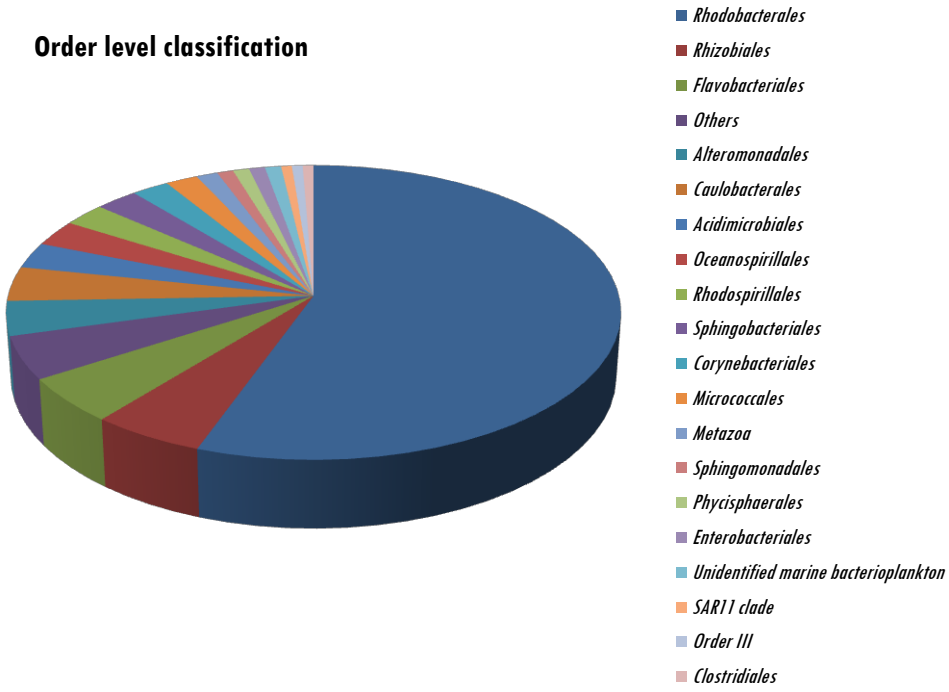
The present study stands for one of the first kind in India which focused on metagenomes associated with marine microalgae, *I. galbana*. Next generation sequencing of 16S rDNA V3 region unravel the entire diversity of bacteria allied with *I. galbana*. A total of 582020 sequence reads were obtained, and after filtering 490869 reads were retained. From 490869 reads 3084 OTUs were identified. From 3084 total OTUs, 1894 singletons were removed and 1190 OTUs were selected for further analysis. The phylum, class, order, family, genus and species distribution of known bacterial groups based on OTU and reads are shown in Fig. 4.1.


Phylum level classification
Cyanobacteria
Proteobacteria
Haptophyta
Bacteroidetes
Actinobacteria
Planctomycetes
Opisthokonta
Firmicutes
Archaeplastida
SAR
BD1-5

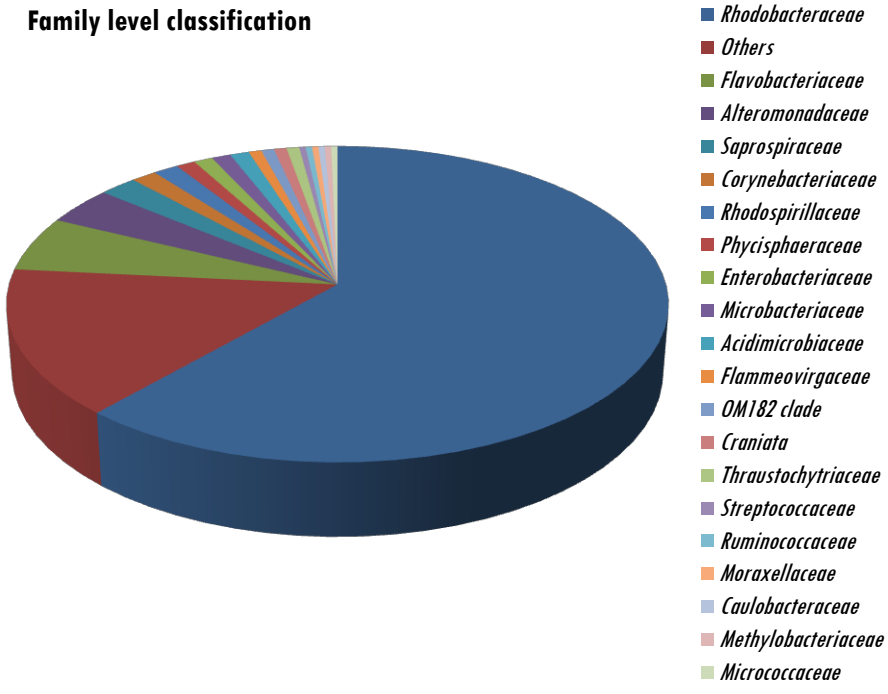
(a)


Class level classification
Alphaproteobacteria
Gammaproteobacteria
Actinobacteria
Flavobacteriia
Acidimicrobiia
Sphingobacteriia
Holozoa
Cytophagia
Deltaproteobacteria
Phycisphaerae
OM190
Chloroplastida
Stramenopiles
Clostridia
Nucletmycea
Bacteroidia
Bacilli

(b)


Order level classification
Rhodobacterales
Rhizobiales
Flavobacteriales
Others
Alteromonadales
Caulobacterales
Acidimicrobiales
Oceanospirillales
Rhodospirillales
Sphingobacteriales
Corynebacteriales
Micrococcales
Metazoa
Sphingomonadales
Phycisphaerales
Enterobacteriales
Unidentified marine bacterioplankton
SAR11 clade
Order III
Clostridiales

(c)


Family level classification
Rhodobacteraceae
Others
Flavobacteriaceae
Alteromonadaceae
Saprospiraceae
Corynebacteriaceae
Rhodospirillaceae
Phycisphaeraceae
Enterobacteriaceae
Microbacteriaceae
Acidimicrobiaceae
Flammeovirgaceae
OM182 clade
Craniata
Thraustochytriaceae
Streptococcaceae
Ruminococcaceae
Moraxellaceae
Caulobacteraceae
Methylobacteriaceae
Micrococcaceae

(d)

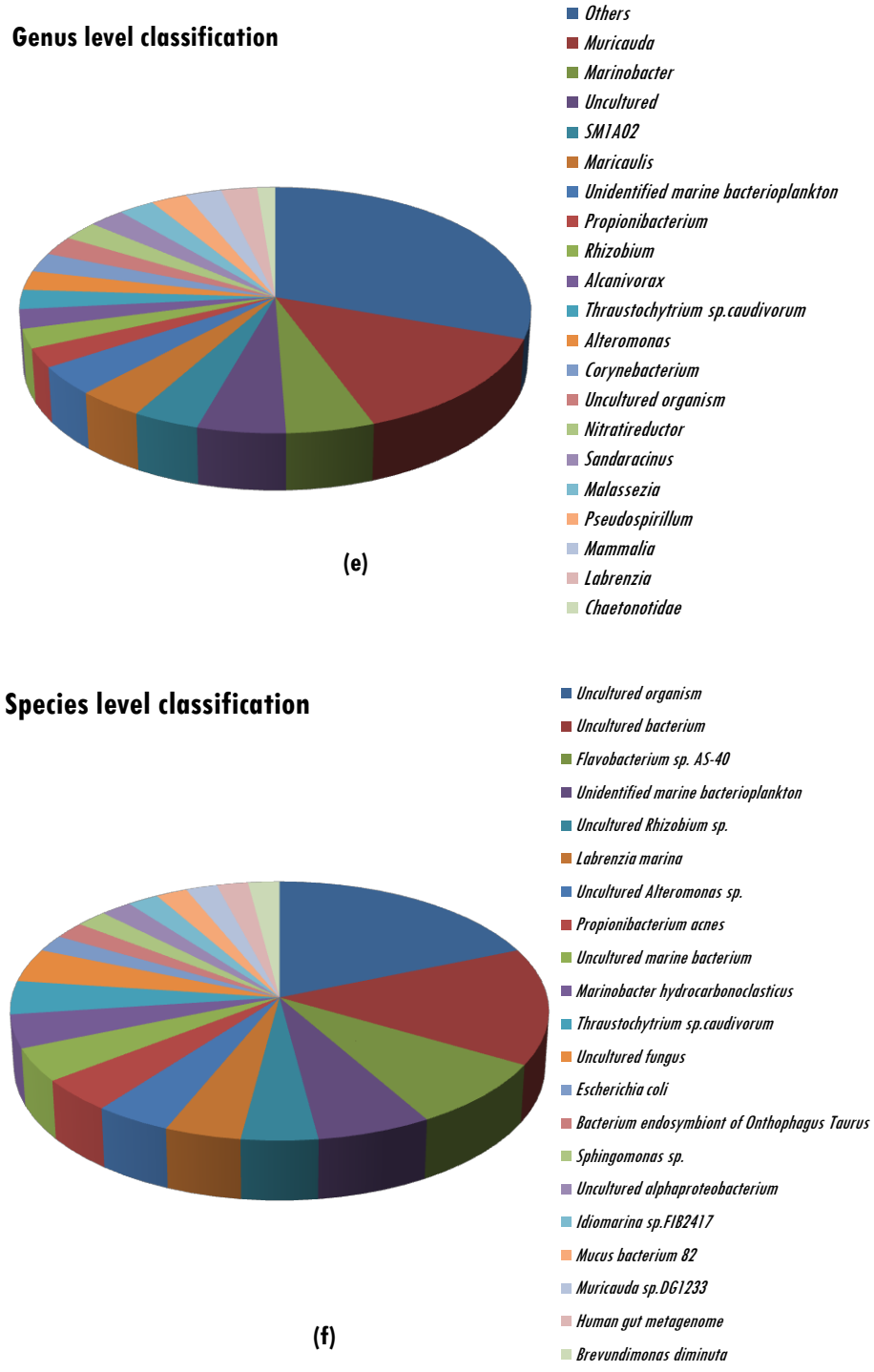


Fig. 4.1. The phylum (a), class (b), order (c), family (d), genus (e) and species (f) distribution of bacterial groups (OTU) associated with *I. galbana*

Table 4.1 Percentage distribution of unknown groups in different taxonomic hierarchy

Category	% OTUs
Phylum	11.68
Class	19.75
Order	73.19
Family	75.97
Genus	93.36
Species	95.57

It should be noted that the taxa other than top 20 are categorized as others. Also, the sequences do not have any alignment against taxonomic database are categorized as unknown. Table 4.1 shows the % OTUs of unknown groups in each taxonomic hierarchy. Further, the microbial diversity was analysed by calculating Shannon, Chao1 and observed species metrics. The rarefaction curve for each of the metric is provided in Fig. 4.2. The chao1 metric estimates the species richness while Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric is the count of unique OTUs identified in the sample.

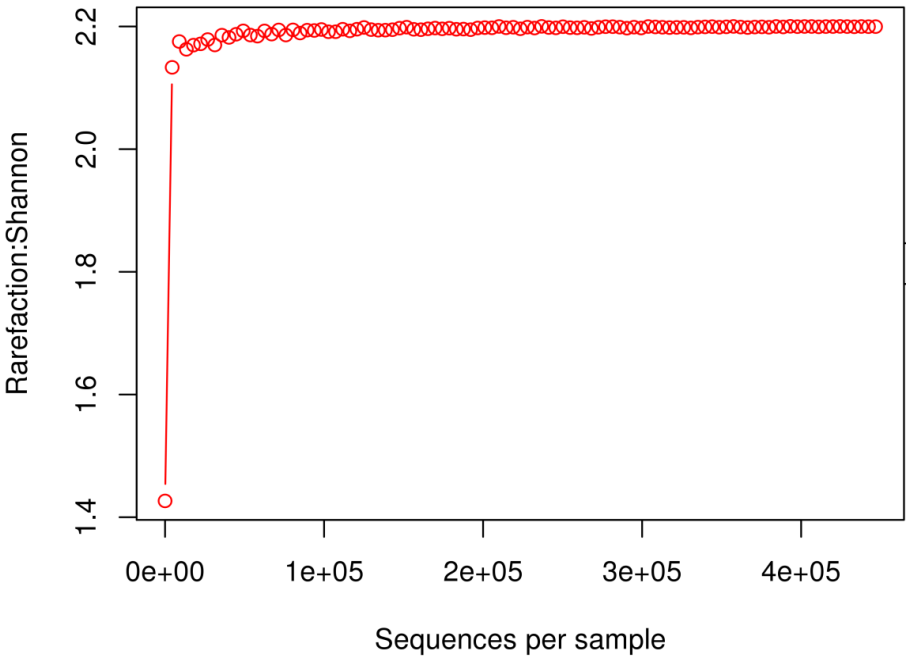
It is widely accepted that more than 99 % of the microorganisms present in many habitats are not readily culturable. Hence, in order to get extensive information about complex microbial communities present in any environment, culture independent approaches are indispensable (Streit and Schmitz 2004). A comparison of bacterial diversity detected in microalgal habitat by culture dependent and independent approaches is given in Table 4.2. Only two bacterial groups belonging to the genera *Alteromonas* and *Labrenzia* were isolated from selected microalgal habitat by culture dependent method (section 2.4). At the same time, the metagenomic approach, as described in this study, has revealed the occurrence of several other bacterial groups in the phycosphere same microalgal strain. A total of 44 different known bacterial genera mostly from the classes *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Flavobacteriia*, *Acidimicrobiia*, *Sphingobacteriia* were detected. Interestingly, these 44 bacterial

genera comprise only 6.64 % of the total OTUs obtained. In addition to these known bacterial groups, numerous unknown bacterial groups were also present in the algal habitat. Thus, the obtained results clearly specify the existence of diverse groups of cultivable and uncultivable bacteria in the phycosphere of *I. galbana*. Most of the bacterial groups identified in this study have previously been found in algal habitat. For example, Amin et al. (2012) reported the consistent association of bacteria belonging to *Alphaproteobacteria* and *Gammaproteobacteria* with different groups of microalgae. Similarly, many previous studies support the occurrence of bacterial genera such as *Marinobacter*, *Alteromonas*, *Flavobacterium*, *Labrenzia* and *Sphingomonas* in algal microhabitat (Jasti et al. 2005, Sapp et al. 2007, Amin et al. 2012, Green et al. 2015, Sandhya et al. 2017).

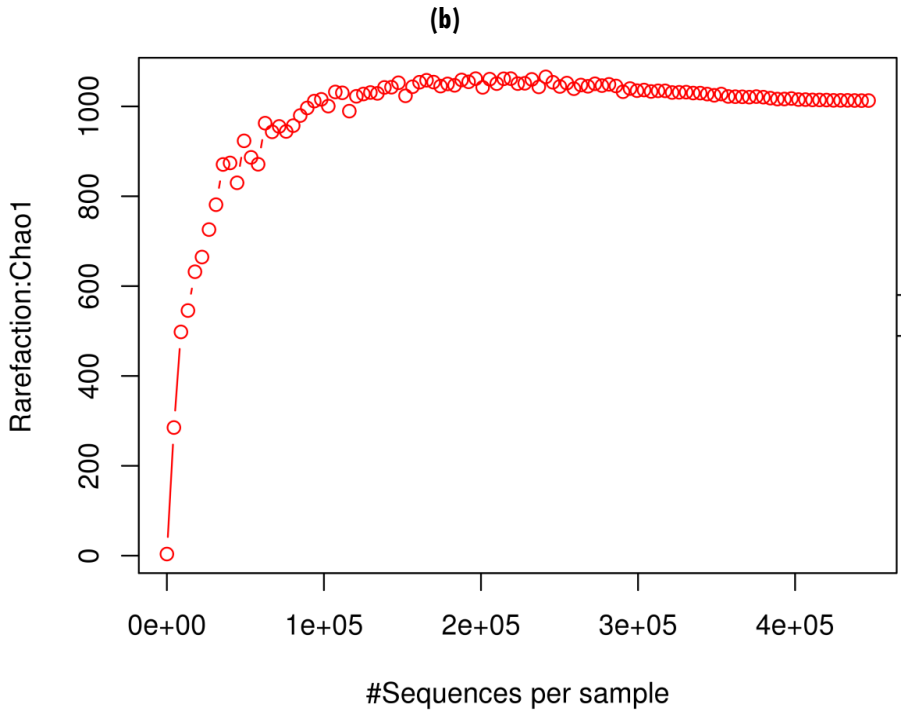
Table 4.2 Comparison of bacterial diversity detected in microalgal habitat (*I. galbana* MBTDCMFRI S002) by culture dependent and independent approaches`

	Bacterial stain distribution on genus level	
Culture dependent approach	*Alteromonas*	*Labrenzia*
Culture independent approach (Next generation sequencing of 16S rDNA V3 region)	*Muricauda*	*Reyranella*
	Marinobacter	*Brevundimonas*
	Uncultured	*Sphingomonas*
	SM1A02	*Tropicimonas*
	Maricaulis	*Ruegeria*
	Unidentified marine bacterioplankton	*Oceanicaulis*
	Propionibacterium	*Rothia*
	Rhizobium	*Roseobacter* clade OCT lineage
	Alcanivorax	*Anaerotruncus*
	Thraustochytrium	AEGEAN-169 marine group
	Alteromonas	*Methylobacterium*
	Corynebacterium	*Liliopsida*
	Uncultured_organism	*Escherichia-Shigella*
	Nitratireductor	*Psychrobacter*
	Sandaracinus	*Youngiibacter*
	Malassezia	*Fodinicurvata*
	Pseudospirillum	*Salinicola*
	Mammalia	*Roseobacter* clade NAC11-7 lineage
	Labrenzia	*Insecta*
	Chaetonotidae	*Klebsiella*
	Streptococcus	*Idiomarina*
	Bacteroides	*Ilumatobacter*
	Muricauda	*Reyranella*

(a)


Rarefaction:Shannon
1.4
1.6
1.8
2.0
2.2
0e+00
1e+05
2e+05
3e+05
4e+05
Sequences per sample

(b)


Rarefaction:Chao1
0
200
400
600
800
1000
0e+00
1e+05
2e+05
3e+05
4e+05
#Sequences per sample

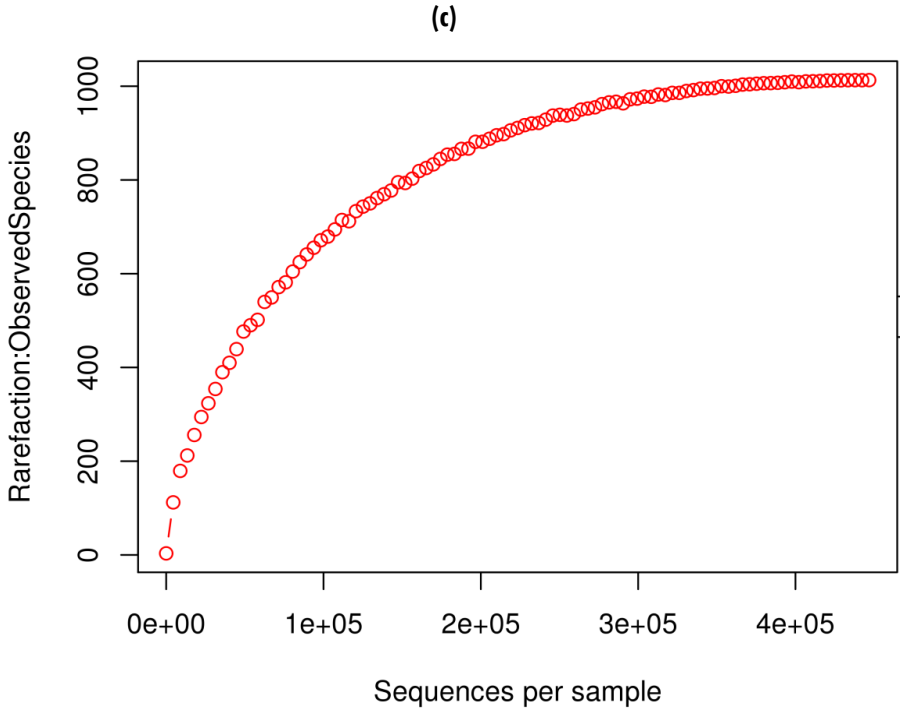


Fig. 4.2. The rarefaction curve for Shannon metric (a) Chao1 metric (b) observed species metric (c)

Metagenome data has been further analysed by PICRUSt (Table 4.3). This evolutionary modelling technique has been used in the present study to predict the functional composition of a metagenome from 16S data and reference genome database (Langille et al. 2013). The obtained results revealed functional role and metabolic capability of bacterial flora associated with *I. galbana*. The major functional modules identified by the analysis include environmental information processing, membrane transport, nutrient metabolism and genetic information processing. These functions might have a significant role in microbial interaction with phytoplankton host. It was found that most number of genes fall into environmental and genetic information processing which reflect the expected abundance of these functions in nature (Nan et al. 2011). Many genes that function in nutrient metabolism (carbohydrate, amino acids, nucleotides), membrane transport (ABC

transporters) and signal transduction were also identified. Improved bacterial survival in any habitat can be facilitated by nutrient uptake and metabolism (Oh et al. 2016). Similarly, signalling between microalgae and bacteria likely a precursor for specific interactions and might enhance bacterial fitness in their phycosphere (Amin et al. 2012). In addition, the presence of extensive energy acquiring mechanism including photosynthesis was also detected in the algal microhabitat. Further, it was noticed that these associated bacteria were able to degrade xenobiotic compounds. The present study opens up the possibilities of using metagenomic approaches for gene mining and bioprospecting.

Overall, the results of the present investigation provided the importance of using metagenomic approaches in the study of phycosphore, where 99 % of the microbes are non-cultivable, and in gene bioprospecting. Here the results showed that phycosphere of *I. galbana* alone harbour a great diversity of microorganisms. By characterising functional profile of associated bacteria, several functional modules with potential positive effects on bacterial interaction with *I. galbana* were identified. Thus, sequence based metagenome analyses in combination with function-based studies significantly enhance our understanding of biodiversity and genetic potential of microbial communities present in algal habitat. In summary, the current study gives detailed insight into algal-bacterial interaction in terms of diversity and functional profile of associated bacteria and many of the characteristics can be further utilised for useful applications.

Table 4.3 Major (list of top 100) KEGG modules present in the phycosphere of *I. galbana*

OTU ID	Count of functional gene	KEGG pathways
Transporters	35447429	Environmental Information Processing; Membrane Transport; Transporters
General function prediction only	23935952	Unclassified; Poorly Characterized; General function prediction only
ABC transporters	21871313	Environmental Information Processing; Membrane Transport; ABC transporters
Photosynthesis proteins	16644527	Metabolism; Energy Metabolism; Photosynthesis proteins
DNA repair and recombination proteins	15114093	Genetic Information Processing; Replication and Repair; DNA repair and recombination proteins
Photosynthesis	13128317	Metabolism; Energy Metabolism; Photosynthesis
Peptidases	13003407	Metabolism; Enzyme Families; Peptidases
Two-component system	12583123	Environmental Information Processing; Signal Transduction; Two-component system
Purine metabolism	12323004	Metabolism; Nucleotide Metabolism; Purine metabolism
Porphyrin and chlorophyll metabolism	12236839	Metabolism; Metabolism of Cofactors and Vitamins; Porphyrin and chlorophyll metabolism
Ribosome	11532093	Genetic Information Processing; Translation; Ribosome
Chromosome	9385660	Genetic Information Processing; Replication and Repair; Chromosome
Oxidative phosphorylation	9078679	Metabolism; Energy Metabolism; Oxidative phosphorylation
Secretion system	8806264	Environmental Information Processing; Membrane Transport; Secretion system
Function unknown	8448876	Unclassified; Poorly Characterized; Function unknown
Others	8315699	Unclassified; Metabolism; Others
Arginine and proline metabolism	8186979	Metabolism; Amino Acid Metabolism; Arginine and proline metabolism
Pyrimidine metabolism	7922248	Metabolism; Nucleotide Metabolism; Pyrimidine metabolism
Amino acid related enzymes	7108754	Metabolism; Amino Acid Metabolism; Amino acid related enzymes
Amino sugar and nucleotide sugar metabolism	7057529	Metabolism; Carbohydrate Metabolism; Amino sugar and nucleotide sugar metabolism
Bacterial motility proteins	6980830	Cellular Processes; Cell Motility; Bacterial motility proteins
Glycolysis / Gluconeogenesis	6946214	Metabolism; Carbohydrate Metabolism; Glycolysis / Gluconeogenesis
Chaperones and folding catalysts	6863116	Genetic Information Processing; Folding, Sorting and Degradation; Chaperones and folding catalysts
Pyruvate metabolism	6665683	Metabolism; Carbohydrate Metabolism; Pyruvate metabolism
Replication, recombination and repair proteins	6438722	Unclassified; Genetic Information Processing; Replication, recombination and repair proteins
Ribosome Biogenesis	6427515	Genetic Information Processing; Translation; Ribosome Biogenesis
Methane metabolism	6383639	Metabolism; Energy Metabolism; Methane metabolism
Protein folding and associated processing	6289864	Unclassified; Genetic Information Processing; Protein folding and associated processing
Aminoacyl-tRNA biosynthesis	6123326	Genetic Information Processing; Translation; Aminoacyl-tRNA biosynthesis
Transcription factors	5685363	Genetic Information Processing; Transcription; Transcription factors

OTU ID	Count of functional gene	KEGG pathways
Alanine, aspartate and glutamate metabolism	5409575	Metabolism; Amino Acid Metabolism; Alanine, aspartate and glutamate metabolism
Starch and sucrose metabolism	5290747	Metabolism; Carbohydrate Metabolism; Starch and sucrose metabolism
DNA replication proteins	5280034	Genetic Information Processing; Replication and Repair; DNA replication proteins
Translation proteins	5210750	Unclassified; Genetic Information Processing; Translation proteins
Other ion-coupled transporters	5202112	Unclassified; Cellular Processes and Signaling; Other ion-coupled transporters
Pentose phosphate pathway	5031210	Metabolism; Carbohydrate Metabolism; Pentose phosphate pathway
Carbon fixation in photosynthetic organisms	4544810	Metabolism; Energy Metabolism; Carbon fixation in photosynthetic organisms
Carbon fixation pathways in prokaryotes	4528574	Metabolism; Energy Metabolism; Carbon fixation pathways in prokaryotes
Cysteine and methionine metabolism	4517463	Metabolism; Amino Acid Metabolism; Cysteine and methionine metabolism
Valine, leucine and isoleucine biosynthesis	4482610	Metabolism; Amino Acid Metabolism; Valine, leucine and isoleucine biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis	4429212	Metabolism; Amino Acid Metabolism; Phenylalanine, tyrosine and tryptophan biosynthesis
Homologous recombination	4410033	Genetic Information Processing; Replication and Repair; Homologous recombination
Fructose and mannose metabolism	4378575	Metabolism; Carbohydrate Metabolism; Fructose and mannose metabolism
Transcription machinery	4325270	Genetic Information Processing; Transcription; Transcription machinery
Glycine, serine and threonine metabolism	4235382	Metabolism; Amino Acid Metabolism; Glycine, serine and threonine metabolism
Protein kinases	4197492	Metabolism; Enzyme Families; Protein kinases
Lipid biosynthesis proteins	4125167	Metabolism; Lipid Metabolism; Lipid biosynthesis proteins
Ubiquinone and other terpenoid-quinone biosynthesis	4040474	Metabolism; Metabolism of Cofactors and Vitamins; Ubiquinone and other terpenoid-quinone biosynthesis
Peptidoglycan biosynthesis	4006813	Metabolism; Glycan Biosynthesis and Metabolism; Peptidoglycan biosynthesis
Mismatch repair	3986206	Genetic Information Processing; Replication and Repair; Mismatch repair
Pores ion channels	3948918	Unclassified; Cellular Processes and Signaling; Pores ion channels
Signal transduction mechanisms	3857111	Unclassified; Cellular Processes and Signaling; Signal transduction mechanisms
Glycosyltransferases	3846287	Metabolism; Glycan Biosynthesis and Metabolism; Glycosyltransferases
Pantothenate and CoA biosynthesis	3807526	Metabolism; Metabolism of Cofactors and Vitamins; Pantothenate and CoA biosynthesis
RNA degradation	3725658	Genetic Information Processing; Folding, Sorting and Degradation; RNA degradation
Nitrogen metabolism	3723136	Metabolism; Energy Metabolism; Nitrogen metabolism
Energy metabolism	3439693	Unclassified; Metabolism; Energy metabolism
Terpenoid backbone biosynthesis	3373341	Metabolism; Metabolism of Terpenoids and Polyketides; Terpenoid backbone biosynthesis
Membrane and intracellular structural molecules	3324443	Unclassified; Cellular Processes and Signaling; Membrane and intracellular structural molecules
Photosynthesis - antenna proteins	3288824	Metabolism; Energy Metabolism; Photosynthesis - antenna proteins
Glyoxylate and dicarboxylate metabolism	3275023	Metabolism; Carbohydrate Metabolism; Glyoxylate and dicarboxylate metabolism
Prenyltransferases	3255574	Metabolism; Metabolism of Terpenoids and Polyketides; Prenyltransferases

OTU ID	Count of functional gene	KEGG pathways
Lysine biosynthesis	3237044	Metabolism; Amino Acid Metabolism; Lysine biosynthesis
Glutathione metabolism	3218649	Metabolism; Metabolism of Other Amino Acids; Glutathione metabolism
Cell cycle - Caulobacter	3208479	Cellular Processes; Cell Growth and Death; Cell cycle — Caulobacter
Protein export	3165319	Genetic Information Processing; Folding, Sorting and Degradation; Protein export
Histidine metabolism	3086446	Metabolism; Amino Acid Metabolism; Histidine metabolism
Butanoate metabolism	2983385	Metabolism; Carbohydrate Metabolism; Butanoate metabolism
Folate biosynthesis	2955062	Metabolism; Metabolism of Cofactors and Vitamins; Folate biosynthesis
Bacterial secretion system	2905789	Environmental Information Processing; Membrane Transport; Bacterial secretion system
Fatty acid metabolism	2863083	Metabolism; Lipid Metabolism; Fatty acid metabolism
Fatty acid biosynthesis	2835300	Metabolism; Lipid Metabolism; Fatty acid biosynthesis
One carbon pool by folate	2832399	Metabolism; Metabolism of Cofactors and Vitamins; One carbon pool by folate
Propanoate metabolism	2791332	Metabolism; Carbohydrate Metabolism; Propanoate metabolism
DNA replication	2777888	Genetic Information Processing; Replication and Repair; DNA replication
Translation factors	2721737	Genetic Information Processing; Translation; Translation factors
Sulfur relay system	2691233	Genetic Information Processing; Folding, Sorting and Degradation; Sulfur relay system
Nicotinate and nicotinamide metabolism	2591603	Metabolism; Metabolism of Cofactors and Vitamins; Nicotinate and nicotinamide metabolism
Tyrosine metabolism	2589543	Metabolism; Amino Acid Metabolism; Tyrosine metabolism
Citrate cycle (TCA cycle)	2534168	Metabolism; Carbohydrate Metabolism; Citrate cycle (TCA cycle)
Thiamine metabolism	2479804	Metabolism; Metabolism of Cofactors and Vitamins; Thiamine metabolism
Streptomycin biosynthesis	2475931	Metabolism; Biosynthesis of Other Secondary Metabolites; Streptomycin biosynthesis
Glycerophospholipid metabolism	2374599	Metabolism; Lipid Metabolism; Glycerophospholipid metabolism
Cytoskeleton proteins	2257504	Cellular Processes; Cell Motility; Cytoskeleton proteins
Valine, leucine and isoleucine degradation	2252466	Metabolism; Amino Acid Metabolism; Valine, leucine and isoleucine degradation
Tuberculosis	2225048	Human Diseases; Infectious Diseases; Tuberculosis
Base excision repair	2138833	Genetic Information Processing; Replication and Repair; Base excision repair
Sulfur metabolism	2109318	Metabolism; Energy Metabolism; Sulfur metabolism
Glycerolipid metabolism	2102887	Metabolism; Lipid Metabolism; Glycerolipid metabolism
Riboflavin metabolism	2061369	Metabolism; Metabolism of Cofactors and Vitamins; Riboflavin metabolism
Selenocompound metabolism	1957284	Metabolism; Metabolism of Other Amino Acids; Selenocompound metabolism
Galactose metabolism	1910964	Metabolism; Carbohydrate Metabolism; Galactose metabolism
Amino acid metabolism	1872989	Unclassified; Metabolism; Amino acid metabolism
Lipopolysaccharide biosynthesis proteins	1774118	Metabolism; Glycan Biosynthesis and Metabolism; Lipopolysaccharide biosynthesis proteins

OTU ID	Count of functional gene	KEGG pathways
beta-Alanine metabolism	1711824	Metabolism; Metabolism of Other Amino Acids; beta-Alanine metabolism
Drug metabolism - cytochrome P450	1711131	Metabolism; Xenobiotics Biodegradation and Metabolism; Drug metabolism - cytochrome P450
Metabolism of xenobiotics by cytochrome P450	1693711	Metabolism; Xenobiotics Biodegradation and Metabolism; Metabolism of xenobiotics by cytochrome P450
Nucleotide excision repair	1693050	Genetic Information Processing; Replication and Repair; Nucleotide excision repair
Cell motility and secretion	1672804	Unclassified; Cellular Processes and Signaling; Cell motility and secretion
Other transporters	1663865	Unclassified; Cellular Processes and Signaling; Other transporters

Chapter 5

Symbiotic association among marine microalgae and bacterial flora: A study with special reference to culturable heterotrophic bacteria of commerically important *Isochrysis galbana* culture

Contents

5.1 Abstract
5.2 Introduction
5.3 Materials and Methods
5.4 Results and Discussion

5.1 Abstract

Bacterial association plays a significant role in massive culture and production system of microalgae, which is used in finfish and shellfish hatcheries as live feed and also as products such as algal paste, spray dried and freeze dried algal powders. In the present study the interactions between *Isochrysis galbana* MBTDCMFRI S002 and its associated bacteria were studied. The nutrient profile of axenic and non-axenic *I. galbana* cultures was compared based on the levels of protein, fatty acid, pigment and carbohydrate. Increased levels of these nutrients in non-axenic culture indicate a positive effect of associated bacteria in algal biomass composition. The interaction between *I. galbana* and associated bacteria was further investigated by co-culturing axenic algal culture with two bacterial symbionts – *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26. There was an increase in algal biomass

accumulation and growth rate in the presence of added bacterial symbionts which specify their growth enhancing role in the algal culture. The heterotrophic growth of these bacterial strains on extra cellular carbon produced by *I. galbana*, a typical symbiotical association between bacterial flora and microalgae, has been evident in the present study. Thus, findings of the present investigation proved the existence of a mutually benefitted interaction and association between *I. galbana* and coexisting bacterial flora.

5.2 Introduction

Microalgae, the primary producers of aquatic food web undertake wide range of mutualistic interactions with bacteria (Cooper and Smith 2015). In addition to nutrient exchange, these interactions might have a dynamic effect on biogeochemical cycling in aquatic world (Ramanan et al. 2015). Thus this mutualistic interaction in which both the symbionts are benefitted do play an important role in natural ecosystem and contribute usefully to the growth and health of algal biomass (Watanabe et al. 2005, Fuentes et al. 2016). However, due to the complexity of microalgal-bacterial interactions, knowledge generated is limited and utilisation is still based on empirical knowledge (Guo and Tong 2014). In this backdrop, a better understanding of these symbiotic mechanisms is crucial to invigorate their combined biotechnological potential (Watanabe et al. 2005, Ramanan et al. 2016).

The bacterial communities associated with microalgal cultures play a ubiquitous role in algal growth and survival (Amin et al. 2015, Ramanan et al. 2016). They may have a positive effect on algal growth through breakdown of organic metabolites or through the production of various algal growth promoting substances (Salvesen et al. 2000). The algal growth stimulatory compounds produced by the bacteria include vitamin like cobalamin, indole-3-

acetic acid, siderophore etc. (Cole 1982, Croft et al. 2005, de-Bashan and Bashan 2008). Moreover, many aerobic bacteria provide favourable environmental condition (eg: reduced oxygen tension) for microalgal growth (Mouget et al. 1995). The stimulatory effect of associated bacteria on physiological state of microalgae can be manifested by increased chlorophyll content, higher biomass production and more stable microalgal culture with delayed death phase (Natrah et al. 2014). The bacterial counterpart in turn may be benefitted by uptake of extracellular organic carbon (EOC) released during microalgal photosynthesis (Watanabe et al. 2005). Thus, they can survive in microalgal habitat without the need of additional carbon source (Natrah et al. 2014). Also, it was reported that many nutrients released by microalgae can act as chemoattractants for marine heterotrophic bacteria which enable them to move towards their phytoplankton host (Miller et al. 2004).

Overall, it is known that microalgae-bacteria interaction can improve and enrich algal biomass production. Nevertheless, in aquaculture, very little attention has been paid to increase the knowledge aspect of microalgal-bacterial symbiotic associations. Many previous investigators considered these bacteria as mere contamination of algal cultures (Watanabe et al. 2005, Fuentes et al. 2016). In this background, the present study is taken up with this objective to explore symbiotic interaction between the marine microalgae, *Isochrysis galbana* MBTDCMFRI S002 and its bacterial associates. The study evaluates nutrient profile of axenic and non-axenic culture of *I. galbana*, effect of bacterial symbionts on microalgal growth and growth of bacterial symbionts on algal EOC.

5.3 Materials and Methods

5.3.1 Bacterial symbionts

Two bacterial symbionts which were isolated from *I. galbana* (MBTDCMFRI S002) culture and preserved as glycerol stocks at microbial culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) were used for this study. They include *Alteromonas* sp. MBTDCMFRI Mab 25 and *Labrenzia* sp. MBTDCMFRI Mab 26.

5.3.2 Axenisation of *I. galbana* culture

Isochrysis galbana MBTDCMFRIS002 maintained at microalgae culture collection of Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) was used for the study. Before axenisation, the associated bacterial strains were screened for their sensitivity towards commercially used antibiotics such as penicillin (P-10 U), streptomycin (S-10 mcg), gentamicin (G-10 mcg) and kanamycin (K-10 mcg) (Himedia, India) by standard disc diffusion method (Bauer et al. 1966). The axenisation of *Isochrysis galbana* MBTDCMFRIS002 was carried out by using a cocktail of above antibiotics at four different concentrations according to the procedure described by Droop (1967) and Andersen (2005). The final ratio of antibiotic concentration in treated culture was 1000:5:5:2.5/ 500:2.5:2.5:1.25/ 250:1.25:1.25:0.6 and 125:0.6:0.6:0.3 µg/ml of penicillin, streptomycin, gentamicin and kanamycin respectively. After 48 h of antibiotic treatment, microalgal culture was inoculated to fresh F/2 medium and incubated under optimum photoautotrophic conditions for 25 days. After incubation, the culture broth was inoculated on Zobell Marine Agar (ZMA) (Himedia, India) to confirm that bacterial symbionts had been removed from *I.*

galbana strain. The obtained axenic culture of *I. galbana* was maintained at microalgae culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin in sterilized sea water (33 ±1 ppt) enriched with F/2 medium (Anderson, 2005) at 22± 1° C under light-dark conditions (16:8 h, 40-50 µmol photons $m^{-2}s^{-1}$).

5.3.3 Comparison of nutrient profile of axenic and non-axenic *I. galbana* culture

For comparison of nutrient profile, equal cell density of axenic and non-axenic *I. galbana* culture were inoculated to sterile F/2 medium and incubated at 22± 1° C under light-dark conditions (16:8 h, 40-50 µmol photons $m^{-2}s^{-1}$). The nutrient profile of both cultures was estimated when they reached at early stationary phase of growth.

5.3.3.1 Total protein analysis

For protein estimation, 20 mg of lyophilised microalgal sample was grind thoroughly with glass beads. It was then homogenised in 10 ml lysis buffer (Triton X: 5 ml/L, EDTA Na: 0.3722 g/L , Phenyl Methyl Sulphonyl Fluoride: 0.0348 g/L) and kept at room temperature for 20 min. 1 ml of the above sample was taken and total protein content was estimated by Lowry's method (Lowry et al. 1951, Wilson and Walker 2000).

5.3.3.2 Total fatty acid analysis

Total lipids from lyophilised microalgal samples (10 mg) were extracted following Bligh and Dyer (1959). From the extracted lipids, fatty acid methyl esters were prepared as per Metcalf et al. (1966). Fatty acid composition of the samples was determined by gas liquid chromatography (GLC) with FID detector (GC 2010^{plus}, Shimadzu, Japan). Fatty acids were identified based on comparison with the retention times of commercial

standards and the concentration of each fatty acid was calculated for the corresponding area of the chromatogram (Supelco FAME 37 standard).

5.3.3.3 Total pigment analysis

The total pigment content of axenic and non-axenic *I. galbana* culture was estimated by methanol extraction method (Lichtenthaler and Wellburn 1983, Henriques et al. 2007). The concentration of pigments was calculated according to the equation,

$$\mu g \text{ pigment/ml medium} = \frac{15.65A_{666} \times \text{volume of extract}}{\text{volume of sample}}$$

Where, A_{666} is the absorbance at 666nm against a blank of methanol (Thermo Scientific, US).

5.3.3.4 Total carbohydrate analysis

Total carbohydrate was estimated separately in the soluble and cell-associated fractions as described by Bruckner et al. (2008). The microalgal culture was centrifuged at 6000 rpm for 10 minutes (16°C) (Remi, India). Then the supernatant containing soluble extracellular polysaccharides was separated from the pellet. The pellet was resuspended in 5 ml sterile distilled water and incubated at 200 rpm for 1 h at room temperature. After centrifugation at 6000 rpm for 10 minutes, the obtained supernatant contained the bound extracellular polysaccharides. Carbohydrate contents of soluble and bound extracellular polysaccharides were measured optically by phenol-sulphuric acid method (Albalasmeh et al. 2013).

5.3.4 Effect of bacterial symbionts on algal growth

To study the effect of bacterial symbionts on microalgal growth, each bacterial strain was precultured as mono cultures on heterotrophic liquid medium (Zobell Marine Broth, Himedia, india). After 48 h of incubation (200 rpm, 30° C), bacterial culture broth was centrifuged at 8000 rpm for 15

minutes (Remi, India), washed three times and finally resuspended in sterile F/2 medium. 1 ml of each symbiont solution ($1x10^8$ cells ml^{-1}) was mixed with 10 ml of axenic *I. galbana* culture in 250 ml Erlenmayer flask containing 100 ml sterile F/2 medium. Effect of mixed culture of associated bacteria (with both symbionts) was also investigated. Axenic and non-axenic *I. galbana* cultures were used as control (Watanabe et al. 2005). The growth of microalgae was monitored by taking cell count at regular time interval using hemocytometer till day 24. The specific growth rate was calculated according to the equation (Guilard 1973)

$$\text{Specific growth rate, } \mu = \frac{\ln (N_t/N_o)}{t_t - t_0}$$

Where N_t - cell density at the end of time interval

N_o - cell density at the beginning of time interval

t_t-t_0 - length of time interval

5.3.5 Growth of bacterial symbionts on algal EOC

The heterotrophic growth of bacterial symbionts on EOC produced by *I. galbana* was examined as described by Watanabe et al. (2005). The culture broth containing algal EOC as carbon source was prepared by cultivating the axenic *I. galbana* using modified ASN III medium (without any carbon sources) (Andersen 2005) under photoautotrophic condition for three weeks. The culture broth was centrifuged (5000 rpm, 10 min, 4°C) to remove the cells, and the obtained supernatant was filtered through a sterile 0.2 μm membrane filter (Pall, USA). This filtrate was mixed with an equal volume of the sterile modified ASN III medium for the supplement of inorganic salt. Sterile medium without algal EOC was kept as control. Each bacterial symbiont was inoculated to both test and control cultures and incubated at 30°C without illumination (200 rpm). The growth of each symbiont was monitored at OD_{600} (Thermo Scientific, US).

5.3.6 Statistical analysis

The obtained results were subjected to statistical analysis and the means of all parameters were examined for significance ($p < 0.01$) using R software (Version 2.3-0).

5.4 Results and Discussion

Survival of bacterial strains such as *Alteromonas* sp. MBTDCMFRI Mab 25 (GenBank Accession No KR004801) and *Labrenzia* sp. MBTDCMFRI Mab 26 (GenBank Accession No KR004822) even in the laboratory maintained culture of *I. galbana* clearly indicates the existence of a strong and close association between them. Thus these bacterial strains such as *Alteromonas* sp. and *Labrenzia* sp. are of special interest; even if they represent a subset of larger symbiotic consortium exist in the microalgal habitat. The focus of the present study is to explore the symbiotic interaction among these isolated bacterial genera and *I. galbana*. Such studies required axenic culture of microalgae which are devoid of any bacterial symbionts. Hence, *I. galbana* culture was axenised using a cocktail of four antibiotics (penicillin, streptomycin, gentamicin and kanamycin) to which the associated bacterial strains were susceptible (Fig. 5.1). The antibiotic concentration of 1000:5:5:2.5 µg/ml of penicillin, streptomycin, gentamicin and kanamycin respectively for 48 h was found to be most effective in inhibiting bacterial growth in microalgal culture.

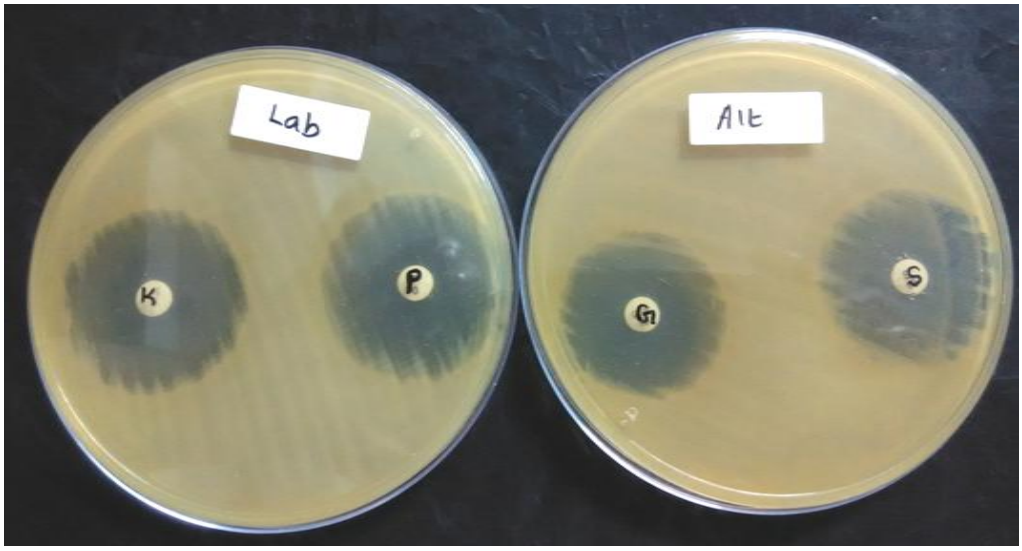
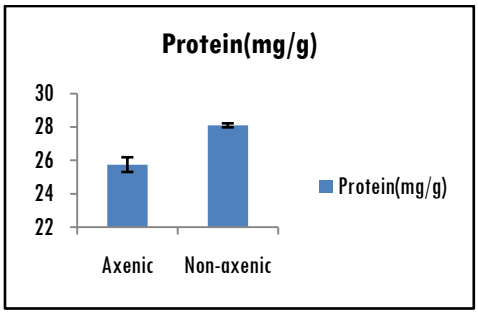


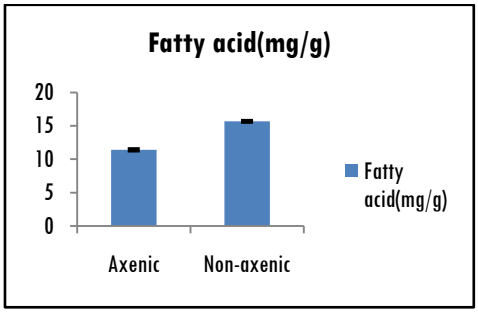
Fig. 5.1. Antibiotic susceptibility pattern of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26) by disc diffusion assay

To evaluate the role of associated bacteria on algal biomass composition, the nutrient profile of axenic and non-axenic *I. galbana* culture was compared. The level of four major nutrients such as proteins, fatty acids, pigment and carbohydrate were estimated and the results showed that non-axenic culture was superior to axenic culture (Fig. 5.2). Total protein content of non-axenic culture was 28.09 ± 0.12 mg/g whereas that of axenic culture was 25.73 ± 0.44 mg/g. Also, the chromatographic analysis demonstrated that non-axenic *I. galbana* culture produces more fatty acid (15.7 ± 0.17 mg/g) in the early stationary phase of growth than axenic *I. galbana* culture (11.4 ± 0.2 mg/g) (Fig. 5.3). Similarly, non-axenic culture possessed more pigment and carbohydrate content (9.32 ± 1.33 µg/ml and 67.59 ± 1.32 µg/ml) than axenic culture (7.01 ± 0.57 µg/ml and 45.85 ± 1.01 µg/ml). The obtained results were statistically analysed using an independent samples t-test. The analysis revealed that there was a significant difference in the level of each nutrient at 99 % confidence level interval

(p<0.01). The improved chemical composition of non-axenic *I. galbana* culture might be due to the presence of associated bacteria. Algal growth stimulation of bacteria coupled with enhancement of the intracellular levels of carbohydrates, lipids and pigments was previously reported (Fuentes et al. 2016). The results of the present work is in agreement with the findings of de-Bashan et al. (2002) who reported that the total lipid and pigment content of the *Chlorella s*pp. was increased when they were co-immobilised in alginate beads with microalgae growth promoting bacterium *Azospirillum brasilense*. Similarly, Bruckner et al. (2008) reported that there was an increased secretion of polysaccharides by the diatom in the presence of *Proteobacteria*. The obtained results in the present investigation found to be in total agreement with the study of Grossart and Simon (2007) in which they obtained lower concentration of dissolved carbohydrates in axenic cultures than in cultures inoculated with natural bacterial community. Also, many fatty acids and their derivatives have antibacterial activity against wide range of pathogenic bacteria (Shin et al. 2007, Nobmann et al. 2010). Hence increased fatty acid profile might also contribute to the defence strategy of microalgal species. In aquaculture hatchery rearing systems, *I. galbana* is normally supplied at the larval stage and nutrient composition of algal diet definitely determines the end nutritional quality of rearing animal (Molina- Cardenas et al. 2014, Fuentes et al. 2016). Thus it is evident that chemical composition of algal live feed is certainly a key factor in aquaculture. From the current study it is clear that it is better to use non-axenic cultures for algal biomass production processes. In this respect, microalgal-bacterial interaction deserves greater attention in various aquaculture systems.



(a)



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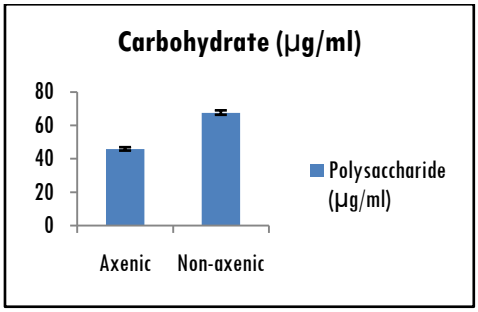

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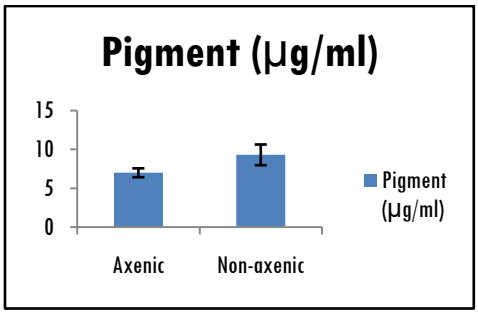

(d)

Fig. 5.2. Nutrient profile of axenic and non-axenic culture of *I. galbana* MBTDCMFRI S002

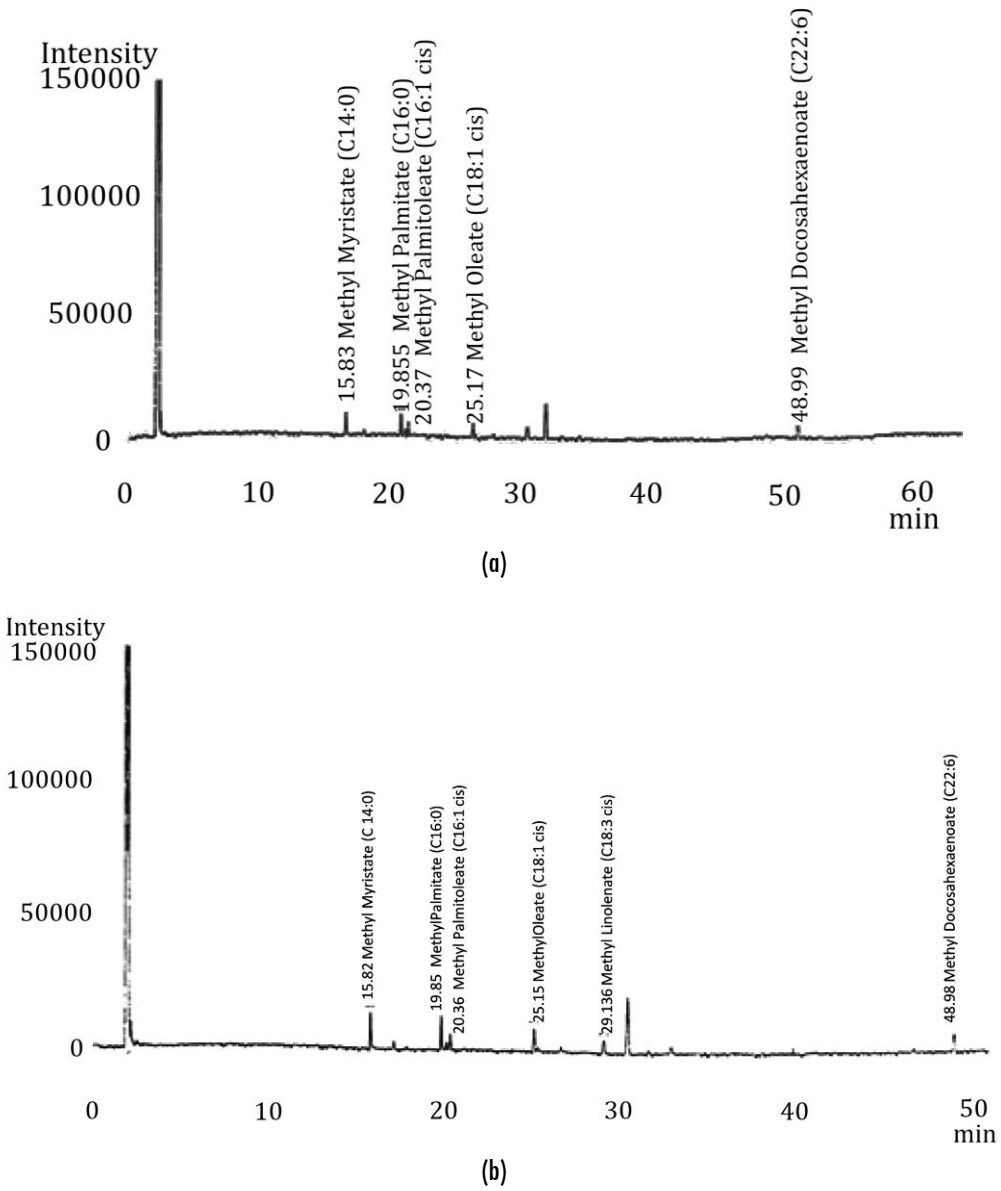

Fig. 5.3. Chromatogram showing fatty acid composition of axenic (a) and non-axenic (b) culture of *I. galbana* MBTDCMFRI S002

Axenic strain of *I. galbana* was cultivated along with bacterial symbionts to investigate the effect of associated bacteria on microalgal growth. Time course of cell count of test and control cultures are shown in Fig. 5.4. Effect of bacterial addition on biomass accumulation and growth rate of *I. galbana* at day 24 are

shown in Fig. 5.5. It was found that there was an increase in both biomass accumulation and growth rate of axenic *I. galbana* culture in the presence of bacterial symbionts. The biomass accumulation of axenic culture of *I. galbana* was increased from 2.54 ± 0.36 x 10^6 cells/ml to 3.87 ± 0.47 x 10^6 cells/ml in the presence of *Alteromonas* sp. Mab 25. Similarly, in the presence of *Labrenzia* sp. Mab 26, the biomass accumulation was increased up to 4.35 ± 0.32 x 10^6 cells/ml. Interestingly, it was noticed that even in the presence of added bacterial symbionts, the growth rate and biomass accumulation of axenic culture was less than that of non-axenic culture (8.55 ± 1.17 x 10^6 cells/ml). The enhanced growth of non-axenic culture might be due to the undetectable symbionts in microalgal habitat. The difference in the growth rate was statistically analysed using a one way ANOVA. The obtained results were found to be statistically significant at 99 % confidence level interval (p<0.01). At the same time, Bonferroni post-hoc test revealed that there was no significant difference in the growth promoting effect of bacterial symbionts when they were inoculated individually and as a consortium (p = 1.00).

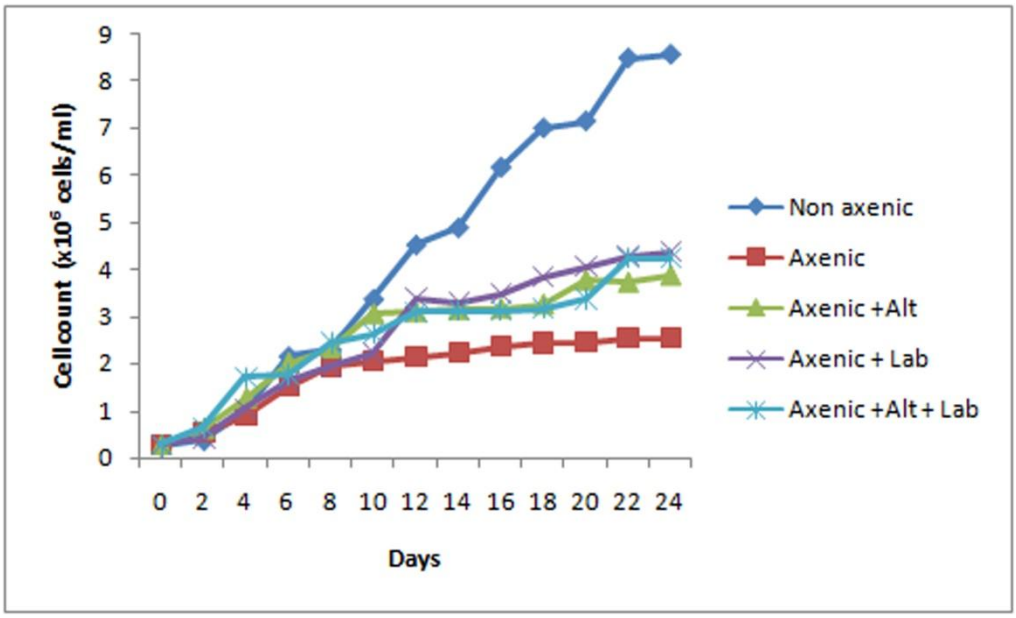

Fig. 5.4. Growth pattern of *I. galbana* MBTDCMFRI S002 in the presence and absence of associated bacteria

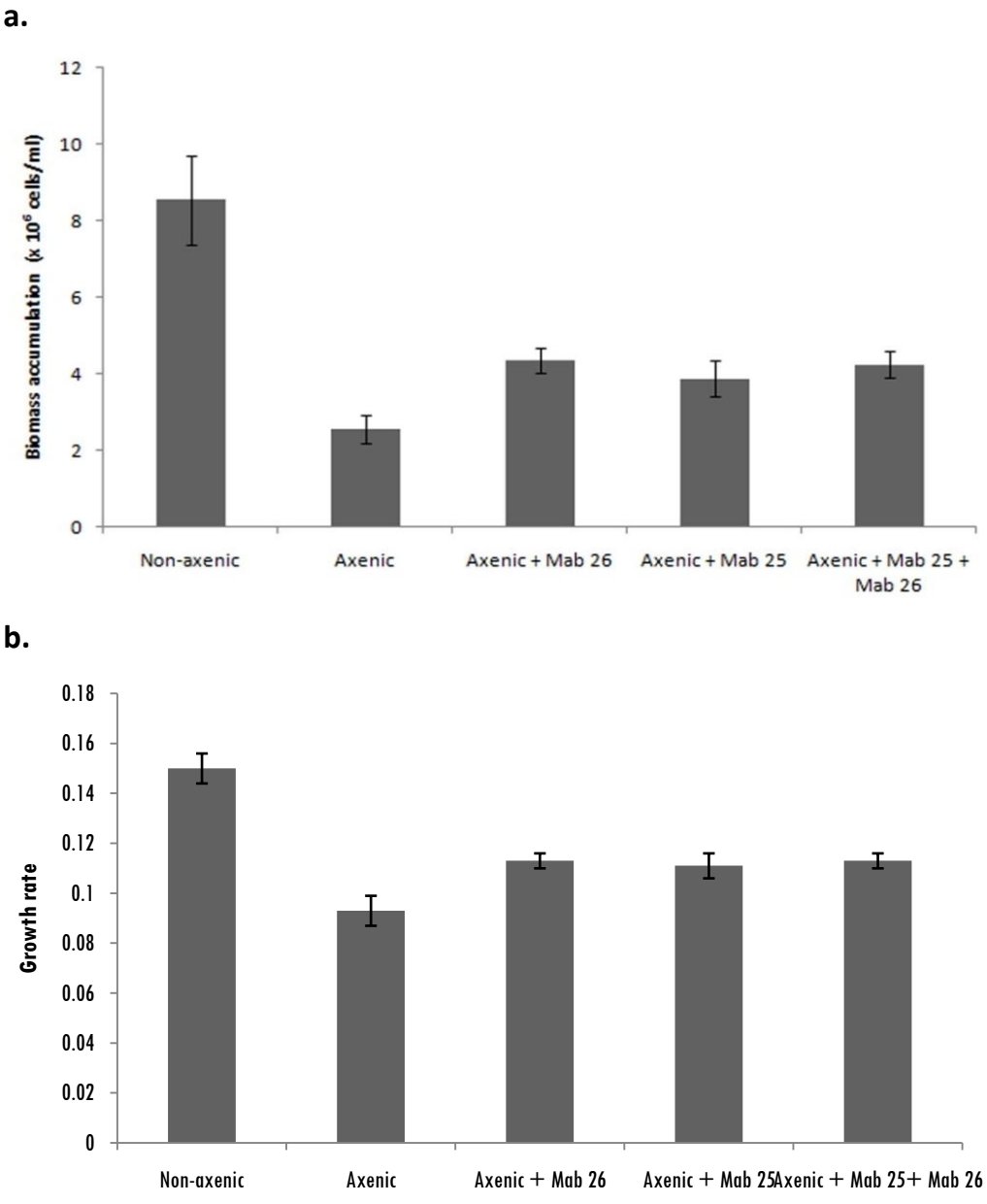

Fig. 5.5. Effect of bacterial addition in biomass accumulation (a) and growth rate (b) of *I. galbana* MBTDCMFRI S002

In this study, growth enhancement of *I. galbana* was observed when co-cultured with bacterial symbionts. The stimulatory effects of bacteria on

microalgal growth have been widely studied. Mouget et al. (1995) reported that there was an increase in the maximum cell density of *Scenedesmus bicellularis* associated with strains of *Brevundimonas diminuta.* Le Chevanton et al. (2013) observed that the strains of bacteria affiliated to *Alteromonas sp*. and *Muricauda sp*. can help for nitrogen accumulation in *Dunaliella sp* and can enhance biomass accumulation. The exact mechanisms of these positive effects bought by associated bacteria are largely unknown (Natrah et al. 2014). But it was reported that bacteria can produce various growth stimulatory compounds, especially in symbiotic situations (Cole 1982, Amin et al. 2015). There was a previous report on bacterial production of vitamin required by microlalgae (Guo and Tong 2014). Durham et al. (2015) reported that vitamin B_{12} produced by *Ruegeria pomeroyi* have a positive effect on the growth of *Thalassiosira pseudonana*. Bacteria can secrete hormones like indole-3-acetic acid (IAA) which can stimulate algal metabolism (Natrah et al. 2014). Similarly siderophores synthesised by bacterial counterparts can provide a steady supply of soluble irons to algal cells (Cooper and Smith 2015). These algal growth promoters produced by associated bacteria might have a significant positive impact on algal growth. In addition to the release of growth factors, bacteria can remineralise inorganic nutrients and CO_2 for the use of microlagae (Natrah et al. 2014).

The growth of bacterial symbionts in algal EOC was also examined. Both bacterial strains were able to grow in culture broth containing algal EOC as carbon source. Concurrently, they showed no growth in the absence of EOC in the medium (control). As shown in Fig. 5.6, *Alteromonas* sp. Mab 25 reached at the stationary phase of growth on day 4 of inoculation whereas *Labrenzia* sp. Mab 26 reached stationary phase on day 6. These results imply

that phytoplankton host can provide organic carbon source for heterotrophic growth of both bacteria under photoautotrophic conditions.

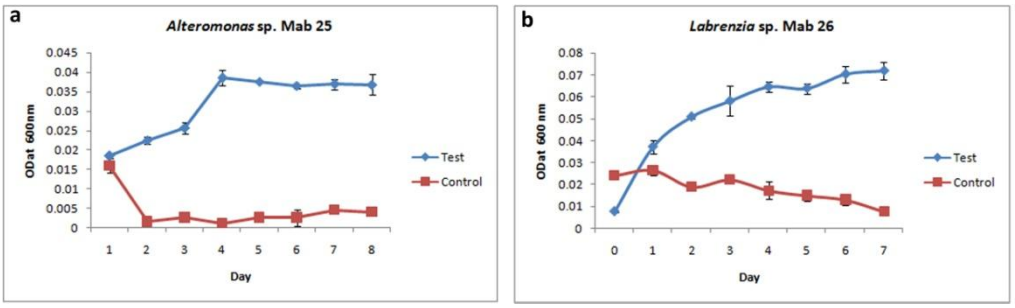

Fig. 5.6. Heterotrophic growth of bacterial symbionts on algal EOC

In conclusion, there exists a symbiotic relationship between *I. galbana* and associated bacteria, which has been proved in the present study. Both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 exhibit mutualism, receiving nutrients from the microalgae while promoting its growth. Also, the associated bacterial communities found to have a significant impact on nutrient profile of *I. galbana*. This report is the first observation on symbiotic association of bacteria in *I. galbana* culture. These findings give a greater insight on microalgal-bacterial interactions which can be further explored to improve productivity and sustainability in aquaculture rearing systems.

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Chapter 6

In vivo evaluation of microalgae associated bacteria on shrimp larval rearing *(Penaeus indicus)* based on survival rate and growth performance

Contents

6.1 Abstract
6.2 Introduction
6.3 Materials and Methods
6.4 Results and Discussion

6.1 Abstract

The objective of the present study was to find out the efficacy of phycosphere bacteria on survival, growth and larval (Mysis) conversion rate in *Penaeus indicus* hatchery rearing system. The axenic and non-axenic *Isochrysis galbana* culture was enriched with added microbial cocktail (*Alteromonas* sp. Mab 25, *Labrenzia* sp. Mab 26, *Marinobacter* sp. Mab 34, *Marinobacter* sp. Mab 18). The larvae of Indian white shrimp *P. indicus* were fed, from the stage of zoea I to mysis I, with axenic *I. galbana*, non-axenic *I. galbana*, axenic *I. galbana* enriched with added microbial cocktail and non-axenic *I. galbana* enriched with added microbial cocktail. Higher suvival of *P. indicus* larvae has been recorded when reared with added microbial cocktail. The length of of protozoea (Z I to Z III) was found to be equal among all experimental treatments ($p>0.01$). For stage mysis I, the largest size were recorded for larvae fed with non-axenic *I. galbana* enriched with microbial

cocktail (3.15 mm) and smallest size were observed on larvae fed with axenic *I. galbana* (2.6 mm) ($p < 0.01$). Higher levels of shrimp larval conversion rate were recorded for larvae fed with non-axenic *I. galbana* enriched with microbial cocktail (90 ± 7.07 %). The results revealed that the use of selected consortia of microalgae associated bacteria, when used as a cocktail, have great potential for improving the growth and health of shellfish and finfish larviculture. The concept proved in the present work can be developed as an application package in shrimp hatcheries.

6.2 Introduction

Seafood including shellfish such as shrimps has been considered as a healthy food, and countries such as India is looking forward to increase the production through aquaculture, to impart the nutritional security of the increasing population. Although there have been some marked changes in catch trends, fish from capture has levelled off over the past three decades (Khojasteh et al. 2013). Therefore, an increase in aquaculture production is the only sustainable solution to meet up with ever increasing demands for fish and fish products, while aquaculture contributes a total of 44.14 % of fish produced globally (Dauda et al. 2018). In India too, half of the fish production is from the aquaculture sector, which is projected to increase on yearly basis in the future. Very often, the larval rearing of marine species represents one of the critical phase, with the survival and health issues. Good nutrition and healthy larval rearing is essential to overcome the risk of survival and disease related mortalities during this hatchery phase of culture (Das et al. 2012, Khojasteh et al. 2013). Live food organisms contain all the nutrients essential for growth and survival of the young ones of finfish and shellfish and hence are commonly known as "living capsules of nutrition". Thus, success of a larval rearing system highly depends on availability of suitable live feed for

larval feeding (Das et al. 2012, Jamali et al. 2015). The major live food organisms used in aquaculture include microalgae, rotifers and brine shrimp (Merchie et al. 1997, Conceição et al. 2010). Microalgae are used as a direct starting diet for all growth stages of bivalve molluscs (e.g., oysters, scallops, clams and mussels), crustaceans and finfish species (Jamali et al. 2015). Moreover, they also serve as indirect food source, in the production of zooplankton (e.g., rotifers and *Artemia*), which in turn used as food for the larvae of many of the marine fish and shrimp species (Conceição et al. 2010). Also, microalgae have an important role in aquaculture as they carry nutrients such as vitamins, essential PUFAs, pigments and sterols—which are transferred to a high level through the food chain (Jamali et al. 2015). Importance of microalgae as larval food in aqua hatcheries not only owes its nutritional attributes but more so for its proper size fitting the smaller mouth size of the hatched out larvae, digestibility and ability to stimulate enzymatic synthesis and on-set of feeding in young larvae (Das et al. 2012). The microalgae that are usually used as live feed in aquaculture are: *Chaetoceros*, *Thalassiosira*, *Tetraselmis*, *Isochrysis*, *Nannochloropsis*, *Pavlova* and *Skeletonema* (Enright et al. 1986, Thompson et al. 1993, Jamali et al. 2015).

The shrimp farming industry which serves the dual purpose of producing high quality seafood and generation of employment has great economic and social importance (Nayak et al. 2011, Cao 2012). It is widely accepted that use of right live feed is one of the most important aspects in the production of quality shrimp post larvae (Lopez-Elias et al. 2008). The feeding protocols of larvae in commercial shrimp hatcheries include wide range of natural feeds and nutritional supplements (Jamali et al. 2015). Nevertheless, live feeds continue to be the principal nutritional basis for culture of larvae (Richmond 2004, Khojasteh et al. 2013). They act as main source of nutrients

for zoea larvae, and to a lesser degree for mysis (Jamali et al. 2015). It has been proven that the growth and survival of shrimp larvae are associated with the composition of food supplied to the larval stage (D'Souza and Loneragan 1999). As revealed in the previous chapter, the associated bacteria can improve growth as well as the chemical composition of their phytoplankton host. The potential of microalgae associated bacteria in preventing invasion and proliferation of pathogenic bacteria in aquaculture systems was also reported (Fuentes et al. 2016). Thus, it is expected that the use of co-cultures, or addition of selected associated bacterial strains have great potential for improving the growth and health of larvae in the hatchery rearing stage. However, a well-selected bacteria and microalgae, as a consortium are rarely used in aquatic industry (Natrah et al. 2014). In this regard, the present study was aimed to find out the efficacy of phycosphere bacteria on survival, growth and larval (Mysis) conversion rate of *Penaeus indicus* larvae.

6.3 Materials and Methods

6.3.1 Microalgal culture

Axenic and non-axenic cultures of *Isochrysis galbana* MBTDCMFRIS002 maintained at microalgae culture collection of Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) were used for this study.

6.3.2 Microbial cocktail

Four bacterial symbionts which were isolated from microalgal cultures and preserved as glycerol stocks at microbial culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) were used for this study. They include *Alteromonas* sp. MBTDCMFRI Mab 25 (GenBank Acc No KR004801),

Labrenzia sp. MBTDCMFRI Mab 26 (GenBank Acc No KR004822) *Marinobacter* sp. MBTDCMFRI Mab 18 (GenBank Acc No KR004794) and *Marinobacter* sp. MBTDCMFRI Mab 34 (GenBank Acc No KR004807). Among the selected bacterial strains, *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 represents the bacterial groups associated with *I. galbana* culture (MBTDCMFRIS002) and *Marinobacter* spp. correspond to the most predominant cultivable bacterium present in microalgal habitat.

To prepare the microbial cocktail, each bacterial strain was precultured as mono cultures in heterotrophic liquid medium (Zobell marine broth, Himedia, India). After 48 h of incubation (200 rpm, 30°C), bacterial culture broth was centrifuged at 8000 rpm for 15 minutes (Remi, India), washed three times and finally resuspended in sterile F/2 medium. Microbial cocktail, at 1 % (v/v), consisting of equal amount each bacterial symbiont solution was inoculated to sterile F/2 medium along with the algal cultures during production processes of microalgae.

6.3.3 Study site

The study was conducted at Muttukadu Experimental Station (MES) of Central Institute of Brackishwater Aquaculture (ICAR), Chennai.

6.3.4 Larval rearing study

The newly hatched out larvae (Nauplii) of *P. indicus* were used for the study. The feeding experiments were conducted in triplicate, at a stocking density of 100 nauplii L^{-1} with a daily water exchange of 20 – 50 % (Fig. 6.1). Four treatments were fed to *P. indicus* larvae from zoea I to mysis I stages which include:

- Non-axenic *I. galbana*
- Axenic *I. galbana*
- Non-axenic *I. galbana* grown along with added microbial cocktail
- Axenic *I. galbana* grown along with added microbial cocktail.

Chaetoceros sp. (live feed, routinely used in commercial hatcheries) was kept as control. Larval development was evaluated daily by microscopic observation. Total length of the larvae at each developmental stage was measured using a binocular microscope. At the end of the experiment, percentage survival and larval (Mysis) conversion rate were quantified.

6.3.5 Statistical analysis

The obtained results were subjected to statistical analysis (one way ANOVA) and the means of all parameters were examined for significance ($p<0.01$) using R software (Version 2.3-0).

6.4 Results and Discussion

Previous studies and the outcome of the present study indicates the possibility of utilizing the algal-bacterial interactions in larval rearing systems to improve algal biomass production and to produce more sustainable live feed (Fuentes et al. 2016). However, only limited studies reported the use of phycosphere bacteria for practical purposes (Natrah et al. 2014). In the current study, the efficacy of phycosphere bacteria, such as *Alteromonas* sp., *Labrenzia* sp. and *Marinobacter* spp. on growth and survival of shrimp larvae was evaluated, in a larval rearing environment. It was noticed that there was no considerable difference in the growth performance of *P. indicus* larvae when they fed with either *I. galbana* or *Chaetoceros* sp. At the same time, the total length, survival and mysis conversion rate of *P. indicus* larvae were

increased when they fed with algal diet enriched with microbial cocktail. The length of all stages of zoea was found to be equal among the entire experimental treatments ($p>0.01$). For mysis I, the largest size were recorded for larvae fed with non-axenic *I. galbana* enriched with microbial cocktail (3.15 mm) and smallest size were observed for larvae fed with axenic *I. galbana* (2.6 mm) (Table 6.1). The difference in the length of the mysis I stage was found to be statistically significant at 99 % confidence level interval ($p<0.01$). As shown in Fig. 6.2, the survival of *P. indicus* larvae was found to be increased in the presence of added microbial cocktail. The maximum survival rate was observed for the larvae fed with non-axenic *I. galbana* culture grown along with added microbial cocktail (87 %). The obtained results were statistically analysed using one way ANOVA at 99 % confidence level interval. The analysis revealed that there was a significant difference in the % survival of larvae in each experimental treatments ($p<0.01$). The survival of *P. indicus* larvae was found to be minimum (33 %) when they have fed with axenic culture of *I. galbana*. At the same time, when the axenic *I. galbana* culture was enriched with added microbial cocktail, the survival rate was increased up to 66 %. Also, Bonferroni post-hoc test revealed that there was no significant difference in survival rate when larvae fed with axenic *I. galbana* + microbial cocktail and non-axenic *I. galbana* ($p =1.00$). These results clearly suggest that the associated bacteria have a positive impact on live feed which in turn are beneficial to the health of rearing animal. Similarly, the maximum larval (Mysis) conversion rate was observed in larvae fed with non-axenic *I. galbana* enriched with microbial cocktail (Table 6.2).

Table 6.1 Total length (mm) of larvae at zoea (Z I, Z II and Z III) and mysis I stages in different experimental set up

Algal Diet	Total Length of larvae (mm)			
	Zoea I	Zoea II	Zoea III	Mysis I
Non axenic *I. galbana*	1.02 ± 0.014	1.29 ± 0.069	2.26 ± 0.107	2.83 ± 0.229
Axenic *I. galbana*	1.05 ± 0.158	1.30 ± 0.076	2.24 ± 0.146	2.60 ± 0.285
Non -axenic + Microbial cocktail	1.01 ± 0.093	1.36 ± 0.084	2.34 ± 0.245	**3.15 ± 0.221**
Axenic + Microbial cocktail	1.00 ± 0.038	1.28 ± 0.040	2.31 ± 0.183	2.95 ± 0.119
Chaetoceros sp.	1.02 ± 0.135	1.23 ± 0.048	2.09 ± 0.107	2.81 ± 0.141

Table 6.2 Mysis conversion rate of *P. indicus* larvae in different experimental set up

Algal Diet	Mysis conversion (%)
Non axenic *I. galbana*	70 ± 7.07
Axenic *I. galbana*	62 ± 2.83
Non -axenic + Microbial cocktail	**90 ± 7.07**
Axenic + Microbial cocktail	75 ± 7.07
Chaetoceros sp.	70 ± 7.07

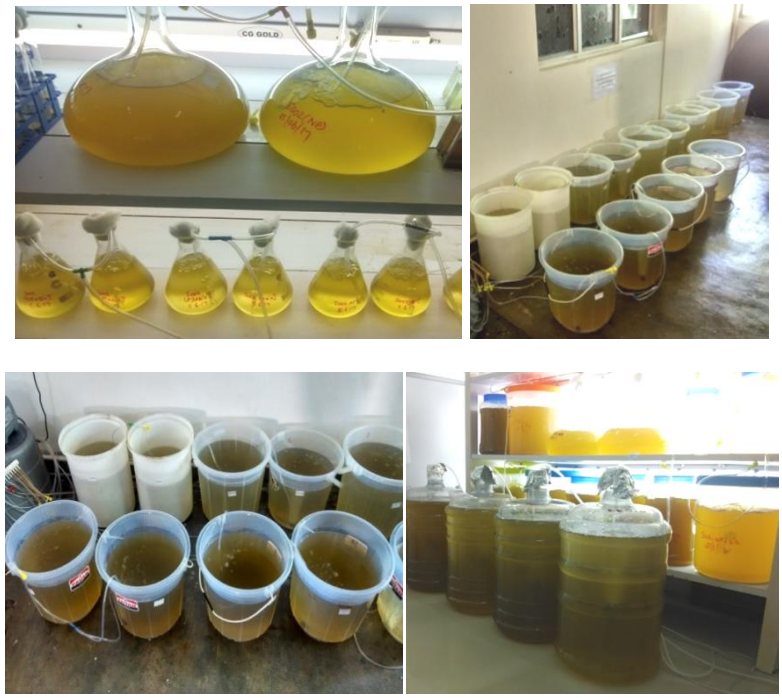

Fig. 6.1. Experimental set up for evaluation of effect of phycosphere bacteria on shrimp larval rearing at Muttukadu Experimental Centre of Central Institute of Brackishwater Aquaculture (ICAR), Chennai

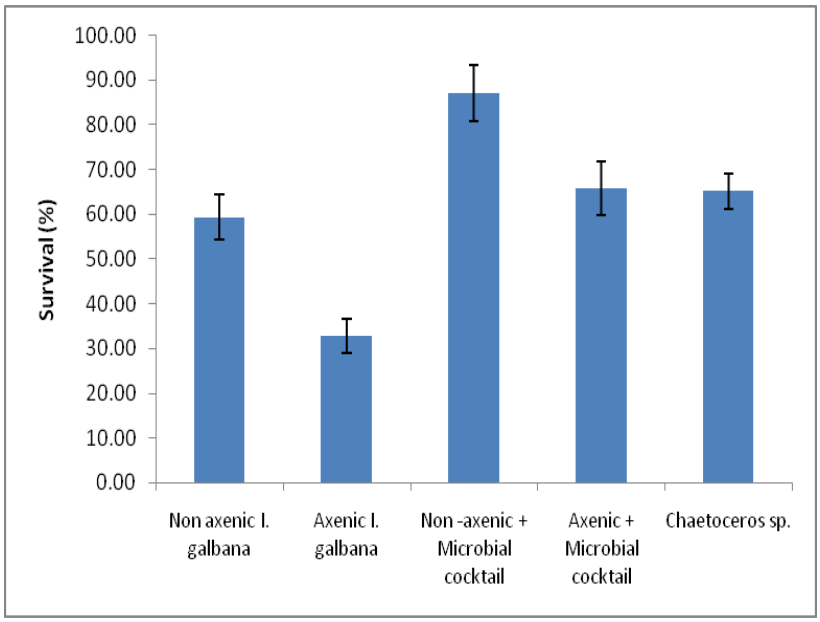

Fig. 6.2. Survival of *P. indicus* larvae in different experimental set up

Advantage of using beneficial bacteria as a food supplements for rearing aquatic animals is widely studied (Farzanfar 2006, Rafiee et al. 2014). Positive effects of these probiotic bacteria on shrimp larviculture include accelerated larval development and survival rate (Rafiee et al. 2014). Liu et al. (2009) reported that the growth of *Litopenaeus vannamei* juveniles was increased when they are fed by diet containing probiotic bacteria, *Bacillus subtilis*. Nevertheless, little attention has been paid to enrich microalgal diets with selected consortium of beneficial bacteria, which are natural associates to the microalgae. The possible role of associated bacteria in improving the productivity and stability of microalgal cultures has been reported (de Kluijver et al. 2010, Arora et al. 2012, Natrah et al. 2014). Thus, the best candidates for enhancing the quality of these live feed would be the bacteria associated with them (Natrah et al. 2014). It was reported that suitable consortia of bacteria

and their favourable effect on microalgal productivity could also promote larval rearing success (Nicolas et al. 2004, Natrah et al. 2014). Moreover, these associated bacteria can grow to high densities and remain dominant within microalgal cultures (Natrah et al. 2014). For example, Avendano-Herrera and Riquelme (1999) reported that bacteria isolated from *I. galbana* not only stimulated the growth of microalgae but were also able to grow well in the microalgal extracellular products.

From the results of the present work, it is clear that phycosphere bacteria can boost up the potential of microalgae in the larviculture of aquaculture species. The bacterial enrichment technique has resulted in greater survival, higher growth performance and increased larval conversion rate in shrimp larviculture. The impact of associated bacteria in larval rearing systems can be substantial (Nicolas et al. 2004). As reported by Moal et al. (1996) bacteria can complement the diet with growth factors and can improve larval growth significantly. In addition, the associated bacteria play a key role in the chemical composition of the microalgal biomass (Fuentes et al. 2016). D'Souza and Loneragan (1999) reported that the larval growth and development were more likely related to the biochemical composition of the diet. In the current study, the larvae fed non-axenic *I. galbana* culture displayed higher survival and development than those fed axenic culture of *I. galbana*. As described in section 5.4, the associated bacteria could improve the nutritional composition of microalgae. The level of four major nutrients such as proteins, fatty acids, pigment and carbohydrate were found to be increased in the presence of associated bacteria. It has been proven that nutritional value of microalgae significantly contributes to the growth rate of shrimp larvae (Jamali et al. 2015). Hence, the difference in the larval growth performance between axenic and non-axenic algal diets might be due to the difference in their nutrient profile.

Furthermore, the obtained results clearly demonstrated that the added microbial cocktail could improve the performance of both axenic and non-axenic culture of *I. galbana*. In particular, the larval growth performance was higher when the microbial cocktail was added to non-axenic culture of *I. galbana*. It could be due to the presence of several undetectable bacterial symbionts in the non-axenic culture which might be removed during axenisation.

On the whole, the present study illustrated that a mixed diet of microalgae and associated bacteria could have a significant positive impact on survival and growth of cultivated animals comparable to monoalgal diets, using *P. indicus* as a model. Thus, the bacterial enrichment of microalgae can be explored to improve microalgal productivity as well as the larval rearing success. The present work would open up new avenues to produce more sustainable and efficient 'novel live feeds' with a well-selected consortium of phycosphere bacteria which will definitely enhance the productivity of aquaculture. There is sufficient scope to develop 'Microbial cocktail', as a product which could be used in aquaculture larval rearing systems.

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Chapter 7

Bioprospecting prospects of heterotrophic bacterial strains isolated from marine microalgae, *Isochrysis galbana* MBTDCMFRI S002

Contents

7.1 Abstract
7.2 Introduction
7.3 Materials and Methods
7.4 Results and Discussion

7.1 Abstract

This study was undertaken to explore two bacterial strains *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26, isolated from *Isochrysis galbana* for various biotechnological applications namely silver nanoparticle biosynthesis, antioxidant activity, siderophore production, indole-3-acetic acid production, biosurfactant production and bacterial exopolysaccharide production. Bacterial isolate *Labrenzia* sp. Mab 26 was to able achieve the production of silver nanoparticles. The synthesised nanoparticles were further characterised by methods viz., UV-vis spectroscopy, XRD analysis, FT-IR spectrum, scanning electron microscopy and transmission electron microscopy. Both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 were exhibited 2,2-Diphenyl-1-picrylhydrazyl scavenging activity. At concentration 2.5 mg ml^{-1} the crude extract of *Alteromonas* sp. Mab 25 had higher scavenging effect (41 %) than that of *Labrenzia* sp. Mab 26 (25.3 %). The IC_{50} value of two bacterial isolates

was 6.38 and 9.55 mg ml^{-1} respectively, showing their potential as antioxidant. *Labrenzia* sp. Mab 26 was found to be positive for indole-3-acetic acid production whereas *Alteromonas* sp. Mab 25 was negative. Both bacterial strains exhibited siderophore, biosurfactant and bacterial exopolysaccharide production potential. The study proved that bacteria associated with microalgae could also serve as potential bioresources for many biotechnological applications.

7.2 Introduction

The marine environment has proven to be an untapped reservoir of diverse natural products with potent biological activities (Penesyan et al. 2010, Balakrishnan et al. 2014). Among the marine organisms, microalgae represent one of the richest sources of valuable bioactive compounds for various industrial applications (Singh et al. 2005, Saranya et al. 2014, Rubavathi and Ramya 2016). Bacterial communities in algal phycosphere have been extensively investigated in several studies (Natrah et al. 2014, Schwenk et al. 2014, Sandhya et al. 2017). These associated bacteria often metabolically linked with their phytoplankton host. It has been suggested that these microbial symbionts produce wide range of bioactive compounds and thus create a suitable chemical microenvironment with their phytoplankton host. These close metabolic associations between microalgae and bacteria can make it difficult to reveal which partner organism is responsible for the production of any particular metabolite (Penesyan et al. 2010). Moreover, the associated bacteria may have a greater potential to produce bioactive agents than that of its free living counterpart (Abdel-Wahab et al. 2013, Horta et al. 2014). Thus, these symbiotic or commensal bacteria could be expected to be an interesting source of valuable bioactive compounds with the potential for providing sustainable human benefits. However, biological wealth of bacteria associated

with the marine microalgae is relatively unexplored. In this background, the present study aims to explore the emerging applications of culturable bacteria associated with the marine microalgae. Here the bacterial strains identified from *Isochrysis galbana* have been evaluated for potential biotechnological applications viz,

- Silver nanoparticle (AgNp) biosynthesis
- Antioxidant activity
- Siderophore production
- Indole-3-acetic acid (IAA) production
- Biosurfactant production
- Bacterial exopolysaccharide production.

7.3 Materials and Methods

7.3.1 Bacterial strains

Two bacterial strains (*Alteromonas* sp. MBTDCMFRI Mab 25 and *Labrenzia* sp. MBTDCMFRI Mab 26) which were isolated from *I. galbana* (MBTDCMFRI S002) culture and preserved as glycerol stocks at microbial culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) were used for this study.

7.3.2 AgNp biosynthesis

The bacterial strains were grown in Zobell Marine Broth (ZMB, Himedia, india). After 48 h of incubation, the biomass was separated by centrifugation at 8000 rpm for 15 minutes (Remi, India). The culture supernatant was then mixed with equal quantity of 5 mM silver nitrate solution

(prepared in sterile distilled water) and incubated at room temperature in a shaker for three days. Sterile ZMB mixed with silver nitrate solution was kept as control. The flasks were observed for synthesis of AgNps by a colour change.

7.3.2.1 Characterisation of AgNps

The formation of AgNps was studied by UV-vis spectroscopy (Thermo Scientific, US). The λ_{max} was measured within the range of 300-800 nm. Further, the crystalline nature of synthesised AgNps was determined by XRD analysis (Bruker, D8 Advance, Germany). The freeze - dried sample was smeared over low back ground sample holder (amorphous silica holder) and fixed on the sample stage in goniometer. The instrument is set with B-B geometry. The current and voltage is set to 40 mV and 35 mA and data has been collected. FT-IR spectroscopic analysis was conducted on a Nicolet Avatar-370 FT-IR spectrophotometer with the resolution of 4 cm^{-1}. Freeze – dried samples were analysed using KBr window. Potassium bromide (KBr) pellets were prepared by mixing 1-2 mg of dried product with approximately 100 mg of powdered KBr. The mixture was ground before being compressed in a special metal KBr die under the pressure of 15-30 tonnes to produce transparent KBr discs. The average number of scans taken per sample was 32 in the spectral region between 400 and 4000 cm^{-1}. The size and shape of obtained AgNps were also studied by Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM). An extremely small amount of freeze – dried sample was suspended in ethanol to obtain a slightly turbid solution and it was then ultrasonicated to disperse the particles. A drop of this solution was casted on carbon-coated grids of 200 mesh and observed under transmission electron microscope at various magnifications (Jeol - Model JM 2100). For SEM analysis, the solution was mounted on specimen stubs with

carbon conductive tape and coated with gold in a sputter coater (TESCAN VEGA 3).

7.3.3 Antioxidant activity

Primary screening of antioxidant potential of selected bacterial strains was done using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma, USA) as described in Pawar et al. (2015). In brief, pure culture of *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 were spotted on Zobell Marine Agar (ZMA) (Himedia, India). The plates were incubated at 30°C for 24 h. A sterilsed Whatman no 1 filter paper was then placed on the agar plates and the plates were further incubated for another 24 h. After incubation, the filter paper was taken out and dried. It was then sprayed with DPPH solution prepared in methanol (80 μg ml^{-1}). The bacterial strains showing white on purple spot were considered as positive and the antioxidant activity was measured as zones of decolourisation around spotted colonies.

After primary screening, to determine the antioxidant activity of the crude extract of the extracellular metabolites, the bacterial strains were inoculated in 100 ml sterile ZMB (Himedia, India) and incubated at 30°C at 200 rpm for 72 h. The culture broth was centrifuged and the supernatant was extracted by using same volume of ethyl acetate, for three times. The organic phase was then concentrated in rotary evaporator at 40°C. The bacterial crude extract dissolved in methanol to prepare different concentrations (2.5, 2.0, 1.5, 1.0, 0.5 mg ml^{-1}) and the antioxidant activity was estimated by DPPH radical scavenging method as described in Abdel-Wahab et al. (2013).

7.3.4 Siderophore production

For the screening of siderophore production potential, pure culture of *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 were spotted on chrome

azurol S (CAS) agar with 2 % NaCl (Lacava et al. 2008, Chaitanya et al. 2014). The plates were incubated at room temperature for 7-10 days and observed for a visual change in colour from dark blue to orange around the colonies.

7.3.5 Indole-3-acetic acid production

The IAA production potential of *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 was determined by the method as described in Vijayan et al. (2012). The bacterial strains were inoculated to sterilized nutrient broth (with 2 % sea salt) supplemented with tryptophan (10 µg/ml) and incubated at room temperature for 3 days at 200 rpm. After incubation, bacterial culture broth was centrifuged at 10000 rpm for 10 minutes (Remi, India). To the culture supernatant (2 ml), 2 drops of orthophosphoric acid was added and incubated at room temperature for 10 minutes followed by addition of 4 ml of Salkowski reagent (1 ml 0.5 M $FeCl_3$, 50 ml 35 % $HClO_4$). Development of pink colour indicates the positive result for IAA production.

7.3.6 Biosurfactant production

The screening of biosurfactant production potential was done oil displacement test using mineral oil (Satpute et al. 2008). In brief, the petriplate base was filled with 30 ml of distilled water and 20 µl of mineral oil was layered uniformly on this water. Further, 10 µl of 24 h bacterial culture grown in ZMB was added at different spots on the crude oil which is coated on water surface. The occurrence of a clear zone indicates the production of biosurfactant.

To determine the emulsification index, 2 ml culture supernatant was mixed with 3 ml of n-hexadecane and vortexed at high speed for 2 minutes. The mixture was allowed to stand for 10 minutes prior to measurement. The emulsification index was calculated according to the equation (Hamed et al. 2012)

Emulsification Index (%) = (Height of emulsion/Total height) X 100

7.3.7 Bacterial exopolysaccharide production

YMG (Yeast extract Monosodium glutamate Glucose) agar plates prepared in 50 % sea water (Glucose 10g/L; Yeast extract 3g/L; Beef extract 3g/L; Peptone 5g/L; Monosodium glutamate 1g/L; Sucrose 30g/L) was used to determine the exopolysaccharide production potential (Vijayan et al. 2012). Pure culture of *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 were streaked on YMG agar plates and incubated at room temperature for 3 days. Oozing out of gummy substances on the periphery of the bacterial colonies indicated the production of exopolysaccharide.

7.4 Results and Discussion

7.4.1 AgNp biosynthesis

Algal microhabitat has opened up unexpected new ways for finding novel microbes with potential of different biotechnological applications. Silver nanoparticles are the noble metal particles that have been studied extensively as they provide superior material properties and functional versatility (Shivakrishna et al. 2013, Selvaraj et al. 2014). They are increasingly used in various fields including medical, food, health care, consumer and industrial purposes (Zhang et al. 2016). The nanoparticle synthesis can be achieved by physical, chemical and biological methods. The problems with most of the physical and chemical methods of nanosilver production are the side effects, use of toxic chemicals and hazardous by-products (Karthik et al. 2014, Selvaraj et al. 2014). Hence, the researchers are now interested in biogenic production of AgNps as it provides an eco-friendly and also a cost effective method (Karthik et al. 2014, Mehta et al. 2014, Vithiya et al. 2014).

In the present study, the culture supernatant of *Labrenzia* sp. Mab 26 incubated with silver nitrate solution showed gradual colour change of dark brown from yellow which indicated the formation of AgNps. *Alteromonas* sp. Mab 25 and control did not show any change in colour after incubation (Fig. 7.1). The AgNp formation by *Labrenzia* sp. Mab 26 was further confirmed by UV-vis spectroscopy which is proved to be a very useful and reliable technique for the primary characterisation of synthesised nanoparticles. AgNps strongly interact with specific wavelength of light due to their unique optical properties. (Zhang et al. 2016). In the UV- visible spectrum, a strong peak was observed at 420 nm which confirmed the successful formation of AgNps (Fig. 7.2). This might be due to the reduction of silver ions into silver particles by secondary metabolites produced by bacterial cells. Combined vibration of electrons at the surface of AgNps in resonance with the light wave give rise to a surface plasmon resonance (SPR) absorption band with a peak at 420 nm (Karthik et al. 2014, Vithiya et al. 2014). The results were similar when compared to earlier published report of biogenesis of AgNps (Shivakrishna et al. 2013, Karthik et al. 2014).

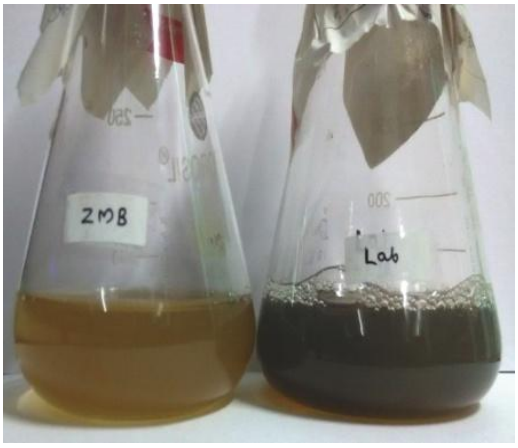


Fig. 7.1. Control and positive of biogenesis of silver nanoparticles

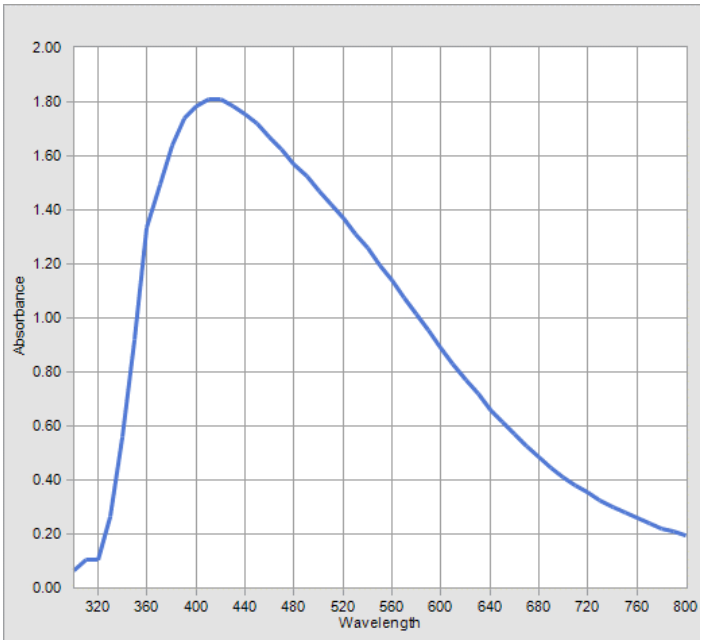


Fig. 7.2. UV-visible spectrum analysis of synthesised silver nanoparticles

XRD analysis of synthesised AgNps showed high crystallanity nature and the intense peaks at 2θ values of 27.51°, 31.87°, 45.57°, 56.56°, 66.26° and 75.25° corresponding to XRD planes from (210), (113), (124), (240) based on the fcc cubic structure of silver nanoparticles (Fig. 7.3). Here the peaks are shifted to higher angles due to the small size of the particles. The mean particle diameter of AgNps was calculated from the XRD pattern using Debye-Scherrer equation:

$$\text{Size} = 0.9\lambda/\beta\cos\theta$$

where λ is the wavelength of the X-ray, β is width of the peak and θ is the half of the Bragg's angle respectively. The calculated average crystallite size of the AgNps was found to be 20.79 nm.

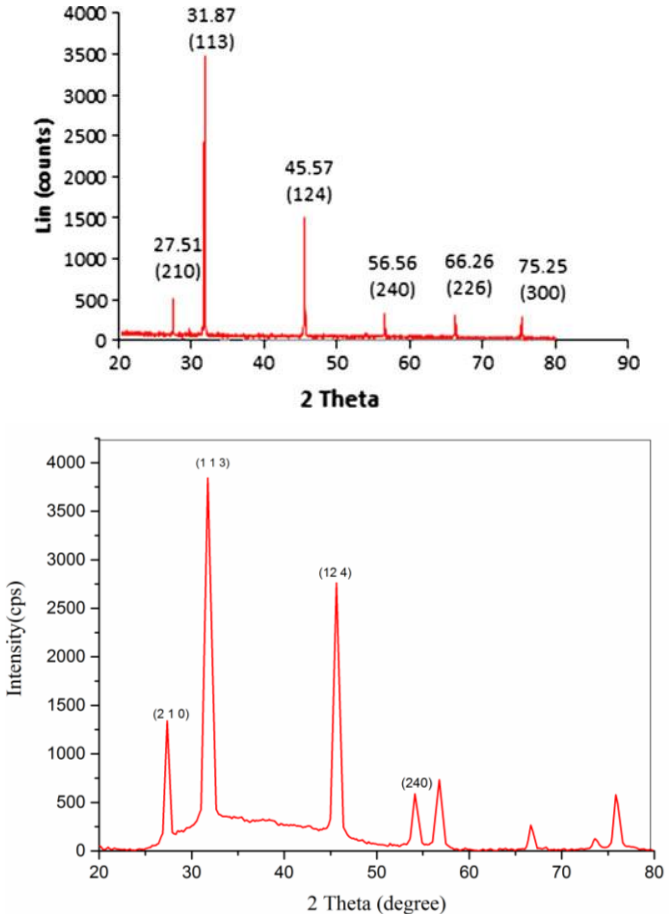


Fig. 7.3. XRD pattern of synthesised silver nanoparticles

The FT-IR analysis of synthesised AgNps showed peaks at 3424.29; 1632.42; 1067.80; 564.99 cm^{-1} (Fig. 7.4). The strong absorption at 3424 cm^{-1} may be attributed to the stretching vibration of O-H group, whereas the absorption at 1632 cm^{-1} is assigned to the presence of carboxylic (C=O) group (Mehta et al. 2014). The peak at 1067 cm^{-1} is ascribed to the stretching vibration of C-O bond of the primary alcohol. The absorption peak at 564 cm^{-1} corresponds to metal-oxygen (silver stretching vibrations) vibrational mode (Baskar et al. 2015). FT-IR analysis data confirms the presence of O–H

stretching (around 3,417 cm^{-1}) which may be responsible for reducing metal ions into their respective nanoparticles (Karthik et al. 2014).

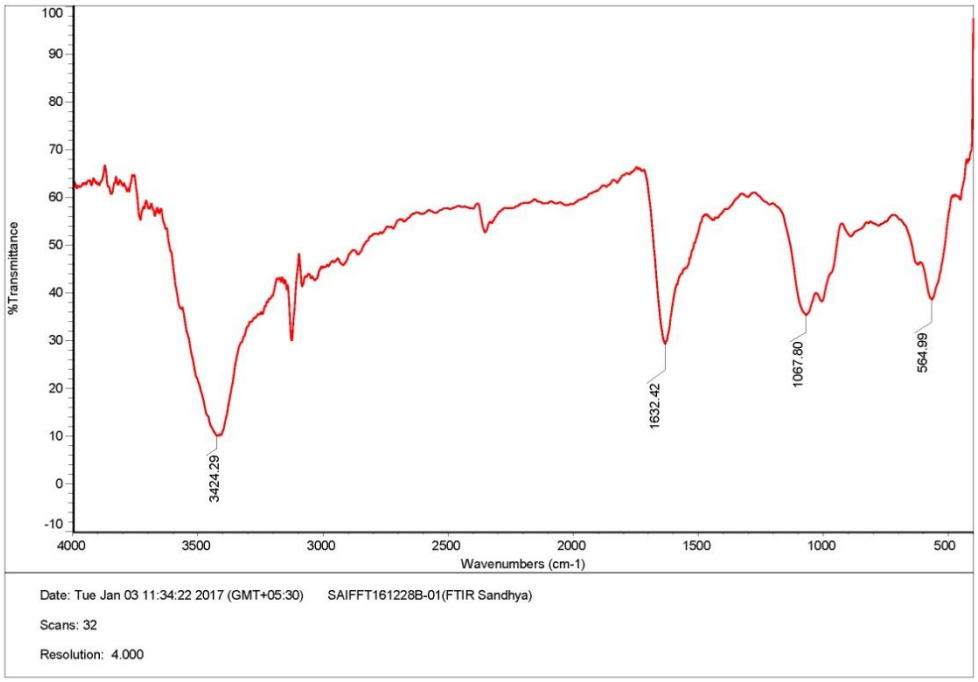
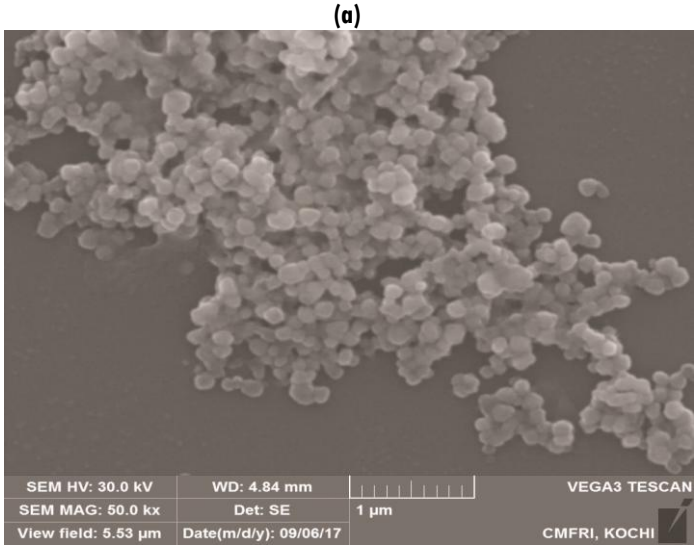


Fig. 7.4. FT-IR spectra of synthesised silver nanoparticles

Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM) study provided further insight into the shape and size of the synthesised nanoparticles (Fig. 7.5). The SEM and TEM analysis images illustrate the individual nanoparticles as well as the clusters. As shown in the figure, biosynthesised nanoparticles are small and spherical in shape. They are fairly dispersed with an average size of 28 ± 9 nm.

(a)



(b)

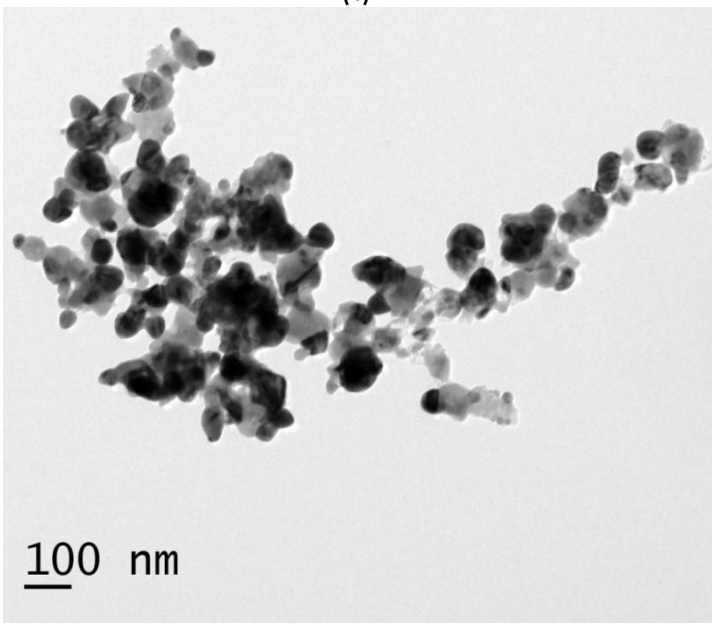


Fig. 7.5. SEM (a) and TEM (b) image of synthesised silver nanoparticles

Recently, biosynthetic methods have emerged as a simple and viable alternative to more complex chemical synthetic procedures to obtain AgNps (El-Nour et al. 2010). The metal nanoparticles have been successfully synthesised by microorganisms such as actinomycetes, bacteria, fungi, yeast,

algae and plants (Karthikeyan et al. 2015). Suja et al. (2016) reported the green synthesis of AgNps using marine microalgae, *I. galbana*. However, from the perusal of available literature, there was no report on the synthesis of nanoparticles from heterotrophic bacteria associated with *I. galbana*. Therefore, the present work highlighted the potential of *Labrenzia* sp. Mab 26 associated with *I. galbana* for green synthesis of biogenic AgNps. In addition to the number of environmental applications, AgNps have been used widely in health industry, food storage and textile coatings (El-Nour et al. 2010). Moreover, nanotechnology has a wide application in aquaculture and seafood industry (Can et al. 2011). They can be used in fish health management, water treatment in aquaculture, animal breeding, harvest and postharvest technology (Selvaraj et al. 2014). The current study is a demonstration of efficient synthesis of AgNps by a phycosphere bacterium, *Labrenzia* sp. Mab 26. The selected bacterial strain can synthesise AgNps extracellularly at room temperature. Thus, the green synthesis of AgNps derived from microbial origin can be further explored for various biomedical, aquaculture and environmental applications.

7.4.2 Antioxidant activity

In general, antioxidant compounds can scavenge free radicals and other reactive oxygen species and thus provide protection against cellular damage and oxidative stress induced by these chemical species. (Valko et al. 2007, Sarma et al. 2010, Abdel-Wahab et al. 2013, Balakrishnan et al. 2014, Rubavathi and Ramya 2016). Several studies suggested that these antioxidant compounds are effective in combating diseases such as cancer, inflammatory disorders, coronary heart diseases, neurological disorders and aging (Balakrishnan et al. 2014, Balakrishnan et al. 2015). One of the major limitations of synthetic antioxidants is its toxicity and issues related to safety

(Rubavathi and Ramya 2016). Currently researchers are more interested in discovering antioxidants from natural sources. Hence the antioxidant potential of culturable bacteria associated with the marine microalgae *I. galbana* was explored in the present study.

When the DPPH solution reacts with an antioxidant compound it gets reduced by donating hydrogen atom. The degree of discolouration is directly proportional to the free radical scavenging activity of that compound (Abdel-Wahab et al. 2013, Elmhdwi et al. 2015). Both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 associated with *I. galbana* showed white on purple spot when sprayed with DPPH solution which indicated that both bacterial strains are able to produce extracellular antioxidants (Fig. 7.6). The decolourisation zone for *Alteromonas* sp. was 70 mm and that of *Labrenzia* sp. was 20 mm. The DPPH scavenging ability was found to be increased with increase in concentration of bacterial crude extract (Table 7.1). The scavenging effect of *Alteromonas* sp. Mab 25 was 41 % at a concentration of 2.5 mg ml^{-1} whereas that of *Labrenzia* sp. Mab 26 was 25.3 % at the same concentration. The IC_{50} of the two bacterial isolates were 6.38 and 9.55 mg ml^{-1} respectively.

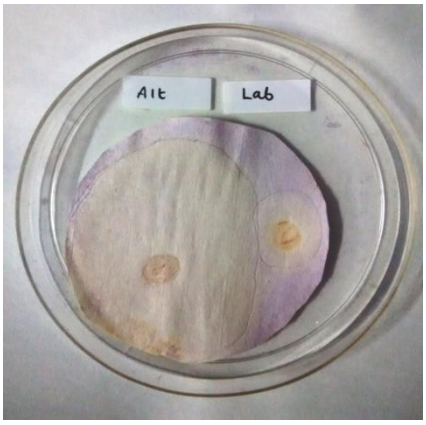


Fig. 7.6. Antioxidant activity of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26) by DPPH assay

Table 7.1 Antioxidant activity of crude extract of bacteria associated with *I. galbana* using DPPH assay (Strain code of both isolates start with MBTDCMFRI)

Concentrations mgml^{-1}	Antioxidant activity (%)	
	***Alteromonas* sp. Mab 25**	***Labrenzia* sp. Mab 26**
2.5	41.0	25.3
2.0	35.7	21.0
1.5	28.0	15.3
1.0	20.1	8.60
0.45	16.7	4.80

Though the antioxidant potential of microalgae is being explored globally, the knowledge on antioxidant activity of its associated bacteria is rather limited at the moment. The obtained results clearly specify the antioxidant property of bacterial strains associated with the microalgae *I. galbana*, a well explored source of natural antioxidant (Saranya et al. 2014, Yingying et al. 2014, Rubavathi and Ramya 2016). Through the production of these bioactive molecules these microbial partners construct a chemical microenvironment and hence maintain a close cross-relationship with their eukaryotic hosts (Penesyan et al. 2010). The associated bacteria often produce such bioactive compounds to inhibit competing organisms and microbial pathogens (Balakrishnan et al. 2014). Thus owing to the antioxidant potential, these bacterial strains could play a role in microlagae defense mechanism. Priyanka et al. (2014) reported the antioxidant activity of *Labrenzia* sp. isolated from deep seawater from offshore of Cochin (India). Similarly, Yeo et al. (2006) isolated antioxidant producing *Alteromonas macleodii* strain from the coast of Korea. Compared to previous studies these bacterial strains exhibited lesser antioxidant activity. However, it is assumed that they can boost up the antioxidant potential of their phytoplankton counterpart and bring in a synergistic effect. Thus this microbial consortium can complement the live feed which in turn exerts an overall positive effect to the aquaculture rearing system.

7.4.3 Siderophore production

Iron is most essential vital element for virtually all living cells owing to its ability to catalyse redox reactions, transfer electrons and transport ligands such as dioxygen (Litwin and Calderwood 1993, Amin et al. 2012, Chaitanya et al. 2014). The scarcity of dissolved iron in the aerobic marine environment has driven microorganisms to adopt a way for iron acquisition by producing iron chelating molecule i.e. siderophore (Amin et al. 2012, Ali and Vidhale 2013). These low-molecular-compounds can bind with ferric ion with high affinity (Lacava et al. 2008). A universal siderophore assay using chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators was developed by Schwyn and Neilands in 1987. Hence, in the present study, CAS assay was performed to evaluate the production of siderophores by *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 associated with *I. galbana*. Both these bacterial strains were CAS positive for siderophore production as there observed a orange halo around the colonies after incubation (Fig. 7.7). The zone diameter for *Alteromonas* sp. Mab 25 was 14 mm and that for *Labrenzia* sp. Mab 26 was 12 mm. These bacterial siderophores are known to be involved in enhancing the growth of their phytoplankton host (Natrah et al. 2014, Fuentes et al. 2016). They provide a steady supply of Fe (III) to algal cells, potentially in exchange for organic carbon (Amin et al. 2009). As an example, majority of the *Marinobacter* spp. which are consistently associated with diverse algal cultures produce the siderophore vibrioferrin, which binds Fe (III), making it available for microalgae and bacteria (Amin et al. 2012, Fuentes et al. 2016). Similarly, the microalgae *Scrippsiella trochoidea* makes use of siderophores produced by bacteria present in its environment (Amin et al. 2009). In addition to the algal growth promoting role, these microbial siderophores have wide applications in

various field such as agriculture to improve soil fertility and biocontrol, environmental application and medicinal application (Ali and Vidhale 2013).

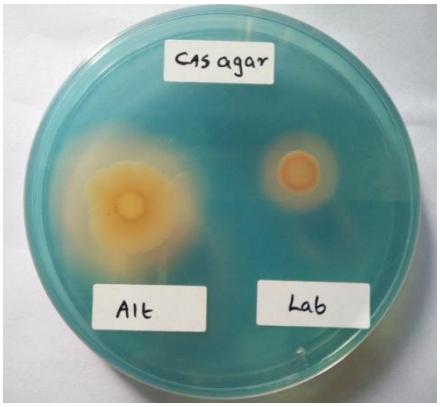


Fig. 7.7. Siderophore production potential of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26) by CAS assay

7.4.4 Indole-3-acetic acid production

On the addition of orthophosphoric acid and Salkowski reagent, *Labrenzia* sp. Mab 26 produced slight pink colouration indicating the production of IAA in their culture filtrates. Concurrently, *Alteromonas* sp. Mab 25 was found to be negative for IAA production (Fig. 7.8). It was reported that bacteria can stimulate the metabolism of microalgae by secreting hormones like indole-3-acetic acid (Natrah et al. 2014, Fuentes et al. 2016). There was a threefold increase in the growth of microalgae *Chlorella vulgaris* in the presence of IAA producing bacterium *Azospirillum brasiliense* (Gonzalez and Bashan 2000, de-Bashan et al. 2008). Furthermore, the production of IAA by *Labrenzia* sp. Mab 26 may offer a chance to use this phycosphere bacterium as a biofertilizer to improve the growth and yield of agricultural crops in coastal saline influenced lands (Vijayan et al. 2012).

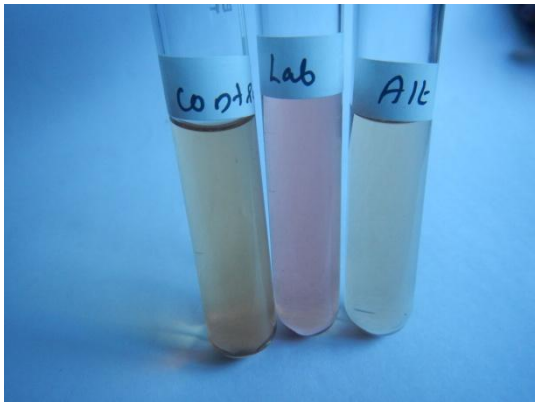


Fig. 7.8. Screening of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26) for IAA production

7.4.5 Biosurfactant production

Many bacteria can produce biosurfactants that can solubilise and assimilate hydrocarbons. In recent years biosurfactants are thought to be very suitable alternative to chemical surfactants as they confer an environmental and health friendly status mainly due to their biodegradability, diversity, low toxicity, biocompatibility and digestibility (Satpute et al. 2008, Hamed et al. 2012). The biosurfactants has received considerable attention in the field of environmental bioremediation as well as in food, cosmetic and pharmaceutical industries (Saravanan and Vijayakumar 2012). In the current study, the screening of biosurfactant production potential was done by oil displacement test and the observed results revealed that both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 have the ability to produce biosurfactant. The emulsification index of *Labrenzia* sp. Mab 26 was 59.34 % and that of *Alteromonas* sp. Mab 25 was 58.75 % (Fig. 7.9). Present investigation showed that these phycosphere bacteria are interesting for both environmental and medical applications and further studies are needed to unravel the molecular architecture of biosurfactant produced by these bacterial strains.

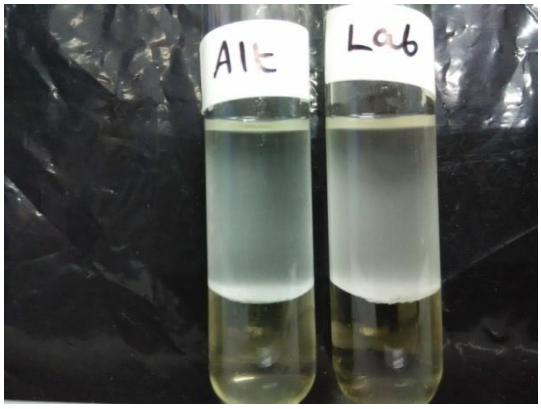


Fig. 7.9. Determination of emulsification index of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26)

7.4.6 Bacterial exopolysaccharide production

Both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 showed gummy secretion on YMG agar medium which indicate the production of bacterial exopolysaccharide (Fig. 7.10). The microbial exopolysaccharides have industrial potential as new biomaterials due to their properties such as gel formation, emulsifying, absorption, cohesion and film formation (Vijayan et al. 2012).

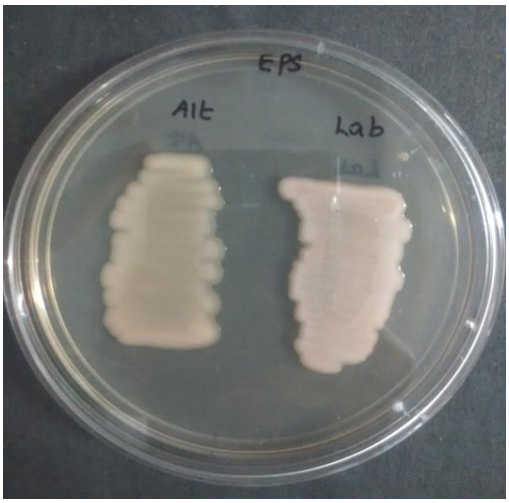


Fig. 7.10. Screening of *Alteromonas* sp. (MBTDCMFRI Mab 25) and *Labrenzia* sp. (MBTDCMFRI Mab 26) for exopolysaccharide production

The present work at large throws lights on characteristics of microalgal habitat and biotechnologically valuable prokaryotic microorganisms. The metabolic or chemical nature of algal-bacterial interaction may explain the abundance of bioactive producing bacteria in phycosphere. Further, the work contributed towards a better insight on underlying mechanisms behind these interactions, which would trigger more studies and the search for novel bioactive compounds. Overall, these findings open up avenues to explore the chemical ecology of microalgae-bacteria associations for welfare of all living beings as well as our environment.

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Chapter 8

Summary and Conclusion

Aquaculture is the fastest growing food producing sector in the world and microalgae are ideal candidates as major live feeds in aquaculture. Other than providing the nutritional input, these microalgal live feeds may have a role with bacterial communities of larval rearing systems since they always coexist in the aquatic ecosystem. Thus, knowledge of microalgae-associated microhabitat is important in the management of a healthy hatchery rearing system. The present research work deals with study on the bacterial association with marine microalgae, their diversity and exploration of these associated bacteria for various aquaculture and biotechnological applications.

The present study has been divided into the following sections:

- The diversity of culturable bacteria associated with established marine microalgal cultures and microalgal production system of a hatchery
- Entire bacterial diversity in microalgal habitat with special reference to *Isochrysis galbana* by using metagenomic approach
- Symbiotic association of culturable heterotrophic bacteria with *Isochrysis galbana*
- Efficacy of microalgae associated bacteria on survival, growth and mysis conversion rate of shrimp larvae (*Penaeus indicus*)
- Screening the heterotrophic bacteria associated with *Isochrysis galbana* for various biotechnological applications

8.1 Salient findings of the study:

- Major groups of bacteria associated with ten aquaculturally important marine microalgae and microalgal production system of a marine finfish hatchery were identified.
- Phylogenetic analysis using 16S rDNA sequencing revealed that the bacterial phylotypes associated with marine microalgae belong to *Gammaproteobacteria, Alphaproteobacteria, Flavobacteriia* and *Bacilli* classes and *Vibrio* spp., a commonly occurring bacterial group in marine environment were totally absent**.**
- Members of *Marinobacter* genus showed higher prevalence followed by *Alteromonas, Labrenzia, Oceanicaulis, Ponticoccus, Stappia, Rheinheimera, Leisingera, Nautella, Halomonas* and *Ruegeria.*
- Bacteria belonging to the genera *Gaetbulibacter, Maritalea, Idiomarina, Albidovulum* and *Staphylococcus* were reported for the first time in microalgal association.
- Most notably, the record of new bacterial groups and the absence of *Vibrio* spp., the major aquaculture pathogen in tested microalgal stains suggest the suitability of these microalgae for use in larval rearing systems such as hatcheries.
- Phycosphere of *Isochrysis galbana* MBTDCMFRIS002 was selected as a representative for further studies on algal-bacterial interactions and metagenomic analysis revealed the entire bacterial diversity associated with *I. galbana*.
- Next generation sequencing technologies have been applied for sequencing of 16S rDNA V3 region. The data set comprised of 1190

Operational Taxonomic Units based on which phylum, class, order, family, genus and species distribution was determined.

- A total of 44 different bacterial genera mostly from the classes *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Flavobacteriia*, *Acidimicrobiia*, *Sphingobacteriia* were detected in the study. In addition to these known bacterial groups several unknown groups (the sequences do not have any alignment against taxonomic database) were also detected in the studied algal habitat.
- By PICRUSt analysis, several functional traits of bacterial communities associated *I. galbana* were identified.
- It was found that these bacterial communities are mainly involved in environmental information processing, genetic information processing, membrane transport and nutrient metabolism. These functions may mediate their interaction with phytoplankton host and thus improve bacterial survival in algal habitat.
- To study the symbiotic interaction between microalgae and bacteria, *I. galbana* culture was axenised using an antibiotic cocktail of penicillin, streptomycin, gentamicin and kanamycin.
- The antibiotic concentration of 1000:5:5:2.5 µg/ml of penicillin, streptomycin, gentamicin and kanamycin respectively for 48 h was found to be most effective in inhibiting bacterial growth in microalgal culture.
- The nutrient profile of axenic and non-axenic *I. galbana* cultures was compared based on the levels of protein, fatty acid, pigment and carbohydrate.

- Total protein content of non-axenic culture was 28.09 ± 0.12 mg/g whereas that of axenic culture was 25.73 ± 0.44 mg/g.
- Chromatographic analysis demonstrated that non-axenic *I. galbana* culture produces more fatty acid (15.7 ± 0.17 mg/g) in the early stationary phase of growth than axenic *I. galbana* culture (11.4 ± 0.2 mg/g).
- Similarly, non-axenic culture possessed more pigment and carbohydrate content (9.32 ± 1.33 µg/ml and 67.59 ± 1.32 µg/ml) than axenic culture (7.01 ± 0.57 µg/ml and 45.85 ± 1.01 µg/ml).
- It was found that addition of bacterial symbionts (*Alteromonas* sp. MBTDCMFRI Mab 25 and *Labrenzia* sp. MBTDCMFRI Mab 26) has a positive effect on biomass accumulation and growth rate of axenic *I. galbana.*
- Both bacterial strains were able to grow in culture broth containing algal extracellular organic carbon (EOC) as carbon source.
- There exist a mutualistic interaction between microalgae and bacteria in which both partners are benefitted. The associated bacteria can enhance the growth and nutrient profile of microalgae and the bacterial counterparts in turn benefitted by utilising organic carbon released by phytoplankton host.
- The efficacy of phycosphere bacteria on survival, growth and mysis conversion rate of shrimp larvae was studied. The larval survival of Indian white prawn, *P. indicus* was found to be increased at significant level when they fed with microbial cocktail of *Alteromonas* sp. MBTDCMFRI Mab 25, *Labrenzia* sp. MBTDCMFRI Mab 26,

Marinobacter sp. MBTDCMFRI Mab 18 and *Marinobacter* sp. MBTDCMFRI Mab 34.

- Mysis fed with non-axenic *I. galbana* enriched with microbial cocktail proved to give higher growth in size (3.15 mm) while larvae fed with axenic *I. galbana* resulted in poor growth (2.6 mm) ($p < 0.01$). Also, among mysis larvae, conversion rate was found to be maximum in larvae fed with non-axenic *I. galbana* enriched with microbial cocktail (90 ± 7.07 %).
- 'Microbial cocktail' developed from the selected algal associated bacteria proven to be effective in the healthy larval rearing of aquaculture species.
- The bacterial strains *Alteromonas* sp. MBTDCMFRI Mab 25 and *Labrenzia* sp. MBTDCMFRI Mab 26, associated with *I. galbana* were screened for various biotechnological applications namely silver nanoparticle biosynthesis, antioxidant activity, siderophore production, inole-3-acetic acid production, biosurfactant production and bacterial exopolysaccharide production.
- Biosynthesis of silver nanoparticles was achieved by using *Labrenzia* sp. Mab 26. The synthesised nanoparticles were further characterised by UV-vis spectroscopy, XRD analysis, FT-IR spectrum, scanning electron microscopy and transmission electron microscopy.
- Both *Alteromonas* sp. Mab 25 and *Labrenzia* sp. Mab 26 exhibited 2,2-Diphenyl-1-picrylhydrazyl scavenging activity. At concentration 2.5 mg ml^{-1} the crude extract of *Alteromonas* sp. Mab 25 had higher scavenging effect (41 %) than that of *Labrenzia* sp. Mab 26 (25.3 %).

The IC_{50} value of two bacterial isolates was 6.38 and 9.55 mg ml^{-1} respectively, proving their potential as antioxidant.

- *Labrenzia* sp. Mab 26 was found to be positive for indole-3-acetic acid production whereas *Alteromonas* sp. Mab 25 was negative.
- Both bacterial strains exhibited siderophore, biosurfactant and bacterial exopolysaccharide production potential.
- This study highlights the significance and scope for exploring microalgae associated bacteria for various industrial and biotechnological applications.

8.2 Conclusion

There exist a strong and close association between bacteria and microalgae which makes the phycosphere a hotspot of complex interactions. These associated bacteria have a significant impact on algal growth and metabolism. Consequently, these microflora could improve the efficiency of microalgal mass culture and associated valuable compounds. In aquaculture hatchery rearing systems, microalgae are normally used as starter feed at the larval stage and nutrient composition of algal diet definitely determines the end nutritional quality of rearing shellfish or finfish. This would open up avenues to use co-cultures, or addition of selected associated bacterial strains for improving the growth and health of aquatic organisms, especially at larval rearing stages. The present study stands as first one of its kind involved in practical application of phycosphere bacteria to produce more sustainable live feeds, which might greatly enhance the productivity and efficiency of larviculture in aquaculture. Moreover, they can also bioprospect for potential bioactive compounds for providing sustainable human benefits. The algal – bacterial interactions are mainly regulated by the chemical nature of exchanged mediator molecules,

including nutrients, cross – signalling and the natural ability of microalgae to adhere to specific phycosphere bacteria. Thus, a greater insight on chemistry behind these interactions should accelerate the search for novel bioactive compounds. The current development in the understanding of these interactions is leading to specific aquaculture and biotechnological applications. However, further research on algal microhabitat is needed for controlled integration of specific bacteria in the scale up production processes of microalgae to obtain desired beneficial and commercial outcomes.

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PUBLICATIONS

[1] **S.V. Sandhya**, K. Preetha, Anusree V Nair, M. Leo Antony, K.K. Vijayan. Isolation, characterisation and phylogenetic diversity of culturable bacteria associated with marine microalgae from saline habitats of south India. Aquatic Microbial Ecology (2017), 79: 21-30.

[2] **S.V. Sandhya**, K. Preetha, K.K. Vijayan. Phylogenetic diversity of culturable bacteria in *Chaetoceros gracilis* mass culture system of a marine finfish hatchery. JMBAI (2017), 59 (2).

Papers and posters presented

[1] **Sandhya S V**, Preetha K, Anusree V Nair, Leo Antony M, Vijayan K K. Role of phycosphere bacteria on the growth of *Isochrysis galbana,* an aquaculturally important microalga. MECOS-2 (2014) International symposium, December 2-5, pp 106.

[2] **S V Sandhya**, K Preetha, Anusree V Nair, K K Vijayan. Bacterial diversity associated with microalgal production system of marine finfish hatchery with special reference to *Chaetoceros gracilis* mass culture. 28th Kerala Science Congress, January 2016, pp 1295-1301.

[3] **S V Sandhya**, K Preetha, Anusree V Nair, Leo Antony M, K K Vijayan. Isolation, Characterisation and Phylogenetic diversity of culturable bacteria associated with marine microalgae from tropical habitats of South India. International Conference on Science & Technology for National Development. October 25-26, 2016, pp 47-48.

[4] **S V Sandhya**, K K Vijayan, P. Vijayagopal. Molecular phylogeny and antioxidant activity of bacteria associated with marine microalgae

Isochrysis galbana. 29th Kerala Science Congress, January 2017, pp 845-848.

[5] **S V Sandhya**, K K Vijayan. Biogenesis of silver nanoparticle by marine bacteria *Labrenzia* sp. MBTDCMFRI Mab 26 associated with Isochrysis galbana. International seminar on recent trends in best management practices of aquaculture organized by KUFOS, DST Govt. of India & NAAS Kochi chapter

[6] **S V Sandhya**, K K Vijayan. Symbiotic association of culturable heterotrophic bacteria in *Isochrysis galbana* culture, 30th Kerala Science Congress, January 2017, pp 209-210.

Genbank submissions

1.	Sequence description	:	Partial 16S rDNA
	Number of submissions	:	103
	Accession No	:	KR004791 – KR004798, KR004801 – KR004826, KU572438, KU572440 – KU572443, KU554452 – KU554462, KU554466 - KU554514, MF991457 – MF991460
	Source	:	Microalgae
2.	Sequence description	:	Raw sequence reads from next generation sequencing platform
	Number of submissions	:	1
	Accession number	:	SRR6740228
	Source	:	Microalgae