9. CAROTENOID IDENTIFICATION OF ASSOCIATED ENDOPHYTIC PIGMENTED BACTERIA FROM D. CUNEATUS

9.1. INTRODUCTION

Due to the complex and dynamic system in the ocean, the marine microorganisms have developed unique metabolic and physiologic capabilities that ensure survival in extreme variations like temperature, salinity and pressure. Adaptation to these stress factors could involve metabolic changes in the production or secretion of chemical substances some of which could be bioactive nature. This could be a major reason for marine microbes being metabolically different in respect to their terrestrial counterparts (Fenical and Jensen 1993; Fenical, 1993). A main characteristic feature of marine bacteria is that a large proportion of them are pigmented (Zobell, 1946).

Microorganisms produce a wide variety of pigments including carotenoids, flavins, monascins, chlorophyll, quinines, prodigioson, violacien etc (Dufosse, 2006). The diversity in pigments occur due to the differences in their chemical structure compositions and the presence of specific chromatophores (Hui and Hurlbert, 1979). Pigmented microorganisms have awakened the interest of the scientific community, because of their role in taxonomic studies and their biotechnological potential in the processes like fermentation and bioprocess (Chatoopadhyay et al., 2008). Thereby, represents a prospective source of natural pigments of commercial interest in several industries as food, feed, cosmetics, pharmaceuticals and nutraceuticals (Varsha and Arpana, 2013). In addition, microbial pigments are safe to use, moreover, they maintain controllable and predictable yield unlike plant pigments which they don’t exhibit seasonal and geographical variations (Joshi et al., 2003; Mohanasrinivasan et al., 2013). The expression of the pigments can be affected by various environmental factors, including oxygen availability, nutritional condition, temperature, age of the colony and strain.
variation (Starr, 1958). Among them, the most commonly distributed pigments are the carotenoids.

Carotenoids play a vital role in bacteria, for example, in photosynthetic processes, by preventing photo-damage and conferring resistance to oxidative damage due to the production of activated forms of oxygen. They are synthesized by plants, algae, some fungi, bacteria and archaea. Carotenoids are relatively hydrophobic molecules typically associated with membranes and/or non-covalently bound to specific proteins. The presence of carotenoids may also change the effectiveness of the membrane as a barrier to water, oxygen and other molecules (Britton, 1995). The pigment may be aiding the bacteria to survive in this stressed habitat. Another defining feature of carotenoids is their chromophore composed of a series of conjugated double bonds. The length of these conjugated bonds determines the colour of the molecule. It renders the ability of carotenoids to confer colour that has fuelled commercial interest in these molecules, predominantly as natural colorants.

Carotenoids are believed to improve immune responses and function as an antioxidant. It has also been suggested that the presence of carotenoids may change the effectiveness of the membrane as a barrier to water, oxygen and other molecules (Britton 1995). Carotenoid pigments such as fucoxanthin, astaxanthin have also been proven to possess strong antioxidative activities and attracted greater attention due to their beneficial effects on human health such as their potential in the prevention of diseases such as cardiovascular complaints (Wollgast and Anklam, 2000). Children who are malnourished often have lower concentrations of serum carotenoids compared with good nourished children. Carotenoids have also been used in the treatment of diseases that are very sensitive to light (Mathews, 1964).
From a commercial point of view, there is an increasing demand of special carotenoids as food colorants, precursors of vitamin and animal feed (Ruther et al., 1997). The demand and market for carotenoids is anticipated to change drastically with the discovery that carotenoids exhibit significant anti-carcinogenic activities (Lee and Schmidt-Dannert, 2002). Moreover, industrial interest is now gradually shifting away from the yellow carotenoids such as β-carotene and lutein towards the considerably more valuable orange-red keto-carotenoids, such as torularhodin and torulene, for which at present no commercially exploitable plant or animal sources exist (Nelis and De Leenheer, 1991). Hence the microbial pigment production is one of the emerging field of research to demonstrate its potential for various industrial and clinical applications.

As per the literature, the diversity of bivalve-associated bacteria and their biological activity, interrelations between themselves and host organism is still limited. It is becoming important to study microbial diversity from unexplored or known marine bivalves as well as to search potential microorganisms to produce bioactive metabolites. Commonly in marine organisms, two categories of bacterial flora have been described, the associated or resident flora, maintaining a stable presence in the host organism, and the transitory flora, which may occur periodically in an organism on a temporary basis (Moriarty, 1990). Marine bacteria have been shown to play important roles in the life cycles of different invertebrates (McFall-Ngai and Ruby, 2000). During the past two decade’s research on marine bacteria has highlighted the tremendous potential of these microorganisms not only as a source of new bioactive secondary metabolites, but also for pigment possessing latent activities (Balraj et al., 2014). Hence, the present study was carried to isolate and characterize endophytic pigment producing bacterial strains for carotenoid production from *D. cuneatus*. 
9.2. MATERIALS AND METHOD

9.2.1. Chemicals

Zobell marine agar (ZMA), Zobell marine broth (ZMB), Muller Hinton agar (MHA), Nutrient agar (NA), Ampicillin (AMP, 10 mcg/disc), Azithromycin (AZM, 15 mcg/disc), Ciprofloxacin (CIP, 5 mcg/disc), Chloramphenicol (C, 30 mcg/disc), Tetracycline (TE, 30 mcg/disc), Erythromycin (10mcg/disc), DPPH (2,2-diphenyl-1-picrylhydrazyl), Potassium ferricyanide, Trichloroacetic acid, Ferric chloride, Butylatedhydroxytoluene (BHT), Astaxanthin, β-Carotene, Lyopene, Ethylene diamine tetra acetic acid (EDTA), 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT). Benzene, Methanol, Hexane, Acetone and Acetonitrile of analytical grade was used.

9.2.3. Isolation of bacteria associated with marine bivalve

The collected specimens were washed with filtered and autoclaved sea water until they are visibly free of debris. Following which, the surface of clams was sterilized by a rapid wash of 70% ethanol and immediately immersed in autoclaved sea water and then aspirated. One gram of tissue was cut with a sterile scalpel and homogenised using sterilized motor and pestle in a laminar flow chamber. The homogenate was serially diluted up to $10^6$ dilution and then spread on Zobell marine agar (ZMA) plates. The plates were incubated at room temperature for 3-4 days. The bacteria’s with different types of colonies were isolated and repeatedly streaked to obtain pure culture. The pure culture was stored in ZMA slants at 4°C for further studies (Anand et al., 2006).

9.2.4. Selection of endophytic pigment producing bacteria

On observing the overall growth of microorganisms the number of pigmented bacteria formed in the plates was found to be more in number inferring that the endophytic pigmented
bacterial (EPB) strains may play a major role in the bivalve system. From the obtained, three endophytic pigmented bacterial strains were selected strains for further studies. The selection of EPB strains was done based upon the appearance of brilliant colour and higher frequency of colonies that appear in the dilution plates.

9.2.5. Cultivation of EPB strains

The selected EPB isolates CPA3 (Culture PA3), CAG6 (Culture AG6) and CAM9 (Culture AM9) were subcultured in ZMA plate and incubated at 30 ± 2 °C for three days. A preculture was developed in 5 ml of Zobell marine broth (ZMB) by inoculating the broth with one loop full of selected strains and incubating at 30 ± 2 °C overnight. 50 ml of freshly prepared ZMB taken in 250 ml Erlenmeyer flasks was inoculated with the preculture and incubated for 3-5 days on a rotary shaker depending on the growth rate at 150 rpm 30 ± 2 °C.

9.2.6. Antibiotic susceptibility of EPB strains

The selected EPB strains CPA3, CAG6 and CAM9 were tested for antibiotic susceptibility activity using disc diffusion method described by Bauer (1966). About 25 ml of molten Mueller Hinton agar (MHA) was poured into sterile petri plates (Himedia, Mumbai, India). The plates were allowed to solidify, after which 100 μl of 18 h grown (OD adjusted 0.6) selected EPB strains were transferred in the plate and made culture lawn using sterile L-rod spreader. After five min of setting, the antibiotic discs were deposited onto the plate. The antibiotic discs used were Ampicillin (AMP, 10 mcg/disc), Azithromycin (AZM, 15 mcg/disc), Ciprofloxacin (CIP, 5 mcg/disc), Chloramphenicol (C, 30 mcg/disc) and Tetracycline (TE, 30 mcg/disc). The sterile disc with water served as control. The plates were incubated at 37 °C for 18 h. The antibiotic susceptibility was determined by measuring the diameter of the zone of inhibition around the well.
9.2.7. Extraction of pigments

The extraction of the pigments from EPB isolates CPA3, CAG6 and CAM9 was performed under dim light according to the method of Slater et al. (2003) with suitable modification. Briefly, One ml of culture broth was taken in a microfuge tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The colourless supernatant was discarded. The harvested coloured cells were resuspended in acetone: methanol (7:2, v/v). The microfuge tube with the suspended cell pellet was then kept in a water bath at 60 °C for 20 min, thereby the pigments were extracted. The samples were centrifuged again for 10 min at 4 °C at 10,000 rpm to remove the white cell pellet. The supernatant contained the total extractable pigment. The cell pellet was again extracted with acetone by repeated centrifugation until the cell debris turned colourless and the supernatant was evaporated to dryness. To avoid possible loss of other chromophores, no saponification or other procedure to remove lipid contaminants from the extracts was performed. All operations were carried out rapidly and the effects of light were avoided by carrying out the extractions in glass covered with aluminium foil. The short exposure to an increased temperature was found to produce no injurious effects on the pigments (Liaaen-Jensen and Andrewes, 1972; Schmidt et al, 1994; Schiedt and Liaaen-Jensen, 1995). The samples were stored at -20 °C until further analysis.

9.2.8. Spectral measurements

The pigment of EPB strains were denoted as PPA3 (Pigment PA3), PAG6 (Pigment AG6) and PAM9 (Pigment AM9) from CPA3, CAG6 and CAM9 cultures respectively. The pigments were analyzed by scanning in a UV-Visible spectrophotometer (Shimadzu, Japan) for detecting the λ max. The scanning range was selected between 300 to 900 nm. Absorption spectra were recorded in spectroscopic grade acetone: methanol mixture (7:2, v/v), which were also used as blanks when appropriate.
9.2.9. Determination of Antibacterial activity

9.2.9.1. Microorganisms and inoculum preparation

The extracted pigments PPA3, PAG6 and PAM9 from EPB isolates were tested against the bacterial pathogens *K. pneumoniae*, *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa*. All microorganisms were obtained from the Department of Microbiology, Annamalai University, India.

The bacterial pathogens were subcultured in nutrient agar. The strains were inoculated and grown to exponential phase in nutrient broth at 37 °C for 18 h and adjusted to a final density of $10^5$ CFU/ml by diluting fresh cultures by comparing with McFarland density.

9.2.9.2. Antibacterial activity

Antibacterial activity of crude pigments (PPA3, PAG6 and PAM9) was determined by agar disc diffusion method (Jorgensen and Turnidge, 2007). Plates of Muller Hinton agar were evenly streaked in three different directions with a sterile cotton swab dipped in the bacterial suspension ($10^5$ CFU/ml). Sterile filter paper disc (5 mm diameter) were immersed in the 50 μl of respective extract (0.5 mg/ml) and allowed to dry at room temperature and placed over the MHA plates. Erythromycin 10 mcg/disc was used as positive control. The plates were incubated overnight at 37 °C and the zone of inhibition around the disc was measured.

9.2.10. DPPH radical scavenging activity

The radical scavenging abilities of the crude pigment PPA3, PAG6 and PAM9 was measured from the bleaching of purple-colour methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) following the method of Yen and Chen (1995). Briefly, 1 ml of pigment extracts (0.5 mg/ml) in methanol was added to 4 ml of 0.004% methanol solution of DPPH.
The mixture was vortexed for 1 min and left to stand at room temperature for 30 min in dark, and the absorbance was read at 517 nm. Lower absorbance indicated higher radical-scavenging activity. Inhibition of free radicals by DPPH in percent (Scavenging activity %) was calculated by the following formula:

\[
\text{Scavenging activity} \% = \frac{\text{(Ab} - \text{As})}{\text{Ab}} \times 100
\]

Where, Ab - the absorbance of the control (DPPH solution without sample), As - the absorbance of the test sample (DPPH solution plus test sample), BHT - positive control.

9.2.11. Identification of carotenoids using Thin layer chromatography

9.2.11.1. Instrumentation

The presence of carotenoids in the pigments PPA3, PAG6 and PAM9 of EPB isolates was identified using CAMAG HPTLC system (Switzerland). Five µl of sample was sprayed with help of N\textsubscript{2} gas in the bandwidth of 6 mm using the sample applicator (Linomat 5) fitted with 100 µl Hamilton syringe on TLC silica gel 60 F 254 aluminium plates (Merck, Germany). The plates were developed using the developing solvents in a pre-saturated CAMAG twin-trough chamber which was lined with filter paper. The developed plates were then dried and bands were visualized in UV-chamber at 366 nm and 254 nm.

9.2.11.2. Detection of carotenoids

The concentrated pigments PPA3, PAG6 and PAM9 of isolates EPB were preliminarily detected for the presence of carotenoid groups. The mobile phase used was benzene for the detection of carotenoid ketones and benzene: methanol (49: 1 v/v) for the detection of carotenoid hydroxylates (Stahl, 1969). The identification of the type of carotenoids present in the EPB pigment extracts were identified using three standards Astaxanthin, β-Carotene and Lyopene. The samples and standards were spotted in the TLC.
plate and developed with mobile phase using hexane: acetone (7: 3) in a saturated twin trough chamber. The plates were air dried and scanned at 330 nm.

9.2.12. HPLC purification

The partially purified fraction (scraped fraction obtained from HPTLC) was further investigated using HPLC by comparing with the standard. The separation was achieved by HPLC on C₁₈ reverse phase column (Phenomenex, HPLC system – Shimadzu, Japan). The standard and sample (25μl) was eluted using Acetonitrile: Methanol (65:35, v/v) at flow rate of 1ml/min and peaks were monitored at 450 nm.

9.2.13. Cytotoxicity assay

9.2.13.1. Cell line

The human breast cancer cell lines (MCF-7) obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10 % fetal bovine serum (FBS). The cells were maintained at 37 °C, 5 % CO₂, 95 % air and 100 % relative humidity. Maintenance of cultures was passaged weekly and the culture medium was changed twice a week.

9.2.13.2. Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspension and viable cells were counted using a hemocytometer and diluted with medium containing 5 % FBS to give a final density of 1x10⁵ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5 % CO₂, 95 % air and 100 % relative humidity. After 24 h the cells were treated with serial concentrations of the test sample (partially purified fraction). They were initially dissolved in
dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum concentration with a serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37 °C, 5% CO₂, 95% air and 100% relative humidity. The medium without samples served as control and triplicate was maintained for all concentrations (Mosmann, 1983; Monks et al., 1991).

9.2.13.3. MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

\[
\text{% Cell inhibition} = \frac{\text{Mean absorbance of the test sample}}{\text{Mean absorbance of control}} \times 100.
\]

9.2.13.4. Analysis

A graph of absorbance (y-axis) against the concentration of the sample (x-axis) was plotted and the IC₅₀ concentration was determined as the sample concentration required to
reduce the absorbance to half that of control. The data was then converted to percentage inhibition curve, to normalize a series of curves.


The isolated EPB strains (CPA3, CAG6 and CAM9) were identified based on cell morphology, growth conditions, gram staining, motility and biochemical tests. The obtained data were compared with standard description provided in Bergey’s manual of determinative bacteriology (Holt et al., 1994).

9.2.14.1. 16S rRNA sequencing and analysis

The genomic DNA was extracted from the cells of 18 hours bacterial culture of EPB isolates CPA3, CAG6 and CAM9 using the InstaGeneTM Matrix Genomic DNA isolation kit (Catalog # 732-6030). The sequences were amplified using 16S rRNA universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT) in MJ Research PTC-225 Peltier Thermal Cycle (Nithyanand and Pandian, 2009). The PCR was performed in a reaction mixture containing 1μl of DNA as template and 20 μl of PCR reaction solution. After the initial denaturation of 3 min at 95 °C, 35 cycles consisting of denaturation at 94 °C for 45 sec, annealing at 55 °C for 60 sec and extension at 72 °C were for 60 sec was carried out. DNA fragments are amplified about 1,400 bp for bacteria. A positive control (E. coli genomic DNA) and a negative control were added in the PCR. The PCR products were electrophoresed on 1% agarose gel and documented (Kumaran et al., 2010). The obtained PCR product was purified by using PCR purification kit (Millipore) and the nucleotide sequences of the PCR product were sequenced using the 518F/800R primers. DNA sequencing was performed in a ABI PRISM® BigDye TM Terminator Cycle Sequencing Kits AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Finally, the obtained
sequence was compared with reference 16S rRNA gene sequences available in NCBI GenBank database BLAST using blastn softwares.

9.2.14.2. Nucleotide accession number

The 16S rRNA sequence of EPB strains CPA3, CAG6 and CAM9 was submitted in GeneBank database and the accession number was assigned as KJ949604, KJ949605, KJ949606.

9.2.14.3. Phylogenetic analysis

Phylogenetic analysis of the isolated bacterial strain was performed with their closest sequences of blast result using the program PhyML 3.0 aLRT. Briefly, the sequences were aligned by the program MUSCLE 3.7 (multiple sequence alignment) and phylogentic tree were constructed by using the program TreeDyn 198.3 (Dereeper et al., 2008).

9.2.15. Statistical analysis

All the data expressed as mean ± standard deviation (SD) of number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 11.5 (SPSS, Cary, NC, USA) and the individual comparison were obtained by Duncan’s Mutiple Range Test (DMRT). A value of P < 0.05 was considered to indicate a significance difference between groups.

9.3. RESULT

9.3.1. Bacterial density of D. cuneatus associated microbes

The marine bivalve D. cuneatus was analysed for associated bacterial population. The bacterial density was estimated as 3.2 x 10^6 CFU/ml to 6 X 10^6 CFU/ml. Fig.22. shows the density of endophytic microbes of beach clam.
9.3.2. Isolation of Pigment producing bacteria culture

The obtained cultures in Zobell marine agar were found to contain 23 pigmented colonies from the tissues of *D. cuneatus*, which were of following colors: pale yellow, dark orange, dark yellow, reddish pink, light brown, cream and pale orange (Fig.23).
9.3.3. Selection of EPB strains

The selection of EPB strains for further studies was selected based on (i) the more number of counts (frequency of culture in plates) and (ii) their vibrant color. Based on the above, three strains were selected like EPB CPA3, EPB CAG6 and EPB CAM9 (Fig.24). The presence of more number of endophytic pigmented strains in the bivalve’s emphasis the major role played by them in the bivalve system.

![Three selected pigment producing EPB strains CPA3, CAG6 and CAM9.](image)

**Fig. 24.** Three selected pigment producing EPB strains CPA3, CAG6 and CAM9.

9.3.4. Antibiotic susceptibility of pigment producing bacteria

The selected three pigment producing EPB strains were tested for antibiotic sensitivity against five commonly used antibiotics belonging to different groups. The antibiotics used were Ampicillin (AMP), Azithromycin (AZM), Ciprofloxacin (CIP), Chloramphenicol (C) and Tetracycline (TE). The endophytic isolate EPB CPA3 showed lesser resistance (susceptible) against all the five antibiotics whereas EPB CAG6 exhibited increased resistance against three antibiotics and EPB CAM9 showed increased activity against four antibiotics (Table.10).
Table. 10. Antibiotic susceptibility activity of EPB strains CPA3, CAG6 and CAM9.

<table>
<thead>
<tr>
<th>Antibiotic Name</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA3</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>26</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>27</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>20</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>28</td>
</tr>
</tbody>
</table>

9.3.5. Pigments Extraction

The pigments extraction from the strains was achieved by extracting the coloured culture pellet CPA3, CAG6 and CAM9 using Acetone: Methanol (Fig.25). The pigment was completely dissolved in the solvent which was then removed by evaporation. The net yield of the pigment of the isolates CPA3, CAG6 and CAM9 were found to be 1.72 mg/lt, 1.22 mg/lt and 1.32 mg/lt respectively.
Fig. 25. Pigment extracted from EPB strains of cell culture pellet of CPA3, CAG6 and CAM9 using acetone: methanol (7:2, v/v).

9.3.6. Absorption Characteristics of Pigments

UV-visible absorption spectra of pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids (Medicharla et al., 1991). The pigment of the culture pellets was completely extractable in Acetone: Methanol. The UV-visible absorption spectra of the pigments showed differing range of absorption from 300- 500 nm. Each pigment exhibited two absorption peaks at different nm. The UV-visible absorption spectra of PPA3 showed maximum absorption at 300 nm and 430 nm, PAG6 showed maximum absorption at 340 nm and 470 nm and PAM9 showed maximum absorption at 340 nm and 460 nm (Fig.26). All the three pigment extracts, the major peak was found between 400nm – 500nm.
**Fig. 26.** UV-visible absorption spectrum of the pigments PPA3, PAG6 and PAM9.

### 9.3.7. Antibacterial activity of the pigments

Antibacterial activity of the extracted pigments was determined by agar disc diffusion method. All the pigments extracted showed antibacterial activity against the tested human pathogens. Among them, PPA3 exhibited lesser zone of inhibition whereas PAG6 and PAM9 exhibited increased zone of inhibition. The results are shown in Fig.27.
Fig. 27. Antibacterial activity of the pigments PPA3, PAG6 and PAM9.

9.3.8. DPPH radical scavenging activity

Carotenoids can effectively act as antioxidants thus protecting the body from oxidative damage caused by internal or external stress (Isaksson et al., 2007). Thus the pigments were found to exhibit significant DPPH radical scavenging activity (Fig.28). The scavenging capacity of the reactive species was dependent on the carotenoid concentration. The pigment PPA6 was found to possess maximum antioxidant activity (41.38%, 100 μg/ml) and minimum activity was observed for pigment PPA3 (37.05%, 100 μg/ml) and PAM9 (18.51%, 100 μg/ml).
Fig. 28. DPPH radical scavenging activity of the pigments PPA3, PAG6 and PAM9.

9.3.9. Detection of carotenoids using HPTLC

The presence of carotenoids in the pigment extracts PPA3, PAG6 and PAM9 was identified using different solvent systems. Benzene was used for the detection of carotenoid ketones and benzene: methanol (49: 1 v/v) for the detection of carotenoid hydroxylates. The three pigments illustrated several bands for both the solvent systems used revealing the presence of respective type carotenoid group with differing bands at varying Rf value (Fig.29). Rather, the presence of type of carotenoid present in the three pigments was identified by comparing with the available three standards Astaxanthin, β-Carotene and Lyopene using hexane: acetone (7:3) as mobile phase scanned at 330 nm (Fig.30 and 31). The bands formed for the two pigments PPA3 and PAM9 did not match with any of the three standards used whereas PAG6 exhibited three bands of which, the first band matched with the standard astaxanthin possessing the same Rf value (0.30) and the other two bands did not match with any other standards used.
Fig. 29. HPTLC Detection of carotenoids in the pigments PPA3, PAG6 and PAM9. A & B. Detection of carotenoid ketones using Benzene as solvent at 366 nm and digital scanning of the pigment. C & D. Detection of carotenoid hydroxylates using Benzene: Methanol at 366 nm and digital scanning of the pigment.
Fig. 30. HPTLC analysis of standard with the three extracted pigments. Standard: 1. Astaxanthin, 2. β-Carotene, 3. Lyopene; Samples: 4. PPA3, 5. PAG6, 6. PAM9. A. UV-chamber at 254 nm, B. White light and C. UV-chamber at 366 nm.

Fig. 31. HPTLC of peak display of standard Astaxanthin (A) and astaxathin present in the pigment PAG6 (B).

9.3.10. HPLC

The partially purified astaxanthin of PAG6 (scraped from HPTLC) was used for HPLC identification. The standard astaxanthin was injected which was compared with the
pigment separated PAG6 and scanned at 450 nm. The HPLC profile of PAG6 exhibited similar retention time with that of standard at 3.9 mins which confirmed the presence of astaxanthin (Fig.32).

Fig. 32. HPLC profile of Standard Astaxanthin (A) and partially purified PAG6 pigment detected at 450 nm (B).
9.3.11. Cell line toxicity assay

The cytotoxicity of the partially purified astaxanthin of PAG6 was studied for human breast cancer cell line (MCF-7) by MTT assay. The partially purified astaxanthin exhibited considerable anticancer activity against MCF-7 cell line. The cytotoxicity activity was detected using MTT assay, in which the yellow terazolium salt is metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark formazan product in which the absorbance is directly proportional to the number of viable cells. The cytotoxic activity of the sample was shown in Fig.33 and 34. The cell line exhibited concentration dependent cell death and their IC$_{50}$ value was calculated (the concentration that kills 50 % cells). The IC$_{50}$ of partially purified astaxanthin fraction of PAG6 in MCF-7 was found to be 9.37 µg/ml, thus proving efficient cytotoxicity activity. The cells were observed using inverted microscope for morphological changes and photographed.

![High Concentration](image1.png) ![Medium Concentration](image2.png)

![Low Concentration](image3.png) ![Control – MCF7 cell line](image4.png)

**Fig. 33.** Cytotoxicity effect of partially purified astaxanthin on human breast cancer cell line (MCF-7).
Fig. 34. The percentage cell inhibition of partially purified astaxanthin on human breast cancer cell line (MCF-7) in different concentrations. The values are given as mean ± SD of three experiments in each group.

9.3.12. Identification of EPB strains

The three EPB strains were maintained in Zobell marine agar slants at 4 °C. The morphological and biochemical characteristics are presented in Table 11. Based on Bergey’s manual of determinative bacteriology the phenotypic characteristics of isolates CPA3, CAG6 and CAM9 found belong to the genus *Kocuria*, *Pontibacter* and *Staphylococcus* respectively. Using 16S rRNA sequencing they were confirmed as *Kocuria flava*, *Pontibacter korlensis* and *Staphylococcus saprophyticus* which was designated as *Kocuria flava* PA3, *Pontibacter korlensis* AG6 and *Staphylococcus saprophyticus* AM9 respectively.
Table. 11. Physiological and biochemical characterization of potential strains EPB CPA3, CAG6 and CAM9 (+: Positive, -: Negative).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>CHARACTERISTICS</th>
<th>CPA3</th>
<th>CAG6</th>
<th>CAM9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Grams staining</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Colony color</td>
<td>Yellow</td>
<td>Orange</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>3.</td>
<td>Cell shape</td>
<td>Cocci</td>
<td>Rod</td>
<td>Cocci</td>
</tr>
<tr>
<td>4.</td>
<td>Motility</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Temperature (°C)</td>
<td>30-45</td>
<td>30-37</td>
<td>28-35</td>
</tr>
<tr>
<td>6.</td>
<td>Nitrate reduction</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Urease</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Oxidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>Carbon source utilization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D- cellobiose</td>
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<td>11.</td>
<td>DNA G+C content (mol%)</td>
<td>71</td>
<td>48.2–48.9</td>
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9.3.12.1. Molecular taxonomy (16S rRNA analysis)

Genomic DNA was isolated from the respective strains of EPB CPA3, EPB CAG6, EPB CAM9 and it was amplified using 16S rRNA Universal primers gene fragments. The molecular weight of amplified DNA fragments of the strains corresponding to the DNA ladder in 1% agarose gel was shown in the figure (Fig. 35). The PCR product was purified to remove unincorporated PCR primers and dNTPs using Montage PCR Clean up kit and then sequenced using 518F/800R primers. Sequenced strains EPB CPA3, EPB CAG6 and EPB CAM9 showed partial 16S rRNA sequences consisting of 1447, 1448 and 1476 nucleotides (Fig. 36, 37 and 38) were submitted to GenBank (National Center for Biotechnology, USA).

![Image](image_url)

**Fig. 35.** Amplified product of 16S rDNA of strains CPA3, CAG6 and CAM9. Lane 1: CPA3, Lane 2: CAG6, Lane 3: CAM9, Lane M: DNA marker.
Fig. 36. Screenshots of NCBI of strain CPA3 (GeneBank accession number: KJ949604).

Fig. 37. Screenshots of NCBI of strain CAG6 (GeneBank accession number: KJ949605).
Fig. 38. Screenshots of NCBI of strain CAM9 (GeneBank accession number: KJ949606).

The obtained 16S rRNA gene sequences of three EPB CPA3, CAG6 and CAM9 strains were preliminarily compared with previously obtained sequences of *Kocuria* sp, *Pontibacter* *sp* and *Staphylococcus* *sp* deposited in Genebank (NCBI) which indicated that this organisms are phylogenetically related to the members of those genus as *Kocuria flava*, *Pontibacter korilensis* and *Staphylococcus saprophyticus*, and those determined in this study permitted to build phylogenetic trees using TreeDyn 198.3 (Fig. 39, 40 & 41).
**Fig. 39.** Neighbour-joining tree based on 16S rRNA sequences, showing relationship between the strain CPA3 (1447 nucleotides) and 11 bacterial species.

**Fig. 40.** Neighbour-joining tree based on 16S rRNA sequences, showing relationship between the strain CAG6 (1448 nucleotides) and 11 bacterial species.
Fig. 41. Neighbour-joining tree based on 16S rRNA sequences, showing relationship between the strain CAM9 (1476 nucleotides) and 11 bacterial species.

9.4. Discussion

The present study was carried out to explore the potential pigment producing bacterial strains of *D. cuneatus*. The endophytic bacterial density of *D. cuneatus* was found to be $3.2 \times 10^6$ CFU/ml to $6 \times 10^4$ CFU/ml of which 23 species are extracellular pigment producing strains of differing colors such as pale yellow, dark orange, dark yellow, reddish pink, light brown, cream and pale orange. Among them, three EPB isolates were selected based on frequency of appearance in the plate and their vibrant color.

The competence of these selected EPB strains was determined by testing against the standard antibiotics such as AMP, AZM, CIP, C and TE by the formation of zone of inhibition. The EPB isolates CPA3 was susceptible to all the five antibiotics, CPA6 was resistant to three antibiotics and CAM9 was resistant against four antibiotics. Similarly, Rashid *et al.* (2014) investigated the isolated pigment producing bacteria against 10
commonly used antibiotics from different groups. Among the strains, most of them were resistant to at least 3 antibiotics and more particularly all the isolates were sensitive to Polymixin B and Nalidixic acid. Many studies have also proved that the pigmented bacterial strains demonstrated strong and broad range of antibiotic activities against other organisms when compared to nonpigmented strains (Holmstrom et al., 2002; Bruhn et al., 2007).

Usually, polar organic solvents such as acetone and methanol have been used for the extraction of carotenoids from bacterial isolates as supported by Medicharla et al. (1991), following the same the carotenoids were extracted from the three EPB strains. The net yield of the pigment of the isolates EPB CPA3, EPB CAG6 and EPB CAM9 were found to be 1.72 mg/lt, 1.22 mg/lt and 1.32 mg/lt respectively. Previously, two strains such as Rhodotorula rubra GED2 and the Lactobacillus casei subsp. casei Ha1 were co-cultured in cheese whey for active synthesis of carotenoids which yielded 2.78 mg/l of total carotenoid (Frengova et al., 2003). Whereas, the amount of carotenoids produced by Sphingomonas astaxanthinifaciens sp. strain TDMA-17T was significantly high when compared with others (2.8 mg/g dry cells) (Asker et al., 2007b). Abbes et al., (2013) reported higher yield of carotenoid from Halobacterium halobium ranging between 5.66 and 7.63 mg/l.

Commonly, all the pigmented strains exhibits multiple absorption peaks between 400 and 550 nm, which are characteristic of carotenoid pigments (Chauhan and Shivaji, 1994; Mueller et al., 2005; Du et al., 2006). In the present study, the three isolates exhibited similar absorption spectrum with the major peaks arising from 400 and 500 nm. Every strain formed two characteristic maximal absorption peaks in differing wavelength. The UV-visible absorption spectra of PPA3 showed maximum absorption at 300 nm and 430 nm, PAG6 showed maximum absorption at 340 nm and 470 nm whereas PAM9 showed maximum absorption at 340 nm and 460 nm. Normally, the polyene chromophores of carotenoids absorb light in the 400 to 550 nm, providing the basis for their characteristic yellow-to-red colors and
their ability to quench singlet oxygen (Umeno et al., 2005). The peak absorption shows that
the pigments contained in the bacterium are carotenoids because carotenoids have maximum
absorption at 300 – 600 nm (Gross, 1991). In addition, Umeno et al. (2002) determined the
carotenoid pigmentation levels in the culture extracts from the height of absorption maxima
(λmax): 470 nm for C30 carotenoids and 475 nm for C40 carotenoids where the pigment PAG6
possessed similar maximum peak absorption around 470 nm which states that it could be the
presence of either C30 or C40 carotenoid. Further, Godinho and Bhosle, (2008) also identified
an orange pigment producing strain possessing maximum uv-visible spectrum at 533 nm,
468 nm and 341 nm with three-band shape of the absorption spectrum.

These, carotenoid pigments normally possess effective antibacterial activity. Similarly,
the D.cuneatus pigment PAG6 was found to exhibit higher antibacterial activity against the
five tested human pathogens S. aureus and K. pneumoniae with maximum activity. The
pigments PAM9 and PPA3 also exhibited pronounced activity against the tested pathogens.
Generally, bacterial pigments act as antagonist by exhibiting antibiotic activity against other
microbial organisms. They are considered as a potent weapon for survival and effective
chemical defenses against eukaryotic predators. This class of bioactive agents includes almost
all pigmented compounds commonly produced by Pseudoalteromonas, Pseudomonas and
Streptomyces species.

Recently, Jafarzade et al. (2013) investigated the antimicrobial activity of 55 pigment
producing bacteria isolated from the marine environment. Of which, 18 isolates exhibited
antimicrobial activity against tested pathogens, 52% against E. coli, 68% against B. subtilis
and 37% against C. albicans. The 18 isolates possessing antimicrobial activity was isolated
from different sources such as: 44% from six species of sponges, 22% from seawater, 22%
from mangrove sediment, 6% from sea cucumber and 6% from mussel. Thus the finding
corroborates with the results of present study. Sometimes, the zone of inhibition formed by
the each pigment differs in size and nature which may be due to differences in composition of
the different pigments (Rashid et al., 2014). These compounds may also inhibit the settlement
of marine invertebrate larvae, gemination of algal spores and may also protect the host
surface by interfering the bacterial colonization and biofilm formation (Holmstrom et al.,
1992; Egan et al, 2001; Holmstrom 2002). They may also inhibit other organisms that
compete for space and nutrients. Recently, Ahmad (2012) presented broad spectrum of
antibacterial activity of the crude violet pigment isolated from Chromobacterium violaceum
exhibited efficient activity against Gram-positive (S. aureus and B. cereus) and the Gram-
negative bacteria (P. aeruginosa and E. coli).

Similarly, the violet pigment containing violacein and deoxyviolacein, isolated from
phototrophic bacterium RT102 strain illustrated competent antibacterial effect against
Balraj et al., (2014) reported that the pigmented marine bacteria isolated from water samples
of south east and south west of India exhibited pronounced antimicrobial activity. The
pigment was found to be more effective against more than 10 pathogens where maximum
zone of inhibition was observed against Shigella sp., Klebsiella sp. and S. aureus. The results
indicated that the purified pigment contains antimicrobial substances and it possesses the
ability to inhibit the growth of human pathogens which validates with the current finding.

Generally, marine bacteria are likely to produce carotenoids to protect themselves
from activated oxygen produced by sunlight. Therefore, their potent antioxidant activities
were expected and reasonable (Shindo and Misawa, 2014). Here, the three pigments PPA3,
PAG6 and PAM9 shows significant antioxidant activity. Among the three pigment extracts,
PAG6 exhibited higher DPPH (41.38%, 100 μg/ml) where the other two pigments exhibited
lesser activity. Of the various antioxidant defences in organisms, protection against singlet
oxygen is mainly afforded by carotenoids (Terao, 1989; Miki, 1991). As supporting the
current finding, the carotenoids extracted from *Phaffia rhodozyma* (yeast) found to exhibit significantly higher ability to scavenge the DPPH radical activity (Gramza-Michałowska and Stachowiak, 2010). Rodriguez-Amaya (2001) isolated a pigment bacterioruberin from *Halobacteria sp.* known to contain 13 pairs of conjugated double carbon bonds which endow biological tissues with effective hydroxyl free-radical scavenging power and singlet oxygen quenching activity. The pigment functions to protect *Halobacteria sp.* from fatal injuries under intensive light (Saito et al., 1997; Mandelli et al., 2012) and confers bacteria with resistance to oxidative DNA damage from radiography, UV-irradiation and H$_2$O$_2$ exposure.

Likewise, Yatsunami *et al.* (2014) extracted bacterioruberin from *Haloarcula japonica* that are found to exhibit noteworthy scavenging capacity which was higher than that of β-carotene. The carotenoids from *Deinococcus radiodurans* scavenged 40.2% DPPH radicals compared to β-carotene (31.7%) at a concentration of 0.5 mg/ml (Tian *et al.*, 2009). Recently, Arulselvi *et al.* (2014) also isolated 24 pigment producing bacteria and investigated for their antioxidant property, in which the strain YCD3b produced carotenoid pigment displaying higher quality of free radical scavenging activity up to 78 %. Thus, proving, carotenoids could significantly enhance the stability of photosynthetic complexes against oxidation and their protective (antioxidant) effect depending on the type of the oxidant (Fiedor *et al.*, 2012).

In case of HPTLC analysis, the presence of carotenoids in the three pigments has been confirmed. All the three pigments showed distinct visible bands for the presence of carotenoid ketones but for the presence of carotenoid hydroxylates only the pigments PPA3 and PAG6 showed bands. Thus, the thin layer chromatographic analysis of the pigments (PPA3, PAG6 and PAM9) revealed the presence of ketone and hydroxylated carotenoids in the pigment which was similarly proved for the pigments isolated from the orange pigmented strain *M. arborescens* (Godinho and Bhosle, 2008). The type of carotenoids was determined by comparing with three available standards. The standards used were Astaxanthin, β-carotene.
and Lycopene. PPA3 and PAM9 pigments did not match with the Rf value of three standards used, whereas PAG6 formed band with the same Rf value 0.30 matching with the standard astaxanthin, proving the presence of astaxanthin carotenoid in the pigment extract of EPB CAG6. Similarly, TLC separation of carotenoid extract from *Penaeus semisulcatus* yielded several distinct bands at which Rf = 0.33 corresponds to astaxanthin as in *D. cuneatus* (Lorenz, 1998). Misawa *et al.* (1995) identified a carotenoid biosynthesising gene cluster responsible for the production of astaxanthin from the marine bacterium *Agrobacterium aurantiacum*. Recently, another astaxanthin-producing marine bacterium was isolated and identified as *Paracoccus haeundaensis* (Lee *et al.*, 2004). The pigment PAG6 also formed two unreliable bands.

The astaxanthin PAG6 which exhibited a band with the Rf value matching to the standard astaxanthin was scraped and analysed in HPLC. The elution was carried using Acetonitrile: Methanol. Both the sample and standard produced peak at similar retention time (3.9 mins). Similarly, Razavi *et al.* (2006) developed a method to identify carotenoids including astaxanthin, canthaxanthin, apocarotenoic ester, torularhodin and β-carotene using UV-HPLC/APCI-MS. In which, the retention time of astaxanthin (3-4th min) was similar to the retention time of partially purified astaxanthin PAG6 (3.9 min) in *D. cuneatus*. Asker *et al.* (2007b) identified astaxanthin and astaxanthin isomers from *Sphingomonas astaxanthinifaciens* sp. using HPLC. As such, astaxanthin was sperated from *P. rhodozyma* extract by HPLC at the retention time of 4th min (Gramza-Michałowska and Stachowiak, 2010). More recently, Sasiidharan *et al.* (2013) reported that the HPLC analysis of pigment of the strains RS7, RSS3, RS13 and RS14 exhibited peaks at various retention time (3.3 min) for astaxanthin.

One of the promising biological activities of marine bacterial isolates was their cytotoxic effect against cancer cells. The cytotoxic effect of partially purified astaxanthin of
PAG6 extract against MCF-7 human cancer cell line was studied. The sample exhibited dose dependent activity ranging from 100 – 6.25 μg/ml inducing cell death or apoptosis. Thus, IC₅₀ value of partially purified sample was found to be 9.37 μg/ml. Earlier, Tanaka et al. (1995) also recorded that axtanthin and canthaxanthin are strong chemo preventers of oral carcinogenesis, which may be partly due to suppression of cell proliferation on oral cancer induced male F344 rats using 4-nitroquinoline 1-oxide (4-NQO). The present findings was also agreement with recent data experimented in different approach. The investigation revealed that the astaxanthin treated cancerous tissues in comparison with cancerous ones (untreated), expressively decreased the accumulation of elements (P, S, Ca, Fe and Zn) on the tumor site. Thus, causing the breast cancer cell membrane to lose their desire to collect the elements from healthy tissues; therefore, the concentration of the elements after injecting axtanthin had decreased in comparison with the cancerous group thus leading to the conclusion (Safaverdi et al., 2009). Astaxanthin is known compound to play many beneficial roles against the development of cancer in each stage. Thus, increased intake of axtanthin may typically lower the risk of cancer (Tanaka et al., 2012).

Earlier, it has been reported that the significant properties of axtanthin helps to reduce the number of precancerous lesions in the colon and smaller tumours when they do develop in the colon and breast (Prabhu et al., 2009; Nakao et al., 2010). Kavitha et al. (2010) isolated prodigiosin from Serratia marcescens which was found to possess potent apoptosis activity against human cervix carcinoma cells (HeLa cells) by observing the decrease in proliferation of treated cells when compared to the untreated controls. Prodigiosin induced apoptosis in HeLa cell lines in a dose dependent manner with a mean IC₅₀ of 700 nM from MTT assay. In the same way, the lycopene carotenoid induced cytotoxic effect in human breast cell line (MCF-7) only at the highest concentration exhibiting minimal membrane damage on MCF-7 cells (68 % cell viability) after 24 h of exposure to the culture control (89
% cell viability). The MIC value of lycopene was of 3.4 μM after 24 h. The MIC values did not change after a prolonged period of lycopene incubation (Fornelli et al., 2007). It was strongly evident through a number of studies that carotenoids possess potent cancer chemo preventive properties (Gerster, 1995; Palozza et al., 2004; Palozza et al., 2005) independent of their antioxidant activity (Peto et al., 1981; Khachik et al., 1995; Britton 1995). Further, Soliev (2012) investigated the cytotoxicity effect of prodigiosins on human U937, K562 and HL60 leukemia cell lines. The red pigment possessed significant activity by decreasing the percentage of the viable cells. The IC50 value of prodigiosins against U937 cells was about 0.7 μM while it was around 1.5 μM in HL60 and around 2.5 μM in K562 cells.

The three EPB strains were first characterized by both morphological and biochemical characters as well as 16S rRNA sequencing. Consequently, they were found to be Kocuria flava, Pontibacter korlensis and Staphylococcus saprophyticus designated as Kocuria flava PA3, Pontibacter korlensis AG6 and Staphylococcus saprophyticus AM9 respectively. The accession number for each strain was assigned by the GeneBank (NCBI, USA) upon submission. The strains were compared with previously obtained sequences of respective species deposited in Genebank which indicated that the organisms are phylogenetically related to the member of their genus by forming a neighbour-joining tree, based on 16S rRNA sequences. The EPB strain Kocuria flava nucleotide sequences exhibited 100% identity to the existing strain deposited in NCBI. The EPB strain Pontibacter korlensis formed a close cluster exhibiting 90% identity related to the same species. The EPB strain Staphylococcus saprophyticus also formed a close cluster exhibiting 90% identity related to the same species. Among the three strains Kocuria flava and Pontibacter korlensis are considered as novel species were much work on carotenoid has not been done yet. So far, only 20 similar submissions have been made for Kocuria flava and for Pontibacter korlensis its upto 8 submissions in NCBI Genebank database. This was the first report for astaxanthin
identification from *Pontibacter korensis*. Also, among the three associated bacteria’s isolated each belongs to phylum Actinobacteria, Bacteroidetes and Firmicutes. Similarly, Nithyanand and Pandian (2009) cultivated bacteria from the coral *Acropora digitifera* belonging to the group Firmicutes, Gammaproteobacteria and Actinobacteria based on the full-length sequences of 16S rRNA gene sequences.

Commonly, most of the animals may have limited access to carotenoids in their diet and they only harbour intracellular bacteria that produce carotenoids where Sloan and Moran, (2014) hypothesized that these endosymbionts could serve as an alternative source of carotenoid biosynthesis. These carotenoids could act as effective antioxidants and scavenge singlet oxygen. Many researchers have showed that people today relay mostly on natural additives rather than synthetic ones due to their potential toxicity and carcinogenicity (Barlow 1990; Prior and Cao, 2000). Approximately, 95% astaxanthin in the commercial use contain synthetic pigment (Lorenz and Cysewski, 2000) which are less stable and expensive. Thus, it has become much more important for the need of microorganisms for the production of natural astaxanthin, which possess nutraceutical apart from acting as a medicinal ingredient against degenerative diseases such as cancer (Chew et al., 1999), skin-related illness and heart disease (Guerin et al., 2003) and thus emphasising the importance of current study. Recently, the Food and Drug Administration of the United States has permitted the use of astaxanthin in the aqua-cultural industry has awaken a greater interest towards the field (Golkhoo et al., 2007). At the outset, the isolated astaxanthin has been proven their potency in terms of antioxidant, antibiotic and cytotoxic activities. Therefore, the isolated astaxanthin could be used as an alternate for existing synthetic antioxidants.
10. BIOACTIVE PROPERTIES OF THE SELECTED ENDOPHYTIC PIGMENTED BACTERIA

10.1. INTRODUCTION

The sources of MNPs are of taxonomically diversified organisms include sponges, tunicates, corals, molluses, fungi and sediment-derived bacteria. However, there is growing recognition that the ‘‘collected source’’ for these molecules is not necessarily the ‘‘metabolic source’’. Precisely, marine bacteria and cyanobacteria, either assimilated by an invertebrate grazer (e.g., sea hare grazing on cyanobacteria) or growing in association with an invertebrate host in a symbiotic or commensal relationship, may be the true origin of these molecules (Hildebrand et al., 2004; Piel, 2006; Simmons and Gerwick, 2008). Many endophytic microbes have the potential to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases (Strobel et al., 2004; Staniek et al., 2008; Aly et al., 2010; Kharwar et al., 2011; Kusari and Spiteller, 2012b). Many prominent researchers have reviewed the literature on marine natural products and unequivocally hailed the bioactive potential of marine microbes. There is an increase in the number of metabolites rapidly every year on an average of approximately 700 novel marine natural products and of which 16 – 18% was of microbiological origin (Blunt et al., 2010). Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstances and diversity of species (Carte, 1996; Rinehart, 2000; Schwartsmann et al., 2001).

Metabolic associations between microorganisms and their host can make it difficult to reveal which partner organism is responsible for the production of a particular metabolite. As a result, many bioactive products, previously ascribed to the eukaryotes, have later been found to be produced by associated microorganisms (Kobayashi and Ishibashi, 1993; Unson and Faulkner, 1993; Unson et al., 1994; Oclarit et al., 1994; Bewley et al., 1996; Stierle et al.,
1988; Schmidt et al., 2000; Schmidt, 2005; Konig et al., 2006). For example, Dolastatin 10, originally isolated from the marine gastropod _D. auricularia_ was later shown to originate from cyanobacteria of the genera *Symploca* and *Lyngbya* upon which they feed on (Pettit et al., 1987; Pettit et al., 1989; Leusch et al., 2001; Williamson et al., 2000). The powerful PKC-activating cancer cell toxin Bryostatin was isolated preliminarily from the Californian bryozoan _Bugula neritina_, but the biosynthetic capacity to make this unique macrolide has been localized to an associated bacterium *Endobugula sertula* (Sudek et al., 2007). Normally, the secondary metabolites isolated from microbes exhibit either antimicrobial (antibacterial, antifungal, antiprotozoal), antitumor and/or antiviral activities used to be called as antibiotics. The antibiotic production is subjected to complex regulatory networks and is generally induced in stationary phase under conditions of nutrient limitation (Martin and Liras, 1989). Antibacterial compounds are thought to confer a selective advantage, when in competition with other bacteria populating for their survival in the same ecological niche. This emphasizes the importance of microorganisms as an ideal source of bioactive compounds. Also, due to the low rate of active compounds in marine animals and plants, as well as limitation of bioresource supply, more and more researchers have been focusing on marine microorganisms as sustainable resources (Bultel-Ponce et al., 1999; Holler et al., 2000; Kelecom, 2002; Isnansetyo and Kamei, 2003).

The mounting appreciation of marine microorganisms associated with invertebrate hosts involved in the biosynthesis of secondary metabolites offers new alternatives to the discovery and development of marine natural products (Berrue, 2011). Thus an alternative strategy targeting the microorganisms associated with bivalve for the screening of bioactive natural products may prove to be an effective approach to circumvent the associated difficulties of dealing with the organism itself. Therefore, the present study focuses the bioactive potential of associated marine bacteria of bivalves.
10.2. MATERIALS AND METHODS

10.2.1. Extracellular production of secondary metabolites of EPB strains

Three EPB strains (CPA3, CAG6 and CAM9) isolated from the marine bivalve was selected for the production of bioactive compound. The seed culture of potential bacterial strain was prepared by inoculating in 50 ml of Zobell Marine Broth (PH 7.5) in a shaker (30 °C/ 150 rpm) for 18 h. Later, inoculation was transferred to 500 ml Zobell Marine Broth in Erlenmeyer flask, cultivating and shaking (250 rpm) for 3-5 days depending on the growth rate at 30 °C.

10.2.2. Extraction

The well grown cultures were centrifuged at 7000 rpm for 20 min/ 4 °C. The cell pellets were discarded. Each of the culture supernatants were extracted with ethyl acetate using separating funnel by vigorous shaking. Then the organic layer was separated and concentrated using rotary evaporator. The obtained extracts were lyophilized and stored at -20 °C until further use.

10.2.3. Culture filtrate activity

10.2.3.1. Microorganisms and inoculums preparation

The samples from EPB isolates were tested against the five bacterial pathogens *S. aureus, E. coli, B. cereus, P. aeruginosa* and *S. abony*. All microorganisms were obtained from the Department of Microbiology, Annamalai University, India.

The bacterial pathogens were subcultured in nutrient agar. The strains were inoculated and grown to exponential phase in nutrient broth at 37 °C for 18 h and adjusted to a final density of $10^5$ CFU/ml by diluting fresh cultures by comparing with McFarland density.
10.2.3.2. Antibacterial activity

Antibacterial activity of crude culture filtrates was determined by agar disc diffusion method (Jorgensen and Turnidge, 2007). The Muller Hinton agar plates were evenly streaked in three different directions with a sterile cotton swab dipped in bacterial suspension (10^5 CFU/ml). Sterile filter paper discs (5 mm diameter) were immersed in to 50 μl of crude extract (0.5 mg/ml) and allowed to dry at room temperature and placed over the bacterial plates. Erythromycin 10 mcg/disc was used as positive control. The plates were incubated overnight at 37 °C and the zone of inhibition around the disc was measured.

10.2.4. Purification of crude compound in column chromatography

Three gm of concentrated active crude ethyl acetate extract was poured in activated silica gel (230-400 mesh, merck) packed on to a glass column (450 mm x 40 mm) with the maximum height of 30 cm. Care was taken not to breathe the silica while preparing column. Then, the fractions were eluted with 100 % Hexane, 75% hexane: 25% ethyl acetate, 50% Hexane: 50% ethyl acetate, 25% hexane: 75% ethyl acetate, 100% ethyl acetate, 75% ethyl acetate: 25% methanol, 50% ethyl acetate: 50% methanol, 25% ethyl acetate: 75% methanol and 100% methanol. The final volume of 25 ml was collected and dried. In total, the 9 fractions (Fr1- Fr9) were again screened for antibacterial activity.

Based on the results obtained, the active bioassay fractions were further investigated for the presence of bioactive constituents using FT-IR spectroscopy, HPTLC and GC-MS analysis.
10.2.5. Characterization of active partially purified fraction

10.2.5.1. High Performance Thin layer chromatography

The further fractioning of active metabolites present in the active fraction obtained from column chromatography was done using CAMAG HPTLC system (Switzerland). 50 μl of concentrated active fraction was loaded on the activated silica gel TLC sheet 20 cm x 20 cm (Merck, Germany) as a band using the sample applicator (Linomat 5) fixed with 100 μl Hamilton syringe with help of N₂ gas. The plates was further developed using various developing solvents like hexane: chloroform (80:20), chloroform: ethyl acetate (90:10), methanol: ethyl acetate (30:70) to determine the appropriate solvent system using CAMAG twin-trough chamber lined with filter paper and pre-saturated with 15 ml of mobile phase. The developed plates were dried and bands were visualized in UV-chamber at 366 nm and 254 nm. The separated fractions were located by exposing the plate to iodine fumes in a developing chamber and the Rf value of each fractions was calculated.

10.2.5.2. FTIR spectral analysis

Sample preparation was carried out as described by Naumann et al. (1991). Briefly, the purified pooled active fractions was taken in a smooth agitator mortar and mixed thoroughly with 2.5 mg of dry Potassium Bromide (KBr) using a pestle. The powder was filled in the microcup of 2 mm interval dia to obtain the diffuse reflectance infrared spectrum to replicate samples. All IR spectra recorded at 24 ± 1°C in along with infrared range (500 – 4000 cm⁻¹) using FT-IR spectrometer (Shimadzu, Japan). Typically, 20 scans signals were averaged for a single spectrum. Each spectrum is displayed in terms of absorbance as calculated from the reflectance-absorption spectrum using the Hyper-IR software. To minimize the difficulties arising from unavoidable shifts, baseline correction was applied.
10.2.5.3. GC-MS analysis

The active fraction was examined for the chemical composition by using GC-MS instrument (GC Clarus 500 Perkin Elmer) to find out the active principle of the fraction. Chromatography was performed on Elite 5 capillary column (5 % Phenyl/ 95 % Dimethyl polysiloxane), (30 m x 250 μm) with mass detector of the company which was operated in EI mode. Helium was used as carrier gas at flow rate of 1 ml/min, split 10:1 and 2 μl of sample was injected. The injector and detector temperatures were 280 °C and 200 °C respectively. The column oven was programmed as follows: initial temperature 110 °C, initial time 2.0 min, program rate 10 °C/min; final temperature 280 °C; final time 9 min. The sample was dissolved in CH₂Cl₂ and a split injection technique was used. The identification of compounds was based on comparison of their relation indexes (RI), obtained using n-alkanes (C₁₁- C₁₃) and retention time. They were also confirmed by comparison of their mass spectra with NIST Version-Year 2008 and literature data.

10.3. RESULTS

10.3.1. Extracellular production of secondary metabolites of EPB isolates

A mass production of pigmented bacterial strain about 500 ml of culture was produced by shake flask method using ZMB in a shaker (30 °C/ 250 rpm) for 3-5 days (Fig.42.). Cells were separated by centrifugation at 7000 rpm at 4 °C for 20 min.
10.3.2. Extraction

The separated supernatant was subjected to separate the secondary metabolites of pigmented strains using ethyl acetate. After vigorous shaking in separating funnel, the organic layer formed was separated, concentrated, lyophilized and stored at -20 °C until further use. The lyophilized samples were named as SPA3, SAG6 and SAM9 (Supematant extract PA3, AG6 and AM9) for CPA3, CAG6 and CAM9 isolates respectively.

10.3.3. Antibacterial activity

The three collected samples (SPA3, SAG6 and SAM9) were tested for antibacterial activity. The three EPB ethyl acetate extracts exhibited significant antibacterial activity, of which SPA3 showed pronounced effect than other extracts against the five tested pathogens (Fig.43.).
Fig. 43. Antibacterial activity of crude ethyl acetate extracts SPA3, SAG6 and SAM9.

10.3.4. Purification using column chromatography

The active ethyl acetate extract SPA3 was fractioned using silica gel column with various concentrations of hexane, ethyl acetate and methanol. The obtained 9 fractions were evaluated for antibacterial activity using disc diffusion assay. Among the nine fractions tested Fr3, Fr6 and Fr7 exhibited prominent activity (Fig.44.). The three fractions exhibiting activity was pooled, lyophilized and used for further studies.
Fig. 44. Antibacterial activity of the three partially purified active fractions Fr3, Fr6 and Fr7.

10.3.5. HPTLC

The active fractions loaded plates were developed using hexane: chloroform (8:2); chloroform: ethyl acetate (9:1) and methanol: ethyl acetate (30:70). The spots were located by exposing the plate to iodine fumes. Among the four solvents investigated methanol: ethyl acetate (30:70) exhibited clear and high number of separation bands. The active pooled fractions separated using silica plate exhibited four unique bands represented as 1, 2, 3 and 4 (Fig.45 and 46) which may denote the type of compounds present in it.
**Fig. 45.** HPTLC analysis of the pooled active fractions - A. White light, B. UV-chamber at 366 nm and C. UV-chamber at 254 nm.

**Fig. 46.** Chromatogram pattern of HPTLC analysis of the pooled active fractions.
10.3.6. FTIR analysis

FTIR is generally carried out to identify the tentative functional groups present in the test fractions. The pooled fractions from column chromatography exhibited totally 16 peaks responsible for different types of groups (Fig. 47 and Table.12.). Of which, 4 out of range peaks were also observed. The represented IR spectra corresponds to the presence of NH stretch heterocyclic amine around 3442 cm\(^{-1}\), methyl and methylene C-H stretch at 2964 cm\(^{-1}\) and 2933 cm\(^{-1}\), organic sulphate, phosphate and nitrates at 1639 cm\(^{-1}\), 1408 cm\(^{-1}\) and 1350 cm\(^{-1}\) correspondingly.

![FTIR spectrum](image)

Fig. 47. FTIR spectrum of the active pooled extract of SPA3.
Table. 12. IR bands recorded for the active pooled extract of SPA3.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Wave number</th>
<th>IR bands indicating the functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3442.94</td>
<td>Heterocyclic amine, NH stretch</td>
</tr>
<tr>
<td>2.</td>
<td>2964.54</td>
<td>Methyl C-H asym./sym. stretch</td>
</tr>
<tr>
<td>3.</td>
<td>2933.73</td>
<td>Methylene C-H asym./sym. stretch</td>
</tr>
<tr>
<td>4.</td>
<td>2096.62</td>
<td>Isothiocyanate (-SCN)</td>
</tr>
<tr>
<td>5.</td>
<td>1851.66</td>
<td>Five membered ring anhydride</td>
</tr>
<tr>
<td>6.</td>
<td>1639.49</td>
<td>Organic nitrates asym./sym. x O₂ stretch (NO₂ &amp; SO₂)</td>
</tr>
<tr>
<td>7.</td>
<td>1408.04</td>
<td>Organic sulphates</td>
</tr>
<tr>
<td>8.</td>
<td>1350.17</td>
<td>Organic phosphates (P=O stretch)</td>
</tr>
<tr>
<td>9.</td>
<td>1240</td>
<td>Aromatic phosphates (P-O-C stretch)</td>
</tr>
<tr>
<td>10.</td>
<td>1111</td>
<td>Organic sulphates asym./sym. x O₂ stretch (NO₂ &amp; SO₂)</td>
</tr>
<tr>
<td>11.</td>
<td>921.97</td>
<td>Aromatic phosphates (P-O-C stretch)</td>
</tr>
<tr>
<td>12.</td>
<td>538.14</td>
<td>Aliphatic Iodo compounds C-I stretch</td>
</tr>
</tbody>
</table>

10.3.7. GC-MS

GC-MS chromatogram of the active pooled fractions represented 11 prominent peaks indicating the presence of 11 compounds was given in Table. 13 and the spectrum are represented in Fig.48. The mass spectra of these compounds were compared with those of the complied data for the known compounds. The peaks representing compounds were identified as Chloroacetic acid, tetradecyl ester, Pyrrole[1,2-A]pyrazine-1,4-Dione, Hexahydro, 1R,2C,3T,4T-tetramethyl-cyclohexane, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, Dibutyl phthalate, 1,2-benzedicarboxylic acid, butyl 2-methylpropyl ester,
1-heptacosanol, N-heptadecanol-1, Hexacosanol, acetate and Phenol, 3,5-bis (1,1-dimethylethyl).

![GCMS chromatogram of the active extract (SPA3).](image)

**Fig. 48.** GCMS chromatogram of the active extract (SPA3).
Table. 13. Identification of compounds present in the active extract (SPA3).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Retention time</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Similarity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>15.93</td>
<td>Chloroacetic acid, tetradecyl ester</td>
<td>C_{16}H_{31}O_{2}Cl</td>
<td>290</td>
<td>849</td>
</tr>
<tr>
<td>2.</td>
<td>16.24</td>
<td>Pyrrole[1,2-A]pyrazine-1,4-Dione, Hexahydro</td>
<td>C_{7}H_{10}O_{2}N_{2}</td>
<td>154</td>
<td>815</td>
</tr>
<tr>
<td>3.</td>
<td>16.54</td>
<td>1R,2C,3T,4T-tetramethyl-cyclohexane</td>
<td>C_{10}H_{20}</td>
<td>140</td>
<td>842</td>
</tr>
<tr>
<td>4.</td>
<td>17.19</td>
<td>1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester</td>
<td>C_{16}H_{22}O_{4}</td>
<td>278</td>
<td>916</td>
</tr>
<tr>
<td>5.</td>
<td>17.66</td>
<td>Dibutyl phthalate</td>
<td>C_{16}H_{22}O_{4}</td>
<td>278</td>
<td>892</td>
</tr>
<tr>
<td>6.</td>
<td>17.82</td>
<td>1,2-benzedicarboxylic acid, butyl 2-methylpropyl ester</td>
<td>C_{16}H_{22}O_{4}</td>
<td>278</td>
<td>885</td>
</tr>
<tr>
<td>7.</td>
<td>19.77</td>
<td>1-heptacosanol</td>
<td>C_{27}H_{56}O</td>
<td>396</td>
<td>848</td>
</tr>
<tr>
<td>8.</td>
<td>21.45</td>
<td>N-heptadecanol-1</td>
<td>C_{17}H_{38}O</td>
<td>256</td>
<td>782</td>
</tr>
<tr>
<td>9.</td>
<td>23.02</td>
<td>Hexacosanol, acetate</td>
<td>C_{28}H_{56}O_{2}</td>
<td>424</td>
<td>862</td>
</tr>
<tr>
<td>10.</td>
<td>31.43</td>
<td>Phenol, 3,5-bis(1,1-dimethylethyl)-</td>
<td>C_{14}H_{22}O</td>
<td>206</td>
<td>544</td>
</tr>
</tbody>
</table>

10.4. Discussion

The widespread of multiresistant human pathogens have urged the scientific community towards the search of novel antibiotics. Many clinical microbes have developed resistance due to exposure to sub-lethal concentration of antibiotics both in hospitals and also in animal farms where antibiotics are used as growth enhancers (Witte, 1999). Generally, microorganisms acquire resistance towards common antibiotics in two ways: by altering their metabolism and genetic structure (Raghunath, 2008; Maragakis et al., 2008). This setback could be overcome by studying the diversity of molluscan associated bacteria and their biological importance to search for the potential bioactive metabolites. However, marine bacteria have been recognized as an important and untapped resource for novel bioactive compounds. The chemical compounds of marine microorganisms are less well known than those of their terrestrial counterparts. However, in the last few years more number of
bioactive compounds had been isolated from the marine bacteria and are new resources for the development of medically useful compounds (Donia and Hamann, 2003; Anand et al., 2006).

Endophytic marine bacteria are potent strains capable of producing bioactive natural compounds that are useful in human health concern (Strobel et al., 2004). Endophytes are believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites. These bacteria provide a broad variety of bioactive secondary metabolites with unique structures including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones and others (Tan and Zou, 2001). These drugs are claimed as new therapeutic agents for effective treatment of diseases in humans, plants and animals.

In this regard, the present study was carried out to investigate the antimicrobial potential of three isolated pigmented bacteria from D. cuneatus. The three selected pigmented EPB strains CPA3, CAG6 and CAM9 were cultured using flask method with ZMB in a shaker (30 °C/ 250 rpm) for 3-5 days. The supernatant was separated by centrifugation at 7000 rpm at 4 °C for 20 min. The separated supernatant was extracted using ethyl acetate by vigorous shaking in extracting funnel. The organic layer separated was collected, concentrated, lyophilized and stored at -20 °C until use. The three samples such as SAP3, SAG6 and SAM9 were used for further assays.

The three EPB extracts (SAP3, SAG6 and SAM9) exhibited significant antibacterial activity against the five tested pathogens. Among the three samples, SAP3 showed pronounced activity than other extracts with maximum activity against S. abony (18 mm) and minimum activity against P. aeruginosa (2 mm), SAG6 exhibited maximum activity against S. abony (17 mm) and no activity against S. aureus and SAM9 extract also exhibited maximum activity against S. abony (14 mm) and minimum activity against S. aureus, B. cereus and P. aeruginosa (3 mm). Similarly, Romanenko et al. (2008) isolated heterotrophic
bacteria associated with the marine ark shell (*A. broughtoni*). Of the 149 strains screened, eight strains were found to possess inhibitory effect against gram-positive *S. aureus*, *E. faecium*, *B. subtilis* and gram- negative phytopathogenic *Xanthomonas sp. pv. badrii* bacteria and less against *C. albicans*, *A. niger* and *F. oxysporum* fungi. Among these, two yellow-pigmented Gram-negative bacteria *P. fulva* and *S. asaccharolytica* were also found to exhibit potential antimicrobial activities. Zheng et al. (2005) explored the bacteria associated with marine macro organism from different marine environment. Of the bacteria isolated, the ethyl acetate extracts of 42 strains possessed pronounced antibacterial activity, the proportion of active bacteria among the community was marine invertebrates (20%), seaweeds (11%), seawater (7%) and sediment (5%). On the whole, the percentage of energetic bacteria isolated from invertebrates was higher than other sources including sea weeds. These specific antibacterial compounds could confer a selective advantage *i.e.*, when in competition with other bacteria populating for their survival in the same ecological niche (Gopi et al., 2012). Likewise, Prem Anand et al. (2006) examined the bacterial symbionts associated with the marine invertebrates such as sponges. Totally, 75 bacterial strains were isolated from four marine sponge species. Of which, 21 % of the strains were found to be antibiotic producers and their activities ranged from board spectral to species specific. It has also been suggested that some of these bacteria chemically defend the host against microbial infection (Engel et al., 2002). The study had ascertained that they belong to the genera *Vibrio*, *Pseudomonas/Marinobacter* and *Bacillus* dominantly. Further, Prem Anand et al. (2011) also isolated a total of 633 marine bacterial strains from various sources of marine environment. Among these, 5 strains only possessed antibacterial activity and were isolated from sea cucumber, sea urchin and jelly fish of which 4 strains exhibited broad spectral activity. Similarly, 6 strains were isolated from gut microflora of gastropods, of which 2 exhibited potent activities. From crab and molluscan eggs, 11 active strains were identified, of which 4 strains exhibited broad spectral activity. In genus level identification the potential strains were found to be *Alteromonas sp.*, *strptomycies sp.*, *Bacillus sp.*, *Flavobacterium sp.* and *Pseudomonas sp.*
The active crude SPA3 extract was further involved in partial purification process using silica column chromatography eluted using hexane, ethyl acetate and methanol in different concentrations. A total of 9 fractions containing 25 ml was lyophilized and screened for antibacterial activity. Among these, the fraction Fr3, Fr6 and Fr7 exhibited significant activity and was selected and pooled for further analysis.

The pooled active fraction was further subjected to HPTLC analysis. The fractions were separated using different solvent systems. Among the solvents investigated, the solvent methanol: ethyl acetate (70:30) was found to separate more efficiently with more number of bands. Totally, four separation bands were observed as 1 - 4 at the Rf value of 0.08, 0.17, 0.62 and 0.82 respectively. As such, recently, Sharon et al. (2013) detected the presence of ester, quinone, macrolide and terpenoids from ethyl acetate of *Streptomyces* sp. (JF751041) using HPTLC with Rf value ranging between 0.01 to 0.95. In a similar way, *Streptomyces* isolates obtained from marine sponges produced antimicrobial compounds that showed Rf values ranging from 0.40 to 0.78 in TLC analysis (Selvakumar et al., 2010).

FT - IR spectroscopy provides valuable information regarding the functional group of compounds present in the extract. Since the pooled fraction analysed is partially purified many chemical bonds are present, as illustrated in spectra. The represented IR spectra corresponds to the presence of NH stretch heterocyclic amine around 3442 cm⁻¹, methyl and methylene C-H stretch at 2964 cm⁻¹ and 2933 cm⁻¹, organic sulphate, phosphate and nitrates at 1639 cm⁻¹, 1408 cm⁻¹ and 1350 cm⁻¹ correspondingly. Aromatic phosphates, isothiocynate and aliphatic iodo compounds were also detected.

The application of gas chromatography coupled with mass spectrum resulted in successful elucidation of the compounds. The GCMS spectrum of the active pooled fraction exhibited 11 major compounds such as Chloroacetic acid, tetradecyl ester, Pyrrole[1,2-A]pyrazine-1,4-Dione, Hexahydro, 1R,2C,3T,4T-tetramethyl-cyclohexane, 1,2-benzene dicarboxylic acid, bis(2-methylpropyl) ester, Dibutyl phthalate, 1,2-benzedicarboxylic acid, butyl 2-methylpropyl ester, 1-
heptacosanol, N-heptadecanol-1, Hexacosanol, acetate and Phenol, 3,5-bis(1,1-dimethylethyl). In addition, Dash et al. (2009) screened novel sponge associated marine bacteria for their antibacterial and antilarval settlement activity. Among these, three bacterial extracts possessing significant activity were subjected to GCMS analysis. The extracts represented various number of compounds in differing retention time by comparing with NIST library. Of which the marine bacteria, A3 and A5 possessed a common compound namely Pyrrolo (1,2-a) pyrazine 1,4-dione, hexahydro 3 (2-methyl propyl) where a similar pyrrole derivative was identified in the extracts of active pooled fraction of SAP3 as Pyrrole[1,2-A]pyrazine-1,4-Dione, Hexahydro. This Pyrrolo-(1,2-a)pyrazine-1,4- dione, hexahydro-3(2-methylpropyl) was also detected in the ethyl acetate cell extract of *V. parahaemolyticus* strain (An3) isolated from the infected goan mullet was found to be one of the major metabolite responsible for antimicrobial activity. Other compounds were also identified such as indole, phenyl acetic acid, N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidinocarboximidamide, phenol, 4-(1,1,3,3-tetramethyl butyl), nonyl-phenol and tetramethyl pyrazine aid in the antimicrobial property (Pandey et al., 2010). Similarly, 1,2-benzenedicarboxylic acid derivative detected in the active pooled fraction was also present in the three molluscan extracts of *P. arabica, A. granosa* and *P. glaucum* which could be produced by the associated microorganisms and then could be assimilated in the host for protection. This particular compound is reported to possess effective antimicrobial and antifouling activity (Christy et al., 2013; Ramasamy and Balasubramanian, 2012; Thilaga et al., 2014). This active pooled fraction produced strain was found to be *K. flava* using molecular techniques in the previous chapter. The GCMS result of the present study similar with the results of Sahadevan et al. (2014) reported for *Kocuria sp. SRS88* (KF975712) isolated from the soil samples. The similar compounds present in the *Kocuria sp. SRS88* were Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, Phenol, 2, 4-bis (1,1-dimethyl ethyl) and Diethyl Phthalate with minor difference in groups present. Recently, Palomo et al. (2013) identified a new anti-MRSA
bioactive compound- (Kocurin) a new member of thiazolyl peptide from sponge associated microorganisms Kocuria sp. F-276,345, a prolific resource of novel drugs.

The discovery of novel antimicrobial metabolites from endophytic bacteria’s is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens (Yu et al., 2010; Song, 2008). So far, number of studies has reported a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids (Yu et al., 2010). Such bioactive metabolites ascertain wide-range of application such as antiparasitics, antioxidants, anticancer, agrochemicals, antibiotics and immuno suppressant (Gunatilaka, 2006). In particular, the antimicrobial compounds could be used not only as drugs for human but also as food preservatives in the control of food spoilage and food-borne diseases, a serious concern in the world food chain (Liu et al., 2008b).

The associated microorganisms supply their host bivalve with bioactive metabolites providing vital functions or chemical protection from colonization by opportunistic microorganisms. The study of marine mollusc-associated bacteria is marked as an important field for the better understanding of their ecological role and interaction between invertebrates and associated bacteria’s. It’s also for their biotechnological application as producers of bioactive compounds (Romanenko et al., 2008). Thus, the current study highlights the fact that the isolation of antimicrobial compounds from endophytic strains is a potential source for natural product research. It has also been suggested that some of these bacteria chemically defend the host against microbial infection. For the proper identification of these antimicrobial extracts, it’s necessary to obtain them in pure form, which requires large scale purification process and different chemical analysis such as HPLC, spectroscopy and other sophisticated techniques. Thus, the marine associated bacteria producing compounds possess notable bioactivity with novel structures which should be explored to generate pronounced biological activity in the near future.