Antibacterial Property of Halobacterial Carotenoids against Human Bacterial Pathogens

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The present study was aimed to find out the antibacterial potential of carotenoids from halobacteria isolated from salt pan sediments against antibiotic resistant pathogens (Klebsiella sp., Staphylococcus aureus, Pseudomonas aeroginosa, Streptococcus pneumoniae and Streptococcus epidermis) and ophthalmic pathogens (E.coli, Staphylococcus aureus, Streptococcus pyogens, Proteus sp. and Acinetobacter sp). Different concentrations of the carotenoids were analyzed by MIC and MBC techniques. The isolates were identified by 16S rRNA gene sequencing and the relationship between the isolates was identified by phylogenetic tree analysis. Among the isolates, KT-02 showed maximum (14±0.65 mm dia.) sensitivity against Klebsiella sp. followed by 13±0.65 mm dia. against E.coli. The MIC and MBC values varied between 128-512 µg.ml-1. The potential isolate KT-02 showed maximum similarity (>95%) with Halomonas sp. (EU 435355) and the 16s rRNA secondary structure showed 15 loops and 23 turns and the free energy value was identified as -121.2 kkal/mol.

Keywords: Antibacterial activity, Antibiotic resistant pathogens, Halomonas sp, Ophthalmic pathogens.

Introduction

Traditionally, many natural products have played an important role in the discovery of therapeutic agents for infectious diseases and other illness. Recently, about 80% of the drugs have been derived from the natural products 1. Particularly, the marine natural products often yield the unexpected chemical structures. These marine natural products derived from mangroves, seaweeds, seagrass, sponges and associated microorganisms have the potency to cure cancer, malaria, dengue bacterial diseases and fungal diseases etc 2-6. Recently, much attention has been paid to the marine microorganisms due to their considerable biodiversity 7. Carotenoids are organic pigments that are naturally occurring in the chloroplasts of plants and bacteria. It can be synthesized by fats and other organic metabolic building blocks. Carotenoids play an important role in physiological role such as photo protection 8 and free radical scavenging activity. People consuming diets rich in carotenoids from natural foods, such as fruits and vegetables, are healthier and have lower mortality from a number of chronic illnesses 9. However, studies related with the antibacterial property of the carotenoids extracted from the halophilic microorganisms are too limited. In view of this, the present study was initiated to find out the antibacterial potential of the carotenoids against the human ophthalmic and antibiotic resistant bacterial pathogens.

Materials and methods

Isolation and identification of halobacterial isolates

Sediment samples were collected from Thamaraikulam (Lat. 8°10’N and Lon. 77° 26’E) and Puthalam salt pans (Lat. 8°06’N and Lon. 77° 28’E) Kanyakumari district, Tamil Nadu, India and brought to the laboratory for analysis. About 1g of sediment sample was transferred into 100 ml of sterilized 50% sea water and kept for continuous shaking (150 rpm) for 30 minutes. About 100 µl of diluted sample was spreaded over the halophilic agar medium (g.l-1) [Sodium chloride- 222; Magnesium sulphate.7H2O-10; Casein hydrolysate- 5; Potassium chloride- 5; Disodium citrate- 3; Potassium nitrate- 1; Yeast extract- 1; Calcium chloride.6H2O- 0.2; Agar- 10;
pH-7.2] and incubated for 2-4 weeks at 37°C. After attaining the visible growth, morphologically different colonies were re-streaked on the appropriate agar medium and further subjected for the molecular identification 10. Morphologically different halobacterial strains isolated from Puthalam salt pan is marked as KP and the isolates obtained from Thamaraikulam salt pan is marked as KT.

Mass cultivation of halobacteria

A loopful inoculum of morphologically different halobacterial strains were transferred into the 500 ml of halophilic broth (g.l-1) [Sodium chloride- 22.2; Magnesium sulphate.7H2O- 10; Casein hydrolysate- 5; Potassium chloride- 5; Disodium citrate- 3; Potassium nitrate- 1; Yeast extract- 1; Calcium chloride.6H2O- 0.2; pH-7.2±0.2] and kept for incubation at 28±2°C until the broth attaining dark yellow colour for 2 weeks. After incubation, the cells were harvested by centrifugation (1000 × g) for 10 min and washed thrice with distilled water by successive centrifugation.

Extraction of Carotenoids

Extraction of carotenoids from the pigmented cells was carried out by following the method of Asker et al. 11. Briefly, dried pellets were mixed with methanol and vortexed until the methanol layer turned into red. Further, the methanol layer was mixed with hexane and distilled water (1:1) in a separating funnel and shaken well for complete recovery of carotenoids. Then the carotenoids were washed thrice by centrifugation (1000×g) for 10 minutes. After that, equal volume of acetone was added for the precipitation of polar lipids. The precipitate was discarded and the supernatant was dried under rotary flash evaporator. The dried carotenoids were weighed and re-dissolved in hexane for further use.

Test Organisms

Antibiotic resistant pathogens viz., Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus epidermis, Pseudomonas aeruginosa and Streptococcus pneumoniae were obtained from Vinayaga Mission hospital, Salem, Tamil Nadu, India and ophthalmic pathogens viz., Staphylococcus aureus, E. coli, Streptococcus pyogenes, Proteus sp and Acinetobacter sp were obtained from Aravind eye hospital, Madurai, Tamil Nadu, and India.

Antibacterial sensitivity assay

The antibacterial activity of carotenoids extracted from Halomonas sp. was performed with well diffusion assay. About 20 ml of molten Mueller Hinton agar (Hi-Media, Mumbai) was poured into the sterile petriplates. After solidification, (10⁶ cells.ml⁻¹) bacterial cell suspension was swabbed on the surface of the Mueller Hinton agar medium. Then the media was gently punctured with the help of sterile cork borer. After that, different concentrations (10, 20, 30 and 40 µg.ml⁻¹) of carotenoids were diluted with 1% DMSO and added into each well. Negative control was maintained without carotenoids. Standard drug streptomycin (10 µg), Ciprofloxacin (10 µg) were used as positive control. All the plates were incubated at 37°C for 24 hrs and the zone of inhibition was measured. Triplicates were maintained for each bacterial isolates.

Determination of Minimum Inhibitory Concentration (MIC)

Different concentration (8, 16, 32, 64, 128, 256 and 512 µg.ml⁻¹) of carotenoids were dissolved in DMSO and mixed with 450 µl of nutrient broth and 50 µl of overnight bacterial inoculum. Nutrient broth alone was served as negative control. Whole setup in triplicate was incubated at 37°C for 24 hrs in thermostat shaker. After incubation, the tubes were examined by turbidity observations 2.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by sub-culturing the above (MIC) serial dilutions after 24 h in nutrient agar plates using 0.01 ml loop and incubated at 37°C for 24 hours. MBC was regarded as the lowest concentration that prevents the growth of bacterial colony on this solid media 2.

16S rRNA gene sequence amplification and sequencing

The genomic DNA was isolated from the halobacterial isolates by using standard protocols 12. The amplification of the genomic DNA with the forward primer (5'- AGAGTTTGA TCCTGGCTCAG -3') and the reverse primer (5'- ACG GCT ACC TTG TTA CGA CTT -3'). The reaction mixture contained 25 to 50 ng of DNA, Ex Taq PCR buffer, 1.5 mM MgCl₂, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer and 0.5 U of Ex Taq polymerase. PCR conditions consisted of an initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min and final 5 min extension at 72°C. The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc. The complete 16S rRNA gene was
sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 3730 automated DNA sequence (Applied Biosystems, Monza, Italy).

Construction of Phylogenetic tree and RNA secondary prediction analysis
The obtained sequences were compared with the NCBI databases (http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) 13. The pylogenetic tree was analyzed by using MEGA 4.0 software program (http://www.megasoftware.net) by using NJ method with 1000 replicates as bootstrap value and NJ belongs to the distance-matrix method 12. The RNA secondary structure was predicted by using Genebee online software (http://genebee.msu.su/services/rna2_reduced.html).

Results and Discussion
Generally, the carotenoids are responsible to prevent the human diseases including the cardio vascular, cancer and other chronic diseases 14. The infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat in the developing and developed countries 15. The development of resistance to the multiple drugs is also a major problem in the treatment of these infectious diseases caused by pathogenic microbes 12. In this connection, the present study was made an attempt to find out the novel antibacterial agents from halophilic microorganisms. A total of four morphologically different halophilic bacterial strains were isolated from the saltpan sediment. The colour of the isolates were varied with sandal, red, pale sandal, pale orange and the size of the colony was varied from 4 to 6 cm. The antibacterial potential of carotenoids reveals that, KT-02 showed maximum antibacterial sensitivity (14±0.65 mm dia.) against Klebsiella sp. followed by 13±0.65 mm dia. against E.coli. The minimum antibacterial sensitivity (9±0.12 mm dia.) was observed against Pseudomonas aeruginosa. Moreover, no sensitivity was noticed against Streptococcus pneumonia, Streptococcus epidermis, Proteus sp and Acinitobacter sp. (Table 1). However, none of the isolates except KT-02 showed antibacterial activity against any of the tested bacterial pathogens. The MIC and MBC result of the KT-02 reveals that, the sensitivity was observed between 128-512 µg.ml⁻¹ concentration against Streptococcus pyogens, Klebsiella sp. Staphylococcus aureus and E.coli (Table 2). This might be due to the inhibition of cell wall synthesis, accumulation of lysozymes or inhibition of cell multiplication 16. The antibacterial

<table>
<thead>
<tr>
<th>Name of the pathogens</th>
<th>Antibiotic resistant pathogens</th>
<th>Ophthalmic pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella sp.</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>E.coli</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Streptococcus pyogens</td>
<td>256</td>
<td>256</td>
</tr>
</tbody>
</table>

Table 1 – Antibacterial sensitivity of different concentrations of carotenoids from Halomonas sp. (HQ 438316) against antibiotic resistant and ophthalmic bacterial pathogens

<table>
<thead>
<tr>
<th>Name of the pathogens</th>
<th>10 µg</th>
<th>20 µg</th>
<th>30 µg</th>
<th>40 µg</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic resistant pathogens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>12±0.31</td>
<td>13±0.49</td>
<td>12±0.62</td>
<td>14±0.65</td>
<td>16±0.63c</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>11±0.26</td>
<td>12±0.31</td>
<td>12±0.14</td>
<td>12±0.38</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>9±0.12</td>
<td>11±0.52</td>
<td>10±0.43</td>
<td>11±0.26</td>
<td>12±0.57s</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus epidermis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14±0.43c</td>
</tr>
<tr>
<td>Ophthalmic pathogens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.coli</td>
<td>12±0.16</td>
<td>12±0.23</td>
<td>12±0.14</td>
<td>13±0.65</td>
<td>14±0.72s</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>10±0.27</td>
<td>11±0.61</td>
<td>10±0.28</td>
<td>11±0.37</td>
<td>13±0.49s</td>
</tr>
<tr>
<td>Streptococcus pyogens</td>
<td>12±0.64</td>
<td>12±0.53</td>
<td>13±0.24</td>
<td>13±0.15</td>
<td>15±0.36s</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7±0.36s</td>
</tr>
<tr>
<td>Acinitobacter sp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

-- No Sensitivity; Values are average of replicates; c-Ciprofloxacin (10 µg); s-Streptomycin (10 µg)
activity of carotenoids extracted from *Halomonas* sp. (KT-02) is represented in Fig. 1. The author’s \(^2\) reported that, the MIC and MBC values (250 µg.ml\(^{-1}\) - 500 µg.ml\(^{-1}\)) with the seagrass associated bacterial isolates against antibiotic resistant bacterial strains. The sediment bacterial isolates from the Parangipettai south east coast of India showed promising antibacterial activity \(^17\). The potential isolate *Halomonas* sp. (KT-02) showed maximum similarity index with *Halomonas* sp. (EU 435355) and it is previously proved to have potential antifungal activity against grey mould \(^18\). The molecular characterization and phylogenetic analysis showed that, all the bacterial isolates showed maximum similarity (>95%) index with *Halomonas* sp (Fig. 2). Further, the results of 16s rRNA gene secondary structure showed that, various number of loops (15), turns (23) and the various free energy values were noticed [(-147.4 kkal/mol) - (-121.2 kkal/mol)] between the *Halomonas* sp. (Plate.1). Generally, the RNA secondary structure used to differentiate among the similar species. In the present study, eventhough all the isolated *Halomonas* sp. seems to be similar, there is some inherent variation might be present in the genetic system. The author’s \(^3\) also reported that, 16S rRNA secondary structure of *streptomyces* sp. showed different numbers of loops and turns and this might be due to the variations of high GC contents. The lowest free energy values of the rRNA secondary structures provides the high relationship with the most primitive organisms, and higher free energy indicates the less stability during the evolutionary period \(^19\). It is concluded from the present study that, the industrially important carotenoids extracted from the *Halomonas*

\[\text{Fig. 1 – Antibacterial activity of the carotenoids extracted from *Halomonas* sp. (KT-02) against antibiotic resistant bacterial pathogen *Klebsiella* sp. at different concentration}\]

\[\text{Fig. 2 – Neighbour-Joining phylogentic representation of isolated *Halomonas* sp.}\]

\[\text{Plate 1 – 16s rRNA secondary structure of the isolated *Halomonas* sp. (HQ438316) isolated from Thamaraikulam saltpan sediments could be used as a novel antibacterial drug after completing the successful in vivo trials.}\]

**Conclusion**

Generally, the natural products have the greater importance in health care and prevention of human diseases. Particularly, the carotenoids have several pharmacological properties which cure several human chronic as well as the microbial diseases. In conclusion, the present study initiates the microbial carotenoids production from the *Halomonas* sp. of saltpan could be used for the treatment of several human bacterial diseases after completion of
successful clinical trials. Moreover, the present study identified that, the *Halomonas* sp. are the dominant in the saltpan of Kanyakumari district which could be confirmed by the molecular identification and differentiate among the *Halomonas* species by RNA secondary prediction method.

**Acknowledgement**

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**Reference**