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Ubiquitousness of *Haloferax* and Carotenoid Producing Genes in Arabian Sea Coastal Biosystems of India

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Abstract: This study presents a comparative analysis of halophiles from the global open sea and coastal biosystems through shotgun metagenomes (n = 209) retrieved from public repositories. The open sea was significantly enriched with *Prochlorococcus* and *Candidatus pelagibacter*. Meanwhile, coastal biosystems were dominated by *Marinobacter* and *Alcanivorax*. Halophilic archaea *Haloarcula* and *Haloquandratum*, predominant in the coastal biosystem, were significantly ($p < 0.05$) enriched in coastal biosystems compared to the open sea. Analysis of whole genomes (n = 23,540), retrieved from EzBioCloud, detected *crtI* in 64.66% of genomes, while *cruF* was observed in 1.69% Bacteria and 40.75% Archaea. We further confirmed the viability and carotenoid pigment production by pure culture isolation (n = 1351) of extreme halophiles from sediments (n = 410 × 3) sampling at the Arabian coastline of India. All red-pigmented isolates were represented exclusively by *Haloferax*, resistant to saturated NaCl (6 M), and had >60% G + C content. Multidrug resistance to tetracycline, gentamicin, ampicillin, and chloramphenicol were also observed. Our study showed that coastal biosystems could be more suited for bioprospection of halophiles rather than the open sea.

Keywords: halophiles; haloarchaea; carotenoid; microbial pigments; *Haloferax*

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1. Introduction

Carotenoids are natural pigments produced by plants, microbes, some fungi and microalgae. More than 750 carotenoids of potential commercial importance have been isolated from microbial sources [1]. In particular, Haloarchaea is a reservoir of unique carotenoid bacterioruberin [2]. Carotenoids synthesized by extreme halophiles are of specific interest for their ease in the extraction process, for saline tolerance, and for their biological applications against infectious diseases, repression of tumors or cancer growth, and cancer growth [3,4–6]. These extreme halophiles are mainly predominant in marine biosystems such as the open sea and coastal regions (salty marshes, salted ponds or similar ecosystems) [7].

Marine biosystems are exposed to unique environmental stress based on geographical location. For instance, coastal biosystems are exposed to continuous abiotic fluctuations such as salinity, pH, temperature, nutrients, and light, which enrich stress-tolerant enzymatic systems [8–11]. In contrast to coastal biosystems with biogenic and abiogenic colonizable particles, the open sea is rich in biogenic particles [12]. For instance, the open ocean has a lower abundance of available iron, which has led to the significant reduction of iron stress genes in *Synechococcus* strains from the

open sea compared to coastal regions [13]. The open sea is also a rich reservoir of bacteria representing over 90% of total marine biomass [14]. The open sea is significantly influenced by seasonal and temperature changes [15], and microbial communities that are the primary energy producers [16].

On the other hand, coastal biosystems are interconnected with freshwater bodies including rivers that constantly carry foreign solutes and microbes into the marine biosystem that could enrich the diverse microbial community. In our recent study, we detected the predominance of Haloarchaea in the Arabian Sea coastal region, India [2]. Compared to nearshore water, the open sea is also exposed to higher Ultraviolet (UV) radiation due to more increased transparency because of lower amounts of suspended particles [17]. Nevertheless, the sediment in the shallow coastal environment is exposed to similar UV radiation and higher salinity. Carotenoid pigments such as bacterioruberin are known to provide metabolic balance via osmoregulation and oxidation stress, respectively, when exposed to salinity and UV radiation [18–21]. Despite the differences between the open sea and coastal region, there is no in-depth comparative study on the diversity of pigmented halophiles to the best of our knowledge. Hence, this study attempts to perform comprehensive research to delineate the microbiome and halophilic microbial community differences between the open and coastal regions. Subsequently, we isolated halophiles and profiled their potential phenotypical, biochemical, and carotenoid production based on the findings.

2. Results

2.1. Microbiome Composition in Open Sea and Seacoast

The open sea and coastal biosystems were composed of 665 operational taxonomic units (OTUs). The microbiome composition between the biosystems was significantly different, which is evident from the separate clustering of the biosystems in the Weighted UniFrac Principal component analysis (PCA) (Figure 1a). Interestingly, several metagenomes from both biosystems overlapped, suggesting similarities between the datasets. To identify the genera contributing to the similarities and differences, we analyzed the top 10 dominant genera in both ecosystems (Figure 1b). *Flavobacterium* and *Gramella* genera were common to both biosystems with strong similarities regarding their abundance. However, the open sea was significantly enriched with *Prochlorococcus* and *Candidatus Pelagibacter*.

In contrast, the coastal biosystems were dominated by *Pseudomonas*, *Marinobacter* and *Alcanivorax*. In addition, the halophilic archaea *Haloarcula* and *Haloquadratum* were also represented among the top 10 predominant genera in the coastal biosystem with significant ($p < 0.05$) enrichment compared to the open sea (Figure 1b). Surprisingly, in the open sea, extreme halophiles were not detected among the top 10 genera. The predominance of the coastal biosystems by halophiles, particularly those belonging to the haloarchaeal genera, could suggest that coastal biosystems are a better avenue for bioprospection of halophiles.

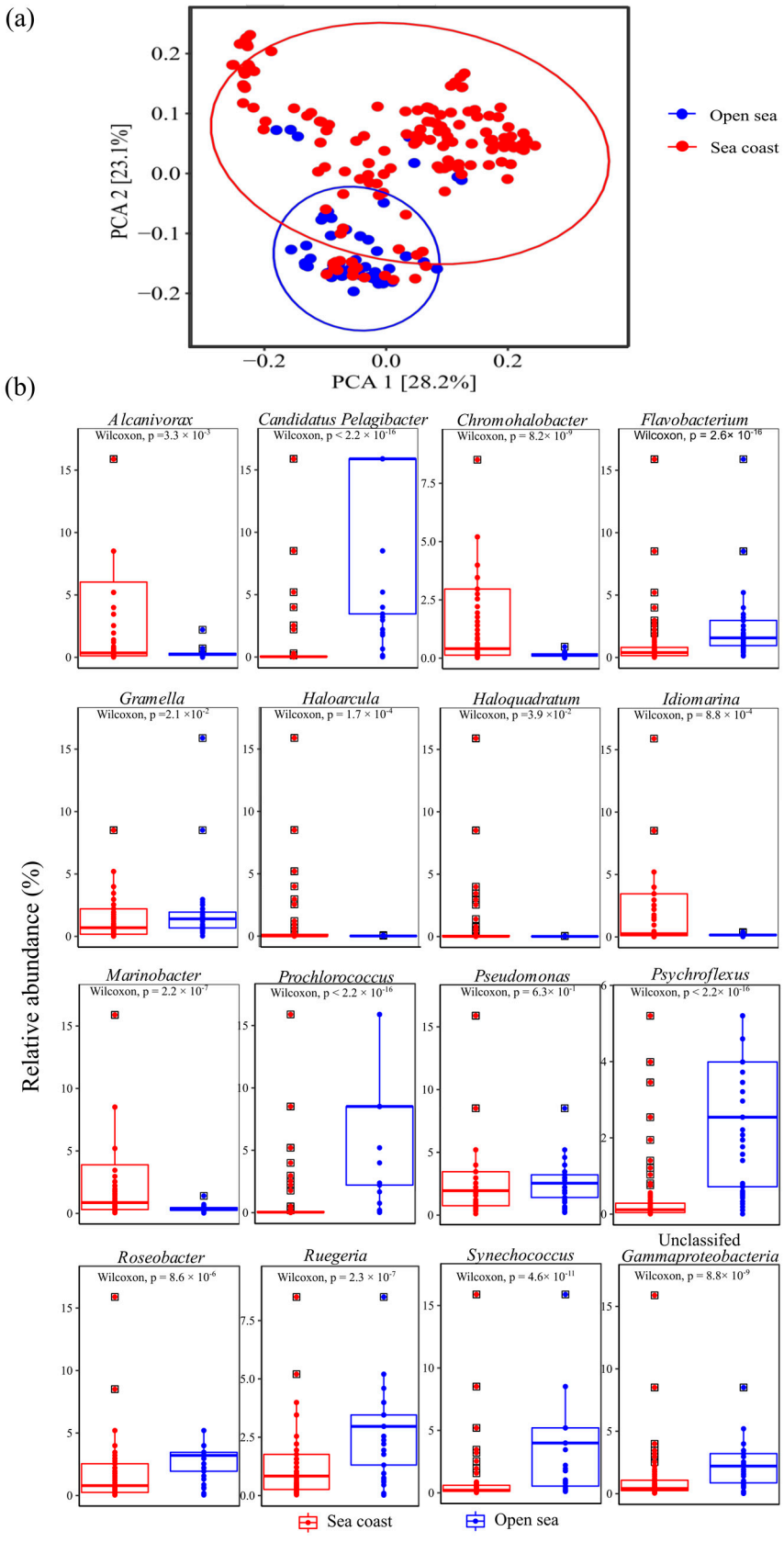

Figure 1. (**a**) Weighted UniFrac PCA plot of the open sea and coastal microbiome. (**b**) Cumulative predominant microbial genera were plotted by retrieving the top 10 genera in the open sea and coastal biosystems.

2.2. Diversity of Halophilic Microbiome in Open Sea and Seacoast

The diversity of the halophiles was further investigated by determining the alpha diversity and composition of the halophiles. Chao1 diversity estimation indicated moderately higher diversity ($p < 0.05$) in the coastal ecosystem compared to the open sea (Figure 2a).

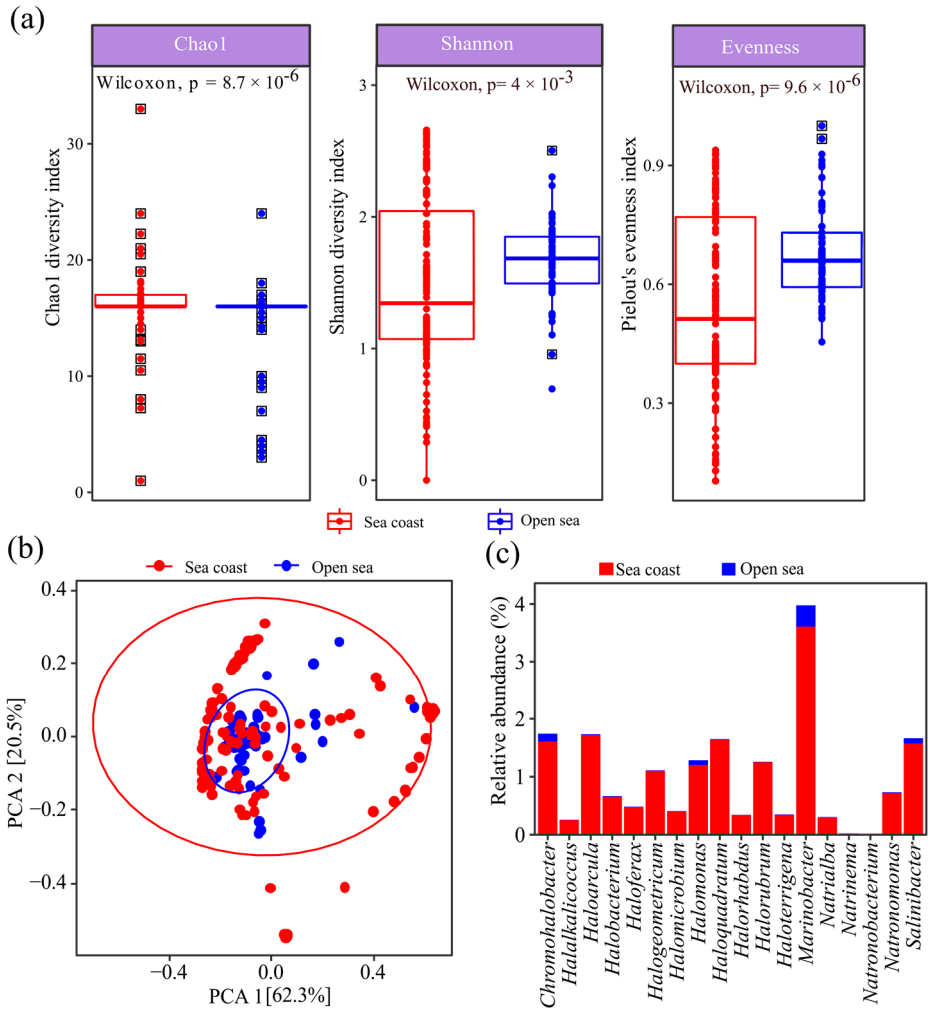

Figure 2. (**a**) Alpha diversity, (**b**) Weighted UniFrac PCA plot and (**c**) relative abundance of the extreme halophilic microbial community in the open sea and coastal metagenomes.

On the contrary, the Shannon index and Pielou's evenness were moderately higher in the open sea, suggesting that the abundance of halophiles in the coastal ecosystem could be unevenly distributed. The weak differences in diversity between the biosystems were further observed in the Weighted UniFrac PCA analysis (Figure 2b). Interestingly, the similarities observed between the microbiome were due to the similarities in the proportion of predominant OTUs. This indicates that halophiles between the biosystems share similarities in diversity yet differences in relative abundance (Figure 2c).

2.3. Diversity of Carotenoid Gene in the Halophiles.

Functional genes (n = 31) in the carotenoid synthesis pathways were similarly distributed in the open sea and seacoast (Figure 3a,b).

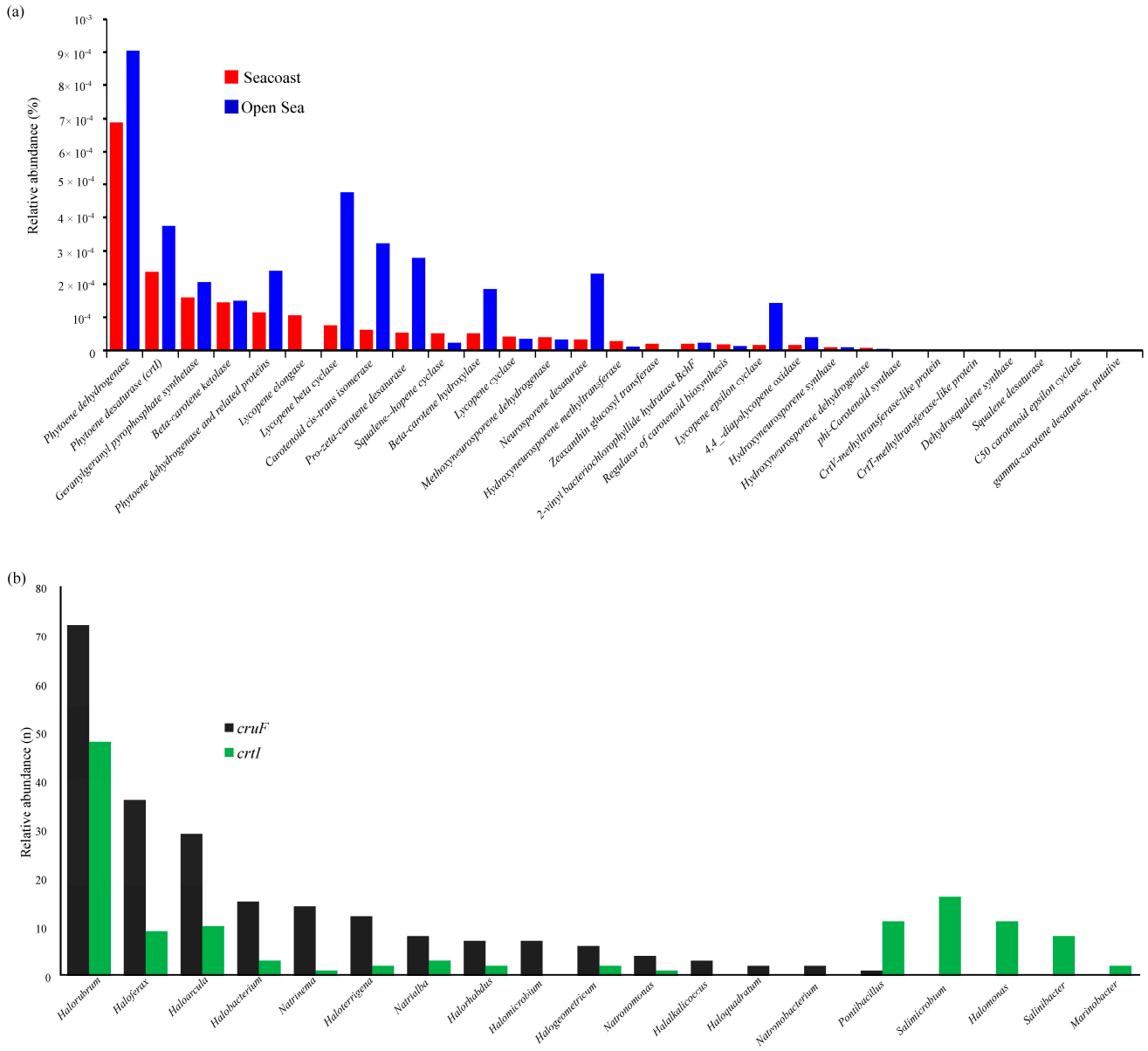

Figure 3. (**a**) Relative abundance (%) of a functional gene within carotenoid metabolism in subsystems level 3 classification. (**b**) *crtI* and *cruF* gene abundance of the whole genome of extreme halophiles detected within the pan-metagenome.

Genes encoding phytoene dehydrogenase, responsible for the lycopene synthesis, were predominant in both biosystems. Despite the significant difference in the functional potential for carotenogenesis, the whole metagenome dataset could provide a biased snapshot for functional genes with low abundance due to insufficient sequencing depth.

Hence, the whole genome of 23,540 species was retrieved from a public repository to circumvent these weaknesses, and the prevalence of significant carotenoid genes viz. *crtI*, *crtL*, *cruF*, and *crtD* genes, were investigated. A total of 64.66% of species harbored the *crtI* carotenoid gene, indicating wide prevalence (Table 1). The *crtI* (phytoene desaturase) and *cruF* (C_{50} carotenoid 2″,3″-hydratase) genes were predominant in *Halorubrum* and *Haloferax* genera. The abundance and distribution of *crtI* and *cruF* gene significantly differed between genera (Figure 3b).

Table 1. Carotenoid gene abundances in bacterial and archaeal strains based on whole-genome sequences.

Domain	**Bacteria**				**Archaea**			
No. of strains	22,615				925			
crt genes	*crtI*	*crtL*	*cruF*	*crtD*	*crtI*	*crtL*	*crtD*	*cruF*
No. of genes	14,795	3001	397	1837	427	0	568	415
No. of unique strains	6766	2684	382	1643	292	0	329	377

2.4. Isolation of Pigmented Halophiles across the Arabian Sea Coastline

As per the metagenomic analysis, coastal biosystems represent a more suitable avenue for the isolation of halophiles than the open sea. In addition, whole-genome analysis further indicated the prevalence of carotenoid genes among the halophiles, indicating the prevalence of carotenoid synthesis. These observations warrant further confirmation through a culture-dependent approach to determine the cultivability and carotenoid production. Since the metagenome was retrieved from public datasets, obtaining the sediments was out of the scope of this study. Hence, coastal sediments were collected in triplicates (n = 410 × 3) along the Arabian coastline of India from seashore (n = 202), estuaries (n = 25), rivers (n = 86), mangroves (n = 51), lakes (n = 28), island (n = 15), and saltpan (n = 3). Halophilic pure cultures (n = 1351) were obtained by enriching the sediments in MSG (Modified Sehgal and Gibbon's) medium with saturated 6M NaCl. Red, yellow, or orange pigmentation was exhibited by 77 isolates (5.69%) (Figure 4). The yellow-pigmented isolates were the most abundant (66.23%), followed by orange (20.78%) and red (12.99%) pigments (Figure S1a). Among the yellow-pigmented isolates, 78.43% originated from seashore sediments, and 1–5% were from other biosystems such as a river, saltpan, island, mangrove, and lakes. Red-pigmented halophiles were isolated from all the biosystems except island samples. The red-pigmented isolates were especially obtained from mangrove sediments (30%). River and lake sediments constituted 20% of the overall red-pigmented isolates, while saltpan and estuary harbored only 10% of the red-pigmented isolates. Interestingly, orange pigmented halotolerant were only observed in the three biosystems, i.e., seashore, river, and mangrove (56.25%, 25%, and 18.75% respectively) (Figure S1b). Sediments from Kerala had the highest evenness in the distribution of pigmented isolates, i.e., yellow (29.41%), orange (47.05%), and red (23.52%). In contrast, sediments from Karnataka, Maharashtra, Goa, and Gujarat were dominated (80–100%) with light, yellow-pigmented isolates. The red pigmented isolates could be isolated mainly from Kerala (n = 8), followed by Maharashtra (n = 1) and Karnataka (n = 1) (Figure S1c).

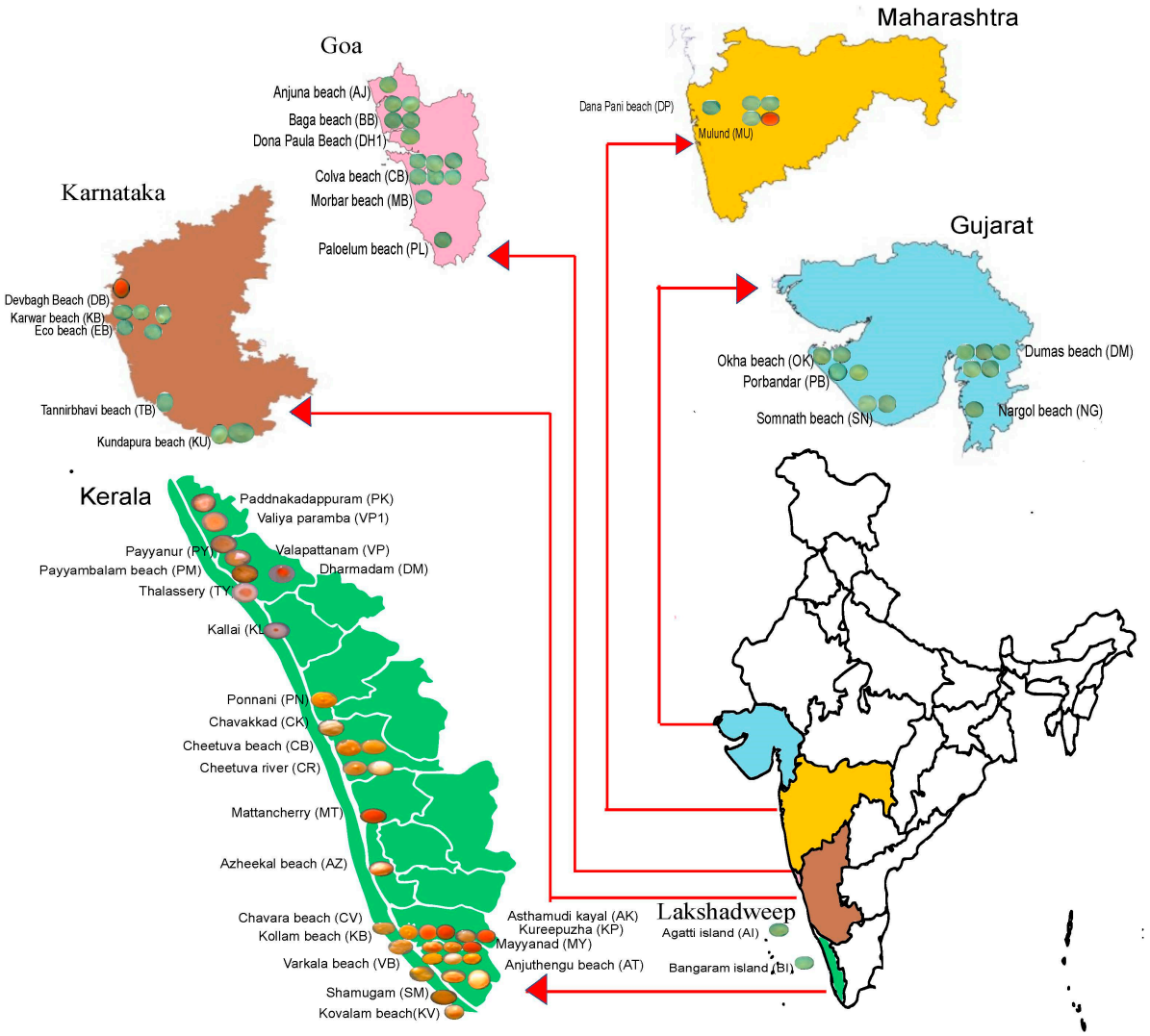

Figure 4. Graphical representation of sediment sampling locations along the Arabian coastline. The stereomicroscopic image of the extreme halophilic pure cultures are shown within the inset map.

2.5. Morphological Diversity across the Halotolerant Organism

Gram-positive isolates were dominant (66.23%) among the isolates. Rod-shaped Gram-positive, rod-shaped Gram-negative, and Gram-positive cocci accounted for 32.46%, 31.16%, and 32.46%, respectively. The rare ones were Gram-positive coccobacillus (1.29%) and Gram-negative irregular cocci (1.29%). Furthermore, it was observed that 77.92% of the isolates were motile, and 46.75% of isolates could survive in both aerobic and aerobic conditions (Table S1).

2.6. Biochemical Diversity across the Halotolerant Organisms

Urease activity was detected in 66.23% of the isolates. Glucose, as the primary source of carbon, was observed in 46.75% of the isolates. Mannitol, lactose, and sorbitol utilization was observed in 36.36%, 32.46%, and 28.57% of the isolates, respectively. Furthermore, oxidase (45.45%) and catalase activities (35.06%) were also widespread among the halophiles. A significant ($p_{Kruskal–Wallis}=1.9 \times 10^{-9}$) difference was observed in the biochemical properties of the isolates based on locations. Out of 32 biochemical tests, isolates RK_AK2, RK_AZ, RK_CR2, RK_KB, and RK_MT from Kerala were positive for 46.87%, 37.5%, 34.37%, 34.37%, and 31.25% of the biochemical test, respectively (Table S2). Furthermore, isolates from Kerala exhibited a similar pattern of oxidase (61.76%) and urease activity (61.76%). All isolates from Lakshad-

weep showed urease, glucose, catalase, lactose and sorbitol activities. H_2S-producing isolates were observed from Karnataka (44.44%), Gujarat (23.07%), and Goa (21.42%). Furthermore, 14–23% esculin activity was also found in Kerala, Karnataka, Gujarat, and Goa isolates.

2.7. Antibiotic Susceptibility Profiling

Most isolates (61.03%) were resistant to gentamicin at a concentration of 25 μg/mL, and 36.36% were resistant to the antibiotics at concentrations up to 100 μg/mL. Similarly, 46.75% of isolates were resistant to tetracycline at concentrations up to 25 μg/mL. However, the isolates were susceptible to ampicillin (72.73%) and chloramphenicol (76.63%) at a concentration of 100 μg/mL (Figure S1d).

2.8. Influence of NaCl Concentration on Pigment Production

The microbial isolates were resistant to 3–6 M NaCl, thus showing an extreme halophilic profile. Interestingly, 94% of the 6 M NaCl resistant halophiles were isolated from Kerala, and 3% of isolates were from Maharashtra and Karnataka. Isolates from Goa and Gujarat were resistant to a maximum salinity of 3 M NaCl (Figure S2a,b). Furthermore, all isolates with red and orange pigments were resistant to 6M NaCl, whilst only 20% of the yellow-pigmented isolates were resistant to 6 M NaCl (Figure S2c). Pigment production was observed after 50 h. incubation and attained optimum levels after 300 h. incubation (Figure S2f,g).

2.9. Identification of the Isolates through 16S rDNA Phylogenetic Analysis

Phylogenetic analysis revealed that 23 isolates belong to Haloarchaea and 54 isolates belong to the bacterial domain (Table S3). In contrast to Haloarcheal diversity observed through the metagenomic approach, the Haloarchea obtained through culturable technique was represented solely by *Haloferax* species such as *Haloferax alexandrinus, Haloferax lucentense, Haloferax chudinovii,* and *Haloferax sulfurifontis.* Halophilic bacterial isolates were represented by *Chromohalobacter, Salimicrobium, Halomonas, Pontibacillus, Staphylococcus, Virgibacillus, Lysinibacillus, Pseudomonas, Bacillus,* and *Acidovorax* genera (Figure S3a–c). Interestingly, 91.3% of the total haloarchaea were isolated from Kerala, whereas only 4.3% were isolated from Maharashtra and Karnataka. Halophilic bacteria were most isolated from Goa (25.92%), Kerala (24.07%), and Gujarat (24.07%). Lakshadweep samples had the lowest percentage of halophilic archaea or bacteria (3.70%). A significant difference in genera distribution was observed between the locations ($p_{\text{Kruskal–Wallis}} = 0.02$).

Isolates from Gujarat samples were represented by *Virgibacillus* (38.46%), *Staphylococcus* (23.07%), *Bacillus* (23.07%), and *Halomonas* (15.38%). Isolates from Goa were represented by well-known salt-tolerant *Halomonas* (42.85%), followed by *Virgibacillus* (14.28%) and *Staphylococcus* (28.57%). Interestingly, *Lysinibacillus* (7.14%) and *Pseudomonas* (7.14 %) were isolated only from the Goa. Sediment from Maharashtra was predominated with *Virgibacillus* (40%), followed by *Haloferax, Staphylococcus,* and *Bacillus*. Lakshadweep sediments were enriched with only two halotolerant organisms, i.e., *Halomonas* and *Staphylococcus*. Kerala biosystem had the highest haloarchaea abundance (61.76% *Haloferax*) in addition to diverse bacterial genera such as *Chromohalobacter, Marinobacter, Salimicrobium,* and *Pontibacillus*. A significant difference in species abundance was observed between geographical locations based on Kruskal–Wallis test ($p < 0.03$) and principal component analysis (Figure 5). *H. lucentense*, *H. alexandrinus,* and *H. chudinovii* were enriched in Kerala, while *Halomonas halophila* was enriched in Gujarat, Goa, and Kerala. (Figure 5). Comparative analysis showed a significant difference in species distribution in each biosystem (Kruskal–Wallis $p = 1.31 \times 10^{-17}$).

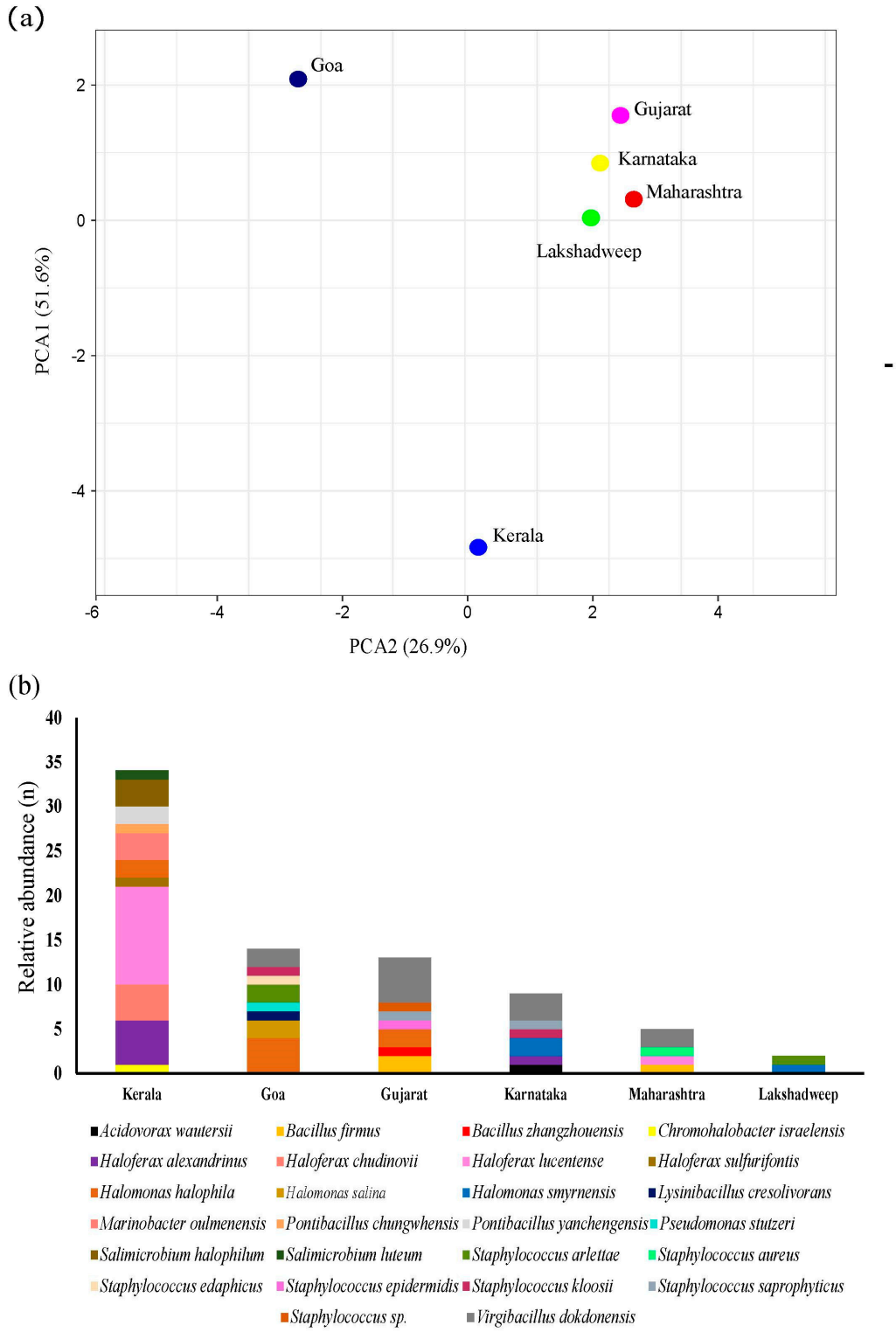

Figure 5. (**a**) PCA plot and (**b**) stacked bar chart of the culturable extreme halophiles isolated from different locations.

Sediments from seashore were represented in a distinct axis in the PCA analysis, enriched with twenty-four diverse species with dominant *Virgibacillus dokdonensis*. However, estuary, island, lake, and saltpan clustered together (Figure 6a,b). Within the *Haloferax* community, *H. lucentense* was predominant in mangroves and rivers.

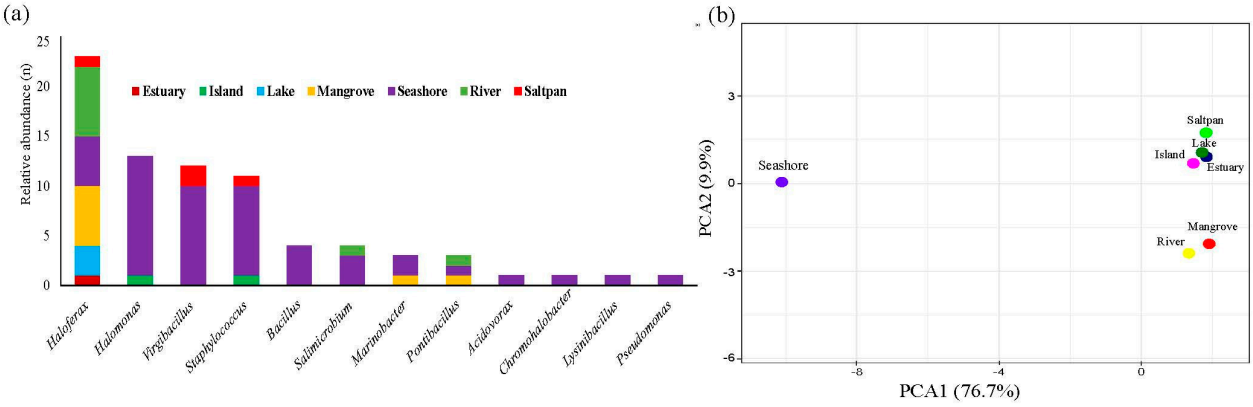

Figure 6. Genera-level distribution of the culturable extreme halophiles represented in (**a**) stacked bar chart and (**b**) PCA plot based on biosystems.

2.10. G + C Diversity across the Halotolerant Organisms

A significant difference in the G + C content was observed between Haloarchaeal and Halobacterial isolates (Epps-singleton $p = 6.3427 \times 10^{-12}$) in line with the results reported in the literature [18,19]. The G + C content for 86.95% of the Haloarcheal isolates was above 60%, whereas only 36.36% of Halobacteria had G+C contents above 60% (Figure S3d,e).

3. Discussion

Marine ecosystems represent one of the largest biosystems with an enormous untapped resource of bioactive molecules. Carotenoids of marine origin have gained interest for their multitude of applications in industrial and pharmaceutical areas. The diversity of carotenoid synthesis genes in marine microbes can vary substantially between biosystems. However, to the best of our knowledge, a broad-scale analysis of the carotenoid synthesis gene diversity between the open sea and coastal regions has never been carried out. In this study, we performed a comparative shotgun metagenome meta-analysis of open sea and coastline halophilic community and further confirmed it through pure culture isolation.

We analyzed the global marine biosystems through shotgun metagenome data (n = 209) and whole-genome (n = 23,540) (see Figure 7 in material and methods section and Table S4). Furthermore, we implemented the culturable technique to isolate halophiles from the Arabian Sea coast of India. As per the metagenomic analysis, halophiles were most diverse in the seacoast biosystems. The carotenoid synthesis genes *crtI* and *cruF* were predominant in the *Halorubrum* and *Haloferax* genera. The significant enrichment of halophiles in coastal biosystems could be due to the salt accumulation upon marine water evaporation [10]. Similarly, the predominance of *Haloferax* was also observed based on culturable techniques. The halophilic isolates from coastal sediments ($n = 410 \times 3$) were further investigated to evaluate the culturable diversity of extreme halophiles (carotenoid production, phenotypic, biochemical, and genotypic characteristics).

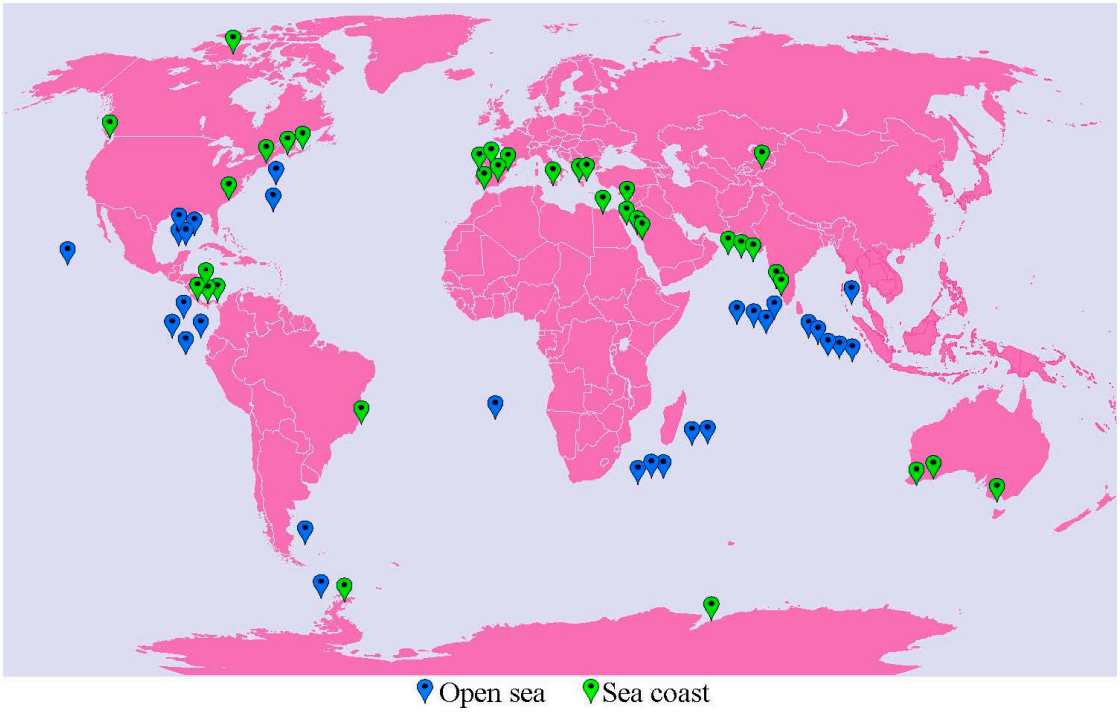

Figure 7. Global map representing the sampling location of the metagenomes retrieved from the MG-RAST server. Sampling sites of coastal and open sea metagenome datasets are highlighted in green and blue, respectively (the global map was generated using https://maps.co, accessed on 7 March 2021).

3.1. Extreme Halophiles Are Enriched in Seacoast Biosystems.

The microbiome structure differed significantly between the open sea and coastal metagenome datasets, although some similarities were also observed. The open sea surface is constantly exposed to sunlight, which provides a suitable condition for photosynthetic bacteria. This leads to the enrichment of photosynthetic genera *Prochlorococcus* (7.8 ± 0.75%) and *Synechococcus* (4.03 ± 0.52%) in the open sea [22]. Furthermore, the unrestricted availability of organic matter enriches *Pelagibacter* (10.5 ± 0.86%), which feeds on organic matter in the open sea. In contrast, the coastal biosystems were enriched with *Alcanivorax* and *Marinobacter*, which are well-known hydrocarbon-degradation indicators [23,24]. In addition, phosphate solubilizer and nitrogen-fixing genera *Pseudomonas* were highly enriched in the seacoast biosystems [25]. The prevalence of such genera could indicate anthropogenic pollutions and microbes related to plant growth promotion. Interestingly, the halophilic archaea *Haloarcula* and *Haloquadratum* were among the predominant genera in the seacoast. *Haloarcula* is an extreme halophile common in saline sediments and saltpan around the globe and is involved in denitrification [26,27]. *Haloquadratum* genus is characterized by its unique flat square shape morphology and an extended doubling time of 10 days [28].

The diversity of halophilic community was slightly higher in the coastal biosystems than in the others, but its abundance was lower. The low Shannon diversity index could be due to the sedimented nature of the coastal biosystems [29]. Sediment microbial communities have strong antagonistic properties to colonize the sediment particles and are exposed to higher concentrations of nutrients and sunlight [30]. Furthermore, coastal biosystems could also be characterized by higher salinity [31]. The relative abundance of extreme halophiles was several folds higher in the coastal biosystems than in the open sea. The predominant genus *Marinobacter* was enriched to a much greater extent than the other genera. The enrichment of *Marinobacter* could indicate anthropogenic pollution in the coastal biosystems on a global scale [32,33]. The coastal biosystems are densely populated and vulnerable to anthropogenic pol-

lutions, climate change, and ecological degradation worldwide [34]. Such circumstances could enrich halophiles with hydrocarbon degradation characteristics.

The other dominant genera in the coastal and open sea were *Chromohalobacter*, *Salinibacter*, *Halorubrum*, and *Haloarcula*. Nonetheless, their abundance was substantially higher on the seacoast. These genera have also been identified in polluted hydrocarbon environments [35]. The adaptation mechanism of the identified genera involves osmoadaptation through carotenoid production, salt-in/salt-out strategy, and compatible solute through glycine Betaine/Carnitine/Choline Transporter (BCCT) [36].

3.2. Archaeal Extreme Halophiles Are Enriched with crtI and cruF

Halophilic microbes produce red, yellow, and orange colored carotenoid pigments. The *crtI* gene is responsible for lycopene production. The lycopene is further processed to produce α-, β-carotene, or bacterioruberin (*cruF*). The carotenoid synthesis pathways in archaeal halophiles have been reported to differ from non-halophiles by producing mostly bacterioruberin, a rare C_{50} carotenoid mainly produced by haloarchaea [4,37]. Bacterioruberin from the bacterial domain has also been reported in the literature, although in paucity, such as psychrotrophic *Arthrobacter agilis* [38], *Rhodospirillaceae* strains [39] and *Azospirillum* species [40]. *cruF* gene was observed in only 1.69% bacteria (*n* = 382/22615) and 40.75% archaea (*n* = 377/925). Despite the lower hit rate ratio of *cruF* in bacterial strains, it is quite interesting to note the presence of *cruF*, because bacterioruberin synthesis from bacterial systems has not received much attention compared to the archaeal counterpart, yet the absolute number of hits is similar. This suggests that bacterial systems could also be as prolific producers as archaea. Among the predominant extreme halophiles under bacterial domain, viz. *Marinobacter*, *Salinibacter*, *Halomonas*, and *Salimicrobium*, *cruF* was not detected, indicating that these genera may not produce bacterioruberin. On the contrary, extreme halophilic archaea encoded *cruF*, thus indicating potential bacterioruberin production. The predominant archaeal genus *Halorubrum* and *Haloferax* have also been reported to produce bacterioruberin [19,41,42]. The wide prevalence of *cruF* among the dominant archaeal genera strengthens the potential to harness bacterioruberin from the coastal biosystems.

3.3. Archaeal Halophiles Exhibit Diverse Pigmentation

Pigmented extreme halotolerant pure cultures from 410 sediments of mangroves, lakes, seashore, saltpan, estuaries, and rivers along the Arabian Sea coast (India) were screened in MSG media enforced with 3–6 M NaCl. To the best of our knowledge, this is the first attempt to isolate pigmented extreme halophilic microbes from various marine biosystems throughout the Arabian coast in this country. Seventy-seven isolates of 1351 extreme halotolerant isolates exhibited pigmentation. Despite the extremely low salinity in river sediment, the presence of pigmented halotolerant warrants further investigation of pigmented halophile diversity in freshwater bodies. This result also sheds light on carotenoid bioprospection and their derivatives from natural environments other than saline ecosystems.

Archaeal isolates exhibited yellow, orange, and red pigments, while bacterial isolates showed yellow and orange but not red pigments. Haloarcheal red pigments are mainly astaxanthin and bacterioruberin, while orange pigments could be salinixanthin [6,37]. Both pigment types have significant antimicrobial and antioxidant properties [6,19,43,44]. Yellow pigments of haloarchaea are compounds such as 4,4'-diaponeurosporene, zeaxanthin, or lutein with applications in food colorants, visual acuity, and antibacterial activity against multidrug-resistant organisms [45,46].

The predominance of yellow-pigmented isolates, especially in the seashore, manifest the occurrence of both autotrophs and heterotrophs in the marine biosystems, which require such pigments to adapt to adverse environmental conditions such as high salinity, radiation, pH, and temperature [47–52]. The predominance of yellow-pigmented isolates could also be explained by the abundance of silica and calcium in the seashore that facilitates absorption of nutrients due to the large surface area that helps develop a diverse microbial community [53].

Compared to bacterial isolates, archaeal isolates had a significant higher doubling and incubation periods for pigmentation. The prolonged incubation for carotenoid production could be because carotenoids are secondary metabolites produced in the late phase of growth. Furthermore, the process (carotenogenesis) comprises complex metabolic networks involving several enzymes, transcriptional regulatory protein, ORC1-type DNA replication protein, and GTP cyclohydrolase III that are induced mainly in the presence of high-saline conditions [26,54]. Extreme halophiles can endure high saline stress mainly through the expression of saline-resistant genes such as *rrnAC2519, cdc6A, gch3, flaC, psp A* and *rpsG* [26].

The isolates showing above 60% G + C contents were predominant in red-pigmented isolate (100%), but only in 50% and 12.5% among the orange and yellow-pigmented isolates. The red pigments were also haloarchaea members (*Haloferax* genus) resistant to saturated NaCl (6M). Previous studies have shown that G + C content is associated with genome stability in high saline and abiotic stress [55–58]. Thus, it is established that high G + C content in haloarchaeal serves as a protective measure from environmental stress, prevention of thymidine dimers and reduces UV-induced mutations [20,57]. The high G + C content is also associated with biased usage of amino acids, leading to acidic proteome [59,60], a hallmark of halophiles [57]. Acidic proteome requires a saline environment for stability, activity, and osmotic balance [61]. Such adaptation could explain the high G + C content in extreme haloarchaeal and halobacterial isolates observed in this study.

The significant enrichment of reddish and orangish pigments among the haloarchaeal group further assures the importance of biomining archaeal communities. Carotenoids and their derivatives are of particular interest because of their commercial value as food additives, colorants, and medicinal applications [62]. Global demand for carotenoid compounds is projected to be $2.0 billion by 2022 [63].

3.4. Biogeography of Extreme Halophiles in Arabia Sea Coast of India

Haloarchaea were solely represented by *Haloferax* species. They were recovered from all biosystems, except islands, indicating their prevalence in Arabian Sea biosystems. Interestingly, all four species of the *Haloferax* genus, i.e., *H. lucentense, H. sulfurifontis, H. chudinovii,* and *H. alexandrinus,* were isolated in Seashore sediments of Kerala. *Haloferax* species are of particular interest as they are hyper producers of bacterioruberin with high antioxidant and pharmaceutical applications [64–66]. *Haloferax* can also convert cheese whey/olive mill wastewater into poly (3-hydroxybutyrate-co-3-hydroxyvalerate), which shows potential applications as biodegradable biopolymer [67–69]. Furthermore, extreme salt tolerance is a desirable attribute for the industrial-scale production of carotenoids or whole-cell biocatalysts due to its ease in extraction, tolerance to salinity, and an array of biological applications [4–6,66].

The bacterial halophiles were represented by eleven genera, predominantly *Salimicrobium, Pontibacillus, Chromohalobacter, Halomonas*, and *Marinobacter*. *Salimicrobium* sp. has been reported to produce glutamate dehydrogenase, which suggests its industrial importance [70]. Extracts from *Pontibacillus* and *Chromohalobacter* have also been shown to exhibit anticancer and α-amylase activity [71–74]. Furthermore, *Chromohalobacter* degrades aromatic hydrocarbons that have a potential role in wastewater treatment and also serves as hydroxyectoine producer [75,76]. *Halomonas* species are known to synthesize ectoine and sulphate exopolysaccharides with biological activities [77,78]. Previous reports from South China Sea sediments have also isolated *Halomonas* species [79]. *Halomonas* species have also been reported to produce emulsifying agents such as P39a, which are of industrial interest [80]. Although most halophiles were isolated from Kerala sediments, several other species (such as *Virgibacillus sp., Pseudomonas sp., Staphylococcus sp., Bacillus sp., Acidovorax sp*.) were only isolated from other locations. Some of these genera are of biotechnological interest: halophilic *Acidovorax* and *Staphylococcus* for instance synthetize enzymes for polyhydroxybutyrate depolymerization and thermo-tolerant alkaline lipases [81,82].

Haloferax dominated the mangrove and *Halomonas* the Seashore sediments. Interestingly, all isolates from Kerala biosystems were resistant to 6M NaCl. The prevalence of extreme halophiles in Kerala could be credited to several factors. Firstly, the high salinity of Arabian Sea coastal regions in western India has been well documented [83]. Furthermore, Kerala has diverse biosystems such as mangroves and brackish water interlinked by 41 freshwater rivers, leading to the formation of numerous salinity gradient estuaries. Finally, most of the halophilic species isolated from the river in the current study have been reported in the literature mainly from a saline environment [26,70]. For instance, *Virgibacillus dokdonensis* is an extracellular proteases producer isolated from Saharan Salt Lake. Similarly, *Chromohalobacter israelensis, Pontibacillus chungwhensis, Haloferax lucentense,* and *Haloferax chudinovii* have been isolated from seawater, saltpans, saline desert, etc. [26,84–87]. To the best of our knowledge, this study describes for the first time that such species are not only exclusive to the saline environment but could also inhabit freshwater sediments. The detection of such halophiles from non-saline sediments could be due to the strong enrichment during the screening step.

3.5. Morphological, Biochemical, and Antibiotic Resistance Profiles of Halotolerant Species

Halotolerant species inhabit harsh environmental conditions that favor diverse biochemical reactions [88]. The extremes in environmental conditions would shape and evolve their enzymes to participate in such biochemical reactions [89]. Nevertheless, salinity has a detrimental effect on enzymatic reactions [90,91]. For instance, catalase and urease activities were reported to be inversely correlated to pH and salinity [92–94]. However, in this study, catalase and urease activities were observed in sev-

eral isolates, indicating that such enzymes in halophiles might have an evolutionary adaptation that confers tolerance to salinity to be functional [95]. Cytochrome oxidase was also highly prevalent among the extreme salt-tolerant isolates. We also observed that 66.23% of isolates were Gram-positive. Previous studies on Salt Lake, the Dead Sea, and the Wadden Sea have also observed a predominance of Gram-positive halotolerant with specific adaptions in the coastal area [96–98]. Furthermore, over 60% of the isolates were rod-shaped. The high proportions of rod shape halotolerant have also been observed in previous studies and could have an evolutionary advantage [97]. In line with the higher diversity of pigmentation among haloarchaea, biochemical activities were also higher in the archaeal halophiles than in the bacterial counterpart.

The landlocked nations in the northern part of India account for about 30% of the world population, which could be sewage and industrial effluent [99–101]. Such anthropogenic activity enriches antibiotic-resistant genes in the coastal environment [102–104]. Furthermore, our previous metagenomic studies identified the prevalence of ARGs in the Kerala mangrove sediments [105]. Antibiotic resistance is a global threat with a mortality of over 700,000 per year, and it is expected to reach 10 million by 2050 [106,107]. Halophiles are reported to harbor multidrug resistance through the mechanism of efflux pumps, beta-lactamase production, etc., including ARG encoded in the plasmid(s) [108,109]. In this study, halotolerant isolates had resistance to gentamycin (61.03%) and tetracycline (46.75%) at 25 μg/mL concentration. Furthermore, multidrug resistances to all four antibiotics (tetracycline, gentamicin, ampicillin, and chloramphenicol) were also observed in three isolates including RK_DM4 (*Bacillus firmus*), RK_OK1 (*Virgibacillus dokdonensis*), and RK_OK3 (*Staphylococcus saprophyticus*). In line with this, our previous study on multidrug-resistant (MDR) bacteria in mangrove sediments from the Arabian coast, India, has also identified *Bacillus firmus* as resistant to all four antibiotics [29]. *Bacillus firmus* and *Staphylococcus saprophyticus* promote plant growth and degrade hydrocarbon, respectively [110–112]. However, they are also well-known as causative agents for food spoilage and urinary tract infections [113,114]. Antibiotic resistance is of grave concern owing to its rapid spread through horizontal gene transfer [115]. The prevalence of MDR isolates observed in this study indicates the potential of antibiotic-resistant gene (ARG) horizontal transfer from environmental to clinically relevant pathogens [116–118].

4. Materials and Methods

4.1. Metagenomic Analysis

Whole metagenome datasets (n = 209; ~1.1 Terabyte) of open sea and coastal region were retrieved from the MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) repository server (Figure 7).

The metagenome datasets originate from various locations such as the Atlantic Ocean, Indian Ocean, Southern Ocean, Pacific Ocean, coastline region of Russia, Australia, Egypt, Pakistan, Mexico, and Antarctica. (Table S4). All raw datasets were processed in the MG-RAST pipeline to avoid differences in the in silico approach. The pipeline includes removing low-quality sequence reads based on the Phred score (less than 20), extracting duplicate reads and host DNA such as human, and then processing for the taxonomic identification and functional profiling. The OUT matrix was batch-normalized with preprocessCore v1.52.0 [119], and the microbiome data were analyzed using phyloseq v1.34.0 [120] and microbiome v1.12.0 package in R v4.0.3 (R Core Team 2020) [121]. We retrieved 23,540 whole-genome CDS profiles from the EzBioCloud database [122] by selecting a single species per genus. In-house R scripts were used to detect the presence or absence of *crtI*, *crtL*, *cruF*, and *crtD* genes from all CDS profiles. These are key genes involved in the carotenogenesis pathway; thus, *crtD* codes for carotenoid 3,4-desaturase; *crtI* codes for lycopene-forming enzyme; *crtL*

encodes lycopene beta cyclase and *cruF* codes for bisanhydrobacterioruberin hydratase [2].

4.2. Sediment Sampling

A total of 410 locations along the Arabian Sea coastal area between Gujarat (Latitude 22°27′34. 7″ N Longitudes; 69°04′01. 8″ E) and Kanyakumari (Latitude; 8°05′11. 8″ N Longitudes; 77°33′16. 0 ″E) (India) (Table S5) were surveyed for the sampling of coastal region sediment. Triplicate sediments were collected in individual sterile plastic bags using a sterile spatula from the upper 5–10 cm during June–November, 2016–2017. The sediment samples were preserved in ice and transported to the lab.

4.3. Enrichment and Isolation of Pigmented Halophiles

The pigmented halophiles were enriched from the sediments in Modified Sehgal and Gibbon's (MSG) media (Table 2) by incubating at 37 °C in a rotary shaking incubator at 100 rpm for 14 days or until pigmentation was observed. The enriched broth was subsequently used as inoculum (10 µL) in modified MSG agar plates [26] augmented with 3 M, 4 M, 5 M, and 6 M NaCl. The plates were further incubated at 37 °C until red, yellow, or orange colonies appeared. The colonies were sub-cultured several times to ascertain the purity, and stereomicroscopic images were obtained using Leica S8 APO with Leica MC 170 HD camera. Pure cultures were stored in MSG slants and glycerol stocks at −80 °C.

Table 2. Media composition of Modified Sehgal and Gibbon's (MSG) used to isolate and enrich halophiles.

Serial Number	Ingredients	g/L or Molar Concentration
1	NaCl	1–6 M
2	Tryptone	5
3	Yeast extract	10
4	Potassium chloride	2
5	Trisodium citrate	3
6	$MgSO_4$	20

4.4. 16S rRNA Gene Identification and Phylogenetic Analysis

Genomic DNA was extracted using HiPurA™ Bacterial Genomic DNA Purification Kit (HiMedia, India). The 16S rRNA gene sequence of archaeal was amplified using Arch344F and Arch915R, while the bacterial 16S rRNA gene was amplified with 8F and 518R (Table 3) [123,124].

The raw sequences were subjected to quality control by trimming off the low-quality reads, and a similarity match was determined using the NCBI BLAST tool (8600 Rockville PikeBethesda MD, 20894 USA). A percentage similar to 97% or higher was considered as the same species [125]. Sequences were deposited in the NCBI gene bank with accession number MT322457-MT322533. Sequence alignment and phylogenetic tree construction were performed using MEGA v7.0 software (Arizona State University and Masatoshi Nei, Pennsylvania State University) with the Neighbor-joining algorithms based on the Tamura 3-parameter method with 1000 bootstrap [126].

Table 3. 16S rRNA gene universal primer used for identification of the archaeal and bacteria extreme halophiles.

Primer name	Universal Primer	Sequence
Arch344F	Archeal Forward	5′-ACGGGGTGCAGGCGCGA-3′
Arch915R	Archeal Reverse	5′-GTGCTCCCCCGCCAATTCCT-3′
8F	Bacterial Forward	5′-AGAGTTTGATCCTGGCTCAG-3′

518R	Bacterial Reverse	5′-ATTACCGCGGCTGCTGG-3′

4.5. Optimization of NaCl Concentration on Growth and Pigment Production

Optimization of growth and pigment production was performed in MSG broth augmented with 3 M and 6 M NaCl at 37 °C for 14 days [26]. Total carotenoid production was determined by culturing in 6 M NaCl MSG broth and absorbance monitored at 490 nm with 12 hour intervals for 14 days using Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Pigment production was further confirmed by streaking the broth on MSG plates with the respective NaCl concentration at 37°C for 14 days.

4.6. Morphological and Biochemical Analysis

All isolates were cultivated at the respective optimum growth condition for biochemical assays. The isolates were stained using the standard Gram Method microscopic observation. The motility by organism was determined with semi-solid agar method by inoculating it in a semi-solid MSG medium and incubating for 14 days. Catalase activity was determined using 3% (*v*/*v*) hydrogen peroxide, and cytochrome oxidases activity was assessed by spotting log phase culture on Whatman No. 1 filter paper followed by adding a few drops of oxidase reagent (Himedia, India) [127,128]. For rapid confirmation of phenotypic characterization, API 20E strips (BioMerieux, Durham, NC, USA) were implemented according to the manufacturer's protocol [129]. The API 20E detection system (BioMerieux, Durham, NC, USA) is an elongated panel with several units containing dehydrated substrates that can be inoculated with a log-phase bacterial suspension. The biochemical tests investigated with the API 20E system are β-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (H2S), urease (URE), tryptophan deaminase (TDA), indole production (IND), Voges–Proskauer (VP), gelatinase (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), saccharose (SAC), melibiose (MEL), amygdalin (AMY), arabinose (ARA), xylose (XYL), esculin (ESC), lactose (LAC), mannose (MNE), salicin (SAL), glycerol (GLY), cellobiose (CEL), melezitose (MLZ), raffinose (RAF), and trehalose (TRE).

4.7. G + C Content Estimation

Genomic DNA was normalized to 5µg/µL in nuclease-free water and diluted 1:100000 with SYBR Green (Origin, Kerala, India). Melting curve genotyping was performed using Roche Light Cycler 480 II (Roche) RT PCR system. The T_m values were calculated from the minimum value of the slope tangent to the melting curve of DNA versus temperature. G + C% was plotted against the calibration curve derived from the whole-genome sequence of *Pseudomonas aeruginosa* PAO1 and *E. coli* K12 [130].

4.8. Antibiotics Resistance Profiling

Antibiotic resistance profiles of all the isolates were evaluated by the serial dilution method. MSG agar plates were supplemented with 25, 50, and 100 µg/mL of ampicillin, gentamicin, chloramphenicol, and tetracycline, individually for each antibiotic.

4.9. Statistical Analysis

The statistically significant difference between the groups was determined by the Kruskal–Wallis test using PAST (PAleontological STatistics) v3.26 software [131]. The difference in G + C content between domains was analyzed with Epps-singleton. A *p*-value of less than 0.05 was considered statistically significant. Principal Component Analysis (PCA) was generated using ClustVis [132].

5. Conclusions

Differences between biotic and abiotic components of the open sea and coastal regions profoundly regulates the microbial community in these biosystems. However, there is a paucity in comparative studies on microbial biodiversity between these ecosystems. In this study, we implemented a whole-genome meta-analysis of the shotgun metagenome and attempted to isolate extreme halophiles from the coastal region. Shotgun metagenomic analysis of the global open sea and seacoast biosystem revealed diverse and abundant halophilic microbes in the seacoast biosystem. The identification of halophilic archaea *Haloarcula* and *Haloquandratum* as signature-predominant genera in the coastal biosystems and also the significant ($p < 0.05$) enrichment of halophilic community ($p < 0.05$) in the coastal regions compared to the open sea provides a strong hit that coastal biosystems could be a reservoir of archaeal halophiles. Whole-genome analysis revealed that *cruF* gene was present in 40.75% of Archaea (n = 377/925) but only in 1.69% of bacterial genomes (n = 382/22,615). However, attempts to isolate halophiles from the coastal sediments of the Arabian coastline through cultivable techniques indicated dominance of the halophilic *Haloferax* genus. This suggested the disparity between culture-dependent and -independent techniques and indicates the need for the development of robust culture media/techniques. Carotenoid pigment production by the pure culture (n = 1351) revealed that all red-pigmented isolates were represented exclusively under the *Haloferax* genus. The halophiles were also multidrug-resistant to tetracycline, gentamicin, ampicillin, and chloramphenicol. Our study shows that bacterioruberin carotenoids are not only exclusive to the archaeal domain, but the bacterial domain could also be a reservoir of bacterioruberin derivatives. Nevertheless, the predominance of archaeal *Haloferax* in coastal biosystems and its extremophilic parameters such as high G + C content (>60%), NaCl tolerance (6 M), and bacterioruberin (red pigments) suggest the crucial roles of haloarchaea in coastal niches and potential implications in pharmaceuticals and biotechnological industry.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/md19080442/s1. Figure S1: Pigmentation in the extreme halophiles grouped according to (a) pigmentation color, (b) biosystems, and (c) geography. (d) Antibiotic resistance profiles of the halotolerant isolates. Figure S2: Distribution of halophilic isolates resistant to (a) 6M and (b) 3M NaCl enforced in MSG media. (c) 3M and 6M NaCl resistance isolated based on pigmentation. Most of the yellow pigmented isolates were resistant only up to 3M NaCl. The G+C content of extreme halotolerant (d) archaea and (e) bacteria. Pigmentation production at various time points in 6M NaCl by halophilic (f) archaeal and (g) bacterial isolates. Figure S3: Phylogenetic tree, constructed using the neighbor-joining method, of halophilic (a) archaea and (b) bacteria isolated from Kerala coastal region, (c) bacterial halophiles from Gujarat, Maharashtra, Goa, Karnataka, and Lakshadweep coastal regions. Table S1: Gram nature, motility, aerobic and pH parameters of the isolated halophiles. Table S2: Biochemical analysis of the microbial isolates: Catalase, Oxidases, urease (URE), gelatinase (GEL), glucose (GLU), arabinose (ARA), amygdalin (AMY), xylose (XYL), esculin (ESC), saccharose (SAC), melibiose (MEL), lactose (LAC), mannose (MNE), β-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H2S production (H2S), tryptophane deaminase (TDA), indole production (IND), Voges–Proskauer (VP), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), salicin (SAL), glycerol (GLY), cellobiose (CEL), melezitose (MLZ), raffinose (RAF), and trehalose (TRE). Table S3: 16S rRNA gene sequence similarity of halotolerant microbial isolates with location and accession number. Table S4: The metagenome data set was retrieved from the MG-RAST server for the comparative analysis of the open sea and coastal microbiome. Sampling with a distance of 100+ km, calculated manually with https://www.freemaptools.com/measure-distance.htm, away from coastline was considered as open sea. Table S5: Sampling locations (n=410) across the Arabian seacoast (India) from seven biosystems. Script: The R script for checking the presence of required gene.

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