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Isolation and characterization of marine actinobacteria associated with the seaweeds, *Codium dwarkense* and *Sargassum cinereum,* collected from the Veraval coastline, Gujarat, India

V. R. Majithiya and S. D. Gohel*
UGC-CAS Department of Biosciences, Saurashtra University, Rajkot-360 005, Gujarat, India.
*Correspondence e-mail: sangeetagohel@gmail.com

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Original Article

Abstract

The marine environment harbour diverse microbial communities. Isolation of actinobacteria from such habitats would facilitate the exploration of new bioactive compounds with novel molecular structures. The diversity of seaweed associated actinobacteria and their metabolites are relatively less explored. Hence, the present study is an attempt to cultivate and characterize the actinobacteria population associated with seaweeds, *Codium dwarkense* and *Sargassum cinereum,* collected from the Veraval coastal area, Gujarat, India. For the cultivation, various growth media, including the International Streptomyces Project (ISP) media, Nutrient agar (NA), Starch agar (SA), Starch casein agar (SCA), Actinomycetes isolation agar (AIA) were prepared using artificial seawater. The seaweed sample *Sargassum cinereum* showed the highest colony forming units (CFU) count on SA, ISP 6, and ISP 1 media plates, while the least CFU was observed on ISP 5, ISP 3, and AIA media plates. The seaweed sample, *Codium dwarkense,* yielded the highest CFU count on ISP 4, ISP 2, and ISP 7 media followed by ISP6, ISP3, and NA media. In comparison, from the seawater sample, the highest CFU count was observed in NA, ISP 6, and SA media. To adjust the high colony-forming units, various dilution strategies were applied. The plates with 0.1 dilutions resulted in crowded growth while very little or no growth was observed with 0.001 dilutions. Various actino morphotypes were observed associated with both the seaweeds as both seaweeds contain different carbon and nitrogen sources. The majority of morphotypes displayed a chalky appearance with varying pigments. Microscopic observation of morphotypes revealed Gram positive nature with filamentous morphology. Thus, the study highlights the isolation strategies and characterization of cultivable actinobacteria associated with seaweed and seawater from the Veraval coastline.

Keywords: *Seaweeds, marine actinobacteria, pigmentation, diversity, distribution*

Introduction

Marine flora is the major benefactor of ocean biodiversity due to their considerable significance in the modern period (Saha *et al.*, 2020). The harvesting and cultivation of the wild seaweed population are largely evolved in Asian countries to render socio-economic benefits to the maritime rural population in the form of aquaculture (Mantri *et al.*, 2020). The global algal diversity reported 72,500 taxa of which 44,000 are already described (Guiry, 2012). However, Kaliaperumal and Chennubhotla (2017) estimated 871 seaweed species from the Indian coastline, which is approximately 7500 km along with a sizable exclusive economic zone (2.5 million km^2) and a vast shelf area (0.13 million km^2). India has reported the highest number of seaweed taxa as compared to all the other nations bordering the Indian Ocean (Mantri *et al.*, 2020). The coastal areas of Gujarat and Tamil Nadu state harbour the highest marine floral diversity (Ganesan *et al.*, 2019). Tamil Nadu has a 1076 km coastline. The survey encountered 282 species of seaweeds in Tamil Nadu (Ganesan *et al.*, 2019). While Gujarat constitutes the longest coastline of about 1600 km in the country which inhabits 62 genera and 198 species of seaweeds (Jha *et al.*, 2009). Although the distribution and diversity of seaweeds have been reported earlier, microorganisms associated with marine seaweeds are very least explored (Mantri *et al.*, 2020).

The marine environment comprises of a widely diverse variety of living organisms including fungi, viruses, firmicutes, yeast and actinobacteria (Singh *et al.*, 2015; Harder, 2009). These microbes also settle on marine flora and fauna, besides occurring in ocean water forming a unique association with their host (Singh and Reddy, 2014). The bacteria associated with seaweed exude beneficial bioactive compounds that regulate the morphogenesis of marine organisms and help them to survive under varied environmental conditions. These bacteria produce signalling molecules, growth-promoting substances, quorum sensing chemicals and other effective molecules which play a crucial role in various developmental stages (reproduction) of seaweed including the promotion of spore settlement, spore germination and correct morphogenesis in adults (Marshall *et al.*, 2006; Matsuo *et al.*, 2003; Tait *et al.*, 2005; Chandini *et al.*, 2008; Singh and Reddy, 2014). Bacteria uptake nutrients produced by seaweed and secret secondary metabolites which protect the host from harmful entities (Lane and Kubanek, 2008).

Within the domain of Bacteria, the phylum Actinobacteria is notable for secondary metabolite production. They produce a wide variety of compounds having diverse biological properties (Subramani and Sipkema, 2019). The search for new bioactive metabolites, enzymes, antimicrobial compounds, and therapeutic agents is essential (Stincone and Brandelli, 2020; Gohel and Singh, 2018). In the current situation, it is more important to find new actinobacteria as it is a crucial source of potent metabolites. The ocean is considered as an enormous reservoir of biochemical diversity. Therefore, the focus on marine habitats has gained more importance in recent years. However, the prokaryotic and eukaryotic diversity of the ocean has not been exploredcompletely (Stincone and Brandelli, 2020).

The present study aimed to isolate beneficial bacterial communities associated with marine seaweeds collected from Veraval, the city of Gujarat, known as the hub of fishing industries in India. The coastal area of this city harbours a wide diversity of seaweed (Patel *et al.*, 2020). Predominately present seaweeds are *Codium dwarkense* and *Sargassum cinereum* in the intertidal zones of the Veraval sea coast and were selected as sample species to study associated marine actinobacteria. The phylum Actinobacteria are well known for the production of bioactive molecules including extracellular enzymes, antibiofilm and antibiotics used for human benefits (Barka *et al.*, 2016). Metabolites obtained from bacteria/actinobacteria are unique, unprecedented, and occasionally complicated with excellent enzymatic and antibacterial potency and usually having low toxicity (Chauhan *et al.*, 2021; Chauhan and Gohel, 2020; Majithiya and Gohel, 2020; Chauhan *et al.*, 2020; Vaghela *et al.*, 2020; Singh *et al.*, 2015). Therefore, the objective of the present study included the isolation of actinobacteria associated with seaweeds by the serial dilution process using different growth media with varying nutrient compositions followed by their morphological and cultural characterization.

Material and methods

Study area and sample collection

In the present study, two seaweeds belonging to different phyla Chlorophyta (*Codium dwarkense*) and Ochrophyta (*Sargassum cinereum*) were collected manually from the inter-tidal zone of the Veraval coastal area (Long 70° 20' 31.2815" E and Lat 20° 55' 1.1594" N) Gujarat, India during January 2019. The seaweed sampleswere placed in a sterile sampling container and immediately transported to the laboratory.

Isolation of seaweed associated bacteria

The procedure for the isolation of bacteria associated with seaweeds was accomplished within 12 hours after sampling. For that, 1.0 g of seaweed samples were washed five times with sterile artificial seawater followed by grinding using a sterile motor and pestle. The serial dilution up to 1:1000 was prepared using ground heat treated seaweed supernatant and seawater (control) followed by spreading of 0.1 ml sample from each dilution on *International Streptomyces Project* (ISP) media 1 to 7, Actinomycetes isolation agar (AIA), Nutrient agar (NA), Starch agar (SA) and Starch casein agar (SCA) prepared using artificial seawater with additional 0-10 % NaCl (w/v). After serial dilution and spreading, the plates were incubated at 28±2 °C for 14 days. The growth was observed every 24 hours. The chalky to leathery colonies were recognized and subcultured on a respective medium plate. All nutrient media were purchased from HiMedia Laboratories Pvt. Ltd., India. All salts were supplied by SRL (Sisco Research Laboratories Pvt. Ltd., India).

Salt tolerance

To check the effect of NaCl on the growth of actinobacterial strain, the isolates were spot inoculated on a starch agar plate containing 0-10 % NaCl (w/v). After the incubation, the growth pattern of the isolates was observed and recorded.

Morphological characteristics

The colony characteristics such as size, shape, colour, elevation, texture, margin and opacity were recorded. Furthermore, the growth cycle of the individual isolate was examined using a light microscope. Briefly, all actinobacterial morphotypes were grown in LB broth and incubated at 28±2 °C at 120 rpm. The Gram staining was performed every 24 h up to 10 days to observe

the growth in terms of filaments formation, fragmentation of filaments and spore formation. The slides were observed in Zeiss microscope under 100X magnification. The pure strains were preserved on a starch agar slant at 4°C temperature and 20% (w/v) glycerol vials at -20°C.

Results and discussion

Two seaweeds (*S. cinereum* and *C. dwarkense*) were collected from the Veraval coastline, Gujarat, India (Fig. 1). Isolation of bacteria was carried out using serial dilution and plating methods. The serial dilutions of samples (up to 10^{-3}) were prepared and spread on various media to obtain a countable number of morphotypes and to avoid overcrowding. A unique pattern of the distribution of bacterial morphotypes distribution was observed in both the seaweeds. The crowded growth pattern was observed with dilution 10^{-1} while very little or no growth was observed in 10^{-3} dilution plate with few exceptions.

Effect of various media on CFU counts

The effect of media composition plays a pivotal role to promote the growth of actinobacteria. Therefore, eleven different growth media with diverse nutrient compositions were used to isolate seaweed associated actinobacteria as suggested by Gohel and Singh (2018). The highest CFU count was observed on SA (4.8022 Log CFU/g), ISP 1(4.131 Log CFU/g), and ISP 4(4.077 LogCFU/g) media plate while the least was observed on ISP 3 (3.000 LogCFU/g), and AIA (3.116 LogCFU/g) media plate from seaweed sample of *Sargassum cinereum* (Fig. 2). The seaweed sample, *Codium dwarkense yielded* the highest CFU count on ISP 4 (4.402 LogCFU/g), ISP 7(4.370 LogCFU/g), and ISP 2 (4.219 LogCFU/g) media while least count was observed on AIA (2.534 LogCFU/g) media (Fig. 2). In comparison, from the seawater sample, the highest CFU count was observed on NA (4.428 LogCFU/g), and ISP 6 (4.334 LogCFU/g), media while the least CFU count was observed on ISP 1 (3.285 LogCFU/g) and ISP 2 (3.345LogCFU/g) (Fig. 2). Actinobacterial growth with different pigmentation on different ISP series mediums was observed. Actinobacterial colonies grew on ISP 1, ISP 4 and ISP 7 had chalky white, black and orange pigmentation. While few actinobacterial morphotypes grown on SA, and AIA showed reverse side pigmentation. The number of actino morphotypes was higher in seawater compared to seaweed sample as chemical composition and variation in metabolite product of seaweed may represent the main parameter driving the dynamic of epiphytic microbial communities (Nylund *et al*., 2009; Lachnit *et al*., 2013). In addition, variation in environmental parameters of seaweed and seawater also seem to be responsible for diverse microbial communities of seaweed halobiont (Marzinelli *et al*., 2018; Morrissey *et al*., 2019).

Fig. 1. Seaweeds collected from Veraval coastline, Gujarat, India; (A) *S. cinereum* and (B) *C. dwarense*

Morphological characterization

The phenotypic features are the foundation for the description of taxa. The majority of actinobacteria are characterized and classified based on their morphological characteristics. The growth pattern and colony morphology of actinobacteria were diverse from other bacterial phyla. In the present study, the majority of actinobacteria form a network of hyphae growing on the surface and under the surface of the agar (Fig. 3A, 3 B; Table 1). Actinobacteria can be differentiated based on various morphological forms including appearance, margin, elevation, texture, and aerial and substrate mycelia pigmentation. Based on the colony morphology, 17 actinobacterial strains (04 strains associated with *S. cinereum*, 03 strains associated with *C. dwarkense* and 10 strains from the seawater sample) were

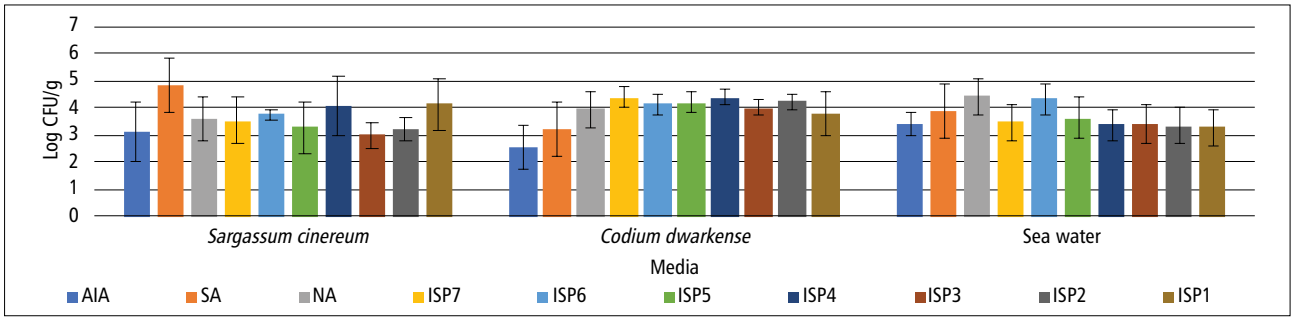

Fig. 2. The effect of various nutrition sources on CFU counts of bacteria associated with seaweeds and ambient water

isolated. Several actinobacteria produced pigmentation of grey, white, orange and black coloured aerial spore mass while some strains also produced brown, red and white substrate mycelium pigmentations (Fig. 3B). Based on colony morphology and microscopic examination, a total of 17 actinobacterial strains were screened and preserved. Microscopic observation of

Table 1. Morphological characteristics of marine actinobacteria.

Colony characteristics		Number of actinobacteria		
		S. cinereum	*C. dwarkense*	Seawater
Appearance	Circular	1	-	2
	Irregular	1	3	5
	Filamentous	2	-	3
Margin	Entire	1	1	5
	Scattered	3	2	5
Elevation	Raised	1	-	2
	Merged	2	1	7
	Flat	1	2	1
Texture	Rough	4	2	8
	Smooth	-	1	2
Aerial mass colour	Orange	1	-	-
	White	2	1	5
	Grey	1	1	4
	Black	-	1	1

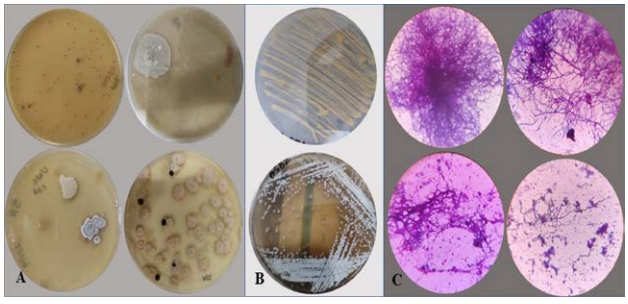

Fig. 3. (A) Colonies of actinobacteria on master plate, (B) Pure actinobacterial strains and (C) microscopic observation of actinobacteria under 100X magnification

actinobacterial strains revealed Gram's positive, long thread-like filamentous growth after 24 h of growth. The filaments displayed fragmentation after 72 h. The microscopic view of actinobacterial growth patterns is depicted in Fig. 3C.

Effect of NaCl concentration

The concentration of NaCl is considered one of the most remarkable factors limiting species distribution and ecological function in aquatic/marine environments (Saha *et al*., 2020). As coastal area changes their salinity also changes. The fluctuations in salinity affect the abundance and growth of microbial life (Gohel and Singh, 2018; Caporaso *et al*., 2011). Therefore, NaCl tolerance of actinobacterial strains was checked at 0 to 10% NaCl (w/v). All actinobacteria grew in 4% NaCl (w/v) while 17.64% isolates among total isolates grew in 10% NaCl (w/v) (Table 2 and Fig. 4). All the isolates associated with *S. cinereum* and *C. dwarkense* were able to tolerate up to 06% NaCl (w/v) while 75% and 25% isolates associated with *S. cinereum* tolerated up to 08 and 10 % NaCl (w/v). In comparison, none of the isolates from *C. dwarkense* grew in 10% NaCl (w/v). Recently, Zhu *et al.* (2020) noted that *S. qinzhouensis* from mangrove soil showed optimum growth at 3% NaCl (w/v). Moreover, Gohel and Singh (2012) reported that *Nocardiopsis alba* from seawater optimally grew and produced protease at 5% NaCl (w/v). They demonstrated that a minute quantity of metallic ions or salts can stimulate the growth of microorganisms with secondary metabolites production.

Growth rate of actinobacteria

All the actinobacterial strains were transferred on a starch agar plate to determine their growth. The visibility of colonies on solid media with reference to incubation time was observed to determine the growth rate of organisms. The growth rate was categorized as fast, moderate, and slow growth (Subramani and Sipkema, 2009). Fast-growing actinobacteria were visible within 2 to 3 days of incubation while moderate needs 3 to 5 days and slow growers require more than 5 days to grow as reported earlier (Bassi and Benson, 2007). Among a total of 17 isolates, 03 isolates showed fast growth, 08 were slow growers

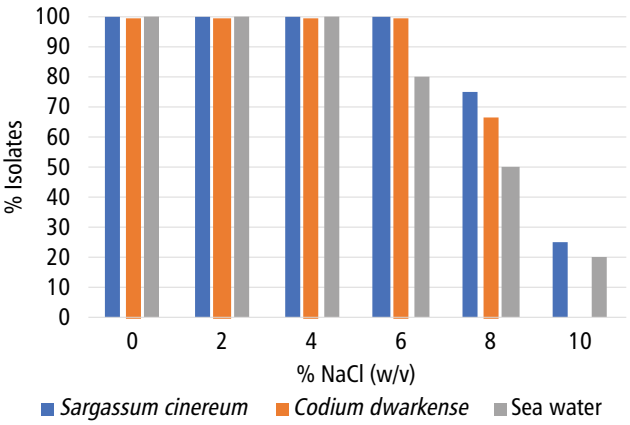

Fig. 4. The effect of % NaCl (w/v) on the growth of actinobacteria

Table 2. The effect of % NaCl (w/v) on growth of actinobacteria.

Sample	% NaCl (w/v)					
	00	02	04	06	08	10
S.cinereum	04	04	04	04	03	01
C. dwarkense	03	03	03	03	02	00
Seawater	10	10	10	08	05	02
Total	17	17	17	15	10	03

and the remaining 06 reveal moderate growth. The results disclosed that all actinobacterial strains were not slow growers.

This study describes the isolation strategy of actinobacterial strains from two different seaweeds (*S. cinereum* and *C. dwarkense*) and marine water. The finding suggested the presence of different actinobacterial strains associated with different species of seaweed that are absent in the surrounding water. NaCl played a crucial role in the isolation of actinobacteria from marine habitats. Despite media with varying nutrient trace elements, NaCl was added to gain osmatic value similar to marine water which would be preferred for isolation of specific genera with maximum diversity of microorganisms. Furthermore, isolated actinobacteria will be screened for their commercially valuable bioactive compounds including enzymes, antibiotics, and pigments.

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