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Isolation strategies, abundance and characteristics of the marine actinomycetes of Kachhighadi, Gujarat, India

Dalip Singh Rathore, Mahejbin Sheikh, Sangeeta Gohel and Satya P. Singh*
Department of Biosciences, Saurashtra University, Rajkot- 360005, Gujarat, India.

*Correspondence e-mail: satyapsingh@yahoo.com

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Original Article

Abstract

A comprehensive and comparative study of actinobacteria of the sea water and sediments of Kachhighadi Coast was carried out during pre-monsoon, monsoon and post-monsoon seasons. During the isolation, physical and chemical parameters were mimicked as those of sea water. Various growth media including International Project on Streptomyces (ISP) media, Actinomycetes Isolation Agar (AIA), Nutrient Agar (NA) and Starch Casein Agar (SCA) media were used for the cultivation ofthe actinomycetes. The samples were subjected to heat exposure and calcium carbonate treatment prior to the isolation of the actinomycetes. Initially, the counts of the Colony Forming Units (CFU) were very high which indicated high microbial load in sea water. The distribution of the actinomorphotypes was adjudged with respect to sample treatment, NaCl concentrations, growth media and dilution strategy. The actinobacterial isolates revealed significant variation in their distribution patterns in response to the applied physical and chemical conditions. The distribution of the actinomycetes in different seasons was substantially diverse. Majority of the morphotypes displayed chalky and amorphous appearance on the growth plates with pigmentation of varying extent. Most of the morphotypes were Gram Positive with filamentous cell morphology under light microscope. The simple filament were transformed into the branched filaments after prolonged incubation. The study represents cultivation and distribution of the marine actinomycetes in response to the cultivation strategies, sample treatment, media selection and seasonal variation.

Keywords: *Actinobacteria, actinomorphotypes, AIA, ISP, marine actinomycetes distribution*

Introduction

Marine ecosystems comprise of largely untapped microbial resources. According to a report by the American Academy of Microbiology entitled "The Microbial World: Foundation of the biosphere, Young (1997), only less than 1% of the bacterial species are so far reported by cultivable approaches and hence isolation and cultivation strategies of the microorganism assumes significance . In recent past, scientists have reported on the marine microbial diversity and their resources (Sharma and Singh, 2016; Datta *et al*., 2015; Adler and Dücker, 2018; York, 2018; Jin *et al*., 2016; Gohel and Singh, 2018; Yang and Song, 2018; Kirthi and Karthik, 2018). Marine water is among many habitats of the halophiles, alkaliphiles and barophilic

microorganisms (Chen and Jiang, 2018, Christiansen *et al.*, 2018; Rao and Durvasula, 2018).

Among the microorganisms, the actinomycetes assume high significance as they are unique and exhibit wide range of adaptation strategies against the ecological stress (Ettoumi *et al.*, 2016; Alvarado *et al.*, 2018; Durvasula and Rao, 2018). Majority of the actinomycetes reported from marine sources are halophilic as well as alkaliphilic in nature as the habitat is alkaline with high salt content (Raval *et al.*, 2018; Ng and Tan, 2018; Salwan and Sharma, 2018).

Actinomycetes are gram- positive, aerobic, spore bearer, filamentous and having high G+C content in their genomic DNA (Lechevalier and Lechevalier, 1967; Srinivasan *et al.*, 1991; Barka *et al.*, 2016). Generally, they appear in white chalky form on the isolation medium and from visible diffusible colored pigments (Chakraborty *et al.*, 2015, Hasnaa *et al.*, 2018; Abraham and Chauhan, 2018). Gujarat State of the Indian Subcontinent accounts for the largest coastline of the country with enormous research possibilities on the microbial diversity and its exploration (Mahapatra *et al.*, 2015; Shinde *et al.*, 2018; Durvasula and Rao, 2018).In the present study, the cultivation, isolation, and distribution of the actinomycetes from the Kachhighadi/ Shivrajpur along the coastline of Gujarat have been described. Cultivation strategies require a thorough knowledge of the habitat and organisms of interest. In the era of non-cultivable approaches, the cultivability of the microorganisms still remains important for the identification and bio-prospecting. Majority of the novel species has been isolated using cultivable approaches as it provides a broad spectrum to adjudge the characteristics apart from molecular techniques. A few reports are available on the cultivation of marine actinomycetes from India including those from the Gujarat State (Gohel and Singh, 2018). However, a significant research has been carried out at the global platform and majority of research on the marine actinomycetes is reported from the South China Sea (Subramani and Aalbersberg, 2013; Subramani and Sipkema, 2019). The present study elaborates the cultural characteristics, cell morphology and distribution of the morphotypes with respect to the techniques employed. The outcome is expected to serve as a baseline for further studies on the diversity and bio-prospecting.

Kachhighadi, near Shivrajpur is part of the Gujarat coast near DevBhumiDwarika (Coordinates: 22.340868 and 68.955960). Consecutivelly, Pre-Monsoon, Monsoon, and Post -Monsoon seasonal samplings were carried out to access the actinobacterial population of the Coastal stretch. CFU load was observed and determined using serial dilution, heat treatment and calcium carbonate treatment (Abdelfattah *et al.*, 2016). Different growth media of varying nutrient compositions were used and the actinomorphotypes were recorded. Diverse cultural, colony characteristics and morphological features were assessed (Bhatt and Singh, 2017; Bhatt *et al.*, 2018, Jenifer *et al.*, 2018; Gobalakrishnan *et al.*, 2018).

Material and methods

Sequentially, Pre-Monsoon, Monsoon and Post-Monsoon samples of the sea water and sediment were collected the low tide in morning hours between March 2015 and August 2016 from the Coastal stretch of Kachhighadi / Shivrajpur (Coordinates: 22.340868 and 68.955960) Gujarat, India. The collected samples were preserved till further processing for physico-chemical analysis and isolation of the actinomycetes (Riley and Chester, 1976; Sharma, 2017). The samples were serially diluted up to 1:1000 as initially lawn growth was obtained with the undiluted samples. The CFUs were recorded on different growth media and the data expressed in terms of CFU/ml of water and CFU/g of the sediment samples. Prior to the isolation using direct plating technique, the samples were treated with heat and Calcium Carbonate and subjected to enrichment. Six different media of ISP series, Starch Casein Agar (SCA), Nutrient Agar and Actinomycetes Isolation Agar (AIA) media were used in this study for the isolation of the actinomycetes (Pascual *et al.*, 2016 ; Sharma, 2017). In heat treatment, the sediment samples were heated at 60°C for 30 minutes to reduce the weedy or vegetative form of the growth, ensuring only spore forming actinomycetes to propagate (Subramani and Aalbersberg, 2013; Claverías *et al.*, 2015; Sharma, 2017). In the enrichment approach, the samples were enriched in actinomycetes isolation broth for seven days to enable the growth of the fastidious actinomycetes. Calcium Carbonate treatment was employed for the sediment samples, ensuring that the treatment will destroy the vegetative forms by desiccation and thus promoting the growth of the actinobacteria in the isolation media (Subramani and Aalbersberg, 2013).

The untreated samples were also processed for the isolation of the actinobacteria without any prior treatment. The isolation was basically carried out using serial dilutions: 1:10 to 1: 1000, varying the NaCl concentrations in the range of 0-15%. Spread plate technique was adapted for the isolation, so that maximum dispersal of the individual cell can take place.

Initially, chalky white appearance of the colonies was the criterion for the selection and identification of the actinomycetes. The selected chalky white colonies with earthy aroma were transferred on the native media from the master plates and pure cultures were obtained after sub-culturing. Colony characteristics were recorded by direct plate observation while cell morphology was monitored under light microscope.

Result and discussion

Overall scenario of the CFU counts

The pH and temperature of the collected samples were determined and found in the range of 7.8-8.4 and 22-26°C respectively. A trend of higher CFUs in the sediment samples as compared to water was observed (Narihiro *et al.*, 2016). One of the prominent causes of this trend may be the high organic contents and presence of other trace nutrients in the sediment compared to sea water (Hedges and Keil, 1995). However, considerably lower CFU counts were observed with the samples treated with $CaCO_3$. Subramani and Aalbersberg (2013) have earlier described that $CaCO_3$ promoted desiccation causing reduction in the vegetative cells.

While slightly reduced CFU counts were observed in heated samples compared to those without any Pre- treatment. Constantly higher CFUs were evident with ISP-6 and ISP-4 media as compared to rest of the other growth media used in the study. CFU counts from water and sediments samples indicate higher density of the microorganisms. Presence of a fair amount of nutrients in water bodies supports higher number of the microorganisms (Alongi, 1988). The observations on the CFU counts directly reflect that sea water and sediments have higher cell density which is an indirect indicator of nutrient richness (Alongi, 1988).

Cultural aspects of the actinomycetes

Determination of the cultural characteristics of the actinomycetes on solid substrate is highly significant step in the assessment of the diversity of the cultivable microbial community.

As mentioned in Fig. 1 and 2(A), microorganisms can be differentiated on the basis of appearance, texture, elevation, margin and the pigmentation of the colonies (Table 1).

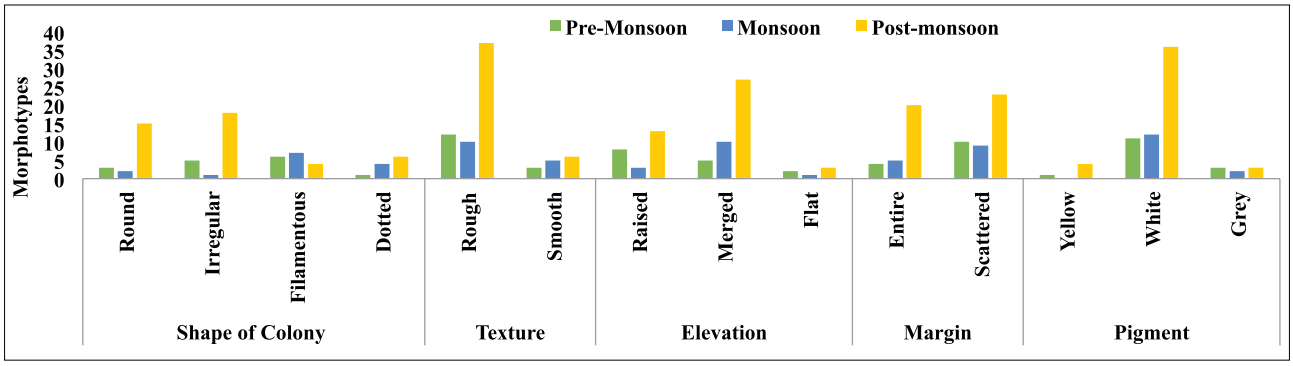

Fig.1. Cultural characteristics of the morphotypes from different seasons

Table 1. Cultural characteristics of the morphotypes from different seasons

Attribute		No. of Morphotypes		
	Sub attribute	Pre-Monsoon	Monsoon	Post-Monsoon
Shape of the Colony	Round	03	02	15
	Irregular	05	01	18
	Filamentous	06	07	04
	Dotted	01	04	06
Texture	Rough	12	10	37
	Smooth	03	05	06
Elevation	Raised	08	03	13
	Merged	05	10	27
	Flat	02	01	03
Margin	Entire	04	05	20
	Scattered	10	09	23
Pigment	Slight Yellow	01	00	04
	White	11	12	36
	Grey	03	02	03

Ambiguity and obsceneness among the isolates can be resolved by analyzing colonies and cultural properties. In all the seasons, majority of the actinomorphic colonies were irregular, filamentous shaped, rough textured, raised and scattered in solid substrate. Pigmentation of the actinomorphotypes were judged as moderate since only few isolates showed pigmentation. As described in Fig. 2 (B), in majority of the cases, slightly yellowish, white and grey colored diffusible pigments were observed. This study describes a wide range of cultural aspects of the actinomorphic microorganisms with visible differentiation.

Long thread like structures, fragmentation of the mycelia and violet colored cell morphology was observed under the microscopic examinations Fig. 2(C) (Chakraborty *et al.*, 2015).

Dilution of the sample and distribution of the morphotypes

Serial dilutions up to 10^{-3} were used to obtain countable number of the actinomorphotypes and to avoid overcrowding (Fig. 3). A typical pattern of the morphotypes distribution was evident in different seasons. Largest number of morphotypes was observed in Pre-monsoon season with 10^{-2} dilution followed 10^{-3} and 10^{-1} dilutions. In case of monsoon samples, the trend was different and maximum morphotypes obtained from 10^{-3} dilution. Simultaneously, 10^{-1} and 10^{-2} appeared with almost equal effectiveness. Post- monsoon CFU's were equally distributed in 10^{-1} and 10^{-2} dilutions, whereas in 10^{-3} dilution, slightly less number of CFU's were observed. The trends, therefore, suggest that 10^{-2} dilution was largely suitable for the isolation of the actinomycetes from the studied habitat.

Fig. 2 (A). Selected photos of the master plates (B). Pure cultures of actinomycetes transferred from master plates (C). Cell Morphology of actinomycetes under light microscopy

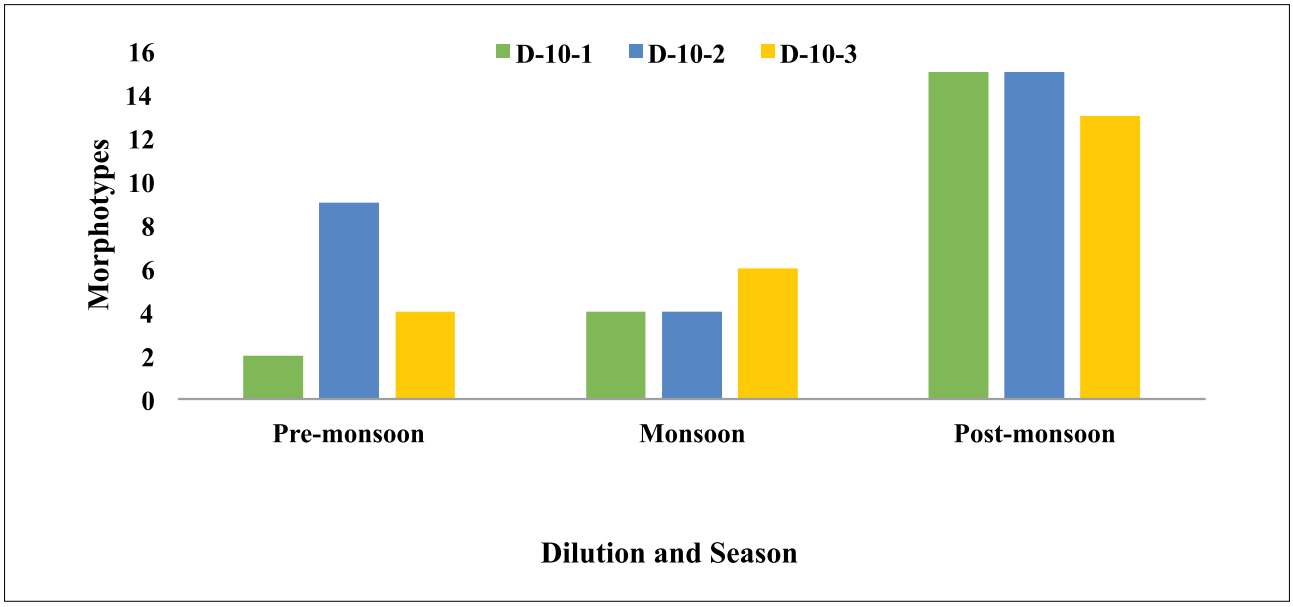

Fig. 3. Dilution wise distribution of the morphotypes of various seasons

Isolation strategies and distribution of the actinomorphotypes

As depicted in Fig. 4, the distribution patterns of the actinomorphotypes from different isolation approaches were random. Heat treated and untreated samples were most efficient in Post - monsoon as majority of the isolates were obtained by these two methods. One of the prominent reasons for this trend appears to be the elimination of the vegetative forms due to heat treatment facilitating the growth of the spore forming actinomycetes. In untreated sample, individual actinomorphotypes might have acquired every possible chance to grow in the absence of any physical or chemical treatment.

Enrichment and $CaCO_3$ treatment methods led to nearly equal distributions of the actinomorphotypes. While in heat and $CaCO_3$ treated samples, vegetative forms will be eliminated allowing the growth of only spore forming actinomycetes.

Distribution of actinomorphotypes on different media

Nine different growth media with diverse nutrient compositions were employed for the cultivation of the actinomorphotypes of different seasons (Fig. 5). In pre-monsoon season, Starch Casein Agar (SCA), ISP-4 and ISP-7 media resulted in highest morphotypes, while for the monsoon season, ISP-5 and ISP-7 media yielded highest number of the morphotypes. ISP-2, ISP-4, ISP-5, ISP-7 and N. Agar showed almost equal number of the morphotypes in Post-Monsoon season. This particular pattern of media preference by actinomorphotypes reveals nutrient priority and requirement of certain growth factor/s for the growth. Applications of wide range of media enhance

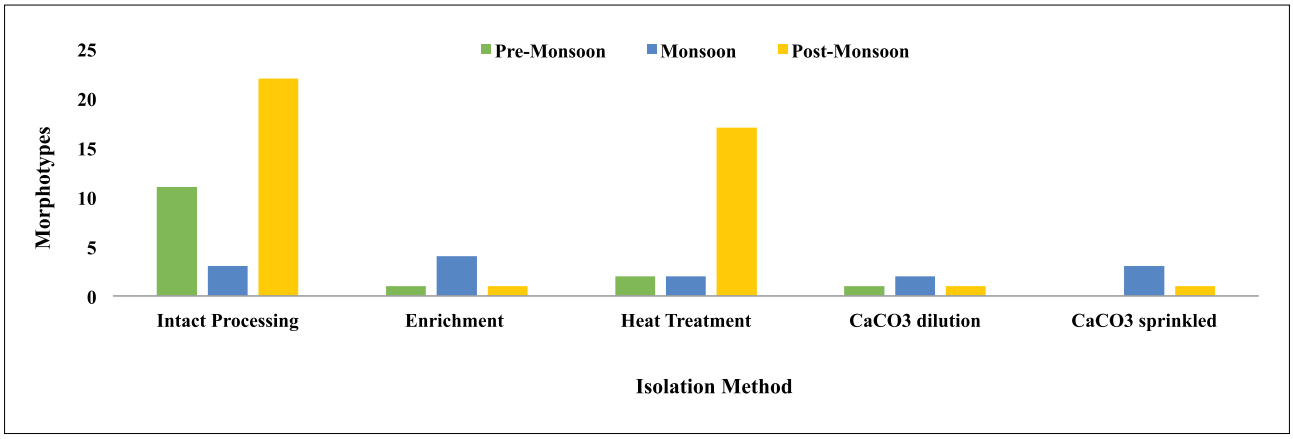

Fig.4. Method wise distribution of the morphotypes of various seasons

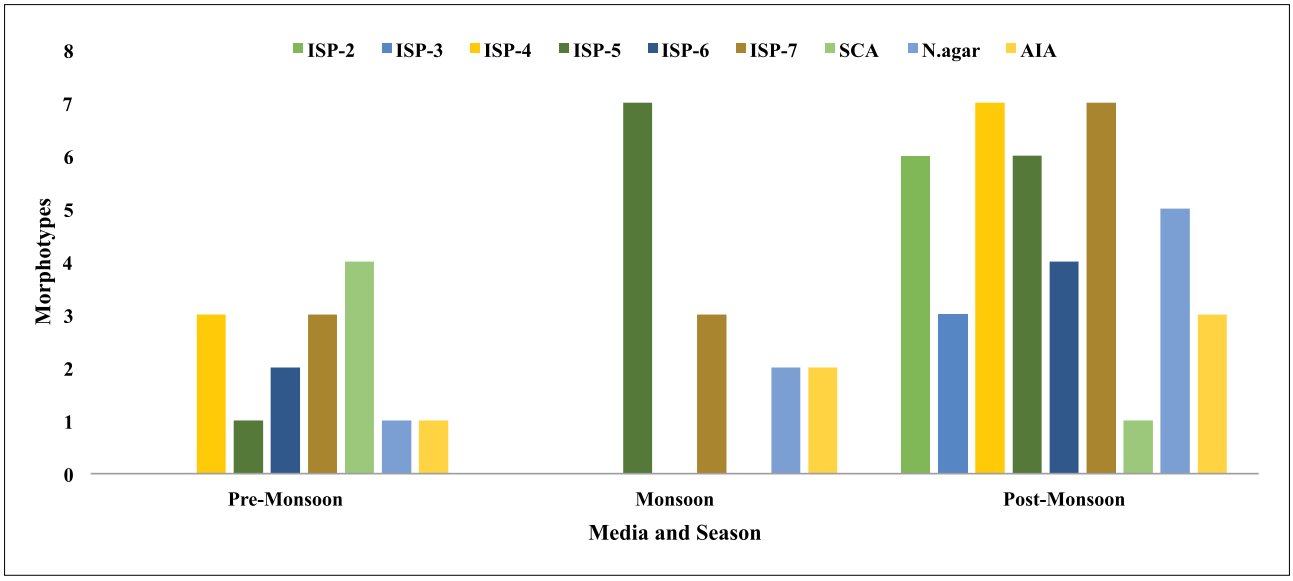

Fig. 5. Media wise distribution of the morphotypes of different seasons

the prospect of the cultivability of the microorganisms from a given habitat (Bhatt and Singh, 2017).

Distribution of the actinimorphotypes with respect to NaCl concentrations

In the present isolation strategy, different NaCl concentrations in the range of 0-15% in the isolation medium were used. Majority of the actinomorphotypes of the post-monsoon season were observed with 0 and 5% NaCl (Fig. 6). Similarly, with respect to monsoon and pre-monsoon season, majority of the actinobacterial isolates were able to grow in the absence and 5% of NaCl. A few isolates were able to grow on 10% and 15% of the salt concentrations. The study establishes that these actinomorphotypes are halotolerant and moderate halophilic in nature, a compatible feature with their habitat.

Distribution of actinomorphotypes in water and sediment samples

The sediments and water samples were collected and processed for the isolation of the actinomycetes (Fig.7). Maximum number of the actinomorphotypes was obtained from water samples, while average numbers obtained from the sediments. Looking into the individual seasons, pre-monsoon sediments yielded slightly higher numbers, while least actinomorphotypes were accounted in the rest of the seasons. Unlike some other studies previously reported, the trends in this report displayed highest actinomorphotypes count in water sample (Ramesh and Mathivanan, 2009; Gobalakrishnan *et al.*, 2018). This pattern can be supported by hydrodynamics and biogeochemistry at the interface of the water and sediment of sea. At the interface of water and sediment in sea, small sediment particles

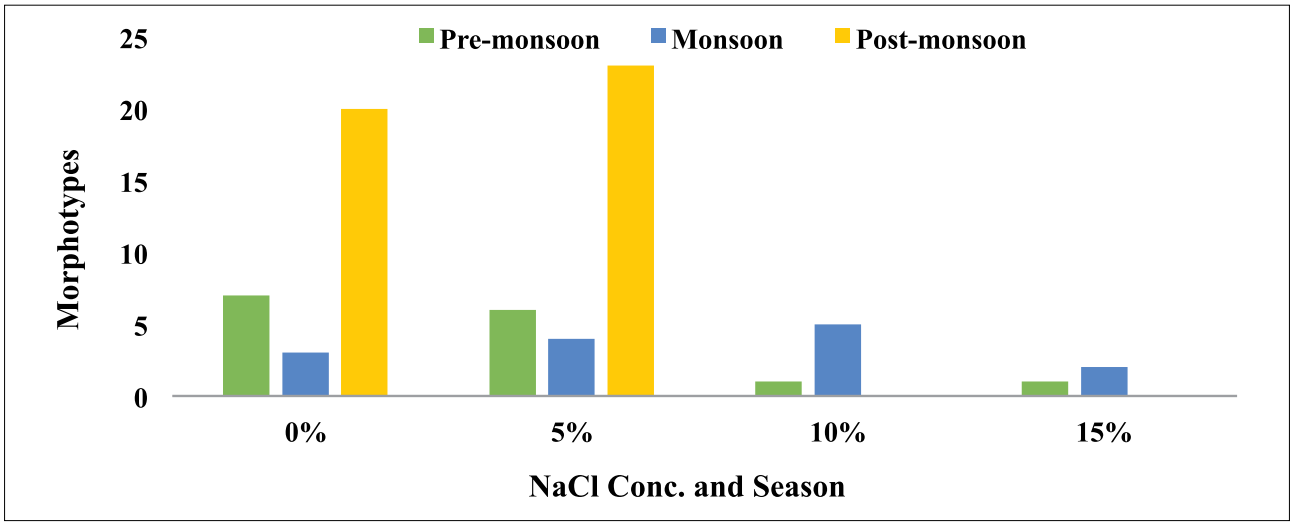

Fig. 6. Effect of salt on the distribution of the morphotypes of different seasons

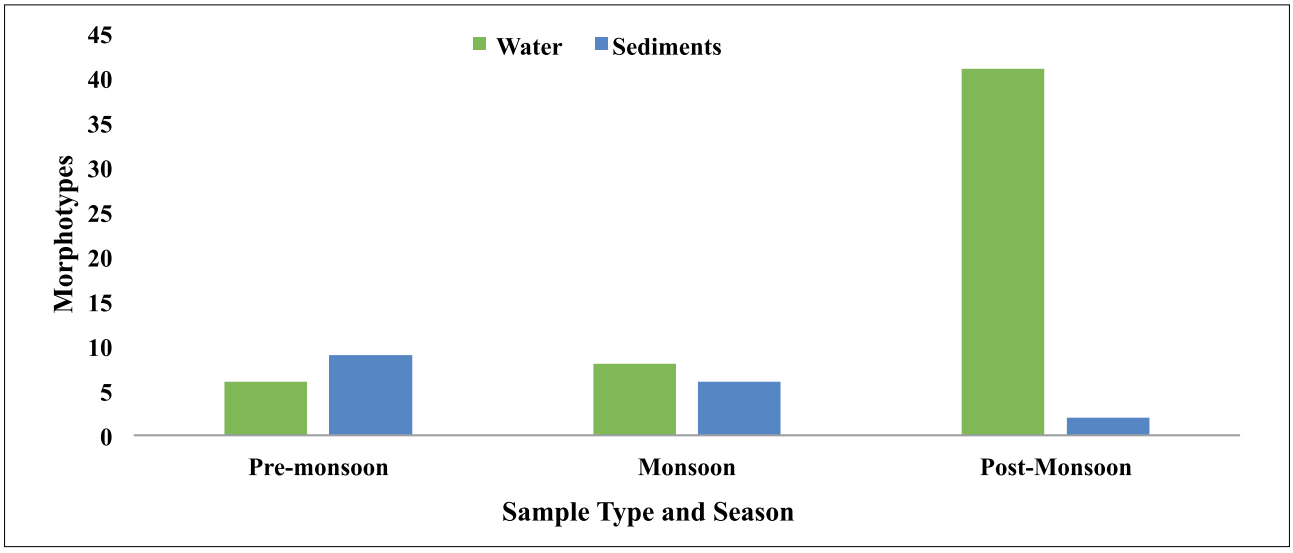

Fig. 7. Variations in the distribution of the morphotypes of different seasons with respect to sediment and water source

get transported and suspended into the water, harbouring undistinguishable water and sediment microorganisms (Boetius *et al.*, 2001). Thus, the above trends of the distribution of the microorganisms can be explained on account of the fact that the samples were collected from the intertidal zone (Boon *et al.*, 1998; Boetius *et al.*, 2001; Feng *et al.*, 2009).

Analysis of the samples from Kachhighadi study site suggests a wide preference of various nutritional media, and physical and chemical treatment techniques. Besides, other treatment techniques, such as, phenol treatment, differential centrifugation of the samples, incorporation of the antimicrobial agents, chemo attractants, surface active compounds and co-culture methods may also be employed depending on the nature of the habitat and physicochemical status of the sample (Subramani and Aalbersberg, 2013; Rusch *et al.* 2001; Subramani and Sipkema, 2019). Usually, the selective methods are preferred for the isolation of the specific genera, while, in order to capture the maximum diversity of the microorganisms, a rather non-specific media and cultural conditions are adapted. The VNBC (Viable but not culturable) actinomycetes can also be retrieved as they are difficult to cultivate (Subramani and Sipkema, 2019). Actinomorphotypes are identified based on their cultural and morphological characteristics. However, for their precise identification various molecular techniques are required. The bio-prospecting of these actinomycetes can further be assessed on the basis of the secretion of extracellular enzymes, antimicrobial agents and biodegradation capabilities. Ultimately, these capabilities can serve as the initiator for the projects, such as EU SeaBioTech: From Seabed to Testbed: Harvesting the potential of marine biodiversity (Edrada-Ebel *et al.* 2018; Poremba *et al.* 1999). Further the frame work like OSMAC (One strain Many Compounds) can be triggered to produce many bio-products from a single strain using a combination of molecular and cultivable approaches (Romano *et al.* 2018). The present study can serve as a reference point for further work.

This study to our best knowledge is the first on the cultivation and morphological variability of the actinobacteria from Kachhighadi / Shivrajpur Coast, Near Dwarika, Gujarat. The study reflected that the abundance of the actinomycetes in sea water of Kachhighadi and that they possess a wide range of nutritional capabilities as reflected by their ability to grow on variety of growth media. The morphotypes were halotolerant and halophilic in nature and reflected diverse but typical morphological and cultural characteristics. Different approaches adapted for the isolation of the actinobacteria suggested diverse patterns of the colony appearance on the solid substrate agar media. The Diverse cultural characteristics suggest towards the strategies for the environmental stress tolerance, invasive ability and survival in the presence of other rapidly growing microorganisms. Dilutions of the samples in the range of 10^{-2} and 10^{-3} were most suitable for the isolation of the actinomorphotypes. Huge differences in the actinobacterial density were evident between the water and sediment samples and their treatments. The study represents a concise and comprehensive account on the occurrence and cultivation of themarine actinomycetes from the sea water and sediments of a pristine coastal habitat in Gujarat.

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