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Cultivation and characteristics of the Marine Actinobacteria from the Sea water of Alang, Bhavnagar

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Marine actinomycetes were isolated from the Alang Sea Water, Gujarat using various isolation strategies.

The methods for the sample treatment and isolation included heat and $CaCO_3$ treatment, enrichment of the samples and use of different growth media. Since the initial microbial load was quite high, the serial dilutions were used for the isolation of the actinomycetes. Morphotypes of different colony morphology and filamentous structure were detected and selected. A distinct variation in the occurrence of the morphotypes on various media using different techniques was evident. Among the three seasons; winter samples had highest number of the morphotypes. The heat treatment of the samples significantly affected the isolation and abundance of the morphotypes. Further, highest numbers of the actinomycetes were obtained on ISP-3 (International Streptomyces Project; Oatmeal agar), ISP-6 (Peptone-yeast extract iron agar) and ISP-2 (Yeast extract-malt extract agar) media, suggesting its suitability for the growth of the marine actinomycetes. Over all, the study highlights the occurrence, cultivability and diversity of the actinomycetes of the Alang Sea water.

[Keywords: Alang; Enrichment; ISP media; Marine Actinomycetes; Microbial diversity]

Introduction

Indian subcontinent has vast geographical diversity. While most of the geographical regions are extensively explored for the biological diversity, the coastal and marine habitats need to be focused on this account[1-3]. Gujarat shares longest coastline of the country and it's a hidden treasure of the microbial diversity as well as bioactive compounds[4,5]. While marine actinomycetes are studied for their antimicrobial activities, only limited attention has been focused on their diversity and enzymatic attributes. Ministry of Earth Science, Government of India has focused on studies related to "Drugs from the Sea" in the search of novel drugs and bioactive compounds from the Indian Coastline. During the recent years, enzymes from marine actinomycetes have been studied and reported in literature[6,7]. Certain novel properties and stability of the enzymes have been recently reported from the marine actinomycetes[8-10].

Alang is one of the largest ship breaking and recycling yard in globe and it's also considered as the polluted coastal stretch[11]. Alang is well known for anthropogenic activities. The objective of this study was to examine the occurrence and distribution of the actinomycetes in sea water of this location. Further, this particular site is largely untapped with respect to the cultivable marine actinomycetes. Successive seasonal sampling from Alang was carried out to cultivate and access the diversity of the actinomycetes. Samples were processed using direct plating, serial dilution, heat treatment and calcium carbonate treatment methods. Ten different growth media with different compositions of carbon and nitrogen sources were used for the isolation and cultivation of the actinomycetes. Varying number of CFU counts and morphotypes were observed with respect to different techniques and media.

Material and Methods

Collection of samples

Water and sediment samples were collected during winter, summer and monsoon seasons from Alang followed by the measurement of the pH and temperature. Various sample treatment strategies such as heat treatment, enrichment with variable salt concentrations and treatment of the sediment samples with calcium carbonate were carried out prior to the isolation of the actinomyctes using the dilution and plate technique.

Dilution and plating method

Serial dilution of the water samples were aseptically done by adding 1 ml of the seawater into 9 ml of sterile distilled water (dilution 1:10). Further dilutions up to 1:1000 were then prepared. Sediment samples were also subjected to the preparation of the dilutions. From each dilution, 0.1 ml of the water and sediment sample was spread over the plates of various media: Tryptone-yeast extract broth (ISP-1), Yeast extract-malt extract agar (ISP-2), Oatmeal agar (ISP-3), Inorganic salts-starch agar (ISP-4), Glycerol-asparagine agar (ISP-5), (Peptone-yeast extract iron agar (ISP-6), Tyrosine agar (ISP-7), Nutrient agar, Starch casein agar (SCA), Actinomycetes isolation agar (AIA)[12]. The pH of all media was adjusted to 9 by adding separately autoclaved Na_2CO_3 and salt concentrations in the range 0-15 %.

Heat and Calcium carbonate Treatment

Water and sediment samples without any prior dilution were subjected to heat treatment in a water bath at 60 ºC for 1 h to reduce the number of unicellular bacteria. After treatment, the samples were diluted and plated over different isolation media as described above and incubation was carried out at 28 ºC for 2-8 weeks[13,14].

It is believed that the treatment of samples with $CaCO_3$ promotes the formation of aerial mycelia in several actinomycetes enhancing the appearance of the actinomycetes on growth media[15].

Sediment samples were mixed with $CaCO_3$ in 1:1 ratio and incubated at 28 ºC for 10 days. After the treatment with calcium carbonate, the samples were sprinkled over different media mentioned in the dilution plating method. The treated samples also diluted up to 1:1000 and used for the isolation of actinomycetes[14].

Enrichment Method

Water and sediment samples were enriched in Actinomycetes Broth with different concentrations of NaCl in the range of 0-15 % and incubated at 28 ºC for 7 days. The enriched samples were then subjected to dilution and plating for the isolation of the actinomycetes.

Typical chalky white colony with rough surface was picked and subculture for getting pure isolates and maintained on their respective media slant at 4 ^oC and 20 % (w/v) glycerol suspensions at -20 ^oC.

Results and discussion

Colony forming units obtained from different seasonal isolation regimes were recorded. In all the seasons, among various techniques employed, lawn growth was observed in lower dilutions. With few exceptions, the CFU counts of the sediment samples were higher compared to seawater (Table 1), which might be due to the higher nutrient contents in sediments[16]. Variable differences were observed between the samples with various treatments and untreated samples at higher dilutions. Heat treatment reduced vegetative or heat sensitive entities, therefore, the CFU counts were less as compared to those without any prior treatment. Calcium carbonate acts as desiccating agent in sediment sample and hence it led to the reduced CFU load compared to untreated sediment samples. The trend, however, appears to be regulated by seasonal variation as enhanced CFU was recorded during winter.

Actinomycetes were selected from the master plates. Majority of the isolates were taken as chalky white colonies for the actinomycetes selection (Fig. 1). These selected colonies were further purified and given serial transfer for maintenance. Some of the isolates showing pinkish pigmentation are shown in Figure 2. Further, microscopic examinations were carried out to determine the morphology and arrangements of the organisms.

The light microscopic examinations of the isolates revealed long filamentous structure of the cells (Fig. 3). The growth of the actinomycetes was monitored at different time interval of the growth. Most of the isolates exhibited filamentous growth

Table 1 — Overall Seasonal CFU counts of Water and Sediment samples

Seasons	Dilution	Water		Sediment		
		Untreated	Heated	Untreated	Heated	$CaCO_3$ Treated
Winter	1:100	2.3×10^5	2.0×10^5	1.4×10^5	1.3×10^5	2.2×10^5
	1:1000	9.5×10^5	3.5×10^5	1.6×10^6	6.9×10^5	1.9×10^6
Summer	1:100	8.9×10^5	8.4×10^5	3.3×10^6	2.9×10^6	2.28×10^6
	1:1000	8.7×10^6	7.7×10^6	1.76×10^7	2.11×10^6	2.2×10^6
Monsoon	1:100	2.8×10^4	2.5×10^4	1.5×10^5	1.1×10^5	9.0×10^4
	1:1000	3.1×10^5	1.9×10^5	2.0×10^6	1.8×10^5	8.0×10^5

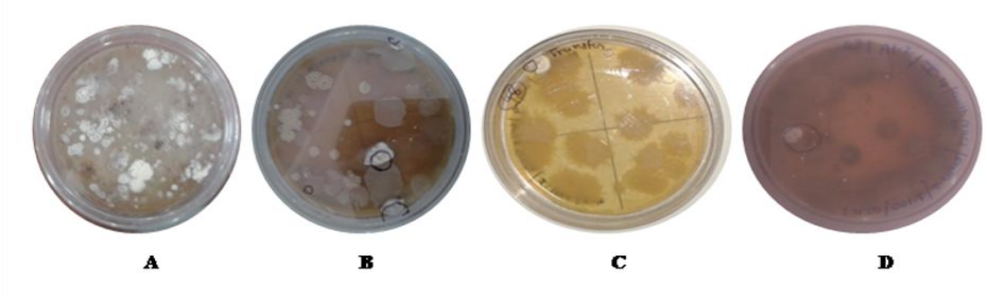

Fig. 1 — Actinomycetes colonies on ISP-6 (A and B), ISP-2(C) and ISP-4(D) medium.

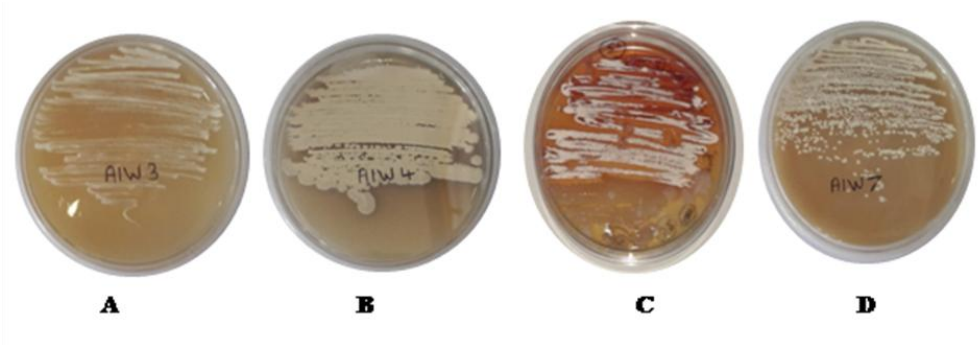

Fig. 2 — Pure isolates of the Actinobacteria. A) Isolates with milky white colony B) Chalky white colony with dark brown substrate mycelium (C) Isolates with pinkish pigment (D) Pin pointed chalky white colony.

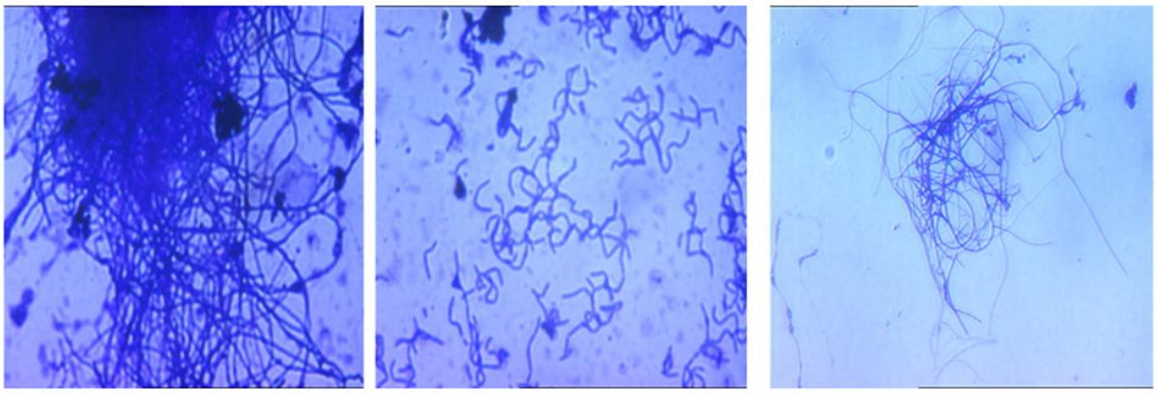

Fig. 3 — Microscopic examinations of some selected actinomycetes

after 48 h of the incubation, while in few isolates, fragmentation of the filaments was observed after 72 h of the incubation.

Varying morphotypes were obtained using different techniques. It is reported that the pretreatment of samples with moist heat can reduce undesirable bacteria and enhance the isolation of the actinobacteria[17]. In the present study as well, heat treatment of the samples appears to eliminate or significantly reduce the bacterial load in terms of CFU count as reflected in Table 1. Heat treatment of the water samples was quite effective as compared to other treatment in winter season. While in case of sediment samples, heat treatment did not have any significant effect (Figs 4 and 5). Highest number of morphotypes was obtained in winter season employing heat treatment method. While the enrichment approach was effective only with the winter season isolation, the untreated samples yielded maximum number of isolates in winter season. Therefore, it can be conclude that the winter season samples yielded highest number of morphotypes

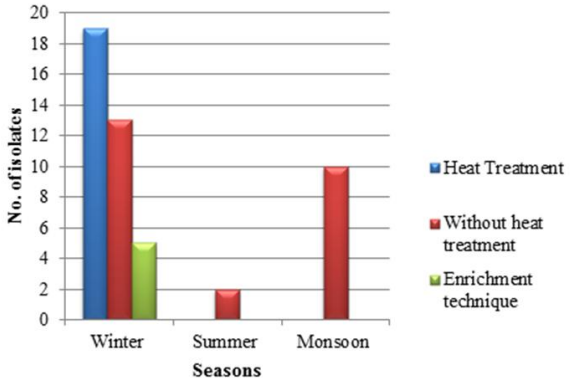

Fig. 4 — Distribution of the sea water isolates with respect to season and sample treatment

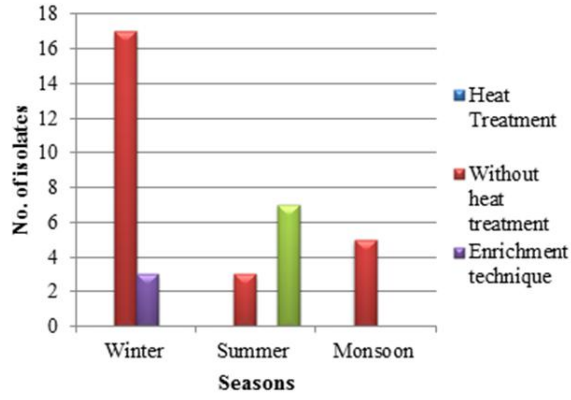

Fig. 5 — Distribution of the sediment isolates with respect to season and sample treatment

followed by those obtained from the monsoon and summer samples.

Majority of the isolates were obtained with the untreated sediment samples of the winter season followed by monsoon and summer seasons. A combination of calcium carbonate treatment and rehydration by centrifugation techniques, rare actinobacteria from the medicinal plants were obtained by Qin et al.[18]. In case of seasonal sediment samples, calcium carbonate treatment was most effective with the summer season samples, while the enrichment method was only effective with the winter season (Fig. 5).

On a larger note, if we compare Figures 4 and 5, it can be concluded that in both water and sediment samples, the winter season samples displayed highest number of the morphotypes compared to other seasons.

Requirement of sodium chloride for the growth of the obligate marine actinobacteria has been suggested[19]. In

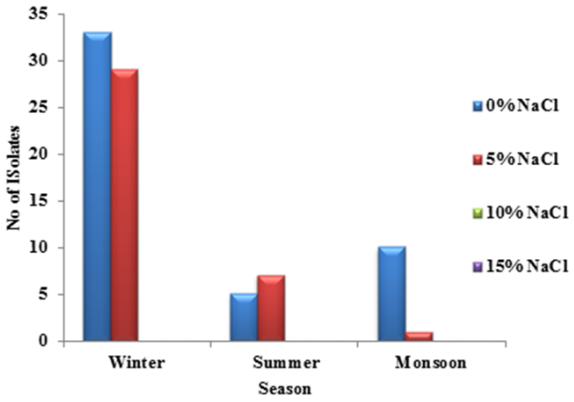

Fig. 6 — Seasonal distribution of the Alang isolates with respect to their salt preferences

our study, salt preferences of the obtained morphotypes and a seasonal comparison have been portrayed in Figure 6. Among the seasons, maximum number of morphotypes was obtained on the media lacking additional NaCl and with 5 % NaCl concentration at pH 9. None of the isolates were obtained from 10 and 15 % salt concentrations in media used.

Occurrence of morphotypes on different media of varying nutrient compositions reflects nutrient preferences of the selected organisms. Actinomycetes with different pigmentation and filamentous growth on different ISP series medium have been described[20]. For the isolation strategy, 10 different media of varying compositions were used to obtain diverse number of morphotypes. In case of winter season samples, the isolates were majorly obtained from ISP-2 medium. While with respect to the monsoon and summer seasons, the ISP-6 and ISP-3 , respectively, emerged as most favored (Fig. 7).

Figure 8 displays the seasonal comparison of actinomorphotypes showing significant variation. Samples of the winter season yielded maximum number of the isolates, whereas summer and monsoon seasons shared almost equal proportion. This variation in the actinobacterial population is likely to be due to the seasonal fluctuations of the nutritional status and physicochemical conditions prevailing in the sea water.

This study depicts a wide occurrence and distribution of the actinomycetes in the sea water of Alang Coast line. The findings of the study suggest seasonal effect on the occurrence of the actinomycetes. Further, the research can be extended towards the biochemical and molecular characterization of the actinobacterial morphotypes, besides exploring their biotechnological potential.

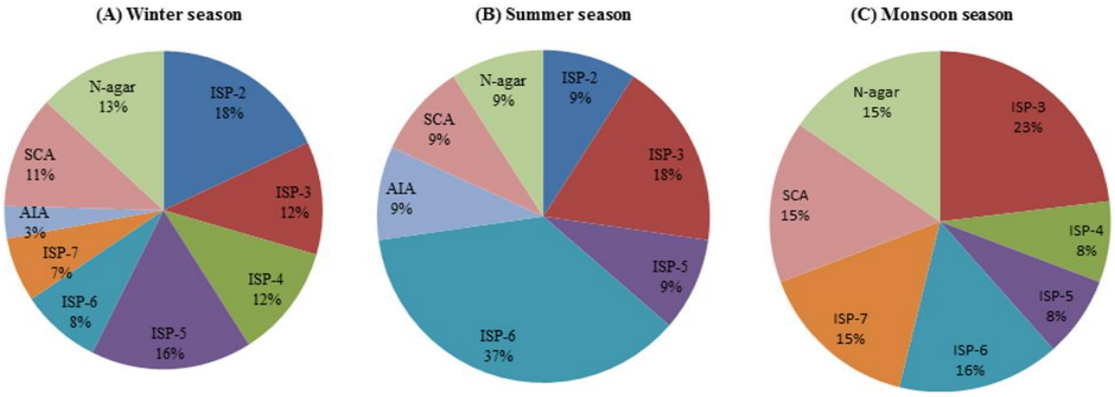

Fig. 7 — Seasonal distribution of the isolates in different media

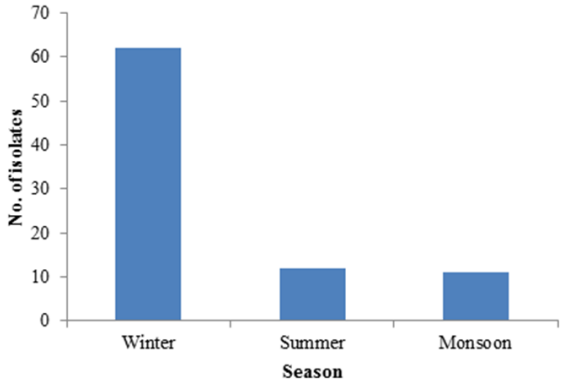

Fig. 8 — Seasonal comparison of the Alang isolates

Conclusion

This study aims to assess the seasonal effect on the occurrence and cultivation of the actinomycetes of sea water. Various isolation strategies, media and salt concentrations were assessed for the isolation of the actinomycetes. Actinomycetes were observed within 2-8 weeks of the incubation as they grow slowly. Highest numbers of isolates were obtained in winter season. Most of the actinomycetes appeared as chalky white colonies on the growth plates, displaying different patterns of pigmentation and filamentous growth. The numbers of actinomycetes varied with respect to the seasons and cultivation techniques. Over all, ISP-3, ISP-2 and ISP-6 media were most favored. The methods and media used in this study were quite effective for the selective cultivation of the slow growing actinobacteria.

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