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**EFFECT OF SITE AND CULTIVATION MEDIA ON ACTINOMYCETES ISOLATED
FROM MANGROVES (SURAT AND NAVSARI) GUJARAT**

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ABSTRACT

Mangrove environments are known to be unique as they are exposed to both terrestrial and marine environments. They were also reported to harbor novel microorganisms and are still one of the least explored regions for biodiversity. Of the various groups of microbiotas that thrive in such environment, actinomycetes are one of the most prolific that are known for their therapeutic potential. Mangroves of Surat and Navsari districts of Gujarat of Western India were least studied in comparison with other mangrove regions of India. In the present study, *Avicennia mariana* rhizosphere samples of various dilutions from eight different locations of Surat and Navsari were analyzed for actinomycetes populations, in three different actinomycetes media (Actinomycetes isolation agar (AA), Starch casein agar (SCA) and Inorganic salt starch agar (ISSA)). Of all the dilutions tested 10⁻⁶ dilutions gave the highest number of morphologically different colonies. There was statistically significant effect of sample site ($p=0.000$) in comparison with culture media (0.058) on the cultivable number of actinomycetes. The beta diversity was also shown to be affected by the media and sample site.

Keywords: Mangroves, Actinomycetes, Culture media and Sample site

INTRODUCTION

The oceans cover more than 70% of the earth's surface. These marine areas are the largest habitat for the living microorganisms including actinomycetes [1]. Mangroves are the tidal forests and high productive ecosystems existing in the intertidal zone of sheltered shores, which contain unique plant communities and are located in tropical and subtropical region of the world [2]. These ecosystems are mainly located in between the terrestrial and marine environment and contain a rich and diverse group of microorganisms [2]. Mangrove ecosystems are known to occupy 15.2 million hectares [3] across the world coastal areas and in Gujarat it covers 1058 sq/km area [4, 5]. Mangroves have high salinity, high temperature, low oxygen, extreme tides, muddy and anaerobic conditions and microbes have been reported to thrive in such conditions [6, 7]. Mangrove marine ecosystems are unique sources of novel microbes with rich potential to produce important active secondary bioactive metabolites. The environment of the mangrove ecosystem is saline and rich in organic matter, nitrogen, potassium, sulfur, phosphorus which can be utilized by microorganisms. Actinomycetes are widely presented throughout the mangrove

environment [8]. They developed vast metabolic and physiological ability to survive in extreme conditions that allows them to produce different kind of metabolites [9] and have the potential of becoming the novel source for actinomycetes species, like *Polymorphos porarubra* spp. nov [10], *Isoptericolla chiayiensis* [11], *Micro-monospora haikuoensis* spp.nov. [12] and *Actinoallomurus acanthiterrae* [13]. In microbial world actinomycetes are characterized by complex life cycle, show filamentous gram positive nature and belong to phylum *Actinobacteria*, that represent taxonomic units with high G+C content [14]. They are the most economically valuable prokaryotes which are responsible for the production of secondary metabolites, antitumor, immunosuppressive agents, enzymes, biofertilizers and especially antibiotics [15]. Till date, more than 10,000 antibiotics have been isolated from actinomycetes [14]. Actinomycetes are able to produce many plant growth promoting compounds and plant protection compounds such as IAA, siderophore, HCN, ACC deaminase, etc. [16, 17]. These group of bacteria have the ability to form spores and can resistun favorable conditions and maintain their population in soil

environment [18]. Actinomycetes also synthesize several enzymes that are able to disturb fungal cell wall this makes actinomycetes an efficient bio control agent [19]. The number and different types of actinomycetes present in different soils would be significantly determined by geographical location such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and moisture content [20]. Located in the country Gujarat have the second largest mangrove cover (1103 sq/km) of the country (4628 sq/km) [4]. The Mangrove cover of the state is distributed over four regions of Kutch, Gulf of Kutch and Saurashtra and South Gujarat (including Dumas, Dandi, Ubhrat areas). However, the mangrove cover distributed unevenly over these four regions and Kutch has the highest mangrove cover(71.5%) of the state [4]. Further, Gulf of Kutch, Saurashtra and South Gujarat (including areas of Dumas, Dandi and Umbhrat) have 15.6%, 0.3% and 12.6% of the total mangrove cover of the state respectively [21]. Studies on actinomycetes from mangroves of Gujarat have largely focused on the Gulf of Kutch and diversity for actinomycetes area in south Gujarat is lacking. In the present study, an attempt was

made to enumerate the actinomycetes populations from the mangroves of Hazira and Navsari followed by the effect of their numbers at different sites using different cultivation media.

MATERIALS AND METHODS

Sampling locations for the collection of soil sample

Soil samples were collected from the eight different sampling sites of South Gujarat region (**Figure 1**). At each location three sediment samples were collected at 0-30 cm depth. Collection of soil sample from Dumas, Junagam, Vansva gam, Rajgiri, Tunda which covers the Hazira Mangrove region of Surat district. Dandi, Borsi, Machwada (represented as machi gam) are part of Navsari district mangrove region. To avoid the recurrence of same actinomycetes, eight sampling sites were set in each sampling site within area of 10 cm for each sample site and the soil samples were collected at different depths and mixed to generate a composite soil sample for future analysis. Soil samples were collected in a dry, clean, and sterilized polythene bags and preserved at 4°C. Physiochemical characteristics of the soil samples were measured at Chalthan Sugar Factory, Kadodra, Gujarat (**Table 1**).

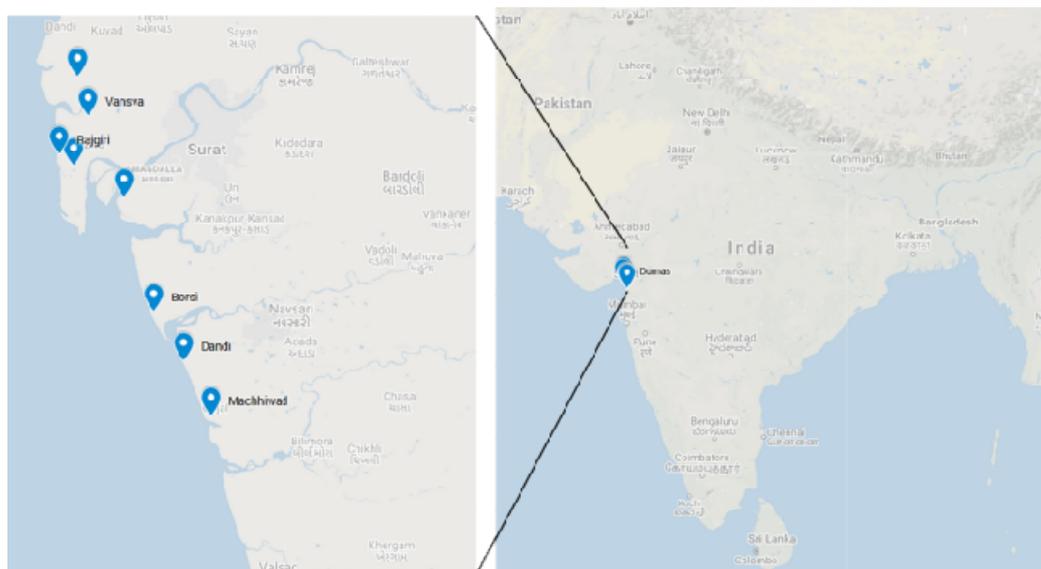


Figure 1: Locations of the sample sites in the South Gujarat region of India

Isolation

Isolation of Actinomycetes was performed by using Actinomycetes Isolation Agar (AA), Starch Casein Agar (SCA) and Inorganic Salt Starch Agar (ISSA) (Himedia, Mumbai) amended with antibiotics Amphotericin B (25 mg/ml) (Himedia). A total of 24 sediment samples were air dried at room temperature for 1 week and the sediments were then ground and sieved to exclude extra-large particle and other organic matter particles before pre-treatment. Further the soil samples were sun dried for one week. Then one gram of soil sample was dissolved in 100ml of distilled water. Serial dilutions (10^{-2} up to 10^{-9}) were spread plated onto the respective media (Actinomycetes Isolation Agar, Starch Casein Agar and Inorganic Salt Starch Agar) by serial dilution plate

technique and incubated at 30°C for 2-3 weeks. After incubation isolates were selected based on their colony morphology with a typical leathery appearance and trans earthy smell and gram staining to see their filamentous hyphae. The isolates were picked up and purified on SCA and stored at 4°C for further use.

Morphological characterization of isolates

Identification of actinomycetes was carried out using cover slip method. The morphological characteristics of the isolates were observed under the microscope (colony morphology like aerial mycelium, substrate mycelium, diffusible pigments, melanin pigments, elevation, surface including odor). All the morphologically different isolates were once again grown on actinomycetes agar in order to rule out the morphologically

looking same isolate from different media (SCA and ISSA).

Statistical analysis

The piechart, scatterplot and bar graphs (standard error) were drawn using Microsoft-Excel 2019. The data was performed in triplicates. As 10^{-6} dilution gave the highest

RESULTS AND DISCUSSION

Out of 8209 isolates that have been enumerated, there were 127 are morphologically different isolates. There was an effect of media on the number of isolates, but no statistical difference was observed. Highest number of isolates were from AA media of 35% followed by 34% in ISSA and least in 31% in SCA (**Figure 2 A**).

Lee *et al.*, (2014) also showed that the actinomycete diversity from the mangrove soil sediments differed based on the different culture media. The total actinomycetes populations in tea plant grown soils were shown to be influenced by soil nutrients such as total organic carbon, nitrogen and phosphorus [23]. The starch casein agar (SCA) was reported to show high amount of diversity (colony morphology) when compared with five other culture media including actinomycetes isolation agar [22]. In a similar study at Kuruvampalayam Electroplating industrial area (Coimbatore, Tamilnadu), maximum number of

number of different morphotypes, the CFU count and beta diversity of this dilution was only represented (data of other dilutions not shown). The statistical significance (p value ≤ 0.05) was estimated using PAST3 (ver1.0.0.0) by ANOVA.

actinomycetes were shown in starch casein agar in comparison with the other media [20]. But in the present study, actinomycetes isolation agar was shown to have highest number of diverse colonies when compared with Inorganic Salt Starch Agar (ISSA) culture media, followed by a similar number in SCA. Not only the diversity of actinomycetes but also there was an effect of media on the inhibitory potential of isolated *Streptomyces* on 12 different organisms of fungi and bacteria together [24]. In a similar study, out of the five tested culture media, actinomycetes populations from the costal marine sediments from Thailand showed highest in SCA and marine soil extract agar [1]. There was an effect of sample sites on the total number of isolates. In total, Dumas sample (15%) site gave the largest number of isolates of all the sample sites and the least was in Vasava and Rajgiri (11%) (**Figure 2B**). There were reported studies that showed there was an effect of site on the diversity of cultivable actinomycetes from different sites

of western China [9]. Sample sites when compared with their respective individual media, there was an effect of site and media (Figure 3). In AA Machigam sample gave highest number of isolates (17%) followed by lowest in Vasava gam (7%). In contrast, Vasava gam showed highest number of isolates (16%) followed by lowest in Rajgiri (8%) when enumerated in ISSA. But whereas in SCA the highest number was observed in Dumas sample (16%) followed by lowest in Rajgiri, Machigam and Borsi (11%) sample sites. Studies have reported that the number of CFUs differed based on the sample site [25-28]. In another reported study, the percentage of distribution of actinomycetes populations varied in between the different samples that were tested across the soil

samples of Chambal territory of Madhya Pradesh region of India; especially SCA was found more suitable in comparison with ISP media [29]. The scatter plot showed that there was an effect of both dilution and the sample site (Figure 4). There was a less or no difference in 'Rajgiri' and 'Borsi' sites when compared across all the three media in 10^{-2} dilution, but contrastingly there was an effect of media in 'Dumas' and 'Vasava gam' sample sites. Similarly, such observations were also observed in the other dilutions with respect to media. But whereas the 10^6 dilution showed maximum number of morphologically different colonies but did not show much difference in the total number of CFUs in between the media (Figure 5A).

Table 1: Physicochemical parameters and sample site descriptions

| Sampling site Information | Dumas | Dandi | Vansva Gam | Junagam | Tunda | Borsi | Machivada | Rajgiri |
|----------------------------------|-------|-------|------------|---------|-------|-------|-----------|---------|
| Latitude (N) | 21.08 | 20.86 | 21.21 | 21.14 | 21.26 | 20.94 | 20.92 | 21.18 |
| Longitude (E) | 72.70 | 72.79 | 72.66 | 72.64 | 72.64 | 72.75 | 72.76 | 72.64 |
| Physicochemical parameters | | | | | | | | |
| pH | 7.3 | 7.2 | 6.9 | 7.3 | 8.2 | 8.40 | 7.7 | 8.2 |
| Salinity (%) | 33 | 32 | 31 | 31 | 29 | 30 | 32 | 15 |
| Temperature (°C) | 30 | 31 | 33 | 32 | 31 | 33 | 32 | 32 |
| Total organic carbon (%) | 0.99 | 0.81 | 0.73 | 0.92 | 0.44 | 0.46 | 1.02 | 0.74 |
| Total Nitrogen (%) | 1.23 | 1.02 | 1.4 | 1.28 | 1.25 | 1.7 | 1.2 | 1.4 |
| Electric Conductivity (mmlol/cm) | 3.73 | 3.74 | 4.95 | 4.31 | 8.50 | 8.20 | 10.07 | 7.66 |
| Available Phosphorus (kg/ha) | 67 | 64 | 72 | 80 | 209 | 123 | 150 | 124 |
| Available Sulphur (ppm) | 20 | 28 | 42 | 33 | 769 | 535 | 625 | 835 |
| Available Potassium (kg/ha) | 1557 | 1556 | 1421 | 1522 | 1361 | 1553 | 1221 | 1344 |

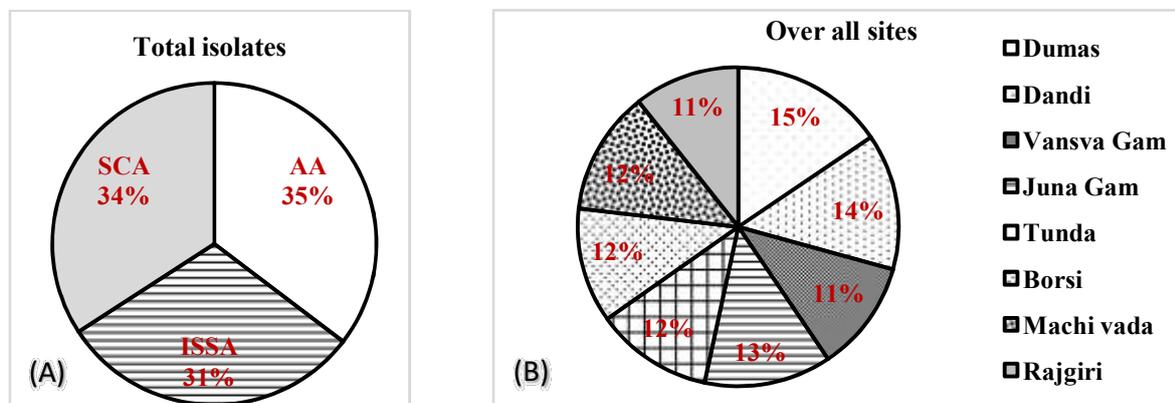


Figure 2: Percentage of morphologically different isolates observed from three different media (AA, SCA and ISSA). (A) Based upon culture media and (B) based upon sample site

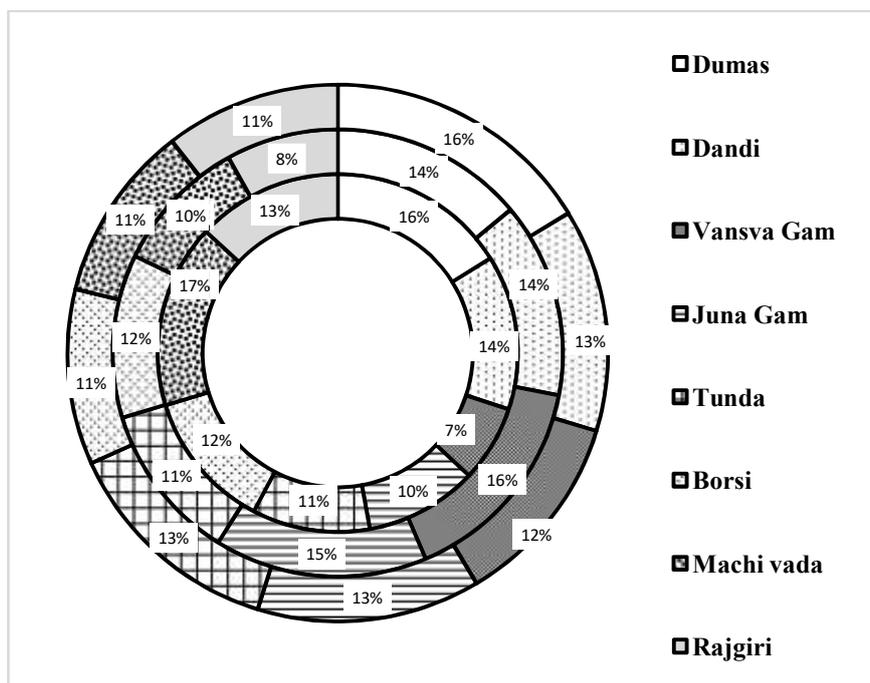


Figure 3: Morphologically different number (in percentage) of isolates observed from different sample sites in accordance with the culture media (outer circle (SCA), middle circle (ISSA) and inner circle (AA))

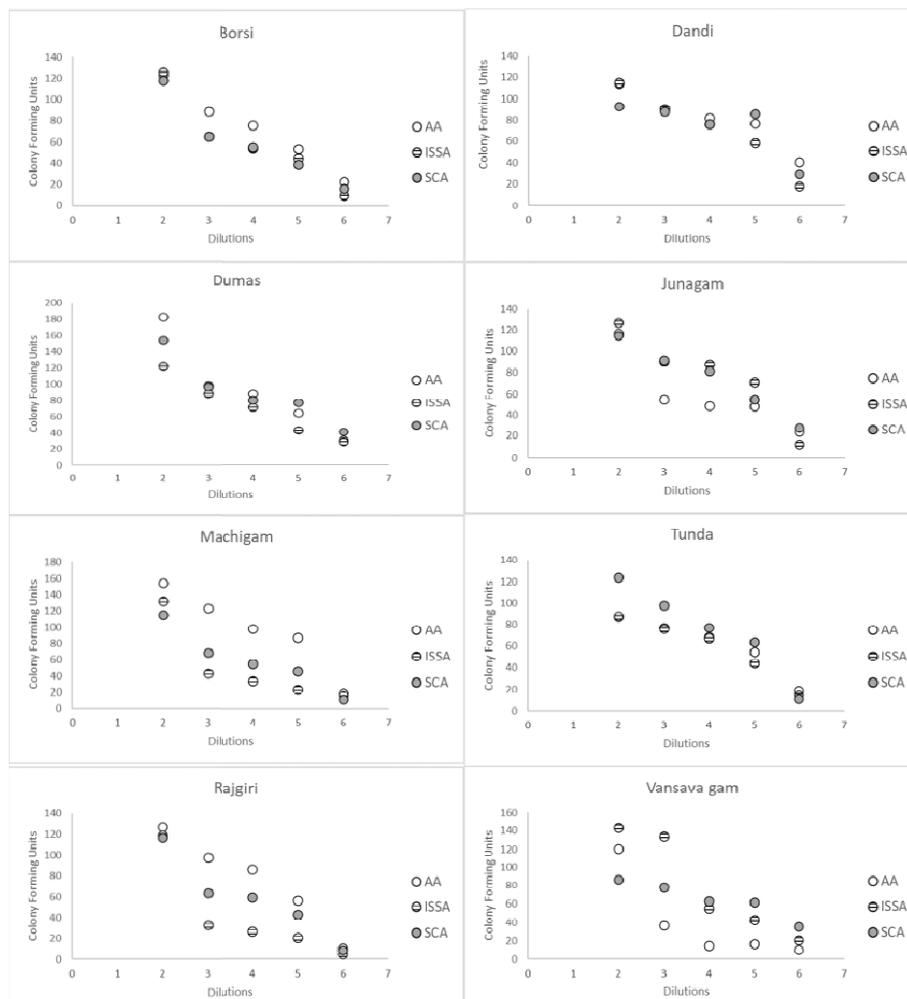


Figure 4: Colony forming units observed in different dilutions (10^2 up to 10^6) in different media from different sample sites

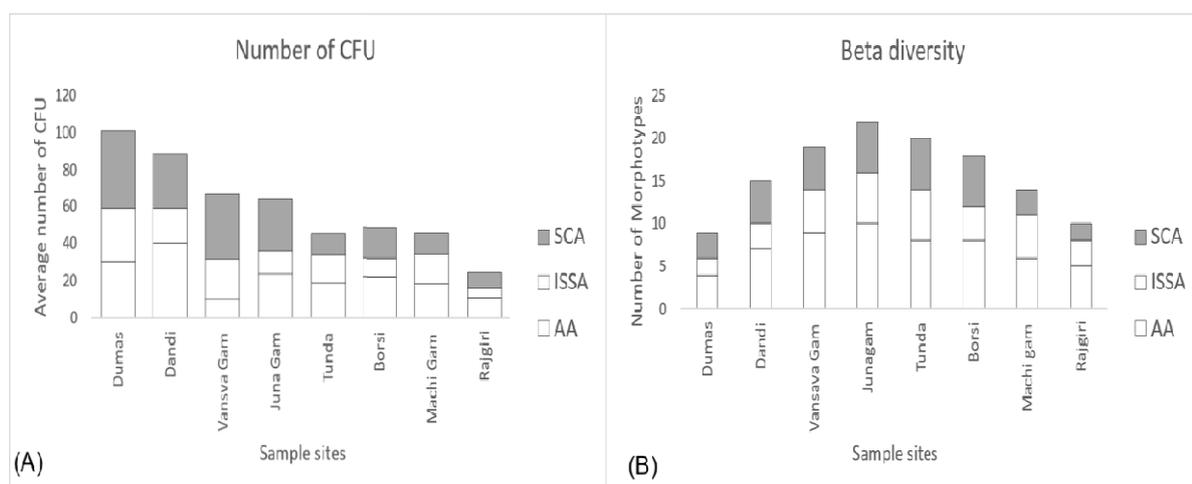


Figure 5: Colony forming units observed in 10^6 dilution from different sample sites and culture media (Figure 5A). Beta diversity as observed from the different sample sites and culture media of 10^6 dilution (Figure 5B)

In a microcosm experiment using sewage microbial community, showed that the diversity decreased after 10^3 dilution factor [30]. The responses of various parameters including diversity was stated to produce results of nonlinear to disturbances or manipulations [30]. The two-way ANOVA of 10^6 dilution showed a statistically significant effect of site (p value ≤ 0.000) and non-significant with respect to media (p value = 0.058). The number of CFUs in between different media (AA, ISSA and SCA) of the individual sample sites did not differ statistically except in 'Junagam' and 'Vasava gam' sample (p value = 0.050). The number of CFUs in actinomycetes agar across all the sample sites 'Dandi' and 'Vasava gam' differed statistically (p value = 0.041), but whereas in 'ISSA' and 'SCA' media no other sample sites differed significantly. In the present study also, 'Rajgiri' sample site showed low number of Colony forming units in comparison with other sample sites. This may be attributed to that 'Rajgiri' is located .001). Beta diversity should be considered as an important component of the diversity as it is connected with both alpha and gamma diversities [33]. In the present study, beta diversity was shown to be dependent on the sample site and the media. High amount of

near to an industrial area (https://www.gpcb.gov.in/pdf/HAZIRA_LNG_EIA_PART_I.PDF). Studies have reported that the pollutants reduced the biological diversity [31]. The meiofaunal diversity was reported to be less in disturbed sites in comparison with the undisturbed sites [6]. In a study involving the coastal marine sediments the bacterial diversity differed due to the gradients of pollution exposure [32]. In the present study, the total number of morphotypes isolated were largest in Junagam followed by lowest in Rajgiri and Dumas. Overall, the actinomycetes agar gave highest number of morphologically different CFUs (Beta-diversity), but there was a 56% decrease in the number of morphotypes of both SCA and ISSA when compared with AA (**Figure 5B**). With respect to sites, overall 'Junagam' gave highest number of morphotypes followed by lowest in 'Dumas' sample site. Two-way ANOVA of the morphotypes in 10^6 dilution showed a statistically significant effect of site (p value = 0.017) and media (p value = 0) beta diversity was found to be from samples collected from 'Junagam', followed by lowest if 'Dumas' and 'Rajgiri' sample sites. In a study involving the North pacific and Caribbean coasts of Costa Rica showed that the beta diversity of culturable actinomycetes

differed in between the sample sites [34]. In a study involving actinomycetes isolated from the coastal multi pond solar saltern of

CONCLUSION

The present study indicates that there is an effect of sample site and media on the culturable actinomycetes populations followed by dilution factors. The actinomycetes agar was shown to represent higher amount of morphologically different isolates among the other tested media. In spite of not having contrast physiochemical parameters the sample sites differed in their diversity. In future, studies involving the culturable methods for diversity purposes should consider the dilution factor and media parameters. But such studies should also be further validated by molecular studies.

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Tuticorin region also reported to differ in their beta diversity [35].

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