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Diversity of Culturable Marine bacteria at Tithal Beach near Valsad and Dumas Beach near Surat in the Coastal Areas of South Gujarat Region

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Abstract*— In the present study bacterial diversity in marine environment from two regions of South Gujarat coastal areas, Tithal beach near Valsad and Dumas Beach near Surat were investigated. Morphological characteristics on Zobell Marine Agar 2216 (Hi Media, Mumbai) and colony characteristic were studied. Organisms showed various pigmented colonies like white, off white, powdery white, cream, pink, orange, grey, brownish, etc. Phenotypic Identification of selected isolates was carried out using the conventional methods as well as detailed characterization was done using VITEK 2 automated microbial identification system. Abiotic parameters including temperature, pH and salinity were measured. The study revealed that the isolates were found to belong to the four phyla, Proteobacteria (Alphaproteobacteria 28.20 % and Gammaproteobacteria (28.20%), Firmicutes (25.64%) and Actinobacteria (7%). 39 isolates belonging to nine different families were reported with Proteobacteria as a dominant phylum. Statistical analysis (Shannon and Simpson Index) was calculated which revealed that diversity of bacteria varied among both sites with same dominance index.

***Index Terms*— Marine culturable bacteria; Diversity of cultivable bacteria; Tithal beach near Valsad; Dumas Beach near Surat.**

I. INTRODUCTION

The Arabian Sea gives its extensive coastine to Gujarat state. Gujarat has the longest coastline of 1600 kms which is approximately 24% of Indian Coast line. Diverse forms of microorganisms are present in the marine environment which exhibit important role. Among the microorganisms, bacteria are found to play a major role in biogeochemical cycles [1].

Yet in natural environments knowledge about existence of bacteria is limited and not at all an easy task [2]. One gram of soil may contain 10^{10} and 1 ml of water may contain 10^6 bacteria [3]. As diverse forms of heterotrophic bacteria present in the marine environment play a major role in biogeochemical cycle and food web [4]. So it is essential to have knowledge about the community structure and diversity. Study about its community structure and diversity will help us to understand the relationship between environmental factor and ecosystem functions. The measurement of diversity helps to study the richness and evenness in a community [5]. Thus studying bacterial abundance and diversity is of utmost importance. In the abundance of microorganisms abiotic parameters (pH, temperature, salinity) and biotic (organic factors) parameters of oceans play a major role? [4], [6], [7], [8]. Changes in temperature, salinity and physiochemical characteristics has importance in distribution of bacteria [9]. Since biotic and abiotic factors are higher in marine environment, marine microorganisms have been found to have a higher potential than terrestrial microorganisms. But as per current knowledge up till now only a small fraction of cultivable bacteria 0.001 to 1% has been isolated through cultivable approaches [10], [11]. It has also being found that it is easy to cultivate aerobic and heterotrophic bacteria belonging to *Alphaproteobacteria* and *Gammaproteobacteria* which constitutes major phyla found in marine bacteria [12].

Culture dependent approaches help to characterize and reveal their ecological importance in the ecosystem [13]. Phenotypic characterization carried out by using culture dependent techniques helps to fill the gap between their identification through molecular approach and their function [14]. Culture dependent approaches can be carried out to study various pigments of bacteria which plays an important role [15]. Such pigmented bacteria can be utilized to extract pigmented compounds which are found to have pharmacological applications [16]. Not only have that marine bacteria had potential to produce biomolecules. These biomolecules are not only industrially and but also environmentally helpful. The Biomolecules produced by marine bacteria can also be helpful to produce human and animal drugs [17].

In South Gujarat coastal region diverse forms of bacteria are present not only on the surface waters but also on the near off shores. The present study was focused to investigate diverse forms of cultivable bacteria in this coastal region. Morphological and phenotypic identification was carried out using culture dependant approaches and automated microbial identification system VITEK 2 compact 30 (bioMerieux) [18]. According to Zbinden *at. el* species level of identification made by VITEK 2 was found in correlation with 16S r RNA Gene sequencing, it was utilized here for phenotypic characterization [19].

II. MATERIALS AND METHOD

A. Sites of sampling and Environmental factors

The sites for sampling were selected from the South Gujarat Coastal region. Both the sites i.e. Tithal Beach near Valsad and the Dumas Beach near Surat were 105 kms apart

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from each other. Marine samples were collected during summer season in the year 2016. Soil samples were collected in a sterile plastic bag and water samples were collected in sterile plastic bottles with a capacity of 500ml. The collected samples were immediately transferred to the laboratory and refrigerated at -20^{0}C until analysis. At each sites pH and temperature of the samples were immediately noted down. pH was measured using a pH meter and temperature was measured using a thermometer. Salinity of the marine samples was measured using a conductometer.

B. Method of Isolation of Bacteria

Enumeration of bacteria was carried out using Standard plate technique. Samples were serially diluted upto 10-6 using sterile distilled water and 0.1 ml of aliquot from each dilution was plated onto Zobell Marine agar 2216 in triplicates. Zobell Marine Agar 2216 (Hi media) having the composition as peptic digest of animal tissue 5 g, yeast extract 1 g, ferric citrate 0.10 g, sodium chloride 19.45 g, MgCl2 8.80 g, Na2SO4 3.24 g, CaCl2 1.80 g, KCl 0.55 g, Sodium bicarbonate 0.16 g, Potassium Bromide 0.08 g, Strontium Chloride 0.034 g, Boric Acid 0.022 g, Sodium Silicate 0.004 g, Sodium flurate 0.0024 g, Ammonium nitrate 0.0016 g, disodium phosphate 0.008 g, agar 15 g having final pH of medium (using 1 Litre of distilled water) 7.6 ±2 was used [20]. All the plates were incubated at 28o ±20C for 5-7 days. The colonies obtained after incubation period were counted as CFU/ml and CFU/g to estimate the abundance of bacteria and then sub cultured for purification of isolates. Well isolated colonies from each isolates were further subjected for the morphological and phenotypic characterization.

C. Morphological characterization and Bacteriological analysis

Colony characteristics of the selected isolates were noted down and Gram reaction as well as motility of isolates was performed. For phenotypic characteristics manual as well as automated microbial identification system VITEK 2 compact 30 (bioMerieux) was used as described. 24 hours fresh isolates which were purified by sub culturing was taken. Sufficient number of colonies were suspended in sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7) in a polystyrene test tubes. Turbidity was adjusted using 0.5 McFarland standards. Gram negative and gram positive cards supplied with the instruments were suspended with respective organisms and incubated at 35.5 to 37^0 C in the instrument. At every 15 minutes turbidity or colored products of substrate metabolism was measured using an optical system which interprets the results using a visible spectrum. The results were obtained as '+', '-'or '(+)' or '(-)'. The reactions appearing in parentheses shows a weak reactions [4], [21, [22].

D. Diversity Indices

Diversity indices like Shannon - Wiener diversity index and Simpson Index and its equitability as well as dominance index were calculated [5], [23], [24]. Shannon Wiener index explains the richness of the sample site and to study the dominance of the sampling site Simpson index can be used [25].

E. Preservation of Isolates

60% Glycerol stock of the Isolates were made to preserve them and stored at -20oC for further study.

III. RESULTS AND DISCUSSION

A. Sample Collection and Environmental parameters

This study increases the knowledge about the diverse form of cultivable bacteria present in the marine environment at Tithal Beach and Dumas beach near the coastal area of South Gujarat region. The pH of the collected marine samples from both the sites varied between 7 and 9. The Electrical conductivity (1:25) of soil of both sites was found to be in the range of 2.5 -3 dsm-1, which suggested that soil was strongly saline. The Electrical conductivity of water sample of Site A was 42 dsm-1 and Site B was 70 dsm-1 which releaved that though salinity of both sites was higher but Site B was found to be hyper saline compared to Site A. Temperature of both sites was ranged between 350C - 450C.

B. Isolation of cultivable bacteria

The marines samples were serially diluted and plated on Zobell Marine agar 2216(Hi Media). After 7 days of incubation abundance of cultivable bacteria from water samples and soil samples was reported as CFU/ml and CFU/gm. (Table 1).

Table 1. Sites, Standard plate count and number of isolates

Sr. No	Sites	CFU/g in soil samples	CFU/ml in water samples	Selected Isolates
1	Tithal Beach, Valsad	2.17×10^7	2.44×10^8	16
2	Dumas Beach, Surat	1.3×10^8	2.23×10^8	23

C. Morphological and Phenotypic characterization

The Selection of isolates for further study was done randomly. Organisms produced various pigmented colonies like white, off white, powdery white, cream, pink, orange, grey, brownish, etc. All the colonies were moist in appearance except the colonies of *Bacillus* species which was dry and *Actinobacteria* was found to be chalk white and powdery. During initial isolation it was observed that organisms showed best pigment after 48 hours of resolution at room temperature. Few isolates showing common pigments were selected for comparison of diversity among the two sites. The glimpse of few isolates are shown in Fig. 1.

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D. Colony Characteristic, Gram Reaction and Motility. Colony characteristics of only 39 isolates are listed in **Table 02** along with the gram reaction which was carried out by standard Hucker's modification method. Molility and other colony characteristics were noted down as mentioned. *Actinomycetes spps*. was seen as filametous under the microscope. Among the total isolates obtained the proportion of gram negative bacteria (59%) was found to be more as compared to gram positive bacteria (41%).

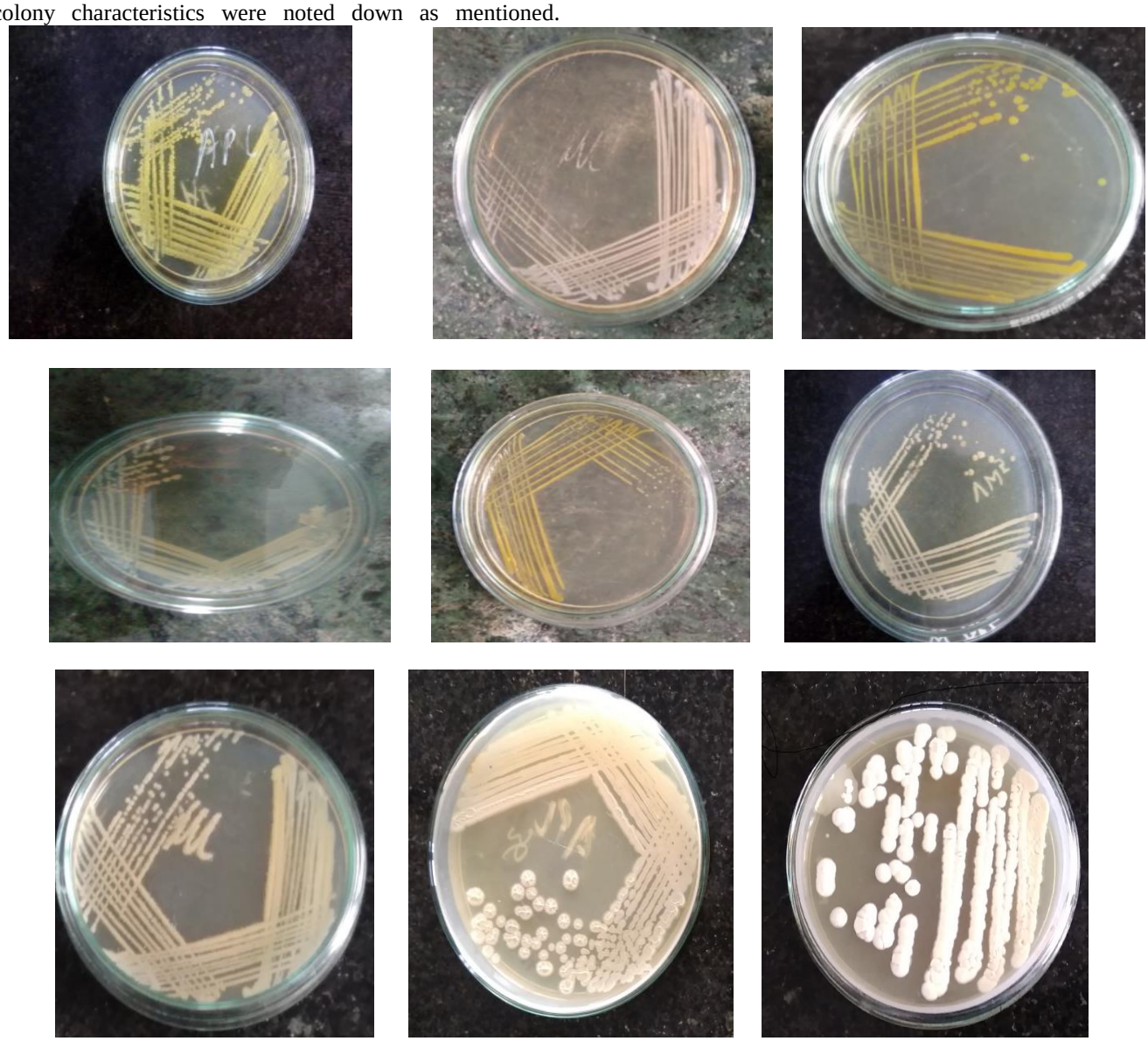
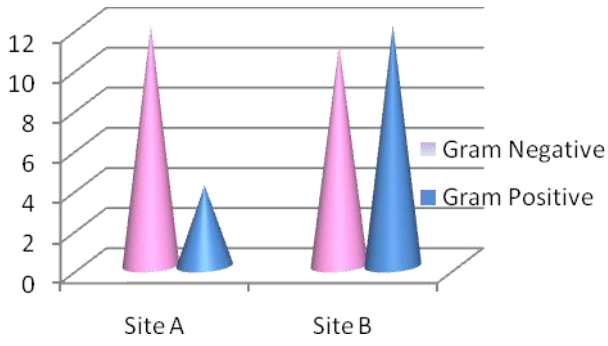


Fig: 1: Glimpse of various Isolates from the two sites

As gram negative bacterial cell wall is more adapted to marine environment it is found to be abundant compared to gram positive bacteria **[26] ,[27].**



Graph 01: Number of Gram Negative and Positive Isolates at each Site

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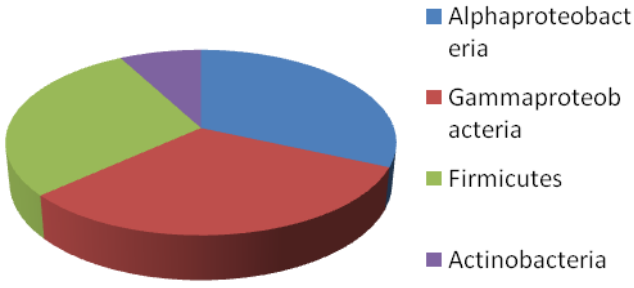
Table 2: Colony Characteristic, Gram Reaction and Motility of Isolates

ID of Isolate	Gram Reaction and Morphology	Motility	Colony Characteristics
SVW2	GNB	Motile	Large, raised, yellow
SVW3	GPC in tetrads	Non - motile	Pin-point, raised, cream
SVW4	GPC	Non - motile	Large, convex, yellow
SVW5	GNB	Sluggishly motile	Large, convex, Off white
SVW7	GNB	Sluggishly motile	Large, convex, Off white
SVW11	GNB	Motile	Small, irregular, convex, yellow
SVW12	GNB	Motile	Large, raised, light pink
SVW13	GPB	Non-motile	Large, Rough, Opaque
SVS2	GNB	Sluggishly motile	Large, Off white, convex
SVS3	GNB	Motile	Small, round, flat, transparent
SVS4	GNB	Motile	Small, round, raised, transparent
SVS5	GNB	Sluggishly motile	Small , convex, bright yellow
SVS7	GPB	Non- Motile	Large, irregular, opaque
SVS8	GPC	Non- Motile	Small, round, opaque
SVS9	GNB	Motile	Small, round, flat, transparent
SVS10	GNB	Motile	Small, round, flat, transparent
SSW1	GNB	Sluggishly motile	Small , convex, bright yellow
SSW2	GPC	Non- Motile	Small, round, convex, yellow
SSW3	GNB	Motile	Small, round, raised, transparent
SSW4	GNB	Sluggishly motile	Small , convex, bright yellow
SSW5	GNB	Motile	Pinpoint, raised, pink
SSW6	GNB	Motile	Small, round, raised, brownish
SSW7	GNB	Motile	Small, round, raised, opaque
SSW8	GPC	Non- Motile	Small, round, convex, yellow
SSW9	GNB	Sluggishly motile	Small , convex, bright yellow
SSW10	GPC	Non- Motile	Small, round, convex, yellow
SSW11	GPC	Non- Motile	Small, round, convex, light yellow
SSW12	GNB	Motile	Pinpoint, raised, pink
SSW13	GPC	Non- Motile	Small, round, convex, light yellow
SSS1	GPC	Non- Motile	Small, round, pink
SSS2	GPC	Non- Motile	Small, round, opaque
SSS3	GNB	Sluggishly motile	Small , convex, bright yellow
SSS4	GPC	Non- Motile	Small, round, opaque
SSS5	GNB	Sluggishly motile	Small , convex, bright yellow
SSS6	GPC	Non- Motile	Small, round, opaque
SSS7	GPC	Non- Motile	Small, round, opaque
SSS9	GNB	Motile	Small, round, raised, transparent
SSS10	GNB	Motile	Small, round, raised, grey
SSS12	GPB	-	Dry, powdery, white

GPC=Gram Positive Cocci, GPB= Gram Positive Bacilli, GNB= Gram Negative Bacilli

Identification of Isolates and their Distribution

During the study it was found that the identified isolates belonged to the phyla Proteobacteria in which the percentage of Alphaproteobacteria and Gammaproteobacteria was 28.20% each, and that of Firmicutes and Actinobacteria was 25.64% and 7% respectively as in **Graph 02**.



Graph: 02: Percent Distribution of Phylum

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Proteobacteria was found to be a major phylum. Similar conclusion for Proteobacteria being a abundant phylum in Coastal area of East China Sea was stated by Feng *et. al*. in 2009 **[1].**

The distribution of culturable bacteria of both the sites is shown in **Table 03** as identified by automated microbial identification system VITEK 2 compact 30 (bioMerieux). 39 isolates found to belong to 9 families were reported which included *Sphingomonadaceae, Pseudomonadaceae, Vibrionaceae, Enterobacteriaceae, Staphylococcaceae, Bacillaceae, Lactobacillaceae, Micrococcaceae* and *Actinomycetaceae.* Percent distribution of all these families showing diversity among two sites is shown bellow in Graph:3. Among the nine families, the percentage of *Sphingomonadaceae* is found to be 31 % and 23 % at site A and site B respectively which is highest among all the other familes. *Sphingomonas paucimobilis* was found abundantly on both the sites.

Common occurrence of *Pseudomonas alcaligenes* and *Staphylococcus species* was reported at both sites. Presence of *Pseudomonas spps.* Often observed in sea water **[28]** was found to play an important role in mineralization and nitrogen cycling as described by *Stabili et. al.* **[29].** Occurrence of *Vibrionaceae, Micrococcaceae* and *Actinomycetaceae* was only observed at site B whereas presence of *Bacillaceae* was found only at site A.

Diversity Indices

Shannon Index (H') for site A was found to be 3.024 and that of site B was found to be 3.303. Shannon Equitability Index for site A was 0.9104 and Site B was 0.9215. According to Pielou H' indicates the population diversity **[30].**

Simpson Index (D) of site A was 0.1 and that of site 2 was 0.0866 whereas dominance Index of both the sites was 0.9. This results indicates that the bacterial diversity was varied at both the sites. But more diverse forms of bacteria were found in site B compared to site A. The study also revealed that dominance index was found to be same on both the sites.

Table 3: Distribution of Culturable Bacteria on Both the Sites

Sr. No.	Phylum	Family	Genus and species	Sampling Sites			
				A1	A2	B1	B2
1.	*Alphaproteobacteria*	*Sphingomonadaceae*	*Sphingomonas paucimobilis*	2	3	4	2
2.	*Gammaproteobacteria*	*Pseudomonadaceae*	*Pseudomonas alcaligenes*	-	1	-	1
			Pseudomonas pseudoalcaligenes	-	-	1	-
		Vibrionaceae	*Vibrio alginolyticus*	-	-	-	1
		Enterobacteriaceae	*Escherichia coli*	-	2	-	-
			Enterobacter aglomerans	2	-		
			Serratia marcescens	-	-	2	-
			Cedecea davisae	-	1	-	-
3.	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus arlettae*	1	-	-	-
			Staphylococcus saphrophiticus	-	-	1	1
			Staphylococcus lentus	-	-	1	-
			Staphylococcus xylosus	-	-	-	1
		Bacillaceae	*Bacillus subtilis*	-	1	-	-
			Bacillus licheniformis	1	-	-	-
		Lactobacillaceae	*Alliococcus otitis*	-	1	-	1
4.	*Actinobacteria*	*Micrococcaceae*	*Micrococcus luteus*	-	-	4	-
			Kochuria rosea	-	-	-	2
			Kochuria kristinae	1	-	-	-
		Actinomycetaceae	*Actinomycetes spps.*	-	-	-	1

A1,A2= Site A (Tithal beach of Valsad) and B2,B3= Site B(Dumas beach near Surat)

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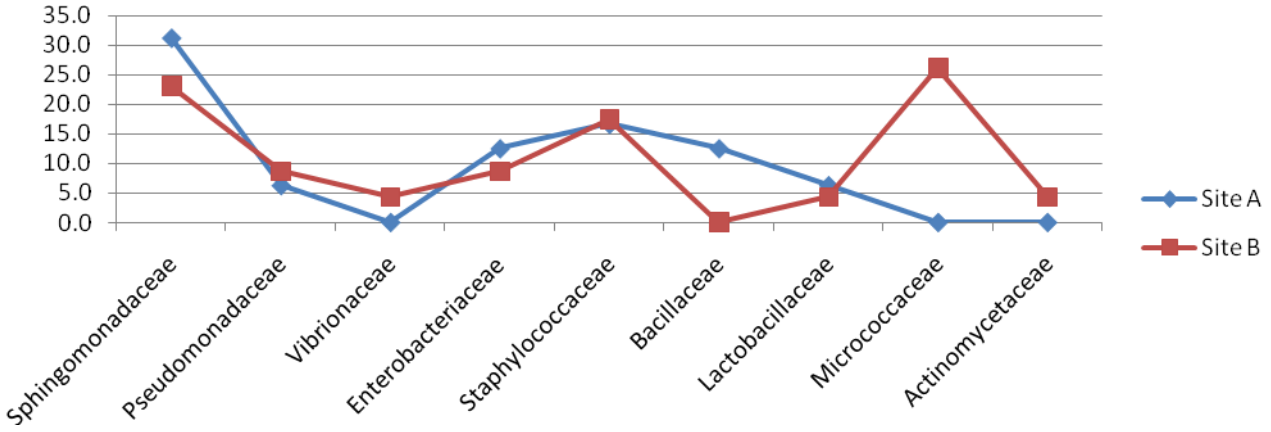

Graph 03: Percent distribution of Families on two sites

IV. CONCLUSION

In conclusion our study reveals the presence of diverse forms of bacteria on the bases of morphological and phenotypic characterization. From this data it can be concluded that *Proteobacteria* was reported as the major phylum followed by *Firmicutes* and *Actinobacteria*.

39 isolates belonging to 9 different families were reported. *Sphingomonadaceae* was found to be highest among all the other families. Abandance of gram negative bacteria in comparison to gram positive bacteria was observed.

Though the diverse forms of organisms were found more at site A in comparison to site B, the dominance index was found to be same. This could help to conclude that the dominant species were found to be same at both the sites as the sites were 150 kms apart from each other. This study was carried out during summer season; similar studies can be carried out in winter and monsoon season to reveal seasonal diversity among cultivable forms. This can also help us to reveal the species richness, evenness and abundance of bacteria during various seasons. The pigments of various bacteria could be extracted and can be used in pharmacology and cosmetology. The organism can be further used for extraction of biomolecule compounds for industrial purposes under standard laboratory conditions. Molecular studies can be carried out to obtain the sequences of bacteria which can be used to construct phylogenetic tree and their relationship.

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