Isolation, Screening and Rapid Identification of Marine Culturable Heterotropic Bacteria

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Abstract: **The present study was attempted to access the diverse forms of heterotrophic marine bacteria using culture dependent techniques. Marine samples were selected from Tithal near Valsad and Ubhrat Near Bardoli. Isolation of the cultivable forms and its characterization was carried out by the conventional methods as well as a detailed characterization by VITEK 2 automated microbial identification system. The identified isolates consisted of 5 phyla *Firmicutes* (35.71%), *Gammaproteobacteria* (25%), *Actinobacteria* (17.85%), *Alphaproteobacteria* (14.28%) and *Bacilli* (7.10%). The predominant forms of the microorganisms were characteristically producing orange, yellow, cream, white and grey pigments.**

***Key words*: Heterotrophic culturable bacteria , Tithal beach near Valsad, Ubhrat.**

I. INTRODUCTION

The oceans occupy 71% of the earth's surface .The oceans have effect on climate of earth and are ultimate reservoir. The marine environment is characterized by the hostile parameters such as pressure, salinity, low temperature, absence of light, etc. The South Gujarat coastal region is a reservoir of diverse microorganisms not only surviving in the surface waters of the sea, but also in the lower and abyssal depths from coastal to the offshore regions. Bacterial diversity is important to understand the evolution of microorganism, their variability and ecological impact. Marine Bacteria are being in the limelight past two decades as they as found to be a potential source of new bioactive secondary metabolites. (Jensen *et. al*.,2000)

From coastal region many bacterial strains have been isolated including the genera *Pseudomonas*, *Vibrio*, and *Flavobacterium*, have been considered to be representative of marine bacteria *(*Harayama *et al., 2004)*. In fact, diverse petroleum degrading bacteria inhabit marine environments such as *Alcanivorax (*Head *et al. 2006), Cycloclasticus (*Kasai *et al., 2002) Marinobacter* (Gerdes *et al.,* 2005), *Neptunomonas (*Hedlund *et al.,1999)* and 'nonprofessional' hydrocarbonoclastic bacteria such as *Vibrio, Pseudoalteromonas* and *Halomonas.*

According to Altug *et al., (2013)* wide diversity of heterotrophic bacteria is found in marine environment and they play a key role in marine biogeochemical cycling and food webs .

Stabili *et al.*(2004) mentioned that the marine bacteria comprises majorly of phyla *Gamma Proteobacteria* and *Alpha Proteobacteria*. Both these groups contains aerobic and heterotrophs which can be cultured easily. Thus the current research was focused to determine the diverse forms of heterotrophic bacteria associated with marine environment. To carry out the initial screening of isolates and study the morphological and biochemical parameters automated microbial identification system VITEK 2 compact 30 (bioMerieux), was used in line up with study carried out by Altug *et al., 2013 and* Kati *et al.* 2013.

The colorimetric VITEK 2 system was also used for identification of gram-negative non fermentative rods by Zbinden *et al*., 2007 and found species level of identification was in correlation with 16S r RNA Gene sequencing.

II. MATERIALS AND METHODS

A. Sites and nature of Sample: Two sites were selected from South GujaratCoastal region , one Tithal Beach near Valsad and the other Near Ubhrat. 4 Soil samples (two from each site) collected from depth of 10 cm , water samples (two from each site) from 20 cm offshore and Sediment samples (two from each site) were collected from these sites.. Soil samples and sediment samples were collected in a sterile plastic bag and water samples were collected in sterile plastic bottles. The samples under refrigerated conditions were transferred to the laboratory where temperature and pH of the samples was determined. The sampling was carried out in winter season in the month of November.

B. Serial Dilution method: All the samples were serially diluted upto 10^{-6} using sterile distilled water and

plated on Zobell Marine Agar 2216 (peptic digest of animal tissue 5 g, yeast extract 1 g, ferric citrate 0.10 g, sodium chloride 19.45 g, $MgCl_2$ 8.80 g, Na_2SO_4 3.24 g, $CaCl_2$ 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 and Distilled water 1000ml) from Hi-media using 50% autoclaved sea water *(*Lee *et al., 2009)*. Triplicate plates from each dilutions were incubated at 28°C for 24 to 48 hours. After 2-3 days of incubation, the colonies were counted as CFU/ml and CFU/g to estimate the number of bacteria and then subcultured for purification of isolates. Well isolated colonies from each isolates were further used to study phenotypic characteristics.

C. *Morphological characteristics and Bacteriological analysis:* Selected isolates were studied for gram reaction and morphological characteristics. The colony characterisitics of isolates were also noted along with pigment production. For phenotypic characteristics automated microbial identification system VITEK 2 compact 30 (bioMerieux) was used as described Altug *et.al.* In this system pure isolates which were identified by gram reaction were evaluated using cards of GN(gram negative fermenting and non fermenting bacilli), GP (gram positive cocci and non spore forming bacilli) and BCL(Gram–positive spore forming bacilli.). The identification cards were used according to manufacturers instructions which were designed by manufacturer taking into consideration various biochemical tests and newly developed substrates. The biochemical tests included 46 tests for BCL, 43 tests for GP and 47 tests for GN. From the pure colonies sufficient colonies from each isolates was mixed with 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in polystyrene test tube. The turbidity was measured and adjusted using turbidity meter called DensiChek™. (GN and GP 0.50-0.63 McFarland, BCL 1.80 to 2.20 McFarland). The microchannels in the identification cards were inoculated with bacterial suspensions. Each reaction was observed after every 15 mins by optical system of instrument automatically for measuring turbidity, or colour produced during substrate metabolism. The results were obtained the as "+"," –", "(–)" or "(+)" depending on the colour development after the reaction was completed. The Reactions that appeared in parentheses indicated weak reactions that are too close to the test threshold.(Pincus, 2005).

D. *Preservation of Isolates:* Isolates selected for the study were preserved in 30% glycerol stocks and stored at -20°C and also were maintained on the slopes of marine agar and preserved at 4°C for further molecular studies of the isolates.

III. RESULTS AND DISCUSSION

The present study was attempted to access the diverse forms of heterotrophic marine bacteria using culture dependent techniques. Marine samples were selected from Tithal near Valsad and Ubhrat Near Bardoli. Both Regions selected were a part of Arabian sea. After serial dilution both the sites showed different count as listed in **Table 01**.

Table 01:- Sites of Collection and CFU/ml obtained at each site:

Sr. No	Sites	CFU/g in sediments	CFU/g in soil samples	CFU/ml in water samples
1	Valsad Tithal Beach	1.15×10^6	1.63×10^6	1.17×10^6
2	Ubhrat Beach	1.34×10^6	1.97×10^6	1.149×10^6

Nearly 25 isolate from Tithal and 32 isolate were obtained from Ubhrat Region. Among the obtained isolates 28 isolates with different pigment were selected from both sites for studying biochemical parameters using VITEK 2 . The number of isolate selected from both sites are listed in **Table 02.**

Table 02:- Number of Isolates at each sites**:**

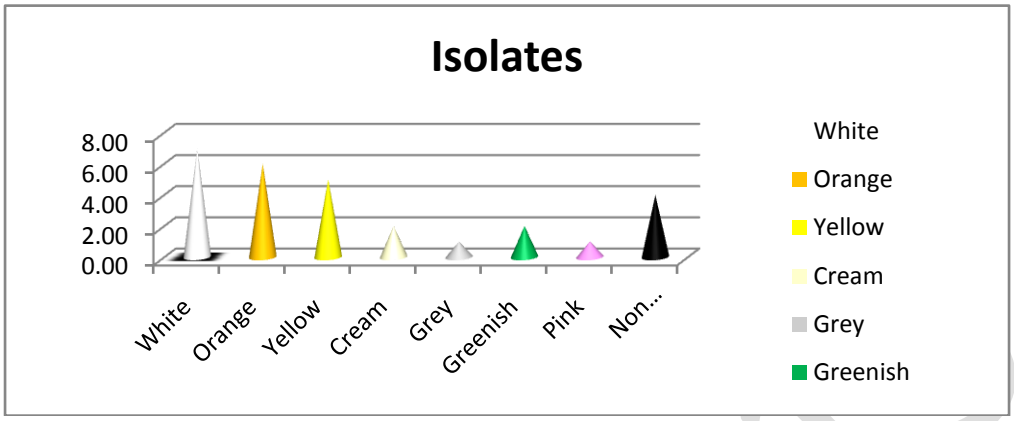
Sr. No.	Sites	No of Isolates	Soil	Water	Sediments
1	Valsad Tithal Beach	13	4	3	6
2	Ubhrat Beach	15	6	7	2

Each pure form of isolate was studied for colony characteristics like size, colour, elevation, consistency, pigmentation , gram reaction and motility . **(Table 03).** Among the obtained isolates 16 were gram positive and 12 were gram negative bacteria**.** As the isolation was carried out in winter season gram positive bacteria predominated the gram negative bacteria.*(*Graciella *et al. 2012).*

Table 03: Morphological observation of the Isolates:

IsolateID	Gram Reaction	Motiliy	Colony Characteristics							
			Shape	Size	Surface	Edge	Elevation	Consistency	Opacity	Pigmentation
VW2	-	Motile	Round	Small	smooth	Entire	Raised	Moist	opaque	Orange
VW4	+	Non motile	Large	Irregular	rough	Irregular	Raised	Dry	opaque	-
VW5	-	Motile	Round	Small	smooth	Entire	Flat	Moist	opaque	Grey
VSd1	-	Non-motile	Round	Small	smooth	Entire	Raised	Moist	opaque	Yellow
VSd2	-	Motile	Round	Small	smooth	Entire	Flat	Moist	opaque	Golden yellow
VSd3	+	Non-motile	Round	Small	smooth	Entire	Flat	Moist	opaque	Cream
VSd4	+	Non-motile	Round	Small	smooth	Entire	Flat	Dry	opaque	White
VSd5	-	Motile	Round	Small	smooth	Entire	Flat	Moist	Transparent	Greenish
VSd6	+	Non-motile	Round	Small	smooth	Entire	Flat	Moist	opaque	White
VS3	+	Non-motile	Round	Small	smooth	Entire	raised	Moist	opaque	Yellow
VS4	+	Motile	Irregular	Medium	rough	Irregular	raised	Dry	opaque	White
VS6	-	Motile	Pin-point	Small	smooth	Entire	Flat	Moist	Semi-transparent	White
VS8	+	Non-motile	Round	Small	smooth	Entire	raised	Moist	opaque	Yellow
UW1	-	motile	Pin-point	Small	smooth	Entire	raised	Moist	opaque	Orange
UW3	+	Non-motile	Pin-point	Small	smooth	Entire	Flat	Moist	Transparent	-
UW4	+	Non-motile	Pin-point	Small	smooth	Entire	convex	Moist	opaque	Orange
UW6	+	Motile	Pin-point	Small	smooth	Entire	Flat	Moist	opaque	Cream
UW7	+	Non-motile	Round	Small	smooth	Entire	Flat	Dry	opaque	White
UW8	-	Motile	Pin-point	Small	smooth	Entire	raised	Moist	opaque	Pink
UW9	-	Motile	Round	Small	smooth	Entire	Flat	Moist	Transparent	Greenish
USd1	+	Motile	Pin-point	Small	smooth	Entire	Flat	Moist	opaque	White
USd2	-	Motile	Round	Small	smooth	Entire	raised	Moist	opaque	Orange
US1	+	Non-motile	Pin-point	Small	smooth	Entire	Flat	Moist	opaque	Orange
US2	+	Non-motile	Pin-point	Small	smooth	Entire	convex	Moist	opaque	Orange
US3	±	Non-motile	Pin-point	Small	smooth	Entire	convex	Moist	opaque	White
US4	+	Motile	Medium	Small	smooth	Entire	Flat	Moist	Transparent	-
US5	-	Non-motile	Round	Small	smooth	Entire	raised	Moist	opaque	Yellow
US6	-	Non-motile	Large	Irregular	rough	Irregular	raised	Dry	opaque	-

In the present study different pigments of the colonies of isolates were observed on both the sites of collection viz. yellow, white, cream, green, pink, etc. Similar studies were carried out by Hailian du *et al.*,(2006) on pigmentation of bacteria. Also Azamjon *et al., (2011)* stated that study of the pigments of the bacteria is helpful as the pigmented compounds from marine origin have pharmacological applications.. Various pigmented isolates are shown in **Graph 01.**



Graph:01 Pigments produced by the isolates.

The isolates after gram reactions were subjected for identification using VITEK 2 and based on the biochemical parameters (Listed in Table 04 and 05) identification of the isolates was carried out.

Table: 04: Biochemical parameters of gram positive bacteria:

Parameters	Isolates Id												
	VSd3	VSd4	VSd6	VS3	VS8	UW3	UW4	UW6	UW7	USd1	US1	US2	US4
D-Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatidylinositol phospholipase C	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	+	+	-	-	-	-	-	-	-	-
Arginine dihydrolase 1	-	+	(-)	+	+	+	+	+	+	+	+	-	-
Beta-galactopyranose	-	-	-	+	+	-	-	-	-	-	-	-	-
Alpha-glucosidase	-	(-)	-	-	-	-	+	+	+	-	+	+	+
Ala-Phe-Pro arilamidaz	-	-	-	-	-	-	-	-	+	-	-	-	-
Cyclodextrin	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Aspartate arilamidaz	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta galactopyranosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Alpha-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Phosphtase	-	-	-	+	+	-	-	-	-	-	-	-	-
Leucine arilamidaz	-	+	-	-	-	-	-	-	+	-	-	-	-
L-Proline arilamidaz	-	+	-	-	-	-	-	-	+	-	-	+	-
Beta glucoro-nidase	(-)	-	+	+	+	-	-	-	-	-	-	-	-
Alpha-galactocidase	-	-	-	-	-	-	-	-	-	-	+	-	-
L-Pyrrolydonyl arilamidaz	-	+	-	+	+	+	+	-	+	-	-	-	-
Beta-glucoronidase	(-)	-	+	-	-	-	-	-	+	-	-	-	-
Alanine arilamidaz	-	+	-	+	+	-	-	-	+	-	-	-	+
Tyrosine arilamidaz	-	-	-	-	-	-	(-)	+	+	-	+	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	-	-	-	-	-	-	-	-
Polymixin B	-	-	-	-	-	-	-	-	-	-	-	-	-

resistance													
D-Galactose	-	-	-	-	-	-	-	-	-	+	-	-	-
D-Ribose	+	-	+	-	-	+	-	-	-	+	-	-	-
L-Lactate alkalinization	(-)	-	-	+	+	+	-	-	+	+	-	-	+
Lactose	+	-	+	+	+	-	-	-	-	-	-	-	-
N- Acetyl D-glucosamine	-	-	-	+	+	-	-	(+)	-	+	+	-	-
D-Maltose	+	-	+	+	+	+	-	+	-	+	+	-	-
Bacitracin Resistance	-	-	-	+	+	-	-	-	-	+	-	-	-
Novobiocin resistace	+	-	+	+	+	-	-	-	-	-	-	-	-
Growth in 6.5% NaCl	+	-	+	+	+	+	+	+	-	+	+	+	-
D-Mannitol	+	-	+	+	+	+	-	+	-	+	-	-	-
D-Mannose	-	-	-	+	+	-	-	-	-	-	-	-	-
Methyl-B-D glucopyranoside	-	-	-	+	+	-	-	+	-	-	+	-	-
Pullulan	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
0/129 Resistance(comp. Vibrio)	+	-	+	+	+	-	-	+	-	+	+	-	-
Salicin	-	-	-	-	-	-	-	+	-	-	-	-	-
Saccharose/sucrose	+	-	+	+	+	+	-	+	-	+	+	-	-
D-Trehalose	+	-	+	+	+	+	-	-	-	-	+	-	-
Arginine dihydrolase 2	-	-	-	-	-	+	-	-	-	+	-	-	-
Optochin resistance	+	-	+	+	+	+	-	+	-	+	+	-	-

+: Growth, -: no growth, (+):weak growth, (-):almost no growth.

Table: 05: Biochemical parameters of gram negative bacteria:

Parameters	Isolates Id										
	VW2	VW5	VSd1	VSd2	VSd5	VS6	UW1	UW9	US3	USd2	US5
Ala-Phe-Pro-Arilamidaz	-	-	-	-	-	-	-	-	-	-	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-
L-Pyrrlydonyl-arilamidaz	-	+	-	-	-	+	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-	-	-	-	-
Beta-galactosidase	-	-	-	-	-	(-)	-	-	-	+	-
H_2S Production	+	-	-	-	-	-	-	-	-	-	-
Beta-N-Acetyl-glucosaminidase	+	-	-	-	-	-	-	-	-	-	-
Glutamyl arilamidaz pNA	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	(+)	-	-	-	+	-	-	+	-	-
Gamma-glutamyl-transferase	-	-	-	-	-	-	-	-	-	-	-
Fermentation/glucose	-	-	-	-	-	-	-	-	-	-	-
Beta-glucosidase	+	(+)	-	-	-	(-)	+	-	-	+	-

D-Maltose	+	-	-	-	-	+	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-
D-Mannose	-	-	-	-	-	-	-	-	-	-	-
Beta-Xylosidase	-	-	-	-	-	-	-	-	-	-	-
Beta-Alanine arilamidaz pNA	-	-	-	-	-	-	-	-	-	-	-
L Proline arilamidaz	-	+	+	+	+	-	-	+	+	-	+
Lipase	-	-	-	-	-	-	-	-	-	-	-
Palatinose	+	-	-	-	-	-	-	-	-	-	-
Tyrosine Arilamidaz	+	-	-	-	-	+	-	-	-	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-
Saccharose/sucrose	+	+	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-
D-Trehalose	+	+	-	-	-	-	-	-	-	-	-
Citrate (sodium)	-	-	-	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	-	-	-	-	-	-	-
5-Keto-D-gluconate	-	-	-	-	-	-	-	-	-	-	-
L-Lactate alkalinisation	-	-	-	-	-	-	-	-	-	-	-
Alpha-glucosidase	-	-	-	-	-	-	+	-	-	+	-
Succinate alkalinisation	-	-	-	-	-	-	-	-	-	-	-
Beta-N-Acetyl-galactosaminidase	-	-	-	-	-	-	-	-	-	-	-
Alpha-Galactosidase	-	-	-	-	-	-	-	-	-	+	-
Phosphatase	-	-	-	-	-	-	-	-	-	-	-
Glycine arilamidaz	-	-	-	-	-	-	-	-	-	-	(-)
Ornithine decarboylase	-	-	-	-	-	-	-	-	-	-	-
Lysine Decarboylase	-	-	-	-	-	-	-	-	-	-	-

L-Histidine assimilation	-	-	-	(-)	-	-	-	-	-	-	-
Courmarate	+	+	+	+	-	-	-	-	-	-	-
Beta-glucoronidase	-	+	-	-	-	-	-	-	-	-	-
()/129 Resistance(comp. Vibrio)	+	-	-	-	-	-	-	-	-	-	-
Glu-Gly-Ary-Arilmidaz	-	(-)	-	-	-	-	-	-	-	-	-
L-Malate Assimilation	-	+	(+)	+	+	-	-	+	-	-	-
Ellman	-	-	-	-	-	(+)	-	-	-	+	-
L-Lactate assimilation	-	-	-	+	-	-	-	-	+	-	-

+: Growth, -: no growth, (+):weak growth, (-):almost no growth.

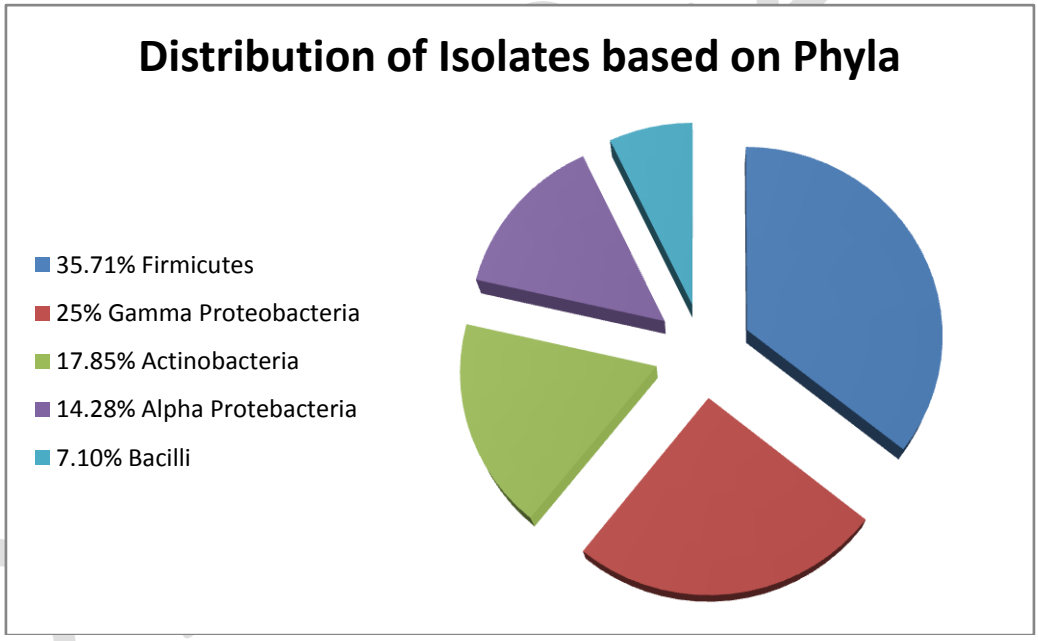
Among the 28 isolates VS4 was found to be an Actinomycetes spps. Bacillus spps. (VW4 andUS6) and Serratia spps. were identified by conventional methods in the laboratory. The remaining isolates which were subjected for identification using VITEK 2 system had probability of identification between 86% to 99%. The isolates identified are listed bellow in Table 06.

Table 06: Identication of isolates along with probability(%):

Isolates ID	**Identified using VITEK 2**	**Probality (%)**
VW2	*Sphingomonas paucimobilis*	86
VW5	*Vibio aliginolyticus*	89
VSd1	*Aeromonas salmonicida*	94
VSd2	*Pseudomonas pseudoalcaligenes*	91
VSd3	*Staphylococcus saprophyticus*	96
VSd4	*Micrococcus luteus*	95
VSd5	*Pseudomonas alcaligenes*	97
VSd6	*Staphylococcus equorium*	91
VS3	*Staphylococcus xylosus*	99
VS6	*Sphingomonas paucimobilis*	98
VS8	*Staphylococcus xylosus*	99
UW1	*Sphingomonas paucimobilis*	98
UW3	*Staphylococcus warneri*	95
UW4	*Granulicatella elegans*	88

UW6	*Staphylococcus vitulinus*	85
UW7	*Micrococcus lylae*	97
UW9	*Pseudomonas alcaligenes*	92
USd1	*Staphylococcus haemolyticus*	85
USd2	*Sphingomonas paucimobilis*	89
US1	*Staphylococcus hominis*	85
US2	*Alliococcus otitis*	89
US3	*Gardnerella vaginalis*	99
US4	*Kochuria varians*	94
US5	*Aeromonas salmonicida*	97

It was also found that *Staphylococus Spps.* was in abundant on both the sites followed by *Sphingomonas paucimobilis*. Common occurance of *Pseudomonas alcaligenes* was also observed at both the sites. The isolates were found to belong majorly to 5 phyla Firmicutes (35.71%), Gammaproteobacteria (25%), Actinobacteria (17.85%), Alphaproteobacteria(14.28%), Bacilli (7.10%). The dominance of Firmicutes was in line with the studies carried out by Irsad *et. al* in 2014 . The percent distribution of the classes recorded during the study was as in graph 2:



In conclusion our above initial study reveals the presence of diverse forms of bacteria. Even though rich diversity is seen between the isolates obtained from Tithal beach and Ubhrat beach few isolates were found common to both sites. This reveals that further study can be carried out to during a timely period to monitor the richness of the species in both sites. And also 16 S rRNA gene sequencing can be carried for the conformation of the identified isolates which could give us the better idea about the distribution of culturable marine heterotrophic bacteria in the marine environment.

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