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ORIGINAL ARTICLE

# Assessment of Microbial Richness in Pelagic Sediment of Andaman Sea by Bacterial Tag Encoded FLX Titanium Amplicon Pyrosequencing (bTEFAP)

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**Abstract** Microbial diversity of 1,000 m deep pelagic sediment from off Coast of Andaman Sea was analyzed by a culture independent technique, bacterial tag encoded FLX titanium amplicon pyrosequencing. The hypervariable region of small subunit ribosomal rRNA gene covering V6–V9, was amplified from the metagenomic DNA and sequenced. We obtained 19,271 reads, of which 18,206 high quality sequences were subjected to diversity analysis. A total of 305 operational taxonomic units (OTUs) were obtained corresponding to the members of firmicutes, proteobacteria, plantomycetes, actinobacteria, chloroflexi, bacteroidetes, and verucomicrobium. Firmicutes was the predominant phylum, which was largely represented with the family bacillaceae. More than 44 % of sequence reads could not be classified up to the species level and more than 14 % of the reads could not be assigned to any genus. Thus, the data indicates the possibility for the presence of uncultivable or unidentified novel bacterial species. In addition, the community structure identified in this study significantly differs with other reports from marine sediments.

**Keywords** Metagenomics · 16S rRNA · Pyrosequencing · Microbial richness · Andaman Sea

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## Introduction

Ocean being the largest part of the biosphere, which covers approximately 71 % of the earth's surface with an average depth of 3,800 m, harbors great numbers and diversity of microorganisms [1]. In the marine environment, microorganisms are not only present in the surface waters, but also in the lower and abyssal depths of the ocean [2]. The marine sediments occupy approximately $3.5 \times 10^8$ $km^2$, which is 48.6 % of the earth's crust, estimated to be the habitat of about $10^6$ microbes/ml [3]. The deep ocean sediments, being the source for a myriad of microbial populations, play an important role in the ecological balance and marine biogeochemical cycles. These microorganisms have special nutritional requirements and physiological characteristics that enable them to thrive in complex conditions such as high salinity, high pressure, low temperature, poor-nutrient environment. For the past two decades, microbial populations of the deep-sea sediments were studied by culture independent 16S rRNA based microbial analysis [4]. Many projects have begun recently to delineate the abundance and diversity of microorganisms present in subsea floor biosphere and their biogeochemical importance [5]. Composition and diversity of the microbial populations in marine environments showed that the environmental microbial community structure is more diverse and complex than originally thought [6]. The increase in the speed and efficiency of the genomic data generation and the sequencing coast plummeted as the next-generation of highly efficient sequencing technologies are available [7]. The microbial diversity of various ecosystems such as deep mines [8], soil [9], deep marine biospheres [10], tidal flats [11], and human microflora [12] were analyzed by employing pyrosequencing technique. On the other hand, potential automated bioinformatics pipelines have been developed to analyze,

achieve consistent, rapid and accurate taxonomic assignments of the 16S rRNA sequence reads [13].

In this study, we explored the microbial diversity of the 1,000 m deep-sea sediment from Andaman Sea, through the bacterial tag encoded FLX titanium amplicon pyrosequencing (bTEFAP) approach. The bTEFAP technique has been selected to determine the microbial community in the pelagic sediment avoiding the culture and cloning bias.

## Materials and Methods

### Sample Collection and Bacterial DNA Extraction

The sediment sample was collected at a depth of 1,000 m from a region 1.2 km away from the Ross Island of Andaman Sea (11°67′24.11″E, 92°77′61.8″N), by the National Institute of Ocean Technology (NIOT) vessels, Government of India. The sample was collected with the grab and stored immediately at −20 °C in a sterile air tight container, until further analysis. DNA was extracted by following a modified method of Zhou et al. [14]. A 5 g of sludge sample was mixed with DNA extraction buffer and proteinase K by horizontal shaking at 225 rpm for 30 min at 37 °C. Cells were lysed with 20 % SDS and the DNA was extracted with chloroform/isoamyl alcohol and precipitated with 100 % ethanol. The extracted DNA was purified using Qiagen DNeasy kit (Hilden, Germany), according to the manufacturer's instructions and the DNA was stored at −20 °C until further analysis.

### Pyrosequencing and Data Analysis

Polymerase chain reaction (PCR) was performed using the universal bacterial primers 926F 5′ AAACTYAAAKGA ATTGRCGG 3′ and 1392R 5′ ACGGGCGGTGTGTRC 3′ covering the 460 bp (V6–V9 region) of the 16S rRNA gene [15]. The amplicon library was generated through one-step PCR consisting of hot start mixture and high fidelity Taq polymerase for 30 cycles and pyrosequencing

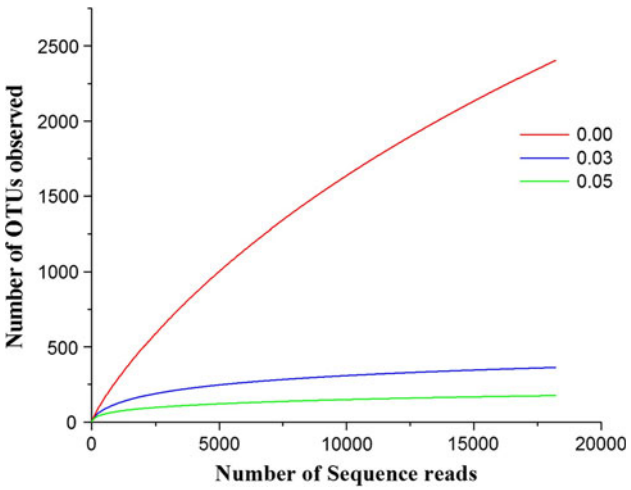



**Fig. 1** Rarefaction curves representing the richness of the pyrosequencing reads with distance values (dissimilarity level) of 0 (unique), 3 (0.03) and 5 % (0.05). The vertical axis shows the number of OTUs that would be expected to be found after sampling the number of tags or sequences shown on the horizontal axis

was performed using 454 Roche FLX instrument following the titanium protocols at the Research and Testing Laboratory (Lubbock, TX, USA). The sequence reads were screened and filtered for quality and length using the programme, MOTHUR [16]. The chimeric sequences were removed by using chimera_check and chimera slayer [17]. High quality sequence reads were aligned against the rdptrainset bacterial database and cluster linkage analysis and other ecological metrics were calculated using the ribosomal database project (RDP). OTUs and rarefaction curves were created from the aligned sequence reads by complete cluster linkage tool and also used to determine richness and diversity indexes Shannon–Weaver, Chao1 and evenness at each dissimilarity level by Shannon index calculator tools of RDP. The taxonomic assignments were given using the RDP classifier program [18] with a bootstrap score of 80 %. The sequences were analyzed by BLAST analysis against SILVA database and the results were represented using the MEGAN4 analysis tool [19].

**Table 1** Phylotype coverage and diversity estimation of the pyrosequencing analysis

| Distance units | Reads | Coverage[a] | OTUs[b] | Chao1 | ACE | Shannon | Evenness[c] |
|---|---|---|---|---|---|---|---|
| Unique | 18,206 | 99 % | 2,403 | 4,904 | 5,340 | 4.09 | 0.525 |
| 0.03 | 18,206 | 99 % | 305 | 372 | 380 | 3.04 | 0.532 |
| 0.05 | 18,206 | 99 % | 143 | 167 | 183 | 2.35 | 0.474 |

[a] The coverage percentages (Good), richness estimators (ACE, Chao1) and diversity indices (Shannon and evenness) were calculated by Good's method and RDP

[b] The operational taxonomic units (OTU) were defined with 0, 3 and 5 % dissimilarity using the RDP classifier

[c] The Shannon index of evenness was calculated with the formula E = H/ln(S), where H is the Shannon diversity index and S is the total number of sequences in that group

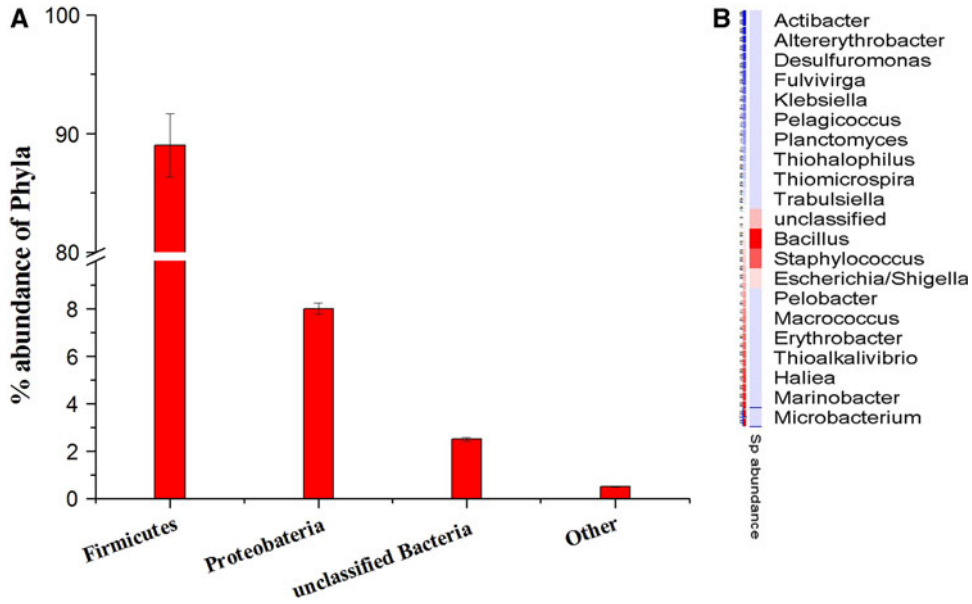



**Fig. 2** Phylogenetic classification obtained by classifying the metagenomic dataset against the RDP database using the RDP classifier. **a** Percentage abundance of the phylum, firmicutes representing 89 % of the sample, while proteobacteria 8 %, Unclassified bacteria 2.5 % and other phylum share the remaining 0.5 %. **b** Heatmap representing the relative abundance of 20 bacterial genera detected in the sample

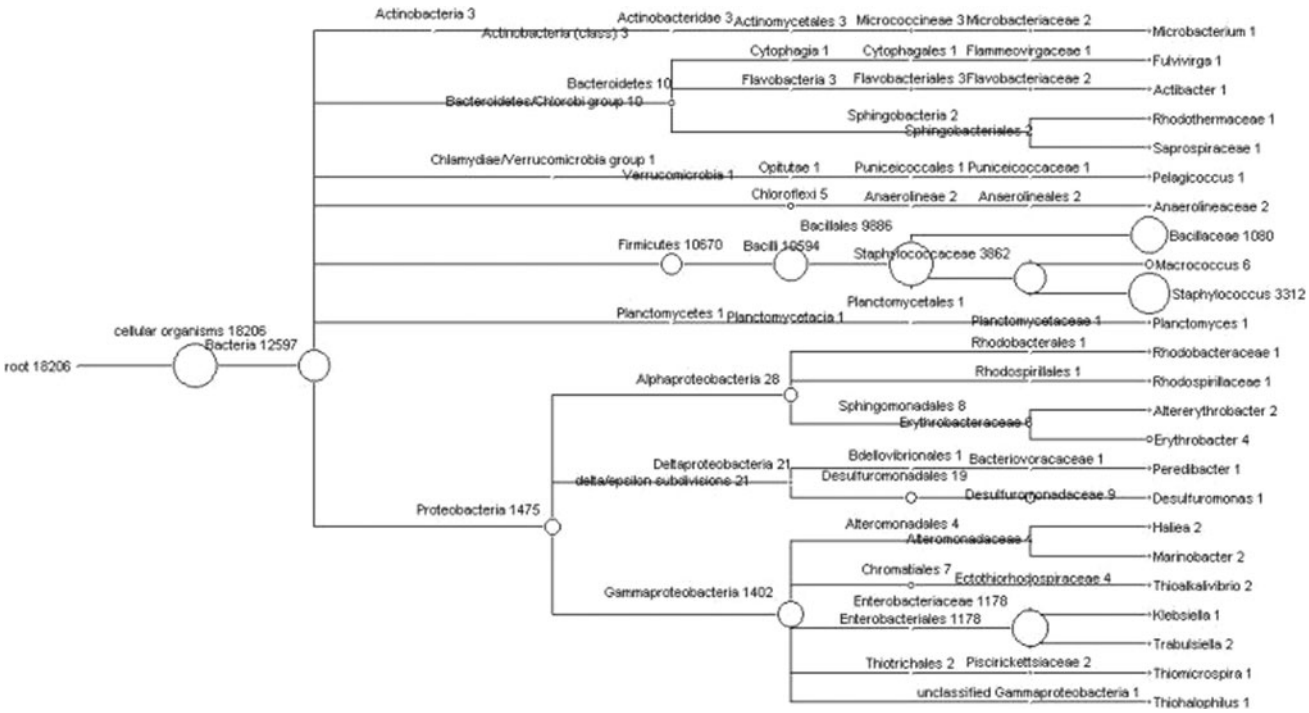



**Fig. 3** Phylogenetic diversity of 18,206 reads of the Andaman Sea sample computed by MEGAN, the classification for the sequence reads were obtained by using Ribosomal Database Project Classifier tool against the RDP database. Each circle represents bacterial taxa in the RDP taxonomy database and the size of the circle represents the number of reads assigned to the particular taxon

## Bacterial Classification

The bacterial populations were classified at their appropriate taxonomic level by BLASTN analysis performed through the web tool VITCOMIC [20]. Sequences with identity scores, to known or well characterized 16S rRNA gene sequence database and the taxonomic assignments were given on the basis of the BLAST average at different similarity level i.e., 80, 85, 90, 95 and 100 %. The overall taxonomic composition of the sample was represented by the vitcomic plot of dots in circle.

## Results

### Pyrosequencing Data

A total of 19,271 sequence reads were obtained from the Genome sequencer FLX system. The sequence has been deposited in the GenBank Sequence Read Archive with the accession number SRA048238.1. We have screened for the quality reads, ambiguous base, and homopolymers and also trimmed the primer sequence by using MOTHUR (http://www.mothur.org), which gave 18,206 high quality sequence reads with the average read length of 390 bp.

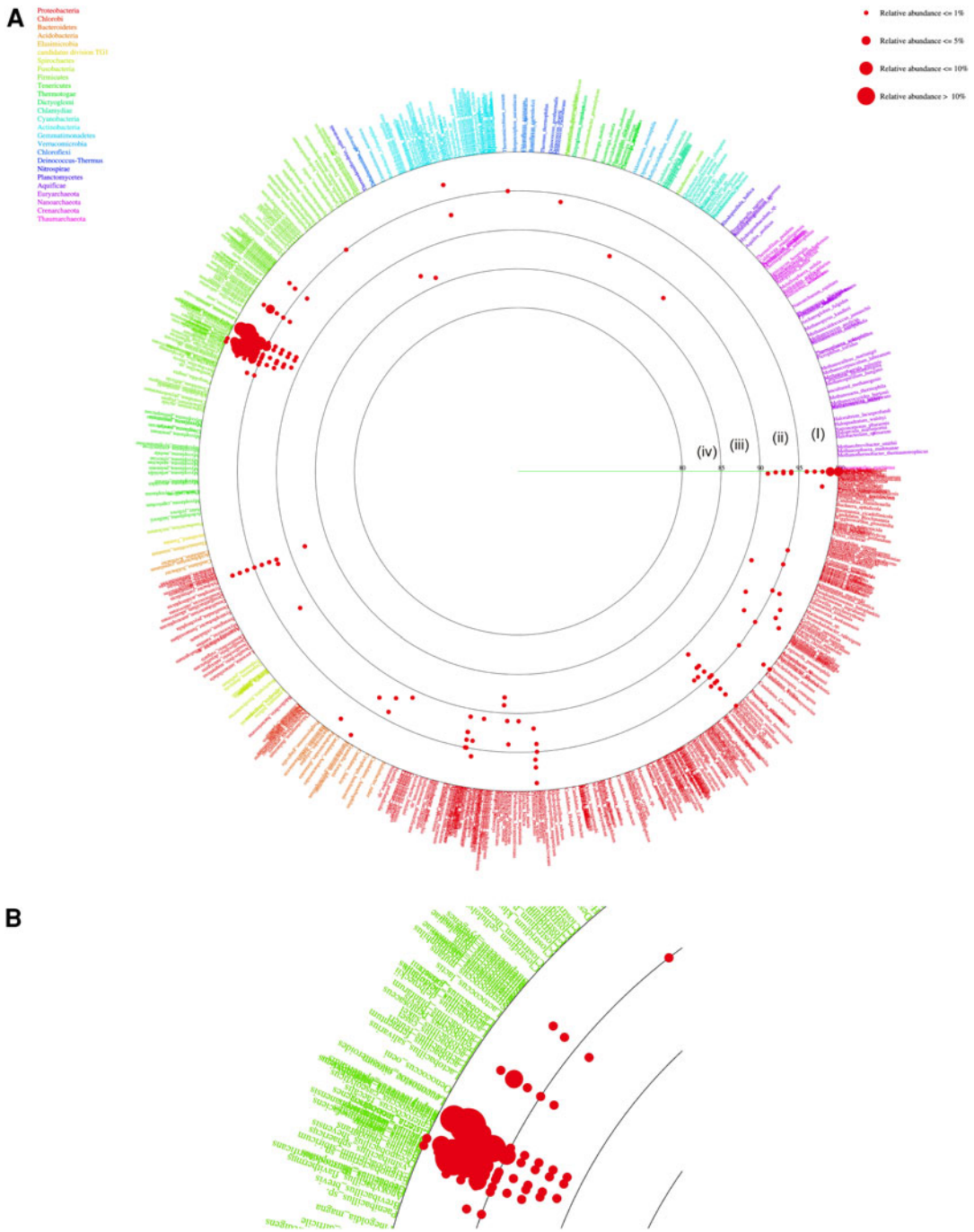
### Microbial Diversity of Pelagic Sediment

The richness of the microbial community present in the sample was estimated by rarefaction curves. The members of the sample or number of organisms assigned at each phylogenetic level depend on the number of sequences analyzed. The rarefaction curve is used to analyze whether complete diversity of the sample was obtained despite the number of sequences. The rarefaction curves based on the OTU values are good indications of the diversity within the sample as various percentage of dissimilarity level are known to differentiate at different taxonomic levels. At a distance level of 3 % is able to differentiate at species level, whereas at a distance level 5 % is able to differentiate at genus level and a distance level 10 % is able to differentiate at family/class level [21]. The rarefaction curves (Fig. 1) indicates that the bacterial richness of the sediment sample was almost completely revealed i.e., very close to the true microbial diversity of the sample. A total of 305 OTUs by clustering at 3 % dissimilarity level (0.03 distance units) and 143 OTUs by clustering a 5 % dissimilarity level (0.05 distance units) were observed. For the total microbial communities clustered at 3 % dissimilarity level, the number of OTUs obtained was very close to the number of OTUs estimated and the statistical estimates of the species richness like Chao1 and ACE diversity indices were obtained (Table 1). The 99 % of Good's coverage indicate the level of coverage of the 16S rRNA sequences identified in these groups represents majority of bacterial sequences present in the tested sample. The phylogenetic classification of the sequences from the sediment sample, at the phylum level is shown in Fig. 2a. The sequences represented eight phyla, where the majority of the sequences were assigned to the phylum firmicutes (89.4 %), followed by proteobacteria (8.1 %) and unclassified phyla (2.4 %). Planctomycetes, actinobacteria, chloroflexi, bacteroidetes, verucomicrobium, gemmatimonadetes were the other phyla identified with relatively low abundance (<1 %). A total of 429 sequence reads (2.4 %) were from unclassified phyla, representing the possibility of the presence of novel unidentified group of microorganism in the pelagic sediment of Andaman Sea.

**Fig. 4** **a** Vitcomic map representing the species diversity of the sample, based on the comparison of the reads against the NCBI non-redundant database using the blastn tool. Large circles indicate boundaries of BLAST average similarities, inner most circle (*iv*) 80–85 %, followed by (*iii*) 85–90 %, (*ii*) 90–95 %, (*i*) 95–100 % similarity of the database sequence. **b** High-resolution view of the region containing the predominant phylum firmicutes. The Larger size dots indicate the relative abundance of the particular taxon is more than 10 % of the sample sequence

The phylogenetic tree represents the BLAST analysis of the 16S rRNA sequence against the SILVA database. The tree was represented at genus level and the species level classification was obtained by creating a mapping file using the ARB-software [22]. At the genus level, 21 different genera were found in the sample (Figs. 2b, 3). Of these, the genera *Bacillus* and *Staphylococcus* roughly constituted about 75 % of the total sequences. The genus *Escherichia* represented 9 % and another 2 % of the sample constituted by other genera such as *Microbacterium, Fulvivirgo, Actibacter, Pelagicoccus, Macrococcus, Planctomycetes, Altererythrobacter, Erythrobacter, Pelobacter, Desulfuromonas, Hailea, Marinobacter, Thioalkalivibrio, Klebsiella, Trabulsiela, Thiomicrospira* and *Thiohalophilus.* Approximately, 14.5 % of the total sequence reads could not be assigned to specific genera.

The total BLAST score was calculated against each sequence of the reference database (http://mg.bio.titech.ac.jp/vitcomic/) to identify the nearest relative of the sample sequence in order to classify at species level (Fig. 4). In Fig. 4, each species name in the reference database is placed in circles with ordered phylogenetic relatedness. Physical distances between nearest species in the plot indicate genetic distances of 16S rRNA genes between them. The circles indicate the boundaries of BLAST average similarities (inner most circle starting at 80 %, followed by 85, 90, 95 and 100 % identity to the database sequence). Each dot represents average similarities of each sequence against the nearest relative species in the reference dataset. The size of these dots indicates the relative abundance of sequences in the sample. The VITCOMIC plot contains four categories of dot size that indicate the relative abundance of the sample sequence. The reads represented by the largest dot (Fig. 4b) i.e., >10 % of relative abundance were *B. licheniformis, M. caseolyticus* and *Staphylococcus epidermidis*. The sequences represented by dots with >5 % similarity were *B. cytotoxicus, B. weihenstephanensis* and *Shigella flexneri.* In addition to these major species, each of *B. halodurans*, *S. aureus, B. anthracis, B. pumilus, B. subtilis,* and *Lactobacillus sakei* were represented by more than 1 % of the total reads. *E. litoralis, Thioalkalivibrio* sp., *B. thuringiensis, Geobacillus kaustophilus, Methylacidiphilum infernorum,*




*Methylococcus capsulatus, Oceanobacillus iheyensis, Pelobacter carbinolicus, Thermomicrobium roseum* and *T. crunogena* were represented by less than 1 % of the total reads (data not shown).

## Discussion

An attempt was made to analyze the bacterial diversity and bacterial community structure of the deep sea sediment of Andaman Sea through bTEFAP pyrosequencing analysis. The accuracy of bacterial diversity of the particular environment is dependent on the size and number of sequences used for analysis. The length of the sequences also strongly affects the phylogenetic affiliation [23–25]. The accuracy of the taxonomic assignment of the microorganisms also depends on the targeted 16S rRNA gene sequence as the required length varies to different hypervariable regions. Different hypervariable regions have different efficacies with respect to species calls in different genera [26].

The V6 hypervariable region is widely used as it has been reported to have good discriminating power. In this study, we have used the V6–V9 variable region with an average read length of 390 bp, which is long enough to classify the reads up to the genus level.

In this study, the obtained sequences and the observed OTUs represented a near saturation of rarefaction curves. In addition, Good's coverage revealed that 99 % of the phylotypes identified in the sediment sample could represent majority of the sequences present in the sample. A total 305 OTUs representing the members of eight different phyla were identified. Firmicutes was the most predominant phylum in the sample, in agreement with the bacterial diversity reported from the subsea floor sediment of Andaman Sea [24]. In contrast, gammaproteobaceria was reported to be the dominant phylum in the marine sediment of China Sea and Arctic Ocean [27–29]. In this study, the proteobacteria was represented by only 8 % of the total reads, whereas firmicutes represented more than 89 %. Microbial communities in deep-sea is dependent on variables such as substrate availability and type, biogeochemistry, nutrient input, productivity and hydrological conditions of the regional scale [30]. Thus, the microbial diversity is impacted by temporal and spatial dimensions of deep-sea [31].

The genus *Bacillus* was most abundant in the sediment sample. The occurrence and distribution of the genus *Bacillus* in marine environment has been extensively studied. The genus *Bacillus* consists of 222 recognized species distributed widely across many aquatic habitats including marine sediments [32–34]. *Bacillus subtilis, B. pumilus, B. licheniformis,* and *B. cereus* were reported to be the common inhabitants of Pacific Ocean habitat [35]. Similarly, marine *Bacillus* sp. have been isolated from shallow marine sediments of Scripps pier, California [36] and from coastal marine sediments in San Diego area [37]. The genus *Bacillus* is capable of producing and secreting many industrially important enzymes and antibiotics. The next abundant genus in the sample was *Staphylococcus,* which might possibly be transported from the surface regions. The sequence reads also showed affiliation to a number of bacteria specifically isolated from the deep sea sediments, such as *A. sediminis, E. litoralis* and *Thiohalospira* sp., etc. *Actibacter sediminis* strain JC2129T was isolated from tidal flat sediment of Dongmak on Ganghwa Island, South Korea [11]. *Erythrobacter* are gram-negative facultative photoheterotrophs, metabolising organic carbon when available, but are capable of photosynthetic light utilization when organic carbon is scarce. They are globally distributed in the euphotic zone and only recently recognized as a component of the marine microbial community that appears to be critical for the cycling of both organic and inorganic carbon in the ocean. *E. litoralis* is a halotolerant aerobic phototroph, an abundant marine organism that has been little studied to date [38]. The genus *Thiohalospira* of the family *Ectothiorhodospiraceae* is of special interest because, unlike other purple sulfur bacteria, these organisms oxidize $H_2S$ and produce $S^{\circ}$ outside of the cell but also because some species are extremely halophilic and are among the most halophilic of all known prokaryotes [39]. Other bacterial genera such as *Microbacterium, Fulvivirgo, Macrococcus, Pelagicoccus, Planctomycetes, Desulfuromonas, Hailea, Marinobacter, Thioalkalivibrio* and *Thiomicrospira* were represented with lower abundance. Interestingly, we have found a number of phylogenetic lineages of unclassified genera that belongs to the phyla proteobacteria, acidobacteria and firmicutes. Altogether 44.5 % of sequence reads could not be classified up to the species level indicating the possibility for the presence of novel uncultivated or unidentified bacterial species.

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