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Full Paper

Metagenomic exploration of bacterial community in marine sediment in Gulf of Mannar (Rameshwaram), Tamil Nadu, India

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Abstract: Metagenomics was used to gain a deeper insight into bacterial diversity of the marine soil sediment of the Gulf of Mannar (Ramanathapuram), Tamil Nadu, India. Sequencing was performed using a MinION Oxford Nanopore Sequencer. Fastq read files of the microbial metagenomic data were processed through the MG-RAST server with default parameters. The metagenome contained 10,245 reads with a total of 2,979,291 bp and a G+C content of 51 ± 5 %. In the analysed bacterial community we found 10,245 species in 14 different phyla. The dominant phyla were Proteobacteria (35.62%), Firmicutes (29.18%), Planctomycetes (9.01%), Bacteroidetes (4.29%), Cyanobacteria (3.86%), Actinobacteria (2.58%), Lentisphaerae (2.58%) and Acidobacteria (0.86%). The distribution of phyla suggested a significant anthropogenic influence affecting the diversity of this marine sediment bacterial community. Comparative analysis of genes (*rfbB*, *rmlB* and *rffG*) active in the biosynthesis of secondary metabolites, xenobiotic degradation and metabolism of sulphur, carbon, and nitrogen revealed that the genes involved in heterotrophic processes such as degradation of carbohydrates, hydrocarbons and aromatics were most highly expressed in the marine soil sediment micro-organisms.

Keywords: metagenomics, marine sediment**,** nanopore sequencing, bacterial, community, Gulf of Mannar, India

INTRODUCTION

The ocean is considered to be the world's largest repository of biological and microbiological diversity. Microbial communities are ecologically important as energy mediators in

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marine biosphere. They play an important part in the nutrient regeneration cycles in marine environments by digesting dead and decaying organic matter. In marine environments, micro-organisms such as bacteria, fungi and bacterial viruses have a well-known ability to develop novel bioactive natural products and various secondary metabolites. High-throughput sequencing has fueled rapid development in microbiome research and huge microbiome data sets are now being generated

The marine ecosystem is our planet's largest habitat. The marine environment is a relatively unexplored source of diverse micro-organisms and novel metabolites [1-3]. The environmental conditions of marine micro-organisms differ significantly from those of their terrestrial counterparts and hence possess unique characteristics and produce different bioactive compounds [4, 5]. Random mutations accompanied by natural selection stress stimulate the evolution of new species of micro-organisms at high frequency [6]. Microbes possess enormous diversity and play a vital role in the preservation of sustainable ecosystems. Marine bacteria are represented within the domains of Archaea and Bacteria, and in the oceanic ecosystem all prokaryotic domains are widespread [7]. Micro-organisms help to maintain the balance and diversity of the ocean environment and are vital for sustaining life through their continuing metabolism of substances in marine habitats and participation in most biogeochemical processes, either directly or indirectly.

The Gulf of Mannar is situated on India's south-east coast and stretches from Rameswaram to Kanyakumari in the south, extending 365 km over four coastal districts, namely Ramanathapuram, Tuticorin, Tirunelveli and Kanyakumari. The gulf has 21 uninhabited islands extending over 140 km along the coast from Mandapam to Tuticorin. For management purposes, these islands are divided into four groups, viz. the Mandapam, the Keezhakkarai, the Vembar and the Tuticorin. The gulf is the first marine biosphere reserve in India as well as in South and South-East Asia. It falls within the Indo-Pacific domain, the richest region in the world from a marine biodiversity standpoint [8].

Metagenomics has been used to study bacterial communities, their adaptability, evolution and function with great success. High-throughput sequencing of targeted in polymer chain reaction (PCR) amplification can characterise microbial community, whose variance can give significant baseline understanding of the microbial ecology and a health assessment in marine environments. Metagenomics can be used to explore the metabolism of microbial communities without any prior knowledge. Results of the metagenomic analysis offer crucial evidence for determining specific ecological processes. Zhang et al. [9] conducted a metagenomic study that revealed the metabolic variety of microbes and their roles in biogeochemical cycles such as nitrogen, carbon and sulphur cycles at Yap Trench in the western Pacific. Metagenomics has also been used to investigate the upper and core portions of the Arabian Sea's oxygen minimum zones, confirming the active nitrogen cycle's genetic potential [10]. The metagenomic data demonstrated that the pollution in the mangrove sediment might reduce micro-organism metabolic capacity and increase greenhouse gas emissions. As a result, metagenomic shotgun sequencing and high-throughput amplicon sequencing can provide a unique viewpoint on microbial communities' complexity and functional traits. Microbial conversion of bioavailable chemicals (e.g. sulfur, nitrogen and carbon) in the marine ecosystem can have a big impact on global biogeochemical cycles, and studying these microbes can help us understand how they survive in this marine environment.

Marine microbial communities, the largest habitat accounting for more than eighty per cent of life on earth, play a significant role in primary energy metabolism and carbon recycling [11]. As many of these organisms are uncultivable, the metagenomics approach can shed information on the

identity and genetic components of a microbial community [12]. It is a very useful tool to screen marine microbes for the synthesis of bioactive compounds with therapeutic potential like anti-cancer, antibiotic and antipyretic compounds [13]. Metagenomics of marine bacterial communities has resulted in a generation of invaluable genetic resources and their potential functions [14].

Recent advances in molecular microbial ecology have provided the means of studying and classifying microbial species in different ecosystems through metagenomic approaches without the need to cultivate them. Next-generation sequencing technology is being successfully applied to the quantitation of bacterial diversity and the discovery of new microbes and secondary metabolites [15]. In spite of this burgeoning interest, the microbial ecology in the marine soil environments of the coastal regions remains one of the most under-studied. It is assumed that the microbial assemblies in the sediment soil and the metabolic pathways in which they are involved would be distinct due to the unique conditions. In order to elucidate the bacterial diversity and recognise how microbial communities are spread in the marine sediment, the microbial diversity in the coastal sediment in Rameshwaram (Gulf of Mannar) is thus investigated in the present study using high-performance sequencing technology.

MATERIALS AND METHODS

Sample Collection and DNA Isolation

Sediment soil samples were collected from the ground sediment of the coastal region of Ramanathapuram (latitude: 9.319078°, longitude: 79.330245°) (Figure 1) using a core sampler. Samples were collected at a depth of 0-10 cm; the surface sediment was of muddy sand and ash and black in colour. Salinity and water temperature were measured using a portable optical salinity meter (Thomas scientific, USA). The ammoniacal-N, nitrate-N, Ca, Mg, phosphate and organic carbon concentrations were measured with a spectrophotometer (Spectronic 200, Thermo Fisher Scientific, India) and the marine sediment sample pH was measured using a digital lab pH meter (Labline, India). Fe and Zn were measured using an inductively coupled plasma – optical emission spectrometer (ICP-OES-5800, Agilent, USA).

The marine sediment samples were collected in sterile plastic bags and transferred at 4°C to the lab for metagenomic analysis. DNA was extracted from 50 mg of sediment soil samples using EXpure microbial DNA isolation kit (BogarBio Bee stores Pvt Ltd, India) and following the manufacturer's protocol. The concentration of DNA was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), and aliquots were stored at -20°C until needed.

Amplification, Purification and Sequencing of Isolated DNA

The amplification of 16S rRNA genes were performed using universal bacterial primers [16] (synthesised by Sigma-Aldrich, India). The reaction mix consisted of 12 µL of PCR master mix (Sigma Aldrich), 1.5 µL of forward and reverse primers (10 picomolar), 5 µL of template DNA and water to make up to 25 µL. PCR cycle conditions were: initial denaturation at 95°C for 2 min. followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min. A final extension was carried out at 72°C for 10 min. The PCR products were purified from unutilised primers and dNTPs using the Montage PCR cleanup kit (Sigma Aldrich). The concentration and quality of the PCR products were checked using Qubit 3.0 fluorometer. Sequencing of the purified PCR products was performed using the commercial service

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of Yaazh Xenomics research laboratory (Coimbatore, Tamil Nadu, India) employing a MinION Oxford Nanopore sequencer (Oxford Nanopore Technologies, UK) [17].

Figure 1. Marine sediment sample collection site

Bioinformatics Protocol

The analysis of 16S-rRNA was performed at the single-read gene level for bacterial identification with reference to database files for detailed investigations at the species and sub-species levels. Phylogeny analysis of the sequence was done with a closely related sequence of basic local alignment search tool (BLAST) results followed by multiple sequence alignment. The workflow was designed for a BLAST baseline sequence against the national 16S bacterial database. Each reading was classified on the basis of per cent coverage and identity. From the raw sequences, QC qualified sequence (8,777) were used for taxonomic and functional genomic analysis. The 16S workflow is useful for identifying beneficial bacteria in a mixed sample or for determining the composition of a microbial community.

Metagenomic Analysis of Bacterial Community

Fastq read files of the microbial metagenomic data were processed through the Metagenomic Rapid Annotations using Subsystem Technology (MG-RAST) server with default parameters [18]. The genes involved in various pathways for the biosynthesis of secondary metabolites and for amino acid, carbohydrate and lipid metabolism, energy production, xenobiotic metabolism and carbon, nitrogen and sulphur metabolism were retrieved from Kyoto encyclopaedia of gene and genomes (KEGG) [19].

Taxonomic Analysis and Diversity

The dataset was analysed on the MG-RAST server by aligning the reads with the RefSeq database, which simultaneously provided searches against different sequence databases (MG-RAST metagenomic analysis server version 4.0.3). The parameters used were: maximum expect value = 0.0001, minimum identity = 60% and total alignment length = 15. The diversity matrices were calculated from the metagenomic data using MG-RAST (Table 1). The adapter sequences were removed using a bit-masked, k-difference matching algorithm.

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Table 1. Details of marine sediment metagenomic data analysis

Upload: sequence bp count	2,979,291 bp
Sequence count (upload)	10,245
Mean sequence length (upload)	291 ± 254 bp
Mean GC (upload)	51 ± 5 %
Artificial duplicate reads: sequence count	1,460
bp count (post QC)	2,328,335 bp
Sequences count (post QC)	8,777
Mean sequence length (post QC)	265 ± 270 bp
Mean G and C (post QC)	51 ± 5 %
Predicted protein features	8,581
Predicted rRNA features	880
Identified protein features (alignment)	272
Identified rRNA features (alignment)	64

RESULTS AND DISCUSSION

Marine Sediment Physico-chemical Parameters

Physico-chemical properties of the marine sediment sample are shown in Table 2. The pH of the sediment sample was 6.57, indicating a slightly acidic condition. The temperature of the marine sediment sample collection site, 28.5°C, was observed.

Table 2. Physico-chemical properties of marine sediment sample in Rameswaram

Property	**Unit**	Result
Salinity	ppt	32
pH	--	6.57
Conductivity	µs/cm	1440.0
Organic carbon	%	0.5
Ammonical-N	kg/Hec	10.0
Nitrate-N	kg/Hec	4.0
Ca	kg/Hec	14.02
Mg	kg/Hec	5.0
Fe	kg/Hec	0.0
Zn	kg/Hec	0.0
Phosphate	kg/Hec	10.0

Microbial Community Composition

The 16S rRNA metagenome sequences of the microbial community in marine sediment contained 10,245 reads comprising 2,979,291 bp with a G+C content of 51 ± 5 %. The details of metagenomic data analysis are given in Table 1. All the sequences were deposited under the accession number SRX7034680 in the National Centre for Biotechnology Information (NCBI)

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sequence read database. After analysing the sequences, a total of 14 phyla were found (Figures 2, 3); the most prevalent phylum was Proteobacteria. From the experimental data, the abundance of Proteobacteria at Ramanathapuram was found to be 35.62%. Next in abundance, the Firmicutes and Planctomycetes formed 29.18% and 9.01% of bacterial population in the sediment zone respectively. Bacteroidetes (4.29%), Cyanobacteria (3.86%), Actinobacteria (2.58%), Lentisphaerae (2.58%) and Acidobacteria (0.86%) were also found (Figure 2). The species-level annotations were from all the annotation source databases used by MG-RAST.

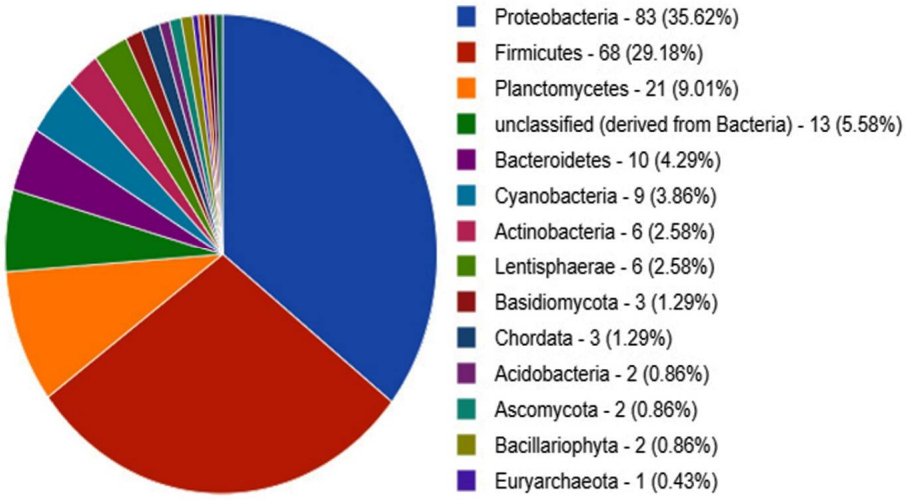

Figure 2. Microbial community composition of marine sediment of the Gulf of Mannar from image analysis system designed within MG-RAST portal

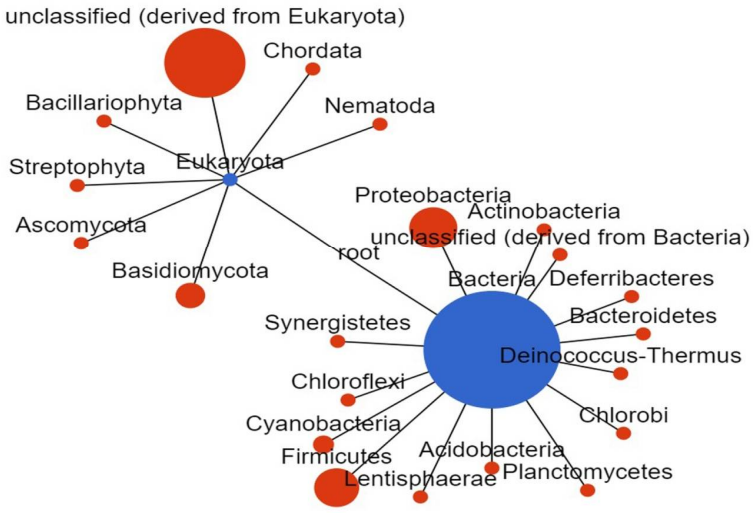

Figure 3. Microbial community composition of marine sediment of the Gulf of Mannar obtained from MG-RAST-phylogenetic distribution of metagenomic sequence.

Source Hits Distribution of 16S Amplicon of Marine Sediment Samples

The source hits distribution was determined by using MG-RAST to map raw sequencing reads against individual 16S sequencing databases (Figure 4). The source hits distribution of 16S amplicon sequencing of marine sediment metagenomic sample shows the number of annotated read

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hits across different databases. It is necessary to distinguish orthologs from paralogs using orthologous group databases such as KO, GO, COG and others. In this case linked gene classification schemes of taxonomic assignments to improve the detection resolution result. The database eggNOG has the highest number of hits annotation among the KO, GO and COG databases, owing to its high phylogenetic resolution, automated annotation scheme, and coverage of more genes and genomes than its counterpart databases, among other factors. On the other hand, according to the data, the KO database is among those with the lowest number of hits annotations.

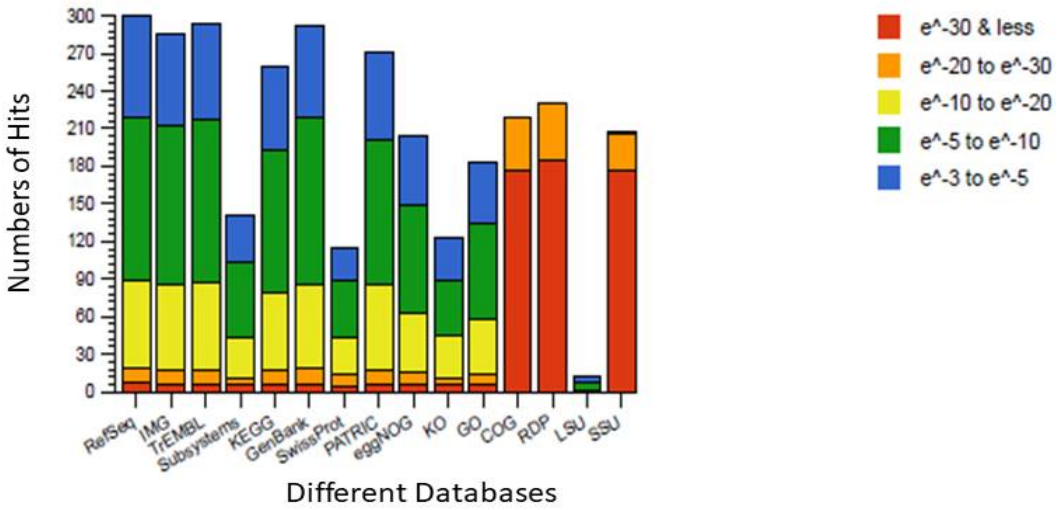

Figure 4. Sample source hits distribution of 16S amplicon sequencing in marine sediment metagenomic sample showing number of annotated read hits across different databases: RefSeq, IMG, TrEMBL, Subsystems, KEGG, Swiss prot, PATRIC, eggNOG, KO, GO, COG, RDP, LSU and SSU. Coloured bars represent observed e-value ranges of annotated reads.

Genes Involved in Biosynthesis of Secondary Metabolites and Degradation and Metabolism of Xenobiotics

The *rfbB*, *rmlB* and *rffG* genes (dTDP-glucose 4,6-dehydratase - EC:4.2.1.46) encoding enzymes for the synthesis of secondary metabolites (bioactive molecules and enzymes, etc.) were detected in the marine sediment. The functional genes found in the metagenome of sediment soil identified using MG-RAST are given in Table 3.

Table 3. Functional genes found in bacteria in sediment soil of Gulf of Mannar

Gene	Function
rfbB, *rmlB* and *rffG*	Biosynthesis of secondary metabolites
Pgl	Carbon metabolism
araB	Pentose and glucuronate interconversion
manB	Amino sugar and nucleotide sugar metabolism
tktA	Microbial metabolism in diverse environment
glsA, GLS	Alanine, aspartate and glutamate metabolism
cysJ	Sulfur metabolism
ndhE	Oxidative phosphorylation

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CES2 genes involved in xenobiotics (complex industrial chemicals, antibiotics and pesticides) degradation and metabolism were detected in the marine soil metagenomes from Rameshwaram, and the data were retrieved from KEGG.

The bacterial community in the Gulf of Mannar is diverse, with representation from 14 phyla. The dominant Proteobacteria phyla identified in this study are similar to those reported in the Bay of Bengal [20]. Tang et al. [21] found 79 fungal species from marine sediment in the south Indian ocean. Marine micro-organisms catalyse the unique and indispensable transformations in the biogeochemical cycle of the biosphere [22]. Marine bacteria can grow in a wide range of temperature, high salt concentrations and conditions of sparse nutrients, and help degrade complex molecules to beneficial molecules [20].

The application of high-throughput metagenomic sequencing has increased the database in marine microbial genes, according to Duarte et al. [23]. The KEGG-based quantitative metagenome approach gives insight into the microbial populations' functional performance present in the Gulf of Mannar sediment. Previous studies have reported marine micro-organisms having functional genes [24, 25]. Using KEGG, the present study highlights the functional genes present in the microbial flora in Rameswaram marine sediment. The *rffG* gene encodes the protein for cell envelope biosynthesis, and the *rfbB* gene encodes enzymes involved in the sugar molecule synthesis. Haro-Moreno et al. [26] reported functional genes in the marine microbial samples from the Mediterranean Sea. The genes coding for enzymes involved in the energy metabolism and degradation of short-chain fatty acids and hydrocarbons such as aliphatic and aromatic hydrocarbons were also identified. Various pathways of degradation of aromatic compounds released into the environment as a result of human activities have been recognised and likely genetic factors coding for enzymes for the degradation are present in the marine sediment bacteria [27, 28].

Genes Involved in Carbohydrate Metabolism

Various genes involved in the degradation of carbohydrates were detected in marine soil metagenomes, with the presence of distinct genes towards specific carbohydrates. Rameshwaram marine soil metagenomes show a comparatively large number of genes for carbohydrate esterases and genes involved in supporting glycoside hydrolases and polysaccharide lyases. A variety of carbohydrate-forming genes, which catalyse the formation of glycoside linkage to generate glycosides, seem to be present in the sediment (Figure 5).

Genes Involved in Sulfur and Nitrogen Metabolism

The genes for nitrate/nitrite removal enzymes, *Cysj*, *nirB*, *napA* / *narG* and *norB*, are present in sediment samples. The nitrification genes are also displayed in the microbiome of soil. In addition, genes for the assimilation of ammonia (*glnA*) and the synthesis of ammonia by the transformation of organic matter were also found in marine sediment metagenomes. In the case of sulphur metabolic pathways, genes associated with sulphate reduction and sulphur oxidation were detected. Several genes involved in thiosulphate oxidation (*soxCD*), sulphide oxidation (*fccAB*) and sulphite oxidation (*soeC*) were found. Certain genes in charge of inorganic and/or organic transformations of sulphur were also found, including genes for alterations of elemental sulphide / sulphide (*psrABC* and *suda*) and thiosulphate / tetrathionate (*tsdA*, *ttrAB* and *doxD*) and genes for sulphate removal from biological molecules.

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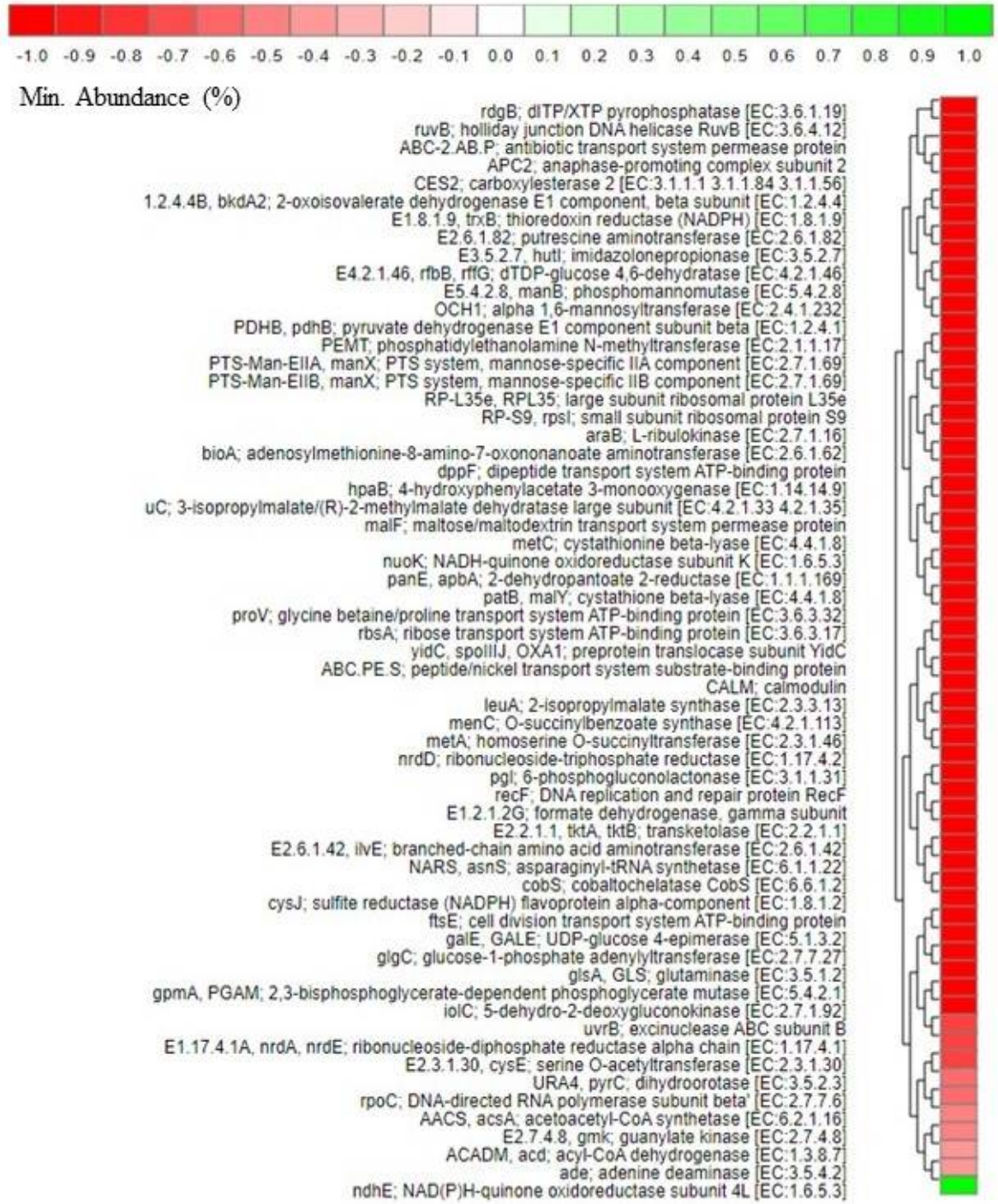

Figure 5. Metabolic enzymes derived from metagenomic data showing functional genes for nitrogen, sulphur, fatty acid, lipid and carbohydrate metabolism**.** Color indicates abundance of functional enzymes in metagenomic sample (maximum enzyme abundance = 1, least enzyme abundance = -1.0).

The results demonstrate a source of genes involved in sulphite oxidation, thiosulphate oxidation, and nitrogen and sulfur metabolism in the marine sediment sample, thus insinuating different microbial populations that dominate and carry out the necessary functional processes at

this site. Based on these results, a further in-depth examination is needed to recognise the ecological functions of the microbes in this marine sediment.

CONCLUSIONS

The results of this metagenomic analysis of marine sediment soil in the Gulf of Mannar (Rameshwaram) show that the microbial species along with their biochemical activities are dominated by Proteobacteria (35.62%) and Firmicutes (29.18%). The results presented here further illustrate the metabolic flexibility of marine bacteria, expanding our knowledge of the ocean's ecosystem and suggesting the potential for exploring the coastal areas for novel bacterial species that may be the sources of useful bioactive compounds. Future screening of these micro-organisms may uncover a trove of new compounds among the microbial metabolites with antiviral, antibiotic or anticancer activities. The rich microbial diversity in the Gulf of Mannar (Ramanathapuram) emphasises the potential inherent in exploring the marine environment for organisms as sources of novel bioactive compounds offering enormous benefits to industry, medicine and basic research.

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