

RESEARCH ARTICLE

Unveiling the Biodiversity of Hyperthermophilic Archaea in Jharia Coal Mines: Potential Threat to Methanogenesis?

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Abstract: Aim: To examine the biodiversity of archaeal sulfate reducers and methanogens present in the underground coal mines of Jharia using metagenomics and pyrosequencing.

Objectives: 1) Bioinformatical analysis of the metagenomic data related to a taxonomic analysis obtained from the coal to investigate complete archaeal taxonomic features of the coal bed methane (CBM) microbiome. 2) Bioinformatical analysis of the metagenomic data related to a functional analysis obtained from the coal to investigate functional features relating to taxonomic diversity of the CBM microbiome. 3) The functional attributes have been examined specifically for ORFs related to sulfite reduction and methanogenesis.

The taxonomic and functional biodiversity related to euryarchaeota will help in a better understanding of the obstacles associated with methane production imposed by the sulfate reducers.

Background: The microbial methanogenesis in the coal microbiome is a resultant of substrate utilization by primarily fermentative bacteria and methanogens. The present work reveals the biodiversity of archaeal sulfate reducers and methanogens present in the underground coal mines of Jharia using metagenomics and pyrosequencing.

Methodology: Bioinformatical analysis for structural and functional attributes was accomplished using MG-RAST. The structural analysis was accomplished using RefSeq database, whereas the functional analysis was done via CoG database with a cut off value, a sequence percent identity, and sequence alignment length cut off of $1e^{-5}$, 60% and 45, respectively.

Results: Attained communities revealed the dominance of hyperthermophilic archaea *Pyrococcus furiosus* along with *Thermococcus kodakarensis* in the coal metagenome. The obtained results also suggest the presence of dissimilatory sulfite reductase and formylmethanofuran dehydrogenase, formylmethanofuran: tetrahydromethanopterin formyltransferase involved in sulfite reduction and methanogenesis, respectively, in the microbiome.

Conclusion: This report is the first attempt to showcase the existence of specific euryarchaeal diversity and their related functional attributes from Jharia coal mines through high throughput sequencing. The study helps in developing a better understanding of the presence of indigenous microbes (archaea) and their functions in the coal microbiome, which can be utilized further to resolve the energy crisis.

Keywords: Euryarchaeota, metagenomics, pyrosequencing, sulfite reductase, SRBs, thermococcales.

1. INTRODUCTION

Different varieties of coal, such as bituminous and subbituminous ranks, are mainly produced from the Jharia coal bed basin in Jharkhand, India [1]. The Jharia coal bed remains one of the highest producers of coal bed methane

(CBM). The Gondwana and Tertiary sedimentary sequences primarily comprise of approximately 60 coalfields, which consist of a widespread coal bed reserve [2]. Extensive ranges of subbituminous and higher rank of coal seams, existing in such sedimentary basins, are a significant source for the biomethane production [3]. The global demand for fuel in the energy sector is increasing with an increase in population.

Thermogenic biomethane production in underground coal mines during coalification has been recently reported [4]. Several reports suggest underground, either abandoned or mined coal beds, to be a rich reserve of biomethane [5, 6].

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Also, sulfate reducing bacteria (SRB) has been observed to play a pivotal role in similar microbiome [7]. The SRBs have a wide range of physiological group, mostly consisting of anaerobic microbes utilizing sulfate as a terminal electron acceptor and petroleum hydrocarbon components as growth substrate [8]. Metagenomics is one of the most significant and widely used techniques to differentiate and exploit the microbial diversity of environmental samples. This technique remains one of the most successful approaches to characterize the microbiome diversity in samples [9]. Culture-independent techniques for genomic DNA isolation avoid microbial culturing and cloning for research and preservation purposes. The metagenomic technique examines the biodiversity and verifies the functional attributes relating to microbial growth and sustenance in a microbiome [10]. The advent of such an independent cultural technique, such as metagenomics, has offered significant insight to the microbial community.

Substantial interest in biogenic methane production has been observed in recent years [11-15], providing an opportunity for energy generation *via* methanogenesis from coal mines. There remains a necessity of collecting more data related to methane generation from the Indian coal bed, due to scarcity of the information. As a result, efficient methane production is restricted largely due to a lack of thorough reservoir characterizations. Hence, with an expanding source of information, coal bed methane productivity can be extended.

Therefore, the current study deals with the collection of coal from the CBM area of Jharia coalfield; DNA extraction from the collected sample followed by 454 pyrosequencing; bioinformatical analysis of the metagenomic data related to a taxonomic and functional analysis obtained from the coal to investigate euryarchaeal taxonomic and functional features of the CBM microbiome. The present study encompasses euryarchaeal functional and taxonomic biodiversity in the coal sample collected from CBM zone of Moonidih colliery through shotgun metagenomics using 454 pyrosequencing, first of its kind from the area. The taxonomic biodiversity related to euryarchaeota will help in a better understanding of the obstacles associated with methane production imposed by the sulfate reducers. The functional attributes have been examined specifically for ORFs related to sulfite reduction and methanogenesis.

2. MATERIALS AND METHODS

2.1. Sample Collection

The coal samples were collected from the West Jharia coal seam, Moonidih, Jharkhand. Samples were obtained at 3.5km inclination along the unexposed coal bed surface of XVIth seam *via* regular drilling method. Samples were initially collected from equidistant sites (≈ 16 locations) from vertical coal seam zone and were placed in autoclaved containers and further stored in liquid nitrogen.

2.2. DNA Extraction and Sequencing

Pyrosequencing requires a minimum of 5 μ g of DNA for successful sequencing and to rule out the extraction bias. DNA extraction from coal samples (5 g) was accomplished using Ultra Clean Mega Soil DNA Kit (MoBio[®] Laborato-

ries Inc., Carlsbad, CA, USA). The DNA from the coal samples was extracted using a rapid bead beating protocol provided as per the manufacturer's user protocol. The sample was vortexed for 1 minute after being added to the bead solution tube. To the solution tube, 1.2 ml of S1 solution was added and vortexed vigorously for 30 seconds. To the mixture, 4ml of Inhibitor Removal Solution and subjected to vortex for 10 minutes and was subsequently shaken for 30 minutes. The mixture was centrifuged at 2500 x g for 3 minutes and the supernatant was transferred to a 2.0 ml centrifuge tube provided with the kit. To the supernatant, solution S2 was added, mixed, and incubated at 4°C for 10 minutes. The supernatant formed after centrifugation at 2500 x g for 4 minutes was transferred to a centrifuge tube and 30 ml of solution S3 was added and thoroughly mixed by inverting. The solution was transferred to spin filter and subsequently centrifuged at 2500 x g for 2 minutes. After discarding the flow through, 6ml of solution, S4 was added and centrifuged at 2500 x g for 3 minutes. The flow-through was discarded and spin filter was transferred in a clean centrifuge tube, to which 8 ml of Solution S5 was added and centrifuged at 2500 x g for 3 minutes. The spin filter was discarded and the DNA was quantified using a NanoDrop spectrophotometer. DNA quality and quantity check were analyzed on Agilent 2100 Bioanalyzer and subsequent pyrosequencing was performed on 454 GS FLX (Roche Applied Science). The raw reads obtained for the coal metagenome were analyzed for taxonomic and functional characterization [16]. The taxonomic classification was done using Reference Sequence (RefSeq) database through MG-RAST server and the microbial richness was determined using best hit classification with a maximum e-value cut off of $1e^{-5}$, a minimum percent identity of 60% and a minimum alignment length cut off of 45. The publicly accessible MG-RAST accession code generated for the coal metagenome was 4516404.3.

2.3. Bioinformatics Analysis

MG-RAST is user friendly, web-based server which is usually used to compare genomic data obtained from environmental samples. This server is supported by the SEED and KEGG framework for better accessibility of structural and functional attributes. MG-RAST allows genomic data comparison by providing various approaches to investigate the taxonomic and metabolic aspects of different metagenomes.

2.3.1. Taxonomic Assignment of the Metagenomic Reads

The taxonomic assessment of quality checked metagenomic sequences were performed using the RefSeq database on the MG-RAST server. The cut-off e-value of $1e^{-5}$ and alignment length with 45 bases, and a sequence identity percent cut off of 60% was assigned to examine the metagenome. The taxonomic analysis for the euryarchaeal community was examined up to species level *via* the RefSeq database. The MG-RAST accession code created for the coal metagenome is 4516404.3 and allowed for worldwide access.

2.3.2. Functional Analysis Using MG-RAST

Metagenomics is a leading approach for examining the microbial diversity obtained from environmental samples.

Obtained sequences, as a result of pyrosequencing, were analyzed for metabolic functions on MG-RAST. The cut-off e-value of $1e^{-5}$ and alignment length with 45 bases, with sequence identity percent cut off of 60%, was assigned to examine the functional attributes related to the coal metagenome. The energy metabolism level was examined to understand various metabolisms related to the coal microbiome via Clusters of Orthologous Groups (COGs) database.

3. RESULTS AND DISCUSSION

3.1. Rarefaction Analysis

The plot (Fig. 1) shows the rarefaction curve of annotated species richness. This curve is a plot of the total number of distinct species annotations as a function of the number of sequences sampled. On the left, a steep slope indicates that a large fraction of the species diversity remains to be discovered. If the curve becomes flatter to the right, a reasonable number of individuals are sampled: more intensive sampling is likely to yield only a few additional species. Sampling curves generally rise very quickly at first and then leveled off towards an asymptote as fewer new species are found per unit of individuals collected.

3.2. Taxonomic Diversity

In the current study, taxonomic assignment for the euryarchaeal community was accomplished at the strain level for coal metagenome via the RefSeq database in MG-RAST based on average percentage identity. The data set of coal contains 78,088 sequences totaling 48,674,212 basepairs with an average length of 623 bps. Of the sequences tested, 26,111 sequences (33.44%) failed to pass the QC pipeline. Out of those, dereplication identified 26,036 sequences as artificial duplicate reads. Out of the sequences that passed QC, 1,400 sequences (3%) contain ribosomal RNA genes, 37,234 sequences (71.75%) contain predicted proteins with known functions, and 13,261 sequences (25.55%) contain predicted proteins with unknown function. The functional

categories with predicted protein functions were annotated to the category metabolism (42%), cellular processes and signaling (21%), information storage and processing (18%), and remaining were poorly characterized (19%) for the COG database.

3.2.1. Alpha Diversity

The α -diversity of this data set is 612 species. Alpha diversity summarizes the diversity of organisms in a sample with a single number. The α -diversity of annotated samples can be estimated from the distribution of the species-level annotations. Annotated species richness is the number of distinct species annotations in the combined MG-RAST data set. The species-level annotations are from all the annotation source databases used by MG-RAST.

3.2.2. Archaeal Diversity

The classified sequences were affiliated to a single archaeal phylum Euryarchaeota via the RefSeq database (Fig. 2). The predominance of *Archaeoglobus fulgidus* was obtained in the coal metagenome, suggesting enhanced sulfate reduction in hyperthermophilic conditions [17]. Sequence identity to *Halalkalicoccus jeotgali* was obtained, which is being reported in this study for the very first time (Table 1). The coal metagenome revealed the presence of genus *Thermococcus* and *Pyrococcus* under the family Thermococcaeae (Fig. 3A).

A sequence similarity match was observed for *Pyrococcus horikoshii*, which is an anaerobic hyperthermophilic chemoorganotroph [18]. Also, the predominance of *Pyrococcus furiosus* and *Pyrococcus abyssi* was found in the metagenome (Table 1). *Pyrococcus furiosus* has been reported to be a potential candidate for rubber desulfurization in anaerobic conditions, whereas *P. abyssi* is an anaerobic, hyperthermophilic sulfur metabolizing archaeon, which was first reported to be isolated from hydrothermal vents of North Fiji basin [19-21]. Sequence match was found for *Thermococcus kodakarensis* of order Thermococcales, which

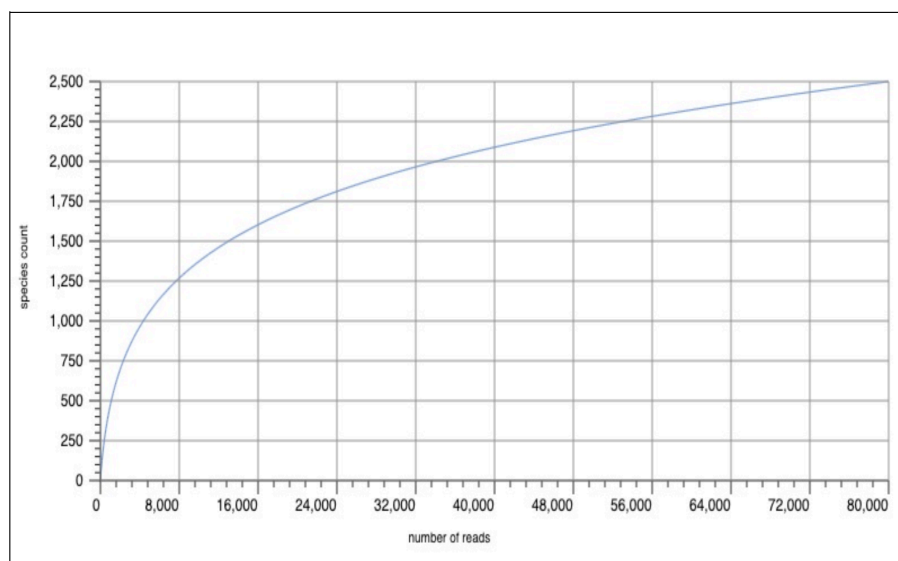


Fig. (1). Rarefaction analysis of coal metagenome. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

is an anaerobe and obligate heterotroph growing in the presence of organic sulfur [22]. The presence of *Thermococcus barophilus* was also observed in the metagenome, which is barophilic, thermophilic, anaerobic, and sulfur metabolizing archaeon [23].

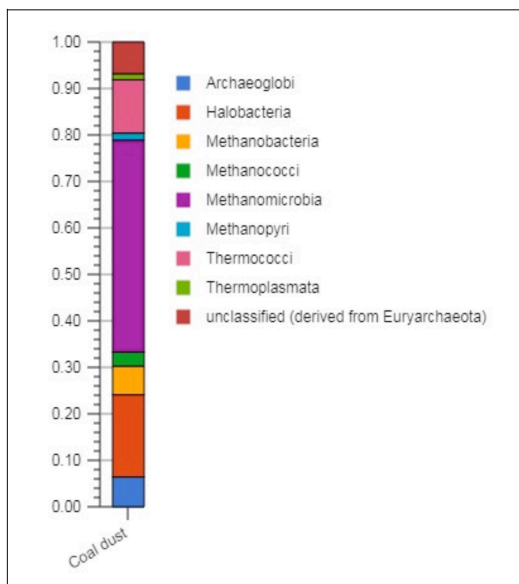


Fig. (2). Stacked bar chart revealing the euryarchaeal diversity in the metagenome. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Sequences were assigned to the known strains of *Methanocella paludicola*, *Methanococcoides burtonii*, *Methanoculleus marisnigri*, *Methanoregula boonei*, *Methanosarcina thermophila*, *Methanosarcina acetivorans*, *Methanosarcina barkeri*, *Methanosphaerula palustris*, *Methanospirillum hungatei*, and *Methanothermobacter thermautotrophicus* (Table 2). Methanobacteriaceae and Methanothermaceae are families under the order Methanobacteriales (Fig. 3B). The family Methanobacteriaceae contains two mesophilic genera *Methanobacterium*, and *Methanosphaera*, along with one extremely thermophilic genus *Methanothermobacter* (Table 2). The coal metagenome indicated the presence of families, such as Methanomicrobiaceae, Methanospirillaceae and Methanosectaceae and Methanosarcinaceae, under Methanomicrobiales and Methanosarcinales, order respectively (Fig. 3C). The presence of *Methanobacterium* and *Methanosarcina* has been found in a study by Yang *et al.* [24] in lignite. The sequence correspondence obtained for *Methanosphaera* and *Methanothermobacter* suggests the predominance of mesophilic to thermophilic conditions in the subsurface microbiome. Hence, the results indicated the prevalence of the hydrogenotrophic methanogenesis under the mesophilic to thermophilic conditions.

3.3. Functional Diversity

The conventional culture-dependent techniques, including 16S rRNA gene based studies, have not been successful in examining structural and functional aspects of the coal microbiome [11, 12]. Hence, employing metagenomic techniques can bypass the cultural dependent approaches to have a further deeper understanding of the subsurface microbi-

ome. Coal being a complex substrate, is considered as substrate and primary organic carbon source for microbial bio-conversion [25]. The community functional process related inorganic ion transport and metabolism under metabolism (Fig. 4). The open reading frames (ORFs) obtained for the genes include the probable presence of sulfite reductase α -, β - subunit. Sequence similarity was found for dissimilatory sulfite reductase α , β , and γ subunits in the metagenome, suggesting sulfite reduction to form hydrogen sulfide in the microbiome (Table 3). The presence of *Archaeoglobus fulgidus* in the microbiome further helps in finding the connection to sulfite reduction *via* dissimilatory sulfite reductase. Sequence matching to that of peptide methionine sulfoxide reductase has been found in the coal metagenome. The report reveals that methionine sulfoxide reductase catalyzes the thioredoxin dependent reduction and is designed to be functional in suboptimal temperature for *T. kodakaraensis* [26].

Sequence match was obtained for formylmethanofuran dehydrogenase, formylmethanofuran: tetrahydromethanopterin formyltransferase, heterodisulfide reductase A and B subunit, 5, 10-methylene-tetrahydrofolate dehydrogenase and 5, 10-methylenetetrahydrofolate reductase (Table 3). These genes are specific for hydrogenotrophic methanogens for catalyzing CO₂ reduction to methane. The taxonomic abundance for euryarchaea suggests the predominance of hydrogenotrophic methanogenesis with respect to the functional abundance obtained for the genes responsible for methane production.

3.3.1. Challenges for Methane Production

One of the most important challenges for methanogens to generate methane is provided by sulfate reducers. The sulfate reducers primarily convert acetate, propionate, and butyrate into hydrogen sulfide and carbon dioxide *via* exergonic reaction having δG° value of -47kJ/ Mol, -37kJ/ Mol and -27kJ/ Mol, respectively. However, methanogens produce methane either *via* hydrogenotrophic or acetoclastic pathway having δG° value of -33kJ/ Mol and -31kJ/ Mol, respectively [27]. This suggests the possibility of both methane and hydrogen sulfide formation with carbon dioxide or sulfur/ sulfate as a terminal electron acceptor under anoxic conditions. A study by Harada *et al.* [28] suggested higher sulfate concentration (30-600 mg SO₄²⁻) restricted methane production due to greater electron flow to sulfate reducers. The present study provides higher hits for methanogens with respect to the SRBs, indicating a positive impact on methanogenesis due to thermodynamic advantages (Tables 1 and 2). Initial reports by Zengler *et al.* [29] suggested the possibility of increased energy recovery from oil and sediment reservoirs using methanogenic consortium for methane production from an alkane. Energy recovery from fossil fuel can be done using microbial consortium *via* bioaugmentation or biostimulation. Biostimulation is one of the most widely utilized techniques for enhancement in methane production from coal bed seams [30]. To overcome the energy crisis and utilization of secondary and tertiary fossil fuel reservoirs, microbial stimulation relating methanogenesis has been implemented by a wide number of profitable undertakings, including Luca Technologies, Next Fuel, and Ciris Energy for the successful commercial outcome on a pilot scale.

Table 1. List of species relating sulfur reducing archaea in coal microbiome.

Domain	Class Name	Species	No. of Hits
Archaea	Archaeoglobi	<i>Archaeoglobus fulgidus</i>	14
		<i>Archaeoglobus profundus</i>	2
	Halobacteria	<i>Halalkalicoccus jeotgali</i>	8
		<i>Haloarcula marismortui</i>	5
		<i>Halobacterium salinarum</i>	6
	Thermococci	<i>Pyrococcus abyssi</i>	5
		<i>Pyrococcus furiosus</i>	8
		<i>Pyrococcus horikoshii</i>	10
		<i>Thermococcus barophilus</i>	3
		<i>Thermococcus gammatolerans</i>	2
		<i>Thermococcus kodakarensis</i>	7
		<i>Thermococcus onnurineus</i>	3
	<i>Thermococcus sp. AM4</i>	2	

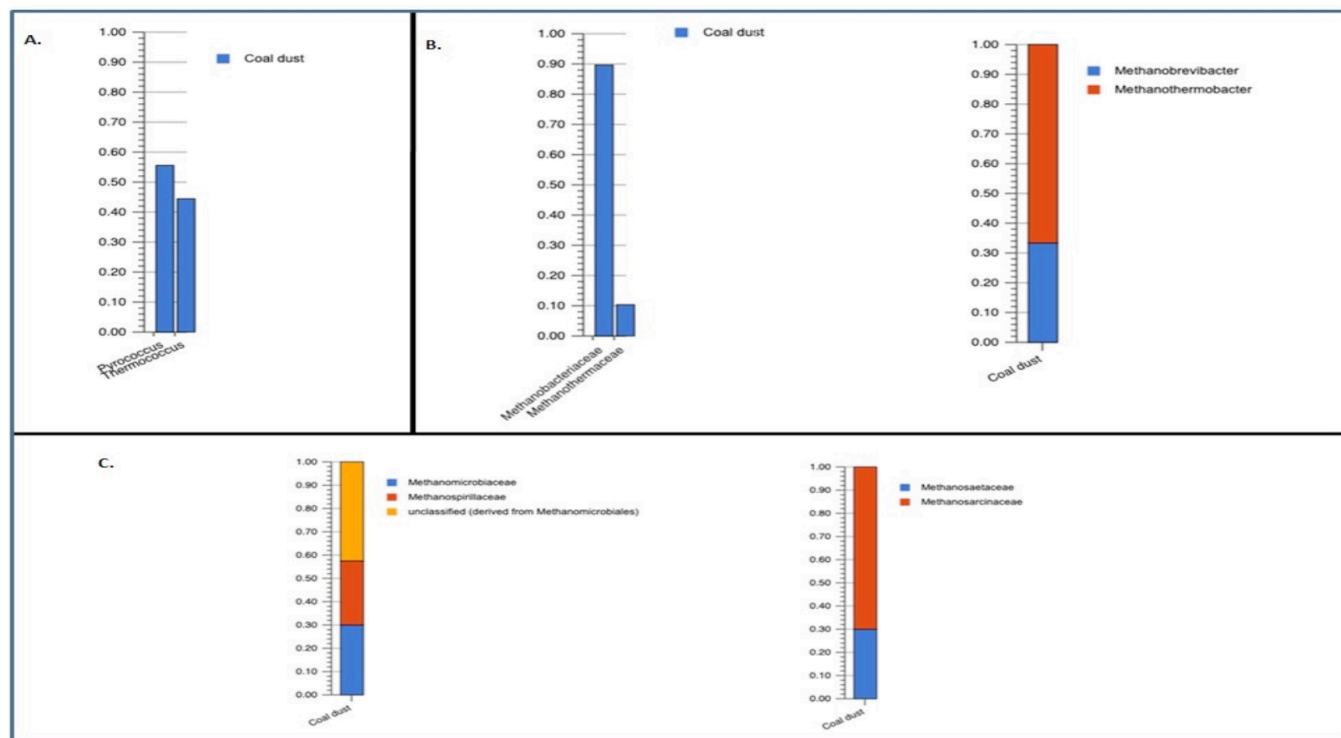


Fig. (3). A. Barchart showing genus distribution in Thermococcaceae family; B. Barchart showing family distribution in Methanobacteriales order and genus distribution in Methanobacteraceae class; C. Stacked barchart revealing family distribution in Methanomicrobiales and Methanosarcinales order. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 2. List of methanogens available in the coal microbiome.

Domain	Class Name	Species	No. of Hits
Archaea	<i>Methanobacteria</i>	<i>Methanobrevibacter ruminantium</i>	1
		<i>Methanobrevibacter smithii</i>	3
	-	<i>Methanothermobacter marburgensis</i>	2
	-	<i>Methanothermobacter thermautotrophicus</i>	10
	-	<i>Methanothermus fervidus</i>	3
	-	<i>Methanosphaera stadtmanae</i>	2
	<i>Methanococci</i>	<i>Methanocaldococcus fervens</i>	2
		<i>Methanocaldococcus jannaschii</i>	1
	<i>Methanomicrobia</i>	<i>Methanocella paludicola</i>	6
		<i>Methanococcoides burtonii</i>	7
		<i>Methanocorpusculum labreanum</i>	2
		<i>Methanoculleus marisnigri</i>	18
		<i>Methanohalobium evestigatum</i>	4
		<i>Methanohalophilus mahii</i>	5
		<i>Methanoplanus petrolearius</i>	5
		<i>Methanoregula boonei</i>	30
		<i>Methanosarcina acetivorans</i>	14
		<i>Methanosarcina barkeri</i>	18
		<i>Methanosarcina mazei</i>	13
		<i>Methanosphaerula palustris</i>	13
		<i>Methanospirillum hungatei</i>	22
	<i>Methanosaeta thermophila</i>	14	
	<i>Methanococci</i>	<i>Methanococcus aeolicus</i>	1
<i>Methanococcus maripaludis</i>		3	
<i>Methanopyri</i>	<i>Methanopyrus kandleri</i>	4	

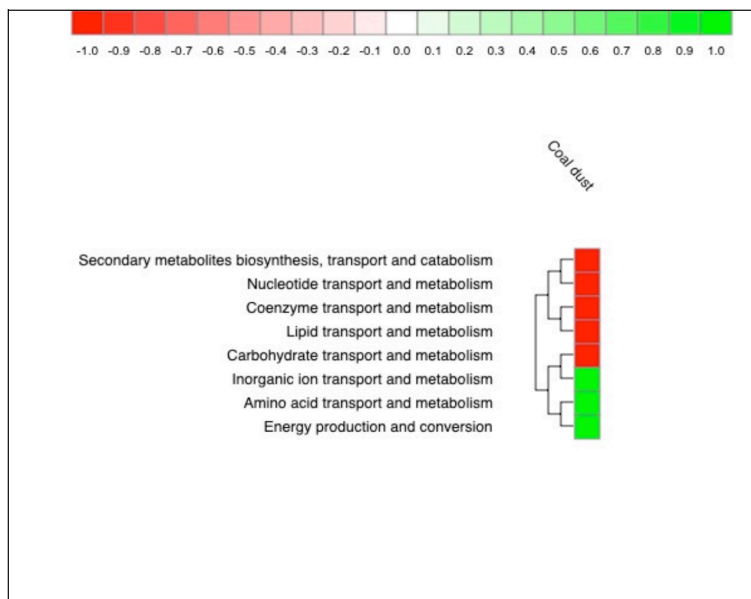


Fig. (4). Heatmap showing the functional distribution of coal metagenome under the metabolism section. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 3. List of functional attributes related to the coal metagenome.

Domain	Level	Function	No. of Hits
Metabolism	Energy production and conversion	Dissimilatory sulfite reductase, alpha and beta subunits	4
		Heterodisulfide reductase, subunit A and related poly ferredoxins	14
		Heterodisulfide reductase, subunit B	2
		Formylmethanofuran dehydrogenase subunit A	3
		Formylmethanofuran: tetrahydromethanopterin formyl-transferase	2
	Inorganic ion transport and metabolism	Sulfite reductase, alpha subunit (flavoprotein)	4
		Sulfite reductase, beta subunit (hemoprotein)	12
		Dissimilatory sulfite reductase, gamma subunit	4
		Formyltetrahydrofolate hydrolase	3
Nucleotide transport and metabolism	Formyltetrahydrofolate synthetase	9	
Cellular Processes and Signaling	Posttranslational modification, protein turnover, chaperones	Peptide methionine sulfoxide reductase	20

CONCLUSION

The potential of coal degrading microbes has not been completely reported primarily due to the lack of evidence relating to microbial metabolic pathways in the microbiome. Ongoing research relating to subsurface microbiome and metabolic processes might help to develop strategies for improved methane production in coal bed methane. Metagenomics, the study of microbial communities directly from environments in which they survive, has become the leading approach of today. To the best of our knowledge, this study represents the first reported set of results for coal metagenome to better understand potential taxonomic and functional diversity relating to euryarchaea. The predominance of *Archaeoglobus*, *Pyrococcus* and *Thermococcus* indicates probable association to sulfate reduction of coal organics in hyperthermophilic conditions and methanogens, such as *Methanospaera* and *Methanothermobacter*, suggesting the possibility of hydrogenotrophic methanogenesis under mesophilic to thermophilic conditions. However, higher hits were obtained for methanogens indicating a positive impact on methanogenesis due to thermodynamic advantages on SRBs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the [MG-RAST server] at [<https://www.mg-rast.org/mgmain.html?mgpage=analysis#>], reference number [4516404.3]”.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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