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3-бөлім

МОЛЕКУЛАЛЫҚ БИОЛОГИЯ ЖӘНЕ ГЕНЕТИКА

Раздел 3

МОЛЕКУЛЯРНАЯ БИОЛОГИЯ И ГЕНЕТИКА

Section 3

MOLECULAR BIOLOGY AND GENETICS

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METAGENOMIC ANALYSIS OF MICROBIAL COMMUNITY IN COAL SAMPLES FROM KAZAKHSTAN USING ILLUMINA NGS TECHNOLOGY

The development of micro- and biotechnological processes for fossil energy utilization has received increasing attention in recent years. There are abundant coal resources in Kazakhstan; in particular, low-rank coal resources of lignite and leonardite. These coal types are not exploited commercially due to their low energetic power. However, they are considered as a rich source of humic substances (HS). The HS in the soil play an important role in physical and chemical quality, carbon capture and stabilization and in the inactivation of pesticides, heavy metals, as well as other polluting agents. Bioprocessing of lignite also involves the production of clean energy.

Research on coal microbes is essential for microbial ecology and applied microbiology with regard to the sustainable utilization of coal resources. Nevertheless, the inability of culturing vast amount (around 99%) of microorganisms in vitro counteract the research procedures. Currently, there is tremendous advances in using non-culturing techniques based on omics to the examination of microbial diversity of environmental compartments, such as soil, sediment, minerals, etc. Different omics tools, including FISH, SIP, next generation sequencing (NGS), microarray, mass spectrometry, etc., evolve instant results to provide comprehensive insight of the coal microbiome.

This paper discusses the findings and challenges in the study of Kazakhstan coal microbes, highlighting Illumina NGS platform. Based on the results of the metagenomic analysis of coal samples (Oikaragai, Lenger, Karaganda, Yekibastuz), 10 taxonomic groups of bacteria belonging to Proteobacteria, Tenericutes, Actinobacteria, Firmicutes, Bacteroidetes, Nitrospirae, Chloroflexi, Gemmatimonadetes, Acidobacteriaand Fusobacteria were identified and analyzed.

Key words: lignite, leonardite, microbial diversity, microbial community, metagenomics, Illumina Miseq sequencing

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Illumina заманауи технологиясын қолдана отырып қазақстандық көмір үлгілеріндегі микробтық қауымдастықтың метагеномикалық анализі

Соңғы жылдары қазба энергоресурстарын қайта өңдеу үшін биотехнологиялық процестерді дамытуға баса назар аударылуда. Қазақстанда көмір қоры жеткілікті екендігі жалпыға мәлім, соның ішінде, сапасы төмен қоңыр және тотыққан қоңыр көмір түрлері көптеп кездеседі. Қоңыр көмір (лигниттер) энергетикалық құндылығы төмен болғандықтан өнеркәсіпте кеңінен пайдаланылмайды. Сонымен қатар, олар гуминді заттардың (ГЗ) ең бай көзі болып табылады, сондықтан көміртегіні бірқалыпты ұстап тұру және тұрақтандыру, пестицидтерді, ауыр металдарды және басқа да ластағыш заттарды инактивациялау сияқты физика-химиялық процестерде маңызды рөл атқарады. Лигнитті биоөңдеу таза энергияны өндіруді де қамтиды.

Көмірдің микробтық алуантүрлілігін зерттеу микробтық экология мен көмір ресурстарын тұрақты пайдалану үшін қолданылатын микробиологияның жалпы міндеті болып табылады. Табиғи жағдайларда тіршілік ететін микроорганизмдердің 99%-ы зертханалық тәжірибеде пайдаланылатын қоректік орталарда in vitro жағдайында өсе алмайтындығы дәлелденді. Қазіргі кезде қоршаған ортаның нысандарын, мысалы, топырақ, жауын-шашын, минералдар және т.б. сияқты микробтық алуантүрлілікті зерттеуге арналған омикаларға негізделген дәстүрлі емес әдістерді қолдану үлкен мүмкіндіктер туғызуда. FISH, SIP, заманауи секвенирлеу (NGS), микрочип, масс-спектрометрия және т.б. әртүрлі әдістер арқылы жедел нәтиже алуға, сонымен қатар көмірдің микробтық пейзаждарының құрылымы мен күйі туралы пайдалы ақпараттармен қамтамасыздандырады.

Бұл жұмыста Illumina NGS технологиялық платформасы пайдаланылып, қазақстандық көмір үлгілерінің микробтық әртүрлілігінің нәтижелері көрсетілген және әрі қарай талқыланады. Қоңыр көмірлердің (Ойқарағай, Ленгір, Қарағанды, Екібастұз) метагеномды сараптамасы бойынша 10 таксономиялық топқа Proteobacteria, Tenericutes, Actinobacteria, Firmicutes, Bacteroidetes, Nitrospirae, Chloroflexi, Gemmatimonadetes, Acidobacteria және Fusobacteria жататын бактериялар идентификацияланды және сараланды.

Түйін сөздер: қоңыр көмір, тотыққан көмір, микробтық алуантүрлілік, микробтық қауымдастық, метагеномика, Illumina Miseq секвенирлеу.

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Метагеномный анализ микробного сообщества в образцах казахстанского угля с использованием технологии секвенирования нового поколения Illumina

В последние годы все большее внимание уделяется разработке биотехнологических процессов для утилизации ископаемых энергоресурсов. Как известно, в Казахстане достаточно угольных ресурсов, в том числе, низкокачественных бурых и окисленных бурых углей. Бурые угли (лигниты) не находят широкого применения в промышленности из-за их низкой энергетической ценности. В то же время, они являются наиболее богатыми источниками гуминовых веществ (ГВ),

и потому играют важную роль в физико-химических процессах, происходящих при улавливании и стабилизации углерода, инактивации пестицидов, тяжелых металлов и других загрязняющих веществ. Биообработка лигнита также включает производство чистой энергии.

Изучение микробного разнообразия углей является общей задачей экологической и прикладной микробиологии для эффективного использования угольных ресурсов. Доказано, что до 99% микроорганизмов, обитающих в природных условиях, не способны расти на питательных средах in vitro, используемых в лабораторной практике. В настоящее время имеются огромные возможности в использовании некультуральных методов, основанных на омикс-технологиях для изучения микробного разнообразия объектов окружающей среды, таких как почва, осадки, минералы и т.д.

В данной работе представлены и обсуждаются результаты микробного разнообразия проб угля казахстанских угольных месторождений, в которых применяется платформа технологии Illumina NGS. По результатам метагеномного анализа образцов угля (Ойкарагай, Ленгер, Караганда, Экибастуз) были идентифицированы и проанализированы 10 таксономических групп бактерий, принадлежащих к Proteobacteria, Tenericutes, Actinobacteria, Firmicutes, Bacteroidetes, Nitrospirae, Chloroflexi, Gemmatimonadetes, Acidobacteria и Fusobacteria.

Ключевые слова: бурый уголь, окисленный уголь, микробное разнообразие, микробное сообщество, метагеномика, секвенирование IlluminaMiseq.

Introduction

Due to the limited oil and gas resources worldwide, coal will become the most important energy source. With the consumption of 2007, in terms of volume, coal reserves will be maintained for 146 years, while oil will be exhausted within 50 years and natural gas within 63 years. After depleting oil and gas reserves, the coal will monopolize the entire fossil energy market. Obviously, coal, especially lignite (brown coal), which accounts for 47.3% of the world's coal reserves, will become an important research issue (Yong et al., 1995: 437-47).

At present, 96,4% of the world's brown coal output is obtained by burning electricity and heat energy. It is a serious environmental pollution. The harmful substances released by the combustion of lignite are mainly sulfur oxides (SOx) and nitrogen oxides (NOx), carbon dioxide, and some trace elements. Statistical results for 2005 show that in Canada25% of sulfur oxides, 10% of nitrogen oxides and 17,2% of carbon dioxide present in atmosphere. All come from the combustion of lignite; in China, 87% of sulfur oxides, 67% of nitrogen oxides and 71% of carbon dioxide is derived from the burning of lignite (Xu et al., 2000: 153-160).Therefore, in terms of environmental protection, combustion is not a suitable technique, currently; chemical methods are mainly used for gasification, liquefaction as fuel or chemicals instead of oil substances to achieve lignite. The conversion is not effective, as it is carried out under high temperature and high-pressure conditions, high-energy consumption, harsh reaction conditions, and cost.

Due to the fact, new conversion technologies are urgently needed to achieve the clean utilization of lignite. Bioconversion technology uses microbes to transform solid lignite into clean, cost-effective products and energy. In contrast, microorganisms have mild conditions of action, simple methods, low equipment, and, more importantly, are environmentally friendly.

At the same time, the complex structure of lignite also indicates that the implementation of this method can encounter greater difficulties. Lignite biotransformation has opened up a new way for efficient and clean use of coal, and it has become a research subject. Most lignite have high ash (about 30%), high moisture (20-50%), low calorific value (about 14 MJ·kg-1), low ash melting point, poor thermal stability, and susceptibility to spontaneous combustion, etc. (Yuan et al., 2002: 13 – 17; Dai et al., 1998: 4-7; Nakagawa et al., 2004: 719-725),meanwhile, it is considered a humic substance (HS) rich source.

HSs are polyelectrolyticmacromolecules which play a crucial role in global C and N cycling and in the regulation of the plant nutrients mobility and environmental contaminants (Weber: 1988: 165–78; Murphy et al., 1995: 103-24; Christl et al., 2000: 617-25). The HS are important for physical and chemical quality of soil, carbon capture and its stabilization (Piccolo et al., 2004: 329-343), and in the neutralization of pesticides, heavy metals, and other polluting agents (Bandeiraet al., 2009: 78-91). HSs also stimulate plant growth (Badis et al., 2009: 997-1007), as they induce root proliferation, and stimulate root system (Barros et al., 2010: 3681-3688). It was reported that some microbes couldgrow on coal and modify it with both non-enzymatic and enzymatic processes by producing humic acid and water-soluble humic material,

including fulvic acids (Fakoussa, 1981: 634-642; Cohen et al., 1982: 437–47).

Research on biological processes for the utilization of fossil energy has received increasing attention in recent years. Microbial treatment has been considered as an economically effective and environmentally safe way of processing low-rank coals via degradation of the macromolecular network into simpler molecular products (Fakoussa et al., 1999: 25-40; Gupta et al., 2000: 103-5; Helena et al., 2002: 17-23). Thus, one of the advantages of biotechnological processing of coalsis to detect, identify and enumerate the microbiota, potential for bioconversion of lignite and leonardite.

There are abundant lignite and leonardite resources in Kazakhstan and coal with low calorific value and high ash content is piled up as rubbish causing a serious waste of resources as well as environmental pollution. Kazakhstan needs to acquire new technologies for coal processing, especially green-based approaches. Certainly, the micro- and biotechnological means of coal processing has a number of advantages, which dictates the need to study microbial diversity of coal as an essential source of environmentally friendly energy and products (Crowford et al., 1991: 577-80; Polman et al., 1995: 249-55; Yong et al., 1995: 437-47; Davison et al., 1990: 447-56).

Relatively little studies have been conducted to evaluate microbiota of Kazakhstan coal. However, no research has addressed the microbial community diversity and structure using novel culture-free molecular techniques, especially Illmina MiSeq sequencing. The present work is intended togenerate an inventory of the microbial diversity, particularly differences in the distribution of specific taxonomic bacterial groups in coal samples by means of Illumina Miseq approaches.

Materials and Methods

Coal samples. Thelignite and leonarditesamples werecollected in four points of Kazakhstan coal deposits. The top layer of 1,5-2,0 cm coal removed with a sterile knife and 500-600 gams of lignitesamples gathered with sterile spatula to the depths of 30 cm. The leonardite samples were placed in a sterile container and transported to the laboratory. Each sample waslabeled indicating the date and sample number. During transportation and storage of coal samples the rules have been followed in order to prevent the possibility of secondary pollution. The coal sampling points are shown in Fig.1.

No.1. Leonardite “KLE”: Karagandy
No.2. Lignite “KLI”: Karagandy
No.3. Lignite “LLI”: Lenger
No.4. Leonardite “LLE”: Lenger
No.5. Leonardite “OLE”: Oikaragai
No.6. Lignite “OLI”: Oikaragai
No.7. Lignite: “YLI”: Yekibastuz

Sequencing:

1. Extraction of genome DNA. Total genome DNA from samples was extracted using CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μL using sterile water (Hess et al., 2011: 463-467; Avershina al, 2013: 211-216).

2. Amplicon Generation. 16S rRNA genes of distinct regions (16SV4 were amplified used specific primer (e.g.16S V4: 515F-806R) with barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, England).

3. PCR Products quantification and qualification. The same volume of 1X loading buffer (contained SYB green) with PCR products were mixed and detected on 2% agarose gel electrophoresis. Samples with bright main strip between 400-450 bpwere chosen for further experiments.

4. PCR Products cleanup and purification. PCR products were mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

5. Library preparation and sequencing. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated.

Data analysis

1. Paired-end reads assembly and quality control

1.1 Data split. Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence.

1.2 Sequence assembly. Paired-end reads were merged using FLASH (V1.2.7,http://ccb.jhu.edu/software/FLASH/) (Caporaso et al., 2011: 4516-4522), a very fast and accurate analysis tool,which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment, and the splicing sequences were called raw tags.

Figure 1 – The location and number of coal (lignite and leonardite) sampling points

1.3 Data filtration. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags (Youssef et al., 2009: 5227-5236) according to the QIIME(V1.7.0,http://qiime.org/index.html) (Hess et al., 2011: 463-467) quality controlled process.

1.4 Chimera removal. The tags were compared with the reference database(Gold database,http://drive5.com/uchime/uchime_download.html)using UCHIMEalgorithm(UCHIME Algorithm,http://www.drive5.com/usearch/manual/uchime_algo.html) (Asnicar et al., 2015: 1029)to detect chimera sequences, and then the chimera sequences were removed (DeSantiset al., 2006: 394-399). Then the Effective Tagsfinally obtained.

2. OTU cluster and Species annotation

2.1 OTU Production. Sequences analysis were performed by Uparse software (Uparse v7.0.1001) (Ondovet al., 2011: 385). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation.

2.2 Species annotation. For each representative sequence, the GreenGene Database(http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (Bulgarelliet al., 2015: 392-403) was used based on RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) (Liet al., 2013: 4207-4216) algorithmto annotate taxonomic information.

2.3 Phylogenetic relationship Construction. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples(groups), multiple sequence alignment were conducted using the MUSCLE software (Version 3.8.31http://www.drive5.com/muscle/) (Lundbergeret al., 2013: 999-1002).

2.4 Data normalization. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

3. Beta Diversity.

Beta diversity analysis was used to evaluate differences of samples in species complexity, Beta diversity on both weighted and unweighted unifrac were calculated by QIIME software (Version 1.7.0).Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software(Version 2.15.3). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of

weighted or unweighted unifrac among samples obtained before it was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Unweighted Pair-group Method with Arithmetic Means(UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (Version 1.7.0).

Results and Discussion

Currently the challenge in isolating the whole microbial biodiversity is not conditional with traditional methods of cultivation, but this approach allows the clearest and most detailed study of the microbial structure and stepwise characteristics, particularly the functional groups of microorganisms.

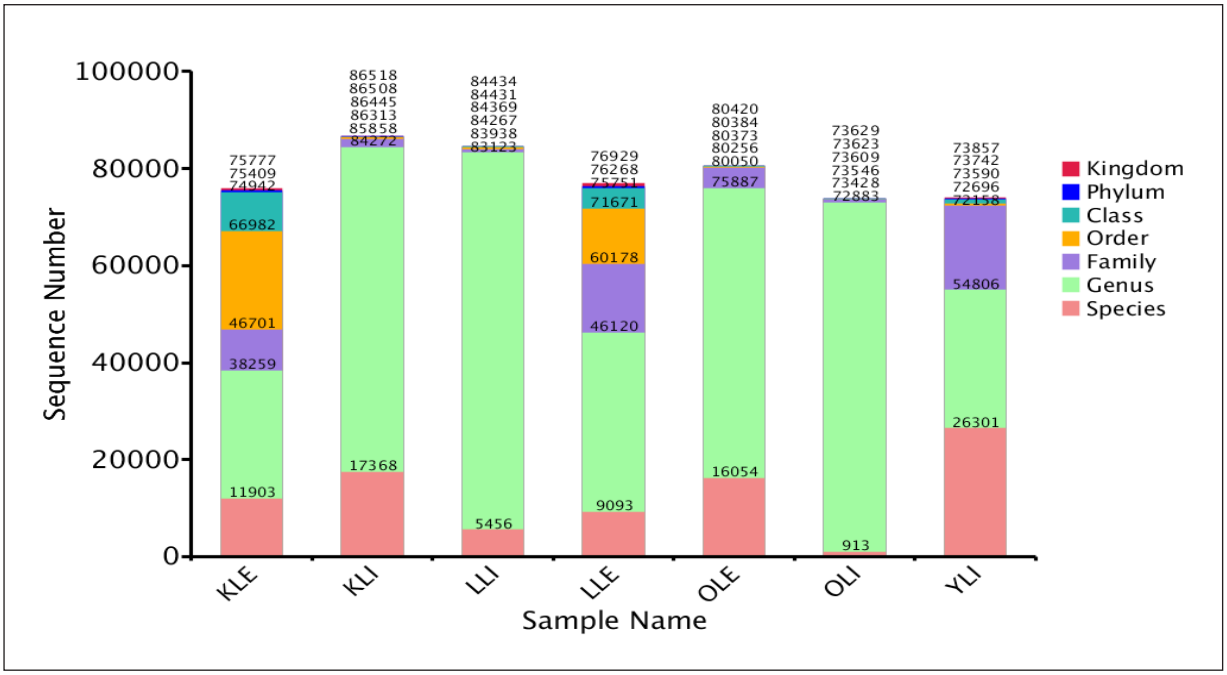

Figure 2 – The statistical amount of sequences of each coal sample at various classification level (Y-axis: the tags number of each classification level, X-axis: the samples name)

In our study, in general, 75777, 86518, 84434, 76929, 80420, 73629 and 73857 sequences were obtained from the seven coal samples, indicated as KLE, KLI, LLI, LLE, OLE, OLI and YLI. As a result, the abundance of bacterial species sequences (BSS) was considerably higher in samples of KLI, LLI and OLE; while lower BBS was observed in OLI and YLI samples (Fig.2.)

(Total Tags number (red) indicates the splicing sequence number. The taxon tags (blue) indicates the number of Tags for building OTUs. Unclassified Tags (green) refers to the building OTUs but without classified information access. Unique Tags (yellow) refers to the frequency is 1, but cannot be clustering to the OTUs. Number of OTUs (purple) refers to the Number of OTUs finally received) (Fig.3.).

It is known that operational taxonomic units (OTUs) can be constructed by clustering sequences *de novo*, essentially based on their similarity, which is computationally much more intensive. Here, in order to study the species composition of each sample, the Effective Tags of all the samples were clustered into OTUs with 97% identity, and species annotations were then performed on the representative sequences of OTUs.

Of those sequences, total 77015, 87037, 84761, 78590, 81889, 73885 and 78690 Tags, and 1019, 709, 628, 1212, 682, 551 and 799 OTUs were found in KLE, KLI, LLI, OLE, OLI and YLI, respectively.

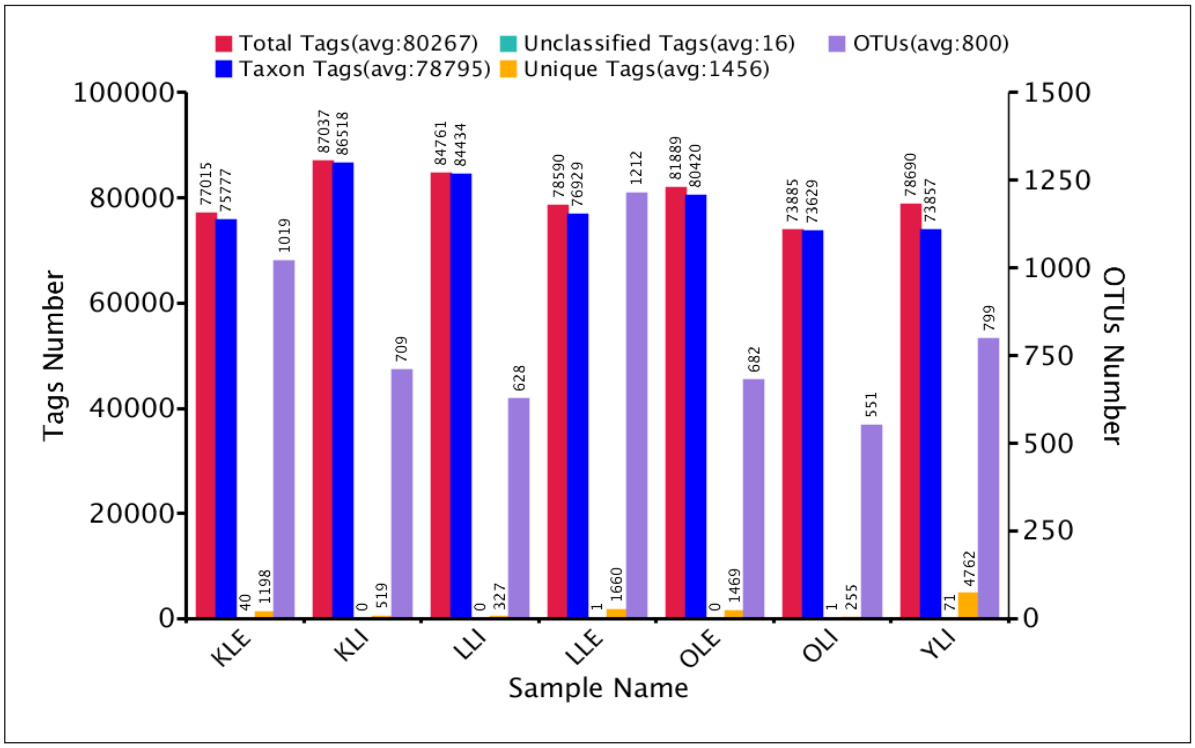

Figure 3 – Tags and OTUs number statistics of coal samples

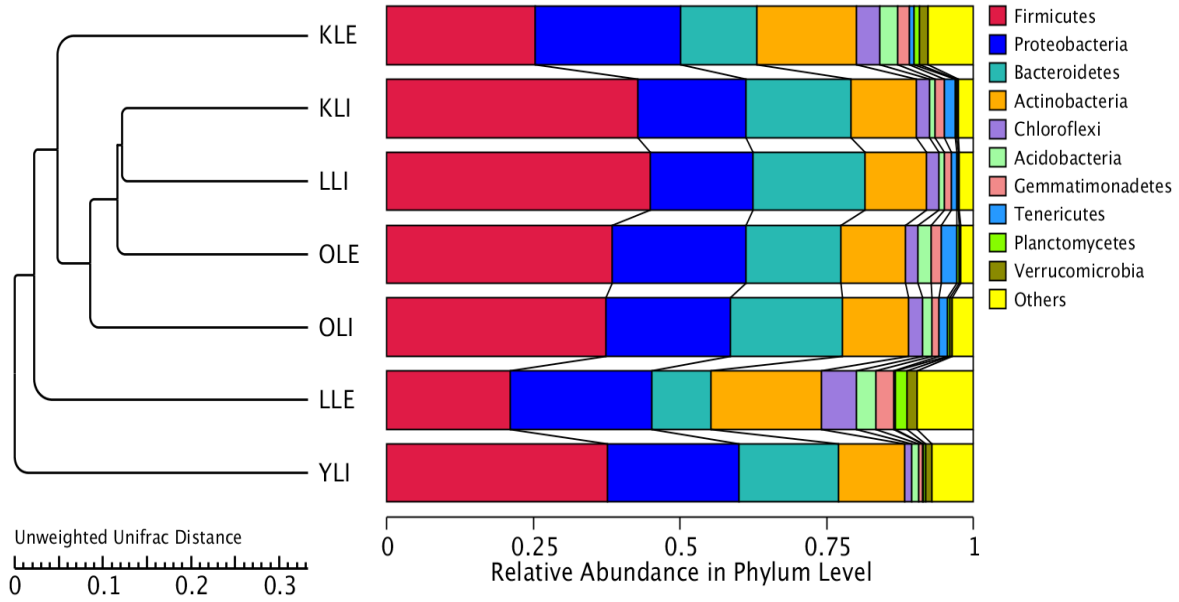

Figure 4 – Hierarchical cluster analysis of coal microbial community

In order to study the resemblance between different coal samples, clustering analysis are used to build a clustering tree. In environmental microbiology, UPGMA (Unweighted Pair-group Method with Arithmetic Mean) is a more commonly adapted cluster technique, which was first used to solve the classification puzzle. Thismethod was used to visualize the interrelationship of test samples. The distance matrices were detected through Unifrac analyses (Fig.4.).

Top ten bacterial genera abundance in phylum level of seven samples were also compared (Fig.5.).

In general, top 10 bacterial phyla were identified in coal samples, with *Proteobacteria, Tenericutes*and *Actinobacteria* mainly being dominant among samples. The relative abundance of *Proteobacteria*, the greatest plentiful phylum in the samples, ranged from 20,3% to 95,5% of the total bacterial 16S rDNA gene sequences. *Actinobacteria* was the second abundant phylum in the samples of KLE, LLE and YLI with a relative abundance of 25,3 – 43,0%. The relative abundance of *Tenericutes* was rich in OLE, showing 65,4%. The relative abundance of *Firmicutes* and *Bacteroidetes*were around 3,2-45%, *Nitrospirae* phylum showed 20% abundance only in LLP. *Chloroflexi, Gemmatimonadetes, Acidobacteria* and *Fusobacteria* were not significant in all samples.

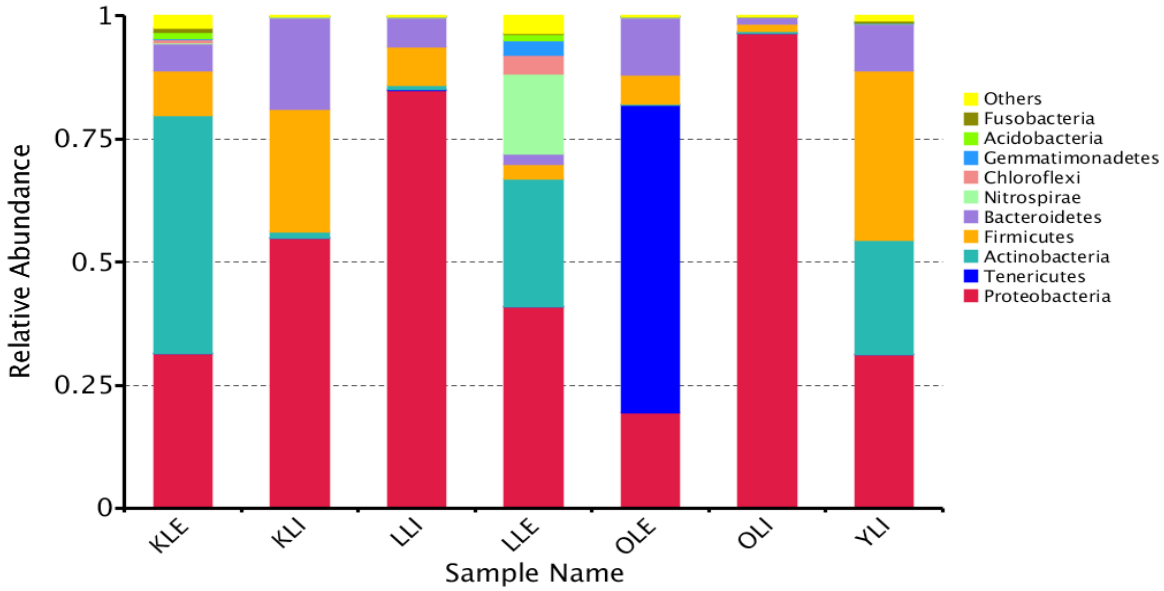

Figure 5 – Relative abundance of various bacterial phyla in coal samples

Moreover, some previous studies also found that *Proteobacteria, Actinobacteria* and *Firmicutes*were also among the most abundant phyla in the wastewater from coal-mining industry analyzed by Illumina high-throughput sequencing (Lozuponeet al., 2005: 8228-8235).

A total of 100 bacterial genera were identified via taxonomic summary. Among the all coal samples, the relative abundance of *Phyllobacterium, Pseudarthrobacter* and *Leptospirillum*were observed, while *Candidatus_Bacilloplasma* showed maximum level in OLE.(Fig.6-8.).

A large majority of *Phyllobacterium* are plant-associated nitrogen-fixing bacteria and occupy diverse ecological niches (Avershinaet al., 2013: 211-216). Their great variety of habitats suggests that these genera have evolved essential adaptive properties to the environment. Additionally, their nonpathogenic status and their ability to promote plant growth has made them attractive.

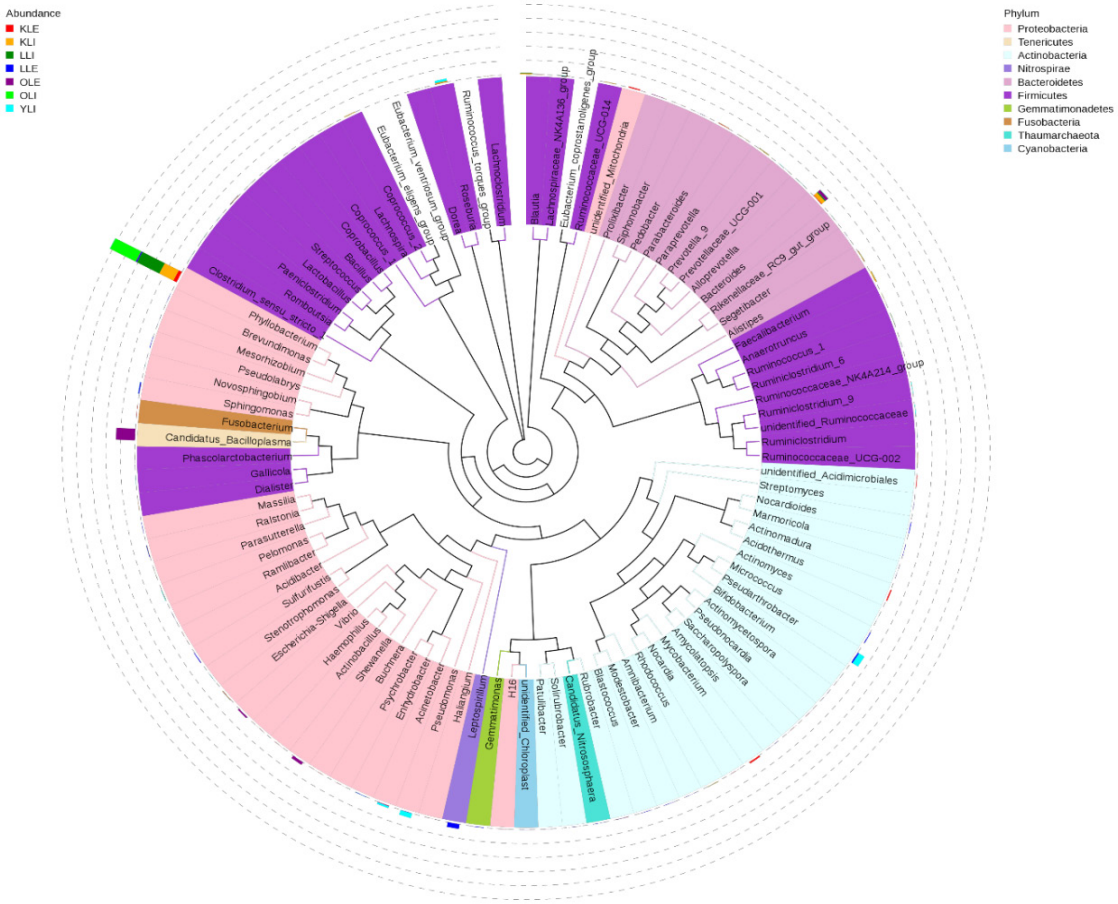

Figure 6 – Major phyla/genera of each coal samples

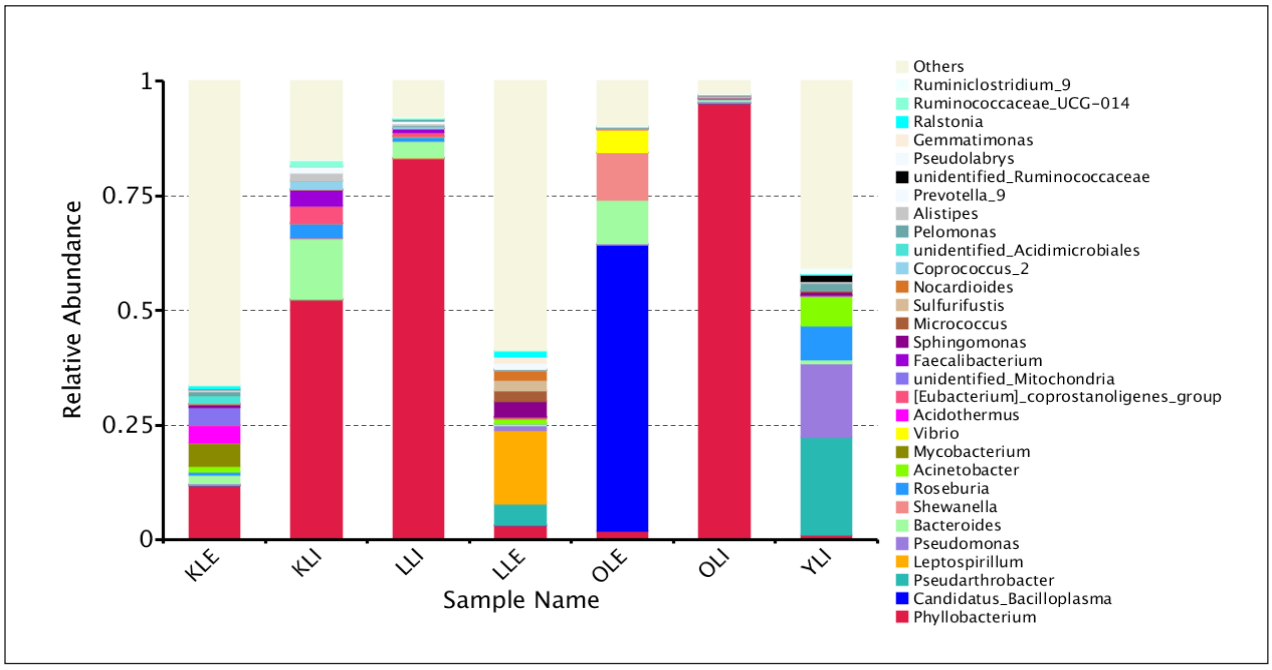

Figure 7 – Relative abundance of bacterial genera in coal samples

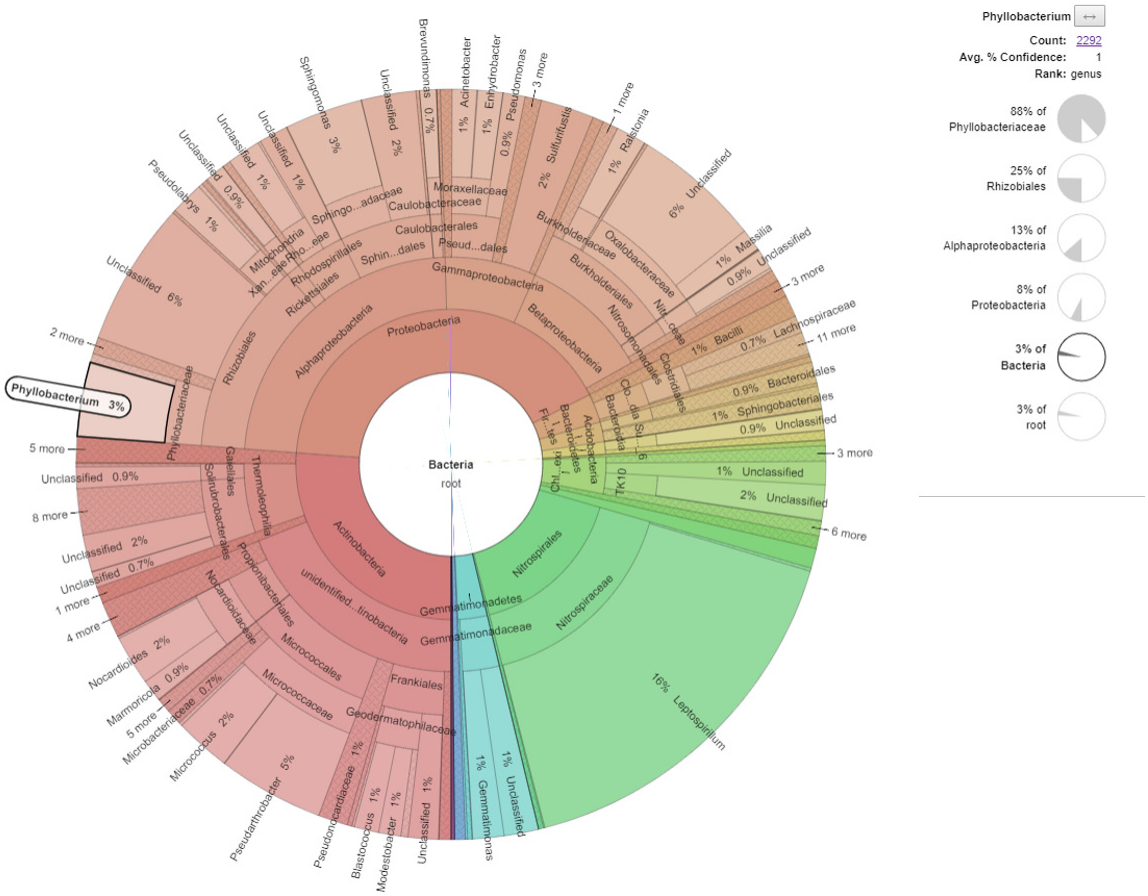

Figure 8 – Krona taxonomy web visualization illustrated by the case of *Phyllobacterium*

Conclusion

Currently, there is a steady trend towards a transition to systemic biology, which is strongly pronounced in the field of ecology of microbial communities. Recognition of the impossibility of considering an integral system from the point of view of the properties of its individual components allowed a completely different look at the approaches used in biological research.

The main advantage of the metagenomic approach is its comprehensive framework – it allows to study not only the functions of individual components of the microbiome, but also the integral roles of the microbial community as a whole, treating it as a set of interacting microorganisms. The conducted in-depth work in this field reveled several fundamentally and crucial features of microbiota in Kazakhstan coal samples.

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