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ISOLATION AND IDENTIFICATION OF COAL ACCLIMATED MICROORGANISMS FROM THE ACTIVATED SLUDGE

Today, Kazakhstan is among the top ten countries in the world in terms of coal production, which is estimated at 38 billion tons. The processing of low-quality coal is a hot topic that requires careful scientific research, since it is one of the main energy carriers of organic origin and is now considered a promising source of raw materials for value-added products, like biofuels or biofertilizers. To increase the bioavailability of coal, various approaches are used, including pre-treatment of coal with exogenous microorganisms. This article demonstrates a method for acclimatizing bacteria from activated sludge, which can then be used for a community of microorganisms as a pre-treatment of coal to improve its bioavailability. Microorganisms adapted to coal were isolated and identified based on the analysis of the 16S rRNA gene, which showed belonging to *Enterobacter bugandensis 247*, *Lysinibacillus macroides LMG 18474*, *Acinetobacter pittii DSM 21653*, *Achromobacter insolitus LMG 6003*, *Achromobacter denitrificans NBRC 15125* strains. Microorganisms actively grew in synthetic cultural media E8, where the only source of carbon was coal. This indicated that acclimatization was successful and in the future this method can be used for acclimatization of exogenous microbial communities.

Key words: coal, bioavailability, bacteria, activated sludge, acclimatization, biodegradation, bioconversion.

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Белсенді тұнбадан көмірге бейімделген микроорганизмдерді оқшаулау және идентификациялау

Бүгінгі таңда Қазақстан көмір өндіру бойынша көлемі 38 миллиард тоннаға бағаланатын, әлемнің алғашқы ондығына кіреді. Төмен сапалы көмірді қайта өңдеу-мұқият ғылыми зерттеуді қажет ететін өзекті тақырып, өйткені ол органикалық тектес негізгі энергия тасымалдаушылардың бірі және қазіргі уақытта биоотын немесе био тыңайтқыш сияқты қосымша құнды өнімдер үшін перспективалы шикізат көзі болып саналады. Көмірдің биожетімділігін арттыру үшін әртүрлі тәсілдер қолданылады, соның ішінде көмірді экзогендік микроорганизмдермен алдын-ала өңдеу. Бұл мақалада белсенді тұнба бактерияларын көмірге бейімдеу әдісі көрсетілген, содан кейін оны микроорганизмдер қауымдастығы үшін көмірдің биожетімділігін арттыру үшін алдын ала өңдеу ретінде пайдалануға болады. 16s рРНҚ генінің талдауы негізінде көмірге бейімделген *Enterobacter bugandensis 247, Lysinibacillus macroides LMG 18474, Acinetobacter pittii DSM 21653*, *Achromobacter insolitus LMG 6003*, *Achromobacter denitrificans nbrc 15125* түрлеріне жататын микроорганизмдер анықталған. Микроорганизмдер E8 синтетикалық қоректік ортасында, белсенді түрде өсті, онда көміртектің жалғыз көзі көмір болды, бұл сәтті акклиматизацияның нәтижесі болды.

Түйін сөздер: көмір, биожетімділік, бактериялар, белсенді тұнба, акклиматизация, биодеградация, биоконверсия.

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Выделение и идентификация акклиматизированных к углю микроорганизмов из активного ила

На сегодняшний день Казахстан входит в первую десятку стран мира по добыче угля, которая оценивается в 38 миллиардов тонн. Переработка низкокачественного угля — актуальная тема, требующая тщательного научного исследования, поскольку он является одним из основных энергоносителей органического происхождения и в настоящее время считается перспективным источником сырья для продуктов с добавленной стоимостью, таких как биотопливо или биоудобрения. Для повышения биодоступности угля применяют различные подходы, в том числе предварительную обработку угля экзогенными микроорганизмами. В данной статье демонстрируется метод акклиматизации бактерий из активного ила к углю, который затем можно использовать для сообщества микроорганизмов в качестве предварительной обработки угля для повышения его биодоступности. Выделены и идентифицированы адаптированные к углю микроорганизмы на основе анализа гена 16S рРНК, который показал принадлежность к штаммам *Enterobacter bugandensis 247, Lysinibacillus macroides LMG 18474, Acinetobacter pittii DSM 21653, Achromobacter insolitus LMG 6003, Achromobacter denitrificans NBRC 15125*. Микроорганизмы активно росли в синтетической культурной среде Е8, где единственным источником углерода был уголь, что являлось показателем успешной акклиматизации.

Ключевые слова: уголь, биодоступность, бактерии, активный ил, акклиматизация, биодеградация, биоконверсия.

Introduction

Due to their low energetic power, low rank coals (LRC) such as peat, leonardite, and lignites are not commercially utilized. Coal bioconversion technology has the ability to convert low-grade and discarded coal into either clean, cost-effective energy raw materials or into value-added goods [1]. Coal treatment methods include physical, chemical, and bio-treatments [2]. It has been demonstrated that the application of alkali and another chemical treatments can disrupt and reestablish distinct coal macromolecule connections and forces [3,4].

Microbial technology will outperform physical and chemical coal processing technologies. Apart from being easier and requiring fewer technical instruments, microorganisms have no environmental impact when compared to typical chemical catalyst particles. Compared with physical methods, the microbiological method has several advantages, namely: the process can be carried out under conditions of atmospheric temperature and pressure, and does not require external energy, and microbial degradation does not produce nitrogen oxides and sulfur oxides making it more environmentally friendly [5].

There are quite a few works devoted to the study of the effectiveness of the use of indigenous an exogenous microorganism for the biodegradation of coal [6-8]. For example, fungal systems have been identified that can modify the structure of coal by various mechanisms [9]. Moreover, because of the alkaline chemicals emitted, bacteria and actinomycetes can represent an effective and inexpensive potential for coal degradation [10,11]. Also, some plant growth-promoting rhizobacteria (PGPR) have been experimentally shown to have coal solubilizing characteristics [12]. Bioaugmentation with coal-acclimated microorganisms from activated sludge could be a strong tool for improving coal degradation processes, such as coal solubilization. For instance, in the majority of investigations on the bioconversion of coal to methane, local microorganisms have been utilized [13-15]. However, it has also been discovered that some exogenous microbial communities were just as more effective than the native populations at converting coal to methane [16,17]. Activated sludge is a mixture of biomass of various microorganisms that is used for wastewater treatment. The bacterial diversity of activated sludge consists of many different functional bacterial groups like aerobic heterotrophic bacteria, nitrate reducers, sulfate-reducers, ammonia-oxidizers, nitrite-oxidizers and etc. [18]. Acid-producing bacteria and methanogenic bacteria are also parts of activated sludge community and the important bacterial groups which are participated in the bioconversion process [19].

Due to the metabolism of so many diverse microorganisms, in particular bacterial taxa, organic compounds, and contaminants such as household waste, pharmaceuticals, pesticides can be degraded

[20]. This feature of activated sludge can be used as a technique to stimulate coal bioconversion.

In this paper, the method of the acclimatization of microorganisms from activated sludge was studied, and microorganisms adapted to the coal were isolated and identified to demonstrate the potential of using microorganisms for successful bioaugmentation for coal degradation.

Materials and Methods

In this study, coal samples from the Oi-Qaragai deposit were used. Coal sampling was carried out manually according to certain rules from the standard document "Hard coal and coke. Manual sampling and ISO 13909-4:2016 Preview Hard coal and coke. Mechanical sampling. Part 4. Coal. Sample preparation for testing" (GOST 10742-71). The coal was crushed to a powder state with a particle size of less than 150 μm, in laboratory conditions, using a grinder. The pulverized coal samples were then dried and stored in a sealed bag for further experiments.

The activated sludge (AS) sample was obtained from a wastewater treatment system in KazNU campus, Almaty, Kazakhstan.

For the acclimatization of AS microorganisms, a modified Ashby's medium [21] was used which contain 60 g of activated sludge, 9 g of glucose, 15 g calcium carbonate, 3 g of yeast extract, 0,6 g of sodium chloride, 0,6 g sodium dihydrogen phosphate, 0,6 g magnesium sulfate and 0,3 g potassium sulfate were added to 3 L of distilled water.

For the isolation of bacteria from AS, which can grow up in coal and convert coal was used synthetic media E8. The composition of E8 media: 2,1 g potassium dehydrogen phosphate, 4,5 g diammonium hydrogen phosphate, 2,4 g magnesium sulfate, 1,5 g sodium chloride, 60 g coal and 3 L of distilled water.

Adapting the AS microorganisms to the coal

For the adaptation of microorganisms was used the method of Wang et al. where to the 10 g AS were added 2,5 g of coal powder with a particle size of less than 150 μm. For continuous acclimatization over a period of 28 days, 0,5 g of coal powder with a particle size of less than 150 m was added every 3 days, and 0,5 g, 0,25 g, and 0,1 g of glucose were added sequentially every 7 days. This was followed by continuous aeration at 35°C [22].

Isolation and identification and of acclimatized bacteria from sludge

Due to the isolation of microorganisms acclimatized to the coal, microorganisms were checked after 28 days on E8 media. 16S rDNA provides sufficient information and contains 10 conservative areas and 9 hypervariable regions (V1-V9), according to a PCR identification test. Five different strains isolated from activated sludge adapted to coal were used in the work.

The universal primer for bacteria 16s (27f/1492r) was used for genomic DNA sequencing, the product was amplified, after that all the results were compared and analyzed. The bacterial genome was extracted using the traditional phenol-chloroform method. The primer set consists of two primers. Primer 27f has a sequence of 5-AGAGTTTGATCCTGGCTCAG-3, while primer 1492r has a sequence of 5-CTACGGCTACCTTGTTACGA-3. The target fragment was seen under blue light after the electrophoresis of the amplification results on 1.5% agarose gel, and the gel was then removed and purified.

The ABI3730-XL sequencing tool was employed to identify the microorganisms. To determine the accurate information regarding the classification of species, all sequencing sequences were checked with the NCBI 16S database and a search was carried out in the NCBI taxonomy database. Aoke Biogel Recovery Kit was used for PCR product recovery. Using the BLAST algorithm, the derived nucleotide chains of bacteria were discovered.

Results and discussion

After 28 days of cultivation, the microflora was observed under a microscope (Microoptix MX-50, Austria) at 100× magnification. On fig.1 it can be seen that the larger black particles (~10 μm) are coal, and the smaller bacilli or coccoid particles (<10 μm) are microbes that randomly and actively rotate/ move around the coal particles. It is noticeable that after inoculation and subsequent acclimatization, the culture medium is rich in microorganisms, mainly bacilli and cocci (Figure 1).

This indicates that the process of acclimatization of microbial cultures to the environment in the presence of coal was successful in obtaining adapted microbial communities.

The microbiological analysis of the obtained samples was carried out by inoculation on a synthetic medium E8 (Figure 2). The growth of colonies adapted to coal was determined on petri dishes with a synthetic medium. The results showed that the samples contain bacteria that use coal as their only source of energy.

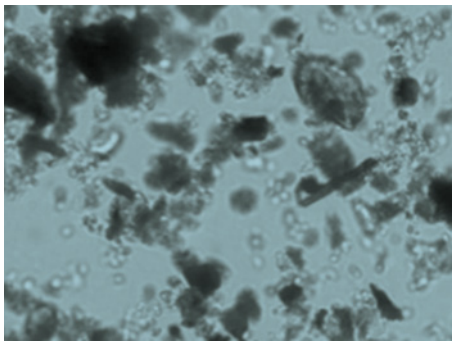


Figure 1 − Microscopy of microbial samples after 28 days (× 100)

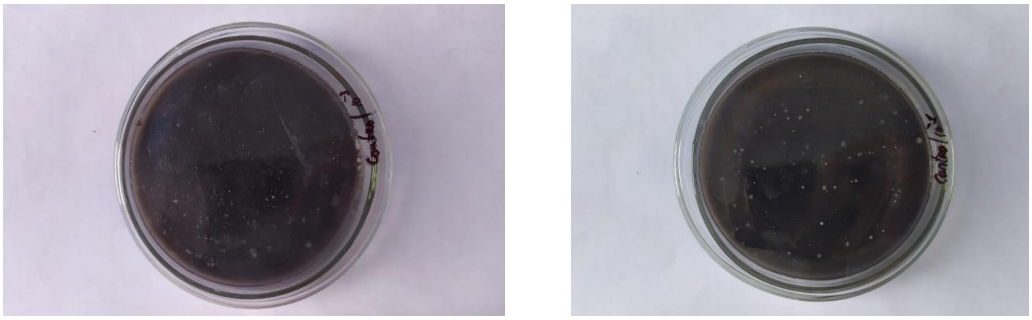
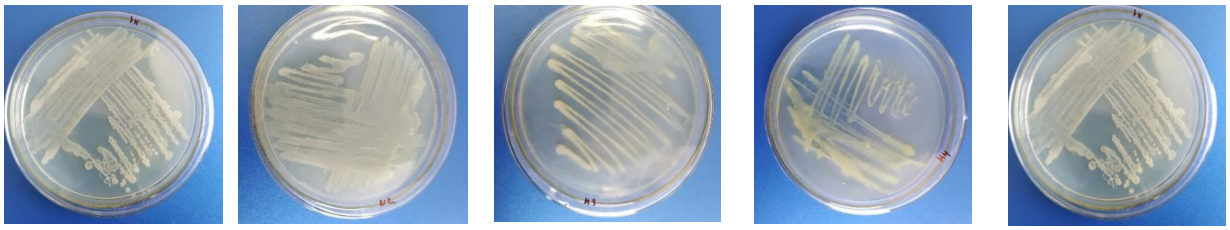


Figure 2 − Colonies of microorganisms isolated from activated sludge after its acclimatization to the coal

There are colonies of round shaped, white and yellowish, creamy and shiny colonies with smooth edges of small size. The number of live bacteria in 1 dose (0,01 ml) growing on E8 is $5{,}8 \times 10^5$ CFU/mg.

For further research and identification, five different strains of microorganisms were selected (Figure 3), the fastest growing strains, and well-grown bacterial monocultures for 24-48 hours.



M1 M2 M3 M4 M5

Figure 3 − Isolation of pure culture microorganisms by streak plate method

In the process of identifying microorganisms to a species, morphological and cultural properties were first studied. The morphological properties of bacterial cultures grown on solid nutrient media were studied under a microscope (Microoptix MX-50, Austria) at 100× magnification.

Microscopy and staining methods were used on the basis of purpose of the study. As a result of staining to determine the morphological structure of cells of isolated bacteria, it was found that microorganisms are gram-negative cocci and gram-positive cocci bacillus (Fig.4).

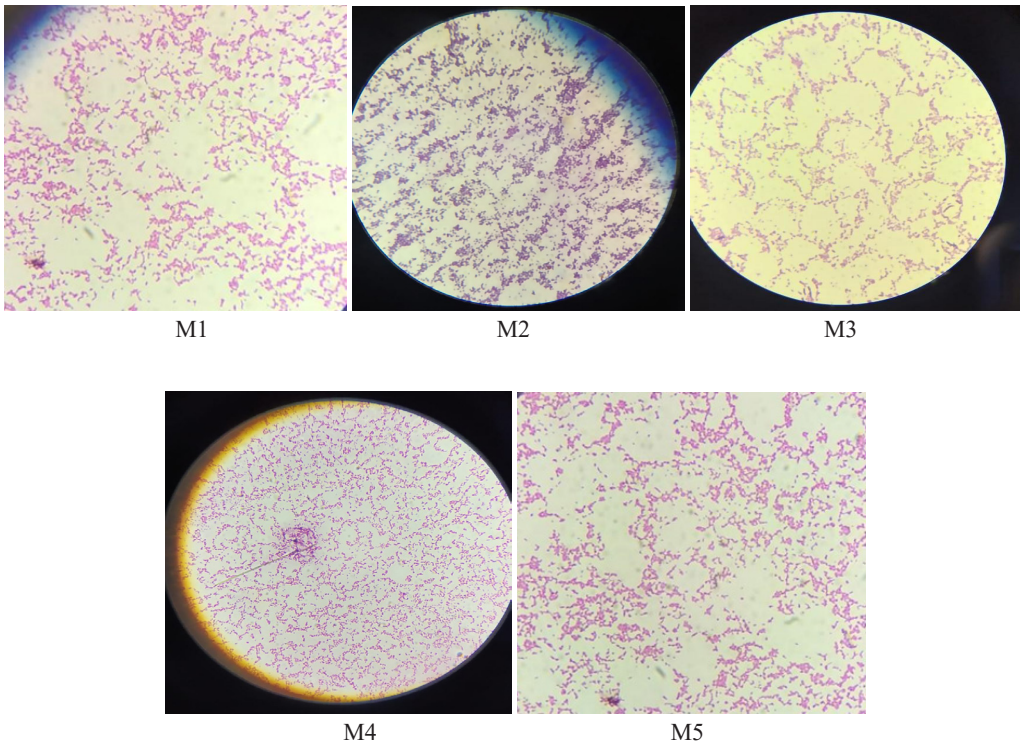


Figure 4 – Morphological characteristics of microorganisms
M1- diplococci, gram positive, 0,7 µm; M2-cocci bacilli, gram negative, 0,7 µm; M3- cocci, gram positive, 0,5µm; M4- cocci, gram positive, 0,5µm; M5 – cocci -0,5µm, gram positive (× 100)

The table below displays the outcomes of strain identification of microorganisms using the ABI3730-XL sequencer (Table 1). The most pertinent information regarding species categorization was then obtained by searching the NCBI taxonomy database and comparing the outcomes with the NCBI 16S database.

Table 1 – Results of identification by the method of nucleotide sequence analysis

The strain name	The sequence nucleotide chains	Name of strain	% identity
M1	ACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGAC-TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGT-CAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTA-CGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCT-GCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTT-GACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTG-CACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCT-GCGGGTAACGTCAATCGACAAGGTTATTAACCTCATCGCCTTCCTCCCC-GCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCT-GCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAG-GAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGAC-CAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTA-ATCCCATCTGGGCACATCTGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTG-GTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTC-CATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCACTCGTCACCCGAGA-GCAAGCTCTCTGTGCTAC	*Enterobacter bugandensis 247*	99.42

Table continuation

The strain name	The sequence nucleotide chains	Name of strain	% identity
M2	AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCG-GTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGATGGCAACTAA-GATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC-GAGCTGACGACAACCATGCACCACCTGTCACCGTTGCCCCCGAAGGGGAAAC-TATATCTCTACAGTGGTCAACGGGATGTCAAGACCTGGTAAGGTTCTTC-GCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGT-CAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCT-TAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACT-CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC-GCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC-CACTGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACACTTGGAATTC-CACTTTCCTCTTCTGCACTCAAGTCCCCCAGTTTCCAATGACCCTCCACG-GTTGAGCCGTGGGCTTTCACATCAGACTTAAAGGACCGCCTGCGCGC-GCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCG-GCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAATAAGGTACCGTCAAGGTA-CAGCCAGTTACTACTGTACTTGTTCTTCCCTTACAACAGAGTTTTACGATCC-GAAAACCTTCTTCACTCACGCGGCGTTGCTCCATCAGGCTTTCGCCCATTGTG-GAAGATTCCCTACTGCTGCCTCCCG	*Lysinibacillus macroides LMG 18474*	99.07
M3	TAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGC-GACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGA-CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC-GCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTA-AAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTG-GACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG-GTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGCGC-GTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATT-GCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGT-GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGC-CATCTGGCCTAACACTGACGCTGAGGTGCGAAAGCATGGGGAGCAAA-CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCC-GTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACC-GCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCC-GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT-TACCTGGCCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTC-GGGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCTCG	*Acinetobacter pittii DSM 21653*	99.63
M4	CTTTCGTGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATC-GGTGTTCCTCCGCATATCTACGCATTTCACTGCTACACGCGGAATTC-CACCTCCCTCTGACACACTCTAGCTCGGTAGTTAAAAATGCAGTTCCAAAGT-TAAGCTCTGGGATTTCACATCTTTCTTTCCGAACCGCCTGCGCACGCTT-TACGCCCAGTAATTCCGATTAACGCTTGCACCCTACGTATTACCGCGGCT-GCTGGCACGTAGTTAGCCGGTGCTTATTCTGCAGGTACCGTCAGTTTCGC-GGGGTATTAACCCACGACGTTTCTTTCCTGCCAAAAGTGCTTTACAACCC-GAAGGCCTTCATCGCACACGCGGGATGGCTGGATCAGGGTTTCCCCCATT-GTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCT-CAGTCCCAGTGTGGCTGGTCGTCCTCTCAAACCAGCTACGGATCGTCGCCTTG-GTGAGCCGTTACCCCACCAACTAGCTAATCCGATATCGGCCGCTCCAATAGTG-CAAGGTCTTGCGATCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTAC-GCTTTCGCGTAGTTATCCCCCGCTACTGGGCACGTTCCGATACATTACTCACCC-GTTCGCCACTCGCCACCAGACCGAAGTCCGTGCTGCCGTTCGACTTGCATGT-GTAAGGCATCCC	*Achromobacter insolitus LMG 6003*	99.44
M5	ACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGC-CGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA-AAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTA-ACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAG-GTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACAC-CGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGC-GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGAT-GTCAACTAGCTGTTGGGGCCTTCGGGCCTTGGTAGCGCAGCTAACGCGT-GAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATT-GACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGC-GAAAAACCTTACCTACCCTTGACATGTCTGGAATCCTGAAGAGATTTAGGAGT-GCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGT-GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTT-GCTACGA	*Achromobacter denitrificans NBRC 15125*	98.81

After the 16S ribosomal RNA gene sequences of the isolates were sequenced and compared to the NCBI database, the isolates were identified as follows: Enterobacter bugandensis 247 (99.42% match), Lysinibacillus macroides LMG 18474 (99.07% match), Acinetobacter pittii DSM 21653 (99.63% match), Achromobacter insolitus LMG 6003 (99.44% match), and Achromobacter denitrificans NBRC 15125 (98.81% match).

Discussion

Microbial community optimization or bioaugmentation is necessary to increase the production of value-added products, such as methane or hydrogen, from the complete biodegradation of coal. An effective method for the degradation of lignite by foreign microorganisms using lignite as a substrate and isolated by acclimation has been reported, where, for example, bioaugmentation has proven to be an effective strategy for stimulation of gas production [23].

The bacteria contained in the activated sludge were successfully adapted, because in the adaptation method, coal as a source of carbon was added gradually, and each time increasing the concentration, the bacteria adapted to the new environmental conditions. It can be assumed that the isolated bacteria are able to solubilize coal and, using the organic matter of coal, they provide themselves with a source of carbon. Since activated sludge accumulates various microorganisms whose metabolism includes a wide range of chemical reactions [24], activated sludge can be used to effectively reduce the recalcitrance of coal.

Adapted and isolated activated sludge microorganisms play important role in the environment as degraders. *Acinetobacter sp.* strains act as the best decomposers for bioremediation of oil-contaminated sites [25]. It was also found that when using *Acinetobacter pitti*. showed a high degree of solubilization of brown coals [26]. *Achromobacter insolitus* participates in the bioremediation of polyaromatic hydrocarbons [27]. Achromobacter denitrificans is also mainly used for the bioremediation soils contaminated with heavy metals [28]. Other strains of *Lysinibacillus sp and Enterobacter sp.* are of ecological importance for the agroecosystem and participate in the cycle of metals [29,30]. Moreover, the identification of isolated microorganisms from activated sludge contributes to a more in-depth study of the biodiversity of the environmental microbial community. Strains of microorganisms adapted to coal can be used in further experiments to study the degree of biosolubilization of coal using these strains.

Conclusion

In conclusion, a method of acclimatization of microorganisms from activated sludge to coal was tested. The current study helped to isolate and identify new bacterial strains in the activated sludge sample. Coal adapted 5 strains have been isolated and identified, based on the analysis of the 16S rRNA gene. These included the strains of *Enterobacter bugandensis 247, Lysinibacillus macroides LMG 18474, Acinetobacter pittii DSM 21653, Achromobacter insolitus LMG 6003, and Achromobacter denitrificans NBRC 15125*. The activated sludge microorganism acclimatization method is an economical and environmentally friendly method. It is suitable method for bioaugmentation in a strategy for optimization the bioconversion of coal in a value-added product. The acclimatization method can be used for the microbial community of activated sludge in field conditions, to stimulate the formation of methane or hydrogen gases in coal deposits. Pure cultures of microorganisms can be used in laboratory conditions as producers of humic acids as a result of coal biosolubilization. In the future, a detailed study of their biochemical properties is required.

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