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Metagenomic Analysis of Microbial Communities from Coal Waste

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Abstract. Coal waste contains few macro-and micronutrients, which makes it less likely to become a growth site for microorganisms. One way to screen coal waste resources is to identify the diversity of microbes in them to study the relationship between these microbial communities in contributing to improving environmental pollution using metagenomics to determine microbial diversity. The purpose of this research is to study the diversity of microorganisms in areas contaminated with heavy metals using a metagenomic method. This study was performed using next-generation sequencing techniques, including DNA extraction, 16S rRNA amplification, and gene sequencing analysis. The results of this research found that the 10 most commonly found species were *Baekduia soli*, *Nocardioides iriomotensis*, *Nocardioides mesophilus*, *Nocardioides pakistanensis*, *Propionibacterium cyclohexanicum*, *Solirubrobacter ginsenosidimutans*, *Gemmatirosa kalamazoonensis*, *Roseisolibacter agri*, *Kosakonia sacchari*, and *Dickeya fangzhongdai*. Based on this research, it can be concluded that most of the microbial communities from coal waste are dominated by the phylum Actinobaceria. The results of this study can be used as an adaptive microbial germplasm for industrial waste management strategies.

Keywords: Coal waste; Metagenomic; *Next Generation Sequencing;* 16S rRNA

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INTRODUCTION

Steam Power Plant (PLTU) activities as a contributor to electrical energy leave behind large amounts of waste every year. The increase in the supply of electrical energy through the construction of new PLTUs has been correlated with an increase in the volume of coal waste produced. When coal waste is not optimally utilized, it becomes an environmental pollution problem (Permatasari et al., 2023). Coal ash is used as a landfill material for mine openings that have entered the mine-out period (Ullah et al., 2020).

Coal ash is still categorized as category 2 hazardous and toxic waste (B3) from specific sources according to Government Regulation No. 101 of 2014, which requires special handling u. Heavy metals are a type of pollutant that has received considerable attention in environmental management. Coal ash contains heavy metals; therefore, it requires serious attention because it is used in landfill materials, particularly in the operation of PLTU (Wibowo et al., 2018). The poor content of macro-and micronutrients in coal waste makes it less likely to become a place of growth for microorganisms, especially bacteria. The presence of bacteria in polluted conditions is very good, particularly as bioremediation agents or agents for reducing the content of dangerous metals in coal ash waste (Kapahi & Sachdeva, 2019).

The level of waste pollution in coal ash *landfills* can be determined by identifying the bacteria found in the area. The more types of bacteria present in coal ash waste, the lower the level of pollution because bacteria that are able to live in polluted environments originating from coal ash can utilize the contents of coal ash waste as nutritional material for growth (Kapahi & Sachdeva, 2019). The microbiota can be considered as a community of microorganisms

such as bacteria, viruses (Berg et al., 2020), archaea, and some unicellular eukaryotes that live in a specific environment. In essence, the use of microorganisms originating from soil has been widely used (Kazi Madina Maraz & Ruhul Amin Khan, 2021). The large role of microorganisms in solving various problems in the environment is the reason for exploring and isolating potential abundant bacteria. Microorganisms are also included in biodiversity and can be isolated from every layer of soil, sea, and even from various types of industrial waste (Ayilara & Babalola, 2023). Therefore, the level of bacterial diversity found in coal ash waste is important to know as a basis for developing biological coal ash waste processing methods such as bioremediation(Akimbekov et al., 2022).

One strategy for screening coal waste resources is to identify microbial diversity to study the relationship between these microbial communities and contribute to improving environmental pollution(Akimbekov et al., 2022). However, this method is limited to cultivating microbes on certain media such as tryptic soy agar (TSA), water yeast extract agar (WYE), and nutrient agar (NA) (Bonnet et al., 2020). Culture methods are considered less efficient in terms of time and energy, and are inadequate for analyzing microbial communities in their natural environment, due to the presence of large numbers of bacteria that culturable, thus providing a more valid and accurate way to identify microbial communities diversity is needed, especially using metagenomics(Mohammed et al., 2022)

Metagenomics is the newest method to address the weaknesses of culture-based methods, and its application has increased sharply in recent years (Prayogo et al., 2020). Metagenomics extracts DNA directly from environmental samples without laboratory culture. The application of DNA to analyze microorganisms has yielded representative and comprehensive results (Aggarwala et al., 2017). Metagenomics has been used in various research fields, such as microbial communities in sugarcane bagasse waste (Tripathi et al., 2018) and high-salt environments (Jacob et al., 2017). In addition to exploring the benefits of natural genetic resources, metagenomic studies can also enhance the knowledge of the associations between microbial communities in natural biogeochemical cycles.

This research was conducted to study the diversity of microorganisms from coal waste that had been buried for a long time at PLTU using the metagenomic method. Local microorganisms isolated under unfavorable conditions at industrial sites can provide new insights into their diversity (Singh & Hiranmai, 2021). In addition, unique species can be identified as adaptive microbial germplasms in industrial waste management strategies, especially for handling heavy metal contamination.

METHODS

Location

This study was conducted between July and September 2023 at PT. Genetica Science Indonesia. Coal waste sampling location in PLTU Tanjung Jati B Jepara, Central Java, Indonesia. The criteria for samples taken were those that had been in the coal landfill for more than five years.

DNA Extraction from Coal Waste Samples

DNA was extracted from the raw samples using the ZymoBIOMICS™ DNA Miniprep Kit extraction protocol. For concentration and purification of gDNA, spectrophotometric absorbance at A_{260}/A_{280} nm was observed using a Nanodrop™ 1000 spectrophotometer and Qubit fluorometer at A_{230}/A_{260} nm.

Amplification of the 16s rRNA Gene

The PCR method employed was the 16S rRNA gene as a target using a specific 16S rRNA primer, namely primers 27F and 1492R for identifying the unknown bacterium as described.

Quantification and Qualification of PCR Products

The quality of gDNA was measured by agarose gel electrophoresis on 1 % (w/v) agarose in 1x TAE buffer and post-stained with Ethidium Bromide. The gel was run at 100V for 25 min, soaked in ethidium bromide solution for 15 min, and also rinsed in 1x TAE buffer. The gel was then placed on a UV transilluminator and documented.

Analysis of 16S rRNA Gene Sequence

Metagenomic analysis was carried out by 16s rRNA gene sequencing using a comprehensive *next-generation sequencing* platform. Library collection was performed using Kits from Oxford Nanopore Technology. using the workstage protocol in accordance with the manufacturer's instructions. Bioinformatics analysis using nanopore sequencing using the MinKNOW software version 23.04.5. The quality of FASTQ files was imaged using Nano Plot, and quality filtering using Nano Filt (Coster et al., 2018)

(Nygaard et al., 2020). Bacterial and archaeal indices were built using the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Downstream analysis for visualization species diversity was carried out with Pavian (https://github.com/fbreitwieser/pavian), and relative abundances with Krona (https://github.com/marbl/Krona).

RESULTS AND DISCUSSION

Isolation of Microbial Communities

DNA analysis began with the main stage of DNA isolation. The extraction process used to obtain high-quality DNA is a basic rule that must be fulfilled in molecular analysis (Jiang et al., 2021). Various molecular biological analyses require DNA isolation with a good level of purity and quality. Isolated DNA must be free from various contaminants, such as proteins and RNA, which can interfere with the PCR process (Boonsrangsom et al., 2023). The results of 16s rRNA amplification are shown in Figure 1.

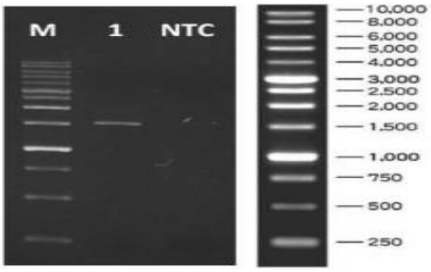

Figure 1. Agarose gel electrophoresis showing the 16S rRNA gene amplicon band of the isolated and most abundant bacterium from coal waste.

Electrophoresis results showed that the 16S rRNA gene was well separated and parallel to the 1,500 bp DNA ladder. This shows that the amplified gene fragment has a size of around ± 1,500 bp which is similar to research (Prihartin et al., 2023) and research (Mohammed et al., 2022). These results indicated that the 16S rRNA gene amplification process was successful. 16S rRNA has been used as a molecular identification method for bacteria because of its distribution in all bacteria and never-changing function (Bahram & Anslan, 2019). This gene was quite large, reaching a size of 1,500 bp. These molecular approaches have resulted in standard procedures for determining the phylogenetic relationships between bacteria.

Analysis of Microbial Communities from Coal Waste

Genomic analysis of environmental microbial communities without the need for cultivation procedures can be carried out using metagenomics (Prayogo et al., 2020). This technique makes it possible to read the microbial diversity of microorganisms from raw samples up to 99% of the total number of microorganisms present in the environment (Alves et al., 2018). Characterization of gene abundance by metagenomic sequencing revealed differences in microbial abundance and diversity under different environmental conditions. Microbial diversity obtained from coal waste isolation obtained 96,766 fragments. The top 10 most abundant bacterial taxa are shown in Figure 2.

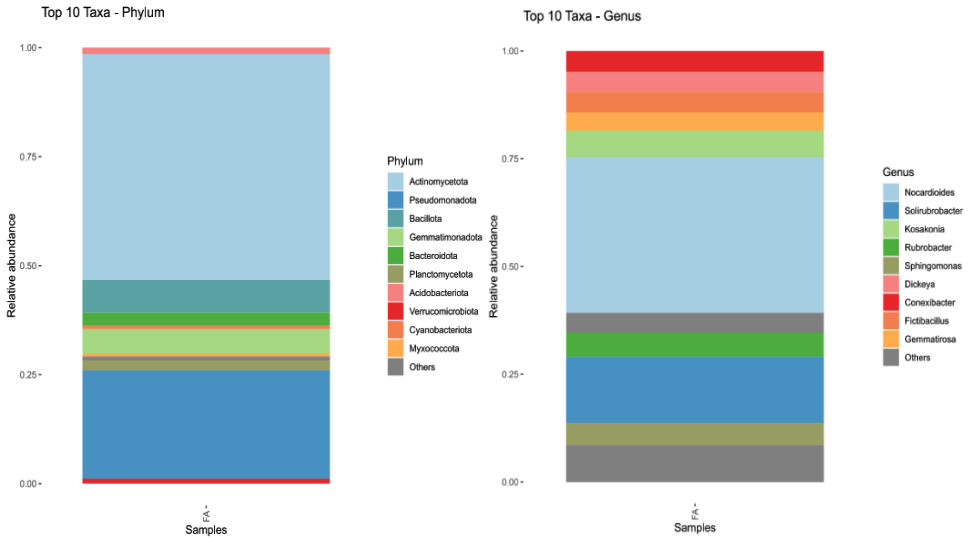

Figure 2. Abundance of phylum and genus level microbial communities

In this study, the bacterial structures of all groups were analyzed at the phylum and genus levels. At the phylum level, the main taxa were Actinobacteria, Proteobacteria, Firmicutes, Gemmatimonadetes, Bacteroidetes, Planctomycetia, Acidobacteria, Verrucomicrobia, Cyanobacteria, and Myxococcota. Actinobacteria was the most dominant phylum. The relative abundance of Actinobacteria (56%) was higher than that of the other phyla. In contrast, the relative abundances of Verrucomicrobia, Cyanobacteria, and Myxococcota (1%) were the lowest. This result is different from that (Song et al. (2022), who found phylum Chloroflexi in lake sediment samples with a relative abundance of Bacteroidetes at (59.76%). The main genera were *Nocardioides, Solirubobracter, Kosakonia, Rubrobacter, Sphingomonas, Dickeya, Conexibacter, Ficitabacillus,* and *Gemmatirosa*.

Actinomycetes are a group of gram-positive bacteria widely distributed in nature and are known as saprophytic microorganisms in soil and litter. Actinobacteria are found in terrestrial and water habitats, but their main population is in soil, where they play a role in converting soil components into organic components through the decomposition of mixed substances and complex organic matter in dead plants, animals, and fungi. They are considered functional bacteria because they can produce secondary metabolites such as enzymes, antibiotics, and pigments (Mondal & Thomas, 2022). The abundance of the microbiota in the coal waste samples is shown in Figure 3.

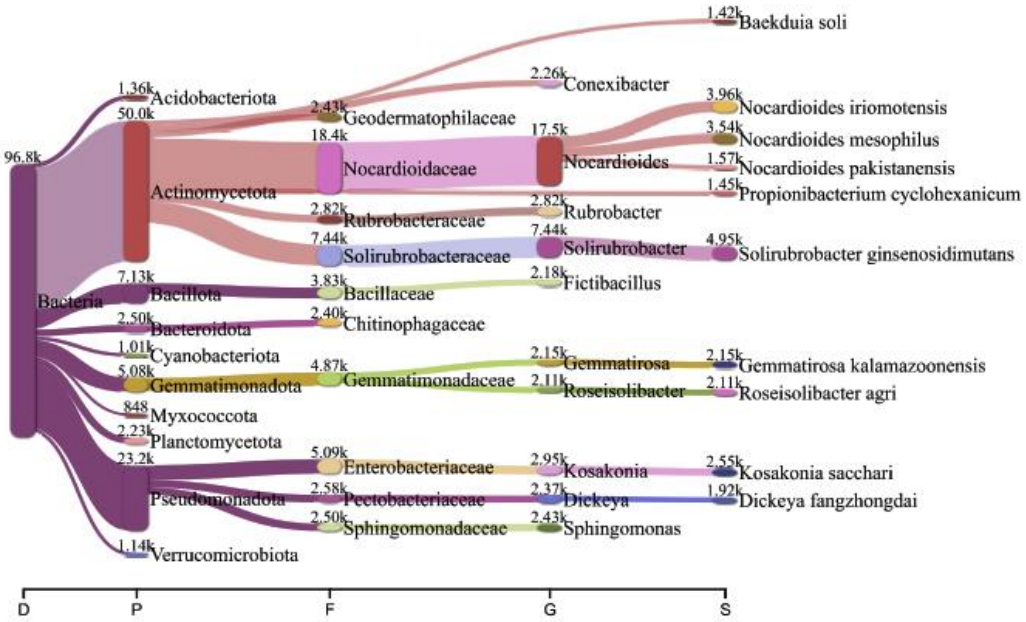

Figure 3. Sankey Diagram of microbial communities of coal waste using Pavian program

Sankey Diagram are used to visualize microbial species of samples. At the species level, it was found that the top 10 dominant species were *Baekduia soli, Nocardioides iriomotensis, Nocardioides mesophilus, Nocardioides pakistanensis, Propionibacterium cyclohexanicum, Solirubrobacter ginsenosidimutans, Gemmatirosa kalamazoonensis, Roseisolibacter agri, Kosakonia saccahari,* and *Dickeya fangzhongdai. Baekduia soli* is a family of *Acidobacteria*. Gram-positive bacterial species do not have spores, are short rod-shaped, do not move, and live aerobically. The morphological characteristics were milky white with a diameter of 0.8-1.1 mm. These bacteria grow optimally at 30˚ C (An et al. 2018).

Nocardioides iriomotensis belongs to the *Nocardioidaceae* family and has the morphological characteristic of developing fragmented mycelia into irregular rods or cocci-like elements (Yamamura et al. 2011). This species can be isolated from various locations such as soil, wastewater, and sediment. The optimum conditions for growing this species are temperature, 30˚ C and pH of 4-12. A strain of the genus *Nocardioides* has been reported to produce the bioactive agent *sandramycin* (Ajani et al., 2022).

Nocardioides mesophilus belongs to the

group of actinomycetes with a characteristic morphology of fungal hyphae, fragmented irregular segments resembling rod- or coccus-like elements, and is a gram-positive bacterium. These organisms are gram-positive, acid-labile, aerobic, and mesophilic (Wang et al., 2021). This species can grow at temperatures between 20 °C and 37 ˚ C with a pH of 7–7.5. *Nocardioides pakistanensis* is a gram-positive, non-spore forming, non-pigmented, and non-motile bacterium that is aerobic, rod-shaped, and grows at an optimal pH of 7.0, and an optimal temperature of 37 °C, including the actinomycetes group (Amin et al., 2016). The genus *Nocardioides* is versatile and has been isolated not only from soil but also from volcanic ash (Lee & Lee, 2014) and seafood samples (Lin et al., 2015).

Propionibacterium cyclohexanicum is a gram-positive, non-motile, rod-shaped bacterium, with a cell size of 1.5-3.0 pm long and 1.1-1.6 pm wide. The cells were club-shaped and bent, some swelling was visible, and spores were absent. Colonies on the surface of PYG agar after three days were circular, white to cream in color, clear, and had a diameter of 0.2 to 0.5 mm. is an anaerobic bacteria, that grows optimally at a temperature of 35°C and a pH of 5.5-6.5(Bronnec et al., 2022). These belong to the phylum Actinomycetes. This group of bacteria is distributed in various environments, such as animal hosts and soil. They are known to produce active compounds such as antimicrobials (Jeantet & Jan, 2021).

Solirubrobacter ginsenosidimutans is a gram-positive, non-spore-forming, non-motile, aerobic bacterium. This type of bacteria was also discovered by An et al. (2011), who isolated it from the soil of a ginseng field at Baekdu Mountain in China. Colonies were smooth, circular, convex, yellow and 0.6–1.2 mm in diameter. It grows at 18–37 °C (optimum, 30 °C). *Gemmatirosa kalamazoonensis*, cells are non-motile and rod-shaped, displaying variable length (1–16 μm). It is an oligotrophic, chemoheterotroph. These bacteria can grow at an optimum temperature of 37 Â °C with an optimum pH of 5.5-6.5. This type of bacteria has also been successfully isolated from agricultural soil in Kalamazoo, Michigan, United States (DeBruyn et al., 2013). *Gemmatirosa kalamazoonensis*, belonging to the phylum Gemmatimonadota, is ubiquitous and particularly common in soils, limnic environments, and sediments (Mujakić et al., 2022).

This species of *Roseisolibacter agri* is also part of the phylum Gemmatimonadota. It includes gram-negative, non-spore-forming, non-capsulated, non-motile, rod-shaped bacteria. The cells are divided into binary fission and budding. The cells are rod-shaped (0.6 to 16 Âμm long and 0.4 to 0.5 μm wide) and appear as single cells. The dominant color was white-pink. Chemoorganotrophs are aerobic but grow slowly and are unable to reduce nitrate and ferment glucose. Optimal growth under normal atmospheric conditions. Growth was observed between 15 and 36 °C (optimum 20–25 °C) and at pH 6.4 to 8.4 (optimum pH 7.0–7.5) (Pascual et al., 2018). These results indicated that members of Gemmatimonadota have specific functions in the environment (Mujakić et al., 2022).

Kosakonia sacchari belongs to the phylum Pseudomonadota and family *Enterobacteriaceae.* is a gram-negative, non-spore-forming motile rod with peritrichous flagella. formed round, convex, and smooth colonies with entire edges on the solid media. It grows best at approximately 30°C and a pH of 7 (Chen et al., 2015). According to (Zhu et al., 2013), the *K. sacchari*, has the ability to act as an endophytic bacterium capable of promoting the growth of agricultural crops through nitrogen fixation.

Dickeya fangzhongdai belongs to the phylum Pseudomonadota and family *Pectobacteriaceae*. Gram-negative bacteria, which are motile rods, grow on yeast peptone dextrose adenine medium. Growth occurs at 39˚ C (Tian et al., 2016). Alič et al. (2019) succeeded in isolating this species from pear trees in China and tested its ability as a bacterial agent to produce plant cell wall-degrading enzymes (PCWDEs), which cause maceration in plant tissue. This research shows that there is microbial diversity in coal waste, where the overall results of phylum diversity in the soil tend to be the same, dominated by *Actinomycetes*. It is hoped that this research will be useful as an adaptive microbial germplasm for industrial waste management strategies, such as handling heavy metal contamination.

CONCLUSION

Based on the results of research using the metagenomic method, the top 10 species that dominate coal waste are *Baekduia soli, Nocardioides iriomotensis, Nocardioides mesophilus, Nocardioides pakistanensis, Propionibacterium cyclohexanicum, Solirubrobacter ginsenosidimutans, Gemmatirosa kalamazoonensis, Roseisolibacter agri,*

Kosakonia sacchari, and *Dickeya fangzhongdai*. Most of these species belong to the Actinobacteria phylum. Further research must be conducted to test the ability of each type of microbe to manage waste.

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