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16S METAGENOMICS OF BRANCH-LEAF COMPOST AT THE EXPO MEMORIAL PARK OSAKA

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ABSTRACT

Compost is one of the potential microbial habitats for the discovery of a wide range of novel biocatalysts. Compost sample originally made of plant litter, were collected at the Expo Park (Osaka, Japan). The temperature of composts is around 50 °C. Metagenomic DNA was isolated from compost and was used to amplify partial 16S rRNA genes (rDNAs) by polymerase chain reaction for investigation of the microbial diversity in the compost. Molecular microbial identification was carried out by metagenome analysis of 16S rRNA using MinION Next Generation Sequencing (NGS). The results indicated that branch-leaf compost sample from Expo Park is dominated by thermophilic microorganism. The majority of bacteria are Caldicoprobacter, Hungateiclostridium, and Thermoclostridium. To conclude, thermophilic microorganism is not only found in extreme environment, they also presence even in moderate temperature.

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Keywords: 16S rRNA genes, compost, metagenome, microbial diversity.

INTRODUCTION

The process of composting is crucial for the recycling and treatment of organic waste. Mesophilic, thermophilic, and cooling stages of the composting process are where organic materials are broken down by aerobic microbes into humus (López et al., 2021). This microbial activity is one of the key driving forces of composting. Microbial communities that have specific abilities to degrade cellulose, lignin, or compost at the thermophilic or maturation stages are often used as a source to isolate microorganisms (Xu et al., 2019). It is generally acknowledged that more than 99% of the environmental microorganisms cannot be cultivated using conventional method. Therefore, a new field of research based on the collective genomes of all microorganisms present in each habitat, the so-called metagenome, has been developed in order to better understand the microbial ecology and response to the increasing biotechnological demands for novel enzymes and biomolecules.

Compost is one of the potential microbial habitats for the discovery of a wide range of novel biocatalysts. It is the end-product of a biological decomposition and stabilization of organic substrates under conditions that allow high temperatures as a result of biologically produced heat. Therefore, screening of metagenomic composts may identify some thermophilic biocatalysts from non-cultured microorganisms.

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The diversity of microbes in an environment is very high but most of these organisms are not easily cultivated (Streit & Schmitz, 2004). Several methods such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) Shyu et al., (2007), fluorescence in situ hybridization (Honeker et al., 2016), and various types of quantitative real time Polymerase Chain Reaction (rt-PCR) have been used for the analysis of an organism or the entire community in an environment (Fierer et al., 2005). The weakness of these methods is the limited detailed information on the entire microbe.

High Throughput Sequencing (HTS) is another technique for identifying microorganism populations molecularly which is currently growing rapidly, namely Next Generation Sequencing (NGS). The NGS method is widely used for metagenomic research which aims to look at the community and phylogenetic dynamics with the samples used can be obtained from the environment regardless of whether the microorganisms can be cultured or not. Microorganisms can be detected by this method provided that there are nonspecific primers for sequence amplification and elongation of the NGS process (Jünemann et al., 2017).

LITERATURE REVIEW

Metagenomics is a scientific discipline that studies the genomes of microorganisms. Through metagenomic analysis, the composition of the microorganism and the role of the functional genome of the microorganism in the sample can be identified. The discovery of the structure of DNA has had a major impact on the development of sequencing and various biological technologies (Akaçin et al., 2022). The technique is based on the hybridization of nucleic acids which will pair with their complements, namely the bases Adenine with Thymine and Guanine with Cytosine.

Microbes are essential to the composting process. The breakdown of organic matter is influenced by the dynamic changes in the microbiota, and the maturity of fermentation is determined by this degradation (Wei et al., 2007). Temperature, moisture, oxygen, and C/N ratio are additional elements that affect the maturity of the fermentation process (Wan et al., 2020). Consequently, the key to establishing connections between compost characteristics, community, and metabolism is the effective detection of dynamic changes in the microbiota and investigation of metabolic pathways during the composting process.

T-RFLP, PCR-DGGE, and 16S rRNA clone libraries were typically employed to evaluate the microbial community during composting, although these techniques are generally limited in their ability to give comprehensive microbial communities and metabolic function features (Asano et al., 2010). Metagenomic libraries have recently become a crucial tool for studying environmental microbiology thanks to the study of phylogeny and function of environmental microbial species (Jovel et al., 2016). Metagenomic sequencing can analyze functional genes, organic matter metabolism, and the microbiome in samples in addition to providing information on the microbiome (Costea et al., 2017).

Using machines, sequencing technology has been mechanized. Following the first generation, also known as dideoxyribonucleotide chain termination sequencing or Sanger, the second generation of sequencing technology is called Next Generation Sequencing (NGS). In this method, one of the DNA strand's segments serves as a DNA template for creating the complementary fragment from the template. The shotgun sequencing technique served as the inspiration for developing NGS devices (Shendure et al., 2004).

One approach to taxonomic classification is to study specific marker genes present in various organisms. This approach involves the characterization of the 16S small subunit ribosomal RNA coding gene (rRNA). The 16S rRNA gene is very useful because it is present in all prokaryotes and is an integral part of the ribosome. The 16S rRNA gene is an integral part of the ribosome, consisting of conserved regions as well as hypervariable regions (HVRs V1-V9) facilitating amplification with universal primers and high specificity for differentiating between organisms (Jünemann et al., 2017).

METHODS

The materials used for the experiment included sample from branch-leaves compost took at Expo Park Osaka. The DNA extraction and analysis materials included: ISOIL 316-06211 (Nippon Gene Co.) for DNA extraction, GoTaq® Green Master Mix for mixture reactions in the Polymerase Chain Reaction (PCR) process, and universal primers 27F and 1492R, which spans nearly full-length of 16S rRNA gene, with an expected amplicon of ~ 1400 bp, for base sequence amplification and elongation of NGS processes.

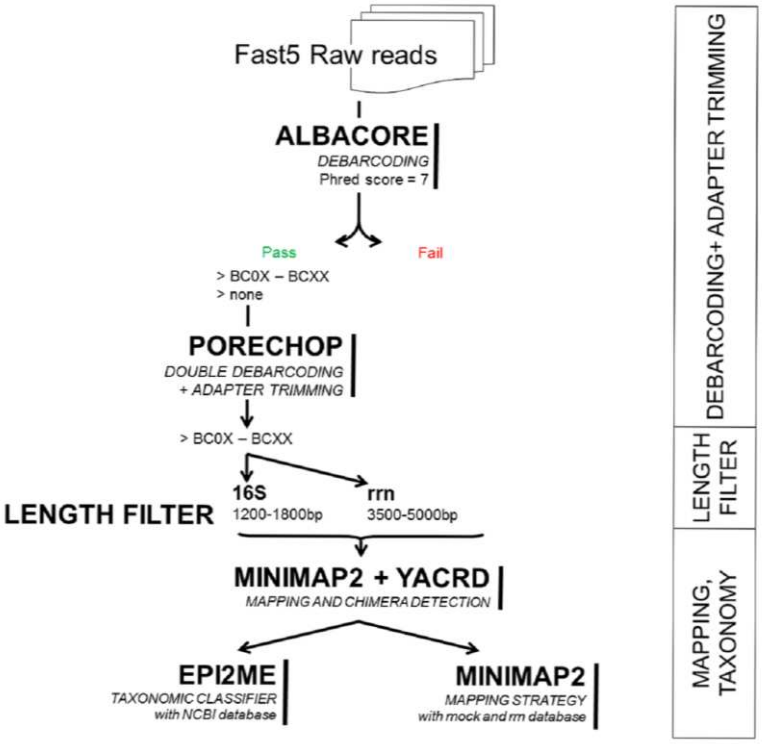

Figure 1 Bioinformatics analysis workflow

Samples

The samples used in this study were: A1, A2, A3 (branch-leaves compost from Expo Parks Osaka) and N1, N2, N3 as the negative control. Compost samples were taken from the core (100 cm deep from the surface) of compost pile, originally made of plant litter and processed at the Expo Park (Osaka, Japan). The temperature of composts is around 50 °C.

DNA extraction

DNA extraction was performed using the ISOIL 316-06211 (Nippon Gene Co.), following the protocol from the manufacturer with several optimizations. 5 gram compost samples were homogenized, lysed and extracted the DNA by following the manufacturer's instructions.

DNA quality and quantity test

The concentration and purity of the extracted DNA were measured using a UV spectrophotometer with a wavelength of 260 nm and 280 nm. Agarose gels stained were photograph by image scanner. For the quantity test, a total of 2 µl of DNA samples were dropped in the Nanodrop tool, then the concentration and purity level of DNA could be seen directly from the instrument by displaying it on the monitor screen.

Polymerase chain reaction (PCR)

The DNA is amplified by PCR using specific 16S primers (27F and 1492R) that contain 5' tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters. PCR assay was carried out in a 25 µl mixture reaction (GoTaq® Green Master Mix). The primers used were 27F and 1429R. The PCR reaction was carried out in a thermal cycler under the following conditions: initial denature 94 °C for 2 minutes, followed by denaturation at 98 °C for 10 seconds, annealing at 55 °C for 30 seconds, extension at 68 °C for 2

minutes, and final extension at 68 °C for 7 minutes. PCR reaction cycles starting from denaturation to extension were carried out in 30 cycles.

Microbial metagenome data analysis

The V3-V4 region of the 16S rRNA gene amplicon was the focus of the microbial metagenome investigation, which was carried out utilizing the NGS method and the MinION sequencing (Oxford Nanopore Technologies) platform. The MinKNOW software, a program for microbial ecology analysis that could be accessed via a Linux-based computer, was used to run the samples. Albacore v2.3.1 was used to base-call and de-multiplex fast5 files after the run. Only the barcodes that agreed with Albacore were kept after a second demultiplexing cycle with Porechop v0.2.330. Moreover, the adapters and barcodes were removed from the sequences using porechop (**Figure 1**) (Cuscó et al., 2019).

RESULTS AND DISCUSSION

Our knowledge of microbial communities in complicated environments has significantly enhanced as a result of recent developments in metagenomics (Kong et al., 2020; Wan et al., 2020). This new information might make it possible to identify important microbes, which would aid in the creation of microbial biotechnologies. In this study, 16S rRNA amplicon sequencing was incorporated for the creation of microbial inoculants to enhance manure composting. The goal of this strategy was to pinpoint important thermophiles that would significantly aid in the breakdown of organic waste.

Compost metagenomic DNA was successfully extracted by ISOIL Large Bead Kit (Nippon Gene). A total yield of about 10-15 µg of DNA per gram sample (wet weight) was obtained. The universal PCR primers (relatively conserved for Bacteria, Archaea and Eukarya), Bacteria-specific PCR primers and Fungi-specific PCR primers amplified rDNA sequences in all of the compost samples. The DNA is amplified by PCR using specific 16S primers (27F and 1492R) that contain 5′ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters.

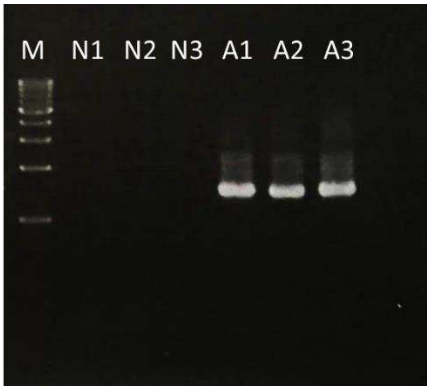

Figure 2 DNA quality analysis by agarose gel electrophoresis

Branch-leaf compost from Expo Parks Osaka was used as the test sample. The PCR product at 55°C revealed that fragments from samples A1, A2, and A3 were successfully amplified specifically and without contamination by other gene fragments, while N1, N2, and N3 served as the negative control and did not show any band (**Figure 2**). The amplification results of the compost samples revealed only one DNA band. If the results of the electrophoresis analysis reveal the presence of a single strand of DNA with the required size based on a previously identified marker, the DNA sample is considered to be specific and successfully amplified (Wiharyani et al., 2014).

Table 1 Genomic DNA quantification by Nanodrop

Sample ID	Nucleic Acid Conc. (ng/µl)	A260	A280	260/280	260/230
A1	94.7	1.894	1.105	1.71	1.46
A2	31.7	0.633	0.377	1.68	1.15
A3	97.4	1.947	1.145	1.70	1.19
N1	0.6	0.012	0.016	0.77	0.06
N2	0.0	0.001	0.007	0.12	0.00
N3	0.0	0.003	0.005	0.15	0.02

NanoDrop is the instrument of choice for DNA concentration measurements and purity indices based on absorbance at 260 nm and 260/280 and 260/230 ratios. The 260/280 ratio is very useful for estimating the approximate purity of DNA and contamination by protein when DNA is extracted from blood or tissues, which have enriched presence of protein. A 260/280 ratio of 1.7–1.9 is expected for pure DNA, while much lower and higher values are indicative of the presence of contaminants like protein and phenol (Onyemata et al., 2021). Based on **Table 1**, it shows that the concentrations of the three compost samples with codes A1, A2, A3 have the best purity index concentration in sample A1. Meanwhile, samples with codes N1, N2, N3, as negative controls, showed low concentrations of nucleic acids, did not show the presence of nucleic acids.

Table 2 Result of MinION sequencing

Reads analysed	43,508
Total yield	64.7 Mbases
Avg. quality score	9.58
Avg. sequence length	1,487

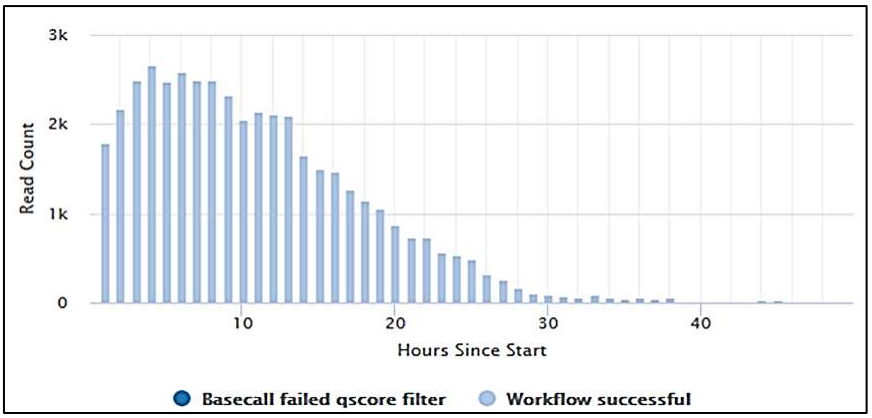

Figure 3 Read count per hour from MinION sequencing

The MinION most likely the first commercially available sequencer that uses nanopores. Nanopore sequencing has been shown to be able to discriminate individual nucleotides by measuring the change in electrical conductivity as DNA molecules pass through the pore (Laver et al., 2015). The device has a low capital cost, is by far the most portable DNA sequencer available and can produce data in real-time. We constructed a sequencing library containing genomic DNA from sample A1, as the best sample based on quality and quantification of the DNA. From **Table 2**, this single MinION run generated template sequence reads for 43,508 different fragments, with total yield 64.7 Mbases. Average of the quality score is 9.58 with average of sequence length is 1,487. As seen in Figure 3, the read count per hour decreases. In the first 12 hours, the read count was still more than 2 thousand, and started to decrease until finally it was less than 1 thousand after 20 hours. Sequencing with minions ran for up to 48 hours with total read analyzed was 43,508.

Table 3 The number of cumulative reads (CR) to each taxon and Characteristics of microorganism

Taxon	Characteristics	CR
Caldicoprobacter	Thermophilic, anaerobic, gram-positive, non-spore-forming	1365
Hungateiclostridium	Thermophilic, anaerobic	1064
Thermoclostridium	Thermophilic, anaerobic	916
Paenibacillus	Thermophilic, aerobic or facultatively anaerobic, Gram-positive	443
Thermoanaerobacter	Thermophilic, anaerobic, saccharolytic	424
Symbiobacterium	Thermophilic, gram-negative, and tryptophanase-positive	380
Desulfofundulus	Thermophilic, thermophilic sulfate-reducing bacterium	266
Desulfotomaculum	Thermophilic, obligately anaerobic, Gram-positive	224
Planifilum	Thermophilic, aerobic, gram-positive	222

Anaerobranca	Alkalithermophilic, anaerobic, fermentative bacterium	217
Desulfallas	Thermophilic, fatty acid-degrading	172
Moorella	Thermophilic, anaerobic and endospore-forming	162

Metagenome analysis with amplicon sequencing of 16S rRNA gene aims to determine the microbial composition in the compost sample. Based on taxon, it shows that there are various types of genera with the relative abundance of the sample. In this study, it is known that the genus distribution based on the cumulative reads of the sample is in accordance with **Table 3**, with the three highest cumulative read is Caldicoprobacter, Hungateiclostridium, and Thermoclostridium.

Based on **Table 3** regarding the characteristics of microorganisms, it shows that the 12 highest taxa have the same main characteristic, namely thermophilic. According to Biyada et al. (2021), the composting process involves mesophilic and thermophilic microorganisms. Microorganisms in compost combine oxygen and carbon to produce carbon dioxide and energy, some of the energy is used for growth, the rest is released as heat (Asano et al., 2010).

The resulting heat energy is a parameter of the activity of cellulolytic microorganisms in the compost. The process of cellulose degradation by cellulolytic microorganisms cannot be separated from the role of the cellulase enzyme complex produced by bacteria. Community characterization of thermophilic cellulolytic bacteria in compost is needed to determine the microorganism community profile of compost.

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CONCLUSION

Based on the analysis of 16s sequencing, branch-leaf compost sample from Expo Park is dominated by thermophilic microorganism. The majority of bacteria are Caldicoprobacter, Hungateiclostridium, and Thermoclostridium. The result indicate that thermophilic microorganism is not only found in extreme environment, they also presence even in moderate temperature.

Author's declaration

Authors' contributions and responsibilities

The authors made substantial contributions to the conception and design of the study. The authors took responsibility for data analysis, interpretation and discussion of results. The authors read and approved the final manuscript.

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Availability of data and materials

All data are available from the authors.

Competing interests

The authors declare no competing interest.

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