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METAGENOMIC EXPLORATION OF ECO-ENZYME PRODUCTION: UNVEILING ENZYMATIC ACTIVITIES AND MICROBIOTA ABUNDANCE FOR SUSTAINABLE ORGANIC WASTE MANAGEMENT

Eksplorasi Metagenomik Produksi Eko-Enzim: Mengungkap Aktivitas Enzim dan Kelimpahan Mikrobiota untuk Pengelolaan Limbah Organik Berkelanjutan

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ABSTRACT

Globally, organic waste is steadily increasing, raising serious environmental challenges. A promising solution is the conversion of organic waste into Eco-Enzyme (EE), a fermented product widely applied in wastewater treatment, soil improvement, and fertilization. Despite its potential, few studies have characterized EE from a metagenomic perspective. This study examined enzymatic activities and microbial communities during EE production. EE was prepared from pineapple and orange peels mixed with palm sugar and water, then fermented for 90 days. The mixture was analyzed for enzymatic activities including amylase, cellulase, protease, chitinase, lignin and Mn peroxidase, peroxidase, glucanase, and catalase, and linked to waste degradation potential. Metagenomic sequencing using Oxford Nanopore Technology revealed Terrabacteria (83%), dominated by *Lacticaseibacillus* and *Lentilactobacillus*, and Proteobacteria (17%) with *Acetobacter fabarum* prevalent in EE sample. The highest activity was catalase (5107 mU/ml), followed by chitinase, protease, and amylase. These results highlight EE's microbial and enzymatic complexity, reinforcing its role in sustainable organic waste management.

Keywords: *Organic waste, Eco-Enzyme, Enzymatic activity, Metagenomics, Waste treatment*

ABSTRAK

Secara global, limbah organik terus meningkat, menimbulkan tantangan lingkungan yang serius. Solusi yang menjanjikan adalah konversi limbah organik menjadi Eco-Enzyme (EE), produk fermentasi yang banyak digunakan dalam pengolahan air limbah, perbaikan tanah, dan pemupukan. Terlihat dari potensinya, beberapa studi telah mengkarakterisasi EE dari perspektif metagenomik. Studi ini meneliti aktivitas enzimatik dan komunitas mikroba selama produksi EE. EE dibuat dari kulit nanas dan jeruk yang dicampur dengan gula aren dan air, kemudian difermentasi selama 90 hari. Campuran tersebut dianalisis untuk aktivitas enzimatik termasuk amilase, selulase, protease, kitinase, lignin peroksidase, Mn peroksidase, peroksidase, glukanase, dan katalase, dan dikaitkan dengan potensi degradasi limbah. Sekuensing metagenomik menggunakan Teknologi Oxford Nanopore menunjukkan bahwa EE mengandung Terrabacteria (83%), didominasi oleh *Lacticaseibacillus* dan *Lentilactobacillus*, dan Proteobacteria (17%) dengan *Acetobacter fabarum*. Aktivitas tertinggi adalah katalase (5107 mU/ml), diikuti oleh kitinase, protease, dan amilase. Hasil ini menyoroti kompleksitas mikroba dan enzimatik EE, yang memperkuat perannya dalam pengelolaan limbah organik berkelanjutan.

Kata Kunci: *Aktivitas enzimatik, Eco-Enzyme, Limbah organic, Metagenomik, Pengolahan limbah*

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INTRODUCTION

As Indonesia's population increases, the consumption of food ingredients also increases. The increase in food consumption was followed by an increase in the amount of organic waste. The percentage of food waste in Indonesia was 44.8% of the total national waste produced (The Ministry of Environment and Forestry, 2022). As much as 51.7% of waste comes from household waste. With such an amount, organic waste needs to have an added value so we can utilize the waste.

One way to leverage the values of organic waste is to convert it into garbage enzyme or colloquially known as Eco-Enzyme (EE) (Galintin *et al.*, 2021, Sethi *et al.*, 2021). The term "Eco-Enzyme" was first used by the Organic Farming Association of Thailand, which has been doing research since the 1980s. A naturopathic researcher from Penang, Malaysia, who studied Eco-Enzymes, popularized them (Novianti A., 2021). It is made mainly from fruit peels and vegetables which are fermented within 3 months (Samriti *et al.*, 2019). The enzyme has been used in various applications such as wastewater treatment (Arun & Sivashanmugam, 2015), Soil ameliorant (Wei *et al.*, 2020), and soil fertilizer (Sethi *et al.*, 2021). The manufacturing of Eco-Enzymes offers two advantages. It has advantages when used in agriculture, animal husbandry, cleaning, and other fields, in addition to reducing the strain of managing organic waste (Samiksha S & Salvi, 2020).

Hitherto, studies on Eco-Enzyme characterization are still few. Arun dan Sivashanmugam (2015) characterized EE produced from vegetables waste. Their study focused on biocatalytic properties and revealed that EE has amylase, lipase, and protease activity. They also calculate the number of microorganisms present in EE using MPN (most probable number) procedures and the value is less than 3 (<3). Another researchers, Samriti *et al.* (2019), also determined the bacterial and fungal composition in EE by spreading EE on Amphotericin B-incorporated Nutrient agar and Ampicillin-incorporated YEPD agar plates, respectively. After several identification step, they suspected *Yersinia* and *Pseudomonas* for bacterial genus and *Aspergillus niger*, *Trichoderma viride*, and *Saccharomyces cerevisiae* for fungal species that present in EE. However, the methods mentioned earlier can only detect culturable microorganism. It cannot detect microorganism that viable but not yet culturable. The method suitable to tackle the issue is by using Metagenomic analysis, harnessing Next Generation Sequencing (NGS) technology.

Metagenomics is the study of genetic material that has been randomly (shotgunly) extracted from environmental materials. There is no longer a requirement to initially cultivate these species to identify whole or almost complete microbial and viral genome sequences from environmental samples as a result of recent breakthroughs enhancing the depth and breadth of metagenomic shotgun sequencing. These sequences, which can be obtained through single-cell sequencing or metagenomic datasets, are referred to as "uncultured genome sequences". Uncultured genome sequences can supplement the genome sequences obtained by sequencing pure or nearly pure cultures of microbial isolates, greatly increasing the amount of data that is available for comparative genome analyses, even though they frequently have a draft status and depending on the approach may represent a locally occurring metapopulation rather than a single clone (Garza & Dutilh, 2015). In food microbial ecology, amplicon-based HTS targeting genes of taxonomic relevance has emerged as the most popular strategy. It was widely used in the last ten years to keep track of the microbial populations while various foods and beverages fermented (De Filippis et al., 2017). Therefore, the aim of this study was to investigate enzymatic activity formed by Eco-Enzyme and characterized its type and microbiota abundance.

MATERIALS AND METHODS

Eco-Enzyme production

A total of 1.5 kg of pineapple peel and 1.5 kg of orange peel was cut into 3x3cm sizes and mixed with 1 kg of palm sugar and 10 L of tap water. The solution is stirred until it reach homogeneity and fermented for 90 days under closed conditions (Hemalatha &

Visantini, 2020). Next, Eco-Enzyme can be harvested by filtering and the filtrate was further analyzed.

Enzymatic activity analysis

Amilase acitvity

Mix 1 ml of sample with 1 ml of 1% starch solution, vortex until homogeneous. Incubate at room temperature for 3 minutes. Add 1 ml of DNS, heat for 15 minutes and cool in ice water to room temperature. Add 9 ml of distilled water then vortex until homogeneous. Read the absorbance at a wavelength of 540 nm. Determine the maltose content of the sample based on the standard curve, and calculate the activity based on following formula:

$$\text{Amylase activity (U/gr)} = \frac{\text{Maltose content x Fp}}{\text{MW Maltose x time x sample weight (gr)}}$$

Cellulase activity

Mix 0.75 ml of sample with 0.75 ml of 1% CMC in 50 mM citrate buffer pH 6.0. Incubate at room temperature for 30 minutes. Add 1.5 ml of DNS, heat for 15 minutes and cool to room temperature for 20 minutes. Read the absorbance at a wavelength of 575 nm. Determine the glucose level of the sample based on the standard curve. Perform the analysis at least twice repetition. Calculate the cellulase enzyme activity with the following formula:

$$\text{Cellulase activity (U/gr)} = \frac{\text{Glucose content x Fp}}{\text{MW Glucose x incubation time}}$$

Lipase activity

Weigh 3 g of CPO and 1 g of polyvinyl alcohol into a 250ml Erlenmeyer. Add 40 mL of pH 5 phosphate-citrate buffer. Incubate at 37 C for 30 minutes. Add 20 mL of acetone : ethanol mixture (1:1 v/v). Titrate using 1 N NaOH using phenolphthalein indicator until the end point is pink.

Protease activity

In a 50 mL tubes, add 350 µL casein substrate, 150 µL phosphate buffer and 50 µL sample. Incubated 30 minutes at 37°C. Add 1 mL of 0.3 M TCA, leave it for 10 minutes at room temperature. Centrifuge at 7000 rpm for 3 minutes. Prepare a clean test tube, add supernatant taken as much as 0.8 mL, 1.7 mL of NaOH, and folin reagent: 0.5 mL of water. Vortex, leave for 15 minutes. Read the absorbance at ʎ 578 nm. Prepare and measure the standard tyrosine solution series. Calculate the sample concentration based on the tyrosine standard curve.

Chitinase activity

Mixed 1 ml of sample with 1 ml of 1% chitin solution, vortex until homogeneous. Incubate at 37°C for 30 minutes. Add 2 ml of DNS, heat for 5 minutes and cool in ice water to room temperature. Centrifuge at 4000rpm for 5 minutes. Read the absorbance at a wavelength of 575 nm.

Lignin Peroxidase activity

Mixed 0.2ml acetate buffer, 0.1ml V alcohol, 0.4ml distilled water, 0.1ml H_2O_2 0.1ml, and 0.2ml enzyme filtrate. Vortex all the solutions until homogeneous. Read at a wavelength of 310 nm

Mn Peroxidase activity

Prepare as many test tubes as used. Add 0.1ml of Na-Lactate buffer, 0.3ml of distilled water, 0.2ml of $MnSo_4$, 0.1ml of H_2O_2, and 0.2ml of enzyme filtrate. Vortex all the solutions until homogeneous. Read at a wavelength of 465 nm

Peroxidase activity

Prepare as many Eppendorf tubes as will be used. Grind ±1 gram sample in phosphate buffer pH 6.5 (cold condition ± 4°C). Centrifuge 8000rpm, 4°C for 15 minutes. The supernatant is transferred to a new tube and stored on ice. Prepare a clean cuvette, add 1.5 ml pyrogalol 0.05M, 0.1ml sample extract, homogenize. Add 0.1ml 1% H_2O_2. Read at a wavelength of 420 nm for 3 minutes (every 30 seconds are recorded).

Glucanase activity

The sample was added with 0.2% laminarin substrate. Incubation at optimum temperature for optimum time. Add 3 ml of DNS solution, heat for 15 minutes. After cooling read at ʎ= 575 nm. Calculate the sample concentration based on the Glucose standard curve.

Catalase activity

Add 50µl sample and 950µl H_2O_2, homogenize. Add 50µl phosphate buffer/PBS and 950µl H_2O_2, homogenize. Read at a wavelength of 240 nm for 2 minutes (every 30 seconds are recorded).

Genomic DNA extraction, library preparation and sequencing

To extract the genomic DNA from Ecoenzyme, ZymoBIOMICS DNA Miniprep Kit (Zymo Research, D4300) was used. Both NanoDrop spectrophotometers and Qubit fluorometers were used to calculate the DNA concentration. Utilizing kits from Oxford Nanopore Technology, library preparations were carried out. The MinKNOW software version 22.05.7 was used to run the nanopore sequencing. Guppy version 6.1.5 with a high-accuracy model was used for basecalling [2]. Utilizing NanoPlot and NanoFilt, the quality of FASTQ files was displayed and filtered [3][4]. Centrifuge classifier was used to categorize filtered readings [5]. The NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/) was used to create the bacteria and archaea index. Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and RStudio with R version 4.2.0 (https://www.R-project.org) were used for downstream analysis and visualizations. Workflow of the study is presented in the Figure 1.

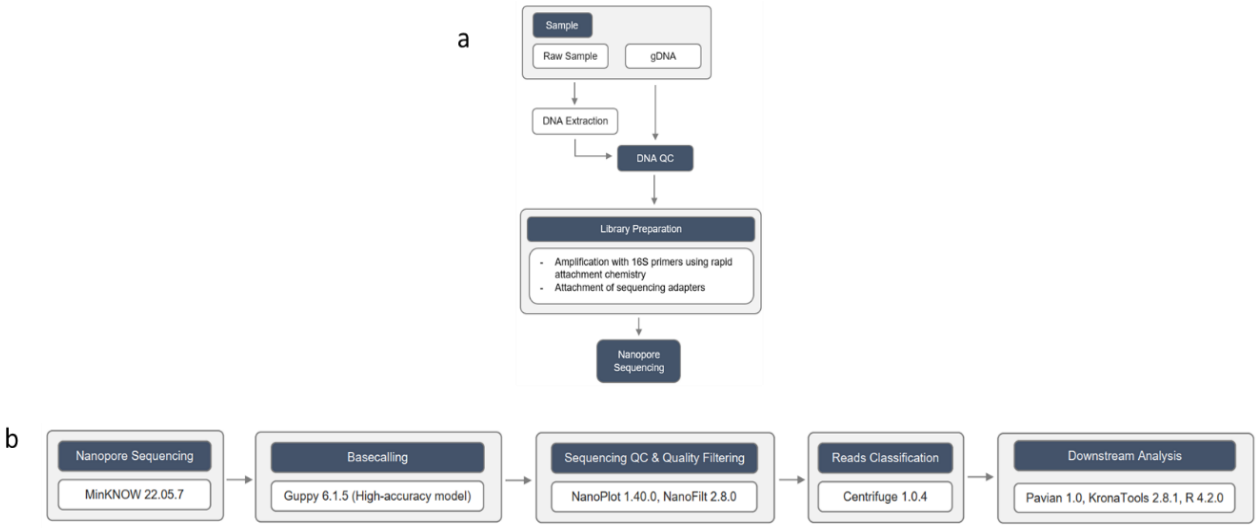


Figure 1. Workflow of the research: (a) sample preparation, extraction, library preparation and sequencing; (b) bioinformatics workflow

RESULTS AND DISCUSSION

Enzymatic activity of Eco-Enzyme

To investigate the enzymatic activity in Eco-Enzyme, we measured the activity of ten (10) enzymes. The highest activity identified in Eco-Enzyme is Catalase with 5107 mU/mL. In several biotechnological fields, including bioremediation, catalase has been exploited as a crucial enzyme. According to Barynin et al. (2001), the basic mechanism by which this enzyme functions is the initial breakdown of the reactive oxygen species, hydrogen peroxide (H_2O_2), into oxygen and water, relieving the oxidative stress brought on by this substrate. Catalase is known as an indicator of hydrocarbon degradation occurring in crude oil pollution (Achuba et al., 2014), an oxygen provider in aerobic bioremediation (Margesin et al., 2000), as well as removal of hydrogen peroxide from bleaching in textile industries (Chico et al., 2013, Amorim et al., 2002).

The second rank is Chitinase with a value of 87.7369 mU/mL, followed by protease activity of 57.4489 mU/mL. The fourth rank is amylase activity with a value of 49.9556 mU/mL, followed by cellulase activity of 44.6159 mU/mL. Glucanase activity value is detected as much as 36.1304 mU/mL. However, the enzyme related to lignin degradation such as Lignin peroxidase is not detected, and Mn peroxidase activity is only 6.1 mU/mL. Peroxidase and Lipase

activity are also detected very low with scores of 5.7139 mU/mL and 0.3430 mU/mL.

However, the Amylase and Cellulase activity in the sample are considered low compared to different types of Eco-Enzyme containing papaya, dragon fruit, and orange peels, with values of 2150 and 1690 mU/mL, respectively (Suliestiyah et al., 2022). Proteins, carbohydrates, and lipids will be significantly reduced throughout the decomposition process due to the presence of the enzyme lipase, amylase, and protease. The enzyme that was obtained has antifungal, antibacterial, and insecticidal properties. It is a versatile cleaning agent. Future Eco-Enzyme production will not only help reduce the amount of waste dumped in landfills but will also provide an alternative to synthetic chemicals that are harmful to both human health and the environment (Muliarta & Darmawan, 2021).

Table 1. Enzymatic activity of Eco-Enzyme in mU/mL unit using spectrophotometry methods. Each activity was conduct in triplicate to ensure the data accuracy

Enzymatic activity	Result	Unit
Amylase	49.9556	mU/mL
Glucanase	36.1304	mU/mL
Lipase	0.3430	mU/mL
Protease	57.4489	mU/mL
Cellulase	44.6159	mU/mL
Chitinase	87.7369	mU/mL
Lignin peroxidase	undetected	mU/mL
Mn peroxidase	6.1	mU/mL
Peroxidase	5.7139	mU/mL
Catalase	5107	mU/mL

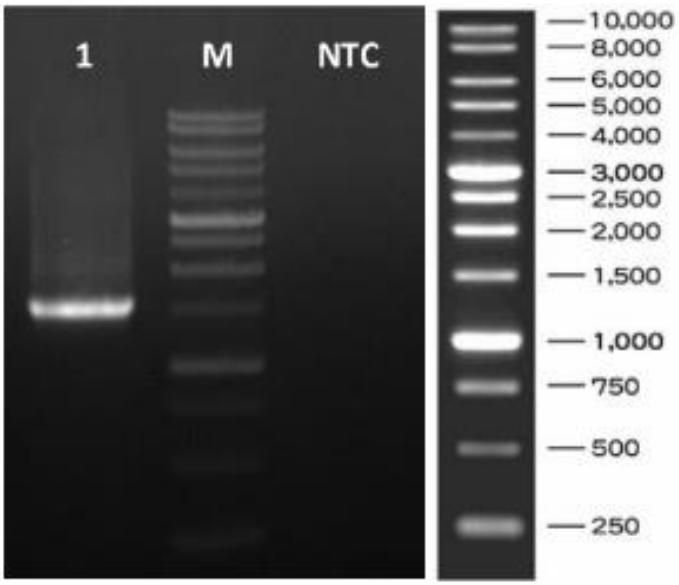


Figure 2. Agarose electrophoresis of Eco-Enzyme PCR product (lane 1)

Sequences data of Eco-Enzyme using NGS

The genomic DNA extracted from the Eco-Enzyme sample resulted in a gDNA concentration of 7.4 ng/µL, with purities of 2.02 (A260/280) and 2.04 (A260/230), as measured using a nanodrop. The amplification of gDNA was clear, supported by electrophoresis data showing a single band with a 1 Kb DNA ladder (Fig 2). Raw sequence reads totaled 70,235, with 13,248 reads remaining after filtering by Nanoplot. The quality of reads after FastQC analysis is reported as 11.5 (Table 2).

Table 2. The quality of FASTQ files of Eco-Enzyme DNA sample from paired-end reads, assessed by NanoPlot.

Sequence	Raw	Filtered
Mean read length	1,578.40	1,525.60
Mean read quality	11.6	11.5
Number of reads	70,235	13,248
Read length N50	1,623	1,558
Total bases	**110,859,611**	**20,207,030**

Relative abundance and Alpha diversities of Eco-Enzyme microbiota

Using the Krona diagram, the relative abundance of microbiota in Eco-Enzyme samples is revealed. The groups are divided into Terrabacteria (83%) and Proteobacteria (17%). Within the Terrabacteria group, the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae are detected in the phylogenetic classification. In the Proteobacteria group, the classification includes phylum Alphaproteobacteria (16%), class Rhodospirillales (16%), order Acetobacteraceae (16%), and family Acetobacter (Figure 3).

Table 3. Alpha diversities including observed, Chao1, se.chao1, ACE, se.ACE, Shannon, Simpson, Inv Simpson, and Fisher in metagenomic analysis of Eco-Enzyme

Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	Inv Simpson	Fisher
211	321.758	32.380	340.098	10.536	2.747	0.892	9.227	35.637

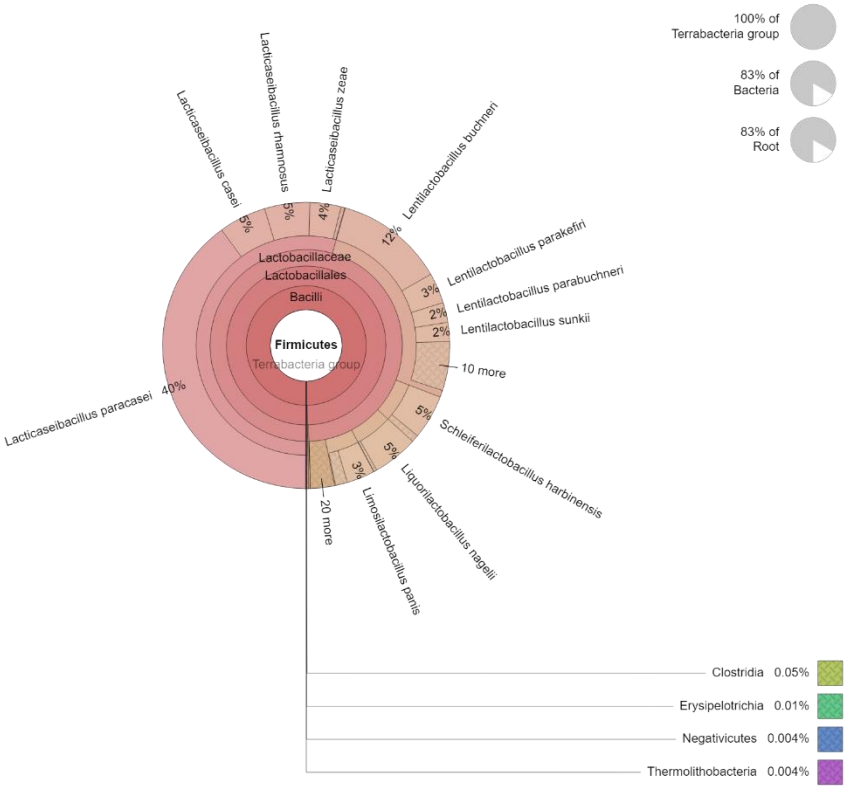


Figure 3. The Krona diagram provides a clear visualization of the relative abundances within the complex hierarchies of metagenomic classifications. The counts in Krona were derived from the numReads obtained from the Centrifuge Report.

At the 7th scale of depth, the Krona diagram exhibits a more diverse and specific genus in the Terrabacteria group. *Lacticaseibacillus* is the most abundant genus (55%), followed by *Lentilactobacillus* (27%), Schleiferilactobacillus (6%), *Liquorilactobacillus* (6%), *Limosilactobacillus* (5%), and 20 other genera (Figure 4a). Moving to the 8th scale of depth, the most abundant species from family Lactobacillaceae is *Lacticaseibacillus paracasei* (40%), with *Lentilactobacillus buchneri* as the second rank (12%) (Figure 4b). In the Proteobacteria group, *Acetobacter fabarum* is identified as the most abundant species, constituting 13% of the population, followed by *Acetobacter lovaniens* at 2% (Figure 3).

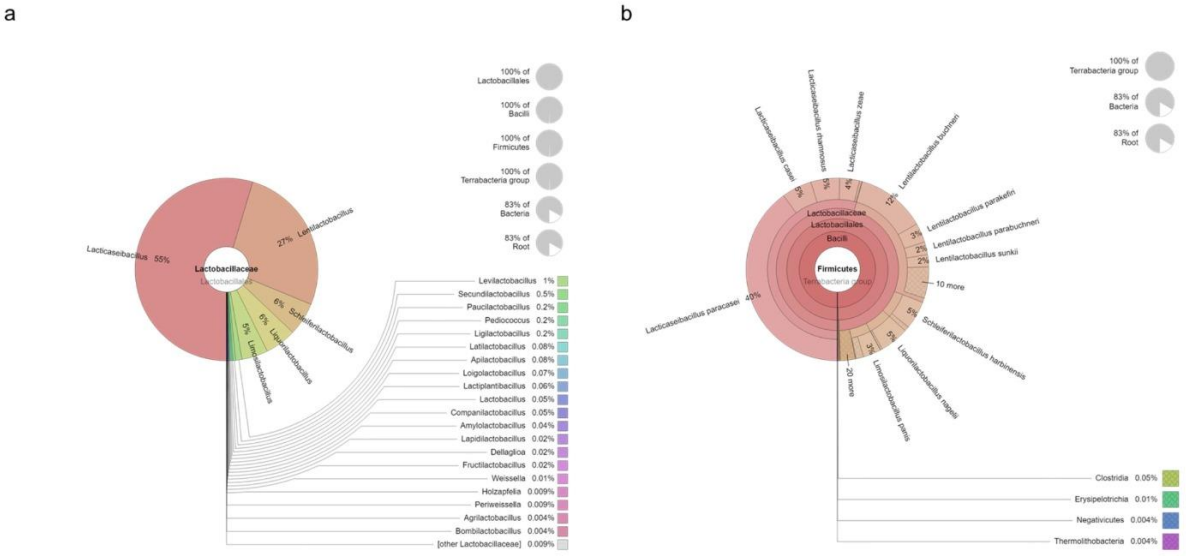


Figure 4. More detailed visualization of relative abundance: (a) Twenty genera detected from terrabacteria group, (b) A depth of scale 8 within the family Lactobacillaceae

The Sankey diagram simplifies the abundance of microbiota, representing the quantity over hierarchy. In the metagenomics analysis, 13.2K bacteria are detected, with proportions of 11.1K Firmicutes and 2.15K Proteobacteria. Other phyla, such as Actinobacteria, Bacteroidetes, and Tenericutes, are also detected but not significantly. At the family level, 11K Lactobacillaceae and 2.13K Acetobacteraceae are identified as the most abundant. At the species level, *Lacticaseibacillus paracasei* is the most dominant (2.82K), followed by *Lentilactobacillus buchneri* (2.13K), and *Lentilactobacillus hilgardii* (118). In the Proteobacteria domain, only two species are perceived: *Acetobacter fabarum* (1.40K) is the most plentiful, followed by *Acetobacter lovaniensis* (169).

The most dominant genus identified in the Eco-Enzyme product is Lacticaseibacillus and Lentilactobacillus. *L. paracasei* (formerly *Lactobacillus paracasei*) species consist of facultative anaerobic lactic acid bacteria (LAB) that are non-motile, non-spore-forming, rod-shaped, and present in a variety of niches, including fermented foods and host-associated microenvironments (Zheng et al., 2020). Mechanistic studies have revealed that *L. paracasei* isolates may exhibit strain-specific antiproliferative (Saxami et al., 2017), immunomodulatory (Chondrou et al., 2020), antibiofilm, and antimicrobial activity (Acurcio et al., 2020). A study found that preactivated *L. buchneri* and urea together increased the fermentation properties, nutritional value, aerobic stability, and methane ratio of corn silages. Additionally, it has been demonstrated that *L. buchneri* preactivation combined with urea treatment significantly improves corn silage's aerobic stability, crude protein, residual water-soluble

carbohydrate, and lactic acid content (Bağcık et al., 2022).

Next, the abundant bacteria from the Proteobacteria domain is *A. fabarum*, which was discovered during the fermentation of a cocoa bean heap in Ghana. *A. fabarum* is a gram-negative bacterium with rounded rod-like shapes that range in size from 0.8 micrometers to 1.2 to 3 micrometers. *A. fabarum* forms spherical, beige colonies on agar plates and reaches 0.8 millimeters in size after three days at 28°C (Cleenwerck et al., 2008). The metabolic properties of *A. fabarum* were initially described by Cleenwerck et al. (2008) in 2008, and they included an intake of ethanol, methanol, D-glucose, and D-xylose as the only carbon sources, acceptance of ammonium as the only nitrogen source, and the presence of the enzyme oxidase and catalase. A study of Korean kefir isolated *A. fabarum* and during kefir fermentation, it creates a barrier that prevents contamination by foodborne bacteria (Kim et al., 2019).

A study mentioned the potential of the biocellulose agent of *Acetobacter fabarum* wo-2 from orange waste and *Acetobacter lovaniensis*, wgr-2 from grapes waste (Kaur and Shah, 2022). Bacterial cellulose/biocellulose (BC) associated with polyethyleneimine (PEI-BC) was used as a heavy metal adsorbent material. Its maximal adsorption capacities for Cu(II) and Pb(II) were determined to be 141 and 148 mg/g, respectively. The polymeric mixture PEI-BC showed good reusability following post-treatment regeneration with Na2EDTA. Although lower than before treatment, the PEI-BC's Cu(II) re-adsorption capacity remained constant after each cycle of use. Overall, PEI-BC showed good reusability in the removal of Cu(II) and Pb(II) from aqueous solutions, demonstrating its promise as a bio-adsorbent for the removal of heavy metal ions from wastewater (Jin et al., 2017).

Since catalase exhibited the highest activity in the Eco-Enzyme, the abundance of *Acetobacter* species is unsurprising, given that members of this genus are generally catalase-positive. Recent characterization of *A. fabarum* confirms its catalase-positive nature, identifying it as an acetic acid bacterium (AAB) involved in fermentation and capable of utilizing diverse carbon sources from biological waste (El-Askri et al., 2022). This suggests that *Acetobacter* species play a crucial role in oxidative metabolism within the Eco-Enzyme matrix.

In addition, the relatively high protease activity observed shows a linear correlation with the abundance of *Lacticaseibacillus* and *Lentilactobacillus* species in the Eco-Enzyme. For instance, *L. paracasei* XJ-003, classified as a lactic acid bacterium, has been reported to maintain stable proteolytic activity. The proteolytic system in lactic acid bacteria comprises proteinases, peptidases, and specific transport proteins that function synergistically. Proteinases initially hydrolyze casein into peptides, which are subsequently degraded by intracellular peptidases into smaller peptides and free amino acids. Transport proteins then facilitate the transfer of these molecules across the cytoplasmic membrane (Kenny et al., 2003). Furthermore, the proteolytic system of lactic acid bacteria not only contributes to nutrient metabolism but also plays a key role in developing the organoleptic properties of fermented products. Beyond that, cellular proteolysis helps regulate polypeptide quality and maintain appropriate levels of regulatory proteins, ensuring proper cellular function (Savijoki et al., 2006).

The use of BC filters to treat colored textile effluents and remove microbiological cells is another intriguing application. The removal of *Escherichia coli*, dye effluents, and solids by BC membranes was successful for up to ten cycles, suggesting that they could be used in different wastewater treatment processes and contribute to the creation of new biotechnological tools like improved filtration techniques using more affordable materials (Alves et al., 2020).

The number of OTUs, the distribution of species abundances, or both can be used to determine how a bacterial community is structured using alpha diversity indices (Willis, 2019). In this study, the Shannon index of Eco-Enzyme is 2.747, and the Simpson index is 0.892. In this case, a larger Shannon index value indicates a higher level of species variety, whereas a smaller Simpson index indicates the dominance of a particular species (Suh & Kim, 2021).

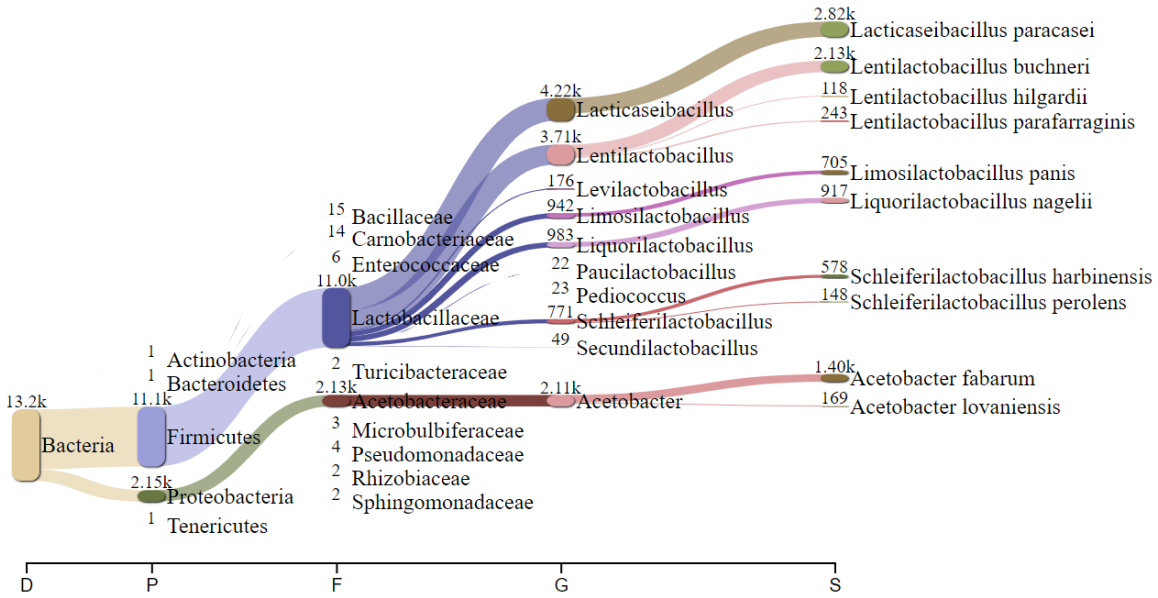


Figure 5. Visualization of microbial species in Eco-Enzyme. The arrow width is proportional to the quantity to depict changes over time hierarchy between nodes

CONCLUSIONS

Eco-Enzyme has successfully demonstrated its high catalase, chitinase, and protease activities, providing insights into how EE can effectively treat waste. Moreover, microbial abundance in EE reveals the dominance of *Lacticaseibacillus* and *Lentilactobacillus*, both classified in Terrabacteria, constituting a significant 83%. The remaining portion belongs to the Proteobacteria group, where *Acetobacter fabarum* is identified as the most abundant species.

CONFLICT OF INTEREST

The authors declare that we are not associated with or engaged in any organization or entity that has financial interests (including honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent-licensing arrangements) or non-financial interests (such as personal or professional relationships, affiliations, knowledge, or beliefs) related to the subject matter or materials covered in this manuscript.

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