



LIGNOCELLULOSE MODIFICATION AND NUTRITIONAL ENRICHMENT OF PALM OIL FRONDS: A STUDY ON OPTIMIZED PRETREATMENT AND MICROBIAL FERMENTATION FOR RUMINANT FEED

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ABSTRACT

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Palm oil fronds are a plantation waste with potential as ruminant feed, but their high lignocellulose content limits widespread use. This study aimed to improve the nutritional quality of palm oil fronds through pretreatment and fermentation, including their amino acid and fatty acid profiles. Three pretreatment methods (steaming, pressure cooking, and eco-enzyme) were combined with fermentation using *Bacillus subtilis*, *Lactobacillus acidophilus*, *Aspergillus niger*, and *Saccharomyces cerevisiae*. Fiber fractions, proximate composition, and amino and fatty acid profiles were analyzed using ANOVA and Duncan's Multiple Range Test. Pretreatment reduced lignin content by up to 52.07±2.58% (30-minute steaming with 100% eco-enzyme) relative to the untreated control (17.76±0.306%), with longer treatment durations giving greater lignin degradation. Fermentation raised crude protein from 4.99% to 16.50% in the T₂M₂ treatment (pretreatment with *A. niger* + *L. acidophilus*), which also had the most favorable amino acid profile. The T₂M₁ treatment (pretreatment with *B. subtilis* + *L. acidophilus*) showed the lowest fiber fraction content and the greatest improvement in fatty acid profile. Because both treatments combined pretreatment and fermentation, the improvements reflect their combined effect rather than the microbial consortium alone. These findings suggest that pretreated and fermented palm oil fronds have potential as a ruminant feed ingredient based on their improved chemical and nutritional profile. However, as the study was limited to laboratory analyses, in vivo feeding trials are needed to validate feed intake, growth performance, and productive efficiency.

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INTRODUCTION

Ruminant animals are composed of a complex digestive system characterized by a four-chambered stomach, namely the rumen, reticulum, omasum, and abomasum. The rumen and reticulum, which are rich in microbial populations, play an essential role in the fermentation of high-fiber feed (Lisnanti & Mukmin, 2020). In tropical regions, fiber sources for ruminant feed are commonly derived from

agricultural and plantation by-products such as palm oil fronds (Wilkinson & Lee, 2018).

Palm oil fronds are a by-product of palm oil cultivation with the potential to be used as feed for ruminants such as cattle (Astuti *et al.*, 2015). However, the high lignocellulose content and low nutritional value pose significant challenges for use as feed (Astuti *et al.*, 2021). Lignocellulose, primarily composed of cellulose, hemicellulose, and lignin, ranging from 17-20.5% of dry biomass, is the main component of palm oil fronds. Lignin that acts as an antinutritional compound hinders feed digestibility (Chanjula *et al.*, 2021). The robust structure of lignocellulose, formed by covalent bonds between lignin and hemicellulose, protects cellulose, making hydrolysis difficult (Rilek *et al.*, 2017). Therefore, pretreatment is carried out to disrupt lignin structure and cellulose crystallinity, facilitating the hydrolysis of hemicellulose and cellulose (Amin *et al.*, 2017).

Pretreatment uses physical methods like grinding, steaming, or adding eco-enzyme to improve digestibility. Grinding reduces particle size of lignocellulose biomass and enhances enzyme access (Moodley, 2021), while fermentation increases feed value from palm oil fronds (Ibarruri *et al.*, 2021). Generally, proper nutrition supports the health, welfare, and productivity of livestock (Kusmiati *et al.*, 2024). Microorganisms such as *Aspergillus niger*, *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, and *Bacillus subtilis* help by breaking down fiber and lignin, improving feed quality (Wenger *et al.*, 2020).

A. niger produces enzymes that degrade plant polysaccharides like cellulose and pectin, including amylase, lipase, and glucose oxidase (Bellaouchi *et al.*, 2021; Dong & Pan, 2023; Li *et al.*, 2020). *S. cerevisiae* generates hydrolytic enzymes that convert complex nutrients into simpler ones for easier absorption (NURHAYATI *et al.*, 2019). *B. subtilis* boosts essential amino and fatty acids in fermented feed (Budi *et al.*, 2011), while *L. acidophilus* improves amino acid content and palatability and helps eliminate antinutritional factors. Therefore, this study aimed to evaluate the effect of pretreatment and fermentation of palm oil fronds by *A. niger*, *S. cerevisiae*, *L. acidophilus*, and *B. subtilis* on fiber fraction content, proximate composition, essential amino and fatty acid profiles, and feed antioxidant activity, in order to assess their potential as a nutritionally improved feed ingredient for ruminants. The improved nutritional quality resulting from this treatment is expected to serve as a foundation for future application in beef cattle feeding strategies, potentially contributing to more cost-efficient feed formulation.

2. METHODOLOGY

2.1 Lignocellulose Pretreatment

Palm oil fronds were peeled, the inner part was cleaned, chopped, and air-dried under direct sunlight for 3–5 days as an initial post-harvest step to reduce moisture content prior to further processing. The resulting moisture content of the dried fronds is presented in Table 3 under the untreated (control) sample. The dried fronds were then ground using a grinder to achieve a particle size of 80 mesh.

Pretreatment in this study was divided into three types: steaming, autoclaving (pressure cooker), and eco-enzyme immersion. The eco-enzyme used was a commercial product formulated from a 50:50 mixture of fruits (orange, mangosteen,

watermelon rind, and pineapple) and vegetables (carrot and green mustard/*caysim*). For steaming and pressure cooker treatments combined with 100% eco-enzyme, the eco-enzyme immersion duration was fixed at 24 hours, allowing the effect of steaming/pressure cooking duration to be evaluated independently. Given the substantial effect of eco-enzyme observed in preliminary trials, eco-enzyme immersion was further tested as a standalone pretreatment across a range of immersion durations (3, 6, 12, and 24 hours; P8–P11) to specifically evaluate its effect over time. Each treatment was conducted in triplicate ($n = 3$), with an untreated sample (P0) serving as control. The resulting lignin and glucose content were analyzed using the DNS (Dinitrosalicylic acid) method, with absorbance measured at 540 nm using a UV-Vis spectrophotometer. The complete treatment combinations are presented in Table 1.

2.1.1 Fiber Fraction Analysis

This study used the Van Soest method to quantitatively analyze lignocellulose content (cellulose, hemicellulose, and lignin) (Lisanti *et al.*, 2021).

Neutral Detergent Fiber (NDF) Analysis

A 1-gram dried sample of palm oil fronds (a), expressed on a dry-matter basis, was placed in a 600 ml beaker, and 100 ml of Neutral Detergent Solution (NDS), consisting of 30 g/l sodium lauryl sulfate, 18.61 g/l disodium ethylenediaminetetraacetate (EDTA) dihydrate, 6.81 g/l sodium borate decahydrate, 4.56 g/l anhydrous disodium hydrogen phosphate, and 10 ml/l triethylene glycol, prepared following the method of Van Soest *et al.* (1991), was added, followed by heating. The sample was extracted for 60 minutes from the boiling point and filtered using a pre-weighed G3 sintered glass crucible (b). After the extraction process, the residue was rinsed with hot water and acetone. The crucible and residue were dried in an oven at 105°C for approximately 4 hours to achieve a constant weight. The sample was removed and cooled in a desiccator, and the crucible was weighed (c). Calculations:

$$NDF \% = \frac{c - b}{a} \times 100\%$$

Acid Detergent Fiber (ADF) Analysis

A 1-gram sample of palm oil fronds (A) was placed in a 600 ml beaker, and added with 100 ml of Acid Detergent Solution (ADS). The sample was extracted for 60 minutes from the boiling point and filtered using a pre-weighed sintered glass crucible (B). The residue was rinsed with hot water and acetone, followed by drying in an oven at 105°C for approximately 4 hours to achieve a constant weight. The crucible was removed and cooled in a desiccator, followed by weighing (C). Calculations:

$$ADF \% = \frac{C - B}{A} \times 100\%$$

Cellulose and Hemicellulose Analysis

For cellulose analysis, ADF sample, which had been weighed (C), was added with 20 ml of 72% sulfuric acid (H_2SO_4) and left to submerge for 3 hours. The residue was rinsed after 3 hours with hot water and acetone, dried in an oven at 105°C for approximately 4 hours until a constant weight was obtained, then removed and cooled in a desiccator. The crucible was removed from the desiccator after cooling and

weighed (D). The amount of cellulose content was calculated using the following equation, adopted from Lisanti et al. (2021), based on the method of Van Soest et al. (1991):

$$\text{Cellulose \%} = \frac{C - D}{A} \times 100\%$$
$$\text{Hemicellulose \%} = \% \text{NDF} - \% \text{ADF}$$

Lignin Analysis

Lignin analysis was a continuation of the ADF and cellulose analyses. The dried sample (D) was burned in a furnace at approximately 600°C for 2 hours. The crucible was cooled in a desiccator and weighed (E). The amount of lignin was calculated using the following equation: Calculations:

$$\text{Lignin \%} = \frac{C - E}{A} \times 100\%$$

2.1.2 Scanning Electron Microscope (SEM) Analysis

The samples used for SEM characterization were palm oil fronds before pretreatment (control) and after pretreatment. Approximately 1 gram of palm oil fronds was prepared, from which a small portion was taken and flattened onto a stage adhesive before being inserted into the SEM instrument. After the sample was evenly distributed on the stage adhesive, morphological analysis was performed using the SEM instrument at magnifications of 200× and 500× to evaluate morphological damage on the palm oil fronds surface resulting from the pretreatment process.

2.1.3 Reducing Sugar Analysis

Reducing sugar analysis aims to measure the amount of reducing sugar in palm oil fronds feed. The method used was the DNS method, with the procedures as follows.

Preparation of DNS and Glucose Stock Solution

DNS solution was prepared by dissolving 1 gram of DNS powder, 20 ml of 2M NaOH, and 30 grams of Ka-Na tartrate in a volume of 100 ml. The solution obtained was stored in a refrigerator. The glucose stock solution for the standard curve was made using glucose solution with concentrations of 0.2, 0.4, 0.6, 0.8, 1, and 1.2 mg/mL. Each standard of glucose stock solution was placed in a test tube in a volume of 1 ml, followed by adding distilled water and DNS reagent. All test tubes were heated in a water bath for 5 minutes to allow the reaction between glucose and DNS, followed by cooling and vortexing to ensure homogeneity. The absorbance of each solution was measured at 540 nm, and the values obtained were used as controls.

Reducing Sugar Test

Reducing sugar test was carried out by taking 1 ml of the sample into a tube, then adding 1 ml of DNS reagent and 2 ml of distilled water to each test tube using a pipette. To enable the reaction between glucose and DNS, the test tubes were heated in a water bath for 5 minutes. The absorbance of each solution was measured at 540 nm (James, 1995), and the values obtained were plotted on a standard curve to determine the glucose concentration in the sample.

2.2 Fermentation of Palm Oil Fronds

Palm oil fronds were weighed at 50 grams and supplemented with 5% urea. The microbial consortium used was *B. subtilis* + *L. acidophilus*, *A. niger* + *L. acidophilus*, and *S. cerevisiae* + *L. acidophilus*. Fermentation with *A. niger* and *S.*

cerevisiae was conducted for 30 days at 27°C, while fermentation with *B. subtilis* was conducted for 10 days at 37°C. This temperature selection reflects the optimal growth range of each microorganism: *B. subtilis* is most efficient in its biological processes within a temperature range of 25–37°C (Kusdini, 2024), while the incubation temperature of 27°C for *A. niger* is consistent with solid-state fermentation conditions previously reported for lignocellulosic substrates (Abduh *et al.*, 2025). *L. acidophilus* was co-inoculated with each respective microbial treatment under the same duration and temperature conditions as its paired microorganism. The preparation of *A. niger* and *S. cerevisiae* cultures used a medium consisting of Potato 50 g/L (dry weight) - Malt extract 20g/L - Yeast extract powder Himedia 0.5 g/L - MgSO₄.7H₂O Merck 2 g/L - FeSO₄.7H₂O Merck 0.1 g/L - CaCl₂.2H₂O Merck 3 g/L. Meanwhile, *B. subtilis* was inoculated with 3-5 loops onto Luria Bertani Broth Himedia medium with a composition of tripton Oxoid 1%, NaCl Puduk 1%, and yeast extract powder Himedia 0.5%. *L. acidophilus* was inoculated into a medium composed of peptone Oxoid 10 g/L, beef extract Oxoid 8 g/L, yeast extract Himedia 4 g/L, glucose Merck 20 g/L, K₂HPO₄ Merck 2 g/L, and C₆H₁₂O₇N₂ 2 g/L, Tween 80 Sigma-Aldrich 1 g/L.

2.2.1 Proximate Analysis

Moisture Content Analysis (AOAC, 2005)

A 3g sample was placed in a pre-dried aluminum crucible of known weight. The sample was then dried in an oven at 105°C for 6 hours, cooled in a desiccator for 15 minutes, and weighed. The drying and weighing process was repeated until a constant weight was obtained. Moisture content was calculated using the following formula:

$$\text{Moisture Content \%} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100\%$$

Procedure Ash Content Analysis (AOAC, 2005)

A 3g sample was placed in a pre-weighed porcelain crucible. The sample was then charred on an electric hotplate and ashed in a furnace at 600°C for approximately 4 hours or until a white ash was obtained. The crucible was cooled in a desiccator to room temperature and weighed. Ash content was calculated using the following formula:

$$\text{Ash Content \%} = \frac{\text{Ash Weight (gr)}}{\text{Sample Weight (gr)}} \times 100\%$$

Procedure Crude Fiber Determination (AOAC, 2005)

A 1g sample of finely ground palm oil fronds was weighed into a 500 mL Erlenmeyer flask, added with 100 mL of 0.255 N sulfuric acid, refluxed for 30 minutes, and filtered. The filtrate was neutralized with distilled water, added with 50 mL of 1.25 N sodium hydroxide, and refluxed for 30 minutes. After cooling, the sample was filtered through filter paper. The residue was washed successively with 25 mL of distilled water, 20 mL of 95% ethanol, and 25 mL of 10% potassium sulfate solution. The residue was dried in an oven at 105°C for 2 hours, cooled in a desiccator, and weighed to a constant mass. Crude fiber content was calculated using the following formula:

$$\text{Crude Fiber Content \%} = \frac{\text{Weight of Dry Residue (gr)}}{\text{Sample Weight (gr)}} \times 100\%$$

Crude Protein Determination (AOAC, 2005)

A 1 g sample was placed in a digestion tube and positioned in a digestion block. Half a Kjeldahl tablet (containing K₂SO₄ and CuSO₄) and 15 mL of concentrated sulfuric acid were added. The solution was mixed by shaking and allowed to stand for 5 minutes. A scrubber cup was attached to the digestion block, and the assembly was placed in a FOSS digester. The sample was digested for 3 hours.

After cooling, 25 mL of distilled water was added, and distillation was carried out by adding 40% NaOH. The ammonia gas produced was trapped in a 3% boric acid solution. This was followed by titration with 0.1 N HCl, and blank determination was performed, without using the sample.

$$N \text{ Content } \% = \frac{\text{Volume of HCl} \times N_{\text{HCl}} \times 14,007 \times 100}{\text{Weight Sample (mg)}} \times 100\%$$

$$\text{Crude Protein } \% = N \times \text{Correction Factor}$$

Procedure Crude Fat Determination (AOAC, 2005)

A 2-gram sample of finely ground palm oil fronds was wrapped in fat-free filter paper and plugged with fat-free cotton. The filter paper containing the sample was placed in a Soxhlet extraction thimble. A pre-weighed fat extraction flask was attached to the extraction thimble of the Soxhlet apparatus, and the system was filled with solvent until the solvent level reached the siphon tube. Cooling water was circulated, and the apparatus was turned on. Extraction was carried out for 5 hours, and solvent containing the fat was evaporated. The fat extraction flask was dried in an oven at 105°C for 30 minutes. Drying was repeated until a constant weight was obtained. The weight of the residue in the flask was recorded as the weight of fat or oil and was calculated using the following formula:

$$\text{Crude Fat } \% = \frac{\text{Fat Weight (gr)}}{\text{Sample Weight (gr)}} \times 100\%$$

Procedure for determining Nitrogen-Free Extract (NFE) and Total Digestible Nutrient (TDN) content

Proximate analysis was conducted to determine the chemical characteristics of the material, including moisture and ash content as well as crude protein, fat, and fiber. Subsequently, the results of proximate analysis were converted to a 100% dry matter basis for the calculation of NFE value using the following equation:

$$\text{NFE}\% = 100\% - (\% \text{Moisture Content} + \% \text{Ash Content} + \text{Crude Fiber Content} + \% \text{Crude Protein Content} + \text{Crude Fat Content})$$

Procedure for Essential Amino Acid Analysis (Saraswanti, 2021)

Amino acid content analysis was conducted at PT. Saraswanti Indo Genetech Laboratory, Bogor. Initially, a 0.1 g sample was hydrolyzed with 5 mL of 6 N HCl at 110°C for 22 hours, transferred to a 50 mL flask, and filtered through a 0.45 µm filter. The filtrate (500 µL) was added with 40 µL of internal standard AABA (Alpha Amino Butyric Acid) solution and 460 µL of deionized water. A 10 µL aliquot of this solution was pipetted, added with 70 µL of AccQ-Fluor Borate, and 20 µL of fluor A reagent. The solution was incubated at 55°C for 10 minutes, then 1 µL of the filtrate was injected into the UPLC under the following operating conditions:

Column Type	: ACC Q-Taq Ultra C18
Temperature	: 49°C
Mobile Phase	: Gradient composition system

Flow rate : 0,7 mL per minute
 Detector : PDA, at 260 nm wavelength
 Injection Volume : 1 µL

Calculation of amino acid content in the sample was performed by comparing the area ratio of the analyte to the internal standard using the following formula:

$$\text{Standard - to - sample ratio} = \frac{A_{spl}}{A_{IS}}$$

$$\text{Amino acid content (ppm, } \frac{mg}{Kg}, \frac{mg}{L} \text{)}$$

$$= \frac{\frac{\text{sample ratio}}{\text{standard ratio}} \times \frac{C_{std}}{1000000} \times BM \times Va \times Fp}{W_{spl} \text{ or } V_{spl}}$$

Keterangan:

A_{Spl} : Peak area of amino acid analyte
 A_{IS} : Peak area of internal standard AABA
 BM : Molecular weight of amino acid (g/mol)
 C_{Std} : Concentration of amino acid standard solution (pmol/µL)
 Va : Final volume of test solution (µL)
 Fp : Dilution factor
 W_{Spl} : Weight of test portion (g)
 V_{Spl} : Volume of test portion (mL)

2.2.3 Antioxidant Test

A 100 ppm DPPH solution was prepared by dissolving 5 mg of DPPH crystals in 50 ml of PA ethanol solvent and stored in a tightly closed container protected from sunlight. Subsequently, 10 mg of the sample was weighed and dissolved in 100 mL of PA ethanol, followed by dilution to several concentrations, namely 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. A 10 mg of vitamin C 1000 were weighed and diluted with 100 ml of PA ethanol. Furthermore, 3 ml of the test solution was placed in a test tube, added with 1 ml of DPPH, and incubated at 27°C. During the reduction process by antioxidants, DPPH radical solution changed color from purple to pale yellow. This decrease in absorbance was measured with a UV spectrophotometer at a wavelength of 515 nm (A_s). Radical inhibition activity was calculated using the formula below:

$$\text{Inhibition (\%)} = \frac{Ab - As}{As} \times 100\%$$

Explanation:

Ab: Blank absorbance

As: Test compound absorbance

2.2.4 Total Plate Count (TPC) and Growth Curve

TPC analysis of bacteria and fungi was performed by taking 1 gram of inoculum and placing in a test tube containing physiological NaCl. The sample was homogenized using a vortex, and dilution was performed to 10⁻⁹ and 10⁻⁷ for bacteria and fungi, respectively. Subsequently, 1 ml of each of the last three dilutions was taken and poured onto sterile Petri dishes. Agar medium was poured onto each Petri dish and gently shaken until evenly distributed. The agar was left to solidify in the Petri dishes, and those containing bacteria samples were stored upside down and

wrapped in paper. This was followed by incubation at 37°C for 24 hours for bacteria and 27°C for 48 hours for fungi. Samples were taken to count the total number of bacteria using the pour plate method.

The data were obtained based on calculations of cell density and the generation time of bacteria and fungi growing on palm fronds. The generation time of bacteria and fungi was calculated once from cell density in the exponential (log) phase. From the data, the growth rate constant (k) and generation time (g) were calculated using the formula by Willey et al. (2008) in the book Microbiology, 7th edition, pages 126-127. The formula for calculating the number of generations is as follows:

$$k = \frac{n}{t} \frac{\log N_t - \log N_0}{0.301}$$

Explanation:

n = Number of generations

N_t = Final cell count (CFU/mL)

N₀ = Initial cell count (CFU/mL)

$$G = \frac{1}{k}$$

Explanation:

G = Generation time

k = Growth rate constant

2.3 Data Analysis

This study was conducted using a Completely Randomized Design (CRD), with 19 treatments and three replications for the pretreatment stage (Stage 1), and 6 treatments with three replications for the fermentation stage (Stage 2). To determine the effect of pretreatment and fermentation on the nutritional quality of feed ingredients, the normality test was initially analyzed using the Liliefors normality test. Data that had been tested for normality were examined for homogeneity using the Bartlett homogeneity test. When the data were declared abnormal or non-homogeneous, transformation was performed before analyzing the variance. For normal and homogeneous data, the variance was analyzed directly using analysis of variance (ANOVA) was analyzed directly using analysis of variance (ANOVA) at a significance level of 0.05 ($\alpha = 0.05$) to determine whether there was an effect of the treatment. When a significant effect was observed, the analysis was followed up with Duncan's Multiple Range Test (Hanafiah, 1991).

3. RESULT AND DISCUSSION

3.1 Pretreatment Lignocellulose

In this study, three types of pretreatment were applied to reduce lignin content and enhance the effectiveness of palm oil fronds hydrolysis. The results of the pretreatment are shown in Table 1.

Table 1. Effect of Pretreatment Type, Duration, and Eco-enzyme Concentration on Lignin Content of Palm Oil Fronds

Pretreatment	Treatment	Lignin Content (%)	Lignin Reduction (%)
Steaming	No Pretreatment	17.76 ± 0.306 ^d	0
	Steaming 15 min + <i>Eco-enzyme</i> 0%	13.75 ± 0.789 ^c	21.09±4.52 ^a
	Steaming 30 min + <i>Eco-enzyme</i> 0%	13.38 ± 0.982 ^c	22.62±5.63 ^{ab}
	Steaming 45 min + <i>Eco-enzyme</i> 0%	11.68 ± 2.090 ^b	32.98±11.99 ^{bc}
	Steaming 15 min + <i>Eco-enzyme</i> 100%	10.13 ± 0.258 ^b	41.84±1.48 ^{cd}
	Steaming 30 min + <i>Eco-enzyme</i> 100%	8.35 ± 0.449 ^a	52.07±2.58 ^{de}
	Steaming 45 min + <i>Eco-enzyme</i> 100%	7.71 ± 0.449 ^a	55.72±2.57 ^e
Pressure Cooker (PC)	PC 5 min + <i>Eco-enzyme</i> 0%	13.43 ± 0.632 ^c	22.95±3.62 ^a
	PC 10 min + <i>Eco-enzyme</i> 0%	13.24 ± 0.642 ^c	23.99±3.68 ^a
	PC 20 min + <i>Eco-enzyme</i> 0%	12.73 ± 0.820 ^{bc}	26.96±4.70 ^{ab}
	PC 30 min + <i>Eco-enzyme</i> 0%	11.76 ± 0.727 ^{bc}	31.81±4.17 ^{ab}
	PC 5 min + <i>Eco-enzyme</i> 100%	11.88 ± 0.128 ^{bc}	31.45±0.73 ^{ab}
	PC 10 min + <i>Eco-enzyme</i> 100%	10.61 ± 1.040 ^{ab}	39.08±5.97 ^{bc}
	PC 20 min + <i>Eco-enzyme</i> 100%	9.74 ± 1.884 ^a	44.08±10.80 ^c
	PC 30 min + <i>Eco-enzyme</i> 100%	9.43 ± 1.900 ^a	45.85±10.90 ^c
Eco-enzyme	<i>Eco-enzyme</i> 100% 3 hours	11.64±0.655 ^c	33.21±0.37 ^a
	<i>Eco-enzyme</i> 100% 6 hours	11.43±0.140 ^c	34.38±0.80 ^a
	<i>Eco-enzyme</i> 100% 12 hours	10.86±0.165 ^b	37.67±0.94 ^b
	<i>Eco-enzyme</i> 100% 24 hours	9.86 ± 0.165 ^b	43.39±0.94 ^c

Note: Pretreatment consisted of 19 treatment combinations, evaluated as a single-factor Completely Randomized Design (CRD) with three replications, comprising: steaming (15, 30, or 45 min) combined with eco-enzyme concentration (0% or 100%); pressure cooker (5, 10, 20, or 30 min) combined with eco-enzyme concentration (0% or 100%); eco-enzyme immersion alone (3, 6, 12, or 24 hours); and an untreated control (P0). Data are presented as mean ± standard deviation. Means followed by different superscript letters within a column indicate significant differences (Duncan's Multiple Range Test, $p < 0.05$).

3.1.1 Lignin Palm Oil Fronds

Duncan's multiple range test showed that pretreatment significantly affected lignin content ($p < 0.05$). As shown in Table 1, the average lignin content decreased in all samples after pretreatment. The results showed that the longer pretreatment process correlated with a higher percentage of lignin reduction. The best treatment was obtained at 45 minutes of steaming with 100% eco-enzyme, producing lignin content of 7.71% and a reduction of 55.72%. A 45-minute steaming time was optimal for breaking down lignin bonds in palm oil fronds. In this study, steaming temperature was maintained constant at 100°C across all treatments, with treatment duration as the sole variable tested. That longer duration and higher temperature increase biodegradability compared to short duration and low temperature treatments (Roy *et al.*, 2020). Therefore, lignin content decreased less in the 15-minute steaming than in the 45-minute, as shorter steaming caused less degradation. A previous study on the effect of thermal pretreatment on lignin content of wheat straw also showed that longer thermal process caused higher reduction (Gabhane *et al.*, 2020).

Based on the three types of pretreatment conducted, eco-enzyme had an effect on reducing lignin content. For example, in the 45-minute steaming without eco-enzyme (0%), lignin content decreased by 32.98%, while the addition of 100% eco-

enzyme reached 55.72%. Eco-enzyme used in this study was made from a mixture of fruits and vegetables in a 50:50 ratio, consisting of oranges, mangosteens, watermelon rind, pineapple, carrots, and green choy sum, with pH of 3. The fermentation process of making eco-enzyme using fruit peels can produce various organic compounds. That lower pH values showed higher organic acids, such as acetic or citric (Nengah Muliarta & Darmawan, 2021). The degradation of lignin in this study could be attributed to organic acid content in eco-enzyme. Similarly, that acid treatment disrupted the hemicellulose structure through hydrolysis of glycosidic bonds and cellulose fiber crystals (Ramírez-Estrada *et al.*, 2024). White rot fungi were found in eco-enzyme after 90 days of fermentation, playing an essential role in the hydrolysis of lignocellulose, particularly lignin (Kartika & Bakti, 2022).

Previous studies have shown that eco-enzyme made from *Cucurbita maxima* has microbial diversity, such as *Yersinia* sp., *Bacillus* sp., and fungi such as *Trichoderma* sp and *Penicillium* sp (Varshini & Gayathri, 2023). *Yersinia* sp., particularly *pestis* and *enterocolitica*, have been identified as capable of degrading lignin. These bacteria have enzyme that facilitates the breakdown of lignin, a complex organic polymer in plant cell walls (Grgas *et al.*, 2023; Singh *et al.*, 2014). Some species, including *Bacillus ligniniphilus* L1, have been found to degrade alkali lignin, producing aromatic compounds such as phenylacetic, 4-hydroxybenzoic, and vanillic acid (Zhu *et al.*, 2017). *Trichoderma* sp. also has the ability to degrade lignin and is known as one of lignocellulose fungi. Specifically, *Trichoderma viridae* can degrade lignin, increase the enzymatic digestibility of biomass, and produce a large amount of degradation products (M & A, 2017). The microbial fermentation process in eco-enzyme will release various types of enzyme such as catalase, amylase, protease, pectinase, glucose isomerase, cellulase, hemicellulase, lipase, and lactase (Benny *et al.*, 2023). Cellulase is an enzyme that can break down cellulose into glucose (Widyaningrum & Parahadi, 2020). Therefore, the decrease in lignin content with the addition of 100% eco-enzyme is attributed to the content of organic acids such as acetic acid, lignin-degrading fungi and bacteria, as well as potential enzyme, which promote lignocellulose hydrolysis.

3.1.2 Reducing Sugar from Palm Fronds

Lignocellulose pretreatment will produce sugar as a form of hydrolyzed cellulose and hemicellulose, the main structural polysaccharides within the lignocellulosic matrix (Loow *et al.*, 2016).

Table 2. Effect of Pretreatment on Reducing Sugar Content

Pretreatment	Treatment	Reducing Sugar (mg/ml)
Steaming	No Pretreatment	0
	Steaming 15 min + <i>Eco-enzyme</i> 0%	0.17 ± 0.003 ^a
	Steaming 30 min + <i>Eco-enzyme</i> 0%	0.19 ± 0.003 ^a
	Steaming 45 min + <i>Eco-enzyme</i> 0%	0.25 ± 0.004 ^b
	Steaming 15 min + <i>Eco-enzyme</i> 100%	0.4 ± 0.008 ^c
	Steaming 30 min + <i>Eco-enzyme</i> 100%	0.67 ± 0.002 ^d
	Steaming 45 min + <i>Eco-enzyme</i> 100%	0.81 ± 0.014 ^e
Pressure Cooker (PC)	PC 5 min + <i>Eco-enzyme</i> 0%	0.2 ± 0.004 ^a
	PC 10 min + <i>Eco-enzyme</i> 0%	0.21 ± 0.004 ^b
	PC 20 min + <i>Eco-enzyme</i> 0%	0.35 ± 0.007 ^c

Pretreatment	Treatment	Reducing Sugar (mg/ml)
	PC 30 min + <i>Eco-enzyme</i> 0%	0.41 ± 0.009 ^d
	PC 5 min + <i>Eco-enzyme</i> 100%	0.43 ± 0.008 ^e
	PC 10 min + <i>Eco-enzyme</i> 100%	0.45 ± 0.004 ^f
	PC 20 min <i>Eco-enzyme</i> 100%	0.61 ± 0.008 ^g
	PC 30 min + <i>Eco-enzyme</i> 100%	0.65 ± 0.004 ^h
Eco-enzyme	<i>Eco-enzyme</i> 100% 3 hours	0.48 ± 0.006 ^a
	<i>Eco-enzyme</i> 100% 6 hours	0.44 ± 0.002 ^b
	<i>Eco-enzyme</i> 100% 12 hours	0.55 ± 0.005 ^c
	<i>Eco-enzyme</i> 100% 24 hours	0.59 ± 0.002 ^d

Note: Data show mean ± standard deviation with p<0.05 (n=3). Superscripts ^{a-h} show significantly different results between eco-enzyme soaking time and eco-enzyme concentration on reducing sugar content using Duncan's test (DMRT). PC= Pressure Cooker

Lignin acts as a physical and chemical barrier that limits cellulose accessibility and inhibits enzymatic hydrolysis, thereby reducing sugar yield. When lignin is degraded through pretreatment, cellulose and hemicellulose become more accessible to hydrolytic enzymes, increasing the rate of hydrolysis and resulting in higher sugar yield (Sun *et al.*, 2014). In this study, reducing sugar content, measured using the DNS (Dinitrosalicylic acid) method and thus representing total reducing sugars rather than glucose specifically, showed a pattern consistent with lignin reduction across treatments. The highest reducing sugar content (0.81±0.014 mg/ml) was obtained in the treatment combining 45-minute steaming and 100% eco-enzyme, which also exhibited the lowest lignin content, supporting the proposed mechanism that lignin degradation enhances the accessibility of cellulose and hemicellulose for hydrolysis into reducing sugars. Similarly, Ong et al. (2019) stated that lignin content decreased by 14.01% after pretreatment with DES for 30 minutes, while the xylose content increased by approximately 58%. Brienzo et al. (2017) also demonstrated that bagasse samples with lower lignin content exhibited higher glucose yields during enzymatic hydrolysis, indicating that reduced lignin content facilitates greater cellulose accessibility for degradation into glucose monomers. Taken together, these findings support the mechanism by which the combined physical (steaming) and chemical (eco-enzyme) pretreatment used in this study facilitated lignin degradation, thereby enhancing the accessibility of cellulose and hemicellulose and increasing overall reducing sugar production.

3.1.3 SEM Palm Oil Fronds

After pretreatment process, palm oil fronds experienced changes in chemical composition and structure. The changes before and after pretreatment are shown in Figure 1.

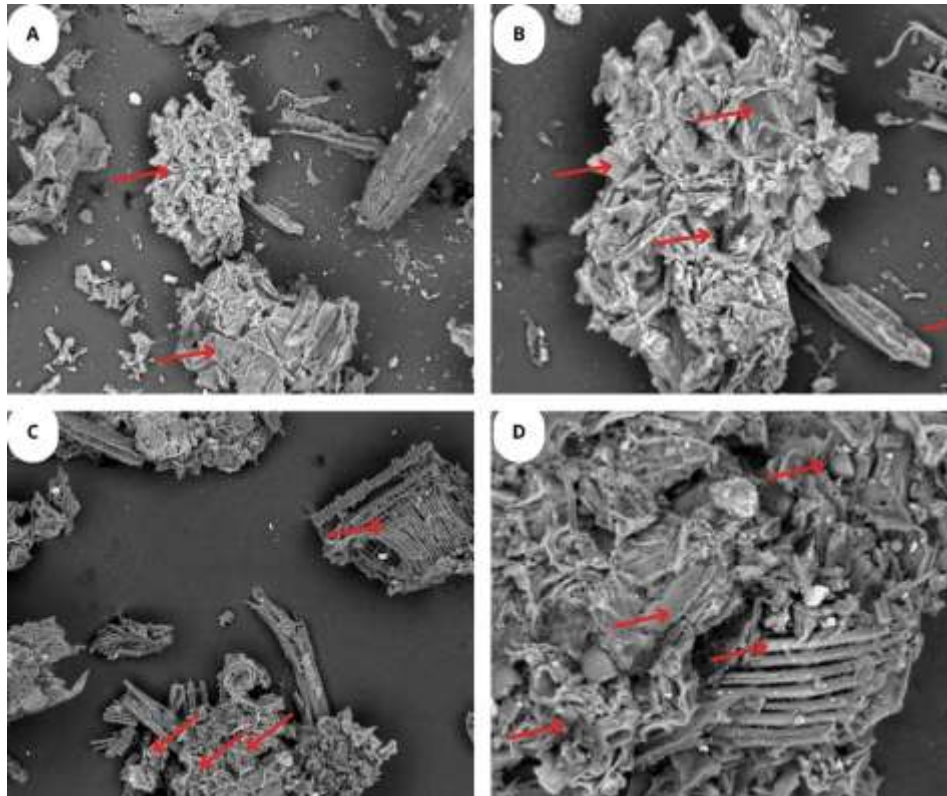


Figure 1. SEM Images of Palm Oil Fronds Before and After Pretreatment.

(A) palm oil fronds before pretreatment at 200x magnification; (B) palm oil fronds before pretreatment at 500x magnification; (C) palm oil fronds after pretreatment at 200x magnification; (D) palm oil fronds after pretreatment at 500x magnification.

Based on Figure 1, palm oil fronds before pretreatment did not show significant damage to cell wall structure (A, B). Palm oil fronds tended to remain intact, characterized by a non-porous structure, while pretreatment samples showed damaged structure, as presented by the presence of pores, visible cellulose, and hemicellulose fibrils. This suggested that the pretreatment process degraded lignin (C, D). Thermal processes at 200, 250, and 300°C caused severe damage, with the bamboo structure becoming damaged and perforated (Ong *et al.*, 2021).

3.2 Palm Oil Fronds Fermentation

Fermentation is an essential process in the production of feed based on plantation by-products to enhance the nutritional value of low-quality feed. Since there was no significant difference between 45 minutes of steaming + 100% eco-enzyme and 30 minutes of steaming + 100% eco-enzyme, the pretreatment used in the fermentation stage was 30 minutes of steaming + 100% eco-enzyme. The microbial consortium used consisted of *B. subtilis* + *L. acidophilus*, *A. niger* + *L. acidophilus*, and *S. cerevisiae* + *L. acidophilus*. The nutrient content after fermentation is shown in Table 3.

Table 3. Nutritional Composition of Palm Oil Fronds Post Solid-State Fermentation

Sample	Moisture Content	Ash Content	Protein Content	Fiber Content	Fat Content	NFE	Energy
SNI	13% (maks)	12% (maks)	13% (min)	24%(maks)	7% (maks)	-	-
T ₀ M ₀	13.22 ± 0.69 ^a	17.15 ± 0.18 ^c	4.99 ± 0.16 ^a	39.16 ± 0.93 ^e	1.75 ± 0.19 ^a	36.94 ± 0.894 ^a	3214 ± 21.58 ^c
T ₁ M ₁	36.21 ± 1.69 ^b	7.14 ± 0.66 ^b	15.61 ± 0.62 ^d	34.30 ± 0.79 ^d	7.08 ± 0.19 ^f	35.86 ± 0.66 ^a	2974 ± 1.13 ^c
T ₁ M ₂	43.54 ± 0.36 ^c	7.26 ± 0.37 ^b	13.68 ± 0.63 ^c	33.72 ± 0.53 ^d	6.23 ± 0.15 ^e	39.08 ± 1.45 ^b	2603 ± 0.66 ^a
T ₁ M ₃	43.19 ± 0.60 ^c	6.90 ± 0.19 ^b	15.52 ± 1.09 ^d	33.12 ± 0.47 ^d	5.38 ± 0.99 ^d	39.08 ± 0.70 ^b	2843 ± 0.47 ^a
T ₂ M ₁	34.86 ± 4.97 ^b	4.863 ± 0.86 ^a	11.44 ± 0.91 ^b	27.87 ± 0.90 ^b	10.57 ± 0.08 ^g	45.12 ± 2.47 ^d	3080 ± 1.05 ^{bc}
T ₂ M ₂	63.96 ± 0.20 ^d	7.26 ± 0.38 ^b	16.50 ± 0.52 ^d	24.13 ± 0.34 ^a	4.07 ± 0.58 ^c	48.08 ± 0.75 ^e	2523 ± 0.85 ^a
T ₂ M ₃	64.09 ± 0.10 ^d	8.27 ± 1.96 ^b	14.08 ± 0.81 ^c	32.10 ± 1.51 ^c	3.21 ± 0.28 ^b	42.34 ± 1.91 ^c	2719 ± 2.76 ^{ab}

Note: Data are presented as mean ± standard deviation with p<0.05 (n=3). Superscripts ^{a-g} show significant differences between steaming time and eco-enzyme concentration on lignin content using Duncan's Multiple Range Test (DMRT). T₀: Untreated palm oil fronds; T₁M₁: Untreated palm oil fronds + Fermentation *B. subtilis* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₁M₂: Untreated palm oil fronds + Fermentation *A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₁M₃: Untreated palm oil fronds + Fermentation *S. cerevisiae* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₁: 30-minute steaming + 100% eco-enzyme + Fermentation *B. subtilis* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₂: 30-minute steaming + 100% eco-enzyme + Fermentation *A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₃: 30-minute steaming + 100% eco-enzyme + Fermentation *S. cerevisiae* + *L. acidophilus* + 5% urea + 50% eco-enzyme; NFE : Nitrogen-Free Extract; SNI: Indonesian National Standard.

3.2.1 Ash

Based on Table 3, fermentation significantly reduced (p<0.01) the ash content of palm oil fronds (expressed on a dry-matter basis). All treatments met the feed standard of not more than 12%. The lowest ash content was obtained in T₂M₁, which included fermentation using *B. subtilis* + *L. acidophilus* (4.863%). This treatment showed the lowest cellulose content (17.46%). The drop in ash content resulted from microbial use of mineral nutrients during fermentation. Similar findings were reported that microbes consume ash minerals for growth (Ogodo *et al.*, 2017). Fermentation also breaks down cellulose into glucose, lowering water-binding capacity and ash levels (Soedjatmiko *et al.*, 2019). *B. subtilis* and *P. polymyxa* have likewise been shown to reduce ash and sulfur in coal by using minerals and altering its chemistry (El-Midany & Abdel-Khalek, 2014).

3.2.2 Protein

Following fermentation, the highest protein content of 16.50% was obtained in the treatment using *A. niger* + *L. acidophilus* + 50% eco-enzyme, representing a 230.66% increase relative to the untreated control (4.99%). The addition of 5% urea contributed to the increase, as it hydrolzed into ammonia and carbon dioxide during

fermentation (Canbolat, 2022). Ammonia provides nitrogen for microbial protein synthesis, while urea enhances nutrient availability and microbial growth. The improvement in nutrient availability supports microbial growth and increases the protein content of the fermented material (Wahyono *et al.*, 2022). *A. niger* is more efficient in using urea nitrogen than *B. subtilis* and *S. cerevisiae*, producing higher protein content (Said *et al.*, 2019). The addition of 5% urea and zeolite additions raised crude protein from 1.13% to 15.49%, with significant increases in glutamic (2.57%) and aspartic (1.24%) acid (Sukria, 2015). The rise was attributed to single-cell protein (SCP) formation from microbial growth (Sharif *et al.*, 2021). After 15 days, *A. niger* reached 1.83×10^8 CFU/ml, contributing to high protein levels (Figure 2).

3.2.3 Fiber

Fiber content of fermented palm oil fronds decreased by 38.38%. with the best treatment applied to pre-treated samples and fermentation using *A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme (T₂M₂). *A. niger* is among cellulolytic fungi capable of producing cellulase, which can hydrolyze cellulose into simpler compounds (Semaun *et al.*, 2016). In addition to the influence of the added microbes in the fermentation process, pretreatment (desizing, steaming, and eco-enzyme) also affected the decrease in crude fiber content. This was because steaming could alter the arrangement of chemical compounds in palm oil fronds, causing changes in the chemical composition of palm oil fronds (Kurniawan *et al.*, 2016). Steam pretreatment was carried out to improve the efficiency of eco-enzyme by providing easier access to cellulose and hemicellulose substrates (Yuansah *et al.*, 2019). The use of eco-enzyme before fermentation was carried out to degrade lignin component contained in palm oil fronds. After degrading lignin component, there was easy access to cellulose and hemicellulose components in lignocellulose substrate. This led to the secretion of enzyme by microbes to hydrolyze cellulose and hemicellulose components as a carbon source for microbial growth (Yuansah, 2019). The complex lignin bond caused cellulose and hemicellulose to be released, which played significant role as energy sources for livestock.

3.2.4 Fat

Based on Table 3, there was a significant increase in fat content. Duncan's test showed that fermentation had a significant effect ($p < 0.05$) on fat content after fermentation. Although all treatments met the feed standards, T₁M₁ and T₂M₁ exceeded the maximum feed standard of 7% for fat content. Excessive fat content can disrupt normal rumen fermentation, leading to decreased fiber digestion and nutrient absorption, alongside metabolic disorders (Ndaru *et al.*, 2021).

3.2.5 NFE

Table 3 shows that fermentation has a significant effect on NFE content of palm oil fronds. The highest NFE content was obtained in T₂M₂, involving fermentation with *A. niger* + *L. acidophilus* on a previously pretreated substrate. As NFE was calculated by difference ($100\% - [\text{moisture} + \text{ash} + \text{crude fiber} + \text{crude protein} + \text{crude fat}]$, on a dry-matter basis), the observed increase in NFE should be interpreted with caution, as it reflects not only a potential increase in soluble carbohydrate content but also the concurrent reduction in ash and fiber content observed in the same treatment. Nevertheless, since NFE nominally represents the

soluble carbohydrate fraction comprising monosaccharides, disaccharides, and readily soluble polysaccharides with high digestibility (Aling *et al.*, 2020). the reduction in structural components such as fiber, resulting from lignocellulose degradation during pretreatment and fermentation, is consistent with a genuine relative increase in the more digestible carbohydrate fraction.

Table 4. Changes in Fiber Fractions of Palm Oil Fronds Post Solid State Fermentation

Sample	Lignin	Celulose	Hemicelulose	NDF	ADF
T ₀ M ₀	17.47±0.18 ^c	34.47 ± 0.25 ^e	20.22± 0.51 ^d	64.95± 0.11 ^f	46.84 ± 0.09 ^e
T ₁ M ₁	14.83 ± 0.14 ^d	31.27 ± 0.36 ^d	16.45 ± 0.41 ^b	62.56 ± 0.38 ^e	46.11 ± 0.24 ^e
T ₁ M ₂	13.49 ± 0.41 ^c	29.44 ± 0.65 ^c	18.10 ± 0.42 ^c	61.03 ± 0.39 ^d	42.93 ± 0.74 ^c
T ₁ M ₃	12.94 ± 0.39 ^c	29.85 ± 1.67 ^c	19.52 ± 1.74 ^c	62.32 ± 0.81 ^e	43.13 ± 0.96 ^c
T ₂ M ₁	6.27 ± 0.87 ^a	17.46 ± 0.16 ^a	15.06 ± 0.29 ^b	41.47 ± 0.16 ^a	26.40 ± 0.23 ^a
T ₂ M ₂	7.04 ± 0.18 ^a	22.64 ± 0.65 ^b	11.58 ± 0.82 ^a	44.27 ± 0.77 ^b	32.69 ± 0.11 ^b
T ₂ M ₃	7.65 ± 0.14 ^b	32.52 ± 0.32 ^d	11.73 ± 0.51 ^a	58.58 ± 0.69 ^c	44.35 ± 0.36 ^d

Note: Data represent mean ± standard deviation with p<0.05 (n=3). Superscripts a-f show significant differences in lignin content among steaming time and eco-enzyme concentrations according to Duncan's Multiple Range Test (DMRT). T₀: Untreated palm oil fronds; T₁M₁: Untreated palm oil fronds + Fermentation *B. subtilis* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₁M₂: Untreated palm oil fronds + Fermentation *A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₁M₃: Untreated palm oil fronds + Fermentation *S. cerevisiae* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₁: Best of Stage 1 + Fermentation *B. subtilis* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₂: Best of Stage 1 + Fermentation *A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme; T₂M₃: Best of Stage 1 + Fermentation *S. cerevisiae* + *L. acidophilus* + 5% urea + 50% eco-enzyme; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber. Best of Stage 1: Steaming for 30 minutes + 100% eco-enzyme.

As shown in Table 4, the lowest fiber fraction content was obtained from the fermentation of *B. subtilis* + *L. acidophilus* on the pre-treated substrate. In this treatment, lignin content, cellulose, hemicellulose, NDF, and ADF decreased by 64.10%, 49.34%, 25.51%, 36.15%, and 42.44%, respectively. The significant decrease was attributed to the pretreatment process and the variation of microorganisms.

3.2.7 Lignin, Cellulose, Hemicellulose

Pretreatment conducted prior to fermentation successfully degraded lignin, the most recalcitrant component of lignocellulosic biomass; however, the resulting lignin content had not yet reached the target maximum threshold of 7% required for ruminant feed. Therefore, fermentation was subsequently applied to the substrate with the best pretreatment outcome (30 minutes steaming + 100% eco-enzyme) to further reduce lignin content and enhance the accessibility of cellulose and hemicellulose. In this study, the lowest lignin content was obtained from the combined pretreatment and fermentation treatment using *B. subtilis* + *L. acidophilus* (T₂M₁). As lignin, cellulose, and hemicellulose contents were only analyzed on pretreated substrates prior to fermentation, the further reduction observed in T₂M₁ reflects the cumulative effect of both pretreatment and fermentation, rather than the contribution of fermentation alone. Nonetheless, the fermentation process is likely to have contributed to further lignin degradation, as *B. subtilis* is known to produce laccase, an enzyme capable of depolymerizing lignin (Shin *et al.*, 2019). Laccase is essential for breaking down the complex structure of lignin into simpler compounds that can

be further degraded by another enzyme. *B. subtilis* also produces peroxidase that has the potential to oxidize lignin-related compounds (Min *et al.*, 2015). This correlated with the study isolating lignolytic bacteria from palm oil fronds, where one of the 19 potential isolates for lignin degradation was *B. subtilis* strain BRM043928 with very good (+++) lignolytic activity (Rupaedah *et al.*, 2019). However, *S. cerevisiae* does not have enzyme level to degrade lignin (Shin *et al.*, 2019). *A. niger* is known to produce various lignin-degrading enzyme, including laccase, lignin peroxidase, and manganese peroxidase. This enzyme is included in lignin degradation, a complex process that breaks down lignin structure into simpler compounds (Afanasevna *et al.*, 2022). The decrease in fiber fractions such as lignin, cellulose, and hemicellulose is also due to the increasingly acidic substrate environment during fermentation. This acidic condition is caused by the presence of lactic acid, a major product of *L. acidophilus*, that can degrade fiber fractions, including lignin.

3.2.8 NDF and ADF

ADF is plant cell wall that is insoluble in acid detergent, while NDF is the part of the cell wall insoluble in neutral detergent. Since the components of ADF are lignin and cellulose, and the components of NDF are lignin, hemicellulose, silica, and cellulose, both ADF and NDF are closely related to feed digestibility (Carrillo-Díaz *et al.*, 2022; Diatta *et al.*, 2021). Low content these components could increase digestibility (Sandi *et al.*, 2020). In this study, *B. subtilis* + *L. acidophilus* showed the lowest content with cellulose, hemicellulose, and lignin. That high NDF and ADF content could decrease in vitro digestibility in palm oil fronds (Rahayu *et al.*, 2015).

3.3 Amino Acids and Fatty Acids in Palm Oil Fronds

In feed, amino acids have potential to enhance meat quality. Based on Table 5, amino acid content in T₂M₁ and T₂M₂ increased after pretreatment and fermentation. This content was obtained from the conversion of amino acid in the dry substrate and water-soluble compounds produced during fermentation. The conversion of soluble amino acid content was carried out by converting the water content in each treatment. This study found that amino acid played an important role in growth and improving meat quality, including lysine, glutamic, aspartic, valine, isoleucine, leucine, and threonine. Moreover, the best amino acid was obtained in the fermentation treatment with *A. niger* + *L. acidophilus* (T₂M₂), with an increase in lysine content of 468.03%, glutamic acid 458.14%, aspartic acid 435.74%, valine 457%, isoleucine 463.29%, leucine 449.32%, and threonine 432.25%. Based on the analysis, the best nutritional content was obtained in the fermentation treatment with *A. niger* + *L. acidophilus* on a previously pretreated substrate with protein content of 16%.

Table 5. Amino acid profile of palm oil fronds

Types of Amino Acid	Parameter (mg/kg)	T ₀	T ₂ M ₁	T ₂ M ₂
Essential amino acid	L- isoleucine	425.65	1244.69	2394.19
	L-leucine	811.89	2366.85	4455.46
	L-lysine	475.38	1505.50	2700.33
	L-valine	596.35	1761.04	3325.22
	L-phenylalanine	476.76	1648.90	2821.73
	L-threonine	651.98	1817.53	3465.08
	L-alanine	872.52	2625.72	4839.50

Types of Amino Acid	Parameter (mg/kg)	T ₀	T ₂ M ₁	T ₂ M ₂
Non-essential amino acid	L-arginine	1506.45	3829.78	8694.79
	L-aspartic acid	1589.31	4588.18	8513.62
	Glycine	1250.05	3679.24	6795.17
	L-glutamic acid	1537.92	4557.17	8583.89
	L-proline	672.42	2002.78	3696.71
	L-serine	825.5	2347.59	4385.50

Note: T₀: Control (untreated palm oil fronds); T₂M₁: Optimized Stage 1 followed by fermentation with *B. subtilis* and *L. acidophilus*, supplemented with 5% urea and 50% eco-enzyme; T₂M₂: Optimized Stage 1 followed by fermentation with *A. niger* and *L. acidophilus*, supplemented with 5% urea and 50% eco-enzyme.

Essential amino acids are among the important nutrients in livestock that support growth rate and performance in beef cattle, as well as optimal milk production. In this study, essential amino acid content increased following fermentation, with the highest values across all essential amino acids observed in T₂M₂. Among these, leucine showed the highest concentration (4455.46 mg/kg), followed by threonine (3465.08 mg/kg) and valine (3325.22 mg/kg). Lysine content also increased substantially, reaching 2700.33 mg/kg in T₂M₂ compared to 475.38 mg/kg in the untreated control (T₀). Amino acid also plays a crucial role in improving meat palatability and flavor (Besung *et al.*, 2019). Specifically, lysine affects growth performance, carcass composition, and meat quality, which influences meat production with characteristics of pH and color (Belloir *et al.*, 2019).

The increase in amino acid concentrations observed in this study is attributed to microbial protein synthesis rather than the release of amino acids already present in the palm fronds, given the inherently low native protein content of this substrate. The addition of 5% urea served as a supplementary nitrogen source, which was hydrolyzed into ammonia during fermentation and subsequently utilized by the microorganisms for protein and amino acid synthesis, thereby enhancing the overall amino acid profile of the fermented product. These BCAAs stimulate the expression and activity of stearoyl-CoA desaturase (SCD) included in the synthesis of monounsaturated fatty acid (MUFA) in skeletal muscle, which increases IMF accumulation and causes marbling (Xu *et al.*, 2022). This finding is particularly relevant to the objective of this study, which aims to develop a feed ingredient capable of influencing marbling composition in beef cattle through enhanced BCAA availability.

Supplementation of valine and isoleucine has been shown to increase subjective muscle marbling scores according to NPPC guidelines (1994), suggesting positive contribution to marbling process (Ma *et al.*, 2020). Methionine and arginine also play an important role in fatty acid synthesis and IMF deposition (Lee *et al.*, 2019). Arginine is an essential stimulator of growth hormone release, increasing muscle mass and weight gain in livestock. It serves as a basic amino acid that stimulates proline, ornithine, glutamine, and creatine (Lisnahan *et al.*, 2021). Glutamic, aspartic, and alanine have been found to contribute to meat flavor and tenderness (Hanagasaki & Asato, 2018).

Table 6. Fatty acid profile of palm oil fronds

Types of Fatty Acid	Parameter	T ₀	T ₂ M ₁	T ₂ M ₂
Essential fatty acid	Linoleic acid	0.173	0.454	0.329
	C 18:2 W6 (Linoleic acid / W6)	0.173	0.454	0.329
	C 18:2 W6C (C-Linoleic acid)	0.173	0.454	0.329
	Omega-6 fatty acids	0.173	0.454	0.329
Non-essential fatty acid	Oleic acid	0.3208	0.870	0.609
	C 18:1 W9C (C-Oleic acid)	0.3208	0.870	0.609
	C 24:1 W9 (Nervonic acid)	0.0744	0.183	0.141
	C 16:1 (Palmitoleic acid)	0.0178	0.048	0.034
	Polyunsaturated fat	0.173	0.454	0.329
	C 18:0 (stearic acids)	0.0816	0.219	0.155
	C 17:0 (Heptadecanoic acid)	0.0113	0.028	0.021
	C 16:0 (Palmitic Acid)	0.3696	0.974	0.702
	Unsaturated Fat	0.586	1.593	1.113
	Omega-9 fatty acids	0.3208	0.870	0.609
	C 15:0 (Pentadecanoic acid)	0.0146	0.039	0.028
	C 14:0 (Myristic acid)	0.0173	0.047	0.033
	C 12:0 (Lauric acid)	0.0069	0.017	0.013
	Monounsaturated fat	0.413	1.139	0.785
	C 10:0 (Capric acid)	0.1028	0.269	0.195
	Saturated fat	0.604	1.594	1.147

Note : T₀: Untreated palm oil fronds; T₂M₁: Steaming for 30 minutes + 100% Eco-enzyme + Fermentation with *B. subtilis* + *L. acidophilus* + 5% urea + 50% Eco-enzyme; T₂M₂: Best from Stage 1 + Fermentation with *A. niger* + *L. acidophilus* + 5% urea + 50% Eco-enzyme.

Based on Table 6, fatty acid content increased after fermentation. This is due to the conversion of amino acid in the dry substrate and water-soluble fatty acid produced during fermentation by changing water content in each treatment. The best fatty acid content was obtained in fermentation with *B. subtilis* + *L. acidophilus* on a previously pretreated substrate. This study found fatty acid that could contribute to meat quality and caused a significant increase compared to non-fermented palm oil fronds. In line with the results, oleic acid, unsaturated fat, and monounsaturated fat increased by 171.19%, 173.24%, and 175.78%, respectively. Meanwhile, linoleic, omega-6 fatty acid, and polyunsaturated fatty acid (PUFA) were found to increase by 162.42%. In Table 3, the fat content in T₂M₂ reached 10.57%, exceeding the maximum feed standard threshold of 7% (SNI), making it highly plausible that the elevated fat content was partly converted into fatty acids via lipase enzyme activity during fermentation. While this indicates an enrichment of the fatty acid profile, it should be noted that excessively high dietary fat content, particularly PUFA, may adversely affect fiber digestibility in the rumen by coating fiber particles, inhibiting cellulolytic bacterial attachment, and disrupting normal ruminal biohydrogenation. Therefore, although T₂M₂ exhibited the most favorable increase in fatty acid content among the tested treatments, its practical application as a feed ingredient would require careful determination of an appropriate inclusion level within a total mixed

ration, rather than use as a sole feed source, to avoid potential adverse effects on rumen fiber digestion.

Table 6 shows that the highest MUFA content reached 1.139% (T2M1), stearic acid reached 0.219%, omega-6 fatty acid reached 0.454%, and omega-9 fatty acid peaked at 0.870%, all observed in T2M1. Additionally, the essential fatty acid detected in fermented palm oil fronds was linoleic acid. Feed rich in linoleic acid could improve carcass characteristics and fatty acid profiles in meat (Nascimento *et al.*, 2021). Fatty acid content in feed can enhance meat quality by increasing muscle mass and reducing fat deposition (Zeng *et al.*, 2023). MUFA acid in meat have been shown to influence palatability (Smith, 2016), with a higher oleic content contributing to meat tenderness. Furthermore, a higher content of PUFA in IMF is positively correlated with marbling in beef cattle (Nguyen *et al.*, 2021). The fatty acid composition can affect flavor and juiciness, which are important characteristics of meat quality (Ladeira *et al.*, 2018). MUFA, particularly oleic acid (18:1), is the dominant fatty acid in beef and contributes significantly to marbling formation and increases the melting point of fat (Smith, 2016). Supplementation with rumen-undegradable MUFA can increase marbling in early-weaned beef bulls (Mangrum *et al.*, 2016). Moreover, saturated fatty acid such as palmitic and stearic contribute significantly to fatty acid composition of beef and fat (Smith, 2016). Stearic acid has a detrimental effect on the melting point of adipose tissue lipids, where higher content causes fat to become harder (Ladeira *et al.*, 2018). Excessive saturated fat content can cause a firmer texture, while higher unsaturated content produces more tender and juicy meat.

Omega-9 fatty acid can be synthesized by the body, with higher content influencing marbling in meat. This acid forms marbling by increasing IMF accumulation, which improves tenderness and flavor (Ladeira *et al.*, 2018). In comparison, Omega-6 fatty acid, including linoleic acid, cannot be synthesized by the body and must be obtained through feed. This fatty acid is included in various metabolic processes, such as the regulation of fat synthesis (Nguyen *et al.*, 2021).

3.4 Antioxidant Levels in Fermented Palm Oil Fronds

Antioxidant in feed play a role in neutralizing free radicals and repairing blood cell structures in livestock. This increase in leukocytes has a positive effect on improving the immune system of ruminants (Fajriyani *et al.*, 2017). In this study, antioxidant activity content in fermented palm oil fronds was measured using the DPPH method, and the results were shown in Table 7.

Table 7 IC₅₀ Values of Fermented Palm Oil Fronds

Sample	LogIC ₅₀	HillSlope	IC ₅₀	Classification
T ₀ M ₀	0	0	0	Very weak
T ₁ M ₁	2.359±0.120	0.3679	228.5	Weak
T ₁ M ₂	2.529±0.055	0.1443	338.1	Weak
T ₁ M ₃	1.984±0.157	0.4619	96.38	Strong
T ₂ M ₁	2.725±0.041	0.1081	531.4	Weak
T ₂ M ₂	2.117±0.082	0.2192	131.0	Moderate
T ₂ M ₃	2.886±0.038	0.09987	769.3	Weak

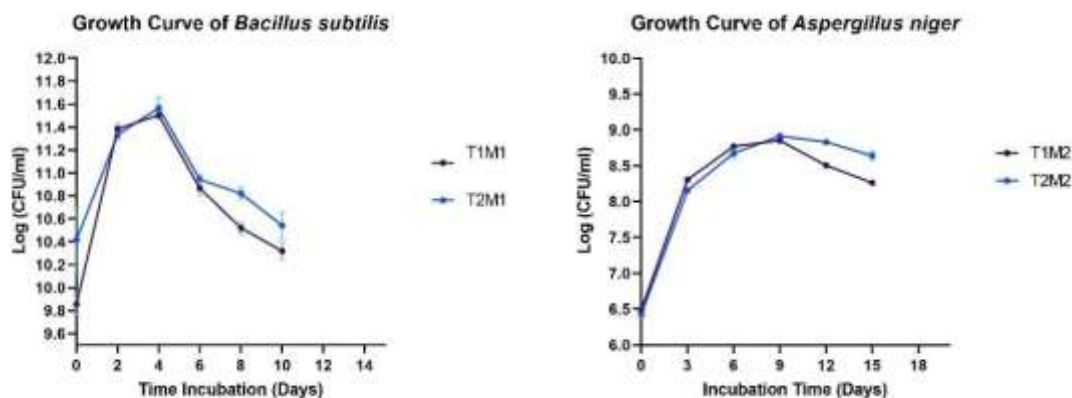
Notes: The values were analyzed using GraphPad Prism 9.0.0 software. The experimental treatments are defined as follows: T₁M₁: palm oil fronds (POF) without pretreatment + Fermentation with *B. subtilis* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₁M₂: POF without pretreatment +

Fermentation with *A. niger* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₁M₃: POF without pretreatment + Fermentation with *S. cerevisiae* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₁: Substrate from Optimized Stage 1 Pretreatment + Fermentation with *B. subtilis* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₂: Substrate from Optimized Stage 1 Pretreatment + Fermentation with *A. niger* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₃: Substrate from Optimized Stage 1 Pretreatment + Fermentation with *S. cerevisiae* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme.

As shown in Table 7, the optimal antioxidant activity was recorded in T₁M₃, achieving a value of 96.38, which was classified as strong. This treatment specifically included fermentation using the *Saccharomyces cerevisiae* + *Lactobacillus acidophilus* consortium on a non-pretreated substrate. The high antioxidant level observed was attributed to the microbial growth kinetics, specifically *S. cerevisiae* colony that was still in stationary phase on Day 15. During the stationary phase, yeast cells experienced significant metabolic changes, enhancing resistance to oxidative stress. This mechanism includes the induction of antioxidant enzyme and the transcription of genes related to stress tolerance (Kocaefe-Özşen *et al.*, 2022). Consequently, the microbial adaptation leads to an observable increase in the substrate's antioxidant activity. *S. cerevisiae* significantly impacted the enhancement of glucan content and antioxidant activity of tempeh, with a mixed inoculum of 1.5% *R. oligosporus* and 1.5% *S. cerevisiae* yielding the highest values (Rizal *et al.*, 2022).

3.5 Microbial Growth Curve Analysis

Microbial growth curve analysis is essential for determining the cellular growth rate and evaluating the influence of fermentation substrate (Sakthiselvan *et al.*, 2019). The growth curve serves as a primary parameter in mathematical models used to describe microbial proliferation over time under a defined set of environmental conditions (Longhi *et al.*, 2017). The duration required for bacteria cell to divide into two is termed generation time (Azhari *et al.*, 2021). Generation time varies significantly across organisms and represents a crucial factor in the organism's life cycle, history, and evolution (Gibson *et al.*, 2018). In this study, growth curve were obtained using the TPC method, performed every 48 hours for 10 days for bacteria, while fungi and yeast required 72 hours for 15 days. Microbial growth generally follows four distinct phases, namely the lag, the exponential or log (rapid), the stationary (static), and the death or decline (Bertranda, 2019). The complete growth curve is shown in Figure 2.



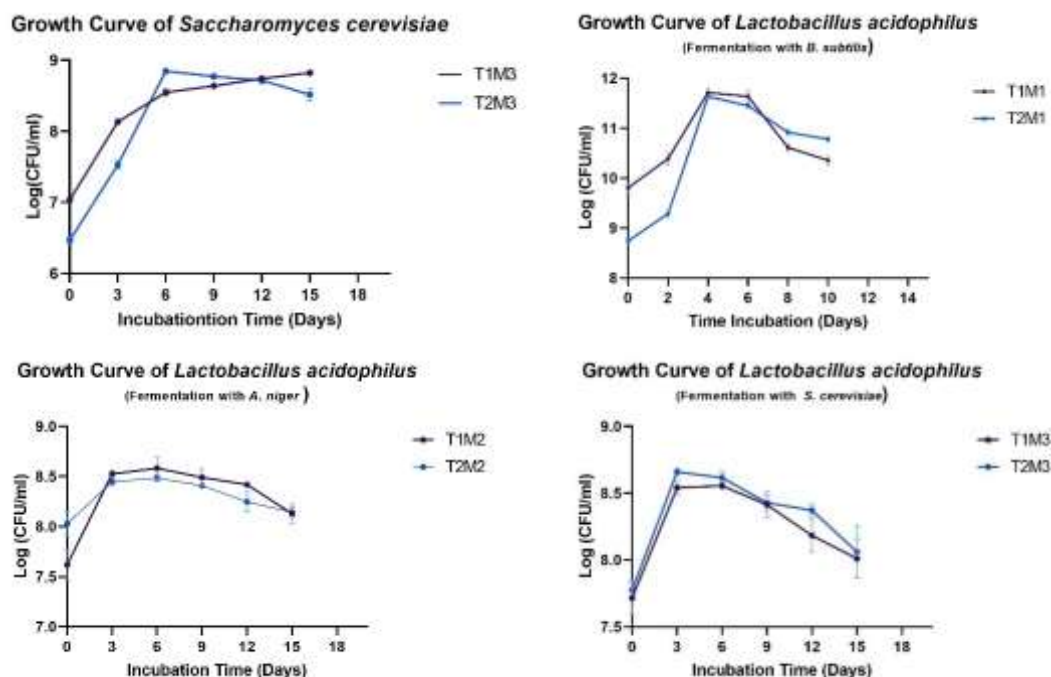


Figure 2. Microbial Growth Curves during Fermentation

Notes: T₁M₁: Palm Oil Fronds (POF) without pretreatment + Fermentation with *B. subtilis* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₁M₂: POF without pretreatment + Fermentation with *A. niger* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₁M₃: POF without pretreatment + Fermentation with *S. cerevisiae* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₁: Optimized Stage 1 Substrate + Fermentation with *B. subtilis* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₂: Optimized Stage 1 Substrate + Fermentation with *A. niger* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme. T₂M₃: Optimized Stage 1 Substrate + Fermentation with *S. cerevisiae* + *L. acidophilus* + 5% Urea + 50% Eco-enzyme.

The growth curve showed that the lag phase for *B. subtilis*, *A. niger*, *S. cerevisiae*, and *L. acidophilus* occurred primarily on Day 0. *B. subtilis* entered the exponential phase on Day 2, followed by the stationary from Day 2 to 4, and death from Day 4 to 10. The growth of *L. acidophilus* was similar to co-culture partner, *B. subtilis*. *A. niger* showed an exponential phase on Day 3. The stationary phase spanned from Day 6 to 9 for the treatment, extending to Day 12. Meanwhile, the death phase was generally observed after Day 12. *S. cerevisiae* reached the exponential phase on Day 3, and the stationary phase occurred from Day 6 to Day 12, extending to Day 15. The death phase for the treatment commenced between Days 12 and 15.

The results showed that microbial colonies exhibited higher proliferation in the pretreated palm oil fronds substrate compared to the non-pretreated substrate. This enhanced microbial growth is likely attributable to multiple factors resulting from the pretreatment process, rather than a single causal factor. Pretreatment was shown to increase reducing sugar content, providing a readily available energy source for microbial growth, while also facilitating the degradation of lignin and thereby increasing the accessibility of cellulose and hemicellulose to microbial enzymes. As changes in pH, moisture, and nitrogen availability following pretreatment were not statistically correlated with microbial colony counts in this study, the relative contribution of each factor to the observed increase in microbial proliferation cannot be conclusively determined and warrants further investigation.

4. Conclusion

In conclusion, this study showed that a combination of pretreatment and fermentation processes on palm oil fronds could significantly improve the nutritional quality of animal feed. The integration of steaming, eco-enzyme addition, and fermentation using various microorganisms and urea produced feed with higher protein, amino acid, and fatty acid content, as well as lower lignin content. The optimal combination was achieved with pretreatment (30-minute steaming + 100% eco-enzyme) and fermentation (*A. niger* + *L. acidophilus* + 5% urea + 50% eco-enzyme), which approximately met the SNI proximate standard for animal feed and showed an improved amino acid profile. Meanwhile, the combination of pretreatment (30-minute steaming + 100% eco-enzyme) and fermentation (*B. subtilis* + *L. acidophilus* + 5% urea + 50% eco-enzyme) showed the lowest fiber fraction content, particularly lignin, along with a higher fatty acid profile. The experimental treatments enhanced the chemical profile and in vitro nutritional quality of the feed, indicating strong potential as a high-quality feed source for livestock. Nonetheless, as this study was limited to laboratory-based assays, future in vivo trials are required to validate its efficacy in terms of actual feed intake, growth rate, and feed conversion ratio in livestock.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this study.

ETHICS STATEMENT

The authors have nothing to report.

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