Role of Vermicomposting Microorganisms in the Conversion of Biomass Ash to Bio-Based Fertilizers

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Abstract: A high pH, low solubility of bound plant nutrients, and negative impacts on microbial communities are common drawbacks of biomass ash (BA) vermicomposting. In this study, nutrient-rich BA mixed with cow manure was tested at three different application rates to obtain final nitrogen (N), phosphorus (P), and potassium (K) contents of 3.5%, 7.0%, and 10.0% for bio-based fertilizers via vermicomposting. The results showed that all BA blends made with cow manure increased fermentation temperatures and allowed successful worm activity during the subsequent vermicomposting phase. The order of indicator enzyme activities in all vermicomposting samples was urease (220 µg NH₄ g⁻¹ h⁻¹) > β-glucosidase (95 µg PNP g⁻¹ h⁻¹) > alkaline phosphatase (91 µg PNP g⁻¹ h⁻¹) > arylsulfatase (83 µg PNP g⁻¹ h⁻¹) > acid phosphatase (60 µg PNP g⁻¹ h⁻¹).

As an indicator of nutrient bioavailability, high correlations were observed between enzyme activities and microbial diversity in vermicompost samples. Determination coefficients (R²) obtained from multiple linear regressions between enzyme activities and bacterial population for T₀, T₁, T₂, and T₃ were determined as 0.90, 0.65, 0.73, and 0.90, respectively. According to a novel metagenome-based approach proposed within the scope of the present study, the stimulatory effects of Flavobacteriales, Burkholderiales, Saccharimonadales, and Pseudomonadales on enzyme activities for the nutrient solubility were found to be significant and positive. The findings of this study demonstrated that worm composting could be a sustainable bio-based technology for the production of slow-release fertilizer from nutrient-rich waste material.

Keywords: biomass ash recycling; vermicomposting; nutrient availability; microbial community; enzyme activity

1. Introduction

The recovery of bio-based plant nutrients from a variety of waste resources to replace synthetic fertilizers is considered a key strategy for more sustainable agriculture [1] and waste management practices [2]. In terms of a circular bioeconomy and sustainable environment, composting is considered to be a unique and cost-effective process that provides high-value-added and useful products from various organic materials [3,4]. In this context, vermicomposting is recognized as an environment-friendly and sustainable biowaste treatment technology since it allows the recovery of nutrients from biowaste [5], the proliferation of beneficial microorganisms [6], and the recovery of plant growth-stimulating substances [7]. However, depending on the feedstock, the nutrient content is generally low, so nutrient enrichment is required to increase end-user acceptance [5,8]. In this regard, biomass power plant ash has the potential to enrich plant nutrients in vermicompost, allowing it to be used as a bio-based fertilizer [9]. On the other hand, the availability
of nutrients in biomass ash to plants is limited as they are typically bound within the crystalline. Therefore, the further processing of BA should be explored in order to improve the nutrient efficiency and enrichment potential of worm compost by BA nutrients.

With the increase in the global demand for renewable biomass for energy production, biomass ash (BA) derived from power plants has been increasing for years. From the perspective of agricultural use, BA contains many plant nutrients such as potassium (K), magnesium (Mg), and phosphorus (P), which are important nutrients for plant growth [10]. Although the limited use of bio-ash in agriculture is attributed to its high pH and heavy metals, which are thought to affect plant growth, returning it to the field as a fertilizer for crop production is one of the most sustainable ways to minimize nutrient depletion in agro-ecosystems [11,12]. Biomass ash, which consists of crop residues, agricultural wastes, and poultry litter, contains significant amounts of fertilizer nutrients, especially phosphorus. In recent years, research on P recovery from waste streams has been triggered by an increased awareness of P scarcity and the need to close the P cycle. However, during the incineration process, 80% of P is converted to the less plant-available apatite form [13].

There are several chemical, physical, and biological ways to solubilize and recover plant nutrients from BA [14]. However, no approach or procedure can effectively recover plant nutrients since there are so many variations in BA materials [15]. In light of this problem, one goal of the present study is to assess the established biological waste treatment methods in order to increase the plant availability of nutrients from BA and release them back into the environment as bio-based fertilizer. The consecutive pre-fermentation and vermicomposting of BA with biowaste could be used as a feasible method to solubilize and recover recalcitrant nutrients from BA [16]. The use of inorganic amendments in the vermicomposting process has become significant regarding the enhancement of composting processes [17] and the nutritional values of the final product [8]. On the other hand, pH is reported to be a key factor in the availability of nutrients in incineration ash [15]. In addition, BA composts are characterized by an alkaline pH, which can have adverse effects on the microbial community and their decomposition activities [18]. To create vermicompost of the highest nutrient content, it is essential to understand the changes in the microbial community, their enzymatic activities, and the nutrient availabilities during the vermicomposting process [12].

Vermicomposting is a worm-mediated biodegradation process using live epigeic worms such as *Eisenia fetida*. Worms play an important role in the process of nutrient dissolution and bioremediation by enhancing the population of beneficial bacteria [19]. The digestive system of worms contains many beneficial microorganisms, nitrogen-fixing bacteria, and enzymes [7]. Although microorganisms are actually responsible for the biochemical degradation of organic matter, worms decompose and condition the substrate, increasing the surface area for microorganism activity [20]. Worms mineralize organic matter through intestinal transit, digest it in the foregut and midgut, and then excrete it through the hindgut, interacting directly with microorganisms [6]. A diversified bacterial community in the gut accelerates the breakdown and mineralization of organic matter and even the accumulation of P and K [21]. Microorganisms involved in the vermicomposting process degrade complex organic compounds (e.g., lignin, cellulose, hemicellulose) into simpler forms and produce a variety of extracellular hydrolytic enzymes such as cellulase, protease, urease, phosphatase, lipase, and β-glucosidase [22], which are responsible for the transformation of nutrients. Therefore, the present study also aimed to clarify the bacterial diversity and enzyme activities responsible for BA nutrients in combination with cow dung.

Several studies have investigated the effectiveness of the aforementioned treatments in improving the leaching and bioavailability of biomass ash nutrients for use as a bio-based nutrient source and their effects on plant growth [10,23]. However, few studies have addressed the specific effects of BA on vermicompost microbial communities and their enzymatic activities related to nutrient availability. Therefore, in order to address the gaps in the literature, a novel metagenome-based approach was used in the present study to
understand the functional bacterial groups and enzymatic function potential of microbial populations in BA-enriched vermicompost samples. Considering the multidimensional effect of earthworms on organic matter, the vermicomposting process was proposed as an effective process by which to improve the bioavailability of BA nutrients (Appendix A). In order to achieve this goal, the following specific objectives were selected for the current investigation: (1) to determine the effects of vermicomposting on the solubility and bioavailability of major plant nutrients; (2) to investigate the effect of BA on bacterial composition and abundance; and (3) to investigate the effect of BA on the bacterial enzyme activities responsible for nutrient dissolution.

2. Materials and Methods

2.1. Vermicomposting Process

In this study, vermicompost samples were prepared by a local vermicomposting company (Anatolian worm compost producers cooperative) using dairy cattle manure and BA from the 15 MW-biomass power plant located in Sakarya province, Turkey. A premixed combination of sawdust, forestry residues, hazelnut shells (20%), and poultry litter (80%) were used as biomass feedstock materials in the plant, which generates approximately 20,000 kg of ash daily. A dewatered form of cattle manure, consisting of a mixture of feces, urine and bedding straw litter, was obtained from a dairy cattle farm in Sakarya, Turkey. A maximum of 7.0% was determined as the limit value for the sum of N, P, and K in the vermicompost of the Turkish compost regulation [24]. Considering this limit value, the amounts of BA were calculated to obtain the final NPK contents of 3.5% (T$_1$), 7.0% (T$_2$) and 10.0% (T$_3$). It is noted that T$_0$ without BA was also included in the study for comparison. The calculated amount of BA was uniformly mixed into the raw cattle manure, and then the mixtures were initially pre-composted for 21 days to remove toxic substances and potential pH shock that could harm the worms [25]. The mixtures were then poured into rectangular open plastic container boxes (length $\times$ width $\times$ depth = 50 $\times$ 34 $\times$ 30 cm) and epigeic (surface-dwelling) worms were added to each box (10 worms per kg of material) to allow for vermicompost production. The boxes were started with 3 kg of feed (on dry weight basis) from each application, and the same amount of material was added to the surface at weekly intervals. For each treatment, vermicompost-forming materials were kept at a 60 $\pm$ 2% moisture level by spraying surface water in a dark place at room temperature (25 $\pm$ 2 $^\circ$C). Composting was monitored using daily temperature and humidity measurements for 60 days until a mature compost was obtained [25].

2.2. Enzyme Activity Analysis

In the present study, enzyme activity analysis was performed on matured 1 g wet vermicompost samples. Fresh samples of $\beta$-glucosidase, acid and alkaline phosphatase, and arylsulfatase were evaluated via incubation with p-nitrophenyl with colorless substrates of each enzyme. The acid phosphatase and alkaline phosphatase activities were analyzed using a colorimetric method and the enzyme activities were expressed as p-nitrophenol g$^{-1}$ vermicompost h$^{-1}$ [26].

$\beta$-glucosidase activity was measured via the quantification of p-nitrophenol obtained after the incubation of samples with the nitrophenyl-$\beta$-D-glucopyranoside substrate. Free p-nitrophenols were measured using a spectrophotometer at 400 nm. The urease activity was analyzed using 1 g of vermicompost with urea solution as a substrate, and the NH$_4$ released was measured using a UV spectrometer at 578 nm and expressed as $\mu$g ammonium g$^{-1}$ vermicompost h$^{-1}$ on a moisture-free basis [8].

2.3. 16S Metagenome Analysis

The vermicomposted samples collected at the end of the vermicomposting experiments from each of the increasing treatments of BA, from T$_0$ (0.0%), T$_1$ (3.5%), T$_2$ (7.0%) to T$_3$ (10.0%), were used for microbial community analysis compared to the pre-composted treatment (K). 16S rRNA gene amplicon analysis was performed using QIIME 2. To create
the library, purification was performed after amplification of the 16S rRNA gene with specific primers [7]. In the index PCR step, illumina binary indexes and adapters were added using the Nextera XT index kit, and then purification was performed. The concentration of the libraries created using real-time PCR (Kyratec, SC-300G, SuperCycler, Caboolture, QLD, Australia) was measured and diluted to 4 nM and normalized. Normalized samples were combined using the pooling method. After the library was prepared, each time a new dNTP (deoxyribose nucleotide triphosphate) was added to the sequencing using the synthesis method, the fluorescence of the added base was optically observed and recorded. The data generated after sequencing were converted to raw sequencing data (FASTA format) for the analysis (Figure 1). It is noted that the FASTA format is a text-based format used to encode nucleotide or amino acid sequences, where nucleotides or amino acids (proteins) are represented by single-letter codes (e.g., A, C, T, G, and N). In summary, raw sequence data are a FASTA or FASTQ text-based data format obtained directly from a sequencing device and used in a next-generation microbiome bioinformatics platform such as QIME.

![Diagram](image-url)

**Figure 1.** 16S rRNA amplicon-based microbiome analysis steps of pre-composted (K) and vermicompost (T₀, T₁, T₂, and T₃). Raw sequencing data were processed using Quantitative Insights into Microbial Ecology (QIIME 2).
2.4. Analytical Processing of Biomass Ash and Vermicompost Samples

The complementary X-ray diffraction (XRD) method was employed to identify the crystalline structure of the ash powder samples. A current of 40 mA was used to perform the XRD measurements on powder samples. The analysis was performed by using the Rigaku diffractometer (Rigaku, Tokyo, Japan) and using Cu K radiation at 40 kV and 130 mA in the scanning angle of 5–50° and at a scanning speed of 0.5° min⁻¹ [5]. The Instrument software was used to analyze the obtained diffraction patterns.

Vermicompost samples were oven-dried at 78 °C until a stable weight and then sieved using 2 mm mesh pieces before being subjected to further chemical analyses. The pH and electrical conductivity (EC) of both the experimental soil and vermicompost samples were measured based on a 1:5 (w/v) soil-to-water suspension using a pH meter (CG 840, Schott Geräte GmbH, Hofheim, Germany) and EC electrode (HQ14D, HACH, Loveland, CO, USA). In addition, organic matter (OM) was determined using an ignition loss technique with an ashing temperature of 550 °C for 4 h. The N in samples were determined according to the Kjeldahl procedure [3]. A colorimetric determination procedure was used to measure the total content and plant available fraction of phosphorus after the extraction of 0.5 M of NaHCO³.

For total element analysis, vermicompost samples (250 mg) were digested in a microwave digester (ETHOS™, Milestone Srl., Sorisole (BG), Italy) by adding an acid mixture of 6 mL of HNO³ (65%) and 1 mL of H₂O₂ (30%). According to the ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA) method, the available nutrients of the plant were determined after the extraction using 0.005 M DTPA. In the next step of this process, the digested samples were cooled and filtered through Whatman filter paper (0.45 µm) and diluted to 50 mL using ultrapure water (Merck Millipore, Molsheim, France). The total and plant available nutrients were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Spectro Arcos, Kleve, Germany) [5].

2.5. Statistical Analysis

Experimental data were subjected to analysis of variance (full block design randomization) using Statgraphics Centurion version XVI (Statpoint Technologies Inc., Warrenton, VA, USA). Means that differed significantly were separated using Tukey’s Honestly Significant Difference (HSD) test using an alpha (α) level of 0.005.

Alpha and beta diversity analyzes were performed according to the data obtained from the sequence results. Beta diversity shows the variation in the distribution of species between five different groups (K, T₀, T₁, T₂, and T₃), or the similarity or difference of the species content detected in the five different groups. Bray–Curtis difference principal coordinate analysis (pCoA) was used for beta diversity analysis. Alpha diversity is the number of different species detected in each sample. The phylogenetic diversity (PD) metrics were used for alpha diversity analysis [27]. To determine the relationship between the bacterial community by genus, the UPGMA unweighted double-group method with arithmetic mean was constructed from its matrix using a phylogenetic tree and Euclidean metrics.

3. Results and Discussion

3.1. Biomass Ash Properties and Composition

Numerous studies have shown that ash particles have a crystalline, amorphous, opaque, round, and porous structure with nanoscale voids [28]. Among these elements, P is important due to its increasing scarcity [5]. However, most biomass combustion retains the P in the ash, which means that it can potentially be recovered. As seen in Figure 2, the main mineralogical composition of BA is as follows [5]: hydroxyapatite (15.6%), feldspars (15.6%), anhydrite (13.8%), calcite (12.5%), akermanite (9.7%), quartz (9.4%), potassium chloride (9.3%), brown millerite (6.2%), aphthalite (4.8%), hematite (2.1%), and diopsite (1.1%). The dominant peak, hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂), is consistent with its high Ca and P contents. The combustion of biomass containing the crystal forms of Ca and P
at temperatures above 800 °C is defined as the main mineral phase, and the solubility of apatite may vary depending on other contents [29].

![XRD pattern of experimental biomass ash and mineralogical main components](image_url)

Figure 2. XRD pattern of experimental biomass ash and mineralogical main components [5].

The retention of elements in ash during the combustion of biomass fuels is important for the reutilization because major plant nutrients, such as K, P, Ca, Mg, and micro nutrients, such as Fe, Mn, Zn, and Cu, accumulate in ash [30]. The pH is influenced by these alkali metals and earth elements, and most biomass ash exhibits a noticeably high pH, as in this study (pH = 13.04). Similarly, the electrical conductivity values for BA were also recorded in a high range of 7.43 mS cm⁻¹. An excessively high pH and EC induced by BA in compost may be harmful for the microbial activity, since they cause a liming effect and osmotic problems [31]. On the other hand, some authors reported that alkaline additives may passivate heavy metals in amended composts [32,33]. Therefore, if successfully established, BA can enrich both the nutrient content of vermicompost and further reduce environmental harm.

It is obvious from Figure 3 that BA enrichment in the worm compost samples from T₀ to T₃ significantly reduced the bioavailable fraction of nutrients in the final product. This result is in agreement with previous findings that indicate that composting or vermicomposting by alkaline additives can have significant effects on nutrient availability [31]. In addition, liming is a common strategy used to passivate the heavy metals in organic waste compost applications [32]. In this regard, BA also helps to reduce the extractable contents and bioavailability of investigated heavy metals or micro plant nutrients in worm compost samples. The improvement of heavy metal passivation by BA is very clear in this vermicomposting system (Figure 3). However, the bioavailability or immobility of plant nutrients primarily depend on specific elements, complexation between metals and the dose of BA itself [34]. The immobilization effect of BA on some nutrients such as K was weak due to the ongoing microbial degradations or solubilizing effect of their metabolism products [21]. Overall, the addition of BA significantly enhanced plant nutrients, the passivation of heavy metals and the solubilization of major plant nutrients during the vermicomposting process. It is important to note that the total concentration of toxic heavy metals, such as Cd or Pb, was either below the detection limits or far below the national compost standards [24].
Figure 3. DTPA extractable percentages (red) of major plant nutrients and some heavy metals from worm compost samples containing increasing doses of biomass ash from T₀ to T₃ (The green bars show non-extractable fractions).

3.2. Enzyme Activities in Vermicompost

There are a number of criteria and parameters based on different characteristics that can be used to evaluate the nutrient transformations in vermicompost. Enzyme activities, which is one of them, are highly effective in the decomposition stages, impact the quality of the chemical components of the raw materials, and increase the diversity of the microbial communities in the soil where it is applied as a fertilizer [35]. Therefore, the enzyme activities are used as an indicator of organic matter conversion and nutrient transformation in vermicompost. Regarding the effects of BA addition on different hydrolytic enzyme activities, the increase in BA significantly reduced all of the enzyme activities studied compared to the T₀ control. All enzymes in the vermicompost showed higher activity in the T₀ that did not contain BA. The order of mean enzyme activities in all vermicompost samples was as follows: urease > β-glucosidase > alkaline phosphatase > arylsulfatase > acid phosphatase (Table 1).

Table 1. Enzymatic activities in vermicompost treatments with increasing dose of biomass ash (values are represented as mean ± standard deviation in three replicates).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Acid Phosphatase (µg PNP g⁻¹ h⁻¹)</th>
<th>Alkaline Phosphatase (µg PNP g⁻¹ h⁻¹)</th>
<th>Arylsulphatase (µg PNP g⁻¹ h⁻¹)</th>
<th>β-Glucosidase (µg PNP g⁻¹ h⁻¹)</th>
<th>Urease (µg NH₄ g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>58.3 ± 2.3 c</td>
<td>90.6 ± 3.2 c</td>
<td>77.3 ± 3.3 bc</td>
<td>98.7 ± 2.1 c</td>
<td>235.0 ± 6.6 c</td>
</tr>
<tr>
<td>T₀</td>
<td>72.3 ± 2.4 a</td>
<td>101.6 ± 7.6 a</td>
<td>100.6 ± 7.2 a</td>
<td>115.7 ± 3.8 a</td>
<td>292.5 ± 6.2 a</td>
</tr>
<tr>
<td>T₁</td>
<td>65.5 ± 2.6 b</td>
<td>97.4 ± 5.6 b</td>
<td>89.4 ± 9.3 b</td>
<td>103.3 ± 3.4 b</td>
<td>263.0 ± 3.6 b</td>
</tr>
<tr>
<td>T₂</td>
<td>57.8 ± 3.1 c</td>
<td>85.2 ± 4.2 d</td>
<td>78.4 ± 3.1 c</td>
<td>88.3 ± 1.8 d</td>
<td>214.6 ± 3.8 d</td>
</tr>
<tr>
<td>T₃</td>
<td>46.3 ± 3.8 d</td>
<td>79.7 ± 4.6 d</td>
<td>61.6 ± 5.4 d</td>
<td>69.4 ± 2.5 e</td>
<td>101.4 ± 5.2 e</td>
</tr>
<tr>
<td>Significance</td>
<td>0.01 **</td>
<td>0.01 **</td>
<td>0.01 **</td>
<td>0.05 *</td>
<td></td>
</tr>
</tbody>
</table>

Significance: * p < 0.05, ** p < 0.01. Means ± standard deviations for each parameter followed by the same letter are not significantly different according to Tukey’s HSD post hoc test at p ≤ 0.05. PNP: p-nitrophenyl phosphate.

Some bacterial groups (e.g., Proteobacteria and Firmicutes) have been used effectively to dissolve the phosphorus found in apatite form in BA into the form that plants can take. In particular, hydrolytic bacterial enzymes such as phosphatase and arylsulfatase can make the P in apatite form soluble and usable [8]. In the present study, phosphatase activities were used to estimate the P mineralization status in worm compost samples. Phosphatase activities tended to decrease as the BA content increased, most probably because of the
liming effect on the microbial activity. In addition, the reason for the high urease enzyme activity in all samples compared to other enzyme activities was attributed to the rapid proliferation of the microbial population, which is capable of producing the urease enzyme. It has been suggested that the decrease in all enzymatic activities due to the increase in the BA may be caused by the decrease in the available substrate ratio and the liming effect of the BA [12,17].

3.3. Microbial Community Structures

Metagenome analysis gives more comprehensive explanations of the genetic complexity of the microbial communities responsible for metabolic activities in the vermicomposting process [36]. According to the metagenomic results of the vermicompost samples prepared using cattle manure and BA, the alpha diversity analysis performed to determine the species richness in a single sample is shown in Figure 4. The fact that the lines become parallel to the right indicates that the number of reads is sufficient for analysis. In beta diversity analysis, the diversity within the microbial community between the experimental samples was calculated. According to Figure 5, the microbial biomass increases in T₀ and T₁, which had undergone worm digestion compared to K without worms. T₀ is the sample with the highest microbial diversity. Samples T₂ and T₃ also contain a similar diversity of microbial communities. Worms played an important role in the microbial diversity of the unmixed T₀ sample compared to the other samples. The increase in BA content negatively affected the number of microbial communities in samples T₂ and T₃ [37].

![Rarefaction curves](image)

**Figure 4.** Rarefaction curves of the five pooled samples at percent (%) sequence similarity illustrate the species richness of vermicomposting microbiota. X-axis represents sequencing depth/samples. Y-axis shows the measures of species richness, determined as the number of distinct features.

After vermicomposting, the bacterial communities of samples T₀ (0%), T₁ (3.5%), T₂ (7.0%) and T₃ (10.0%) clustered, as shown in Figure 5. The Bacterial communities of samples T₂ and T₃ showed a similar distribution. The results revealed that the BA rates could affect bacterial communities with regard to their dispersal patterns in the vermicompost application.
3.4. Bacterial Communities of Samples

Microorganisms are recognized as the main force of nutrient conversion, which takes place during the vermicomposting process. During the formation of vermicompost, the surface area that microorganisms can affect increases with the activity of worms, and accordingly, it facilitates the production of fertilizers with a high plant nutritional value [6,38]. As a result of the analysis performed to provide information about the diversity and functional potential of vermicompost bacteria, the bacterial community structure at the phylum level is shown in Figure 6. *Proteobacteria* (K-23.77%, T₀-18.44%, T₁-24.34%, T₂-23.5%, T₃-27.4%) *Bacteroides* (K-30.5%, T₀-15.7%, T₁-29%, T₂-11.49%, T₃-13.94%) and *Planctomycetota* (K-9.44%, T₀-15.32%, T₁-12.5%, T₂-20.3%, T₃-19.85%) were identified as the dominant bacterial phyla in all samples. This was in line with previous work on vermicompost applications [7,39].

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**Figure 5.** (a) Phylogenetic tree and (b) bacterial pCoA plots of beta-diversity estimates (Bray–Curtis) for treatment (K, T₀, T₁, T₂, and T₃) groups for 16S metagenomic strategies.
Figure 6. The stacked bar chart shows the taxonomic abundance at the phylum level of vermicompost microbes in the species of the samples. X-axis represents biological replicates of samples for each species, which are K, T0, T1, T2, and T3. Y-axis represents the taxon abundance.

Some studies have suggested that worms may form a microbial bacterial subpopulation that reflects the composition of ingested food [26,40,41]. The dominant bacterial phylum Bacterioids, Proteobacteria and Planctomycetota in samples T0, T1, T2 and T3 were similar in non-worm-digested sample K. A decreasing trend was found in the proportion of Bacterioids when comparing the pre-fermented treatment K and sample T1 with T0, T2 and T3. Most of the Bacterioids commonly seen in animal intestines and skins were represented by cattle manure used as raw material [42]. The fact that the genus Flavomacterium, which is called an opportunistic pathogen in the Bacteriodata family, was prominent in treatment K and in sample T1 among all samples was thought to be a result of the high temperature during composting and the inability to resist the alkaline pH value of BA for the pre-fermented treatment K. It can also be concluded that the added BA ratio for T3 is ideal to ensure P transformation during vermicomposting. It has been reported that Chryseolinea bacteria play significant roles in nitrogen fixation as important remineralizers that convert organic materials into micronutrients due to the relative abundance of Chrysochlamydia among all samples were thought to be a result of the high temperature during composting and the inability to resist the alkaline pH value of BA for the pre-fermented treatment K. It can also be concluded that the added BA ratio for T1 is ideal to ensure P transformation during vermicomposting. It has been reported that Chryseolinea bacteria play significant roles in nitrogen fixation as important remineralizers that convert organic materials into micronutrients due to the relative abundance of Chyphagales, which is determined as the dominant genus belonging to the Bacterioid phylum, with a signifi-
icant positive correlation in T₀, T₁, T₂, and T₃ applications [43]. Gram-negative bacterial communities include most of the phyla, including Proteobacteria. Considering the effects of worms on microorganisms, it has been shown that gram-negative bacteria can survive in the worm gut at higher rates than gram-positive bacteria [39,43].

A hierarchical heat map was created by comparing the relative abundances of the 20 most common phyla from five samples (pre-fermented K, and worm-composted T₀, T₁, T₂, and T₃) (Table 2). At the phylum level, 47 bacterial phyla were estimated among the samples, and most of the samples shared similar phyla.

**Table 2.** Heatmap of the top 20 classified bacteria genera from samples (K, T₀, T₁, T₂, and T₃). The color gradient from blue to red indicates increasing species abundance. Numbers within the figure mean the level of species abundance (%).

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>K (%)</th>
<th>T₀ (%)</th>
<th>T₁ (%)</th>
<th>T₂ (%)</th>
<th>T₃ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidota</td>
<td>30.58</td>
<td>15.78</td>
<td>29.30</td>
<td>11.50</td>
<td>13.94</td>
</tr>
<tr>
<td>Planctomycetota</td>
<td>9.45</td>
<td>15.33</td>
<td>12.58</td>
<td>20.31</td>
<td>19.85</td>
</tr>
<tr>
<td>Patescibacteria</td>
<td>4.94</td>
<td>22.52</td>
<td>5.26</td>
<td>12.61</td>
<td>8.08</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>23.78</td>
<td>18.44</td>
<td>24.34</td>
<td>23.35</td>
<td>27.41</td>
</tr>
<tr>
<td>Verrucomicrobiota</td>
<td>8.10</td>
<td>3.79</td>
<td>1.87</td>
<td>3.46</td>
<td>4.09</td>
</tr>
<tr>
<td>Gemmatimonadota</td>
<td>3.13</td>
<td>1.82</td>
<td>3.71</td>
<td>3.82</td>
<td>2.91</td>
</tr>
<tr>
<td>Actinobacteriota</td>
<td>2.33</td>
<td>1.71</td>
<td>8.01</td>
<td>4.10</td>
<td>4.89</td>
</tr>
<tr>
<td>Nanoarchaeota</td>
<td>0.03</td>
<td>4.16</td>
<td>1.02</td>
<td>2.64</td>
<td>1.87</td>
</tr>
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<td>Fibrobacterota</td>
<td>2.16</td>
<td>0.26</td>
<td>1.53</td>
<td>0.32</td>
<td>0.24</td>
</tr>
<tr>
<td>Latescibacterota</td>
<td>-</td>
<td>1.75</td>
<td>0.05</td>
<td>0.20</td>
<td>0.27</td>
</tr>
<tr>
<td>Myxococcota</td>
<td>4.97</td>
<td>1.98</td>
<td>0.55</td>
<td>2.97</td>
<td>2.41</td>
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<tr>
<td>Crenarchaeota</td>
<td>-</td>
<td>0.44</td>
<td>0.02</td>
<td>0.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Acidobacterota</td>
<td>2.21</td>
<td>3.11</td>
<td>1.35</td>
<td>2.67</td>
<td>3.55</td>
</tr>
<tr>
<td>Deinococcota</td>
<td>0.10</td>
<td>0.28</td>
<td>1.38</td>
<td>0.92</td>
<td>1.07</td>
</tr>
<tr>
<td>Hydrogenedentes</td>
<td>0.26</td>
<td>0.37</td>
<td>0.06</td>
<td>1.18</td>
<td>0.77</td>
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<tr>
<td>Chloroflexi</td>
<td>1.64</td>
<td>2.42</td>
<td>3.81</td>
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<td>2.76</td>
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<td>Halobacterota</td>
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<td>0.21</td>
<td>0.10</td>
<td>0.06</td>
<td>0.19</td>
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<tr>
<td>Spirochaetota</td>
<td>0.36</td>
<td>0.15</td>
<td>0.38</td>
<td>0.31</td>
<td>0.24</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>3.3105</td>
<td>1.2473</td>
<td>1.4015</td>
<td>1.5638</td>
<td>1.9464</td>
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<tr>
<td>Bdellovibrionota</td>
<td>1.4373</td>
<td>1.5168</td>
<td>0.6069</td>
<td>0.3561</td>
<td>0.2749</td>
</tr>
</tbody>
</table>

### 3.5. Bacterial Communities and Enzyme Activity Relationships

Worms have digestive enzymes in their guts that help digest and break down macromolecules. These enzymes play an important role in the dissolution of organic matter and are considered an indicator for microbial activity [12]. By increasing the availability of P in BA, the phosphatase enzymes produced by many microorganisms make P soluble. The production of hydrolytic enzymes increases the release of P from fly ash [26]. In the vermicomposting process, the phosphatase enzyme activity dissolves the P in apatite form and makes it available to the plant. Phosphatases can be divided into different classes based on their substrate range (mixed or specific) and pH optimum (acid or alkaline). The phyla of Proteobacteria, Bacteroidota, Firmicutes, and Actinobacteriota, and the genus Pseudomonas, Bacillus, Rhizobium, and Burkholderia, were identified as phosphorus-solubilizing bacteria [38,44].

The population of these bacterial classes in the samples are shown in Figure 7. The abundance of Proteobacteria increased to 18.44%, 24.34%, 23.55%, and 27.41% for T₀, T₁, T₂, and T₃, respectively, with the increase in the amount of BA in the compost samples. Proteobacteria include a variety of bacteria associated with the carbon, nitrogen, and sulfur cycle. The variation in enzyme activity may be due to the organic matter substrate that is available to the distinctive bacterial population [45]. Studies have shown that some microorganisms that play a vital role in the nitrogen cycle are Proteobacteria. In light of this information, Proteobacteria caused an increase in urease enzyme activity in all samples [31,46]. Bacteroidota and Planctomycetota were the other dominant phyla. The abundance of Bacteroidota was 17.78%, 29.30%, 11.50%, and 13.94% for T₀, T₁, T₂, and
T3, respectively. The phyla of Planctomycetota accounted for 15.33%, 12.58%, 20.31%, and 19.85% in T0, T1, T2, and T3. Previous studies have reported that Planctomycetota are well adapted to different habitats, maintain high numbers in microbial communities and have the remarkable ability to participate in nitrogen metabolism in natural environments [47,48]. The significant concentration of the Bacteriodata phylum in the T1 sample also indicated that it had an impact on the alkaline phosphatase enzyme activity value. The dominant population of Flavobacterium, belonging to the Bacteriodata phylum, showed higher activity than T0, T2, and T3 by activating P availability in T1, with 11.50% [49]. The T0, T2, and T3 values for the dominant strain of Flavobacterium were 3.43%, 2.31%, and 3.23%, respectively. Considering these values, although worm digestion had a negative effect on Flavobacterium, the addition of BA to cattle manure showed a positive effect on phosphatase enzyme activity.

Figure 7. Bacterial abundance at phylum level of vermicompost samples collected at the end of vermicomposting experiments from each of the increasing BA applications from K (pre-composted), T0 (0.0%), T1 (3.5%), T2 (7.0%) to T3 (10.0%).

The urease enzyme activity showed the highest activity values in all samples. The urease enzyme plays an important role in converting complex organic nitrogenous matter into simpler forms. The population of Cytophagales producing hydrolytic enzymes in order to convert nitrogen forms, which are one of the most important organic substances of plant growth, to urea forms accounted for 8.44%, 6.14%, 5.97% and 8.09% in T0, T1, T2, and T3, respectively. The urease enzyme activity (Table 1) was determined as T0 > T1 > T2 > T3. The gradual decrease in the urease enzyme activity in the samples showed a parallel decrease for the bacterial population. The reason for this may be related to the diversity of nitrogen-solving bacteria, the abundance of bacteria, the increase in BA content, and the decrease in nutritional value.

The results of the enzyme activity and metagenomic analysis performed on the samples that were vermicomposted by adding cattle manure and BA, as well as the results obtained using the multiple linear regression application, are presented in Table 3. This table shows that the above bacterial species stimulate the enzymes. It is evident from the results that during the vermicomposting process, worms indirectly stimulate microbial populations via the degradation and homogenization of organic matter, which increase the surface area that is available for microbial colonization and decomposition [20,22]. In this study, it was observed that the enzyme activity in vermicomposts was related to the bacterial population, and that the bacterial phylum listed in Table 2 significantly supported
acid phosphatase, alkaline phosphatase, arylsulphatase, β-glucosidase and urease activities. Positive correlations between enzyme activities and the microbial community can increase the yield of recovered phosphorus during the vermicompost process using dominant bacteria (Figure 7).

Table 3. Results of enzyme and metagonomic analyses using multiple linear regression.

<table>
<thead>
<tr>
<th>Enzymatic Activities</th>
<th>Treatments</th>
<th>Bacteria Population (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Candidatus Kaiserbacteria</strong> (4.55%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Candidatus Magasanikbacteria</strong> (4.24%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Woesearchaales</strong> (4.16%)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Saccharimonadales</strong> (4.04%)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Flavobacteriales</strong> (3.43%)</td>
<td>0.90</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>$T_0$</td>
<td>Rhizobiales (3.63%)</td>
<td>0.90</td>
</tr>
<tr>
<td>(µg PNP g$^{-1}$ h$^{-1}$)</td>
<td></td>
<td>Planctomycetales (3.60%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonadales (2.88%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alphaproteobacteria (2.67%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actinomarinales (2.41%)</td>
<td>0.90</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>$T_1$</td>
<td>Saccharimonadales (3.11%)</td>
<td>0.90</td>
</tr>
<tr>
<td>(µg PNP g$^{-1}$ h$^{-1}$)</td>
<td></td>
<td>Flavobacteriales (2.31%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actinomarinales (2.01%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Planctomycetales (2.43%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burkholderiales (3.98%)</td>
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<tr>
<td>Arylsulphatase</td>
<td>$T_2$</td>
<td>Saccharimonadales (5.11%)</td>
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</tr>
<tr>
<td>(µg PNP g$^{-1}$ h$^{-1}$)</td>
<td></td>
<td>Flavobacteriales (3.23%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonadales (3.16%)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhizobiales (3.21%)</td>
<td>0.90</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>$T_3$</td>
<td>Burkholderiales (3.51%)</td>
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<tr>
<td>(µg PNP g$^{-1}$ h$^{-1}$)</td>
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<td>Flavobacteriales (3.23%)</td>
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<tr>
<td>Urease</td>
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<td>(µg NH$_4$ g$^{-1}$ h$^{-1}$)</td>
<td></td>
<td>Rhizobiales (3.21%)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

3.6. Strengths and Limitations of the Present Application

This study proved that during the vermicomposting process, plant nutrients that are strictly bound to the biomass ash are transformed by worms, due to their gut microbial composition and their digestive enzymes, into a more bioavailable form for plant growth. Besides composting bacterial flora, the worm compost itself increased the bacterial composition of the BA vermicompost samples. As a biotechnological system, several physical, chemical and biological reactions play a critical role in nutrient transformation. Although biological activities are prone to nutrient dissolution from biomass ash, the results of the present study do not distinguish the mechanism responsible for nutrient bioavailability. Nonetheless, this study suggests that vermicomposting BA is one of the best options, as it offers a low-cost and environmentally sound strategy by which to obtain bioactive compounds enriched with plant fertilizer nutrients.

3.7. Future Recommendations for Current Research Topic

It is recommended that some future research in this area is still required to provide new process-related insights and to understand the relevant methodology with more certainty. Thus, issues that are not included in the present analysis, but that are important to include in a future study, are summarized below.

(1) A future investigation is needed to evaluate and focus on the entire microbial community, including yeasts and fungi genera, next to the bacterial community that is responsible for the transformation of plant nutrients. It may provide a good insight into the mechanism involved in the reaction to dissolution and help to describe the system’s behavior in terms of adverse alkalinity and electrical conductivity during earthworms’ ingestion of BA-enriched bio-waste substrates.

(2) Future research is encouraged to provide data on the possible dissolution of phosphorus after the enrichment of the vermicomposting process by acidifying bacteria groups.
This is important in order to gain an understanding of the outcome of the transformation and plant availability of less labile Fe- and Al-bound P fractions that specifically need acid dissolution.

(3) It will be interesting for future research to look into the subject of nutrient use efficiency and the response of crops to the nutrient-rich worm compost samples reported in the present study. This will provide information regarding the justification of the product in terms of the crop response, its economic applicability and the acceptance of the final user.

(4) The study also needs to be extended to other domains, such as food wastes, that specifically contain high levels of moisture and generate acidic conditions during the composting process. It may provide different findings (e.g., the optimization of pile moisture and the determination of what happens to major plant nutrients, including P, Ca, Mg, and Fe, during the BA composting process) from various perspectives.

4. Conclusions

The vermicomposting of cattle manure by adding different proportions of biomass ash had a strong effect on worm activity and the enzyme activities of the microbial community. This was seen in the high correlations between enzyme activities and the bacterial population. The results of this study shed light on the relationship between microbial diversity and the enzyme activities that dissolve phosphorus in the form of apatite. According to the bacteria populations (%), the stimulating effect of Flavobacteriales (T₀-3.43%, T₂-2.31%, T₃-3.23%), Burkholderiales (T₂-3.98%, T₃-3.51%), Saccharimonadales (T₀-4.04%, T₂-5.11%, T₃-2.91%) and Pseudomonadales (T₁-2.88%, T₃-3.16%) showed a positive effect on enzyme activities. The determination coefficients (R²) obtained from multiple linear regressions between the enzyme activities and bacterial population for T₀, T₁, T₂, and T₃ were determined as 0.90, 0.65, 0.73, and 0.90, respectively. As a result of the experiments supporting each other with regard to the samples containing biomass ash after vermicompost application, the importance of microorganisms in the solubilization of phosphorus in the apatite form in the biomass was demonstrated. In addition, the presence of microorganisms proved that P was dissolved and that its dynamics continued.


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Conflicts of Interest: The authors declare no conflict of interest.
Appendix A. Conversion of Biomass Ash (BA) to Bio-Based Fertilizer

**Figure A1.** An illustrated presentation showing the solubilization of BA nutrients during the vermicomposting process.

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