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Fermentation of Washed Rice Water Increases Beneficial Plant Bacterial Population and Nutrient Concentrations

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Abstract: Washed rice water (WRW) is said to be a beneficial plant fertilizer because of its nutrient content. However, rigorous scientific studies to ascertain its efficiency are lacking. The purpose of this study was to determine the effect of fermenting WRW on the bacterial population and identification, and to measure how fermentation affects the nutrient composition of WRW. Rice grains were washed in a volumetric water-to-rice ratio of 3:1 and at a constant speed of 80 rpm for all treatments. The treatments were WRW fermented at 0 (unfermented), 3, 6, and 9 days. Bacterial N fixation and P and K solubilization abilities in the fermented WRW were assessed both qualitatively and quantitatively. The isolated bacterial strains and the WRW samples were also tested for catalase and indole acetic acid (IAA) production ability. Significantly greater N fixation, P and K solubilization, and IAA production were recorded after 3 days of fermentation compared with other fermentation periods, with increases of 46.9–83.3%, 48.2–84.1%, 73.7–83.6%, and 13.3–85.5%, respectively, in addition to the highest (2.12×10^8 CFU mL^{-1}) total bacterial population. Twelve bacteria strains were isolated from the fermented WRW, and the gene identification showed the presence of beneficial bacteria *Bacillus velezensis*, *Enterobacter* spp., *Pantoea agglomerans*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia* at the different fermentation periods. All the identified microbes (except *Enterobacter* sp. Strain WRW-7) were positive for catalase production. Similarly, all the microbes could produce IAA, with *Enterobacter* spp. strain WRW-10 recording the highest IAA of up to 73.7% higher than other strains. Generally, with increasing fermentation periods, the nutrients N, S, P, K, Mg, NH_4^+, and NO_3^- increased, while pH, C, and Cu decreased. Therefore, fermentation of WRW can potentially increase plant growth and enhance soil health because of WRW's nutrients and microbial promotional effect, particularly after 3 days of fermentation.

Keywords: bacteria; catalase; fermentation; indole acetic acid; nutrients; wastewater

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1. Introduction

Rice is the second most widely grown cereal, and it is eaten by the majority of the world's population [1]. Rice is a staple food for Malaysians, and it is estimated that Malaysians consume nearly 3 million tons of rice per year [2]; the figure is expected to increase due to the continual rise in population. As a conservative estimate, washing every 1 kg of rice grains with at least 1 L of water would work out to 3 billion L of washed rice wastewater produced yearly. Rice is usually washed to remove dust and dirt before cooking and the water after the rice is washed (hereafter referred to as washed rice water or WRW) is often discarded into the environment. However, rice washing can also remove a significant amount of water-soluble nutrients from the rice. Many studies, as reviewed by Juliano [3], have shown that rice washing can reduce Ca, P, Mg, K, protein, crude fat, crude fiber, thiamine, riboflavin, and niacin by up to 26%, 47%, 70%, 41%, 7%, 65%, 30%, 59%, 26%, and 60%, respectively, through leaching from the rice. These leached nutrients could be used as a liquid plant fertilizer and soil amendments.

Rather than being merely discarded, unused, into the environment, WRW ought to be reused as part of water governance. By the 2050, the global freshwater demand is expected to increase by 55% [4]. This increase is largely caused by the detrimental climate change and continuous increase in world population, driving WWAP [5] to advocate for more effective water governance so that wastewater, rather than just being discarded into the environment, is instead reused. Furthermore, the AQUASTAT database of the FAO of the United Nations estimates that more than half of the global freshwater withdrawals are simply discarded as wastewater into the environment [5]. Only 11% of the global freshwater withdrawal corresponds to municipal water demand, of which only 3% is consumed, while the remaining 8% is simply discarded as wastewater. Therefore, as part of water governance, WRW, like any other wastewater, ought to be reused. The practice of reusing WRW can potentially lead to considerable savings in water, as well as fertilizer use.

WRW is often claimed to be a beneficial plant fertilizer and soil amendment due to the leached rice nutrients in the WRW, but claims of WRW's benefits are without strong scientific evidence. Nabayi et al. [2] reported that scientifically rigorous studies to determine the benefits of reusing WRW for agriculture are severely lacking. Nabayi et al. [2] found only 41 papers on WRW studies, only 10% of them were published in citation index journals. Out of these citation index journals, only about 3% were on microbes. The nutrient contents of WRW (ethanol, P, N, and S) are reported to increase with fermentation for 6 days [6]. Similarly, Nurhasanah et al. [7] reported that WRW was a better medium than the popular nutrient-rich Luria-Bertani (LB) broth to promote the growth of *Pseudomonas fluorescens*, a plant growth-promoting rhizobacteria (PGPR). Several studies also reported WRW to support the growth of beneficial bacteria *Rhizobium*, *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescence*, *Lactobacillus*, and *Bacillus*, as well as beneficial fungi *Trichoderma*, *Penicillium*, and *Saccharomyces* [6,8,9].

This study aimed to determine the effect of fermentation periods on the macro- and micronutrient contents of WRW and whether (and to what degree) fermentation of WRW promotes the growth of beneficial soil bacteria, particularly N-fixing and P- and K-solubilizing bacteria, as well as catalase- and IAA-producing bacteria. The identification and the biochemical characterization of the bacteria in the WRW is necessary to determine if WRW could potentially increase soil health by introducing beneficial soil bacteria [2]. To our knowledge, this could be the first study to report how the fermentation affects both the micro- and micronutrient content of the WRW. Sairi et al. [9] only morphologically identified the microorganisms in the WRW, but this study identified the bacteria in the WRW using gene sequencing. Therefore, the objectives of the study were (1) to determine the potential of fermented WRW to fix N, solubilize P and K, and produce catalase and IAA; (2) to isolate, identify, and test the bacteria present in the fermented WRW for catalase and IAA potential; and (3) to determine the effect of fermentation periods on nutrient content of WRW.

2. Materials and Methods

The rice grain used in the study is 'Rambutan' (Padiberas Nasional Berhad, Malaysia) because of its popularity and availability in Malaysia. For washing the rice, distilled water was used with a mixer (Bossman Kaden matte BK-100S, Tokyo, Japan) at 80 rpm (0.357 g Force) for 90 s to ensure uniformity and repeatability in washing. After washing the rice, the mixture was sieved (500-micron sizes) to separate the rice grains from the WRW, after which the WRW was allowed to ferment for 0 (unfermented), 3, 6, and 9 days. These fermentation periods are labeled as F0, F3, F6, and F9, respectively. The fermentation was carried out indoors, under room temperature, and without additives in the Soil Physics Laboratory, Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia. After the rice was washed at the required water to rice ratio, the resultant WRW was kept in a series of plastic vials for predetermined periods of 3, 6, and 9 days to allow for fermentation, while the freshly prepared WRW was used as the 0-day fermented WRW.

The WRW culture was aerated daily for 20–30 min by opening the vials caps throughout the fermentation period.

2.1. Chemical Analyses

Both the rice grains and the WRW at different fermentation periods were subjected to chemical analyses in addition to the pH and EC measurements. C, N, and S were analyzed by CNS analyzer (LECO Corp., St. Joseph, MI, USA). P, K, Ca, Mg, Cu, Zn, and B were analyzed using AAS (Perkin Elmer, PinAAcle, 900T, Waltham, MA, USA), after dry-ashing in the case of the rice grains [10]. Ammonium and nitrate were determined by the Kjeldahl procedure [11]. pH and EC were measured using a pH and EC lab meter (Metrohm, 827, Riverview, FL, USA) [12].

2.2. Culture Media Preparation and Bacterial Population

Tryptic soy agar (TSA) was used to determine the bacterial population in the various fermented WRW samples, following the method of Tan et al. [13]. After the inoculation of the WRW culture, plates were incubated for 24 h at 33 °C. The bacterial population was counted from each fermented WRW type (in triplicates). Each plate that has a range of 30 to 300 colonies was selected and counted as colony-forming units (CFU) per mL of sample [14].

2.3. N Fixation, Phosphate, and Potassium Solubilization Ability of the WRW Culture

The N fixation was determined qualitatively by growing the WRW on Nfb medium (N-free solid malate medium) following Döbereiner and Day [15]. The quantitative assessment of the N fixing bacteria were determined using the Acetylene Reduction Assay (ARA) method to quantify the N fixation rate of the WRW culture [16,17].

The qualitative phosphate solubilizing ability was determined by observing a halo zone around the colony (10 µL inoculum, 1×10^8 CFU mL^{-1}) after 24 h of incubation [18]. The phosphate solubilizing index (PSI) of the fermented WRW culture was determined as outlined in Sitepu et al. [19]. The vanadomolybdophosphoric acid method was used to quantify the amount of soluble phosphate in the culture supernatants of NBRIP (National Botanical Research Institute's Phosphate) broth [20,21]. A standard curve was prepared using a stock solution containing a mixture of KH_2PO_4 and 5 mL of concentrated H_2SO_4, making up to 1 L using distilled water. Then, 10 µL of each WRW culture suspension was inoculated into each NBRIP broth; 100 mL of NBRIP culture medium in a flask without inoculum served as the control. The flasks were continuously incubated for 12 days (i12) at room temperature under constant agitation at 100 rpm, as outlined in Tan et al. [13]. The pH of each culture medium was also checked accordingly at 6 and 12 days (i6 and i12). The assessment of the solubilized phosphate of the culture was performed at two different times (after i6 and i12 of incubation). Then, 25 mL of each culture medium was transferred to 50 mL tubes and centrifuged at 8000× *g* for 20 min at each assessment time. Next, 2.5 mL of the supernatant was transferred into a 50 mL beaker, followed by the addition of 20 mL of distilled water. Afterwards, 2.5 mL of Barton's reagent [22] was added quickly for mixing action, which was allowed for color development for 10 min. The absorbance was determined calorimetrically using a spectrophotometer at 430 nm.

Aleksandrov agar medium was used to determine the qualitative potassium solubilization [23] of the WRW culture. The quantitative assessment of the potassium solubilization rate was determined based on the ability of the bacteria to release K from the supplemented muscovite mica in the media. For the procedure, 1 mL of an overnight culture of fermented WRW was inoculated into 100 mL of Aleksandrov broth. The quantity of K released in the broth was measured at three different days after incubation (i5, i10, and i15) from three flasks' replicates as outlined in Tan et al. [13]. Centrifuging the incubated broth cultures at 10,000 rpm for 10 min was carried out to separate the supernatant from the muscovite mica and bacterial cells. Then, 1 mL of the supernatant was transferred into a 50 mL volumetric flask and the volume was gradually increased to 50 mL using distilled water and

mixed thoroughly. The available K content in the supernatant was measured by Atomic Absorption Spectrometer (AAS) (Perkin Elmer, PinAAcle, 900T, Waltham, MA, USA).

2.4. Production of Indole Acetic Acid (IAA)

The ability of the fermented WRW and the bacterial isolates to produce IAA were determined following Gordon and Weber [24]. The isolates and WRW culture were grown in Tryptic Soy Broth (TSB) in a conical flask for 3 days in a shaker incubator at 30 °C. Next, 1 mL of a fully grown of either WRW culture or an isolate was transferred into a fresh 100 mL of TSB containing 5 mL of tryptophan (2 µm sterilized Whatman No. 2 filter paper). The mixture was incubated for an additional 24 h in a shaker incubator. Afterwards, 1.5 mL of the mixture was pipetted into Eppendorf tube and centrifuged at 8000 rpm for 15 min. Then, 1 mL of the supernatant was transferred into a new tube and 2 mL of Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL of distilled H_2O, 7.5 mL of 0.5 M $FeCl_3 \cdot 6H_2O$) [25] was added and mixed vigorously and incubated for 25 min. Quantitatively, the IAA was ascertained using a spectrophotometer at 535 nm. A standard was prepared using pure IAA at 0, 5, 10, 20, 30, 40, 50, and 100 ppm. The quantity of the IAA produced was estimated from the standard curve.

2.5. Catalase Test

The isolates and the fermented WRW culture were tested for their ability to produce catalase enzyme following Khalifa et al. [26]. Hydrogen peroxide (5%) was added drop by drop to an aliquot of an incubated cultures from either WRW or isolate culture (after 24 h of incubation) after spreading on a clean glass slide. Positive results were indicated when gas bubbles evolved within a few seconds.

2.6. Bacterial Isolations

Following the bacterial growth and population count, a direct spreading method was used to isolate the different bacteria from different samples (fermentation period) based on shape, color, and size [27].

2.7. Bacterial Identification Using 16S rRNA Gene Sequence

Bacterial inoculum from overnight streaked culture plate was re-cultured overnight in nutrient broth. Genomic DNA was isolated from the bacterial culture (WRW) by using the Genomic DNA Mini Kit (Favorgen) (Pingtung Agricultural Biotechnology Park, Pingtung, Taiwan). Thereafter, the DNA was stored at −20 °C for further analyses. The 16S rRNA gene was amplified using universal forward 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers (Apical Science Sdn. Bhd., Seri Kembangan, Selangor, Malaysia). Then, 30 µL reaction mixture was prepared each containing 2 µL of DNA template, 15 µL of Master mix (containing 10X PCR Reaction Buffer, dNTPs mix, Taq polymerase, MgCl2, and ultra-pure water), 10 µL of Nuclease free water, and 1.5 µL each of forward and reverse primers. PCR reactions, were carried out using a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad, Hercules, CA, USA) with the following cycles: denaturation for 4 min at 95 °C, 45 s at 95 °C, 45 s at 58 °C for annealing, 1 min at 72 °C for initial extension, and final extension for 10 min at 72 °C. The amplified 16S rRNA gene was purified with a Gel/PCR DNA Fragments Extraction Kit (Favorgen) and outsourced for sequencing (Apical Scientific Sdn. Bhd., Selangor, Malaysia). The sequenced data were aligned and analyzed to identify the bacterium and its closest neighbors using BLAST (NCBI, Bethesda, Rockville, MD, USA). The sequences obtained for selected bacterial isolates were manually analyzed using Sequence Scanner Software v1.0 by Applied Biosystems® (Forster City, CA, USA). The interpretation of the sequences was performed by comparing them with information in the BLAST database online (NCBI, Bethesda, MD, USA).

2.8. Sequence Submission and Phylogenetic Analyses

The partial 16S rRNA gene sequences of the identified strains in this study were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) accessed on 15 December 2020, as reported by Nabayi et al. [28]. The sequence was further used to construct a phylogenetic tree using the Maximum Likelihood method. All the 16S rRNA gene sequences were aligned using ClustalW2 with the most closely related bacteria sequences obtained from the NCBI database using the MEGA software version 7.

2.9. Data Analysis

All data obtained were subjected to analysis of variance using the general linear model (GLM). The treatment means were separated by the Honest Significant Difference (HSD) test at 5% with Minitab (version 19) software package (Minitab, LLC, College Town, PA, USA). All treatments were in triplicate unless otherwise stated.

3. Results

3.1. Microbial Analyses

3.1.1. Effect of Fermentation on Bacterial Population and Biochemical Tests of WRW

The highest bacterial population was recorded in F3 (3-day fermentation) with 2.12×10^8 (CFU mL^{-1}), while the least was found in the F0 (0-day or unfermented) (Figure 1A). However, the pH of the WRW culture decreased in the order of F0 > F3 > F9 > F6 (Figure 1B). Compared with the other fermentation periods, F0 had a range increase in pHof 30.7–49.2%. For each level of fermentation, P solubilization was assessed at 6 and 12 days of incubation (i6 and i12) (Figure 2). Generally, irrespective of the fermentation period, greater P solubilization was observed at the i12 incubation period, differing significantly ($p < 0.01$) from the i6 at all fermentation periods (except at F0). Among the fermentation periods, the F3 had a greater P solubilization rate of 48.9%, increased at both i12 and i6 of incubation, compared to other fermentation periods. Similarly, the WRW culture for the P solubilization was assessed for pH at i6 and i12 incubation days, as shown in Figure 2C. Across the same fermentation period, there was no significant difference ($p > 0.05$) (except at 0-day fermentation) in the pH of the culture between the incubation days; however, the pH decreased with an increase in incubation days.

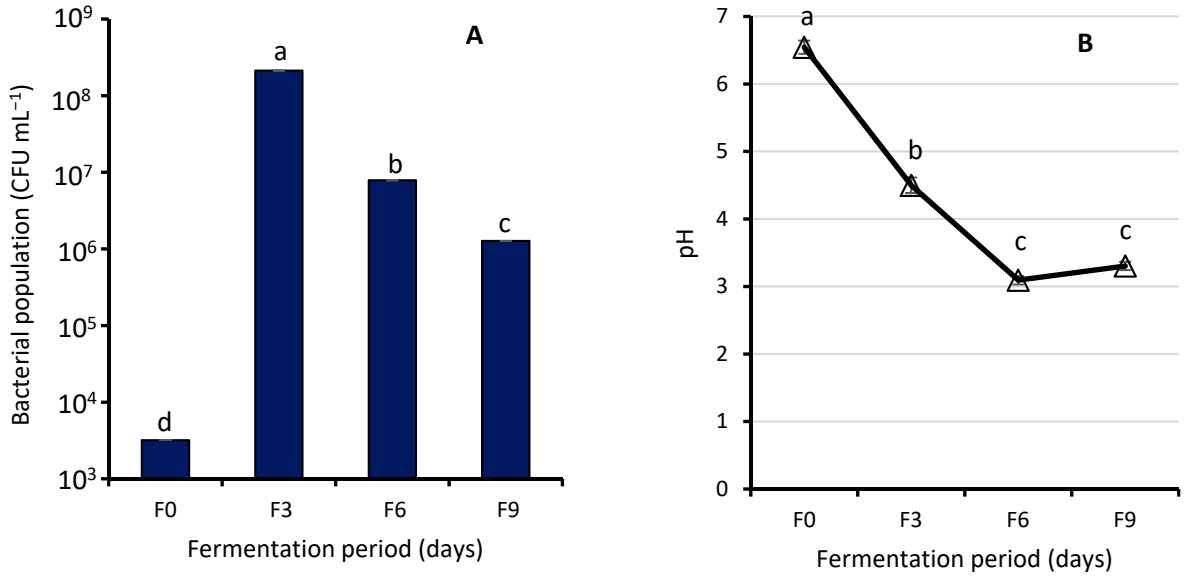

Figure 1. Means (±SE) of (**A**) total bacterial population, (**B**) pH of the WRW at different fermentation periods. Means with the same letters within the same chart are not significantly different from one another at the 5% level using HSD.

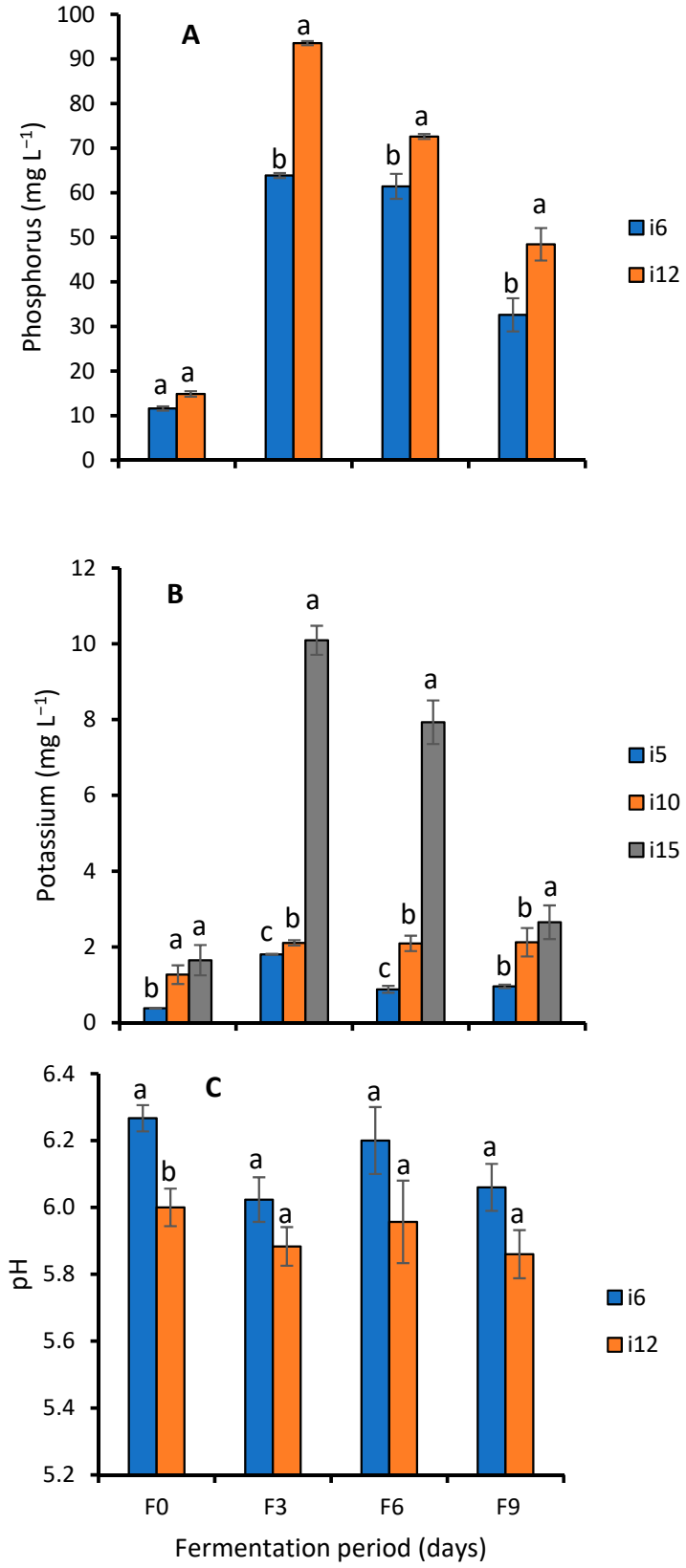

Figure 2. Means (±SE) of (**A**) solubilized phosphorus, (**B**) solubilized potassium, and (**C**) change in culture pH, for different fermentation periods at different incubation days. i6, i12 and i5, i10, i15 represent incubation days. For the same fermentation period, within the same chart, means with the same letters are not significantly different from one another at the 5% level according to HSD.

Within the same fermentation period, a significant difference ($p < 0.05$) was observed between the incubation days of i5, i10, and i15 for K solubilization (Figure 2B). The K solubilization increased in the order of i15 > i10 > i5 irrespective of the fermentation period. Across the same fermentation period, at i15 incubation days, F3 had significantly ($p < 0.05$) greater K solubilization than other fermentation periods, with an increase of 80.3–81.5%.

Similar to P and K solubilization, significantly ($p < 0.05$) greater ethylene production was obtained in the F3 fermentation period (9.6 Nmol C_2H_4 mL^{-1} h^{-1}), which did not differ significantly ($p > 0.05$) from F6, which had 8.7 Nmol C_2H_4 mL^{-1} h^{-1} (Figure 3). The result was in the order of F3 ≥ F6 > F9 > F0, with the least in 0 days of fermentation (1.6 Nmol C_2H_4 mL^{-1} h^{-1}). Remarkably, the F3 had increases in ethylene production of 9.3%, 46.8%, and 83.3% compared to F6, F9, and F0, respectively.

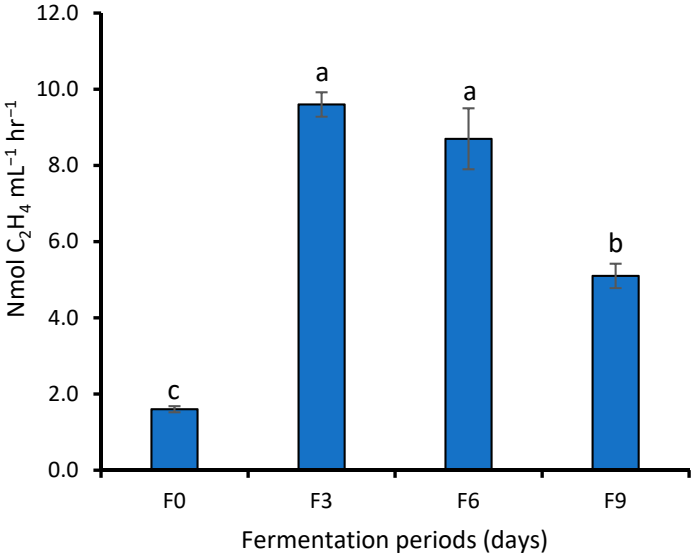

Figure 3. Means (±SE) of acetylene reduction assay of the selected isolates. Means with the same letters are not significantly different from one another at the 5% level using HSD.

3.1.2. Biochemical Test of WRW Fermented at Different Periods

Based on the agar test, the presence of N-fixing and P- and K-solubilizing bacteria were pronounced in the F3, F6, and F9 fermentation periods after observing the cultures' incubated plates for 24 h (Figure 4 and Table 1). Notably, the intensity of the blue color and the diameter of the halo zones were greater in F3, which decreased with the increase in fermentation periods. The fermented WRW and the identified isolates were found to be positive for N fixation and P and K solubilization as well as catalase enzyme production (Tables 1 and 2). Among the fermentation periods, the P and K solubilization indexes were highest in F3 and decreased with the increase in fermentation (Table 1). Similarly, more IAA was also recorded in the F3, followed by F6, and the least was in F9. Interestingly, IAA production at F3 was 13.2% and 30.1% greater than F6 and F9, respectively (Table 1).

Table 1. Biochemical characterizations and IAA production of the fermented WRW.

Fermentation Periods (Days)	N fix	PS	KS	Catalase	PSI	KSI	IAA (μg mL^{-1})
F0	–	–	–	–	0	0	1.2
F3	++	++	+	++	4.2	3.4	8.3
F6	++	++	+	++	2.3	1.9	7.2
F9	+	+	+	++	2.4	2.0	5.8

Note: PS is phosphorus solubilization; KS is potassium solubilization; PSI is phosphorus solubilization index; KSI is potassium solubilization index; IAA is indole acetic acid; – is negative; +, ++ indicate the intensity of the color or size of the halo zones as low and high, respectively.

Table 2. Bacterial identification using 16S rRNA gene amplification, morphological and qualitative biochemical characterizations, and IAA production of the identified WRW strains.

Isolates	Fermentation Period	Accession Number	Closest Identity in NCBI	Similarity (%)	N Fixation	PS	KS	Catalase	PSI	KSI	Gram Stain
WRW-1	F3	MW365554.1	*Bacillus velezensis* strain HSB1	99.35	–	+	+	++	1.3	1.2	Positive
WRW-3	F3	MW365555.1	*Enterobacter ludwigii* strain SDI-19	98.75	++	+	–	++	1.4	0	Negative
WRW-4	F3	MW365556.1	*Enterobacter* sp. Strain LSB19	99.10	++	+	–	++	1.2	0	Negative
WRW-6	F0	MW365557.1	*Enterobacter* sp. Strain LSB3	97.59	+	+	–	+	1.2	0	Negative
WRW-7	F6	MW365558.1	*Enterobacter mori* strain BC1	98.51	–	++	–	–	1.2	0	Positive
WRW-8	F6	MW365559.1	*Bacillus velezensis* strain 2656	99.70	–	+	+	+++	1.4	1.3	Positive
WRW-9	F6	MW365560.1	*Stenotrophomonas maltophilia* strain JM11	99.87	++	+	++	+	1.5	1.3	Negative
WRW-10	F9	MW365561.1	*Enterobacter* sp. Strain LSB10	99.49	+	+	–	+	1.6	0	Negative
WRW-11	F9	MW365562.1	*Enterobacter mori* strain BC1	99.19	–	+	–	++	1.16	0	Negative
WRW-12	F3	MW365563.1	*Klebsiella pneumoniae* strain LB-AMP3KSU	99.87	++	++	++	+++	1.8	2.3	Negative
WRW-13	F3	MW365564.1	*Pantoea agglomerans* stain SVMR	97.92	++	++	++	++	1.6	1.2	Negative
WRW-14	F9	MW365565.1	*Stenotrophomonas maltophilia* strain F41	99.47	+	+	+	+	1.3	1.1	Negative

Note: F0, F3, F6, and F9 are the WRW fermentation periods of 0, 3, 6, and 9 days, respectively; PS is phosphorus solubilization; PSI is phosphorus solubilization index; KS is potassium solubilization, KSI is potassium solubilization index; – is negative; +, ++, +++ indicate the intensity of the color or clarity of the halo zones as low, moderate, and high, respectively.

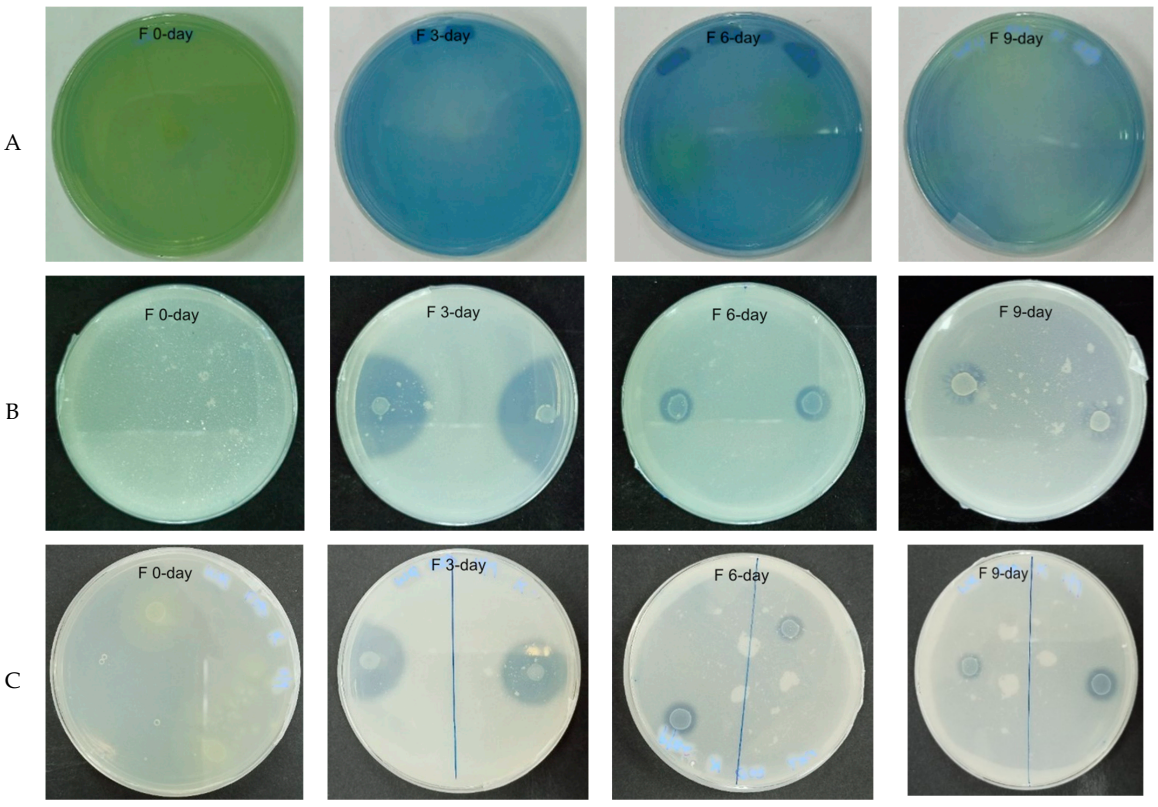

Figure 4. N-free media (**A**), Pikovskaya (**B**), and Aleksandrov (**C**) media agar plates after inoculation for 24 h with WRW fermented at different fermentation periods. Change in color from green to blue (**A**) and halo zone formation around the colony (**B**,**C**) indicate the positiveness of the WRW culture to N-fixing and P- and K-solubilizing bacteria.

3.1.3. Phylogenetic Tree and Biochemical Test of the Isolated Microorganisms from WRW

A total of 12 strains were isolated from different fermentation periods of WRW (F0, F3, F6, and F9) in this study and were identified as *Bacillus velezensis* strain WRW-1, *Enterobacter ludwigii* strain WRW-3, *Enterobacter* sp. strain WRW-4, *Enterobacter* sp. strain WRW-6, *Enterobacter mori* strain WRW-7, *Bacillus velezensis* strain WRW-8, *Stenotrophomonas maltophilia* strain WRW-9, *Enterobacter* sp. strain WRW-10, *Enterobacter mori* strain WRW-11, *Klebsiella pneumoniae* strain WRW-12, *Pantoea agglomerans* strain WRW-13, and *Stenotrophomonas maltophilia* strain WRW-14. The F3 fermentation period had a higher number of bacteria (five strains), but the number decreased with an increase in the fermentation period. The F6 and F9 have three different strains each, while the F0 recorded only one strain. The phylogenetic tree of each of the identified strain was grouped into their respective genus and species (Figure 5). All the isolates were positive for catalase enzymes (except *Enterobacter mori* strain WRW-7), as shown in Table 2. In addition, all the isolates were positive for IAA production; *Enterobacter* spp. strain WRW-10 recorded the highest, with a range increase of 73.7–48.7% compared to other isolates (Figure 6).

3.2. Nutrient Analyses

Effect of Fermentation on Nutrient Contents of WRW

The nutrient content of the rice grains showed a high total carbon percentage, 40%, followed by N, S, P, K, Ca, and Mg (Table 3). In terms of the micronutrients, Cu, Zn, and B are present in the order of Cu > Zn > B with 5.2, 5.01, and 1.4 mg kg^{-1}, respectively. The effect of fermentation periods on the pH, EC, NH_4^+, NO_3^-, TC, and TN of the WRW is shown in Figure 7. The total carbon (Figure 7E) decreased with fermentation periods in the order of F0 > F3 > F6 > F9, which differ significantly ($p < 0.002$) from one another. The F0 had a percent increase in carbon of 22.8–62.5% compared to other fermentation periods. However, the highest TN was found in F3 and decreased with the progression of fermentation period, which did not differ significantly ($p > 0.05$) from F6 and F9. The F3 had a range increase in N of 4.5–27.3% compared to other fermentation periods. Meanwhile, the NH_4^+ and NO_3^- increased with fermentation with the highest in F9, which did not differ significantly ($p > 0.05$) from F6. The F9 and F6 had percent increases in NH_4^+ and NO_3^- of 15.1–24.5% and 1.6–52.5%, respectively, compared to other fermentation periods. The EC increased with the fermentation period similar to the increased in S, P, K, Ca, Mg, and B (Figures 8 and 9).

Table 3. Means (±SE) of nutrient analyses of rice grains and unfermented WRW and percent elements leached from the rice grains into the WRW.

Elements	Rice Grains	Freshly Prepared WRW	% Elements Leached from Rice Grains (%)
Ash (%)	0.95 ± 0.036	–	–
Moisture (%)	14.39 ± 0.057	99.32 ± 0.310	–
TC (%)	40.30 ± 0.008	3.87 ± 0.371	9.6
TN (%)	1.25 ± 0.012	0.02 ± 0.001	1.6
S (%)	0.10 ± 0.003	0.01 ± 0.001	10.0
P (mg kg^{-1})	1320.83 ± 34.044	90.94 ± 4.761	6.9
K (mg kg^{-1})	1130.83 ± 22.639	118.16 ± 2.55	10.4
Ca (mg kg^{-1})	427.08 ± 5.717	18.17 ± 1.891	4.3
Mg (mg kg^{-1})	244.93 ± 10.257	27.91 ± 1.754	11.4
NH_4^+ (mg kg^{-1})	215.45 ± 4.410	18.52 ± 1.281	8.6
NO_3^- (mg kg^{-1})	100.82 ± 8.530	4.92 ± 1.112	4.8
Cu (mg kg^{-1})	5.25 ± 0.236	0.18 ± 0.001	3.4
Zn (mg kg^{-1})	5.02 ± 0.044	0.10 ± 0.002	2.0
B (mg kg^{-1})	1.40 ± 0.152	0.12 ± 0.002	8.6

Note: WRW is washed rice water; TC is total carbon; TN is total nitrogen.

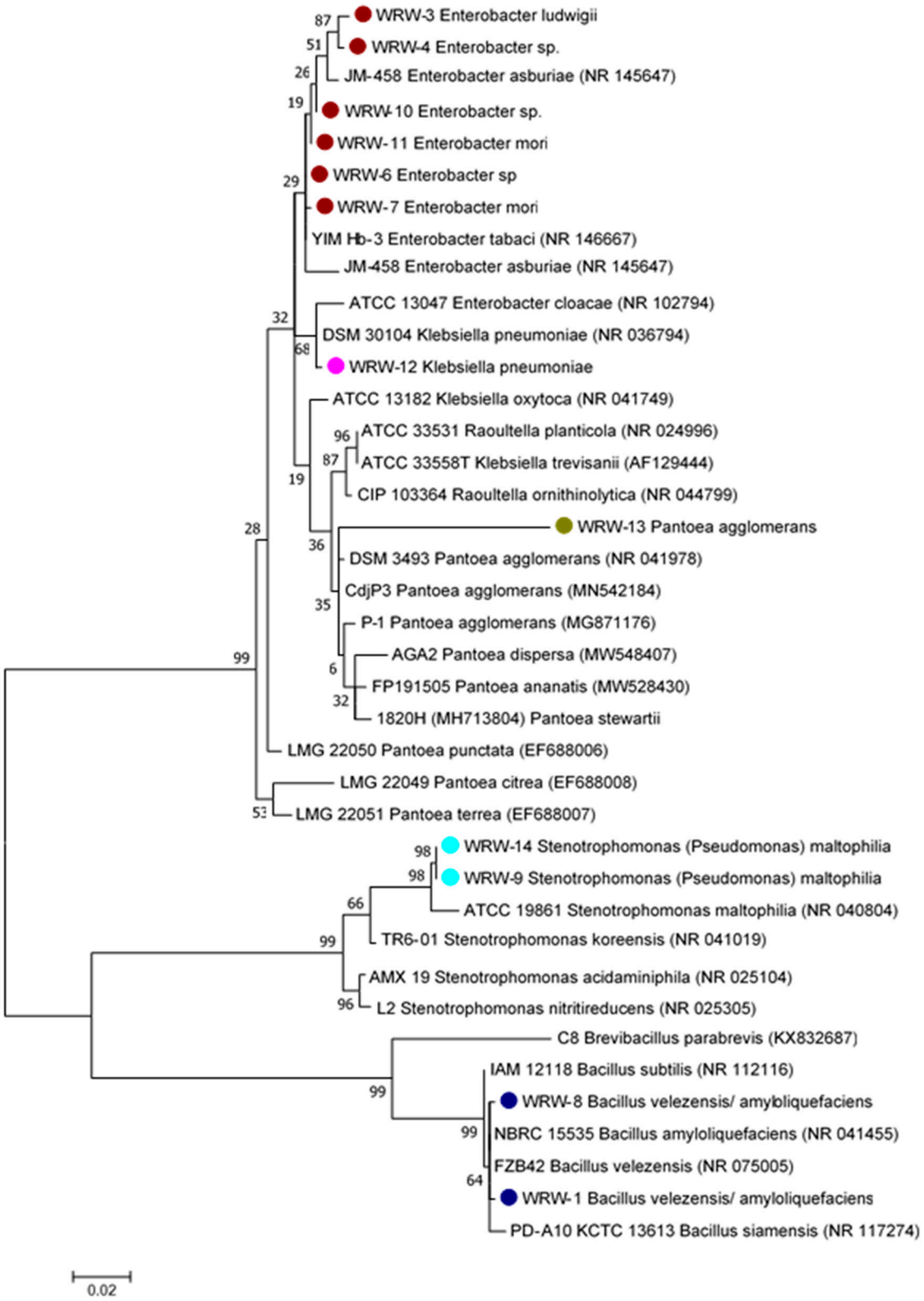

Figure 5. Molecular phylogenetic analysis by the Maximum Likelihood method of different types of bacteria isolated from WRW derived from partial 16S rRNA gene sequencing and other bacterial species from the database. The database accession numbers are indicated after the bacterial names. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position (adapted from Nabayi et al. [28]).

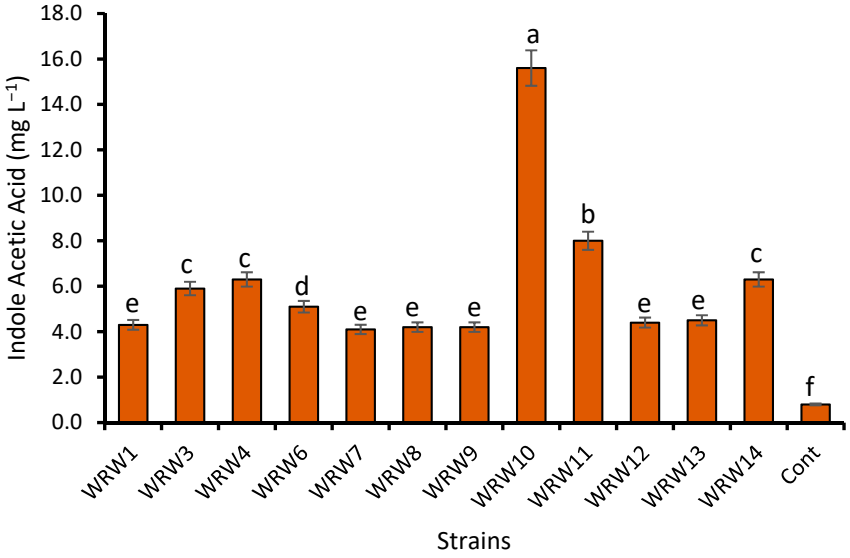

Figure 6. Means (±SE) of indole acetic acid produced by the strains isolated from different fermentation periods of WRW. Means with different letters are significantly different from one another using HSD at the 5% level of significance.

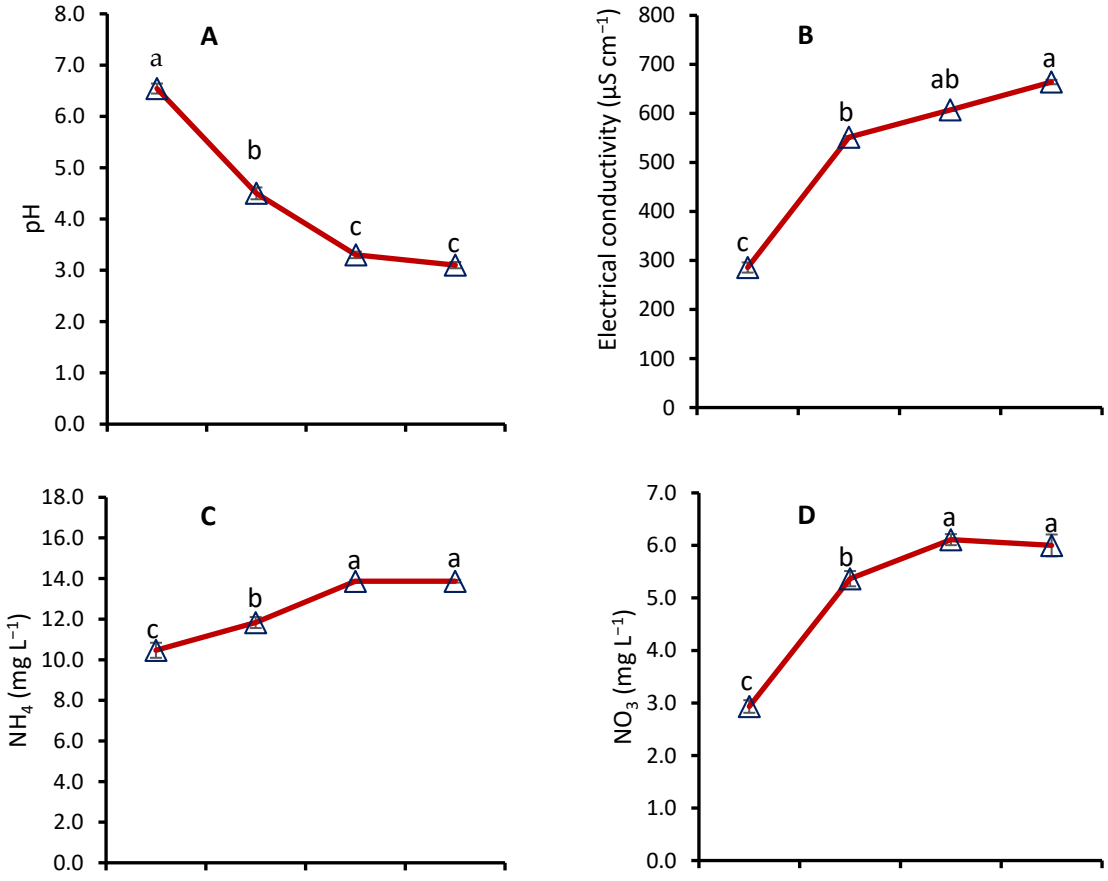

Figure 7. *Cont.*

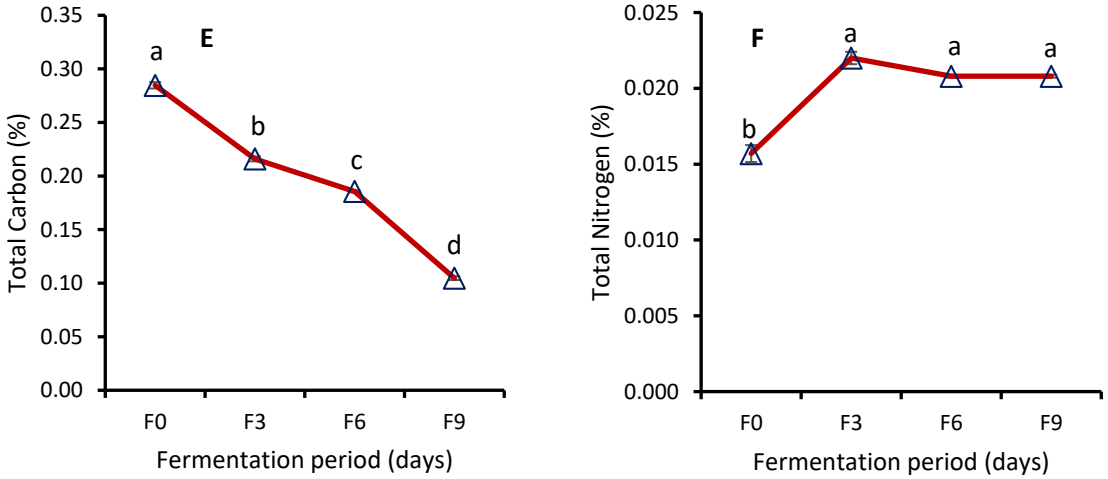

Figure 7. Means (±SE) of (**A**) pH, (**B**) electrical conductivity, (**C**) NH_4^+, (**D**) NO_3^-, (**E**) total carbon, and (**F**) total nitrogen content of WRW as influenced by fermentation periods. Within the same chart, means with the same letters are not significantly different from one another at the 5% level using HSD.

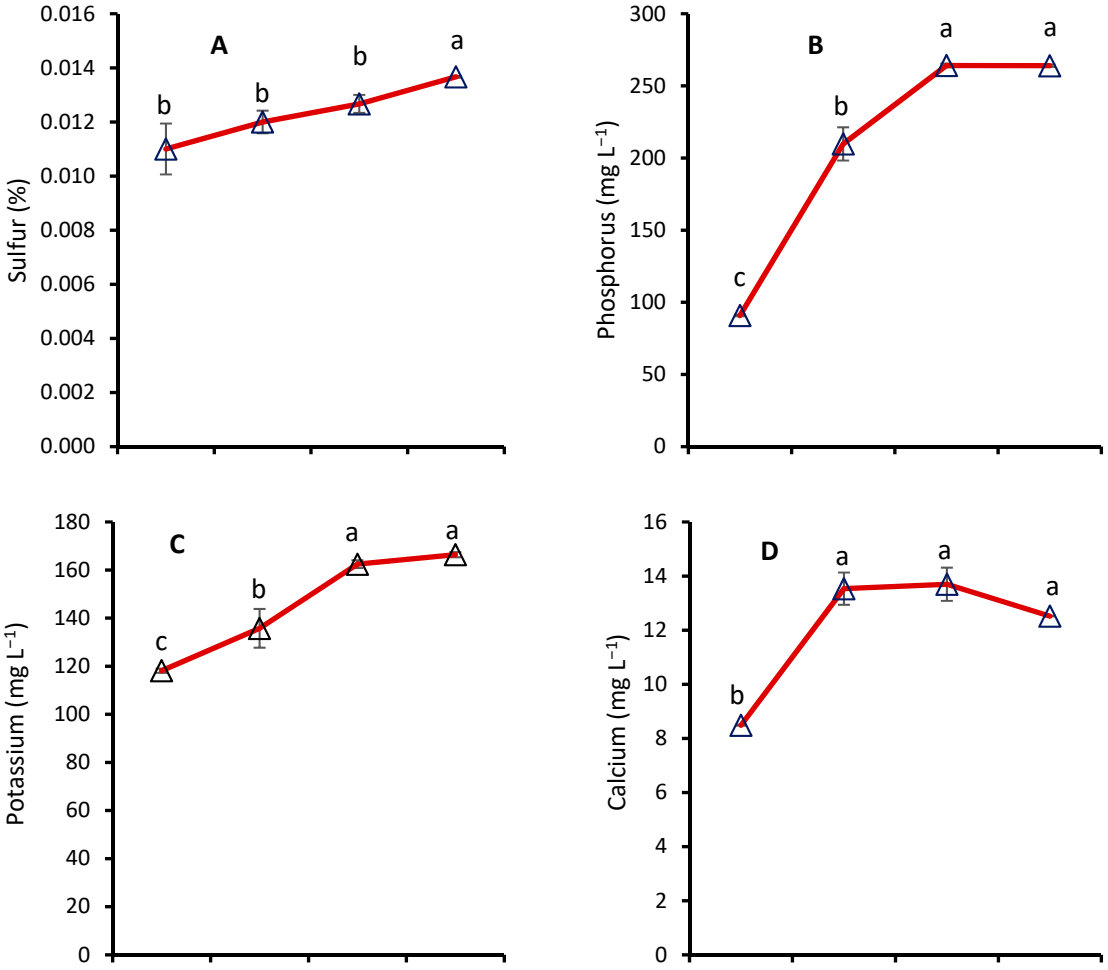

Figure 8. *Cont.*

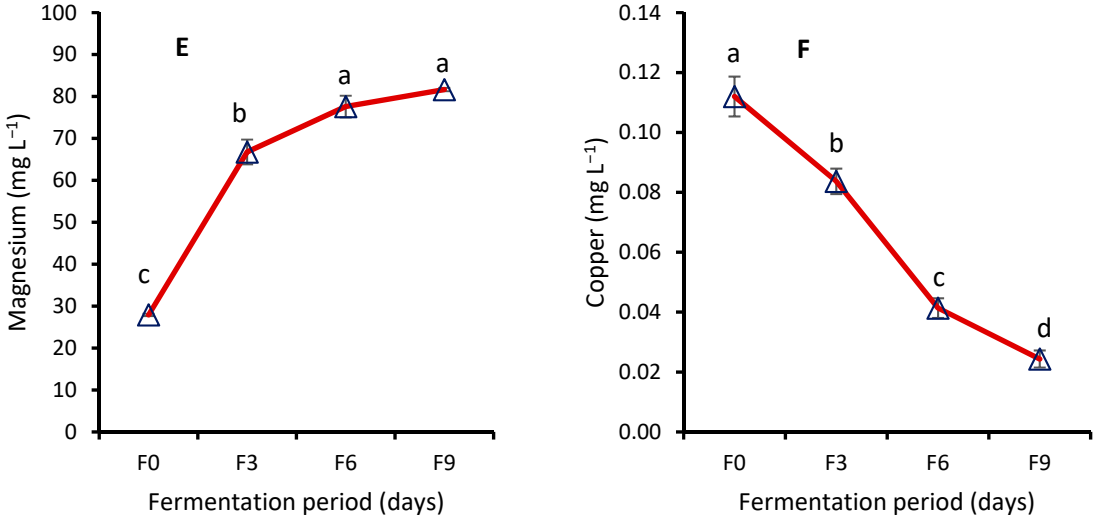

Figure 8. Means (±SE) of (**A**) sulfur, (**B**) phosphorus, (**C**) potassium, (**D**) calcium, (**E**) magnesium, and (**F**) copper content of WRW as influenced by fermentation periods. Within the same chart, means with the same letters are not significantly different at the 5% level using HSD.

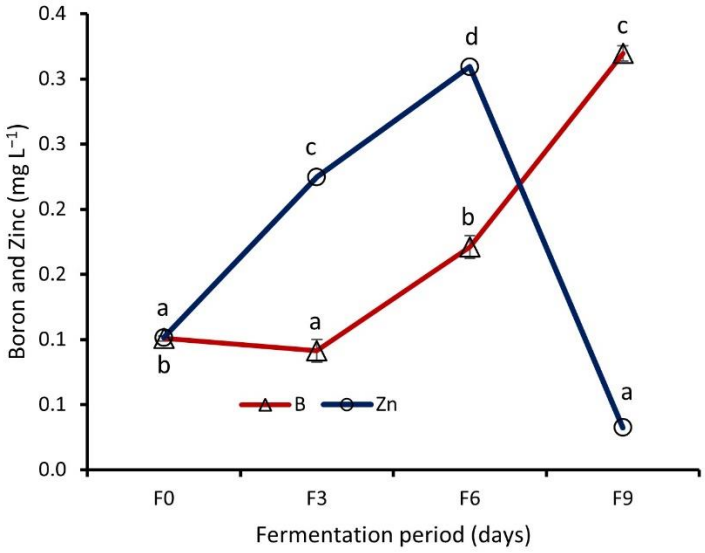

Figure 9. Means (±SE) of zinc, and boron content of WRW as influenced by fermentation periods. Within the same chart, means with the same letters are not significantly different at the 5% level using HSD.

4. Discussion

4.1. Effect of Fermentation on Bacterial Population and Biochemical Tests of WRW

Fermentation introduced bacteria into WRW, and bacterial population increased immediately after fermentation until F3, after which the bacterial population declined because the bacteria had started to deplete the C in the WRW (Figure 7E). Microorganisms from WRW used C as an energy source, which led to its reduction with time [29]. C decreased by 63% from F0 to F9. This indicates insufficiency of C in the WRW to sustain or increase the bacterial population after F3. The bacterial population at all the fermentation (except at F0) are greater than the minimum population level expected for maximum microbial colonization (1×10^6 CFU mL^{-1}) [14]. Similarly, the decrease in pH with fermentation corresponded to the decrease in the total bacterial population (Figure 1A). The decreased in the total bacterial population could be attributed to the decline in pH

because most bacteria thrive at a relatively higher pH (4–8) relative to fungi [30]. The pH reduction was because of the production of organic acids by the microbes in WRW [31,32].

Greater P solubilization at F3 was because of more P-solubilizing bacteria present, which decreased with time because of the competition for food as the C content decreased with time. Greater pH at i6 incubation days irrespective of the fermentation period could indicate lower microbial activity, which led to lower P solubilization compared to the pH of incubation at i12 (Figure 2A). Panhwar et al. [33] reported that microbes release organic acids (metabolites), leading to a reduced media pH and the solubilization of the insoluble tricalcium phosphate ($Ca_3(PO_4)_2$). The decrease in culture pH is directly proportional to the inorganic phosphate solubilization [34], which was attributed to the organic acids and acid phosphatases production by the microbes [35,36]. A similar conclusion with the above reasons was reached in this study. Using fermented WRW could reduce the need for inorganic fertilizer, as the WRW contained microbes that can transform the insoluble P into plant-available P.

Similarly, greater K solubilization at F3 was because of the presence of more K solubilizing bacteria, as indicated by the bacterial population (Figure 1A). Therefore, the lower bacterial population in F0 explained the lowest K solubilization recorded. *Bacillus* and *Enterobacter* species are classified as beneficial plant bacteria that could solubilize P and K [13,28,37,38]; therefore, the K solubilization recorded is because of the presence of these PGPB in the fermented WRW. Potassium solubilization rates for the fermented WRW are within the range of isolates used by Tan et al. [13], which solubilized 10.7 to 14.15 mg L^{-1} after 5 days of incubation. Conversely, the solubilized K (for F0, F3, F6, and F9) in this study were greater than the 4.29 mg L^{-1} solubilized by *Bacillus mucilaginous* MCRCp1 as reported by Sugumaran and Janarthanum [39] after 4 days of incubation. Similarly, our results agree with many studies [13,28,40] that reported an increase in P and K solubilization with a consequent decrease in pH of the culture as the incubation days increased. The increased release of potassium from muscovite is associated with the production of acids, alkalis, or chelates by the bacterial isolates [13].

The greater ethylene production at F3 is because of more N-fixing bacteria being present compared to other fermentation periods (Table 2). The ability to fix N is an essential basis for characterization because it is crucial to the plants as a potential alternative to applying chemical N fertilizer. Diazotrophs can convert atmospheric nitrogen into ammonium, which plants absorb via the biological N fixation process [41]. PGPR and rhizobia play more important roles, particularly in providing plants with nutrients in less fertilized soils [13]. The ethylene produced by the fermented WRW levels are within those reported by Tan et al. [13], ranging from 2.1 to 11.2 Nmol C_2H_4 mL^{-1} h^{-1}, but much higher than those reported by Katupitiya [42] and Naher [43], with 2.3 nmol C_2H_4 $plant^{-1}$ h^{-1} from *Azosprillum* inoculation and 6.1 × 10^{-8} to 1.2 × 10^{-3} Nmol C_2H_4 cfu^{-1} h^{-1} from a diazotroph isolated from rice.

4.2. Effect of Fermentation on the Indole Acetic Acid Production

The increase in IAA in F3 relative to the F6 and F9 was because of the greater bacterial diversity compared to other fermentation periods (Table 2), which indicates more IAA-producing microbes. In this study, the production of IAA by the WRW culture and isolates at different fermentation period (Tables 1 and 2), particularly at F3 and F6, fall within the IAA range produced by the selected isolates of *Bacillus* spp. from rhizospheric soils of different crops, which ranged from 7.03 to 22.02 μg mL^{-1} [44]. Using the same concentration of tryptophan as the precursor (1 mL/20 mL broth), Ng et al. [45] found that *Enterobacter gergoviae* and *Bacillus amyloliquefaciens* produced IAA in the of range of 3.56–24 μg mL^{-1}. IAA act as a phytohormone to regulate plant growth and development, which leads to a persistent search for IAA-producing microbes for a potential application in promoting plant growth [46]. *Enterobacter* sp. was found to have produced up to 200 mg L^{-1} of IAA using 1-tryptophan in NB medium [46]. The IAA production is vital in plant root elongation and root hair development [13]. Several IAA-producing bacteria

were selected based on their IAA-producing capabilities, which showed increased rice plant shoot and root length [13].

4.3. Phylogenetic Analysis of the Isolated Microbes

The phylogenetic tree showed there are two main clusters, with *Bacillus velezensis* and *Stenotrophomonas maltophilia* as one group, and the second group composed of genus of *Enterobacter* spp., *Pantoea agglomerans*, and *Klebsiella pneumoniae*. The identified *Bacillus* species have greater similarities (99%) with *Bacillus* species of *siamensis* and *subtilis*. According to the phylogenetic tree, the identified microorganisms were all grouped into their reference organisms with greater similarities. Phylogenetic analysis and phenotypic tests identified strains as members of the genus *Bacillus* and closely related to *Bacillus subtilis* and *Bacillus amyloliquefaciens* [47]. Therefore, *Bacillus velezensis* plantarum was synonymized with *Bacillus subtilis* and *Bacillus siamensis*, due to the substantial phenotypic and genotypic coherence of the taxa [48]. Fan et al. [49] reported that strains of *Bacillus* could also be found in fermented foods and fermented plants in addition to soils. *Bacillus subtilis* has been used as a biocontrol agent in many crops by inducing systemic resistance (ISR) in plants for pests and pathogens [50–52].

4.4. Effect of Fermentation on Nutrient Contents of WRW

The WRW nutrient analyses show that washing rice led to the removal of nutrients into the WRW (Table 3). The decrease in the total carbon is because of the microbial activity, which similarly decreased with time (Figure 1). Conversely, the reduction in total carbon corresponded to an increase in the total nitrogen, NH_4^+, and NO_3^- due to the mineralization of the WRW by the microbes. The increase in NH_4^+ and NO_3^-. with fermentation could be due to the ammonification and nitrification by the microbes present in the WRW, which led to a reduction in total N and total C because of their use by the microbes as a source of energy. The results indicated that the nutrient contents increase with fermentation, as they were higher at F9.

The increase in P, K, Ca, and Mg with fermentation is because of the presence of N-fixing and P- and K-solubilizing bacteria in the fermented WRW. More elements were recorded in the fermented WRW relative to the F0 (unfermented) because the bacterial population increased with fermentation, indicating the presence of more N-fixing and P- and K-solubilizing bacteria. The results agree with Nabayi et al. [28], who reported an increase in S, P, K, Mg, NH_4^+, and NO_3^- elements with a consequent decrease in C content as the fermentation period progressed. The increase in P, K, Ca, Mg, Zn, NH_4^+, and NO_3^- with fermentation of WRW agrees with Nkhata et al. [32], who reported increased P, Ca, Mg, Zn, and Cu as cereals were mineralized by bacteria. Similarly, the results agree with several studies [53–55] on cereals fermentation that reported increase in P, Ca, Mg, and Zn with increase in the fermentation period, mainly due to the loss of dry matter as the microbes mineralize carbohydrate and protein, leading to the availability of elements.

5. Conclusions and Recommendations

Fermented WRW after 3 days had a greater bacterial population of 2.12×10^8 which decreased with a longer fermentation period. The greater bacterial population led to more IAA, N fixation, and P and K solubilization, which increased by 13.2–85.5%, 9.4–83.3%, 22.4–84.1%, and 21.4–83.6%, respectively, compared to other fermentation periods. Similarly, the study showed an increase in nutrients with an increase in fermentation, indicating the presence of plant beneficial microorganisms such as N-fixing and P- and K-solubilizing bacteria in WRW. The isolation and identification showed the presence of *Bacillus subtilis* and *Enterobacter* spp., among others, that are N-fixing and P- and K-solubilizing microorganisms, and biocontrol agents. Fermenting WRW for 3 days produced greater diversity of beneficial microbes. This study showed that WRW, rather than being discarded, can be reused, as the nutrient and microbial analyses showed the presence of nutrients and beneficial bacterial strains that could promote plant growth and yield and

soil fertility. Therefore, it is recommended that WRW should be fermented for 3 days before its use as plant fertilizer for more beneficial microorganisms and plant nutrients.

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