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Chemical and Microbial Characterization of Washed Rice Water Waste to Assess Its Potential as Plant Fertilizer and for Increasing Soil Health

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Abstract: The wastewater from washed rice water (WRW) is often recommended as a source of plant nutrients in most Asian countries, even though most current research on WRW lack scientific rigor, particularly on the effects of rice washing intensity, volumetric water-to-rice ratio (W:R), and condition of the WRW before plant application. This research was thus carried out: (1) to determine how various rice washing intensities, fermentation periods (FP), and W:R would affect the nutrient content in WRW, and (2) to isolate, identify, and characterize the bacterial community from fermented WRW. The WRW was prepared at several rice washing intensities (50, 80, and 100 rpm), FP (0, 3, 6, and 9 days), and W:R (1:1, 3:1, and 6:1). The concentrations of all elements (except P, Mg, and Zn) and available N forms increased with increasing FP and W:R. Beneficial N-fixing and P- and K-solubilizing bacteria were additionally detected in WRW, which helped to increase the concentrations of these elements. Monovalent nutrients NH_4^+-N, NO_3^-−N, and K are soluble in water. Thus, they were easily leached out of the rice grains and why their concentrations increased with W:R. The bacteria population in WRW increased until 3 days of fermentation, then declined, possibly because there was an insufficient C content in WRW to be a source of energy for bacteria to support their prolonged growth. While C levels in WRW declined over time, total N levels increased then decreased after 3 days, where the latter was most possibly due to the denitrification and ammonification process, which had led to the increase in NH_4^+-N and NO_3^-−N. The optimum FP and W:R for high nutrient concentrations and bacterial population were found to be 3 to 9 days and 3:1 to 6:1, respectively. WRW contained nutrients and beneficial bacterial species to support plant growth.

Keywords: bacteria; fermentation; water to rice ratio; nutrients contents; wash rice water; soil amendments

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1. Introduction

It is often claimed, but without strong scientific evidence, that washed rice water waste is a beneficial plant fertilizer and soil amendment. Milled rice is very often washed prior to cooking to remove the bran, dust, and dirt [1]. However, rice washing can also remove a significant amount of water-soluble nutrients from the rice grains, and the water after rice washing is often simply discarded into the environment. This discarded wastewater is called washed rice water (WRW), and this wastewater has been found to contain several essential plant nutrients, such as (in mg L^{-1}) 40 to 150 of N, 4.19 to 10.14 NO_3^-−N, 2.57 to 39.72 NH_4^+-N, 43 to 1630 P, 51 to 200 K, 8 to 2944 Ca, 36 to 1425 Mg, and 27 to 212 S [1–6].

In addition, several studies have shown that plant watering with WRW had increased the height, stem diameter, and yield of tomato, water spinach, eggplants, pak choy, lettuce, mushroom, adenium, chilli, and mustard green plants [7–14]. Furthermore, WRW was

found to contain plant growth-promoting bacteria (PGPB), such as *Bacillus* and *Lactobacillus* spp. [11]. Their presence in WRW is particularly noteworthy because these bacteria can inhibit plant pathogens, produce phytohormones and siderophores, solubilize potassium and phosphate, and fix nitrogen [15,16].

Unfortunately, nearly all WRW studies come from gray literature. Most of these WRW studies either lacked scientific rigor or lacked essential information or additional measurements needed to explain their experimental observations. There is also a dearth of WRW studies. Nabayi et al. [5] found only 41 papers on the reuse of WRW specifically for agriculture, and of this total, only 10% of them were published in indexed journals, and the rest, either in non-indexed journals (61%) or undergraduate research reports (29%).

Consequently, the benefits of WRW remain inconclusive. Nevertheless, the advocacy for its reuse for irrigation and liquid plant fertilizer remains popular, particularly in Asia. In Indonesia, for instance, the village of Polo Geulis in Central Bogor practices a centralized water-saving system where WRW is collected from the village citizens, after which the water is used to irrigate and fertilize their neighborhood crops of herbs and vegetables [17]. A similar WRW-reuse communal program is also implemented, as part of the local government program, in the village of Lambangkuning, Indonesia [18].

WRW is wastewater, and like any other wastewater, it ought to be reused as part of water governance. Large amounts of WRW are produced, as rice, the second most widely grown cereal in the world is eaten by nearly half of the world's population [19]. Between 2020 and 2021, about 504 million tons of rice were consumed worldwide [20], and the figure is expected to increase with population growth. Even with a conservative estimate of using only 1 L of water to wash every 1 kg of rice grains, this would work out to at least 504 billion L of WRW being produced within this period. Furthermore, global freshwater demand is expected to increase by 55% by 2050 [21]. This increase is mainly due to detrimental climate change and increasing world population, driving WWAP [22] to advocate more effective water governance so that wastewater, rather than just being discarded into the environment, is instead reused, treated, or recycled. The AQUASTAT database of the Food and Agriculture Organization of the United Nations (FAO) additionally estimated that more than half of the global freshwater withdrawals are simply discarded as wastewater into the environment [22]. Municipal water demand, in particular, corresponds to 11% of the global freshwater withdrawal, but out of this, only 3% is consumed, with the remaining 8% simply discarded, unused, as wastewater.

But if WRW is to be advocated for reuse for agriculture, its benefits must be shown in at least two stages: first, that the liquid WRW itself has the properties and nutrient levels that are beneficial to plant growth, and second, that the application of WRW will increase the crop growth and yield in both the short- and long-term, as well as improve the biological and physicochemical soil properties [5].

This paper addressed the first stage; that is, to characterize the chemical and biological properties of the liquid WRW to assess its use as plant fertilizer and for promoting soil health. First, this study measured both the macro and micronutrients in WRW, as well as the plant-available N forms of NH_4^+-N and NO_3^-−N (typically, only the macronutrients are analyzed by other studies). Second, this paper determined how washing rice with several volumes of water and washing intensities would affect the chemical properties and nutrient content in WRW. Third, WRW will ferment over time, so this study additionally determined whether fermentation would lead to higher nutrient content and higher microbial count in WRW, as well as encourage the presence of beneficial bacteria, particularly the N-fixing and P- and K-solubilizing bacteria. That WRW could promote beneficial soil bacteria would increase WRW's worth as a natural fertilizer and soil amendment. The knowledge obtained from this study will provide basic information on the potential use of WRW in agriculture, and perhaps lead to follow-up studies that will rigorously evaluate the benefits of plant watering with WRW on crop growth and yield.

2. Materials and Methods

2.1. Chemicals and Media

All the chemicals and microbiological media utilized in the experiment were of analytical grade. Nutrient agar and nutrient broth were purchased from Merck (Darmstadt, Germany) and supplied via Sigma-Aldrich (Selangor, Malaysia).

2.2. Sample Preparation

The rice brand used was 'Rambutan' (Padiberas Nasional Berhad, Malaysia), which is a commercially available medium-grained rice in Malaysia. The WRW was prepared in a volumetric water-to-rice (W:R) ratio of 1:1, 3:1, and 6:1. The mixture was obtained using a stand mixer (Bossman Kaden matte BK-100S, Tokyo, Japan) and at three (3) different washing intensity of 50 (0.139 g Force), 80 (0.357 g Force), and 100 (0.559 g Force) rpm at a constant time of 90 s. The mixture (rice grains and water) was then separated using sieves (500-micron sizes). For the fermented batches, the same water was kept at room temperature in a container for periods of either 3, 6, or 9 days for fermentation before use. After every selected fermentation cycle, the fermented water was subjected to chemical analyses.

2.3. Chemical Analyses

The total C, N, and S content of the rice samples were determined using CNS analyzer (LECO Corp., St. Joseph, MI, USA); and P, K, Ca, Mg, Cu, Zn, and B were analyzed using graphite furnace atomization atomic absorption spectrophotometer (AAS) (Perkin Elmer, PinAAcle, 900T, Waltham, MI, USA) after dry ashing the rice samples following Nelson and Sommers [23], where 1 g of the oven-dried (at 105 °C) ground rice grains were used. The samples were put into a muffle furnace and subjected to a series of temperatures from 200 °C, to 550 °C, for 6 hrs where complete ash was obtained for further assays. Samples from the WRW at different speeds, fermentation periods, and W:R ratios were filtered through a Whatman 1 filter paper (11 µm size) and analyzed for pH, EC, total N, nitrate, ammonia, C, S, P, K, Ca, Mg, Cu, Zn, and B. Total N, C, and S were analyzed using a CNS analyzer (LECO Corp., St. Joseph, MI, USA); and P, K, Ca, Mg, Cu, Zn, and B were analyzed using atomic absorption spectrophotometer (AAS) (Perkin Elmer, PinAAcle, 900T, Waltham, MI, USA). The detection and quantification limits of the AAS were 0.01–1 ng mL^{-1} and 4 nM, respectively. Ammonium and nitrate were determined by the Kjeldahl procedure [23]. pH and EC were measured using the 827 pH and EC lab meter (Metrohm AG, Zurich, Switzerland) [24]. The different batches of the WRW were also subjected to bacterial population, isolation, and characterization.

2.4. Culture Media and Bacterial Growth

Tryptic soy agar (TSA) was used for the bacterial population growth of the various fermented washed rice water following Tan et al. [25]. The bacterial growth was counted from each fermented WRW type (in triplicates) to determine the bacterial population. Each plate with a range of 30 to 300 colonies was selected and counted as colony-forming units (CFU) per mL of the sample [26].

2.5. Bacterial Isolations

Different bacteria were isolated from different samples (of different fermentation periods) based on shape, color, and sizes following the bacterial growth and population count. The isolates were sub-cultured several times to obtain the pure colony, which was subsequently subjected to a series of tests. The isolated bacteria were grouped based on fermentation period irrespective of the W:R ratio and washing intensity used.

2.6. N_2 Fixation, Phosphate Solubilization, and Potassium Solubilization

The qualitative N_2 fixation ability was ascertained by growing the isolates on Nfb medium (N-free solid malate medium) following [27]. While phosphate and potassium

solubilizing ability of the isolated microbes were determined using Pikovskaya [28] and Aleksandrov agar media [29], respectively.

2.7. Bacterial Identification Using 16S rRNA Gene Sequence

The selected isolates were identified by partial sequencing of the 16S rRNA gene. Genomic DNA was isolated from the (WRW) bacterial culture by using the Genomic DNA Mini Kit (Favorgen) (Pingtung Agricultural Biotechnology Park, Pingtung, Taiwan). 16S rRNA gene was amplified using universal forward 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers (Apical Science Sdn. Bhd. Selangor, Malaysia). 30 µL reaction mixture was prepared, each containing 2 µL of DNA template, 15 µL of Master mix (containing 10× PCR Reaction Buffer, dNTPs mix, Taq polymerase, $MgCl_2$ and ultra-pure water), 10 µL of Nuclease free water, 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) with cycles as follows: 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) (Pingtung Agricultural Biotechnology Park, Pingtung, Taiwan) 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, Maryland, WA, USA).

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 10 November 2021) on 15 December, 2020 under accession numbers; MW365555.1 (*Enterobacter ludwigii*), MW365556.1 (*Enterobacter* sp.), MW365557.1 (*Enterobacter* sp.), MW365558.1 (*Enterobacter mori*), MW365561.1 (*Enterobacter* sp.), MW365562.1 (*Enterobacter mori*), MW365564.1 (*Pantoea agglomerans*), MW365565.1 (*Stenotrophomonas maltophilia*), MW365560.1 (*Stenotrophomonas maltophilia*), MW365563.1 (*Klebsiella pneumoniae*), MW365554.1 (*Bacillus velezensis*), MW365559.1 (*Bacillus velezensis*).

2.8. Phylogenetic Analysis

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. The similarities between the nucleotide sequences were computed using Hasegawa–Kishino–Yano model [30]. All positions containing gaps and missing data were eliminated from the dataset. The reference sequences were downloaded in FASTA format from the NCBI database, and a phylogenetic tree was constructed by the Maximum Likelihood method using MEGA7 software [31]. Tree topologies were evaluated by performing bootstrap analyzes using 1000 replications.

2.9. Data Analysis

Completely Randomized Design (CRD) in a factorial arrangement was used in the analysis of the WRW nutrient content study. A two-factor analysis between rice washing speed and W:R ratio was carried out to assess their effect on 0-day WRW (unfermented WRW). To include the effect of the fermentation period (3, 6, and 9 days), three-factor analysis was carried out with speed and W:R ratio factorially. All data were analyzed by analysis of variance (ANOVA) using R software package (version 4.1.1). Means were separated by the Tukey HSD test procedure at a threshold significance level of 5%. A uniform manifold approximation and projection (UMAP) analysis and interaction trends were further carried out on the measured chemical variables using the R-studio interface (version 1.4.1717) using the 'uwot' package (version 0.1.10) to provide additional meaningful information to the mean separation test [32].

3. Results

3.1. Chemical Properties and Elemental Concentrations in WRW

Washing rice at different speeds and various water:rice volumetric ratios (W:R) had leached out between 1.4 to 35.2% of all nutrients, with most of these losses from S (35.2%), NO_3^- −N (14.5%), and K (11.1%), while the least element leached was Zn with 1.4% (Table 1). Specifically, washing rice lost nutrients by the following percentages: 35.2 S, 14.5 NO_3^- −N, 11.1 K, 10.0 Mg, 9.7 C, 8.6 B, 8.1 NH_4^+-N, 4.9 P, 3.6 Cu, 3.3 Ca, 2.4 total N, and 1.4 Zn.

The unfermented WRW contained plant-available N forms, NH_4^+-N and NO_3^- −N, at 24.1 and 19.9%, respectively, of its total N. As expected, tap water contained very little nutrients, as this was the municipal tap water that had been treated for safe human drinking and use, according to the National Water Standards [33].

Only the main effect of W:R was significant ($p < 0.01$) on the pH, C, C:N, NH_4^+-N, NO_3^- −N, Ca, and S (Table 2a). Washing rice with the highest water volume (6:1) leached out the most C, available N (NH_4^+-N and NO_3^- −N), Ca, and S, as well as producing the least change in pH. This was generally followed by W:R of 3:1. Interaction between washing intensity and W:R was significant on EC and the concentrations of total N, P, K, Mg, Cu, and Zn (Table 2b). Averaging across all washing intensities revealed that increasing W:R from 1:1 to 6:1 resulted in lower EC from 72 to 57.5% and lower concentrations in P, Mg, and Zn from 80 to 41, 62.8 to 46.9, and 87.5 to 58.3%, respectively. However, for K and Cu, their concentrations increased from 7.2 to 14.2% and 44 to 53%, respectively.

Table 1. Means (± SE) element analyzes of medium-grained rice and the tap water used for washing the rice.

Parameters	Rice Grain	WRW *	Tap Water †
pH	–	6.53 ± 0.02	6.58 ± 0.02
EC (μS cm^{-1})	–	372.83 ± 34.53	125.36 ± 28.21
Ash (%)	0.95 ± 0.04	–	–
TOC (%)	30.30 ± 0.21	2.64 ± 0.72	Trace
Moisture (%)	14.39 ± 0.06	99.32 ± 0.31	–
Total C (%)	40.30 ± 0.01	3.87 ± 0.24	0.03 ± 0.002
Total N (mg kg^{-1})	12,500 ± 100.70	80.50 ± 5.20	30.20 ± 4.12
NH_4^+-N (mg kg^{-1})	215.45 ± 4.41	18.88 ± 1.68	1.44 ± 0.04
NO_3^- −N (mg kg^{-1})	100.82 ± 8.53	16.02 ± 1.41	1.45 ± 0.03
C:N	32.24 ± 0.02	48.3 ± 5.64	0.50 ± 0.001
S (mg kg^{-1})	1000 ± 38.12	452 ± 62.15	100 ± 9.64
P (mg kg^{-1})	1320.83 ± 34.04	64.64 ± 5.76	0.05 ± 0.02
K (mg kg^{-1})	1130.83 ± 22.64	130.66 ± 2.55	5.74 ± 0.15
Ca (mg kg^{-1})	427.08 ± 5.72	23.97 ± 2.68	10.95 ± 0.06
Mg (mg kg^{-1})	244.93 ± 10.26	25.23 ± 1.78	0.97 ± 0.06
Cu (μg kg^{-1})	5250 ± 120.40	188.52 ± 11.50	2.4 ± 1.02
Zn (μg kg^{-1})	5020 ± 97.04	73.77 ± 7.73	5.3 ± 1.30
B (μg kg^{-1})	1400 ± 96.45	121.18 ± 23.93	1.2 ± 1.02

Note: All % for rice grains are based on dry weight basis;—not determined; † measured in mg L^{-1}; * unfermented washed rice water averaged across all washing intensities (50, 80, and 100 rpm) and volumetric water-to-rice ratios (W:R) (1:1, 3:1, and 6:1).

Note that Table 2 is only for unfermented WRW. Our full data set involved three W:R levels (1:1, 3:1, and 6:1), three washing intensities (50, 80, and 100 rpm), and four fermentation periods (0-day, 3-day, 6-day, and 9-day, with 0-day as the unfermented WRW). These many levels of factors and their various combinations had resulted in very intricate and unclear trends (3-way ANOVA results not shown). Consequently, a data visualization technique known as Uniform Manifold Approximation and Projection (UMAP) [34] was used to determine the influence of W:R, washing intensity, and fermentation on the chemical properties of WRW (Figure 1). This technique was much more effective in revealing trends. UMAP works similarly to Principle Component Analysis (PCA) in reducing large datasets by representing the data with fewer components or factors. Unlike PCA, however, UMAP does not assume data linearity.

Table 2. Chemical properties of unfermented washed rice water (WRW) due to the: (**a**) main effect W:R and (**b**) interaction effect between washing intensity (R) and volumetric water-to-rice ratio (W:R).

(a)								
W:R		**pH**	**C**	**C:N**	**NH_4^+-N**	**NO_3^-−N**	**Ca**	**S**
			%			mg kg^{-1}		
1:1		6.48b	2.48c	35.17b	9.94c	9.78b	9.05c	110.68c
3:1		6.47b	3.76b	38.68b	17.74b	14.94b	21.02b	449.33b
6:1		6.63a	5.37a	68.05a	28.95a	23.35a	41.84a	797.13a
SE (±)		0.04	0.13	5.35	1.23	1.48	0.55	49.80
(b)								
Washing Intensity (R)	**W:R**	**EC**	**TN**	**P**	**K**	**Mg**	**Cu**	**Zn**
rpm		μS cm^{-1}				mg kg^{-1}		
100	1:1	607.0b	115.0a	98.0a	129.7c	35.4a	0.111e	0.105ab
80		551.3c	67.0bc	90.8ab	118.1de	34.9a	0.112e	0.101ab
50		671.7a	56.6c	93.3ab	117.5de	36.5a	0.133de	0.144a
100	3:1	332.0d	101.0ab	82.0b	141.9b	25.7bc	0.243a	0.060bcd
80		291.1d	93.0ab	57.7c	123.8cd	19.3cd	0.193bc	0.060bcd
50		283.4d	100.6ab	79.2b	139.6b	30.0ab	0.161cd	0.089bc
100	6:1	223.5e	50.0c	19.6e	112.9e	13.5d	0.244a	0.046cd
80		212.7e	47.0c	35.4d	153.1a	17.2d	0.260a	0.040d
50		182.7e	35.4c	25.3de	138.9b	14.2d	0.240ab	0.018d
W:R		***	***	ns	*	ns	*	ns
R		***	***	***	***	***	***	***
R × (W:R)		***	***	**	***	**	***	*
SE (±)		10.90	7.14	3.13	1.76	1.47	0.0098	0.0095

Within the same column, means with the same letters are not significantly different from one another according to the Tukey test ($p > 0.05$). *** significant at 0.1%, ** significant at 1%, * significant at 5%.

UMAP shows that W:R and fermentation largely explained the data variance, with washing intensity having little or no impact on explaining the data variance (Figure 1). The UMAP revealed there were three main clusters, distinguished by the three W:R of 1:1 (solid line), 3:1 (long dashed line), and 6:1 (dotted line). Additionally, within each of these clusters, there were further four subclusters that were distinguished by the four fermentation groups: 0d (⊗ marker), 3d (●), 6d (□), and 9d (▲), where 0d, 3d, 6d and 9d denote fermentation at 0 (unfermented), 3, 6, and 9 days, respectively.

Longer WRW fermentation led to higher levels of NH_4^+-N, NO_3^-−N, P, K, Ca, Mg, and Zn in the WRW (Figure 2). Longer WRW fermentation also increased EC (Figure 2) but decreased pH (Figure 3). Unlike other nutrients, longer WRW fermentation lowered Cu levels (Figure 3). C levels also generally declined with increased fermentation. How W:R affected the nutrient levels depending on the nutrient type. Generally, higher W:R ratios led to higher levels in WRW for NH_4^+-N, NO_3^-−N, K, Ca, C, Cu, and S but lower levels for P, Mg, Zn, and EC.

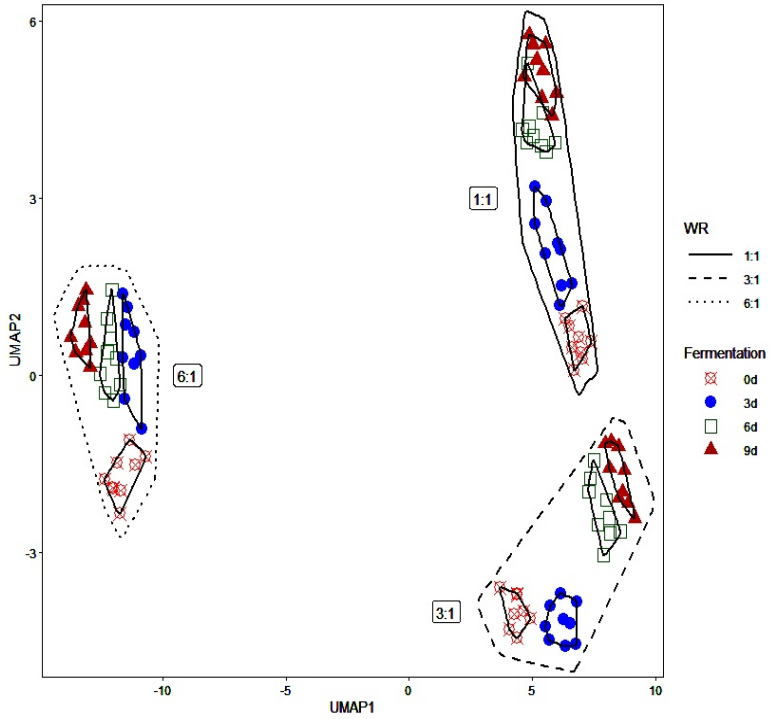

Figure 1. Uniform manifold approximation and projection (UMAP) analysis of fermentation, washing intensity, and volumetric water-to-rice ratio (W:R) on the measured chemical properties and nutrient content in washed rice water (WRW). The variance in data was mostly explained by W:R and fermentation, with little or no contribution by the washing intensity factor. Note: 1:1, 3:1, and 6:1 denote W:R.

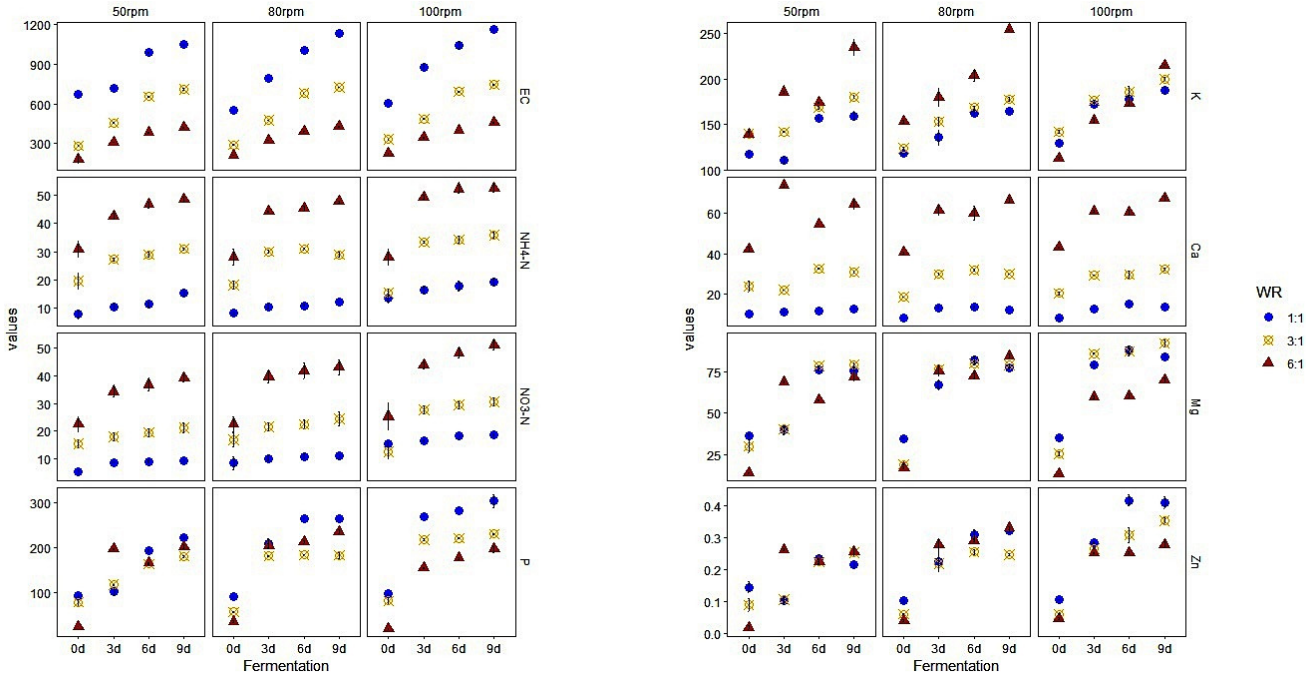

Figure 2. Interaction between washing intensity, volumetric water-to-rice ratio (W:R), and fermentation on the means (±SE) of EC, NO_3^-−N, 2.57 to 39.72 NH_4^+-N, P, K, Ca, Mg, and Zn of WRW. 0d, 3d, 6d, and 9d are the fermentation periods for 0-, 3-, 6-, and 9-day, respectively, and 1:1, 3:1, and 6:1 are the water-to-rice volume ratios. Except for EC (which is in μS cm^{-1}), all units are in mg kg^{-1}.

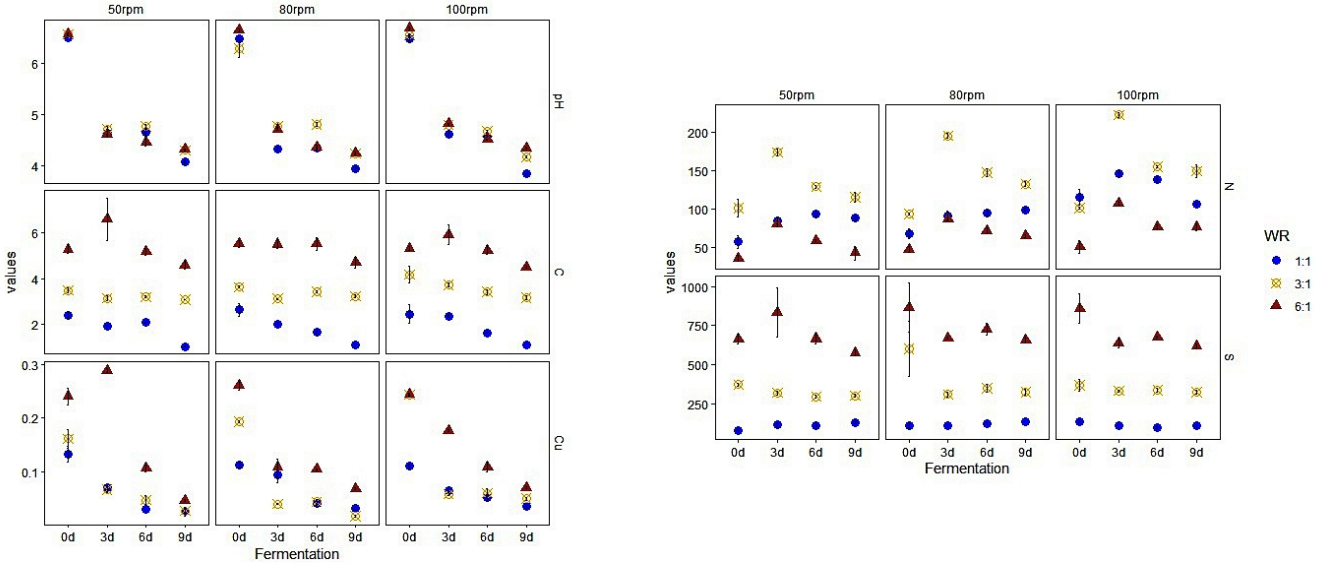

Figure 3. Interaction trend between washing intensity, volumetric water-to-rice ratio (W:R), and fermentation period on the means (±SE) of pH, C, Cu, N, and S content of WRW. 0d, 3d, 6d, and 9d are the fermentation periods for 0-, 3-, 6-, and 9-day, respectively, and 1:1, 3:1, and 6:1 are the W:R ratios. Except for C (%) and pH, all units of the elements are in mg kg^{-1}.

Figures 2 and 3 reveal that washing intensity had little to no effect on the nutrient levels, agreeing with the earlier UMAP analysis (Figure 1) and that data variance was mostly explained only by W:R and fermentation factors. One exception was total N (Figure 3). Total N levels peaked at 3d of fermentation, then declined thereafter, and total N level was the highest for 3:1 W:R. This was exceptional because the levels of other nutrients either increased or decreased with increasing W:R or fermentation period.

3.2. Bacterial Population and Identification of the WRW

The bacterial population declined with increasing W:R (Figure 4). The bacterial population also peaked at 3 days of fermentation, then declined thereafter. Washing rice at either 80 or 100 rpm had a similar effect on the bacterial population (Figure 4a,b), but both of them produced a higher bacteria population than at the 50 rpm (Figure 4c).

Based on 16S rRNA gene sequencing (Table 3), the 12 total strains found were identified under several genera of *Bacillus, Enterobacter, Pantoea, Klebsiella* and *Stenotrophomonas*. The phylogenetic tree of the identified microbes was clustered into clades to their respective genus and species, as shown in Figure 5. It is worth noting that at 0-day fermentation, only *Enterobacter* sp. strain was found in the WRW. However, after further fermentation, in the 3-day fermentation, five different strains were found: *Bacillus velezensis*, *Enterobacter ludwigii*, *Enterobacter* sp., *Klebsiella pneumoniae* and *Pantoea agglomerans.* Similarly, *Enterobacter mori*, *Bacillus velezensis* and *Stenotrophomonas maltophilia* were isolated from a 6-day fermentation, while *Enterobacter* sp., *Enterobacter mori* and *Stenotrophomonas maltophilia* were isolated from the 9-day fermentation. Interestingly, *Enterobacter* spp. was the common strain found irrespective of the fermentation period (Table 3). However, *Pantoea agglomerans and Klebsiella pneumoniae* were unique to only 3-day fermentation, similar to the presence of only *Stenotrophomonas maltophilia* in 6-day and 9-day fermentation. In addition, the presence of *Bacillus velezensis* was only found in the 3-day and 6-day fermentation.

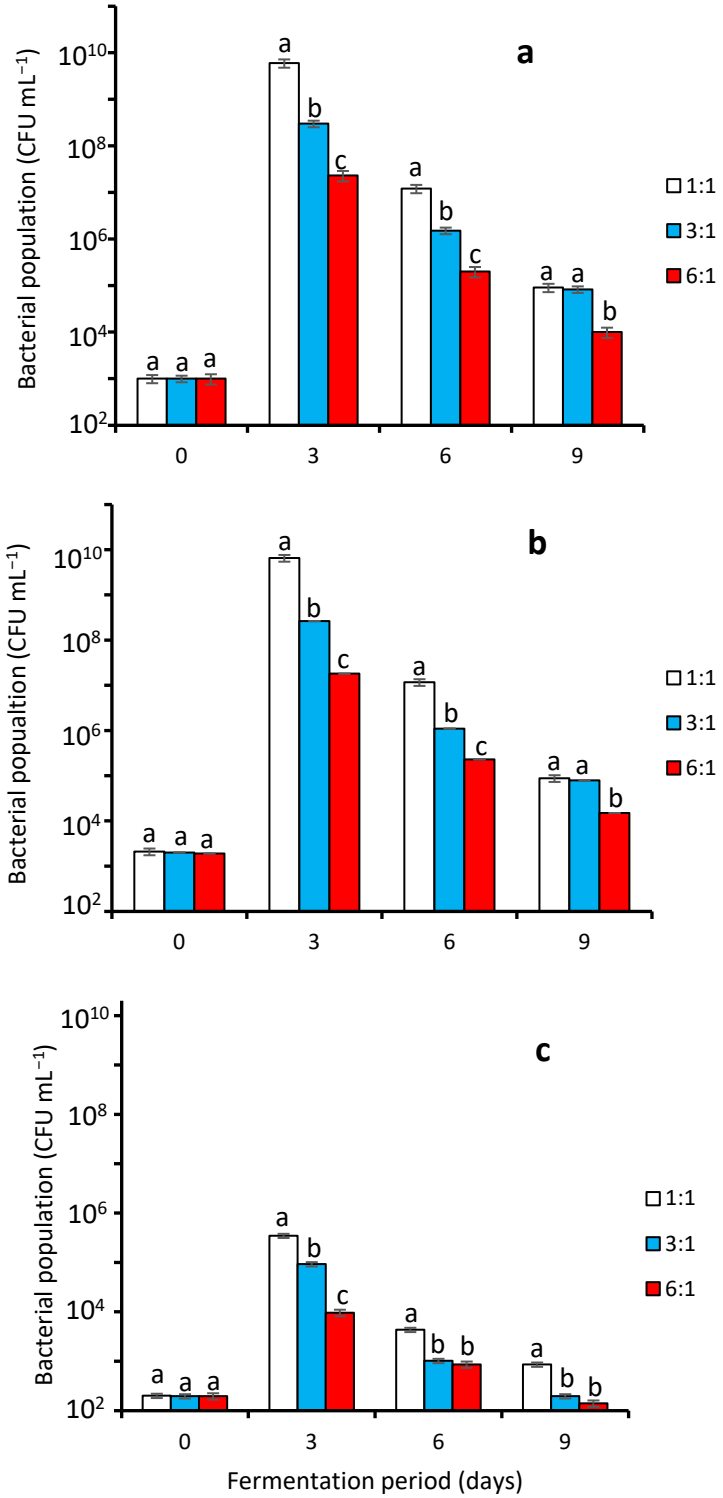

Figure 4. Interaction means (±SE) of total bacterial population in WRW at different W:R ratio within fermentation periods of (**a**) 100 rpm, (**b**) 80 rpm and (**c**) 50 rpm. Means with the same letters within the same column are not statistically different from each other based on Tukey test ($p > 0.05$).

Table 3. Bacterial identification using 16S rRNA gene amplification.

Molecular Identification				
Strain	**Fermentation Period**	**Accession Number**	**Close Relatives in NCBI**	**Similarity (%)**
WRW-1	3d	MW365554.1	*Bacillus velezensis* strain HSB1	99.35
WRW-3	3d	MW365555.1	*Enterobacter ludwigii* strain SDI-19	98.75
WRW-4	3d	MW365556.1	*Enterobacter* sp. Strain LSB19	99.10
WRW-6	0d	MW365557.1	*Enterobacter* sp. Strain LSB3	97.59
WRW-7	6d	MW365558.1	*Enterobacter mori* strain BC1	98.51
WRW-8	6d	MW365559.1	*Bacillus velezensis* strain 2656	99.70
WRW-9	6d	MW365560.1	*Stenotrophomonas maltophilia* strain JM11	99.87
WRW-10	9d	MW365561.1	*Enterobacter* sp. Strain LSB10	99.49
WRW-11	9d	MW365562.1	*Enterobacter mori* strain BC1	99.19
WRW-12	3d	MW365563.1	*Klebsiella pneumoniae* strain LB-AMP3KSU	99.87
WRW-13	3d	MW365564.1	*Pantoea agglomerans* stain SVMR	97.92
WRW-14	9d	MW365565.1	*Stenotrophomonas maltophilia* strain F41	99.47

All the broth containing isolates showed a decrease in pH upon bacterial inoculation from 6 to 12 days of incubation (Figure 6). Generally, however, there was a slight (but non-significant) decrease in the culture pH with an increase in the incubation days. To assess whether the 12 strains in the WRW fermented at different period possessed N_2 fixation and nutrient solubilizing potential, these strains were screened for N_2 fixation and P and K solubilization activities using the appropriate media (as mentioned in the methodology). The results of the strains' capability as N_2 fixation and P and K solubilizers are shown in Table 4. Only 8 out of the total 12 showed were positive to N_2 fixation based on the N-free solid malate medium (Nfb medium) after 5 days incubation period. Four strains from 3-day showed N_2 fixation ability, followed by 9-day with two strains, whereas, for 0-day and 6-day fermentation had one strain each. The amount of ethylene produced by the WRW strains ranged from 2.1–11.2 nmol C_2H_4 mL^{-1} h^{-1} (Figure 7). The significantly higher ethylene was produced by *Enterobacter* sp. Strain WRW-10 with 11.2 nmol C_2H_4 mL^{-1} h^{-1}, which differ significantly ($p < 0.01$) from other strains, while the least was produced by *Stenotrophomonas maltophilia* strain WRW-9 (2.1 nmol C_2H_4 mL^{-1} h^{-1}).

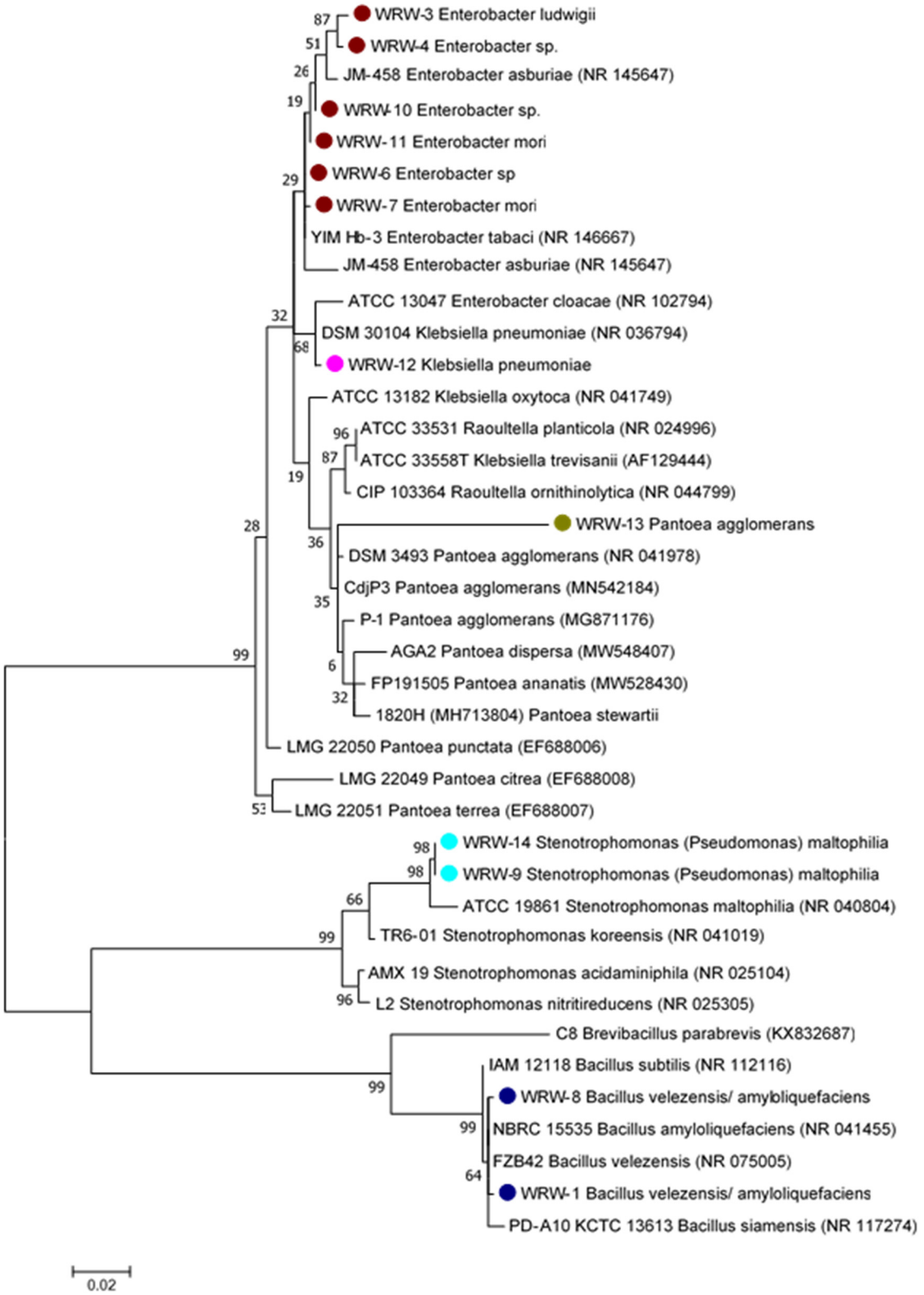

Figure 5. Molecular Phylogenetic analysis by Maximum Likelihood method of Phylogenetic tree derived from analysis of the partial 16S rRNA sequences of WRW1, WRW3, WRW4, WRW6–14 and related sequences obtained from NCBI database. Scale bar, 0.02 substitutions per nucleotide position.

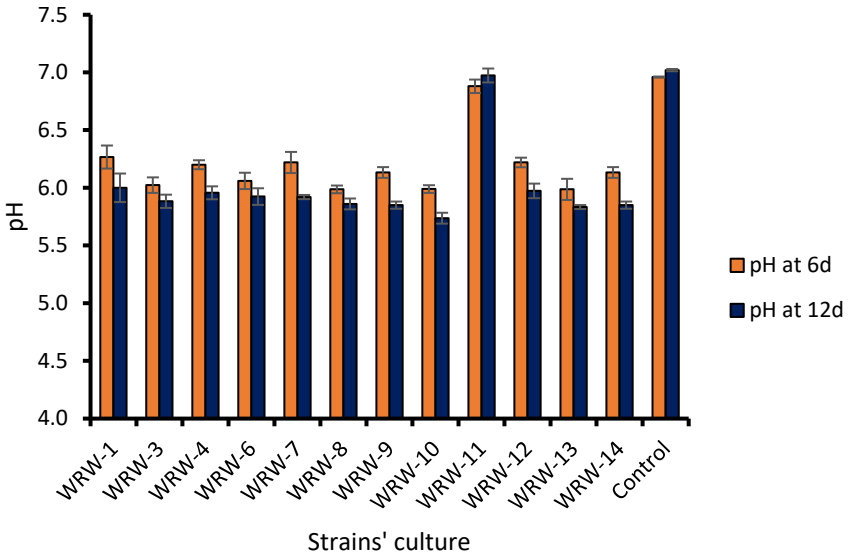

Figure 6. Means (± SE) of the pH of the medium by isolates inoculations. 6d and 12d are 6 and 12 days of culture incubation periods.

Table 4. Qualitative biochemical characterizations of the screened strains.

Strains	N_2 Fixation	PS	KS	PSI	KSI	Gram Stain
Bacillus velezensis strain WRW-1	−	+	+	1.3	1.2	−
Enterobacter ludwigii strain WRW-3	++	+	−	1.4	0	+
Enterobacter sp. strain WRW-4	++	+	−	1.2	0	−
Enterobacter sp. strain WRW-6	+	+	−	1.2	0	−
Enterobacter mori strain WRW-7	−	++	−	1.2	0	+
Bacillus velezensis strain WRW-8	−	+	+	1.4	1.3	+
Stenotrophomonas maltophilia strain WRW-9	++	+	++	1.5	1.3	−
Enterobacter sp. strain 10	++	+	−	1.6	0	−
Enterobacter mori strain WRW-11	−	+	−	1.16	0	−
Klebsiella pneumoniae strain WRW-12	++	++	++	1.50	2.3	−
Pantoea agglomerans strain 13	++	++	++	1.53	1.2	−
Stenotrophomonas maltophilia strain WRW-14	+	+	+	1.2	1.1	−

Note: PS is phosphorus solubilization, PSI is phosphorus solubilization index, KS potassium solubilization, KSI is potassium solubilization index, −is negative, +, ++, +++ indicates the intensity as low, moderate, and high of the color or clarity of the halo zones.

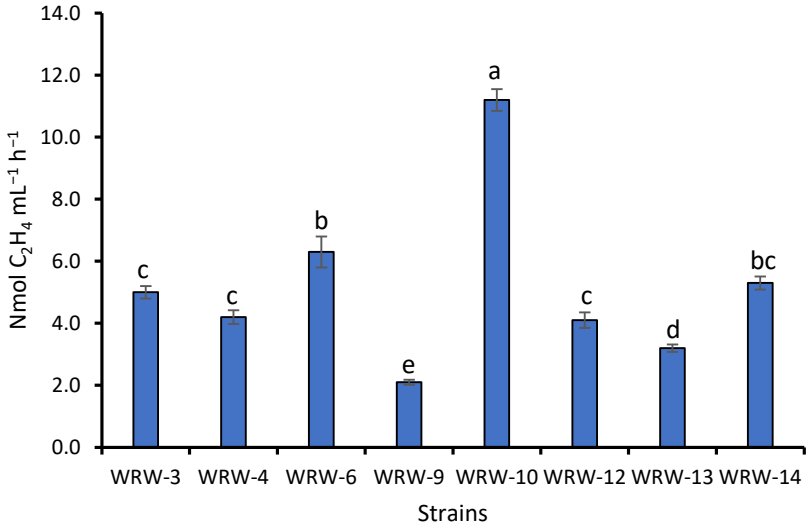

Figure 7. Means (±SE) of acetylene reduction assay of selected strains. Means with different letters are significantly different from one another using HSD at 5% level of significance.

However, based on the agar test, all the 12 strains in the WRW, irrespective of the fermentation periods, showed signs of P solubilization. Similarly, the phosphorus solubilization index (PSI) increased gradually with an increase in incubation days. Overall, at the 6 days of the incubation, higher PSI was observed in *Stenotrophomonas maltophilia* strain WRW-9, *Enterobacter* sp. strain WRW-10, *Klebsiella pneumoniae* strain WRW-12, and *Pantoea agglomerans* strain WRW-13 with 1.50, 1.6, 1.50, and 1.53, respectively. The quantitative P solubilization increased with time irrespective of the strains (Figure 8A). The *Enterobacter mori* strain WRW-7 had higher P solubilization of 38 mg L^{-1}, with 37.1–91.0% increased than other strains, while the least was recorded in *Stenotrophomonas maltophilia* strain WRW-14 with 3.57 mg L^{-1}. In terms of potassium solubilization index (KSI) only six strains were positive based on the agar test (Table 4) with 3-day, 6-day and 9-day fermentation periods having three, two and one positive microorganisms, respectively. *Klebsiella pneumoniae* strain WRW-12 produced the highest KSI of 2.3, while the least was recorded in *Stenotrophomonas maltophilia* strain WRW-14 with 1.1. The amount of K solubilized (quantitatively) by the bacterial strains ranged between 1.65 mg L^{-1} and 11.16 mg L^{-1}, from 5 to 15 days of incubation (Figure 8B). However, across the same strain, a significant difference ($p < 0.05$) between the incubation days (15d, 10d, 5d) was observed. However, the highest K solubilization was produced by *Pantoea agglomerans* strain WRW-13 with a range of percent increased between 10.1 and 71.9% than other strains (Figure 8B).

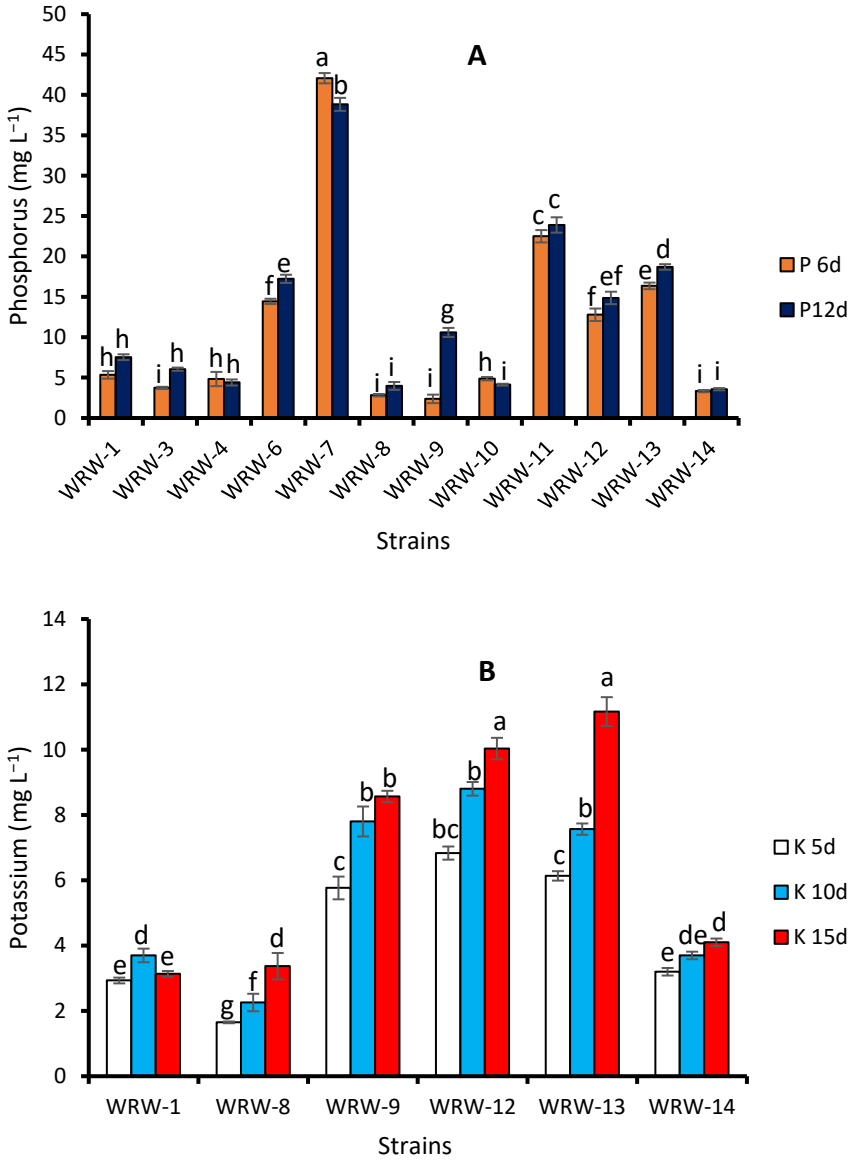

Figure 8. Means (±SE) of solubilized phosphorus (**A**) and potassium (**B**) of the isolated microbes at different days. Means with different letters within the same chart are significantly different using HSD at 5%. 6d, 12d, 5d, 10d, and 15d represent 6-, 12-, 5-, 10-, and 15-day of incubation period.

4. Discussion

4.1. Chemical Characteristics of WRW

The nutrient levels in WRW (Table 1) were near or within the range obtained by other studies [1–4,6]. The elements NH_4^+-N, NO_3^-−N, P, K, Ca, Mg, Zn, and EC increased (except Cu) with longer fermentation due to the activity of the microorganisms in the WRW. However, B was not significantly affected by either fermentation or W:R, possibly because rice is low in B (Table 1). Atique-ur-Rehman et al. [35] reported that rice is deficient in B due to their cultivation in either highly acidic or alkaline soils which are classified as B deficient soils. NH_4^+−N, NO_3^-−N, K, and C increased with higher W:R, which could be due to their high solubility in water. Monovalent elements or ions, such as NH_4^+-N, NO_3^-−N, and K have a higher solubility in water due to their weaker bonds than divalent cations [36], and therefore, easier to be leached out.

The increase in P and K with the increase in fermentation was because of the presence of P and K solubilizing bacteria (Figure 8). *Bacillus* and *Enterobacter* species are PGPB that could solubilize P and K [16,25,37]. Furthermore, the increase in P and K with fermentation also agrees with the total bacterial population (Figure 4) which shows an increasing trend

with the increase in fermentation periods until 3 days, then decline thereafter. The results agree with several studies of cereals fermentation that reported an increase in P, Ca, Mg, Zn with an increase in fermentation period mainly due to the loss of dry matter as microbes mineralized the carbohydrate and protein contents of the cereals, leading to the availability of these elements [38–40].

Higher P, Mg, and Zn concentrations at lower W:R were mainly due to their low solubility in water [36]. Similarly, the decrease in P, Mg and Zn at higher W:R could also be attributed to their precipitations by Ca. Diaz [41] reported that Ca affected the availability of P by precipitation. Significant correlations between Mg and water-extractable P obtained by Kleinman et al. [42] supported our observations on the possible association of Mg and P in WRW. In this study, we also found a significant ($p < 0.01$) positive correlation between the P and Mg (r = 0.93), P and Zn (0.95), and Mg and Zn (0.93), and this indicates that the availability of one element could lead to the availability of the other. For instance, Mg and Zn content in laundry wastewater in the study by Tan [43] were found to decrease with higher water volume. Phytate is the main form of organic P in grains (including rice) and the phytate form of P is not soluble [44]. Phytate binds strongly to many elements during fermentation, and phytates are non-soluble in water [38,45,46].

The decrease in C content with an increase in fermentation periods was due to the bacterial activity in the WRW. Bacteria use C as an energy source [47], which leads to a reduction in C as fermentation progressed. Similarly, the decrease in C and total bacterial population (Figure 4) led to the increase in NH_4^+-N, NO_3^-−N, P, K, Mg and Cu indicated the utilization of the C content by the microbes for the mineralization process, as reported by Pranoto et al. [38].

The higher N at 3-day was because of the presence of N-fixing bacteria (*Bacillus* and *Enterobacter* spp.) when the bacterial population was high (Figure 4 and Table 3). The decrease in N with longer fermentation (3 to 9-day) could partly be attributed to the lower initial N content of the rice. Osman [46] and Pranoto et al. [38] reported a decrease in the protein content of cereals with fermentation due to the lower initial protein content of cereals. The decrease in N as fermentation progressed was most possibly because of the denitrification and ammonification process which had led to an increase in NH_4^+-N and NO_3^-−N (Figure 2). In addition, however, the decrease in the N corresponded to the increase in the NH_4^+-N and NO_3^-−N content (Figure 3), which agrees with Neina [48] who reported that N can be converted to NH_4^+ and NO_3^- during mineralization. A concurrent decrease in WRW pH with an increase in NH_4^+-N and NO_3^-−N observed in this study agrees with Musa et al. [49], where they reported a negative relationship between nitrate production and the pH of the soil. In this study, the higher the W:R ratio, the greater the pH, and the longer the fermentation, the lower the pH. The decrease in pH was because of organic acids formation, such as bicarbonate acids [44,49,50]. The decrease in pH with a longer fermentation period corresponded to the decrease in the bacterial population (Figure 4). Rousk et al. [51] likewise reported a decrease in bacterial growth with pH reduction.

EC increased with fermentation because the concentrations of nearly all elements increased (only Cu declined). The formation of organic acids through the decomposition of organic substances increases EC [52]. The EC, however, decreased with higher W:R because of greater dilution. Volatilization of ammonia and precipitation of mineral salts would lead to lower EC values [53]. EC indicates plant-available nutrients [54,55], which in this study showed that increasing the water proportion in the water and rice mixture had led to the dilution of the nutrient concentrations in WRW.

4.2. Identification and Characterization of the Bacterial Strains

In this study, 12 different PGP strains (*Bacillus velezensis* WRW-1, *Enterobacter ludwigii* WRW-3, *Enterobacter* sp. WRW-4, *Enterobacter* sp. WRW-6, *Enterobacter mori* WRW-7, *Bacillus velezensis* WRW-8, *Stenotrophomonas maltophilia* WRW-9, *Enterobacter* sp. WRW-10, *Enterobacter mori* WRW-11, *Klebsiella pneumoniae* WRW-12, *Pantoea agglomerans* WRW-13 and *Stenotrophomonas maltophilia* WRW-14) were isolated from fermented WRW (0-, 3-, 6-, and

9-day). The phylogenetic tree clearly showed there are two main clusters with *Bacillus velezensis* and *Stenotrophomonas maltophilia* as one cluster, and the second cluster composed of genus of Enterobacter, Pantoea and Klebsiella. The lower fermentation period (3-day) had higher bacterial diversity which decreased as the fermentation progressed. The presence of the unique bacteria at 3-day (*Pantoea agglomerans* and *Klebsiella pneumoniae*) agrees with Figure 4 which shows a higher bacterial population at the same fermentation period. The decrease in C content with progression of fermentation could be the reason for the absence of *Pantoea agglomerans* and *Klebsiella pneumoniae* at the higher fermentation period (6- and 9-day) which might be attributed to their inability to resist competition as compared to *Bacillus velezensis*, *Enterobacter* spp. and *Stenotrophomonas maltophilia*. Adugna [56] reported that the effectiveness and availability of microbes depend on a supply of available carbon. The identified *Bacillus velezensis* have higher similarities (99%) with *Bacillus* spp. of siamensis and subtilis which agrees with Dunlap [57] who reported *Bacillus velezensis* to synonymized with *Bacillus subtilis* and *Bacillus siamensis* due to their texa's high phenotypic and genotypic coherence. The *Enterobacter* spp. [58], *Bacillus velezensis* [59], *Klebsiella pneumoniae* [60], *Pantoea agglomerans* [61], and *Stenotrophomonas maltophilia* [62] strains have been identified as PGP microorganisms employed in the cultivation of a variety of crops.

Therefore, the utilization of microbes as components of biofertilizers is considered an alternative to chemical fertilizers to improve soil health and crop productivity [63]. Park and DuPonte [64] opined that PGP microorganisms have considerable biopotentials and are a new means for providing substantial benefits to agriculture because the organisms can colonize roots and rhizospheres to stimulate the growth and development of plants. Similarly, Yadav et al. [65] reported that PGP microorganisms possess tremendous characteristics that are directly related to plant growth via the production of plant growth hormones and N_2 fixation and the solubilization of P, K, and Zn or indirectly by the production of ammonia, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, antibiotics, siderophores, hydrocyanic acid, and lytic enzymes.

Enterobacter sp. strain WRW-10 had a range of percent increase in ethylene production of 43.7 to 81.2% than other strains, which shows a greater nitrogenase activity as compared to the other strains. The ability to fix N_2 is a vital criterion for characterization because it is crucial to the plants as a potential alternative to applying chemical N fertilizer. Only eight of the 12 bacterial strains (66%) are able to perform biological N_2 fixation (Table 4). PGP and rhizobia play more important roles, particularly in providing plants with nutrients in less fertilized soils [25]. *Bacillus* sp. was reported to have provided 67% of the nitrogen to a young oil palm via biological N_2 fixation [66]. The ethylene produced by these bacterial strains were within those reported by Tan et al. [25], ranging from 2.1 to 11.2 nmol C_2H_4 mL^{-1} h^{-1} but much higher than those reported by Katupitiya et al. [67] and Naher et al. [68] with 2.3 nmol C_2H_4 $plant^{-1}$ h^{-1}, and 6.1 $\times$ 10^{-8} to 1.2 $\times$ 10^{-3} nmol C_2H_4 cfu^{-1} h^{-1} from Azosprillum inoculation and a diazotroph isolates from rice, respectively.

Studies have shown that certain bacteria function as PGP through soil nutrient solubilizing ability [69,70]. Bacteria that can solubilize nutrients, such as P and K are vital because they can convert insoluble P and K in soils into soluble P and K. The increase in the P and K release is associated with the production of acids, alkalis, or chelates by the bacterial strains [25]. Talaat et al. [71] and Yadav et al. [65] reported that soluble P and K, which are converted by beneficial microorganisms, can be easily taken up by plants for growth and development. The decrease in culture pH is directly proportional to the inorganic phosphate solubilization [72] which is attributed to the organic acids and acid phosphatases production by the microbes [73,74]. Meanwhile, the gradual increase in phosphate solubilization index value for invariably all the strains (excepts *Klebsiella pneumoniae* strain WRW-12 and *Stenotrophomonas maltophilia* strain WRW-13) showed that more insoluble phosphate could still be solubilized beyond 6 days incubation time. Therefore, these isolates could benefit the plants more considering the longer period the insoluble phosphate takes (relatively immobile) before been solubilized. Potassium solubilization rates for these bacterial strains were all lower than the strains used by Tan et al. [25], which

solubilized a range of 10.7 to 14.15 mg L^{-1} after 5 days of incubation. Conversely, the solubilized K in this study was higher than 4.29 mg L^{-1} solubilized by *Bacillus mucilaginous* MCRCp1 reported by Sugumaran and Janarthanum [75] after 4 days of incubation. The use of fermented WRW could reduce the need for inorganic fertilizer as the WRW contained beneficial microorganisms that can solubilize the insoluble P and K into the available form. The differences in the availability and activity of the microorganisms indicate that there is a higher probability that the same raw materials and techniques used to produce the WRW may not produce common species as the ones isolated in this study.

4.3. Potential Use of WRW for Agriculture

Domestic and municipal wastewaters have been used for plant fertilization, and they are reported to have element concentrations in the ranges of (in mg L^{-1}): 39.3–53 TN, 3.7–25 TP, 2.84–12.0 PO_4^{2-}, 0.51–41.0 K, 10–44 NH_4^+-N, 0.3–18.05 NO_3^-−N, 45–130 Ca, and 18–39 Mg [76–84]. The fermented WRW nutrient concentrations are generally within the range of the reported values obtained in the domestic and municipal wastewater and palm oil mill effluent (POME). WRW has lower N and K by 43.9 and 45.2%, respectively than that in POME. However, WRW has a higher P (64.64 mg L^{-1}) than POME by 82% [85]. Interestingly, WRW has a higher C than in POME and domestic wastewaters by between 67.9 to 89.1%.

C is a source of energy for microorganisms [47,86], and this study showed that the nutrient contents of WRW increased (except C) with fermentation (Figures 2 and 3). This shows that WRW has the potential to be used as a source of plant nutrients similar to municipal and domestic wastewater, POME, and other wastewaters.

Over 95% of WRW composition is water. Therefore, for WRW reuse, the wastewater must be applied in small doses but applied frequently to minimize leaching losses and to gradually build up soil health. Our study showed that, if applied correctly, the benefits of WRW can be consequential over the long term, where WRW has the potential to gradually build up both the soil nutrient concentrations and beneficial soil biota. Nevertheless, it is important to note here that the reuse of WRW is not to replace or even substitute other amendments.

5. Conclusions

WRW contained essential nutrients to support plant growth and development. Fermentation and W:R were the two most important factors in determining the WRW nutrient contents. The concentrations of C, K, Ca, S, NH_4^+-N, and NO_3^-−N increased while P, Mg, and Zn decreased with higher W:R, indicating elements with higher solubility in water would increase in higher W:R. However, with a higher fermentation period, all the measured elements (except C) increased, indicating the mineralization of WRW. Fermented WRW (as compared with unfermented WRW) had higher elemental concentrations, particularly, N, P, and K with 59.7, 60.2, and 25.0%, respectively, due to the presence of beneficial microorganisms, such as *Bacillus velezensis*, *Klebsiella pneumoniae*, and the variety of *Enterobacter* spp. which are N fixing, and P- and K-solubilizing bacteria. However, to validate the potential of WRW as an organic amendment, field trials involving various crops and soils are required to evaluate the effects of WRW, particularly against conventional mineral fertilizers, on improving soil health and increasing crop growth and yield in both the short and long term (such as over several planting cycles).

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