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ORIGINAL PAPER

Effects of Cellulolytic Bacteria on Nitrogen-Fixing Bacteria, 16S rRNA, *nifH* Gene Abundance, and Chemical Properties of Water Hyacinth Compost

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Abstract

This research investigated alterations to and the interdependency of nitrogen-fixing bacteria, 16S ribosomal ribonucleic acid gene (16S rRNA) and nitrogenase reductase gene (*nifH*) gene abundance, and chemical properties of water hyacinth compost when using cellulolytic bacteria isolated from soil and leaf litter (CSL) inoculum. The un- and inoculated treatments in the compost were designed with three replications. Microbiological analysis involved examination of the total number of bacteria and gene abundance in the compost based on quantitative real-time polymerase chain reaction (qPCR). Some chemical properties of the compost were also analyzed. The results indicated that applying cellulolytic bacteria into compost could increase the amounts of bacteria, especially nitrogen-fixing bacteria. The pH of the compost increased slightly for the first 4 weeks. The amount of nitrogen and organic matter (OM) in the compost increased continuously during the composting period. The concentration of ammonium changed markedly in the range 1.5–2 times at the 4th and 10th weeks of the composting process, which was consistent with an increase of nitrogen-fixing bacteria. The concentration of nitrate doubled at the 12th week. The abundance of 16S rRNA and *nifH* genes was significantly correlated with the number of bacteria, total nitrogen, ammonium, nitrate, and OM. The inoculated cellulolytic bacteria not only accelerated the nitrogen mineralization process but also promoted bacterial numbers in the compost. These bacteria also affected the transformation of nutrients and correlated positively with gene abundance.

Keywords Water hyacinth compost · Cellulolytic bacteria · Nitrogen-fixing bacteria · *nifH* gene abundance

Abbreviations

CSL	Cellulolytic bacteria isolated from soil and leaf litter
CMC	Carboxyl methyl cellulose
CFU	Colony-forming unit
NF	Number of nitrogen-fixing bacteria
Cel	Number of cellulolytic bacteria
TB	Number of total bacteria
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
Dw	Dry weight
OM	Organic matter
nifH	Nitrogenase reductase gene
16S rRNA	16S ribosomal ribonucleic acid gene
DNA	Deoxyribonucleic acid
TISTR	Thailand Institute of Scientific and Technological Research

1 Introduction

Water hyacinth (*Eichhornia crassipes*) is one of the most widespread aquatic plants in the world. However, in the last century, it has become a water weed problem in many countries, including Thailand. The amount of water hyacinth in Thailand reportedly amounted to roughly 4.52 million t, causing a budget loss of USD 0.27 million per year to eliminate

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477,000 t using a water hyacinth harvester (Ayuttaya 2015). In 2016, the Department of Public Works and Town & Country Planning surveyed the spread of water hyacinth in five regions of Thailand: North, Central, Eastern, Southern, and Northeastern, with the amounts being 0.25, 2.78, 0.25, 0.61, and 2.42 million t, respectively (Ministry of Interior 2016). Nakhon Pathom province previously suffered from thick mats of water hyacinth that blocked the water flow and air-water interface, thus turning the water putrid and toxic (Ministry of Foreign Affairs 2017). The Department used satellite images in 2020 to estimate that there were up to 42,000 t of water hyacinth in the period February–June each year in the Tha Chin River of Sam Phran district, Nakhon Pathom province (Geo-Informatics and Space Technology Development Agency 2020). Water hyacinth is composed of a high percentage of cellulose and hemicellulose, accounting for 44–66.9% of dry weight (Dw), and a low lignin content (Kumar et al. 2009). The cellulose content of the water hyacinth was reported in the range 18–35% (Rezania et al. 2017) and at 25% (Istirokhatun et al. 2015). Analysis of the composition of cellulose in parts such as the roots, stems, and leaves of water hyacinth from the Tunal River in México indicated values of 16, 8.4, and 8.7%, respectively (Lara-Serrano et al. 2016). Cellulose is a polysaccharide of D-glucose that is linked by β-1, 4 glycosidic bonds, which consist of more than 12,000 glucose units (Himmel et al. 2007). The cellulose-containing materials are decomposed by a complex cellulase activities including (1) endoglucanase activity, (2) exoglucanase activity (also called cellodextrinase or cellobiohydrolase), and (3) β-glucosidase activity (Lakhundi et al. 2015). These cellulase systems act with or without carbohydrate-binding modules and dissolve the β-1, 4 glycosidic bond to release glucose in the metabolic process (Lynd et al. 2002). Many cellulolytic bacteria with cellulase activities have been reported belonging to several different phyla such as Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Chloroflexi (Koeck et al. 2014). Using cellulolytic bacteria is an alternative to accelerate the water hyacinth removal for producing the compost. The cellulolytic bacteria can decompose cellulose in the municipal solid waste and water hyacinth (Parveen and Padmaja 2010), which resulted in higher contents of nitrogen, phosphorus, and potassium with reducing the composing period (Mahanta et al. 2014).

In cellulose degradation process, cellulose was degraded by the cellulolytic bacteria (primary microorganisms), resulting in cellobiose and glucose under aerobic condition, and propionate, butylate, lactate, and acetate under anaerobic condition (Leschine 1995; Schellenberger et al. 2012). All of these products can be used as substrates or energy sources for other bacteria. The nitrogen-fixing bacteria are one of secondary microorganisms, which can utilize these substrates to survive through the nitrogen cycle (Emmyrafedziawati and Stella 2018) as they cannot directly use cellulose in nitrogen fixation process (Ladha and Peoples 1995). Furthermore, the nitrogen-fixing bacteria community can be studied based on the nitrogenase reductase gene (*nifH*) gene, using quantitative real-time polymerase chain reaction (qPCR) to compare the fluctuations with 16S ribosomal ribonucleic acid gene (16S rRNA; (Silva et al. 2013).

The objective of the current study was to test the hypothesis that the cellulolytic bacteria will release nutrients in water hyacinth decomposition. Thereafter, other microorganisms will utilize these nutrients (especially nitrogen-fixing bacteria), resulting in the alteration of the chemical properties in the water hyacinth compost. Furthermore, interdependency microbes, chemical properties, and *nifH* gene abundance within the compost pile have never been investigated. This research was carried out to better understand the changes in the chemical and biological properties of water hyacinth compost when using cellulolytic bacteria isolated from soil and leaf litter (CSL) inoculum, as well to investigate the correlation of nitrogen-fixing bacteria and gene abundance with cellulolytic bacteria.

2 Material and Methods

2.1 Inoculum Preparation

The CSL inoculum obtained from 17.408871° N and 101.432435° E (Loei province, Thailand) was used, and it was isolated using carboxyl methyl cellulose (CMC) agar (Yodying et al. 2019). The CSL inoculum contained mixed cultures and was kept at the culture collection of Thailand Institute of Scientific and Technological Research (TISTR) as TISTR 2970 and TISTR 2971. Both isolates were classified as *Bacillus cereus* phu01 and phu02 accession number LC582804 and LC582805, respectively. Cellulase activity was detected by the appearance of transparent zones on the CMC agar after staining with 0.3% Congo red for 20 min and washing with 1-M NaCl. The average values for the clear zone width per colony and the width ratio of each isolate were 2.80 and 2.33 cm, respectively. At 50 °C, cellulase activity using water hyacinth substrate was 138.37 and 107.26 mg ml^{-1}, respectively. The mixed culture had a highest decomposition rate of 27.22 mg day^{-1}. The cultures were stored in nutrient broth with 20% glycerol at − 20 °C. Each isolate was cultured in a CMC broth medium and diluted to a cell concentration of 10^8 colony-forming unit (CFU) ml^{-1}. The cultures were mixed with 100-g peat moss as a carrier. The CSL inoculum was determined at 10^8 CFU g^{-1} peat moss for this experiment.

2.2 Composting Process and Sampling

A completely randomized design was used involving 3 replications and 2 treatments: the control or un-inoculated

treatment (without the CSL inoculum) and inoculated treatment (with the CSL inoculum). The water hyacinth compost pile was prepared by making 4 layers of water hyacinth, corncob, rice husk, and coconut coir with a ratio of 5:3:1:1, respectively, by fresh weight. The dimensions of each pile were approximately 100 cm (width) × 100 cm (length) × 50 cm (height). A sample of 100 g of the CSL inoculum was used with 1 t of water hyacinth compost using peat instead of the CSL inoculum for the control. The compost piles were left outside under a rainproof material. The moisture content of the compost was controlled at 60% and turned every 7 days (for aeration) for 3 months. Samples were collected from each treatment every 2 weeks during the composting for chemical, microbiological, and molecular biology analysis.

2.3 Chemical Analysis of Water Hyacinth Compost Samples

Samples (each 10 g) of the compost were put into separate 250-ml beakers, after which 100 ml of distilled water was added and then shaken for 5 min before being left to stand for another 30 min. The pH of the supernatant was measured using a pH meter (Cao et al. 2013). The Kjeldahl method was used to determine total nitrogen, as described in the ASTM D2973-16 (2016), as well as ammonium and nitrate (Thompson et al. 2002). Organic carbon was analyzed using the Degtjareff method (Walkley and Black 1934). Oxidation involved heating with a potassium dichromate solution in sulfuric acid, which was determined using titration with ferrous sulfate.

2.4 Microbiological Analysis of Water Hyacinth Compost Samples

The number of bacteria in the compost pile was calculated using the dilution plate count method. Briefly, 10 g of compost was transferred to a 250-ml Erlenmeyer flask containing 90 ml of sterile distilled water and shaken. Serial dilutions were made and 0.1-ml aliquots (10^3–10^6 dilution) were spread on plates with each specific medium containing Burk's N-free medium, pH 7.0 (Park et al. 2005) for the nitrogen-fixing bacteria, CMC agar, pH 7.0 (Behera et al. 2014) for the cellulolytic bacteria, and nutrient agar, pH 7.0 for total bacteria determination. The plates were incubated for 7 days at room temperature and then counted using 30–300 colonies.

2.5 Sample Extraction and qPCR

For molecular biology analysis, deoxyribonucleic acid (DNA) was extracted from 0.25 g of the compost using a NucleoSpin® soil kit (Macherey-Nagel, Germany) and was purified using NucleoSpin® gDNA Clean-up (Macherey-Nagel, Germany) according to the manufacturer's instructions. The quality of the extracted DNA was determined by the ratio of A260/A280 nm using a nanophotometer. The electrophoresis was prepared with 1% agarose gel solution containing 4 μl of RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, Sangdaewon-Dong, Korea) and then monitoring the bands under UV illumination at 50 V for 40 min; then, the DNA was stored at − 20 °C before use.

The qPCR used primer sets for *nifH* gene and 16S rRNA gene amplification (Table 1). The *nifH* gene qPCR used a nested protocol, whereas the 16S rRNA used a single amplification step. The first (non-qPCR) of the PCR reactions was performed in a total volume of 50 μl at a final concentration containing a 2-μl DNA template (10 ng), 5 μl of 1 × reaction buffer, 1.25 μl of 2.5 mM $MgCl_2$, 4 μl of 200 μM dNTP mixture, 0.5 μl of 1 μM each primer (*nifH*-Fv, *nifH*-Rv), 0.2 μl (1 U) of Taq DNA polymerase, and some distilled water make up to the final volume. For the first polymerase chain reaction (PCR), the PCR conditions consisted of initial denaturation for 6 min at 94 °C, pre-denaturation for 11 s at 94 °C, and 30 amplification cycles performed for 15 s at 92 °C (denaturation), 8 s at 54 °C, and 30 s at 56 °C for the first reaction and 25 s at 72 °C (extension). For the nested reaction (annealing of qPCR), the conditions were 8 s at 51 °C and 30 s at 53 °C (Levy-Booth and Winder 2010). The qPCR was carried out using MyGo Pro equipment (iScience Technology Co., Ltd.). The reaction mixture was prepared in a total volume of 20 μl at a final concentration using 5 μl of the first PCR product (only for the *nifH* gene), 10 μl of Sygreen, 1.5 μl of 0.75 μM each primer (*nifH*-B-F, *nifH*-B-R), and 2 μl of distilled water. For the 16SrRNA gene, the reaction mixture contained 2 μl of DNA template, 10 μl of Sygreen, 1 μl of μM each primer (338F-518R-F, 338F-518R-R), and 6 μl of distilled water. The *nifH* and 16S rRNA qPCR conditions are shown in Table 2.

2.6 Standard Curve Preparation

Genomic DNA from bacterial pure cultures of *Azospirillum brasilense* Sp7 (ATCC 29729) was used to develop the universal *nifH* standard curve. It has been used quantitatively with success to detect the *nifH* gene (Levy-Booth and Winder 2010; Widmer et al. 1999). This bacterium was cultured on spirillum nitrogen-fixing (ATCC® medium 838) broth for 48 h. DNA was extracted from cultures using a NucleoSpin® tissue kit (Macherey-Nagel, Germany). The DNA was stored at − 20 °C prior to qPCR. For quantification of the target gene, *A. brasilense* (ATCC 29729) was used to develop the *nifH* gene and the 16S rRNA standard curve (Levy-Booth and Winder 2010) by serially diluting genomic DNA in the range 10^9 to 10^{13} gene copies for both the 16S rRNA and the *nifH* genes. The standard curves were generated by plotting the logarithm of each gene target copy number and the averages of the threshold cycle. The gene copy number was determined using the

Table 1 Primer sets used in quantitative real-time PCR analysis of water hyacinth compost

Gene target	Sequence (5′-3′)	Primer name	Reference
nifH gene	ForA, GCI WTI TAY GGN AAR GGN GG Rev, GCR TAI ABN GCC ATC ATY TC	*nifH*-Fv *nifH*-Rv	Widmer et al. 1999
	ForB, GGI TGY GAY CCN AAV GCN GA Rev, GCR TAI ABN GCC ATC ATY TC	*nifH*-B-F *nifH*-B-R	Bürgmann et al. 2003
16S rRNA gene	For, ACT CCT ACG GGA GGC AGC AG Rev, ATT ACC GCG GCT GCT GG	338F-518R-F 338F-518R-R	Muyzer et al. 1993

regression equation: $y = -2.8801x + 51.366$ ($r^2 = 0.998$) for the 16SrRNA gene and using $y = -2.4958x + 52.627$ ($r^2 = 0.997$) for the *nifH* gene. The gene copy number was calculated assuming an average base pair weight of 650 Da and Avogadro's number (6.022×10^{23}) using the following equation: gene copy number = (DNA ng amount × 6.022×10^{23} molecules mol^{-1})/(length of DNA in base pairs × 1×10^9 ng g^{-1} × 650 g mol^{-1}). The DNA amplicon size was approximately 180 bp for the 16S rRNA gene (Fierer et al. 2005) and 370 bp for the *nifH* gene (Widmer et al. 1999). Gene abundance was determined using logarithm gene copy number gDw^{-1}.

2.7 Correlation and Statistical Analysis

Analysis of variance was carried out using the SPSS version 16.0 software package with difference comparisons between mean values using Duncan's new multiple range test. The difference between treatments was indicated as either significant ($P < 0.05$) or highly significant ($P < 0.01$). The correlation coefficient was used to test the relationships between quantitative variables with the Minitab (version 16.2.0) statistical software.

3 Results

3.1 Alteration of Chemical Properties in Inoculated Water Hyacinth Compost

The properties during the composting process are shown in Table 3. Both the un-inoculated and inoculated treatments were slightly acidic at the beginning of the compost process. At the 8th week, the highest pH (slightly alkaline at 7.63) was recorded and then the pH decreased slightly at the end of the composting process in the un-inoculated treatment, while the maximum pH in the inoculated treatment increased to slightly alkaline (7.53) at the 6th week, and then the pH gradually reduced to neutral. The initial total nitrogen content of both treatments was 0.56%. In the inoculated treatment, the percentage of total nitrogen was 2 times higher for 2–12th weeks than that in the control and was the highest at the 12th week. There were significant differences compared with the control. The ammonium concentrations in the inoculated treatment were markedly different from the control being 17.46 and 26.17 ppm at the 4th and 10th weeks, respectively. The nitrate concentrations of both treatments remained constant throughout the composting period. There were no significant differences except with the inoculated treatment, where the value of 17.44 ppm at the 12th week was greater than the nitrate concentration of the control. The OM in the compost pile changed in the starting stage to 25.09 and 26.62% for un-inoculated and inoculated treatments, respectively. The inoculated treatment was significantly different from the control from weeks 2–12; the greatest OM content was 66.65% at the 12th week.

3.2 Alteration of Bacterial Numbers in Inoculated Water Hyacinth Compost

The bacterial numbers during composting of the water hyacinth are shown (Fig. 1). Initially, there were higher numbers of bacteria in the inoculated treatment than in the un-

Table 2 Conditions used in quantitative real-time PCR analysis of water hyacinth compost

Gene target	Conditions	Reference
nifH gene	Initial denaturation step: 6 min at 94 °C Pre-denaturation: 11 s at 94 °C	Modified from Levy-Booth and Winder 2010
	30 cycles: 15 s at 92 °C, 10 s at 51 °C, 30 s at 53 °C	
	Extension: 25 s at 72 °C	
16S rRNA gene	Initial denaturation step: 5 min 94 °C 40 cycles: 45 s at 94 °C, 45 s at 56 °C, 90 s at 72 °C	Zhou and Wu 2013
	Extension: 10 min at 72 °C	

Table 3 Effects of cellulolytic *Bacillus cereus* phu01 (LC582804) and phu02 (LC582805) on altering chemical properties of water hyacinth compost

Treatment	pH every 2 weeks						
	0	2	4	6	8	10	12
T1	6.35 ± 0.01	7.22 ± 0.05	7.33 ± 0.03	7.54 ± 0.03	7.63 ± 0.02**	7.54 ± 0.05*	7.42 ± 0.04**
T2	6.42 ± 0.02**	7.36 ± 0.03*	7.45 ± 0.05*	7.53 ± 0.02	7.49 ± 0.01	7.36 ± 0.06	7.22 ± 0.03
Treatment	Total nitrogen (%) every 2 weeks						
	0	2	4	6	8	10	12
T1	0.56 ± 0.02	0.62 ± 0.04	0.64 ± 0.04	0.65 ± 0.04	0.62 ± 0.06	0.64 ± 0.06	0.61 ± 0.08
T2	0.56 ± 0.04	0.66 ± 0.06**	0.69 ± 0.04**	0.70 ± 0.06**	0.66 ± 0.03**	0.74 ± 0.07**	0.77 ± 0.04**
Treatment	Ammonium concentration (ppm) every 2 weeks						
	0	2	4	6	8	10	12
T1	0.00 ± 0.00	8.73 ± 0.00	8.73 ± 0.00	17.46 ± 0.00	17.45 ± 0.00	17.45 ± 0.01	26.19 ± 0.02
T2	0.00 ± 0.00	8.73 ± 0.00	17.46 ± 0.01**	17.45 ± 0.01	17.45 ± 0.01	26.17 ± 0.01**	26.16 ± 0.01
Treatment	Nitrate concentration (ppm) every 2 weeks						
	0	2	4	6	8	10	12
T1	0.00 ± 0.00	8.73 ± 0.00	8.73 ± 0.00	8.73 ± 0.00	8.72 ± 0.00	8.73 ± 0.00	8.73 ± 0.01
T2	0.00 ± 0.00	8.73 ± 0.00	8.73 ± 0.00	8.72 ± 0.00	8.73 ± 0.00	8.72 ± 0.00	17.44 ± 0.01**
Treatment	Organic matter (%) every 2 weeks						
	0	2	4	6	8	10	12
T1	25.09 ± 4.17	41.14 ± 2.54	40.05 ± 2.67	41.97 ± 2.73	45.26 ± 2.06	46.87 ± 5.47	56.61 ± 7.18
T2	26.62 ± 2.24	50.11 ± 2.15**	48.48 ± 3.01**	52.01 ± 3.76**	54.83 ± 2.83**	62.90 ± 5.56**	66.65 ± 6.15**

T1: un-inoculated; T2: inoculated. Significant difference is indicated by *, $P < 0.05$, and **, $P < 0.01$, between treatments according to Duncan's multiple range test

inoculated treatment. During the composting process, the total numbers of bacteria, cellulolytic bacteria, and nitrogen-fixing bacteria (Fig. 1a, b, and c, respectively) increased significantly. It was clear that the total bacterial numbers (Fig. 1a) in the inoculated treatment were larger than in the un-inoculated treatment between weeks 6 and 12. The numbers of cellulolytic bacteria (Fig. 1b) and nitrogen-fixing bacteria (Fig. 1c) constantly increased between weeks 4 and 12. The highest numbers of total bacteria, cellulolytic bacteria, and nitrogen-fixing bacteria were 286, 47.7, and 39.1 ($\times 10^5$) CFU gDw^{-1}, respectively, at the 12th week in the inoculated treatment, while in the un-inoculated treatment, they were 95.4, 24.3, and 3.1 ($\times 10^5$) CFU gDw^{-1}, respectively. This research indicated that the CSL inoculum affected the population of bacteria in the compost pile.

3.3 Alteration of Gene Abundance in Inoculated Water Hyacinth Compost

The abundance of genes in the compost piles decomposed by cellulolytic bacteria was evaluated using qPCR. The abundance of the 16S rRNA and *nifH* genes in the compost piles during the experimental period is shown (Fig. 2). The 16S rRNA gene abundance presented a range of 6.72–7.20 log gene copies gDw^{-1} in the un-inoculated treatment, while offering a range of 6.73–7.40 log gene copies gDw^{-1} in the inoculated treatment. The differences between the 16S rRNA gene abundance were compared with the un-inoculated treatment with 6.85 and 7.40 log gene copies gDw^{-1} at the 8th and 10th weeks, respectively (Fig. 2a). The *nifH* gene abundance of the inoculated treatment was not significant (Fig. 2b). The number of *nifH* gene copies ranged between 2.97–3.20 and 2.93–3.38 log gene copies gDw^{-1} in the un-inoculated and inoculated treatments, respectively. These results showed that the CSL inoculum caused the increase 16S rRNA gene abundance in the compost pile.

3.4 Correlation of Various Parameters in Water Hyacinth Compost

The criteria for separating correlation levels determined followed (Hinkle et al. 2003). The correlation coefficients are presented in Table 4. In the un-inoculated treatment, nitrogen-fixing bacteria numbers (NF) had a highly significant positive correlation with cellulolytic bacteria numbers (Cel), total bacteria numbers (TB), ammonium, and OM in the range 0.77–0.86. On the contrary, the NF showed low and very low correlations to nitrate, total nitrogen, and *nifH* gene abundance. There were moderate correlations of NF with pH and 16S rRNA gene abundance. There were high levels of correlation for Cel with TB, ammonium, and OM in the range 0.74–0.86. In contrast, there was a negative correlation between Cel and total nitrogen. Cel had low and very low correlations with nitrate, 16S rRNA, and *nifH* gene abundance.

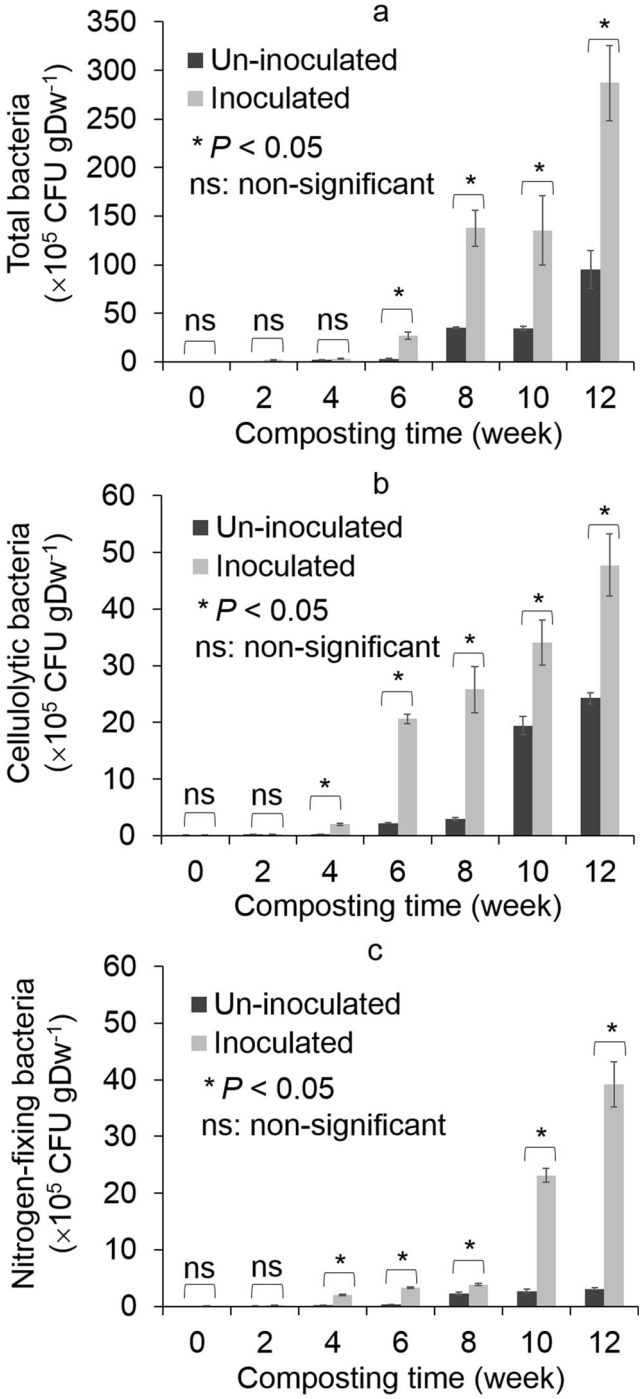

Fig. 1 Changes of bacterial number of total bacteria (**a**), cellulolytic bacteria (**b**), and nitrogen-fixing bacteria (**c**) in water hyacinth compost. The black bars (■) represent an un-inoculated treatment, whereas the gray bars (■) represent an inoculated treatment with cellulolytic *Bacillus cereus* phu01 (LC582804) and phu02 (LC582805). Significant differences are indicated as * ($P < 0.05$) and ns non-significant

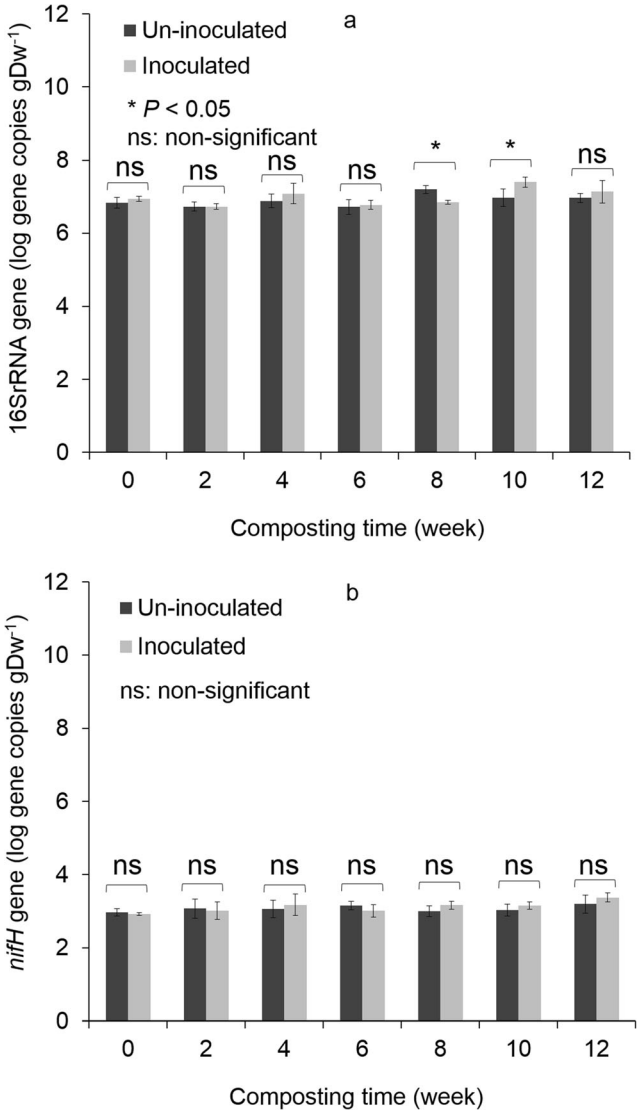

Fig. 2 Changes of 16S rRNA (**a**) and *nifH* (**b**) gene abundance in water hyacinth compost. The black bars (■) represent an un-inoculated treatment, whereas the gray bars (■) represent an inoculated treatment with cellulolytic *Bacillus cereus* phu01 (LC582804) and phu02 (LC582805). Significant differences are indicated as * ($P < 0.05$) and ns non-significant

TB had high correlations to ammonium (0.79) and OM (0.83). In contrast, the abundance of both genes was slightly correlated with TB. The pH values with nitrate, ammonium, and OM were correlated at very high (0.94), high (0.79), and moderate (0.67) levels, respectively. The total nitrogen was highly correlated with nitrate (0.81) and pH (0.87), while it was associated moderately with ammonium (0.51). The ammonium had a very high correlation with OM (0.90) and a high correlation with nitrate (0.71). There was a moderate link between nitrate and OM. The abundance of the 16S rRNA and *nifH* genes had low and very low correlations with ammonium, OM, pH, total nitrogen, and nitrate in the range 0.15–0.34. The abundance of the 16S rRNA gene had a negative correlation with the *nifH* gene (− 0.36).

In the inoculated treatment (Table 5), NF had a very high level of correlation with TB (0.92) followed by high levels with Cel, total nitrogen, ammonium, and nitrate in the range

Table 4 Correlation coefficients of biological and chemical properties of water hyacinth compost in un-inoculated treatment of cellulolytic *Bacillus cereus* phu01 (LC582804) and phu02 (LC582805) during the composting period

Parameter	Un-inoculated									
	NF	Cel	TB	Total N	NH_4^+	NO_3^-	OM	pH	16S rRNA	*nifH*
NF[a]										
Cel[b]	0.86**									
TB[c]	0.86**	0.86**								
Total N[d]	0.25	− 0.00	0.02							
NH_4^+	0.80**	0.74**	0.79**	0.51*						
NO_3^-	0.39	0.30	0.29	0.81**	0.71**					
OM[e]	0.77**	0.78**	0.83**	0.32	0.90**	0.69**				
pH	0.53*	0.35	0.34	0.87**	0.79**	0.95**	0.67*			
16S rRNA	0.52*	0.26	0.36	0.17	0.31	0.15	0.34	0.29		
nifH	0.15	0.22	0.29	0.21	0.30	0.23	0.23	0.18	− 0.36	

[a] *NF* number of nitrogen-fixing bacteria

[b] *Cel* number of cellulolytic bacteria

[c] *TB* number of total bacteria

[d] *Total N* total nitrogen

[e] *OM* organic matter

[f] *16S rRNA* 16S ribosomal ribonucleic acid gene

[g] *nifH* nitrogenase reductase gene

Minitab (version 16.2.0) statistical software was used for correlation testing and significant differences are indicated as * ($P < 0.05$) and ** ($P < 0.01$)

0.70–0.83. Moderate levels of OM and 16S rRNA and *nifH* gene abundance with NF were apparent. Cel had a very high correlation with TB (0.93). There were high levels of correlation of Cel with total nitrogen, ammonium, nitrate, and OM. The *nifH* gene abundance was moderately correlated with Cel. There were high correlations of TB with total nitrogen, ammonium, and nitrate (0.72–0.75). The highest correlation for TB was the moderate relation with the OM and *nifH* gene abundance. The correlations of total nitrogen with ammonium and OM were at very high levels and total nitrogen was related to nitrate and pH at a high level and connected to *nifH* gene abundance at a moderate level. Ammonium had a high level of correlation with nitrate (0.81) and OM (0.88). A moderate relationship was shown for ammonium with pH and gene abundance. There was a high association between nitrate and OM (0.88), while there was a moderate correlation of nitrate with pH (0.60) and *nifH* gene abundance (0.63). There were low and very low correlations of 16S rRNA gene abundance with Cel, total nitrogen, nitrate, OM, pH, and *nifH* gene abundance (0.2–0.38). There was a low correlation (0.26) between the abundance of the *nifH* gene with pH. In addition, pH had a negative correlation with the abundance of the 16S rRNA gene (− 0.03).

These results indicated that NF was more positively related to the chemical properties of the compost in the inoculated treatment. The 16S rRNA gene abundance correlated with NF, TB, and ammonium more than that of the un-inoculated treatment. The *nifH* gene abundance was more closely related to NF, Cel, TB, total nitrogen, ammonium, nitrate, and OM that of the un-inoculated treatment. However, the correlations of ammonium and pH in the inoculated treatment were lower.

4 Discussion

The effect was investigated of CSL inoculum on changes of chemical properties, bacterial numbers, and gene abundance. The pH changes were in the ranges 6.35–7.63 and 6.42–7.53 for the un-inoculated and inoculated treatments, respectively. Both treatments clearly increased pH during the water hyacinth composting process. This also was apparent from the pH increase from 6.5–7.5, 6.2–7.2, 6.4–7.2, and 7.1–7.8 in the compost pile of the water hyacinth collected from four different locations in Guwahati, India (Singh and Kalamdhad 2015). The range of pH values suitable for bacterial development is 6.0–7.5, while fungi prefer an environment in the pH range 5.5–8.0 (Gajalakshmi and Abbasi 2008). The current results identified the highest pH of the inoculated treatment was earlier than for the un-inoculated treatment. This was due to the intensive decomposition from the higher microbial activity with increased aeration resulting in a much higher pH (Sundberg and Jönsson 2008). The release of ammonia through ammonification due to the initiation of the proteolytic process also caused a rise in the pH values (Batham et al.

Table 5 Correlation coefficients of biological and chemical properties of water hyacinth compost in inoculated treatment of cellulolytic *Bacillus cereus* phu01 (LC582804) and phu02 (LC582805) during the composting period

Parameter	Inoculated									
	NF	Cel	TB	Total N	NH_4^+	NO_3^-	OM	pH	16S rRNA	*nifH*
NF[a]										
Cel[b]	0.83**									
TB[c]	0.92**	0.93**								
Total N[d]	0.70**	0.78**	0.72**							
NH_4^+	0.74**	0.82**	0.74**	0.95**						
NO_3^-	0.75**	0.72**	0.75**	0.88**	0.81**					
OM[e]	0.64**	0.73**	0.67**	0.93**	0.88**	0.88**				
pH	0.08	0.34	0.17	0.72**	0.67**	0.60**	0.78**			
16S rRNA[f]	0.58**	0.38	0.46*	0.33	0.50*	0.20	0.26	−0.03		
nifH[g]	0.60**	0.56**	0.63**	0.57**	0.58**	0.63**	0.55*	0.26	0.21	

[a] *NF* number of nitrogen-fixing bacteria

[b] *Cel* number of cellulolytic bacteria

[c] *TB* number of total bacteria

[d] *Total N* total nitrogen

[e] *OM* organic matter

[f] *16S rRNA* 16S ribosomal ribonucleic acid gene

[g] *nifH* nitrogenase reductase gene

Minitab (version 16.2.0) statistical software was used for correlation testing and significant differences are indicated as * ($P < 0.05$) and ** ($P < 0.01$)

2014; De Nobili and Petrussi 1988). The production of organic compost from water hyacinth in Lake Victoria Basin also resulted in an alkaline pH between 7.38 and 8.13 (John and Kakamega 2016). Subsequently, we found that the pH decreased gradually until the 12th week. The pH decreased during the decomposition process due to acid production by microbial metabolic activity (Elvira et al. 1996).

Our results showed an increased total nitrogen throughout the composting process except in the 8th week. The increased total nitrogen was consistent with dry matter loss due to the loss of organic carbon as carbon dioxide during composting through organic decomposition and N-mineralization (Jusoh et al. 2013; Viel et al. 1987). The result was the same for the various formulas of water hyacinth compost with increased N content in the composting process (John and Kakamega 2016; Singh and Kalamdhad 2015). The decreased N in the 8th week was due to N assimilation through glutamine synthetase and the glutamate synthase pathways of the microbes (Wang et al. 2020). They also transformed N to gas such as ammonia, nitrous oxide, and dinitrogen (Guo et al. 2020; Zainudin et al. 2020).

Our experiment indicated that the amount of ammonium released from the compost with the CSL inoculum was 4–5 times higher than that of a report on compost of water hyacinth + cattle manure and water hyacinth + effective microorganisms at 60 days (John and Kakamega 2016). Ammonification is the conversion of organic N (including proteins, amino acids, and nucleic acids) into ammonium, which is released into the ecosystem (Bernhard 2010). On the other hand, the N also increased due to the activity of nitrogen-fixing bacteria commonly found in compost piles (Bishop and Godfrey 1983) through nitrogen fixation, which converts atmospheric N into ammonia. The ammonium cation is formed by the protonation of ammonia, which is available for plant uptake. Rotary drum composting of water hyacinth is enriched with numerous microbial groups such as *Enterobacter*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus badius*, and *Bacillus thuringiensis* (Vishan et al. 2017). Some cellulolytic bacteria, including *Bacillus cereus*, also can fix N (Harindintwali et al. 2020). Later in our composting process, the ammonium content decreased in the 8th week while the amount of nitrate increased due to N transformation during co-composting (Wu et al. 2010). This resulted from nitrification, converting ammonium to nitrate by nitrifying bacteria (Li et al. 2018). The peak levels of nitrate concentration occurred in the 3rd week for water hyacinth composted with molasses at 25% or 50% total sugar content, in the 4th week for water hyacinth co-composted with cattle manure, and in the 5th week for water hyacinth co-composted with poultry manure (Beesigamukama et al. 2018). These results were the opposite of our experiment in which the nitrates were released slowly at the 12th week due to differences in the types of organic materials mixed in the compost.

Our results showed that the breakdown of cellulose from plant residues by the CSL inoculum throughout the composting process resulted in a continuous increase in the OM. The OM was higher than that in the un-inoculated treatment. Similarly, maize straw decomposition resulted in C and N release by decomposers (Tian et al. 2019). Similarly, mulched water hyacinth compost (WHCM) throughout the cropping period resulted in soil OM being in the range 0.64–3.57% higher than that without composting (Balasubramanian et al. 2013). Nevertheless, in our experiment, the OM reduced at the 4th week during composting due to organic C assimilation by the heterotrophic communities as a source of energy (Purkamo et al. 2015).

The applied CSL inoculum in our study of water hyacinth compost promoted the numbers of total bacteria, cellulolytic, and nitrogen-fixing bacteria. The organic carbon composition was important leading to shifts in the bacterial community structure (Liu et al. 2020). The observation of microbial diversity during the composting of municipal solid waste, agro-industrial waste, and organic materials including water hyacinth was studied (Umsakul et al. 2010; Vishan et al. 2017; Yan et al. 2015). Mesophilic and thermophilic microorganisms appeared and were the dominant bacteria in the compost followed by actinomycetes and fungi. There are several diverse groups of bacteria such as ammonia-oxidizing bacteria (AOB), ominant bacteria in the compost, followed by actinomycetes and fungi. There are several diverse groups of bacteria such as AOB and nitrogen-fixing bacteria bearing pectinolytic, amylolytic, and cellulolytic microorganisms. Microbial enrichment with nitrogen fixers, phosphorus solubilizers, and cellulose decomposers is one of the possible ways of increasing the nutrient content of the final compost product (Manna et al. 1997). The nitrogen-fixing bacteria are important in decomposition processes and nutrient cycling. It is possible that synergistic relationships of cellulolytic, nitrogen-fixing, and other bacteria occur in the compost pile ecosystem. The cellulolytic bacteria supply metabolites (glucose or cellobiose). The source of carbohydrate is important to allow for nitrogen fixation activity, which requires large amounts of energy and reducing equivalents (Chan et al. 1994) whilst the nitrogen-fixing bacteria supply ammonia or ammonium as a nitrogen source for other bacteria. Beneficial microorganisms can enhance plant growth, protected pathogens, and improve the biochemical properties of the soil solution (Przemieniecki et al. 2019). The compost applied into soil can improve the chemical and microbiological properties of the soil in the rhizosphere.

The bacterial diversity in the compost pile can be predicted based on monitoring the 16S rRNA gene, while the abundance of one-in-seven nitrogen-cycling genes using *nifH* gene as a marker gene can be used to identify nitrogen-fixing bacteria and archaea (Gaby and Buckley 2012; Henry et al. 2006; Wang et al. 2014; Zhang et al. 2013). The 16S rRNA gene abundance changed at weeks 8 and 10 in our experiment. This suggested that total microbial biomass increased, similar to increasing 16S rRNA gene abundance during incubation in both pig manure and manure + biochar (Ngigi et al. 2020). We found that the *nifH* gene abundance was linked with the number of nitrogen-fixing bacteria and the nitrogen nutrients. This indicated that genes can lead to plant-beneficial functions such as promoting plant growth and improving soil fertility (Bruto et al. 2014). The *nifH*-g1 gene copy number was reported to be stronger with soil carbon and nitrogen in the organic horizon, indicating that the asymbiotic nitrogen-fixing bacteria are greatly influenced by their habitat (Levy-Booth and Winder 2010). No research report has directly identified the abundance of genes in water hyacinth compost. However, the compost used in agricultural management promoted gene abundance and microbial diversity as well as improved soil quality (Li et al. 2017; Nelson et al. 2015; Schmidt et al. 2019). Genes related to the nitrogen cycle were found in soil amended with compost (Pereg et al. 2016). The average number of copies of the 16S rRNA gene increased and often had positive effects on *nifH* gene diversity and activity (Orr et al. 2011). The abundance of *nifH* copies decreased in the rhizosphere of two sorghum cultivars when a high amount of chemical fertilizer was used (Coelho et al. 2009). That result was different from our research because the water hyacinth compost did not contain chemical fertilizers. The natural relationships between the nitrogen-cycling functional gene abundance and soil nutrient status were apparent.

The correlation coefficient is a measure of the linear relationship between two variables (Ratner 2009). Notably, the Cel of inoculated treatment was high related to NF, total nitrogen, and nitrate. These increased more than for the un-inoculated treatment. This suggested that the inoculation of compost by cellulolytic bacteria encouraged an increase in nitrogen-fixing bacteria and other bacteria as well as promoting the decomposition of OM through the mineralization process. The results were consistent with *Azotobacter* bioinoculant (a nitrogen-fixing bacteria) being synergistic to the growth of *Pleurotus eous* that had cellulolytic enzyme activity (Eyini et al. 2005). In particular, the *nifH* gene had moderately positive correlations with NF, Cel, TB, total nitrogen, ammonium, nitrate, and OM for the inoculated treatment. This revealed that the abundance of the *nifH* gene was linked to the increasing amount of nitrogen-fixing bacteria. These bacteria are vital in the nitrogen cycle in an ecosystem. Thus, the nitrogen can be transformed within the compost pile. N transformation is one of the factors that determine the correlation with the nitrogen-fixing gene during chicken manure composting (Liu et al. 2019). The increasing total nitrogen and nitrate concentrations were in accordance with Keshri et al. (2015), who studied the abundance of functional genes in soil. Nutrient types and concentrations impacted on the biodegradation of microcystin in water bodies of cyanobacteria and influenced the dynamics of gene abundance (Li et al. 2011). Log *nifH* gene copies were correlated to total nitrogen at very high levels (0.92).

5 Conclusions

The cellulolytic bacteria isolated from soil and leaf litter (CSL) inoculum (*Bacillus cereus* phu01 and phu02) influenced the changes in the chemical and biological properties of water hyacinth compost. The degradation of cellulose in water hyacinth was considered to be the main function of cellulolytic bacteria responsible for releasing contents of carbon and nitrogen in the compost. Their activity also had an impact on other microorganisms, which were associated with the nitrogen cycle. In addition, the synergy was observed between the CSL inoculum and nitrogen-fixing bacteria. This confirmed by the increased amounts of nitrogen-fixing bacteria, which was correlated to the nitrogenase reductase (*nifH*) gene abundance. Therefore, the CSL inoculum can release nutrient contents in water hyacinth with promoting nitrogen-fixing bacteria, resulting in the increased beneficial substances for plant production.

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Data Availability The datasets produced and analyzed in the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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