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ORIGINAL ARTICLE

Effect of *Micromonospora* sp. KSC08 on nitrogen conservation throughout composting

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Abstract

Composting includes a series of reactions resulting in alterations on organic content and nitrogen amount. NH_3 volatilization via ammonification and N_2 and N_2O losses through nitrification are the major sources of nitrogen loss during composting. Amendment with microorganism inoculation was among recently adopted approaches to compensate for nitrogen losses and improve compost quality. Compost amendment via *Micromonospora* sp. KSC08, an asymbiotic free-living-(N_2)-fixing microorganism, was conducted in the present study in order to investigate microorganism's potential for nitrogen regulation. Twenty windrow systems with varying olive pomace%, microorganism amount, and addition time were prepared for statistical determination of the changes in total C, total nitrogen, and nitrate-nitrogen amounts. Analyses were evaluated in accordance with pH, humidity, and temperature measurements obtained during composting. Final part of the study included maturity evaluation and PCA modeling of FT-IR data. *Micromonospora* sp. KSC08 was shown to improve microbial activity and regulate nitrogen content by providing exogenous nitrogen to compost mixture. PCA models revealed entirely different structures between untreated and *Micromonospora* sp. KSC08-treated compost samples at the end of 120 days. The variant molecular structure of samples inoculated with *Micromonospora* sp. KSC08 was attributed to reactions between carboxylic acid units and nitrogenous compounds leading to a significant increase in amide content compared to untreated mixtures. Higher amide content was due to higher nitrogen content of *Micromonospora* sp. KSC08-treated compost, and based on the findings, it was concluded that *Micromonospora* sp. KSC08 had been effective in nitrogen regulation and proposed as a possible component of microbial consortium for use in conventional composting systems.

Keywords *Micromonospora* sp. · Olive pomace · Compost · Nitrogen · FT-IR

1 Introduction

Composting is a process composed of a series of reactions that resulted in the stabilization of organic matter. The product is used as fertilizer to improve soil and increase soil water retention and carbon capacity [1]. This process gained an increasing attention due to Landfill Directive implemented by European Council. The directive mandates the reduction of wastes removed by landfilling and utilization of alternative methods to remove biodegradable wastes. Composting, as an alternative, is a relatively simple procedure that could be applied both for industrial and household wastes as long as the waste is of biological origin [2]. The number of household wastes could be reduced down to 77%, as previously accomplished with a study conducted in Spain [3].

Reactions involved in the process are conducted by microorganisms that became active at different temperature intervals. These intervals determine the phases of composting and are named mesophilic, thermophilic, and maturation phases. Constant increase in temperature leads to thermophilic phase. Organic matter was mostly degraded near completion in this phase by microorganisms activated during the process [4]. The increase in temperature also enables

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the elimination of pathogens. Maturation is the last phase of composting in which mineralization of organic matter proceeds for an indefinite amount of time [4]. The quality of the final product in composting depends on the control of process parameters such as temperature, moisture content, carbon/nitrogen (C/N) ratio [5], and aeration [6].

Microorganism addition to compost mixtures increases reaction rate during composting, which was the case in a study conducted by Manu et al. [7]. Results indicated a 7-day decrease of thermophilic phase duration for compost mixtures amended with microorganisms. The amendment with microorganisms enabled the completion of active degradation on the 36th day with microorganisms added compost mixtures while degradation lasted 54 days for control [7].

Microbial activity is also responsible for the loss of nitrogen during composting. Organic nitrogen in biomass is degraded into ammonium through ammonification in the early stages of composting. Initial loss of nitrogen is via volatilization in NH_3 form, the remaining part is either lost as N_2 and N_2O or utilized in nitrification. Some part of the nitrate is further involved in denitrification. Consequently, the nitrogen amount in the final product is inadequate most of the time, and nitrogen is added via chemical fertilizers to maintain efficient crop production [8]. Preserving nitrogen or decreasing the amount of nitrogen loss during composting are areas of research which recently gained an increasing attention. Apart from chemical fertilizer addition, it is possible to compensate for nitrogen loss by means of additives or microorganism utilization.

The addition of carbon sources to compost mixtures is proven to be useful in nitrogen regulation; examples included utilization of biochar [9], glucose, sucrose, and starch [10]. Pig manure with acidic substrates [11], the addition of woody peat [12], and diatomite addition to pig manure and sawdust [13] are other examples which have been proven to be useful in delaying nitrogen loss during composting. Nitrogen turnover bacterial agent (NTB), identified as a bacterial consortium consisted of ammonifiers, nitrobacteria, and *Azotobacter*, was used in the work of Jiang et al. [14] for pig manure composting. The results indicated that adding NTB at the beginning of the composting process effectively reduced the nitrogen loss and increased maturity [14].

Nitrogen transformation in compost mixture is basically a series of reactions including ammonification, nitrification, denitrification, and nitrogen fixation. Inoculation of ammonium-oxidizing, nitrite-oxidizing, and free-living-(N_2)-fixing bacteria to biomass mixtures is among the latest trend adopted to improve the quality of compost [5, 15]. These microorganisms fix and include atmospheric nitrogen to nitrogen cycle which may result in extension of time spent in thermophilic phase. Accumulation of ammonium via exogenous microorganism addition increase the amount of nitrogen transformed during composting which lead to the increase in total nitrogen content of the compost [5].

Micromonospora genus are a member of *Micromonosporaceae* family, which were widely distributed in different environments such as soil, plants, rhizosphere soil, plant nodule, water (fresh, marine, and river), and sediment [16, 17]. Studies on nitrogen fixing potential of *Micromonospora* spp. were scarce due to slow growth rate of the microorganism. However, recent data indicated their presence especially in the nodules of leguminous and actinorhizal plants. The genetic diversity of *Micromonospora* spp. pointed out the presence of *Micromonospora saelicesensis* as the most frequent species along with *Micromonospora lupini* relating with the host plant. These findings implied that *Micromonospora* spp. had potential as nitrogen fixing microorganism [18]. Nitrogen fixing potential of symbiotic *Micromonospora* spp. was known; however, members of the genus isolated from rhizosphere and soil also have the potential to fix nitrogen and studies on the effect of free-living asymbiotic *Micromonospora* spp. on nitrogen transformation are scarce. Hence, investigation of asymbiotic free-living-(N_2)-fixing *Micromonospora* spp. for their nitrogen-fixing potential was adopted as a novel approach in the present study.

Olive pomace, besides manure and green wastes, is among the organic amendments which could be found in massive quantities in Mediterranean countries. Olive pomace, when added directly into the soil, is a pollutant with phytotoxic wastes. The high content of phenol and waste in its structure inversely affects soil and groundwater quality, and their removal via composting is crucial in maintaining sustainable production [19]. The study is organized and presented in three consecutive sections containing isolation, identification of *Micromonospora* spp. along with selection of a single *Micromonospora* sp. for use in composting experiments in order to evaluate microorganism's nitrogen-fixing potential, and composting maturity. The samples were obtained in the 21 and 120th days of composting. The details are given in the proceeding sections.

2 Material and methods

2.1 Isolation of *Micromonospora* spp. and elimination of strains for use in compost

Samples collected from soil and rhizosphere of different regions are illustrated in Table S1. Samples were initially dried at room temperature for 15 days and sieved. Dilution plate methods were utilized in isolation with 10^{-3} and 10^{-4} dilutions to obtain the highest possible number of microorganisms. Soil suspensions prepared with 10^{-1} dilution were also treated with 1.5% phenol at 30 °C for 30 min which was an applied procedure specific to *Micromonospora* spp. isolation [20, 21]. Incubation was conducted at 28 °C for 14–21 days. The selective medium used for *Micromonospora* spp. isolation is given in Table S2.

The number of strains obtained at the end of the isolation procedure was very high for use in compost mixtures (Table S3). Hence, an elimination step based on the determination of nitrogen-fixing ability was applied for the selection. The nitrogen-fixing ability of strains was determined based on a previously developed method [22]. The method contained the activation of strains in the "Isolation *Streptomyces* Project 2" (ISP2) medium (Yeast extract (Difco) 4.0 g, malt extract (Difco) 10.0 g, dextrose (Difco) 4.0 g, agar 20.0 g). The activated strains were then inoculated in semisolid agar without nitrogen by point inoculation. Nitrogen-containing semisolid agar with 2 g/L $(NH_4)_2SO_4$ was also utilized as a control medium. The selection was conducted based on higher production in the absence of nitrogen [22].

2.2 Selection of single *Micromonospora* sp. for composting experiments

Visual inspection based on colony morphology and determination of nitrogen-fixing ability were initial steps leading to selection of the microorganism for use in compost. Thirteen microorganisms were selected for 16S rRNA sequencing, and these were further investigated via enzyme tests for final selection. Microorganisms were tested for starch degradation [23], cellulose [24], and caseinase activity [25]. These tests were applied to determine their ability when utilized in compost mixture. The indole acetic acid (IAA) test was applied both as an additional selection criterion and to determine the microorganism's plant growth potential [25].

2.3 Identification of selected *Micromonospora* spp. via 16S rRNA sequencing analysis

The initial step of the analysis was chromosomal DNA isolation, which was conducted by utilizing a DNA isolation kit. 27f and 1525r primers were utilized for PCR amplification [26], and 16S rRNA sequence analysis was conducted according to literature [27]. Sequences were aligned and analyzed in the Mega 7.0 program for the identification of strains. The phylogenetic tree was constructed via neighbor-joining tree algorithms [28, 29]. PCR conditions and the primers utilized for sequencing are illustrated in Tables S4 and S5, respectively.

2.4 Composting experiments and statistical analyses in the presence of *Micromonospora* strain

Composting experiments were conducted with feed mixtures composed of manure, olive pomace, and garden wastes. Manure and olive pomace were supplied from cow farms located in Kızıldamlar province in Bilecik (Turkey) and olive oil factories in Aydın (Turkey). Garden wastes were collected from the gardens in Bilecik Sheikh Edebali University.

Experiments were performed by varying microorganism amount, olive pomace % in the mixture, and adding time of microorganism which were predetermined as experimental parameters. In an experimental design with three parameters, a total of 27 experiments without replication should be performed. Response surface methodology as a versatile statistical approach was applied to decrease the number of experiments and labor force associated with the number of composting mixtures. The total number of experiments, including replicates, was 20 in this method, provided that central composite design methodology was applied for experimental design. The parameters applied in composting experiments and the order of experiments are illustrated in Tables S6 and S7 (Supplementary File), respectively. The effect of parameters on total carbon, total nitrogen, and nitrate-nitrogen, designated as responses, was investigated in the study.

The dry matter in composting systems was determined as 100 kg, and garden wastes were taken as 1 kg in all experiments. The change in temperature values were monitored and recorded at 12-h intervals. Samples were prepared by taking 10–15 subsamples from the top, middle, and core of the compost pile. These subsamples were mixed to maintain homogeneity. Total nitrogen (N), nitrate-nitrogen, total carbon (C), pH, and moisture values were recorded in 3-day intervals for the first 6 days (0, 3, 6th day), 14th day, and 21st days of the thermophilic phase. Composts were turned in every 3 days till the end of (21st day) of the experiment. Compost turning intervals and sampling procedures were determined according to EPA (40 CFR Part 503) requirements. The microorganism was added with Ringer's solution to prevent lysis.

Statistical analyses were performed by ANOVA via Minitab software. Parameter significance was recognized with a $P < 0.05$ threshold. A second-order quadratic response model was constructed according to the below equation:

$$Yt \text{ and } Yn = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i,i>j}^{k} \sum_{j}^{k} \beta_{ij} \beta_{ij} X_i X_j$$

Y indicated responses (t for total nitrogen and n for nitrate-nitrogen), i and j were the linear and quadratic coefficients, and β was the regression coefficient. Finally, k was designated as the number of optimized factors.

2.5 Determination of compost maturity

Compost maturity was evaluated via three separate sets of analyses. Initial characterization was conducted to samples

obtained at the end of the 21st day of composting. Total organic content was determined with samples consecutively dried at 105℃ and ashed at 550℃ for 4 h. The pH of the samples was determined with a pH meter, according to the 1/10 potentiometric method. Total humic and fulvic content of the samples were determined according to TS5869 ISO5073. Organic carbon was determined via the Walkley–Black method. Total nitrogen and nitrate-nitrogen were determined based on previously developed procedures developed by Bremner et al. [30].

Compost samples obtained at the end of 120 days were further analyzed to determine phytotoxicity. The mixtures' germination index was determined with compost extracts obtained by mixing 20 g of compost in 200 ml of deionized water for 5 h. Samples were extracted via centrifugation at 10,000 rpm. Maturity assessment was conducted with *Cucumis sativus* seeds, which were evenly distributed on Whatman No 1 filter paper placed inside 10 cm diameter Petri dishes. A total of 10 seeds were placed in each petri dish, and experiments were conducted in two replicates with three separate runs to assure biological and technical repeatability. Seeds were soaked with 5 ml of distilled water and extract for comparison [31]. The phytotoxicity evaluation method was also slightly modified with the inoculation of the seeds for 2 h in compost extract. Inoculated seeds were soaked with 5 ml of distilled water compared with control and seeds soaked with compost extract.

2.6 Determination of biochemical constituents in mature compost

The third step in evaluation of compost maturity was determination of biochemical constituents. Composts obtained at the end of 120 days of maturation were analyzed using a Cary 630 Fourier transform infrared spectrometer (Agilent, USA) equipped with attenuated total reflectance (ATR) accessory. The spectrum of air was used as a reference spectrum. The raw spectra of untreated (UT group) and microorganism-treated (MOP group) composts were collected at room temperature in the 4000–650 cm^{-1} infrared region with 32 scan numbers. The resolution of spectra was 4 cm^{1}.

The spectral data were analyzed using OPUS 5.5 (Bruker, USA) software. The peaks' absolute intensities, mainly reflecting the compost biomass's biochemical constituents, were quantified using second derivative and vector-normalized spectra (smoothing point 9). The spectral data were statistically compared using the unpaired T-test in GraphPad Prism 6.01 V (GraphPad, USA). The degrees of significance was denoted as less than or equal to $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***, and $P < 0.0001$ ****. The results were expressed as means ± standard error of the mean.

Unsupervised chemometric analysis, namely principal component analysis (PCA), was performed to bring out the most prominent biochemical changes in compost. The analysis was done in the Unscrambler X 10.4 V (Camo Software, NO). The model was created in the whole spectral region (4000–650 cm^{-1}) by applying the cross-validation method (full with 17 segments) to the mean-centered datasets containing raw spectral data of both UT and MOP groups. The singular value decomposition (SVD) algorithm and Hotelling's T2 statistics were used in the modeling of datasets [32].

3 Results and discussion

3.1 Isolation of *Micromonospora* spp.

Strains with pink, orange, red, brown, and purple colors and having a rough colony morphology were purified for further elimination. A total of 168 strains resembling to *Micromonospora* spp. were transferred to glucose yeast extract agar (GYEA), triptone yeast glucose agar (TYGA), and SM3 agar and incubated at 28 °C for 7–14 days to obtain pure isolates. The list of strains obtained after isolation is given in Table S3, and some strains that were defined to be *Micromonospora* spp. are illustrated in Figure S1.

An elimination step based on the determination of nitrogen-fixing ability was applied for the selection due to high number of strains. The strains were classified according to three different growth behavior (Table 1). Group 1 included strains with a higher growth rate compared to $(NH_4)_2SO_4$ containing media. Group 2 belonged to microorganisms with similar behavior in both media, and group 3 had the rest of the microorganisms with a weaker growth rate than the control. 16S rRNA sequencing was conducted with microorganisms in the first group.

3.2 Identification of selected *Micromonospora* spp. via 16S rRNA sequencing analysis, and selection of a single strain for use in compost mixtures

16S rRNA sequencing results were presented by utilization of neighbor-joining tree algorithm. Results are given in Fig. 1. *Micromonospora* spp. GBT09, KSC08, KSC09, and VYN17 (2–3 nt difference; 99.79% similarity) were determined to be closely related with *Micromonospora vinacea* type strain. *Micromonospora* spp. KSC07 and KSC50 (4–8 nt difference; 99.44–99.75% similarity) were found to be close relatives of *Micromonospora parathelypteridis* type strain. *Micromonospora* spp. KSC37, KSC38, KSC41, and KSC43 (1–24 nt difference; 98.0–99.93% similarity) isolates were related to the *Micromonospora saelicesensis* type strain. KSC06 and SLV02 were the only isolates related to *Micromonospora coriariae* and *Micromonospora phytophila* with 4 nucleotide difference 99.7% similarity and 5 nucleotide difference 99.64% similarity.

Table 1 Classification of isolates according to semi solid test

1. Group (−>+)	2. Group (−=+)	3. Group (−<+)	3. Group Cont
SLV02	VYN09	VYN35	KSC31
SLV11	VYN34	VYN26	KSC36
GBT09	SLV01	VYN25	KSC52
KSC41	GBT07	VYN13	KSC57
KSC37	GBT14	VYN05	KSC53
KSC08	GBT02	VYN12	KSC59
KSC09	VYN16	VYN20	KSC28
KSC50	KSC01	VYN36	KSC01
KSC43	KSC14	SLV20	KSC16
KSC38	KSC58	VYN36	KSC48
KSC06	KSC49	GBT17	KSC56
KSC07	KSC32	GBT24	KSC50
VYN17		GBT11	GBT07
		GBT08	
		GBT13	
		KSC10	

-, Microorganism growth in the presence of semi solid agar without $(NH_4)_2SO_4$+; Microorganism growth in the presence of control

In the case of nitrogen fixation from atmosphere, there had been opposite findings stating incapability of *Micromonospora* spp. in fixing nitrogen. Some members of *Micromonospora* spp. could not grow in nitrogen-free culture media. These microorganisms also did not have nitrogenase activity or confirmed genes related to nitrogen fixation. 16S rRNA sequencing of some isolates revealed high resemblance to *Micromonospora lupini* and *Micromonopsora saelicesensis* which, in the present study, was controversially determined to have higher growth rate in nitrogen free culture media [33]. *Micromonospora* spp. including the ones with high resemblance to *M. vinacea*, *M. phytophila*, *M. lupini*, *M. saelicesensis*, and *Micromonopsora zamorensis* were also among the species with no contribution to either nitrogen fixation or nodule formation in alfalfa plant [34]. Based on contradictory results in the literature, enzyme tests were added as criteria for the selection of a single microorganism. Tests were organized to determine both the composting and plant growth-promoting ability of the strains. Results illustrated in Table 2 revealed all strains' ability to degrade starch while none of the strains was found to be capable of cellulose degradation which implied that microorganism addition during composting is useful only on nitrogen fixation, and reactions during composting be mostly governed by microorganisms already existed in the compost mixture. The majority of microorganisms could produce IAA indicating the potential of *Micromonospora* spp. in plant growth promotion. Caseinase activity of the strains implied the possible role of the strains against protection from pathogens. Except for cellulose degradation, results indicated *Micromonospora* sp. KSC08 as the single microorganism with positive results against all tests.

3.3 Composting experiments and statistical analyses in the presence of *Micromonospora* strain

Composting experiments were conducted with twenty systems which were prepared based on experimental design obtained by response surface methodology. The parameters altered in experiments were microorganism amount, adding time of the microorganism, and olive pomace % in the mixture. Statistical analyses were performed to determine the effect of these parameters on total C, total N, and nitrate-nitrogen amounts. Temperature and humidity measurements were monitored at 12-h intervals. The pH of the mixtures was determined in the 0, 3, 6, 14, and 21st days of the experiment. Microbial activity in terms of total C % decrease and nitrogen change in terms of total and nitrate-nitrogen % increases were evaluated via the statistical analyses of the data obtained from the % changes of total C, total N, and nitrate-nitrogen between 0 and 21 days.

Windrow composting was applied in the course of the study. This method is basically static pile composting with periodical agitation or turning in regular basis. Regular turning of organic waste increases passive aeration and accelerates organic decomposition of biomass [35]. These systems are practical and easy to prepare in small scales and heat losses inside the mixture could be substantially reduced depending on the amount of organic waste utilized in mixture. Moisture content remains in the vicinity of 40–55% depending on the heat loss [36]. Windrow composting often results in high amounts of NH_3 volatilization and therefore considered as one of the most convenient methods to validate compensation of nitrogen via free-living-(N_2)-fixing microorganisms [37].

Humidity measurements revealed long-lasting moisture varying between 60 and 70% for 15–18 days. Moisture amounts were decreased to 50–55% in the last 3 days of composting. Moisture amounts remained higher than the threshold (> 40%), and water addition was not necessary for the investigated time interval. Systems were turned in 3-day intervals, which was the only intervention besides microorganism addition in predetermined experiment times. Sampling from windrow systems requires extra effort to maintain homogeneity in measurements. Numerous factors could affect the results, and a statistical approach considering possible inconsistencies should be adopted to reach a decisive conclusion. Statistical analysis results were utilized in system selection for simultaneous evaluation of pH, temperature, total C, total N, and nitrate-nitrogen results.

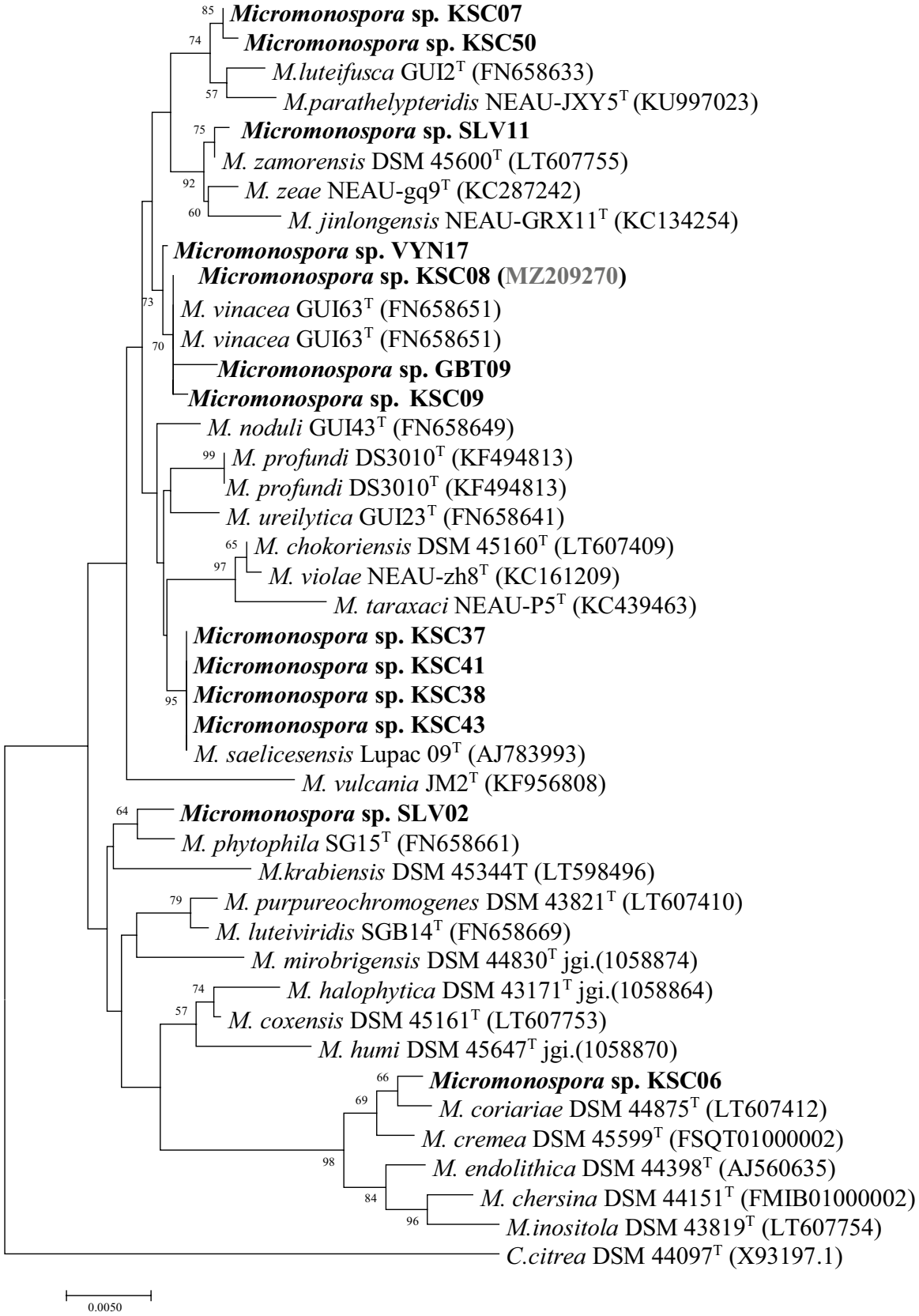

Fig. 1 Neighbor-joining tree based on 16S rRNA sequencing of *Micromonospora* sp. and their closely related strains

The statistically significant results obtained from variance analysis were presented in bold, in Table 3.

The initial response to be interpreted in statistical analyses was the decrease in total C% values. Effects of microorganism amount, addition time, and olive pomace% on total C were statistically determined, and results are given in Table 3 and Fig. 2, respectively. The regression equation obtained for the change in total C% was given below:

$$\begin{aligned}\textbf{T.C.21day} = & -44.85 - 14.27\ \text{M. A.} + 7.79\ \text{A.T (day)} \\ & - 5.95\ \text{OP \%} + 13.00\ \text{M.A*M.A} \\ & + 2.98\ \text{A.T(day)*A.T (day)} \\ & + 8.04\ \text{OP\%*OP \%} - 13.83\ \text{M A.*A.T (day)} \\ & - 4.98\ \text{M.A*OP \%} + 1.35\ \text{A.T (day)*OP \%}\end{aligned}$$

All parameters had been effective on the change of total C%, as seen in Table 3. The regression coefficient (93.1%) indicated that the proposed quadratic model had been appropriate. 2D contour plots revealed that the highest decrease of total C had occurred in the earliest addition time, which was an interesting result considering the inability of *Micromonospora* sp. KSC08 on cellulose degradation. Results validated that *Micromonospora* sp. KSC08 had no inhibiting effect on microbial activity and implied the possibility of a symbiotic relationship between *Micromonospora* sp. KSC08 and microbial flora. Total C decreases were higher in the presence of higher olive pomace% (> 20), and microorganism amount also higher than 0.25 g. Based on the results, systems 6, 15, and 17 were selected to evaluate further the periodic changes in pH and temperature (Fig. 2).

pH and temperature changes for 21 days are given in Fig. 3. The increase in temperature was limited, with the highest value of 49 °C for the systems, which was an expected result considering the amount of low dry matter utilized in experiments. The time to reach the thermophilic phase depended on the amount of olive pomace utilized in systems. On the other hand, the microorganism amount was also effective on the progress of composting, and results implied the presence of a threshold in its utilization based on the times spent to reach the thermophilic phase (Fig. 3).

The increase of pH values between 0 and 6 days could be observed for systems 15 and 17. These increases depended on organic matter degradation and mineralization of organic nitrogen which resulted in ammonia accumulation. This was an expected result considering the losses in total carbon content and a simultaneous increase in temperature values. In a traditional composting system, NH_4^+-N in the form of ammonia should have been lost, which would typically result in the decline of pH values [38]. However, that was not the case for the selected systems, and pH values remained almost stable during the thermophilic phase. These stable pH values implied the presence of an external factor affecting the progress of composting. Nitrogen fixation from the atmosphere could compensate for the losses that occurred from ammonia volatilization. The stabilization of pH values during the thermophilic phase implied a change of ammonia balance in the systems via *Micromonospora* sp. KSC08 addition (Fig. 3).

The effect of parameters on the change of total N% was calculated according to the equation below:

$$\begin{aligned}\textbf{T.N.21day} = & 39.39 + 5.19\ \text{M.A} + 20.66\ \text{A.T (day)} \\ & - 5.28\ \text{OP \%} - 4.96\ \text{M.A*M.A} \\ & - 2.07\ \text{A.T (day)*A.T (day)} \\ & - 22.61\ \text{OP \%*OP \%} - 3.41\ \text{M.A*A.T (day)} \\ & + 2.84\ \text{M.A*OP \%} - 0.79\ \text{A.T (day)*OP \%}\end{aligned}$$

Variance analyses for total nitrogen change indicated the effect of all parameters as seen from Table 3. 2D contour plots illustrating the effects of parameters are given in Fig. 4. Results revealed that the greatest change in total N had been in the presence of medium olive pomace% and latest addition times. It was also statistically shown that microorganism amount had been influential in the increase of total nitrogen. Based on the results, system 9 was selected for further evaluation (Fig. 4).

pH, temperature, and % change in total nitrogen for system 9 are illustrated in Fig. 5. The temperature remained stable until the 10th day, and a slight increase in temperature with the highest value of 42 °C was observed for 7 days. System 9 was prepared with the latest microorganism addition (6th day), and the activity increase after addition revealed the effect of *Micromonospora* sp. KSC08 on microbial activity. The pH values decreased in the first 6 days, as seen from Fig. 5. Based on the temperature profile, it would be fair to point out that the activity for the first 6 days had been negligible.

The decline in pH values was related to ammonia volatilization already existed in manure. pH values increased starting from the 6th day, which was due to microbial activity enhanced by *Micromonospora* sp. KSC08 addition. The temperature profile of the system indicated a stable high-temperature zone, which lasted for 7 days. Consequently, the increase of pH values between 6 and 14 days was probably due to the degradation of organic matter and mineralization of organic nitrogen. So far, pH change indicated a pattern that was expected in regular compost systems. However, instead of a rapid decline, the pH values between 14 and 21 days remained almost unchanged, related to ammonia compensation due to *Micromonospora* sp. KSC08 addition (Fig. 5).

Mass reduction during organic matter degradation was not enough to explain the sharp increase in total nitrogen % values (Fig. 5). pH values varied between 8.3 and 8.6 during the thermophilic stage of composting, and these

Table 2 Enzyme tests applied to *Micromonospora* spp

Code	N fixation	Starch deg	Cellulase deg	IAA production	Caseinase activity
KSC06	+	+	-	–	–
KSC07	+	+	-	-	-
KSC08	+	+	-	+	+
KSC09	+	+	-	+	-
KSC37	+	+	-	+	-
KSC38	+	+	-	+	-
KSC41	+	+	-	-	+
KSC43	+	+	-	–	–
KSC50	+	+	-	+	-
VYN17	+	+	-	–	–
SLV02	+	+	-	+	-
SLV11	+	+	-	-	+
GBT09	+	+	-	–	–

+, pozitif; -, negatif; –, not growth

values established a suitable condition for the high release of ammonia. Temperature values in the thermophilic stage were just a little higher than the threshold for nitrification, implying low transformation of ammonia via nitrification. Hence, this sharp increase in total nitrogen values depended on an external effect changing the nitrogen balance in compost mixtures.

The change of nitrate-nitrogen amount was formulated as follows:

$$\begin{aligned}\textbf{N.N.21day} =& 24.09 + 2.48\ \text{M.A} + 17.80\ \text{A.T (day)} \\ & - 5.45\ \text{OP \%} - 4.25\ \text{M.A*M.A} \\ & + 4.04\ \text{A.T (day)*A.T (day)} \\ & - 6.93\ \text{OP \%*OP \%} + 1.48\ \text{M.A*A.T (day)} \\ & + 1.65\ \text{M.A*OP \%} - 0.95\ \text{A.T (day)*OP \%}\end{aligned}$$

Analysis results revealed the effect of addition time and olive pomace% on nitrate-nitrogen % change during 21 days of composting (Table 3). The increasing effect of addition time on nitrate-nitrogen change was expected. However, the irrelevance of microorganism amount on nitrate-nitrogen change was an interesting result. Olive pomace values varying between 20 and 40% with the latest addition of microorganisms resulted in the greatest nitrate-nitrogen changes as observed from Fig. 6. Consequently, system 9 was further evaluated to explain the changes that occurred in nitrate-nitrogen levels during composting.

Nitrate-nitrogen levels changed according to a pattern similar to the % increase in total nitrogen levels (Fig. 5D). Temperature change indicated initialization of the thermophilic phase in the 10th day, and pH values increased between 6 and 14th days (Fig. 5B). This increase of pH during the thermophilic phase should be due to increased ammonia production through organic nitrogen mineralization. The decrease of pH values between days 14 and 21 was related to increased nitrification levels, and usually, an increase in nitrate-nitrogen levels should also be observed in traditional composting systems. However, that was not the case, and a decrease of nitrate-nitrogen was observed. This vice versa trend of nitrate-nitrogen % change implied

Table 3 Results obtained from variance analyses for the change of total C%, total nitrogen%, and nitrate-nitrogen%

Responses	Total C%		Total nitrogen		Nitrate-nitrogen	
Source	F-value	P-value	F-value	P-value	F-Value	P-Value
Model	14.99	0.001 <	29.24	0.001 <	20.81	0.000
Linear	19.38	0.001 <	48.59	0.001 <	56.60	0.000
M.A	**39.49**	**0.001 <**	**8.14**	**0.017**	2.95	0.116
A.T (day)	**11.78**	**0.006**	**129.17**	**0.001 <**	**152.51**	**0.000**
OP %	**6.86**	**0.026**	**8.45**	**0.016**	**14.32**	**0.004**
Square	14.32	0.001 <	37.49	0.001 <	5.10	0.021
M.A*M.A	**9.01**	**0.013**	2.05	0.183	2.39	0.153
A.T (day)*A.T (day)	0.47	0.507	0.36	0.563	2.16	0.172
OP %*OP %	3.44	0.093	**42.55**	**0.001 <**	**6.36**	**0.030**
2-way interaction	11.26	0.002	1.64	0.241	0.75	0.549
M.A*A.T (day)	**29.66**	**0.001 <**	2.82	0.124	0.84	0.380
M.A*OP %	3.84	0.078	1.96	0.192	1.05	0.330
A.T (day)*OP %	0.28	0.606	0.15	0.704	0.35	0.568
Lack-of-fit	4.65	0.058	1.13	0.448	3.05	0.123
R-sq (%)	93.10		96.34		94.93	
R-sq (adj) (%)	86.88		93.04		90.37	
R-sq (pred) (%)	37.60		80.60		58.45	

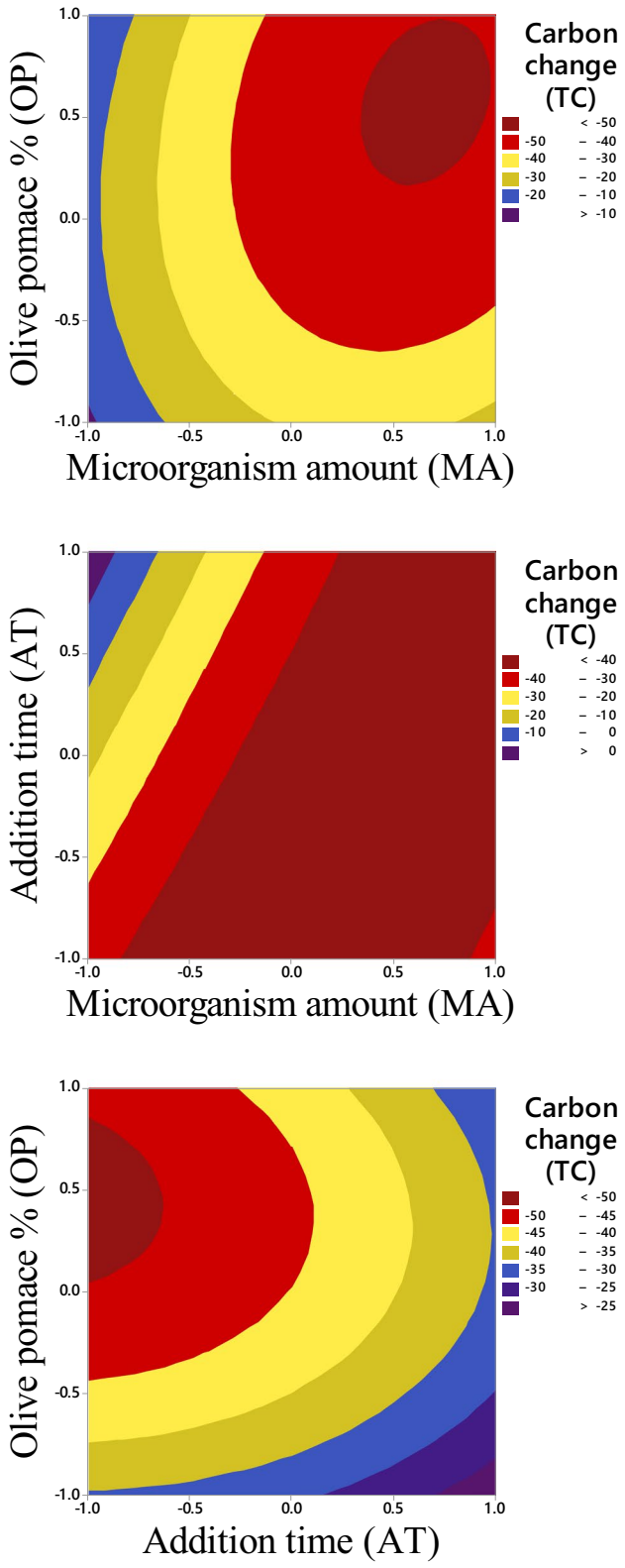

Fig. 2 2D contour plots for total C changes in compost systems

a different pattern affecting the balance between ammonia and nitrate-nitrogen. Ammonia production could increase during the thermophilic phase, which would emanate from organic nitrogen mineralization during this stage. The temperature level remained very stable at values higher than 40 °C during the thermophilic phase, which would inhibit nitrification. Considering that total nitrogen levels should remain the same, the mass balance dictates a decrease of nitrate-nitrogen during the thermophilic stage, which was also not the case. In other words, the changing trends of nitrate-nitrogen between 6–14 and 14–21 days should be the opposite to those expected in regular composting systems. Organic matter degradation could explain the increase of total nitrogen levels up to a certain point.

On the other hand, an increase of nitrate-nitrogen levels between days 14 and 21 should be observed if organic matter degradation was solely responsible for the changes. As seen from Fig. 5D, a vice versa trend in nitrate-nitrogen levels was observed. Total nitrogen changes between 14 and 21 days was negligible, and the decrease of nitrate-nitrogen level in this interval should be due to the increase in ammonia production. The temperature interval between 14 and 21 days indicated the termination of the thermophilic phase, and nitrification would increase during this period. In our opinion, that was also true for our system since composting has proceeded as expected. However, the vice versa trend of nitrate-nitrogen levels implied the simultaneous presence of both organic nitrogen mineralization and nitrification, which was impossible without an outside nitrogen source. This source, in our opinion, was *Micromonospora* sp. KSC08, which acquired nitrogen from the atmosphere. It would be fair to state that organic nitrogen mineralization and nitrification had occurred in accordance during composting based on the effect of statistically shown latest addition.

3.4 Determination of compost maturity

The results obtained from statistical analyses should be carefully interpreted to select a single system for maturity evaluation. Total C changes indicated the effect of microorganism on activity provided that a threshold had been used in systems. Higher microorganism amounts had a negligible effect on composting. Addition time, on the other hand, indicated a variant effect for total C, total N, and nitrate-nitrogen amounts. Latest addition times were effective for total and nitrate-nitrogen increase while earliest addition of microorganism increased microbial activity. Olive pomace higher than 20% was required in the systems to obtain highest microbial activity along with the highest total and nitrate-nitrogen amounts. The effect of microorganism amount on total nitrogen was statistically shown, yet any amount of microorganism could have been employed based on the results. Consequently, addition time should be maintained as high as possible to achieve 2

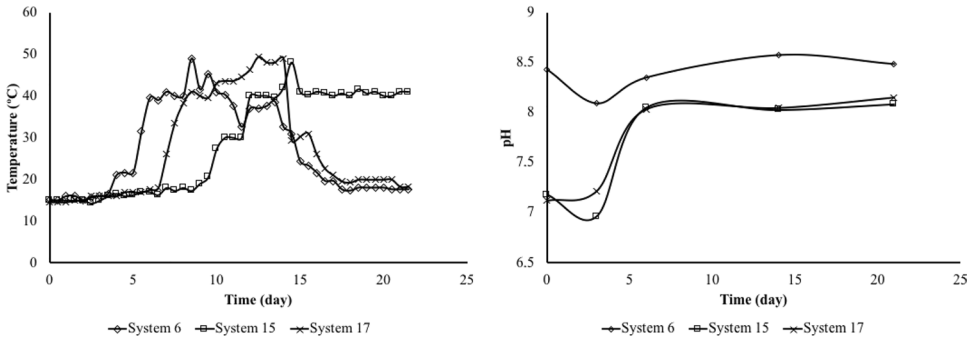

Fig. 3 **A** Temperature and **B** pH changes of selected systems

of three goals which were the increase in total and nitrate-nitrogen amounts (1). It would also be logical to apply medium olive pomace % (0) in systems. The choice of microorganism amount should also be medium (0) to achieve both microbial activity and increase of total and nitrate-nitrogen levels (see Supplementary File Table S6 for parameter coding). Hence, system 9 which was previously evaluated for total and nitrate-nitrogen changes was given as an example for maturity validation. Analyses were conducted on samples acquired at the 21st day of experiment.

Compost maturity was evaluated by a series of analyses illustrated in Table 4. Results indicated that selected system had met the parameters required for maturity and harmlessness [38]. Sampling was conducted in the 21st day of the experiment and results were promising considering relatively low duration of the compost mixture. Olive pomace, as previously stated, was not adequate for direct use in soil. On the other hand, composting in the presence of *Micromonospora* sp. KSC08 enabled olive pomace removal.

System 9 was kept for 120 days for determination of germination index (GI) values. Two separate analyses with triplicate runs were conducted to ensure technical and biological repeatability. Seeds were also inoculated in compost extracts with the intent to illuminate the possible toxic effects of microorganisms inside the extract. A GI value of 80% or higher revealed the absence of phytotoxicity [31, 39] which was validated with the results obtained for both regular and inoculated seeds. Although higher GI values were observed in the case of inoculation, the change of root length was statistically insignificant. Nevertheless, results implied accumulation of microorganism promoting the growth and a detailed future research elaborating the effects of compost microorganism on the seed be conducted.

3.5 Determination of biochemical constituents in matured compost

Biochemical constituent analyses of both UT and MOP compost groups are presented in Figs. 7, 8, and Fig S2 (Supplementary File). In this context, the PCA model was developed on the infrared (IR) spectral dataset to discriminate the UT group from the MOP one. As shown in the scores plot in Fig. 7A, the MOP group located on the left side of the plot along PC1 was separated from the UT group located on the right side. The major variation (PC1 + PC2) in data was 99%; however, only PC1 was counted in the analyses as 98% of throughput ratifying the model was represented by PC1. A loadings plot of PC1 indicated that the discrimination seen in the scores plot had emerged from variations in prominent and sharp negative discriminators/peaks. These variations represented the specific biomolecule changes (Fig. 7B). The variance plot indicating the almost identical calibration and validation plots for all PCs is provided in Fig. 7C, to demonstrate the accuracy of the model and the discriminatory potency of PC1 (98%).

The biochemical constituents of mature compost were studied based on quantification of absolute intensities, i.e., the concentration of spectral marker bands indicative of lignocellulosic and organomineral components. As shown in Fig S2, these marker bands were specifically chosen to track down the utilization and/or production of major agriculturally important biomolecules found in matured compost. The spectral bands located at 1733 cm^{-1}, 1450 cm^{-1}, and 1511 cm^{-1} were assigned for hemicellulose (C = O stretching/ester groups) [24], cellulose (in-plane bending vibrations of the CH2 groups) [39], and lignin (C = C stretching) [40], respectively (Fig S2). The reduction in the concentration of these generally so-called lignocellulosic components and also nitrate content (band at 1385 cm^{-1} for N–O vibrations in nitrate) [41] was significant in the MOP group compared to UT compost (Fig. 8). On the other hand, the amide (bands at 1636 cm^{-1} and 1626 cm^{-1} for Amide I) [42, 43] and silica mineral (huge band at 1029 cm^{-1} for Si-0-Si vibrations) [43, 44] contents (specified here as organomineral components) were significantly increased in the MOP group. In other words, *Micromonospora* sp. KSC08 treatment encouraged other soil microorganisms to decompose

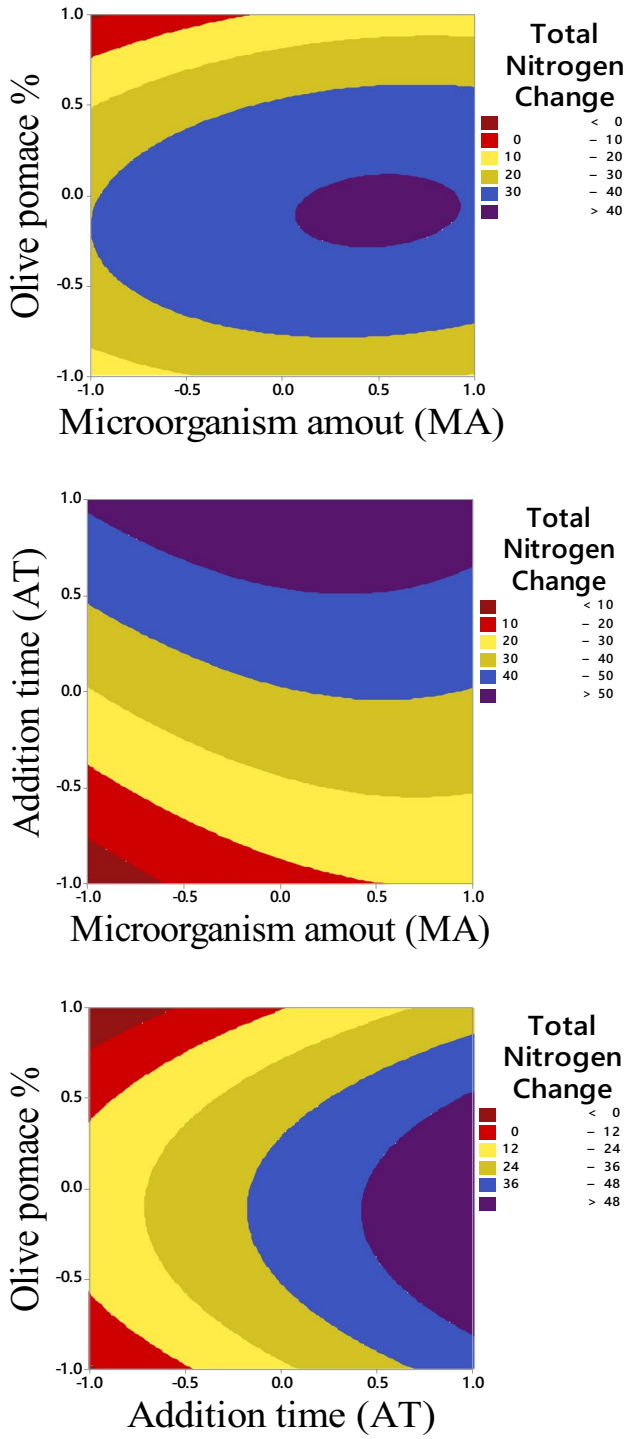

Fig. 4 2D contour plots for total N changes in compost systems

the lignocellulosic components in favor of the increase in the concentration of organomineral components.

In nature, lignocellulose accounts for a major part of biomass and, consequently, its degradation are essential for the operation of the global carbon cycle. Lignocellulose, such as wood, is mainly composed of a mixture of cellulose, hemicellulose, and lignin. Lignin is an integral cell wall constituent, which provides plant strength and resistance to microbial degradation [45]. It is known that the chemical degradation of lignocellulosic components yields carboxylic acids [46–48] and CO_2 [46]. Besides, many soil bacteria and fungi were shown to accomplish the biological solubilization of lignin by producing certain enzymes [45, 49]. The process involves complex reactions and generates different carboxylic acid units [50]. Ammonia's classical condensation reaction (ammonolysis) with carboxylic acids is known to give amide formation [51].

The supplemented bacteria (*Micromonospora* sp. KSC08) probably promoted the microbial degradation of lignocellulosic components and nitrate in the soil, yielding carboxylic and nitrogenous products, respectively [52, 53]. The measured reduction in nitrate can be related to the synthesis of organic nitrogen via fractional immobilization of ammonia and/or incomplete denitrification process since it is known that the ammonium/ammonia transformation is mutual [54]. It was also reported that the high content of organic ingredients inhibits the ammonia oxidation stage of the nitrification process [55]. In turn, the amides might be generated as an end-stage by-product due to the ammonolysis reaction between carboxylic acids and nitrogenous compounds/ammonia. Enhanced production of amides and reduction in carboxylic acids were previously shown in decomposed (10 weeks) manure [42]. Silica is considered a mineral and nutrient needed for soil health and sustainable agriculture, especially for rice production [56]. The increased bioavailability of silica minerals in MOP compost is a favorable condition possibly achieved due to system's pH [57]. A clay-like matter of the silica-rich manures provides a base to assemble aggregates. Clay minerals, including silica, also play critical functions in the catalysis of abiotic polymerization and stabilization of humic substances [58].

The eventual process of bio molecular turnover could also be related to the circulation of different nitrogen forms through the natural ecosystem. The biochemical transformation of the inert atmospheric nitrogen into a biologically active state to utilize lifeforms is defined as a nitrogen cycle. The process is quite complicated and is generally accomplished through several essential phases. Briefly, the first phase is called a nitrogen fixation process accomplished via certain nitrogen-fixing bacterial species. The inert nitrogen gases precipitate into the soil and aqueous environments and

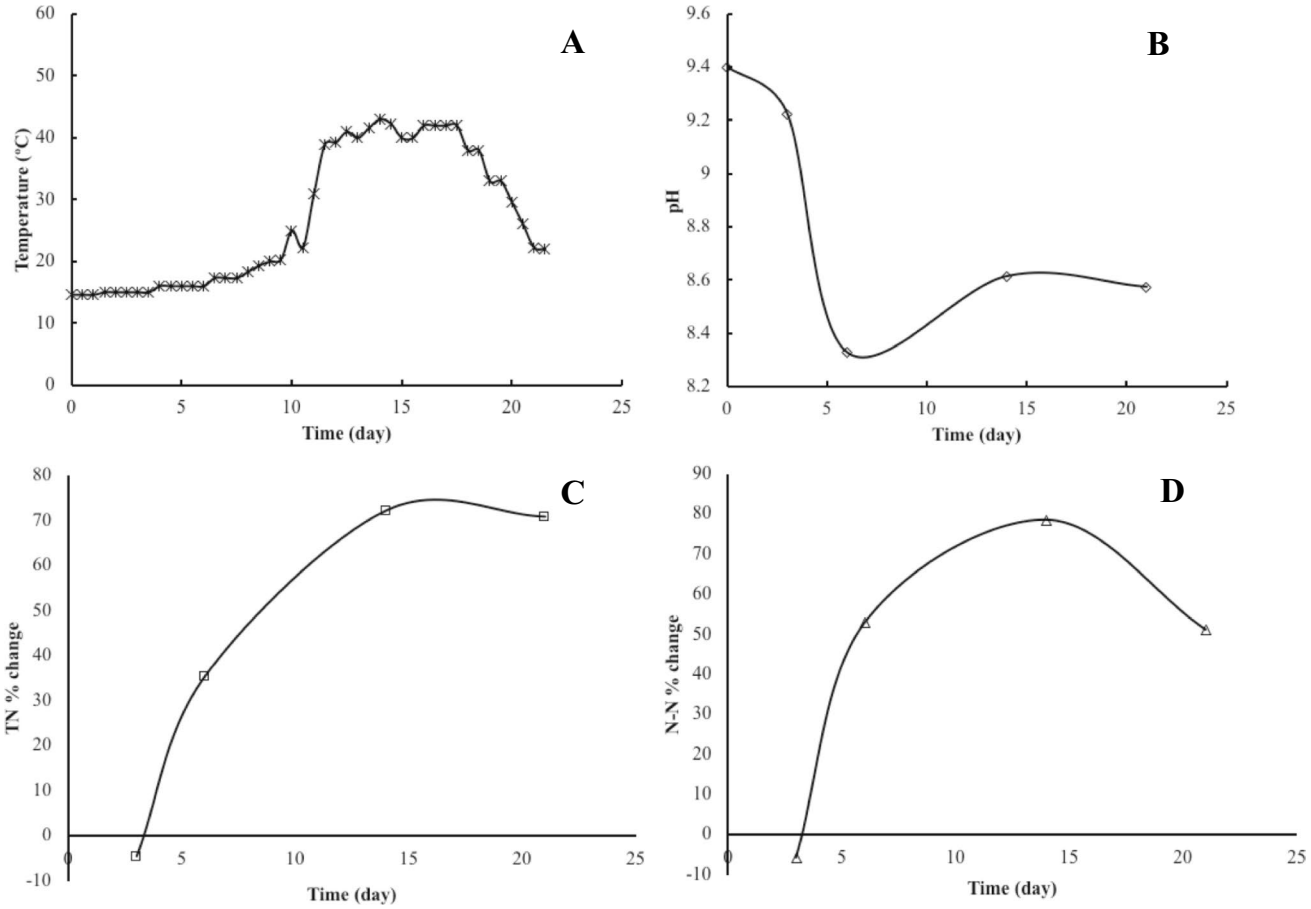

Fig. 5 **A** Temperature, **B** pH, **C** total nitrogen %, and **D** nitrate-nitrogen % changes for system 9

are subsequently converted into ammonium. In other words, ammonium is a usable form of nitrogen gas that can be used by living organisms. During the nitrification phase governed by aerobic nitrifying bacteria in soil, the ammonium is first oxidized into a very toxic nitrite form and later to a non-toxic nitrate form. In the assimilation phase, photosynthetic plants, the leading creators of organic matter, assimilate the different nitrogenous substances (ammonia, nitrite, nitrate, and ammonium ions) from the rhizosphere to produce chlorophylls needed for the realization of photosynthetic activities. The organic molecules such as amino acids and nucleic acids are also synthesized from plants' soil, ammonium, and nitrate. After animals and/or plants' death, the soil bacteria and fungi decompose the organic materials present in these organisms back into ammonium. The re-produced ammonium in this ammonification phase is utilized in different biological activities in soil. Finally, the denitrification phase happens when the nitrogenous substances are anaerobically recycled into the atmosphere by denitrifying bacteria, primarily metabolizing the soil nitrate to acquire oxygen.

During this biochemical reaction, the nitrogen is released into the air as a bacterial by-product. As an assumption, the outside nitrogen sources might be reacted with organic substances to form amide structures. The increased concentrations of amides are presumably due to this phenomenon. In other words, during the first stage of maturation (21 days), one part of the externally supplied nitrogen sources might be utilized in ammonium production. In contrast, the other contributors to the system were via nitrate formation. The amide formation probably caused the remaining part of the externally supplied nitrogen at 120 days of maturation. In this aspect, the clear-cut distinction of UT and MOP groups from each other was based on the dissimilarities of biochemical structures in PCA scores and loadings plots, and the accuracy of the developed model in the PCA variance plot can be considered as rational. Indeed, this phenomenon and the assumption put forward above requires further experimental proof and deserves a more comprehensive future investigation.

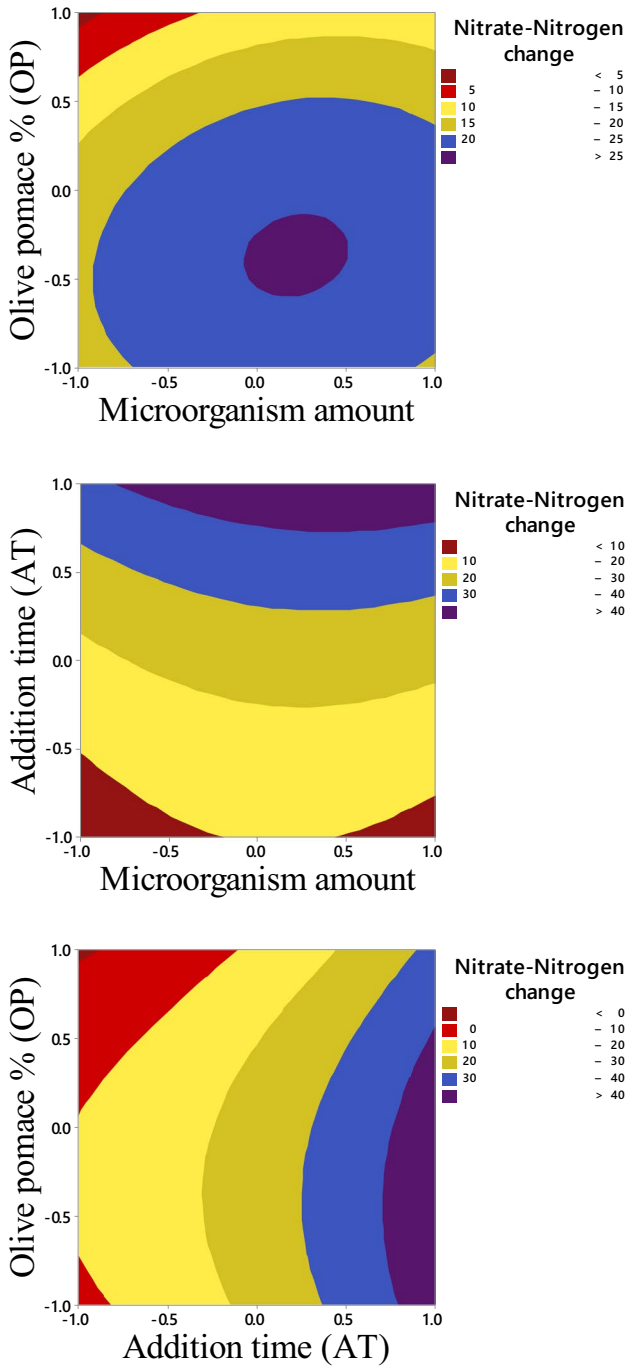

Fig. 6 2D contour plots for nitrate-nitrogen changes in compost systems

Table 4 Evaluation of compost maturity for selected system

	System 9	Threshold
pH	8.6	5.0–9.0[1]
EC (mS/cm)	2.64	< 3[1]
Organic matter (%)	57.1	≤ 40[2]
Organic C (%)	14.74	
Humic + fulvic acid	30.52	
Total nitrogen (%)	2.89	> 1[1]
Nitrate-nitrogen (%)	1.88	
C/N ratio	11.4*	< 25[1]
T value	0.56	
GI % 1st trial	110	> 80%[3]
GI % 2nd trial	149	> 80
GI % 1st trial (inc.)	133	> 80
GI % 2nd trial (inc.)	155	> 80

[1]Martinez-Salgado et al. 2019

[2]Chen et al. 2019

[3]Jagadabhi et al. 2019

4 Conclusions

Micromonospora sp. KSC08 inoculation to compost systems was proposed as an amendment to achieve nitrogen regulation and enhance microbial activity. This microorganism had higher growth rate in the absence of a nitrogen source and therefore had a potential to fix nitrogen from atmosphere. Nitrogen fixation from atmosphere provided a fundamental support to the life cycle of the microbial flora in need of biological nitrogen. Hence, an increase in microbial activity of compost mixture due to *Micromonospora* sp. KSC08 addition should be expected. The increase in microbial activity was validated with the highest change in total C with the earliest microorganism addition. *Micromonospora* sp. KSC08 was introduced as an asymbiotic free-living-(N_2)-fixing microorganism, and theoretically, this microorganism would act as an outside nitrogen source contributing to ammonia formation during composting. In regular composting systems, pH values normally decrease during thermophilic phase due to gradual NH_3 volatilization. However, in systems inoculated with *Micromonospora* sp. KSC08, pH values were stable during thermophilic phase. This result combined with statistically shown increase in total nitrogen indicated the presence of an outside nitrogen source compensating the loss of nitrogen during composting. The presence of *Micromonospora* sp. KSC08 as an exogenous nitrogen source was further validated with opposite trends of total nitrogen and nitrate-nitrogen % values during composting. The % ratio of nitrate-nitrogen, which should increase in regular systems during composting, decreased due to NH_3 addition via *Micromonospora* sp. KSC08.

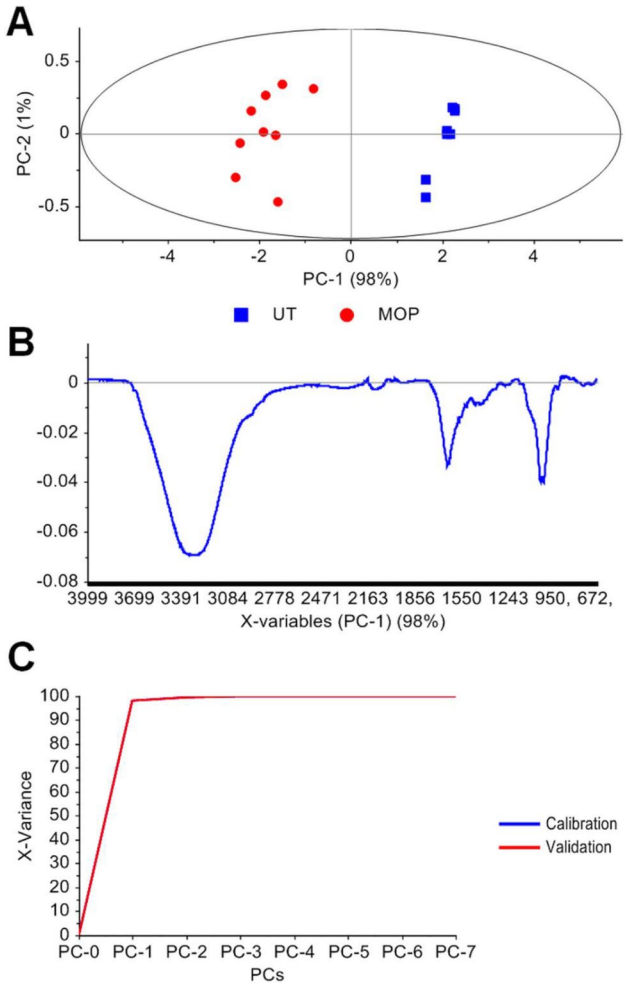

Fig. 7 Chemometric/PCA model for composts obtained after 120 days of maturation. **A** Scores plot, **B** loading plot, and **C** explained variance plot of PCA model obtained at the 4000–650 cm^{-1} infrared spectral region in untreated (UT) and microbially treated (MOP) composts

As a result of microbial activity, lignocellulosic components were involved in a serious of complex reactions resulted in generation of carboxylic acids which further reacted with nitrogenous compounds to generate amides as an end-stage by-product. PCA models revealed a clear-cut distinction of biochemical structures between *Micromonospora* sp. KSC08-treated and regular compost systems. The dissimilarity of biochemical structures at the end of 120 days was related and shown to be due to amide formation in *Micromonospora* sp. KSC08-treated system. A total of 120 days of composting was more than enough to achieve gradual completion of composting and the increase in amide and silica mineral contents in compost system inoculated with *Micromonospora* sp. KSC08 was concluded to be an additional validation of its effect on long-term microbial activity. The increase of amide structures in *Micromonospora* sp. KSC08 added systems

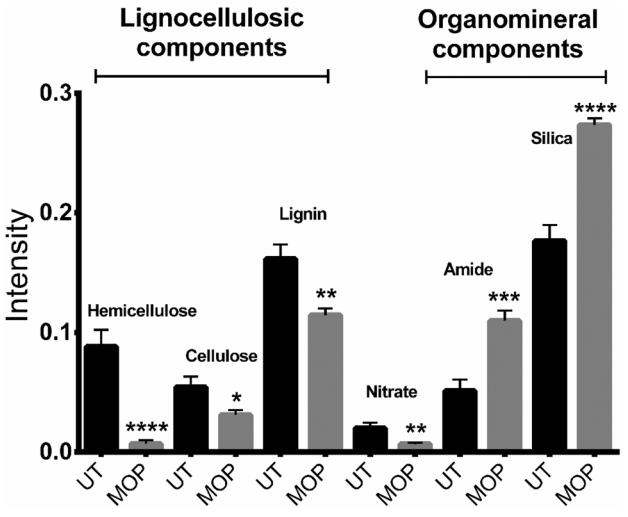

Fig. 8 Quantitative analysis of biochemical constituents in untreated (UT) and microbially treated (MOP) composts after 120 days of maturation

could also be attributed to the increase in nitrogenous compounds. Hence, it was concluded that *Micromonospora* sp. KSC08 had been effective both in composting and nitrogen regulation. This microorganism was concluded to be a suitable candidate for use as a member of microbial consortium in conventional composting systems.

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Data availability Not applicable.

Code availability Statistical analyses were conducted via Minitab software. OPUS 5.5 (Bruker, USA) software was utilized in the analysis of spectral data. Principal component analysis (PCA) was done by Camo Software, NO.

Declarations

Ethics approval Not applicable.

Consent to participate All authors agreed with the content of the work.

Consent for publication The present work was submitted based on considerations given in authorship principles of the journal.

Conflict of interest The authors declare no competing interests.

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