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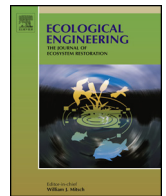
Nitrification of vegetable waste using nitrifying bacteria

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ABSTRACT

Current inorganic chemical-based fertilizers for soils have adverse impacts on human health and environment. In the present work, two nitrifying bacteria, i.e. *Nitrosomonas* sp. DSM 28437 and *Nitrobacter* sp. DSM 10236, were explored for the production of nitrate in vegetable wastes. For this purpose, the nitrite production by *Nitrosomonas* sp. and nitrate production by *Nitrobacter* sp. in two synthetic media containing ammonium and nitrite, respectively were investigated. Also, simultaneous growth of both bacteria in a single synthetic medium containing ammonium was investigated. Finally, the performance of both bacteria in a mixture of vegetable wastes, where a consortia of microorganisms present in cattle-based compost produced ammonium, were investigated. Highest concentration of nitrite (13.2 ppm) and nitrate (1600 ppm) in *Nitrosomonas* sp. and *Nitrobacter* sp. inoculated synthetic media were achieved after 12 and 28 days, respectively. The simultaneous growth of both bacteria in synthetic media and both bacteria along with compost microorganisms in vegetable wastes resulted in 12 and 20 ppm nitrate, respectively. Also, it was observed that with increasing time, the optical density of *Nitrobacter* sp. population increased from 0.04 to 0.72 after 35 days, but for *Nitrosomonas sp.,* until 12 days, the optical density increased 0.3 from to 0.6 and then decreased to 0.2 on day 21 because of nitrite toxicity to the microorganism. The transformation of ammonium to nitrate using *Nitrosomonas* sp. and *Nitrobacter* sp. in synthetic media and a mixture of vegetable wastes was studied to develop a new organic fertilizer containing nitrogen source.

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1. Introduction

Conventional agriculture has led to an increasing dependence on pesticides and chemical fertilizers to address the food demands of the ever-growing human population (Santos et al., 2012; Tu et al., 2006). Pollution of air and water media, such as eutrophication of water bodies is caused by overuse of such chemical fertilizers enriched with known quantities of nitrogen, phosphorus and potassium, as well as excessive quantities of animal manure (Bhardwaj et al., 2014). For example, around 13%–28% of nitrogen used for growing crops is released to the atmosphere (Liu et al., 2014). Although chemical fertilizers may make a country self-dependent in agricultural products, but it disturbs the environment with its harmful impacts on living organisms. Furthermore, using excess amounts of chemical fertilizers is costly and can lead to depletion of water holding capacity of soils and

disparity in nutrients (Bhardwaj et al., 2014; Raja 2013; Youssef and Eissa 2014). Therefore, developing some affordable, effective and environmentally-benign fertilizers has been the focus of researchers for long time. Nowadays, some species of microorganisms are broadly used due to their significant contribution to providing a natural substitute to chemical fertilizers (Megali et al., 2014; Sahoo et al., 2014). Encouraging organic farming by using biofertilizers ensures food safety and improves the biodiversity of soils (Araújo et al., 2008).

One of the most important processes in natural fertilization process is nitrification in which gaseous nitrogen is transformed into ammonia and then to nitrite and finally to nitrate which is the preferred form of nitrogen for plants. This process is carried out through a complex, sensitive and slow set of reactions and the involved microorganisms require strict aerobic conditions. Therefore, selection of suitable strains and providing related conditions based on the nature of the substrate are necessary (Subbarao et al., 2006, 2009).

Nitrosomonas sp. and *Nitrobacter* sp. are important components of the soil microbial population that play major role in

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biological conversion of ammonia to nitrite and then to nitrate (Norton et al., 2002). Verhamme et al., oxidized ammonia to nitrite by both ammonia oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) by incubating soil samples for 28 days with different ammonium concentrations (0–20 μg NH_4^+-N per gram of soil). Their analysis showed considerable growth of AOA at all concentrations, whereas AOB growth was important only at higher concentration (Verhamme et al., 2011). Grunditz and Dalhammar used *Nitrosomonas* sp. and *Nitrobacter* sp. isolated from activated sludge. The highest activity was reported to be at pH 8.1 and 35 °C for *Nitrosomonas* sp. and pH 7.9 and 38 °C for *Nitrobacter sp.* (Grunditz and Dalhammar, 2001). Blackburne et al., investigated the capability of *Nitrospira* sp. and *Nitrobacter* sp. for transformation of nitrite to nitrate. They found that *Nitrospira* had lower inhibition threshold concentrations for free nitrous acid (<0.03 ppm) and free ammonia (0.04–0.08 ppm) compared to *Nitrobacter* sp. (Blackburne et al., 2007).

Agro-wastes, such as manures, hulls, leaves, plant stalks and vegetable residues are produced from different agriculture and food processing activities, such as dairy production, protein extraction and brewery. The agro-wastes, such as brewery spent grains, cheese processing residues and soy waste are produced in large volumes. They are often useless for farmers and companies and they will often be discarded representing a cost for producers (Lim and Matu 2015). Agro-wastes contain water soluble compounds e.g., sugar and amino acids and insoluble compounds e.g., cellulose and lignin. These compounds are the main natural source of organic matter in soil (Caprara et al., 2011). Therefore, due to their low cost, no adverse environmental impacts because of their biodegradability and richness in wide range of nutrients, the agro-wastes have a potential to be used by farmers to improve the fertility of soil.

To the best of our knowledge, there is no paper discussing the performance of nitrifying bacteria in vegetable waste samples. In this research work, the performance of *Nitrosomonas* sp. and *Nitrobacter* sp. in transformation of ammonium to nitrite and nitrite to nitrate were investigated in synthetic media and a mixture of vegetable wastes (a combination of soya, brewery, and whey) in order to develop a new organic fertilizer.

2. Material and methods

2.1. Strains and growth condition

An enriched *Nitrosomonas europaea* (DSM 28437) and *Nitrobacter vulgaris* (DSM 10236) culture were obtained from Leibniz-Institut (DSMZ-Deutsche Sammlung von). The composted cattle manure mixed with garden soil (Nitrogen 0.5%, Phosphate 0.5%, Potash 0.5%) was purchased from Circle H Farms (USA). *Nitrosomonas europaea* was grown on 1583 medium for ammonia oxidizing bacteria that had the following composition:

Solution A: NH_4Cl, 535 mg; KH_2PO_4, 54 mg; KCl, 74 mg; $MgSO_4 \times 7H_2O$, 49 mg; $CaCl_2 \times 2H_2O$, 147 mg; NaCl, 584 mg; Trace element solution, 1 mL; Cresol red solution, 2 mL; Distilled water, 1000 mL.

Trace element solution: Distilled water, 975 mL; HCl, 1 M 25 mL; $MnSO_4 \times 4H_2O$, 45 mg; H_3BO_3, 49 mg; $ZnSO_4 \times 7H_2O$, 43 mg; $(NH_4)_6 Mo_7O_{24} \times 4H_2O$, 37 mg; $FeSO_4 \times 7H_2O$, 973 mg; $CuSO_4 \times 5H_2O$, 25 mg.

Cresol red solution: Cresol red, 50 mg; Distilled water, 100 mL.
The pH was adjusted to 7.8 by addition of 10% $NaHCO_3$.

Nitrobacter vulgaris was grown on 756 Heterotrophic nitrobacter medium that had the following composition:

Solution A: 2 g $NaNO_2$, Yeast extract, 1.50 g; Peptone, 1.50 g; Na-pyruvate, 0.55 g; Trace element solution, 1 mL; Stock solution, 100 mL; Distilled water, 899 mL.

Stock solution: $CaCO_3$, 0.07 g; NaCl, 5 g; $MgSO_4 \times 7H_2O$, 0.5 g; KH_2PO_4, 1.5 g; Distilled water, 1000 mL;

Trace element solution: $MnSO_4 \times H_2O$, 33.8 mg; H_3BO_3, 49.4 mg; $ZnSO_4 \times 7H_2O$, 43.1 mg; $(NH_4)_6Mo_7O_{24}$, 37.1 mg; $FeSO_4 \times 7H_2O$, 97.3 mg; $CuSO_4 \times 5H_2O$, 25 mg; Distilled water, 1000 mL.
The pH was adjusted to 7.4 with NaOH or KOH.

2.2. Growth in standard media

The culture media was prepared as described in Section 2.1. Each strain was revived two times in 150 mL liquid standard media at 28–30 ± 1 °C and 150 rpm for 3 days. Also, compost was inoculated into two culture media to compare with the strains. The optical density (OD_{600}) and colony forming units (CFU) per milliliter were measured after second revival for both strains and compost in the two media.

2.3. Resistance to high ammonia concentration

Five flasks containing 100 mL of media 1583 without NH_4Cl and with different concentration of ammonia i.e. 100, 250, 500, 750 and 1000 ppm were prepared and autoclaved at 121 ± 1 °C for 20 min. Later, they were inoculated with three ml of *Nitrosomonas* sp. inoculum and incubated for six days at 37 ± 1 °C and 175 ± 1 rpm. The concentration of nitrite and ammonium were measured after 6 days and the tests were done in duplicates. These tests were carried out to find maximum concentration of ammonia that *Nitrosomonas* sp. can tolerate.

2.4. Kinetics study of nitrification

Media 1583 at 150 mL with optimized concentration of NH_4Cl (obtained from Section 2.3) was prepared in 250 mL flasks and autoclaved at 121 ± 1 °C for 20 min and the kinetics of growth, residual ammonium and production of nitrite for *Nitrosomonas* sp. were studied for three weeks. Similarly, 150 mL of media 752 was prepared in 250 mL flasks, autoclaved at 121 ± 1 °C for 20 min and the kinetics of growth and production of nitrate for *Nitrobacter* sp. were studied for five weeks. Also, kinetics of residual ammonium and production of nitrite in media 1583 inoculated with compost and production of nitrate in media 756 inoculated with compost were studied for three and five weeks, respectively.

2.5. Vegetable waste as growth substrate

In order to evaluate the potential of vegetable waste to be transformed into a useful fertilizer, vegetable wastes were used as substrate without autoclaving. The substrate was composed of 36% soya waste, 54% brewery waste and 10% liquid whey. The total nitrogen content of the substrate was 2.98 g/kg. The media were placed in two 2-L flasks and their volumes were adjusted to 1 L using milli-Q water. One flask was inoculated with 10 g compost. The second flask was inoculated with 10 g compost, 25 mL of *Nitrosomonas* sp. and 25 mL of *Nitrobacter sp.* The procedure was carried out in duplicate. During fermentation, media were aerated. After 1 week, the final concentrations of nitrate were measured.

2.6. Measurement of ammonium, nitrite and nitrate concentrations

To measure the ammonium, nitrite and nitrate, colorimetric titration was employed. Ammonium was determined following the modified method of indophenol in which phenol is combined with hypochlorite as it reacts in the presence of ammonium to form

Table 1
Concentration of ammonium, optical density and nitrite for *Nitrosomonas* sp.

Parameter	Unit	Value				
Initial ammonium	ppm	100	250	500	750	1000
Residual ammonium	ppm	80	240	480	740	980
Optical density (660 nm)	–	0.362	0.072	0.056	0.008	0.002
Produced nitrite	ppm	0.3	0.1	0.1	0.1	0.1

Table 2
Nitrite and nitrate production using *Nitrosomonas* sp. and *Nitrobacter* sp.

Days	Nitrite Production (ppm)	Nitrate Production (ppm)
0	0.1	–
4	0.3	–
8	6.4	–
12	13.2	–
18	–	5
24	–	7
30	–	10
35	–	12

indophenol blue. The color intensity is proportional to the amount of total ammonium present.

For nitrite, the method of modified diazotization was used. The sample reacted with 4-aminobenzensulfonic acid to produce varying red shades. The color intensity was proportional to the amount of nitrite present. Similarly, nitrate concentrations were determined by transforming it by using a reducing agent (Nutrafin TM) to produce a color with the intensity proportional to the amount present.

3. Results and discussion

3.1. Resistance to ammonia

The *Nitrosomonas* sp. was grown at six levels of ammonium concentration to observe its nitrification rate. The concentrations of residual ammonium and produced nitrite against initial concentration of ammonium are given in Table 1. The results showed higher growth of *Nitrosomonas* sp. at lower ammonium concentration (100 ppm) compared to intermediate or high levels of ammonium concentration (250–1000 ppm). Similarly, nitrification rate was higher at lower concentration of ammonium. It seemed that free ammonia inhibited the activity of *Nitrosomonas* sp. at a concentration higher than 100 ppm. Kim et al., and Yang et al., observed similar trends and reported that high free concentration of ammonia (NH_3-N) inhibited heterotrophic, nitrite oxidizing and ammonia oxidizing bacteria (Kim et al., 2006; Yang et al., 2004). Also, Vadivelu et al., observed that free ammonia up to 16.0 ppm, had no inhibitory effect on catabolic or anabolic processes of the *Nitrosomonas* sp. (Vadivelu et al., 2006). Due to high permeability of free ammonia through cell membrane, it was hypothesized that free ammonia may affect the cells by changing intracellular pH, increasing energy requirements, or inhibit their enzymatic reactions (Gallert and Winter 1997; Wittmann et al., 1995).

According to Table 2, the initial concentration of ammonium was adjusted to 100 ppm for the rest of the experiments.

3.2. Nitrite and nitrate production

The kinetics study of *Nitrosomonas* sp. and compost in media 1583 and *Nitrobacter* sp. and compost in media 756 were performed. The trends of ammonium consumption and production of nitrite for both compost and *Nitrosomonas* sp. in media 1583 are shown in Fig. 1A and B, respectively. According to Fig. 1A, concentration of nitrite continuously increased up to 8 ppm within the test period in compost inoculated medium. However, for *Nitrosomonas* sp. inoculated medium, a maximum nitrite concentration of 13.2 ppm was observed after 12 days. The maximum yield of nitrite production after 12 days was calculated to be 0.9 mg nitrite/mg ammonium. The reduction in production of nitrite can be attributed to the toxicity of nitrite for bacterial growth. Weon et al., observed same inhibitory effect during growth of *Acinetobacter* sp. and they reported that removing nitrite from cultures reversed the inhibitory effect. They attributed the toxicity of nitrite to its effect on bacterial membranes and energy generation (Weon et al., 2002). Also it is reported that pH affects this toxicity by formation of protonated species with capability of crossing the membrane (Anthonisen et al., 1976).

The variation of nitrate concentration within test period in media 756 inoculated with compost and *Nitrobacter* sp. are shown in Fig. 2. According to this figure, it took 35 and 28 days respectively for compost and *Nitrobacter* sp. inoculated media to reach to the maximum nitrate concentration of 1600 ppm. The maximum yield of nitrate production after 28 days was calculated to be 0.8 mg nitrate/mg nitrite. Guo et al., investigated the nitrification process in a 10 L sequencing batch reactor and observed decrease in ammonium and increase of nitrite and nitrate concentrations under aerobic condition. Finally, they achieved 25.7 ppm nitrate and 6.2 ppm nitrite (Guo et al., 2009). Grigatti et al., incubated soil, bark and manure with different volume of activated sewage sludge for 72 h. They found that the nitrate concentra-
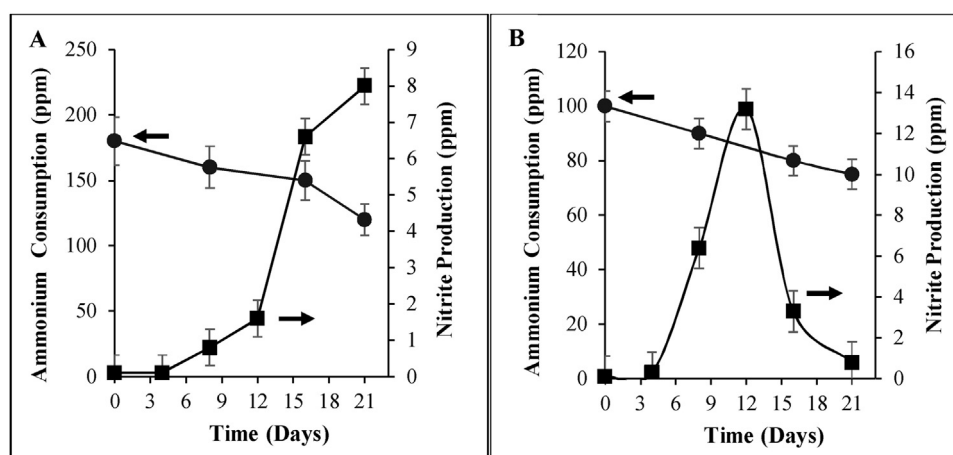

Fig. 1. Concentration of nitrite and ammonium at different times for: (A) compost; and (B) *Nitrosomonas* sp. in media 1583 (Circle and square legends represent ammonium consumption and nitrite production, respectively).
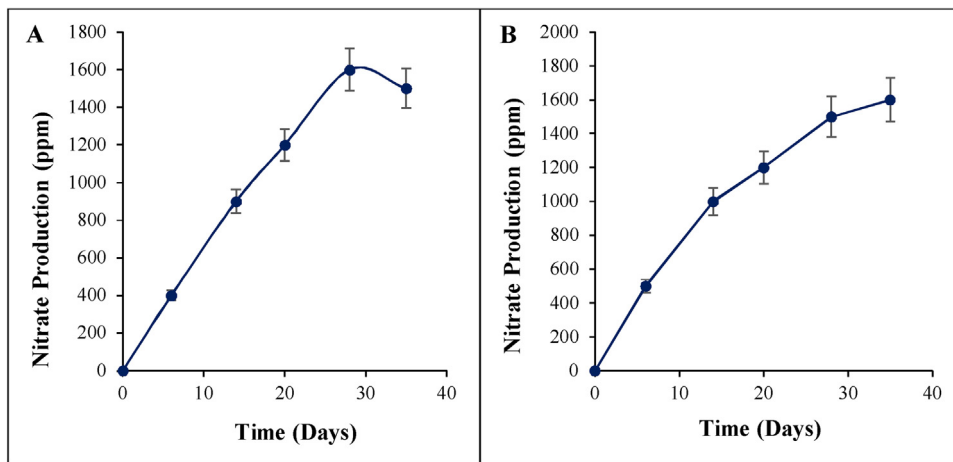
Fig. 2. Variation of nitrate concentration in media 756 inoculated with: (A) compost; and (B) *Nitrobacter* sp.

tion increased with increasing amounts of added sewage sludge. For soil, bark and manure incubated with 10 mL of sewage sludge, the total nitrate production was 5073, 4801 and 7530 mg N/kg volatile solids, respectively (Grigatti et al., 2007). Accordingly, the obtained result for nitrate production in this research was within the range obtained by other researchers though they used diverse microorganisms (present in sewage sludge) instead of using single microorganism.

3.3. Growth rate of bacteria

The effect of time on the growth rate of *Nitrosomonas* sp. medium 1583 and *Nitrobacter* sp. in medium 756 are illustrated in Fig. 3. For *Nitrosomonas* sp. (Fig. 3A), a maximum bacterial density was obtained in 12 days and soon after, the population of bacteria decreased significantly ($p < 0.03$). But for *Nitrobacter* sp. (Fig. 3B), the bacterial population reached a saturation point after 20 days.

The bacterial growth in batch system can be modeled with four distinguished phases i.e. lag phase (A), exponential phase (B), stationary phase (C), and death phase (D). In lag phase, cells are adapted to their new environment and in exponential phase, the cells multiply rapidly. The exhaustion of nutrients ($S \approx 0$) and build-up of metabolites and waste materials lead to stationary phase in which there is no net growth. In death phase, the number of living organism decreases, due to toxic metabolites and lack of nutrients

(Farges et al., 2012). For this phase, the balance of cell mass in a batch culture gives, by Eqs. (1) and (2):

$$\frac{dX}{dt} = \mu_{net}X \quad and \quad X = X_0 \, (at \, t = 0) \tag{1}$$

$$ln\frac{X}{X_0} = \mu_{net} \, (t - t_0) \tag{2}$$

Where μ_{net} is the maximum specific growth rate (1/time) and X_0 and X are cell concentration at time $t = 0$ and t. The parameter μ_{net} was calculated to be 0.1 and 1.1 (1/day) for *Nitrosomonas* sp. and *Nitrobacter sp.*, respectively.

3.4. Nitrification in medium 1583 with both bacteria

According to Section 3.2, the maximum nitrite and nitrate production were 13.2 and 1600 ppm in media 1538 and 756, respectively. The possibility of production of nitrate was investigated in medium 1583 so that after 12 days of inoculation of medium 1583 with *Nitrosomonas* sp., *Nitrobacter* sp. was added to the medium to convert nitrite to nitrate. The results of this experiment are shown in Table 2. According to this Table, the concentration of nitrate reached 12 ppm after 35 days.

For comparison, the nitrification test in medium 756 with *Nitrobacter* sp. was studied by setting the initial concentration of nitrite to 13.2 ppm and it was observed that the nitrate concentration reached 16 ppm after 35 days. Accordingly, the ratio
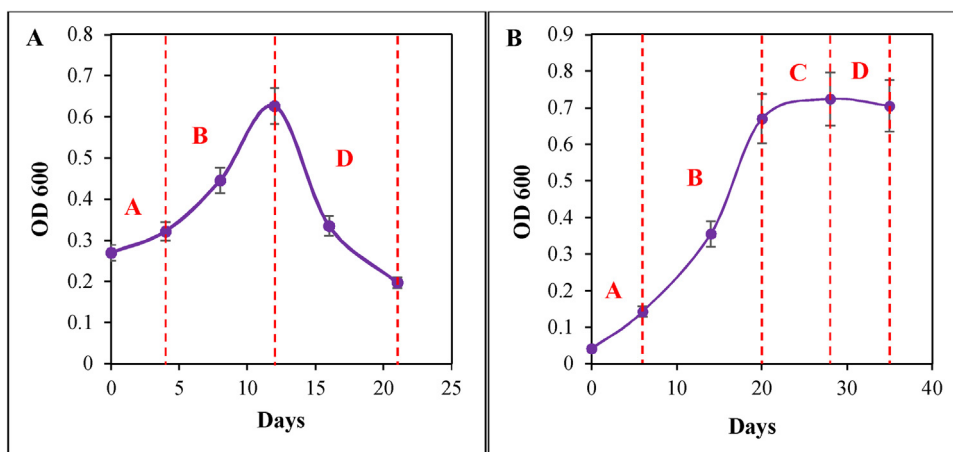

Fig. 3. Effect of storage time on the growth of: (A) *Nitrosomonas* sp.; and (B) *Nitrobacter* sp. (Lag phase (A), logarithmic or exponential phase (B), stationary phase (C), and death phase (D)).

Table 3
comparative performance of bacterial consortia in the production of soluble nitrate starting from different agro industrial waste combinations.

Substrate composition	Strains	Ammonium/Nitrate (mg)	mg N/g soil	Reference
Sorghum bagasse (88%) + pine bark (11%) + Urea (1%)	*Nitrobacter* sp. + *Nitrosomonas* sp.	0.1	–	Sánchez-Monedero et al. (2001)
Sorghum bagasse (86%) + pine bark (11%) + brewery sludge (3%)	*Nitrobacter* sp. + *Nitrosomonas* sp.	0.4	–	Sánchez-Monedero et al. (2001)
Rice straw and cow dung (5:1:0.2)	Geobacillus	–	1.6	Sarkar et al. (2010)
Dairy-waste compost or ammonium sulfate	Geobacillus	–	19.4	Sarkar et al. (2010)
Compost	Unidentified consortium	0.4	–	Shi et al. (2001)
Compost + ammonium sulfate	Unidentified consortium	0.1	–	Shi et al. (2001)
Soybean residues + with leaves + sawdust (1:1:3)	Unidentified consortium	25	–	Joseph et al. (2001)
Soya waste + brewery waste + whey (36%:54%:10%)	*Nitrobacter* sp. + *Nitrosomonas* sp.	0.35	20	Present study
Soya waste + brewery waste+ whey (36%:54%:10%))	Compost+ *Nitrobacter* sp. + *Nitrosomonas* sp.	0.7	13	Present study

of nitrite consumption to nitrate production was 1.48 mol/mol N which is within the range obtained by other researchers. Trigo et al., studied oxidation of ammonium in a sequencing batch reactor using Van de Graaf medium. Their results showed that the ratio of nitrite consumed to nitrate produced was 5.54 mol/mol N (Trigo et al., 2006). In a similar research, Farges et al., studied the performance of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in nitrification and observed that the ratio of nitrite production to ammonium consumption was 0.95 mol/mol N for *Nitrosomonas europaea* and the ratio of nitrite consumption to nitrate production was 0.975 mol/mol N for *Nitrobacter winogradskyi* (Farges et al., 2012). It seemed that the presence of *Nitrosomonas* sp. did not interfere the conversion of nitrite to nitrate by *Nitrobacter* sp.

3.5. Nitrification in vegetable waste

As explained in Section 2.5, the synthetic media was replaced by vegetable waste to study the nitrification performance of selected bacteria. After one week, the final concentrations of nitrate were 5 ppm and 20 ppm (Fig. 4) for samples inoculated with compost and compost/*Nitrosomonas sp./Nitrobacter* sp., respectively. At this point, the process stopped due to simultaneous production of

Fig. 4. Comparison of NO_3 and Sulfur production in ppm by commercial compost vs compost + *Nitrobacter* sp. and *Nitrosomonas* sp.

soluble sulfurous compounds that reached 11.8 and 30 ppm in each medium, respectively.

As shown in Fig. 4, the higher the nitrate production, there was higher the release of sulfur from the selected media mixture. In both cases, the release of sulfur was slightly higher than the nitrate. In the case of soybean, it is reported to have 5.7 ppm of sulfur content (He et al., 2009) while brewery wastes contains up to 20 ppm sulfate (Sudarjanto et al., 2011). Moreover, whey is reported to contain large amount of sulfur-containing amino acids. Since sulfur uptake by plants is approximately 0.25 times of nitrogen, it is recommended to avoid whey and maximize soya waste use in the media in order to obtain the best nitrate sulfur proportion. Accordingly, it is expected to yield up to 60 ppm nitrate. The final solution can be further concentrated to increase the nutrients per liter to match synthetic fertilizers. However, further studies are needed to achieve a maximum uptake by the targeted plants, otherwise the nutrients will end up in the environment which could generate ecological problems for several decades as recently reported (Meter et al., 2016).

From Table 3, it is possible to see that the addition of compost does not necessarily imply an advantage. As seen in the table, the higher the ammonium/nitrate, the less efficient the result. Since the used media was not sterilized prior to use, it already contained bacteria that were able to carry out the ammonification step needed before *Nitrobacter* can start producing nitrite. Moreover, the consortium that forms compost contains many other large bacterial populations than can lead to undesired results like the high release of sulfur contained in the amino-acids of the media that leads to nitrification arrest due to toxicity. In this sense, it is very important to ensure that the source used has a useful proportion of N/S as well as to control the participating bacterial strains in order to obtain a proper final concentration of nitrate to be used as fertilizer and avoiding by-products at the same time.

4. Conclusion

The transformation of ammonium to nitrite and nitrite to nitrate using *Nitrosomonas* sp. and *Nitrobacter* sp. in synthetic media and a mixture of vegetable wastes was studied to develop a new organic fertilizer containing nitrogen source. The results showed that the highest concentration of nitrite (13.2 ppm) and nitrate (1600 ppm) in synthetic media inoculated with *Nitrosomonas* sp. and *Nitrobacter* sp. were obtained after 12 and 28 days, respectively. Also, the presence of both bacteria in synthetic media and both bacteria with compost microorganisms in vegetable wastes resulted in 12 and 20 ppm nitrate, respectively. Therefore, vegetable wastes can be considered as a promising candidate for nitrogen rich bio-fertilizer.
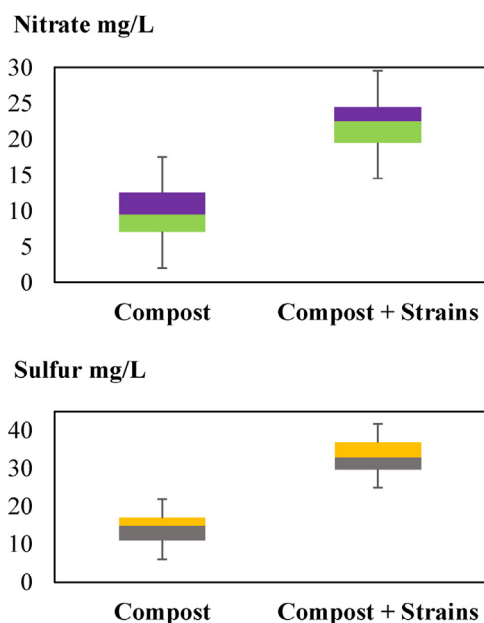
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