Research Article

Method for Rapid Labeling of Waste Sludge from a Food Factory with ^{15}N-Glycine and Evaluation of N Use Using Komatsuna (*Brassica rapa* Var. *perviridis*)

Mizuho Miura-Ido,[1] **Yuzuri Iwamoto** [iD]**,**[1,2] **Hiroi Kouya,**[1] **Yoshihiko Takahashi,**[1] **Amjad Hassan,**[1,3] **Norikuni Ohtake,**[1] **Hidetaka Hori** [iD]**,**[1,4] **and Takuji Ohyama** [iD]**[1,5]

[1]*Applied Bioscience, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan*
[2]*Department of Applied Chemistry and Bioscience, Graduate School of Engineering, Kanagawa Institute of Technology, Atsugi, Kanagawa 243–0292, Japan*
[3]*Biotechnology Program, Department of Environmental Science, COMSATS University Islamabad, Abbottabad 22060, Pakistan*
[4]*AAG Corporation, Daigiri, Fujisawa, Kanagawa 251-0002, Japan*
[5]*Department of Agricultural Chemistry, Graduate School of Agriculture, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan*

Correspondence should be addressed to Yuzuri Iwamoto; iwamoto@bio.kanagawa-it.ac.jp

The waste sludge from food factories has rich nutrients and useful material for fertilizer or animal feed, but quick treatments and recycling of the waste sludge are difficult due to its higher water content. We have developed a rapid composting system to make sludge fertilizer using mix of waste sludge and shredded newspaper (Sludge Fertilizer Made by Paper Mixing Method, SF-PMM). The mixture was incubated in a box reactor, continuously aerated with warm air around 35°C, and changed to mature SF-PMM, in only two weeks. To search movement of N from the SF-PMM to plants, we developed a new method to label small amounts of SF-PMM with ^{15}N-glycine. 50 L of wastewater from a food factory was incubated with 1 L of active sludge and 3 g of ^{15}N-glycine (98 atom% ^{15}N), and 175 g of labeled sludge was obtained in a day. This sludge was mixed with 25 g of newspaper chips, packed between two steel meshes, and placed at 20 cm depth in the reactor composting 200 kg of unlabeled sludge-paper mixture. Composting was restarted, and after about 7 days of reaction, ^{15}N-labeled SF-PMM 7.03 atom% ^{15}N was obtained. The surrounding unlabeled compost contained 4.0, 4.0, and 0.8% of N, P_2O_5, and K_2O, respectively. C/N and pH were 10 and 7.4, respectively. Komatsuna (*Brassica rapa* var. *perviridis*) was cultivated in a pot with 50 and 100 mg N of SF-PMM, and healthy plants were obtained as in the Control experiments containing 50 mg N ammonium sulfate. No growth inhibition was observed in these experiments. Even in 100 mg SF-PMM, excellent growth of the roots was observed. About 56% of the N in the plant was shown to come from ^{15}N-SF-PMM, and about 6% of the total^{15}N in the ^{15}N-SF-PMM was also shown to be incorporated into the plant.

1. Introduction

In 2018, in Japan, 376 million tons (Mt) of industrial waste was disposed of, and in almost recent 20 years, 1996 to 2018, about 380~430 Mt of industrial waste has been disposed of every year [1, 2]. These industrial wastes contain around 45% of sludge containing nonorganic materials and various heavy metals, so that the reduction of the disposition of the sludge is important issue for the sustainable industries.

On the other hand, in 2018, 8.8 Mt of the wastes was disposed of by the food industrial activity in Japan, along with 6.5 Mt of the waste sludge corresponding to 75% (w/w) of the total weight of the waste materials from the food industries [1]. The sludge from the food industry is generally

not contaminated with dangerous materials, such as heavy metals and chemicals. Therefore, the recycle of the sludge is very important issue for the development of the food industry. Waste sludge from food factories is rich in nutrition and contains organic materials and less amounts of dangerous contaminations, but the waste sludge in the food industries generally contains 80~90% water [3]. Due to the higher water content, to use the waste sludge for animal feed or organic fertilizer is not easy. Waste sludge has been used as a combustion improver or an additive to fertilizer because principally landfill and incineration of sludge were prohibited in 1992 by the law, and the law for the advancement of recycles society was enacted in 2000.

Active sludge is used to clarify contaminated wastewater in food factories, as an eco-friendly and efficient method to treat a large volume of wastewater. Active sludge contains various microorganisms which uptake various compounds from wastewater for their growth and clarify the wastewater [4]. These microorganisms eventually die and form sediments with the addition of coagulants (e.g., poly-aluminum). The precipitate containing cadavers of microorganisms, along with some living organisms, is removed from the active sludge system as waste sludge. As mentioned above, the sludge is relatively free from harmful contamination. Therefore, the dried active sludge is accepted as normal ordinary fertilizer by convention of the Japanese Government if the dried materials contained more than 4% N, 1% P, and 1% K. Besides, the N, P, and K contents of the sludge from various food factories in Japan are relatively consistent at about 7, 4, and 0.8% (based on dry weight), respectively, irrespective of the kinds of food factories [5].

Because of the higher water content in waste sludge from food factories, the recycling rate is low; in addition, sludge burning in open fields is prohibited as mentioned above. To improve the recycling rate of waste sludge, we developed a rapid and easy method to convert waste sludge from the bean curd, confectionery, and fish meat factories into well-matured fertilizer during only two weeks [6, 7].

About 200 kg of waste sludge from food factories was mixed with newspaper chips through a shredder to reduce the water content to a certain level suitable for composting reaction. The mixture was placed in a 400 L reactor with continuous aeration at 35°C from the bottom of the reactor. A few days after the beginning of aeration, the composting reaction began, and the temperature throughout the reactor increased to 70°C in a few more days. After two weeks of composting reaction, maturation of the sludge fertilizer was completed. The composting period is very short compared with an ordinary composting method piling it in the open air. Our method of mixing waste sludge with paper chips was termed the paper mixing method (PMM); therefore, the resulting well-matured sludge fertilizer (SF) is termed SF-PMM [8, 9].

The SF-PMM is produced from inexpensive small-scale equipment with low energy consumption; therefore, the system can be installed to produce SF-PMM in various small food factories. The waste management in food factories has become an important issue to establish zero emission of food industries in the world including developing countries;

therefore, this new composting system may contribute to waste management in various food factories.

We have reported the effects after the application of SF-PMM to various plants and plant parts, for instance, root elongation [6, 8, 10], expansion of flowering period (unpublished data), and increase of flower bud formation of viola and marigold (unpublished data). However, the contribution of SF-PMM to vegetable growth as an N source has not been reported. Therefore, we estimated the effects of the SF-PMM on the growth of komatsuna.

Using fertilizer labeled with ^{15}N is a reliable method for the evaluation of N use from fertilizer in soil to plants. Previous studies used ^{15}N-labeled manure [11] and dried active sewage sludge [12]. In the former, ^{15}N-labeled compost was prepared using a slow composting process from cow manure, and the authors clarified that tomato plant and rice plant adsorbed about 5.9% of the whole N from the cow manure [11]. In the latter, by using sewage sludge and ^{15}N-labeled ammonium chloride (^{15}N-NH_4Cl), the efficiency of the uptake of N from the sewage sludge and the chemical fertilizer was determined as 20 and 12%, respectively. The relative efficiency of the sludge fertilizer to the chemical fertilizer was about 60% [12].

The ^{15}N-labeled urea was also used to investigate the remaining soil N content after crop harvesting [13]. ^{15}N-labeled ammonium sulfate, $(NH_4)_2SO_4$, was used to label waste sludge from a food factory [14].

In this study, we report a new method developed to prepare a small amount of ^{15}N-SF-PMM. A small volume of ^{15}N-waste sludge (WS)-PMM was placed in a 400 L reactor performing the composting reaction to make SF-PMM, and after two weeks, labeled ^{15}N-SF-PMM was obtained, which was enough to determine the utilization of N from ^{15}N-SF-PMM by plants, komatsuna in a pot experiment. We expect that the results in this study might support the adoption of the PMM for waste sludge management in various small food factories.

In this report, we described the method for the production of SF-PMM and the easy and speedy way to label the SF-PMM with ^{15}N-glycine. We also reported briefly the evidence of the uptake of ^{15}N from ^{15}N-SF-PMM into komatsuna.

2. Materials and Methods

We and the owner of the bean curd factory, located next to Niigata University, collaborated to recycle and reuse the sludge wasted from the factory. We equipped reactors in the factory producing 100 kg of waste sludge in wintertime and 200 kg in summertime every day.

2.1. Preparation of Active Sludge Labeled with ^{15}N-Glycine.
The 50 L of wastewater of the bean curd factory and 1 L of active sludge from the same factory were mixed by aeration at a 25 L·min^{-1} from the bottom of the cylindrical plastic tank (62 cm$\phi \times$ 72 cm high) at 25°C for 2~5 days (Figure 1). At the start of aeration, 3 g of ^{15}N-glycine (98 atom% ^{15}N; Mass Trace Inc., Woburn, MA, USA) was added.

3 g of glycine or
^{15}N-glycine

↓

Condensed active
sludge

↓

50 L of wastewater
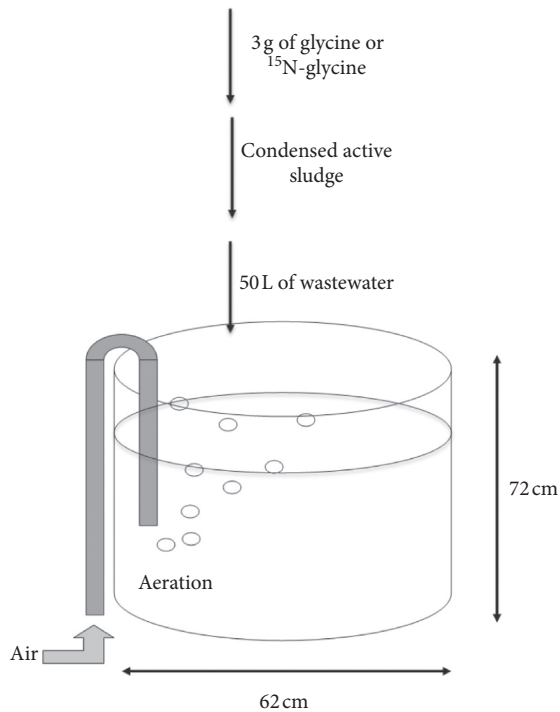
↓

Aeration

Air

72 cm

62 cm

FIGURE 1: The cylindrical tank used to prepare the labeled sludge with ^{15}N-glycine. 50 L of wastewater from a bean curd factory was mixed with 1 L of active sludge and 3 g of ^{15}N-glycine (98 atom% ^{15}N) and aerated at 25 L·min^{-1} from the bottom of the cylindrical tank, 72 cm h × 62 cm ϕ, at 25°C for 5 days. During the aeration, samples were collected and allowed to stand for 1 h for floc sedimentation, and the supernatant was recovered to estimate various parameters described in Section 2.4. Experiments without the addition of glycine were done several times. The mixing with cold glycine and with ^{15}N-glycine was performed 3 times and 1 time, respectively.

During the 5 days of aeration, 1 L of aliquots of wastewater was frequently collected and allowed to stand for 1 h for floc sedimentation. In the first 24 h of incubation, samples were taken in 3 h intervals; in the next 48 h, they were taken in 12 h intervals; and in the final period of 48~120 h, the sampling was done every 24 h. Various parameters such as pH and concentrations of amino acid, protein, and NH_4^+ in the supernatant were measured. When agitation was ceased, 40 mL of 0.2% poly-aluminum chloride, $[Al_2(OH)_nCl_{6-n}]_m$, was added to the suspension in the reactor and allowed to stand for 1 h to precipitate solid materials. The supernatant was discarded with gentle suction using a water pump, and the precipitate was finally recovered by centrifugation at 1,500 ×g for 10 min. The precipitate was filtered through filter paper on a Buchner funnel with suction until the water content of the precipitate reached around 85%. The water content was measured with a water content meter (FD-600; Kett Electric Laboratory, Tokyo, Japan), and 175 g of precipitate was obtained. The addition of the coagulant poly-aluminum chloride did not increase the aluminum concentration in the SF-PMM (data not shown).

2.2. Preparation of ^{15}N-Labeled Waste Sludge Fertilizer, ^{15}N-SF-PMM, with the Paper Mixing Method in a Nurse Reactor.

The nurse reactor was prepared according to the method in [6, 8], and the air was blown from the bottom of the reactor at 40~50 L·min^{-1} with an inverter-controlled blower (FVR-C11, Fuji Electric, Tokyo). The waste sludge, 170 kg, from the bean curd factory was mixed with 30 kg of shredded newspaper (WS-PM) and placed in a box reactor (60 cm l × 60 cm b × 120 cm h) (Figure 2). The reactor was continuously aerated with warm air (around 35°C) through the bottom of the reactor using a blower. The temperature was monitored with sensors in the center of the reactor at 10, 40, and 70 cm below the surface of the WS-PM, and temperature data were collected hourly for 20 days with a data logger. The nurse reactor had been started 2~4 days before the insertion of the ^{15}N-WS-PM. The ^{15}N-WS-PM was sandwiched between two stainless steel mesh sheets (30 cm × 30 cm, 5 mm mesh) and embedded horizontally 10 cm below the surface in the nurse reactor warmed up beforehand, and aeration was restarted (see Section 2.3) (Figure 2). The timing of the embedding of the ^{15}N-WS-PM was flexible by a few days because the water contents and temperature of the waste sludge in the nurse reactor differed in each occasion.

The WS-PM was composted for around 12 days, dried at 60°C overnight, and subjected to nutrient analysis. The N, P (as P_2O_5), and K (K_2O) contents were 4.0, 4.0, and 0.8%, respectively, based on dry weight; the C/N ratio was about 10; and the pH was about 7.4. These values were repeatedly obtained with this reactor [6, 8].

2.3. Temperature Fluctuation during Composting of Waste Sludge Labeled with ^{15}N-Glycine.
The ^{15}N-WS-PM, 200 g, was incubated in the upper part of the nurse reactor (Figure 2), because such a small amount of waste sludge could not spontaneously begin the composting reaction, even after mixing with shredded newspaper. The stainless mesh sheet containing ^{15}N-WS-PM was placed in the nurse reactor on day 3 or 4 after the starting of aeration when the temperature was increased to 40~50°C, and aeration was restarted after the setting (see Section 2.2). The timing to set the mesh sheet differed a little bit from one batch to another. Figure 3 presents typical temperature fluctuations of WS-PM in the 400 L reactor. The timing to reach the highest temperature and duration of the plateau varied by a few days. Such differences were expected, because of the variations in various parameters in the WS-PM. Regardless of these differences, Figure 3 showed temperature fluctuations of 35 to 70 to 40°C over the course of two weeks; the highest temperature lasted for 2-3 days.

2.4. Analytical Methods

2.4.1. Measurement of Various Parameters in the Wastewater.
Incubation of ^{15}N-glycine in the wastewater was ceased at an optimum time to obtain solid material having the utmost ^{15}N concentration. The experiments were performed three times with the different wastewater. Each replicate showed different fluctuation patterns, with different times for the maximum and/or minimum values. However, importantly, the inverse trends between the amino
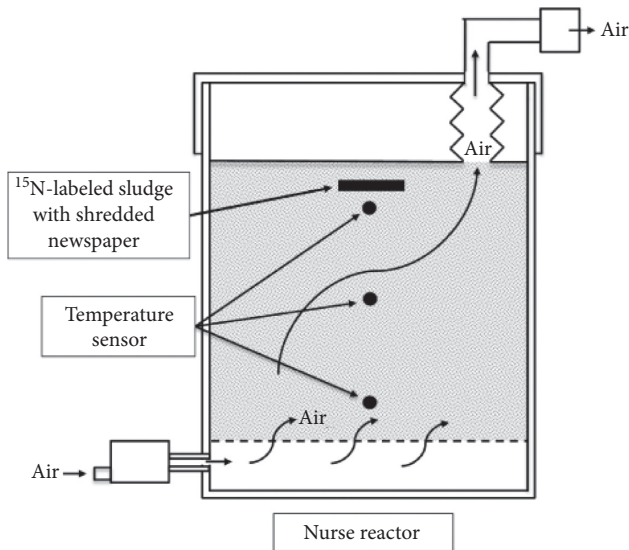
FIGURE 2: The nurse reactor for preparing the ^{15}N-SF-PM. Waste sludge of 170 kg that came from a bean curd factory was mixed with 30 kg of shredded newspaper and placed in a box reactor (60 cm l × 60 cm b × 120 cm h). The composting reaction starts by aeration with warmed air at around 35°C, and 3~5 days after the start of aeration when the temperature reached around 40~50°C, the sandwiched ^{15}N-WS-PM was placed horizontally at the area marked with a black bar at 10 cm below the top of the WS-PM. Right after the insertion of the sandwich, the aeration was restarted (for details, see Section 2.2).

acid and protein concentrations were reproducible, as was the acute increase in the NH$_4$ concentration.

(1) The Fluctuations of Parameters of Biological Oxygen Demand, BOD, in the Wastewater. The dissolved oxygen concentration in the wastewater was measured with the modified Winkler method [15]. The wastewater was diluted with a mixture of phosphate buffer (0.12 M K$_2$HPO$_4$, 0.062 M KH$_2$PO$_4$, 0.12 M Na$_2$HPO$_4$, and 0.032 M NH$_4$Cl; pH 7.2), 0.09 M MgSO$_4$, 0.25 M CaCl$_2$, and 9.2 mM FeCl$_3$ (III). The diluted wastewater was adjusted to 1 L with the same buffer, and the dilution degree was factored into all subsequent calculations. Five aliquots of 170 mL suspension from the 1 L of diluted wastewater were transferred to five 170 mL screw-cap test tubes, and they were capped tightly after the removal of air bubbles. One 170 mL tube was used immediately for the measurement of dissolved oxygen as time zero, and the rest were kept in an incubator for 5 days at 20°C. Biological oxygen demand (BOD) was calculated as follows:

$$BOD\left(mg \cdot L^{-1}\right) = \left(DO_1 - DO_5\right) \times \text{dilution degree}, \quad (1)$$

where DO$_1$ is the concentration of dissolved oxygen at time 0 and DO$_5$ is that at after 5 days of storage.

(2) Amino Acid and Protein Concentrations in the Wastewater. The wastewater was centrifuged (1,500 ×g, 10 min), and 10% (v/v, final concentration) trichloroacetic acid (TCA) was added to the recovered supernatant. To precipitate denatured proteins, the sample was centrifuged for
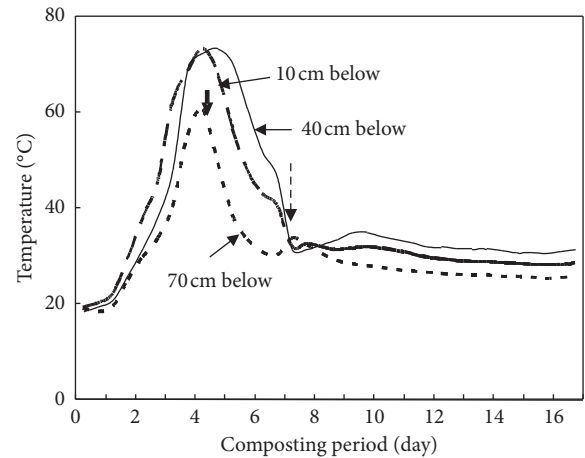

FIGURE 3: The changes of temperature in the reactor during composting of WS-PM. The waste sludge from a bean curd factory was mixed with newspaper chips as described in Figure 2, and the temperature of the reactor was monitored as described in Sections 2.2 and 2.3. On day 7, indicated with a broken line arrow, the aeration was ceased, and the WS-PM in the reactor was collected and replaced again after the thorough mixing of the sludge to check the subsequent 2nd composting reaction. After setting the remixed WS-PM, aeration was started with the same condition as those in the first aeration. The broken line ---, the solid line –, and dotted line ···· represent fluctuation of the temperatures at depth of 10 cm, 40 cm, and 70 cm, respectively, from the surface of the mixture in the reactor.

20 min at 12,500 ×g. To remove TCA, two volumes of ethyl ether were mixed vigorously in the supernatant. The ethyl ether washing was repeated two additional times, and the amino acid concentration was determined with the ninhydrin method [16] using a calibration curve of L-leucine.

Floc was removed by sedimentation (see Section 2.1), and the protein concentration in the supernatant was determined with the Bradford method [17] using bovine γ-globulin as the standard.

(3) pH and Ammonium Ion (NH$_4^+$) in the Wastewater Supernatant. The pH and ammonium ion (NH$_4^+$) concentration in the wastewater during incubation in the tank were measured with a pH meter (GST-2729; TOA Denpa, Tokyo, Japan) and NH$_4^+$ electrode (AE2041; TOA Denpa), respectively.

(4) Weight of Dry Solid Materials in the Wastewater. During the incubation of the wastewater in the tank, 1 L aliquot was taken, and solid materials were collected by centrifugation (1,500 ×g, 10 min) and dried for 24 h at 105°C. The weight was measured with an electric balance.

(5) Water Content. The water contents in various fractions were measured with a water content meter (FD-600; Kett Electric Laboratory, Tokyo, Japan).

2.4.2. Determination of the C/N Ratio, Phosphate and K Concentrations, and ^{15}N Abundance. After completing the composting reaction, the SF-PMM was dried, and 50 mg of

pulverized sample was hydrolyzed with 1 mL of concentrated sulfuric acid according to the Kjeldahl method [18]. The C/N ratio was determined with a C/N recorder (MT700 Mark II; Yanaco Co. Ltd., Kyoto, Japan). Phosphate was determined with the ammonium vanadomolybdate absorptiometry [19] and expressed as P_2O_5. K was measured with flame photometry using an atomic absorption photometer (Z-6100; Hitachi, Tokyo, Japan) and expressed as K_2O.

An aliquot of hydrolyzate in the Kjeldahl method [18] was mixed with an equal volume of 1 N NaOH and adjusted to 300 μL with water. 200 μL of phenyl nitroprusside solution (0.64 M C_6H_5OH, 0.76 mM $Na_2[Fe(CN)_5NO]$ in 18 mM Na_3PO_4, 12 mM $Na_3C_6H_5O_7$, and 8.1 mM EDTA) and 300 μL of alkaline sodium hypochlorite (25 mL NaClO in 1 L 0.4 M NaOH) were added and reacted for 45 min. The N concentration was measured calorimetrically at 635 nm with a calibration curve based on ammonium sulfate $(NH_4)_2SO_4$. The ^{15}N concentrations in the compost and plant materials were determined by an emission spectrometry with a ^{15}N analyzer (NIA1; Jasco Inc.) [20].

2.4.3. Measurement of the Uptake of the SF-PMM and Evaluation of N Use from ^{15}N-Labeled Compost in Komatsuna.

SF-PMM or ^{15}N-SF-PMM was dried and finely powdered with a vibration mill (TI-100; Irie-Shokai, Tokyo, Japan) and mixed well with sand dune soil from Ikarashi, Niigata, Japan. The texture of the soil was analyzed as follows: loamy sand, pH (H_2O): 6.75; cation exchange capacity: 4.60 cmolc·kg^{-1}; total N: 0.03%; available N: 20.4 mg·kg^{-1}; available P_2O_5: 72.8 mg·kg^{-1}. The available N is low and good to estimate the N movement from exogenous fertilizer to plant. We assumed that the application of fine powder fertilizer is more effective than coarse particle or flake formulations; future experiments are required to evaluate this assumption and optimize the formulation. Each pot in the treatment, 590 g of the sandy soil was used (see Section 2.4.4).

In the preexaminations, the culture system with the soil amended with $(NH_4)_2SO_4$-50 mg N supported the growth of komatsuna for more than 5 weeks without any abnormal inhibitory effects. According to these observations, we designed experiments to estimate the fertility of the SF-PMM for 5-week culture as follows: Control: $(NH_4)_2SO_4$ 50 mg N; experiments: SF-PMM-50 mg N and SF-PMM-100 mg N·kg^{-1} soil designed to estimate the plant growth.

In these treatments, calcium dihydrogen phosphate (calcium phosphate, monobasic) $(CaH_4O_8P_2)$ and potassium chloride (KCl) were also added to make up 50 or 100 mg of P and K kg^{-1} soil if they were less than 50 or 100 mg, respectively. In the Control treatment, no exogenous nutrient was used.

The effects of these three treatments in supporting the growth of komatsuna were estimated with increase of the weight in the shoot (above ground part) of the plants.

2.4.4. Culture of the Plants.

In the culture of the plants for the three treatments, six seeds of komatsuna were sown in one pot (10,000^{-1} a, Neubauer pot), and the pots were incubated for 5 weeks, in the longest case, in a climate chamber under a 12 h photoperiod with 130 μmol·m^{-2}·s^{-1} photons. In the measurements of the weight of shoots, the 4 plants were used to make an average and statistical analyses.

At 5 weeks after the sowing, 4 plants in each treatment were harvested randomly from 4 pots. The total four plants were used to calculate the average in each treatment. These averages of each weight coming from four plants weights were statistically analyzed using group comparison analyses. The water content in the soil was maintained at 50~60% of the field capacity with deionized water throughout the experiment.

The percentage of N derived from ^{15}N-labeled compost (^{15}N%) in the plants was calculated using the following equation [21], where ^{15}N atom% excess is ^{15}N atom% minus natural abundance of 0.37 atom%.

$$^{15}\text{N}\% = \frac{^{15}\text{N atom\% excess in komatsuna}}{^{15}\text{N atom\% excess in compost}} \times 100. \quad (2)$$

The N content derived from ^{15}N-compost in the plant was calculated as ^{15}N% in komatsuna × total N content.

2.4.5. Statistical Analyses.

The data of the weight of four plants in each treatment described in Sections 2.4.3 and 2.4.4 were analyzed with Tukey–Kramer multiple comparison procedure in $P < 0.05$ [22]. To determine P value, Student's t-test was also done, but data was not shown.

3. Results

3.1. Changes of Various Parameters in the Wastewater during Incubation with Active Sludge.

The BOD was 900 mg·L^{-1} at the start of the incubation and drastically decreased to 200 mg·L^{-1} in the first 24 h. The weight of floc increased slightly to 0.5 g·L^{-1} after a 12 h lag in the first 24 h of incubation and decreased to 0.4 g·L^{-1} during further 5 days of incubation (Figure 4(a)). We assumed that an increase and subsequent decrease in protein concentration would follow a decrease and subsequent increase of amino acid concentration and that these trends would mirror one another. This assumption was verified, as shown by the changes in protein and amino acid concentration over time in Figures 4(a) and 4(b). Because it is easier to measure amino acid concentration than to measure protein concentrations, we used the fluctuations in amino acid concentration to determine the optimal time to stop the incubation. The NH_4^+ concentration was low, around 1 mg·L^{-1}, and steady in the first 24 h but increased dramatically to 9 mg·L^{-1} in the subsequent 24 h (Figure 4(c)). Importantly, increase of the NH_4^+ was similar in pattern to that of amino acid as shown in Figure 4(b).

3.2. Effects of the Addition of Glycine to the Mixture of Wastewater and Active Sludge.

We assessed whether the addition of 3 g of glycine to 50 L of wastewater altered the fluctuation trends in the parameters shown in Figure 4. The
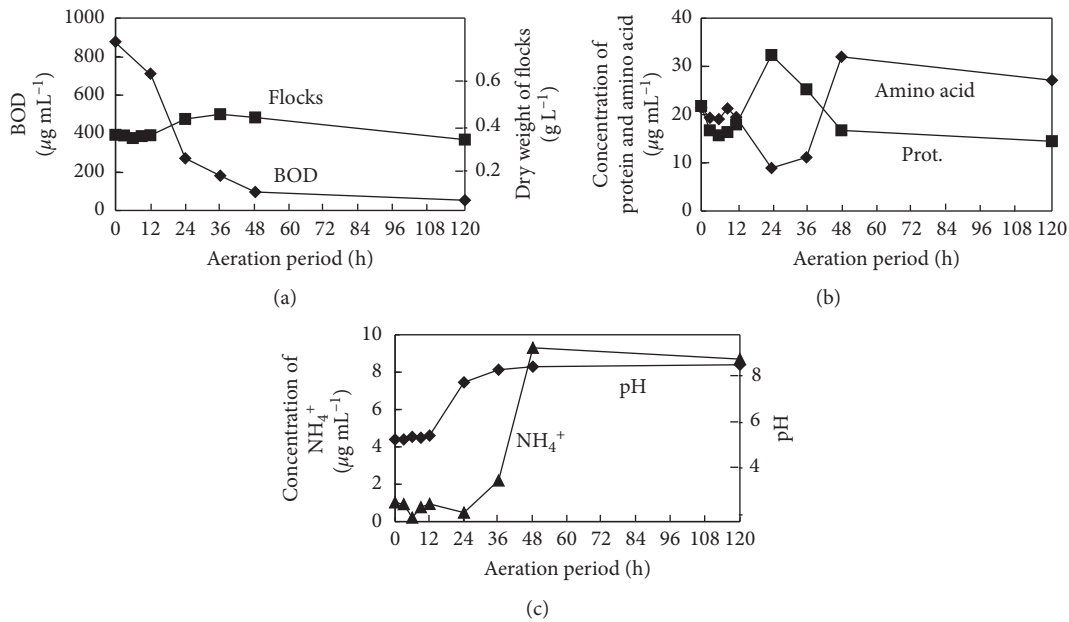
(a)

(b)

(c)

FIGURE 4: Changes of various parameters in the wastewater during incubation with active sludge in the plastic tank. 50 L of the wastewater from a bean curd factory was mixed with 1 L of active sludge and aerated as described in Section 2.1. (a) Flocs (■) and BOD (♦), (b) amino acid (♦) and protein (■), and (c) NH_4^+ (▲) and pH (♦) were measured. Although not all parameters were always tested, the experiment was repeated several times as described in Figure 1. For the details about the method to determine the various parameters, see Section 2.4.2.

trends obtained in the experiment with glycine (Figure 5) were similar to those without glycine (Figure 4). Of particular importance, the inverse trends between the amino acid and protein concentrations and the sharp increase in NH_4 (Figure 5) were essentially the same as those without glycine and were reproducible in three experiments (data not shown). Interestingly, the minimum amino acid concentration was observed 12 h earlier in the experiment with glycine than that without glycine (Figures 4(b) and 5(a)). However, the time required to reach the maximum or minimum values of each parameter differed among the replicates (data not shown).

The amino acid concentration at the start of aeration (Figure 5(a)) was 90 mg·L^{-1} in the experiments with exogenous glycine and decreased to about 40 mg·L^{-1} during the initial 12 h of incubation. Therefore, a large amount of added glycine, 2.5 g, was estimated to be incorporated into the microorganisms. This amount of incorporated amino acid was about 4 times higher than the value of about 13 mg·L^{-1} estimated from the results presented in Figure 4(b), in which the lowest concentration reached at 24 h after the start of amino acid incorporation.

This suggested that the addition of glycine significantly enhanced the proliferation of microorganisms during the initial 12 h of incubation, and that autolysis of microorganisms and protein degradation occurred 12 h earlier in the presence of glycine than in its absence. These results suggested that fluctuations in amino acid concentration can be used to monitor the appropriate timing to obtain a higher yield of ^{15}N-labeled sludge.

3.3. Incorporation of ^{15}N-Labeled Glycine into Microorganisms. In the sludge labeling with ^{15}N-glycine,

along with the expected fluctuations in amino acid and protein concentrations, the NH_4^+ concentration increased slightly during 18 and 21 h of incubation (data not shown). As described in Section 3.2, after adding ^{15}N-glycine in the mixture of the wastewater and the active sludge, the amino acid content rapidly decreased in the initial 12 h. At 18 h after the addition of ^{15}N-labeled glycine, the rapid decrease in amino acid ceased (data not shown).

The incubation was terminated at 24 h after the addition of ^{15}N-glycine, and 175 g of labeled sludge containing 85% water was recovered. Using emission spectrometry, the resulting precipitate was evaluated to have a ^{15}N content of 10.2 ± 0.03 atom% excess ($n = 3$), and approximately 0.9 g of ^{15}N-glycine was suggested to be incorporated into the sludge. Consequently, about 30% of the added ^{15}N-glycine appeared to be assimilated into microorganisms, suggestive of high incorporation efficiency. The total N content of the waste sludge was about 6% and the same as the value of the sludge routinely produced in this factory (data not shown).

3.4. Changes in the Reactor Temperature during the Composting of Waste Sludge-Paper Mixture, WS-PM. The changes of temperature in the WS-PM during composting in the reactor were similar to those reported previously [6] and reached the highest temperature, 60~70°C, on day 5 after the starting of aeration (Figure 3). The maximum temperature, around 70°C, was recorded for 2 days (on days 4~5) at 10, 40, and 70 cm below the surface of the mixture, and then the temperature acutely decreased to around 30°C during the subsequent 2 days at all sensing points. Although a
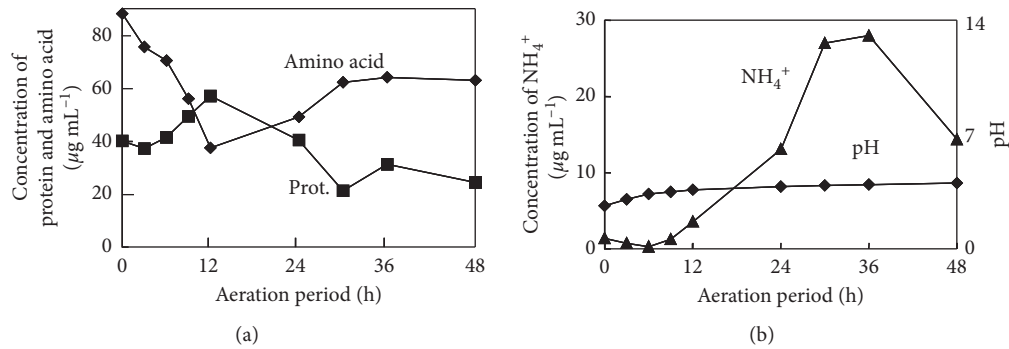
(a)

(b)

FIGURE 5: Effects of addition of glycine on the change of various parameters in the wastewater during incubation with the active sludge in the plastic tank. 50 L of the wastewater was treated as described in Section 2.1, and 3 g of glycine was added at the start of the incubation. (a) Concentrations of protein (■) and amino acid (♦) were measured according to the description in Section 2.4.2. (b) NH_4^+ concentration (▲) and pH (♦) were measured according to the methods described in Section 2.4.2. The experiment using cold glycine was done three times as mentioned in Figure 1, and the experiment of the addition of ^{15}N-glycine was done one time.

difference of a few days was often observed, the fluctuation of the temperature was observed with very high fidelity in many experiments.

At time indicated with arrow with a dotted line in Figure 3, the sludge in the reactor was remixed and a second composting reaction was attempted, but almost no second composting reaction was observed. These observations indicated that the reactor could be used as a nurse reactor for the labeled fertilizer in terms of high temperature and short period reactions. Therefore, ^{15}N-WS-PM was placed at the center of the nurse reactor on day 3 or 4 after starting aeration (see Section 2.3).

3.5. Composting of ^{15}N-SF-PM. We obtained about 175 g of ^{15}N-labeled waste sludge from the tank and composted it in the nurse reactor as described in Section 2.2. The temperature of the nurse reactor was measured as shown in Section 2.3, and a similar temperature fluctuation to that in Figure 3 was observed at the three sensing ports (data not shown). Finally, 300 g of ^{15}N-SF-PMM was obtained. Total N content was 6.06%, and 7.03 atom% of ^{15}N abundance was shown with the Kjeldahl method and the ^{15}N analyzer, respectively. Of note, although glycine seemed to enhance the proliferation of the microorganisms (see Section 3.2), almost the same amount of sludge (floc) was produced with or without glycine, i.e., at 4 h and 48 h of incubation, about 180 and 250 g flocs, respectively (data not shown).

3.6. Effects of the SF-PMM on the Growth of Komatsuna. The effects of the SF-PMM on the growth of komatsuna were estimated in comparison with the treatment of Control of $(NH_4)_2SO_4$-50 mg N. All data were statistically analyzed.

Komatsuna was cultivated in the SF-PMM-50 mg N and -100 mg N treatments for 5 weeks (Table 1). The weights of the shoots in SF-PMM-50 and -100 treatments at 5-week were 3.136 ± 0.326 and 4.299 ± 0.445 g, respectively. Compared to the weight in Control, the shoot weights in SF-PMM-50 and -100 treatments were 2.2 and 3.1 times heavier. These values of the weights in the three treatments were statistically significant with Tukey–Kramer analysis [22].

TABLE 1: Effects of SF-PMM on the growth of komatsuna shoots in 5-week culture.

Treatments	Control	SF-PMM	
	Chem-50	50 mg N	100 mg N
Weight (g)	1.404	3.136	4.299
SD deviation	0.606	0.326	0.445
Significance	a	b	c

The photographs of the plants cultured in 5-week experiments are shown in Figure 6. The three plants were harvested at 5 weeks in the treatments of Control (Figure 6(a)), SF-PMM-50 (Figure 6(b)), and SF-PMM-100 (Figure 6(c)), respectively.

The plants in SF-PMM-50 (Figure 6(b)) and SF-PMM-100 (Figure 6(c)) treatments looked very healthy and showed luxuriant growth in roots. Compared to these two photos, the plant in Control was slightly small, but it was average feature.

3.7. The Movement of the ^{15}N from ^{15}N-SF-PMM to the Komatsuna Cultured in ^{15}N-SF-PMM-100 Treatment. The uptake and accumulation of ^{15}N from ^{15}N-SF-PMM into komatsuna were assessed in pot experiments (see Sections 2.4.2 and 2.4.3). In the komatsuna cultured in SF-PMM 100 mg N treatment in 5-week experiment, about 56% of the total N in the plant was composed of ^{15}N from ^{15}N-SF-PMM exogenously given, and the amount of ^{15}N in the plant was estimated as 6% of the total ^{15}N of ^{15}N-SF-PMM (Figure 7).

These data suggested that our labeling method and nurse composting system offered useful tools for analyzing N movement in plants and soil and could be adopted in future fields experiments. However, it will be necessary to increase the amount of the N released from the SF-PMM.

4. Discussion

4.1. The Composting Sludge from Food Factory Wastewater. The composting of the waste sludge from food factories to well-matured fertilizer usually takes long time, due to its

(a)   (b)   (c)

FIGURE 6: Photos of the komatsuna harvested in 5-week experiments. In the fifth week, one plant was taken randomly from each pot in the Control treatment: (a) chemical fertilizer of 50 mg N, SF-PMM-50 treatment; (b) SF-PMM 50 mg N and SF-PMM-100 treatment; (c) SF-PMM 100 mg N. In Control treatment, N, P, and K were supplied from $(NH_4)_2SO_4$, $CaH_4P_2O_8$, and KCl. In SF-PMM-50 and -100 treatments, N was supplied from SF-PMM, and the lack of P and K was compensated for by $CaH_4P_2O_8$ and KCl, respectively, if necessary. See for detail, Section 2.4.4.

higher water contents [3]. Besides, the higher water content in the waste sludge interferes with the start of composting reaction. However, it is not difficult to start the composting reaction using sawdust or rice husk mixing method [3, 6, 23]. In these treatments, the temperature often reached around 70°C, but it was very difficult to convey these high temperatures all over the reactor [23], because these subsidiary materials, sawdust and rice husk, are too rigid to make "flexible thin air streams" all over the reactor. These subsidiary materials are also not fluffy to keep sufficient air in the mixture of sludge having large volume water which is necessary for the growth of microorganisms. Therefore, these materials cannot make a long random air stream in the mixture [23].

On the other hand, thin paper chips, such as shredded newspaper, were a good subsidiary material to get stable starting of a composting reaction even in the mixture with 75% water. In this paper, with the chips mixing method, it was possible to convey the high temperature all over the reactor [7]. Generally, in the waste sludge-paper mixing method, WS-PMM, after slow increase of the temperature next to the lag time, the temperature was going up steeply regardless of the length of lag time (Figure 3). The highest temperature in the reactor of WS-PMM was found throughout the reactor, but as mentioned above, in the ordinary composting reactions, such high temperature could be detected only in the surface area of the reactor (unpublished data).

It is important to attribute the reason for the high fidelity to the rapid and stable increase of such high temperature in the WS-PMM for the stable sludge recycle. Here, we point out a few theories. Fujita indicated the importance of a porous structure in the substrate in the reactor [23]. He insisted that the porous structure in the sludge could make longer random air streaming, and through this stream, aerobic atmosphere could be spread all over the reactor. The PMM reduced the water contents to around 75% from around 90%; under this water content, the air pumping urged the composting reaction voluntarily in the whole area of the reactor; and shortly 70°C temperature was recorded (Figure 3) [6, 8]. Several paper chips in a large amount of the chips added make a tiny compound composed of a small volume of the sludge and paper chips in the first mixing step. In other words, the SW-PMM in the reactor is the gigantic
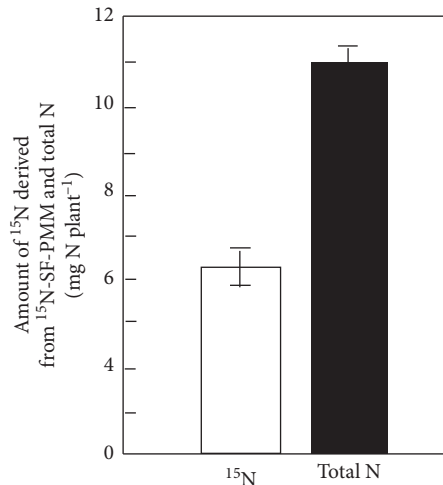
FIGURE 7: The concentrations of ^{15}N (□) and the total amount of N (■) in the whole plant of komatsuna cultured in the ^{15}N-SF-PMM-100 treatment in 5-week experiment. An accumulation of ^{15}N from the ^{15}N-SF-PMM into komatsuna was briefly assessed (for the details, see Section 2.4.4). Komatsuna was cultured in the ^{15}N-SF-PMM-100 treatment in 5-week experiment. In week 5, the two plants were harvested and lyophilized. The lyophilized samples were pulverized, and the aliquots were subjected to ^{15}N determination with a ^{15}N analyzer. White column: average of the amount of ^{15}N incorporated into a plant. Black column: the amount of total N in a plant. Two plants were used to calculate the average. See for details, Section 2.4.3.

mass of aggregates of those particles made by several paper chips and a tiny sludge particle. Inside the aggregate, an infinite flexible random stream exists like tiny capillary vessel. Then, pumped air can move along the stream randomly all over the reactor, and homogeneous aerobic conditions in the reactor could be maintained during the whole process of the composting reaction [23]. "We think this is a porous structure," Fujita said.

Porous structure guarantees even distribution of heat, water, and air which are necessary for the proliferation of the microorganisms in a reactor. Bacteria can catabolize the nutrition in the all particles, and this guarantees the homogeneous characteristics in the mass. To obtain high-quality sludge fertilizer, the composting reaction must reach and keep around 70°C for at least 2~3 days in the whole area of the reactor [3]. During these periods, various thermophilic bacteria digest the sludge and secrete various metabolites including unknown molecules. Future work is required to analyze precisely the mechanisms of SF-PMM and determine the utility of sludge fertilizer in agricultural applications. Undoubtedly, the PMM will be applied to other organic materials such as livestock feces.

4.2. Changes of Various Parameters in the Wastewater during Incubation with Active Sludge.

We obtained 175 g of labeled sludge with 10.2 ± 0.03 ^{15}N atom% excess and with 85% water content from the mixture of 50 L wasteawater, active sludge, and ^{15}N-glycine. Around 0.9 g of ^{15}N-glycine was incorporated into the sludge, so about 30% of the added ^{15}N-glycine was calculated to be assimilated into microorganisms, suggestive of high incorporation efficiency. In the labeling experiments, we noticed that a rapid increase of NH_4^+ occurred almost concurrently with the decrease of proteins (Figure 4). The monitoring of NH_4^+ is very easy and accurate, so improvement of the incubation method to get higher yield of the labeled materials will be possible by monitoring NH_4^+.

Microorganisms in the sludge are essentially steady even under frequently changing conditions in the water clarifying system. This hypothesized theory is supported by the fact that the relatively stable N, P, and K contents were observed among the food factory waste sludge, FF-WS [5]. That said, it is slightly difficult to expect that the wastewater from such different factories would have stable pH, BOD, COD, ingredient concentrations, organic molecules, etc. In either case, it is very important future work to determine quantitatively and qualitatively what compounds are labeled with ^{15}N in our labeling system. To determine those molecules will bring epoch in the research about the N movement among soils, fertilizers, and plants.

4.3. Development of a Nursing Reactor to Compost Small Amounts of ^{15}N-WS-PM.

A small volume of reactor cannot make a matured small volume of compost, because the heat rapidly dissipates and it is difficult to maintain an appropriate high temperature without an addition of heat [23]. In our reactor, the temperature around 60°C was maintained for around 4 days (Figure 1), and this was thought to fit a condition of nurse reactor for the small sized materials. Using our tank system shown in Figure 1, about 30% (w/w) of the ^{15}N-glycine was incorporated into microorganisms. In the subsequent nursing system, ^{15}N-labeled SF-PMM resulted from ^{15}N-labeled WS-PMM, and the contents of the label were estimated as 7.03 atom% ^{15}N. Thus, during the composting in the nursing reactor, about 3% of the ^{15}N was lost (see Sections 3.3 and 3.5). It is important to reduce these losses during the nursing to get highly labeled SF-PMM.

Takahashi et al. [11] estimated the ^{15}N content in the manure compost as 1.81 atom%. Meanwhile, Sørensen [24] evaluated the N mineralization in the sheep manure compost using ^{15}N-labeled compost with ^{15}N of 3.70 atom% and found that 12% of N was mineralized in 7 days. Sørensen and Amato [25] searched ^{15}N movement using ^{15}N-labeled pig manure with ^{15}N of 3.07 atom%. Compared to these reports, content of the label in the ^{15}N-SF-PMM was more than two times higher.

To get more highly labeled fertilizer, longer labeling period with ^{15}N seemed to be better. In our labeling system, 12~24 h was required for the incorporation of ^{15}N into the waste sludge, and in the subsequent nursing reaction, ^{15}N-SF-PMM 10 atom% ^{15}N was obtained. To increase the contents of ^{15}N in the SF-PMM, higher amount of ^{15}N-compound and longer incubation will be better. Moreover, in the subsequent nursing reactor, higher temperature and short incubation are necessary. In future, to elucidate precise ^{15}N movement between plant, soil, and fertilizer, it is necessary to produce the fertilizer highly labeled with ^{15}N.

PMM with nursing reactor must be one of the useful technologies to get such highly labeled compound.

As mentioned above, we suggested the possibility of applying the PMM to composting of livestock manures. In this case, the point to get such highly labeled fertilizer is to develop the method to mix homogeneously the ^{15}N-compounds and shredded paper chips with a small amount of feces having appropriate fluidity.

4.4. Effects of SF-PMM Treatments on the Growth of Komatsuna and Photo Observation.

To determine the efficiency of the SF-PMM, we used the sandy dune soil which had no history of exogenous fertilization and had a relatively low fertility. The soil elements are shown in Section 2.4.3.

The weights of komatsuna in 50 mg N and 100 mg N experiments are higher than that of the Control treatment, Chem-50, 2.36 and 3.06 times, respectively (Table 1). These three weights were statistically significant and clearly suggested that 50 and 100 mg N of SF-PMM supported the growth of komatsuna shoots for 5 weeks without any inhibitory effects compared to that in 50 mg N Control treatment. Any significant abnormal growth was not shown in the photo observations (Figure 6). Interestingly, very magnificent roots were observed in the plants grown in SF-PMM-50 and -100 treatments (Figures 6(b) and 6(c)), suggesting that, even in the rather higher concentration of SF-PMM-100, komatsuna might not receive inhibitory effects from SF-PMM in the growth of the roots.

Here, we introduce research showing the SF-PMM fertilizer's advantage in root growth. Honma et al. [10] used SF-PMM to proliferate and grow the cuttings of the Japanese cider Sugi, *Cryptomeria japonica* D. Don, which was a mutant making no pollen. Therefore, the ordinary proliferation of the Sugi was the cutting, and the fertilization was started one year later after the cuttings. In the simultaneous application of any fertilizers, including commercially available organic fertilizers, almost all cuttings were killed. However, even in simultaneous application of the SF-PMM with the cutting, more than 90% of the cuttings survived, and they gained statistically significant increase in the height of twigs and the root weight [10].

Rooting in the cutting must be one of the most delicate phenomena in the agricultural proliferation. The report of Honma et al. [10] suggested that the SF-PMM was something mild, and even in SF-PMM-200 it allowed the rooting even in simultaneous application with cuttings. This must be the most difference point from the Control treatment with $(NH_4)_2SO_4$.

4.5. N Use from SF-PMM and Uptake of ^{15}N from ^{15}N-SF-PMM into Komatsuna.

In the culture of plants with ^{15}N-SF-PMM-100 mg N treatment, 6.2 mg of ^{15}N was accumulated into a plant, and this amount was about half of the total amount of N in a plant grown in the pot. This suggests that SF-PMM was easily decomposed in the soil and provided N for the plant growth. However, the total amount of ^{15}N incorporated into the plant was only 6.2% of whole amount of ^{15}N-SF-PMM applied in the treatments, suggesting that only several percent of the compounds labeled with ^{15}N in the ^{15}N-NF-PMM were adsorbed into a plant. We believe that, under more appropriate conditions, higher amount of ^{15}N can be incorporated into the plant.

In a previous study, Matsuda et al. [14] attempted to describe ^{15}N-labeled compounds in a plant. As future work, it is important to determine the ^{15}N-labeled molecules in both the plants and the soil. These experiments to determine the compounds will explain the movement of N from SF-PMM to plants. To estimate the appropriate amount of nutrients that should be applied in the field, it is necessary to measure the amount of the residual fertilizer in farmland on a yearly basis [13, 26]. Such research must be undertaken using highly labeled ^{15}N-SF-PMM in future pot experiment as well.

About 50% of N was incorporated from SF-PMM applied; this relatively higher value could have resulted from the use of low fertility soil in this study. In this pot tests, we used the sandy soil having a value of available N as 20.4 mg·kg^{-1}. On the other hand, the question of why the availability rate was as low as 6% must be answered. The answer must be very important to obtain well designed SF-PMM, but we can just speculate now. First, the volume of the SF-PMM applied was too high, and almost 90% of the N in SF-PMM remained. Second, very high nitrification and/or denitrification occurred during the pot treatment. We must determine the amount of remaining ^{15}N in the pots. This must be very important to design useful SF-PMM.

5. Conclusion

In this study, we developed a rapid and efficient method to label the sludge in the wastewater of the food factory with ^{15}N-glycine. A small volume of wastewater was mixed with active sludge and ^{15}N-glycine for half a day, and the resulting active sludge was embedded into 400 L reactor tank carrying high temperature composting reaction at ~70°C. After around 10 days of more composting, sludge fertilizer labeled with 7.03 atom% ^{15}N was obtained. From the labeled ^{15}N-SF-PMM, about 6% of the label applied was incorporated into komatsuna. Nonlabeled SF-PMM at 50 or 100 mg N supported the growth of komatsuna, and healthy shoots with flourishing roots were obtained in 5 weeks. The method proposed herein and related results were expected to establish the recycle of waste sludge from food factories with efficient sludge fertilizer.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

References

[1] Japan Ministry of the Environment, "Discharge and treatment status of industrial waste, (FY2018 results)," http://www.env.go.jp/recycle/waste/sangyo.html, 2021, in Japanese.

[2] Japan Ministry of the Environment, "Annual report on the environment, the sound material-cycle society and biodiversity in Japan 2021," http://www.env.go.jp/policy/hakusyo/r03/pdf.html, 2021, in Japanese.

[3] A. Ushikubo, "Waste sludge from food industries," in *Dictionary for Recycle of Organic Waste, (Yuki Haikibutsu Sigenka Daijiten)*, N. Date, Ed., The Committee for Promotion of Recycle of Organic Waste, Noubunkyo Publishing, Tokyo, Japan, 4th edition, pp. 177–182, Tokyo, Japan, 2000, in Japanese.

[4] *Microorganisms in an Aeration Tank*, T. Murata, Ed., Incorporated Association of Japan Sewerage, Tokyo, Japan, 3rd edition, 1991, in Japanese.

[5] CJC, *Report of Search for the Recycle of Wasted Sludge from Food Factories*, Clean Japan Center, Incorporated Foundation, Tokyo, Japan, 1998.

[6] H. Hori, K. Iwabuchi, A. Azwan, and T. Mitsui, "Ultra high performance of composting of sludge from food industry by novel newspaper mixing-method: comparative study of the composting with sawdust mixing-method," *Bulletin of the Faculty of Agriculture*, vol. 53, no. 2, pp. 133–143, 2001.

[7] H. Hori and K. Iwabuchi, "Composting of organic waste materials and use of resulting materials," Japan Patent Office, Tokyo, Japan, Patent description, Japanese Patent 3499484, 2003.

[8] A. Hassan, H. Ono, M. Miura et al., "Ultra high-performance composting of sludge from food industry: comparative study of fermentation in sawdust and paper mixing methods," in ", I. A. Raja and R. Farooq, Eds., in *Proceeding of 1st International Conference on Environmentally Sustainable Development*, pp. 786–792, University of COMSATS Press, Abbottabad, Pakistan, 2005.

[9] H. Hori, A. Hassan, and H. Taira, "Novel method for composting of waste sludge and impact of the resulted compost on plant growth," in *Proceeding of the 3rd International Conference for Development of Integrated Pest Management in Asia and Africa*, J. Lumbanraja, F. X. Susilo, Purnomo et al., Eds., University of Lampung Press, Bandar Lampung, Indonesia, pp. 225–239, 2010.

[10] R. Honma, H. Ono, M. Akutsu, T. Horikawa, H. Hori, and H. Taira, "Compost made from waste sludge from food industry enhanced significantly rooting and growth of cuttings of Sugi (*Cryptomeria japonica* D.don)," *Journal of the Japanese Forest Society*, vol. 90, no. 3, pp. 194–197, 2008.

[11] S. Takahashi, S. Yamamuro, and S. Ono, "Nitrogen uptake by rice and tomato from nitrogen-15 labeled composted cattle dung," *Japanese Journal of Soil Science and Plant Nutrition*, vol. 71, no. 2, pp. 246–248, 2000.

[12] N. Asagi and H. Ueno, "Determination of application effects of sewage sludge on growth, soil properties, and N uptake in Komatsuna by using the indirect ^{15}N isotope method," *Communications in Soil Science and Plant Analysis*, vol. 39, no. 13-14, pp. 1928–1942, 2008.

[13] W. Zhao, B. Liang, X. Yang, and J. Zhou, "Fate of residual ^{15}N-labeled fertilizer in dryland farming systems on soils of contrasting fertility," *Soil Science and Plant Nutrition*, vol. 61, no. 5, pp. 846–855, 2015.

[14] A. Matsuda, K. Ohno, T. Nagakubo, and T. Matoh, "Labelling of food industry sludge compost with ^{15}N and its availability to Italian ryegrass (*Lolium multiflorum* L.) plants," *Japanese Society of Soil Science and Plant Nutrition*, vol. 78, no. 3, pp. 317–321, 2007.

[15] L. W. Winkler, "Die Bestimmung des im Wasser gelösten Sauerstoffes," *Berichte der Chemischen Gesellschaft*, vol. 21, no. 2, pp. 2843–2854, 1888.

[16] E. W. Yemm, E. C. Cocking, and R. E. Ricketts, "The determination of amino-acids with ninhydrin," *The Analyst*, vol. 80, no. 948, pp. 209–214, 1955.

[17] M. M. Bradford, "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[18] J. M. Bremner, "Inorganic forms of nitrogen," in *Methods of Soil Analysis*, C. A. Black, D. D. Evans, J. L. White, L. E. Ensminger, and F. E. Clark, Eds., pp. 1179–1237, ASA, Madison, WI, USA, 1965.

[19] R. E. Kitson and M. G. Mellon, "Colorimetric determination of phosphorus as molybdivanadophosphoric acid," *Industrial & Engineering Chemistry Analytical Edition*, vol. 16, no. 6, pp. 379–383, 1944.

[20] T. Ohyama, "Emission spectrometric ^{15}N analysis of amino acids," *Radioisotopes*, vol. 31, no. 4, pp. 212–221, 1982.

[21] D. Clay, C. Reese, S. A. H. Bruggeman, and J. Moriles-Miller, "The use of enriched and natural abundance nitrogen and carbon isotopes in soil fertility research," in *Soil Fertility, Management in Agroecosystems*, A. Chatterjee, D. Clay, ASA, and S. S. S. A. CSSA, Eds., pp. 111–123, Guilford Rd., Madison, WI, USA, 2014.

[22] C. Y. Kramer, "Extension of multiple range tests to group means with unequal numbers of replications," *Biometrics*, vol. 12, no. 3, pp. 307–310, 1956.

[23] K. Fujita, "The technology for composting," in *The Technology for Effective Utilization of Waste Materials*, Gihodo Shuppan, Tokyo, Japan, 3rd edition, 1998.

[24] P. Sørensen, "Short-term nitrogen transformations in soil amended with animal manure," *Soil Biology and Biochemistry*, vol. 33, no. 2, pp. 1211–1216, 2001.

[25] P. Sørensen and M. Amato, "Remineralisation and residual effects of N after application of pig slurry to soil," *European Journal of Agronomy*, vol. 16, no. 2, pp. 81–95, 2002.

[26] M. Maeda, B. Zhao, Y. Ozaki, and T. Yoneyama, "Nitrate leaching in an Andisol treated with different types of fertilizers," *Environmental Pollution*, vol. 121, no. 3, pp. 477–487, 2003.