Isolation of A *Nocardiopsis Chromatogenes* Strain That Degrades PLA (Polylactic Acid) From Pig Waste–Based Compost

Nobuyuki Kawashima (nobuyuki.kawashima@mitsuichemicals.com)
Minato-ku Tokyo https://orcid.org/0000-0002-0554-7086
Junko Tokuda
Mitsui Chemicals Inc.
Tadashi Yagi
Minato-ku Tokyo
Katsuyuki Takahashi
Minato-ku Tokyo

Research Article

Keywords: polylactic acid, compostability, biodegradability, actinomycete, Nocardiopsis chromatogenes, Circular Economy

Posted Date: October 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-990367/v1

Abstract

A new *Nocardiopsis* species that degrades polylactic acid (PLA) was isolated from pig dung–based compost from a municipal composting facility in Japan. To obtain strains capable of efficient PLA degradation, we minimized the effect of non-enzymatic degradation of PLA by maintaining the temperature at 37°C or below. After screening a total of 15 animal waste–based compost samples, consisting of pig dung, cow dung, horse dung, or chicken droppings, we found that compost derived from pig dung was most efficient for degradation of PLA film, and used it for isolation of PLA-degrading microorganisms. Screening for PLA-degrading microorganisms in compost was performed using an agar plate–based method; an emulsifier was omitted to avoid selection of strains that assimilated the emulsifier instead of PLA in the medium. After repeated enrichment, six strains were obtained. One strain that exhibited stable PLA degradation on agar plates was subjected to genomic analysis and identified as *Nocardiopsis chromatogenes,* an actinomycete.

Introduction

Plastic products contribute to many aspects of daily life, and the global community is harnessing their benefits. Annual production of various types of plastics reached 368 million tons in 2019 [1]. With the rise in consumption, the risk of resource depletion and elevation of greenhouse gas (GHG) emissions due to increased use of resources and energy throughout the supply chain have become major issues. Furthermore, environmental burdens due to insufficient recovery of plastic products after use, in particular environmental pollution outflowing into the ocean, has become a significant social issue around the world. To realize a resilient and sustainable economy, the idea of “reduce, reuse, and recycle” to focus on what is appropriate for each application, development of innovative technologies, and designs for social systems are required in order to enhance resource utilization and prevent outflow. These holistic concepts will be realized through effective collection and sorting after use, followed by mechanical recycling, chemical recycling, and energy and thermal recovery. At the same time, there is an urgent need to convert the economy from fossil resources to renewable resources [2].

Circular Economy is an integrated scheme involving resource circulation, carbon neutrality, and social systems. Future models of fossil-based plastics and bioplastics must encompass raw materials, plastic products, applications, and waste/byproduct management from the standpoint of Circular Economy [3]; this will be enhanced not only by reuse and recycling but also by maintenance, repair, and longer life, as well as sharing, throughout the value chain. This transition will be accelerated by digitalization and knowledge sharing such as trading platforms that identify the environmental impacts of individual materials and online databases that track the use of a material across its lifecycle [4]. Furthermore, this direction is expected to be enhanced by an introduction of a regulatory and legislative framework, collaboration with other industries such as agriculture, and incorporation of ambitious and innovative methods for carbon dioxide capture, utilization, and storage (CCUS).

To realize an essential and true Circular Economy for plastics, it is also necessary to take into account people’s needs and pains, as well as the hidden struggles of society: convenience, enjoyment, financial benefits, and social prestige. This requires responsible efforts that utilize traceability throughout the value chain, rather than just part of the circulation of objects.

In Europe, composting to convert organic waste into fertilizer rather than placing it into a landfill is becoming more common [5]. In this context, organic wastes are being collected together with packaging and cutlery made of biodegradable or compostable materials, which are hard to separate after use; these materials are then composted, and the resultant compost is used as a fertilizer for plant growth. Furthermore, by using biomass instead of fossil resources as the raw material, it is possible to realize a carbon cycle and reduce GHG emissions, in addition to preventing outflow to the environment.

Biodegradable plastics include polylactic acid (PLA), polyhydroxyalkanoates (PHA), and polybutylene succinate (PBS). Among them, PLA, a bioplastic made from biomass, has been under development since the 90's [6, 7] and has recently

attracted renewed attention. PLA is a transparent plastic with high glass transition temperature (Tg) and high crystallinity. These characteristics were widely exploited, and PLA is commercially manufactured and used in food packaging, containers, agriculture, hygiene, and durables [8, 9].

There are various types of composts, based on the wastes from which they are derived: food waste, leaves, farm waste, animal manure, and sewage sludge. For animal waste–based compost, a previous study examined cow dung and pig dung–composts for the degradation of food waste [10]. We previously conducted a pilot-scale composting study of PLA products to confirm that there were no adverse effects on the composting process as well as the growth of plants cultivated using the obtained compost [11]. To date, studies have examined the efficiency of PLA degradation in compost at pilot and commercial scales [12–16].

The degradation of PLA in compost proceeds in two phases [6, 8, 17–20]. First, PLA is disintegrated and fragmented via chemical or enzymatic hydrolysis. Second, the fragmented PLA is further hydrolyzed by microorganisms. Some studies, however, have reported that PLA degradation is mostly performed chemically, with only a limited contribution from microorganisms [21]. A previous study [22] concluded that degradation depended on temperature, not the presence of microbes. PLA sheet decomposition was demonstrated on a lab scale using yard waste compost; the results showed that an excessive amount of PLA input produced lactic acid via hydrolysis, resulting in toxicity to microorganisms [23]. Thus, PLA degradation mechanisms should be studied from the standpoints of chemistry, enzymes, and microorganisms, not only for scientific analysis but also for efficient social implementation of composting.

After Tokiwa et al. isolated and identified *Amycolatopsis*, a genus of PLA-degrading actinomycetes, in 1997 [24], the same research group isolated a variety of PLA-degrading microorganisms from environmental sources, soil, and compost. Some of these microorganisms belong to the genera of *Rhizobium, Bacillus*, and *Tuberibacillus*, identified in 2008 [25]. Others belong to the family *Pseudonocardiaceae* and related genera such as *Amycolatopsis, Lentzea, Kibdelosporangium, Streptoalloteichus*, and *Saccharothrix*, described in 2006 [26]. These discoveries were subsequently reviewed in 2017 [27] and 2019 [28].

A study attempted to improve the efficiency of PLA degradation in the compost by spraying a mixture of potent PLA-degrading bacterial strains in 2016. In these experiments, a mixture of four strains, classified as species, *Penicillium chrysogenum, Cladosporium sphaerospermum, Seratia marcescens*, and *Rhodotorula mucilaginosa*, was sprayed onto compost made of vegetable waste, wood chips, and fruit peels. Addition of the bacterial cocktail facilitated PLA degradation and degraded 44 wt% of PLA in 30 days under laboratory conditions. The results demonstrated the importance of microorganisms in PLA degradation during composting [29].

The composition of composts varies depending on the types of waste and the facility where composting is performed. Further, even at the same composting facility, the degradability of PLA is expected to change if the composting conditions, including the microbiota, are different. Thus, in order to optimize degradation conditions, it is important to study the chemical and biological factors of the compost to be used in waste management. In this study, 15 different animal waste–based composts consisting of pig dung, cow dung, horse dung, or chicken droppings from nine different composting facilities in Japan were screened for efficient PLA degradation at a relatively low temperature, e.g., 37° C. In general, when the temperature of cow dung compost rises, ammonia is generated by the decomposition of proteins, and hydrolysis of PLA is accelerated. To focus on the isolation of microorganisms capable of degrading PLA, the temperature was regulated at 37°C to avoid promoting chemical decomposition. Emulsifiers such as those developed for PLA by Tokiwa et al. in 1997 [24] have been widely used to isolate PLA-degrading microorganisms. Emulsifiers were removed afterwards to avoid the possibility of selecting microorganisms that assimilate these compounds From the most efficient pig dung–based compost, we isolated a strain of *Nocardiopsis chromatogenes*. This is the first study focusing on screening animal waste–based composts from farms and facilities leading to isolation of a strain of *Nocardiopsis chromatogenes*.

Materials

- Compost

Fifteen different types of matured compost originating from nine municipal composting facilities in Japan were collected and used. Among 15 types of compost, eight were based on pig dung, four on cow dung, one on horse dung, one on chicken droppings, and one on a mixture of cow dung and chicken droppings.

- λ PLA

A PLA cast film was used in the compost screening test; the size was 3 × 5 cm with a thickness of 20–30 μm. The film was produced by lab-scale T-die extrusion using the PLA powder LACEA (produced by Mitsui Chemicals), which is a BPS-certified Green Plastic (PL#40701). BPS was formerly the Biodegradable Plastics Society, but changed its name to the Japan Bioplastics Association (JBPA). Green Plastic is a brand name of biodegradable plastics. PLA powder (40–50 μm radius, Mw 120,000–150,000) was used to prepare PLA microspheres (1–5 μm radius).

- Reagents

Plysurf A 210G, produced by Dai-ichi Kogyo Seiyaku or DKS, was used as an emulsifier for dispersion of PLA microspheres.

Methods

- Screening of composts for PLA film degradation [primary screening]

Fifteen composts from nine municipal composting facilities were placed in separate jars with PLA films. Jars with PLA films were placed in an incubator at 37°C for 3 months to minimize the effect of non-enzymatic degradation of PLA.

Two-thirds of the 5-cm PLA film was inserted vertically into the jar with the compost (Figure 1).

- Further evaluation of selected composts and collection of microorganisms [secondary screening]

One compost selected from the primary screen was subject to PLA film degradation at 37°C for 6 months.

During test periods, the sample was occasionally sprayed with water to avoid drying.

- Single-colony isolation of PLA-degrading microorganisms on agar plates

For screening of the strains, glycerol/asparagine-based medium was used, as described previously [30].

Two-layer agar plates were prepared as follows.

(Bottom layer) Glycerol/asparagine-based medium was prepared with a composition of 0.5 g/L glycerol, 0.5 g/L L-asparagine, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 1 mg/mL $FeSO_4 \cdot 7H_2O$, 1 mg/mL $MnCl_2 \cdot 4H_2O$, and 1 mg/mL $ZnSO_4 \cdot 7H_2O$ at a pH of 7.0. To the medium was added 20 g of Bacto agar; the resultant mixture was autoclaved at 120°C for 20 min and poured into petri dishes.

(Top layer) One gram of PLA powder (40–50 μm radius, MW 120,000–150,000), as shown in Figure 2, was dissolved in 40 mL of methylene chloride. The resultant solution was emulsified with 1 L of 100 mg/L of emulsifier Plysurf A 210G. The emulsion was subjected to a warm bath and evaporator to evaporate the methylene chloride. The resultant PLA microsphere particles were collected by filtration, followed by repeated washing and filtration to remove the emulsifier. The collected PLA microsphere particles, as shown in Figure 2, were mixed with 1 L of the same glycerol/asparagine broth as

for the bottom layer, and following the addition of 20 g of Bacto agar, the PLA-containing medium was autoclaved and poured onto the bottom layer of the petri dishes.

- Isolation of strains from the agar plates

After 6 months of secondary screening, degradation of PLA film and growth of microorganisms on the degraded portion was observed. The microorganisms were collected with an inoculation loop and streaked on a two-layer agar plate of which the top layer contained PLA.

The plates were incubated at 37°C for about 2 weeks until clear zones were visible. The clear zone was scraped with an inoculation loop and streaked onto new agar plates to enrich and isolate single colonies. This process was repeated four times.

- Characterization of isolated strains and phylogenetic analysis

Physiological characterization was conducted by Japan Food Research Laboratories.

To extract 16S rDNA, colonies on plates were collected and resuspended in TE buffer and disrupted by vortexing with glass beads. The debris was removed by centrifugation (7,000 *g*), and the clear lysate was collected as the DNA solution. TE: Tris and EDTA, Tris: 2-Amino-2-(hydroxymethyl) propane-1,3-diol; EDTA: 2,2',2",2‴-(Ethane-1,2-diyldinitrilo) tetraacetic acid.

The genomic DNA of 16S rRNA was amplified and isolated using the QIAamp DNA Mini Kit (Qiagen). The 16S rRNA gene was sequenced by chromosomal walking using the dRhodamine Dye Terminator Cycle Sequencing Kit on an ABI 377 sequencer. Sequence analysis was performed using the Genetyx software.

The primers used for the amplification of 16S rRNA gene were: 27f (5’-GAGTTTGATCCTGGCTCAG-3’), 357f (5’-CTCCTGCGGGAGGCAGCAG-3’), 536f (5’-CCAGCAGCCGCGGTAATAC-3’), 803f (5’-GATTAGATACCCTGGTAGT-3’), 1114f (5’-GCAACGAGCGCAACCC-3’), 519r (5’-GTATTACCGCGGCTGCTGGC-3’), 907r (5’-CCGTCAATTCCTTTGAGTTT-3’), 1385r (5’-CGGTGTGTACAAGGCCC-3’), and 1525r (5’-AGGAGGTGATCCAGCC-3’).

Phylogenetic analysis was conducted using the MEGA 5 software. The sequence for MT-20147 is registered as LC648363 in the DNA Data Bank of Japan (DDBJ).

Results And Discussion

- Screening of animal waste–based composts (primary screening)

To select the composts to be used for isolation of PLA-degrading microorganisms, 15 composts collected from nine operating municipal compost facilities in Japan were screened for PLA film degradation. Out of 15 composts, eight were based on pig dung, four on cow dung, one on horse dung, one on chicken droppings, and one on a mixture of cow dung and chicken droppings.

The PLA films were checked visually every 2 weeks. The pig dung–based composts No. 2, 3, 9, 12, 13, and 15 degraded the PLA film, whereas two composts of the same type, No. 11 and 14, did not. The cow dung–based composts (No. 1, 5, 7, 8), the horse dung–based compost (No. 4), the chicken dropping–based compost (No. 6), and the mixture (No. 10) did not degrade PLA film. After 3 months of composting in the 37°C incubator, degradation of PLA film was observed in six composts, as summarized in Table 1 and Table 2.

One possible reason why the PLA film was degraded in pig dung compost, but not in composts based on other material including cow dung, is as follows. Because these were mature composts and tests were conducted in small jars, it is probable that the temperature did not rise. In general, when the temperature of cow dung compost rises, e.g., to 58°C, the Tg

of PLA, ammonia is generated by the decomposition of proteins and the hydrolysis of PLA is accelerated. However, in these trials, the temperature was controlled at 37°C, so chemical decomposition was not promoted and degradation by microorganisms did not occur. In other words, in this experiment, no microorganisms that degraded PLA at 37°C were present in compost derived from cow dung, but such organisms were present in compost derived from pig dung.

A previous report examined pig dung– and cow dung–based composts for degradation of food wastes [10]. The authors of that study compared the process of composting between cow dung/food waste and pig manure/food waste. The results revealed that the number of bacteria was about two orders of magnitude higher in the pig dung system than in the cow dung system, and the pH was also lower. This result relates to our assumptions regarding the comparison of cow and pig dung.

The standard test method for evaluating biodegradable plastic with compost (ISO14855) is performed at 58 ± 2°C. Many studies have the degradability of PLA products in a pilot scale compost, e.g., compost with fiber, fat, and protein in animal fodder at 58°C [31]; compost with cow manure and wood waste at 60–65°C [12,13]; and compost with green yard waste at about 60°C [14]. In addition, in the PLA degradation test with our pilot scale compost [11], we used compost containing horse manure and plants. Because this was a fresh and large-scale compost with a total weight of 110 kg, the internal temperature was 70°C or higher at the beginning of the composting process. Despite the use of horse manure, degradation proceeded because hydrolysis was promoted at the early stage.

In our screening of the composts, one compost derived from pig dung (No. 2), which was provided by a municipal composting facility in Kita-hiroshima, Hokkaido, had the highest PLA degradation activity. Accordingly, we selected it for further study.

- Further evaluation of selected composts and collection of microorganisms (secondary screening)

The selected compost (No. 2) was subject to further evaluation of PLA degradation. PLA film was placed in the compost, and degradation was monitored.

After 6 months of incubation at 37°C, degradation of the films was observed in the compost as shown in Figure 3. Notably, in our other experiment, the T-dye–extruded film was more susceptible to degradation than the biaxial-oriented film, which has higher crystallinity.

- Isolation of the strain

A cluster of microorganisms was observed on the degraded portions of the films. The cluster was collected with an inoculation loop to isolate the strain responsible for the degradation of PLA film.

The cluster was streaked on agar film, and clear zones were spotted after 1.5 months. To avoid isolating microorganisms that assimilate and grow on the emulsifier, we removed the emulsifier during plate prep. As described in the Methods, after PLA powder was dissolved in CH_2Cl_2, the emulsifier was added, and the resultant emulsified solution was warmed to evaporate the CH_2Cl_2. PLA microspheres were collected by repeated filtration and washing to remove the emulsifier. Conversion of PLA powder to microspheres was predicted to increase contact with the microorganisms.

Although many studies have used emulsifiers to form microspheres (Tokiwa et. al. in 1997 [24], 2004 [33], and 2006 [17]; and by others in 2001 [32], 2008 [34], 2009 [35], 2014 [36], 2016 [28], and 2018 [37]), microspheres can also be formed without emulsifiers, as follows. After PLA pellets were dissolved in chloroform, methanol ([38] in 2004 and [39] in 2019) or ethanol in 2014 [21] was added for homogenization, and the microspheres were obtained through filtration.

To further purify the microorganisms, the clear zone was scraped and streaked onto a new plate (Figure 4). This process was repeated four times. Ultimately, six colonies were isolated that produced a clear zone on the plate. Because a large

halo formed as shown in Figure 5, we speculated that enzymes were secreted. We chose one strain, MT-24107, for further analysis and identification. The edge of the white fungus that formed the halo shown in Figure 5 was observed by phase-contrast microscopy. The thread-like spreading pattern is characteristic of Actinomycetes (Figure 6).

The reason for using the two-layer screening system in this experiment is as follows. Because it takes about 1 month to decompose PLA, it is difficult to detect the decomposition halo in a one-layer system in which PLA microspheres are dispersed over the entire agar medium. By adopting a two-layer system, when the upper microspheres decompose and halos are generated, it is easier for light to pass through, and detection is easier.

We employed PLA microspheres in our plate screen for PLA-degrading microorganisms in order to shorten the overall time required. Although PLA powder (40–50 μm radius) took 4 weeks to form a clear zone (i.e., halo), the process took only 2 weeks when PLA microspheres (1–5 μm radius) were used.

MT-20147 was suspended in medium with the same composition as during agar plate testing after secondary screening, except for the addition of Bacto agar, and the suspended liquid was poured onto a PLA film placed in a Petri dish. After incubation for several days, the film surface was washed and observed by electron microscopy. A characteristic degradation path was observed on the film surface, as shown in Figure 7.

- Identification of the strain

As a result of physiological characterization shown in Table 3, the strain was presumed to be *Nocardiopsis*, classified as an actinomycete because of its cell wall and quinone types. However, spore formation was not observed, and the genus could not be confirmed from this result alone.

Colonies of isolated strain MT-20147 was scraped and collected. The harvested cells were physically disrupted by glass beads and the genomic DNA was extracted.

Sequencing started with the primer 27f. Using the resultant sequence data, the new primers 519r, 357f, and 536f were designed. Sequence analysis was performed for the region read by these primers, and subsequent primers were designed in the same manner to cover a 1,489-bp fragment in the 16S rRNA gene by assembling contigs (Figure 8).

The sequence of the assembled contigs was analyzed using BLAST and exhibited 99.2% of similarity with *Nocardiopsis chromatogenes*, order Streptosporangiales, as summarized in Table 4. A phylogenetic tree was created with the MEGA5 software as shown in Figure 9.

- Meanings and issues of compost treatment of PLA products after use

PLA has been attracting attention as a representative commercially produced biodegradable plastic derived from renewable resources [8, 9]. To achieve a Circular Economy, it is necessary to perform appropriate and efficient treatment of PLA products after use. Composting is the second-best approach for handling PLA composites; the best approach is recycling, as with fossil-based plastics [40]. In Europe, the role of composting is becoming more important as a means to treat organic wastes efficiently rather than become landfill [5], and it will be worthwhile to use compostable products such as food packaging and cutlery when it is difficult to separate and recover those products from organic waste such as food residues.

Because the resultant compost contributes to plant growth, it should be regarded as an effective use of waste and by-products in the circular system. The CO_2 emissions are totally suppressed because the CO_2 generated by degradation is absorbed by the plant [40, 41].

In regard to social implementation, we published a paper that describes degradation of PLA products in compost on a pilot scale. The results revealed that the presence or absence of PLA products does not adversely affect the degradation

process or the quality and safety of the resultant compost [11]. In addition, pilot and commercial-scale composts, which contain cow dung as the main component [12–14] or green yard waste as the main component [15, 16, 42] were used to test the degradability of PLA products. In all these papers, the temperature condition of the compost was 58°C or higher.

The social implementation of composting has been widely studied. For examples, studies that elucidated the mechanism by which PLA is degraded in compost were published in in 1998 [22], 2001 [23], and 2014 [21]. Papers describing the bacteria and enzymes that promote degradation were published in 2004 [33], 2005 [43], and 2006 [26]. A review of microbial degradation of polymers was published in 2017 [27].

To construct a feasible framework for PLA waste management, it is important to further study the factors and mechanisms affecting biodegradability, for example, types of composts, microorganisms involved in degradation, physical properties of PLA, temperature, pH, and time.

- Isolation of *N. chromatogenes*

In this study, we isolated and identified PLA-degrading microorganisms using animal-based composts (pig, cow, horse, and chicken). Multiple reports have described the isolation of PLA-degrading microorganisms, but to date no study has identified *N. chromatogenes* as a PLA-degrading microbe.

In the 90's, actinomycetes isolated from the natural environment (e.g., soil) were identified as PLA-degrading microorganisms, and multiple actinomycetes have been described not only in soil but also in compost. Furthermore, *Bacillus subtilis* and fungi capable of degrading PLA have also been reported.

PLA-degrading microorganisms were reviewed as follows based on the classification of the cited references. We expect to convey that the species we identified had not been previously reported in the literature.

In 1997, Tokiwa et al. isolated and identified *Amycolatopsis*, a genus of actinomycete whose members are capable of degrading PLA, from 45 types of soil samples in Tsukuba City, Japan [24]. This bacterium also degrades silk, as reported in 1999 [44]. Using actinomycetes obtained from public institutions, *Kibdelosporangium aridum* [45] and *Saccharothrix waywayandensis* [46] were shown to degrade PLA in 2003, and *Amycolatopsis orientalis* was shown to produce PLA-degrading enzymes in 2006 [17]. Furthermore, in 2004, Tokiwa et al. reviewed the importance of actinomycetes as PLA-degrading microorganisms in conjunction with active enzymes [33]. In 2001, the same type of microorganism, *Amycolatopsis sp.*, was isolated from 300 soil samples [32].

In addition to actinomycetes, *Laceyella sacchari* isolated from forest soil in 2014 [36] and strains of *Pseudomonas* and *Bacillus*, both isolated from sludge in 2017 [20], are capable of PLA degradation. In 2016, a paper showed that three out of four microbes isolated from 300 soil samples from various sources were fungi: *Penicillium chrysogenum sp.*, *Cladosporium sphaerospermum sp.*, and *Rhodotorula mucilaginosa sp.*; the exception was *Serratia marcescens* [29].

PLA-degrading microorganisms in compost are diverse. The raw materials used in the compost test for isolation and identification of PLA-degrading microorganisms are mainly animal feed, food waste, and plant residue, and animal manure has been used in only a few cases. *Bacillus smithii* of the order Bacillales was obtained from a garbage fermentor, and the PLA-degrading enzyme was identified as a serine protease in 2001 [47]. In 2008, *Bacillus licheniformis* of the order Bacillales was isolated from compost made from animal fodder and identified as a PLA-degrading microorganism [34].

Microorganisms that form biofilms were shown to degrade PLA in compost in 2015 [48]. These organisms were of the genera *Acidovorax, Aeromonas, Arthrobacter*, and *Chryseobacterium*. All four are Bacteria, and *Arthrobacter* is a type of actinomycete. *Thermopolyspora flexuosa*, another actinomycete, was identified from lab-scale compost in 2014 [21].

As reviewed above, it is clear that PLA-degrading microorganisms range from prokaryotes such as actinomycetes to eukaryotes such as fungi.

A comprehensive review on PLA-degrading microorganisms was published in 2019 [27]. That paper described five families, eleven genera, and twenty-five species for which enzyme classification was also available. Furthermore, enzymes related to PLA-degrading microbes were reviewed in a paper from 2006 [25]. A comprehensive review article of microorganisms that degrade fossil-based plastics as well as biodegradable plastics, including PLA, was published in 2017 [26].

In the long history of research on PLA-degrading microorganisms, *Nocardiopsis chromatogenes* has not been identified.

Recent studies have examined microorganisms that degrade PET have been conducted. Among them, *Nocardiopsis chromatogenes* was shown to be a PET-degrading species in 2018 [49]. Although PET is aromatic and PLA is aliphatic, and the two compounds have different chemical structures, it is noteworthy that microorganisms from the same genus are involved in the degradation of both polyesters.

As previously described, the PLA degradation mechanism consists of hydrolysis, enzymatic degradation, microbial degradation, and combinations thereof. To carry out the degradation in compost more efficiently and economically, future work should elucidate the mechanisms, microbes, and enzymes secreted during the process of degradation.

Conclusions

In this study, we used two specific approaches to isolate PLA-specific degrading microorganisms. First, we attempted to rule out the possibility of PLA degradation by ammonia, which promotes proteolysis at high temperatures, by limiting the temperature below 37°C in compost derived from various types of livestock manure. Normally, the composting of organic waste by cow dung compost is performed at high temperature. Because we suppressed the temperature at or below 37°C, hydrolysis at the initial stage of degradation did not proceed and PLA was not degraded, i.e., PLA-degrading microorganisms could not be obtained. On the other hand, PLA-degrading microbes were obtained only from pig dung compost. Furthermore, to avoid the possibility of selecting microbes that assimilate the emulsifier, it was removed after the screening process.

As a result, a microorganism capable of degrading PLA was isolated from pig dung–based compost. Based on the properties of the microorganism and a genetic analysis, it was identified as *Nocardiopsis chromatogenes*, an actinomycete of order Streptosporangiales. This is the first time that *N. chromatogenes* has been shown to degrade PLA. Further research is needed to characterize the microbe we isolated, the enzymes it secretes, and the mechanism of degradation.

Declarations

Acknowledgments

The authors thank Shin Fukuda, Shingo Shibata, Kiyoshi Ito, Hiroyasu Yamaoka, Tetsuya Hamada, Ken Migita, Ryohei Ogawa, and Toshie Sato for their support and insight. The authors also thank Tadahisa Iwata of The University of Tokyo, Hiroyasu Yamaguchi of Osaka University, Masahiro Sakamoto of Kyoto University, and Mari Takahara of National Institute of Technology, Kitakyushu College, for their advice and instruction.

Funding: This research received no external funding.

Conflicts of interest/Competing interests: The authors declare no conflict of interest or competing interests.

Availability of data and material (data transparency): Not applicable

Code availability (software application or custom code): Not applicable

References

[1] Plastics – the Facts 2020 (2020) PlasticEurope, Brussels, Belgium.

https://www.plasticseurope.org/application/files/8016/1125/2189/AF_Plastics_the_facts-WEB-2020-ING_FINAL.pdf. Accessed 1 June 2021

[2] Kawashima N, Yagi T, Kojima K (2019) How do bioplastics and fossil-based plastics play in a circular economy? Macromol Mater Eng. https://doi.org/10.1002/mame.201900383

[3] Towards the Circular Economy (2013) The Ellen MacArthur Foundation.

https://www.ellenmacarthurfoundation.org/assets/downloads/publications/Ellen-MacArthur-Foundation-Towards-the-Circular-Economy-vol.1.pdf. Accessed 1 June 2021

[4] Kawashima, N (2020) How do fossil-based plastics and bioplastics play in a circular economy? Polymer Preprints. In: Proceedings of the 70th SPSJ Annual Meeting, 26 May 2020

[5] Relevance of Biodegradable and Compostable Consumer Plastic Products and Packaging in a Circular Economy (2021) https://circulareconomy.europa.eu/platform/sites/default/files/relevance_of_biodegradable.pdf. Accessed 1 June 2021 doi:10.2779/497376

[6] Kawashima N, Ogawa S, Obuchi S, Matsuo M, Yagi T (2004) In: Doi Y, Steinbüchel A (eds) Biopolymers, Polylactic acid "LACEA", Wiley-VCH, Weinheim, Germany, 2004, Volume 4, pp. 251–274.

[7] Gruber PR, O'Brien M (2005) In: Steinbüchel, A (ed) Biopolymers Online, Polylactides "NatureWorks® PLA", Wiley-VCH, Weinheim, Germany, 2005, pp. 235–239.

[8] Iwata T (2015) Biodegradable and bio-based polymers: Future prospects of eco-friendly plastics. Angew Chem Int Ed. https://doi.org/10.1002/anie.201410770

[9] Castro-Aguirre E, Iniguez-Franco F, Samsudin H, Fang X, Auras R (2016) Poly(lactic acid) - Mass production, processing, industrial applications and end of life. Adv Drug Deliv Rev. https://doi.org/10.1016/j.addr.2016.03.010

[10] Adegunloye DV, Adetuyi FC (2009) Composting of Food Wastes Using Cow and Pig Dung as Booster. African Journal of Basic & Applied Sciences 1:70–75

[11] Kawashima N, Yagi T, Kojima K (2021) Pilot-Scale Composting Test of Polylactic Acid for Social Implementation. Sustainability. https://doi.org/10.3390/su13041654

[12] Kale G, Auras R, Singh SP (2006) Degradation of commercial biodegradable packages under real composting and ambient exposure conditions. J Polym Environ.

https://doi.org/10.1007/s10924-006-0015-6

[13] Kale G, Aurus R, Singh SP, Narayan R (2007) Biodegradability of polylactide bottles in real and simulated composting conditions. Polym Test.

https://doi.org/10.1016/j.polymertesting.2007.07.006

[14] Kijchavengkul T, Kale G, Auras R (2009) Degradation of biodegradable polymers in real and simulated composting conditions. In: Celina MC, Wiggings JS, Billingham NC (eds) Polymer Degradation and Performance of the ACS

Symposium Series, Division of Polymer Chemistry in American Chemical Society, Washington, DC, USA, 2009; Volume 1004, pp. 31–40. DOI: 10.1021/bk-2009-1004.ch003

[15] Greene J (2007) Biodegradation of compostable plastics in green yard-waste compost environment. J Polym Environ. https://doi.org/10.1007/s10924-007-0068-1

[16] Zhanga H, McGill E, Gomez CO, Carson S, Neufeld K, Hawthorne I, Smukler SM (2017) Disintegration of compostable foodware and packaging and its effect on microbial activity and community composition in municipal composting. Int Biodeterior Biodegrad.

https://doi.org/10.1016/j.ibiod.2017.09.011

[17] Jarerat A, Tokiwa Y, Tanaka H (2006) Production of poly(L-lactide)-degrading enzyme by Amycolatopsis orientalis for biological recycling of poly (L-lactide). Appl Microbiol Biotechnol.

https://doi.org/10.1007/s00253-006-0343-4

[18] Agarwal M, Koelling KW, Chalmers JJ (2008) Characterization of the Degradation of Polylactic Acid Polymer in a Solid Substrate Environment. Biotechnol Prog.

https://doi.org/10.1021/bp980015p

[19] Karamanlioglu M, Robson GD (2013) The influence of biotic and abiotic factors on the rate of degradation of poly(lactic) acid (PLA) coupons buried in compost and soil. Polym Degrad Stab.

https://doi.org/10.1016/j.polymdegradstab.2013.07.004

[20] Kim MY, Kim C, Moon J, Heo J, Jung SP, Kim JR (2017) Polymer Film-Based Screening and Isolation of Polylactic Acid (PLA)-Degrading Microorganisms. J Micro Biotech.

https://doi.org/10.4014/jmb.1610.10015

[21] Husárová L, Pekařová S, Stloukal P, Kucharzcyk P, Verney V, Commereuc S, Ramone A, Koutny M (2014) Identification of important abiotic and biotic factors in the biodegradation of poly(l-lactic acid). Int J Biol Macromol. doi: 10.1016/j.ijbiomac.2014.04.050.

[22] Agarwal M, Koelling KW, Chalmers JJ (1998) Characterization of the Degradation of Polylactic Acid Polymer in a Solid Substrate Environment. Biotechnol Prog. https://doi.org/10.1021/bp980015p

[23] Ghorpade VM, Gennadios A, Hanna MA (2001) Laboratory composting of extruded poly(lactic acid) sheets. Bioresour Tech.

https://doi.org/10.1016/S0960-8524(00)00077-8

[24] Pranamuda H, Tokiwa Y, Tanaka H (1997) Polylactide Degradation by an Amycolatopsis sp. Appl Environ Microbiol 63:1637–1640

[25] Sangwan P, Wu DY (2008) New insights into polylactide biodegradation from molecular ecological techniques. Macromol Biosci. https://doi.org/10.1002/mabi.200700317

[26] Tokiwa Y, Calabia BP (2006) Biodegradability and biodegradation of poly(lactide). Appl Microbiol Biotechnol.

https://doi.org/10.1007/s00253-006-0488-1

[27] Pathak VM, Navneet (2017) Review on the current status of polymer degradation: a microbial approach. Bioresour Bioprocess.

DOI 10.1186/s40643-017-0145-9

[28] Butbunchu N, Pathom-Aree W (2019) Actinobacteria as Promising Candidate for Polylactic Acid Type Bioplastic Degradation. Front Microbiol.

https://doi.org/10.3389/fmicb.2019.02834

[29] Nair NR, Sekhar VC, Nampoothiri KM (2016) Augmentation of a Microbial Consortium for Enhanced Polylactide (PLA) Degradation. Indian J Microbiol.

https://doi.org/10.1007/s12088-015-0559-z

[30] Nishida H, Tokiwa Y (1993) Distribution of poly(β-hydroxybutyrate) and poly(ε-caprolactone)aerobic degrading microorganisms in different environments. J Environ Poly Degrad 1:227–233

[31] Yang HS, Yoon JS, Kim MN (2005) Dependence of biodegradability of plastics in compost on the shape of specimens. Polym Degrad Stab.

https://doi.org/10.1016/j.polymdegradstab.2004.07.016

[32] Nakamura K, Tomita T, Abe N, Kamio Y (2001) Purification and characterization of an extracellular poly(L-lactic acid) depolymerase from a soil isolate, *Amycolatopsis sp.* strain K104-1. Appl Environ Microbiol.

doi: 10.1128/AEM.67.1.345-353.2001

[33] Tokiwa Y, Jarerat A (2004) Biodegradation of poly(L-lactide). Biotechnol Lett 26:771–777

[34] Kim MN, Kim WG, Weon HY, Lee SH (2008) Poly(L-lactide)-Degrading Activity of a Newly Isolated Bacterium. J Appl Polym Sci.

https://doi.org/10.1002/app.26658

[35] Sukkhum S, Tokuyama S, Tamura T, Kitpreechavanich V (2009) A novel poly (L-lactide) degrading actinomycetes isolated from Thai forest soil, phylogenic relationship and the enzyme characterization. J Gen Appl Microbiol.

https://doi.org/10.2323/jgam.55.459

[36] Hanphakphoom S, Maneewong N, Sukkhum S, Tokuyama S, Kitpreechavanich V (2014) Characterization of poly(L-lactide)-degrading enzyme produced by thermophilic filamentous bacteria *Laceyella sacchari* LP175. J Gen Appl Microbiol.

https://doi.org/10.2323/jgam.60.13

[37] Bubpachat T, Sombatsompop N, Prapagdee B (2018) Isolation and role of polylactic acid-degrading bacteria on degrading enzymes productions and PLA biodegradability at mesophilic conditions. Polym Degrad Stabil.

https://doi.org/10.1016/j.polymdegradstab.2018.03.023

[38] Jarerat A, Tokiwa Y, Tanaka H (2004) Microbial Poly(L-lactide)-Degrading Enzyme Induced by Amino Acids, Peptides, and Poly(L-amino Acids). J Poly Environ.

https://doi.org/10.1023/B:JOOE.0000038545.69235.f2

[39] Panyachanakul T, Sorachart B, Lumyong S, Lorliam W, Kitpreechavanich V, Krajangsang S (2019) Development of biodegradation process for Poly(DL-lactic acid) degradation by crude enzyme produced by *Actinomadura keratinilytica* strain T16-1. Electron J Biotechnol. https://doi.org/10.1016/j.ejbt.2019.04.005

[40] Beigbeder J, Soccalingame L, Perrin D, Bénézet JC, Bergeret A (2019) How to manage biocomposites wastes end of life? A life cycle assessment approach (LCA) focused on polypropylene (PP)/wood flour and polylactic acid (PLA)/flax fibres biocomposites. Waste Manag. https://doi.org/10.1016/j.wasman.2018.11.012

[41] Vink ETH, Rabago KR, Glassner DA, Gruber PR (2003) Applications of life cycle assessment to NatureWorks™ polylactide (PLA) production. Polym Degrad Stab. https://doi.org/10.1016/S0141-3910(02)00372-5

[42] Sintim HY, Bary AI, Hayes DG, English ME, Schaeffer SM, Miles CA, Zelenyuk A, Suski K, Flury M (2019) Release of micro- and nanoparticles from biodegradable plastic during in situ composting. Sci Total Environ.

https://doi.org/10.1016/j.scitotenv.2019.04.179

[43] Lim HA, Raku T, Tokiwa Y (2005) Hydrolysis of polyesters by serine proteases. Biotechnol Lett.

https://doi.org/10.1007/s10529-005-2217-8

[44] Tokiwa Y, Konno M, Nishida H (1999) Isolation of Silk Degrading Microorganisms and Its Poly(L-lactide) Degradability. Chem Lett. https://doi.org/10.1246/cl.1999.355

[45] Jarerat A, Tokiwa Y, Tanaka H (2003) Poly(L-lactide) degradation by *Kibdelosporangium aridum*. Biotechnol Lett.

https://doi.org/10.1023/B:BILE.0000004398.38799.29

[46] Jarerat A, Tokiwa Y (2003) Poly(L-lactide)degradation by *Saccharothrix waywayandensis*. Biotechnol Lett.

https://doi.org/10.1023/A:1022450431193

[47] Sakai K, Kawano H, Iwami A, Nakamura M, Moriguchi M (2001) Isolation of a Thermophilic Poly-L-Lactide Degrading Bacterium from Compost and Its Enzymatic Characterization. J Biosci Bioeng.

https://doi.org/10.1016/S1389-1723(01)80266-8

[48] Walczak M, Brzezinska MS, Sionkowska A, Michalska M, Jankiewicz U, Deja-Sikora E (2015) Biofilm formation on the surface of polylactide during its biodegradation in different environments. Colloids Surf B,

https://doi.org/10.1016/j.colsurfb.2015.09.036

[49] Joo S, Cho IJ, Seo H, Son HF, Sagong HY, Shin TJ, Choi SY, Lee SY, Kim KJ (2018) Structural insight into molecular mechanism of poly(ethylene terephthalate) degradation. Nat Comm. https://doi.org/10.1038/s41467-018-02881-1

Tables

Table 1: Origin of compost, its main components, and the results of the PLA film degradation test

Municipal composting facility in Japan		Main component of the compost	Degradation
Hokkaido A (Hayakita)	#1	cow dung–based	N
Hokkaido B (Kita-Hiroshima)	#2	pig dung–based	Y
Aomori	#3	pig dung–based	Y
	#4	horse dung–based	N
Yamagata	#5	cow dung–based	N
	#6	chicken dropping–based	N
Niigata	#7	A cow dung–based	N
	#8	B cow dung–based	N
	#9	pig dung–based	Y
Chiba	#10	cow dung– and chicken dropping–based	N
Nagano	#11	pig dung–based	N
	#12	pig dung–based	Y
Aiichi	#13	pig dung–based	Y
	#14	pig dung–based	N
Kagoshima	#15	pig dung–based	Y

Composting facilities provided the compost at the authors' request.

Y:degraded, N:not degraded.

Table 2: Numbers of compost facilities and their ID numbers that provided samples capable of degrading PLA film

Main components of the compost	Number of composting facilities	Numbers of compost facilities and the ID numbers of samples capable of degrading PLA film
Pig dung	8	6 (#2, #3, #9, #12, #13, #15)
Cow dung	4	0
Chicken droppings	1	0
Horse dung	1	0
Cow dung and chicken droppings	1	0

Table 3: Physiological characterization of strain MT-20147

Items	Results
Cell wall composition	Type Ⅻ
LL- Diaminopimelic acid	−
meso – Diaminopimelic acid	−
Diaminobutyric acid	−
Glycine	−
Aspartic acid	−
Ornithine	−
Lysine	−
Arabinose *1	−
Galactose *1	−
Quinone system	MK-10(H_6), -10(H_8)
Substrate Mycelium	−
Aerial Mycelium	−
Spore formation	Not observed
Color of colony	Grayish orange
Production of water-soluble pigments	− Brownish orange
Nitrate reduction	−
Growth at 30℃	−
Growth at 40℃	−
Growth at 50℃	−
Carbon source utilization	
Aesculin	−
Casein	−
DNA	−
Gelatin	−
Guanine	−
Hypoxanthine	−
Starch	−
Testosterone	−
Tyrosine	−
Xanthine	−
Intracellular DNA GC content（mol%）*2	73

*1 Estimated using sulfuric acid hydrolysate of whole cells.

*2 According to the HPLC method.

Tested by Japan Food Research Laboratories

Table 4: Results of BLAST homology search of strain MT-20147

Rank	Name	Strain	Authors	Accession	Pairwise Similarity (%)	Mismatch/Total nt	Completeness (%)
1	Nocardiopsis chromatogenes	YIM 90109	Li et al. 2006	AY619715	99.22371	11/1417	97.39726027
2	Nocardiopsis baichengensis	YIM 90130	Li et al. 2006	ANAS01000053	98.9011	16/1456	100
3	Nocardiopsis halophila	KCTC 9825	Al-Tai and Ruan 1994	AF195411	98.83242	17/1456	100
4	Nocardiopsis coralliicola	SCSIO 10427	Li et al. 2012	JN006759	98.07281	27/1401	96.36737491
5	Nocardiopsis composta	KS9	Kämpfer et al. 2002	AF360734	97.70992	33/1441	99.10775566
6	Nocardiopsis potens	DSM 45234	Yassin et al. 2009	ANBB01000116	97.6584	34/1452	100
7	Nocardiopsis sediminis	1SS5-02	Muangham et al. 2016	LC110387	97.38652	38/1454	100
8	Nocardiopsis rosea	YIM 90094	Li et al. 2006	AY619713	97.11142	42/1454	100
9	Nocardiopsis gilva	YIM 90087	Li et al. 2006	AY619712	97.03857	43/1452	99.79437971
10	Nocardiopsis mwathae	No.156	Akhwale et al. 2016	KF976731	96.97595	44/1455	100

Figures

Figure 1

Figure 1

PLA film degradation test with the compost in a jar.

Figure 2

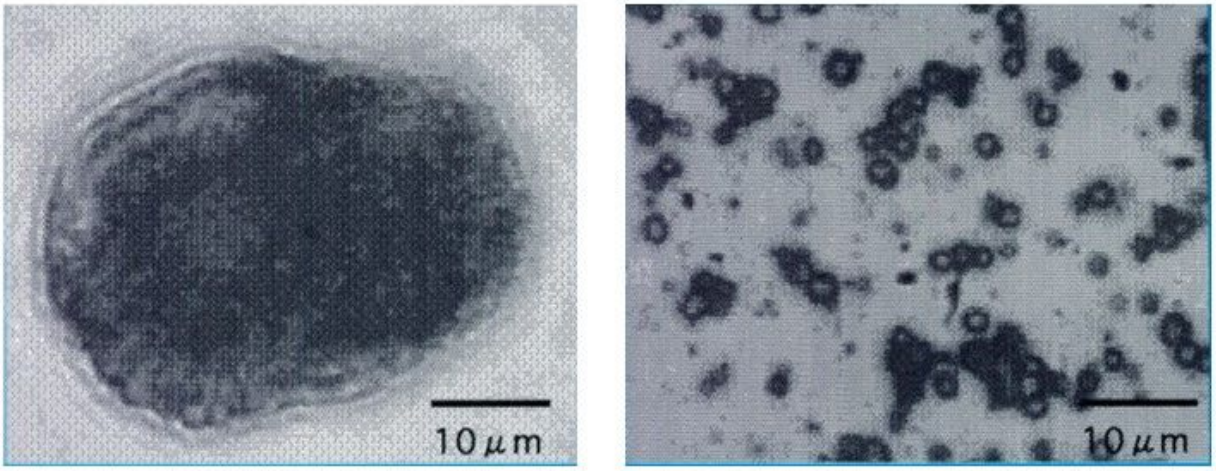


Figure 2

Photos of the PLA powder and microspheres. The average size of PLA powder (left) is 40–50 μm and that of a PLA microsphere (right) is 1–5 μm.

Figure 3

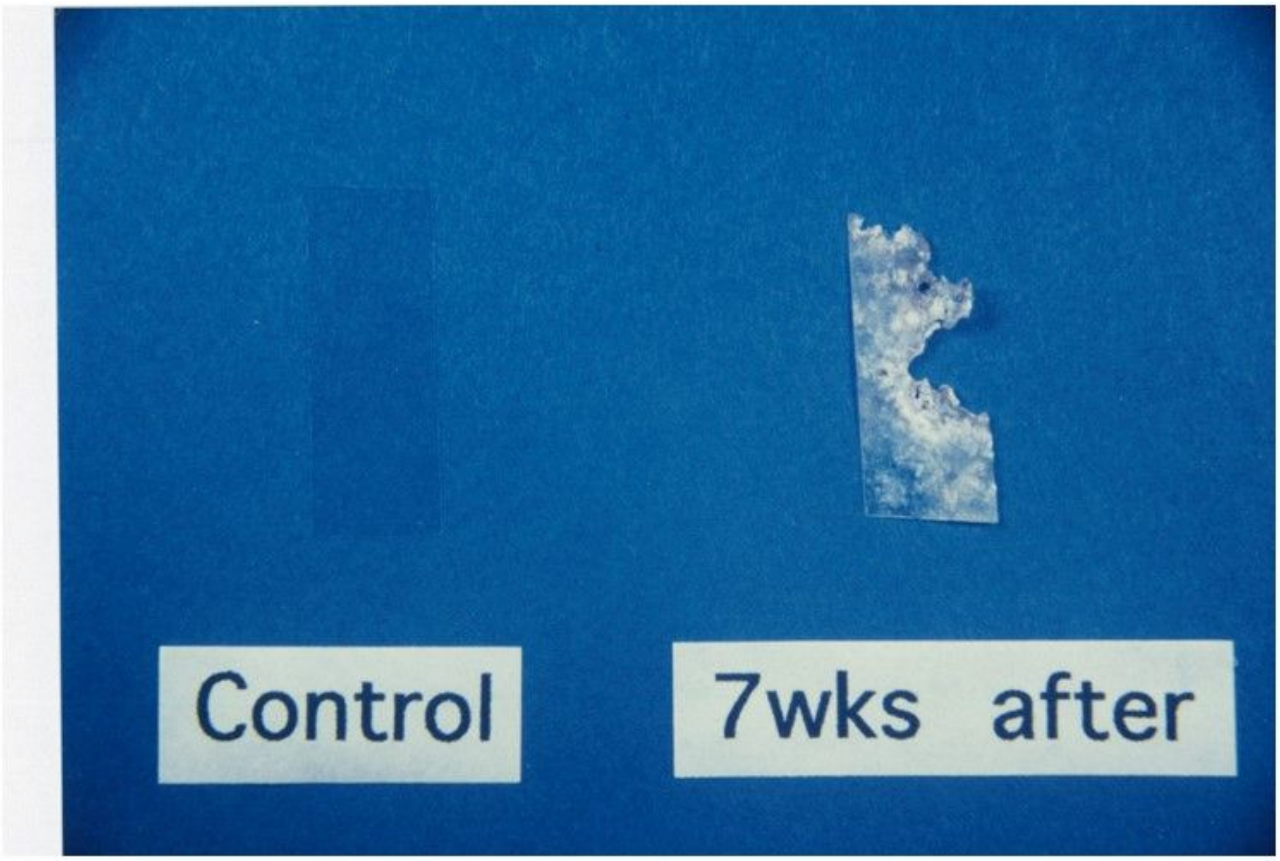


Figure 3

A photo of PLA film before and after the degradation test. PLA films: Control before the testing (left), which is hard to see because of its transparency, and the film after 7 weeks in the compost (right).

Figure 4

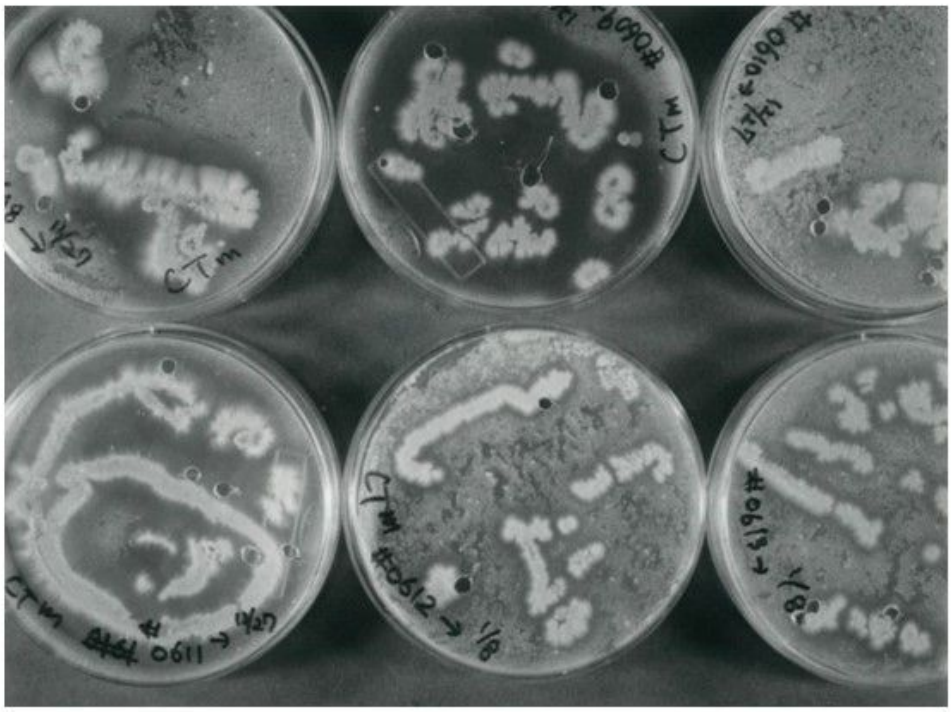


Figure 4

Photo of six strains isolated from pig dung–compost. Six strains isolated from the pig dung compost that exhibited halo-forming ability. White indicates a colony of microorganisms, and the transparent part is the halo.

Figure 5

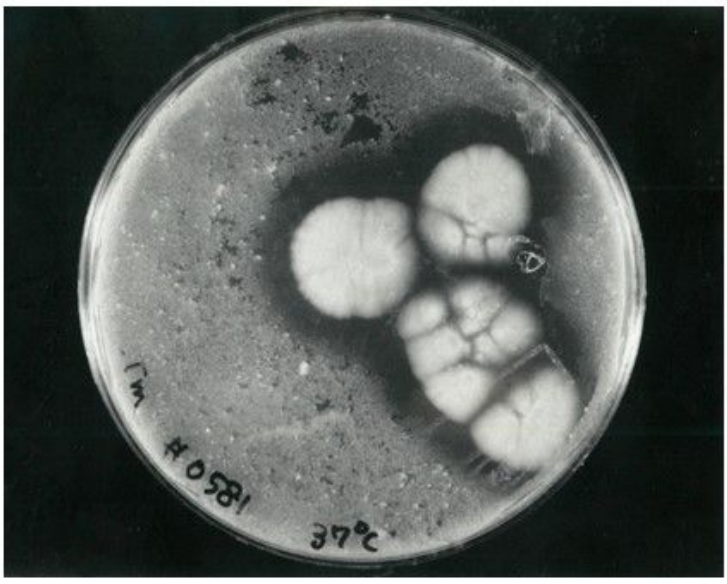


Figure 5

Photo of halo formation on a PLA agar plate by strain MT-20147. It took 2–3 weeks for strain MT-20147 to form a halo on a PLA agar plate. This image was acquired 1.5 months after cultivation started.

Figure 6

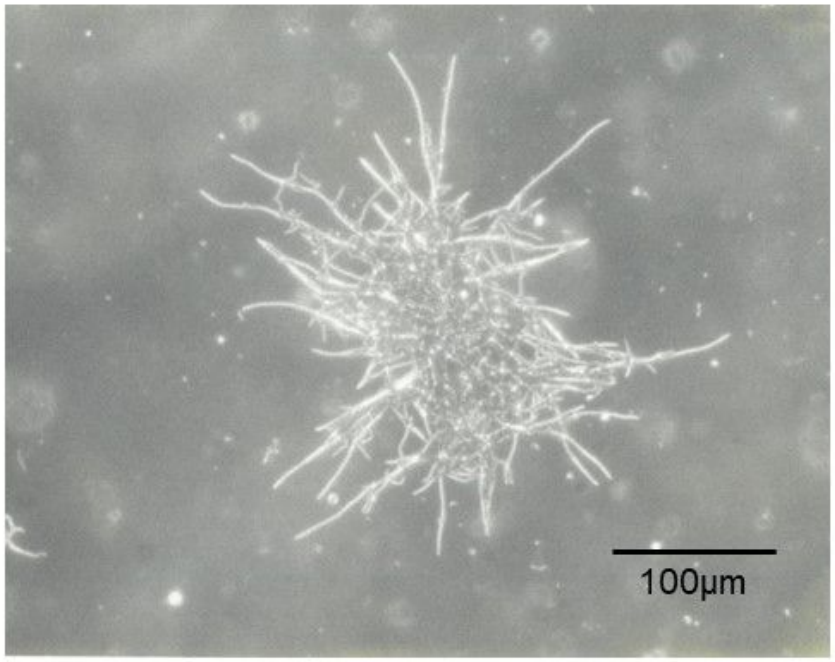
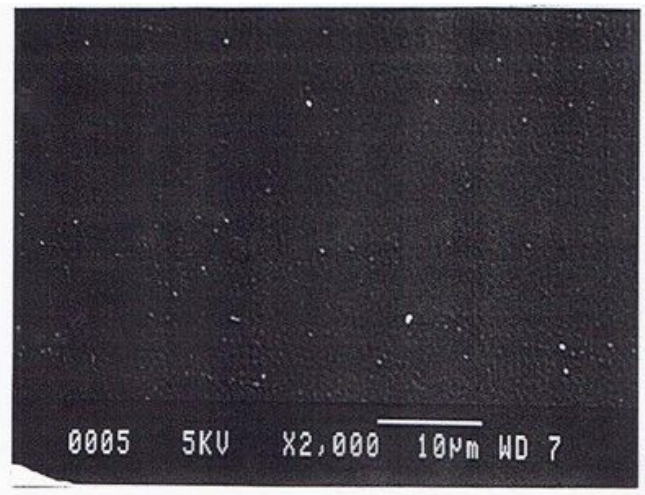


Figure 6

Phase-contrast microscopy of strain MT-20147.

Figure 7



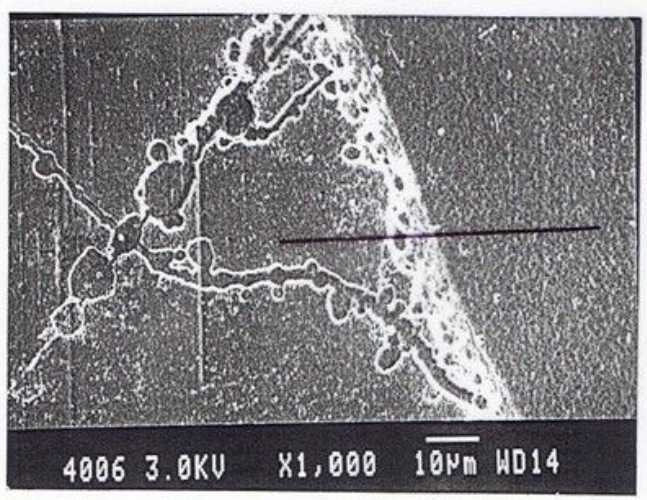


Figure 7

Photo of PLA film degradation by suspended media of strain MT-20147. Surface of PLA film before suspension in the media of strain MT-20147. Surface of PLA film after suspension in the media of strain MT-20147

Figure 8

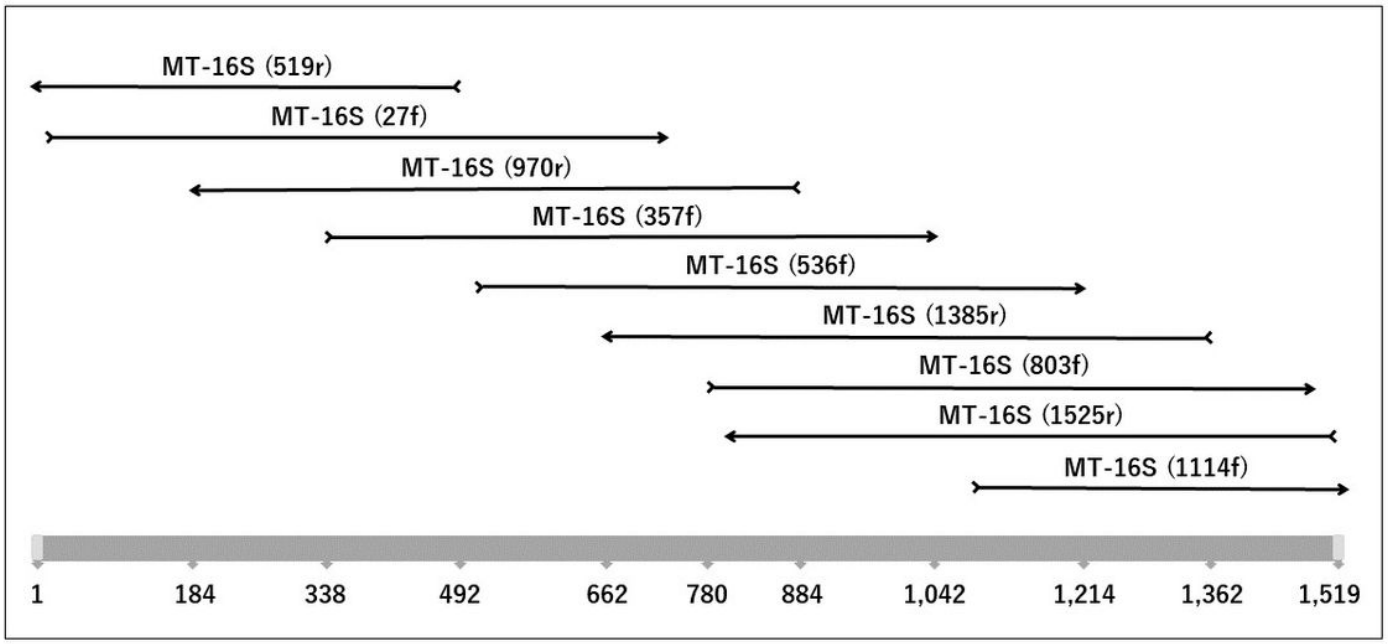


Figure 8

Primers and contigs used sequencing analysis of the 16S rRNA gene of the obtained strain.

Figure 9

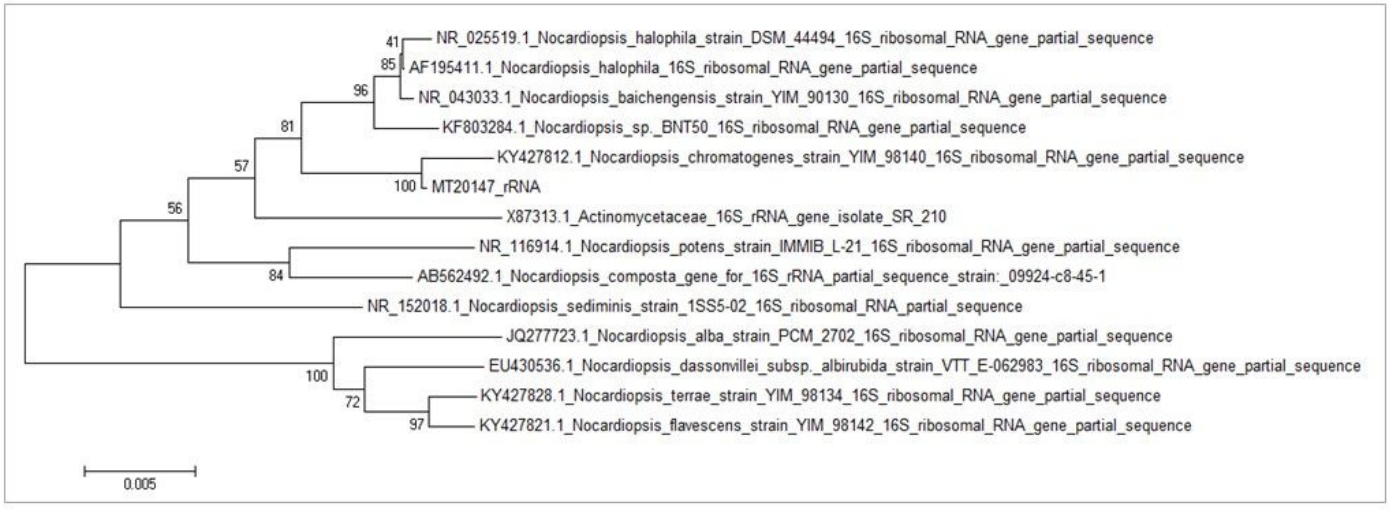


Figure 9

Phylogenetic tree analysis of strain MT-20147.