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Inactivation of *Botrytis cinerea* During Thermophilic Composting of Greenhouse Tomato Plant Residues

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Abstract

The effectiveness of in-vessel thermophilic composting on the inactivation of *Botrytis cinerea* was evaluated. The bioreactor operated on an infected mixture of tomato plant residues, wood shavings, and municipal solid compost (1:1.5:0.28). Tap water and urea were added to adjust the moisture content and C:N ratio to 60% and 30:1, respectively. Used cooking oil was added as a bioavailable carbon source to compensate for heat losses from the system and extend the thermophilic composting stage. The controlled thermophilic composting process was successful in inactivating *B. cinerea*. During all experiments, the average reactor temperature increased gradually, reaching its peak after 31 h of operation. Temperatures in the range of 62.6–63.9°C were maintained during the thermophilic stage by the intermittent addition of used cooking oil. The results of the enzyme-linked immunosorbent assay test indicated that the initial concentration of *B. cinerea* in the compost samples (14.6 μg of dried mycelium/g of compost) was reduced to 12.9, 8.8, and 2.4 μg/g after 24, 48, and 72 h of thermophilic composting, respectively. Plating assay indicated that the mold was completely inactivated in samples after 48 h of thermophilic composting. No significant reduction in *B. cinerea* was observed during the transient phase (first 30 h of rising temperature) because the temperature reached the lethal level of 55°C after 23 h, thus allowing only 7 h of exposure to temperatures higher than 55°C during this phase. The relatively short time required for complete inactivation of *B. cinerea* was achieved by maintaining a constant high temperature and a uniform distribution of temperature and extending the duration of the thermophilic stage by the addition of the proper amount of bioavailable carbon (used cooking oil).

Index Entries: Compost; temperature; inactivation; tomato remains; *Botrytis cinerea*.

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Introduction

Tomatoes are the most commonly grown vegetable crop in greenhouses, accounting for 58% of the total greenhouse vegetable production in Canada in 2000, with a production area of 1550 ha, yielding 182,736 t *(1)*. As a result of trimming and harvesting the crop, greenhouse vegetable operations typically generate 40–-60 t of organic residues/ha annually, which must be disposed of properly *(2)*. Because environmental conditions within greenhouses are optimal for the growth of most pathogens, tomato plants are susceptible to a variety of bacterial, fungal, and viral diseases. The most reported plant pathogens affecting greenhouse tomato crops are fungi from the genus *Botrytis (3)*. Because the debris from infected plants is a common source of inoculum for disease transmission within greenhouses, improper disposal of plant residues can contribute to the spread of plant pathogens *(4)*.

According to the Ontario Department of Agriculture and Food *(2)* and the Nova Scotia Department of Agriculture, Food and Fisheries *(5)*, composting of greenhouse wastes is the preferred organic waste management method, especially for the destruction of plant pathogens. Composting is the aerobic biologic decomposition of organic matter *(6,7)*, whose end product (compost) can be used to restore and preserve the environment *(8)*. It converts the unstable carbonaceous and nitrogenous materials into more stable organic forms. The end result is a product that is safer to use than the raw organic material and one that improves soil fertility, tilth, and water-holding capacity *(9)*. In addition, composting reduces the volume of organic material to be spread; improves its handling properties; and reduces odor, flies, and pathogens *(10)*.

The objective of the present study was to investigate the inactivation of the mold *Botrytis cinerea* during controlled thermophilic composting of infected tomato plant residues.

Materials and Methods

Apparatus

The composting system shown in Fig. 1 consisted of a frame, three bioreactors (each with a mixing unit and an air supply unit), and a data acquisition system. The frame was made of two parts. The main part consisted of three aluminum sheets (3.2 mm thick). The central one measured 330 × 1100 mm and the two side sheets measured 140 × 1100 mm each. They were soldered together, making a vertical channel (U-shaped) with a length of 1100 mm, a width of 330 mm, and a depth of 140 mm. This U-shaped stand held the mixing motors, flow meters, air and exhaust gas manifolds, tubing, and thermocouple wires. The second part of the frame was a horizontal supporter made of three 50- × 50-mm aluminum angles (3.2-mm thick), two of which measured 700 mm and were fixed to the main part by means of four 6-mm diameter stainless steel bolts and nuts, whereas the

Fig. 1. In-vessel composting system.

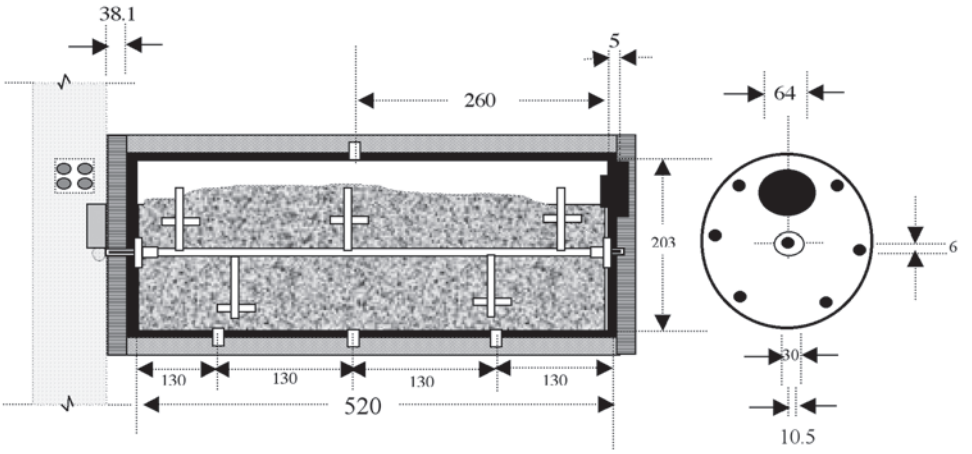


Fig. 2. Detailed schematic of bioreactor.

third one measured 328 mm and kept the other two angles 330 mm apart. The three aluminum angles were permanently soldered together.

Each bioreactor (Fig. 2) was constructed of 520-mm long and 203-mm (ID) polyvinyl chloride (PVC) tube having a wall thickness of 5 mm. A removable circular Plexiglas plate of 203 mm diameter and 6 mm thickness was recessed and secured into one side of the cylinder by means of six stainless steel screws (6 mm). A rubber gasket lining (2.5-mm thick o-ring) was added to the inner side of the circular plate to keep it tight. There was a small circular window (64 mm in diameter) on the removable circular plate, which was closed with a rubber stopper (no. 13) and used as a sampling port. A fixed circular PVC plate of 203-mm diameter and 6-mm thickness was glued into the other side of the tube and secured by means of six stainless steel screws (6 mm) and fitted into an aluminum ring, which was fastened into the frame with four bolts (6 mm) and nuts. A removable 10.5-mm diameter solid stainless steel shaft (having five stainless steel collars in which five bolts of 69 mm length and 6 mm diameter each were mounted) was mounted on two bearings inside each bioreactor. The shaft was rotated by a thermally protected electric motor (Model No. 127P1486/B, D.C.; Sigma Instruments, Braintree, MA). There were three holes at the bottom and one at the top of the bioreactor, which were drilled and threaded to take a 12-mm nylon hose barb. The holes at the bottom were connected to a manifold by 6.4-mm diameter Tygon tubing and used for aeration, whereas the one at the top was used for the exhaust gas. Air was supplied continuously to the bottom of the bioreactor from the laboratory air supply. It passed through a pressure regulator and a pressure gage (to maintain the pressure at about 5 kPa), then through a water bath (to humidify the inlet air to nearly 100% saturation), and finally through a flow meter (Model 32461-14, Cole-Parmer, Vernon Hills, IL) capable of measuring a flow in the range of 0.0566–0.566 m^3/h. Both circu-

Table 1
Characteristics of Tomato Plant Residues, Wood Shavings, Municipal Solid Compost and Used Cooking Oil

Characteristic	Tomato residues	Wood shavings	Municipal compost	Used cooking oil
Moisture content (%)	76.0	8.0	58.6	—
Total solids (mg/g DM)				
Volatile solids	693.0	997.4	854.6	999.45
Ash	307.0	2.6	145.4	0.55
Nitrogen (mg/g DM)				
Total Kjeldahl nitrogen	27.0	1.0	18.0	0.22
Ammonium nitrogen	2.2	0.2	5.2	0.004
Carbon (mg/g DM)				
Total	327.0	499.0	440.0	—
Organic	260.0	390.0	350.0	—
Elemental composition (mg/g DM)				
Ca	51.0	0.8	20.0	0.057
Na	0.7	0.0	6.2	0.301
Fe	0.4	0.0	2.8	0.14
Mg	4.7	0.1	1.8	0.008
Zn	0.0	0.0	0.1	—
K	57.6	0.6	7.8	0.01
Cl	0.07	0.0	0.3	0.742
P	10.5	0.0	2.7	0.01
S	7.9	0.9	2.3	2.324
Others	174.1	0.2	101.4	0.161

DM, dry matter.

lar plates were insulated with a 38.1-mm thick Styrofoam layer, and the tube was insulated with 38.1-mm thick fiberglass.

The data acquisition system consisted of a master unit (Multiscan 1200; Omega, Stamford, CT), a thermocouple scanning card with 24 isolated differential input channels (MTC/24; Omega), software, type T (copper-constantan) temperature sensors (Cole Parmer), a personal computer (IMB Pentium IV), and a printer (Hewlett Packard Laser Jet 4). Three thermocouples were located at the bottom of the bioreactor and were used to measure the temperature of the compost mass, and a fourth was located at the top of the bioreactor, near the outlet air exit (21 mm away), and was used to measure the temperature of the exhaust gas.

Preparation of Compost Mixture

Tomato plant residues, wood shavings, municipal solid compost, urea, and used cooking oil were used. Table 1 provides characteristics of these materials. The tomato plant residues (leaves, stems, and some fruits) were collected from a greenhouse at an average moisture content of 90% and left overnight at room temperature (≈25°C) to dry (moisture content [MC] 76%).

They were then chopped into small pieces using a shredder (Model 242A645-515, SHP; Briggs and Stratton, Plainfield, NJ), mixed with wood shavings (1:1.5 dry basis), and ground in a hammer mill (Model C-H; Horvick, NCC, Moorhead, MN) to an average size of 6.0 mm. A 15-d-old municipal solid waste compost was added to the mixture of tomato plant residues and wood shavings in order to introduce a wide range of active microorganisms. Urea ($CO[NH_2]_2$) was added to adjust the C:N ratio to 30:1, water was used to adjust the moisture content to 60%, and used cooking oil was added as a bioavailable carbon source. Eighteen milliliters of used cooking oil was added to the bioreactor every 12 h to compensate for heat losses and extend the thermophilic composting stage. The amount of used cooking oil added to the bioreactor was determined from the total heat losses from the system, which was 51.2 kJ/h and the energy content of the used cooking oil, which was 36 kJ/mL *(11)*.

Preparation of Inoculum

B. cinerea (ATCC #12481) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The double vial containing the culture of *B. cinerea* was carefully broken. Five milliliters of sterile water was added to the inner vial and the culture was left to rehydrate for 2 h. The contents of the vial were then transferred into a test tube containing 9 mL of sterile potato dextrose broth (PDB) (Difco, Detroit, MI) and incubated for 3 d at room temperature (~20°C). The rehydrated mold was streaked onto 100 plates containing potato dextrose agar (PDA) (Difco) using an inoculating loop. The plates were incubated in the dark at room temperature (~20°C) for 6 d, at which time the *B. cinerea* isolate had developed abundant hyphal swelling. The culture was then transferred into sterile 250-mL Erlenmeyer flasks containing 120 mL of PDB (two plates per flask). The flasks were sealed and placed on a shaker (at 150 rpm) in the dark at room temperature for 6 d. The contents of the flasks were blended and combined together (~6 L) to ensure a uniform inoculum. The inoculum was then divided into 200-mL portions, placed in small sterile containers, and stored at 4°C until required.

The final inoculum was prepared by mixing 250 mL of the liquid inoculum with 500 g of the final compost mixture. Tap water and urea were added to the mixture to adjust the moisture content and C:N ratio to 60% and 30:1, respectively. All materials were thoroughly mixed and placed in an incubator in the dark at 18°C for 3 d to allow the growth of *B. cinerea* before use. The mixture was removed from the incubator, thoroughly mixed, divided into 50-g portions, and placed into sachets (7 × 12 cm) made of porous fabric (muslin mesh). The sachets were placed in ziplock bags and refrigerated t 4°C until needed.

Composting Protocol

Approximately 64 mL of used cooking oil was initially added to each 1.8-kg mixture of tomato plant residues:wood shavings:municipal solid

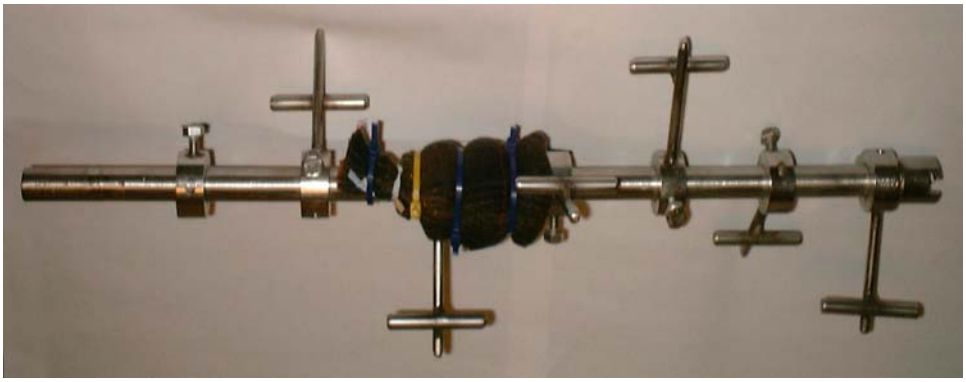


Fig. 3. Position of inoculum sachet on mixing shaft.

waste compost (1:1.5:0.28). Tap water and urea were added to adjust the moisture content and C:N ratio to 60% and 30:1, respectively. A single inoculum sachet of the plant pathogen *B. cinerea* was securely fixed to the mixing rod as shown in Fig. 3. The final compost mixture (3.5 kg) was then placed in the bioreactor. It occupied 75% of the total volume of the bioreactor (or 0.012 m^3). The Plexiglas side wall was put in place and the insulation cover was placed on the side wall. The system was operated at a mixing speed and an aeration rate of 5 rpm and 0.15 m^3/h (0.17 vvm), respectively. The temperature was continuously monitored. Once the temperature peaked (after 31 h), 18 mL of used cooking oil was added to the bioreactor every 12 h in order to maintain the temperature above 55°C *(11)*.

Identification of B. cinerea

The sachets were retrieved from the bioreactors at predetermined times (24, 48, 72, 96, 120, and 144 h after the peak temperature was reached in reactors 1–6, respectively), and an enzyme-linked immunosorbent assay (ELISA) (Adegen Limited; Auchincruive, AYR, Scotland, UK) was used to identify/quantify the mold. A standard curve was generated (using precoated strips supplied by the manufacturer) according to the procedure supplied by the manufacturer in order to quantify the amount of *B. cinerea* antigen present in the samples (Fig. 4). Because an ELISA test cannot distinguish between viable and nonviable cells *(12,13)*, a plating assay was utilized to provide information about the time at which *B. cinerea* was eradicated from the infected mixtures.

Five-gram samples of the infected untreated and treated (24, 48, 72, 96, 120, and 144 h of thermophilic composting) mixtures were thoroughly shaken in 5 mL of deionized distilled water according to the procedure described by Paul and Clark *(14)* and Harrigan and McCance *(15)*. A 1-mL volume of the prepared slurry was transferred into a test tube containing 9 mL of sterilized distilled water ($10^{!1}$) and serially diluted. A 0.1-mL volume of the supernatant of each dilution ($10^{!3}$, $10^{!4}$, and $10^{!5}$) was trans-

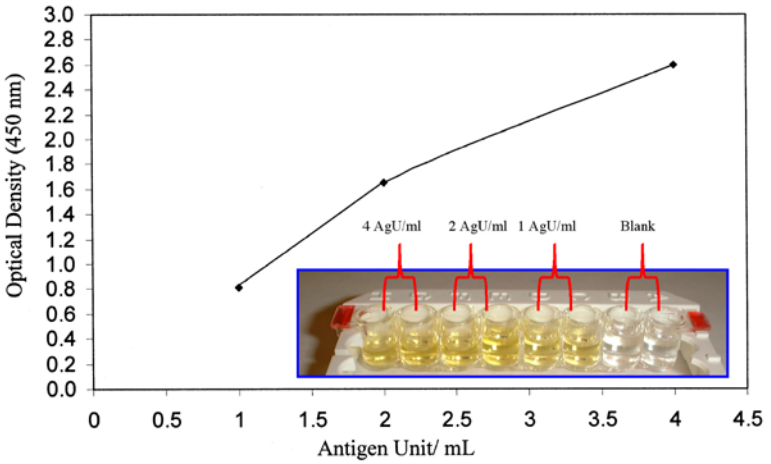


Fig. 4. Standard curve of *B. cinerea*. AgU, antigen units.

ferred onto freshly prepared PDA plates and spread with a sterile bent glass rod. The inoculated plates were inverted and incubated at 20°C for 48 h. This incubation temperature was chosen because it is within the range of optimum temperature for the growth of *B. cinerea* (15–22°C) and because microorganisms typically present in compost experience a long lag period when incubated at temperatures <20°C *(6)*. All plates were visually and microscopically (at 40 magnification) inspected for the target mold (*B. cinerea*).

Results and Discussion

Temperature

Figure 5A presents the temperature profiles. During all experiments, the average reactor temperature increased gradually, reaching its peak after 31 h of operation. Average thermophilic temperatures of 63.9 ± 1.8°C, 63.5 ± 1.1°C, and 62.6 ± 1.5°C were maintained in the first, second, and third bioreactors for 24, 48, and 72 h, respectively.

ELISA Assay

Samples were immediately tested for the presence of *B. cinerea* using an ELISA test kit. The variations in absorbance values (OD_{540nm}) between samples on the same plate were 0.014 ± 0.12%. Using the developed standard curve, the absorbance readings were converted into antigen units , which are equivalent to micrograms of dried mycelium per milliliter of buffer (phosphate-buffered saline). The final values are presented as micrograms of dried mycelium per gram of compost mixture. Figure 5B presents the concentration of *B. cinerea* in the compost samples obtained

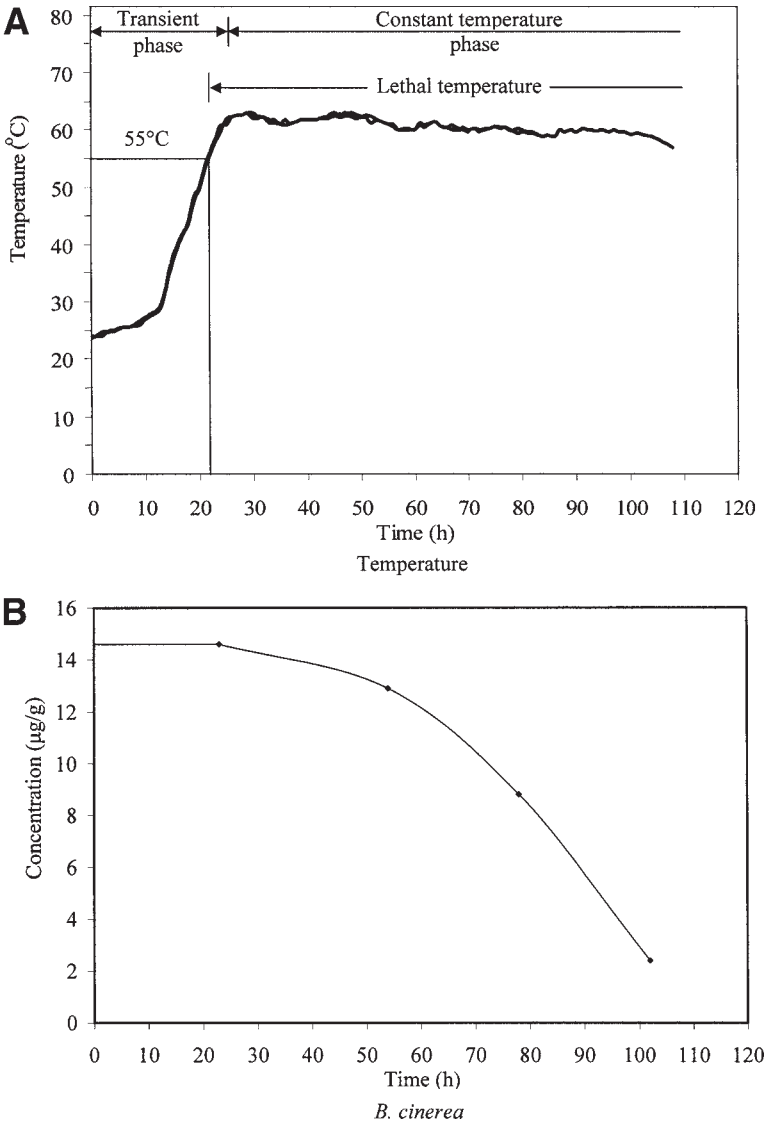
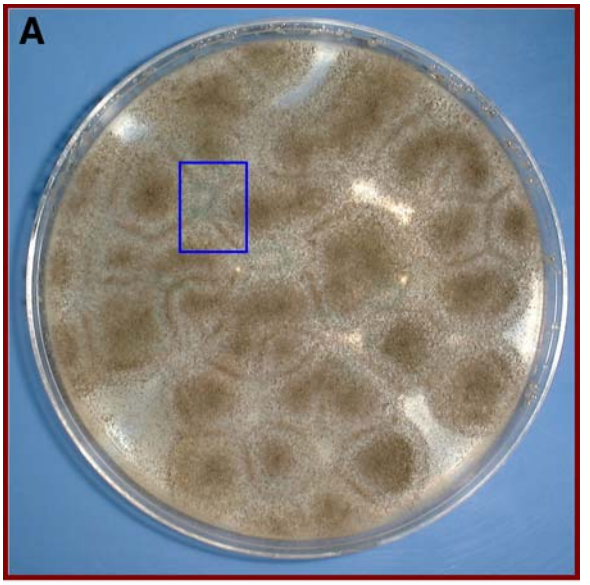
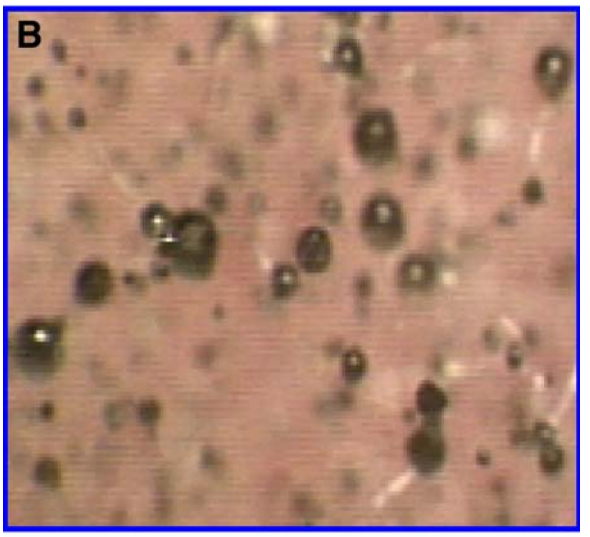


Fig. 5. **(A)** Temperature and **(B)** *B. cinerea* profiles.

after 24, 48, and 72 h of thermophilic composting. The initial quantity of 14.6 µg/g was reduced to 12.9, 8.8, and 2.4 µg/g after 24, 48, and 72 h of thermophilic composting, respectively. This amounted to reductions of 11.6, 39.9, and 83.4% after 24, 48, and 72 h, respectively. The results also showed that no significant reduction in *B. cinerea* occurred during the transient phase (first 30 h of rising temperature) because the temperature reached the lethal level of 55°C *(16)* after 23 h, thus allowing only 7 h of exposure to temperatures higher than 55°C during this phase.



Colonies after 48 h.



Grape-like clusters of spores (40X).

Fig. 6. Pure culture of *B. cinerea* before composting: **(A)** colonies after 48 h; **(B)** grape-like clusters of spores (H40).

Plating Assay

Figure 6 shows a pure culture of *B. cinerea* (10^{-4} dilution). The developed gray colonies were filamentous and circular in shape with raised elevation, as described by Agrios *(17)*. At ×40 magnification, the gray mycelia were visible and the conidiophores were long and branched with rounded apical cells bearing grapelike clusters, as described by Willetts

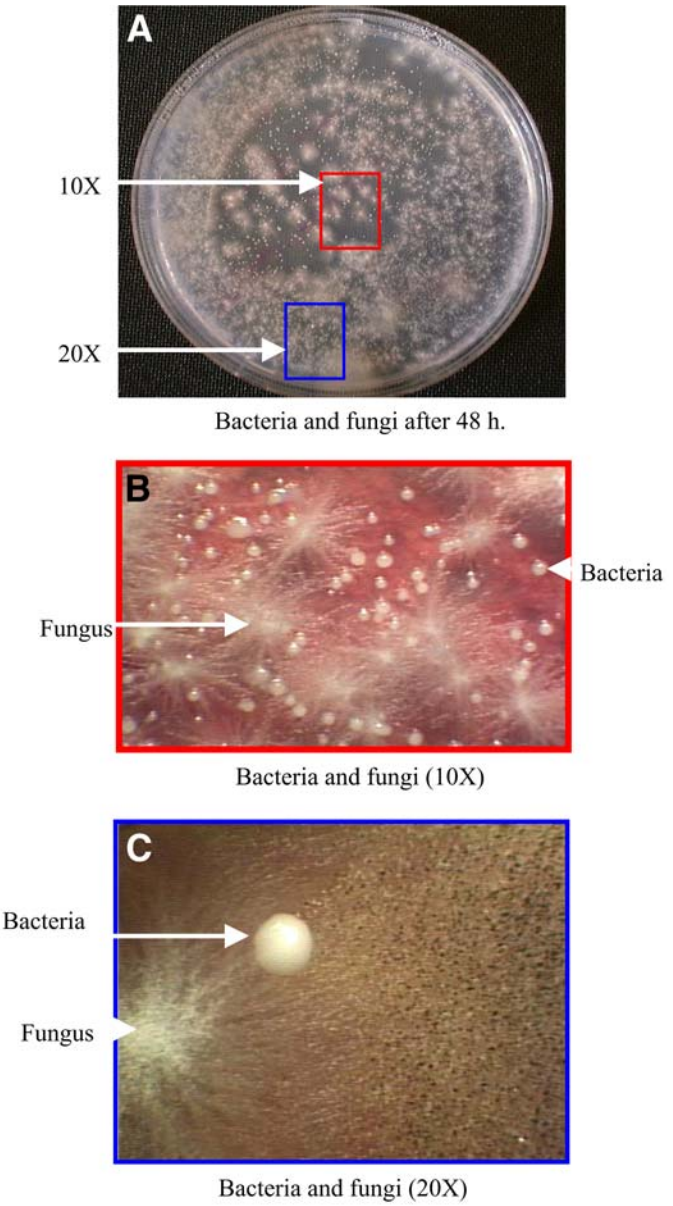


Fig. 7. Microbial populations found in initial compost mixture: **(A)** bacteria and fungi after 48 h; **(B)** bacteria and fungi (H10); **(C)** bacteria and fungi (H20).

(18). Plating of the initial infected compost mixture (10^{-4} dilution) after 48 h at 20°C showed an intensive growth of psychrophilic fungi and bacteria in addition to *B. cinerea* (Fig. 7). *B. cinerea* was present in samples that had undergone thermophilic composting for 24 h (Fig. 8). However, the mold was completely inactivated in samples that had been composted for 48 and 72 h at thermophilic temperatures. These samples were dominated primarily by bacterial colonies (Figs. 9 and 10).

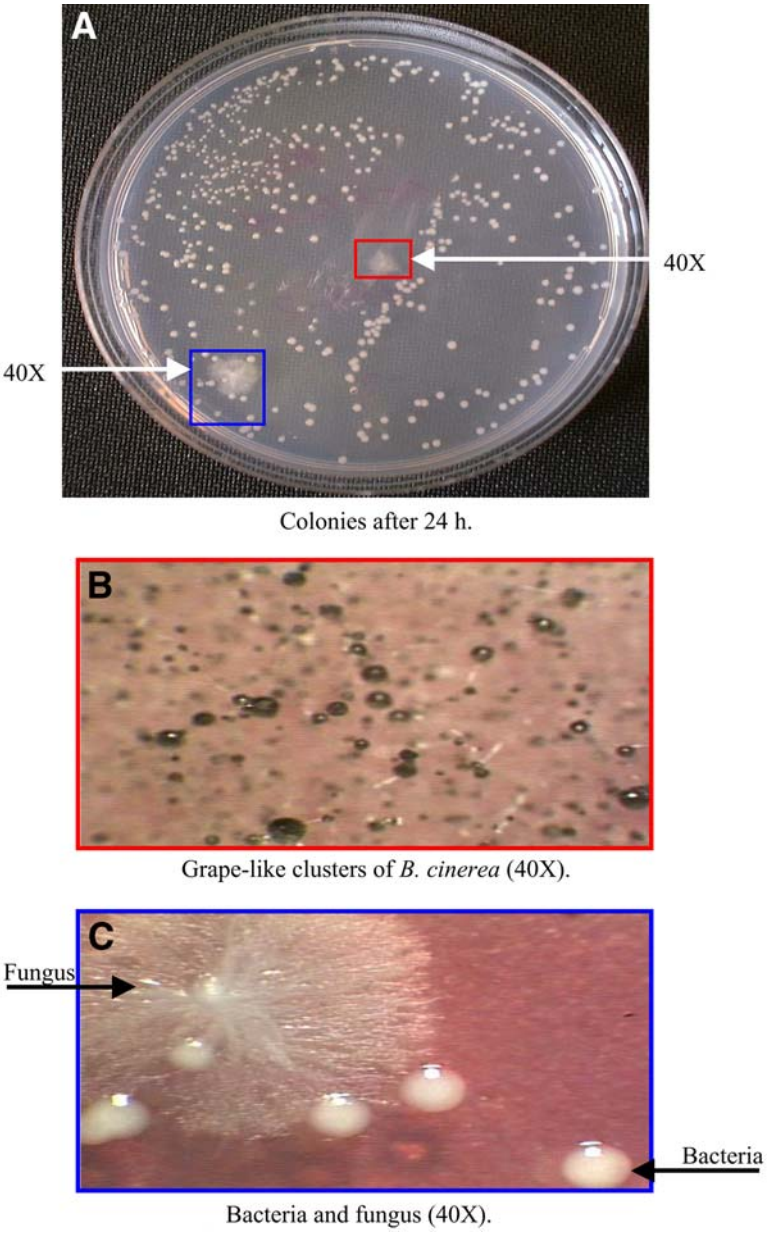


Fig. 8. Plating assay after 24 h of thermophilic composting: **(A)** colonies after 24 h; **(B)** grapelike clusters of *B. cinerea* (H40); **(C)** bacteria and fungus (H40).

According to Bollen et al. *(19)*, temperature is considered the most important factor for the elimination of plant pathogens, and its effect is mainly attributed to the denaturation of cell proteins. Lopez-Real and Foster *(20)* obtained complete inactivation of *Plasmodiophora brassica* spores in plant tissue after 24 h of exposure in compost at 54°C. Suárez-Estrella et al.

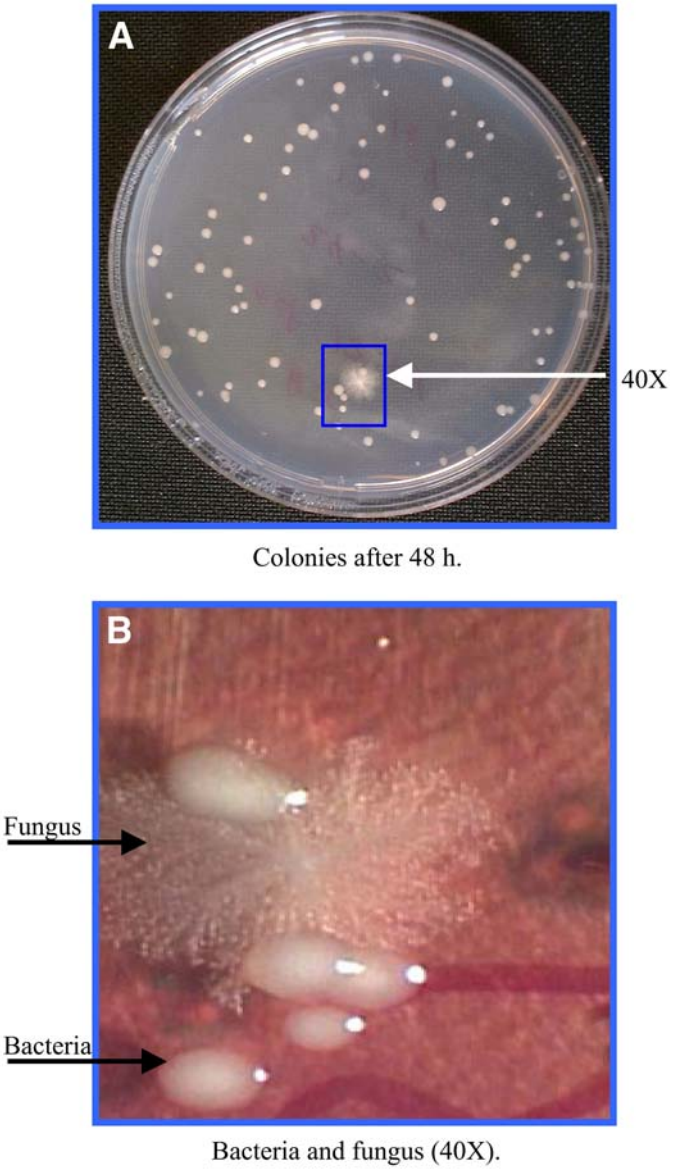


Fig. 9. Plating assay after 48 h of thermophilic composting: **(A)** colonies after 48 h; **(B)** bacteria and fungus (H40).

(21) studied the effect of the composting process on the fungus *Fusarium oxysporum* f. sp *melonis* and found that complete inactivation was achieved in high temperature regimes (55 and 65°C) after 2 to 3 d. Hoitink et al. *(22)* reported complete destruction of *B. cinerea* in an incubator after 7 and 21 d at temperatures of 50 and 40°C, respectively. Lopez-Real and Foster *(20)* investigated the survival of *B. cinerea* in vitro and found that it was completely inactivated at an average temperature of 54°C within 72–96 h.

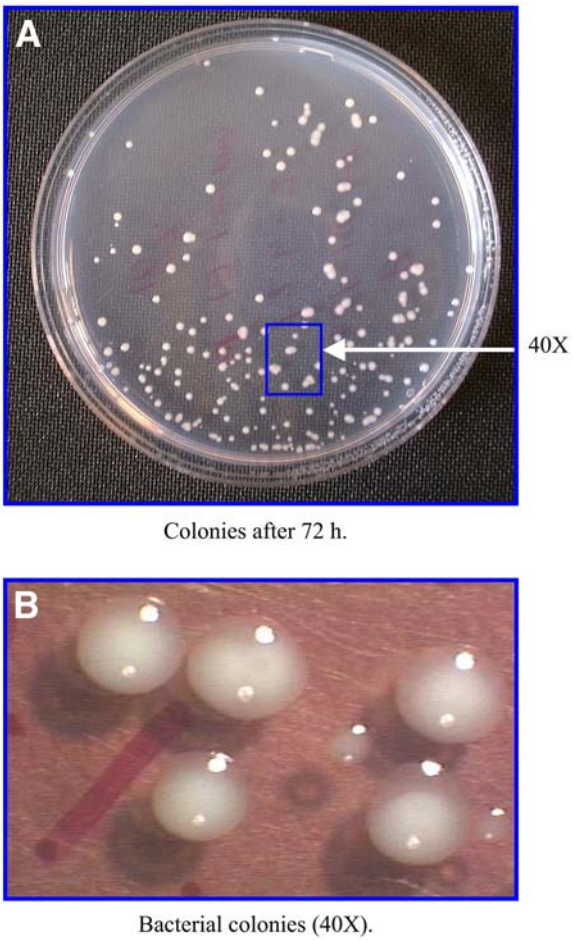


Fig. 10. Plating assay after 72 h of thermophilic composting: **(A)** colonies after 72 h; **(B)** bacterial colonies (H40).

According to Hogland et al. *(23)* and Ryckeboer et al. *(24)*, the high temperature (about 60°C) that can be reached during the thermophilic stage of composting is the most important factor for inactivation of plant pathogens. Although higher temperatures will decrease pathogens dramatically, temperature fluctuations, clumping of solids, and improper mixing of raw material and amendment are the most negative characteristics of solid mixtures during the composting process *(6)*. Bruns et al. *(25)* reported that complete inactivation of plant pathogens depends on the kind of pathogen, the type of raw composted material, and the composting method (system) utilized.

In the present study, composting microorganisms were limited to bacteria after the second day of composting at thermophilic temperatures (>60°C). Strom *(26)* stated that high bioreactor temperatures are very selec-

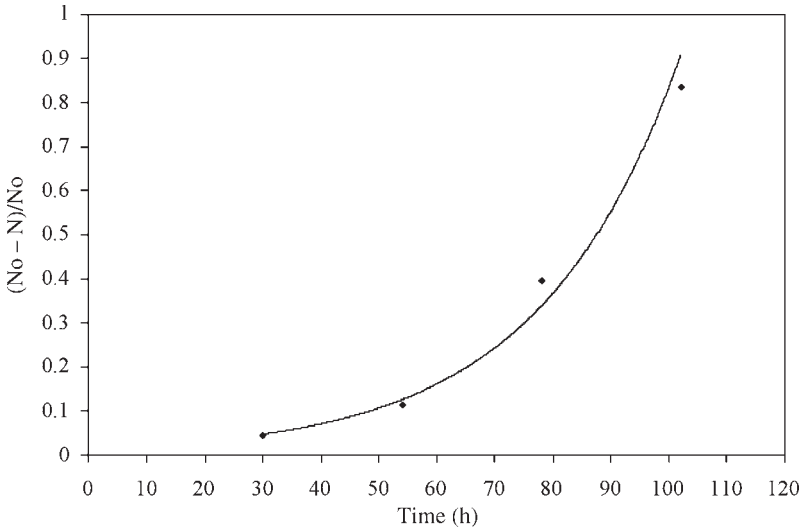


Fig. 11. Inactivation of *B. cinerea* as function of time.

tive for thermophilic bacteria (*Bacillus*), and when temperatures are above 65°C, composting microorganisms are reduced to a pure culture of *Bacillus strearothermophilus*. Bacteria are considered the most important group of composting microorganisms and typically account for 80–90% of the microbial activity *(6,27,28)*. Haug *(6)* stated that bacteria dominate the composting process because they have a high surface area–to–volume ratio (owing to their small sizes of 0.3–0.5 μm diameter), which accelerates the exchange of soluble substrates with their environment.

The constant thermophilic temperature (~63°C) achieved in the present study, the uniform temperature distribution in the bioreactor (owing to the use of continuous mixing), and the extension of thermophilic stage (by the continuous addition of used cooking oil as a bioavailable carbon source) contributed to the effectiveness of the composting process in destroying *B. cinerea*. Because the ELISA test could not distinguish between the viable and nonviable cells of *B. cinerea*, a plating assay was used to ensure that the remaining cells were inactivated (dead). The inactivation (destruction) of *B. cinerea* (measured by the mold-specific protein using the ELISA test) as a function of time (Fig. 11) can be described by the following equation ($R^2 = 0.99$):

$$\frac{N_0 - N}{N_0} = e^{kt}$$

in which N_0 is the initial mold concentration (μg of dried mycelium/g of compost), N is the mold concentration at any time (μg of dried mycelium/g of compost), and $t$is the time (h). The deactivation coefficient (k) was determined to be 0.0416 h^{-1}.

Conclusion

The average reactor temperature increased gradually, reaching its peak after 31 h of operation. The intermittent addition of a bioavailable carbon source (used cooking oil) maintained the temperature in the range of 62.6–63.9°C during the thermophilic stage. The results of the ELISA test indicated that the initial concentration of *B. cinerea* in the compost samples (14.6 µg/g) was reduced to 2.4 µg/g after 72 h of thermophilic composting, but the results of the plating assay indicated that the mold was completely inactivated after 48 h of thermophilic composting. No significant reduction in *B. cinerea* was observed during the transient phase (first 30 h of rising temperature) because the temperature reached the lethal level of 55°C after 23 min, thus allowing only 7 h of exposure to temperatures above 55°C during this phase. The relatively short time required for complete inactivation of *B. cinerea* in this study was achieved by maintaining a constant high temperature and a uniform distribution of temperature and extending the duration of the thermophilic stage by continuously adding the proper amount of bioavailable carbon (used cooking oil).

Acknowledgments

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