J Microbiol Immunol Infect.
2007;40:462-473

Original Article

Application of thermotolerant microorganisms for biofertilizer preparation

Kuo-Shu Chen[1], Yann-Shying Lin[1], Shang-Shyng Yang[1,2]

[1]Institute of Microbiology and Biochemistry and [2]Department of Biochemical Science and Technology, National Taiwan University, Taipei, Taiwan

Received: June 30, 2006 Revised: August 15, 2006 Accepted: August 30, 2006

Background and Purpose: Intensive agriculture is practised in Taiwan, and compost application is very popular as a means of improving the soil physical properties and supplying plant nutrition. We tested the potential of inoculation with thermotolerant microorganisms to shorten the maturity and improve the quality of biofertilizer prepared by composting.

Methods: Thermotolerant microorganisms were isolated from compost and reinoculated for the preparation of biofertilizer. The physical, chemical and biological properties of the biofertilizer were determined during composting. The effects of biofertilizer application on the growth and yield of rape were also studied.

Results: Among 3823 colonies of thermotolerant microorganisms, *Streptomyces thermonitrificans* NTU-88, *Streptococcus* sp. NTU-130 and *Aspergillus fumigatus* NTU-132 exhibited high growth rates and cellulolytic and proteolytic activities. When a mixture of rice straw and swine manure were inoculated with these isolates and composted for 61 days, substrate temperature increased initially and then decreased gradually during composting. Substrate pH increased from 7.3 to 8.5. Microbial inoculation enhanced the rate of maturity, and increased the content of ash and total and immobilized nitrogen, improved the germination rate of alfalfa seed, and decreased the content of total organic carbon and the carbon/nitrogen ratio. Biofertilizer application increased the growth and yield of rape.

Conclusions: Inoculation of thermotolerant and thermophilic microorganisms to agricultural waste for biofertilizer preparation enhances the rate of maturity and improves the quality of the resulting biofertilizer. Inoculation of appropriate microorganisms in biofertilizer preparation might be usefully applied to agricultural situations.

Key words: Bacteria; Fungi; Humic substances; Soil microbiology; Time factors

Introduction

Intensive agriculture is practised in Taiwan. More than 60% of farmland soils were acidic and the organic matter content was below 2% [1]. It is estimated that each hectare requires 20 tons of organic matter per year to supplement decomposition in the field under the prevailing conditions of high temperature, humidity and microbial activity in Taiwan [2]. Compost application is very popular in Taiwan and Asian countries as it has the potential to improve soil physical properties, supply plant nutrition, recycle waste materials, and reduce environmental pollution. Many biological materials show active decomposition accompanied by rise in temperature and are considered suitable for composting, such as agricultural byproducts, crop residues, animal wastes, vegetable market wastes, waste mushroom media, food processing wastes, and municipal refuse [3].

In 2004, there were 339,283 tons of rice straw, 30,723 tons of rice hull, 59,162 tons of corncob, 763,128 tons of vegetable market wastes, 6,199,149 tons of poultry feces, 6,341,528 tons of livestock feces and 84,000 tons of waste mushroom media in Taiwan [4,5]. These materials are sometimes processed separately or as mixed materials. During composting, thermophilic, thermotolerant and mesophilic microorganisms decompose cellulose, hemicellulose and lignin of substrates [6]. The composting process is an exothermal

Corresponding author: Professor Shang-Shyng Yang, Institute of Microbiology and Biochemistry, National Taiwan University, Taipei 10617, Taiwan.
E-mail: ssyang@ntu.edu.tw

biological oxidation of organic matter carried out by a dynamic and quick succession of aerobic microorganism populations. The heterogeneous organic matter of the raw material is transformed, after a suitable composting period which includes bio-oxidative and maturation phases, into a stabilized end-product through partial mineralization and humification [3,7].

In this study, thermotolerant and thermophilic microorganisms were isolated from the compost of mixtures of vegetable market wastes and agricultural wastes. The organisms were identified and their cellulolytic and proteolytic activities determined. We then evaluated the effects of inoculation of thermotolerant and thermophilic microorganisms on the maturity and quality of compost, and the effect of biofertilizer application on rape growth.

Methods

Raw materials

Vegetable market wastes containing 84.1-92.1% moisture, 9.8-10.1% ash, 0.2-0.5% soluble nitrogen, 1.7-2.5% total nitrogen, 5301-9800 ppm soluble biochemical oxygen demand, and 30,666-53,146 ppm soluble chemical oxygen demand were collected from Wan-Da Market, Taipei. Corncob had 10.5% moisture, 48.9% total carbohydrate, 32.5% crude fiber, 2.2% crude protein, and 3.5% ash. Rice straw comprised 38.7% cellulose, 18.3% hemicellulose, 15.0% lignin, 4.1% crude protein, and 12.2% ash. Swine manure had 28.0 ± 1.2% total organic carbon, 2.3 ± 0.2% total nitrogen, and a carbon/nitrogen ratio of 12.2.

Culture media and conditions

Thermotolerant microorganisms were isolated at 50°C in modified M3 medium at pH 7.0 ± 0.1 [8]. ISP3 medium contained (g/L) oatmeal 20 and agar 18 at pH 7.0 ± 0.1. CYC agar medium comprised (g/L) sucrose 30, yeast extract 2, casamino acid (vitamin free) 6, sodium nitrate 3, dipotassium hydrogenorthophosphate 1, hydrated magnesium sulfate 0.5, potassium chloride 0.5, ferrous sulfate heptahydrate 0.01 and agar 20 at pH 7.0 ± 0.1. Cellulase (EC 3.2.1.4) activity was measured in Mandels-Reese medium with carboxymethylcellulose (CMC; Sigma Chemicals Co., St Louis, MO, USA) as carbon source at pH 7.0 ± 0.1 [9]. Amylase (EC 3.2.1.1) activity was determined in soluble starch-yeast extract medium at pH 7.0 ± 0.1 [10]; protease activity was measured in protease analysis medium at pH 7.0 ± 0.1 [11].

Isolation of thermotolerant cellulolytic microorganisms

Vegetable market waste, rice straw and corncob were composted in a compost box (1 m × 1 m × 1 m) or compost bioreactor (3 m × 2 m × 2 m). During composting, samples were collected periodically for isolation of thermotolerant cellulolytic microorganisms with modified M3 medium or Mandels-Reese medium at 50°C. After cultivation, carboxymethyl cellulase (CMCase) [EC 3.2.1.4] activity was determined by the dinitrosalicyclic method. One unit of CMCase activity was defined as the amount of enzyme causing the release of 1 μmole of glucose in 1 min under the assay conditions [12].

Identification of thermotolerant isolates

Thermotolerant isolates were cultivated on nutrient agar or potato dextrose agar. The following characteristics were determined: optimal growth temperature, morphology, color, spore stain and biochemical properties. Actinomycete isolates were identified with the International *Streptomyces* Project [13]. Bacterial isolates were identified with BBL™ Crystal™ Identification Systems (BD Diagnostic Systems, Franklin Lakes, NJ, USA), Gram-positive microbe kits and computer identification software. Fungal isolates were identified according to the proposals of Raper and Fennell [14], and Ainsworth et al [15]. Species identification was done by use of the Biological Resources Collection Center of Food Industry and Development Institute (Hsinchu, Taiwan).

Preparation of biofertilizer

Rice straw and swine manure were mixed in the ratio of 4:1 (w/w), adjusted to 70% moisture content, inoculated with tested microorganisms (about 10^5 colony-forming units [CFU]/g dry substrate), and composted in a pile (1 m × 1 m × 1 m compost box) for 61 days. The physical, chemical and biological properties were determined during composting.

Germination test

After composting, the sample was extracted with five washings of distilled water (w/v) and shaking at 180 rev/min and incubated at 30°C for 30 min. Three mL of an appropriate dilution compost extract was applied to filter paper (Whatman No. 1) and then 25 alfalfa seeds were placed on the filter paper and incubated at 25°C for 5 days. Percentage germination was counted at day 5, and distilled water was used as the control.

Chemical analysis

pH was measured directly or in a 5-fold dilution of distilled water with a pH meter (Good digital pH model 2002, Taiwan). Substrate temperature was measured daily at a depth of 15 cm with a thermometer. Moisture content was determined by drying a sample at 105°C for 24 h to a constant mass, and ash content was measured at 550°C for 24 h. Total nitrogen was determined with a modified Kjeldahl method [16]. Soluble nitrogen extracted with 2N potassium chloride was also measured with a modified Kjeldahl method. Immobilized nitrogen was calculated from the difference between total nitrogen and soluble nitrogen contents. Total organic carbon was determined as follows: a 0.05-g sample, 1N potassium dichromate 10 mL, and concentrated sulfuric acid 20 mL were mixed thoroughly and left to stand for 30 min. Distilled water (200 mL) and 85% phosphoric acid (10 mL) were added. After cooling, 1 mL diphenylamine was added as indicator and the reaction mixture was titrated with 5N ferrous (II) ammonium sulfate [17]. Experiments were carried out in triplicate and the data subjected to analysis of variance and Duncan's multiple range tests (p=0.05) using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) [18].

Results

Isolation of thermotolerant microorganisms

During composting, each gram of substrate contained 6.3×10^6-5.5×10^7 CFU (compost box) and 1.0×10^6-9.8×10^7 CFU (compost bioreactor) on nutrient agar medium. The total microbial count had a maximum value at 14 to 28 days of composting in the static compost box, and after 28 days of composting in the turning and aeration compost bioreactor (Table 1). 3823 colonies (183 colonies from the compost box system and 3640 colonies from the compost bioreactor system) were picked from modified M3 medium at 50°C for successive cultivation and enzyme activity assay. There were 912 isolates with cellulolytic activity on Mandels-Reese medium. After testing for amylolytic activity, proteolytic activity and antimicrobial potency,

Table 1. Populations of thermotolerant microorganisms during composting of vegetable market wastes, rice straw and corncob

Composting period (days)	Total count (CFU/g)	Isolate colonies tested	Isolates able to grow at 50°C	Isolates suitable for further testing[a]	Isolate codes
Compost box					
7	6.27×10^6	19	18	4	NTU-1-NTU-4
14	4.95×10^7	37	15	5	NTU-5, NTU-7-NTU-10
28	5.45×10^7	49	13	9	NTU-14-NTU-16, NTU-18, NTU-19, NTU-21-NTU-24
42	2.63×10^7	26	15	8	NTU-25, NTU-26, NTU-28-NTU-32, NTU-34
56	1.37×10^7	36	13	8	NTU-36, NTU-38-NTU-44
70	7.00×10^6	16	5	5	NTU-45-NTU-48
Compost bioreactor					
7	-	148	10	4	NTU-49-NTU-52
14	2.20×10^7	200	26	11	NTU-53-NTU-63
21	1.30×10^6	327	8	1	NTU-64
23	1.00×10^6	302	14	1	NTU-65
28	9.70×10^6	155	16	1	NTU-67
35	1.50×10^7	793	20	8	NTU-68- NTU-75
42	9.80×10^7	118	78	14	NTU-76-NTU-78, NTU-80, NTU-81, NTU-83-NTU-89. NTU-92, NTU-93
49	4.80×10^7	396	62	17	NTU-94, NTU-95, NTU-97-NTU-111
Random sampling during composting					
		1201	599	15	NTU-112-NTU-114, NTU-117-NTU-120, NTU-123, NTU-129, NTU-130, NTU-132-NTU-134, NTU-137, NTU-138
Total		3823	912	96	

Abbreviation: CFU = colony-forming units

[a]Microorganisms with cellulolytic, amylolytic, proteolytic activities and antimicrobial activities.

96 isolates were used for further tests. Morphological observation on agar plate and under the microscope showed 24 isolates from the compost box, comprising 18 actinomycetes, 5 bacteria and 1 fungus, and 72 isolates from the compost bioreactor, comprising 47 actinomycetes, 24 bacteria and 1 fungus.

Metabolic activity of isolated organisms

The hydrolytic enzyme activity and the antimicrobial potency of 96 isolates were measured at 50°C. Sixty seven isolates had cellulolytic activity, 68 isolates had amylolytic activity, 52 isolates had proteolytic activity, 46 isolates had antimicrobial potency and 23 isolates had all 4 of these activities. From the morphological observation, 65 actinomycete isolates, 29 bacterial isolates and 2 fungal isolates with hydrolytic enzyme activity were isolated. Due to the high temperature during composting, 72 isolates from the compost bioreactor and 24 isolates from the compost box were detected with hydrolytic enzyme activity.

The ratios of clear zone to colony size on Mandels-Reese medium, soluble starch-yeast extract medium, skim milk medium and antibiotic medium no. 1 were used as the parameters of microbial activity. Twenty three isolates with all 4 kinds of activity included 18 actinomycetes, 3 bacteria and 2 fungi. In addition, 7 isolates gave a clear zone on Mandels-Reese medium larger than 30 mm, including isolates NTU-1, NTU-47, NTU-62, NTU-88, NTU-118, NTU-130 and NTU-132. Isolates displaying all 4 kinds of activities (NTU-51, NTU-52, NTU-53, NTU-56, NTU-59, NTU-65, NTU-67, NTU-88, NTU-130 and NTU-132) were selected for further investigation; these comprised 8 actinomycete isolates, 1 bacterial isolate and 1 fungal isolate. The actinomycete isolate NTU-88 grew rapidly on Mandels-Reese medium. Thus, actinomycete isolate NTU-88, bacterial isolate NTU-130 and fungal isolate NTU-132 were chosen for further investigation.

Identification of actinomycete isolate NTU-88

The biochemical properties and morphology of mycelia and conidia are very important in identification of actinomycetes. Actinomycete isolate NTU-88 had an optimal growth temperature of 50°C. The colony diameter was 5, 16, 30, 41 and 20 mm at 30, 40, 50, 55 and 60°C, respectively, after 9 days of incubation on nutrient agar.

The thermotolerant actinomycete isolate NTU-88 grew on nutrient agar, CYC agar and ISP 3 agar at 55°C, and after 5 days produced irregular margin circular colonies with rough surface, non-luminance, small upper surface, wrinkled with some circular radiation light yellow hyphae, yellowish dark brown pigment deposits, aerial hyphae and conidiospores with gray black and white margin. It had 0.8-1.4 μm substrate mycelium, and 0.5-1.0 μm aerial mycelium spiral spores on CYC agar; in submerged cultivation, it had fragmented mycelium. The morphological and physiological characteristics of NTU-88 and other related actinomycetes are summarized in Table 2 [19]. According to the thermophilic actinomycete classification, the isolate NTU-88 belongs to *Streptomyces thermonitrificans*.

Identification of thermotolerant bacterial isolate NTU-130

The thermotolerant bacterial isolate NTU-130 had an optimal growth temperature of 50°C. Spores were of

Table 2. Comparative properties of the thermotolerant actinomycete isolate NTU-88 and other thermophilic actinomycetes

Characteristic	*Streptomyces thermovulgaris*	*Streptomyces thermoviolaceus*	*Streptomyces megasporus*	Actinomycete isolate NTU-88
Spore morphology	Spirales (16/19), *Retinaculiaperti* (3/19)	Spirales (7/7)	Spirales (1/9), *Retinaculiaperti* (8/9)	Spirales[a]
Color of aerial mycelia and spores	Gray (18/19), white (1/19)	Gray (5/7), red (2/7)	Yellow (5/9), white (4/9)	Gray[a]
Substrate mycelia pigment	No distinctive pigment (19/19)	Red/orange (6/7), no distinctive pigment (1/7)	No distinctive pigment (9/9)	No distinctive pigment[a]
Diffusible pigments	None found	Red/orange (2/7), blue (1/7)	Yellow/brown (1/9)	Yellow/brown[a]
Protease activity	Proteolysis on egg yolk (19/19)	Proteolysis on egg yolk (7/7)	Proteolysis on egg yolk (9/9)	Proteolysis on skim milk
Reference	Goodfellow et al [19]	Goodfellow et al [19]	Goodfellow et al [19]	Present study

[a]Growth on CYC agar (CYC agar medium comprised [g/L] sucrose 30, yeast extract 2, casamino acid [vitamin free] 6, sodium nitrate 3, dipotassium hydrogenorthophosphate 1, hydrated magnesium sulfate 0.5, potassium chloride 0.5, ferrous sulfate heptahydrate 0.01 and agar 20 at pH 7.0 ± 0.1).

green color with 5% malachite green and 0.5% safranin staining. The colonial diameter was 10, 47, 70, 56 and 13 mm at 30, 40, 50, 55 and 60°C, respectively, after 9 days of incubation on nutrient agar. The morphological and physiological characteristics of NTU-130 and other thermotolerant bacteria are summarized in Table 3. Using BBL Crystal commercial kits, computer identification system and *Streptococcus pyogenes* American Type Culture Collection (ATCC) 19615 as a reference strain, thermotolerant bacterial isolate NTU-130 was shown to belong to a *Streptococcus* sp.

Identification of thermophilic fungal isolate NTU-132

Thermophilic fungal isolate NTU-132 had an optimal growth temperature of 40°C. The colonial diameter was 60, 70, 34 and 13 mm at 30, 40, 50 and 55°C, respectively, after 9 days incubation on potato dextrose agar. It could not grow at 10°C or 60°C. The morphological and physiological characteristics of NTU-132 and *Aspergillus fumigatus* ATCC 1012 are summarized in Table 4 [20]. According to the definition of thermophilic fungi, the maximal growth temperature was 50°C or more, and the minimal growth temperature was 20°C or less. The growth temperature of the isolate was between 15 and 55°C, and the growth temperature of *A. fumigatus* ranged from 12 to 55°C, with an optimal growth temperature of 40°C. Therefore, the fungal isolate NTU-132 was a thermophile. From the culture and morphological characteristics, growth temperature, and comparison with the characteristics of *A. fumigatus*

Table 3. Comparative characteristics of the thermophilic bacterial isolate NTU-130 and other thermophilic bacteria

Characteristic	*Streptococcus pyogenes* ATCC 19615	*Bacillus brevis* ATCC 8246	Bacterial isolate NTU-130
Optimum growth temperature (°C)	37	37	50
Spore stain with Wirtz-Conklin method	–	+	–
Fluorescent negative control[a]	–	–	–
4MU-beta-D-glucoside[a]	–	+	+
L-valine-AMC[a]	+	+	–
L-phenylalanine-AMC[a]	+	+	+
4-MU-alpha-D-glucoside[a]	+	+	+
L-pyroglutamic acid-AMC[a]	+	+	+
L-tryptophan-AMC[a]	+	+	+
L-arginine-AMC[a]	+	+	V
4-MU-N-acetyl-beta-D-glucosaminide[a]	–	+	+
4-MU-phosphate[a]	+	V	V
4-MU-beta-D-glucuronide[a]	–	–	–
L-isoleucine-AMC[a]	+	V	+
Trehalose[a]	+	–	–
Lactose[a]	+	–	–
Methyl-alpha- and beta-glucoside[a]	+	–	–
Sucrose[a]	+	–	–
Mannitol[a]	–	–	–
Maltotriose[a]	+	–	–
Arabinose[a]	–	–	–
Glycerol[a]	+	–	–
Fructose[a]	+	–	–
pNP-beta-D-glucoside[a]	V	V	–
pNP-beta-D-cellobioside[a]	–	–	–
Proline and leucine-p-nitroanilide[a]	+	V	–
pNP-phosphate[a]	V	V	–
pNP-alpha-D-maltoside[a]	–	V	–
ONPG and pNP-galactoside[a]	–	–	–
Urea[a]	–	V	–
Esculin[a]	–	V	–
Arginine[a]	V	+	–

Abbreviations: ATCC = American Type Culture Collection; 4-MU = 4-methylumbelliferone; AMC = amino-4-methylcoumarin; ONPG = ortho-nitrophenyl-beta-D-galactopyranoside; pNP = p-nitrophenyl; – = negative; + = positive; V = variable

[a]BBL™ Crystal™ Identification System, Gram-positive ID kit.

Table 4. Comparative characteristics of thermal fungal isolate NTU-132 and *Aspergillus fumigatus*

Characteristic	*Aspergillus fumigatus* Fresenius[a] ATCC 1022	Fungal isolate NTU-132
Growth temperature	12-55°C	15-57°C
Morphology on Czapek agar	Velvety, floccose; conidial heads columnar, slate-olive to storm gray; Reverse uncolored, yellowish, reddish or light greenish-brown to dark brown	Velvety, floccose; conidial heads columnar, slate-olive to deep green Reverse dull yellowish
	Borne in very well defined columns	Borne in very well-defined columns
	Vesical distinctly spathulate to pyriform, pale green to olive brown, 6.4-24.0 μm	Vesical distinctly spathulate to pyriform, 15.0-22.5 μm;
	Aspergilla uniseriate; stipe 4.0-12.0 × 88-440 μm uncolored to olive brown, smooth, straight to sinuous	Aspergilla uniseriate; stipe 5.0-8.0 × 150-350 μm smooth
	Phialides covering the upper one-half to two-thirds of the vesicle, 2.0-4.0 × 4.4-11.2 μm; conidia globose to ovoid, very small, smooth to spinulose, 2.4-3.8 μm in diameter	Phialides covering the upper one-half to two-thirds of the vesicle, 2.3-3.0 × 5.0-10.0 μm; conidia globose to ovoid, to spinulose, 2.0-3.5 μm in diameter
Colony morphology on malt extract medium	Plain or velvety; mycelium white; conidial heads slate-olive to castor gray; reverse dull yellowish-green	Plain or velvety; mycelium white; conidial heads slate-olive to deep green

Abbreviation: ATCC = American Type Culture Collection
[a]Tzean et al [20].

Fresenius ATCC 1012, NTU-132 was identified as *A. fumigatus* by the Biological Resources Collection Center of Food Industry and Development Institute (Hsinchu, Taiwan).

Composting of agricultural wastes by thermotolerant and thermophilic microorganisms

The mixture of swine manure and rice straw in the ratio 4:1 (w/w) was composted with the inoculation of thermotolerant or thermophilic microorganisms, and the properties during composting were determined.

Temperature profile

Compost temperature increased to 50°C at day 3, decreased to 39-40°C at day 5, and then increased again after turning. However, the temperature decreased gradually after 15 days of composting (Fig. 1A). Inoculation of thermotolerant or thermophilic microorganisms was associated with higher temperatures than control (without inoculation) due to the heavy growth of inoculants and the more rapid decomposition of substrate.

Moisture content

The moisture content increased from 65.6-67.7% to 68.0-72.0% at 61 days of incubation (Fig. 1B). The moisture content of compost increased during incubation because of the use of the closed bioreactor with cover. Inoculation of thermotolerant or thermophilic microorganisms was associated with higher moisture content than the control (without inoculation) due to the production of metabolic water.

pH

The pH of the substrate increased gradually during composting due to the degradation of nitrogen-containing materials in the raw materials to soluble organic nitrogen, the formation of ammonium ion (NH_4^+) and the release of hydroxide by hydrolysis (Fig. 1C). Therefore, the pH increased during composting because of the immobilization of NH_4^+. In the later stages, some NH_4^+ was converted to NH_3 in the alkaline conditions, and the pH decreased slightly.

Ash content

Ash content increased from 17.5 ± 0.6% to 28.3 ± 0.4% with inoculation of thermotolerant and thermophilic microorganisms, and was 19.6% in the control. The mixed cultures had the highest value (21.77%) for the stimulation of organic matter decomposition (Fig. 1D). Ash content was conserved, and the stability of ash content can be used as a parameter of compost maturity.

Total organic carbon and nitrogen content

Organic compounds were degraded to carbon dioxide during composting, and total organic carbon decreased. After 61 days of incubation, total organic carbon decreased from 38.2 ± 1.38% to 31.6 ± 0.83%, 30.8 ± 1.13%, 32.4 ± 1.35%, 31.1 ± 1.04% and 31.8 ± 2.13% with inoculation of mixed cultures, *S. thermonitrificans*

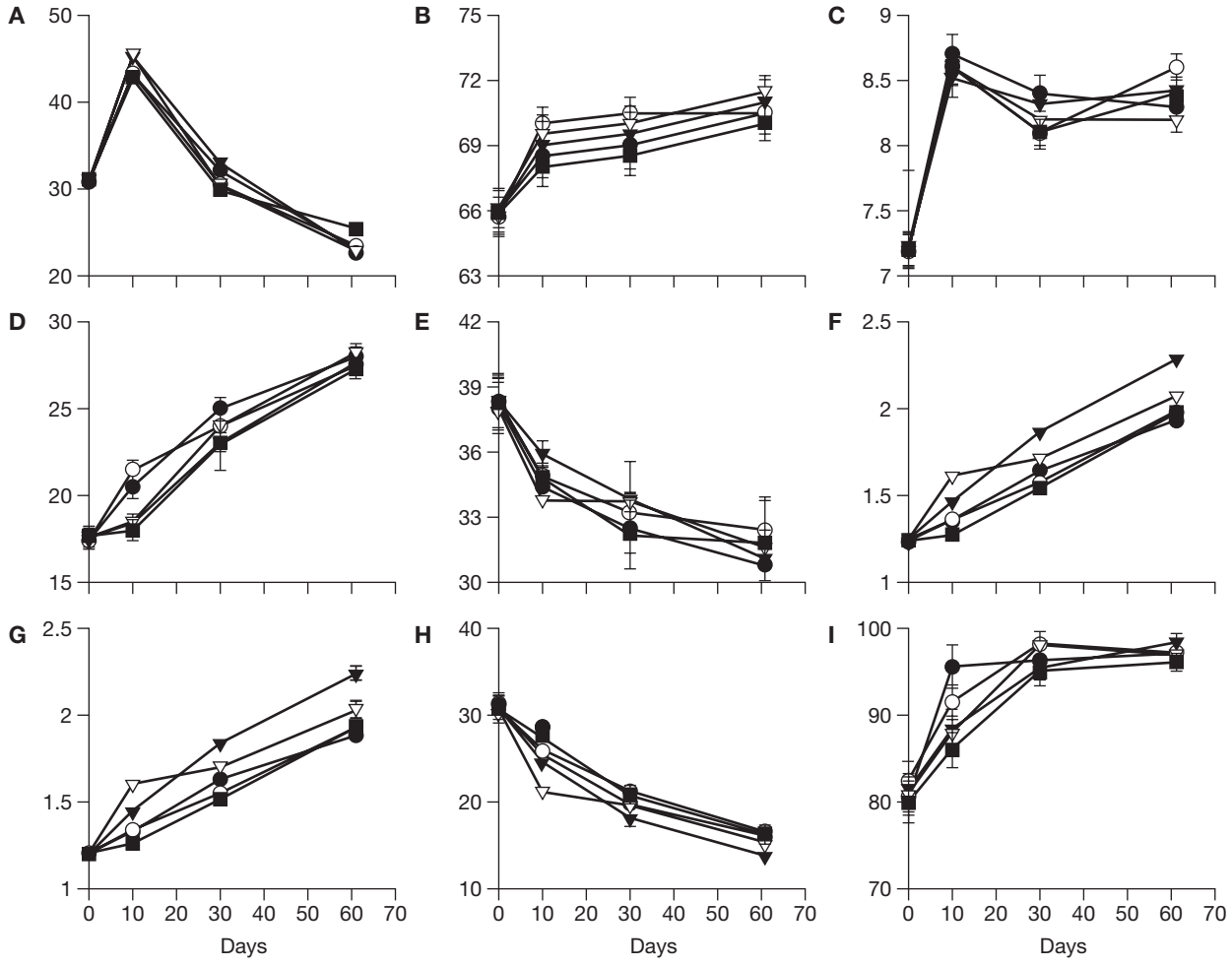

Fig. 1. Properties of biofertilizer preparation with the inoculation of thermotolerant microorganisms during composting. (A) Temperature (°C); (B) moisture content (%); (C) pH; (D) ash content (%); (E) total organic carbon (%); (F) total nitrogen (%); (G) immobilized nitrogen (%); (H) carbon/nitrogen ratio; (I) germination rate (%). ● = *Streptomyces thermonitrificans* NTU-88; ○ = *Streptococcus* sp. NTU-130; ▼ = *Aspergillus fumigatus* NTU-130; ▽ = mixed cultures; ■ = control (without inoculation).

NTU-88, *Streptococcus* sp. NTU-130, *A. fumigatus* NTU-132, and control, respectively (Fig. 1E). Total nitrogen content increased from 1.24 ± 0.04% to 2.07 ± 0.01%, 1.93 ± 0.01%, 1.97 ± 0.03%, 2.28 ± 0.01% and 1.97 ± 0.04%, respectively (Fig. 1F). Immobilized nitrogen increased from 1.21 ± 0.04% to 2.03 ± 0.05%, 1.89 ± 0.02%, 1.93 ± 0.05%, 2.24 ± 0.04% and 1.93 ± 0.05%, respectively (Fig. 1G). Carbon/nitrogen ratio decreased from 31.2 ± 1.35 to 15.3 ± 0.40, 16.0 ± 0.59, 16.5 ± 0.69, 13.7 ± 0.46, and 16.2 ± 1.08, respectively (Fig. 1H). Mixed cultures and *A. fumigatus* NTU-132 inoculation resulted in higher total nitrogen and immobilized nitrogen than the control.

Germination rate

The germination rate of alfalfa seed was 95% after 10 days of composting with the inoculation of thermotolerant *S. thermonitrificans* NTU-88; 91% with the inoculation of thermotolerant *Streptococcus* sp. NTU-130; and 90% with the inoculation of thermophilic *A. fumigatus* NTU-132, mixed cultures and the control (Fig. 1I). It was shown that the inoculation of thermotolerant or thermophilic microorganisms could enhance the degradation of organic raw material, and remove the phytotoxic compounds that inhibit the germination of alfalfa seeds, such as ammonia, ethylene oxide and organic acids.

Odor of compost

The odor of compost with thermotolerant or thermophilic microorganisms inoculation was lower than that of the control in the early stages. After 10 days of incubation, the foul odor became 'earthy' or odorless with *S. thermonitrificans* NTU-88 inoculation, and the foul

Table 5. Effect of inoculants on the odor and color of compost

Inoculation microorganisms	Period of composting (days)					
	5	10	15	20	25	30
Odor						
Streptomyces thermonitrificans NTU-88	F	E	E	E	E	E
Streptococcus sp. NTU-130	F	S	S	S	E	E
Aspergillus fumigatus NTU-132	F	F	S	E	E	E
Mixed cultures	F	S	E	E	E	E
Control (without inoculation)	F	S	S	S	E	E
Color						
Streptomyces thermonitrificans NTU-88	B	B	B	DB	DB	DB
Streptococcus sp. NTU-130	B	B	DB	DBB	DBB	DBB
Aspergillus fumigatus NTU-132	B	B	DB	DBB	DBB	DBB
Mixed cultures	B	DB	DBB	DBB	DBB	DBB
Control (without inoculation)	B	B	DB	DB	DBB	DBB

Abbreviations: F = foul; B = brown color; E = earthy or odorless; S = smelly; DB = deep brown color; DBB = dark brown color

odor was reduced to smelly with *Streptococcus* sp. NTU-130 inoculation (Table 5).

The foul odor was reduced to earthy or odorless at day 15 with mixed cultures inoculation, at day 20 with *A. fumigatus* NTU-132 inoculation, and at day 25 with *Streptococcus* sp. NTU-130 inoculation and the control.

Color change

Inoculation of thermotolerant or thermophilic microorganisms enhanced the color change from brown to deep-brown or dark-brown color. The brown color of compost converted to deep-brown color after 10 days of incubation with the mixed cultures; after 15 days of incubation with *Streptococcus* sp. NTU-130, *A. fumigatus* NTU-132 and the control; and after 20 days of incubation with *S. thermonitrificans* NTU-88. The deep-brown color became dark-brown after 15 days of incubation with the mixed cultures; after 20 days of incubation with *Streptococcus* sp. NTU-130 and *A. fumigatus* NTU-132; and after 25 days in the control (Table 5). The color of compost with *S. thermonitrificans* NTU-88 remained deep-brown after 30 days of incubation.

Microbial population during composting

The effect of inoculation of thermotolerant microorganisms on the microbial population during composting is demonstrated in Fig. 2. The changes in total microbial count and thermotolerant cellulolytic microorganisms were both similar with the inoculation of *S. thermonitrificans* NTU-88 and the mixed cultures. Each gram of dry material contained 1.21×10^8 CFU thermotolerant cellulolytic microorganisms and 1.16×10^8 CFU thermotolerant actinomycetes with the inoculation of thermotolerant *S. thermonitrificans* NTU-88 after 10 days of incubation. In the mixed cultures inoculation, each gram of dry material had 3.50×10^8 CFU thermotolerant cellulolytic microorganisms and 1.41×10^8 CFU thermotolerant actinomycetes. Each gram of dry material had 1.13×10^7, 3.15×10^6, and 7.19×10^6 CFU thermotolerant cellulolytic microorganisms with the inoculation of *Streptococcus* sp. NTU-130, *A. fumigatus* NTU-132 and in the control, respectively. In the case of thermotolerant actinomycetes, the populations were 4.38×10^6, 2.35×10^6 and 5.55×10^6 CFU, respectively.

Maturity of compost

The inoculation of the mixed cultures and *A. fumigatus* NTU-132 at the initial stages had significant stimulatory effects on the maturity of compost. However, the inoculation of *S. thermonitrificans* NTU-88 was the most effective in terms of odor reduction. Inoculation of mixed cultures and *Streptococcus* sp. NTU-130 resulted in the shortest period to color change.

Effect of biofertilizer on the growth of rape

The plant height, number of leaves and dry weight of rape increased with the application amount of biofertilizer preparation from the inoculation of mixed cultures (Table 6). The potency of biofertilizer proved to be 50% that of the chemical fertilizer based on application rates.

Discussion

Due to the higher temperatures achieved during composting, the percentage of thermotolerant actinomycete isolates was higher with the compost bioreactor method

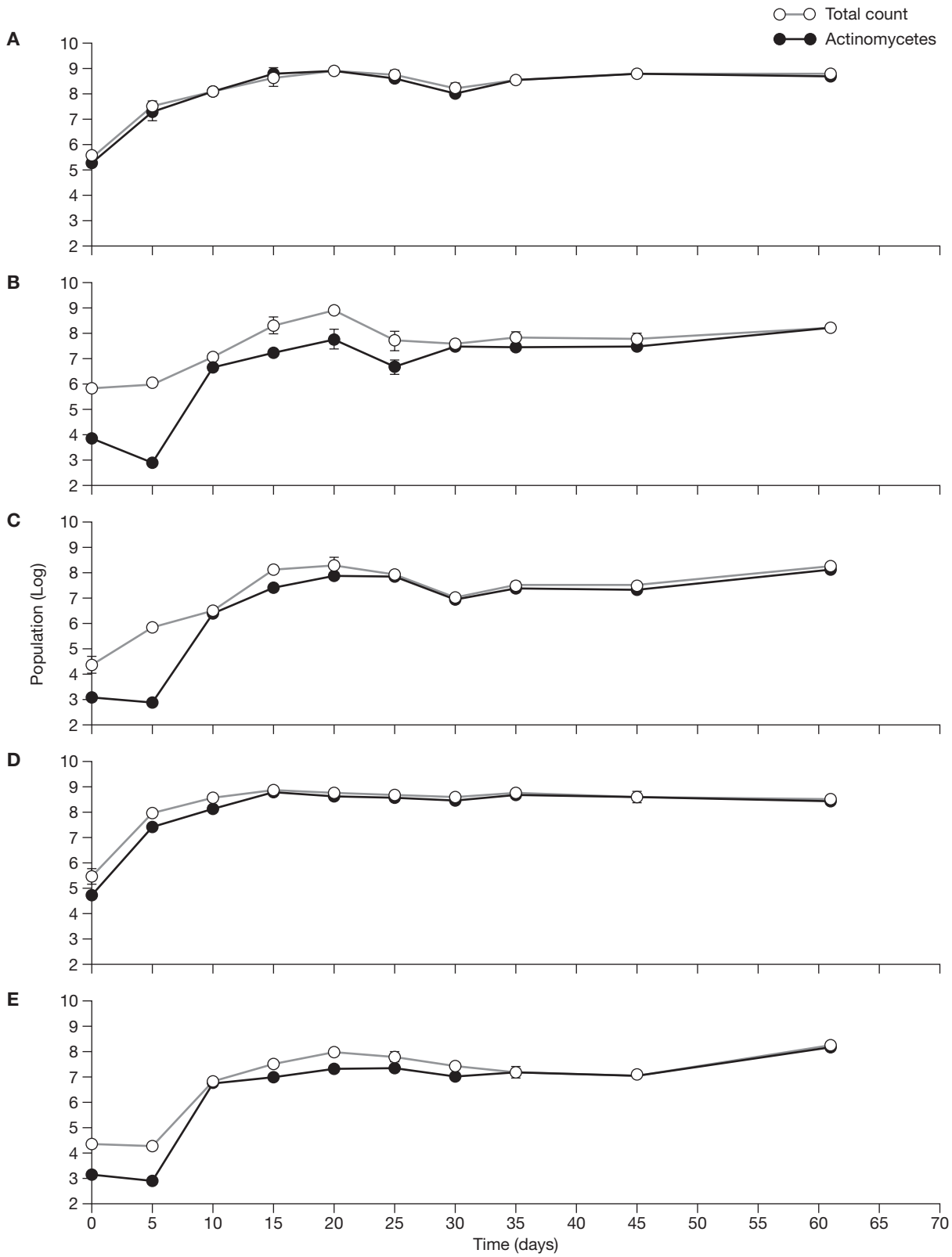

Fig. 2. Populations of thermotolerant microorganisms in biofertilizer preparation with the inoculation of thermotolerant microorganisms during composting. (A) *Streptomyces thermonitrificans* NTU-88; (B) *Streptococcus* sp. NTU-130; (C) *Aspergillus fumigatus* NTU-130; (D) mixed cultures; (E) control (without inoculation). Values are the mean of triplicate experiments and error bars represented the standard deviation. ○ = total count; ● = actinomycetes.

Table 6. Effect of biofertilizer on plant height, number of leaves and dry weight of rape [mean ± standard deviation (n = 3)].

Treatment	Plant height (cm)	No. of leaves	Yield	
			(g/pot)	Index (%)
Control	16.4 ± 0.7^{e}	7.2 ± 0.7^{c}	20.25 ± 0.33^{e}	100.0
Chemical fertilizer	23.7 ± 0.9^{b}	$8.9 \pm 0.3^{a,b}$	63.91 ± 0.97^{b}	315.6
Biofertilizer (× 1)	17.9 ± 0.4^{d}	6.5 ± 0.2^{d}	33.32 ± 0.51^{d}	164.5
Biofertilizer (× 2)	20.8 ± 0.4^{c}	8.6 ± 0.2^{b}	63.36 ± 0.34^{c}	312.9
Biofertilizer (× 4)	26.5 ± 1.0^{a}	9.3 ± 0.2^{a}	70.54 ± 0.67^{a}	348.3

[a-e]Values followed by the same superscript in the same column are not significantly different ($p<0.05$) as determined by Duncan's multiple range test.

(82%) than with the compost box method (63%). Actinomycetes were the dominant organisms, followed by bacteria, with fungi much less common. Huck et al [8] indicated that modified M3 medium favored actinomycetes growth. Strom [21] used trypticase soy agar for isolation of thermophilic microorganisms from sawdust and waste paper and showed that 87% of isolates were *Bacillus*. Therefore, types of thermotolerant or thermophilic microbial isolates depended on the raw materials, isolation media and conditions. In this study, modified M3 medium at 50°C was used; therefore, thermotolerant actinomycetes were the dominant organisms.

Identification of actinomycetes followed the International *Streptomyces* Project method in standard culture medium, and colony growth, color, shape, luminance, aerial mycelia, spore formation and pigment production were observed [13]. For species determination, chemical analyses of amino acids and sugars in the cell wall [22], and of phospholipid, mycolate, and menaquinone are required [23]. In this study, the actinomycete isolate was identified by physiological properties, morphological observations and then following the guidelines of Nonomura [13] and Bergey's Manual [24]. According to Henssen's definition [25], actinomycete isolate NTU-88 belongs to the thermotolerant microorganisms. From the thermophilic actinomycete classification of Goodfellow et al [19], the isolate NTU-88 belongs to *Streptomyces thermonitrificans.*

During composting, the temperature increased to around 50°C, and then decreased gradually; it increased again after turning. The temperature patterns were similar to those reported by other researchers in compost preparation [7,26]. Proper composting effectively destroys pathogens and weed seeds through the metabolic heat generated by microorganisms during the process [3]. Such biofertilizer is not only suitable for use as a soil conditioner and fertilizer, but can also suppress soil-borne and foliar plant pathogens [3,27]. The moisture content increased with the closed bioreactor process. However, the moisture content usually decreases gradually in open field composting due to evaporation [7,26]. Substrate moisture content also increased in protein enrichment, and in enzyme, antibiotic and polyunsaturated fatty acid production in solid state fermentation, via the production of metabolic water [11,28-31]. The change of pH during composting was also similar to that observed in composting of commercial compost plant operation [7]. Ash content increased gradually, as noted by Harada et al [26] in the composting of cow wastes. Ash content is conserved during composting and can be used as a parameter of compost maturity.

Inoculation of thermotolerant or thermophilic microorganisms decreased the odor. Ohta and Ikeda [32] also showed that *Actinomycetes* reduced the odor of compost. Tanaka et al [33] isolated thermophilic *Streptomyces* sp. No. 101, *Thermoactinomyces* sp. No. 64 and *Micromonospora* sp. No. 604, organisms that could degrade the yeast cell debris and indigestible rice protein bodies and remove the odor of volatile fatty acids. Similarly, we found that both *S. thermonitrificans* NTU-88 and the mixed cultures of thermotolerant and thermophilic microorganisms reduced the odor of compost.

Inoculation of thermotolerant and thermophilic microorganisms enhanced the color change of composting material to dark-brown. Shekhar Sharma and Johri [34] reported that microorganisms could transform plant residues and animal components to humate substances, especially *A. fumigatus* and *Humicolor insolens*. The color change of compost could be used as a parameter of maturity.

The thermophilic microbe count was higher than that of the mesophilic microorganisms in biofertilizer preparation with the inoculation of the thermophilic lipolytic organism *Brevibacillus borstelensis* SH 168

[35]. The same phenomenon was found in our study. Populations of both thermotolerant and thermophilic microorganisms can be established by inoculation during composting.

In conclusion, inoculation of appropriate microorganisms during compost preparation might be a potential and practical process. The procedure could stimulate organic matter decomposition, improve the quality of compost and shorten the period for maturity.

Acknowledgments

The authors thank the National Science Council and Council of Agriculture for financial support [NSC 90-2317-B005-008, NSC 91-2317-B005-006, NSC 92-2317-B005-021, 90-AGRI-2.1.2-Food-Z1(4), 91-AGRI-3.1.2-Food-Z1(3) and 92-AGRI-4.2.2-Food-Z1(4)].

References

1. Tan CW, Liu CC, Chen KN. Soil fertility and rational fertilization. In: Tai CY, Lin CY, Tsai WH, Liao CL, eds. Rational fertilization: Special issue. Taichung: Taiwan Agriculture Research Institute and Chinese Sustainable Agriculture Association; 2005:43-62.
2. Lee CC. Organic waste treatment and sustainable agriculture. In: Huang SF, Lu HS, Chu C, eds. Treatment and application of agricultural organic wastes. Taipei: The Biomass Energy Society of China; 1999:1-7.
3. Yang SS. Development and application potential of biofertilizers. Agric Biotechnol Indust Quart. 2005;4:9-17.
4. Yang SS, Lin CF, Wang CK. Waste treatment and re-utilization. Taipei: National Air University; 2003:59-116.
5. Council of Agriculture. Annual report of Taiwan agriculture — 2004. Taipei: Council of Agriculture, Executive Yuan; 2006.
6. Yang SS, Chen KS. Application of thermophilic microbes for preparing biofertilizers. Plant Protect Bull Spec Public New. 2003;5:267-91.
7. Yang SS. Preparation and characterization of compost. J Biomass Energy Soc China. 1997;16:47-62.
8. Huck TA, Porter N, Bushell ME. Positive selection of antibiotic-producing soil isolates. J Gen Microbiol. 1991;137:2321-9.
9. Mandels M, Mrdeiro JE, Andreotti RE, Bisset FH. Evaluation of cellulose culture filtrates under use conditions. Biotechnol Bioeng. 1981;23:2009-26.
10. Cheng CW, Lin JS, Liu YT, Yang SS. Cloning and expression of the α-amylase gene and oxytetracycline production in *Streptomyces rimosus*. World J Microbiol Biotechnol. 2000; 16:225-30.
11. Yang SS, Huang CI. Protease production by amylolytic fungi in solid state fermentation. J Chin Agric Chem Soc. 1994;32: 589-601.
12. Acebal C, Castillon MP, Estrada P, Mata I, Costa E, Aguado J, et al. Enhanced cellulase production from *Trichoderma reesei* QM 9414 on physically treated wheat straw. Appl Microbiol Biotechnol. 1986;24:218-23.
13. Nonomura H. Key for classification and identification of 458 species of the *Streptomycetes* included in ISP. J Ferment Technol. 1974;52:78-92.
14. Raper KB, Fennell DI. The genus *Aspergillus*. Baltimore: Williams & Wilkins; 1965:686.
15. Ainsworth GC, Sparrow FK, Sussman AS. The fungi. An advanced treatise. Vol. IVA. A taxonomic review with keys: *Ascomycetes* and Fungi imperfecti. New York: Academic Press; 1973:133.
16. Yang SS, Chang SL, Wei CB, Lin HC. Reduction of waste production in the Kjeldahl methods. J Biomass Energy Soc China. 1991;10:147-55.
17. Nelson DW, Sommers LE. Total carbon, organic carbon and organic matter. In: Page AL, Miller RH, Keeney DR, eds. Methods of soil analysis, Part 2. Chemical and microbiological properties. Wisconsin: The American Society of Agronomy; 1982:539-80.
18. SAS Institute. SAS/STAT User's Guide. Cary, NC: SAS Institute Inc; 1999.
19. Goodfellow M, Lacey J, Todd C. Numerical classification of thermophilic *Streptomycetes*. J Gen Microbiol. 1987;133: 3135-49.
20. Tzean SS, Chen JL, Liou GY, Chen CC, Hsu WH. *Aspergillus* and related teleomorphs from Taiwan. In: Annual report of Food Industrial Research and Development Institute. Hsinchu: Food Industrial Research and Development Institute; 1970;39-40.
21. Strom PF. Identification of thermophilic bacteria in solid-waste composting. Appl Environ Microbiol. 1985;50:906-13.
22. Uchida K, Aida K. Acyl type of bacterial cell wall; its simple identification by colorimetric method. J Gen Appl Microbiol. 1977;23:249-60.
23. Lechevalier MP, Lechevalier HA. The chemotaxonomy of *Actinomycetes*. In: Dietz A, Thayer DW, eds. Actinomycete taxonomy. Virginia: Society for Industrial Microbiology; 1980: 227-91.
24. Garritty GM, Boone DM, Castenholz RW, eds. Bergey's Manual of Systematic Bacteriology. 2nd ed. Vol. 1. New York: Springer-Verlag; 2001.
25. Henssen A. Morphology and system of thermophilic *Actinomycetes*. Arch Mikrobiol. 1957;26:373-414. [Article in German]
26. Harada Y, Haga K, Osada T, Koshino M. Quality aspects of animal waste compost. In: Proceedings of symposium on pig waste treatment and composting II. Tainan: Taiwan Livestock

Research Institute; 1991:54-76.
27. Zang W, Han DY, Dick WA, Davis KR, Hoitink HA. Compost and compost water extract-induced systemic acquired resistance in cucumber and *Arabidopsis*. Phytopathol. 1998; 88:450-5.
28. Yang SS. Protein enrichment of sweet potato residue with amylolytic yeasts by solid state fermentation. Biotechnol Bioeng. 1988;32:886-90.
29. Yang SS, Yuan SS. Oxytetracycline production by *Streptomyces rimosus* in solid state fermentation of sweet potato residue. World J Microbiol Biotechnol. 1990;6:236-44.
30. Yang SS, Swei WJ. Oxytetracycline production by *Streptomyces rimosus* in solid-state fermentation of corncob. World J Microbiol Biotechnol. 1996;12:43-6.
31. Jang HD, Lin YY, Yang SS. Polyunsaturated fatty acid production with *Mortierella alpina* by solid substrate fermentation. Bot Bull Acad Sin. 2000;41:41-8.
32. Ohta Y, Ikeda M. Deodorization of pig feces by *Actinomycetes*. Appl Environ Microbiol. 1978;36:487-91.
33. Tanaka Y, Murata A, Hayashida S. Accelerated composting of cereal Shochu-distillery wastes by *Actinomycetes*: promotive composting of Shochu-distillery waster (I). Seibutsu-kogaku Kaishi. 1995;73:365-72.
34. Shekhar Sharma HS, Johri BN. The role of thermophilic fungi in agriculture. In: Arora DK, Elander RP, Mukerji KG, eds. Handbook of applied mycology. Vol 4. Fungal Biotechnology. Marcel Dekker Inc; 1992:707-27.
35. Tsai SH, Liu CP, Yang SS. Microbial conversion of food wastes for biofertilizer production with thermophilic lipolytic microbes. Renewable Energy. 2007;32:904-15.

