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Isolation and Identification of Citrus–Juice–Residue Composting Bacteria

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Microbial analysis was carried out on citrus–juice–residue compost. Microflora of the compost was composed of bacteria only and no other microorganism from the compost fermented at 60 °C. Twelve meso–thermophilic bacterial strains were isolated and identified from the compost. Nine bacterial strains of them were identified as *Bacillus stearothermophilus*, two were *Thermus* and one was *Thermoactinomyces*. To reveal whether the identified bacterial strains belonged to the residential microflora of composting or not, isolated strains tested by growth on the residue medium and the digestive capability of the residue. From the results, it was confirmed that the composting microflora composed of the isolated strains which were dominant strains of the composting. It, therefore, is possible to convert citrus–juice–residue to compost by using present isolates.

INTRODUCTION

More than 400 thousand tons per year of citrus (*Citrus unshu* Maric. L) juice residue (Miyake *et al.*, 1991) are discharged from fruit juice factories in Japan. This useless residue is a troublesome waste because it pollutes the environment. One possible solution is to treat the residue by composting and to use the compost as an organic fertilizer on citrus plantations. The residue, as a raw constituent of compost, is not suitable for compost because of its high moisture content, low pH, low nitrogen content and low fermentable sugars content. However, it has been found that it is possible to convert the residue to compost by the addition of calcium carbonate, ammonium sulfate and acclimated bacteria as seed (Kume *et al.*, 1994). There are many commercial seed cultures in Japan, however, the seed cultures have not been revealed their microflora.

This paper deals with isolation and identification of bacterial flora from a compost by using citrus–juice–residue supplemented with calcium carbonate and ammonium sulfate.

MATERIALS AND METHODS

Citrus–residue composting

Raw citrus–juice–residue composed of 85% moisture and 15% solid (pH 3.5). The dried solid composed of 1.1% nitrogen, 47% carbon (C/N ratio=39) and 4% ash. Organic composition of the dried solid also composed of 68% soluble non–nitrogen matter, 14% crude protein, and 12% crude fiber. Composting medium was prepared by 900 g the

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residue, 40 g ammonium sulfide, and 200 g calcium carbonate. The medium was inoculated 200 g of sewage sludge compost (Fujio and Kume, 1991) as the first inoculum. After 10 days incubation at 60 °C, matured citrus–residue compost was used as seed culture of next citrus–residue compost. Composting to prepare seed culture was carried out in a bench scale composter (10 L working volume) with aeration (300 mL/min), moisture (controlled to 55–60% (w/w) during composting by moistured airation) and temperature adjusted to 60 °C by an electric heater.

Composting of citrus–juice–residue was done by using the same medium composition as the seed culture preparation except addition of seed culture instead of sewage sludge compost (Fujio and Kume 1991). Composting fermentation was carried out without any agitation for 7 days fermentation at 60 °C to obtain citrus–residue compost.

Isolation of microorganisms

Isolation source was used the citrus–residue compost. Isolation media were Tryptone–Soy Agar (TSB agar, Eiken Chemicals Co. Ltd., Tokyo) and Nutrient Agar (NB agar, Difco Laboratories, Detroit, MN, USA), both adjusted to the desired pH (4.5 and 7.0). From a diluted suspension of the compost with sterilized water (10 mL) isolation was done by the streak–plate method followed by repeated streaking until a pure culture was obtained. The plates were incubated at 60 °C for 2–3 days under aerobic and anaerobic conditions. Anaerobic incubation was done using anaerobic–jar (BBL Microbiology Systems, Becton, Dickinson and Co., Cockeysville, MD, USA). Purified colonies grown on the agar plates were transferred to an agar slant prepared using the same isolation medium in a test tube.

Identification of isolates

Physiological and biochemical tests on the isolates were conducted by the Minitek system method and CHB–50 method (BBL Microbiology systems) (Flockton and Cross, 1975). Constitutive enzyme production of isolates was also tested by the API–ZYM method (API system S.A., Montalieu vercieu, France) (Logan and Berkely, 1981). After characterization of the test results, the identification of isolates was based on Bergey's Manual of Systematic Bacteriology, vol. 1 (1984) or vol. 2 (1986) and on a report by Walker and Wolf (1971). In this identification procedure, *Bacillus stearothermophilus* IFO 12550, IFO 12983 and IFO 13737 were used as the reference strains (as authentic strain).

Digestion test of citrus residue

Based on the report by Sato *et al* (1984), mashed citrus residue 80 g (wet basis), yeast extract 2 g, and ammonium sulfate 6 g (adjusted pH to 7) were mixed thoroughly in a 30 mL Erlenmeyer flask plugged with cotton wool. After autoclaving at 121 °C for 20 min, 10 g of seed (matured citrus–residue compost including 12 strains (see section 2.2)) were inoculated into the flask medium. Cultivation was done at 60 °C and for maximum of 15 days. A control culture was done without inoculation. A sample of 3.0 g was taken just after inoculation and at appropriate time intervals with culture. Deionized water (400 mL) was added to the sample and then stirred for 10 min. After filtration to separate solid residue, the solid residue was dried at 105 °C for 24 h and weighed to

determine the dried solid fraction. The solid weight decrease of the culture medium was caused by consumption by the inoculated strain. The degree of digestion was defined as decrease in solid weight from the initial medium as a percentage.

Bacterial growth on the residue medium of digestion test

Bacterial growth of the digestion test was by enumeration using the dilution–plating method on NB agar plates. A sample compost, 1 g (wet weight) was taken from the citrus–juice compost at time intervals during composting and added to 10 ml sterilized water. After shaking thoroughly, 10 times dilutions of the sample were made. A 0.1 mL of each diluted sample was plated on NB agar and incubated at 60 °C for 24 h. Colonies formed were then counted and indicated by CFU (colony forming unit).

RESULTS

Isolation

Isolation work from the citrus–residue compost was carried out repeatedly by the plating method. An isolated strain was classed a residential strain if it was re–isolated following repeated (more than 3 times) plating. As a result, 12 isolates classed as residential bacteria were obtained from the compost and the residential bacterial flora of the compost may be composed of these 12 isolates. Twelve bacterial strains isolated were numbered tentatively from No. 1 to No. 9, and 1T, 2T and 1A.

Morphological, physiological and biochemical characteristic tests

Table 1 summarizes the results of the morphological, physiological and biochemical tests. All isolates show smooth, opaque and non–pigmented colonies on the NB agar except strain No. 9 which produced a pinkish diffusible pigment.

Identification of the isolated strains

Nine strains were Gram–positive endospore–forming bacilli (strains 1, 2, 3, 4, 5, 6 and 9), and two strains were Gram–negative non–endospore forming bacilli (1T and 2T). The nine strains (strains No. 1–9) should be classified into the genus *Bacillus* according to their characteristics (Gram–positive rods, endospore forming, motile, aerobes, catalase positive and growth pH range). From the description in Bergey's Manual (Claus and Berkeley, 1986), these 9 strains of the genus *Bacillus* were identified as *Bacillus stearothermophilus*, based on their growth temperature range of 40–75 °C (no growth below 40 °C), optimum growth temperature of 60–65 °C, oval endospore formation, growth inhibition with 3% NaCl, growth inhibition with NaN_3, and the similarity of their physiological and biochemical tests with three reference strains including the type strain. Walker and Walf (1971) classified *Bacillus stearothermophilus* strains into three distinct groups based on their response to physiological and biochemical tests. The isolated *Bacillus stearothermophilus* strains could be categorized into group 1 (strains No. 7 and 8) and group 2 (strains No. 1, 2, 3, 4, 5, 6 and 9) by their classification.

On the other hand, strains 1T and 2T were tentatively identified as being members of genus *Thermus* as due to their biochemical and physiological tests, in accordance with the description in Bergey's Manual of Systematic Bacteriology, vol. 1, section 5 (Block,

Table 1. Morphological, physiological and Biochemical Characteristics

Strain no.	IFO 13737	IFO 12550	IFO 12983	No. 1	2	3	4	5	6	7	8	9	1T	2T	1A
Morphology															
Cell size															
length of rods (μm)	1 2.5	1 2	1-2.5	1.5-2.5	1.5-2.5	1-2	2-4	2-3	1.5-2.5	2-3	2-4	1.5-2.5	2-20	1.5-20	1-20
filaments	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
width of rods (μm)	0.7	0.8	0.8	0.8	0.8	0.6	0.6	0.5	0.8	0.5	0.6	0.7	0.7	0.7-1.0	0.6-0.9
Endospore															
length of spore (μm)	1-2	1-1.5	1-1.5	1-1.5	1-1.5	1.2	1.0	1.2	1.5	1.0	1.0	1.2	–	–	0.9
width of spores	1	1-1.2	1-1.2	1.0	1.0	0.8	0.8	1.0	0.6	0.8	0.9	1.0	–	–	0.6
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–
Color of colonies	–	–	–	–	–	–	–	–	–	–	–	p	–	–	y
Biochemical and physiological reaction															
NaCl tolerance (%)	1	1	1	3	2	4	4	3	4	3	2	3	2	3	3
Sodium azide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Catalase	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
Nitrate	+	+	+	–	+	+	–	–	–	–	–	–	+	+	–
Hydrolysis of															
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	w	+	+	–	+	–	–	–	+	–	–	+	–	–	–
Cellulose	–	–	–	–	–	w	w	–	–	–	–				
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	–	–	–	–	–	w	+	–	w	–	w	w
Temperature of growth	40	40	40	40	40	37	37	40	37	40	40	40	40	40	40
Max	78	75	75	75	75	72	72	70	70	75	75	75	75	72	72
Optimum	65	65	65	60	60	60	60	60	60	60	60	60	63	60	60

w: weak; y: yellow; p: pink

1984) and in a report by Hudson *et al.* (Hudson, *et al.*, 1986). They grew in aerobic conditions at a pH range of 7–8 and temperature range of 40–75 °C (optimum growth temperature was 63 °C). They were oxidase and catalase positive, penicillin sensitive, actinomycin D sensitive, OPNG degradable, and have other biochemical features similar to those of *Thermus* strains as reported by Hudson *et al.*, (Hudson, *et al.*, 1986). Members of the genus *Thermus* form yellow, orange or reddish colonies (description in Bergey's Manual, vol. 1 (Block, 1984)), but the colonies of the two isolated strains showed no pigmentation. Ramaley and Hixson (1971) reported a bacterium that resembles *Thermus aquaticus* these described in morphologically but appears to lack the carotenoid pigment. Based on the features observed here and the literature, isolated strains 1T and 2T were tentatively classified as *Thermus*. sp..

Isolated strain, 1A, was a bacillus form that was Gram negative, endospore forming and formed filaments with a distinct branched mycelium. The growth was good on NB agar, but was poor on PDA. Growth on NB broth was excellent with turbidity appearing within 2–3 days. It was a facultative aerobe with a growth temperature range of 40–70 °C and an optimum growth temperature at 60 °C. Strain 1A formed light yellowish colonies on NB agar and also TBS agar. The strain 1A formed compact colonies that could spread

Table 2. Constitutive Enzyme Production by Api Zym Test of Authentic and Isolated Strains

Strain no.	IFO 13737	IFO 12550	IFO 12983	No. 1	2	3	4	5	6	7	8	9	1T	2T	1A
Constitutive enzyme	Color development in reaction cupules (color intensity level from 0 to 5)														
1. Negative control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2. Alkaline phosphatase	4	4	4	2	2	1	1	1	1	2	1	4	1	1	1
3. Esterase (C4)	1	2	2	2	4	5	5	3	3	4	4	2	3	2	3
4. Esterase lipase (C8)	1	4	2	2	3	3	2	3	3	3	3	3	1	1	2
5. Lipase (C14)	0	0	0	0	1	0	1	1	1	1	1	1	0	1	1
6. Leucine arylamidase	1	0	1	1	1	0	1	1	1	1	2	1	1	0	3
7. Valine arylamidase	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
8. Cystine arylamidase	0	0	0	1	0	1	0	1	0	1	1	0	0	0	1
9. Trypsine	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1
10. Chymotrypsine	1	1	2	0	1	1	1	1	1	0	0	0	0	0	2
11. Acid phosphatase	2	3	3	2	2	1	1	1	2	2	2	1	2	2	1
12. Phosphoamidase	3	3	3	3	4	4	4	3	4	3	4	4	3	3	3
13. α-galactosidase	3	0	5	0	0	0	0	0	0	0	0	0	0	0	0
14. β-galactosidase	1	1	2	0	0	0	0	1	0	1	0	0	0	0	0
15. β-glucronase	0	1	1	1	0	0	1	0	0	1	0	0	0	0	0
16. α-glucosidase	4	4	5	3	1	3	4	3	4	4	0	0	4	0	0
17. β-glucosidase	0	2	3	1	0	1	1	1	1	0	0	0	1	0	1
18. N-acethyl-β-glucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19. α-mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20. α-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

all over surface of an agar plate in a petri dish during 16 hour of culture. Strain 1A formed smooth, glistening, compact colonies on most medium tested. In accordance with their morphological and biochemical characteristics, the 1A strain was identified tentatively as genus *Thermoactinomyces* based on description in Bergey's Manual, vol. 1 (Block, 1984).

Constitutive enzyme test by API-ZYME

In accordance with a report described by Sharp *et al.* (1980), the API ZYM system (Logan and Berkely, 1981) was applied to constitutive enzyme production of isolated strains of *Bacillus stearothermophilus*. Table 2 shows the constitutive enzyme production of the isolated *Bacillus stearothermophilus* in comparison with the enzyme production of three authentic *Bacillus stearothermophilus* (IFO 12550, IFO 12983, IFO 13737). There were similarities in the enzyme production between the isolated strains and the reference strains. The constitutive enzyme similarities were esterase (C4), esterase lipase (C8), acid phosphatase, phosphoamidase and α-glucosidase. Two reference strains (IFO 12550 and IFO 13737) showed a high level of α-glucosidase, while IFO 12938 strain did not produce the enzyme. All of isolated *Bacillus stearothermophilus* also did not produce the α-glucosidase. These results were further confirmed by finding that the isolated strains Nos. 1-9 were *Bacillus stearothermophilus* due to their production of esterase (C4) and the comparative similarity in constitutive enzyme production with the reference strains.

Digestion activities by mixed culture of isolated strains on the citrus residue

Figure 1 shows the result of the digestion test by isolated strains. After culture for 15 days, the digestion degree of the control was only 3–4%, while the degree, was up to 22% for the inoculated culture. The results presented in Fig. 1 show that isolated strains can solubilize (and then consume) some substance containing in the citrus residue. From the results, the isolated strains were confirmed as the dominant flora of the citrus residue composting process.

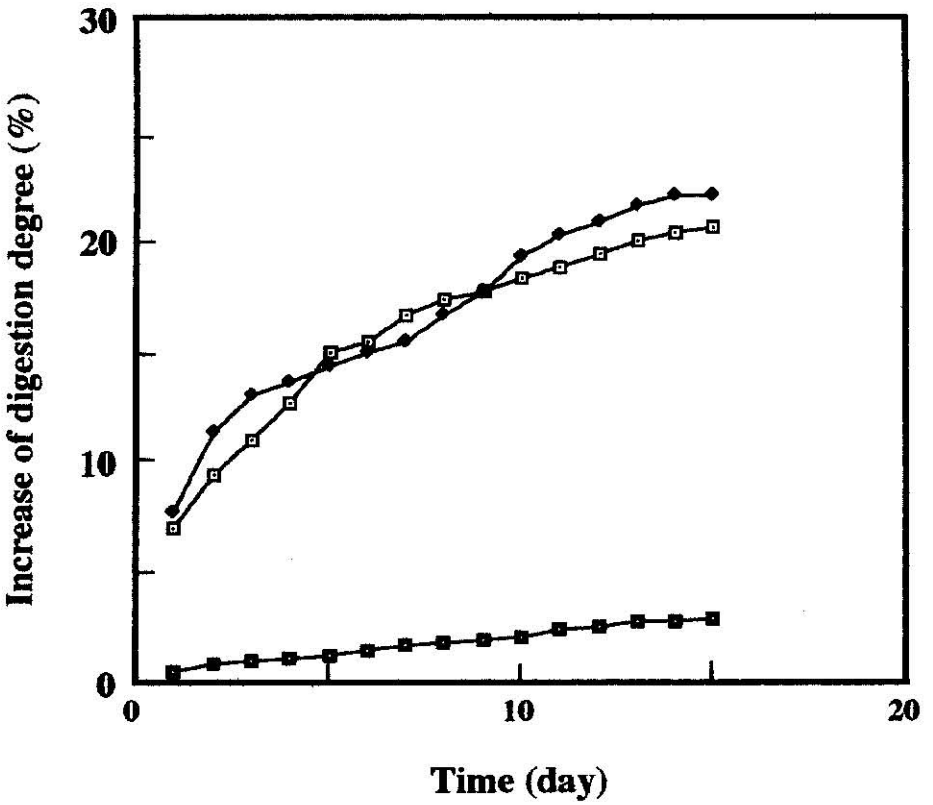

Fig. 1. Digestion of citrus–residue by the isolates

—□— 1st digestion test by mixture of the isolates (1st run)

—◆— 2nd digestion test by mixture of the isolates (2nd run)

—■— Control run

Digestion degree was defined as following equqtion and the result was given in percent.

$$\frac{\text{(initial solid–solid at an incubation time)}}{\text{initial solid}} \times 100$$

sample solid was suspended with water and mixed throughly then centrifuged. Solid part was dried at 105 °C for 48 h and weighed.

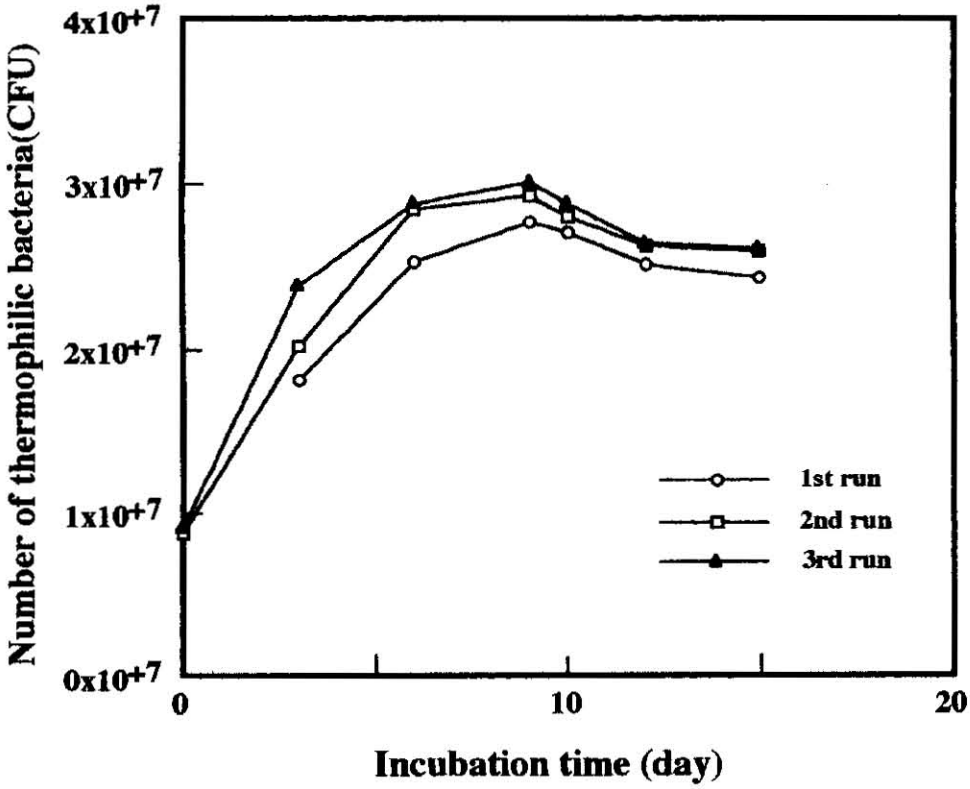

Fig. 2. Changes of thermophilic bacterial number during digestion culture of citrus-residue

—○— 1st run
—□— 2nd run
—▲— 3rd run

Citrus-residue medium with isolates were cultured at 60 °C for 15 day and the culture was repeated three times (run 1, run 2, and run 3).

Bacterial growth on the residue medium

Figure 2 shows the change with culture time of bacterial number per 1 g (wet weight) of the residue medium. Because of large inoculum size, the initial bacterial number was almost 10^7 and with the culture time, the bacterial number increased to a maximum of 3×10^7 in 10 days culture and then decreased to 2.5×10^7 after 15 days culture. The bacterial growth test was repeated for 3 times (1st, 2nd and 3rd in Fig. 2), however, the tendency of bacterial growth seemed to be almost the same as shown in Fig. 2.

DISCUSSION

In the case of microorganism analysis for septic fermentation such as composting, one of the problems is that the isolated microorganism may belong to residential flora or be transients to the medium. In the present isolation work, isolated strains were confirmed as dominant composting bacteria by digestion capability (Fig. 1, maximum 22%) and growth (Fig. 2, maximum number 3×10^7) on the residue. Isolated strains were classified as (meso-)thermophilic bacteria whose growth optimum was at 60–65°C (Table 1).

Usually, bacterial composting of organic waste is carried out at a high temperature, with a maximum of 80–90°C. The composting of citrus residue supplemented with a nitrogen source, therefore, may take place at higher temperatures than 60°C in a large scale by using isolates here as a seed. High temperature composting is preferable because the composting will cease after a short time and be accompanied by ample bacterial growth. Because of the high temperature of composting, the isolated strains may be useful in the treat ment of organic waste. In the past, the composting treatment of the citrus-juice-residue was not examined, so the confirmation of the microflora of the citrus residue compost may be helpful to composting treatment.

REFERENCES

Block, T. D. 1984 Genus *Thermus* Block and Freese 1969, 295, *In* "Bergey's manual of systematic bacteriology", The Williams and Wilkins Co., Baltimore, USA, pp. 333–339

Claus, D. and R. C. W. Berkeley 1986 Genus *Bacillus* Cohn 1872, **174**, *In* "Bergey's manual of systematic bacteriology", vol. 2, The Williams and Wilkins Co., Baltimore, USA, pp. 1105–1141

Flockton, H. I. and T. Cross 1975 Variability in *Themoactinomyces vulgaris*. J. Appl. Bacteriology, **38**: 309–313

Fujio, Y. and S. Kume 1991 Isolation and Identification of thermophilic bacteria from sewage sludge compost. J. Ferment. Bioeng., **72**(No. 5): 334–337

Hudson, J. A., H. W. Morgan and R. M. Daniel 1986 A numerical classification of some *Thermus aquaticus* isolate. J. Gen. Microbiol., **132**: 531–540

Kume, S., Y. Fujio, H. Maeda and Y. Ifuku 1994 Composting of juice residue of Citrus unshu (In Japanese), Proceedings of 1994-Annual Meeting of Nippon Shokuhin Kogyo Gakkai, Tokyo, pp. 122

Logan, N. A. and R. C. W. Berkely 1981 Classification and identification of members of the genus *Bacillus* using Api tests. *In* "The aerobic endospore-forming bacteria; classification and identification", ed. by R. C. W. Berkely and M. Goodfellow, Academic Press, London, pp. 105–140

Miyake, M., N. Inaba, K. Nakayama, H. Maeda and Y. Ifuku 1991 Mikan sakujukasu no kanso oyobi shoyoryoka ni yuko na biseibutsu no kensaku (in Japanese). Nippon Shokuhin Kogyo Gakkaishi, **38**(No. 5): 398–404

Ramaley, R. F. and J. Hixson 1970 Isolation of a nonpigmented thermophilic bacterium similar to *Thermus aquaticus*. J. Bacteriology, **103**: 527–528

Sato, K., J. Antheunisse and E. G. Mulder 1984 A possible effect of cellulose decomposition on soil bacterial flora. Journal of General Microbiology, **30**: 1–14

Sharp, R. J., K. J. and A. Atkinson 1980 Phenotypic and genotypic characterization of some thermophilic of bacillus. Journal of General Microbiology, **117**: 201–210

Walker, P. D. and J. Wolf 1971 The taxonomy of *Bacillus stearothermophilus*. Spore research,: 247–264