

Isolation and Characterization of Soybean Waste-Degrading Microorganisms and Analysis of Fertilizer Effects of the Degraded Products

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Two microorganisms which could degrade soybean lees efficiently were isolated and identified as *Bacillus circulans* and *B. stearothermophilus*. These two strains secreted thermostable proteases into the medium and could digest soybean lees rapidly and completely at 50°C. Initially, the soybean lees were degraded to proteins in approximately 20 h by these two strains, after which time the concentrations of peptides in the medium gradually increased. The degraded products from soybean lees contained abundant nitrogen compounds, such as peptides, amino acids, and amides. Approximately 10 times more fresh plant weight was obtained (in the case of *Brassica campestris*) when these degraded products were applied than when water was applied for 42 days. These stimulatory effects of the soybean lees products were almost equal to those of a chemically synthesized fertilizer.

It is estimated that approximately 160 billion tons of organic biomass are produced annually worldwide by photosynthesis (16, 21). Conversion of organic biomass into proteins, peptides, and other products by microorganisms is a process of increasing importance for the future (4, 6, 8, 14). As components of the biomass, soybeans are well known as a source of excellent-quality vegetable proteins and have several functional properties, so that they are widely cultured in the world and are utilized for various foods and cooking oil (17, 18).

Soybean lees, however, which are by-products and/or waste materials from soybeans after abstraction of the oil and which contain rich nitrogen compounds, such as vegetable proteins, are currently only reutilized as feedstuff for animals and media for microorganisms (22). Soybean lees were previously used directly as a useful nitrogenous fertilizer in large quantities (19). However, ammonium sulfate, urea, and chemically synthesized fertilizers took the place of fertilizer made from soybean lees because soybean lees were degraded too slowly by microorganisms in the soil and the fertile effects on plants were milder than those of chemically synthesized fertilizers.

However, because of increasing environmental concerns, the potentially detrimental effects on soil of the use of too much chemically synthesized fertilizer have recently received increased attention. Therefore, the development of new fertilizers by use of natural materials, such as amino acids and natural nitrogen compounds, has become the focus of much research interest (1, 9, 20). We point out that soybean lees are inexpensive, are natural products, and are rich in nitrogen compounds (vegetable proteins). We thus embarked on a research project attempting to isolate microorganisms which could degrade soybean lees efficiently at a high speed. In this report, we describe the stimulatory

effects of degraded soybean lees products (DSP) upon the growth of plants.

MATERIALS AND METHODS

Bacterial strains and media. *Bacillus circulans* HA12 and *B. stearothermophilus* HA19 are new soybean lees-degrad-

TABLE 1. Properties of strains HA12 and HA19

Property	Result for:	
	HA12	HA19
Cell morphology	Rod	Rod
Gram staining	+	+
Spore formation	+	+
Motility	-	-
Growth at:		
25°C	+	-
37°C	+	+
Strict aerobic reaction	+	+
Oxidase reaction	+	+
Catalase reaction	+	+
Nitrate reduction	-	+
H ₂ S production	-	-
Indole production	+	-
Methyl red reaction	+	-
Growth in urease	+	+
Growth in NaCl		
0%	+	+
3%	+	+
7%	-	-
10%	-	-
Hemolysis	-	-
Decarboxylation from ornithine	-	+
Decarboxylation from lysine	-	-
Decarboxylation from arginine	+	+
Gas from glucose	+	+
Gas from mannitol	+	-
Gas from lactose	+	-
Gas from sucrose	+	-
Gas from maltose	+	+

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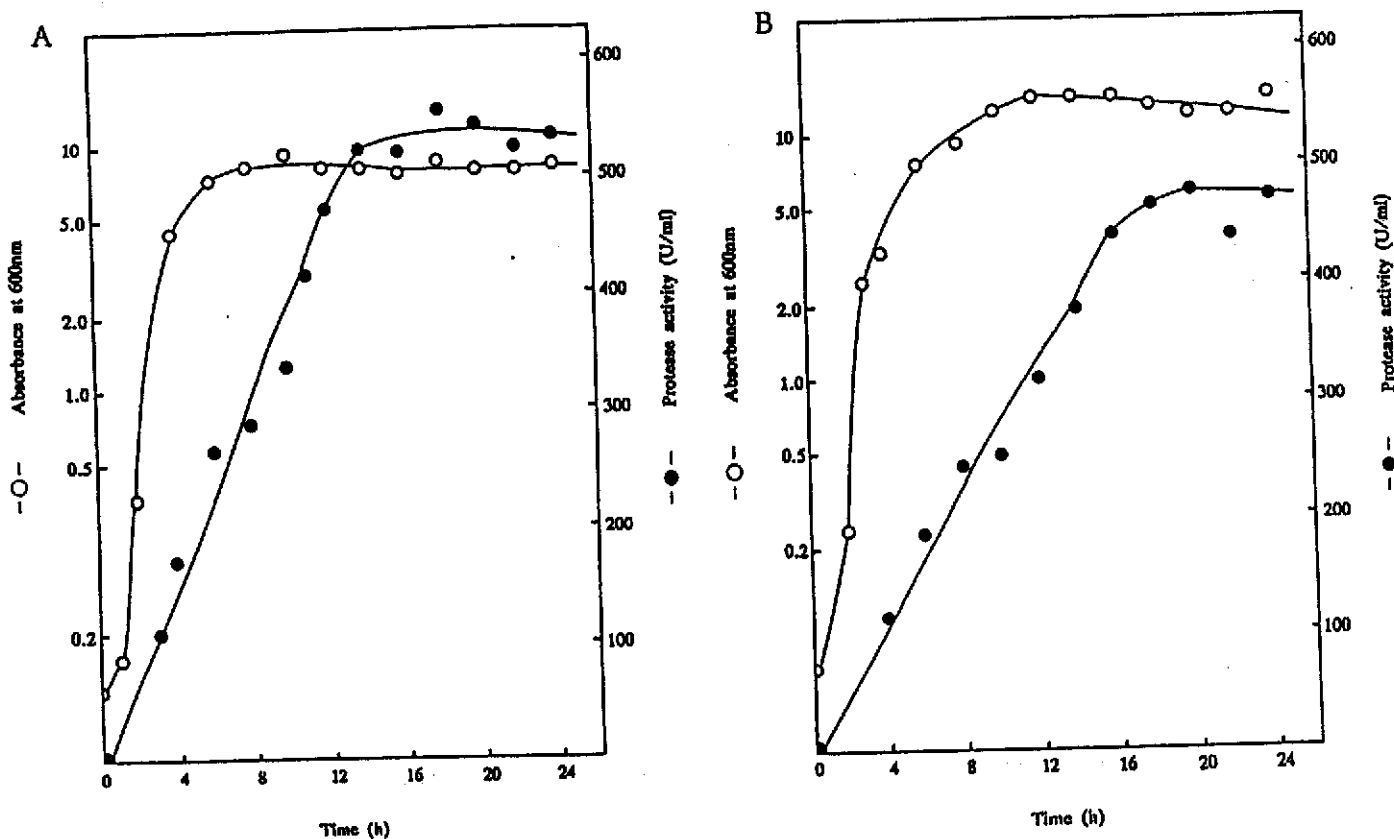


FIG. 1. Time courses of protease production and patterns of cell growth for *B. circulans* HA12 (A) and *B. stearothermophilus* HA19 (B). Cells were grown at 50°C in L broth on a rotary shaker. The culture supernatants were used for enzyme assays (●). Bacterial growth (○) was measured as the optical density at 600 nm.

ing strains isolated during this study. The characteristics of these strains are described below, and the compositions of L broth, L agar, and LC agar (L agar plus 1% casein) have been described elsewhere (10). Soybean lees medium, consisting of 1% soybean lees (Honen Corp., Tokyo, Japan), was used as the screening medium.

Detection of protease-producing colonies and assay of protease activity. Protease-producing colonies were detected on LC agar, and protease activity was assayed for casein hydrolytic activity as described previously (5, 7). For enzyme production, the bacteria were cultivated at 50 or 60°C in L broth in a 500-ml flask on a rotary shaker (11). The culture supernatants were used for enzyme assays. We defined 1 U of protease as the quantity required to increase the A_{275} at a rate equivalent to the formation of 1 μ g of tyrosine per min at 37°C (12).

Screening of soybean lees-degrading microorganisms. Samples taken from natural environments, such as compost or hot springs, were incubated in 5 ml of L broth at 37 or 50°C for 4 h with vigorous shaking; 1-ml samples from each culture were reinoculated into 100 ml of soybean lees medium and incubated at 37 or 50°C for 48 h. After cultivation, 100- μ l samples from each culture were spread on LC agar. Single colonies which produced protease(s) and degraded soybean lees (48 h) were screened, isolated, and characterized (13).

Protein and peptide concentrations. Protein concentrations were determined by use of bicinchoninic acid protein assay reagent from Pierce Chemical Co., Rockford, Ill., and crystalline bovine serum albumin as the standard (24). Peptide

concentrations were determined by the ninhydrin reaction after removal of proteins by trichloroacetic acid (final concentration, 3%) precipitation (15).

Cultivation of plants. Seeds were placed in distilled water for 15 h to promote generation. The soil was soaked in water and sterilized in an autoclave (121°C, 15 min). The water was then removed, and the soil was dried for 5 h at 180°C. Eight plants of *Brassica campestris* were sown in a planter (25 by 13 by 11 cm), and on each day, 200 ml of DSP from HA12 or HA19, chemically synthesized fertilizer 50 (Richell, Horikawa, Toyama, Japan), or water was given to the appropriate planter. Cultivation was carried out at 25°C and 20,000 lx for 42 days in a plant incubator (model MLR-350T; Sanyo, Tokyo, Japan).

Rock wool (Nichiasu, Tokyo, Japan) was used in the cultivation of Japanese radish. The rock wool was placed in a planter (19 by 14 by 3 cm), and 20 seeds of Japanese radish were sown on it. Each planter was filled with 300 ml of the appropriate fertilizer solution, and cultivation was carried out at 25°C for 9 days in a dark incubator.

RESULTS

Isolation of soybean lees-degrading bacteria. Samples taken from natural environments, such as compost or hot springs, were incubated with soybean lees medium at 50 or 60°C for 48 h with vigorous shaking. Following growth, 100- μ l samples from each culture were spread on the surface of LC agar plates, and the plates were incubated at 50 or 60°C overnight. About 5,000 mesophilic and thermophilic bacteria were

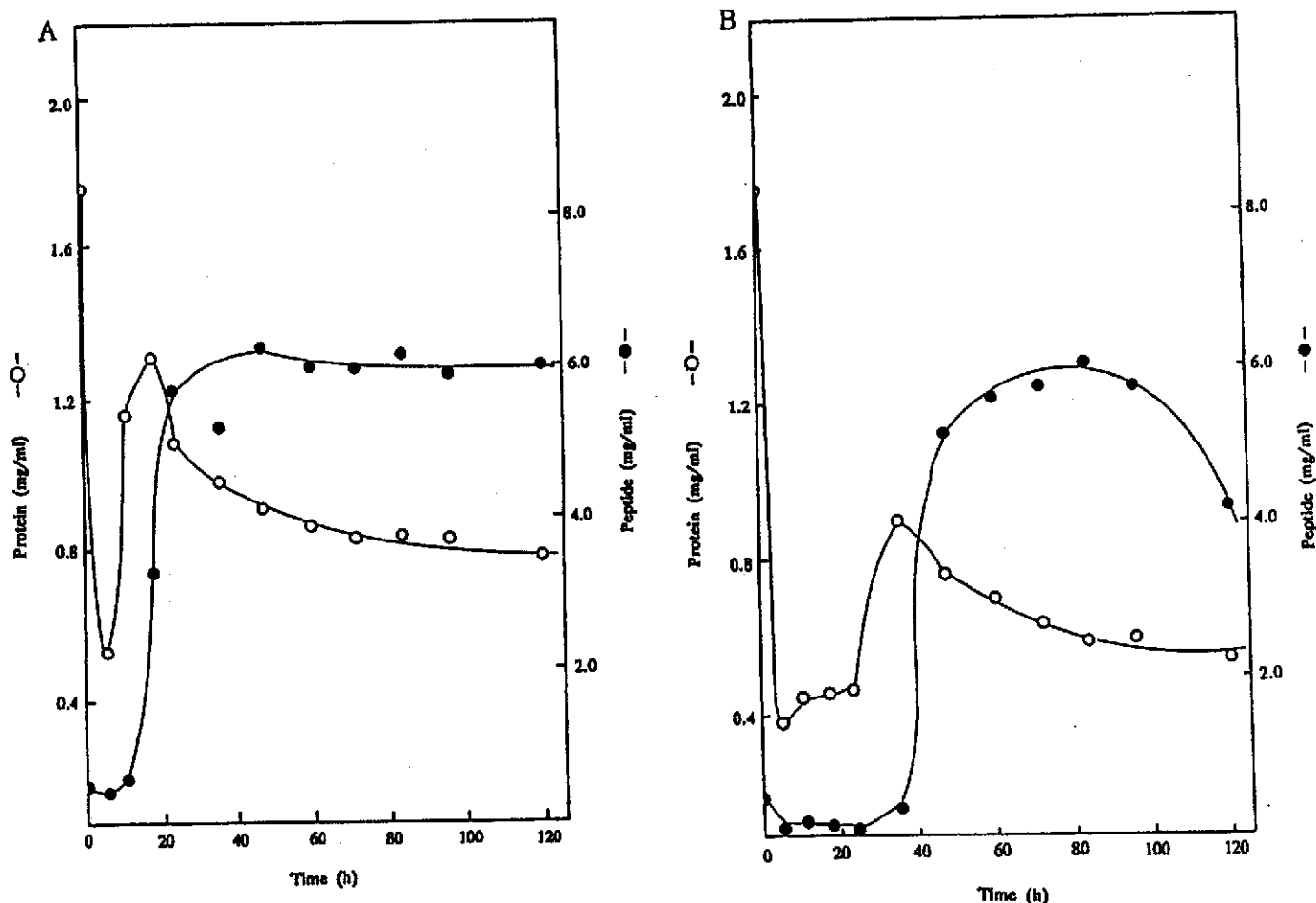


FIG. 2. Time courses of protein and peptide production for *B. circulans* HA12 (A) and *B. stearothermophilus* HA19 (B). Cells were grown at 50°C in soybean lees medium on a rotary shaker. The culture supernatants were used for the measurement of protein (O) and peptide (●) concentrations.

isolated. Among these isolates, about 50 bacteria could produce protease(s) on LC agar plates; these isolates differed morphologically as well as microscopically. Each isolate was further subcultured in 100 ml of soybean lees medium at 50 or 60°C for 48 h. As a result, mesophilic bacterium HA12 and thermophilic bacterium HA19 were isolated; they exhibited the capacity to degrade soybean lees rapidly and efficiently.

Characterization of bacteria HA12 and HA19. Strains HA12 and HA19 were classified by standard methods (3). Since both strains were strictly aerobic, gram positive, catalase producing, and endospore forming, they were classified in the genus *Bacillus*. The maximum temperatures for the growth of strains HA12 and HA19 were 55 and 70°C, respectively. The characteristics of these strains are listed in Table 1. According to *Bergey's Manual of Determinative Bacteriology* (3), strains HA12 and HA20 are *B. circulans* and *B. stearothermophilus*, respectively.

Production of protease(s) by HA12 and HA19. *B. circulans* HA12 and *B. stearothermophilus* HA19 formed clear and distinct haloes on LC agar plates, and both strains degraded soybean lees efficiently. To demonstrate the existence of a proteolytic enzyme(s), the time course of the protease activity of both strains was investigated. Figure 1A shows the patterns of cell growth of and enzyme production by *B.*

circulans HA12 at 50°C. The maximum cell concentration and enzyme activity were an optical density at 600 nm of 10 (10 h) and 550 U/ml (16 h), respectively (Fig. 1A). On the other hand, the maximum cell concentration and enzyme activity of *B. stearothermophilus* HA19 were an optical density at 600 nm of 12 (12 h) and 490 U/ml (18 h), respectively (Fig. 1B). The proteases from *B. circulans* HA12 and *B. stearothermophilus* HA19 were both secreted into the medium, and the optimum temperature for the activities of these enzymes was about 70°C (data not shown).

Analysis of patterns of degradation of soybean lees by *B. circulans* HA12 and *B. stearothermophilus* HA19. To investigate the degradation of liquified soybean lees by *B. circulans* HA12 and *B. stearothermophilus* HA19, the products formed in soybean lees medium were analyzed. Figure 2 shows the accumulation of proteins and peptides from soybean lees in medium by cultures of *B. circulans* HA12 and *B. stearothermophilus* HA19. In the first stage, dissolved proteins from soybean lees were consumed, suggesting that these proteins were used for primary metabolism by both strains. In the next stage, soybean lees were digested by the proteases from both strains, and proteins accumulated gradually in the medium. The stage of increasing amounts of proteins corresponded to the formation of proteases by both strains. Subsequently, these proteins were themselves fur-

TABLE 2. Fertile effects on *B. campestris*

Fertilizer	Harvest wt (g) in expt:			Avg harvest wt (g)
	1	2	3	
Water	2.3	0.8	1.3	1.5
Chemically synthesized	12.3	17.0	17.7	17.3
DSP from HA12	11.9	16.1	21.5	16.5
DSP from HA19	11.6	10.9	20.0	14.1

ther digested to smaller molecules, including peptides and amino acids. The concentrations of peptides and amino acids in the medium incubated with *B. stearothermophilus* HA19 decreased gradually after about 80 h. Maximum concentrations of peptides produced in the presence of *B. circulans* HA12 and *B. stearothermophilus* HA19 were 6.5 and 6.3 mg/ml, respectively. Approximately the same concentration of peptides were maintained in the medium by *B. circulans* HA12 (Fig. 2A).

Growth-stimulating effects of DSP. Soybean lees have frequently been utilized as a fertilizer for plants (19). However, because many chemically synthesized fertilizers have more immediate effects, soybean lees have become less utilized as a fertilizer (19). It is well known that N, P, and K are essential elements for plant growth (2, 23), and since DSP include amino acids and peptides (Fig. 2), the fertile effects of DSP on plants were investigated.

B. campestris and Japanese radish were chosen to examine the fertile effects of DSP from the two strains, since the cultivation of these species requires little time or care. Table 2 shows the fertile effects on *B. campestris*. Each of three seedlings of *B. campestris* was grown with either water, chemically synthesized fertilizer, or DSP from the two strains for 42 days, and the final harvest weights were measured. As shown in Fig. 3, the weights of *B. campestris* were 10 to 11 times higher with DSP from *B. circulans* HA12 and *B. stearothermophilus* HA19 than with water. Approximately equal harvest weights of *B. campestris* were observed when DSP from the two strains and chemical fertilizer were applied (Table 2).

Likewise, the fertile effects were investigated with Japanese radish and various concentrations of DSP from the two strains (Tables 3 and 4). When high concentrations (above 50%) of DSP from the two strains were supplied to Japanese radish, growth was inhibited. However, remarkable growth-stimulating effects were found upon the application of low concentrations of DSP from both strains.

DISCUSSION

Soybean lees are inexpensive, natural materials, but they have not been efficiently utilized in recent years. In attempting to develop new methods for the utilization of soybean lees, we have isolated microorganisms from nature that could degrade soybean lees. Many soybean lees-degrading microorganisms, including two strains (HA12 and HA19) with the potential to rapidly degrade soybean lees, were isolated. These two strains were identified as *B. circulans* and *B. stearothermophilus*, respectively. Both strains formed haloes on LC agar plates and secreted large quantities of thermostable proteases into the medium. It is believed that the proteases secreted by these strains into the medium were responsible for the formation and digestion of proteins from soybean lees.

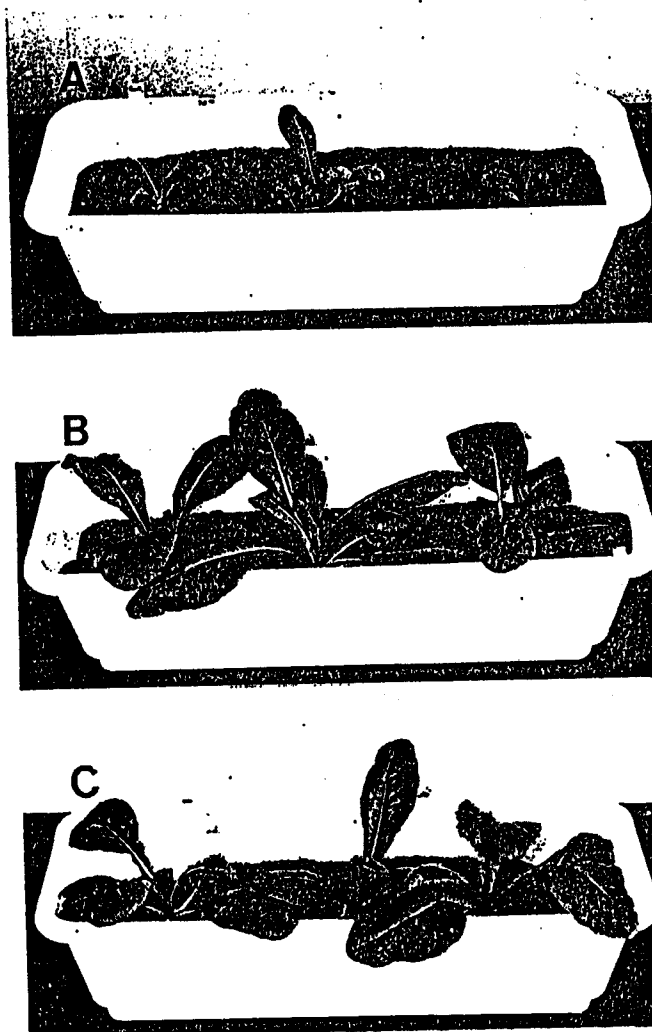


FIG. 3. Fertile effects of DSP on *B. campestris*. Cultivation was carried out for 42 days. (A) Water. (B) DSP from *B. circulans* HA12. (C) DSP from *B. stearothermophilus* HA19.

The time courses of degradation by *B. circulans* HA12 and *B. stearothermophilus* HA19 were investigated. The amounts of dissolved proteins from soybean lees in the medium decreased following cell growth in the first stage, suggesting that these proteins were metabolized during the initial growth of the microorganisms. These two microorganisms secreted larger quantities of proteases in the next stage, and the soybean lees in the medium were digested gradually by these proteases. The quantities of peptides increased

TABLE 3. Fertile effects of DSP from HA12 on Japanese radish

DSP concn (%)	Plant length, in cm, on the following day ^a :							Plant final wt, in g ^b
	3	4	5	6	7	8	9	
0	0.3	1.2	3.6	4.7	5.8	6.3	7.0 (100)	2.3 (100)
1	0.0	0.3	2.0	2.6	3.7	4.6	5.1 (73)	1.2 (73)
10	0.4	2.9	5.1	6.5	7.9	8.7	8.9 (127)	3.2 (136)
25	0.0	0.9	3.1	4.0	5.4	6.5	7.5 (107)	2.5 (107)
50	0.2	0.8	1.6	1.9	2.2	2.4	2.7 (39)	0.9 (40)
100	0.0	0.2	0.4	0.4	0.4	0.5	1.1 (16)	0.5 (20)

^a Numbers in parentheses indicate percentages of length.

^b Numbers in parentheses indicate percentages of weight.

TABLE 4. Fertile effects of DSP from HA19 on Japanese radish

DSP concn (%)	Plant length, in cm, on the following day ^a :							Plant final wt, in g ^b
	3	4	5	6	7	8	9	
0	0.3	1.2	3.6	4.7	5.8	6.3	7.0 (100)	2.3 (100)
1	0.8	3.5	5.1	5.9	10.2	10.5	11.4 (175)	3.1 (134)
10	0.2	1.3	2.6	3.5	5.3	5.8	7.1 (101)	2.3 (99)
25	0.0	0.6	2.3	3.5	5.2	5.2	6.4 (91)	2.3 (99)
50	0.2	1.0	2.9	3.0	3.8	4.5	5.1 (73)	2.0 (83)
100	0.0	0.2	0.4	0.4	0.7	0.8	1.2 (16)	0.5 (20)

^a See Table 3, footnote a.

^b See Table 3, footnote b.

concomitantly with decreases in protein concentrations in the medium. As can be seen from the patterns of degradation of soybean lees by these two strains (Fig. 1), the soybean lees were degraded efficiently by these two strains. An estimate of the complete decomposition time for 1% soybean lees in water at 50°C was 30 to 40 h.

The fertile effects on plants of DSP from *B. circulans* HA12 and *B. stearothermophilus* HA19, which consisted of rich nitrogen sources, were investigated. As expected, the DSP obviously contributed to the growth of the plants (*B. campestris* and Japanese radish), in comparison with the effects of adding only water. Comparable stimulatory effects were observed when either a chemically synthesized fertilizer (ammonium sulfate as a principal ingredient) or DSP were used as a fertilizer. These results indicate that the components of DSP from these two strains may become a new effective fertilizer for plants because these products originated from natural materials and had fertile effects equivalent to those of the chemically synthesized fertilizer. With various combinations of proteases used to digest soybean lees may come increases in the fertile effects on plants. Further experiments are in progress to characterize proteases from *B. circulans* HA12 and *B. stearothermophilus* HA19 more fully.

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