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Isolation and Numerical Identification of *Streptomyces humidus* strain S5-55 Antagonistic to Plant Pathogenic Fungi

Song Won Lim, Jeong Dong Kim, Beom Seok Kim and Byung Kook Hwang*

Department of Agricultural Biology, Korea University, Seoul 136-701, Korea

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To search for the antifungal substances, various actinomycete isolates were obtained from various soils of Korea using plate dilution method on the humic acid vitamin agar plates. In the screening procedures using a dual culture method, 32 actinomycete isolates were selected, which showed the inhibitory activity against mycelial growth of plant pathogenic fungi *Alternaria mali*, *Colletotrichum gloeosporides*, *Fusarium oxysporum* f.sp. *cucumerinum*, *Magnaporthe grisea*, *Phytophthora capsici*, and *Rhizoctonia solani*. Bioassay of the crude extracts from culture filtrates and mycelial mets revealed that 12 antagonistic actinomycetes produced highly active antifungal substances. Actinomycete strain S5-55 which showed the substantial antifungal activity against the tested fungi was selected for production of the antifungal substances. Based on the cytochemical and morphological characteristics, strain S5-55 was identified as a *Streptomyces* species. The results of the numerical identification using the TAXON program confirmed that *Streptomyces* strain S5-55 was identical with *Streptomyces humidus* including in TAXON major cluster 19. The production of antifungal substance was most favorable when *S. humidus* strain S5-55 was cultivated for 10 days, on soluble starch broth supplemented with K_2HPO_4. The antifungal substances active against the plant pathogenic fungi *P. capsici* and *M. grisea* were partially purified using C_{18} reversed-phase column chromatography.

Keywords : numerical identification, *Streptomyces humidus*, antifungal activity, plant pathogenic fungi.

The more intensive crop protection has been demanded to increase crop yields in modern agriculture. Integrated crop management such as crop rotation, crop breeding for resistance to disease, accurate diagnosis and early detection methods of disease developments, etc. have been recommended to the farmers for the control of plant diseases in the sustainable agriculture (Evans, 1998). For the control of fungal diseases on crop plants, however, use of fungicides may be a primary strategy.

Japanese groups have tried to discover various antibiotic fungicides for agricultural use. Since blasticidin S from *Streptomyces griseochromogenes* was introduced for control of rice blast caused by *Magnaporthe grisea* (Takeuchi et al., 1957), kasugamycin (Umezawa et al., 1965), polyoxin (Isono et al., 1965), validamycin (Iwasa et al., 1970), mildiomycin (Harada and Kishi, 1978), etc. have practically been used for control of the plant pathogenic fungal diseases.

Since isolation of streptomycin from *Streptomyces griseus* in 1943, the new antibiotics have been discovered from microorganisms in rapid succession. Based on the data of Miyadoh (1993), more than 10,000 antibiotics have been isolated from various antibiotic-producing microorganisms, in which 70% antibiotics were produced by actinomycetes. Among the antibiotic-producing actinomycetes, 47% was identified to be *Streptomyces* spp. Most actinomycetes were found to be producers of antibiotics such as peptides, aminoglycoside, non-polyene and polyene macrolides, polyether or anthracycline antibiotics, and nucleosides (Betina, 1994).

Yarbrough et al. (1993) demonstrated that soil microorganisms are an important source for the antifungal metabolites. In a screening procedure for a novel antibiotic compound, soils rountinely sampled from different geographical areas and ecological habitats were used for selective isolation of the certain antibiotic-producing microorganisms after various pretreatments.

Since most of the microbially derived antibiotics are produced by the members actinomycetes and filamentous fungi, these taxonomic groups become a target for the screening of antibiotics,. The isolation methods and culturing conditions such as type of media and incubation temperature etc. seem to be the most important factors for a large scale production of antibiotic metabolites. In particular, the culturing process is the most important factor, because the antifungal substances including antibiotics may be produced mainly by the secondary metabolite pathway under the nutritionally limited conditions. The nutritional factors such as carbon and nitrogen source, ammonia, inorganic phosphate, and metal ions such as maganese, cobalt,

*Corresponding author.
Phone) +82-2-3290-3061, FAX) +82-2-925-1970
E-mail) bkhwang@mail.korea.ac.kr

zinc, calcium, and magnesium in the media may also affect the production of secondary metabolites during the cultivation of the microorganisms.

Screening for lead fungicidal compounds from various microorganisms has resulted in the rediscovery of some antibiotics which showed antifungal activity against plant pathogenic fungi. For instance, some antibiotics such as capsimycin (Aizawa et al., 1982), concanamycin B (Kim et al., 1993), tubercidin (Hwang and Ahn, 1994), manumycin-type antibiotic (Hwang et al., 1996), phthoramycin (Omura et al., 1988) and streptimidone (Kim et al., 1999) have been demonstrated to be very effective in controlling *P. capsici* diseases.

In the present study, the actinomycete strain S5-55 isolated from mountain soils in Korea was identified as *Streptomyces humidus* using TAXON program. The antifungal substances were purified from the culture filtrates of *S. humidus* strain S5-55.

Meterials and Methods

Isolation of antagonistic actinomycetes. Soil samples were collected from 5 locations of Kangwon Province such as Hoengsung (M, sea sand), Jungsun (G, sea mud), Pyungchang (N, paddy soil), Yangyang (K, pepper-growing soil), and Youngwol (S, mountain soil). The soil samples air-dried for 7 days at room temperature were heat-treated following the method of Hayakawa et al. (1987). Five gram soil sample was suspended in 50 ml of sterilized water in a 250 ml-Erlenmeyer flask and then shaken at 150 rpm for 30 min. The soil suspension (0.5 ml) was spread on the humic acid-vitamine agar (1 g humic acid, 0.5 g Na_2HPO_4, 1.71 g KCl, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCO_3$, 0.5 mg thiamine-HCl, 0.5 mg riboflavin, 0.5 mg niacin, 0.5 mg pyridoxin-HCl, 0.5 mg inositol, 0.5 mg Ca-panthothenate, 0.5 mg *p*-aminobenzoic acid, and 0.25 mg biotin, 50 mg cycloheximide, 18 g agar, 1L water, and adjusted to pH 7.2). After incubation for 4 weeks at 30°C, actinomycete colonies appearing on the humic acid-vitamin agar were transferred to the yeast-malt extract agar (YMA, 4 g yeast extract, 10 g malt extract, 4 g glucose, 20 g agar, 1 L water, and pH 7.3 before autoclaving). The antifungal activity of the actinomycete isolates was tested on the V8 juice agar (20% V8 juice, 15 g $CaCO_3$, 18 g agar, and 1 L water) against 6 plant pathogenic fungi such as *Alternaria mali*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f.sp *cucumerinum*, *Magnaporthe grisea*, *Phytophthora capsici*, and *Rhizoctonia solani*. Each actinomycete strain was streaked in the center of V8 juice agar plate, followed by incubation for 7 days at 28°C. The mycelial disks (7 mm in diameter) of the test fungi were placed on both sides 30 mm from the streak-inoculated actinomycetes. After incubation for 5-8 days at 28°C, the inhibition zone (mm in length) of mycelial growth was measured. Thirty-three actinomycetes which showed antifungal activity were maintained in the YMA slants until used for further *in vitro* bioassay.

***In vitro* bioassay of antifungal activity.** The culture extracts from 12 antagonistic actinomycete strains which showed a high antifungal activity against 6 plant pathogenic fungi in the dual culture test were tested for antifungal activity *in vitro* by paper disk method. Actinomycetes were inoculated in 70 ml yeast-malt extract broth (4 g yeast extract, 10 g malt extract, 4 glucose, and 1 L water) in 250 ml-Erlenmeyer flask and incubated in a rotary shaking incubator (120 rpm) for 3 days at 29°C. The 5 ml-seed culture was transferred to 500 ml of modified soluble starch broth (5 g starch, 10 g glycerol, 2 g yeast extract, 1 g NH_3SO_4, 0.2 g KH_2PO_4, 0.5 g $MgSO_4$, 500 ml H_2O, and pH 6.5 after autoclaving) in 1L-Erlenmeyer flask and incubated in a rotary shaking incubator (170 rpm) for 14 days at 29°C. Packed cell volume (PCV) and pH of the culture filtrates of 12 antagonistic actinomycetes were monitored after the batch-fermentation. PCV was measured after centrifugation at 1,250 g for 30 min. The supernatant of culture broth was extracted with 500 ml of *n*-buthanol and the pellet was extracted with 100 ml of methanol. The extracts of culture filtrate and mycelial mats and the remaining aqueous phase were concentrated to 5 ml, 10 ml, and 1 ml *in vacuo*, respectively. Antifungal activity of each extract was evaluated by paper disk method against 6 plant pathogenic fungi on PDA. The inhibition zone (mm in length) was measured after incubation for 24-48 h at 28°C. Among the 12 actinomycetes, strain S5-55 which showed high antifungal activity was selected for further species identification and antibiotic production.

Generic identification of antagonistic actinomycete strain S5-55. For the generic identification of actinomycete strain S5-55, 2,6-diaminopimelic acid (DAP), known as a cell wall component of actinomycete mycelia, was analyzed by the methods of International *Streptomyces* Project (Shirling and Gottlieb, 1966) and Williams et al. (1989).

The spore chain morphology of the actinomycete strain S5-55 which was grown for 10 days on yeast-malt agar, oatmeal agar and inorganic salt starch agar was examined under the light microscopy. Spore ornamentation was observed by scanning electron microscopy (SEM, JSM-5410 LV, JEOL, Japan) interfaced with a cryo-transfer system (CT 1500 Cryotrans, Oxford instruments, UK) at 20 keV. The categories of Pridam et al. (1948) were employed with the modified terminology of Shirling and Gottlieb (1968a) for evaluation of spore chain morphology. Cultural characteristics of the actinomycete strain S5-55 were examined based on the guide of International *Streptomyces* Project (ISP) made by Shirling and Gottlieb (1966).

Numerical identification of actinomycete strain S5-55 using TAXON program. To numerically identify the species of actinomycete strain S5-55, TAXON program (Ward, 1991) was used. TAXON is the computer program that calculates the identification scores and similarity coefficient by comparing an unknown strain with the database of unit characteristics of *Streptomyces* species. Tests of 50 major unit characters were done following the method of Williams et al. (1983 a, b).

Determination of identification scores. Identification scores of actinomycete strain S5-55 were determined using TAXON program. The results of 50 unit characters of unknown (*u*) were input into TAXON program and compared with each taxon in turn. Identification scores and the best match taxonomic group were

calculated.

i) Wilcox probability (L_{UJ}) (Wilcox et al., 1973). This is the likelihood of unknown strain (u) against taxon J devided by the sum of the likelihood of u against all q taxa, i.e., $L_{UJ} / \Sigma^q L_{UJ}$. The nearer the score approaches 1.0, the better is the fit of an unknown strain with a group in the matrix.

ii) Taxonomic distance (d). This is given by $\sqrt{[\Sigma(u_i - P_{ij})^2/m]}$. This expresses the distance of an unknown strain from the centroid of the group with which it is being compared ; hence low scores indicate relatedness.

iii) 95% taxon radius. This represents the radius of taxonomic groups including 95% group of taxon J.

iv) % probability of strain further away. This indicates that % of all the cluster groups is represented by strains in cluster groups outside the identified strain.

v) Simple matching coefficient (S_{SM}). This is for use on binary characters only. It is simply the proportion of characters that have the same state (both negative and both positive). This coefficient has the great virtue of intuitive simplicity.

Production of antifungal substances. The antifungal activity of actinomycete strain S5-55 was tested with the 5 media for selection of the suitable medium for antifungal substance production. The five media used were 500 ml of the modified soluble starch broth (SSB, 5 g soluble starch, 10 g glycerol, 4 g yeast extract, 0.2 g KH_2PO_4, and 0.5 g $MgSO_4$), SSB without glycerol, SSB without starch, SSB with 0.3 g K_2HPO_4, and yeast-malt extract broth in a 1L-Erlenmeyer flask. The strain S5-55 was incubated for 15 days at 29°C and antifungal activity of each butanol-extracted culture filtrate was measured against *P. capsici* and *M. grisea* by paper disk method. Actinomycete strain S5-55 identified as *Streptomyces humidus* was precultured in 500 ml of SSB supplemented with 0.3 g K_2HPO_4 (pH 6.8) in a 1L-Erlenmeyer flask on a rotary shaker at 167 rpm for 2 days at 28°C. The 5 ml-aliquot of culture broth was transferred to 500 ml of SSB with 0.3 g of K_2HPO_4 in a 1 L-Erlenmeyer flask. Batch fermentation was done for 14 days on a rotary shaker under the same condition as above preculture. To determine the culture time optimal for antifungal substance production, 500 ml culture broth each was collected every 2 days after inoculation and extracted with *n*-butanol. Each butanol extract was concentrated *in vacuo* and the antifungal activity was monitored against *P. capsici* by paper disk method. Also, packed cell volume (PCV) and pH were recorded.

Isolation of antifungal substances. The culture broth (100 L) was centrifuged at 1,250 g for 30 min, and filtered through Whatman No.2 filter paper. The culture filtrate was extracted with *n*-butanol (100 L). The butanol phase was concentrated *in vacuo* by the rotary evaporator (Büchi, Switzerland). The oily residue was dissolved with 100 ml of methanol. The crude extracts were purified by C_{18} flash column chromatography. The open glass column (150 × 200 mm) was packed with C_{18} resin (C_{18} resin, Lichroprep RP-18, 40-63 μm, Merck, Darmstadt, Germany). The column loaded with crude extracts was eluted with stepwise gradients of methanol and water (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0, v/v). Each fraction (2.5 L) was concentrated *in vacuo* to 10 ml. The antifungal activity of each fraction against *P. capsici* and *M. grisea* was measured by paper disk method.

Results

Isolation of antibiotic-producing actinomycetes. Among the 32 thermophilic actinomycetes which showed antifungal activity against 6 plant pathogenic fungi on V8 agar (Table 1), the G strains from sea-mud inhibited the growth of *A. mali* and *M. grisea*. The strain M1-46 from sea sand also inhibited mycelial growth of tested fungi, except *R. solani*. The K strains from pepper-growing soil were inhibitory to *A. mali*, *M. grisea*, and *P. capsici*. Among the actinomycetes from sea sand (M) soil and phenol-treated sea sand soil (PM), strain PM-24 showed strong antifungal activity against plant pathogenic fungi, but the strain PM-12 did not show antifungal activity. The N strains isolated from paddy soil strongly inhibited the growth of tested fungi, except *F. oxysporum* f.sp *cucumerinum*. The 12 actinomycete strains were selected for further *in vitro* bioassay for antifungal activity of culture filtrates.

***In vitro* bioassay for antifungal activity.** To determine the antifungal activity of selected 12 actinomycetes by paper disk method, each strain was batch-fermented in the modified soluble starch broth. The butanol extract, water layer from culture filtrate, and methanol extract of mycelial mats were concentrated *in vacuo* and bioassayed against 6 plant pathogenic fungi, respectively (Table 2).

Among the tested actinomycetes, the butanol and methanol extracts of the actinomycete strain S5-55 showed a broad spectrum of antifungal activity against *A. mali*, *C. gloeosporioides*, *M. grisea*, *P. capsici*, and *R. solani*. The packed cell volume of the strain S5-55 was 19 ml and the pH was 3.7 after fermentation for 14 days (Table 3). The packed cell volume of strain KP-19 was highest (48 ml), but the antifungal activity of the culture extracts was not so good as the other tested actinomycetes. The packed cell volume of strain N1-278 was lowest, but the culture extracts showed moderate antifungal activity against *M. grisea*, *P. capsici*, and *R. solani*, as compared with those of the other tested actinomycetes. Since the strain S5-55 showed the highest antifungal activity, it was chosen for further large-scale production of antifungal substances.

Generic identification of antagonistic actinomycete strain S5-55. The whole cell hydrolysates of strain S5-55 were developed on the cellulose TLC plate. The major cell wall chemotype was identified to be L-diaminopimelic acid (cell wall chemotype I) as a diagnostic diamino acid of the peptidoglycan. Compared with the standard DAP, ninhydrin-stained band appeared at the same position as a standard L-DAP. The scanning electron microscopic observation showed that the mycelia were well branched and 20-30 spores usually included in the chains of *spirales* (Fig. 1). Individual spores also were well detached from the spore chains and had a smooth surface.

Table 1. Antifungal activity of antagonistic actinomycete isolated from soils of various locations in Kangwon Province, Korea

Actinomycete strain	Inhibition zone length (mm)[a] of					
	A. mali	*C. gloeosporioides*	*F. oxysporum* f. sp *cucumerinum*	*M. grisea*	*P. capsici*	*R. solani*
G1 – 12	12.5 ± 0.7	16.0 ± 2.8	3.5 ± 2.1	18.5 ± 2.5	10.5 ± 1.6	1.0 ± 1.4
G1 – 15	5.0 ± 4.2	0.0 ± 0.0	2.5 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 3.5
G1 – 16	4.0 ± 1.4	3.0 ± 1.4	0.0 ± 0.0	5.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
G4 – 23	2.5 ± 0.7	2.0 ± 1.8	0.0 ± 0.0	9.0 ± 1.4	0.0 ± 0.0	0.0 ± 0.0
G4 – 35	8.5 ± 0.7	7.5 ± 2.1	0.5 ± 0.7	8.5 ± 1.4	1.5 ± 2.1	0.0 ± 0.0
G5 – 15	15.5 ± 0.7	16.0 ± 2.8	1.0 ± 1.4	10.0 ± 4.2	1.5 ± 2.1	17.0 ± 2.8
M1 – 46	9.5 ± 3.5	15.0 ± 5.3	6.0 ± 1.4	16.5 ± 4.9	12.0 ± 1.4	2.0 ± 1.4
M2 – 6	2.0 ± 0.0	3.0 ± 0.0	0.0 ± 0.0	12.0 ± 0.0	6.0 ± 1.4	1.0 ± 1.4
M2 – 146	5.5 ± 2.1	4.5 ± 2.1	0.5 ± 0.7	5.0 ± 0.0	0.5 ± 0.7	0.5 ± 0.7
K1 – 76	9.5 ± 0.7	9.0 ± 0.0	6.0 ± 0.0	12.0 ± 0.0	7.0 ± 0.7	10.5 ± 2.1
K1 – 129	7.5 ± 0.7	4.5 ± 0.7	2.0 ± 2.8	13.5 ± 0.7	5.5 ± 7.8	22.0 ± 1.4
K1 – 145	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.0	3.0 ± 4.2	0.0 ± 0.0
K1 – 196	11.5 ± 2.1	12.5 ± 0.7	6.5 ± 0.7	18.0 ± 2.8	17.0 ± 1.4	9.0 ± 5.7
K1 – 197	14.0 ± 0.0	11.5 ± 0.7	4.0 ± 4.2	9.5 ± 0.7	9.5 ± 0.7	14.0 ± 1.4
K1 – 223	1.0 ± 0.0	0.5 ± 0.7	0.0 ± 0.0	3.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
K1 – 256	12.0 ± 0.0	16.5 ± 0.7	5.0 ± 0.0	15.0 ± 0.0	13.0 ± 2.8	0.0 ± 0.0
N1 – 65	8.0 ± 2.8	13.0 ± 1.4	1.0 ± 0.0	12.0 ± 2.8	12.0 ± 0.0	0.0 ± 0.0
N1 – 251	5.0 ± 0.0	8.5 ± 6.4	0.0 ± 0.0	15.0 ± 0.0	13.5 ± 2.1	0.5 ± 0.7
N1 – 278	20.0 ± 0.0	21.0 ± 0.0	8.0 ± 0.0	21.5 ± 0.7	10.5 ± 0.7	22.0 ± 7.1
N2 – 51	7.0 ± 0.0	8.0 ± 1.4	0.0 ± 0.0	7.5 ± 0.7	8.5 ± 0.7	2.5 ± 0.7
N2 – 232	1.5 ± 0.7	1.0 ± 1.4	0.0 ± 0.0	10.5 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
N2 – 270	8.5 ± 0.7	6.0 ± 0.0	0.0 ± 0.0	10.5 ± 3.5	5.5 ± 0.7	0.0 ± 0.0
S5 – 54	4.0 ± 0.0	6.5 ± 0.7	3.0 ± 0.0	14.5 ± 0.7	8.5 ± 0.7	13.0 ± 4.2
S5 – 55	10.5 ± 0.7	11.5 ± 0.7	1.5 ± 0.7	14.0 ± 1.4	11.0 ± 5.7	1.5 ± 2.1
PM – 12	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
PM – 24	14.0 ± 0.0	18.5 ± 2.1	6.5 ± 0.7	20.0 ± 0.0	11.0 ± 0.0	8.0 ± 0.0
KSS – 16	0.5 ± 0.7	0.0 ± 0.0	1.5 ± 1.4	5.5 ± 3.8	0.0 ± 0.0	0.0 ± 0.0
KSS – 19	4.5 ± 0.7	5.0 ± 1.4	0.0 ± 0.0	6.5 ± 2.1	4.5 ± 0.7	0.0 ± 0.0
KP – 10	9.5 ± 2.1	8.0 ± 2.8	6.0 ± 1.4	11.0 ± 0.0	8.0 ± 0.0	0.0 ± 0.0
KP – 10PS	11.0 ± 2.8	12.5 ± 3.5	0.0 ± 0.0	15.5 ± 3.8	11.5 ± 4.9	0.0 ± 0.0
KP – 12SM	2.0 ± 2.8	2.0 ± 2.8	1.0 ± 1.4	7.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
KP – 19SM	12.0 ± 1.4	16.5 ± 2.1	0.0 ± 0.0	19.0 ± 0.0	15.5 ± 2.1	4.0 ± 1.4

[a]Mycelial disks (7 mm in diameter) were placed on both sides of 30 mm far from streak-inoculation of antagonistic actinomycetes each in the middle of V8 juice agar plates. Inhibition of mycelial growth of each plant pathogenic fungus was rated 5 days after inoculation of the test fungus.

The cultural characteristics of strain S5-55 on various agar media are shown in Table 4. The color of aerial and vegetative mycelia was from light brown to black. The mycelial growth was poor on the trypton-yeast extract agar. The reverse side colors varied on the tested media to be colorless, pale yellow, or light olive gray on trypton-yeast extract agar, ISP 4 agar with glucose, ISP 4 with yeast extract, ISP 5 agar, ISP 8 agar, nutrient agar, and bennett's agar. The strain S5-55 did not produce any soluble pigments in the tested media.

Based on the morphological and cytochemical characteristics, actinomycete strain S5-55 was confirmed to be *Streptomyces* species.

Numerical identification of actinomycete strain S5-55 using TAXON program. The color of spore mass were gray without soluble pigment. The melanoid pigments were not produced on the ISP 6 and ISP 7 media. The strain S5-55 showed antibiosis against *Micrococcus luteus* NCIMB 3610, *Saccharomyces cerevisiae* CBS 1171, and *Streptomyces murinus* ISP 5091. The negative results were found in xanthine and arbutin degradation test. No growth occurred in the media containing each of 7% NaCl, 0.01% NaN_3, 0.1% phenol, and 0.001% thallous acetate. The strain S5-55 was able to utilize not only nitrogen sources such as L-

Table 2. Antifungal activity[a] of culture filtrates of 12 selected antagonistic actinomycetes against six plant pathogenic fungi on the agar medium

Strain	Inhibition zone length (mm) of																	
	Alternaria mali			*Colltotrichum gloeosporioides*			*Fusarium oxysporum* f. sp *cucumerinum*			*Magnaporthe grisea*			*Phytophthora capsici*			*Rhizoctonia solani*		
	B[b]	W	M	B	W	M	B	W	M	B	W	M	B	W	M	B	W	M
G1-12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K1-129	9	0	0	20	0	0	0	0	0	21	0	12	40	0	37	12	0	0
K1-196	9	0	11	25	0	32	0	0	0	12	0	24	11	0	20	10	0	10
K1-256	0	0	8	23	0	30	0	0	0	15	0	11	11	0	14	14	0	10
M1-46	0	0	0	25	0	28	0	0	0	11	0	0	12	0	0	0	0	9
N1-251	0	0	0	9	0	0	11	11	9	11	0	0	12	13	18	17	0	0
N1-278	0	0	0	11	0	0	0	0	0	18	10	14	9	0	19	15	0	0
S5-54	11	0	9	0	0	10	0	0	0	10	0	11	17	11	16	0	0	20
S5-55	18	0	22	26	0	31	0	11	0	30	0	38	13	15	21	28	0	17
KP-10	11	0	0	16	0	0	9	12	0	18	0	0	16	13	10	12	0	0
KP-19	0	0	0	11	0	20	0	13	0	15	0	13	15	24	11	15	0	16
PM-24	11	0	14	11	0	16	0	11	0	5	3	0	12	12	22	0	0	0

[a] Antifungal activity was measured by paper disk method.
[b] B, butanol extracts; W, water extracts; M, mycelial extracts.

Table 3. Packed cell volume and pH of cultures of antagonistic actinomycetes after batch fermentation[a] in soluble starch broth

Actinomycete strain	pH	Packed cell volume (ml)
G1-12	4.3	18
M1-46	5.2	20
K1-129	4.9	19
K1-196	4.3	22
K1-256	4,2	20
N1-251	3.8	21
N1-278	7.3	5
KP-10	3.7	17
KP-19	3.7	48
S5-54	4.0	15
S5-55	3.7	19
PM-24	5.4	12

[a] The 12 actinomycete strain each was inoculated into 1 L Erlenmeyer flask containing 500 ml modified soluble starch broth (5 g starch, 10 g glycerol, 2 g yeast extract, 1 g NH_3SO_4, 0.2 g KH_2PO_4, 0.5 g $MgSO_4$, and 500 ml H_2O) and cultured in a rotary shaking incubator for 14 days at 29°C.

Fig. 1. Scanning electron micrograph of spore chains of actinomycete strain S5-55 cultured on oatmeal agar for 10 days.

valine and L-hydroxyproline, but also carbon sources such as *meso*-inositol, mannitol, L-rhamnose, raffinose, and D-melibiose. There was no growth of the strain S5-55 on sucrose, D-melezitose, adonitol, dextran, and xylitol (Table 5).

Numerical identification of *Streptomyces* sp. strain S5-55 was conducted using TAXON program, based on the data of characters of *Streptomyces* spp. classified systematically by Williams et al. (1983a). The results of *Streptomyces* sp. strain S5-55 obtained from the major 50 unit characters were input to TAXON program in the order of code name to compare with the data of other strains (Table 5). In general, correct identification of an unknown strain requires that the willcox probability approaches 1.0, that taxonomic distance is included within 95% taxon radius and has a low score, and that % probability of strain further away has a high score. Wilcox probability showed that the strain S5-55 might be involved in the taxon major cluster 19 (*Streptomyces diastaticus*) with the value of 0.9982. The value of taxon distance (0.4448) was confirmed to exist within the

Table 4. Cultural characteristics of antagonistic actinomycete strain S5-55 on different media

Medium	Cultural characteristic		Medium	Cultural characteristic	
Trypton-yeast extract agar (ISP 1)	G[a]	Poor	Glycerol-asparagine agar (ISP 5)	G	Moderate
	A	None		A	Moderate, dark brown
	R	Colorless		R	Colorless
	S	None		S	None
Yeast extract malt extract agar (ISP 2)	G	Moderate	Peptone-yeast extract iron agar (ISP 6)	G	Moderate
	A	Moderate, black		A	None
	R	Light brown		R	Yellow
	S	None		S	None
Oatmeal agar (ISP 3)	G	Moderate	Tyrosine agar (ISP 7)	G	Moderate
	A	Moderate, black		A	Poor, ivory
	R	Ivory		R	Light brown
	S	None		S	None
Inorganic salt starch agar (ISP 4)	G	Good	Nitrate agar (ISP 8)	G	Moderate
	A	Abundant, black		A	Moderate, black
	R	Ivory		R	Colorless
	S	None		S	None
ISP 4 with glucose	G	Moderate	Nutrient agar	G	Moderate
	A	Moderate		A	Poor, black
	R	Colorless		R	Colorless
	S	None		S	None
ISP 4 with yeast extract	G	Good	Bennett's agar	G	Good
	A	Poor, black		A	Moderate, black
	R	Colorless		R	Colorless
	S	None		S	None

[a] G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.

value of 95% taxon radius (0.4508). The value of % probability of strain further away (6.7895) was the highest than the other taxon major clusters. The identification scores were also compared with hypothetical median organism (HMO), centrotype (*Streptomyces phaeoviridis*), outer most member (*Streptomyces coralus*), best matched stain (*Streptomyces humidus*) and unidentified strain S5-55 (Table 6). The value of Wilcox probability (0.999785) of best matched strain was higher than that of the centrotype (0.997509). The value of taxonomic distance (0.3356) of best matched strain also existed within the value of 95% taxon radius (0.4508). The value of taxonomic distance was larger than that of the centrotype, but smaller than that of the outer most member. Based on the value of % probability of strain further away, the best match strain (90.5971) was placed near the center of taxon major cluster 19.

Simple matching coefficients (S_{SM}) of the strain S5-55 to the member organisms in *Streptomyces* major cluster 19 were calculated based on 50 unit characters. S_{SM} of the 2 strains such as *Streptomyces minoensis* (S_{SM} value = 86%) and *S. nigellus* (S_{SM} value = 86%) were similar to that of the best matched stain *S. humidus* (S_{SM} value = 84%). Based on the description in Shirling and Gottlieb (1968), however, the spore chain morphology of *S. minoensis* was *retiaculiaperti* on yeast-malt extract agar and oatmeal agar, but open *spirales* on inorganic salt-starch agar and glycerol asparagine agar. The spore chain morphology of *S. nigellus* was *spirals* and the mature chains have 3 to 10 spores on the yeast-malt agar, oatmeal agar, inorganic salt-starch agar, and glycerol asparagine agar. This strain was also able to utilize sucrose and raffinose. The spore chain morphology of *S. humidus* was *spirales* when incubated on yeast-malt extract agar, oatmeal agar, inorganic salt-starch agar, and glycerol asparagine agar. Other morphological characters of the strain S5-55 resembled those of *S. humidus* (Shirling and Gottlieb, 1968b). Therefore, strain S5-55 was designated *Streptomyces humidus* Nakazawa, Shibata, Tanabe, and Yamamoto.

Production and isolation of the antifungal substances. To select the media favorable for antifungal substance production, *Streptomyces humidus* strain S5-55 was incubated in five different media. The inhibitory effects of culture filtrates were measured against the mycelial growth of *P. capsici* and *M. grisea* by paper disc method (Fig. 2). Soluble starch broth supplemented with K_2HPO_4 was capable of producing a large amount of antifungal substances. The

Table 5. Physiological and biochemical characteristics of actinomycete strain S5-55

Characteristic	Code name	S5-55
Spore chain morphology *Rectiflexibles*	RFS	–[a]
Spore chain morphology *Spirales*	SPI	+
Color of spore mass red	RED	–
Color of spore mass gray	GRY	+
Mycelial pigment red-orange	ROS	–
Diffusible pigment produced	PIG	–
Diffuslble pigment yellow-brown	YBP	–
Melanin production on ISP6 medium	MPI	–
Melanin production on ISP7 medium	MTY	–
Antibiosis against:		
Bacillus subtilis NCIMB 3610	SUB	–
Micrococcus luteus NCIMB 196	LUT	+
Candida albicans CBS 562	ALB	–
Saccharomyces cerevisiae CBS 1171	CER	+
Streptomyces murinus ISP 5091	MUR	+
Aspergillus niger	NIG	–
Enzyme activity:		
Lecithinase	LEC	+
Lipolysis	LIP	–
Pectin hydrolysis	PEC	+
Nitrate reduction	NO3	+
H_2S production	H2S	+
Hippurate hydrolysis	HIP	+
Elastin degradation	ELA	+
Xanthine degradation	XAN	+
Arbutin degradation	ARB	–
Growth at 45°C	45C	–
Antibiotic resistance to:		
Penicillin G (10 i.u)	PEN	–
Neomycin (50 μg/mL)	NEO	–
Rifampicin (50 μg/mL)	RIF	+
Oleandomycin (100 μg/mL)	OLE	–
Growth with (%, w/v):		
NaCl (7.0)	7NA	–
NaN_3 (0.01)	01Z	–
Phenol (0.1)	PHN	–
Pottasium tellurite (0.001)	01T	+
Thallous acetate (0.001)	T01	–
Growth on sole nitrogen source (0.1%, w/v):		
DL-α-amino-n-butyric acid	BUT	–
L-Cysteine	CYS	–
L-Valine	VAL	+
L-Phenylalanine	PHE	–
L-Histidine	HIS	–
L-Hydroxyproline	HYD	+
Growth on sole carbon source (1%, w/v):		
Sucrose	SUC	–
meso-Inositol	INO	+
Mannitol	MAN	+
L-Rhamnose	RHA	+
Raffinose	RAF	+
D-Melezitose	MEZ	–
Adonitol	ADO	–
D-Melibiose	MEB	+
Dextran	DEX	–
Xylitol	XYT	–

[a]Symbols '+' and '–' represent positive and negative results, respectively

time course for the production of antifungal substance, PCV, and pH are presented in Fig. 3. The production of antifungal substances by the strain S5-55 started 6 days after incubation, reaching the maximum at 10 days after incubation. Soluble starch medium supplemented with K_2HPO_4 was selected for large-scale production of antifungal substances. Antifungal substances active against plant pathogenic fungi were purified from the culture (100 *l*) of *S. humidus* strain S5-55 using different chromatography procedures. The antifungal activity against *M. grisea*, *P. capsici*, and *R. solani* was found in the crude butanol extracts of culture broth. The 40% methanol eluates showing antifungal activity were obtained from C_{18} flash column chromatography of the butanol extracts (Fig. 4)

Discussion

Actinomycetes are producers of various bioactivie compounds including antifungal antibiotics. Among the antibiotic-producing microorganisms, actinomycetes produced over two-third of the naturally occurring antibiotics (Tanaka and Omura, 1990). The most common actinomycetes in soils are the *Streptomyces* sp. Various physical and chemical methods were developed to selectively isolate antifungal substance-producing actinomycetes from soils (Porter et al., 1960; Hayakawa and Nonomura, 1987; Hayakawa et al., 1988). To enhance the isolation rate of rare actinomycetes, the soil samples air-dried at room temperature were heated at 100°C before inoculation of soil suspension on the humic acid vitamin agar plates, which may result in the isolation of thermophilic actinomycetes by killing the eubacteria and heat-labile *Streptomyces* sp., consequently, humic acid-vitamin agar seemed to be appropriate for selective isolation of *Actinomadura, Dactylosporangium, Micromonospora, Microbispora*, and *Streptosporangium*.

The antifungal activity of each antibiotic-producing actinomycete was measured by the dual culture against 6 plant pathogenic fungi, because the actinomycetes that did not show the antifungal activity *in vitro* might not have an antifungal ability in soils. The strain S5-55 which showed a high antifungal activity against 6 tested fungi among the 32 thermophilic actinomycetes was screened for a large scale production of antifungal substances.

The identification of actinomycetes is in general performed, based on the morphological, physiological and biochemical characteristics. The compositions of cell wall amino acids and whole cell sugars have been known to be useful for the chemotaxonomical identification. The cell wall composition of strain S5-55 was confirmed to be LL-diaminopimelic acid (Fig. 2). The genera *Arachinia, Pemelobacter, Nocardioides, Intrasporangium, Kineospo-*

Table 6. Identification of the taxon major cluster of *Streptomyces* sp. strain S5-55 using taxon program

Taxon major cluster	Taxonomic distance[a]	95% Taxon radius[b]	% Probability of strain further away[c]	Willcox probability[d]
19 (*Streptomyces diastaticus*)[e]	0.4448	0.4508	6.7895	0.998244
21 (*S. griseoruber*)	0.4713	0.3709	0.0001	0.000043
20 (*S. olivaceoviridis*)	0.5035	0.3720	0.0000	0.000008
23 (*S. microflavus*)	0.5052	0.3931	0.0000	0.000001
6 (*S. violaceus*)	0.5070	0.4126	0.0009	0.000003
31 (*S. antibioticus*)	0.5204	0.4131	0.0002	0.000001
12 (*S. rochei*)	0.5219	0.4173	0.0003	0.001397
32 (*S. violaceoniger*)	0.5235	0.3854	0.0000	0.000000
18 (*S. cyaneus*)	0.5278	0.4497	0.0127	0.000274
1C (*S. albidoflavus*)	0.5339	0.3883	0.0000	0.000026
37 (*S. griseoflavus*)	0.5406	0.3658	0.0000	0.000000
17 (*S. griseoviridis*)	0.5433	0.3943	0.0000	0.000000
1B (*S. alvidoflavus*)	0.5445	0.4404	0.0006	0.000001
40 (*S. phaechromogenes*)	0.5486	0.3805	0.0000	0.000000
29 (*S. lydicus*)	0.5489	0.3831	0.0000	0.000000
15 (*S. chromofuscus*)	0.5527	0.4271	0.0000	0.000000
5 (*S. exfoliatus*)	0.5545	0.4455	0.0004	0.000000
3 (*S. atroolivaceus*)	0.5620	0.3631	0.0000	0.000000
42 (*S. rimosus*)	0.5644	0.3507	0.0000	0.000000
30 (*S. filipinensis*)	0.5663	0.3845	0.0000	0.000000
33 (*S. chromogenus*)	0.5769	0.3955	0.0000	0.000000
10 (*S. fulvissimus*)	0.5960	0.4036	0.0000	0.000000
61 (*S. lavendulae*)	0.6111	0.4118	0.0000	0.000000
1A (*S. albidoflavus*)	0.6163	0.3782	0.0000	0.000000
16 (*S. albus*)	0.6247	0.3347	0.0000	0.000000
65 (*Kitasatoa* spp.)	0.6327	0.3374	0.0000	0.000000

[a] The distance of an unknown strain from the centroid of the group which is being compared.
[b] Taxon radius which contains 95% strains of taxon.
[c] The % of strains which exist outside the identified, unknown strain in taxon.
[d] This is the likehood of unknown strain against taxon J devided by the sum of the likehood of unknown strain (u) against all q taxa. The nearer the score approaches 1.0, the better is the fit of unknown strain with a group in the matrix.
[e] *Streptomyces* spp. in parenthesis are representative species belonging to the taxon major cluster of *Streptomyces*.

Table 7. Identification of hypothetical median organism (HMO)[a], centrotype, outer-most member, best matched *Streptomyces* strain, and *Streptomyces* sp. strain S5-55 based on the major cluster 19 of *Streptomyces* using TAXON program

Cluster 19 member	Taxonomic distance	95% Taxon radius	% Probability of strain further away	Wilcox probability
HMO	0.2658	0.4508	99.9072	0.997032
Centrotype : *Sterptomyces phaeoviridis*	0.3296	0.4508	92.9354	0.997509
Outer most member : *Streptomyces coralus*	0.4295	0.3931	0.3293	0.022383
Best matched strain : *Streptomyces humidus*	0.3356	0.4508	90.5971	0.999785
Unidentified strain S5-55	0.4448	0.4508	6.7895	0.998244

[a] HMO which has the best identification scores achievable by an entirely typical example of each group in an identification matrix.

ra, *Streptomyces*, and *Streptoverticillium* possess cell wall chemotype of LL-DAP (Williams et al., 1989). The morphology of spore and various cultural and physiological characteristics were the most important factors for the identification of actinomycetes. In general, the spore surface type of *Streptomyces* spp. was classified into 4 groups in terms of smooth, warty, spiny, or hairly (Tresner et al., 1961). The spore surface of the actinomycete strain S5-55 was found to be smooth (Fig. 3). However, the morphological and cultural characteristics confirmed that the strain S5-55 belongs to the genus *Streptomyces*.

The numerical identification using a wide range of char-

Table 8. Simple matching coefficients (S_{SM})[a] of actinomycete strain S5-55 to the member organisms in *Streptomyces* major cluster 19 based on 50 unit characters

Cluster 19 member	ISP No.	ATCC No.	S_{SM} (%)
Streptomyces humidus	5263	12760	84
S. minoensis	5031	19787	86
S. nigellus	5490	27450	86
S. olivochromogenes	5451	3336	77
S. glomeraurantiacus	5429	19839	72
S. rishiriensis	5489	14812	72
S. vastus	5309	25506	66
Streptomyces sp.	F1		66
S. diastaticus	5496	3315	61
S. flavus	M315	15332	56
S. bottropensis	5262	25435	56
S. coralus	5256	23901	56
S. mirabilis	5553	27447	56
S. phaeoviridis	5285	23947	56
S. tauricus	5560	27470	56
M. cinerea	M301	15840	50
S. galilaeus	5481	14969	50
S. achromogenes	5028	12767	44
S. diastatochromogenes	5449	2309	44

[a]The proportion of phenotype coincidence between the unidentified strain S5-55 and the strains in cluster 19, which includes both positive and negative matches.

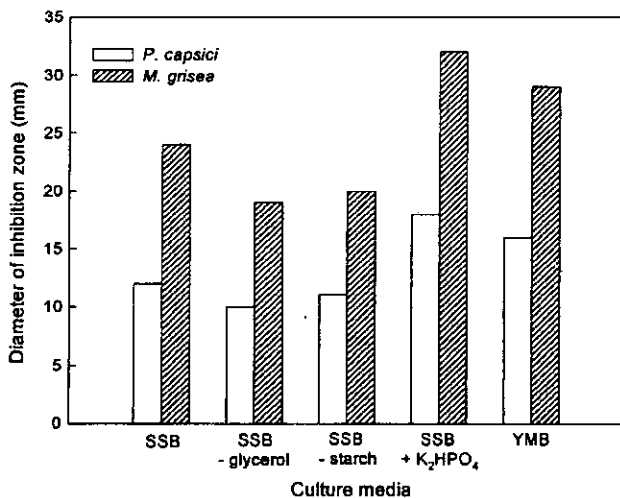

Fig. 2. Inhibitory effects of butanol-extracted culture filtrates of *Streptomyces humidus* strain S5-55 against mycelial growth of *Phytophthora capsici* and *Magnaporthe grisea*. SSB and YMB represent soluble starch broth and yeast-malt extract broth, respectively.

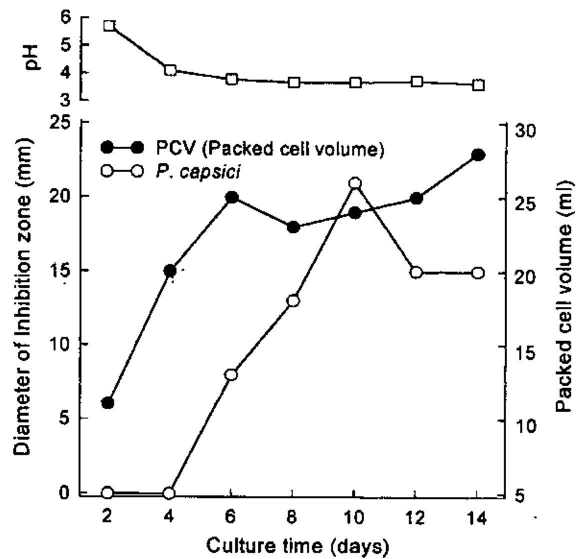

Fig. 3. Time course for production of antifungal substances from *Streptomyces humidus* strain S5-55 cultured in 500 ml-soluble starch broth with 0.3 g K_2HPO_4 in a 1L-Erlenmeyer flask. The 2.5 ml-culture extracts were bioassayed for antifungal activity against *Phytophthora capsici* by paper disk method.

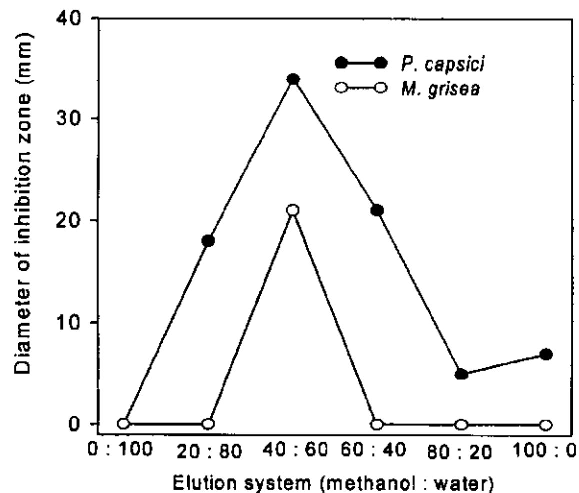

Fig. 4. C_{18} reversed-phase flash chromatogram of the crude culture extracts from *Streptomyces humidus* strain S5-55. The antifungal activity of each fraction was tested by paper disk method against *Phytophthora capsici* and *Magnaporthe grisea*.

acteristics was performed in the genus *Streptomyces* and some related genera taxa (Williams et al., 1983a, b). Twenty-two major clusters (19 *Streptomyces*, 2 *Streptoverticillium*, and one *Norcardia mediterranea*) and one minor cluster (*Streptomyces fradiae*) were included in TAXON program. The numerical identification of the strain S5-55 was performed, based on the probabilistic identification matrix. The Wilcox probability of the strain S5-55 to the cluster 19 was 0.9982. The taxonomic distance and the value of % probability of strain further away indicated that strain S5-55 was in the cluster 19 and the best matched strain was *Streptomyces humidus* with the S_{SM} value of 84%. The S_{SM} value of *S. minoensis* and *S. nigellus* in the cluster 19 was higher than that of the best matched strain *S. humidus* (data not shown). The *Streptomyces* strains in

TAXON program were described in International *Streptomyces* Project (ISP) made by Shirling and Gottlieb (1966, 1968a and b, 1969). The morphological and cultural characteristics of 3 strains were compared with the description in ISP. Based on this ISP characterization, actinomycete strain S5-55 was confirmed to be *S. humidus*.

The secondary metabolites such as antibiotics were produced, when the cell reached its idiophase during the fermentation process (Bu'Lock et al., 1965). Carbon and nitrogen sources, and aeration should be considered for the culture of microorganisms. In general, the biomass of the cell, production of CO_2, amounts of products (e.g. secondary metabolites), etc. have been evaluated during the fermentation process. During the culture process of strain S5-55, packed cell volume for the biomass of the cell and antifungal activity of culture filtrates against plant pathogenic fungi were examined to confirm the production of antifungal metabolites. During the broth culture, the relationship between the antibiotics production and the morphology of *Streptomyces* spp. was well examined following the method of Pickup et al. (1993). When the mycelial fragmentation by physical damage, e.g., shaking, occurred in broth culture, antibiotic production was usually inhibited. The production of antifungal secondary metabolites was measured by the diameter of inhibition zone using paper disk method (Fig. 4). The mycelial fragmentation did not occur during the culture process. Among the tested media, SSB with K_2HPO_4 was found to be suitable for a large-scale production of antifungal substances. Growth of the strain S5-55 reached the idiophase after incubation for 10 days (Fig. 3).

The antifungal substances active against *P. capsici* was partially purified from the culture filtrates of *S. humidus* strain S5-55 using C_{18} reversed-phase flash column chromatography. Chemical structures of the antifungal substances are being identified. The potential of the compounds in the control of plant diseases should also be further evaluated.

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