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Isolation and Antifungal and Antioomycete Activity of *Streptomyces scabiei* Strain PK-A41, the Causal Agent of Common Scab Disease

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The actinomycete strain PK-A41 was isolated from a soil sample from pepper fields in Ko-yang, Korea. The strain PK-A41 inhibited the mycelial growth of some plant pathogenic fungi and oomycete, *Alternaria mali, Colletotrichum orbiculare, Fusarium oxysporum* f.sp. *lycopersici, Magnaporthe grisea, Rhizoctonia solani,* and *Phytophthora capsici.* The presence of LL-diaminopimelic acid in the cell wall extract and the nucleotide sequence of the 16S rDNA region of the strain PK-A41 was assigned to *Streptomyces scabiei.* Further morphological, biochemical, and pathological analyses also confirmed the strain PK-A41 to be *S. scabiei,* which is pathogenic to potato tubers. The maximum antibiotic production of the strain PK-A41 was achieved when grown on the glycerol peptone broth (GPB) medium for 9 days.

Keywords **:** *Streptomyces scabiei*, antibiotic activity, identification, culture condition

Actinomycetes are antibiotic producers that are capable of generating 75% of all known antibiotic products. The actinomycetes are a group of filamentous, gram-positive bacteria with a high G+C content in their DNA (Williams et al., 1993). These organisms are aerobic, saprophytic, and mesophilic forms whose natural habitat is the soil. Several members of the actinomycetes are known to be producers of important secondary metabolites, including antibiotics, herbicides, and growth-promoting substances (Connell, 2001). In particular, *Streptomyces* is a major producer of antibiotics. Since the discovery of streptomycin (Schatz et al., 1944), a large number of antibiotics have been isolated from *Streptomyces* and *Streptoverticillium* cultures. Over 55% of antibiotics have been isolated from the genus *Streptomyces* (Embley, 1994), and more than 400 species of the genus *Streptomyces* are mostly soil saprophytes. Among them, only a small number of *Streptomyces* species are known to infect plant tissues. Plant pathogenic *Streptomyces* infect a wide range of crop species and cause diseases on the plant underground structures (Loria et al., 1997).

The most studied pathogenic *Streptomyces* species is *S. scabiei* (formerly, *S. scabies*), which causes not only common scab in potatoes, but also similar symptoms on other plant species. *S. scabiei* is distributed worldwide and grows saprophytically or on the roots of various vegetables and weeds in the soil (KenKnight, 1941; Hooker and Kent, 1946). As a major causal agent of the common scab disease on potatoes in dry, neutral to alkaline soils, *S. scabiei* was first isolated in 1890 and called *Oospora scabies* (Thaxter, 1891). After Güssow (1914) renamed the strain *Actinomyces scabies*, the strain was named *Streptomyces scabies* by Waksman and Henrici (1943). Based on the pathogenicity, Waksman and Henrici (1948) recommended the strain IMRU 3018 (= ISP 5078) as a neotype. In the 1960s, the strain IMRU 3018 represented *S. scabies* in the International *Streptomyces* Project (ISP), but lacks the spiral spore chains and melanin production. A number of "*S. scabies*" reference strains were subsequently isolated from the potato. Although *S. scabiei* belongs to one of the first isolated Streptomycetes, its taxonomy had been in a chaotic state for long time. This is because the lack of correlation between the plant pathogenicity and taxonomy. They could be listed and considered to be *incertae sedis* (type strain does not exist, but many taxonomically different strains are available). The name, *S. scabies* (*S. scabiei*), was redefined by Lambert and Loria (1989). *S. scabies* is characterized as a melanin producer with smooth grey spores in spiral chains. *S. scabies* uses L-arabinose, D-fructose, D-glucose, D-mannitol, rhamnose, sucrose, D-xylose, and raffinose as the nutrient sources, it is sensitive to streptomycin and does not grow at < pH 4.5. More recently, Trüper and De'Clari (1997) proposed that the classification of *S. scabies* be corrected to *S. scabiei* based on rule 12c of the International Code of Nomenclature of Bacteria.

Infections by the potato common scab pathogen, *S. scabiei*, reduce the yield, degrade the market quality, and

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increase the waste in peeling. The symptoms of the common scab are small, brownish, raised spots on the young potato tubers. These spots gradually develop and later enlarge, darken and become corky. Superficial lesions, deep cracking and pitting appear on the surface of potatoes. Sometimes, symptoms cover almost the entire tuber surface. The pathogen spread through soil water, by wind-blown soil, splashing rain, farm machinery, tools, and on infected potato seed tubers. Generally, the pathogen remains indefinitely once the soil becomes infested. The severity of the common scab of potato increases as the pH of the soil increases. Scab disease causes most damage on potatoes in slightly acid to alkaline soils of pH 5.5 to 7.5. Accordingly, scab control can be achieved by lowering the soil pH through the use of acid-producing fertilizers or the application of sulfur at pH 4.8 to 5.2 (Loria et al., 1997). *S. scabiei* can grow with an optimum of 25°C to 30°C, which is higher than optimal potato-growing temperature (Bouchek-Mechiche et al., 2000). The scab severity can increase by maintaining the soil temperature higher than a normal potato season. The incidence of potato scab is reduced by high soil moisture during the period of tuber initiation, so that high soil moisture can lead to the use of irrigation for disease control (Davis et al., 1976). The incidence of scab disease is lower in fields after crop rotation and plowing of certain green manure crops. The best control is accomplished by the use of scab-resistant potato cultivars or by treating the seeds with either pentachloronitrobenzene (PCNB) or maneb-zinc dust (Wilson et al., 1999). However, many of the preferable cultivated potato cultivars lack resistance. Biological measures have shown potential for the control of potato scab (Neeno-Eckwall et al., 2001). Treatment with nonpathogenic, antibiotic-producing streptomycetes has been shown to reduce the incidence of potato scab. This suppression was achieved by their ability to colonize on the potato tuber (Neeno-Eckwall et al., 2001).

The actinomycete strain PK-A41 that showed antifungal and anti-oomycete activity against various plant pathogenic fungi was isolated from a soil sample from pepper fields in Ko-yang, Korea (Lee and Hwang, 2002). During the screening procedure for potent antifungal metabolites against some plant pathogenic fungi, the methanol extract from the strain PK-A41 exhibited antifungal and anti-oomycete activity against various plant pathogenic fungi and oomycetes. Based on the methods reported by Waksman (1961), Shirling and Gottlieb (1966), Lechevalier and Lechevalier (1970), and Holt et al. (1994) in addition to Bergey's manual of Systematic Bacteriology (Williams et al., 1989), the strain PK-A41 was identified to be *Streptomyces scabiei*. This paper describes the isolation and the characteristics of the strain PK-A41 as well as the optimal cultural conditions for producing antifungal compounds from its culture filtrates.

Materials and Methods

Isolation of antibiotic-producing actinomycete strain PK-A41. The actinomycete strain PK-A41 was isolated from a soil sample from pepper fields in Ko-yang, Korea (Lee and Hwang, 2002). The antifungal and anti-oomycete activity of the strain PK-A41 was evaluated against various plant pathogens, *Alternaria mali, Colletotrichum orbiculare, Fusarium oxysporum* f.sp. *lycopersici, Magnaporthe grisea, Rhizoctonia solani*, and *Phytophthora capsici* on V8 agar (20% V8 juice, 18 g agar, and 1 L distilled water) plates. The strain PK-A41 was grown at 28°C on yeast-malt extract agar (YMA) (10 g malt extract, 4 g yeast extract, 4 g glucose, 18 g agar, and 1 L distilled water). In addition, the strain PK-A41 also was grown in yeast-malt extract broth (YMB) (10 g malt extract, 4 g yeast extract, 4 g glucose, and 1 L distilled water). The spore suspensions were preserved in 15% glycerol at -70°C.

Identification of strain PK-A41. The taxonomical studies used to identify the strain PK-A41 were performed according to the methods suggested by Waksman (1961), Shirling and Gottlieb (1966), Lechevalier and Lechevalier (1970), Holt et al. (1994), and the Bergeys Manual of Systematic Bacteriology (Williams et al., 1989).

Analysis of 16S rDNA gene sequence of strain PK-A41. For genomic DNA extraction, a single colony of strain PK-A41 on the YMA plate was sub-cultured in 5 ml YMB at 28°C for 2 days. Genomic DNA was isolated from a PK-A41 culture using a slight modification of the method reported by Pospiech and Neumann (1995). The isolated genomic DNA (ca. 100 ng) was used as the template DNA for the PCR amplification of 16S rDNA of the strain PK-A41 using the universal primers fD1 (AGAGT TTGAT CCTGG CTCAG) and rP2 (ACGGC TACCT TGTTA CGACTT) (Weisburg et al., 1991). The amplified PCR product was purified from 0.8% agarose gel using the method of Heery et al. (1990), and then was ligated into the pCR®2.1-TOPO® T vector (Invitrogen™ Co., Carlsbad, CA, USA) according to the manufacturers instruction. Plasmids containing the PCR product were isolated using Wizard® plus SV Minipreps DNA Purification system (Promega, Madison, WI, USA). Nucleotide sequence of the cloned PCR product was determined by an AB1310 DNA sequencer (Applied Biosystems) and analyzed using the NCBI BLAST search (http://www.ncbi.nlm.nih.gov). Alignment of nucleotide sequences was done using a cluster method of the DNASTAR software program (DNASTAR Inc., Madison, WI, USA). In order to determine the genetic relationship between these strains, a phylogenetic tree was generated based on the percentage difference between the sequences.

Chemotaxonomic characteristic of strain PK-A41. The whole cell wall hydrolysate was analyzed using the methods of Becker et al. (1964). The hydrolysates were spotted on a cellulose-coated TLC aluminum sheet (Cellulose, 0.1 mm thick, Merck). For the comparisons, the hydrolysates of the cell wall, a standard diaminopimelic acid (DAP) solution (1% DAP in water, w/v), and standard glycine (1% glycine in water, w/v) also were spotted on the same TLC sheet. The sheet was developed in a solvent system (methanol-pyridine-H_2O-10 N HCl = 80:20:26:2.5, v/v/v/v) (Becker et al., 1965) and stained with a ninhydrin solution (0.1%

ninhydrin in acetone, w/v), which was followed by air-drying and heating at 100°C for 2 min.

The Guanine plus Cytosine (GC) content in the deoxyribonucleic acid of PK-A41 were using the thermal melting methods of Marmur and Doty (1962), and Mandel et al. (1970), and Johnson (1985). The melting temperature was measured with a UV/Visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech, UK). The mean values ± standard deviations were from three determinations. The G+C content was calculated using a formula: G + C% = 2.08(Tm-69.4/x); x=specific to each batch of 0.1X SSC buffer.

Morphological observation of strain PK-A41. The spore chain morphology, spore size, spore mass, and spore surface ornamentation of the strain PK-A41 were determined by scanning electron microscopy (SEM). The organism was cultured on inorganic salts-starch agar (ISP 4, 10 g soluble starch, 2 g $CaCO_3$, 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 20 g agar, and 1 L distilled water) for 7 days (cross-hatched inoculation). The gold-coated samples of the agar blocks were viewed under a Philips SEM515 (Eindhoven, Netherlands) with an accelerated voltage of 20 kV.

Cultural characteristics of strain PK-A41. Cultural characteristics of PK-A41 was examined using the method of the International Streptomyces Project (ISP) that was described by Shirling and Gottlieb (1966), Waksman (1961), and Locci (1989). Various ISP media used for analyses of the cultural characteristics were yeast extract-malt extract agar (ISP 2, 4 g yeast extract, 10 g malt extract, 4 g glucose, 20 g agar, and 1 L distilled water), oatmeal agar [ISP 3, 20 g oatmeal, 18 g agar, 1 ml trace salts solution (0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$ per 100 ml distilled water), and 1 L distilled water], inorganic salts-starch agar (ISP 4, 10 g soluble starch, 2 g $CaCO_3$, 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 20 g agar, and 1 L distilled water), glycerol-asparagine agar (ISP 5, 10 g glycerol, 1 g L-asparagine, 1 g K_2HPO_4, 20 g agar, 1 ml trace salts solution, and 1 L distilled water), peptone-yeast extract iron agar (ISP 6, 15 g peptone, 5 g proteose peptone, 1 g K_2HPO_4, 1 g yeast extract, 0.5 g ferric ammonium citrate, 0.08 g sodium thiosulfate ($Na_2S_2O_3$), 15 g agar, and 1 L distilled water), tyrosine agar (ISP 7, 15 g glycerol, 0.5 g L-tyrosine, 1 g L-asparagine, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 20 g agar, 1 ml trace salts solution, and 1L distilled water), and Bennetts agar (10 g glucose, 2 g pancreatic digest of casein, 1 g yeast extract, 1 g beef extract, 15 g agar, and 1L distilled water). Strain PK-A41 was inoculated with a cross-hatched pattern on each plate. Mycelial growth (G), the properties of the aerial mycelium (AM), the properties of the substrate mycelium (SM), the production of soluble pigment (SP), and the reverse side color (R) were observed 7 days after incubation.

Physiological and biochemical characterization of strain PK-A41. The physiological and biochemical characteristics of strain PK-A41 were tested according to the Manuals suggested by Williams et al. (1983a, 1983b). The results were analyzed based on the data reported by Williams et al. (1983a).

The pigments secreted from the mycelium and diffusible pigments were observed through the reverse side of the inoculated ISP 5 medium. The pigment production was recorded as a positive reaction. Melanin pigment production was tested on the ISP 6 and 7 media. Degradation activities of strain PK-A41 were observed on various media containing arbutin, aesculine, casein, starch, gelatin, xanthine, and tyrosine. The extent of PK-A41 growth was determined at different temperatures, pH, and NaCl.

Strain PK-A41 was examined for its ability to utilize nitrogen (DL-α-amino-n-butyric acid, L-cysteine, L-histidine, L-hydroxyproline, L-phenylalanine, and L-valine) (0.1%, w/v) and carbon (adonitol, arabinose, dextran, fructose, *meso*-inositol, mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, sucrose, xylitol, and xylose) (1%, w/v) source. L-asparagine and D-glucose was added as positive controls for N and C, respectively.

The strain PK-A41 was examined for its resistant activities against four antibiotics, neomycin, oleomycin, penicillin G, and rifampicin. The filter paper soaked with the antibiotic solutions was placed on the basal medium inoculated with PK-A41. The inhibitory effect against six test microorganisms, *Aspergillus niger*, *Bacillus subtilis* NCIMB 3610, *Candida albicans* CBS562, *Micrococcus luteus* NCIMB 196, *Saccharomyces cerevisiae* CBS 1171, and *Streptomyces murinus* ISP 5091 was tested. The inhibition zone was recorded as a positive result.

Pathogenicity assay. A pathogenicity assay of *S. scabiei* strain PK-A41 was performed following the method of Loria et al. (1995). Immature potato tubers, cv. Daejima, were peeled and sterilized in 0.05% NaOCl containing 0.1% $CaCO_3$ for 3 min, and rinsed twice in sterile H_2O. The tubers were punched with a sterile cork borer (3 cm in diameter), and sliced into large disks (0.5 cm in thickness) with a sterile knife. Pieces of the potato tubers were sterilized and rinsed as described above, and placed on a moist sterile filter paper in a sterile petri dishes (9 cm in diameter). The tested strains were grown on oatmeal agar (OMA) for 7 days at 28 °C. The agar blocks (0.3 cm in diameter) from the sporulating colonies were punched with a cork borer and inverted onto the tuber disks. *S. scabies* ATCC 49173 and *S. acidiscabies* ATCC 49003 were included as controls. The inoculated disks were incubated in a moist dew chamber at 26°C for 4 days in the dark. Necrosis and the collapse of the tuber cells were observed from the tuber disks inoculated. Three replicated tuber disks per treatment were used.

Optimization of culture conditions for antibiotic production. In order to select the medium and culture time favorable for the antibiotic production from the *S. scabiei* strain, PK-A41, four media containing different carbon and nitrogen sources were tested. The media tested were glycerol dextrin broth (GDB) (20 g glycerol, 20 g dextrin, 10 g soytone, 3 g yeast extract, 2 g $(NH_4)_2SO_4$, 4 g K_2HPO_4, and 1 L distilled water) (Igarashi et al., 1997), glycerol peptone broth (GPB) (20 g glycerol, 10 g polypeptone, 5 g beef extract, and 1 L distilled water) (Komaki et al., 1999), starch casein broth (SCB) (20 g soluble starch, 0.6 g tryptone peptone, 4 g K_2HPO_4, 4 g KNO_3, 4 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.04 g $CaCO_3$, and 1 L distilled water) (Sugawara et al., 1997), and starch glucose broth (SGB) (20 g soluble starch, 10 g glucose, 5 g yeast extract, 5 g casamino acid, and 1 L distilled water) (Momose et al., 1998).

A pre-cultured broth of strain PK-A41 was incubated in YMB on a rotary shaker, and 2 ml of the pre-cultured broth was transferred into the 200 ml of each medium in a 1 L Erlenmeyer flask. Changes in the pH, dried cell weight, and antibiotic production were examined after incubation in each medium at 28°C for 2-14 days. Antibiotic production from the culture filtrates (200 ml) of the strain PK-A41 in each medium was monitored at various time intervals after incubation, based on an evaluation of the antifungal activity against plant pathogens.

Each of the 4 culture broths (200 ml) was centrifuged at 30,000 g for 15 min to remove the mycelial mats every two days after inoculation. The supernatant was loaded on an open column packed with Diaion HP-20 resion. The column was washed with 300 ml H_2O and eluted with 100 ml methanol. The methanol eluent was concentrated *in vacuo* by a rotary evaporator at 40°C. The antifungal and antioomycete activity of the eluent was evaluated against *C. orbiculare, M. grisea,* and *P. capsici* using a paper disk assay method.

Results

Isolation of antibiotic-producing actinomycete strain PK-A41.

The actinomycete strain, PK-A41, isolated from a soil sample from pepper fields in Ko-yang, Korea inhibited the mycelial growth of *A. mali, C. orbiculare, F. oxysporum* f.sp. *lycopersici, M. grisea, R. solani,* and *P. capsici on* V8 agar (Fig. 1).

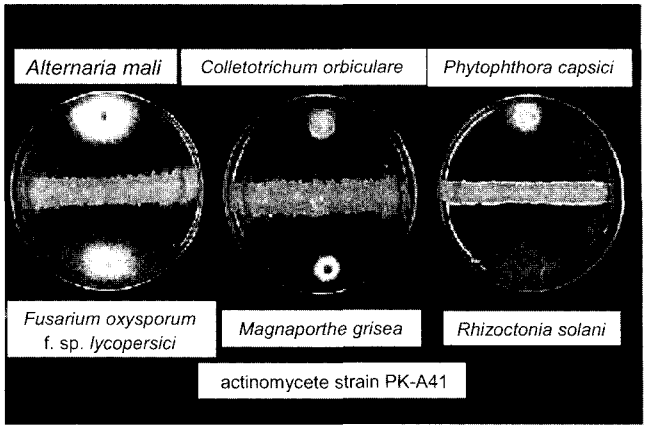

Fig. 1. Inhibitory activities of strain PK-A41 against six plant pathogenic fungi and oomycetes.

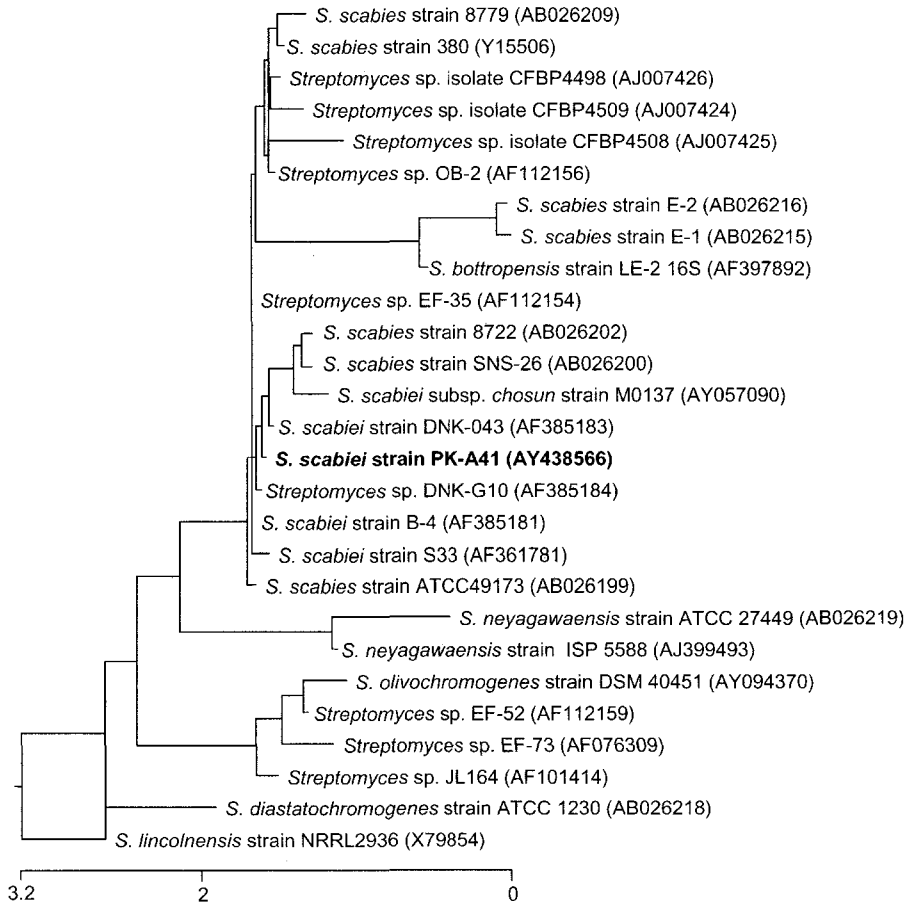

Fig. 2. Phylogenetic tree of strain PK-A41 and 26 *Streptomyces* strains based on the16S rDNA sequence data. The length of each pair of branches represents the distance between the sequence pair, while the units of the bottom of the tree indicate the number of substitution events.

Analysis of 16S rDNA gene sequence of strain PK-A41. The 16S rDNA region of PK-A41 was sequenced and aligned with the streptomycete nucleotide sequences derived from GenBank of the BLAST network services at the NCBI. The Megalign program was used to determine the generic relationship between strain PK-A41 and its neighborhood strains, and a phylogenetic tree was generated based on the percentage difference between the sequences (Fig. 2). The neighborhood strains of the strain PK-A41 in the phylogenetic tree were clustered in the groups of *S. scabiei* and *Streptomyces* sp.. These results indicate that strain PK-A41 belongs to the *Streptomyces scabiei*.

Chemotaxonomic characteristic of strain PK-A41. Compared to the standard DAP, the DAP in the cell wall extract of strain PK-A41 was LL-diaminopimelic acid (Fig. 3). The melting temperature and G+C content were 87.6°C and 67.4%, respectively (data not shown).

Morphological observation of strain PK-A41. The strain PK-A41 formed an aerial mycelium on various ISP media. Spore chain and surface morphology of strain PK-A41 was determined by scanning electron microscopy (Fig. 4). The spore chain had the *rectiflexibles* and *spirales* type and the spore surfaces were smooth.

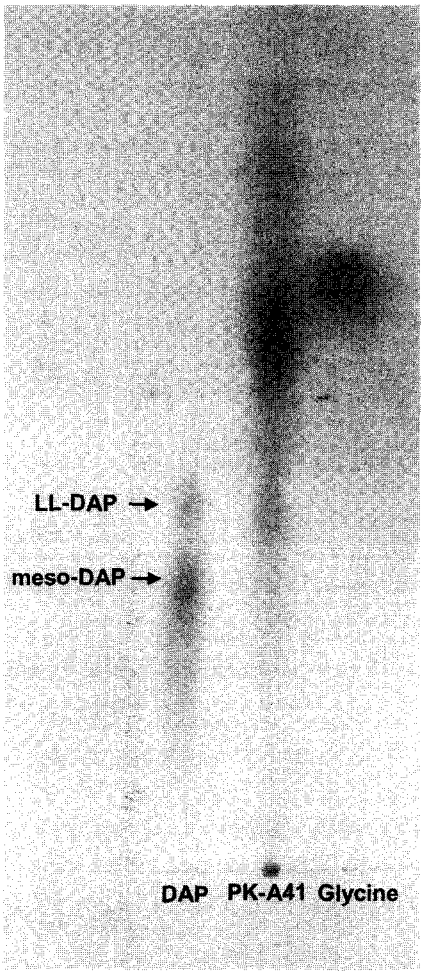

Fig. 3. Thin layer chromatogram of the cell wall hydrolysates of strain PK-A41. DAP : diaminopimelic acid.

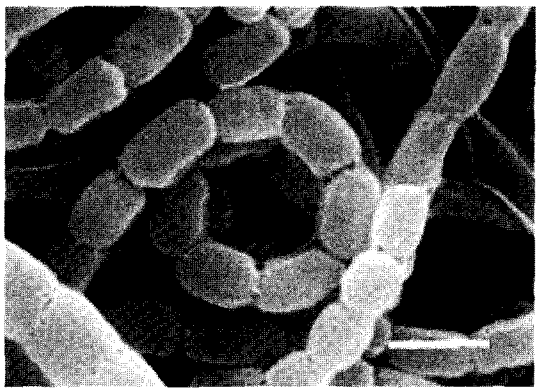

Fig. 4. Scanning electron micrograph of the mycelia and spores of strain PK-A41 incubated in inorganic salt starch agar. Bar = 1 μm.

Cultural characteristics of strain PK-A41. The cultural characteristics of the strain PK-A41 and *S. scabies* ATCC 49173 on the seven media tested are presented in Table 1. The aerial mycelium, substrate mycelium, and reverse side of the plate color were observed. The aerial mycelium colors were almost white to gray on the tested media. The substrate mycelium colors were brown and creamy. The strain PK-A41 produced soluble pigments on the ISP 2, 6 media, and the Bennetts agar, but not on the ISP 3, 4, 5, and 7 media. The growth was good on the tested media, except for the ISP 5 medium.

Physiological and biochemical characteristics of strain PK-A41. The physiological and biochemical properties of strain PK-A41 are shown in Table 2. The strain PK-A41 did not produce diffusible pigments on the ISP 5 medium. Melanin production on the ISP 6 medium was observed, but not on the ISP 7 medium. The strain PK-A41 could degrade the arbutin, esculin, starch, tyrosine, and xanthine, but did not degrade casein and gelatin. The strain PK-A41 did not grow at 4 and 45°C, and in the medium containing 9% NaCl. In addition, strain PK-A41 grew well in pH 8. The strain PK-A41 was evaluated for its ability to utilize nitrogen and carbon sources. The strain PK-A41 was able to utilize DL-α-amino-n-butyric acid, L-histidine, L-hydroxyproline, L-phenylalanine, and L-valine as nitrogen sources, but not L-cysteine. Adonitol, arabinose, dextran, fructose, *meso*-inositol, D-melibiose, sucrose, xylitol, and xylose were utilized as the carbon sources, but mannitol, D-melezitose, raffinose, and L-rhamnose were not. The strain PK-A41 was sensitive to the antibiotics, neomycin, oleomycin, penicillin G, and rifampicin. The inhibitory effect of strain PK-A41 was examined against six

Table 1. Cultural characteristics of the actinomycete strain PK-A41 and *Streptomyces scabies* ATCC 49173 on the different media

Medium		Characteristic	
		Strain PK-A41	*Streptomyces scabies* ATCC 49173
	AM[a]	White to gray	Gray
ISP 2 medium	SM	Dark brown	Dark brown
(Yeast-malt extract agar)	SP	Light brown	Light brown
	R	Dark brown	Dark brown
	G	Good	Good
	AM	Gray	White to gray
ISP 3 medium	SM	Creamy	Creamy
(Oatmeal agar)	SP	None	None
	R	Creamy	Creamy
	G	Good	Good
	AM	White to gray	White to gray
ISP 4 medium	SM	Creamy	Creamy
(Inorganic salt-starch agar)	SP	None	None
	R	White to gray	White to gray
	G	Good	Good
	AM	White to gray	White to gray
ISP 5 medium	SM	Creamy	Creamy
(Glycerolasparagines agar)	SP	None	None
	R	Creamy	Creamy
	G	Moderate	Moderate
	AM	None	None
ISP 6 medium	SM	Creamy	Creamy
(Peptone-yeast extract iron agar)	SP	Brown	Brown
	R	Light brown	Light brown
	G	Good	Good
	AM	White to gray	White to gray
ISP 7 medium	SM	Brown	Dark brown
(Tyrosine agar)	SP	None	None
	R	Dark brown	Dark brown
	G	Good	Good
	AM	White to gray	Gray
	SM	Dark brown	Brown
Bennetts agar	SP	Light brown	Light brown
	R	Creamy	Light brown
	G	Good	Good

[a] AM: aerial mycelium, SM: substrate mycelium, SP: soluble pigment, R: reverse color, and G: growth.

microorganisms. Positive results were recoded against *A. niger* and *M. luteus* NCIMB 196.

Pathogenicity of strain PK-A41. The strain PK-A41 and the tested type strains produced necrotic reactions on surfaces of the potato tuber slices (Fig. 5). The symptoms began to appear 2 days after inoculation. The symptoms were small, and there was a brown halo zone on the surface of the tuber slice around the mycelial agar disks. After 4-day incubation, symptoms developed in different shapes, particularly covering with mycelia and becoming brown around the deep-pitted lesion. The non-inoculated tuber slices did not produce any symptoms. *S. scabies* ATCC 49173 and *S. acidiscabies* ATCC 49003 also showed necrotic symptoms on the tuber slices. The scab disease was more severe on the tuber slices inoculated with *S. acidiscabies* ATCC 49003.

Optimal culture conditions for antibiotic production. Four media containing different carbon and nitrogen sources were tested to select the medium and culture time favorable for the antibiotic production (Fig. 6). The media inoculated with the *S. scabiei* strain PK-A41 were cultured at 28°C for 14 days. Each of four media was harvested at

Table 2. Physiological and biochemical characteristics of the actinomycete strain PK-A41 and the *Streptomyces scabies* ATCC 49173

Characteristic	Strain PK-A41	*Streptomyces scabies* ATCC 49173[a]
Formation of aerial mycelium	+	+
Spore chain morphology *Rectiflexibles*	+	+
Spore chain morphology *Spirals*	+	+
Melanin production on ISP6 medium	+	+
Melanin production on ISP7 medium	–	–
Degradation of		
Arbutin	+[b]	+
Casein	–	–
Esculin	+	+
Gelatin	–	–
Starch	+	+
Tyrosine	+	+
Xanthine	+	+
Max NaCl tolerance (%, w/v)	9%	9%
Resistance to antibiotcs		–
Neomycin	–	–
Oleomycin	–	–
Penicillin G	–	–
Rifampicin	–	–
Growth temperature range (°C)		
4	–	–
10	+	+
30	+	+
40	+	+
45	–	–
Growth on sole carbon source (1%, w/v)		
L-Cysteine	–	–
L-Histidine	+	+
L-Hydroxyproline	+	+
L-Phenylalanine	+	+
L-Valine	+	+
Growth on sole carbon source (1%, w/v)		
Adonitol	+	+
Arabinose	+	+
Dextran	+	+
Fructose	+	+
meso-Inositol	+	+
Mannitol	–	–
D-Melezitose	–	–
D-Melibiose	+	+
Raffinose	–	–
L-Rhamnose	–	–
Sucrose	+	+
Xylitol	+	+
Xylose	+	+
Growth of pH		
3	–	–
5	–	–
8	+	+
11	–	+
13	–	–
Antibiosis against		
Aspergillus niger	+	+
Bacillus subtilis NCIMB 3610	–	–
Candida albicans CBS562	–	–
Micrococcus luteus NCIMB 196	+	+
Saccharomyces cerevisiae CBS 1171	–	+
Streptomyces murinus ISP 5091	–	–

[a] Result from the data of Lambert and Loria (1983).
[b] Symbols '+' and '–' represent positive and negative reactions, respectively.

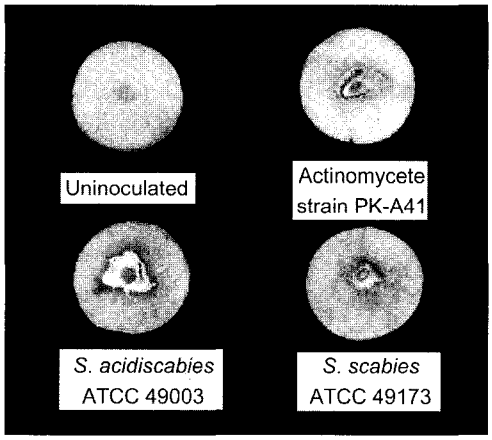

Fig. 5. Necrogenic phenotypes on the potato tuber disks (cv. Daejima) infected with strain PK-A41, *Streptomyces scabies* ATCC 49173, and *S. acidiscabies* ATCC 49003.

different time intervals after incubation, which was followed by loading on an open column packed with Diaion HP-20 resion. To examine the antifungal activity, the methanol eluent was concentrated *in vacuo* and loaded on paper disk (8 mm in diameter). The strain PK-A41 grew well in all the media tested. All culture filtrates of the strain PK-A41 had an inhibitory effect against the tested pathogens *C. orbiculare*, *M. grisea*, and *P. capsici*. In particular, among the tested pathogens, *P. capsici* was most sensitive to the culture filtrates. The antifungal activity of all the four culture filtrates was generally weak against *M. grisea*. The pH of the cultures in the different media was not drastically altered during the culturing of the strain PK-A41 for 8 days

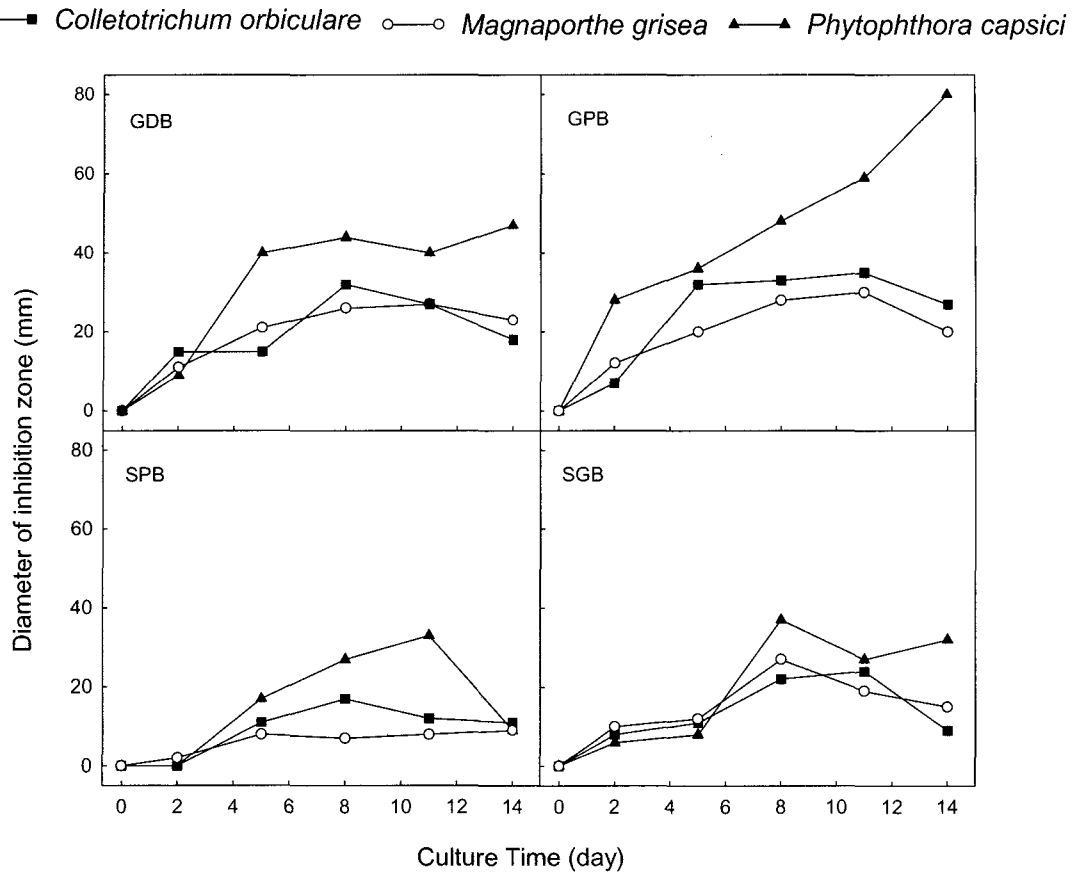

Fig. 6. Time courses of inhibitory effects of the culture filtrates of strain PK-A41 grown in glycerol dextrin broth (GDP), glycerol peptone broth (GPB), starch peptone broth (SPB), and starch glucose broth (SGB) against *Colletotrichum orbiculare, Magnaporthe grisea, and Phytophthora capsici.* The inhibition of mycelial growth of the test fungi was measured using a paper disk bioassay method.

(Fig. 7). After 10 days incubation, the pH gradually increased to an alkaline state of pH 9 in the GPB, SPB, and SGB cultures, but decreased to an acidic state of pH 5 in the GPB cultures. During culturing in the different media, the dried cell weight of the strain PK-A41 increased gradually to 11 days after inoculation, but declined drastically thereafter, with the exception for of the SGB cultures.

The glycerol peptone broth (GPB) cultures showed the most remarkable antifungal activity among all the four culture filtrates tested. It was particularly effective against *P. capsici* (Fig. 6). Based on the antifugal activity, the GPB medium was selected for antibiotic production. The time courses of antibiotic production by the strain PK-A41 cultured on a glycerol peptone broth (GPB) are presented in Figure 8. The pH was neutral before incubation, but changed to alkaline during culturing for 14 days. The production of antibiotics by PK-A41 in the GPB cultures gradually increased to 11 days after incubation, but decreased thereafter. In particular, the antibiotics produced in the GPB by the strain PK-A41 were most effective against the mycelial growth of *P. capsici*.

Discussion

The actinomycetes are well known as a producer of many useful secondary metabolites, including antibiotics (Williams et al., 1993). The isolation of actinomycetes in nature is the first step of screening for natural products such as secondary metabolites. The actinomycetes exist as a major component of the microbial population in most soils. The isolation of actinomycetes in the soil was performed using the dilution plate procedure, which does not differentiate between the forms of growth in the natural habitats. The actinomycete strain PK-A41 was isolated from a soil sample from pepper fields in Ko-yang, Korea. The strain PK-A41 grown on the V8 agar was tested for its antifungal and anti-oomycete activities against the mycerial growth of various plant pathogenic fungi and oomycetes. The strain PK-A41 inhibited the mycerial growth of *A. mali, C. orbiculare, F. oxysporum* f.sp. *lycopersici, M. grisea, R. solani*, and *P. capsici*. Therefore, the strain PK-A41 was selected for production and purification of antibiotic substances.

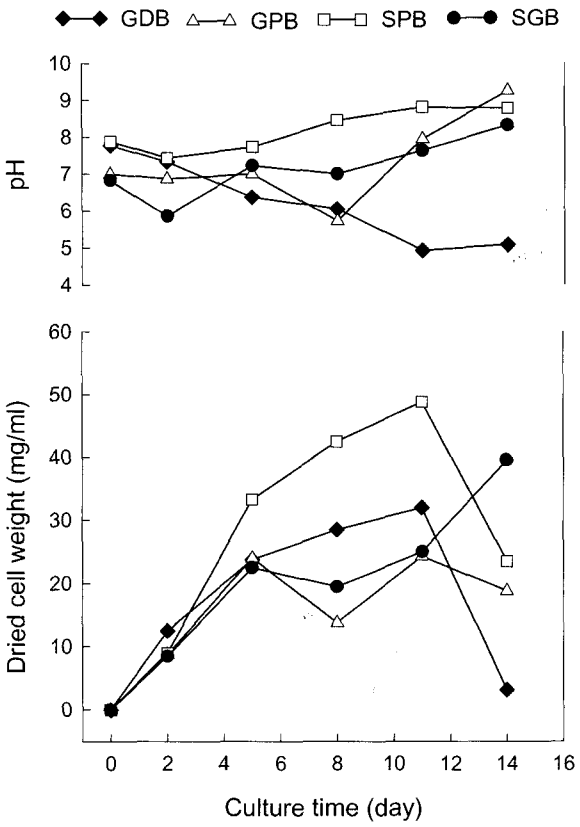

Fig. 7. Time courses of changes in the pH and dried cell weight cultures of strain PK-A41 in each 200 ml of glycerol dextrin broth (GDP), glycerol peptone broth (GPB), starch peptone broth (SPB), and starch glucose broth (SGB).

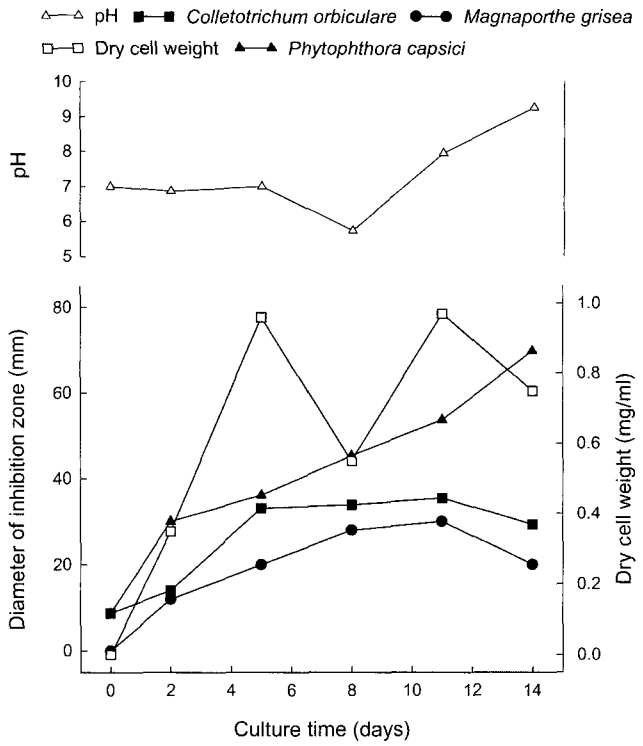

Fig. 8. Time courses of antibiotic production from strain PK-A41 cultured in glycerol peptone broth (GPB). The antifungal activity of each fraction was evaluated against *Colletotrichum orbiculare*, *Magnaporthe grisea*, and *Phytophthora capsici* using paper disk bioassay method.

The idea that sequence analysis of the proteins and nucleic acids could be used to determine the evolutionary relationships was first expound by Zuckerkandl and Pauling (1965). The molecular criteria were used in the classification, identification, and recognition of the phylogenetic relationships among the actinomycetes (Korn et al., 1978). The analysis of the partial or complete sequences of the 5S and 16S rDNA genes was performed to determine the generic relationship between the microorganisms (Olsen and Woese, 1993; Stackebrandy et al., 1997). The sequences of the 16S ribosomal DNA have provided a phylogenetic tree useful to examine the evolution and taxonomy of the actinomycetes. The strain PK-A41 shares high nucleotide sequence similarities with *Streptomyces* sp. EF-35 (98%) and *S. scabiei* (98%), suggesting that the strain PK-A41 belongs to *S. scabiei*. Almost all the neighborhood strains on the phylogenetic tree were *S. scabiei* strains.

The chemical compositions of the cell wall were available for the classification and identification of actinomycetes. Lechevalier and Lechevalier (1970) clearly demonstrated that *Streptomyces* and the other genera of the family Streptomycetaceae contained LL-diaminopimelic acid in the cell wall. The presence of LL-diaminopimelic acid represents the best chemical criterion for identifying the family Streptomycetaceae. Compared to the standard DAP, the DAP in cell wall extract of strain PK-A41 was LL-diaminopimelic acid (Fig. 3). Another chemical character of the Streptomycetaceae is the high GC content of the DNA (69-78 mol%) (Williams et al., 1983a). The result of the GC content of strain PK-A41 was 67.4%, indicating that it belongs to a high GC content group.

Morphology is also important for the characterization and description of *Streptomyces* (Cross and Alderson, 1988). Morphological criteria include the fragmentation pattern of the substrate mycelium, the formation of spore chains, the formation type of the aerial mycelium, and the spore-surface ornamentation. The spores themselves have been called conidia or arthrospores, because of their similarity to fungal spores. Scanning electron microscopy (SEM) was found to be the best technique for detecting the growth sites on natural substrates and for the observation of *Streptomyces* surface (Mayfield et al., 1972; Goodfellow and Williams, 1983). Morphological analysis by SEM confirmed that the spore chain of the strain PK-A41 formed in the *rectiflexibles* and *spirals* type (Fig. 4). The morphological characteristics

of *S. scabiei* were first descrived by Lambert and Loria (1989). The *S. scabiei* cultures were branched and formed spore chains, which were generally loose spirals. The spore chain of the strain PK-A41 is consistent with that of *S. scabiei*.

The chemical and morphological properties shown by the strain PK-A41 are consistent with the classification of the genus *Streptomyces*. The cultural, physiological and biochemical properties of strain PK-A41 shown in Tables 1 and 2 suggest that the strain PK-A41 was similar to *S. scabies* ATCC 49173. In addition, the analysis of the 16S rDNA showed that the strain PK-A41 belongs to *S. scabiei* and its closely related species. In view of all the taxonomic results, strain PK-A41 was identified as *S. scabiei*.

The seven media containing different nutrient sources were used to compare the cultural characteristics of strain PK-A41 and *S. scabies* ATCC 49173. In Table 1, each medium has an International Streptomyces Project (ISP) number and a general name. The strain PK-A41 has cultural characteristics similar to *S. scabies* ATCC 49173. *S. scabiei* was found to produce melanin on peptone iron agar and tyrosine agar (Lambert and Loria, 1989). The strain PK-A41 produced soluble pigments on the peptone-yeast extract iron agar, but not on the tyrosine agar. However, the reverse color of strain PK-A41 was dark brown. The substrate mycelium produced melanin, which could not diffuse through the agar plate. Taylor and Decker (1947) suggested that melanin production is consistently associated with the pathogenicity. However, it is neither essential (Gregory and Vaisey, 1956) nor invariable (Elesaway and Szabó, 1979) on *S. scabiei*.

Various physiological tests used in the earlier studies on Streptomycetes have been found to be rather unreliable and of little taxonomic value (Korn-Wendisch and Kutzner, 1992). The formation of the melanin pigment and the utilization of the nine carbon sources have been employed in the International Streptomyces Project (ISP) (Korn-Wendisch and Kutzner, 1992). However, numerous physiological tests have been used to evaluate significant characteristics of *Streptomyces*. Various physiological tests were also applied to the strain PK-A41 and *S. scabies* ATCC 49173. The strain PK-A41 and *S. scabies* ATCC 49173 were sensitive to a number of toxin compounds such as neomycin, oleomycin, penicillin G, and rifampicin. The sensitivity of *S. scabiei* to penicillin G and streptomycin has been reported (Lambert and Loria, 1989).

The *S. scabiei* species are well known plant pathogens. The strain PK-A41 was tested for its pathogenicity in comparison with other pathogenic strains, such as *S. scabies* ATCC 49173 and *S. acidiscabies* ATCC 49003. The strain PK-A41, *S. scabies* ATCC 49173, and *S. acidiscabies* ATCC 49003 produced typical scab symptoms on the potato tubers. The symptoms began to appear as small, brown specks on the surface of the tuber slice and enlarged. The surface of the potato tuber inoculated with strain PK-A41 was raised and deeply corky, but the diameter of lesions was small. The diameter of the lesion inoculated with *S. scabies* ATCC 49173 was small and pitted. *S. acidiscabies* ATCC 49003 produced the most severe lesions. The disease lesion spread over the surface of the tuber slices and formed deep cracks and depressions.

Based on the chemical and morphological characteristics, strain PK-A41 was confirmed to belong to the genus *Streptomyces*. In addition, the genetic, cultural, physiological and biochemical characteristics also indicated that strain PK-A41 belong to the *S. scabiei*. Therefore, strain PK-A41 is *S. scabiei* and is pathogenic to potatos.

Four media containing different carbon and nitrogen sources were examined to select the medium and culture time most favorable for achieving the maximum antibiotic production. The selected medium was a glycerol peptone broth (GPB) and the most favorable culture time was 9 days after incubation. The secondary metabolites are usually produced during the stationary phase. The inhibitory effects of strain PK-A41 also were strong at the later time after incubation. The factors that affect the growth of the microorganisms and antibiotic production are the temperature, pH, as well as the oxygen, and nutrient levels. In general, the growth media used for large-scale production appears to be rich in nutrients. Microbial cells produce metabolites when the culture media usually becomes acidic (Flower and Williams, 1977). However, the pH of the glycerol peptone broth (GPB) inoculated with the strain PK-A41 was neutral before incubation, but became alkaline after incubation for 14 days. The purpose of shaking the cultures is to supply oxygen and nutrients to the growing cells. *Streptomycetes* are aerobic organisms, and require a high oxygen level for a large-scale culture.

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