Isolation, Identification and Optimal Culture Conditions of *Streptomyces albidoflavus* C247 Producing Antifungal Agents against *Rhizoctonia solani* AG2-2

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(Received April 24, 2009. Accepted June 11, 2009)

Streptomyces albidoflavus C247 was isolated from the soil of the Gyeongsan golf course in Korea. Physiological, biochemical and 16S rDNA gene sequence analysis strongly suggested that the isolate belonged to *Streptomyces albidoflavus*. Preliminary screening revealed that the isolate was active against fungi and bacteria. Self-directing optimization was employed to determine the best combination of parameters such as carbon and nitrogen source, pH and temperature. Nutritional and culture conditions for the production of antibiotics by this organism under shake-flask conditions were also optimized. Maltose (5%) and soytone (5%) were found to be the best carbon and nitrogen sources for the production of antibiotics by *S. albidoflavus* C247. Additionally, 62.89% mycelial growth inhibition was achieved when the organism was cultured at 30°C and pH 6.5. Ethyl acetate (EtOAc) was the best extraction solvent for the isolation of the antibiotics, and 100 µg/ml of EtOAc extract was found to inhibit 60.27% of the mycelial growth of *Rhizoctonia solani* AG2-2(IV) when the poison plate diffusion method was conducted.

KEYWORDS : Antifungal agent, *Rhizoctonia solani* AG2-2(IV), *Streptomyces albidoflavus* C247

Numerous antibiotics have been isolated from a variety of microorganisms; however, studies are still being conducted to identify novel antibiotics effective against pathogenic fungi and bacteria. The number and species of microbes in soil vary directly in response to environmental conditions such as nutrient availability, soil texture, and type of vegetation cover (Atlas and Bartha, 1998). Filamentous Actinomycetes are known to have the ability to produce a wide variety of secondary metabolites. Indeed, each strain of Actinomycetes likely has the genetic potential for the production of 10 to 20 secondary metabolites (Bentley *et al.*, 2002; Sosio *et al.*, 2000).

Actinomycetes are useful biological tools for the production of antimicrobials against fungi and bacteria (Okami and Hotta, 1988). In general, *Streptomyces* are saprophytic and are commonly associated with soils, where they contribute significantly to the turnover of complex biopolymers and antibiotics (Woese, 1987). However, in the past two decades there has been a decline in the discovery of new lead compounds from common soil-derived actinomycetes. As a result, the cultivation of various taxa of actinomycetes has become a major focus in the search for the next generation of pharmaceutical agents (Mincer *et al.*, 2002).

Additionally, *Streptomyces* are especially prolific (Locci, 1989; Nolan and Cross, 1998). The problems of drug resistance, patient sensitivity and an inability to control

certain infectious diseases have driven a continuous search for new antibiotics worldwide. To combat multidrug resistant organisms, it is essential to develop new antimicrobial compounds or antibiotics.

Marten *et al.* (2001) reported that Rhizovit[®] from *Streptomyces rimosus* was used to control a wide range of fungi including *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Alternaria brassicicola*, and *Botrytis* sp. In addition, Liu *et al.* (2004) reported that *S. rimosus* showed a high antagonistic activity against *Fusarium solani*, *F. oxysporum* f. sp. *cucumarinum*, *Verticillium dahliae*, *R. solani*, *Fulvia fulva*, *Botrytis cinerea*, *A. alternata*, *Sclerotinia sclerotiorum* and *Bipolaris maydis*.

In the present study, we reported the media and culture conditions of *S. albidoflavus* C247 and the extraction methods for the isolation of antibiotics.

Materials and Methods

Soil sample and isolation of actinomycetes. A soil sample was collected from a golf course in Korea. Five grams of soil were then suspended in 45 ml of sterile double distilled water, and the suspension was then serially diluted to 10⁻⁵. Next, aliquots (100 µl) were spread on TSA and actinomycetes isolation agar (MTCC, 1994). To minimize the fungal and bacterial growth, actidione (20 mg/l) and nalidixic acid (100 mg/l) were added. The plates were then incubated at 27°C for 7~14 days, after which actinomycetes were selected based on their mor-

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Table 1. Cultural characteristics of *Streptomyces albidoflavus* C247

Media	Mycelial growth ^a	Areal mycelium color	Substrate mycelium color ^b	Spores	Soluble pigment ^b
ISP 2	+++	White	Pale yellow (89)	Brown Yellow	Dark grayish blue (187)
ISP 3	+	White	Dark yellowish pink (30)	No spores	Absent
ISP 4	+++	White	Grayish yellow (90)	Brown	Greenish gray (155)
ISP 4-G	+++	White	Grayish yellow (90)	Brown	Grayish green (150)
ISP 4-Y	+++	White	Grayish yellow (90)	Brown	Yellowish green (151)
ISP 5	++	White	Pale yellowish pink (31)	Brown	Absent
ISP 6	++	White	Strong yellow (84)	Brown Yellow	Grayish green (150)
GYM	+++	White	Grayish yellow (90)	Brown Yellow	Absent
LBA	++	White	Pale yellow (89)	No spores	Absent
TSA	++	White	Pale yellow (89)	Brown	Absent
PDA	+	White	Yellowish white (92)	No spores	Absent

ISP: International Streptomyces Project medium; ISP 4-G: ISP-4 medium with glucose; ISP 4-Y: ISP-4 medium with yeast extract; GYM: Glucose, yeast and malt extract agar; LBA: Luria broth agar; TSA: Tryptic soy agar; PDA: Potato dextrose agar.

^a+: poor; ++: moderate; +++: good.

^bISCC-NBS color centroid system.

Streptomyces albidoflavus C247 was incubated at 27 ± 2°C for 14 days.

phology and then cultured on ISP4 medium. The stock culture was preserved in 15% (v/v) glycerol at -20°C (Maniatis *et al.*, 1989).

Microorganism and its maintenance. The common turfgrass pathogen, *Rhizoctonia solani* AG2-2(IV) (KACC 40152), was used as a test organism in this study. The organism was stored at 4°C in tubes containing potato dextrose agar (PDA) for routine use. For long term preservation, it was stocked in glycerol solution at -70°C.

Screening of antibiotic producing actinomycetes. The antagonistic effect and antibiotic production by the isolate was evaluated using a slightly modified version of the dual culture method (Landa *et al.*, 1997). Isolated actinomycetes were streaked on a 4 cm wide TSA plate and incubated for 48 h at 27°C. A mycelial disk (5mm in diameter) of *R. solani* AG 2-2 (IV) was then inoculated on the plate and incubated for 72 h at 27°C. The the mycelial growth of *Rhizoctonia solani* AG2-2 (IV) towards (a) and away (b) from the actinomycetes were then measured and used to calculate the percentage growth inhibition rate (IR %) as follows:

$$IR (\%) = 100 - (a/b \times 100)$$

Molecular and morphological characterization. Strain C247 was identified through morphological, physiological and biochemical analysis as well as 16S rDNA gene sequence analysis.

Optimization for maximum antibiotic production. Various media were used to determine the optimal media and culture conditions for the growth and production of antibiotics. LB medium was supplemented with different carbon and nitrogen sources, after which 5 ml of mycelial suspen-

sion was inoculated into the medium (100 ml in 500 ml Erlenmeyer flask) and incubated on a rotary shaker at 150 rpm and 28°C for 5 days. The effects of various temperature (25, 30, 35 and 40°C), pH (5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) and incubation time (48, 72, 96 and 120 h) on the growth and antibiotic production were then evaluated.

Organic solvent extraction. For isolation of the antibiotics, the culture was stopped when it reached the maxi-

Table 2. Characteristics of *Streptomyces albidoflavus* C247

Characteristics	Responses
Gram reaction	+
Lacto phenol cotton blue	Arial mycelia with sporangium
Production of diffusible pigment	+
Range of growth temperature	20–35°C
Optimum growth temperature	30°C
Range of initial pH	4.5–8.5
Optimum initial pH	7.0
NaCl tolerance	0.03–0.20 M
Optimum NaCl for growth	0.05 M
Catalase production	+
Oxidase production	–
Protease	–
Amylase	–
Gelatinase	–
H ₂ S production	–
Optimum carbon source	5% Corn starch for cell growth but maltose (5%) is suitable for antibiotic production
Optimum nitrogen source	5% Soytone is suitable for both cell growth and antibiotic production
Optimum culture time	96 hours

All of the above experiments were performed according to Bergey's Manual of Systematic Bacteriology.

+: positive; –: negative.

Fig. 1. Morphological micrograph of isolate C247 after 14 days of culture on ISP4 medium. A; Light microscopic observation ($\times 400$), B; Scanning electron micrographs (SEM).

mum activity. The culture broth (5 liter) was centrifuged and the supernatant containing antibiotics was extracted using solvents such as hexane, chloroform, ethyl acetate and dichloromethane. The solvents were added to the culture supernatant at a ratio of 1 : 3 (v/v) and then shaken vigorously for 20 min. This process was repeated three times. The solvent layer was then collected and evaporated to dryness in a vacuum evaporator at 40°C, after which it was stored at 4°C.

In vitro antimicrobial test. The crude extract was dissolved in methanol (MeOH) and then added to a molten PDA plate. Freshly grown mycelial discs (7 mm in diameter) of *R. solani* AG2-2(IV) were then placed in the centre of the plates and incubated at $27 \pm 2^\circ\text{C}$. Plates were prepared in triplicate for each treatment. Positive and neg-

ative controls (MeOH) were also included. The mycelial growth of *R. solani* AG2-2(IV) was recorded after 3 to 5 days. Measurement of the colony diameter was conducted according to the growth rate of the fungal pathogen. The inhibitory activity of each treatment was expressed as the percentage growth inhibition when compared to the negative control (0%) using following calculation:

$$\text{Growth inhibition (\%)} = \frac{\text{DC} - \text{DT}}{\text{DC}} \times 100$$

where, DC = the diameter of the control colony and DT = the diameter of the treated fungal colony (Pandey *et al.*, 1982).

Table 3. Inhibitory effect of *Streptomyces albidoflavus* C247 on the mycelial growth of phytopathogenic fungi grown in dual culture on TSA

Pathogenic fungi	Inhibition rate (%)
<i>Rhizoctonia solani</i> AG2-2	97.1 $\pm$ 0.6
<i>Rhizoctonia solani</i> AG2-2(A) (KACC 40132)	95.9 $\pm$ 4.3
<i>Rhizoctonia solani</i> AG2-2(B) (KACC 40151)	79.0 $\pm$ 4.4
<i>Rhizoctonia solani</i> AG4-4 (KACC 40141)	59.5 $\pm$ 0.9
<i>Rhizoctonia cerealis</i> (KACC 40154)	100.0 $\pm$ 0.0
<i>Sclerotinia homeocarpa</i>	91.3 $\pm$ 2.2
<i>Pythium graminicola</i> (KACC 40155)	35.3 $\pm$ 3.1
<i>Collectotrichum gloeosporioides</i> (KACC 40003)	90.7 $\pm$ 1.3
<i>Collectotrichum circinans</i> (KACC 40641)	90.6 $\pm$ 5.8
<i>Fusarium oxysporum</i> (KACC 40052)	47.7 $\pm$ 0.9
<i>Botrytis cinerea</i> (KACC 40573)	100.0 $\pm$ 4.7
<i>Phytophthora cactorum</i> (KACC 40166)	82.9 $\pm$ 2.1
<i>Penicillium ailli</i> (KACC 41337)	94.1 $\pm$ 0.9
<i>Chaetomium globosum</i> (KACC 40308)	50.7 $\pm$ 3.5
<i>Glomerella cingulata</i> (KACC 40299)	86.5 $\pm$ 0.6
<i>Corynespora cassiicola</i> (KACC 40964)	88.4 $\pm$ 1.0

The *S. albidoflavus* A247 were streaked (35 mm) on one side of a TSA plate, and 48 hr after incubation at 27°C a mycelial disk (5 mm in diameter) of the test fungus was placed 35 mm from the antagonistic bacteria. The plates were then incubated at 27°C.

GCTTACACATGCAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGCGCA
ACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACCTCTGGGACAAGCCCTGGA
AACGGGGTCTAATACCGGATATGACTGTCCATCGCATGGTGGATGGTGTAAAG
CTCCGGCGGTGCAGGATGAGCCCGCGGCTATCAGCTTGTTGGTGAGGTAGTG
GCTCACCAAGGCGACGACGGGTAGCCGGCTGAGAGGGCGACCGGCCACACT
GGGACTGAGACACGCCCCAGACTCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGAAAGCCTGATGCAGCGACGCCGTGAGGGATGACGGCCTTCG
GGTTGTAAACCTCTTTTCAGCAGGAAGAAGCGAAAGTGACGGTACCTGCAGAA
GAAGCGCCGGCTAACTACGTGCCAGCAGCCGGTAATACGTAGGGCGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTGGTT
GTGAAAGCCCCGGGCTTAACCCCGGGTGTGACGTGATACGGGAGGCTAGAG
TTCGGTAGGGGAGATCGGAATCTGTTGTAGCGGTGAAATGCGCAGATATCA
GGAGGAACACCGGTGGCGAAGGCGGATCTTGGGCCGATACTGACGCTGAGG
AGCGAAAGCGTGGGAGCGAACAGGATTAGATACCTGGTAGTCCACGCCGTA
AACGGTGGGCACTAGGTGTGGGCAACATTCACGTTGTCCGTGCCGCACTAA
CGCATTAAGTGCCCCCGCTGGGGAGTACGG

Fig. 2. The 16S rDNA sequences of isolate C247.

Fig. 3. Antagonistic activity of isolate C247. A, Antagonistic activity of isolate C247 against *R. solani* AG2-2(IV); B, Normal growth of *R. solani* AG2-2(IV) on TSA.

Results

Isolation and identification. Strain C247 was isolated from the soil and found to have antimicrobial activity against the turfgrass pathogen, *R. solani* AG2-2(IV). The isolate was aerobic, gram positive and had areal mycelia with sporangium (sporophore). During culture, grayish red pigment diffused into the surrounding medium (Table 1). The strain was also mesophilic and catalase positive, but did not produce oxidase or hydrogen sulphide. The strain also did not produce amylase, protease or gelatinase (Table 2). Based on the morphological, physiological, and biochemical results, isolate C247 was found to be a member of the genus *Streptomyces*, and 16S rDNA gene

Table 4. Antifungal activity of EtOAc extracts (100 $\mu\text{g/ml}$ PDA) of *Streptomyces albidoflavus* A247 against 8 isolates of *Rhizoctonia solani* AG2-2(IV)

Isolate	Inhibition rate (%)	
	Cell extract	Supernatant
R1	63.2	71.5
R2	56.2	66.8
R3	61.3	72.3
R4	61.2	68.5
R5	56.0	67.1
R6	59.7	74.2
R7	54.8	65.6
R8	50.9	66.8

Fig. 4. Effect of tryptic soy broth (TSB) concentration on the production of antibiotics by *Streptomyces albidoflavus* C247. *S. albidoflavus* C247 was cultured in 3 different concentration of TSB (A, 10 g/l; B, 20 g/l; C, 30 g/l) for 72 hours at 30°C and the cell-free filtrates were then collected and subjected to aseptic 0.4 μm -filtration. A A freshly growing *R. solani* AG2-2(IV) mycelial disk was placed on the center of a TSA plate containing 10% (v/v) of each culture filtrate. D; A normal TSA plate culture of *R. solani* AG2-2(IV), Control.

sequence analysis suggested that it was most closely related to *Streptomyces albidoflavus* (Fig. 1 and 2).

Screening of antibiotics. Preliminary screening revealed that *Streptomyces albidoflavus* C247 was an excellent producer of antifungal and moderate antibacterial compounds (Table 3 and 5). The antagonistic effect of isolate C247 was most evident on *R. solani* AG2-2(IV). The EtOAc extract of *S. albidoflavus* C247 inhibited the mycelial growth of various isolates of *R. solani* by 65 to 75% (Fig. 3 and Table 4).

Effect of media and nutrients. Two thirds strength tryptic soy broth (TSB) was found to be a favorable basal media for the growth and production of antimicrobial materials from *S. albidoflavus* C247. Culture supernatant from C247 cultured in 10, 20 and 30 gm/l TSB induced 50.94, 59.03 and 49.58% mycelial growth inhibition, respectively (Fig. 4).

Fig. 5. Effect of carbon sources on cell growth and production of antibiotics by *Streptomyces albidoflavus* C247.

Fig. 6. Effect of nitrogen sources on cell growth and production of antibiotics by *Streptomyces albidoflavus* C247.

To screen for a suitable carbon and nitrogen source, strain C247 was incubated in basal medium containing various carbon and nitrogen sources. Each of the following carbon sources was added to the basal medium (LB broth) at a concentration of 1 to 9% (w/v): maltose, fructose, corn starch, sucrose, glycerol, water soluble starch, glucose, galactose, lactose and dextrin. Various nitrogen sources including urea, glycine, soytone, beef extract, peptone, malt extract, tryptone, yeast extract and poly peptone were also added to the basal medium at concentrations of 1 to 9% (w/v).

Maltose and sucrose resulted in the highest antibiotic production, with maltose inducing 74.33% growth inhibition and sucrose inducing 70.67% (Fig. 5). Soytone was favorable for the cell growth and production of antibiotics (Fig. 6). When compared with inorganic nitrogen sources, organic nitrogen sources gave a relatively higher production of antibiotics.

Effect of initial pH and temperature. Strain C247 was cultivated in the above described medium at different initial pHs (pH 3.5 to 8.5) and temperatures (25 to 40°C).

Fig. 7. Effect of initial pH and culture temperature on the production of antibiotics by *Streptomyces albidoflavus* C247.

Table 5. Antibacterial susceptibility test of EtOAc extract of *S. albidoflavus* C247

Microorganism	Streptomycin (µg/ml)	MIC (mg/ml)
<i>Bacillus subtilis</i> IFO 3026	2.30	0.25
<i>Staphylococcus aureus</i> ATCC 25923	5.25	1.00
<i>Escherichia coli</i> ATCC 25922	13.25	0.50
<i>Enterococcus faecalis</i> ATCC 29212	27.00	0.50
<i>Pseudomonas aeruginosa</i> ATCC 27853	26.00	1.00
<i>Klebsiella pneumonia</i> ATCC 10031	6.75	1.00
<i>Xanthomonas</i> sp. IAM 1671	6.25	0.50

MIC. Minimum inhibitory concentration.

Fig. 8. Effect of solvents used for the extraction of antibiotics from *Streptomyces albidoflavus* C247.

The results revealed that the optimal pH and temperature were 6.5 at 30°C, respectively, and the corresponding inhibition rate was 62.89% (Fig. 7). It is believed that this occurred because strain C247 has adapted to the slightly acidic soil from which it was isolated.

Solvent extracts. Hexane, ethyl acetate, dichloromethane and chloroform extracts of strain C247 were tested against *R. solani* AG2-2(IV). All extracts showed antifungal activity against the test pathogen and EtOAc extract was effective against 8 isolates of *R. solani* AG2-2(IV) isolated from diseased turf grass (Table 4). The minimum inhibitory concentration (MIC) of the crude EtOAc extract against *Bacillus subtilis* IFO 3026 was 0.25 mg/ml (Table 5). Treatment with 10 and 100 µg/ml of the crude EtOAc extracted resulted in 30.88 and 60.27 % inhibition of the mycelial growth of *R. solani* AG2-2(IV), respectively (Fig. 8).

Discussion

In the present study, the optimal conditions for the growth of isolate C247 were found to be pH 6.5 and 30°C. Additionally, maltose (5%) and soytone (5%) were found to be the best carbon and nitrogen sources. These results were similar to the findings of a study conducted by Sujatha *et al.* (2005). Additionally, it has been reported that environmental factors such as temperature, pH and incubation have a profound influence on antibiotic production (Iwai *et al.*, 1973). In this study, the extracellular products (ECPs) of *S. albidoflavus* C247 showed potent antimicrobial activity against various pathogenic fungi and bacteria (Table 4 and 5). Furthermore, the MIC of the ethyl acetate extracts against tested bacteria indicated that it is a broad spectrum antibiotic (Sujatha *et al.*, 2005). Finally, the efficiency with which the EtOAc extract inhibited the mycelial growth of turfgrass pathogens clearly indicates that *S. albidoflavus* C247 is an effective antagonist against

turfgrass pathogens.

In conclusion, the actinomycete isolated in this study was found to be a potential antimicrobial agent and the optimal media, nutrients and culture conditions promoted the antifungal activities of *S. albidoflavus* C247.

Acknowledgments

This work was financially supported by the BK21 project at Daegu University, Korea.

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