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Taxonomy, Fermentation, Isolation and Characterization of a Herbicidal Compound, 3D5

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The herbicidal compound, 3D5 was isolated from the culture broth of strain 3D5, a soil isolate. Based on taxonomic studies, this culture was identified as *Streptomyces* sp. 3D5.

In the course of screening for herbicidal compounds produced by actinomycetes, the active compound was isolated from the culture of strain 3D5, a soil isolate.

The present paper deals with the taxonomy of the producing strain and with the production, isolation, and characterization of this active compound.

Structural elucidation of this compound will be reported in the accompanying paper (7).

MATERIALS AND METHODS

Taxonomic Study

The cultural and physiological characteristics of the producing microorganism were determined by the use of the media and methods described by Shirling and Gottlieb (5). Observations of the culture were made after incubation at 28℃ for two weeks, except where otherwise mentioned.

Utilization of carbon sources was examined according to the method of Pridham and Gottlieb (3). The taxonomic key of Bergey's manual of determinative bacteriology (4) and of Waksman in the Actinomycetes (6), were used to compare culture with recognized genera and species of actinomycetes.

The color terms recorded for each culture were described according to color Harmony Manual (2).

Fermentation

The strain 3D5 was isolated from a soil sample collected at Kangwon-Do, Korea. Strain 3D5 was grown at 28℃ for 14 days on Bennett's agar plate.

For the production of plant growth inhibitor for the primary screening, an aerial mass from the agar slant cultures was inoculated into 100 ml of seed medium in a 500 ml Erlenmeyer flask. The seed culture was incubated at 27℃ for 3 days on a rotary shaker and 3 ml of the culture broth was transferred into 500 ml Erlenmeyer flasks containing 100 ml of production medium. The fermentation was carried out at 28℃ for 5 days on a rotary shaker operating at 200 rpm. The composition of seed and production medium, Glucose-Soluble starch-Soybean meal (GSS) medium, contains 2% glucose, 1% soluble starch, 2.5% bactosoytone, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl and 0.005% K_2HPO_4. The pH of the medium was 7.3 before autoclaving.

Larger quantities of the broth required for isolation and purification studies were produced in a 50-liter fermentor containing 35 liters of the medium with the same composition. Fermentation was performed by inoculation of 2 liters of seed culture fermented in 5-liter fermentor for 2 days at 28℃, followed by agitation at 200 rpm and aeration (20 liters per minute) for 5 days at 28℃.

Isolation

The fermentation broth thus obtained was separated into culture filtrate and mycelium cake by centrifugation. Mycelium cake was extracted with 70% acetone solution. After evaporation of acetone, the mycelium extracts were combined with culture filtrates, then followed by the butanol extraction. The butanol extracts were concentrated at 45℃ under reduced pressure, and subjected to Diaion HP-20 column eluting with 70% acetone. Concentrated eluate was extracted with ethylacetate. The Organic phase was dried over anhydrous sodium sulfate and

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concentrated *in vacuo* to a brown oil. Further purification was carried out by silica gel chromatography with a linear gradient solvent system consisting of chloroform to chloroform:methanol (8:2), then followed by MCI CHP 20P gel chromatography eluting with 70% acetonitrile. Final purification was achieved by semipreparative HPLC on C_{18} reverse phase column (Waters, μ bondapak) eluting with 70% acetonitrile. Detection was made by UV absorption at 210 nm.

The steps leading to the isolation and purification of 3D5 compound from culture broth were outlined in Fig. 1.

Antimicrobial Activity

Antimicrobial activity was measured by the conventional paper disc diffusion method. Antimicrobial activity was observed after 24 hours of incubation at 37℃ for bacteria or longer incubation at 27℃ for fungi and yeast.

Seed Germination Assay

To examine the inhibition of seed germination and growth, ten seeds of radish were spread on whatman No. 2 filter paper placed in 9 cm petri dishes, to which 5 ml of testing solutions or distilled water was added. After incubating the seeds at 27℃ for 5 days with moisture under the photoperiod of 12 hour light and 12 hour dark, the inhibition of germination or growth was evaluated.

Whole Plant Assay

Whole plant assay was conducted by the following method. Plastic pots were planted with seeds of various monocotyledonous and dicotyledonous plants.

Herbicidal activities were tested two weeks after germination by foliar spraying and the effects were examined two weeks after treatment.

Culture broth + mycelium extracts
↓
butanol extraction
↓
Diaion HP-20 column, 70% acetone elution
↓
ethylacetate extraction
↓
silica gel column chromatography
gradient elution from 100% chloroform
to chloroform:methanol (8:2)
↓
MCI CHP 20P gel chromatography
70% acetone elution
↓
HPLC C_{18} reverse phase column
eluting with 70% acetonitrile
solution

Fig. 1. Isolation procedure of 3D5 compound.

Instrumentation

The UV absorption spectrum was measured with a Shimazu model 260.

Proton NMR spectrum in $CDCl_3$ was recorded on a Jeol JNM GX-400 spectrometer using tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

Taxonomy of the Producing Organism

Morphological observations were made by optical and electron microscopy using cultures grown at 28℃ for 14 days on inorganic salt-starch agar and tyrosine agar.

The spore chains formed spirals, and the surface of spore was smooth. The size of spore was approximately 0.7×1.0 μm in diameter (Fig. 2).

Cultural characteristics were shown in Table 1. The physiological properties and utilization of carbon sources were summarized in Table 2 and 3, respectively.

Cell wall analysis for amino acids and sugars was performed as described by Becker *et al.* (1). L.L-diaminopimelic acid was found as a component of the cell wall of strain 3D5. The morphological and chemical charac-

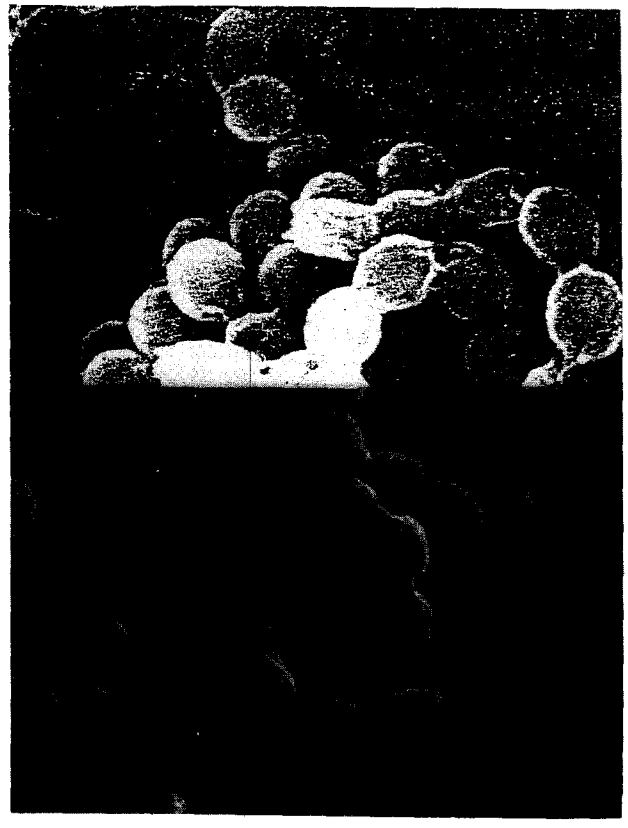

Fig. 2. Scanning electron micrograph of strain 3D5 (tyrosine agar, 28℃ for 14 days).

Table 1. Cultural characteristics of strain 3D5

Medium	Growth	Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar	Good	Yellow	Good:Greenish grey	Yellowish brown
Oatmeal agar	Good	Yellow	Good:Grey	None
Inorganic salts-starch agar	Poor	White	Poor:Brownish grey	None
Glycerol-asparagine agar	Moderate	Yellow	Moderate:Grey	Yellowish brown
Tyrosine agar	Good	Yellow	Good:Reddish grey	None
Nutrient agar	Poor	White	None	None

Table 2. Sugar utilization by strain 3D5

Response	Sugars
Positive	D-glucose, L-arabinose, D-xylose salicin, mannitol D-fructose, maltose, lactose
Negative	rhamnose, raffinose sucrose

Table 3. Physiological characteristics of strain 3D5

Starch hydrolysis	positive
Gelatin liquefaction	negative
Milk petonization	positve
Milk coagulation	negative
Melanoid pigment production	negative
Casein hydrolysis	positive
H_2S production	negative
Temperature range	25~37℃
NaCl tolerance	≤10%
Indole production	positive
Catalase production	positive
Urease production	positive

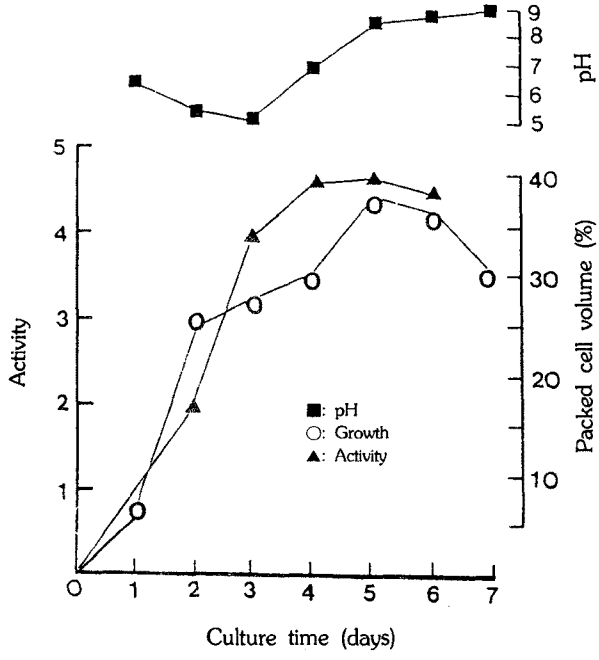

Fig. 3. Typical fermentation profile for 3D5 compound.

teristics of strain 3D5 identified this organism as a member of the genus *Streptomyces*.

Consequently, strain 3D5 was named *Streptomyces* sp. 3D5 and has been deposited in Korean Collection for Type Culture, Genetic Engineering Research Institute, Korea under the accession No. KCTC 0001BP.

Fermentation

A number of complex and synthetic media were evaluated for the production of the 3D5 compound. Among the complex media, GSS medium (see Materials and Methods) was chosen for further studies.

Typical fermentation profile for 3D5 compound was shown in Fig. 3. Herbicidal activity, pH and packed cell volume were monitored at regular intervals. Mycelial growth was expressed as packed cell volume obtained after centrifugation of 5 ml of the culture broth at 3000 rpm for 5 minutes. The production of the herbicidal compound was determined by the seed germination test and by HPLC. The amount of active compound increased during the logarithmic phase of cell growth, and reached maximum after 5 day cultivation and decreased with concomitant lysis of cells. For isolation of the compound, fermentation was terminated in 5 days.

Biological Activity

3D5 compound showed no antimicrobial activity against bacteria, fungi and yeast at the concentration of 1 mg/ml.

3D5 compound inhibited the seed germination completely at the concentration of 25 μg/ml, and was found to have potent herbicidal activity against most of mono- and di-cotyledous plants, especially *Portulaca oleracea* L. and *Digitaria sanguinaris* L., which were the most abundant weeds in Korea.

Physico-chemical Properties

The purified 3D5 compound migrated on silica gel (Kieselgel $60F_{254}$, Merck) as a single spot in several TLC

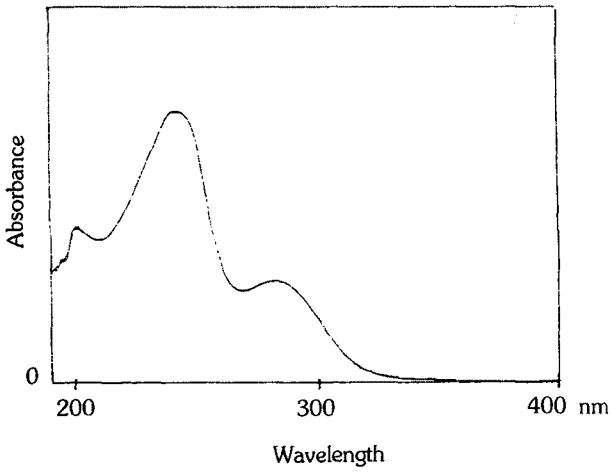

Fig. 4. UV spectrum of 3D5 compound (CH_3OH).

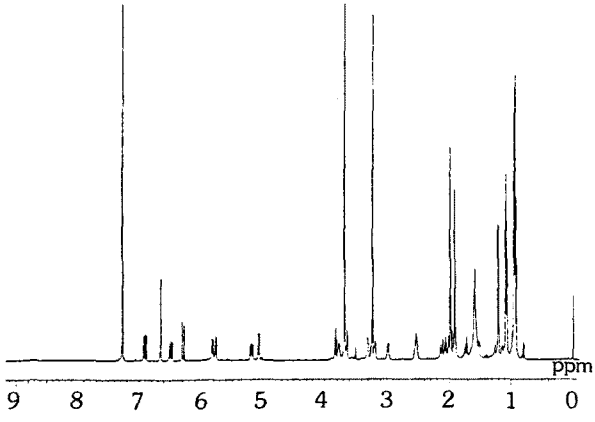

Fig. 5. Proton NMR spectrum of 3D5 compound (400 MHz, $CDCl_3$).

system, and eluted at 28 minutes as a single peak with an isocratic solvent system, 70% acetonitrile solution, in HPLC.

The compound was isolated as a white powder, relatively stable in neutral, acidic or alkaline solutions, giving no loss of activity in the pH range of 2 to 11 by heating for 15 minutes at 90℃. It was soluble in acetone, chloroform, ethylacetate, slightly soluble in methanol, and insoluble in water.

The UV and proton NMR spectra of 3D5 compound were shown in Fig. 4 and Fig. 5, respectively. The UV spectrum of this compound showed absorption maxima at 240 and 280 nm, suggesting the presence of a conjugated double bond.

The molecular formula was established to be $C_{35}H_{56}O_8$ by the high resolution mass measurement, ^1H NMR and ^{13}C NMR spectroscopy.

The structure has been determined by the spectroscopic methods. Full details will be described in the accompanying paper.

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