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Production of Sepedonin by *Sepedonium chrysospermum* NT-1 in Submerged Culture

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Strains of *Sepedonium chrysospermum* and the anamorph strain of *Hypomyces chrysospermus* (≡*Apiocrea chrysosperma*) were isolated and purified from parasitic filamentous fungi on the fruiting bodies of *Boletaceae*, such as the *Gyroponus* and *Suillus* genera in Japan, and identified from formations of conidia and chlamydospores. It is known that these strains produce sepedonin. *S. chrysospermum* NT-1 strain was selected from these strains and isolated. As the optimum medium □(CY-1 medium), 0.1% yeast extract was added to the fruiting-body-forming medium (C medium) of *Schizophyllum commune*. After 8 days of growth on CY-1 medium, the yield of sepedonin was about 34 mg per 2 g of glucose added. This sepedonin seemed to inhibit the growth of various gram-positive and gram-negative bacteria, yeasts and molds.

1. Introduction

We have studied many fruiting-body-forming microorganisms for use as unexploited natural resources, and have stored and identified the isolated strains and collected fruiting bodies. We noted that specific forms of mycoparasitic fungi grew on fruiting-body-forming fungi and that these microorganisms were particularly parasitic to Basidiomycetes. Among these parasitic fungi, the *Sepedonium* genus grew on the Basidiomycete *Boletaceae*. This strain is well known as a poor growth or deformity indicator and a producer of physiological

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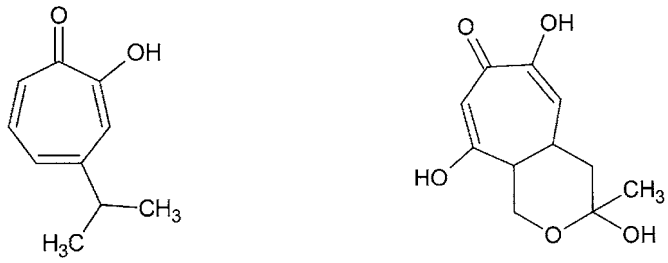
compounds that cause various deformities in fungi. We expected a physiological activity of sepedonin for microorganisms, since sepedonin is analogous to the ring structure of hinokitiol produced by *Chamecyparis obusta*[1] (Fig. 1). In this paper, we report on the production of sepedonin by *Sepedonium chrysospermum* isolated from the fruiting bodies of *Boletaceae*.

2. Experimental Procedures

Strains of *S. chrysospermum* were isolated from parasitic mycelia on the fruiting bodies of *Boletaceae* collected from some fields in Okayama prefecture, Japan. *Sepedonium* species were identified on the basis of the confetto-like chlamydospore and the shape of conidia using a microscope. The composition of the CY-1 medium for submerged and solid cultures was the following in 100 ml distilled water: 2.0 g, glucose; 0.2 g, polypeptone; 0.1 g, yeast extract; 0.046 g, KH_2PO_4; 0.1 g, K_2HPO_4; and 0.05 g, $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.8 with 1N HCl. Agar of 2.0 (w/v)% was added for solid culture. One loopful of seed strain from a plate culture was inoculated into 100 ml of CY-1 medium in a 300-ml preculture flask, and cultivation was carried out on a rotary shaker (28°C, 200 rpm). Seed cultivation was performed using cells of the late-logarithmic-growth phase before pigmentation. The cultivation temperature was 28°C.

The cell mass weight was measured after drying for 12 h at 85°C. After filtration, the mass of sepedonin was estimated from a standard curve using purified crystal sepedonin, which formed plates and showed yellow by a spectrometer at 382 nm a wavelength of after dilution with 50 mM Tris-HCl buffer solution (pH 7.1). Sepedonin in culture broth was extracted with ethyl acetate and the crude compound was dried by evaporation under reduced pressure. A crystal preparation was prepared according to the Handbook of Secondary Fungal Metabolites, Volume II.[1] The identification of the isolated strains used was carried out in accordance with the method described by Tubaki.[2] Glucose reduction was measured using the Somogyi-Nelson method. The detection and identification of



Hinokitiol Sepedonin

Fig.1. Structures of hinokitiol and sepedonin.

sepedonin were carried out using a spectrometer and thin-layer chromatography (TLC) (absorbent reagent; Silica gel 60 G (Merck, width: 20×20 cm, thickness: 0.5 mm); solvents: petroleum ether, acetone, benzene, acetic acid = 20 : 5 : 5 : 2 by volume). The color development reagents used for detection were 1% $FeCl_3$-methanol reagent and bromophenol blue (BPB). The sepedonin produced was identified using UV, IR, MASS and NMR analyzers according to the Handbook of Secondary Fungal Metabolites, Volume II.[1] The detection of antibacterial activity towards bacteria and fungi was carried out by the paper-disk method.

3. Results and Discussion

3.1 *Collection of* Boletaceae *parasites and parasite strains*

Boletaceae parasites were collected in Okayama, Japan. These fungi were white and yellow (Fig. 2) and they exhibited various deformities. Growth inhibition of bacteria and fungi was observed. Therefore, these *Boletaceae* parasites were determined as *S. chrysospermum*, which is an anamorph strain of *Hypomyces chrysospermus*, on the basis of the lack of sexual organs and the many yellow confetto-like chlamydospores[2] (Fig. 2).

3.2 *Isolation and identification of strains isolated*

We selected two strains from the ten strains isolated. The two strains were isolated by streak culture from the white verticillium type of *Boletaceae* parasite. These strains showed good growth on CY-1 agar medium, but poor growth on Czapek-Dox agar medium. The surface of the colonies was initially the verticillium type of white conidia such as aleuriospores and phialospores, and then it formed yellow confetto-like chlamidospores. This was observed on the CY-1 agar plate only (Fig. 2). These strains were identified as *S. chrysospermum* since the sexual organs present during the life cycle were not observed. One strain that was isolated was named *S. chrysospermum* NT-1. The optimal temperature for the cultivation of this strain was 28°C. Sepedonin production has been investigated on Czapek-Dox and Raulin-Thom media.[3]

3.3 *Production conditions of sepedonin*

Sepedonin-like pigment was produced from almost all sugars (Fig. 3). In the flask cultivation, polypeptone was present as a nitrogen source and yeast extract as a growth factor. Sepedonin-like pigment was produced at about 34 mg per 2 g of glucose (Fig. 4, p. 4). The optimum production conditions of sepedonin-like pigment were as follows: temperature, 28°C; carbon source, sugars; amount of sugar, 2.0 (w/w)%; nitrogen/polypeptone 0.2 (w/w)%; forced aeration, 1 L of air per 1 L of medium per minute. Aeration influenced the production of this pigment. In the above conditions, useful sugars for the production of sepedonin-like pigment were xylose, fructose, galactose, glucose, mannose, saccharose, starch and dextrin (Fig. 3). After the culture broth was diluted with 50 mM Tris-HCl buffer solution (pH 7.1), the absorbance at a wavelength of 382 nm was measured to detect the sepedonin-like pigment produced. The production period in the culture reflected that this pigment was a secondary metabolite (Fig. 4). This pigment was extracted by ethyl acetate. The Rf value by TLC of this pigment, which was yellow, was 0.08 (Fig. 4). After 8 days

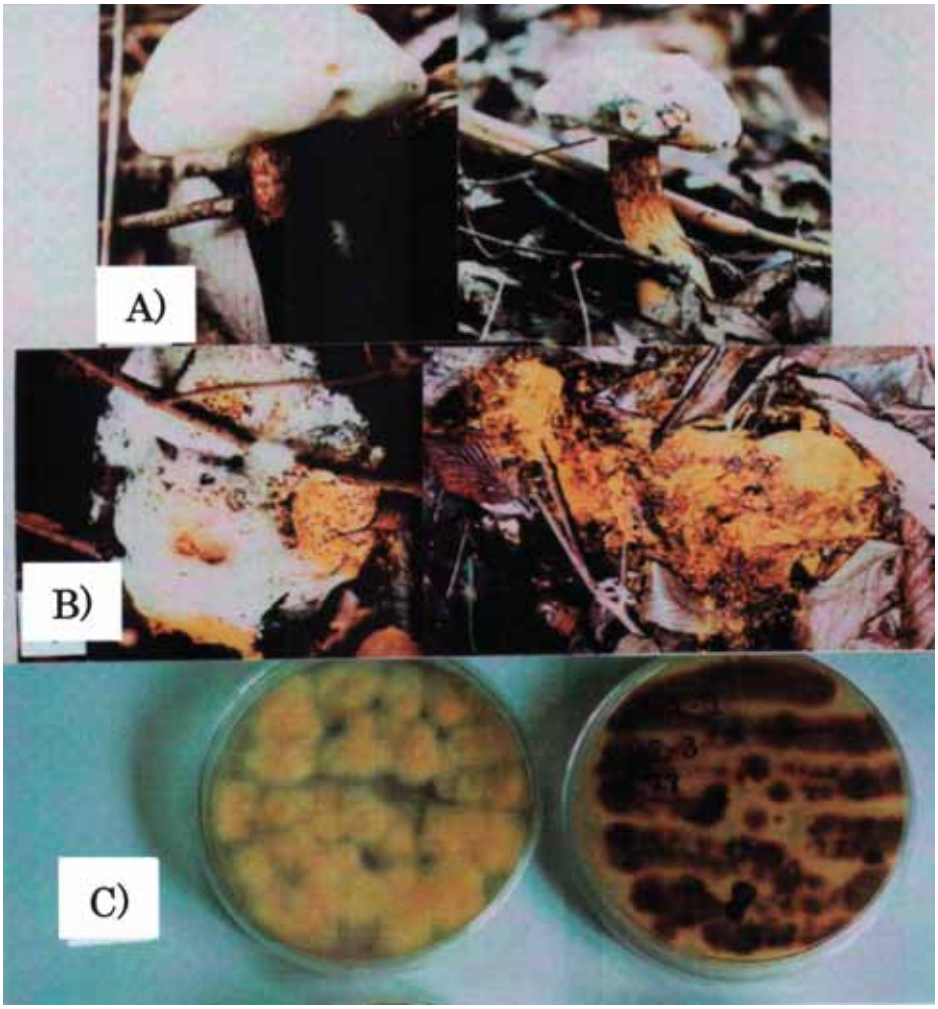


Fig. 2

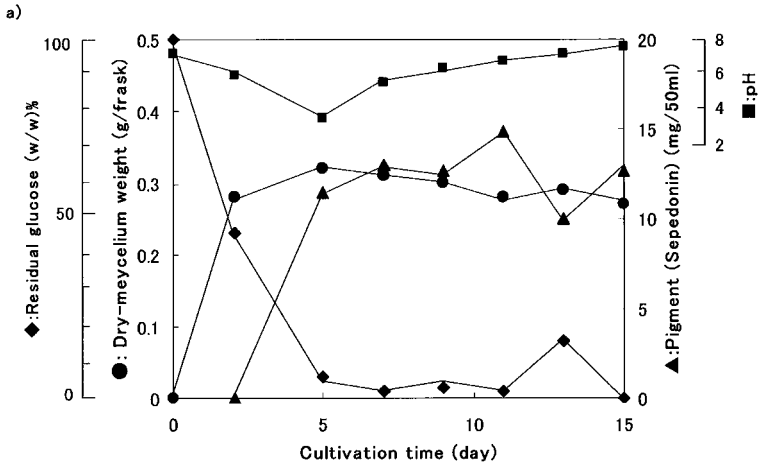


Fig. 4 (a)

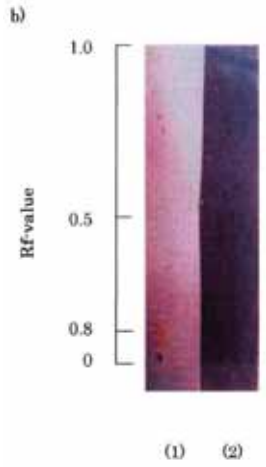


Fig. 4 (b)

Fig. 2. *S. chrysospermum* as a *Boletaceae* parasite and isolated stain *S. chrysospermum* NT-1. A) Verticillium-type. B) Formation of chlamydospore. C) Isolated strain *S. chrysospermum* NT-1 (sepedonin production).

Fig. 4. Time course of growth and sepedonin production. a) Time course, cultivation conditions: 50 ml of CY-1 medium/500-ml Sakaguchi flask. b) TLC of sepedonin extracted by ethyl acetate after 8 days: (1) negative (original extract); (2) sprayed with BPB solution.

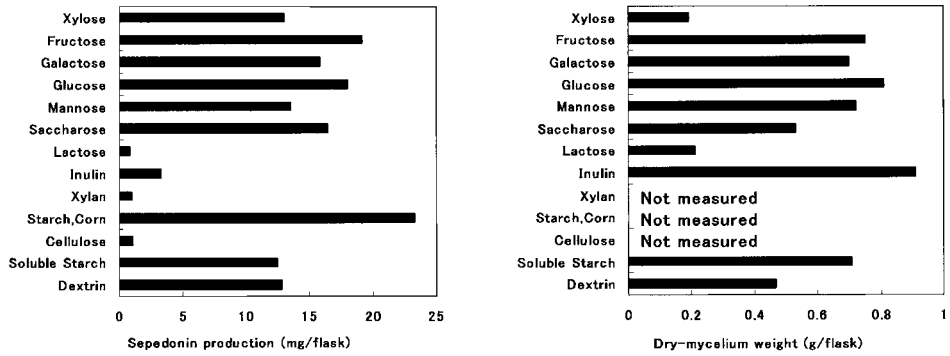


Fig. 3. Effect of sugars on sepedonin production. 2 g of sugar in 300-ml flask containing 100 ml of medium.

of TLC separation, the extracts of this spot were nonacidic. This was also shown by spraying BPB solution on the TLC film. Almost all amounts in the extracts resulted from sepedonin-like compounds. Reaction with the spot of $FeCl_3$–methanol solution was positive (deep-green). This reaction occurred between the adjacent hydroxyl group and the ketone group in the ring. This yellow pigment was extracted with ethyl acetate and then dried by evaporation. The dry compound was dissolved in ethyl acetate and then cooled. Yellowish plate crystals were observed. The product was identified as sepedonin by UV, IR, MASS and NMR according to the Handbook of Secondary Fungal Metabolites, Volume II.[1] The product in the culture broth was predominantly sepedonin. The quantity of sepedonin produced compared to that in a previous study.[3]

Forced aeration in the flask cultivation was necessary. Therefore, we tried jar fermenter and airlift-type fermenter cultivations. The results of these cultivations are shown in Table 1. For effective aeration, the airlift-type culture was better than the flask and jar fermenter cultivations. After 8 days of growth on CY-1 medium with the airlift-type cultivation, the yield of sepedonin was about 34 mg per 2 g of glucose.

3.4 *Physiological significance of sepedonin production by* S. chrysospermum

Antimicrobial activity was examined for gram-positive and gram-negative bacteria, yeast and molds (Table 2). The growth of many bacteria, yeasts and molds was inhibited by sepedonin. Accordingly, sepedonin produced by the *Sepedonium* genus (a *Boletaceae* parasite) could be a depressed putrefaction of the fruiting body. Therefore, *S. chrysospermum* showed better growth than other microorganisms on the fruiting bodies of *Boletaceae*.

Table 1
The sepedonin production by cultivation methods.

	Sakaguchi flask		Jar fermenter	Airlift type fermenter
Medium	25 ml	50 ml	8 L	1 L
Cultivation day	5	5	6	8
Glucose added (g)	0.5	1	160	20
Yield				
/glucose added (%)	1.3	0.8	0.51	1.7
/glucose consumed (%)	1.3	0.8	1.8	1.7

%: (w/w)%. The conditions of cultivation were as follows: reciprocal shaking speed, 110 strokes/min; span, 6 cm; 500-ml sakaguchi flask or jar fermenter; aeration, 1 L/1 L medium/min; rotation speed, 2×10^2 rpm; and airlift fermenter; aeration speed, 1 L/1 L medium/min.

Table 2
The relative antimicrobial activity of sepedonin.

	Strain used	Relative anti-microbial activity
Prokaryote		
Gram-positive	*Bacillus subtilis* RM125	100
bacteria	*Bacillus circulans* IAM1105	75
	Staphylococcus aureus IAM1098	56
Gram-negative	*Escherichia coli* C600	39
bacteria	*Escherichia coli*	88
	Pseudomonas aeruginosa PAO590	38
	Azotobacter vinelandii IFO13581	50
	Alcaligenes faecalis AFK2	56
	Klebisiella aerogenes W70	18
Eukaryote		
Yeast	*Saccharomyces cerevsiae* HUT7099	75
	Candida albicans	100
Mold	*Mucor racemosus*	39
	Aspergillus oryzae	100
	Neurospora crassa	100
	Penicillium chrysogenum	100

Paper-disk method (ϕ8 mm).
Sepedonin used: 2.5 mg per paper disk.

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