A NEW ALKALOID AM-2282 OF *STREPTOMYCES* ORIGIN TAXONOMY, FERMENTATION, ISOLATION AND PRELIMINARY CHARACTERIZATION

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(Received for publication February 14, 1977)

AM-2282, a new alkaloid has been isolated from cultures of *Streptomyces* sp. AM-2282 by solvent extraction and silica gel chromatography. The compound exhibits a strong absorption maximum at 292 nm and shows antimicrobial activity against fungi and yeast. The LD_{50} of its hydrochloride (i.p. in mice) is 6.6 mg/kg. The molecular formula of AM-2282 has been determined as $C_{28}H_{26}N_4O_3$. The producing strain, AM-2282 was classified as a new species and the name, *Streptomyces staurosporeus* AWAYA, TAKAHASHI and ŌMURA, nov. sp. is proposed.

We have previously reported on the isolation of new alkaloids such as pyrindicin[1], NA-337A[2], TM-64[3] *etc.* from actinomycetes. The continuing search for the alkaloid productivity of actinomycetes led to the discovery of AM-2282, a basic compound having antimicrobial and pharmacological activity. The producing organism, *Streptomyces* sp. AM-2282, was isolated from a soil sample obtained in Iwate Prefecture, Japan.

The present paper deals with taxonomy of the producing strain, fermentation, isolation, and physico-chemical and biological properties of the new alkaloid.

Taxonomy

The morphology of spore chains and spores developed on yeast extract-malt extract agar was observed using light microscopy and electron microscopy (JEM-100U, JEOL Co., Ltd.), respectively. The cultural characteristics were determined by the methods of WAKSMAN[4], and SHIRLING and GOTTLIEB[5]. Each culture was incubated at 27°C for 14 days except for the case of skimmed milk (37°C) and gelatin(22°C). Aerial mass colors were determined by comparison with Color Harmony Manual[6].

1. Morphological Characteristics

Strain AM-2282 forms well-developed aerial mycelia with simple branching and straight spore-bearing hyphae (Plate 1). Sporangia and flagellated spores are not observed. Spores are cylindrical or oblong, 0.4~0.6 μ by 1.8~2.4 μ, with smooth surface (Plate 2). Sclerotic granules are produced on some organic agar such as yeast-malt agar and range in diameter, from 10 to 30 μ (Plate 3).

2. Cultural and Physiological Characteristics

Cultural and physiological characteristics of the strain on various media are listed in Tables 1 and 2, respectively. The utilization of carbon source by the strain is shown in Table 3. The cultural and physiological characteristics can be summarized as follows: Growth is grayish white to pale yellowish orange or redish yellow; aerial mass colors variegate white, yellowish or pinkish color on various agar media; melanoid pigments are not formed in peptone-yeast-iron agar, tyrosine agar, and tryptone-

Plate 1. Sporophores of *Streptomyces* sp. AM-2282 on oatmeal agar (×55.0)

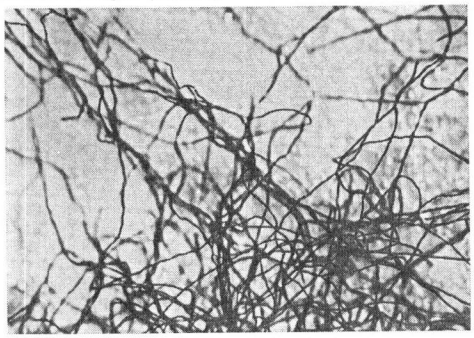


Plate 2. Spores of *Streptomyces* sp. AM-2282 on oatmeal agar (×3583)

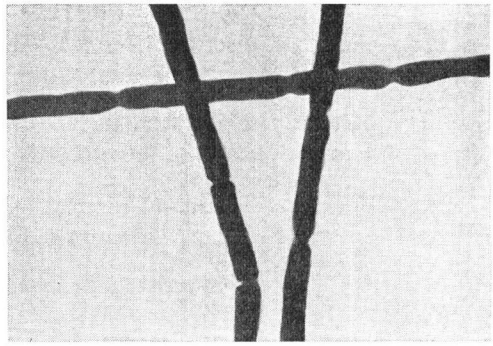
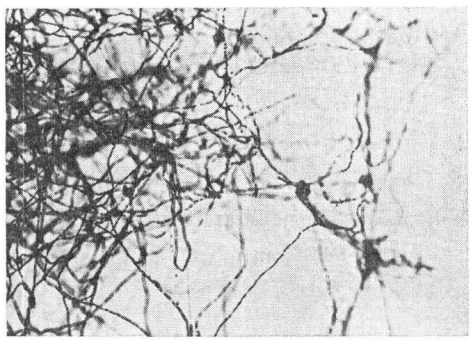
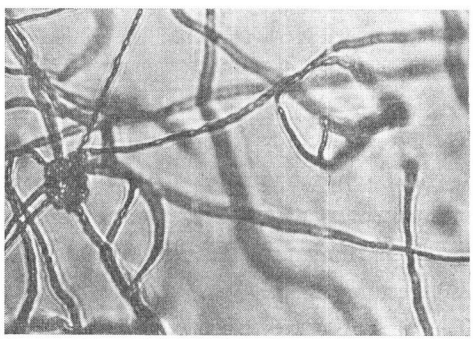


Plate 3. Sclerotic granules, dotting the aerial mycelium of *Streptomyces* sp. AM-2282 on yeast-malt agar (A: ×56, B: ×224)

A



B



yeast broth. When these descriptions were compared with those of known series of *Streptomycetes* described in "BERGEY's Manual of Determinative Bacteriology (8th, ed.)"[7] and other sources, an agreement was found in the following five species: *Streptomyces roseofulvus* PRIDHAM *et al.* 1958[8], *Streptomyces pluricolorescens* OKAMI, and UMEZAWA 1961[9], *Streptomyces moderatus* REUSSER 1967[10], *Streptomyces baarnensis* PRIDHAM *et al.* 1958[8] and *Streptomyces tolypophorus* SHIBATA *et al.* 1971.[11] However, these strains were distinguished from strain AM-2282 by the following descriptions of their morphological and cultural characteristics.

The spore of *S. roseofulvus* is about 1 μ, and pale grayish yellow or pale brownish gray pigment is found in yeast extract-malt extract agar, oatmeal agar and salts-starch agar. This strain utilizes D-xylose, D-fructose and rhamnose. The spore of *S. pluricolorescens* is also short (about 1 μ) and the reverse side of the colony appears grayish yellow or yellowish brown on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar. Aerial mycelium of *S. moderatus* appears grayish yellow pink on yeast-malt agar, oatmeal agar and salts-starch agar, and it produces purple soluble pigment. *S. baarnensis* and *S. tolypophorus* resemble *Streptomyces* sp. AM-2282 in having long spore. However, *S. baarnensis* has poor aerial mycelia, its color is white to gray on various organic media and the reverse side of colony appears light ivory. *S. tolypophorus* has poor and white aerial mycelia, and the reverse side of the colony has no distinctive pigment on salts-starch agar and has an orange yellow pigment on calcium-malate agar. In carbon-source utilization notable growth is observed with fructose, xylose and rhamnose.

On the basis of the above data, it is reasonable to conclude that *Streptomyces* sp. AM-2282

Table 1. Cultural characteristics of *Streptomyces* sp. AM-2282

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	good, melon yellow (3ga)	light melon yellow (3ea)	poor, velvety, light melon yellow(3ea)	light melon yellow (3ea)
Glucose-nitrate agar	good, wrinkled, melon yellow (3ga)	light melon yellow (3ea)	poor, velvety, light melon yellow(3ea)	light melon yellow (3ea)
Glycerol-calcium malate agar	good, light melon yellow(3ea)	pearl pink (3ca)	moderate, velvety, pearl pink (3ca)	—
Glucose-asparagine agar (ISP)	good, apricot (4ga)	light apricot (4ea)	moderate, velvety, flesh pink (4ca)	—
Glycerol-asparagine agar (ISP)	good, light melon yellow (3ea)	light melon yellow (3ea)	moderate, velvety, white	—
Inorganic salts-starch agar (ISP)	good, light melon yellow(3ea)	pearl pink to light tan (3gc)	abundant, velvety, white to pearl pink(3ca)	—
Tyrosine agar (ISP)	good, light melon yellow(3ea)	light ivory (2ca)	moderate, velvety, pearl pink (3ca)	—
Nutrient agar	good, bamboo (2fb)	maize (2hb)	poor, velvety, ivory tint (2cb) to pearl pink (3ca)	bamboo (2fb)
Glucose-peptone agar	moderate, wrinkled, light maize (2ea)	melon yellow (3ga)	moderate, velvety, flesh pink (4ca)	—
Yeast extract-malt extract agar (ISP)	good, amber (3pc)	melon yellow (3ia)	moderate, velvety, white to pearl (3ba)	—
Oatmeal agar (ISP)	good, light melon yellow (3ea)	light melon yellow (3ea)	moderate, velvety, white to pearl pink (3ca)	—
Peptone-yeast extract-iron agar (ISP)	good, ivory (2db)	maize (2hb)	—	—
Tryptone-yeast extract broth (ISP)	surface growth light ivory (2ca)		light ivory (2ca)	colonial yellow (2ga)

Table 2. Physiological properties of *Streptomyces* sp. AM-2282

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	−
Hydrolysis of starch	+
Liquefaction of gelatin	+
Peptonization of milk	+
Coagulation of milk	±
Cellulolytic activity	±
Temp. range for growth	20～40°C

Table 3. Utilization of carbon sources by *Streptomyces* sp. AM-2282

Response	Carbon source
Positive	L-Arabinose, D-mannitol, sucrose, D-glucose, *i*-inositol, raffinose
Doubtful	D-Xylose, D-fructose, rhamnose

is classified as a new species of *Streptomyces*, and the name, *Streptomyces staurosporeus* AWAYA, TAKAHASHI and ŌMURA, nov. sp. is proposed. The proposed species epithets *"stauros"* and *"spora"* are the Greek nouns meaning "stave" and "spore", respectively.

A detailed characterization of the new taxon follows: *Streptomyces staurosporeus* AWAYA, TAKAHASHI and ŌMURA, nov. sp.

Spore chain morphology: Section *Rectiflexibiles*. Spore chains moderately long, usually 10 to 50, or often more than 50, spores per chain. This morphology is seen on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar. Many sclerotic granules (10～30 μ) are formed on yeast-malt agar. Spores are cylindrical or oblong, 0.4～0.6 μ by 1.8～2.4 μ.

Spore surface: Smooth.

Color of colony: Aerial mass color in the white or red color-series on oatmeal agar, salts-starch agar and glycerol-asparagine agar; white or yellow color-series on yeast-malt agar.

Reverse side of colony: No distinctive pigment.

Color in medium: Melanoid pigments are not formed in peptone-yeast-iron agar, tyrosine agar and tryptone-yeast broth. Pigments other than melanoids not formed in yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Carbon utilization: D-Glucose, L-arabinose, D-mannitol, sucrose, *i*-inositol and raffinose are utilized for growth.

Mesophilic.

Aerobic.

Habitat: Soil.

Type strain: Strain, AM-2282 is designated as the type of this species and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan, and assigned as *Streptomyces* sp. AM-2282 with an accession number of FERM-P 3725.

Fermentation

The culture of *Streptomyces* sp. AM-2282 was maintained on glucose-asparagine agar or as freeze-dried stock.

Several sources of carbon and nitrogen were examined for production of AM-2282. The best ones were glucose as a carbon source and soybean meal as a nitrogen source (Table 4).

The stock culture was inoculated in a 500-ml SAKAGUCHI's flask containing 100 ml of a seed medium composed of 2.0% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dried yeast, and 0.4% calcium carbonate (pH adjusted to 7.0 prior to sterilization), and cultured for 2 days at 27°C. The seed culture (400 ml) was transferred into a 30-liter jar fermentor containing 20 liters of the fermentation medium composed of 3.0% glucose, 1.5% soybean meal and 0.4% calcium carbonate (pH adjusted to 7.0 prior to sterilization). It was maintained for about 65 hours at 27°C under the following conditions: Temperature, 27°C; aeration, 10 liters/min; agitation, 250 r.p.m.; and pressure, 0.5 kg/cm^2. Adecanol LG-109 (Asahi Electro-Chemical Co., Ltd.) was used as antifoam agent.

A large-scale fermentation was carried out using a 400-liter tank fermentor containing 200 liters of the fermentation medium described above.

The titer of alkaloid accumulated in the culture medium was determined as following. After the culture broth (50 ml) was made basic with aqueous ammonia, the alkaloid was extracted with one half volume of butyl acetate and then transferred into one-fifth volume of 0.1 N hydrochloric acid. The water layer was diluted ten times with methanol. From the OD values of the acidic methanol measured by UV-spectrometer at 292 nm, the amounts of alkaloid accumulated in the culture broth were calculated.

A typical time course of AM-2282 production in a 400-liter tank fermentor is shown in Fig. 1. The concentration of the alkaloid produced reached a maximum about 45 hours after incubation when 25 mcg/ml of the compound was observed in the broth.

Isolation

Cultured broth (200 liters) of *Streptomyces* sp. AM-2282 obtained by incubation in a 400-liter tank fermentor was used as starting material for the isolation of alkaloid AM-2282; the titer of the broth was 23 mcg/ml.

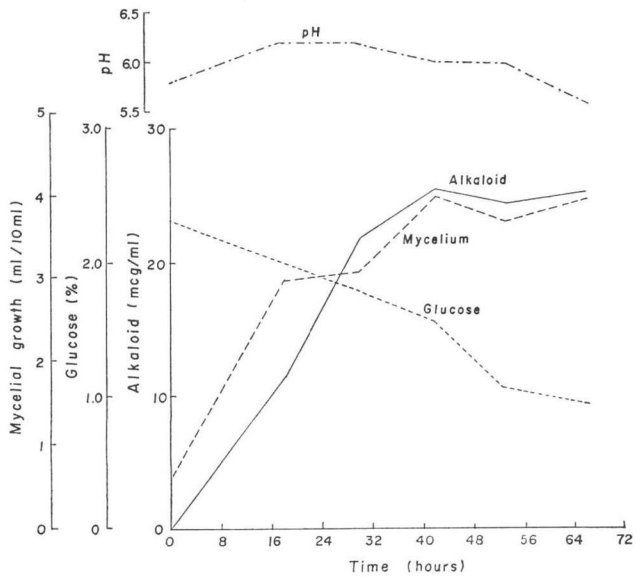
The broth containing mycelia was adjusted to pH 10 with aqueous ammonia. The alkaloid produced was extracted with 60 liters *n*-butyl acetate and then transferred into 30 liters 0.1 N hydrochloric acid. The water layer was subsequently adjusted to pH 10 with aqueous ammonia and extracted twice with 8 liters of ethyl acetate. The combined extracts were dried with anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and then chromatographed on silica gel (120 g, Merck, Kieselgel G) eluting with a solvent mixture of chloroform and methanol (60: 1, v/v). Alkaloid fractions, which gave a positive test with DRAGENDORFF's reagent and whose Rf value was 0.55 on silica gel thin-layer chromatography, eluted with chloroform and methanol (10: 1, v/v), and were collected and evaporated to dryness *in vacuo* to yield yellowish powder (1.7 g). The powder was recrystallized from chloroform - methanol (50: 1, v/v) mixture to afford pale yellow needles (1.1 g) of alkaloid AM-2282. The hydrochloride of the compound for biological tests was prepared as following. After the crystals were dissolved in anhydrous chloroform, dry hydrogen chloride gas was introduced into the solution until no more precipitate formed. A pale yellow powder (AM-2282 hydrochloride)

Table 4. Effects of various carbon and nitrogen sources on production of AM-2282

Carbon sources (%)	Nitrogen sources (%)	pH	Mycelial growth ml/10ml	Titer of alkaloid mcg/ml
glucose 3.0	yeast extract 0.5 peptone 0.5	6.6	2.3	7.4
starch 3.0	〃	6.6	1.9	2.6
glycerol3.0	〃	6.6	1.2	1.8
oatmeal3.0	〃	6.7	6.5	6.0
glucose 1.0 starch 2.0	yeast extract 0.5	6.8	1.8	2.9
〃	〃 1.0	6.7	3.1	4.3
〃	peptone 0.5	6.6	1.4	4.5
〃	〃 1.0	6.7	0.9	2.3
〃	meat extract 0.5	6.8	1.0	2.0
〃	〃 1.0	7.0	1.3	2.5
〃	corn steep liquor 0.5	6.3	1.0	2.1
〃	〃 1.0	6.5	1.2	2.7
〃	soybean meal 1.0	6.6	2.6	14.2
〃	〃 2.0	6.7	2.6	12.6

Each medium contained 0.4% $CaCO_3$ and was adjusted to pH 7.0 before sterilization.
Volume: 100 ml/500-ml SAKAGUCHI's Flask.
Cultured for 4 days at 27°C.

Fig. 1. A typical time course of AM-2282 fermentation



was thus obtained.

Physical and Chemical Properties

Alkaloid AM-2282 is obtained as basic and lipophilic crystals. Its physical and chemical properties are summarized in Table 5. The molecular weight and the molecular formula of the compound were proposed on the basis of mass spectrometry of the alkaloid and its acetyl derivative.

A characteristic physical property of AM-2282 is its unique ultraviolet absorption (Fig. 2). The infrared spectrum in a KBr tablet is shown in Fig. 3. Conspicuous bands were observed at 3200~3500 cm^{-1} corresponding to amine and hydroxyl groups; 2850~3000 cm^{-1} corresponding to methyl and methylene groups; 1675 cm^{-1} to carbonyl and double bond groups. The PMR spectrum (Fig. 4) measured in DMSO-d_6 shows signals at δ 1.55, 2.30, 3.23, 2.9~3.3, and 7.5~8.5 ppm.

Biological Properties

Hydrochloride of AM-2282 was used for biological tests. The compound, isolated as an alkaloid, has also antimicrobial activity. Its activity was determined by conventional agar dilution method using nutrient agar for bacteria (37°C, 18 hours), and glucose-potato agar for fungi and yeast (27°C, 72 hours). As can be seen in Table 6, AM-2282 is active toward fungi and yeast, but it had no significant effects on bacteria. The acute toxicity (LD_{50}) of the compound by intraperitoneal administration in mice was 6.6 mg/kg.

Table 5. Physical and chemical properties of AM-2282

Appearance	Pale yellow crystals
Melting point	270°C (decomp.)
Elemental analysis	C 70.03%, H 6.03%, N 11.21%, no halogen, phosphorus
Molecular weight	466(M^+, *m/e*). acetyl derivative: 508 (M^+, *m/e*)
Molecular formula	$C_{28}H_{26}N_4O_3$
Optical rotation	$[\alpha]_D^{25}$ +35.0° (*c* 1, MeOH)
Ultraviolet absorption	λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$), 243 (537), 267 (sh. 552), 292 (1228), 322 (sh. 537), 335 (313), 356 (220), 372 (254)
Color reaction	Positive: ninhydrin, RYDON-SMITH, DRAGENDORFF, MOLISCH, EHRLICH Negative: BEILSTEIN, aniline phthalate, ferric chloride
Solubility	Soluble in dimethyl sulfoxide, dimethyl formamide Slightly soluble in chloroform, methanol
Rf values on silica gel TLC (Merck, kieselgel G)	$CHCl_3$ - MeOH (10: 1) 0.55 Benzene - acetone (1: 2)0.43 Butanol - acetic acid - water (8: 1: 1) 0.24

Discussion

It is known that some alkaloids, such as nigrifactin[12,13], abikoviromycin[14,15], pyrindicin[1], NA-337A[2] and TM-64[3] are produced by actinomycetes. AM-2282 was differentiated from these alkaloids by comparison of IR spectra and thin-layer chromatographic behavior. Since AM-2282 is an alkaloid having antimicrobial activity and characteristic ultraviolet absorption at 292 nm, it was also compared with other known antibiotics such as oxamicetin[16] and pyridomycin[17,18]

Fig. 2. UV spectra of AM-2282

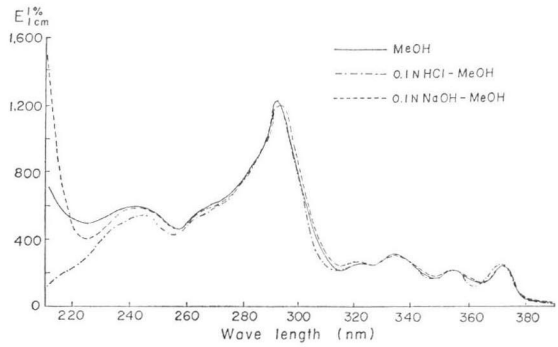


Fig. 3. Infrared spectrum of AM-2282 (KBr)

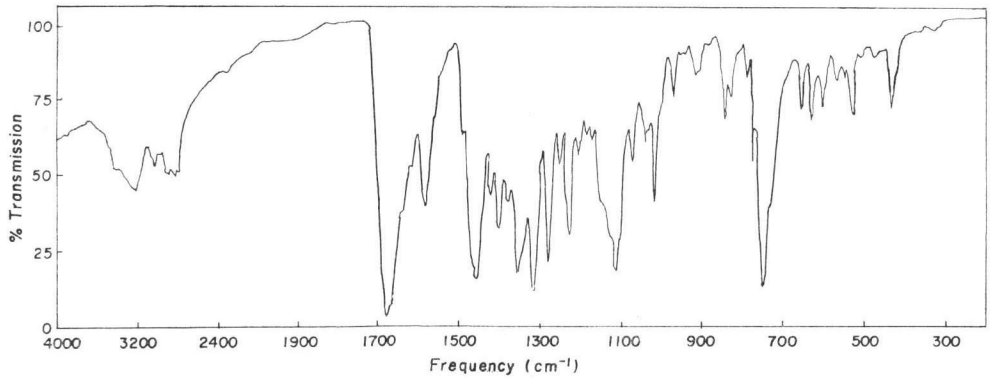
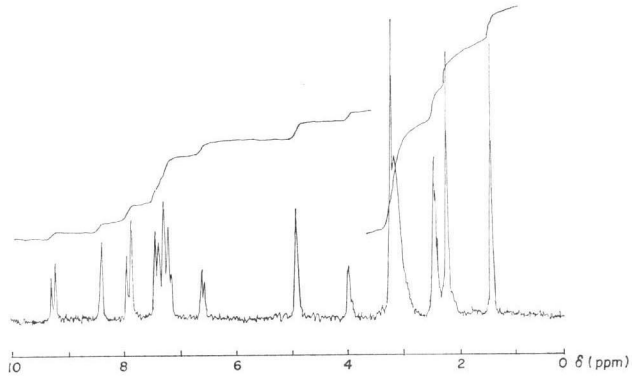


Fig. 4. NMR spectrum of AM-2282 (100 MHz, DMSO-d_6)



which have ultraviolet absorption around 292 nm. However, none of their physico-chemical properties were identical with those of the compound. Consequently, it is reasonable to conclude that AM-2282 is a novel alkaloid.

In addition, the compound was found to possess strong hypotensive activity by primary test. Further investigation on its pharmacological activities is now in progress.

Acknowledgements

The authors wish to thank Asahi Chemical Industry Co., Ltd. and Toyo Jozo Co., Ltd. for assay of biological activities. Thank is also due to Mr. K. MATSUMOTO of Kitasato Institute for his technical assistance.

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Table 6. The antibacterial spectrum of AM-2282

Test organism	MIC (mcg/ml)	Medium*
Staphylococcus aureus FDA 209P	>200	N
Bacillus subtilis PCI 219	>200	N
Sarcina lutea PCI 1001	25	N
Mycobacterium smegmatis ATCC 607	50	N
Escherichia coli NIHJ	>200	N
Pseudomonas aeruginosa P-3	>200	N
Proteus vulgaris IFO 3167	>200	N
Xanthomonas oryzae	200	N
Candida albicans	6.25	P
Candida pseudotropicalis	3.13	P
Saccharomyces sake	3.13	P
Aspergillus niger	25	P
Aspergillus brevipus	3.13	P
Aspergillus fumigatus	12.5	P
Trichophyton rubrum	6.25	P
Trichophyton mentagrophytes	25	P
Cryptococcus neoformans	50	P
Microsporum gypseum	>100	P
Sclerotinia cinerea	0.78	P
Piricularia oryzae	0.78	P

*N: peptone 0.5%, meat extract 0.5%, agar 1.2%, pH 7.0.
P: potato extract containing glucose 1.0% and agar 1.2%, pH 6.8.

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