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Antifungal antibiotic effect of saccharides produced by the *Bacillus sp*. of YJH-1 isolated from soils in Korea

Jae Hong Yoo

National Institute of Agricultural Sciences and Technology, Rural Development Administration, Suwon 441-707, Republic of Korea

Abstract

After isolating and screening the antifungal strain from the soil in Korea, effective antifungal strains were selected after testing the paper disk method. A strain was selected after it was found highly effective against *Pyricularia oryzae* and *Pellicularia filamentosa* and named as YJH-1. For morphological characteristics of YJH-1 were rod-shaped, Gram positive and has a good motility. The temperature for the growth of YJH-1 ranged from 15~50^{o}C, pH ranging from 3~10, and NaCl concentration of 6%. Physiological characteristics of YJH-1 showed positive catalase, negative oxidase, and negative propionate and also hydrolyzed starch. All sugar components except cellulose were utilized for the growth of YJH-1. The antifungal antibiotic was extracted three times using *n*-butanol from the culture broth of *Bacillus* sp. Bioassay-guided column chromatography with silica gel and Sephadex LH-20 yielded 62 mg of the original active compound from 1 litre of culture broth. The minimal inhibitory concentration (MIC) values were 25 µg/ml and 50 µg/ml against *Pyricularia oryzae* and *Pellicularia filamentosa* respectively. Based on results from the structure analysis of the antibiotic using MS, NMR and IR spectroscopy, the antifungal antibiotic was found to consist of only six of fructose.

Keywords: Antifungal; saccharide; *Pyricularia oryzae*; *Pellicularia filamentosa*

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Introduction

Antibiotics have long been widely studied since Fleming discovered in 1929 that the growth of *Staphylococcus aureus* was inhibited by *Penicillium notatum.* Thereafter, the antibiotic studies have actively been researched and various types of antibiotics with effects on a wide spectrum of pathogens have been developed (Pelaez, F 2006). Even with development of the antibiotics with significant effects on prokaryotic pathogens, the antibiotics with effects on eukaryotic pathogens such as fungal infections have not been much satisfactory up to date (Xiong et al., 2000). The treatment and prevention of pathogenic fungal infections in human and plants were initially studied by Elizabeth and Brown (1950) and they isolated fungicidin from soils which were effective against *Candida albican* and *Cyptococcus neoformans*. Griseofulvin isolated from *Penicillium griseofulvum* was proven its effect on plant pathogens and amphotericin B was found antibiotic to yeast (Donovick et al., 1956). It was reported pyrrolnitrin produced by *Pseudomonas pyrrocinia* was effective against yeast and Gram positive bacteria by Arima et al. (1965). Isono et al. (1965) found polyoxin has no toxicity on livestock, human, fish and crops but has antifungal effects on rice pathogens. For antibiotics used in rice pathogens especially for the rice blast, Umezawa et al. (1965) found kasugamycin that well infiltrated the rice cells with prevention and treatment effects on those pathogens. Thereafter, antibiotics with low-toxin components but well-controlling effects on fungi and yeasts such as aculeacin (Mizuno et al., 1977) and mycoversilin (Samanta et al., 1984) with the minimum-

Corresponding author: Jae Hong Yoo, National Institute of Agricultural Sciences and Technology, Rural Development Administration, Suwon 441-707, Republic of Korea.
Phone: +81-31-290-0379; Fax: +82-31-290-8488

growth inhibition value for the filamentous fungi, *Trichophyton rubrum*. A nucleoside antibiotics of dapiramicin produced by *Micromonospora* was found effective for the sheath blight in rice by Nishizawa et al. (1984). And also, various types of antibiotics with antifungal effects such as albopeptin a (Isono et al., 1965), fengycin (Vanittanakom et al., 1986), and octacosamicin (Dobashi et al., 1988) were developed. Among those antibiotics, large quantities of kasugamycin, validamycin, and polyoxin have been imported up to date. In Korea, the pesticide products have not been much developed and most of them are being imported, which causes a high cost to domestic users. One of the highly significant problems in use of pesticides is the residue of organic pesticide that smeared into the soils, causing the pesticide poisons to human. The toxin residue also is one of the most important social issues. The organic chloride products such as dichlorodiphenyltrichloro ethane (DDT) and benzene hexachloride (BHC) was already found to cause cancers and seriously threatened human health. In this study, I have isolated and screened the microbic strains from the soils in Korea that have antifungal effects by analyzing the structure of the antifungal components to develop the potential bio-pesticides as was described in the study of Walsh (2003).

Materials and Methods

Isolation of antifungal microorganisms

The microorganisms were isolated and screened from soils in Korea. They were inoculated into NB liquid broth (meat extract 0.3%, peptone 0.5%) and cultivated in the rotary shaker for 3 days at 30°C. The cultivation broth was centrifuged at 12,000×g for 20 min and then, *Pyricularia oryzae* IFO 30517 and *Pellicularia filamentosa* IFO 8985 were used as test strains. The supernatants of the broth were tested for their antifungal effects by the paper disk method. Among those test strains, those with significant antifungal effects were screened and used in this study.

Identification of the antifungal antibiotic strains

The identification of strains was done following the methods described in Manual of Methods for General Bacteriology (Gerhardt et al., 1981), Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974), and Microbiological Methods (Collins et al., 1976).

Isolation and purification of antibiotic

A volume of 250 ml *of Bacillus sp.* in an optimal medium (3.0% soluble starch, 0.8% yeast extract, 0.2% KCl, pH 8.0) was cultivated in a 1 litre flask with 1% at 37°C for 72 h. The culture was centrifuged at 12,000 × *g* for 20 min, and the resulting supernatant was extracted with *n*-butanol three times. The n-butanol alcohol layer was decolorized with charcoal and concentrated *in vacuo*. The residue was redissolved in water and lyophilized to get crude powder. The crude powder was subjected to chromatography on silica gel. The major active compound was found in the MeOH:EtOH (1:1, v/v) eluate. This fraction was subjected to chromatography on the Sephadex LH-20 resin and eluted with MeOH: dichloromethane (5:1, v/v). After evaporation of the solvent *in vacuo*, a yellow powder was obtained.

Physio-chemical characteristics of the antifungal components

Thin layer chromatography

The antifungal solution was spotted onto the TLC plate and a mixed solvent was developed. And then, UV illuminator (UVGL-25, UVP) and spray of alkaline $KMnO_4$ were used to detect the spots.

Colour identification

After antifungal solution was spotted onto the TLC plate and developed in the n-BuOH-AcOH- H_2O (4:1:5, upper phase), colouring agent was sprayed and mixed with the antifungal solution for colour identification (Dawson et al., 1974).

Measurement of melting point

The melting point of the antifungal components was measured by the electrothermal device (Electrothermal Co.) at an increase of 1°C/min.

UV spectrum

After melting the antifungal components in the water (1mg/ml), UV/Vis spectro-photometer (Perkin-Elmer 552S) was used for the UV absorption spectrum.

IR spectrum

IR spectrum of the antifungal components was measured by the high-pressure method (KBr) using the IR spectrophotometer (Perkin-Elmer 1430).

NMR spectrum

H NMR and C NMR spectrum of the antifungal components were dissolved in D_2O under the conditions of 400MHz and 100MHz using the NMR spectrometer (Bruker Co.).

Mass spectrum

The antifungal components were acetylated and were measured by the mass spectrometer (Bruker Co.). The antifungal spectrum was conducted by the paper disc agar diffusion method. For bacteria and candida, 0.1 ml of the test solution was inoculated onto the soft agar (agar 0.8% (w/v)) and the overlay- plate was made. The spores of the fungi were evenly suspended and inoculated into 0.1 ml of PDA. And then, the overlay-plate was made. The solution of the antifungal (100μg/ml) was tested using the disk paper (diameter 8mm) for 24 to 48 hrs at 30°C to check the clear zones.

Table 1: Morphological properties of the strain YJH-1

Shape	Rod
Cell size	1.5μm×3.6μm
Motility	Positive
Gram stain	Positive
Flagella	Positive
Spore shape	Ellipsoidal
Spore position	Central
Sporangium swollen	Negative

Table 2: Cultural characteristics of the strain YJH-1

Form	Circular
Surface	Smooth
Edge	Undulate
Elevation	Umbonate
Opacity	Opaque
Colour	Creamy
Brilliancy	Glistening

Nutrient broth (30°C, 1-2days); Growth abundant, turbid with pellicle and sediment

Table 3: Physiological properties of the strain YJH-1

Temperature range for growth		15-50°C
pH range for growth		3-10
NaCl tolerance for growth		< 6%
Catalase		+
Oxidase		-
Urease		+
Lipase (Tween 80)		-
β-Galactosidase		+
Arginine dihydrolase		-
Phenylalanine deaminase		-
Hydrolysis of :	Starch	+
	Casein	+
	Cellulose	-
	Esculin	+
Indole production		-
H_2S production on TSI agar		-
Levan formation from sucrose		+
NH_3 production from peptone		+
NH_3 production from arginine		+
Gelatin liquefaction		+
Utilization of citrate		-
Utilization of propionate		+
Methyl red test		+
Voges-Proskauer reaction		+
Nitrate reduction		+
Denitrification		+
Action on milk :	Coagulation	-
	Peptonization	+
Hemolysis, human blood		+
O-F test		Fermentative
Degradation of tyrosine		-

+: Positive - : Negative

In vivo measurement of antifungal effects

After growth of rice until they have 3 to 4 leaves out, the pathogens of rice such as rice blast were artificially infected and then, the antifungal solutions of 100 μg/ml and 50 μg/ml were sprayed on the infected rice. After 14 days, the rice growth was compared with the control group vs. the treated groups.

Results

After isolating and screening the antifungal strain from the soil in Korea, effective antifungal strains were selected after testing the paper disk method. Among those strains, a strain was selected for its best effect on *Pyricularia oryzae* and *Pellicularia filamentosa* and was named as YJH-1. For morphological characteristics of YJH-1, it is rod-shaped, Gram positive, and has a good motility. The details of the characteristics were shown in Table 1.

Culture characteristics

The culture characteristics of YJH-1 culture are described in Table 2. Colonial form of YJH-1 in the agar plate was circular, creamy-coloured, shiny surface and slimy. In the liquid medium, YJH-1 well grew, formed pellicle, and showed the precipitation of the strain.

Physiological characteristics

The physiological characteristics of YJH-1 were well documented in Table 3. The temp for the growth of YJH-1 ranged from 15~50°C, pH ranging from 3~10, and NaCl concentration of 6%. YJH-1 showed positive catalase, negative oxidase, and negative propionate and well hydrolyzed the starch. All sugar components except cellulose were utilized for the growth of YJH-1 as described in Table 4. The starch fermentation was also examined. Acid was produced but gases were not produced from all the sugar sources, which was described in Table 5.

Identification of antifungal antibiotic

The antifungal activity of the compound was assessed using *Pyricularia oryzae* IFO 30517 and *Pellicularia filamentosa* IFO 8985 by using the cup method (Iwasa et al., 1971). The melted bottom-layer medium (0.1% sucrose, 1.0% beef extract, 1.0% peptone, 0.8% agar) was maintained either at 45~55°C with 0.5 ml of the *P. oryzae* IFO 30517 hyphal suspension added, or at 30°C with 0.5 mL of the *P. filamentosa* IFO 8985 hyphal suspension added, and then incubated for 48 h. The plate was overlaid with 10 ml of melted upper-layer medium (0.25% sucrose, 0.1% NaCl, 0.45% peptone, 1.2% agar). After the medium solidified, the cup (6 mm I.D. x 10 mm height) was placed on the surface of the upper layer and a solution of test materials was added inside the cup. The inhibition zone was measured after incubation for 48 hrs at 30°C. The minimal inhibitory concentration (MIC) was determined using the serial agar dilution method. For structure identification, the isolated compound was hydrolyzed with 1N HCl for 10 h at 100°C and was neutralized with 1N NaOH. The reaction mixture was developed by thin layer chromatography (TLC) with a

Table 4: Sugar utilization by the strain of YJH-1

Sugar	Utilization
Arabinose	+
Cellobiose	+
Cellulose	-
Dextrin	+
Fructose	+
Galactose	+
Glycerol	+
Inositol	+
Inulin	+
Lactose	+
Mannitol	+
Mannose	+
Raffinose	+
Soluble starch	+
Sorbitol	+
Sucrose	+

+ : Utilized - : Not Utilized

Table 5: Sugar fermentation by the strain YJH-1

Sugar	Acid	Gas
Arabinose	-	-
Cellobiose	+	-
Cellulose	-	-
Dextrin	-	-
Fructose	+	-
Galactose	-	-
Glycerol	+	-
Inositol	-	-
Inulin	-	-
Lactose	-	-
Mannitol	+	-
Mannose	+	-
Raffinose	+	-
Soluble starch	-	-
Sorbitol	-	-
Sucrose	+	-

+: Positive - : Negative

developing solution consisting of *n*-BuOH: AcOH:H_2O (4:1:5, v/v). The spot was observed after spraying with alkaline $KMnO_4$ solution. Seliwanoff reagent was added to the hydrolysate, fructose, and glucose solutions, and the colour change was evaluated. The hydrolysate and standard sugar were injected into a XBridge Amide HPLC column (Waters, Milford, MA), with a refractive index detector, following filtration with a membrane filter. The column used was specific for carbohydrate analysis. The mobile phase was 85:15 (acetonitrile:water, v:v) at a flow rate of 2 ml/min. The *n*-butanol extract from culture broth of *Bacillus* sp. showed an inhibition zone of 34 mm for *P.. oryzae* and 31 mm for *P. filamentosa*. After purification with silica gel and Sephadex LH-20 column chromatography, one spot was shown by TLC. The purification yield was 29% and the purification fold was 12. Inhibition zones of the isolated compound for *P. oryzae* were 15.2, 19.7, 24.5, 28.1, and 32.2 mm at concentration of 62.5, 125, 250, 500, and 1000 µg/ml, respectively. There are many antifungal compounds for these two particular fungi that have been reported, such as octacosamicin (Dobashi et al., 1988), fengycin-A (Vanittanakom et al., 1986). The MIC of the isolated compound was comparable to those of these compounds. From the TLC results following hydrolysis of the isolated compound, there was one spot at R_f = 0.30, the same as fructose. The Seliwanoff test is a chemical test that is used to distinguish between aldose and ketose sugars. The isolated compound showed a positive result from the Seliwanoff test, indicating that this chemical contains fructose, not glucose. The hydrolysate from the isolated antifungal compound, fructose, and hydrolyzed Hexocin were injected into the HPLC column. Hexocin is a six-saccharide consisting only of fructose. Each sample showed a peak at the same retention time. The UV spectrum of the antibiotic showed no absorption from 200 to 400 nm. The isolated compound had strong, broad absorption at frequencies of 2800–3700 and 1100–1700/cm, and the IR spectrum indicated that there were no specific functional groups, such as an aromatic ring, except a hydroxyl (OH) group present. MS analysis of the isolated antibiotic showed an M^+ peak at m/z 989. This matched with the mass of a hexamer of sugar. ^{13}C NMR spectrum showed six anomeric carbons at δ of 100.63, 100.50, 100.43, 100.31, 96.63, and 92.74 ppm. Based on these results, the antifungal compound produced from the *Bacillus sp.* is a homo-oligosaccharide consisting of only six fructose.

Activity of *in vivo* test

This chemical has the *in vivo* antifungal activity against *P. oryzae* and *P. filamentosa* in rice plants at concentrations of 50 and 100 µg/ml, respectively as shown in Fig. 1. This compound could be very useful for agricultural purposes.

Discussion

After examining the optimum mass production condition, the antifungal antibiotic was best produced at pH 8 at 30°C. The sorbistin production by *Pseudomonas sorbicini* was maximized at temp of 28°C. Nakajima et al. (1991) also reported that cornexistin produced by *Paecilomyces variotii* was best when pH in the early stage of the cultivation was 6.0. Antibiotic produced by bacteria, e.g., *Bacillus sp,* was most effective when pH in the early cultivation stage was 7.0 (Vanittanakom et al., 1986) and 7.0 for *Pseudomonas genus* (Nielsen et al., 2000, Isnansetyo et al., 2009). In my study, pH levels mostly ranged around 8.0. With addition of 3% (w/v) of soluble starch, the maximum production of antifungal anitibiotic was observed. However, the effects were decreased when the soluble starch added was over 3% (w/v). As reported by Kawai et al. (1983), the antibiotic of arugomycin was maximum produced when 2% (w/v) of soluble starch was added (El-Enshasy et al., 2008). For

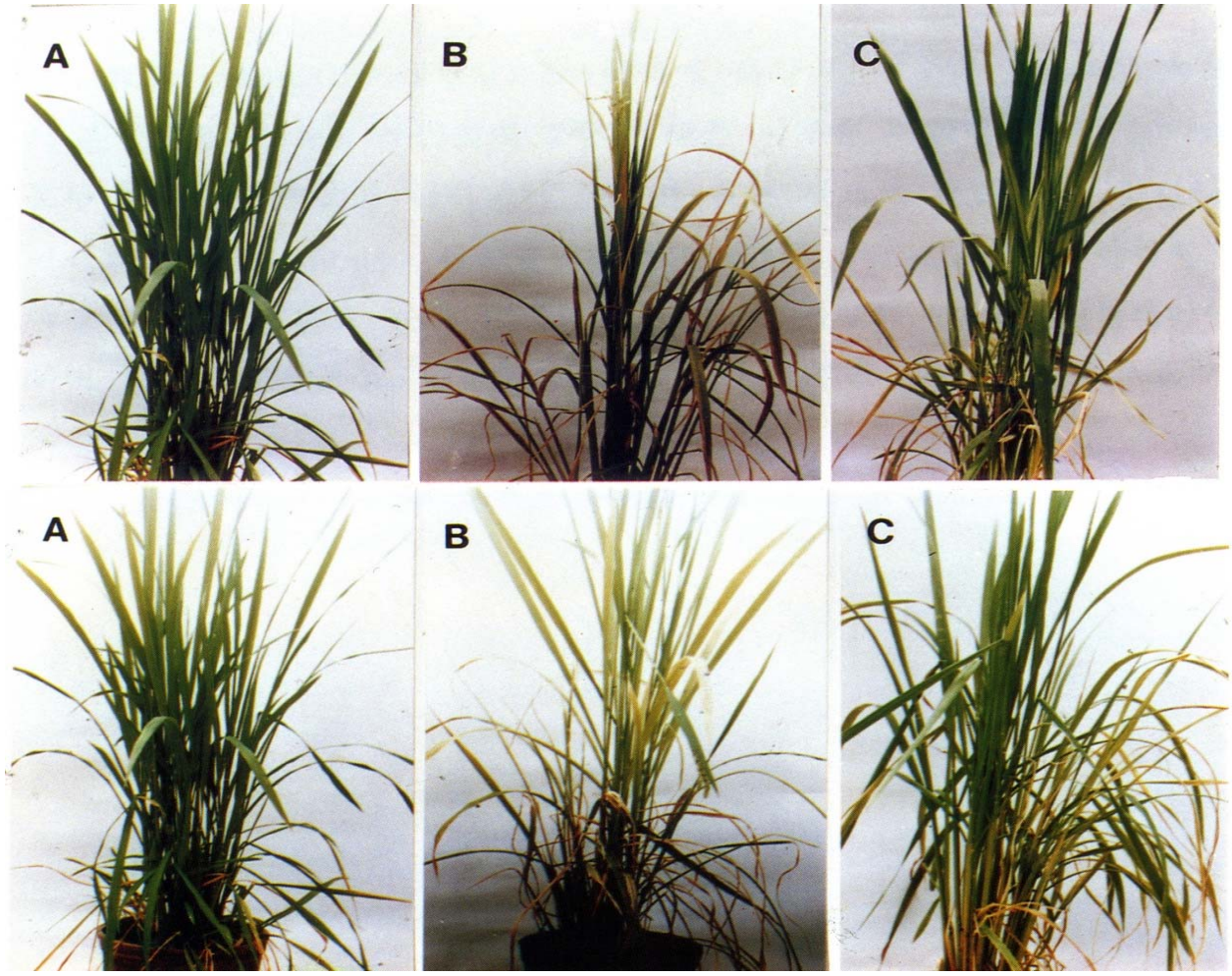

Fig. 1: *In vivo* antibiotic effects of the isolated compound on plant pathogenic microorganisms Top: Effect on *Pyricularia oryzae*; A= plants that were not inoculated with any pathogens, B= plants infected with *Pyricularia oryzae*, C= Plants inoculated with *Pyricularia oryzae* and treated with antibiotic (50 µg/mL). Bottom: Effect on *Pellicularia filamentosa*; A= plants that were not inoculated with any pathogens, B= plants infected with *Pellicularia filamentosa*, C= Plants inoculated with *Pellicularia filamentosa* and treated with antibiotic (100 µg/mL)

organic nitrogen sources, antibiotic was most produced when yeast extracts were added, which was observed in this study (Gillespie et al., 2002). The MICs of the isolated compound were 25 µg/ml for *P. oryzae* and 50 µg/ml for *P. filamentosa*. Polyoxin showed a MIC of 6.25 µg/ml and 1.6 µg/ml for *P. oryzae* and *P. filamentosa*, respectively (Isono et al., 1986). Nishizawa et al. (1984) reported that dapiramycin had a MIC of 400 µg/mL and 100 µg/mL for *P. oryzae* and *P. filamentosa*, respectively. There are many antifungal compounds for these two particular fungi that have been reported (Isnansetyo et al., 2009) such as octacosamicin (Dobashi et al., 1988) and fengycin-A (Vanittanakom et al., 1986).

In conclusion, the saccharides produced by the *Bacillus sp.* of YJH-1 have significant effects of *in vivo* antifungal activity against *P. oryzae* and *P. filamentosa* in rice plants.

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