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Research article

Characterization of the newly isolated antimicrobial strain *Streptomyces goshikiensis* YCXU

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Abstract
A rhizosphere bacterial strain coded as *Streptomyces goshikiensis* YCXU with broad spectrum antifungal activity was isolated from a cucumber field infested with *Fusarium oxysporum* f. sp. *niveum*. The strain YCXU showed antagonism to a broad range of phyto-pathogenic fungi and bacteria as well as strain YCXU produced volatile organic compounds that could reduce the fungal growth up to 40% compared to control, concluding that it can be used as biocontrol agent. Because of little information about the newly isolated strain, we further characterized the strain YCXU. The strain YCXU showed maximum growth on glucose containing yeast-malt extract (YME) medium at pH 7 and pink spores were produced after 7 days of incubation at 28°C. The strain YCXU exhibited nitrate reduction, melanin production, blood hemolysis, and casein, gelatin, starch, tyrosine, and hypoxanthine hydrolysis. This characterization will aid further research regarding the strains of *S. goshikiensis.*
Key words: Characterization, growth, hydrolysis, *Streptomyces goshikiensis* YCXU.

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Introduction

The genus *Streptomyces* has been proposed by Waksman and Henrico [1] as a term to encompass Gram positive, aerobic, spore-forming actinomycetes and is a unique source of novel antibiotics [2]. At present, the genus *Streptomyces* contains a large number of described species and nearly 600 were validly published [3]. Most of the recently described *Streptomyces* species have been delineated using polyphasic taxonomic approaches [4, 5]. *Streptomycetes* mostly represent a considerable proportion of the actinomycetes community in soils [6]. *Streptomycetes* are also widely distributed in a variety of natural and man-made environments, constituting a significant component of the microbial population in most environments [7]. They were commonly believed to be intermediate between bacteria and fungi [8].

At the present time with several thousand of described microbial metabolites, new strategies must be introduced into the screening programs to increase the chances of discovering the novel compounds [9]. The filamentous actinomycetes share significant fraction of microbial metabolites and among them; *Streptomyces* is most prolific genus and generally synthesize a huge number of diverse natural secondary metabolites e.g. platensimycin [10], daptomycin, streptomycin, the best known of which are antibiotics used worldwide as pharmaceutical and agricultural products [11, 12]. The actinomycetes produce ~3,000 known antibiotics and 90% of those are from *Streptomyces* species [13, 14]. In addition, actinomycetes have been found to form intimate association with plants and colonize their internal tissue. The actinomycetes inhabit a wide range of plants as either symbionts or parasites [15]. *Streptomyces* strains were isolated from the rhizosphere of different plant species in Italy and from maize in Brazil, which showed antagonistic activities against Gram positive bacteria and fungi [16, 17].

Streptomyces goshikiensis YCXU was isolated from a healthy cucumber plant in a wilt diseased field because of its strong antimicrobial activity against *Fusarium oxysporum* f. sp. *niveum.* Due to its little known characteristics, we decided to further characterize this strain for future use. Thus the aim of our study was to characterize *Streptomyces goshikiensis* YCXU, so that we could further utilize this antifungal and antibacterial strain as an antagonist against different plant diseases efficiently.

Material and methods

Isolation of microbial strain

Tenfold dilution series method was used to isolate *Streptomyces goshikiensis* YCXU on PDA (potato infusion 200g, dextrose 20g, agar 15g in 1L of distilled water) medium from soil as an antifungal strain against *Fusarium oxysporum* f. sp. *niveum*, After isolation from plant rhizosphere, the strain was grown on YME media (yeast extract 4g/L, malt extract 20g/L, glucose 4g/L, pH 7) and stored at -20°C for future use.

Microscopic study

Morphological studies were done under high resolution light microscope and individual colony pictures were taken with mycelial structures. The mycelia of strain YCXU were stained with aniline crystal violet dye and were observed under light microscope to confirm its mycelial structures like other *Streptomyces* strains.

Morphological identification on different media

Morphological characters were noticed on different media including tryptone yeast media (TYG), Luria-Bertani (LB) media, potato dextrose agar (PDA) media, Czapek media, Bennets agar media, soil agar media, inorganic starch salt agar media, potato carrot (PC) agar media, Gauss media and International Streptomyces Projects (ISP) 1, 2, 3, 4, 5, 6, 7 media [18]. Bacterial colony color and growth habits were recorded after 3 and 7 days.

Degradation activity assay

The degradation of adenine, tyrosine, hypoxanthine, xanthine and casein were detected by the method of Washington and Koneman [19]. Basal medium was prepared (beef extract 3g/L, peptone 5g/L and agar 15g/L) with 1% of each compound. Transparent zones present around bacterial colonies were considered as positive. Gelatin hydrolysis was detected in basal medium amended with 1% gelatin [20] and starch degradation was detected in the same basal medium amended with 1% starch by flooding plates with iodine solution [21]. Blood agar hydrolysis analysis was done on blood agar plates (Chengdu Rich Science Company Co. Ltd).

Nitrate reduction and hydrogen sulfide production assay

For nitrate reduction test, nitrate broth was prepared (beef extract 0.3g/L, peptone 0.4g/L, protease peptone no. 3 0.1g/L and potassium nitrate 0.1g/L) and color change to pink or red was an indicator of the presence or absence of nitrite. Hydrogen sulfide production was tested by preparing sulfide-indole-motility (SIM) medium (pancreatic digest of casein 2g/L, beef extract 6.1g/L, ferrous ammonium sulfate 0.2g/L and agar 3.5 g/L) and production of black precipitates was considered as positive for hydrogen sulfide reduction [22].

Melanin pigment production

For melanin pigment production, tyrosine-casein agar plates (L-tyrosine 1g/L, casein hydrolysate 2.5g/L, sodium nitrate 1g/L and agar 20g/L; pH 7) were prepared and black color around colonies was considered as positive [23].

Salt utilization assay

Citrate, private, benzoate and tartrate salts utilization were also examined by following Pridham and Gottlieb [24] method with slight modification. For this, TYG medium (tryptone 3g/L, yeast extract 5g/L, glucose 5g/L, and agar 20g/L) was prepared with respective salts. Bacterial growth in media was considered as positive.

Antifungal and antibacterial activity assay

The liquid culture (100 ml) of strain YCXU was centrifuged (12000×g) after 7 days growth at 28^{o}C in YME medium and antimicrobial compounds were extracted twice from cell free liquid culture with two volumes of ethyl acetate. The ethyl acetate was dried in rotatory evaporator and residues were dissolved in 1 ml of methanol, which was used to evaluate its antagonistic activity against fungal and bacterial strains. From the PDA medium plates, an 8 mm agar plug was removed and filled with 100 µl of cell free liquid culture after filtering through 20 µm filter and in the middle an 8 mm agar plug taken from the edge of actively growing fungal pathogen strain *Fusarium oxysporum* f. sp. *niveum* was placed. For antibacterial activity, bacterial pathogen *Ralstonia solanacearum* overnight cultures were spread on the PDA medium. Plates were incubated at 28^{o}C and inhibition zones were determined after three days for fungal strain and after two days for bacterial strain.

Antifungal volatile compounds assay

For antifungal volatile compounds production assay, divided plates were used. Both compartments of plates were added with PDA medium. One compartment was inoculated with a plug of freshly grown *Fusarium oxysporum* f. sp. *niveum* and other compartment was inoculated with newly isolated bacterium YCXU. The plates were sealed with Parafilm and fungal growth was measured on daily basis. Two control treatments were included: one was containing *Escherichia coli* DH5ά in place of strain YCXU and second without any bacterial inoculation.

Results and Discussion

Streptomyces goshikiensis YCXU was Gram-positive, non-motile and aerobic like other Actinobacteria. *Streptomyces* species produced mycelia with the aerial hyphae and mobility is achieved by the dispersion of spores [25]. Single colony (Fig.1A) and stained mycelia (Fig.1B) were

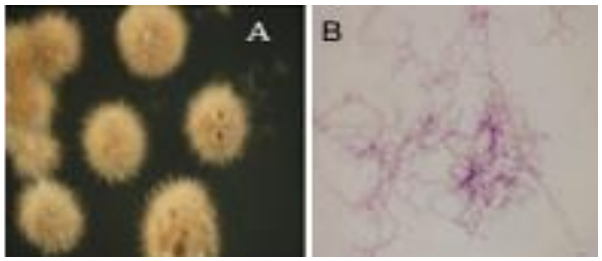

Fig. 1 Single colony structure on TPC agar media under microscope **(A)**, stained mycelia of *Streptomyces goshikiensis* YCXU **(B)**.

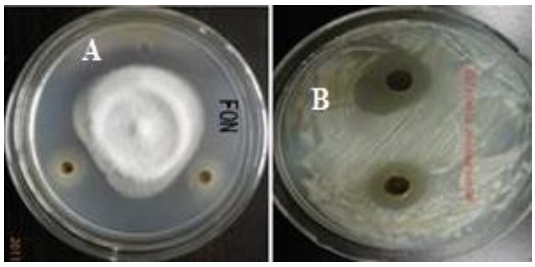

Fig. 2 Antifungal **(A)** and antibacterial **(B)** activity of liquid culture extract of *Streptomyces goshikiensis* YCXU.

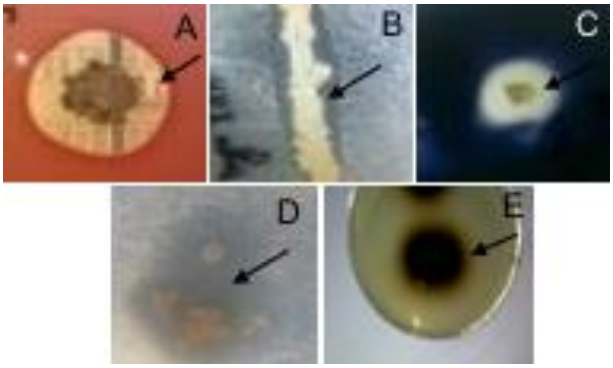

Fig. 3 *Streptomyces goshikiensis* YCXU showed blood agar hydrolysis **(A)**, hypoxanthine hydrolysis **(B)**, starch hydrolysis **(C),** casein hydrolysis **(D)** and melanin production **(E)**. Black arrows indicate the zone of degradation or hydrolysis.

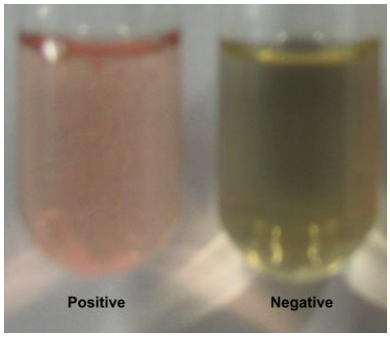

Fig. 4 Nitrate reduction by *Streptomyces goshikiensis* YCXU. The red coloration showed the positive results while yellow coloration showed the negative results.

observed under light microscope. For individual colony structure and microscopic study, TPC agar showed the best result and the characteristic color on this media was in-between light grayish yellowish brown and grayish yellowish pink as described by Niida [26]. The strain YCXU was able to produce antimicrobial compounds that significantly inhibited the growth of fungal pathogen *Fusarium oxysporum* f. sp. *niveum* and bacterial pathogen strain *Ralstonia solanacearum* (Fig. 2).

The strain YCXU showed degradation activity on blood agar medium (Fig. 3A), and positive results for hypoxanthine (Fig. 3B), starch (Fig. 3C), casein (Fig. 3D) and tyrosine hydrolysis but with xanthine was unable to show any transparent zone of hydrolysis. The strain YCXU showed positive result for melanin production as shown in Fig. 3E. Niida isolated *S. goshikiensis* strain showed gelatin and starch hydrolysis [26]. The strain YCXU showed positive results for nitrate reduction that was observed due to the color change from yellow to red (Fig. 4), while there was no hydrogen sulfide production (data not shown). Similar results for nitrate reduction and hydrogen sulfide and melanin production by another *S. goshikiensis* strain were reported by Niida [26]. The volatile compounds assay was conducted in split plates. The strain YCXU produced volatile compounds that inhibited the growth of *Fusarium oxysporum* by 40% (Fig. 5). The production of antimicrobial diffusible compounds and antimicrobial volatile compounds is an important characteristic of biocontrol strains [27]. Our results showed that the strain YCXU can be used as a biocontrol agent to control soil borne pathogenic diseases of plants. The strain YCXU showed 70% control of Fusarium wilt of watermelon caused by *Fusarium oxysporum* f. sp. *niveum* in a pot experiment [28]. This strain should be tested against other plant diseases.

Fig. 5 The antifungal activity of volatile organic compounds produced by *Streptomyces goshikiensis* YCXU against *Fusarium oxysporum* f. sp. *niveum*

Citrate, pyruvate, benzoate and tartrate salts utilization results showed that the strain YCXU was able to grow in citrate and pyruvate salts while was unable to grow in benzoate and tartrate salts containing medium. The results of the morphological

Table 1 Morphological features of *Streptomyces goshikiensis* YCXU after 1-3 and 4-7 days on different ISP media.

ISP medium	1-3 days colony color	4-7 days colony color	Reverse side colony color
1	Brown beige	White grayish spores	Brown beige
2	Ochre yellow	White pinkish spores	Light Cream brown
3	Brown beige	White pinkish spores	Brown beige
4	Yellowish, brown beige	White grayish spores	Brown beige
5	Brown beige	Ivory	Brown beige
6	yellowish Brown beige	White pinkish	Olive brown
7	Yellowish Brown beige	Ivory color spores	Brown beige

characteristics of strain YCXU showed that the ISP2 medium showed the best result for the growth of bacteria and colony color was ochre yellow in the first three days then it started to produce spores of white color. The TYG medium also showed good growth with yellowish brown colony color and on the LB medium, strain YCXU showed turner yellow color after three days then turned white at maturity because of the spores. The PDA medium showed yellow ochre light color of strain YCXU during first two days, then converted to light pink because of the spore production. The Czapek medium colony color was very light lavender to pinkish, Bennets agar medium was having a yellowish brown color with very fine growth of individual colonies and soil agar medium showed whitish to pinkish colony color at maturity. The inorganic starch salt agar medium showed low growth rate of strain YCXU as compared to other media and colony color was brown beige at maturity after 7 days. Gauss medium showed white to light grayish color colonies. The colony colors on ISP 1, 2, 3, 4, 5, 6 and 7 media are shown in Table 1.

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