

Full Length Research Paper

***In vitro* antifungal activity of *Streptomyces spororaveus* RDS28 against some phytopathogenic fungi**

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One hundred and twenty-eight strains of actinomycetes were isolated from different soils in Riyadh region, Saudi Arabia. All isolates were screened for their antifungal activities against *Rhizoctonia solani*, *Fusarium solani*, *Fusarium verticillioides*, *Alternaria alternata* and *Botrytis cinerea*. A potent antagonist against all tested phytopathogenic fungi, designated RDS28, was selected and identified as *Streptomyces spororaveus* RDS28 according to analysis of 16S rRNA gene sequence. Factors affecting the production of the antifungal compounds were investigated. The results showed that incubation of *S. spororaveus* RDS28 for 72 h at 31°C and initial pH 7.5 on a medium containing glucose as a carbon source and proline as a nitrogen source gave the best antifungal antibiotic production. The culture filtrate of *S. spororaveus* RDS28 was extracted and purified and rechecked for their *in vitro* antifungal activities against the tested fungi. It can be concluded that, the antifungal antibiotic produced by *S. spororaveus* RDS28, demonstrated an obvious inhibitory effect on the tested pathogenic fungi.

Key words: *Alternaria alternata*, antibiotic, *Botrytis cinerea*, *Fusarium solani*, *Fusarium verticillioides*, *Rhizoctonia solani*.

INTRODUCTION

Phytopathogenic fungi cause serious and economically important diseases worldwide including Saudi Arabia. They affect the growth and productivity of economically important crops. *Rhizoctonia solani*, *Fusarium solani*, *Fusarium verticillioides*, *Alternaria alternata* and *Botrytis cinerea* cause root rot, collar or root rots, stalk rot, leaf spots and gray-mold rot or *Botrytis* blight on a wide variety of agricultural crops, respectively (Al-Kassim and Monawar, 2000; Abd-El-Kareem, 2007; Tesso et al., 2009; Vagelas et al., 2009; Abdel-Fattah et al., 2011).

Chemical control may be available to reduce the effects of different plant diseases effectively and extensively, but field applications of these fungicides present a menace to the health of humans, animals and environments (Arcury and Quandt, 2003). Health concerns and environmental hazards associated with the use of chemical pesticides have resulted in an increasing interest in biological control as a promising alternative or a supplemental way

of reducing the use of agro-chemicals (Gnanamanickam, 2002). Microbial antagonists are widely used for the biocontrolling of fungal plant diseases. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Anitha and Rebeeth, 2009).

Streptomyces is the largest antibiotic producing genus in the microbial world discovered so far. The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades. Recent reports show that this group of microorganisms still remains an important source of antibiotic (Watve et al., 2001). Biological control of fungal plant diseases using *Streptomyces* spp. has been studied by many investigators in the last three decades (Hwang et al., 2001; Jeong et al., 2004; Prapagdee et al., 2008; Oskay, 2009; Degtyareva et al., 2009). However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their biocontrol effectiveness. The aim of this study is to isolate an antagonistic actinomycete isolate from Saudi soil, and to investigate the *in vitro*

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antagonistic activity of this isolate against *R. solani*, *F. solani*, *F. verticillioides*, *A. alternata* and *B. cinerea* and optimum conditions for its maximum antifungal potentiality.

MATERIALS AND METHODS

Isolation of soil-borne actinomycetes

Twenty random rhizosphere soil samples were collected from different agricultural fields (tomato, cucumber, alfalfa and onion) in Riyadh region, Saudi Arabia and stored in sterile plastic bags, labeled in the field and stored at 4°C until use. Soil samples (\approx 300 g) were carefully taken with spatula down to a 10 cm depth into the soil (around the plant root). Ten grams of air-dried soil sample were suspended in 100 ml of basal salt solution (5 g/L KH_2PO_4 and 5 g/L NaCl) and shaken in a rotary shaker (150 rpm) at 28°C for 30 min.

The soil suspension was then diluted and 1 ml of diluted soil suspension was spread onto starch-nitrate-agar plates (Waksman, 1961) that contained (g/L): Starch; 10, NaNO_3 ; 2.5, K_2HPO_4 ; 1, KH_2PO_4 ; 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5, KCl; 0.5, trace salt solution 1 ml [$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.64 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.11 g/L), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.79 g/L), and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.15 g/L)], agar; 20 and distilled water, 1 L. The medium was adjusted to the initial pH 7 prior to sterilization using 0.1 N NaOH or 0.1 N HCl solution, supplemented with 50 $\mu\text{g}/\text{ml}$ of filter-sterilized cycloheximide to inhibit fungal growth, and incubated at 28°C for one week. Colonies of actinomycetes on the agar plates were picked on the basis of their morphological characteristics and purified and then transferred to starch nitrate/NaCl slants for further use (Shirling and Gottlieb, 1966).

The fungal pathogens *Rhizoctonia solani*, *Fusarium solani*, *F. verticillioides*, *Alternaria alternata* and *Botrytis cinerea* were isolated originally from different naturally diseased plants collected from different agricultural fields in Saudi Arabia. The isolated fungi were grown on potato dextrose agar (PDA) (Difco, USA) plates and incubated at 28°C for 4 to 6 days. Purification of the resulting isolates was done using the hyphal tip or single spore techniques to obtain them in pure cultures; the detected isolates were then transferred into slant of PDA and kept at 4°C for further studies. Pure cultures of the isolated fungi were identified according to the cultural properties, morphological and microscopical characteristics of each fungus (Domsch et al., 1980).

Screening for the antifungal activity

All isolates were screened for their *in vitro* antifungal activity against the tested pathogenic fungi. A 7 mm diameter disk from 5-day-old culture of the actinomycete isolate being tested was placed in the centre of starch nitrate agar plate seeded with the tested fungus. Three replicates of each treatment were used. The starch-nitrate plates were then incubated at $30 \pm 1^\circ\text{C}$. The inhibition zone, if any, was measured in mm diameter after 24, 48 and 72 h (Waksman, 1961).

Identification of isolated antagonist

Genus identification of the selected isolate was identified by 16S rRNA sequencing. 16S rRNA was amplified in a thermocycler (Perkin Elmer Cetus Model 480) by using universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525r (5'-AAG GAG GTG ATC CAG CC-3') under the following condition: 94°C for 5

min, 35 cycles of 94°C for 60 s, 55 for 60 s, 72°C for 90 s and final extension at 72°C for 5 min. The product was directly sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems, USA) on an ABI 310 automated DNA sequencer (Applied Biosystems, USA). Homology of the 16S rRNA sequence of isolate was analyzed by using BLAST program from Genbank database (Prapagdee et al., 2008).

Determination of optimum conditions for production of the antifungal compound(s)

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate medium each were inoculated with the selected isolate and incubated on rotary shaker (160 rpm) at $30 \pm 1^\circ\text{C}$ for various incubation periods (e.g 1, 2, 3, 4, 5, 6 and 7 days). At each incubation period, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used within each incubation period for each fungus (Isenberg, 1992).

Effect of incubation temperature

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate medium each were inoculated with the selected isolate and incubated on rotary shaker (160 rpm) for the optimum incubation period (3 days), at different temperatures (25, 28, 31, and 34°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used for each temperature incubation for each fungus (Isenberg, 1992).

Effect of pH

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate medium each were adjusted at various levels of pH (6, 6.5, 7, 7.5 and 8) using a phosphate buffer before the sterilization and then inoculated with the selected isolate and incubated for the optimum incubation period (3 days) at the optimum temperature (31°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used for each pH level for each fungus (Isenberg, 1992).

Effect of carbon source

In this experiment, glucose, fructose, sucrose, lactose, manose or galactose were tested as substitute carbon sources. Carbon source of starch-nitrate medium was substituted with one of the tested sources (containing the same quantity of carbon). Erlenmeyer flasks containing starch substituted starch-nitrate medium were inoculated with the selected isolate. The initial pH of the various media was adjusted at 7.5, before sterilization and the flasks were incubated for 3 days at 31°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each carbon source for each fungus (Vanderzant and Splittstoesser, 1992).

Effect of nitrogen source

In this experiment, potassium nitrate, ammonium sulphate, proline,

Table 1. Antifungal activity of the isolated actinomycetes against the tested fungi.

Antifungal activity*	Number of isolates of soil-borne actinomycetes possessing antifungal activity against the tested fungi **					
	<i>F. verticillioides</i>	<i>F. solani</i>	<i>A. alternata</i>	<i>B. cinerea</i>	<i>R. solani</i>	All of them
+++	5	1	2	2	6	1
++	7	1	4	3	11	1
+	12	3	8	7	23	3
-	104	123	114	116	88	123

*Antifungal activity was determined by the inhibition zone. ** Each isolate was tested using three replications. The rating of antifungal activity was modified from those of El-Tarabily et al. (2000). +++ = inhibition zone \geq 20 mm; ++ = inhibition.

alanine, or histidine were tested as substitute nitrogenous sources. Nitrogenous source of starch-nitrate medium was substituted with one of the tested sources (containing the same quantity of nitrogen). Erlenmeyer flasks containing starch-nitrate medium (sodium nitrate substituted with one of the tested sources) were inoculated with the selected isolate. The initial pH of the various media was adjusted at 7.5, before sterilization and the flasks were incubated for 3 days at 31°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each nitrogenous source for each fungus (Vanderzant and Splittstoesser, 1992).

Fermentation and extraction of the bioactive compounds

Twenty Erlenmeyer flasks (1000 ml) containing 400 ml sterile starch-nitrate medium each were inoculated with a 14-day-old colony of *Streptomyces spororaveus* RDS28 grown on starch-nitrate slants. The medium was adjusted to the initial pH 7.5. The inoculated flasks were then incubated on a rotary shaker at 31°C and 300 rpm for 7 days. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 15 min at 5°C (Z 36 HK High speed centrifuge, Hermle, Germany), and the supernatant was filtered (Milipore filter, 0.45 μ m). The supernatant was extracted twice with an equal volume of ethyl acetate. The organic extract was concentrated using a rotary evaporator at 50°C (Heidolph WB2000, Germany) to give 2 g of crude extract.

After the extraction, the antifungal activity of the crude extract was determined. Ten milligrams of the crude extract were dissolved in 1 ml of dimethyl sulfoxide (DMSO), and 50 μ l extract were loaded on to sterile filter paper discs (8 mm diameter). Fungal discs (8 mm diameter) from 5-day-old cultures on PDA plates at 28°C were placed at the center of the PDA plates. The loaded filter paper discs were placed on the opposite sides of the plates, 3 cm away from the fungal disc. The plates were incubated at 28°C for 10 days. Bioactivity was determined by measuring the inhibitory zones (mm) (Khamna et al., 2009).

Statistical analysis

Standard errors of the inhibition levels were calculated using the statistical analysis software "CoStat 6.34" (CoStat, 2005).

RESULTS AND DISCUSSION

Isolation of actinomycetes and *in vitro* antifungal activity

One hundred and twenty-eight strains of actinomycetes

were isolated from rhizosphere soils. Among 128 strains, forty isolates (31.3%) inhibited the growth of *R. solani*, twenty-four isolates (18.8%) inhibited the growth of *F. verticillioides*, fourteen isolates (11%) inhibited the growth of *A. alternata*, twelve isolates (9.4%) inhibited the growth of *B. cinerea*, and five isolates (4%) inhibited the growth of *F. solani*. Only 5 isolates showed antagonistic ability against all of the tested fungi and only one isolate designated R28, had the strongest antagonistic activity against all of the tested fungi. This isolate was selected and used in the next experiments (Table 1).

Analysis of the 16S rRNA gene sequences showed that RDS28 is identical to *S. spororaveus* (100% similarity) with GenBank database accession number (HQ834290) and then designated *S. spororaveus* RDS28. The degree of antifungal activity varied greatly among the actinomycetes. Several researchers have already reported similar antimicrobial activity of actinomycetes against fungal pathogens. Prapagdee et al. (2008) found that out of 146 strains of indigenous actinomycetes isolated from rhizosphere soils (paddy and orchards fields) only 10 strains exhibited antifungal activity. Khamna et al. (2009) isolated 396 *Streptomyces* strains from 16 rhizosphere soil samples (medicinal plants). 27 (6.8%) isolates showed antifungal activity. Bharti et al. (2010) obtained 316 actinomycete strains from different soil samples, of which, 31% exhibited antifungal activity. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Errakhi et al., 2007; Khamna et al., 2009). The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (Fguira et al., 2005; Atta, 2009) and/or extracellular hydrolytic enzymes (Mukherjee and Sen, 2006; Prapagdee et al., 2008).

Factors affecting the production of the antifungal compound

The nutritional sources like carbon and nitrogen, as well as the environmental factors such as incubation period, pH and temperature are known to have a profound effect on antibiotic production by actinomycetes (Himabindu

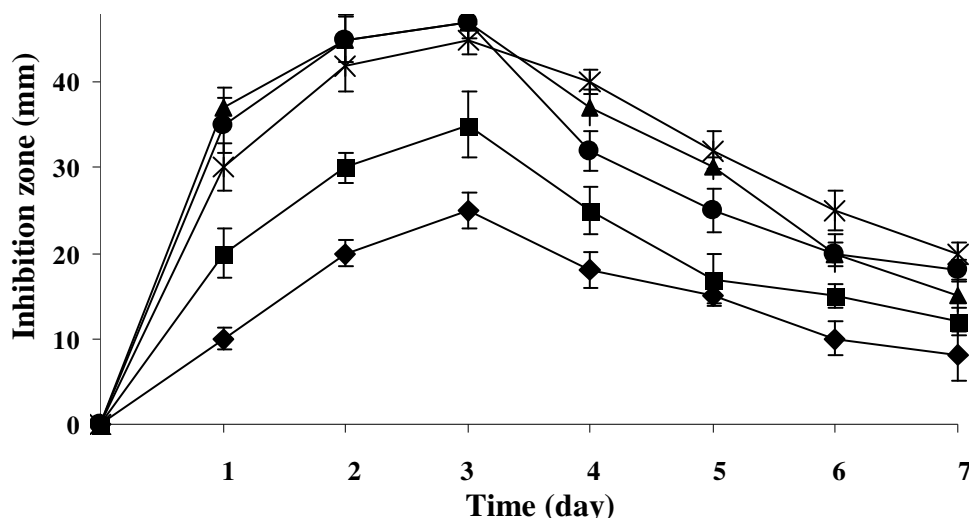


Figure 1. Effect of incubation period on the antifungal activity of *S. spororaveus* RDS28 against the tested fungi, *F. verticillioides* (●), *F. solani* (■), *A. alternata* (×), *B. cinerea* (♦) and *R. solani* (▲).

and Jetty, 2006). Optimization of culture conditions is essential to get high yields of the antimicrobial metabolites. Hence, the present study described the optimization of culture conditions for the production of antifungal metabolites by *S. spororaveus* RDS28. Results illustrated in Figure 1 show the antifungal activity during 7 days of incubation period. The obtained results indicated that the antifungal activity of *S. spororaveus* RDS28 against all of the tested fungi was initially observed after 24 h of incubation and reached its maximum levels after 72 h of incubation. Antifungal activity was found to decline as the incubation period further extended.

The obtained result is accordance with that reported by Srinivasulu et al. (2002) who found that the highest antibiotic production by *Streptomyces marinensis* was obtained after 72 h of incubation. At longer incubation periods, the antibiotic production also decreased gradually. Influence of different temperature on the antifungal activity of *S. spororaveus* RDS28 against all of the tested fungi is depicted in Figure 2. Increasing temperature up to 31°C led to an increase in the antifungal production and antagonistic activity, which was the optimum temperature for *F. solani*, *F. verticillioides* and *A. alternata*, whereas the optimum temperature for *R. solani*, and *B. cinerea* was 28°C. Above the optimum temperature the increase in temperature was accompanied by a decrease in the antifungal production and a decrease in the antagonistic activity. The obtained results were in agreement with that achieved by Sujatha et al. (2005), who found that the best growth and antibiotic production by *Streptomyces psammoticus* were recorded when incubation was carried out at temperature of 30°C.

Incubation temperature directly affects the activity and growth of cells; every species has an ideal temperature

for growth that is influenced by its physiology (Shuler and Kargi, 1992). *S. spororaveus* RDS28 is a mesophilic actinomycetes and the optimum temperature (31°C) is suitable for its growth and consequently for the activity of enzymes responsible for the secondary metabolite biosynthesis. Influence of pH on the antifungal activity of *S. spororaveus* RDS28 against all of the tested fungi is presented in Figure 3. Antifungal activity varied as medium pH changed between 6 and 8. Increasing the medium pH led to an increase in the antifungal production up to a certain limit above which any increase in the pH value was accompanied by a decrease in the antifungal production and activity. The optimum pH value that gave the maximum antifungal activity of *S. spororaveus* RDS28 against all of the tested fungi was 7.5. The obtained result is in agreement with that of El-Naggar et al. (2003) who recorded that the highest antibiotic production by *Streptomyces violatus* was obtained at initial pH value of 7.5. El-Mehlawy et al. (2005) found that highly acidic or basic media, whether adjusted initially or buffered after autoclaving were not suitable for the antifungal production by many *Streptomyces* species and that neutral media (pH 7) were the most favorable for antifungal production. The same result was obtained by Guimarães et al. (2004) who reported that the highest retamycin production by *Shewanella olindensis* was achieved at pH 7. Similar to temperature, pH of the media influences the growth rate of bacteria by affecting the activity of cellular enzymes (Shuler and Kargi, 1992). Influence of additional carbon source on the antifungal activity of *S. spororaveus* RDS28 against all of the tested fungi is illustrated in Figure 4. It is shown that glucose was the best carbon source for antifungal production and consequently the antagonistic activity, followed by fructose, while lactose

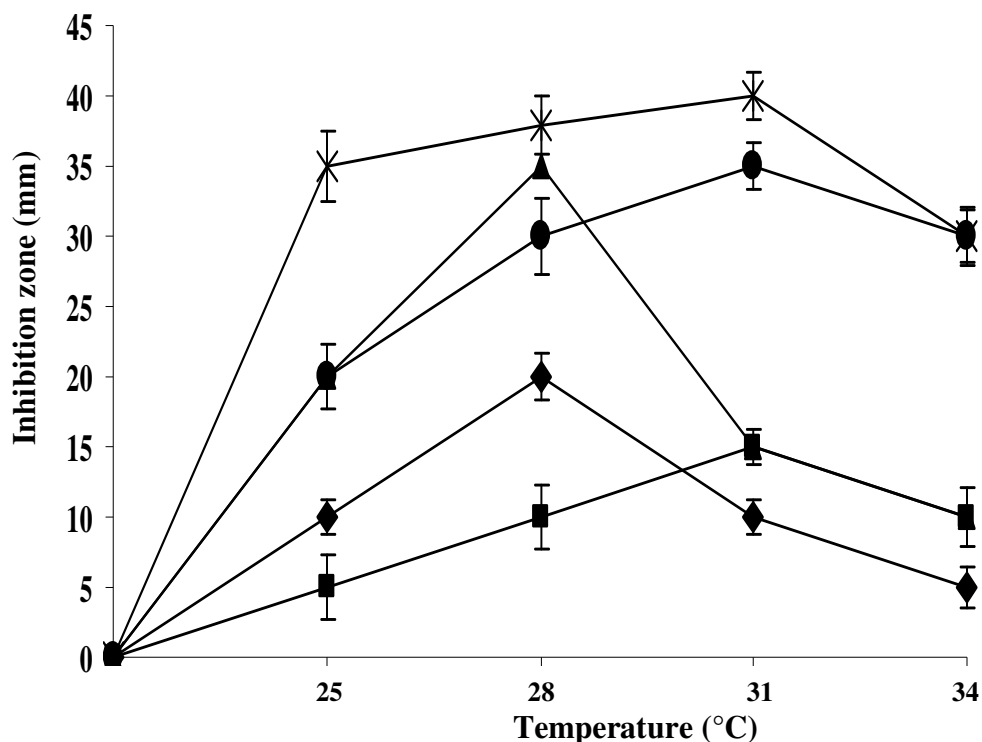


Figure 2. Effect of temperature on the antifungal activity of *S. spororaveus* RDS28 against the tested fungi, *F. verticillioides* (●), *F. solani* (■), *A. alternate* (×), *B. cinerea* (◆) and *R. solani* (▲).

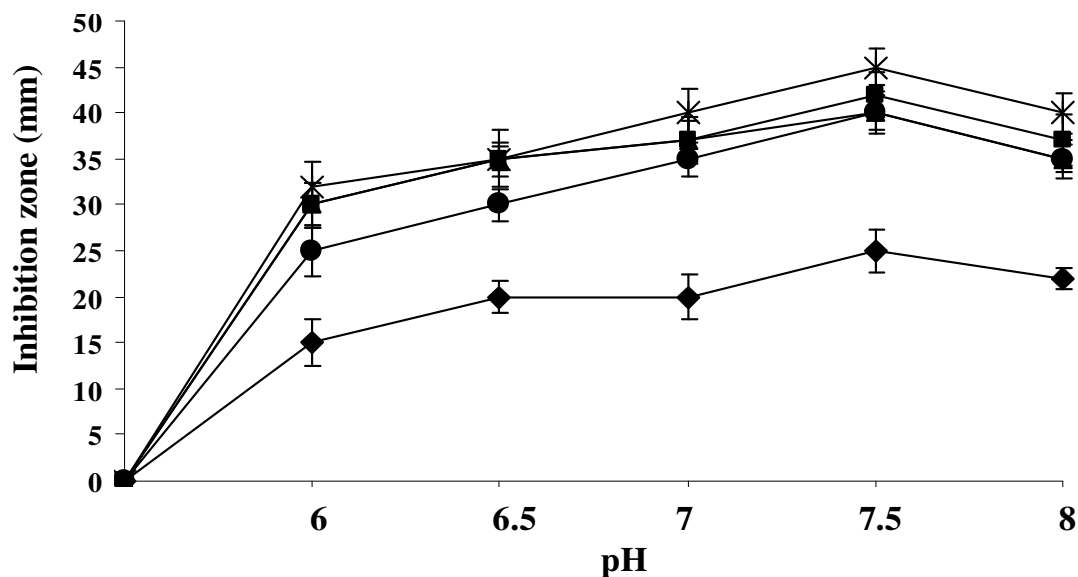


Figure 3. Effect of pH on the antifungal activity of *S. spororaveus* RDS28 against the tested fungi, *F. verticillioides* (●), *F. solani* (■), *A. alternate* (×), *B. cinerea* (◆) and *R. solani* (▲).

gave the poorest antifungal yield. This result is in agreement with that obtained by Tarhan et al. (2011) who found that, antibiotic production of *Streptomyces* sp. M4018 was higher in glucose medium when compared with glycerol and starch. The same result was obtained

also by Vasavada et al. (2006) who reported that the highest antibacterial activity of *Streptomyces sannanensis* strain RJT-1 was obtained when glucose at 1% (w/v) was used as a carbon source followed by xylose and arabinose. The obtained result is also in

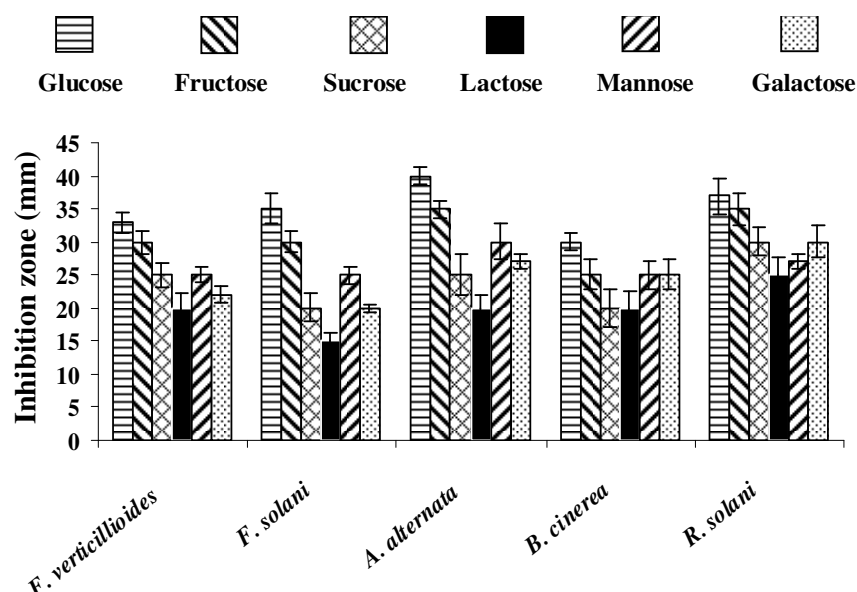


Figure 4. Effect of different carbon sources on the antifungal activity of *S. spororaveus* RDS28 against the tested fungi.

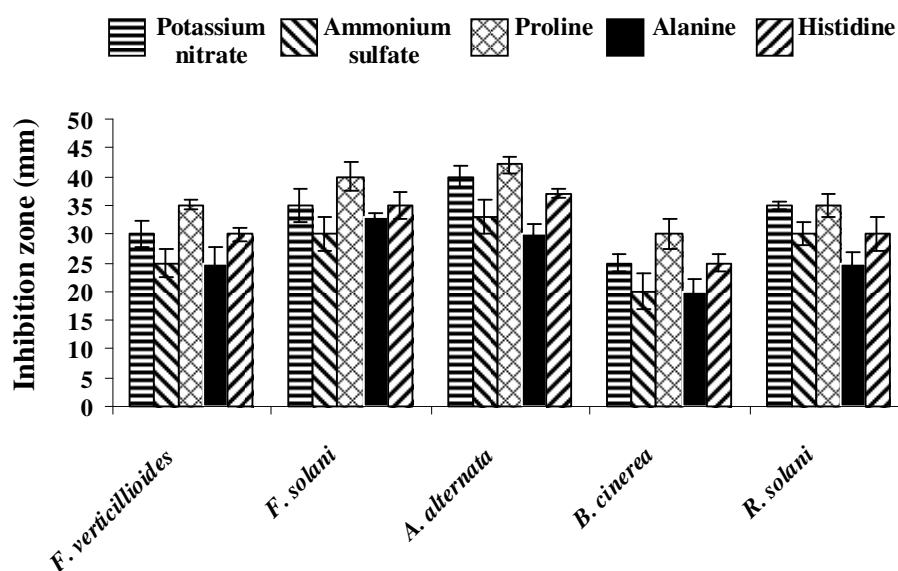


Figure 5. Effect of different nitrogen sources on the antifungal activity of *S. spororaveus* RDS28 against the tested fungi.

agreement with that of Pandey et al. (2005) and Ripa et al. (2009), in which glucose proved to be the best carbon source for antibiotic production.

Work by Jakeman et al. (2006) indicated that carbon source added to the media has an impact on the antibiotic production by *Streptomyces venezuelae*. The effect of carbon source on growth and antibiotic production is dependent upon several factors such as pH (Kontro et al., 2005) and carbon concentration (Chen et al., 2008). In this connection, Zhu et al. (2007) studied

the effects of glucose concentration on avilamycin biosynthesis in *Streptomyces viridochromogenes* and found that high concentrations of glucose led to the absence of the precursors for avilamycin biosynthesis and affected antibiotic synthesis. Influence of additional nitrogen source on the antifungal activity of *S. spororaveus* RDS28 is presented in Figure 5.

Proline was the best nitrogen source for antifungal production, followed by potassium nitrate, while alanine was the poorest nitrogen source for supporting antifungal

production. The obtained result is comparable with that reported by Soliveri et al. (1988), who described the effect of different nutrients on the production of the macrolide polyene antibiotics (PA-5 and PA-7) produced by *Streptovercillium* sp. 43 to 16. Optimal production yields have been achieved with L-proline and glycine as nitrogen sources, respectively. Huck et al. (1991) reported that proline and humic acid (0.1 %) had been listed as selective carbon and nitrogen sources for the isolation of actinomycetes. Moreover, proline had also been recommended for the selection of antibiotic producing actinomycetes. Dastager et al. (2006) showed that proline was one of the best nitrogen sources for production of melanin pigment (secondary metabolite) in *Streptomyces* species.

The culture filtrate of *S. spororaveus* RDS28 was extracted, concentrated and rechecked for their *in vitro* antifungal activity using discs method against the tested fungi. It can be concluded that, the antifungal antibiotic produced by *S. spororaveus* RDS28, demonstrated obvious inhibitory effects against the tested pathogenic fungi. However, more studies should be conducted with regard to field application, formulation and mass production of the biocontrol agent, in order to develop a biofungicide that can be used easily, efficiently for the large scale.

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