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Streptomyces griseorubens E44G: A Potent Antagonist Isolated from Soil in Saudi Arabia

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In this study, a wide survey was conducted along Saudi Arabia. One hundred soil samples were collected from different 18 governorates representing different climatic conditions. Five hundred and seventy strains of actinomycetes were isolated from the collected soil samples. Among them, 225 were found to be antagonistic to the pathogenic fungus (*Fusarium oxysporum* f. sp. *lycopersici*) with varying degrees. Only one isolate designated E44G, had the strongest antagonistic activity against the tested fungus. The taxonomic status of this isolate was established using Phenotypic and molecular methods. The optimum nutritional and environmental conditions were studied to produce the maximum yield of antifungal activity. The highest antifungal activity was obtained at 2nd day of incubation, 7.5 pH, and 30 °C. Glucose was the best carbon source and yeast extract was the best nitrogen source. In the present study, we report on a potential *Streptomyces* strain which has antifungal and antibacterial activities and holds the potential for use in studies of bioactive compounds as well as for possible use in biological control of fungal and bacterial diseases of important crops.

Key words: Antibacterial, Antifungal, Optimization, Streptomycetes.

Fusarium wilt disease, caused by *Fusarium oxysporum* f. sp. *lycopersici*, is one of the most important tomato diseases in the world. It can be a serious disease causing a considerable yield loss in tomato affecting its growth and productivity. Chemical control may be available to reduce the effects of this disease effectively and extensively. However, field application of these fungicides may not always be desirable. The persistent, injudicious use of chemicals has been discouraged owing to their toxic effects on non-target organisms and due to the undesirable changes they inflict upon the humans, animals and environment[1]. However, recent researches led to the possibility of biological control as an increasingly realistic option for tomato disease management[2].

Streptomycetes are one of the most abundant microorganisms in the soil. They are of universal occurrence in nature, living and multiplying in both cold and tropical zones, and

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have been reported to occur even under the most extreme conditions of the desert[3]. The temperate zones are, however, generally most favorable for their development[4]. In fact, Streptomycetes are able to maintain themselves in a complex competitive environment due to prolific sporulation, a broad carbon utilization pattern and the ability to form antibiotics, explaining their wide distribution[5]. The metabolic diversity of Streptomycetes is due to their extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs[6].

Streptomyces is the largest antibiotic producing genus in the microbial world discovered so far. The majority of antibiotics in current uses, as well as drugs with anticancer, antifungal, and immunosuppressant activities, are obtained from *Streptomyces* species[7]. Because of their ability to synthesize numerous compounds that exhibit extreme chemical diversity, *Streptomyces* strains are major part of industrial strain collections used in screening for new bioactive molecules[8].

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal agents that inhibit several pathogenic fungi[9,10] and antibacterial agents[11,12]. The antimicrobial mechanisms may involve physical contact (hyperparasitism), synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition[13,14]. However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their antimicrobial effectiveness. The need for new, safe and more effective antifungal agents are a major challenge to the plant protection industry today. The present study aimed at isolating a wide range of actinomycetes from the tomato rhizospheric soil, screening them for their *in vitro* antagonistic activity against *F. oxysporum*, investigating their cultural, biochemical and physiological properties and determining the optimum conditions for its maximum antimicrobial activity, extracting, purificating and characterizing of the antifungal compound(s). This research is the primary investigation in a long-term project that is aiming at developing effective and eco-friendly bio-fungicides to control *Fusarium* wilt disease of tomato.

MATERIALS AND METHODS

Soil sampling

One hundred soil samples were collected from different governorates in Saudi Arabia. The survey area included 18 governorates representing different climatic conditions namely, Al-Ahsaa, Al-Jouf, Al-Kharj, Al-Madenah, Al-Qaseem, Al-Qatif, Al-Quwayiyah, Al-Sulayyil, Al-Ta'if, Hail, Jeddah, Gazan, Makkah, Najran, Riyadh, Shagra, Tabuk and Wadi Al-Dawasir. Soil samples (≈300g) were carefully taken with spatula down to a 10 cm depth into the soil and kept in sterile plastic bags, labeled in the field and stored at 4°C until use.

Isolation and maintenance of soil-borne actinomycetes

For each sample, 10 g of air-dried soil were suspended in 100 ml of basal salt solution (5 g KH_2PO_4, 5 g NaCl and 1000 ml H_2O) and shaken using a rotary shaker (150 rpm) at 28°C for 30 min. The soil suspension was then diluted and 1 ml of the diluted soil suspension was used. Isolation trails from the soil suspension were carried out on starch nitrate agar plates[15] with an incubation period of 6 days at 30°C and pH 7. Colonies of actinomycetes on agar plates were picked up on the basis of their morphological characteristics. The isolates were maintained on starch nitrate/NaCl slants at 4°C and as glycerol suspensions (20%, v/v) at -20°C for further use.

Screening for antifungal activity

All isolated actinomycetes were screened for their *in vitro* antimicrobial activity against *F. oxysporum*. Spore suspension of the tested fungus was incorporated in starch nitrate agar plates by adding an appropriate amount of the suspension to the melted medium just before solidification. Seeded plates containing 20 ml of the medium were prepared. A 7 mm-diameter disk from 5-day-old culture of each isolate was placed in the center of starch nitrate agar plate. Three replicates of each treatment were used. The starch-nitrate plates were then incubated at 30±1°C. The antimicrobial activity was estimated after 2 days of incubation.

Cultural, biochemical and physiological characterization of strain E44G

Cultural characteristics including aerial spore-mass color, substrate mycelial pigmentation and the production of diffusible pigments were observed in 10-day-old cultures of strain E44G

grown on differential agar media at 30°C[16]. The isomers of diaminopimelic acid and cell wall sugar pattern were analyzed by TLC following procedures described by Hasegawa *et al*.[17] and Lechevalier and Lechevalier[18]. Broad range of biochemical and physiological properties of strain E44G were investigated as described by Williams *et al*.[19] and Kämpfer *et al*.[20] including, enzymes production, degradation of some chemicals and NaCl tolerance. In addition, resistance to antibiotics was examined as described by Al-Tai *et al*.[21].

Microscopic observations using scanning electron microscope (SEM)

The spore-chain morphology and spore-surface ornamentation of strain E44G were studied by examining gold–coated, dehydrated specimen using SEM according to Erdos[22]. Cultures of 14-days age grown on starch nitrate agar plate were first excised and trimmed to approximately 10 mm x 10 mm specimens as thin as possible (1 - 2 mm), and fixed before they were further reduced into smaller (approx. 5 mm x 5 mm) specimens. Solid specimens are fixed in a buffered (0.1 M, pH 6.5-7.0) fixative such as 2-3% glutaraldehyde for periods ranging from 5 min to 24 h[23]. Then, the specimens were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded series of acetone. Critical-point dried specimens were coated with gold palladium in a Polaron E500 sputter coater (Polaron Equipment Ltd., England) and viewed in SEM (JEOL JSM 35C).

Genotypic identification of strain E44G

Total genomic DNA of strain E44G was extracted using wizard genomic DNA purification kit (QIAGENE DNA kit, Hilden, Germany) according to the manufacturer procedure. PCR amplification of the 16S rRNA gene was conducted in a thermocycler (Perkin Elmer Cetus Model 480) by using the universal primer; the forward primer 5'-AGA GTT TGA TCC TGG CTC AG -3' and the reverse primer 5'-AAG GAG GTG ATC CAG CC-3' [24]. PCR reaction was performed in a total volume of 25 µl containing: 2.5 µl PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 U *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer), 2.5 µl of 10 pmol each primer and 2.5 µl of the extracted bacterial DNA and the volume was completed to 25 µl using sterile distilled H_2O. PCR reaction conditions were approached as; one cycle of 94°C for 5 min followed by 35 cycles; each cycle consists of. 94°C for 1 min, 55°C for 1 min, 72°C for 90 s and a final extension step at 72°C for 5 min. The PCR product (1500bp) was directly sequenced by a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI 310 automated DNA sequencer using both the reverse and forward primers (Applied Biosystems, Foster City, CA, USA). Homology of the 16S rRNA sequence was analyzed by using the BLAST algorithm, available in Genbank (http: www.ncbi.nlm.gov/BLAST/).

Assay of antimicrobial activity

Antimicrobial activity of strain E44G was investigated against some pathogenic fungi and bacteria. Ten fungi kindly provided by Plant Pathology Institute, Agricultural Research Center, Egypt namely; *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina*, *Alternaria radicina*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Nigrospora oryzae*, *Phoma destructive*, *Penicillium notatum* and *Aspergillus niger*. Five bacterial isolates (*Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis*) obtained from the American type culture collection (ATCC) were used in this investigation. The antimicrobial activity was estimated using the disc diffusion method formally described.

Optimum conditions for maximum antimicrobial activity

Effect of incubation period

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate medium each were inoculated with strain E44G and incubated on rotary shaker (160 rpm) at 30 ± 1°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 antimicrobial activity was determined using the inhibition zone method. Three plates were used within each treatment[25].

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 (2 days), at different temperatures (20, 22, 24, 26, 28, 30. 32, 34, 36 and 38°C). For each, 5 ml of the culture filtrate were then taken aseptically

and the antimicrobial activity was determined using the inhibition zone method. Three plates were used for each treatment [25].

Effect of pH

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch-nitrate medium each were adjusted at various levels of pH (5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) using a phosphate buffer before the sterilization and then inoculated with the selected isolate and incubated for the optimum incubation period (2 days) at the optimum temperature 30°C. For each, 5 ml of the culture filtrate were then taken aseptically and the antimicrobial activity was determined using the inhibition zone method. Three plates were used for each treatment[25].

Effect of carbon source

Glucose, galactose, fructose, sucrose, lactose, maltose and ribose were individually 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 2 days at 30°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antimicrobial activity was determined by the inhibition zone method described earlier. Three plates were used for each treatment[26].

Effect of nitrogen source

Potassium nitrate, ammonium sulphate, urea, casein, yeast extract, beef extract, or malt extract were individually 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 2 days at 30°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antimicrobial activity was determined using the inhibition zone method. Three plates were used for each treatment[26].

RESULTS AND DISCUSSION

In this study, a wide survey was conducted along Saudi Arabia. One hundred soil samples were collected from different 18 governorates representing different climatic conditions. Saudi Arabia has a desert dry climate with high temperatures in most of the country, but there are also wide variations between the seasons and the regions. Variation in the climatic conditions and soil properties may results in a wide biodiversity in the soil actinomycetes content, particularly *Streptomyces*.

Five hundred and seventy strains of actinomycetes were isolated from the collected soil samples. The obtained isolates showed a wide variation in its cultural color. These isolates were screened for their antagonistic activity against *F. oxysporum*. A high variation in the antifungal activity was recorded between the isolates. Among the 570 actinomycete isolates, 225 were found to be antagonistic to the tested fungus with varying

Table 1. Cultural characteristics of *S. griseorubens* E44G

Medium	Growth	Color of aerial mycelia	Color of substrate mycelia	Color of diffusible pigment
Yeast-malt extract agar (ISP-2)	Moderate	10-PK. Gray	70-l.oy	No
Oatmeal extract agar (ISP-3)	Good	263-L. Gray	76-l.yBr	79-l.gy.YBr
Inorganic salts starch agar (ISP-4)	Moderate	10-PK. Gray	79-l.gy.YBr	No
Glycerol asparagine agar (ISP-5)	Good	264.L. Gray	79.l.gy.YBr	No
Peptone yeast extract iron agar (ISP-6)	Poor	10-PK. Gray	79.l.gy.YBr	No
Tyrosine agar (ISP-7)	Poor	10-PK. Gray	79.l.gy.YBr	No

PK = pinkish, l.oy = light orange yellow, L. = light, l.yBr = light yellowish brown and l.gy.YBr = light gray yellowish brown.

Table 2. Phenotypic characteristics of *S. griseorubens* E44G

Characteristics		Result
Morphological characteristics	Shape of spore chains	Open hook
	Color of spore mass	Gray
	Spore surface	Smooth
	Color of substrate mycelium	Creamy
	Color of diffusible pigment	No
	Motility	Non motile
Cell wall hydrolysis	Diaminopimelic acid (DAP)	LL-DAP
	Sugar pattern	Not detected
Physiological characteristics	Protease, chitinase and catalase	+
	Lipase, amylase and cellulase	-
	Pectinase, lecithinase, and urease	-
	Melanoid pigment	+
	Degradation of esculine and xanthine	-
	H_2S production	+
	Nitrate reduction and citrate utilization	-
	Coagulation of skim milk	-
Utilization of different carbon sources	D-glucose, D-galactose, D-fructose and sucrose	+
	L-arabinose, xylose and *meso*-inositol	-
	Raffinose and rhamnose	+
Utilization of different nitrogen sources	L-cystiene, L-valine, L-alanine, and L-leucine	+
	L-histidin, L-lysine, L-tyrosine, and L-proline	+
	L-phenylalanine	-
NaCl tolerance	10 %	+
	15 %	-
Antibiotics resistance	Streptomycin	-
	Amoxicillin	-
Growth on different temperatures	<20	-
	20 – 45	+
	>45	-
Growth on different pH	<4.5	-
	5.5 – 9.9	+
	>10.5	-

(+) = growth or activity, and (-) = no growth or activity.

Table 3. Antimicrobial activity of *S. griseorubens* E44G

Fungi	Activity	Bacteria	Activity
Fusarium solani	+	*Staphylococcus aureus*	-
F. oxysporum	+		
Macrophomina phaseolina	+	*Streptococcus pneumoniae*	-
Alternaria radicina	+		
Rhizoctonia solani	+	*Pseudomonas aeruginosa*	+
Sclerotium rolfsii	+		
Nigrospora oryzae	+	*Escherichia coli*	+
Phoma destructive	-		
Penicillium notatum	-	*Bacillus subtilis*	-
Aspergillus niger	-		

(+) = low activity and (-) = no activity

degrees. Only one isolate designated E44G, had the strongest antagonistic activity against the tested fungus. This isolate was used in the next investigations. Variation in the antagonistic activity between actinomycetes was already reported by several researchers[27,28,29]. The antimicrobial mechanisms may involve physical contact (hyperparasitism), synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition[13].

The investigated strain formed an extensively branched substrate mycelium, aerial hyphae which carried smooth -surfaced ellipsoidal spores in open hooked spore chains (Fig. 1). Strain E44G contains LL-diaminopimelic acid in the wall peptidoglycan, lacks characteristic major sugars and formed a grayish aerial spore mass on the seven standard media used (Table 1) which is a characteristic of the genus *Streptomyces*. Therefore, E44G was preliminary characterized as belonging to *Streptomyces*. The assignment of the strain to the genus *Streptomyces* is also supported by the results of the 16S rRNA gene sequence studies. The 16S rRNA gene sequence (1096bp) was determined for strain E44G. Comparison of the 16S rRNA gene sequence with corresponding Streptomycete sequences using DNA BLASTn (NCBI website) clearly showed that strain E44G is a member of the genus *Streptomyces* with a maximum similarity percentage 99% with *S. griseorubens*. The 16S rRNA gene sequence of strain E44G was deposited in the GeneBank under the accession number (KJ605118).

Description of *S. griseorubens* E44G

Streptomyces griseorubens E44G is aerobic, mesophilic, halotolerant, Gram-positive actinomycete that forms an extensively branched substrate mycelium and aerial hyphae. Open

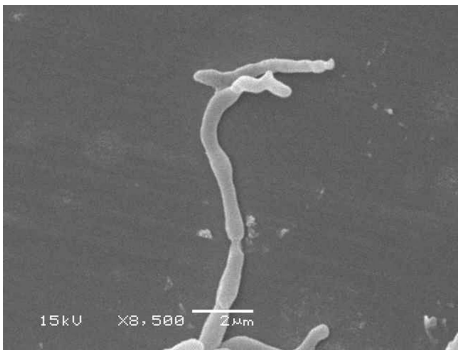

Fig. 1. Scanning electron micrograph showing spore chains and spore-surface ornamentation of *S. griseorubens* E44G grown on starch nitrate agar for 14 days at 30 ºC. Bar = 2 µm.

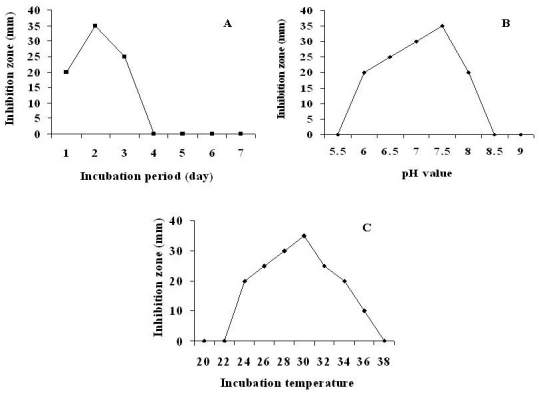

Fig. 2. Effect of different environmental conditions on antifungal activity of *S. griseorubens* E44G, incubation period (A), pH value (B) and incubation temperature (C).

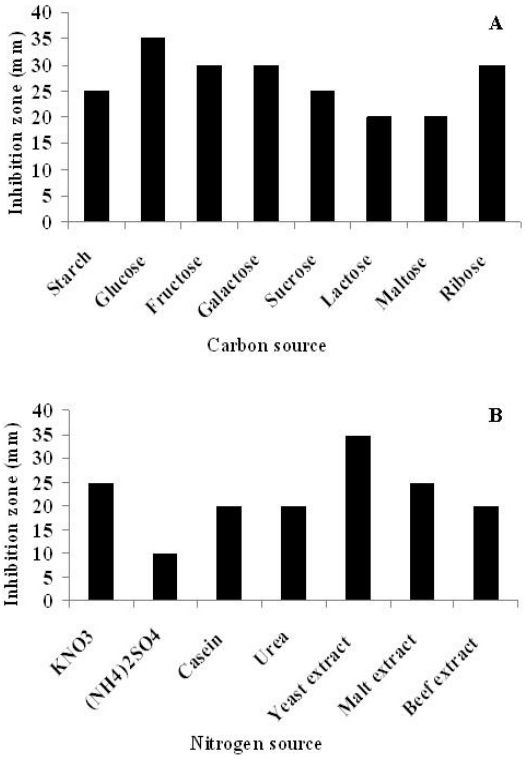

Fig. 3. Effect of different nutritional conditions on antifungal activity of *S. griseorubens* E44G, carbon sources (A) and nitrogen sources (B).

hooked spore chains with ellipsoidal, smooth surfaced spores are frequently formed. A creamy substrate mycelium and a grayish aerial spore mass are formed on starch nitrate agar medium. Diffusible pigments are not formed, but melanin pigments are produced on peptone/yeast extract/iron or tyrosine agars. The culture grows well between 20 to 45°C, but does not grow below 20°C and above 45°C. It grows at pH values from 5.5 to 9.5, but not at d" 4.5 or e" 10.5. Growth occurs at 10% NaCl but not at 15%. Protease, catalase, chitinase, are produced but, amylase, cellulose, pectinase, lipase, lecithinase and urease are not. The investigated strain produces hydrogen sulfide but does not reduce nitrate, utilize citrate, produce coagulase enzyme or degrade esculine and xanthine. D-glucose, D-fructose, D-galactose, sucrose, raffinose and rhamnose are used as sole carbon sources for energy and growth, but L-arabinose, xylose and *meso*-inositol are not. The organism uses L-cystiene, L-lysine, L-tyrosine, L-valine, L-alanine, L-leucine, L-histidin and L-proline as nitrogen sources but, L-phenylalanine is not. The described strain is sensitive to streptomycin and amoxicillin (Table 2).

It shows antifungal activity with various degrees against isolates of phytopathogenic fungi namely; *F. solani, F. oxysporum, M. phaseolina, A. radicina, R. solani, S. rolfsii* and *N. oryzae* but not *P. destructive, P. notatum* and *A. niger.* It shows antibacterial activity against strains of *P. aeruginosa, E. coli* but not *S. aureus, S. pneumonia,* and *B. subtilis* (Table 3). Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal agents that inhibit several pathogenic fungi[9,10] and antibacterial agents[11,12].

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[30]. Optimization of growth conditions is essential to maximize the antimicrobial potentiality.

In this connection, effect of different incubation periods was investigated. The antifungal activity was observed at the first three days of incubation. However, the highest activity was recorded at the second day as the best incubation period. Thereafter, the antifungal activity was dramatically declined and disappeared at the fourth day (Fig. 2A). The obtained result is in agreement with that reported by Prapagdee *et al*.[31]. The maximum antifungal activity after 48 hrs of incubation may be attributed to the fact that *S. griseorubens* E44G entered the stationary phase of growth. It has been reported that the antibiotic production by *Streptomyces* takes place in the stationary phase of the growth. The decrease in the antifungal activity after 48 hrs can be attributed to the decrease in the supply of nutrients to the microorganism or the accumulation of toxic by-products.

Effect of different pH values on the antifungal activity was studied using phosphate buffer with wide range of pH (Fig. 2B). No activity was recorded at pH 5.5. Afterward, the activity was increased with the increase in pH value and reaches its peak at pH 7.5 then any increase in pH caused a decrease in the antifungal activity and totally disappeared at pH 8.5. This means that the antifungal bio-compound was very sensitive to high acidity and alkalinity. According to these results, the antifungal bio-compound produced by *S. griseorubens* E44G has high efficiency at around neutral pH. These results are in agreement with those of Al-Askar *et al*.[10], who recorded pH 7.5 as the optimum pH for *S. tendae* RDS16. Changes in pH affect growth kinetics of microorganisms as enzymes activities. Most of bacterial strains have their optimum growth on neutral conditions. As a result, most antibiotics are optimally produced in pH close to (7) [32].

Incubation temperature was one of optimum factors which affected the antifungal activity. Wide range of incubation temperatures was tested in this study (20 to 38°C). The antifungal activity was observed between 24 and 36°C. No activity was recorded at 20, 22 or 38°C. The highest antifungal activity was observed at 30°C as the best incubation temperature (Fig. 2C). The obtained results are in agreement with those reported by Islam *et al*.[33] who found that the maximum inhibition of the fungal growth was achieved when *S. albidoflavus* C247 was cultured at 30°C. Temperature affects on growth rate and enzymatic reactions involved in metabolic synthesis. Lesser growth of microorganism at higher temperature is due to the fact that high temperature retards the metabolic processes of the microorganism by

denaturing enzymes, transport carriers and other proteins[32].

Bacterial growth is strongly related to carbon components and their concentrations in culture media[34]. These compounds enter in different metabolic processes resulting in the production of primary and secondary metabolites, including antifungal compounds[35]. The antifungal activity was observed with all carbon sources, but the best carbon source was glucose at which the highest antifungal activity was recorded followed by galactose, fructose and ribose (Fig. 3A). These results are in agreement with that of Vasavada *et al*.[36] who recorded that *S. sannanensis* RJT-1 utilized glucose as the best carbon source for antibiotic production. Optimal antifungal activity has generally been achieved by cultivating organisms in media containing slowly used nutrient sources, or under conditions which allow a slow supply of these nutrients[37,38].

Nitrogen constitutes the second major requirement for growth since it is involved in the synthesis of cell structural and functional proteins. Many studies have proved that antibiotic synthesis strongly related to nature and concentration of nitrogen source in the culture medium. Effect of different nitrogen sources on the antifungal activity of *S. griseorubens* E44G was studied. The highest activity was observed with yeast extract (Fig. 3B). Quickly metabolized nitrogen sources, usually decrease antibiotic production in different microorganisms as well as streptomycetes. Different studies have shown that complex nitrogen sources such as yeast extract, soybean meal and corn meal can increase the antibiotics due to slow decomposition of the compounds in the medium[39].

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