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Research Article
Antibacterial Activity of a New Strain of *Streptomyces maritimus* MSQ21 against *Ralstonia solanacearum*

Marwa A. Zaki, El Sayed A. Saleh, Mahmoud M. Zaki, Abdalla S. Korayem and Shimaa A. Amin

Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shubra, 11241 Cairo, Egypt

Abstract
Background and Objective: Actinobacteria represent the most prominent group of microorganisms, which produce a vast number of bioactive compounds especially antibiotics. The present study investigated the antibacterial activity of some actinomycete isolates against *Ralstonia solanacearum* type 3 biovar 2 (phytopathogenic bacterium that causes tomato wilt disease and brown rot of potatoes). **Materials and Methods:** The most potent actinomycete isolates in the antibacterial activity was further identified up to species based on its phenotypic and molecular characteristics. Additionally, the most suitable carbon and nitrogen sources for increasing the antibacterial activity were also investigated. **Results:** Interestingly, *Streptomyces* isolate MSQ21 achieved the highest antibacterial activity against *R. solanacearum* with an inhibition zone of 18 mm. 16S rRNA gene analysis suggested that *Streptomyces* MSQ21 was identified as a strain of *S. maritimus* Glycerol (2.25%, w/v) and $(NH_4)_2SO_4$ (0.13%, w/v) were the most suitable carbon and nitrogen sources for increasing the antibacterial activity. **Conclusion:** It could be concluded that the maximum antibacterial activity (30mm) produced by *S. maritimus* strain MSQ21 against *R. solanacearum* could be obtained by using the modified starch nitrate medium containing (g L^{-1}): Glycerol, 25: Ammonium sulphate, 1.6: Dipotassium hydrogen phosphate, 1: Magnesium sulphate, 0.5: Sodium chloride, 0.5: Calcium carbonate, 3: Ferrous sulphate and 0.01: Distilled water up to 1 L and under the following conditions: Temperature 30°C, agitation speed 250 rpm, inoculum size 1-50 mL medium, incubation period 4 days and pH 8.5.

Key words: *Streptomyces maritimus*, *Ralstonia solanacearum*, antibacterial activity, identification, response surface methodology

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Corresponding Author: Marwa A. Zaki, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shubra, 11241 Cairo, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The phylum actinobacteria (Phyl. nov) is one of the major phyla in the domain: Bacteria, as inferred from its branching pattern in the 16srRNA gene tree and taxon-specific 16srRNA signature[1]. The phylum includes phenotypically diverse organisms which show diverse morphological properties that range from cocci to highly differentiated mycelia[2]. The majority of actinobacteria are free-living organisms that are widely distributed in both terrestrial and aquatic (including marine) ecosystems[3]. Among Prokaryotic members of the class: Actinobacteria, notably *Streptomyces* strains are the richest source of novel natural products especially antibiotics, enzymes, vitamins, hormones and other bioactive compounds[4].

Ralstonia solanacearum is widespread in tropical and subtropical regions. Its harmfulness, wide host range, persistence and huge genome plasticity have made it one of the world's most important phytopathogenic bacteria and one of the most intensively studied[5]. Bacterial wilt, *Ralstonia solanacearum* attacks more than 200 plant species, including more than 50 plant families[6,7]. In Egypt, bacterial wilt is caused by *Ralstonia solanacearum* is a serious and common disease of economically important crops mainly solanaceous family such as tomato, potato and pepper[8,9].

Microbial secondary metabolites represent the most important sources of natural compounds with potential bioactivities being used in different fields such as agriculture and medicine. The representative sources of these bioactive secondary metabolites are gram-positive soil-living bacteria actinomycetes, particularly *Streptomyces*, which produce approximately 80% of commercially available antibiotics[10]. Therefore, members of novel *Streptomyces* species are in demand as sources of the novel, environmentally friendly, commercially significant, natural bioactive compounds. Recently, several studies point to the importance of *Streptomyces* as a source of antimicrobial metabolites against *Ralstonia solanacearum*[11,12]. The present study investigated the antibacterial activity of actinomycete isolates against *Ralstonia solanacearum*. The taxonomic identity of the most potent isolate was determined by cultural, morphological and physiological characteristics, as well as16S rRNA gene sequence analysis. Additionally, the most suitable carbon and nitrogen sources for increasing the antibacterial activity of the selected *Streptomyces* strain were also investigated.

MATERIALS AND METHODS

Study area: The study was carried out from 2018 until 2021 in the microbiology lab in the Department of Agricultural Microbiology at the Faculty of Agriculture, Ain Shams University.

Isolation, purification and maintenance of actinomycetes: Soil samples were collected in clean bags from different localities in Egypt (Qalioubia, Sharkia, Fayoum, Giza, Menoufia, Alexandria, Ports Said and Dakahlia Governorates), from 10-15 cm depth and passed through a 2 mm sieve to remove debris and plants. The samples were air-dried, mixed with 10% (w/w) CaCO and then incubated at room temperature for 15 days. Following the incubation, 10 g of each soil sample were mixed with 90 mL sterile distilled water. The soil suspension was incubated at 28°C and 250 rpm on a rotary shaker for 30 min. The supernatant was collected and subjected to serial dilutions from 10^{-2}-10^{-5}. Aliquots (1 mL) was speared on a plate of Starch Nitrate Agar (SNA)[13]. The inoculated plates were incubated at 28±2°C for 4-7 days. Following the incubation, the actinomycete colonies were picked up and sub-cultured several times until pure cultures were obtained. Purified actinomycete isolates were maintained on the same medium at 4°C until used. Subculturing of completely purified actinomycete isolates was usually carried out every 2 months. All purified actinomycete isolates were identified up to the genus according to Goodfellow *et al.*[2].

Screening of actinomycete isolates for antibacterial activity: The actinomycete isolates were screened for their antibacterial activity against *Ralstonia solanacearum* type 3 biovar 2 (obtained from Potato Brown Root Project (PBRP), Dokki, Giza, Egypt) by agar well diffusion method according to Ahsan *et al.*[14]. Each actinomycete isolate was growing on SNA for 15 days at 28±2°C. Following the incubation, 5 agar discs from each actinomycete culture were separately transferred to a 250 mL ask containing 50 mL starch nitrate broth[13]. The inoculated flasks were incubated for 7 days at 30°C on a rotary shaker at 200 rpm. After incubation, each culture was centrifuged at 12000 rpm and aliquots (0.1 mL) of the supernatant of each actinomycete culture were loaded into nutrient agar plates (Oxoid) previously inoculated with overnight culture *Ralstonia solanacearum* (adjusted to 1×10^{6} cfu mL^{-1}) and cut the well using 0.6 mm sterile cork borer. The plates were incubated overnight at 37°C. Next, the antibacterial activity of each extract was evaluated by measuring the diameter (mm) of the clear zone around each well. The most potent actinomycete isolates in the antibacterial activity were selected for the subsequent study.

Identification of the most efficient actinomycete isolate classical identification: The most efficient actinomycetes isolate was completely identified, based on its cultural, morphological, physiological and biochemical characteristics according to the standard methods adopted by Goodfellow *et al.*[2]. The description of streptomycete species of the International Streptomycetes Project (ISP) introduced by Goodfellow *et al.*[2]. was consulted.

The selected actinomycete isolate was also investigated for its ability to produce amylase enzyme[15], lipase[16], cellulase[17], gelatinase[18] and chitinase enzymes[19].

Molecular identification: Molecular identification was used to confirm the bacterial species previously identified by classical techniques. Genomic DNA extraction, Polymerase Chain Reaction (PCR) mediated amplification of the 16S ribosomal DNA, purification of PCR products and sequencing of the PCR products for the selected actinomycete isolate under study was carried out according to the method previously described by Antony-Babu *et al.*[20]. The 16S rRNA gene sequencing was performed using 2 consensus primers: forward primer, U8-27 AGAGTTTGATC (AC) TGGCTCAG and reverse primer R1494-1514 CTACGG (T/C) TACCTTGTTACGAC then amplified using AmpliTaq® DNA polymerase (Macrogen Genomics, Korea). The amplified product was sequenced using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Bio-Systems, USA) and also analyzed in the National Centre for Biotechnology Information, USA database using Basic Local Alignment Search Tool for Nucleotides (BLASTN).

Phylogenetic analysis sequences were aligned with those of the reference strains using Bio Edit program version 7.0.4.01. The phylogenetic tree was constructed using the MEGA program with the neighbour-joining method (www.megasoftware.net). Bootstrap values were determined according to the method previously described by Tamura *et al.*[21] with 1000 replicates.

Antibacterial enhancement of the selected strain: The most suitable carbon and nitrogen sources for increasing the antibacterial activity of the selected *Streptomyces* strain against *Ralstonia solanacearum* were studied. Therefore, trials were done by replacing the source in productive basal medium with the equivalent amount of the tested source to eliminate errors which may occur as a result of differences in concentrations in each source as follows briefly, of dextrin, galactose, glucose, glycerol, sucrose, corncobs, rice straw and wheat straw were separately added to SNA instead of starch. Similarly, ammonium nitrate, calcium nitrate, ammonium sulphate, sodium nitrate, beef extract, peptone, urea and yeast extract were separately added to SNA instead of potassium nitrate. The growth conditions and antibacterial activity of the selected strain against *Ralstonia solanacearum* were carried out as mentioned before.

Optimization of antibacterial activity by *Streptomyces* strain MSQ21 using Plackett-Burman experimental design: Plackett-Burman design[22] was used to evaluate the integrated effects of different variables on antibacterial activity production by *Streptomyces* strain MSQ21. For each variable, 2 different concentrations, high (+) and low (-), were tested. The experimental design was prepared with the help of software design expert trial 12.0.3.0 (Stat Ease Inc USA). Antibacterial activity production was carried out in 250 mL Erlenmeyer flasks containing 50 mL medium and each run was done in triplicates.

Plackett-Burman experimental design is based on the first-order model, which was determined by the following Eq. 1:

$$Y = B_0 + \sum Bixi \quad (1)$$

Central composite design of response surface methodology (RSM): After identifying the significant variables for antibacterial activity by *Streptomyces* isolate through a Plackett-Burman design, a Central Composite Design (CCD) was adopted to optimize the major variables (carbon source concentration, nitrogen source concentration and agitation speed). The 3 selected independent variables were studied at 3 different levels (as -1, 0 and +1), 6 replications at the central point and sets of 20 experiments (batch experiments) were carried out in Table 3. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 3. The statistical software package design expert trial 12.0.3.0 (Stat Ease Inc USA), was used to analyze the experimental data.

The optimal values of the independent variables that gave a theoretical maximum response in Eq. 2 were obtained by maximizing the equation within a definite boundary condition.

Antibacterial activity was taken as the response (Y) and multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The relationship of the independent variables and the response was calculated by the second-order polynomial Eq. 2:

$$Y_i = b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_{12}+b_{22}X_{22}+b_{33}X_{32}+b_{12}X_1X_2+ b_{23}X_2X_3+b_{13}X_1X_3 \quad (2)$$

Where, Y_i is the predicted response, X_1, X_2, X_3 are independent variables, b_0, is the offset term, b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{33} are squared effects and b_{12}, b_{23}, b_{13} are interaction terms.

Statistical analysis: Statistical analysis and graph plotting was performed using design expert software design expert trial 12.0.3.0 (Stat Ease Inc USA). Statistical analysis of variance (ANOVA) through fisher's test was used to evaluate the effect of independent variables on the response and significant results were identified by a p-value of <0.05. Multiple correlation coefficient (r) and adjusted r^2 were used as quality indicators to evaluate the fitness of the 2nd-order polynomial equation. Contour plots (3D) and response surface curves were employed to demonstrate the relationship and interaction between the coded variables and the response. The optimal points were determined by solving the equation derived from the final quadratic model. The data analysis was performed using the software of IBM® SPSS® statistics server version 23.0. (2015), which applied Duncan's test[23] at a 5% level to compare between mean values.

RESULTS AND DISCUSSION

Isolation, identification and screening of actinomycete isolates: Actinomycetes are widely distributed in natural ecosystems and can produce a wide variety of biologically active metabolites especially antibiotics. Figure 1 show that 60 actinobacterial isolates which differed in their colonies feature were isolated from different localities in Egypt. All isolates were purified and identified up to genus according to their cultural, morphological and physiological characteristics. All these isolates were found to belong to genus *Streptomyces*, as they form well-developed branching, non-septate, non-fragmented aerial mycelium bearing along spore chains and non-motile spores which not borne in verticillate sporophores[2]. The antibacterial activity of these isolates against *Ralstonia solanacearum* showed that only 9 (15%) of these isolates exerted antibacterial activity, whereas 51 (85%) of these isolates have no antibacterial activity against *Ralstonia solanacearum*. *Streptomyces* MSQ21 achieved the highest antibacterial activity against *R. solanacearum*, with inhibition zone 18 mm, followed by *Streptomyces* MCA10, FJB70, GBC21, QDE10, QAD61, QAF01, SFG10 and QMH51, with inhibition zone 16, 16, 15, 14, 12, 11, 11 and 8 mm, respectively shown in Fig. 2.

The data of the present study are in agreement with those reported in previous studies, which revealed that actinomycetes are capable of surviving in different habitats and are widely distributed in natural ecosystems such as soil, rhizosphere soil and hypersaline soil. They are part of the normal microflora of the soil and constitute a significant

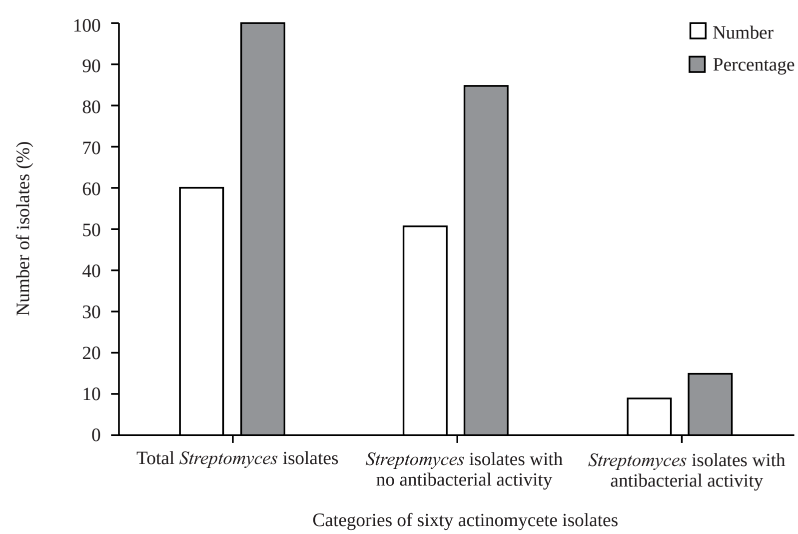

Fig. 1: Screening of sixty actinomycete isolates against *Ralstonia solanacearum*

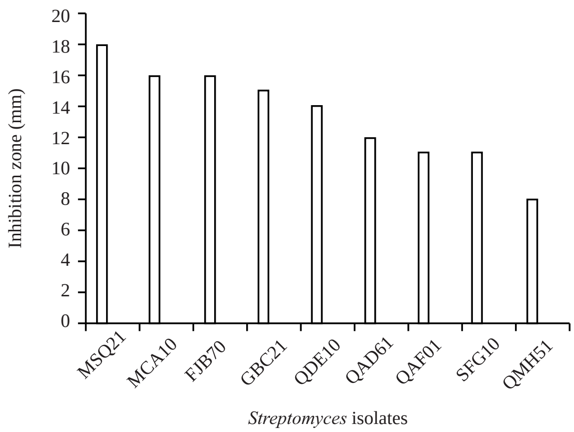

Fig. 2: Antibacterial activity of nine *Streptomyces* isolates against *Ralstonia solanacearum*

Table 1: Culture, morphological, physiological and biochemical characteristics of *Streptomyces* MSQ21

Parameters	Characteristics
Cultural characteristics	
Colour of aerial mycelium	Gray
Colour of substrate mycelium	Colourless
Diffusible pigments	-
Morphological characteristics	
Spore surface ornamentation	Smooth
Spore chain morphology	(RF)
Physiological characteristics	
Melanoid pigment produced	-
Growth on Czapek's medium	+
Sodium chloride tolerance	Up to 7%
Sensitivity to streptomycin (50 µg mL^{-1})	-
Antifungal activity[1]	+
Antibacterial activity[2]	+
Biochemical characteristics	
Amylase	+
Lipase	+
Gelatinase	+
Cellulase	+
Chitinase	+
Catalase	+
Oxidase	+
Utilization of different carbon sources	
No carbon	-
D-glucose	+
D-xylose	-
L-arabinose	-
L-rhamnose	+
D-fructose	+
Galactose	+
Raffinose	-
D-mannitol	+
Inositol	-
Sucrose	+

1: Against (*Rhizoctonia solani* ATCC 76144, *Alternaria alternata* MTCC 1362 and *Fusarium oxysporum f.* sp. *lycopersici*), 2: Against (*Ralstonia solanacearum* type 3 biovar 2, *Bacillus cereus* ATCC 11778, *E. coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27853), RF: Rectus-flexibilis, +: Presence and -: Absence

component of the soil microbial community. Actinomycetes especially *Streptomyces* species produce antimicrobial activities against bacteria, fungi and viruses. Approximately, 80% of known antibiotics have been produced from genus *Streptomyces*[10,24]. In the light of the aforementioned results, it could be stated that the most effective *Streptomyces* isolate MSQ21 in antibacterial activity was selected for sequential studies.

Identification of *Streptomyces* MSQ21: The maximum antibacterial activity against *Ralstonia solanacearum* in the present study was achieved *Streptomyces* MSQ21. The cultural, morphological, physiological and biochemical characteristics of *Streptomyces* MSQ21 are summarized in Fig. 3 and Table 1. The results showed that the colour of aerial mycelium was grey and colourless of vegetative mycelium and no soluble pigments were produced in Fig. 3a. It has straight and long spore chains are in section Rectus-Flexibilis (RF) in Fig. 3b and the spores are characterized by smooth surface in Fig. 3c. Melanoid pigments are not produced on all selected media used. The isolate was also characterized by positive growth on Czapek's agar medium, tolerant against sodium chloride up to 7% NaCl concentration and not inhibited by streptomycin (50 µg mL^{-1}). It was found that D-glucose, L-Rhamnose, d-fructose, galactose, d-mannitol, sucrose are all utilized as the sole carbon source for growth, whereas the other carbon sources used did not support any growth. *Streptomyces* isolate MSQ21 was produced many enzymes such as amylase, lipase, gelatinase, cellulase, chitinase, catalase and oxidase. *Streptomyces* MSQ21 exhibited antibacterial and antifungal activities against different pathogenic microorganisms.

It's interesting to mention that in all standard references examined[2], no similar species was found to *Streptomyces* MSQ21. Therefore, the phylogenetic analyses based on 16SrRNA gene sequencing of the selected *Streptomyces* isolate MSQ21 were studied.

Intestinally, PCR amplification, Basic Local Alignment Search Tool (BLAST), phylogenetic analysis and neighbour-joining method indicated that the *Streptomyces* MSQ21sequence showed 99% similarity with *Streptomyces maritimus* and received National Center for Biotechnology Information (NCBI) Genbank accession number LC638499.1 in Fig. 4. Thus, *Streptomyces* MSQ21 could be identified as new strains of *Streptomyces maritimus*. To knowledge, this is the first report describing the antibacterial activity of *Streptomyces maritimus* against *Ralstonia solanacearum*. Generally, the obtained results were supported by recent

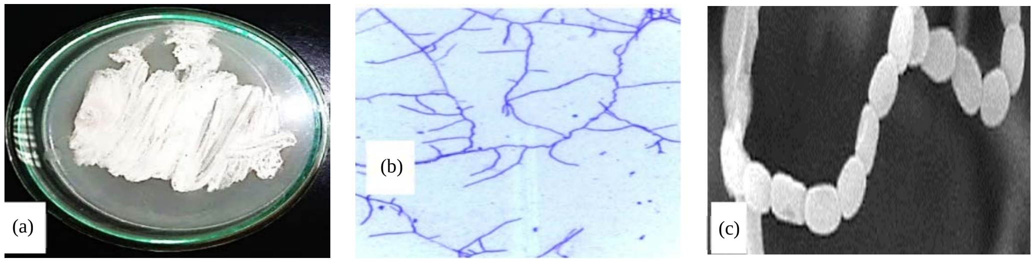

Fig. 3(a-c): Cultural and morphological characteristics of *Streptomyces* MSQ21, (a) Image of *Streptomyces* MSQ21 grown on starch nitrate agar, (b) Microphotograph of spore chains morphology of *Streptomyces* MSQ21 (1000x) and (c) Electron micrograph of spore surface ornamentation of *Streptomyces* MSQ21 (10000x)

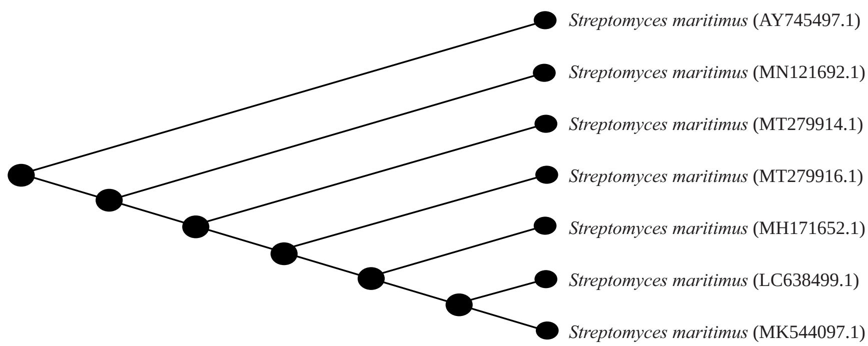

Fig. 4: Phylogenetic analysis based on 16S rRNA gene sequencing showing the position of *Streptomyces* MSQ21 in the neighbour-joining tree

studies, which demonstrated the importance of *Streptomyces* as a source of antimicrobial metabolites against *Ralstonia solanacearum*[11,12].

Effect of different carbon and nitrogen sources on the antibacterial activity of *S. maritimus* MSQ21: Figure 5a clearly shows that the best suitable carbon source for increasing the antibacterial activity of *Streptomyces maritimus* MSQ21 against *Ralstonia solanacearum* was glycerol because it gave the highest inhibition zone (20 mm) and the lowest mycelial dry weight (90 mg/50 mL), while dextrin gave the lowest inhibition zone being (5.5 mm). When compared to other carbon sources. When studying the effect of different nitrogen sources on the antibacterial activity in Fig. 5b, it was found that the best nitrogen source for antibacterial activity was ammonium sulfate because it gave the highest antibacterial activity (27 mm of inhibition zone) and lowest mycelial dry weight (90 mg/50 mL), while potassium nitrate gave the lowest inhibition zone being (20 mm), as compared to other nitrogen sources.

Optimization of antibacterial activity by statistical experimental designs

Selection of the most significant variables using Plackett-Burman design: Table 2 showed that the inhibition zone diameters formation by *S. maritimus* MSQ21 against *R. solanacearum* was ranged from 4-28 mm. The largest inhibition zone diameter (28 mm) was measured in run number 1 with the medium consisting of glycerol (22.5 g L^{-1}), $(NH_4)_2SO_4$ (1.3 g L^{-1}) and adjusted pH to 8.5, inoculated with 1.0% and then incubated at 30°C and 225 rpm for 4 days followed by run number 12 which the inhibition zone diameter was 26 mm. In contrast, the smallest diameter inhibitory zone (4 mm) was seen in run number 10, which was composited with glycerol (17.5 g L^{-1}), $(NH_4)_2SO_4$ (0.7 g L^{-1}) and adjusted pH to 8.5 and inoculated with 1.0% before incubating at 37°C and 175 rpm for 4 days.

Data in Table 2 also revealed that the ANOVA was used to evaluate the effect of independent variables on the response and the significant results were identified by a p-value <0.05. The model F-value of 91.44 implies that the model is significant for antibacterial activity. The smaller p-value

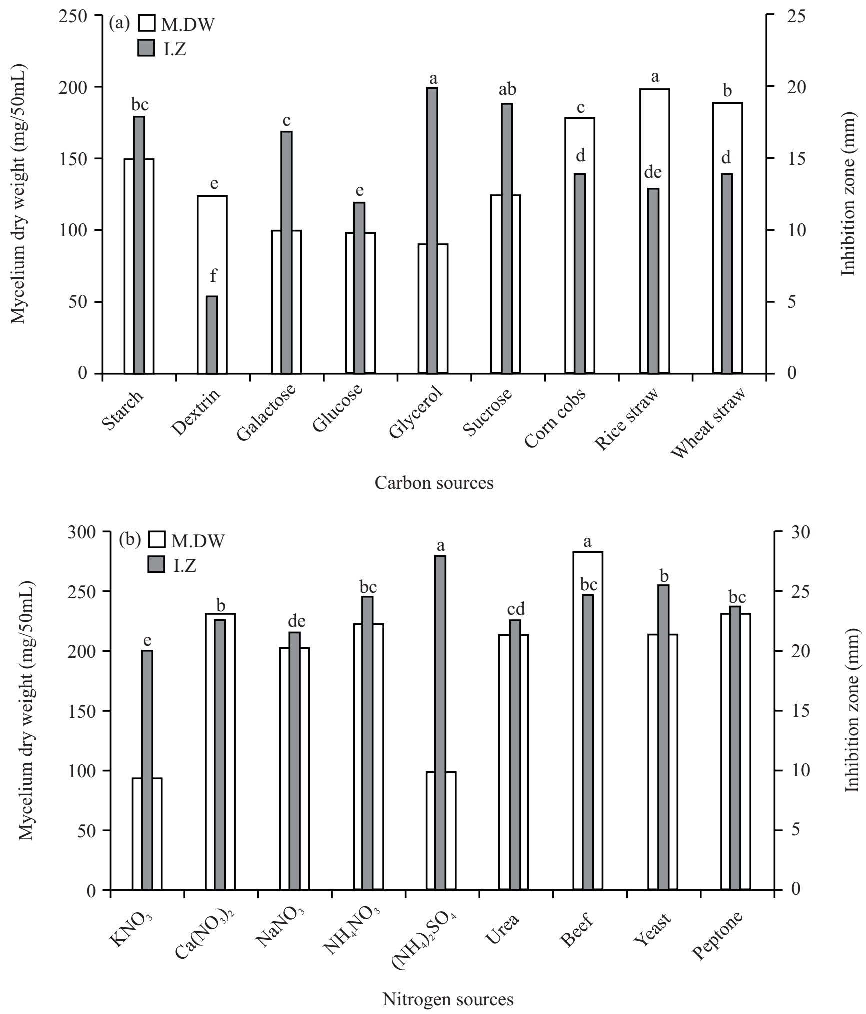

Fig. 5(a-b): Effect of different carbon and nitrogen sources on antibacterial activity of *S. maritimus* MSQ21 against *Ralstonia solanacearum*, (a) Carbon source and (b) Nitrogen source

[a,b]values different in the same column are difference significant according to Duncan's test, I.Z.: inhibition zone and M.D.W.: Mycelium dry weight (mg /50 mL) *Growing at 28±2°C on a rotary shaker (200 rpm) and initial pH 7.7

indicates the high significance of the corresponding coefficient[25]. The analyzed results of *S. maritimus* MSQ21 suggest that out of 7 different independent variables, only 3 (glycerol, $(NH_4)_2SO_4$ and agitation speed) were the most significant variables that affected the antibacterial activity. While the remaining variables were insignificant ($p \geq 0.05$). The standard division and mean were 0.87 and 15.9, respectively.

The coefficient of determination (R^2) was 0.995 specified a high correlation between the experimental and predicted values.

By using design-expert, the equation obtained for Plackett-Burman design (first order model) of *S. maritimus* MSQ21 was as follows:

Y antibacterial activity = -31.53968+2.16667, Glycerol+11.94444, $(NH_4)_2SO_4$-0.452381, Temperature+0.036667, Agitation speed+0.250000, Inoculum size-0.250000, Incubation period+0.083333, Dummy 3+0.250000 Dummy 4

In this study, glycerol was proved to be the best carbon source for producing antibacterial activity. These results are somewhat similar to the findings of Ilić *et al.*[26]. Also, the $(NH_4)_2SO_4$ was the best nitrogen source for producing antibacterial activity. These results are somewhat similar to the findings of Zhu *et al.*[27] reported the importance of optimal concentration of $(NH_4)_2SO_4$ in antibiotic production by *Streptomyces viridochromogenes*. Cultural parameters also influence the production of bioactive secondary metabolites[28].

Table 2: Plackett-Burman experimental design matrix and ANOVA for screening variables affecting the *S. maritimus* MSQ21 antibacterial activity

		Variables											
		A	B	C	D	E	F	G	H	J	K	L	
Run	Real levels Low (-1)	17.5	0.7	30	175	7.7	0.5	4	-	-	-	-	
numbers	High (1)	22.5	1.3	37	225	8.5	1	7	-	-	-	-	IZD (mm)
1		1	1	-1	1	1	1	-1	-1	-1	1	-1	28
2		1	-1	1	1	-1	1	1	1	-1	-1	-1	17
3		-1	-1	-1	1	-1	1	1	-1	1	1	1	10
4		1	-1	-1	-1	1	-1	1	1	-1	1	1	18
5		-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8
6		1	1	1	-1	-1	-1	1	-1	1	1	-1	22
7		-1	1	1	-1	1	1	1	-1	-1	-1	1	12
8		-1	1	-1	1	1	-1	1	1	1	-1	-1	15
9		1	-1	1	1	1	-1	-1	-1	1	-1	1	17
10		-1	-1	1	-1	1	1	-1	1	1	1	-1	4
11		-1	1	1	1	-1	-1	-1	1	-1	1	1	14
12		1	1	-1	-1	-1	1	-1	1	1	-1	1	26
ANOVA	Model												
Coefficient means squares	68.58	352.08	154.08	30.08			0.75				0.0833	0.75	
Df	8	1	1	1			1				1	1	
F-value	91.44	469.44	205.44	40.11			1				0.1111	1	
p-value	0.0017	0.0002	0.0007	0.008			0.391				0.7608	0.391	
Std. Dev.					0.87								
Mean					15.9								
R^2					0.995								

IZD: Inhibition zone diameter, A: Glycerol, B: $(NH_4)_2SO_4$, C: Temperature, D: Agitation speed, E: pH, F: Inoculum size, G:Incubation period, -1: Low level of the variable, +1: High level of the variable, 0: Medium level of the variable, Df: Degree of freedom and *Significant at 5% level ($p \leq 0.05$)

In the present study, fermentation conditions such as pH, temperature and incubation time were optimized through conventional means. *Streptomyces maritimus* MSQ21 showed the best growth and antibacterial activity at pH 8.5 and temperature of 30°C after an incubation period of 4 days. These results are somewhat similar to the findings of Jose and Jebakumar[29], who reported the maximum antibacterial activity of pH 7.5 at 32°C after 11 days with a rare actinomycete, *Nonomuraea* sp. JAJ18. From earlier reports, it is also evident that the maximum antibiotic production by *Streptomyces* cultures required at least 96 hrs.

Improvement of antibacterial activity production by central composite design (CCD): Table 3 reveal that the inhibition zone diameters formation (actual and predicted) by *S. maritimus* MSQ21 against *R. solanacearum* were ranged from 5-30 and 3.35-30.4 mm, respectively. The largest inhibition zone diameter in actual form (30 mm) and predicated form (30.4 mm) was appeared in run number 7 with the medium consisting of glycerol (25 g L^{-1}), $(NH_4)_2SO_4$ (1.6 g L^{-1}) and incubated at 250 rpm, followed by run number 14 which the inhibition zone diameter was 28.75 mm. Whereas, the smallest diameter inhibition zone was recorded in run number 2 being 5 in actual form and 3.35 mm in predicated form, respectively which is composited with glycerol (17.5 g L^{-1}) and $(NH_4)_2SO_4$ (1.0 g L^{-1}) and then incubated at 200 rpm, respectively.

Table 3 clearly show that ANOVA was used to assess the effect of independent variables on the response and the significant results which were identified by a p-value <0.05. The model F-value of 15.01 implies that the model is significant for antibacterial activity. The smaller p-value indicates the high significance of the corresponding coefficient Tanyildizi *et al.*[25]. The coefficient of determination (R^2) was 0.931. This indicates a high correlation between the experimental and predicted values. The standard division and mean were 2.51 and 19.60, respectively.

The mathematical model of *S. maritimus* MSQ21 describing the relationship between variables (A, B and C) and response (Y) for antibacterial activity could be obtained by the following second order polynomial Eq.:

$$Y \text{ antibacterial activity} = -222.17433+13.87849, \text{ Glycerol}+ 1.80054, (NH_4)_2SO_4+0.702849, \text{ Agitation speed}+0.902363, \text{ Glycerol}*(NH_4)_2SO_4-0.005172, \text{ Glycerol*Agitation speed}- 0.009764, (NH_4)_2SO_4 \text{ *Agitation speed}-0.288534, \text{ Glycerol}^2- 4.75928\ (NH_4)_2SO_{42}- 0.001285 \text{ Agitation speed}^2$$

Table 3: Central composite design matrix and ANOVA for optimizing variables affecting the *S. maritimus* MSQ21 antibacterial activity

		Variables				
		A	B	C		
	Real levels					
	Low (-1)	17.5	0.4	150	Antibacterial activity (mm)	
	Medium (0)	20	1	200		
Run numbers	High (1)	22.5	1.3	225	Actual	Predicted
1		1	-1	1	19	22.51
2		-1	0	0	5	3.35
3		0	0	0	22	21.59
4		-1	1	1	15	17.01
5		-1	-1	1	10	13.49
6		0	0	0	22	21.59
7		1	1	1	30	30.4
8		1	1	-1	26	25.76
9		-1	1	-1	13	12.74
10		1	0	0	22	21.59
11		-1	-1	-1	8	8.92
12		1	-1	0	18	14.85
13		1	1	0	25	24.9
14		1	1	1	28	28.75
15		1	0	0	22	21.59
16		1	0	0	22	21.59
17		1	0	0	27	25.4
18		1	-1	-1	18	19.24
19		1	0	-1	14	14.6
20		1	0	1	26	22.15

ANOVA

Variables	Coefficient means squares	Df	F-value	p-value
Model	94.64	9	15.01	0.0001*
A	499.11	1	79.17	<0.0001*
B	103.7	1	16.45	0.0023*
C	58.53	1	9.28	0.0123*
AB	4.61	1	0.7317	0.4123
AC	1.05	1	0.1669	0.6915
BC	0.054	1	0.0086	0.9281
A^2	85.57	1	9	15.01
B^2	4.83	1	0.7658	0.4021
C^2	16.98	1	2.69	0.1318
Std. Dev.			2.51	
Mean			19.60	
R^2			0.931	

A: Glycerol, B: $(NH_4)_2SO_4$, C: Agitation speed, -1: Low level of the variable, +1: High level of the variable, 0: Medium level of the variable, DF: Degree of freedom and *Significant at 5% level ($p\leq0.05$)

The interaction between 3 variables was displayed by 2-dimensional contour plots and 3D contours that are graphical charts based on the model equation to determine the optimum level of each variable for antibacterial activity in Fig. 6a-c. Each contour presented the interaction among 2 factors while the others were set at zero level[25]. The maximum response score was obtained when there is a perfect interaction between the independent variables[30] and it can be observed by the central point within the highest red contour area which represents the optimum condition and concentrations of respective variables[25].

Fig. 6a-c reveal that antibacterial activity was highly and interactively influenced by all selected variables. Antibacterial activity was predicted to be increased with high concentrations of glycerol (25 g L^{-1}) and $(NH_4)_2SO_4$ (1.6 g L^{-1}) when agitation speed was maintained in the selected high level at 250 rpm in Fig. 6a. The optimum antibacterial activity could be possible with a high in each glycerol concentration (25 g L^{-1}) and agitation speed at 250 rpm in Fig. 6b. A similar response curve in Fig. 6c showed the interaction of $(NH_4)_2SO_4$ concentration and agitation speed at 250 rpm by glycerol concentration at optimum value.

In the present study, after optimization through RSM, the antibacterial activity has been notably increased when compared to the un optimized medium. Antibacterial activity against *R. solanacearum* in terms of zone of inhibition was

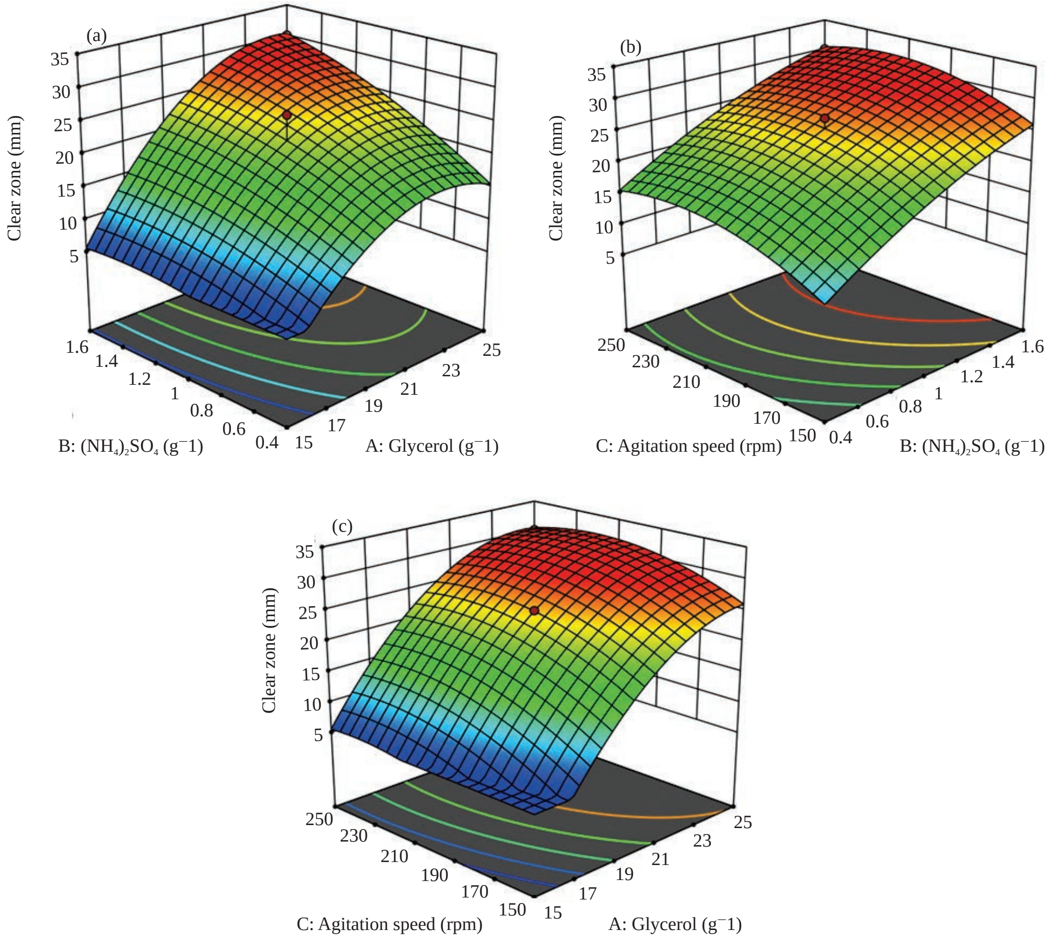

Fig. 6 (a-c): Three-dimensional response surface and contour plots showing the effect of glycerol, $(NH_4)_2SO_4$ and agitation speed and their interacted effect on antibacterial activity, (a) Interaction between glycerol vis. $(NH_4)_2SO_4$, (b) Interaction between glycerol vis. agitation speed and (c) Interaction between $(NH_4)_2SO_4$ vis. agitation speed

increased from 4-30 mm. An 86.66% increase from modified medium strongly suggests that the quantity of medium components affects antibacterial metabolite activity against *R. solanacearum*. Hence, the current statistical experimental design was found to be accurate in optimizing the significant medium components. It is the first report from a *Streptomyces* sp. where an 86.66% increase in antibacterial activity against *R. solanacearum*. These results are somewhat similar to the findings of Jacob *et al.*[31], who found that using RSM, antibacterial activity against *S. epidermidis* in terms of zone of inhibition was increased from 15±1.5-28±1.5 mm.

CONCLUSION

It is concluded here that the *Streptomyces maritimus* strain MSQ21 is a novel strain and has efficiency in the production of antibacterial activity and the best medium for antibacterial activity against *R. solanacearum* contains (g L^{-1}): glycerol, 25: NH4)2SO4, 1.6: K_2HPO_4, 1: $MgSO_4.7H_2O$, 0.5: NaCl, 0.5: $CaCO_3$, 3: $FeSO_4.7H_2O$, 0.01 and distilled water up to 1 L and under the following conditions: Temperature 30°C, agitation speed 250 rpm, inoculum size 1-50 mL medium, incubation period 4 days and pH 8.5, where it gave clear zone of 30 mm.

SIGNIFICANCE STATEMENT

Due to the efficiency of *Streptomyces maritimus* strain MSQ21 in the production of antibacterial activity, it could be used in biological control against *Ralstonia solanacearum* (Phytopathogenic bacterium that causes tomatoes wilt disease and brown rot of potatoes).

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