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Detection of the Antifungal Activities and Halotolerance Genes of Some *Streptomyces* Bacteria using PCR Technique

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This study reported that, there thirty halotolerant *Streptomyces* strains were isolated from the saline soil of Alnatron valley in Egypt. These strains were screened against five phytopathogenic fungi to detect their antifungal activities. The screening test showed that sixteen strains were found have different antifungal activities, however, only two strains (S-1 and S-2) had been recorded as most producers of antifungal activities, however, the later has a higher activity than the former. On the other hand, the salt-tolerance range of all *Streptomyces* strains was determined. The results proved that, twenty three, five, and two strains were found have the ability to grow at 7, 11, and 15% NaCl respectively. Fortunately, the two most halotolerant strains are the same two that mentioned before. Therefore, they were identified as *Streptomyces gardneri* (S-1) and *Streptomyces platensis* (S-2). Eventually, the halotolerance genes (*P5CR* and *mtlD*) were detected in the DNA extracts of five strains included *S. gardneri* and *S. platensis*, while only *P5CR* gene was detected in the DNA extracts of seventeen strains, and *mtlD* gene was detected in the DNA extracts of thirteen strains.

Key words: Gene amplification, Halotolerance, Salt-stress resistance genes, Streptomycetes.

Some Gram-positive *Streptomyces* bacteria habitat and abundantly grow saline environments so they are called halotolerants, which had been known as a main source of useful primary and secondary metabolites particularly novel compounds[1,2,3]. Halotolerant *Streptomyces* bacteria can grow at a wide range of sodium chloride concentration (5 to 25%). Halotolerance refers to some native adaptations including a highly saline cytoplasm, specialized salt-requiring proteins, and the unique light-driven proton as well as chloride pumps bacteriorhodopsin and halorhodopsin[4]. Halotolerance mechanisms could be detected by virtue of haloadaptation study at the gene level[5,6,7,8,9,10,11]. RAPD-PCR technique was successfully used for screening the gene sequence variation within different microorganisms such as *Plasmopara viticola*[12], *Candida albicans*[13] and *Streptomyces* spp[14,15,16,17,18,19]. Phytopathogenic fungi are a most common cause of plant diseases, which lead to constriction of plant growth and productivity. *R. solani, F. solani*, *F. verticillioides*, *A. alternata* and *B. cinerea* cause root rot, collar or root rot, stalk rot, leaf spots and gray-mold rot and *Botrytis* blight, respectively[20,21,22,23,24]. Chemical control of phytopathogenic fungi is an effective attitude but leads to many health complications and environmental hazards[25]. Therefore, the biological control is recently used as a promising alternative way which is an environment friendly[26,27,28]. This study aims to detect the antifungal activities and halotolerance genes of some halotolerant *Streptomyces* bacteria by using PCR.

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MATERIALS AND METHODS

Collection of soil samples

The saline soil samples were collected from Alnatron valley aseptically in sterilized polythene bags at three different depths ranging from 10, 20 and 30 cm. These samples were rapidly transported to the laboratory in iced condition.

Isolation of *Streptomyces* spp and determination of salt-tolerance range

Halotolerant *Streptomyces* strains were isolated on starch nitrate agar medium[29] separately contained different concentrations of sodium chloride (0.05% as a normal concentration, 5, 7, 9, 11, 13, and 15%). The inoculated triplicate plates were incubated at 28°C ± 2 for 15 days to obtain a good growth. Plates containing salt-free medium were used as negative control. The salt-tolerance range of isolated *Streptomyces* bacteria was determined according to Saleh[30] and Mohamed[31].

Collection of phytopathogenic fungi

Five well identified strains of phytopathogenic fungi were collected from Agriculture Research Center. These fungi are *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata*, *Rhizoctonia solani*, and *Botrytis cinerea* which had already been isolated from different diseased crops.

Identification of *Streptomyces* bacteria

The *Streptomyces* bacteria were identified according to Shirling and Gottlieb[32]. Glycerol asparagine agar[33] was used for electron micrograph; this medium was inoculated by *Streptomyces* spores and then incubated at 28°C ± 2 for 7 days. A plug of the culture was removed and fixed in glutaraldehyde (2.5% v/v), washed with water and post-fixed in osmium tetraoxide (1% w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLISM 541OLV scanning electron microscope at 15 kv. On the other hand, *Streptomyces* strains were also identified by using 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 60s, 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[34].

Phylogenetic analysis

Pure subculture of the two strains was inoculated in freshly prepared LB broth and incubated for six days in a shaker incubator at 28°C ± 2 and 160 rpm. Total genomic DNA was extracted using Ultra-Clean Microbial DNA Isolation Kit (Mo Bio Laboratories, Calif. USA) according to the manufacturer's specifications, based on the method of Stach[35]. The DNA was semi quantified on a 1% agarose gel in 1 x TAE buffer and visualized under UV by staining with ethidium bromide[36]. Total DNA from each isolate was used as a template for amplification of the 16S rRNA gene. This was done using the HotStar Taq Master Mix Kit (Qiagen, USA) according to the manufacturer's instructions. Nearly full length 16S rRNA gene sequences was PCR amplified using bacterial primer pair 27F forward 5'-TAG AGT TTG ATC CTG GCT CAG-3') and 1392R reverse, 5'-GAC GGG CGG TGT GTA CA-3' (Sigma) according to the position in relation to *E. coli* gene sequence[37,38]. Amplification was performed using a model PTC-100 thermal cycler (MJ research inc., USA) according to the procedure described by Roux[39]. Amplification products (20 ml) were separated on a 1% agarose gel in 1 x TAE buffer and visualized by ethidium bromide staining[36]. Sequencing of purified PCR products was done in both directions without cloning, using a commercial service provider. The CHECK CHIMERA program (http://rdp.cme.msu.edu/html/) of the Ribosomal Database Project[40] was used to check for the presence of possible chimeric artifacts[41]. Sequence data was analyzed with ARB software package version 2.5b, O. Strunk and Ludwig, Technishe Universitat Munchem (http://www.arb-home.de). The new sequences were added to the ARB database and aligned with the Fast Aligner Tool (version 1.03). Alignments were checked and corrected manually where necessary, based on conserved regions. The 16S rRNA gene sequences were compared to sequences in the public database using basic local

alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) in order to determine similarity between sequences in the GenBank database[42,43]. The 16S rRNA gene sequences with high similarities to those determined in the study were retrieved and used in the construction of the phylogenetic tree. Phylogenetic trees were constructed by Maximum likelihood method. Bootstrap analysis using PHYLIP for 100 replicates was performed to attach confidence estimates for the tree topologies[44,45].

Detection of halotolerance genes

The DNA of two identified halotolerant *Streptomyces* strains was used as a template in a trail to isolate some halotolerant genes using PCR. For *P5CR* gene, two primers named F1: 5'GGA GAT CTA ACA ATG GAG ATT CTT CCG ATT CCG GCG G3' and R2: 5'GGG ATA TCT TAG CTC TGT GAG AGC TCG CGG3' flanking a size of about 831 bp were used. Regarding *mtlD* gene F2: 5'CGA GAT CTA ACA ATG AAA GCA TTA CAT TTG GCG C3' and R2: 5'GGG ATA TCT TAT TGC ATT GCT TTA TAA GCG G3' flanking 1150 bp were used. Amplification was performed as reported by Mohamed and Chaudary[46] in GeneAmp PCR System 2400 Thermocycler for 35 cycles after initial denaturation for 4 min at 95°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by electrophoresis on 1.5% agarose gel in 1X TAE buffer at 80v for 1 h[36]. PCR fragments were visualized as mentioned before.

RESULTS AND DISCUSSION

It is well known that Egypt includes many areas suffering from high salinity such as Alnatron valley. Some actinobacteria are habitat these saline soils so they are called halotolerants or halophites. The moderate halotolerant actinobacteria can grow up to 8% sodium chloride, but over this concentration they are designated extreme halotolerants such as *Actinopolyspora halophila*[47,48,49]. Notable, the halotolerance adaptation is usually carried out by either of the two strategies; compatible solute and salt-in strategies. Compatible solute strategy is usually employed by the majority of moderately halophilic and halotolerant actinobacteria. This strategy depends on the maintaining of low ionic strength in the cellular cytoplasm by balancing osmotic potential through the synthesis or uptake of low molecular weight, highly water soluble and osmoregulatory organic compatible solutes such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine and ectoines, this strategy. Whilst that, the salt-in strategy is employed by true halophiles and extremely halophilic bacteria, which cannot survive at moderate or lower concentrations of sodium chloride. The mechanism of this strategy depends on the maintaining of the osmotic equilibrium by uptake of the selective influx of K^+ ions into the cytoplasm [4,50,51,52].

In this work, thirty halotolerant *Streptomyces* strains were isolated at varied salt-tolerance range, where, twenty three, five, and two strains had been isolated at 7%, 11%, and 15% NaCl as a maximum level respectively "as shown in Table 1" by using serial dilution method. Sonya and Zubeda[53] reported that, sixteen halotolerant *Streptomyces* strains were isolated from different locations in Egypt, these strains varied in their salt tolerance range, in particular, with increasing NaCl concentration in the growth medium up to 140g.L^{-1}. It was also noted that all the applied *Streptomyces* strains tolerated NaCl concentrations up to 70g.L^{-1}. When NaCl concentration was raised to 105g.L^{-1}, strains except *S. melanogenes* Si-11, gave moderate growth. On the contrary, NaCl concentration of 140g.L^{-1} inhibited the growth of 50% of strains under investigation, but the other 50% of these strains gave moderate growth. Moreover, all strains were screened on PDA medium against five phytopathogenic fungi to determine the antifungal activity. The results proved that, sixteen halotolerant *Streptomyces* strains were found have varied antifungal activities against all test phytopathogenic fungi "as shown in Table 2". The results showed that, S-1 and S-2 were the highest antagonistic strains among the others; however, the later one was higher than the former. Furthermore, the most halotolerant strains were identified as *S. gardneri* (S-1) and *S. platensis* (S-2). The cultural characteristics of the two strains were reported in "as shown in Table 3". Moreover, morphological, physiological and biochemical

characteristics were recorded in "as shown in Table 4". Despite *S. gardneri* and *S. platensis* were similar in their morphology and cell wall analysis; they have many differences in their physiological characteristics. *S. gardneri* and *S. platensis* have ellipsoidal spores with smooth surfaces that were arrayed in spiral chains, also both strains are non-motile and their cell walls were found composed of LL-Diaminopimelic acid and the sugar pattern was not detected. Both strains were found have the ability to produce amylase, protease, cellulase, and pectinase enzymes; whilst they couldn't be produced lecithinase enzyme. Furthermore, *S. gardneri* was found has the ability to produce catalase, coagulase, nitrate reductase, and melanin pigment but didn't have the lipolytic activity. On the contrary, *S. platensis* able to produce lipase without catalase, coagulase, nitrate reductase, and melanin pigment. The results showed that, both strains didn't have the capacity for H_2S production; however, xanthin and esculin were degraded. Both strains were sensitive to streptomycin and amoxicillin antibiotics at 50µg/ml that represent aminoglycosides and â-lactams respectively. Because these strains were isolated from saline soil, they could grow at high concentrations of sodium chloride ranged from 5 to 15 %. D-glucose, D-galactose, D-fructose, sucrose, mannitol, and *meso*-Inositol had been utilized by both strains; while L-arabinose and

Table 1. Halotolerance range of *Streptomyces* strains isolated from Alnatron valley

Strains	NaCl concentration (%)					
	5	7	9	11	13	15
S. gardneri (*S-1*)	+++	+++	+++	++	++	++
S. platensis (*S-2*)	+++	+++	++	++	++	+
S-03	+++	++	-	-	-	-
S-04	+++	++	-	-	-	-
S-05	+++	+	-	-	-	-
S-06	+++	+	-	-	-	-
S-07	+++	+++	-	-	-	-
S-08	+++	+++	-	-	-	-
S-09	+++	+++	-	-	-	-
S-10	+++	++	-	-	-	-
S-11	+++	+	-	-	-	-
S-12	+++	++	-	-	-	-
S-13	+++	++	-	-	-	-
S-14	+++	+++	-	-	-	-
S-15	+++	+	-	-	-	-
S-16	+++	++	-	-	-	-
S-17	+++	++	-	-	-	-
S-18	+++	++	-	-	-	-
S-19	+++	+++	-	-	-	-
S-20	+++	+++	-	-	-	-
S-21	+++	+++	-	-	-	-
S-22	+++	+++	-	-	-	-
S-23	+++	+	-	-	-	-
S-24	+++	+	-	-	-	-
S-25	+++	++	-	-	-	-
S-26	++	++	+	+	-	-
S-27	+++	+++	++	+	-	-
S-28	+++	+++	++	++	-	-
S-29	++	++	++	+	-	-
S-30	++	++	++	++	-	-

-: No growth. +: Weak growth. ++: Moderate growth. +++: Abundant growth.

xylose hadn't; raffinose and rhamnose had been utilized only by *S. gardneri*. Eventually, L-cysteine, L-valine, L-histidine, L-alanine, L-lysine, L-leucine, L-tyrosine, and L-phenylalanine had been utilized by both strains; however, L-proline was utilized only by *S. gardneri*.

These two strains were identified at molecular level by determination the sequence of 16S rRNA gene by using PCR technology. Moreover, phylogenetic study was performed to exactly detect the similarities and dissimilarities with all reference strains that are recorded in GenBank. The phylogenetic tree "as shown in Figure 1" illustrated that, presence of 95% similarities and 5% dissimilarities between the two concerned strains. When the full length DNA sequence of our two strains was aligned using DNA BlastN on GenBank, results showed that the strains belong to *Streptomyces* genus with identity not exceeded more than 95%. Different 28 species of *Streptomyces* were selected (published on GenBank) for construction a phylogenetic tree to study the evolution of our strains. The topology of the resultant phylogenetic tree shows two distinct lineages including all the examined isolates. The maximum nucleotide sequence divergence was observed in the first lineage which including 27 isolates out 28. This lineage consists of 9 clusters. The first cluster contains five different *Streptomyces* species; *Streptomyces awyekz* E44G, *Streptomyces flavoviridis* ZG084/GQ985452, *Streptomyces brasiliensis* A1/JN207922,

Table 2. Screening test for antifungal activities of *Streptomyces* bacteria

Strains	The antifungal activity by the mean value of inhibition zone (mm)				
	F. oxysporum	*F. solani*	*A. alternata*	*R. solani*	*B. cinerea*
S. gardneri (*S-1*)	25.4	28.2	26.5	22.7	29.1
S. platensis (*S-2*)	30.2	33.4	28.1	25.0	35.6
S-03	None	None	None	None	None
S-04	None	None	None	None	None
S-05	None	None	None	None	None
S-06	None	None	None	None	None
S-07	22.4	25.7	15.2	26.2	20.0
S-08	25.1	24.4	20.4	22.3	17.2
S-09	12.2	15.4	20.1	10.2	10.6
S-10	15.2	15.7	18.2	14.4	16.2
S-11	10.2	10.5	10.7	14.3	13.5
S-12	None	None	None	None	None
S-13	None	None	None	None	None
S-14	None	None	None	None	None
S-15	17.2	18.2	15.8	12.2	10.0
S-16	14.2	15.1	17.2	18.2	12.2
S-17	None	None	None	None	None
S-18	18.1	20.2	16.4	12.4	10.5
S-19	None	None	None	None	None
S-20	None	None	None	None	None
S-21	15.4	14.7	17.5	15.2	13.6
S-22	None	None	None	None	None
S-23	None	None	None	None	None
S-24	None	None	None	None	None
S-25	20.1	22.4	25.4	21.6	18.7
S-26	22.0	25.4	14.6	16.4	10.4
S-27	None	None	None	None	None
S-28	10.2	12.4	17.2	14.2	10.4
S-29	12.2	12.2	14.1	17.2	15.3
S-30	15.2	18.2	17.3	18.2	13.6

F. is *Fusarium*, A. is *Alternaria*, R. is *Rhizoctonia*, B. is *Botrytis*, None is no activity

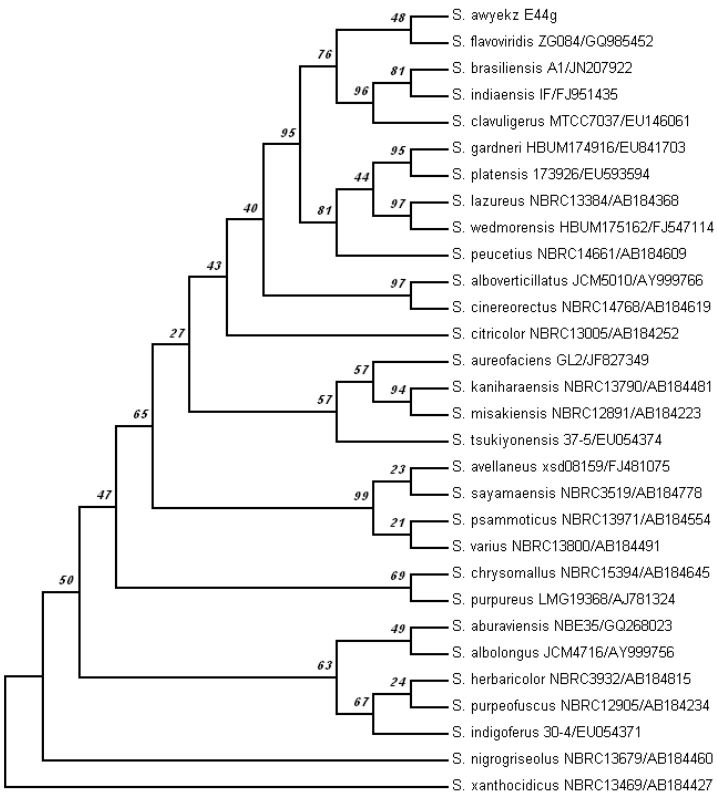

Fig. 1. Phylogenetic tree showing evolutionary relationship between the two strains and also between them and other *Streptomyces* strains presented in GenBank.

Table 3. Cultural characteristics of *S. gardneri* and *S. platensis*

Strain	Media	Growth	Color of mycelia		Color of diffusible pigment
			Aerial	Substrate	
S. platensis	TY extract broth	Good	263 LG	79-l.gy.YBr	76-LyBr
	YM extract agar	Poor	263 LG	90-gy.Y	None
	OM extract agar	Good	263 LG	60-l.gy.Br	61-gy.Br
	ISS agar	Poor	10PkG	76-l.yBr	None
	GA agar	Good	264 LG	79.l.gy.YBr	None
	PY extract iron agar	Moderate	263 W	73-p.oy	None
	Tyrosine agar	Good	9 PkW	76-LyBr	79-l.gy.YBr
S. gardneri	TY extract broth	Poor	264 LG	79.l.gy.YBr	None
	YM extract agar	Moderate	10PkG	70-l.oy	None
	OM extract agar	Good	263 LG	76-LyBr	79.l.gy.YBr
	ISS agar	Moderate	10PkG	79.l.gy.YBr	None
	GA agar	Good	264 LG	79.l.gy.YBr	None
	PY extract iron agar	Poor	10PkG	79.l.gy.YBr	None
	Tyrosine agar	Poor	10PkG	79.l.gy.YBr	None

TY = Tryptone Yeast; YM = Yeast Malt; OM = Oat Meal; ISS = Inorganic Salt Starch; GA = Glycerol Asparagine; PY = Peptone Yeast; LG = Light Gray; PkG = Pinkish Gray; W = White; PkW = Pinkish White; l.gy.YBr = Light Gray Yellowish Brown; gy.Y = Grayish Yellow; l.gy.Br = Light Grayish Brown; p.oy = Pall Orange Yellow; LyBr = Light Yellowish Brown; gy.Br = Grayish Brown; l.oy = Light Orange Yellow;

Streptomyces indiaensis IF/FJ951435 and *Streptomyces clavuligerus* MTCC 7037/EU146061. The second lineage contains only the species *S. xanthocidicus* which showed high diverge for the other species. The two lineages have one ancestor and all the strains have the same origin. Phylogenetic analysis demonstrates that most of the isolates shared a sequence identity of between 97 - 100% with known *Streptomyces* species. The study confirms the findings by other researchers[54] who also isolated antagonistic *Streptomyces* species from reserved areas in Pakistan.

Isolation of halotolerant genes (*P5CR* and *mtlD*) from the DNA extracts of *S. gardneri* and *S.*

Table 4. Morphological, physiological and biochemical characteristics of *S. gardneri* and *S. platensis*

Item	Characteristics	Results	
		S. gardneri	*S. platensis*
Morphology	Spore chain	Spiral	Spiral
	Spore surface	Smooth	Smooth
	Motility	None motile	None motile
Cell wall hydrolysis	Diaminopimelic acid	LL-DAP	LL-DAP
	Sugar pattern	Not detected	Not detected
Physiology	Amylase production	+	+
	Protease production	+	+
	Lipase production	-	+
	Cellulase production	+	+
	Pectinase production	+	+
	Catalase production	+	-
	Lecithinase production	-	-
	Coagulase production	+	-
	H_2S production	-	-
	Melanoid production	+	-
	Nitrate reduction	+	-
	Degradation of xanthin	+	+
	Degradation of esculin	+	+
Antibiotics resistance	Streptomycin 50 µg/ml	-	-
	amoxicillin 50 µg/ml	-	-
Utilization of different carbon sources	D-glucose	+	+
	D-galactose	+	+
	Sucrose	+	+
	Mannitol	+	+
	L-arabinose	-	-
	Raffinose	+	-
	meso-Inositol	+	+
	D-fructose	+	+
	Xylose	-	-
	Rhamnose	+	-
Utilization of different nitrogen sources	L-cystiene	+	+
	L-valine	+	+
	L-histidine	+	+
	L-alanine	+	+
	L-lysine	+	+
	L-leucine	+	+
	L-tyrosine	+	+
	L-phenylalanine	+	+
	L-proline	+	-
NaCl tolerance	5-15%	+	+

+ is growth, - is no growth

platensis was carried out by using PCR. Results "as shown in Table 5" showed that the *P5CR* gene was detected in the DNA extracts of seventeen strains included *S. gardneri* and *S. platensis* "as shown in Figure 2a", while, *mtlD* gene was found in the DNA extracts of thirteen strains included *S. gardneri* and *S. platensis* "as shown in Figure 2b". These results indicate the differences in the genetic make up of different *Streptomyces* strains, as well as different mechanisms that are involved in conferring salt tolerance in these strains. The results showed that the salt tolerance of *S. gardneri* and *S. platensis* was higher than the others due to their genome which involved the

Table 5. PCR detection of halotolerance genes from the DNA extracts of thirty *Streptomyces* strains isolated from Alnatron valley

Strains	Halotolerance genes	
	P5CR	*mtlD*
S. gardneri (*S-1*)	+	+
S. platensis (*S-2*)	+	+
S-03	-	+
S-04	-	-
S-05	+	+
S-06	-	-
S-07	+	-
S-08	+	-
S-09	-	+
S-10	-	+
S-11	+	-
S-12	-	-
S-13	+	-
S-14	-	+
S-15	+	+
S-16	+	+
S-17	+	-
S-18	+	-
S-19	+	-
S-20	-	+
S-21	-	+
S-22	-	+
S-23	+	-
S-24	-	-
S-25	-	-
S-26	+	-
S-27	-	+
S-28	+	-
S-29	+	-
S-30	+	-

+: Detected, -: Not detected.

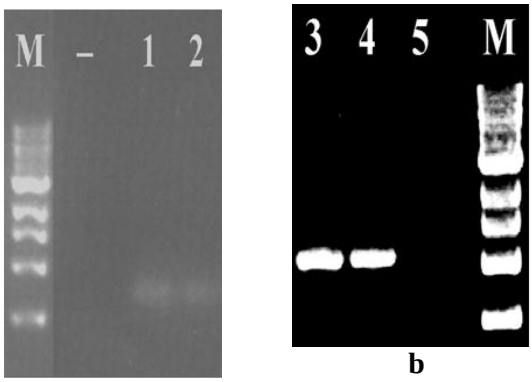

Fig. 2. Agarose gel electrophoresis of amplified halotolerance genes extracted from *S. gardneri* and *S. platensis* (**a**) *P5CR* gene -: Negative control (PCR mixture with no template). M: 1 Kb DNA ladder, (**b**) *mtlD* gene. Lane 5: Negative control (PCR mixture with no template). M: 1 Kb DNA ladder.

two halotolerant genes *P5CR* and *mtlD*. Ronde[55] showed that the L-DELTA1-pyrroline-5-carboxylate reductase (*P5CR*) gene controls the common step in the both pathways governing the biosynthesis of proline from ornithine and glutamic acid. Abebe[56] showed that ectopic expression of the *mtlD* gene of *Escherichia coli* for the biosynthesis of mannitol in wheat (*Triticum aestivum* L. cv Bobwhite) improved tolerance to water stress and salinity. Mohamed[57] reported that, the DNA of four halotolerant *Streptomyces* stains that are isolated from sea water and tolerated a salt range of 14 to 21% NaCl in the growth medium were used. A trial was conducted to detect two salt tolerance genes (*P5CR*, and *mtlD*) in the DNA extracted from the applied *Streptomyces* strains using PCR. The *P5CR* gene was detected in the DNA extracts of three species, namely, *S. cirratus*-02; *S. rishiriensis*-04; *S. luteogriseus*-08, while, *mtlD* gene was found in the DNA extracts of *S. cirratus*-02.

CONCLUSION

Only two *Streptomyces* strains among thirty ones were the most antifungal and halotolerant which were identified as *S. gardneri* and *S. platensis*. However, the later was found have antifungal activity and salt-stress tolerance more

than the former. The halotolerance genes (*P5CR* and *mtlD*) were detected in the DNA extracts of both strains, contrary to what is an object with other strains, and this is the reason for being the most halotolerants.

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