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Full Length Research Paper

Isolation of Hg and Cu resistant *Streptomyces* from marine sediments in different regions of the Caspian Sea

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Expansion of industrial activities; mining and metal plating could cause release of high amount of heavy metals into seawater and marine sediment resulting in heavy metal resistant microorganisms. Due to the important role of *Streptomyces* in production of secondary metabolites, the reduction ability of enzymes and elements was used to study the resistance exhibited by *Streptomyces* in the isolation of heavy metals (copper and mercury) from marine sediments and coastal waters of the Caspian Sea. Marine sediments samples were collected from coastal locations aseptically. The media of Starch Casein agar (SCA), and Kusters agar (KUA) were used for the isolation of *Streptomyces* and biochemical testes like lipid, casein, starch and gelatin hydrolysis and etc. was carried out for identification of this bacteria and determination of heavy metals (Hg, Cu) resistant strains was performed with well diffusion assay and for detection of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) Micro dilution assay was used. Also, amounts of heavy metals in different parts of coastal waters were measured with atomic absorption spectrophotometry assay. A total of 41 *Streptomyces* were isolated from coastal locations 7 strains (17.07%) showed copper resistant to 1000 mg/L, 4 strains (9.75%) showed mercury resistant. The strains C9, D11 and E16 to 20 mg/L and C2 to 40 mg/L were mercury resistant. Due to existence of heavy metals (Hg, Cu) resistant *Streptomyces* in the Caspian Sea, researcher can use them as well suited agents for Bioremediation.

Key words: *Streptomyces*, heavy metals, resistant, sediment.

INTRODUCTION

Streptomycetes are filamentous bacteria having high G+C content about 60-70 mol%. There are most of 500 *Streptomyces* species characterized by formation of aerial mycelium and spores on solid mediums, they were easily identified with their different colors, dry and wrinkle mature colony. *Streptomycetes* are Actinomycetes with cell wall type I, belonging to the family *Streptomycestaceae* a member of the order Actinomycetales with complex life cycle (Yadav et al., 2009).

Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* a count for 90% of the total Actinomycetes population (Poopal and Laxman, 2009). A few strains have also been reported from deep sea sediments. *Streptomyces* species are generally isolated from terrestrial habitats. In addition to other representatives of Actinomycetales, their ubiquitous presence in marine and estuarine sediments is now well documented (Ravel et al., 1998).

Heavy metals pollution is spreading throughout the world with the expansion of industrial, mining, tanning

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activities and battery. Many industrials, especially plating and battery, release heavy metals like nickel and copper in waste waters. These metals which find many useful applications in our life are very harmful if they are discharged into natural water resources and may pose a serious health hazard (ضztürk et al., 2004).

Copper is an essential element at low concentration for all animals, plants and organisms. However copper is toxic at higher concentration as it inhibits synthesis of macromolecules and other enzymatic reactions. Mercury contamination in environment is arising from human activities, such as burning coal and petroleum products (Senthilkumar et al., 2005). Many microorganisms show adaptation to the toxic materials constantly released into their environment. They have developed strategies to resist, tolerate, metabolize and detoxify these toxic substances (Yadav et al., 2009).

The tolerance of bacteria to heavy metals has been proposed as an indicator of potential toxicity to other forms of life (Rifaat et al., 2009).

Due to their ability to produce bioactive compounds, enzymes and their role on bioremediation of pesticides and heavy metals Actinomycetes group is the subject of interest for scientist. In addition, some *Streptomyces* species exhibit multiple tolerances against different metals (Yadav et al., 2009; Rho and Kim, 2002). The present study was carried out for isolation, identification of *Streptomyces* from sediment in 5 regions of the Caspian Sea and was survived potent heavy metals (copper and mercury) resistant *Streptomyces.*

MATERIALS AND METHODS

Sample collection

Marine sediments were collected from 5 coastal locations of the Caspian Sea at a depth of 10, 20 and 30 cm into sterile 50 mL tubes. Also, at each of locations sediment samples were collected from estuarine. Salinity, temperature and pH of samples were measured with salinometer, thermometer and pH meter respectively. Then, they were transported to the laboratory and stored in the refrigerator at 4°C until use.

Isolation of *Streptomyces*

The sediment samples were dried aseptically. Heat treatments were performed by holding sediment samples in a water bath at 50°C for 60 min (Senthilkumar et al., 2005). Then, the samples were diluted with 50% of sterilized filtered sea water and 50% of sterilized distilled water, and then spread on Starch Casein Agar (SCA) and Custer's Agar (KUA) that provides with 50% of sterilized filtered sea water. However, these media were supplemented with 75 µg/mL of Cycloheximide and Tetracycline to inhibit fungi and bacteria growth respectively. Plates were incubated for 10-30 days at 28°C (Jensen et al., 1991). Through types of created colonies, doubtful colonies of *Streptomyces* were isolated by their unique characteristics and were purified by streaking on SCA medium without antibiotics. *Streptomyces* were surveyed and recognized by morphological properties (tough, leathery, wrinkle, colony color, gram and acid fast staining) according to the guidelines of the International *Streptomyces* Project (Morakchi et al., 2009). For biochemical characterization lipid, casein, starch, gelatin hydrolysis test, nitrate reduction test, assimilation of carbon sources, antibiotic sensitivity, salt tolerance were observed. The different carbon sources were used to study like xylose, dextrose, raffinose, fructose, glucose, mannitol, maltose, galactose, sucrose. The antibiotic sensitivity assay was done by agar diffusion assay. Antibiotic discs were placed on the surface of agar plates pre inoculated with spores of isolates to be tested and zone of inhibition were measured after incubation at 28°C for 3 days. To study the salt tolerance spores were inoculated in trypticase soy broth amended with 1, 3, 5 and 7% NaCl were incubated at 28°C for 3 days.

Determination of Cu and Hg in water samples

A 25 ml of Millipore filtered water sample was acidified with 5 ml of concentrated HNO_3 and was concentrated to 25 ml by evaporation. Metal contents were determined using Thermo S series model of Atomic Absorption Spectrometer to determine the presence of copper and mercury (Baldwin et al., 1999).

Screening of copper and mercury resistant *Streptomyces*

Primary qualitative screening assay was carried out in plates containing minimal medium Agar (MMA) supplemented with 20 mg/L $CuSO_4$ and $HgCL_2$ independently. The sensitivity of the strains was tested by well diffusion assay. The spore suspensions of all the isolates were prepared as described earlier by Kieser et al., (2000) and were uniformly spread using sterile cotton swab on a sterile Petri dish Mueller Hinton Agar (MHA) containing some wells of 6 mm diameter. Seven serial dilutions yielded concentrations of 640, 320, 160, 80, 40, 20 and 10 mg/L for $CuSO_4$ and $HgCl_2$. 50 µL of each concentration were added to each of wells. The Petri dishes were incubated for 24 h at 28°C. After incubation, confluent bacterial growth was observed; inhibition of the bacterial growth was measured in mm. Sensitive strains showed zones of inhibition of > 10 mm, whereas zones of inhibition of resistant strains were < 7 mm (Yadav et al., 2009; Senthilkumar et al., 2005).

Microdilution broth assay

$CuSO_4$ and $HgCl_2$ were independently diluted with Mueller Hinton Broth (MHB) to obtain concentrations of 1200, 1100, 1000, 900, 800, 700, 600 mg/L for $CuSO_4$ and concentrations 160, 80, 40, 20 and 10 mg/L for $HgCl_2$. 95 µL of each dilutions were brought into microtitre plate (96 wells, round bottom), 95 µL of MHB without metal were brought into the last well of each columns as control well. 5 µL of each spore suspensions of resistant *Streptomyces* were added to wells. The inoculated microtitre plates were incubated at 28°C for 48 h. The MIC was defined as the lowest metal concentrations that prevented visible growth as detected by unaided eyes. To confirm MIC and to establish minimum bactericidal concentrations (MBC), 10 µL of each well with no visible growth were removed and inoculated on ISP2 (International of *Streptomyces* Project) plates, the plates were incubated for 24 h at 28°C.

RESULTS

Forty one *actinomycetes* were isolated from 80 sediment samples collected in different coastal regions in the Caspian Sea from the basis of morphological, microscopy

Table 1. Morphological characteristics of isolated resistant *Streptomyces.*

Isolates	Color of mass	Soluble pigment
C2	White	Brown
C9	Whitish grey	-
C12	Whitish blue	-
D8	Cream	Yellowish green
D11	Whitish brown	Cream
E14	Light grey	-
E16	Grey	-
E17	Grey	-

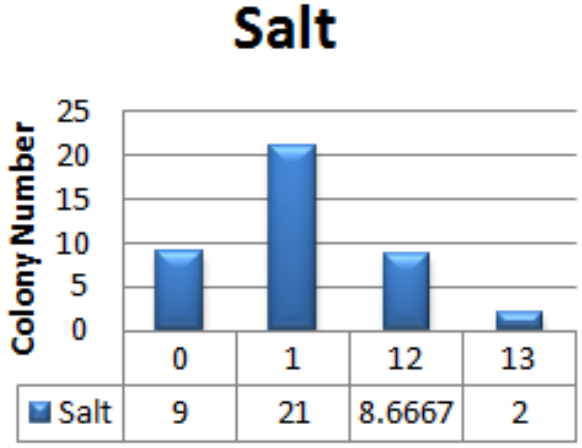

Figure 1. Relationship between salt concentration with dispersion of isolated *Streptomyces.*

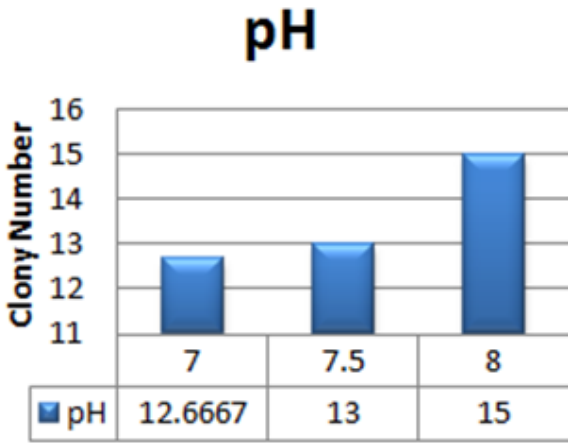

Figure 2. Relationship between pH with dispersion of isolated *Streptomyces.*

and biochemical characterizations. From these, 39 colonies were isolated on Starch Casein Agar medium and 2 colonies on Kuster`s Agar medium. The isolated *actinomycetes* grew well at 28°C on SCA producing pinpoint to slow growing, powdery, wrinkle and raised colonies possessing an earthy dour characteristic of *actinomycetes*. On SCA the isolates produced white, cream, gray, whitish blue colored aerial spore mass, these properties of the isolates are presented in the Table 1. Microscopic observation revealed that all strains have branching filaments and aerial hyphea were abundant, well-developed with spores, the strains showed positive for Gram and acid fast reaction. 5 strains were produced the soluble pigments on the media. Based on the cultural characters and morphology all of them were assigned to the genus *Streptomyces*. The biochemical characterizations of isolated resistant strains were shown in Table 2; nine numbers of carbon sources used in this study and all resistant strains utilized fructose, these strains have not been utilized raffinose, whereas these strains showed variability for utilization of some carbon sources like manitol, maltose, xylose, dextrose, glucose, galactose, sucrose. In the study of salt tolerance, the strains were found to grow up to 5% of NaCl concentration, but no growth was observed up 7%. For antibiotic sensitivity of the strains, seven different antibiotics were used, out of these antibiotics all the resistant strains showed variable sensitivity to nitrofurantion, nalidixic acid, gentamaycin, erythromycin and rifampicin, while penicillin and ampicillin have no activity against of these strains. Only seven (17.07%) isolates (D8, C9, D11, C12, E14, E16 and E17) observed maximum copper resistance up to 1000 mg/L and 4 (9.75%) isolates (C2, C9, D11 and E16) were observed mercury resistance, that among isolates, 3 strains (C9, D11 and E16) were found to be resistant to mercuric chloride up to 20 mg/L but strain C2 was resisted up to 40 mg/L. results of MIC and MBC determinations are presented in Table 3 using micro dilution. Atomic Absorption Spectrometry (AAS) of water samples at each of regions were shown in Figure 4, however that concentration of Hg in these samples was very little, could not be analyzed with AAS.

DISCUSSION

This study isolated *Streptomyces* resistant to Cu and Hg from the sediments of coastal and also surveys the relationship between salt concentration an pH and temperature with rate of dispersion *Streptomyces* may be accompanied with regard to statistical methods performed with SPSS software and using the Analysis of variance (ANOVA) (Figures 1, 2 and 3). There is a significant difference between *Streptomyces*, temperature, pH and salt concentrations ($P < 0.01$) ,in temperature 26°C and pH= 8 and salt concentration 1ppt are the most common ,of course, whatever the salt concentration increased the number of colonies is reduced. Yadav et al. (2009) reported the best conditions for growth of *Streptomyces* isolated from soil were temperature 28°C

Table 2. Biochemical characteristics of resistant *Streptomyces* isolates.

Test \ Isolates	C2	C9	D8	D11	C12	E14	E16	E17
Fructose	+	+	+	+	+	+	+	+
Manitol	+	+	-	+	+	+	-	+
Maltose	+	+	-	-	+	+	-	-
Xylose	+	+	+	+	+	-	-	-
Galactose	-	+	+	-	-	+	+	-
Raffinose	-	-	-	-	-	-	-	-
Glucose	-	+	+	-	-	+	+	-
Dextrose	+	+	-	-	+	+	+	-
Sucrose	+	+	+	+	+	+	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+
dipiL sisylordyh	+	+	+	+	+	+	+	+
esaerU	-	-	-	-	-	-	-	-
esalataC	+	+	+	+	+	+	+	+
noitcuder etartiN	-	+	-	-	+	-	-	-
noitazilitu etartiC	+	-	-	+	+	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Antibiotic sensitivity								
Rifampicin (5 mcg)	R*	R	21 mm	R	15 mm	R	R	R
Nitrofurantion (300 mcg)	R	R	R	R	22 mm	23 mm	R	R
Nalidixic acid (30 mcg)	23 mm	18 mm	15 mm	R	R	R	R	R
Gentamycin (10 mcg)	17 mm	16 mm	26 mm	30 mm	34 mm	30 mm	R	20mm
Penicillin (10 mcg)	R	R	R	R	R	R	R	R
Erythromycin (15 mcg)	R	R	R	R	15 mm	17 mm	R	R
Ampicillin (10 mcg)	R	R	R	R	R	R	R	R

Table 3. MIC and MBC value for metals by the micro dilution method.

Metal	Hg(mg/L)		Cu(mg/L)	
Isolates	MBC	MIC	MBC	MIC
C2	160	80	900	800
C9	80	40	1200	1100
C12	-	S*	1200	1100
D8	-	S	1200	1100
D11	80	40	1200	1100
E14	-	S	1200	1100
E16	40	40	1200	1100
E17	-	S	1200	1100

* Sensitive.

and pH about 6-7. A total of 41 actinomycete colonies were observed. From these, 39 colonies were isolated in SCA medium. Only two colonies were isolated in KUA medium, in regard to, the two media have been use the same ingredients and only the amount of NaCl (KUA= 2% and SCA=0.2%), it seems that the SCA for isolation of actinomycetes is best in the Caspian Sea. Remya and Vijaykumar (2008) isolated actinomycets from Indian shore and SCA was observed to be the best medium for maximal growth.

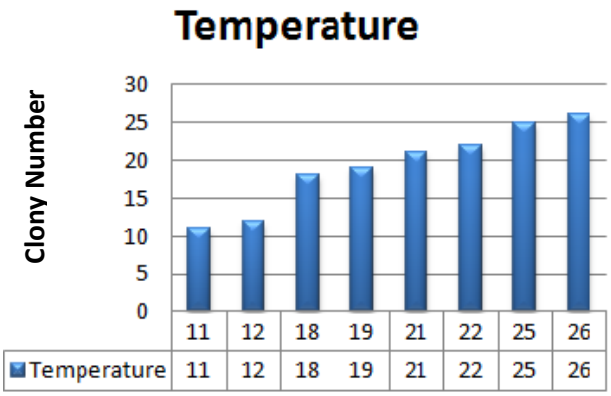

Figure 3. Relationship between temperatures with dispersion of isolated *Streptomyces.*

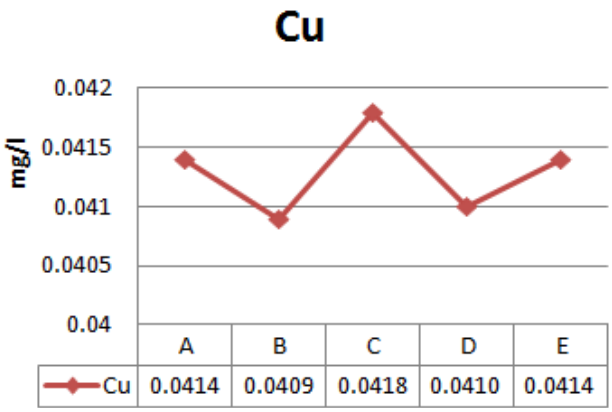

Figure 4. Concentration of Cu in water of 5 locations.

Yadav et al. (2009) 3 strains *Streptomyces* (A160, A161 and A164) identified from Bay of Bengal, India. These showed copper resistance up to 480 mg/l (Yadav et al., 2009). In this study seven strains D8, C9, D11, C12,E14,E16 and E17 observed copper resistance up to 1000 mg/l and 4 strains C2, C9, D11 and E16 were mercury resistance up to 20 mg/l (7.35.10-5 mol), strain C2 showed maximum resistance up to 40 mg/l (14.7×10^{-5} mol). Earlier a strain of halophilic *actinomycets* was also isolated from the salt marsh environment of Vellar estuary, southeast coast of India by Senthilkumar et al. (2005) which showed resistance towards mercuric chloride up to 100-150 nmol. Also they reported SCA medium can be better used for isolation of *actinomycetes* than the other media used GAA, KUA and SGA2. The strains showed high salt tolerance up to 5% of NaCl concentration. This is important trait for their proliferation and colonization and for biological control of disease in crops in agricultural field for coast area with high salt nature (Vasada et al., 2006), also isolated a *S. sannanensis* strain RJT-1 from the alkali soil of Rajkot, India which grew 9% NaCl.

Conclusion

These strains may be useful for copper, mercury and salt resistance and utilize a variety of carbon sources. Actinomycetes constitute a significant component of the microbial population in most soil. Their metabolic diversity and particular growth characteristic, mycelia from and relatively rapid colonization of selective substrates, indicate them as well suited agents for bioremediation of metal and organic compounds.

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