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Phylogenetic Diversity and Anti- MRSA Activity of Halotolerant Actinobacteria from sediments in Great Salt Plains, Oklahoma

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Abstract

Studies have been surged on extreme environments and hypersaline ecosystems through the recent decades. Despite the apparent severity of the environmental conditions in the Great Salt Plain, recent phylogenetic studies carried on its soil samples have revealed a wide diversity of microorganisms. In this current study, we present the actinobacteria as one of the largest and important phenotypic groups. The Great Salt Plain of Oklahoma is an extreme region and a hypersaline environment from marine origin. Thirty soil samples were collected from vegetated and salt flat sites of the Great Salt Plain area and used for isolation. Actinomycetes were selectively isolated by employing four different media. A total of 358 actinomycetes isolates obtained from the Great Salt Plain soil samples. Based on morphological and physiological characterization 155 representative isolates were chosen for phylogenetic diversity, salt tolerance, and antimicrobial activities. Results from the 16S rRNA gene sequence analysis indicate that, the isolates could be grouped into two genera; phylotypes were detected at high frequency, and affiliated to the genus *Streptomyces*, and phylotypes were detected at low frequency, and affiliated to the genus *Nocardiopsis*. The majority of the isolates were of hypersaline or marine origin. 38% of actinomycetes isolates were able to grow at 10% salinity, and only 7% of actinomycetes shown salient ability at 15% salinity. Of the 155 isolates, 44 strains exhibited anti-MRSA bioactivities. To our knowledge, this research paper is the first report on the isolation of actinomycetes members from hypersaline environment of the Great Salt Plain of Oklahoma. This current study confirms that the Great Salt Plain harbors significant diversity of actinobacterial communities, and illustrates their potential for production biologically

active molecules. Due to the high bioactivity percentage, broad bioactivities against both MRSA strains, the isolated actinomycetes presented their capability in pharmaceutical application.

1. Introduction

The Great Salt Plains (**GSP**) is a unique area of the Salt National Wild Refuge in the north-central region of Oklahoma. It is distinguished by a very broad area of salt flat (**~65km^2**) of mud flats and sand bars, these structures are covered by a small layer of thasassohaline salt evaporates (**Johnson 1980; Reed 1982**). The GSP is punctuated by few sparse scrub brushes. The GSP is frequently moistened at the soil surface a result of the groundwater brine which is moved from the subterranean salt deposit upward to the surface by artesian flow. The GSP encounters rain, rapid shift in salinity, water availability, surface temperature and flooding (**Kirkwood and Henley, 2006; potter et al, 2006**). Rain temporarily washes the salt crust. Salt Chloride is the main component of the salt brine. There is a shift change in the salt concentration of the GSP surface. The GSP contains shallow ponds of saturated brine of mainly sodium chloride; on the other hand it also has ephemeral pools that are associated with shift in microbial diversity (**Major et al., 2005**). Although the salt source exists in the form of buried marine deposits, but there is no recent association with marine system, and the nearest marine system is the gulf of Mexico which is located at ~900 km. therefore the GSP considers an athalasic environment (**Herbst, 2001**). The dynamic change in salinities from zero to saturation, the high temperature of the surface, the freezing winter, alkaline pH, desiccation, and unlimited intense UV irradiation pressed the selection of microbial structure that led to novel taxa. It was reported that, environment unruly force changes in microbial diversity; the intermediate disturbance hypothesis states that, very stable or very unstable environments will generate less diversity than moderately unstable environments (**Petraitis 1989**). Therefore the GSP considers an extreme environment with a moderate level of disturbance, and harbors more microbial diversity than regular environments, and is a part of an interconnected group of microbial observatory, sponsored by the National Science Foundation, and has named the Salt Plains Microbial Observatory, to help researchers to study the microbial community there.

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Studies on marine microorganisms have still fallen behind due to the difficulties in samples collection from both water column and sediments in compared to the terrestrial environments. The microbial diversity at hypersaline environments close to saturation is very low (**Oren 2002; Burns et al., 2004**), even though there is a significant microdivesity, hence the presences of many closely related clones of microorganisms were detected in those environments (**Benlloch et al., 2002**). It is worth to mention that the marine environments are expected to have an excellent microbial diversity in the seawater and sediments (**Sugita et al., 1993**). It has been known that halophilic bacteria could endure and grow at high salinity. However, the existence of of halophilic actinomycetes was not known until 1970s. The analysis of 16S rRNA gene, characteristic gene rearrangements, presence of conserved insertions, and deletions in certain type of proteins, support classification of phylum *actinobacteria* **(Goodfellow and Fiedler, 2010).** *Actinomycetes* (order *Actinomycetales* within *Actinobacteria* subdivision) are ubiquitous highly diverse microbes (**Holmfeldt et al., 2009**), known for their biotechnological applications, and involved in the turnover of organic matter. Halophilic actinomycetes have been recently isolated from marine environments, which require a salt concentration similar to marine bacteria. Halophilic bacteria and actinomycetes have been classified according to salinity requirements by scientists such as (**Kushner 1978; Vreeland 1987; Lasern 1986**), and the method of (**Kuschner 1978**) seems to be the most common. Recently, it has shown clearly that actinomycetes diversity is much wider than believed previously (**Bredholdt et al, 2007**), they exist in association with aquatic macrophytes (**Wohl and McArthur 1998**) with members of actinomycetes existed in the marine environment (**Fenical and Jensen 2006**), in sand dunes (**Kurtboke et al., 2007**), in hypogen layers (**Carlsohn et al., 2007**), in alkaline (**Selyanin et al., 2005**), and in acidic soil (**Zakalyukina et al., 2002**). Most studies on actinobacteria have been mainly focused on terrestrial isolates. However researches on different kinds of terrestrial bacteria belonging to *Actinomycetes* have led to compounds of almost the same chemical structure and almost come to an end in recent years. In order to discover new natural substances which can be developed for the treatment of human disease and biotechnological aspects, researches has switched towards the study of microbes in extreme and unexplored environments. Therefore, a great deal of interest has been paid

for microorganisms inhabited extreme niches, due to their adaptation to extreme environments, production of various biologically active compounds **(Solingen et al., 2001; Margesin and Schinner 2001),** and a new era of researches initiated to carry on microbes in extreme environments such as high salinity (NaCl) environments (**Chen et al., 2004**) are increasing. It is noteworthy to mention that the work of Waksman and coworkers led to *Streptomycetes* have been the most important bio-factory of antibiotics since the discovery of streptothricin, streptomycin, and Actinomycin D. in the 1940s **(Hopwood, 2007).** It was shown that *Streptomycetes* exist in marine sediment habitats **(Cross, 1981b; Goodfellow and Haynes, 1984; Weyland and Helmke, 1988)**. **Okami and Okazaki (1978)** found that *Streptomycetes* are almost found in sediments of shallow sea (70-520 m deep) with 300-1270 colonies per cm^3. *Streptomycetes* which inhabit drastically changing environments, have the high mechanism of genome adaptation towards the extreme environments **(Kampfer, 2012).** Many studies were shown that a great number of antibiotic-producing *Streptomycetes* have been isolated from marine habitats **(Hotta et al., 1980; Busti et al., 2006; Jinyuan et al., 2009; Goodfellow and Fiedler 2010; Becerril-Espinosa et al., 2013.** Genus *Nocardiopsis* harbors many species that are considered thermotolerant microorganism that can grow at 45-50°C, with an optimal temperature of 37 °C **(Kampfer et al., 2002; Li et al., 2006).** Members of genus *Nocardiopsis* can tolerate NaCl concentration up to 20% (w/v). The *Nocardiopsis* halotolerant species can grow in media supplemented with 15-18 % (w/v) NaCl (**Kampfer et al., 2002; Li et al., 2006).** Certain *Nocardiopsis* species are true halophilic as NaCl (at least 3% w/v is necessary for growth with an optimum concentration of 10-15% (w/v) **(Li et al., 2003a, and 2004).** It was found that many *Nocardiopsis* strains have been recovered from soils with high salt concentrations **(Li et al., 2004, and 2006; Chen et al., 2008),** and deep sea sediments **(Zhang and Zeng, 2008).** Different studies have revealed the distribution of *Nocardiospsis* in marine habitats (**Shin et al., 2003; Sabry et al., 2004).** It is noteworthy to report that *Nocardiospsis* was the second dominant genus after *Streptomyces* in most cases of study on microbial diversity of culturable actinobacteria isolated from certain type of marine sponges **(Zhang et al., 2006; Jiang et al., 2007, and 2008).** Recently, it was reported that some marine *Nocardiopsis* strains could produce some bioactive substances **(Schumacher et al., 2001;**

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Shin et al., 2003). Another study were recovered that two new FKBP12-binding macrolide polyketides were produced by sediment-isolated *Nocardiopsis* strain **(Raju et al., 2010).** Many previous studies have been carried on the GSP to study the microbial diversity **(Caton et al., 2004, and 2009; Wilson et al., 2004; Major et al., 2005; Litzner et al., 2006; Potter et al., 2006; Nicholson and Fathepure 2005; Schneegurt 2013).** In the early studies, members belonging to Streptomyces and other genera were recovered from the marine sediments **(Grein and Meiers, 1958)** and a significant number of the isolated species was exhibited antibiotic activities, indicating that marine environments harbor microorganisms that can be an interesting source for bioprospecting. Recently, new genera and new species of marine actinomycetes have been characterized **(Meldonado et al., 2005a). Fenical and Jensen (2006)** found that some of the new species could produce unique compounds. The use of selective isolation techniques is frequently an important method to reveal new actinomycetes. In the current study we have used several isolation media to investigate the diversity of actinobacteria in the GSP. This current study addressed fundamental points: (1) How does the actinomycetes community structure differ between the vegetation sites (rhizosphere areas) and the salt bare regions? And (2) Have the different habitats i.e. vegetation and salt flat been influenced the structure of actinomycete members? (3) What is the salient potentiality of the isolated actinomycetes (4) Do the recovered actinomycetes have the potential to produce anti-MRSA biological molecules? To address prospective answer for these questions, we set **Our hypothesis are;** the GSP sediment soils harbors diversity of actinomycetes, the majority of them would be related to hypersaline and marine origin, and other members are not related to hypersaline or marine environment (genetic links and mixing with terrestrial actinomycetes). Rhizosphere areas have a complex relationship between plant roots and soil particles providing a variety of carbon sources, amino acids, organic acid and carbohydrates, that consumed by actinomycetes to generate energy. Actinomycete isolates would have salient tolerance, and some of them have antagonism bioactivities against MRSA strains. We support our hypothesis by previous studies which recovered actinobacterial diversity from marine sediments by culture dependent methods and found they possess antimicrobial bioactivity. The purpose of the present study was to reveal the biodiversity of actinomycetes at the GSP by isolation and

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taxonomic identification to evaluate the ability of the actinomycete isolates to tolerate different salinity concentration spectrum, and to exploit them for production of anti-MRSA compounds. A total of 358 actinomycetes isolates have been isolated, and 155 isolated have been chosen according to morphological and biochemical characterization for further study for phylogenetic analysis, screening of salinity potential and the ability to produce anti- MRSA compounds. Since there were no previous reports on the actinobacteria diversity present in the GSP, we undertake this current study to be the first one to isolate and characterize actinomycetes at the GSP.

2. Materials and Methods

2.1. Ethics Statement

A specific permission was required for this present study. The location is a protected area. This study did not involve any protected or endangered species.

2.2. Site Description

The GSP sampling sites are located within 600 m of each other, unvegetated samples were collected at north of Clay Greek, near the western and eastern edges of the barren salt flats, and towards the side of Sandpiper Trail and Observation Tower. Vegetated samples were collected at south of Clay Greek, near the western and eastern edges of barren salt flats, and towards Crystal Digging Area and Observation Tower **(Fig. 1).** The GSP are barren sandy mud area covering almost 65 km^2, the sample sites are in areas of the GSP, which show surface salt crusts from continues reform of saturated NaCl brine resulting from underlying strata. The sampling sites are experienced flooding with freshwater during rain events. The salt flat is permanently covered by salty surface despites, except in the event of rainfall. Salt deposits are subject to sharply dilution by rainfall, which lowers the salinity of those areas. Saline system at the GSP is considered as a poikiloenvironment, hence, it is expected the rainfall events result dramatically unexpected changes in saline content of the flat soil **(Caton et al., 2009).** Hence, salinity is gradually regained by the influence of evaporation. The soil appears in patchy appearance, with areas of mud flats and sandy soils. The soil surface topology characterization is subject to change within days to months. The GSP harbors temporary streams and pools with different salinities concentration. The maximum annual rainfall is

115.3 cm, with 78.6 cm falling in 2009, and 11.2 cm falling in the month of sampling. The surface temperatures are 55.5ºC, with median daily reaches to 45.5 ºC. The variation of median day-night temperature was 22.4 ºC. The range of speed of wind is from 0 to 69 km h^{-1}, with 25th percentiles (8.6 km h^{-1}) and 75th percentiles (21 km h^{-1}). The salinity of the ground water reached to 4% to 37%, however, salinity of surface soil ranged from 0.3% to at least 27%. Soil pH varied from 7.34 to 9.23 with a mean of 8.75, and a median of 9.06. Samples were collected on June and December 2009 on a dry day. The sampling sites are located in regions poor in organic matter. 21 unvegetated sampling sites (salt-crusted sandy) and 9 vegetated sampling sites (Rhizosphere) were obtained.

2.3. Sample Collection

Thirty soil samples were collected including 21 unvegetated salt flat sites, and 9 vegetated rhizosphere sites of the GSP, during time scale between June and December 2009. The sampling dates were15 June, and 20 December 2009. Samples were collected from the top of 12-15 cm of soils of the unvegetated and vegetated areas of the GSP. The weights of soil samples (700-900 g) were taken with sterile Petri dishes, and hand spades, put in sterile pages. Samples were transported to laboratory at 25ºC in a cooler. In the lab, the collected samples were labeled, dated, and categorized according to the site of collection and then dried for 2 weeks at ambient temperature. 50 g of each sample was maintained in sterile tubes and preserved in -20ºC.

2.4. Isolation of actinomycetes

10 g of dried soil sample was adding to 90 ml of autoclaved water. After vigorous shaking for 20 minutes, the soil suspension was allowed to settle for 5 minutes and diluted to 10^{-3} using autoclaved water. The diluted sample was then variously shaken for 1 minute before 100 µl of 10^{-3} dilution was inoculated onto agar media and spread with sterilized glass rod. All soil samples were inoculated onto four different isolation media. All agar media were princely prepared and autoclaved at 121ºC for 20 minutes. The isolation media consisted of the following: Starch Nitrate Agar, soluble starch 20.0 g, KNO_3 2.0 g, K_2HPO_4 1.0 g, $MgSO_4$ 0.5 g, $CaCO_3$ 3.0 g, $FeSO_4$ 0.01g, trace salt solution 1.0 ml, agar 22.0 g, and sterilized water 1 l; Starch Casein Agar, soluble starch 10.0 g, casein 0.3 g, KNO_3 2.0g, NaCl 2.0g, KH_2PO_4 2.0 g, $MgSO_4 . 7 H2O$ 0.5 g, NaCl 2.0g; $CaCO_3$ 0.02 g, $FeSO_4.7 H2O$ 0.01 g, agar 22.0 g, and sterilized water 1 l; Yeast Malt

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Extract Agar- ISP-2, yeast extract 4.0 g, Malt extract 10.0g, dextrose 4.0, agar 22.0g and sterilized water 1 l; and TSA 30.0 g, and sterilized water 1 L. and 15 µg naldixic acid were added after autoclaving to inhibit the growth of the other fast growing bacteria. Starch Nitrate Agar: soluble starch 20.0 g, KNO_3 2.0 g, K_2HPO_4 1.0 g, $MgSO_4$ 0.5 g, $CaCO_3$ 3.0 g, $FeSO_4$ 0.01g, trace salt solution 1.0 ml, agar 22.0 g, and sterilized water 1 l; Starch Casein Agar: soluble starch 10.0 g, casein 0.3 g, KNO_3 2.0g, NaCl 2.0g, KH_2PO_4 2.0 g, $MgSO_4 . 7 H2O$ 0.5 g, NaCl 2.0g; $CaCO_3$ 0.02 g, $FeSO_4.7 H2O$ 0.01 g, agar 22.0 g, and sterilized water 1 l; Yeast Malt Extract Agar- ISP-2: yeast extract 4.0 g, Malt extract 10.0g, dextrose 4.0, agar 22.0g and sterilized water 1 l; and TSA 30.0 g, and sterilized water 1 L. and 15 µg naldixic acid were added to inhibit the growth of the other fast growing bacteria, then incubated at 30°C for 3 weeks. Inoculated plates were then incubated at 30ºC for up to 3 weeks, and all well-separated, developed actinomycete colonies, observed by naked eye or under light microscope at a magnification power of up to x30 were therefore picked and removed from the original isolation plates and subcultured on starch nitrate agar. The inclined coverslipe culture technique **(Williams et al. 1989; Peela et al., 2005)** was served to determine the morphological characters of the recovered isolates. All recovered actinomycetes were grouped according to colony color, morphological characters, and pigment production, and the chosen representatives from each phenotypic suite were then subjected to phylogenetic analysis. The purified actinomycete cultures were cryopreserved in autoclaved 30% glycerol vials at -80°C.

2.5. Nucleic acid extraction

Genomic DNA was prepared as follows: actinomycete isolates grow on Starch Nitrate Agar for 5 to 7 days at 30°C. The aerial and vegetative growth were harvested and macerated, and used to extract the genomic material following the protocol described by **(Bürgmann et al., 2001).**

2.6. 16S rRNA gene amplification and sequencing

16S ribosomal DNA (rDNA) sequencing gene templates were amplified using primers 27F (5'-AGAGTTTGATCACTGCCTCAG-3'), and 1492R (5'-GGTTACCTTGTTACGACTT-3') **(Edwards et al., 1989).** The PCR reaction was carried out in 25 µl reaction. Each 25 µl reaction contained 12.5 µl GoTaq® Green Master Mix (Promega, Madison, WI, USA), 3.5 µl sterile water (Promega, Madison, WI,

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USA), 1 µl (25 pmol) of each primer (IDT, Coralville, IA, USA), and 3 µl DNA of extracted genomic DNA. The PCR program conditions consisted of step (1) 94°C for 5 min, step (2) 30 cycles of at 94°C for 30 sec, 60°C for 30 sec, and 72 °C for 1 min and followed by a final extension step at 72°C for 7 min. After the completion of the cycles, reactions were maintained at 4°C until the electrophoretic run. Amplified PCR products were examined by 0.8% agarose gel electrophoresis using horizontal electrophoresis in a 1x Tris-acetate EDTA (TAE) buffer. A 1kb plus ladder (Bioneer, Alameda, CA, USA) was served as the molecular ladder. Loaded electrophoretic gels were visualized and detected by ultraviolet transillumination using UV imager (UVP). The amplicon products were purified using ExoSAP-IT enzyme (Affymetrix, Santa Clara, CA, USA). The purified amplicons then subjected to preparing process for the sequencing reaction with a modified ABI 3130*xl* manufacturer's sequencing protocol (Applied Biosystem, Foster City, CA, USA). The sequencing reaction was prepared according to the following, the full sequencing reaction was prepared up to 10 µl volume containing 3 µl purified PCR amplicon, 1.5 µl primer (27 F, 1429R, plus flanking and internal primers ;-TU108: 5'-AAACTCAAAGGAATTGACGG-3'; TU108r: 5'-ccgtcaattcctttgagttt-3') **(Edwards et al., 1989: Caton et al., 2004),** 0.5 µl sequencing buffer, 2 µl betaine, o.5 µl BigDye, and 2 µl RNase-free water. The cycling conditions of the sequencing reaction were fulfilled according to the ABI capillary sequencer instructions (Applied Biosystems, Foster City, CA, USA). The sequenced products were then read by utilizing the ABI 3130*xl* (Applied Biosystems)

2.7. Characterization, identification and phylogenetic analysis of the 16S rRNA gene

All phylogenetic analysis was performed on almost nearly complete 16S rRNA sequences in SILVA databases. To affiliate the sequences to the species level assignment, 97 % similarity was chosen as a threshold, and was determined according to their closest relative reference OTUs in SILVA database. The representative OTUs generated the cutoff ($OTUs_{0.03=}0.97$) were then classified using SINA web aligner tool (SINA 1.2.11), imported in the SILVA SSURef databases **(Quast et al., 2013).** To construct the phylogenetic tree, the classified sequences were retrieved into MEGA 6 for Windows (**Tamura et al., 2013),** and the phylogenetic reconstructions were calculated with

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Neighbor-joining statistical method, bootstrap method of phylogeny at 1000 samples to assess the support for inferred phylogenetic relationship. Model of phylogeny was conducted by Maximum composite likelihood, substitution to include use with d: Transition + Transversions, rates among sites used with uniform rates, pattern among lineages were same (Homogenous), gaps/missing data treatment method was pairwise deletion. Cluster analysis, and heat map visualization to investigate, and compare the phyla abundance, was constructed by PermutMatrix Version 1.9.3.En (http://www.limm.fr/~caraus/PermutMatrix/), dissimilarity was conducted by euclidean distance (L2), hierarchical clustering average linkage (UPGMA).

2.7.1. Electron microscopy

Actinomycete cells were grown for 5 to 10 days onto Starch nitrate agar. The aerial growth was touched by stamp and then sputter coated with gold-palladium alloy under vacuum. Samples were then visualized at a magnification of x4, 000 by using a (FEI Inspect S50) scanning electron microscope.

2.7.2. Nucleotide sequence accession number

16S rRNA gene sequences were deposited in BioProject under the accession numbers: PRJNA356259 ID: 356259; www.ncbi.nlm.nih.gov/bioproject/?term=prjna356259

2.8. Screening the GSP actinomycete for salinity potential

The capabilities of 155 actinomycete isolates were investigated. Suites of Starch glycerol nitrate plates were prepared and supplemented with salinity ranged from (0, 3, 5, 10, 15, 20, 25, and 30%). A total of 155 actinomycete cultures were grown in Starch glycerol liquid culture for at 30°C and 200 rpm for 7 days. The culture cells were transferred to 96-multiwell plate and labeled. Using autoclaved 96 multi-applicator combs actinomycete cell were moved and plotted onto the plates and incubated at 30°C. The growth of actinomycete cells on plates were detected after 5 to 7 days and considered a positive result.

2.9. Production media for anti-MRSA and Screening the GSP actinomycete using disk diffusion method for anti- MRSA bioactivities

The antimicrobial potentialities of a total of 155 were tested against two MRSA strains methicillin resistance strains of (*Staphylococcus aureus* B-8-41-D-4 and methicillin

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resistance strains *Staphylococcus aureus* 4656), using agar disc diffusion method. A total of 155 actinomycetes were inoculated in 30 ml of SGN broth (soluble starch 10.0 g, glycerol 5.0 g, yeast extract 5.0 g, potassium nitrate 2.0 g, dipotassium hydrogen sulphate 1.0 g, magnesium sulphate 0.5 g, calcium carbonate 3.0 g, ferrous sulphate 0.01 g, and water 1000 mL).), and incubated in orbital shaker at 30°C and 200 rpm for 5 days. The fermented broth was centrifuged at 10,000 rpm for 5 mints. The culture filtrates were examined for antimicrobial activities using disk diffusion method. Late exponential phase of a pure colony of the test bacterial strains of MRSA were prepared by inoculating 1% (v/v) of the cultures into fresh Muller-Hinton broth (Merck) and inoculating on an orbital shaker at 37°C and 100 rpm overnight. Then 50 µl of the bacterial growth uniformly spread onto Muller-Hinton plates and incubated at 37°C for 2 days. Upon using the test culture, the culture suspensions were standardized with a final cell density of visible turbidity and density equal to that of 0.5 McFarland. After adjusting the turbidity, sterile cotton swab was dipped into the bacterial suspension and streaked onto Muller-Hinton Agar Plates. Using a cork borer, 6 mm diameter wells were made and 50 µL of the actinomycetes filtrate was added per well, incubated at 37°C for 2 days, and then the results were determined by estimating the diameter of the clear zones.

3. Results and Discussion

3.1. Selective isolation of culturable actinomycetes

Recent decades have been drawn surge in research on extreme environments including hypersaline environments **(Hedi et al., 2009).** The GSP have areas which contains shallow ponds of saturated brine of mainly sodium chloride, whereas, other regions have only ephemeral pools that are associated with remarkable shift in microbial community structure **(Major et al., 2005).** The GSP is a formidable area, and the climate data have been previously reported, the daily winds were moderate speeds and ranges from 12 to 19 km h^{-1} along the flats, and with daily maxima ranging from 54 to 137 km h^{-1}. The rainfall were lowest in summer and winter months, with maxima resulting in October (18.03 cm) and March, May and June of (>6 cm). the average incident solar radiation levels were ranging from 9 to 28 MJ m^{-2}, and being lowest in winter and highest

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in summer season, this fluctuation was due to a combination of daylength and maximum middy solar flux which were high higher (53% and 76% respectively) on June than December. The average day/night temperatures revealed the maximum degree of variation in summer and early fall (July-September), estimating in 15-degree differences between daytime and nighttime hours **(Major et al., 2005).** Due to the dynamic, and drastic environmental disturbance of the GSP, it is considered an extreme environment with an intermediate level of disturbance, which in turn is expected to harbor more microbial diversity The current study was investigated recovering and analysis of culturable actinomycetes which grew under aerobic condition at regular atmospheric pressure. It was revealed that the recognizable marine source of actinomycetes is sediments **(Goodfellow and Haynes, 1984).** However, it was reported that actinomycetes mostly account for a small fraction of the bacterial communities (0-9%) in marine sediments **(Bull et al., 2005).** However, the actinomycetes proportion in this study is high (46%). A total of 30 soil samples (21 soil samples from salt flat areas and 9 soil samples from vegetation areas) were collected from the GSP along summer and winter of 2009. Four selective isolation media supplemented with salt concentration ranged from 0, 5 and 10% were used; Starch nitrate agar (SNA), Starch-casein agar (SCA), Yeast malt extract agar (YMEA), and Trypticase soy agar (TSA). Based on the distinct morphology of actinobacteria isolates on the media plates, three hundred fifty eight presumptive actinomycetes were successfully isolated from the GSP (actinomycetes could be recovered from 21 soil samples, and 9 soil samples were not be able to produce actinobacteria). The 9-vegetated soil samples and 12 out of 21-salt flat soil samples were be able to produce actinomycetes using serial dilution method. It was shown that 51(14%) isolates were recovered from salt flat soil samples and 307 (86%) isolates were recovered from vegetation soils samples. This may be due to the presence of exudates in the rhizosphere area which enhance the proliferation of actinobacteria communities. A frequency of 52 % of the total isolates were recovered by SCA, 28%, 14%, and 8% were recovered by SNA, YMEA, and TSA respectively. This current study revealed that SCA medium is the most effective isolation medium for marine actinomycetes. Starch-casein agar medium was earlier devised by **(Grein and Meyers, 1958)** and was shown by **(Peela et al, 2005; Adinarayana et al., 2006)** that it is more effective medium for marine

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actinomycetes. The actinomycetes population recorded in this study are consistent with those reported by **(Meena et al., 2013).** In addition to that it is difficult to compare the date obtained in this study with those estimated in previous reports as confirmed by **(Pathom-aree et al., 2006)**. It was shown that an adverse environmental condition is reflected in genetic and metabolic diversity of marine actinomycetes, which still behind our full understood **(Bull et al., 2005). Jensen et al., (1991)** studied the physico-chemical factors such as salinity, pH, sediment nutrients which influence the diversity of marine actinomycetes in marine sediments. In our study it is likely that the important physico-chemical parameters influencing the distribution of actinomycetes were temperature, porosity, salinity, pH, and total organic carbon. Soil actinomycetes prefer the pH 5 to 9 to grow as they functioning in an acceptable way as neutrophiles in culture with an optimum close to neutrality **(Goodfellow and Williams 1983)**. Therefore, in this study the soil was near neutrality in all sites. Another important physico-chemical factor that controls the distribution of actinomycetes is the temperature **(Goodfellow and Williams 1983)**. It is well known that most actinomycetes functioning properly as mesophiles at 25-30°C. However, the temperature was quite higher in this study than the range, and the effect of temperature on distribution of the actinomycetes between underneath vegetation and salt flat sites in this study was observed. We hypothesize that shrubs shooting system alleviate the high temperature effect on soil underneath vegetation sites, which significantly influence and encourage more actinomycetes frequency than the salt flat sites do, and our results emphasize our hypothesize. Total organic carbon is another physico-chemical factor which governs the actinomycetes diversity. Actinomycetes are saprophytic microbes (prefix *sapro*-means rotten) that depend on the availability of carbon source in environment. Actinomycetes are able to degrade organic matter in environment and accomplish the supply of carbon and other nutrients in the soil. We also hypothesize that underneath the vegetation sites, shrubs roots in the rhizosphere release exudates, which consider polysaccharide compounds in soil. These exudates naturally furnish good porosity, and organic matter that would be decomposed by actinomycetes, lead to a better proliferation, remarkable frequency and diversity of actinomycetes than the salt flat sites have, that hypothesize was confirmed by the results of this study

3.2. Phylogenetic diversity and characterization of culturable actinomycetes

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One of the objectives of this study was to determine the phylogenetic assignment of the culturable actinomycetes isolated from the GSP. In total 155 actinobacteria isolates were chosen to be representatives according to employing morphological, physiological characterization for preliminary grouping. The nearly full-length (>1300) 16S rRNA genes of actinomycete isolates could be accomplished for ~96, were sequenced and subjected to phylogenetic analysis. The utilization of microbiological and molecular studies has recovered the presence of moderately to extremely halophilic microorganisms **(Oren, 2002; Ventosa 2006).** Consequently, in our study, the using of 16S rRNA gene sequence similarity value ≥ 97%, which considered to be the cutoff value for species for species identity **(Wayne et al., 1987).** The molecular characterization defining by the nearly full sequence of 16S rRNA gene sequence and distances of 0.02 indicated that the isolated formed ~96 OTUs. Neighbor-joining trees with estimated phylogenetic placement of the GSP actinomycetes sequences (Figures 12 and 13) have been established. Phylogenetic assignment showed that all isolates were members of two genera of the domain bacteria, including high frequency members affiliated to the genus *Streptomyces*, and low frequency members affiliated to the genus *Nocardiopsis*. Results showed that the actinobacteria isolates belonging to two families and two genera *Streptomycetaceae* (*Streptomyces*), and *Nocardiopsaceae* (*Nocardiopsis*). The relative abundance of isolates from these genera were as follows: *Streptomyces* (115 isolates: 74%), *Nocardiopsis* (40 isolates: 26%). Our study is the first to report the presence of these actinobacteria genera in the GSP soil samples. Members of the both genera are considered aerobic microorganisms. Nearly all of these isolates were recovered from the surface of the GSP surface and in the first centimeters down (~12-15 cm) of each biotope. All these isolated microorganisms may use various spectrums of organic compounds and considered chemoorganitrophic. The rRNA sequences fall into two categories: (1) sequences belonging to, but not identical with organisms that previously have been isolated from hypersaline and marine environment, and (2) sequences belonging to microorganisms which have not been related to hypersaline or marine origin. The first category harbors *Nocardiopsis* isolates (Figure 13: Clusters 20-22) and a large number of *Streptomyces* isolates (Figures 12; Clusters 1-18) than the second category. The second category contains *Streptomyces* and *Nocardiopsis* isolates not belonging to hypersaline

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environment, suggesting that the GSP soils harbored some halotolerant wash off *Streptomyces* due to the dynamic environmental conditions. The phylogenetic analysis pattern resulted that the distribution of *Streptomyces* isolates fell into 18 clusters, according to their 16S rRNA gene sequence similarity to the most closely reference strains by consulting the publicly databases (SILVA). Isolates SGR-26-1-0% and SGR-20-2-0% were found most closely to the thermophilic isolate recovered from hot spring *Streptomyces sp.* GP2 (GenBank accession number AY566558) **(Lal R., M. Dadhwal, M. Kumar, S. Bala, S. R. Prabagaran, and S. Shivaji, Unpublished)** (Figure 1: Cluster-1). Isolates SGR-22-4-5%, SGN-30-1-0%, SGR-26-8-0%, and SGR-27-1-0% were recovered from vegetation site and SGR 8-3-0% which recovered from salt flat site were found most closely related to rDNA sequence from thermo-strain *Streptomyces sp.* VTTE-062974 (GenBank accession number EU430546) **(Suihko et al., 2009)** (Figure 12: Cluster-2). Isolate SGR-8-7-0% was found very similar to *Streptomyces actinobacterium BH0951* (GenBank accession number GU265720, Huang D., Y. Xu, and S. Chen, Unpublished) which isolated from marine environment and showed antibacterial activities (Figure 12: Cluster-3). In cluster-4 related to (Figure 12), phylogenetic analysis showed that isolate SGN-8-1-5% representing most closely resemblance to EU430564 *Streptomyces sp. VTT E-062988* which isolated from thermophilic environment **(Suihko et al., 2009).** On the other hand, other isolates that recovered from vegetation and salt flat sites were shown similarities to reference strains not belonging to marine and hypersaline environments (Figure 12: Clusters with red circle shapes). In Figure 12, cluster-5, four *Streptomyces* isolates recovered from vegetation sites and an isolate SGR-8-9-0% recovered from salt flat site were clustered and distantly more closely related to *Streptomyces sp.* NPA1 (GenBank accession number JN565291) isolated from sediment **(Xing, Unpublished)**, which support our hypothesis of either isolate wash off or genetic flow between short distance niches. Seven *Streptomyces* isolates recovered from vegetation places distributed and clustered closely to thermophilic uncultured *Streptomyces sp.* (GenBank accession number JQ358562) isolated from **compost (Wang, Asparagus straw compost microorganisms, Submitted)** (Figure 12; cluster 6). Two *Streptomyces* isolates from different isolation regions clustered together and showed close similarity to cellulose-degrading isolate *Streptomyces albogriseolus* (GenBank

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accession number AJ494865) **(Wirth and Ulrich, 2002)**, and to *Streptomyces* uncultured bacterium (GenBank accession number FJ152784) which isolated from alkaline saline soil of former lake **(Valenzuela-Encinas et al., 2009).** Isolate SGR-25-4-0% in (Figure 12; Cluster-8) was exhibited most closely related to endoxylanase and endoglucanase production-isolate *Streptomyces* F2621 (GenBank accession number DQ205517) **(Tuncher et al., 2004).** In cluster 9 of Figure 12, two isolates from vegetation and salt flat sites were showed distantly related to a bioactive compound production-isolate *Streptomyces sp.* NEAU-TLv1 (GenBank accession number KF887912) **(Frang, B, and C. Liu, submitted).** In cluster-10, it harbors 11 isolates recovered from vegetation sites were found most closely related to thermophilic isolate *Streptomyces sp.* VTT E-062985(GenBank accession number EU430556) **(Suihko et al., 2009)**, and marine isolate *Streptomyces parvus* (GenBank accession number EU741180) which recovered from Pacific and Caribbean waters **(Solano, G., K. Rojas-Jimenez, G. Tamayo-Castillo, M. Jaspars, and M. Goodfellow, Submitted).** It was found that two vegetated isolates and one isolate were found more closely similar and distantly related to *Streptomyces sp.* 136470 (GenBank accession number EU741145) obtained from Pacific and Caribbean waters **(Solano, G., K. Rojas-Jimenez, G. Tamayo-Castillo, M. Jaspars, and M. Goodfellow, Submitted)** in cluster-11. In cluster-12 one vegetated isolate was found most closely related to an isolate *Streptomyces globisporus* subsp. *Globisporus* obtained from Pacific and Caribbean waters (GenBank accession number EU741221) **(Solano, G., K. Rojas-Jimenez, G. Tamayo-Castillo, M. Jaspars, and M. Goodfellow, Submitted).** One vegetated isolated was being harbored in cluster-13 and found most closely related to a lead resistant isolateStreptomyces sp. CPA1 (GenBank accession number DQ784091) **(Day D. M., J. L. Seabaugh, and J. E. Champine, Submitted)**. Cluster-14 includes isolates SY-25-10-0% and SR-24-1-0% were recovered from vegetated areas, and most closely related to Zinc-solubilizing actinomycetes isolate *Streptomyces roseofulvus* (GenBank accession number JF792522) **(Sharma, S. K., A. Ramesh, S. Shivaji, and M. P. Sharma, Submitted).** The 16S rRNA gene analysis showed that (Cluster-15) three salt flat isolates; SGN-1-1-5%, SGN-1-2-5%, and SBA-1-1-5% were found having similar sequencing and most closely related to isolate *Streptomyces sp.* VITTK3 (GenBank accession number GU808333) which isolated from marine sediment and has a

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novel antifungal activity **(Thenmozhi, M., K. Kannabiran, and S. Madhu, Submitted)**. Isolate SY-26-2-5% was found genetically most related to the type strain *Streptomyces sp.* MTCC8377 (GenBank accession number GU808333) that has antimicrobial activity (Cluster-16). In Cluster-17, the16S rRNA gene sequencing analysis revealed that four vegetated isolates were shown a close similarities to an isolate Streptomyces sp. SM3501(GenBank accession number KF006349) which isolated from salt lake soil **(Tatar, D., A. Sazak, and N. Sahin, Submitted)**. Two vegetated isolates and one salt flat isolate were found most closely related to an isolate Streptomyces sp. 042403 (GenBank accession number FJ842613) which isolated from marine fish (Chao, D., Q. Wei, Submitted). Additionally, they were shown distantly related to an antimicrobial isolate Streptomyces sp. SXY46 (GenBank accession number GU045530) **(Wen, C., J. Chen, and D. Zheng, Submitted)**. Phylogenetic assignment using 16S rRNA gene sequencing analysis was able to genetically group *Nocardiopsis* isolates into five clusters; cluster-19 includes five vegetated isolates were found most closely related to an isolate *Nocardiopsis sp.* YIMC555 (GenBank accession number EU135692) which isolated from haloalkaline soil of lake bank **(Cui, X.-L., Y.-G. Chen, W.-J. Li, L.-H. Xu, and C.-L. Jiang, Submitted)**. In cluster-20, five vegetated isolated were found most closely related to *Nocardiopsis aegyptia* (GenBank accession number AJ539401) which isolated from marine sediment **(Sabry et al., 2004).** Isolates SW-19-3-5%, SGN-19-6-5%, and SGN-19-7-10% in cluster-20, were isolated from vegetated sites, found more closely related to a cytotoxic peptide-producing isolate *Nocardiopsis lucentensis* (GenBank accession number EF392847) **(Cho et al., 2007),** and also to an isolate *Nocardiopsis lucentensis* DSM 44048 (GenBank accession number EF392847) that recovered from salt marsh soil **(Li et al., 2013).** Four isolates were recovered from vegetated sites and found most closely related to two reference isolates; one of them is: a bioactive and alkaliphilic isolate *Nocardiopsis sp.* YIM (GenBank accession number AY299630) **(Zhang Y. Submitted)**, and the other is marine sediment-isolate *Nocardiopsis sp.* WBF4 (GenBank accession number EU099408) **(Ye, L., T. Xi, G. S. Na, and J. S. Yang, Submitted)**

Rhizosphere microbes have an importance to benefit plant growth by suppression of pathogens, degradation of phototoxic compounds, increasing the production of phytohormones, availability of mineral nutrients **(Singh et al., 2004; Lugtenberg and**

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Kamilova, 209). The bacterial community structure of rhizosphere is more diverse than the root bacteria **(Jin et al., 2014).** The root region harbors a complex habitat furnishing a large variety of carbon sources, organic acids, amino acids, and carbohydrates which use by rhizosphere microorganism to get energy **(Wawrik et al., 2005).** Thus these conditions enhance the proliferation and more diversity of rhizosphere microorganisms. It is obvious that bacterial 16SrRNA sequences of the actinomycete isolates of the GSP presented high similarities percentage with sequences retrieved from hypersaline, marine and other environments might support the point of the populations of halotolerant species were not isolated by biogeographic barriers, and gene flow may occur between microbes in different niches with distance. These findings contribute to the evidence that the diversity of actinomycetes in the GSP is not exclusively encountered hypersaline related actinomycetes. It is also interesting that phylogenetic analysis of the isolate sequences indicated that the majority of *Streptomyces* and all *Nocardiopsis* isolates have been assigned to 16S rRNA subclades which corresponded to the sites from which the strains were isolated. These observations might reflect the viewpoint of environmental selection of *Streptomyces* and *Nocardiopsis* isolates. The majority of actinobacteria isolated in this study were *Streptomyces* species and our result is in consistence with **(Zhang et al., 2006)**. The frequency and dominance of *Streptomyces* in various locations have been shown in studies by **(Vijayakumar et al., 2007; Zhang et al., 2006).** The absence of other renowned hypersaline and marine actinobacteria isolates in our study is puzzling, but not unusual. We can hypothesis that the most likely explanation is the type of isolation media we have used resulted in high abundance of *Streptomyces spp*. which obscures the presence of the growth of other actinobacterial less prevalent genera. Additionally, it is most likely that the types of isolation media we have used have favored *Streptomyces* and *Nocardiopsis* growth, but thus do not reflect their real distribution in GSP soils. Carrying out a comparison of phylogenetic trees of our isolated *Streptomyces* cultures and the references strains in database, it was revealed that all actinobacteria isolated in this study were most closely related to culturable species, thus our result is in agreement with **(Meena et al., 2013).** It was found that *Streptomyces* was between the dominant genera and their frequency in Bay of Bengal **(Suthindhiran and Kannabiran, 2010).** Contrarily to previous study **(Mincer et al., 2002)** none of the isolate

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actinomycetes in this study required salt for growth. However, herein, we point out to the biotechnological importance of the recovered actinomycetes from environment of marine origin **(Bull et al., 2002).** Similar to other hypersaline ecosystems, the GSP is prone to drastic physico-chemical conditions including sharp changes in temperature and dryness, high radiation (UV), and high salinity which make shape it to be a demandable region to study the microbial communities. The combines results of this study suggest that the perspective point of actinobacteria diversity in the GSP needs more research work to determine more comprehend view of the assemblage dominance. It is noteworthy to mention that none of the isolates recovered in this current study required salt for growth. Our results support the previous studies that *Streptomyces* are metabolically active in marine sediments **(Moran et al., 1995).** We confirm our hypothesis that there is a link of genetic mixing with terrestrial strains synchronized with the adaptation required for survival in the hypersaline environment of marine origin, has emerged the evolution of actinomycetes isolates belonging to *Streptomyces* and *Nocardiopsis*. It is appear that the GSP actinobacterial communities are composed of a broad scaffold of closely related species which are best identified by the combined utilization of culture dependent as employed in this study, and need continued efforts to improve cultivation techniques and along with culture independent techniques that would serve in other of our article to reveal the true extent of hypersaline actinomycete diversity.

3.3 Scanning electron microscopy

The coverslipe culture technique for spore chain morphology investigation was followed **(Williams et al. 1989; Peela et al., 2005).** The selected actinomycete isolates investigates for spore surface ornamentation showed that the majority have smooth (Non-hairy) spore surface (Figures 6:11). It is worth mentioning that smooth surface spores were characteristic of 75%-80% of *Streptomyces* genus. It was reported that the high percentage of smooth surface spore ornamentation by **(Peela et al., 2005: Adinarayana et al., 2006).**

3.4 Screening of actinobacteria isolates salinity range potential

A total of 155 actinomycete isolates were screening on Starch nitrate agar plates medium supplemented with salinity range (0, 3, 5, 10, 15, 20, 25 and 30%). Almost all isolates were be able to grow up to 5% salinity range and 38% of the total isolates

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(*Streptomyces spp* "18 isolates'', and *Nocardiopsis spp* "21 isolates") were belonging to vegetation regions except one *Streptomyces* isolated, and be able to grow at 10% salinity, and only 7% of the total actinomycete isolates (One isolate belongs to *Streptomyces*, and 3 *Nocardiopsis* isolates) were recovered from vegetation sites and shown ability to grow at 15% salinity **(Figure 5).** No actinomycete isolates could grow at salinity range of 20, 25, and 30%. We hypothesize that the halotolerant isolates were washed as spores with soil from the vegetation and rhizosphere areas, thus all isolates were able to survive and grow in the presence of absence of salinity in the growing media. More extreme environments decrease diversity **(Hacine et al., 2004).** We have revealed that there is linking between actinobacteria diversity (vegetation and salt flats) and salt tolerance. We may attribute the broad salt tolerance of *Actinbacteria* due to; actinobacteria may tolerate a wide range of salinity due to the effect of shrub exudates, and actinobacteria may attach to sediment particles in rhizosphere area. It was reported that most microorganisms may probably be attached to the surface of mineral particles, thus when examine salinity potentiality may not represent the true salinity capability of these microbes. Additionally, there are certain groups of the actinobacteria can respond to salinity change, but this response was not reliable for phylogenetic assignment **(Jinyuan et al., 2009)**.

3.5 Screening of actinobacteria isolates for anti-MRSA compounds

The problem of high spectrum of antibiotic-resistance bacterial pathogen demands discovery of new antimicrobial compounds effective against resistant pathogenic bacteria. In this scenario it is important to screen actinomycetes from different sources of effective compounds. Isolation of microorganisms from extreme environments such as hypersaline areas, deep sea sediment, glacier regions, ant hot sulphur **(Tiwari and Gupta, 2012).** Along the past decades, a large number of actinomycetes have been isolated and screened, thus accounting for 70-80% of relevant secondary substances that available commercially **(Baltz, 2008; Kumar et al., 2010).** It was shown that the fermentation products from the marine-derived actinobacteria have biological activities against pathogenic microorganisms **(Magarney et al., 2004: Peela et al., 2005).** The antagonism characterization of actinobacteria isolates against two MRSA strains were determined using well plate method. The SGNA medium was employed for anti-MRSA substance production, and Millur–Hinton agar media was used for assay. The inhibition zones

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(estimated by mm) of the bioactive actinomycete isolates against the two MRSA strains and the numerical cluster analysis for the inhibition zones of the bioactive isolates is shown in (Figures 6 and 7.) A total frequency (44 isolates: 28%) of the actinobacteria isolates showed antibacterial potential against MRSA strains, the majority of them were recovered from vegetation samples. The reason may be the complex biochemical pathways belonging to our isolates as a result of the available nutrients and osmotic flux in sampling sites (**Meena et al., 2013)**. Within the anti-MRSA bioactive actinobacteria isolates, *Streptomyces spp.* represented (40 isolates: 91%), and that is higher occurrence than compared with **(Xi et al., 2012).** It was interesting to find that the bioactive isolates of *Nocardiopsis spp.* represented (4 isolates: 9%), that was not in agreement with **(Meklat et al., 2011; Xi et al., 2012)** as the frequency of the bioactive *Nocardiopsis spp* isolates in their study was 29%. The most bioactive actinomycete isolates which exhibited anti-MRSA bioactivities were; isolate SY-27-5-0% was shown the highest anti-MRSA bioactivities, and found most closely related to strain recovered from saline water *Streptomyces parvus* (GenBank accession number EU741180) **(Salano, G., K. Rojas-Jimenez, G. Tamayo-Castillo, M. Jaspars, and M. Goodfellow. Submitted)**, then isolate SGR-26-2-0% was shown most closely related to strain isolated from saline soil *Nocardiopsis sp.* 10030 (GenBank accession number AY297777-partial sequences) **(Zhang, L., P., Y. H. Li, Z. T. Lu, and R. L. Yang. Submitted)**, and the third isolate SGR 26-1-0% `was having similarities with themophilic strain recovered from hot spring and found most closely related to *Streptomyces sp.* GP2 (GenBank accession number AY566558) **(Lal, R., M. Dadhwal, M. Kumar, S. Bala, S. R. Prabagaran, S. Shivaji. Unpublished).** The noteworthy point in this study was the serving of SGNA medium for the production of anti-MRSA compounds. While it would not undoubtedly be surprising that the employment of other suite of production media would reveal more bioactive isolates against MRSA strains. The higher frequency of anti-MRSA bioactive actinomycete isolates tested in this current study including *Streptomyces spp*, and *Nocardiopsis spp*. could be evidence of the high capacity of halotolerant actinomycetes for producing wide spectrum of anti-MRSA active compounds.

Conclusions and future perspective

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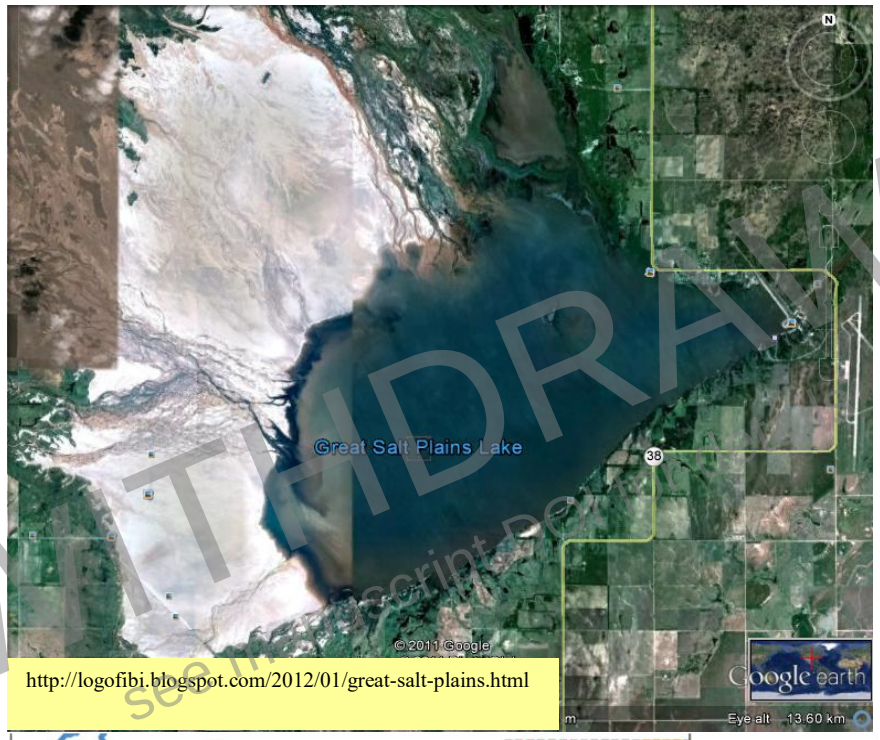
In the current studies, research mainly focuses on hypersaline actinomycetes isolated from GSP which considers of marine origin. This started with isolation of actinobacteria on different isolation media, study the phylogenetic diversity of actinomycetes, examine the salt tolerance, and investigate the anti-MRSA bioactivities of the recovered isolates.

To our knowledge, our current study is the first microbiological study on halotolerant actinomycetes from the GSP. We did also further characterization on the phylogenetic analysis, salt tolerance, and anti-MRSA bioactivities of hypersaline actinomycete for futuristic industrial and pharmaceutical byproducts. The capacity of the isolated actinomycetes to salt tolerance, and along with their capability to secrete valuable secondary metabolites in terms of anti-MRSA compounds, initiates an attractive feature of these microorganisms. The industrial application of these microorganisms has been reported in detail i.e. bioremediation, biopolymers, production of compatible solutes **(Margesin and Schinner, 2001; Mellado and Ventosa, 2003),** that inventively pushing us to screen our culture collection of actinomycetes for molecular of industrial interest as they constitute a potent source of halostable enzymes (work in progress). Further studies should be conducting towards more selective media and molecular characterization approach of these biologically active substances to undoubtedly illustrate new horizon in the secondary metabolites production and to avoid downstream process associated with the current conventional technologies. Concisely, the continuous and systematic screening of marine actinobacteria from different sources and locations in the GSP would probably facilitate recovering more species, novel, with desirable bioactive molecules of high demands.



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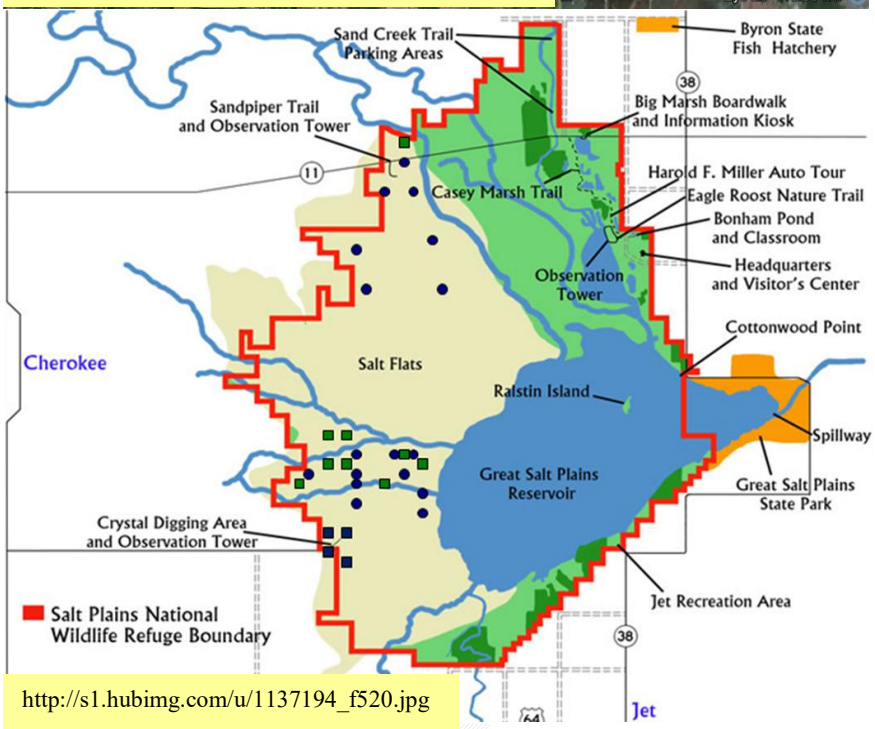

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Figure 1. Satellite image and Map of the Great Salt Plains National Wildlife Refuge showing the GSP and sampling sites in this current study. Blue circles indicate samples site in salt places-June 2009, Green squares indicate the sampling sites in vegetation places-June 2009, and blue squares indicate the sampling site in salt places in December 2009.

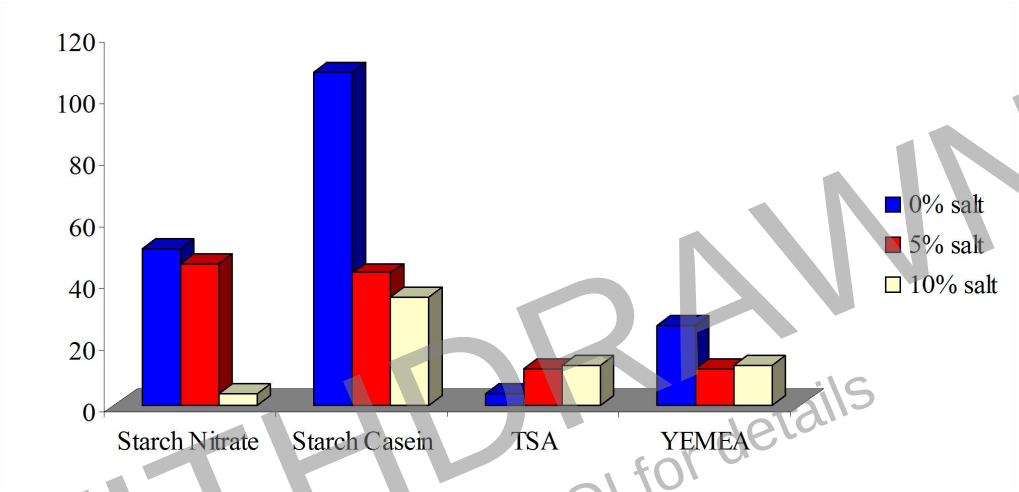


Figure 2. Number of actinomycete isolates produced on different isolation media supplemented with range of salinity.

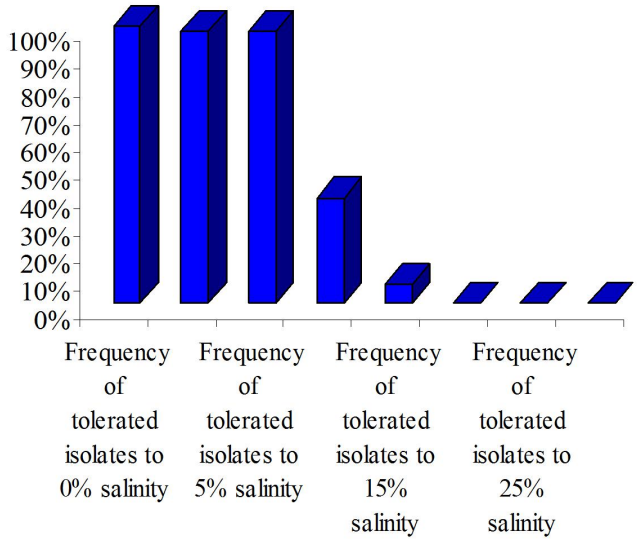


Figure 3. Frequency of actinomycete isolates for salinity rage potential.

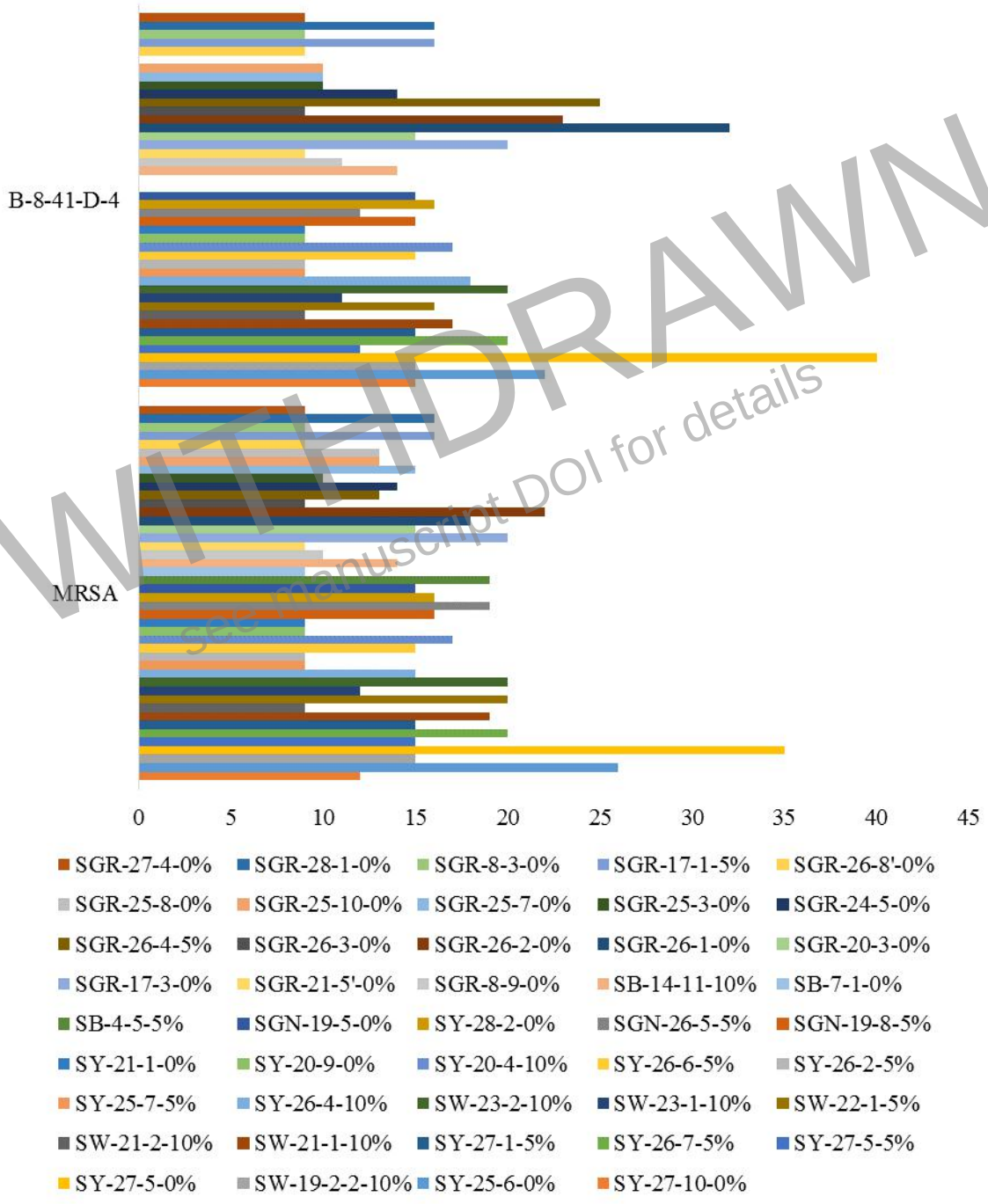


Figure 4. Inhibition zones of actinomycete isolates against two MRSA strains.

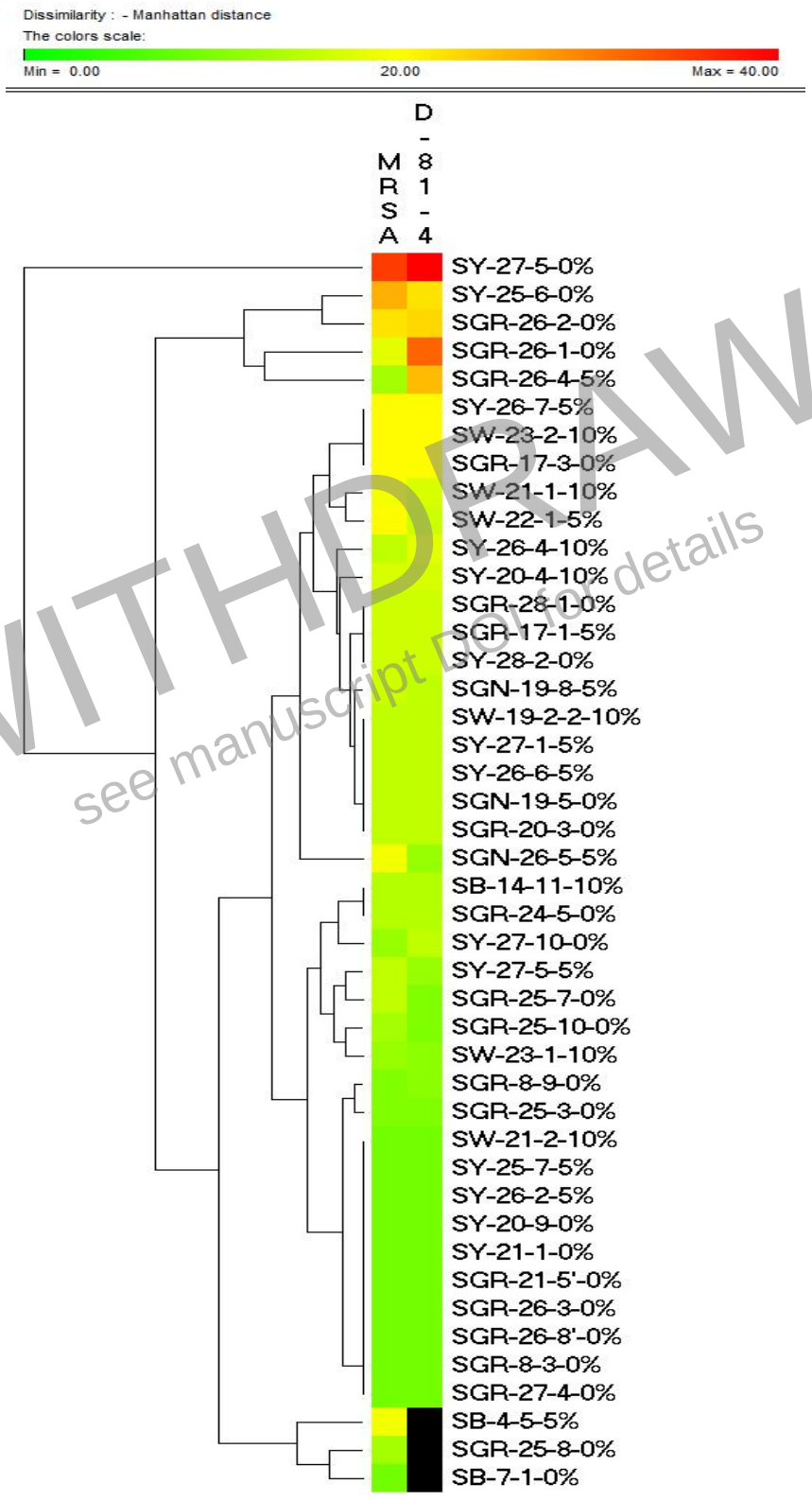


Figure 5. Cluster analysis hierarchy and heat map visualization of the anti-MRSA producing actinomycetes.

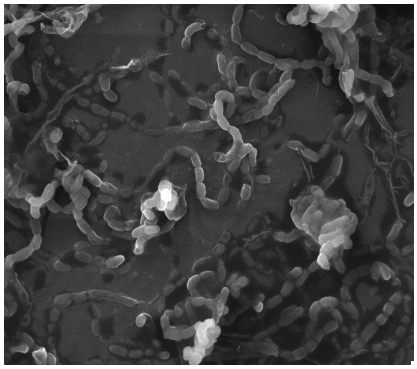


Figure 6. Scanning electron micrograph of Nocardiopsis isolate SGR-20-6-5% (smooth surface spores).

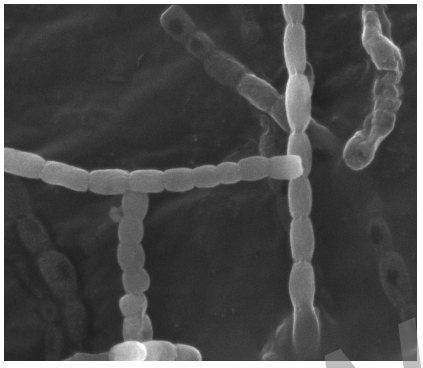


Figure 7. Scanning electron micrograph of *Nocardiopsis* isolate SY-24-4-10% (smooth surface spores).

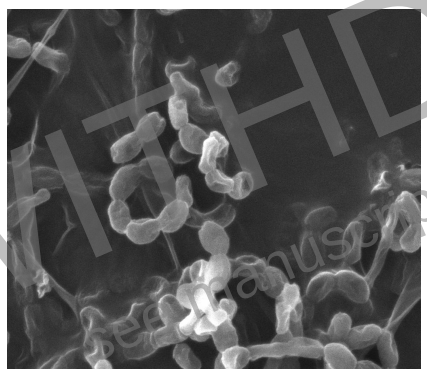


Figure 8. Scanning electron micrograph of *Streptomyces* isolate SR-25-1-0% (smooth surface spores).

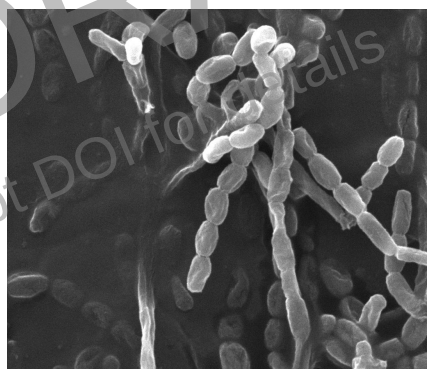


Figure 9. Scanning electron micrograph of *Streptomyces* isolate SGR-8-2-0% (smooth surface spores).

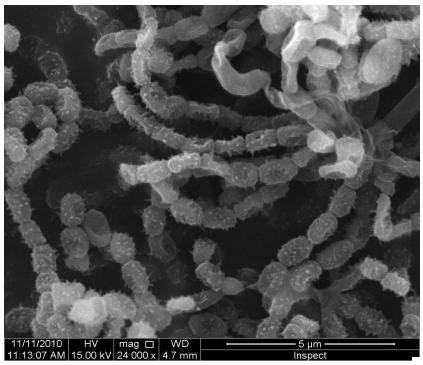


Figure 10. Scanning electron micrograph of *Streptomyces* isolate SGR-26-2-0% (spiny surface spores).

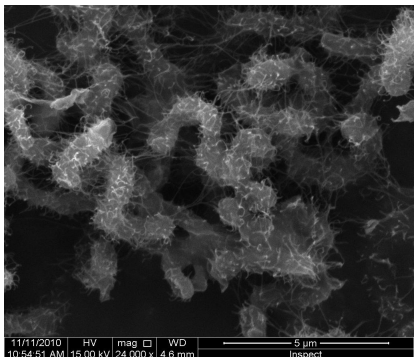


Figure 11. Scanning electron micrograph of *Streptomyces* isolate SGR-14-1-0% (spiny surface spores).

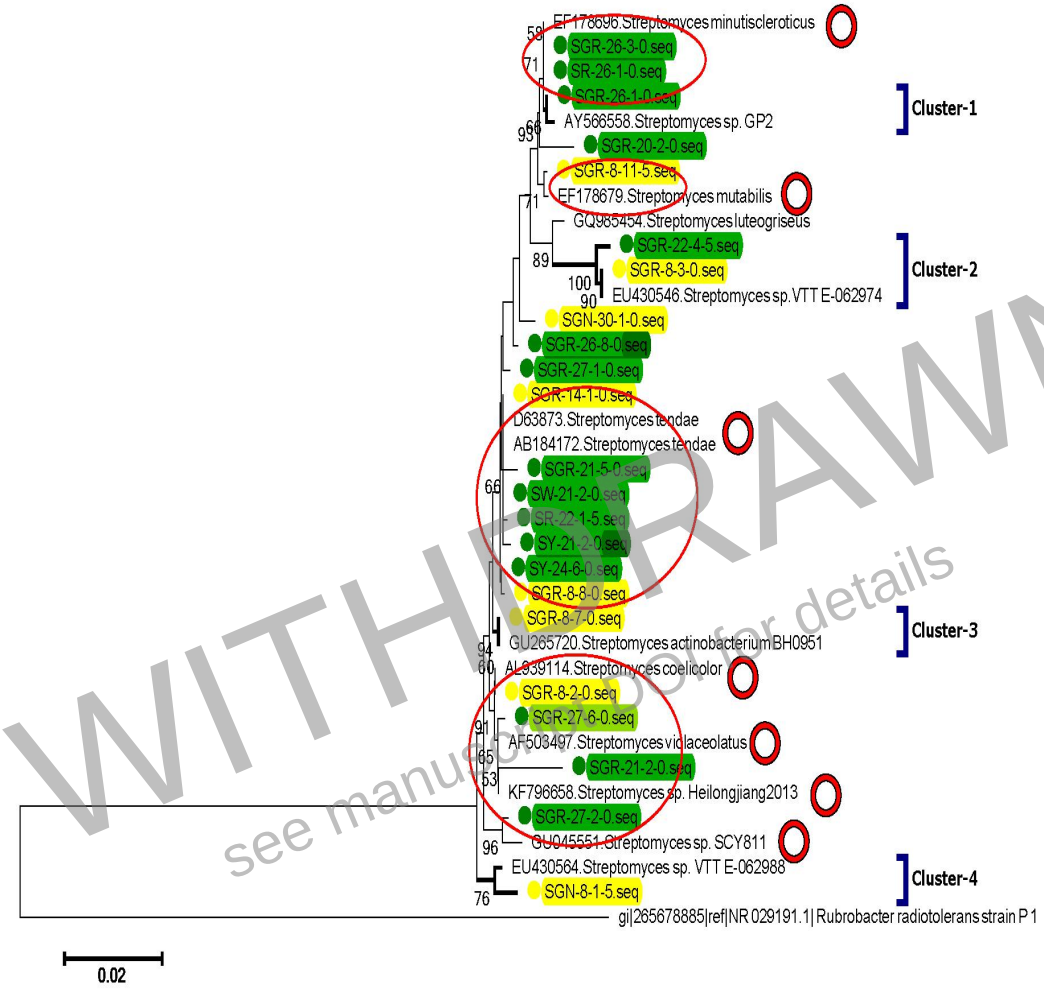


Figure 12. Neighbor-joining distance tree constructed in MEGA using the aligned almost complete 16S rRNA gene sequences of representing strains of *Streptomyces spp.* and the type strains of the most closely related species. Green shapes indicate the vegetated isolates. Yellow shapes indicate salt flat isolates. Red circles indicate the isolates that related to reference strains not belonging to hypersaline or marine environment. Bootstrap values (in percent) calculated from 1,000 reseamplings using the neighbor-joining method are shown at the nodes for values of ≥50%. *Rubrobacter radiotolerance p1* was used to position the root. Genus- and species-level affiliations were carried out using maximum likelihood treeing methods.

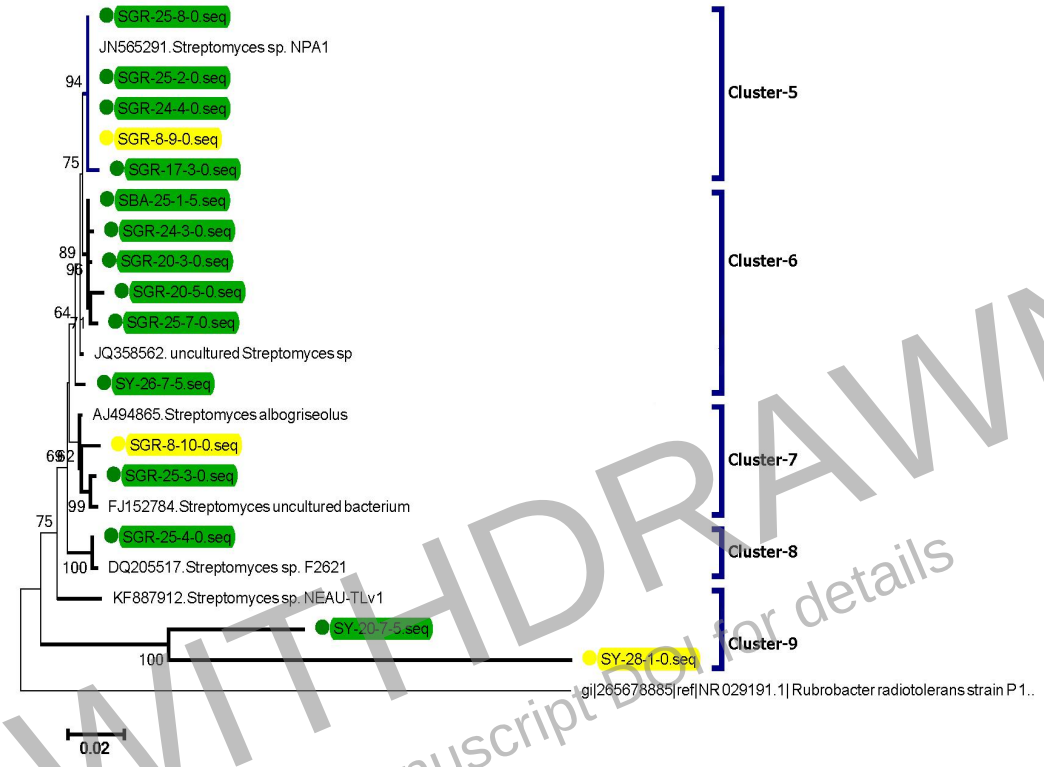


Figure 12-continue. Neighbor-joining distance tree constructed in MEGA using the aligned almost complete 16S rRNA gene sequences of representing strains of *Streptomyces spp.* and the type strains of the most closely related species. Green shapes indicate the vegetated isolates. Yellow shapes indicate salt flat isolates. Red circles indicate the isolates that related to reference strains not belonging to hypersaline or marine environment. Bootstrap values (in percent) calculated from 1,000 reseamplings using the neighbor-joining method are shown at the odes for values of ≥50%. *Rubrobacter radiotolerance p1* was used to position the root. Genus- and species-level affiliations were carried out using maximum likelihood treeing methods.

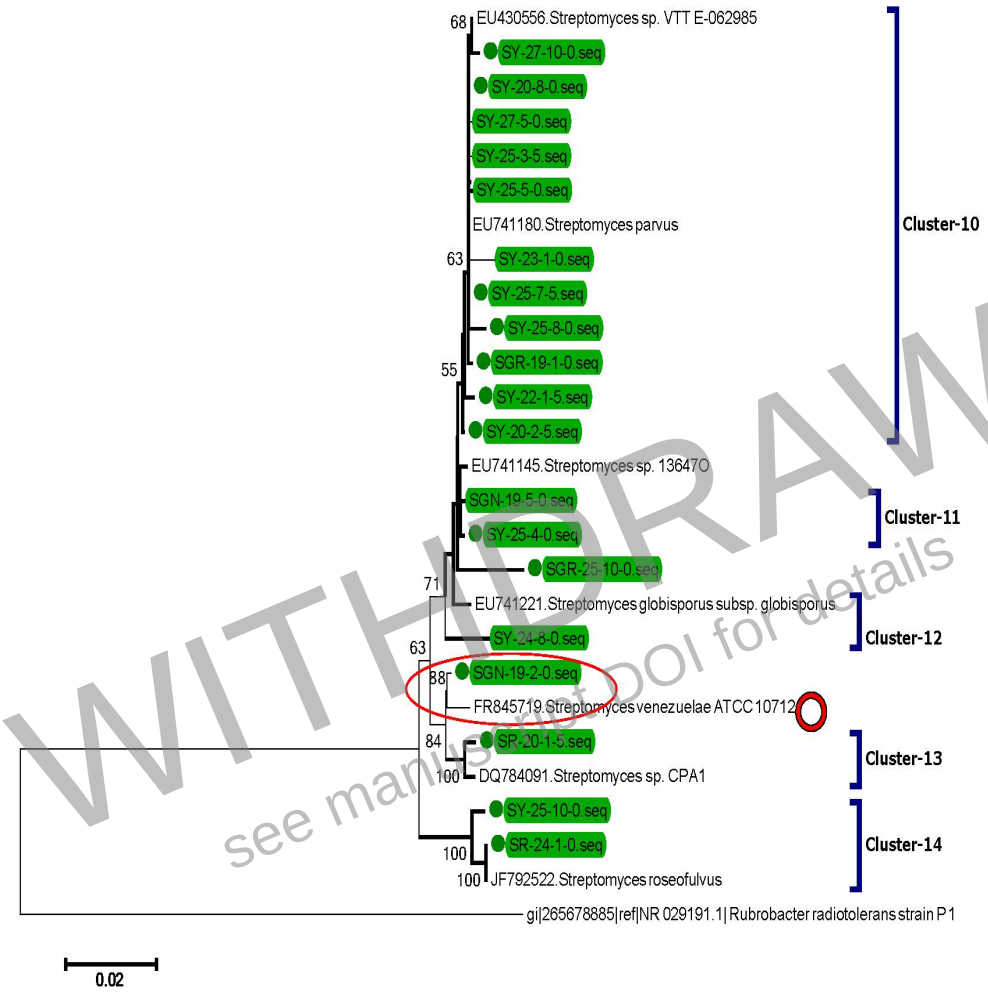
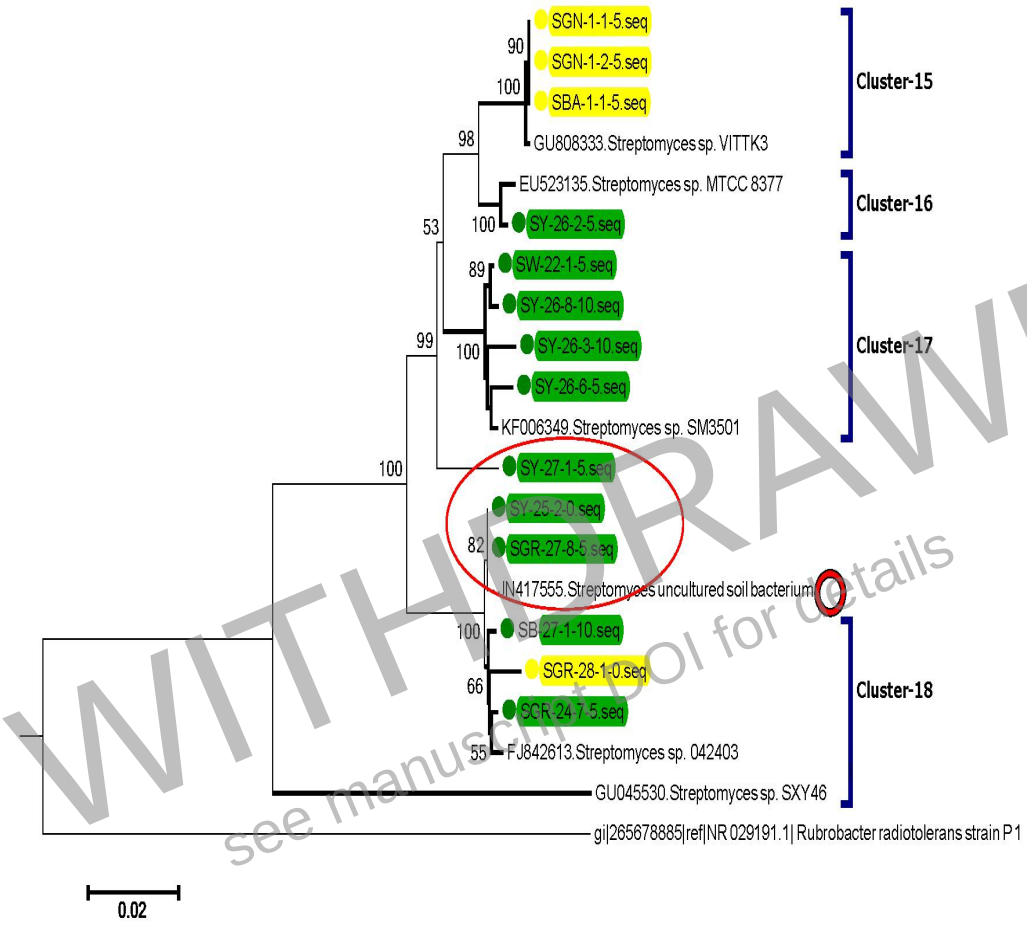


Figure 12-continue. Neighbor-joining distance tree constructed in MEGA using the aligned almost complete 16S rRNA gene sequences of representing strains of *Streptomyces spp.* and the type strains of the most closely related species. Green shapes indicate the vegetated isolates. Yellow shapes indicate salt flat isolates. Red circles indicate the isolates that related to reference strains not belonging to hypersaline or marine environment. Bootstrap values (in percent) calculated from 1,000 reseamplings using the neighbor-joining method are shown at the nodes for values of ≥50%. *Rubrobacter radiotolerance p1* was used to position the root. Genus- and species-level affiliations were carried out using maximum likelihood treeing methods.



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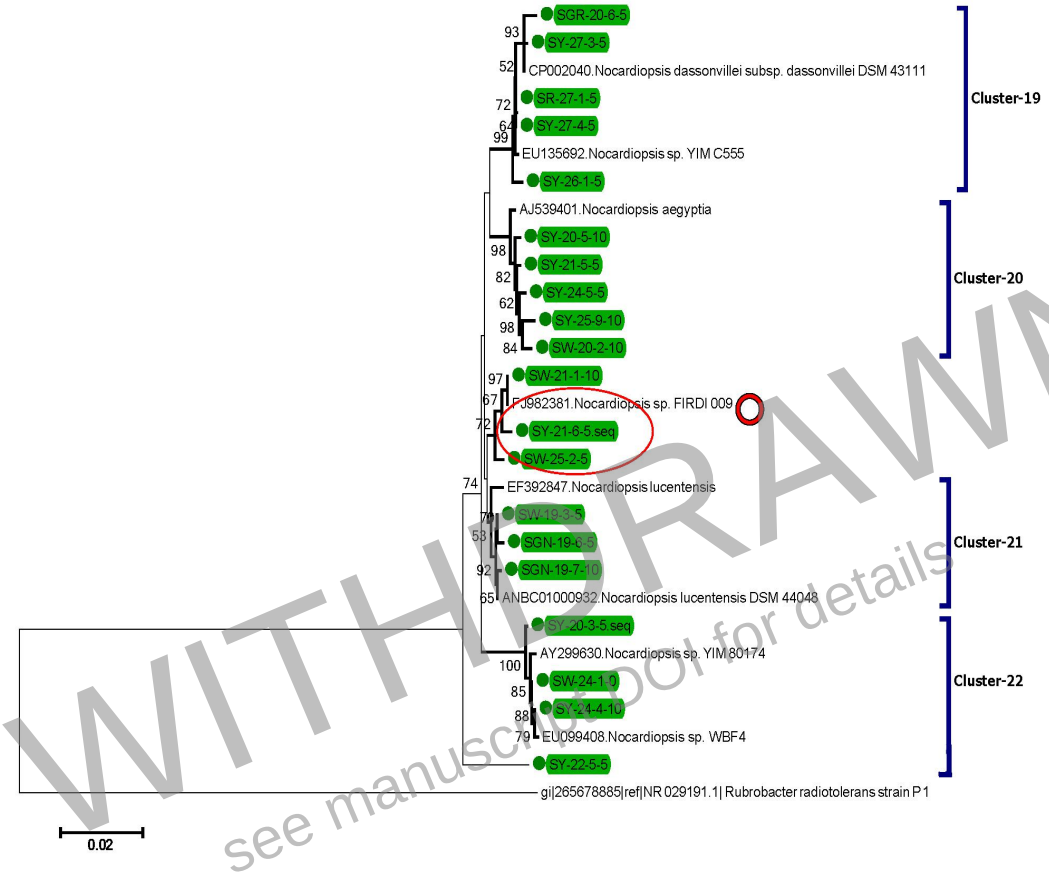

Figure 13. Neighbor-joining distance tree constructed in MEGA using the aligned almost complete 16S rRNA gene sequences of representing strains of *Nocardiopsis spp.* and the type strains of the most closely related species. Green shapes indicate the vegetated isolates. Yellow shapes indicate salt flat isolates. Red circles indicate the isolates that related to reference strains not belonging to hypersaline or marine environment. Bootstrap values (in percent) calculated from 1,000 reseamplings using the neighbor-joining method are shown at the nodes for values of ≥50%. *Rubrobacter radiotolerance p1* was used to position the root. Genus- and species-level affiliations were carried out using maximum likelihood treeing methods.

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