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Bacteria from Hypersaline Environments: A Bioactivity Reservoir of Anti-Methicillin Resistant *Staphylococcus aureus*

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Abstract

In the past decades, novel pharmaceutical compounds have been produced by a wide diverse groups of marine bacteria. These marine bacteria are potential reservoirs for antimicrobial products. In this study, we investigated **40** soil samples collected from the Great Salt Plain of Oklahoma **GSP** for anti-methicillin resistant *Staphylococcus aureus* bioactivities. A total number of **499** heterotrophic bacterial isolates (**202** mesophilic halotolerant isolates, **125** thermophilic isolates, **84** halophilic isolates and **88** thermophile-halophile isolates) were recovered by culture dependent isolation and subjected to high-throughput screening to investigate their bioactivities against two strains of methicillin resistant *Staphylococcus aureus* **MRSA**. A total of **101** isolates **(20.2%)** out of **499** isolates possessed bioactivities against MRSA strains. They included; **eighty (40%)** isolates out of the **202** mesophilic halotolerant isolates showed anti-MRSA bioactivity. **Twenty one** bioactive **(7%)** isolates out of the **297** enrichment isolates showed anti-MRSA bioactivity. They involved; **eleven** bioactive **(11%)** isolates out of **125** of the thermophilic group and **ten** bioactive isolates **(10%)** out of **84** halophilic group isolates. No anti-MRSA bioactivity was revealed by the **88** isolates of the thermophile-halophile group. These **101** bioactive isolates (**80** mesophilic halotolerant, **11** thermophilic and **10** halophilic) exhibited bioactivities against at least one *Staphylococcus aureus* MRSA using well diffusion technique. In regard to biogeographical distribution, a total of **29 (29%)** and **72 (71%)** bioactive isolates were isolated from vegetation and salt flat areas respectively. **Thirty four (34%)** isolates showed bioactivity against both methicillin resistant *Staphylococcus aureus* strains and **fourteen (14%)** isolates showed antimicrobial bioactivity against *Staphylococcus aureus* B-8-41-D-4, whereas **fifty two (52%)** isolates revealed antagonism against *Staphylococcus aureus* 4656. Furthermore, 16S rRNA-based study exposed that, Firmicutes harbored the highest number of bioactive isolates **77**

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(77%) including *Bacillus* (n=**45** isolates), *Halobacillus* (n=**13** isolates), *Virgibacillus* (n=**7** isolates), *Brevibacillus* (n=**7** isolates), *Paenibacillus* (n=**1** isolates), *Sediminibacillus* (n=**2** isolates), *Oceanobacillus* (n=**1** isolates) and *Staphylococcus* (n=**1** isolate). Proteobacteria-Gammaproteobacteria contained **seven** bioactive isolates **(7%)**, including *Halomonas* (n=**5** isolates), *Marinobacter* (n=**1** isolate) and *Pseudomonas* (n=**1** isolate). Actinobacteria were the third group and contained **two** bioactive isolates **(2%)**, including, *Cellulomonas* (n=**1** isolate) and *Micrococcus* (n=**1** isolates). To our knowledge, this is the first study to explore the anti-methicillin resistant *Staphylococcus aureus* bioactivities of bacteria isolated from **GSP**. We consider our findings promising for further research to develop novel antimicrobial antibiotics.

Introduction

The increasing rate of multidrug-resistant microorganisms is currently a public heath problem worldwide **(Bush, 2010). Nussbaum (2006**) reported an increasing rate of antibiotic resistant pathogens due to the lack of potential antibiotic substance. Methicillin-resistance *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) drug resistant bacteria and others are of great importance, because they contribute to the incidence of infectious disease in both community and hospital settings **(Rice, 2006)**. Methicillin resistant *Staphylococcus aureus* has become resistant to almost all available antibiotics except vancomycin and tricoplanin (**Witte, 1999)**. Methicillin-resistance *Staphylococcus aureus* (MRSA) susceptibility to vancomycin has decreased, furthermore, vancomycin-intermediate and vancomycin-resistant *S. aureus* infections have been increasingly reported in many countries (**Hanaki, 1998; Patersen, 1999)**. Natural source antimicrobial screening is an important demand for the medical application. **(Schmidt, 2004).** As a result of pathogenic bacteria are distinctly acquired resistance to commonly used antibiotics, there is a demand for new antimicrobials to challenge the threatening rise of infections **(Coates et al., 2002)**.The recent attainment of the bioactive natural products generated from marine microorganisms may answer that demand and offer new antibiotics to overcome the increasing incidence of multidrug-resistance human pathogens **(Calfee, 2012)**. Over two third of the clinically used antibiotics have been discovered from natural sources or they were semi-synthetic derivatives of natural antibiotics **(Newman and Cragg 2007)**. In the past 30 years, bio-screening for new marine natural products produced thousands of chemically different substances; in 2009 to 2010 alone 2,014 novel marine natural products were discovered **(Blunt**

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et al., 2011 and 2012). In the past decades, marine microorganisms have gained a remarkable interest as being a source for natural substances (**Imhoff et al., 2011).** It was reported that more than 300 structurally bioactive substances were discovered from marine microorganisms and phytoplankton members in 2010 **(Blunt et al., 2011 and 2012)**. The marine environments are endless reservoir of microbial diversity and harbor microorganisms with important active metabolites **(Gurgui and Piel 2010)**. Microorganisms in hypersaline environment had to generate survival strategies to cope the harsh conditions and to compete other microbes for food.

marine microorganisms evidenced to have the ability to be a potential source for bioactive substances. It was demonstrated that the heterotrophic bacteria from Palk bay sediment showed antibacterial bioactivity against methicillin resistant *Staphylococcus aureus* and other species **(Nithya and Pandian, 2009).** Antimicrobial bioactivities of culture supernatants of marine bacteria exhibited antagonism against hospital-acquired methicillin resistant *Staphylococcus aureus* **(Wilson et al., 2010)**. Mediterranean sponges bacteria revealed antimicrobial bioactivities against methicillin resistant *Staphylococcus aureus* **(Hentschel et al., 2001)**. Moreover, marine actinobacteria exhibited anti-MRSA activities **(Kumar and Rao, 2012).** Extremophiles include different categories of microorganisms according to the environmental conditions where they arise from. For instance, thermophiles are organisms having an optimum growth temperature ranging from 45°C or higher and halophile indicates organisms that require at least 0.2 M (3-30%) salt requirement for growth. **Muhammad et al., (2009)** antibiotic production was investigated by thermophilic *Bacillus* species SAT-4. Antimicrobial bioactivity was detected against methicillin resistant *Staphylococcus aureus* and *E. coli* by thermotolerant bacteria recovered from coal mine spoil **(Sethy and Behera, 2012)**. Halotolerant and halophilic actinobacteria isolated from marine salterns were found to produce antimicrobial against methicillin resistant *Staphylococcus aureus* and other bacteria **(Ballav et al., 2014).** Hypersaline environments are being occupied by members of one of the domain prokaryotes bacteria **(Gad, 2017)**. Many of the hypersaline bacterial species are halophiles and most are moderately halophilic, proliferating best at salt concentrations up to 10-20% L^{-1}. However, some species can grow up to salt saturation **(Ventosa et al., 1998; Oren, 2002a).** The Great Salt Plain (GSP) is a terrestrial saline environments in North America which has sharp change in salinities from zero to saturation, the high surface temperature (~50ºC), desiccation, alkaline pH, unlimited intense UV irradiation, and freezing winter. These harsh and dynamic environmental stressors have

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pressed the selection of microbial structures to harbor unique microorganisms, survival mechanisms to cope with these extremism. Therefore, an extreme environment with an intermediate level of disturbance, is expected to harbor more microbial diversity. As a response to the current challenge of widespread antibiotic resistance, we have undertaken this investigation. To our knowledge, this is the first study to investigate the isolated heterotrophic bacteria from the GSP for bioactivities against two strains of hospital, and community acquired methicillin resistant *Staphylococcus aureus* respectively. We aimed to explore the bioactivity of bacteria (mesophiles, halophiles, and thermophiles) isolated from the GSP, Oklahoma, against hospital and community acquired methicillin resistant *Staphylococcus aureus*. Anti-methicillin resistant *Staphylococcus aureus* bioactivity was detected in **20.2%** of the **499** isolates. This study revealed the importance of hypersaline and extremophilic bacteria for bioactivity against, hospital and community-acquired methicillin resistance *Staphylococcus aureus* (MRSA).

Materials and Methods

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, towards Crystal Digging Area and Observation Tower. 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 deposits, except in the event of rainfall. Salt deposits are subject to sudden dilution by rainfall, which lowers the salinity of those areas. Saline system at the GSP is considered as a extreme environment, therefor, it is expected the rainfall produces dramatically unexpected changes in saline content of the flat soil **(Caton et al., 2009)**. So, 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 salinity concentrations. The maximum annual rainfall is 115.3 cm, with 78.6 cm falling in 2009, and 11.2 cm falling in

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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 in June, December 2009 and June 2012 on a dry day. The sampling sites are located in regions poor in organic matter. A total of 24 unvegetated sampling sites (salt-crust sand layers) and 16 vegetated sampling sites (Rhizosphere) were obtained.

Sample Collection

Forty soil samples were collected including 24 unvegetated salt flat sites and 16 vegetated rhizosphere sites of the GSP. Sampling was performed in June, December 2009 and June 2012. Samples were collected from the top of soils (12-15 cm). The soil samples (700-900 g) were collected with sterile Petri dishes and hand spades and put in sterile bags. 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 allowed to dry for 2 weeks at room temperature. 100 g of each sample was maintained in sterile tubes and preserved in -20ºC.

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Isolation of mesophilic halotolerant bacteria

10.0 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 provided with different sets of salinity (0%, 5%, 10% and 15%). All agar media were prepared and autoclaved at 121ºC for 20 minutes. The isolation media consisted of the following; **Starch Nitrate Agar**, soluble starch 20.0 g, KNO3 2.0 g, K2HPO4 1.0 g, MgSO4 0.5 g, CaCO3 3.0 g, FeSO4 0.01 g, 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, KNO3 2.0 g, NaCl 2.0 g, KH2PO4 2.0 g, MgSO4 7 H2O 0.5 g, NaCl 2.0 g; CaCO3 0.02 g, FeSO4 7 H2O 0.01 g, agar 22.0 g, and sterilized water 1 l; **Yeast Malt Extract Agar-**

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ISP-2, yeast extract 4.0 g, Malt extract 10.0 g, dextrose 4.0 g, agar 22.0 g and sterilized water 1 l; and **Tryptic Soy Agar (TSA)** 30.0 g, and sterilized water 1 L. 15 µg naldixic acid was added to the sterile media to inhibit the growth of the fast growing bacteria. Inoculated plates were then incubated at 30ºC for 3 weeks. All well-separated, developed bacterial colonies, observed by naked eye or under light microscope at a magnification power of x30 were, therefore picked up from the isolation plates and transferred to **TSA** plates with the corresponding salt concentration. Isolated colonies were purified using streak plate method. The purified cultures were cryopreserved in autoclaved 30% glycerol vials at -80°C.

Enrichment techniques and isolation of extremophiles

A total of 40 soil samples were served for enrichment process to recover halophilic and thermophilic bacteria, three different techniques were applied; **1)** To select the **halophilic** bacteria, 20.0 g of each soil samples was placed in Petri dish, then each sample maintained moistening with 10% sterile saline solution if needed and incubated at 30 ºC for 3 weeks. **2)** To enhance the growth of **thermophilic** bacteria, 20.0 g of each soil samples was placed in Petri dish, then maintained moistening with sterile water and at 55ºC for 3 weeks. **3)** To select the **halophilic-thermophilic** bacteria, 20.0 g of each soil samples was placed in Petri dish, then allowed to be to moistened with a sterile saline solution (10%) and incubated at 55ºC for 3 weeks.

Isolation procedures were performed by dilution plate method on **Tryptic soy agar plates**, 10.0 g of enrichment 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. Serial dilution was performed and 10^{-3} dilution was selected for plating. 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. Agar media were previously prepared and autoclaved at 121ºC for 20 minutes. Inoculated plates of the halophilic group were provided with NaCl (10%) then incubated at 30ºC for up to 2 weeks. **Thermophilic group** inoculated plates were wrapped with aluminum foil, and incubated at 55 ºC for 7 days. **Thermophilic-Halophilic group** inoculated plates were provided with NaCl (10%), wrapped with aluminum foil and incubated at 55 ºC for 7 days. All well-separated, developed bacterial colonies, observed by naked eye or under light microscope at a magnification power of up to x30 were therefore picked up from the isolation plates and transferred to tryptic soy agar medium with the corresponding isolation conditions .

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Isolated colonies were purified using streak plate method. The purified cultures were cryopreserved in autoclaved 30% glycerol vials at -80°C.

Nucleic acid extraction

Genomic DNA was prepared from bacterial isolates using the single cell lysing buffer (SCLB) technique **(Marmur, 1961)**.

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, USA), 1 µl (25 p mol) 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 3130xl 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

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read by utilizing the ABI 3130xl (Applied Biosystems).

Plating of bacterial isolates on agar media

A total of **499** bacterial isolates were obtained. Out of them **202** mesophilic halotolerant bacterial isolates and **297** bacterial isolates were obtained by enrichment techniques. **499** bacterial isolates were inoculated onto **Starch glycerol nitrate agar** (**SGNA**; 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, agar 22.0 g, 10% NaCl in case of the halophilic strain, and water 1000 mL) plates by uniformly spread method. The plates were incubated at 30°C and 55°C for mesophilic and thermophilic respectively for 7 days.

Production medium and screening of antibiotic activities using well diffusion method

The antimicrobial bioactivities of a total of **499** were screened against two methicillin resistance strains of *Staphylococcus aureus,* (*Staphylococcus aureus* B-8-41-D-4 and *Staphylococcus aureus* 4656), using agar well diffusion method. A total of **499** bacterial isolates were inoculated in 30 ml of Starch Glycerol Nitrate broth SGN (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 (mesophilic) /or 55°C (thermophilic) at 200 rpm for 5 days. The fermented broth was centrifuged at 10,000 rpm for 5 mints. The culture filtrates were examined for antimicrobial bioactivities using well diffusion method. A pure colony of test organisms (methicillin resistant *Staphylococcus aureus* MRSA-C3 and methicillin resistant *Staphylococcus aureus* MRSA-A4656) was transferred into fresh Muller-Hinton agar (Merck), spread uniformly through the entire media, and incubated at 37°C for 24 hours until the visible growth appeared. Late exponential phase of a pure colony of the test organisms of methicillin resistant *Staphylococcus aureus* 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

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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 **(Bennet et al., 1966; Perez et al., 1990)** were made and 50 µL of the bacterial filtrate was added per well, incubated at 37°C for 2 days, then the bacterial activities were estimated by measuring the diameter of inhibition zone (mm) on the surface of plates and the results were reported.

Results

Classification and the anti-methicillin resistant *Staphylococcus aureus* screening of the GPS bacteria

A total of **499** isolates were recovered from **40** soil samples (**16** vegetated samples and **24** salt flat samples). Streak plate method was performed at 30° C and produced **202** halotolerant isolates. Eighty **(40%)** strains out of **202** strains showed anti-methicillin *Staphylococcus aureus* bioactivity. Eleven **(9%)** isolates out of **125** isolates of the thermophiles group (isolated by enrichment at 55°C) showed bioactivity against anti-methicillin *Staphylococcus aureus* and ten isolates **(12%)** out of **84** isolates of the halophilic group (isolated by enrichment at 10% NaCl) exhibited anti-methicillin *Staphylococcus aureus* bioactivity. The **88** isolates of the thermophile halophile group (isolated by enrichment at 10% NaCl, and 55°C) showed no anti-methicillin *Staphylococcus aureus* bioactivity **Fig. 1**. As regards to the enrichment bacterial isolates, **twenty one (7%)** isolates out of the **297** enriched strains showed anti-methicillin *Staphylococcus aureus* bioactivity. Out of **499** bacterial isolates, the **101** heterotrophic bacterial isolates **(20.2%)** (mesophilic halotolerant bacteria and enrichment ones) showed activities against at least one methicillin *Staphylococcus aureus* using well diffusion technique **Fig. 2, Fig. 4** and **Table 1**. In regard to biogeographical distribution, a total of **29 (28.7%)** bioactive bacterial isolates were isolated from vegetation areas, and **73 (71.2%)** bioactive bacterial isolates were isolated from salt flat areas. **Thirty eight** bacterial **(37.6%)** isolates were revealed bioactivity against both anti-methicillin *Staphylococcus aureus* strains, and **fifty six (55.4%)** bacterial isolates were shown antimicrobial bioactivity against methicillin *Staphylococcus aureus* B-8-41-D-4, however,

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thirteen (12.8%) isolates were revealed antagonism bioactivity against methicillin *Staphylococcus aureus* 4656. *Firmicutes* comprised the highest number of bioactive isolates **74 (73.2%)** including *Bacillus* (n=**37** isolates), *Halobacillus* (n=**13** isolates), *Virgibacillus* (n=**7** isolates), *Brevibacillus* (n=**2** isolates), *Paenibacillus* (n=**2** isolates), *Sediminibacillus* (n=**2** isolates), *Oceanobacillus* (n=**1** isolates), and *Staphylococcus* (n=**1** isolate). *Proteobacteria-Gammaproteobacteria* contained seven bioactive isolates **(7%)**, including *Halomonas* (n=**5** isolates), *Marinobacter* (n=**1** isolate), and *Pseudomonas* (n=**1** isolate). On the other hand, *actinobacteria* were the third group and harbored six bioactive isolates **(6%)**, including *Brevibacterium* (n=**4** isolates), *Cellulomonas* (n=**1** isolate), and *Micrococcus* (n=**1** isolates). Some bacterial isolates (n=**14** isolates) could not be sequenced or have bad sequences **Fig. 1**. The **12** strongest bioactive anti-methicillin resistant *Staphylococcus aureus* isolates were chosen to be represented and their phylogenetic assignment were shown in **Fig. 3** and **Table 2**.

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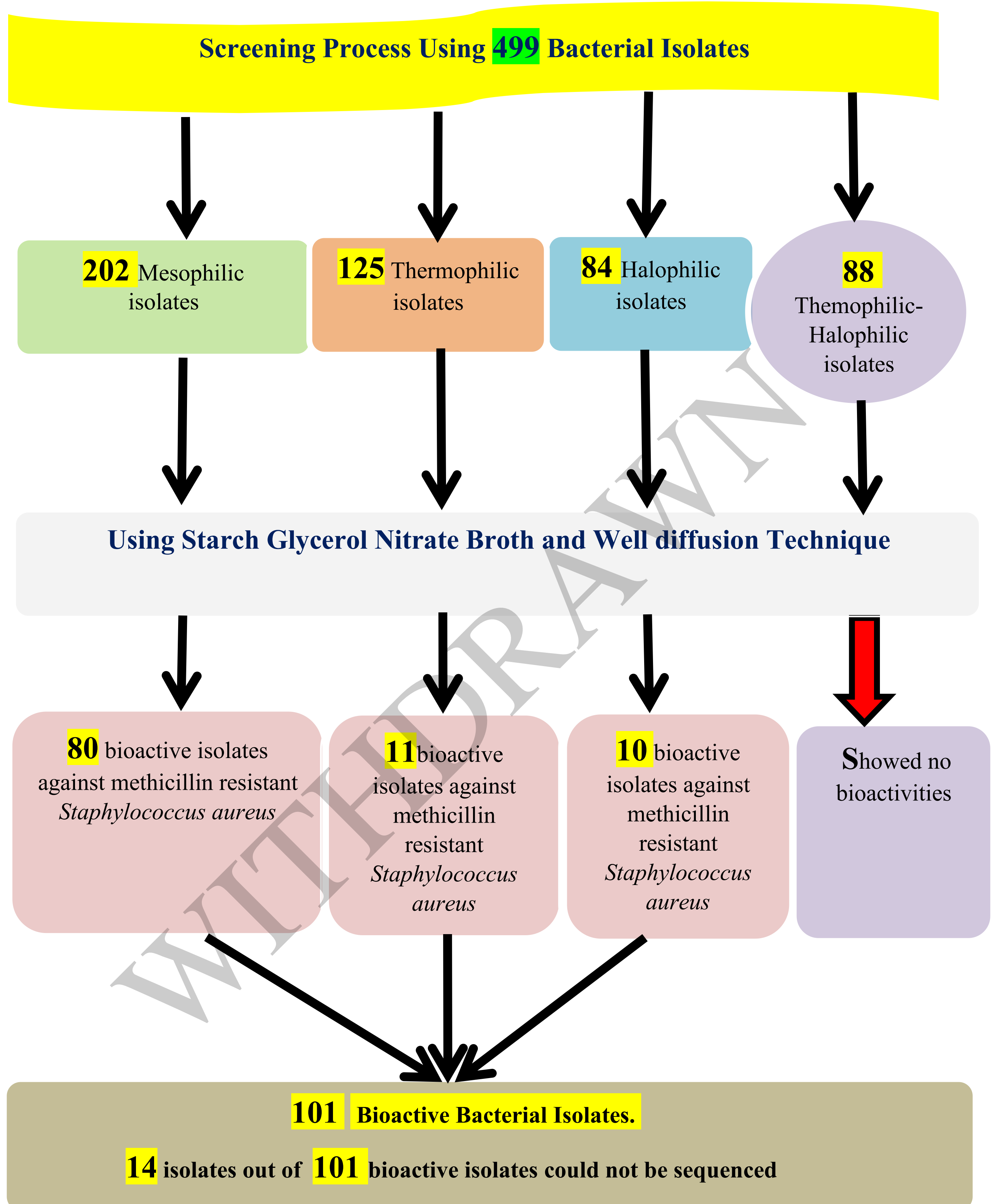

Figure 1. Flowchart process of bioactive screening of bacterial isolates obtained from the GSP soils, for anti-methicillin resistant *Staphylococcus aureus* compounds

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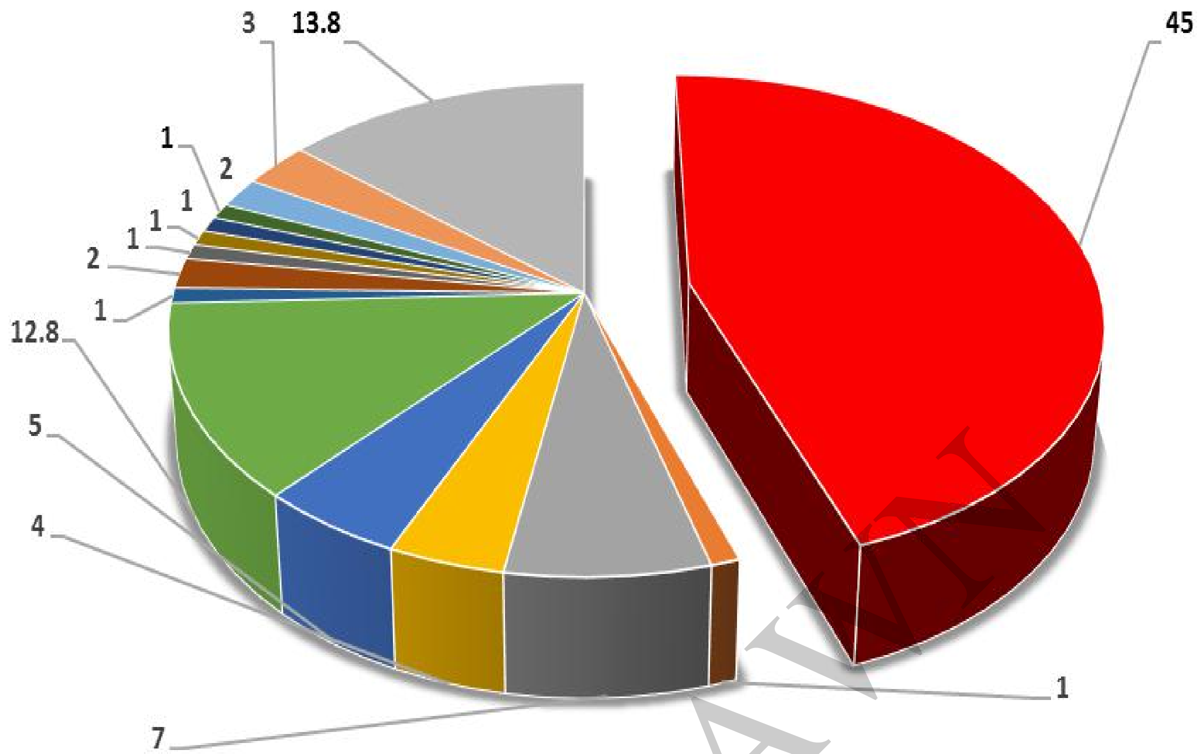

Figure 2. Frequency of bioactive bacterial genera obtained from the GSP soils, against methicillin resistant *Staphylococcus aureus* strains

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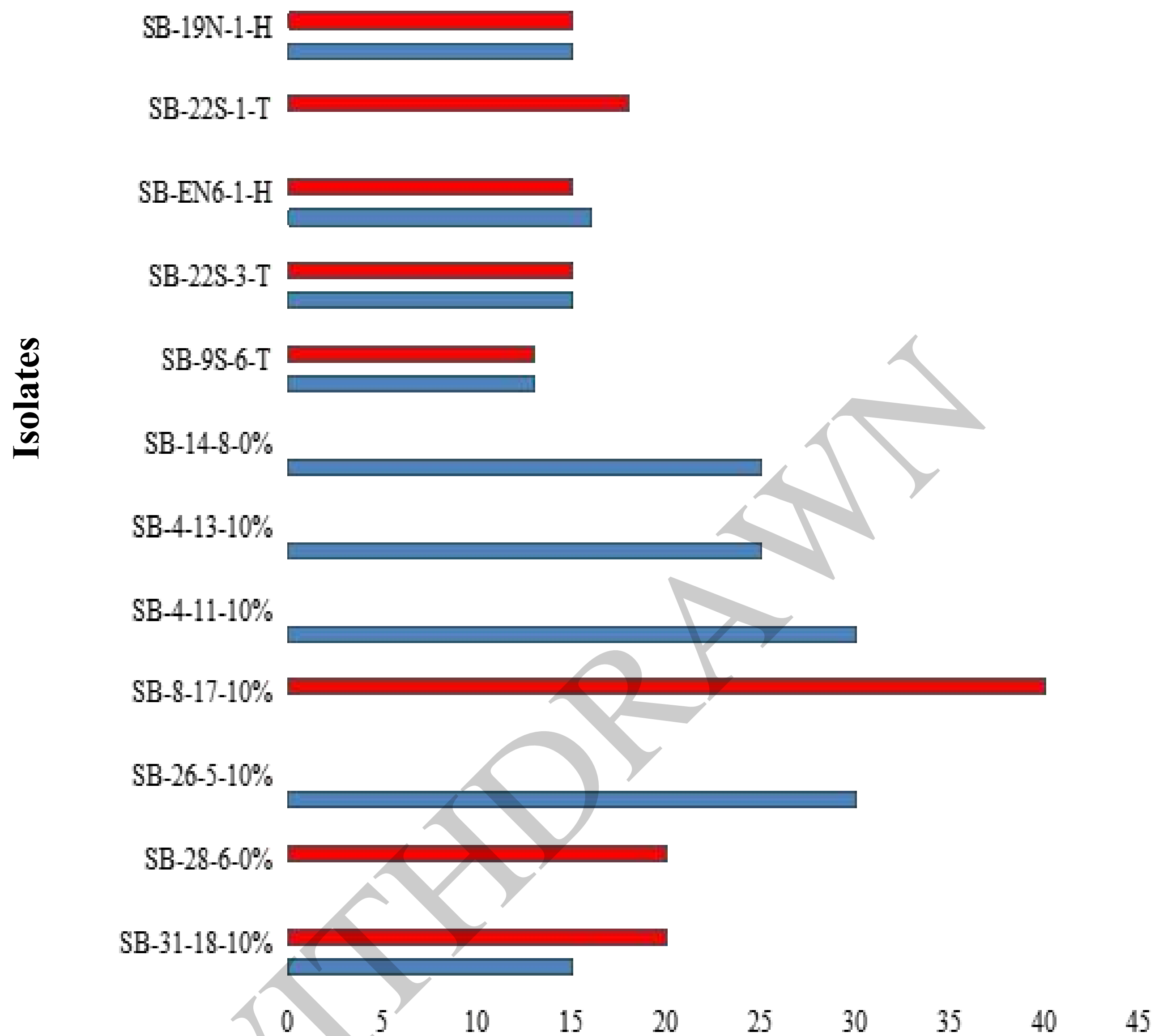

Figure 3. The highest 12-bioactive anti-methicillin resistant *Staphylococcus aureus* bacterial isolates, and their anti-MRSA compound representing in clear zones in mm

MRSA-B-8-41-D-4 MRSA-4656

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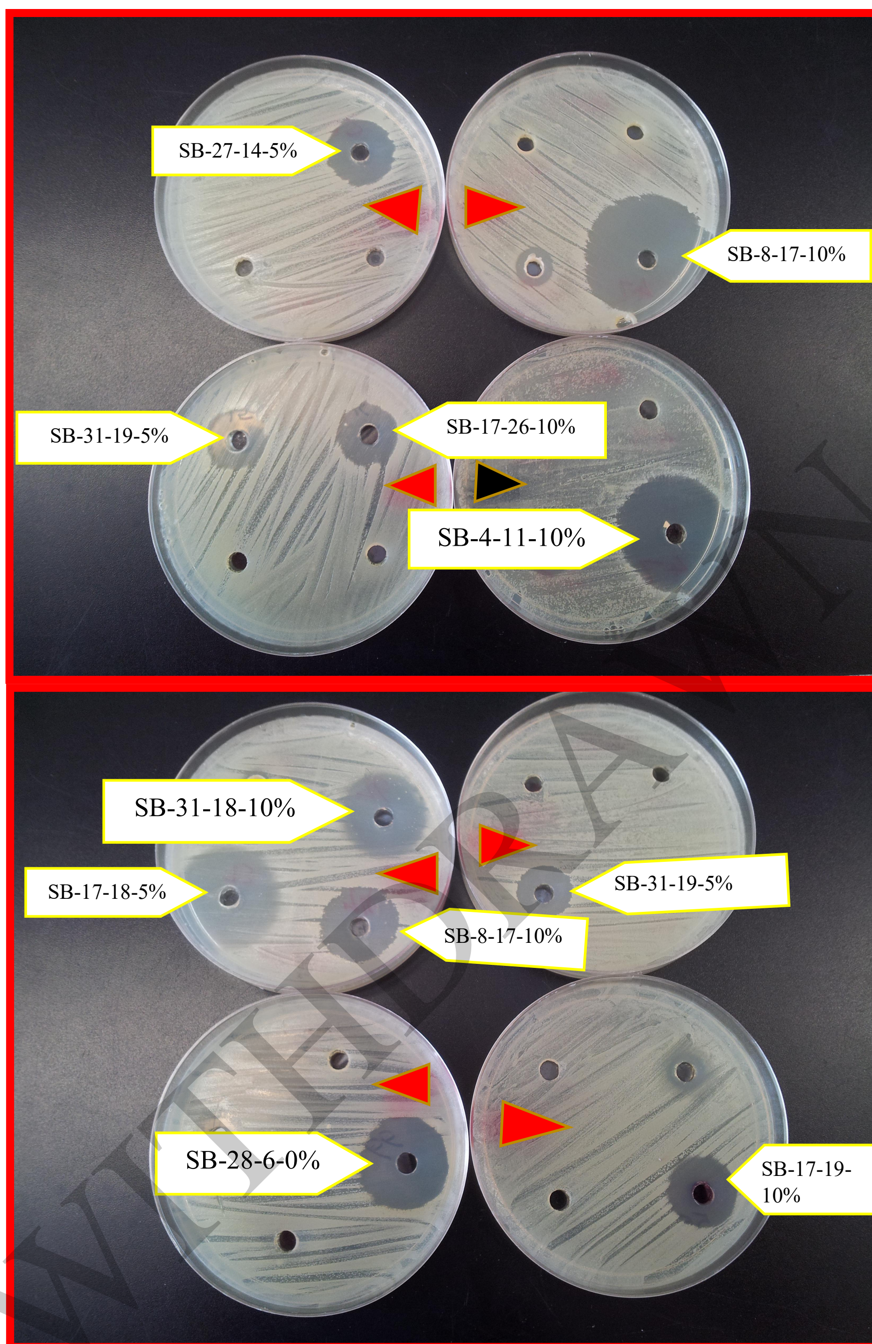

Figure 4. The anti-methicillin resistant *Staphylococcus aureus* bioactivities of some heterotrophic bacteria isolated from the GSP using well-plate technique, showing the clear zones. Red arrows indicate anti-MRSA bioactivity against MRSA B-8-41-D-4, and black arrow indicates anti-MRSA bioactivity against MRSA-4656

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Table 1. Taxonomic affiliation of the bioactive isolates from the GSP soils, soil type and results of bioactivity against the test methicillin resistant *Staphylococcus aureus* strains in mm

Isolate	Sample	*Genus*	Phylum	Inhibition	Inhibition
SB-31-19-5%	Salt flat	*Staphylococcus*	Firmicutes	15	10
SB-26-9-5%	Vegetation	*Oceanobacillus*	Firmicutes	25	0
SB-12-12-0%	Salt flat	*Sediminibacillus*	Firmicutes	15	0
SB-N7-1-H	Salt flat	*Sediminibacillus*	Firmicutes	9	9
SB-31-18-10%	Salt flat	*Virgibacillus*	Firmicutes	15	20
SB-27-18-10%	Vegetation	*Virgibacillus*	Firmicutes	18	13
SB-28-7-10%	Salt flat	*Virgibacillus*	Firmicutes	13.5	8
SB-4-13-10%	Salt flat	*Virgibacillus*	Firmicutes	25	0
SB-25-9-10%	Vegetation	*Virgibacillus*	Firmicutes	11.5	0
SB-28-11-10%	Salt flat	*Virgibacillus*	Firmicutes	18	14
SB-28-8-10%	Salt flat	*Virgibacillus*	Firmicutes	16.5	15
SB-13-17-5%	Salt flat	*Brevibacillus*	Firmicutes	20	8
SB-1-13-0%	Salt flat	*Brevibacillus*	Firmicutes	15	0
SB-15-11-0%	Salt flat	*Brevibacillus*	Firmicutes	11	0
SB-29S-1-T	Salt flat	*Brevibacillus*	Firmicutes	10	11
SB-19N-2-T	Vegetation	*Brevibacillus*	Firmicutes	9	9
SB-21S-3-T	Vegetation	*Brevibacillus*	Firmicutes	9	10
SB-17-27-0%	Salt flat	*Brevibacillus*	Firmicutes	0	10
SB-28-5-0%	Salt flat	*Halobacillus*	Firmicutes	13	8
SB-26-5-10%	Vegetation	*Halobacillus*	Firmicutes	30	0
SB-29-10-10%	Salt flat	*Halobacillus*	Firmicutes	10	0
SB-14-10-10%	Salt flat	*Halobacillus*	Firmicutes	15	0
SB-14-15-10%	Salt flat	*Halobacillus*	Firmicutes	20	0
SB-21-13-10%	Vegetation	*Halobacillus*	Firmicutes	15	8.5
SB-24-5-10%	Vegetation	*Halobacillus*	Firmicutes	20	0
SB-EN6-1-H	Vegetation	*Halobacillus*	Firmicutes	16	15
SB-N5-1-H	Salt flat	*Halobacillus*	Firmicutes	0	9
SB-8S-2-H	Salt flat	*Halobacillus*	Firmicutes	12	11
SB-EN9-1-H	Salt flat	*Halobacillus*	Firmicutes	10	10
SB-13-13-10%	Salt flat	*Halobacillus*	Firmicutes	13	0
SB-13-12-10%	Salt flat	*Halobacillus*	Firmicutes	11.5	0
SB-13-12-10%	Salt flat	*Halobacillus*	Firmicutes	11.5	0
SB-31-12-5%	Salt flat	*Bacillus*	Firmicutes	11.5	0
SB-17-18-5%	Salt flat	*Bacillus*	Firmicutes	0	20

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SB-17-24-10%	Salt flat	*Bacillus*	Firmicutes	23	9.5
SB-17-21-0%	Salt flat	*Bacillus*	Firmicutes	0	10.5
SB-17-22-5%	Salt flat	*Bacillus*	Firmicutes	0	10
SB-17-19-10%	Salt flat	*Bacillus*	Firmicutes	0	12.5
SB-13-11-0%	Salt flat	*Bacillus*	Firmicutes	20	9
SB-13-18-5%	Salt flat	*Bacillus*	Firmicutes	11.5	11
SB-13-10-0%	Salt flat	*Bacillus*	Firmicutes	15	0
SB-13-6-5%	Salt flat	*Bacillus*	Firmicutes	18	10
SB-28-4-0%	Salt flat	*Bacillus*	Firmicutes	9	0
SB-28-6-0%	Salt flat	*Bacillus*	Firmicutes	0	20
SB-28-6-2-0%	Salt flat	*Bacillus*	Firmicutes	0	17.5
SB-30-10-0%	Salt flat	*Bacillus*	Firmicutes	18	0
SB-30-15-5%	Salt flat	*Bacillus*	Firmicutes	14	0
SB-1-9-0%	Salt flat	*Bacillus*	Firmicutes	9	0
SB-1-14-0%	Salt flat	*Bacillus*	Firmicutes	15	0
SB-29-7-5%	Salt flat	*Bacillus*	Firmicutes	8.5	0
SB-29-6-0%	Salt flat	*Bacillus*	Firmicutes	10	0
SB-14-8-0%	Salt flat	*Bacillus*	Firmicutes	25	0
SB-14-11-10%	Salt flat	*Bacillus*	Firmicutes	16	0
SB-3-6-5%	Salt flat	*Bacillus*	Firmicutes	25	0
SB-3-11-5%	Salt flat	*Bacillus*	Firmicutes	11	0
SB-3-5-0%	Salt flat	*Bacillus*	Firmicutes	25	0
SB-3-9-0%	Salt flat	*Bacillus*	Firmicutes	13	0
SB-3-4-5%	Salt flat	*Bacillus*	Firmicutes	20	0
SB-5-4-10%	Salt flat	*Bacillus*	Firmicutes	11.5	9.5
SB-28-4-0%	Salt flat	*Bacillus*	Firmicutes	12	0
SB-7-3-0%	Salt flat	*Bacillus*	Firmicutes	14	0
SB-11-13-0%	Salt flat	*Bacillus*	Firmicutes	15	0
SB-11-8-5%	Salt flat	*Bacillus*	Firmicutes	13.5	0
SB-3-9-0%	Salt flat	*Bacillus*	Firmicutes	20	0
SB-7-1-0%	Salt flat	*Bacillus*	Firmicutes	11	0
SB-28S-2-T	Salt flat	*Bacillus*	Firmicutes	11	11
SB-EN4-3-T	Vegetation	*Bacillus*	Firmicutes	9	11
SB-EN7-1-T	Vegetation	*Bacillus*	Firmicutes	11	12
SB-22S-3-T	Vegetation	*Bacillus*	Firmicutes	15	15
SB-EN2-2-H	Vegetation	*Bacillus*	Firmicutes	0	9
SB-13S-1-H	Salt flat	*Bacillus*	Firmicutes	0	9

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SB-EN7-2-H	Vegetation	*Bacillus*	Firmicutes	14	0
SB-19N-1-H	Vegetation	*Bacillus*	Firmicutes	15	15
SB-4-7-0%	Salt flat	*Bacillus*	Firmicutes	11	0
SB-4-11-10%	Salt flat	*Bacillus*	Firmicutes	30	0
SB-4-12-5%	Salt flat	*Bacillus*	Firmicutes	20	0
SB-28-10-10%	Salt flat	*Bacillus*	Firmicutes	20	14
SB-3-10-0%	Salt flat	*Paenibacillus*	Firmicutes	13	0
SB-27-20-10%	Vegetation	*Marinococcus*	Firmicutes	20	0
SB-27-15-10%	Vegetation	*Halomonas*	Proteobacteria	18.5	0
SB-27-14-5%	Vegetation	*Halomonas*	Proteobacteria	18.5	13
SB-21-12-10%	Vegetation	*Halomonas*	Proteobacteria	20	0
SB-24-12-10%	Vegetation	*Halomonas*	Proteobacteria	10	0
SB-EN1-1-H	Vegetation	*Halomonas*	Proteobacteria	10	10
SB-3-12-1-0%	Salt flat	*Pseudomonas*	Proteobacteria	14	0
SB-24-4-5%	Vegetation	*Marinobacter*	Proteobacteria	15	0
SB-24-14-5%	Vegetation	*Cellulomonas*	Actinobacteri	11.5	0
SB-3-8-0%	Salt flat	*Micrococcus*	Actinobacteri	15	0
SB-17-26-10%	Salt flat	Bacterial isolate		0	11
SB-30-8-0%	Salt flat	Bacterial isolate		14	0
SB-12-16-5%	Salt flat	Bacterial isolate		22.5	14
SB-12-11-0%	Salt flat	Bacterial isolate		21.5	15
SB-19-10-0%	Vegetation	Bacterial isolate		0	16.5
SB-19-14-10%	Vegetation	Bacterial isolate		25	0
SB-8-17-10%	Salt flat	Bacterial isolate		0	40
SB-8-18-0%	Salt flat	Bacterial isolate		25	0
SB-24-11-10%	Vegetation	Bacterial isolate		25	0
SB-24-8-5%	Vegetation	Bacterial isolate		25	0
SB-9S-6-T	Salt flat	Bacterial isolate		13	13
SB-EN8-1-T	Salt flat	Bacterial isolate		9	9
SB-EN4-1-T	Vegetation	Bacterial isolate		12	10
SB-22S-1-T	Vegetation	Bacterial isolate		0	18

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Table 2. The highest 12 bioactive anti-methicillin resistant *Staphylococcus aureus* bacterial isolates, their phylogenetic assignments, and clear zones in mm

Isolate	Soil Type	Closest strain; Accession number	Similarity	MRSA-4656 (inhibition zone in mm)	MRSA-B-8-41-D-4 (inhibition zone in mm)
SB-31-18-10%	Salt Plain	*Virgibacillus marismortui; NT-6; EU095647*	97%	15	20
SB-28-6-0%	Salt Plain	*Bacillus subtilis; AMM202; AB092795*	100%	0	20
SB-26-5-10%	Vegetation	*Halobacillus sp. SB115_1; EU308338*	98%	30	0
SB-8-17-10%	Salt Plain	Bacterial isolate		0	40
SB-4-11-10%	Salt Plain	*Bacillus licheniformis; C32; DQ153970*	100%	30	0
SB-4-13-10%	Salt Plain	*Virgibacillus salarius; AN-R37; AB523705*	99%	25	0
SB-14-8-0%	Salt Plain	*Bacillus subtilis; D-39-25-2; AB190126*	100%	25	0
SB-9S-6-T	Salt flat	Bacterial isolate		13	13
SB-22S-3-T	Vegetation	*Bacillus licheniformis strain OKF02; gb KC969075.1*	99%	15	15
SB-EN6-1-H	Vegetation	*Halobacillus trueperi strain GSP062; gb DQ157162.1*	99%	16	15
SB-22S-1-T	Vegetation	Bacterial isolate		0	18
SB-19N-1-H	Vegetation	*Bacillus sp. Zh168.gb|FJ851424.1|*	99%	15	15

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Discussion

Natural bioactive compounds have been used since the beginning era in traditional medicine **(Donnelly, 2006)**. They are produced by the secondary metabolism of the microorganisms. These natural products include antibacterial compounds that can be used in treatment. The search for new drugs, novel antibiotics are in urgent demand due to the increase rate of multidrug resistant microorganisms to the current antibiotics. The **40** soil samples of the GSP allowed the isolation of large number of heterotrophic bacteria (n=**499** isolates). A total of **499** bacterial isolates were used for anti-methicillin *Staphylococcus aureus* MRSA screening using fermentation technique in a 50 mL Erlenmeyer flask. Bacterial isolates were fermented in starch glycerol nitrate broth, and the obtained filtrations were screened for bioactivity against the tested methicillin resistant *Staphylococcus aureus* strains. Of the total **499** bacterial filtrations, **20.2%** (**101** isolates) showed bioactivity. Out of the mesophilic halotolerant isolates (n=**80** bioactive isolates), a total of **17** bioactive bacteria were recovered from the vegetation regions, and mainly belonging to the following genera; *Halomonas*, *Virgibacillus*, *Marinococcus*, *Halobacillus*, *Oceanobacillus*, *Cellulomonas*, and *Marinobacter*. The enrichment bacterial isolates were as follows: **7** bioactive bacterial isolates related to the thermophilic group, **5** bioactive bacterial isolates of the halophilic group and were belonged to *Bacillus, Brevibacillus, Halobacillus, Sediminibacillus* and *Halomonas* and were recovered from the salt flat regions. A total of **29** **(29%)** bacterial isolates out of the **101** bioactive bacterial isolates were isolated from vegetation areas. Using 16S rRNA gene analysis for the bioactive isolates **Table 1 and Table 2**, **seventy seven** isolates of Firmicutes **(77%)** belonging to the genera *Bacillus* **45%** (n=**45** isolates), *Halobacillus* **13%** (n=**13** isolates)*, Virgibacillus* **7%** (n=**7** isolates)*, Oceanobacillus* **1%** (n=**1** isolate), *Brevibacillus* **7%** (n=**7** isolates)*, Sediminibacillus* **2%** (n=**2** isolates), *Staphylococcus* **1%** (n=**1** isolate) and *Paenibacillus* **1%** (n=**1** isolates). **Two** bacterial isolates of Actinobacteria **2%** (n=**2** isolates) belonging to the genera *Cellulomonas* **1%** (n=**1** isolate) and *Micrococcus* **1%** (n=**1** isolate). **Seven** bacterial isolates of Proteobacteria (Class: Gammaproteobacteria) **(7%)** belonging to the genera; *Halomonas* **5%** (n=**5** isolates)*, Marinobacter* **1%** (n=**1** isolate) and *Pseudomonas* **1%** (n=**1** isolate). In addition, non-sequenced bacterial isolates **14%** (n=**14** isolates) showed anti-MRSA bioactivity against one or both of the used test organisms. A total of **52 (52%)** isolates were active against the hospital acquired strain *Staphylococcus aureus* MRSA-4656 strain, **14 (14%)** bacterial isolates were active against the community acquired strain

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Staphylococcus aureus MRSA-B-8-41-d-4 and **34 (34%)** bacterial isolates were active against both methicillin resistant *Staphylococcus aureus* strains. **Table 1** shows the taxonomic affiliation of all bioactive isolates, and the correlation between the taxonomic position and their bioactivity against the test microorganisms representing by clear zone diameter. A higher number of bacterial isolates belonging to Firmicutes demonstrated bioactivity against methicillin resistant *Staphylococcus aureus* strains, followed by the Proteobacteria and then Actinobacteria. Interestingly to our findings, since 1997 to 2008, most of the new marine bacterial compounds were mainly produced by Actinobacteria (40%), Proteobacteria (12%), Firmicutes (5%), and others **(Williams, 2009)**. Moreover, our study revealed that bacterial isolates belonging to the genera; *Marinococcus, Oceanobaillus, Cellulomonas, Marinobacter, Micrococcus, Pseudomonas*, and *Paenibacillus* showed anti-methicillin resistant *Staphylococcus aureus* bioactivities against only the hospital-acquired *Staphylococcus aureus* MRSA-4656. However, there was not a clear cut correlation between the isolates species and its bioactivity against the test microorganisms. A suite of antimicrobial antibiotics was observed in members of *Bacillus* spp. from marine environment (**Muscholl-Silberhorn, 2008**). Furthermore, members of *Bacillus* spp. were displayed activities against set of fouling bacteria, some luminescent *Vibrio, Photobacterium* and a group of pathogenic bacteria **(Kanagasabhapathy et al., 2008)**. Members of *Bacillus* spp. **(Romanenko, 2008**) revealed antimicrobial bioactivities against *Staphylococcus aureus, Bacillus subtilis, Candida albicans, Xanthomonas sp.* pv. Badrii*, Enterococcus faecium, Aspergillus niger, Fuzarium oxysporum* and *Citricoccus sp.* High antimicrobial potentiality against *Staphylococcus aureus, Klebsiella pneumonia, Streptococcus pyogenes*, *Salmonella typhi*, *Trichophyton rubrum*, *Aspergillus niger, Aspergillus flavus* and *Fusarium* sp. by halophilic isolate of *Halomonas* species was reported **(Donio et al., 2013)**. **Velmurugan et al., (2013)** revealed that the halophilic isolate of *Halomonas salifodinae* MPM-TC was recovered from solar salt and showed antibacterial bioactivity against *Vibrio harveyi, Vibrio parahaemolyticus, Pseudomonas aeuginosa* and *Aeromonas hydrophila.* Medical substances were affiliated to marine *Pseudomonas spp.* (**Isnansetyo and Kamei 2009)**. **Wratten et al., (1977)** isolated a marine antibiotic-producing *Pseudomonas* sp. 102-3 with bioactivity against *Vibrio anguillarum, Vibrio harveyi, Staphylococcus aureus* and *Candida albicns*. Bioactive compounds produced by marine *Pseudomonas* species against *Bacillus subtilis, Escherichia coli* and *Candida albicans* were reported **(Anand, 2006). Santos (2010**) showed antimicrobial bioactivity by *Bacillus* spp.

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against vancomycin-resistant *Enterococcus faecium*. Variable antimicrobial activities of the gram positive strain *Micrococcus* against *Escherichia coli* was described by **(Hentschel et al., 2001)**. Marine isolates of *Paenibacillus* were reported showing activities against indicator strains in a study conducted by **(Romanenko et al., 2008). Tupinamba et al., (2008)** found antimicrobial bioactivity of *Paenibacillus polymyxa* SCE2 against *Micrococcus* sp. and *Aspergillus versicolor. Staphylococcus* sp. isolated from marine origin exhibited antimicrobial bioactivity against *Bacillus subtillus* and *Candida albicans* **(Anand, 2006)**. Antimicrobial metabolites of *Virgibacillus* sp. were reported against *Botrytis cinerea* **(Essghaier et al., 2014)**. **Sulistiyani et al. (2010)** reported two marine strains of *Virgibacillus* spp. showed antimicrobial bioactivity to inhibit the growth of multidrug resistant *Staphylococcus aureus*. *Halobacillus* of marine origin showed capability to produce antimicrobial cyclopeptides **(Yang et al., 2002)**. Furthermore, *Halobacillus* strain obtained from a sea grass sample revealed secondary metabolites to inhibit quorum sensing and bioluminescence production by *Vibrio harveyi.* Two *Halobacillus* metabolites inhibited quorum sensing-regulated violacein biosynthesis by *Chromobacterium violaceum* CV026 and green fluorescent protein production by *Escherichia coli* JB525 **(Teasdale et al., 2009)**. *Oceanobacillus picturae* secondary metabolites exhibited antimicrobial bioactivity against *Fuzarium sp.* **(Pakpitcharoen et al., 2008)**. *Oceanobaclillus* sp BRI 10 isolated from antarctic sea water showed antimicrobial bioactivity of a biosurfactant against *Escherichia coli* **(Jadhav et al., 2013)**. *Marinobacter* bacteria was reported as a producer of bioactive substances against *Collectotrichum gloeosporioides* 40003 and *Alternaria alternara* 42131 **(Irshad et al., 2013)**. **Anand et al. (2006)** showed antibacterial bioactivity of *Marinobacter* isolate against *Bacillus subtillus* and *Escherichia coli.* A suit of actinobacteria isolates harbored and clearly showed anti-MRSA bioactivities **(Gad 2017b)**. In our study, *Sediminibacillus* and *Cellulomonas* revealed bioactivity at least against one of the test methicillin resistant *Staphylococcus aureus* strains, but we did not find a related references or citations for antimicrobial production by either *Sediminibacillus* or *Cellulomonas.* Our findings are promising, clearly 20.2% of the isolates exhibited remarkable anti-methicillin *staphylococcus aureus* bioactivity and several isolates showed specificity to either one or the other of the test MRSA. Several studies indicated the predominance of gram-negative producers in the marine environment **Fenical, (1993)**. Moreover, antimicrobial production by marine bacteria was reported that 36 % of the strains were Gram-negative rods **(Bernen et al., 1997)**. Contrarily to the previous studies, our findings showed that

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20.2% out of the total **499** isolates were bioactive against MRSA. Interestingly, starch glycerol nitrate broth as a production media revealed more bioactive mesophilic halotolerant bacteria than the enrichment isolates. Supposedly, the stressed conditions which applied for enrichment isolation deactivated or did not reveal more bioactive isolates. We found it was unrealistic to compare our results with previous studies, due to the fermentation conditions plays a direct role for microbial metabolites production **(Bode et al. 2002)**, moreover, growth condition and growth media have a great impact on the production of biologically active compounds and also depending on the microbial strains **(Chen et al. 2000)**. Thus, it is apparent that fermentation medium (SGNB) used may not be providing the nutrients and anti-MRSA production conditions to induce anti-MRSA production in many of the GSP isolates **(Learn-Han et al 2014)**. Our study declared hypersaline gram-positive bacteria were more bioactive against methicillin *staphylococcus aureus*. This is possibly related to a broad bacterial community observed in the GSP soils using a metagenomic study **(Gad 2017a)**. Therefor some bacterial members have the ability to produce bioactive metabolites continuously when they are in contact with the target microbe (**Sánchez-Hidalgo et al. 2012)**. This is the first report to investigate the bioactivities of anti-methicillin *staphylococcus aureus* bioactivities by heterotrophic bacteria recovered from the GPS, and belonging to these genera; *Bacillus, Halobacillus, Virgibacillus, Paenibacillus, Brevibacillus, Sediminibacillus, Oceanobacillus, Staphylococcus, Halomonas, Marinobacter, Pseudomonas, Cellulomonas* and *Micrococcus*. It is worth to mention that the results of isolation of the heterotrophic bacteria showed that **31** bacterial isolates were recovered from isolation media provided with 0% salinity, **21** bacterial isolates were recovered from isolation media provided with 5% salinity and **29** bacterial isolates were obtained from isolation media provided with 10% salinity. The **12** strongest isolates which exhibited inhibition effect against MRSA and their taxonomic assignments were shown in **Table 2**. Moreover, **Fig. 3** illustrated the inhibition zones inn mm of some of them. **Seven** strains of mesophilic isolates were belonging to *Virgibacillus*, *Bacillus*, and *Halobacillus* genera. However, 5 isolates of extremophiles were belonging to *Bacillus* and *Halobacillus* genera. In our screening study, starch glycerol nitrate broth was served as a fermentation medium for Anti-methicillin resistant *Staphylococcus aureus* substances production. We highlighted the importance of extreme environments represented an endless reservoir for isolation of Anti-methicillin resistant *Staphylococcus aureus* compounds by bacterial isolates, which are potential sources for discovery of antimicrobial compounds.

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suggesting the broad distribution of antimicrobials biosynthetic pathway among the different lineages of the phyla; Firmicutes, Proteobacteria, and Actinobacteria.

Conclusion

This study performed a robust screening for **anti-methicillin resistant** *Staphylococcus aureus* potential of hypersaline bacteria isolated from the Great Salt Plain **GSP** of Oklahoma, United States. The substantial findings of this study confirmed the importance of bacteria isolated from genera such as *Bacillus, Halobacillus, Virgibacillus, Paenibacillus, Brevibacillus, Sediminibacillus, Oceanobacillus, Staphylococcus, Halomonas, Marinobacter, Pseudomonas, Cellulomonas* and *Micrococcus.* Our study demonstrated the first association of genera *Sediminibacillus* and *Cellulomonas* with anti-MRSA bioactivity. Starch glycerol nitrate broth remarkably served for the production of **anti-methicillin resistant** *Staphylococcus aureus* compounds. However, this study highlights the need for employing multiple culture conditions in antibacterial screening assays of GSP associated bacteria. Extreme environments are a valuable source to produce potent antimicrobial compounds which could be important in future drug discovery programs.

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