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Elucidation of antibacterial activity of *Bacillus* sp. and *Alcaligenes* sp. metabolites against multidrug-resistant bacteria

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Abstract

Different resistance mechanisms are involved in exhibiting resistance to different groups of antibiotics. Researchers are searching for new therapeutic options to encounter the emerging trend of microbial resistance. Bacteria were isolated from the extreme environment of Cholistan Desert and were screened for characterization. Potential metabolites that showed broad-spectrum activity were partially purified using silica gel chromatography and determined their minimum inhibitory concentration. A collection of 50 bacterial isolates from soil samples was screened for metabolite production and among them isolate R19 of *Bacillus* sp. and isolate A8 of *Alcaligenes* sp. had high similarity with strong antimicrobial metabolite producers. The growth of A8 was stable at slight acidic pH while R19 was best at neutral pH. Similarly, the best growth of A8 was observed at 37 °C while R19 at 35 °C. Minimum inhibitory concentration of purified compounds of *Bacillus* sp. were determined at concentration range of (3.12 – 100 %) against multidrug-resistant (MDR) strains of *Shigella*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia* and produced 10 – 25 mm zone of inhibition. Metabolites of *Alcaligenes* sp. were sufficient to inhibit the growth of all selected MDR bacteria at concentrations 12.25 – 100 % and shows 10 – 20 mm zone of inhibition. *Bacillus* sp. and *Alcaligenes* sp. can be used as producers of potential antibacterial metabolites. Proper utilization of selected metabolites can be helpful in combating emerging drug resistant pathogenic bacteria. In addition, further proteomic analysis and structural insight should be considered to elaborate their active ingredients and its efficacy.

Introduction

Microbes are potential sources of different types of important active metabolites. In search of potential metabolites producer microbial strains, several studies have been conducted to explore different habitats for potential metabolites producer microbes. It can be deduced from different studies that microbes of diverse environments carrying strong antimicrobial potential according to Andayani *et al.* (2015), observed by others (Masand *et al.* 2018; Rajasabapathy *et al.* 2020). Microbial metabolites due to their increasing applications have allure the attention of scientists to

explore different untouched sites for potential metabolites producer microbes Kumar *et al*. (2018). Metabolites have various therapeutic applications in the treatment and eradication of different types of bacterial, fungal and parasitic diseases Demain (1999). In pre-antibiotic era, the treatment of infectious diseases was challenging due to unavailability of antibiotics Adedeji (2016). The discovery and commercialization of penicillin give new hope because morbidity and mortality associated with infectious diseases were reduced in infected population. Unfortunately, with passage of time resistant strains like emergence of methicillin Resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* and multidrug resistant (MDR) strains of gram negative bacteria were reported worldwide particularly in developing world Hesse and Adhya (2019). World Health Organization estimates that annually millions of people lost their lives due to drug-resistant bacterial strains. According to Shrivastava *et al*. (2018) the World Health Organization enlist priority pathogens for which new and effective drugs need to be explored having bactericidal activity. Among these pathogens, carbapenem resistant strains are global threats Le Thanh Dong and Espinoza (2020). Carbapenems drugs are considered the last choice for treatment of MDR bacteria. Recently, few extreme drug resistant (XDR) strains of *Salmonella* sp. were isolated from infected patients in Pakistan, which revealed resistance to most of the available drugs including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, and ceftriaxone while fortunately sensitive to azithromycin and carbapenems Rasheed *et al*. (2019). In relation to the resistance, the induction of new effective drugs rate is low as compared to previously approved drugs that lose their efficacy Ribeiro da Cunha *et al*. (2019). To tackle antibiotic resistance issue, scientists are trying to explore new avenues for the isolation of potential metabolite producer microbes having strong inhibitory potential.

Bacillus a gram positive spore forming bacteria, having potential to cope harsh environment has been frequently isolated from extreme habitats Nicholson *et al*. (2000). Their secondary metabolites exhibit strong antibacterial activity and many of their compounds have been commercialized to use as antibiotics such as bacteriocins Morikawa *et al*. (1992). Beside *Bacillus*, actinomycetes, fungi are also sources of antibiotics Singh *et al*. (2019). In the current scenario, researchers demonstrate key interest in bacterial characterization from the unexplored harsh environment like deserts and marine with the hope to explore strong bioactive metabolites producer strains according to Abdelkader *et al*. (2018); observed by Cita *et al*. (2017). Indeed, by 2008, more than 100 specialized metabolites had been isolated and identified from microorganisms isolated from such habitats**.** In addition, in the previous decade, 129 putative strains, producing 186 drug gable metabolites, were isolated from extreme strata Sayed *et al*. (2020).

The current study was conducted for the exploration of antibacterial metabolites producer bacterial strains from the unexplored habitat of Cholistan Desert (Punjab, Pakistan) with harsh environmental conditions like high temperature, scarcity of nutrients and water depletion.

Experimental

Collection and isolation of bacteria from soil samples

Samples from different locations of Cholistan Desert were collected in sterile bottles using standard procedure as previously described by Elbendary *et al*. (2018). All samples were brought to the Medical Laboratory, Department of Microbiology, KUST and were stored at -80 °C for further analysis. All samples were serially diluted up to 10^{-9} in sterilized distilled water. Aliquot of 100 µL was spread from last three dilution on tryptone soya agar (TSA) and nutrient agar (NA) and plates were incubated for 48 h at 37 °C. After incubation different colonies appeared on different culture media.

Resistant bacterial strains profile

MDR bacterial strains of *P. aeruginosa, E. coli, E. aerogenes, K. pneumonia, S. aureus*, and *Shigella* sp. were obtained from medical laboratory, Department of Microbiology, Kohat University of Science and Technology. To assess the

antibacterial potential of broth extract of metabolite producer strains, its inhibitory effects were screened against the resistant pathogens. All the selected bacterial strains revealed resistance toward different antibiotics like penicillin, cephalothin, tetracycline, amoxicillin, cefoperazone, ceftriaxone, and cefepime based on CLSI guidelines.

Evaluation of Antibacterial activity of cell-free extract

Isolated bacteria were grown in Luria Bertani broth (LB) for 5 to 7 days in shaking incubator at 170 rpm and 30 °C. After incubation, centrifugation was performed, and supernatants of each broth were filtered. The cell-free filtrates were assayed for antibacterial activity using well diffusion assay Oskay (2011).

Growth optimization at different temperature and pH

The isolates were incubated in nutrients broth at various temperatures like 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, and 75 °C. Growth conditions were optimized at different pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. The results were recorded using spectrophotometer at 600 nm OD. For proper growth, pH of the media was adjusted according to bacterial needs as previously described Khalil *et al*. (2009).

Extraction of antibacterial metabolites

Bacterial isolates from desert soil samples (n = 50) were screened for their antibacterial metabolites production. Among these isolates, A8 and R19 exhibit broad-spectrum activity against MDR bacteria and were selected for further analysis. An equal amount of ethyl acetate was added to the extracted filtrate and then metabolites were concentrated using rotary evaporator. Bacterial metabolites were purified using silica gel column chromatography, chloroform/methanol in the ratio of (100 : 0, 80 : 20, 60 : 40, 50 : 50, 40 : 60, 20 : 80, 0 : 100) as an eluent. Further each fraction were concentrated using rotary evaporator Rao *et al*. (2007).

Minimum inhibitory concentration (MIC) determination of antibacterial metabolites

Activity of each fraction of selected metabolites was examined on Mueller Hinton agar (MHA) against drug resistant strains by using agar well diffusion process. Fractions of best restraining were selected for MIC determination against resistant pathogens. Active fractions (2 mL) were considered 100 % and were serially diluted into two-fold to make 50 %, 25 %, 12.5 %, 6.25 %, and 3.125 %, and concentration respectively. Further 100 µL from each concentration was used for the MIC determination.

Biochemical and molecular characterization of metabolites producer strains

Strong metabolites producer strains were biochemically characterized using standard procedures of (Rahimnahal *et al*. 2017; Marathe *et al*. 2018). For molecular characterization, DNA from the selected active antimicrobial metabolites producers strains were extracted by standard phenol chloroform method (Neumann *et al*. 1992) using overnight culture. PCR reactions were carried out for the amplification of 16S rRNA gene using universal primers forward primer 5'-AGAGTTTCCTGGCTCAG-3' and reverse primer 5'-AAGGAGGTGATCCAGCC-3' (Lim *et al*. 2016). PCR conditions were set for amplification of 16S rRNA gene. The thermal cycling reactions were performed as: Initial denaturation at 94 °C for 5 min, then 25 cycles of 94 °C for 1 min, 50 °C for 40 s and 72 °C for 60 s and final extension was 72 °C for 5 min.

The amplified products of PCR of the selected isolates were confirmed on 2 % agarose by adding 3.5 µL ethidium bromide as a staining dye and visualized by the digital gel doc system (Analytik Jena System, Jena, Germany). The products were subjected to sequencing and reactions were conducted at Macrogene (Seoul, South Korea) using the universal 16S rRNA forward primer. The resulted sequences were extracted using Bioedit software. NCBI BLAST analysis was performed for sequence alignment. Moreover, phylogenetic tree was constructed using MEGA 7 software.

Results

Antibacterial activities of A8 and R19 metabolites

Soil samples (n = 50) were screened for their antibacterial metabolites production and among these isolates A8 and R19 were expressed broad spectrum activity against resistant bacterial culture and were selected for further analysis. Morphologically isolate A8 stained gram-negative rod shape while R19 was identified as gram-positive rod shape and was also spore positive Fig. 1. Biochemically both isolates were catalase, oxidase, sugar fermenting, and motility recorded positive.

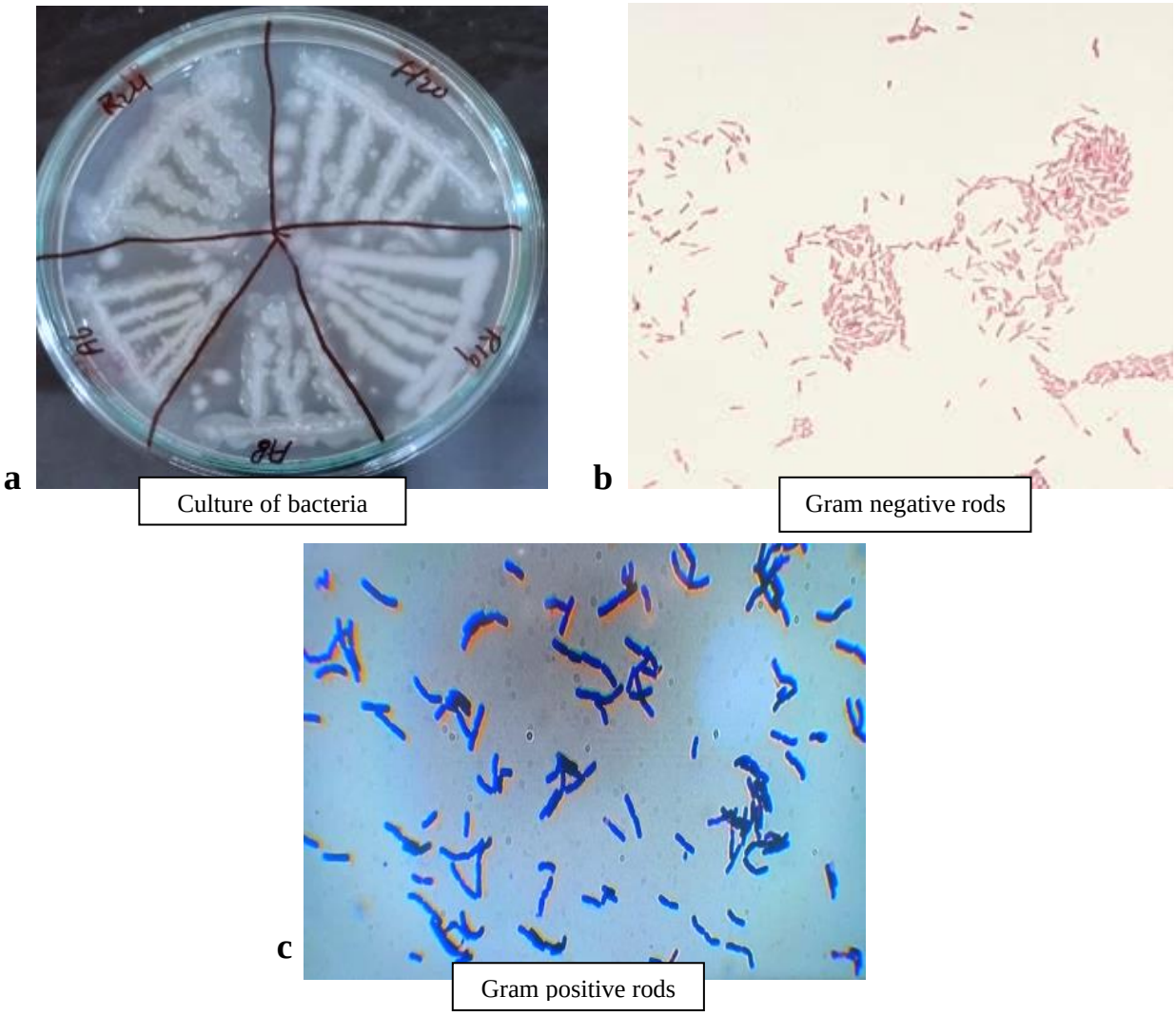

Fig. 1. Morphological study of the selected isolates from Cholistan Desert. **a** – culture of bacteria on nutrient agar; **b** – pink colour Shows gram negative bacteria; **c** – the blue colour shows gram positive bacteria.

Growth optimization of selected isolates on different temperature and pH

Best growth of selected isolate A8 was observed at 37 °C and lowest growth were observed at 15 °C. Similarly isolate R19 have shown maximum growth at temperature 35 °C as shown in Fig. 2a. Similarly, pH also has a huge impact on the bacterial growth. A8 shows maximum growth at neutral pH while R19 showed maximum activity at pH 9.0. Both isolates growth was restrained at acidic pH as shown Fig. 2b.

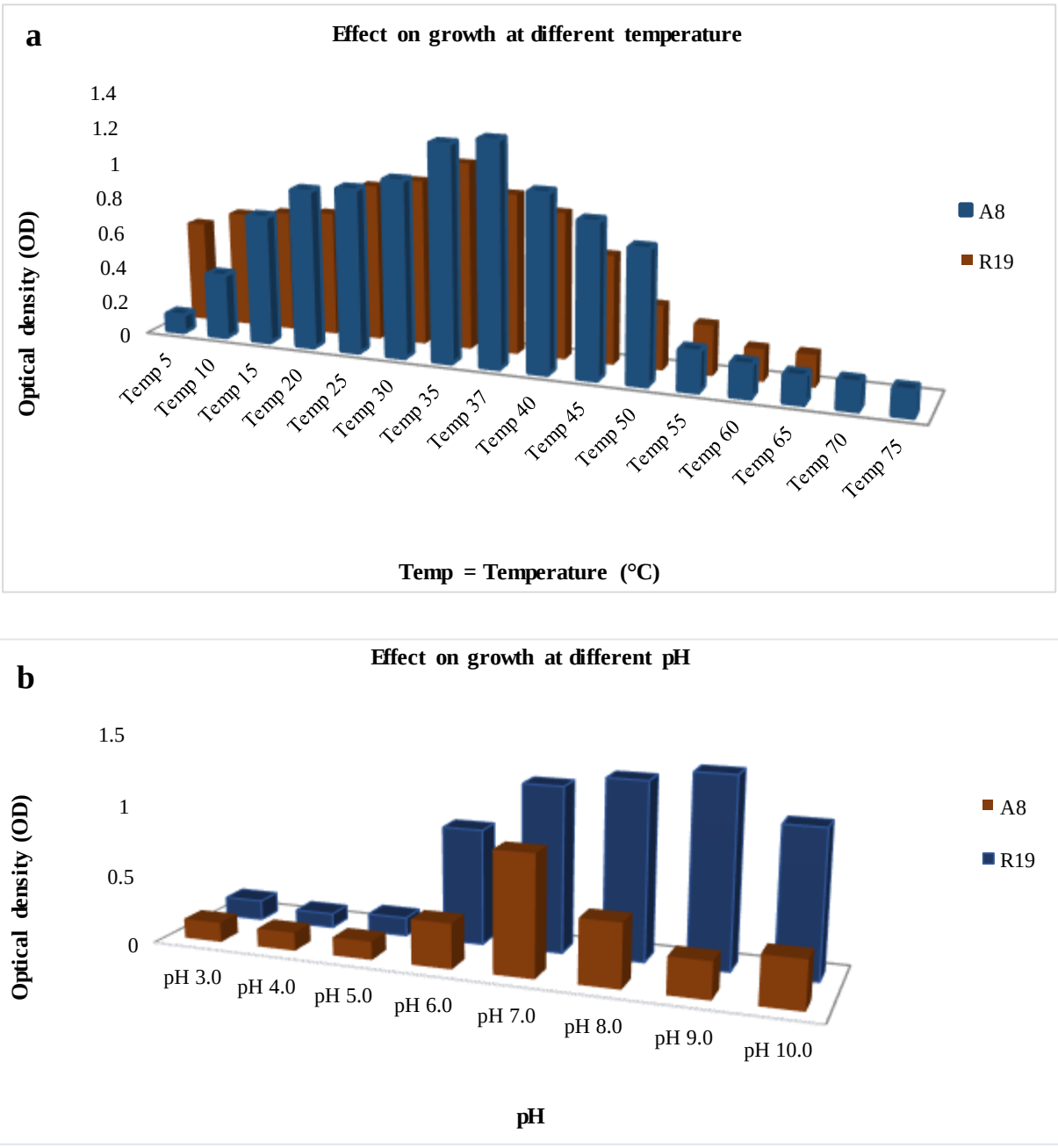

Fig. 2. Effects of temperature (**a**) and pH (**b**) on growth of isolates (A8 and R19).

MIC determination of isolate A8 collected through silica gel at different concentrations.

The metabolites activities of strain A8, after silica gel purification, were observed for antibacterial activity against *Shigella*, *S. aureus*, *E. aerogenes*, *P. aeruginosa*, *E. coli*, and *K. pneumonia* MDR strains respectively at different concentration as shown in Table 1 and Fig. 3. Isolate A8 metabolites has the maximum zone of inhibition against MDR *Shigella* and *S. aureus* of about 25 mm, 24 mm, respectively. Similarly, it has strong inhibitory activity toward *P. aeruginosa* and 19 mm zone of inhibition while in case of *E. aerogenes* 20 mm zone of inhibition was recorded. While less inhibitory activity was observed against *E. coli* and *K. pneumonia*.

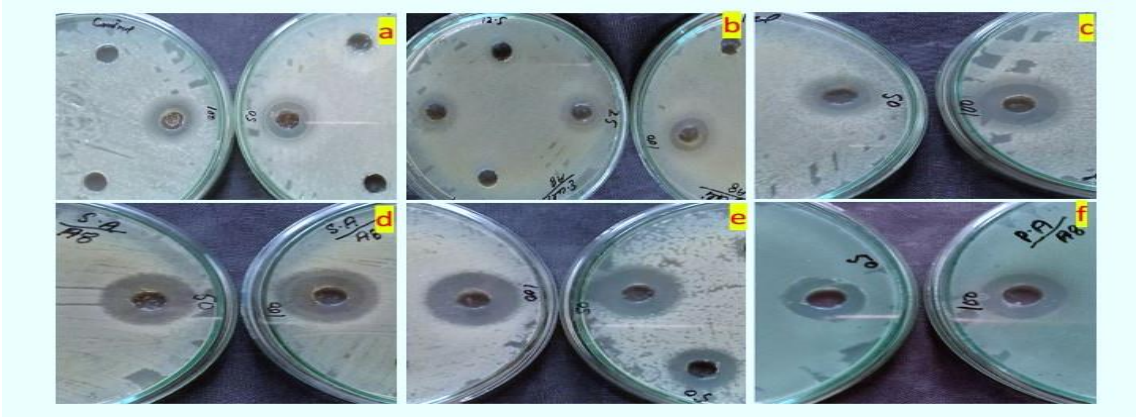

Fig. 3. Antibacterial activity of A8 isolate against (**a**) *Klebsiella pneumonia*, (**b**) *E. coli*, (**c**) *E. aerogenes*, (**d**) *S. aureus*, (**e**) *Shigella*, (**f**) *P. aeruginosa*.

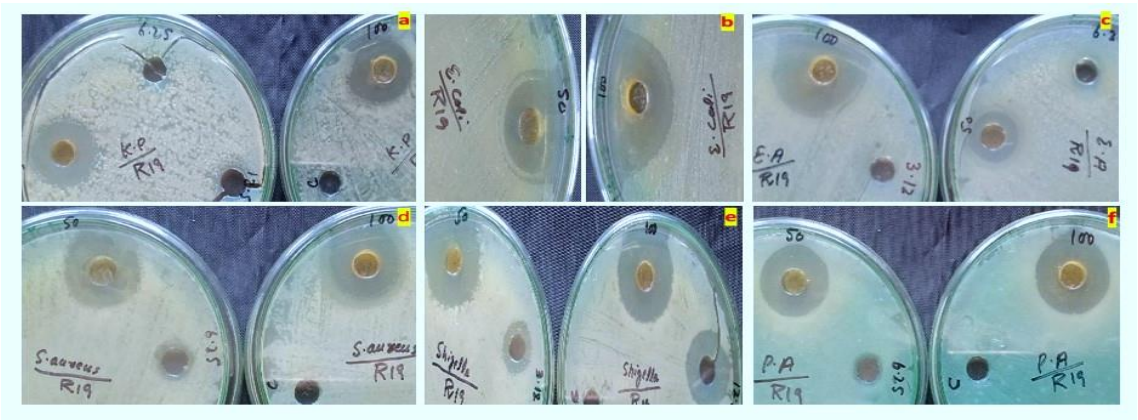

Fig. 4. Antibacterial activity of R19 isolate against (**a**) *K. pneumonia*, (**b**) *E. coli*, (**c**) *E. aerogenes*, (**d**) *S. aureus*, (**e**) *Shigella*, (**f**) *P. aeruginosa*.

Table 1. Inhibition zones of A8 metabolites against selected bacterial strains at different concentration.

Tested bacterial strains	**3.15 % [mm]**	**6.25 % [mm]**	**12.5 % [mm]**	**25 % [mm]**	**50 % [mm]**	**100 % [mm]**
Shigella	--	--	13	15	22	25
S. aureus	--	--	10	12	21	24
E. aerogenes	--	--	--	11	14	20
P. aeruginosa	--	--	10	15	17	19
E. coli	--	--	--	11	12	15
K. pneumonia	--	--	--	--	14	15

Antibacterial activity of R19 isolate

The antibacterial activity of R19 metabolite was observed against *Shigella*, *S. aureus*, *E. aerogenes*, *P. aeruginosa*, *E. coli*, and *K. pneumonia* MDR strains at different concentration as shown in (Table 2, Fig. 4). R19 bacterial metabolites were more effective against all selected resistant bacterial strains as compared to A8. It has shown a strong inhibitory pattern from lower to higher concentration against *Shigella* and *E. aerogenes*. At lower concentration (3.12 %) it produced 10 mm zone of inhibition while at higher concentration (100 %) it generated 19 mm and 20 mm zone of inhibition against selected MDR strains respectively. Similarly, it has strong inhibitory action against MDR *E. coli* at concentration (50, 100 %) and manifest 19 mm and 23 mm zones of inhibition toward selected strains. Further their action against other tested isolates including *K. pneumonia* and *P. aeruginosa* revealed 16 – 18 mm zone of inhibition at concentration (100 %) as shown in Table 2.

Table 2. Inhibition zones of R19 metabolites against selected bacterial strains at different concentration measured in diameter (mm).

Test bacterial strains	3.12 % [mm]	6.25 % [mm]	12.5 % [mm]	25 % [mm]	50 % [mm]	100 % [mm]
Shigella	10	13	15	16	18	19
S. aureus	--	8	9	12	16	20
E. aerogenes	10	11	12	13	14	20
P. aeruginosa	--	8	9	11	15	18
E. coli	--	5	6	10	19	23
K. pneumonia	--	--	--	10	15	16

Molecular characterization of A8 and R19 strains using 16S rRNA gene

Further identification of both A8 and R19 metabolite producer strains were characterized through PCR amplification of 16S rRNA gene, size 1399 bp, as shown in Fig. 5. Amplified product of A8 and R19 were sequenced at Macrogen (Seoul, South Korea) using forward primer. Homology analysis of both isolates were conducted at NCBI BLAST and there were 97.7 % identity with *Alcaligenes* sp. for A8 isolate and 98.3 % identity with *Bacillus* sp. for R19. Based on sequencing results, phylogenetic trees were constructed using MEGA7 for the selected isolates (A8 and R19) as shown in Fig. 6 and 7**.**

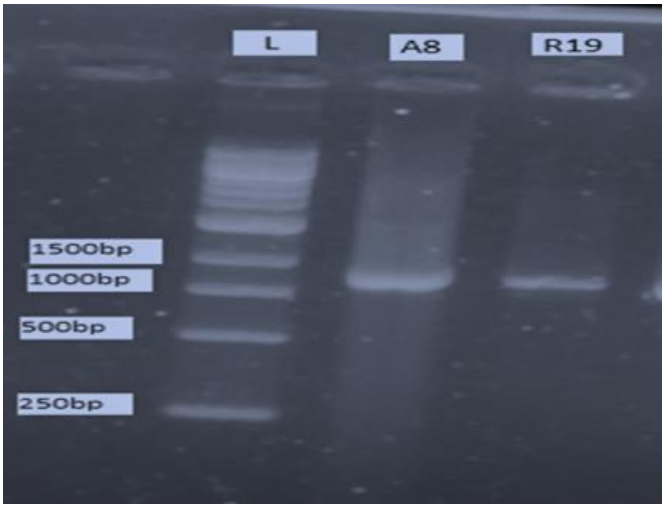

Fig. 5. Gel image of amplified product of 16S rRNA gene. Lane L shows ladder, lanes A8 and R19 bacterial isolates A8 and R19, respectively.

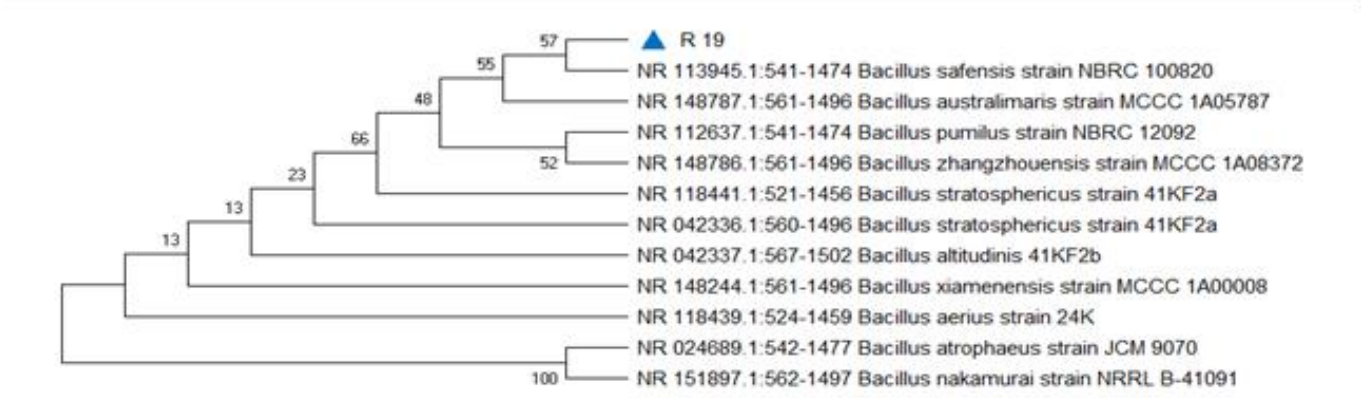

Fig. 6. R19 phylogenetic tree using MEGA7.

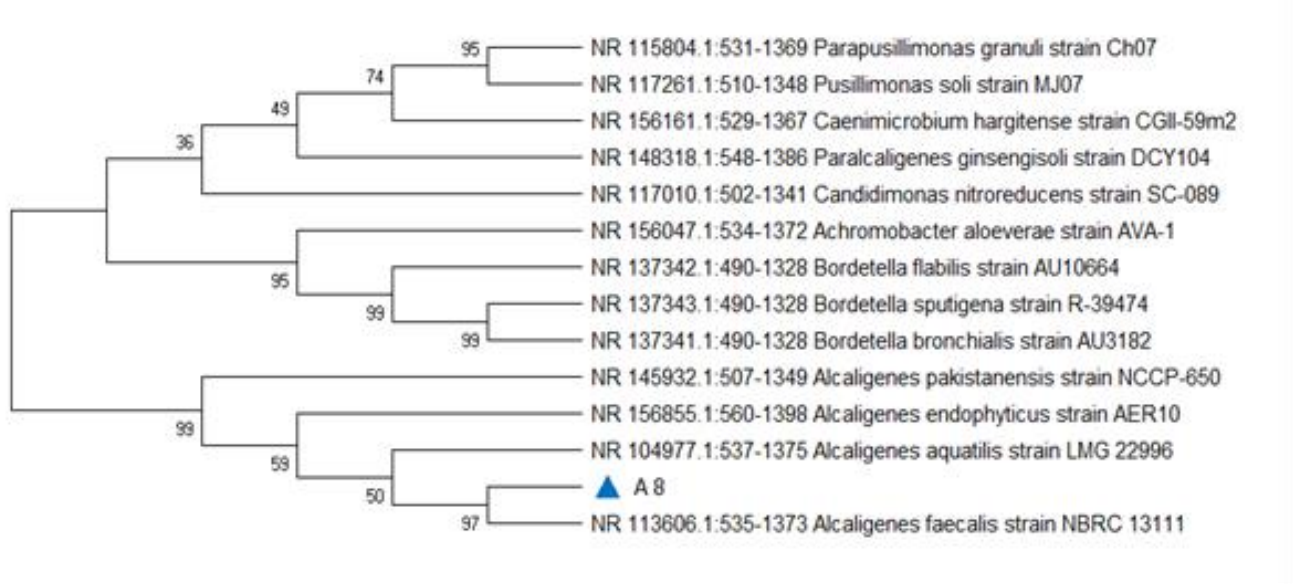

Fig. 7. A8 phylogenetic tree using MEGA.

Discussions

To combat extending bacterial resistance, exploration of new drugs is indispensable of a current scenario. Scientists are in search of new metabolites that have strong potential to address resistant strains. Soil harbour diverse microbial community which has potential to cherish strong antibacterial potential. Previously bacterial isolates particularly *Bacillus* sp. have been reported from different habitats having strong antibacterial activity. In recent study efforts were made to explore microbial community for potential of strong antibacterial compounds, from diverse environmental conditions. Deserts have diverse environmental conditions and have mixed microbial flora. Cholistan Desert of Pakistan has wide range of microbial community and have potential to cope with challenging environmental conditions Amin *et al*. (2020). In current study two isolates (A8 and R19) emerged as strong antibacterial metabolites producer strains that have been characterized based on ribotyping which revealed that A8 showed 97.74 % sequence similarity to *Alcaligenes* sp. and R19 unveil 98.33 % sequence similarity to *Bacillus* sp. Similar active producer strains have been screened from the unexplored unique habitat of mountain Himalaya which produced strong antibacterial metabolites according to Hussain *et al*. (2018). According to Das *et al*. (2018) study, streptomyces strains were isolated from protected forest land and most of these isolated strains carried broad spectrum antibiotic activity and were stable at 37 °C while some of the isolated strains were stable 42 °C.

A8 and R19 isolate showed broad spectrum activity, against MDR clinical bacterial strains. Maximum growth of A8 and R19 isolates were observed on normal nutrient media. Other growth conditions like temperature and pH were also analysed, isolates that the best grown at temperature 37 ℃ and the lowest below 20 ℃ have been recorded. Our result are in line with the study conducted by Bharali *et al*. (2011) in which the *Alcaligenes* sp. isolates growth was documented at 42 °C. A8 showed maximum growth at neutral pH while R19 was most stable at slight alkaline pH. These results confirmed previously reported by Farag *et al*. (2019) in which metabolites of *Bacillus* manifest promising activity was observed at slight alkaline pH. In recent development, growth of MDR strains of *Shigella* and *E. aerogenes* were restricted at concentration of 3.12 % of the partially purified compound of *Bacillus* sp. Similarly, for *S. aureus*, *P. aeruginosa* and *E. coli* were determined 6.25 % while 25 % for *K. pneumonia*. The partially purified metabolites of isolated strain of *Bacillus* sp. exhibited promising activity against gram negative bacteria and it has also excellent inhibitory activity against *S. pyogenes* MTCC 442, *B. cereus*, *E. faecalis*, and *S. epidermidis* ATCC 12228.

Similarly, Ramachandran *et al*. (2014) isolated *Bacillus* sp. from soil samples and found a strong producer of metabolites that showed strong effective against broad-spectrum microbes. The metabolites of *Alcaligenes* sp. manifest strong activity at lowest concentration (12.25 %) against *Shigella*, *S. aureus* and *P. aeruginosa* while at concentration (25 %) growth of *E. aerogenes* and *E. coli* were inhibited. A thermophilic strain of *Alcaligene* sp. was isolated from petroleum contaminated soil having potency of antibacterial activity against *Bacillus* sp., *K. pneumonia*, *E. coli*, *P. aeruginosa*, and *S. aureus* observed by Bharali *et al*. (2011). Similarly, isolated strains of *Alcaligenes* sp. were reported from common effluent treatment plant which demonstrated antibacterial activity against MDR *Enterobacter* sp. and *Serratia* sp. being supported by the production of active a nucleoside antibiotic according to Kapley *et al*. (2016). *Bacillus* sp. are predominantly soil bacteria and can be found in different other habitats that their existence in soil in huge numbers as a result of their capacity to form resistance endospores and develop bioactive compounds which promote their resistance to variable environmental conditions reported by Onajobi *et al*. (2020). A study reported by Abd Sharad *et al*. (2016) of the *Alcaligenes* sp. isolated from marine habitat has strong antibacterial activity at 3 $mg.mL^{-1}$ against resistant bacteria.

Conclusions

Metabolites of *Alcaligenes* sp. (A8) and *Bacillus* sp. (R19) have shown the maximum zone of inhibition against MDR clinical bacterial strains. The active fractions of *Alcaligenes* sp. metabolites revealed strong inhibitory effects against all resistant pathogens. This study provides initial layout for researchers and drug developers to further characterize active ingredients. Furthermore, its growth was optimized on the best pH and temperature that will be helpful in extraction and stability of metabolites. Proper utilization of selected metabolites can be helpful in combating emerging drug resistance mess in pathogenic bacteria. In addition, further proteomic analysis and structural insight should be considered to elaborate their active ingredients and its efficacy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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