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SCREENING AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM BACTERIA ISOLATED FROM MARINE SEDIMENTS OF KARACHI, PAKISTAN

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Abstract

This study explores marine actinomycetes from Karachi coastal sediments as potential sources of novel antimicrobial agents. Three isolates (K3.7N, K3.2S, and K2.4ES) were selected from marine sediment samples, characterized biochemically and morphologically, and identified through 16S rRNA gene sequencing as Alcaligenes sp. SV/09-02 (FJ654476.1) (2) and Bacillus sp. strain csj15 (KY970075.1), i.e., K3.7N and K3.2S, respectively, while the isolate K2.4ES could not be sequenced. Antibiotic susceptibility test results revealed that both K3.2S and K2.4ES were sensitive, whereas K3.7N was resistant. All three isolates tolerated 25 minutes of 254 nm UV irradiation and varying salt concentrations, though the UV did not affect growth or bioactivity. The metabolites displayed stable antibacterial activity up to 45°C even after Proteinase K treatment, indicating the non-proteinaceous nature. Antimicrobial production was affected by various growth parameters and the crude extracts of Alcaligenes sp. SV/09-0 and Bacillus sp. strain csj15 demonstrated significant antibacterial, antifungal, and anticandidal activity, with a bacteriostatic effect. These findings highlight the untapped potential of Karachi's marine ecosystems for novel antibiotic discovery.

Key Words: *Anticandidal, Antifungal, Antimicrobial, Crude extract, Metabolites, Proteinase K*

INTRODUCTION

Marine microorganisms are an exotic source for marvelous bioactive compounds possessing human utility potential (1). These microorganisms synthesize intricate chemicals with different biological characteristics for versatile applications in industrial and biotechnological sectors, where some of these organisms can live in hostile marine environmental conditions (2, 3). Therefore, different marine microorganisms, i.e., (microalgae, bacteria, fungi, and myxomycetes), have been explored previously that produce compounds exhibiting antiviral, antibacterial, antitumoral, antioxidant, and apoptotic activity (4).

Approximately 22,000 bioactive metabolites have been identified from the marine biota, where the microbe-derived bioactive compounds from the marine biosphere, comparatively, still remain an underexplored site of interest. Hence, the exploration of microorganisms for the production of novel bioactive metabolites continues to be a fascinating and emerging approach in advanced drug discovery programs (5).

The marine biosphere is nature's treasure of bacteria for drug discovery and biomedical research (6-8). Within marine microbiota, actinomycetes play a pivotal role because among naturally occurring antibiotics, about one-third are produced by Streptomyces through the process of secondary metabolism (9, 10).

Optimization of the culture and growth conditions of the bacteria can hone the fermentation profile, i.e., incubation period, inoculum size, pH of the media, and temperature (11, 12). Thus, optimum variables, i.e., physicochemical parameters are the paramount significant in enhancing the bioactive compounds production (13, 14).

Nowadays, antibiotic resistance is increasing with the passage of time and has become a global concern. Thus, not only did it affect millions of people, but also infectious diseases became difficult to treat (15-18). Therefore, it is an utmost essential to develop new antimicrobial agents to cope with the dilemma of antibiotic resistance. Currently, the investigation of potential antibiotics from the marine biosphere with minimal lethal effects and low cost has become indispensable biomedical research (19, 20). Marine environment fascinates researchers to unravel this underexplored site for novel metabolites, due to the versatile diversity, hostile conditions, and comparatively less proportion of actinomycetes in the marine biosphere in comparison to the terrestrial environment.

Throughout the years, vast chemical programs were established globally for bioactive metabolites production to determine their mechanism of action (21). The increasing requirement of antibiotics for combating evolving infections or superbugs resulted in oceans exploration (22). Therefore, countless struggles have been made targeting the isolation of novel bioactive metabolites from marine microbiota (23). The objective of this study was to screen marine actinomycetes from Karachi coastal sediments for the production of bioactive compounds with antimicrobial properties. Additionally, the study aimed to optimize growth conditions, characterize the bioactive metabolites, and identify the selected bacterial isolates at the genetic level.

MATERIALS AND METHODS

All chemicals, media, and solvents used in this study were of high analytical grade and were purchased from Merck, Hi-Media, and Sigma-Aldrich with a purity of 99%.

SAMPLE COLLECTION AND PROCESSING

Marine sediment samples were collected from Clifton beach, Karachi, Pakistan at coordinates 24.8132 °N, 67.0305 °E. The sediment was collected from a depth of approximately 10-15 cm using a sterile corer, placed in a sterile container, and immediately transported to the laboratory under cold conditions within 6 hours. Following serial dilution, sediment samples were processed on Nutrient Agar media.

PRELIMINARY SCREENING OF ISOLATES

A total of 42 distinct microbial isolates were obtained on morphological differences. Antibacterial activity of the isolates was checked out primarily through point inoculation method on Mueller Hinton Agar (MHA) (Merck, Germany) plates against different American Type Culture Collection (ATCC) strains. The obtained isolates were also checked against different fungal pathogens on Sabouraud dextrose agar (SDA) plates through point inoculation where the inhibitory zones were noted and measured after every 24 hours of incubation. All bacterial isolates were incubated aerobically at 30°C for 48 hours, while fungal isolates were incubated at 28°C for 5-7 days under aerobic conditions. Out of 42 isolates, three isolates (K3.7N, K3.2S and K2.4ES) were selected for further study based on their significant antimicrobial activity in preliminary screening. Preliminary screening of isolates was conducted in triplicate and results are expressed as mean ± standard deviation. Table I represents the bacterial and fungal strains used in this study.

Table I. Different bacterial ATCC and fungal strains used during the study

S. No.	Bacterial Pathogen	ATCC No.	Fungal Pathogen
1	*Staphylococcus aureus*	2593	*Rhizopus solani*
2	*Escherichia coli*	25922	*Aspergillus niger*
3	*Staphylococcus epidermidis*	12228	*Pythimum ultimum*
4	*Salmonella enterica*	14028	*Fusarium fujikuroi*
5	*Pseudomonas aeruginosa*	27853	*Rhizopus oryza*
6	*Bacillus spizizenii*	6633	*Candida albican*
7	*Enterococcus faecalis*	29212	*Candida krusei*
8	*Klebsiella pneumoniae*	clinical isolate	*Candida glabrata*

SECONDARY SCREENING OF ISOLATES

Secondary screening of the isolates was accomplished through agar well diffusion method on Mueller-Hinton Agar (MHA) (Merck, Germany) plates against different American Type Culture Collection (ATCC) strains. The inhibitory zones were measured after 24 hours of incubation at 37 °C (24). Secondary screening was also carried out against different fungal pathogens. The experiment was carried out in duplicate, and the mean zone of inhibition was measured.

PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF ISOLATES

Morphological determination of the isolates was done based on cultural characteristics (shape, size, color, and margins, etc.) and Gram-staining. Biochemical tests were conducted as per Berge's Manual of Systematic Bacteriology. Genomic DNA was extracted using the Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The phylogenetic tree was constructed according to protocols.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). The isolates were streaked on Mueller-Hinton Agar plates, and different broad-spectrum antibiotics discs (OXOID, UK) of different classes were placed with the help of a sterile syringe. Inhibitory zones were examined and noted after 24 hours of incubation at 37 °C. The experiment was carried out in triplicate and the results are expressed as mean ± standard deviation.

TOLERANCE OF ISOLATES TO SALTS AND UV LIGHT

The capability of isolates to tolerate different salts was checked by using different salts (NaCl, MgSO4.7H2O, CaCl2.2H2O, KH2PO4, MgCl2, and KCl) in different concentrations. Similarly, the potential of isolates to tolerate UV light was checked by exposing the streaked plates to UV light of 254nm wavelength in a UV chamber for different time intervals. The experiment was carried out in triplicate, and the results are expressed as mean ± standard deviation.

OPTIMIZATION

The three selected bacterial isolates were grown in Nutrient Broth, and their incubation period was checked for optimum growth and antimicrobial production by taking 1 mL of the sample in a glass cuvette for checking optical density (OD) at 654 nm wavelength on the y-axis and incubation period on the x-axis. The agar well diffusion method was used for antibacterial activity after every 24 hours of incubation. It will give an idea about the production of metabolites, the maximum production of antimicrobials, and the decline of metabolite production.

Other growth parameters, i.e., media, temperature, Inoculum size, and pH, were observed for maximum biomass and antimicrobial production. For maximum extraction of metabolites, liquid-liquid extraction was carried out in a separating funnel by using different solvents of different polarity indexes to check the solvent in which maximum extraction occurs.

EXTRACTION OF CRUDE METABOLITES IN OPTIMIZED SOLVENT

Butanol and n-hexane were used as optimized solvents for isolating K3.7N and K3.2S, respectively. The culture supernatant was extracted with Butanol and n-hexane, respectively in a 1:1 (v/v) ratio, shaken vigorously for 1 hour at room temperature, and left undisturbed for at least 3 hours.

GENERAL CHARACTERIZATION OF BIOACTIVE METABOLITES

Thermal stability was accomplished after centrifugation of the cell-free supernatant and kept at different temperatures, i.e., (0, 4, 15, 25, 30, 37, 45, and 60) °C for 1 hour. Antibacterial activity was determined through the agar well diffusion method (25).

The cell-free supernatant of isolates K3.7N and K3.2S was treated with Proteinase K (0.005 mL) and left undisturbed for 2 hours at room temperature i.e., 25°C. Antibacterial activity of the metabolites was checked before and after Proteinase K treatment through agar well diffusion method by taking Proteinase K and Distilled water as a Control. The experiment was carried out in triplicate and the results are expressed as mean ± standard deviation.

Antibacterial activity of the crude extract was examined by dissolving the metabolite in different concentrations per 1 mL of DMSO, where 100 µL was loaded in each sealed well. DMSO was taken as a negative control, and an antibiotic disc as a positive control. Inhibitory zones were measured after 24 hrs. of incubation. The experiment was performed in duplicate, and the mean zone of inhibition was measured.

Anticandidal activity of the crude extract was accomplished in duplicate through agar well diffusion assay on SDA plates by taking Nystatin as a positive control and DMSO as a negative control. Mean Inhibitory zones were noted and measured after every 24 hours of incubation at 25 °C.

Antifungal activity of the crude extract was carried out in duplicate by using agar well diffusion method on SDA plates by taking Nystatin as a positive control and DMSO as a negative control. Mean Inhibitory zones were noted and measured after every 24 hours of incubation at 25 °C (26).

In order to analyze the effect of bacterial crude extracts of both the isolates against pathogens, samples were taken from clear zones of inhibition through a sterile cotton swab and streaked on sterile Nutrient agar plates in duplicate and were subjected to incubation.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism (Version 8.0). Results are presented as mean ± SD. Statistical comparisons were made using one-way ANOVA followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS

From the collected isolates, three samples, K 3.7N, K3.2S, and K2.4 ES, were selected for further processing on the basis of the results of point inoculation in the present study.

PRIMARY AND SECONDARY SCREENING OF ISOLATES

The selected three isolates were subjected to primary screening against different ATCC bacterial strains, Phytopathogens, and candidal strains through the point inoculation method and are depicted in Fig. 1. A-D. These isolates were also subjected to secondary screening by agar well diffusion method, where the results are depicted in Table II.

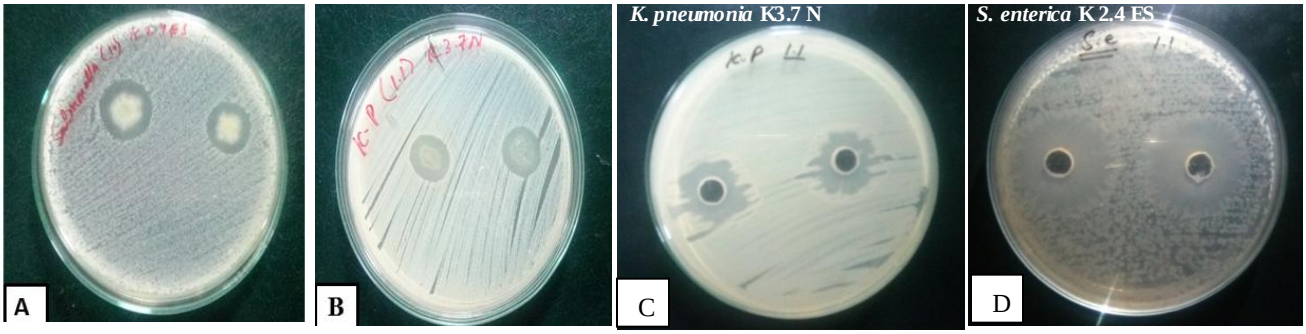

Fig. 1 (a & b). Antibacterial activity of isolates through point inoculation; **(c & d).** Secondary screening of supernatant through agar well diffusion method

Table II. Antibacterial activity profile (zone of inhibition in mm) of selected isolates through secondary screening

S. No.	Bacterial pathogen	Isolate K3.7N	Isolate K3.2S	Isolate K2.4ES
1	*Staphylococcus aureus*	27.3 ± 0.6^{a}	**26.8** ± 0.5^{a}	23.2 ± 0.4^{b}
2	*Escherichia coli*	20.5± 0.5^{a}	21.1 ± 0.6^{a}	10.3 ± 0.3^{b}
3	*Staphylococcus epidermidis*	30.2 ± 0.7^{a}	0.0 ± 0.0^{c}	23.5 ± 0.5^{b}
4	*Pseudomonas aeruginosa*	27.1 ± 0.4^{a}	15.4 ± 0.6^{b}	**30.3** ± 0.5^{c}
5	*Klebsiella pneumonia*	22.4 ± 0.5^{a}	17.2 ± 0.4^{b}	10.6 ± 0.3^{c}
6	*Salmonella enterica*	**30.6** ± 0.6^{a}	19.5 ± 0.5^{b}	17.3 ± 0.4^{c}

Values represent mean ± SD (n=3). Different superscript letters within a row indicate statistically significant differences ($p<0.05$)

MORPHOLOGICAL IDENTIFICATION OF ISOLATES

The morphological and biochemical profiles of isolates are depicted in Table III. An isolated colony of isolate K3.7N on Nutrient agar was found; orangish/greenish in color, circular in shape, elevated, without margins, punctate form, shiny colony, mucoid, and opaque. The isolate K3.2 S was observed as filamentous with whitish /brownish pigmentation, a flat, large, dry colony, opaque and without margins, whereas the isolate K2.4 ES was noted as circular, orangish, elevated, small, shiny, opaque, and mucoid.

Table III. Phenotypic characteristics of selected isolates

S. No.	Test name	K 3.7 N	K 3.2 S	K 2.4 ES
	Gram stain	**Gram -ve**	**Gram +ve**	**Gram +ve**
	Biochemical tests		**Result**	
1	Oxidase	+	+	+
2	Catalase	+	+	+
3	MR	-	-	-
4	VP	-	+	-
5	Indole	-	-	-
6	Citrate	+	-	-
7	Lactose fermentation	-	-	+
8	Nitrate Reduction	+	+	+
9	DNase	-	-	+
10	Aesculin	-	+	+
11	Acetate	+	-	+
12	Gelatinase	+	-	-
13	Acid Production for Defense	-	-	-
14	H_2S Production	+	-	-
15	Mannitol Fermentation	-	+	+
16	Urease	+	-	-
17	Amylase	-	+	-
18	Cellulase	+	+	-
19	Protease	+	+	+
20	Lipase	+	+	+

*Absence of activity indicated as (-), and presence of activity as (+)

OPTIMIZATION OF THE GROWTH PARAMETERS

The different tested growth parameters for maximum antimicrobial production of the selected isolates are shown in Table IV.

Table IV. Different tested growth parameters of the selected isolates

S. No.	Test Name	K 3.7 N	K 3.2 S	K 2.4 ES
	Temperature (°C)		**Antimicrobial production**	
1	25	+	+	+
2	30	++	+	++
3	35	+	++	+
4	40	+	+	+
5	50	-	-	-
	pH		**Antimicrobial production**	
1	5	++	+	-
2	6	+	++	-
3	7	+	+	++
4	8	+	+	+
5	9	-	+	+
6	10	-	+	+
	Media		**Antimicrobial production**	
1	NB	++	++	++
2	TSB	+	+	-
3	LB	+	+	-
4	ZMB	+	-	+
5	MSM	-	-	+

	Incubation period (hr.)	Antimicrobial production		
1	24	-	-	-
2	48	+	+	+
3	72	+	+	+
4	96	++	++	++
5	120	+	+	+
6	144	+	+	+
7	168	-	-	-
	Inoculum size (%)	**Antimicrobial production**		
1	0.5	+	+	+
2	1	+	+	+
3	3	+	+	+
4	5	++	++	+
5	7	+	+	++

*Absence of inhibitory zone indicated as (-), Moderate zone of inhibition (+), and Maximum zone of Inhibition (++)

POTENTIAL OF SELECTED ISOLATES

The potential tolerance of selected isolates to UV light of 254 nm wavelength and different salts is shown in the given Table V.

Table V. Potential of selected bacterial isolates to UV and salts

S. No.	UV light	Tolerance		
1	**UV**	**K 3.7 N**	**K 3.2 S**	**K 2.4 ES**
		> 25 mints	> 25 mints	> 25 mints
2	**Salt concentration**	**% Tolerance**		
i	NaCl	5	10	10
ii	$MgSO_4$	7	>12.5	>12.5
iii	$CaCl_2$	2.6	5	7
iv	KH_2PO_4	2	2	2
v	$MgCl_2$	3.5	>12.5	>12.5
vi	KCl	7	10	15

ANTIBIOTIC SUSCEPTIBILITY TESTING

Sensitivity of isolates against different classes of antibiotics was checked. The result of antibiotic susceptibility is tabulated in Table VI.

Table VI. Antibiogram of selected isolates

Different classes of antibiotics	Concentration (µg)	Isolate K3.7N
Amoxicillin- clavulanate	20/10	Resistant
Piperacillin –tazobactam	100/10	Resistant
Cefepime	30	Resistant
Cefotaxime	30	Resistant
Ceftriaxone	30	Resistant
Cefuroxime	30	Resistant
Meropenem	10	Resistant
Azithromycin	15	Resistant
Tigecycline	15	Sensitive
Norfloxacin	10	Sensitive
Ofloxacin	5	Sensitive
Gentamycin	10	Sensitive
Phosphomycin	200	Sensitive
Different classes of antibiotics	**Concentration**	**Isolate K3.2S & K2.4ES**
Gentamicin	10	Sensitive
Doxycycline	30	Sensitive
Chloramphenicol	30	Sensitive
Clindamycin	2	Sensitive
Erythromycin	15	Sensitive
Linezolid	30	Sensitive
Ciprofloxacin	5	Sensitive
Trimethoprim	1.25/23.75	Sensitive
Nitrofurantoin	300	Sensitive

GENOTYPIC CHARACTERIZATION OF ISOLATES

DNA of the selected isolates was extracted and sequenced. The tree was generated by neighbor neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0. Sequence analysis of the isolate K3.7 N showed 96% similarity with *Alcaligenes sp.* SV/09-02 (FJ654476.1) (2) and depicted in Fig. 2.

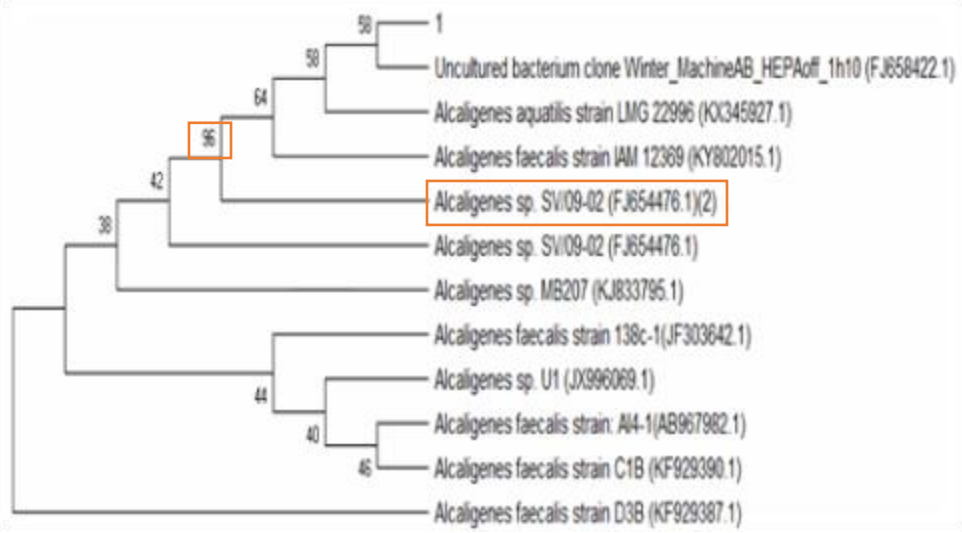

Fig. 2. Phylogenetic tree of isolate K 3.7 N

The isolate K 3.2 S showed similarity of 70% with *Bacillus sp.* strain csj15 (KY970075.1) and is depicted in Fig. 3; however, the extracted DNA of isolate K 2.4 ES failed in sequencing.

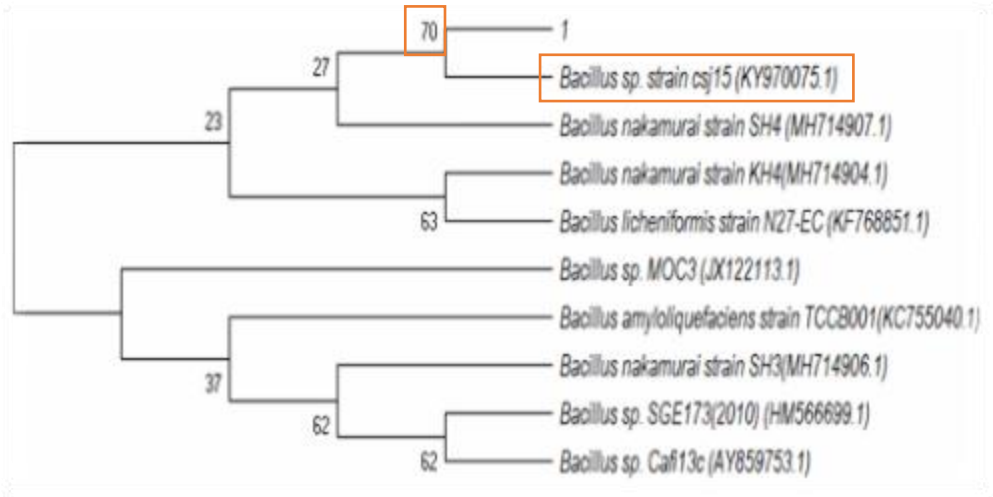

Fig. 3. Phylogenetic tree of isolate K 3.2 S

OPTIMIZATION OF ORGANIC SOLVENT FOR BIOACTIVE METABOLITES EXTRACTION

Isolate K 3.7 N showed antimicrobial activity in all the solvents used but showed maximum antibacterial activity with 20 mm inhibitory zone against *S. aureus* in Butanol where isolate K 3.2 S showed maximum antimicrobial activity in n- Hexane with inhibitory zone of 19 mm against *S. aureus* while didn't show any antibacterial activity in Ethyl acetate, Chloroform and in combination of Ethyl acetate with Hexane and Butanol. Isolate K 3.2 ES showed no extraction in all of the used organic solvents.

EXTRACTION OF CRUDE METABOLITES IN OPTIMIZED SOLVENT

The crude metabolite of isolate K 3.7 N was found to be dark brown in color, sticky in nature, and heavy weighted, whereas the crude metabolite of isolate K 3.2 S was whitish in color, dry in nature, and light weighted. The recovery of crude metabolite in the case of isolate K 3.7N was higher than that of isolate K 3.2S.

GENERAL CHARACTERIZATION OF BIOACTIVE METABOLITES

Metabolites of the selected isolates showed significant activity at all mentioned temperature values till 37 °C; however, activity was slightly decreased at 45 °C, and no antibacterial activity was observed at 60 °C, which means that metabolites lost their activity at high temperature. Proteinase K treatment didn't exhibit any significant change on the antibacterial activity of the metabolites which revealed that the metabolites may not be peptides/ proteinaceous in nature.

Isolate K 3.7 N exhibits maximum antibacterial activity of 20 mm at 30 mg/mL against *E. coli*, while the crude extract of isolate K3.2 S displayed 23 mm at 10 mg/mL against *E. coli*, as clearly depicted in the given Fig. 4.

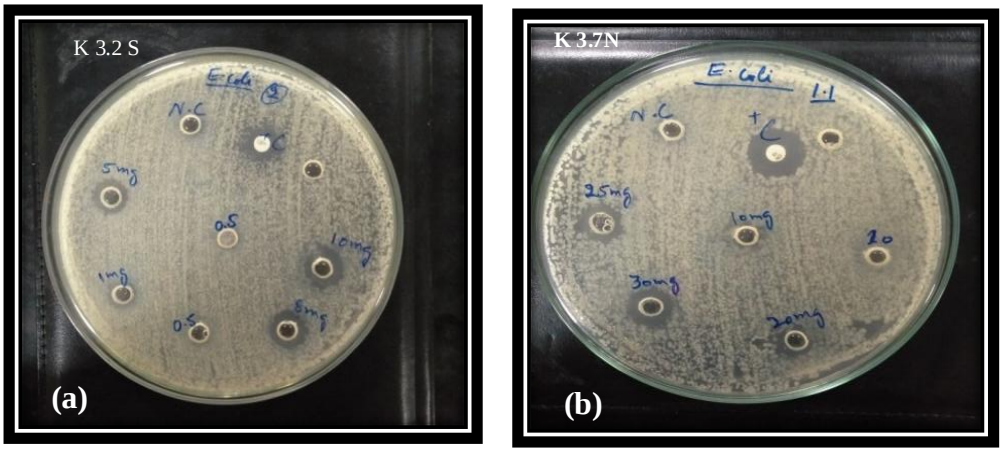

Fig. 4 (a). Antibacterial activity of crude extract of K 3.2 S against *E. coli*; **(b).** Antibacterial activity of crude extract of K 3.7 N against *E. coli*

The crude extract of both the isolates exhibits significant anticandidal activity, i.e., *C. glabrata* and *C. krusei*, and was observed even higher than the positive control, i.e., Nystatin. Interestingly extract of isolate K 3.7 N showed selective activity against *C. krusei*. The antifungal activity of both the extracts is tabulated in Table VII.

Table VII. Anticandidal activity of the crude extracts in different concentrations

S. No.	Crude Extract	Candidal spp.	Concentration (mg/mL)	Zone of inhibition (mm)
1	K3.7N	*C. krusei*	10	16.2 ± 0.4^{b}
			15	20.1 ± 0.5^{a}
			20	16.4 ± 0.3^{b}
			25	20.3 ± 0.4^{a}
			30	22.5 ± 0.6^{a}
2	K3.2S	*C. glabrata*	5	20.2 ± 0.5^{a}
			8	20.4 ± 0.5^{a}
			10	20.1 ± 0.4^{a}
		C. krusei	5	20.3 ± 0.5^{a}
			8	20.2 ± 0.4^{a}
			10	20.4 ± 0.5^{a}

*Values represent mean ± SD (n=3). Different superscript letters within a column indicate statistically significant differences ($p < 0.05$)

ANTIFUNGAL ACTIVITY OF THE CRUDE EXTRACT AGAINST PATHOGENIC FUNGI

Both the extracts displayed significant antifungal activity against the tested phytopathogens, where the K 3.2S metabolite displayed selective activity against *Rhizopus solani* only. Antifungal activity is tabulated in the given Table XIII.

Table XIII. Antifungal activity exhibited by the extracted crude metabolites against pathogenic fungi

S. No.	Crude Extract	Fungal spp.	Concentration (mg/mL)	Zone of inhibition (mm)
1	K3.2S	*Rhizopus solani*	5	14.2 ± 0.4^{c}
			8	19.1 ± 0.5^{b}
			10	20.4 ± 0.6^{a}

			10	15.3 ± 0.4[c]
2	K3.7N	*Rhizopus solani*	15	20.2 ± 0.5[b]
			20	22.1 ± 0.6[a]
			25	17.5 ± 0.4[c]
			30	25.3 ± 0.5[a]
			10	0.0 ± 0.0[c]
		Pythimum ultimum	15	0.0 ± 0.0[c]
			20	0.0 ± 0.0[c]
			25	0.0 ± 0.0[c]
			30	21.4 ± 0.6[a]

Values represent mean ± SD (n=3). Different superscript letters within a column indicate statistically significant differences ($p < 0.05$)

DISCUSSION

Bioactive compounds may sluggish bacterial growth by disrupting cell wall structure. The pharmacodynamics of alkaloids as antibiotics is stated by (27) disrupt bacterial membranes, causing respiratory and enzyme inhibition in bacteria, and affects cell multiplication along with virulence genes.
The current study was targeted to characterize three marine bacterial isolates designated as K 3.7 N, K 3.2 S, and K 2.4 ES for bioactive compounds production, which were previously isolated from the marine sediments of different locations in Karachi, Pakistan. These isolates displayed remarkable antagonistic activity against different pathogens through both point inoculation (cellular activity) and agar well diffusion method (supernatant activity). The inhibitory zone formation around the target bacterial growth displayed the synthesis of bioactive metabolites by the selected isolates, which diffused in the medium, thus inhibiting the growth of the selected bacterial pathogen in the diffused area (28). The potency of the bacteria for inhibiting the growth of target microorganisms is a kind of antagonistic activity that is believed to be accompanied by the synthesis of bioactive metabolites (28).

Upon 16S rRNA sequencing, isolate K 3.7N displayed 96 % similarity with *Alcaligenes sp.* SV/09-02 (FJ654476.1) (2), isolate K 3.2 S showed 70 % similarity with *Bacillus sp.* strain csj15 (KY970075.1) while the designated isolate K 2.4ES failed during sequencing. The 16S rRNA gene sequencing can be employed for the determination of phylogenetic studies, i.e., evolutionary relationships, taxonomy, along the array of variety among bacterial species, including the rate of species divergence. Moreover, 16S rRNA gene sequencing is useful for bacterial identification and differentiation among interrelated bacteria (29). The branches of the Phylogenetic tree displayed the relationship between biological organisms. The usage of 16S rRNA gene sequencing has evaluated several assumptions, though appropriate and powerful, i.e., the recent historical assumptions that gene sequences with >95% similarity showed the same genus, where the sequences possessing identity >97% depict the same species (30).

Recently, the occurrence of *Bacillus* in the marine environment has been fascinating, with strong antimicrobial characteristics. Some of the marine *Bacillus* strains i.e., *B. firmus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, and *B. mojavensis*, are reported by (31) possessing potent antimicrobial potential. Syed *et al* reported a marine *bacillus* identified as *Bacillus flexus* upon 16S rRNA sequencing. The marine *bacillus* strain was isolated from the soil sediment samples collected from various regions of the Bay of Bengal, India. This study revealed that *Bacillus flexus* produced maximum antibacterial metabolite production at pH 6 and pH 7, incubation period of 96 hrs. and 120 hrs. in Nutrient Broth at a temperature of 40 °C, where there was a direct correlation between the culture growth, i.e., OD, and antibacterial metabolite production (Syed, Sairam et al. 2019). Our results also depicted that isolate K 3.2 S identified as *Bacillus sp.* strain csj15 (KY970075.1) synthesizes maximum antimicrobial metabolites at pH 6, incubation period of 96 hrs. in Nutrient Broth at a temperature of 35 °C (Table 6). In contrast to the study of Syed *et al*, the optimum temperature for the isolate K 3.2 S was 35 °C, and there was no direct correlation between growth OD and antimicrobial metabolites production (32). The selected bacterial isolates in our study produce bioactive compounds in the stress phase.

Optimization of various cultural parameters aims to excel the microbial growth, hence increase the probability of efficient biomass production along with bioactive compounds (5).

The marine *Alcaligenes faecalis* was isolated by Romano *et al.* from marine water samples of the Machilipatnam sea coast of Andhra Pradesh, India. Analyzing the influence of different physicochemical parameters on *Alcaligenes faecalis*, Romano *et al.* revealed that the bacteria displayed optimal biomass and metabolite production at a temperature of 35 °C, pH 7, and incubation period of 72 hours (5,10). These results were in contrast to our obtained results i.e., *Alcaligenes sp.* SV/09-02 (FJ654476.1) (2) designated as K 3.7N displayed substantial metabolite production at a temperature of 30°C, pH 5, and an incubation period of 96 hours.

The extraction of crude metabolites at optimum culture conditions from the cell-free supernatant was accomplished under the strategy of liquid-liquid extraction, where the *Bacillus sp. Strain* csj15 (KY970075.1) displayed maximum recovery in n-hexane with a maximum inhibitory zone of 19 mm against *S. aureus*, where no recovery and antagonistic activity were found in ethyl acetate and in its combination solvents. These findings are in contrast with the results of Wibowo *et al*, where all the *Bacillus sp*., including *Bacillus subtilis, Bacillus velezensis*, and *Bacillus paralicheniformis,* showed extraction in ethyl acetate with substantial antagonistic activity against *E. coli,* followed by *S. aureus* (28). The crude extracts of *Bacillus sp. Strain* csj15 (KY970075.1) exhibits significant antibacterial, antifungal, and anticandidal activity, which are relevant to the results reported by Wibowo *et al* (28). The isolated *Alcaligenes sp.* SV/09-02 (FJ654476.1) showed optimum recovery and antimicrobial activity in Butanol, with maximum antagonistic activity of 20 mm against *S. aureus*, and its crude extracts displayed remarkable antibacterial, antifungal, and anticandidal activity. Upon the antibiotic susceptibility test, the *Alcaligenes* sp. SV/09-02 (FJ654476.1) (2) showed high resistance to many of the antibiotic discs used, and minimal sensitivity was observed against 5 kinds of antibiotics/compounds (e.g. Tigecycline, Norfloxacin, Ofloxacin, Gentamycin, and Phosphomycin (Table 8). These results were relevant to the findings of De & Ramaiah *et al*, where the isolated marine *Alcaligenes species* displayed high resistance to many of the used antibiotics, and the least sensitivity was reported (33). The isolate *Bacillus sp. Strain csj*15 *(KY970075.1*) didn't display any resistance to the used antibiotics, which are in contrast to the results of Radisic, & Marathe *et al* (34).They reported a marine *Bacillus toyonensis* strain 4HC1 isolated from the polyethylene in Øygarden, Norway, carrying various antibiotic resistance genes and displaying phenotypic antibiotic resistance to various antibiotics.

Antibiotic resistance is one of the serious global concerns. In comparison to terrestrial habitats, there is little knowledge regarding the presence and diversity of antibiotic-resistant genes in the marine biosphere. One of the studies conducted about this aspect concluded that about 44 % of the antibiotic-resistant genes were predominant in a few marine bacterial taxa, e.g. *Pelagibacter*, *Prochlorococcus*, and *Vibrio (35).*

Biochemical potential of *Alcaligenes* sp. SV/09-02 (FJ654476.1), showed tolerance to different salts in different concentrations, where maximum tolerance was observed for KCl and $MgSO_4$, i.e., 7 %, lagging by NaCl, i.e., 5%, $MgCl_2$ 3.5%, $CaCl_2$, 2.6% and minimal salt tolerance was displayed towards KH_2PO_4 i.e., 2 %. However, the influences of different salts on bacterial growth i.e., OD and bioactive compounds production wasn't studied. Similar to our obtained results *Alcaligenes faecalis* D334, a salt tolerant bacterium, was isolated from mangrove sediment (36). From physiological experiments, it was revealed that the bacterial strain was able to grow up to 12 % (w/v) NaCl. Similarly, the biochemical profile of *Bacillus sp.* Strain csj15 (KY970075.1) displayed a halophilic nature and comparatively high tolerance to different salts than *Alcaligenes sp.* SV/09-02 (FJ654476.1). The highest salt tolerance observed for $MgSO_4$ and $MgCl_2$ was 12.5 % lagging by NaCl and KCl 10%, $CaCl_2$ 5 % and KH_2PO_4 2%. A salt tolerant *Bacillus* sp. Strain Ba9, isolated from Asian Seabass Cage Sediment, Thailand showed 1.5 % -4 % NaCl tolerance (37).

CONCLUSION

Alcaligenes sp. SV/09-02 (FJ654476.1) (2) and *Bacillus sp.* strain csj15 (KY970075.1) labeled as isolate K 3.7 N and K 3.2 S, are marine bacteria exhibiting remarkable antibacterial, antifungal, and anticandidal activities. Adaptation of the marine bacteria to mesophilic conditions, i.e., halophilic, thermal, and osmotic stability, are a positive feature for the investigation of novel bioactive compounds of pharmaceutical significance. Future studies on the purification of the crude extracts to characterize the bioactive components,

their detailed mechanism of action, and various biological assays would be required to reveal the potent utility of the *Alcaligenes sp.* SV/09-02 (FJ654476.1) (2) and *Bacillus sp.* strain csj15 (KY970075.1), and will definitely offer a scope in the field of drug discovery with exotic and potent bioactive metabolites. In conclusion, this study highlights the seroprevalence of hepatitis C infection in educational institutes in Quetta, Pakistan. The infection recorded were more in female than male and more productive 15-25 years age group was more vulnerable. Other chronic diseases, surgical procedures, blood transfusion and rural/urban life style were the most common risk factors findings in positive patients. This emphasizes how common is the HCV infection in educational institutions of Quetta, Balochistan and need stern action to educate the society and adopt hygienic measures to ease the health care burden in the area.

Conflict of interest:

The authors declare no conflict of interest.

Authors' contribution:

HN conceived study; GA & SK carried out bacterial isolation and screening; SR & AS performed biochemical and molecular analyses; MK data interpretation; HN & GA drafted the manuscript.

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