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Isolation, Characterization and Screening of Marine *Pseudomonas Sp.* for Bioactive Compounds and their Potential Use as Anti-Microbial and Anti-Oxidant Agent

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ABSTRACT:
Marine ecosystem is known for its diversity and for novel bioactive metabolites with potential applications in the field of pharmaceutical industry. To explore, 18 marine water samples were collected and inoculated onto isolated medium. A total of 170 isolates were obtained which were screened for their anti-bacterial activity against gastro-intestinal pathogens. n-butanol, hexane, ethyl acetate, chloroform were used to extract the bioactive compounds. Escherichia coli, Salmonella typhi, Shigellaboydii, Salmonella typhi A, Salmonella typhi B, Pathogenic Pseudomonas aeruginosa, Pathogenic Escherichia coli, Salmonella enterica were used as test pathogens. These pathogens were tested for their ability to resist against antibiotics as mentioned by Clinical and Laboratory Standards Institute (CLSI). Results of ethyl acetate extract of isolates proved to be potential anti-bacterial and anti-oxidant agent. SWA14 showed significant antibacterial activity and 50.6% of anti-oxidant activity. Bioactive compounds were identified using gas chromatography and mass spectroscopy (GC-MS), and the chromatogram reveals as multi-component bioactive compounds. Therefore, SWA14 as potential organism and other isolates were subjected to 16S rRNA sequencing and SWA14 was identified as Pseudomonas stutzeri ATCC 17588(T) with 99.66% similarity.
Keywords: *Bioactive compounds, Pathogens, Anti-oxidant activity, GC-MS, CLSI.*

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INTRODUCTION

Oceans cover more than 70% of the earth's surface. About 97% of all the water available on the earth is in the oceans. Oceanic waters have a very rich wealth of marine life because oceans serve as major reserve for food, energy, and minerals. They also control the global climate [1]. Marine ecosystem is characterized by the many biotic and abiotic components. Biotic components are: Organisms and their species, Predators, Parasites, Competitors and Mates. Abiotic Components (Physical and Chemical) are: Temperature, Concentration of Nutrients, Sunlight, Turbulence, Salinity and density[1].The marine environment also represents a largely unexplored source for isolation of new microbes (bacteria, fungi, actinomycetes, microalgae-cyanobacteria and diatoms) that are potent producers of bioactive secondary metabolites with a wide range of activities. Extensive research has been done to unveil the bioactive potential of marine microbes (free living and symbiotic) and the results are amazingly diverse and productive, providing solutions for many medical problems. Bioactive compounds from marine flora and fauna have extensive past and present use in the treatment of many diseases/infections and serve as compounds of interest both in their natural form and as templates for synthetic modification (changes in side chains/functional groups – transformation/biotransformation). Several molecules isolated from various marine organisms (microorganisms, algae, fungi, invertebrates, and vertebrates) are currently under study at an advanced stage of clinical trials, some of them have already been marketed as drugs for treatment of various diseases/infections. Marine microbial products have attracted the attention of the biologists and chemists all over the world for the last five decades and researchers are much interested to explore marine microbial diversities and their products[7].Bioactive substances derived from marine bacteria have bright prospect in marine drugs development and research. Many of these compounds were found to have inhibitory effect on other microorganisms like bacteria, fungi, viruses etc. and hence are termed as antibacterial, antifungal and antiviral compounds. The search for unique drugs from the marine organisms particularly from marine bacteria because of its unexplored area has attracted attention of the scientists and researchers in the

research laboratories and clinics throughout the world [17].The development of MDR is a complicated issue which has become an international dreadful concern. To decrease the rise and spread of MDR, cooperative efforts are requisite [18].The current research focus on isolation and characterization of *Pseudomonas strains* producing bioactive compounds with anti-bacterial and anti-oxidant activity followed by identification of efficient strains by 16S rRNA sequencing.

MATERIAL AND METHODS

Isolation of microorganisms from marine sample

Total 18 samples of marine water sample has been collected from Alibag, Panvel and Mumbai region of Maharashtra state. Samples were collected in sterile polythene bags and transported to laboratory. The samples were serially diluted using physiological saline and spreaded over the surface of sterile zobell marine agar, sterile sea water agar and sterile *Pseudomonas* agar. Post inoculation, the plates were incubated at room temperature for five days.

Morphological characterization of isolates and production of bioactive compounds

Total 170 isolates were obtained after five days of incubation. Morphologically distinct colonies were selected and characters like size, shape, margin, pigmentation, elevation, opacity, gram nature were recorded. Isolates were subjected to production of bioactive compounds by inoculating one percent of culture adjusted to 0.1 optical density.Incubation at room temperature under shaker conditions adjusted at 100 revolutions per minute.

Extraction of bioactive compounds

Centrifugation of production media at 10,000 rpm for 10 minutes. Supernatant was collected and subjected to extraction. Polar solvents like n-butanol, hexane, ethyl acetate, chloroform were used for extraction. Each polar solvent was added to supernatant in 1:1 ratio, mixture was agitated using separating funnel and organic phase was collected.

Screening of bioactive compounds for anti-microbial activity against gastro pathogens

Agar cup method was used to test the potency of bioactive compounds against gastro-intestinal pathogens. Test organisms used were *Escherichia coli, Salmonella typhi, Shigellaboydii, Salmonella typhi A, Salmonella typhiB, Pathogenic Pseudomonas aeruginosa, Pathogenic Escherichia coli, Salmonella enterica*.0.1 optical density of the culture was adjusted and 15 ml of sterile Muller Hinton agar was used. n-butanol, hexane, ethyl acetate, chloroform were test controls. Pathogens were tested for multi drug resistance against ampicillin, Piperacillin, Tobramycin, Cefotaxime, Tetracycline, Gentamycin, Co-trimoxazole, Amikacin, Nalidixic acid, Ofloxacin, Nitrofurantoin, Chloramphenicol. All the tests were carried in triplicates.

Screening of bioactive compounds for anti-oxidant activity

0.1mM solution of DPPH (1,1- diphenyl-2-picrylhydrazyl) in ethanol was prepared. 1.0ml of DPPH solution was added to 0.5ml of samples in different concentrations. After 5, 10, 15 & 20 minutes, the absorbance was measured at 525nm. DPPH· scavenging activity (%) = $[(A_0-A_1)/A_0]$ x 100. Where, A_0 was the absorbance of the blank i.e. no sample, DPPH solution only) and A_1 was the absorbance in the presence of the test compound

Characterization of efficient bioactive compounds by GC-MS

Bioactive compounds were subjected to gas chromatography - mass spectroscopy. Bioactive compounds were completely miscible in acetonitrile solution.

Identification of efficient isolates using 16S rRNA sequencing

Genomic DNA was isolated by the standard phenol/chloroform extraction method, followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation &directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per instructions. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read at least twice. Assembly was carried out using Lasergene package, identification using the EzBio-Cloud database.

RESULT AND DISCUSSION

Isolation, characterization of microorganisms and extraction of bioactive compounds

Overall, 170 isolates were obtained and these isolates were characterized based on the distinct morphology.Isolates were both pigmented and non-pigmented in nature. Based on the unique feature in them, they were selected and further proceeded for production of bioactive compounds. Bioactive compounds were extracted into organic solvent like n-butanol, hexane, ethyl acetate, chloroform.

Multi-drug resistance of test pathogens: As per the Clinical and Laboratory Standards Institute (CLSI) guidelines for testing susceptibility of antibiotics against gastro-intestinal pathogens. Disc diffusion method was performed to determine the antibiotic susceptibility. Gastrointestinal pathogens showed

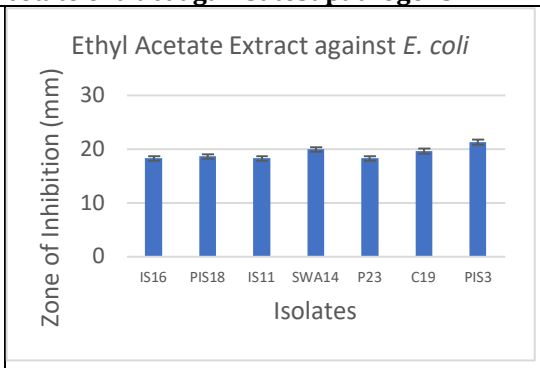
resistance to Ampicillin, Piperacillin, Cefotaxime, Gentamycin, Co-trimoxazole, Nalidixic acid, Ofloxacin, Nitrofurantoin, Tobramycin, Tetracycline. Co-trimoxazole and Amikacin showed significant zone of inhibition against gastrointestinal pathogens indicating sensitivity of test pathogens towards antibiotics. Biological compounds are to be investigated against the pathogens while organisms are gaining resistance to anti-bacterial compounds.

Screening of bioactive compounds for anti-microbial activity against gastro- pathogens

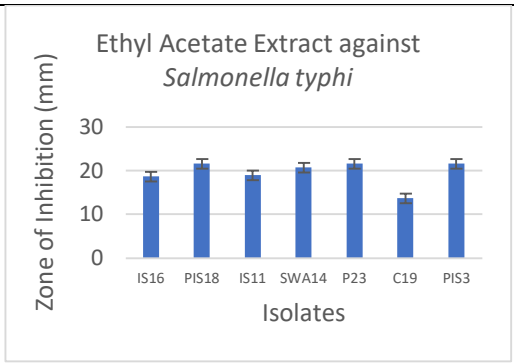
n-butanol, hexane, ethyl acetate, chloroform extracts of bioactive compounds was tested against gastro-intestinal pathogens. Ethyl acetate extract showed utmost anti-bacterial activity indicating its potential use in formulations for treating infections. The analysis result found potential anti-bacterial compounds through the screening of extracts of 170 isolates. Out of 170 isolates, n-butanol, hexane, chloroform extracts showed unsatisfactory results while ethyl acetate extract of bioactive compounds showed significant result. The results of extracts obtained from IS16, PIS18, IS11, SWA14, P23, C19, PIS3 represented below.

Table and Graph 1: Result of ethyl acetate extract against test pathogens

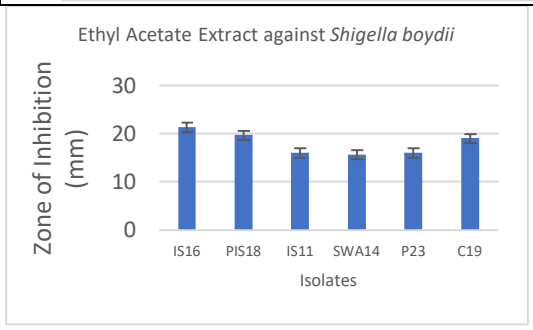
Extract of Isolates (Ethyl Acetate)	*Escherichia coli* (zone of inhibition)	Std. Dev.	Std. Err.
IS16	18	0.5774	0.3333
PIS18	19	1.5275	0.8819
IS11	18	0.5774	0.3333
SWA14	20	1.0000	0.5774
P23	18	0.5774	0.3333
C19	20	0.5774	0.3333
PIS3	21	0.5774	0.3333



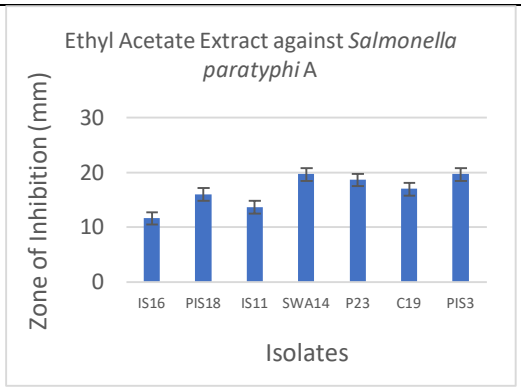
Extract of Isolates (Ethyl Acetate)	*Salmonella typhi* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	19	0.5774	0.3333
PIS18	22	0.5774	0.3333
IS11	19	0.0000	0.0000
SWA14	21	0.5774	0.3333
P23	22	0.5774	0.3333
C19	14	0.5774	0.3333
PIS3	22	1.5275	0.8819



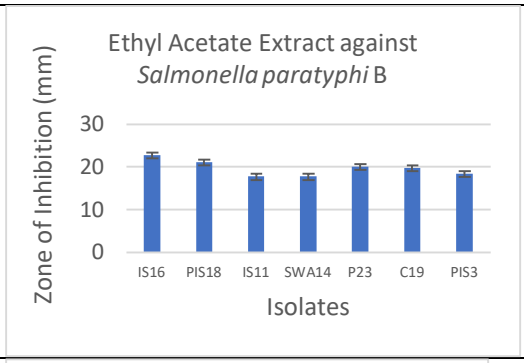
Extract of Isolates (Ethyl Acetate)	*Shigellaboydii* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	16	1.0000	0.5774
PIS18	21	1.1547	0.6667
IS11	20	1.5275	0.8819
SWA14	16	2.0000	1.1547
P23	16	0.5774	0.3333
C19	16	1.0000	0.5774
PIS3	19	1.0000	0.5774



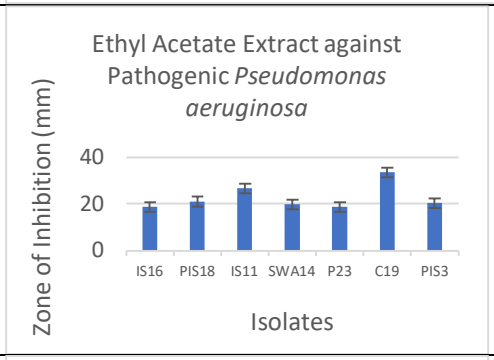
Extract of Isolates (Ethyl Acetate)	*Salmonella paratyphi A* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	12	0.5774	0.3333
PIS18	16	0.0000	0.0000
IS11	14	2.0817	1.2019
SWA14	20	1.5275	0.8819
P23	19	0.5774	0.3333
C19	17	1.0000	0.5774
PIS3	20	0.5774	0.3333



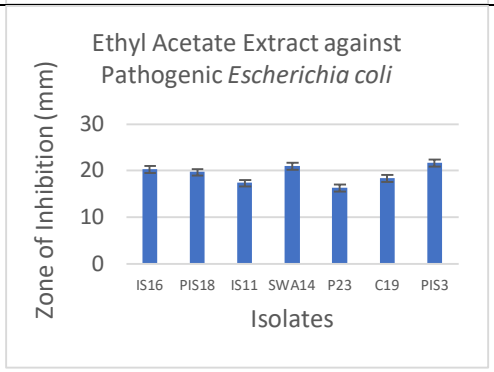
Extract of Isolates (Ethyl Acetate)	*Salmonella paratyphi B* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	23	0.5774	0.3333
PIS18	21	0.0000	0.0000
IS11	18	1.5275	0.8819
SWA14	18	1.5275	0.8819
P23	20	1.0000	0.5774
C19	20	0.5774	0.3333
PIS3	18	0.5774	0.3333



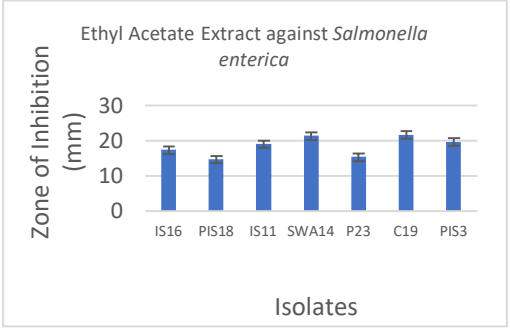
Extract of Isolates (Ethyl Acetate)	*Pathogenic Pseudomonas aeruginosa* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	19	0.5774	0.3333
PIS18	21	1.7321	1.0000
IS11	27	1.5275	0.8819
SWA14	20	0.5774	0.3333
P23	19	0.5774	0.3333
C19	33	1.1547	0.6667
PIS3	20	1.1547	0.6667



Extract of Isolates (Ethyl Acetate)	*Pathogenic Escherichia coli* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	20	1.5275	0.8819
PIS18	20	0.5774	0.3333
IS11	17	1.1547	0.6667
SWA14	21	2.0000	1.1547
P23	16	0.5774	0.3333
C19	18	0.5774	0.3333
PIS3	22	0.5774	0.3333



Extract of Isolates (Ethyl Acetate)	*Salmonella enterica* (zone of inhibition)	*Std. Dev*	*Std. Err.*
IS16	17	1.1547	0.6667
PIS18	15	2.3094	1.3333
IS11	19	1	0.5774
SWA14	21	1.1547	0.6667
P23	15	1.1547	0.6667
C19	22	0.57735	0.3333
PIS3	20	0.57735	0.3333



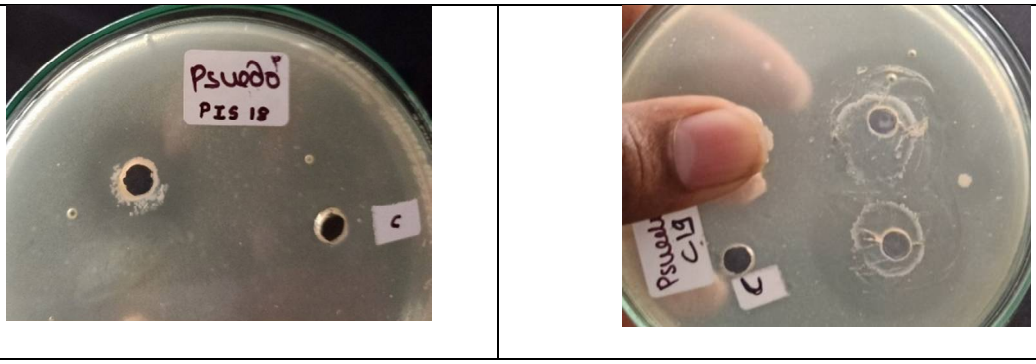


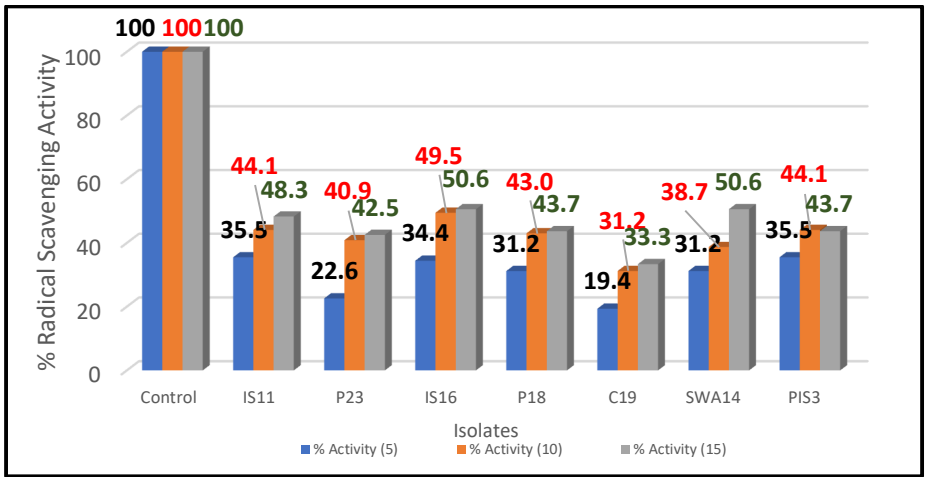
Figure 1: Plate showing zone of inhibition: ethyl acetate extract against test pathogens

Anti-oxidant activity

Free radical scavenging activity of the bioactive compounds were tested using DPPH. The results projected here are performed in triplicates. Extract of SWA14 and IS16 showed maximum free radical activity as 50.6%. The details of assay are mentioned below:

Table 2: Results of anti-oxidant assay

Sample	5 Minutes	10 Minutes	15 Minutes	Sample	% Activity (5)	% Activity (10)	% Activity (15)
Control	0.93	0.93	0.87	Control	100	100	100
IS11	0.6	0.52	0.45	IS11	35.5	44.1	48.3
P23	0.72	0.55	0.5	P23	22.6	40.9	42.5
IS16	0.61	0.47	0.43	IS16	34.4	49.5	50.6
P18	0.64	0.53	0.49	P18	31.2	43.0	43.7
C19	0.75	0.64	0.58	C19	19.4	31.2	33.3
SWA14	0.64	0.57	0.43	SWA14	31.2	38.7	50.6
PIS3	0.6	0.52	0.49	PIS3	35.5	44.1	43.7



Graph 2: Results of radical scavenging activity of ethyl acetate extract

Characterization of efficient bioactive compounds by GC-MS

Bioactive compounds were extracted and subjected for identification using gas chromatography – mass spectroscopy. Based on the retention time of the compounds, the compounds were identified as decane, 1-Decene and so on. Here, the compound is identified as multi-component bioactive compound. Details of spectrum and table represent the extract of SWA14 as *Pseudomonas stutzeri* ATCC 17588(T), which is the focus organism of this study.

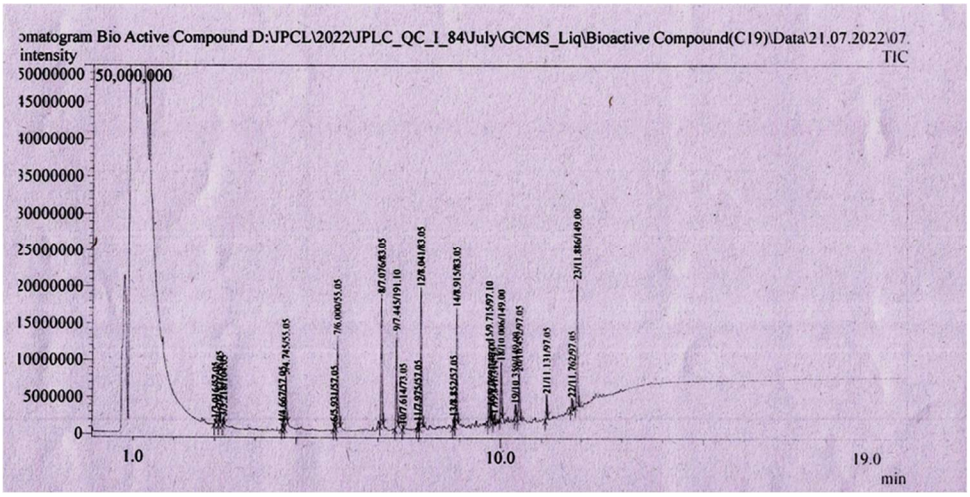


Figure2: Chromatogram of bioactive substance extracted from SWA14

Table 3: Multi component bioactive compounds of SWA14 identified using GC-MS

Peak	Name of the Compound	Retention Time
1	Decane	3.016
2	1-Decene	3.121
3	1,3,5,7-Cyclooctatetraene	3.218
4	Dodecane	4.667
5	1-Dodecene	4.745
6	Tetradecane	5.931
7	1-Pentadecen	6.000
8	1-Heptadecene	7.076
9	2,4-Di-tert-butylphenol	7.445
10	Cyclononasiloxane, octadecamethyl	7.614
11	Heneicosane	7.975
12	1-Nonadecene	8.041
13	Nonacosane, 3-methyl	8.852
14	1-Nonadecene	8.915
15	1-Nonadecene	9.715
16	Dibutyl phthalate	9.769
17	14-Octadecenoic acid, methyl ester	9.841
18	1,4-Dibutyl benzene 1,4-dicarboxylate	10.006
19	1,4-Dibutyl benzene 1,4-dicarboxylate	10.359
20	1-Heptacosanol	10.452
21	1-Heptacosanol	11.131
22	1-Heptacosanol	11.762
23	Bis (2-ethylhexyl) phthalate	11.886

Identification of efficient isolates using 16S rRNA sequencing

Efficient isolates after testing their potency as anti-bacterial and anti-oxidant agent were subjected to identification by 16S rRNA sequencing. The identified isolates are listed below:

Isolate	Taxonomic Designation	% Similarity	Accession No.
P23	*Bacillus stercoris* JCM 30051(T)	100	MN536904
IS16	*Bacillus tequilensis* KCTC 13622(T)	99.91	AYTO01000043
	Bacillus cabrialesii TE3(T)	99.91	MK462260
C19	*Bacillus safensis subsp. safensis* FO-36b(T)	99.92	ASJD01000027
	Bacillus safensis subsp. osmophilus BC09(T)	99.92	KY990920
P18	*Shigellaflexneri* ATCC 29903(T)	99.92	X96963
PIS3	*Acinetobacterpittii* CIP 70.29(T)	99.73	APQP01000001
SWA14	*Pseudomonas stutzeri* ATCC 17588(T)	99.66	CP002881

DISCUSSION

18 marine water samples were collected and subjected isolation by spread plate method. Total of 170 isolates were obtained and subjected to morphological characterization and production of bioactive compounds. n-butanol, hexane, ethyl acetate, chloroform were used to extract the bioactive compounds. *Escherichia coli, Salmonella typhi, Shigellaboydii, Salmonella typhi A, Salmonella typhi B, Pathogenic*

Pseudomonas aeruginosa, Pathogenic Escherichia coli, Salmonella enterica were used as test pathogens. These pathogens were tested for their ability to resist against antibiotics as mentioned by Clinical and Laboratory Standards Institute (CLSI). During screening stage, 3.5% (06 isolates) were found having potential antimicrobial compounds because they showed inhibitory activity toward tested microorganisms. Extract of ethyl acetate of SWA14 showed significant result as anti-bacterial agent against test pathogens showing their use as active pharmaceutical ingredients and showed 50.6% anti-oxidant activity that can be utilised as natural anti-oxidants. These extracts were subjected to GC-MS and the retention time based on chromatogram proved it to be a multi-component bioactive compound. The isolates were identified by 16S rRNA sequencing.

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