European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 11, 2020

ISOLATION AND SCREENING OF MARINE MICROBE AGAINST CLINICAL PATHOGENS

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Abstract

The marine microbes isolated from sedimented specimens from various places of Pichavaram, Parankipettai, and Muthupet in India. Antibacterial substances from marine bacteria were identified by cross streak method and agar plate technique. The potent marine bacteria species were inoculate on production medium and filtered. The extract was screened for antibacterial activity. Nine marine bacteria species were isolated from the selected marine sediment sample. All the nine isolates were active against the test organisms. Chemical screening strongly suggested that presence of alkaloids, Carbohydrates and Glycosides. The result of marine bacteria is composed of potent secondary metabolites. Isolation, screening of marine bacteria can be useful in discovery of novel metabolites.

Keywords: Marine bacteria, Primary screening, Antibacterial activity, secondary metabolites.

Introduction

The explosive development in the chemistry of marine species throughout the last 15 years have resulted in the discovery of a plethora of new frameworks, most of which have no precedent among systems of terrestrial region and possesses previously unknown pharmacokinetic and pharmacodynamic properties (Selvin *et al*., 2004). Marine microbes have special properties because they need to adapt to severe environments in the marine climate, like higher or lower temperatures, alkaline or acidic water, higher pressure and small deep ocean water substrates. The distinguishing properties have gained many researches to discover in depth since there is the possible of microbes utilized in biotechnological applications. Baharum *et al*., (2010) reported that Marine microbes have become a significant site of research in search for new antibiotics. These are attributable to the decline in findings from terrestrial bacterial species, and also the appearance of antibiotic sensitive to clinical bacterias like *Mycobacterium tuberculosis*, *Enterococcus*, *Pseudomonas* sp., *Streptococcus pneumoniae*, and *Staphylococcus aureus* lead to continuous necessity to identify novel resources of active antibiotics.

Pseudomonas is a various genus of Gamma proteobacteria with nearly 60 species possessing wideranging life styles in an extensive environment, such as soil, water, plant surfaces, and animals. They are familiar for their appearing in the organic world, capability to use an arresting diversity of natural substances as energy sources, resistant to a broad variety of clinically - and agriculturally-significant bacterial substances, and creation of a notable array of 2º metabolites (Harald Gross and Joyce E. Loper, 2009). The variety of marine bacterial cells producing antibiotics extracted in the current study indicates that sponges are valuable sources of innovative bacteria. R. Saravanakumar *et al.,* (2010).

Cyclo-peptides metabolite produced by *Pseudomonas sp.* and it showed promising Inhibiting agent against *S. aureus, M. luteus, B. subtilis, E. coli* and *Vibrio anguillarum* (Kang, H.K.; Seo, C.H., (2015), Mondol, M.*et al*., (2013), Srivastava, A. and Mishra, V (2018), Rungprom et al., (2008). In this work, an attempt was made to explore the secondary metabolites produced by marine *Pseudomonas sp.* isolated from pichavaram sediment samples. Secondary metabolites are nitrogen-containing heterocyclic compounds, with strong physiological activity. The secondary metabolites are used as a drug for various applications and are well known for its high bioactivity.

Materials and method Isolation of Marine Microbes

Sedimented specimens were taken from different marine environments like Pichavaram,Parankipettai and Muthupet and were examined for the isolation of Marine Bacteria. In the present study Zobell Marine Agar 2216 and Modified Nutrient agar (MNA) were used for isolation of marine bacteria by serial dilution technique

(Gokulkrishnan et al, 2011). Microscopic observation was done for confirmation and sub cultured again three to four times to get the pure cultures. Pure cultures were chosen and preserved on Modified Nutrient agar (MNA) slants for another analysis

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Screening of Isolates for Antibacterial applications

In the initial stage of screening, evaluation of the antibacterial action of pure isolates were executed by zone of inhibition technique on Nutrient agar (NA) utilizing *Escherichia coli* MTCC 64, *Salmonella typhi* MTCC 734, *Klebsiella pnemoniae* MTCC 10 and *Staphylococcus aureus* MTCC 96 as pathogens. The plates were kept incubation for 2 days at 28ºC and then sowed with "test" species by one streak at a 90º angle to the strip of the "producer strain" and eventually the plates are kept incubation for 2 days sat 28ºC. The bacterial connections were examined by defining the distance of suppression calculated in mm. Bacterial species displaying "moderate" to "good" suppression action were chosen for 2º screening that was accomplished via agar well technique, utilizing 100 µl of their fermented culture towards *Escherichia coli* MTCC 64, *Salmonella typhi* MTCC 734, *Klebsiella pnemoniae* MTCC 10 and *Staphylococcus aureus* MTCC 96. All these trials are accomplished in triplicates and then an average values are taken for further study.

Molecular Identification of Isolated Strains

The potent isolate chosen from the secondary screening were molecular authenticated by 16S r DNA analysis. The genomic DNA was separated from the microbes and then they obtained hugely purified DNA was enlarged in a thermocycler at conditions: 35 cycles of 94°C for 1 min, 55°C for 1 min and at 72°C for 2 min which is suitable for the amplification of the 16S r RNA sequences explicitly by employing the primers (A.F. Fouad, 2002). Forward (16F27):5 -AGAGTTTGATCCTGGCTCAG-3; Reverse (16R1522):5 AAGGAGGTGATCCAGCCGCA-3. DNA sequencing was achieved in an extremely automated gene sequencer. Then the corresponding sequences were recited in Genbank databases (BLAST) and in comparison, with other sequences to examine the microbial class and its phylogeny. Sequences were further corelated with other unconstrained sequences in the GenBank database utilizing BLAST program (National Center for Biotechnology Information).

Metabolite Production and Extraction

The potent isolate chosen from the secondary screening were grownup in a 500 mL Erlenmeyer Flash that comprises liquid media of ZoBell 2216E and nurtured in a temperature of 30ºC for 3–5days and cell development was validated via visible pellets, bunches, accumulates or turbidness in the culturing broth. After 5 days of fermentation time, the bacterial growth media was sieved to isolate the biological mass from fermentation solution. The fermentation solvent was then separated twice utilizing natural solvent of n hexane, ethyl acetate and n-buthanol to acquire the needed extract. The extract was then disappeared in an evaporator to yield thick extract and then kept in a desiccator for further process.

Purification and Partial Characterization of the Substances

The active substances were cleansed by adsorption chromatography utilizing silica gel (poresize60Å, meshsize:230–400, particle size 40–63 µm) as a stationary stage and gel filtration chromatography utilizing sephadex LH- 20. An eluted fraction was assessed for their biological activity towards *Escherichia coli* MTCC 64, *Salmonella typhi* MTCC 734, *Klebsiella pnemoniae* MTCC 10 and *Staphylococcus aureus* MTCC 96. The purity of the bioactive fractions was further assessed by high pressure liquid chromatography (HPLC) by employing the reverse phase silica column (RP18). Eventually, the UV spectral (Perkin Elmer Lambda-25 UV spectrophotometer) of diverse antimicrobial substances were determined in methanol at 200–500 nm wavelength

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Overall Antioxidant Action

Overall antioxidant behaviour of the proportions are examined as per the technique of Prieto *et al* (1999) 0.3mL of each fraction (100µg/ml) was combined with 3.0 mL of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammoniummolybdate). Reaction mix was nurtured at 95ºC for 90 minutes in a

H_2O bath. Absorbance of total specimens were evaluated at 695 nm using LAB INDIA UV 3000 double beam spectrophotometer. Quercitin (100, 200,300,400 and 500µg/ml) was utilized as standard.

DPPH Radical Scavenging Activity

The free radical scavenging action of total solvent was examined by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) as per the already stated technique 14. Shortly, a 0.1mM solution of DPPH in methanol was processed, and 1mL was introduced to 3 mL solution of total extracts at diverse concentration (10, 20, 30, 40 and 50 µg/mL). The combinations were agitated strongly and permitted to stand at room T for 30 mins. Then the absorbance was noted at 517 nm utilizing a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was utilized as the reference. Lesser absorbance values of scavenging activity. The capacity to scavenging the DPPH radical was examined by employing the following formula. DPPH scavenging impact (% inhibition) = {(A0 –A1)/A0)*100} Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in existence of total of the extract specimens and reference. All the trials were executed in triplicates and the outcomes were averaged (Shen *et al.*, 2010).

Results and Discussion

Therapeutic dosage form from the natural source have been achieved nowadays could be possible and succeeded utilizing infrastructure of the resources in research and development on biomaterials. In this investigation of the research sediment sample were collected from different marine environments like Pichavaram, Parankipettai and Muthupetthat represented different ecological habitats as per the previous activity on bacterial research reported by Solingen *et al*., (2001). Sediment specimens were chosen at the depths of 0.5 cm to 10 cm under the exterior that is enrich in natural matter.

Diverse physico-chemical feature was employed for the careful separation of marine bacteria from the composed specimens. (**Table 1)** showed that digital value of the selected sediment from Pichavaram has different crucial essentials and physical characteristics that is indispensible for significant development of marine bacteria that was similar cum co-related with previous report (Saseeswari, 2015).

Table 1. Physico chemical analysis of selected sediment samples.

S. No.	Physico-chemical parameters	Pichavaram	Parankipettai	Muthupet
1	pH	8.3±1.02	8.2±1.15	7.9±0.53
2	Soil texture	Black in colour	Black brown	Black in colour
3	Electrical Conductivity (dsm^{-1})	6.6±1.05	8.5±1.02	7.24±1.05
4	Salinity (ppm)	32.4 ±0.49	35.2±0.12	29.05 ±0.09
5	Turbidity	3.2±2.80	2.58±0.14	2.36±0.18
6	TDS (mg/l)	3.9±1.36	2.48±0.84	1.96±0.86

7	DO (mg/litre)	1.5±0.12	1.9 ± 0.14	1.7 ± 0.09
8	Available phosphorus	BDL	1.7 ± 0.84	1.8 ±0.02
9	Nitrogen (ppm)	1.7±1.02	0.6±0.12	0.8±0.14
10	Potassium (ppm)	53±1.05	47±1.15	50±0.14
11	Organic carbon (ppm)	213±0.16	198±0.21	234±0.17

Value represents Mean ± SD; n=3

ppm - (parts per million): **BDL** - Below the detectable level, **DO**-Dissolved oxygen, **TDS**-Total dissolved solids

Isolation of Marine Bacteria:

Nine strains were observed with different colony morphology were streaked on Zobell Marine Agar 2216 and Modified Nutrient agar (MNA) plates to obtain pure culture with identity simultaneously. The isolated marine bacteria were named as according to the selected sediment samples. The isolated marine bacteria were named as according the areas as (PV-1, 2, 3, 4, MP-1, 2, 3 and PP-1, 2, 3) and they were taken for further study.

Screening of Isolated strains:

All the isolated species were separated for the confirmation of secondary metabolites producing bacteria in which three strains showed maximum activity in the primary screening by cross streak method and these three (PV-4, MP-3 and PP-2) were taken for the further study (**Table 2).** In 2^{o} filtering, the metabolites processed through marine bacteria was examined towards the bacterias (Dhanasekaran*et al*., 2009) by agar well technique on Muller Hinton agar medium. The metabolites PV-4 displayed higher zone of inhibition towards all the species *viz*., *Salmonella typhi* MTCC 734, *Escherichia coli* MTCC 64, *Klebsiella pnemoniae* MTCC 10, *Staphylococcus aureus* MTCC 96 as 17mm, 18mm, 16mm, and 19mm respectively (**Table 3)** and the activity above mentioned was higher than the result previously obtained from the marine sample Jagan Mohan (2013).

Table 2. Primary screening of Marine Bacteria.

S. No	Isolates	Screening of antagonistic activity of isolated marine bacterial strains			
		Salmonella typhi MTCC 734	*Escherichia coli MTCC 64*	*Klebsiella pnemoniae MTCC 10*	*Staphylococcus aureus MTCC 96*
1	PV-1	-	-	-	-
2	PV-2	+	-	-	-
3	PV-3	-	-	+	-
4	PV-4	++	++	++	++
5	MP-1	-	-	+	-
6	MP-2	+	-	-	-
7	MP-3	-	+	+	-
8	PP-1	-	+	-	-
9	PP-2	-	+	-	+

- : No inhibition,+:moderate inhibition, ++: high inhibition

Table 3. Antagonistic effect of cell free supernatants from selected bacterial isolates against test organisms.

Selected bacterial isolates	Antagonistic effect (zone of inhibition, diam, in mm) Test organisms			
	Salmonella typhi MTCC 734	*Escherichia coli MTCC 64*	*Klebsiella pnemoniae MTCC 10*	*Staphylococcus aureus MTCC 96*
PV-4	17	18	16	19
MP-3	NZ	11	10	NZ
PP-2	NZ	10	NZ	11
Standard (cifrofloxin)	23	21	22	23

Diameter of zone of inhibition including well diameter 6mm, NZ – No zone of inhibition

Characterization and Molecular Identification of the marine bacterial strain

The marine bacterial strain PV-4 which inhibit maximum pathogens was selected and characterized by 16 S ribosomal DNA genes study and were determined phylogenetically as, *Pseudomonas* sp. The bacterial cultures were submitted in NCBI GenBank and Accession no. was obtained (GenBank Accession Nos:MK027024). *Pseudomonas* are well-known prevalent microbe that have been separated from diverse of organic sources, soil, plants, mineral waters and clinical sample and they are categorized by a higher extent of metabolic assortment (Romanenko et al., 2005). Production of the protease and lipase was reported in *Pseudomonas sp.* with both the enzyme being secreted concomitantly (Mohammed *et al.*, 2014).

Partially Chemical Characterization of the Bioactive Substances

The biologically active substances are cleaned from chosen *Pseudomonas sp.* PV-4 strain utilizing silica column, by methanol: chloroform incline as an eluted solvent scheme. The cleaned segments were partly characterized through detecting below UV-absorbance and its absorption range together few preliminary ideas concerning the framework of the compound(s) (Figure 1). The bioactive compounds (5:5) produced specific characteristic absorbance maximum of 200 and 250 nm for the bioactive molecules. The area of the peak occupied 227.9 and 243.9 revealed that this bioactive molecule in large quantity synthesised was observed from the Table 4. Absorbance inside UV variety verified the existence of unsaturated in total biologically active substances.

Figure 1. UV spectrum of bioactive compounds (5:5).

Table 4. UV analysis of bioactive compounds (5:5).

S. No.	Peak (nm)	Peak (AU)
1.	200	227.9
2.	250	243.9
3.	900	896.0

Total antioxidant activity

The antioxidant are the compounds which prevent the oxidation process induced by the free radicals by oxidation reduction principle here the two fractions were involved in the antioxidant activity as total effect of the sample.

Quercitin the standard reference was used for the comparison activity between the fractions F1and F2 at the dose of 100 to 500 µg/ml. From the Table 5, F1 showed increasing activity in the dose dependant manner was higher than the F2. So, the F1 activity was considered to be comparable with standard reference Quercitin and the inhibition DPPH activity produced by F1- fraction was the maximum compare to standard IC_{50} value 6.8 µg/ml (Table. 6) which dominated the activity obtained from isolates of previous study (Massoumeh Farasat, 2014).

Table 5. Total antioxidant activity.

Fraction	Total Anti-oxidant Activity				
	100µg/ml	**200µg/ml**	**300µg/ml**	**400µg/ml**	**500µg/ml**
F-1	65.853±0.05	72.097±0.01	78.341±0.06	82.12±0.08	86.09 ±0.06
F-2	60.813±0.02	66.027±0.09	70.31±0.05	72.22±0.11	74.23±0.17
Quercitin	75.09±0.01	82.621±0.03	84.203± 0.57	87.02 ± 0.01	92.08 ± 0.45

Value represents mean ± SD

Table 6. Percentage Inhibition of DPPH Activity of the fractions.

Fractions	Percentage Inhibition of DPPH Activity					
	10µg/ml	**20 µg/ml**	**30µg/ml**	**40 µg/ml**	**50µg/ml**	**IC_{50}**
F1	73.23±0.03	78.23±0.01	84.32±0.01	88.32±0.04	91.32±0.01	6.8 µg/ml
F2	49.11±0.10	55.41±0.13	57.58±0.09	64.21±0.11	51.66±0.06	10.20µg/ml
Vitamin C	60.92±0.01	72.69±0.02	84.75±0.01	94.53±0.01	98.57±0.01	**8.33 µg/ml**

Conclusion

Recently, the growth of drug resistant, transmittable microbes has advised the request for discovering the substances formed by the marine bacteria from unfamiliar areas. In our work, the species separated from marine ecology have the capability to suppress the development of infectious bacteria like MRSA and other human pathogens. In future the secondary metabolites could be isolated from the selective marine bacteria using advanced technology in the research and development for the novel therapeutic ligands.

References

1. Selvin, J., & Lipton, A. P. (2004). Biopotentials of secondary metabolites isolated from marine sponges. Hydrobiologia, 513(1-3), 231-238.
2. Baharum, S. N., Beng, E. K., & Mokhtar, M. A. A. (2010). Marine microorganisms: potential application and challenges.
3. Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical biochemistry, 269(2), 337-341.
4. Saseeswari, A., Kanimozhi, G., & Panneerselvam, A. (2015). Studies on physicochemical parameter and bacterial populations in sediment soil at Karankadu Mangrove forest, Ramanathapuram (Dt), Tamilnadu. International Journal of Science, Environment and Technology, 4(3), 886-889.
5. Dhanasekaran, D., Selvamani, S., Panneerselvam, A., & Thajuddin, N. (2009). Isolation and characterization of actinomycetes in Vellar estuary, Annagkoil, Tamil Nadu. African Journal of Biotechnology, 8(17).
6. Farasat, M., Khavari-Nejad, R. A., Nabavi, S. M. B., & Namjooyan, F. (2014). Antioxidant activity, total phenolics and flavonoid contents of some edible green seaweeds from northern coasts of the Persian Gulf. Iranian journal of pharmaceutical research: IJPR, 13(1), 163.
7. Mohan, Y. S. Y. V. J., Sirisha, B., Haritha, R., & Ramana, T. (2013). Selective screening, isolation and characterization of antimicrobial agents from marine actinomycetes. Int. J. Pharm. Pharm. Sci, 5, 443-449.
8. Solingen, P., Meijer, D., Kleij, W. A., Barnett, C., Bolle, R., Power, S. D., & Jones, B. E. (2001). Cloning and expression of an endocellulase gene from a novel streptomycete isolated from an East African soda lake. Extremophiles, 5(5), 333-341.
9. Gross, H., & Loper, J. E. (2009). Genomics of secondary metabolite production by Pseudomonas spp. Natural product reports, 26(11), 1408-1446.
10. Kang, H. K., Seo, C. H., & Park, Y. (2015). Marine peptides and their anti-infective activities. Marine drugs, 13(1), 618-654.
11. Mondol, M. A. M., Shin, H. J., & Islam, M. T. (2013). Diversity of secondary metabolites from marine Bacillus species: chemistry and biological activity. Marine drugs, 11(8), 2846-2872.
12. Srivastava, A., & Mishra, V. (2018). Marine peptides act as novel chemotherapeutic agent. J. Microbiol. Exp, 6, 267-270.
13. Rungprom, W., Siwu, E. R., Lambert, L. K., Dechsakulwatana, C., Barden, M. C., Kokpol, U., ... & Garson, M. J. (2008). Cyclic tetrapeptides from marine bacteria associated with the seaweed Diginea sp. and the sponge Halisarca ectofibrosa. Tetrahedron, 64(14), 3147-3152.
14. Saravanakumar, R., Moomeen, H. S., Ronald, J., & Kannan, M. (2010). Control of fish bacterial pathogens by antagonistic marine actinomycetes isolated from Gulf of Mannar coast. World Journal of Fish and Marine Sciences, 2(4), 275-279.
15. Gokulkrishnan, K. (2011). Antimicrobial activity of marine bacteria isolated from the Mangalore coast, west coast of India. Recent Research in Science and Technology.
16. Fouad, A. F., Barry, J., Caimano, M., Clawson, M., Zhu, Q., Carver, R., ... & Radolf, J. D. (2002). PCR-based identification of bacteria associated with endodontic infections. Journal of clinical microbiology, 40(9), 3223-3231.
17. Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X., & Li, P. (2010). Antioxidant activity in vitro of the selenium-contained protein from the Se-enriched Bifidobacterium animalis 01. Anaerobe, 16(4), 380386.
18. Marhoon, A. A., Aziz, I. H., & Altaai, M. E. (2014). Identification Pseudomonas aeruginosa by 16s rRNA gene for Differentiation from Other Pseudomonas Species that isolated from Patients and environment. *Baghdad Science Journal*, *11*(2), 1028-1034.