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RESEARCH MANUSCRIPT

Screening of Marine bacteria for multiple Biotechnological applications

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Abstract

This study was undertaken to isolate bacteria from marine environment of Chennai beaches and to screen them for various biotechnological applications namely antibacterial activity, production of extracellular enzymes, solubilization of phosphate, production of exopolysaccharides (EPS) and degradation of chemical dyes. A total of four bacteria, of which were actinobacteria, were isolated in pure culture form and subjected to study their biotechnological potential. Among four bacteria studied, two bacteria were protease producers, one produced alkaline protease and one produced cellulase. All the four bacteria have produced amylase. None of them showed lipase activity. Qualitative tests for EPS production indicated that all the four isolates have the ability to produce EPS while quantification study exhibited that strain 3B produced EPS in higher amounts. All the bacterial isolates have produced IAA quantitatively and among them strain 3B produced more amount of IAA. None of the bacteria have exhibited antibacterial activity against *Staphylococcus aureus*. Similarly none of them have solubilized tri-calcium phosphate. All the four marine bacteria have decolorized and degraded the tested dyes saffranin and crystal violet. The study has overall concluded that marine bacteria could serve as potential bioresources for many biotechnological applications.

Keywords: Marine environment, antibacterial activity, exopolysaccharides, biotechnological potential.

Introduction

Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses. With the help of different molecular and biotechnological techniques, humans have been able to elucidate many biological methods applicable to both aquatic and terrestrial organisms (Jha and Zi-rong, 2004). Marine microbes offer great opportunities for biodiscovery (Bull *et al.,* 2000), yet that potential has yet to be realized. Despite a huge microbial diversity, there is a lack of laboratory cultures of the microbes that are most abundant in the environment that severely limits development of biodiscovery research.

Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water. These distinctive characteristics have attracted many researchers to explore in depth since there is the potential of marine microorganisms used in industry (Baharum *et al.,* 2010). In their review, Baharum *et al*. (2010) have focused on marine microorganisms that provided biotechnological applications in enzymes industry and pharmaceutical products and also provided an overview of the challenge faced by researchers in order to explore and exploit the marine reservoir.

Research into natural products from the marine environment, including microorganisms, has rapidly increased over the past two decades. Despite the enormous difficulty in isolating and harvesting marine bacteria, microbial metabolites are increasingly attractive to science because of their broad-ranging pharmacological activities (Azamjon *et al.,* 2010). There has been a great interest from researchers to explore marine microorganisms as new source of antibacterial compounds as increasing resistance of pathogen to present antibiotics. One example of studies that has been carried out is purification and partial characterization of marinocine, a new broad-spectrum antibacterial protein produced by *Marinomonas mediterranea* (Lucas-Elio *et al.,* 2005). Marine microorganisms were proven already to have many beneficial bioactivities such as production of industrial enzymes (Chatellier *et al.*, 2011; Manasi, 2011), plant growth promotion potentials such as production of phytohormones and phosphate solubilization (Jayaprakashvel *et al.,* 2011), antifungal activity (Jayaprakashvel *et al.,* 2010), biocontrol activity for plant disease control (Gobalakrishnan *et al.,* 2010; Bhagat *et al.,* 2010a), antibacterial and probiotic activity (Kaarthikeyan *et al.,* 2010). Actinomycetes, the filamentous, high G+C rich gram positive bacteria, are the most economically and biotechnologically valuable prokaryotic microorganisms.

Prokaryotic microorganisms were found to produce nearly half of the discovered bioactive secondary metabolites. These secondary metabolites include antibiotics, antitumor agents, immunosuppressive agents and enzymes (Lam, 2006). Since environmental conditions of the sea are extremely different from terrestrial conditions, they produce different types of antibiotics. Marine actinomycetes have a diverse range of enzyme activities that are capable of catalyzing various biochemical reactions. Different commercial enzymes viz. L-glutaminase, α-galactosidase, amylase, cellulase, protease, L-asparaginase, have also been obtained from the marine actinobacteria (Lakshmanaperumalsamy, 1978; Jayaprakashvel *et al.,* 2012). The diverse chemical compounds of marine actinomycetes have been found to have various biological activities such as antimicrobial, anti-tumor, anti-malarial, anti-algal, antioxidant, anti-inflammatory etc. These various bioactive metabolites of marine actinomycetes are having scope for developing as potent therapeutic agents. The potential of marine actinomycetes is rightly realized though the current biological wealth of these organisms is relatively unexplored (Jayaprakashvel, 2012). In this context, this study was attempted to isolate and screen marine bacteria from Chennai coastal area for antibacterial activity, antifungal activity, production of phytohormones, solubilization of phosphate and dye degradation.

Materials and methods

Isolation of marine bacteria: Surface sediment soil samples were collected from Chennai coastal area and brought to the laboratory in aseptic plastic containers. Two samples were subjected for the isolation of marine bacteria. Soil samples were serially diluted in sterile distilled water and pour plated both in nutrient agar medium and starch casein agar medium at room temperature (35 ± 2°C) for five days. After five days morphologically distinct bacteria from nutrient agar medium and actinomycetes from starch casein agar medium were subcultured to purity on the respective culture media (Jayaprakashvel *et al.,* 2010).

Screening of marine bacteria for the production of extracellular enzymes: All the four marine bacteria were subjected for the screening of extracellular enzymes namely protease (neutral and alkaline), lipase, amylase and cellulase using a simple qualitative plate assay. Half strength nutrient agar medium was prepared with different substrates of the tested enzymes at 0.5% concentration such as gelatin for protease, tween-80 for lipase, starch for amylase and CM cellulose for cellulase. This substrate amended medium was streaked with the two marine bacteria. For alkaline protease screening, the medium pH was adjusted to reach 10 and gelatin was used as substrate at 0.5% concentration. In case of actinomycetes, instead of nutrient agar half strength starch casein agar medium was used with same substrates as stated above. The actinomycetes were streaked as a straight line in the sterilized medium. The inoculated plates were incubated for 48 h in room temperature. After 48 h incubation, different visualization tests were followed to observe enzyme activity. For protease test, 0.3% mercuric chloride solution prepared in 1N HCl was poured onto the plate so that it reaches all parts of the media and after 20 min, zone of clearance indicating proteolytic activity, if any was observed around the streak of bacteria. For amylase test, grams iodine was used as indicator. Iodine reacts with the starch in the remaining area to give it a blue-black color. A clear zone around the strain shows that amylase produced by the specimen has digested the surrounding starch. For lipase test, if the microbe breaks down the tween-80 by the production of lipase, a opaque zone should appear around the streak. To visualize cellulase activity, plates were flooded with 0.3% aqueous congo red solution for 15 min followed by flooding with 10% NaCl for 15 min. Clear zone around the reddish background indicates the production of cellulase by the test bacteria (Ramesh and Mathivanan, 2009).

Qualitative detection of EPS production: Qualitative detection of EPS production was studied by modifying the protocol of Sayyed *et al.* (2011). YMG Agar medium was prepared by dissolving glucose: 10 g; yeast extract: 3 g; malt extract: 3g; peptone: 5 g; monosodium glutamate: 1 g; sucrose: 30 g in 1 L of 50% seawater and pH was adjusted to 7.0 and was poured in petri plates after sterilization. The bacterial strains to be tested for EPS production were streaked on the solidified medium. The plates were incubated at room temperature for 3 d. Oozing out of gummy substances on the periphery of the bacterial colonies indicated the production of EPS.

Quantification of EPS production: Quantitative detection of EPS production was studied by modifying the protocol of El-Tayeb and Khodair (2006). One loop full of bacterial strain was inoculated in 5 mL YMG broth medium in test tube and incubated at room temperature for 3 d. After 3 d of incubation 250 µL of the culture broth was taken and centrifuged at 8000 rpm for 5 min. Supernatant (200 µL) was taken and transferred in vials and 600 µL of ice-cold ethanol was added and kept in refrigerated condition. After 30 min, the solution was centrifuged at 10000 rpm for 10 min and the supernatant was discarded. The vial was kept for air drying to remove excess alcohol for 30 min. Then, the amount of EPS in the eppendorf was quantified by using a fresh eppendorf as reference for empty.

In vitro screening of bacterial isolates for IAA production: The strains were tested for IAA production by the method of Bric *et al.* (1991). The strains were inoculated in sterilized nutrient broth supplemented with tryptophan (10 µg/mL) and incubated at 37°C for 3 d in shaking conditions. After incubation period, fully grown bacterial cultures were centrifuged at 10,000 rpm for 10 min.

To the supernatant (2 mL), 2 drops of ortho phosphoric acid was added and incubated at room temperature for 10 min, followed by addition of 4 mL of Salkowski reagent (50 mL, of 35% sulphuric acid, 1 mL of 0.5 M $FeCl_3$). Development of pink colour indicates the positive result for IAA production and no color change indicates the negative result for IAA production.

Quantitative of IAA: The quantification of IAA was performed according to Gordon and Weber (1951). For this purpose, nutrient broth with 0.3% tryptone was prepared. Each of the isolated bacterial cultures was inoculated in 5 mL of the sterilized nutrient broth. These tubes were incubated at room temperature for 3 d. After incubation, the cultures were centrifuged at 3000 rpm for 30 min. For measuring the IAA equivalents, 1.5 mL of the fluid supernatant were pipetted into test tubes and 4 mL of Salkowski reagent (50 mL, of 35% sulphuric acid, 1 mL of 0.5 M $FeCl_3$) were added as coloring agent. The tubes containing the mixture were left for 30 min. The IAA produced by the strains was measured spectrophotometrically at 530 nm. The OD values were compared with a standard IAA graph and quantification was done.

Screening for antibacterial activity: Nutrient agar was prepared and autoclaved and poured in petriplates. The bacterial pathogen, *Staphylococcus aureus* obtained from the Infectious Diseases Laboratory, YRG Care, was inoculated as lawn culture on the agar plates. All the 4 bacterial strains were placed at 4 corners of the petridish as patch inoculums and kept for incubation. After 24 h, the zone of inhibition around the bacterial colonies was observed if any.

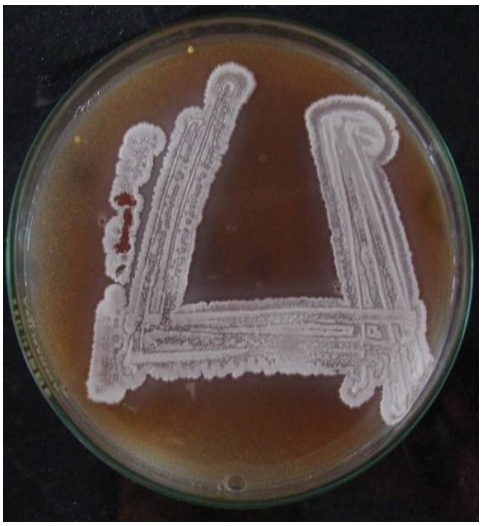
Phosphate solubilization activity: For the screening procedure, quarter strength of nutrient agar was prepared and 1% of Tricalcium Phosphate was added before autoclaving the medium. This resulted in a milky white medium. The medium was poured on petriplates and made to solidify. After which the bacteria was patched on 4 corners of the plate and incubated for 7 d at room temperature (Nautiyal and Mehta, 2001). A clear zone around the bacterial patches indicates the ability of the bacterium to solubilize phosphate in agar medium.

Degradation of dyes: Nutrient broth (4 mL) was prepared and 1 mL of chosen dyes (0.1% of crystal violet and saffranin) were added in test tubes and sterilized. They were then inoculated with the respective bacteria and actinomycetes. Control was prepared with 4 mL of nutrient broth added with 1 mL of sterile distilled water. Tubes were incubated for 48 h after which they were visually for decolorization if any. In addition, the growth of bacteria in the presence of dye was estimated by taking absorbance of the culture broth at 600 nm in Elico UV Vis Spectrophotometer.

Results

Isolation of marine bacteria: A total of four marine bacteria including four marine actinomycetes were isolated as pure culture in this study. Both strains MB1 and MB2 are eubacteria with medium sized, mucoid pale brown colored colonies on nutrient agar medium. Strain MB3 is an actinomycete with powdery, grey colored aerial mycelium and dry colonies with brown colored substrate mycelium. Strain MB4 is also a marine actinomycete with powdery, white colored aerial mycelium and dry colonies with pale brown colored substrate mycelium (Fig. 1).

Fig. 1. Colony morphology of marine actinomycete strain MB4 on starch casein agar medium.



Screening of marine bacteria for the production of extracellular enzymes: All the 4 bacteria were screened for their ability to produce 5 different enzyme activities extracellularly. Both the bacterial strains MB1 and MB2 produced good proteolytic activity both at neutral and alkaline pH indicates the production of neutral and alkaline protease.

Table 1. Production of extracellular enzymes by selected marine bacteria.

Strain code	Production of extracellular enzymes				
	Neutral protease	Alkaline protease	Lipase	Amylase	Cellulase
MB1	+	+	-	+	+
MB2	+	+	-	+	-
MB3	-	-	-	+	-
MB4	-	-	-	+	-

+ indicates the production of the enzyme while – indicates no production.

Fig. 2. Amylase activity exhibited by actinomycete strain MB3.

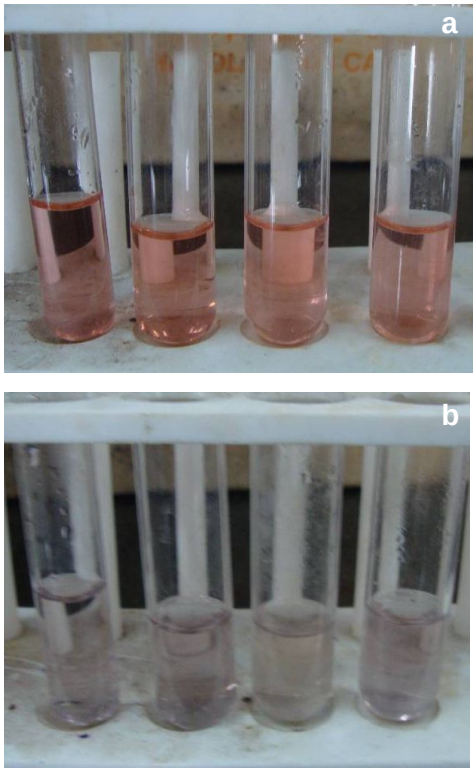
None of the marine actinomycetes exhibited proteolytic zone with the substrate gelatin. However, they cannot be considered as non protease producers unless they were studied with different protein substrates. All the 4 marine bacteria have failed to produce lipase activity. Interestingly all the 4 marine bacteria have exhibited good amylase activity (Fig. 2). In case of cellulase, except MB1, other 3 strains did not produce any cellulolytic zones. Overall analysis concluded that strain MB1 is capable of producing multiple extracellular enzymes (Table 1).

EPS production by marine bacteria: All the 4 marine bacterial strains produced gummy secretions either around the colony periphery or by whole colony in YMG Agar medium. Quantification experiment concluded that all the 4 bacteria produced considerable amounts of EPS. Strain MB1 produced 12 mg/mL, MB2 produced 8 mg/mL and strain MB3 produced 4 mg/mL. Strain MB4 which is an actinomycete produced a maximum of 16 mg/mL of EPS in YMG broth (Table 2).

IAA production by marine bacteria: All the 4 marine bacterial strains have produced slight pink coloration when qualitative assay was done indicating the production of IAA in their culture filtrates. Quantification experiment revealed that strain MB2 has produced relatively higher amounts of IAA (Table 2).

Phosphate solubilization activity: None of the strains produced any zone of clearance around their colony growth even after 5 d in tri-calcium phosphate amended medium which indicates that none of them were having the ability to solubilize phosphate extracellularly.

Fig. 3. Dye decolorizing ability of marine bacteria: Decolourized spent liquid media.



a. Saffranin; b. Crystal violet.

Antibacterial activity: None of the bacterial strains have exhibited antibacterial activity against the tested human pathogen *S. aureus.* However, the bacteria are to be tested by other modes of screening antibacterial activities such as well diffusion/disc diffusion assay with their culture filtrates for more clarity of results.

Degradation of dyes: All the 4 bacteria decolorized the tested dyes considerably. However, growth analysis indicated that the actinomycete strain MB3 has grown very well even in the presence of both the dyes when compared to other 3 bacteria (Fig. 3 and 4).

Discussion

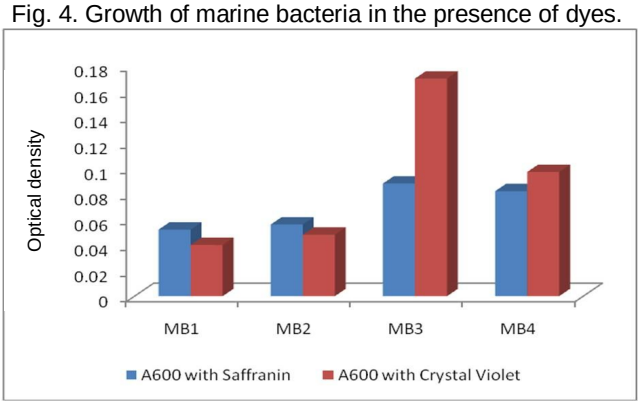
The marine environment is the largest habitat on Earth, representing more than 70 % of the surface of our planet. Oceans include the greatest extremes of temperature, light and pressure encountered by life (Munn, 2004). Adaptation of marine bacteria to the harsh environments has led to a rich biological and genetic diversity. Marine bacteria are attracting attention as new biotechnological resources.

Table 2. Production of extracellular enzymes by selected marine bacteria.

Strain code	Exopolysaccharide		Indole acetic acid	
	Production	Quantity (mg/mL)	Production	Quantity (µg/mL)
MB1	+	12	+	7.3
MB2	+	8	+	8.6
MB3	+	4	+	8.0
MB4	+	16	+	6.1

+ indicates the production of the substance while – indicates no production.

Fig. 4. Growth of marine bacteria in the presence of dyes.



These marine bacteria can be a potential source of new bioactive compounds for industrial, agricultural, environmental, pharmaceutical and medical uses (Debnath *et al.,* 2007). Culture dependent methods are much suited for the exploration studies to use marine microorganisms for biotechnological applications. Despite a huge microbial diversity, there is a lack of laboratory cultures of the microbes that are most abundant in the environment that severely limits development of biodiscovery research. It has been known for more than 30 years that many more bacteria are present in the surface ocean than can be cultured by the traditional microbiological approach of plating a sample onto selective media (Hobbie *et al.,* 1977). In this study only very limited number of marine bacteria were isolated from marine samples using conventional media and isolation practices. Only a small fraction of naturally-occurring microbial assemblages were cultured on conventional selective media and standard plating technique recovered a very small proportion, 0.001% to 1% of the total assemblage (Joint *et al.,* 2010). In this study, 4 marine bacteria including 2 marine actinomycetes were isolated from the surface sediment soil samples collected in the interidal zones of Chennai coastal area. Microorganisms from intertidal zones must be able to tolerate rapid and repeated fluctuations in environmental conditions including temperature, light and salinity, and are exposed to wave action, ultraviolet radiation, as well as periods of drought (Dionisi *et al.,* 2012). Hence, microbes from such harsh environments may exhibit potential properties which can be exploited for biotechnological applications. Marine bacteria including actinomycetes were already isolated from such intertidal sediments and studied for various biotechnological applications by our research group (Bhagat *et al.,* 2010b; Gopalakrishnan *et al.,* 2010; Jayaprakahsvel *et al.,* 2011). Marine microorganisms are increasingly becoming an important source in the search for industrially important molecules. They are considered highly valuable as they produce various antibiotics and other therapeutically useful compounds with diverse biological activities (Ramesh and Mathivanan, 2009). In the marine environment, extracellular enzymes play a central role in the recycling of organic carbon and nitrogen compounds.

High molecular weight organic compounds cannot be transported directly into bacteria to be catabolized. Thus, bacteria must hydrolyze these organic polymers to smaller molecules before they are incorporated into the cell for subsequent metabolism. This extracellular hydrolytic activity is performed by bacterial extracellular enzymes (Belanger *et al.,* 1997). Thus, in this study, the marine bacteria were screened for their ability to produce one or more extracellular hydrolytic enzymes using substrate amended plate assay. A particular strain MB2 produced more number of enzyme activities comparatively. Our earlier studies have also proved that marine bacteria including marine actinomycetes are exhibiting diverse pattern in secreting extracellular enzymes (Ramesh *et al.,* 2008; Jayaprakashvel *et al.,* 2008) Similarly, potential capability of heterotrophic bacteria for extracellular enzyme synthesis and their activity were determined in a transect from dunes to a water depth of 1 m in a sandy beach near spot on the southern Baltic coast. Among studied enzymes, alkaline phosphatase, esterase lipase, and leucine arylaminase were synthesized in a higher degree, whereas α-fucosidase, β-glucouronidase and α-galactosidase had only low level (Mudryk and Podgórska, 2006).

Exopolysaccharide, designated as EPS frequently, is the material construction of biofilm matrix, serving as a multipurpose functional element for adhesion, structure, protection, and recognition. This microbial EPS has been used in a wide range of industries due to its functions such as gel formation, emulsifying, absorption, cohesion, and film formation. Furthermore, heavy-metal ion accumulating, anti-tumor active, and anti-ulcer active EPSs have also been reported. These EPSs have industrial potential as new biomaterials due to these activities (Ko *et al.,* 2000). In this study all 4 screened bacteria exhibited EPS production while a particular strain designated as MB3 produced more amounts of EPS in YMG broth. Many marine bacteria produce exopolysaccharides (EPS) as a strategy for growth, adhering to solid surfaces, and to survive adverse conditions. There is a growing interest in isolating new EPS producing bacteria from marine environments, particularly from extreme marine environments such as deep-sea hydrothermal vents characterized by high pressure and temperature and heavy metal presence (Poli *et al.,* 2010). Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non-rhizospheric soils (Ahmad *et al.,* 2005). Our research group has reported earlier that marine bacteria which are no way related to terrestrial environment are having the ability to produce auxins (indole acetic acid-IAA) (Jayaprakashvel *et al.,* 2011). The production of auxins by marine bacteria may offer a chance to use these bacteria as biofertilizers to improve the growth and yield of agricultural crops in coastal saline influenced lands.

In this context, this study investigated IAA production by selected marine bacteria and found that all of them hadthe ability to produce IAA while few produced more amount in culture filtrates. Phosphorous is one of the major essential macro-nutrients for plants which largely remain in unusable form. However, phosphate solubilization ability of the microorganisms is considered as one of the most needed qualities of a biofertilizer organism. Phosphate solubilization by marine bacteria has been studied very infrequently (Jayaprakashvel *et al.,* 2011). However, in this study none of the tested marine bacteria exhibited phosphate solubilizing activity. Many marine heterotrophic bacteria are known to produce antibacterial substances which inhibit or kill other bacteria. Research studies have shown that these antibacterial compounds are not only inhibitory to terrestrial bacteria but also to indigenous bacterial strains, which is of considerable ecological significance (Nair and Simidu, 1987). Many studies have concentrated on the screening of marine bacteria for antibacterial activity against human pathogenic bacteria (Blunt and Prinsep, 2006). However, the screening of marine bacteria for antibacterial activity in this study has not resulted in amtibacterial activities.

Triphenylmethane dyes such as Malachite Green and Crystal Violet are used extensively in textile, paper and leather, food and cosmetic industries. Some of the triphenylmethane dyes are used as medicine and biological stains. Dyes are recalcitrant molecules which are difficult to degrade biologically. From the treatment point of view, the degradation of dyes has received considerable attention. Nowadays, biodegradation of various dyes are getting considerable attention because of its cost effective, environment friendly nature and does not produce large quantities of sludge. Biodegradation of dyes like crystal violet has always under interest (Ayed *et al.,* 2009). Hence, in this study, the comparative abilities of marine bacteria for decolorization of crystal violet and saffranin was evaluated. However, these organisms are to be studied for their ability to degrade the dyes rather than decolorization so as to make it biotechnologically relevant in environmental applications. There has been very few studies reported on the decolorization of Triphenylmethane dyes by marine bacteria and this report is a preliminary and primary study on this aspect. Hence, this study assumes much significance.

Conclusion

All species have a role in our biosphere. However trying to understand that role is not an easy job. With the help of experimentations we can try to understand the activities of microbes and implement them to our advantage in the industrial field or bioremediation. The research findings about the marine bacteria gave insight into the ecology of microbes in aquatic sediments. Further analysis will give us a broader aspect of understanding. However, a major limitation is culturing of certain strains have posed a difficulty. Once cultured, further studies can give information on the applications of these microbes. Since the marine bacteria obtained are not from deep sea levels, it proves that these microbes are easy to access and can be subjected to bioprospecting.

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