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RESEARCH ARTICLE

Isolation and partial characterization of marine actinomycetes

Navami, S. S., Lekshmi, M. and Ayona Jayadev*
Department of Environmental Sciences, All Saints' College, Thiruvananthapuram

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Corresponding Author

Ayona Jayadev

Abstract

Appreciating the potential of actinomycetes in the present biotechnological scenario, a research work was carried out for isolation and screening of marine actinomycetes and the preliminary findings of isolation and steps for partial characterization of marine actinomycetes are presented. A total of 20 actinomycetes were isolated from the three sampling site, 10 from Sanghumukham, 7 from Vizhinjam and 3 from Veli coast which comes to about 50%, 35 % and 15% are from the sampling sites respectively. The morphological characters of the strains were identified using cover slip method. Biochemical characterizations (gram staining, Methyl red and Voges Proskaeur test (MR-VP), Gelatinase test, Oxidase test, Urea hydrolysis, Catalase test and Starch hydrolysis) of the isolated actinomycetes strains were done using standard procedures and the results are shown. The methodologies of the tests are also elaborated in the paper.

INTRODUCTION

Marine biotechnology, one of the rapidly expanding branch uses marine organisms fully or partially to make bioactive compounds, the modify products or to improve the environmental conditions. This is one of the unique ecosystems which offer great opportunities for biodiscovery (Bull *et al*., 2000). Actinomycetes are one of the active groups which form stable population among marine microbial communities with unlimited potential for production of bioactive metabolites as well as enzymes (Asolkar *et al*., 2010; Rahman *et al.*, 2010). They are Gram positive, filamentous, sporulating bacteria with DNA rich in G+C content ranging from 55-75%. They contribute immensely to the field of biotechnology with the capacity to produce products ranging from antibiotics to enzyme inhibitors and anti-cancer agents to various alkaloids. The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000 of which 10,000 are produced by actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered (Berdy 2005).Over time since the origin of earth and oceans, the marine actinomycetes have evolved genetic diversity and greatest metabolic activities in an attempt to adapt to extreme conditions of this unique environment which is very different from any terrestrial ecosystem, (Kijjoa and Sawangwong, 2004). There are only limited screening efforts to screen marine actinomycetes but even this has recently surpassed that of their terrestrial counterparts (Subramani and Aalbersberg, 2012). Marine microorganisms were already proven to have many beneficial bioactivities in the production of industrial enzymes, (Chatellier *et al*., 2011; Manasi, 2011, Lekshmi *et al.*, 2014), plant growth promotion potentials such as production of phytohormones and phosphate solubilisation (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). They are also reported to contribute to the breakdown and recycling of organic compounds and can be used for bioremediation processes. Hussain *et al.*, (2002) reported that actinomycetes play a significant role in biological control of insects by producing insecticidal active compounds. Realizing the importance of marine actinomycetes in research and industry, a preliminary work was carried out for isolation and partial characterization of actinomycetes from selected marine samples.

Materials and Methods

Collection of samples

Marine water samples for the isolation of actinomycetes were collected from Sanghumukham, Vizhinjam and Veli coast (TTPL effluent discharge point), Thiruvananthapuram, Kerala. All the three sampling sites were with human activities. The first one is a beach, second one, a fish harbor with so many motor boats active and the third site is an effluent discharge point of an industry. The samples were collected in sterile bottles and were brought to laboratory maintaining a cold chain and refrigerated.

Enrichment and isolation of actinomycetes

For enrichment, 1 mL of each selected sample was transferred to 100 mL of starch casein broth supplemented with 25 mg/mL cycloheximide and 25 mg/mL nalidixic acid (Kumar and Kannabiran, 2010) and incubated at 30°C for 7 days in shaker at 200 rpm. Isolation of actinomycetes was done by the serial dilution and pour plate technique. A loopful of inoculum from the starch casein broth was streaked onto the starch casein agar (SCA) supplemented with 50 µg/mL fluconazole and incubated at 30°C for 7 days (Savitri and Azmi, 2003). Single separated colonies were selected and the subcultures were maintained on starch casein slants at 4°C until further use.

Identification of actinomycetes by coverslip method

The isolated strains were confirmed as actinomycetes by observing their morphology under microscope. The starch casein agar was poured on sterile slide and allows solidifying. Then the organisms were streaked on it and incubate at 37°C for 48 hrs. After that added 2 drops of methylene blue dye and allow it for a minute. Then the slide was covered with cover slip and observed their morphology under microscope (Prazeres *et al.*, 2004).

Biochemical characterization of isolates

Biochemical characterization of the actinomycete cultures was carried out based on Bergeys Manual of Determinative Bacteriology (1994) and Cappucino and Sherman, (1999).

Gram staining

Gram staining of isolates was done as per Bergeys Manual of Determinative Bacteriology (1994).

Reagents used were crystal violet, gram‟s iodine, 95% ethyl alcohol and safranin. The actinomycete cultures were stained with crystal violet for one minute and excess stain was washed off with tap water. Gram‟s iodine application was carried out for one minute and then washed off with tap water. 95% alcohol was added drop by drop and washed off with tap water. Counter stained with safranin for 45 seconds. Washed off the safranin with tap water, and examined under oil immersion. Development of purple colour represented positive test and pink colour represented negative test.

Methyl red and Voges Proskaeur test (MR-VP)

Glucose phosphate medium was taken in two sets of test tube. Inoculated 100µL of actinomycete cultures to each of the test tube and incubated at 37°C for 48 hours. Added a few drops of methyl red after incubation, to one set of tubes and noticed the colour change. Positive reaction was indicated by the appearance of red colour. The glucose phosphate medium having neutral pH in the experiment was converted to acidic pH, when the microbes introduced fermented glucose releasing acid into the medium. Methyl red indicator shows red colour at pH 6.9.

Barrits reagent Solution A (0.5 mL) was added to the second set of tubes, and shaken vigorously till pink/ red colour appeared. Voges-Proskeaur test differentiates the actinomycete producing large amounts of acid. These organisms grow anaerobically on glucose via respiratory mechanism and shift to butenediol fermentation via EMP. Acetion formed in the culture is oxidized into diacetyl in the presence of oxygen and potassium hydroxide. Diacetyl reacts with VP reagent gives a crimson red colour complex.

Gelatinase test

Inoculum of 18-24h old test actinomycetes was inoculated into tubes containing nutrient gelatin. The inoculated tubes along with uninoculated control were incubated at 25°C for 7-10 days. After incubation, the tubes were placed in refregirator for 15 to 30 minutes. Afterwards, hydrolyzed gelatin showed liquified medium against the uninoculated control medium in solid form (Stapp, 1953 and Shejul, 1999).

Oxidase test

Reagent- Dissolve 100 mg (1 mg in 10 mL) of N, N, N, N tetramethyl-P-Phenyl-enediamine dihydrochloride in 100 ml distilled water. Filter paper soaked with oxidase reagent was placed on glass slides. Streaked actinomycete cultures on the filter paper. Positive reaction was indicated by the appearance of purple colour. This test was used to detect the production of oxidase enzyme by microorganisms.

Urea hydrolysis

Cultures were grown in media containing (gL-1) yeast extract (0.1), monopotassium phosphate (9.1), dipotassium phosphate (9.5), urea (20), and phenol red (0.01). pH was adjusted to 6.8. Filter sterilized the medium and dispensed 5 ml volume in tubes. Inoculated the culture and incubated for 48-72 hours. Positive reaction was indicated by cherry red colour.

Catalase test

A drop of 3% hydrogen peroxide was placed on a clean glass slide. The actinomycete colonies were transferred into it. Formation of effervescence was considered as a positive catalase test.

Starch Hydrolysis

Cultures were grown in media containing (gL^{-1}) beef extract, peptone, starch and agar. Prepared the starch agar medium and sterilized at 120°C for 20 minutes. Streak the actinomycete cultures on it and incubated for 48 hours at 37°C. Iodine solution was poured into it. A positive result was indicated by the streaked culture as colourless and medium in blue black colour.

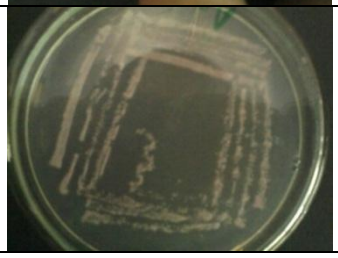
Results and Discussion

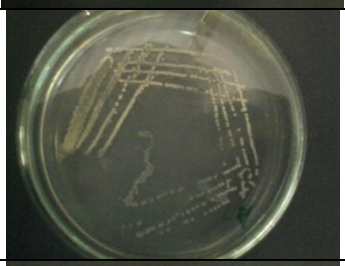
Basics of isolation and characterization of actinomycetes from the marine samples are studied and presented. Marine samples when inoculated in the enrichment media showed heavy growth of microorganisms after 7 days of incubation. Standard Plate Count was made to enumerate and isolate actinomycetes from enrichment. A total of 20 actinomycetes strains were isolated from the selected marine samples. Ten isolates (A1, A2, A3, A4, A5, A6, A7, A8, A9 and A10) from Sanghumukham, seven isolates (B1, B2, B3, B4, B5, B6 and B7) from Vizhinjam and three isolates (T1, T2 and T3) from Veli, coast. The morphological characters of the strains were identified using cover slip method. The results are shown in Table 1. Previous studies have shown that, actinomycetes are ubiquitous in nature including water and sediments of marine and estuarine environments. The use of cover slip method enables the visualization of the fragmented spores in the agar media without disruption and breakage.

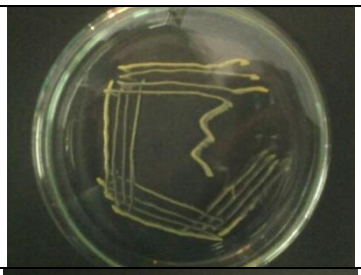
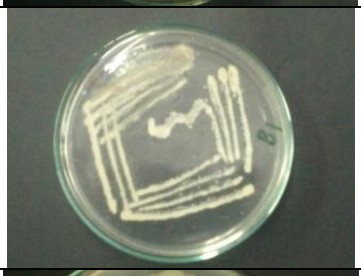
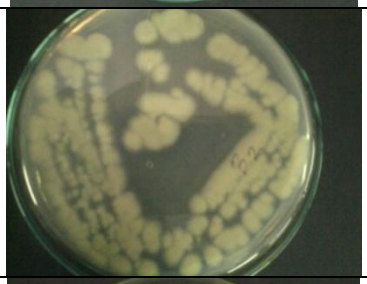
Biochemical characterizations of the isolated actinomycetes strains were done using standard procedures and the results are shown in Table 2. In the present study out of the 20 isolated actinomycetes strains 50% of the strains were isolated from Sanghumukham coast, 35 % from Vizhinjam coast and the least number of isolated strains i.e. 15% are from Veli coast. This difference may be due to the nature of the sample.

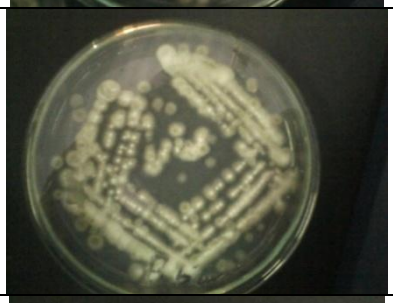
The ecological features of the habitat in which marine organisms dwell, effects the metabolic functions of them, enabling their bio molecular machinery. Veli coast is sited near to an industrialized area which in turn affects the nature of the locale and there by the organisms existing there. There are reports that the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physiological conditions. The ability to utilize a wide range of substrates suggests better survival in different environments. These views are better supported in the present study also. So the present study reveals that marine ecosystem can be considered as a good source of actinomycetes strains. Previous studies also shown that marine environment is still an untapped source of diverse group of actinomycetes with unique biological functions (Maldonado *et al.,* 2005). Further studies on the molecular characterization of the isolates are in progress. The part of work involving screening of marine actinomycetes for bioactives is published, (Lekshmi *et al.,* 2014).

Table: 1. Colony morphology of isolated actinomycetes

Name of the Colony isolated	Morphology	Figure
A1	Branching, Off white, slightly creamy, irregular	
A2	Pink, Branching ,Irregular, Dry	

A3	Off white, Irregular, Powdery, Slightly creamy	
A4	Grey, Powdery, Irregular, Dry, Early stage- off white	
A5	Grey, Powdery, Irregular, Branching, Dry	
A6	Slight grey, Powdery, Branching, Irregular	
A7	Dry, Off white, Irregular, white margin, Dark cream dots at centre	
A8	Off white, cottony, Irregular, Dry, yellow pigment producing	

A9	Yellow, Creamy, opaque	
A10	Greyish white, Branching, Irregular	
B1	Slightly pink, branching	
B2	Off white, Branching, Powdery, Irregular	
B3	Off white, Waxy surface	
B4	White, Dotted, Slimy, Non branching	

B5	White, Become grey when mature, Branching, opaque, Dry, Irregular	
B6	Off white, Grey at centre, Branching, opaque, dry, irregular, medium size	
B7	Creamy, Branching, Irregular, opaque, Medium sized	
T1	Yellow, Immature colonies-off white, Creamy, Dotted	
T2	Brown pigment producing, Dry, cottony surface, irregular, opaque	

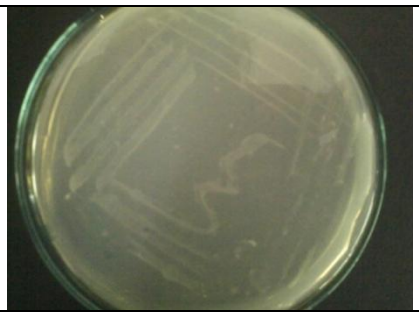
T3	Off white, creamy, Irregular	

Table: 2 Biochemical characterization of the isolated actinomycetes strains

Strains	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	B1	B2	B3	B4	B5	B6	B7	T1	T2	T3
Gram's staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
Voges proskauer	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	-	-
Gelatinase test	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
Oxidase Test	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Urea hydrolysis	-	+	-	-	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	-
Catalase Test	+	+	-	-	-	+	+	-	+	+	+	-	-	+	+	+	-	+	+	+
Starch hydrolysis	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	-

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