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An Investigation On Morphological Characterization Of Actinomycetes Isolated From Marine Sediments.

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ABSTRACT

Marine actinomycetes have immense potential as producers of distinctive bioactive compounds due to its extraordinary adaptation in the insensitive environment in the ocean. The isolate was examined on the basis of their color, surface morphology, formation of aerial and substrate mycelia, production of enzymes and characterization. Serial dilution technique was used in order to reduce the bacterial growth. Starch casein nitrate agar was used for isolation and propagation of actinomycetes from marine soil. The biochemical test starting from the Gram staining test, Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Lipase test, Urease utilization test, IMVIC test, Salt tolerance test and MacConkey agar test were performed. Finally the isolation and investigation revealed that the selected strain is an Actinomycetes.

Keywords: Actinomycetes, characterization, propagation, adaptation, morphology, bioactive.

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INTRODUCTION

The marine ecosystem severs as a wealthy source of compounds possessing novel structures and biological behaviors. Marine flora, fauna and their biological compounds are an attractive sources of bioactive molecules with applications in a wide channel across agricultural, pharmaceuticals, agrochemicals, nutritional supplements, cosmetics, enzymes, molecular probes and fine chemicals, etc,[1,2]. Actinomycetes have been considered as a separate group of microorganism, positioned in between the true bacteria and the true fungi [6]. Actinomycetes, belonging to the class actinobacteria and the order actinomycetales, are ubiquitous, free living, slow growing, filamentous, gram positive, saprophytic eubacteria having DNA with a high GC content [3]. The ecological parameters such as soil temperature, soil pH, soil nature, cultivation, organic matter content, exposure to air and moisture content, influences the number and the type of actinomycetes present in a meticulous soil [4]. However, alkaliphilic actionmycetes are widely distributed and are easily isolated from the marine surroundings. They are capable of synthesizing, wide varieties of biologically active secondary metabolites, which include more than 90% of practical potent antibiotics, vitamins, herbicides, anti-parasitic inhibitors, immunosuppresent, enzyme inhibitors, pesticides, enzymes, cosmetics and nutritional supplements, etc, [3]. They diverge significantly in their physiology, morphology and biochemical and biological activities such as antibacterial, antifungal, antialgal, antiviral, anticancer, antimalarial, antioxidant, anti-inflammatory and neuritogenic activities [5]. The genus, *streptomyces*, which are predominantly salt tolerant, have been responsible for the production of more than 80% of known antibiotics [7]. It is fascinating to point that the chemical constituents of biologically active metabolites synthesized by microorganisms vary predominantly as the result of their extreme living environments, signifying marine actinomycetes might produce a broad range of secondary metabolites when compared to those of the same species at the less exigent environments. Thus, the objective of the present study was to isolate actinomycetes from soil habitats of Muttukadu coast, Chennai. It is a backwater area of the Bay of Bengal located 36 km from the city centre and 23 km from Adyar on the way to Mamallapuram. It lies between 11° 00' to 12° 00' latitudes and 77° 28' to 78° 50' longitudes. Further to characterize and identify them on the basis of cultural, physiological, morphological and biochemical features in a brief manner.

MATERIALS AND METHODS

Collection of Soil Sample

Marine soil sample were collected from Muttukadu coastal region, Chennai, India. The samples were collected from 2 – 3 inches below the soil surface and were packed in ziplock covers and stored in container with icepacks (~4°C) for the transportation. For further processing, the soil samples were air dried for 3-4 days in shade [8,9].

Isolation of Actinomycetes from Soil Sample

Actinomycetes were isolated by serial dilution plate technique using Starch Casein Nitrate Agar Media. The medium were composed of (g/l): starch- 10.0; casein- 0.3; dipotassium hydrogen phosphate- 2g; potassium nitrate- 2.0; magnesium sulphate- 0.02; sodium chloride- 2.0; calcium carbonate- 0.05 and agar- 20.0[10]. The media were supplemented with a pinch of nalidixic and cycloheximide to inhibit unwanted bacterial and fungal contamination respectively [11]. An aliquot of 0.1ml sample were serially diluted upto 10^{-9} and a pour plate technique were performed and incubated for 3-4 days at 28°C. The actinomycetes colonies were identified by their chalky, powdery colonies and leathery texture [12]. These colonies were sub cultured and maintained at 4°C for further characterization.

Microscopic Characterization

The Gram stain is a differential stain, which allows the majority of bacteria to be categoriesed into two classification, gram-positive bacteria and gram-negative bacteria [13]. The cover slip culture method was used for microscopic characterization which includes the following procedure; the glass slides were cleaned and dried with tissue paper. Drops of inoculum were spread over the centre of the slide and were allowed to air dry. The smear were fixed over flame and flooded with Crystal Violet and kept for one minute. Then the slides were repeatedly washed with tap water to remove the excess stain. The washed smear was flooded with Gram's Iodine and treated with decolourization 95% ethanol. Further the final reagent, the counter stain

Saffranin was flooded for a second and washed [14]. The slides were completely dried and observed under microscope.

Starch Hydrolysis Test

The salt hydrolysis test determines the ability of microorganisms to degrade polysaccharide starch in the media by producing hydrolytic extra cellular enzymes. Starch Agar Medium (starch – 2g/L, peptone - 5g/L, beef extract – 3g/L, agar – 30g/L) was prepared and sterilized. The single streak inoculation technique was adopted; the sample organism was streaked in the centre of the plate and incubated for 24 hrs at 37°C. After incubation the plates were flooded with Gram's iodine solution and observed for clear zone.

Imvic Test for Enteric Bacteria

On the basis of differentiation of the gram negative enteric bacteria from the family of enterobacteriaceae, the IMVIC is performed. It includes four types of test such as (i) Indole production from tryptophan, (ii) Methyl Red, (iii) Voges Proskauer test, (iv) Citric acid (citrate) utilization test.

Indole Production Test

The Indole Tryptone Broth (tryptone – 10g/L) was pre-sterilized and inoculated with the sample culture. The test tubes were incubated at 37°C for 48 hrs, finally tested with Kovac's reagent. With care, test tubes were shaked vigorously for 2mins and observed for colored bands.

Methyl Red Test

The Methyl Red broth (peptone -7g/L, glucose- 5g/L, potassium phosphate- 5g/L) was autoclaved at 15lb/inch² for 15mins, inoculated with sample and incubated at 37°C for 48hrs. After incubation five drops of methyl red indicator were added and observed for color change.

Voges Proskauer Test

The Voges Proskauer broth (peptone -7g/L, glucose- 5g/L, potassium phosphate- 5g/L) was autoclaved at 15lb/inch² for 15 mins, inoculated with sample and incubated at 37°C for 48hrs. After incubation, few drops of Barritt's reagent were added and the tubes were gently shaked for 2 mins and incubated for 15mins for the complete reaction. Further the color changes were observed.

Citrate Test

The slants were prepared for Simmon's Citrate Agar Medium. By stabbing and streaking method the samples were inoculated in the respective slants and incubated at 37°C for 48 hrs. The results were examined.

Casein Hydrolysis Test

Casein is a complex protein which is responsible for the fair color of milk. The milk agar medium was autoclaved and poured in petri-plates. The milk agar composition is as follows, skim milk powder-100g/L, peptone -5g/L, agar -15g/L. Inoculation of the sample as a single streak was done and incubated at 37°C for 48hrs. The results were noted.

Urea Hydrolysis Test

The christensen's urea agar medium (peptone- 1g/L, dextrose -1g/L, sodium chloride- 5g/L, potassium phosphate monobasic- 2g/L, phenol red- 0.012g/L, agar- 15g/L) was prepared and sterilized in autoclave at appropriate parameters. After sterilization the medium was cooled to palm bearable temperature and 20g/L of urea was added. Mixed well, inoculated and incubated at room temperature for four days. The results were observed.

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Lipase Test

This test determines the capacity of the particular organism to produce the lipase enzyme in the medium**.** The Tributryin agar medium (peptone -5g/L, yeast extract- 3g/L, agar- 12g/L) was pre sterilized and poured in petri plates at the required rates. The samples were streaked on the medium and incubated for four days at room temperature.

Gelatin Hydrolysis Test

Gelatin is composed of short amino acid polymers and their derivatives. They are considered as nitrogen and carbon source for a wide variety of microbes. Nutrient gelatin agar medium(gelatin- 120g/L, beef extract- 3g/L, peptone-5g/L, agar-15g/L) were sterilized and inoculated by the sample organism and incubated at 35°C for ten days. After incubation the tubes were kept in ice to check liquefaction of the medium.

MacConkey Agar Test

MacConkey agar test is a differential test which supports only the gram negative bacteria. Hence Macconkey \4120gar medium (peptone-17g/L, protease peptone-3g/L, lactose- 10g/L, bile slats- 1.5g/L, sodium chloride- 5g/L, neutral red- 0.03g/L, crystal violet- 0.001g/L, agar-13.5g/L) were autoclaved and inoculated by organism. The results were observed after four days of incubation at room temperature.

Salt Tolerance Test

This determines the capability of an organism to withstand and grow at high salt concentration rates. Thus the sodium chloride concentration in the starch casein nitrate agar medium was increased up to 6.5 times higher and the results were examined after incubation for 3- 4 days at 37°C.

RESULTS

The samples which were serially diluted, spread over by the spread plate technique and incubated for 48hrs, a single colony was chosen for future work based upon the appropriate morphology. These were further subcultured using the continuous streaking method. In order to obtain the single pure colony, a quadrant streaking was done and preserved in glycerol agar medium.

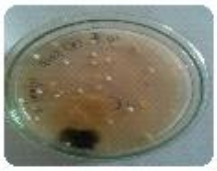

Figure 1(a): Pour Plate Method

Figure 1(b): Continuous Streak Plate Method

Figure 1(c): Quadrant Streak Method

Figure 1: Isolation of Actinomycetes; 1(a) Pour plate method, 1(b) Continuous Streak plate method, 1(c) Quadrant streak method

Gram Staining

The organism were confirmed as gram positive due to their purple colour cells, which reveals that they accept and retained the Gram dye like Grams crystal violet.

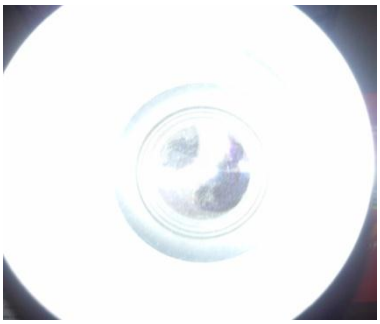

Figure 2: Gram Staining result

Starch Hydrolysis

After the incubation period, the iodine solution was flooded over the surface and observed for 10mins, a clear zone around the organism was seen while the other area were covered with iodine solution. This is because, the organism was surrounded by the amylase enzyme produced by them. The amylase was produced in order to degrade the starch present in the medium.

Figure 3(a): Before iodine incubation

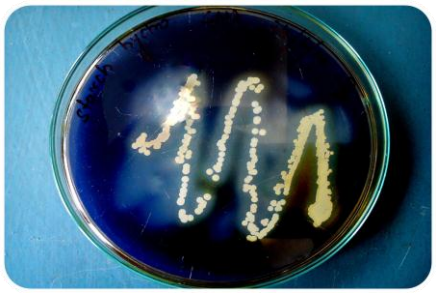

Figure 3(b): After iodine incubation

Figure 3: Starch Hydrolysis Result; 3(a) Before iodine incubation, 3(b) After iodine incubation

IMVIC Test

Indole Test

Tryptophan is an essential amino acid that can undergo oxidation by the enzymatic activities of majority of bacteria. Conversion of tryptophan into metabolic end products is mediated by the enzyme tryptophanase. The presence of indole is detectable by adding Kovac's reagent, which develops a strong cherry red reagent layer. The color is produced by the reagent, which is composed of P- dimethylaminobenzaldehyde,

butanol and hydrochloric acid. Indole is extracted from a medium into the reagent layer by the acidified butanol component and forms a complex with the P- dimethylamino benzoldehyde, yielding a cherry red color (Rasindole dye). Figure 6 represents the absence of the red coloration which demonstrates that the substrate tryptophan is not hydrolyzed and indicates an indole negative reaction.

Figure 4: Indole test result with absence of cherry red ring

Methyl Red Test

Methyl red is a pH indicator with a range between 6(yellow) to 4(red). It is a quantitative test for the detection of mixed acid production such as lactic, formic, acetic and pyruvic acids, etc., from glucose through mixed acid fermentation pathway. Thus to produce a color change, the organism must generate large amount of acid from the carbohydrate substrate being used. Figure 5 represents the presence of pink color which indicates a positive result.

Figure 5: Methyl Red result indicating the positive pink color change

Voges Proskauer Test

The active product formed after bacterial metabolism is acetyl methyl carbinol (acetoin) a neutral reacting end product. It is a product of butylene glycol pathway. Pyruvate is formed during the fermentation degradation of glucose, which is further metabolized by bacterial enzymes through butylene glycol pathway and produce acetoin. In the presence of oxygen and potassium hydroxide, acetoin gets converted into diacetyl and creatine. α- naphthol serves as a catalyst to bring out the final red color change. Figure 6 indicates the pale yellow color after incubation hence it indicates the negative result.

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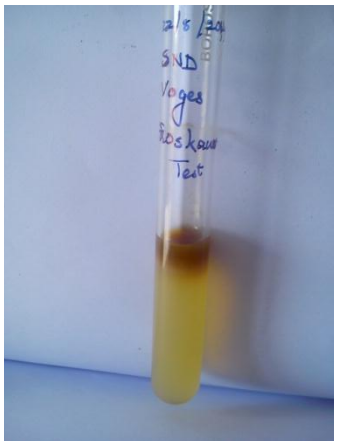

Figure 6: Voges Proskauer result indicating negative result

Citrate Utilization Test

Figure 7 represents a positive result by the development of a deep blue color within 24-48 hrs, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products.

Figure 7: Citrate utilization indicating a positive result

Casein Hydrolysis Test

The casein present in the media is degraded by the protease. This presence of enzyme is indicated by the clear zone, around the organism. Thus it clearly reveals that the test organism is capable of producing protease.

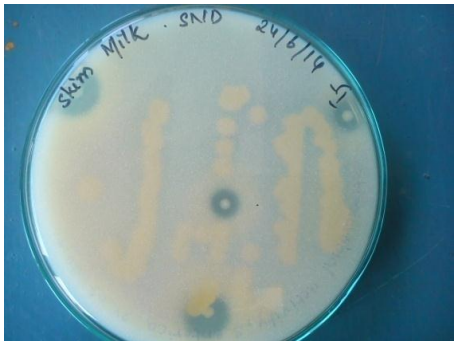

Figure 8: Casein Hydrolysis result proves positive result by zone formation around the organism

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Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea, to produce ammonia and carbon dioxide. But in the figure 9, the organism has not utilized the urea present in the medium as a substrate to produce the urease enzyme resulting in the absence of a red color zone, which shows that the organism does not have the ability to produce the enzyme.

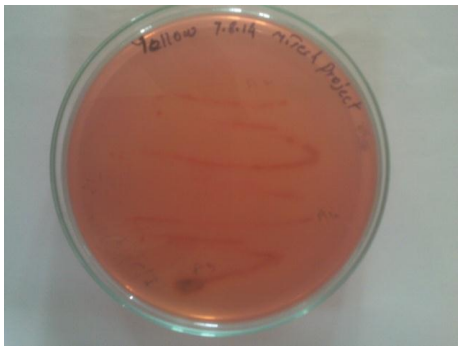

Figure9: Urease test

Lipase Test

Tributryin oil serves as a fat molecule. The lipase test is used to test whether the organism has the capability to produce lipase enzyme to degrade the complex fat molecules present in the media, while in the figure12, it indicates a negative result by showing no change after incubation.

Figure 10: lipase test

Gelatinase Test

The organism was tested for the gelatinase production, where the gelatin is used in the media. After the incubation, the samples were kept in ice for 1-2 hrs where the media still remains unsolidified which indicates the gelatin have been hydrolyzed by the organism by producing gelatinase enzyme.

Figure 11: Gelatin Hydrolysis test before Incubation

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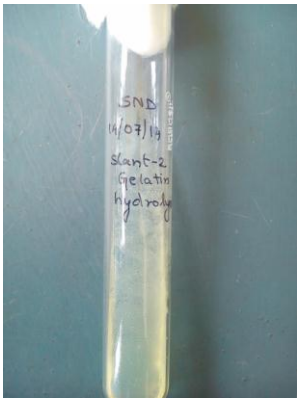

Figure 12: Gelatin Hydrolysis after incubation

MacConkey Test

After the incubation period of inoculums in MacConkey agar media, the growth of the organism was not seen, which concludes that the isolated test organism are Gram positive bacteria.

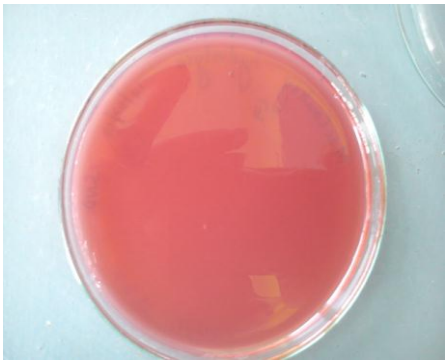

Figure 13: MacConkey agar test

Salt Tolerance Test

The ability of the organism to tolerate the maximum amount of salt was tested using the salt tolerance test. The organism survived at high amount of salt concentrations. Thus they were found to be salt tolerant.

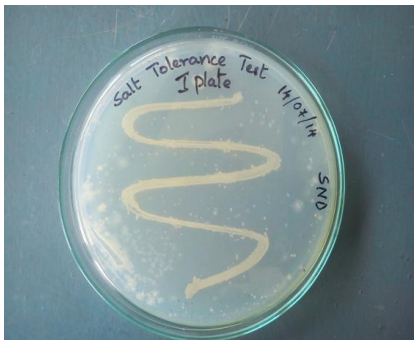

Figure 14: Salt Tolerance result

DISCUSSION

The growth of actinomycetes was observed in the 10^{-4} dilutions. In the biochemical characterization such as MacConkey agar test, growth of the actinomycetes was not observed. In urease test a negative result

was obtained which proves that it does not support the organism. The gelatin test yields a positive result by unsolidifying the medium. The isolated strain was capable of utilizing the casein (an exoenzyme) and a higher concentration of salt in the medium. While performing the gram staining test, it showed purple color indicating the positive result. The isolated actinomycetes showed the ability to produce the amylase enzyme when iodine solution was flooded into the medium surface.

CONCLUSION

In the present study, the organism was isolated from the marine coastal soil and the various biochemical characterization studies were performed such as gram staining, starch hydrolysis, IMVIC test, casein hydrolysis, lipase hydrolysis, gelatin hydrolysis, urease test and salt tolerance test. The isolated strain showed up the production of gelatinase, protease, amylase etc., in the appropriate substrate medium by the zone formation. The microscopical, physiological, morphological and cultural studies reveal that the isolated and investigated strain is an actinomycetes. More studies should be done on the isolated actinomycetes to utilize potential actinomycetes for anti-cancer studies.

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