ANTAGONISTIC ACTIVITY OF MARINE ACTINOMYCETES AGAINST DENTAL PATHOGEN *Streptococcus mutans.*

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Received: July 12, 2018 Accepted: August 21, 2018

ABSTRACT *The present study was an effort to isolate and determine Antagonistic property of Marine Actinomycetes against Dental Pathogen 'Streptococcus mutans'. Here, six marine sediment samples collected from the coast of Shangumugham and Valiyathura of Thiruvananthapuram district and Muzhappilangad of Kannur district Kerala. 112 actinomycetes isolates were obtained. Out of 112 marine actinomycetes isolates, 45 isolates were selected for the study of antagonistic activity against the dental pathogen Streptococcus mutans. Streptococcus mutans strain was isolated from plaques of dental caries patients. One actinomycete isolate showed significant antagonistic property against Streptococcus mutans, this isolate was mass cultivated by solid state fermentation and the ethyl acetate extract of its secondary metabolite was partially purified by TLC.*

Keywords: *Marine Actinomycetes, Dental Pathogens, Streptococcus mutans.*

INTRODUCTION

Dental caries is one of the most prolonged infective diseases in the world. The initial stage of dental caries is considered by a damage of dental structures caused by acids and its by-products of carbohydrate metabolism by cariogenic bacteria (Botelho *et al*., 2007). *Streptococcus mutans* is the major causative agent of dental caries in men and its virulence property includes development of dental plaque (Hamada *et al.,* 1980; Kawabata *et al.,* 1999). Dentists recommended seven to eleven percentages of all common antibiotics for the treatment of dental caries (Cleveland and Kohn, 1998). But one-third of all out-patient antibiotic recommendations are unnecessary (Swift and Gulden, 2002). Continued exposure to antibiotics at its inappropriate dosages results in serious side effects. The increasing antibiotic resistance problems of recent years may be due to overdose or misuse of antibiotic agents like cephalosporins and fluoro-quinolones (Wise *et al.,* 1998; Buke *et al.,* 2005). In this situation discovery of novel antibiotics and new therapeutic agents for control the dental caries pathogen *Streptococcus mutans* is essential.

The ocean covers more than 70% of earth and little is known about its microbial diversity (Atta *et al.,* 2011). Among marine microorganisms, actinomycetes have the principal genomics and metabolic diversities to produce complex metabolites with unusual biological properties. The actinobacteria have significant role in the marine microbes, for of its diversity and capability to produce novel chemical compounds of high commercial value (Hopwood, 2007; Amador *et al.*, 2003). The study 'Studies on Marine Actinomycetes Antagonistic to Dental Pathogen *Streptococcus mutans*' concentrate to identify the antagonistic property of marine actinomycetes against *Streptococcus mutans.*

MATERIALS AND METHODS

Sample Collection

Six marine sediment samples were collected in sterile, disposable plastic containers from three to five feet depth of Shangumugham and Valiyathura coast of Thiruvananthapuram district and Muzhappilangad coast of Kannur district Kerala state, India. The samples were tightly packed and transported to the Laboratory (Sirisha *et al*., 2013).

Isolation of Actinomycetes from Marine Sediment Samples

The samples were serial diluted in sterile sea water and spread on to starch casein agar plates. The plates were incubated at 28^0 C for one to three weeks. After incubation, actinomycetes colonies were morphologically analyzed and selected.

Purification and Preservation of Marine Actinomycetes

By repeated sub-culture techniques the selected actinomycete isolates were purified and the purified isolates were streaked on starch casein agar slants and preserved at 4^0 C (Deepa *et al.,* 2012).

Collection of Dental swab samples

Dental swab collected form dental caries patients, soaked in sterile saline were transported to the laboratory stored in the refrigerator at 4^0Cfor further analysis.

Isolation, Selection, Purification and Preservation of Bacterial isolates *Streptococcus mutans.*

The dental swabs were streaked on to mitis salivarius (MS) agar plates. The plates were incubated at 37^0 C for 24 – 48 hours and after proper incubation, plates were observed and the colonies formed were morphologically analysed (Raja *et al.,* 2010). *Streptococcus mutans* colonies were selected by microscopic and macroscopic analysis. *Streptococcus mutans* colonies were purified by repeated sub-culture on MS agar plates. The purified bacterial isolates streaked on to Nutrient agar slants and stored at 4^0 C.

Antibiogram Study

Kirby-Bauer disc diffusion method used for Antibiogram study of *Streptococcus mutans* against antibiotics which is used for dental caries. The isolate was effectively swabbed on Muller Hinton Agar plates and antibiotic disc was placed. Then the plates be incubated at 37^0 C for 24 hours.

Antibacterial Activity of Actinomycetes:

a. Primary Screening by Agar overlay method.

Actinomycete broth culture was single streaked on the Muller Hinton Agar plates and incubated at 28^0 C for three days and soft agar medium contained *Streptococcus mutans* culture was poured over the actinomycete growth on Muller Hinton Agar plates then the plates were incubated at 37^0C for 24 hours (Kumar *et al*., 2010).

b. Secondary Screening by Well Diffusion Method and Disc Diffusion Method

12 hours young over-night *Streptococcus mutans* culture was effectively swabbed on the Muller Hinton Agar plates, made wells on it and 50 µl cell free culture filtrate and cell lysate extract were loaded in separate wells. The plates were incubated at 37^0 C for 24 hours. (Kumar *et al*., 2010). In the same time, 12 hours young *Streptococcus mutans* culture was swabbed on another Muller Hinton Agar plates and the 100µl cell free culture filtrate and intra cellular extract were loaded discs were placed aseptically on pre-inoculated agar plate surface using sterile forceps. The plates were kept incubator at 37.0^0C for 24 hours. After incubation the plates were observed and measured the zone of growth inhibition around the wells and discs (Bauer *et al.,* (1966).

Microscopic, Macroscopic, Biochemical Tests and Physiological Characterization of Marine Actinomycete Isolate

The isolate was microscopically analysed by Gram's staining, Motility Test, Capsule staining. Morphological characters were observed macroscopically. The biochemical tests include Indole Production Test, Methyl Red Test, Vogus- Proskaur Test, Catalase Test, Oxidase Test, Citrate Utilization Test, Nitrate Reduction Test, Triple Sugar Iron Test, Urease Test, Carbohydrate Fermentation Test, Salt Tolerance Test, Starch Hydrolysis, Casein Hydrolysis, Gelatin Hydrolysis, Lipid Hydrolysis, Esculin Hydrolysis, and Hemolytic Activity.

Ethyl acetate extraction of bioactive compound of marine actinomycetes

Marine actinomycete isolate was inoculated into ISP1 broth and grown up for five days in a shaker cum incubator at 28.0^0C and 120rpm. The isolate was swabbed on 50 numbers of starch casein agar plates and incubated for five to seven days at 28.0^0C. After proper massive growth of actinomycetes, the biomass along with the agar was sliced into small pieces using sterile spatula and it was saturated with ethyl acetate for 24 hours in shaker cum incubator at 150rpm. The extract was filtered (Adinarayana *et al*., 2007).

Thin layer chromatography and Antibacterial activity study by well diffusion method

The ethyl acetate extract of actinomycete isolate with antibacterial activity was further purified by thin layer chromatography. The elute of actinomycete isolate was spotted on the TLC sheet on the origin spot. The development tank was saturated with chloroform and methanol as mobile phase in the ratio of 9:1. The final solvent front was marked and TLC sheet was dried. The spots observed by exposing to Iodine vapour as well as UV light. The fractions showing the same R_f were combined together by scrapping of TLC sheet.

The antimicrobial activity of the ethyl acetate extract was checked by well diffusion method as bacterial cultures were swabbed on Muller Hinton agar plates. Six mm diameter wells were made on it. 100 µl elute was loaded in separate wells and the plates were kept at room temperature for one hour. The plates were incubated at 37.0^0C for 24 hours (Dhanasekaran *et al.,* 2008).

RESULT AND DISCUSSION

Isolation and selection of Marine Actinomycetes

112 actinomycete isolates were obtained and 45 isolates were selected for further studies based on their colony morphology and predominance. Bulks of the colonies were moderate to small size but few large colonies. Round, irregular, filamentous and spindle shaped colonies were observed. Colour of the colonies were white at the beginning later the colour changed to off white, creamy and grey or cement colour on attaining full growth. Majority of the isolates were with entire margin in addition to curled and undulate margin. All colonies selected were opaque. In case of elevation many colonies were flat and few rose elevations also observed. Sujatha *et al.* (2005) identified 88 isolates of actinomycetes and based on sporophore morphology and arrangement of the spore chain, 64 isolates were assign to the genus *Streptomyces*, 8 isolates to the genus *Micromonospora,* 5 to the genus *Nocardia*, 7 to the genus *Streptoverticilium* and 4 to the genus *Saccharopolyspora.*

Isolation, Selection and antibiogram properties of *Streptococcus mutans.*

The selection of *Streptococcus mutans* was purely based on their distinct morphological characters and predominance. Selected strains of *Streptococcus mutans* were slight blue coloured colony with translucent, white mucoid appearance (figure 1) and the purified strains were characterized by microscopic (Table 1). Raja *et al*., (2010) isolated three oral pathogenic bacteria from decayed tooth sample.

Figure. 1 *Streptococcus mutans* on MS agar

Table 1. Microscopic Characterization of *Streptococcus mutans*

S No	Test	Response of *Streptococcus mutans*
1	Gram's staining	Positive, cocci
2	Capsule staining	Capsule present
3	Motility test	Non motile

Antibiogram of *Streptococcus mutans strain.*

Streptococcus mutans showed resistance towards antibiotics such as Amoxicillin, Ampicillin, and Penicillin-G Intermediary response was noticed in case Tetracycline also Sensitivity was noticed in Chloramphenicol, Ciprofloxacin, Kanamycin, and Vancomycin evidenced by a growth inhibitory zone diameter (Table 2).

Table 2.Antibiogram of *Streptococcus mutans strains.*

S No	Antibiotic Discs	Zone of inhibition
1.	Amoxicillin	No zone
2.	Ampicillin	6mm
3.	Chloramphenicol	18mm
4.	Ciprofloxacin	30mm
5.	Kanamycin	18mm
6.	Penicillin-G	No zone
7.	Tetracycline	12mm
8.	Vancomycin	20mm

Primary screening by Agar overlay method.

In primary screening by agar overlay method of 45 actinomycete isolates were screened to detect their antibacterial activity against *Streptococcus mutans.* Among 45 marine actinomycete, 36 (80%) isolates were antagonistic and 9 (20%) were non-antagonistic to the *Streptococcus mutans*. Of the 34 actinomycete isolates antagonistic to the *Streptococcus mutans* five isolates showed significant activity, the remaining isolates showed slight to moderate response. Narendrakumar *et al.,* (2010) screened 117 actinomycetes for its antagonistic property by agar spot method, among them 15 isolates showed significant antibacterial

activity. Raja *et al*., (2010) verified the inhibitory effect of five actinomycetes isolates against *Streptococcus mutans. A*mong those five D*ectylosporanium species* showed maximum activity against *Streptococcus mutans*.

Secondary screening

Well and Disc difussion method.

Well and disc diffusion methods were carried out using the cell free culture filtrate of selected five actinomycete isolates as crude extract against *Streptococcus mutans* (figure 2 and 3). Among the five actinomycete isolates screened, one isolate showed significant activity in terms of production of zone of growth inhibition against *Streptococcus mutans* hence it was selected for further studies.

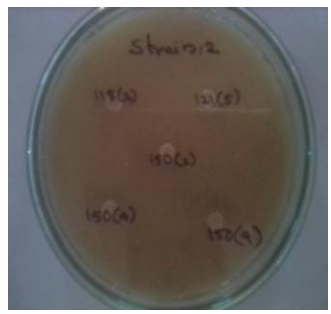

Figure 2. Antibacterial activity study by Well difussion method.

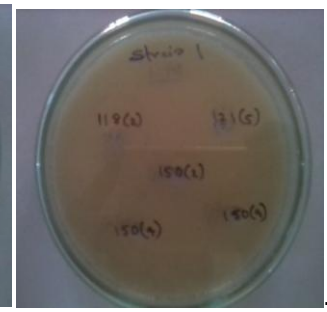

Figure 3.Antibacterial activity study by Disc difussion method.

Characterization of Actinomycete isolate.

The antagonistically significant marine actinomycete was characterized by Gram positive, rod shaped, capsulated and non-motile cells (Table 3). The biochemical test showed positive response to Methyl red, Nitrate, TSI, Citrate, Urease, Oxidase test. But it was negative to indole and voges-proskauer, catalase and it showed gas production in carbohydrate fermentation test. This isolate hydrolyzed bile esculin, casein, starch, gelatin, and lipid. In case of haemolysis it was beta haemolytic. Comparison of these properties to that of the Bergy's manual of Determinative Bacteriology, second edition, the isolate was identified as *Streptomyces sp.* Rofiq and Bambang, (2010) isolated 29 actinomycete isolates and screened for antibacterial activity by disc diffusion method. Eight among them showed antimicrobial activity against both Gram's positive as well as Gram negative bacteria. Morphology of all isolates was same like genus of *Streptomyces*.

Table 3. Microscopic Characterization of Actinomycete isolate

S No.	Test	Marine actinomycete
1	Gram's staining	Positive , rod
2	Capsule staining	Capsule present
3	Motility test	Non motile

Antagonistic activity of Ethyl acetate extract of secondary metabolites of actinomycete isolate.

Ethyl acetate extract of the actinomycete isolate analysed for detection of antagonistic activity against *Streptococcus mutans*, by well diffusion method zone of growth inhibition 38 mm disc difussion showed 35mm (Table 4) exposed that the elute contains bioactive compounds with antagonistic property. Arasu *et al*., (2009) isolated and screened 15 actinobacteria for antimicrobial activity against four multi drug resistant bacteria. Only two isolates were effective in cross streak method. The potential isolate inoculated in SS Medium, the bioactive compound was extracted in different polarity solvents like ethyl acetate and chloroform and the extracts were screened for antimicrobial activity towards drug resistant strains by well diffusion method.

Table 4. Antagonistic property of ethyl acetate extracts of actinomycete isolate against *Streptococcus mutans.*

S No	Method	Response of *S. mutans* (in mm)
1	Well diffusion	38
2	Disc diffusion	35

Thin layer chromatography

The thin layer chromatography of the ethyl acetate extract of secondary metabolite of actinomycete isolate revealed the presence of several spots with very low resolution. Among the spots noticed the rf value of five prominent spots were calculated (Figure 4) (Table 5).

Figure 4. TLC analysis of ethyl acetate extracts of actinomycete isolate

Table 5.rf values of prominent bioactive compounds in the ethyl acetate extracts of actinomycete isolate by TLC.

S No	Solute Front (cm)	Solvent Front (cm)	Rf value
1	0.9	7.1	0.13
2	2.1		0.30
3	3.6		0.51
4	4.9		0..69
5	6.0		0.85

Antagonistic activity of TLC purified bioactive compounds of actinomycete isolate by Auto bioassay method.

Formation of growth inhibitory zone around the spots on the chromatogram evidenced the presence of antibiotic bioactive compounds in the ethyl acetate extract obtained from the actinomycete and hence it was further confirmed by well difussion method (Figure 5)

Figure 5. Antagonistic activity of TLC purified bioactive compounds of actinomycete isolate by Auto bioassay method.

Antagonistic activity of TLC purified bioactive compounds of actinomycete isolate by well difussion method.

The result of antimicrobial activity of the scrap of spots on the TLC plates of the ethyl acetate extract of actinomycete isolate against *Streptococcus mutans* revealed the antagonistic property of the bioactive compounds of the isolate by the production of zone of inhibition 21mm against *Streptococcus mutans* (Figure 6).

(Figure 6). Antagonistic activity of TLC purified bioactive compounds of actinomycete isolate by well difussion method.

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