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Isolation and Identification of Bioactive Compound from Bacillus Megaterium from South East Coastal Region of India against Human Dental Caries

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Abstract*— periodically, marine bacteria are producer of secondary metabolites in the harsh ocean. In this present study, a marine bacterium was isolated from marine cone snail in the Gulf of Mannar. The potential isolates was tested for various biochemical test and 16Sr RNA gene sequencing, which leads to their identification as Bacillus megaterium producing antibiotic compound for treating human diseases. Further, the selected isolates were cultured, purified and screened for antimicrobial activity against a battery of decay causing cariogenic pathogen Lactobacillus acidophilus. This pathogen were identified and analysed by Pg and Research Department of Zoology, Division of Microbial Technology, Chikkanna Govt. Arts College, Tirupur, collected from Fenn Dental Clinic in Tirupur District. Among these, 16 isolates exhibited strong bactericidal properties against tested pathogen Lactobacillus acidophilus. One promising strain, designated as MTBMVG07, with strong antimicrobial activity against cariogenic pathogen.

Keywords*— Antagonistic activity, Bacillus megaterium, Dental caries, L. acidophilus and Secondary metabolites.

I. INTRODUCTION

Sea microbiom has prosperous genetic variety to produce various unique molecules which is not created by their counterparts in worldwide. The organisms existing in marine surroundings have been evaluated to adapt stress conditions also they exert choice of chemical substance to protect itself from predators. Predominantly the invertebrate associated microbes in high antagonism for their resident and nourishment which makes them as an appropriate candidate for exploring pharmacologically promising molecules. A lot of antimicrobial, antifouling substance has been found amongst these kinds of bacteria owing to the dedicated role they participate in their particular hosts (Holmstrom et al. 2002). It is suggested that the chief role of these antibiotic substances could be related to ecological competition.

The naval bacteria *Bacillus megaterium* forever play a very important role in the improvement of numerous drugs and also manufacture highly exhaustive antimicrobial protein against gram-negative bacteria, yeasts, fungi and gram-positive bacteria as well (Abriouel et al, 2011). The natural products from bacteria, mainly the cone snail related organisms, stay after one of the most significant sources of lead compound for the pharmaceutical industry and various have been making the bioactive analogs (Radwana and EL-Sherbiny, 2007) for treating human diseases.

Dental caries are an infection associated with microbes implanted in a template of polymers of bacterial and salivary derivation. The incidence of dental caries is frankly linked to the capability of microbes to colonize the tooth surface and form biofilm to cause dental diseases. Formation of biofilm begins with the attachment of free-floating microbes to a surface.

These initial colonists assist the appearance of others by providing linkage sites and the matrix that holds the biofilm jointly. *Streptococcus mutans*, *Lactobacilli* spp. and *Candida albicans* are the major microorganisms found in dental plaque related with a caries lesion (Signoretto et al., 2009). *Streptococcus mutans*, *Lactobacilli* spp. are measured crucial for the initiation

and progression of dental caries by their additional acidogenic and acidophilic properties than those of other oral bacteria (Shu et al. 2000). They can convert dietary carbohydrates into acid, which lowers the *p*H of the environment, and solubilizes the calcium phosphate of the enamel to create the decay.

Presently various caries preventive strategies are in use like oral health education, chemical and mechanical control of plaque, use of fluorides, application of pit and fissure sealants etc.A numerous of these approaches can be mostly effective. But moreover, most of the antimicrobial agents that are currently in use have been rendered unsuccessful by a wide incidence of multiple drug challenging strains of microorganisms (Owhe – Ureghe *et al.,* 2010).

The emergence of transmittable diseases and drug resistance device developed by communicable microbes makes the natural product scientist to find successful molecules from marine background to delight the disease accurately. The pathogens built-up remedy resistance mechanism increasingly to the bare curative agents caused remerging of infection (Cetina et al. 2010).

Recurrently, bacteria have residential alteration mechanisms next to the action of antimicrobial drugs (Al-Haj *et al.*, 2009). However, economic, behavioral or cultural barriers to their use have sustained the pandemic of disease in the mouth of several people on a overall level. This is a major concern and an urgent need for searching new and safe antibacterial agents to treat dental diseases. Considering above mentioned strategies, the present work was aimed to isolate an effective antimicrobial drug from marine epiphytic bacterium like *Bacillus megaterium* for the latest approach for combating dental caries. That is well suited for public health applications especially in environments that do not lend themselves to regular health care. Our results provide insights into the wide spectrum antimicrobial ability of the identified *Bacillus* species from the Indian coastal environment.

II. MATERIALS AND METHODS

Collection of marine cone snail *Conus amadis*:

The specimens were collected from coral reef islands of the Gulf of Mannar, Rameshwaram, and Tamilnadu, India (Fig. 1). After the sample was then transferred to a sterilized Polyethylene bag stored at -4°C. It was kept in ice box and was brought to the laboratory for further analysis.

Laboratory analysis:

The collected cone snails were killed and venom duct of *Conus amadis* were dissected out in sterile conditions. The dissected sample was kept in mortar and pestles and grind well. The homogenate suspension was serially diluted. After the appropriate dilution was taken for the isolation of associated bacteria from the sample. The different dilutions were drawn and poured onto the surface of SPERBER agar media for isolation (Fig. 2).

Identification of antibiotic producing strain - *Bacillus megaterium*

Phenotypic and Genotypic Characterization of *Bacillus megaterium*

The isolates were identified by gram staining, biochemical test and selective media, 16SrDNA sequencing.

Colony morphology and microscopic examination

During microscopic examination all the isolates were found to be gram positive rods detected by gram staining technique.

Genomic DNA Extraction

The selected colonies were inoculated in Luria Bertani broth and incubated overnight at 28◦C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400µl of Sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and the solution was incubated for 1hr at 37°C. To the tube,100µl of 0.5M EDTA (*p*H 8.0), 60µl of 10% SDS and 3µl of proteinase K were added and incubated at 55ºC overnight. Extracted with equal volume of phenol: chloroform (1:1), centrifuged (10000 rpm; 10 min) and the supernatant was transferred to a sterile tube. The supernatant was extracted twice with phenol: chloroform and once with chloroform: isoamyl alcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water and stored at4°C for immediate use and at 20°C for long-term storage (Willy, 2006).

Collection of test bacteria from dental caries

The fifty different dental clinical samples from various dental clinics and from the patients having dental caries who come for the dental treatment with different age group ranging from 5-17yrs. were collected with the help of an excavator and immediately transferred to 3 ml of saline solution in sterile glass vials.

Information of patient's dental case history was also recorded along with his/her consent. After inoculation, vials were capped and sealed by Parafilm. The packed vials were brought to the laboratory immediately and kept in incubator at 37°C for 24 hours for bacterial enrichment.

Identification and maintenance of test organism

The cariogenic *Lactobacillus acidophilus* were screened, identified and purified by series of sub-culture on specific media such as Man Rogosa Sharpe agar and Nutrient agar were incubated aerobically at 37°C for 24 hours. The identification of all the microbes was confirmed by standard biochemical and staining methods (Aneja, 2003). All the pure cultures were stored and maintained in nutrient broth at 4˚C for further use.

Screening for marine bacteria for antagonistic activity

Primary Screening:

A total of 16 bacterial strains isolated (Marine Agar 2216, High Media) from the venom duct marine cone snail *Conus amadis* were screened for the production of antibacterial substance. In the primary screening, antimicrobial activity was assessed against the target pathogenic microorganisms (Garrity et al. 2006).

From the wholesome cultures, naval bacteria were spotted on intention organisms swabbed in Muller Hinton Agar plate. The zone of inhibition, for all bacterial strain was selected for secondary screening activity.

Secondary screening:

Secondary screening was done by agar well diffusion assay for testing the antagonistic activity of marine bacterial isolates against decay pathogen. The isolated bacteria were inoculated in production media composed of marine broth for the production of derived metabolites. It was incubated on the rotary shaker at 250 rpm at 37ºC for 7 days. The broth was centrifuged at 5000rpm for 30 min. Further, the supernatant was collected and extracted thrice with ethyl acetate crude extract was concentrated by evaporation at 37◦C. The crude extract was used for antimicrobial activity screening. The wells were cut using a sterile cork of 6 mm diameter and 50µl, 100µl and 150µl of supernatant was loaded into each well for assay of antagonistic activity by well diffusion assay against decay pathogen. The results were recorded after incubation at 37◦C for 24 hrs. The isolated marine bacterial strain with antibacterial activity was identified to the species level by observing its morphology and biochemical reaction according to the methods described by Bergey's manual for systemic of bacteriology (Felsenstein, 1989).

III. RESULTS AND DISCUSSION

Preface

The world's ocean, which cover almost 70% of the earth's surface and over 90% volume of its crust, encompass a diverse array of fauna and flora, many of which have no terrestrial counterparts. Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have more novels and unique structure owing to the complex living circumstances and diversity of species and the bioactivities are much stronger.

Collection of marine sample

For above said reason the approach was taken in this present study isolate 16 strains of marine *Bacillus megaterium* from venom duct of cone snail. Among them 16 isolates used in this study one isolates of *B.* megaterium (MTBMVG07) was exhibited the maximum capability of producing antibiotic compounds compare than other isolates.

Identification of Marine Epiphytic Bacteria

The associated bacterium was identified by based on cellular morphology, growth condition, gram staining (Fig. 3), biochemical tests (Fig. 4) and 16S r-DNA gene sequencing and the bacterium was identified as *Bacillus megaterium*.

Genomic DNA extraction

The antibiotic producing *B. megaterium* carrying C-DNA. The molecular size of the C-DNA was calculated to be 8000bp to 10,000bp respectively (Fig.5).

Antagonistic activities – primary and secondary screening

Antibacterial activity of cone snail associated *B. megaterium* was performed by agar well diffusion assay against dental pathogen *L. acidophilus.* Three different concentrations of 50µg, 100µg and 150µg were used. The maximum zone of inhibition 18mm, 20mm, and 22mm was observed in strain No. MTBMVG07 (Fig. 6) followed by minimum zone of inhibition 10mm, 10mm and 11mm was observed in strain No. MTBMVG05 at 150µl concentration of different µg – 50, 100and 150.

The world's ocean has been viewed as the most important and productive source of biomedical compound by the pharmaceutically industry. The marine discovery has a bright future as has been visualized by the current research activities (Fenical, 1997). The marine microbes have been the source of potential pharmaceutical activities. In this study, the antibiotic producing strain *Bacillus megaterium* isolated from marine cone snail (*Conus amadis*), Tamilnadu, and India. The isolates were identified by gram staining, IMViC test, fermentation test, hydrolysis test and selective media. Through, this systematic study couples with molecular study revealed the true identity of the organism. Since the organism has pharmaceutical potentiality it was identified by 16Sr-RNA method. It was identified as *Bacillus megaterium*.

In this present study, the isolates from coastal region of Rameshwaram were identified as *Bacillus megaterium* (our laboratory reference MTBMVG07) have proved pharmaceutically active in nature. The author stating that many members of *Bacillus* group continue to be dominated bacterial workhorse in microbial fermentation for the production of novel proteins (Barbosa et al. 2005).

The present study *Bacillus megaterium* proved that peptides of these microbes exerted a wide range of both antimicrobial effects on gram positive cariogenic pathogen. Sanders *et al*., (2003) reported that dozens of different peptide antibiotics exhibiting antagonism against a broad spectrum of microbes identified from the *Bacillus* genus and Berditsch *et al*., (2007) also reported an antibiotic substance from *B. brevis* destroyed pathogens.

In this study, to obtain the extract of antibiotic compound was checked by well diffusion method for its activity against cariogenic pathogen *L. acidophilus*. It showed the maximum zone of inhibition 18mm, 20mm and 22mm was observed in strain no. MTBMVG07 followed by the minimum zone of inhibition 10mm, 10mm and 11mm was observed in strain no. MTBMVG05. Mirac Yilmaz et al. (2006) found that a total of 29 *Bacillus* species isolated from the soil was analyzed using the agar diffusion method in terms of their general inhibition effects to some test bacteria.

It has been found that isolates are effective against Gram-positive and Gram-negative bacteria whereas their extensive inhibition effect is particularly against Gram-positive bacteria. Furthermore some isolates are more effective against test bacteria when compared to some antibiotics.

IV. FIGURES AND TABLES

Figure 1: Marine Cone Snail
CONUS AMADIS

Figure 2: Colony morphology of *Bacillus megaterium* on different agar media

Figure 3: Gram staining of *Bacillus megaterium*

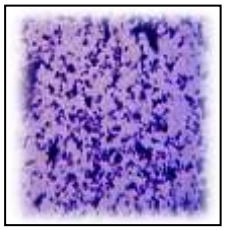
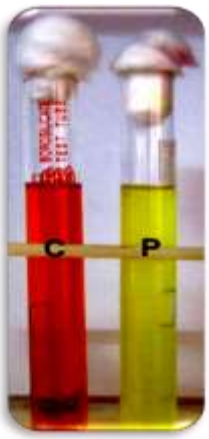


Figure 4: Biochemical characterization of marine *Bacillus megaterium*

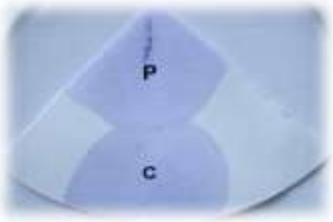
I – Indole; MR – Methyl Red; VP – Voges Proskauer; C – Citrate utilization

Sugar fermentation test

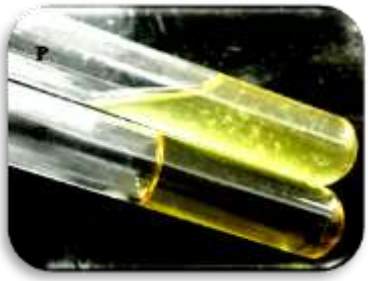


C: Control; P: Positive

Oxidase and Nitrate Reduction Test



Hydrolysis Test



Control; P: Positive

i. Gelatin

Starch

C:

Casein

Trible Sugar Iron Test

C: Control; P: Positive

Urease and Arginine Test

N: Negative

Figure 5: Genomic DNA extraction from *Bacillus megaterium*

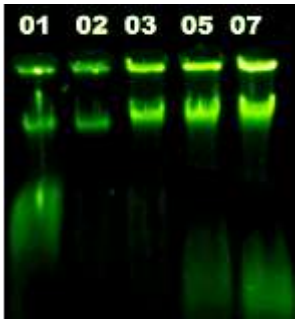


Figure 6: Antibacterial activity of *B. megaterium* against dental pathogen

Table 1: Antibacterial activity of the *B. megaterium* against on 50% resistant *Lactobacillus acidophilus*

S. No	Strain. No	Extract of B.M (Concentration (50µg)		
		50µl	100µl	150µl
1	MTBM05	10mm	10mm	11mm
2	MTBM07	18mm	20mm	22mm

CONCLUSION

Since the beginning of mankind nature has been contributing considerably to drug discovery for human beings against infectious diseases. Natural products are of major importance in the discovery of new therapeutic agents. Apart from plants, bacteria and fungi are the most important producers of such compounds. These natural product resources of marine biotopes occupy considerable place in drug discovery. In this study antibiotic producing marine *Bacillus megaterium* isolated from *Cone snail Conus amadis*, further the strain identified through phenotypic and genotypic characterization. Due to the two methods the strains confirm *Bacillus megaterium.* Further the strain extract chosen for antimicrobial activity against dental pathogen using well diffusion technique.

This research proved that the venomous animal associated microbe has ability to produce novel antibiotic production, biomedical property to treat multi drug resistant isolates and also it act as a weapon of eliminate cariogenic organism in and around oral micro biota.

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