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Molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers

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ABSTRACT

Bacteria from marine environment are an underutilized source of novel antibiotics. Gram-positive bacteria are part of the normal flora of fish from different aquatic environments. The aim of the present study was to isolate and screen enterotoxic pathogenic strains of Bacillus from gut region of local marine tropical fish varieties and their genetic diversity analysis. Tropical marine fishes selected in this study were shark, anchovy, ribbion fish, sole, mackerel, seer, tuna, snapper, sardine, and pomfret commercially available in Bangalore region. The bacteria were isolated on to marine agar. All the strains were morphologically and biochemically characterized to be Bacillus cereus. A total of 10 primers of the OP series were used in the present study. RAPD PCR using primers OPB-08 and OPD-20 generated maximum banding pattern in this study showed high polymorphism between selected Bacillus cereus species. Data obtained from this study will help in understanding genetic variation among the various Bacillus cereus strains isolated form tropical marine fishes which has great economic importance in food industry.

Key words: *Enterotoxic, Bacillus cereus, Lecithinase, RAPD, Polymorphism.*

INTRODUCTION

Nowadays, the study of bacteria from marine origin and their potential role in the production of bioactive compounds is becoming a new topic for research[1]. The number of natural products isolated from marine organism's increases rapidly and now exceeds 18,000, with hundreds of new compounds being discovered every year. The emergence of resistance of bacteria to antibiotics is a common phenomenon. Therefore, there has been a great concern from scientists to investigate marine microorganisms as new source of antibacterial compounds[2]. A number of bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances[3].

Marine fishes are able to produce bioactive compounds with antibacterial activity to protect them from dangerous pathogens. Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. The importance of intestinal bacteria in the nutrition and well–being of their hosts has been established for homeothermic species, such as birds and mammals. However, there is limited information for fish, the poikilothermic vertebrates[4]. With the increasing intensification and commercialization of aquaculture production, disease is a major problem in the fish farming industry[5]. It has been well documented that the use of antibiotics develops drug-resistant microorganisms with antibiotic residues retained in fish flesh and environment. In addition, antibiotics can affect the normal microflora of the digestive tract which is beneficial to host and may be inhibited by treatment with the antibiotics[6]. In this respect, use of probiotic bacteria is a new approach, which is gaining acceptance in aquaculture to control potential pathogens[7,8].

The genus *Bacillus* is comprised a phylogenetically and phenotypically diverse species; they are ubiquitous in terrestrial and freshwater habitat and are also widely distributed in sea water[9]. Bacillus is an interesting genus to investigate for antimicrobial activity as Bacillus species produce a large number of

peptide antibiotics representing several different basic chemical structures[10]. The *Bacillus cereus* group comprises closely related gram-positive bacteria that exhibit highly divergent pathogenic properties[11]. The antibacterial effect of bacteria is generally due to any of the following factors, either singly or in combination: production of antibiotics, bacteriocins, siderophores, lysozymes or proteases, and alteration of pH values by the organic acids produced. The widespread use of antibiotics has provoked an exponential increase in the incidence of antibiotic resistance in several bacterial groups in recent years. Thus, multidrug-resistant Bacteria are rather common in hospital settings and farms but have been also detected in food animals and in food like fish products[12]. The food chain is considered a potential route of transmission of antibiotic-resistant bacteria to humans[13].

RAPD (random amplified polymorphic DNA) analysis is wide used for the genetic mapping, taxonomic and phylogenetic studies of many organisms[14]. It can be also applicable for the detection of DNA alterations after influence of many genotoxical agents as well[15]. The present work aimed to evaluate the genetic diversity of Enterotoxigenic *Bacillus cereus* strains isolated from marine fish varieties viz. shark, anchovy, ribbion fish, sole, mackerel, seer, tuna, snapper, sardine, and pomfret and using Random Amplification of Polymorphic DNA by PCR method and Dendrogram analysis. Data obtained from this study will help in understanding genetic variation among the various *Bacillus cereus* strains isolated form tropical marine fishes which has great economic importance in food industry.

MATERIALS AND METHODOLOGY

Sample Collection and Processing

10 samples of Marine tropical fishes were collected from Shivajinagar area of Bangalore belonging to shark, anchovy, ribbion fish, sole, mackerel, seer, tuna, snapper, sardine, and pomfret varieties. Only fresh fishes were used for experimental analysis. Fish samples were dissected in order to expose the gut region. The gut region was separated and macerated using a sterile mortar-pestle.

Isolation and Screening of Enterotoxigenic strains

The macerated gut region was serially diluted and spread on MYP media plate for the selective isolation of *Bacillus cereus*. Typical suspected *B. Cereus* colonies showing lecithinase reaction were confirmed by Gram's staining and biochemical characterization according to Bergey's manual for Bacterial identification. Selected strains were maintained on nutrient agar prepared in sea water slants.

DNA Extraction

The DNA was extracted and isolated according to the procedure described by[16]. Selected strains were inoculated into nutrient broth and incubated at 37 °C for 48 hours. Broth was centrifuged at 6000rpm for 10minutes. Cells as pellet were collected in the form of pellets at the bottom and the supernatant was discarded. Repeat the above step to give an increased concentration of cells. Pellet was resuspended in 1ml of lysis buffer and incubated at 45°C in boiling water bath for 10 minutes. 1ml of phenol: chloroform mixture was added and centrifuged at 10,000rpm for 10 minutes. To the supernatant (upper aqueous layer) equal volume of chloroform: isoamyl alcohol mixture was added with $1/20^{th}$ volume of 3M sodium acetate and centrifuged at 10,000 rpm for 10 minutes. To the upper aqueous layer double volume of chilled ethanol was added and incubated at -20°C for 20minutes. Ethanol treated samples were centrifuge at 12,000rpm for 10minutes and the pellet was air dried. The DNA pellet was dissolved in 20-50µl TE buffer and stored at 4^{o}C.

Qualitative and Quantitative estimation of DNA

Quantitative analysis DNA samples were carried out spectrophotometrically by calculating the A260/A280 ratios providing a value of 1.8 which determines pure DNA preparation. Purity of isolated DNA samples were qualitatively analyzed using 0.8% Agarose Gel electrophoresis with Ethidium bromide (1µl/10ml) staining using 1X TAE buffer at 50 V for 45 mints. A 500 base pair ladder (Chromas biotech) was used as molecular size marker. DNA bands were observed under UV light using Gel Doc System (Alpha Imager, Innotech, USA).

RAPD-PCR Amplification

Random Amplification of Polymorphic DNA using PCR method was performed as described by[17]. PCR was set up for 25µl Reaction Mixture volume as, 10X buffer (2.5 µl), dNTPs (1.5 µl), Primer (1 µl), isolated DNA (100 ng/ µl) (1 µl), Taq polymerase (1U/ µl) (2 µl) and Nuclease Free water (17 µl) for each sample. The samples were amplified with two arbitary primers namely OPB- 8 (GTCCACACGG) and OPD- 20 (ACCCGGTCAC) using Corbett Research CG1- 96 PCR Palm Cycler. The thermal cycle profiles for 36 cycles were as follows denaturation at 95ºC for 45sec, annealing at 36ºC for 1 min, extension at 72ºC for 1 min. There was also an initial denaturation step for 5 min at 95ºC and, at the end of the 37cycles, a final extension at 72ºC for 10 min and finally hold at 4ºC.

Visualization of RAPD-PCR Products

PCR Products of both the primers were separated by 1.5% Agarose gel electrophoresis stained with EtBr using 1X TBE buffer in Protean II xi Cell (Bio-Rad, USA) Electrophoresis unit at 100 V for 90 minutes. l0 µl (6 µl of amplified sample and 4 µl cf tracking dye) of sample was loaded into each well. Gel after electrophoresis were observed under UV light and photographed by gel documentation system alpha imager hp (Innotech, USA). The sizes of the amplified products were determined by comparison with a 500 bp ladder purchased from Chromous Biotech, Bangalore.

Dendrogram Analysis

Phylogenetic variations were determined using Dendrogram analysis by converting RAPD-PCR data into a frequency similarity Matrix and analyzed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a phylogenetic tree.

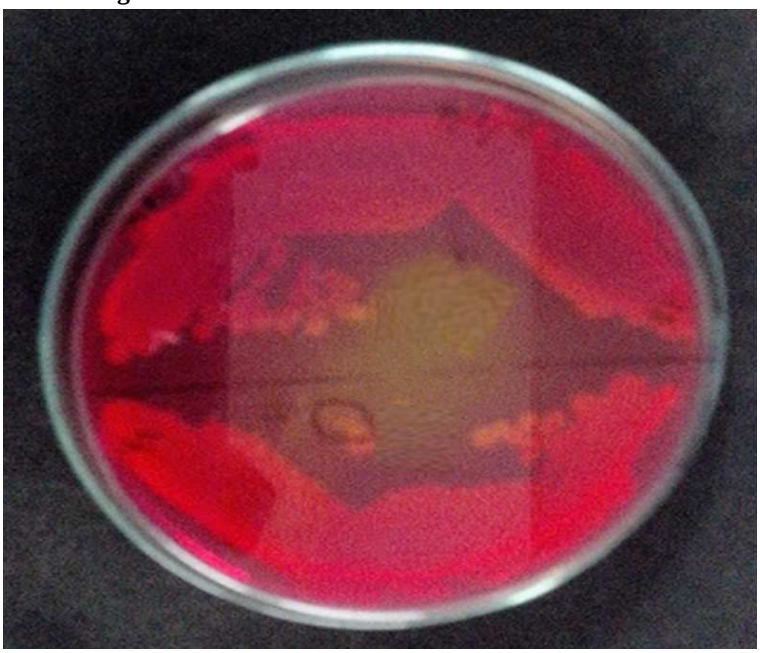
RESULTS

Isolation and Screening of *Bacillus cereus*

Five colonies of *Bacillus cereus* which showed lecithinase reaction on MYP plates were selected, isolated and maintained on Nutrient Agar slants prepared using sea water. Colonies which appeared as pink (due to the production of Lecithinase enzyme) in MYP Agar plates suspected to be *Bacillus cereus* were identified and subcultured on Nutrient agar slants. The selected isolates were morphologically characterized by observing colony morphology and Gram's staining. These isolates were further biochemically characterized.

Lecithinase Activity on MYP Media

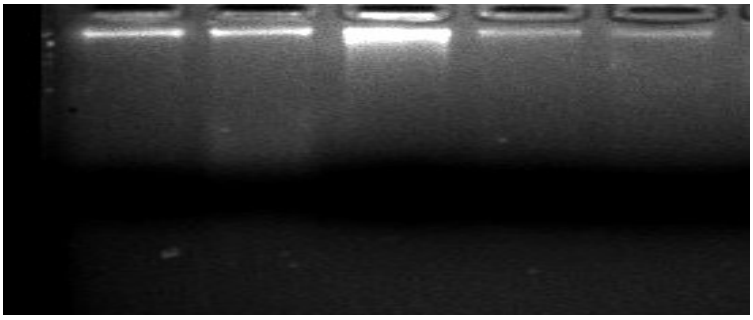
Fig.1: Bacterial Strains innoculated on MYP media



Qualitative Analysis of DNA by Agarose Gel Electrophoresis

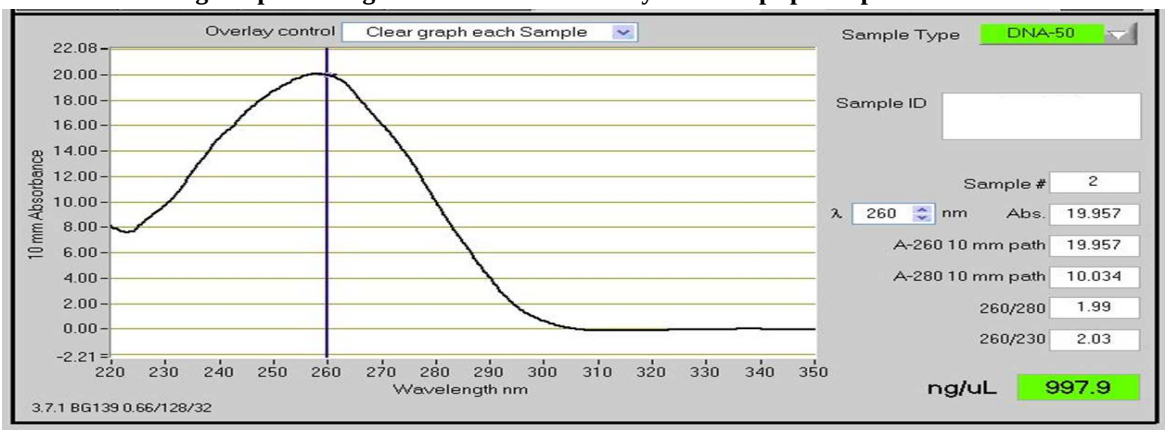
The qualitative analysis of DNA by gel electrophoresis is shown in Figure 2. The obtained DNA showed sharp single bands on 0.8% agarose gel without any degradation or RNA contamination.

Fig.2: Qualitative analysis of isolated DNA samples



Quantification of DNA Using Nano Drop Spectrophotometer

Fig.3: Spectra of genomic DNA obtained by Nanodrop spectrophotometer



The graphs show the DNA quantification by NanoDrop ND 1000. The graphs represent the DNA quantity in ng/ul. Also it shows the 260/280 and 260/230 ratios which represent the purity of DNA. All the samples showed good amount and purity of DNA. Concentrations of isolated DNA were found to be in the range of 900µg/ml to 1200µg/ml.

RANDOM AMPLIFICATION OF POLYMORPHIC DNA USING PCR

The samples were amplified with two arbitary primers namely OPB-8 and OPD-20. The amplified product was run on a 1 % agarose gel. After which the bands were analysed using Gel Doc Alpha Imager and UPGMA method for dendogram analysis.

Amplification using primer OPD-20

Fig.4: Gel Doc Image for Primer OPB-08 PCR Amplification

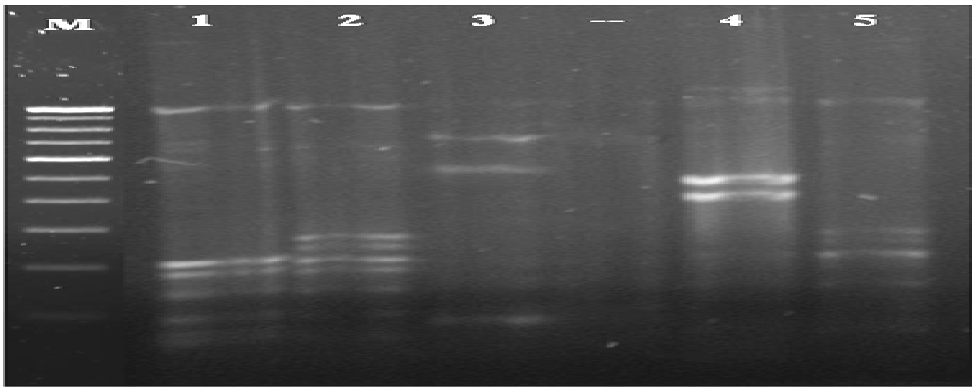
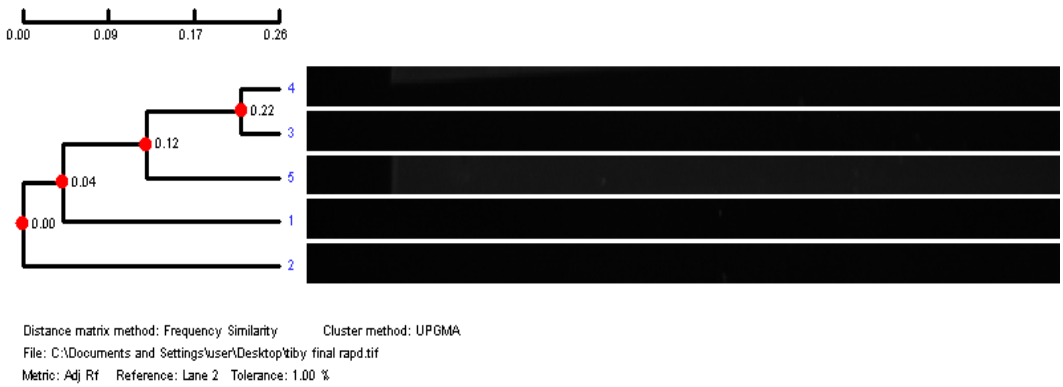


Fig.5: Dendrogram for OPB-8 Primer amplification



	1	2	3	4	5
1	**100.00**	53.85	53.85	61.54	53.85
2	53.85	**100.00**	38.46	46.15	53.85
3	53.85	38.46	**100.00**	92.31	69.23
4	61.54	46.15	92.31	**100.00**	76.92
5	53.85	53.85	69.23	76.92	**100.00**

Similarity Matrix Calculated by: Frequency Similarity using OPB-8

Amplification using primer OPD-20

Fig.6: Gel Doc Image for OPD-20-PCR Amplification

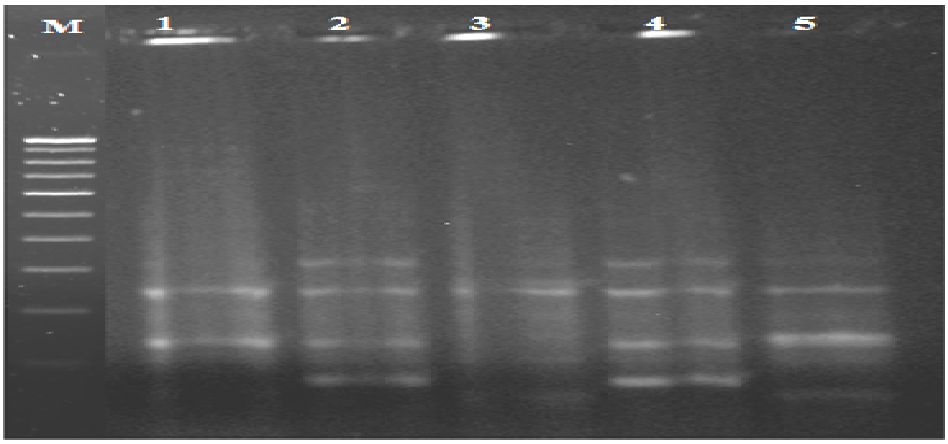


Fig.7: Dendrogram for OPD-20

	1	**2**	**3**	**4**	**5**
1	**100.00**	20.00	40.00	20.00	20.00
2	20.00	**100.00**	80.00	100.00	60.00
3	40.00	80.00	**100.00**	80.00	40.00
4	20.00	100.00	80.00	**100.00**	60.00
5	20.00	60.00	40.00	60.00	**100.00**

Similarity Matrix calculated by: Frequency Similarity using OPD-20

Table 1: Pattern of polymorphism and uniqueness (2 primers) between 5 strains of *Bacillus cereus*

Pattern of polymorphism	**OPB-08**	**OPD-20**	**Total**
Total No. of bands	28	16	44
Total No. of polymorphic bands	24	9	33
Total No. of monomorphic bands	0	5	5
Total No. of unique bands	4	2	6
Polymorphism, %	85.71	56.25	75.00
Monomorphism, %	0.00	31.25	11.36
Uniqueness, %	14.29	12.50	13.64

DISCUSSION

The present study deals with the determination of genetic diversity among the enterotoxigenic *Bacillus cereus* isolated from tropical fishes using RAPD markers. The selected *Bacillus cereus* colonies were subcultured and confirmed on MYP Agar, morphological and biochemical characterization.

Genomic DNA was isolated from 5 strains of *Bacillus cereus* by Phenol-chloroform extraction method with some modifications. The qualitative estimation of the DNA on 0.8% agarose gel gave single, sharp and distinct bands devoid of any smear. The genomic DNA were obtained in high concentration for all the samples and they showed a good 260/280 ratio (i.e. between 1.8 and 2.0) indicating absence of any protein or RNA contaminants.

In the present study, 85.71% and 56.25% polymorphism was observed in OPB-8 and OPD-20 respectively, which indicates high genetic variation among the *Bacillus cereus* strains isolated from tropical marine fishes. According to Dendrogram analysis of band pattern generated by Primer OPB-8, *Bacillus cereus* Strain 4 and 3 were closely related whereas strain 4 and Strain 2 were genetically more diverse. On the other hand, Dendrogram obtained from OPD-20 Primer RAPD-PCR data showed that *Bacillus cereus* Strain 2 and Strain 4 were genetically more similar whereas Strain 4 and Strain 1 were least similar to each other.

In a study by[18], Intestinal bacteria isolated from seven coastal fish were examined for their antibacterial ability against *Vibrio vulnificus* RIMD 2219009 using a double-layer method. This bacterium efficiently produced an antibacterial substance and inhibited it inhibited the growth of 227 (62.5%) of 363 intestinal bacteria from seven coastal fish. These results suggest that *Bacillus* sp. strain NM 12 may be a suitable strain as a biocontrol agent in fish intestines and culture water. Similarly, enterotoxigenic *Bacillus cereus* strains isolated in our study can be further tested for its application as biocontrol agent.

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