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Genetic Variability among Protease producing Microorganism using RAPD Technique

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Abstract: The water and the sediment were collected from different fish farms. The organism should be isolated from the samples and that organism should be protease producing. The protease is the enzyme that breaks down the protein molecules. The protease producing organism is that the microorganism which can able to break down the protein molecule. Eg: Bacillus spp, Aeromonas hydrophila, Aeromonas sobria. This bacteria were grown on the milk agar media. The colonies that are having the zones around it are separated and subcultured in the nutrient agar plates. Then the gram staining can be done for the identification of morphological characteristic of the bacteria. The organism can be subcultured in the LB broth. The DNA is then isolated from the organism and that isolated DNA is used for the RAPD analysis using the primers OPA-13,OPU-18,OPA-20. The samples were run in the agarose gel and the amplified DNA bands are observed. The genetic variability between the closely related organism can be studied by using this RAPD technique.
Keywords: fish farms, protease, Bacillus spp**,** RAPD analysis, genetic variability.

Introduction

Proteases are enzymes which catalyze the hydrolysis of peptide bonds. Microorganisms are capable of producing these enzymes intracellularly and extracellularly. The isolation of proteases especially the extracelluar proteases of microbial origin is easy and economical.[1] Seasonal fluctuations in the availability of raw material usually do not affect the enzyme production by microbes. They find application in the food, pharmaceutic and detergent industries and are important tools in studying the structure of proteins and polypeptides present[2]. The study describes a qualitative method for detection of extracellular proteases on agar plates and the proper culture medium for their production. [3] *Bacillus* sp. beingindustrially important organisms produces a wide variety of extra-cellular enzymes including proteases.[4]

Genetic variability is different from genetic diversity (or genetic variation) in a way that the former measures how much the trait or the genotype will tend to vary whereas the latter measures the number of the actual variation of species in a population[5] Compared with genetic diversity, is more difficult to measure. At the molecular level, genetic variability may be measured by determining the rate of mutation[6]. It is a measure of the tendency of individual genotypes in a population to vary from one another. Variability is different from genetic diversity, which is the amount of variation seen in a particular population.[7,8] This genetic heterogeneity is interesting in view of the production of extracellular enzymes. Use of the random amplified polymorphic DNA (RAPD) method has extensive application for rapid typing of microorganisms at the species and

subspecies levels.[9,10] In this study, the RAPD method was used to investigate the genetic heterogeneity of *Bacillus* strains isolated from different habitats and characterization was done in relation to production of extracellular enzymes.[11,12,13,14]

The polymerase chain reaction, comes from the DNA polymerase used to amplify (replicate many times) a piece of DNA by *in vitro* enzymatic replication.[15] PCR is used in research laboratories in DNA cloning procedures, Southern blotting, DNA sequencing, recombinant DNA technology, to name but a few. Random Amplified Polymorphic DNA (RAPD) which is a Polymerase Chain Reaction (PCR)-based technique in which amplification of random DNA segments with single primers of arbitrary nucleotide sequence is conducted and used as powerful tool for genetic studies involving molecular genetic variability of microorganisms.[16] RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications.[17]
The PCR based method of gene typing based on genomic polymorphism is a recent approach which is widely used for the assessment of inter and intraspecific genetic variation and uses a single short random oligonucleotide primer. In most cases of bacterial genetics, RAPD assay generated the best DNA pattern for differentiation of bacteria.[18]

Materials and Methods

Isolation of Protease Producing Microorganism

For the isolation of protease producing bacteria, water samples were collected from different fish farms Ulsoor, Bangalore. It was taken care that the place of sample collection had ample spillage of growth nutrients for the organisms to grow. Isolation of the protease producing organism was done from water by serial dilution. The water sample was serially diluted using distilled water 10^{-1} to 10^{-5}…etc from each dilution 0.1ml of sample was inoculated onto Milk Agar plates followed by incubation for 24 hrs at 37^{o}C. When an organism produces protease and breaks down the Protein molecules, a clear halo surrounds the areas where the protease-producing organism has grown.

Identification of the organism through colony morphology and staining characteristics:

The colonies were isolated on the basis of the clear zone produced by the organism, morphology (shape, size, structure, texture, appearance, elevation and color). Further identification was done on the basis of staining. Differential staining i.e. Gram Staining was performed.

Isolation of DNA

After incubation at 37 ºC for 24 hours the discrete or isolated colonies had been examined and some of the cells from one of the colony were sub cultured on separate agar plates with a sterile needle or loop for further examination.The microorganisms were cultured in LB broth. This broth is used before DNA isolation & in other microbial genetic manipulations as this media is believed to recover the cells from shocks and provides ready nutrients for the growth .DNA was extracted from 24hr old culture grown in Luria Bertani (LB)broth using a modified method of DNA isolation and the quality and quantity was determined by agarose gel electrophoresis.

Qualitative analysis of DNA (Agarose Gel Electrophoresis)

Qualitative analysis was done using Agarose gel electrophoresis. This procedure examines the integrity of the isolated DNA and checks the contaminations. Agarose gel electrophoresis is based on the principle that biological molecules are charged species, which can move in an electric field.

Quantitative analysis of DNA (Nanodrop Spectrophotometry)

The Nanodrop ND-1000 UV-Vis Spectrophotometer enables highly accurate analyses of 1 µl samples with remarkable reproducibility. The patented sample retention system eliminates the need for cuvettes and capillaries which decreases the measurement cycle time. In addition, the high absorbance capability eliminates the need for most dilutions. The quantity of DNA was estimated by Nanodrop spectrophotometer. At 260/280nm if the optical density value is between 1.6-1.8 then the DNA will be in the pure form.

PCR Assay using RAPD:

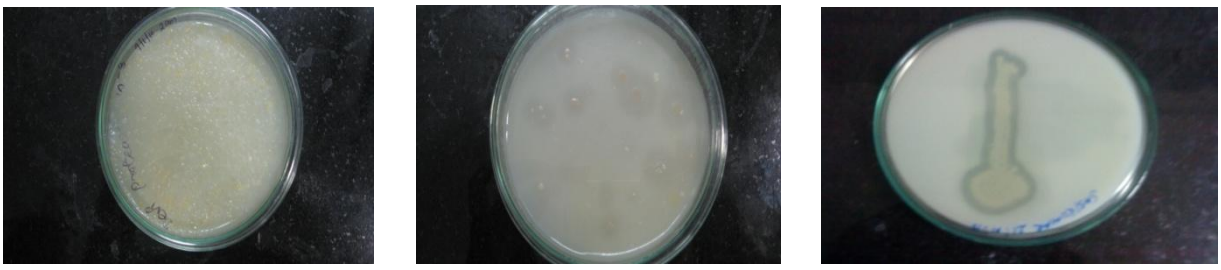
PCR is invitro nucleic acid synthesizing reaction, it amplifies the template DNA flanked by primers. Thermocycler controls the temperature at various stages of the replication cycle. Three primers were screened to amplify the DNA isolated. 3 RAPD primers were used for the analysis. OPA 13- CAGCACCCAC, OPA 20-GTTGCGATCC,OPU 18-GAGGTCCACA. RAPD assays were carried out in 25 µl reaction mixture and the components. Initial denaturation at 94º C for 1 min consisting 30 repeats. Final denaturation at 94 ºC for 1 min. Annealing temperature is 36 ºC for 1 min. Extension at 72 ºC for 2 min. Final extension at 72 ºC for 10 mins.Storage at 4 ºC for 1 min. PCR products were analyzed on 1.5% agarose gel and visualized by ultraviolet illumination.

Result

RAPD-PCR was carried out to determine the genetic variability among same bacterial genus of different species collected from water.

Figure 1: (a) Colonies of Serially diluted water sample with 10^{-1} concentration (b) Colonies of Serially diluted water sample with 10^{-5} concentration and (c) Isolation of colony from10^{-5} concentration.

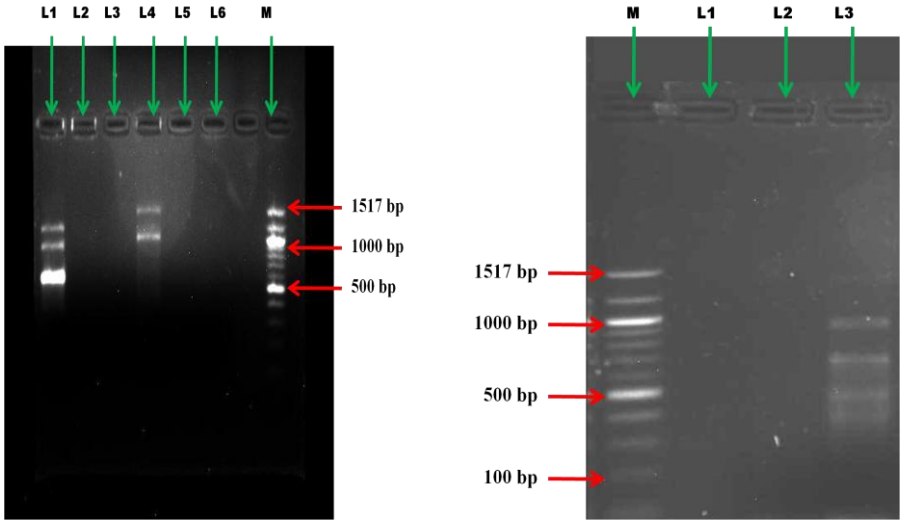
Figure 1: Colony isolation.



(a) (b) (c)

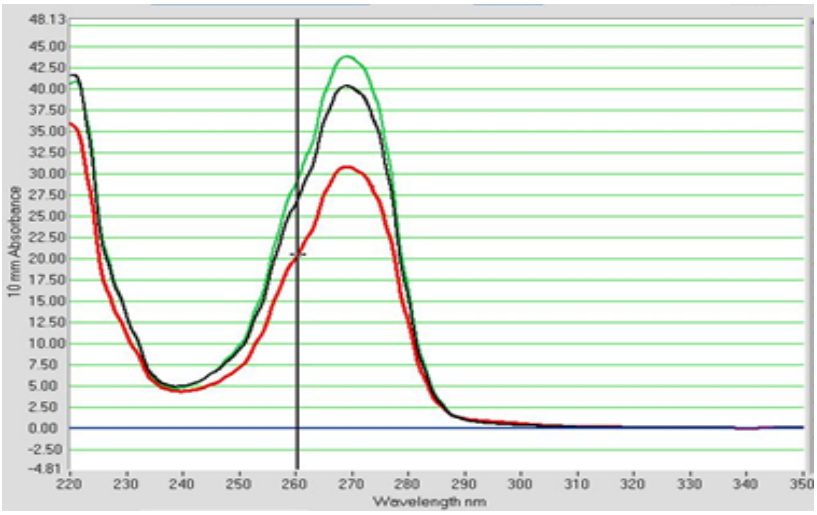
Figure 2: (a) M – 100 bp ladder, L1 - Bacillus spp 1(OPA 20), L2 - Bacillus spp 2 (OPA 20), L3 - Bacillus spp 3 (OPA 20), L4 - Bacillus spp 1(OPU 18), L5- Bacillus spp 2 (OPU 18), L6 - Bacillus spp 3 (OPU 18). RAPD –PCR for Bacillus spp 1 using OPA 20 primer amplified 3 fragments between 1500 bp – 500 bp. Bacillus spp 1 using OPU 18 primer amplified 2 fragments between 1517 bp- 1000bp (b) M – 100 bp ladder,L1 - Bacillus spp 1, L2 - Bacillus spp 2, L3 - Bacillus spp 3 RAPD –PCR for Bacillus spp 3 using OPA 13 primer amplified 5 fragments between 1200 bp – 300 bp.

Figure 2: Qualitative analysis of DNA by Agarose gel electrophoresis.



(a) (b)

Figure 3: Quantative analysis of DNA by Nanodrop spectrophotometer.



Discussion

Random Amplified Polymorphic DNA (RAPD) is a method of producing a biochemical fingerprint of a particular species. Relationships between species` may be determined by comparing their unique fingerprint information. The following research involved the isolation of DNA from approximately twenty bacteria using the Qiagen, Inc. protocol. PCR was accomplished using RAPD primers, OPA-02 and OPA-06. RAPD-PCR has been used to differentiate strains of various species, various serotype within species, and various subtypes within a serotype. Interestingly, the current study clearly demonstrated the possibility of discrimination, at the molecular level, that RAPD-PCR markers help in diversity analysis as well as fingerprinting of *P.aeruginosa* isolated. The results showed that some primers gave main and for all isolates such as primers OPT-19, OPA-11, OPF-16, OPG-13, this indicates that all *P. aeruginosa* isolates have the same primer annealing sites on the template DNA of the tested isolates Agarose gel electrophoresis was done producing favorable banding results for most samples. Bands were then analyzed using a binary system and a phylogenetic tree created. The phylogenetic relationships shown were not as expected. Additional primers and possibly different primer are needed to improve the accuracy of the results.[19]

In the present study 3 RAPD primers (OPA 13, OPA 20 and OPU 18) were used to detect the variability among three different Bacillus spp. The amplification patterns generated with OPA 13, OPA20 & OPU 18 were distinct. Bacillus spp 1 was amplified with OPA 20 & OPU 18 whereas Bacillus spp 3 was amplified with OPA 13 .bacillus spp 2 did not show any amplification.

Serratia marcescens FS-3, isolated from a soil sample in the southwestern area of Korea, exhibited a strong protease activity of 60.7 U/ml after 3 days of incubation. The protein removal from natural crab shell wastes with 10% *S. marcescens* FS-3 inoculum was 84% after 7 days of fermentation. At the same time, 47% demineralization occurred. When the shell waste was treated with 1% Delvolase as a reference, deproteinization rate was 90%. In combination of 10% *S. marcescens* FS-3 culture supernatant and 1% Delvolase, deproteinization rate of the shell waste was 85%, while the rate was 81% in 10% FS-3 culture supernatant only. These results suggest that bio-deproteinization of the crab shell wastes using *S. marcescens* FS-3 could be applicable to the chitin production process.[20]

The bacterial strain was isolated and identified as Bacillus spp based on the results from the morphological characteristics and gram staining. Milk agar plates were used for screening of Protease production. The presence of clear zone around the colony was observed as protease producers. DNA of the isolates obtained in this study was extracted by Phenol chloroform method.

Conclusion

Classification of specific microbes using random amplified polymorphic DNA (RAPD) analysis revealed genetic similarities and differences among isolates belonging to the same genus. Because of the simplicity and low cost of the RAPD technique, it has found a wide range of applications in many areas of biology. RAPD markers have found a wide range of applications in gene mapping, population genetics,

molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications. Despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labor.

The present study was an approach to provide the information about the genetic variability among three morphologically different Bacillus isolates by RAPD. Bacillus spp 1 showed amplification with primers OPA 20 & OPU 18. Bacillus spp 3 showed amplification with OPA 13 but Bacillus spp 2 was not amplified with any of these 3 primers. RAPD PCR for Bacillus spp 3 using OPA 13 amplified 5 fragments between 1200-300 bp. Similarly RAPD –PCR for Bacillus spp 1 using primers OPA 20 & OPU 18 amplified 3 & 2 fragments in the range of 1500-500 bp and 1517-1000 bp respectively. This reveals that they are genetically variable.

This investigation supports previous reports that RAPD analysis is efficient, reproducible, and capable of detecting genomic polymorphisms among various microbial species without previous knowledge of the nucleotide sequence on the target DNA.

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