European Journal of Biotechnology and Bioscience

www.biosciencejournals.com

E J B B European Journal of Biotechnology and Bioscience

ISSN: 2321-9122
www.biosciencejournals.com
EJBB 2014; 2 (3): 38-42
Received: 30-08-2014
Accepted: 12-09-2014

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Molecular characterization of protease producing *Bacillus sp.* isolated from kitchen soil using RAPD markers

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Abstract
Amongst the industrial enzymes, proteases hold a major significance and are used in various industries like soaps and detergents, brewing, meat processing, leather, dairy, pharmaceutical and food. Proteases are one of the many extra-cellular enzymes that are produced by Bacillus sp., which are a group of industrially important microorganisms. In the present study 22 isolates were obtained from the kitchen soil samples. These isolates were then screened for their protease-activity through the plate assay using skimmed Milk Agar. Five isolates showing high protease-activity were selected, which were further characterized based on their colony characteristics, morphological and biochemical characteristics. The genetic variability of these protease producing Bacillus sp. were analysed using RAPD-PCR technique. In the bands obtained using primer A11, 92.30% were polymorphic and only 7.69 % were monomorphic and with C9, 92.7% were polymorphic and only 7.27 % were monomorphic. This method of molecular characterization using random primers implements a rapid identification and the diversity of the Bacillus sp.

Keywords: Protease, *Bacillus species,* RAPD, Diversity analysis.

1. Introduction:

Enzymes are proteins and certain class of RNA (ribozymes) which enhance the rate of a thermodynamically feasible reaction and are not permanently altered in the process. Some microorganisms were found to produce enzymes similar to that of plants and animals in terms of its activity. Ever since then microorganisms have been used as bio-factories for production of enzymes [1]. Microorganisms are found everywhere, they can be observed in the deepest ocean sediments, at high atmospheric pressure, at unusually high and cold temperatures and also in highly polluted environments. Microorganisms for a potential process can be isolated from a variety of sources, most commonly soil. The decomposing sites and industrial effluents, the semi moist layer of soil deposited by run-off water in the water streams are chosen for isolating the novel microbial organisms. These types of soils can be used specifically to identify & isolate the microbial organisms as industrially important strains producing the secondary metabolites or enzymes such as proteases, cellulases, pectinases, amylases, lipases etc. Screening of these organisms depends on the type of desired organism, the product of interest and the source from where this microorganism can be obtained. Kitchen area soil and garden area soil has been used as a source of organism producing protease because kitchen soil and garden soil contain waste from protein rich food products, hence the natural predisposition of the presence of protein degrading or protease producing bacteria. Protease, also termed peptidase or protease, refers to a group of enzymes whose catalytic function is to hydrolyze proteins i.e. begins protein catabolism by hydrolysis of the peptide bond that link amino acids together in the polypeptide chain forming the protein [2]. Proteases are classified according to their structure or the properties of the active site. There are several kinds of proteases such as serine-, metallo-, carboxyl-, acidic-, neutral-, and alkaline proteases. Proteases have evolved multiple times, and different classes of protease can perform the same reaction by completely different catalytic mechanism. Proteases can be found in animals, plants, bacteria, archea and viruses [3]. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. Proteases are one of the most important groups of industrial enzymes, and commercial proteases account for nearly 60% of the total industrial enzyme market.

The extracellular proteases have commercial value and find multiple applications in numerous industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers [4]. Proteases are one of the most important group of industrial enzymes and account for nearly 60% of the total enzyme sale [5, 6]. The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds [7]. The present study involves the isolation, screening of protease producing *Bacillus sp* from kitchen soil, followed by its diversity analysis using RAPD technique.

2. Materials and Methods

Sample collection:-

The soil samples for the study were collected from the kitchen soil surroundings in C.V. Raman Nagar, Bangalore and transferred into sterile plastic covers. The collected soil sample was maintained under aseptic conditions in the laboratory at room temperature.

Isolation and Identification of *Bacillus* species:-

The collected kitchen soil samples were serially diluted using autoclaved sterile distilled water in the range of 10^{-1} to 10^{-7}. 0.1 ml of the serially diluted sample was spread on Luria Bertani (LB) agar plates, using sterile L-shaped glass rods. The plates were incubated overnight. The colonies obtained were purified by sub-culturing them and storing them on LB agar slants for further analysis.

Screening of the isolates for extracellular protease production:

Pure cultures obtained from the LB agar plates were spot inoculated on Skimmed milk agar plates and incubated for 48h. These isolates were observed for a zone of clearance indicative of protease synthesis. The isolates showing protease activity were stored for further analysis [8].

Morphological and Biochemical characterization:

Morphological characterization of the isolated colonies was determined by Gram's staining. The isolated colonies were also biochemically characterized by the following tests as per Bergeys Manual of Determinative Bacteriology: Catalase test, Citrate utilization test, Mannitol motility test, Indole test, Methyl red and Voges Proskauer test.

Isolation of genomic DNA:

Genomic DNA was isolated using the phenol-chloroform isolation method. 2 ml of fresh bacterial cell suspension (18 h old bacterial cell suspension grown in Luria–Bertani broth) was centrifuged at 15,000 g for 10 min at 4 °C. The pellets obtained were resuspended in 500 µl lysis buffer containing 0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol. Further extraction was carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000 g for 15 min at 4 °C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000 g for 15 min at 4 °C to separate the aqueous phase which was then transferred to a fresh tube. Then the DNA was precipitated by incubation at -20 °C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 g for 15 min at 4°C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes left open until the pellet dried. The DNA pellet was dissolved in autoclaved distilled water.

The isolated DNA was quantified using a Nanodrop spectrophotometer (Abs260) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs260/Abs280). Agarose gel electrophoresis was performed to qualitatively determine the isolated DNA.

Determination of Genetic variability using RAPD-PCR:

The alkaline protease producing bacterial isolates were subjected to RAPD screening using two random RAPD oligonucleotide primers for the assessment of their genetic variability. The amplification reaction was prepared in 25 µl volume using 1µl of the template DNA, 2.5µl of 10x Taq polymerase buffer with $MgCl_2$, 1.5µl dNTPs of 2.5 mM, 2 µl Taq polymerase, 1µl of 10 pmole RAPD primers. The amplification was carried out in a thermal cycler, programed for 4 min of initial denaturation at 95 °C followed by 35 cycles at 94 °C for 1 min, 37 °C for 1 min, 72 °C for 1 min and final extension for 10 min.

Analysis of amplicons using Agarose gel electrophoresis:

The amplified PCR products were separated by agarose gel electrophoresis in 1.2% run in 1x TBE buffer at 85-90 V till amplified fragments are separated and visualized under UV transilluminator.

Data Interpretion:

Binary data was analyzed based on the presence or absence of bands. The similarity matrix thus obtained was subjected to cluster analysis by UPGMA and dendrogram was generated to determine the genetic relationship of the bacterial strains.

3. Results

3.1 Isolation and identification of the organism:

The colonies from master plates with colony characteristics like white or creamish, dry, flat or elevated, and irregular were selected and subjected to gram staining. Colonies showing positive gram staining results were named and subcultured further.

3.2 Screening of the isolates for extracellular protease production:

The pure isolates showing maximum zone of clearance on Skimmed milk agar were indicative of the protease synthesis. These six isolates were assigned culture nos. X_4, X_9, X_{12}, X_{13}. $PA_1$and PA_2 was selected for further analysis.

3.3 Morphological and biochemical Characterization:

The selected bacterial strains were characetrized morphologically and biochemically to identify the organism and according to the Bergey's manual of determinative bacteriology [9] the organisms were considered to be Bacillus sp.

3.4 Qualitative and Quantitative estimation of genomic DNA:

The DNA bands when observed under the UV trans-illuminator showed the presence of clear band devoid of any RNA or protein contamination. The yield of genomic DNA obtained varied from 43.3- 2190.5 ng/µl. The 260/280 ratio of the samples were found which indicated the presence of pure DNA.

3.5 RAPD-PCR analysis of the selected strains:

The RAPD-PCR analysis performed using two random primers A11 and C9 determined the polymorphism amongst the protease producing bacterial isolates. Determination of the banding pattern score was based on the presence or absence of clear, visible and reproducible bands. The results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals. In this study, both the primers produced a total of 52 and 55 bands respectively.

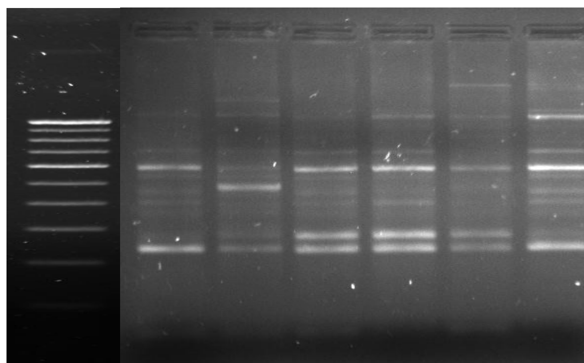

Fig 1: RAPD profile of six protease producing Bacillus sp. using primer A11

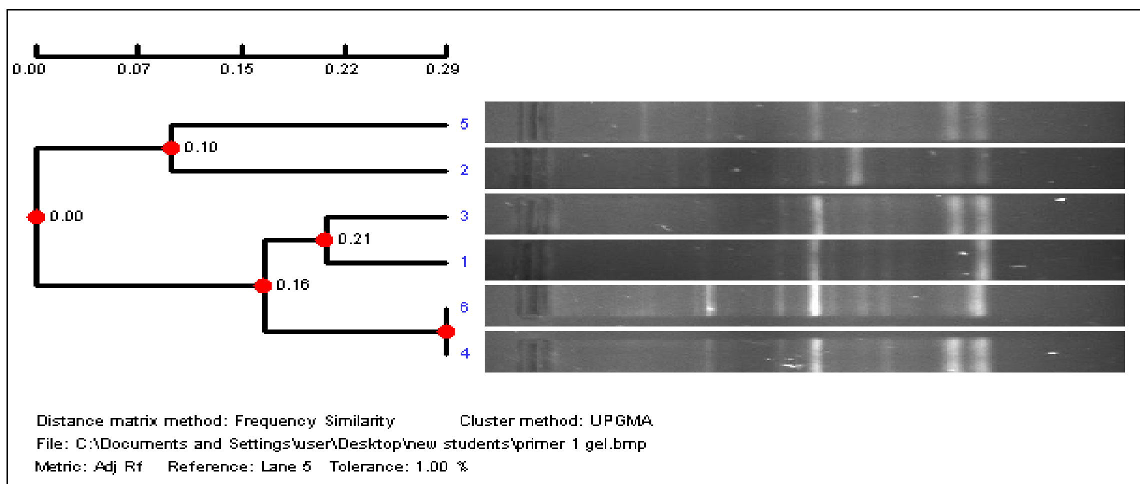

Fig 2: Dendogram derived from cluster analysis (UPGMA) showing relationship between six protease producing *Bacillus sp.*

In the bands obtained using primer A11, 92.30% were polymorphic and only 7.69 % were monomorphic. The obtained RAPD data was further utilized for construction of dendrogram and similarity matrix.

Table 1: Genetic variability obtained using RAPD primer A11 using Frequency Similarity coefficient

1	100.00	46.15	38.46	30.77	38.46	53.85
2	46.15	100.00	76.92	23.08	46.15	61.54
3	38.46	76.92	100.00	30.77	38.46	53.85
4	30.77	23.08	30.77	100.00	61.54	46.15
5	38.46	46.15	38.46	61.54	100.00	38.46
6	53.85	61.54	53.85	46.15	38.46	100.00

Similarity Matrix Calculated by: Frequency Similarity

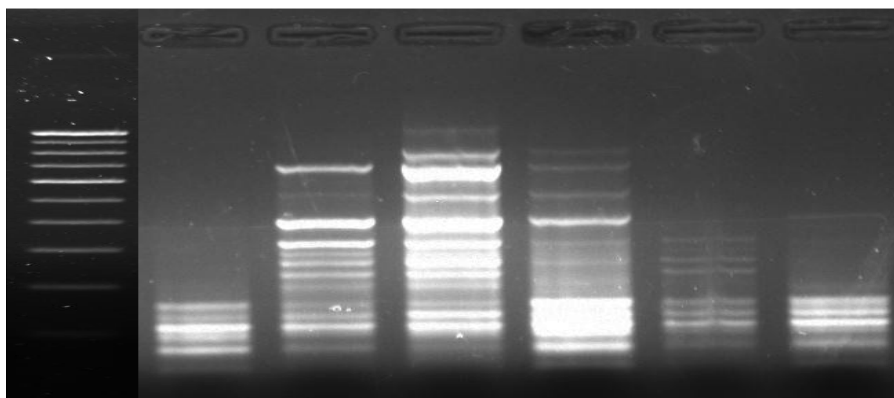

Fig 3: RAPD profile of six protease producing Bacillus sp. using primer C9

In the bands obtained using primer C9, 92.7% were polymorphic and only 7.27 % were monomorphic. The obtained RAPD data was further utilized for construction of dendrogram and similarity matrix.

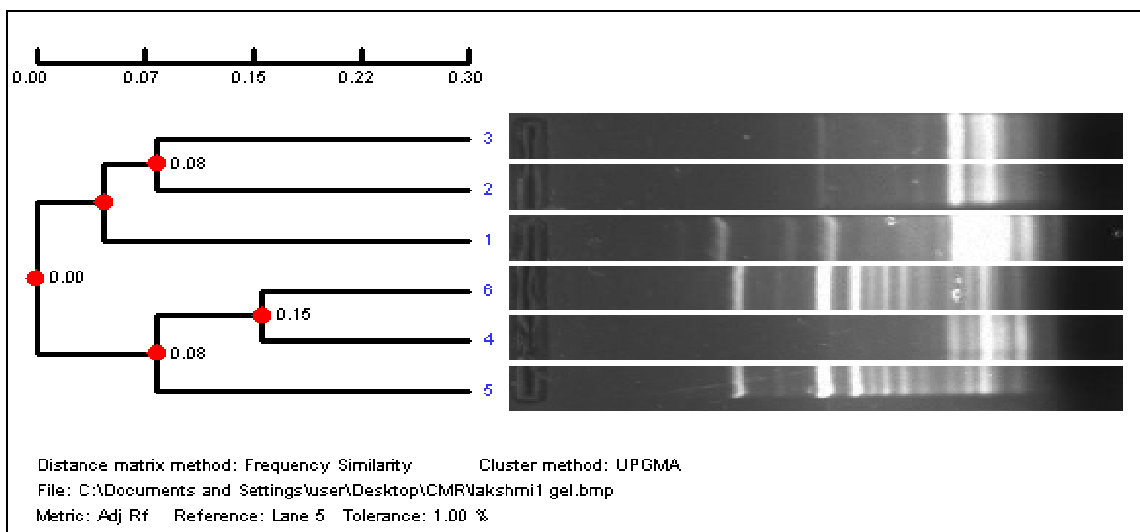

Fig 4: Dendogram derived from cluster analysis (UPGMA) showing relationship between six protease producing *Bacillus sp*

Table 2: Genetic variability obtained using RAPD primer A11 using Frequency Similarity coefficient

1	100.00	46.15	38.46	30.77	38.46	53.85
2	46.15	100.00	76.92	23.08	46.15	61.54
3	38.46	76.92	100.00	30.77	38.46	53.85
4	30.77	23.08	30.77	100.00	61.54	46.15
5	38.46	46.15	38.46	61.54	100.00	38.46
6	53.85	61.54	53.85	46.15	38.46	100.00

Similarity Matrix Calculated by Frequency Similarity

4. Discussion

Bacteria of the genus *Bacillus sp.* are among the most widespread microorganisms in nature. The source used in this study was kitchen soil sample, the preliminary characterization was done using Bergey's Manual of Determinative Bacteriology as mentioned by [9]. The novelty in the present study lies in the molecular characterization of the selected protease producing isolates using RAPD-PCR to ascertain considerable genetic variability. The different isolates obtained were qualitatively screened using the Skimmed Milk agar plate assay [10] which showed six organisms to be effective producers of protease. DNA fingerprinting experiments of the selected isolates were done to obtain a phylogenetic relantionship and for analysis of the diversity amongst them. The RAPD technology is well suited to DNA fingerprinting [11] although it suffered from a certain lack of reproducibility due to mismatch annealing [12] the substantial degree of polymorphism was obtained using both the oligonucleotide primers A11 and C9 indicated that these two primers can be used as suitable markers for diversity analysis of the protease producing organisms. The

dendrograms obtained using the RAPD data separated the protease producing *Bacillus sp.* samples into different clusters. The primary clusters were then further sub divided into subclusters as seen in dendrograms.

5. Conclusion

In the present study PCR amplication and RAPD studies has been used as a major tool to ascertain phylogenetic relationship between the protease-producing bacterial stains. The RAPD results shows a proven result that the

oligonucleotide primers A11 and C9 can be used as a promiment marker to determine the genetic variability among the bacterial species at a genetic level in a large scale. They also provide a suitable alternative to specific markers and also the polymorphism pertaining to protease producing bacterial isolates, that has emerged as a complex group due to their genetic specificity. Therefore, this study might be a vital step in the evolutionary and diversity determination studies of protease producing bacterial isolate.

6. References

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